The Evolution of Planetary Protection Implementation on Mars Landed Missions

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***Abstract*—** NASA has developed requirements dedicated to the prevention of forward and backward contamination during space exploration. Historically, international agreements provided guidelines to prevent contamination of the Moon and other celestial bodies, as well as the Earth (e.g., sample return missions). The UN Outer Space Treaty was established in 1967 and the Committee on Space Research (COSPAR) maintains a planetary protection policy complying with Article IX of this treaty. By avoiding forward contamination, the integrity of scientific exploration is preserved. Planetary Protection mission requirements are levied on missions to control contamination. These requirements are dependent on the science of the mission and on the celestial bodies encountered or targeted along the way. Consequently, categories are assigned to missions, and specific implementation plans are developed to meet the planetary protection requirements. NASA missions have evolved over time with increasingly more demanding scientific objectives and more complex flight systems to achieve those objectives and, thus, planetary protection methods and processes used for implementation have become much more intricate, complicated, and challenging. Here, we will portray the evolution of planetary protection implementation at JPL in several important areas throughout the course of NASA sponsored robotic Mars lander or rover missions, starting from Mars Pathfinder through the beginning of Mars 2020. Highlighted in the discussion will be process changes in planetary protection requirements development and flow down. Development and implementation of new and improved methods used in the reduction of spacecraft bioburden will be discussed as well as approaches and challenges that come along with setting up remote laboratories to perform bioassays. The consequences and forward planning of delays on missions will be highlighted as well as lessons learned on the impact of communication and training in achieving planetary protection requirements. The evolution of methods used for the detection of microbial bioburden on spacecraft hardware will be considered. These methods use standard microbiology as well as the adaptation of advances in biotechnology, molecular biology, and bioinformatics. Technical approaches developed for the prevention of contamination and recontamination of hardware during Assembly, Test, and Launch Operations will be discussed.

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1. INTRODUCTION

The question of whether or not there is life on other planetary bodies other than the Earth has sparked human curiosity for centuries. Humans have always been the curious wanderer – and wonderer. The question of life existing in the solar system other than on our planet has been an ancient topic for philosophers going back to the 4th century BC, if not even earlier. During the

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Renaissance, when Galileo made significant discoveries and confirmed planets orbit the Sun, the question of extraterrestrial life became directly relevant to astronomy.

As human exploration spread to the "New World" starting in the 15th century, a race to map and dominate the world was begun, contributing to the interaction and transfer of organisms from one land mass to another. Although this was not intentional, the consequences of forward contamination usually had catastrophic consequences on the local ecosystem, which had existed untouched up to this point. As an example, entire populations of endogenous natives were almost lost in the Americas as an outcome of not having an immune system resistant to smallpox, which was brought along with explorers from Europe. Not only were there dire consequences such as these, similar interactions occurred with back contamination.

Planetary protection is NASA's policy of addressing the consequences, which might occur when material is actually transferred between different planets – if life *does* exist on other worlds. Not only does planetary protection want to protect solar system bodies (planets, moons, asteroids, and comets) from Earth life, it also intends to protect the Earth from extraterrestrial life, which, if it exists, could be brought back from other solar system bodies. Here, we wish to describe the historical development of the practice and philosophy of planetary protection as it relates to Mars missions.

2. BEGINNINGS OF PLANETARY PROTECTION

In the 1950's, a new frontier was added to those of land and sea exploration: space. The burning "need to know" was (and is) real, and was instrumental in the launch of the Space Age to determine if we are alone in the universe, i.e., the existence of life on other planets.

Although life exists in different extreme environments on Earth, there is no data whatsoever on alternative chemistries that may support life – not as we know life to be, but of an entirely exotic, unrelated form – elsewhere in the Universe. To determine these alternative chemistries, extraterrestrial environments – and any living organisms that exist within them – must be protected until the space community has the opportunity and technology to identify them. Today's planetary protection policy is dominated by the principle of safeguarding scientific investigations and instrumentation… "It's strictly to protect science. Planets for the sake of science [1]."

As early as 1956, there were rumblings of concern regarding possible lunar and planetary contamination as the race for space exploration was just beginning. This led to an attempt to coordinate international efforts so that such things did not happen – particularly since there were two launches that surprised most Americans and instigated the U.S. Space Program: the successful launches of Sputnik I and of Sputnik II towards the end of 1957 by the Soviet Space Program. These dual successful launches heralded the beginnings of a new era in technological achievement, which had serious consequences for the balance in the Cold War. In response, the United States Congress – in addition to the military establishment and the American public itself – saw the urgent need for scientific and technological advancements to match the Soviets. The conquest of space became a new Cold War imperative for the U.S., bringing with it concerns about planetary protection.

Instrumental in voicing these alarming concerns were a handful of scientists – pioneers – who were key visionaries of the importance of practicing what we now identify as planetary protection. Stanford geneticist and Nobel Laureate Joshua Lederberg, University of California at Berkeley Chemist and Nobel Laureate Melvin Calvin, and British biologist J. B. S. Haldane were all essential in identifying the ease by which space exploration, could in theory, contaminate the environments which were being explored. At the time, there was the fear that living organisms from Earth could grow and spread in a new environment, thereby affecting the characteristics and ecologies of any existing life forms which might be natural habitants of the satellite under question. Lederberg's concerns and warnings to the scientific community at large were eventually recognized by the U.S. National Academy of Sciences (NAS), which took the first action of adopting resolutions paving the way to international and national treaties and agreements [2].

3. INTERNATIONAL TREATIES AND AGREEMENTS

In 1958, the NAS called for the International Council of Scientific Unions (ICSU) to develop a process where spacecraft would not jeopardize or compromise the integrity of any scientific investigation [3]. As a result, ICSU established an ad hoc Committee on Contamination by Extraterrestrial Exploration (CETEX) primarily to provide findings on vulnerabilities to contamination. CETEX recommended precautions be taken against space vehicles landing on Mars or Venus (accidentally or deliberately) without first sterilizing them. CETEX reasoned that if precautions were not taken, then future explorations would have devastating effects on any type of life form – if they existed. Consequently, it was apparent that a code needed to be drafted to protect future interplanetary exploration as well as the protection of extraterrestrial environments. The ICSU gathered international experts to address these issues [4], leading to the establishment of the Committee on Space Research, COSPAR [5].

During this same time, Lederberg's efforts contributed to the establishment of the Space Science Board (SSB) in June of 1958 [6]. Not only was the NAS instrumental in establishing the SSB, it also directed the SSB to work in

collaboration with civilian and Government agencies, in particular, the newly established National Aeronautics and Space Administration (NASA), the National Science Foundation (NSF), the Advanced Research Projects Agency (ARPA), and foreign partners in this field. The establishment of NASA was a direct outcome of the recent political unease, which had arisen from the launch of Sputnik in 1957. Within just a few months, COSPAR was in the middle of encouraging joint international collaboration and information exchange in interplanetary endeavors. COSPAR had to deal with fundamental space research, and explore and understand biological aspects of interplanetary exploration, including spacecraft sterilization and planetary quarantine. COSPAR was to serve as an arena for open dialog between space scientists from the Eastern bloc and those from the rest of the world – mostly from the United States. During the Cold War, COSPAR selected vice-presidents from the USSR and from the US, assuring both countries of equal representation. COSPAR actively enforced that both of these countries examine approaches, which would avoid transfer of terrestrial organisms to other planets. This was the first step in implementing a program to protect planets of biological interest so that life detection experiments would not be disqualified by contamination, leading to irreversible and permanent changes. In essence all spacefaring nations had to share details for spacecraft engineering and fabrication. Planetary protection could only be achieved with full cooperation of all countries participating in space exploration [7].

Similar to COSPAR, the United Nations General Assembly established another ad hoc committee in reaction to the launch of Sputnik and for the prevention of contamination of celestial environments. In 1959, the Committee on the Peaceful Uses of Outer Space (UNCOUPUOS) was asked to identify and report on legal issues arising from human exploration of space, including forward and back contamination dangers. This led to the recommendation of the development of international agreements and guidelines – similar to COSPAR. Due to these efforts, President Lyndon Johnson proposed the basis of such a treaty in July 1966. The *Outer Space Treaty* was issued in January 1967. This international agreement provided articles supporting space exploration, but specifically claimed that all exploration should be conducted to preserve the environments being explored, particularly in Article IX [8].

Joshua Lederberg and Carl Sagan's lobbying efforts were very effective in identifying methods to prevent forward and back contamination. Consequently, the first anti-contamination panel was formed in 1963, under COSPAR's jurisdiction. Lederberg's stature for being a Nobel Laureate helped make a strong case for implementing rigorous planetary protection measures. NASA's first spacecraft sterilization policy statements are all attributed to Lederberg's concerns and lobbying. Along with NASA's Director of Space Flight Programs, Abe Silverstein's efforts, the first sterilization policies and approaches were officially implemented in November 1959 [9].

