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# Impact of physical conditions of deep space on the infectivity of coliphage MS2

Graduation Thesis

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# Utjecaj fizikalnih uvjeta dubokog svemira na infektivnost kolifaga MS2

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Posljednjih nekoliko godina demonstrirane su opasnosti koje virusne bolesti predstavljaju za javno zdravstvo. Kada bi se virusna epidemija dogodila u svemirskoj bazi, posljedice bi bile mnogo gore nego na Zemlji. U ovom istraživanju, virioni kolifaga MS2, surogata za enteričke i respiratorne viruse, izloženi su nekim fizikalnim uvjetima dubokog svemira – temperaturama -80 °C i do 80 °C, simulantu mjesečeve prašine, tlaku bliskom vakuumu, visokom intenzitetu rendgenskih zraka i disperziji u hladnim kapljicama u prisutnosti UV-a. Rezultati pokazuju da većina testiranih uvjeta dubokog svemira značajno inaktivira viruse. Na neke od uvjeta, virusi su doduše bili otporniji nego što je očekivano npr. ekstremno niske temperature, nizak tlak i rendgenske zrake. Također, simulant mjesečeve prašine dao je određenu razinu zaštite virusima na termičku inaktivaciju, ali pri niskim temperaturama, djelovala je negativno na viruse. Uz to, pokazano je da se MS2 može širiti preko kapljica suspendiranih u zraku, čak i uz prisutnost UV-a što doprinosi ideji o stabilnosti virusa u kapljicama vode koje nastaju u izbojima vode na ledenim mjesecima sunčevog sustava poput Europe ili Enceladusa. Ovo istraživanje pokazuje da da fizikalni uvjeti dubokog svemira mogu značajno inaktivirati visoko rezistentni virus MS2 i postavlja temelje za buduća istraživanja u području svemirske virologije.

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# Impact of physical conditions of deep space on the infectivity of coliphage MS2

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Recent years have demonstrated the dangers that viral diseases pose to public health. If an outbreak happens in a space habitat, the consequences would be more devastating than on Earth. In this research, virions of coliphage MS2, a surrogate for enteric and respiratory viruses have been exposed to some of the physical conditions of deep space – temperatures of -80 °C, and up to 80 °C, lunar dust simulant, near-vacuum pressure, high X-ray doses, and dispersion in cold droplets under presence of UV. Results show that most tested conditions of space rapidly inactivate viruses. However, to some of those conditions like extremely low temperatures, near-vacuum, or X-rays, viruses showed a higher degree of resistance than expected. Also, lunar dust simulant showed some degree of protection for viruses at high temperatures but aids their inactivation at low temperatures. Additionally, MS2 has been shown to be transmissible in air droplets and aerosols, even under UV light, aiding the idea of viral stability in water plumes of solar system's icy moons like Europa or Enceladus. Taken together, this study shows that physical conditions of deep space inactivate highly environmentally resistant MS2 virus to a great extent and paves the way for future research in space virology.

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Key words: virus stability, extreme conditions, MS2, radiation, low pressure, thermal inactivation, lunar dust.

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# List of Abbreviations

| | |
|---|---|
| 95%CI | 95% confidence interval |
| ANOVA | Analysis of variance |
| cDNA | Complementary DNA |
| DLR | Deutsches Zentrum für Luft- und Raumfahrt |
| DNA | Deoxyribonucleic acid |
| DSMZ | Deutsche Sammlung von Mikroorganismen und Zellkulturen |
| EAC-1 simulant | European Astronaut Centre-1 simulant |
| ESA | European Space Agency |
| EVA | Extra-vehicular activity |
| F-test | Fisher test |
| FAM | 6-carboxy-fluorescein (at 5’ end of a probe in qPCR) |
| GCR | Galactic cosmic ray |
| HR | Homologous repair |
| ISS | International Space Station |
| LD50 | 50% lethal dose (dose that reduces infectivity by 50%) |
| LEO | Low Earth orbit |
| MS2 | Male-specific 2 coliphage |
| NASA | National Aeronautics and Space Administration |
| NER | Nucleotide excision repair |
| NFQ | Non-fluorescent quencher (at 3’ end of a probe in qPCR) |
| NHEJ | Non-homologous end joining |
| NuSTAR | Nuclear Spectroscopic Telescope Array |
| OSIRIS-REx | Origins Spectral Interpretation Resource Identification Security - Regolith Explorer |
| PFU | Plaque-forming units |
| RBS | Ribosome-binding site |
| RNA | Ribonucleic acid |
| RNase | Ribonucleotidase |
| RT-qPCR | Reverse transcription-quantitative polymerase chain reaction |
| SD | Standard deviation |
| SDO | Solar Dynamics Observatory |
| SIRV-1 and -2 | *Sulfolobus islandicus* rod viruses 1 and 2 |
| SPE | Solar particle event |
| STIV | *Sulfolobus islandicus* icosahedral virus |
| $T_H2$ lymphocytes | Thymus “Helper”-2 lymphocytes |
| T-lymphocytes | Thymus lymphocytes |
| UV radiation | Ultraviolet radiation |

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# 1. Introduction

In recent years, there has been an increased interest in human space travel. In 2019, National Aeronautics and Space Administration (NASA) officially announced plans for sending the first woman and next man to the moon by 2024 under the project named Artemis (Pearlman, 2019). It is widely believed that this project will pave the way for future human missions to Mars (Dunbar, 2018). Besides, new technologies and high involvement of private companies into spaceflight has allowed cheaper, more efficient mission designs and opportunities for even more ambitious missions in the future. This means that currently, there are high investments and opportunities in the aerospace sector to allow human propagation beyond low Earth orbit (LEO), that is into deep space.

Newly planned prolonged missions for humans to the moon and Mars call for research of deep space conditions on biological factors. This means not just human bodies, but also behavior of microbes – both symbiotic and pathogenic. This is important to reduce the chances of future mission complications or even failures due to unpredicted biological reasons like astronauts falling ill or biological contamination of celestial bodies.

As recent years have shown, viral pandemics pose an increased risk to public health and society. Only a few of the most recent examples of viral diseases that caused significant concern around the world are Influenza A (H1N1)pdm09 (also known as swine flu) in 2009, Middle East respiratory syndrome in 2012, Ebola virus disease in 2014, Zika virus disease in 2015, and most recently, Coronavirus disease 2019 (COVID-19) which is still ongoing as of 2020. The rapid spread of COVID-19 all around the world clearly demonstrated the impact that viruses can have on all humankind. It pointed out the importance of researching viruses, methods for their detection, and prevention of spread of viral diseases. It also showed the importance of social distancing in prevention of respiratory virus spread (Lewnard and Lo, 2020).

In space habitats, humans are enclosed in compact spaces with limited possibilities of isolation, and extremely limited resources for treating the ill (Palinkas, 2001). In addition, the space environment possesses a set of stressful factors, both for viruses and human bodies, which can complicate the disease outcomes as will be discussed further throughout the text. Hypothetically, a viral disease outbreak in a space habitat, as prone to spread as those witnessed recently on Earth, would have devastating consequences for astronauts, and potentially, the future of space exploration. Hence, it is important to understand viral stability in the space environment to assess the true risks of viral infections occurring in astronauts.

Coliphage *Male-specific 2* (MS2) is a commonly used surrogate for enteric and respiratory RNA viruses (Helmer and Finch, 1993; Dawson *et al.*, 2005; Coulliette *et al.*, 2014). Coliphages are viruses whose natural host is well-studied bacteria *Escherichia coli*. Many of them are therefore easy to culture, and consequently, also studied in detail, as opposed to human viruses which are a health hazard and require complex conditions to grow. The specifics of MS2 replication cycle and its value as a surrogate is discussed in detail in section 1.5. The insights gained from the stability studies of MS2 can be inferred to human viruses to prevent or sustain future outbreaks, both on Earth and in space missions.

## 1.1. Stressful physical conditions of deep space

Human space travel poses a set of significant challenges. One of the greatest problems is overcoming the stressful conditions of space and their consequences on human missions. This includes the effects on human bodies, technology needed to aid humans on such missions, and human microbiome (Mars, 2018). Those stressful conditions include high intensity of solar energetic particles, cosmic and UV radiation, extreme temperatures and pressures found on various objects throughout the solar system, extreme g-forces (during liftoff, achieving the orbit and orbital transfer), very fine, razor-sharp and cancerogenous dust particles on some rocky bodies such as moon, and psychological stress of being far from Earth. Special technologies are developed to protect humans from such stresses. Examples of those technologies include implementation of radiation-impermeable materials like aluminum or polyethylene in spacecraft and space suits (Naito *et al.*, 2020), use of indium tin oxide protective layer in space suit fiber to prevent lunar dust from adhering (Jenner, 2019), or design of the windowed Cupola module on International Space Station (ISS) so astronauts can have a constant view on planet Earth.

All such technologies, aiding in human space travel, are first tested on ISS in LEO, at an altitude of 400-420 km before they will be deployed in human missions beyond. Currently, the only crewed missions beyond LEO were nine Apollo missions - Apollo 8 and 10-17, all of which achieved the lunar orbit and six of them also landed on the lunar surface – Apollo 11, 12 and 14-17. Though limited in numbers, those missions offered some valuable insight into the effects of deep space conditions on human bodies which cannot be explored in LEO due to big differences. For example, ISS orbits close enough to Earth that it is protected from charged particles by the action of Earth's magnetic field, but in deep space, this natural protection lacks. Apollo missions showed that radiation level on such short-term missions, though higher than in LEO, does not pose a significant threat from radiation poisoning, as long as there are no solar particle events (SPEs, massive ejections of hazardous charged particles from the Sun) during missions (English *et al.*, 1973), which are unfortunately challenging to forecast even today (Hu, 2017).

The other example of a deep space condition that differs from LEO would be temperature changes. The ISS is locked in Earth's orbit. It makes one orbit approximately every 90 minutes. When it is on the day side, temperatures on the surface can get up to 93 °C, and on the night side down to -129 °C (Wright, 2015). Passive thermal control of the ISS allows humans to be productive and comfortable inside, but the question remains if it would be true for distinguishable thermic conditions of the moon, Mars, interplanetary space, or any other place in the solar system. The other problem with passive thermal control on the ISS is that it does not allow for temperature changes that humans experience on Earth, but rather holds a constant temperature range throughout any mission. Changes in habitat temperature therefore need to be controlled artificially, and it's not known if those artificial temperature variations as opposed to natural seasonal variations have any long-term effect on the human body. ISS also has an active thermal control, which takes over when the thermal conditions exceed the capabilities of passive control and it is mostly used for waste heat rejection.

### 1.1.1. Extreme temperatures

In various places of the solar system, temperatures greatly vary. Heat in any star system comes mostly from radiation of the host star(s). Hence, the central star is radiating heat in the form of light (mostly infrared) and its intensity drops with squared distance from the star, according to the inverse-square law (Weik, 2000). This results in a popular notion of a habitable zone around stars, referring to the region around the central star where the heat-bearing light intensity is such that the temperature range is suitable for liquid water to exist (Gurnett, 2009).

While true, this model alone does not explain all temperature ranges in the solar system. This is because the host star is not the only source of heat in a star system. Also, there are planetary bodies that can absorb or trap some heat and they can interact gravitationally resulting in heat transfer. All this complicates the distribution of temperature ranges in the solar system resulting in deviations from the ideal heat distribution. For example, on the night side of Mercury, the closest planet to the Sun, temperatures can reach down to -185 °C and on the day side up to 430 °C (Wild, 2019). This means that, if the conditions allow it, temperature ranges could be suitable for liquid water to exist even in some places on Mercury. Another well-known example would be Jupiter's moon Europa which, despite being far outside the habitable zone, has mounted evidence of liquid water under its surface (Mann, 2017). The heat for this icy moon comes from its tidal interactions with Jupiter. Also, despite being the hottest planet in the solar system, Venus has a temperate area in its atmosphere at ~50 km height where temperatures range between 0 and 100 °C. Recently, there has been some evidence of phosphine present in this temperate area of Venusian atmosphere (Greaves *et al.*, 2020) which is on Earth only produced artificially or by some bacteria, making it a potential biomarker. This evidence is however under debate (Voosen, 2020).

In the vacuum of deep space, temperature is harder to define. This is because the temperature of anything is a function of average motion speed of particles making it up. In the vacuum of space, the temperature of a body would depend on the density of heat-transferring light rays and, according to heat transfer theory, body's heat conduction, convection, and radiation. This means that if a biological entity like an infectious virus particle (virion) finds itself in a vacuum of space, the temperature it experiences highly depends on its surrounding environment. In other words, it makes a difference if a virion is dry in a vacuum, on a surface, or in liquid droplet.

There is a lot of recent evidence of water plumes on Europa (Roth *et al.*, 2014; Sparks *et al.*, 2017; Jia *et al.*, 2018), which has no atmosphere. This means that all those water droplets end up in vacuum of space around Europa. Currently, NASA is seriously considering sending a probe around the Jupiter's satellite to fly through one of those plumes and sample it (Howell and Pappalardo, 2020) in search for presence of biomarkers. Hypothetically, if a microbe finds itself in one of such water droplets in deep space, and we for now ignore evaporation of the droplet due to low pressure, it will probably experience fast cooling of the surrounding solution because water radiates heat. In addition, density of light rays at that distance from the Sun ($778 \times 10^6$ km) is too low to heat it efficiently (Li *et al.*, 2018). Therefore, the droplet temperature drops drastically. Hence, it would make sense to investigate transmissibility of viruses and other microbes in cold water droplets to try to model potential for their presence in Europa's and other icy satellites' water plumes.

Microbes generally sustain extremely low temperatures well, though they usually do not replicate and abate their metabolism in such conditions (Nedwell, 1999). In fact, temperatures of -80 °C and lower are usually used for long-term preservation of microbial samples, including viruses (De Paoli, 2005). For viruses, the lack of replication and slower metabolism of their host is not a limiting factor to their stability. From a perspective of a virus, the only relevant question is if it can stay infectious until a new host arrives so they can infect it, and afterwards finds itself in conditions that allow viral

replication. Most studies that explored viral stability in low temperatures confirm that low temperatures reduce the drop in viral infectivity over time as opposed to room temperature (Merrill *et al.*, 2008; Varianytsia and Vysekantsev, 2017). The most significant influence of low temperatures on an organism is that metabolism slows down or gets disrupted due to lack of heat. But viruses lack the active metabolism in their virion form, so this effect is not as significant for them as for other microbes. However, there are other indirect effects of low temperatures that pose a stress both to viruses and cells. Usually, biological entities are surrounded by water, and need it to aid in shaping their biomolecules. Freezing of the surrounding water causes freeze-induced desiccation of cells and viruses which can disrupt the shapes of biological macromolecules (Clarke *et al.*, 2013). Cells can fight this effect to some extent with chaperons, preserving the protein shape, but viruses have no way of counteracting it so the viral titer can drop due to this effect. It is therefore advised to add a cryoprotectant such as glycerol for long-term preservation of virus solutions.

Extremely high temperatures are also prevalent in the solar system. The problem that organisms face at high temperatures is mainly thermal denaturation of their proteins. Shape of most proteins is crucial for their biological function (Berg, Tymoczko and Stryer, 2002). At temperatures significantly higher than an organism is adapted to, its proteins change shape and consequently lose their ability to perform tasks - be it structural components, catalyzing reactions, or something else. Protein shape and heat stability highly depend on its primary structure, that is its amino acid sequence. Some organisms have evolved such protein sequences which are more resistant to heating than other proteins (Szilágyi and Závodszky, 2000). Many organisms have specialized proteins called chaperons which function by attaching to misfolded proteins and aid their folding. In this way, those organisms can handle temperature variations in their environment. Viruses, however, do not possess chaperons, unless they are inside a host. They can interact with host chaperons to aid their folding (Xiao, Wong and Luo, 2010; Geller, Taguwa and Frydman, 2012; Chamakura, Tran and Young, 2017). Coliphage MS2 has also been shown to interact with host DnaJ chaperon to aid it with cell lysis (Chamakura, Tran and Young, 2017). Hence, if the host can sustain high temperatures, its viruses might also persist. In addition, there are many examples of thermophilic viruses - mostly archaean (Le Romancer *et al.*, 2006; Munson-Mcgee, Snyder and Young, 2018). They can possess a variety of distinct virion shapes, not seen in mesophilic viruses - like e.g. *Sulfolobus islandicus rod viruses 1* and *2* (SIRV1 and 2) which have shapes of helical rods, or members of spindle-shaped *Rudiviridae* family (Rice *et al.*, 2001). However, not all thermophilic viruses have unusual shapes – e.g. *Sulfolobus turreted icosahedral virus* (STIV) which has a shape similar to many animal and bacterial viruses (Rice *et al.*, 2004). In fact, STIV virions have the same shape as MS2, which propagates at 37 °C, but they have ~2x larger diameter than MS2. Therefore, the question remains open if unusual capsid shapes aid viruses in sustaining high temperatures.

Nevertheless, virions of mesophilic viruses are susceptible to thermal denaturation so thermal treatment at >80 °C for 10 min is routinely used to eliminate many viral contaminations (Kampf, Voss and Scheithauer, 2020), though some pathogenic viruses like *Hepatitis B virus* (HBV) have been shown to survive such thermal treatments (König *et al.*, 2019). Naturally, longer treatments at higher temperatures have better efficiencies of viral inactivation. The experiments on HBV have shown that treatment at temperatures >98 °C for 5 min can sufficiently inactivate viruses (König *et al.*, 2019). All taken together, mesophilic viruses, like human viruses and coliphages, show extremely low stability at temperatures >100 °C (Feng *et al.*, 2003; Tuladhar *et al.*, 2012). According to European Space Agency (ESA), spacecraft which are sent to land or sample objects of high risk of contamination (Mars, Europa, asteroids) are sterilized at 125 °C for several days to ensure absolute sterility (ESA, 2020). This treatment is more than enough to inactivate any viruses potentially present on a spacecraft surface. This treatment is in fact aimed to inactivate all or most of the spores (bacterial and fungal) which can be far more resistant to physical conditions than viruses and might be present on spacecraft. They could potentially sustain such high temperatures and pose a risk for space missions.

Various temperature ranges can be observed throughout the solar system regardless of the distance from the Sun. This means that a lot of places can be suitable for life in terms of temperature, especially organisms tolerating wide temperature ranges like bacterial or fungal spores (Jesenská, Piecková and Bernát, 1993; Kort *et al.*, 2005) and their viruses which might survive with them. Those microbes pose various threats to space exploration – from human health hazard to planetary contamination. Hence, any research on heat stability of different biological entities, especially microorganisms and their viruses, contributes to the risk assessment for contamination of missions in space.

### 1.1.2. Extreme pressures

As is the case for temperatures, various pressures can be found throughout the solar system. In search for life in the universe, both extremely high and extremely low pressures are relevant. The relevance of low pressure is easy to see, considering that many rocky bodies in the Solar system either do not have an atmosphere or have a very thin one. Hence, if organisms can sustain such low pressures, there is a chance they can propagate through space on asteroids which adds significance to the idea of panspermia - propagation of life throughout the solar system. Therefore, it is especially interesting to test resistance of organisms to extreme pressures.

Pressures near vacuum are especially interesting, not just because they offer insight into the possibility of panspermia, but also because future planned human missions are set to exploit some of the microbes or their enzymes in biological life support systems and biotechnology (Karouia, Peyvan and Pohorille, 2017). Also, the first celestial targets of future astronaut missions are moon and Mars, both of which have extremely low pressures on their surfaces. While on the moon there is no atmosphere to offer any significant pressure, average pressure on Mars is between 4 and 9 mbar (Grayzeck, 2020). Therefore, the exploration of microbes at low pressures is important in space exploration.

There is scarce amount of data available on how microorganisms react to low pressures even though this is a very important field of study in terms of space application. The data that is available however, suggests that few genera can be metabolically active, and growth in such conditions seems to be unique to bacteria (Schuerger and Nicholson, 2016; Verseux, 2020) but this notion may change as more organisms are tested. In contrast to high pressure, the places on Earth with low pressure-conditions are hard to find so organisms did not have a lot of chances to adapt to such conditions. The only naturally occurring place on Earth where low pressure is present is high in the atmosphere. Unfortunately, microbiome of high atmospheric layers is not researched in detail. At extremely low pressures, microbes face desiccation and hence changing of capsid protein shape. This is because at near-vacuum pressures, water sublimates at room temperature so viruses dry fast. This is a problem because water usually gives shape and interacts with macromolecules on membrane surfaces of cells and on viral capsids. The research of virus stability at extremely low pressures is lacking. Nevertheless, it is known that many viruses, especially nonenveloped, can sustain drying to some extent (Malenovská, 2014). Lyophilization (vacuum drying at low temperatures) is even used as a preservation method for many viruses but it requires stabilizing additives to reduce desiccation (Malenovská, 2014; Manohar and Ramesh, 2019). However, the pure response of viruses to vacuum has not been researched in detail yet and it would be both interesting and relevant.

On the other hand, there are numerous places in the Solar system with extremely high pressures. Such examples include the interior of gas giants like Jupiter and Saturn, deep oceans of Europa and Enceladus, or the surface of Venus with a dense atmosphere. High pressures can also be found deep in Earth's oceans. Microbial life which thrives at high pressures, known as piezophiles, has been found flourishing there (Kato *et al.*, 1998; Li *et al.*, 2020). Due to the importance of high hydrostatic pressure

technologies in biotechnology and food industry, the effect of high pressures on microbes, including viruses has been well studied (Mota *et al.*, 2013).

Under constant temperature, the effects of high pressure stem in reduction of reaction volume. Due to their already small volumes, survival of microbes under high pressure is not as challenging as for multicellular life. It seems that microbes are generally very adaptable to high pressures since many mutations have been shown to aid in survival of such conditions in various microbes (Mota *et al.*, 2013). However, even single-cellular organisms can be distorted and hence destroyed by high pressures. In addition, it has been shown that with rising pressure, UV-absorbing molecules leak from cells both in bacteria and fungi (Park, Park and Park, 2003). In combination with low temperatures, high pressure significantly reduces membrane fluidity and its water permeability. In response, cells increase the content of unsaturated fatty acids in their membranes to combat reduced membrane fluidity at high pressure (Valentine and Valentine, 2004). Viruses however do not possess regulatory mechanisms to combat increasing pressure. At extremely high pressures, virions are inactivated by disintegration (Oliveira *et al.*, 1999; Araud *et al.*, 2015). High pressures are also used for inactivation of viruses for vaccine development (Ishimaru, Sá-Carvalho and Silva, 2004; Silva *et al.*, 2004; Gaspar *et al.*, 2008; Shearer *et al.*, 2016).

