A Ratio of Spore to Viable Organisms: A case study of the JPL – SAF cleanroom

Final Report

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ABSTRACT

Spacecraft surfaces that are destined to land on potential life-harboring celestial bodies are required to be rigorously cleaned and continuously monitored for spore bioburden as a proxy for spacecraft cleanliness. The NASA standard assay (NSA), used for spacecraft bioburden estimates, specifically measures spores that are cultivable, aerobic, resistant to heat shock, and grow at 30˚C in a nutrient-rich medium. Since the vast majority of microorganisms cannot be cultivated using the NSA, it is necessary to utilize state-of-the art molecular techniques to better understand the presence of all viable microorganisms, not just those measured with the NSA. In this study, the nutrient-deprived low biomass cleanrooms, where spacecraft are assembled, were used as a surrogate for spacecraft surfaces to measure the ratio of NSA spores in relation to the total viable microorganism population in order to make comparisons with the 2006 Space Studies Board (SSB) estimate of 1 spore per approximately 50,000 viable organisms. Ninety-eight surface wipe samples were collected from the Spacecraft Assembly Facility (SAF) cleanroom at the Jet Propulsion Laboratory (JPL) over a 6-month period. The samples were processed and analyzed using classical microbiology along with molecular methodology. Traditional microbiology plating methods were used to determine the cultivable bacterial, fungal, and spore populations. Molecular assays were used to determine the total organisms (TO, dead and live) and the viable organisms (VO, live). The TO was measured using adenosine triphosphate (ATP) and quantitative polymerase chain reaction (qPCR) assays. The VO was measured using internal ATP, propidium monoazide (PMA)-qPCR, and flow cytometry (after staining for viable microorganisms) assays. Based on the results, it was possible to establish a ratio between spore counts and VO for each viability assay. The ATP-based spore to VO ratio ranged from 149–746, and the bacterial PMA-qPCR assay–based ratio ranged from 314–1,491 VO, per spore. The most conservative estimate came from fluorescent-assisted cell sorting (FACS), which estimated the ratio to be 12,091 VO per 1 NSA spore. Since archaeal (<1%) and fungal (~2%) populations were negligible, the spore to VO ratios were based on bacterial population estimates. The most conservative ratio from this study can be used as a replacement for the SSB estimate on nutrient-deprived (oligotrophic) desiccated spacecraft surfaces, to estimate the VO from NSA measurements without utilizing state-of-the art molecular methods that are costly and require more biomass than is typically found on spacecraft surfaces.

Executive Summary

Since the beginning of planetary protection efforts in the 1960s, the aerobic, cultivable, heat shock resistant spore has been used as a marker of spacecraft cleanliness. This spore population was viewed as the most hardy and resistant microbial population, so if a sampled spacecraft had few, or even zero spores, it was assumed that the spacecraft was "clean." However, we now understand that the majority of microorganisms (99%) cannot be cultivated with standard cultivation methods or the NASA standard assay (NSA) (1). Recent planetary protection efforts have begun to explore and utilize more comprehensive molecular methods, to estimate and measure a more representative overall population. While some of these methods have been approved by NASA as a means to estimate, but not officially verify, spacecraft surface bioburden, the primary standard to measure spacecraft cleanliness over the life of a mission is still the NSA.

While still utilizing the NSA, we wanted to establish a way to estimate the viable microbial population using spore measurements. In this report, we describe how we established a ratio between NSA spore measurements and viable organisms. Since the majority of the Mars 2020 spacecraft is still in development and unavailable for sampling, we had to find a proxy surface for this study. It was decided that the Spacecraft Assembly Facility (SAF) cleanroom was the most representative environment to make these measurements because it has low biomass and the construction of the Mars 2020 mission will primarily take place in this cleanroom. Additionally, it has been shown that spacecraft surfaces have about ~2 orders of magnitude less microorganisms than the SAF floors, which allow us to provide a conservative estimate of the spore to VO ratio (2). Aside from the NSA, we utilized other traditional microbial methods on tryptic soy agar (TSA) and potato dextrose agar (PDA) plates to measure the cultivatable bacterial and fungal populations, respectively. Additionally, several molecular techniques, including adenosine triphosphate (ATP; total and viable) assay, quantitative polymerase chain reaction (qPCR; total and viable), and fluorescent-assisted cell sorting (FACS; viable), were used to assess the temporal and spatial microbial abundance in the SAF cleanroom environment, to establish a conservative spore to VO estimate. Furthermore, volume two of this report will discuss three additional components of this study: the characterization of spore-forming bacterial diversity, microbiome (16S rRNA gene-targeted iTag sequencing) analysis, and metagenome analysis (shotgun sequencing). These methodologies were used to gain a better insight into the taxonomic identity of the organisms present in the SAF, which enabled us to understand the types of spore-forming microorganisms that could not be cultured using the NSA method.

Conclusions

Three widely used viability assays were employed in this study to establish the most conservative and comprehensive measurement of spore and VO ratio counts and to compare with the previous Space Studies Board (SSB) estimation (1 spore to 50,000 VO)(3). As demonstrated in Section 6.8, internal ATP and propidium monoazide (PMA) qPCR provided the lowest spore to VO estimates, with an ATP-based estimate of 1 spore to 149–746 VO and a qPCR-based estimation of 1 spore to 314–1,491 VO. The third viability assay used in this study, FACS, provided the most conservative spore to VO estimate of 1 spore to 12,091 VO. Based on the empirical data generated during this study and the desire to utilize the most conservative method, it is recommended that the FACS-based ratio be used as a replacement for the SSB estimate for the Mars 2020 planetary protection samples.

1 Introduction

Since the 1960s, the planetary protection group has been utilizing the NASA standard assay (NSA) as a gold standard for assessing spacecraft biological cleanliness (1, 4-14). The NSA is a heat shock–based colony count method that involves collecting spacecraft samples, heat shocking at 80˚C for 15 minutes, plating on nutrient rich agar, and growing for 72 hours at 32˚C (15). However, standard plate culture methods have been shown to only detect ~1% of the total microbial population of various indoor and outdoor environmental samples and are unable to detect fastidious microorganisms that may require varying cultivation conditions, such as temperature, pH, and salt concentration (1). The NSA detects an even smaller percentage of what standard plate culture does as it selects for only those microorganisms that are heat resistant, aerobic, viable, and spore-forming (3, 14, 16). On the other hand, culture-independent molecular methods are better at estimating microbial bioburden as it detects both cultivable and non-cultivable organisms.

The Mars 2020 mission has science requirements that limit the probability that a single viable organism is present in a sample tube, to prevent false positive life detection if samples were ever to be returned to Earth. The most recent rover mission, Mars Science Laboratory (MSL), utilized molecular methods along with the NSA to measure microbial burden from 5,000 wipes collected from spacecraft surfaces (17). While rapid molecular methods would be preferable for assessing biological contamination on spacecraft surfaces, numerous limitations, such as cost and total number of samples required, prevent it from being the only viable option. The NSA is currently the only NASA approved option for monitoring the overall bioburden load of spacecraft throughout the entire mission. Although it is labor intensive, it does not require advanced technologies and is a more affordable option. Since the Mars 2020 mission will continue to use the NSA as the primary method of monitoring bioburden, it is important to establish a ratio between NSA measurements and the viable microbial population that will allow for an indirect estimation of viable organisms on spacecraft surfaces. Since the majority of the components for the 2020 mission are not available for sampling, the Spacecraft Assembly Facility (SAF) was used as a proxy environment to establish a spore (i.e., detected by NSA) to viable organism (VO) ratio. The SAF was chosen as the proxy environment because the majority of the Mars 2020 mission will be constructed in this cleanroom and the sampled floors would represent a “dirtier” surface, allowing enough microbial material to construct an accurate ratio that can then be applied to spacecraft surfaces. This ratio can be used during the construction of the Mars 2020 mission to accurately estimate the number of viable microorganisms from the NSA samples that will be taken throughout the assembly, test, and launch operations (ATLO) activities.

Currently, a spore to VO ratio has been recommended based on assumptions from a 2006 Space Studies Board (SSB) report (3). In this SSB report, it was estimated that the cultivable population represents 50× the spore population determined by NSA. It was also suggested that each microbial subpopulations’ abundance is underestimated “by a factor of 1,000.” Based on SSB recommendations, when cultivable organisms are viewed as a subset of the viable population, the ratio is assumed to be 1 spore to 50,000 VO (3). This ratio was established with generalizations and approximations based on various aquatic and terrestrial environments, creating a need for an empirically backed and standardized estimate for the Mars 2020 mission, based on samples taken in a relevant environment such as the SAF. To accomplish this, three viability assays (internal adenosine triphosphate [ATP], propidium monoazide [PMA] quantitative polymerase chain reaction [qPCR], and fluorescent-assisted cell sorting [FACS]) were used, along with the NSA,

to comprehensively estimate viable organisms and conservatively establish an empirically backed spore to VO ratio. They will be described in detail in Section 4.

This final report is intended to be a comprehensive documentation of the entire spore to VO task, including the objectives, technical approach, results, recommendations, and lessons learned. A project workflow illustrating the activities throughout the spore to VO task is provided in Figure 1.

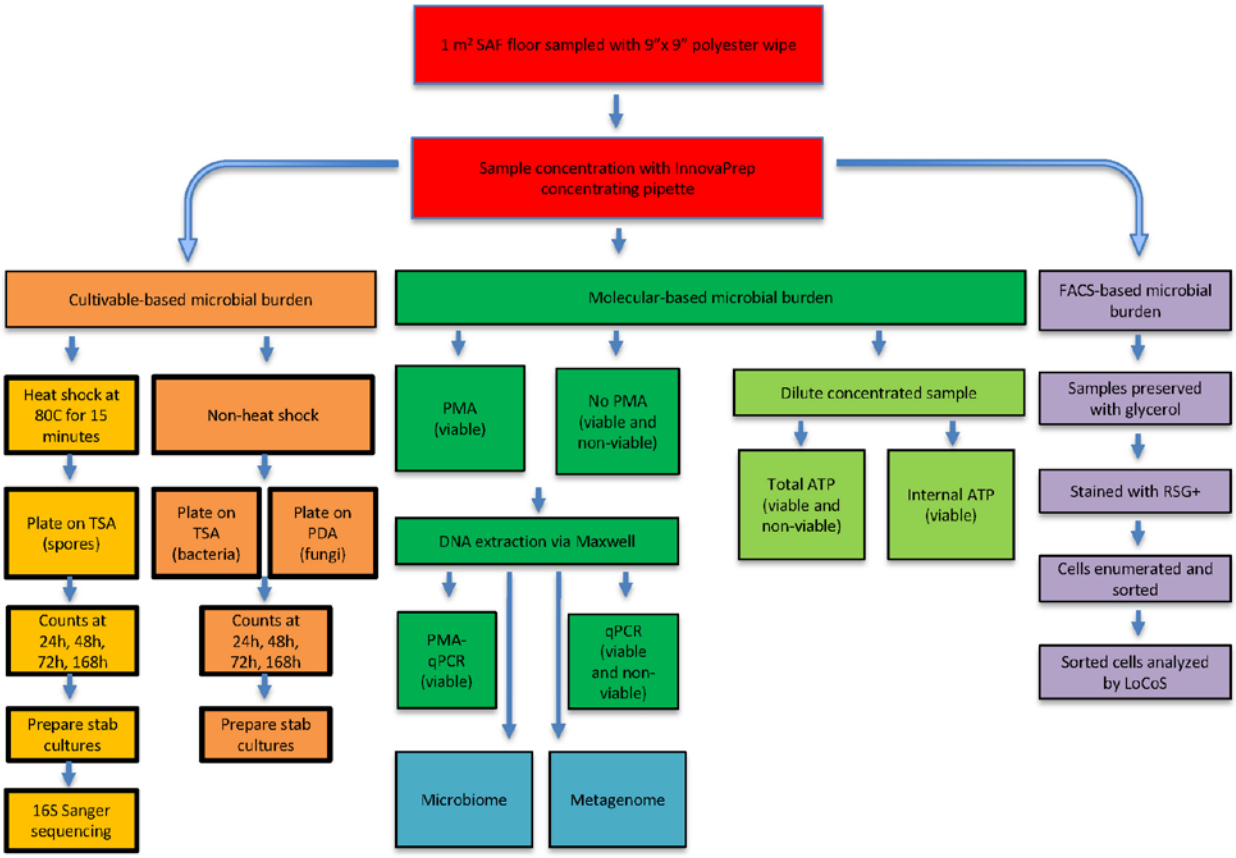

Figure 1: Workflow for collecting and processing spore to VO samples.
This schematic shows the workflow of the spore to VO from sample collection to analysis. A 1 m^2 sample was collected from a 9" × 9" polyester wipe of the SAF (Red, Section 4.2.1). Phosphate buffer saline (200 mL) was added to each sample and concentrated to 5 mL using a 0.45 µm hollow fiber polysulfone (Red, Section 4.2.2). An aliquot of the concentrated sample (1.2 mL) was used for cultivation assays (Orange). That aliquot was split for the NAS (Light Orange, Section 4.3.1.3) and non-heat shock assays (Orange, Section 4.3.1.1 and Section 4.3.1.2). Another 3 mL of the concentrated sample was taken and split to undergo PMA and non-PMA treatment (Green, Section 4.2.3), followed by DNA extraction (Section 4.3.3.1) and qPCR (Section 4.3.3.3). Additionally, extracted DNA was sent for microbiome and metagenome (Blue, Volume 2) analysis. A 200 µL aliquot of the concentrated sample was used for ATP analysis (Light Green, Section 4.3.2). Of the 98 samples analyzed, 25 were analyzed by FACS (Purple, Section 4.3.4).

All relevant conference posters, workshop reports, peer-reviewed publications (abstracts and links to full publications online), and miscellaneous supporting materials are included as appendices of Volume 2. To provide more information on the microbial population, the microbiome and metagenome were assessed to estimate the relative abundance of spore-forming and non-spore-forming populations and how they varied throughout the cleanroom and over the

period of the study, all of which are detailed in Volume 2 of this report. In addition, single-cell genomics was carried out for a subset of population to generate whole genome sequences of predominant microorganisms.

2 Strategic Perspective

The next NASA rover mission, Mars 2020, will be the first Mars mission capable of collecting regolith samples that will be stored on the surface of Mars. These samples could potentially be brought back to Earth by a future mission. As such, to protect the return sample science integrity, NASA requires the sample caching system to be cleaned to the level of less than 1 viable Earth organism for each sample tube. This requirement necessitates the ability to understand and estimate the number of viable organisms present on various areas of the spacecraft prior to launch to help understand the probability that they could end up in the sample tube during the process of constructing the rover, or during entry, descent, and landing (EDL) or surface operations (surface ops).

The current method used to measure microbial contamination of spacecraft surfaces is the traditional NASA microbiology technique, the NSA, which has been used since planetary protection efforts began in the late 1960s (1, 4-14). The NSA leaves the vast majority of microorganisms undetected and is unable to measure anaerobic spores and viable, but yet-to-be cultivated, microbial populations. Because NASA needs to understand the viable contamination of the spacecraft, modern, culture-independent methods are needed to measure the microorganisms that are not detected with the NSA.

This report documents a variety of microbial assays used to measure the various microbial populations in the SAF, where the majority of the Mars 2020 mission will be assembled. The primary goal of the spore to VO task was to utilize the various methodologies together to empirically estimate the ratio of NSA spore to viable organisms to use as a replacement of the SSB estimate of 1 NSA spore being equal to 50,000 viable organisms (3). This estimate will provide the Mars Exploration Program and other NASA stakeholders with an estimate of the viable organism burden of the spacecraft from collected NSA samples.

3 Objectives and Expected Impact

3.1 Definition of Spore to VO

In the context of this report, spore to VO refers to the ratio of spore-forming organisms, as measured by the NSA, to the viable organisms (VO), as estimated by three different molecular assays. The VO was estimated by targeting deoxyribonucleic acid (DNA), ATP, and single cell after staining with a viability dye.

3.2 Objectives

The main objective of this study was to determine ratio of a spore to VO by utilizing the traditional, culture-based NSA techniques concurrently with modern, molecular analyses. This study will allow us to conservatively estimate the VO present on the Mars 2020 spacecraft surfaces by utilizing the results from NSA spore method results on spacecraft-associated assembly surfaces. Subsequent objectives are to provide a detailed understanding of the spatial and temporal distribution of the various populations in the Mars 2020 SAF cleanroom (total, viable, cultivable, and spore) surface samples. Furthermore, state-of-the-molecular techniques

such as next generation sequencing methods were also employed to study the microbiome and metagenome to allow for the identification of spore-forming and viable genera. The results of the microbiome and metagenome are presented in Volume 2 of this report and will provide insight into the unique microbial community of the SAF.

3.3 Assumptions

The assumptions made for the respective assays used in this study are depicted in Table 1.

Table 1: Methods used to determine microbial burden from SAF, and the corresponding assumptions with each method.

Assay	Assumptions
Total ATP	• Detects ATP from both living and dead cells: fungi, bacteria, archaea, but not spores (minimal ATP in spores) • 1 RLU of ATP is equivalent to 1 CFU [1] • Fungi = ~100 RLU/cell, Gram-positive = ~5 RLU/cell, Gram-negative = 1 RLU/cell, spores no ATP [1, 12]* • Gram-positive and Gram-negative bacteria occur in equal proportion in the SAF [22]
Internal ATP	• Detects only metabolically active, viable cells (VO): fungi, bacteria, archaea • ATP eliminating reagent enzymatically degrades free ATP • 1 RLU of ATP is equivalent to 1 viable CFU [1] • Fungi = ~100 RLU/cell, Gram-positive = ~5 RLU/cell, Gram-negative = 1 RLU/cell, spores no ATP [1, 12] *
16S rRNA qPCR	• Detects both living and dead cells (TO): only bacteria • 16S rRNA gene copy numbers per cell (1–15, average: 4.2, SD: 2.7) [19]
16S rRNA PMA-qPCR	• Detects living cells (VO): only bacteria • 16S rRNA gene copy numbers per cell (1–15, average: 4.2, SD: 2.7) [19] • Propidium monoazide (PMA) intercalates with free DNA and DNA from compromised cells, preventing downstream amplification and detection
Non-Heat Shock	• Detects aerobic, cultivable bacteria that grow at 32°C on TSA • 1 CFU = 1 Cell
NSA Heat Shock	• Detects aerobic, cultivable spores that can survive 80°C for 15 minutes and grow at 32°C on TSA (NSA) • 1 CFU = 1 Cell
FACS	• Detects viable cells capable of being stained with RSG+ dye and showing reductase activity (VO) • ~20% of counts are positive for low coverage sequencing (LoCoS)** • RSG+ dye is a conservative indicator of viability, has no known taxonomic bias, is compatible with cryopreserved sample analysis, has low background fluorescence, and is compatible with downstream genomics analysis.

***Actual estimates of RLU/CFU: Fungi/Yeasts (263.5 RLU/CFU), Gram-positive (4.49 RLU/CFU), Gram-negative (1.34 RLU/CFU), Spore (0.0016 RLU/CFU).**

**** According to Table 14, 14.3% of counts were positive for LoCoS.**

3.4 Expected Impact on Present Knowledge

Currently, the spore to VO ratio recommendation is based on assumptions in a 2006 SSB report (3). In this SSB report, it was estimated that the cultivable population represents 50× the spore

population determined by using the NSA. It was also suggested that each microbial subpopulations' abundance is underestimated "by a factor of 1,000." Based on SSB recommendations, when cultivable organisms are viewed as a subset of the viable population, the ratio is assumed to be 1 spore to 50,000 VO (3). This conservative ratio was established with generalizations and approximations based on aquatic and other terrestrial environments. Because the NSA only detects heat shock resistant, viable, aerobic organisms, a variety of molecular approaches were used and compared to calculate a spore to VO ratio. This empirically backed ratio could be used in place of the current SSB estimate to more accurately estimate the VO populations for the Mars 2020 mission in a relevant environment.

Due to the scale of this study, it was possible to gain a better understanding of the microbial composition of the SAF environment and how it changes over the 6-month period of the study, as well as how different locations of the cleanroom may vary in microbial population abundance. This knowledge can help engineers to better understand the changes in the microbial populations that may occur over the lifespan of a mission, providing empirical data to make risked based biological health assessments to implement engineering protocols and procedures (e.g. identify areas that need additional cleaning, implementation of other facility controls, etc.) to accommodate hardware critical activities.

Additionally, VO estimates were generated from several assays, including FACS. This is the first time that FACS along with single-cell whole genome amplification (WGA) has been used to measure VO in a low-biomass cleanroom environment. Additionally, the microbiome and metagenome of the SAF was measured for the first time using standard 1 m^2 polyester wipe samples with the help of the recently developed protocol by the University of California, San Diego (UCSD) sample analysis pipeline (20). The development of these procedures used for this study could lead to potential future studies of the SAF, spacecraft surfaces, or similar low-biomass environmental samples.

4 Spore to VO Methodology

This section summarizes the materials and methods associated with the numerous traditional and molecular analysis techniques that enabled the spore to VO task. Additional details and scientific results are reported in Sections 5 and 6.

4.1 General Approach

The overall objectives of the spore to VO task were to (1) estimate a spore to VO ratio and effectively assess the (2) temporal and (3) spatial effects on microbial diversity and abundance in the SAF using molecular techniques.

Three assays capable of measuring viable microorganism (ATP, qPCR, FACS) were used with NSA measurements to establish spore to VO ratios and systematically compared to determine which assay method provided the most conservative ratio in the low-biomass samples. Several samples (98 times) were collected over a period of 6 months from 13 locations in the SAF in order to determine whether fluctuations occur in viable and spore populations over time and whether different locations in the SAF have different abundances of viable and spore populations. The methodological flow of the spore to VO task is displayed in Figure 1.

Traditional microbiology procedures were utilized to estimate the cultivable bacterial and fungal populations. In addition, spore population was measured using the NSA. Subsequently, a variety

of molecular methods were used that target universally common molecules (ATP and DNA) to measure the total and viable microbial population.

Molecular methods utilize universally common cellular compounds such as DNA and ATP for microbial detection and can be modified to distinguish the total and viable populations (12, 21-23). Utilization of both molecular assays and traditional microbiology methods for future missions is ideal, but not practical, due to the unavailability of large areas on spacecraft surfaces that are required to collect enough biomass, and various project constraints such as mission schedule and budget. However, the SAF cleanroom environmental surfaces, where spacecraft are built, are available for analysis and can be used as a surrogate to conservatively estimate VO that may be present on spacecraft.

4.2 Sample Collection and Processing

Over a period of six months, between March 2016 and August 2016, 98 floor samples were collected during 11 sampling time periods in the JPL-SAF. The specific location for each sampling event and collection date are given in Figure 2. The total surface area of the SAF cleanroom is 921.1 m^2 with controlled conditions such as: temperature (20 ± 4°C), humidity (30 ± 5%), stringent gowning requirements, and weekly cleaning (24, 25). Although the SAF is capable of becoming an ISO-7 (10k) cleanroom, at the time of sampling, the SAF was certified as an ISO-8 (100k) cleanroom. A maximum measurement of 8,287 0.5 µm particles/ft^3 and 159 5.0 µm particles/ft^3 were recorded during the 6 months of the study.

