FACULDADE DE ENGENHARIA DA UNIVERSIDADE DO PORTO

INSTITUTO CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

Sustainable Space Habitats: Cyanobacterial Biomass as Feedstock for Fungal Biotechnology

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September, 2025

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MSc in Bioengineering - Molecular Bioengineering

8th September 2025

Resumo

O próximo passo na jornada da Humanidade pelo cosmos é o estabelecimento de bases em órbita e na superfície da Lua e, eventualmente, em Marte. No entanto, a distância acarreta uma necessidade urgente de independência dos recursos da Terra. A presença de humanos a longo termo nestes postos extraterrestres só será possível com uma produção e reciclagem autónoma da maioria dos recursos essenciais para a sobrevivência – comida, água, oxigénio, materiais de construção, medicamentos, entre outros. As tecnologias desenhadas para esta finalidade são conhecidas como Sistemas de Suporte de Vida (SSV) e, quando processos biológicos são empregues, são designados de Sistemas de Suporte de Vida Biorregenerativos (SSVB).

A investigação em SSVB tem-se focado extensivamente em plantas e cianobactérias, visto que ambas podem ser transportadas a massas iniciais reduzidas, diminuindo assim os custos associados. Para além disso, apresentam elevada versatilidade de aplicações, como a produção de oxigénio, fixação de dióxido de carbono, produção de comida, reciclagem de resíduos, entre outros. Pelo contrário, os fungos filamentosos têm sido relativamente negligenciados neste domínio. No contexto de exploração espacial, os fungos têm sido estudados para aplicações como proteção contra radiação, produção de biomateriais, síntese de metabolitos secundários de interesse (e.g. ácido cítrico) e até mesmo como fontes alimentares. No entanto, a sua integração direta em SSVB continua amplamente inexplorada.

O objetivo desta dissertação é, portanto, colmatar esta lacuna, ao estudar a integração de *Aspergillus niger* num SSVB, utilizando recursos disponíveis *in situ*, como rególito Lunar ou Marciano e biomassa de cianobactérias. A hipótese central é que *A. niger* consegue utilizar biomassa de cianobactérias, suplementada com minerais disponíveis nos simulantes de rególito, como fonte de nutrientes para sustentar o seu crescimento e produzir metabolitos de valor biotecnológico. Para testar isto, quatro estirpes de *Anabaena sp*. – PCC 7120, PCC 7938, LEGE X-002 e LEGE 00250 – foram testadas como substratos orgânicos para o crescimento deste fungo, diluídas numa solução de simulante de rególito Lunar ou Marciano. A sua biomassa foi usada sem tratamento ou após lise mecânica através de um protocolo simples e facilmente automatizado. O crescimento fúngico foi quantificado através de medições da biomassa seca após 7 dias de crescimento. A produção de ácido cítrico foi monitorizada através

de medições de pH do meio de cultura e Cromatografia Líquida acoplada à Espectrometria de Massa.

Os resultados demonstraram que *A. niger* não apresentou crescimento no meio com biomassa de cianobactérias não tratada, mas a biomassa lisada suportou o seu crescimento. Entre as concentrações testadas (0.5–2 g/L), 1 g/L de biomassa lisada foi identificada como a condição ótima, dado que os rendimentos de biomassa fúngica em 1 g/L e 2 g/L não foram estatisticamente diferentes. Para além disso, a utilização de 1 g/L reduz a quantidade de biomassa de cianobactéria necessária para o crescimento de fungos, maximizando a eficiência de utilização de recursos. Das quatro estirpes de *Anabaena*, PCC 7938 emergiu como o substrato mais eficiente nas condições testadas. Assim, esta estirpe foi selecionada para experiências subsequentes do crescimento de *A. niger* em microgravidade simulada. Os resultados indicam que a microgravidade não afeta significativamente o crescimento de *A. niger* em cultura líquida, e que PCC 7938 diluída numa solução preparada a partir de simulantes de rególito Marciano ou Lunar consegue suportar cerca de 30% do rendimento de biomassa do controlo positivo. A produção de ácido cítrico foi confirmada em culturas de *A. niger* com lisados de PCC 7938 diluído na solução de simulante de rególito Marciano.

Estes resultados evidenciam o potencial de crescimento de *A. niger* em condições relevantes para o espaço, utilizando recursos disponíveis *in situ.* O trabalho fornece, assim, um *proof-of-concept* para a viabilidade da integração de *A. niger* em futuros SSVB, promovendo uma exploração espacial sustentável.

Abstract

The next step on the journey of Humanity throughout the cosmos is the establishment of settlements in orbit and on the surface of the Moon, and, eventually, on Mars. Yet, this distance brings an increasingly urgent need of independence from Earth resources. Long-term human presence in such extraterrestrial outposts will only be feasible with an autonomous production and recycling of most of the essential resources for survival – food, water, oxygen, construction materials, medicine, among others. Technologies designed for this purpose are known as Life Support Systems (LSS) and, when biological processes are employed, they are named Bioregenerative Life Support Systems (BLSS).

Research in BLSS has extensively focused on plants and cyanobacteria, as both can be transported at initial low masses, thus reducing associated costs. Moreover, they present a high versatility of applications, such as oxygen generation, carbon dioxide fixation, food production, waste recycling, among others. In contrast, filamentous fungi remain comparatively overlooked. In the context of space exploration, fungi have been studied for applications such as radiation shielding, biomaterial production, synthesis of secondary metabolites of interest (e.g. citric acid), and even as food sources. However, their direct integration into a BLSS remains largely unexplored.

The aim of this dissertation is, therefore, to address this gap, by studying the integration of *Aspergillus niger* into a BLSS, using *in situ*-available resources, such as Lunar or Martian regolith and cyanobacterial biomass. The central hypothesis is that *A. niger* can use cyanobacterial biomass, supplemented with minerals available in regolith simulants, as a nutrient source to sustain its growth and produce metabolites of biotechnological value. To test this, four strains of *Anabaena sp*. – PCC 7120, PCC 7938, LEGE X-002 and LEGE 00250 – were tested as organic substrates for fungal growth, diluted in a solution of Lunar or Martian regolith simulants. Their biomass was used either untreated or mechanically lysed through a simple and easily automated protocol. Fungal growth was quantified through dry biomass measurements after 7 days of growth. Production of citric acid was further monitored through pH measurements of the culture medium and Liquid Chromatography–tandem Mass Spectrometry.

Results show that *A. niger* was unable to thrive on media with untreated cyanobacterial biomass, but lysed biomass successfully supported growth. Among tested concentrations (0.5–2 g/L), 1 g/L of lysed biomass was identified as optimal, since fungal biomass yields at 1 g/L

and 2 g/L were not statistically different. Using 1 g/L, in turn, reduces the amount of cyanobacterial biomass needed for fungal growth, maximizing resource efficiency. Of the four *Anabaena* strains, PCC 7938 emerged as the most effective substrate under the tested conditions. As such, this strain was selected for further experiments of *A. niger* growth in simulated microgravity. Results indicate that microgravity does not significantly affect *A. niger* growth in liquid culture, and PCC 7938 diluted in solutions of Martian or Lunar regolith simulants can support approximately 30% of the biomass yield of the positive control. Citric acid production was confirmed in *A. niger* culture with PCC 7938 lysates diluted in the solution of Martian regolith simulant.

These results highlight the potential for *A. niger* to grow under space-relevant conditions, using resources available *in situ*. This work provides, thus, a proof-of-concept for the feasibility of *A. niger* integration into future BLSS, promoting sustainable space exploration.

UN Sustainability Development Goals

The United Nations (UN) Sustainable Development Goals (SDGs) offer a universal framework to guide global efforts toward a more sustainable and equitable future. Adopted in 2015 by all UN member states, the SDGs comprise 17 interconnected goals that address urgent global challenges such as poverty, climate change, peace and justice.

This project particularly aligns with one of these goals, by presenting a proof-of-concept sustainable life-support system, applicable both in space and Earth-based contexts, mainly in areas of limited resource access. The specific SDG supported by this work, as mentioned in **Table i**, is SDG 12: "Ensure sustainable consumption and production patterns".

Table i - Identification of SDG and work contribution.

SGD	Target	Contribution	Performance Indicators and Metrics
12	**12.2**: By 2030, achieve the sustainable management and efficient use of natural resources.	Proof-of-concept integration of cyanobacteria and fungi in a sustainable closed-loop system for space exploration, capable of producing and recycling resources *in situ*, thus diminishing reliance on transported resources, which would contribute to overall polluting emissions.	Successful fungal growth using cyanobacterial biomass and regolith (~30% of control), which could be used for biocomposite production, radiation shielding, citric acid production, among others.
	12.5: By 2030, substantially reduce waste generation through prevention, reduction, recycling and reuse.		

Agradecimentos

Lembro-me de um dia, no secundário, estar a ver vídeos sobre astrobiologia. Lembro-me de encontrar uma TED Talk chamada "Astrobiology: The Search for Life Beyond Earth", apresentada por uma cientista portuguesa. Lembro-me de pensar o quão incrível deveria ser, um dia, poder aprender com alguém como ela.

Anos passados e aqui estou, a começar os agradecimentos da minha Dissertação de Mestrado por agradecer a essa mesma cientista – Dra. Marta Cortesão. Estas primeiras palavras não podiam de deixar de ser para ela, que incansavelmente me orientou, apoiou e fez com que todo este trabalho fosse possível. A ela, que partilha o mesmo fascínio pelo espaço, o mesmo brilho no olhar – não podia ter pedido outra pessoa para me acompanhar ao longo destes meses.

Estas palavras estendem-se à Dra. Catarina Magalhães, que, desde o primeiro momento, há quase dois anos, me apoiou e orientou incondicionalmente no meu desejo de fazer investigação em astrobiologia. Por ter confiado que seria capaz de aprender e crescer. Sem esse voto, não poderia nunca ter chegado aqui.

Ao Space Microbes Lab (e membros honorários, Inês e Alex), por todo o apoio nestes últimos meses; por tudo o que me ensinaram; pelos almoços em arquitetura e por todos os desenhos no quadro de giz.

To Dr. Daniela Billi; to Gio, Costi, Andrea, Anto and Lo. For truly showing me what a team is like; for making me feel at home, feel like I truly belonged; for making the days go by as fast as those *pizzette rosse* disappeared in the mornings; for teaching me wholeheartedly – lab-wise and napolitano-wise. *Dal profondo del mio cuore*, *no no, grazie a te*.

À minha família; Mou, Mabel, Catita, Nês, Nené, primos, Guidinha, vô Manel, tios, pais. No fundo, ao Copacabana. Pelo assado de domingo, pela chave da sala, e por cada vez que não se fez silêncio para o avô dormir; pela mesa de 4 pessoas onde cabem sempre 40 (ou mais); por me terem visto crescer e terem deixado a minha cabeça na Lua.

Aos meus amigos. À Pepa, e aos retiros bianuais; ao berbequim e aos filmes búlgaros a preto e branco; aos almoços na creche e às francesinhas sem batatas fritas; por terem estado ao meu lado todos estes anos. Às shades + ponchi, e as manhãs na sala de estudo; aos almoços na churrasqueira, à hipertensão, às vírgulas em falta; por serem os que me acolheram. À Bea, por continuar a ser o Evan no 4º mundo paralelo. A M&B; aos Tubbies e à Debas; ao Pergaminho da Verdade, a Madalena e d'Arrasto; por me ensinarem o que significa "segredos desta cidade levo comigo pra vida".

E, por fim, as minhas últimas palavras não podiam deixar de ser para o Afonso. Ele, que esteve comigo desde que li Carl Sagan pela primeira vez; que me ouviu dizer "quero ser astrobióloga" e nunca, nem um só segundo, duvidou que isso seria possível; que me viu entrar neste curso, e agora me vê sair. A prova que o entrelaçamento quântico é real. Em todas as linhas temporais, em todas as possibilidades, és tu.

Obrigada,

Isabel

“The cosmos is within us. We are made of star-stuff.
We are a way for the universe to know itself.”

— Carl Sagan

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Abbreviations

BLSS	Bioregenerative Life Support System(s)
CID	Collision-Induced Dissociation
COSPAR	Committee on Space Research
dH_2O	Deionized sterile water
DLR	German Aerospace Center
ESA	European Space Agency
FEP	Fluorinated Ethylene Propylene
G	Gravitational Constant
ISRU	*In Situ* Resource Utilization
ISS	International Space Station
lWRM	Lunar Water-Released Minerals
LC-MS/MS	Liquid Chromatography–tandem Mass Spectrometry
LEO	Low Earth Orbit
LSS	Life Support Systems
MCC	Mission Control Centre
MM	Minimal Media
mWRM	Martian Water-Released Minerals
NASA	National Aeronautics and Space Administration
NC	Negative Control
NS	Not Significant
NT	No Treatment
PC	Positive Control
PCC	Pasteur Culture Collection
RT	Room Temperature
WRM	Water-Released Minerals
µG	Microgravity

Chapter 1

Introduction

1.1. Context

Where is Humanity going?

Certainly, to the stars.

Thus, the fundamental question should not be “where,” but rather “how.” How do we carry life across the void? How do we build not just vessels, but worlds?

The International Space Exploration Coordination Group, a colligation of 28 space agencies around the globe, including the Portuguese Space Agency, has outlined the main objectives for the future of space exploration as the establishment of outposts on the Moon and on Mars until 2050 (ISECG, 2024).

The first steps toward this long-term vision are already underway. The National Aeronautics and Space Administration (NASA) has designed the Artemis Program, in collaboration with international partners such as the Canadian Space Agency, the European Space Agency (ESA), and the Japan Aerospace Exploration Agency. Artemis seeks to establish a sustainable and permanent human presence on the Moon, as a testbed for future missions to Mars and for the unprecedented reality of continuous deep-space scientific research (Creech *et al*., 2022; Smith *et al*., 2020).

A central element of this endeavor is the Lunar Gateway (**Figure 1**), a modular space station designed to orbit around the Moon. The Gateway will function as a hub for surface expeditions, a platform for scientific research and a demonstration site for new technologies essential for long-term exploration. Already under development, the first two modules of the Gateway are scheduled for launch in 2027, with full assembly around the Moon expected to follow in subsequent years (Crusan *et al*., 2019; Fuller *et al*., 2022).

The continuous human presence in space since the year 2000 aboard the International Space Station (ISS) might suggest that present-day technology is sufficient to sustain Lunar – and eventually Martian – outposts. However, several critical technological and logistical obstacles remain unsolved. The ISS is located approximately 400 km from Earth, a distance that allows for regular resupply of essential consumables, including food, medicine, fuel and

repair materials (Lynch *et al*., 2024). The Moon, however, lies 384,000 km away, and Mars is at a distance of more than 140 million km. These vast distances impose clear constraints, including extended travel times, communication delays and significant economic burdens.

Thus, to establish long-term human settlements on the Moon or Mars, systems that reduce dependence on Earth must be developed. Future astronauts will need to rely on technologies capable of guaranteeing the local production and recycling of essential consumables – such as oxygen, water, food and medicine – while simultaneously addressing waste management, radiation protection among other environmental hazards. The future of space exploration depends not merely on rockets, but on Bioregenerative Life Support Systems (BLSS) – closed, resilient ecosystems that allow for life to be carried and flourish across the Solar System (H. Liu *et al*., 2021; Verseux *et al*., 2022).

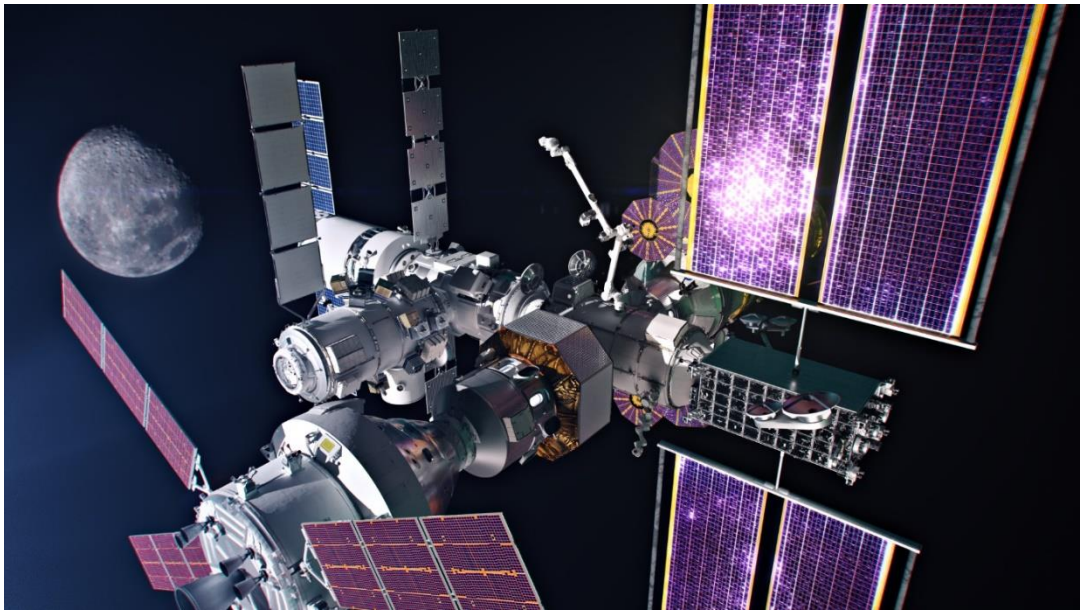

Figure 1. Artist's concept of the Lunar Gateway, orbiting the Moon. Credits: NASA, Johnson Space Center; Alberto Bertolin, Bradley Reynolds.

1.2. Motivation and Objective

The establishment of sustainable human habitats on the Moon and on Mars will require robust Bioregenerative Life Support Systems (BLSS) capable of recycling waste, producing essential resources, and minimizing dependence on resupply from Earth.

Cyanobacteria and plants have been extensively studied for their roles in such systems (De Micco *et al*., 2023; H. Liu *et al*., 2021). Filamentous fungi have a great potential for BLSS, including for radiation protection (Cordero, 2017; Koch *et al*., 2023), biocomposite production (Alaneme *et al*., 2023), synthesis of bioactive molecules (R. Yu *et al*., 2021), among others (Kaur & Verma, 2020; Vivek & Venkitasamy, 2023). Despite this remarkable versatility, fungi incorporation into BLSS remains limited.

Thus, the central objective of this Master Dissertation is to perform a proof-of-concept

study showcasing that filamentous fungi can be integrated into a BLSS, to support future Lunar and Martian outposts. The working hypothesis is that *Aspergillus niger* can be efficiently incorporated into BLSS technologies by relying on resources likely available *in situ*. To test this, the study pursues three main goals:

1. Demonstrate the feasibility of *In Situ* Resource Utilization (ISRU): test whether resources available in Lunar and Martian habitats (namely Lunar/Martian regolith, and cyanobacterial biomass from previously established BLSS) can sustain the growth of the filamentous fungi *A. niger*;
2. Assess *A. niger* growth in media formulated with substrates relevant for space exploration under simulated microgravity conditions;
3. Explore functional integration in BLSS: establish how *A. niger* can contribute to key BLSS functions, namely through the production of molecules of biotechnological interest.

By addressing these objectives, this work seeks to elevate fungi from overlooked organisms to key players in the architecture of future space habitats. In doing so, it contributes not only to the scientific foundation of upcoming space missions – such as Artemis – but also to the broader vision of humanity's next great adventure: carrying life into the cosmos.

1.3. Document Outline

The remainder of this work is structured as follows: Chapter 2 provides the background and state-of-the-art on space exploration and Bioregenerative Life Support Systems (BLSS), with a particular focus on the applications of cyanobacteria and fungi. Chapter 3 describes the methods employed throughout this work. Chapter 4 provides a comprehensive analysis and discussion of the results, in the light of current literature. Finally, Chapter 5 concludes the dissertation by summarizing the key findings and outlining future research directions related to the concepts here explored.

1.4. Outputs

The results and relevance of this work were shared with both the scientific community and the general public through the following events:

- Poster presentation at the *Encontro de Investigação Jovem da Universidade do Porto* (IJUP), Porto, Portugal (May 2025);
- Oral presentation for science communication and dissemination at *Fantasy Basel*, Basel, Switzerland (May 2025);
- Oral presentation at the *Blue Think Conference*, Porto, Portugal (September 2025);
- Oral presentation in the Space Factor contest of the *European Astrobiology Network Association (EANA) Conference*, Lisbon, Portugal (October 2025).

Chapter 2

Literature Review

2.1. Current Challenges of Space Exploration

Fifty-six years have passed since humanity first landed on the Moon; fifty-three have gone by since the last crewed mission left its surface. While space technologies have advanced significantly since the Apollo program, human exploration beyond Low Earth Orbit (LEO) remains hindered by a complex interplay of technical, economic, and physiological challenges.

The Apollo missions were never intended to establish a sustainable human presence on the Moon. Modern efforts, however, focus on long-term habitation, which requires a vastly different approach. Achieving this goal, both for future Lunar and Martian bases, involves overcoming a broad spectrum of interrelated obstacles.

2.1.1. Physiological Hazards of Space

Microgravity, often termed “weightlessness,” occurs when the gravitational forces are significantly diminished, due to a state of continuous free fall. This is the condition experienced by astronauts aboard the ISS, and will also characterize future missions to the Moon and Mars.

Human physiology has evolved under Earth's constant gravitational force. Consequently, even minor alterations to normal gravity can induce systemic dysfunction (**Figure 2**). In the absence of gravity pulling blood downwards, bodily fluids shift toward the upper body (Thornton & Hoffler, 1977), leading to facial puffiness, nasal congestion and positional displacement of the brain within the skull (Ong *et al*., 2023). These changes can cause Spaceflight-Associated Neuro-Ocular Syndrome, a condition reported in over 60% of long-duration spaceflight crew members, featuring optic disc edema, choroidal folds and hyperopic refractive shifts (Aleci & Dutto, 2025; Sibony *et al*., 2023).

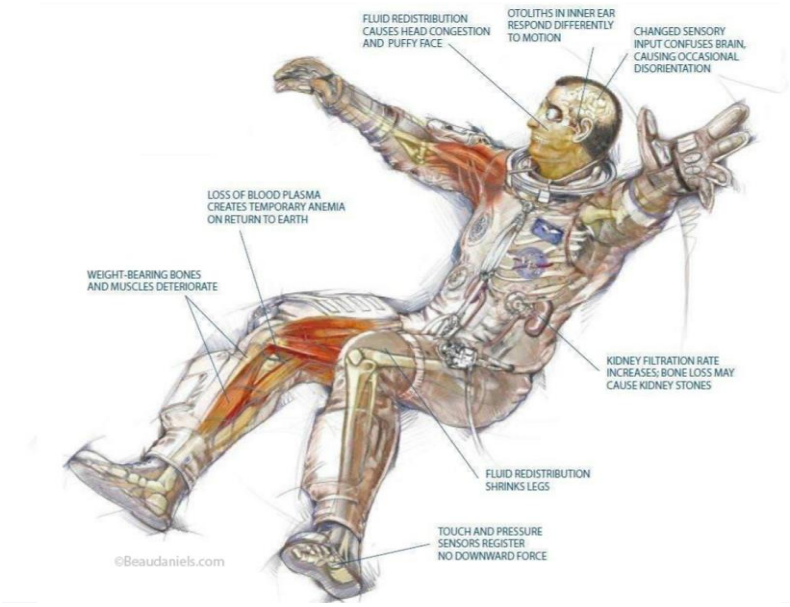

Figure 2. Effects of spaceflight on the human body. Credits: Beau and Alan Daniels.

Another well-documented physiological consequence of prolonged exposure to microgravity is the accelerated degradation of the musculoskeletal system, namely skeletal muscle atrophy and reduction in bone mineral density (Stavnichuk *et al*., 2020). These losses are primarily due to the absence of mechanical loading and gravitational stimuli, which are normally generated during activities such as walking (Klein-Nulend *et al*., 2003).

Radiation exposure is another major concern. Beyond the protective influence of the Earth's magnetosphere, astronauts are exposed to a combination of galactic cosmic rays and sporadic solar particle events. These exposures contribute to DNA damage (Moreno-Villanueva *et al*., 2017), increased lifetime cancer risk (Guo *et al*., 2022), immune dysregulation (Marchal *et al*., 2024) and potential cardiovascular diseases (Mircea *et al*., 2024). While on Earth humans are exposed to 2.4 mSv a year, on the ISS, which is shielded from part of the harmful space radiation by the Earth's magnetosphere, this value increases to 0.5 mSv a day. On a voyage to Mars, the radiation dose is estimated to increase to 1 mSv a day. Although the legal limits of radiation exposure for astronauts differ across space agencies, NASA astronauts would likely be unable to safely travel to Mars. As the maximum cumulative career dose allowed for a NASA astronaut is 600 mSv, the possible duration of such missions would be narrowed to approximately 600 days, which is not compatible with most current estimations of such missions (NASA, 2023; R. L. Ramos *et al*., 2023).

2.1.2. Economical Constrains

Transportation costs of humans and essential resources to space have diminished since the beginning of the space era. However, even with reportedly more affordable launch systems

such as SpaceX's Falcon 9 and Falcon Heavy, the cost per kilogram of payload to LEO is approximately $2k and $1.3k, respectively (Weinzierl *et al*., 2020).

When considering farther destinations, such as the Moon or Mars, these costs increase drastically. Astrobotic, a company with expertise in Lunar landers and rovers, presents a cost of $300k/kg for delivery to the Lunar orbit, and $1200k/kg to the Lunar surface (Astrobotic, 2021). More optimistic estimations based on the Falcon Heavy predict the cost of delivering payload to the Moon surface at around $10.8k/kg (Jones, 2023). As for crewed Mars missions, estimates place total costs at nearly half a trillion dollars, with launch expenses alone projected to 60 billion dollars (Jones, 2016).

Apart from transportation costs, current recycling technologies are equally expensive. Analyses estimate that existing recycling systems for water and oxygen incur operating costs of approximately $1,125/day and $19,210/day, respectively. Transporting these supplies from Earth could cost as much as $280,400 per crew-member-day for water and $28,010 for oxygen, leading to breakeven points of ~1,208 days and ~402 days, respectively, beyond which recycling becomes more cost-effective (Jones, 2023). As such, there is a compelling need for cost-effective recycling technologies, capable of minimizing logistical dependence on Earth.

2.1.3. Planetary Protection

Whenever humanity ventures beyond Earth into other celestial bodies, there is a risk of cross contamination. Forward contamination, the unintentional transfer of terrestrial life to other celestial bodies, can compromise astrobiological investigations and disturb potential extraterrestrial ecosystems. Backward contamination, the risk of potential extraterrestrial organisms or materials returning to Earth, can pose a risk to our own ecosystems (Benardini & Moissl-Eichinger, 2022).

To address these issues, the Committee on Space Research (COSPAR) has established the Planetary Protection Policy, which defines mission-specific requirements to minimize biological contamination of celestial bodies, spacecraft, crew, and Earth. For instance, flyby, orbiter and lander missions to the Moon are classified under Category II, as there is an interest regarding the chemical evolution and the origin of life on Earth, but the likelihood of harmful contamination remains remote. Mars, however, resides in Category III or IV, for flyby and orbiters or lander missions, respectively. This classification was attributed in the light of the potential past habitability of the red planet (Coustenis *et al*., 2023; Kminek *et al*., 2017).

With the approaching prospect of crewed missions to the Moon and, eventually, Mars (Smith *et al*., 2020), the existing Planetary Protection framework will require substantial revision. Namely, human presence on Mars would inevitably result in the production of

chemical and material contamination, a scenario that is currently not fully addressed in existing policies (Hader *et al*., 2023). Moreover, crewed surface operations will inevitably introduce high microbial loads from human-associated microbiota, making it challenging to distinguish between terrestrial contaminants and potential extraterrestrial biosignatures. Thus, it is equally necessary to improve the understanding of contaminant dispersal, persistence, and potential ecological interactions with indigenous life forms (McKaig *et al*., 2024; Spry *et al*., 2024).