In the early 1960's, a special Life Science Program was established within NASA, which included spacecraft sterilization and exobiology programs within its mandate. The Jet Propulsion Laboratory (JPL) led the effort for constructing a facility to identify fundamental issues in decontaminating and sterilizing space probes [10]. JPL's role in the implementation of planetary protection practices began at that time, and was focused on two major issues: to determine which spacecraft components were compatible to sterilization treatments, and to develop new spacecraft components or materials which were more compatible to sterilization treatments, because testing had proven that not all components were compatible. Adding to this level of complexity was the matter of mission categorization, which was defined in essence, by the science the mission would carry out.

4. MISSION IMPLEMENTATION

Mission planetary protection requirements are defined by the NASA Procedural Requirements (NPR) 8010.12, PP Categorization Letter from the NASA Planetary Protection Officer (PPO), and the NASA Level 1 requirements.

The requirements from NPR 8020.12 detail the general mission requirements for target body and mission type, monitoring and verification requirements, gate product documentation and review schedules, detailed documentation and review requirements, microbial reduction requirements, launch and post launch operations, and roles and responsibilities. Process requirements are also detailed in NPR 8020.12 in the Appendices to include communications with the PPO for each mission phase and process specification sheets for bioburden assumptions and microbial reduction processes.

As part of the NASA PP process, the mission is required to submit a request for a PP categorization. This request is to include a matured mission plan, description of the primary mission, science objectives, and payload investigations to include particular detection sensitivities of biochemical molecules. From a PP stance, the desired landing site capabilities should also be discussed to include (or exclude) a Mars Special Region (SR) as defined by the Mars Exploration Program Analysis Group (MEPAG-SR) Report. The categorization should include the projects' best knowledge of the project's likely PP categorization and build upon that assertion. Detailed analysis may be required for the mission specific engineering or science needs (e.g. Radioisotope Power System (RPS) assessment for creation of a spacecraft induced special region in an off-nominal landing or contamination / recontamination analysis). The PP Officer (PPO) will then evaluate the mission categorization request letter and issue a mission categorization letter

detailing the mission categorization formally assigned, reiterate the relevant NPR 8020.12 requirements, and notify the project of any mission specific requirements.

NASA HQ can directly impose Level 1 requirements specifically related to the mission in the Program Level Requirements Appendix. These requirements are brokered between the Project and NASA Program Management and are a direct path into the Project's System Engineering requirements.

Thus, PP requirements come to a mission in some combination of the NPR 8020.12, PP categorization from the PPO, and the NASA Level 1 requirements. In general, the mission type defines the PP implementation approach for Mars-based missions. These requirements then flow into the Project System Engineering requirements system and the PP approach is defined in the Project PP Plan. If relevant, the detailed implementation approaches to the probability of impact analyses, bioburden assessment, unique project analysis, and the closed loop verification process is described in the PP Implementation Plan and subsidiary Plans (if relevant).

5. MISSION COMPLEXITY: PP IMPLEMENTATION PLANS AND CHALLENGES

An Introduction to PP Implementation – NASA Robotic Mars Missions at JPL

Planetary protection implementation requirements have continued to evolve from one mission to the next as knowledge of the red planet and other celestial bodies of biological interest continues to expand based on what is learned in preceding projects. In general, PP requirements become more restrictive as more is learned about life in extreme environments and new ways to detect it.

Mars Pathfinder (MPF)—In the 22 years between the launch of Viking in 1975 and the launch of Mars Pathfinder late in 1996, there were no NASA lander missions. Hence, when the development of MPF began, the formal NASA planetary protection requirements for Mars were as they were for Viking [11]. There was, however significant activity to modify these requirements in the intervening period. The findings of Viking biology experiments and the measurements by other instruments suggested that spacecraft sterility was an excessively rigorous requirement. In 1978, the Space Science Board (SSB) of the National Research Council[1] issued a sharply reduced (less stringent) set of values for the key parameter in the analytical approach to the estimation of the probability of contaminating Mars, the probability of growth P_g [12]. The SSB was particularly reluctant to set a value for P_g, especially for the surface at subpolar latitudes, because terrestrial organisms could not grow there except in oases. No oases (early version of "special regions") were detected by orbiters, and the evidence indicated that none could exist.

An alternative approach was developed in the 1980s [13], [14]. The previous method employed the Coleman-Sagan formula in a form apparently never formally published, which allowed an estimation of the probability of contamination P_c of Mars versus a requirement (10^{-4} for Viking):

$$P_c = N_0 R P_s P_I P_R P_g \quad (1)$$

Where N_0 = the number of microorganisms on the spacecraft initially, R = reduction due to conditions on the spacecraft before and after launch (include Dry Heat Microbial Reduction, DHMR), P_S = Probability the microorganisms on the spacecraft reach the surface of the planet, P_I = Probability that the spacecraft will impact the planet (one for a lander), P_R = Probability of microorganisms being released in the environment after landing (usually set to one for all bioburden for crash-landing, specification values for surface, mated, and encapsulated otherwise), and Pg = Probability of growth (for targets with liquid water typically set to one).

This calculation was performed for each of the exposed, mated and encapsulated components of the total burden, which had unique values for the probability of release.

The new requirements for Mars were placed on the burden N_0 itself (no calculations required). The logic was to invert the formula into a formula for the allowed N_0. More importantly, the requirements for landers without in situ life detection experiments were set to the burden values for the Viking landers prior to terminal heat sterilization. For landers with life detection experiments, the requirements were set to the best estimate for the Viking landers post the terminal sterilization (not zero). This general approach was accepted and recommended by the SSB in 1992 [15]. Further work [16] provided the specification values for the allowed (maximum) burden, $3x10^5$ spores on exposed and mated surfaces, and $5x10^5$ spores total (all surfaces, mated and encapsulated). The two values reflect the thought behind the probability of release. These values were adopted for use by MPF through its PP documentation, which refers to a final draft form of the new NASA Planetary Protection Requirements document [17], [18]. Also, the average bioburden on the exposed surfaces of the landed system was not to exceed 300 spores/m^2.

MPF satisfied the burden requirements by selected dry heat microbial reduction (DHMR) with the use of the existing specifications. There was no system level sterilization. The central electronics module was exempted for the burden requirement by isolation behind a high-efficiency particulate arresting (HEPA) filter, a unique approach first done for this project. The spore count was estimated per the NASA standard assay [19] as

[1] Now the Space Studies Board (SSB)

used for Viking, except only heat-shocked samples were assayed, to represent spores. The requirements were silent on statistics; MPF used estimated standard deviations and 3-sigma upper limit in counts applied against burden requirement. This conservative measure was intended to account for the sampling. A major departure from the burden requirements applying to the values at launch was approved through the MPF PP plan document. The external surfaces of the aeroshell and the entirety of the cruise stage were shown sterilized by atmospheric heating during entry at Mars. This precedent was employed in later missions as well. More details on the microbiological cleanliness of MPF are available in the public domain [20].

Following the developments in the 1980s and early 1990s, changes were made to other requirements, such as for probability of impact P_I of Mars (that also apply to orbiters and flybys) [17], [18]. A new structure to the requirements for all solar system bodies was also established (but beyond the subject of this paper). The specification values for the launch vehicle and the spacecraft P_I were made less stringent during the MPF PP activities. The value for the launch vehicle (or any part of it) was 10^{-5} at time the PP plan was written, but became 10^{-4} for PP prelaunch report. The P_I (for a hard impact) by the lander was 10^{-3}.

The method for the estimation of P_I for a spacecraft during the interplanetary cruise phase was improved from the previous practice by the introduction of system reliability and meteoroid kill parameters. The formula for P_I

$$P_I = \sum_{i=1}^{n} p_i \, q_{i+1} \qquad (2)$$

where p_i is the probability that the i^{th} maneuver leaves the spacecraft in an impact trajectory and q_{i+1} is the probability that the next maneuver (after the i^{th} maneuver) would fail. Prior to MPF, the value of all q_{i+1} were taken to be 0.01, except for i=n, the last maneuver, for which the value was one, of course. Of course, the probability of a system failure or a meteoroid impact depends on the duration between maneuvers. Values for these two parameters were obtained by a reliability analysis of Mars Global Surveyor (a Mars orbiter) and a study of one spacecraft and some historical data [11]. This analysis set the precedent for later projects. The values were never adopted as NASA specifications, but their use was explicitly permitted by way of project PP plans. Of course, project specific analyses are preferred, but costly.

Mars Exploration Rover (MER)—The principal advancement in the PP implementation of the Mars Exploration Rover mission (MER) was the development of a spreadsheet of all of the parts of each MER spacecraft system, the PP Equipment List (PPEL). This EXCEL workbook provided a method for tracking the PP status and estimated or measured value of the spores on each component and to feed the rolled up estimates at the subsystem level. The PPEL contained surface areas, volumes, materials, and cleaning/microbial reduction processes, organized based on the project's mass equipment list to facilitate updates as the flight system matured. This methodical approach was necessitated by the vastly increased complexity and size of the MER spacecraft, compared to MPF. The MER PP team developed another new tool to support bioburden estimation: the Barcode system. The Barcode system was an Access database containing all data relative to bioassay sampling from descriptions and pictures of each sample taken through the 24, 48, and 72-hr plate counts for each sample.