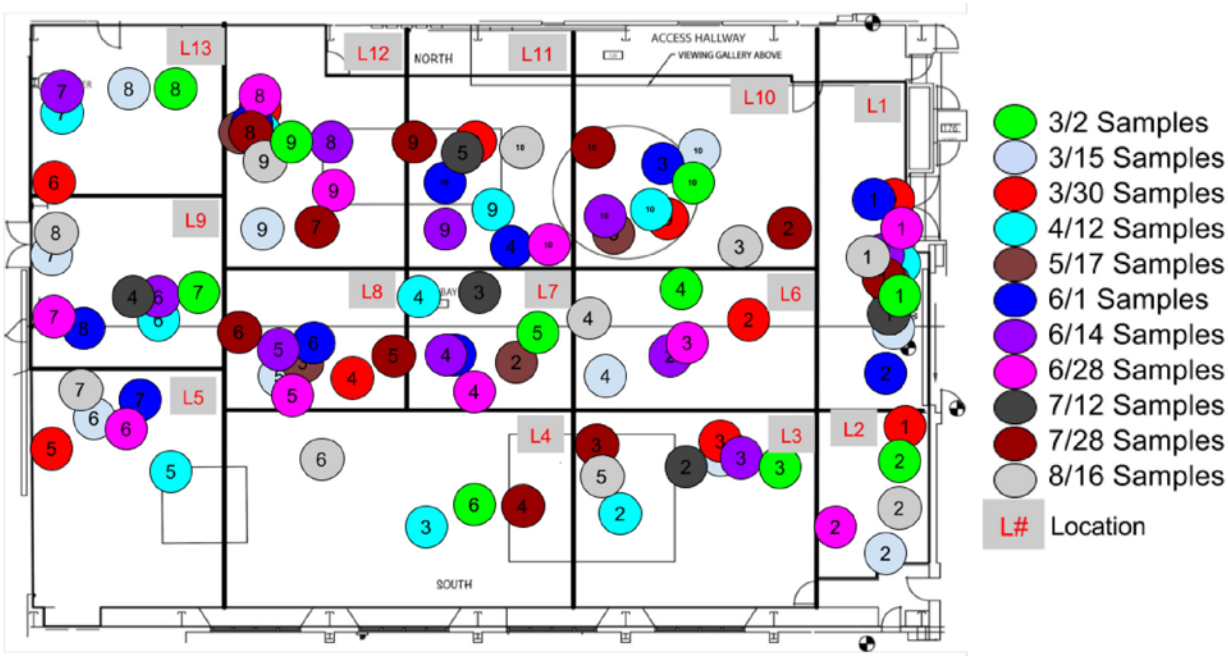

Figure 2: Schematic of the dates and locations sampled in the Spacecraft Assembly Facility.
A total of 98 samples were collected over a 6-month period from the SAF. The schematic above shows the date and the location of each sample that was collected. The circles represent the sample location, and the number inside the circles represent the numerical order that the samples were taken. The color of the circle represents the corresponding day that the sample was collected. The graph is sectioned into artificial quadrants based on sample grouping and foot traffic, depicted by a gray box, to look for location-specific differences in results. In total, there are 11 sampling dates and 13 sampling locations.

4.2.1 Collection of Sample

Sterile 9" × 9" polyester wipes (Texwipe; TX1009, NC, USA) were prepared and used to collect 1 m^2 floor surface area particulates as previously described (3). After sample collection, wipe samples were deposited into sterile 500 mL glass bottles and transferred to a lab for further processing (26, 27).

4.2.2 Concentration of Sample

Immediately after samples arrived in the lab for processing, 200 mL of sterile phosphate buffer saline (PBS; pH 7.4; Sigma Aldrich, MO, USA) solution was added and shaken for 30 seconds to thoroughly mix the solution and release any collected particulates and associated microorganisms. In parallel, an InnovaPrep concentrating pipette device was primed for operation by decontaminating the instrument as per the manufacturer's standard maintenance kit (InnovaPrep Drexel, MO, USA). This kit includes a sodium hydroxide decontamination fluid and a rinse fluid (containing 25 mM of Tris and 0.075% Tween 20). Following the decontamination and rinse steps, 200 mL of sample was concentrated to approximately 5 mL using an InnovaPrep concentrating pipette with 0.45 µm hollow fiber polysulfone (HFPS) concentrating pipette tips (InnovaPrep Drexel, MO, USA). The exact amount of concentrated sample was weighed on a tared scale and appropriately recorded. Samples were then used for culture-dependent and culture-independent analyses as outlined below.

4.3 Sample Analysis

Each individual wipe sample was analyzed using the methods listed below. Of the 98 samples, only 25 were analyzed by FACS. Of those 25 FACS samples, only 1 sample was analyzed by single-cell whole genome analysis (SCWGA). All unused FACS samples are preserved appropriately. Once suitable resources become available, SCWGA can be performed on the preserved samples if needed.

4.3.1 Cultivation Assays

Several cultivation assays were employed to determine various microbial populations, as described below. The NASA standard assays for standard cultivation on TSA (Section 4.3.1.1) and heat shock spore assays (Section 4.3.1.3) were slightly modified to accommodate small volumes for this study and to observe CFUs after 7 days.

4.3.1.1 Modified Standard Cultivation on TSA

To measure the cultivable bacterial population, suitable aliquots of samples prepared above were plated on tryptic soy agar (TSA) medium as described below. Procedures modified from the standard procedure described in HNB 6022 are *italicized.*

- *100 µl aliquots* of concentrated sample, as prepared in Section 4.2.2, were deposited into four replicate, sterile petri dishes for quadruplicate measurements.
- Molten, sterile TSA was then added using a standard plate pouring technique.
- Once solidified, plates were incubated at 32°C, and CFUs were counted after 24h, 48h, 72h, and *7 days* of incubation time.

Results from this assay are described in Section 6.3.1. The procedure used for choosing colony isolates for storage and further analysis is described in Section 4.3.1.4.

4.3.1.2 Cultivation on PDA

To measure the cultivable fungal population, suitable aliquots of samples prepared above were plated on potato dextrose agar (PDA) medium as detailed below.

- Aliquots of concentrated sample (100 µl), as prepared in Section 4.2.2, were deposited into four replicate, sterile petri dishes for quadruplicate measurements.
- Molten, sterile PDA was then added using a standard plate pouring technique.
- Once solidified, plates were incubated at 32°C, and CFUs were counted after 24h, 48h, 72h, and 7 days of incubation time.

Results from this assay are described in Section 6.3.2. The procedure used for choosing colony isolates for storage and further analysis is described in Section 4.3.1.4.

4.3.1.3 Modified NASA Standard Spore Assay)

The NSA was performed to measure the cultivable, heat shock resistant spore population. Procedures modified from the standard procedure described in HNB 6022 are *italicized.*

- An aliquot of *425 µl* from concentrated sample, as prepared in Section 4.2.2, underwent the NSA treatment.
- This consisted of a heat shock treatment (80˚C +/- 2 ˚C; 15 min), followed by pour plating (27).
- Aliquots of the heat-shocked concentrated sample (100 µl) were then deposited into four replicate, sterile petri dishes for quadruplicate measurements.
- Molten, sterile TSA was then added using a standard plate pouring technique.
- Once solidified, plates were incubated at 32°C, and CFUs were counted after 24h, 48h, 72h, and *7 days* of incubation time.

Results from this assay are described in Section 6.3.3. The procedure used for choosing colony isolates for storage and further analysis is described in Section 4.3.1.4.

4.3.1.4 Identification of Isolates

Single colonies from various TSA (heat shock and non-heat shock) and PDA plates were picked and stored in stab cultures (1:10 dilution of given media) at room temperature for future analysis, including molecular identification of 140 isolates targeting ribosomal RNA (rRNA) gene sequencing via Sanger method as described in Section 4.3.5. All of the NSA isolates were picked and stored for future identification. However, isolates from TSA and PDA were chosen more stringently. If isolates were found from a PDA or TSA, only 1 representative plate was chosen out of the four replicate plates to pick strains for further molecular identification. Additionally, up to 5 colonies were chosen from that specific plate. If more than 5 colonies were present, the 5 most morphologically unique colonies were chosen.

4.3.2 ATP Assays

ATP assays were performed to estimate total microorganisms (TO) and VO. An aliquot of concentrated sample (200 µl), as prepared in Section 4.2.2, was added to 1.8 mL of sterile phosphate buffered saline (PBS) to create a 1:10 dilution. Samples were then processed with the following protocols to measure internal and total ATP using the CheckLite HS ATP kit (Kikkoman, Japan) (26).

4.3.2.1 Total ATP

TO were estimated using the total ATP procedure. This consisted of 4 replicates of 100 µl of the diluted sample (as described in Section 4.3.2) added to individual lumi tubes provided by the manufacturer. The following steps were followed:

- Prior to each measurement, 100 µl of cell lysing detergent (benzalkonium chloride) was added to the lumi tubes.
- The tubes were incubated at room temperature for 30 minutes.
- 100 µl aliquot was added to the lumi tube.
- 100 µl of cell lysing detergent (benzalkonium chloride)
- The lumi tube was incubated for 1 minute at room temperature.
- The lumi tube was briefly vortexed (~5 seconds).
- 100 µl of luciferin-luciferase reagent was added.
- The lumi tube was immediately measured for luminescence using a luminometer.

The photon count, which is proportional to ATP concentration, was measured with a luminometer (Lumitester K-200, Kikkoman, Japan) as relative luminescence units (RLU). Results were recorded and can be seen in Section 6.4.1.

4.3.2.2 Internal ATP

VO were estimated using the internal ATP procedure. This was accomplished by eliminating free ATPs and ATPs associated with dead cells before measuring the amount of intracellular ATP, which has been shown to be a biomarker of VO (12, 28). This consisted of 4 replicates of 100 µl of the diluted sample (as described in Section 4.3.2). Prior to each measurement, the following steps were followed:

- 50 µl of apyrase/adenosine deaminase (ATP-eliminating) reagent step was added to 500 µl of the diluted samples.
- The lumi tubes were then incubated at room temperature for 30 minutes.
- 100 µl alliquot was added to the lumi tube.
- 100 µl of cell lysing detergent (benzalkonium chloride) was added to each lumi tube.
- The lumi tubes were incubated for 1 minute at room temperature.
- Lumi tubes were briefly vortexed (~5 seconds).
- 100 µl of luciferin-luciferase reagent was added
- The lumi tube was immediately measured for luminescence using a luminometer.

The photon count, which is proportional to ATP concentration, was measured with a luminometer (Lumitester K-200, Kikkoman, Japan) as RLU. Results were recorded (Section 6.4.2) and later converted to CFU/m^2 and spore to VO ratio estimates (Section 6.4.3).

4.3.3 qPCR Assays

The qPCR assay was performed to estimate TO and VO by using the non-PMA and PMA treated samples, respectively, as prepared in Section 4.2.2. A 3 mL aliquot was taken from the 5 mL concentrate of each sample and split into two, 1.5 mL portions for PMA and non-PMA treatment.

4.3.3.1 Non-PMA and PMA Treatment

For PMA samples, the following procedure was followed:

- One 1.5 ml aliquot of the concentrated surface sample was treated with 18.75 µl of 2 mM PMA (25 µM - 2 mM; Biotium, Inc., Hayward, CA, USA) (21, 25, 29, 30).
- Each sample was vortexed and incubated in the dark for 5 minutes at room temperature.
- Samples were then exposed to PhAST Blue-Photo activation system for 15 minutes (GenIUL, S.L., Terrassa, Spain) (21, 25, 29, 30).

For non-PMA, 1.5 ml aliquot of concentrated samples were directly used for DNA extraction. DNA suspensions for both conditions were stored in molecular grade water (50 µl each) at –20°C for further analysis (qPCR, microbiome, metagenome characterization) (31).

4.3.3.2 Quantitative PCR

Quantitative PCR were run in a CFX-96 thermal-cycling qPCR instrument (Bio-Rad, California, USA) as described below:

- 1 µl of template DNA from each sample was tested in triplicate.
- The 1369F and 1492 R universal bacterial primers targeting the 16S rRNA gene were used.
  - The 1369 F sequence used is as follows: 5′-CGG TGA ATACGT TCY CGG-3′
  - The 1492 R sequence used is as follows: 5′-GGW TAC CTTGTT ACG ACT T-3′ (27, 32).

Each 25 µl reaction in the 96-well plate consisted of:

- 12.5 µl of 2 × iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA)
- 1 µl each of forward and reverse oligonucleotide primers (10 µM each)
- 9.5 µl DNase/RNase free water (Ultrapure, Gibco)
- 1 µl of template DNA to be quantified

Reaction conditions were set to the following (27):

- 3 minutes 95°C denaturation
- Followed by 39 cycles of denaturation at 95°C for 15 seconds
- Annealing at 55°C for 30 seconds
- Extension at 72°C for 30 seconds

Ribosomal ribonucleic acid (RNA) gene standards, spanning 10^2–10^8 gene copies/µl, were generated by serially diluting 4 NMOLE ULTRAMER® DNA OLIGO (Integrated DNA Technologies, Coralville, Iowa, USA). Results are described in Section 6.5.

4.3.4 FACS Analysis and Single-Cell Whole Genome Amplification

Among 98 samples collected, only 25 were analyzed for FACS analysis. Only one sample was analyzed for WGA. Viable cell counting, bacterial single amplified genome (SAG) generation, and SAG low coverage sequencing (LoCoS) were performed at the Bigelow Laboratory Single Cell Genomics Center (Maine, USA).

4.3.4.1 Fluorescence-Assisted Cell Sorting (FACS)

After the initial InnovaPrep concentration of the sample, an aliquot was preserved at –80°C in glyTE to preserve reductase activity until FACS analysis. Once processing began, samples were diluted threefold with filtered (0.2 µm pore size) 1× PBS and stained with RedoxSensor Green (RSG; Thermo Fisher Scientific) to identify viable cells. Individual particles that showed

reductase activity were sorted using an inFlux sorter, with index sort capabilities, into three, 384-well plates, containing 0.6 µl of Tris EDTA (TE) buffer per well. 64 negative control wells and 3 positive control wells with 10 cells each were included in the plate. The DNA for each cell was amplified using WGA-X (33). Cell diameters were determined using the FACS light forward scatter signal, which was calibrated against cells of microscopy-characterized laboratory cultures (33). Cell sorting and robotic liquid handling were performed in a cleanroom environment. The FACS-sorted viable cells were randomly sorted, and 384 individual droplets were collected from each sample tested and sequencing was performed to authenticate the presence of biological particles. Out of the total number of viable cells that were sorted, only 20% were biological particles and were able to be taxonomically assigned. A summary of the FACS-based VO population is described in Section 6.6.

4.3.4.2 Single-Cell Whole Genome Amplification

SAGs were taxonomically identified as previously described (34), and the workflow is shown in Figure 3. Small subunit (SSU) ribosomal DNA (rDNA) gene analysis was completed using GenBank BLASTN, Ribosomal Database Project (RDP) Classifier, and Seqmatch (35-37). Following automatic sequence alignment using ClustalX, phylogenetic trees were assembled with PHYLIP (38, 39). For LoCoS, uniquely barcoded libraries of ~370 base pair (bp) fragments were generated from each SAG, followed by Illumina sequencing of ~300,000 reads of 2×150 bp. These were then analysed by FastQC for read-level quality control (QC). The reads were then trimmed, filtered, and normalized, and de novo assembly was performed (SPAdes). Quality trimming and filtering of contigs were performed, followed by analysis via CheckM (for taxonomy, completeness, and contamination), 16s rRNA gene extraction and phylogeny, Tetramer principal component analysis (PCA) (for contamination, infections, horizontal gene transfer [HGT]), and BLASTn against nucleotide (nt) database (for additional QC). The entire SAG generation and analysis workflow was benchmarked using previously sequenced microbial cultures with diverse genome complexity and %GC (33). Results are shown in Section 6.6.2.

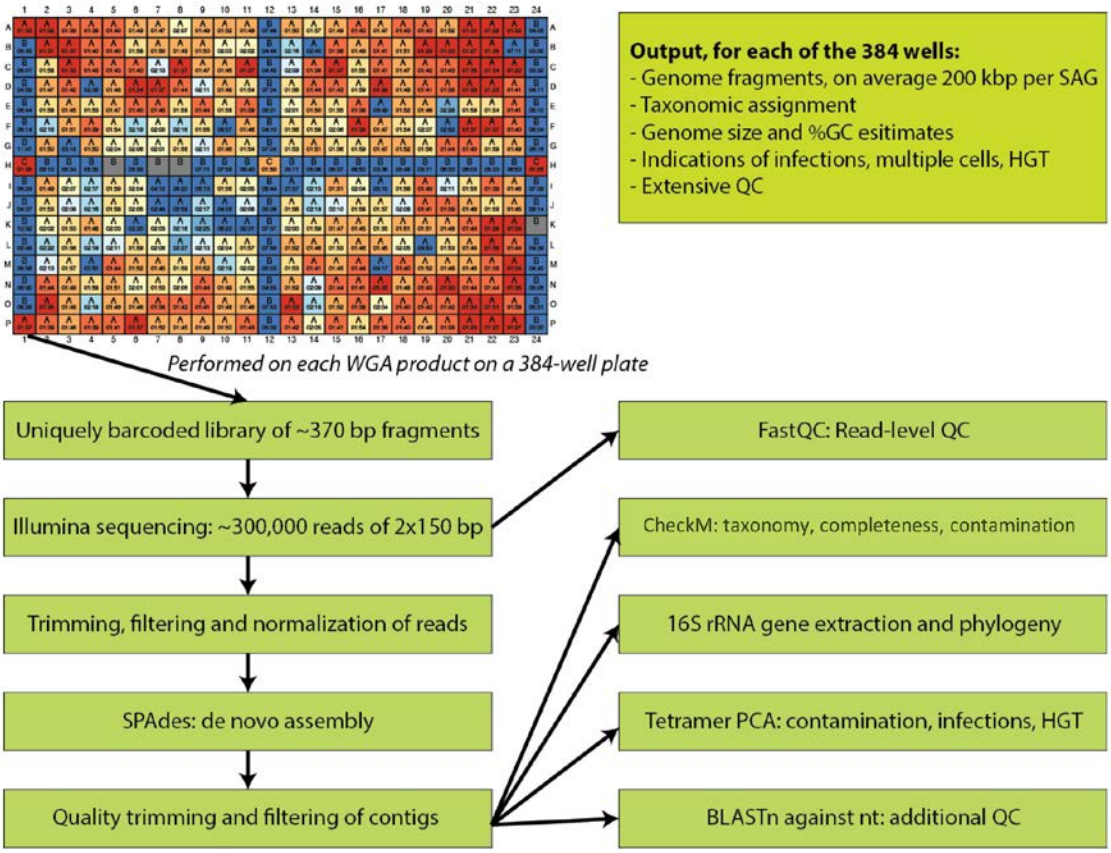

Figure 3: Whole genome amplification workflow.
This image demonstrates the workflow of processes that follow WGA. The top left shows a standard 384 reaction plate with a varying scale of colors. The scale ranges from dark blue (no reaction) to dark red (large reaction). Gray color represents a failed reaction. The right and left edges, along with the cross in the middle are negative controls with a few positive controls mixed in.

4.3.5 Identification of Bacterial Isolates

Isolates, cultured from surface wipes, were streaked from 10% stab culture on their respective plates to check the purity of the strains. Single colonies were picked from the plate and inoculated in respective broth followed by incubation at 30˚C overnight. Genomic DNA was extracted from the overnight culture using the DNeasy UltraClean Microbial Kit extraction kit (Cat No: 12224-250, Qiagen, Germany).

The extracted DNA was used for PCR to amplify the 1.5 kb 16S rRNA gene in order to identify bacterial strains. The following primers were used for the 16S rRNA amplification: the forward primer, 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and the reverse primer, 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (40, 41). The PCR was run using the following parameters:

- Denaturation at 95˚C for 5 minutes
- Followed by 35 cycles consisting of denaturation at 95˚C for 50 seconds
- Annealing at 55˚C for 50 seconds
- Extension at 72˚C for 90 seconds

- Final extension at 72˚C for 10 minutes

The amplified PCR products were sent for Sanger sequencing (ChunLab, Fort Lee, NJ) and assembled using SeqMan Pro from DNAStar Lasergene Package (DNASTAR Inc., Madison, WI). The bacterial sequences were searched against ChunLab EzTaxon type strain database. Phylogenetic trees were reconstructed using MEGA7 via a neighbor-joining algorithm. Results are discussed in Section 6.3.4.

5 Assay Selection and Various Considerations

This section summarizes testing of various assays and methods commonly used and associated with the numerous analysis techniques that enabled the spore to VO task along with other considerations for this study.

5.1 General Approach

It was important to establish the best sampling method for this study (biological sampling kit [BiSKit] or wipe), along with the verification of a sample concentrating method (InnovaPrep).

Time and resources did not permit the Spore to VO team to assess every sampling device available to microbiology researchers. However, previously, Mars Program Office (MPO) funded a Genetic Inventory (GI) project where several sampling devices were tested, and the results recommended that BiSKit and polyester wipes shall be used for collecting materials from large surfaces (42). Therefore, the team narrowed the field by selecting the sampling tools commonly used by NASA for planetary protection and by surveying relevant literature to select two additional methods to explore (FACS and microscopy).

5.2 Sampling and Analysis Selection

This section describes how various sampling, or analysis, methodologies were tested for verification of success on low-biomass samples.

5.2.1 Sample Collection Method

To establish the most effective collection method, both 9” × 9” polyester wipes (Texwipe; TX1009, NC, USA) and macrofoam BiSKits (Quicksilver Analytics Inc.; Abingdon, MD) were tested for this study. Previous research had shown that BiSKits had a higher level of recovery (~5×) than polyester wipes of mixed microbial community (MMC) rDNA on stainless steel surfaces (27). This study showed that total ATP, internal ATP, total qPCR, PMA-qPCR, cultivable bacteria, cultivable fungi, and cultivable spores values were higher in wipe samples than BiSKit samples when taken side by side in 5 locations in the SAF (Table 2).

Table 2: Comparison of wipe and BiSKit in adjacent sample locations in the SAF.

Sample Type 5/17/16	Total microbes[a] (RLU/m^2)	Viable microbes[a] (RLU/m^2)	Total microbes[a] (Copies/m^2)	Viable microbes[a] (Copies/m^2)	Cultivable[a] (CFU/m^2)	Cultivable Spores[a] (CFU/m^2)	Cultivable Fungi[a] (CFU/m^2)	Spore to VO[b,c] ATP	Spore to VO[b,d] qPCR
Wipe (n = 5)	1.2×10^5	5.7×10^3	8.4×10^6	5.1×10^4	6.9×10^2	8.3×10^1	3.3×10^1	68	618
BiSKit (n = 5)	1.1×10^5	2.2×10^3	6.6×10^6	3.9×10^4	3.8×10^2	3.7×10^1	5.1×10^0	60	1,061

[a] Values calculated by taking the average of given assay values on individual sampling locations. See Section 4 for detailed explanations of individual assays.
[b] Ratio calculated for a given date by taking the sum of a given viable assay divided by the sum of cultivable spore of all relevant samples. If an individual sample from a date had a viability assay measurement of BDL (below detection limit), it along with the corresponding cultivable spore, were not included in the calculation.
[c] Average represents a 1 RLU = 1 CFU assumption.
[d] Average represents a 1 16S rRNA copy = 1 CFU assumption.

To comprehensively compare sample collection methods, samples were taken both inside the SAF cleanroom and in the adjacent gowning room used by personnel prior to entry to the SAF. Comparison of averages from the various assays are shown in Table 3. Similar to the side-by-side comparison described above, all assays showed higher values in wipe SAF samples than in BiSKit SAF samples. Gowning results showed similar differences, except that BiSKits showed slightly higher fungal numbers.

Table 3: Comparison of wipe and BiSKit samples in SAF and gowning room.

Sample Type	Total microbes[a] (RLU/m^2)	Viable microbes[a] (RLU/m^2)	Total microbes[a] (Copies/m^2)	Viable microbes[a] (Copies/m^2)	Cultivable[a] (CFU/m^2)	Cultivable Spores[a] (CFU/m^2)	Cultivable Fungi[a] (CFU/m^2)	Spore to VO[b,c] ATP	Spore to VO[b,d] qPCR
BiSKit SAF (n = 15)	4.2×10^4	1.4×10^3	2.3×10^6	3.1×10^4	2.0×10^2	1.8×10^1	1.1×10^1	79	1,693
BiSKit Gowning (n = 15)	3.1×10^4	9.7×10^2	8.8×10^3	6.4×10^3	3.1×10^1	1.0×10^1	5.2×10^0	87	634
Wipe Gowning (n = 5)	1.0×10^6	2.1×10^4	6.9×10^6	4.7×10^5	7.9×10^3	1.6×10^2	2.8×10^0	132	2,976
Wipe SAF (n = 98)	3.3×10^5	2.8×10^4	5.3×10^6	7.9×10^4	4.4×10^2	3.6×10^1	1.7×10^1	750	2,121

[a] Values calculated by taking the average of given assay values on individual sampling locations. See Section 4 for detailed explanations of individual assays.
[b] Ratio calculated for a given date by taking the sum of a given viable assay divided by the sum of cultivable spore of all relevant samples. If an individual sample from a date had a viability assay measurement of BDL, it along with the corresponding cultivable spore, were not included in the calculation.
[c] Average represents a 1 RLU = 1 CFU assumption.
[d] Average represents a 1 16S rRNA copy = 1 CFU assumption.

