Space Biology Beyond LEO
Instrumentation & Science Series
Science Working Group
2021 Annual Report

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INTRODUCTION

THRIVING IN DEEP SPACE: A NEW DIRECTION FOR BIOLOGICAL RESEARCH

Human space exploration was never intended to stop within low Earth orbit (LEO). Although nearly all of biological research in space has taken place in LEO, on the Space Shuttle, International Space Station (ISS), and free-flyer CubeSat missions, NASA's recent shift in emphasis toward human exploration of the Moon and ultimately Mars necessitates a shift in the focus of its research in the biological sciences [1]. Specifically, in 2022 and beyond, the Division of Biological and Physical Sciences seeks to pivot toward a focus on Thriving In DEep Space (TIDES), furthering the fundamental research necessary for understanding risks and mitigation strategies for deep-space stressors on human crew, plants, and their microbiomes. This effort entails both research on model organisms to elucidate the molecular processes underlying the biological consequences of deep-space exposure, and research on the organisms that will be necessary companions to sustain life and facilitate resource utilization in long-duration missions.

TO DATE, BIOLOGICAL RESEARCH BEYOND LEO HAS BEEN EXTREMELY LIMITED

The Apollo era saw several experiments investigating the effect of the beyond LEO environment on organisms: for instance, Russia's Zond 5, which sent a diverse group of organisms on a 6-day circumlunar mission and the Biostack experiments I and II on Apollo 16 and 17, which measured the effect of high atomic number and energy (HZE) particle radiation on immobilized organisms such as bacterial spores during lunar transit [2]. However, these works were necessarily limited by the technology of the time to phenotypic and physiological assessments of spaceflight effects, and the end of the Apollo program in 1972 marked the last time that NASA sent any organisms beyond LEO for decades. Recent technological advances have dramatically changed the nature of the research that can be conducted, and, with renewed beyond LEO flight opportunities, have the potential to generate substantial new insights into beyond LEO biology. In 2019, China's Chang'e-4 lunar lander was the first mission to germinate plant seeds on the moon, in an attempt to demonstrate a closed biological life support system. NASA's first beyond LEO biological experiments in decades will launch on Artemis 1: BioSentinel and BioExpt-1. BioSentinel will send an autonomous microfluidic culturing device on a SmallSat to measure the effect of deep-space radiation on two actively growing yeast (Saccharomyces cerevisiae) strains in heliocentric orbit [3,4,5]. BioSentinel will leverage extensive ground work in yeast genetics and metabolic biochemistry, as well as advanced microfluidics, optics, and radiation detection technology.

STATEMENT OF TASK

The Beyond LEO Instrumentation & Science Series Science Working Group (BLISS-SWG) was established in December 2020 to provide NASA's Space Biology Program with sustained input
from a group of subject matter experts from the space biosciences community in its strategy for developing research priorities and tools for beyond LEO exploration. The work of the BLISS-SWG is an extension of that of a prior Science Working Group, the Life Beyond Low Earth Orbit (LBLEO) SWG, which met in July 2016 and published a report in 2018 [6]. The LBLEO report formed a comprehensive survey of nine space life sciences disciplines and a statement of broad research goals for beyond LEO research in each. While the work of the BLISS-SWG builds on that of LBLEO, the newer group differs primarily in that it is a standing committee that will exist, with rotating membership, and issue annual reports for the lifetime of the Beyond LEO Instrumentation & Science Series (BLISS); and that its aims are to report upon near-term scientific goals and technological developments that will be accessible within the upcoming few years, to best inform the direction of the Space Biology Program.

Specifically, the two specific aims of the BLISS-SWG, as stated in the charter, are:

1. To define the technical capabilities that should be sought in order to enable biological research beyond LEO.
2. To report on the potential scientific gains of various experimental organisms in future research beyond LEO.

And while this first report lays the groundwork for long-term planning, the ultimate focus is on describing research goals that can be achieved within the next 2-5 years.

In creating this work, the members of the BLISS-SWG surveyed the following prior strategic documents describing NASA's priorities for research in the space life sciences:

- Space Biology Science Plan 2016-2025 (Chapter X in the SLPSRA Integrated Research Plan)

Research questions and topics presented in these documents as being high priority were then considered in light of 1) relevance to the beyond LEO environment, and 2) feasibility of addressing these questions in the next 2-5 years, given current technological capabilities.
SCIENCE BACKGROUND

RESEARCH QUESTIONS OF HIGH RELEVANCE ADDRESS FEATURES THAT ARE UNIQUE TO THE LIFE BEYOND LEO ENVIRONMENT.

This report focuses on research that cannot be conducted within LEO (e.g. on the International Space Station) or through ground simulations. The 2018 LBLEO report[6] defined the two most salient characteristics for research done beyond LEO: the radiation environment and the long duration of missions. The BLISS SWG group identified several additional important factors of the beyond LEO environment. Collectively, in summary these are:

- **Radiation**, including galactic cosmic radiation (GCR), solar particle radiation, and secondary radiation experienced on the surfaces of the Moon and planets. While radiation research has been conducted within LEO, protection from the Earth's magnetic field dramatically reduces the impact of deep-space radiation. While the physics of deep-space radiation are well understood, few empirical measurements have been made of their effects on organismal growth, metabolism, and genetic stability. Ground simulation facilities have several limitations, for instance the common use of unrealistically high dose rates due to time and technology constraints at the available space radiation facilities at the NASA Space Radiation Laboratory (NSRL). Radiation is considered a substantial health threat to human crew on long-duration missions, and is likely to affect the biology of plants and microorganisms in unique ways.

- **Long-duration spaceflight.** The length of time required for the exploration of deep space, for instance the transit to Mars, far exceeds the time most human crew currently spend in space, and introduces novel biological challenges. Further research is needed to understand the effects of space stresses such as microgravity on the human body for such long periods of time; and for shorter-lived organisms, such as plants and microorganisms, multigenerational exposure to such stresses may have evolutionary implications. Moreover, sustaining crew health for long time periods in space will require the further development of biological life support systems, *in situ* resource utilization, and space food and pharmaceuticals production, applications that will all be built upon further fundamental biological research.

- **Altered gravity combined with other beyond LEO stresses.** While microgravity is not unique to the beyond LEO environment, moving from Earth’s gravity to microgravity during spaceflight, to partial gravity once a destination has been reached is a feature specific to beyond LEO. Some studies of partial gravity have been conducted on the ISS using centrifuges, but the scale of such studies is necessarily extremely limited. In addition, the combined effects of altered gravity and radiation experienced on the Moon and Mars can only be studied in those settings.

- **Non-terrestrial chemical environments.** Many of the environments experienced by organisms during missions beyond LEO may harbor specific chemistries that affect biological functioning. It is predicted that even habitable environments such as the Gateway will have unique environmental conditions that-- as has been demonstrated in the ISS-- would affect the growth of plants and microorganisms, such as atmosphere composition, frequency of chemical cleaning, etc. But perhaps of greatest interest are the
properties of naturally occurring environments such as lunar regolith: health risks to organisms, and potential for *in situ* resource utilization.

**Life beyond LEO biological work faces several feasibility challenges.**

Because the goal of this group is to discuss how to maximize science return in near-term studies, the limitations of current platforms must be taken into account. Some of the primary limitations recognized by this group include:

- **Long pre-launch and transit times.** Existing beyond LEO flight opportunities typically entail periods of months to over a year between the time that a payload is prepared and the time that it reaches its destination. A primary consequence of this is that experimental organisms must have the capacity to remain in stasis and viable for extended time periods and then to be reliably reactivated. This limits the range of candidate organisms.

- **Extreme environmental conditions.** While the environmental conditions of beyond LEO are the focus of biological research, experimental platforms must have the capacity to mitigate those that are not of scientific interest in a particular experiment (for example, the thermal environment), while simultaneously exposing to and accurately measuring the parameters of interest (such as radiation).

- **Limited or no crew involvement.** The majority of beyond LEO flight opportunities in the near future, such as SmallSats or CLPS autonomous lunar landers (see following section on mission opportunities) involve no human crew. Experiments must therefore be conducted autonomously.

- **Limited or no sample return.** At the time of writing, SmallSats and CLPS landers do not offer an opportunity for sample return; experiments must therefore involve assays that will yield valuable science only from data gathered *in situ* and transmitted to Earth.
THE LIFE BEYOND LEO ENVIRONMENT

The major stress factors living organisms experience in space relate to altered gravitational force in combination with radiation and the spacecraft/planet environment. In orbit around the Earth or during transit to the Moon or Mars, organisms experience microgravity ($10^{-3}$ to $10^{-6}$ of Earth’s $g$) and on Mars and the Moon the gravitational force is reduced to 1/3rd and 1/6th Earth’s $g$, respectively. The radiation environment in beyond LEO is more complex and involves exposure to GCR and solar particle events (SPE), while other environmental conditions relate to the beyond LEO living environment and include temperature, pressure, regolith and atmospheric composition.

1. THE LUNAR AND DEEP SPACE ENVIRONMENTS

The thermal environment on the lunar surface changes drastically throughout the lunar “day” (28 earth days). Minimum night-time temperature is about 80 K at all latitudes and rises to maxima of 390 K at the equator, 330 K at $60^\circ$ latitude and 290 K at $75^\circ$. The poles are colder, 110-180 K in summer and 40 K in winter. A spacecraft orbiting the moon will be heated to about 100 K in the moon’s shadow and 300 K on its illuminated side.

![Figure 1. Model calculations of lunar surface temperature variations as a function of local time and latitude [7]. Local time is expressed in lunar hours which correspond to 1/24 of a lunar month. At 89° latitude (lunar poles), diurnal temperature variations are shown at summer and winter solstices. (From [8]).](image)

There are two chemical environments to consider: The “built environment” of space habitats and the lunar regolith. The atmospheric pressures and compositions of the built environment may not necessarily be identical to that experienced on ISS. Plants, for example, will be sensitive to humidity and CO$_2$ levels, and this may drive crop plant selection and/or breeding (genetic modification). Lunar regolith is primarily fine grey dust with a density of about 1.5 g/cm$^3$ and scattered breccia and fragments of bedrock. The maria consist mainly of basalt, and some 20% of the grain size distribution consists of particles less than 20 μm in size with the average being...
about 70 μm [9]. This aspect of lunar dust, which is so easily suspended, has significant health implications [10]. Apart from the fused soil, pyroxene, plagioclase feldspar and glasses dominate the mineral composition. The soil agglutinate glass particles, relative to the bulk regolith, are higher in alumina (Al₂O₃), calcium and feldspar (K₂O), but FeO, metallic Fe and MgO are also present. Typical bulk regolith composition is about 44% SiO₂, 17% Al₂O₃, 12% FeO, 12% CaO, 9% MgO, 2% TiO₂ and less than 1% total feldspar, phosphate and sulfur and a few ppm of minor components [9].

The ionizing radiation environments in deep space and on or around the moon differ from that in LEO, where trapped protons and electrons are present and the Earth’s magnetosphere mitigates the flux of energetic solar and galactic particles. GCR consists of protons (~87%), helium (12%) and heavier ions (1%) [11], while the main concern from SPE is proton exposure. SPE can also consist of heavier ions and helium. Exposure to GCR and SPE depends on sunspot activity during the 11-year solar cycle. During a solar minimum the sunspot activity is at its lowest. However, GCR is at a maximum and SPE are at a minimum. The opposite is true during a solar maximum. Another concern when astronauts are on the surface of the Moon or Mars are back scattered (albedo) particles generated from the GCR or SPE colliding with the surface of the planet. The most dangerous albedo particles for biological systems are neutrons. As is well known neutrons and heavy ions, while a small fraction of the spectrum, are more biologically damaging -- 3 to 20-fold, depending on the biological endpoint.