In space biology, one of the most tempting goals is to find conditions where liquid water can exist. This is because of the observation that water in liquid state is crucial for Earth life to exist due to many reasons which are not the topic of this work, but are extensively discussed elsewhere, e.g. (Hanslmeier, 2011). Though the necessity of liquid water for life in the universe is still heavily debated (Benner, Ricardo and Carrigan, 2004; Ball, 2017), it is at least known that life similar to Earth's requires it. As for all other substances, the state of aggregation for a defined volume of water depends on environmental temperature and pressure. Hence, there is a phase diagram for water, that can be interpreted to determine in which state will a constant volume of water be in tested conditions. The phase diagram in Figure 1.1 shows that under high pressures, water can be found in liquid state even at temperatures lower than 0 or higher than 100 °C. At extremely low pressures on the other hand, water becomes either gaseous or solid, depending on temperature – at temperatures <-80 °C, water is solid at 10 µbar pressure. This points out that given the right pressure, liquid water can exist in a range of temperatures broader than 0-100 °C. Hence, studying microbial and viral stability at different temperatures and pressures has a great astrobiological value.

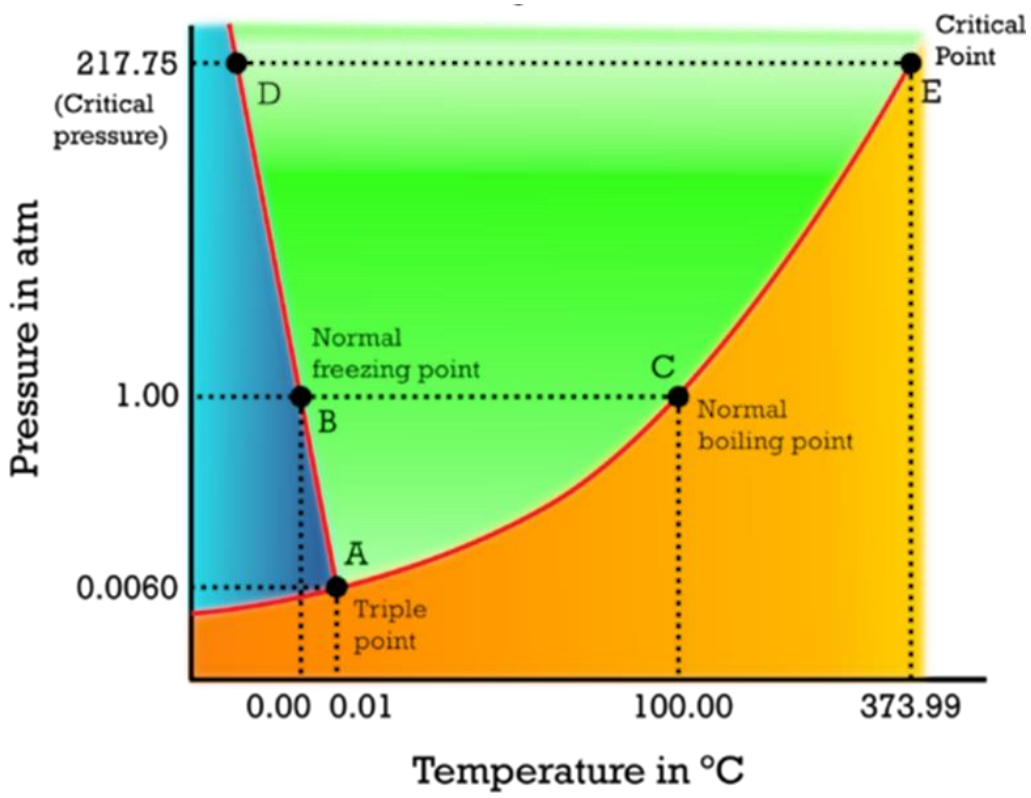


**Figure 1.1.** A simplified phase diagram of water. In the temperature and pressure conditions of the blue area, water is solid, conditions of the green area liquid, and conditions of orange area gas. At extremely high pressures, water can be liquid even below 0 °C, and above 100 °C. Modified from source: https://courses.lumenlearning.com/cheminter/chapter/phase-diagram-for-water/. Credit: CK-12 Foundation – Christopher Auyeung

### 1.1.3. Radiation

Radiation is defined as light waves or high-energy particles traveling through space. Even though some types of radiation can be seen (visible light and high-energy particles) or even felt (infrared light), most types cannot be perceived without specialized detectors or dosimeters. Some types of radiation are more dangerous than others for humans and microbes. For instance, light waves that are absorbed by biomolecules like DNA, lipids or proteins are especially dangerous since they directly cause problems in biological reactions. At the same time, visible light has only minor damaging effects (Mahmoud *et al.*, 2008), and some visible wavelengths are even crucial for life and ecosystems on Earth as they are required for photosynthesis. Radiation that ionizes molecules, causing breaks in molecular bonds and producing free radicals is called ionizing radiation and it is one of the most dangerous conditions of deep space environment. Earth's atmosphere and magnetic field protect the planet's surface from most of the damaging space radiation. However, organisms are still exposed to natural sources of radiation, like radioactive minerals or radon present in soil, as well as to artificial ones, like nuclear weapons, reactors, and medical equipment for cancer treatment. From here on, there will be focus on radiation in space environment.

Ionizing radiation present in outer space is one of the most dangerous aspects of space travel. Significant efforts are taken by various space agencies to tackle this problem and to protect astronauts and equipment from its consequences (Furukawa *et al.*, 2020). There are three primary sources of ionizing radiation in solar system: extrasolar sources, the Sun, and Van Allen belts. All of them pose

significant problems in space travel, and their intensity varies over time unpredictably. Consequently, there is a term for variable radiation-related conditions in space – space weather. Of those three sources, Van Allen belts, charged particles trapped in Earth's magnetic field, are the only ones that are not present in deep space, so they will not be discussed here.

Extrasolar radiation largely consists of galactic cosmic rays (GCRs) passing through the solar system. They come from various supernovae, stars, galaxies and accretion disks of black holes (Blasi, 2013). Due to large distances of other stars and light dispersion, radiation in form of light waves coming outside the solar system is not intense enough to pose a concern. However, high-energy atomic nuclei created in supernovae, quasars, and around black holes are a threat to human space travel (Pissarenko, 1993). Those particles travel through space at relativistic speeds. They can rip apart molecules they hit and decompose, releasing energy in form of light rays which can excite electrons in surrounding molecules, further propagating the damage. Those nuclei are mostly from hydrogen (87%) and helium (12%) (Chancellor, Scott and Sutton, 2014) but can vary up to uranium (<1%). Being atomic nuclei, those particles are positively charged, which means that their path is affected by magnetic field. Hence, solar magnetic field protects the solar system from them to some extent, but due to their extremely high energies, magnetic field has little effect. During peak solar activity however, the amount of GCRs is at its minimum, and vice versa. This is because when the Sun is active, it discharges large amount of high-energy charged particles during SPEs which, while traveling, strengthen the solar magnetic field, protecting the solar system from GCR particles.

High intensities of radiation are coming from the Sun both in a form of light waves and charged particles. Sun irradiates white light, meaning it is a spectrum of wavelengths. Most of those wavelengths are in the visible part of the spectrum (wavelength 400-700 nm), but other wavelengths are also observable. From a biologist's point of view, ultraviolet (UV, wavelength 100-400 nm) radiation and X-rays (wavelength 0.01-10 nm) are among the most damaging light waves coming from the Sun. Both have high energies and are efficiently blocked by Earth's atmosphere. This means that while they do not pose a big threat on the ground, they are a major problem for space travel.

Figure 1.2 summarizes Sun's spectral irradiance in a $10\text{-}10^4$ nm wavelength range. It is visible that there is a peak in the amount of 100-200 nm wavelengths coming from the Sun which falls into the UV spectrum. The Sun has a surface temperature of ~6 000 K and its emission spectrum is approximated by 5770 K-black body radiation (Sakurai, 2017). In theory, such black body is expected to be extremely weak source of X-rays because its temperature is too low. Nevertheless, high intensities of X-rays are constantly observed coming from the Sun with X-ray telescopes, with highest intensities inside solar flares (Figure 1.3). It turns out that those X-rays are not coming from the Sun's surface, but from solar corona, which has a temperature of $\sim 2 \times 10^6$ K (Vand, 1943). Because X-rays are formed in the solar corona, their intensity is highly dependent on solar activity.

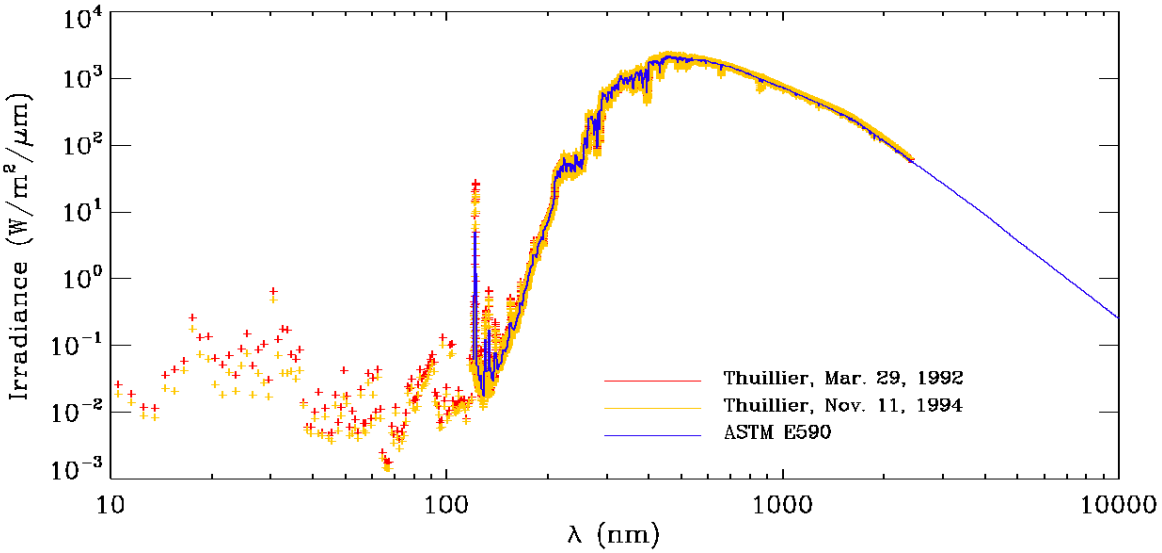


**Figure 1.2.** Irradiance of various wavelengths coming from the Sun in range from X-rays to infrared as determined by three different models. While visible light is irradiated at highest intensity, there is also a peak at UV wavelengths. Wavelengths in X-ray spectrum are also observable. Source: https://www.nasa.gov/mission_pages/sdo/science/solar-irradiance.html. Credit: NASA.

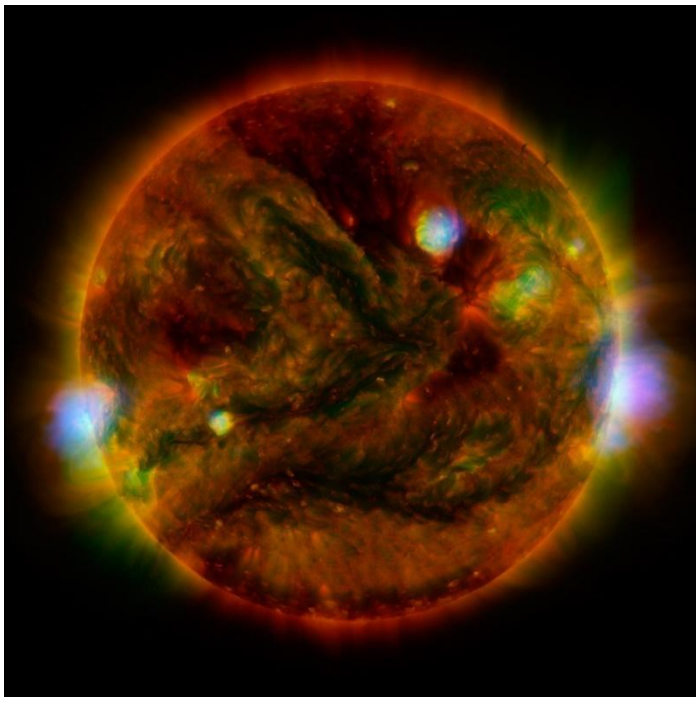

**Figure 1.3.** Combined image of the Sun in X-ray and UV spectrum as imaged by several telescopes: NuSTAR (blue), Hinode (green), and SDO (yellow and red). Blue and green represent high- and low-energy X-rays respectively, and yellow and red represent high- and low-energy UV radiation. It is evident that X-rays are irradiated in high-intensity patches, most intensive at solar flare spots, suggesting their origin under the solar surface. On the other hand, UV radiation is distributed more homogenously across Sun's surface suggesting its origin there, where the temperature is much lower. Source: https://www.nasa.gov/jpl/nustar/searing-sun-seen-in-x-rays ; Credits: NASA/JPL-Caltech/GSFC/JAXA.

Light of the UV spectrum wavelengths is divided according their biological activity to UVA (wavelength 315-400 nm), UVB (wavelength 280-315 nm), and UVC (wavelength 100-280 nm). Of those types, UVC is the most damaging because it is best absorbed by DNA (Olson and Morrow, 2012). Thankfully, UVC is also most efficiently blocked by Earth's ozone layer, meaning that it does not create problems for life on the ground. Other two types of UV are also filtered by ozone layer to some extent, and they also cause damage to biomolecules, but organisms are generally well adapted to their action. However, along UVC, there is a significantly higher intensity of UVA and UVB in space than on Earth's surface and most organisms, including humans, are not well adapted to such conditions. UV radiation can be absorbed by bipyrimidine sequences in nucleic acids (CC, CT, TT or TC) to form dimeric photoproducts which are often skipped by polymerases, causing mutations. In addition, those dimers cause changes in nucleic acid conformation. The conformation of nucleic acids is very important for them to perform their biological tasks – for DNA sequences to be recognized by DNA-binding proteins/RNA, and for RNA sequences to perform enzymatic reactions and specifically bind to other nucleic acids. Most organisms on Earth have evolved different ways of protection from this kind of damage. Pyrimidine dimer lesions in DNA are both in prokaryotes and eukaryotes repaired by nucleotide excision repair (NER). However, this process intrinsically differs in prokaryotes and eukaryotes in the mode of action (Friedberg *et al.*, 2005). While in many prokaryotes like *E. coli*, NER is completed by only 3 genes (UvrA, B, and C) (Tang, Lieberman and King, 1982), eukaryotes can have ~30 genes involved in this process. In addition, prokaryotic NER proteins are highly conserved among bacteria and archaea, while eukaryotes do not possess those homologues. In addition to NER, many organisms possess an enzyme photolyase which uses the energy of light to separate formed dimers to their original state in a process called photoreactivation. Photolyase has diverse functions in different organisms, but it seems that its ancestral function was DNA repair from UV damage or even sensing of UV radiation (Ozturk, 2017). Bacteriophages can utilize the host genome-repair machinery if they insert their genome into the host's (Menouni *et al.*, 2015). Many eukaryotic viruses on the other hand, either inactivate or hijack the cell's DNA repair machinery to alter cell cycle and help them replicate (Hollingworth and Grand, 2015). Virions are very prone to damage by UV radiation because they lack the genome-repair machinery and UVC is routinely used for their inactivation (Reed, 2010). DNA viruses tend to be more resistant to UV damage than RNA viruses, demonstrating a big role of genome damage in inactivation of viruses with UV (Tseng and Li, 2007).

X-rays on the other hand cause single and double stranded DNA breaks. Their energy is high enough to break molecular bonds. In DNA, they do it at random spots, causing randomly distributed strand breaks. If not repaired, those breaks have severe biological consequences. The higher the X-ray intensity, the more double stranded breaks are created, causing worse consequences for an organism. They do not only cause mutations, but also severely impair genome integrity, stopping many essential processes for life, like RNA transcription and genome compaction. In many eukaryotes, the cells that sustained such damage and were not successful in repairing it efficiently, are immediately sent to apoptosis to protect the organism (Nakano and Shinohara, 1994; Zhang *et al.*, 2017). Otherwise, in vertebrates, those cells cause inflammation, leading to other health consequences. The basic mechanism of repairing double stranded DNA breaks is similar in prokaryotes and eukaryotes but differs in detail. Both take advantage of nonhomologous end joining (NHEJ) and homologous repair (HR) mechanisms to repair double stranded breaks (Lieber, 2010). HR is a more reliable mechanism, but since it requires a non-damaged copy of a sequence to be present, it is generally less associated with monoploid organisms - like prokaryotes usually are. Therefore, while prokaryotes often use NHEJ, eukaryotes commonly use HR. Eukaryotes have more complex protein machineries participating in double stranded break repairs than prokaryotes. Even though there are significant dissimilarities between X-rays and fast charged particles, specifically in the mechanism of causing biological damage, the biological consequences are similar – single/double stranded genome breaks and production of free radicals. Hence, X-ray experiments may be used to make some conclusions about biological consequences of fast charged particles - with extreme caution in interpretation of results. Even though it is reasonable to

assume that X-rays also cause strand breaks in RNA, the truth is that their effect on RNA is not well studied. Though viruses are vulnerable to damage caused by ionizing radiation, proteins and nucleic acids do not absorb it well. The result is that some viruses can sustain high levels of radiation due to their small size – most of the radiation rays miss them. Nevertheless, virions proteins and genome can be damaged by ionizing radiation – directly and indirectly (Pollard, 1954; Hume *et al.*, 2016).

X-rays are ionizing radiation, and their dose is measured in Grays (Gy). One Gy is equivalent to 1 J/kg. Another popular measuring unit for the ionizing radiation dose is rad, which is equivalent to 0.01 Gy. The dose of X-rays received is a function of distance from the source of radiation and time of exposure. Additionally, the presence of a material that blocks X-rays between the source and an irradiated object also plays a role in the dose received.

Both X-rays and UV radiation can form radicals in biomolecules and water. When they cause damage to biomolecules, this is referred to as direct damage, and when they produce radicals in surrounding molecules like water, which then damage biomolecules, this is referred to as indirect damage. Since X-rays have higher energies, they have higher potential for exciting electrons.

### 1.1.4. Lunar regolith

Lunar regolith is the material making up most of the surface of the moon. It is mostly composed of silicon dioxide and aluminum oxide. Though the subsurface of the moon is solid, the surface material is mostly composed of ultra-fine dust due to 4 billion years of asteroid bombardment of the lunar surface. Lunar dust creates many problems for the astronauts and rovers because it has a high tendency to become airborne, especially in low lunar gravity or inside modules in-orbit as the lessons from Apollo show (Lam *et al.*, 2013). Lunar dust is known to cause immunologic reactions and damage to lungs (Wagner, 2006; Scheuring *et al.*, 2008; Lam *et al.*, 2013).

To perform the experiments with lunar regolith on Earth, the regolith simulants are often used since the real lunar material is very limited (Taylor, Pieters and Britt, 2016). There are many versions of lunar regolith simulants, with different properties suitable for different kinds of research and tests. In this research, lunar regolith simulant European Astronaut Center-1 (EAC-1) (Engelschiøn *et al.*, 2020) has been chosen due to its fine structure - simulating the lunar surface dust, and its availability at DLR. EAC-1 is a light-gray fine-structured powder (Methods, Figure 2.13) with density of 1.54 g/cm$^3$. Its composition is listed and compared with lunar samples from Apollo and Luna programs (Laul and Schmitt, 1973; Morris *et al.*, 1983) in Table 1.1. EAC-1 contains grains between 0.02 and 1 mm in diameter but is mostly composed of grains 0.2-0.5 and 0.5-1 mm in ~50/50 ratio.

**Table 1.1.** Comparison of the major element composition by % weight (wt%) of the EAC-1 lunar regolith simulant used in this study with the lunar soil samples brought to Earth during Apollo and Luna programs. Data from EAC-1 simulant documentation by ESA and DLR.

| | EAC-1 (wt%) | Lunar soil – Mare (wt%) | Lunar soil – Highlands (wt%) |
|---|---|---|---|
| **$SiO_2$** | 43.70 | 46.70 | 43.70 |
| **$TiO_2$** | 2.40 | 1.70 | 3.50 |
| **$Al_2O_3$** | 12.60 | 13.20 | 17.40 |
| **$Fe_2O_3$** | 12.00 | 16.30 | 12.20 |
| **MnO** | 0.20 | 0.21 | 0.16 |
| **MgO** | 11.90 | 10.90 | 11.10 |
| **CaO** | 10.80 | 10.40 | 11.30 |
| **$Na_2O$** | 2.90 | 0.38 | 0.42 |
| **$K_2O$** | 1.30 | 0.23 | 0.09 |
| **$P_2O_5$** | 0.60 | 0.16 | 0.08 |

The lunar soil and EAC-1 simulant both have grains of various shapes, hypothetically enabling microbes to fit into structural irregularities. Hence, microbes could be protected there from various environmental hazards such as radiation, or extreme temperature changes inside the dust. The microbial protection potential of lunar dust has not been researched in detail, which would be interesting, especially for viruses. This is not only because of the future return of humans to the moon, but also because there is a potential that, over the 4 billion years that the moon exists, there has been exchange of material between Earth and the moon. Some of this material might have carried biological entities such as viruses or other microbes to the lunar surface. Therefore, microbes might be present on the moon, protected by the lunar dust, for some time after they are potentially delivered there. However, due to the lack of nutrients, and light inside the lunar dust, it is unlikely that microbes would be able to propagate there, and certainly not viruses, which require a host for replication. Nevertheless, the possibility of microbial survival in the lunar environment represents an interesting astrobiological perspective worth of extensive research.

## 1.2. Spaceflight weakens the immune system

Despite all the protective measures, astronauts are nevertheless exposed to stresses associated with space travel, and they can have negative impacts on human body (Blaber *et al.*, 2011). It has been shown in multiple studies that the stress of space travel weakens human immune system. This holds for space shuttle flights, 6-month orbital missions, analogue missions in Siberia and Antarctica, underwater analogues, and bed rest studies (Crucian *et al.*, 2018). The exact mechanism of that effect is not entirely clear, but there are some insights into the matter (Crucian *et al.*, 2018). Those insights led to interesting hypotheses to explain the negative effects of spaceflight on immune system. It is certain that combination of stress conditions present during space travel plays a major role in immune suppression.