Due to these empirical data, it was decided that wipes were the preferred method to collect particulates from the SAF floors. Aside from the higher recovery shown here, wipes are the preferred sampling tool and are flight certified by NASA to sample large spacecraft surfaces for

planetary protection purposes. Additionally, BiSKits are not compatible for use on spacecraft surfaces and they failed in electrostatic discharge certification; thus, they were not the choice as the sample collection method.

5.2.2 Sample Concentration

The sample processing utilized an InnovaPrep concentrating pipette with 0.45 µm HFPS concentrating pipette tips (InnovaPrep Drexel, MO, USA) to concentrate the 200 mL samples to 5 mL for further processing. Once the 200 mL of sample has been passed through the concentrating pipette, the sample needs to be eluted out to a total volume of 5 mL. Even on the large elution setting (~0.5 mL), the InnovaPrep elution volumes can vary as the elution can is being used. Also, the cost of the elution cans did not make it practical to perform 10+ elutions in order to gain the desired volume. If only a few elutions were required, sterile PBS could be added post-elution to get the sample to the desired 5 mL. We wanted to observe the recovery rate of extractions to verify that we could use this method.

The manufacturer suggests that the recovery rate of two extracts is 55.7–80.7% using a 0.1 µm polyethersulfone (PES) tip. The MPO-funded GI project documented ~60% recovery of the spiked cells and DNA when 0.45 µm HFPS tips were used (42). In addition, the recovery of known concentrations of bacterial cells were also tested using 0.45 µm HFPS tips during this study. An overnight culture of ~10^7 CFU/mL of *B. pumilus* was first measured using a total ATP kit before and after concentration. Groups of three individual elutions were pooled into each sample tested (i.e., elutions 1–3, 4–6, 7–9). The first 3 elutions yielded 60% recovery. After the initial three elutions, a 3-log reduction was seen in subsequent results (Table 4). It was recommended that the best sample concentration method to use would be three large elutions, followed by the addition of sterile PBS until the total volume reached 5 mL.

Table 4: Effectiveness of back washes in the elution of trapped microorganisms from the InnovaPrep tips.

	Before Concentration (dilute sample)	Group 1[a] (Elution 1–3)	Group 2[a] (Elution 4–6)	Group 3[a] (Elution 7–9)
Average RLU[b]	9.4×10^3	3.5×10^4	7.3×10^1	2.9×10^1

[a] Values calculated by taking the average of two replicate measures of the pooled elutions listed. See Section 5.2.2 for detailed explanations of individual assays.

[b] Values are the average of all individual samples from the given dates. Viability average was calculated by taking the sum of all samples from Internal ATP values and dividing by the sum of Total ATP values of all relevant samples. If an individual sample had a measurement of BDL, it along with the corresponding counterpart value, were not included in the calculation.

5.2.3 Microscopy

This section describes how samples were tested using various microscopy techniques and instruments by the Center for Biofilm Engineering at Montana State University (MSU), as well as JPL, to determine the ability to enumerate viable cells in the SAF wipe samples.

5.2.3.1 Montana State University Analysis

Microscopy was also investigated as a potential method to measure and enumerate viable cells using BacLight staining (Thermo Fisher Scientific, USA). Five wipe samples (including a negative control) were collected from the SAF floor on 4/12/16 and concentrated using the procedures outlined in Sections 4.2.1 and 4.2.2. Samples were stained with a Live/Dead stain (Thermo Fisher Scientific, USA). Live/Dead stain is a fluorescent molecular probe that allows for a two-color discrimination of viable (green) and dead (red) cells. Samples were viewed with epifluorescent and confocal microscopy techniques.

The negative control (Figure 4), sample 1 (Figure 5), and sample 5 (Figure 6) were tested by taking the entire 1 mL sample, staining with Live/Dead for 1 hour, filtered onto a 0.1 µm membrane, and imaged on the epifluorescent microscope at 100×. Images were taken at 63× on an inverted confocal microscope using a combination of the red and green channels, black and white of the green channel, and black and white of the red channel. Additionally, the confocal images of the samples were rendered in 3D with a 63× oil objective. Because of the large debris observed, the remaining two samples (6 and 9) were diluted prior to analysis.

Sample 6 (Figure 7) and sample 9 (Figure 8) were diluted to 1:10 prior to the previously described procedure. Images were taken of 20 fields of view to obtain total cell count, but no identifiable cells were detected (images not shown). The same slide was also imaged at 63× with an inverted confocal microscope (images C and D). In addition, 500 µL of the original sample were stained with SYBR Green for 30 minutes, filtered onto a 0.1 µm membrane, and imaged on the inverted confocal microscope. Images were taken at 63× on an inverted confocal microscope using a combination of the red and green channels, black and white of the green channel, and black and white of the red channel. Additionally, the confocal images of the samples were rendered in 3D with a 63× oil objective.

Images taken via epifluorescent microscopy proved that this method is not a viable option because of the low biomass and background fluorescence found in the samples. Confocal microscopy was able to show individual cells and cell clusters in samples 1, 5, 6, and 9, while showing no cells in the negative control. Enumeration of cell counts require a disaggregated/ homogenous sample that can be filtered on a flat membrane and observed on one focal plane; thus, the microbial contamination in these samples were not able to be quantified when the confocal microscopy method was used. This methodology could potentially be used for future enumeration of viable cells if more time and resources were used to develop a method for these low-biomass surfaces.

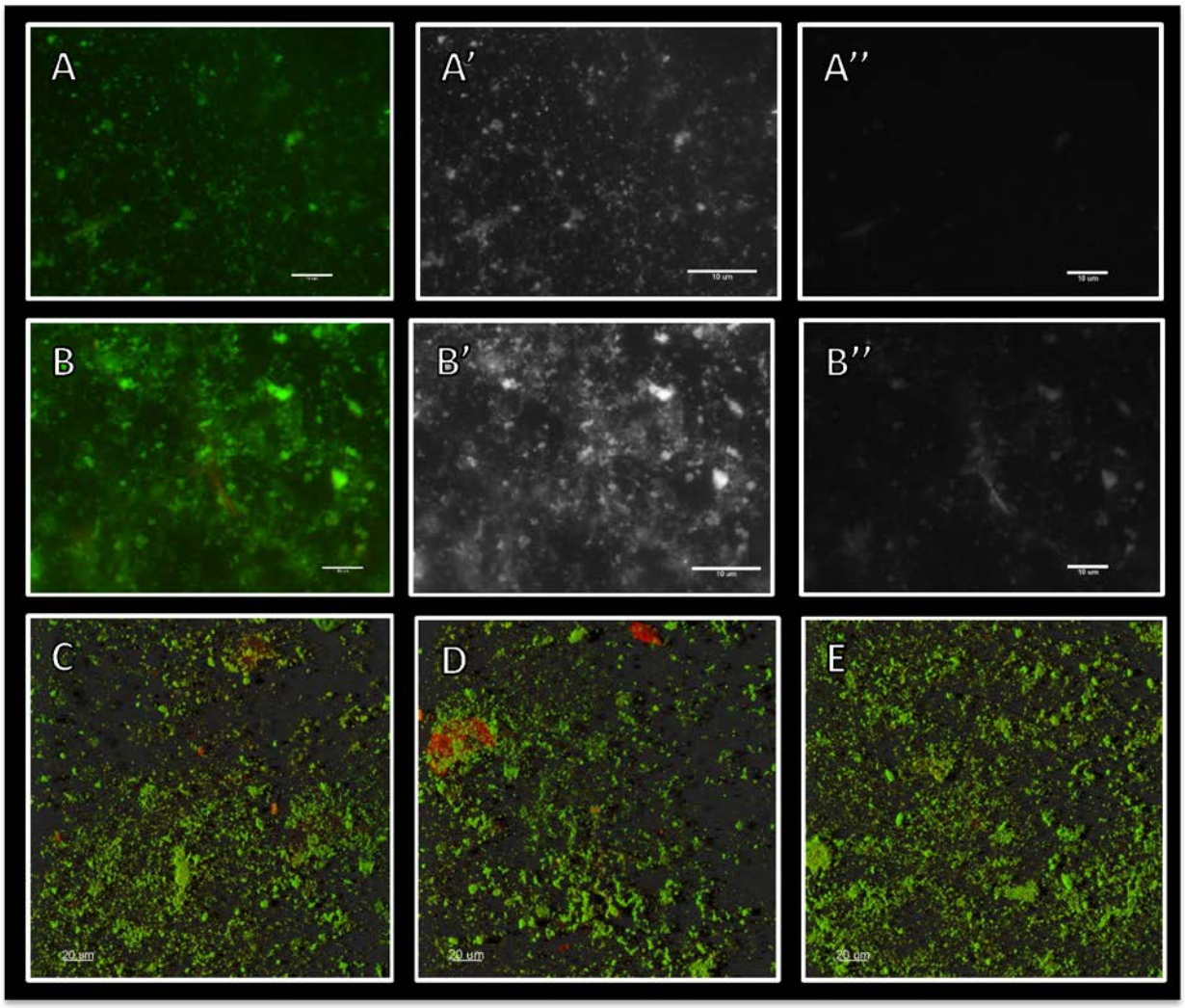

Figure 4: Epifluorescent and confocal images of the negative control sample collected on 3/15.
The sample was spotted after being stained with Live/Dead for 1 hour, and then filtered onto a 0.1 um membrane. Figure shows two 100x fields of overlay (A and B) of the selected field of epifluorescence images of the negative control sample, followed by the black and white of the green channel (A', B') and the black and white of the red channel (A'', B''). The same slide was imaged using confocal microscopy with a 63x magnification (C, D, and E).

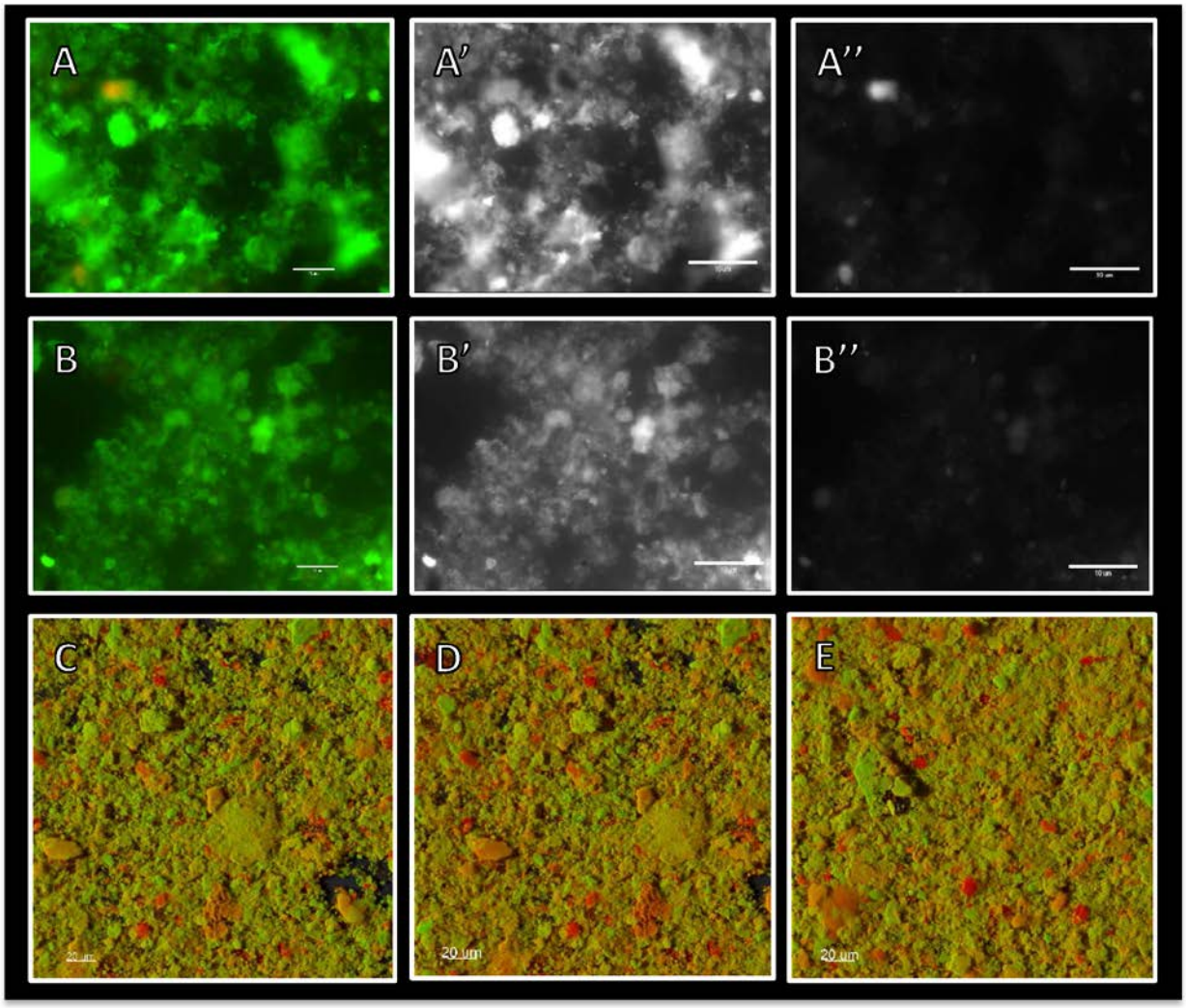

Figure 5: Epifluorescent and confocal images of sample 1 collected on 3/15.
The sample was spotted after being stained with Live/Dead for 1 hour, and then filtered onto a 0.1 um membrane. The figure shows two 100x fields of overlay (A and B) of the selected field of epifluorescence images of the surface wipe sample, followed by the black and white of the green channel (A', B') and the black and white of the red channel (A'',B''). The same slide was imaged using confocal microscopy with a 63x magnification (C, D, and E).

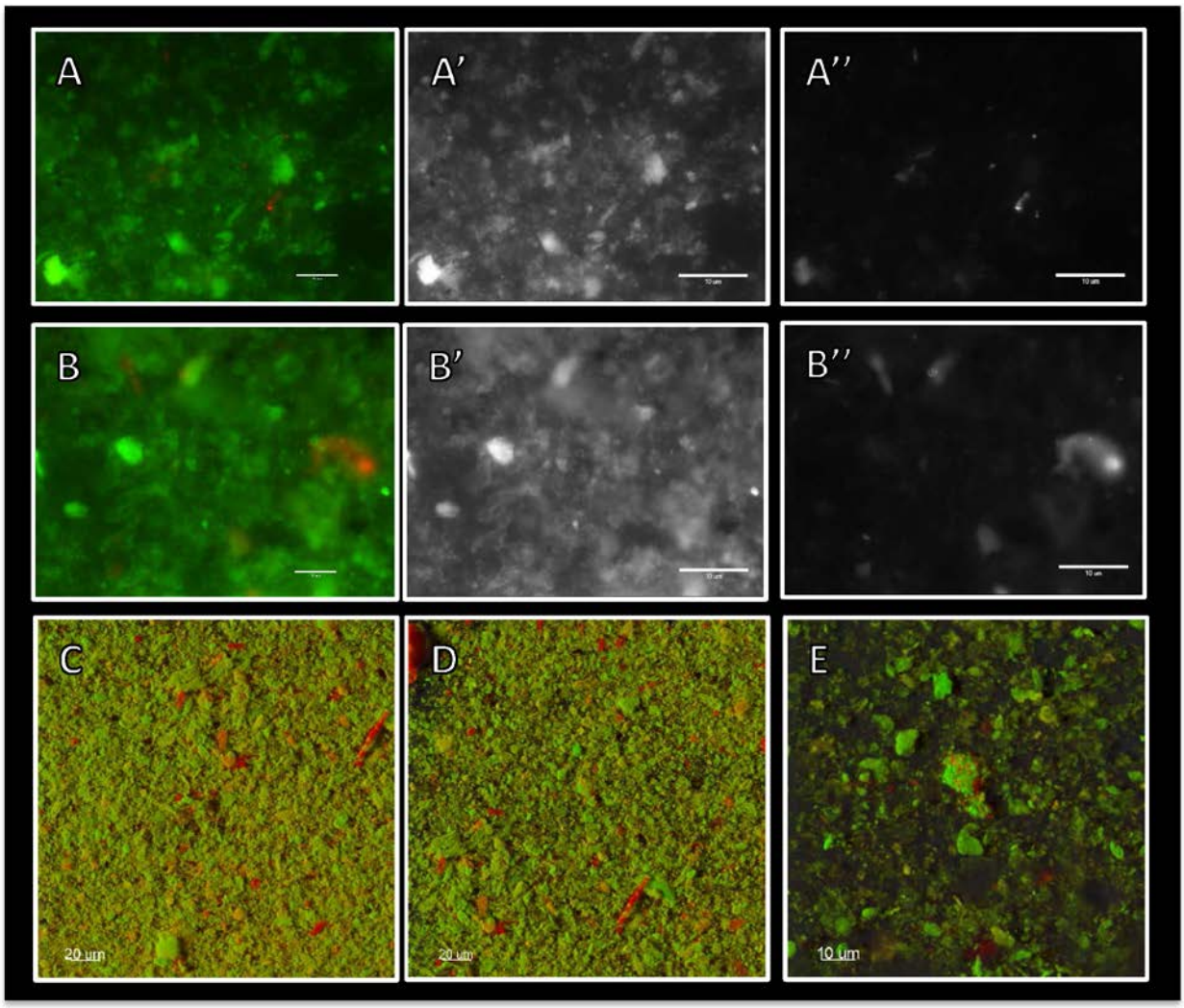

Figure 6: Epifluorescent and confocal images of sample 5 collected on 3/15.
The sample was spotted after being stained with Live/Dead for 1 hour, and then filtered onto a 0.1 um membrane. Two fields of view at 100x are shown for epifluorescent images (A and B). These images are shown with the color combine of the red and green channels (A, B), followed by the black and white of the green channel (A', B') and the black and white of the red channel (A'', B''). The same slide was imaged using confocal microscopy with a 63x magnification (C, D). Image E shows a cluster of cells, with the assistance of a 2.37x digital zoom.

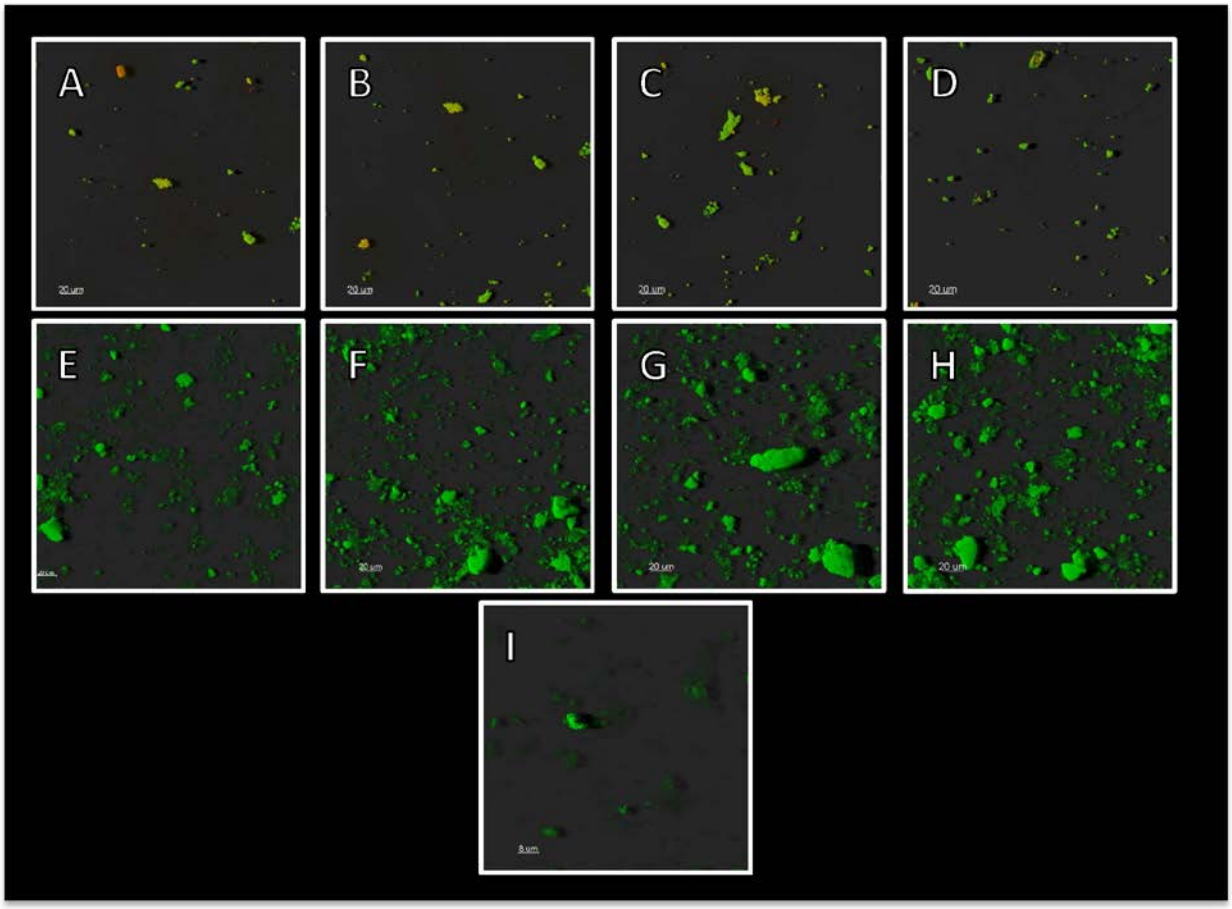

Figure 7: Epifluorescent and confocal images of sample 6 collected on 3/15.
The sample was spotted after being diluted (1:10), stained with Live/Dead for 1 hour, and filtered onto a 0.1 um membrane. The fields of view shown are at 100x using epifluorescent microscopy (A–D). These images are shown with the color combine of the red and green channels. Subsequently, 500uL of the remaining original sample was stained with SYBR Green for 30 minutes, filtered onto a 0.1 um membrane, and imaged on the inverted confocal microscope. The images below (E–H) show the 3D rendering of those confocal images obtained with a 63x oil objective. Image I shows a cluster of cells, with the assistance of a 2.5x digital zoom.

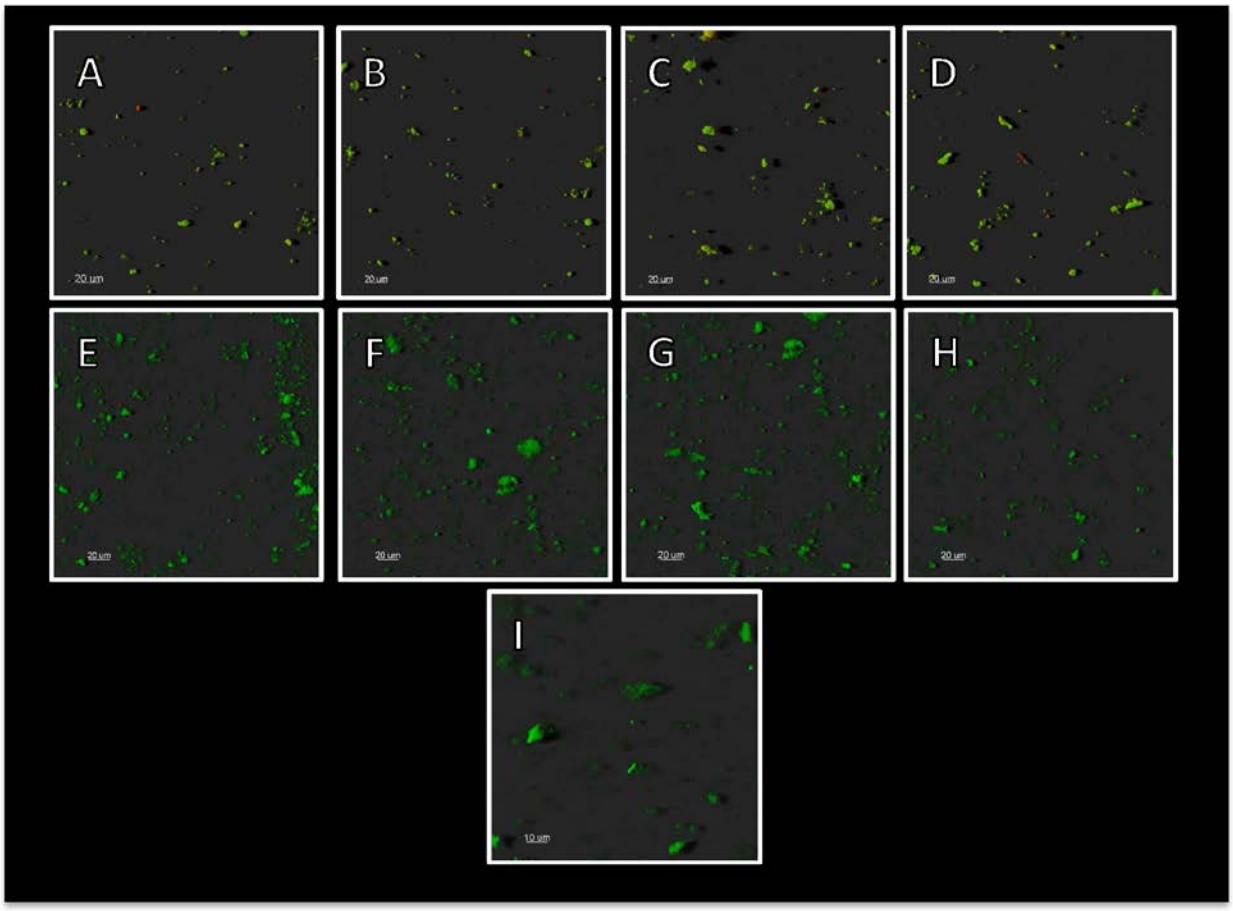

Figure 8: Epifluorescent and confocal images of sample 9 collected on 3/15.
The sample was spotted after being diluted (1:10), stained with Live/Dead for 1 hour, and filtered onto a 0.1 um membrane. The fields of view shown are at 100x using epifluorescent microscopy (A–D). These images are shown with the color combine of the red and green channels. Subsequently, 500uL of the remaining original sample was stained with SYBR Green for 30 minutes, filtered onto a 0.1 um membrane, and imaged on the inverted confocal microscope. The images below (E–H) show the 3D rendering of those confocal images obtained with a 63x oil objective. Image I shows a cluster of cells, with the assistance of a 2.00x digital zoom.