Note that the total maximum number of spore forming organisms, and the population density per square meter requirements were unchanged from MPF. In addition, there were two complete spacecraft to account for. The spreadsheet was based on the analogous Mass Equipment List (MEL), used to track and estimate the mass of all components and the system mass. In addition, MER greatly extended the use of HEPA filter isolation first employed with MPF, for many electronic modules.

The MPF Project did not consider PP issues/requirements until the critical design phase of spacecraft development. The MER Project chose to actively include PP in design considerations beginning in the Pre-Project phase and continuing through launch. The PP requirements for MER were the same as for MPF with one exception: the probability of impact by the flight system requirement was 10^{-2} rather than 10^{-3}. The probability of impact requirements were met using the same methodology as for MPF.

The MER Project had the added complexity of two essentially identical flight systems, each consisting of approximately 4.5 x 10^3 m^2 of accountable surface area. The biological cleanliness requirements were met using a combination of dry heat microbial reduction (DHMR), alcohol-wipe cleaning, bioassay sampling, and HEPA filter isolation. DHMR was used on components with large surface area (e.g., airbags, parachute, multi-layer insulation), and those with surfaces that were difficult to clean or not cleanable (e.g., open-cell foam, motor interior surfaces, aluminum honeycomb). HEPA filter isolation was used on the main rover chassis and smaller electronics modules on the rover and lander. Alcohol-wipe cleaning was used on easy-to-clean surfaces prior to integration and to maintain cleanliness during integration and after system-level testing. Bioassay sampling was performed periodically to assess cleanliness protocols, and at last available access during integration. The bioassay sampling goal was to sample, at last available access, 10% of those surfaces accountable by bioassay. The MER PP team chose to include the results of the NASA Planetary Protection Officer's (PPO) verification samples in the final flight system bioburden estimate.

The MER PP team developed two new tools to support bioburden estimation: the Planetary Protection Equipment List (PPEL) and the Barcode system. The PPEL was an Excel workbook containing surface areas, volumes, materials, and cleaning/microbial reduction processes; organized based on the project's mass equipment list to facilitate updates as the flight system design matured. The Barcode system was an Access database containing all data relative to bioassay sampling from descriptions and pictures of each sample taken through the 24, 48, and 72-hr plate counts for each sample.

The MER Project took credit for entry heating of the cruise stage and heat shield as was done by MER and Mars Polar Lander (MPL); however, entry heating of the backshell was not expected to raise the backshell (BS) external surfaces to sterilization temperatures. The MER PP team then had to consider all backshell recontamination risks and the bioburden encapsulated in the backshell thermal protection system (TPS). Backshell recontamination risks consisted of cruise stage (CS) surfaces, the heat shield (HS) outboard surface, launch vehicle (LV) fairing, payload attachment fitting (PAF), upper stage, and fairing air conditioning. To limit recontamination of the backshell, the PP team imposed a cleanliness requirement of a maximum surface spore density of 1000 spores/m^2 on all of these surfaces, necessitating cleaning and bioassay sampling of these surfaces. The fairing air conditioning duct was also sampled, and HEPA filters were required in the air conditioning flow. Bioburden in the backshell TPS was reduced by DHMR of the backshell and the TPS.

The following table provides the details of the various sources of bioburden accountability.

Table 1. Details of Source of Bioburden

Flight System Zone	Fraction of accountable area represented by:		Fraction of worst case estimated surface spore burden represented by completed assays
	Completed Assays	DHMR	
Rover	28.4%	61.8%	46.0%
Lander	3.6%	93.8%	24.8%
HS	5.3%	93.1%	1.5%
Parachute & BS	6.0%	92.5%	59.4%
CS	8.2%	89.4%	16.4%

Phoenix—Phoenix PP implementation was essentially similar to MER, with one major difference: Phoenix was targeted to sample ice at the polar cap. The spore requirements for the robotic sampling arm and associated instruments were more stringent than otherwise. In order to allow the rest of the spacecraft to comply with the less rigorous constraints (i.e., like MER), a deployable biobarrier was designed to isolate the robotic arm. The biobarrier was deployed after landing. This was the first instance of a split system requirement and a possible precursor to future Mars sampling projects.

Mars Science Laboratory (MSL)—The Mars Science Laboratory mission (MSL) had the same spore constraints as MER did, while the entire flight system and the rover grew even larger in size and complexity than MER. Considering evolving requirements from the joint work of NASA and ESA, getting a fully functioning flight system to meet PP requirements became more difficult than ever.

PP requirements for a mission formally begin with a request for categorization from the Planetary Protection Officer, the PPO. For MSL there were additional requirements: landing sites had to be addressed as well as the ability to access special regions on Mars. MSL was designated a Category IVb mission. After the landing site was selected and changes to some of the hardware, it was ultimately launched as a Category IVa mission.

Biological cleaning or microbial reduction of the hardware was accomplished through the use of several techniques including mechanical cleaning using precision cleaning, alcohol wiping, as well as dry heat microbial reduction. All flight hardware was cleaned and bioassayed prior to entering the Assembly, Test and Launch Operations (ATLO) flow. For MSL, almost 90% of all surfaces saw standard or non-standard Dry Heat Microbial Reduction (DHMR). During ATLO as hardware inaccessibility was achieved, bioburden levels were assessed by sampling surfaces, and if values were above limits, hardware was subjected to re-cleaning and re-sampling until acceptable values were obtained. MSL was the first project for which the Adenosine Tri-Phosphate (ATP) assay was utilized as a cleanliness predictor, prior to the closeout of critical hardware. (See Section 8, subparagraph 3, ATP and LAL Assays).

Prior to final encapsulation, sets of samples were taken and provided to the PPO for microbial bioburden estimation by an external team of microbiologists, by which an independent verification of the spore levels would be provided. Ultimately the final access sample data was used to determine the final spore counts. In addition to the flight hardware sampling, all supportive GSE and facilities were assessed for microbial bioburden during the assembly flow, to ensure they also remained biologically clean and within specification.

Prior to any environmental testing on the spacecraft, bioassays are collected before and after such tests to ensure that no gross contamination occurs. These tests simulate extreme conditions the spacecraft can encounter during its journey in the vacuum of space to the satellite of interest. MSL underwent exposures to simulate space in a solar/thermal vacuum chamber and acoustic testing in

large chambers or rooms; these environments did not meet the standard cleanroom requirements. To verify that MSL hardware was not contaminated during these testing activities, the spacecraft was bioassayed to establish a baseline prior to being moved. Each environmental testing facility was thoroughly cleaned, bioassayed and evaluated for particle counts. Witness plates were used during the testing for both biological and particulate contamination. After the test was completed the facility was resampled, and pre- and post-testing results were compared. Even the backfill process of the chamber took into consideration the fill rate, and by keeping the fill rate very slow, the potential of contamination was kept at a minimum.

MSL used accounting methods to obtain volumetric spore specification values, in much the same way MER did. A PPEL was used to account for all of the parts by name on the spacecraft. This included all of the electric components as well as other large area and volume components. The large number of electronic components totaled a significant volume to be included in the total bioburden calculations. Utilizing the temperatures the components were exposed to during manufacture, DHMR, burn in and testing, and verifying by part number which were manufactured in a cleanroom, allowed the Project to significantly reduce the specification bioburden. Using the thermal curing process needed by various components – such as the propulsion system carbon fiber wrapping, adhesives and the internal volumes of the heat shield and back shell TPS material – allowed additional large spore specification values to be significantly reduced.

Additionally, embedded bioburden in areas and volumes such as electric components, carbon fiber wrapping on propulsion tanks, paints, adhesives, liquids and gasses had accepted conservative spore specification values since it was not possible to sample the flight materials. The large size of the MSL hardware would have made it extremely difficult to meet the maximum number of spore forming organisms. With the approval of the PPO, a series of studies was performed by the Project to determine what the actual spore levels were in the flight materials. These studies were performed on hydrazine liquid and vapor, carbon fiber, thermal paint, adhesives, insulation, Freon and helium. The studies were performed using materials from the exact batches used on the flight hardware. Results demonstrated that the materials used had spore levels vastly lower that the specification numbers.

In comparison to the Viking and Pathfinder missions, MSL had the largest number of bio-samples ever taken on a U.S. spacecraft to date. A total of 3472 swab and 1283 wipe samples were taken to determine the spacecraft microbial burden. The large size of the flight hardware allowed more wipe samples to be taken than on previous missions, which resulted in a larger surface area sampled.