Surpassing the challenge of planetary protection will further rely on the development of new sterilization technologies. Advanced decontamination methods – such as plasma sterilization (Roy *et al*., 2024), gamma irradiation, and other emerging techniques initially developed for terrestrial applications (Gradini *et al*., 2019) – can be complemented with real-time contamination detection systems (Khodadad *et al*., 2021).

2.1.4. Communication Delays

Crewed missions have always relied on a team of ground-based experts – the Mission Control Centre (MCC) – who actively monitor mission data, command spacecraft systems, oversee onboard operations and coordinate the activities of the crew. However, as missions extend beyond LEO, real-time communication is no longer a guarantee. For the Artemis program, communication delays are expected to vary between 5 to 14 seconds. As for future Mars missions, delays will be approximately 20 minutes one-way (Parisi *et al*., 2024), with communication blackouts during solar conjunctions lasting up to two weeks (Morabito & Hastrup, 2002; Quade *et al*., 2020).

The impact of communication delays has been highlighted during the Apollo program, where even short delays hindered MCC's ability to intervene effectively during real-time crew task execution (Parisi *et al*., 2023). Recently, an analog study showed that in a simulated Mars mission with a 20-minute one-way delay, MCC task performance declined and error rates on procedural record forms increased (Diamond *et al*., 2025). Overall, the future of space exploration depends on a paradigm shift in operational design, with enhanced onboard decision-making capabilities and robust autonomous systems.

2.1.5. Habitat Design and Maintenance

The challenges outlined in the previous subsections are not isolated issues. Rather, they converge within a single, overarching requirement: the design, construction, and maintenance of habitats capable of sustaining human life beyond Earth (**Figure 3**).

A functional extraterrestrial habitat must protect the crew from external threats such as

radiation, micrometeoroids, and extreme temperature fluctuations, while also ensuring the continuous supply of essential resources – such as oxygen, water, food, and materials – without excessive reliance on Earth. In parallel, it must maintain stable environmental parameters (pressure, humidity, temperature, gas composition) and support advanced contamination control to prevent biological or chemical compromise, both for planetary protection and crew health (Choate *et al*., 2023; H. Sun *et al*., 2024).

How these systems can be conceived and implemented remains one of the central questions in advancing human space exploration. This question is precisely the focus of the following section.

Figure 3. Artist's concept of the Artemis Base Camp on the Lunar Surface. Credits: NASA.

2.2. Life Support Systems

Since the year 2000, more than 200 astronauts have inhabited the ISS, which constitutes the largest extraterrestrial outpost humanity has ever constructed. This is essentially possible due to the presence of Life Support System (LSS) technologies. LSS are artificial ecosystems capable of aiding the survival of humans in space, usually through a regeneration cycle based on chemical or physical processes.

For future Lunar and Martian bases, LSS technologies will be coupled with *In Situ* Resource Utilization (ISRU), which consists of using resources directly from the environment, such as the regolith – the layer of loose, fragmented material covering solid rock on planetary surfaces – to produce consumables (Sacksteder & Sanders, 2007).

Ideally, a LSS should be capable of producing all essential resources for survival, including food, oxygen, and water. However, current technologies remain far from achieving this vision, highlighting the urgency of advancing research in this field to ensure the long-term sustainability of missions such as Artemis.

2.2.1. Environmental Control and Life Support System

As previously noted, the continuous human presence aboard the ISS is made possible by two key factors: its proximity to Earth, which allows for regular resupply missions, real-time communication, and emergency evacuation; and the existence of the Environmental Control and Life Support System (ECLSS).

The ECLSS is a collection of subsystems designed to maintain resources and a habitable environment for astronauts, relying exclusively on mechanical, electrical, and physicochemical processes (**Figure 4**). These modules include: the Atmosphere Control and Supply; Temperature and Humidity Control; Fire Detection and Suppression; Atmosphere Revitalization; Water Recovery and Management; and the Vacuum System (Balistreri *et al*., 2024; Vega, 2021).

The Water Recovery System recycles water from astronaut urine (solid waste is discarded), flush water, cabin humidity, and water generated by the Carbon Dioxide Reduction System. Recovery of potable water is achieved through distillation, filtration and chemical oxidation. This water can either be consumed directly or used for oxygen production in the Oxygen Generation Assembly, via electrolysis. Current technologies achieve a recovery rate above 95% from urine (Kayatin *et al*., 2017; Williamson *et al*., 2023).

The Atmosphere Revitalization System removes excess carbon dioxide (CO_2) and trace contaminants through adsorbent beds that chemically bind CO_2 during air circulation (Cmarik & Knox, 2019). It is integrated with the Condensing Heat Exchanger and water

separator, which regulate cabin temperature and humidity, while collecting condensate for recycling. This system also includes the Oxygen Generation System, which produces breathable oxygen either through water electrolysis (from the Water Recovery System) or through carbon dioxide reduction, which presents a recovery rate of oxygen of about 50% (Schneider & Shull, 2017; Sivolella, 2016).

Despite its effectiveness on the ISS, the ECLSS has critical limitations for long-duration exploration missions. Essential resources such as food, medicines, and spare parts are still entirely dependent on Earth resupply (Ichimura & Yamashiki, 2025; Jones *et al*., 2014). Furthermore, the system was designed specifically for the ISS environment, characterized by microgravity and partial protection from space radiation by the Earth's magnetosphere. Thus, it is not optimized for partial gravity (Moon or Mars), where changes in pressure, fluid dynamics, and radiation exposure would require significant redesign and ground-based testing (Gentry, 2021). Additionally, the system's mass, volume, and power requirements are tailored for the ISS, and will need to be thoroughly adapted for smaller platforms, such as the Gateway or interplanetary spacecraft (Barrett *et al*., 2023).

To address these challenges, NASA is currently testing an exploration-class ECLSS aboard the ISS, with the aim of adapting it for Artemis and Mars transit missions. The development strategy prioritizes increased automation, greater commonality of parts, and improved subcomponent repairability. However, because the current design is deeply tied to ISS architecture, major reconfiguration will be needed (Gentry, 2021; Ridley *et al*., 2024).

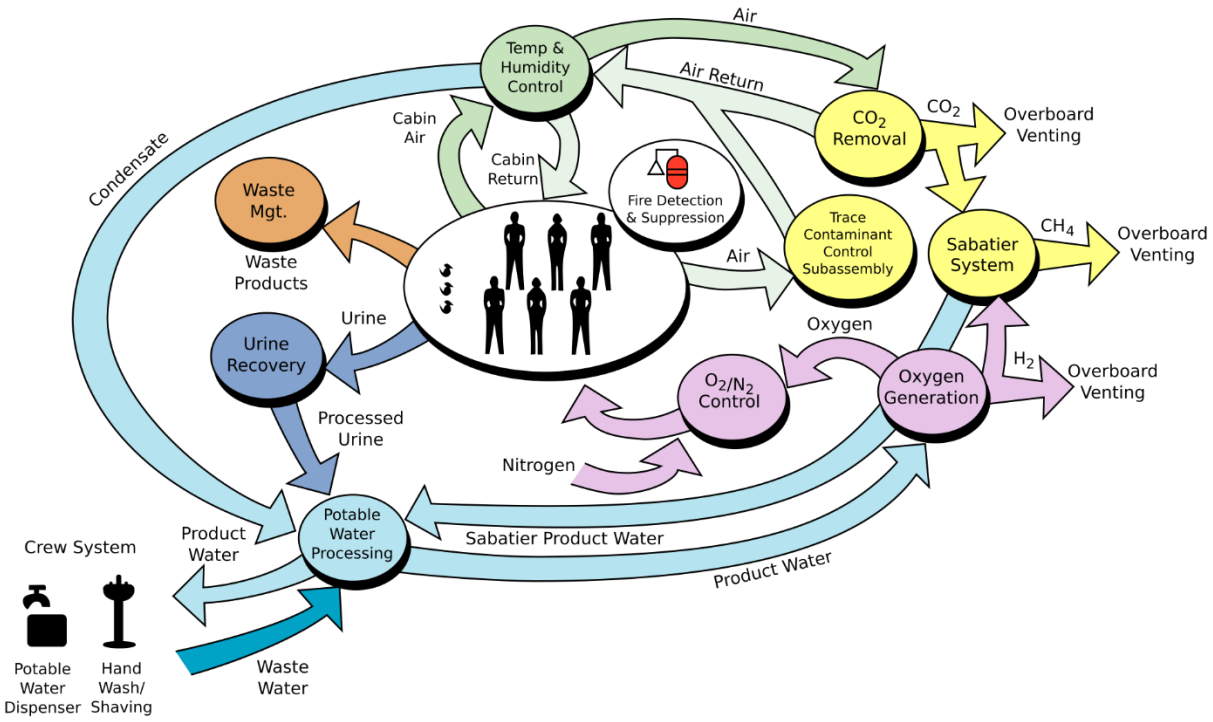

Figure 4. Schematic representation of the ISS Environmental Control and Life Support System (ECLSS), and interactions between the different modules. Credits: NASA.

As the ECLSS cannot independently sustain crews on long-duration or interplanetary missions, research has focused on alternative LSS that adopt different recycling approaches – namely the incorporation of biological processes, which are currently absent in the ECLSS.

2.2.2. Bioregenerative Life Support Systems

On Earth, human survival is inherently dependent on other living organisms. There is, thus, no reason to expect this interdependence to be different in autonomous extraterrestrial outposts.

Unlike the ECLSS, which depends solely on physicochemical processes, Bioregenerative Life Support Systems (BLSS) incorporate the efficiency of physicochemical processes with the versatility and reliability of biological processes. The integration of these two domains can enable a more complete closed-loop system, capable of regenerating vital resources such as oxygen, water, and food.

According to H. Liu *et al*. (2021), numerous BLSS concepts have been studied and tested on Earth as potential candidates for future space missions. The following subsections will highlight the most promising approaches so far presented.

2.2.2.1. MELiSSA: The Micro-Ecological Life Support System Alternative

MELiSSA is a program that was first presented by ESA in the eighties, with the aim of developing an autonomous, closed-loop habitat capable of sustaining human life during long-term space missions. The main inspiration for this initiative was a typical Earth ecosystem, characterized by an interconnection of different life forms, which exchange products between them. Similarly, MELiSSA is composed of five interconnected compartments, all of which rely on living organisms (**Figure 5**; Gòdia *et al*., 2002; Walker & Granjou, 2017).

Compartment I is the liquefying compartment. It functions as a composting chamber, where all the produced waste – such as human solid and liquid waste, as well as plant waste – are fed to anaerobic thermophilic bacteria. These will convert the waste into carbon dioxide, hydrogen, ammonium and fatty acids.

The fatty acids are then fed to compartment II, the photoheterotrophic compartment, where *Rhodospirillum rubrum* will use them to produce ammonia as well as biomass, which can serve as food for the astronauts.

The ammonia is then directed to compartment III, the nitrifying compartment, whose main function is to cycle nitrogen. *Nitrosomonas europaea* and *Nitrobacter winogradsky* will use the ammonia to produce nitrate, which is the preferred nitrogen form for the growth of

higher plants.

As such, the nitrate will constitute the input of compartment IV, the photoautotrophic compartment. This fourth compartment can be further divided into compartment IVa, where there is a culture of cyanobacteria; and IVb, where higher plants are grown. Both will provide oxygen and consume carbon dioxide, thus contributing to the maintenance of breathable air; as well as biomass, which can be used as a food source.

Humans themselves constitute the fifth compartment, using the resources from compartment IV and producing the inputs of compartment I.

Compared to the ECLSS, MELiSSA offers several advantages that are highly relevant for life support in space. One of its most important strengths is redundancy, which is intrinsically correlated with the nature of biological ecosystems. Redundancy is critical in space missions, as it ensures that the end-products of a system are produced, even if one component fails. For instance, in MELiSSA, the carbon dioxide required to feed Compartment IV can be supplied by both Compartment II and Compartment V; similarly, food is produced not only by higher plants in Compartment IVb, but also by microorganisms in Compartments IVa and II. Another key advantage is precisely the production of food, which was not guaranteed by the ECLSS and will be of utmost importance in future space missions.

Despite these advantages, MELiSSA is not yet capable of fully sustaining a human crew. Current performance estimates indicate that while it can cover the respiratory needs of a

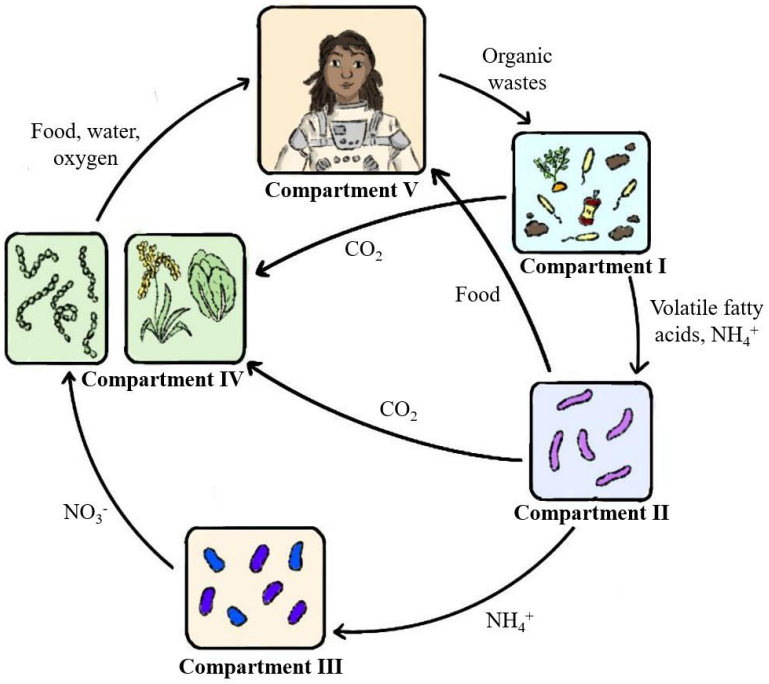

Figure 5. Simplified representation of the MELiSSA loop illustrating the interconnections between the different biological compartments. Credits: author's own work.

single person, it only meets 20–40% of dietary requirements (Garcia-Gragera *et al*., 2021). Moreover, the system does not address the production of medicines or construction materials – resources that would be fundamental for the long-term establishment of extraterrestrial habitats. At present, MELiSSA is primarily a testbed for BLSS technologies, requiring further development and optimization to overcome these limitations and to approach the efficiency necessary for long-duration space missions (Lasseur *et al*., 2010; Molist, 2023).

2.2.2.2. Eu:CROPIS: *Euglena gracilis* and Combined Regenerative Organic-food Production In Space

Eu:CROPIS mission was a compact satellite developed by the German Aerospace Center (DLR), launched in 2018. Its objective was to investigate the feasibility of growing tomato plants using human urine as a nutrient source, under Martian and Lunar gravitation conditions (Kottmeier *et al*., 2018).

The rationale behind Eu:CROPIS was the recognition that physicochemical and biological LSS offer complementary strengths. Physicochemical processes provide reliability, while biological processes contribute with versatility and the possibility of on-demand production. Combining the two into hybrid LSS could, therefore, enhance overall performance (Hauslage *et al*., 2018).

A particular challenge addressed by Eu:CROPIS was urine processing. As previously mentioned, on the ISS, the ECLSS recovers water from urine but discards the remaining solutes. Eu:CROPIS sought to valorise these components by nitrifying urine into a plant fertilizer solution, which would be used to grow tomato plants. These would consequently produce oxygen and edible biomass (Hauslage *et al*., 2018).

In the system (**Figure 6**), water circulated from a storage tank through three main modules, before returning to the tank: the C.R.O.P.® biofilter, a greenhouse, and an exchanger containing *Euglena gracilis*. The C.R.O.P.® biofilter contained a bacterial consortium responsible for converting urea from the urine into ammonia, nitrite and, ultimately, nitrate – which is the preferred nitrogen source for higher plants (Bornemann *et al*., 2015, 2018). *E. gracilis* played a crucial role in the system, by detoxifying excess ammonia – which would otherwise be toxic to the plants. Additionally, it provided oxygen to sustain bacterial nitrification until the tomato plants became photosynthetically active. Once the solution was enriched with nitrate, it flowed into the greenhouse to nourish the tomato seeds, which would then grow and produce oxygen (Zabel *et al*., 2019). Throughout this process, different sensors monitored key parameters, including nitrogen species and pH, to ensure an automated system stability (Kottmeier *et al*., 2018).

The mission ended in December 2019, but failed due to a software malfunction (DLR, 2020). Despite this, Eu:CROPIS served as a proof-of-concept for integrating biological and physicochemical processes in space-based LSS. While not as comprehensive as MELiSSA or the ISS ECLSS, it demonstrated the feasibility of urine recycling, biological fertilization, and hybrid system design, paving the way for future bioregenerative technologies.

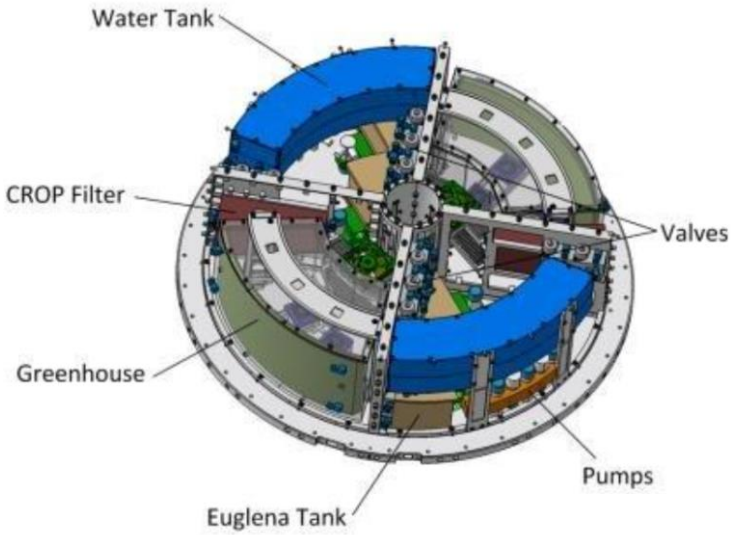

Figure 6. Primary Payload Module of the Eu:CROPIS mission, illustrating its main functional components. Credits: Kottmeier *et al*. (2018).

2.2.2.3. Lunar Palace 365

The Lunar Palace 365 experiment was a 370-day mission designed to test the long-term feasibility of a BLSS. The experiment was conducted in the Lunar Palace 1 facility (with P.A.L.A.C.E. standing for Permanent Astrobase Life-support Artificial Closed Ecosystem), at Beihang University, China, between 2017 and 2018. The experiment was built upon the earlier Lunar Palace 105 experiment, which lasted 105 days (Fu *et al*., 2016). There was a total of eight volunteer crew members, organized into two rotating groups. Once inside the facility, the group would remain completely sealed from the outside environment, simulating the isolation of a Lunar base (Fu *et al*., 2021).

The Lunar Palace 1 complex (**Figure 7**) covers an area of 160 m², divided into two plant cabins and one comprehensive cabin, which served as the crew's main living space. The ecosystem was designed to recycle matter in a closed loop: plants absorbed CO_2 exhaled by the crew, animals, and microorganisms, and generated O_2 through photosynthesis. This oxygen was then used by all living organisms inside the habitat (Fu *et al*., 2025).

Water recycling was achieved through multiple stages. Domestic wastewater was purified using a membrane biological activated carbon reactor. Urine underwent rotary

evaporation, recovering nitrogen and water, which was subsequently treated in the same membrane. The water was then sent to a plant nutrient solution tank, disinfected by UV light, and used for irrigation. Water transpired by plants and condensed in the plant cabins was collected, purified, and disinfected again. It could be then used as drinking water, sanitary water and domestic water (Zhao *et al*., 2022).

Solid waste recycling also played a central role. Dried grain straw was mixed with feces and placed into a solid fermentation unit, inoculated with straw-decomposing microbial agents. Some straw was also used to grow mushrooms, while the leftover substrate and fermented straw were mixed with vegetable residues to feed yellow mealworms. These insects were used as a protein source for the crew. Feed residues and insect waste were returned to the fermenter, thus closing the cycle (Fu *et al*., 2025).

Besides the animal protein, 35 different types of plants – including crops, vegetables and fruit – were cultivated under optimized light conditions. One cabin relied on hydroponic cultures, while the other used soil-like substrates prepared from recycled solid waste (Fu *et al*., 2025).

Apart from studying the circularity of all these resources, the Lunar Palace 365 experiment further tested the resilience of the system when faced with operational failures, such as temporary power loss (Fu *et al*., 2025).

In general, the results were highly promising. Atmospheric conditions remained stable throughout the whole period: CO_2 concentrations ranged from 246 to 4131 ppm, and O_2 concentrations between 20.11% and 21.52%, with an O_2 regeneration of 100%. Harmful gases were detected only at trace levels. When photosynthesis stopped due to the blackout, CO_2 levels returned to normal within 24 hours, once power was restored. Similarly, water recycling was 100%, and food regeneration was 73% in dry weight, with plants presenting a 100% regeneration rate. Urine treatment recovered 99.7% of nitrogen, and microbial degradation achieved a 67% solid waste recovery rate (Fu *et al*., 2021, 2025).

Overall, the Lunar Palace 365 experiment demonstrated that it is possible to maintain a crew for one year using a closed-loop system integrating biological and physicochemical processes. The experiment further validates the intent of Eu:CROPIS of creating a hybrid LSS, but at a larger scale and with direct crew involvement.

It is, however, relevant to acknowledge the limitations of this experiment. As it was conducted on Earth, critical factors such as altered gravity, radiation exposure and reliance on extraterrestrial resources were not tested. In addition, the effects of communication delays with Earth, energy demands of such a system and the feasibility of implementing power generation on the Moon were not contemplated.

The Lunar Palace 1 was not the first facility of its kind, being preceded by BIOS-3, in Russia (Salisbury *et al*., 1997), and Biosphere 2, in the United States (Marino & Odum, 1999). Both experiments were conducted in the 20th century, constituting important milestones in the history of BLSS, and serving as foundations for technologies later developed. In contrast, the Lunar Palace experiments represent a more recent effort, incorporating updated technologies and methodologies that better reflect current approaches to BLSS (H. Liu *et al*., 2021).

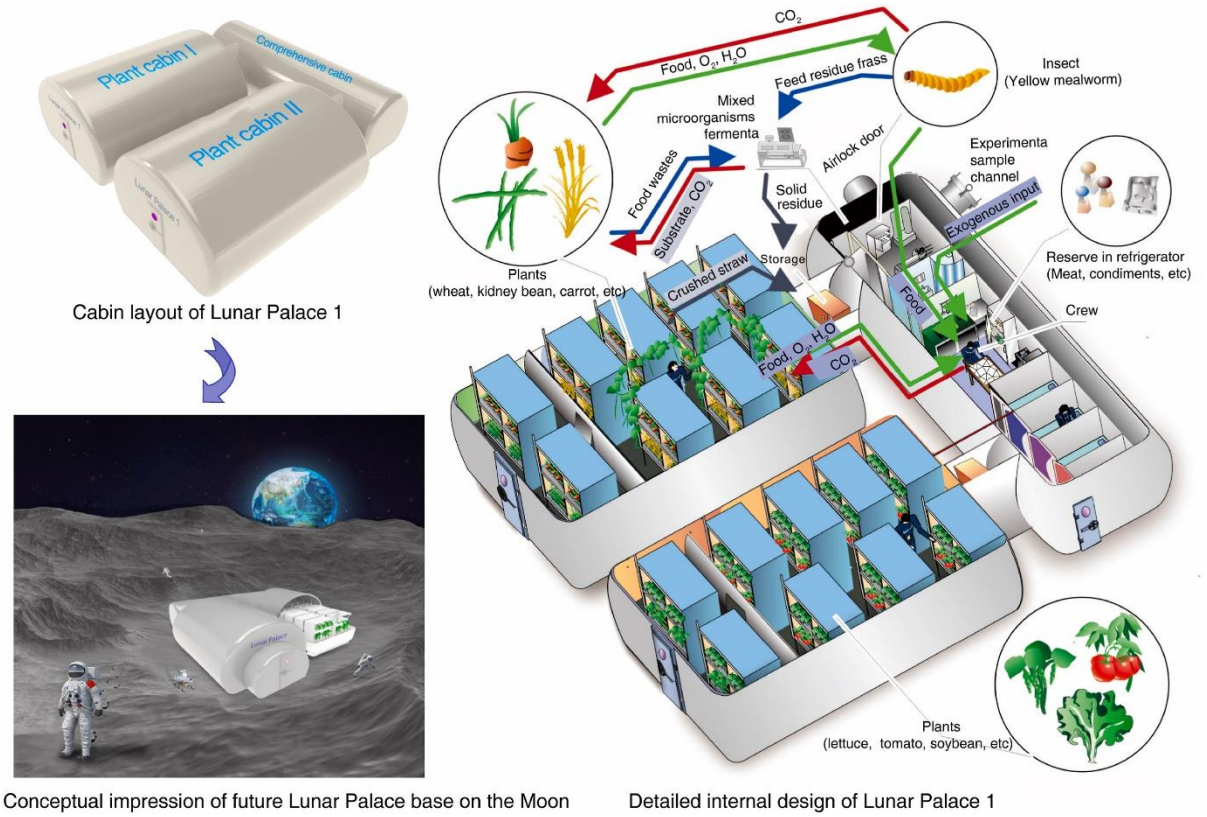

Figure 7. Model of the Lunar Palace 1 facility. Credits: Fu *et al*. (2021).

2.2.2.4. EDEN-LUNA Project

The LUNA Analog Facility, inaugurated in 2024, in Cologne, stems from a collaboration between ESA and the DLR. It was designed as a testbed for Lunar operations, ranging from robotic expeditions to habitable modules, further aiding the preparation of new technology and knowledge for the upcoming NASA's Artemis program. The interior of the LUNA facility features a 700 m^2 regolith testbed area, black-painted walls to minimize reflections and a Sun Simulator. This simulator can reproduce the illumination conditions of different Lunar surface locations, from polar to equatorial landing sites (Uhlig *et al*., 2025).

Several projects and expansions are planned for the LUNA facility. New external modules aim to be constructed in connection with the regolith room; one example includes a new habitable module for astronauts, enabling realistic training scenarios for Lunar surface

operations (Uhlig *et al*., 2024). From the perspective of BLSS, however, the most relevant addition will be the integration of the EDEN-LUNA Project.

The EDEN ISS (Evolution & Design of Environmentally-closed Nutrition-sources, **Figure 8**) is an advanced space-analog greenhouse. It employs advanced Controlled Environment Agriculture technologies, such as remote monitoring systems, sensor networks, and robotic automation for tasks including crop harvesting. The greenhouse has already been successfully demonstrated in the extreme conditions of Antarctica, near the Neumayer III station, for 5 years (between 2018 and 2022). In these conditions, EDEN ISS was able to produce fresh food products, such as lettuce, cucumbers, tomatoes, among others (Zabel *et al*., 2020).

In the upcoming EDEN-LUNA Project, the greenhouse will be tested not only for its biological role in future BLSS, but also for its interaction with a crew of astronauts and the surrounding environment within the analog facility (Kim, 2025).

Compared to the Lunar Palace 365, the EDEN ISS introduces key innovations, such as the advanced agricultural technologies; and the fact that all plants are cultivated within a single shared environment and, thus, subjected to the same conditions. This configuration more closely resembles the actual conditions expected in future space missions. Testing the EDEN-ISS system within the LUNA facility will contribute to current knowledge on BLSS for long-duration Lunar and Martian exploration (Franke *et al*., 2024; Zabel *et al*., 2020).

Figure 8. EDEN ISS greenhouse in Antarctica. Credits: Zabel *et al*. (2020).