2. ANTICIPATED RADIATION ENVIRONMENTS

Historically, direct dosimetric measurements have come from the Radiation Assessment Detector on the Mars Science Lander [12], the Lunar Reconnaissance Orbiter [13], and the Advanced Composition Explorer probe located at lunar Lagrange point L1 [14]. In lunar orbit the instantaneous dose rate, dominated by galactic particles, varies between 4 and 6 x 10⁻⁷ cGy/s and somewhat less during solar maxima and in the moon’s GCR “shadow”. Integrated dose in lunar orbit was observed to be 12 cGy/y or 0.12 Gy, consistent with earlier estimates based on particle flux analysis [15]. During periods just preceding and just following solar maxima there will be coronal mass ejections resulting in energetic particle storms known to deliver up to 150 cGy, more than half of which may be attenuated by spacecraft structures. NASA’s Human Research Project (HRP) Radiation Element investigators are currently working together to establish multipliers (Relative Biological Effectiveness, RBE) applicable to this radiation spectrum and to specific human end-points. Dose in Gy x RBE = dose in Sieverts (Sv). Terrestrial radiation biological experiments applying ground-based analogs (accelerators and radioisotope sources) are both mechanistic and empirical. Near-term spaceborne radiobiological experiments in the beyond LEO environment, limited to model organisms, would have strictly mechanistic objectives.
Platforms & Types of Missions

Several platforms are available now or will be shortly for conducting experiments beyond LEO. Within the next five years, research will be conducted on Commercial Lunar Payload Services (CLPS) landers, on crewed Artemis missions, and in free flyer spacecraft. These platforms provide unique opportunities and have various constraints. Further out there will be other opportunities, potentially with some limited crew involvement, within the Lunar Gateway and planned human-rated landers and lunar habitats. These are still in the planning phase and science capabilities have not yet been determined.

Near-term Missions

1. CLPS Lunar Landers
The CLPS program offers opportunities for experiments to be conducted on the surface of the Moon. Payloads are offered a power and data interface but are largely responsible for controlling their own environment and conducting their own experiments. Users can make unique requests to the CLPS program for specific placement within the lander, or to be deployed onto the lunar surface. The commercial landers are not currently planned to survive the lunar night, so the maximum duration of a designed experiment is a single lunar day. Since no sample return will be available, data must be collected and processed in-situ and telemetered to Earth. Hardware with SmallSat pedigree and similar designs are well suited for CLPS missions as they are typically sealed, temperature controlled, and completely autonomous.

2. Internal Artemis Missions
There will be some limited capacity inside the crewed Orion capsule on future Artemis missions for science experiments. Since payloads will be inside the vehicle with crew, the environment will be controlled by an Environmental Control and Life Support System (ECLSS). Payloads will therefore not be required to control their own oxygen and temperature environment if what is provided to the crew is sufficient for the planned experiment. Crew time will be very limited, so experiments should be largely passive or autonomous. Sample return is available for internal missions, so this platform does offer support for experiments that require post-processing of samples on the ground. Simple ISS-style missions are well suited for this platform, with the caveat that they do not require extensive interaction or conditioned sample stowage. Autonomous payloads in CubeSat configurations are also appropriate as there will likely be some limited volume provided with a power/data interface.

3. Free Flyer Missions
Each Artemis mission will also have CubeSat deployers, which offer free flyer opportunities to be placed in several beyond LEO orbits. BioSentinel will be the first biological CubeSat deployed by
such a mission and will be placed in heliocentric orbit on Artemis I. Free Flyers are responsible for controlling their own environments similar to the CLPS lunar lander payloads but must also generate their own power and have their own telemetry capability. Autonomous missions with SmallSat heritage and similar designs that do not require sample return are well suited for these missions, particularly if longer durations are desired. Once these spacecrafts are placed in a stable orbit they can support experiments months-to-years long.

**Figure 2.** Summary of future platforms and the increasingly complex organisms each can likely support, including microbes, cells, plants, organs-on-a-chip, invertebrates, and vertebrates.

**Future Platforms**

1. **Gateway**

The Lunar Gateway may offer more capabilities for other types of experiments once it is complete. Limited sample return and crew tending will likely be available. There also may be an opportunity for payloads to be placed outside the Gateway itself to expose experiments to the space environment. Given the limited sample return, in-situ processing and autonomy will still need to be emphasized in payload design. ECLSS will be available during the time the Gateway is crew-inhabited, but the environment when uninhabited is currently unknown and it may be necessary
for payloads to provide their own environmental control. ISS-style experiments may be well-suited for Gateway, particularly those that are shorter duration and require minimal crew time and sample return. Closed system CubeSat heritage and similar hardware may be appropriate, particularly for longer-duration missions that take advantage of periods where the Gateway is uninhabited.

2. **Human-rated landers/habitats**

There may be some experimental opportunities within the habitable space of human-rated landers and crewed habitats planned for the surface of the moon. While ECLSS and crew tending may be available, sample return may continue to be limited. Autonomy and *in-situ* sample processing will likely continue to be important factors for these opportunities.

**Existing and Planned Gateway Radiation Detector Systems**

The Artemis and Gateway space systems are expected to support radiation studies beyond LEO. Some of the plans are listed here.

There will be internal and external dosimeter arrays, IDA (Internal Dosimeter Array) and ERSA (European Space Radiation Sensor Array), respectively. IDA is an international collaborative payload involving ESA and JAXA. It will be located inside the NASA-commissioned Habitation and Logistics Outpost or HALO module of the Gateway from where it will continuously monitor internal radiation levels. Its instruments will evaluate how well the vehicle shields from radiation and the intensity of protons, neutrons and other particles ejected due to spallation. Radiation measurements will be cross-referenced with those taken by ERSA. ERSA will be mounted on the outside of the lunar outpost. The two payloads share similar instruments, which will allow for a more comprehensive overview of the radiation environment in lunar orbit. ERSA in particular will enable researchers to more accurately forecast radiation events [16]. The M-42 thin silicon (Si) detector developed by the German Aerospace Center (DLR) will be widely used. It is essentially an "energy-loss" (dE) detector that provides a dE spectrum and integrates energy-loss data to provide the dose in Gy [17].

The NASA-led heliophysics investigation, HERMES, is the second instrument to fly aboard the Gateway. This is a Sun-oriented space weather experiment to observe solar particles and solar wind. HERMES (Heliophysics Environmental and Radiation Measurement Experiment Suite) is led by Goddard Space Flight Center and consists of four instruments: A magnetometer to measure magnetic fields around Gateway, the Miniaturized Electron pRoton Telescope, or MERIT, which measures ions and electrons; the Electron Electrostatic Analyzer, or EEA, which measures the lower energy electrons that make up most of the solar wind, and the Solar Probe Analyzer for Ions, or SPAN-I, which measures protons and ions including oxygen.

The Matroshka AstroRad Radiation Experiment (MARE) is a radiation science payload proposed to fly on Artemis-1 by German Aerospace Center (DLR) and the Israel Space Agency (ISA) supported by Lockheed Martin and accepted for manifestation by NASA. MARE consists of two female tissue-equivalent phantom torsos instrumented with radiation detectors and located inside the Orion spacecraft. Both phantom torsos are integrated by DLR with active and passive radiation detectors provided by DLR and other international participants including a real-time COTS dosimeter system “ALMAR” from the Greek company HERADO that independently records gamma, electron, neutron and heavy-ion dose (Products – HERADO). [18].

A White Paper from Marshall Space Flight Center suggests the use of two existing space-based neutron spectrometers to (1) explore lunar surface composition, (2) confirm results of Lunar
Prospector and Lunar Reconnaissance Orbiter missions and (3) provide surface neutron radiation data. The Advanced Neutron Spectrometer (ANS-LSM) is a fast neutron spectrometer used on ISS, and Neutron Measurements at the Lunar Surface (NMLS) measures thermal and epithermal neutron count rates [19].

A BioSentinel nanosatellite will be deployed by Artemis-1 and eventually enter a solar orbit. It is designated to study yeast cell responses to the flight (especially radiation) environment as biosensor, and it carries a TimePix LET spectrometer [20]. This spectrometer is, a miniaturized charge-coupled Si device that records the passage of each high-energy particle as a track through its 256 x 256 pixel array giving a dE spectrum, and it accumulates total integrated dose (TID) within its 59 µL volume [3].

HERA (Hybrid Electronic Radiation Assessor) system consists of one processing unit and two sensor units based on Timepix radiation detector technology. It is built to operate as the primary radiation detection system for Orion, is currently undergoing testing on ISS and is certified for flight on the Artemis missions [21].

**COMPONENTS FOR FUTURE HARDWARE**

Design of experiments that may require incorporated sensing and recording of the radiation environment could utilize existing space-qualified devices listed in "2019-BLEO Bio Instrumentation Trade Study_v7" [22]. These include:

- Oxford RadFET, semiconductor: Records TID passively; active readout
- Hamamatsu PIN Diode: Measures instantaneous dose rate only
- Teledyne μDOS TID solid state: Measures and stores TID
- JSC Single-Board LET Radiation Spectrometer: Measures LET, records spectra.

The first three are commercial items, and all are qualified for flight on ISS. Based on these systems and the details available to date it appears that near-future research will include radiation studies directed at the safety of human deep space missions. With this availability of physical measurements and data, future biological experiments should be closely coordinated with radiation detection and/or measurements to the extent possible.
Life Beyond LEO: Questions of Importance for Future Investigations

SECTION A: How does the Beyond LEO Environment Impact Cellular Functions?

There are fundamental common processes within cells that are critical to the functioning of the cell and these are found in prokaryote, eukaryote, single cell and multicellular organisms. Examples relevant to beyond LEO include DNA structure, transcription, metabolism, and oxidative stress responses. Alterations in these critical functions or pathways can result in changes in phenotype or the ability of the cell to survive. Cells will alter signaling and activate survival pathways when exposed to stress, and high energy space radiation and changes in gravitational forces impose stress on cells. This section aims to consider questions that need to be addressed to understand how essential cell processes could be changed by conditions beyond LEO and in particular by living conditions on the moon. Understanding how these fundamental cell processes change will be essential to proposing and testing countermeasures that will support humans Thriving In DEep Space (TIDES).

Critical Questions in this Research Area

1. How does exposure to Beyond LEO influence DNA and DNA-related functions?

DNA is prone to damage, and ionizing radiation can directly damage the DNA by ionizing the DNA molecule or by generating reactive oxygen species (ROS) that oxidize the bases and deoxyribose backbone. The severity of damage induction can be modulated by the DNA structure. For example, transcribed DNA has a more open structure and is more prone to damage than non-transcribed DNA, but transcribed DNA is also repaired faster [23, 24]. The DNA structure also influences the transcription of genes and the ability to repair the DNA, and DNA repair influences cell survival and mutation rate. Although these processes are intertwined, they are separated into questions below to allow the relevance of each to be highlighted.

1.1 How does exposure to beyond LEO alter DNA structure?

DNA in all organisms is protected by proteins that bind and wrap the DNA. Eukaryotic nuclear
DNA is wrapped around an octamer of basic histone proteins to form nucleosomes [25]. In *Escherichia coli*, the proteins that form the DNA nucleoid structure include H-NS, HU, Fis, Hfq, StpA, Dps and CbpB [26,27,28]. Proteins bound to DNA can change DNA compaction and transcription. In *E. coli* Dps increases compaction during stationary phase and protects the DNA from oxidative damage by hydrogen peroxide [29]. Hfq, which is known as a global gene regulator, is decreased by low shear modeled microgravity (LSMMG) and LEO spaceflight [30,31,32,33]. Alterations in the levels of these nucleoid proteins could change the compaction of the DNA and the sensitivity to radiation-induced damage. The highly condensed nucleoid structure of the DNA in *Deinococcus radiodurans* is implicated in the radioresistance of the organism, possibly because double strand breaks can be easily ligated due to the close proximity of DNA fragments in the DNA structure [34].

1.2 How does exposure to beyond LEO alter the epigenome?

The DNA and eukaryotic histones can be modified: DNA can be methylated and histone modifications include acetylation, phosphorylation, methylation, and ubiquitinylation. These modifications form the epigenome. DNA in eukaryotes and prokaryotes can be methylated and exposure to radiation changes DNA methylation patterns [35,36], although dramatic alterations were not detected during LEO spaceflight in the Twins Study [37]. Alterations to DNA methylation and chromatin modifications are biologically relevant as they alter gene expression and DNA repair. Chromatin remodeling and chromatin modifying proteins are important for the initiation, accuracy and completion of DNA repair [38,39,40]. The most common studied chromatin modification in radiation biology is the phosphorylation of H2AX (γH2AX), as this is part of the signaling to repair DNA double strand breaks. There are many different types of chromatin modifications [41] but few have been studied following exposure to simulated microgravity or during space flight. A chromatin modification (H3K27me3) associated with neurogenic differentiation capacity was suppressed in human mesenchymal stem cells grown under simulated microgravity [42], demonstrating that alterations in gravitational force could change the epigenome. Changes to the epigenome due to living conditions beyond LEO will be important for cell survival and possibly evolution as the epigenome is heritable and has been linked to human disease [43].