5.2.3.2 JPL Analysis

Since MSU's method were not suitable for this study, we also explored using microscopy at JPL using epifluorescence microscopy (Olympus, JPL) and field emission scanning electron microscope (FE-SEM, Sirion, KNI, Caltech). Phase contrast and epifluorescent microscopy was used to analyze two samples collected on 3/15, samples 8 and 10. Microscopy images from sample 8 can be found in Figure 9, and images from sample 10 can be found in Figure 10. It was difficult to visually detect any microbial cells in both samples. In Figure 9, we were only able to detect one rod-shaped particle. In Figure 10, we also were only able to detect one rod-shaped particle. As seen in quadrant B' of Figure 10, it appeared that the bacillus structure had been in the process of sporulation. With only two potential microbial cells detected in two SAF floor samples, it was clear that epifluorescence would not work for microbial cell enumeration of these samples.

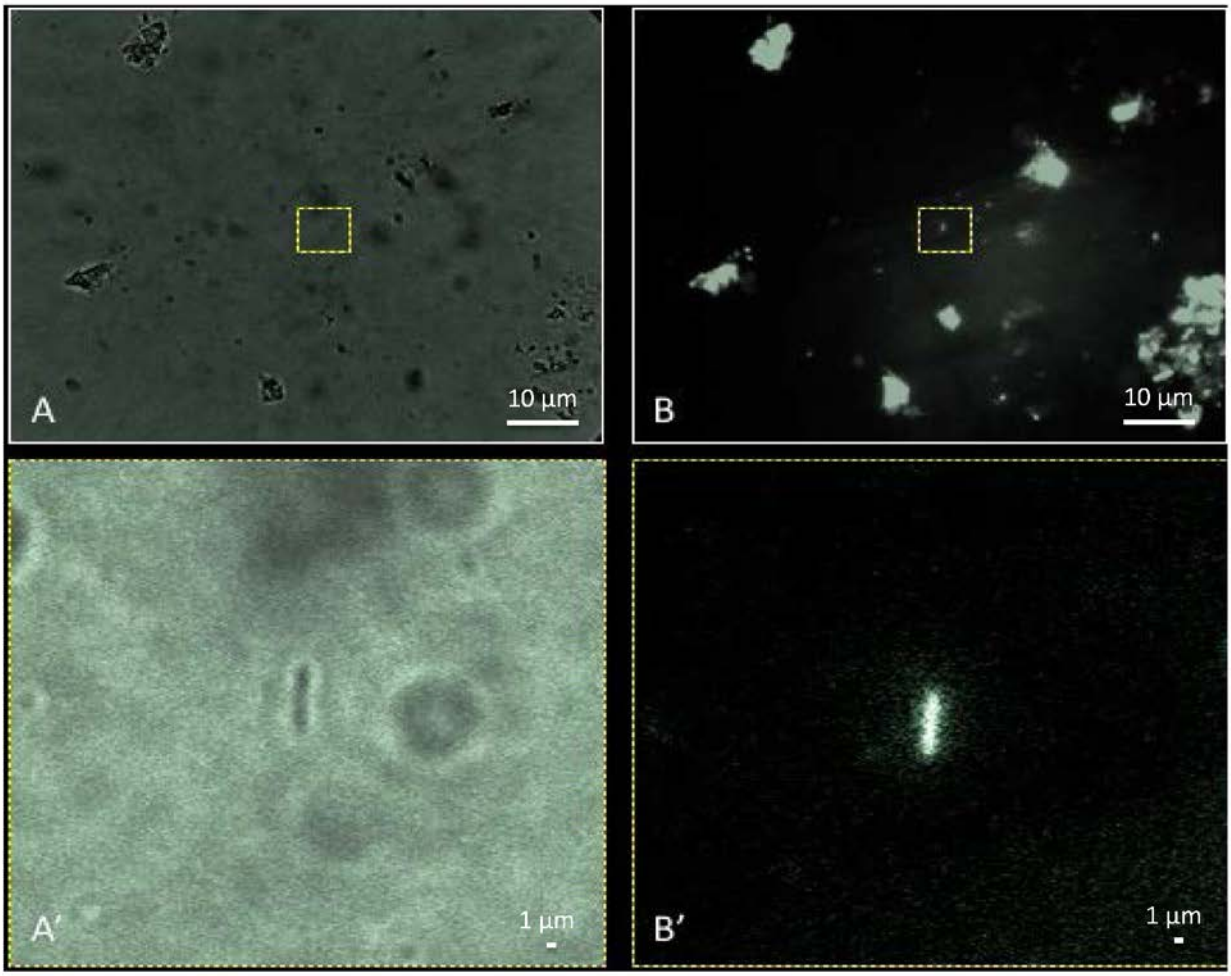

Figure 9: Phase contrast and epifluorescent images of sample 8 collected on 3/15.
Image A shows a rod-shaped bacterium. The yellow box in Image A is blown up and shown as Image A'. Image B shows the same field of view as A, but with epifluorescence. The yellow box in Image B is blown up to show the same rod-shaped bacteria in B'.

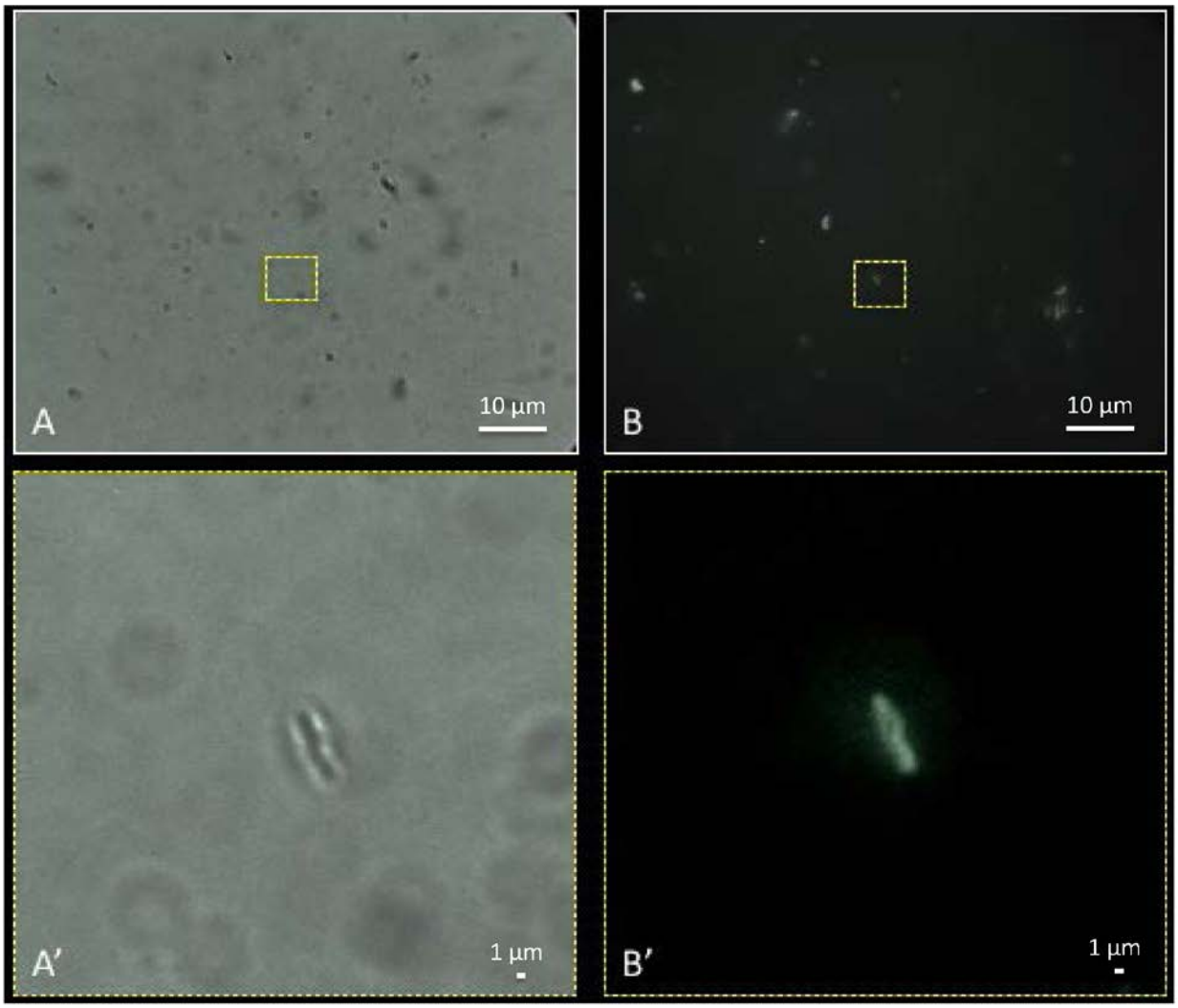

Figure 10: Phase contrast and epifluorescent images of sample 10 collected on 3/15.
Image A shows a rod-shaped bacterium. The yellow box in Image A is blown up and shown as Image A'. Image B shows the same field of view as A, but with epifluorescence. The yellow box in Image B is blown up to show the same rod-shaped bacteria in B'.

We used the FE-SEM on samples collected on 8/16, and observed that a lot of debris was present in the samples and made it difficult to locate and enumerate the cells. Also, when the FE-SEM method is used, the viability of the cells could not be discerned. All tested microscopy methods determined that quantitative microscopy would not be a viable option given the time and budgetary restraints. A 100 uL sample was spotted onto the polycarbonate filter without fixation, and the non-coated sample was visualized under Sirion SEM and is shown in Figure 11.

Overall, it was determined that quantitative microscopy would not be a practical method for spacecraft and cleanroom surfaces due to the low abundance of microbes in these areas and high levels of debris, which promote false positive results from autofluorescence as well as issues with insufficient and nonspecific binding of strains (1, 2, 43)

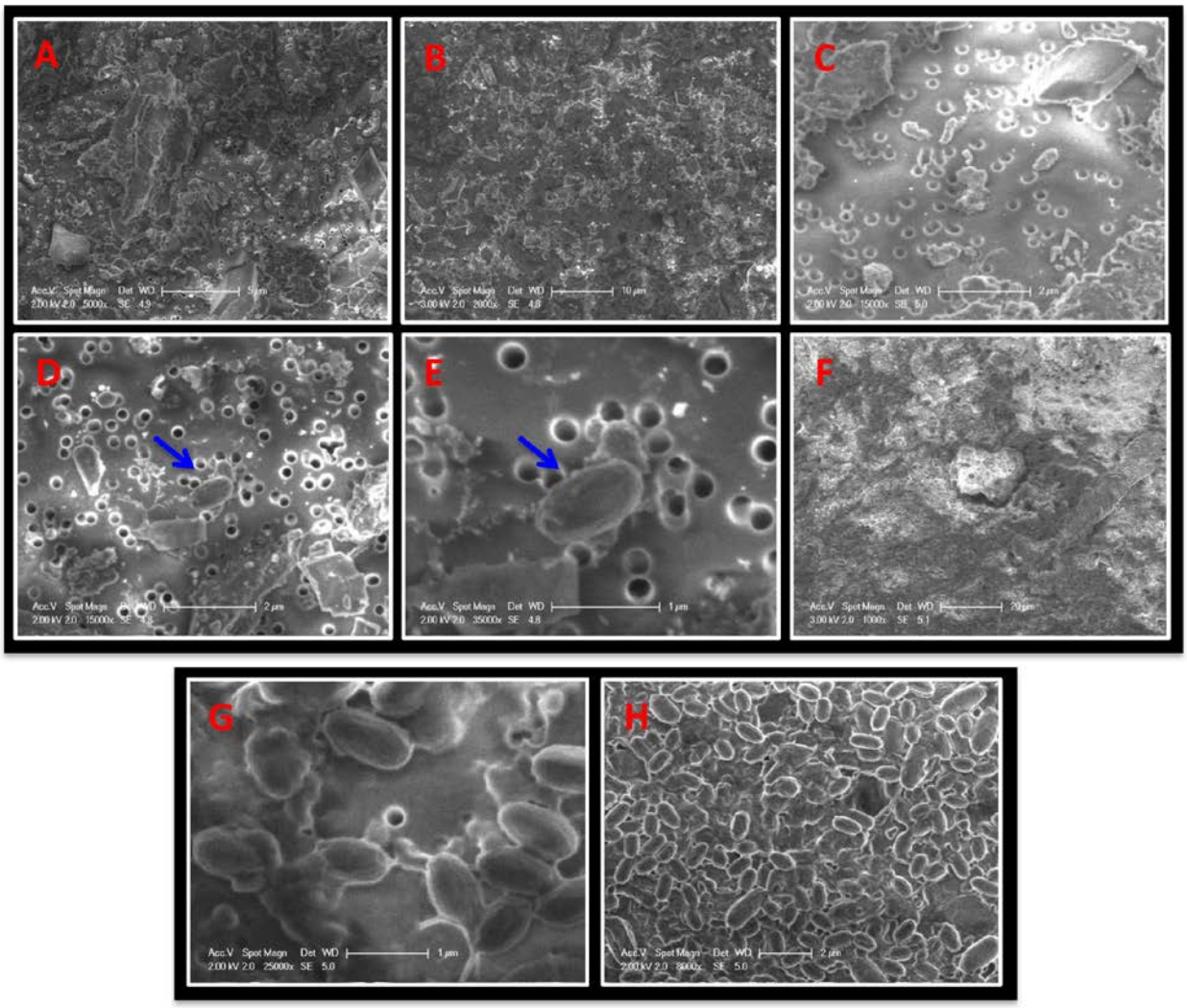

Figure 11: SEM images of the JPL-SAF surface wipe sample spotted onto the polycarbonate filters without fixation and non-coated sample.
SEM Images A and B were scanned at different magnifications of the selected fields of inert particles. Images D and E show bacterial spores (highlighted with the blue arrow) and inert particles. Images C and F were scanned at a 30º tilt of the selected field of interest. SEM images of a spore culture are also shown as a reference to spore morphology (Images G and H).

5.2.4 FACS and WGA Validation

This section describes the various tests utilized to validate FACS and WGA.

5.2.4.1 Model Microbial Community Identification

A model microbial community (MMC), a previously prepared representative mixture of cleanroom organisms, was sent to test the ability of the FACS and WGA pipeline to identify unknown organisms (27). Figure 12 depicts the forward and side scatter plot generated from the MMC blindly processed by FACS and WGA assessments. The FACS methodology combined with WGA validation was able to successfully identify 10 of the 11 strains in the mixture. The one organism that were not identified was *M. luteus*. It was hypothesized that *M. luteus* was missed due to the clusters that it forms, which are excluded in this FACS analysis. MMC was used to help establish a sort gate for viable cells and spores.

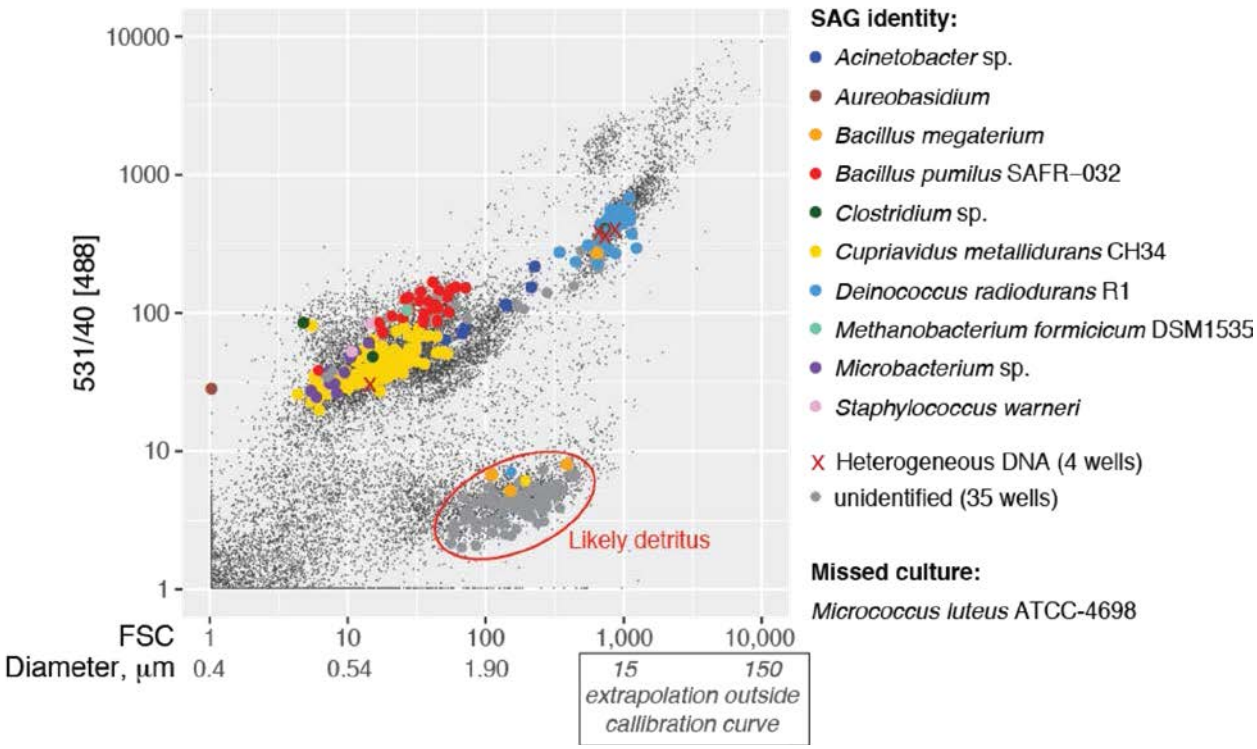

Figure 12: The forward and side scatter plot used in the identification of Model Microbial Community (MMC) organisms.
A MMC mixture, as previously prepared (44), was sent for FACS and WGA analysis. Bigelow Laboratories were not aware of the composition of the MMC prior to analysis. The unknown MMC was sorted using FACS and several random cells were picked and identified using WGA. Of the 11 organisms present in MMC, 9 were positively identified. The y-axis is the green fluorescence (correlates with reductase activity), and the x-axis is the light forward scatter (correlates with particle size).

5.2.4.2 Viability Dye Selection

It was determined that RSG was the best-suited dye for viability identification due to the elaborate validation study conducted by Bigelow (44). RSG was suitable because it is cultivation-independent, has a conservative viability definition, no known taxonomic biases, works on cryopreserved samples, has low background fluorescence, and is compatible with genomics. The compatibility to work with cryopreservatives was beneficial because collected samples could be immediately frozen using glyTE after sampling and stored until they could be processed. There was no significant effect of glyTE on marine samples after one day of treatment (Figure 13).

Once processing began, it was found that 30 minutes of incubation time with RSG was the optimal amount of incubation. The addition of propidium iodide was also explored as an additive, but it was shown to not improve results. For potential future analysis, some alternative probes were suggested: CTC, FDA, CFDA, etc., propidium iodide + SYTO-9, $DiOC_2(3)$, $DiBAC_4(3)$.

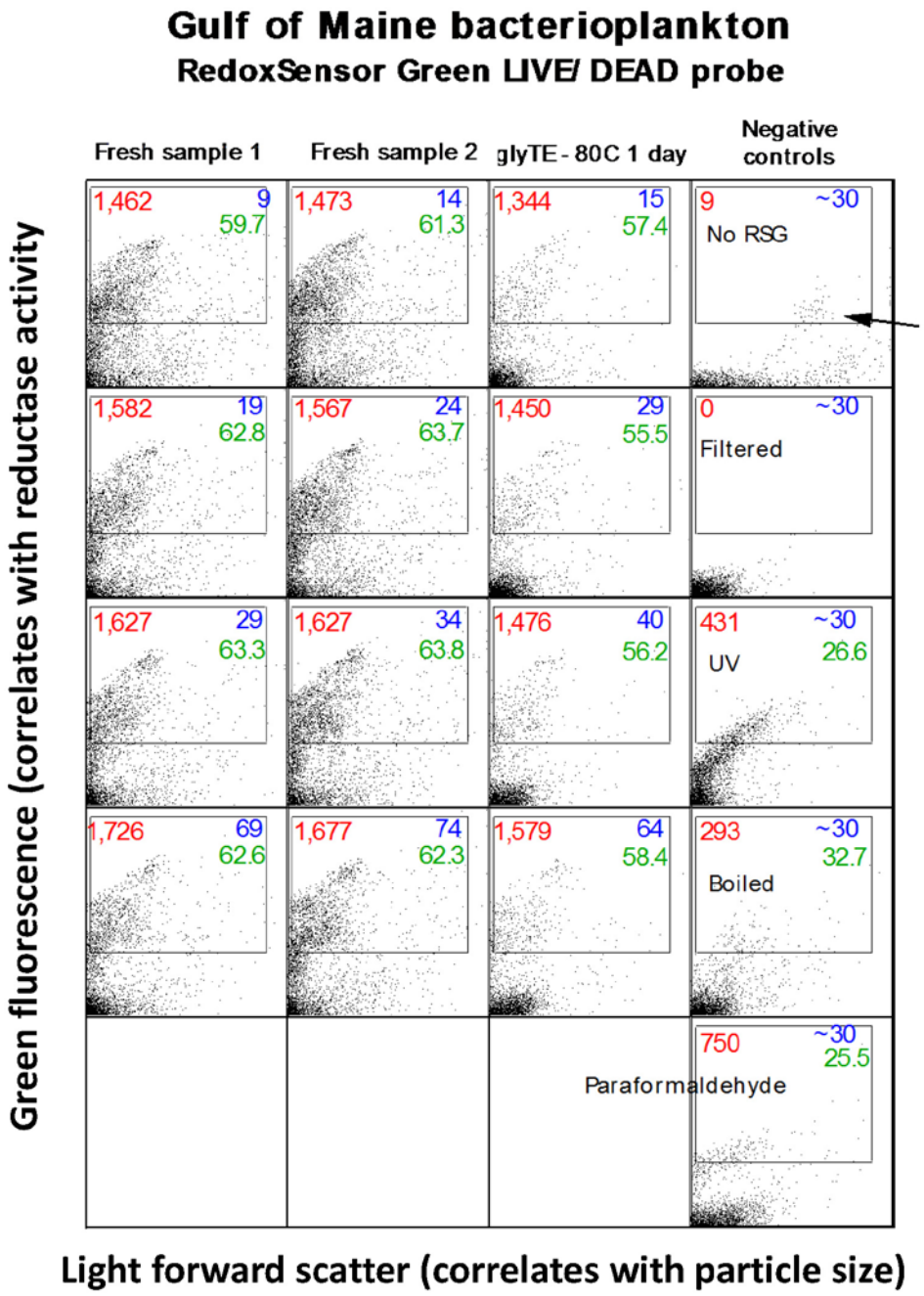

Figure 13: Validation of RSG in marine samples with and without glyTE preservation, as well as various treatments to create truly negative controls.
The red values in each box represent the cells present per uL inside the sorting gate. Blue numbers represent the length of RSG incubation in minutes. The green numbers represent the geometric mean of green fluorescence of RSG positive counts. The arrow in the top right negative control box is pointed at cells, likely *Synechococcus*, a common reagent contaminant. Fresh marine samples (columns 1 and 2) were compared to the one-day glyTE –80C treatment (column 3) to find that the glycerol treatment did not affect the viable cell measurements. The fourth column shows a variety of methods used to establish a negative control that did not results in viable counts occurring in the sort gate established for viable cells. The y-axis is the green fluorescence (correlates with reductase activity), and the x-axis is the light forward scatter (correlates with particle size).

