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Life beyond the borders: the resistance of the black fungus *Cryomyces antarcticus* to radiation and space environment

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"Science is much more than a body of knowledge. It is a way of thinking. A way of skeptically interrogating the universe with a fine understanding of human fallibility."
CARL SAGAN (1934-1996)

Abstract

Our understanding of potential life in extraterrestrial environment and its biosignature has mainly focused on studying extreme environments on Earth. Terrestrial extreme environments, indeed, offer a rich source of information allowing us to determine how extreme conditions affect life and molecules associated.

Extremophilic organisms, inhabiting these environments, have adapted to the most stunning conditions on Earth environments; among these, the Antarctic cryptoendolitic black fungus *Cryomyces antarcticus* CCFEE 515, isolated from the McMurdo Dry Valleys in Antarctica, is one of the most resistant eukaryotic microorganism known to date, survived over 1.5 years real space during the LIFE experiment.

In the frame of different astrobiological projects (BIOMEX and STARLIFE), the fungus was exposed to different kind of stressors, including vacuum, temperature ranges, Martian atmosphere, simulated space conditions and even real space exposure, which included various types of radiations as UVC, whole UV spectrum, ionizing radiation (γ- and x-rays), deuterons and heavy ions (helium); these rays, being part of the cosmic radiation environment, are space-relevant radiations.

The role of the melanin, a pigment composing the fungal cell wall, in the photo-protection was investigated and demonstrated, by comparing melanized and non-melanized strain of the fungus; melanin as biosignature molecule was analyzed too.

In conclusion, this research demonstrated the strong endurance of the Antarctic cryptoendolitic black fungus *Cryomyces antarcticus* in simulated and real space conditions contributing to our knowledge on the limits of life on Earth, on the potential survival of this eukaryotic microorganism on other planets and to the identification of biosignatures for searching life beyond Earth.

Contents

1 Introduction

1.1 Astrobiology

Astrobiology is a rather young field of research gathering scientists from different backgrounds around the questions of the origin, evolution, distribution and future of life on Earth and in the Universe. This interdisciplinary field encompasses researches on the origin and evolution of planetary systems, origins of organic compounds in space, rock-water-carbon interactions, abiogenesis on Earth, planetary habitability, search of biosignatures for life detection, and studies on the potential for life to adapt to challenges on Earth and in outer space (Sullivan and Baross, 2007). Astrobiology is not only diverse in terms of disciplines. It also traverses a very wide spectrum of spatial and temporal scales: from the molecular level to ecosystems and planetary systems, at scales ranging from Earth's (sub)surface to planetary objects detected thousands of lightyears away, and from understanding the origins of life to its future evolution and destiny (Horneck et al., 2016). Platforms placed in Low Earth Orbit (LEO) and ground based simulators have been developed to expose organisms and molecules to the space environment. New generation astrobiological experiments have been carried out by using nanosatellites, e.g. cubesats (Shiroma et al., 2011); that orbit where the radiation doses are significantly higher (at least one order of magnitude) than on the ISS (Woellert et al., 2011). All the efforts should answer to the astrobiological questions: What is life? How did it start? Are we alone in the Universe? Did living organisms arise on the planet Mars and do they survive today? Does life exist in the subterranean oceans of Europa, one of the Moons of Jupiter? What is the future of life on Earth and beyond?

1.2 Dry Valleys in Antarctica are considered one of the extraterrestrial analogue environments

As reviewed by Cockell et al. (2016) we have to define all the conditions that life require in order to investigate deeply the origin of life on Earth, its persistence on the planet since its emergence, and the search for evidence of life on other planets. To answer these points many astrobiologists have attempted to further explain habitability and the requirements for presence of life. They focused mainly on defining the basic requirements for life to be

metabolically active or to reproduce in planetary environments and the processes to be sustained over geological periods within the lifetimes of planetary bodies.

Our knowledge of the boundaries for life on Earth defines the standard parameters that define these boundaries include, for example, extremes of temperature, pH, salinity, pressure, redox states, radiation, gravitation, the availability of electron donors and acceptors, and thermodynamic laws (Stevenson et al., 2015; Horneck et al., 2016). Some extreme environments on Earth are characterized by only one or two of these parameters. However, several environments are characterized by multiple extremes (Harrison et al., 2013).

Recent findings (Lin et al., 2006; Pointing et al., 2009; Shtarkman et al., 2013) reveal that life may thrive in environments we thought previously uninhabitable, suggesting we have not yet encountered the limits of life on our own planet. Extreme environments on Earth, previously thought to be incompatible with active life, are perfect models for studying the limits of habitability on Earth. A large number of analogue sites have been identified similar to the environmental conditions we would expect elsewhere in our Solar System: hot deserts, cold and dry polar regions, permafrost soils, deep seas, alkaline and hypersaline habitats (González-Toril et al., 2003; McKay et al., 2003; Gunde-Cimerman et al., 2005; Gilichinsky et al., 2007; Onofri et al., 2007a; Stevenson et al., 2015). Examples from hot deserts such as the Atacama and the Tunisian Sahara desert Gypsum crusts (Dong et al., 2007; Stivaletta et al., 2010) suggest that life is possible in deserts localizes and specializes towards areas that offer protection from the harsh UV radiation, and dry conditions.

The super-arid and cold location of the McMurdo Dry Valleys in Antarctica are analogue of Mars environment. Subglacial Antarctic lakes as Vostok or Vida, ice-bound systems presumably isolated with solar-derived organic carbon and coincident microbial life which has survived for millennia since isolation, are analogue to the icy moons around Jupiter and Saturn, Europa and Enceladus. Such aphotic and anoxic ecosystems provide potential analog for habitats on other icy worlds where water-rock reactions may co-occur with saline deposits and subsurface oceans (Murray et al., 2012).

After the discovery of life in evaporates, halite is now the new frontiers for astrobiologists: from the drought of the Atacama Desert to salt deposits up to Permian in age and 2000 meters in burial depth, living microbes have been found. Because halite is geologically

stable and impermeable to ground water, the microbes allegedly are the oldest organisms known to live on Earth (Jaakkola et al., 2016). The now-frozen water on Mars may have been liquid in the past, offering a prerequisite for life; sometimes microbes become trapped inside these inclusions, where liquid water allows them to remain viable and wait for the environmental conditions to become more hospitable (Oren et al., 1995). There is evidence of evaporitic minerals on the planet (Osterloo et al., 2008), and if there ever was life on Mars, remnants of it could now be in slumber under the surface. Other potential targets for searching for halophilic life in the Solar System are Jupiter's moon Europa and Saturn's moon Enceladus (Jaakkola et al., 2016).

Among the environments described above, the McMurdo Dry Valleys, located in Southern Victoria Land in Antarctica, being the coldest hyper-arid desert environment on Earth (Cowan et al., 2014), are considered to be, among the terrestrial 'extreme' environments, the closest analogue of Mars (Fig 1.1). A combination of very cold and very dry conditions, very poor nutrient availability, and large fluxes of UV-light characterizes the environment (Horneck, 2000; Finster et al., 2007; Onofri et al., 2007a, b).

They have a total ice-free area of 4500 km^2, making them the largest (15%) ice-free land portion of the continent (Cary et al., 2010; Levy, 2013). This region has a mean annual surface temperature of near -20°C (Doran et al., 2002) with temperatures dropping down to -60°C in the winter (Horowitz et al., 1972; Cary et al., 2010). Frequent daily temperature fluctuations of >20°C often result in multiple freeze-thaw cycles (Aislabie et al., 2006; Barrett et al., 2008).

Further environmental factors that pose extreme stresses on microbial life are the low bioavailability of water [<10 cm yr^{-1} water equivalent precipitation (Witherow et al., 2006)], high salt concentrations, low nutrient availability (<1% by weight) (Vishniac, 1993; Burkins et al., 2000) and high radiation, including UV (Onofri et al., 2007b). Microbial biomass is low in these regions but just like in other extreme environments, life tends to seek for shelter in areas that provide protection against the most destructive effects of desiccation, freeing and radiation.

All the environments characterized by these kind of stressors are hostile for organisms; however, a small group of microbes, the so called "extremophiles" are adapted to live even in the Martian-like region of the McMurdo Dry Valleys and to cope with extreme

desiccation. For this they are supposed to have the potential to survive some extra-terrestrial conditions.

Obviously, our knowledge of life and resistance of living microorganism on Earth will be essential for the new missions searching for past or present life on Mars, Europa, Enceladus and other planetary bodies.

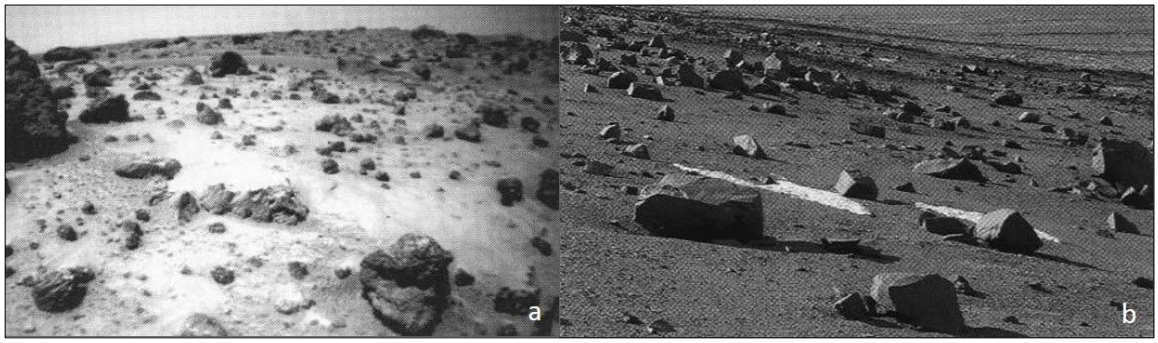

Figure 1.1 a) Mars Landscape **b)** Mc Murdo Dry Valleys landscape.

1.3 The black fungi of the Antarctic cryptoendolitic communities

As explained above, Earth is our only reference for studying the possibility of life on other planetary bodies: extreme terrestrial environments host specifically adapted organisms, among which anhydrobionts are generally considered the best models for exobiological studies (Finster et al., 2007; Stevenson et al., 2015). The extremely arid McMurdo Dry Valleys, therefore, represent a natural source of stress resistant microorganisms. In this hostile environment, fungi and cyanobacteria have adopted a strategy to escape most of the stress parameters by colonizing the inside of rocks (Friedmann, 1982). When conditions become too harsh and epilitic life is not possible on the surface, cracks, fissures and porosity within the rocks represent the main sites for colonization. Cryptoendolithism is one of the most spectacular adaptation of microbes to the environmental pressure and the predominant life-form in the inner part of continental Antarctica (Friedmann et al., 1993). The lichen dominated community is one of the most complex among the endolithic communities (Friedmann, 1982): these communities, showed in Figure 1.2, represent a borderline adaptation,but actually the last chance to survive in that area.

The cryptoendolithic lichen dominated communities include several prokaryotic and eukaryotic organisms that live at the limit of their biological potential (Onofri et al., 2004; Ruisi et al., 2007): black meristematic fungi are invariably present in these communities (Selbmann et al., 2005, 2008) and are one of the most impressive examples of adaptation (Gunde-Cimerman et al., 2005; Sterflinger, 2005).

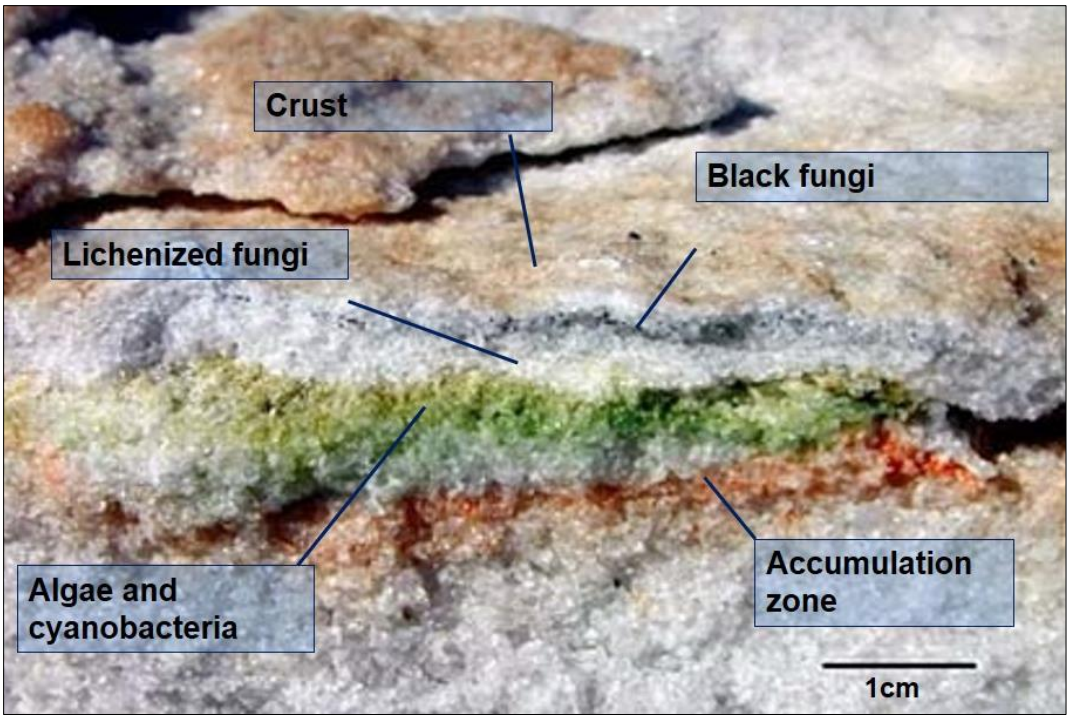

Figure 1.2 Cryptoendolithic lichen dominated community (Battleship Promontory, Convoy Range, McMurdo Dry Valleys, Antarctica).

For long time, Exobiology has focused on prokaryotic models (Horneck et al., 1994; Nicholson et al., 2000) because of their less complex organization, their earlier emergence, and putative higher resistance to stresses compare to eukaryotes. Nowadays, eukaryotes demostrated to be able to survive or even thrive in different extreme environments (Zettler et al., 2002), and are increasingly attracting interest for astrobiological studies. In particular, the black meristematic fungi have been suggested as the best eukaryotic model for exobiological speculations (Onofri et al., 2007b). Nevertheless, data on their tolerance to different stresses and about actual limits of surviving are scant or even missing.

Rock Inhabiting Fungi (RIF) are extremophilic or extremotollerant organisms sharing peculiar features allowing them to survive in oligotrophic environments characterized by

extremely high or low temperatures, high UV radiation and osmotic stress, combined together. They are usually melanized, often able to reproduce by unicellular growth, at least for a part of their life cycle and organized in microscopic colonies on the rocky substrate, so they are also called black yeasts or microcolonial fungi (MCF) (Staley et al., 1982; Sterflinger, 2005; Selbmann et al., 2014b); because of their ability to form cells' clumps with a peculiar isodiametric expansion, they are also called meristematic fungi (Sterflinger et al., 1999).

They are characterized by thick and melanized cells wall, which protect cells against extreme temperatures and desiccation, as well as UV irradiation. Melanins are high molecular weight pigments responsible of the characteristic dark, brown or black color of RIF, largely contributing to their resistance to chemical and physical stresses. Melanins are negatively charged idrophobic molecules, often aggregated to proteins and carbohydrates, formed by phenols and indolic compounds polymerization (Butler and Day, 1998). They can be DOPA-melanin (3,4-dihydroxyphenylalanine) or DHN-melanin (1,8-dihydroxynaphtalene) (Butler and Day, 1998). They are synthesized in the cell wall (Butler and Day, 1998) or released as extracellular polymers (Kogej et al., 2004). Some melanized fungal species have been found in nuclear reactors and their cooling water systems, suggesting that melanins could confer a remarkable tolerance to ionizing radiation (Zhdanova et al., 2000), even being responsible of ionizing gamma radiation's conversion into chemical energy by still unknown mechanisms (Dadachova et al., 2007).

Furthermore solutes such as trehalose and sucrose, which possess water-retention properties, have been widely detected in endolithic microorganisms, including RIF (Friedmann et al., 1993). In particular trehalose is very efficient for its cryoprotective effects during freezing or desiccation (Weinstein et al., 2000), acting as stabilizer of enzyme conformation and phospholipid bi-layers of membranes allowing these surprising organisms to survive complete dehydration (Onofri et al., 2012).

RIF have also very slow growth rate; meristematic growth (i.e. isodiametric cellular expansion) represents an additional advantage resulting in a minimal surface/volume ratio, which allows survival in dry conditions (Wollenzien et al., 1995). The ability to modify cellular polarity (Yoshida et al., 1996), a scarce morphological differentiation and the capacity to rely on air-borne sparse nutrients exclusively (oligotrophism) are crucial

features for extreme environments inhabitants (Gunde-Cimmerman et al., 2005; Zettler et al., 2002).

Regulation of metabolic activities is a strategy to balance energy expense according to changes in the composition of the atmosphere and climate. Sterflinger et al. (2012) suggests that in polar environments for large part of the year fungi incur dormancy, a reversible state that stops only when temperature rises and melting water is available. Rock inhabiting fungi are invariably asexual since the genetic machinery for recombination active implies too high energetic expense. Moreover, in order to decrease energy expense, the life-cycle in these fungi is extremely simplified, usually limited to just a few cells that subdivide and fall apart for passive dispersal.

A paradigmatic example of rock inhabiting fungi tolerance to environmental stresses is represented by the genus *Cryomyces*, isolated from cold Antarctic and Alpine rocks, among which the Antarctic endemic *C. antarcticus* Selbmann et al. (2005) is one of the most-stress-tolerant organisms known to date.

1.4 The black fungus *C. antarcticus*, an eukaryotic model for astrobiology

As reviewed by Selbmann et al. (2013, 2015), during the last years strains of *Cryomyces* spp. have been subjected to a number of experiments in order to test stress tolerance.

Figure 1.3 *Cryomyces antarcticus* at light microscope.

Antarctic strains, which have typical psychrophilic profiles (Van Uden, 1984) with optimal growth temperatures around 10 °C or at least 15 °C, are unable to grow above 20 °C. In the Antarctic deserts, during summer temperature fluctuactions on rock surfaces can be wide and sudden and cause a repeated freeze-thawing stress to lithobionts; during Austral winter organisms live in permanently frozen conditions. Antarctic black fungi may actually easily tolerate this stress: repeated treatment to – 20 + 20 °C did not affect growth ability (Onofri et al., 2007b; Onofri et al., 2008). Moreover, strains were proved to tolerate even very high temperatures, since germination ability of *Cryomyces* spp. is not affected after exposition at 90 °C for 1 hour (Onofri et al., 2008). In cold environments, resistance to osmotic stress represents an additional challenge, since water availability decreases during ice crystals formation. Moreover, rock fungi evolved specific adaptation to tolerate considerable high salt concentration. For instance, strains of *Cryomyces* spp. are still able to grow at NaCl concentration of 25% (Onofri et al., 2007b), demonstrating a remarkable tolerance to osmotic stresses. Moreover, using a proteomic approach, it was demonstrated that *C. antarcticus* did not not actively respond to stress temperature or even Mars simulated conditions; yet it just to down-regulates its metabolism, suggesting that both trehalose and mannitol might play a cell protective role in those fungi (Tesei et al., 2012; Zakharova et al., 2012). The ability to survive long-term desiccation makes these isolates pre-adapted to the extreme conditions of space, since high-vacuum conditions produce an extreme dehydrating effect.

Resistance to radiation has been largely reported in *Cryomyces* spp. *C. antarcticus* maintains its ability to germinate after high UV exposition (Onofri et al., 2007b) and even after space radiation (Onofri et al., 2012) by resisting, rather than repairing potential DNA damages (Selbmann et al., 2011). Therefore, *C. antarcticus* is able to withstand short-term, Mars-simulated ground-based exposition when actively growing (Zakharova et al., 2014) and long-term exposition (up to 1.5 years) when dehydrated (Onofri et al., 2015).

To summarize *C. antarcticus* (Figure 1.3) is able to resist extremes of temperatures, high salt concentration, UV radiations and even real space exposure and simulated Martian conditions (Onofri et al., 2007a, 2007b, 2008, 2012, 2015; Selbmann et al., 2011) and for these reasons it has been chosen as best eukaryotic model for the astrobiological experiments, including this study.

1.5 Astrobiological Projects

With the development of space technology, many experiments were performed to simulate the harsh conditions expected in space. The impossibility to completely reproduce on the ground the full-spectrum of solar irradiation or the combined effects of all the space constraints, namely vacuum, radiations and temperature cycles, necessarily implies to perform experiments in real space conditions.

As reviewed by Horneck et al., (2010) outer space is a harsh and inhospitable environment for terrestrial organisms due to lethal effects of vacuum, solar and galactic cosmic radiations and temperature extremes. The record of survival in space remains that of 6 years of *Bacillus subtilis* spores (Horneck et al., 2010). Ultimately, organisms from the three domains of life (Bacteria, Archaea and Eukaryota) have survived space exposure in either the BIOPAN or the more recent EXPOSE missions (Horneck et al., 2012; Onofri et al., 2012, 2015; Tepfer et al., 2012; Brandt et al., 2014).

Among the last astrobiological projects, BIOMEX and STARLIFE, two experiments of real space exposure and simulated environments experiments, have been performed.

1.5.1 BIOlogy and Mars EXperiment Project

BIOlogy and Mars Experiment (BIOMEX) is one of the four space experiments on the exposure facility EXPOSE-R2 onboard the ISS, using ground based facilities for reference studies. The experiment focuses on desiccation-tolerant organisms, including halophyles, bacteria and cyanobacteria, fungi, and their cellular components, such as pigments, membranes and proteins. The aim of the project is to get new insights about stability and degradation of the exposed extremophiles and their constituents, grown on terrestrial, lunar and Martian analogue mineral substrates. Investigating the degradation of microorganisms and their metabolites, which may be induced by the space environment (radiation, vacuum and Mars atmosphere), BIOMEX will provide an efficient characterization and list of organic compounds (e.g., amino acids, nucleobases, lipids) for searching of extant or extinct life. These molecules are essential for life on Earth and are also prime targets in the search for life beyond Earth, with a special focus on Mars (de Vera et al., 2012). Biosignatures degrade over time; *in situ* environmental conditions influence the preservation of those molecules. Nonetheless, upon shielding (e.g., by mineral surfaces),

particular biosignatures can persist for billions of years, making them of vital importance in answering questions about the origins and limits of life on early Earth, Mars or other habitable worlds.

Hence, the choice of targets to detect life (namely biosignatures) and the understanding of their degradation under different extraterrestrial conditions is a key feature for future missions to recognize life when encountered (Gómez and Parro, 2012). Methods and analytical tools in the field of life science are continuously improving; in particular, amplification methods are very useful for the detection of low concentrations of genomic material but most other organic molecules are not prone to amplification methods (Aerts et al., 2014). Putative biosignatures have been identified by Raman spectroscopy in photoprotective and antioxidant molecules, UV screening compounds in rock-inhabiting communities from hot and cold deserts which are perceived as critically important for the forthcoming ExoMars mission for the robotic search of life on Mars (Jorge-Villar and Edwards, 2013; Vítek et al., 2010).

Furthermore, BIOMEX samples, selected microbes intermixed with Martian and lunar mineral analogues, will be investigated to both have insights of potential effects of substrates on microbes and to consider life endurance in the contest of the Litho-Panspermia theory (Arrhenius, 1908; Martins et al., 2008). This theory based on the idea of the possibility of an interplanetary travel of microbe inside meteorites.

Taking advantage of the combined harsh conditions present in LEO (UV and ionizing radiations, temperature extremes, space vacuum and microgravity), in the EXPOSE-R2 space mission selected organisms have been exposed for 1.5 years not only to space conditions, but also to a simulated Mars-like environment (CO_2 atmosphere and UV > 200 nm). The space mission was successfully launched to the ISS on July 24th, 2014 on board the space cargo Progress 56P, whereas on August 18th, was installed outside the ISS on the Russian Svezda module (Figure 1.4). The experiment ended in June 2016.

A set of microorganisms, representing species of all three main branches of the tree of life were exposed, including microorganisms which are known to be relevant for Mars (e.g. methane producing archaea, cyanobacteria and iron bacteria) and to corroborate the Litho-Panspermia theory (Figure 1.4, de Vera et al., 2012).

Figure 1.4 Top: EXPOSE-R2 launch onboard the space cargo Progress 56P from the Baikonur Cosmodrome, Kazakhstan (credit Roscosmos). Bottom: Retrieve EXPOSE-R experiment from the Universal Work Platform on the Russian Zvezda module (credit NASA).

Among the microorganisms, BIOMEX included the Antarctic cryptoendolithic black fungus *C. antarcticus*, as eukaryotic model organism for investigating astrobiological topics. In the preparation of EXPOSE-R2 space mission, selected space and Martian simulations (EVTs for Experiment Verification Tests and SVT for Science Verification Tests) were scheduled to test whether the selected samples could withstand real exposure. Colonies of *C. antarcticus* were grown on substrates including Lunar, P-MRS, S-MRS analogues, or sandstone, which is the Original Substrate (OS). The results on EVTs and SVT tests were analyzed in this thesis (Chapters 2 and 3).

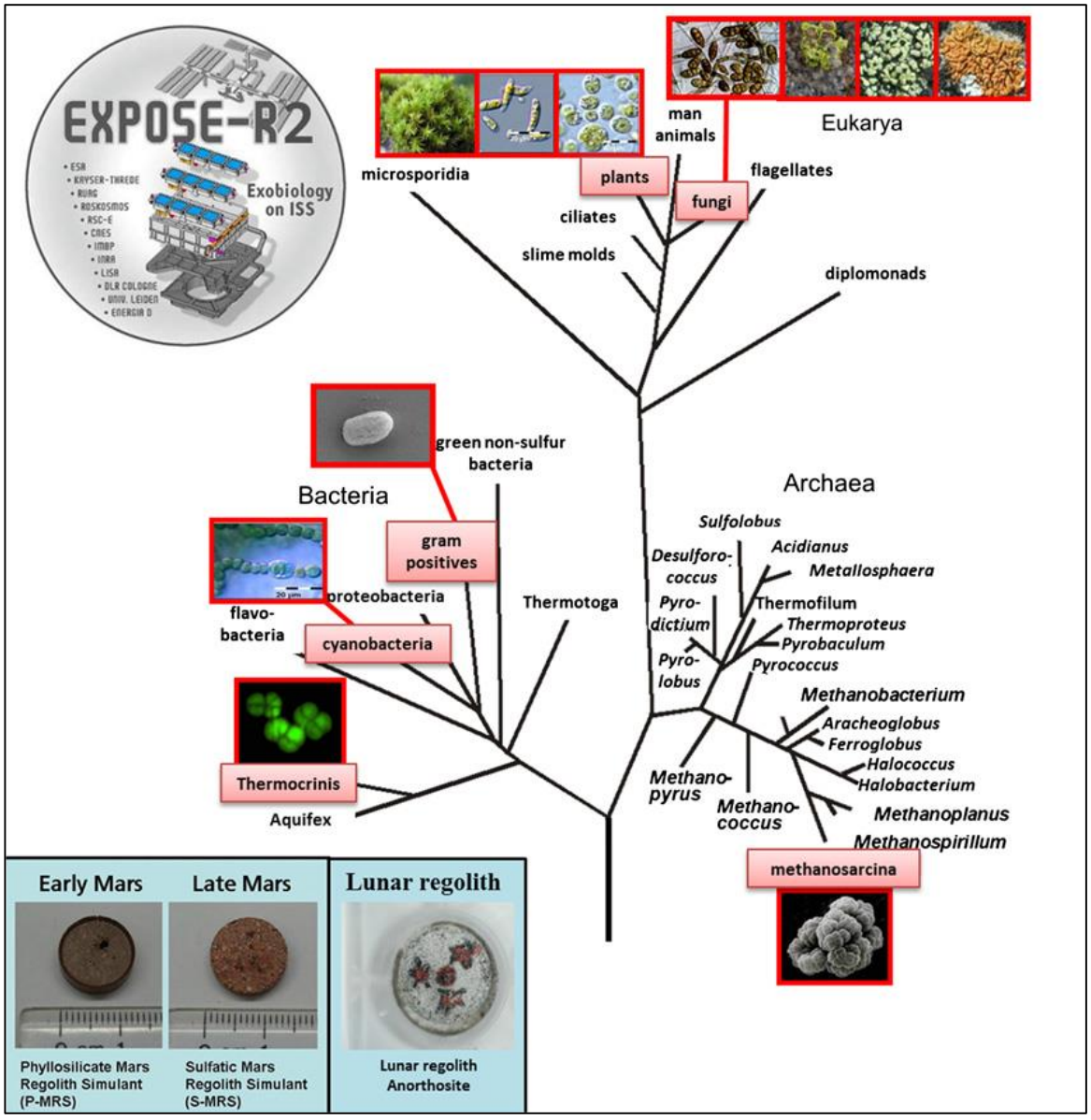

Figure 1.5 The rDNA analysis-based phylogenetic tree of terrestrial life including the selected organisms (highlighted in the tree) for the BIOMEX space experiments onboard the ISS. Bottom left: mineral pellets selected for BIOMEX (de Vera et al., 2012).

1.5.2 STARLIFE Project

Among other space parameters as extreme vacuum, desiccation and strong thermal contrasts (Nicholson et al., 2000), exposure to space environment includes numerous types of ionizing radiation as high-energy photons and particles of different masses, charges, and energies (Moeller et al., 2010; Dartnell, 2011).

The complex space radiation has been considered one of the main hazardous components of space environment for any biological system to survive long periods in space (Ferrari and Szuszkiewicz, 2009); yet, a deeper understanding of the biological effects of this parameter is required for assessing the radiation risks in space. This pertains to astronauts, the cabin microflora and accompanying bioregenerative life support systems during long-term exploratory missions (Badhwar and O'Neill, 1994; Cucinotta, 2015) as well as to any microorganism accidentally traveling through space after being ejected from its planet's surface by a meteorite impact, as described in the scenario of Litho-panspermia.

To address these questions, a set of different astrobiological model systems have been studied within the STARLIFE radiation campaigns (Moeller et al., *in press*). The STARLIFE group aims to investigate the responses of different astrobiological model systems to the different types of ionizing radiation (x-rays, gamma-rays, heavy ions), which represent major parts of galactic cosmic radiation spectrum. Low and high-energy charged particles radiation experiments have been performed at the HIMAC facility (Heavy Ion Medical Accelerator) at the National Institute of Radiological Sciences (NIRS) in Chiba, Japan. X- or γ-rays were used as reference radiation at DLR (German Aerospace Center, Cologne, Germany).

Exposure to this particulate radiation in space causes a wide range of different types of genomic lesions, e.g., single and double strand breaks, abasic sites, modified (mainly oxidized) bases or interstrand crosslinks (Asaithamby and Chen, 2011; Friedberg, 2003; Yokoya et al., 2008) with consequences of gene mutations, chromosome exchanges, cancer induction and cell death (Cacao et al., 2016).

Various biological endpoints have been investigated with a combination of different biochemical and molecular biological methods, e.g., colony formation assays, most probable number, vitality staining, different microscopic analysis, RAMAN spectroscopy, PCR/RT-PCR, metabolism, and key biosignatures stability. They allowed gaining a clearer understanding and broader spectrum of the effects of galactic cosmic rays on astrobiological model systems.

In the frame of STARLIFE, three Antarctic endolithic microorganisms, namely *C. antarcticus, Umbilicaria* sp. mycelium and *Stichococcus* sp., were exposed to high doses of γ-rays; findings will give insights to define the limit of life under radiation environment.

1.6 Aim of the thesis and chapters description

The present thesis aims to describe the responses of the Antarctic meristematic black fungus *Cryomyces antarcticus* CCFEE 515 to stresses, in the context of astrobiological studies; focus was on resistance to the ground-based simulations, both in the preparation of the BIOMEX LEO experiments, STARLIFE project and to 1.5 years of real space exposure outside the ISS, under anydrobiotic condition. The role of melanin pigments in the protection of the fungus, comparing melanized and non-melanized strain of *C. antarcticus* under physiological condition, were investigated too.

The first chapter is an introduction to the astrobiology research, focusing on the importance of extreme environments on Earth as references for the space environment, especially the McMurdo Dry Valleys, in Antarctica, which is the closest terrestrial analogue to Mars. The introduction continues with the description of the cryptoendolitic communities, which live inside the rocks, and of *C. antarcticus,* one of the most resistant eukaryote know to date. The fungus, being perfect eukaryotic astrobiological model, after the high survival reported after the LIFE project, was investigated in BIOMEX and STARLIFE projects, which are deeply described at the end of the introduction.

The following chapters of this thesis describe our understanding on the survival of the fungus *C. antarcticus* after the ground-based simulations performed in the preparation for the ISS exposure of BIOMEX experiment. The ground-based simulations (second and third chapters) were carried out in the framework of Experiment Verification Tests (EVTs) and Science Verification Test (SVT), respectively, and performed using the Planetary and Space Simulation facilities of the Institute of Aerospace Medicine (German Aerospace Center, DLR, Köln, Germany).

In chapter 2, the BIOMEX experiment is described with the first results obtained during the EVTs on survival, DNA and ultrastructural damage of *C. antarcticus* grown of Martian and Lunar regulites, to selected space and Martian simulated conditions. The main techniques used in the following chapters are also presented as a proof of concept in this chapter. Chapter 3 deals with the second ground test, the SVT, looking at the survivability of the fungus for the real space mission. XTT assay has been optimized for the black fungus, to evaluate the fungal metabolic activity too. This chapter focuses also on the suitability of melanin as fungal biosignature, using the RAMAN spectroscopy; limits and

limitation of this technique on our model is put forward. Results shown in chapter 4 are achieved in the contest of STARLIFE project, a simulation experiment which includes numerous types of ionizing radiation as high-energy photons and γ-radiation. In this contest we have exposed three different Antarctic microorganisms to very high level of space relevant ionizing radiation (^{60}Co) to evaluate the microbes survival and the persistence of DNA, as biosignature. Again, *C. antarcticus* has been showed as the most resistant among our test microorganisms, both in term of survival and DNA resistance. In the fifth chapter, the protective role of fungal melanin is investigated after exposure to space-relevant densely and sparsely ionizing radiation, by comparing melanized and non-melanized strains of *C. antarcticus*, under physiological condition. Survival was analyzed by plating CFU's, the metabolic activity both by XTT and MTT assays, while the ATP content in the cells was analyzed by the ATP assay.

General conclusions and synthesis are presented in chapter 6, supplementary data and preliminary results on samples exposed to real space and simulated Martian atmosphere in space for 1.5 years are reported in the Appendixes.

Chapter 2

2 BIOMEX experiment: Ultrastructural alterations, molecular damage and survival of the fungus *Cryomyces antarcticus* after the Experiment Verification Tests

Abstract

The search for traces of extinct or extant life in extraterrestrial environments is one of the main goals for astrobiologists; due to their ability to withstand stress producing conditions, extremophiles are perfect candidates for astrobiological studies. The BIOMEX project aims to test the ability of biomolecules and cell components to preserve their stability under space and Mars-like conditions, while at the same time investigating the survival capability of microorganisms. The experiment has been launched into space and is being exposed on the EXPOSE-R2 payload, outside of the International Space Station (ISS) over a time-span of 1.5 years. Along with a number of other extremophilic microorganisms, the Antarctic cryptoendolithic black fungus *Cryomyces antarcticus* CCFEE 515 has been included in the experiment. Before launch, dried colonies grown on Lunar and Martian regolith analogues were exposed to vacuum, irradiation and temperature cycles in ground based experiments (EVT1 and EVT2). Cultural and molecular tests revealed that the fungus survived on rock analogues under space and simulated Martian conditions, showing only slight ultra-structural and molecular damage.

Keywords: BIOMEX, cryptoendolithic black fungus, DNA damage, Mars, space simulations, survival.

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2.1 Introduction

The question whether extraterrestrial life exists has always intrigued scientists. Extremophilic and extreme-tolerant microorganisms have, as their natural niches, environments previously thought to be incompatible with active life; for this reason they are perfect models for studying the limits of habitability on Earth. The McMurdo Dry Valleys of Antarctica, Arctic regions, permafrost soils and cold deserts, for instance, are considered to be good analogues of Mars environments due to their permanently cold and dry conditions (Hansen et al., 2007). Microbes living there are pushed to the absolute limits of adaptability and represent a perfect tool for astrobiological research (Finster et al., 2007). The resistance of these terrestrial extremophiles under both space simulation and LEO (Low Earth Orbit) has been documented (Horneck et al., 2010). Both ground based and space experiments on the International Space Station (ISS), i.e. the LIFE experiment on Expose-E (Rabbow et al., 2012, 2014), showed that some organisms, such as spores of bacteria, meristematic black fungi, and lichens, are able to survive and reactivate their metabolism after space simulations or direct exposure to space (Demets et al., 2005; Horneck et al., 1994; Olsson-Francis et al., 2009; Onofri et al., 2008, 2012; Raggio et al., 2011; Sancho et al., 2007, 2008) and even simulated Martian conditions (Baqué et al., 2013; Meeßen et al., 2013a; Moeller et al., 2012a; Sánchez et al., 2012).