In addition to the risk involved in contaminating the spacecraft during environmental testing, there were also other sources, which could contribute to contamination. Not only were facilities evaluated for cleanliness levels, any facility participating in ATLO activities were also candidates for study. These included facilities at assembly locations, and at the launch sites – be they at KSC or elsewhere. Trailblazer activities were performed to identify the microbial bioburden including launch pads and cleanrooms. As with MER, unique ducting specifically manufactured for the Payload Launch Fairing were also tested and verified. Additional testing and verification of the cleanliness of the portable HEPA filtered transport vehicle and the transfer ducting system used in raising the encapsulated spacecraft to the top of the rocket were also carried out. These activities were conducted to demonstrate that the spacecraft would not be re-contaminated from the time it left the clean assembly facility through arrival at the pad and launch.

However, the interior surface area of the payload launch faring and isolation diaphragm were additional potential sources of spacecraft contamination post-encapsulation and during launch. A study was conducted at KSC early in the project life for MSL when an identical faring was being processed. This work demonstrated that the interior could be easily cleaned and decontaminated to acceptable spore levels of less than the required 1000 spores/ m^2. The surface area was over 400 m^2, thus this would still be a large number of spores. With diligent effort by the technicians working on the flight unit, the interior of the actual flight faring was cleaned to a level of 4 spores/m^2.

Biobarrier isolation of large and small volumes using HEPA filters was used in several parts of the spacecraft, as with Phoenix. This allowed pressure venting while preventing movement of spores isolated in internal volumes.

All the effort performed by the MSL project to meet the PP requirements resulted in having a flight system, which was given launch approval by the PPO. The final total of spore forming organisms on the spacecraft was 278,000 out of the less than 500,000 spore requirement. The actual spore density/m^2 was 22, well below the 300 spore/m^2 requirement.

InSight—The requirements and PP procedures for the Discovery mission InSight (Interior Exploration using Seismic Investigations, Geodesy and Heat Transport) followed many of the methods of the previously stated projects. A major advancement was the adoption of system engineering. InSight was the first mission to instill the use of systems engineering processes and practices for planetary protection. While the mission has yet to be launched, thus far, it has been deemed an overwhelming success in terms of the utilization of systems engineering within the domain of planetary protection, clearly showing that it does work. (See Section 6, below.)

Mars 2020—In addition to the PP categorization requirement process, as mentioned above, there are biologically-relevant contamination issues that may be associated with an in-situ payload's detection level of biological molecules of interest, or would be applicable to a restricted Earth-return mission. The Mars 2020 mission has several payloads in which in-situ payload biological contamination could possibly impact the detection threshold and thus could be considered both a PP and science-based requirement. NASA has established the in-situ payload biological contamination requirements for Mars 2020, a Project Science based sample integrity requirement. NASA HQ and the Mars Exploration Program have defined a Return Sample Science Board as defined that are imposing additional science based microbial contamination assessment requirements such as identifying, quantifying, documenting, and archiving potential pre-launch terrestrial contamination sources. Hence, for the specific Mars 2020 mission there are additional requirements that are imposed based on the evolving science investigations and based on the needs of samples that could potentially be returned.

The approach taken by the Mars 2020 project to ensure that both planetary protection and return sample science requirements are met go above and beyond the measures that were taken for MSL, which in and of itself was an unprecedented effort. The implementation path taken for MSL relied heavily on microbial reduction and biological assays to verify cleanliness compliance against the NPR 8020.12D bioburden levels. As M2020 has evolved to collecting samples for potential return from Mars, an enhanced approach must be taken on the contamination sensitive hardware. There is a three-tiered approach to the M2020 PP implementation, which was driven by the addition of the sampling and caching system: 1) PP solutions inherent to the engineering design, 2) a more stringent controlled build environment with a cleanliness monitoring plan, and 3) allocating witness coupons/hardware for process verification. The most impactful implementation approach to ensure that the hardware is clean and protected from recontamination is through the engineering design. Elements such as the fluid mechanical particle barrier (FMPB) restricts the flow of particles to the contamination sensitive hardware and protects recontamination from occurring. Covers and late integration of hardware also prevent critical pieces of the sampling and caching system hardware from recontamination.

Thus, the biological cleanliness requirements for the Mars 2020 hardware are motivated both by preventing forward contamination as well as science integrity should the sample be returned to Earth.

Furthermore, the addition of a genetic inventory has also evolved from a study during MSL to a project-supported formal effort. This enhancement is necessary to identify and document the potential contaminants on the journey to Mars and will serve as a point of comparison should a future mission discover signs of life.

6. SYSTEMS ENGINEERING

In recent years there has been a dramatic paradigm shift in the way planetary protection has been implemented at JPL. On previous missions, including MSL, planetary protection requirements were often only documented in planetary protection plans. Hardware cognizant engineers had to rely on reading this documentation and communication and oversight of the planetary protection team to make sure the requirements were met on the mission. As missions became progressively more complex over time, it became very difficult for the planetary protection team to ensure proper coverage to all of the hardware engineers particularly given resource constraints to ensure requirement compliance. In the more extreme example of MSL, this lack of formal process for requirement flow down ultimately in part caused the mission to be re-categorized from a IVc mission to a IVa mission right before launch. Fortunately for MSL, this did not impact the science investigations planned for the mission. After MSL's launch, a thorough review of lessons learned revealed that the project would have benefit from a more rigorous process for planetary protection requirement flow down and verification and validation (V&V) efforts for requirement close-out and compliance. Thus, a paradigm shift emerged in JPL planetary protection to implement a formal systems engineering process within the domain.

The shift towards utilization of systems engineering practices and processes within planetary protection is advantageous since planetary protection is a cross-cutting domain in that planetary protection requirements affect the entire flight system and launch vehicle. Given this, it is appropriate to place the planetary protection team directly under the oversight of the project systems engineer. This allows for planetary protection to be "visible" across the entire flight system. As per the formal systems engineering process, planetary protection requirements are also involved in a rigorous top down process in which the top level requirements given to a mission by NASA at the agency level are flowed down all the way to the subsystem level for which the hardware engineers are responsible for ensuring full compliance. The requirements are written in a fashion that a verification and validation activities can be planned for each individual requirement in which evidence can be provided to show compliance with the requirement, which is helpful in showing progress and compliance during planetary protection audits and reviews with the NASA Planetary Protection Officer. Planetary protection requirements are not only contained in planetary protection documentation but also are now maintained and tracked in the mission's formal requirements tracking tool similar to all the other mission requirements which allows for personnel to track the requirements they are

responsible for and enable linking of plans for verification and validation activities for each requirement to be described as well as linkages to the evidence that show the requirements have been closed out. The requirements are under formal change control and any requirement changes must undergo a Change Control Board review in which the case for the change must be presented and the appropriate authorities must approve before any changes can be made to the requirements.

Throughout the formal requirement flow down process on a mission, the planetary protection team works with each hardware engineer to understand which requirements are applicable to that piece of hardware. These conversations help the hardware engineers understand the planetary protection requirements they will ultimately be responsible for meeting. In addition, these conversations allow for development planetary protection implementation plans for that hardware. Critical within this negotiation process is determining what hardware may need special attention because they are not amenable to standard planetary protection processes for whatever reason. Knowing this information allows for an end to end implementation strategy to be developed for each individual piece of hardware which will meet planetary protection requirements and generate evidence that the requirements were met.

The Discovery mission InSight was the first mission to instill the use of systems engineering processes and practices for planetary protection. While the mission has yet to be launched, thus far, it has been deemed an overwhelming success in terms of the utilization of systems engineering within the domain of planetary protection, clearly showing that it does work. Some lessons learned have already been noted from InSight and these items have already started to be incorporated in the next mission, Mars 2020. Given that the mission is much more complex with a mixture of heritage and new hardware, bigger overall flight system and team, and stricter requirements for planetary protection and contamination control because of the collection of samples for possible future return to Earth, it is evident that utilization of systems engineering processes and practices within the domain of planetary protection will be critical to getting the job done.

7. BIOBURDEN REDUCTION – PATH TO PP IMPLEMENTATION

DHMR

Reducing the microbial bioburden on spacecraft hardware is a primary planetary protection objective. Although complete testing and evaluation of all spacecraft hardware is not practical, when microbes embedded inside of polymers or between mated surfaces cannot be accessed, PP heat reduction procedures are employed.

The most important remediation measure in reducing the bioburden is the application of heat. Meeting the overall bioburden requirements for spacecraft hardware cannot be achieved without subjecting structures, parts and instruments to dry heat microbial reduction. Heat and radiation are the only two forms of microbial reduction that can penetrate to the center of hardware. Chemicals, plasmas, ultraviolet light and gaseous methods can only reduce microbes on surfaces. The trade-off for many microbial reduction techniques is to reduce the microbial population without damaging the hardware or materials. As an example for electronic systems the microbes are about as resistant as the circuits, therefore exposure time and temperatures must be adjusted to protect the hardware.

Since the earliest days of microbiology, it has been known that bacterial spores are the most difficult organisms to kill by any method. Thus, the bacterial spore has been the benchmark for methods of reducing microbial populations. By eliminating the presence of viable bacterial spores, which are difficult to kill, other less robust cells are also eliminated. The current assessment of microbial bioburden of the spacecraft is based upon detecting bacterial spores.