5.2.4.3 Establishment of a Negative Control

To establish a "true" negative control, a subset of MMC, *B. subtilis,* and *B. pumilus* spores sent for analysis were treated with ultraviolet (UV) light to kill the cells. Cells were treated using the following procedure. Purified *B. pumilus* spores were first exposed to 1,500 J m^{-2} dose (UV-C), but subsequent plating showed survival of spores. The UV-C dosage was increased to 4,000 J m^{-2} and was verified to have no growth on TSA or any visual turbidity in TSB. However, FACS analysis, as seen in Figure 14, showed that our UV treatment was reducing the population of all three cultures, but not successfully eliminating all of the cells.

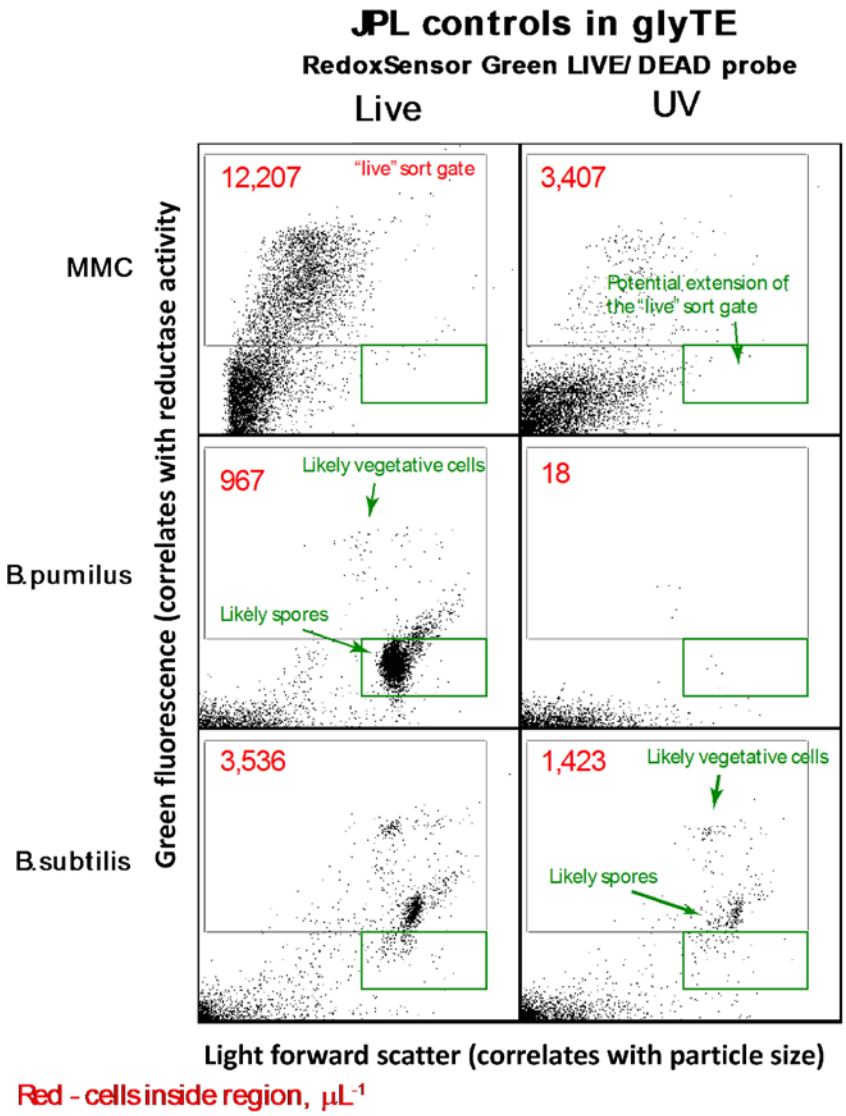

Figure 14: Comparison of MMC, B.pumilus, and B.subtilis samples before and after UV treatment.
To help determine sort gates, three different samples were sorted before and after UV treatment (4,000 J m^{-2}). The red numbers represent the number of cells counted in the standard sort gate. An additional sort gate, identified in green, was added to capture the likely spore population. UV treatment of MMC was able to reduce the cells in the sort gate by about ~25%. UV treatment of *B.pumilus* was able to reduce counts by ~98%. UV treatment of *B.subtilis* was able to reduce cell counts by ~60%. It was determined that UV treatment was not a sufficient method to kill all cells in a sample. The y-axis is the green fluorescence (correlates with reductase activity), and the x-axis is the light forward scatter (correlates with particle size).

Bigelow tested several other methods (filtration, UV, boiling, and paraformaldehyde) on marine samples to help determine a good method to establish a negative control sorting gate (Figure 13). Additionally, paraformaldehyde was added to MMC and marine samples to observe the effect. Although paraformaldehyde seemed to work well on marine samples, it had little effect on MMC samples (Figure 15). Filtration was determined to be the most successful method to establish confident negative controls.

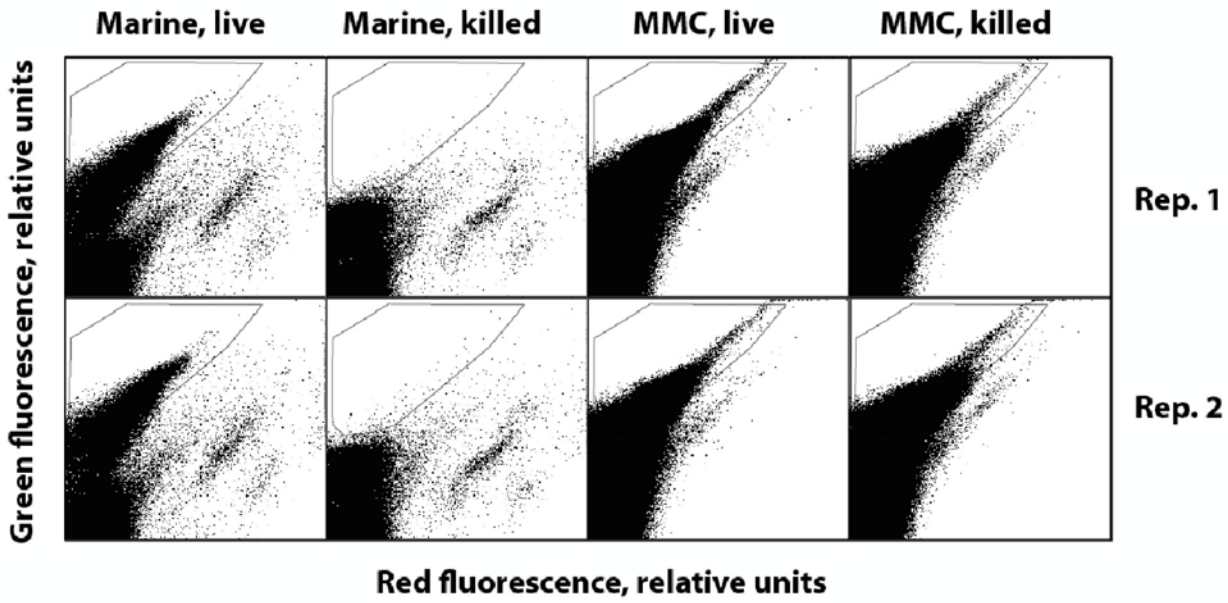

Figure 15: Impact of paraformaldehyde addition on marine and MMC samples.
This image shows the effectiveness in using paraformaldehyde in marine samples, and the ineffectiveness of using paraformaldehyde with MMC samples. It was determined that paraformaldehyde was not effective to use with our low-biomass SAF samples. The y-axis is the green fluorescence, and the x-axis is the red fluorescence.

5.3 Fungal and Archaea Populations

5.3.1 Fungi and Archaea

Although spacecraft cleanroom microbial communities have been shown to be dominated by bacteria, it is important to include estimates of the fungal and archaea populations (45). This section describes the assumptions made from the empirical data generated for both of these populations.

5.3.2 Fungal Population

The fungal population was estimated in this study by fungal qPCR and cultivation on PDA (Table 5). These estimations showed that the fungal populations in the SAF were ~2% of the microbial community, which were consistent with other studies (46, 47).

Fungal qPCR was performed as previously described (48). For all reactions, 1 µl of purified genomic DNA was added to 23 µl of PCR cocktail containing 1× Power SYBR-Green PCR Master Mix (Applied Bios, Foster City, CA), as well as NS91 forward (5‘-GTC CCT GCC CTT TGT ACA CAC-3‘) and ITS51 reverse (5‘-ACC TTG TTA CGA CTT TTA CTT CCT C-3‘) primers, each at 0.02 M final concentration. These primers amplify a 203 bp product spanning the 18S/ITS1 region of rRNA encoding genes.

The reactions steps for qPCR were as follows:

- Hold at 95˚C for 10 minutes
- Followed by 40 cycles of 95˚C for 15 seconds
- Annealing at 58˚C for 20 seconds
- Elongation at 72˚C for 15 seconds

Measurements were recorded at the end of each annealing step. After the 40th cycle, a melt curve analysis was performed by recording changes in fluorescence as a function of raising the temperature from 60˚C to 95˚C in 0.5˚C per 5 s increments. Ribosomal RNA gene standards, spanning 10^8–10^2 gene copies/µl, were generated by serially diluting 4 NMOLE ULTRAMER® DNA OLIGO (Integrated DNA Technologies, Coralville, Iowa, USA). The sequence used as the standard was *Aureobasidium pullulans* 28v1 and is listed as follows: (5'-GTC CCT GCC CTT TGT ACA CAC CGC CCG TCG CTA CTA CCG ATT GAA TGG CTG AGT GAG GCC TTC GGA CTG GCC CAG GGA GGT CGG CAA CGA CCA CCC AGG GCC GGA AAG TTG GTC AAA CTC CGT CAT TTA GAG GAA GTA AAA GTC GTA AC-3').

The number of cultivable fungi ranged from below detection limit (BDL) to 1.7×10^2 CFU/m^2. The average incidence of fungal burden was 1.7×10^1 CFU/m^2. In order to assess the temporal and spatial distribution of the microbial population, samples collected at different dates and locations were analyzed and summarized in Figure 16 (graphs E and F). The highest cultivable fungi was on day 3/1/16 (7.4×10^1 CFU/m^2). Similarly, the lowest load for fungi was on days 6/14/16 through 8/15/16 (BDL). Location-wise, the highest load for fungi was in location 10 (Fungi: 4.2×10^1 CFU/m^2). Correspondingly, the lowest load for fungi was in location 11 (5.0×10^0 CFU/m^2). Fungal burden on the most abundant date, 3/1/16, was statistically significant to all dates ($p < 0.05$) aside from 5/17/16. No other significant temporal or spatial distribution was noticed among cultivable microbial population. Moreover, cultivable fungi were detected in similarly low quantities as that of spores between sampling dates 3/1/16 and 6/1/16, but were not recovered from 6/14/16 onward. Spatially, fungi were consistently distributed in low quantities in all 13 locations.

Table 5: Comparison of Average Fungal and Bacterial results from various assays.

Assay	Fungi	Bacteria
Cultivable	1.7×10^1 CFU/m^2 (n=98)	4.4×10^2 CFU/m^2 (n=98)
Total qPCR	2.4×10^3 copies/m^2 (n=19)	5.3×10^6 copies/m^2 (n=97)
PMA-qPCR	2.4×10^3 copies/m^2 (n=19)	7.9×10^5 copies/m^2 (n=97)

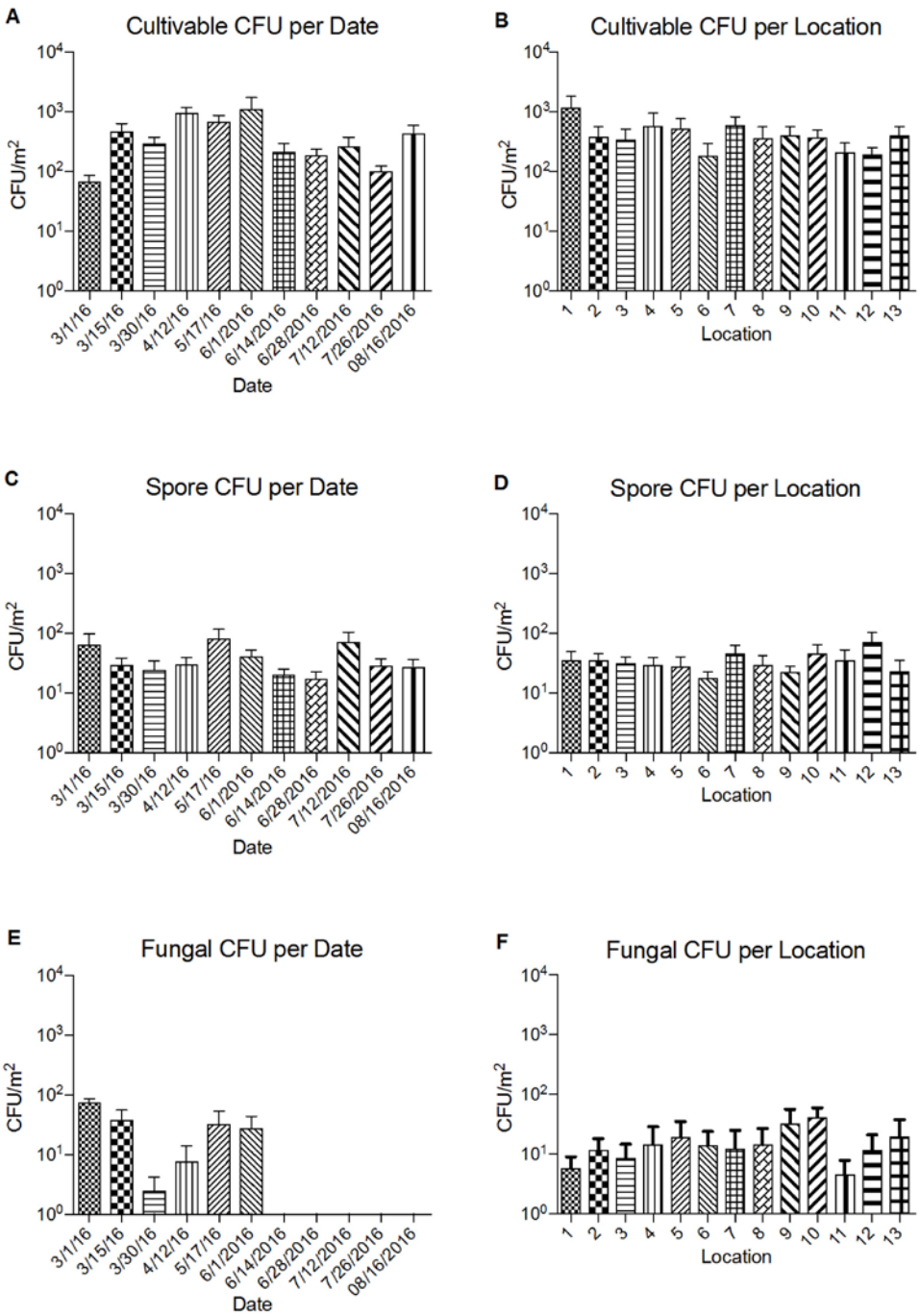

Figure 16: Cultivable microbial burden at each date and location sampled in the SAF.
Samples were plated on TSA (for bacteria) and PDA (for fungi) and incubated at 32˚C for 7 days, at which time the CFU were counted. Spores were determined by NSA, a 15-minutes, 80˚C heat shock of the samples, followed by plating on TSA plates and incubation at 32˚C for 7 days to measure bacterial spores. Cultivable burden based on date: (A) CFU/m^2 of bacteria, (C) CFU/m^2 of NSA spores, and (E) CFU/m^2 of fungi. Each bar represents the average of all samples collected at each sampling date. Cultivable burden based on location: (B) CFU/m^2 of bacteria, (D) CFU/m^2 of NSA spores, and (F) CFU/m^2 of fungi. Each bar represents the average of all samples collected at each location (i.e., 1–13). Error bars for all graphs represent the standard error of the mean.

5.3.3 Archaea Population

Archaeal populations, although not measured directly in this study, have previously been shown to exist in extremely low quantities (BDL-1%) in spacecraft cleanrooms (46). However, archaea-specific qPCR assay was attempted in the early stages and found to show no amplification of the desired archaeal products. Hence, during this study, it was considered that archaea are either BDL or negligible when determining the ratio of spore to VO.

6 Sample Analysis

This section summarizes the culture-dependent and culture-independent sample analysis and results of the 98 samples collected in the SAF.

6.1 Objectives

The objectives of this study were to measure the various microbial populations (NSA spore, cultivable, viable and total) present in the SAF floor samples, using a variety of available assays. The metadata collected for each individual sample (date and location) were recorded to allow for further computational analysis of how the microbial populations varied by date or location in the SAF cleanroom. From this information, we were able to establish several spore to VO ratios with various viability measuring methods, as well as by sample location and sample date to get the most comprehensive view of the microbial environment.

6.2 General Approach

Measurements from various assays were recorded and compared between, and among samples. Each individual assay was analyzed by sample date and location. Overall, each assay provided both an average and a range of values throughout the 6 months of sample collection. Additionally, assays were compared with results from previous studies.

6.3 Cultivation Analysis

In order to assess the temporal and spatial distribution of the microbial population, samples collected at different dates and locations were analyzed and visualized in Figure 16. The temporal distribution of the microorganisms is summarized in Table 6, and spatial distribution given in Table 7.

6.3.1 Modified NASA Standard Assay (Spores)

NSA was performed to estimate the cultivable spore population. The SAF floor samples showed a much smaller range than cultivable organisms. The spore population ranged from BDL to 3.6×10^2 CFU/m^2, with an average of 3.6×10^1 CFU/m^2. Temporal analysis showed that the highest cultivable spore load of 8.3×10^1 CFU/m^2 were found on 5/17/16. The lowest spore load was on 6/28/16, with 1.7×10^1 CFU/m^2. Spatial analysis showed that the highest spore load was in location 12 (7.2×10^1 CFU/m^2) and lowest load in location 6 (1.8×10^1 CFU/m^2). No other significant temporal or spatial distribution was noticed among the cultivable microbial population.

6.3.2 Cultivation on TSA (Bacteria)

Cultivation on TSA was performed, as outlined in Section 4.3.1.1. Overall, cultivable bacteria ranged from 1.2×10^1 to 6.6×10^3 CFU/m^2. The average bacterial burden was 4.4×10^2 CFU/m^2.

When analyzed by date, the highest average cultivable population was found in the 6/1/16 (1.1×10^3 CFU/m^2) samples, while the lowest average was seen in the 3/1/16 samples

(6.9×10^1 CFU/m^2). Although the maximum difference between the highest and lowest average dates was ~2 orders of magnitude, there was no significant difference found between any dates.

Location-wise, the highest bacterial population was in location 1 (1.2×10^3 CFU/m^2), which happens to be at the entrance of the facility. This area would be expected to experience the highest foot traffic and would be expected to have the highest bacterial load. Correspondingly, the lowest load for bacteria was in location 6 (1.8×10^2 CFU/m^2). This location was closer to the back of the cleanroom that had lower levels of foot traffic. Similar to analysis by date, no significant differences were observed.

Table 6: Comparison of cultivation assays by date.

Date	Cultivable[a] (CFU/m^2)	Cultivable Fungi[a] (CFU/m^2)	Cultivable Spores[a] (CFU/m^2)
3/1/16	6.9×10^1	7.4×10^1	6.4×10^1
3/15/16	4.8×10^2	3.8×10^1	3.0×10^1
3/30/16	3.0×10^2	3.0×10^0	2.4×10^1
4/12/16	9.6×10^2	8.0×10^0	3.1×10^1
5/17/16	6.9×10^2	3.3×10^1	8.3×10^1
6/1/16	1.1×10^3	2.8×10^1	4.2×10^1
6/14/16	2.2×10^2	BDL[c]	2.0×10^1
6/28/16	1.9×10^2	BDL[c]	1.7×10^1
7/12/16	2.7×10^2	BDL[c]	7.2×10^1
7/26/16	1.0×10^2	BDL[c]	2.9×10^1
8/15/16	4.4×10^2	BDL[c]	2.8×10^1
Average[b]	4.4×10^2	1.7×10^1	3.6×10^1

[a] Values calculated by taking the average of given assay values on individual sampling date. See Section 4 for detailed explanations of individual assays.
[b] Values are the average of all individual samples from the given dates. Viability average was calculated by taking the sum of all samples from Internal ATP values and dividing by the sum of Total ATP values of all relevant samples. If an individual sample had a measurement of BDL, it along with the corresponding counterpart value, were not included in the calculation.
[c] Below detection limit.

6.3.3 Cultivation on PDA (Fungi)

Cultivable fungal estimation proved to have the most interesting results, even though the average population during the entirety of sampling was only 1.7×10^1 CFU/m^2, or about 2 orders of magnitude less than cultivable bacteria, or about 4% of the cultivable microbial burden in the SAF. The overall range of measured fungi was from BDL to 1.7×10^2 CFU/m^2.

When viewed temporally, fungal isolates were only found in the first 6 of the 11 sampling dates. Of the 6 dates with detectable levels, the highest average was seen on 3/1/16, with 7.4×10^1 CFU/m^2. Because the final 5 sampling dates had no detectable cultivable fungi, they shared the lowest average of BDL. Fungal burden on the most abundant date, 3/1/16, was statistically significant to all dates ($p < 0.05$) aside from 5/17/16.

When viewed by cleanroom location, the fungal populations were fairly consistent. The highest fungi average was seen in location 10 (fungi: 4.2×10^1 CFU/m^2), while the lowest fungal load was found in location 11 (5.0×10^0 CFU/m^2). There were no significant differences observed by cleanroom location.

Table 7: Comparison of cultivation assays by location.

Location	Cultivable Bacteria[a] (CFU/m^2)	Cultivable Fungi[a] (CFU/m^2)	Cultivable Spores[a] (CFU/m^2)
1	1.2×10^3	6.0×10^0	3.5×10^1
2	3.8×10^2	1.2×10^1	3.5×10^1
3	3.5×10^2	9.0×10^0	3.2×10^1
4	5.8×10^2	1.5×10^1	2.9×10^1
5	5.2×10^2	2.0×10^1	2.8×10^1
6	1.8×10^2	1.4×10^1	1.8×10^1
7	5.9×10^2	1.2×10^1	4.6×10^1
8	3.7×10^2	1.5×10^1	2.9×10^1
9	4.0×10^2	3.8×10^1	2.3×10^1
10	3.7×10^2	4.2×10^1	4.6×10^1
11	2.1×10^2	5.0×10^0	3.5×10^1
12	2.0×10^2	1.2×10^1	7.2×10^1
13	4.0×10^2	2.0×10^1	2.3×10^1
Average[b]	4.4×10^2	1.7×10^1	3.6×10^1

[a] Values calculated by taking the average of given assay values on individual sampling locations. See Section 4 for detailed explanations of individual assays.
[b] Values are the average of all individual samples from the given locations. Viability average was calculated by taking the sum of all samples from Internal ATP values and dividing by the sum of Total ATP values of all relevant samples. If an individual sample had a measurement of BDL, it along with the corresponding counterpart value, were not included in the calculation.

6.3.4 Identification of NSA Isolates

Figure 17 depicts a phylogenetic tree of 140 SAF bacterial isolates identified by 16S rRNA sequencing. These 140 heat-shocked isolates were represented by 13 genera and 52 species. Four isolates are potentially novel species (≤98% sequences similarity to any type-strain 16S rRNA sequence). The identities of these 4 isolates will be confirmed by sequencing the gyrB gene. Out of the 136 identifiable strains, 79 of these isolates (58%) belong to 26 known species of the genus *Bacillus.* The most abundant species represented within these samples was *Virgibacillus pantotheticus*, with 16 isolates recovered.