This work is a part of BIOMEX, whose main goal is to detect signatures of extinct or extant life on Mars, investigating the fate of selected extreme-tolerant organisms and the stability of associated biomolecules, after exposure to actual space and simulated Mars conditions. Investigations will be based on sensitive and non-destructive approaches such as Infrared (Igisu et al., 2006, 2009) and Raman spectroscopies (Böttger et al., 2012, 2013; de Vera et al., 2012), using for comparison an international Raman library whose construction is in progress.

Another main objective of BIOMEX is to test survival in extra-terrestrial conditions of selected extreme-tolerant/extremophilic lithobionts, such as bacteria, meristematic black fungi and lichens grown on Mars and Lunar regolith analogues.

Among the selected organisms, the cryptoendolithic black fungus *Cryomyces antarcticus* CCFEE 515, from the McMurdo Dry Valleys in Antarctica, is an excellent eukaryotic model due to its exceptional stress resistance and ability to grow inside the rock. Its survival

in dried conditions after 18 months of exposure to actual space outside of the ISS, as well as to simulated Mars conditions in space, which was recently demonstrated, gave new insights to the Litho-Panspermia theory (transfer of life between neighbor planets within a meteorite) (Onofri et al., 2012).

On July 24th 2014 the EXPOSE-R2 facility (Fig. 2.1): was launched onboard a Russian Progress cargo spacecraft (Fig. 2.2) from the Baikonur Cosmodrome, Kazakhstan to the ISS and mounted outside the ISS Zvezda module.

Fig. 2.1 BIOMEX launch on July 24th 2014.

The EXPOSE-R2 facility carried BIOMEX, along with the Biofilm Organisms Surfing Space (BOSS), Photochemistry on the Space Station (PSS) and an experiment from the Russian Institute of Biomedical Problems (IBMP).

This work focuses on the preparatory ground-based EVTs (Experiment Verification Tests) which include space and Martian simulations, performed on *C. antarcticus* in support of the actual space exposure.

Results give clues in searching for life in future Mars exploration missions (de Vera et al., 2012) in detecting putative biosignatures.

2.2 Material and methods

2.2.1 Fungal strain preparation

Cryomyces antarcticus CCFEE 515 was isolated by R. Ocampo-Friedmann from sandstone collected at Linnaeus Terrace (Southern Victoria Land) by H. Vishniac, in the Antarctic expedition 1980-81.

For the EVT tests, cell suspensions were spread on MEA (malt extract agar: malt extract, powdered 30 g/L; peptone 5 g/L; agar 15 g/L; Applichem, GmbH) in Petri dishes, mixed with Antarctic sandstone (15 g/L), Lunar and Martian analogues (1 g/L), prepared to optimize mineral/microorganisms interactions. Sandstone was the original substrate (OS) for the test fungus, Lunar analogue (L) was constituted mainly of anorthosite (Mytrokhyn et al., 2003) and two specific Martian analogues were composed of sulfatic Mars regolith (S-MRS) and phyllosilicatic Mars Regolith (P-MRS), simulating late basic Mars and early acidic Mars surface lithosphere composition, respectively (Böttger et al., 2012). Mars analogue composition was developed and produced by the Naturkundemuseum Berlin, according to the data of Mars research missions (Bibring et al., 2005; Chevrier and Mathé et al., 2007; Poulet et al., 2005). Colonies were grown at 15 °C for 3 months. Disks, cut to fit within the wells of exposure carrier (12 mm diameter) (Fig. 2.2b), were drilled under sterile conditions.

Untreated samples, prepared as above and stored in the dark at room temperature, were used as controls in all the tests performed.

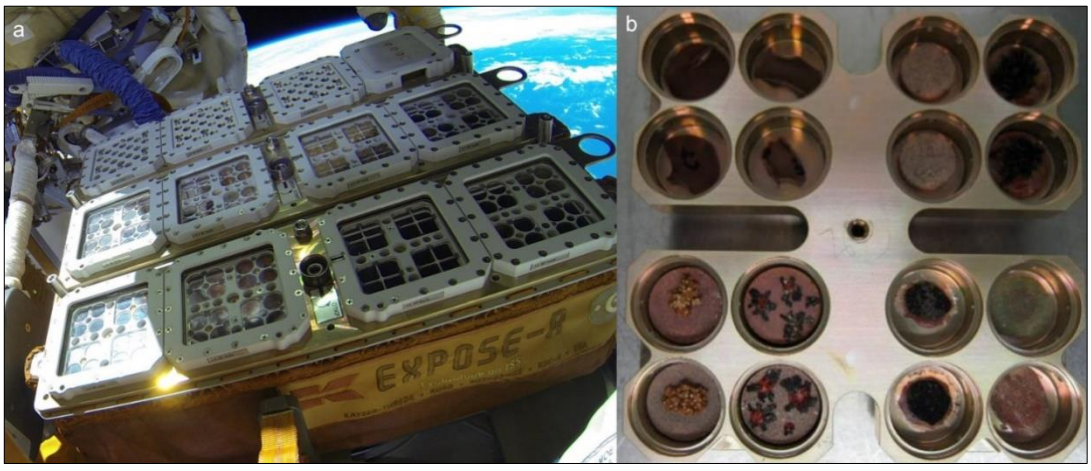

Fig. 2.2 a) EXPOSE R2 Facility mounted outside the ISS from October 22th 2014; **b)** Microorganisms integrated in the sample carriers.

2.2.2 Tests facilities and exposure conditions

Ground-based simulations (EVTs) were performed using the Planetary and Space Simulation facilities (PSI) at the Institute of Aerospace Medicine (German Aerospace Center, DLR, Köln, Germany). Tests were performed in triplicate and exposure conditions were as reported in Table 2.1.

EXPOSE-R2 EVT part 1 exposure experiments	
Test Parameters	**Performed**
Vacuum Vacuum	1 h, pressure 3.86 x 10^{-3} ± 0.12 Pa 7 h, pressure 8.50 x 10^{-5} ± 0.12 Pa
Mars atmosphere Mars atmosphere	1 h, pressure 6.08 x 10^{2} ± 0.12 Pa 7d, pressure 6.00 x 10^{2} ± 0.12 Pa
Temperature min and max: –10 °C to +45 °C	66 cycles 2 h –10 °C ± 1 °C; 2 h +45 °C
Temperature min and max: –25 °C / +60 °C	1 h –25 °C ± 0.5 °C; 1 h +60 °C ± 0.5°C
Irradiation UVC (254 nm) irradiation with Hg low pressure lamp at 80 mW/cm^2	0 J/m^2 12 s, 9.6 J/m^2 2 min, 5 s, 96 J/m^2 20 min, 50 s, 1000 J/m^2 208 min, 20 s 10,000 J/m^2
EXPOSE-R2 EVT part 2 exposure experiments	
Test Parameters	**Performed**
Irradiation Polychromatic UV irradiation (200-400 nm) with SOL2000 at 1271 Wm-2	28 d dark 0 kJ/m 5,5 x 10^{2} kJ/m (7min 12 sec) (0,1%ND) 5,5 x 10^{3} kJ/m (1 h 12 min) (1%ND) (1 wd) 1,4 x 10^{5} kJ/m (1 h 12 min) (1%ND) (1 wd) 2.7 x 10^{5} kJ/m (30 h)(4 wd)(60 h)(9 wd) 5,5 x 10^{5} kJ/m (120 h) (18 wd @ 7 h /wd)

Table 2.1 Exposure conditions during the Experiment Verification Tests (EVTs).

2.2.3 Survival Tests

Cultivation test

Survival of *C. antarcticus* was determined by its colony forming ability as percentages of CFU (Colony Forming Units). For the test, three of the treated colonies was suspended in 1 mL of physiological solution (NaCl 0.9%), and diluted to a final concentration of 3,000 cells/mL, 0.1 mL of the suspension was spread on Petri dishes supplemented with MEA (5 replicates), incubated at 15 °C for 3 months and counted.

PMA assay

The test was performed by adding the Propidium MonoAzide (PMA, Biotium, Hayward, CA) at a final concentration of 200 μM to 1-2 re-hydrated fungal colonies. PMA penetrates only damaged membrane cells, crosslinks to DNA after light exposure and thereby prevents Polymerase Chain Reaction (PCR).

Following DNA extraction and purification, quantitative PCR (Biorad CFX96 real time PCR detection system) was used to quantify the number of fungal Internal Transcribed Spacer (ITS) ribosomal DNA fragments present in both PMA treated and non-treated samples. Five μL of purified genomic DNA (0.1 ng/ml) was added to 12 μL of PCR cocktail containing 1X Power Sybr-Green PCR Master Mix (Applied Bios, Foster City, CA), as well as NS91 forward (5‘-gtc cct gcc ctt tgt aca cac-3‘) and ITS51 reverse (5‘-acc ttg tta cga ctt tta ctt cct c-3‘) primers, each at 5 pmol final concentration. Sterile water was added to reach the final volume of 25 μL. These primers amplify a 203 bp product spanning the 18S/ITS1 region of rRNA encoding genes.

A standard Q-PCR cycling protocol, consisting of a denaturation steps at 95°C for 2 min, followed by 35 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s, was performed. Fluorescence measurements were recorded at the end of each annealing step. After forty cycles, a melt curve analysis was performed by recording changes in fluorescence as a function of raising the temperature from 60-90 °C in 0.5 °C per increments. All tests were performed in triplicate.

Statistical analyses

For multiple data points, the calculation of the mean and standard deviations were performed. Statistical analyses were performed by one-way analysis of variance (Anova)

and pair wise multiple comparison procedure (Tukey test), carried out using the statistical software SigmaStat 2.0 (Jandel, USA).

2.2.4 DNA extraction and PCR analyses

DNA was extracted from rehydrated colonies, using Nucleospin Plant kit (Macherey-Nagel, Düren, Germany) following the protocol optimized for fungi.

ITS and LSU amplification were performed using BioMix (BioLine GmbH, Luckenwalde, Germany) adding 5 pmol of each primer and 20 ng of template DNA at final volume of 25 μL. The amplification was carried out using MyCycler Thermal Cycler (Bio-Rad Laboratories GmbH, Munich, Germany) equipped with a heated lid.

The rDNA regions were amplified as follows: for the ITS region the first denaturation step at 95 °C for 2 min was followed by denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s and for the LSU region the first denaturation step at 95 °C for 3 min was followed by denaturation at 95 °C for 45 s, annealing at 52 °C for 30 s, extension at 72 °C for 3 min. The last three steps were repeated 35 times, with a last extension 72 °C for 5 min for ITS and 7 min for LSU. Primers ITS5, ITS4 (White et al. 1990), LR5 and LR7 (Vilgalys and Hester 1990) were employed to amplify ITS and LSU rDNA portions, respectively.

2.2.5 Random amplification of polymorphic DNA assay

RAPD was performed using BioMix (BioLine GmbH, Luckenwalde, Germany) adding 5 pmol of the primer and 1 ng of template DNA at final volume of 25 μL. The primer used for RAPD was GGA_7 (GGA GGA GGA GGA GGA GGA GGA) (Kong et al., 2000). The conditions for amplification were: first denaturation step at 94 °C for 2 min followed by denaturation at 94 °C for 20 s, annealing at 49 °C for 60 s, extension at 72 °C for 20 s. The last three steps were repeated 40 times, with a last extension 72 °C for 6 min.

2.2.6 Transmission Electron Microscopy

Controls and UV-irradiated colonies were treated with 5% glutaraldehyde/cacodylate sucrose buffer 0.1 M (pH 7.2) for 12 h at 4 °C, washed three times in the same buffer for 1 h each at 4 °C and fixed with 1% OsO_4 + 0.15% ruthenium red in 0.1 M cacodylate buffer

(pH 7.2) for 3 h at 4 °C. Samples were washed in distilled water (2 times for 30 min at 4 °C), treated with 1% uranyl acetate in distilled water for 1 h at 4 °C and washed in distilled water (2 times, 30 min at 4 °C). Samples were then dehydrated in ethanol solutions: 30%, 50%, 70% (15 min each, at room temperature) and 100% EtOH (1 h at room temperature), critical point dried and infiltrated in ethanol 100%: LR White series with accelerator, in rotator, at 4 °C (2:1 for 3 h; 1:1 for 3 h, 1:2 overnight) and embedded in pure resin for 1 day and overnight; as final step, samples were included in pure resin in gelatinous capsule for 2 days at 48–52 °C.

2.3 Results

2.3.1 Cultivation test

C. antarcticus retained the colony-forming ability after both EVT1 and EVT2 treatments. In EVT1 (Fig. 2.3) the fungus showed a similar trend of survival on all the substrates tested: in general, survival decreased with increased UV-irradiation doses. Yet, colonies formed even at the highest dose, 10.000 J/m², where percentage of survival was 5%, 18%, 38% and 46% in Lunar, Original Substrate, P-MRS and S-MRS analogues, respectively. The fungus, grown on Martian analogues, was able to survive exposure to the Martian atmosphere with no significant decrement on S-MRS with respect the control and almost 60 % of survival on P-MRS. Surprisingly, lower vitality was observed, for all substrates tested, at -25 °C than at 60 °C.

In the EVT2 treatments (Fig. 2.4), a progressive increase of mortality was observed with increasing of UV-irradiation doses, but 48%, 38% and 72% of germination was recorded even at highest doses for colonies grown on Original substrate, S-MRS and P-MRS, respectively. Colony-forming ability was maintained in most cases after EVT2 treatments; the only exceptions was the highest irradiation in Lunar sample.

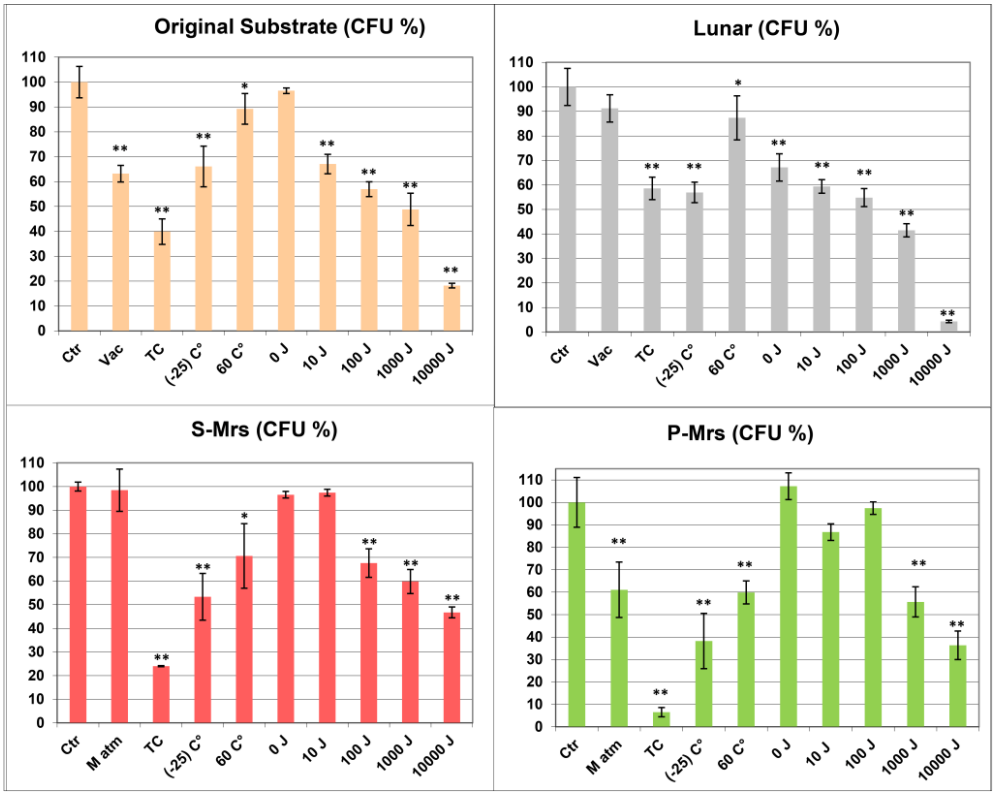

Fig. 2.3 Cultural test after EVT1 treatments: Percentages of CFU's of *C. antarcticus* on different substrates, relative to controls on the same substrate.Control (Ctr), vacuum 7h (Vac), Martian atmosphere 7g (M atm), temperature cycles (TC), minimum temperature (-25 °C), maximum temperature (+60 °C), irradiation at different intensities (0, 10, 100, 1000 and 10,000 J). Significant differences were calculated by Tukey test with * = $p > 0.05$. and ** = $p > 0.001$.

Vitality, specifically, the integrity of the plasma membrane, was also tested through PMA assay on control and EVT2 samples treated with medium and maximum irradiation doses for each substrate (Fig. 2.5). This test gave a higher percentage of possible survival (measured as cells with intact membrane) compared to the results from the cultural test, even at the highest dose; for instance, no colonies were recorded under Lunar conditions but 35% was observed with PMA assay. No significant differences between treated samples and control were obtained on P-MRS.

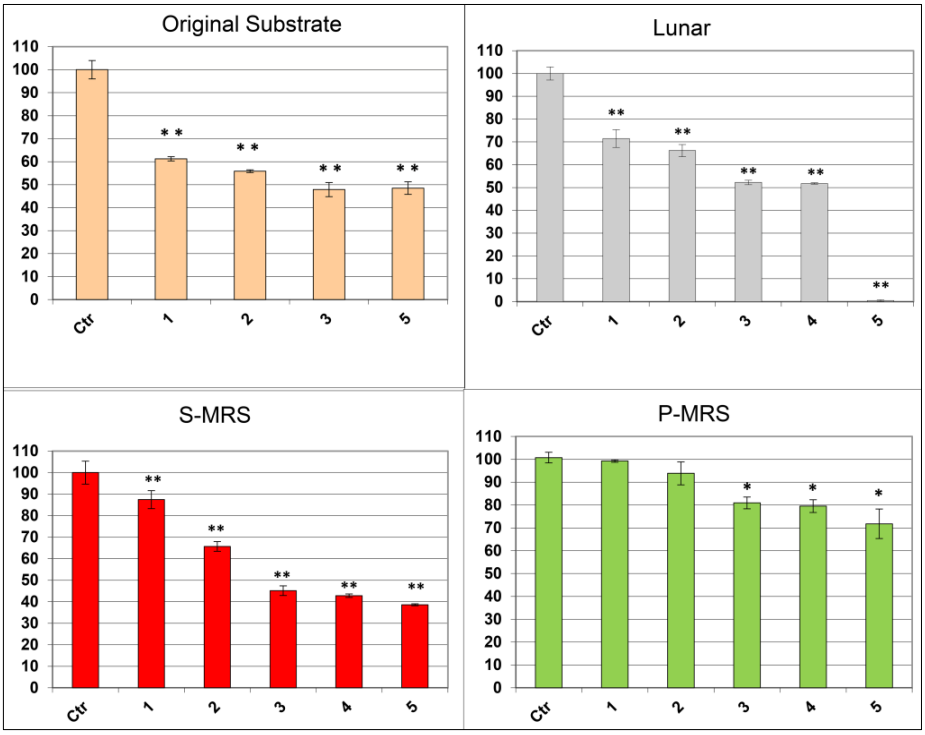

Fig. 2.4 Cultural test after EVT2 treatments of *C. antarcticus* grown on different substrates: CFU's following exposure to UV light relative to controls on the same substrate. Control (Ctr), increasing polychromatic UV irradiation doses **1**: $1.5x10^3$, **2**: $1.5x10^4$, **3**: $1.5x10^5$, **4**: $5.0x10^5$, **5**: $8.0x10^5$kJ/m^2. The statistical analyses were performed as Figure 2.3.

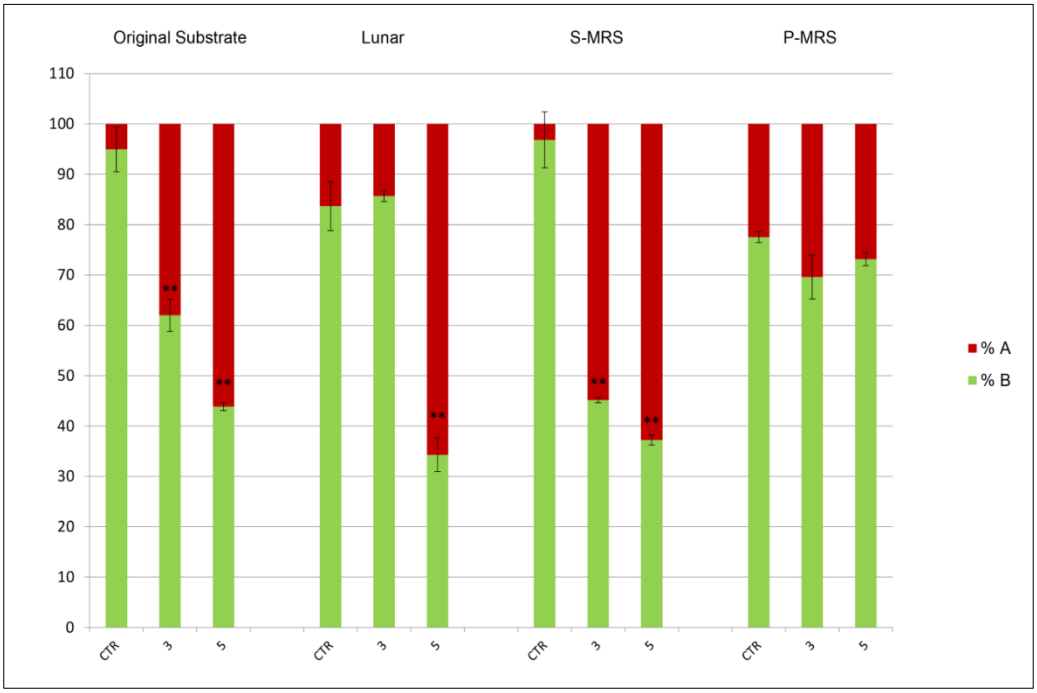

Fig. 2.5 Results of PMA assay coupled with qPCR after EVT2 treatments in a selection of samples (Samples correspond with those in Fig. 4): **A**: Percentages of *C. antarcticus* cells with damaged membrane **B**: Percentages of *C. antarcticus* cells with intact membrane. The statistical analyses were performed as in Figure 2.3.

2.3.2 DNA damage

The integrity of genomic DNA in treated samples was tested by assessing its ability to serve as a PCR template both after EVT1 and EVT2 treatments.

All the EVT2 samples were analyzed, while for EVT1, on the basis of colony forming results, only samples exposed at maximum treatments were chosen: Temperature Cycles, Vacuum 7h, Martian atmosphere 7d, maximum dose of UV (254 nm) irradiation at 10^4J/m². Amplifications worked out for all the gene-lengths in EVT1 (Fig. 2.6a, b, c) and EVT2 (Fig. 2.7a, b, c) exposed samples. A reduced intensity of PCR bands was evident in panel c, with the largest gene length, in Lunar EVT1 samples exposed to 10,000 J/m^2 (Fig. 2.6c, Lane L 10^4) and for colonies grown on S-MRS at the highest dose of UV (>200 nm) irradiation in EVT2. Although there was an overall decrease in band intensity, mainly for the highest molecular weight (MW) bands (about 2200 bp) the RAPD profiles were well preserved in all samples both after EVT1 and EVT2 treatments (Fig. 2.6d, 2.7d), demonstrating a good preservation of the whole genomic DNA.

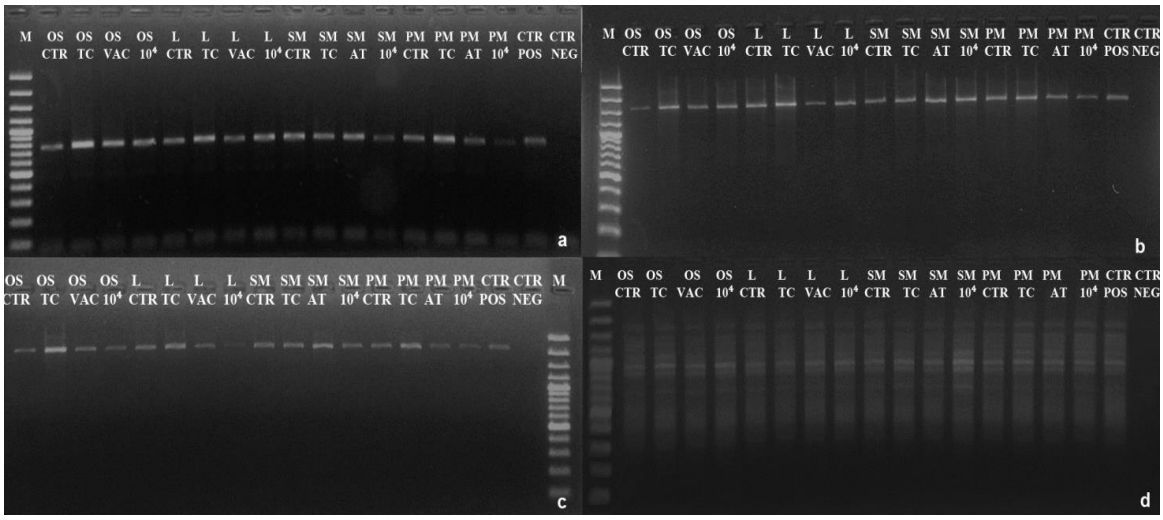

Fig. 2.6 Increasing detrimental effect of EVT1 treatments on the DNA integrity of genes of different lengths **a)** ITS rRNA gene (ITS5-ITS4 primers)(700bp) **b)** LSU rRNA gene (ITS5-LR5 primers) (1600bp). **c)** LSU rRNA gene (ITS5-LR7 primers) (2000 bp). **d)** RAPD profile of *C. antarcticus.* Treatments were as follows: Control, Thermal Cycles (TC), Vacuum (VAC) and 10^4 J/m², Positive PCR Control (CTRL POS), Negative PCR Control (CTRL NEG), DNA ladder (M). Substrates: OS, Original Substrate; L, Lunar; SM, S-MRS; PM, P-MRS.

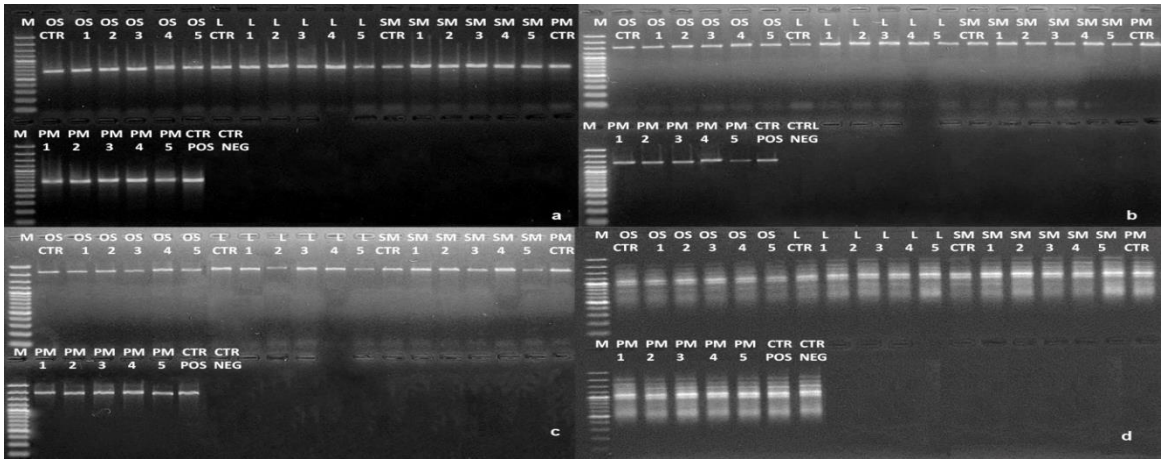

Fig. 2.7 Increasing detrimental effect of EVT2 treatments on the DNA integrity on genes of different lengths **a)** ITS rRNA gene (ITS5-ITS4 primers) (700bp). **b)** LSU rRNA gene (ITS5-LR5 primers) (1600bp). **c)** LSU rRNA gene (ITS5-LR7 primers) (2000 bp). **d)** RAPD profile of *C. antarcticus* after EVT2 treatments. Order as follows: DNA ladder (M), irradiation (sample correspondence as for Fig. 2.3), Control (CTR). Substrates: OS, Original Substrate; L, Lunar; SM, S-MRS; PM, P-MRS.

2.3.3 Ultrastructural Damage

Ultrastructural damage in *C. antarcticus* cells was observed by TEM. The samples treated at the highest irradiation dose of EVT2, for which the effect on vitality and DNA damage were evident (Fig. 2.8) were compared with laboratory controls

Control cells maintained mostly an intact cell membrane and a well-organized and defined cytoplasm after dehydration (Figs. 2.8a, c, e, g, i). By contrast, the majority of cells in the irradiated samples showed extended damage with irregular shapes, damaged cell walls, discontinuous cell membranes and a compromised organization of the cytoplasm (fig. 2.8b, d, f, h, j).

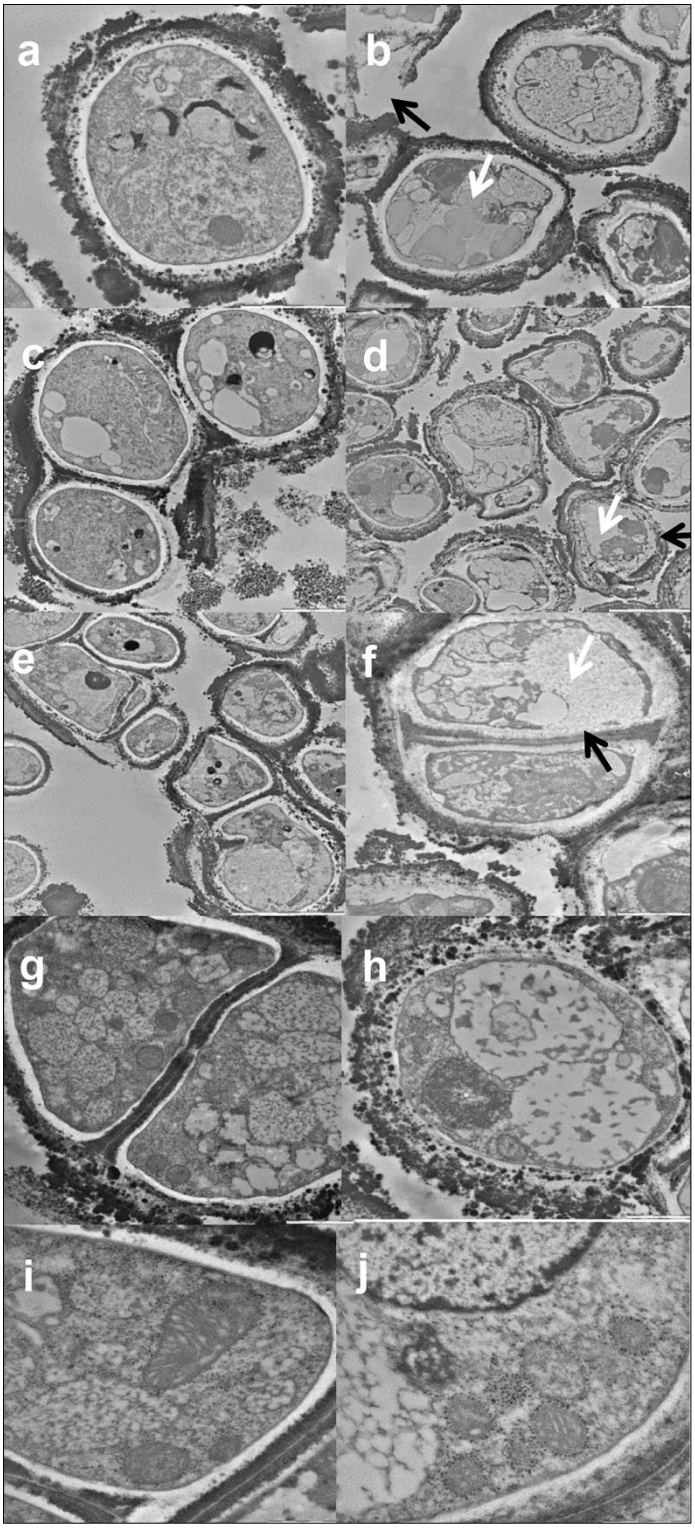

Fig. 2.8 TEM micrographs. Untreated (CTR) and treated (maximum irradiation of EVT2, $8.0x10^5$ kJ/m^2) microcolonies of *C. antarcticus* on sandstone (OS) (**a, b**); Lunar (L) (**c, d**); S-MRS (**e, f**); P-MRS CTR (**g, i**) Unirradiated (a,c,e,g,i) and irradiated (b,d,f,h,j) respectively. The structure of the cytoplasm was not conserved in many cases in the irradiated samples, the organelles are not visible (b, d, f, white arrows) and the continuity of the cell membrane (f, black arrow) and of the cell wall (b, d, black arrow) interrupted. The irradiated cells were better preserved in colonies cultivated on P-MRS where the structure of mithocondria were still well discernable (j, white arrow).

2.4 Discussion

Ground-based simulations, EVT1 and EVT2, performed in the frame of the BIOMEX experiment currently on-board the EXPOSE-R2 platform fixed outside the ISS, were preliminary experiments to validate samples for the space mission. One of the aims of the project was to study dried cells of *C. antarcticus,* grown on lunar regolith analogue rocks and two Mars regolith analogue mixtures, to test survival as well as DNA and ultra-structural damage.

Although a reduced survival was observed after exposure to the most stressing parameters, *C. antarcticus* retained some colony forming ability after UV irradiation as well as after simulated treatment with other outer space stressors. These results were confirmed by TEM observations showing that, in addition to cells showing ultrastructural damage after the highest irradiation dose, a number of cells were still in good condition (not shown), which agrees with the survival rate recorded in the colony forming assays. The main damaging factors of the EVTs were UV irradiation and temperature cycles. *C. antarcticus* survival was higher after +60 ºC than after -25 ºC treatment. The surprising ability of this psychrophilic fungus to tolerate very high temperatures was previously observed, when it was found to retain 100% survival after exposure to 1 h at 90 °C (Onofri et al. 2008). Survival was high even under simulated Mars atmosphere and vacuum since more than 50% of colonies developed in all cases. If survival was comparable in the irradiated samples on Martian analogues, values were different on the two substrata when the fungus was exposed to Martian atmosphere: no significant decrement was recorded on S-MRS with respect the control whereas here was less than 60 % of survival for P-MRS (Fig. 2.3). This apparently incongruent result is difficult to explain and requires additional investigations.

Most samples showed a higher percentage of apparent survival in PMA assays as compared to colony forming tests. This apparent inconsistency may be due to the coincidental preservation of cell membrane integrity in cells that have lost the ability to multiply that have, preventing PMA to penetrate and react with DNA; this could have led to an overestimation of vitality in some cases, i.e. Lunar sample. The membrane may be less susceptible to damage than DNA or the DNA replication enzymes under some of these conditions. Similar results were reported by Bryan et al. (2015) who also obtained higher

values with the indirect XTT assay method with respect the clonogenic approach for testing survival for irradiated fungal cells of *Cryptococcus* neoformans. Data obtained in Lunar sample ($8x10^5$ kJ/m^2), where survival was 35% in PMA assay compared to zero in cultural test, may be due to the loss of the ability to multiply in some cells where UV treatment caused extensive molecular damage, since UV targets more specifically the DNA rather than the membrane.

It is worth noting that the highest survival, both from cultural analysis and PMA assay, was obtained for *C. antarcticus* grown on P-MRS; the same fate was recently reported for the mycobiont of *Buellia frigida* suggesting a protective role of the substratum (Meeßen et al., 2015). The same authors observed that the highest viability was obtained when the lichen was exposed on the original rock substratum. Differently, in the frame of BIOMEX experiment, Baquè et al. (2014) reported a higher survival of the cyanobacterium *Chroococcidiopsis* when mixed with S-MRS regolith. These contrasting results led us to conclude that a possible protective role of the analogues is difficult to be sustained for now and needs to be further studied. Our results clearly demonstrate the high resistance of *C. antarcticus* to all EVT treatments, including exposure to vacuum, simulated Mars atmosphere, and different doses of monochromatic (254 nm) and polychromatic (>200 nm) UV radiation.

Further studies on *C. antarcticus* in the last ground tests (Science Verification Test, SVT) are still in progress and will clarify the real role of substrates in the protection; it was suggested that survival in space could benefit from the shielding provided by melanin. Melanin is a biological macromolecule mainly known for its protective role against UV, extreme temperatures, desiccation and osmotic stress (Sterflinger 2006; Plemenitaš et al. 2008). The high tolerance to UV-B exposure of single cells of black fungi has been reported by Onofri et al. (2007b). It is known that melanin strongly absorbs UVB, UVA, and PAR, thereby protecting fungi and lichens against those stressors (Nybakken et al., 2004, Meeßen et al., 2013b). Moreover, it was observed that melanized fungal spores, in addition to resistance to UVR, even resist γ-ray and X-ray treatment better than melanin-deficient ones (Bell and Wheeler, 1986) suggesting a role for this pigment in radioprotection of fungi (Henson et al., 1999; Dadachova et al., 2007). In this study, the presence of either Martian and Lunar analogues did not affect molecular analyses since genomic DNA was

successfully extracted and amplified even from samples that had lost the ability to form colonies. In agreement with what is reported in the literature, PCR band intensity decreased mainly in the highest molecular weight fragments in single-gene PCR (Atienzar et al., 2002). However, most of the amplicons were still obtained even at highest doses of UV-irradiation and RAPD profiles were well preserved in all samples. This surprisingly high DNA resistance to UV-irradiation, if protected by screening pigments, the outer cell envelop or a dust layer, suggests DNA as a possible biosignature candidate in future exploration missions (Lyon et al., 2010). Of course its resistance to UV-radiation needs to be proved over much longer timescales.