1.3 How does exposure to beyond LEO alter DNA damage, DNA repair and DNA mutations?

Deep space radiation can induce complex clustered DNA damage that is more lethal [44] and mutagenic [45,46] than individual damages introduced by ROS generated from metabolism on Earth. The steady state level of DNA damage in a cell is determined by the induction of the damage and the removal of the damage by DNA repair. The repair of oxidative base damage and double strand break repair are relevant to removal of complex radiation damage. Studying DNA repair capacity in cells of different organisms has uncovered the importance of DNA repair capacity to survival after radiation exposure. The radiation extremophile *D. radiodurans* [47] has DNA repair systems that work efficiently and contribute to the radiation resistant phenotype, and alterations to the DNA repair system were key to generating radiation resistant *E. coli* from populations subjected to 50 cycles of radiation treatment [48]. Many experiments have examined the effects of radiation, microgravity, or LEO space flight on γH2AX or 53BP1 foci formation, both of which relate to double strand break repair in eukaryotes [49]. The level of these foci in blood cells has been proposed as a biomarker of radiation exposure and a predictor of how well an astronaut may respond to the stresses of space travel [50].

Misrepaired and unrepaired DNA damage can result in DNA mutations if cells survive. Extensive
studies have examined chromosome aberrations and micronuclei formation in mammalian cells [51] and mutations in genes such as the adenine phosphoribosyl transferase (APRT) in mice [52] using ground-based simulated space radiation. These studies demonstrated an increase in mutations and genetic instability at astronaut-relevant doses of particle radiation. Studies are needed to determine if space radiation and altered gravity are synergistic with respect to increasing mutation frequency and inducing cell death. Experiments have found that even though double strand break repair is not altered by simulated microgravity, the combination of radiation and microgravity results in a synergistic detrimental effect on the cell [49]. The combined effects of simulated microgravity and radiation also induced higher chromosomal aberrations in human fibroblasts [53], and an increase in chromosome aberrations in-flight and post-flight was detected in the Twins Study and in astronauts with ISS missions of 6 months or 1 year [37, 54]. The synergistic effect of microgravity and radiation is believed linked to enhanced ROS production, alterations in signal transduction and changes in the transcriptome [49]. The synergy between reduced gravity and radiation may initiate at a threshold gravitational and/or radiation level, which may be cell type dependent. DNA damage, signaling, DNA repair and DNA mutations need to be examined to determine possible long-lasting effects of living beyond LEO on humans, and other organisms as beyond LEO living conditions may induce inheritable mutations, increase virulence, or increase drug resistance of organisms.

1.4 What changes occur to the transcriptome of cells due to the beyond LEO environment?

Alterations to the transcriptome have the potential to change every characteristic of the cell as changes in transcript levels can alter the proteome. As discussed above, transcription is influenced by DNA structure, the epigenome and by the presence of DNA damage. Excessive DNA damage in transcribed genes in eukaryotes and prokaryotes can result in transcription stress where RNA polymerase stalls at damage or by-passes DNA damage producing mutant transcripts and potentially mutant proteins [55]. Transcription stress will not be addressed here, as the focus is on alterations in global gene expression that can reveal information about how the cell is adapting to the stress conditions of living beyond LEO. DNA microarrays and more recently RNASeq have allowed studies to probe changes in gene expression in prokaryotes and eukaryotes either in single cells, mammalian cells in culture, cells in animals and plants, and host-pathogen or host-symbiotic partners. Transcriptomics is a powerful tool. Alterations in pathways related to a specific factor can be detected even if the transcript level of the factor itself is not significantly altered. The change in a pathway also indicates that the protein controlling the pathway has altered in function. In prokaryotes, a common factor identified as responding to simulated microgravity and to LEO stress is Hfq [30, 31, 32, 33, 56, 57]. Hfq is an RNA chaperone that binds small regulatory RNAs (sRNA) and promotes the binding of sRNAs to target RNAs. This changes the half-life and the translation of the target RNA and Hfq is therefore classed as a global gene regulator [58]. Other pathways altered by simulated microgravity or spaceflight include stress responses, chemotaxis, motility, and metabolic pathways [59, 60, 61, 62, 63]. Pathways found to change in mammalian cells also include, but are not limited to, oxidative stress, DNA repair, metabolism, circadian regulated genes and NF-κB [64,65,66,67]. Few published studies have examined partial gravity, which has required the use of centrifuges on the ISS or the use of microgravity simulation devices, such as a random positioning machine on Earth. Two transcriptome studies examining cell growth and cell proliferation in Arabidopsis thaliana seedlings at different gravity levels did identify gene expression changes that were different at microgravity, partial gravity (lunar or Mars) and normal gravity [68,69]. This demonstrates the importance of examining the transcriptome of different types of cells exposed to different gravitational forces. Other beyond LEO conditions, such as higher CO₂ level, human/ animal
isolation during spaceflight, and lunar dust on the lunar surface, may induce synergistic changes and add to the combined effect of radiation and partial/ microgravity on biological systems. Venturing further to the moon and beyond to reveal transcriptome modifications will be essential to understanding the stress pathways activated under conditions of living beyond low Earth orbit.

2. **How does exposure to beyond LEO influence metabolism?**

Transcriptomic studies of prokaryotes and eukaryotes subjected to LEO spaceflight or simulated microgravity have revealed changes in transcript levels of genes involved in metabolic pathways. These pathways include oxidative phosphorylation [70,71], lipid metabolism [59,71,72], carbohydrate metabolism [71,73,74] and anaerobic metabolism [33]. Few studies have measured metabolites or the activity of specific metabolic pathways. Suzuki et al. (2020) did find that mouse plasma levels of glycerol, glycine and succinate were altered in a similar way by LEO spaceflight and aging in humans on Earth [71]. This suggests that countermeasures may be required to prevent metabolic human aging on long missions. Ground-based radiation studies have identified metabolite disturbances in the intestines of irradiated mice for nucleotides, amino acids and metabolic markers of inflammation [75] and differences were detected between γ-ray and 56Fe irradiated mice. GCR and SPE could therefore induce specific changes to metabolism. Metabolism directly affects the ability of the cell to generate energy and produce cell components required for growth and cell maintenance. Metabolism is also important for generating NADPH, which is needed to maintain the epigenome and to maintain antioxidants such as thioredoxin and reduced glutathione. Specific types of metabolism such as microbial carbon fixation [76,77] and plant photosynthesis will be useful to humans for developing technology and growing food, and so will be essential to life beyond LEO.

3. **Does exposure to beyond LEO increase oxidative stress in cells?**

Cells have protective mechanisms to combat the day-to-day ROS generated by living, but an imbalance in ROS production or ROS removal results in oxidative stress. Microorganisms exposed to LEO spaceflight [78], or simulated microgravity [31, 59] do elicit oxidative stress responses and have altered sensitivity to exogenous oxidative stress. Ground-based analogs for microgravity and radiation, and LEO spaceflight studies have detected oxidative stress in mammalian cells by staining for lipid peroxidation [79,80,81], analyzing the transcriptome [70] and measuring enzymes and antioxidants [82]. Transcriptome studies have also implicated oxidative stress in plant responses to space flight [83]. In eukaryotic cells, mitochondria are the predominant site for ROS production and cells use antioxidants and enzymes to limit oxidative damage to lipid, protein and DNA. A decrease in antioxidant capacity, altered mitochondrial gene expression and an increase in DNA damage was detected in the LEO Twins Study [84]. Exposure to low dose particle radiation also results in a persistent oxidative stress [85] that can last weeks to months. 56Fe ion irradiation of mice resulted in increased ROS in the cerebral cortex for up to 12 months [86], and increased mitochondrial ROS production was detected in mouse intestinal epithelial cells one year after irradiation of mice with 56Fe ions [87]. Increasing the antioxidant capacity in the mitochondria by overexpressing catalase did protect from oxidative stress generated from 0.5 Gy proton radiation [88], which supports the idea that the radiation-induced increased ROS and oxidative stress originates in the mitochondria in eukaryotes. Oxidative stress is implicated in multiple pathophysiological human conditions including neurodegeneration, osteoporosis, cardiovascular disease, diabetes and cancer. Exposure to deep space radiation and partial or microgravity on long missions beyond LEO on the moon or Mars could result in persistent oxidative stress and increase the risk of astronauts developing early-onset degenerative diseases.
Feasible research beyond LEO in the next 5 years.

1. How does living beyond LEO alter DNA structure and the epigenome?

Monitoring the expression of nucleoid proteins, chromatin proteins, DNA methyl transferases or chromatin modifying factors could indirectly provide information about the DNA structure and epigenome of the DNA. Visualization of DNA compaction requires highly specialized microscopy techniques. However, fluorescent tagging of proteins can be used to visualize regions of DNA. Fluorescent-tagged MeCP2 and MBD1 proteins bind methyl cytosines and have been used to visualize regions of heterochromatin in living eukaryotic cells using fluorescence microscopy [89]. Specific DNA regions can also be visualized by integrating arrays of the lac operator (LacO) sequence into a specific site in the genome and expressing a GFP-tagged LacI repressor that binds to the LacO sequence [90,91]. This GFP-LacI/LacO system can be used in bacteria, yeast and mammalian cells. These techniques can be used to monitor changes in structure due to DNA damage and DNA repair. Chromatin modifications such as H3 lysine 9 acetylation and H4 lysine 20 monomethylation can also be monitored in living cells using fluorescence microscopy by introducing a vector into the cell that expresses a “mintbody” [92,93]. A mintbody, or modification of a specific intracellular antibody, is a fluorescent-tagged antibody produced in the cell.

2. Do living beyond LEO conditions induce DNA damage and can the damage be repaired?

DNA repair proteins and certain DNA damage signaling proteins localize at sites of DNA damage. Fluorescent-tagged proteins involved in double strand break repair such as 53BP1, MDC1, Rad52 and Ku80 can be visualized by microscopy in mammalian cells as foci when these proteins bind to DNA damaged sites [94,95]. The quantitation of foci provides a measure of DNA damage induction. When repair occurs, the foci resolve and hence repair can be monitored using time-lapse microscopy [96]. This technique can also be used for yeast to detect Rad51 and Rad52 foci, but yeast need to be immobilized prior to microscopy [97,98] and this adds extra technological considerations for autonomous experiments. Double strand break repair by homologous recombination in replicating cells or non-homologous end-joining in all cells can be monitored using specially designed DNA substrates integrated into the genome. Following repair of a double strand break in the substrate, a fluorescent molecule is expressed and repair is quantitated by measuring fluorescence using microscopy or flow cytometry. Repair of low and high LET radiation induced breaks has been quantitated using an EGFP direct repeat homologous recombination substrate integrated as a single copy in the genome of mammalian cells [99]. The more common double strand break repair assay requires an enzyme such as I-SceI or HO to induce a double strand break in the integrated DNA substrate [100] and repair of the break is then measured by fluorescence. In yeast, the I-SceI or HO can be expressed from a vector and induced by galactose. This allows the experiment to be initiated by changing the growth medium once beyond LEO has been reached. To evaluate the ability of cells beyond LEO to activate DNA damage response pathways and to perform DNA repair, cells could be challenged with chemical DNA damaging agents. Also cells deficient in DNA repair or DNA damage response pathways (e.g. Rad51 or p53) will sensitize the cells to radiation. This may be useful to examine certain end-points as the radiation dose rates are very low in the deep space environment.

3. Do beyond LEO living conditions increase mutation frequency?

Commonly used mutation assays include the forward mutation assay, where the loss of function mutation is identified by resistance to a compound, and the reverse mutation assay, where mutations result in restoration of function of a selectable marker [101]. Both assays require bacteria and yeast to be grown on solid medium with the selection agent so that the surviving
number of colonies can be quantitated. Growth on solid medium to determine survival by colony forming ability will not be feasible in an autonomous experiment. The restoration of function assay can be adapted for use with a fluorescent protein. The organism carries a vector with an open reading frame for a fluorescent protein that is disrupted by a stop codon or multiple repeat sequences that prevent production of the fluorescent protein. Mutation at the stop codon or instability at the repeat sequence results in production of a fluorescent protein and fluorescence can be monitored by microscopy, a fluorescence detector or flow cytometry [102]. This type of assay could be used in a variety of cell types or organisms.