Immune system is divided into innate and adaptive immunity. Innate immunity is the part of immune system that responds in the same way for any pathogen – therefore, it is often called nonspecific immunity. This is body's first protection from pathogens. In contrast, adaptive immunity has a specific response to any antigen that invades human body - it is usually also referred to as specific immunity. Adaptive immunity usually has a delayed response, after innate immunity, due to its appreciable complexity and since it is activated by innate immunity through action of T-lymphocytes.

Due to its high complexity and diversity of processes, some aspects of the immune system differ from individual to individual. For example, there is a strong difference in infection susceptibility

between age groups, but the exact differences between children, adults and elderly individuals remain poorly understood (Simon, Hollander and McMichael, 2015). Also, it is well established that immune cell frequencies and serum protein concentrations vary heavily between individuals (Brodin and Davis, 2017). These interconnections and variations of immune subsystems are very curious research topics of systems immunology (Davis, 2020). It is evident that the whole immune system is an extremely complex system composed of various cellular and molecular subpopulations and is hence very susceptible to internal and external stress factors. Those factors are usually grouped in three categories: lifestyle, environment, and genetics – all of which are significant factors in space flight. Both innate and adaptive component of the immune system seem to be altered during space flight in various aspects, as well as in their mutual interaction (Crucian *et al.*, 2018).

One experimentally well backed-up hypothesis states that spaceflight disrupts the adaptive immune system by influencing lymphocyte development and their antigen-specific response (Crucian *et al.*, 2015, 2018; Akiyama *et al.*, 2020). For instance, it has been shown that levels of some cytokines are elevated in astronaut's blood, which was correlated with a shift in $T_H2$ lymphocytes which in turn resulted in shedding of reactivated herpesviruses (Mehta *et al.*, 2013). $T_H2$ lymphocytes help regulate adaptive immune response, thus keeping all pathogens protruding an organism at bay. With lymphocyte function impaired, different opportunistic pathogens can cause severe disease. In addition to lymphocytes, dysregulation of other leukocytes has also been reported during early space shuttle flights in 1980s: elevated level of neutrophils and reduced level of eosinophiles (Taylor and Dardano, 1983). Dysregulation of lymphocyte maturation and function is probably caused by various factors of spaceflight, mainly acting on bone marrow and thymus.

Microgravity affects immune system via bone, muscle, and thymus atrophy. This is due to lack of activity in compact space station and constant floating, which means that bones and muscles perform significantly less work during space flight than on Earth. Therefore, bones slowly degrade, changing the bone marrow environment in the process. This has an impact on the formation of blood cells - and leukocytes are no exception. However, studying those effects in humans is challenging because currently, a few people go to space missions, and there are no non-invasive methods to study bone marrow and thymus directly. Therefore, rodent, and *in vitro* studies are valuable models in space physiology research. Studies carried on mice and human bone marrow stem cells in simulated and real microgravity showed changes in gene expression that inhibit differentiation of mesenchymal stem cells in bone marrow (Blaber *et al.*, 2014; Li *et al.*, 2019). Additional experiments on mice also suggest that thymus experiences enhanced atrophy in altered gravity which is accompanied by lymphocyte maturation defects (Gridley *et al.*, 2003, 2013; Novoselova *et al.*, 2015). Since lymphocytes are created in bone marrow, and T-lymphocytes mature in thymus, it is easy to appreciate how gene-expression and morphological changes in bone marrow and thymus environment can affect lymphocyte maturation and function.

Radiation is another factor of spaceflight that has a significant impact on the immune system. Despite radiation protection, astronauts inside space modules are exposed to elevated doses of ionizing radiation (Cucinotta, 2014). This will be especially dangerous in long-term deep space missions which are currently planned. Effects of radiation to the human immune system are better understood than microgravity's effects since radiation is used to treat cancer. Ionizing radiation directly inhibits cellular activities by rising oxidative stress in its environment and inducing protein and nucleic acid damage. During extra-vehicular activities (EVAs), there is additional danger of exposure to increased intensity of UV radiation. Both UV and ionizing radiation have a direct impact to immune cells and organs.

## 1.3. Passenger microbes pose a problem for spaceflight

Even though at first glance it seems that biological contamination of spacecraft should not be a big problem since harsh space conditions should inactivate contaminants, the truth is that this is a major concern in spaceflight (Horneck, Klaus and Mancinelli, 2010). Increasing number of biological entities are being identified that can sustain extreme conditions of space. It's not just extremophiles, that require extreme conditions to grow, but also organisms present around us in everyday life – like tardigrades (Weronika and Łukasz, 2017) or black mold - *Aspergillus niger* (Romsdahl *et al.*, 2018). As was pointed out previously, if the host can survive in extreme conditions, there is a chance that the viruses it is carrying can survive too. Therefore, there is a high risk of biological contamination in space missions which is a problem due to multiple reasons.

First, there is an issue of planetary protection from biological contamination. From ultraclean spacecraft assembly facilities, microbes have been isolated that pose a threat of contaminating the surface of other celestial bodies where humans send their spacecraft – mostly spore-forming bacteria of *Bacillus* genus (Link *et al.*, 2004). Consequently, there has been a tremendous research tackling the resistance of *Bacillus* species and their spores to space conditions. However, some analysis of such facilities suggests that there may be many still unculturable and hence unstudied microbes (Venkateswaran *et al.*, 2003; La Duc, Kern and Venkateswaran, 2004). Bacterial, fungal and even moss spores have been shown to be resistant to exposure to outer space conditions to some degree (Takahashi *et al.*, 2011; Horneck *et al.*, 2012). It is a big issue if such spores contaminate spacecraft and are transferred to other planetary bodies. Significant measures are taken to ensure absolute sterility of spacecraft when sending them to other worlds, especially if probing is planned, like in OSIRIS-REx mission to the asteroid Bennu, or even landing, like in Mars Perseverance rover mission (Fairén *et al.*, 2019). In addition to protecting other celestial bodies from Earth's microbes, it is also important to plan protection of Earth from potential microorganisms found on those objects. For example, both aforementioned missions include sample returns from Bennu and Mars to Earth. If they sample a new lifeform, there is a potential that it may be hazardous for Earth's life. For that reason, there are strict rules for handling any samples returned from outer space which include constant enclosure of the samples and handling in sterile conditions until appropriate disinfection methods are not applied (Crawford, 2005). In this context, viruses do not pose a significant threat due to their low resistance to disinfection methods and generally low surface stability. For this reason, spacecraft sterilization methods are focused on removal of bacteria, fungi, and spores, while viruses are inactivated in the process. In addition, viruses need a specific host to reproduce, which means that even if a virus survives a trip to other planetary body, the lack of host will make this contamination insignificant from a planetary protection point of view.

Second issue that arises with passenger microbes is due to opportunistic pathogens that can infect humans with compromised immune systems during space missions. It has been briefly discussed in section 1.2. how space travel negatively affects the immune system. Humans inevitably carry whole communities of microbes on skin surface and inside their bodies. Some of those microbes can be pathogenic but held inactive by the immune system. When the immune system weakens, pathogens, which normally would not pose a health concern, can cause serious disease. Though this has not yet been observed in 60 years of human spaceflight history, this may be due to short mission times. No human has been in space for longer than one year, and astronauts are usually on ISS for 3-6 months. This will however change with currently planned prolonged missions to moon and Mars so there is a concern that such issues will become more visible. The problem with pathogens is that even though they usually cannot survive space conditions, they stick and travel with their host. During space missions, humans are mostly protected from harsh space conditions, together with pathogens. It is important to study the effects of spaceflight conditions to pathogenic microorganisms, including viruses to understand their potential to cause disease and propagate in such environments. The effect of

spaceflight conditions on bacterial cells has been extensively studied over the last three decades (Tixador *et al.*, 1985, 1994; Wilson *et al.*, 2007; Zea *et al.*, 2017; Gilbert *et al.*, 2020). The evidence suggests that during spaceflight, bacterial cell wall thickens, increasing virulence, ability to form biofilms, and resistance to antibiotics. In addition to the compromised immune system, this is a big concern for future of space travel. However, resistance of bacteria to bacteriophages during spaceflight has not been thoroughly researched. If susceptibility of bacteria to bacteriophages does not change, bacterial viruses might be a useful tool for fighting bacterial contaminations and diseases in space. Therefore, it is important to study how bacteriophages and other viruses respond to spaceflight conditions.

## 1.4. Viruses in conditions of outer space

When talking about viruses, the usual association are infectious viral particles - virions. It must be noted that a virion is only one phase of viral replication cycle, and viral response to spaceflight is interesting to study in all stages. However, in other stages of their replication cycle, viral response to spaceflight is heavily dependent on the response of their host. It is only in the virion stage that virus responds independently. It is hence interesting to start the studies of viral response to spaceflight with the virion stage, so the host response can be ignored, and build on the acquired knowledge with further studies of host response combined with other stages of viral replication cycle. In this study, viruses were exposed to physical conditions of space in virion form and change in infectivity was tested without the exposure of the bacterial host.

The resistant microbes and their spores can survive the harsh conditions of space due to their robust DNA-repair mechanisms, fast RNA and protein turnover, thick protective layers of polysaccharides, and low amount of water inside them (spores). Viruses however do not possess any of those characteristics - at least not in their virion form. Therefore, virions tend to be less stable in space conditions than many cellular organisms. Also, viruses are not generally stable on dry surfaces compared to bacteria. In a recent metagenomic analysis of the surfaces on ISS, viral sequences composed only 1.15% of all identified sequences (Mora *et al.*, 2019). Due to their low environmental stability, there is currently a lack of experiments of virion exposure to real spaceflight conditions. Nevertheless, various media where virions can find themselves in the environment may influence their stability on surfaces (Vasickova *et al.*, 2010). It is therefore important to study viral stability in different media in respect to varying environmental conditions.

On the other hand, there is plenty of research of latent virus reactivation and shedding in astronauts (Crucian *et al.*, 2018). For instance, there is mounted evidence that herpes viruses often reactivate in astronauts, which is observed both in real spaceflight and spaceflight analog missions (Rooney *et al.*, 2019). Reactivation of latent herpes viruses is a complex process that depends on many factors, only some of which are known. Weakened immune system is undoubtedly one of the factors aiding viral reactivation. Viral reactivation in space missions is supported by the lack of vitamin D (Zwart *et al.*, 2011). This is probably related to how vitamin D affects the immune system. It reduces the production of inflammatory cytokines and boosts regulatory T-cell activity which increases anti-inflammatory cytokines (Aranow, 2011).

Considering that virions of different viruses can have varying environmental stability, some of which can retain infectivity after 180 days in granular soil with 10% moisture (Bosch, Pintó and Abad, 2006) it would not be surprising if virions were able to travel from Earth to other bodies such as moon or Mars – either though spacecraft contaminations or meteorites. Depending on the launch time and orbital transfer method, a trip to Mars lasts for several months. An average Hohmann transfer orbit from Earth to Mars lasts for 259 days, but hyperbolic orbital transfer performed when Mars passes closest to

Earth (approx. every two years) can reduce this trip to 90-150 days, as calculated by NASA Trajectory Browser (Foster, 2013), which is, in theory, short enough time for an enteric virus contamination to persist and reach Mars. Though this poses a high risk for human missions to Mars (human-mediated contamination), it is highly unlikely that a meteor originating from Earth will find itself in such trajectory (natural contamination). However, exchange of material between Earth and Mars has been a common occurrence throughout the history of the solar system (Belbruno *et al.*, 2012). Natural contamination of the moon is even more likely due to its constant proximity to Earth. Still, human-mediated risk of contamination of celestial bodies is real and it is of great interest to address the problem.

Studying virus behavior in space conditions is also interesting from evolutionary biology point of view. Viruses represent ancient group of simple biological entities. Their roots possibly stem from RNA world (Koonin, Senkevich and Dolja, 2006; Hulo *et al.*, 2017). How viruses react in space conditions might provide insights into behavior of life at its origin and evaluate the idea of panspermia.

## 1.5. Coliphage MS2: a surrogate for enteric and respiratory viruses

Coliphage MS2 is a nonenveloped, icosahedral bacterial virus (bacteriophage or "phage") of 25-28 μm in diameter (Kuzmanovic *et al.*, 2003) (Figure 1.4 A). It belongs to family *Leviviridae*, genus *Levivirus*. Its natural host is *E. coli* with F-pilus, sometimes referred to as "male" *E. coli*, because the bacteria with F-pilus are donors of genetic material in conjugation – hence the name for the MS2 virus ("male-specific"). The phage virion consists of a capsid and one copy of (+)ssRNA genome. The capsid is composed of 89 *capsid protein* (cp) dimers, to form an icosahedron with triangulation number T = 3, and one *maturation protein* A (matA) inserted in capsid, to attach for the bacterial F-pilus (Rolfsson *et al.*, 2016). In addition, matA binds both ends of the genome, circularizing it inside a virion (Rolfsson *et al.*, 2016). The small genome, 3.569 kb in size, is coding for four proteins: matA, cp, *lysis* (lys), and *replicase* (rep) (Figure 1.4 B). This genome structure is characteristic for *Leviviridae* family (Tars, 2020). Interestingly, lys gene is encoded in part by the 3'-end of the cp, and in part by the 5'-end of the rep gene. The genome contains a hairpin structure between the cp and rep genes which prevents too early expression of lys, but also the late translation of the rep gene, and serves as the basis for virion assembly.

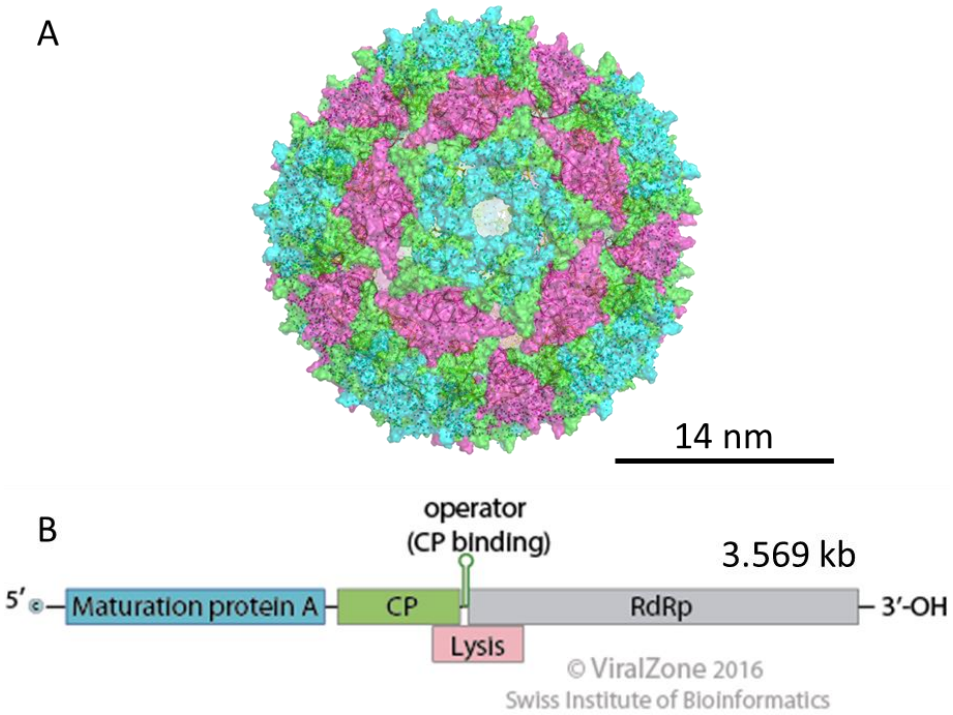


**Figure 1.4. A** MS2 virion has an icosahedral, almost circular shape and the diameter of 25-28 nm. Though capsid is formed only of cp protein, various colors of protein subunits indicate the geometry of cp assembly (PDB ID: 2IZ9). **B** Coliphage MS2 (+)ssRNA genome codes for four proteins and has a length of 3 569 bp. Source B: https://viralzone.expasy.org/291. Credit: ViralZone, Swiss Institute of Bioinformatics,

MS2 virion infects the target bacterial cell by attaching to the F-pilus by matA receptor. The mechanism of virus entry is not fully understood, but it is believed that the virion gets to the basis of the pilus and somehow utilizing the mechanism for transfer of nucleic acids by F-pilus (Tars, 2020). After the attachment of the virion, matA is cleaved, linearizing the genome, and enters the cell, while carrying the viral genomic RNA (Dent *et al.*, 2013). This genome linearization allows the beginning of translation by bacterial ribosomes. However, being an RNA genome, it is compactly folded by a verity of secondary RNA structures which help regulate gene expression and help differentiate the viral genome from other RNAs present in a cell. At first, only the ribosome-binding site (RBS) of the cp gene is exposed. The ribosome binds and initiates translation of the cp, and in the process unfolds part of the genome, exposing the RBS of rep. The rep gene, which codes for a subunit of RNA-dependent RNA polymerase (RdRP), is hence translated. Once the rep protein is in the cell, it interacts with three bacterial proteins – EF-Tu, EF-Ts and ribosomal protein S1 – to form a functioning RdRP. The MS2 RdRP is extremely specific for the MS2 genome, and it is believed this is due to the combination of sequence- and secondary genome structure-specificity. RdRP begins the replication cycle, temporarily exposing the matA RBS. This exposure of matA only lasts briefly and only once every replication cycle – ensuring only approximately one copy of matA is produced per genome. During the translation of the cp gene, in 5% of times, the ribosome does not terminate, but continues to translate, which leads to production of lys protein. In this way, lys slowly builds up during the cycle until it's time for cell lysis at the end of the cycle, when enough of it builds up. In the late stage of replication cycle, when a high amount of cp and viral genome copies have been produced, cp binds a hairpin loop upstream of the rep gene, preventing its further synthesis. In addition to cp, matA also binds the RNA genome, circularizing it, and stemming the virion assembly (Dai *et al.*, 2017). When enough of the lysis protein accumulates,

it inserts itself into the membrane of *E. coli*, forming pores in a cell and causing lysis and release of virions.

Although it is hard to compare environmental stability of different viruses due to lack of standardization in virus environmental stability research, enteric viruses are believed to be amongst the more environmentally stable eukaryotic viruses (Alidjinou *et al.*, 2018). They vary in size from 25-80 μm in diameter throughout various families. They are nonenveloped, mostly RNA viruses, which replicate in epithelial cells of the small intestine (Bishop and Kirkwood, 2008). To reach the site of infection, they usually pass through a diverse environment of human, mammalian or avian digestive system which is harsh due to biological, chemical, and physical conditions. In the small intestine, enteric viruses can cause inflammation and damage (Ahmed *et al.*, 2014). Due to their high resistance and efficient spread, enteric viruses cause significant issues for public health. Unfortunately, many of them are currently difficult to grow, or even unculturable - like noroviruses (Hennechart-Collette *et al.*, 2020) which are the leading cause of foodborne infections worldwide (Mattison, Cardemil and Hall, 2018).

This points out the importance of surrogate viruses, like MS2. By researching those viruses, we can infer a lot about non-cultivable enteric viruses which, in turn, helps us to prevent outbreaks and limit viral spread. MS2 is very similar to many enteric viruses – e.g. noroviruses and astroviruses, in shape, size and genome type (ssRNA) (Bishop and Kirkwood, 2008). However, since MS2 is an *E.coli* virus, methods for its growth are well-established and it's easy to grow. On the other hand, enteric viruses usually require specific human epithelial cell line grown in conditions mimicking human intestine which is hard to simulate. Even then, enteric viruses cannot be replicated in such high concentrations as MS2 due to much slower replication cycle, both of the host and the virus. Therefore, MS2 is widely used as a surrogate for enteric viruses (Kim, Kim and Kang, 2017).

In addition to enteric viruses, MS2 can be used as a conservative surrogate for respiratory viruses because it can stay infectious in air droplets and is more resistant to disinfection methods than most respiratory viruses (Walker and Ko, 2007). This is because respiratory viruses usually have a lipid envelope. The viral receptor proteins in an envelope are more susceptible to environmental denaturation than those in a highly structured, compact protein coat as the one of MS2. Also, many problematic respiratory viruses are RNA viruses (Hodinka, 2016), as is MS2, making it a good surrogate for antimicrobial methods that induce genome damage. MS2 has been used to test and develop antimicrobial technologies and protective equipment against influenza (Vo, Rengasamy and Shaffer, 2009; Fisher and Shaffer, 2011; Coulliette *et al.*, 2014) and SARS-CoV-1 and -2 (Walker and Ko, 2007; Beaudry *et al.*, 2020). Taking all this into account, MS2 is a valuable surrogate for conservative tests of disinfection methods and respiratory virus stability.

## 1.6. Aims of the study

This study aims to test the stability of coliphage MS2 in some of the physical space conditions to draw conclusions about hypothetical stability of viruses in space conditions. The results obtained here are valuable in terms of risk assessment that viruses pose for human space missions. In addition, the results hold an astrobiological value for evaluating the stability of simple biological entities such as viruses in space, giving insight into the possibility of life's propagation throughout the solar system.

Though treatments performed here are not exact simulations of deep space conditions, but rather basic stability and transmission experiments, they provide a reference and methodology for future detailed space simulation research.

# 2. Materials and Methods

All tests have been conducted in a way that the solution of phages with known titer (± 95% confidence interval, 95%CI), was treated. After that, 10-fold serial dilutions from $10^{-1}$ - $10^{-8}$ were prepared in SM buffer. The dilutions were plated on a freshly prepared *E. coli* bacterial lawn in a double layered plate. Every experiment was performed two times.

First experiment was always a preliminary test without negative controls or replicates. First, 10 µL of the phages was plated in eight serial decimal dilutions per plate ($10^{-1}$ - $10^{-8}$ or $10^{0}$ – $10^{-7}$) to determine the range of dilutions where plaques are countable after treatment. Next day, the range of dilutions would be determined, and an experiment would be repeated with three replicates and a negative control. This time, 20 µL of only four serial dilutions was plated in a determined range. This way, the confidence in the results was increased, the experimental error was reduced, and a rough check of the reproducibility of the results was performed. The plaques were easier to distinguish at larger area and higher sample volume was used than in preliminary experiments. The plates that were not countable, or were outliers were removed from the datasets so in such treatments, number of replicates was 2 instead of 3. The work was performed in sterile conditions. The experimental procedure for all treatments is summarized in Figure 2.1.

