

Culture-Independent Microbial Air Profiling using a Spaceflight-Compatible Nanopore Sequencing Method

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Microbial monitoring of spacecraft air is critical toward assessing the efficacy of microbial controls within the environmental control and life support systems to protect the crew and vehicle environment. Currently, onboard the International Space Station (ISS), the air is monitored every quarter using an impaction air sampler. With this method, microbial cells and spores are pulled onto plates containing a growth medium. Following onboard incubation, the crew reports approximate microbial levels to the ground, but sample return is required for identification. Upon return of the plates, the isolates present are identified for crew health risk assessments. As NASA moves beyond low-Earth orbit, sample return will be impractical, and a near real-time monitoring capability is essential. Significant strides have been made in recent years to utilize a molecular-based method for microbial profiling of ISS surfaces. The developed method is independent of microbial culture, thus removing the bias toward detecting only culturable organisms, eliminating the need for sample return, and reducing the risk to crew health from exposure to high microbial levels. The work described here details the evaluation of three different air sampling platforms whose product is amenable to downstream molecular processing. The three samplers were compared in terms of mass and power requirements, ease of use, and the resulting data. For the two highest-ranking samplers, a basic concept of operations was developed to transfer the sample into the already established preparation and sequencing process. Using these concepts of operations, an in-depth comparison of the molecular data generated was compared to the historical culture-based method. Data from both methods detailed similar microbial profiles, while the molecular method detailed microbial identifications that were lacking from the culture data. The developed method will enable the generation of near real-time microbial profiles of the spacecraft atmosphere

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Nomenclature

<i>CHeCS</i>	=	Crew Health Care System
<i>CFU</i>	=	Colony Forming Units
<i>dBA</i>	=	A-weighted Decibels
<i>DNA</i>	=	Deoxyribonucleic Acid
<i>ECLSS</i>	=	Environmental Control and Life Support System
<i>HVAC</i>	=	Heating, Ventilation, and Air Conditioning
<i>ISS</i>	=	International Space Station
<i>JSC</i>	=	Johnson Space Center
<i>LPM</i>	=	Liters per Minute
<i>MapQ</i>	=	MAPping Quality Threshold
<i>MAS</i>	=	Microbial Air Sampler
<i>MMOP</i>	=	Multilateral Medical Operations Panel
<i>MORD</i>	=	Medical Operations Requirement Document
<i>NCBI</i>	=	National Center for Biotechnology Information
<i>OGA</i>	=	Oxygen Generation Assembly
<i>PCR</i>	=	Polymerase Chain Reaction
<i>rDNA</i>	=	Ribosomal Deoxyribonucleic Acid
<i>SAS</i>	=	Surface Air System
<i>SASS</i>	=	Smart Air Sampler System
<i>SDAC</i>	=	Sabouraud Dextrose Agar containing Chloramphenicol
<i>SOP</i>	=	Standard Operating Procedure
<i>TSA</i>	=	Trypticase Soy Agar
<i>WRS</i>	=	Water Recovery System

I. Introduction

Microbial monitoring of spacecraft air has been an integral aspect of microbiological risk reduction since early International Space Station (ISS) missions¹⁻³. To provide sustainable oxygen for the ISS crew, the Oxygen Generation Assembly (OGA), which is part of the Environmental Control and Life Support System (ECLSS), electrolyzes potable water generated from the Water Recovery System (WRS)⁴. High-volume exchange and HEPA filtration are utilized as engineering controls to minimize the concentration of microbial bioburden in the air. However, as with terrestrial closed habitable environments, atmospheric microorganisms are ubiquitous and will accompany the human occupants, biological payloads, foods, and other cargo onto spacecraft destined for the ISS^{1,5}. Additionally, each visiting vehicle contains its own unique atmospheric microbiome that is introduced and mixed with that of the ISS microbiome upon hatch opening. The ability to monitor the atmospheric microbiome and glean pertinent information regarding the functionality of engineering controls and subsequent air quality is crucial in sustaining long-term human spaceflight¹.

The ISS atmospheric microbiome is made up of bioaerosols, which most commonly include airborne bacteria, fungi, and viruses, that are originating from a given source (humans, ECLSS hardware, payloads, cargo, etc.) and can be observed using both culture-dependent and culture-independent methods^{6,7}. Even though bioaerosols have the potential to contribute to contagious infectious diseases, research into bioaerosols is limited⁸. Presently, there are no set dose-response estimates for bioaerosol exposure and no standardization of air sampling or analytical methods⁶⁻⁹. Despite the lack of terrestrial standards, the Multilateral Medical Operations Panel (MMOP) Microbiology Subgroup established the use of colony forming units (CFU) to guide the acceptability of microbial air quality. The limits are defined in the ISS Medical Operations Requirement Document (MORD) as 1,000 CFU/m³ for bacteria and 100 CFU/m³ for fungi¹.

Currently, per the ISS MORD, the ISS cabin air is monitored every three months using the Microbial Air Sampler (MAS) (Burkhard Manufacturing Co. Ltd. Hertfordshire, UK), an impaction air sampler using plated growth medium. Prior to use, a standard Petri dish containing either trypticase soy agar (TSA) or on Sabouraud dextrose agar containing 0.5% chloramphenicol (SDAC) to culture bacteria or fungi, respectively, is inserted into the MAS. The MAS is activated, and ISS air is pulled onto the Petri dish. Following sample collection, the dishes are stored at ambient ISS temperature and read after five days of incubation by comparison to a colony density chart, which eliminates the need

for the crew to physically count colonies. The approximate microbial levels are then reported to the Johnson Space Center (JSC) Microbiology Laboratory.