2.3. The Role of Microorganisms in BLSS

From the previous examples of BLSS, it is evident that organisms selected to integrate such systems must comply with a specific set of characteristics, which make them suitable for long-term space missions. Although not formally codified, it is possible to outline some general criteria: a) resilience to space-specific conditions, including altered gravity and increased radiation; b) the ability to be transported at a low initial mass and volume, thereby optimizing payload efficiency and reducing launch costs; c) versatility in their applications for sustaining crew survival – for instance, plants can simultaneously contribute to oxygen regeneration, food production, and waste management; and d) the capacity to utilize resources readily available onboard or at the extraterrestrial destination, such as recycled waste or *in situ* materials.

Microorganisms align remarkably well with these requirements. Many have demonstrated the ability to survive and even thrive under the harsh conditions of space (Deshevaya *et al*., 2024; Salido *et al*., 2025). They can be transported as small aliquots or spores, representing negligible mass and volume, and later be propagated with minimal infrastructure once onboard. Moreover, their versatility allows them to be used for a variety of purposes, such as food and medicine production, waste treatment, creation of biofuel and materials, among others. Lastly, some of them can even use urine and regolith as their nutritional sources (Koehle *et al*., 2023).

Because of these qualities, microorganisms represent an invaluable cornerstone in the design of sustainable BLSS, complementing higher organisms such as plants.

2.3.1. Cyanobacteria

Plants will undoubtedly be essential in future BLSS, providing psychological advantages and nutritional balance in a way that cyanobacteria cannot mimic. Nonetheless, cyanobacteria are considered strong complementary candidates (Verseux *et al*., 2016).

Similarly to plants, their photosynthetic nature allows them to contribute to CO_2 removal and O_2 production. As previously mentioned, this function is harnessed in MELiSSA, using *Arthrospira sp.*, commercially known as Spirulina (Walker & Granjou, 2017).

Several cyanobacterial strains can further produce protein-rich, edible biomass, which is more easily processed than plant biomass, resulting in reduced waste generation. For example, Spirulina typically contains 55-70% of protein in dry weight; presents all essential amino acids; offers crucial nutrients, such as fatty acids and vitamins; and exhibits high digestibility due to the absence of cellulose cell walls (Richmond, 2004; Suwono & Suryoprabowo, 2025). Although Spirulina cannot serve as a complete substitute for plant-based foods, its biomass could play an important complementary role in meeting the crew's dietary

needs, particularly since plant-derived foods are often limited in protein (Lupatini *et al*., 2017). Another example of a cyanobacterium that presents high protein content is *Chlorella vulgaris*, which has even been intended to be tested in a photobioreactor aboard the ISS (Detrell *et al*., 2019) and is a strong candidate for future space missions (Detrell, 2021; Revellame *et al*., 2021). Strains such as *Chloromonas brevispina* and *Dunaliella salina* are also of interest, as they can grow at lower pressures (characteristic of a future Martian base, for instance) and be used for food and oxygen production (Cycil *et al*., 2021). As previously mentioned, the Lunar Palace experiment used insects (yellow mealworms) as a protein source (L. Li *et al*., 2015); replacing these insects with cyanobacteria could reduce waste production, lower space requirements, and simplify resource management within a BLSS.

Moreover, some cyanobacteria can use *in situ* resources, available on extraterrestrial settings (**Figure 9**). *Nostoc sp*. HK-01 has demonstrated survival under vacuum and growth on Martian regolith simulants (Arai *et al*., 2008). *Synechococcus sp*. PCC 7335 and *Chroococcidiopsis sp*. 029, exhibit far–red light adaptation, enabling them to perform photosynthesis under limited light, as is typical of Mars surface (di Stefano *et al*., 2024; Liistro *et al*., 2024). *Chroococcidiopsis sp*. 029 has been shown to grow both on Martian and Lunar regolith simulants, if supplemented with a nitrogen source, which could be found in astronaut's urine (Fernandez *et al*., 2023). Owing to its far-red light photoacclimation, *Chroococcidiopsis* is particularly advantageous for use in bioreactors where Water-Released Minerals (WRM) from Martian regolith could partially shield incident light (Santos de Sousa *et al*., 2025). *Anabaena sp*. PCC 7120 has also been shown to grow using Martian regolith simulants, presenting even some degree of resistance to the toxic perchlorates that exist in the regolith (Ramalho, *et al*., 2022a). Recently, *Desmonostoc muscorum* UTAD N213 was shown to grow using Martian regolith simulants and survive short irradiance periods of UV-B, common in the Martian surface (Arribas Tiemblo *et al*., 2025).

Among the different ISRU applications of cyanobacteria, their capacity to promote bioweathering is particularly relevant. As lithotrophs, cyanobacteria can produce extracellular metabolites that can solubilize and disintegrate rocks, improving, for instance, their predisposition for cultivation of plants (Mapelli *et al*., 2012; Olsson-Francis *et al*., 2012). Brown *et al*. (2008) investigated the bioweathering activity of siderophilic cyanobacteria exposed to Lunar and Martian regolith simulants. Their results showed that cyanobacterial growth was stimulated in contact with these substrates and that the organisms produced organic acids, including 2-ketoglutaric acid, which contributed to mineral dissolution.

Cyanobacterial biomass also represents a versatile platform for biotechnological applications, particularly in the production of biofuels. Considerable research on Earth has focused on the fermentation of cyanobacterial biomass into ethanol, due to its commercial value as a renewable biofuel (Arias *et al*., 2021; Y. Huang *et al*., 2021; Möllers *et al*., 2014).

Cyanobacteria are especially attractive in this regard because of their rapid growth rates and higher photosynthetic efficiencies, compared to other organisms traditionally employed for biofuel production (Majhi, 2025). Beyond bioethanol, cyanobacteria can also be exploited for biohydrogen production, with *Anabaena sp.* PCC 7120 being a potential candidate (Nayak *et al*., 2014). The potential to harness cyanobacteria for biofuel production in space is appealing, as it could provide sustainable, *in situ*, and on-demand energy sources (Kruyer *et al*., 2021; Meng, 2023).

Finally, beyond their direct edible potential, cyanobacterial biomass can also be valorized as a nutrient source for heterotrophic producers, enabling the synthesis of food, pharmaceuticals, biomaterials, and other valuable products. For example, *Chroococcidiopsis sp*. 029 – which, as previously mentioned, has been shown to grow on Lunar and Martian regolith supplemented with nitrogen – produces a biomass which can sustain *Escherichia coli* growth, opening possibilities for a wide array of biotechnological applications in spatial environments (Fernandez *et al*., 2023). Similarly, *Anabaena sp*. PCC 7938 grown on Martian regolith simulant produces a biomass which can support both *E. coli* and the higher plant *Lemna sp*. (Ramalho, *et al*., 2022a).

Importantly, this concept is not restricted to bacteria alone. Cyanobacterial biomass has also been tested as a growth substrate for fungi. Studies unrelated to space exploration have demonstrated that strains such as *Aspergillus tubingensis* and *Saccharomyces cerevisiae* can grow on cyanobacterial lysates (Gupta *et al*., 2024; Mishra & Kumar, 2007), underscoring the potential of integrating cyanobacteria with fungi in closed-loop systems.

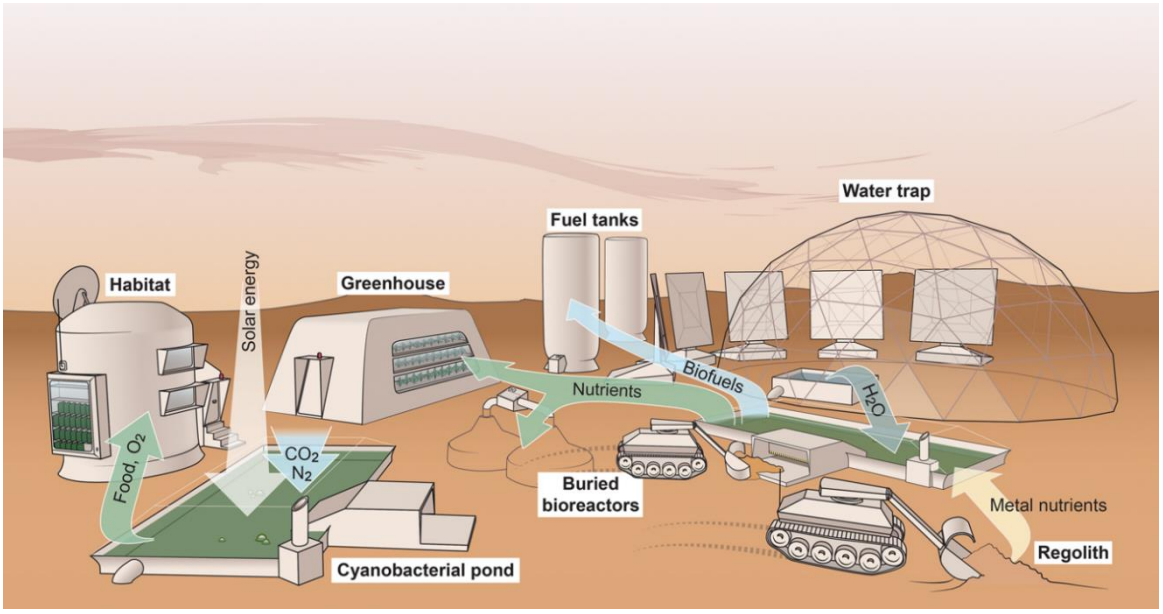

Figure 9. Artist's concept of a BLSS based on many of the applications of cyanobacteria. Credits: Cyprien Verseux (ZARM, Bremen University) and Sean McMahon (Yale University); layout by Sean McMahon; (Verseux *et al*., 2016).

2.3.2. Fungi

Fungi thrive in virtually every known ecosystem on Earth, from the deep sea (Zain ul Arifeen *et al*., 2019) and permafrost (da Silva *et al*., 2020) to hot springs (K.-H. Liu *et al*., 2018) and even acidic mine drainage (B. K. Das *et al*., 2009). Remarkably, they have also been detected aboard the ISS, where they are exposed to microgravity, UV radiation, X-rays, and cosmic rays (Checinska Sielaff *et al*., 2019).

On Earth, fungi have long been exploited for their biotechnological versatility, with applications ranging from the production of pigments, antibiotics, enzymes, and antibacterial agents to food fermentation, edible biomass production and waste degradation (Aaronson, 2000; Kaur & Verma, 2020; Vivek & Venkitasamy, 2023). Naturally, fungal biotechnology is increasingly recognized as relevant for space exploration (Cortesão, *et al*., 2020a; Simões *et al*., 2023).

2.3.2.1. Radiation protection

One of the most promising space applications of fungi is their capacity to produce melanin, a pigment with radiation-shielding properties. Melanin is strongly associated with fungal survival in extreme environments, providing a mechanistic explanation for the persistence of certain fungi in the ISS (Dadachova & Casadevall, 2008; Qin & Xia, 2024).

Recent studies have begun to explore fungi as a means of radiation protection, by using its melanin to coat spacecraft and habitable modules. For instance, *Cladosporium sphaerospermum* was cultivated aboard the ISS for 26 days, where it not only survived exposure to microgravity and cosmic radiation, but also displayed enhanced growth compared to ground controls (Averesch *et al*., 2022). Similarly, *A. niger* melanin pigments, such as pyomelanin, have been studied for their UV-C radiation shielding potential (Koch *et al*., 2023). Other extremotolerant fungi, such as *Cryomyces antarcticus* (Pacelli *et al*., 2020) and *Aspergillus fumigatus* (Blachowicz *et al*., 2020) have also been investigated for their capacity to resist and protect from space radiation.

From a bioengineering perspective, melanin nanoparticles derived from fungi are considered an innovative approach to radiation shielding (Stewart *et al*., 2022; Waghmode *et al*., 2023). This line of research is particularly relevant in the context of future crewed missions to Mars, where mission duration alone would expose astronauts to radiation levels likely exceeding the acceptable limit of 600 mSv defined by NASA (NASA, 2023; R. L. Ramos *et al*., 2023).

2.3.2.2. Food production and fermentation

On Earth, fungi play a central role in the food industry, both as direct sources of nutrition and as essential agents in fermentation processes (Dufossé *et al*., 2014; Pouris *et al*., 2024). Furthermore, *A. niger* is widely used for the industrial production of citric acid, a compound broadly used as a food preservative (Behera, 2020).

These functions can, naturally, be translated into space habitats. In fact, mushrooms were included as a dietary component in the Lunar Palace 365 project (Fu *et al*., 2016). Beyond this, several studies have investigated fungal fermentation under space-related conditions. For instance, *Fusarium flavolapis* was successfully cultivated in a bioreactor under simulated microgravity, generating a protein-rich "biomat" suitable as a nutritious food source for long-term missions (Pisarski & Light, 2021). Similarly, the BioNutrients-1 project developed on-demand nutrient production packs containing desiccated *Saccharomyces cerevisiae* and edible substrates. Once hydrated, the engineered yeast strains produced carotenoids such as β-carotene and zeaxanthin, offering a model for *in situ* nutrient biosynthesis in space (Ball *et al*., 2020).

Fermentation of traditional foods has also been demonstrated beyond Earth. *Aspergillus oryzae* was exposed to real microgravity and cosmic radiation aboard the ISS for 30 days, successfully producing a "space miso" comparable to terrestrial controls (Coblentz *et al*., 2024). A kombucha culture containing the fungal strains *Pichia sp*., *Brettanomyces/Dekkera anomala*, and *Zygosaccharomyces bailii* was exposed to Mars-like conditions aboard the ISS for 18 months. Although the culture experienced DNA degradation and membrane alterations under extraterrestrial UV radiation, revival and subculturing restored part of its structure and metabolic functions, suggesting resilience of such communities in space environments (Podolich *et al*., 2019).

Similar to cyanobacterial protein, fungi could also provide a sustainable and nutritious alternative to meat in space, requiring fewer resources (Majumder *et al*., 2023). This could be particularly critical during the initial stages of colonization on the Moon and Mars. All these applications could be further coupled with molecular tools such as CRISPR, to improve the nutritious value of fungi and safeguard the health of astronauts (Strong *et al*., 2022; Y. Zhang *et al*., 2023).

2.3.2.3. Production of biocomposites

Current approaches for the construction of habitable modules on the Moon and on Mars involve using *in situ* resources – such as Lunar and Martian regolith – to produce materials through additive manufacturing (Clinton *et al*., 2022; Y. Wang *et al*., 2022).

A complementary and increasingly attractive alternative is the use of biocomposites, which integrate biological components with structural matrices. Using fungal biomass to produce biocomposites is an increasingly relevant alternative, due to their capability of producing lightweight and biodegradable materials, which can be transported to extraterrestrial destinations at initial low masses (below milligrams). Once at the site, fungal growth could be propagated in automated systems, scaled up according to construction needs, and adapted to use locally available biomass as feedstock (Herzog *et al*., 2024).

Elsacker *et al*. (2018) explored this concept using *Schizophyllum commune, Trametes multicolor* and *Pleurotus ostreatus*, fed on fern. These fungi were cultivated under simulated microgravity using a random positioning machine, after which the resulting biomass was subjected to mechanical and physical tests, to assess its viability for composite production. The authors inclusively proposed a methodology for the automation of biocomposite production. Among the tested strains, *S. commune* emerged as the most promising candidate, being able to grow under microgravity and radiation exposure. Its biomass was subsequently used for proof-of-concept 3D bioprinting.

Another innovative project that has been developed in this field is the NASA Myco-Architecture Off-Planet Project, which aims to develop self-growing habitats for the Moon and Mars utilizing *in situ* resources (**Figure 10**; Brandić Lipińska *et al*., 2022; Rothschild *et al*., 2018, 2019). Briefly, the concept of this project is to build habitats that function as a biological shell. These structures would consist of two layers of plastic, with the space in between filled with dried fungal mycelia and organic nutrient sources, such as cyanobacteria or algae biomass. Upon activation with water and increased temperature, both the fungal and photosynthetic

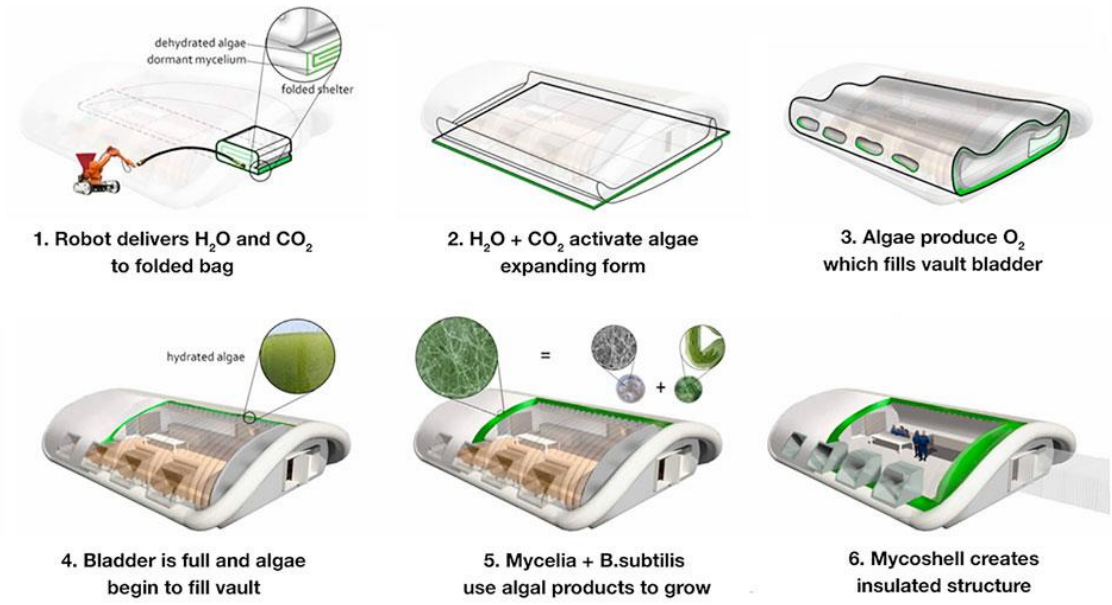

Figure 10. Representation of a self-grown habitable structure proposed by NASA's Myco-Architecture off-planet project. Credits: Rothschild *et al*. (2018).

components would initiate their metabolic activity. The fungi would use oxygen produced by the photosynthetic organisms to grow and colonize the interior of the shell, thereby forming a solid, living barrier. This system could be further combined with bacteria such as *Bacillus subtillis*, which could be used for a variety of applications, such as a producer of biocement; as a sensor for monitoring oxygen concentrations; or even as a promoter of fungal growth in areas where structural cracks emerge. This project is still in a proof-of-concept phase, having already tested the physical properties of the biocomposites produced from fungal mycelium.

The application of fungal mycelium to produce biocomposites for construction is intrinsically correlated with their ability to shield against harmful space radiation that would, otherwise, reach the interior of habitable modules. A recent project, MelaRad, was initiated with the goal of developing lightweight biocomposite shielding to coat spacecraft and satellites (Gulacsi *et al*., 2022). Different biocomposites – namely polylactic acid infused with fungal, synthetic or animal melanin – were sent to the ISS for 6 months and tested for different physical property indicators. Notably, fungal melanin presented effective shielding effects, and was even able to reduce surface wrinkle formation (Cordero *et al*., 2025).

Other relevant research in this field includes the use of red reishi mushrooms (*Ganoderma Lucidum*) to produce interlocking bricks for LEO and Moon habitats (Ramirez *et al*., 2025). Another example is the mWALLd project, which proposes using a 3D bioprinting approach to produce biocomposites from the mycelium of *Cryomyces sp.*. This extremophilic fungus can be combined with engineered bacterium capable of producing reinforcing fractions, such as calcium carbonate (Pawlicki & Bałka, 2022).

2.3.2.4. Biomining

Another relevant strategy for ISRU in space is biomining. Biomining consists of the recovery of minerals and metals through biological processes, which are more sustainable than chemical methods. This process is possible through the secretion of organic acids by microorganisms, which will consequently solubilize metals from the ores into the surrounding liquid medium (Gumulya *et al*., 2022; Santomartino *et al*., 2022). On Mars, for instance, the high abundance of iron makes biomining especially attractive (Carissimo *et al*., 2024).

Several filamentous fungal strains have been studied for their biomining potential in extraterrestrial habitats. *Penicillium simplicissimum* has been tested in a lunar regolith simulant using a low-nutrient, diluted potato dextrose broth. The fungus demonstrated the ability to secrete organic acids, including citric acid, enabling the extraction of magnesium, calcium, aluminium, among others (Figueira *et al*., 2025). This fungal strain was also used on the BioAsteroid experiment, aboard the ISS. In this setting, *P. simplicissimum* was demonstrated

to be capable of extracting minerals from L-chondrite material, a common type of meteorite, under real microgravity conditions (Santomartino *et al*., 2024).

Similarly, *Aspergillus spp.* presents promising candidates for biomining in space. *Aspergillus tubingensis* was shown to efficiently solubilize manganese and other elements from a Martian regolith simulant within just 5 days, primarily through the production of oxalic and citric acids (Vezzola *et al*., 2023). *A. niger* has also been tested for biomining in ground-based experiments, in preparation for future ISS studies (Martin *et al*., 2025). It has even been reported that microgravity stimulates the production of organic acids by *Aspergillus carbonarius*, which could enhance biomining efficiency – though it can equally accelerate the corrosion of metals (Jiang *et al*., 2019, 2022).

2.3.2.5. Production of biotechnologically relevant molecules

On Earth, fungi are major producers of bioactive molecules, many of which are used in the pharmaceutical industry (S. Das *et al*., 2024). As such, the field of fungal bioremediation in space is particularly relevant. While small amounts of medicine might suffice for short-term missions to the ISS, long-term habitation on the Moon and on Mars would necessitate local production (Cortesão, *et al*., 2020a; McNulty *et al*., 2021).

It is known that spaceflight conditions – such as microgravity and radiation – influence microbial growth and metabolism (B. Huang *et al*., 2018). These environmental factors can, thus, alter the biosynthetic pathways of secondary metabolites. For this reason, it is relevant to study metabolite production in space.

In this context, *Humicola fuscoatra* has been tested for the production of the antibiotic monoderm under real microgravity and space radiation, aboard the ISS. It was shown that flight samples produced more monoderm than ground controls (Lam *et al*., 1998). Similarly, a strain of *A. niger* isolated from the ISS showed increased production of various secondary metabolites relevant for the pharmaceutical industry, including naphtho-γ-pyrones – which present antitumoral and antimicrobial activity – and pyranonigrin, which presents antioxidant properties, among others (Romsdahl *et al*., 2020). Moreover, *Penicillium chrysogenum*, a strain from the genus used on Earth for penicillin production, has been tested under simulated microgravity. The experiment showed an increased expression of enzymes involved in the production of penicillin (Sathishkumar *et al*., 2016).

2.3.2.6. Integration of fungi in BLSS

To evaluate current attempts at integrating fungi in BLSS, a targeted search was performed on Web of Science (topic: *fungi* OR *fungal* AND *LSS* OR *BLSS*), yielding 28 results as of August 20, 2025. Of these, 15 were discarded as irrelevant to the topic. Seven studies focused exclusively on fungi as potential hazards to LSS, for instance through air contamination or as targets for anti-fungal measures.

The remaining six studies directly addressed the potential of fungi in BLSS. One explored the use of Soil-Like Substrate, obtained by successive conversion of wheat straw by oyster mushrooms (*Pleurotus spp*.) and worms, as a candidate for plant cultivation in BLSS (Gros *et al*., 2005).

The other five studies were all related to the Lunar Palace 365 experiment. Two studies were related to the rhizospheric microbial communities during wheat cultivation, in which fungi naturally play a role (Liao, Yao, *et al*., 2025; R. Sun *et al*., 2022). In this case, fungi were indeed present within the BLSS, but only as part of the plant-associated microbiome, not as deliberately introduced agents.

The remaining studies delved into the process of solid waste recycling employed in the Lunar Palace 365. This process was initially described as relying on "microbial inoculants that are able to degrade plant waste" (Fu *et al*., 2016). D. Liu, Xie, Dong, *et al*. (2020) characterized the microbial diversity in this compartment but focused solely on bacterial identification techniques. This is consistent with the design assumption that waste recycling would be performed exclusively by bacterial consortia. Later follow-up studies using inoculum derived from Lunar Palace 365 compost revealed the presence of fungi in the samples (Liao, Feng, *et al*., 2025; D. Liu, Xie, Liu, *et al*., 2020; Z. Wang *et al*., 2025).

Taken together, these findings show that fungi have already emerged in BLSS contexts, but mostly indirectly: either as unavoidable components of plant microbiomes or as unplanned yet functionally relevant contributors to waste degradation. This gap further highlights the novelty and importance of exploring the plethora of fungal applications previously presented into new BLSS.

2.3.2.7. *Aspergillus niger*

On Earth, *A. niger* has long been regarded as a cell factory to produce many relevant secondary metabolites. Its industrial relevance spans for centuries, thus being one of the most studied fungal species in biotechnology (Cairns *et al*., 2018; R. Yu *et al*., 2021). In the context of space, *A. niger* is increasingly considered a model organism for space microbiology

(Cortesão, 2021; Koch *et al*., 2023; Mota *et al*., 2025). This derives from several factors, as described followingly.

Firstly, as highlighted in the previous subsections, *A. niger* is a potential candidate for nearly all the fungal applications envisioned for space habitats. Indeed, the only subsection of fungal applications for space where it was not mentioned was for biocomposite production. However, even here, Earth-based studies have already demonstrated its potential (K. Li *et al*., 2023). Moreover, its molecular machinery can be engineered to expand its functional repertoire, making it a strong candidate for an on-demand factory on distant worlds (Arentshorst *et al*., 2023; Kuivanen *et al*., 2016; Rojas-Sánchez *et al*., 2020).

Additionally, *A. niger* is very resistant to space conditions. Its spores have been shown to exhibit remarkable resistance, which would allow them to remain viable after long-term exposure to the space environment (Cortesão, *et al*., 2020b). *A. niger* has even been shown to withstand the harsh conditions of the ISS, where it is frequently detected as one of the most common naturally occurring fungal colonizers (Blachowicz *et al*., 2022; Romsdahl *et al*., 2018).

It is precisely this unique combination of biotechnological versatility and resilience that makes *A. niger* an attractive candidate for integration into BLSS. For these reasons, *A. niger* was selected as the species to be employed in this work.

Chapter 3

Materials and Methods

3.1. Strains and Maintenance

In this study, ***Aspergillus niger* wild type (N402)** was used for all experiments. *A. niger* spores were harvested from 3-day-old cultures incubated on Complete Medium (55 mM glucose, 11 mM KH_2PO_4, 7 mM KCl, 178 nM H_3BO_3, 2 mM $MgSO_4$, 76 nM $ZnSO_4$, 70 mM $NaNO_3$, 6.2 nM Na_2MoO_4, 18 nM $FeSO_4$, 7.1 nM $CoCl_2$, 6.4 nM $CuSO_4$, 25 nM $MnCl_2$, 174 nM EDTA, 0.5% (w/v) yeast extract and 0.1% (w/v) casamino acids) at 30 ºC, by flooding the agar plates with 10 mL of saline solution (0.9% NaCl) and harvesting the spores using a cotton stick. Spore suspensions were filtered using Miracloth (Millipore), to remove hyphal fragments, and were kept at 4°C. Spore counting was performed using a Neubauer chamber. All experiments were performed with fresh spore suspensions, no older than 2 weeks.

Four strains of cyanobacteria (**Table 1**) were tested as nutrient sources for *A. niger*. ***Anabaena sp.* PCC 7120** (hereby referred to as PCC 7120) was kindly provided by Dr. Paula Tamagnini (Institute for Research and Innovation in Health, University of Porto, Porto, Portugal). The strain was grown in $BG\text{-}11_0$ (BG-11 medium without nitrate), under a continuous light regime of 30–40 µmol photons.$m^{-2}s^{-1}$, at 28 °C, and mild shaking conditions (60–80 rpm).