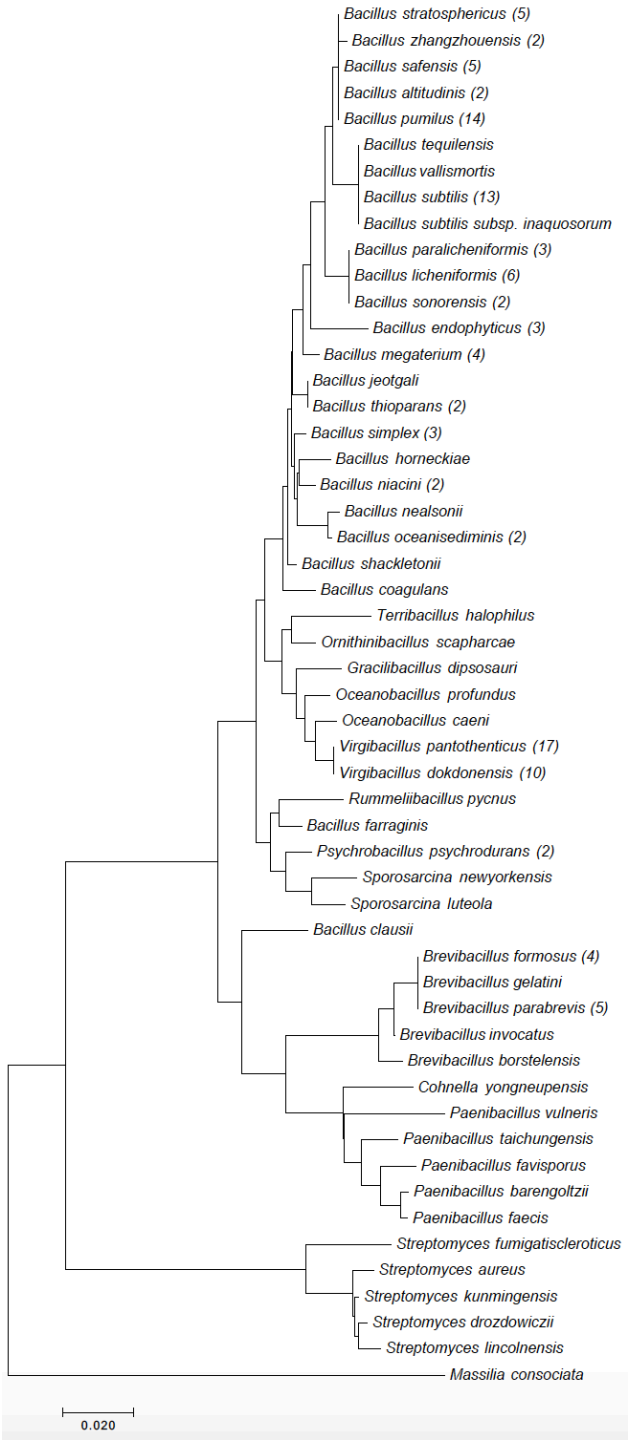

Figure 17: Phylogenetic tree (neighbor-joining) based on 16S rRNA gene sequences of heat-shocked cultivable bacteria isolated from various locations within the SAF at JPL.
The type strain designation is given for each isolate. Numbers in parenthesis represent the number of isolates that had that identification. The three most abundant NASA standard assay isolate found was *Virgibacillus pantothenticus*, *Bacillus pumilus*, and *Bacillus subtilis*. The scale bar shows a 1% estimated difference in nucleotide sequence positions.

6.4 Adenosine Triphosphate (ATP) Analysis

Samples were analyzed with ATP and internal ATP assays to measure total and viable populations, respectively (Figure 18). When converting RLU/m^2 to CFU/m^2, the sizes of various microbial populations were considered, since ATP is directly proportional to the size of microorganisms. The assumptions for RLU/cell are described in Section 3.3. Since fungi (2%) and archaeal population (1%) were negligible in this environment, they were not included in the conversion. Since Gram-positive (5 RLU per cell) and Gram-negative (1 RLU per cell) bacterial composition were equally present, a range of 1–5 RLU/CFU was used in spore to VO estimates (12). The temporal distribution of the ATP content is summarized in Table 8, and the spatial distribution is given in Table 9.

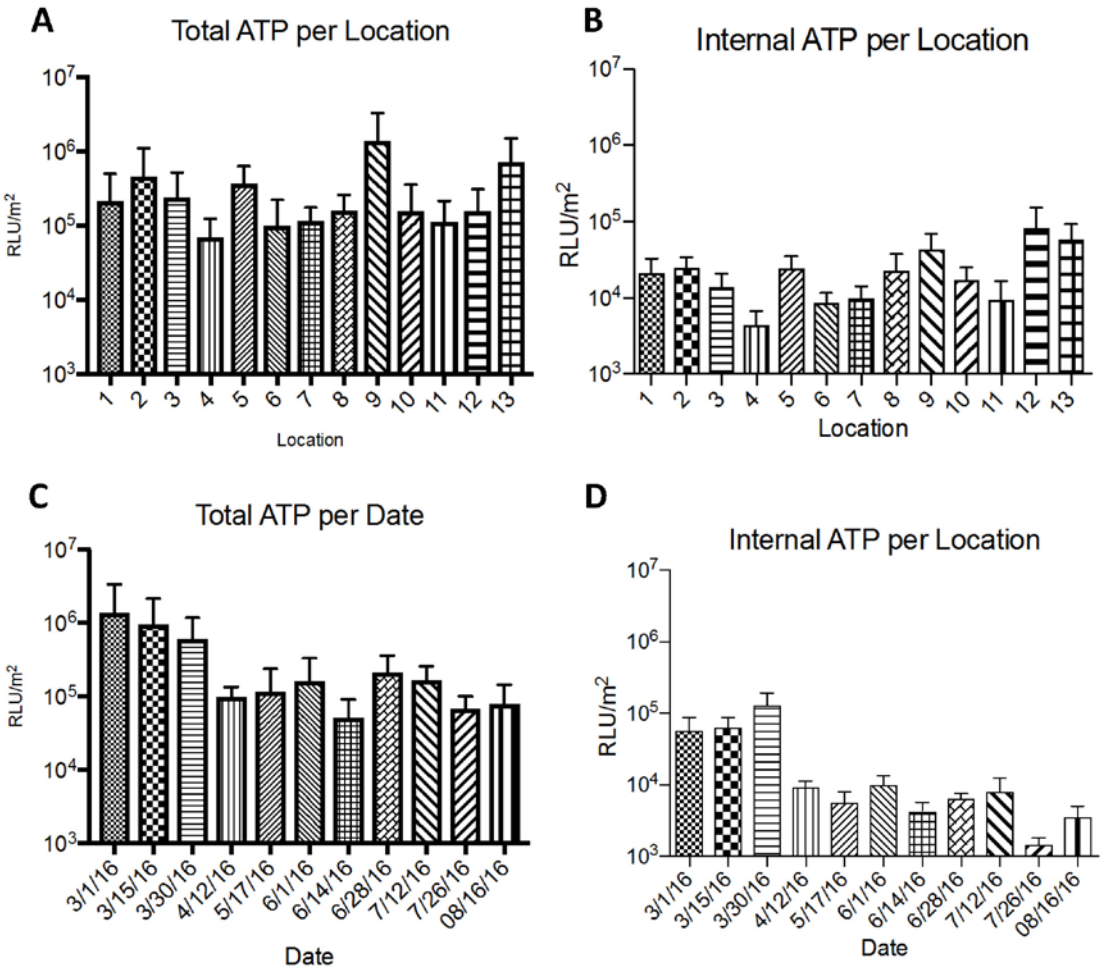

Figure 18: Microbial burden as measured by ATP analysis at each date and location sampled in the SAF. Total ATP was measured using the Kikkoman total ATP kit as outlined in Section 4.3.2.1. Internal ATP was determined by applying an apyrase/adenosine deaminase (ATP-eliminating) reagent to the samples prior to measurements to remove extra-cellular ATP as outlined in Section 4.3.2.2. Microbial burden by location: (A) Total ATP (dead and viable) RLU/m^2, (B) Internal ATP (viable) RLU/m^2. Each bar represents the average of all samples collected at each location (i.e., 1–13). Microbial burden by date: (C) Total ATP (dead and viable) RLU/m^2, (D) Internal ATP (viable) RLU/m^2. Each bar represents the average of all samples collected at each sampling date. Error bars for all graphs represent the standard error of the mean.

Table 8: Comparison of total and internal ATP assays by date.

Date	Total microbes[a] (RLU/m^2)	Viable microbes[a] (RLU/m^2)	% Viable[b]
3/1/16	1.4×10^6	5.7×10^4	6.07
3/15/16	9.5×10^5	6.3×10^4	4.98
3/30/16	6.0×10^5	1.3×10^5	23.22
4/12/16	9.8×10^4	9.3×10^3	13.05
5/17/16	1.2×10^5	5.7×10^3	4.93
6/1/16	1.6×10^5	1.0×10^4	6.25
6/14/16	5.1×10^4	4.3×10^3	8.39
6/28/16	2.1×10^5	6.3×10^3	2.78
7/12/16	1.7×10^5	7.9×10^3	4.82
7/26/16	6.8×10^4	1.5×10^3	2.20
8/15/16	7.8×10^4	3.6×10^3	3.92
Average[c]	3.3×10^5	2.8×10^4	14.7

[a] Values calculated by taking the average of given assay values on individual sampling dates. See Section 4 for detailed explanations of individual assays.

[b] Viability percent is calculated by taking the sum of all Internal ATP values, which was divided by the sum of Total ATP values of all relevant samples.

[c] Values are the average of all individual samples from the given dates. Viability average was calculated by taking the sum of all samples from Internal ATP values and dividing by the sum of Total ATP values of all relevant samples. If an individual sample had a measurement of BDL, it along with the corresponding counterpart value, were not included in the calculation.

Table 9: Comparison of total and internal ATP assays by location.

Location	Total microbes[a] (RLU/m^2)	Viable microbes[a] (RLU/m^2)	% Viable[b]
1	2.1 x 10^5	2.1 x 10^4	10.02
2	4.5 x 10^5	2.5 x 10^4	5.55
3	2.4 x 10^5	1.4 x 10^4	6.19
4	6.9 x 10^4	4.3 x 10^3	2.32
5	3.7 x 10^5	2.4 x 10^4	7.42
6	9.9 x 10^4	8.5 x 10^3	13.74
7	1.1 x 10^5	9.9 x 10^3	9.48
8	1.6 x 10^5	2.3 x 10^4	14.31
9	1.4 x 10^6	4.4 x 10^4	3.18
10	1.6 x 10^5	1.7 x 10^4	9.41
11	1.3 x 10^5	9.3 x 10^3	8.11
12	1.7 x 10^5	8.2 x 10^4	48.61
13	7.1 x 10^5	5.8 x 10^4	8.13
Average[c]	3.3 x 10^5	2.8 x 10^4	9.27

[a] Values calculated by taking the average of given assay values on individual sampling locations. See Section 4 for detailed explanations of individual assays.
[b] Viability percent is calculated by taking the sum of all Internal ATP values, which was divided by the sum of Total ATP values of all relevant samples.
[c] Values are the average of all individual samples from the given locations. Viability average was calculated by taking the sum of all samples from Internal ATP values and dividing by the sum of Total ATP values of all relevant samples. If an individual sample had a measurement of BDL, it along with the corresponding counterpart value, were not included in the calculation.

6.4.1 Total ATP

For total ATP analysis, 14 of the 98 samples were not included in the calculations because they were below control values. The total ATP ranged from BDL to 4.2×10^6 RLU/m^2, with an average of 3.3×10^5 RLU/m^2. The calculated average RLU/m^2 was 6.2×10^4 to 3.3×10^5 cells/m^2. The highest total ATP was seen on samples collected on 3/1/16 (1.4×10^6 RLU/m^2), which was significantly different from three other dates, including the date with the lowest average measurement (6/14/16; 5.1×10^4 RLU/m^2). When samples were compared spatially (location-wise), the only significant difference ($p < 0.05$) observed was between samples from highest average, location 9 with 1.4×10^6/m^2 RLU/m^2, and six other locations (1, 6, 8, 10, 11, 12). The lowest total ATP average was seen in location 4 (4.1×10^4/m^2).

6.4.2 Internal ATP

Of the 98 internal ATP samples analyzed, 14 were not included in the calculations because they were below control values. These values were later used for ATP-based VO estimation, as seen in Figure 23. Of the 98 samples analyzed, 11 showed values below control and were not included in the average. The intracellular ATP from the SAF floors averaged 2.7×10^4 RLU/m^2, which was equivalent to 5.6×10^3 to 2.8×10^4 viable cells/m^2. The range was BDL to 7.2×10^5 RLU/m^2. The only significant difference ($p < 0.05$) observed by date was from samples collected on 3/30/16, which had the highest average of 1.3×10^5 RLU/m^2, and four other dates (4/12/16, 6/1/16, 6/14/16, 7/26/16). The lowest intracellular ATP contents were from 7/26/16 samples (1.5×10^3 RLU/m^2). There were no significant differences observed by location. The highest

average of intracellular ATP was 8.2×10^4 RLU/m^2 in location 12, and the lowest was in location 4 (4.34×10^3 RLU/m^2).

6.5 Quantitative-PCR Analysis

Samples were analyzed with non-PMA qPCR and PMA qPCR assays to measure total and viable populations, respectively (Figure 19). The 16S rRNA gene copy numbers were converted to CFU based on the average 16S rRNA gene copies per cell, 4.2 +/- a standard deviation of 2.7, for a range of 1.5 to 6.9 copies/cell (19). Since >98% of the microbial burden was due to the bacterial abundance, the TO and VO numbers mentioned below were for bacteria. The temporal distribution of the qPCR values are summarized in Table 10, and the spatial distribution is given in Table 11.

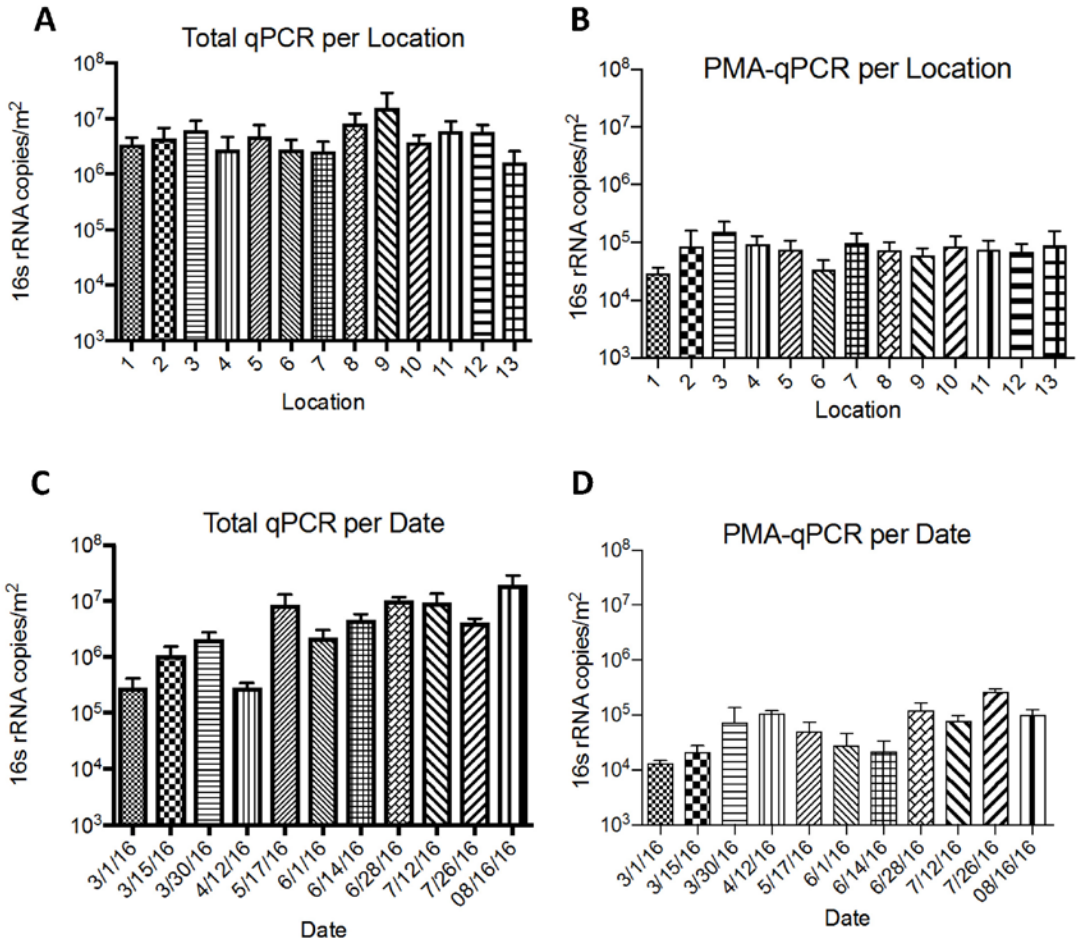

Figure 19: Microbial burden as measured by 16S qPCR analysis at each date and location sampled in the SAF.

Total qPCR was measured as outlined in Section 4.3.3. Viability was assessed via PMA-qPCR performed on samples, which allows for only viable (and not dead) cells to be detected, and as outlined in Section 4.3.3.1. Microbial burden by location: (A) Total qPCR (dead and viable) 16S rRNA gene copies/m^2, (B) PMA-qPCR (viable) 16S rRNA gene copies/m^2. Each bar represents the average of all samples collected at each location (i.e., 1–13). Microbial burden by date: (C) Total qPCR (dead and viable) 16S rRNA gene copies/m^2, (D) PMA-qPCR (viable) 16S rRNA gene copies/m^2. Each bar represents the average of all samples collected at each sampling date. Error bars for all graphs represent the standard error of the mean.

Table 10: Comparison of non-PMA and PMA qPCR assays by date.

Date	Total microbes[a] (16s/m^2)	Viable microbes[a] (16s/m^2)	% Viable[b]
3/1/16	2.8×10^5	1.3×10^4	4.68
3/15/16	7.9×10^5	2.1×10^4	2.78
3/30/16	2.1×10^6	7.4×10^4	3.49
4/12/16	2.8×10^5	1.1×10^5	37.70
5/17/16	8.4×10^6	5.1×10^4	0.61
6/1/16	2.2×10^6	2.9×10^4	1.28
6/14/16	4.6×10^6	2.2×10^4	0.47
6/28/16	1.0×10^7	1.2×10^5	1.19
7/12/16	9.3×10^6	7.8×10^4	0.84
7/26/16	4.0×10^6	2.7×10^5	6.55
8/15/16	2.0×10^7	1.0×10^5	0.51
Average[c]	5.3×10^6	7.9×10^4	1.45

[a] Values calculated by taking the average of given assay values on individual sampling locations. See Section 4 for detailed explanations of individual assays.

[b] Viability percent is calculated by taking the sum of all PMA qPCR values, which was divided by the sum of non-PMA qPCR values of all relevant samples.

[c] Values are the average of all individual samples from the given dates. Viability average was calculated by taking the sum of all samples from PMA qPCR values and dividing by the sum of non-PMA qPCR values of all relevant samples. If an individual sample had a measurement of BDL, it along with the corresponding counterpart value, were not included in the calculation.

Table 11: Comparison of non-PMA and PMA qPCR assays by location.

Location	Total microbes (16s/m^2)	Viable microbes (16s/m^2)	% Viable[b]
1	3.4×10^6	2.9×10^4	0.85
2	4.6×10^6	8.5×10^4	5.67
3	6.2×10^6	1.5×10^5	2.45
4	2.8×10^6	9.3×10^4	3.31
5	4.8×10^6	7.5×10^4	1.55
6	2.8×10^6	3.4×10^4	1.23
7	2.6×10^6	9.9×10^4	3.80
8	8.2×10^6	7.2×10^4	0.88
9	1.6×10^7	6.0×10^4	0.38
10	2.3×10^6	8.5×10^4	2.18
11	6.1×10^6	7.5×10^4	1.23
12	5.7×10^6	6.9×10^4	1.20
13	1.7×10^6	9.1×10^4	5.46
Average[c]	5.3×10^6	7.9×10^4	1.45

[a] Values calculated by taking the average of given assay values on individual sampling locations. See Section 4 for detailed explanations of individual assays.
[b] Viability percent is calculated by taking the sum of all PMA qPCR values, which was divided by the sum of non-PMA qPCR values of all relevant samples.
[c] Values are the average of all individual samples from the given dates. Viability average was calculated by taking the sum of all samples from PMA qPCR values and dividing by the sum of non-PMA qPCR values of all relevant samples. If an individual sample had a measurement of BDL, it along with the corresponding counterpart value, were not included in the calculation.

6.5.1 Quantitative Polymerase Chain Reaction

Non-PMA-treated samples underwent qPCR to estimate the TO population. The TO averaged 5.3×10^6 copies/m^2. This was equivalent to 7.7×10^5 to 3.6×10^6 cells/m^2. The range of copies/m^2 varied from 1.1×10^4 to 9.7×10^7 copies/m^2. A significant difference in TO was noticed between samples taken on 8/15/16 and seven other dates (3/1/16, 3/15/16, 3/30/16, 4/12/16, 6/1/16, 6/14/16, 7/26/16), due to the high TO incidence observed on 8/15/16 (2.0×10^7 copies/m^2). The lowest TO average, 2.8×10^5 copies/m^2, was seen from the 3/1/16 samples. When compared spatially (location-wise), the average TO burden had no significant differences. The lowest TO average was seen in location 13 (1.7×10^6 copies/m^2), and the highest (1.6×10^7 copies/m^2) was observed in location 9.

6.5.2 Propidium Monoaziade Quantitative Polymerase Chain Reaction

The same samples that measured TO were analyzed after treatment with PMA dye to measure VO. The average VO was 7.9×10^4 copies/m^2, which was converted to an estimate of 1.2×10^4 to 5.4×10^4 viable bacterial cells /m^2. The VO average was at least 2-orders of magnitude less than the TO values. The temporal (date-wise) distribution of VO as measured by PMA-qPCR is shown in Table 10. The average VO varied slightly between dates collected, but samples collected on 7/26/16 were significantly different ($p < 0.05$) than all other dates. The lowest VOs were seen on 3/1/16 (1.3×10^4 cells/m^2) and the highest VOs on 7/26/16 (2.7×10^5 cells/m^2).

Spatially (by location), no significant differences were observed (Table 11). The lowest VO average was from location 1 (2.9×10^4 cells/m^2), and the highest VO average was from location 3 (1.5×10^5 cells/m^2).

6.6 Fluorescent-Assisted Cell Sorting Analysis

Viable cells were stained with RSG and measured using a FACS. Results were confirmed with LoCoS.

6.6.1 FACS Viable Estimation

The average VO estimate was 4.8×10^5 cells/m^2. However, approximately 20% of sorted "viable" particles were able to be amplified from sequence analyses. When the 20% factor is applied, the average estimate was 9.5×10^4 viable cells/m^2. Both raw counts and counts with the 20% factor can be found in Figure 20. The date-wise results are shown in Table 12, and the location-wise results are shown in Table 13. Sorting of individual samples, with the chosen sorting gate, are shown in Figure 21.

