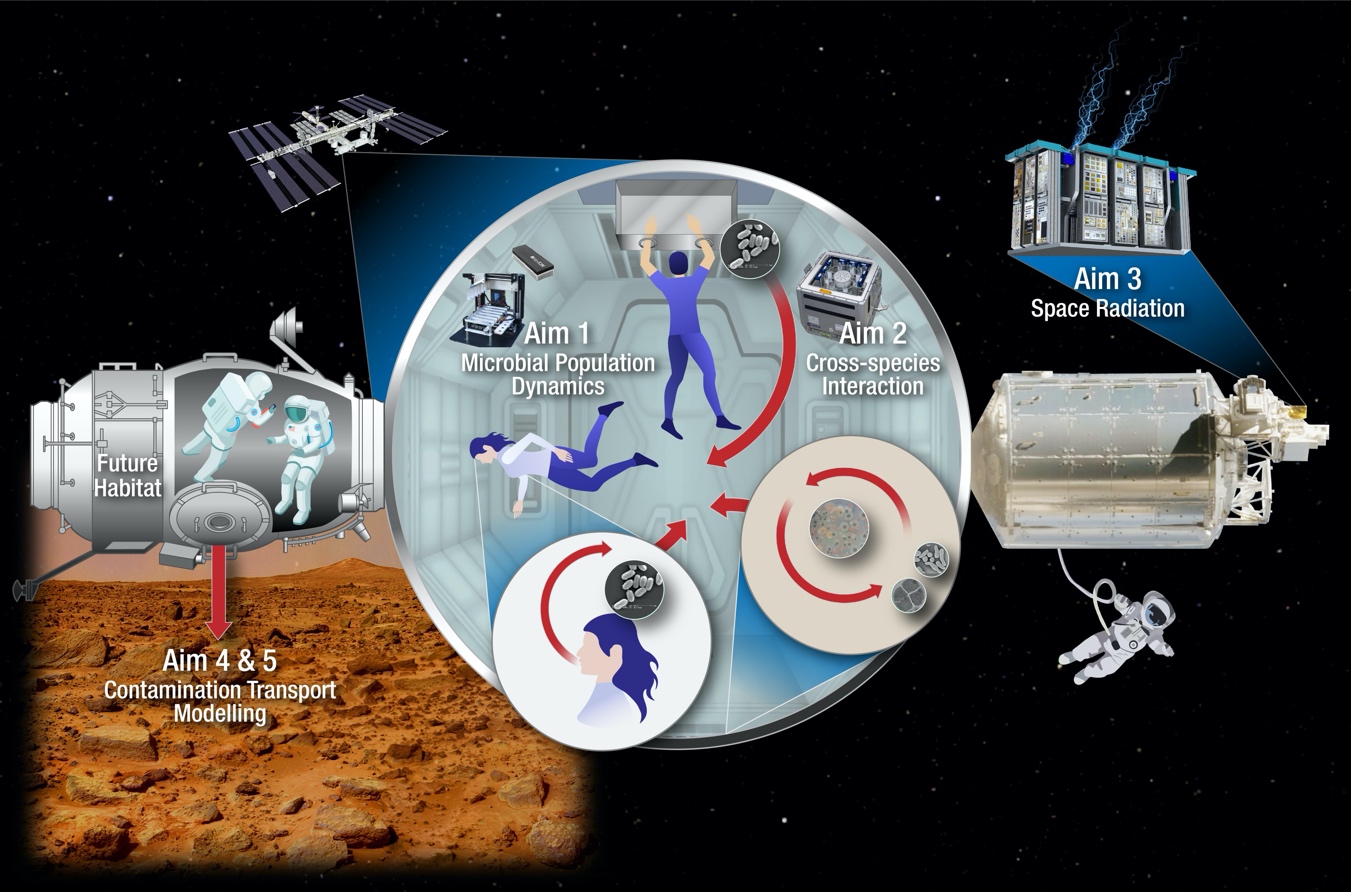
# Cover Page of the Research Campaign White Paper