***Anabaena sp.* PCC 7938** (hereby referred to as PCC 7938) was kindly supplied by Dr. Cyprien Verseux (Center of Applied Space Technology and Microgravity (ZARM), University of Bremen, Bremen, Germany) and was obtained from the Pasteur Culture Collection of Cyanobacteria (Paris, France). The strain was cultivated in $BG11_0$ medium, using 5L Schott bottles, bubbled at a rate of approximately 1 L/min with N_2 enriched with 1% of CO_2, under a photon flux density of 10 photons.$m^{-2}s^{-1}$ (16:8h day:night cycle).

***Anabaena aphanizomenoides* LEGE 00250** and ***Anabaena sp.* LEGE X-002** (hereby referred to as LEGE 00250 and LEGE X-002, respectively), provided by the Blue

Biotechnology and Ecotoxicology Culture Collection (LEGE-CC, CIIMAR, Porto, Portugal, https://lege.ciimar.up.pt/), were grown in Z8 medium at 19 ºC and 140 rpm, under a continuous light regime of 30–40 µmol photons.$m^{-2}s^{-1}$.

Table 1. Cyanobacterial strains tested as nutrient sources for the growth of *A. niger*.

Strain	Description	References
Anabaena sp. PCC 7120	Multicellular, filamentous, freshwater and heterocyst-forming cyanobacterium, used as a prokaryotic model for the study of cell differentiation and cell-cell interactions	(Videau & Cozy, 2019; Xing *et al*., 2022)
Anabaena sp. PCC 7938	Multicellular, filamentous cyanobacterium, used as a model for biological ISRU on Mars	(Ramalho, *et al*., 2022a)
Anabaena aphanizomenoides LEGE 00250	Multicellular, filamentous, freshwater/aquatic cyanobacterium isolated from the Maranhão dam reservoir, Portugal	(Osswald, 2002)
Anabaena sp. LEGE X-002	Multicellular, filamentous, freshwater/aquatic cyanobacterium, anatoxin-a producing strain	(Osswald *et al*., 2007; Sivonen *et al*., 1989)

3.2. Water-Released Minerals

Solutions of Water-Released Minerals (WRM) were prepared from the Lunar regolith simulant LHS-1 (Exolith Lab, 2021) or the Martian regolith simulant MGS-1 (Cannon *et al*., 2019), whose compositions are reported on **Table 2.** WRM preparation was performed according to a protocol adapted from Fernandez *et al*. (2023). Briefly, simulants were weighted in an analytical scale and transferred to flasks containing 1 L of deionized sterile water (dH_2O). Mixtures at a final concentration of 40 g/L were shaken overnight at 200 rpm and room temperature (RT). Followingly, the solutions were centrifuged at 100 g for 10 minutes, and the supernatant was collected and filtered with a 0.2 µm filter to a sterile glass flask. Solutions were preserved throughout the work at 4 ºC.

Table 2. Composition of the Lunar and Martian regolith simulants, LHS-1 and MGS-1, on weight percentage (Wt. %). Data acquired from Long-Fox & Britt, (2023).

Oxide	LHS-1 Lunar Highlands Simulant	MGS-1 Mars Global Simulant
SiO_2	49.12	43.9
TiO_2	0.63	0.46
Al_2O_3	26.29	12.84
FeO	3.20	10.60
MnO	0.06	0.11
MgO	2.86	14.81
CaO	13.52	7.91
Na_2O	2.55	1.49
K_2O	0.34	0.29
P_2O_5	0.17	0.17

3.3. Cyanobacterial Cells Pre-Treatment

After sufficient growth was observed, cyanobacterial cells were centrifuged at 6000 g for 10 minutes in sterile Eppendorf tubes. Two cycles of rinsing the resulting pellet with 1 mL of distilled water and centrifugation at 6000 g for 10 minutes were performed. The biomass was dried for 3 to 5 days in an incubator at 60 to 70 °C. Biomass weight in each Eppendorf tube was calculated by subtracting the difference between the Eppendorf with the dried biomass and the empty Eppendorf.

To assess the effect of different pretreatments on cyanobacterial cells on the growth of *A. niger*, two experimental groups were set: a) no treatment group (NT), in which cyanobacterial cells were used to prepare the fungal growth media directly after drying; and b) mechanical lysis group. Mechanical lysis was performed by bead beating, adapting the protocol described by Santos de Sousa *et al*. (2025). Silica beads (0.1 mm) were used at a 1:1 ratio relative to the dried pellet size. Each pellet was resuspended in 1 mL of dH_2O, Martian WRM (mWRM) or Lunar WRM (lWRM), and the mixture was subjected to three cycles of 2-min homogenization at 6800 rpm (Precellys Evolution Homogenizer, Bertin Technologies), with 1-minute intervals on ice between cycles. Lysates were examined under an optical microscope to verify successful cell disruption. If intact cyanobacterial cells were still observed, the bead-beating procedure was repeated until complete lysis was confirmed. The resulting mixtures were then centrifuged at 6000 g for 5 minutes, and the supernatants were collected to be used as fungal growth media. Solutions were kept at -20 ºC until further usage.

3.4. Fungal Growth Under Standard Earth Gravity

Fungal growth experiments were conducted in flat-bottom 12-well plates (Frilabo II), sealed with parafilm to minimize evaporation. Each well was inoculated with 1×10^6 spores of *A. niger* per mL, in a final volume of 3 mL of test medium. Cultures were incubated at RT on an orbital shaker set at 120 rpm, for 7 days.

pH was monitored at timepoints t = 0, 3, and 7 days. For each timepoint, 10 µL aliquots were collected per well and analysed using pH indicator strips (Universal Indicator Paper pH 1–14, METRIA). All measurements were performed in triplicate for each well. Experiments were performed with a minimum of three biological replicates. Experimental media compositions are detailed in the following subsections.

After the designated incubation period, cultures (biomass and supernatant) from each well were transferred to previously weighed sterile 1.5 mL Eppendorf tubes, and centrifuged at 6000 g for 10 minutes. To ensure complete collection of the total culture volume (3 mL), the transfer and centrifugation steps were performed in two rounds. Supernatants were discarded and biomass pellets were dried at 60 to 70 °C for 3 to 5 days, until constant weight was achieved. Total fungal dry biomass was determined by subtracting the weight of the empty Eppendorf tube from the weight of the Eppendorf tube containing the dried pellet.

3.4.1. Lunar and Martian Water-Released Minerals Media Assays

The first experimental media conditions were designed to evaluate whether *A. niger* could utilize the inorganic nutrients from mWRM or lWRM. As *A. niger* is not an autotrophic organism, it is unable to grow in media composed exclusively of the inorganic nutrients of WRM.

As such, Lunar/Martian WRM were diluted in Minimal Media (MM; 55 mM glucose, 11 mM KH_2PO_4, 7 mM KCl, 178 nM H_3BO_3, 2 mM $MgSO_4$, 76 nM $ZnSO_4$, 70 mM $NaNO_3$, 6.2 nM Na_2MoO_4, 18 nM $FeSO_4$, 7.1 nM $CoCl_2$, 6.4 nM $CuSO_4$, 25 nM $MnCl_2$, 174 nM EDTA), to achieve final WRM concentrations of 99%, 95%, 90%, 75%, and 50% (v/v). In a parallel experiment, MM was diluted in dH_2O to obtain equivalent MM concentrations of 1%, 5%, 10%, 25%, and 50% (v/v). As dH_2O does not contain inorganic nutrients, the dH_2O–MM conditions served as negative controls for nutrient supplementation. Direct comparison of fungal biomass produced in WRM–MM and dH_2O–MM with the same MM concentration enabled assessment of whether *A. niger* could utilize the inorganic components of WRM to support growth (**Figure 11**).

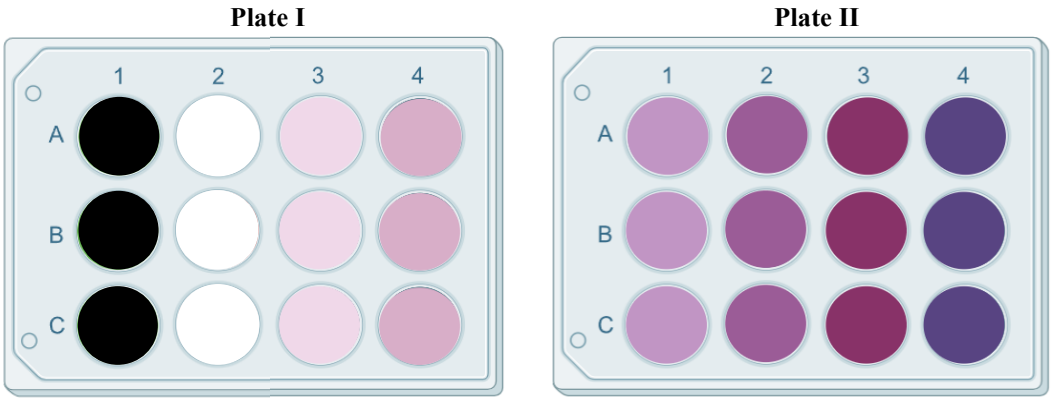

Plate	Column	Experimental Conditions		
		Martian WRM–MM	**Lunar WRM–MM**	**dH₂O–MM**
I	**1**	Positive control (MM)		
	2	Negative control (dH₂O)		
	3	Negative control (mWRM)	Negative control (lWRM)	-
	4	99% mWRM 1% MM	99% lWRM 1% MM	99% dH₂O WRM 1% MM
II	**1**	95% mWRM 5% MM	95% lWRM 5% MM	95% dH₂O WRM 5% MM
	2	90% mWRM 10% MM	90% lWRM 10% MM	90% dH₂O WRM 10% MM
	3	75% mWRM 25% MM	75% lWRM 25% MM	75% dH₂O WRM 25% MM
	4	50% mWRM 50% MM	50% lWRM 50% MM	50% dH₂O WRM 50% MM

Figure 11. Composition of the experimental media tested for *A. niger* growth in the Lunar and Martian WRM dilution assays. Percentage of concentration corresponds to v/v. WRM: Water-Released Minerals; mWRM: Martian Water-Released Minerals; lWRM: Lunar Water-Released Minerals; MM: Minimal Media; dH₂O: deionized sterile water.

3.4.2. Supplementation of Lunar and Martian Water-Released Minerals with Different Organic Sources

Following the evaluation of mWRM and lWRM as potential inorganic nutrient sources for *A. niger*, a subsequent set of experiments was conducted to identify an appropriate complementary organic nutrient source. This would eliminate the need to use MM, which would not be readily available during Lunar or Martian missions.

As such, undiluted WRM (100%) was supplemented with either the equivalent (55 mM) or half the amount (27.5 mM) of glucose present in standard MM (**Figure 12**). This experiment was performed to assess whether an easily assimilable organic carbon source would be sufficient to support fungal growth in an otherwise nutrient-poor mineral media.

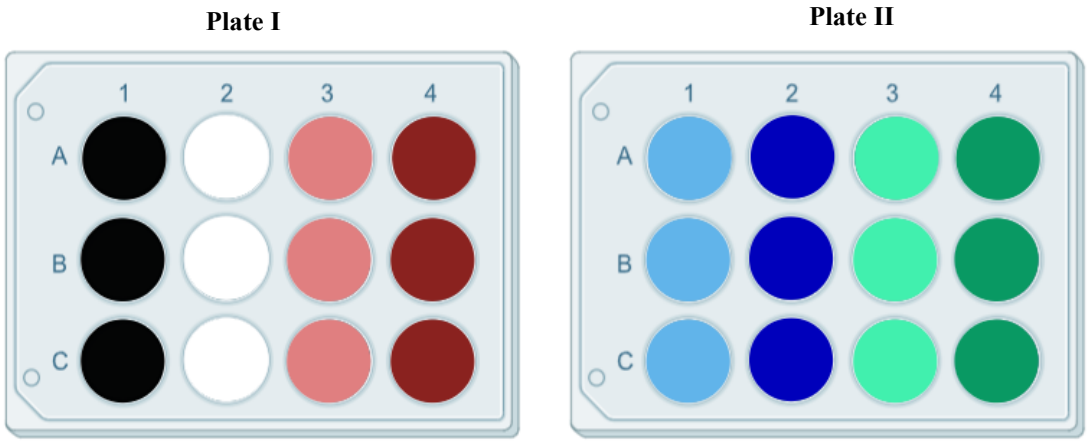

Plate	Column	Experimental Conditions
I	1	Positive control (MM)
	2	Negative control (dH_2O)
	3	Negative control (dH_2O + 27.5 mM of glucose)
	4	Negative control (dH_2O + 55 mM of glucose)
II	1	mWRM + 27.5 mM of glucose
	2	mWRM + 55 mM of glucose
	3	lWRM + 27.5 mM of glucose
	4	lWRM + 55 mM of glucose

Figure 12. Composition of the experimental media tested for *A. niger* growth in the Lunar and Martian WRM glucose supplementation assays. Percentage of concentration corresponds to v/v. mWRM: Martian Water-Released Minerals; lWRM: Lunar Water-Released Minerals; MM: Minimal Media; dH_2O: deionized sterile water.

Followingly, another set of experiments was performed to evaluate cyanobacterial biomass as a potential alternative organic nutrient source. As such, cyanobacterial biomass from the strain *Anabaena sp*. PCC 7120 was used as proof-of-concept, to identify the optimal

biomass concentration and pre-treatment method to support fungal growth. Biomass was tested in two forms: intact (NT: No Treatment) and mechanically lysed via bead beating (as described in **Section 0.**). Biomass was added at final concentrations of 0.5, 1, and 2 g/L to either Martian or Lunar WRM. Experimental conditions and respective controls are described in **Figure 13**.

Based on fungal growth performance, mechanical lysis and a biomass concentration of 1 g/L were selected as optimal conditions for fungal growth. Using these optimized parameters, three additional cyanobacterial strains – PCC 7938, LEGE X-002, and LEGE 00250 (listed in **Table 1**) – were tested, in combination with Martian or Lunar WRM. Details of the experimental design are presented in **Figure 14**.

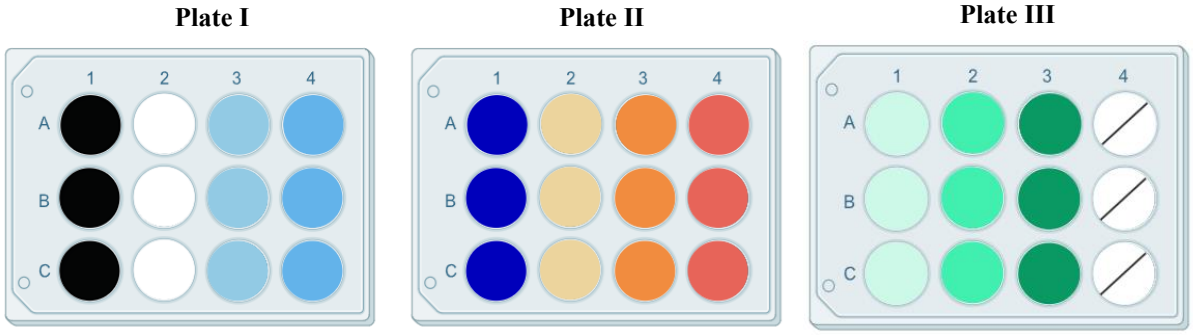

Plate	Column	Experimental Conditions	
		WRM and NT PCC 7120	**WRM and lysed PCC 7120**
I	**1**	Positive control (MM)	
	2	Negative control (dH_2O)	
	3	dH_2O + 0.5 g/L NT PCC 7120	dH_2O + 0.5 g/L lysed PCC 7120
	4	dH_2O + 1 g/L NT PCC 7120	dH_2O + 1 g/L lysed PCC 7120
II	**1**	dH_2O + 2 g/L NT PCC 7120	dH_2O + 2 g/L lysed PCC 7120
	2	mWRM + 0.5 g/L NT PCC 7120	mWRM + 0.5 g/L lysed. PCC 7120
	3	mWRM + 1 g/L NT PCC 7120	mWRM + 1 g/L lysed PCC 7120
	4	mWRM + 2 g/L NT PCC 7120	mWRM + 2 g/L lysed PCC 7120
III	**1**	lWRM + 0.5 g/L NT PCC 7120	lWRM + 0.5 g/L lysed PCC 7120
	2	lWRM + 1 g/L NT PCC 7120	lWRM + 1 g/L lysed PCC 7120
	3	lWRM + 2 g/L NT PCC 7120	lWRM + 2 g/L lysed PCC 7120
	4	-	-

Figure 13. Composition of the experimental media tested for *A. niger* growth in the Lunar and Martian WRM supplemented with *Anabaena sp.* PCC 7120 preliminary assays. NT: No Treatment. WRM: Water-Released Minerals; mWRM: Martian Water-Released Minerals; lWRM: Lunar Water-Released Minerals; MM: Minimal Media; dH_2O: deionized sterile water.

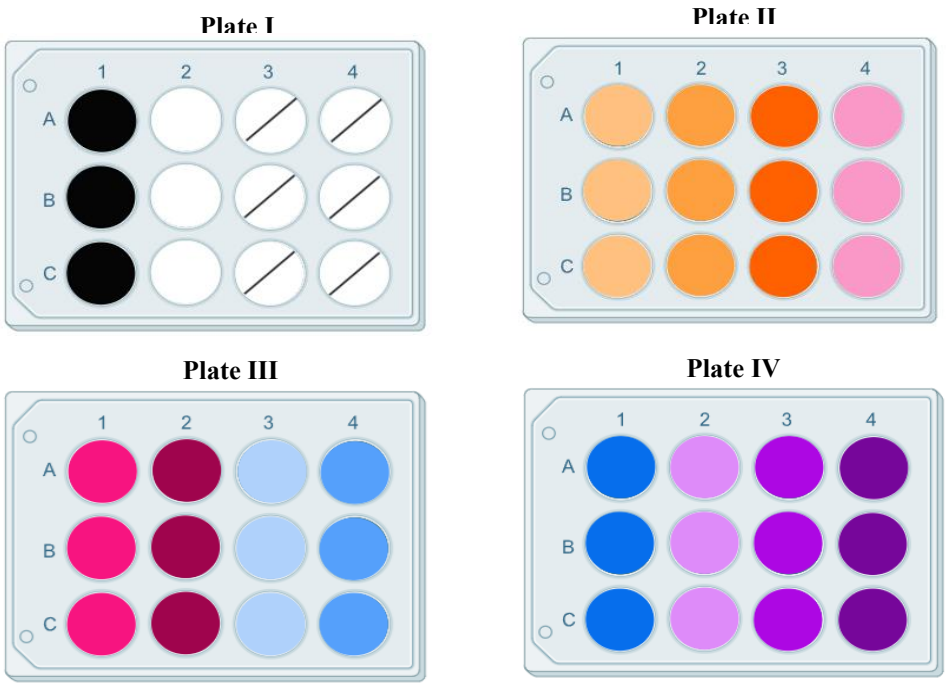

Plate	Column	Experimental Conditions
		WRM and 1 g/L lysed cyanobacterial biomass
I	1	Positive control (MM)
	2	Negative control (dH_2O)
	3	-
	4	-
II	1	dH_2O + 1 g/L lysed PCC 7120
	2	mWRM + 1 g/L lysed PCC 7120
	3	lWRM + 1 g/L lysed PCC 7120
	4	dH_2O + 1 g/L lysed PCC 7938
III	1	mWRM + 1 g/L lysed PCC 7938
	2	lWRM + 1 g/L lysed PCC 7938
	3	dH_2O + 1 g/L of lysed LEGE X-002
	4	mWRM + 1 g/L lysed LEGE X-002
IV	1	lWRM + 1 g/L lysed LEGE X-002
	2	dH_2O + 1 g/L lysed LEGE 00250
	3	mWRM + 1 g/L lysed LEGE 00250
	4	lWRM + 1 g/L lysed LEGE 00250

Figure 14. Composition of the experimental media tested for *A. niger* growth in the Lunar and Martian WRM supplemented with different strains of *Anabaena sp.*. WRM: Water-Released Minerals; mWRM: Martian Water-Released Minerals; lWRM: Lunar Water-Released Minerals; MM: Minimal Media; dH_2O: deionized sterile water.

3.5. Fungal Growth in Simulated Microgravity

To assess the impact of microgravity on fungal growth, experiments using 1 g/L of cyanobacterial lysate of *Anabaena sp.* PCC 7938 in mWRM and lWRM media were replicated in simulated microgravity conditions.

Experiments were conducted in Fluorinated Ethylene Propylene (FEP) gas-permeable bag-bioreactors (C-bags, Saint Gobain), which have previously been validated as proof-of-concept cultivation systems for altered gravity simulations (Nielsen *et al*., 2021). The input tube was aseptically cut, and sealed with Parafilm. The different experimental media, as enumerated in **Table 3**, were inoculated with 1×10^6 spores/mL of *A. niger*, to a total volume of 45 mL. A 50 mL syringe was used to transfer the mix to the FEP bag. Bubble formation was avoided by careful removal of any air with the plunger of the syringe. All conditions were tested in biological duplicates.

Bioreactor bags were incubated for 7 days at RT under simulated microgravity conditions, using a Modified IKA roller-Clinostat (IKA-Werke GmbH & Co. KG, Germany). The 2D clinostat is widely used for microgravity simulations. It operates by continuously rotating the samples at a constant speed around a horizontal axis (90º). This rotation minimizes sedimentation of cells and particles, thereby creating a functional microgravity environment (Nishimura, 2023; Thombre *et al*., 2022). For this experiment, the rotation speed was 10 rpm, to allow for buoyancy-driven functional simulation of microgravity, according to Equation 1.1 and 1.2:

$$a_c = \omega^2 r \qquad (1.1)$$

$$\omega = 2\pi \times \frac{v}{60} \qquad (1.2)$$

where a_c is the centripetal acceleration (m^2/s), ω is the angular velocity (rad/s), v is the linear velocity (rpm), and r is the radius of the trajectory. Bags were wrapped around the bars of the clinostat with clamps, as pictured in **Figure 15**. Control bags, incubated under standard Earth gravity, were set in a rack with agitation (120 rpm), at RT, next to the clinostat.

pH was measured in triplicates at the beginning and end of the period of incubation (t = 0 and t = 7 days, respectively). After the designated incubation period, cultures (biomass and supernatant) from each bag bioreactor were transferred to previously weighed sterile 50 mL Falcon tubes, and centrifuged at 6000 g for 10 minutes. Supernatants were discarded and biomass pellets were dried at 60 to 70 °C for 3 to 5 days, until constant weight was achieved. Total dry fungal biomass was determined by subtracting the weight of the empty Falcon tube from the weight of the Falcon tube containing the dried pellet.

Table 3. Composition of the experimental media tested for *A. niger* growth under simulated microgravity. All growth settings were additionally tested in control conditions (standard Earth gravity, 120 rpm of agitation). MM: Minimal Media; mWRM: Martian Water-Released Minerals; lWRM: Lunar Water-Released Minerals.

Media composition
Positive control (MM)
75% mWRM + 25% MM
75% lWRM + 25% MM
mWRM + 1 g/L lysed PCC 7938
lWRM + 1 g/L lysed PCC 7938

Figure 15. 2-D clinostat adapted for bag bioreactors, used for simulated microgravity experiments.

3.6. Citric Acid Analysis by LC-MS/MS in Full and CID Modes

To assess possible production of citric acid by *A. niger* under key growth conditions, as an indicator of its biotechnological potential in future BLSS, Liquid Chromatography–tandem Mass Spectrometry (LC-MS/MS) analysis was performed.

As such, 1 mL of the supernatant of cultures from the microgravity experiment was collected, lyophilized and resuspended in 100 µL of methanol 50% acidified with 0.1% formic acid. The analytical system used was a Liquid Chromatograph Thermo Finnigan Surveyor High-Performance Liquid Chromatography System (Thermo Scientific, MA, USA), coupled

with a Mass Spectrometry LCQ Fleet™ Ion Trap Mass Spectrometer (Thermo Scientific, MA, USA).

Methodology followed Fernández-Fernández *et al*. (2010), with some modifications due to the different mass spectrometer detector and chromatographic column. Instrument tuning was performed via direct injection of a citric acid standard (~5 µg/mL in methanol), to find the most intense detector response. The mass spectrometer operated in electrospray negative polarity mode, using full scan (50-300 m/z) and Collision-Induced Dissociation (CID) modes, allowing for both precursor ion detection and fragmentation analysis.

Optimized MS parameters included: spray voltage set to 4 kV; capillary temperature at 275 ºC; capillary voltage and tube lens maintained at 35 and 120 kV, respectively. Nitrogen gas was used as both sheath gas (35 arbitrary units) and auxiliary gas (10 arbitrary units). Pure helium was applied as the collision buffer gas.

Chromatographic separation was achieved on a ACCQ-TAGTM ULTRA C18 column (100 × 2.1 mm internal diameter, 1.7 µm particle size, serial number 04603515418405, Waters), maintained at 25 °C. A constant flow rate of 0.15 mL/min was applied, and the injected volume was 10 µL, performed in a no-waste mode. The eluents used were methanol (A) and water (B), both acidified with formic acid at 0.1% (v/v). A 15-minute isocratic program was done with 10% methanol. Mass spectra were acquired and processed using the software XcaliburTM version 2 (Thermo Scientific, MA, USA).

3.7. Statistical Analysis

All statistical analyses were performed using GraphPad Prism (version 8.0.1, GraphPad Software, San Diego, CA, USA). Fungal growth data from standard gravity experiments were tested for normality using the Shapiro-Wilk test, and all datasets conformed to a normal distribution, allowing for the use of parametric tests.

For experiments with a single independent variable – namely, fungal growth in varying dilutions of MM, mWRM and lWRM – a one-way ANOVA was applied to assess differences across groups.

For experiments with two independent variables, a two-way ANOVA was conducted, to assess the main effects of each variable and their interaction. This included the glucose supplementation assay, the cyanobacterial biomass assays and the comparison of MM diluted in water, mWRM, or lWRM. Positive controls and negative controls were separately analysed using one-way ANOVA, as performed for single independent variable experiments.

For all cases, post-hoc comparisons were performed using Tukey's test, to identify statistically significant differences between specific conditions. Statistical significance was defined as $p < 0.05$ in all analyses.

For the microgravity experiment, as only two replicates were used, it was not possible to perform a normality test. Therefore, the data was analysed using a two-way ANOVA followed by Tukey's post-hoc test, as well as a non-parametric Kruskal–Wallis test, followed by Dunn's multiple comparison test.

For pH monitorization values of experiments under standard Earth gravity, a nonparametric Friedman test was applied to assess overall differences across timepoints (day 0, day 3, and day 7), followed by Dunn's multiple comparison post-hoc test to evaluate pairwise differences. A nonparametric approach was selected as most datasets included repeated values (same pH value in different biological replicates), which thus did not follow a normal distribution. For the pH monitoring of the microgravity experiment, only two timepoints (day 0 and day 7) were available; therefore, comparisons were performed using the nonparametric Mann–Whitney test.

Chapter 4

Results and Discussion

The aim of this work was to establish a proof-of-concept for the integration of filamentous fungi in Lunar and Martian-based BLSS, using resources available *in situ*, such as regolith and cyanobacterial biomass. The choice of using cyanobacterial biomass was motivated by its central role in BLSS research, where extensive efforts have already highlighted its potential as a primary biological resource (Koehle *et al*., 2023; Santomartino *et al*., 2023; Verseux *et al*., 2016). The fungal strain selected for this work was *Aspergillus niger*, due to its remarkable resistance to space conditions and consequent extensive study within the context of space exploration (Cortesão, 2021; Koch *et al*., 2023; Romsdahl *et al*., 2018).

As previously mentioned, to demonstrate the feasibility of *A. niger* integration in BLSS, aside from assessing its survival using only resources available *in situ*, fungal growth was assessed under simulated microgravity conditions. It is worth mentioning that this work was not intended to reassess the broad range of fungal applications – which are already well documented – but rather to show that their incorporation into BLSS is technically feasible. Nonetheless, as a bridge to future work, citric acid production was additionally evaluated, serving as an example of how fungal metabolism could be harnessed within future BLSS.