While wet saturated heat is the most effective means of killing microbes and reducing the bioburden, steam is detrimental to most flight hardware. Oxidation, rusting, mineral deposition and electrical short-circuiting are the principle drawbacks of steam-based microbial reduction. Spacecraft parts and materials can tolerate moderate heat if the atmosphere is dry. Investigations into the efficacy of dry heat revealed that bacterial spores were least susceptible to killing if there was a moderate quantity of moisture, but spores were most effectively killed by either very wet or very dry humidity conditions. This is the reason for controlling humidity. Humidity can be controlled by flowing a dry inert gas such as nitrogen or by reducing the moisture content under vacuum conditions. The historic specification requires controlling relative humidity to less than 25% at 0°C at 760 torr.

NASA scientists were faced with many obstacles earlier in the Space Age. Most importantly, based on early concerns for the prevention of cross contamination between satellites, spacecraft sterilization was very challenging and proved to be very difficult to implement effectively. Some spacecraft materials were not compatible with high temperatures. Studies had to identify components, which did withstand extreme exposures, and parameters needed to be established for the inactivation of microbial organisms. Extension for the parameters to allow processes for ambient humidity without a normal atmosphere (a regular oven) or for temperatures differing from a prescribed set of temperatures was the result of years of research investigations into the microbial inactivation of spores. In addition to decades of research for microbial inactivation, the exceedingly extraordinary heat resistance of spores from some species described as “hardies” was identified

as an important factor to be considered. The potential presence of these difficult to kill spores limited the "accounting credit" that may be assigned to dry heat processes. Since the frequency of occurrence is not known (it is assumed that 1 in 1000 are super-tolerant to heat), and the understanding that the frequency may vary by location, season or activities, it is prudent to account for these variables.

Years of research proved that there were temperatures and durations, which did not have damaging effects on hardware, but were effective at reducing bioburden. This data suggested that 125°C and dry-heat exposures times of 24 hours was sufficient for NASA's needs at the time. Earlier attempts at using gaseous ethylene oxide as a sterilization process on spacecraft did not fare very well. Multiple mission failures were directly blamed on this method, and the use of this gas fell out of favoritism. A direct consequence of these failures was the development of a very lenient attitude towards the use of sterilization procedures – particularly at a time when attitudes towards the U.S. space program were changing. Consequently, JPL changed its sterilization process by initially, not performing any dry-heat sterilization on the components, and then eventually dropping terminal gaseous surface sterilization completely from the plan in 1963. The policy now, was based on the NASA's Management Manual NMI-4-4-1, *NASA Unmanned Spacecraft Decontamination Policy*, who's primary objective was to prevent contamination until further notice. Although this policy lifted the requirement for sterilization requirements for the Moon, it did not lift the requirements for planetary missions, since they were still considered potentially contaminable. In the mid 1960's, COSPAR provided recommendations resulting in Resolution 26.5, which developed at planetary protection standard, stating "a sterilization level such that the probability of a single viable organism aboard any spacecraft intended for planetary landing or atmospheric penetration would be less than $1 \times 10^{-4,}$ and a probability limit for accidental planetary impact by unsterilized flyby or orbiting spacecraft of 3×10^{-5} or less…during the interval terminating at the end of the initial period of planetary exploration by landing vehicles." In addition to these recommendations and subsequent resolutions and guidelines, NASA was also in the midst of analyzing potential contamination sources for Mars missions – including examining possible sources of contamination, to carrying out mathematical models and mechanisms of the survivability of microorganisms during the sterilization processes under study. The space science community was most concerned with those microorganisms, which were the most heat-resistant, and would be a typical contaminate of space hardware. Such a microorganism could be used as a standard to measure the effectiveness of sterilization approaches; *Bacillus subtilis* variety niger (or *Bacillus globiggi*, or BG) became the standard test organism mentioned in the NASA Handbook NHB 8020.12. Years of research were dedicated to this; thermal sterilization procedures and techniques were evaluated, D-values (kill rates) were determined, new sterilization technologies were evaluated and methods of contamination were identified – all leading to an understanding that there is a level of complexity which grows continuously, or from one mission to the other.

The mission perspective had to be noted as well. NASA, and in particular JPL, began a selection and development process which helped identify more than 40,000 of the best parts and materials available for spacecraft development at the time. Solder, conformal coatings, electronic parts, epoxy resins – all needed to be able to withstand sterilization or have their microbial bioburden reduced during manufacturing activities. The use of cleanrooms, the control of contamination, cleaning subassemblies on a regular basis – these, and other useful steps improve the microbial bioburden reduction processes during manufacturing.

The Viking mission saw a terminal sterilization procedure, which took place after it was delivered to the Kennedy Space Center, Florida. These were performed at KSC's Spacecraft Assembly and Encapsulation Building. Exposure parameters were determined by the bioburden results obtained from the last assay done on the lander just prior to encapsulation into the bioshield. Based on NASA-funded research, the parameters which would result with the most lethality were determined to be at 111.7°C for 30 hours after the coldest point reached that specific temperature. NASA had established specifications ranging from 104°C to 125°C for sterilization.

The Planetary Protection specifications for dry heat microbial reduction (DHMR) called for exposures of hardware to elevated temperature, vacuum (at least $1x10^{-4}$ Torr), and extended durations to reduce microbial bioburden ideally by 4-orders of magnitude. These specifications are summarized in the table below.

Table 2. Dry Heat Microbial Reduction (old Specifications)

Surface Bakeout		Encapsulated Bakeout	
T°C	Time (Hrs)	T°C	Time (Hrs)
110	50	110	250
125	5	125	25

However, the specifications were updated due to isolation and identification of heat resistance of "hardy" bacteria from spacecraft cleanroom assembly facilities, as mentioned previously. The parameters for the DHMR process had to be extended as indicated in the following two tables.

Table 3. Dry Heat Microbial Reduction Parameters for Surface Bakeouts

Temperature (^{o}C)	Time (Hrs)		
	3-log	4-log	6-log
112	15.60	132.18	-
114	12.53	124.07	-
126	3.36	85.98	257.95
155	0.19	4.76	14.29

Table 4. Dry Heat Microbial Reduction Parameters for Encapsulated Bakeouts

Temperature (^{o}C)	Time (Hrs)		
	3-log	4-log	6-log
112	-	660.89	-
114	-	623.33	-
126	16.8	429.91	1289.74
155	0.93	23.83	71.45

With longer durations and higher temperature exposures, the "Dry" variable of DHMR has been eliminated upon usage of these new specifications. These extended conditions – new specifications – have eliminated the need for vacuum overall.

Precision Cleaning

Precision cleaning of materials for spacecraft and support equipment are normally carried out in (Class 100 and Class 10000) clean rooms. Although the cleaning procedure is generally tailored for individual hardware, the process includes a selected combination of the following steps: ultrasonic cleaning in alkaline detergent, rinsing in water, ultrasonic vapor degrease in Vertrel XF, ultrasonic cleaning with selected organic solvents (iso-propyl alcohol, acetone, or dichloromethane), rinsing with fresh and filtered iso-propyl alcohol (IPA), drying process with hot filtered gaseous Nitrogen (GN_2), and sealed in anti-static bags. Upon requirement, the particle count from final IPA rinse is used to determine the cleanliness of the hardware.

Alcohol Cleaning

The use of wipes soaked in alcohol has been routinely used for cleaning surfaces of spacecraft components. Conceptually, the practice involves saturating a cleanroom wipe with 100% IPA and wiping the hardware surface – all while donned in cleanroom garments and with gloves taped to the sleeve of the garment. The wipes are swept across the surface in one uni-directional motion, then folded in half and swept across at a 90^{o} turn of the wipe and swept across the surface in a uni-direction motion, again. Afterwards, the wipe is folded one last time, and the same motion repeated. Although the cleaning action of this process is due mainly to the IPA's solvent properties, the mechanical action that is performed in affect, increases the probability or likelihood of lifting off contamination from a surface.

VHP Microbial Reduction

The DHMR process, used to sterilize earlier lander and spacecraft components, is an approved and efficient process used for microbial reduction. However, with the utilization of highly sophisticated electronics and sensors in modern spacecraft, this process presents significant materials challenges. Another technique utilizing lower temperatures is needed to augment DHMR and provide a viable alternative. Several techniques including ethylene oxide gas, ultraviolet radiation, paraformaldehyde, and chlorine dioxide have been considered. Whereas these methods had material compatibility issues, in addition to material hazards, the use of vapor hydrogen peroxide (VHP) presented promising results.