***Title*: Integrative Investigation on Microbial Systems of the International Space Station**

* Primary author: Nitin Singh (8183545799); Jet Propulsion Laboratory, California Institute of Technology; nitin.k.singh@jpl.nasa.gov
* Co-authors:
  + A. Scott Howe, William Hoey; Jet Propulsion Lab, Pasadena, CA
  + Stefan Green; Rush University, Chicago
  + Scott Tighe; Univ of Vermont Cancer Center, Burlington, VT
  + Season Wong; AI BioSciences, College Station, TX
  + Fathi Karouia; Blue Marble; ARC, Mountain View, CA
  + Ashish Mahabal; California Institute of Technology, Pasadena, CA



**Potential Impact:** The Integrative Investigation on Microbial System (IIMS) project will address NASA Decadal Survey committee #P2 and #P3 recommendations by determining biological adaptations to spaceflight and understanding how biological life support systems may function in support of exploration. Multigenerational studies of microbial populations (#P1) of the IIMS project are for continued monitoring (Aim 1) to support the Space Biology and Planetary Protection Roadmap. The study of the cross-species interactions within the community (bacteria and fungi) (Aim 2) is to discover principles that apply to exploration life support (food, bioactive compounds production, etc., #P2 and P3). Empirical data about space radiation survival (Aim 3) generated in the IIMS project will aid in the development of a contamination transport control model (Aim 4). Multi-omics data generated during this study should be placed in the Space Biology administered GeneLab database (Aim 5), which will help future space researchers predict commonality and differences across microbial species for specific responses to the spaceflight environment.

# Project Description of the Research Campaign

The proposed research campaign, *Integrated Investigation on Microbial Systems (IIMS)*, will require both ground simulation and flight investigations. The previous NASA projects have illuminated many space biology science effects, strongly suggesting that spaceflight has a significant influence on biological systems and that biology in space is not the same as biology on the Earth (1). To understand the dynamics of microbial populations in space, "omics" characterization will be performed on board the International Space Station (ISS) instead of bringing the samples back to Earth for analysis.

## Hypothesis and Specific Aims

The overarching hypothesis of the IIMS research campaign is whether principles of built-environment microbiome formation in microgravity/outer space, including human-microbe, microbe-microbe, and microbe-environment interactions, are fundamentally different from Earth. The ISS is dominated by microbes that respond to changes in microgravity and radiation, enabling them to shape the environment at local and habitat levels. Recent advances in experimental and computational biology have provided tools to generate large biological datasets and formulate hypotheses-driven research that must be tested, eventually leading to improved algorithms and predictive models. Integrating large multi-omics data sets (including the incorporation of historical and new data) to provide a systems-level view will reveal previously unattainable information on the microbial dynamics of the ISS environment in ecological and evolutionary contexts. The specific aims of the IIMS project are to link data generated using metagenomics, transcriptomics, functional genomics, bioinformatics, & computational modeling to address Space Biology and Planetary Protection (PP) programs' guiding questions.

## Proposed Research Plan and Science Objectives

The hypothesis-driven research plan of the IIMS project consists of five aims and corresponding tasks (Fig. 3-1) that would fulfill the gaps of both the Space Biology and PP roadmaps.

## Objectives and Expected Significance

### Objectives/Aims

**Aim 1:** *Microbial Population Dynamics*: Detect microbial diversity onboard the ISS related to crew health, and functional characteristics. **Aim 2:** *Cross-species Interaction:* Discover individual microbial adaptations and their impact on cross-species interactions within the community. **Aim 3:** *Space Radiation:* Expose extremophiles to the outside of the ISS to address the combined effects of radiation and altered gravity on microorganisms. **Aim 4:** *Microbial Transport Modeling:* Develop a model to predict microbial contamination sourcing, transport, survival, and burden level of a closed habitat. **Aim 5:** *Microbial Response to Spaceflight:* Predict commonality and differences among microbial species for specific responses in the spaceflight environment.