4.1. Growth of Cyanobacteria

In the present study, four strains of *Anabaena sp.* (PCC 7120, PCC 7938, LEGE X-002, and LEGE 00250) were cultivated under standard laboratory conditions to assess their potential as biomass sources for *A. niger* in future BLSS. The choice of *Anabaena sp.* is correlated with its increasingly recognized potential for ISRU on Mars, particularly for oxygen production, nitrogen fixation and production of a biomass which can be used to feed heterotrophic producers (Ramalho, *et al*., 2022a; Ramalho, *et al*., 2022b).

In terms of oxygen needs, one astronaut requires approximately 1.020 kg of oxygen per day, including 2 hours of intensive exercise, which is needed to reduce bone density loss, as previously mentioned (Horneck *et al*., 2003; Stavnichuk *et al*., 2020). To meet this demand solely through cyanobacterial photosynthesis, ~1.020 kg of O_2 must be produced daily. Based on photosynthetic stoichiometry (1 mol of fixed CO_2 per 1 mol of released O_2), this corresponds to ~1.4 kg of CO_2 fixed per astronaut per day, as calculated followingly:

$$1.020\ kg\ O_2 = 1020\ g\ O_2 = \frac{1020g}{32\ g/mol} O_2 = 31.817\ mol\ O_2 \quad (2.1)$$

$$31.817\ mol\ CO_2 = 31.817\ mol \times \frac{44g}{mol} CO_2 = 1402.5gCO_2 \approx 1.4\ kg\ CO_2 \quad (2.2)$$

CO_2 fixation rates in *Anabaena sp*. depend on many factors, including growth conditions and strain specificities, but reported values range from 0.677 g CO_2 L^{-1} day^{-1} (Nayak & Das, 2013) for *Anabaena sp*. PCC 7120, to 1.01 g CO_2 L^{-1} day^{-1} for *Anabaena sp*. CH 1 (Chiang *et al*., 2011) and even up to 6.6 g CO_2 L^{-1} day^{-1} for *Anabaena sp*. ATCC 33047 (González López *et al*., 2009). Meeting astronaut O_2 needs would therefore require bioreactors with volumes of approximately 0.2-2 m^3 per crew member.

It is worth mentioning that optimizing culture parameters – such as light intensity and spectrum, aeration, mixing, temperature, and nutrient supply – could substantially improve photosynthetic performance. Coupled with bioengineering approaches to enhance photosynthetic efficiency, these strategies may enable cyanobacteria to realistically contribute to crew O_2 requirements (Hu *et al*., 2023; Luan *et al*., 2020).

In terms of biomass production, for the proposed BLSS to sustainably work long-term, the biomass output of the cyanobacterial bioreactor should match the demands of the downstream fungal reactor, guaranteeing a steady nutrient supply. Among the tested strains, PCC 7938 has been reported to present growth rates corresponding to an average of 32 mg/L/day in BG-11_0 medium (Ramalho, *et al*., 2022b). Similarly, *Anabaena sp.* PCC 7120 reportedly presents a growth rate corresponding to an average of 46 mg/L/day in standard BG-11 medium (G. Yu *et al*., 2011). Growth rate values for LEGE X-002 and LEGE 00250 could not be found in the literature. However, during this work, it was observed that biomass yield was much lower for these two strains than for PCC 7938 and 7120, likely influenced by culture conditions. Although growth rate estimations were not formally determined in this work, such data will be essential for early BLSS design, where high productivity is critical for biomass supply and oxygen generation.

Moreover, future work should investigate whether the *Anabaena* strains tested here can grow using Martian or Lunar regolith as the sole nutrient source, or whether additional supplementation is required. Previous studies demonstrated that both *Anabaena sp*. PCC 7120 and PCC 7938 can grow in Martian regolith simulants, identifying PCC 7938 as the most promising candidate for integration into Martian BLSS (Ramalho, *et al*., 2022a; Ramalho, *et al*., 2022b). LEGE-X002 and LEGE-00250 have never been evaluated under such conditions, and none of these strains have been tested with Lunar regolith simulants. Prioritizing such experiments will be essential to determine their suitability for space-based applications and to guide future BLSS design.

Taking all this into account, as cyanobacterial growth optimization was not assessed in this work, it was not possible to identify the most promising strain for BLSS. For this reason, all four *Anabaena* strains were tested as nutrient sources for *A. niger* under Earth-standard gravity conditions.

4.2. Water-Released Minerals

To assess the potential *in situ* use of regolith as a nutrient source for fungal growth, Martian and Lunar regolith simulants were used to prepare aqueous extracts, herein referred to as mWRM for Martian and lWRM for Lunar Water-Released Minerals.

The protocol employed in this work was adapted from previous studies (Fernandez *et al*., 2023; Ramalho, *et al*., 2022b). Following the centrifugation step, visual analysis of the media revealed that the supernatant still contained small mineral grains in suspension (**Figure 16**). While these residual solids are not problematic under standard Earth gravity, their presence could pose significant issues in simulated microgravity environments, since the clinorotation-induced cell buoyancy can be interrupted due the suspended particles and potentially introduce random movements to the cells (Anken, 2013).

Moreover, the WRM solutions may, in the future, also serve as nutrient sources for autotrophic organisms, such as *Anabaena sp*. (Ramalho, *et al*., 2022a; Ramalho, *et al*., 2022b). Given its phototrophic nature, the turbidity caused by suspended particles could reduce light penetration, thereby limiting photosynthetic efficiency and overall productivity. Such an effect was already predicted by Rzymski *et al*. (2023), who flooded Martian regolith simulants with water and monitored turbidity through time.

Although the filtration step increases protocol complexity, it is a critical optimization to ensure system functionality in space-based bioreactors. To reduce astronaut workload, future designs should incorporate automated filtration systems, such as filter-based separation using pressure differentials (Ibrahim *et al*., 2016, 2018).

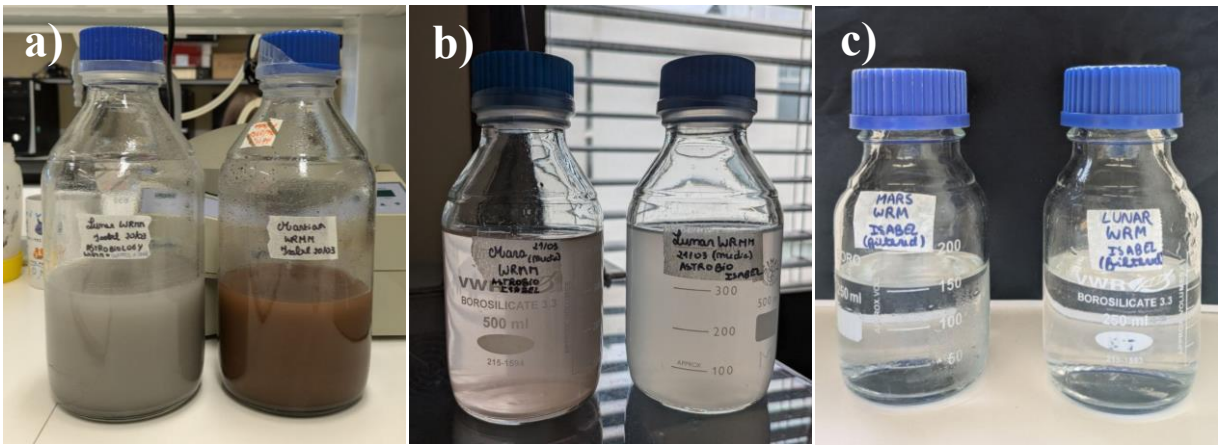

Figure 16. Preparation of Lunar and Martian Water-Released Minerals (WRM). From left to right: (a) precursors of lWRM and mWRM after 24 h of shaking; (b) precursors of mWRM and lWRM after centrifugation; and (c) mWRM and lWRM after filtration, ready for use as nutrient sources.

4.3. Cyanobacterial Cells Pre-Treatment

To ensure complete cell disruption of the different *Anabaena sp*. strains, the procedure described in Section 3.3. was performed three consecutive times, totaling 18 minutes of homogenization with 1 minute of cooling intervals between sessions. This extended treatment was necessary as initial cycles left intact filaments visible under the microscope. Upon completion, the pellets exhibited a brownish-green coloration, consistent with the release of intracellular pigments and indicative of lysis. Microscopic imaging confirmed the absence of intact cells, as illustrated in **Figure 17**, which shows *Anabaena sp*. PCC 7120 and PCC 7938 before and after cell disruption.

Protocols employing mechanical lysis have previously been used for cyanobacterial biomass preparation as a nutrient source for heterotrophic producers, in the context of BLSS. For instance, Verseux *et al*. (2021) used a liquid nitrogen-cooled mortar and pestle for manual grinding of biomass. This process, however, might not be easily translated into extraterrestrial habitats, as liquid nitrogen will most likely not be available and manual grinding is not feasible in microgravity, where material could disperse uncontrollably. Similarly, Fernandez *et al*. (2023) combined freeze-thaw cycles with bead beating, again requiring liquid nitrogen. Santos de Sousa *et al*. (2025) demonstrated that a simplified protocol relying solely on bead beating, vortexing and ice cooling was sufficient to obtain biomass capable of supporting heterotrophic growth.

The present study validated a simplified version of the protocol described by Santos de Sousa *et al*. (2025), to minimise required resources and enhance its applicability to space research. As samples remain sealed within closed Eppendorf tubes during bead beating, it mitigates the risks of dispersion in microgravity. Furthermore, a cooling system could be

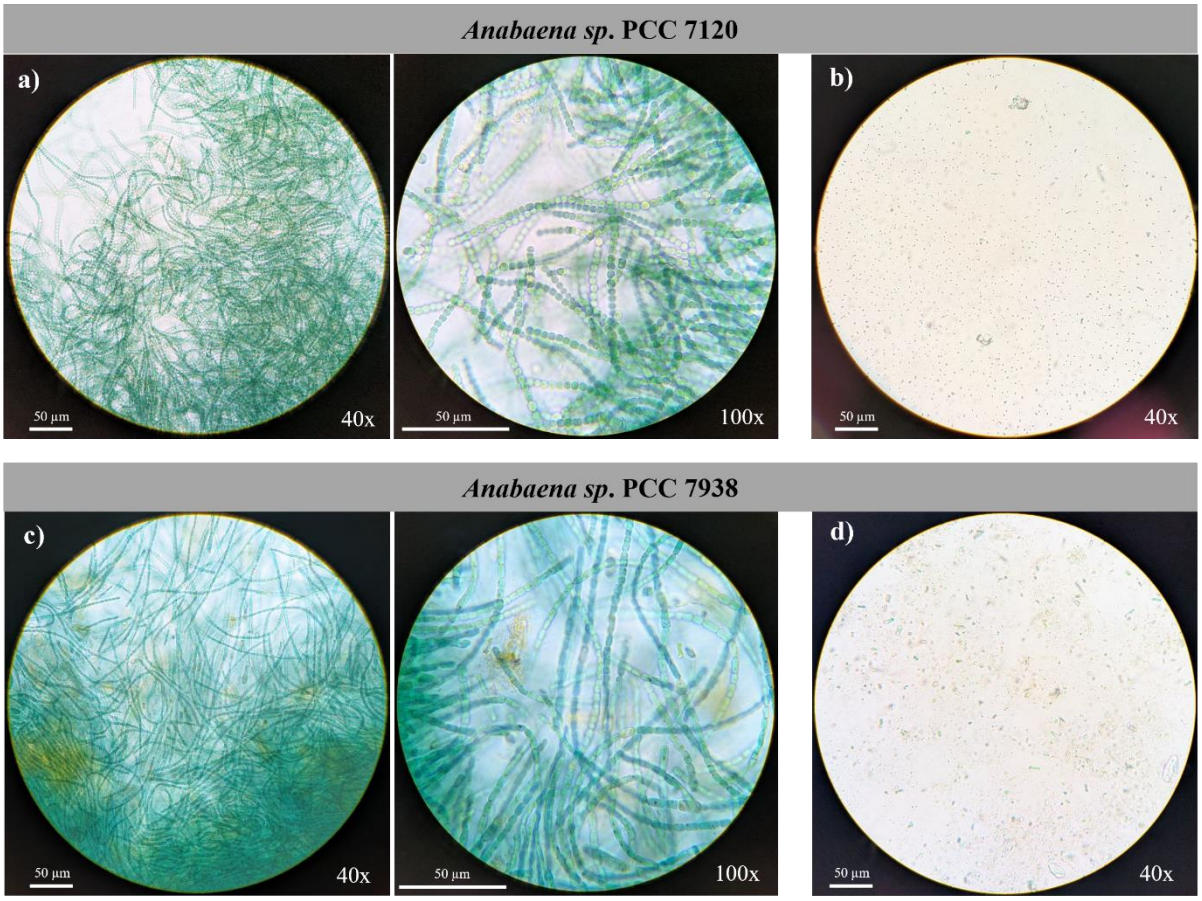

Figure 17. Microscopic images (Primostar 3, Zeiss) of: *Anabaena sp*. PCC 7120 prior to (a) and following (b) bead beating cell lysis; *Anabaena sp.* PCC 7937 prior to (c) and after (d) bead beating cell lysis.

integrated into the bead-beating itself. Centrifugation remains feasible in microgravity environments, as demonstrated by the existence of centrifuges aboard the ISS (Brinckmann, 2012). Consequently, the protocol here employed could be performed in space with relatively minor adjustments, ideally incorporating greater automation.

An alternative strategy could involve microfluidics-based lysis (Grigorov *et al*., 2021). The cyanobacterial bioreactor could be directly coupled to the fungal bioreactor, with an outlet channel containing nano-obstacles which would induce cyanobacterial cell lysis prior to biomass transfer. To ensure compatibility with the high volumes of the bioreactors, parallelization of microfluidic channels could be implemented, allowing scalability according to nutrient input demands (Yadavali *et al*., 2019).

4.4. Fungal Growth in Standard Earth Gravity

One of the main objectives of this work was to identify the most favorable growth conditions for *A. niger* using resources that could be realistically available in future Lunar and Martian habitats. In this context, minimizing the input of resources while still achieving robust fungal growth was a central priority, ensuring compatibility with closed-loop and resource-

limited BLSS. Notably, all fungal growth experiments were conducted at RT, both to reflect potential temperature fluctuations within extraterrestrial habitats and to avoid reliance on additional energy-demanding equipment for temperature control.

Following this rationale, the first step of this study was to determine the minimal requirements for fungal growth. Given the heterotrophic nature of fungi, Martian and Lunar Water-Released Minerals alone cannot sustain *A. niger* growth. Therefore, it was investigated whether supplementation of these mineral solutions with additional, readily available resources could support fungal biomass production. Once the optimal growth conditions were established, *A. niger* growth was further assessed under simulated microgravity, to validate the performance of the system under a space-relevant condition. For all assays, detailed statistical results can be found in Annex A.

4.4.1. Lunar and Martian Water-Released Minerals Media Assays

Results of the Minimal Media (MM) dilution assay are presented in **Figure 18**. Biomass data was normally distributed across all tested medium dilutions.

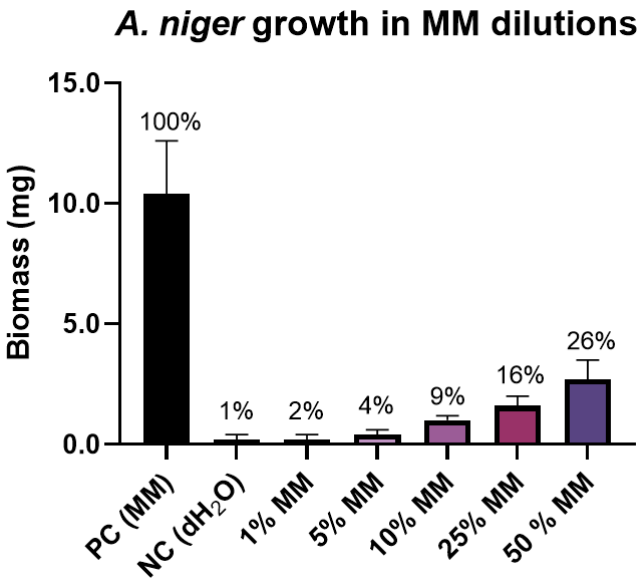

Figure 18. Dry biomass of *A. niger* acquired after 7 days of growth under standard Earth gravity in different concentrations of Minimal Media (MM), diluted in sterile water. Percentages indicate growth compared to the positive control. PC: Positive Control (100% MM); NC: Negative Control (sterile water). n=6.

According to ANOVA test results, the growth of *A. niger* across different dilutions of MM showed a strong dependence on nutrient concentration, with statistically significant differences among treatments ($p < 0.0001$). Post-hoc Tukey comparisons revealed that for low-concentration of MM, where biomass yields were similar, there was no statistically significant difference (negative control vs. 1% – 25% MM). From a bioprocessing perspective, these results highlight that extremely diluted MM (<25%) yields minimal biomass, rendering such conditions inefficient for biomass production applications.

For the Martian Water-Released Minerals (mWRM) dilution assay (**Figure 19 a)**), all groups passed normality testing. Differences in biomass yields were almost entirely explained by treatment conditions ($p < 0.0001$, one-way ANOVA). Tukey's post-hoc test revealed that undiluted or nearly undiluted mWRM (100%, 99%, and 95%) did not statistically differ from the negative control (dH_2O) in biomass yield. Significant growth differences only emerged at concentrations of mWRM lower than 90%, with biomass decreasing progressively as the mWRM concentration increased.

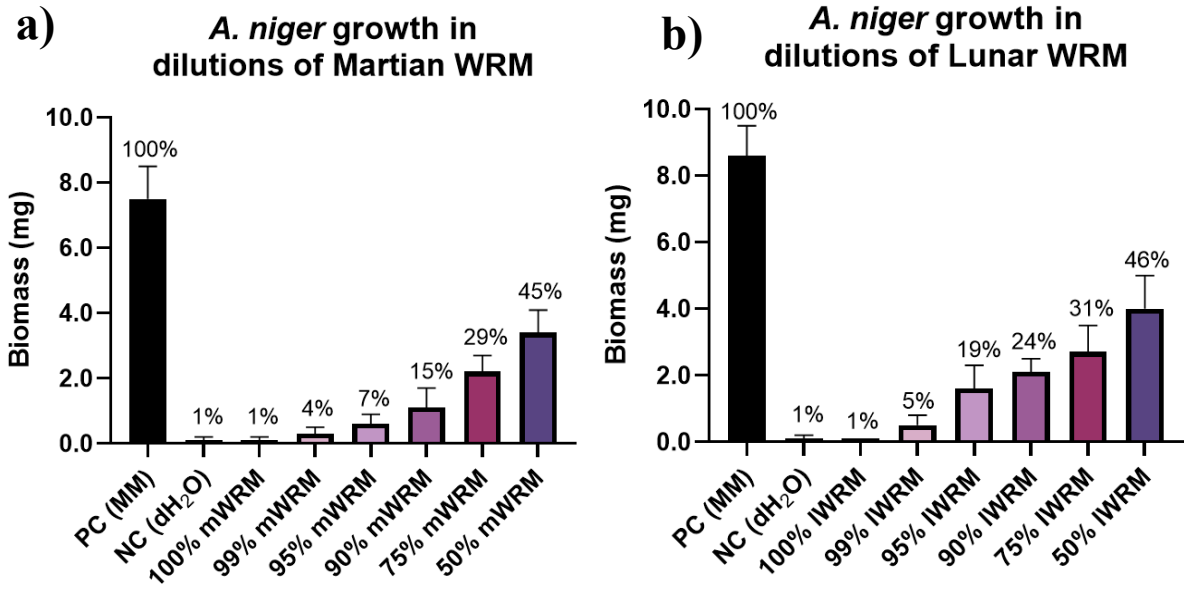

Figure 19. Dry biomass of *A. niger* acquired after 7 days of growth under standard Earth gravity in different concentrations of a) mWRM and b) lWRM, diluted in Minimal Media (MM). Percentages indicate growth compared to the positive control. PC: Positive Control (100% MM); NC: Negative Control (sterile water); WRM: Water-Released Minerals; mWRM: Martian Water-Released Minerals. lWRM: Lunar Water-Released Minerals. n=6.

The same interpretation unfolded in the Lunar Water-Released Minerals (lWRM) dilution assay (**Figure 19 b)**), where the treatment was statistically confirmed to be the primary determinant of biomass yield ($p < 0.0001$, one-way ANOVA). Similarly to mWRM dilution assay, post-hoc Tukey's test revealed no significant differences between negative control and high concentrations of lWRM (100%, 99%) were present.

These results, therefore, support the hypothesis that undiluted or minimally diluted lWRM and mWRM are unable to sustain measurable growth of *A. niger*. This observation is consistent with the organism's heterotrophic metabolism, rendering it incapable of surviving solely on the inorganic nutrient profile of the regolith simulants.

When comparing fungal biomass yields between MM diluted in water, lWRM or mWRM, a growth advantage was observed in the regolith-supplemented treatments across all MM concentrations. A two-way ANOVA confirmed that both the dilution medium (water, mWRM, or lWRM) and MM concentration significantly influenced *A. niger* growth

($p < 0.0001$). Moreover, the effect of MM concentration on growth was dependent on the dilution medium used ($p = 0.0313$). Tukey's post-hoc analysis showed that, at lower MM concentrations (0–1%), there were no significant differences between media types. This implies that minimal supplementation levels are insufficient to reveal performance differences between water and WRM. From 5% MM onwards, however, growth in lWRM was significantly higher than in water. Although **Figure 18** and **Figure 19** suggest that MM supplemented with mWRM tends to yield higher average biomass than MM diluted in water, the post-hoc analysis confirmed that this difference is not statistically significant. For instance, for 50% MM, biomass yields were 2.7 ± 0.8 mg when diluted in water, 3.5 ± 0.7 mg in mWRM and 4.0 ± 1.0 mg in lWRM.

The superior performance of lWRM over mWRM may be rooted in the mineralogical differences of the regolith simulants used. Indeed, as previously shown in **Table 2**, the Lunar regolith simulant contains higher levels of most oxides, potentially offering a more balanced mineral profile for fungal metabolism. In contrast, the Martian simulant presents considerably higher FeO (10.60% vs. 3.20% in the Lunar regolith simulant) which may become detrimental for fungal growth at elevated concentrations by inducing oxidative stress through fungal ferroptosis (Abdel-Wareth & Abdel-Wahed, 2018; Shang *et al*., 2025).

While the observed differences in *A. niger* growth between lWRM and mWRM can be attributed, at least in part, to their distinct mineralogical compositions, the specific mechanisms underlying these effects require further investigation. It is also noteworthy that the Martian regolith simulant used in this study was not supplemented with perchlorates, which are naturally present in the Martian surface and are known to exhibit strong toxicity toward most life forms, including *A. niger* (Fischer *et al*., 2024).

These findings suggest that, although *A. niger* cannot grow solely on the inorganic nutrients present in regolith simulants, it can utilize some of these nutrients when combined with MM, resulting in a synergistic enhancement of biomass production. Thus, replacing water with WRM can enhance *A. niger* growth, with lWRM providing the most pronounced benefit.

Nonetheless, to ensure the viability of the proposed BLSS module without reliance on resupply of MM from Earth, it will be necessary to identify alternative organic nutrient sources to complement WRM. In this study, glucose supplementation was first evaluated to assess its potential as a replacement for MM. Subsequently, biomass from *Anabaena sp*. was tested as an alternative nutrient source. In future work, other options – such as plant biomass or organic waste derived from crew activities – could also be considered to further close the nutrient loop in the system.

4.4.2. Supplementation of Lunar and Martian Water-Released Minerals with Different Organic Sources

The glucose supplementation assay aimed to evaluate whether the addition of a simple organic carbon source could substitute the role of MM in sustaining *A. niger* growth, when coupled with WRM. The glucose concentrations used in this experiment were calculated based on the amount present in MM. To replicate this concentration in the 3mL total volume of the multi-well plates, 60 µL of glucose were added to achieve the same concentration of 55 mM. Additionally, a lower concentration of 27.5 mM glucose was tested.

Biomass yields showed a marked decrease in all conditions compared to the positive control (MM, 9.1 mg), with values ranging between 0.1 mg and 0.5 mg (**Figure 20**).

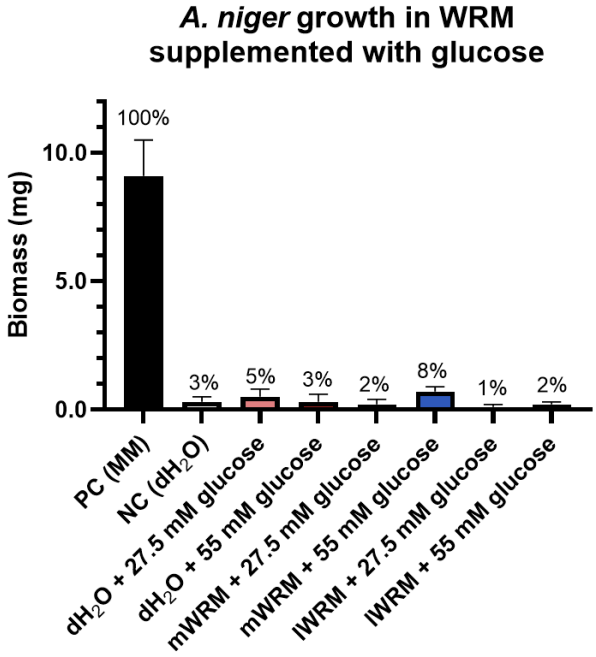

Figure 20. Dry biomass of *A. niger* acquired after 7 days of growth under standard Earth gravity in Martian or Lunar Water-Released Minerals, supplemented with different concentrations (55 mM, 27.5 mM) of glucose. Percentages indicate growth compared to the positive control. PC: Positive Control (100% MM); NC: Negative Control (sterile water); MM: Minimal Medium; WRM: Water-Released Minerals; mWRM: Martian Water-Released Minerals; lWRM: Lunar Water-Released Minerals. n=6.

Two-way ANOVA results suggest that both the media (water, lWRM or mWRM, $p = 0.0015$), the glucose concentration (27.5 mM or 55 mM, $p = 0.0416$) and their interaction ($p = 0.0006$) presented a significant effect on determining fungal growth. This indicates that the effect of glucose supplementation on growth depends on the type of medium used (water, lWRM or mWRM). Tukey's post-hoc analysis further highlighted that, at 27.5 mM of glucose, *A. niger* growth in lWRM was significantly lower than in water ($p = 0.0108$). Conversely, at 55 mM of glucose, mWRM supported significantly higher biomass than both lWRM ($p = 0.0002$) and water ($p = 0.0017$), although not enough to be desirable as an alternative to MM.

Although statistical significance was detected between some growth conditions, the practical relevance of these differences is limited, as the biomass values were exceedingly low and approached the limit of detection of the weighing scale used. Thus, quantitative results should be interpreted with caution, due to the high relative error associated with measurements near the method's limit of detection.

Overall, these results demonstrate that glucose supplementation alone is insufficient to sustain *A. niger* growth, at levels comparable to those obtained with MM. This further reinforces the need for more complex nutrient sources, such as cyanobacterial or plant-derived biomass. These findings are consistent with previous reports, which demonstrated that glucose alone is insufficient to support *A. niger* conidial germination, and that lower glucose concentrations result in a reduced proportion of germinating spores (Ijadpanahsaravi *et al*., 2021).