In addition to the microbial levels, the identifications of the colonies present are a critical piece of data needed for risk assessment. To obtain these microbial identities, sample return is currently required. Following the return of the samples, identification-based analyses include microscopy, biochemical evaluation, and Sanger sequencing¹. The average time from sample collection onboard the ISS to availability of identifications for risk assessment can span weeks to months¹⁰. This correlates with a substantially longer timeframe toward when remediation could occur based on the presence of harmful microbes.

With the knowledge that sample return will not be possible on exploration-class missions, and that providing both microbial quantities and identifications will be an in-flight requirement for all future programs, research has been underway to enable near real-time microbial profiling of ISS surfaces. A molecular method has been developed around the small, portable, spaceflight-compatible platforms miniPCR™ (miniPCR bio, Cambridge, MA, USA) and the MinION™ (Oxford Nanopore Technologies (ONT), Oxford, UK)^{11,12}. The first demonstration of the applicability of this method involved the identification of bacteria that had been collected and cultured onboard the ISS as part of routine microbial surface monitoring¹³. Identifications obtained onboard the ISS were identical to those of the same colonies following return to Earth and implementation of the laboratory's standard operating procedures (SOP)¹³. Building on the success of the first off-Earth, culture-dependent microbial identifications, a culture-independent, swab-to-sequencer method was implemented to remove the bias toward detecting only culturable organisms and to reduce the risk to crew health from exposure to high microbial levels¹⁰. This investigation concluded that, while the molecular method revealed greater diversity, similar microbial profiles were observed as compared to the traditional culture-based method, and organisms of importance were not missing from the culture-independent data¹⁰. Furthermore, it was determined that this data was appropriate for meeting NASA's microbial identification requirements, and this method is currently in use onboard the ISS under the Crew Health Care Systems (CHeCS) as a medical operations system demonstration (known as the BioMole Facility).

Air is intrinsically more difficult to monitor with a molecular-based approach, as additional hardware is required for sample collection. There are also more complexities involved with transferring the air sample into a format that will fit downstream molecular processing. Three lightweight, portable, high-ease-of-use air samplers were selected for evaluation toward method development centered on the previously validated nanopore sequencing method. The investigation here was conducted specifically to compare a new culture-independent monitoring method with that of the NASA Microbiology Laboratory's SOPs for air sampling and analysis. As a first step, the objectives include evaluating the ability to generate culture-independent microbial air profiles and determining if important culturable organisms are absent from the data. Here we will compare the three samplers and discuss the data generated as compared to the current culture-based method.

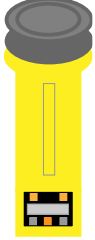
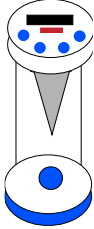
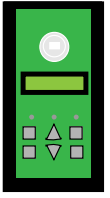
II. Materials and Methods

A. Air Samplers and Sampling Locations

Three air samplers designed for molecular-based workflows, the Coriolis Compact (Bertin Instruments, Montigny-le-Bretonneux, France), the Smart Air Sampler System (SASS)® 3100 (Research International, Monroe, WA) and the Bio-Pump Plus® (Zefon International, Ocala, FL), were chosen for detailed evaluation. Data from these samplers were compared to culture-based data collected by the Surface Air System (SAS) Super 180 (Bioscience International, Rockville, MD), which is used by the JSC Microbiology Laboratory for all ground-based air assessments. The SAS 180 was not evaluated for spaceflight and served as the culture-based control for comparison. All four of these air samplers use inertial impaction, but each has different collection rates, collection media, and specifications, as shown in **Table 1**. Device specifications (except for sound level) in **Table 1** were provided by the manufacturer. A calibrated acoustic dosimeter was used at 1 m from each device to determine the sound level in A-weighted decibels (dBA). Based on the ISS MORD, all air samplers were set to collect a total volume of 1000 L of air per sampling session from moderately populated environments. For each sampling session, the Coriolis Compact, the SASS 3100, or the Bio-Pump Plus was operated in tandem with the SAS 180 for a culture-independent and culture-dependent comparison. Each device was disinfected with 70% ethanol before each sampling session. Samples were collected from the NASA JSC Gilruth Fitness Center or the JSC Human Health and Performance Laboratory breakroom (Building 21, Houston, TX). Samples from the fitness center were collected at midday on a 4'1" mid-wall centered in the room. This wall separates the cardio section of the fitness center from the free weight section and is below an industrial-sized fan. The heating, ventilation, and air conditioning (HVAC) system was active during sampling. On average, there were between

2 to 10 people actively exercising at the time of sampling. Additionally, the men's showers were in proximity, with only a single wall separating the two rooms. The samples that were collected from the Building 21 breakroom were collected midday on a 2' 9" table centered in the room. On average, there were between 3 to 4 people actively eating, and the HVAC system was active during sampling.

Table 1. Air samplers evaluated in this study. All specifications provided by the manufacturers except for the noise level.

				
	Surface Air Sampler (SAS) Super 180	Coriolis Compact	Smart Air Sampler System (SASS)® 3100	Bio-Pump Plus®
Collection Method	Inertial Impaction	Inertial Impaction	Inertial Impaction	Inertial Impaction
Air Collection Rate (liters per minute)	180	50	50 – 300	15
Collection Media	Growth Media	Polyethylene Cone	Electret Filter	Water Soluble Media
Collection Media Shelf Life	6 months	N/A	N/A	N/A
Device Dimensions (W x H x D)	4.3" x 11.4" x 4.1"	5.31" x 10.04" x 5.12"	6.14" x 7.8" x 6.71"	4.5" x 2.75" x 8"
Weight (kg)	1.8	1.4	3.3	0.8
Operating Temperature (°C)	0 – 50	5 – 45	- 40 – 70	0 – 28
Storage Temperature (°C)	0 – 50	0 – 40	- 40 – 70	15 – 28
Humidity	N/A	10% - 90%	All-weather	N/A
Decontamination	Autoclave / 70 % EtOH	H ₂ O ₂ / 70% EtOH	1-5% Bleach / 70% EtOH	70% EtOH
Power Source	Rechargeable Nickel Metal Hydride	Rechargeable Lithium Ion	Rechargeable Lithium Ion	Rechargeable Nickel Metal Hydride
Noise Measurement (dBA)	63.8	68.9	49.9	37.6
Cost	\$5,500	\$9,500	\$8,250	\$749