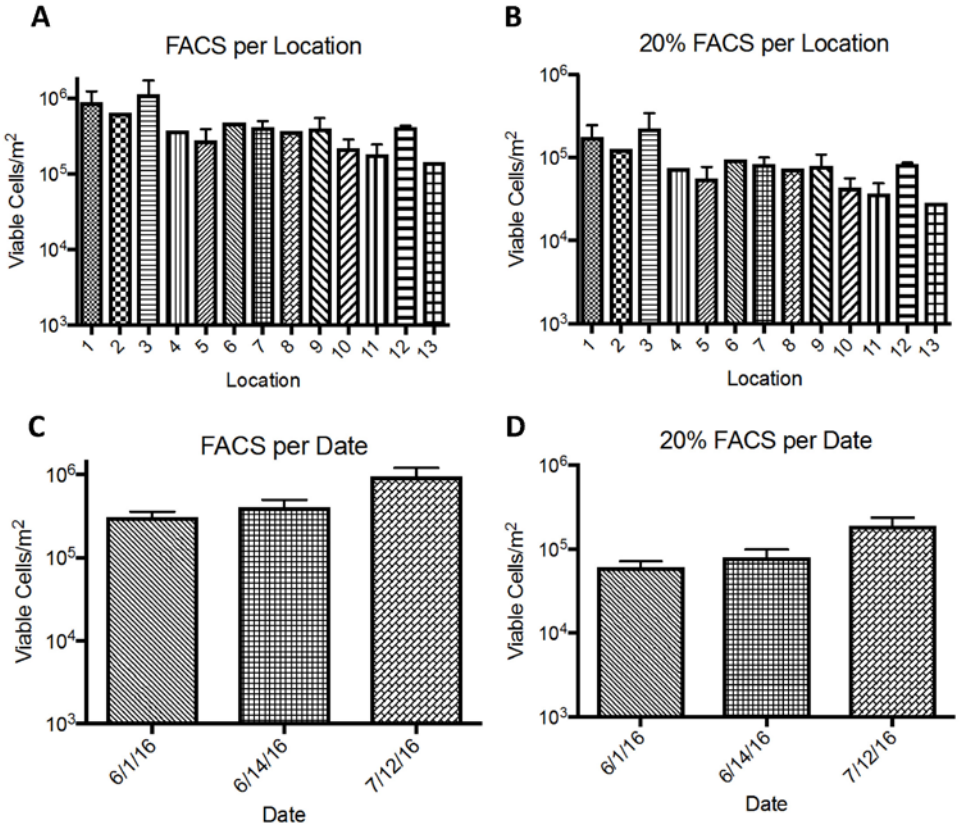

Figure 20: Microbial burden as measured by FACS analysis at each date and location sampled in the SAF as outlined in Section 4.3.4.

Viable cells were estimated using the RedoxSensor Green dye to specifically tag viable cells for counting during cell sorting. Approximately 20% of particles identified as viable via FACS were confirmed by low coverage sequencing. Viable microbial burden by location: (A) FACS-estimated viable cell/m^2, (B) 20% FACS-estimated viable cell/m^2. Each bar represents the average of all samples collected at each location (i.e., 1–13). Viable microbial burden by date: (C) FACS-estimated viable cell/m^2, (D) 20% FACS-estimated viable cell/m^2. Each bar represents the average of all samples collected at each sampling date. Error bars for all graphs represent the standard error of the mean.

When the VO population from FACS was viewed by date, the lowest value was on 6/1/16 ($6.1 \times 10^4/m^2$), and the highest value was seen on 7/12/16 (1.9×10^5 cells/m^2). The 7/12/16 values were significantly different ($p < 0.05$) than 6/1/16 and 6/14/16 samples. When the spatial distribution of the VO population was viewed, the lowest value was at location 13 (2.9×10^4 cells/m^2), and the highest VO was at location 3 (2.3×10^5 cells/m^2).

Table 12: Temporal distribution of FACS-based VO of SAF floors.

Sampling Date	Viable microbes[a] (RSG+ CFU/m²) [B3]	Cultivable Spores[a] (CFU/m²) [D]	Spore to VO (FACS)[b] [B3/D]	20% Viable microbes[a] (RSG+ CFU/m²) [B4]	20% Spore to VO (FACS)[b] [B4/D]
6/1/16	1.1×10^5	4.2×10^1	7,338	6.1×10^4	1,468
6/14/16	4.0×10^5	2.0×10^1	19,889	8.1×10^4	3,978
7/12/16	9.6×10^5	7.2×10^1	13,218	1.9×10^5	2,644
Average[c]	4.8×10^5	3.6×10^1	12,091	9.5×10^4	2,418

[a] Values calculated by taking the average of given assay values on individual sampling locations. See Section 4 for detailed explanations of individual assays.

[b] Ratio calculated for a given location by taking the sum of a given viable assay divided by the sum of cultivable spore of all relevant samples. If an individual sample from a location had a viability assay measurement of BDL, it along with the corresponding cultivable spore, were not included in the calculation.

[c] Values are the average of all individual samples from the given dates. Spore to VO ratios were calculated by taking the sum of all samples from viable assays and dividing by the sum of cultivable spore of all relevant samples. If an individual sample from a location had a viability assay measurement of BDL, it along with the corresponding cultivable spore, were not included in the calculation.

Table 13: Spatial distribution of FACS-based VO of SAF floors.

Sampling Location	Viable microbes[a] (RSG+ CFU/m^2) [B3]	Cultivable Spores[a] (CFU/m^2) [D]	Spore to VO[b] (FACS) [B3/D]	20 %Viable microbes[a] (RSG+ CFU/m^2)[a] [B4]	20% Spore to VO[b] (FACS) [B4/D]
1	8.8×10^5	3.5×10^1	12,090	1.8×10^5	2,418
2	6.4×10^5	3.5×10^1	8,483	1.3×10^5	1,697
3	1.1×10^6	3.2×10^1	34,754	2.3×10^5	6,951
4	3.8×10^5	2.9×10^1	29,124	7.5×10^4	5,825
5	2.8×10^5	2.8×10^1	44,400	5.6×10^4	8,880
6	4.8×10^5	1.8×10^1	9,825	9.6×10^4	1,965
7	4.2×10^5	4.6×10^1	6,452	8.3×10^4	1,290
8	3.7×10^5	2.9×10^1	9,825	7.4×10^4	1,965
9	4.0×10^5	2.3×10^1	18,805	7.9×10^4	3,761
10	2.2×10^5	4.6×10^1	5,695	4.4×10^4	1,139
11	1.8×10^5	3.5×10^1	5,617	3.7×10^4	1,123
12	4.1×10^5	7.2×10^1	13,133	8.3×10^4	2,627
13	1.4×10^5	2.3×10^1	11,354	2.9×10^4	2,271
Average[c]	4.8×10^5	3.6×10^1	12,091	9.5×10^4	2,418

[a] Values calculated by taking the average of given assay values on individual sampling locations. See Section 4 for detailed explanations of individual assays.

[b] Ratio calculated for a given location by taking the sum of a given viable assay divided by the sum of cultivable spore of all relevant samples. If an individual sample from a location had a viability assay measurement of BDL, it along with the corresponding cultivable spore, were not included in the calculation.

[c] Values are the average of all individual samples from the given dates. Spore to VO ratios were calculated by taking the sum of all samples from viable assays and dividing by the sum of cultivable spore of all relevant samples. If an individual sample from a location had a viability assay measurement of BDL, it along with the corresponding cultivable spore, were not included in the calculation.

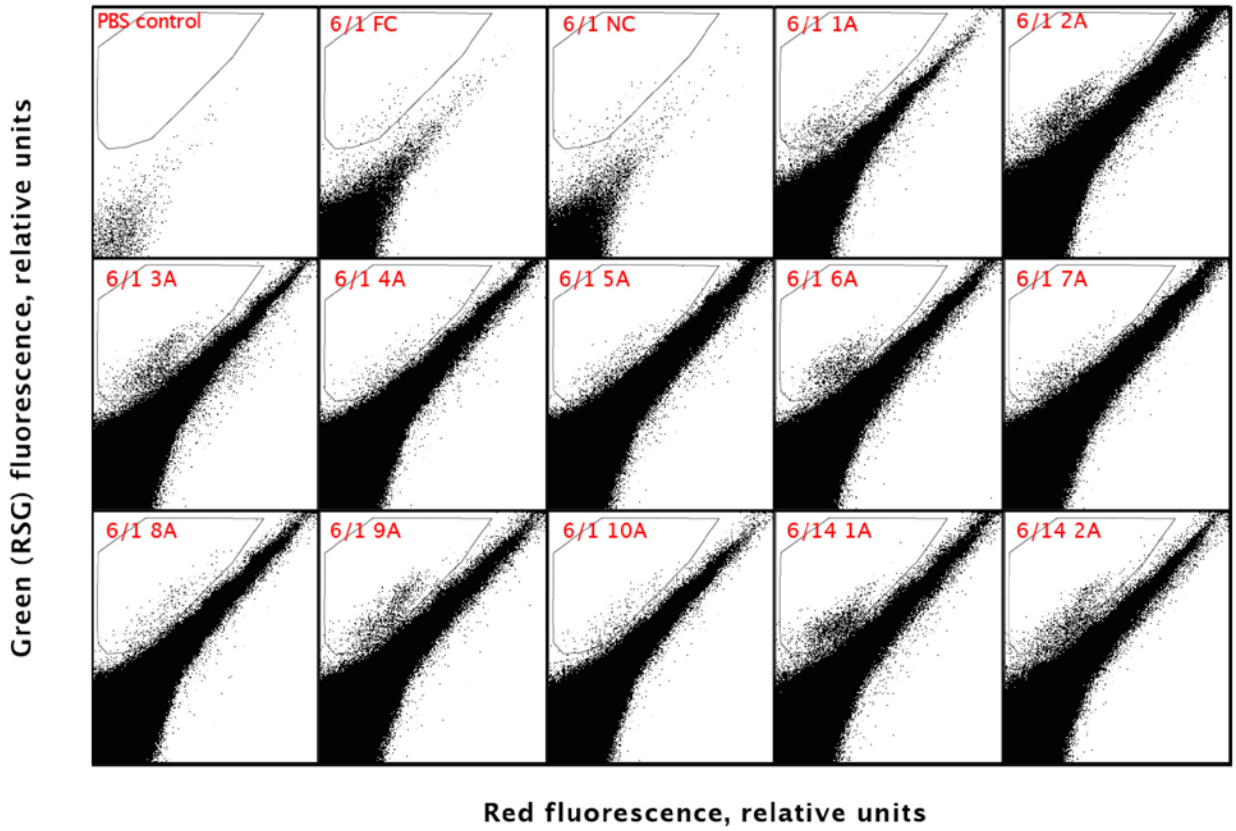

Figure 21: FACS sorting of SAF samples.
This image shows several SAF samples that were sorted and enumerated. The chosen sort gate is shown in the top left of each sample box, with each dot representing one count. Dots residing inside the chosen sort gate were counted as viable cells. The negative control and field control also showed some counts in the chosen sort gate used for this study. The y-axis is the green fluorescence (RSG) in relative units, and the x-axis is the red fluorescence in relative units.

6.6.2 SAG Results

One sample (7/12/16, location 1) was chosen for whole genome amplification. Table 14 shows the results of the whole genome amplification of this sample and the identification of organisms.

Table 14: Results of sample 1 collected on 7/12/16 and processed by WGA.

Sample	Trimmed Read Count	Final Contigs Assembled Length	CheckM Classification
AG-822-A06	5,391,754	17,158	NA;unresolved
AG-822-A15	6,551,840	37,572	NA;unresolved
AG-822-A19	5,518,524	107,436	NA;unresolved
AG-822-A23	6,571,067	20,889	NA;unresolved
AG-822-B20	6,728,970	25,531	Fusobacteriales
AG-822-C06	5,740,890	50,675	NA;unresolved
AG-822-C11	6,204,490	47,248	*Paracoccus zeaxanthinifaciens*
AG-822-C15	5,410,852	45,207	NA;unresolved
AG-822-D04	6,535,819	6,757	NA;unresolved
AG-822-D07	7,059,299	147,636	*Acinetobacter lwoffii*
AG-822-D09	5,956,169	5,302	NA;unresolved
AG-822-E16	7,286,648	9,778	NA;unresolved
AG-822-F10	6,295,129	23,903	NA;unresolved
AG-822-F23	6,523,426	9,633	NA;unresolved
AG-822-J05	5,601,585	143,863	NA;unresolved
AG-822-J08	4,238,629	75,268	NA;unresolved
AG-822-L11	6,571,323	82,652	NA;unresolved
AG-822-L18	6,873,563	56,533	NA;unresolved
AG-822-M10	6,342,831	53,071	NA;unresolved
AG-822-M19	6,870,658	50,257	*Gemmatimonas aurantiaca*
AG-822-N17	7,017,309	10,081	NA;unresolved
AG-822-N21	5,689,787	68,963	NA;unresolved
AG-822-O07	6,370,440	61,837	NA;unresolved
AG-822-O16	7,325,383	30,147	NA;unresolved
AG-822-O22	6,322,438	17,935	NA;unresolved
AG-822-P01	6,360,809	503,009	Paracoccus
AG-822-P09	6,334,203	79,175	NA;unresolved
AG-822-P20	3,856,588	149,907	NA;unresolved

6.7 Viability Assays

All three viability assays were able to establish estimates on the viable microbial population. The comparisons of the three methodologies sorted out by date and location are depicted in Figure 22.

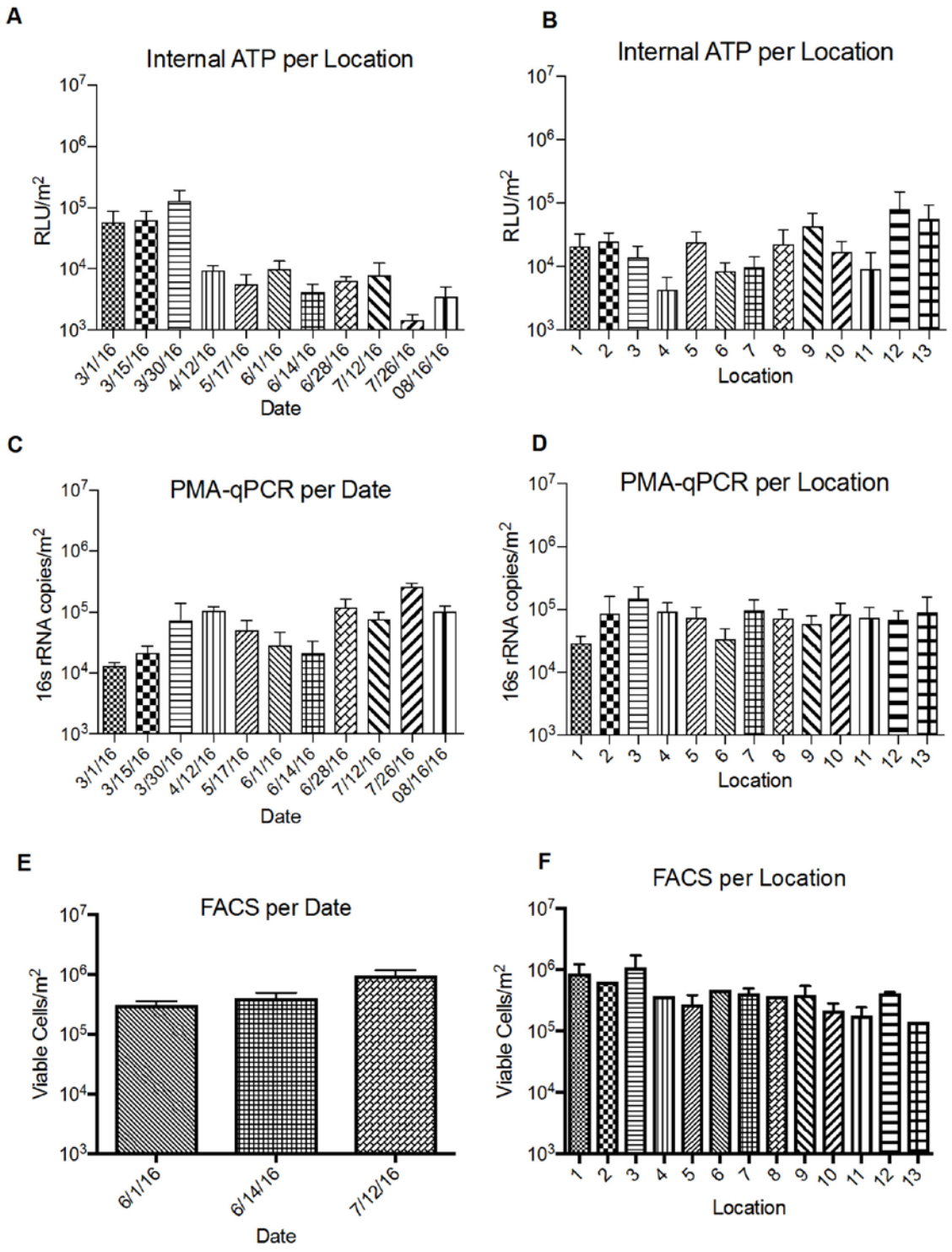

Figure 22: Viable microbial burden at each date and location sampled in the SAF.
Samples were analyzed via Internal ATP (A and B, Section 6.4.2), PMA-qPCR (C and D, Section 6.5.2), and FACS (E and F, Section 6.6). Viable burden by date: (A) Intracellular ATP, (C) PMA-qPCR, (E) FACS. Each bar represents the average of all samples collected at each sampling date. Viable burden by location: (B) Intracellular ATP, (D) PMA-qPCR, (F) FACS. Each bar represents the average of all samples collected at each location (i.e., 1–13). Error bars for all graphs represent the standard error of the mean.

6.8 Spore to VO Ratios

This section describes the three different spore to VO calculations (ATP, qPCR, and FACS). The temporal and spatial results for all three methodologies can be found in Figure 23.

6.8.1 ATP-Based Spore to VO Ratio

The ATP-based spore to VO ratio was established by taking the sum of all internal ATP RLU/m^2 values and dividing by the sum of all spore CFU/m^2 values (Figure 23). A range was then created from the average value by separately dividing the average by 1 and 5 to account for the different RLU/cell for Gram-negative and Gram-positive cells. Spore values from samples that had below control internal ATP measurements were not included in this calculation. The spore to VO measured by intracellular ATP range was 149 to 746. No significant differences were seen spatially. Location 13 showed the highest spore to VO range (501 to 2,504), and location 4 showed the lowest spore to VO range (30 to 148). The spore to VO ratio measured by ATP assay showed significant ($p < 0.05$) temporal distribution when samples were compared (3/15/16 with 4/12/16, 5/17/16, 6/1/16, 6/14/16, 6/28/16; 3/30/16 with 6/1/16, 6/14/16; 3/30/16 with 7/26/16). The highest range was observed on 3/30/16 (1,052 to 5,261), and the lowest ratio range was documented on 7/26/16 (10 and 52). Temporal results are found in Table 15, and spatial results are found in Table 16.

6.8.2 Quantitative PCR-Based Spore to VO Ratio

The qPCR-based spore to VO ratio was established by taking the sum of all PMA-qPCR 16S rRNA gene copies/m^2 values and dividing by the sum of all spore CFU/m^2 values (Figure 23). The calculated spore to VO value of 2,176 was then converted to a range by accounting for the numbers of 16S rRNA gene per bacterial cell (4.2 ± one standard deviation, 2.7). The resulting spore to VO ratio was in the range of 314 to 1,491. No significant differences were observed in this ratio by samples collected either date-wise or location-wise. The highest range was on 7/26/16 (1,332 to 6,339) and the lowest on 3/1/16 (30 to 141). Location 3 showed the highest spore to VO range (688 to 3,274), and location 11 showed the lowest (113 to 539).

Table 15: Microbial burden of Spacecraft Assembly Facility floors by date.

Sampling Date	Viable microbes[a] (RLU/m^2) [B1]	Viable microbes[a] (16s/m^2) [B2]	Cultivable[a] (CFU/m^2) [C]	Cultivable Spores[a] (CFU/m^2) [D]	Cultivable ATP %[b] [C/B1] x 100	Cultivable qPCR %[b] [C/B2] x 100	Spore %[b] [D/C] x 100	Spore to VO[c,d] (ATP) [B1/D]	Spore to VO[c,e] (qPCR) [B2/D]
3/1/16	5.7×10^4	1.3×10^4	6.9×10^1	6.4×10^1	0.7	0.5	55.8	708	206
3/15/16	6.3×10^4	2.1×10^4	4.8×10^2	3.0×10^1	1.3	4.9	22.9	1,923	718
3/30/16	1.3×10^5	7.4×10^4	3.0×10^2	2.4×10^1	0.6	2.4	17.1	5,261	3,036
4/12/16	9.4×10^3	1.1×10^5	9.6×10^2	3.1×10^1	11.4	1.1	6.9	306	3,484
5/17/16	5.7×10^3	5.1×10^4	6.9×10^2	8.3×10^1	14.5	2.3	15.0	68	618
6/1/16	1.0×10^4	2.9×10^4	1.1×10^3	4.2×10^1	11.7	8.6	18.2	241	683
6/14/16	4.3×10^3	2.2×10^4	2.2×10^2	2.0×10^1	7.2	1.7	21.5	211	1,071
6/28/16	6.3×10^3	1.2×10^5	1.9×10^2	1.7×10^1	3.5	0.3	14.6	326	7,043
7/12/16	8.0×10^3	7.8×10^4	2.7×10^2	7.2×10^1	7.2	0.7	40.6	110	1,080
7/26/16	1.5×10^3	2.7×10^5	1.0×10^2	2.9×10^1	9.2	0.04	35.5	52	9,217
8/15/16	3.6×10^3	1.0×10^5	4.4×10^2	2.8×10^1	19.4	0.5	6.2	158	3,866
Average[f]	2.8×10^4	5.3×10^6	4.4×10^2	3.6×10^1	7.7	2.2	22.9	750	2,121

[a] Values calculated by taking the average of given assay values on individual sampling dates. See Section 4 for detailed explanations of individual assays.

[b] Percentage calculated using average of all samples calculated percentage values on a given sampling date. Individual samples that were BDL were not included in the calculations.

[c] Ratio calculated for a given date by taking the sum of a given viable assay divided by the sum of cultivable spore of all relevant samples. If an individual sample from a date had a viability assay measurement of BDL, it along with the corresponding cultivable spore, were not included in the calculation.

[d] Average represents a 1 RLU = 1 CFU assumption.

[e] Average represents a 1 16S rRNA copy = 1 CFU assumption.

[f] Values are the average of all individual samples from the given dates. Spore to VO ratios were calculated by taking the sum of all samples from viable assays and dividing by the sum of cultivable spore of all relevant samples. If an individual sample from a location had a viability assay measurement of BDL, it along with the corresponding cultivable spore, were not included in the calculation.

Table 16: Microbial burden of Spacecraft Assembly Facility floors by location.

Sample Location	Viable microbes[a] (RLU/m^2) [B1]	Viable microbes[a] (Copies/m^2) [B2]	Cultivable[a] (CFU/m^2) [C]	Cultivable Spores[a] (CFU/m^2) [D]	Cultivable ATP %[b] [C/B1] x 100	Cultivable qPCR %[b] [C/B2] x 100	Spore %[b] [D/C] x 100	Spore to VO[c,d] (ATP) [B1/D]	Spore to VO[c,e] (qPCR) [B2/D]
1	2.1×10^4	2.9×10^4	1.2×10^3	3.5×10^1	14.8	6.4	11.1	491	825
2	2.5×10^4	8.5×10^4	3.8×10^2	3.5×10^1	2.4	4.1	33.0	714	2,462
3	1.4×10^4	1.5×10^5	3.5×10^2	3.2×10^1	2.6	1.0	27.5	552	4,761
4	4.3×10^3	9.3×10^4	5.8×10^2	2.9×10^1	14.6	0.9	13.3	148	3,150
5	2.4×10^4	7.5×10^4	5.2×10^2	2.8×10^1	3.0	2.5	5.9	765	2,667
6	8.5×10^3	3.4×10^4	1.8×10^2	1.8×10^1	1.4	1.9	21.5	663	1,863
7	9.9×10^3	9.9×10^4	5.9×10^2	4.6×10^1	13.2	2.2	25.3	188	2,142
8	2.3×10^4	7.2×10^4	3.7×10^2	2.9×10^1	7.0	1.2	13.9	778	2,468
9	4.4×10^4	6.0×10^4	4.0×10^2	2.3×10^1	4.1	0.7	15.5	1,931	2,665
10	1.7×10^4	8.5×10^4	3.7×10^2	4.6×10^1	9.7	2.5	21.0	376	1,369
11	9.3×10^3	7.5×10^4	2.1×10^2	3.5×10^1	10.1	1.2	33.7	263	784
12	8.2×10^4	6.9×10^4	2.0×10^2	7.2×10^1	6.0	1.0	46.1	1,053	961
13	5.8×10^4	9.1×10^4	4.0×10^2	2.3×10^1	5.0	3.1	17.6	2,504	3,947
Average[f]	2.8×10^4	5.3×10^6	4.4×10^2	3.6×10^1	7.7	2.2	22.9	750	2,121

[a] Values calculated by taking the average of given assay values on individual sampling dates. See Section 4 for detailed explanations of individual assays.
[b] Percentage calculated using average of all samples calculated percentage values on a given sampling date. Individual samples that were BDL, were not included in the calculations.
[c] Ratio calculated for a given date by taking the sum of a given viable assay divided by the sum of cultivable spore of all relevant samples. If an individual sample from a date had a viability assay measurement of BDL, it along with the corresponding cultivable spore, were not included in the calculation.
[d] Average represents a 1 RLU = 1 CFU assumption.
[e] Average represents a 1 16S rRNA copy = 1 CFU assumption.
[f] Values are the average of all individual samples from the given dates. Spore to VO ratios were calculated by taking the sum of all samples from viable assays and dividing by the sum of cultivable spore of all relevant samples. If an individual sample from a location had a viability assay measurement of BDL, it along with the corresponding cultivable spore, were not included in the calculation.