Also, when it is stated in this work that a culture was put to grow in the incubator overnight, this means 18-20 h as it was determined that this is optimal time for preparing fresh bacterial lawn in NZCYM medium for MS2 phages to grow. All the incubations were done at 37 °C. Liquid cultures were incubated in 10 mL volume (inside 100 mL-Erlenmeyer flasks) in shaking incubator (Infors Multitron HT AJ103S Incubator Shaker) at 200 RPM.

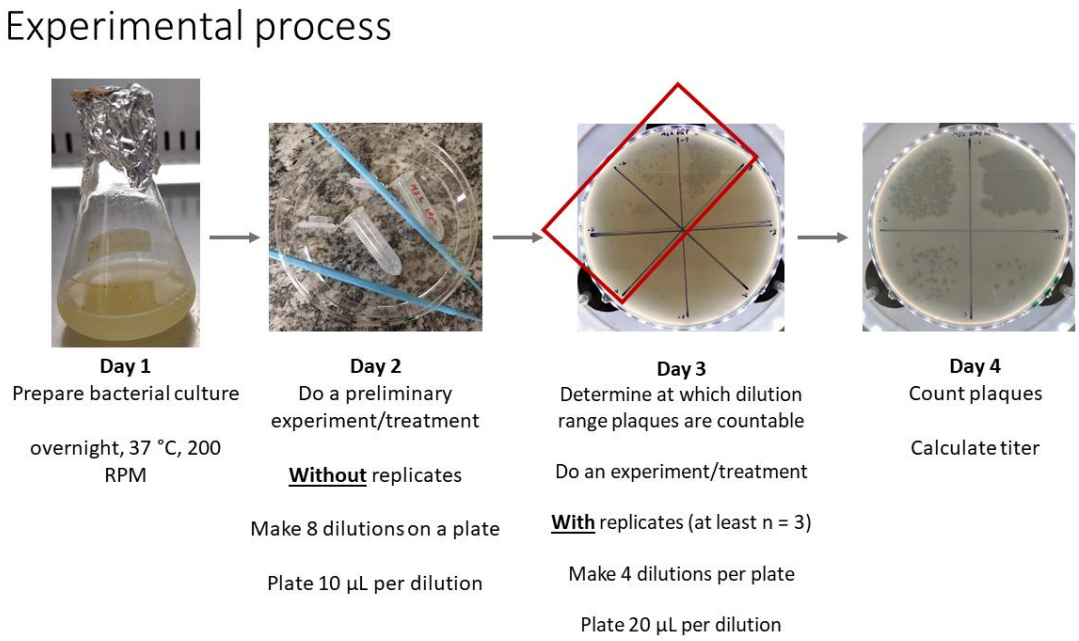


**Figure 2.1.** A summary of the experimental procedure for each phage treatment in this work. On the pictures, there is an example for air-drying of phages, but steps were the same for all treatments. On day 1, bacteria would be put for overnight culture to have bacteria in an exponential growth phase the next day in the morning. On day 2, a known amount of viruses would be treated, plated afterwards and left to grow overnight – without replicates. On day 3, the range of dilutions where plaques are countable after a treatment would be determined (red square), and the treatment of the same virus stock as the day before would be repeated, but with 3 replicates. Then, the viruses would be plated in a determined range of dilutions and left to grow overnight. On day 4, the plaques would be counted.

## 2.1. Preparation of media, plates, and buffer

For growth of the bacterial host and viruses, NZCYM medium (Sigma-Aldrich, Table 2.1) was used. The medium was prepared by dissolving22 g of NZCYM powder in1 L of sterile ultrapure water. The solution was autoclaved for 20 min at 121 °C.

**Table 2.1.** The contents of NZCYM medium (Broth N3643) obtained from Sigma Aldrich

| **Chemical** | **Concentration (g/L)** |
|---|---|
| Casamino Acids | 1 |
| MgSO4 | 15 |
| NaCl | 5 |

For the liquid medium, two 0.25 L bottles of stock medium were prepared. The lids of the bottles were closed after autoclaving and stored at room temperature on the working bench for a maximum of one month. By always storing two smaller stocks of liquid medium, the backup stock was always present, which reduced the wasted medium in case of contamination.

When preparing the solid medium, agar was added into the bottle together with NZCYM powder before autoclaving. The hard medium had 1.5% (w/v) agar and the soft medium had 0.7% (w/v) agar. The hard medium was used to grow bacteria and sustain the pure culture and as a solid base for the bacterial lawn plates. The soft medium was used only as an overlay for bacterial lawn plates.

While the hard and soft media were autoclaving, the UV lamp in a sterile room was turned on for 1 h. After the autoclavation was finished, the lids of the media were closed, and the media were put on a magnetic stirrer in the sterile room to cool down for 30-45 min (Figure 2.2). When it cooled down, the hard medium was distributed into plates while it was still liquid – 50-60 plates were prepared from 1 L of hard medium. The soft medium was distributed into tubes (Figure 2.3), 10 mL into each - 50 tubes were prepared from 0.5 L of soft medium. Then, the media was put to harden and afterwards, everything was left in the incubator at 37 °C overnight to check for contamination. The next day, the non-contaminated plates and tubes were put into the fridge. The media inside plates and tubes stayed in the fridge for maximum of one month before use.

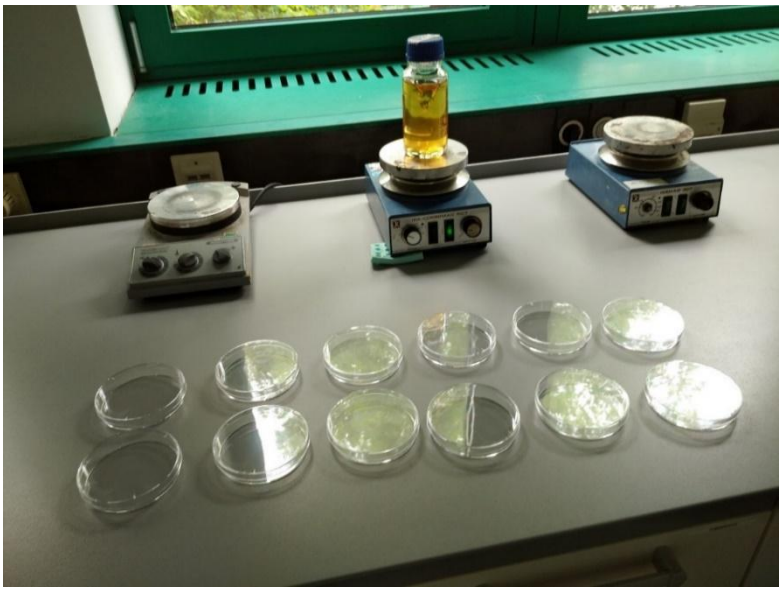

**Figure 2.2.** Cooling and stirring of hard NZCYM medium in sterile room before pouring it into plates.

**Figure 2.3.** Tubes with 10 mL soft agar each, used as a top layer for double layer cultures.

The SM buffer was prepared according to Table 2.2. Stock of 500 mL was prepared. First, powders were weighted and added, together with the calculated amounts of solutions and then the bottle was filled with sterile ultrapure water up to 500 mL. The magnetic stir bar was put inside the mixture and altogether was put on a magnetic stirrer for 5 min. The lid of the bottle was loosened and the bottle was put for autoclaving for 20 min at 121 °C. After autoclaving was finished, the lid was closed and the bottle with the buffer stock was stored at the working bench at room temperature for up to two months. The picture of used materials and chemicals is shown in Figure 2.4.

**Table 2.2.** Chemicals and amount used for preparation of 500 mL SM buffer.

| Chemical | Amount | Final concentration | Brand |
|---|---|---|---|
| NaCl | 2.9 g | 0.100 mol/L | Supelco Sodium chloride for analysis EMSURE® 1.06404 |
| $MgSO_4 \times 7H_2O$ | 1 g | 0.008 mol/L | Supelco Magnesium sulfate heptahydrate or analysis EMSURE® 1.05886 |
| Gelatin | 0.05 g | 0.01% (w/v) | Sigma-Aldrich Gelatin from porcine skin G1890 |
| Tris-Cl buffer (1 mol/L) | 25 mL | 0.050 mol/L | Sigma-Aldrich Tris-EDTA buffer solution T9285 |
| Distilled water | Filled to 500 mL | - | - |

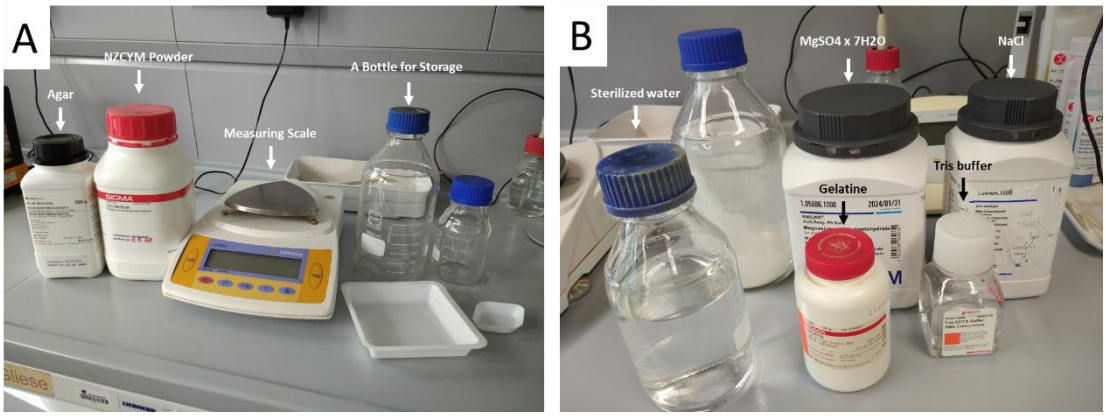


**Figure 2.4. A** Setup for preparing NZCYM medium. **B** Setup for preparing SM buffer.

## 2.2. Preparation of bacterial lawn

All steps were performed in sterile conditions. First, 10 mL of liquid NZCYM medium was transferred into a 100 mL-Erlenmeyer flask. Then, one clear and isolated colony was picked with an inoculation loop and transferred into the prepared 10 mL liquid medium. The medium was covered with an aluminum foil and put in the shaking incubator overnight.

The next day, it was assessed if the bacteria grew by checking the medium turbidity. If the medium became homogeneously turbid in comparison to the negative control, which was a pure medium in an Erlenmeyer flask incubated together with bacterial culture, that was a sign that the bacteria grew and there was no contamination (Figure 2.5). The plates with hard NZCYM medium and the tubes with soft NZCYM medium were taken out from the fridge. The plates were put at room temperature in the sterile room for >1 h so the media is not too cold for bacteria. The water bath was filled with water, turned on, and the temperature was raised to 48 °C. During that time, all the tubes were put in a glass filled with water on the heater (Figure 2.6). The water was heated to boiling, and the soft medium inside the tubes melted down. Every few minutes the tubes were slightly rotated to check if the medium is liquid. When the medium melted, the hot tubes were transferred in the water bath at 48 °C to cool down (Figure 2.7). When a tube cooled down to hand temperature, 500 µL of the overnight bacteria culture was transferred inside the tube, mixed gently by rotating it a few times, and then poured quickly onto the plate with hard medium. After 5-10 min, the soft medium hardened. The result was double layered agar plate, ready for plating phages (Figure 2.8). Until use, the bacterial lawn plates were kept at room temperature which was never more than 1 h.

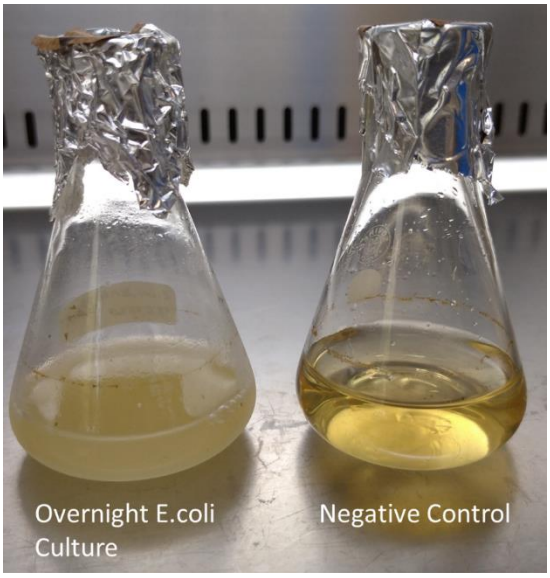


**Figure 2.5.** Overnight culture of *E. coli* and negative control which is NZCYM medium incubated together with the bacterial cultures, but not inoculated.

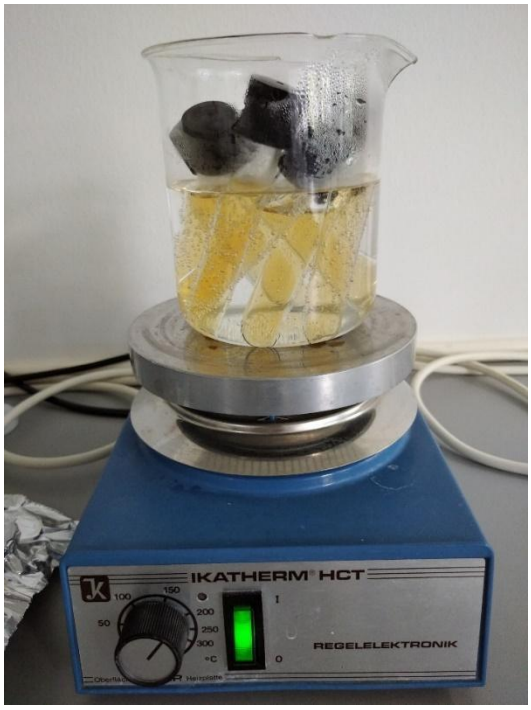


**Figure 2.6.** Melting down of the soft agar in boiling water before cooling it down in warm water bath.

**Figure 2.7.** Tubes with soft agar cooling down at 48 °C after melting and before adding bacteria inside.

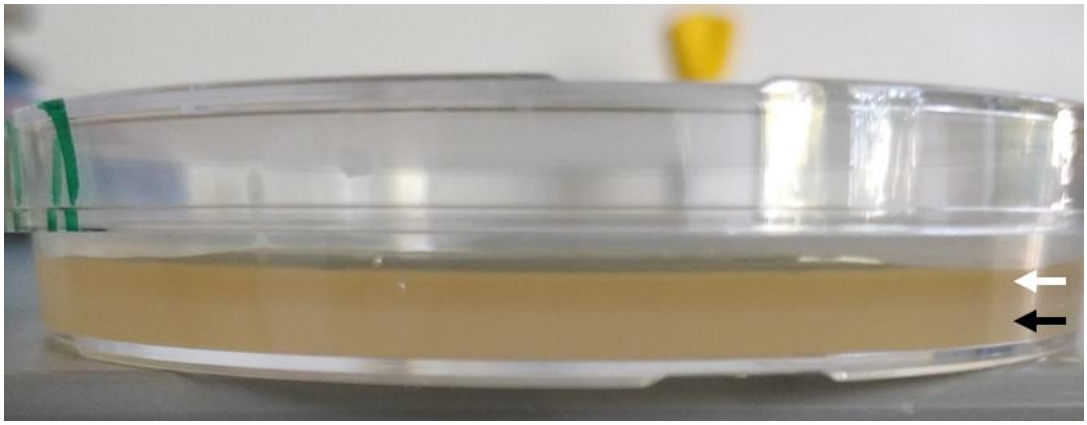

**Figure 2.8.** Double layered plate for viral growth. White arrow indicates the soft, top layer agar with bacteria, and black arrow indicates the hard, bottom layer agar.

## 2.3. Preparation of bacterial and viral cultures

The F+ *Escherichia coli* strain DSM 5695 (Figure 2.9) was ordered from the DSMZ. The bacteria arrived lyophilized and they were revived by adding 500 µL of NZCYM medium and keeping them for 30 min at room temperature. After that, 10 µL of the prepared stock was spread across the plate on hard NZCYM medium and put to grow overnight in the incubator. The rest of the stock was transferred to cryotube and 500 µL of 50% (v/v) glycerol was added. The contents of a cryotube were vortexed thoroughly for >30 s and stored at -80 °C. Every week, a new subculture was prepared. For this, the bacteria from one isolated colony from the previous week's culture was transferred into 10 mL liquid

culture and put to grow overnight in a shaking incubator at 200 RPM. The next day 10 µL of liquid bacterial culture was spread on another hard NZCYM medium plate and grown overnight to get new colonies for that week.

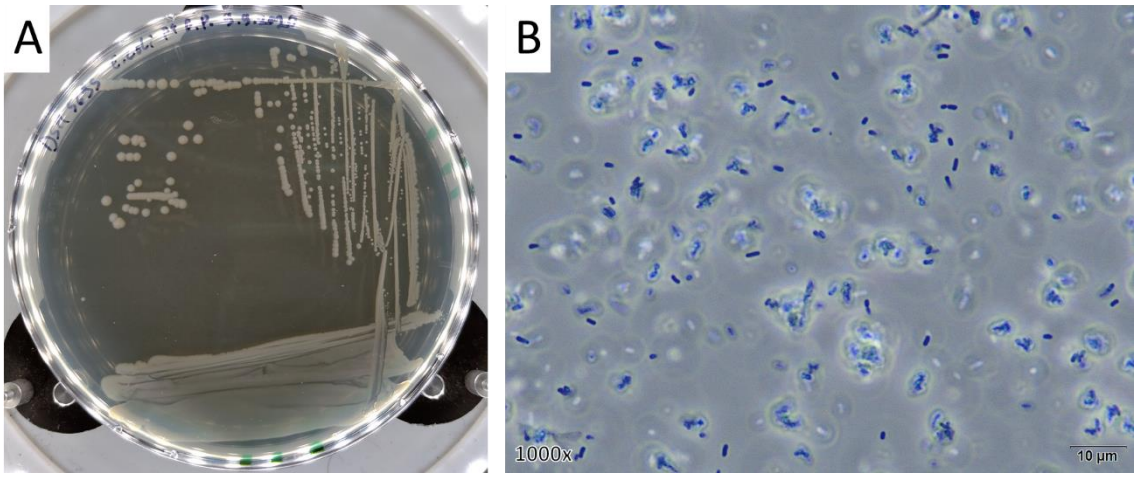


**Figure 2.9.** F+ *E.coli* (strain DSM 5695) used in this study. **A** It forms clear white, round colonies on NZCYM medium. **B** Bacteria under light microscope, 1 000x magnification. They have a rod-like shape, and F-pilus is not visible.

The Coliphage MS2 strain DSM 13767 (Figure 2.10) was ordered from the DSMZ. The phages arrived lyophilized on a filter paper. They hence needed to be revived. First, a fresh bacterial lawn was prepared. After that, the filter paper containing virions was cut under sterile conditions, with sterile scissors and one half of the filter was put in the center of the bacterial lawn. The other half of the filter paper was stored in the Eppendorf tube in the fridge. Then, 10 µL of liquid NZCYM medium was put on top of the filter paper in the bacterial lawn, left for 15 min at room temperature and put in the incubator overnight. The next day, a clear area was formed around the filter paper. The viruses were extracted by pouring 5 mL of SM buffer in the plate and left slowly shaking (50-100 RPM, Figure 2.11, shaker: Edmund Bühler KL2) for 4 h at room temperature. After that, the buffer was collected, transferred to a 15 mL-falcon tube and centrifuged at room temperature for 30 min at 4 000 RPM (centrifuge: HERMLE Z 36 HK). After that, the supernatant was transferred to the disposable syringe and filtered through a 0.2 µm-porous filter into a new tube to get rid of remaining bacterial cells and cellular debris. The resulting filtrate was distributed into two 15 mL-falcon tubes - 1.5 mL of filtrate into each tube. The tubes were then filled with SM buffer up to 15 mL volume. The phage stock solutions were stored in the fridge for a maximum of one month. An example plate with MS2 viruses grown is shown in Figure 2.10.

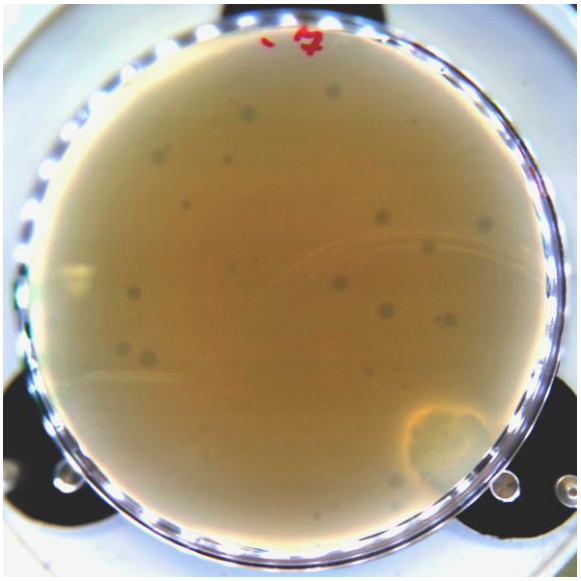

**Figure 2.10.** A bacterial lawn with plaques formed by MS2 virus (strain DSM 13767). It forms regular, round, turbid plaques, varying in size.


**Figure 2.11.** Shaker used for virus isolation and preparation of stock solution. The black arrow indicates the plate containing viruses that grew overnight at 37 °C in double layered agar.

When a viral stock almost ran out, a new one was prepared. First, a bacterial lawn plate was prepared. Then, 100 µL of the previous stock was plated on it, and put for incubation overnight. Henceforth, the method for isolating viruses was as described above.

## 2.4. Determination of MS2 stability in SM buffer

A stock solution of MS2 ($3.58\times10^{9}$ $\pm9.12\times10^{8}$ PFU/mL) was prepared that was constantly kept in the fridge at ~4 °C. The same day the stock was prepared, the phages were plated to determine their titer. That day is marked as day 0. Whenever the stock was taken out, it would be instantly put on ice.

From there, the work was performed in sterile conditions. Every seven days, the stock was mixed gently by rotating it, three 30 µL-samples were taken, and decimal dilutions prepared of each in SM buffer. Then, 20 µL of every dilution was plated on the bacterial lawn. The experiment lasted for 35 days.

## 2.5. Testing virion interaction with lunar dust simulant

For all experiments with lunar dust simulant, EAC-1 lunar dust simulant (Figure 2.12) was used, which was available at DLR. Composition is listed in Table 1.1 (Section 1.1.4, EAC-1).