It is worth noting that ancient DNA was actually recovered on Earth from samples between 400 thousand and 1.5 million years old (Sankaranarayanan et al., 2014); it was also postulated that present Mars conditions (in terms of dryness and low temperatures) may even preserve ancient DNA much better than Earth conditions (Sephton, 2010) with a theoretical preservation of a 100 bp fragment of DNA along a timescale of 3.4×10^9 years at -50 °C and 3×10^{21} years at -110 °C at the Martian polar ice caps (Willerslev et al., 2004). Moreover, some specific conditions could improve the long-term preservation of ancient DNA in halite crystals, permafrost, amber depositions and marine sediments (Panieri et al., 2010). Of course, some other damaging factors, such as ionizing radiation (Hassler et al., 2014) and oxidative environments (Yen et al., 2000; Hecht et al., 2009) on Mars, must be taken into consideration; but our simulation experiments suggest that we may have a good chance to reveal the presence of DNA, in present, in putative extraterrestrial samples, by using some low-specificity based approach such as random primers (as for RAPD) or non-specific staining such as orange acridine or non-toxic ones such as gel red and sytox green.

2.5 Conclusions

C. antarcticus is an astonishingly resistant fungus, able to withstand even long term exposure to actual Space conditions (Onofri et al., 2012); the present study proved that the fungus survives space simulated stressors even when grown on extraterrestrial rock analogues. The awareness that a terrestrial microbe may survive extraterrestrial conditions is an important clue in searching for life on other planets, above all on Mars. Many efforts

are now devoted to the definition of proper biosignatures to detect whether life was ever present in an extraterrestrial sample from a putatively habitable region. Our results show that genomic DNA can be successfully extracted even in the presence of Martian or Lunar analogues; the ease of isolation and detection are key characteristics for a suitable biosignature, and optimizing the extraction is an important challenge in detecting biomarkers (Aerts et al., 2014). PCR was also successful and amplifications were obtained for most of the genes even at a length of up to 2000 bp; this was regardless of the treatments, revealing a high DNA persistence. Further analyses on samples treated in more stressing ground based experiments (SVT) or exposed to actual space conditions in the frame of the BIOMEX experiment, will provide further information on the detectability of this molecule and its suitability as biomarker in future exploration missions. Studies are in progress to define additional biomolecules to be used as good biomarkers using non-destructive approaches as Raman and Infrared spectroscopies, according to the instruments available on ExoMars.

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Chapter 3

3 BIOlogy and Mars Experiment: responses of the black fungus *Cryomyces antarcticus* to the EXPOSE-R2 Science Verification Test

Abstract

The BIOlogy and Mars Experiment (BIOMEX) is part of the ESA space mission EXPOSE-R2 in Low Earth Orbit (LEO). In the frame of this international and interdisciplinary experiment, dried colonies of the Antarctic cryptoendolithic black fungus Cryomyces antarcticus CCFEE 515, grown on Martian and Lunar analog regolith pellets, were exposed for 1.5 years to LEO space and simulated Mars conditions on the International Space station (ISS). In preparation for this mission, several preflight tests, Experiment Verification Test (EVT) and Science Verification Test (SVT), were performed to investigate the space mission candidate organisms' resistance to space stressors, the potential interference of extraterrestrial rock analogues on fungal survival and the detection of biomolecules as potential biomarkers. The present results demonstrate that C. antarcticus was able to tolerate the conditions of the SVT experiment, regardless of the substratum where it was grown. DNA demonstrated high stability after treatments and it was confirmed as a possible biosignature molecule, while melanin, a molecule chosen as a target for biosignature detection, was impossible to unambiguously detect by Raman spectroscopy.

Keywords: Astrobiology, Radiation resistance, Biosignatures, Extremophilic microorganisms, Raman spectroscopy.

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3.1 Introduction

One of the main scientific challenges for our time is to answer the question whether life ever existed, or still exists on Mars or on other planets or satellites. The related question of "habitability" is another challenge to investigate (Horneck et al., 2016). As reviewed by Cockell et al. (2016), the investigation of the origin of life on Earth, its persistence on the planet since it appeared, and the search for evidence of life on other planets sheds light on the conditions that permit life.

Astrobiologists strive to understand the limits of habitability and to define the requirements for metabolic activity and reproduction in planetary environments (Cockell et al., 2016). A second main focus in astrobiology is the definition of significantly distinguishable biosignatures, i.e. molecules indicating the presence of putative extinct or even extant life (de Vera et al., 2012; Horneck et al., 2016). Melanins are promising biosignature candidates: they are distributed among all the kingdoms of life suggesting an early emergence in the course of evolution. Melanins confer on organisms a number of useful characteristics, allowing them to adapt to extreme environments. In addition to tolerance to heat, cold, extreme pH, UV radiation, heavy metals, salt and dryness (Gadd and de Rome 1988; Gunde-Cimerman et al., 2000; Gorbushina et al., 2008; Onofri et al., 2008; Selbmann et al., 2008, 2011; Sterflinger et al., 2012), many melanized fungi are highly radiation resistant, requiring radiation doses exceeding 5 kGy to reduce cell survival to 10%, roughly 1000-fold higher than the lethal dose for humans (Dadachova and Casadevall, 2008). Melanins also possess unique physical, chemical, paramagnetic and semi-conductive properties (Meredith and Sarna, 2006) acting as possible energy harvesting pigments (Dadachova et al., 2007; Gessler et al., 2014). This double role of protection and energy transduction may have been crucial for microbes in the early history of life on Earth, when radiation was higher than today.

Extreme environments on Earth, previously thought to be incompatible with active life, are perfect models for studying the limits of life and habitability. Among them, the McMurdo Dry Valleys in Antarctica are the coldest hyper-arid desert environments on Earth (Cowan et al., 2014) and represent a terrestrial analog of Mars. In this location, where the environmental conditions limit the colonization of rocky surfaces, endolithic life, and cryptoendolitic in particular, is the predominant life-form, (Friedmann, 1982), representing

the last chance for life before extinction (Friedmann and Weed, 1987). Recently, the geographical limits for endolithic life in the Victoria Land were defined (Zucconi et al., 2016). Endolithic communities are of astrobiological significance because putative life on Mars may have adopted an endolithic habitus before its potential extinction, when conditions of the Red Planet become harsher and harsher (Friedmann, 1986).

Our astrobiological model organism, the cryptoendolithic black fungus *Cryomyces antarcticus* CCFEE 515, from the McMurdo Dry Valleys, is able to resist extreme and long term desiccation, a wide temperature fluctuation, and high doses of radiation (Onofri et al., 2004; 2008; 2012; 2015; Selbmann et al., 2011).

For all these reasons, it is an excellent eukaryotic model for astrobiological space research and was already selected for the LIFE experiment where it survived outside the ISS in dried conditions, exposed to space for 18 months (Onofri et al., 2012). In the frame of the same experiment, it was also exposed to simulated Martian conditions (95% carbon dioxide, ultraviolet >200 nm, and cosmic radiations. The results showed that *C. antarcticus* maintained good viability, with minimal DNA damage (Onofri et al., 2015).

C. antarcticus is one of the microorganisms selected for the BIOMEX experiment and it was exposed for 1.5 year outside the ISS on the EXPOSE R2 facility. This project is one of the EXPOSE-R2 mission astrobiology experiments, launched on July 23rd, 2014. The organisms were exposed outside the ISS until February 3rd, 2016, when samples were retrieved from the outside platform of the Russian Svezda module to the inside of the ISS, awaiting return to Earth, which occurred on June 18th, 2016.

BIOMEX aims to test the resistance and stability of biomolecules, as well as the endurance of selected extremophiles under space and Mars-like conditions (de Vera et al., 2012). In particular, the experiment aims to define a list of reliable biosignatures (biomolecules including pigments) and to build up a biosignature database. To mimic real planetary conditions, microorganisms were grown on lunar regolith analogue rocks like anorthosite (Mytrokhyn et al., 2003) and on two Mars regolith analogue mixtures, namely Phyllosilicatic Mars Regolith Simulant (P-MRS) and Sulfatic Mars Regolith Simulant (S-MRS); the latter reflect two separate evolutionary epochs associated with environmental changes on Mars (Böttger et al., 2012). In preparation for the mission, several preflight tests were performed to test and select the most promising organisms for this

astrobiological experiment, to optimize the sample preparation and to improve the hardware integration procedures; EVT and SVT tests were used to assess the resistance of selected organism to the abiotic stressors experienced under space and Mars-like conditions. The results of the EVT tests, simulating the Martian atmosphere, the extreme temperatures, vacuum and UV radiation, showed a high survival rate of *C. antarcticus*, as well as the preservation of the fungal DNA (Pacelli et al., 2016).

In the present study, we report and discuss the results of the last set of experiments, the SVT, a dress rehearsal of the mission as planned, providing a combination of simulated space vacuum and UV radiation and simulated Martian conditions. The post-exposure viability was tested by both cultural and molecular methods as previously and successfully applied on samples of the EVTs (Pacelli et al., 2016). Ultrastructural damage was investigated by Transmission Electron Microscopy (TEM) and metabolic activity was evaluated by the use of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT assay, Kuhn et al., 2003). After exposure to the SVT conditions, the detectability of melanin, our target biosignature molecule, was checked by Raman spectroscopy.

3.2 Materials and Methods

3.2.1 Organisms and culture conditions

Cryomyces antarcticus CCFEE 515 was isolated by R. Ocampo-Friedmann from sandstone collected at Linnaeus Terrace (Southern Victoria Land) by H. Vishniac, in the Antarctic expedition 1980-81.

For the SVT tests fungal cells were grown for three months at 15°C on malt extract agar (MEA) Petri dishes mixed with Antarctic sandstone, which is the Original Substrate (OS), and with Lunar analogues (L), which mainly consists of anorthosite. To mimic two distinct Martian soil compositions representing soil from two different Martian epochs, colonies were grown on the Sulfatic Mars Regolith (S-MRS) and Phyllosilicatic Mars Regolith (P-MRS) Simulants, respectively. The mineral compositions are reported in Table 3.1 and all samples were prepared in order to maintain their integrity in the exposure facility EXPOSE R2, according to the optimized protocol reported in Pacelli et al. (2016).

Table 3.1 Mineralogical composition of Sulfatic and Phyllosilicatic Mars Regolith Simulants (S- and P-MRS) and Lunar Analog Anorthosite (Lunar) in weight/volume % (modified from Böttger et al., 2012).

S-MRS		**P-MRS**		**Lunar**	
mineral	*weight (%)*	*mineral*	*weight (%)*	*mineral*	*weight (%)*
gabbro	32	montmorillonite	45	plagioclase	67
gypsum	30	chamosite	20	volcanic slag	10
dunite	15	quartz	10	diopside	9
hematite	13	iron(III)-oxide	5	hypersthenes	6
goethite	7	kaolinite	5	olivine	6
quartz	3	siderite	5	apatite	1
		hydromagnesite	5	illmenite	1
		gabbro	3	iron	1
		dunite	2		

3.2.2 Ground-based simulations

Like the EVT, the SVT tests were performed at the PSI at the Institute of Aerospace Medicine (German Aerospace Center, DLR, Köln, Germany).

Basically, SVT were designed to ensure that all samples were prepared and suitable for hardware integration for the conditions experienced during the mission and for post-flight de-integration. The EXPOSE R2 ground hardware and the scheme of samples accomodation are reported in Figure 2.3a, b.

To simulate space-like test conditions, the samples grown on OS and L analogues were exposed to vacuum (10^{-5} Pa) and cycling temperatures between -25 °C (16 h in the dark) and + 10 °C (8 h during irradiation). In parallel, Mars test parameters were simulated by low temperature (- 25 °C), Mars atmosphere (95.55% CO_2, 2.70% N_2, 1.60% Ar, 0.15% O_2, ∽ 370 ppm H_2O, Praxair Deutschland GmbH), and Mars-like pressure of 10^3 Pa for S-MRS and P-MRS analogues. All conditions were simulated for a period of 28 days.

Additionally, the upper layer sample sets (TOP) were irradiated with UVR, by using the solar simulator SOL2000 applying a total fluence of 5.5×10^5 kJ m^{-2}. The applied fluency corresponds to one year of exposure outside the ISS, as estimated from previous EXPOSE

data and simulations (Rabbow et al., 2012) and the use of Neutral Density filter as planned for the real mission.

Below, an identical set of samples (BOTTOM) was kept in the dark and experienced all simulation parameters except UVR exposure. Controls (CTR) were kept at DLR in the dark, at room temperature. The exposure conditions are summarized in Table 3.2 and the experimental setup and sample accommodation are shown in Figure 3.1 a, b. For SVTs, the real space accommodation plan was followed and only one replicate per sample was consequently possible.

Table 3.2 Exposure conditions during the Science Verification Tests (SVTs).

Test parameter	Duration
Vacuum 10^{-5} Pa + UV irradiation, polychromatic 200-400 nm, Fluence 5,5 x 10^5 kJm^{-2}	28 d SOL2000 120 h* (18 wd @ 7 h/wd)
Simulated CO_2 Mars atmosphere 10^3 Pa + UV irradiation, polychromatic 200-400 nm, Fluence 5,5 x 10^5 kJm^{-2}	28 d SOL2000 120 h* (18 wd @ 7 h/wd)
Control experiment, 1 atm air, dark, room temperature	28 d

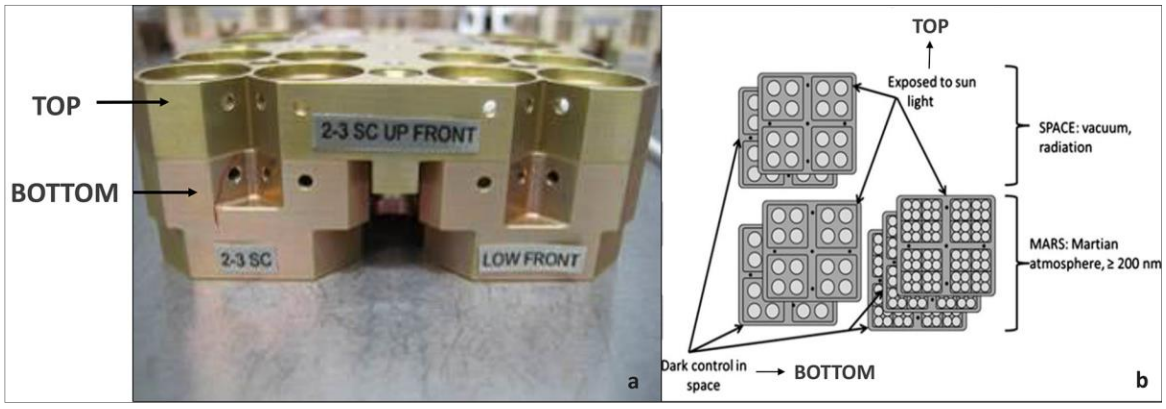

Fig. 3.1 a) Sample carriers for trays in EXPOSE-R2 ground hardware; **b)** experimental setup of TOP and BOTTOM treatment and sample accomodation.

3.2.3 Survival assessment

Cultivation test

After re-hydratation for 3 days in 1 mL of physiological solution (NaCl 0.9%), the treated colonies were diluted to a final concentration of 50,000 cells/mL and 0.1 mL of suspension was spread on Petri dishes supplemented with MEA (5 replicates). Plates were incubated at 15 °C for three months and CFUs (Colony Forming Units) counted.

PMA assay

The test was performed by adding the Propidium MonoAzide (PMA, Biotium, Hayward, CA) at a final concentration of 200 μM to re-hydrated fungal colonies. PMA penetrates only damaged membrane cells, crosslinks to DNA after light exposure and thereby prevents Polymerase Chain Reaction (PCR). DNA extraction, purification and quantitative PCR, used to quantify the number of fungal Internal Transcribed Spacer (ITS) ribosomal DNA fragments present in both PMA treated and non-treated samples, were performed according to Onofri et al. (2012). Before qPCR, DNA was quantified using QUBIT system and diluted at the same concentration (2 ng/mL). All tests were performed in triplicate.

Determination of metabolic activity by XTT assay

Colorimetric assay of cellular viability, namely XTT assay, was performed according to the protocol in Kuhn et al. (2003); the XTT is converted to a coloured formazan in the presence of cell metabolic activity. After SVT treatment colonies of *C. antarcticus* were re-hydrated in 1 mL of Malt Extract (30 gr/L). After 10 days fungal cells were washed, suspended in PBS and placed into 96 well plates, 3 wells for each condition. The XTT assay was performed adding 54 μL XTT(10mg/ml)/menadione (2mM) to each well. Plates were covered with foil and incubated, in agitation, at room temperature (24°C). Formazan product in the supernatant was detected by measuring the optical density at 492 nm (Labsystem Multiskan, Franklin, MA) after 2, 3, 4 and 12 hours of incubation.

Statistical analyses

Means and standard deviations were calculated. Values obtained for each analogue (OS, L, S-MRS, P-MRS) were normalised to each control, respectively. Statistical analyses were performed by one-way analysis of variance (Anova) and pair wise multiple comparison procedure (Tukey test), carried out using the statistical software SigmaStat 2.0 (Jandel, USA).

3.2.4 DNA damage revealed by PCR-based assays

DNA was extracted from rehydrated colonies, using Nucleospin Plant kit (Macherey-Nagel, Düren, Germany) following the protocol optimized for fungi (Onofri et al., 2012). Before amplification, DNA was quantified using QUBIT system and diluted at the same concentration (2 ng/mL).

ITS and LSU amplification were performed using BioMix (BioLine GmbH, Luckenwalde, Germany) adding 5 pmol/ng of each primer and 20 ng of template DNA at final volume of 25 μL. RAPD was performed using BioMix (BioLine GmbH, Luckenwalde, Germany) adding 5 pmol of the primer and 1 ng of template DNA at final volume of 25 μL. The primer used for RAPD was GGA_7 (GGA GGA GGA GGA GGA GGA GGA) (Kong et al., 2000). The amplification was carried out using MyCycler Thermal Cycler (Bio-Rad Laboratories GmbH, Munich, Germany) equipped with a heated lid.

ITS, LSU and RAPD amplification conditions were as reported in Pacelli et al. (2016). Band intensity was measured and compared by using ImageJ software (Schneider et al., 2012).

3.2.5 Evaluation of cell integrity through Transmission Electron Microscopy

After re-hydration, control and TOP colonies were prepared for Transmission Electron Microscopy, according to the protocol in Pacelli et al. (2016) and the image acquisition was performed at the Center for High Instruments, Electron Microscopy Section of University of Tuscia (Viterbo, Italy).

3.2.6 Raman Confocal Spectroscopy

Raman measurements were performed in the Astrobiology-Raman-Lab at the DLR Institute of Planetary Research (Berlin) with a confocal Raman microscope (Witec alpha300 R system, http://www.witec.de/en/products/raman/alpha300r/) at room temperature under laboratory atmospheric conditions, according to the optimizations made by Böttger et al. (2012). The Raman laser excitation wavelength was 532 nm, which is the value proposed for the Raman Laser Spectroscopy instrument on ExoMars, and the surface laser power of 1 mW, was a compromise between measurement time in the lab and the RLS irradiance values (Hutchinson et al., 2014). The spectral resolution of the spectrometer

was 4–5 cm^{-1}. A Nikon 10x objective was used to focus the laser on a 1.5 μm spot. A spectral calibration was performed by using Raman measurements of a pure silicon test sample and paracetamol.

3.3 Results

3.3.1 Survival: Cultivation and PMA tests

Germination ability was not significantly different on the controls of each analogue (OS, L, S-MRS, P-MRS) compared to data obtained on Malt Agar alone, suggesting that substrates do not exert any reliable toxic effect (data not shown). *C. antarcticus* retained significant-colony-forming ability after SVT treatments (Fig. 3.2), showing a similar trend of survival in all the substrates tested.

In general, radiation clearly represent an additional stress to the cell respect to the vacuum or Mars atmosphere alone of BOTTOM samples. For example, a decrease of 22% of the vitality was measured in BOTTOM OS (Vacuum only) compared to OS control, and an additional decrease of 25%, compared to the BOTTOM, was recorded for TOP samples (Vacuum plus radiation). Similar trends were obtained for L (same conditions) and P-MRS (Mars atmosphere BOTTOM and plus radiation TOP). Differently, for S-MRS samples no statistically significant differences were observed either for BOTTOM or TOP treatments compared to the control. Yet, for all substrates, a considerable capacity of germination was still maintained; 44%, 23%, 88% and 57% of survival was recorded for colonies exposed at TOP treatments grown on OS, L, S-MRS and P-MRS, respectively.

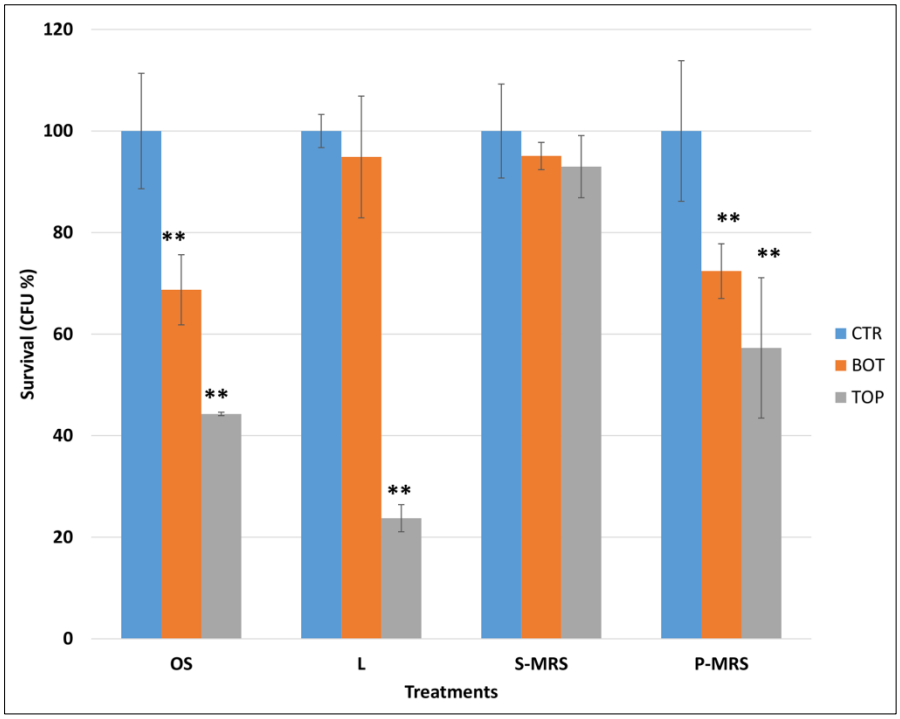

Fig. 3.2 Cultivation test after SVT treatments: percentages of CFU of *C. antarcticus* on different substrates, relative to controls on the same substrate. Significant differences were calculated by Tukey test with * = p > 0.05. and ** = p > 0.001.

Viability, specifically the integrity of the plasma membrane, was tested through PMA treatment followed by qPCR (Fig. 3.3). The PMA test too indicated the TOP treatments, including vacuum plus radiation (OS and L) and Mars atmosphere plus radiation (S-MRS and P-MRS), as the most deleterious for the fungus, especially on OS and L; besides, a higher percentage of viable cells (measured as cells with intact membrane) was observed compared to the results of the cultivation test. No significant differences between treated samples and control were obtained on S-MRS, corroborating the cultivation test.

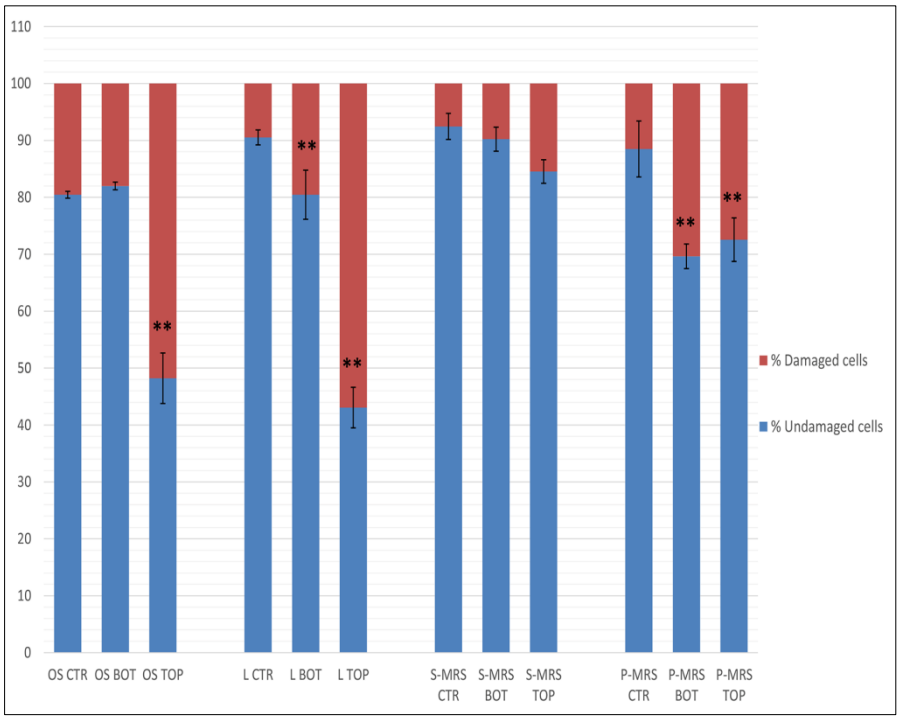

Fig. 3.3 Results of PMA assay coupled with qPCR after SVT treatments in colonies grown on different substrates. A: percentages of *C. antarcticus* cells with damaged membrane B: percentages of *C. antarcticus* cells with intact membrane. The statistical analyses were performed as in Fig. 3.2.

3.3.2 Metabolic activity analyses

For the XTT analysis the most representative results were obtained after 4 hours and are shown in Figure 3.4. The XTT activities decreased accordingly to the intensity of irradiation from the BOTTOM to the TOP for colonies grown on OS, Lunar and P-MRS; the sample Lunar TOP showed again the highest decrease. The fungus grown on S-MRS retained the same metabolic activity (92%, no statistical difference from control) for BOTTOM and a slight decrease (80%) in the TOP sample. All the results were again in general agreement with the cultivation tests.

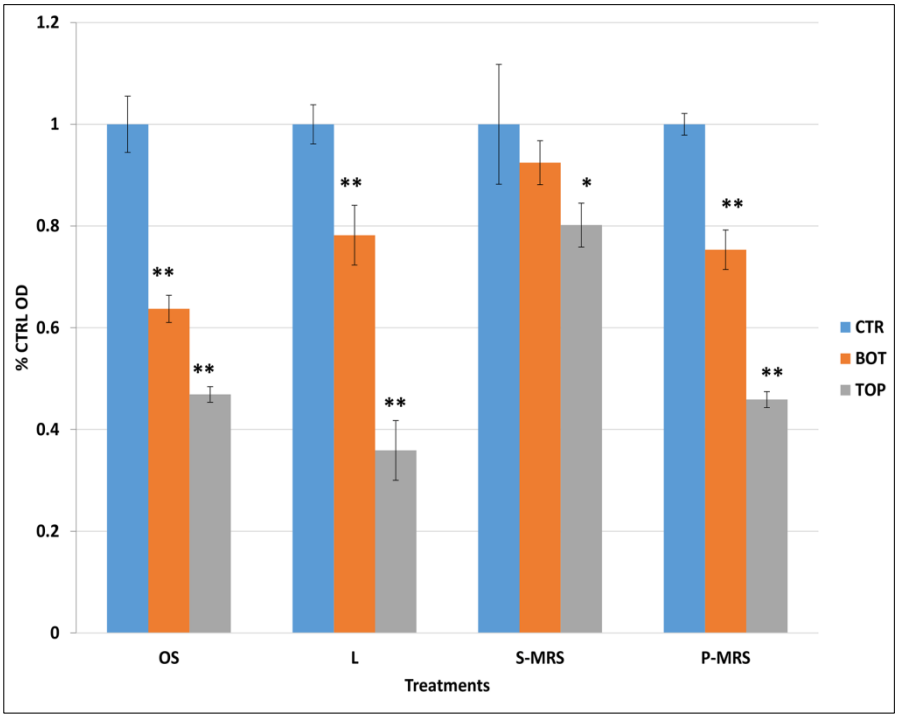

Fig. 3.4 Effects of irradiation on *C. antarcticus* XTT reducing activity after SVT treatments. The statistical analyses were performed as Fig. 3.2.

3.3.3 DNA damage

The integrity of genomic DNA in treated samples was tested by assessing its ability to serve as a PCR template after SVT treatments. All the amplifications worked out for the ITS and LSU region (Fig. 3.5a-c). In particular, for the 700 bp gene length, a reduced intensity of PCR bands was recorded in TOP samples, except for S-MRS (SM in Fig. 3.5a), where the gel analyses measured around 85% relative density of the band both for TOP and BOTTOM samples (Fig. 3.5a). For the 1600 bp gene length the disappearance of the sole Lunar TOP sample was observed (Fig. 3.5b). For 2000 bp fragments the band disappearance of Lunar and P-MRS (PM in Fig. 3.5) TOP samples was recorded and 3% of band intensity was obtained for OS TOP sample, besides around 85% of intensity was still maintained in TOP and BOTTOM S-MRS samples (Fig. 3.5c). The RAPD profiles were preserved in all the conditions tested (Fig. 3.5d). A reduction of intensity was measured for L and P-MRS TOP samples only, demonstrating an overall good preservation of the whole fungal genomic DNA.

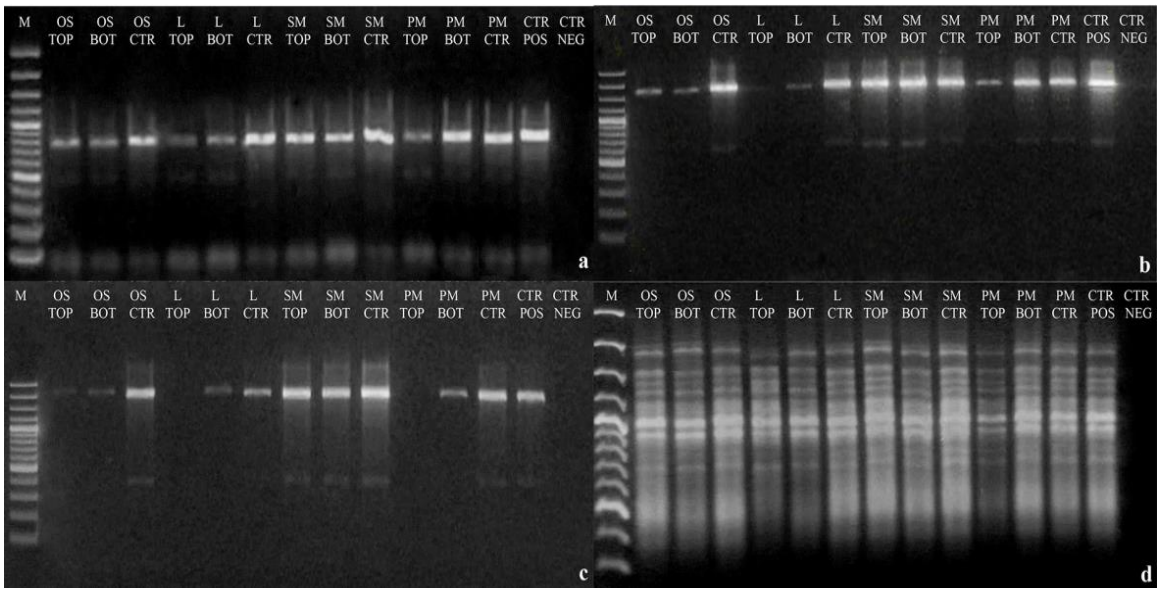

Fig. 3.5 Assessment of the DNA damage on *C. antarcticus* after SVT treatments; single gene PCR **a)** 700 bp; **b)** 1600 bp; **c)** 2000 bp and **d)** RAPD complete genomic fingerprinting. DNA ladder (M), Positive PCR Control (CTR POS), Negative PCR Control (CTR NEG). Substrates: OS, Original Substrate; L, Lunar; SM, S-MRS; PM, P-MRS.

3.3.4 Ultrastructural Damage

TEM observations were performed on samples of the TOP samples, exposed to the highest UVR doses, where the effects on vitality and DNA damage were the most pronounced compared to the laboratory controls (Fig. 3.6). The ultrastructural damage in the cells of *C. antarcticus* was visible in all the irradiated samples, mostly in terms of loss of coherence and organization of the cytoplasm and cell membrane integrity. This was particularly evident in OS and L samples where the organelles were not visible in some cases and cell membranes were discontinuous (Fig. 3.6b, d, arrows). The same damage was also observed in P-MRS and S-MRS samples but to a lesser extent in terms of number of cells involved, particularly for the latter (Fig. 3.6f, h, arrows). These results are again consistent with data coming from the previous tests where the colonies grown on S-MRS were less affected by the treatment.

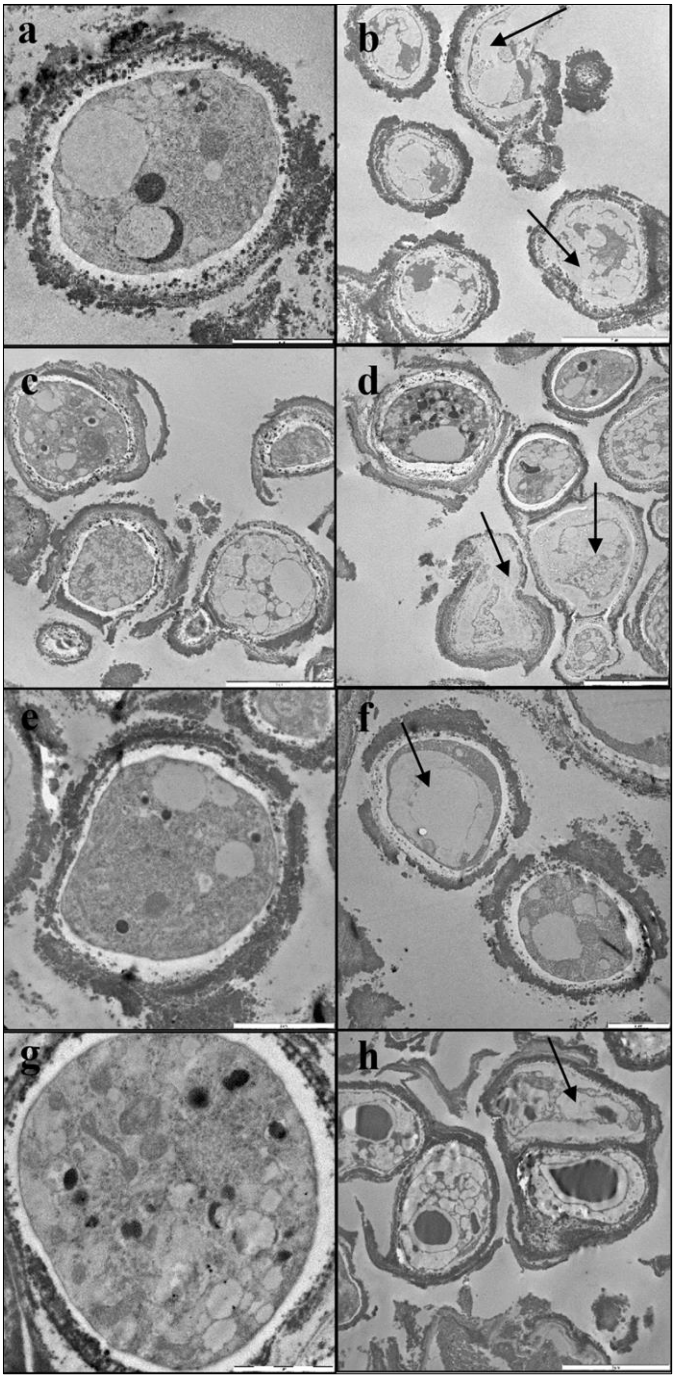

Fig. 3.6 TEM micrographs. Untreated (CTR) and treated (TOP samples) microcolonies of *C. antarcticus* on sandstone (OS) (**a**, **b**); Lunar (L) (**c**, **d**); S-MRS (**e, f**); P-MRS CTR (**g**, **h**); not irradiated (**a**, **c**, **e**, **g**) and irradiated (**b**, **d**, **f**, **h**) samples, respectively.

3.3.5 Raman Spectroscopy

As for TEM, the Raman spectroscopy was performed on samples treated with TOP treatment and laboratory controls only. Minerals from the analogues and melanins from the fungal colonies were well detectable both in control samples (results not shown) and irradiated samples (Fig. 3.7). In particular the Raman spectrum of melanin in *C. antarcticus* was identified on irradiated samples on OS, S-MRS and P-MRS substrates (Fig. 3.7a, b, c). The melanin specific Raman peaks were 1349 cm^{-1} and 1599 cm^{-1} (Fig. 3.7d). On the other hand, the irradiated Lunar samples gave a high fluorescence, so that any other peaks, including melanin, which might be there, were undetectable (Fig. 3.7e, f).