### Expected Significance

As stated in the Space Biology Science Element Objectives and the National Research Council's 2011 Decadal Survey Report (2), the effects of spaceflight environment on microbial populations dynamics are largely unknown. Analysis of microbial growth in space and their physiological responses to the spaceflight environment (#P2) and demonstrating the roles of microbial systems in long-term life support (#P3) are the highest priorities relevant to the exploration of deep space (2). More specifically, the IIMS project will address if exposure to space environments affect genetic and metabolic regulation (Aim 3), and/or altered potential for disease (Aim 1), enhanced virulence, and genetic changes underlying physiological responses to spaceflight by performing multi-omics and physiological studies of model organisms (Aim 2). Empirical data generated on space radiation survival (Aim 3) will aid in the development of contamination transport control models (Aim 4)

outlined as a gap in the PP Roadmap for future human missions. Multi-omics data generated during this study and placed in the NASA GeneLab database (Aim 5) will help future research to predict commonality and differences across microbial species for specific responses to the spaceflight environment. The project will address the Human Research Program (HRP) risk: *adverse health effects due to alterations in microorganism interaction under microgravity*. By measuring the microbial pathogens onboard, the HRP community will be able to characterize virulence and pathogenicity, thereby revealing the alterations due to microgravity and subsequent effects on crew health. Instrumentation validation for in situ environmental and health monitoring will enable Lunar and Mars exploration missions. In addition, the formation of biofilms on surfaces, with consequent biofouling/ biocorrosion of space hardware and life-support systems, is of significant concern to NASA.

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| **Figure 3-1.** Hypotheses, task descriptions, performing organizations, and end deliverables of the IIMS project. |

## Description of Experimental Methods and Procedures

### Aim 1: Microbial Population Dynamics

To ensure reproducible results and higher throughput, an automated nucleic acid system, like the µTitan, is necessary. The system should run multiple extractions at the same time using the self-contained cartridges, enabling biosafety and significantly reducing the crew time needed to perform the work. The µTitan is a compact system that extracts nucleic acids (DNA/RNA) from most human, air, surface, and water samples (3-5). Furthermore, the µTitan system was tested using parabolic flight and in summer 2021, a technology demonstration at the ISS is planned. We will use this tested and validated µTitan system onboard the ISS to extract total DNA/RNA from a variety of sample types, followed by MinION sequencing to assess microbial diversity. Details of crew protocols were established, and field-testing procedures were published by this team (6). Our hypotheses are to *assess the benefits of low-temperature sample storage to preserve sample integrity over a long duration; measure differential microbial composition using metagenomics (habitat and crew); and curate molecular data and biospecimens.*

To generate rapid microbial diversity assessment on ISS, a microgravity compatible approach for the metagenome library preparation should be implemented that employs space-proven Nanopore sequencing. Since rapid analysis is essential, and PCR should be avoided on ISS because of the potential to contaminate the entire station, non-PCR amplification is essential. A randomly fragmented genomics approach that provides microbial identification and burden estimates will be useful. Assessing microbial bioburden using RAD004 rapid DNASeq reagents and LRK001 field kit by Oxford Nanopore to detect small amounts of DNA might be a good choice (7-9). Using a multiple titration approach including sample DNA mixed with either 50 ng, 5 ng, 1 ng, 500 pg, and 0 ng of Lambda DNA, a DNASeq library can be generated using the RAD004 / LRK001 nanopore reagents and sequenced using the MinION MK1B or MK1C to calculate microbial content and taxonomy. Calculation of microbial populations shall be carried out bioinformatically to remove background DNA and determine microbial diversity and content. The requirement to increase sequencer detection and performance is because most samples collected on the ISS by swabs will be well below the minimum RAD/LRK library input. Low input nanopore sequencing causes auto termination of sequencing pores due to excessive ion flux when no DNA is in the pore, so Lamda DNA can be added to keep the nanopores actively sequencing.

### Aim 2: Cross-Species Interaction

Microgravity fundamentally changes the way microbes encounter metabolites from other species and from themselves (10, 11). Microbes experience low shear stress and encounter signaling molecules by mass diffusion and hence cellular wastes are unable to settle (12). This can profoundly influence many factors, including the development of antimicrobial resistance (AMR), virulence, and cross-species interactions. The hypotheses of Aim 2 are to understand *whether microgravity enhances AMR and virulence in microbes; and whether cross-species interactions within the community (bacteria and fungi) have antagonistic behavior by altering physical structures or suppressing biofilm formation*. In Aim 2, we will grow microbes on the ISS, fix cells, and characterize cross-species interaction using molecular assays.