Hydrogen peroxide has been used successfully as a sterilant in the medical industry with no discernable consequences. In the typical medical application, an excessive process is preformed to ensure sterility. However, for spaceflight hardware, with potential material and system compatibility issues, knowledge of the minimum adequate process is vital. Studies at JPL determined the minimum VHP process conditions to achieve microbial reduction levels acceptable for planetary protection, with the goal to include this technique in NPR 8020.12 as a low temperature complementary technique to the dry heat sterilization process. Optimal VHP process parameters – including hydrogen peroxide concentration, number of pulses, exposure duration, and numerous environmental parameters - were determined. The derivation of D-values permitted conservative recommendations for a planetary protection specification. The recommended VHP process specification was validated with naturally occurring organisms. The approved specifications for NASA and ESA planetary protection are as follows (ECSS-Q-ST-70-56C-DFR1):

Controlled Vacuum—The procedure for controlled vacuum environment shall be used for a 2 to 6 order of magnitude bioburden reduction. The hydrogen peroxide vapour concentration for surface bioburden reduction under controlled vacuum conditions shall be from 0.5 mg/L to 1.1 mg/L. D-value for surface bioburden reduction under controlled vacuum conditions shall be 200 (mg/L)sec.

Controlled Ambient—The procedure for controlled ambient environment shall be used for a 2 to 6 order of magnitude bioburden reduction. The hydrogen peroxide vapour concentration for surface bioburden reduction under controlled ambient conditions shall be ≥ 1.1 mg/L. D-value for surface bioburden reduction under controlled ambient conditions shall be 200 (mg/L)sec.

Controlled Bioburden Overkill—The procedure for overkill shall be used under controlled vacuum conditions. The hydrogen peroxide vapour concentration for surface bioburden overkill under controlled vacuum conditions shall be from 6 mg/L to 8.6 mg/L. Ct-value for surface bioburden overkill under controlled vacuum conditions shall be ≥ 14000 (mg/L)sec.

VHP compatibility with more than 100 spacecraft materials was evaluated at the Jet Propulsion Laboratory. The VHP material compatibility exposure condition was based on a six-log reduction as the treatment requirement for hardware. Considering a rework and qualification scenario, each material treatment was repeated four times. For the majority of the materials tested, the VHP sterilization process had little or no impact on the materials' properties. Although some materials showed changes more than 15%, these might still be acceptable for a particular engineering application. However, the VHP effect should be addressed during engineering design.

Unconventional Cleaning methods

Instruments for recent Mars missions are capable of detecting organic molecules up to parts per billion (ppb). To ensure the integrity of science instruments and their measurements, stringent cleanliness requirements must be achieved and maintained. There are urgent needs to improve current spacecraft hardware cleaning technologies.

Cleaning and sterilization are distinctly different operations. Sterilization is the process to kill live microbes, while cleaning is a process that physically removes live and dead microbes and debris from hardware surfaces. The most commonly used current spacecraft hardware cleaning methods are precision cleaning and alcohol wiping. While these methods are efficient for cleaning massive contamination, they are not effective for removing micron and submicron sized microbes and debris from hardware surfaces. NASA planetary protection regulations state that a surface may be considered "sterile" if a microbial burden of less than 300 aerobic bacterial spores per meter^2 can be treated to achieve a 10^4 fold reduction in viable endospores (spores). The following three methods have been evaluated and have achieved a four-log reduction on viable spore counts. However, the latter two of these methods have not been universally approved by the PPO, and depending on the science objectives of future missions, projects may ask for acceptance of use.

The Liquid Boundary Layer Disruption System—This process manipulates vapor pressure of cleaning solution to initiate bubble formation or cavitation on the surface of spore inoculated coupons. Formation and collapse of the bubbles at nucleation sites (surface imperfections or spores) is intended to disrupt the boundary layer on the material surface and dislodge particles. The process continues until the desired level of cleanliness is achieved. The method completely cleaned all the coupons and parts that were inoculated with a deposition of 1.0 x 10^5 spores. Thus a log reduction of over 4 orders of magnitude was achieved with a 99.9% confidence level.

CO_2 Jet Composite Cleaning—This method is distinct from other commercial CO_2 snow spray technologies: it delivers both solid crystal CO_2 and CO_2 gas to the surface under question. The CO_2 crystals can be made into different sizes and can be released at various speeds to generate enough kinetic energy to shear particulate contaminants (including spores and their remnants) off sample surfaces. When the CO_2 ice pellets bounce off the surfaces, they become liquid and gas. The shockwave from the expansion due to the phase transformation further enhances the shearing power. By adjusting CO_2 crystal size, spray speed, distance and angle of incidence and cleaning duration, targeted bioburden reduction can be achieved on coupons if cleaned with the CO_2 composite spray.

Laser induced plasma shockwave cleaning (LSC)—In this method of cleaning, a laser is focused above the surface of a substrate to be cleaned by a lens. The focused laser creates a localized plasma which, in turn, creates a shockwave front. This shockwave front interacts across a localized portion of the surface, which then by momentum transfer, dislodges particles. The LSC method has shown to be effective to remove submicron particles. Particles, as small as 40 nm, can be removed with the LSC method. Using a Quanta-Ray pulsed Nd:YAG laser at a wavelength of 1064 nm, up to six-log reduction of spore counts have been achieved. This method also has the potential to be further developed as an *in situ* cleaning method for sample return missions.

8. BIOBURDEN LIMITS AND DETECTION METHODS

Throughout the course of the history of planetary protection and the attempts to create a baseline method to detect and assess microbial bioburden on Mars-bound missions, the space community has seen the development of methods primarily for the detection and enumeration of heterotrophic, mesophilic, and aerobic microorganisms. Procedures for detection of other classes of microorganisms have also been developed, as mission specific needs have ev. Below, is a brief description of the most extensively used assessment method that has withstood the test of time: the NASA Standard Assay, in addition to a few molecular-based detection methods which have been introduced with the advancement of molecular technologies.

NASA Standard Assay

Missions having bioburden constraints require that microbial bioburden levels be monitored and documented with approved methods. Approved procedures for the

microbiological assay of spacecraft hardware and their associated environments are provided in the NASA HDBK 6022, "NASA Standard Procedures for the Microbiological Examination of Space Hardware." The long standing NASA Standard Assay has evolved through time and now uses sonication to dislodge particles from the sample and heat shock to eliminate vegetative bacteria and selectively choses bacterial endospores as an indicator of microbial bioburden. It is used to assess microbial contamination on spacecraft during mission ATLO activities. This method uses both the swab method to determine microbial bioburden, and also the wipe method to assess the same. Sterile swabs are used to sample small surfaces, 25cm^2, and sterile cleanroom polyester wipes (23 cm x 23 cm) sample surfaces, which are generally closer to 1m^2 in surface area. The entire assay takes over 72 hours to complete. Both sampling methods use sterile water for moistening purposes.

Rapid Spore Assay

Time is a precious resource in an ATLO environment. The development of a rapid endospore assay is an important contribution to the field of planetary protection. A Rapid Microbiology Detection System (RMDS) developed by Millipore, was modified for detecting endospores, and thus permitted the system to be used for planetary protection applications, detecting and counting viable microorganisms using membrane filtration. The standard RMDS was modified for detecting endospores, and changes included heat shock treatment to eliminate non-spores, background reduction techniques, and modifications of the bioluminescence reagent mix. The technique combines membrane filtration, adenosine triphosphate (ATP) bioluminescence reagents, and image detection and analysis based upon photon detection. A procedure was developed to compare the Rapid spore assay (RSA) to the NASA standard assay (NSA). The RSA proved to be a quick and highly sensitive technique, which reduced the bacterial endospore assay time from over 72 hours to less than 8. Both methods show equivalent sensitivity and are able to detect one Colony Forming Unit, CFU.

ATP and LAL Assays

Both the Adenosine Tri-Phosphate (ATP) and Limulus Amoebocyte Lysate (LAL) assays were new technologies certified during the MER mission, and later approved by NASA to be used as a proxy assays during ATLO activities. They are used for the detection of total microbial burden on spacecraft surfaces and materials. Since all living organisms – including microorganisms – use ATP as an energy source, this molecule can be employed as an indicator for the presence of living organisms (past or present) on any surface or in any given sample. Both assays are rapid, enzyme-based detection methods to evaluate the "microbial burden cleanliness" of spacecraft surfaces for planetary protection purposes. These non-culture based assays, are capable of extreme sensitivity, and able to assess cleanliness levels much faster than the three days required by the NASA Standard Assay.

The ATP assay measures microbial biomass using biotechnology based on the detection and quantification of ATP with firefly enzyme luciferin/luciferase and a luminometer.

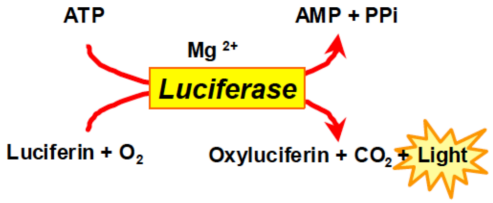

Figure 1: The bioluminescence reaction of firefly luciferase illustrating how light is generated with the consumption of ATP.