B. Surface Air Sampler (SAS) Super 180

The SAS 180 impaction sampler was used for all culture-based air sampling assessments to allow for comparisons to the culture-independent data. This sampler was not assessed for use in the developed spaceflight method. The SAS 180 has a flow rate of 180 liters per minute (LPM), functions similarly to the one currently onboard ISS, and is used in all JSC Microbiology Laboratory SOPs for culture-based microbial air analysis. Additionally, the SAS 180 is calibrated annually by a third-party specialist to ensure that the flow rate is within the acceptable tolerance. Prior to the day of use, the SAS 180 pump head was detached and autoclaved at 121 °C for 30 minutes to ensure sterility and hinder cross-contamination between sampling time points. Before sample collection, the unit was disinfected with 70% ethanol, set to collect 1000 L of air, equipped with a TSA plate (Hardy Diagnostic, Santa Maria, CA), and was placed mid-room approximately 8 inches from either the Bio-Pump Plus, Coriolis Compact, or the SASS 3100. The units were started simultaneously. After sampling, the TSA plate was incubated at 35 °C. Following 72 hours of incubation, only bacteria were enumerated and typified by colony morphology and abundance. The sorted bacterial

colonies were then streaked onto new TSA plates to obtain isolated and pure colonies. Pure isolates were then identified following the JSC Microbiology Laboratory SOPs for the Fast MicroSeq 500 16S rDNA PCR and Sequencing Kits coupled with the ABI 3500 genetic analyzer (ThermoFisher Scientific, Waltham, MA). Sequencing results were analyzed by the MicroSeq ID database following the JSC Microbiology Laboratory SOP.

C. Coriolis Compact

The Coriolis Compact is a lightweight, portable, dry-collecting air sampler that uses the Coriolis force to centrifuge microbial cells onto the inner wall of its attached cone. The Coriolis Compact has a fixed flow rate of 50 LPM, and the sampling time can be adjusted on the unit and saved for future sampling sessions. As the unit was new, it was within the manufacturer's calibration date. If further testing occurs, calibration will be performed annually. Prior to use, the cones, air intake, and caps (Bertin Instruments) were cleaned by submersion in a 0.1 M NaOH solution for 15 minutes to eliminate DNase/RNase. Then, in a laminar flow cabinet, they were rinsed with sterile nuclease-free water (ThermoFisher Scientific), rinsed once more with 70% ethanol, and then allowed to dry. After the cones, air intakes, and caps were dry, they were packaged in sterile bags. Immediately prior to sample collection, the unit was disinfected with 70% ethanol, equipped with the collection cone, and then the collection protocol was initiated. Following sampling, the cone was detached from the unit, capped hand-tight with a sterile cap, and bagged in a sterile plastic bag until processing in the lab. To process the sample, 2 mL of Butterfield's buffer (Weber Scientific, Hamilton, NJ) was added to the cone and then vortexed for 30 seconds. The aqueous sample was then filtered through a 0.22 μ m filter (EMD Millipore Corporation, Burlington, MA). Using sterile forceps, the 0.22 μ m filter was folded and transferred into a sterile PCR tube containing 120 μ L of QuickExtract™ DNA Extraction Solution (Lucigen Corporation, Middleton, WI).

D. Smart Air Sampler System (SASS) 3100

The SASS 3100 is a compact, portable, dry-collecting air sampler that utilizes a high efficiency centrifugal fan to collect bioaerosols onto an electret filter (Research International). Through the connection of the SASS 3100 to an external computer, the SASS 3100 software was programmed to collect 1000 L at 180 LPM, mimicking the SAS 180. As the unit was new, it was within the manufacturer's calibration date. If further testing occurs, calibration will be performed annually. Prior to use, the particle extraction fluid (Research International) was sterilized using a 90 mm diameter, 0.22 μ m pore size filter unit (ThermoFisher Scientific). Prior to sample collection, the electret filter was removed from its sterile packaging and aseptically attached to the SASS 3100. Following sample collection, the filter was detached from the unit and placed in a sterile bag until processing in the lab. To process the sample, the electret filter was dislodged from its plastic housing and transferred into the sterile particle extractor (Research International). Sterile particle extraction fluid (Research International), 10 mL, was passed through the electret filter, eluting the particles into an aqueous form. The aqueous sample was then filtered through a 0.22 μ m filter. Using sterile forceps, the 0.22 μ m filter was folded and transferred into a sterile PCR tube containing 120 μ L of QuickExtract DNA Extraction Solution (Lucigen Corporation).

E. Bio-Pump Plus

The Bio-Pump Plus is a portable air sampling pump designed to be used with the Via-Cell® cassette (Zefon International). The Bio-Pump Plus has a fixed flow rate of 15 LPM; thus, the sampling time was adjusted to collect air for 66.6 minutes. As the unit was new, it was within the manufacturer's calibration date. If further testing occurs, calibration will be performed annually. The Via-Cell cassette contains a tacky, water-soluble collection media that bioaerosols adhere to when the Bio-Pump Plus is active. With program control on the Bio-Pump Plus sampler, the unit was set to collect 1000 L, and the Via-Cell cassette was removed from its sterile packaging and attached to the Bio-Pump Plus. Following sample collection, the Via-Cell cassette was detached from the unit and aseptically stored for return to the lab for processing. The Via-Cell cassette water-soluble collection media was removed using a sterile swab and transferred into 120 μ L of QuickExtract DNA Extraction Solution (Lucigen Corporation) and incubated at room temperature for 10 minutes.