6.8.3 FACS-Based Spore to VO Ratio

The FACS-based spore to VO ratio is seen in Figure 24 (Graphs E and F). The ratio was determined by using the sum of FACS-based viable counts and dividing by the sum of the applicable spore CFU/m^2 values. When the raw FACS viable counts are used, the ratio is 12,091. Of the three dates observed, there were significant differences ($p < 0.05$) between 7/12/16 and samples collected from 6/1/16 and 6/14/16. There were no significant differences by location.

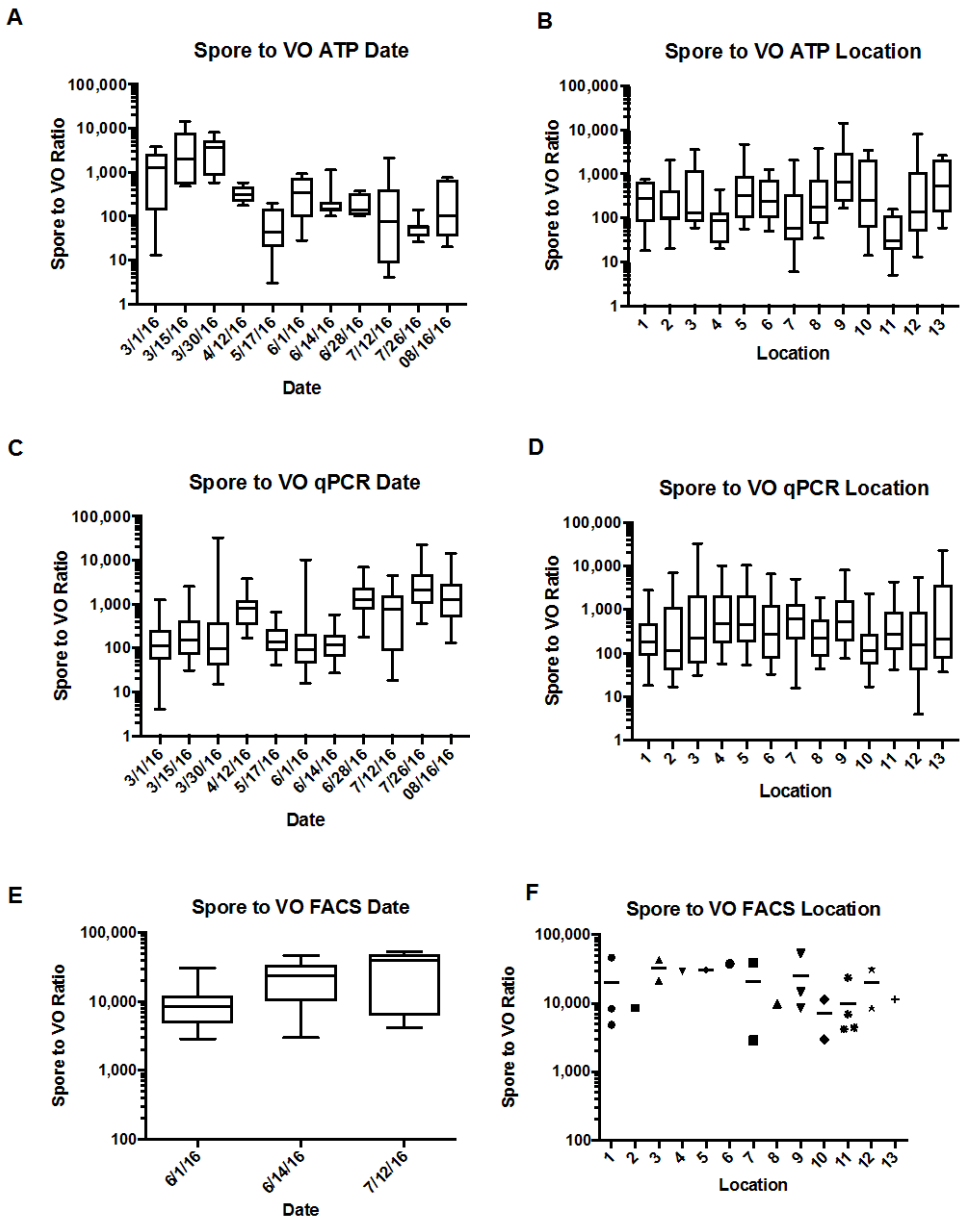

Figure 23: Spore to viable organism ratio as determined by Internal ATP (A and B), bacterial 16S rRNA gene PMA-qPCR (C and D), and FACS (E and F) by date and location in SAF.
The spore to VO ratio was calculated by dividing the sum of assay-specific values/m^2 by the sum of spore/m^2 of the same samples. For ATP spore to VO ratios, two numbers were included for each sample to account for the equal abundance of Gram positive (5 RLU = 1 CFU) and Gram negative cells (1 RLU = 1 CFU) (18). This ATP spore to VO range was calculated by sample date (A) and sample collection location (B). For PMA-qPCR based spore to VO ratio, average 16s rRNA copies per cell used (4.19 copies +/– a standard deviation of 2.73 gene copies) (18). The PMA-qPCR based spore to VO ratio is shown by date (C) and location (D). The FACS-based spore to VO ratio is shown by date (E) and location (F). Each box plot shows the minimum and maximum values (whiskers) and first quartile, third quartile, and median (box).

6.9 Statistical Analysis

All statistical analyses were performed by Prism 7. Prism 7 was used to perform One-Way ANOVA (and Nonparametric) analysis with Tukey multiple comparisons. All statistical tests that had a $P = <0.05$ were considered significant.

6.10 Controls and Lower Detection Limits of Assays Employed

Appropriate environmental controls were included at all stages of sample collection, processing, and downstream analysis. Negative controls, handling controls (sampling wipes briefly exposed to the ambient sampling environment, and Maxwell DNA extraction controls were prepared and analyzed by all methods.

Polyester wipes that were exposed to the sampling environment, but not used for active sampling, were placed (using sterile gloves) directly into sterile rinse solution and processed in blind fashion as handling (negative) controls in all molecular assays. Purified DNA from *B. pumilus* ATCC 7061 was included in all PCR amplification protocols as a positive control. In the same manner, ultraclean, molecular-grade sterile water served as a blank (i.e., negative control) to monitor reagent cleanliness.

7 Conclusion

This section summarizes the key conclusions and recommendations resulting from the spore to VO task. Pertinent research challenges and lessons learned are discussed.

7.1 Spore to VO Conclusion and Summary

The spore counts measured during this study were consistent with those obtained from previous studies (1, 23) (Table 17). Notably, average spore counts were observed across various locations in the SAF within the same order of magnitude (~10^1 CFU/m^2). However, when compared between individual samples, differences in spore counts were in the range of 2% to 99% variation from the average. This would suggest that spore populations fluctuate nominally at various locations spatially, and further confirmed that there are temporal variations potentially related to assembly activities and human traffic. More metadata are necessary to measure the influence of human activities on the incidence of spore counts. The routine cleaning and maintenance procedures followed in the JPL-SAF are effective at reducing the spore burden but not in eradicating them. In contrast, the average cultivable bacterial burden showed variability of up to two orders of magnitude between each sample collection date. Spatially, the cultivable populations were much more consistent. However, location 1, the site that sees the most foot traffic, had an average cultivable bioburden approximately double that of any other individual location. Moreover, cultivable fungi were detected in similar low quantities as that of spores between sampling dates 3/1/16 and 6/1/16, but were not recovered from 6/14/16 onward. Spatially, fungi were consistently distributed in low quantities in all 13 locations.

In addition to the cultivation-based assays, a variety of molecular methods were utilized to assess microbial burden. Compared to other non-systematic studies of SAF, ATP and qPCR values obtained in our study had a slightly larger range of VO (1, 24, 25, 49) (Table 17). This could be the result of our larger sample size, which would potentially increase the likelihood of finding a microbial rich sampling location that was not detected by the prior studies.

Table 17: Microbial burden of Spacecraft Assembly Facility surfaces.

Assay (Units)	This Study	Previous Studies
Cultivable (CFU / m^2)	1.2×10^1 – 6.6×10^3	BDL – 5.4×10^5 [1, 23]
Spores (CFU / m^2)	BDL – 3.6×10^2	BDL – 4.0×10^3 [1, 23]
Total ATP (RLU / m^2)	BDL – 4.2×10^6	BDL – 4.5×10^6 [1, 23, 24]
Internal ATP (RLU / m^2)	BDL – 7.2×10^5	1.4×10^3 – 4.8×10^4 [1, 24]
Total qPCR (copies / m^2)	1.1×10^4 – 9.7×10^7	BDL – 3.3×10^7 [1, 24, 25]
PMA qPCR (copies / m^2)	3.8×10^3 – 6.5×10^5	6.2×10^3 – 4.9×10^4 [24, 25]
FACS (CFU / m^2)	8.7×10^4 – 1.7×10^6	—

The spatial and temporal estimation of internal ATP-based VO values were consistently lower than PMA-qPCR based VO values. These low results can be the result of inherent lower metabolic activity demonstrated by microbes in the cleanroom environment, along with the reduced metabolic activity caused by the floor cleaning reagents used during weekly cleaning in the SAF. Furthermore, it has been previously suggested that the shorter half-life of ATP, compared with DNA, could also be responsible for lower ATP values (24). ATP could be underestimating VO in low biomass conditions compared with qPCR methods that utilize a more resilient and metabolic independent molecule, DNA.

This was the first time that the third molecular assay, FACS, was used in SAF to assess viable burden thus rendering to generate VO estimates. After these samples that were sorted using FACS were further analyzed and sequenced (data not shown), only 20% of the cells were amplified and capable of being assigned to taxonomic affiliations. Since the WGS was performed in order to generate enough material to sequence for the taxonomic identity, other factors, such as too low of a DNA concentration or DNA extraction procedures that are not compatible with cells from hardy populations, might explain the FACS-based taxonomic identity. However, FACS studies previously conducted by others exhibited similar percentage outcomes as was observed during this study (50-53). Since the FACS methods are proven to sort only viable cells, in order to calculate the ratio of spore to VO, counts of all sorted viable cells, irrespective of their identification, were taken into account.

Although SAF microbial communities have been shown to be dominated by bacteria, it is important to include estimates of the fungal and archaea populations (45). The fungal population was estimated in this study by fungal qPCR (Table 5) and cultivation on PDA. These estimations showed that the fungal populations in SAF were ~ 2% of the microbial community, which were consistent with other studies (46, 47). Archaeal populations, although not analyzed directly in this study, have previously been shown to exist in extremely low quantities (BDL-1%) in spacecraft cleanrooms (46). Even though it is not reported here, the microbiome and metagenomics data (not shown) revealed no archaeal genetic signatures. Although the combined fungal and archaeal communities have a small presence in the SAF, it is important to understand the entire viable microbial population in order to assess contamination for the spacecraft.

Three viability assays were used to establish a ratio between spore counts and VO to compare with the SSB estimation (1 spore to 50,000 VO) (3). As demonstrated in Section 6.8, internal ATP and PMA-qPCR provided the lowest spore to VO estimates with the ATP-based estimate of

1 spore to 149 – 746 VO and the qPCR-based estimation of 1 spore being equal to 314 – 1,491 VO. The third viability assay used in this study, FACS, provided the most conservative spore to VO estimation of 1 spore being equivalent to 12,091 VO. Based on the empirical data generated during this study and the desire to utilize the most conservative method, it is recommended that the FACS-based ratio of 1 spore being equal to 12,091 VO be used as a replacement of the SSB estimate. The original SSB estimate was created based on generalizations and assumptions observed in various environments, while the conservative FACS-based ratio from this study provides an empirically backed value that should be used for samples collected from Mars 2020 and associated SAF surfaces.

It is also important to consider that this conservative ratio is representative of the SAF floors at JPL and could potentially be different in other spacecraft cleanrooms, and on spacecraft surfaces. In fact, microbial burden on cleanroom floors has previously been shown to be typically two orders of magnitude greater than spacecraft surfaces (2). This could potentially be answered with additional studies in those environments, and in fact, a future study (at the Kennedy Space Center [KSC] Payload Processing Facility [PPF]) is planned to establish the ratio in a spacecraft cleanroom that will house Mars 2020 in the months leading up to its launch. Future work should also consider additional FACs samples to increase the sample size for analysis and minimize the variance seen in the results presented in this report.

In addition to the results presented here, future results will present the iTag-based microbiome and multigene-based metagenomics to further explore the microbial burden and diversity of the SAF environment.

7.2 Lessons Learned

1. SAF and other relevant, low-biomass cleanrooms create restrictions that do not allow every microbial assay to be utilized. It is important to verify that new tools and their limits of detection do not interfere with the results in these cleanroom environments.
2. In the spore to VO task, microscopy and FACS were explored as useful tools to measure various microbial populations (total, viable, etc.). Microscopy proved to be difficult to quantify microbial cells because of debris present in samples and the incompatibility of current procedures and processing of low-biomass cleanroom samples. FACS, on the other hand, was able to provide useful results, but continued modifications would be recommended to more finely tune the process to handle low-biomass samples and achieve more positive taxonomic identification of the FACS-sorted cells.
3. An adequate amount of time should be spent in the (a) design and logistics of experimental procedures and (b) interpretation and all-inclusive analyses involved in converting raw data from various assays into comparable measurements. This will allow a time-efficient and comprehensive understanding of the microbial populations. Without the proper handling of data, investigations can rapidly become too data-rich and analysis-poor without proper planning and logistics.

7.3 Future Considerations

1. The results from this study are representative of the SAF floors at JPL. To better understand the spore and viable populations associated with spacecraft, a future study on applicable spacecraft surfaces should be considered. Additionally, the Mars 2020 mission

will spend a significant period of time prior to launch at KSC, so a relevant study in that environment would help create an overall picture of the spore and VO that the spacecraft will experience during ATLO.

2. While early phase mission data is important to gather engineering judgement and project biological performance data actual data is recommended to be collected during ATLO to assess the actual spore to VO ratio overserved during the hardware life cycle. While this may not be done to such an extensive monthly basis a spot check or portions of the spore to VO study should be implemented to assess whether results are within anticipated engineering judgement predictions. If they are not it may require more extensive testing and analysis.
3. It is also important to consider that this ratio is representative of the SAF floors at JPL and could potentially be different in other spacecraft cleanrooms, and on spacecraft surfaces. In fact, microbial burden on clean room floors is two orders of magnitude greater than spacecraft surfaces (2). This could potentially be answered with additional studies in those environments.
4. Future investigators are encouraged to consider emerging methodologies that can enumerate VO.
5. The scope of future spacecraft-associated biodiversity assessments should consider whether attention should also be given to fungi and archaea. Future investigators could continue to expand the limits of resolvable biodiversity across the three domains of life, to achieve an even more comprehensive understanding of the various microbial populations in spacecraft and their associated cleanrooms.
6. Future investigators are encouraged to monitor metadata and apply biostatistics and bioinformatics approaches, in order to detect potential changes in data (i.e., over time or as a consequence of another external factor such as a reagent batch change), in order to preserve the consistency and validity of the collected data set.

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9 Acronyms

ATLO	=	assembly, test, and launch operations
ATP	=	adenosine Triphosphate
BDL	=	below detection limit
BiSKit	=	biological sampling kit
bp	=	base pair
CFDA	=	carboxyfluorescein diacetate succinimidyl ester dye
CFU	=	colony forming units
COSPAR	=	Committee on Space Research of the International Council for Science
CTC	=	5-cyano-2,3-ditolyl tetrazolium chloride dye
$DiBAC_4(3)$	=	bis-(1,3-dibutylbarbituric acide) trimethine oxonol
$DiOC_2(3)$	=	3,3' –diethyloxacarbocyanine iodide
DNA	=	deoxyribose nucleic acid
EDL	=	entry, descent, and landing
ESA	=	European Space Agency
FACS	=	fluorescent-assisted cell sorting
FDA	=	fluorescein diacetate dye
FE-SEM	=	field emission scanning electron microscope
GI	=	Genetic Inventory
glyTE	=	glycerol
HFPS	=	hollow fiber polysulfone
HGT	=	horizontal gene transfer
ISO	=	International Organization for Standardization (a set of cleanliness codes to quantify particulate contamination levels per milliliter of given sample)
ITS	=	Internal Transcribed Spacer
JPL	=	Jet Propulsion Laboratory
KSC	=	Kennedy Space Center
KSC PHSF	=	Kennedy Space Center Payload Hazardous Servicing Facility
KSC PPF	=	Kennedy Space Center Payload Processing Facility
LBNL	=	Lawrence Berkeley National Laboratory
LoCoS	=	low coverage sequencing
MDx-16	=	Maxwell-16 automated DNA extraction system
MMC	=	model microbial community
MPO	=	Mars Program Office
MSL	=	Mars Science Laboratory
NASA	=	National Aeronautics and Space Administration
NHS	=	non-heat shock
NSA	=	NASA standard assay
PBS	=	phosphate buffered saline
PCA	=	principal component analysis
PCoA	=	principal coordinates analysis
PCR	=	polymerase chain reaction
PDA	=	potato dextrose agar
PES	=	polyethersulfone
PI	=	propidium iodide

PMA	=	propidium monoazide
QC	=	quality control
qPCR	=	quantitative polymerase chain reaction
rDNA	=	ribosomal DNA
RDP	=	Ribosomal Database Project
RH	=	relative humidity
RLU	=	relative luminescent unit
RNA	=	ribonucleic acid
rRNA	=	small subunit ribosomal ribonucleic acid
RSG	=	RedoxSensor Green
SAC	=	spacecraft assemble cleanroom
SAF	=	Spacecraft Assembly Facility
SAFR-032	=	*Bacillus pumilus* strain isolated from the Spacecraft Assembly Facility
SAG	=	single amplified genome
SCWGA	=	single-cell whole genome analysis
SSB	=	Space Studies Board
SSU	=	small subunit
SYTO-9	=	SYTO-9 green fluorescent nucleic acid stain
TE	=	Tris EDTA
TO	=	total organisms
TSA	=	tryptic soy agar
UCSD	=	University of California, San Diego
UV	=	ultraviolet
VO	=	Viable Organisms
WGA	=	Whole Genome Amplification

10 Glossary of Terms

16S rRNA gene
The 16S ribosomal RNA (16S rRNA) is a component of the 30S small subunit of prokaryotic ribosomes, and is commonly used in phylogenetic studies as it is highly conserved between different species of bacteria and archaea.

454 tag-encoded pyrosequencing
A method of molecular analysis that provides an in-depth evaluation of the microbial diversity present in samples by examining the highly informative hypervariable regions on the 16S ribosomal RNA gene. 454 refers to 454 Life Science, the company that developed this technology.

Actinobacteria
A dominant group of Gram-positive bacteria having high guanine and cytosine content in their DNA. These bacteria can be terrestrial or aquatic, and typically have hardy cell walls that help them persist in harsh environments.

biodiversity
The degree of variation of life forms within a given species, ecosystem, or microbiome.

biological sampling kit (BiSKit)
A macrofoam-based sampling device, which is protectively encased in a sterile plastic unit meant to minimize potential risk of sampling device contamination. In the Genetic Inventory experiment, it serves as one of two materials used for sample collection of large surface areas (i.e., stainless-steel sheets).

bioinformatics
A branch of biological science that deals with the study of methods for storing, retrieving and analyzing biological data, such as biochemical pathways, genetic interactions, and nucleic acid (DNA/RNA) and protein sequence, structure, and function.

biostatistics
The application of statistics for designing biological experiments, summarization, and analysis of data from those experiments and the interpretation of these results.

cloning and Sanger-sequencing
A somewhat conventional approach to elucidating sample microbial diversity via clone libraries and subsequent DNA sequencing. Cloning refers to the shuttling of PCR-amplified ribosomal RNA genes from noncultivable microorganisms into genetically amenable lab strains of *E. coli* via plasmid vectors. Sanger was the scientist that devised the regimen of DNA sequencing used in this approach, wherein 4 reservoirs each containing a different radiolabeled dNTP are used in concert to render a DNA sequence read of approximately 600-bp.

Committee on Space Research (COSPAR)
The COSPAR was established by the International Council for Science in 1958. Among

COSPAR's objectives are the promotion of scientific research in space on an international level, with emphasis on the free exchange of results, information, and opinions, and providing a forum, open to all scientists, for the discussion of problems that may affect space research. These objectives are achieved through the organization of symposia, publication, and other means. COSPAR has created a number of research programs on different topics, a few in cooperation with other scientific unions.

cotton swab
A sampling device consisting of an organic, cellulose-rich (>95%) material that is wrapped around one end of a wooden stick. In the Genetic Inventory task, cotton swabs served as one of two materials used to collect samples from small surface areas (i.e., stainless-steel coupons).

field control (FC)
An experimental negative control in which a sampling wipe exposed to the ambient sampling environment (without any contact with the surface of interest).

genetic inventory
An extensive census, a.k.a. "passenger list," of microorganisms associated with a given sample, achieved by numerous surveys implementing systematic sample collection, processing, analysis, and cataloging of rRNA gene sequences.

Greengenes
A web application developed by Lawrence Berkeley National Laboratory (LBNL) that enables access to comprehensive 16S rRNA gene sequence alignment tools.

low-biomass
A characteristic of a sample that contains very few living or dead microorganisms (i.e., bacteria, archaea, and fungi).

macrofoam
A sponge material provided as part of the BiSKit. In the Genetic Inventory experiment, the macrofoam sponge is used to sample large surface areas such as 2500-cm^2 stainless-steel sheets.

model microbial community (MMC)
A mixed microbial assemblage of known phylogenetic composition and cellular/endospore density. In the Genetic Inventory experiment, MMC is synthesized to serve as the positive control.

negative control
A group in a scientific experiment in which the results are known to be negative; used to isolate any variables from the experiment results.

polyester wipe
A sampling device of continuous tightly interwoven long polyester fibers . In the Genetic Inventory experiment, it serves as one of two materials used for sample collection of large surface areas (i.e., stainless-steel sheets).

polymerase chain reaction
An enzyme-catalyzed molecular reaction used to amplify DNA molecules. This reaction transforms unusable, insignificant numbers of DNA molecules into highly robust and overly abundant template DNA concentrations, which are required for many downstream molecular analyses.

spacecraft assembly cleanroom (SAC)
A cleanroom is an environment, typically used in manufacturing (in this case, spacecraft assembly) or scientific research, that has a low level of environmental pollutants such as dust, airborne microbes, aerosol particles, and chemical vapors. More accurately, a cleanroom has a controlled level of contamination that is specified by the number of particles per cubic meter at a specified particle size. To give perspective, the ambient air in a typical class 100k cleanroom environment contains 3,520,000 particles per cubic meter in the size range 0.5 μm and larger in diameter, corresponding to an ISO 8 cleanroom.

Spacecraft Assembly Facility (SAF)
A spacecraft assembly cleanroom situated at JPL, Pasadena, CA.

species evenness
A quantitative measure of the extent of equality (i.e., evenness) in species abundance among samples.

species richness
The number of distinct species present in a sample set.

standard assay
The NASA standard assay (NSA) method, designed to assess planetary protection risk for spacecraft bound for Mars, is culture-based. Technicians collect samples, allow the organisms in the sample to grow for 3 days, and then count resulting colonies. The standard assay technique is designed to enable a count of organisms that are capable of forming endospores, respire aerobically, are resistant to heat processing, and are able to be cultivated on tryptic soy agar media. This technique does not address the issue of microbial diversity of spacecraft.

taxonomy "calling"
Applying a given set of taxonomic criteria to assign taxonomic affiliations to observed DNA sequences.

UniFrac tool
A software suite used to perform environmental clustering and principal coordinates analysis. Phylogenetic information is used to statistically compare microbial community diversity between samples.

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