4. **How does living beyond LEO alter the transcriptome?**

Current technology prevents the interrogation of the transcriptome on an autonomous mission without sample return. However, it will be possible to determine how transcription of a specific gene of interest is altered by beyond LEO by using the promoter of the gene in a promoter-reporter construct that is carried by cells/organisms. The reporter protein needs to be short-lived to increase the sensitivity of the assay. Destabilized GFP has been developed for bacteria [103], yeast [104] and mammalian cells [105] to monitor promoter activity by fluorescence. By using promoter-reporter constructs carrying the DNA binding sequence of a specific transcription factor or sigma factor, the activity of the specific factor can be monitored in the cell while beyond LEO. Promoters known to be induced by a specific stress, such as oxidative stress, can also be used to determine whether the cells are under that type of stress beyond LEO (see question number 6 below).

5. **How does living beyond LEO alter metabolism?**

Measuring the activity of specific metabolic pathways will not be possible without sample return or astronaut involvement. It will be possible to monitor the expression of specific enzymes using promoter-reporter assays as described above.

6. **Does living beyond LEO induce oxidative stress?**

Oxidative stress can be studied by monitoring the activity of promoter-reporter constructs using promoters known to be activated by oxidative stress. These promoter-reporter constructs have been developed for bacterial genes regulated by SoxRS and OxyR [106] and the thioredoxin promoter in yeast [107]. For mammalian cells and yeast, genetically-encoded redox sensitive fluorescent molecules (roGFP, Hyper, SypHer) are also available to detect ROS [108,109,110,111]. These proteins fluoresce when oxidized and have been targeted to the cytosol and mitochondria to assess ROS. A biosensor has also been developed for mammalian cells to measure the mitochondrial NADPH levels (iNAP) [111]. NADPH is required for redox cycling of antioxidants such as glutathione and so will provide information concerning the ability of the cell to protect itself from oxidative stress.

**Model Organisms**

Single cell prokaryotes and eukaryotes can be used to address the questions posed above. Yeast has many similarities to mammalian cells and has proven to be able to survive spaceflight. Mammalian cells in culture can also be used although technology will need to be developed to maintain cultures during missions. Cell types related to the Human Research Program risk gaps are recommended.
**BEYOND LEO TECHNOLOGY - NEEDS FOR THE NEXT 5 YEARS**

- **Growth/maintenance of cells.** Temperature, pressure and oxygen/carbon dioxide levels will need to be regulated. For long-term studies, cells in suspension such as bacteria, yeast or certain mammalian cells will need to be diluted and maintained in fresh growth medium. Adherent mammalian cells could be provided with new medium but will need to be removed from the surface prior to dilution and re-attachment. Differentiated adherent mammalian cells could be autonomously maintained by providing growth medium. Adherent mammalian cells will be more useful for microscopy studies.

- **Microscopy** (bright/dark field, phase contrast, and fluorescence) with still imaging, time-lapse imaging and/or video capture.

- **Fluorescence measurement** using spectrophotometry, microscopy and flow cytometry.
SECTION B: HOW DOES THE BEYOND LEO ENVIRONMENT IMPACT MICROORGANISMS AND MICROBIAL COMMUNITIES?

Microorganisms can serve as models to elucidate fundamental biological effects of beyond LEO conditions on higher organisms, and are important for human Microbiomes. For example, experiments can be conducted autonomously in deep space or on the lunar surface with microorganisms to determine how beyond LEO conditions impact different aspects of cellular growth and physiology (see SECTION C). This information may indicate the potential impacts of space radiation and other beyond LEO conditions on the similar biological processes of human cells and other organisms.

Because microorganisms on Earth primarily exist as communities of interacting species/strains it is also paramount to understand how microbial community interactions are impacted by beyond LEO conditions. For example, the coordinated metabolisms of microbial communities are responsible for key services, including nutrient cycling and plant growth promotion. It is currently not known how the space environment beyond LEO will influence microbial dynamics, their interspecies/interkingdom interactions and the overall ecology of the microbial community. To answer this question, it is important to take advantage of model microbial systems that have sufficient simplicity to allow experimental control in beyond LEO conditions [112].

Single microbes and/or communities of microbes can also provide essential services that are needed for life support during long-duration missions. These services include water recycling, waste management, vitamin production [113], human probiotics, and plant growth promotion. Thus, it is important to understand how beyond LEO conditions will impact these critical services.

The next level of complexity is understanding microbial ecosystems where the environmental context is important. Relevant questions concern how microbes and microbial communities interact with plants and animals. These questions are covered in sections D and E.

CRITICAL QUESTIONS IN THIS RESEARCH AREA

1. WHAT ARE THE IMPACTS OF DEEP-SPACE RADIATION AND PARTIAL GRAVITY ON MICROBIAL BIOLOGY?

It is currently not known how partial gravity, deep-space radiation, and the combination of these factors, will impact different types of microorganisms. For studies of individual microbial species, investigations of interest include studies of beyond LEO conditions on genetics, growth, reproduction and physiology. Note that evolution will be discussed in a separate section (see section F). Prior studies have examined the impact of microgravity on microbial cultures [section A above, 114], and a number of microbes alter growth, aggregation and resistance to antibiotics in liquid culture under simulated microgravity or LEO spaceflight [30,70,115,116,117]. Therefore, additional investigations of interest include studies of growth dynamics, susceptibility or resistance to antibiotics, biofilm formation, and synthesis of secondary metabolites under beyond LEO conditions.
2. HOW DO CONDITIONS ON THE LUNAR SURFACE IMPACT FUNDAMENTAL MICROBIAL PROPERTIES?

Specific to the lunar surface, studies of interest include determination of the effect of albedo particles, lunar dust and the lunar chemical environment on microbial biology.

3. WHAT IS THE POTENTIAL FOR MICROBIAL PATHOGENS TO EMERGE BEYOND LEO?

Another important area for beyond LEO research concerns understanding the threat of microorganisms as pathogens. Some microorganisms that are typically non-pathogenic in Earth environments, may pose a threat when conditions change beyond LEO. Opportunistic pathogens may be able to survive and colonize new niches in the beyond LEO environment that could pose a threat to human, animal or plant health. For example, *Salmonella typhimurium* was significantly more virulent when grown in space, when compared to grown on the ground [32]. This topic was also highlighted as a future space microbiology NRA in the Space Biology Science Plan as follows: “Under the reduced microbial-diversity conditions of space habitats, do opportunistic pathogens have a greater survival capacity, and do they have a greater propensity to infect as compared with ground controls?” [118].

4. HOW ARE COMMUNITIES OF MICROORGANISMS (SYNTHETIC COMMUNITIES ‘SYNCOMS’, OR CHARACTERIZED ASSEMBLIES) IMPACTED BY BEYOND LEO CONDITIONS?

It is important to understand how beyond LEO conditions impact interactions between members of microbial communities and their coordinated functions. On Earth, microbial communities have evolved to coordinate metabolic and other interactions between species. Interactions vary between beneficial mutual interactions, including commensalism and symbiosis, to negative interactions, including competition and predation [119,120]. Direct examples of microbe-microbe interactions include biomass turnover, production of extracellular polysaccharides and competitive exclusion. Molecular interactions include syntrophic interactions that can be either directional or commensal, quorum sensing, production of antibiotics and metabolic division of labor. Questions to address for microbial communities beyond LEO include: Do microbial communities persist over time? Are they stable? Does biodiversity remain stable, or change? Do commensal, cooperative or competitive interactions (2+ populations at a time) differ in beyond LEO conditions compared to those on the ground? Questions relevant to microbial systems biology can intersect with human microbiome and plant microbiome ecosystems (see sections E and F).

FEASIBLE RESEARCH BEYOND LEO IN THE NEXT 5 YEARS

Many of the questions discussed above can be addressed in the next five years with small advances in technology. Because the experiments must be conducted autonomously, without sample return, all data will be gathered *in situ* and transmitted to Earth. For example, autonomous microbiological research platforms exist for culture-based assays [121,122]. Capabilities are, however, limited and would need expansion to address all questions above. In addition, some technologies that currently require sample return, such as untargeted proteomics and metabolomics, are not yet feasible to perform autonomously. Note that crewed experiments on Gateway and lunar surface may change these points in the longer term. However, because most experimental opportunities in the near future will not involve crew, there will be long pre-launch and transit times that require organisms to remain viable in stasis. For microorganisms, options to consider include lyophilization, use of dormant/inactive cells and spore formers that can be activated into a viable state once the destination has been reached. Technologies should also be
able to mitigate extreme environmental conditions, while exposing to and accurately measuring the environmental parameters of interest.

Types of experiments could include fluorescent biosensor strains, or experiments with complementary colored fluorescently tagged cells for study of microbial interactions. Note that fluorescent activity-based probes are available for detection of specific enzyme activities and these would be feasible for adoption to existing technology in the next 5 years [123]. Use of bioluminescence is another option with sensitive optical sensors to detect the output from luminescent biosensors or growth of specific luminescent cells. In addition, Raman spectroscopy is an option for determining the physiological state of microbial cultures [124]. Chemical monitoring, for example by mass spectrometry, could be an option for determining metabolic processes in pure cultures or in microbial communities [125].

Autonomous sequencing of DNA should also be feasible in the next five years, using recent advances in microfluidics for PCR amplification and small single molecule sequencing platforms, such as nanopore (MinION) sequencing [126].

**MODEL ORGANISMS**

Relevant organisms for autonomous study include model eukaryotic microbes, such as yeasts and filamentous fungi. Different prokaryotic microorganisms are also of interest. For this report they are classified into functional categories; including but not limited to the following: nitrogen fixers, denitrifiers, photosynthetic microbes - cyanobacteria/ purple sulfur/non-sulfur bacteria, chemotrophs, halotrophs, ect. (Table 1 - Organism Summary below).

For study of microbial community interactions, systems can be constructed with characterized species into synthetic model communities, or “Syncom’s” [120]. The advantage of this approach is that use of well-defined species can allow for ease of genetic and physiological assessment of community interactions. The other option is to use naturally evolved communities of microorganisms. The advantage of natural systems is that the member species have been adapted to naturally interact and experimental assessment of beyond LEO conditions on those interactions may sometimes be more impactful. Examples include microbial mat communities that harbor hundreds of robust taxa that can survive a range of environmental stresses. Other relevant microbial communities/systems include Earth relevant systems that will be mimicked on the Lunar surface or during long-duration space flight; such as soil, lunar dust, space flight surfaces, plants and bioreactors.

**BEYOND LEO TECHNOLOGY - NEEDS FOR THE NEXT 5 YEARS**

Relevant microbial investigations for the 2022–2027-time frame include measurement of effects of deep-space radiation, altered atmospheric conditions of spacecraft (and lunar surface), weightlessness, and other factors of the spaceflight environment, during long-duration missions and on the lunar surface, on fundamental biology of microorganisms, microbial communities and microbial ecosystems.

Beyond LEO technology needs for the next five years include equipment for autonomous measurement of microbial cell numbers, microbial growth and ideally also microbial interactions. The Space Biology Science Plan mentions development of a multi-fluorescence microscopy technology platform that was designed for microbial culture experiments on the ISS (Space Biology Science Plan). This type of technology, that relies on instrument control from the ground,
could be applicable for autonomous sampling of fluorescently-tagged microorganisms and microbial communities beyond LEO.
SECTION C: HOW DOES LIFE BEYOND LEO IMPACT THE PHYSIOLOGY OF MULTI-CELLULAR ANIMALS?

As discussed in section A above, life beyond LEO is expected to impact cellular biology. Since cells make up organs which in turn make up multi-cellular animals, life beyond LEO is expected to impact physiology. Thus, it is important to consider studying beyond LEO impact on multi-cellular animals at the level of cells as discussed above and in terms of host-microbe interactions and evolution as discussed in sections E and F below; note that such studies can, in some instances, be combined with studies also targeting physiology in the same organism(s).

The impact of LEO on human and animal physiology has been well studied and is regularly reviewed by the National Academy of Sciences via decadal surveys, the most recent being published in 2011 with one currently under way. Additionally, the NASA Life Below Low Earth Orbit Science Working Group has recently reviewed the physiologic systems of particular interest for research beyond LEO [6].

CRITICAL QUESTIONS IN THIS RESEARCH AREA

1. HOW DOES BEYOND LEO IMPACT PHYSIOLOGIC SYSTEMS?

This topic was extensively reviewed by the NASA Life Beyond Low Earth Orbit Science Working Group which highlighted the Immune, Muscle and Skeletal, Cardiovascular, and Central Nervous Systems to be of particular interest for animal physiology research beyond LEO [6].