F. Molecular Processing and Nanopore Sequencing of Collected Air Sample

To extract DNA, the sample contained in the QuickExtract DNA Extraction Solution (Lucigen Corporation) in a PCR tube was placed into a miniPCR and heated to 65 °C for 30 minutes followed by heating to 98 °C for 10 minutes. To purify the DNA, the cell lysate underwent a 1:1 magnetic bead purification with Agencourt AMPure XP beads (Beckman Coulter Genomics, Brea, CA) following the manufacturer's protocol with the exception of 5% polyethylene

glycol containing 1.25 mM sodium chloride being used as a wash buffer in place of ethanol¹⁰. Purified DNA was amplified using LongAmp® Hot Start Taq 2X Master Mix (New England Biolabs (NEB), Ipswich, MA) and primers from the 16S Barcoding SQK-RAB204 Kit (ONT). Amplification was achieved within the miniPCR using the following thermal parameters: initial denaturation at 95 °C for 120 s, 30 cycles of 95 °C for 20 s, 55 °C for 30 s, and 65 °C for 60 s, with a final extension of 65 °C for 300 s¹⁰. The quality of the amplicons was assessed using the Qubit™ 1X dsDNA High Sensitivity kit (ThermoFisher Scientific) and the Agilent D5000 ScreenTape (Agilent Technologies, Santa Clara, CA). The barcoded DNA was combined for multiplexing and purified using a modified 0.6X Agencourt AMPure XP bead (Beckman Coulter Genomics) cleanup and eluted in 10 µL of 10 mM Tris-HCl (pH 8.0) with 50 mM NaCl, as described above. Purified DNA was prepared for sequencing and loaded into a flow cell following the spaceflight-compatible procedures as described in Burton *et al.* and Stahl-Rommel *et al.*^{10,13}. Briefly, 10 µL of DNA was added to 5 ng of a sequencing control DNA consisting of a ~750 bp *Triticum aestivum* amplicon compatible with the ONT sequencing adaptor. The combined DNA was then added to 5 µL of rapid adaptor buffer (ONT) and incubated for 5 minutes at ambient temperature. Following incubation, the multiplexed, sequencing-ready sample was added to sequencing buffer (ONT), and 150 µL was injected into the sample port of the R 9.4.1 flow cell within the MinION Mk1C (ONT). Sequencing was performed for 12 hours with live high-accuracy basecalling and barcode demultiplexing running concurrently with GPU-enabled Guppy for MK1C Software 4.3.4 (ONT).

G. Nanopore Sequencing Analysis

A targeted detection strategy was used to identify bacterial taxon. Basecalled and demultiplexed data was removed from the Mk1C for detailed analysis. Length filtering was completed using NanoFilt Version 2.5.0¹⁰ to 1350-1650 bp for targeted 16S rRNA or 400-900 bp for the spike in positive sequencing control. Using minimap2 version 2.17-r941 (-ax map-ont), the positive sequencing control barcoded reads were mapped to a non-microbial positive control reference, while the air samples and negative control barcodes were mapped to the curated 16S database from NCBI Reference Sequence (RefSeq) Database^{14,15}. Primary alignments were extracted using SAMtools version 1.9 and filtered with a MAPping Quality (MAPQ) threshold of 1¹⁶. Sequencing alignment identity was calculated per read using MarginStats (marginStats.py 0.1)¹⁷. Data was filtered to ≥ 90% alignment identity. Positive sequencing control DNA confirmed successful nanopore sequencing run performance. To optimize the reporting for air samples, the negative controls were used to identify contamination within the sample set, and the contaminating reads were removed. Data visualizations were created in R using the ggplot package, pheatmap, and correlation network package¹⁸⁻²¹.

III. Results and Discussion

A. Comparison of Technical Specifications

There are many factors to take into consideration when evaluating spaceflight-compatible air sampling devices; these include technical specifications, ease-of-use, and how well the particle collection and extraction methods align with the culture-independent nanopore sequencing method^{10,13}. All three of the selected commercially available devices use an inertial impaction collection method but vary in their collection media, collection rates, size, and technical specifications, as described in **Table 1**. The SAS 180 air sampler was selected for culture-based microbial profile comparison because of its use in the JSC Microbiology Laboratory as a ground air sampling unit with comparable performance to the MAS used onboard the ISS. For the three samplers under assessment, the Coriolis Compact, SASS 3100, and Bio-Pump Plus all are described by their developer as being compatible with culture-independent microbial analysis. Areas evaluated during the trade space phase included sampling rate, mass, volume, power, shelf stability of additional consumables, emitted noise level during operations, and compatibility with downstream nanopore sequencing.

In terms of sampling rate, while lower volumes could be extrapolated to 1000 L, sampling a larger volume is ideal for low biomass molecular investigations. The SASS 3100 has an adjustable flowrate, with a maximum of 300 LPM, which is higher than the Coriolis Compact and the Bio-Pump Plus that are fixed at 50 LPM and 15 LPM, respectively (**Table 1**). Programming the SASS 3100 to its maximum flow rate and collecting 1000 L of air results in a sampling time of approximately 3 minutes. It takes approximately 20 and 67 minutes for the Coriolis Compact and Bio-Pump Plus to sample the same volume of air, respectively. Beyond sampling rate, the Bio-Pump Plus is the smallest and lightest unit at 0.8 kg with the Coriolis Compact at 1.4 kg and SASS 3100 at 3.3 kg (**Table 1**). Again, the volume for the Bio-Pump Plus is the smallest at ~ 99 in³, while the Coriolis Compact is ~ 273 in³ and SASS 3100 ~ 321 in³. While

the Bio-Pump Plus seems to trade well as compared to the other samplers, the Via-Cell cartridges add mass and volume that result in all units being roughly equivalent. The Coriolis Compact requires collection cones and the SASS 3100 requires a filter and sample particle extractor. Shelf life is not a concern with any of the consumables associated with the three samplers. Moreover, power was not a strong influence toward a given sampler, as all have rechargeable batteries, similar to the current ISS MAS. The Bio-Pump Plus did prove to have the lowest noise output at 37.6 dBA; however, all devices measured noise level below 70 dBA (**Table 1**), which is the maximum recommended noise level by the World Health Organization and NASA for prolonged exposure²².

