The Future of In-Situ Sequencing-based Microbial Monitoring: Development of a Shelf-Stable Method for Artemis and Beyond

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Microbial monitoring onboard the International Space Station (ISS) is essential for assessing the efficiency of the Environmental Control and Life Support Systems (ECLSS) and providing insight into potential risk to both crew and spacecraft. Historically, this monitoring required the need to culture organisms onboard, return these cultures to Earth, and then complete the identifications, a process that would take months. Over the past decade, and through numerous payloads, advances in molecular biology have enabled in-flight microbial identifications using nanopore sequencing. The swab-to-sequencer method resulting from these efforts was transitioned from research to operations for microbial monitoring under the Crew Health Care Systems (CHeCS) BioMole. Collectively, these accomplishments have propelled the swab-to-sequencer method to be selected as the Microbial Surface Monitor (MSM) for Gateway, as well as a payload on Artemis IV. However, the lack of cold stowage availability for Artemis requires modifications to the entire method due to the thermal instability of the reagents required for sample preparation. To achieve this, new development, optimization, and validations were undertaken. Key considerations included enzyme concentration, buffer compatibility, and equal or enhanced sensitivity and specificity. At each step, thorough side-by-side comparisons with the current ISS method were performed. The development of a robust shelf-stable method will ensure continued sequencing-based microbial monitoring for Artemis and beyond, providing data in near real-time, enhancing risk response time, and yielding clear insight into the microbiome of spacecraft.

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Acronyms and Nomenclature

ADB = Adapter Buffer
ATCC = American Type Culture Collection
BEST = Biomolecule Extraction and Sequencing Technology
CFU = Colony Forming Unit
CHeCS = Crew Health Care Systems
DNA = Deoxyribonucleic Acid
EB = Elution Buffer
ECLSS = Environmental Control and Life Support System
g = Gravitational Rotation
JSC = Johnson Space Center
LEO = Low Earth Orbit
LIS = Library Solution
MSM = Microbial Surface Monitor
MoBE = Microbiome of the Built Environment
NEB = New England Biolabs
NEEMO = NASA Extreme Environment Mission Operations
NFW = Nuclease Free Water
OD = Optical Density
ONT = Oxford Nanopore Technologies
PCR = Polymerase Chain Reaction
PEG = Polyethylene Glycol
R2A = Reasoner's 2 Agar
RA = Rapid Adapter
SB = Sequencing Buffer
TSA = Tryptic Soy Agar
TSB = Tryptic Soy Broth

I. Introduction

Microbial monitoring of crewed spacecraft is a critical component for maintaining crew health and vehicle integrity. Onboard the International Space Station (ISS), routine monitoring mitigates potential health risks to the crew and prevents microbially-induced degradation of spacecraft systems. Additionally, while the Environmental Control and Life Support Systems (ECLSS) have various means of microbial control, monitoring exposes occasional process escapes. Historically, ISS-based monitoring has relied on in-flight microbial culture, followed by return to Earth for identification in the Johnson Space Center (JSC) Microbiology Laboratory. Identification, required for NASA risk assessment, involves subculturing for isolation, characterization of cell wall morphology, biochemical assays, and/or Sanger sequencing (Figure 1).[1] While this has served the ISS well, the process takes weeks-to-months from sample collection to identification, results in data biased towards only those organisms that can be cultured in the given conditions, and potentially exposes the crew to high levels of pathogens.

Advances in molecular biology tools and developments in spaceflight-compatible methodologies have fundamentally changed the paradigm that sample return is required for sophisticated analyses. In 2016, the Genes in Space-1 payload demonstrated that DNA amplification via polymerase chain reaction (PCR) is feasible in microgravity using the miniPCR thermal cycler (miniPCR bio, Cambridge, MA), which is capable of rapid and precise temperature cycling.[2] That same year, the Biomolecule Sequencer payload validated in-flight sequencing using the handheld Oxford Nanopore Technologies (ONT) MinION device by sequencing genomic DNA prepared on the ground (Figure 1).[3] Building on these milestones, the Genes in Space-3 payload marked a pivotal advancement in space-based microbial identification when crew members extracted, amplified, and sequenced DNA from routine monitoring cultures (Figure 1).[4] Ground-based identification of the returned culture matched the in-flight sequencing results, confirming the accuracy of the approach.[4] Despite this success, the reliance on culture-based methods remained a limitation.