Microbes exhibit enhanced AMR under microgravity, potentially posing problems to crew health (13-15). Multiple instances of AMR have been identified in bacterial species isolated from ISS surfaces (16, 17). When tested in space conditions, we hypothesize that AMR will be increased in microbial strains exposed to antibiotics under microgravity to a greater degree than would occur at 1G. Examining transcriptomics and gene methylation changes will allow for a systemic understanding of enhanced AMR in microgravity.

Likewise, exposure to both simulated and true microgravity has induced changes in virulence in numerous microbial species (10, 18-21), including environmental fungi (22, 23). *Aspergillus* species are ubiquitous environmental fungi that can cause diseases in immunocompromised humans, many of which are caused by *A. fumigatus* (24). Multiple *A. fumigatus* strains have been isolated from the ISS which are closely related to clinical isolates (22). When compared with Earth clinical strains, *A. fumigatus* ISS strains showed no difference in the accumulation of mutations or stress tolerance but were significantly more virulent (22). We hypothesize that Earth strain *A. fumigatus* Af293 will acquire virulence similar to that of ISS strain *A. fumigatus* ISSFT-021 when grown under microgravity, while this will not occur in 1G controls.

To study cross-species interactions, an *in silico* metabolic network model of space microbiome data (25) predicted that ISS isolated *Klebsiella pneumoniae* suppresses growth (amensalism) of *Aspergillus* and *Pencillium* species. Such antagonistic interaction has been confirmed on Earth (26). *K. pneumoniae,* an opportunistic pathogen that most frequently causes nosocomial infections (27), has been isolated from the ISS (28), and physical alterations of *A. fumigatus* (mycelial development and conidial germination) by *K. pneumoniae* were shown when grown under simulated microgravity. When fungal species are grown in the presence of the microbial strain to evaluate whether antagonistic interactions occur in microgravity (26), transcriptomic analyses are needed to identify genes coding for possible bioactive compounds, which would enable the development of countermeasures for eradicating fungal burden on ISS (29).

### Aim 3: Space Radiation

Extremophilic microorganisms will be exposed to outer space radiation (OSR) conditions of the ISS. The IIMS team will use the JPL-developed novel metalmembrane (MM) system to expose multiple microbial entities to the outside ISS environment. After specified exposure time (e.g. ~6 months) of low Earth orbit (LEO) and return to Earth, extremophiles' survival, adaptation, biological functions, and omics shall be characterized using traditional microbiology and molecular biology techniques. Extremophiles have been shown to withstand radiation recorded at high altitudes (30) and outside the ISS (31). Our hypotheses are about *what could be learned by exposing extremophiles to OSR conditions since it is important to understand their biological functions and characterize their survival mechanisms on other planets for the Space Biology and PP programs* (32-34).

Utilization of infrastructure capable of exposing microorganisms outside ISS would help for exposure to OSR conditions. These EXPOSE trays (32) consist of several compartments, each accommodating 16 sample stacks beneath an optical filter window. The tray can be exposed to either solar electromagnetic radiation or kept in darkness. Afterwards, appropriate sample stacks shall be assigned to different microorganisms. During the mission period, several samples (n=225) can be exposed to different OSR conditions. Example: UV-Space, UV-Mars, Dark-Space, and Dark-Mars, including space vacuum (10−7 to 10−4 Pa); filled with simulated Mars atmosphere at Martian pressure (600–1,000 Pa); galactic cosmic radiation (130–190 mGy); and either full-spectrum solar extraterrestrial electromagnetic radiation (λ>110 nm) with fluences of (5.5–6.1) ×108 Jm−² (100% transmission insolated samples), or a simulated Martian UV radiation climate (λ>200 nm) with fluences of (4.0–4.3) ×108 Jm−²; 100% transmission insolated samples. All fluences can be calculated for the biologically active ultraviolet wavelength region 200–400 nm. The temperature should be maintained between −20°C and +59.6°C depending on the orientation of the ISS to the Sun (35).