While physical attributes did not propel any sampler to a higher ranking, the Bio-Pump Plus displayed the lowest mass, volume, and emitted noise level. However, due to its low sampling flow rate, it either must be operated for over an hour or the data resulting from the lower collected volume would have to be extrapolated. Despite this, the Bio-Pump Plus was the first unit thoroughly investigated for compatibility with the previously developed nanopore sequencing process. Per the manufacturer's protocol, the water-soluble collection media should fully dissolve in 5-10 minutes. However, during protocol development using sterile molecular water, as directed by the manufacturer's protocol, substantial issues with dissolving the adhesive material were noted. Following this, attempts were made to use a spaceflight-compatible buffer, as protocols indicated other buffers could be used. Despite the use of water or an appropriate substitute, and allowing for the allotted time for dissolving, the liquid was cloudy and resulted in insufficient DNA extractions and subsequent amplification. The final attempted assessment used a sterile swab to scrape off the collected material followed by submerging it into QuickExtract DNA Extraction Solution (Lucigen Corporation). After 10 minutes, cloudiness was still observed, ultimately yielding poor genomic DNA concentrations and an inability to perform amplification. It is not clear why these issues were encountered, but, due to the complexity of the process, it was beyond the scope of the comparison to further troubleshoot this issue. As such, the culture-independent detection of microorganisms from this device was unachievable, and this device was abandoned from future testing.

The Coriolis Compact and SASS 3100 are relatively new dry air sampling technologies. While the characteristics of both compare well, their sample collection and extraction designs differ. The Coriolis Compact uses the Coriolis force to centrifuge atmospheric contaminants onto the inner wall of its attached cone, while the SASS 3100 utilizes a high efficiency centrifugal fan to collect material onto an electret filter. While both devices have simple collection methods, the particle extraction method of the SASS 3100 is more complex, requiring the addition of a sterile particle extractor to manipulate the sample into an aqueous form for down-stream processing. Biomass manipulation associated with the Coriolis Compact is simple, as only the addition of 2 mL of buffer and vortexing is required. Extraction of the liquid from both samplers resulted in sufficient DNA for amplification and downstream sequencing.

B. Culture-Independent Microbial Profiles from the Coriolis Compact and SASS 3100

The Coriolis Compact and SASS 3100 are both capable of serving as sampling devices for culture-independent atmospheric microbial profiling (**Figure 1**). Upon examination of the generated profiles, the most abundant genera shared between the samplers and locations were *Streptococcus* and *Staphylococcus* (**Figure 1**), which are both members of the human microbiome²³. Additionally, these organisms seem to be prevalent in most indoor air samples when using culture-dependent or culture-independent methodologies²⁴. The prevalence of the Gram-negative bacterial genera, *Pseudomonas* and *Pantoea*, are expected due to the proximity of the fitness room to the men's shower and have previously been identified in indoor air samples collected from fitness centers and residential built environments²⁴⁻²⁶. Difficult-to-culture and/or anaerobic bacterial genera including *Bacteroides*, *Blautia*, *Eubacterium*, *Faecalibacterium*, and *Haemophilus* were present in the culture-independent profiles (**Figure 1**) and would not be expected to be observed in culture-dependent data sets. As mentioned above, samples taken from both the fitness center and the breakroom include the oral commensal microbe, *Streptococcus*, but other anaerobic oral bacterial genera, *Fusobacterium* and *Moraxella*, were also included in the culture-independent profiles from the breakroom (**Figure 1**).

Culture-independent assessments reveal a more accurate depiction of the microbiome as compared to culture-based examination, as difficult-to-culture organisms are captured^{10,27-29}. While culture-dependent monitoring has resulted in a healthy crew and ISS, a true understanding of the spacecraft microbiome is only now emerging^{10,30,31}. While it is exciting to harness molecular technology for in-flight monitoring and an increased understanding of the microbiome, it is necessary to understand how this data compares with historical culture data.