When carrying out the ATP assay, surface samples should be considered nondetect (ND) or zero if the values reported are less than the detection method limit of 7.0x10^{-14}mmol/sample. A suggested cleaning level for the assay is 2.57x10^{-11}mmmol/sample. Whereas, a value of 3.51x10^{-11}mmol/sample indicates required cleaning and re-sampling of surfaces.

The LAL assay was developed for use in the pharmaceutical industry to monitor the presence of Lipopolysaccharide (LPS), an endotoxin, in injectable drugs and on medical devices. LPS is a biomolecule found only in the cell wall of Gram-negative bacteria, and β-glucan, a cell wall component of most yeasts and molds. These sources typically constitute about half of the microbial bioburden in the spacecraft assembly environment. The LAL assay is quantified using either a laboratory-based microplate reader or a portable instrument. Results can be reported in less than an hour.

The LPS concentration in a laboratory sample is often expressed in "endotoxin units" (EU, 1 EU ~ 10^{-11} g *E. coli* LPS). LAL samples should be considered to be a "non-detect" (ND) if the value reported is less than the Method Detection Limit, 0.005 EU/ml. Warning limits have been designated for use with the assay: at 0.075 EU/sample**,** re-cleaning of the surface is suggested. However, when detection limits are 0.127 EU/sample or greater, re-cleaning and re-sampling is required.

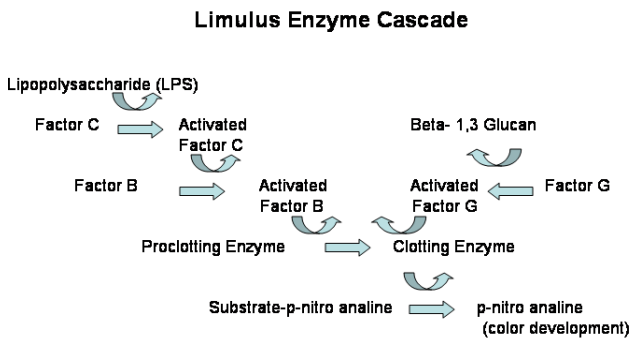

Figure 2: The Limulus Amebocyte Lysate enzyme cascade for Gram-negative bacteria triggered by LPS (left) and yeast and molds triggered by Beta- 1,3 Glucan (right). This cascade is part of the innate microbial defense system carried out by blood cells (amebocytes) of the primitive horseshoe crab, *Limulus polyphemus*.

MSL had a successful planetary protection campaign by meeting all applicable requirements, despite being the largest rover sent to Mars to date. This was the first NASA mission for which the ATP assay was utilized as a cleanliness predictor, prior to NASA Standard Assay (NSA) sampling of critical hardware and/or vital spacecraft testing. Both ATP cleanliness verifications and source specific encapsulated microbial bioburden studies ultimately were essential in enabling MSL to be compliant with PP requirements. During the entire MSL ATLO campaign 586 ATP swabs were collected for 16 operations, with over 60% of the samples occurring within the launch operations phase at Kennedy Space Center. ATP swabs were an outstanding predictor of spore cleanliness, as all subsequent NSA samples exhibited spore bioburden levels within acceptable tolerance limits. Currently, the InSight and Mars 2020 missions continue to use this assay as a proxy for the NASA Standard assay. Although the ATP assay was first implemented during MSL ATLO, particularly during the launch campaign, the LAL assay was not. The LAL assay may be used as a proxy assay to determine levels of spacecraft cleanliness, with the Mars 2020 mission.

9. CURRENT CHALLENGES

Delays and their Consequences

Mars missions with bioburden requirements have to be considered when there is a postponed launch. Specifically, any hardware that is closed out (e.g. where lost access) having already undergone microbial reduction processing and bioburden surface verification must be maintained within an ISO 8 (or better) environment and appropriately protected against recontamination. This has potential to add additional scope to the mission in the sense that a baseline pre-storage bioburden assessment is conducted on the hardware accessible surfaces and a post-storage bioburden assessment is performed to verify that the storage period did not introduce or provide a conducive environment to harbor organisms on the hardware. Additionally, PP engineering support is also required to ensure that all hardware is adequately stored, maintained, and hardware access is defined and granted immediately before and after storage. It is also best practice that all stored hardware undergo a through cleaning prior to the restart of mission.

Despite additional planning, coordination and hardware time for cleaning and sampling, the relative risk to the hardware if stored properly and monitored for PP is low. Full flight system examples include the Mars ’01 Lander being stored and re-flown for the Phoenix ’05 mission and the Mars Science Laboratory 2008 to 2011 launch delay. Sub-system examples include the following flight spare items that are repurposed for a future mission:

- Mars ’01 Lander arm refurbished and used for InSight 2018,
- Mars Exploration Rover HazCam and NavCam hardware being utilized on MSL and InSight,
- MSL descent stage structure and propulsion components inherited for Mars 2020, and
- InSight small deep space transponder and solid state power amplified transferred to Mars 2020.

Both the entire flight system and subsystem examples have illustrated that stowage of flight hardware is possible provided that PP is involved in the monitoring storage conditions, bioburden assessments pre- and post-storage, and a hardware cleaning is conducted as a standard practice post storage.

Lessons Learned

Documentation of lessons learned has been key in knowledge transfer and progressing the discipline from mission to mission. The primary high level categories of lessons learned from Mars missions, dating back to Viking, focus on improving hardware throughout its lifecycle, process improvements, and philosophical discussions to integrate/mainstream PP into the engineering organization [21-25].

Integrating PP into the hardware lifecycle has been an evolving lessons learned from mission to mission. The main lesson learned focuses on the implementation of microbial reduction processes and the concept of recontamination prevention. Microbial reduction processes include hardware compatibility to time and temperatures, development of standard practices for hardware cleaning (e.g. IPA wiping/swabbing and precision cleaning) and implementation of the heat reduction process to ensure minimal hardware exposures by aligning PP, contamination control and environment requirements. After microbial reduction, recontamination prevention has been a major lessons learned for the engineering staff as it is often easy to microbially reduce

the hardware but more of an effort to ensure that it stays clean. Covering the hardware when not in use, establishing packaging requirements, and increasing PP surveillance activities have been most effective implementation approaches to the recontamination prevention lessons learned.

Additionally, training, establishing multiple communication pathways, and PP data tacking systems have also been vital for improving the overall PP process. Development of the NASA HQ PP course, general PP overview training, microbiology fundamentals and more tailored project specific training packages have been useful in getting the project teams vested into the PP requirements and process. In general, high school biology was the last life science course that is taken by most engineering disciplines. Establishing a foundation of microbiology and its role in hardware development has proven effective in applying hardware practices and procedures conducive for meeting PP requirements. Multiple communication pathways are also a major lesson learned. Given that PP compliance for a bioburden based mission entails everyone from the cleanroom technical facilities management service to the project manager establishing open communication is critical at all levels within the engineering chain of custody. Notably, communication with the project systems engineering team, direct 1:1 communication with the hardware engineers, contamination control engineering (if separate from PP), and mission assurance are particularly helpful for success. Finally, PP data tracking systems have been useful for time-savings in documenting the PP process. With the large amount of biological performance data (~1.3 million data points for MSL and 1.0 million data points for MER) a laboratory information management systems that has the capability to capture hardware sample metadata, associate its biological performance data, and analyze the data to generate a current best bioburden estimate has been a major resource savings. An end-to-end requirement verification closure tracking processes has also been a key lesson learned. This includes an approach to adequately document the hardware requirements, applicable hardware processes for PP, bioburden performance data, and PP engineering assessment.

The significant philosophical discussion to integrate PP into the engineering organization lessons learned originated from the MSL mission, which was to integrate PP requirements in the projects systems engineering flow. This approach has been adopted by NASA and is now implemented in the InSight and Mars 2020 missions. In general, requirements are accepted by the project into the project management tracking system; requirements are passed down to lower levels, closed loop verification activities established and all are tracked in the project's dynamic requirements database by the project system engineer. This has also resulted in PP being directly managed by the project system engineer for the mission as opposed to the flight system manager. (See Section 6, above).

Remote Laboratory Assembly

The challenges with setting up remote laboratories are several and the complexity varies from mission to further challenging mission. Ongoing discussions between the host organization and the mission are necessary and vital to establish an understanding of the infrastructure capabilities of the host. The capabilities dictate the requirements will be stated in the Ground Support Requirements Document (GSRD), which is a controlling agreement between the mission and the ATLO site. Careful planning must take place in order to carry out a smooth transfer of support equipment to and from the remote laboratory.