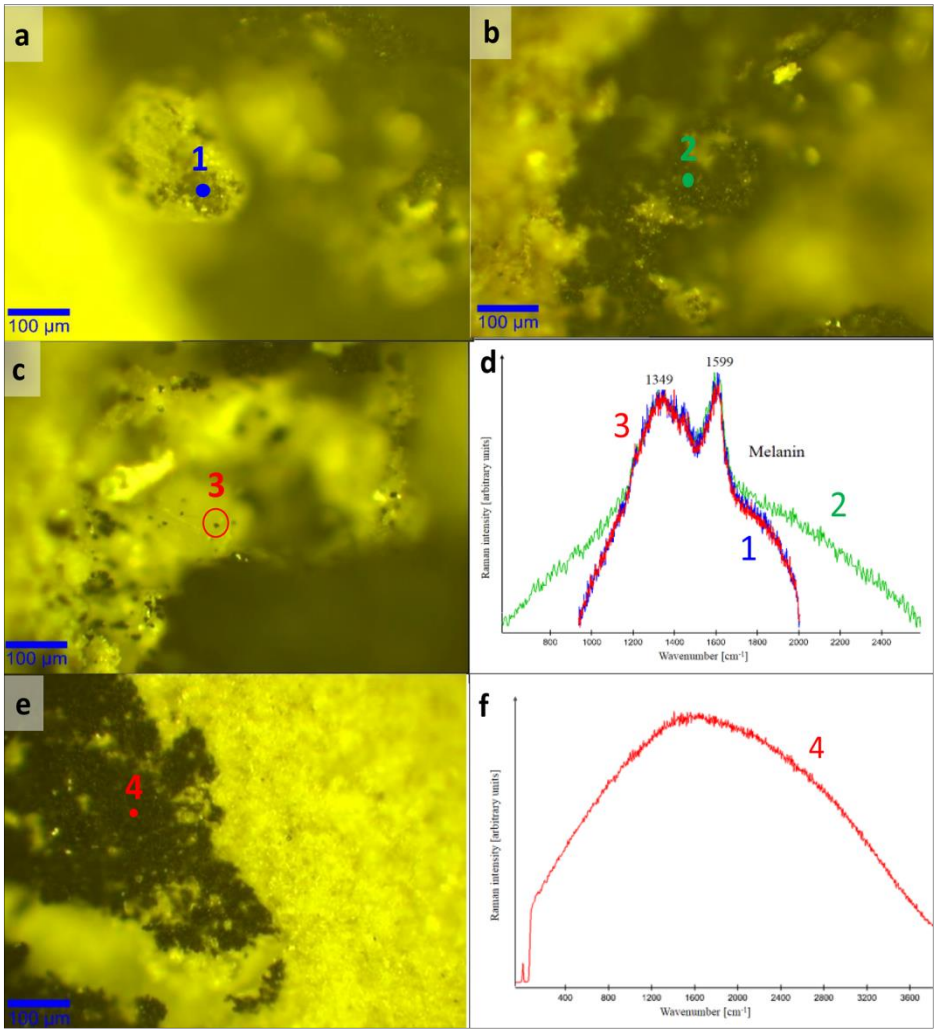

Fig. 3.7 Melanin Raman spectra (**d**, peaks 1349 cm^{-1} and 1499 cm^{-1}) obtained from *C. antarcticus* grown on OS, S-MRS and P-MRS at maximum irradiation (respectively **a**, **b** and **c**) on selected point. Not detectable spectra of melanin from Lunar irradiated sample (**e**, **f**). Measurement time and number of spectra per point on sample were (10s, 5 x).

3.4 Discussion

The study of microbial communities adapted to the harshest conditions on Earth, and the assessment of limits for life on our own planet, could help in getting insights into the potential for other worlds to support life, and determining whether life, as we know it, may exist elsewhere in the galaxy (Saffary et al., 2002). In this work, we investigated the ability of the extremophilic black fungus *C. antarcticus* to survive and resist stressing conditions, simulating the space and Mars-like environment, experienced during the pre-flight tests SVT of the BIOMEX experiment. In order to mimic the real conditions in space, fungal colonies were exposed to the stressors expected over a year of exposure outside the ISS, as estimated from Rabbow et al. (2012), and also were grown on Lunar and Martian rock analogues.

The described experiments aimed to investigate fungal resistance in term of survival, metabolic activity, DNA integrity and ultrastructural damage. After exposure, fungal survival was assessed both by testing the colony forming ability and qPCR coupled with PMA. The metabolic activity and the ultrastructural damage were analyzed by XTT assay and TEM, respectively, to corroborate the survival results. The preservation of biosignatures, namely DNA and melanins, was evaluated by using PCR-based assay and RAMAN spectroscopy.

The results revealed good viability after the SVT treatments. None of the conditions tested caused complete inhibition of growth or metabolic activity, with a higher viability under Mars-analogue conditions compared to space vacuum and irradiation. The highest resistance was obtained from colonies grown on S-MRS analogues, where we did not record any statistical difference between treated sample, both TOP and BOTTOM, compared to the controls. Previous ground-based experiments, namely EVTs in the frame of BIOMEX too, showed the highest survival of *Buellia frigida* and *C. antarcticus* when grown on P-MRS analogues (Meeßen et al., 2015; Pacelli et al., 2016), while Baqué et al. (2013) and Johnson et al. (2011) reported a higher survival of the cyanobacterium *Chroococcidiopsis* sp. when mixed with S-MRS regolith. Overall, these results are of utmost importance, indicating that none of the analogues seems really to interfere with the survival of the biological models we are using for checking the limits of life and the quest for checking the habitability of other planets like Mars.

TEM observations were performed after the treatment to gain insight into the potential damage to the ultrastructure. The ultrastructural analyses confirmed the survival results; even if some injured cells were undoubtedly present in the treated samples, intact cells were also observed and were more frequent in S-MRS, where the survival and metabolic activity was the highest.

These results were consistent with the analyses on DNA damage. Amplicons were obtained in almost all samples for the shortest length analyzed. The disappearance of DNA was recorded only for Lunar and P-MRS TOP samples and still 3% of band intensity was obtained for OS TOP; yet, the fingerprinting analyses were successful for all samples, showing a good preservation of the whole genome. These findings were in accordance to the previous analyses performed in the EVT ground-tests, in the frame of BIOMEX (Pacelli et al., 2016), and further support the use of DNA, an unequivocal proof of life, as a biosignature. In fact, the DNA extraction was efficient even for colonies grown on regolith analogues with no interference in the PCR reactions. Recent discoveries of nucleic acids or their precursors within meteorites (Martins et al., 2008; Schmitt-Kopplin et al., 2010; Callahan et al., 2011; Cooper et al., 2011) and in interstellar space (Hollis et al., 2000) could steer the development of life toward these biomolecules. Thus, it makes sense to search for RNA or DNA based life within potential habitable zones and on Mars. New approaches to search for life in the universe for the ExoMars mission include Polymerase Chain Reaction (PCR)-based methods for targeting ribosomal RNA genes and DNA sequencing (Isenbarger et al., 2008; Carr et al., 2013) are e.g. also one possibility to be used for the search for life.

Previous studies of *C. antarcticus* have demonstrated this organism's high capacity to resist, when dehydrated, the extreme stresses of simulated space and Martian conditions exposure (Pacelli et al., 2016), real space exposure (Onofri et al., 2012, 2015) and high doses of ionizing radiation up to 55.81 kGy (Pacelli, unpublished data). The key factor allowing microorganisms to survive extreme environmental conditions is anhydrobiosis (Crowe et al., 1992; Billi et al., 2000; Kranner et al., 2008); for melanized fungi this protective function is undoubtedly supported by the abundant presence of melanin in the outer cell wall layers (Gorbushina, 2003). Radiation-resistant melanized fungi can even survive in contaminated nuclear reactors (Zhdanova et al., 2000).More recently, it was

demonstrated that both melanized *Cryptococcus neoformans* and *Cryomyces antarcticus* strains showed higher survival after high acute radiation doses under physiological conditions, compared to the non-melanized strains (Pacelli, unpublished data). The high viability of the fungus in the present study can be - at least partially - explained by the anhydrobiotic and resting state of the fungus and by the presence of melanin in cell walls. How long they might retain their viability in the interplanetary environment remains a guess for now and challenge for the future; the analyses of real space experiments would give new insights.

We decided to use Raman Spectroscopy to detect the presence and persistence of melanin, our target molecule as a potential a potential biosignature, after SVT exposure since a Raman spectroscope is among the miniaturized facilities planned as part of the Pasteur payload in the ESA-Roscosmos ExoMars mission (Vago et al., 2006). We used anorthosite (Mytrokhyn et al., 2003; Kozyrovska et al., 2006), the main component of lunar regolith, to simulate real exposure of putative biosignatures to an extraterrestrial environment and P-MRS and a S-MRS analogues, reflecting minerals formed during the Noachian (Early Mars) and Hesperian/Amazonian (Late Mars), respectively (Böttger et al., 2012; de Vera et al., 2012). We performed a set of measurement along strict intervals of time and number of repetitions in order to optimize the detection of minerals and biological markers and to reduce the disturbing effect of cosmic rays (Böttger et al., 2012). We obtained the mineral spectra in the tested sample (results not shown). Melanins were dominant in the spectrum gained from fungal colonies. We obtained melanin spectra both for CTR and TOP samples of OS, P-MRS and S-MRS; however the melanin peaks were too similar to amorphous carbon, 1352 cm^{-1} and 1613 cm^{-1} (Böttger et al., 2012), and this made it impossible to distinguish the presence of our target unambiguously. The Raman approach on Lunar irradiated samples was further affected by presence of high fluorescence, covering all Raman peaks, including melanin. The same problem with high fluorescence in Lunar regoliths after irradiation was already observed in other experiment within BIOMEX (Baqué M., personal communication). Moreover, it was already reported that radiation might lead to degradation of Raman biosignatures (Dartnell et al., 2012). The effect of fluorescence of minerals on Raman spectra points out the need for taking into account their geological and chemical characteristic when developing biosignature databases.

3.5 Conclusions

As a preparative phase of the BIOMEX experiment of the EXPOSE-R2 space mission onboard the ISS, dried cells of *C. antarcticus* CCFEE 515 were mixed with lunar and Martian regolith analogues and exposed to simulated Mars and space conditions. Results demonstrated that *C. antarcticus* retains metabolic activity and is even capable of forming colonies, after SVT treatment on extraterrestrial regolith analogues without significant DNA or ultrastructure damage. These results are essential to substantiate and validate the analyses from space exposed samples.

The high resistance of this fungus justifies its use in a space experiment to test its responses *in situ* to the real conditions of outer space. It is expected that it will be capable of coping with the complex space environment and simulated Martian conditions. DNA has been proposed as a valid biosignature, while the use of Raman Spectroscopy with parameters foreseen for the Exomars mission is inadequate to analyze melanin. Further investigation on this is necessary to derive the appropriate parameter set for Raman spectroscopy of melanin. Future analyses to search for biosignatures on our samples will focus on gas chromatography mass spectrometers, as it is one of the miniaturized facilities for the Pasteur payload of the ExoMars mission.

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Chapter 4

4 STARLIFE V: Survival, DNA integrity and ultrastructural damage in Antarctic cryptoendolithic eukaryotic microorganisms exposed to ionizing radiation

Abstract

Life dispersal between planets, planetary protection and searching for biosignatures are main topics in astrobiology. Under the umbrella of STARLIFE project three Antarctic endolithic microorganisms, the melanized fungus *Cryomyces antarcticus* CCFEE 515, a hyaline strain of *Umbilicaria* sp. (CCFEE 6113, lichenized fungus), and a *Stichococcus* sp. strain (C45A, green alga), were exposed to high doses of space relevant gamma radiation (^{60}Co), up to 117.07 kGy. After irradiation survival, DNA integrity and ultrastructural damage were tested. The first was assessed by clonogenic test; viability and dose responses were reasonably described by the linear-quadratic formalism. DNA integrity was evaluated by PCR and ultrastructural damage was observed by Transmission Electron Microscopy (TEM). The most resistant among the tested organisms was *C. antarcticus* both in term of colony formation and DNA preservation. Besides, results clearly demonstrate that DNA was well detectable in all the tested organisms even if microorganisms were dead. This high resistance provides support for the use of DNA as possible biosignature during the next exploration campaigns. Implication in planetary protection and contamination during long-term space travel are put forward.

Keywords: Biosignatures, ionizing radiation, DNA integrity, eukaryotic microorganisms, fingerprinting, Mars exploration.

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4.1 Introduction

The priority for Mars Science Laboratory (NASA), ExoMars (ESA) and for the next Mars exploration missions is the search for organic molecules as biosignatures to indicate the presence of putative extinct or even extant life. A task force is planned to explore the Martian surface and subsurface and even to bring back Martian samples to Earth for comprehensive analyses (Bada, 2001). Therefore, a key step for interpretation of future data is to shed light on the possibility of preservation of organic matter in Martian environment and space.

DNA sequences of halophilic archaea, 22,000 to 34,000 years old, can be detected in crystals of halite from Death Valley, California. PCR-based approaches detected DNA in even older halites, 10^6 to 10^8 years old (Sankaranarayanan et al., 2014), while microbial nucleic acids hundreds of millions of years old were obtained from Siberian and Antarctic permafrost (Willerslev et al., 2004). The surface of Mars is continuously exposed to high levels of cosmic radiation because the planet lacks a thick atmosphere and a global magnetic field and ionizing radiation is deleterious for both survival and persistence of molecular biosignatures such as DNA (Dartnell et al., 2007). The effect of ionizing radiation on biological systems has to be taken into consideration when analyzing the survival of microorganisms and looking for stable biosignatures. Deleterious effects of radiation on DNA and other biological molecules can be induced either directly or indirectly; the energy can be absorbed directly by the key biomolecules, such as protein or nucleic acids. Alternatively, the energy can be absorbed first by other molecules, producing a radiation-induced radical which secondarily damages the biosignatures.

Several studies are addressed to test both microbial survival and persistence of biosignatures following irradiation. A comprehensive review of microorganisms survival in space has been published (Horneck et al., 2010), dealing with microbial survival under different extreme conditions. A great deal of interest in astrobiology has recently been devoted to different biomolecules, including DNA, to establish a suitable biosignature. Recent studies proved the persistence of both carotenoids and DNA to polychromatic UV irradiations (Baquè et al., 2016), while eukaryotic DNA remained well detectable after both polychromativ UV irradiaton and cosmic rays in real space conditions (Pacelli et al., 2016, Onofri et al., 2015). The STARLIFE Project (Moeller et al., 2016, *in press*) aims to

characterize the effect of ionizing radiation of astrobiological relevance on different microorganisms. In this frame, we analyzed survival, ultrastructural damage and DNA persistence at different ^{60}Co irradiation doses, in three eukaryotic models from Antarctic cryptoendolithic communities, widespread in the Antarctic desert of the McMurdo Dry Valleys, the closest Terrestrial analogue for Mars. Our models were: (i) *Cryomyces antarcticus* CCFEE 515, a black fungus, (ii) a hyaline fungus, strain CCFEE 6113, related to the lichenized genus *Umbilicaria*, and (iii) *Stichococcus* sp. strain C45A, a green alga. *C. antarcticus*, in particular, was already selected in the past for astrobiological studies for its exceptional stress resistance and long-term survival when exposed to actual space conditions and simulated Martian conditions in space (Onofri et al., 2012, 2015). From October 2014 to February 2016, *C. antarcticus* was exposed anew on the EXPOSE-R2 platform, outside the International Space Station, in the frame of the BIOMEX project (de Vera et al., 2012). Less information are available on the resistance of the selected alga and *Umbilicaria* sp. Photosynthetic organisms are known to be very sensitive to ionizing radiation since they can directly interfere with photosynthesis along with other metabolic functions (Kovacs and Keresztes, 2002). Algal cells in *Cladonia verticillata* treated with 10,000 Gy ionizing radiations showed cytoplasmic breakup in 20-30% of cells and altered growth (de la Torre, this issue). Besides, both an Antarctic strain of *Stichococcus* sp. and a lichenized hyaline ascomycete were isolated from rocks colonized with cryptoendolithic communities after long-term permanence in outer Space (Scalzi et al., 2012). The responses of taxonomically and functionally distant organisms to the same treatment in terms of survival and DNA persistence is of astrobiological significance giving indication on resistance to some space parameters and searching for biosignatures.

4.2 Materials and Methods

4.2.1 Samples preparation

Three different eukaryotic models from Antarctic cryptoendolitic communities were chosen for testing the effect of ionizing radiation: (i) *Cryomyces antarcticus* CCFEE 515, an Antarctic cryptoendolithic black yeast-like micro-colonial fungus isolated from sandstone collected at Linnaeus Terrace (McMurdo Dry Valleys, Southern Victoria Land),

Antarctic expedition 1980-81. The fungus was described by Selbmann et al. (2005) as new genus and species endemic for the Antarctic. (ii) A sterile hyaline fungus CCFEE 6113, hyphae 2.5-3.0 µm diam. Based on ITS, SSU, and LSU ribosomal DNA sequences comparison it belongs to a possible new species in the lichen genus *Umbilicaria*; (iii) a green one-celled alga, with nearly cylindrical cells, 11-12x5.0 µm, strain C45A; the BLASTn analysis based on ITS and SSU sequences indicated this strain as a new species in the genus *Stichococcus*. Both the *Umbilicaria* sp. and *Stichococcus* sp. were isolated from sandstone collected in the McMurdo Dry Valleys, Antarctica during the Italian expedition 2010-2011.

Samples were prepared as follows: cell suspensions (1000 CFU) were spread on Petri dishes of MEA medium (malt extract agar: malt extract, powdered 30 g/L; peptone 5 g/L; agar 15 g/L; Applichem, GmbH). *C. antarcticus* was incubated at 15 °C for 3 months, *Umbilicaria* sp. at 25 °C for 1 month and *Stichococcus* sp. at 15 °C for 1 month. Once grown, colonies were dried under laminar flow in a sterile cabinet overnight and then irradiated.

4.2.2 Tests facilities and exposure conditions

Dried samples were exposed to γ-radiation from a ^{60}Co source (γ-rays at 1.17 MeV, low LET of 0.3 keV/µm). The doses were reported in Table 4.1. Irradiation was performed at the Institute of Aerospace Medicine (German Aerospace Center, DLR, Köln, Germany). Tests were performed in triplicate.

Table 4.1 Gamma radiation doses applied for each sample.

Sample	**Applied/received doses**
DLR Lab control	non-irradiated
Transport control	non-irradiated
Irradiated sample	^{60}Co: 6.66 kGy
Irradiated sample	^{60}Co: 12.72 kGy
Irradiated sample	^{60}Co: 19.04 kGy
Irradiated sample	^{60}Co: 27.15 kGy
Irradiated sample	^{60}Co: 55.81 kGy
Irradiated sample	^{60}Co: 81.11 kGy
Irradiated sample	^{60}Co: 117.07 kGy
Positive control	non-irradiated wet sample

4.2.3 DNA extraction and PCR reactions

DNA was extracted from colonies, rehydrated in 1 µL of physiological solution for 48h, using Nucleospin Plant kit (Macherey-Nagel, Düren, Germany) following the protocol optimized for fungi (Selbmann et al., 2014a).

ITS, LSU and SSU amplifications were performed using BioMix (BioLine GmbH, Luckenwalde, Germany) adding 5 pmol of each primer and 20 ng of template DNA at final volume of 25 µL. The amplification was carried out using MyCycler Thermal Cycler (Bio-Rad Laboratories GmbH, Munich, Germany) equipped with a heated lid. The fungal rDNA regions were amplified according to Selbmann et al. (2011). For *Stichococcus* sp. the SSU regions of different lengths (500, 1000 and 1500 bp) were amplified using NS1-NS2, NS1-NS4 and NS1-18L, respectively. Conditions for amplification were: first denaturation step at 94 °C for 3 min followed by denaturation at 94 °C for 45 s, annealing at 55 °C for 60 s, extension at 72 °C for 3 min. The last three steps were repeated 40 times, with a last extension at 72 °C for 5 min. Gene lengths amplified, primer pairs and primer sequences for each test organism are reported in Table 4.2 and Table 4.3.

Band intensity was measured and compared by using Image J software (Schneider et al., 2012).

Table 4.2 rDNA region lengths and relative primer pairs.

Samples	DNA region	Primers
Cryomyces antarcticus	ITS (700 bp)	ITS4-ITS5
	LSU (1600 bp)	ITS5-LR5
	LSU (2000 bp)	ITS5-LR7
Umbilicaria sp.	ITS (700 bp)	ITS4-ITS5
	LSU (1600 bp)	ITS5-LR5
	LSU (2000 bp)	ITS5-LR7
Stichococcus sp.	SSU (500 bp)	NS1-NS2
	SSU (1000 bp)	NS1-NS4
	SSU (1500 bp)	NS1-18L

Table 4.3 Primer sequences.

Primers	Sequence (5'->3')	References
ITS4	TCCTCCGCTTATTGATATGC	White et al., 1990
ITS5	GGAAGTAAAAGTCGTAACAAGG	White et al., 1990
LR5	TCCTGAGGGAAACTTCG	Vilgalys et Hester, 1990
LR7	TACTACCACCAAGATCT	Vilgalys et Hester, 1990
NS1	GTAGTCATATGCTTGTCTC	White et al., 1990
NS2	GGCTGCTGGCACCAGACTTGC	White et al., 1990
NS4	CTTCCGTCAATTCCTTTAAG	White et al., 1990
18L	CACCYACGGAAACCTTGTTACGACTT	Hamby et al., 1988).
$(GGA)_7$	GGA GGA GGA GGA GGA GGA GGA	Kong et al., 2000
OPA 13	CAGCACCCAC	Ho et al., 1995

4.2.4 Random amplification of polymorphic DNA (RAPD) assay

For both fungi, the RAPD protocol was according to Selbmann et al. (2011). For *Stichococcus* sp. the protocol was optimized, using OPA13 primer, as follow: first denaturation step at 94 °C for 4 min followed by denaturation at 96
°C for 30 s, annealing at 49 °C for 60 s, extension at 72 °C for 30 s. The last three steps were repeated 40 times, with a last extension at 72 °C for 6 min.

4.2.5 Clonogenic assay

Microorganisms survival was determined by measuring colony forming ability as percentages of CFU (Colony Forming Units). For the test, three of the treated colonies were rehydrated for 72h in 1 mL of physiological solution (NaCl 0.9 %), and diluted to a final concentration of 3000 cells/mL; 0.1 mL of the suspension was spread on Petri dishes supplemented with MEA (5 replicates). *C. antarcticus* was incubated at 15 °C for 3 months; *Umbilicaria* sp. was incubated at optimal growth temperature (25 °C) for three weeks; the alga was incubated at 15 °C temperature (temperature of isolation) for 1 month exposed to light 24h/day. After incubation the colonies were counted. Means and standard deviations were calculated. Statistical analyses were performed by one-way analysis of variance

(Anova) and pair wise multiple comparison procedure (Tukey test), carried out using the statistical software SigmaStat 2.0 (Jandel, USA).

The dose responses for clonogenic survival were modeled by the linear-quadratic (LQ) formalism, which is a simple mechanistically-plausible radiobiological model (Sachs et al., 1997; Brenner et al., 1998). According to this model, the cell surviving fraction S (relative to un-irradiated controls) can be described by the following equation, where d is radiation dose and α and r rare adjustable parameters:

$$S = \exp(-\alpha d - \left(\frac{\alpha}{r}\right) d^2) \quad (1)$$

Parameter α is a linear dose response term, and parameter r quantifies the quadratic dose response component: low values of r generate a strongly "downwardly curving" dose response (on a log scale). Best-fit values of α and r were found by maximizing the log-likelihood for the binomial distribution where the number of plated cells represented the number of "trials" and the number of observed colonies represented the number of "successes" (Shuryak et al., 2016). Uncertainties (95% confidence intervals, CIs) for parameter values were estimated by profile likelihood.

4.2.6 Ultrastructural damage

After re-hydration, controls and colonies irradiated with maximum irradiation dose (117.07 kGy) were prepared according to the protocol reported by Pacelli et al. (2016). TEM observations were performed using a JEOL 1200 EX II electron microscope at the Center for High Instruments, Electron Microscopy Section of University of Tuscia (Viterbo). Micrographs were acquired by the Olympus SIS VELETA CCD camera equipped the iTEM software.

4.3 Results

4.3.1 DNA integrity

The integrity of DNA template after ionizing irradiation was assessed by amplifying three different gene lengths and by fingerprinting analysis.

Cryomyces antarcticus

Amplicons were obtained both for ITS and for LSU regions, fragments of 700 bp, 1600 bp and 2000 bp *C. antarcticus* DNA respectively, after irradiation treatments (Fig. 4.1a, b and c). All bands were well preserved in 700 bp and 1600 bp gene length; the gel analysis for ITS measured 100% relative density of the band till the highest irradiation dose while a reduction ranging from 34% to 31% was measured in LSU from 55.8 to 117.07 kGy. For 2000 bp fragments a reduction up to 50% was measured starting from 19.04 kGy with a progressive decrease; besides 20% of intensity was still maintained at 117.07 kGy (Fig. 4.1c). The RAPD profiles were mostly preserved in all the conditions tested with a visible reduction of the highest MW band at the two last irradiation doses. Yet, a reduction was also recorded at 27.15 kGy. (Fig. 4.1d).

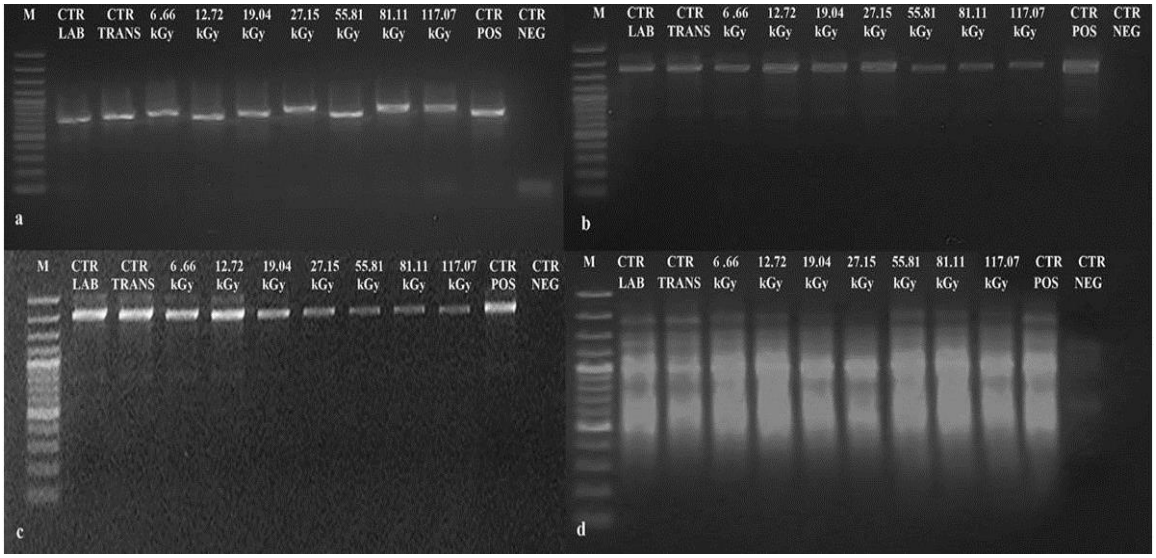

Fig. 4.1 Assessment of the DNA damage in *C. antarcticus* irradiated with gamma-rays doses (^{60}Co); single gene PCR **a)** 700 bp; **b)** 1600 bp; **c)** 2000 bp and **d)** RAPD genomic fingerprinting.

Umbilicaria sp.

All amplifications worked out for all the gene-lengths for *Umbilicaria* sp. (Fig. 4.2a, b and c).

Band intensities were well preserved (round 100%) in 700 bp fragments with a relative density reduction of 8% at highest dose only. In the LSU gene (1600 bp), a decrease of 23% in relative density was measured starting from 55.81 kGy and 50% was still measured at maximum dose. For 2000 bp fragments the DNA was more affected by treatments and almost no signal was present at maximum dose (round 1% only of relative density was

maintained, Fig. 4.2c). The RAPD profiles were well preserved till 55.81 kGy, while the disappearance of the highest molecular weight (MW) band was observed at 81.11 kGy and 117.07 kGy doses (Fig. 4.2d).

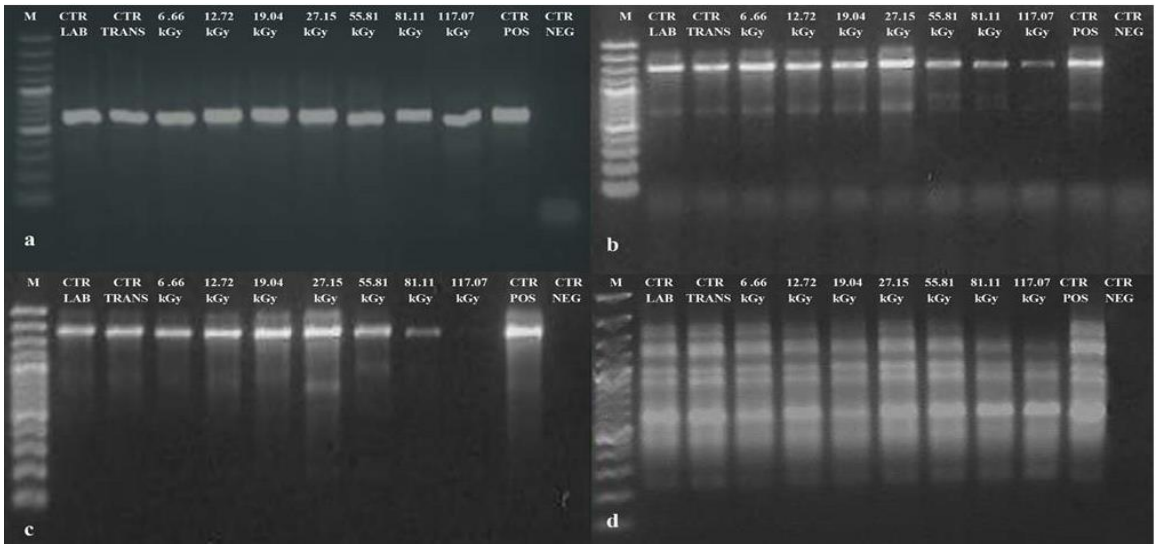

Fig. 4.2 Assessment of the DNA damage *Umbilicaria* sp. irradiated with gamma-rays doses (^{60}Co); single gene PCR **a)** 700 bp; **b)** 1600 bp; **c)** 2000 bp and **d)** RAPD genomic fingerprinting.

Stichococcus sp.

The effect on single genes on DNA of the alga was tested using three different lengths of the SSU portion of ribosomal genes: perfectly visible single bands were obtained at all the exposures (Fig. 4.3). Band intensities were well preserved (round 100%) for the shortest gene lengths, while a reduction up to 50% was measured for the medium gene length starting from 55.81 kGy till the maximum dose applied (Fig. 4.3a, b). The highest effect was observed for the longest genes where a decrease of 88% was measured at 55.81 kGy and an almost complete disappearance of the band at 117.07 kGy where 5% only of band relative intensity was recorded (Fig. 4.3c). The RAPD profiles preserved in all the conditions tested excepted for an intensity reduction of the highest MW band starting from 27.15 kGy onward (Fig. 4.3d).

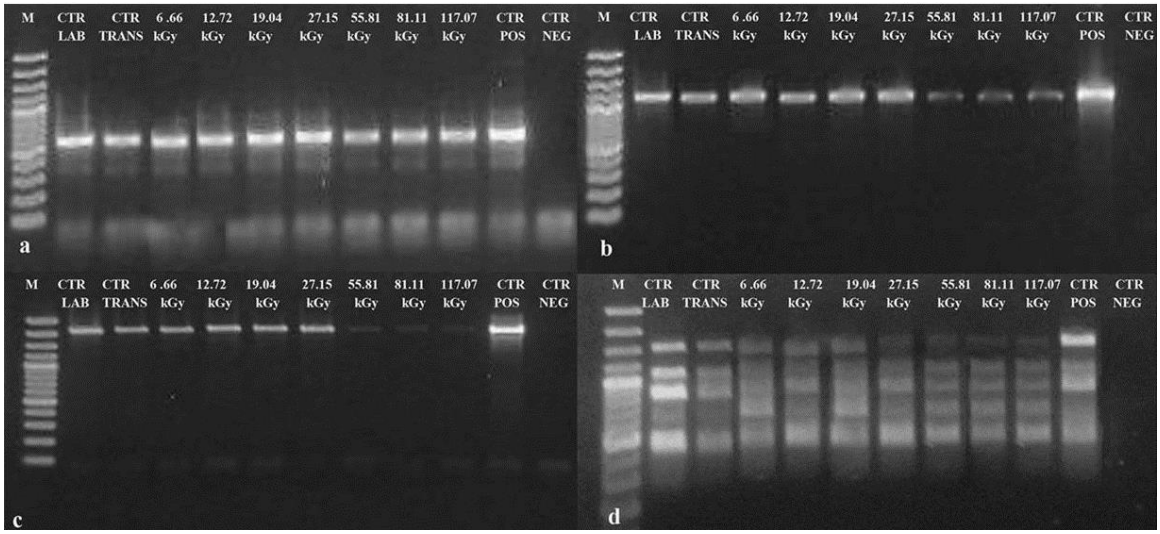

Fig. 4.3 Assessment of the DNA damage in *Stichococcus* sp. irradiated with gamma-rays doses (^{60}Co); single gene PCR **a)** 500 bp; **b)** 1000 bp; **c)** 1500 bp and **d)** RAPD genomic fingerprinting

4.3.2 Clonogenic survival

The cultivation tests indicated that the melanized *C. antarcticus* maintained the highest capacity to multiply after the irradiation treatment with no statistically significant decrease after the first irradiation dose; a progressive decrease was observed until 55.81 kGy where 12% survival was still maintained (Fig. 4.4a). *Umbilicaria* sp. showed a statistically significant decrease in CFU formation at the first irradiation dose already; in general CFU declined more rapidly with the increase of irradiation compared to *C. antarcticus*; 20% of survival was still present at 27.15 kGy but a few colonies were still recorded at 55.81 kGy already (Fig. 4.4b). *Stichococcus* sp. resulted much more sensitive to the treatment with a reduction of CFU to 63% at the first irradiation dose. The decrease of survival was rather sharp with a few colonies at 27.15 kGy already (Fig. 4.4c). The data and LQ model fits to clonogenic survival dose responses for all three organisms are shown in Fig. 4.4d. The LQ model described the data reasonably – substantial discrepancies between best-fit model predictions and observed data occurred only at high doses/low surviving fractions for each organism. This probably occurs because the LQ model is a simplified approximation of more detailed DNA repair models (Sachs et al., 1997; Brenner et al., 1998) and, therefore, its accuracy tends to decrease at high doses/low surviving fractions (Garcia et al., 2006; Kirkpatrick et al., 2008).

C. antartcticus was the most radioresistant of the tested organisms (Fig. 4.4d), and its best-fit dose response parameters were: $\alpha = 6.92$ (95% CIs: 5.57, 8.28) $\times 10^{-3}$ kGy^{-1}, r = 11.03 (8.39, 14.0) kGy. *Umbilicaria* sp. was somewhat more sensitive, and had a more strongly curved dose response shape: $\alpha = 2.39$ (95% CIs: 0.00, 5.07) $\times 10^{-3}$ kGy^{-1}, r = 1.21 (0.51, 2.74) kGy. *Stichococcus* sp. was the most sensitive: $\alpha = 34.17$ (95% CIs: 28.98, 39.40) $\times 10^{-3}$ kGy^{-1}, r = 10.60 (8.22, 13.50) kGy.

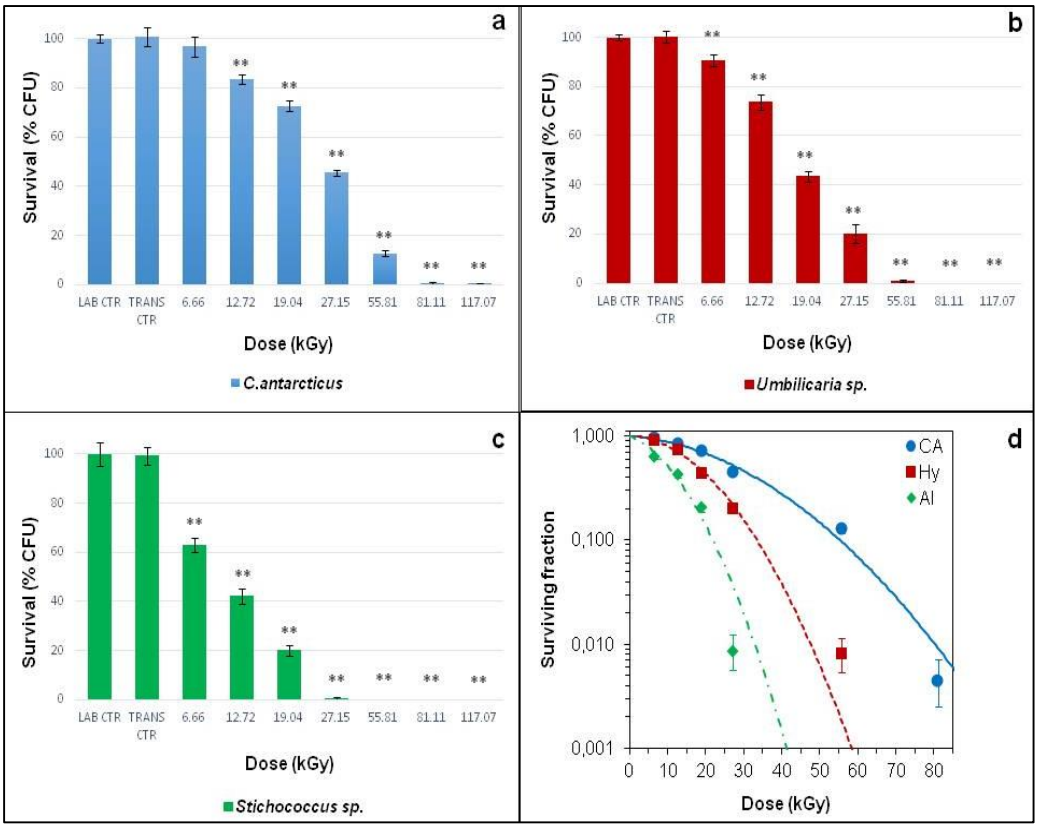

Fig. 4.4 Clonogenic test after ionizing irradiation (^{60}Co), CFU% of **a)** *C. antarcticus*, **b)** *Umbilicaria* sp., **c)** *Stichococcus* sp. Significant differences were calculated by Tukey test with ** = p > 0.001. **d)** Clonogenic survival for *C. antarcticus* (CA), *Umbilicaria* sp. (Hy) and *Stichococcus* sp. (Al). Symbols represent data points, and error bars represent 95% CIs. Curves represent LQ model fits.