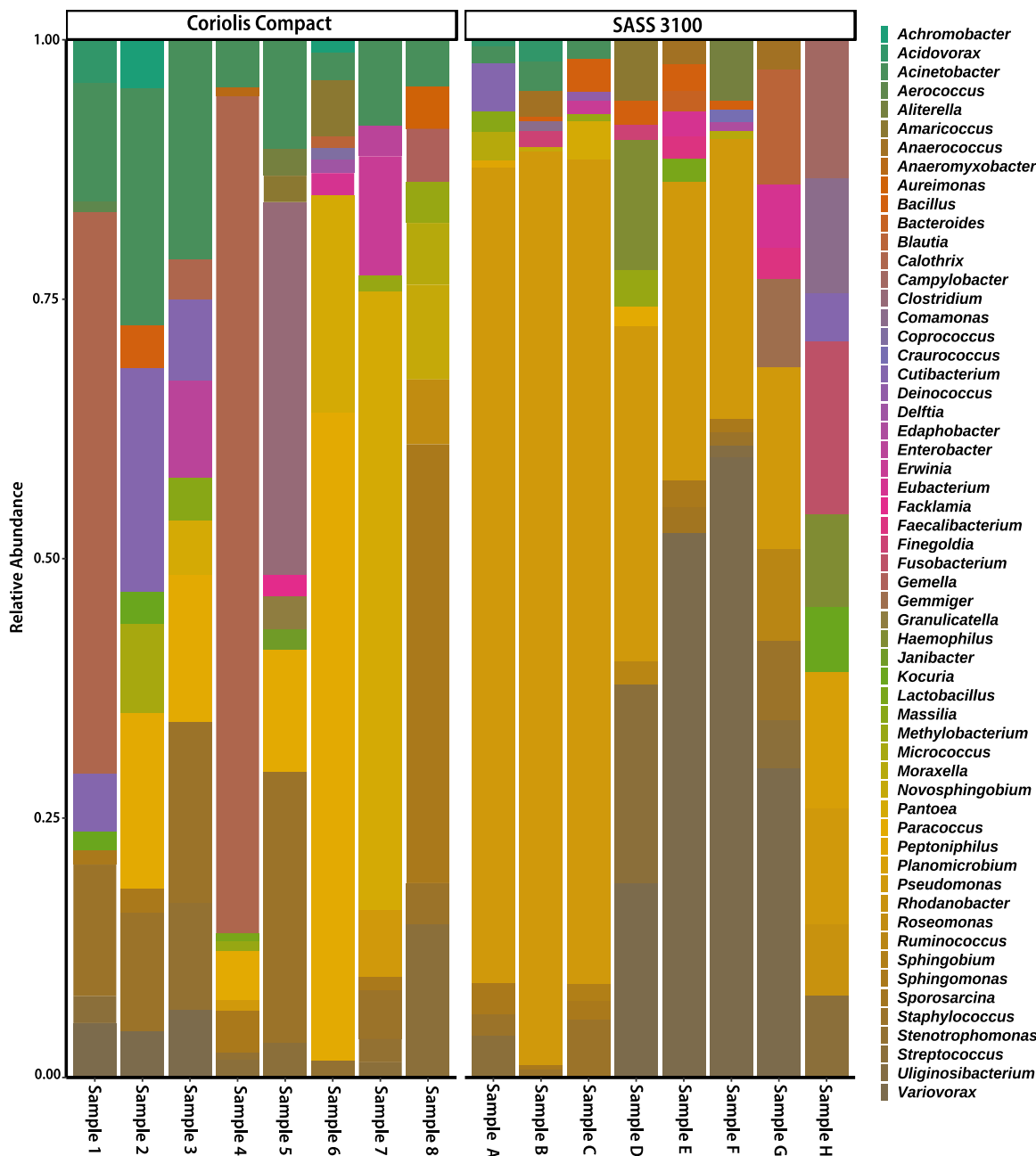


Figure 1. Microbial profile of the top 10 genus-level identifications generated from the Coriolis Compact and SASS 3100. Air samples collected from the NASA JSC Gilruth Fitness Center are denoted as samples 1-7 and A-G, while those collected from the JSC Building 21 breakroom are denoted as samples 8 and H. Samples collected with each instrument were processed and identified using a culture-independent nanopore sequencing method.

C. Comparison of Culture-Dependent and Culture-Independent Methods for the Coriolis Compact and SASS 3100

NASA has applied culture-based monitoring to the in-flight assessment of microbial air quality since early ISS missions¹⁻³. The current investigation was conducted specifically to compare the new culture-independent monitoring method with that of the JSC Microbiology Laboratory SOPs for air sampling and analysis. Side-by-side sample collection with the SAS 180 and either the Coriolis Compact or the SASS 3100 allowed for a detailed comparison