**Figure 2.12.** Lunar dust simulant EAC-1 used in this study.

The simulants interaction with water was tested by diluting 200 mg in 500 µL of sterile distilled water, and vortexed until the homogenous dilution was created. From that point, the speed of separation from water was observed.

For testing viral interaction with the simulant, 200 mg of lunar dust simulant was transferred to an Eppendorf tube. Then, 25 µL of MS2 stock solution ($7.17\times10^9 \pm 6.17\times10^9$ PFU/mL) was added in sterile conditions and vortexed for >30 s. After that, 500 µL of SM buffer was added and the solution was vortexed for >30 s. From that point, two approaches for sampling were tested, based on the hypotheses of phage interaction with lunar dust: 1) extracting virions by centrifugation and 2) sampling virions together with the lunar dust. Both experimental procedures are summarized in Figure 2.13.

### 2.5.1. Extracting virions by centrifugation

The mixture of lunar dust simulant, SM buffer and viruses at 5 000 RPM were centrifuged at room temperature for 30 min (centrifuge: Eppendorf Centrifuge 5417 R). A 30 µL-sample of the supernatant was collected in triplicates, serial dilutions were prepared, 20 µL of each dilution was plated, and put for incubation overnight. The day after, the plaques were counted.

### 2.5.2. Sampling virions together with the lunar dust simulant

Immediately after the vortex, a 30 µL-sample of lunar dust was suspended in 270 µL SM buffer in triplicates and decimal dilutions were prepared in SM buffer. Then, 20 µL of each dilution was plated. The plates were put for incubation overnight. The next day, the plaques were counted.

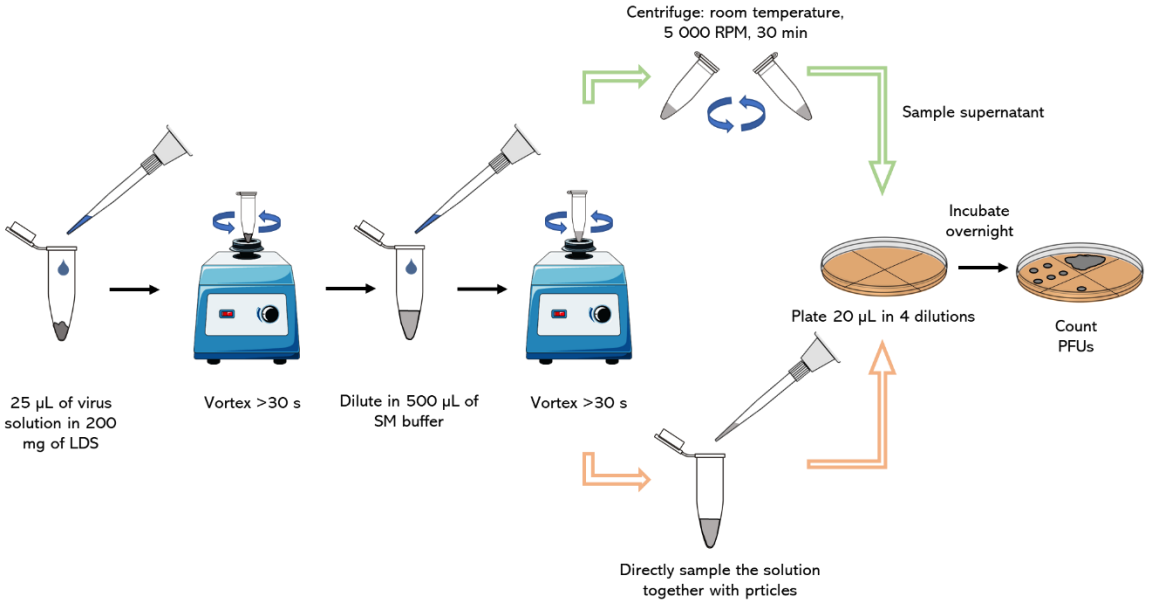


**Figure 2.13.** Summary of the two methods for testing interaction of MS2 virions with lunar dust simulant (LDS). Green arrows indicate the procedure with centrifugation, and orange arrows indicate the procedure without centrifugation.

## 2.6. Determination of virion thermal stability

The MS2 virions were treated at five different temperatures: 40, 50, 60, 70, and 80 °C. Two series of treatments were performed. The first was for 10 min (starting titer: $3.58\times10^9$ $\pm9.12\times10^8$ PFU/mL), and the second was for 30 min (starting titer: $1.23\times10^9$ $\pm3.56\times10^8$ PFU/mL). For each treatment test, three 200 µL-samples were used. Each sample was put on ice until treatment. A negative control, 200 µL SM buffer without viruses, was also treated to check whether the final temperature of SM buffer visibly affects the growth of *E. coli* in bacterial lawn. After each treatment, the samples were immediately put on ice until dilutions ready for plating were made. Plating was performed in sterile conditions and put for incubation overnight.

After that, MS2 virions inside the lunar dust simulant were treated at 60, 70 and 80 °C for 10 min (starting titer: $3.43\times10^9$ $\pm5.21\times10^8$ PFU/mL). The experiment was done in triplicates. The phages and lunar dust were mixed as described in section 2.5. After that, the samples were put on ice until treatment. After the treatments, the solution was sampled, diluted and plated according to the approach described in section 2.5.2 (since this approach proved more effective).

The stability of MS2 virions exposed to -80 °C was also tested. In sterile conditions, 25 µL samples in triplicates were prepared from the stock MS2 solution ($7.17\times10^9$ $\pm6.17\times10^9$ PFU/mL), put in an Eppendorf tube and left in deep freeze at -80 °C for 24 h. After the treatment, the samples were put on ice to melt down slowly. All 25 µL were sampled from each tube and serial decimal dilutions were prepared in 225 µL SM buffer in sterile conditions. The dilutions were plated in sterile conditions and put for incubation overnight.

Along with it, a triplicate of MS2 virion controls was prepared with a cryoprotectant – 25% (v/v) glycerol. From the stock solution, 50 µL of virions was sampled and 50 µL of 50% (v/v) glycerol was added. The samples were put inside the deep freeze at -80 °C for 24 h. After the treatment, the samples were put on ice so they could melt slowly. Then, samples were diluted and plated in sterile conditions as described at the beginning of section 2.

Also, triplicate samples of MS2 virions were mixed with lunar dust simulant as described in section 2.5 and put at -80 °C for 24 h along with the other samples. After the treatment, the virions were sampled, diluted and plated as described in section 2.5.2.

## 2.7. Determination of virion stability after air and vacuum drying

For air drying experiments, a sample of 20 µL phage stock solution ($1.23\times10^9$ $\pm3.56\times10^8$ PFU/mL) was transferred to an Eppendorf tube in sterile conditions. The experiment was performed in triplicates. Replicates were placed together, opened in a petri dish and covered with the lid held with two inoculation loops to allow airflow (Figure 2.14). The petri dish was placed in a protected area with airflow. They stayed there for 18 h. The next day, the droplets evaporated and 500 µL of SM buffer was added. Then, samples were diluted in decimal dilutions, plated in sterile conditions and put for incubation overnight. The starting and final titers were corrected for the dilution of dried viruses in a new volume (500 µL). The starting titer was corrected by calculating the number of viruses that was collected in a 30 µL sample, and then the titer was calculated for this number of viruses if they were diluted in 500 µL. The final titer was determined by plating and it was compared to the starting titer.

**Figure 2.14.** Setup for air drying of phages. Two inoculation loops hold the lid of a petri dish and inside is a triplicate of phage solution and a negative control to track possible contamination. The Eppendorf tubes are held open to allow airflow.

For vacuum drying, a triplicate of 20 µL virus stock solution ($4.18\times10^7$ $\pm1.53\times10^7$ PFU/mL) were put in opened Eppendorf tubes in a vacuum centrifuge (Eppendorf Concentrator Plus) to spin at 1 400 RPM, 24 °C, and pressure of <20 mbar for 3 h (setting *V-AQ* for water solution). Before and after using the centrifuge, the centrifuge ran without samples for 15 min to dry from any condensed liquid. After the centrifugation was done, 500 µL of SM buffer was added. Dilutions were prepared and plated in sterile conditions as described at the beginning of section 2. After that, the plates were put for incubation overnight. The correction of titer for a new starting volume was performed in the same way as for air-drying.

## 2.8. Irradiation of virions with X-rays

Before irradiation of the samples, the time of radiation needed to get the required dose of radiation was determined. This was done by putting an X-ray dosimeter inside, at the same distance from the source of radiation as the samples would be. The machine was turned on, and determined how many Gy per minute dosimeter reads. The time of radiation needed to get the required radiation doses was hence calculated.

In sterile conditions, 50 µL of phage stock solution ($5.3\text{x}10^7$ $\pm1.61\times10^7$ PFU/mL) in triplicates was taken. Immediately, they were put on ice until treatment. Before any X-ray treatment, the tubes were wiped, so they are completely dry from outside.

The virions were treated with various X-ray doses: 0, 100, 250, 500, 1 000, and 2 000 Gy. The radiation was a mix of wavelengths with energies 59 and 67 keV. Inside the machine, samples were at room temperature. The triplicates of tubes were placed horizontally in a star-like formation so that the solutions inside were in the same spot as the dosimeter (indicated by laser light inside the machine). When the time of irradiation passed for one triplicate, the next triplicate was put inside and irradiated. The experiment was performed this way until the final dose treatment (2 000 Gy).

The radiation machine (Gulmay, Figure 2.15) used a tungsten cathode as an X-ray source. It also filters the *bremsstrahlung* radiation, so the resulting radiation is mixed radiation of tungsten cathode spectrum wavelengths: 59 and 67 keV which corresponds to 0.0210 and 0.0185 nm wavelengths, respectively.

**Figure 2.15.** The radiation machine (Gulmay) used to irradiate MS2 phage with X-rays.

After irradiation, the samples were immediately put on ice until decimal dilutions were prepared and plated in sterile conditions. The plates were put for incubation overnight.

## 2.9. Testing MS2 transmissibility by air-suspended droplets and aerosols

The 50 mL aluminum spray cans (Figure 2.16) were used for dispersing virions into air droplets and aerosols. To optimize the use of materials, the minimal volume of sprayed liquid needed to get efficient dispersion was first tested. This test was performed by adding ultrapure sterile water into one of the cans and spraying it horizontally. The volumes of water tested were 1, 2, 3, 4, 5 and 10 mL. It was determined that there is no significant difference in spray dispersion when spraying 4 mL and 10 mL of liquid. Hence, 4 mL of liquid was always used for spraying.


**Figure 2.16.** Aluminum spray cans used for spraying phages.

Aluminum spray cans were sterilized by wiping them and filling ¼ of their volume with 70% (v/v) ethanol. Ethanol was sprayed out 5 times, so it passes the spraying tube. The cans were turned gently so the ethanol reached all spaces inside the cans. The cans were then left for ethanol to evaporate for >1 h in a closed sterile cabinet with UV lamp turned on. After that, ethanol was discarded and the cans were filled to ½ of their volume with sterile ultrapure water, turned gently, and sprayed out 10 times. The cans were then left in a sterile cabinet overnight to dry.

First, the test was performed to determine if the viruses could survive being dispersed in spray and checked the distance virions can travel when sprayed. In sterile conditions, 4 mL of MS2 stock solution ($4.18x10^7$ $\pm 1.61\times10^7$ PFU/mL) and 4 mL of SM buffer (negative control) was added into sterilized aluminum spraying cans. While the experiment lasted (~30 min), both cans were held at room temperature. The solutions were sprayed in a sterile cabinet. Fresh, opened bacterial lawns were placed at 0.5 and 1.5 m distances from the spray. The negative control was sprayed five times from the marked position. The cabinet was closed and left for 5 min for droplets to settle down. Then, the plates were covered and put for incubation overnight. After that, the experiment was repeated with the MS2 stock solution and three plates at 0.5, 1, and 1.5 m distances. In the end, MS2 solution was sprayed two times directly at a fresh bacterial lawn at ~10 cm distance which served as positive control (Figure 2.17). The experiment summary is shown in Figure 2.18. The same experiment was carried with different phage dilutions: $10^{-2}$, $10^{-4}$ and $10^{-6}$. The dilutions were prepared by adding 3.96 mL of SM buffer into three cans and adding 40 µL of phage stock solution into the first can. It was mixed well, and 40 µL of the resulting solution was transferred to the second spraying can and so on. After finishing the experiment, the cabinet was sterilized with UVC for 1 h.

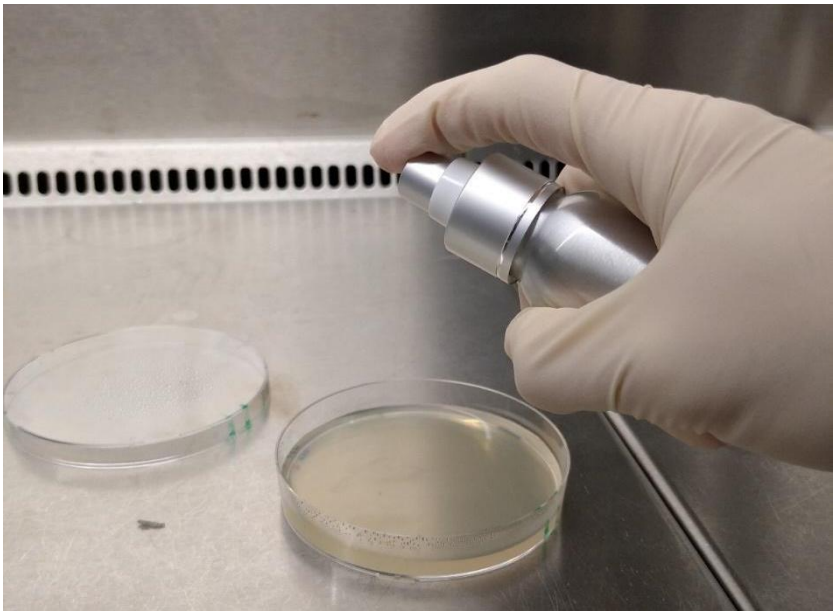

**Figure 2.17.** Applying a positive control for testing of MS2 phage transmission in aerosols. The phages ($4.18x10^7$ $\pm 1.61\times10^7$ PFU/mL) were sprayed onto the fresh bacterial lawn at ~10 cm distance.

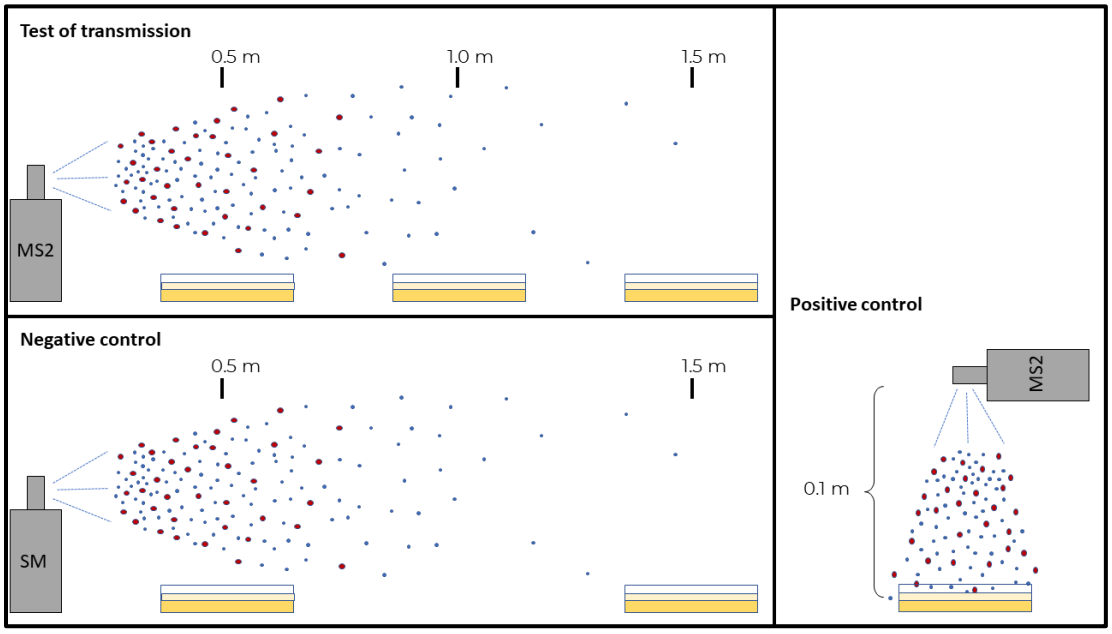


**Figure 2.18.** Schematic representation of the experimental setup for testing aerosol- and air-droplet-transmission of MS2 phages. For the experiment, fresh double-layered plates were put at 0.5, 1, and 1.5 m distance from the spray can and spraying was performed five times. For a negative control, SM buffer was sprayed, and plates were put at distances 0.5 and 1.5 m. For positive control, the phage solution was sprayed directly onto a plate at 0.1 m distance. While large droplets (red) travel lower distances (<1 m), aerosols (blue) can travel much further.

Also, the stability of phages in the air was assessed after 5 and 35 min post spraying. First, a $10^{-2}$ dilution of phages was prepared as described above (stock titer: $3.43\times10^{9}$ $\pm9.20\times10^{8}$ PFU/mL). The spraying position was marked and distances of 0.5 and 1 m were measured. The solutions were sprayed 5 times, the cabinet was closed, and left for 5 min. Then, fresh bacterial lawns were added at 0.5 and 1 m distance and waited 5 min for droplets and aerosols to settle down. After 35 min passed, the procedure was repeated. The solutions were tested in room temperature buffer, and in buffer close to the freezing point. The experiments were also performed with and without the UVC lamp turned on. For each experiment – UV and cold buffer, there was a positive control, where the viral solution was directly sprayed at the bacterial lawn at 10 cm distance. After each experiment, the cabinet was sterilized with UVC for 1 h.

## 2.10. Statistical analysis

Experiments were done in independent triplicates. In each plate, two values could be determined for the titer according to Equation 2.1. The two values of each plate were dependent on the original infectivity since plaques were usually countable in two different dilutions on each plate (e.g., Results, Figure 3.1). Therefore, the mean infectivity was calculated for every replicate plate. Since virus sampling from a solution is a random process, there is no reason that the chances of sampling a certain titer of viruses is not normally distributed around a true mean titer. Therefore, assuming a normal distribution of the data for every titer, a standard deviation (SD) was calculated as well as 95% confidence interval (95%CI). Before any statistical analysis was performed, the final infectivity values (measured in PFU/mL) were transformed by taking $\log_{10}$ of the values and hence normalized the data distribution even more and reducing experimental error.

$$infectivity\left(\frac{PFU}{mL}\right) = \frac{N(PFU)}{V(mL)\times 10^{-D}}$$

**Equation 2.1.** Calculation of viral infectivity after plating and incubation. N(PFU) is number of counted plaque-forming units [between 1 and 250], V(mL) is volume of phages taken from any sample [usually 0.03 mL], and D is the number of a serial dilution in which plaques were counted [between 0 and 10].

The analysis was carried with means, which are very sensitive to outliers. Therefore, all outliers were removed from each dataset. For each experiment, the data distributions were checked if they are close to normal distribution by plotting the values in density plots. Figure 2.19 shows one example of a density plot for a dataset with virus heat stability experiments in SM buffer. When the outliers were detected, they were removed from a dataset. Then a mean value and standard deviation (SD) for each treatment was determined.

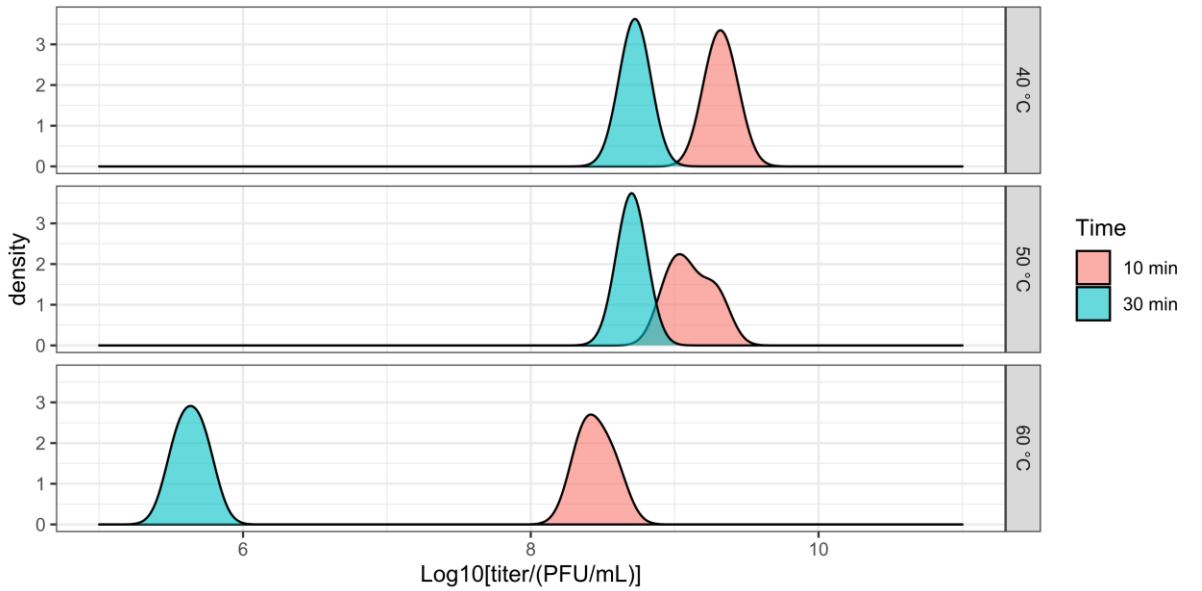


**Figure 2.19.** An example of a density plot to check if the data is approximately normally distributed. This is an example for heat stability of phages in SM buffer, but other experiments showed similar distribution shapes after log-transformation and removal of outliers.

To detect differences between groups, analysis of variance (ANOVA) was a method of choice. ANOVA assumes normal data distribution, hence $\log_{10}$ transformation and density-plot checking was performed as described above. It also assumes homogeneity of variances between compared groups, so before performing ANOVA, Levene test for homogeneity of variances was performed on each dataset. For *post hoc* analysis, Tukey test was performed to determine which groups differ significantly.