FEASIBLE RESEARCH BEYOND LEO IN THE NEXT 5 YEARS

Multicellular animals, including Astronauts, require life support systems to support them in space. Additionally, for mouse and most fish experiments it is likely that no sample return would be deemed unethical. This largely rules out vertebrate experiments in the short term. Further, as life support systems and habitats themselves can alter physiology [127,128] it is important that model systems research take place in environments as similar/identical to Astronauts as possible.

In the short term, adaptation of worm, fly, and organ on a chip hardware for ISS physiology experiments could be considered. Alternatively, or in addition, calls for payloads using existing small SAT technology for worm experiments could be made. For example, behavioral analysis beyond LEO.

MODEL ORGANISMS

In keeping with Earth based translational projects funded by the NIH and larger programs such as the monarch initiative [129], genomic model organisms including yeast, worms, flies, fish and mice should be employed. This approach enables forward and back translation in the interest of improving Astronaut health. Such an approach has already been called for in the literature [130] and is fully in keeping with the focus on Thriving in Deep Space.

Previously, worms and flies have been used to study the immune, muscle, cardiac, and nervous systems in LEO [131,132,133,134], fish for the muscle and nervous system [135] and for aquaculture considerations [136], and mice all of these priority physiologic systems [137]. Of note,
rats are a long-standing model for physiology and have previously been used in spaceflight experiments [138] and there is still considerable debate about mice versus rats for studying/modeling human physiology. Additionally, organs on a chip are being utilized to study some aspects of physiology ex vivo and there is currently high interest in using these in joint NIH/National Labs projects. Lastly, DARPA has expressed considerable interest in flatworms as a model for regeneration, a capability not usually found in other animal’s physiology.

**Beyond LEO Technology - needs for the next 5 years**

For the majority of physiologists, rodent models are a priority. Thus, development of mouse habitats for beyond LEO is a priority in the short and medium term. These habitats should take advantage of the latest advances in physiological monitoring in terms of smart and metabolic cages as well as monitoring (e.g. wearables). Wherever possible this monitoring should be identical/parallel to human subjects monitoring. This may be an appropriate time to explore designs for remote *in-vivo* fluorescence monitoring.

For shorter term and longer distance missions, worm and fly models should be prioritized. Thus, development of habitats for use on the moon, Mars, or in deep space should be a priority. Video return is expected to be the primary outcome.
SECTION D: HOW DOES LIFE BEYOND LEO IMPACT PLANT DEVELOPMENT AND PHYSIOLOGY?

Plants are a vital and valuable component of bioregenerative life support systems for long duration space missions. Plants provide several crucial functions from production of food to helping with air purification, and recycling of water [139] as well as psychological benefits [140]. However, there are challenges to growing plants in LEO and beyond [141]. These include providing the essential requirements for optimal plant growth such as lighting, water and nutrients. Additionally, strategies are needed to mitigate the detrimental effects of radiation and microgravity that are particular hazards of the beyond LEO environment. In order to maximize the potential of plants for bioregenerative life support systems, several key science questions will need to be addressed concerning seed viability, plant quality and growth in space.

CRITICAL QUESTIONS IN THIS RESEARCH AREA

1. WHAT ARE THE EFFECTS OF DIFFERENT G LEVELS ON GERMINATION, GROWTH, TROPISMS, SECONDARY METABOLITE PRODUCTION AND FOOD QUALITY?

The moon, Mars and spacecraft with artificial gravity represent intermediate g levels between that of earth and that of orbital flight. At about 1/6th g the lunar surface would be an ideal venue for exploring this question. So far, intermediate g levels have been simulated in the laboratory, finding, for example, lunar gravity impacting root growth parameters in a similar manner to microgravity and Mars gravity impacting root growth in a similar manner to Earth gravity [142].

2. HOW CAN ROOT ZONE WATER, NUTRIENT AND O₂ PROVISION BE OPTIMIZED FOR PLANT QUALITY AND GROWTH IN SPACE?

The optimization of water, nutrients, and O₂ to the root zone is critical for plant health and the behavior of water and nutrient solutions under partial gravity conditions needs to be understood. While numerous plant species have been grown on orbit, some with astounding success, root matrix selection and design require continued exploration, and the relative merits of porous media, hydroponic seal and aeroponic mist (which is of rising interest) are still under discussion. NASA is implementing a Passive Orbital Nutrient Delivery System (PONDS) prototype into a flight-qualified Enhanced Passive Water Delivery System (EPWDS) for the eventual purpose of most effectively delivering aqueous nutrient solutions to the roots of plants intended for food. A lunar settlement might use regolith as porous root-zone media to minimize equipment, upmass and energy. Seed germination tests with lunar regolith simulant and deionized water [143], as well as root zone aeration by oxygen producing polymers [144], have yielded encouraging results and need to be explored further.

3. HOW DO PLANT-MICROBE INTERACTIONS AFFECT PLANT QUALITY AND GROWTH IN SPACE (BENEFICIAL AS WELL AS PATHOGENS)?

Beneficial microbes can promote plant growth, increase resistance to pathogens and reduce the need for fertilizer input [145,146]. Therefore, they would be valuable additions to increase plant productivity in space. In nature, the plant microbiome is varied and diverse, and more ground based studies are needed to develop minimal synthetic consortia to supplement non-soil-based growth media in space. Beneficial microbial strains will need to be carefully vetted to ensure safety and efficacy.
Furthermore, more studies are needed to understand the response of plants in space to opportunistic pathogens. Zinnia plants growing in Veggie hardware on the ISS were more susceptible to *Fusarium* infection when their roots were under hypoxia and excess water \[147\]. Currently, plant seeds are sanitized to minimize crew health risks. However, this could lead to a higher susceptibility to opportunistic pathogens from the unique microbiome of a transit vehicle. Additionally, some bacterial pathogens were found to be more virulent in space which could increase the risk of plant disease.

Preparation for space travel beyond LEO is a very good reason to aggressively pursue studies to understand the impact of long duration culture and fractional gravity on interactions between the microbe, the host and the environment. This includes studies of pathogenic and commensal microbial responses (genotypic, molecular genetics, metabolomic and phenotypic).

4. **What are the Effects of Different Radiation Levels on Plant Quality and Growth in Space?**

Numerous published findings have shown that the effect of ionizing radiation on plants depends upon species, cultivar, development stage, tissue architecture and genome organization, as well as radiation features, e.g. quality, dose, and duration of exposure \[148,149,150\].

In deep space, GCRs present as an extremely low dose background radiation which may have less impact on short term plant growth experiments (i.e. during early seedling development, the maximum accumulative GCR dose is at milligray range for a 10 day exposure). Thus, for imbibed seeds, protons released from a large SPE, pose a more significant impact than GCRs. On the other hand, dry seeds in long-term storage during deep space missions will be exposed to a much higher accumulative GCR dose, which will affect seed viability over a long-duration mission.

Long-duration exposure of seeds to the space environment have been carried out using MISSE, EXPOSE-E and R, and LDEF platforms. In general, these studies have shown that seed viability and germination are negatively impacted, although the severity of the response varied between experiments and plant species tested \[151,152,153\]. Following the EXPOSE-E mission, *Arabidopsis* seed survival was 23%; however germination dropped to 3% with no survival, following EXPOSE-R mission where total UV and cosmic radiation doses were >1.4 times higher.

In a very recent experiment (CRESS 1U CubeSat), *Arabidopsis* seeds (under 1 atm) were exposed to Stratosphere (36-40 km) environment above Antarctica in a 30 day long-duration high altitude balloon mission. In a parallel experiment, seeds were exposed to 40 cGy GCRs 1 simulation at NSRL. GCR and Stratosphere exposed seeds showed significantly reduced germination rates of 76.4% & 82.5%, respectively compared to 98% for the controls. Significantly elevated somatic mutation rates (& developmental aberrations) were also revealed in these GCR or Stratosphere exposed seeds with the GCR exposure generating significantly higher mutation rate than that of Antarctica. These mutations also resulted in the death or delayed growth of certain plant organs. Heritable mutations were found in the second generation of the GCR irradiated seeds \[154\]. Heritable epigenetic changes were also detected in rice seeds following space flight \[155\].

It is clear that more studies need to be conducted, on a variety of space crops to determine the impact of deep space radiation on critical developmental stages in the plant life cycle.
5. **HOW CAN LEGGING STRATEGIES BE USED TO BOTH MONITOR AND MAXIMIZE PLANT QUALITY AND GROWTH IN SPACE?**

Maximizing the lunar environment for crop growth would involve a minimally pressurized containment, maximum use of natural ambient light, and lunar regolith as root matrix [156]. Challenges faced by plants in a pressurized enclosure on the moon include sunlight intensity (1.37 vs. 1.0 kW/cm² on Earth), spectrum (UV below 250 nm) and cycle (14 d vs. 12 h on/off), temperature (+120°C) and its fluctuations (to -170°C), day length (14 d), and regolith composition (basalt, pyroxene, olivine).

6. **HOW DOES ATMOSPHERIC COMPOSITION AND PRESSURE AFFECT PLANT QUALITY AND GROWTH IN SPACE?**

Maintaining atmospheric pressure during long duration missions imposes costs associated with mass and energy requirements. Defining the limits of pressure and composition that are needed for optimal plant growth is therefore of great interest [157]. Much of our current understanding of plant adaptations to low atmospheric pressure comes from experiments conducted at high altitude locations as well as in hypobaric chambers. These studies have revealed that low atmospheric pressure results in hypoxia as well as increased water loss by transpiration. Transcriptional studies have shown that the effects of hypobaria can be partially mitigated by sufficient O₂ and water availability [158,159]. However, hypobaria also constitutes a unique stress and more studies are needed to enable plants to adapt and thrive under these unfamiliar environmental conditions.

7. **WHAT PLANTS AND NOVEL ORGANISMS SHOULD BE USED AND OR DEVELOPED FOR FOOD PRODUCTION AND BIOREGENERATIVE LIFE SUPPORT SYSTEMS IN SPACE?**

The ideal plants for food production would be high yielding (high harvest index) with minimum hardware requirements, small upmass and energy provision. A fully consumable plant with less waste would be valuable (i.e. 10-day aeroponic beet). Microgreens are good candidates [160] as well as tuberous crops with high edible biomass such as potatoes [161,162]. Additionally, Cyanobacteria or unicellular algae could be used to recycle oxygen from CO₂ as well as provide food at the end of their growth cycle; however, palatability issues will need to be solved by further research for the feasibility of crew consumption.

Research will also be needed to generate crop cultivars with improved traits either by breeding/selection or genetic engineering. Traits of interest include the ability to withstand stress, enhanced plant performance under unfavorable conditions, resistance to pathogens/pests and improved nutritional content.

8. **WHAT ARE THE EFFECTS OF DIFFERENT MAGNETIC FIELD LEVELS ON PLANT QUALITY AND GROWTH IN SPACE?**

Although some claims have been made concerning the effects of modified magnetic environments on plant processes there has been no evidence that removal of plants from the earth’s 3 x 10⁻⁵ Tesla field will have a catastrophic effect on plant performance.

9. **MULTI-STRESSOR EFFECTS (COMBINED EFFECTS).**

It is clear that plants in nature are exposed to multiple stressors simultaneously, which may have antagonistic or synergistic interactions. Recent work has shown that plant responses to multiple stress combinations are unique and cannot be extrapolated from the response to a single stress treatment [163,164].
Similarly, plants in spaceflight are exposed to a combination of unfavorable conditions, such as radiation, altered gravity, non-optimal growth conditions (including water stress, high CO\textsubscript{2} and VOC levels, and altered air pressure). To date, combined effects have not been studied in crop plants and other candidate biology for deep space bioregenerative life support systems. Ground-based simulation studies are able to provide some insight, however, to obtain high fidelity data, seeds and plants still need to be tested in the true deep space environment to prove the knowledge base and validate mitigation concepts developed from ground-based studies.

10. **What are the Comparative Effects of Ambient vs. Built-Environment (LED) Illumination on Photosynthesis and Tropisms?**

While spectrally ideal combinations of LEDs have been identified, it would still be valuable to investigate a means of using the ambient continuous daylight of interplanetary space to potentially save energy and spacecraft complexity.

**Feasible research beyond LEO in the next 5 years**

While it is conceivable, that some parameters listed above could be tested on the ground or on the ISS, (such as optimizing LED lighting and low atmospheric pressure), it is clear that altered g levels and radiation cannot be adequately simulated. Furthermore, the combination of multiple stresses will be hard to replicate.

In the absence of sample return and limited options for downstream analysis, only some of the questions can be feasibly addressed in the near term. These are listed below.