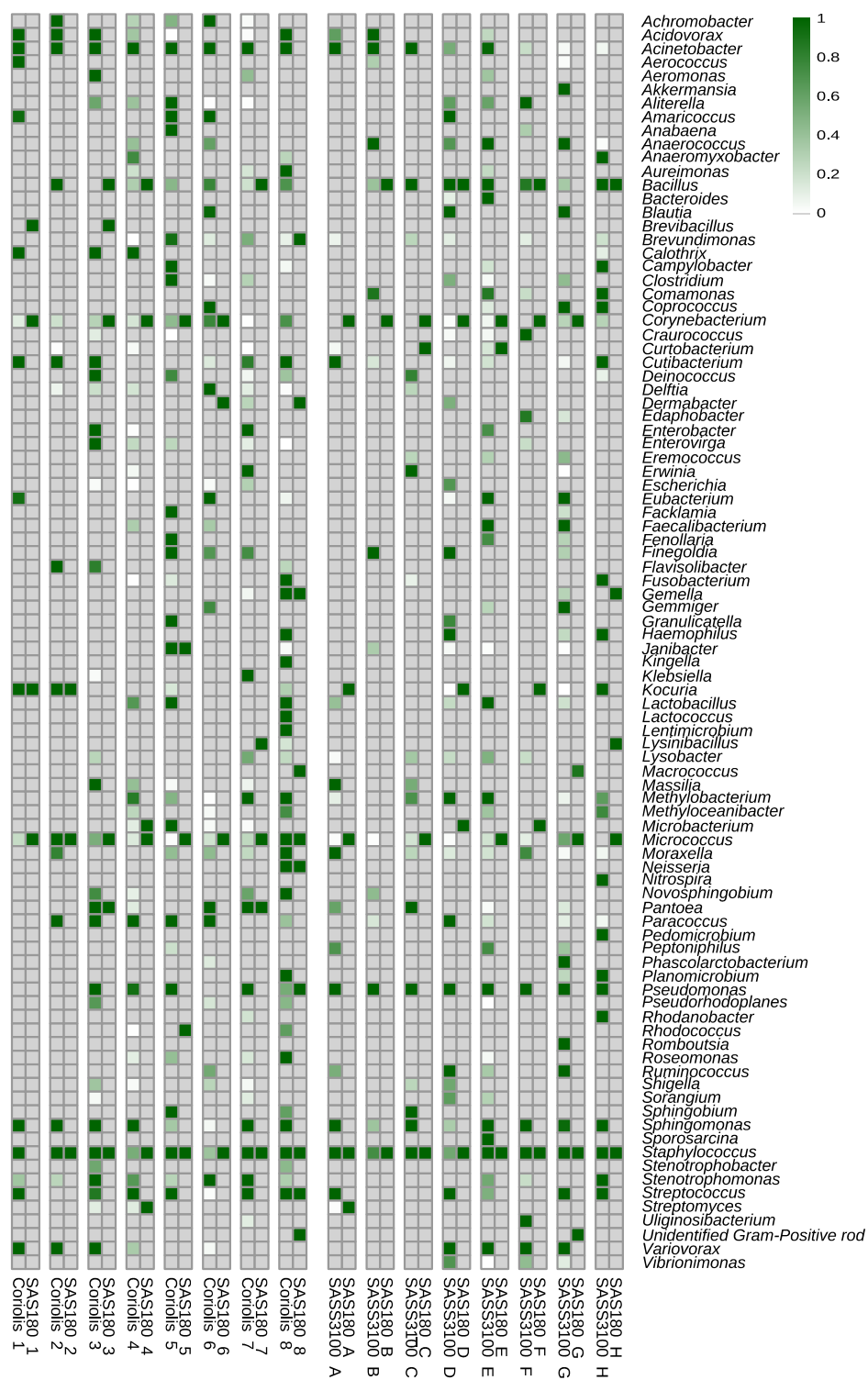


Figure 2. Heatmap of the relative abundance for bacterial genera identified from the SAS 180 as compared to the culture-independent process from the Coriolis Compact or SASS 3100. Samples collected from the NASA JSC Gilruth Fitness Center are denoted samples 1-7 and A-G, while samples collected from JSC Building 21 breakroom are denoted samples 8 and H. Genera less than 1% in abundance for all samples were excluded. The least abundant to the most abundant is represented from white to dark green with grey as zero abundance. Colony forming units (CFU) were used to calculate the relative abundance of culture-based isolates.

between culture-dependent and culture-independent microbial profiles of a fitness center and office building (**Figure 2**). Eight tandem sampling sessions were conducted using each device from August 2021 to February 2022 for a total of 16 samples. Each sampling session took place around the same time of day and with 2 - 10 individuals present. Of the 16 SAS 180 samples, 79 culture isolates were observed. Of these, 77 isolates were identified to at least genus-level using Sanger sequencing, while 2 isolates could not be resolved beyond unidentified Gram-positive rods. As commonly seen due to both the association with human-occupied environments and the type of growth media used, *Bacillus*, *Staphylococcus*, *Micrococcus*, and *Corynebacterium* were the most abundant genera in all the culture-dependent samples (**Figure 2**). These top genera were also identified from the culture-independent nanopore sequencing data from the Coriolis Compact and the SASS 3100 and are consistent with other studies that collected air and surface samples from a fitness center using a culture-independent swabbing method and culture-dependent air sampling method^{24,25}.

While not surprising, the culture-independent method revealed greater microbial diversity. However, samples from the SAS 180 demonstrated the presence of *Brevibacillus*, *Bacillus*, *Macrococcus*, *Rhodococcus*, and *Dermabacter* that were not identified in the Coriolis Compact data (**Figure 2**). For some, this was an isolated incidence, as in the example of *Bacillus*, where the mismatch with the culture data only occurred at a singular time. Typically, the other genera were only seen on occasion (one or two isolations out of the eight samples). There was greater misalignment when comparing the SASS 3100 molecular data to the SAS 180 cultures. *Lysinibacillus*, *Corynebacterium*, *Kocuria*, *Curteobacterium*, *Microbacterium*, *Gemella*, *Micrococcus*, and *Macrococcus* were all identified through culture but were not represented in the SASS 3100 data (**Figure 2**). While some of these were singular occurrence offsets, *Corynebacterium* was missed in 50% of the SASS 3100 samples, which suggests that some inherent bias may occur with this sampler. Overall, out of 16 total samples, the Coriolis Compact showed closer similarity to the SAS 180 at 81% alignment while SASS 3100 only demonstrated 67% alignment to its corresponding SAS 180 samples. While further assessment is required, it is worth noting that perfect alignment between the culture isolates and the corresponding culture-independent data was not expected, as these were not truly split samples from the same air sampler for each session. While the air samples were collected synchronously, they are two distinct and unique air samples collected with two differing methods. As such, differing results were anticipated³².

A Pearson correlation coefficient was used to visualize relationships among the atmospheric microbial profiles obtained (**Figure 3**). Not unexpectedly, the culture-based SAS 180 profiles clustered closely together and distantly from the Coriolis Compact and SASS 3100 profiles. This likely results from the high degree of similarity of the culture taxonomy, where 23 genera were observed from 79 isolates. The lack of diversity noted in the culture profiles is common, as only one type of media, TSA, and one set of environmental conditions were used for cultivation (aerobic incubation at 35 °C). The trend of further disassociation between the SASS 3100 and culture-based profiles, as noted with the heat map, is apparent. Furthermore, several of the Coriolis Compact and culture-based distributions are more closely associated. Interestingly, the Coriolis Compact “8” and SASS 3100 “H” profiles, both from the Building 21 breakroom, cluster in nearer proximity to each other than profiles from the fitness center from same device (**Figure 3**). This same association is not noted with the SAS 180 breakroom profiles (8 and H). While additional samples are required, this trend suggests that culture-independent profiling may provide a more accurate representation of the atmospheric microbiome than culture-dependent profiling.