4.3.3 Ultrastructural damage

Fig. 4.5 shows the ultrastructural damage observed by TEM in samples exposed at maximum doses compared to untreated controls. All the control cells (Figs. 4.5a, c, e) maintained cell membrane and wall integrity with a well organized cytoplasm even after

dehydration. Irradiated samples of *C. antarcticus* showed a number of empty cells and dispersed cell wall fragments (Fig. 4.5b, white arrows); besides, some cells maintained an appreciable membrane integrity even if suvival was not observed in clonogenic test (Fig. 4.5b, dark arrow). Damaged cells were present in both the irradiated *Umbilicaria* sp. and *Stichococcus* sp., with broken cells discharging cytoplasm (Fig. 4.5d, white arrow) and irregular shaped cells with disorganized cytoplasm, (Fig. 4.5f), respectively.

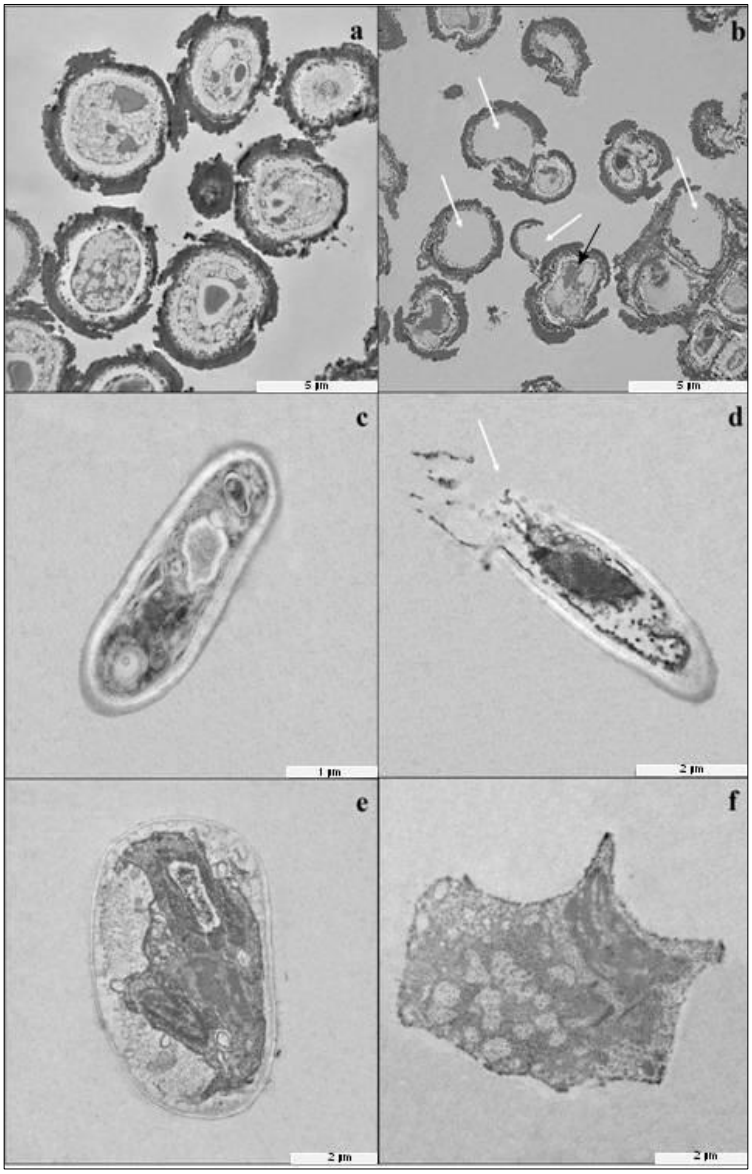

Figure 4.5 TEM micrographs; comparison of untreated colonies (controls) and colonies irradiated at maximum gamma-irradiation dose (117.07 kGy). *C. antarcticus* **a)** control, **b)** irradiated; *Umbilicaria* sp. **c)** control, **d)** irradiated; *Stichococcus* sp. **e)** control, **f)** irradiated.

4.4 Discussion

The survival capacity and DNA damage of organisms in space is of high astrobiological significance to speculate life dispersal between planets, planetary protection and searching for biosignatures.

In the frame of the STARLIFE consortium, which aims to compare the response of different astrobiological model systems to different types of ionizing radiation, three eukaryotic cryptoendolitic Antarctic microorganisms were irradiated, while dried, with high ionizing irradiation doses of gamma-rays, representing part of the galactic cosmic radiation spectrum.

Survival, ultrastructural damage and DNA stability were tested by cultivation test, TEM observations and PCR approaches, respectively; the last was based on the fact that damaged DNA is not suitable for amplification (Selbmann et al., 2011).

The black fungus *C. antarcticus* showed the highest survival ability both in terms of CFU% and gamma-rays dose resistance; in fact 12% of survival was still recorded at 55.81 kGy while *Umbilicaria* sp. and *Stichococcus* sp. were barely able to multiply at 55.81 and 27.15 kGy, respectively. Even the LQ model applied to analyze the survival dose responses confirmed the highest radio resistance of *C. antarcticus* among the tested organisms (Fig. 4.4d). None of the tested organisms were able to multiply after maximum dose of irradiation (117.07 kGy), confirming what observed with TEM, where a consistent ultrastructural damage in all the irradiated test organisms was observed (Fig. 4.5). The scattered intact cells in *C. antarcticus* (Fig. 4.5b) have most probably accumulated some other damage limiting their vitality, since no growth was observed in samples irradiated at maximum dose.

This higher survival is likely related to the conspicuous amount of melanin in the thick cell wall of *C. antarcticus* (Fig. 4.4a) which presence confers to the fungus a notable capacity to withstand ionizing radiation (Pacelli et al., unpublished data). Melanins in black fungi are different types of high molecular weight pigments produced by enzymatic coupling of phenolic units reported as 1,8-dihydroxynaphthalene (Kogej et al., 2004); they can play many different roles in microorganisms because of their unique physical-chemical properties. These pigments are responsible for the typical dark green to brown or totally black colour of these fungi and confer them the ability to survive a number of different

external pressures, such as excessive heat or cold, extreme pH or osmotic conditions, polychromatic UV radiation; melanins also seem to mediate tolerance toward metals (Selbmann et al., 2014b). Many melanized fungi are also very radioresistant, requiring radiation doses exceeding 5 kGy to reduce cell survival to 10% (Dadachova et al., 2008). Such doses are roughly 1000-fold higher than the lethal dose for humans, showing that extreme radioresistance is not limited to prokaryotes such as *Deinococcus radiodurans*, and can be achieved by eukaryotic cells. Dadachova et al. (2007) even reported the surprising results that low levels of gamma radiation even stimulate growth in melanized fungi.

Stichococcus sp. resulted the most affected with a rapid decrease of survival even at the lowest dose: no CFU were recorded starting from 55.81 kGy. This high susceptibility of eukaryotic phototrophs was already reported for the photobionts of the lichen *Circinaria gyrosa* showing a sudden reduction of photosynthetic activities at the same irradiation dose (de la Torre, this issue).

The analyses on DNA stability confirmed the data observed in the cultivation test; *C. antarcticus* DNA showed the highest stability maintaining 20% of band relative density even at the maximum dose applied and the longest gene considered. The DNA of *Umbilicaria* sp. was also well preserved but a relative density reduction of the bands was already well visible in the second gene length tested (LSU) at high doses, while the SSU amplicons completely disappeared at the maximum irradiation dose applied.

The most susceptible to the treatment was *Stichococcus* sp. showing a reduction up to 50% for the medium gene length starting from 55.81 kGy and an almost complete disappearance of the highest MW bands starting from 55.81 kGy.

Except for the disappearance of the highest MW bands at high irradiations, the RAPD profiles were well preserved in all the tested organisms. These results are in concordance with Atienzar et al. (2002) reporting that DNA damage is visible in highest molecular weight amplicons first, because of the higher probability to accumulate mutations.

Notably in our experiments DNA could be easily detected in all the tested organisms even after high irradiation treatment and even when microorganisms lost their viability and ultrastructural damage is extended. A good biosignature for life detection must have both high specificity and high intrinsic stability; DNA would provide unequivocal proof of the

presence of extant life, or at least its presence in the recent past (Aerts et al., 2014). Here we found that DNA has also a high intrinsic stability and that it could be regarded as biosignature even after life extinction.

Previous criticisms of the use of such biosignatures asserted that life on other planets does not have to be based on an exact replica of terrestrial DNA, and common gene sequences used as primer targets for Earth organisms may not be present in extraterrestrial DNA (Röling and Head, 2005) making it difficult to detect putative extraterrestrial DNA. The use of primers with very low specificity could overcome this problem, allowing the amplification of short, repeated sequences of eukaryotic genome.

The discovery of nucleotide bases in the Murchison meteorite lends credence to the possibility of life emerging on another planet and travelling via meteorite to Earth (Martins et al., 2008). The detection of nucleobases in meteorites indicates that fundamental building blocks of complex biopolymers are present beyond Earth and could potentially be involved in the evolution of life on other planets (Aerts et al., 2014). There is a high potentiality to spread material from one planet to another, due to the large number of meteorites within our solar system, theoretically paving way for an interplanetary ancestor of all life (Mileikowsky et al., 2000a, 2000b).

One concern is the life-span of biomolecules. The timescales that DNA can persist in the fossil record are still debated although lifetimes of at least several tens of thousands to a hundred thousand years (Lindahl, 1993; Wayne et al., 1999) are generally accepted (Aerts et al., 2014). Critics assume that reports of ancient DNA dating from millions of years ago are the result of flawed experiments or contamination (Pääbo et al., 2004; Willerslev et al., 2004). Nonetheless, certain conditions, such as halite crystals, permafrost and marine sediments, could improve the conservation of ancient DNA (Sankaranarayanan et al., 2014).

The low temperatures in space, of about 80 K, reduce DNA breaks by half as compared to ambient Earth temperatures (Lindahl, 1993), and the dry space environment preserves DNA (Lyon et al., 2010). These findings encourage to postulate that the low temperatures and dry conditions on Mars may better preserve DNA, even in long-term, than on Earth (Kanavarioti and Mancinelli 1990; Sephton, 2010). Other studies on *C. antarcticus* DNA

damage after exposure to Martian atmosphere demonstrated that fungal DNA was not affected and remained perfectly detectable by PCR (Pacelli et al., 2016).

This preliminary study on survival and DNA resistance of eukaryotic extremophiles to gamma radiation, which are part of the cosmic radiation spectrum, is a first step in the investigation of the fate of microorganism and their biomolecules in space. Survival ability of microorganisms to space conditions and radiations in particular is of great interest in space biological research to evaluate potential contamination risks in the frame of planetary protection. Fungi in particular are of high importance on one hand for their potentiality to adapt to human body behaving as opportunists that could danger the health of the crew and, on the other hand, for their strong metabolic and degradative abilities that could affect the integrity of spacecraft materials in long-distant space journeys.

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Chapter 5

5 Melanin is effective in protecting fast and slow growing fungi from various types of ionizing radiation

Abstract

Melanin is a ubiquitous pigment with unique physicochemical properties. The resistance of melanized fungi to cosmic and terrestrial ionizing radiation suggests that melanin also plays a pivotal role in radioprotection. In this study, we compared the effects of densely-ionizing deuterons and sparsely-ionizing x-rays on two microscopic fungi capable of melanogenesis, fast-growing *Cryptococcus neoformans* (CN) and slow-growing *Cryomyces antarcticus* (CA); melanized and non-melanized counterparts were used as comparison. CA was more resistant to deuterons than CN, and similar resistance was observed for x-rays. Melanin afforded protection against high-dose (1.5 kGy) deuterons for both CN and CA (p-values $<10^{-4}$). For x-rays (0.3 kGy), melanin protected CA (p-values $<10^{-4}$) and probably CN. Deuterons increased XTT activity in melanized strains of both species, while the activity in non-melanized cells remained stable or decreased. For ATP levels the reverse occurred: it decreased in melanized strains, but not in non-melanized ones, after deuteron exposition. For both XTT and ATP, the largest and statistically-significant differences by melanization status occurred in CN. Our data show, for the first time, that melanin protected both fast-growing and slow-growing fungi from high doses of densely-ionizing charged particles under physiological conditions. These observations may give clues for creating melanin-based radioprotectors.

Keywords: black fungi, melanin, ionizing radiation, deuterons, x-rays.

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5.1 Introduction

Melanin is a ubiquitous pigment in nature and possesses a variety of unique physical, chemical, paramagnetic and semiconductive properties (Meredith and Sarna, 2006). The melanin amount in *C. neoformans* and other melanized fungi constitute 10-15% of the fungal cellular biomass dry weight (Wang et al., 1996; Revskaya et al., 2012). Many melanized fungi are very radioresistant, requiring radiation doses exceeding 5 kGy to reduce cell survival to 10% (Dadachova and Casadevall, 2008). Such doses are roughly 1000-fold higher than the lethal dose for humans, showing that extreme radioresistance is not limited to prokaryotes such as *Deinococcus radiodurans*, and can be achieved by eukaryotic cells. Moreover, since fungi are eukaryotes, the main features of their cellular machinery share important similarities with the human counterparts; this makes them better microbial models for human radiation responses, than prokaryotes. The resistance of melanized fungi to cosmic radiation in Mir Spacecraft and the presence of numerous melanized fungal species in the damaged nuclear reactor at Chernobyl suggest that melanins also play a pivotal role in protection from ionizing radiation (Dadachova and Casadevall, 2008, Vember et al., 1999, Novikova, 2004).

During the last decade, some important insights into the interaction of fungal melanin with ionizing radiation have been reported. One of the studies indicated that the radioprotective properties of fungal melanin result from a combination of physical shielding, chemical composition, spherical arrangement and free radical quenching (Dadachova et al., 2008) and another demonstrated that irradiation of melanin alters its oxidation-reduction potential (Turick et al., 2011). Radiotropism, the propensity of fungi to grow towards a radiation source was described in the late 1990-s (Vember et al., 1999). Since then the increased growth of melanized fungi was demonstrated after irradiation with γ-rays and x-rays (Dadachova et al., 2007; Robertson et al., 2012; Shuryak et al., 2014) and γ-rays have been shown to alter the electronic properties of melanins (Dadachova et al., 2007; Robertson et al., 2012; Shuryak et al., 2014; Khajo et al., 2011). In addition, recent transcriptomic investigation revealed that ionizing radiation stimulates protein biosynthesis with input from melanin (Robertson et al., 2012). The radioprotective properties of melanins are of high interest for a number of different potential applications, from the development of novel and effective radioprotective materials for saving cancer patients undergoing

radiation therapy from the side effects of ionizing radiation treatment to astronauts protection from space radiation in manned space missions. Data confirm that radioprotective properties of fungal melanins can be exploited in mammalian systems; in fact, it was recently reported that internal administration of melanin protected mice against experimental lethal irradiation (Revskaya et al., 2012; Schweitzer et al., 2010; Kunwar et al., 2012; Rageh et al., 2014).

Most research on radioresistance mechanisms and radioprotection involves the use of high-energy photons (γ- and x-rays) which are sparsely ionizing, so that energy deposition and ionizations are relatively randomly distributed throughout the irradiated material (Sachs *et al*., 1997). However, more recently, densely ionizing high-energy protons and heavy ions which produce a definitive dense track of radiation events in their path (Stewart et al., 2011; Hada and Georgakilas, 2008) are attracting interest in both radiation oncology field and space radiation protection in manned space missions (Durante, 2014). In this study, we compared the effects of highly ionizing deuterons and sparsely ionizing x-rays on two microscopic fungi capable of melanogenesis–pathogenic basiodiomycete forming an induced DOPA-melanin *Cryptococcus neoformans* and slow-growing and space-resistant environmental rock-inhabiting ascomycete sprouting a constitutive DHN-melanin *Cryomyces antarcticus* (Onofri et al., 2012; Onofri et al., 2015). In this study we investigated the effects of melanin on both fungal species exposed to both types of radiation.

5.2 Material and Methods

5.2.1 Microorganisms

Cryomyces antarcticus CCFEE 515 was isolated by R. Ocampo-Friedmann from sandstone collected at Linnaeus Terrace (Southern Victoria Land) by H. Vishniac, in the Antarctic expedition of 1980-81. It has been supplied by the CCFEE, Mycological Section of the Italian Antarctic Museum, (DEB, University of Tuscia, Italy). To obtain non-melanized cells of *C. antarcticus*, tricyclazole [5-methyl-1,2,4-triazolo(3,4-b) benzothiazole] was added to cultures for inhibition of the DHN-melanin pathway, at final concentrations of 10 mg/L, according to Cunha, 2005. Melanized and non-melanized *C. antarcticus* were grown on MEA (malt extract agar: malt extract, powdered 30 g/L; peptone 5 g/L; agar 15 g/L;

Applichem, GmbH) in Petri dishes for three months; then cells were transferred into suspensions for three days before the irradiation. American Type Culture Collection (ATCC, Rockville, MD) strain *Cryptococcus neoformans* H99 and its non melanized laccase mutant deficient Lac(-) (kind gift from Dr. A. Idnurm, Duke University, NC) were used in all experiments. *C. neoformans* was grown in Sabouraud dextrose broth (Difco Laboratories, Detroit, MI) for 24 hrs at 30°C with constant shaking at 150 rpm. Melanized *C. neoformans* cells were generated by growing the fungus in minimal medium with 1 mM 3,4-dihydroxyphenylalanine (L-dopa) for 5–10 days.

5.2.2 Irradiation conditions

Both x-ray and deuteron irradiations were performed at the Radiological Research Accelerator Facility, Columbia University, New York. Fungal cell concentration was adjusted to 10^7 per mL (*C. antarcticus*) and 10^8 per mL (*C. neoformans*) before the irradiation. For x-rays, 1.3 mL of each strain were placed in 1.5 mL Eppendorf tubes for each dose and subjected to 50 kVp x-ray irradiation at dose rate of 5 Gy/min. The deuteron irradiations were performed in customized Mylar-bottom dishes at the dose rate of 1.2-2.5 Gy/sec and at Linear Energy Transfer (LET) of 40.8 keV/μm. The doses and the dose rates are described in Table 5.1. Shortly before irradiation, a small volume (30 μl) of culture medium containing suspended fungal cells was applied to the center of the Mylar surface within the dish, and a glass cover slip was floated on top of the liquid to ensure the formation of a layer with uniform thickness. The dishes (up to 20 at a time) were placed into an aluminum wheel, which was rotated by a computer-controlled motor at a rate defined by the desired dose for each wheel (including zero dose as a control). As the wheel was rotated, a beam of particles sequentially traversed each dish vertically from bottom to top, through the Mylar film and through the overlying cell suspension.

Clonogenic survival assay. Survival of *C. antarcticus* and *C. neoformans* was determined by measuring the number of colony forming units (CFUs) following treatment. After irradiation *C. antarcticus* cells were suspended in 1.3 mL of PBS and diluted to a final concentration of 5,000 cells/mL, and 0.1 mL of the suspension was spread on Petri dishes supplemented with MEA (3 replicates), incubated at 15°C for 3 months and counted. The

C. neoformans cells were diluted to 4,000 cells/ml, 0.1 ml was plated and the colonies were counted after two to three days.

Table 5.1 Deuterons and x-rays radiation doses used in the study.

Deuterons	Applied doses (Gy)	x-rays	Applied doses (Gy)
LET: 40 KeV/um Dose Rate: 2 Gy/sec	0	Dose Rate: 5 Gy/min	0
	50		
	250		150
	500		
	1500		300

5.2.3 Mechanistic modeling of clonogenic survival data.

The most commonly used mechanistic model for the dose responses of clonogenic survival of irradiated cells is a linear quadratic (LQ) model (Sachs et al., 1997; Brenner et al., 1998; Hall and Giaccia, 2006). It has been quite successful at describing the data (particularly for mammalian cells) and making robust predictions over a wide range of irradiation scenarios, including dose fractionation or protraction (Brenner et al., 1998; Brenner et al., 2002). However, there is growing evidence that the inducibility of some DNA repair pathways, and the effects of inter- and intra-cellular signaling can alter the shape of the clonogenic survival dose response, particularly at radiation doses where the damage is not lethal to the majority of cells (Fernandez-Palomo et al., 2016; Xue et al., 2015; Coic et al., 2008; Singh and Krishna, 2006; Bauchwitz and Holloman, 1990). The induced repair (IR) model (Marples and Joiner, 1993) which assumes that cells become progressively more radioresistant as radiation dose increases, is a convenient and mechanistically-plausible approach for accounting for the effects of these factors on the survival dose response. Although measurements of DNA damage repair are beyond the scope of the current study, we applied the IR model to the clonogenic survival data for the purpose of quantifying the dose responses for the tested organisms.

We chose the following parametrization for the IR model: linear dose response coefficients for situations when repair and/or pro-survival signaling are fully induced (α_{min}) or not

induced (α_{max}), the dose dependence for the induction of repair and/or pro-survival signaling (δ), the quadratic dose response coefficient (β), and the plating efficiency PE. The equation for cell surviving fraction S(d) after radiation dose d is:

$$S(d) = PE\ e^{-(\alpha_{min} + (\alpha_{max} - \alpha_{min})\ \exp[-\delta\ d])d - \beta\ d^2} \quad (1)$$

At very low doses, when induction of repair has not yet occurred, the term exp[–δ d] remains close to 1 and the dose response predicted by Eq. 1 is dominated by the parameter α_{max}. In contrast, at higher doses induction occurs and the term exp[–δ d] becomes close to 0, so the dose response is dominated by the parameter α_{min}. Finally, at very high doses the dose response is dominated by the parameter β. If the inducible processes play little/no role in determining survival, then the IR model reduces to the LQ model: $\alpha_{min} = \alpha_{max}$ and δ=0.

Statistical analysis of clonogenic survival data. To analyze the clonogenic survival data, we viewed them as a set of Bernoulli trials, where the number of "successes" is the counted number of colonies on the i-th plate ($k(d,i)$) after radiation dose d, and the number of "trials" is the number of seeded cells ($n(d,i)$) on the same i-th plate. The "success" probability is assumed to have a binomial error distribution (Shuryak *et al.*, 2016).

The log-likelihood function (LL) for this scenario is below, where S(d) is the surviving fraction described by the IR model (Eq. 1):

$$LL = \sum_d \left(\sum_i k(d,i)\ \ln\left[S(d)\right] + \sum_i \left[n(d,i) - k(d,i)\right] \ln\left[1 - S(d)\right]\right) \quad (2)$$

Here, all constant terms which do not involve S(d) and therefore do not change the results of the optimization were omitted for simplicity.

The LL expression from Eq. 2 is theoretically correct, but the exact values of $n(d,i)$ are not known. Instead, only the average values for the numbers of seeded cells N(d) are available. Consequently, we modified Eq. 2 as follows, to produce LL_e which is the LL expression using only the data that are available:

$$LL_e = \sum_d \left(\sum_i k(d,i)\ \ln\left[S(d)\right] + \sum_i max[0, N(d) - k(d,i)] \ln\left[1 - S(d)\right]\right) \quad (3)$$

Here, the expression $max(0, N(d) - k(d,i))$ is used to account for the instances when $k(d,i)$ could, by chance, become > N(d). Such instances would be rare for radiation doses substantially larger than zero, where S(d) would be << 1.

To implement this data analysis approach, we determined the best-fit values of parameters which enter into the model for S(*d*), for which the LL_e function (Eq. 3) was maximized. The maximization was performed using the sequential quadratic programming (SQP) algorithm in Maple 2015® software. The plating efficiency (PE), which is unrelated to radiation, was allowed to be different for different experimental dates, whereas all four radiation dose response parameters (α_{min}, α_{max}, δ, β) were kept constant for all experiments using the given fungal strain and radiation type. The parameter values were restricted to ≥ 0 to maintain mechanistic plausibility.

The probability of finding the global maximum (rather than local maxima) was enhanced by using 100 random initial conditions for the model parameters. Uncertainties (95% CIs) for best-fit model parameter values were estimated by profile likelihood as follows: 10,000 Monte-Carlo-generated parameter values in the vicinity of the best-fit values were used to estimate the critical contour of the log likelihood function, which is based on the asymptotic X^2 behavior of the log likelihood distribution.

To quantify the effect of melanization status on the clonogenic survival of each tested species, we compared the plating efficiencies for melanized and non-melanized strains at the same radiation dose. The observed plating efficiency is the ratio of the number of counted colonies to the number of plated cells. Uncertainties (95% CIs) for the plating efficiency were calculated by the score confidence interval approach for binomial proportion (Agresti and Coull, 1998). Ratios of plating efficiencies for melanized vs non-melanized strains and corresponding p-values were estimated by Monte Carlo simulation.

5.2.4 Determination of metabolic activity of melanized and non-melanized cells subjected to deuteron and x-rays radiation by XTT and MTT assays.

After irradiation, melanized and non-melanized fungal cells were washed, suspended in PBS and 100 μL cells at 10^5 - 10^6 cells/ml concentration were placed into wells in 96 well plates, 3 wells for each condition. For XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) assay 40 μL (1 mg/mL XTT in PBS) and 4 μL 1mM menadione in acetone were added to each well, the plates were covered with foil and incubated at room temperature for 2, 3, 4 and 12 hours. We show the readings obtained after 4 hours. The absorbance (Labsystem Multiskan, Franklin, MA) was read at

492 and 650 nm, and the absorbance at 650 nm was subtracted from the absorbance at 492 nm. For MTT (2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide) assay, a MTT solution in PBS was added to the wells with the cells, so that the final MTT concentration was 0.5 mg/mL. After incubation at room temperature for 24 hours and 72 hours, the cells were spun down at 2,000 rpm, supernatant was discarded, followed by addition of 100 µL 10% Sodium dodecyl sulfate (SDS) in 50% dimethylformamide (DMF) to each well. The absorbance was read at 550 and 650 nm, and the absorbance at 650 nm was subtracted from the absorbance at 550 nm. The results show the 24 hours readings. Both XTT and MTT results were normalized by the number of cells per well and these values were again normalized by dividing by the control values which were the non-irradiated cells at time zero.

5.2.5 ATP assay

Following experimental treatments, the cellular extracts were prepared for adenosine triphosphate (ATP) measurements by freezing 0.2 mL cells in 0.5 mL tube in a dry ice-ethanol bath. TE buffer (0.1 mL) was added to the frozen cells and the mixture was boiled for two minutes. Cell debris was pelleted by centrifugation at 1000 rcf for 10 min, and 10 µL/well of the cell supernatants were assayed for ATP using the luciferase/luciferin kit from Molecular Probes (Eugene, Oregon, USA). Emitted light was measured in a Wallac Perkin Elmer Victor 2 luminometer/plate reader (Waltham, MA, USA). Measurements were made in triplicate for each treatment. The assay was linear over a range of extracts derived from 0.3 to 2.6 x 10^5 cells. The luminometer readings in the luciferase assay increased for about 10 min after addition of the substrate and enzymes, followed by gradual decrease in luminescence. Readings recorded at 5 min are shown. ATP concentrations were calculated using a standard curve for every assay. The ATP concentrations were then normalized as for XTT and MTT assays, first by dividing the ATP concentration by cell number, and then dividing that value by the control value which were the non-irradiated cells at time zero.

Statistical analysis and descriptive modeling of metabolic assay data. To the best of our knowledge, mechanistic modeling of metabolic assay data after irradiation is not as developed as modeling of clonogenic survival. Consequently, we used simple descriptive

modeling (multiple linear regression) on the metabolic assay data. The response variable (either XTT, MTT or ATP) measured in each sample was normalized by the mean for non-irradiated control samples, and logarithm transformed. The transformation was intended to bring the error distribution closer to the normal distribution, and to avoid biologically impossible negative values. The independent variables were radiation dose and dose squared. The linear regression was performed separately for each fungal strain and radiation type.

5.3 Results

5.3.1 Protective effects of melanin on both fungal species

The clonogenic survival of melanized and non-melanized *C. neoformans* and *C. antarcticus* after deuteron irradiation is shown in Fig. 5.1A and 1B, respectively. *C. neoformans* H99 and its non melanized laccase mutant deficient Lac(-) were used to generate melanized and non-melanized cells, respectively, while to obtain non-melanized cells of *C. antarcticus,* tricyclazole [5-methyl-1,2,4-triazolo(3,4-b) benzothiazole] was added to cultures for inhibition of the DHN-melanin pathway, as melanin deficient mutants for this fungus are not available. Data, which encompassed a wide range of doses (0-1.5 kGy) suggested that both melanized and non-melanized *C. neoformans* strains showed fairly high (>10%) survival at doses <0.5 kGy (Fig. 5.1A), but at 1.5 kGy melanized cells survived much better than non-melanized ones. At this high dose of deuterons, melanization clearly resulted in increased survival of *C. neoformans* in all replicate experiments: the magnitude of this protective effect was quite large (10-fold) (Fig. 5.1C), and there was robust statistical significance (Table 5.2). *C. antarcticus* was clearly more resistant to killing by deuteron radiation than *C. neoformans* (Fig. 5.1B). The melanized strain showed substantially higher survival at 1.5 kGy (Fig. 5.1C), and this effect had robust statistical significance (Table 5.2).

Because of the technical reasons, the dose range delivered by x-rays exposure (0-0.3 kGy) was much narrower and even much lower than the deuteron dose rate (Table 5.1). However, the results revealed that in contrast to deuteron irradiation, non-melanized *C. neoformans* was more resistant to x-rays than non-melanized *C. antarcticus* (Fig. 5.2A, B). In fact, it was as resistant as the melanized strain (Fig. 5.2C, Tables 5.2-3). For C.

antarcticus the high sensitivity of the non-melanized strain to x-rays resulted in statistically significant difference in survival between the melanized and non-melanized cells (Fig. 5.2C, Tables 5.2-3). Overall, these results for the first time show that in both *C. neoformans* and *C. antarcticus* the presence of melanin provided protection against high doses of deuterons.

Table 5.2 Comparison of clonogenic survival of melanized vs non-melanized strains of *C. neoformans* (black) and *C. antarcticus* (green) after the highest tested doses of deuteron and x-rays irradiation. For each replicate experiment, the ratio of plating efficiencies (and its 95% CIs) for melanized vs non-melanized cells represents the ratio of clonogenic survival: values >1 indicate that melanized cells had higher survival than their non-melanized counterparts. The p-value represents the probability that the plating efficiency ratio was ≤1.

Organism	Radiation type and dose	Replicate experiment	Plating efficiency ratio: melanized vs non-melanized			p-value
			value	95% CIs		
C. neoformans	deuterons, 1.5 kGy	A	10.75	5.72	29.62	$<10^{-4}$
		B	4.50	2.12	15.72	$<10^{-4}$
C. antarcticus	deuterons, 1.5 kGy	A	1.73	1.59	1.89	$<10^{-4}$
C. neoformans	x-rays, 0.3 kGy	A	2.54	2.12	3.04	$<10^{-4}$
		B	1.11	0.86	1.42	0.212
		C	1.16	0.92	1.45	0.106
C. antarcticus	x-rays, 0.3 kGy	A	87.94	65.33	131.35	$<10^{-4}$
		B	4.94	4.37	5.62	$<10^{-4}$

Table 5.3 Best-fit model parameters for clonogenic survival of irradiated *C. neoformans* and *C. antarcticus*. Italic font indicates parameter values, which are significantly affected by melanization status. Parameter meanings are: linear dose response coefficients for situations when DNA repair and/or pro-survival signaling are fully induced (α_{min}) or not induced (α_{max}), the dose dependence for the induction of DNA repair and/or pro-survival signaling (δ), the quadratic dose response coefficient (β).

Organism	Radiation type	Melaniza-tion	Best-fit model parameters											
			α_{min} (kGy^{-1})			α_{max} (kGy^{-1})			δ (kGy^{-1})			β (kGy^{-2})		
			Value	95% CIs		value	95% CIs		value	95% CIs		value	95% CIs	
C. neoformans	Deuterons	Yes	0.00	0.00	0.00	*13.84*	*12.9*	*15.06*	*3.58*	*3.37*	*4.56*	1.24	0.62	1.33
		No	0.00	0.00	0.00	*5.54*	*5.07*	*6.04*	*1.00*	*0.63*	*1.62*	1.20	0.60	1.62
	x-rays	Yes	1.28	0.64	2.56	8.06	4.23	16.07	8.36	4.19	16.68	1.95	0.98	3.89
		No	0.71	0.14	3.57	2.75	0.55	13.79	7.99	1.60	40.06	7.72	1.54	11.47
C. antarcticus	Deuterons	Yes	0.00	0.00	0.00	*1.99*	*1.85*	*2.14*	1.10	0.86	1.68	0.27	0.03	0.34
		No	0.00	0.00	0.00	*3.70*	*3.57*	*3.86*	0.80	0.76	0.94	0.00	0.00	0.04
	x-rays	Yes	0.40	0.04	3.99	*0.94*	*0.09*	*9.44*	2.12	0.21	21.22	5.13	0.51	7.71
		No	1.64	0.26	9.24	*10.96*	*10.0*	*69.12*	1.03	0.32	6.52	1.42	0.23	8.96

5.3.2 Comparison of the IR and LQ models

The best-fit induced repair (IR) model prediction curves for deutron-irradiated *C. neoformans* and *C. antarcticus* were consistent with the experimental data (Fig. 5.1). The survival curves for melanized vs. non-melanized strains of *C. neoformans* diverged at the highest tested dose of 1.5 kGy. The melanized strain clearly survived this dose better, than the non-melanized one (Fig 5.1A, C). A similar pattern was observed for *C. antarcticus* (Fig. 5.1B, C). These melanization-dependent

differences in survival were mathematically described by the best-fit parameter values for the IR model (Table 5.3).

Overall, the IR model described the data adequately, much better than the simpler linear quadratic (LQ) model (by ≥14 sample size corrected Akaike information criterion units). However, the IR model is not the only plausible explanation for the shape of the dose responses we observed here.

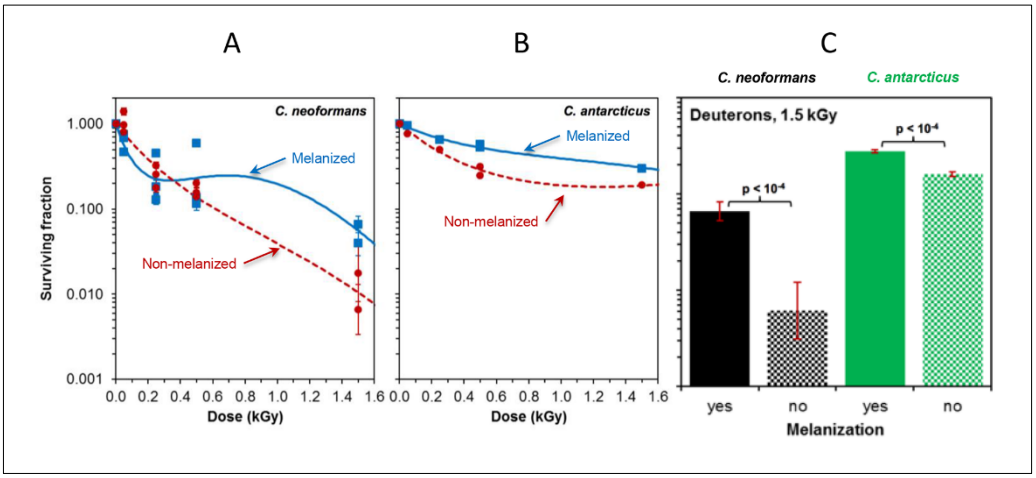

Fig. 5.1 Clonogenic survival of melanized and non-melanized *C. neoformans* and *C. antarcticus* after deuteron irradiation: A) *C. neoformans*; B) *C. antarcticus*. Symbols represent data and curves represent IR model fits. Several symbols at the same radiation dose and fungal strain represent data from different replicate experiments. Error bars represent 95% CIs. Blue = melanized, red = non-melanized; C) Comparison of clonogenic survival of melanized vs non-melanized strains of *C. neoformans* (black) and *C. antarcticus* (green) after the highest tested doses of deuteron and x-ray irradiation. Error bars represent 95% CIs.