Student's t-test was used for comparing two groups. Again, Levene test was done to check for homogeneity of variances. When the significance of phage inactivation was tested, the $\log_{10}$(infectivity) after treatment was compared with $\log_{10}$(starting infectivity) using one-sided student's t-test since the expectation was that the final infectivity is lower or equal to the starting one. On the other hand, when the change in infectivity was compared after two different treatments, two-sided t-test was used since there was no reason to assume that any treatment would result in higher or lower change in viral infectivity.

For lunar dust simulant treatments, the efficiency of virus extraction was determined by dividing final concentration with the starting concentration in virion extraction described in section 2.5.2. The factor of 0.0248 was determined to be a sufficient correcting factor. This factor was used to correct all results of the lunar dust simulant treatments so they can be compared with other treatments.

For change in infectivity over time and x-ray exposure, linear models were calculated by linear regression. F-test was conducted for the models to determine 95%CIs and *p*-values.

All the statistical analysis and plotting was performed with programming language and environment R version 4.0.3 (R Core Team, 2020) inside RStudio version 1.3.1093 (RStudio Team, 2020) with the aid of packages *dplyr* (Hadley *et al.*, 2020) and *car* (Fox and Weisberg, 2019) for statistical analysis, and *ggplot2* (Hadley, 2016) for plotting.

# 3. Results

## 3.1. MS2 stability in SM buffer

Figure 3.1 shows example plates after counting plaques from the same stock solution every week while constantly kept in the fridge. The reduction in titer is clearly visible. While at day 0, the plaques were clearly visible at $10^{-7}$ dilution (-7), by day 35, the maximum dilution for visible plaques was $10^{-5}$ (-5), suggesting roughly 2 $\log_{10}$ reduction.

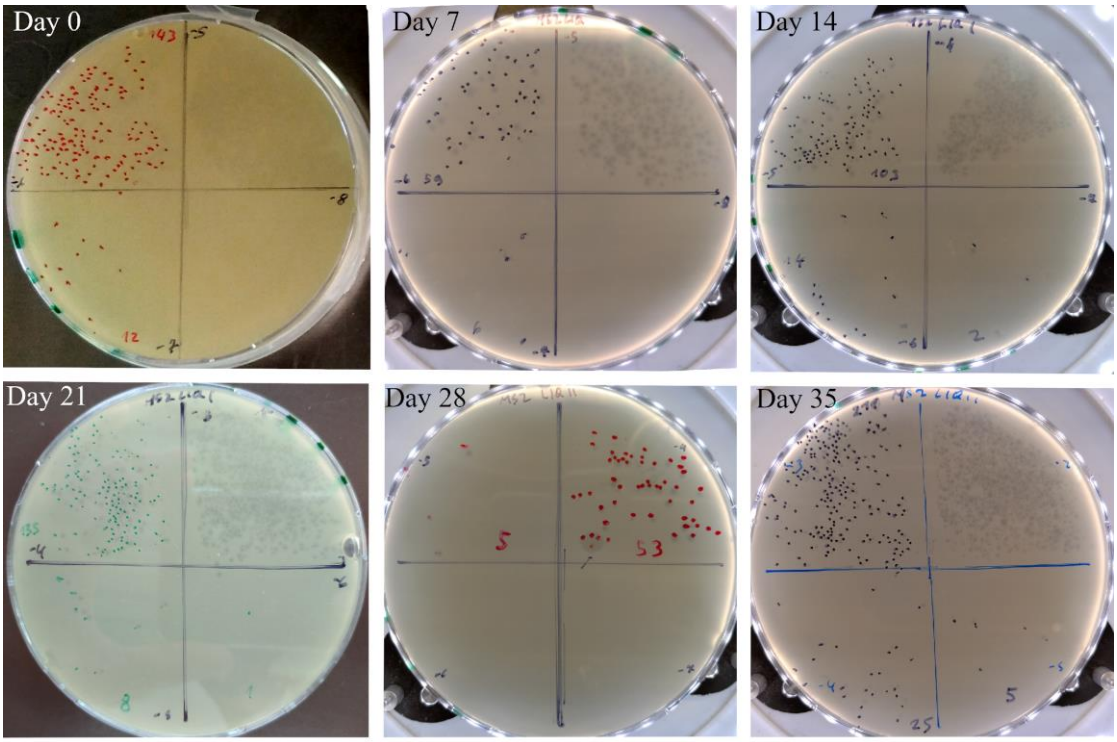


**Figure 3.1.** Representative plates showing how MS2 phage titer changes during storage in fridge. It is visible that each plate had 2-3 dilutions at which plaques were countable.

Figure 3.2 shows the loss of bacteriophage titer over time. Every seven days, MS2 phage infectivity roughly halves. Over 35 days in the fridge, there has been a total 2.52 $\log_{10}$ reduction in phage titer. Table 3.1 shows the statistics for the calculated linear model.

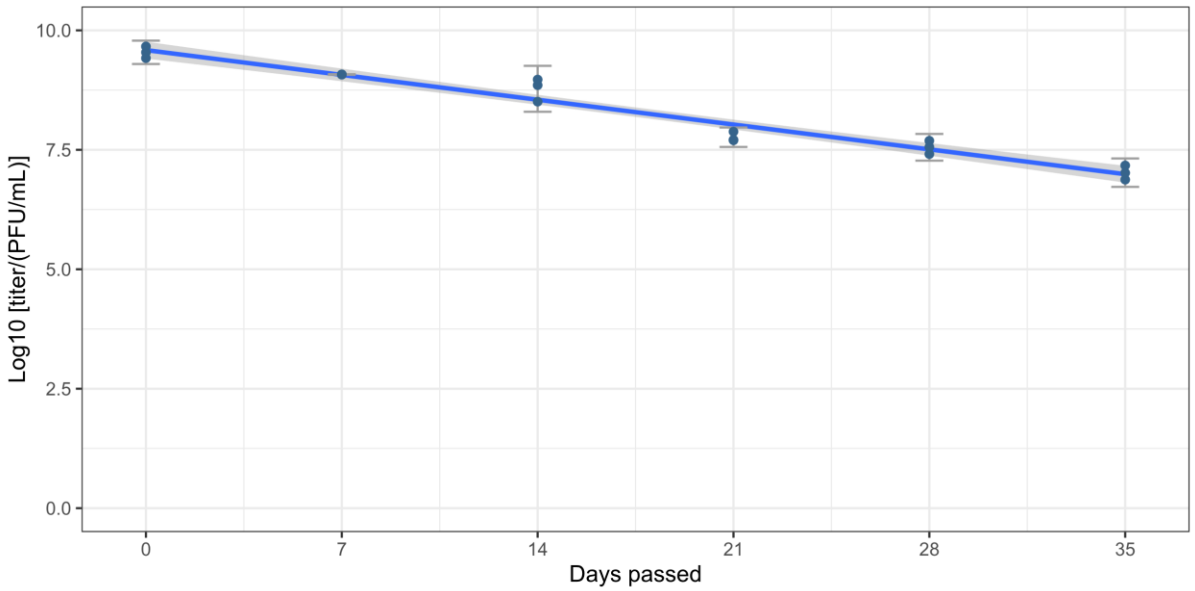


**Figure 3.2.** Change in $\log_{10}$ of MS2 phage titer during storage in the fridge in SM buffer over 35 days. Error bars indicate the SD (n = 2 or 3). The blue line represents the trendline as calculated by linear regression. The gray area around the line represents 95%CI for the linear model as determined by F-test.

**Table 3.1.** Values relevant for the calculated linear model of phage stability in SM buffer in fridge.

| | |
|---|---|
| **Formula** | $f(x) = -0.074183x + 9.586692$ |
| **Adjusted $R^2$** | 0.9545 |
| ***p*-value (as determined by F-test)** | $2.271 \times 10^{-12}$ |
| **Correlation coefficient** | -0.9783 |

## 3.2. Virion interaction with lunar dust simulant

Before tests of virion interaction with the lunar dust simulant were done, the lunar dust simulant interaction with pure SM buffer was assessed. SM buffer (500 µL) and lunar dust simulant (200 mg) fully segregated from each other approximately 10-15 s after vortexing (Figure 3.3).

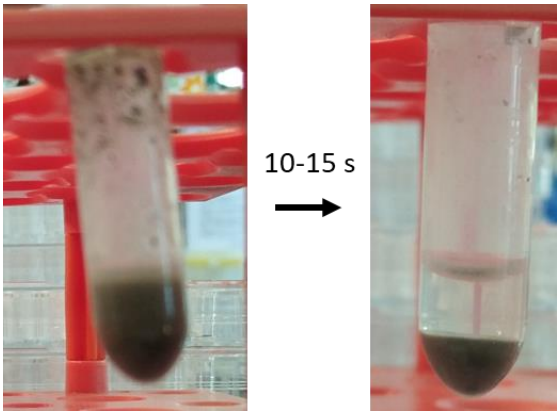


**Figure 3.3.** Observation of lunar dust simulant diluted in SM buffer. Only 10-15 s after mixing, two clear phases formed.

With centrifugation (described in section 2.5.1), the efficiency of virion extraction was determined to be 0.2496%. However, when phages were sampled together with lunar dust simulant, the efficiency raised to 2.4764% (described in section 2.5.2).

## 3.3. Thermal stability of MS2

To determine thermal stability of MS2 phages, change in infectivity was observed after 24-h treatment at -80 °C, 10- and 30-min treatment at room temperature, 40, 50, 60, 70, and 80 °C in SM buffer and lunar dust simulant.

### 3.3.1. MS2 infectivity after 24 h at -80 °C

Figure 3.4 shows the change in titer after treatment of phages at -80 °C for 24 h. After the treatment, there was no significant loss of MS2 phage infectivity when virions were held in SM buffer – with or without cryoprotectant. However, there was a significant loss of infectivity for phages in lunar dust simulant as determined by one-way ANOVA and Tukey test. This showed that only phages held in lunar dust differed so after that, a one-sided t-test was performed of the final infectivity of phages in lunar dust simulant (Table 3.2).

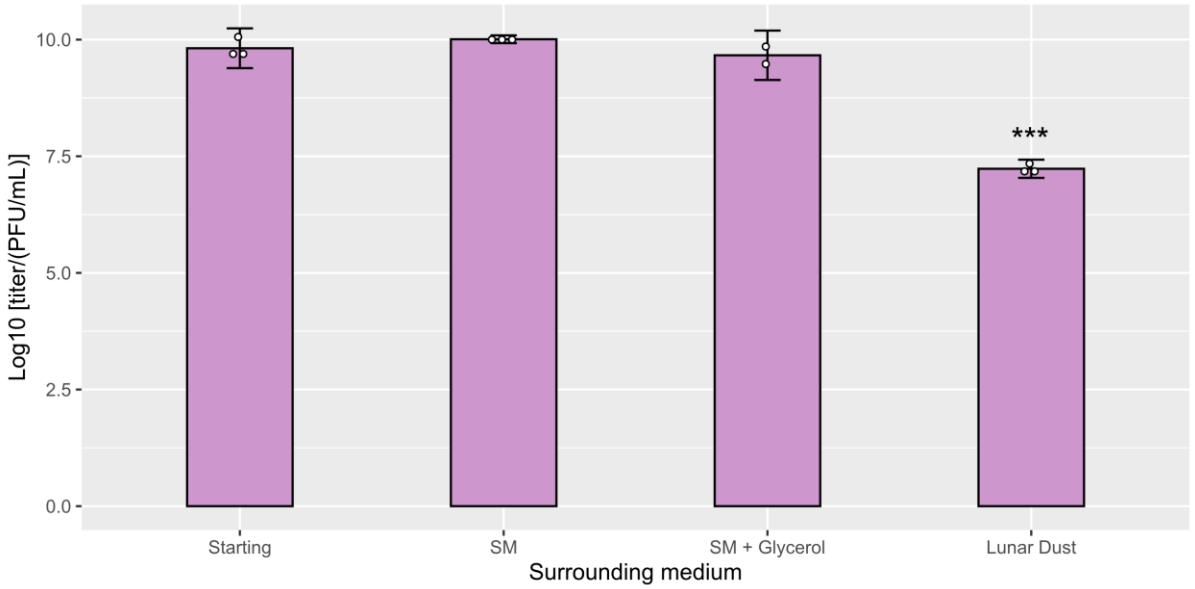


**Figure 3.4.** Change in MS2 phage $\log_{10}$ infectivity after 24 h at -80 °C. There were no significant changes in infectivity when phages were held in SM buffer or SM buffer with added glycerol (cryoprotectant), but there was a significant decrease in infectivity when phages were held in lunar dust simulant. Error bars represent SD, and *** represents *p*-value < 0.001 as determined by one-way ANOVA with Tukey *post hoc* test and one-sided t-test of Lunar Dust sample against Starting sample.

**Table 3.2.** Statistical values after performing one-sided t-test for the mean $\log_{10}$(infectivity) of phages after treatment in lunar dust simulant at -80 °C with the starting mean $\log_{10}$(infectivity).

| | |
|---|---|
| **t-value** | -19.077 |
| ***p*-value** | $2.224 \times 10^{-5}$ |
| **Degrees of freedom** | 4 |
| **Mean (lunar dust simulant $\log_{10}$(infectivity))** | 7.231 |
| **Mean (starting $\log_{10}$(infectivity))** | 9.815 |

### 3.3.2. MS2 infectivity after heat treatment in SM buffer and lunar dust simulant

Phage inactivation after heat treatments is shown in Figure 3.5 from room temperature up to 70 °C. Treatment was also performed on all groups at 80 °C, but no PFUs were detected in any of them so this treatment is not plotted. Even though after treatment in SM buffer at 70 °C there were no plaques detected, they were detected after treatment in lunar dust simulant. Inactivation of phages was significantly higher than at 50 °C in SM buffer – both for 10 and 30 min. Also, there was some phage inactivation in lunar dust simulant after 10 min at room temperature. Figure 3.6 shows the matrix of *p*-values after performing ANOVA and Tukey *post hoc* test to determine which treatments significantly differ from each other - gray and blue circles represent significant difference, yellow and orange low significance, and red circles no significant difference in $\log_{10}$ infectivity.

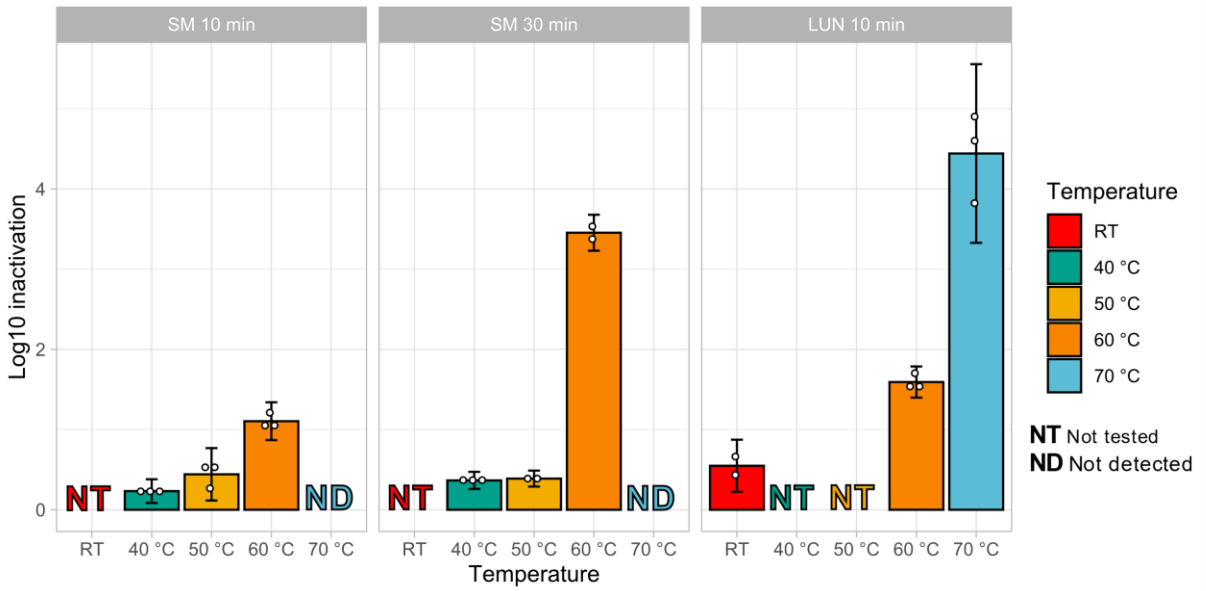


**Figure 3.5.** Loss of MS2 phage infectivity after treatment for 10 and 30 min at room temperature (RT), 40, 50, 60 and 70 °C. Phages were treated in SM buffer (**SM 10 min** and **SM 30 min**) and lunar dust simulant (**LUN 10 min**). Error bars represent SD (n = 2 or 3).

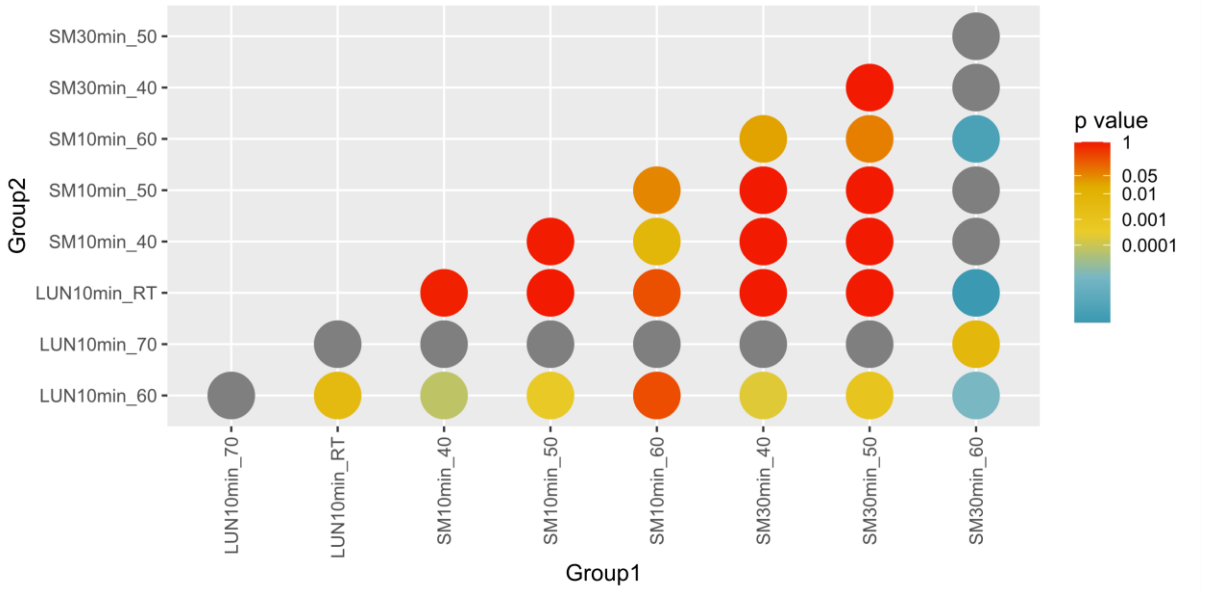


**Figure 3.6.** Matrix of *p* values when comparing the loss of infectivity after heat treatment of all experiments with one-way ANOVA and Tukey *post hoc* test. Gray and blue circles represent significant difference (*p*-value <<0.001), yellow and orange low significance (*p*-value <0.05), and red circles no significant difference (*p*-value > 0.05).

## 3.4. MS2 infectivity after drying

Both after air and vacuum drying, phages experienced significant decrease in infectivity as inferred by one-sided student's t-test with 4 degrees of freedom (Figure 3.7). Table 3.3 shows the values relevant for the two t-tests conducted – one for air drying, and one for vacuum drying.

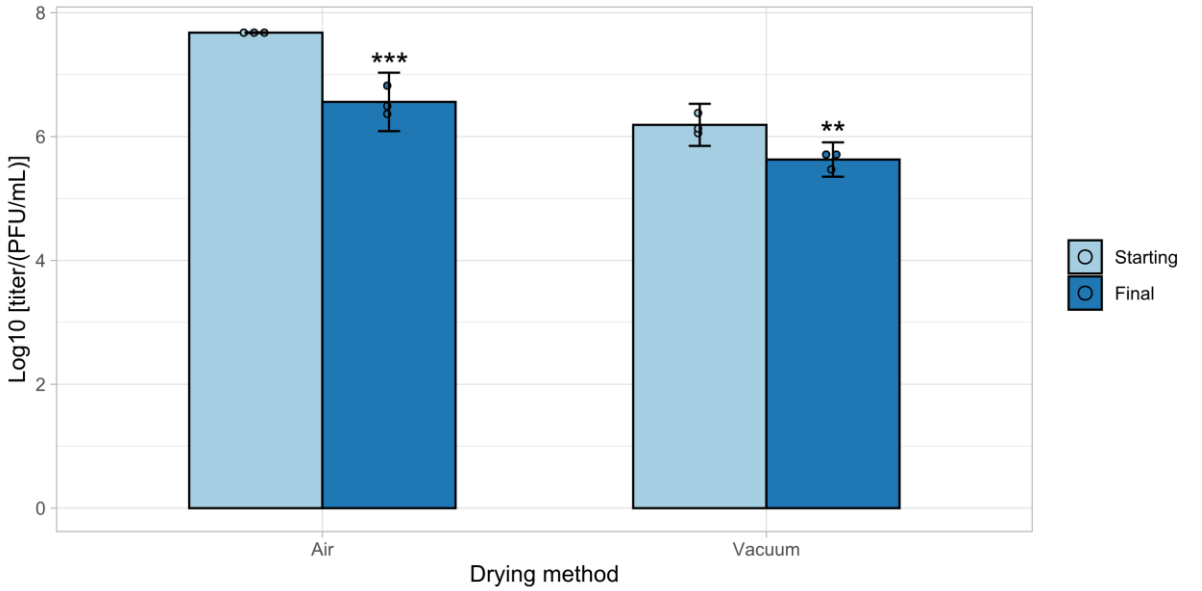


**Figure 3.7.** MS2 phage infectivity before and after drying on air and in vacuum. Both methods significantly reduced phage infectivity. Error bars represent SD, ** represents a *p*-value < 0.01, and *** a *p*-value < 0.001 as inferred from one-sided student's t-tests (final against starting log10 (titer)).