ATLO

The Assembly, Test and Launch Operations (ATLO) process for any mission begins with detailed planning and forecasting of activities, starting from engineering and designing, all the way through launch. More specifically, there are six main processes making up ATLO. The development and details of a mission must be identified based on the size and complexity of the project. This also includes the selection of test sets, staffing, scheduling constraints, and the general flow of the spacecraft or scientific instrument test program, from testbed testing through launch. Testbed testing involves testing at the earliest practical time within the project schedule first by using simulations and then transition incrementally to hardware configurations. Mechanical operations consist of the assembly, handling and shipment of the flight or flight-like hardware to the launch site. Electrical integration and functionality testing will occur after systems have been delivered and are ready for integration. Flight and flight-like systems have to be tested for environmental conditions to prove reliability and sturdiness of the hardware. This will include stress tests, thermal and solar thermal vacuum tests, as well as vibration tests. Finally, the spacecraft or instrument will be transported, tested, fueled, and integrated to the launch vehicle and launched, usually either from the Kennedy Space Center in Florida, or Vandenberg Air Force Base in California. With any mission that has a planetary protection component to it, state-of-the-art methodologies must be utilized to provide an assessment of spacecraft cleanliness. Novel technologies have to be certified and approved by NASA in order to be used during ATLO campaigns (as was done with MER, consequently leading to the use of the ATP assay for MSL) to experimentally determine hardware bioburden values.

Launch Vehicle

The launch vehicle system can pose a particular challenge to PP as the launch vehicle and launch environment may serve as a recontamination source. Early communication with the launch systems engineer, launch service program

and launch service contractor is key in the understanding, and development and implementation of PP requirements. Trailblazers are practically effective in buying down risk to establish as baseline bioburden to a launch provider standard process. Launch hardware that is contained within the payload launch fairing envelope and the environmental control systems should be considered as contamination sources to the mission. Thus, mission unique cleaning procedures will need to be in place for the hardware and appropriately assessed for biological cleanliness prior to coming in contact with clean mission hardware. These mission unique process should be captured as launch vehicle requirements and formally dispositioned at reviews and in working groups.

Spacecraft encapsulation, transportation to launch pad, rocket integration (upper stage to payload adapter), and on pad spacecraft integration and testing are key events in launch processing that must be considered for recontamination. These pose a risk to recontamination due to the amount of hardware needed for these activities, the ability to control ISO environments, and the necessity for white room construction and cleaning to biological requirements. These risks can be overcome by trailblazer support by PP, integration of PP in the hardware flow (both spacecraft and launch vehicle), and developing a PP risk strategy for recontamination. The PP risk strategy for prevention of recontamination may include detailed cleaning of closely associated hardware, inline air sampling of environmental control systems, surface samples of the air handling system, and particulate air monitoring [26].

10. NEW TECHNOLOGIES FOR FUTURE IMPLEMENTATION

It is a fascinating challenge to plan the implementation path forward for a mission that will take part in the possible return of samples over a decade down the road. Considering that technology will evolve greatly between these two time points, one must anticipate both the technological enhancements that will be available as well as the overcome the a priori knowledge that governs how we collect and document information. In an effort to be prepared to answer questions pertaining to the science integrity of the sample, several technologies have been developed to aide in minimizing contamination throughout the course of the mission and contamination budgets have been put into place along with guidance to the future missions to help in this effort. The developed technologies pertaining to maintaining sample integrity include new transport model development, including all of the inputs to the model (e.g. the number of microbes that exist on a particle given the particle size, the adhesion forces of microorganisms, etc.), the fluid mechanical particle barrier (FMPB) which is an engineering solution that keeps the viscous fluid from flowing into the clean hardware and thus maintaining its sterility, and the collection of samples to build a genetic inventory of all microorganisms isolated from biological assays of the spacecraft and associated hardware.

BioVigilant

Cleanroom certifications and relative cleanliness are determined by the particle counts per unit volume of air sampled. Yet, a majority of the particle counters assess particle size distribution and relative abundance of particles but cannot discern inert particles from bioaerosols. It was hypothesized that humans are key contributors of biological and inert particles in a cleanroom but the impact of human presence on cleanroom biological particle counts has not been systematically assessed.

The correlation between bioburden risk and particle counts as applicable to spacecraft assembly facilities has yet to be elucidated. A weak relationship between class level, particle concentrations, and bioaerosol levels needs further verification. Additionally, the same can be said for a weak relationship between particles less than 0.5 microns, particles less than 1 micron, and viable airborne fungi. Yet, no correlation between microbiological and inert particle counts has been made – although there are suggestions that there could be an inverse relationship between particle size and bacterial counts. A majority of these studies were performed in surgical clean rooms, where the microbial profile may be different from spacecraft assembly facilities. The limitation of these studies lies in the limited target microbes (aerobic bacteria), particle size and the uncontrolled sampling environments as compared to highly regulated cleanroom environments.

During a recent study (under review), real-time monitoring and quantification of bioaerosols and inert particles along with their size distribution in spacecraft assembly cleanrooms in JPL was performed using the BioVigilant IMD-A 350. The IMD-A 350 air monitoring system is based on optical spectroscopy that can differentiate inert particles and biological particles based on the detection of three microbial metabolites. Comprehensive and rigorous air monitoring was performed in six cleanrooms (ISO Class 6, 7, and 8) for six hours during normal operational activities (at work) and during no activities (at rest). A positive correlation was established between human activities and elevated bioaerosol counts primarily of 0.5 to 1 micron size that was consistent across all the clean rooms. This study represents the first continuous air monitoring of spacecraft assembly clean rooms for simultaneous detection of 'biological' and inert' particles. The results of this study will help to reassess current modeling standards for bio-aerosol transport in spacecraft assembly cleanrooms.

The use of the BioVigilant IMD-A 350 could provide a more comprehensive assessment of biological particle and particle size distribution as applicable to spacecraft assembly facility environments. The BioVigilant IMD-A 350 is a non-cultivation based particle counter with real time operation, simultaneous measurement of particle size and biologic status, immediate data reporting, continuous monitoring, and synchronized video and data collection. Capable of simultaneously detecting particle size (range 0.5 – 10 microns) and intrinsic fluorescence of biological markers in airborne particulates, this event-based method can resolve particle counting events to 10 second intervals. Data obtained from this continuous air monitoring system could help to develop a more reliable model of bio-aerosol particle transport, to accurately predict and determine points of cross contamination in spacecraft assembly facility cleanrooms.

Metagenome Sequencing

Using conventional and state-of-the-art molecular techniques, a wide range of investigations have examined cultivable [27, 28], and non-cultivable microbial diversity [29] associated with spacecraft and associated cleanrooms (SAC). Despite numerous characterizations of microbial populations in SAC [30], [31], understanding metabolic traits responsible for persistence and survival remains a significant challenge. Functional capabilities required for survival in harsh and extreme environments might be found only in "problematic" microbial strains or species [30]. As such, not all microorganisms pose an equivalent threat to forward contamination and the confounding of life-detection experiments. Understanding the resistance traits of these microbial populations, would factor significantly in the ability to accurately assess forward contamination risk for NASA missions. Metagenomics is a culture-independent genomic analysis of entire microbial communities inhabiting a particular niche [31], [32]. A metagenome study was undertaken at JPL, to provide new insights into the genetic variability and functional capabilities of unknown or uncultured microorganisms of spacecraft associated surfaces. Such knowledge will promote NASA's ability to gauge the probability of transfer of organisms with functional attributes relevant to microbial survival in extraterrestrial environments. A recent publication from our group [33], constitute the literature's first ever account of the spacecraft assembly cleanroom metagenome derived from DNA originating solely from the potential viable microbial population. Understanding the natural status (i.e., viable vs. non-viable) of source organisms is crucial when inferring risk to human health from environmental samples (intensive care units) via nucleic acid based analyses. Results demonstrate that the cleanroom microbiome consists of bacteria, eukaryotes, and even viruses, and as such, is much more complex than was previously posited [34]. Sequence abundance and correlation analyses suggest that the viable indoor microbiome is influenced by both the human microbiome and the surrounding ecosystem(s). In a recent publication from our group [35], we have reported the first functional metagenomics study describing the microbial flora in cleanroom environments. The results of this study should be considered for microbial monitoring of enclosed environments including spacecraft and more isolated habitats such as the International Space Station and considerations for the possibility of future manned missions to Mars.

11. SUMMARY

The pace of scientific exploration of our solar system provides ever-increasing insights into potentially habitable environments, and associated concerns for their contamination by Earth organisms. Biological and organic-chemical contamination has been extensively considered by the COSPAR Panel on Planetary Protection (PPP) and has resulted in the internationally recognized regulations to which spacefaring nations adhere, and which have been in place for more than 40 years. The only successful Mars lander missions with system-level "sterilization" were the Viking landers in the 1970s. Since then different cleanliness requirements have been applied to spacecraft based on their destination, mission type, and scientific objectives. The Planetary Protection Subcommittee of the NASA Advisory Council has noted that a strategic Research & Technology Development (R&TD) roadmap would be very beneficial to encourage the timely availability of effective tools and methodologies to implement planetary protection requirements. New research avenues in planetary protection for ambitious future exploration missions can best be served by developing an over-arching program that integrates capability-driven developments with mission-driven implementation efforts. Microbial reduction and cleaning methods, recontamination control and bio-barriers, operational analysis methods, are all always at the forefront of technology development and possible applications for the future in the field of PP.

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