Culture-independent profiles can be obtained from an air sample collected with either the Coriolis Compact or SASS 3100. Moreover, the data suggests that these profiles are more accurate than those obtained using traditional culture-based analysis. While both samplers missed some organisms identified through culture, the Coriolis Compact most closely paralleled the culture-dependent profiles and did not indicate a bias toward specific genera. Beyond the data, the SASS 3100 sampling parameters cannot be changed in real-time and there is no visual indicator of how long the unit has been actively sampling or an estimated time of sample completion. Programming of the Coriolis Compact on the unit is possible and increases its overall ease-of-use. Following the analysis of additional datasets, one unit will be selected for further testing. The liquid obtained from the down-selected sampler will be split to provide a true comparison between standard culture and the developed culture-independent method.

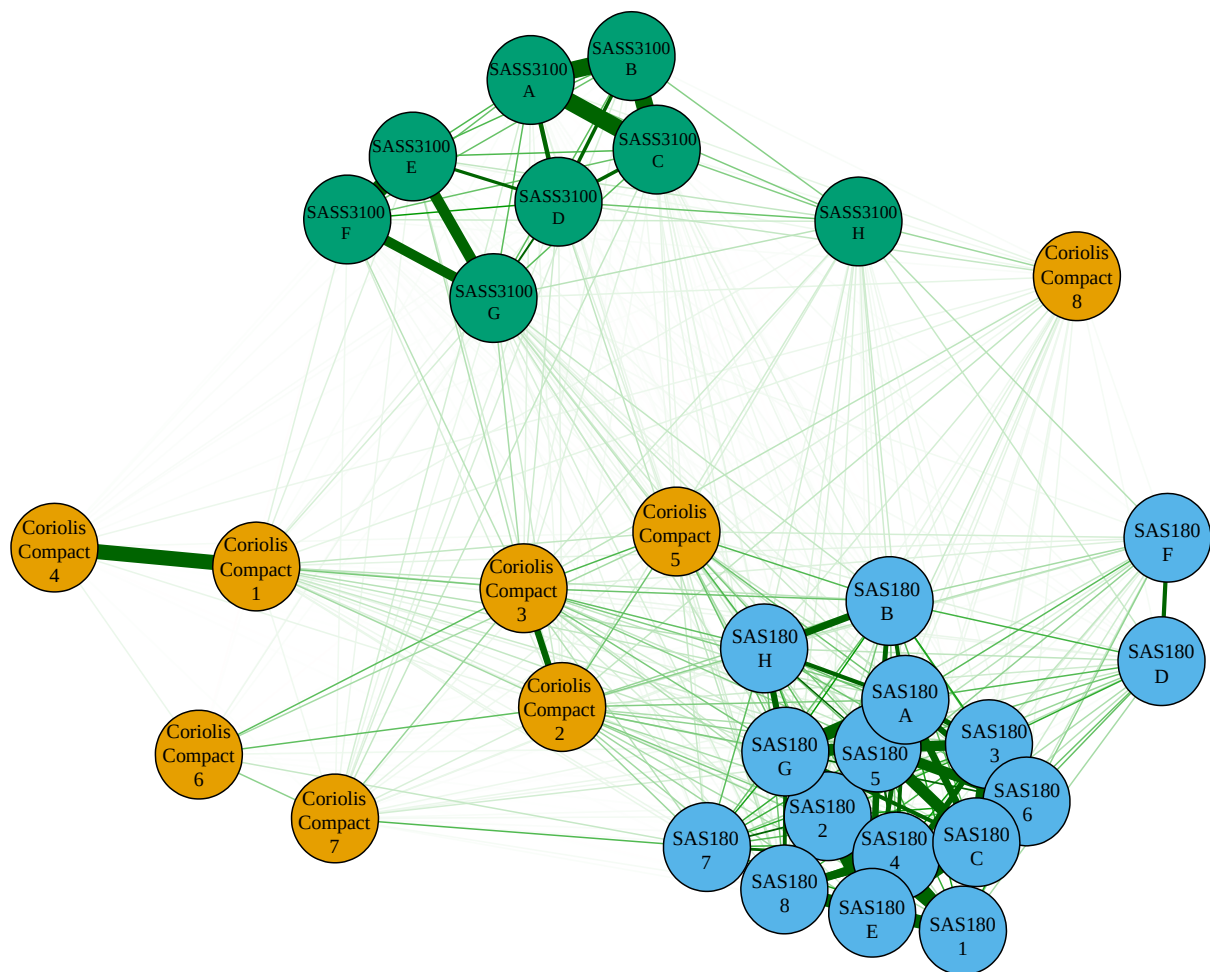


Figure 3. Pearson correlation network demonstrating the relationship between culture-based SAS 180 atmospheric microbial profiles (blue) and culture-independent Coriolis Compact (orange) and SASS 3100 (green) profiles. Air samples collected from the NASA JSC Gilruth Fitness Center are denoted 1-7 and A-G, while samples collected from the JSC Building 21 breakroom are denoted 8 and H.

IV. Conclusion

Microbial monitoring of ISS air has been achieved through culture-based methods that require the return of samples to Earth for identification. In recent years, small, portable molecular technologies have enabled the in-flight identification of microorganisms from both culture-dependent and culture-independent processes. Building upon those investigations, three air samplers were identified that could support a direct sample-to-sequencer process for atmospheric microbial air profiling. While the technical specifications of the samplers did not significantly set them apart in a trade space, DNA was unable to be extracted from the Bio-Pump Plus adhesive matrix. Microbial profiles generated from culture-independent, sample-to-sequencer processing for both the Coriolis Compact and SASS 3100 support the potential for the near real-time assessment of spacecraft air. The implementation of a molecular-based method would reduce the sample-to-answer time, remove the bias towards the identification of culturable organisms, and reduce crew exposure to high levels of potential pathogens. Future work will continue to optimize and validate this method, as well as define a concept of operations to enable utilization beyond the ISS to exploration missions.

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