Removing the culture requirement was achieved through analog-based testing and development followed by ISS validation. During the NASAs Extreme Environment Mission Operations (NEEMO) -21 and -22, a swab-to-

sequencer-based workflow was created, thoroughly analyzed, and optimized. The subsequent 2018 Biomolecule Extraction and Sequencing Technology (BEST) payload on the ISS validated the method in spaceflight and resulted in the first culture-independent microbial identifications in the spaceflight environment (Figure 1).[5] These successes paved the way for the establishment of the ISS BioMole Facility, which transitioned the method from a research payload to medical operations hardware owned and operated by the Crew Health Care Systems (CHeCS). Through the operational use of BioMole, the swab-to-sequencer method was fully validated for surface-based monitoring, and a culture-independent method for water analysis was also validated. Building off ISS successes, the swab-to-sequencer method was selected as the Microbial Surface Monitor (MSM) for Gateway and as an Artemis IV payload (Microbiome of the Built Environment, MoBE). While these advances remove the requirement for sample return, a continued difficulty remains, in that the consumable reagents to support in-situ sequencing require cold stowage. For long-duration exploration missions beyond low Earth orbit (LEO), such cold storage capability will be unavailable.

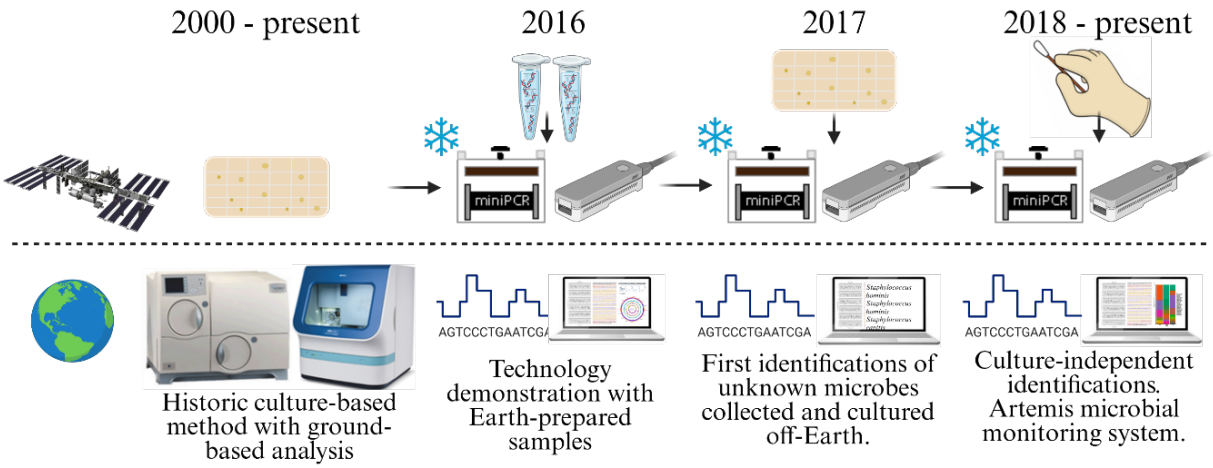


Figure 1. Timeline of microbial monitoring methods for the International Space Station from 2000 to present. Culture-based microbial monitoring was initiated in 2000, with ground-based identification performed through biochemical or Sanger sequencing analysis. In 2016, molecular methods were deployed with successful amplification of DNA using miniPCR,[2] and successful sequencing, using the MinION device, of ground-prepared genomic DNA.[3] In 2017, a combination of the two technologies resulted in the first ever identification of an unknown microorganism from culture in space.[4] In 2018, the need for culture was eliminated with the validation of the swab-to-sequencer method, a culture-independent microbial identification process.[5] For all molecular workflows, cold stowage is required, but sample return is not, with only data transmitted to Earth for analysis.