**Table 3.3.** Statistical values for one-sided t-test of final phage infectivity compared to starting infectivity for air and vacuum drying. Both dryings significantly reduced infectivity.

| | **Air drying** | **Vacuum drying** |
|---|---|---|
| **t-value** | -8.2072 | 4.4217 |
| ***p*-value** | 0.0006 | 0.0057 |
| **Degrees of freedom** | 4 | 4 |
| **Mean (Final $\log_{10}$(infectivity))** | 6.5597 | 5.6294 |
| **Mean (Starting $\log_{10}$(infectivity))** | 7.6780 | 6.1891 |

Loss of infectivity was determined to be higher after air drying (Figure 3.8), with low statistical significance as inferred by two-sided student's t-test (Table 3.4).

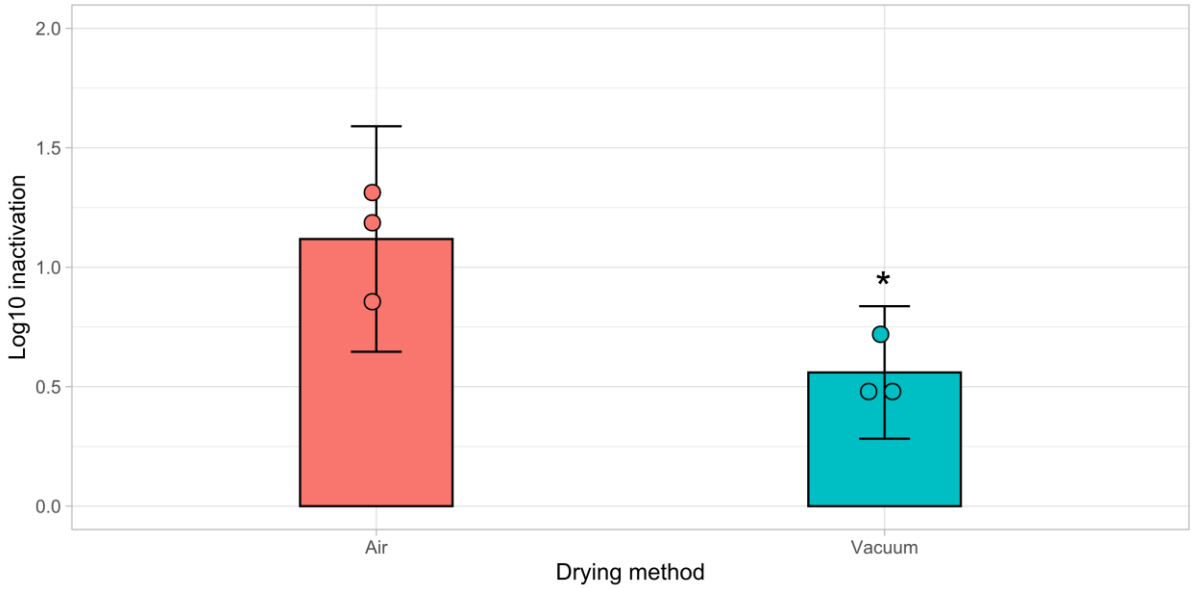


**Figure 3.8.** Difference in MS2 phage inactivation after air- and vacuum-drying. Inactivation after air drying seems to be higher, though with questionable significance. Error bars indicate SD (n = 3), and * indicates a *p*-value < 0.05 as inferred by two-sided student's t-test.

**Table 3.4.** Statistical values for two-sided t-test comparing inactivation of phages after air drying and vacuum drying.

| | |
|---|---|
| **t-value** | -3.5350 |
| ***p*-value** | 0.0241 |
| **Degrees of freedom** | 4 |
| **Mean (Air drying $\log_{10}$(inactivation))** | 1.1183 |
| **Mean (Vacuum drying $\log_{10}$(inactivation))** | 0.5597 |

## 3.5. MS2 infectivity after X-ray exposure

MS2 phages showed exponential drop in infectivity with exponential increase in X-ray dose. Therefore, logarithmic dependence of decrease in phage infectivity was determined (Figure 3.9). It was determined that LD50 of X-rays for MS2 phages is 120 Gy (Table 3.5).

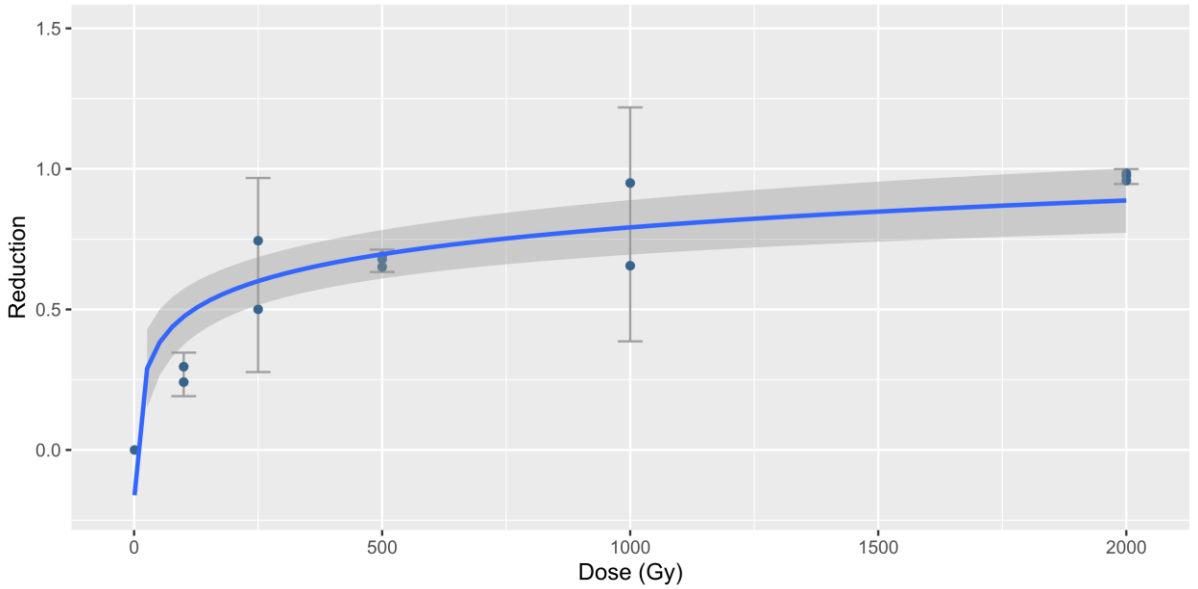


**Figure 3.9.** Logarithmic curve describing the relationship between X-ray dose and reduction in MS2 phage infectivity. Error bars represent SD (n = 2 or 3), and gray area around the line is 95%CI for the logarithmic model.

**Table 3.5.** Values relevant for the calculated logarithmic model of phage inactivation during exposure to increasing X-ray doses.

| | |
|---|---|
| **Formula** | $f(x) = 0.31764\log_{10}(x) - 0.16095$ |
| **Adjusted $R^2$** | 0.8001 |
| ***p*-value (as determined by F-test)** | $2.261 \times 10^{-5}$ |
| **Correlation coefficient** | -0.9038 |
| **LD50** | 120.4522 Gy (95%CI = 96.4208…142.3331 Gy) |

## 3.6. MS2 transmissibility by air-suspended droplets and aerosols

Table 3.6 shows a schematic of the phage spraying transmissibility results. After spraying, PFUs were detected at a distance of 0.5 m at original titer (0), as well as dilutions -2 and -4, but not -6. Also, transmission was not observed for 1 and 1.5 m distances. Starting viral titer was $4.18 \times 10^7 \pm 1.61 \times 10^7$ PFU/mL.

Figure 3.10 shows a representative result (stock concentration) on plates. The projection of the spray cone in 2D is visible in form of plaques in the plate that was 0.5 m from the source.

**Table 3.6.** Summary of results of testing MS2 phage transmissibility in air droplets and aerosols. Starting viral titer was $4.18x10^7 \pm 1.61\times10^7$ PFU/mL. Detection of PFUs is indicated by green fields and +, while failure of PFU detection by red fields and -.

| **Distance** | **0.5 m** | | | | **1 m** | | | | **1.5 m** |
|---|---|---|---|---|---|---|---|---|---|
| **Dilution** | 0 | -2 | -4 | -6 | 0 | -2 | -4 | -6 | 0 |
| Room temperature buffer | + | + | + | - | - | - | - | - | - |

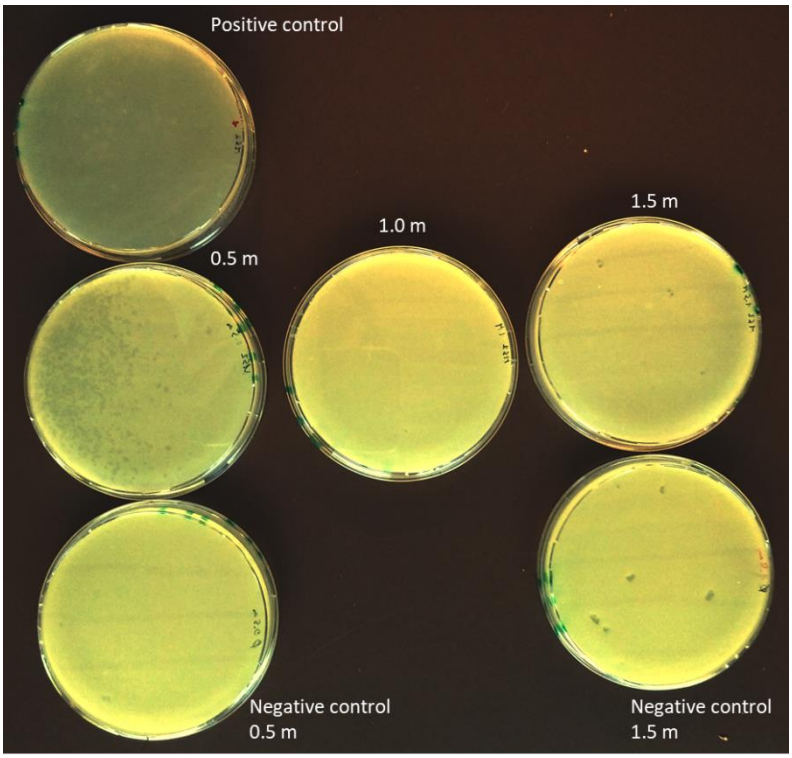


**Figure 3.10.** Results of testing MS2 phage transmissibility after spraying in original stock solution (dilution 0, $4.18x10^7 \pm 1.61\times10^7$ PFU/mL). For all tested dilutions, results were similar except for -6 where PFUs were not detected at 0.5 m. Also, all dilutions showed PFUs in the positive control except for -6.

After the results of transmissibility were observed, stability experiments were performed in relation to presence of UV radiation and buffer temperature. The experiments were additionally performed in cold SM buffer (~0 °C) and with UV after 5 and 35 min in the air. Phages were sprayed in -2 dilution. Table 3.7 shows the results. While PFUs were detected in room temperature buffer consistently both after 5 and 35 min, they were never detected in cold buffer. It must be noted however that after spraying viruses in cold buffer, PFUs were not detected in the positive control either, hinting that viruses might have failed to be sprayed when in cold buffer.

**Table 3.7.** Summary of the results of testing MS2 phage transmissibility by air droplets and aerosols after 5 and 35 min in air. Only -2 dilution was used (original stock: $4.18x10^7$ $\pm 1.61\times10^7$ PFU/mL) and collection was performed at a distance 0.5 m. Detection of PFUs is indicated by green fields and +, while failure of PFU detection by red fields and -.

| **Time of sampling after spraying** | **5 min** | **35 min** |
|---|---|---|
| Room temperature buffer | + | + |
| Cold buffer | - | - |
| Room temperature buffer + UV | + | + |
| Cold buffer + UV | - | - |

# 4. Discussion

## 4.1. Methodology used

In this work, MS2 stability was determined only in terms of infectivity. However, it would be beneficial if other methods were also used to assess the effect of various stress factors on the phages. There are various methods that could be applied for that purpose.

For instance, the damage to the phage genome could have been determined by isolating the RNA after each treatment (along the untreated control), running it on native gel electrophoresis and dyeing it with RNA-specific dye. For RNA isolation, it would be first necessary to treat virion solution with RNase to degrade all the RNA outside virions. Then, inactivation of RNase and treatment with protease would be necessary, to release the RNA from virions, and then isolating RNA from the solution. This could be done with one of the standard RNA isolation kits (Nanassy *et al.*, 2011). MS2 phage genome has the length of 3.569 kb according to its GenBank entry (GenBank accession number: NC_001417). For untreated control, it would be expected for one clear band to be present between 3.5 and 3.6 kb length. That would mean that most of the viral genome is intact. If on the other hand, the schmear of dye stays on the gel after applying the dye, that would mean that the phage genome has been damaged.

Similar experiment could be done for the phage capsid integrity which has been demonstrated before (Caldeira and Peabody, 2011). Virions would be first treated with non specific nucleases to prevent any tampering of surrounding nucleic acids with electrophoresis. Then, virions would be directly put to into a gel electrophoresis along with the control virions which were not subjected to extreme conditions, and dyed with a protein-specific dye, such as Coomassie Brilliant Blue, to see if there is a difference in the formed bands between treated and control virions. If denaturation of the viral capsid occurred, and protein dissociation from the capsid took place, the band specific for virions would fade from the gel, depending on the severity of damage to the coat.

A well-established method for assessing viral stability is RT-qPCR (Rodríguez, Pepper and Gerba, 2009). First, the virion solution would be treated with RNase to remove any RNA in solution which would give a false positive. Then, RNA would be isolated from viral capsids and subjected to reverse transcription to make cDNA copies of phage ssRNA genomes using random hexamer primers. After that, qPCR would be performed on the cDNA with primers specific for MS2 Lysis protein (RefSeq Gene ID: 1260897, Table 4.1) and a specific fluorescently-labelled probe for the gene to track the replication process, as it has been used previously for MS2 phage quantification (O'Connell *et al.*, 2006; Nanassy *et al.*, 2011). In this way, the amount of undamaged phage genome sequences can be determined. Advantage of this method in comparison to PFU test is that it is highly objective with low variability of the results. Additionally, in contrast to the PFU test, RT-qPCR can be employed for non-culturable viruses. However, the disadvantage is that it usually overestimates the viral stability (Nuanualsuwan and Cliver, 2002; Rodríguez, Pepper and Gerba, 2009). This is because infectivity of a virion is not only dependent on the genome damage, but also protein damage, which cannot be accounted for in RT-qPCR tests. Hence, PFU test is better at determining the real infectivity, but since it is subjective, and prone to experimental errors, it yields higher variability of the results and lower statistical significance. It would be therefore beneficial to use it together with RT-qPCR test to assess the viral infectivity. Additionally, the differences between the two tests could be explained for each treatment if protein and RNA damage tests are also performed.

All these tests can be performed in future research to validate the results obtained in this work, and to find more about the mechanisms that cause drop in phage infectivity after each treatment.

**Table 4.1.** Sequence of the MS2 Lysis gene which could be used for RT-qPCR to determine phage stability after various treatments in this work. The replicated sequence (115 bp, weight: 70.927 kDa) is indicated in red. Tm indicates the melting temperature for the sequences.

| | Sequence | Tm (°C) |
|---|---|---|
| **Target gene (MS2 Lysis)** | 5'ATGGAAACCCGATTCCCTCAGCAATCGCAGCAAACTCC GGCATCTACTAATAGACGCCGGCCATTCAAACATGAGG ATTACCCATGTCGAAGACAACAAAGAAGTTCAACTCTT TATGTATTGATCTTCCTCGCGATCTTTCTCTCGAAATTT ACCAATCAATTGCTTCTGTCGCTACTGGAAGCGGTGAT CCGCACAGTGACGACTTTACAGCAATTGCTTACTTAA3' | 83.8 (Replicated sequence, red) |
| **Forward primer** | 5'CCTCAGCAATCGCAGCAAA3' | 64.2 |
| **Reverse primer** | 5'GGAAGATCAATACATAAAGAGTTGAACTTC3' | 63.6 |
| **Probe** | 5'FAM-TGTCTTACCCATGTCGAAGACA-NFQ3' | 64.2 |

## 4.2. MS2 stability in SM buffer

Since MS2 phages were constantly kept in the fridge, it was interesting to evaluate the loss of infectivity over time. Loss of the phage infectivity over weeks has been expected, however not to the observed extent. There was a more than 2.5 $\log_{10}$ reduction after 35 days, which is more than 99.6% reduction in infectivity. At that pace, the phage infectivity roughly halved every week spent in the fridge. There was nevertheless still a high titer of MS2 phages left and the stock would still be usable for experiments after 35 days. However, at this rate, phages would be unusable for significant experimentation after ~1 month more in the fridge.

A very interesting observation is a high consistency in reduction of phage infectivity over time. The reduction was clearly exponential since the linear model shows that $\log_{10}$ in infectivity correlates with the days in the fridge with a correlation coefficient of ~ -0.98 and highly significant $R^2$ value of 0.95 (*p* value <<0.001, Figure 3.2 and Table 3.1). This consistency in reduction suggests that all or most of the factors influencing the phage stability in fridge were held constant and since this was not a highly controlled experiment, it also suggests that there might not be many factors influencing the phage stability in SM buffer stored in fridge.

Considering there is no precise temperature control in the fridge where phages were held, changes in temperature might have added to the loss of infectivity. Since the fridge is constantly opened and closed, it would not be surprising if temperatures in the fridge fluctuated significantly. This would vastly contribute to rise in the temperature inside the fridge even though it should be around 4 °C. Other authors also showed MS2 phage instability during storage in fridge (Olson, Axler and Hicks, 2004). They observed 20% loss of infectivity after 8 days in the fridge. My results show ~50% drop every seven days. One significant difference between the study conducted here and (Olson, Axler and Hicks, 2004) is that in their research, the temperature was highly controlled and held constantly at 4 °C. This shows that variations in temperature inside the fridge might indeed be a hazardous factor for virion stability. Hence, those temperature changes could have caused the phage infectivity to drop significantly. Either way, holding phages in such conditions showed that under regular conditions in the fridge, phages are not very stable, and this pointed out the importance of preparing cryo-stocks whenever working with MS2 phages, especially for a longer period than 1-2 months.

## 4.3. MS2 interaction with lunar dust simulant

From the experiments with isolation of MS2 phages from lunar dust simulant, it was observed that virions interact with lunar dust. If virions did not interact with the simulant, approximately the same efficiency of phage isolation with and without centrifugation would be observed. However, efficiency was ~10x higher without the centrifugation, suggesting that phages adhere to the simulant particles. Extraction was still low efficiency even without centrifugation (2.48%), hinting that phages adhere to the simulant which may prevent them from infecting the host or are inactivated by the simulant in some way. The mechanism of virion interaction with lunar dust simulant as well as real lunar dust would be very interesting to research further, in detail.

It was also observed that EAC-1 lunar dust simulant, sediments from dilution in SM buffer or distilled water very fast, suggesting the hydrophobic nature of the particles. Though this could also be attributed to the effects of gravity, this is improbable since the EAC-1 simulant is very fine dust simulant with most particles being significantly smaller than 1 mm (and down to 0.02 mm). Such particles would be expected to sediment slowly. After diluting 200 mg of the fine dust simulant in 0.5 mL of SM buffer or water, clear two phases appeared after only 10-15 s (Figure 3.3). Also, at the pH 7.5 of the SM buffer, phages likely had a negative surface charge because this is higher than their pI of 3.9 (Langlet, Gaboriaud and Gantzer, 2007). Hence, it is likely that virions interacted with water droplets isolated inside the lunar dust simulant. Due to the losses during virion isolation from the lunar dust simulant, the infectivity after treatment in it was always corrected by dividing the infectivity with the isolation efficiency factor (0.0248) that was determined in this experiment.

Even though developing a virion extraction method from the lunar dust simulant was not the aim of this research, this might be an interesting topic for future experiments, and the results obtained here can help guide such experiments. There is also a possibility that low efficiency of phage isolation from the lunar dust simulant is due to lunar dust directly damaging the phages through yet-unknown mechanism. Therefore, development of better phage extraction methods from the lunar dust and its simulant would be highly interesting.

## 4.4. Thermal stability of MS2

From freezing experiments, it is evident that phages in SM buffer do not lose their infectivity after 24 h at -80 °C. This result is in line with expectation. Ultra-low temperatures like -80 °C temperature are usually used to store viruses for long term usage (Gould, 1999). In addition, numerous studies showed that phages and other viruses do not reduce their infectivity significantly when stored in deep freezer, especially not in short-term, as they were in this experiment (Halfon *et al.*, 1996; Ackermann, Tremblay and Moineau, 2004; Merrill *et al.*, 2008; Gonzalez-Menendez *et al.*, 2018). While phages did not lose infectivity after storage at -80 °C for 24 h, this time is too short to observe any differences in phage stability. Therefore, it would be interesting to repeat the experiment, but with longer periods of time like weeks, months, and years. It is expected that in such time periods, the effect of cryoprotectant would be visible. Nevertheless, this was a preliminary study to see how the extremely low temperatures would affect viral infectivity, and the aim was not to determine viral long-term stability in such conditions. In addition, those results show that if viruses are frozen inside a solution for 24 h, this should not affect their infectivity. This result is relevant because satellites like Europa and Enceladus, as well as many comets, contain frozen water on and under their surface (Bernstein, 1998). MS2 phage is a relatively simple biological entity consisting of proteins and RNA. From that point of view, it is exciting to see that such simple systems can be sustained in a saline buffer such as SM for 24 h, even without cryoprotectant. This aids the idea of panspermia.

In contrast to the SM buffer, it is evident that lunar dust simulant negatively affects phage stability at -80 °C (Figure 3.4). While there was no significant inactivation after 24 h in SM buffer, with or without cryoprotectant, in lunar dust simulant there was a significant decrease in infectivity according to the one-sided t-test with alternative hypothesis that the phage infectivity in lunar dust simulant is lower than the starting. The alternative hypothesis was accepted with *p*-value <0.001. After one day in lunar dust simulant at -80 °C, there has been 2.6 $\log_{10}$ reduction in infectivity. This may be because while the virions were in lunar dust simulant, they were incorporated in wet solid medium which may not be ideal for them to sustain infectivity. In drying experiment, it was found that MS2 virions are susceptible to drying. This means that virions are negatively affected by lack of water around them. Therefore, it would be helpful if I had a dried control also treated at -80 °C for 24 h. However, it must be noted that even in buffer, samples are frozen so there is minimal liquid water present around viruses. In addition, viruses in SM buffer did not show significant difference in loss of infectivity with or without a cryoprotectant which further points that it's not the lack of water that caused inactivation. Therefore, the inactivation of viruses in lunar dust simulant (and potentially real lunar dust) at low temperatures needs to be investigated further. In either case, those results are encouraging since in real world environment, viruses that are present on dried surfaces and fomites pose a big threat to public health (Springthorpe and Sattar, 1990; Aitken and Jeffries, 2001; Boone and Gerba, 2007). To my knowledge, this is the first study of the viral stability in lunar dust simulant in deep-freeze environment.