1. **What are the Effects of Different G Levels on Germination, Tropisms, Secondary Metabolite Production and Food Quality?**

In the context of the above questions, only lunar gravity would be explored. Holding other variables (pressure, illumination, moisture) constant will be a challenge, but even 1/6\textsuperscript{th} g will make water management easier than on ISS. Note the other two items below.

2. **How can Root Zone Water, Nutrient and O\textsubscript{2} Provision be Optimized for Plant Quality and Growth in Space?**

The selection process involving porous media vs hydroponic vs aeroponic approaches might be resolved by testing the suitability of fresh lunar regolith as root zone media with artificial grey water. This combination would reduce upmass for a lunar settlement and could be tested by depositing a permanently pressurized growth chamber at a specified depth into the regolith and using solar-powered environmental controls and image and data telemetry.

3. **How can Legging Strategies be Used to Both Monitor and Maximize Plant Quality and Growth in Space?**

Initially a small number of food crop plants should be selected for study. A remote means of scooping regolith into a growth chamber will be needed. Pressure and composition of an artificial atmosphere needs to be optimized and supplied by a pressure bottle. Solar-powered environmental controls and image and data telemetry, with real-time remote control from earth will optimize operations. Light control will require a combination of heavily filtered sunlight supplemented (during lunar night) by battery-powered LED illumination.
MODEL ORGANISMS

Model organisms (Arabidopsis, moss species, green algae Chlorella) and crop species (lettuce, tomato, peppers, maize), for seed storage and seedling development at the early deep space exploration stage, and adult plants and multigenerational studies in later exploration stages when platforms and hardware are available. (Maybe also possible to conduct limited experiments with cell cultures).

BEYOND LEO TECHNOLOGY - NEEDS FOR THE NEXT 5 YEARS

1. OPERATIONS
   - Passive storage and/or active water delivery,
   - LED lighting to initiate germination and growth,
   - Carefully calculated energy budgets for remote environment control.

2. ENDPOINTS

Morphological, physiological, and molecular data collection (Some of the large scale “OMICs” approaches would require sample return and may not be feasible in the near term). Numerous endpoints can be quantified using image data.

3. ANALYTICAL TOOLS REQUIRED ON-BOARD THE MISSIONS
   - Cameras/video cameras for automated phenotyping,
   - Automated hyperspectral and or thermal infrared imaging,
   - Fluorescence imaging, (chlorophyll fluorescence as a measure of stress),
   - Sensors for O₂/CO₂/moisture,
   - Centrifuge facility with hardware for growing plants under partial g levels.

ANALYTICAL TECHNOLOGY FROM THE LIFE BEYOND LEO REPORT

New bio-analytical instruments suitable for launching to and operation in LEO are becoming available at an almost monthly frequency. NASA’s Wetlab projects have been attempting to follow this trend. Coming with each new instrument is a reduced amount of effort required to adapt it for space flight. Indeed, a Nanopore (single-molecule) DNA sequencer has been tested on ISS. Thanks to powerful ELISA (Enzyme-Linked Immunosorbent Assay) adaptation to microfluidic systems thousands of proteins can be quantified without an electrophoresis step. A hand-held microelectronic microfluidic cell analyzer can be expected. These developments impact beyond LEO research in two ways: Analytical data can be collected in space, including beyond LEO, without any on-the-ground involvement, and the chemical reagents, not the instrument, constitute nearly all of the upmass. The selections from among these technologies will depend on beyond LEO priorities.
Gravity represents one of the few constant evolutionary drivers of life on Earth [165], yet it has been a consistent vector through the history of life. How multicellular organisms respond to gradients in gravity or how these gradients shape the evolution of life is not fully understood [166]. Compounding our understanding of the mechanisms underlying the effects of changing gravity conditions on eukaryotic health is the lack of understanding of the impact of changes in gravity on host-associated microbiomes [167]. A microbiome is typically defined as the sum of the microbes, genomes and community interactions that interact with the body [168,169,170]. The term has been quickly adopted to represent the connectivity and interactions between complex host-microbe associations [170]. Initial surveys indicate that for every host gene there are hundreds of microbial genes, thereby providing the host with millions of genes of additional metabolic functional potential [171].

Another confounding variable of beyond LEO conditions is radiation. Radiation is known to have negative impacts on human physiology [49,172]. Because of the intricate interplay between the host and its associated microbiome it is imperative to also understand how radiation impacts the host microbiome and if the microbiome can be harnessed to counteract some of the negative impacts.

Together, these efforts to understand the diversity and stability of host-microbe interactions under changing gravity and radiation conditions will provide important insight into the resiliency of the host microbiome to withstand the stress of spaceflight. Regular disturbances and perturbations may result in a loss of biodiversity or extirpation (i.e., the extinction of a species in a localized area within the host) that may potentially drive the community towards dysbiosis and disease of the host. Therefore, it is critical to provide a comprehensive assessment not only of the complement of microbiota associating with plant and animal hosts in the space environment, but how the interactions between a host and its associated microbiome are initiated, persist, and are maintained over long-duration spaceflight. Through the examination of these processes, it is likely that signatures of host-microbe co-evolution within the spaceflight environment will emerge and may be used to help mitigate and attenuate any negative impacts on host health.

CRITICAL QUESTIONS IN THIS RESEARCH AREA

1. **How does the host microbiome change over long duration space travel?**

As the awareness of microbiome health has increased in recent years, so too has the realization that for long duration space travel microbiome research needs to be a critical area of study. There has been a rapid rise in the number of microbiome studies conducted under spaceflight or modeled microgravity conditions, especially regarding astronaut health [37,173,174,175]. However, most of these studies have either focused on short-term changes in hosts or there have included very small sample sizes.

Soon there will be rapid increase in the number of commercial (e.g., Axiom) and government-led (e.g., Gateway, Tiangong) space stations beyond ISS, and as these new stations become
functional and inhabited it will be important to monitor the microbiome of the crew as well as the station to understand the changes and exchanges that occur between the human host and the habitat. Key questions include:

- Are host microbiomes stable over time and how do diversity and function change?
- What is the extent of exchange between habitats and hosts over time and space?
- What is the efficacy of probiotics as supplements for hosts if key taxa are extirpated?
- Does the stability of the space station habitat microbiome mitigate the spread of pathogens for plant and animal hosts?

2. **How are beneficial interactions with microbes established in the space environment?**

Although studying established microbial consortia with hosts is valuable, understanding whether the space environment negatively impacts the formation of host microbe interactions will be essential for long-duration space flight and ecosystem maintenance. For example, as the growth of food crops likely diversify beyond lettuce and chili peppers, the initiation and establishment of the rhizosphere and host microbiome will be necessary under spaceflight or lunar gravity conditions. Evidence using partial gravity simulations of plants have found distinctive thresholds of cell growth and proliferation [142] but the impact on the associated microbes has yet to be fully explored.

Likewise, animal physiology under a changing gravity continuum also shows changes [176]; however, only a few studies have examined the initiation of animal-microbe interactions in modeled microgravity conditions [177,178]. To more fully understand whether, or how, symbiont colonization occurs under key areas of study include:

- Evaluate whether there are gravity thresholds for successful colonization of host tissues.
- Assess whether there are changes in colonization phenotypes across the gravity continuum (e.g., changes in competition between taxa/strains?)
- Determine whether the specificity of host-microbe interactions change under changes in the space environment due to changes in gravity and/or radiation. (e.g., are animals/plants more, or less, permissive to other strains/taxa of microbes?)

3. **How are functional activities of beneficial interactions with microbes maintained throughout the life of the host organism in the space environment?**

Once an association is established in the space environment, it is unknown whether the long-term impact of spaceflight conditions would negatively impact the persistence and normal healthy functions of the host-microbe interactions. There is very little data on the metabolic activity and exchange that occurs between a host and its microbiome in the space environment over long-periods of time (e.g., > six months). Key areas of study include:

- Evaluate whether microbes and their hosts use previously unknown signaling pathways to communicate under the stress of the space environment and whether these pathways change under a gravity continuum and/or changes in radiation.
- Assess whether microbes regulate and control host processes differently under a gravity continuum and/or changes in radiation.
Feasible research beyond LEO in the next 5 years.

The selection of the host organisms will be critical to ensure the feasibility of examining host-microbe interactions autonomously and without sample return. Invertebrate organisms that are able to survive long-durations without crew intervention will be critical to enable real-time in situ analyses of changes that occur in the host-microbe association. Additionally, there could be a focus on conserved response pathways (e.g. innate immune or stress responses) that are conserved across eukaryotes could be the initial focus for study.

Model Organisms

To address these questions, simplified model systems are needed where the interactions between microbes and their hosts can be examined in the space environment.

1. Potential Plant Models

1.1 Food crops. Food crops (e.g., lettuce, mizuna, chili peppers) are now being regularly grown on ISS using Veggie and will likely be included in beyond LEO; therefore, these crops represent a valuable model for examining how plant-associated microbiome with rhizome, leaves, stems change over time and under various stress conditions. Recent publications on developing techniques for the rapid assessment of the metagenome and microbiome are now emerging [179].

1.2 Arabidopsis. Techniques are well established for maintaining this plant for long periods of time in the space environment [180,181,182].

2. Potential Animal Models

Use animal models where cells or animals can be frozen and reanimated after extended periods of low temperature. Or target animals that can live autonomously with minimal care for >6 months.

2.1 Hydra. Essentially immortal and cells can regenerate extensively. Valuable for studies of genome stability as well as symbioses with various strains of photosynthetic algae (e.g. Chlorella). The presence of the photosynthetic symbiont enables the animal’s cells to withstand long periods of starvation [183].

2.2 Rotifers. Rotifers can deliver beneficial microbes to other animals in ecosystems. Rotifers typically colonize habitats and facilitate the transition of energy from primary producers to secondary consumers and are important members of the ecological cycling of nutrients [184]. Rotifers have also become the go-to animal for water toxicity testing [184,185].

2.3 Termites. The termite symbiosis is one of the longest-studied beneficial insect-microbe symbiosis [186]. Termites are social insects and provide important ecosystem services and are often sources for therapeutic drugs (e.g. antibiotics), biofuel production using cellulose-degrading species, and nutrient cycling [187]. Termites can also pose as a valuable food source as they are high in essential minerals (e.g. Fe-Mn-Zn-Cu-Mg) [186,188] and in the remediation of plant waste material aboard spacecraft. Termites have been shown to be highly amenable to
laboratory cultures and are valuable models for the study of the manipulation of the microbiome [189].

**BEYOND LEO TECHNOLOGY - NEEDS FOR THE NEXT 5 YEARS**

- **Microbiome monitoring.** Real-time monitoring of changes in microbes on model hosts that can be assessed remotely. Recent advances in DNA and cDNA sequencing using MinION technology have made it possible to conduct real-time sequencing of the taxonomic and metabolic activities of the host- and spacecraft associated microbes [179,190]. Expanding this capability to include the automated nucleic acid extraction and processing would facilitate the automated and more regular monitoring of how host-associated microbes and microbiomes change over time.

- **Universal animal cultivation habitats.** Autonomous monitoring, maintenance and potential reanimation of host organisms. To accommodate long-duration incubations autonomously, habitats will be needed that are versatile and can house numerous types of invertebrates. Ideally, if the habitat could house both aquatic and terrestrial animals. Reanimation of cells from a frozen state and then maintaining temperature control, feeding conditions, and overall monitoring would be needed.
SECTION F: HOW DOES THE BEYOND LEO ENVIRONMENT IMPACT THE EVOLUTIONARY PROCESS?

Exploration scenarios in the beyond LEO/TIDES context will expose Earth life to new mutagenic sources and selection pressures. These include micro- and variable gravity, radiation, non-terrestrial physicochemical environments, the extreme built environment of the spacecraft and exploration habitats, and the interaction effects of all these factors. For the crew and associated manifested biology (e.g., seeds and plants for fresh food), it is most likely that physiological acclimation will dominate over evolutionary processes. However, the co-occurring microbial bio-load, be it viruses, fungi and other small eukaryotes, or bacteria, will be exposed to these stressors on evolutionary-relevant timescales. With their large population sizes and short generation times and the inability to completely control the microbiota of spacecraft and crew, understanding how these microbes adapt evolutionarily to life beyond LEO is critical.