As such, the next step was testing whether cyanobacterial biomass could substitute MM as an organic nutrient source, when glucose alone failed. As mentioned in Chapter 3, *Anabaena sp*. PCC 7120 was used for preliminary experiments, and three biomass concentrations (0.5 g/L, 1 g/L, and 2 g/L, dry weight) were tested. Reported concentrations of cyanobacterial biomass used to support heterotrophic growth vary widely in the literature, ranging from 1 g/L (Fernandez *et al*., 2023), 13-14 g/L (Markou *et al*., 2013), 25 g/L (Verseux *et al*., 2021), and up to 80 g/L (Karatay, 2015). As such, in this study, lower concentrations were chosen, due to the slower growth rates of the used cyanobacterial strains compared to *A. niger*, ensuring potential long-term compatibility within a BLSS framework.

Two variables were simultaneously assessed: different concentrations of PCC 7120 and different pretreatments of the biomass (no treatment, NT; and mechanically lysed cells, **Figure 22**). Normality was verified for all groups of data. Two-way ANOVA revealed that both biomass pretreatment and its concentration, as well as their interaction, had statistically significant effects on fungal growth ($p < 0.0001$).

Tukey's post-hoc analysis showed that NT PCC 7120 did not result in significant differences compared to the negative control. Consistently, as illustrated in **Figure 21 b)** and **Figure 22**, fungal biomass accumulation under NT conditions was negligible, confirming that untreated cyanobacterial biomass cannot sustain *A. niger* growth.

In contrast, lysed PCC 7120 supported higher fungal biomass (**Figure 21 b)**). At both 1 g/L and 2 g/L, lysed biomass diluted in mWRM or lWRM promoted statistically higher *A. niger* growth compared to the corresponding NT groups ($p < 0.0001$). Importantly, the same concentrations diluted in water did not achieve similar results. At 1 g/L, lysed PCC 7120 in mWRM and lWRM supported significantly higher fungal growth than in water ($p < 0.0001$ and $p = 0.0118$, respectively). No statistical differences were observed between

1 g/L lysed PCC 7120 in lWRM and the same concentration in mWRM, nor between 2 g/L lysed PCC 7120 in the two media. Similarly, within each medium, fungal biomass did not differ significantly between 1 g/L and 2 g/L of lysed PCC 7120.

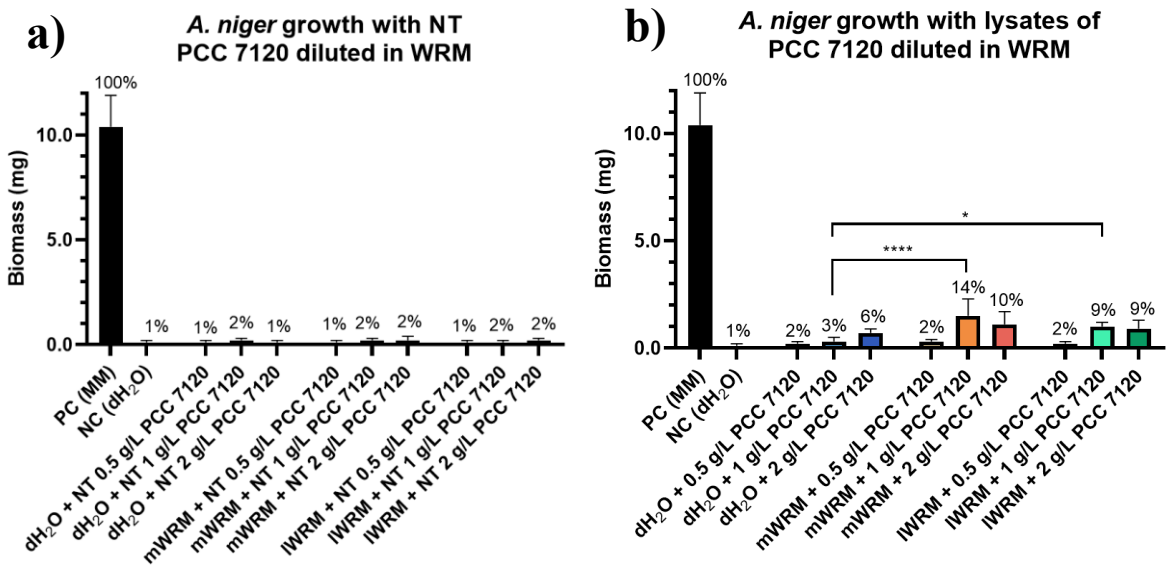

Figure 21. Dry biomass of *A. niger* acquired after 7 days of growth under standard Earth gravity in a) *Anabaena sp.* PCC 7120, whether with no pre-treatment or b) previously lysed, diluted in sterile water, Martian or Lunar Water-Released Minerals. Percentages indicate growth compared to the positive control. PC: Positive Control (100% MM); NC: Negative Control (sterile water); MM: Minimal Medium; WRM: Water-Released Minerals; mWRM: Martian Water-Released Minerals. lWRM: Lunar Water-Released Minerals. NT: No Treatment. n=6.

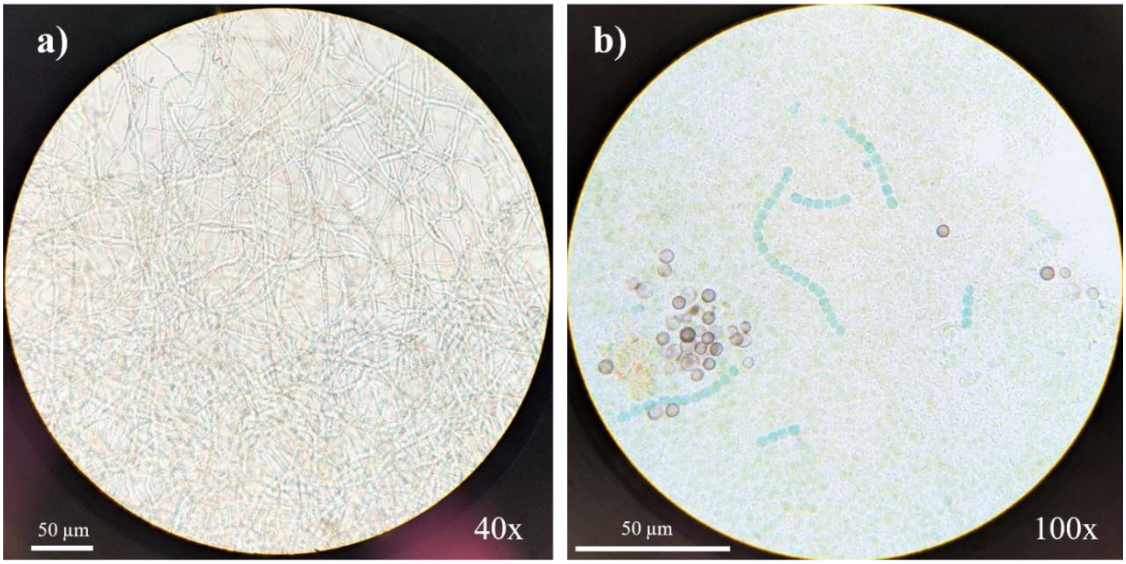

Figure 22. Microscopic images (Primostar 3, Zeiss) of *A. niger* grown in a) Minimal Media (positive control) and b) *Anabaena sp.* PCC 7120 without pretreatment, diluted in water.

These results indicate that both the pretreatment of cyanobacteria through cell lysis and the presence of mWRM or lWRM are essential to support the growth of *A. niger*. It is further possible to conclude that increasing the biomass concentration from 1 g/L to 2 g/L does not confer additional benefits.

Given these results, subsequent experiments with the other three selected *Anabaena sp.* strains were performed using a pretreatment step (cell lysis) and a final cyanobacterial biomass concentration of 1 g/L. This concentration was chosen over 2 g/L, as no statistically significant differences in *A. niger* growth were observed between 1 g/L and 2 g/L of lysed PCC 7120. Furthermore, to minimize biomass consumption, and thus ensure future compatibility in a BLSS, preserving cyanobacteria for additional applications such as oxygen production and biohydrogen generation is highly relevant. The concentration of 0.5 g/L was excluded, as it did not result in significant growth in either lWRM or mWRM.

Results of the experiments with the four *Anabaena sp.* strains are shown in **Figure 23** and **Figure 24**. Normality of each dataset was confirmed. The two-way ANOVA revealed that the main determinant of *A. niger* growth was the cyanobacterial strain used as a nutrient source, which accounted for 76.6% of the total variation in the dataset ($p < 0.0001$). In contrast, neither the culture medium (water, mWRM or lWRM) nor the interaction between cyanobacterium and medium had a significant effect on fungal growth ($p = 0.27$ and $p = 0.44$, respectively). This indicates that the choice of cyanobacterial strain is the primary driver of biomass availability for fungal metabolism, while the surrounding medium composition has a comparatively negligible influence under the tested conditions.

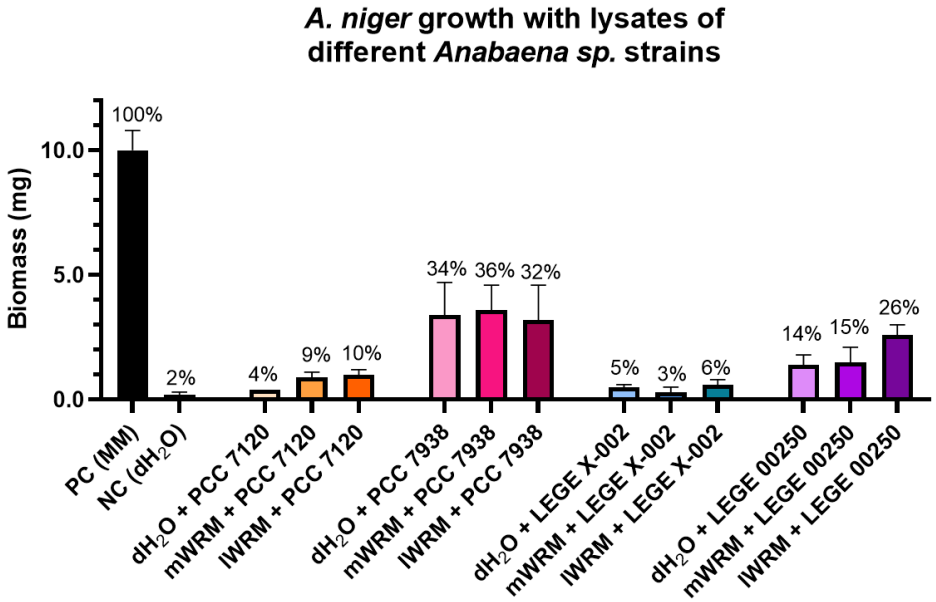

Figure 23. Dry biomass of *A. niger* acquired after 7 days of growth under standard Earth gravity with different strains of lysed *Anabaena sp*. at a final concentration of 1 g/L, diluted in sterile water, Martian or Lunar Water-Released Minerals. Percentages indicate growth compared to the positive control. PC: Positive Control (100% MM); NC: Negative Control (sterile water); MM: Minimal Medium. mWRM: Martian Water-Released Minerals. lWRM: Lunar Water-Released Minerals. n=3.

Post-hoc Tukey's test consistently identified PCC 7938 as the superior strain across all media (**Figure 23**). In water, mWRM and lWRM, PCC 7938 supported significantly higher *A. niger* growth compared to PCC 7120, LEGE X-002 and LEGE 00250 – which achieved comparable results between them. The only exception was observed between PCC 7938 and LEGE 00250 diluted in lWRM, where no significant difference was detected ($p = 0.7276$).

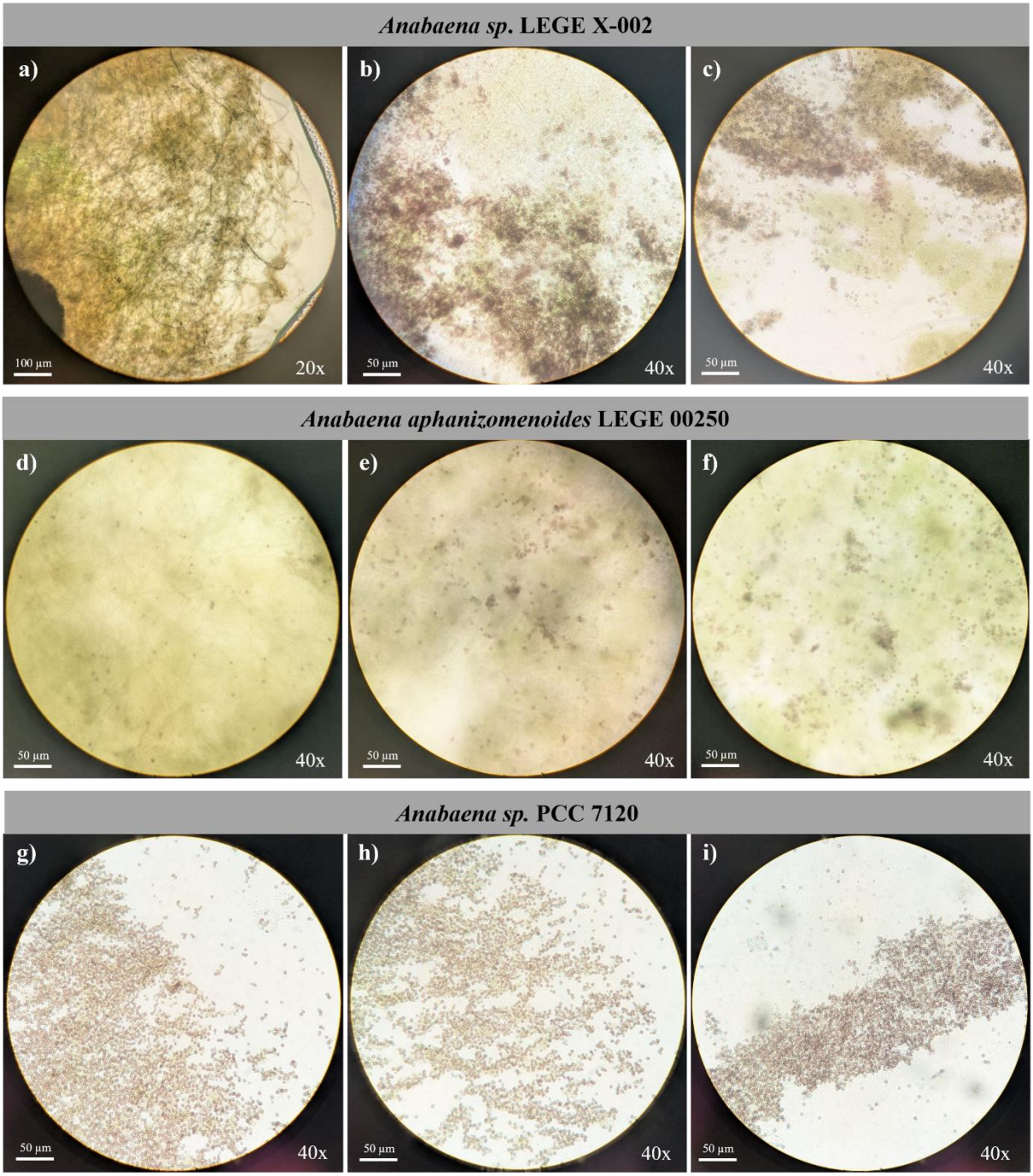

Figure 24. Microscopic images (Primostar 3, Zeiss) of *A. niger* grown with lysates of: *Anabaena sp*. LEGE X-002 diluted in a) water, b) lWRM or c) mWRM; *Anabaena aphanizomenoides* LEGE 00250 diluted in d) water, e) lWRM and f) mWRM; *Anabaena sp.* PCC 7120 diluted in g) water, h) lWRM and i) mWRM.

When considering the fungal biomass yield, lysed PCC 7938 yielded on average 3.4 mg in water, 3.6 mg in mWRM, and 3.2 mg in lWRM. In contrast, PCC 7120 and LEGE X-002 achieved only around 0.3–1.0 mg of biomass depending on the medium, while LEGE 00250 reached 1.4 mg in water, 1.5 mg in mWRM, and 2.6 mg in lWRM. Thus, PCC 7938 consistently sustained the highest fungal biomass across conditions, reaching values twofold higher than any other strain.

Overall, these results demonstrate that *A. niger* can grow using resources that could be locally available in future Lunar and Martian habitats, thereby fulfilling the first objective of this dissertation. However, to select the most suitable *Anabaena* strain to support fungal growth, several factors must be taken into consideration, aside from the presented results of the biomass yield.

Firstly, as previously mentioned, culture conditions directly influence cyanobacterial biomass composition, which, in turn, determines the availability of nutrients for fungal development (González-Fernández & Ballesteros, 2012; Kushwaha *et al*., 2018). Therefore, to confidently select the best strain to support *A. niger* growth, an optimization of cyanobacterial growth conditions should have been performed.

Secondly, the safety of the selected strain is highly relevant for future BLSS applications. Among the tested strains, LEGE X-002 produces anatoxin, a cytotoxin with harmful effects against humans (Sivonen *et al*., 1989). This trait deems LEGE X-002 particularly undesirable for future BLSS, as potential risks can be avoided by selecting a non-toxic alternative.

Finally, it is essential that the chosen strain can be cultivated on *in situ* resources. It was already mentioned that both PCC 7120 and PCC 7938 can grow on Martian regolith simulant. Particularly, PCC 7938 has been established as a model organism for ISRU on Mars, with its lysates at a concentration of 25 g/L being able to support overnight *Escherichia coli* growth to levels significantly higher than in the standard growth medium of *E. coli* (Ramalho, *et al*., 2022a). In the present study, fungal growth on PCC 7938 lysates achieved approximately 30% of the positive control. This may be correlated with the substantially lower concentrations of cyanobacterial biomass used – an additional parameter that could be optimized in future work.

Taking all of this into account, PCC 7938 was thus selected as the most promising strain to support *A. niger* growth. This choice was based not only on its superior performance in sustaining fungal biomass under the tested conditions, but also on its demonstrated ability to grow on Martian regolith simulant and its non-toxic nature. As such, biomass of PCC 7938 was used as a nutrient source for *A. niger* in the final experiment – the microgravity test.

4.5. Microgravity Experiment

The final test performed involved the growth of *A. niger* under simulated microgravity, using the optimal growth conditions previously identified: lysed PCC 7938 at a final concentration of 1 g/L, diluted in either mWRM or lWRM. For comparison, additional conditions of 75% mWRM or lWRM in MM were also included.

The ultimate goal of this dissertation is to demonstrate the feasibility of incorporating fungi into Lunar and Martian BLSS. Consequently, it would be highly relevant to investigate fungal growth under Lunar and Martian gravity. Nonetheless, priority was given to testing growth under microgravity, as it represents a more extreme condition. Demonstrating successful growth in microgravity would provide strong evidence for a higher likelihood of survival and adaptability under comparatively milder gravitational environments, such as those of the Moon and Mars.

After the growth period, macroscopic morphological differences were observed in cultures grown in simulated microgravity and standard Earth gravity (**Figure 25**). Under normal gravity, *A. niger* tended to form a biofilm that adhered to the inner surfaces of the bag bioreactor, while under simulated microgravity, fungal biomass aggregated into clumps suspended in the medium. This outcome was expected, as the absence of sedimentation in microgravity reduces cell contact with the bag surface, thus hindering adhesion and biofilm formation.

Results of the fungal biomass obtained after 7 days of growth are presented in **Figure 26**. As previously stated, no normality test was performed due to the limited number of biological replicates (n = 2). Consequently, both parametric and non-parametric analyses were carried out, to ensure a robust interpretation of the data. Detailed results of the statistical analysis can be found in Annex A.

The two-way ANOVA revealed that media composition was the dominant factor influencing the biomass yield of *A. niger* ($p < 0.0001$), explaining more than 96% of the observed variation. In contrast, gravity did not significantly affect biomass accumulation ($p = 0.65$). A small but significant interaction effect ($p = 0.035$) was detected, indicating that while gravity itself was not a primary driver of growth, it may subtly modulate the effects of specific media formulations. Tukey's post-hoc analysis revealed no statistically significant differences between growth under identical media conditions but different gravity regimes.

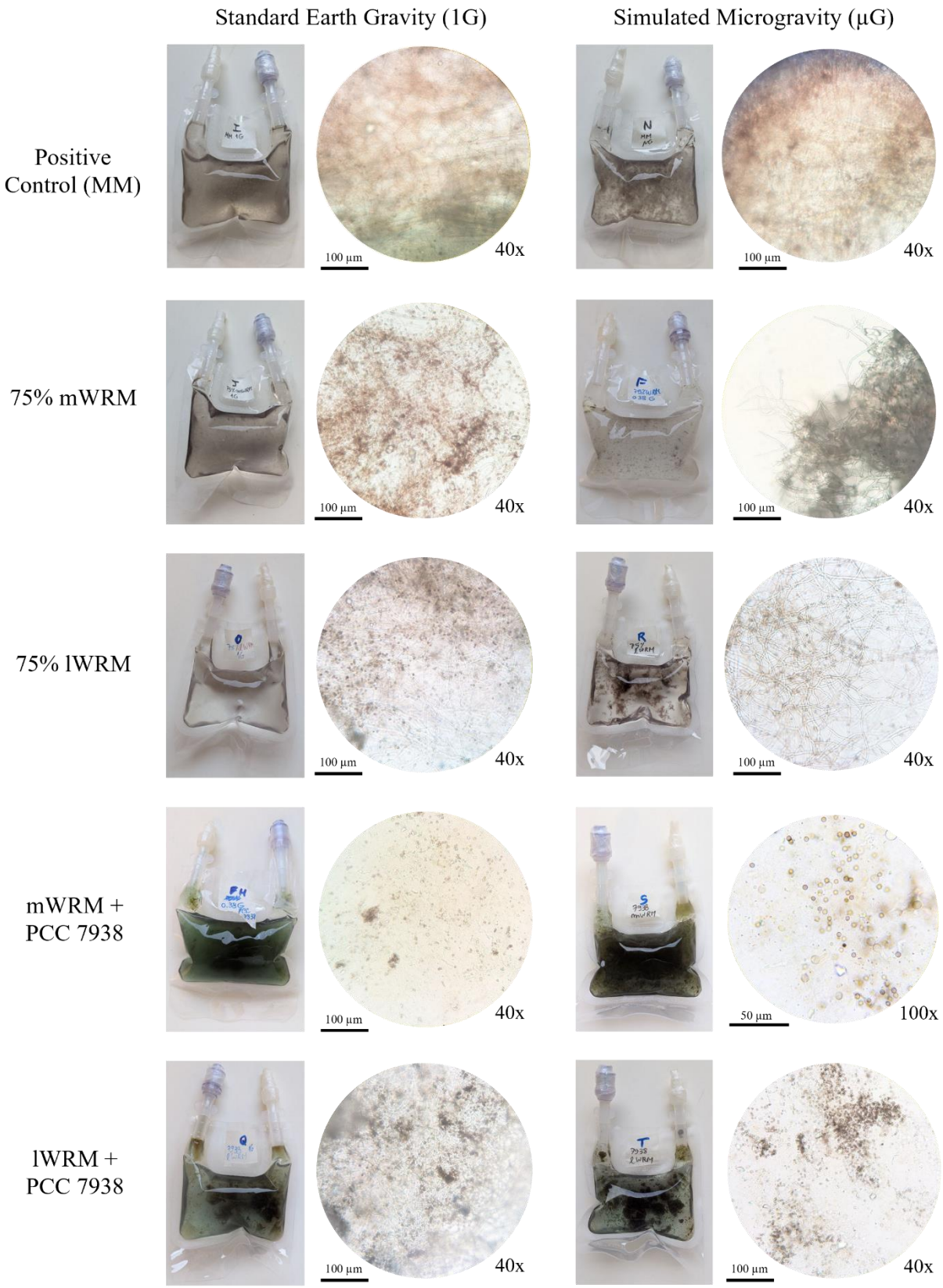

Figure 25. Bioreactor bags used in the simulated microgravity experiment, alongside optical microscopy images (Primostar 3, Zeiss) showing the growth of *A. niger*. The composition of the culture media and the gravity condition (standard Earth gravity or simulated microgravity) are indicated for each sample. Microscopy images include the corresponding magnification. MM: Minimal Media. mWRM: Martian Water-Released Minerals. lWRM: Lunar Water-Released Minerals.

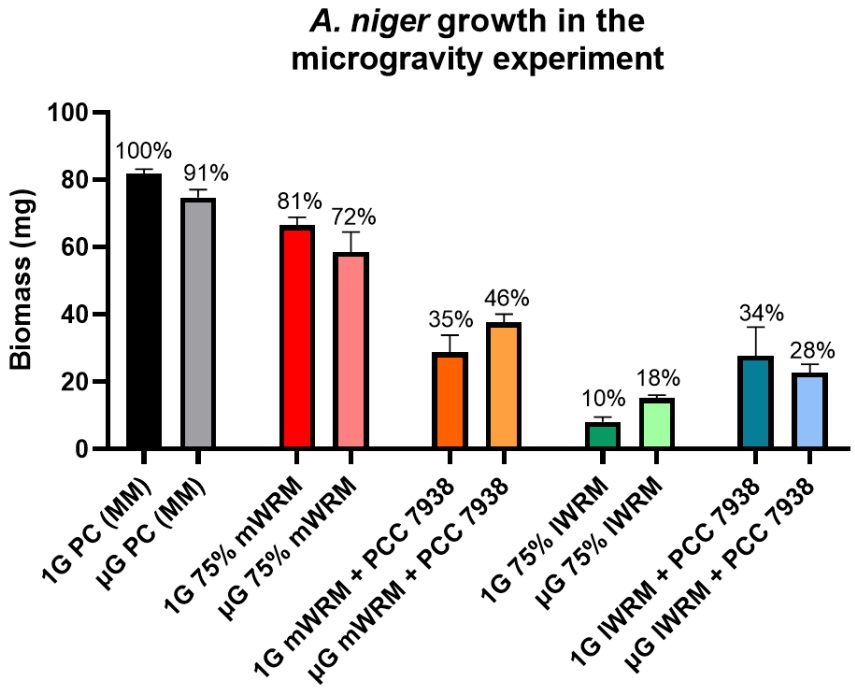

Figure 26. Dry biomass of *A. niger* acquired after 7 days of growth under standard Earth gravity or simulated microgravity, with lysed *Anabaena sp*. PCC 7938, at a final concentration of 1 g/L, diluted in sterile water, Martian or Lunar Water-Released Minerals. Percentages indicate growth compared to the positive control. PC: Positive Control (100% MM); MM: Minimal Media; mWRM: Martian Water-Released Minerals; lWRM: Lunar Water-Released Minerals; µG: Microgravity; 1G: Standard Earth Gravity. n=2.

Both at 1G and µG, biomass yield was significantly higher in 75% mWRM compared to 75% lWRM. This result contrasts with the previous conclusion from the lWRM and mWRM dilution assays, where fungal growth was significantly higher in lWRM. Indeed, when comparing results of the microgravity experiment with preliminary multi-well plates experiments, a statistically significant difference is observed. Normalization of biomass values to the respective positive controls revealed a statistically significant increase in fungal growth in 75% mWRM within FEP bag bioreactors, but a significant decrease of growth in 75% lWRM (see Annex A, **Figure 30** for detailed results).

These discrepancies could be related to differences in culture hardware. In this experiment, FEP bag bioreactors were used, which provide greater oxygen availability and increased surface area for fungal growth compared to multi-well plates (Saint-Gobain, 2022). These conditions may have favored mWRM over lWRM. In fact, no significant difference was observed between the positive control and the 75% mWRM media formulation, under 1G. However, to test these hypotheses, an examination of the mineralogical composition of both mWRM and lWRM would need to be performed, to assess its composition and potential beneficial or toxic effects on fungal growth.

Conversely, there was no statistical difference between growth in mWRM supplemented with PCC 7938 and lWRM supplemented with PCC 7938, both in normal and

microgravity. These results are in line with the previous experiment (**Figure 23**), where the presence of cyanobacterial biomass appeared to mitigate the effect of the diluent medium on fungal growth.