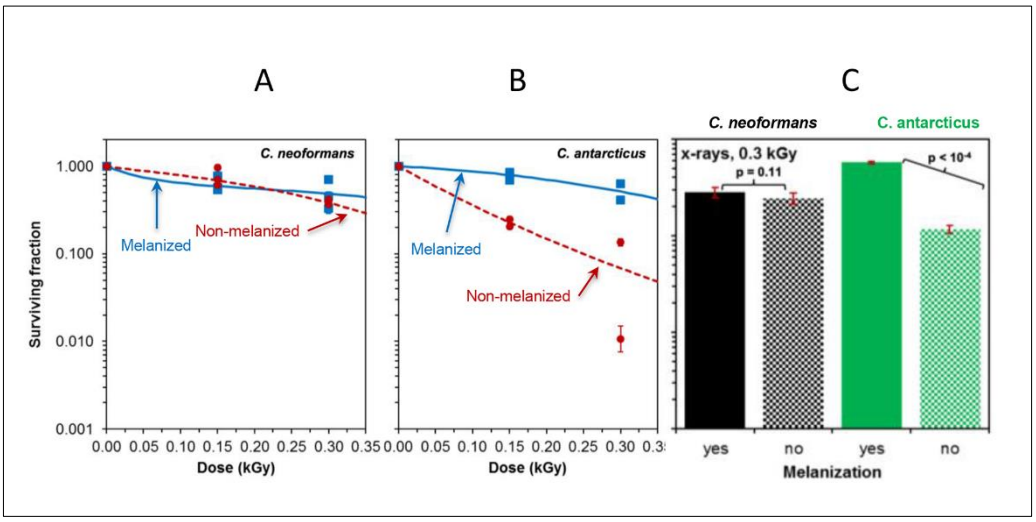

Fig. 5.2 Clonogenic survival of melanized and non-melanized *C. neoformans* and *C. antarcticus* after x-rays irradiation: A) *C. neoformans*; B) *C. antarcticus*. Symbols represent data and curves represent IR model fits. Error bars represent 95% CIs. Blue = melanized, red = non-melanized; C) Comparison of clonogenic survival of melanized vs non-melanized strains of *C. neoformans* (black) and *C. antarcticus* (green) after the highest tested doses of deuteron and x-ray irradiation. Error bars represent 95% CIs.

5.3.3 Metabolic activity assays

XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) and MTT (2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide) results demonstrate differences in metabolic activity for melanized vs non-melanized cells post irradiation. The use of XTT and MTT assays in parallel can help to define the location of the melanin-mediated electron transfer in the cells; positively charged MTT is taken into the cells via the plasma membrane potential and is reduced intracellularly while the negatively charged XTT is cell impermeable and its reduction occurs at the cell surface (Berridge et al., 2005). Our results (Fig. 5.3, Table 1S) suggest that deuteron irradiation of *C. neoformans* increased XTT levels in melanized cells, but not in non-melanized ones. The effects of x-rays were less clear, probably due to limitations of the dose range, as previously mentioned. For *C. antarcticus*, deuterons and x-rays increased XTT in melanized cells, but in non-melanized ones there was no statistically-significant change (Fig. 5. 4, Table 1S).

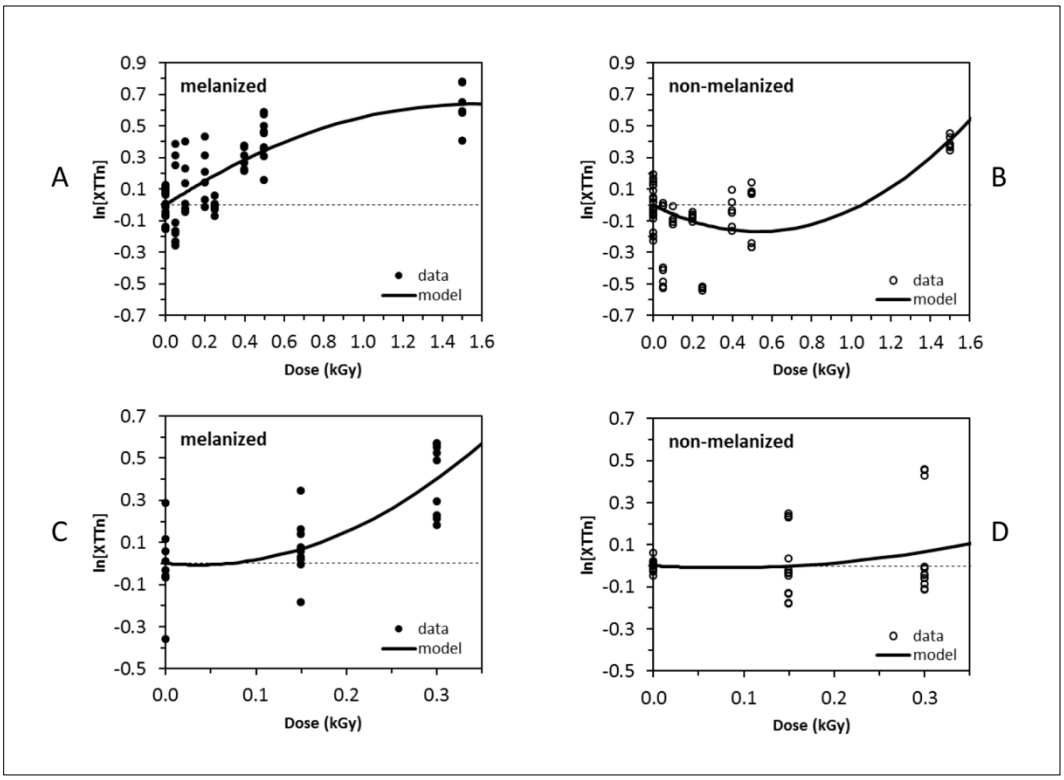

Fig. 5.3 Effects of irradiation on *C. neoformans* XTT reducing activity. A) Melanized cells exposed to deuterons. B) non-melanized cells exposed to deuterons. C) melanized cells exposed to x-rays. D) non-melanized cells exposed to x-rays. Closed symbols represent melanized cells, and open symbols represent non-melanized cells. The results were normalized by the number of cells per well and these values were again normalized by dividing by the control values which were the non-irradiated cells at time zero. n stands for normalized.

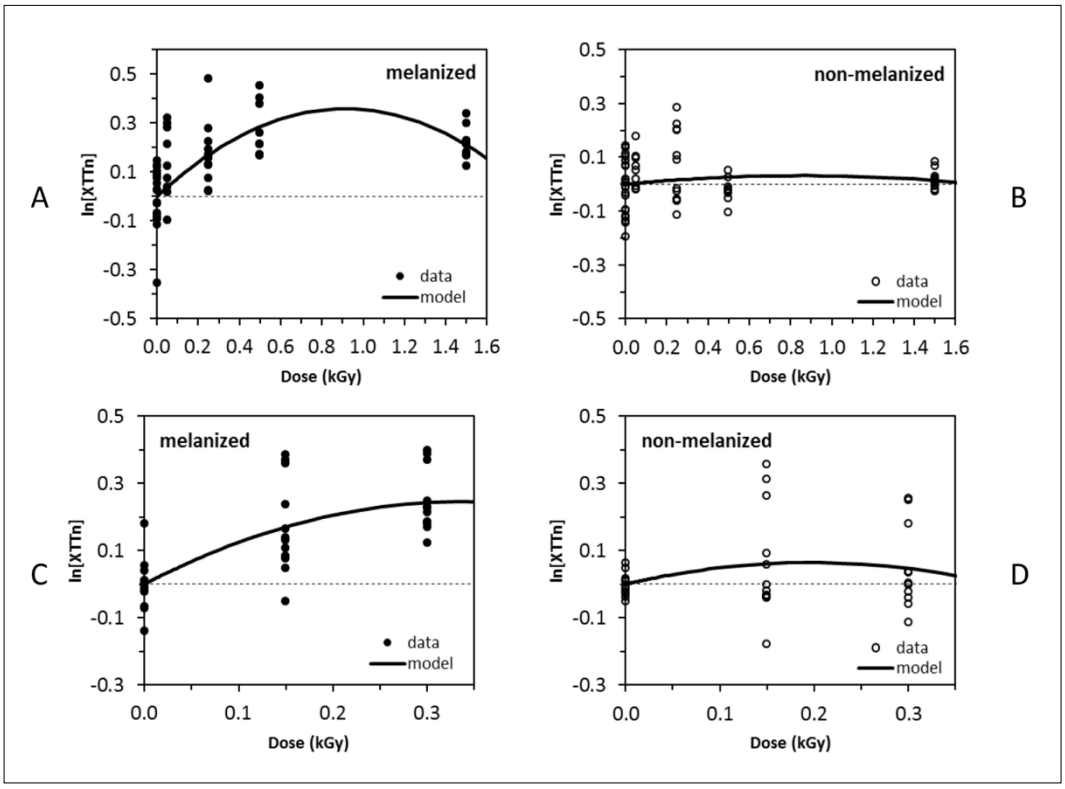

Fig. 5.4 Effects of irradiation on *C. antarcticus* XTT reducing activity. A) Melanized cells exposed to deuterons. B) non-melanized cells exposed to deuterons. C) melanized cells exposed to x-rays. D) non-melanized cells exposed to x-rays. Closed symbols represent melanized cells, and open symbols represent non-melanized cells. The results were normalized by the number of cells per well and these values were again normalized by dividing by the control values which were the non-irradiated cells at time zero, n stands for normalized.

MTT levels in melanized cells were somewhat increased as a result of deuteron irradiation in non-melanized *C. neoformans* cells and in -melanized ones as well. However, the differences between irradiated and control were not statistically significant (both the linear and quadratic coefficients are positive, but their 95% CIs overlap zero; Fig. 5.5, Table 1S). MTT in *C. antarcticus* did not provide a clear pattern (Fig. 5.6, Table 1S) but overall there was not much change in MTT levels for both melanized and non-melanized cells.

Radiation exposure resulted in decreased ATP (adenosine triphosphate) level in melanized cells post irradiation. Figs. 5.7 and 5.8 and Table 1S displays the changes in ATP concentration in melanized and non-melanized cells post irradiation. Deuterons caused significant decreases in ATP levels in melanized *C. neoformans* and *C. antarcticus*, whereas the effect was weaker/inconclusive in non-melanized cells of both species. This is consistent with the report of decrease in ATP in melanized *C. neoformans* cells post exposure to γ-rays, UV and visible light (Bryan et al., 2011).

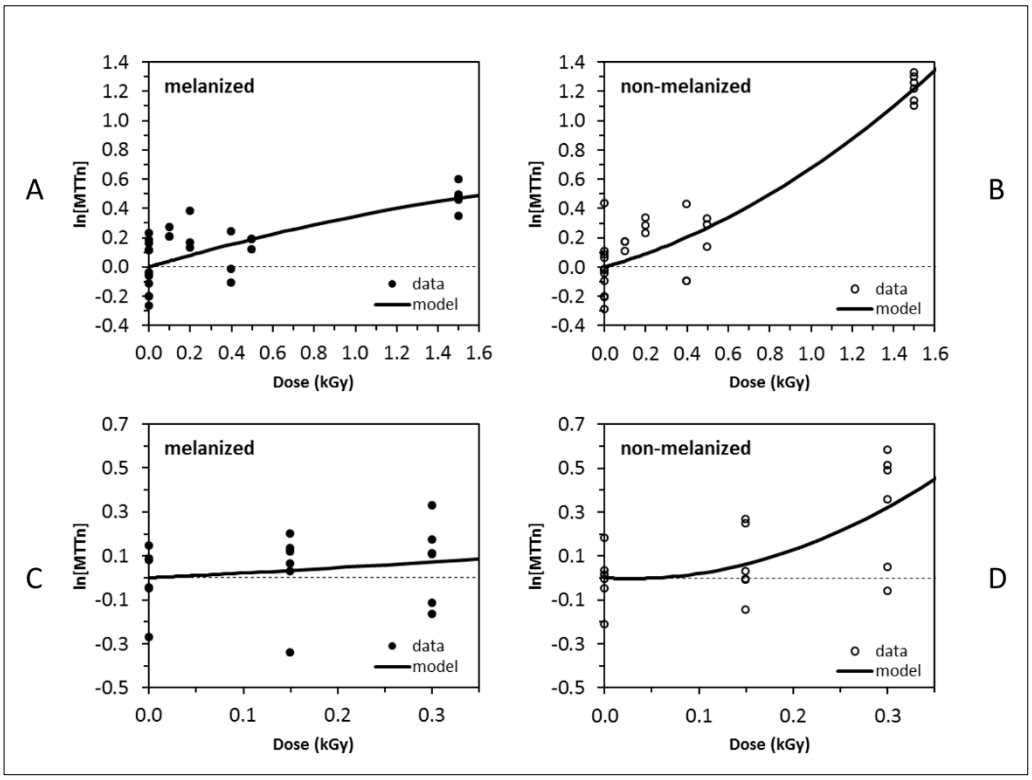

Fig. 5.5 Effects of irradiation on *C. neoformans* MTT reducing activity. A) Melanized cells exposed to deuterons. B) non-melanized cells exposed to deuterons. C) melanized cells exposed to x-rays. D) non-melanized cells exposed to x-rays. Closed symbols represent melanized cells, and open symbols represent non-melanized cells. The results were normalized by the number of cells per well and these values were again normalized by dividing by the control values which were the non-irradiated cells at time zero, n stands for normalized.

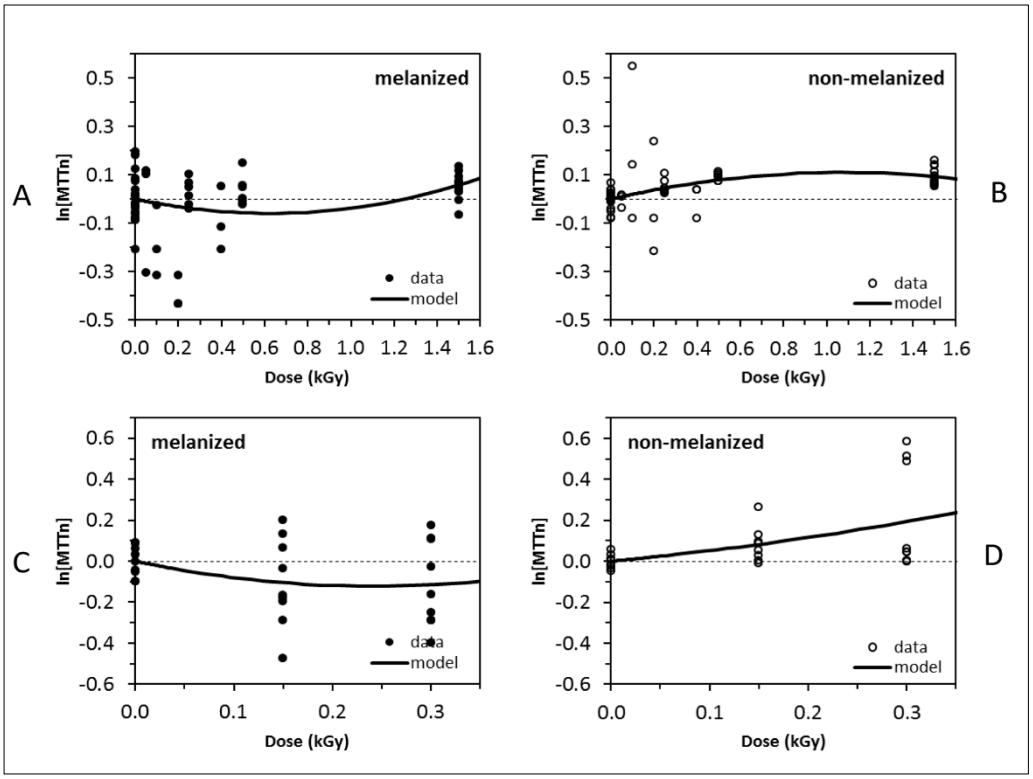

Fig. 5.6 Effects of irradiation on *C. antarcticus* MTT reducing activity. A) Melanized cells exposed to deuterons. B) non-melanized cells exposed to deuterons. C) melanized cells exposed to x-rays. D) non-melanized cells exposed to x-rays. Closed symbols represent melanized cells, and open symbols represent non-melanized cells. The results were normalized by the number of cells per well and these values were again normalized by dividing by the control values which were the non-irradiated cells at time zero, n stands for normalized.

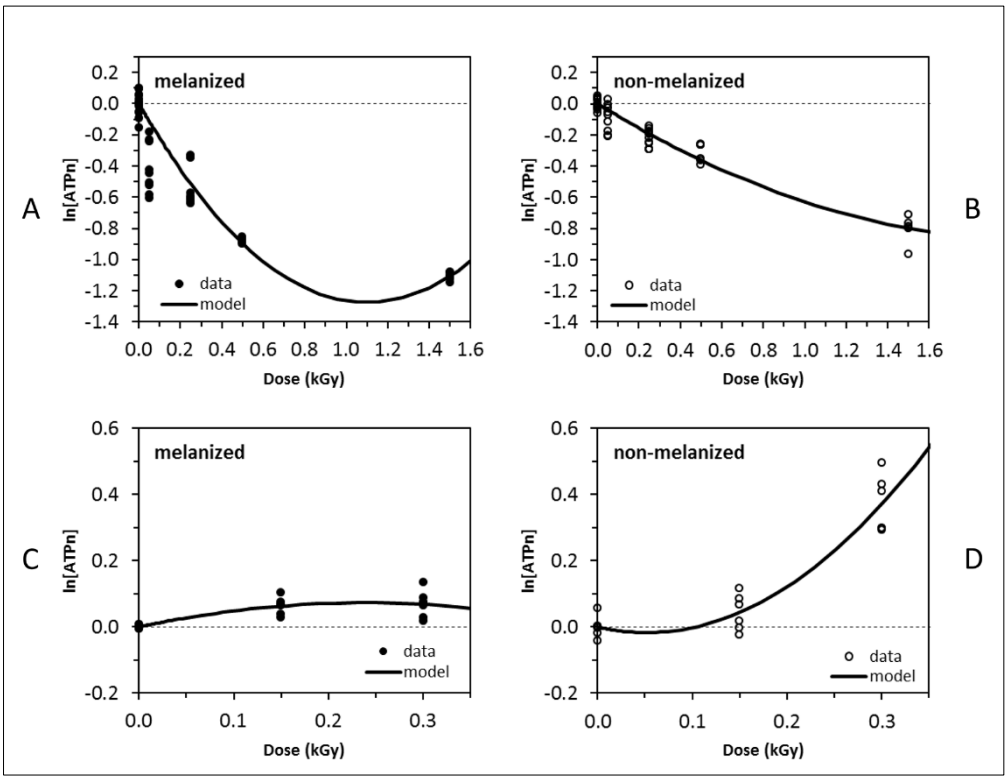

Fig. 5.7 Effects of irradiation on *C. neoformans* ATP level. A) Melanized cells exposed to deuterons. B) non-melanized cells exposed to deuterons. C) melanized cells exposed to x-rays. D) non-melanized cells exposed to x-rays. Closed symbols represent melanized cells, and open symbols represent non-melanized cells. The results were normalized by the number of cells per well and these values were again normalized by dividing by the control values which were the non-irradiated cells at time zero, n stands for normalized.

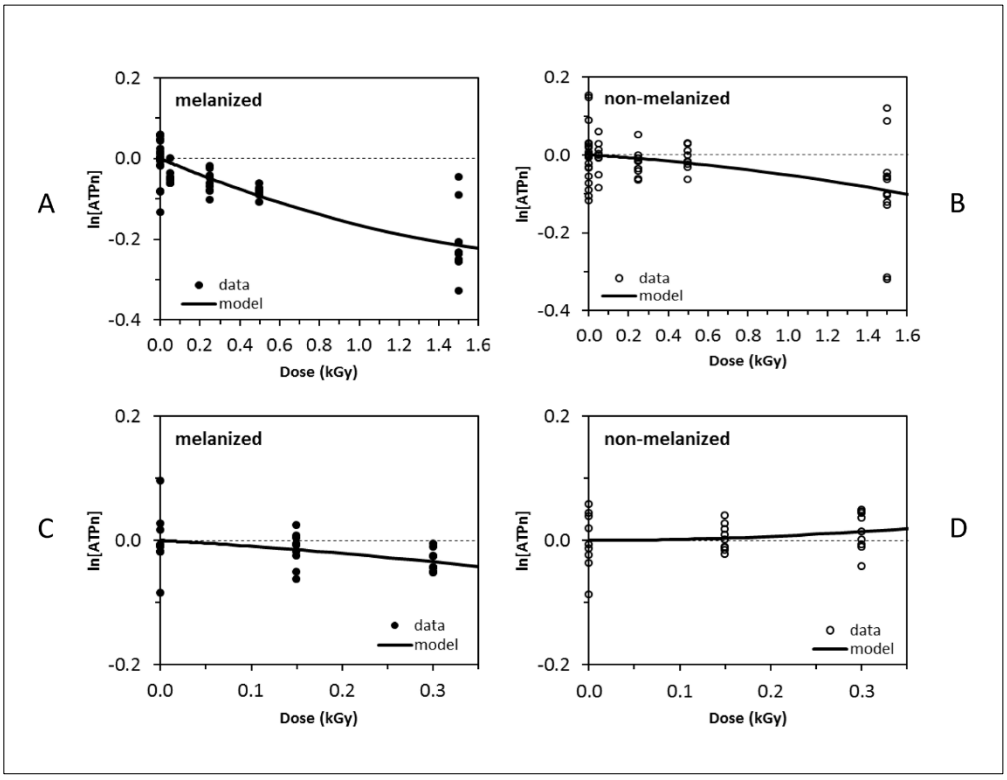

Fig. 5.8 Effects of irradiation on *C. antarcticus* ATP level. A) Melanized cells exposed to deuterons. B) non-melanized cells exposed to deuterons. C) melanized cells exposed to x-rays. D) non-melanized cells exposed to x-rays. Closed symbols represent melanized cells, and open symbols represent non-melanized cells. The results were normalized by the number of cells per well and these values were again normalized by dividing by the control values which were the non-irradiated cells at time zero, n stands for normalized.

5.4 Discussion

In this study we compared the effects of deuterons and x-rays on two microscopic fungi capable of melanogenesis – fast growing *C. neoformans* (doubling time 2 hrs) and slow growing *C. antarcticus* (doubling time is approximately 2 weeks). *C. neoformans* is a ubiquitous soil dwelling microorganism able to survive at 37 ^{o}C, which makes it an opportunistic human pathogen. *C. neoformans* can produce melanin in presence of melanin precursors such as L-dopa. *C. antarcticus* is a cryptoendolithic intrinsically melanized fungus from McMurdo Dry Valleys in Antarctica which grows best at 12-15 ^{o}C (Selbmann et al., 2005).

The most important findings of this study are: 1) Melanin affords significant protection against high doses (1.5 kGy) of deuterons for both *C. neoformans* and *C. antarcticus*. To the best of our knowledge, this is the first time when the role of melanin in protection of eukaryotic cells against deuterons under physiological conditions (full hydration, temperatures allowing growth) has been reported. 2) Another novel observation of this study is that for both slow growing *C. antarcticus* and fast growing *C. neoformans*, exposure to deuterons resulted in similar metabolic patterns: strong changes in XTT and ATP levels in melanized cells, but not in non-melanized ones. Non-melanized *C. neoformans* and *C. antarcticus* demonstrated various sensitivity to deuterons versus x-rays – while non-melanized C. antarcticus was more resistant to deuterons than non-melanized *C. neoformans*, the latter was more resistant to x-rays.

The explanation possibly lies in the rate of DNA and protein synthesis, which allows for quick repair of single strand breaks post x-rays irradiation by fast growing *C. neoformans*, but cannot help against the double DNS breaks induced by deuterons, and in this case the slow growing *C. antarcticus* has the advantage. According to classical radiobiology, dividing cells are more prone to radiation damage (Hall and Giaccia, 2006). However, even if the different growth rates may have a role in the different resistance observed, some other mechanisms related to the peculiar ecology and adaptations could be involved. This is something that needs to be investigate in the future.

We used metabolic XTT, MTT and ATP assays in parallel to assess the responses of melanized and non-melanized cells to deuterons and x-rays. Interaction of negatively charged XTT which is largely cell-impermeable and which is reduced extracellularly, at the cell surface with melanin, a known electron-shuttle, post irradiation resulted in elevated XTT levels for both types of radiation and both fungi. The XTT assay reflects primarily the interaction of radiation with melanin. The same elevation in XTT levels in melanized *C. neoformans* cells was observed in Dadachova et al. (2007) when the cells where subjected to gamma radiation with the doses being thousands times lower than it this study. The MTT assay goes deeper into the cell as MTT is taken into the cells via the plasma membrane potential and is reduced intracellularly. In non-melanized cells *C. neoformans* cells both

deuterons and x-rays caused elevation in MTT levels which is caused by the cells increasing their metabolic rates in response to the radiation insult to repair the damage. In melanized *C. neoformans* cells this effect was less pronounced because melanin protects the cells from the damage and plus it could impede the penetration of MTT into the cell to some extent. In Dadachova et al. (2007) no changes in MTT levels were observed post-irradiation in either melanized or non-melanized *C. neoformans* cells as the radiation doses were very low and non-fungicidal, in contrast to this study. As *C. antarcticus* is inherently more resistant to deuterons than *C. neoformans*, its non-melanized cells practically did not have the elevated levels of MTT as not much cellular repair was needed. In contrast, since non-melanized *C. antarcticus* is more sensitive to x-rays, its non-melanized cell had to elevate their metabolism and MTT levels to repair the damage. Finally, the damaging deuterons caused decrease in ATP levels in both melanized and non-melanized cells of both fungi, reflecting high-energy expenditure for damage repair. X-rays exposure did not affect the ATP levels of *C. antarcticus*, probably, because changes in ATP production by this very slow growing fungus are beyond the sensitivity levels of our assay. In melanized *C. neoformans* cells the x-rays did not produce any changes in ATP levels, and increased in non-melanized. This is in contrast to the observations by Bryan et al., (2011) with very low levels of gamma radiation, which decreased the ATP levels in melanized cells and did not change in non-melanized cells.

High resistance to different kinds of radiation was reported for dried samples of *C. antarcticus* during preparatory ground-based tests for ESA LIFE EXPOSE-E and BIOMEX EXPOSE-R2 experiments on the ISS (Experiment Verification Tests, EVTs) (Onofri et al., 2008; Pacelli et al., 2016). Dried *C. antarcticus* was able to withstand real space vacuum and radiations exposure during LIFE EXPOSE-E experiment on the ISS (Onofri et al., 2012; Onofri et al., 2015). Previous studies have found that other organisms that exhibit high resistance to desiccation were also resistant to radiation (Billi et al., 2000; Rainey et al., 2005; La Duc et al., 2007; Daly, 2009) and the survival of *C. antarcticus* after extreme desiccation under 10^{-5} Pa vacuum has been reported (Onofri et al., 2012). *C. antarcticus* can also survive high doses of UV-B irradiation, with no DNA damage detectable afterwards using PCR approaches (Selbmann et al., 2011). The procedure was set to minimize any possible involvement of the repair system: therefore, the remarkable resistance of *C. antarcticus* was explained by its ability to avoid DNA damage. Yet, the involvement of the DNA repair system under physiological conditions is highly probable as suggested by the statistical model fit. Since the whole genome of *C. antarcticus* was recently sequenced (Sterflinger et al., 2014) new insights on the DNA repair potentialities of this fungus will be achieved.

These observations are also of utmost importance for space biological research and planetary protection because the ability to survive at high radiation doses enables microorganisms to potentially

contaminate or invade extraterrestrial niches if accidentally transported (Moeller et al., 2012b). The ability of fungi to withstand harsh outer space conditions and their tendency to contaminate spacecraft has to be taken into consideration also because some of them are potential human pathogens capable of endangering well-being of the astronauts during manned flights. In addition, fungi have strong enzymatic systems and secrete various metabolites, which can degrade structural materials of the spacecraft (Novikova, 2004). The protective role of melanin in fungal cells, coupled with increase in fungal metabolic activity under high-energy radiation observed in this study, could open interesting scenarios for searching extraterrestrial life and biosignatures in the Universe (de Vera et al., 2012). Melanins are ubiquitous in all biological kingdoms, suggesting that they emerged early in the course of evolution having a double role of protection and possible energy harvesting pigments, when the basic background radiation was higher than today. The same or similar pigments might have enabled life to settle elsewhere in the Universe, exploiting putative extraterrestrial environments, e.g. by using melanin to protect themselves as well as capture and utilize ionizing radiation as an energy source (Dadachova and Casadevall, 2008). It is also worth mentioning, that because these fungi produce different types of melanin – DHN melanin by *C. antarcticus* and DOPA melanin by *C. neoformans* – it was impossible to compare the influence of melanin type on radiation protection. This is the subject of further investigation.

In conclusion, we have demonstrated that melanin pigment present in the fast growing *C. neoformans* and slow growing *C. antarcticus* fungi efficiently protects them against high acute radiation doses. Specifically, our data suggest that melanin is protective not only against sparsely-ionizing photons, but also against deuterons under physiological conditions. These observations are important for basic space radiobiology and for creating melanin-based radioprotectors for humans.

Acknowledgments and funding sources

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Chapter 6

6 Synthesis and Conclusions

This thesis aimed to study the resistance of the Antarctic cryptoendolitic black fungus *Cryomyces antarcticus* to exposure under ground-based simulations to space and Martian conditions in preparation for the BIOMEX-EXPOSE-R2 space mission and to cosmic radiation simulations in the contest of STARLIFE project; the role of melanin in the high radiation resistance of the fungus was also investigated by comparing responses of melanized strains and their non-melanized counterparts. Chapter 1 describes the characteristics of this eukaryotic model and the potential implications for astrobiological research. This work perfectly fits with the aim of the key objectives of the research topic 4, e.g. life and habitability, part of the "The European AstRoMap project" (Horneck et al., 2016) which will drive the European astrobiological researches for the next ten years. AstRoMap aims to deepen knowledge on the diversity, adaptability, and boundary conditions of life on Earth. For this purpose the study of organisms thriving in extreme environments on Earth, Antarctica in this thesis, is of particular interest in astrobiology; the persistence of active biota in almost any environment containing transient liquid water, an energy source and nutrients, even under extreme physico-chemical conditions, extends the possibilities for life on other planets of our Solar System and beyond (Rothschild and Mancinelli, 2001).

The stunning ability to survive, and even thrive, in environments normally neglected by or precluded to most life-forms, makes the black fungus *C. antacticus*, member of the Antarctic cryptoendolithic communities, one of the best eukaryotic model for astrobiological purposes. It was already reported that the fungus is able to withstand: **(i)** different kinds of radiation during preparatory ground-based tests for ESA-LIFE EXPOSE-E (Onofri et al., 2008); **(ii)** real space vacuum and radiations exposure during LIFE EXPOSE-E experiment onboard the ISS (Onofri et al., 2012; 2015); **(iii)** extreme desiccation under 10^{-5} Pa vacuum (Onofri et al., 2012). This thesis is performed in the frame of BIOMEX and STARLIFE Projects that are extensively described at the end of the first chapter.

Chapters 2 and 3 describe the ground-based simulations performed in the context of the BIOMEX-EXPOSE-R2 space mission; these studies allowed us to test further the resistance of *C. antarcticus* and its cellular constituents under space and Martian simulated conditions, when grown on Lunar and Martian mineral analogues. The preparation of the samples used during these experiments is illustrated in Appendix A.A. The EVTs and SVT simulations were focused on reproducing specific stressors found in the space environment in LEO and Martian simulated conditions in LEO.

In chapter 2 are reported the first results gathered in the context of EVTs BIOMEX experiment. The EVTs reproduce: EVT1, single stressor parameters (space vacuum, Martian atmosphere, UVC

radiation and temperature extremes); EVT2, increasing doses of UV irradiation simulating the whole Solar UV spectrum expected in LEO for the mission duration (up to 18 months).

Cultivation tests, PMA-qPCR assay and the ultrastructural analyses reported a high survival of *C. antarcticus* that was able to withstand UV irradiation as well as simulated treatments with other outer space stressors. Therefore, a reduced survival was observed after exposure to the most stressing parameters and the main damaging factors of the EVTs were UV irradiation and temperature cycles. The PCR analyses showed that the DNA is still amplified even after the highest doses of UV-irradiation and RAPD profiles were well preserved in all samples, indicating a good preservation of the template. The easy extraction and the high resistance of DNA were confirmed for other microorganisms (Baquè et al., 2013, de la Torre, personal communication), suggesting that molecule as a possible biosignature candidate in future exploration missions. Results from chapter 2 were published in "Origins of Life and Evolution of Biospheres" journal (Pacelli et al., 2016).

Chapter 3 focuses on the last BIOMEX ground-based experiment, the SVTs: they simulated the whole LEO mission by applying either a simulated space vacuum or a Martian simulated atmosphere (for 3 months) in combination or not with polychromatic UV irradiation (200-400nm) with the expected dose of a 12 months mission. As for EVTs, a high fungal survival after the treatments was observed, even after vacuum or Martian atmosphere coupled with irradiation; the results were confirmed by cultivation test, PMA-qPCR and ultrastructural analysis. A new method, i.e. XTT assay, to analyze the fungal metabolic activity after treatments was optimized for the fungus and the results were concordant with the previous analyses.

The highest survival, in term of CFU's, was reported for *C. antarcticus* grown on S-MRS for SVT and on P-MRS in the EVTs (chapter 2, Pacelli et al., 2016); the same result was recently reported for *Buellia frigida* suggesting a protective role of the substratum (Meeßen et al., 2015). On the contrary, in the frame of BIOMEX experiment, Baquè et al. (2014) reported a higher survival of the cyanobacterium *Chroococcidiopsis* when mixed with S-MRS regolith. These contrasting results led us to conclude that a possible protective role of the analogues is not supported for now and needs to be further investigated.

Melanin as a possible biosignature was investigated in Chapter 3 with RAMAN spectroscopy, the promising not-destructive tool for the remote detection of biosignatures in the context of planetary exploration (Tarcea et al., 2008). Indeed, the ExoMars rover will incorporate a Raman Laser Spectrometer, with green excitation light at a wavelength of 532 nm, which covers both mineralogical and biological Raman regions (Edwards et al., 2013). Results indicate that mineral and melanin spectra were detectable from each fully exposed samples, except for the fungus grown on Lunar analogues: this sample gave high fluorescence that covered all Raman peaks.

The signal saturation produced by the increased fluorescent background was also reported by other groups within BIOMEX, and the dramatic increase in background fluorescence appears to be correlated to the increase of UV dose received (Baqué, pers. communication). This must to be taken in consideration for next exploration mission, when detecting biosignatures *in situ*. Unfortunately, the melanin peaks were too similar to amorphous carbon: consequently, it was impossible to distinguish the presence of our target unambiguously. To conclude, melanin spectra is not useful to develop biosignature databases for now, and we need to optimize some parameters. These results were submitted to Astrobiology journal and are still under revision.

Analyses of other putative fungal biosignatures have been performed with Gas Chromatograph-Mass Spectrometry, also installed on ExoMars: analyses on EVTs samples, are still ongoing.

One of most dangerous component of the space or Martian environment is the ionizing radiation. In that context, chapter 4 describes the purpose of the STARLIFE campaign to improve our understanding of the biological efficiency of ionizing radiation in space by studying the responses of selected astrobiological model systems - most of them being "space veterans" - to radiation mimicking single components of cosmic radiation, mainly accelerated heavy ions and high doses of x- and γ-rays. In this frame three different cryptoendolithic Antarctic microorganisms, i.e. *C. antarcticus*, *Umbilicaria* sp. and *Stichococcus* sp., were exposed to high doses of space relevant γ-rays (^{60}Co) up to 117.07 kGy (chapter 4, Pacelli et al., *in press*) and Helium up to 1.000 Gy (unpublished data). Survival and ultrastructural integrity was analyzed by cultivation test and TEM analyses, respectively; DNA damage was investigated through PCR.

The main findings of this work are **(i)** *C. antarcticus* was the most resistant among our test microorganisms: fungal colonies were still recorded at 55.81 kGy. None of the tested organisms was able to multiply after maximum dose of irradiation, 117.07 kGy; **(ii)** no DNA damage was detected for the microorganisms even in sample were the viability was lost. These results, together with the analysis of the previous chapters (2 and 3), support the use of DNA as biosignature.

Due to their indispensability for life on Earth, DNA is also prime target in the search for life on Mars or other planets. Biosignatures degrade over time and *in situ* environmental conditions influence the preservation of those molecules. The degradation of DNA is mediated via three factors: **(i)** space radiation, mainly UV, **(ii)** vacuum or low pressures, which encourage strand breaks, mutations and cross-linking, and **(iii)** the level of hydration, with more hydrated DNA being vulnerable to greater damage (Folkard, et al., 1999). Nonetheless, both shielding by mineral surfaces, such as halite crystals, permafrost and marine sediments, coupled to specific conditions (i.e. low temperatures) could improve the conservation of ancient DNA. As demonstrated in chapters 2, 3 and 4, DNA was easily detected in all the tested organisms even after high irradiation treatments (up to 117.07 kGy)

and even when viability was not recorded. Here we found that DNA has a high intrinsic stability, which is important for a good biosignatures (Aerts et al., 2014) and that it could be regarded as biosignature even after life extinction. Results from chapter 4 were accepted for publication in Astrobiology journal and are *in press*.