The results suggest that it would be hard for viral contaminations on the lunar surface to be sustained there. However, it may be a different outcome if viruses in lunar dust contaminate the surfaces of rovers or inside spacesuits where the dust can settle, and viruses can be held warm (Kawamoto and Hara, 2011).

In heat stability experiments, there was a significant reduction of infectivity at all treated temperatures: 40, 50, 60, 70 and 80 °C both after 10- and 30-min treatment. Though mean inactivation was always somewhat higher at 50 than 40 °C, the difference between the two treatments was never significant (Figure 3.5).

On the other hand, after treatment at 60 °C, phage infectivity started to drop drastically. Inactivation was significantly higher at 60 than at 40 and 50 °C in SM buffer. This is in agreement with the previous research of MS2 virion thermal stability (Caldeira and Peabody, 2011) where stability was tested by presence of viral particle band in agarose gel electrophoresis. Caldeira and Peabody observed that at temperatures higher than 50 °C, the band's visibility drops, suggesting that at those temperatures, the phage capsid starts to disintegrate (Caldeira and Peabody, 2011). Other studies suggest that the limiting temperature for MS2 phage is 72 °C (Nuanualsuwan and Cliver, 2002; Pecson, Martin and Kohn, 2009). In one of those studies, 3-min treatment at 72 °C was enough to cause 8 $\log_{10}$ reduction in infectivity (Pecson, Martin and Kohn, 2009). Considering that in this study, the starting $\log_{10}$ infectivity was ~9, it is not surprising that after 10- and 30-min treatments at 70 °C, no PFUs were detected, especially not at 80 °C.

Phages were also treated for 10 min at room temperature, 60, 70 and 80 °C in lunar dust simulant. It is known that lunar regolith has low thermal conductivity (Park *et al.*, 2018). Therefore, it is interesting to research the possibility that it can provide thermal protection for MS2 virions. The results show that at 60 °C, phage inactivation in lunar dust simulant is not different than in SM buffer. However, at 70 °C, infectivity was detected for all three replicates. The infectivity loss was ~4.5 $\log_{10}$ which is the highest observed. This contrasts with the treatment in SM buffer where at 70 °C, no infectivity was detected neither after 10- nor 30- min treatment. This suggests that the lunar dust might provide thermal protection to viruses which has not yet been discussed in literature and requires further investigation.

Major loss of infectivity after heat treatment in lunar dust also suggests that at such high temperatures, viruses would not be stable for a long time. Since there was a high reduction of phage infectivity at 60, 70 and 80 °C, this suggests that at places on the moon with such temperature ranges,

phages would probably not be stable for more than 30 min, but more experiments are required to tell exactly how long they could be sustained there. There is also a chance that it would apply also for those meteors that have a similar composition to the EAC-1 simulant. Since most asteroids have fine, granular regolith on their surface (Cheng, Yu and Baoyin, 2017), phage stability might be similar in those conditions to the lunar dust. This means that if an asteroid reaches such high temperatures during its lifetime, there is low possibility that viruses could propagate on it from Earth to other worlds. Since this idea is still under investigation (Berliner, Mochizuki and Stedman, 2018), results obtained here help to shed light on the topic.

In heat stability experiments, I also treated phages in lunar dust simulant for 10 min at room temperature. Interestingly, loss of 0.5 $\log_{10}$ infectivity was also observed, though not as high as for 24 h at -80 °C in lunar dust. This suggests that temperature plays a role in viral inactivation by lunar dust. At higher temperatures, lunar dust seemingly has a protective effect, while at lower ones acts to inactivate virions.

There is a question if viruses could persist trips to the moon or Mars. Here, I showed that a highly environmentally resistant virus – coliphage MS2, rapidly drops infectivity in lunar dust simulant at extremely low temperatures. Also, I observed that in lunar dust simulant after 10 min at room temperature, there is a moderate loss of infectivity. But at high temperatures in lunar dust, there is a high inactivation of phages – at 60 °C equivalent as in SM buffer, but at 70 °C lower than in SM buffer solution. From these experiments, it is evident that the chances of viral propagation through the solar system are low due to low stability even of one of the more resistant viruses - MS2 phage, in granular medium like lunar dust simulant. However, better-controlled tests with other simulants and more replicates are required to prove or disprove this hypothesis.

The results that viruses can survive in lunar dust simulant after treatment at 70 °C is worrying for future lunar missions. Lunar dust is already known to pose big problems in moon missions due to its magnetic properties, and very fine, sharp structure (Kawamoto and Hara, 2011). To my knowledge there has not been any experiments or discussion in literature on the topic of virus stability during heat treatment in regolith simulants. Thermal protection of viruses, and possibly other microbes, by lunar dust poses an additional problem in lunar missions because space suits are sterilized thermally. Lessons from Apollo missions teach us that lunar dust associates tightly with spacesuits (Christoffersen *et al.*, 2008). This means that it will be harder to sterilize spacesuits after each use. One seeming solution to this problem could be to constantly hold spacesuits outside the habitats (Figure 4.1) to prevent human contact. Also, longer exposure of microbes to the outside environment of space has better chances of inactivation. However, if the spacesuits are held in lunar environment, they will build up the dust inside of them much faster. Therefore, the most feasible solution would be creating a “buffer zone” between the habitat and the outside environment with regular disinfection of spacesuits with a combination of heat and some other method like UV.

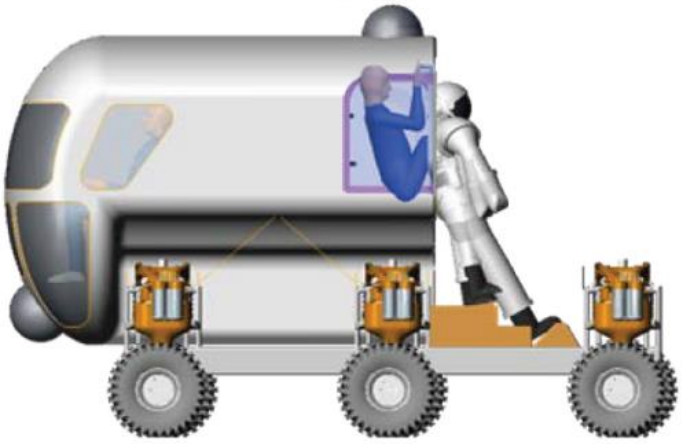

**Figure 4.1.** The model of constant holding of the spacesuits outside the habitats and rovers for constant isolation from the outside environment. This method prevents any microbial contaminations from the surface of spacesuit to come into contact with astronauts. Source: http://ntrs.nasa.gov/archive/nasa/casi.ntrs.nasa.gov/20080014281_2008013625.pdf. Credits: NASA.

## 4.5. MS2 infectivity after drying at air- and near-vacuum pressure

In this study the MS2 virions were dried in low pressure and in air. MS2 showed low resistance to drying in general, independently of the method. Both dryings had significant reduction in infectivity ($p < 0.001$ for air drying and $p$-value $< 0.01$ for vacuum) according to one-sided t-tests. The alternative hypothesis for air drying was that starting infectivity was greater than final infectivity. The alternative hypothesis for vacuum drying was that final infectivity was lower than starting. Both alternative hypotheses were accepted.

With air drying, there has been 1.1 $\log_{10}$ reduction in infectivity and with vacuum drying 0.6 $\log_{10}$ reduction. Drying experiments of other phages show that, depending on the phage species, loss of infectivity can go from 0.4 up to 2.5 $\log_{10}$ after drying (Matinkhoo *et al.*, 2011; Vandenheuvel *et al.*, 2013; Leung *et al.*, 2016; Chang *et al.*, 2017). This implies that phages usually do not sustain drying very well. To my knowledge, there is no available data on drying stability of MS2 phages. However, compared to other phages in the literature and based on the results obtained here, MS2 phage seems to be among more resistant viruses to drying.

Inactivation was higher during air drying, implying that MS2 virions possess at least some level of resistance to near-vacuum pressure. However, this was inferred with low statistical significance ($p < 0.05$), so this experiment needs to be repeated with a higher number of replicates to challenge the obtained results. Also, it would be beneficial if I had a "g-force control" that would be air-dried in a centrifuge since during vacuum drying, phages were exposed to unusually high g-forces, but not so in air drying. Another difference between the methods is that air drying lasted for 18 h (overnight), and vacuum drying only 3 h. This difference is due to the time needed for evaporation of the surrounding buffer in each method. Both drying methods were performed at room temperature. Vacuum drying results in dry viruses much faster since the buffer evaporates rapidly. At atmospheric pressure, however, a droplet of buffer needs much longer time to evaporate. There is a possibility that the higher inactivation of viruses after air drying is because air-dried samples were dry for a longer period. However, this explanation is unlikely since phages are usually dried for long-term preservation

(Manohar and Ramesh, 2019). Therefore, the difference between the time periods that phages spent dry is too small to cause the difference in inactivation efficiency between the two drying methods.

Unfortunately, the effects of extremely high pressure on virus stability has not been researched in this work. However, a lot of research has already been conducted in that domain since this is an important virus inactivation method in food industry and vaccine development (Oliveira *et al.*, 1999; Ishimaru, Sá-Carvalho and Silva, 2004; Silva *et al.*, 2004; Araud *et al.*, 2015). Still, it presents an interesting opportunity for future research for application in astrobiology.

From these results, it is visible that phages might be resistant to near-vacuum pressure. If protected from other influences from the space environment, phages would suffer the loss of infectivity due to drying but would probably survive low pressures while dry. However, in this study, I checked their stability in vacuum after only 3 h, and it would be very interesting to perform additional experiments lasting for days, months or years, as this would be relevant for space travel. The equipment necessary for such tests would be complicated and expensive since vacuum must be held for long periods of time. It is important in future research to test thermal stability of microbes combined with vacuum because currently, moon and Mars are primary targets for future human missions. Moon has no atmosphere, and the Martian atmosphere is extremely thin (discussed in section 1.1.2.). This means that if astronaut's equipment or planetary surface gets contaminated, it would be valuable to know how long microbes could be sustained there.

## 4.6. MS2 X-ray stability

The phage inactivation curve has been drawn according to the logarithmic model in relation to X-ray dose (Figure 3.9). The highest irradiation dose resulted in 1.5 $\log_{10}$ reduction. The dose where 50% inactivation is observed, LD50, is 120 Gy. Some other authors found even higher radiation doses for inactivation of bacteriophages. For example, in one study, to inactivate 63% of phages HP1c1, the dose of 90 krad (900 Gy) of X-rays was necessary (Boling and Randolph, 1977). Potentially due to their small size, MS2 phages can sustain high intensities of X-rays. This hypothesis states that X-ray needs to hit a viral particle to inactivate it, and since there are a lot of small particles in solution, there are low chances of inactivating every one. The diameter of MS2 phage virion is 27-28 nm (Dika *et al.*, 2011) which is smaller than virions of many other species, generally ranging from 20 to 200 μm (Louten, 2016). It must be noted however that phages were irradiated in a small volume of SM buffer (50 μL) which contained 2% (w/v) gelatin. Gelatin and water can absorb some ionizing radiation, protecting the phages, but to low extent (Polatnick and Bachrach, 1968). In addition, the low volume of solution and high phage concentration counteracted the protective effects of water and gelatin. Nevertheless, it would be interesting to make an experiment to test the X-ray stability of phages in buffer with and without gelatin, as well as dry phages.

It is clear from the logarithmic fit that the drop of phage infectivity is exponential. Exponential inactivation of phages is not surprising, considering MS2 is an ssRNA virus with a simple protein envelope. Both proteins and RNA can take direct damage from X-rays (Bury *et al.*, 2016). Having a single stranded genome, every hit of X-rays that breaks an RNA strand, cuts the genome in two, rendering it inactive and irreparable. Virions do not have a genome-repair mechanism, making them susceptible to ionizing radiation such as X-rays. In addition, since the phage capsid depends on a high organization of proteins making it up, damage to those proteins may destabilize the capsid. This would in turn compromise the phage entry into the cell because it is hypothesized that MS2 relies on the stability of its capsid to insert its genome to the bacterial cell (Tars, 2020). Therefore, one-hit hypothesis of phage inactivation by X-rays is sensible to assume and is consistent with the results. If there was more than one hit necessary to inactivate a viral particle, a sigmoidal curve would be a better fit to the

data. However, a rapid decrease in phage survival is evident from the start with asymptomatic approach to 100% inactivation with exponential increase in radiation dose (Figure 3.9), suggesting one-hit inactivation mechanism according to target theory (Zhao *et al.*, 2015). In addition to the direct damage of the molecules, ionizing radiation can cause ionization of water which in turn can damage biological macromolecules, so phages are also susceptible to that effect. However, with exponential increase in the number of inactivated virus particles, the chances of inactivating a new particle decrease at the same pace.

Remarkably, phage infectivity was still detectable after irradiation with 2 kGy of X-rays. As discussed above, this is mostly attributed to their small size, and to some extent the protective effect of the surrounding solution. Nevertheless, the results suggest that phages might be able to sustain high radiation intensities related to space travel.

There is a lack of new studies of X-rays on viruses, and specifically, phage stability. This may be due to hazardous health effects of X-rays and lack of interest in using them for virus decontamination. Such studies are interesting in terms of space travel since viruses might pose a great threat for astronauts in future space missions.

## 4.7. Transmissibility of MS2 by aerosols and air-suspended droplets

In this work, it has been shown that phage MS2 can be transmitted by aerosols and air-suspended droplets (Figure 3.10). In addition, I tested if its transmissibility in air depends on the presence of UVC radiation or the temperature of the solution, they are suspended in. The results show that phages are transmissible in room temperature buffer, but not in the cold buffer, independently of the presence of UVC radiation – at least for up to 35 min in air.

The study conducted here was a preliminary study to assess MS2 phage transmissibility in air. Since the study was only preliminary, the interpretation of the results must be cautious. For instance, the spray density, average droplet velocity and size were not evaluated in this study, hindering the reproducibility of the results. Also, with the method used here for collection of viral aerosols, it is not possible to quantitatively determine the infectivity of virions after dispersion in air because the difference in determined number of PFUs is highly susceptible to chance. Therefore, it is only possible to qualitatively ascertain if phages are transmissible or not by success/failure in detection of PFUs, which has been determined here. Also, the intensity of the UVC lamp used here was not determined, which is an additional problem in terms of reproducibility.

Nevertheless, these results show that phages can be propagated in air-suspended droplets, making them a potential surrogate for testing respiratory virus spread. Other studies have also confirmed that phages can be transmitted by air-droplets and aerosols though they are inactivated to some extent by being air-suspended (Trouwborst and De Jong, 1973; Eninger *et al.*, 2009; Zuo *et al.*, 2014). In this experiment, only phage transmissibility in the air, and not stability, was tested. Also, even though the exact UVC intensity is not known, the results still show an interesting potential that virions might be able to sustain UV radiation while in droplets suspended in space - like in droplets from water plumes on Europa. Therefore, this study paves the way for future, better controlled research of viral stability in solution droplets in extreme conditions.

Some other authors came to the conclusion that MS2 is strongly inactivated by various wavelengths of UV radiation from 210-290 nm while suspended in water solution (Havelaar *et al.*, 1990; Misstear and Gill, 2012; Beck *et al.*, 2016). On the other hand, tests of phage surface- and air-inactivation with UV show that they are better inactivated at lower relative humidity (Tseng and Li, 2005, 2007), hinting that air droplets might offer phages protection from UV. Though in this work phage UV stability was

not tested, this hypothesis could explain the detection of PFUs even after 35-min UVC radiation as determined by experiments here but needs to be further investigated. In addition, there is evidence that MS2 is more resistant to UV when aerosolized than many other viruses (Walker and Ko, 2007; Verreault *et al.*, 2015). Phage sensitivity to UV as a function of relative humidity is in agreement with some animal respiratory viruses - like vaccinia, influenza, or poxviruses - which are more susceptible to UV inactivation at lower relative humidity (McDevitt *et al.*, 2007, 2008; McDevitt, Rudnick and Radonovich, 2012). The exact levels of UVC that MS2 phages can sustain while suspended in air-droplets and aerosols, and on what factors their sensitivity depends (temperature, pressure, droplet size…), remains to be tested. Such research is important to question the suitability of MS2, and other phages, as surrogates for air-inactivation of respiratory viruses by UV radiation.

The inability to detect MS2 PFUs in cold buffer is extremely interesting (Table 3.7), especially because, to my knowledge, there is no research of MS2 stability in low-temperature air-droplets and aerosols. However, it must be noted that for cold buffer, PFUs were not detected in positive control. This means that there might have been a problem with the spraying of phages. The spraying cone was clear and present, showing that the spraying mechanism worked fine. The spraying cans were sterilized with ethanol and washed thoroughly with sterile water afterwards. There is a possibility that some sterile water stayed in the spraying tube and was sprayed instead of viruses. While possible, this is also less likely explanation since the positive control was sprayed after the experiments, meaning that even if some water stayed inside the tube, it would be sprayed out quickly so phages would be sprayed afterwards. Another possibility is that some ethanol stayed in the tube, inactivating the viruses, but this is extremely unlikely since 70% (w/v) ethanol used for sterilization would be highly diluted after washing with water and adding the virus-containing buffer in the spraying can. Another possibility is that viruses in a cold buffer redistribute less efficiently, so only a very small amount of viruses was sprayed, making them undetectable. They would not be detectable because even in the positive control, the whole spray cone was not collected since in previous tests with room temperature buffer, phages were distributed across the whole spraying cone (Figure 3.10, Positive control). In either case, this experiment must be repeated for cold (near 0 °C) buffer, especially because of the lack of evidence for stability of viruses in cold air-suspended solution.

There is strong evidence that temperature affects virus stability and transmissibility in air suspension for human respiratory viruses, like influenza, respiratory syncytial virus and SARS-CoV-2 (Lowen *et al.*, 2008; Yang and Marr, 2012; Paynter, 2015; Dabisch *et al.*, 2020). Data from different experiments is consistent: higher ambient temperatures negatively affect viral stability. This also stands for bacteriophages, like MS2, Φ6, PR772 and Φ174 (Verreault *et al.*, 2015). In those studies, ambient temperature is reported, and no study researched viral stability at low temperatures, so this data is lacking. In future experiments, it would be of interest to research viral stability in aerosols in extremely low and high temperatures to assess the possibility of their stability in space conditions. Considering high resistance of some viruses like MS2 to humidity and UV while aerosolized, it would not be surprising if they could propagate in such conditions. Future research in this topic is needed and seems very interesting.

# 5. Conclusion

Conditions of deep space are hard to sustain for viruses, even for one of the most resistant known viruses like MS2. The physical conditions explored here all inactivated MS2, except for aerosolization, whose viral inactivation capability was not explored here but only viral transmissibility.

Even though in this work not all the physical conditions of deep space were explored (e.g., microgravity and fast charged particles), viruses showed relatively low tolerance to the conditions tested here with lowest observed reduction in infectivity of 0.6 $\log_{10}$ (in near-vacuum treatment), and highest >4 $\log_{10}$ reduction (in heat treatment).

The highest inactivation was observed with heat treatment at >60 °C where viruses were not detectable after treatment, except in the lunar dust simulant. This suggests that lunar dust may offer thermal protection for viruses and this possibility needs to be explored further.

MS2 showed some degree of resistance to 3 h-drying in near vacuum pressure. However, longer treatments are needed to certainly assess phage stability in vacuum during space travel. Nevertheless, the results show that, even if phages are inactivated by low pressure, some virions might sustain the vacuum of space.

MS2 virions showed remarkable stability under X-ray radiation since their infectivity was still detectable even after radiation with 2 000 Gy. However, they showed rapid inactivation with exponential increase in X-ray dose. The observed resistance is probably due to their extremely small size, which means that there is a possibility that low concentrations of viruses might be able to sustain high ionizing radiation doses of deep space, especially in the outer solar system where radiation density is lower.

Phage MS2 also showed to be transmissible while suspended in droplets and aerosols, with and without UV light but not in cold solution. Such droplets might exist on Europa and Enceladus, but are also generated when astronauts breathe, talk, sneeze or cough. Therefore, MS2 is a good surrogate to test viruses that can spread by aerosols in space missions.

This research introduced viral research in space conditions and showed that MS2 could be used as a surrogate for viruses in future space virology research. The suitability of this surrogate for space experiments, however, has yet to be confirmed. Nevertheless, this work settled the base for such research.

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# 8. Curriculum vitae

Bruno Pavletić earned his BS in Molecular Biology in 2018. In his Bachelor Thesis, he explored the evolution of alternative RNA splicing. Since 2015, he was doing practice on Ruđer Bošković Institute, RBI, Zagreb in the Laboratory for Evolutionary Genetics, performing bioinformatics research. In 2018, he started working on detection of grapevine viruses and a method for curing viruses from the grapevine. He received the Rector's award for that research. Due to his interest in aerospace, in 2018, he also started working on the project for one of the first Croatian satellites - FERSAT. He gained a lot of group-work experience there and learned a lot about orbital dynamics, Earth observation, teamwork, and project management. In 2019, he started an Astrobiology group at his university to spread his interest in the field with his colleagues. In 2020, he did an internship at German Aerospace Center, DLR, Cologne in Space Microbiology Research Group. He researched viral stability in aerospace-related environments and based his Master Thesis on that research. Also, he participated in Mars Society million-people settlement design, where together with his team, he got to the finals, and their work will be published in 2021. Currently, he is using his experience in Earth-observation and coding skills to develop machine learning-based model for prediction and tracking of COVID-19 spread based on satellite data to help policymakers predict viral spread in real time, all around the world. In addition, with a group of exceptional teammates, he is working on a space ISRU startup – Darkoob space. The group's first product is LEXICON – a 3D-printable system for economic and safe ISRU on moon and Mars. With that innovation, they won the Asclepios Hackathon in 2020 and their prototype is set for testing in an analog mission in late 2021.

Memberships: European Association for Cancer Research (EACR), Space Generation Advisory Council (SGAC)