To complement the ANOVA, a non-parametric Kruskal–Wallis test was also performed. This analysis confirmed the presence of significant differences between groups ($p = 0.0288$, $H = 18.60$, $df = 9$), thereby supporting the conclusions obtained through the parametric approach. Post-hoc analysis using Dunn's multiple comparisons results yielded results largely consistent with Tukey's test, with the only relevant difference being the absence of statistical difference between 75% mWRM and 75% lWRM in microgravity; and between the positive control in 1G and 75% mWRM in microgravity.

Previous research had proved that *A. niger* N402 – the strain used in this work – was able to grow under simulated microgravity on solid media, exhibiting increased spore production (Cortesão *et al*., 2022). Similarly, S. Zhang *et al*. (2025) reported that exposure to real microgravity for 90 days aboard the Chinese Space Station promoted the growth of *A. niger* ATCC 16404 on solid media.

The results of this experiment indicate that *A. niger* is also unaffected by microgravity when cultivated in liquid culture, further demonstrating its viability for use in automated bioreactors within future BLSS. Moreover, these results confirm that *Anabaena sp*. PCC 7938 can support *A. niger* growth under microgravity, at lysate concentrations as low as 1 g/L diluted in Martian or Lunar WRM. Therefore, this fulfills the second objective traced for this dissertation.

It is, nonetheless, relevant to acknowledge that this experimental work is a proof-of-concept, subject to certain limitations. Regarding data reliability, only duplicate experiments were performed, and additional replicates would be necessary to strengthen confidence in the results. Furthermore, other environmental stressors, such as radiation, must be evaluated to determine whether *A. niger* growth in liquid media remains sufficiently unaffected under space conditions. Extending fungal cultivation to longer periods with intervallic renewal of nutrient sources is also essential to assess the feasibility of maintaining continuous growth. Finally, it would also be relevant to analyse the fungal biomass composition, to further study its biochemical profile and determine the potential presence of toxic molecules.

4.6. Citric Acid Production

A. niger is industrially exploited as a cell factory for citric acid production, which is extensively used as a food preservative (Behera, 2020). In the context of BLSS, food preservation becomes particularly relevant, given the limited availability of fresh products from onboard cultivation and the need to consume them within a limited timeframe before spoilage. Another key application of citric acid is in the pharmaceutical industry, namely as a stabilizing or flavouring agent of prescription medications (Vandenberghe *et al*., 2017). This can also be relevant for long-term extraterrestrial human settlements.

Given these reasons, citric acid was selected as a model metabolite to illustrate one of the potential applications of *A. niger* in future Martian and Lunar BLSS. To evaluate its production, two complementary approaches were performed: pH monitorization and direct detection with LC-MS/MS.

The production of organic acids by *A. niger* typically results in acidification of the growth medium (Odini, 2017). Accordingly, the pH of all experiments was measured at day 0, 3, and 7, with the exception of the microgravity experiment, where measurements were performed only at day 0 and 7. **Figure 27** presents the experimental culture conditions in which pH alterations were detected (results of statistical analysis are provided in the Annex B); for all other conditions, pH remained neutral (7) at all timepoints.

As expected, a pH decrease was consistently observed in the positive control (100% MM) of all experiments. For MM diluted in water, mWRM, or lWRM (**Figure 27 a) – c))**, the conditions that exhibited a pH reduction were those corresponding to higher MM concentrations, which were precisely the conditions under which higher fungal biomass was obtained. This likely reflects greater metabolic activity and, consequently, increased production of organic acids.

In the glucose supplementation experiment, however, the results were strikingly different (**Figure 27 d)**). While fungal growth was negligible, a pronounced decrease in pH was consistently observed across all glucose-containing media, including the glucose–water control. This effect suggests that the pH shift did not arise from chemical interactions between glucose and the WRM, but rather from fungal metabolic activity. It is likely that a fraction of spores underwent limited germination, enabling glucose uptake and triggering metabolic processes. However, in the absence of essential nutrients, biomass development was hindered. Carbon flux may have been redirected into overflow metabolism, where glucose catabolism exceeds the capacity of the tricarboxylic acid cycle and respiratory pathways. This imbalance likely resulted in the secretion of organic acids and proton extrusion, resulting in media acidification (Legiša & Mattey, 2007).

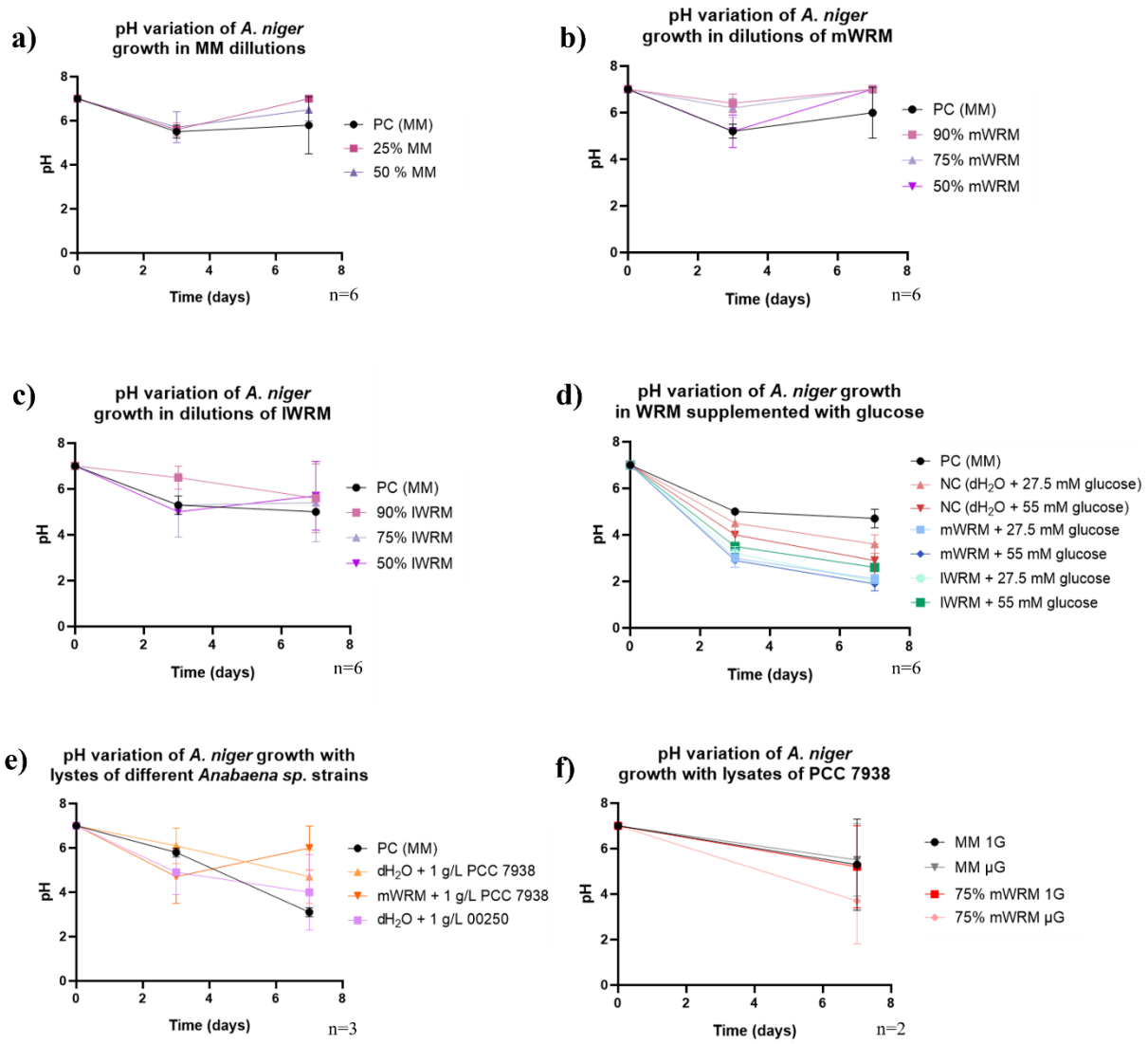

Figure 27. pH variations observed during *A. niger* growth under different conditions: a) MM dilutions; b) dilutions of mWRM; c) dilutions of lWRM; d) MM supplemented with glucose; e) lysates of different strains of *Anabaena sp*.; and f) lysates of PCC 7938, in Standard Earth Gravity or simulated microgravity. PC: Positive Control (100% MM); NC: Negative Control (sterile water); MM: Minimal Medium; mWRM: Martian Water-Released Minerals; lWRM: Lunar Water-Released Minerals. 1G: Standard Earth Gravity; µG: Simulated Microgravity. pH measurements were performed with technical triplicates. Number of biological replicates are represented next to each graph.

Regarding experiments of fungal growth with cyanobacterial lysates, although PCC 7120 was able to support *A. niger* growth, no measurable pH changes occurred, suggesting that the amount of organic acid secreted was below the detection threshold of pH measurements with strips. pH decreases were only detected with PCC 7938 (in water and mWRM) and LEGE 00250 (in water) (**Figure 27 e)**).

Finally, for the microgravity experiments, pH decreases were observed only for the positive control and for the 75% mWRM condition, both under 1G and µG, although variations were not statistically significant (**Figure 27 f)**). The lack of difference between standard Earth gravity and microgravity conditions further supports the earlier hypothesis that *A. niger*

metabolism in liquid culture is not substantially affected by microgravity within the tested timeframe.

It is further relevant to mention that, in some cases, pH initially decreased from day 0 to day 3, but increased again from day 3 to day 7. This phenomenon has previously been reported (Cai *et al*., 2013) and is likely correlated with a metabolic shift associated with sugar availability in the medium. During early growth, under conditions of abundant sugar availability, fungi often undergo overflow metabolism, secreting large amounts of organic acids and decreasing the medium pH. Once the available sugars are depleted, these organic acids can themselves be metabolized by the fungi, leading to a subsequent increase in pH (Legiša & Mattey, 2007; Netik *et al*., 1997; Upton *et al*., 2017).

Overall, it is important to consider that pH was measured using pH strips, which are less precise than other analytical methods. Therefore, conditions where pH decreases were not observed or they were not statistically significant may reflect the limitations of colour-based pH measurement rather than a true absence of organic acid production.

Because pH monitoring alone cannot distinguish between citric acid and other organic acids (e.g., gluconic, oxalic, or malic acids), LC-MS/MS was performed to detect citric acid production. Only aliquots from the supernatant of culture conditions of the microgravity experiment were analysed.

LC-MS/MS revealed citric acid was present in the supernatant of only two conditions: 75% mWRM and PCC 7938 diluted in mWRM, both under 1G and µG, as presented in **Table 4**.

The identification of citric acid is possible by analysing the resulting spectra of LC/MS-MS. **Figure 28** presents an example of a positive result on the identification of citric acid (additional graphs can be found in Annex B). The top graph corresponds to the full scan spectrum, in which the precursor ion of citric acid $[M–H]^-$ is observed at 191 m/z (retention time of 4.01 min for standard solution, and 4.14-5.42 min for supernatant of *A. niger* cultures); in the lower panel, which correspond to the CID spectrum, two fragment peaks consistently appear – one at m/z ~173, corresponding to $[M–H–H_2O]^-$; and another at m/z ~111, corresponding to $[M–H–CO_2–2H]^-$. The conserved relative intensity between these fragment peaks, comparable across samples and matching the positive control, provides additional confirmation that the detected compound is citric acid (Fernández-Fernández *et al*., 2010).

Table 4. List of samples analysed through LC-MS/MS and corresponding results on the identification of citric acid, presence of precursor ion and respective fragments on the full scan and CID mass spectrum, respectively. MM: Minimal Media; mWRM: Martian Water-Released Minerals; lWRM: Lunar Water-Released Minerals.

Sample	Citric acid was present?	Precursor ion $[M-H]^-$ at 191 m/z	Fragment 1 $[M-H-H2O]^-$ at 173 m/z	Fragment 2 [M-H-CO2-2H]- at 111 m/z
Citric acid solution at 5 µg/mL	Yes	Yes	Yes	Yes
1G MM	No	-	-	-
1G 75% mWRM	Yes	Yes	Yes	Yes
1G PCC 7938 mWRM	Yes	Yes	Yes	Yes
1G 75% lWRM	No	-	-	-
1G PCC 7938 lWRM	No	-	-	-
µG MM	No	-	-	-
µG 75% mWRM	Yes	Yes	Yes	Yes
µG PCC 7938 mWRM	Yes	Yes	Yes	Yes
µG 75% lWRM	No	-	-	-
µG PCC 7938 lWRM	No	-	-	-
Blank (methanol 50%)	No	-	-	-
Negative control (MM)	No	-	-	-

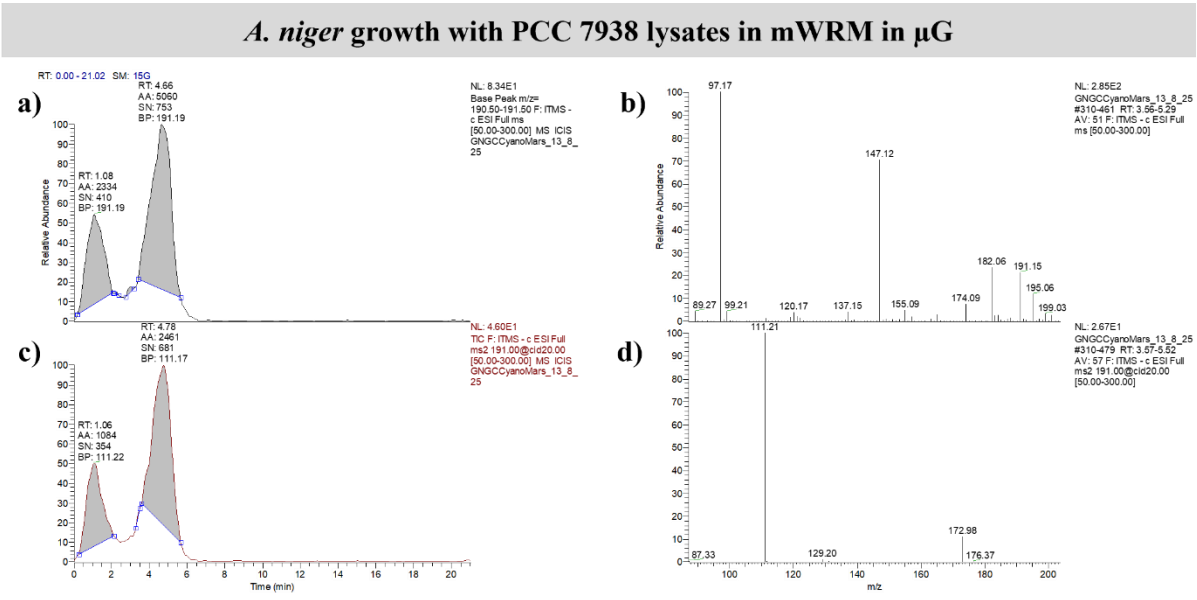

Figure 28. Results of LC-MS/MS analysis for citric acid detection on the supernatant of culture of *A. niger* growth with lysates of PCC 7938 in mWRM, grown in microgravity. a) Total Ion Chromatogram (TIC); b) Mass spectrum obtained in electrospray negative polarity mode, using Ful scan (50-300 m/z, top graphics); c) Collision-Induced Dissociation (CID) Chromatogram; and d) Mass spectrum of CID. mWRM: Martian Water-Released Minerals; µG: Microgravity.

From a general perspective, it is possible to conclude that the gravitational environment (1G vs μG) had no effect on citric acid production. Indeed, organic acid production by *A. niger* in microgravity has previously been reported in the literature, in which *A. niger* strain ATCC 16404 was shown to produce organic acids capable of corroding different metals (Jiang *et al*., 2019, 2022). This work further contributes by demonstrating that cyanobacterial biomass can support organic acid production by *A. niger* in microgravity, thus achieving the third and final objective defined for this dissertation.

A second relevant interpretation is that there seems to be no direct correlation between pH decrease and citric acid production. For instance, for the positive control (MM) of the microgravity experiment, there was a pH decrease through time, but no citric acid was detected in LC-MS/MS. This could be correlated to the production of other types of organic acids, such as oxalic acid, due to the influence of the medium on *A. niger* metabolism (Aboyeji *et al*., 2020; Hamdy, 2013; Leangon *et al*., 1999). Conversely, in the culture with lysates of PCC 7938 diluted mWRM, citric acid was detected, but with no pH decrease. This could be explained by the fact that the limit of detection of citric acid in LC-MS/MS is lower than the concentration of organic acids needed to change the pH of the media in a way that could be detected with a pH strip.

Finally, one last notable result is that no citric acid was detected in any condition with lWRM, mirroring the earlier observation that pH did not decrease in these assays. This suggests that the presence of cyanobacterial biomass in lWRM may interfere with fungal metabolism, diminishing organic acid production. Alternatively, it is possible that pH fluctuations occurred during the intermediate stages of the experiment, initially decreasing due to acid production and subsequently returning toward neutrality, as previously observed in multi-well plate assays.

Future directions should focus on full method validation of LC-MS/MS, to allow for accurate quantification of citric acid and other organic acids. Furthermore, monitoring citric acid production over time through daily sampling would be relevant, to inform on the dynamics of organic acid production during fungal growth from cyanobacterial lysates.

Overall, these results demonstrate that while pH changes are indicative of organic acid production by *A. niger*, they are not a reliable marker for citric acid biosynthesis. Acidification of the medium may result from the accumulation of other organic acids – such as gluconic, oxalic, or malic acids (Zhursinali & Kurmanbaev, 2020). Moreover, only when organic acids reach millimolar concentrations does their effect on bulk pH become measurable with pH strips. These results further support that organic acid production by *A. niger* is highly dependent on media composition.

Chapter 5

Conclusions and Future Work

5.1. Conclusions

The future of space exploration depends on the development of sustainable Bioregenerative Life Support Systems (BLSS), capable of producing fundamental resources for astronauts, such as water, oxygen, food, construction materials and medicine. While cyanobacteria and plants have been extensively studied for BLSS applications, the integration of filamentous fungi remains limited. Previous research has highlighted their versatility of applications for space exploration, yet few studies have attempted to integrate them into closed loops for resource recycling and regeneration.

This dissertation aimed to present a proof-of-concept for the feasibility of integrating fungi into BLSS, by showcasing that *A. niger* can grow utilizing resources expected to be available in future space habitats – namely Lunar/Martian regolith and cyanobacterial biomass – under simulated space conditions. Furthermore, their utility was explored through the assessment of citric acid production.

Overall, this work demonstrated that *A. niger* can grow using a combination of Lunar or Martian Water-Released Minerals and lysates of cyanobacterial biomass. Among the four strains tested, *Anabaena sp*. PCC 7938 proved to be the most suitable substrate, supporting higher fungal biomass production – approximately 30% of the positive control. Moreover, *A. niger* was further shown to grow in these media conditions under simulated microgravity conditions, with no statistically significant difference in biomass production compared to standard Earth gravity controls.

Additionally, *A. niger* grown in lysates of PCC 7938 diluted in mWRM was shown to produce citric acid, which could be used for food preservation. Interestingly, no citric acid production was detected for cultures grown with PCC 7938 lysates diluted in Lunar WRM, nor in the Minimal Media (MM) positive control. For the MM cultures, the absence of citric acid is likely attributable to a metabolic shift in *A. niger*, as a pH reduction was observed throughout the experiment, suggesting production of other organic acids. In contrast, for the PCC 7938 diluted in Lunar WRM, the lack of citric acid production is more plausibly explained by a suppression of organic acid synthesis, potentially due to interactions between the cyanobacterial lysates and the mineral composition of the Lunar simulant.

These results provide strong evidence supporting the feasibility of integrating *A. niger* into future BLSS for Martian and Lunar exploration, utilizing *in situ* resources. By proving both growth and citric acid production in simulated space-relevant conditions, this work provides a foundation for future research into bioregenerative and sustainable space habitats.

5.2. Future Work

This work served as a proof-of-concept for the integration of fungi into BLSS. As such, a fundamental next step is to determine whether cyanobacterial strains could also be cultivated in space habitats with resources available *in situ*, namely Lunar and Martian regolith. While it has already been demonstrated that *Anabaena* sp. PCC 7120 and PCC 7938 can grow using Martian regolith simulants (Ramalho, *et al*., 2022a), their growth was not tested with Lunar regolith.

Furthermore, exploring additional strains besides *Anabaena sp*. may provide new alternatives with advantages not contemplated by the four strains tested here. For example, *Gloeocapsopsis crepidinum* LEGE 06123, isolated from an intertidal diazotrophic mat in Southern Portugal, tolerates harsh environmental conditions such as high salinity and temperature fluctuations. It is closely related to thermophilic strains from Yellowstone and Zerka Ma'in hot springs, deeming it a promising candidate for growth on extreme extraterrestrial environments (V. Ramos *et al*., 2010). This is particularly relevant considering the presented results, where Lunar regolith supported less *A. niger* growth and citric acid production compared to Martian regolith. Consequently, investigating alternative cyanobacterial sources that may perform better in combination with Lunar regolith constitutes an important direction for future research.

Aside from cyanobacteria, research should also investigate the ability of fungi to grow on alternative nutrient sources, particularly plant organic waste, which will inevitably be produced in future BLSS.

Once cyanobacterial and fungi bioreactors are established, their interconnectivity must be tested, and the performance of this integrated system must be evaluated under continuous operation. Key parameters to monitor would include a) production of fungal and cyanobacterial biomass; b) oxygen generation; and c) synthesis of relevant products. At this stage, it will be crucial to define and prioritize the most relevant target products, such as biocomposites, melanin, or citric acid. In parallel, sterilization and operational protocols must be developed and validated.

Finally, once the system demonstrates stable operation and all parameters are thoroughly defined, experiments should transition to real space environments, for instance aboard the ISS, to validate its feasibility under real microgravity and space radiation.

Overall, these experiments should first prioritize Lunar applications, given the near-term goals of the Artemis program and the need to develop life-support alternatives for the Lunar Gateway.

Given these priorities, a new BLSS concept has been designed: "SELENA: Sustainable microbial Ecosystem for Lunar ExploratioN" (**Figure 29**). This project is envisioned to address much of the future work previously outlined, and to advance the integration of cyanobacteria and fungi into a functional bioregenerative loop. SELENA will be developed in the context of a forthcoming PhD project, giving continuation to the work herein developed.

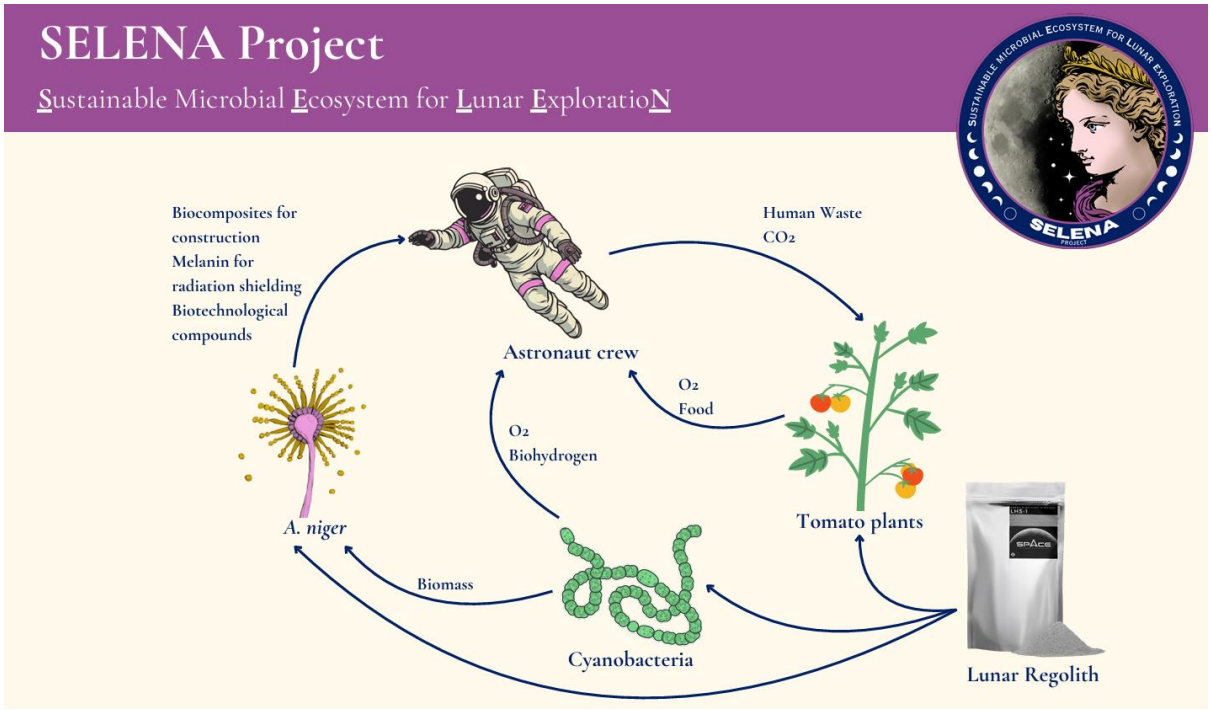

Figure 29. Conceptual representation of the SELENA bioregenerative loop, illustrating the integration of cyanobacteria, tomato plants and fungi within a closed microbial ecosystem for Lunar applications.

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Annex A

Statistical Analysis of Biomass Yields

Table 5. Summary of the results of the ordinary One-Way Anova analysis. Conditions with statistical significance (*p* value < 0.05) are highlighted in green.

Experiment	One-Way ANOVA Results		
	F value	*p* value	R^2
MM dilutions assay	95.79	<0.0001	0.9426
mWRM dilutions assay	144.5	<0.0001	0.9620
lWRM dilutions assay	120.7	<0.0001	0.9548

Table 6. Summary of the results of the Two-Way Anova analysis. Conditions with statistical significance (*p* value < 0.05) are highlighted in green.

Experiment	Source of Variation	% of Total Variation	F (DFn, DFd)	*p* value
MM dilutions assay (water vs mWRM vs lWRM)	Interaction	3.031	F (10, 90) = 2.112	0.0313
	Medium used for dilution	6.953	F (2, 90) = 24.22	<0.0001
	Concentration of MM	77.10	F (5, 90) = 107.4	<0.0001
Glucose supplementation assay	Interaction	27.22	F (2, 30) = 9.497	0.0006
	Medium used for dilution	23.30	F (2, 30) = 8.130	0.0015
	Concentration of glucose	6.493	F (1, 30) = 4.531	0.0416
Supplementation with NT or lysed *Anabaena sp*. PCC 7120 at different concentrations	Interaction	19.41	F (8, 90) = 7.686	<0.0001
	Pre-treatment (lysed vs NT)	30.25	F (1, 90) = 95.81	<0.0001
	Concentration of cyanobacterial biomass	21.92	F (8, 90) = 8.678	<0.0001
Supplementation with lysates of different *Anabaena sp.*	Interaction	4.350	F (6, 24) = 1.017	0.4381
	Medium used for dilution	1.957	F (2, 24) = 1.372	0.2727
	Strain of the cyanobacterial lysate	76.58	F (3, 24) = 35.79	<0.0001
Microgravity experiment	Interaction	2.057	F (4, 10) = 3.967	0.0351
	Gravity conditions	0.02814	F (1, 10) = 0.2170	0.6513
	Medium used for dilution	96.62	F (4, 10) = 186.3	<0.0001

Table 7. Results of the post-hoc Tukey test of the MM dilutions assay. Only comparisons with a statistically significant difference (*p* value < 0.05) were included.

MM dilutions assay	
Comparison	***p* value**
PC (MM) vs. NC (dH_2O)	<0.0001
PC (MM) vs. 1% MM	<0.0001
PC (MM) vs. 5% MM	<0.0001
PC (MM) vs. 10% MM	<0.0001
PC (MM) vs. 25% MM	<0.0001
PC (MM) vs. 50% MM	<0.0001
NC (dH_2O) vs. 50% MM	0.0004
1% MM vs. 50% MM	0.0007
5% MM vs. 50% MM	0.0020
10% MM vs. 50 % MM	0.0300

Table 8. Results of the post-hoc Tukey test of the 2-way ANOVA of MM dilutions in water/mWRM/lWRM, and the glucose supplementation assay. Only comparisons with a statistically significant difference (*p* value < 0.05) were included.