New insights on the biological effects of radiation in space are of importance for assessing the radiation risks during space missions. In last years, most researches focused on the use of high-energy photons (γ- and x-rays, sparsely ionizing), but more recently densely ionizing high-energy protons and heavy ions are being used. Thus, in the last chapter we investigated the role of melanin on fungal species after exposition to different kind of radiation, by comparing survival and metabolic activity of melanized and non-melanized strains. In this study, we compared the effects of highly ionizing deuterons and sparsely ionizing x-rays on two microscopic fungi, *C. neoformans* and *C. antarcticus*, characterized by two different kind of fungal DOPA and DHN melanin.

We demonstrated that melanin affords significant protection against high doses (1.5 kGy) of deuterons for both *C. neoformans* and *C. antarcticus* under physiological conditions (full hydration, temperatures allowing growth). Another novel observation of this study is that for both slow growing *C. antarcticus* and fast growing *C. neoformans*, exposure to deuterons resulted in similar metabolic patterns: strong changes in XTT and ATP levels in melanized cells, but not in non-melanized ones, suggesting a role of melanin as electron acceptor. These results were perfectly in concordance to what reported for *C. neoformans* in Dadachova et al. (2007), when cells where subjected to gamma radiation. To summarize, chapter 5 demonstrated the protective role of melanin in fungal cells: the mechanism is still to be deeply investigated. Interestingly, melanins, having double role of photoprotection and possible energy harvesting pigments, by increasing the fungal metabolic activity, may have improved fungal resistance to radiation in the past, even in an hypothetical travel or life outside the Earth. These results were accepted with minor revision and already re-submitted in Environmental Microbiology journal.

To conclude, this thesis contributes to the understanding of the survival limits of the Antarctic black fungus *C. antarcticus* under simulated space and Martian conditions and to various types of space relevant radiation. It was demonstrated its ability to resist the stressor in ground-based simulations in the frame of the BIOMEX and STARLIFE projects. A role of melanin in protection against radiation was deeply reported. Future investigations on samples actually exposed to space condition mission will give us further insights on the mechanisms of its resistance and the preservation of fungal biosignatures in extraterrestrial conditions, with implications for astrobiological research and for the next exploration mission.

List of Publications

Pacelli, C., Selbmann, L., Zucconi, L., De Vera, J. P., Rabbow, E., Horneck, G., de la Torre, R., and Onofri, S. (2016) *BIOMEX Experiment: Ultrastructural Alterations, Molecular Damage and Survival of the Fungus Cryomyces antarcticus after the Experiment Verification Tests.* Origins of Life and Evolution of Biospheres, 1-16.

Pacelli, C., Selbmann L., Zucconi L., Raguse M., Moeller R., Shuryak I., and Onofri S. *STARLIFE V: Survival, DNA integrity and ultrastructural damage in Antarctic cryptoendolithic eukaryotic microorganisms exposed to ionizing radiation.* (accepted for publication to Astrobiology, *in press*).

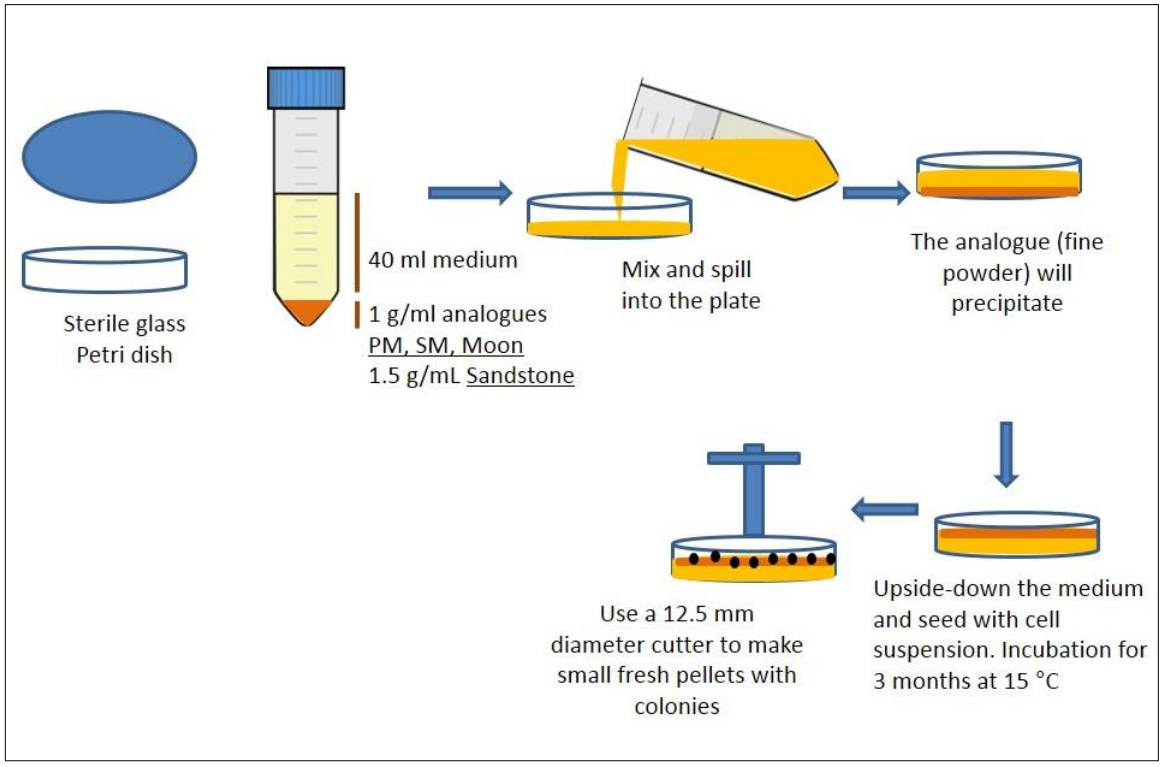
Pacelli, C., Selbmann L., Zucconi L., Coleine C., de Vera J.P., Rabbow E., Dadachova E., Böttger U., and Onofri S. *BIOlogy and Mars Experiment: responses of the black fungus Cryomyces antarcticus to the EXPOSE-R2 Science Verification Test.* (submitted for publication to Astrobiology).

Pacelli, C., Bryan R., Onofri S., Selbmann L., Shuryak I., and Dadachova E. *Melanin is effective in protecting fast and slow growing fungi from various types of ionizing radiation* (accepted with minor revision for publication to Applied and Environmental Microbiology).

7 Appendixes

A.A Preparation of samples

The preparation of the samples for the BIOMEX experiment is illustrated in Figure A.1. As briefly described in the corresponding chapters, cell suspensions were spread on MEA (malt extract agar:



malt extract, powdered 30 g/L; peptone 5 g/L; agar 15 g/L; Applichem, GmbH) in Petri dishes, mixed with Antarctic Sandstone (1.5 g/L), Lunar and Martian analogues (1 g/L), and prepared to optimize mineral/microorganisms interactions. Colonies were grown at 15 °C for 3 months. Disks were cut to fit within the wells of exposure carrier (12 mm diameter) and then air-dried under a sterile hood for 18 hours under sterile conditions.

A.B Mechanism of PMA treatments

Propidium monoazide (PMA) assay (Mohapatra and La Duc, 2012) was used for evaluating percentage of cells with undamaged cell membranes by quantifying fungal DNA extracted from colonies of C. antarcticus This was performed by adding PMA, at a final concentration of 200 µM, to fungal colonies after rehydration. PMA penetrates only damaged cell membranes, cross-linking to DNA after light exposure and thereby preventing PCR, as illustrated in Fig A.2. Following DNA extraction and purification, quantitative polymerase chain reaction (qPCR; BioRad CFX96 real-time PCR detection system) was employed to quantify the number of fungal ribosomal DNA fragments present in samples either treated or not treated with PMA. Genomic DNA was added at a concentration of 0.1 ng to final volume of 25 µL of PCR cocktail containing 1X Power Sybr-Green PCR Master Mix, NS91 forward (5¢-GTC CCT GCC CTT TGT ACA CAC-3¢) and ITS51 reverse (5¢-ACC TTG TTA CGA CTT TTA CTT CCT C-3¢) primers, each at 0.02 M final concentration. These primers amplify a 203 bp product spanning the 18S/ITS1 region of rRNA encoding genes. A standard qPCR cycling protocol consisting of a hold at 95 °C for 10 min, followed by 40 cycles of denaturing at 95 °C for 15 s, annealing at 58 °C for 20 s, and elongation at 72 °C for 15 s was followed. Fluorescence measurements were recorded at the end of each annealing step. At the conclusion of the 40th cycle, a melt curve analysis was performed by recording changes in fluorescence as a function of raising the temperature from 60 °C to 95 °C in 0.5°C per 5 s increments.

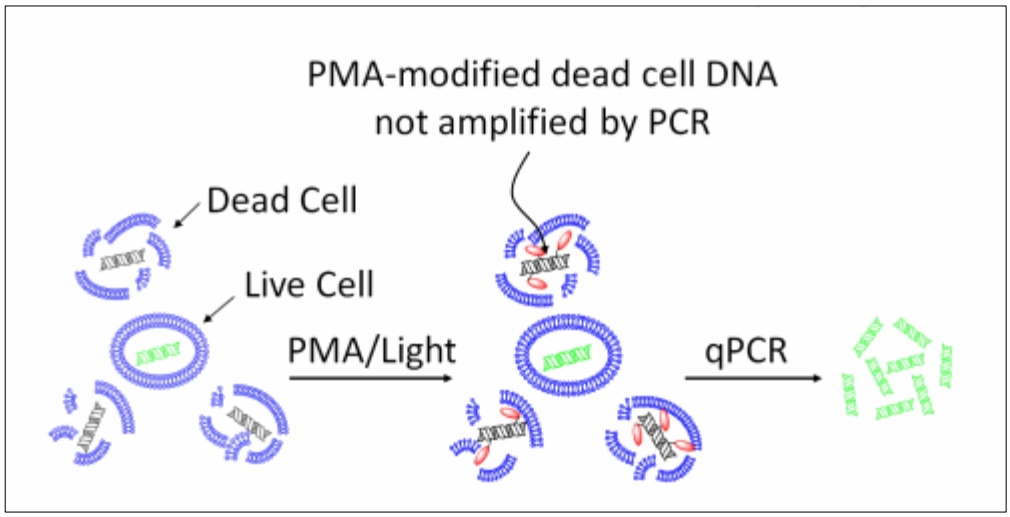


Figure A.2 Explanation of PMA-qPCR test.

A.C Preliminary results on samples exposed to actual space and Martian simulated condition in space.

This paragraph reported the preliminary results after samples exposition for 1.5 years to actual space and Martian simulated condition in space, in the contest of BIOMEX-EXPOSE R2 mission. The fungus *C. antarcticus* survives all the conditions tested, in all analogues as reported in table A.1. The amplicons revealed a good preservation of DNA, for the gene lenght tested (ITS region, 700 bp) and a good preservation of the whole genome, with a reduction of the highest molecular weight bands visible in some cases (Figure A.3).

Table A.1 Survival reported for *C. antarcticus* after space mission.

	BOTTOM	TOP
Original Substrate	+	+
Lunar Analogue	+	+
P-MRS	+	+
S-MRS	+	+

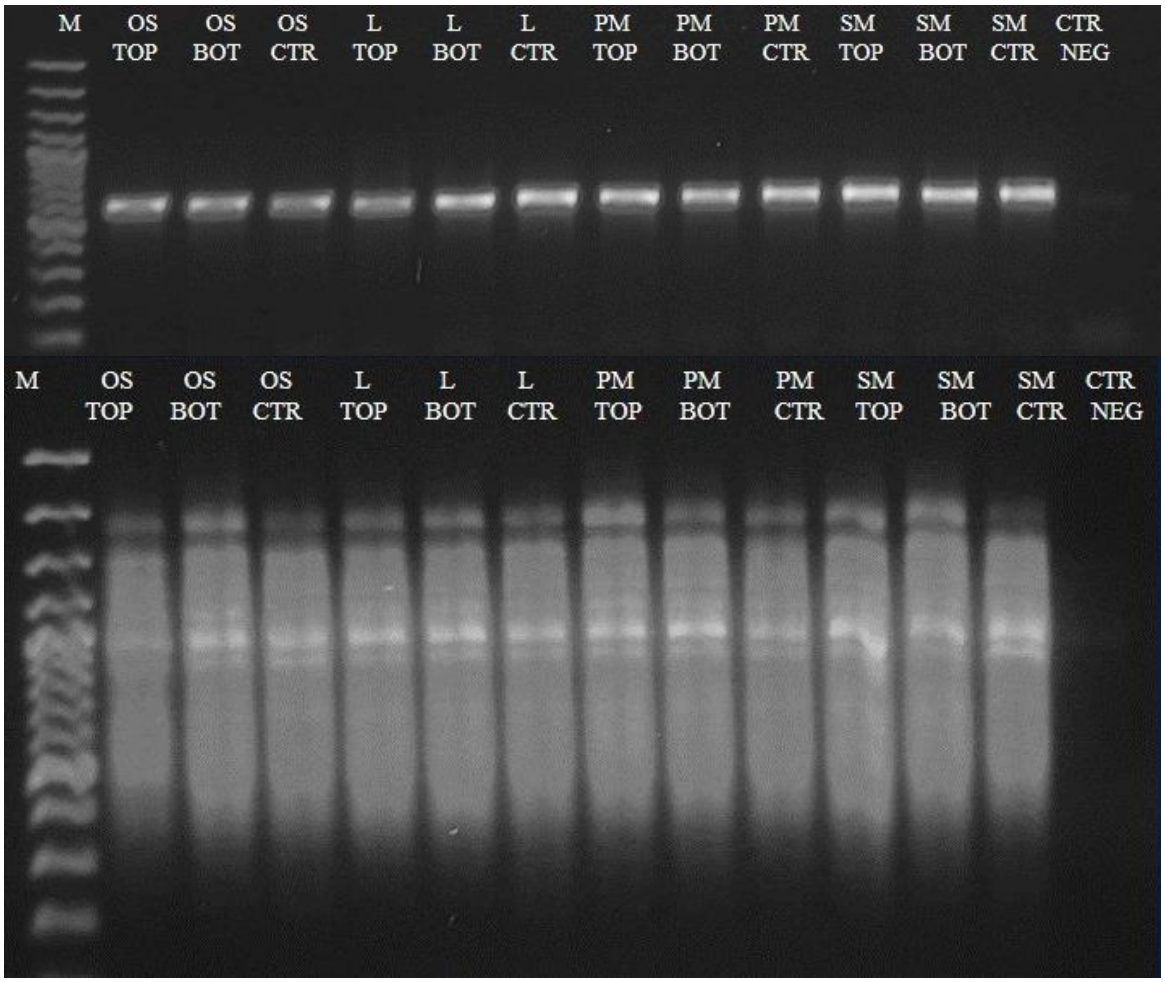


Figure A.3 Assessment of the DNA damage on *C. antarcticus* after real space exposure and simulated Martian condition treatments. **Up:** single gene PCR-ITS Region 700 bp; **Above:** RAPD complete genomic fingerprinting. DNA ladder (M), Positive PCR Control (CTR POS), Negative PCR Control (CTR NEG). Substrates: OS, Original Substrate; L, Lunar; SM, S-MRS; PM, P-MRS.

The ultrastructural analysis performed by TEM revealed on TOP samples the presence of loose cytoplasm, damaged membranes or gosts; intact cells were well visible in the top samples, with nucleus, mitochondria and melanine granules in some cases, justifying the cultivation data (Fig. A.4-5.). Some controls were more injuried than the treated counterparts; the same was obtained by cultivation test and Dna damage, highlighting a possible problems related with the controls. Same problem with controls are reported from other group within the BIOMEX team and need to be further investigated togheter with the preservation on fungal biosignatures.

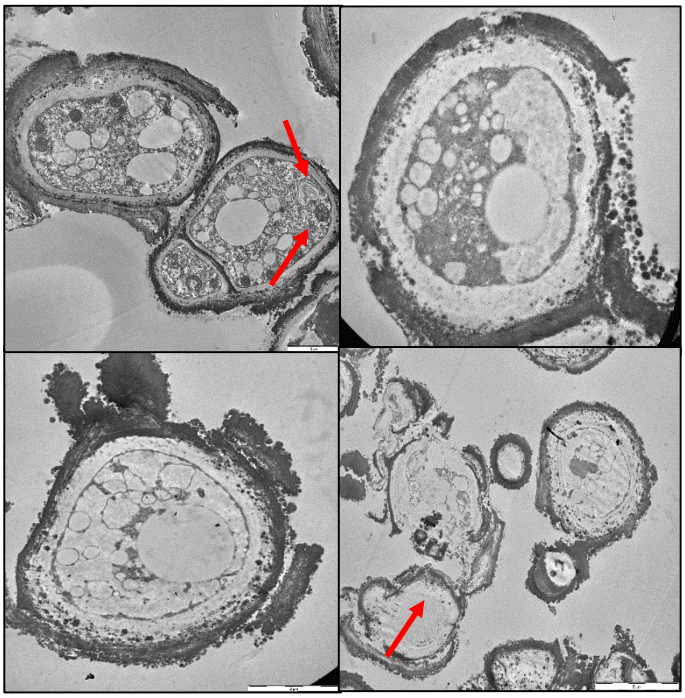


Fig. A.4 TEM micrographs. Untreated (CTR).

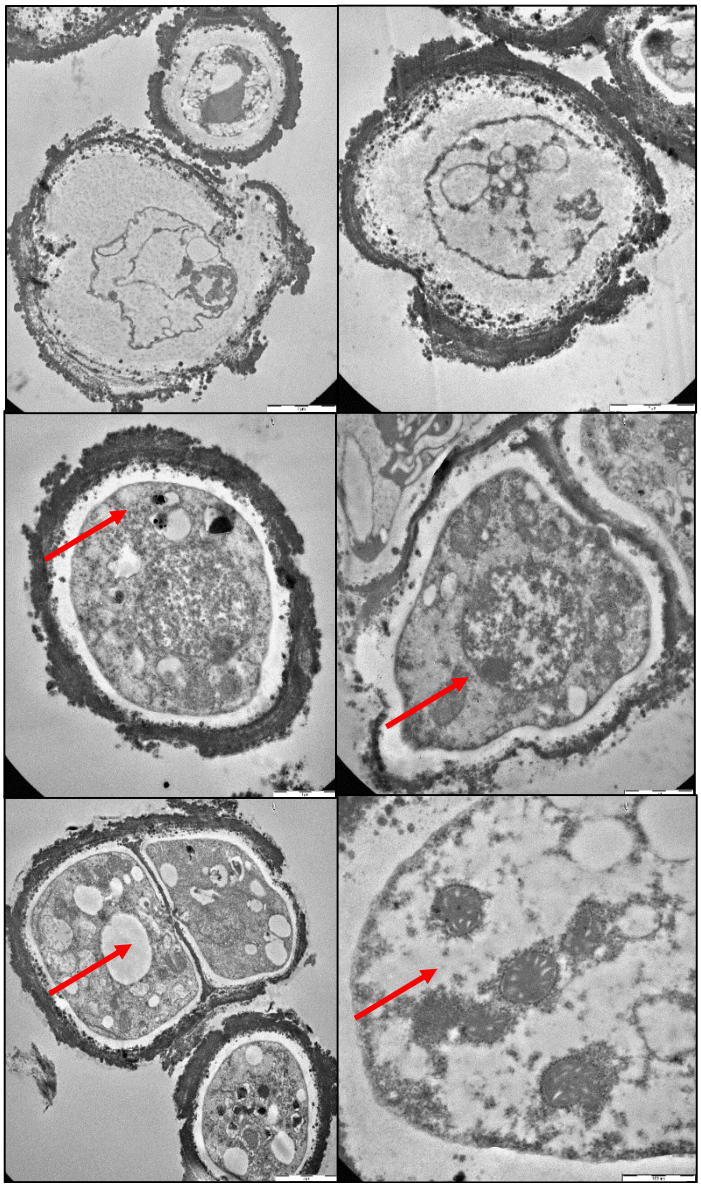


Fig. A.5 TEM micrographs. Treated (TOP sample), with space vacuum or a Martian simulated atmosphere in combination with polychromatic UV irradiation .

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8 References

Aerts, J.W., Röling, W.F., Elsaesser, A., and Ehrenfreund, P. (2014) Biota and Biomolecules in Extreme Environments on Earth: Implications for Life Detection on Mars. *Life* 4:535-565.

Agresti, A., and Coull, B.A. (1998) Approximate is better than "exact" for interval estimation of binomial proportions, The American Statistician.

Aislabie, J.M., Chhour, K.L., Saul, D.J., Miyauchi, S., Ayton, J., Paetzold, R.F., and Balks, M.R. (2006) Dominant bacteria in soils of Marble point and Wright valley, Victoria land, Antarctica. *Soil Biol Biochem* 38(10):3041-3056.

Arrhenius, S. (1908) Worlds in the Making: The Evolution of the Universe. Harper and Row, NewYork.

Asaithamby, A., and Chen, D.J. (2011) Mechanism of cluster DNA damage repair in response to high-atomic number and energy particles radiation. *Fund Mol Mech Mut* 711(1):87-99.

Atienzar, F.A., Venier, P., Jha, A.N., and Depledge, M.H. (2002) Evaluation of the random amplified polymorphic DNA (RAPD) assay for the detection of DNA damage and mutations. *Mutat Res* 521:151-163.

Bada, J.L. (2001) State-of-the-art instruments for detecting extraterrestrial life. *Proc Natl Acad Sci* 98:797-800.

Badhwar, G.D., and O'Neill, P.M. (1994) Long-term modulation of galactic cosmic radiation and its model for space exploration. *Adv Spa Res* 14(10):749-757.

Baqué, M., de Vera, J.P., Rettberg, P., and Billi, D. (2013) The BOSS and BIOMEX space experiments on the EXPOSE-R2 mission: Endurance of the desert cyanobacterium Chroococcidiopsis under simulated space vacuum, Martian atmosphere, UVC radiation and temperature extremes. *Acta Astronaut* 91:180-186.

Baquè, M., Verseux, C., Rabbow E., de Vera, J.P., and Billi, D. (2014) Detection of Macromolecules in Desert Cyanobacteria Mixed with a Lunar Mineral Analogue After Space Simulations. *Orig Life Evol Biosph* 44:209-221.

Baqué, M., Verseux, C., Böttger, U., Rabbow, E., de Vera, J. P. P., and Billi, D. (2016) Preservation of Biomarkers from Cyanobacteria Mixed with MarsLike Regolith Under Simulated Martian Atmosphere and UV Flux. *Orig life evol biosph* 46(2-3):289-310.

Barrett, J.E., Virginia, R.A., Wall, D.H., Doran, P.T., Fountain, A.G., Welch, K.A., and Lyons, W.B. (2008) Persistent effects of a discrete warming event on a polar desert ecosystem. *Glob Change Biol* 14(10):2249-2261.

Bauchwitz, R., and Holloman, W.K. (1990) Isolation of the REC2 gene controlling recombination in Ustilagomaydis. *Gene* 96(2):285-8.

Bell, A.A., and Wheeler, M.H. (1986) Biosynthesis and functions of fungal melanins. *Ann Rev Phytopath* 24:411-451.

Berridge, M.V., Herst, P.M., and Tan, A.S. (2005) Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol Annu Rev* 11:127-52.

Bibring, J.P., Langevin, Y., Gendrin, A., Gondet, B., Poulet, F., Berthé, M., Soufflot, A., Arvidson, R., Mangold, N., Mustard, J., Drossart, P., and the OMEGA team (2005) Mars surface diversity as revealed by the OMEGA/Mars Express observations. *Science* 307:1576-1581.

Billi, D., Friedmann, E.I., Hofer, K.G., Caiola, M.G., and Ocampo-Friedmann, R. (2000) Ionizing-radiation resistance in the desiccation-tolerant cyanobacterium *Chroococcidiopsis*. *Appl Environ Microb* 66(4):1489-1492.

Böttger, U., de Vera, J.P., Fritz, J., Weber, I., Hübers, H.W., and Schulze-Makuch, D. (2012) Optimizing the detection of carotene in cyanobacteria in a Martian regolith analogue with a Raman spectrometer for the ExoMars mission. *Planet Space Sci* 60:356-362.

Böttger, U., de Vera, J.P., Hermelink, A., Fritz, J., Weber, I., Schulze-Makuch, D., and Hübers, H.W. (2013) Application of Raman Spectroscopy as In Situ Technology for the Search for Life. In Habitability of Other Planets and Satellites (pp. 331-345). Springer Netherlands.

Brandt, A., Posthoff, E., de Vera, J. P., Onofri, S., and Ott, S. (2016) Characterisation of Growth and Ultrastructural Effects of the *Xanthoria elegans* Photobiont After 1.5 Years of Space Exposure on the International Space Station. *Orig Life Evol Bios* 46(2-3):311-321.

Brenner, D.J., Hlatky, L.R., Hahnfeldt, P.J., Huang, Y., and Sachs, R.K. (1998) The linear-quadratic model and most other common radiobiological models result in similar predictions of time-dose relationships. *Radiat Res* 150(1):83-91.

Brenner, D.J., Martinez, A.A., Edmundson, G.K., Mitchell, C., Thames, H.D., and Armour, E.P. (2002) Direct evidence that prostate tumors show high sensitivity to fractionation (low alpha/beta ratio), similar to late-responding normal tissue. *Int J Radiat Oncol* 52(1):6-13.

Bryan, R., Jiang, Z., Friedman, M., and Dadachova, E. (2011) The effects of gamma radiation, UV and visible light on ATP levels in yeast cells depend on cellular melanization. *Fungal Biol* 115:945-9.

Bryan, R.A., Shuryak, I., Broitman, J., Marino, S.A., and Dadachova, K. (2015) Irradiation of Cryptococcus neoformans with high doses of gamma rays or alpha particles leads to capsule dependent increases in XTT reducing activity. American Society for Microbiology 115th General meeting.

Burkins, M.B., Virginia, R.A., Chamberlain, C.P., and Wall, D.H. (2000) Origin and distribution of soil organic matter in Taylor Valley, Antarctica. *Ecology*, 81(9):2377-2391.

Butler, M.J., and Day, A.W. (1998) Fungal melanins: a review. *Can J Microbiol* 44(12):1115-1136.

Cacao, E., Hada, M., Saganti, P.B., George, K.A., and Cucinotta, F.A. (2016) Relative Biological Effectiveness of HZE Particles for Chromosomal Exchanges and Other Surrogate Cancer Risk Endpoints. *PLoS One* 11:e0153998.

Callahan, M.P., Smith, K.E., Cleaves, H.J., Ruzicka, J., Stern, J.C., Glavin, D.P., House C.H., and Dworkin, J.P. (2011) Carbonaceous meteorites contain a wide range of extraterrestrial nucleobases. *P Natl Acad Sci* 108(34):13995-13998.

Carr, C.E., Rowedder, H., Vafadari, C., Lui, C.S., Cascio, E., Zuber, M.T., and Ruvkun, G. (2013) Radiation resistance of biological reagents for in situ life detection. *Astrobiology* 13(1):68-78.

Cary, S.C., McDonald, I.R.,Barrett, J.E., and Cowan, D.A.(2010) On the rocks: the microbiology of Antarctic Dry Valley soils. *Nat Rev Microbiol* 8:129-138.

Chevrier, V., Mathé, P.E. (2007) Mineralogy and evolution of the surface of Mars: a review. *Planet Space Sci* 55:289-314.

Cockell, C.S., Bush, T., Bryce, C., Direito, S., Fox-Powell, M., Harrison, J.P., Lammer, H., Landenmark H., Martin-Torres, J., Nicholson, N., Noack, L., O'Malley-James, J., Payler, S.J., Rushby, A., Samuels, T., Schwendner, P., Wadsworth, J., and Zorzano M.P. (2016) Habitability: A Review. *Astrobiology* 16(1):89-117.

Coic, E., Feldman, T., Landman, A.S., and Haber, J.E. (2008) Mechanisms of Rad52-independent spontaneous and UV-induced mitotic recombination in *Saccharomyces cerevisiae. Genetics* 179(1):199-211.

Cooper, G., Reed, C., Nguyen, D., Carter, M., and Wang, Y. (2011) Detection and formation scenario of citric acid, pyruvic acid, and other possible metabolism precursors in carbonaceous meteorites. *P Natl Acad Sci* 108:14015-14020.

Cowan, D.A., Makhalanyane, T.P., Dennis, P.G., and Hopkins, D.W. (2014) Microbial ecology and biogeochemistry of continental Antarctic soils. *Front Microbiol* 5:154.

Crowe, J.H., Hoekstra, F.A., and Crowe L.M. (1992) Anhydrobiosis. *Annu Rev Physiol* 54(1):579-599.

Cucinotta, F.A. (2015) Review of NASA approach to space radiation risk assessments for Mars exploration. *Health Phys* 108(2):131-142.

Cunha, M. M., Franzen, A. J., Alviano, D. S., Zanardi, E., Alviano, C. S., De Souza, W., Rozental, S. (2005) Inhibition of melanin synthesis pathway by tricyclazole increases susceptibility of Fonsecaea pedrosoi against mouse macrophages. *Micros Res Tech* 68(6):377-384.

Dadachova, E., Bryan, R.A., Huang, X., Moadel, T., Schweitzer, A.D., Aisen, P., and Casadevall, A. (2007) Ionizing radiation changes the electronic properties of melanin and enhances the growth of melanized fungi. *PloS one 2*(5):e457.

Dadachova, E., Bryan, R.A., Howell, R.C., Schweitzer, A.D., Aisen, P., Nosanchuk, J.D., and Casadevall, A. (2008) The radioprotective properties of fungal melanin are a function of its chemical composition, stable radical presence and spatial arrangement. *Pigment Cell Melanoma Res* 21:192-199.

Dadachova, E., and Casadevall, A. (2008) Ionizing radiation: how fungi cope, adapt, and exploit with the help of melanin. *Curr Opin Microbiol* 11(6):525-531.

Daly, M.J. (2009) A new perspective on radiation resistance based on Deinococcus radiodurans. *Nat Rev Microbiol* 7:237-245.

Dartnell, L.R., Desorgher, L., Ward, J.M., and Coates, A.J. (2007) Martian sub-surface ionizing radiation: biosignatures and geology. *Biogeosci Discuss* 4:455-492.

Dartnell, L. (2011) Biological constraints on habitability. *Astron Geophys* 52(1):1-25.

Dartnell, L.R., Page, K., Jorge-Villar, S.E., Wright, G., Munshi, T., Scowen, I.J., Ward, J.M., and Edwards, H.G. (2012) Destruction of Raman biosignatures by ionising radiation and the implications for life detection on Mars. *Anal Bioanal Chem* 403(1):131-144.

de la Torre R., Sancho, L.G., Pintado, A., Rettberg, P., Rabbow, E., Panitz, C., Deutschmann, U., Reina, M., and Horneck, G. (2007) BIOPAN experiment LICHENS on the Foton M2 mission: Pre-flight verification tests of the *Rhizocarpon geographicum*-granite ecosystem. *Adv Space Res* 40:1665-1671.

de La Torre, R., Sancho, L.G., Horneck, G., de los Rìos, A., Wierzchos, J., Olsson-Francis, K., Cockell, C., Rettberg, P., Berger, T., de Vera, J.P., Ott, S., Frías, J.M., Melendi, P.G., Lucas, M.M., Reina, M., Pintado, A., and Demets, R. (2010) Survival of lichens and bacteria exposed to outer space conditions–results of the Lithopanspermia experiments. *Icarus* 208:735-748.

de la Torre, R., Miller, A., Cubero, B., Martín-Cerezo, M.L., Raguse, M., and Meessen, J. (2016) STARLIFE VII: The effect of high-dose ionizing radiation on the astrobiological model lichen Circinaria gyrosa. *Astrobiology, in press*.

de Vera, J.P., Horneck, G., Rettberg, P., and Ott, S. (2002) The potential of the lichen symbiosis to cope with extreme conditions of outer space–I. Influence of UV radiation and space vacuum on the vitality of lichen symbiosis and germination capacity. *Int J Astrobiol* 1:285-293.

de Vera, J.P., Horneck, G., Rettberg, P., and Ott, S. (2004) The potential of the lichen symbiosis to cope with the extreme conditions of outer space II: germination capacity of lichen ascospores in response to simulated space conditions. *Ad Space Res* 33:1236-1243.

de Vera, J.P., Boettger, U., de la Torre Noetzel, R., Sánchez, F.J., Grunow, D., Schmitz, N., Lange, C., Hübers, H.W., Baqué, M., Rettberg, P., Rabbow, E., Reit, G., Berger, T., Möller, R., Bohmeier, M., Horneck, G., Westall, F., Jänchen, J., Frizt, J., Meyer, C., Onofri, S., Selbmann, L., Zucconi, L., Kozyrovska, N., Leyal, T., Foing, B., Demets, D., Cockell, C., Bryce, C., Wagner, D., Serrano, P., Edwards, H.G.M., Joshi, J., Huwe, B., Ehrenfreund, P., Elsaesser, A., Ott, S., Messen, J., Feyh, N., Szewzyk, U., Jaumann, R., and Spohn, T. (2012) Supporting Mars exploration: BIOMEX in Low Earth Orbit and further astrobiological studies on the Moon using Raman and PanCam technology. *Planet Space Sci* 74:103-110.

Demets, R., Schulte, W., and Baglioni, P. (2005) The past, present and future of Biopan. *Adv Space Res* 36:311-316.

Doran, P.T., McKay, C.P., Clow, G.D., Dana, G.L., Fountain, A.G., Nylen, T., and Lyons, W.B. (2002) Valley floor climate observations from the McMurdo Dry Valleys, Antarctica, 1986–2000. *J Geophys Res-Atmos* 107(D24).

Dong, H., Rech, J.A., Jiang, H., Sun, H., and Buck, B.J. (2007) Endolithic cyanobacteria in soil gypsum: Occurrences in Atacama (Chile), Mojave (United States), and Al-Jafr Basin (Jordan) Deserts. *J Geophys Res Biogeosci* 112(G2).

Durante, M. (2014) New challenges in high-energy particle radiobiology. *Br J Radiol* 87(1035):20130626.

Edwards, H.G.M., Hutchinson, I.B., Ingley, R., Parnell, J., Vítek, P., and Jehlička, J. (2013) Raman Spectroscopic Analysis of Geological and Biogeological Specimens of Relevance to the ExoMars Mission. *Astrobiology* 13:543-549.

Fernandez-Palomo, C., Seymour, C., and Mothersill, C. (2016) Inter-Relationship between Low-Dose Hyper-Radiosensitivity and Radiation-Induced Bystander Effects in the Human T98G Glioma and the Epithelial HaCaT Cell Line. *Radiat res* 185(2):124-33.

Ferrari, F., and Szuszkiewicz, E. (2009) Cosmic rays: a review for astrobiologists. *Astrobiology* 9(4):413-436.

Finster, K., Hansen, A.A., Liengaard, L., Mikkelsen, K., Kristoffersen, T., Merrison, J., Nörnberg, P., and Lomstein, B.A. (2007) Mars simulation experiments with complex microbial soil communities. In: ROME: Response of Organisms to the Martian Environment (Cockell C, Horneck G, ed). ESA Communications. ESTEC, Noordwijk, The Netherlands: 59-71.

Friedberg, E.C. (2003) DNA damage and repair. *Nature* 421:436-440.

Friedmann, E.I. (1982) Endolithic microorganisms in the Antarctic cold desert. *Science* 215(4536):1045-1053.

Friedmann, E.I. (1986) The Antarctic cold desert and the search for traces of life on Mars. *Adv space res* 6:265-268.

Friedmann, E.I., and Weed, R. (1987) Microbial trace-fossil formation, biogenous, and abiotic weathering in the Antarctic cold desert. *Science* 236(4802):703-705.

Friedmann, E.I., Kappen, L., Meyer, M.A., and Nienow, J.A. (1993) Long-term productivity in the cryptoendolithic microbial community of the Ross Desert, Antarctica. *Microb Ecol* 25(1):51-69.

Gadd, G.M., and de Rome, L. (1988) Biosorption of copper by fungal melanin. *Appl Microbiol Biotechnol 29:610-617.*

Garcia, L., Leblanc, J., Wilkins, D., and Raaphorst, G. (2006) Fitting the linear–quadratic model to detailed data sets for different dose ranges. *Phys med biol* 51(11):2813

Gilichinsky, D.A., Wilson, G.S., Friedmann, E.I., McKay, C.P., Sletten, R.S., Rivkina, E.M., Vishnivetskaya, T.A., Erokhina, L.G., Ivanushkina, N.E., Kochkina, G.A., Shcherbakova, V.A., Soina, V.S., Spirina, E.V., Vorobyova, E.A., Fyodorov-Davydov, D.G., Hallet, B., Ozerskaya, S.M., Sorokovikov, V.A., Laurinavichyus, K.S., Shatilovich, A.V., Chanton, J.P., Ostroumov, V.E., Tiedje, J.M. (2007) Microbial populations in Antarctic permafrost: biodiversity, state, age, and implication for astrobiology. *Astrobiology* 7: 275-311.

Gessler, N.N., Egorova, A.S., and Belozerskaya, T.A. (2014) Melanin pigments of fungi under extreme environmental conditions (Review). *Applied Biochem Micro* 50(2):105-113.

Gómez, F., and Parro, V. (2012) Applications of extremophiles in astrobiology: Habitability and life detection strategies. In Adaption of Microbial Life to Environmental Extremes, H. Stan-Lotter, and S. Fendrihan, eds. (Springer Vienna), pp. 199–229.