Current knowledge about microorganisms in confined built habitats including hospitals, cleanrooms, and the International Space Station (ISS) has been reported [191], but beyond LEO offers new challenges, even compared to the ISS, with respect to duration, isolation, variable gravity, and radiation. Further, the evolution of bacteria, fungi, plant-microbe interactions, and population-level genetics in the context of in-situ resource utilization (ISRU), food-production and human health in spaceflight are long-term targets for fundamental research. Understanding the evolutionary process in the beyond LEO environment will link in with cellular functions, microbial ecosystems, plant growth and plant-microbe interactions, and microbial ecosystems (sections a, b, d, and e of this report).

Microbes will play key roles in the development of biologically based closed-loop regenerative life support, food production, ISRU, and will have extensive interactions with human and plant hosts. Further, microbes will pose challenges through contamination, as nuisance factors such as biofilms, and through enhanced pathogenicity and antibiotic resistance [192]. Previous spaceflight experiments with microbes have documented striking physiological and phenotypic changes including differences in growth rates, enhanced antibiotic resistance and virulence [193,194,195,196,197,198,199]. There appears to be a nascent microbial ecology on the ISS [200], with new bacterial species first identified on the ISS [201], and evidence of colonization of crew microbiomes by ISS microbes [202,203]. Potentially virulent bacteria exist onboard ISS, with some evidence of persistence and even an increase in virulence factors [204].

Although many studies have detailed physiological adaptation to the space environment [205], studies that examine underlying genetic changes that might also occur via evolutionary change or adaptation are lacking. Long-term evolutionary studies are a logistical and technical challenge in the context of spaceflight, where experimental requirements specify automation with minimal to no human intervention, and dictate limitations on experimental duration, power, mass, storage, and sample return.

Evolution is complex and includes multiple aspects, including epigenetics, methylation, as well as neutral and population-level processes, and the co-evolution of microbes with the built environment, and plant and human hosts. In order to advance understanding of how life evolves in the space exploration environment, fundamental science questions will need to be addressed concerning microbial evolution and adaptation, microbe-host interactions, and risks and countermeasures in space.
CRITICAL QUESTIONS IN THIS RESEARCH AREA

1. HOW DOES LONG-DURATION SPACEFLIGHT AND EXPLORATION AFFECT RATES OF EVOLUTIONARY CHANGE?

Experimental evolution studies with bacteria on Earth have revealed general rates and processes for mutation, adaptation, and bacterial evolution in laboratory settings [206,207]. These studies have shown that adaptation to a new, benign environment, as indicated by clear increases in growth rate, can take up to 1,000 generations to be clearly observable, with examples of even faster adaptation occurring under selective conditions, and it has been previously noted that increasing growth rate is a hallmark of adaptation to selective conditions [197,208,209,210]. Comparable evolution studies in spaceflight are lacking. In space, particularly with ISS-based microbial studies, a wealth of information on the diversity and distribution of microbial taxa has been reported, including the collection of microbial isolates, sequences, and genomes [204]. However, there is little to no ability to know the provenance of an individual sequence, genome or isolate; is it representative of a lineage that has persisted and evolved for decades onboard the ISS, or is it representative of a microbe newly arrived with the latest crew transfer or resupply mission? Controlled multi-generational evolution studies that explore the mechanistic nature of the evolutionary process (rates of mutation and change, including indels, gene loss and duplication, horizontal gene transfer) and selection can clarify the effects of spaceflight on the evolutionary process. In particular, understanding the evolutionary responses to variable gravity and radiation will be foundational in understanding how life is impacted across generations at the molecular genetic level.

2. WHAT ARE THE TARGETS OF GENETIC, MOLECULAR, AND BIOCHEMICAL PROCESSES THAT ARE SELECTED UPON IN THE BEYOND LEO SPACE ENVIRONMENT?

More specific to a general understanding of changes in rates of mutation and the evolutionary process in spaceflight is the question of what genes, pathways and processes are specifically affected? Does the space environment cause epigenetic changes, and which genes are susceptible or affected, and how does this impact biological function in space and after return to Earth gravity (see section A for potential experiments to study gene expression and epigenetic changes). Studies that target specific phenotypic traits in an evolutionary context (e.g., antibiotic resistance and virulence, motility, membrane transport, cell adhesion) will be of particular interest. Further, population-level selection will occur on microbial communities in the beyond LEO environment, including microbe-microbe and microbe-host interactions. Studies that can elucidate how these microbial communities adapt to spaceflight will be important (see sections B and E). Adaptation of microarray technology to flight, or targeted gene-expression studies will be invaluable, although linking the data expected to be collected to evolution (versus acclimation) may be a challenge without the possibility of sample return.

FEASIBLE RESEARCH BEYOND LEO IN THE NEXT 5 YEARS

In the absence of sample return, and advancement in capabilities in automation, microfluidics and sequencing technology, comprehensive sequence-based studies (protein and nucleic acid) in beyond LEO missions cannot be performed, limiting the portfolio of experiments that can currently explore evolution in deep-space. Although nucleotide sequencing has been demonstrated onboard the ISS [190], the technology is currently lacking for a fully automated “experiment to sequence” approach in space. However, flight-proven optical methods, including direct imaging,
transmittance and optical density, and fluorescence-based methods (e.g., measurement of competing reporter genes) will provide direct evidence of evolutionary changes in the absence of direct sequence data or sample return. Recent microbial evolution studies on Earth have visually demonstrated the drift of neutral alleles in microbial populations as they propagate across surfaces [211], and have shown the adaptive evolution of antibiotic resistance in time and space using agar matrices dosed with increasing concentrations of antibiotics [212]. Similar studies could be adapted to spaceflight, including observations of varying growth rates, and direct competition of differing genotypes. Quantitative genomics approaches, such as the competition (direct or indirect) and/or assessment of growth rates of a set of genotyped organisms with known single nucleotide polymorphisms (SNPs), indels, or alleles could identify adaptive features in spaceflight without sample return. The upcoming BioSentinel mission, scheduled to fly with Artemis I, will assess DNA damage in the yeast *Saccharomyces cerevisiae* as it is exposed to the beyond LEO radiation environment using metabolic dye and an LED detection system [5]. Though not an evolutionary study per se, BioSentinel will indirectly measure DNA (and cellular) damage, which is a key component of the evolutionary process, and this mission represents the current and near-future technological capabilities for integrating microfluidics, biology, and imaging/measurement in a beyond LEO context.

**MODEL ORGANISMS**

Model bacteria with flight heritage (e.g., *Bacillus*, *Escherichia*, *Deinococcus*, *Pseudomonas*, *Salmonella*), as well as small eukaryotes (e.g., tardigrades and nematodes, such as *Caenorhabditis*, green algae including *Chlorella*, yeasts, and filamentous fungi). Small plants with the potential for multi-generational and plant-microbe interaction studies (e.g., *Brassica* cultivars, *Arabidopsis*). Prokaryotes based on functional capabilities (e.g., diazotrophs, denitrifiers, and other N-cycle bacteria). Photosynthetic bacteria, including cyanobacteria and purple bacteria. Microbiome-associated bacteria, specifically human microbiome (skin, oral, gut), and plant-associated and plant growth promoting bacteria. Microbial assemblages and experimental communities.

**BEYOND LEO TECHNOLOGY - NEEDS FOR THE NEXT 5 YEARS**

**TECHNOLOGY / OPERATIONS**

- In situ monitoring of cells: direct imaging, optical density, absorbance, fluorescence, biosensors (fluorescent, electrical or optical), spectroscopy (fluorescence, luminescence, Raman, UV, IR, etc)
- Chemical monitoring - Mass Spectroscopy, Imaging (metabolic markers, gene expression, growth)
- Microfluidics
- Autonomous Microscopy
- Capability to support long-term studies (>20 days) autonomously.
SECTION G: HOW DOES THE BEYOND LEO ENVIRONMENT IMPACT BIOTECHNOLOGICAL PROCESSES?

MOTIVATIONS FOR BIOTECHNOLOGY IN SPACE

Biotechnological processes have unique features that make them appealing in the deep space environment (low temperature, low pressure, regenerable, expandable, programmable) and are the only means of manufacturing certain products (e.g., protein products such as enzymes and biologics). They can also make a far wider range of products or chemicals (e.g., drugs) available on a space mission to address contingencies than could be manifested as cargo. Because of this, NASA and other space agencies are developing new biological technologies to fill defined technology gaps [213] and enable new mission architectures. For example, CUBES (Center for the Utilization of Biological Engineering in Space, https://cubes.space) is a 5-year $15 M multi-institute effort to develop concepts and technologies to support a biotechnology ecosystem on Mars [214], and various perspectives on the utility of biotechnology for space are available [214,215,216,217,218,219,220].

EXAMPLES OF BIOTECHNOLOGICAL PROCESSES UNDER DEVELOPMENT TO ADVANCE SPACE TRAVEL

To date, biotechnological processes have not been utilized in space. However, a number of systems have been advanced to various TRLs. Below we discuss a limited set of biotechnology applications to communicate the breadth and potential of this technology. This section is not exhaustive and many promising technologies are not discussed.

The MELiSSA (Micro-Ecological Life Support System Alternative) project aims to develop a closed-loop system for air, water and waste management in space habitats. MELiSSA relies upon four subsystems: an anaerobic liquifying compartment that converts heterogeneous wastes to ammonium ion, H₂, CO₂, volatile fatty acids and minerals, a photoheterotrophic compartment that removes the remaining volatile fatty acids, a nitrifying compartment that converts ammonium ion to nitrates, and a photoautotrophic compartment responsible for regenerating oxygen. MELiSSA has operated a pilot process on earth to improve integration between these systems [221], and a set of spaceflight experiments has assessed the performance and stability of individual components [222].

In-space additive manufacturing could enable new mission architectures and 3D printing is under continual development. Currently, Made In Space operates a 3D filament printer on the ISS capable of utilizing various input substrates (presently ABS, HDPE, and PEI-PC polymers are authorized). Future deep space 3D printing operations could be constrained by the need for continual resupply of substrate from earth. To relieve these constraints, various approaches for generating these substrates from locally sourced materials are being investigated. Amongst these are microbially generated polyhydroxyalkanoates (PHAs). PHAs serve as a source of stored carbon for multiple microbial species and in some conditions PHAs can make up >50% of cellular
dry mass. Over 150 different varieties of PHAs have been discovered, all with different material characteristics [223]. Using a variety of input materials, microbially generated PHAs could be procured in-space at sufficient scale to improve missions’ architectures [216].

Microbes can be used for processes that can support sustainable human exploration of space. For example, bacteria and fungi can be used to extract and recover valuable metals from minerals [224]. In fact, this is commonly done on Earth and 15% and 5% of copper and gold, respectively, currently on the market comes from biomining processes. Additionally, microorganisms can be used to extract rare earth elements from ores (e.g. asteroid regolith) as well as electronic waste (printed circuit boards). The effectiveness of biomining processes has already been tested on ISS under a European project, including reduction of vanadium [225].

Another biotechnological process that may be implemented beyond LEO using bacteria is the bioremediation of habitat air (CO2 removal, O2 generation) and water (removal of human- and machine-produced toxic compounds) [226].

Microbes can also aid in soil formation efforts to enable crops to grow on regolith (unconsolidated and heterogeneous rock deposits, such as on the lunar surface and Mars). An additional application is biocrete production (microbiologically induced calcite precipitation (MICP) [227].

In-space repair/manufacture/assembly of (certain) human organs would improve in-space medical capabilities. Moreover, the microgravity environment of space may result in improved organ characteristics, which could lead to a terrestrial market for in-space manufactured organs. As such, in-space organ printing is being pursued. Recently, a scaffold-free and nozzle-free magnetic levitation-based process has successfully generated tissue spheroids (chondrospheres) on this ISS [228].

Beyond these examples, biotechnology promises to be flexible enough to provide multiple services including generation of edible nutrients, pharmaceuticals, materials, catalysts, and fuels. As bioengineering and synthetic biology tools continue to improve, biotechnology will become more desirable and competitive to traditional approaches for obtaining key materials (i.e. resupply or strictly physicochemical systems) and resources during space missions.

**Critical Questions in this Research Area**

In the following section, we discuss the central questions that need to be addressed for the effective deployment of biotechnological processes to the Moon and other deep space environments. We focus on questions not covered in more general sections (e.g. the Cellular Functions section), as it is assumed that these general issues will affect the biological components of biotechnological processes in a related way.

1. **How does the lunar gravity environment affect biotechnological processes?**

As discussed in section A, reduced gravity could directly or indirectly impact cellular and biochemical processes. These changes would impact biotechnological processes by altering the ambient baseline conditions under which a cellular factory would operate. These changes may impair or improve biological processes. For example, production of valuable secondary metabolites were alternately increased or decreased in distinct strains of *Aspergillus nidulans* grown on the ISS [229].