MM dilution in water/mWRM/lWRM	
Comparison	***p* value**
5% MM in Water vs. lWRM	0.0003
5% MM in mWRM vs. lWRM	0.0014
10% MM in Water vs. lWRM	0.0005
10% MM in mWRM vs. lWRM	0.0024
25% MM in mWRM vs. lWRM	0.0014
50% MM in mWRM vs. lWRM	0.0002

Glucose supplementation assay	
Comparison	***p* value**
27.5 mM of glucose in lWRM vs. dH_2O	0.0108
55 mM of glucose in mWRM vs. lWRM	0.0002
55 mM of glucose in mWRM vs. dH_2O	0.0017

Table 9. Results of the post-hoc Tukey test of the mWRM and lWRM dilutions assays. Only comparisons with a statistically significant difference (p value < 0.05) were included.

mWRM dilution assay	
Comparison	***p* value**
PC (MM) vs. NC (ddH_2O)	<0.0001
PC (MM) vs. 100% mWRM	<0.0001
PC (MM) vs. 99% mWRM	<0.0001
PC (MM) vs. 95% mWRM	<0.0001
PC (MM) vs. 90% mWRM	<0.0001
PC (MM) vs. 75% mWRM	<0.0001
PC (MM) vs. 50% mWRM	<0.0001
NC (dH_2O) vs. 90% mWRM	0.0336
NC (dH_2O) vs. 75% mWRM	<0.0001
NC (dH_2O) vs. 50% mWRM	<0.0001
100% mWRM vs. 90% mWRM	0.0252
100% mWRM vs. 75% mWRM	<0.0001
100% mWRM vs. 50% mWRM	<0.0001
99% mWRM vs. 75% mWRM	<0.0001
99% mWRM vs. 50% mWRM	<0.0001
95% mWRM vs. 75% mWRM	<0.0001
95% mWRM vs. 50% mWRM	<0.0001
90% mWRM vs. 75% mWRM	0.0162
90% mWRM vs. 50% mWRM	<0.0001
75% mWRM vs. 50% mWRM	0.0064

lWRM dilution assay	
Comparison	***p* value**
PC (MM) vs. NC (dH_2O)	<0.0001
PC (MM) vs. 100% lWRM	<0.0001
PC (MM) vs. 99% lWRM	<0.0001
PC (MM) vs. 95% lWRM	<0.0001
PC (MM) vs. 90% lWRM	<0.0001
PC (MM) vs. 75% lWRM	<0.0001
PC (MM) vs. 50% lWRM	<0.0001
NC (dH_2O) vs. 95% lWRM	0.0041
NC (dH_2O) vs. 90% lWRM	<0.0001
NC (dH_2O) vs. 75% lWRM	<0.0001
NC (dH_2O) vs. 50% lWRM	<0.0001
100% lWRM vs. 95% lWRM	0.0047
100% lWRM vs. 90% lWRM	<0.0001
100% lWRM vs. 75% lWRM	<0.0001
100% lWRM vs. 50% lWRM	<0.0001
99% lWRM vs. 90% lWRM	0.0014
99% lWRM vs. 75% lWRM	<0.0001
99% lWRM vs. 50% lWRM	<0.0001
95% lWRM vs. 50% lWRM	<0.0001
90% lWRM vs. 50% lWRM	0.0002
75% lWRM vs. 50% lWRM	0.0215

Table 10. Summary of the results of the post-hoc Tukey test of the supplementation with NT and lysed *Anabaena sp.* PCC 7120. Only the most relevant comparisons were included.

Comparison	*p* value
NT dH_2O + 1 g/L vs. Lysed dH_2O + 0.5 g/L	ns
NT dH_2O + 1 g/L vs. Lysed dH_2O + 1 g/L	ns
NT dH_2O + 1 g/L vs. Lysed dH_2O + 2 g/L	ns
NT mWRM + 1 g/L vs. Lysed mWRM + 0.5 g/L	ns
NT mWRM + 1 g/L vs. Lysed mWRM + 1 g/L	<0.0001
NT mWRM + 1 g/L vs. Lysed mWRM + 2 g/L	<0.0001
NT lWRM + 1 g/L vs. Lysed lWRM + 0.5 g/L	>0.9999
NT lWRM + 1 g/L vs. Lysed lWRM + 1 g/L	0.0002
NT lWRM + 1 g/L vs. Lysed lWRM + 2 g/L	0.0005
Lysed dH_2O + 0.5 g/L vs. Lysed mWRM + 0.5 g/L	ns
Lysed dH_2O + 0.5 g/L vs. Lysed lWRM + 0.5 g/L	ns
Lysed dH_2O + 1 g/L vs. Lysed mWRM + 1 g/L	<0.0001
Lysed dH_2O + 1 g/L vs. Lysed lWRM + 1 g/L	0.0188
Lysed dH_2O + 2 g/L vs. Lysed mWRM + 2 g/L	ns
Lysed dH_2O + 2 g/L vs. Lysed lWRM + 2 g/L	ns
Lysed mWRM + 0.5 g/L vs. Lysed mWRM + 1 g/L	<0.0001
Lysed mWRM + 0.5 g/L vs. Lysed mWRM + 2 g/L	0.0003
Lysed mWRM + 2 g/L vs. Lysed lWRM + 2 g/L	ns
Lysed lWRM + 0.5 g/L vs. Lysed lWRM + 2 g/L	0.0015
Lysed lWRM + 1 g/L vs. Lysed lWRM + 2 g/L	ns
Lysed mWRM + 1 g/L vs. Lysed mWRM + 2 g/L	ns
Lysed mWRM + 0.5 g/L vs. Lysed lWRM + 0.5 g/L	ns
Lysed mWRM + 1 g/L vs. Lysed lWRM + 1 g/L	ns

Table 11. Results of the post-hoc Tukey test of the 2-way ANOVA of the assay of different *Anabaena sp.* strains as nutrient sources for *A.* niger. Only comparisons with a statistically significant difference (p value < 0.05) were included.

Comparison	*p* value
PCC 7938 vs. X-002, diluted in water	0.0001
PCC 7938 vs. 00250, diluted in water	0.0061
PCC 7938 vs. PCC 7120, diluted in water	<0.0001
PCC 7938 vs. X-002, diluted in mWRM	<0.0001
PCC 7938 vs. 00250, diluted in mWRM	0.0029
PCC 7938 vs. PCC 7120, diluted in mWRM	0.0002
PCC 7938 vs. X-002, diluted in lWRM	0.0005
PCC 7938 vs. PCC 7120, diluted in lWRM	0.0025
X-002 vs. 00250, diluted in lWRM	0.0061
00250 vs. PCC 7120, diluted in lWRM	0.0295

Table 12. Summary of the results of the post-hoc analysis of the microgravity experiment. Only the most relevant comparisons were included. Comparisons in which results of the Tukey's multiple comparison test and the Dunn's multiple comparison test differed are highlighted in red.

Comparison	Tukey's multiple comparison test	Uncorrected Dunn's multiple comparison test
Positive control (MM): 1G vs. µG	ns	ns
75% mWRM: 1G vs. µG	ns	ns
75% lWRM: 1G vs. µG	ns	ns
mWRM + PCC 7938: 1G vs. µG	ns	ns
lWRM + PCC 7938: 1G vs. µG	ns	ns
1G Positive control (MM) vs. 1G 75% mWRM	ns	ns
µG Positive control (MM) vs. µG 75% mWRM	$p = 0.0459$	ns
1G 75% mWRM vs. 1G 75% lWRM	$p < 0.0001$	$p = 0.0180$
µG 75% mWRM vs. µG 75% lWRM	$p < 0.0001$	ns
1G mWRM + PCC 7938 vs. 1G lWRM + PCC 7938	ns	ns
µG mWRM + PCC 7938 vs. µG lWRM + PCC 7938	ns	ns

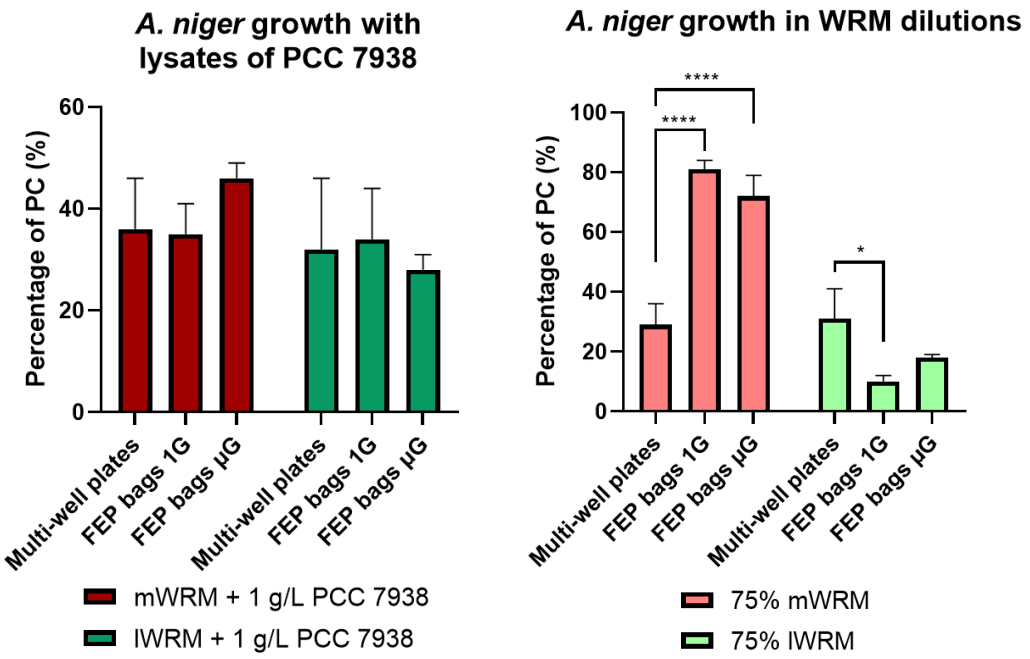

Figure 30. Comparison of *A. niger* biomass yields between the microgravity experiment and previous assays in multi-well plates. Biomasses are represented as percentages of the positive control of the respective experiment. Statistically significant differences between data groups with the same medium composition are indicated.

Annex B

Results of the monitorization of citric acid production

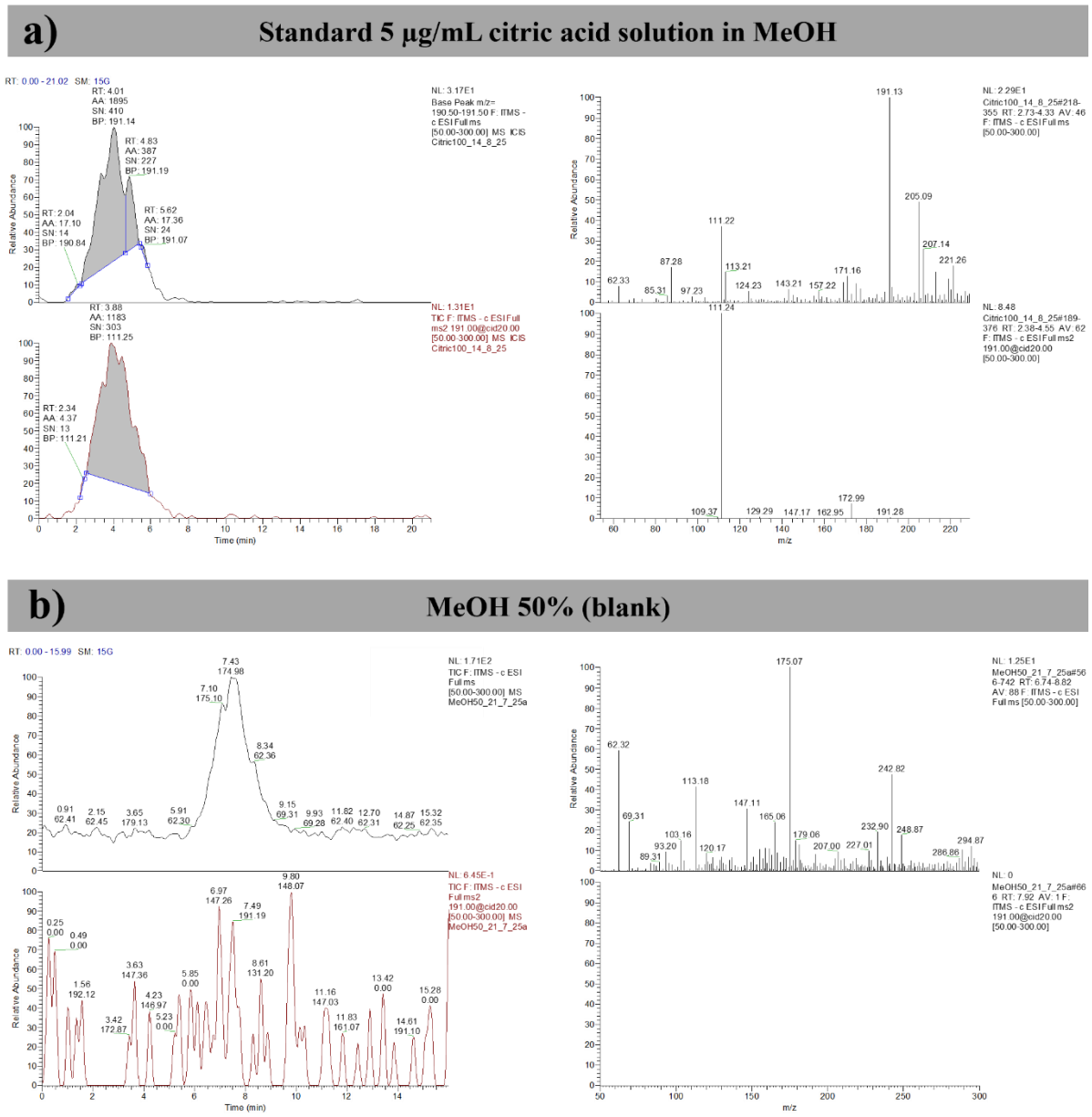

Figure 31. Results of LC-MS/MS analysis for citric acid detection in a) a standard 5 µmg/mL solution of citric acid in methanol; b) solution of 50% methanol, used as blank. For each panel, the top-left graph is the Total Ion Chromatogram (TIC); top-right graph is the Mass spectrum obtained in electrospray negative polarity mode, using Ful scan (50-300 m/z); bottom left graph is the Collision-Induced Dissociation (CID) Chromatogram; and bottom-right graph is the Mass spectrum of CID.

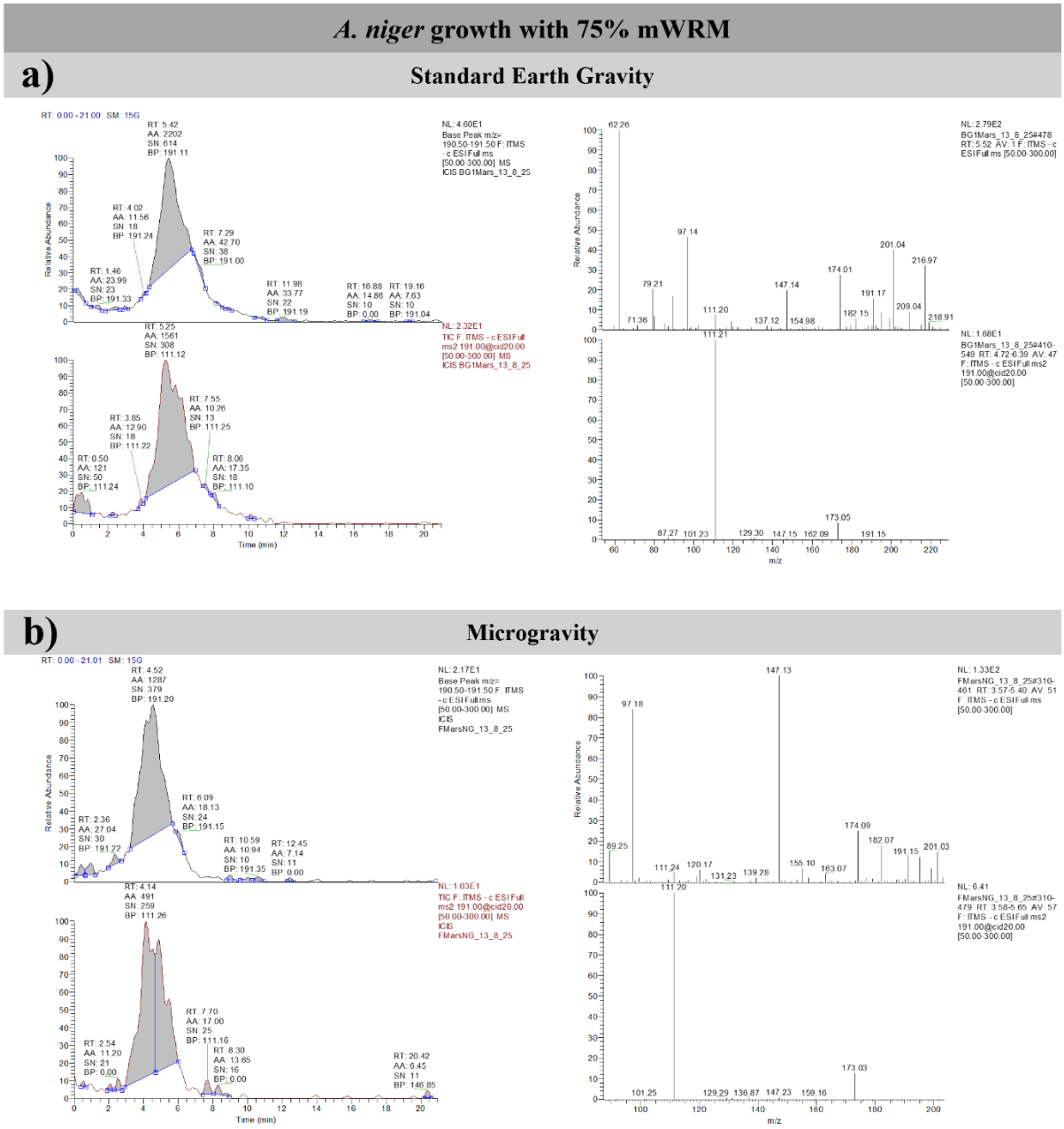

Figure 32. Results of LC-MS/MS analysis for citric acid detection in *A. niger* grown with 75% mWRM in a) Standard Earth Gravity; b) Microgravity. For each panel, the top-left graph is the Total Ion Chromatogram; top-right graph is the Mass spectrum obtained in electrospray negative polarity mode, using Ful scan (50-300 m/z); bottom left graph is the Collision-Induced Dissociation (CID) Chromatogram; and bottom-right graph is the Mass spectrum of CID.

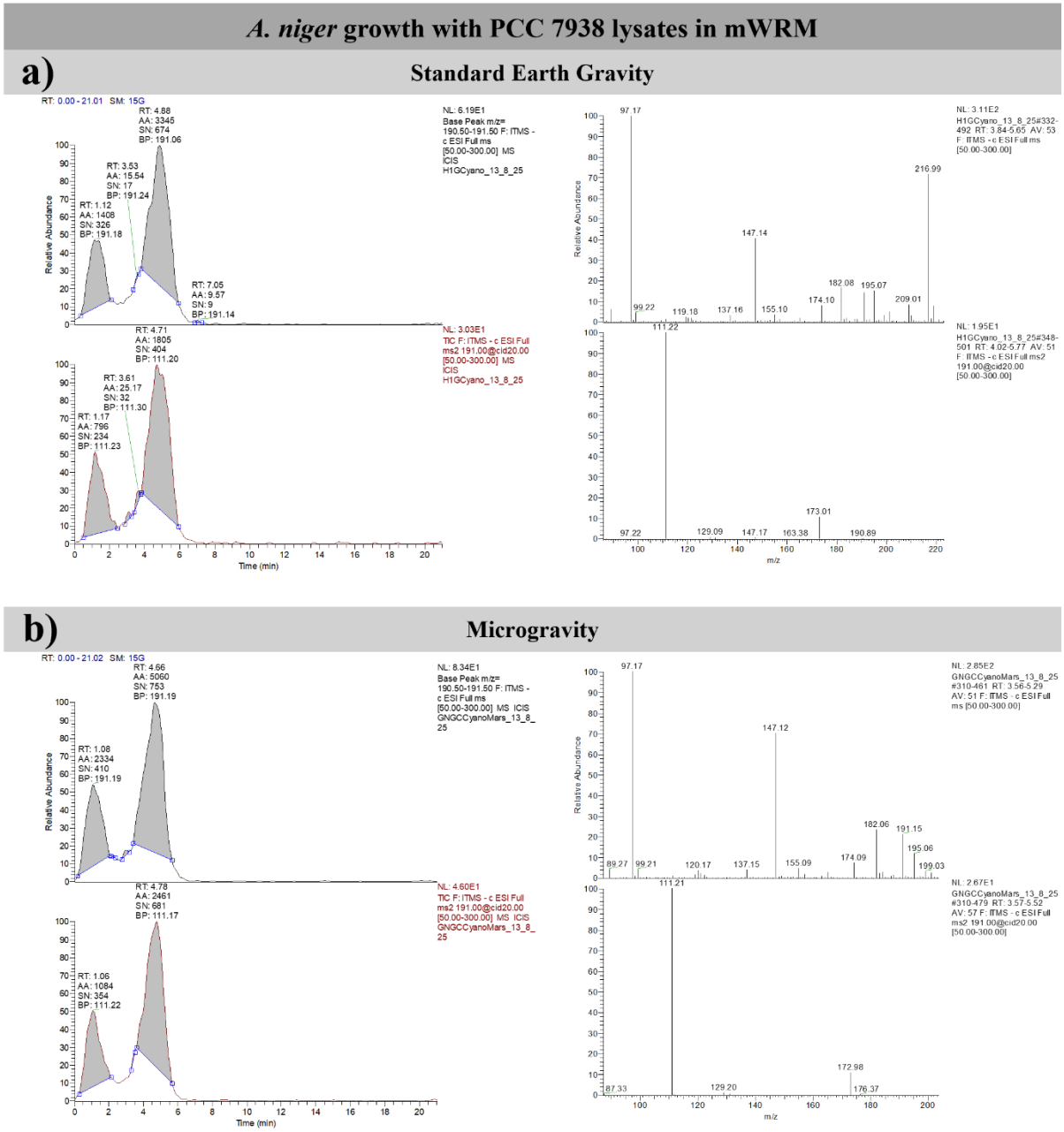

Figure 33. Results of LC-MS/MS analysis for citric acid detection in *A. niger* grown with lysates of *Anabaena sp.* PCC 7938 diluted in mWRM in a) Standard Earth Gravity; b) Microgravity. For each panel, the top-left graph is the Total Ion Chromatogram; top-right graph is the Mass spectrum obtained in electrospray negative polarity mode, using Ful scan (50-300 m/z); bottom left graph is the Collision-Induced Dissociation (CID) Chromatogram; and bottom-right graph is the Mass spectrum of CID.

MM without *A. niger* (Negative Control)

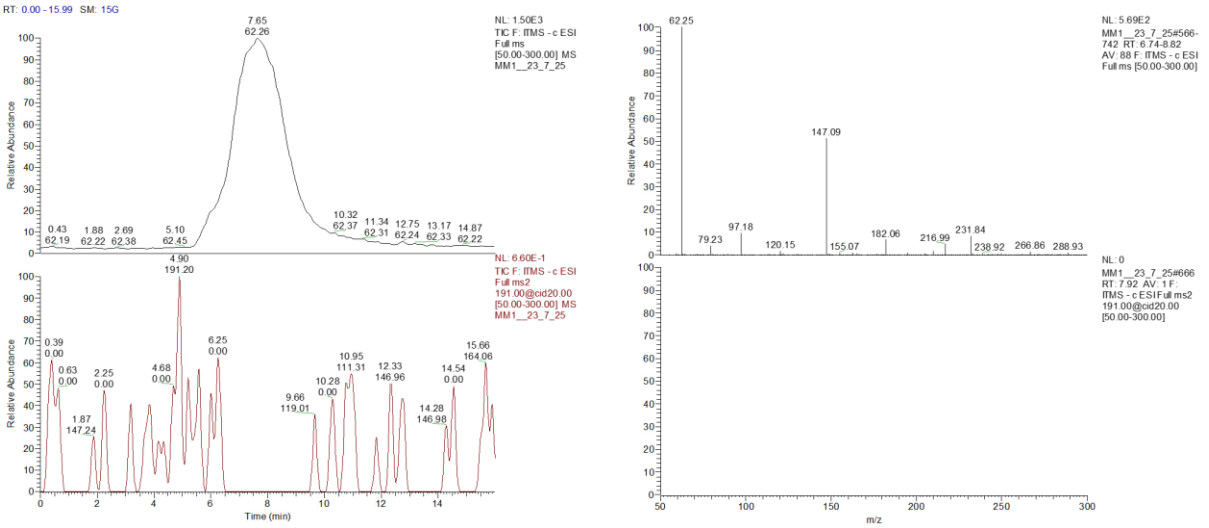

Figure 34. Results of LC-MS/MS analysis for citric acid detection in a sample of Minimal Media, used as a Negative Control. The top-left graph is the Total Ion Chromatogram; top-right graph is the Mass spectrum obtained in electrospray negative polarity mode, using Ful scan (50-300 m/z); bottom left graph is the Collision-Induced Dissociation (CID) Chromatogram; and bottom-right graph is the Mass spectrum of CID. MM: Minimal Media.

Table 13. Results of the statistical analysis of pH monitorization of experiments in Standard Earth Gravity. Only conditions where a pH decline was observed are included.

Experiment	Culture composition	Friedman Test Result	Multiple Comparison
MM dilutions	MM (PC)	* (p=0.0247)	ns
	25% MM	** (p=0.0041)	Significant variation from day 0 to day 3, and day 3 to day 7
	50% MM	* (p=0.0484)	ns
mWRM dilutions	MM (PC)	** (p=0.0082)	Significant variation from day 0 to day 3
	90% mWRM	** (p=0.0041)	Significant variation from day 0 to day 3, and day 3 to day 7
	75% mWRM	Approximate p value	ns
	50% mWRM	** (p=0.0041)	Significant variation from day 0 to day 3, and day 3 to day 7
lWRM dilutions	MM (PC)	** (p=0.0082)	Significant variation from day 0 to day 3
	90% lWRM	* (p=0.0278)	ns
	75% lWRM	* (p=0.0370)	ns
	50% lWRM	* (p=0.0484)	ns
Glucose supplementation	MM (PC)	** (p=0.0021)	Significant variation from day 0 to day 7
	Water + 27.5 mM glucose	*** (p=0.0005)	Significant variation from day 0 to day 7
	Water + 55 mM glucose	*** (p=0.0003)	Significant variation from day 0 to day 7
	mWRM + 27.5 mM glucose	*** (p=0.0001)	Significant variation from day 0 to day 7
	mWRM + 55 mM glucose	*** (p=0.0001)	Significant variation from day 0 to day 7
	lWRM + 27.5 mM glucose	*** (p=0.0001)	Significant variation from day 0 to day 7
	lWRM + 55 mM glucose	*** (p=0.0001)	Significant variation from day 0 to day 7
NT vs lysed PCC 7120	MM (PC)	*** (p=0.0003)	Significant variation from day 0 to day 7
Different *Anabaena sp.* Strains	MM (PC)	* (p=0.0278)	Significant variation from day 0 to day 7
	PCC 7938 in Water	ns	ns
	PCC 7938 in mWRM	ns	ns
	LEGE 00250 in Water	ns	ns