### Aim 4: Microbial Transport Modeling

Prevention of forward contamination from Earth organisms to celestial bodies is the prime focus of the NASA PP program. However, only a fraction of microbes that could withstand interplanetary transfer and extreme conditions of exploring planets will survive. The hypothesis of Aim 4 is *to predict these PP-relevant microbes using a datamining approach and also to know whether a contamination transport model could aid in predicting the transport of the microorganisms from a human habitat?*

Modern molecular biology techniques help identify viable space hitchhikers and have deepened our understanding of microbial populations and their interactions. The same is true for detection and enumeration/quantification of microorganisms surviving spacecraft sterilization and ultra-low nutrient clean room environments. A computational strategy for rapidly detecting microbes that are relevant to PP, including anaerobes (36), psychrophiles (37), and radiation-resistant organisms (32) capable of surviving spaceflight is needed. Artificial Intelligence (AI) models such as Convolutional Neural Networks (CNN) and Deep Belief Networks (DBN) can be used on data extracted from curated and published sequences associated with PP-relevant (anaerobes, psychrophiles, and radiation-resistant) organisms. Since each organism has a specific genetic feature to withstand PP-relevant conditions, such traits shall be targeted. Furthermore, each organism can have multiple variants and detecting them using traditional methods is time-consuming but AI techniques will be rapid to detect PP-relevant microbes. The CNNs search for trait patterns, whereas DBNs explore the structure of the data hierarchically. The AI application can be trained using known PP-relevant microbes. It is applicable to any genetic study and reduces manual curation time, saving flight preparation time, and associated cost. The application will be able to process hundreds of samples simultaneously in a single run. Currently, available metagenomics data from NASA spacecraft assembly cleanrooms and ISS shall be used to test the effectiveness of the analysis. Furthermore, in order to develop a model to predict microbial contamination transport of the ISS environment several empirical data is needed. Some of them are: microbial burden of the surfaces and air of the habitat, its size, leak rates to the external environment, crew (spacesuits) and instrument (e.g., rover) movements, etc.

### Aim 5: Microbial Response to Spaceflight

Whole-genome sequences (WGS) of >3,000 microorganisms were generated (17, 28, 38-43). The Aim 5 hypothesis is whether *the commonality and differences can be predicted among microbial species by datamining existing omics data in the GeneLab and NCBI databases?* In addition to microbial speciation, a visualization program can be invaluable (44) in determining the genotypic differences between closely related microbial species. Therefore, multi-omics data of extremophiles that survive space conditions will directly address the guiding questions of the Space Biology: *How do alterations in gravity affect microbial cells? What are the genetic changes underlying physiological responses to space flight demonstrated in biological systems? Do the genetic changes observed with microbial cultures flown in space occur in other species?*

## Relevance to the Proposed Research Campaign and Related Work

NASA performs routine microbial monitoring on the ISS, but culture-based methods currently in use do not provide a comprehensive assessment of microbial diversity. NASA is actively working to increase monitoring capabilities by testing different *in situ* protocols for nucleic acid extraction for downstream molecular analyses on the ISS (45, 46). High-throughput sample processing onboard the ISS followed by NGS would enable astronauts to know what microbes and their properties are in the environment in real-time. This is monumental considering that omics analyses of ISS samples (performed on Earth) have detected both novel organisms and known organisms with unique properties (23, 47-51). Many terrestrial species sent from Earth to grow on the ISS have become more virulent (22), more antibiotic-resistant (13), and have formed more biofilms (52, 53), properties that can affect astronauts' health and the stability of the spacecraft. A matured micro(µ)gravity tested instrument for automated nucleic acid extraction (µTitan) system will be instrumental for monitoring crew and environmental microbiome. Having the µTitan system on the ISS to process samples and generate data immediately will enable inflight detection and measurement of several biomolecules related to physiological and immunological effects induced by spaceflight. The ability to quantify microbial load associated with the crew in real-time, will provide an efficient means to monitor immune function and to allow for proper countermeasures to be implemented, such as medication strategies, prophylactic antibiotics, or stress-reduction therapies (54, 55).

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**Acknowledgement:**

The work was carried out at the Jet Propulsion Laboratory, California Institute of Technology, under a contract with the National Aeronautics and Space Administration (80NM0018D0004).