González-Toril, E., Llobet-Brossa, E., Casamayor, E.O., Amann, R., Amils, R. (2003) Microbial ecology of an extreme acidic environment, the Tinto river. *Appl Environ Microbiol* 69: 4853-4865.

Gorbushina, A. (2003) Microcolonial fungi: survival potential of terrestrial vegetative structures. *Astrobiology* 3:543-554.

Gorbushina, A.A., Kotlova, E.R., and Sherstneva, O.A. (2008) Cellular responses of microcolonial rock fungi to long-term desiccation and subsequent rehydration. *Stud Mycol* 61:91-97.

Gunde-Cimerman, N., Zalar, P., de Hoog, S., and Plemenitaš, A. (2000) Hypersaline waters in salterns: natural ecological niches for halophilic black yeasts. *FEMS Microbiol Ecol* 32:235-240.

Gunde-Cimerman, N., Oren, A., Plemenitaš, A. (2005) Adaptation to life at high salt concentrations in Archaea, Bacteria, and Eukarya. Springer, The Netherlands.

Hada, M., and Georgakilas, A.G. (2008) Formation of clustered DNA damage after high-LET irradiation: a review. *J Radiat Res* 49(3):203-10.

Hall, E.J., and Giaccia, A.J. (2006) Radiobiology for the Radiologist. Lippincott Williams & Wilkins.

Hamby, R.K., Sims, L.E., Issel, L.E., and Zimmer, E.A. (1988) Direct RNA sequencing: optimization of extraction and sequencing techniques for work with higher plants. *Plant Mol Biol Rep* 6:179-97.

Hansen, A.A., Herbert, R.A., Mikkelsen, K., Jensen, L.L., Kristoffersen, T., Tiedje, J.M., Lomstein, B.A., Finster, K.W. (2007) Viability, diversity and composition of the bacterial community in a high Arctic permafrost soil from Spitsbergen, Northern Norway. *Environ Microbiol* 9:2870-2884.

Harrison, J.P., Gheeraert, N., Tsigelnitskiy, D., and Cockell, C.S. (2013) The limits for life under multiple extremes. *Trends Microbiol* 21:204-212.

Hassler, D.M., Zeitlin, C., Wimmer-Schweingruber, R.F., and MSL Science Team (2014) Mars' surface radiation environment measured with the Mars Science Laboratory's Curiosity Rover. *Science* 343:6169.

Hecht, M.H., Kounaves, S.P., Quinn, R.C. and Smith P.H. (2009) Detection of perchlorate and the soluble chemistry of Martian soil at the Phoenix lander site. *Science* 325:64-67.

Henson, J.M., Butler, M.J., and Day, A.W. (1999) The dark side of the mycelium: melanins of phytopathogenic fungi. *Annu Rev Phytopathol* 37:447-471.

Ho, C.L., Phang, S.M., and Pang, T. (1995) Molecular characterisation of Sargassum polycystum and S. siliquosum (Phaeophyta) by polymerase chain reaction (PCR) using random amplified polymorphic DNA (RAPD) primers. *J Appl Phycol* 7(1):33-41.

Hollis, J.M., Lovas, F.J., and Jewell, P.R. (2000) Interstellar glycolaldehyde: the first sugar. *Astrophys J Lett* 540.2:L107.

Horneck, G., Bücker, H., Reitz, G. (1994) Long-term survival of bacterial spores in space. *Adv Space Res* 14:41-45.

Horneck, G. (2000) The microbial world and the case for Mars. *Planet Space Sci* 48(11):1053-1063.

Horneck, G., Klaus, D.M., and Mancinelli, R.L. (2010) Space microbiology. *Microbiol Mol Biol Rev* 74:121-156.

Horneck, G., Moeller, R., Cadet, J., Douki, T., Mancinelli, R.L., Nicholson, W.L, Panitz, C., Rabbow, E., Rettberg, P., Spry, A., Stackebrandt, E., Vaishampayan, P., and Kasthuri J. (2012) Resistance of bacterial endospores to outer space for planetary protection purposes—experiment PROTECT of the EXPOSE-E mission. *Astrobiology* 12(5):445-456.

Horneck, G., Walter, N., Westall, F., Grenfell, J.L., Martin, W. F., Gomez, F., Leuko, S., Lee, N., Onofri, S., Tsiganis, K., Saladino, R., Pilat-Lohinger E., Palomba, E., Harrison, J., Rull, F., Muller, C., Strazzulla, G., Brucato, J.R., Rettberg, P., and Capria M.T. (2016) AstRoMap European Astrobiology Roadmap. *Astrobiology* 16:201-243.

Horowitz, N.H., Cameron, R.E., and Hubbard, J.S. (1972) Microbiology of the dry valleys of Antarctica. *Science* 176(4032):242-245.

Hutchinson, I.B., Edwards, H.G.M., Ingley, R., Harris, L., McHugh, M., Malherbe, C., Jehlicka, J., Marshall, C., and Parnell, J. (2014) Preparations for the Launch of the EXOMARS Raman Laser Spectrometer — A Review of Recent Studies Which Highlight the Astrobiological and Geological Capabilities of Portable Raman Instrumentation. *LPI Contributions* 1783, 5093.

Igisu, M., Nakashima, S., Ueno, Y., Awramik, S.M., and Maruyama, S. (2006) In situ infrared microspectroscopy of ~850 million-year-old prokaryotic fossils. *Appl Spectrosc* 60:1111–1120

Igisu, M., Ueno, Y., Shimojima, M., Nakashima, S., Awramik, S.M., Ohta, H. and Maruyama, S. (2009) Micro-FTIR spectroscopic signatures of bacterial lipids in Proterozoic microfossils. *Precambrian Res* 173:19–26.

Isenbarger, T.A., Carr, C.E., Johnson, S.S., Finney, M., Church, G.M., Gilbert, W., Zuber M.T., and Ruvkun, G. (2008) The most conserved genome segments for life detection on Earth and other planets. *Orig Life Evol Biosph* 38.6:517-533.

Jaakkola, S.T., Ravantti, J.J., Oksanen, H.M., and Bamford, D.H. (2016) Buried Alive: Microbes from Ancient Halite. *Trends Microbiol* 24(2):148-160.

Johnson, A.P., Pratt, L.M., Vishnivetskaya, T., Pfiffner, S., Bryan, R.A., Dadachova, E., Radtke, K., Chan, E., Tronick, S., Borgonie, G., Mancinelli, R.L., Rothschild, L.J., Rogoff, D.A., Horikawa, D.D., and Onstott, T.C. (2011) Extended survival of several organisms and amino acids under simulated Martian surface conditions. *Icarus* 211:1162-1178.

Jorge-Villar, S., and Edwards, H. (2013) Microorganism Response to Stressed Terrestrial Environments: A Raman Spectroscopic Perspective of Extremophilic Life Strategies. *Life* 3:276-294.

Kanavarioti, A., and Mancinelli, R.L. (1990) Could organic matter have been preserved on Mars for 3.5 billion years? *Icarus* 84:196-202

Khajo, A., Bryan, R.A., Friedman, M., Burger, R.M., Levitsky, Y., Casadevall, A., Magliozzo, R.S., Dadachova, E. (2011) Protection of melanized Cryptococcus neoformans from lethal dose gamma irradiation involves changes in melanin's chemical structure and paramagnetism. *PLoS One* 6(9):e25092.

Kirkpatrick, J.P., Meyer, J.J., and Marks, L.B. (2008) The linear-quadratic model is inappropriate to model high dose per fraction effects in radiosurgery. *Semin radiat oncol* 18(4):240-243

Kogej, T., Lanišnik-Rižner, T., and Gunde-Cimerman, N. (2001) Black yeast from the salterns: the effect of the salt on melanization. In: International conference on halophilic microorganisms. Ventosa A, Arahal DR Eds, Sevillia, Spain.

Kogej, T., Wheeler, M.H., Rižner, T.L., and Gunde-Cimerman, N. (2004) Evidence for 1, 8-dihydroxynaphthalene melanin in three halophilic black yeasts grown under saline and non-saline conditions. *FEMS Microbiol Lett* 232(2):203-209.

Kong, L., Dong, J., and Hart, G.E. (2000) Characteristics, linkage-map positions, and allelic differentiation of *Sorghum bicolor* (L.) *Moench* DNA simple-sequence repeats (SSRs). *Theor Appl Genet* 101(3):438-448.

Kovacs, E., and Keresztes, A. (2002) Effect of gamma and UV-B/C radiation on plant cells. *Micron* 33(2):199-210.

Kozyrovska, N.O., Lutvynenko, T.L., Korniichuk, O.S., Kovalchuk, M.V., Voznyuk, T.M., Kononuchenko, O., Zaetz, I., Rogutskyy, I.S., Mytrokhyn, O.V., Mashkovska, S.P., Foing, B.H., and Kordyum, V.A. (2006) Growing pioneer plants for a lunar base. *Adv Space Res* 37(1):93-99.

Kranner, I., Beckett, R., Hochman, A., and Nash, T.H. (2008) Desiccation-tolerance in lichens: a review. *Bryologist* 11:576-593.

Kuhn DM, Balkis M, Chandra J, Mukherjee PK, and Ghannoum MA (2003) Uses and limitations of the XTT assay in studies of Candida growth and metabolism. *J Clin Microbiol* 41:506-508.

Kunwar, A., Adhikary, B., Jayakumar, S., Barik, A., Chattopadhyay, S., Raghukumar, S., Priyadarsini, K.I. (2012) Melanin, a promising radioprotector: mechanisms of actions in a mice model. *Toxicol appl pharmacol* 264(2):202-11.

La Duc, M.T., Benardini, J.N., Kempf, M.J., Newcombe, D.A., Lubarsky, M., and Venkateswaran, K. (2007) Microbial diversity of Indian Ocean hydrothermal vent plumes: microbes tolerant of desiccation, peroxide exposure, and ultraviolet and γ-irradiation. *Astrobiology* 7(2):416-431.

Langfelder, K., Streibel, M., Jahn, B., Haase, G., and Brakhage, A.A. (2003) Biosynthesis of fungal melanins and their importance for human pathogenic fungi. *Fungal Genet Biol* 38(2):143-158.

Levy, J. (2013) How big are the McMurdo Dry Valleys? Estimating ice-free area using Landsat image data. *Antarct Sci* 25(01):119-120.

Lin, L.H., Wang, P.L., Rumble, D., Lippmann-Pipke, J., Boice, E., Pratt, L.M., Lollar, B.S., Brodie, E.L., Hazen, T.C., Andersen, G.L., DeSantis, T.Z., Moser, D.P., Kershaw, D., Onstott, T.C. (2006) Long-Term Sustainability of a High-Energy, Low-diversity Crustal Biome. *Science* 314:479-482.

Lindahl, T. (1993) Instability and decay of the primary structure of DNA. *Nature* 362:709-715.

Lyon, D.Y., Monier, J.M., Dupraz, S., Freissinet, C., Simonet, P., and Vogel, T.M. (2010) Integrity and Biological Activity of DNA after UV Exposure. *Astrobiology* 10:285-292.

Marples, B., and Joiner, M.C. (1993) The response of Chinese hamster V79 cells to low radiation doses: evidence of enhanced sensitivity of the whole cell population. *Radiat Res* 133(1):41-51.

Martins, Z., Botta, O., Fogel, M.L., Sephton, M.A., Glavin, D.P., Watson, J.S., Dworkin, J.P., Schwart, A.W. and Ehrenfreund, P. (2008) Extraterrestrial nucleobases in the Murchison meteorite. *Earth Planet Sci Let* 270:130-136.

McKay, C.P., Friedmann, E.I., Gomez-Silva, B., Caceres-Villanueva, L., Andersen, D.T., Landheim, R. (2003) Temperature and moisture conditions for life in the extreme arid region of the Atacama Desert: four yr of observations including the El Niño of 1997–1998. *Astrobiology* 3:393-406.

Meeßen, J., Sánchez, F.J., Brandt, A., Balzer, E.M., de la Torre, R., Sancho, L.G., de Vera, L.P., and Ott, S. (2013a) Extremotolerance and resistance of lichens: comparative studies on five species used in astrobiological research I. Morphological and anatomical characteristics. *Origins Life Evol B* 43:283-303.

Meeßen, J., Sánchez, F.J., Sadowsky, A., de la Torre, R., Ott, S., and de Vera, J.P. (2013b) Extremotolerance and Resistance of Lichens: Comparative Studies on Five Species Used in Astrobiological Research II. Secondary Lichen Compounds. *Origins Life Evol B* 43:501-526.

Meeßen, J., Wuthenow, P., Schille, P., Rabbow, E., de Vera, J.P., Ott, S. (2015) Resistance of the lichen *Buellia frigida* to simulated space conditions during the preflight tests for BIOMEX—viability assay and morphological stability. *Astrobiology* 15.

Meredith, P., and Sarna, T. (2006) The physical and chemical properties of eumelanin. *Pigment Cell* Res 19(6):572-94.

Mileikowsky, C., Cucinotta, F.A., Wilson, J.W., Gladman, B., Horneck, G., Lindegren, L., Melosh, J., Rickmanh, H., Valtoneni M., and Zheng, J.Q. (2000a) Natural transfer of viable microbes in space: 1. From Mars to Earth and Earth to Mars. *Icarus* 145:391-427.

Mileikowsky, C., Cucinotta, F.A., Wilson, J.W., Gladman, B., Horneck, G., Lindegren, L., Melosh, J., Rickman, H., Valtonen, M., and Zheng, J.Q. (2000b) Risks threatening viable transfer of microbes between bodies in our solar system. *Planet Space Sci* 48:1107-1115.

Mytrokhyn, O.V., Bogdanova, S.V., Shumlyanskyy, L.V. (2003) Anorthosite rocks of Fedorivskyy suite (Korosten Pluton, Ukrainian Shield). In: Current Problems in Geology. Kyiv National University, Kyiv, pp 53–57.

Moeller, R., Reitz, G., Berger, T., Okayasu, R., Nicholson, W. L., and Horneck, G. (2010) Astrobiological aspects of the mutagenesis of cosmic radiation on bacterial spores. *Astrobiology* 10(5):509-521.

Moeller, R., Reitz, G., Wayne, L., Nicholson, the PROTECT Team, and Horneck, G. (2012a) Mutagenesis in bacterial spores exposed to space and simulated Martian conditions: data from the EXPOSE-E spaceflight experiment PROTECT. *Astrobiology* 12:457-468.

Moeller, R., Schuerger, A.C., Reitz, G., and Nicholson, W.L. (2012b) Protective role of spore structural components in determining Bacillus subtilis spore resistance to simulated Mars surface conditions. *Appl Environ Microbiol* 87:8849–8853.

Moeller, R., Raguse, M., Leuko, S., Berger, T., Hellweg, C., Fujimori, A., Okayasu, R., and Horneck, G. STARLIFE – an international campaign to study the role of galactic cosmic radiation in astrobiological model systems. *Astrobiology*, in press.

Murray, A.E., Kenig, F., Fritsen, C.H., McKay, C.P., Cawley, K.M., Edwards, R., Kuhn, E., McKnight, D.M., Ostrom, N.E., Peng, V., Ponce, A., Priscu, J.C., Samarkin, V., Townsend, A.T., Wagh, P., Young, S.A., Yung, P.T., Doran, P.T. (2012) Microbial life at −13 °C in the brine of an ice-sealed Antarctic lake. *Proc Natl Acad Sci* USA 109:20626-20631.

Novikova, N.D. (2004) Review of the knowledge of microbial contamination of the Russian manned spacecraft. *Microb Ecol* 47:127-132.

Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J., and Setlow, P. (2000) Resistance of Bacillus endospores to extreme terrestrial and extraterrestrial environments. *Microbiol Mol Biol R* 64(3):548-572.

Nicholson, W.L. (2009) Ancient micronauts: interplanetary transport of microbes by cosmic impacts. *Trends Microbiol* 17:243-250.

Nienow, J.A., and Friedmann, E.I. (1993) Terrestrial litophytic (rock) communities. Antarctic Microbiology Wiley-Liss Eds., New York. pp 343-412.

Nybakken, L., Solhaug, K.A., Bilger, W., and Gauslaa, Y. (2004) The lichens *Xanthoria elegans* and *Cetraria islandica* maintain a high protection against UV-B radiation in Arctic habitats. *Oecologia* 140(2):211-216.

Olsson-Francis, K., de La Torre, R., Towner, M.C., and Cockell, C.S. (2009) Survival of akinetes (resting-state cells of cyanobacteria) in Low Earth Orbit and simulated extraterrestrial conditions. *Origins Life Evol B* 39(6):565-579.

Onofri, S., Selbmann, L., Zucconi, L., and Pagano, S. (2004) Antarctic microfungi as models for exobiology. *Planet Space Sci* 52:229-237.

Onofri, S., Zucconi, L., Selbmann, L., Hoog, G.S., de los Ríos, A., Ruisi, S., and Grube, M. (2007a) Fungal Association at the cold edge of life. In: Algae and Cyanobacteria in Extreme Environments. Series: Cellular Origin, Life in Extreme Habitats and Astrobiology (Seckbach J, ed.). Springer, Berlin.

Onofri, S., Selbmann, L., De Hoog, G.S., Grube, M., Barreca, D., Ruisi, S., and Zucconi, L. (2007b) Evolution and adaptation of fungi at boundaries of life. *Adv Space Res* 40:1657-1664.

Onofri, S., Barreca, D., Selbmann, L., Isola, D., Rabbow, E., Horneck, G., de Vera, J.P., Hatton, J., and Zucconi, L. (2008) Resistance of Antarctic black fungi and cryptoendolithic communities to simulated space and Mars conditions. *Stud Mycol* 61:99-109.

Onofri, S., de la Torre, R., de Vera, J.P., Ott, S., Zucconi, L., Selbmann, L., Scalzi, G., Venkateswaran, K.J. Rabbow, E., Sánchez Iñigo, F.J., and Horneck, G. (2012) Survival of rock-colonizing organisms after 1.5 years in outer space. *Astrobiology* 12:508-516.

Onofri, S., de Vera, J.P, Zucconi, L., Selbmann, L., Scalzi, G., Venkateswaran, K.J., Rabbow, E., de la Torre, R., and Horneck G. (2015) Survival of Antarctic Cryptoendolithic Fungi in Simulated Martian Conditions On Board the International Space Station. *Astrobiology* 15(12):1052-1059.

Oren, A., Gurevich, P., Anati, D.A., Barkan, E., and Luz, B. (1995) A bloom of Dunaliella parva in the Dead Sea in 1992: biological and biogeochemical aspects. *Hydrobiologia* 297(3):173-185.

Osterloo, M.M., Hamilton, V.E., Bandfield, J.L., Glotch, T.D., Baldridge, A.M., Christensen, P.R., Tornabene, L., and Anderson, F.S. (2008) Chloride-bearing materials in the southern highlands of Mars. *Science* 319(5870):1651-1654.

Pääbo, S., Poinar, H., Serre, D., Jaenicke-Després, V., Hebler, J., Rohland, N., Kuch, M., Krause, J., Vigilant, L., and Hofreiter, M. (2004) Genetic analyses from ancient DNA. A*nn Rev Genet* 38:645-679.

Pacelli, C., Selbmann, L., Zucconi, L., De Vera, J.P., Rabbow, E., Horneck, G., de la Torre, R., and Onofri, S. (2016) BIOMEX experiment: Ultrastructural alterations, molecular damage and survival of the fungus *Cryomyces antarcticus* after the Experiment Verification Tests. *Orig life evol biosph* 1-16.

Panieri, G., Lugli, S., Manzi, V., Roveri, M., Schreiber, B. C., and Palinska, K. A. (2010) Ribosomal RNA gene fragments from fossilized cyanobacteria identified in primary gypsum from the late Miocene, Italy. *Geobiology* 8(2):101-111.

Plemenitaš, A., Vaupotič, T., Lenassi, M., Kogej, T., and Gunde-Cimerman, N. (2008) Adaptation of extremely halotolerant black yeast Hortaea werneckii to increased osmolarity: a molecular perspective at a glance. *Stud Mycol* 61:67-75.

Pointing, S.B., Chan, Y., Lacap, D.C., Lau, M.C., Jurgens, J.A., and Farrell, R.L. (2009) Highly specialized microbial diversity in hyper-arid polar desert. *Proc Natl Acad Sci* 106:19964-19969.

Poulet, F., Bibring, J.P., Mustard, J.F., Gendrin, A., Mangold, N., Langevin, Y., Arvidson, R.E., Gonde, B., and Erard, S. (2005) Phyllosilicates on Mars and implications for early Martian climate. *Nature* 438(7068):623-627.

Rabbow, E., Rettberg, P., Barczyk, S., Bohmeier, M., Parpart, A., Panitz, C., Horneck, G., von Heise-Rotenburg, R., Hoppenbrouwers, T., Willnecker, R., Baglioni, P., Demets, R., Dettmann, J., and Reitz, G. (2012) EXPOSE-E: an ESA astrobiology mission 1.5 years in space. *Astrobiology* 12:374-386.

Rabbow, E., Rettberg, P., Barczyk, S., and Bohmeier (2015) The astrobiological mission EXPOSE-R on board of the International Space Station. *Int J Astrobiol* 14(01):3-16.

Rageh, M.M., El-Gebaly, R.H., Abou-Shady, H., and Amin, D.G. (2014) Melanin Nanoparticles (MNPs) provide protection against whole body irradiation in mice via restoration of hematopoietic tissues. *Mol Cell Biochem* 399:59-69.

Raggio, J., Pintado, A., Ascaso, C., de La Torre, R., De Los Rios, A., Wierzchos, J., Horneck, G., and Sancho, L.G. (2011) Whole lichen thalli survive exposure to space conditions: results of Lithopanspermia experiment with *Aspicilia fruticulosa*. *Astrobiology* 11(4):281-292.

Rainey, F.A., Ray, K., Ferreira, M., Gatz, B.Z., Nobre, M.F., Bagaley, D., Rash, B.A., Park, M.J., Earl, A.M., Shank, N.C., Small, A.M., Henk, M.C., Battista, J.R., Kämpfer, P., and da Costa, M.S. (2005) Extensive diversity of ionizing-radiation-resistant bacteria recovered from Sonoran Desert soil and description of nine new species of the genus Deinococcus obtained from a single soil sample. *Appl Environ Microb* 71(9):5225-5235.

Revskaya, E., Chu, P., Howell, R.C., Schweitzer, A.D., Bryan, R.A., Harris, M., Dadachova, E., and Casadevall, A. (2012) Compton scattering by internal shields based on melanin-containing mushrooms provides protection of gastrointestinal tract from ionizing radiation. *Cancer Biother Radiopharm* 27(9):570-6.

Robertson, K. L., Mostaghim, A., Cuomo, C. A., Soto, C. M., Lebedev, N., Bailey, R. F., and Wang, Z. (2012) Adaptation of the black yeast Wangiella dermatitidis to ionizing radiation: molecular and cellular mechanisms. *PloS one* 7(11):e48674.

Röling, W.F.M., and Head, I.M. (2005) Prokaryotic Systematics: PCR and Sequence Analysis of Amplified 16S rRNA Genes. In Molecular Microbiology Ecology; Garland Science: New York, NY, USA.

Ruisi, S., Barreca, D., Selbmann, L., Zucconi, L., and Onofri, S. (2007) Fungi in Antarctica. *Rev Environ Sci Biotechnol* 6(1-3):127-141.

Sachs, R.K., Hahnfeld, P., and Brenner, D.J. (1997) The link between low-LET dose-response relations and the underlying kinetics of damage production/repair/misrepair. *Int j radiat biol* 72(4):351-374.

Saffary, R., Nandakumar, R., Spencer, D., Robb, F.T., Davila, J.M., Swartz, M., Ofman, L., Thomas, R.J., and DiRuggiero, J. (2002) Microbial survival of space vacuum and extreme ultraviolet irradiation: strain isolation and analysis during a rocket flight. *FEMS Microbiol Lett* 215(1):163-168.

Sánchez, F.J., Mateo-Martí, E., Raggio, J., Meeßen, J., Martínez-Frías, J., Sancho, L.G., Ott, S., and de la Torre, R. (2012) The resistance of the lichen *Circinaria gyrosa* (nom. provis.) towards simulated Mars conditions—a model test for the survival capacity of an eukaryotic extremophile. *Planet Space Sci* 72(1):102-110.

Sancho, L. G., De la Torre, R., Horneck, G., Ascaso, C., de los Rios, A., Pintado, A., Wierzchos, J., and Schuster, M. (2007) Lichens survive in space: results from the 2005 LICHENS experiment. *Astrobiology* 7(3):443-454.

Sancho, L.G., de la Torre, R., and Pintado, A. (2008) Lichens, new and promising material from experiments in astrobiology. *Fungal Biol Rev* 22(3):103-109.

Sankaranarayanan, K., Lowenstein, T.K., Timofeeff, M.N., Schubert, B.A., and Lum, J.K. (2014) Characterization of Ancient DNA Supports Long-Term Survival of Haloarchaea. *Astrobiology* 14:553-560.

Scalzi, G., Selbmann, L., Zucconi, L., Rabbow, E., Horneck, G., Albertano, P., and Onofri, S. (2012) LIFE Experiment: isolation of cryptoendolithic organisms from Antarctic colonized sandstone exposed to space and simulated Mars conditions on the International Space Station. *Orig life evol biosph* 42(2-3): 253-262.

Schmitt-Kopplin, P., Gabelica, Z., Gougeon, R.D., Fekete, A., Kanawati, B., Harir, M., Gebefuegia, I., Eckel, G., and Hertkorn, N. (2010) High molecular diversity of extraterrestrial organic matter in Murchison meteorite revealed 40 years after its fall. *Proc Natl Acad Sci* 107(7):2763-2768.

Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012) NIH Image to ImageJ: 25 years of image analysis, *Nat Methods* 9(7): 671-675.

Schweitzer, A.D., Revskaya, E., Chu, P., Pazo, V., Friedman, M., Nosanchuk, J.D., Cahill, S., Frases, S., Casadevall, A., and Dadachova, E. (2010) Melanin-covered nanoparticles for protection of bone marrow during radiation therapy of cancer. *Intern J Rad Oncol Biol Physics* 78(5):1494-502.

Sephton, M.A. (2010) Organic geochemistry and the exploration of Mars. *J Cosmol* 5:1141-1149.

Selbmann, L., De Hoog, G.S., Mazzaglia, A., Friedmann, E.I., and Onofri, S. (2005) Fungi at the edge of life: cryptoendolithic black fungi from Antarctic desert. *Stud Mycol* 51:1-32.

Selbmann, L., de Hoog, G.S., Zucconi, L., Isola, D., Ruisi, S., Gerrits van den Ende, A.H.G., Ruibal, C., De Leo, F., Urzì, C., and Onofri, S. (2008) Drought meets acid: three new genera in a dothidealean clade of extremotolerant fungi. *Stud Mycol* 61:1-20.

Selbmann, L., Isola, D., Zucconi, L., and Onofri, S. (2011) Resistance to UV-B induced DNA damage in extreme-tolerant cryptoendolithic Antarctic fungi: detection by PCR assays. *Fungal biol* 115: 937-944.

Selbmann, L., Egidi, E., Isola, D., Onofri, S., Zucconi, L., de Hoog, G.S., Chinaglia, S., Testa L., Tosi, S., Balestrazzi, A., Lantieri, A., Compagno, R., Tigini, V., and Varese, G.C. (2013) Biodiversity, evolution and adaptation of fungi in extreme environments. *Plant Biosyst* 147(1):237-246.

Selbmann, L., Isola, D., Egidi, E., Zucconi, L., Gueidan, C., de Hoog, G.S., and Onofri, S. (2014a) Mountain tips as reservoirs for new rock-fungal entities: Saxomyces gen. nov. and four new species from the Alps. *Fungal Divers* 65:167-182.

Selbmann, L., de Hoog, G.S., Zucconi, L., Isola, D., and Onofri, S. (2014b) Black Yeasts In Cold Habitats. In: Yeasts from cold Habitats, Buzzini, P. Margesin, R. (Eds), Springer-Verlag, Berlin, pp 173-189.

Sephton, M.A. (2010) Organic geochemistry and the exploration of Mars. *J Cosmol* 5:1141-1149.

Shiroma, W.A., Martin, L.K., Akagi, J.M., Akagi, J.T., Wolfe, B.L., Fewell, B.A., and Ohta, A.T. (2011) CubeSats: A bright future for nanosatellites. *Cent Eur J Eng* 1, 9–15.

Shtarkman, Y.M., Koçer, Z.A., Edgar, R., Veerapaneni, R.S., D'Elia, T., Morris, P.F., and Rogers, S. O. (2013) Subglacial Lake Vostok (Antarctica) accretion ice contains a diverse set of sequences from aquatic, marine and sediment-inhabiting bacteria and eukarya. *PLoS One* 8(7):e67221.

Shuryak, I., Bryan, R.A., Nosanchuk, J.D., and Dadachova, E. (2014) Mathematical modeling predicts enhanced growth of X-ray irradiated pigmented fungi. *PLoS One* 9(1):e85561.

Shuryak, I., Sun, Y., and Balajee, A.S. (2016) Advantages of Binomial Likelihood Maximization for Analyzing and Modeling Cell Survival Curves. *Radiat Res* 185(3):246-56.

Singh, R.K., and Krishna, M. (2006) DNA damage induced nucleotide excision repair in *Saccharomyces cerevisiae. Mol Cell Biochem* 290(1-2):103-12.

Shuryak, I., Sun, Y., and Balajee, A.S. (2016) Advantages of Binomial Likelihood Maximization for Analyzing and Modeling Cell Survival Curves. *Radiat Res* 185(3):246-256.

Staley, J.T., Palmer, F., and Adams, J.B. (1982) Microcolonial fungi: common inhabitants on desert rocks?. *Science* 215(4536):1093-1095.

Sterflinger, K., De Hoog, G.S., and Haase, G. (1999) Phylogeny and ecology of meristematic ascomycetes. *Stud Mycol* 5-22.

Sterflinger, K. (2005) Black yeasts and meristematic fungi: ecology, diversity and identification. In Yeast Handbook: Biodiversity and Ecophysiology of Yeasts, edited by C. Rosa and P. Gabor, Springer, New York, pp 505-518.

Sterflinger, K. (2006) Black yeasts and meristematic fungi: ecology, diversity and identification. In: Seckbach J, editor. The yeast handbook. Biodiversity and ecophysiology of yeasts Berlin: Springer-Verlag. p. 501-14.

Sterflinger, K., Tesei, D., and Zakharova, K. (2012) Fungi in hot and cold deserts with particular reference to microcolonial fungi. *Fungal ecol* 5:453-462.

Sterflinger, K., Lopandic, K., Pandey, R.V., Blasi, B., and Kriegner, A. (2014) Nothing special in the specialist? Draft genome sequence of *Cryomyces antarcticus*, the most extremophilic fungus from Antarctica. *PLOS One* 9(10):e109908.

Stevenson, A., Burkhardt, J., Cockell, C.S., Cray, J.A., Dijksterhuis, J., Fox-Powell, M., Kee, T.P., Kminek, G., McGenity, T.J., Timmis, K.N., Timson, D.J., Voytek, M.A., Westall, F., Yakimov, M.M., Hallsworth, J.E. (2015) Multiplication of microbes below 0.690 water activity: implications for terrestrial and extraterrestrial life. *Environ Microbiol* 17(2):257-277.

Stewart, R.D., Yu, V.K., Georgakilas, A.G., Koumenis, C., Park, J.H., and Carlson, D.J. (2011) Effects of radiationquality and oxygenonclustered DNA lesions and celldeath. *Radiat Res* 176(5):587-602.

Stivaletta, N., Lopez-Garcia, P., Boihem, L., Millie, D.F., and Barbieri, R. (2010) Biomarkers of endolithic communities within gypsum crusts (southern Tunisia). *Geomicrobiol J* 27(1): 101-110.

Sullivan III, W.T., and Baross, J. (2007) Planets and life: the emerging science of astrobiology. *Camb Univ Press.*

Tarcea, N., Frosch, T., Rösch, P., Hilchenbach, M., Stuffler, T., Hofer, S., Thiele, H., Hochleitner, R., and Popp, J. (2008) Raman spectroscopy—A powerful tool for in situ planetary science. *In Strategies of Life Detection* (pp. 281-292). Springer US.

Tepfer, D., Zalar, A., and Leach, S. (2012) Survival of plant seeds, their UV screens, and nptII DNA for 18 months outside the International Space Station. *Astrobiology* 12(5):517-528.

Tesei, D., Marzban, G., Zakharova, K., Isola, D., Selbmann, L., and Sterflinger, K. (2012) Alteration of protein patterns in black rock inhibiting fungi as a response to different temperatures. *Fungal Biol* 116:932-940.

Turick, C.E., Ekechukwu, A.A., Milliken, C.E., Casadevall, A., and Dadachova, E. (2011) Gamma radiation interacts with melanin to alter its oxidation-reduction potential and results in electric current production. *Bioelectrochem* 82(1):69-73.

Vago, J., Gardini, B., Kminek, G., Baglioni, P., Gianfiglio, G., Santovincenzo, A., Bayón, S., and van Winnendael, M. (2006) ExoMars-searching for life on the Red Planet. *ESA Bulletin* 126:16-23.

van Uden, N. (1984) Temperature profiles of yeasts. *Adv Microb Physiol* 25:195-251.

Vember, V.V., Zhdanov, N.N., and Tugai, T.I. (1999) The effect of gamma irradiation on the physiological-biochemical properties of strains of *Cladosporium cladosporioides* (Fres.) de Vries differing by the trait of radiotropism. *Mikrobiolohichnyi zhurnal* 61(2):25-32.

Vilgalys, R. and Hester, M. (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several Cryptococcus species. *J Bacteriol* 172:4238-4246.

Vishniac, H.S. (1993) The microbiology of Antarctic soils. *Antarct Microbiol* 297-341.

Vítek, P., Edwards, H.G.M., Jehlička, J., Ascaso, C., Ríos, A.D.L., Valea, S., Jorge-Villar, S.E., Davila, A.F., and Wierzchos, J. (2010) Microbial colonization of halite from the hyper-arid Atacama Desert studied by Raman spectroscopy. *Phil Trans R Soc A* 368:3205-3221.

Wayne, R.K., Leonard, J.A., and Cooper, A. (1999) Full of sound and fury: the recent history of ancient DNA. *Annu Rev Ecol Syst* 457-477.

Weinstein, R.N., Montiel, P.O., and Johnstone, K. (2000) Influence of growth temperature on lipid and soluble carbohydrate synthesis by fungi isolated from fellfield soil in the maritime Antarctic. *Mycologia* 222-229.

White, T.J., Bruns, T., Lee, S., and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications 18:315-322.

Witherow, R.A., Lyons, W.B., Bertler, N.A., Welch, K.A., Mayewski, P.A., Sneed, S.B., Nylen, T., Handley, M.J., and Fountain, A. (2006) The aeolian flux of calcium, chloride and nitrate to the McMurdo Dry Valleys landscape: evidence from snow pit analysis. *Antarct Sci* 18(04):497-505.

Willerslev, E., Hansen, A.J. and Poinar, H.N. (2004) Isolation of nucleic acids and cultures from fossil ice and permafrost. *Trends Ecol Evol* 19:141-147.

Wollenzien, U., De Hoog, G.S., Krumbein, W.E., and Urzi, C. (1995) On the isolation of microcolonial fungi occurring on and in marble and other calcareous rocks. *Sci Total Environ* 167(1):287-294.

Woellert, K., Ehrenfreund, P., Ricco, A.J., and Hertzfeld, H. (2011) Cubesats: Cost-effective science and technology platforms for emerging and developing nations. *Adv Space Res* 47:663-684.

Xue, L., Furusawa, Y., and Yu, D. (2015) ATR signaling cooperates with ATM in the mechanism of low dose hypersensitivity induced by carbon ion beam. *DNA Repair* 34:1-8.

Yen, A.S., Kim, S.S., Hecht, M.H., Frant, M.S., and Murray, B. (2000) Evidence that the reactivity of the Martian soil is due to superoxide ions. *Science* 289:1909-1912.

Yokoya, A., Shikazono, N., Fujii, K., Urushibara, A., Akamatsu, K., and Watanabe, R. (2008) DNA damage induced by the direct effect of radiation. *Radiat Phys Chem* 77:1280-1285.

Yoshida, S., Takeo, K., De Hoog, G.S., Nishimura, K., and Miyaji, M. (1996) A new type of growth exhibited by Trimmatostroma abietis. *Antonie van Leeuwenhoek* 69(3):211-215.

Zakharova, K., Marzban, G., de Vera, J.P., Lorek, A., and Sterflinger, K. (2014) Protein patterns of black fungi under simulated Mars-like conditions. *Sci Rep* 4.

Zettler, L.A.A., Gómez, F., Zettler, E., Keenan, B.G., Amils, R., and Sogin, M.L. (2002) Microbiology: eukaryotic diversity in Spain's River of Fire. *Nature* 417(6885):137-137.

Zhdanova, N.N., Zakharchenko, V.A., Vember, V.V., Nakonechnaya, L.T. (2000) Fungi from Chernobyl: mycobiota of the inner regions of the containment structures of the damaged nuclear reactor. *Mycol Res* 104:1421-1426.

Zucconi, L., Onofri, S., Cecchini, C., Isola, D., Ripa, C., Fenice, M., Madonna, S., Reboleiro-Rivas, P., and Selbmann, L. (2016) Mapping the lithic colonization at the boundaries of life in Northern Victoria Land, Antarctica. *Polar Biol* 39(1):91-102.