Beyond this, there are additional concerns with reduced gravity that only become especially relevant in the context of a biotechnological process. For example, foaming within terrestrial
bioreactors is a major concern that must be managed and it is reasonable to expect that the severity of this problem and the effectiveness of different mitigation strategies may be altered in the lunar gravity environment. The same is true for all aspects of gas or fluid management in a biotechnological process, particularly those related to mass transport. Thus, there would be great value in experiments designed to test and validate these aspects of a biotechnological process.

2. **How Does the Lunar Radiation Environment Affect Biotechnological Processes?**

As discussed in sections A and F, the lunar environment - particularly the lunar radiation environment - could lead to increased mutation rates and an altered biologically selective landscape. This could be of particular concern for biotechnological processes that need to be reliably operated within specified parameters. Even on Earth, continuously operated systems face issues with culture stability, as the metabolic burden associated with production can select for cells with reduced productivity [230]. This is because high production output necessitates diversion of carbon and protein synthesis capacity away from core processes necessary for cell growth and replication and towards the synthesis of pathway enzymes and/or products. Thus, cells with reduced productivity will usually grow faster. Developing methods to measure and respond to cellular burden is a major goal of synthetic biology [231]. Approaches include the development of “anti-mutator” strains of *E. coli* [232], pathway synthesis on orthogonal ribosomes [233], feedback control circuits [234,235], metabolic switching through two-stage fermentation [236,237], population quality control with sensor-selector [238,239] or growth-coupled production approaches [240].

3. **How Can Biotechnological Processes Best Utilize Lunar Resources?**

Any biotech process at scale will need to acquire resources (carbon, oxygen, nitrogen, water) on site to avoid costly delivery from Earth. As the moon effectively lacks an atmosphere, all resources must be sourced from the lunar regolith. The lunar surface can be subdivided into the ancient lunar highlands and the younger lunar mare (‘seas’). The lunar highlands are rich in calcium, aluminum, silicon, and oxygen in the form of anorthite (CaAl₂Si₂O₈) [241], but poor in magnesium and iron. The lunar maria are relatively rich in magnesium, iron and titanium in the form of anorthite (CaAl₂Si₂O₈), orthopyroxene ((Mg,Fe)SiO₃), clinopyroxene (Ca(Fe,Mg)Si₂O₆), olivine ((Mg,Fe)₂SiO₄), and ilmenite (FeTiO₃), but poorer in calcium and aluminum. At the surface these minerals exist as a layer of loose regolith several meters thick with an average grain size of 60 µm.

The lunar surface is constantly bombarded by the solar wind, which consists primarily of hydrogen and helium nuclei (by number) with heavier elements making up less than 0.1%. These solar wind particles accumulate in the regolith with volatile carbon present at a concentration of ~125 ppm (µg/g). In 2009 the Lunar Crater Observation and Sensing Satellite (LCROSS) impacted Cabeus crater on the Moon’s south pole and revealed the presence of CO₂, light hydrocarbons (CH₄, C₂H₆), and CO [242].

Overall, the moon is highly depleted of water but recent discoveries show its presence within permanently shadowed craters at the poles where water delivered from comets or formed through reactions with the solar wind has been trapped. In addition, there is evidence for hydrated minerals outside of the PSR at high latitudes, likely formed through reaction with the solar wind.

Oxygen is present within the various sources of water but also within anhydrous oxide and silicate minerals, making up >40% of lunar regolith by mass.

Extraction of these resources for use in a biological process would take place in the context of a larger In Situ Resources Utilization (ISRU) system focused on the extraction and generation of
critical life- and mission-support resources (e.g. oxygen, propellant). Biotech processes would comprise one component of the larger ISRU ecosystem that would rely on lunar regolith [244]. For example, over 20 processes have been identified to extract oxygen from lunar regolith, with two having been developed to TRL 4-5 and demonstrated at human-relevant scales. These are a Hydrogen Reduction process where iron oxide is reduced to iron and water with hydrogen at 900°C and a Carbothermal Reduction process where silicates are reduced at 1600°C to generate CO and H₂, which are then converted to CH₄ and water. The water is then electrolyzed to O₂ and H₂ [244].

As the larger lunar ISRU framework is further developed, it would be valuable to test the integration of biotechnological processes with this infrastructure on the moon. This could include experiments that are directly attached to future ISRU validation hardware, or stand-alone missions that have dedicated mechanisms for the sampling and processing of lunar regolith.

**Feasible research beyond LEO in the next 5 years.**

Given the potential of biotechnology to provide important in-space capabilities, it will be important to address critical knowledge gaps and to perform essential engineering tests on the moon and elsewhere in deep space. The questions to be answered will overlap those in other, more general sections. For example, it will be important to understand how the lunar environment affects microbial evolution and metabolism as discussed in the “Evolution”, “Cellular Functions”, and “Fundamental Microbial Biology and Ecology” sections. However, certain questions become particularly relevant in the context of biotechnology development, including specialized culturing and analytical capabilities and the capacity to provide the inputs relevant to a biology-based ISRU process.

It is completely feasible to answer important biotechnological questions within a 2022-2027 timeframe. Many questions, including basic information on the growth kinetics of key manufacturing chassis could be addressed with existing LEIA hardware (BioSensor). Upgrades or modifications to LEIA instrumentation would allow a better assessment of process performance, while substantial upgrades and redesign would be required to enable the end-to-end test of processes that use waste and lunar regolith as an input.

**Model Organisms**

Terrestrial biotechnology utilizes diverse host cells for production [245]. While *E. coli* and *S. cerevisiae* are popular engineering chassis, many economically important processes rely upon alternate species and cell types. For example, cellulases are harvested from the filamentous fungi [246]; insect and plant cells are used to generate proteins [247, 248]. With current rapid improvements in genomic and synthetic biological tools, additional species and cells are becoming viable engineering components [249], including halophilic [250] and thermophilic microbes [251], complex microbial communities, and biofilms [252]. On Earth, chemolithoautotrophs (iron- and sulfur-oxidizing microbes) are commonly used for biomining processes and may serve as a basis to interrogate and mature these technologies beyond LEO [224,253]. Bioprinting of replacement human organs relies upon human stem cells. In summary, the list of relevant organisms is large and continues to grow. While some of these organisms may have very similar requirements, special capabilities should be planned for in order to incorporate certain species.
BEYOND LEO TECHNOLOGY - needs for the next 5 years

TECHNOLOGY / OPERATIONS

1. Culturing capabilities

As discussed above, biotechnological processes already utilize diverse organisms and cell types; this diversity will increase as engineering tools develop. This makes it difficult to make precise recommendations. Instead, a more general set of suggestions is outlined below. Whenever possible, hardware should support a broad range of temperatures, pressures, pH and salt ranges to support cultures and processes beyond the typical conditions used with model organisms (*E. coli*, *S. cerevisiae*). Hardware should enable use of cells that are not planktonic and instead may take on filamentous forms (fungi) or may be embedded within biofilms. Finally, some consideration should be given to the highly specialized case of bioprinting of human tissue and organs.

2. Analytical capabilities

Terrestrial bioreactors are commonly heavily instrumented and monitored in order to allow for process optimization. Regularly collected metrics include culture density, pH, salinity, dissolved oxygen content and head gas composition. Beyond culture monitoring, analysis of the quantity and quality of a target product generated is critically important to assessing the performance of a process. Depending upon the identity of the target product, this can include analysis by gas or liquid chromatography, mass spectrometry, or various forms of spectroscopy. Therefore, specialized analytical capabilities could be required to support future lunar studies focused on biotechnological processes above and beyond what would be expected for some general biological experiments.

While traditional instrumentation technologies may not be easily repackaged into a deployable payload, alternate methodologies continue to emerge including electrochemical [254] and fluorescent (https://www.presens.de/shop/o2/sensors/oxygen-sensor-spots/?p=1) approaches for reporting on culture conditions, and bio-informational frameworks for reporting on cellular states [255]. Development and integration of these approaches could substantially increase the breadth and depth of science that could be deployed in future LEIA missions.

3. ISRU capabilities

Certain in-space biomanufacturing processes will only make sense if they can harness resources available on-site. Therefore, initial feasibility demonstration of gathering the necessary elements and compounds from mission-generated wastes and lunar regolith within reasonable energy and time constraints will be important.
### Table 1. A high-level overview of the sample types, motivation and organisms for biological research in beyond LEO environments in the next 5 years that are discussed in this report is provided. This table is informative, not definitive. Specifically named organisms, genera or sample types are listed as examples from the main text and do not indicate any priority for research or exclude other possibilities. Cells labeled ‘open’ indicate areas of potential research that were not explicitly addressed in this report. The table provides a categorical list of sample types, with motivation for each type, and application to the research questions outlined in each of the major biological research sections of this report.
Figure 3. Summary of Questions of Importance. Evolution and cellular functions are foundational as they impact everything above. Biotechnology is applied and relies on everything.
SUMMARY OF TECHNOLOGY REQUIREMENTS

TECHNICAL REQUIREMENTS ARE FORMULATED TO ENABLE ANSWERING THE SEVEN DESIGNATED TOP-LEVEL SCIENCE QUESTIONS:

- Measure cellular functions, mutation rates, gene expression
- Characterize ecologies, phenotypes, and dynamics of microorganisms, microbial communities, microbial ecosystems
- Track physiological status and changes in multi-cellular systems
- Follow plant development, monitor plant physiology/function
- Assess host - microbe interactions
- Track the evolutionary process
- Monitor biotechnological processes

REQUIREMENTS COMMON TO MANY/ALL CLASSES OF SCIENCE EXPERIMENTS:

1. SUPPORT STASIS OF BIOLOGICAL SAMPLES DURING PRE-LAUNCH STORAGE
   - provide methods and supportive environments for biological specimen stasis
   - durations of days to months (delivery/integration, pre-launch, transit, deployment)

2. SUPPORT GROWTH/METABOLIC ACTIVITY THROUGHOUT SCIENCE EXPERIMENTS IN A BIOCOMPATIBLE ENVIRONMENT THAT PROVIDES:
   - media/nutrients, (dissolved) gases; waste management
   - physical containment
   - illumination for plants, (micro)algae
   - pH, ionic conductivity, temperature, pressure, humidity control
   - through stages of growth, division, reproduction; multi-generational if required

3. PROVIDE CRITICAL REAGENTS
   - drugs / agonists, stains/dyes
   - reagents for analytical processes
   - standards, controls, reagents to support analytical measurements
• at specified concentrations/doses

4. PROVIDE PROCESSING CAPABILITIES
• sample prep for analytical processes
• homogenization, lysis, capture, clean-up, concentration, desalting, filtration, etc.

5. MONITOR THE AMBIENT
• radiation (dose/spectrum/flux; ionizing and UV/visible)
• temperature, pressure, humidity, gases (esp. O2, CO2)

6. MEASURE THE BIOLOGICAL PARAMETERS / PROCESSES OF INTEREST
• from single reporters to multiplexed measurements to -omic analyses
• for monocultures or some/all members of communities/ecologies
• measurement frequency according to anticipated rates of change (and method)
• General and physical measurement parameters
  o Morphology, morphometry
  o Overall cellular metabolic activity, metabolic products
  o Photosynthetic activity/efficiency
  o Cell/organism population, replication
  o Reproductive processes
  o Cell membrane physical integrity/morphology
  o Cell/organism/community size/morphology
  o Pairwise and multi-partner interactions between cells, organisms, proteins
• Molecular parameters
  o DNA sequence (incl mutations), RNA expression
  o Epigenetic modifications e.g. DNA methylation
  o protein expression & state (incl post-translation mods)
  o metabolites / physiological indicators
  o receptor / ion channel status/expression

BEYOND LEO FEASIBILITY AND TECHNOLOGY AVAILABILITY
• A significant portion of the technology needs identified above, along with many of their existing and potential solutions, have been described elsewhere [256,257,258].
• Most of the above are feasible in the next 5 years, with constraints on organism types, measurement duration, extent of analysis (e.g., not all full -omics analyses will be possible)
• Existing technologies already practiced in space flight form a basis for meeting many of the above requirements (microscopes, reactors, cameras, analyzers, meters, fluorescence measurement systems, spectrophotometers, etc.)
• If the capabilities are presented in tabular format, minor, moderate, or extensive development needs may be identified for each
• As experiments are specified within each experiment class, specific hardware items can be mapped onto the experiment matrix
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