The development of a fully shelf-stable nanopore sequencing workflow is essential for sustained microbial monitoring during deep-space missions. This challenge is particularly significant for nucleic acid extraction, as extraction buffers typically contain enzymes responsible for cell wall degradation and the breakdown of intracellular components. While enzymes are indispensable tools in molecular biology, they generally exhibit low thermodynamic and kinetic stability,[6] necessitating storage at temperatures ranging from −80 °C to 4 °C. Efforts to improve enzyme stability through bioengineering have yielded multifunctional enzymes with enhanced robustness. One notable example is Proteinase K, a widely used protease that degrades nucleases and other proteins that could otherwise compromise DNA integrity. Multiple commercially available formulations of Proteinase K now demonstrate kinetic stability at room temperature for up to two years, depending on the manufacturer.[7, 8]

Achieving shelf stability for DNA extraction reagents addresses only the first step of a molecular identification workflow. Subsequent DNA amplification via PCR remains essential, particularly for targeted sequencing approaches such as the 16S rRNA gene analysis currently used with the swab-to-sequencer method.[5] Central to PCR is DNA polymerase, the enzyme responsible for synthesizing new DNA strands by incorporating nucleotides into a growing target DNA segment (amplicon). DNA polymerases, like most enzymes, typically require cold storage to maintain activity. However, previous studies have demonstrated successful DNA amplification using lyophilized PCR reagents,[9] in which DNA polymerase, cofactors, and stabilizing buffers are lyophilized into a format that can be

reconstituted with the addition of high-purity water, primers, and DNA template. These approaches offer a promising pathway toward a fully shelf-stable sequencing workflow suitable for deployment beyond LEO.

The work presented here provides a significant step forward in developing a shelf-stable nanopore sequencing approach through the evaluation of multiple extraction buffers and their concentrations, lyophilized PCR reagents, and overall compatibility for a robust continued microbial identification method for Artemis and beyond.

II. Materials and Methods

A. Control Organisms Growth Conditions

Two structurally-diverse microorganisms, *Ralstonia pickettii* (Rp) and *Staphylococcus aureus* (Sa), were chosen to serve as standards, as they represent both ends of the spectrum in terms of ease-of-lysis.[10] These two organisms were cultured separately at 35 °C on Reasoner's 2A Agar (R2A) media (Hardy Diagnostics, Santa Maria, CA and Springboro, OH) or Tryptic Soy Agar (TSA) (Hardy Diagnostics, Santa Maria, CA and Springboro, OH) respectively, for approximately 24 hours. Following overnight culture, a 10 µl loop of inoculum for each organism was taken and resuspended in 10 mL R2A liquid media, for *R. pickettii*, or Tryptic Soy Broth (TSB) (Hardy Diagnostics, Santa Maria, CA and Springboro, OH), for *S. aureus*, and incubated at 35 °C with shaking at 150 RPM for approximately 17 hours. Following incubation, samples were centrifuged at 3220 × g for 10 minutes, the supernatants were removed, and the cells were resuspended in 10 mL of nuclease free water (NFW). This 10 mL wash with NFW was then repeated two additional times. Following this washing, cells were resuspended in NFW to achieve an OD600 of 0.5 using a BioTek Synergy H1 Plate Reader (Agilent Technologies, Santa Clara, CA). This process resulted in a final concentration of approximately 10^9 CFU/mL for both organisms. Each solution underwent 10-fold serial dilution and plating in triplicate to confirm the concentration. For each organism, a final estimated 10^5 CFU concentration in pellet form was prepared and stored at -80 °C.

B. Extraction Buffer

Two shelf-stable extraction buffers, referred to as E1 and E2, were compared to the standard swab-to-sequencer extraction buffer, QuickExtract DNA Extraction Solution (ES) (LGC BioSearch Technologies, Middlesex, UK) . DNA extraction optimization was tested for E1 and E2. The E1 extraction buffer was tested with various concentrations of Proteinase K and compared to ES. The E2 extraction buffer was tested for various heating conditions and compared to the ES standard heating method.

To determine the optimal concentration of Proteinase K for E1, the following concentrations were tested in a total volume of 150 µl: 0.1, 0.2, 0.5, 1, 2, and 5 mg/mL. Briefly, extractions were prepared by resuspending a pellet of *S. aureus* and *R. pickettii* in duplicate in either 150 µl of ES or E1 extraction buffer with the varying Proteinase K concentrations. Extraction was performed using the standard thermal conditions used in the ISS method.[5] DNA was purified using shelf-stable SPRIselect beads (Beckman Coulter Genomics, Brea, CA) in a 1.25 M NaCl/5% polyethylene glycol (PEG) wash solution. Duplicate samples were combined and then separated into two equal DNA concentration samples for amplification. Targeted amplification of the 16S rRNA gene was performed using the standard procedure.[5] Analysis included an assessment of the efficiency of each extraction buffer through quantitative and qualitative results post amplification using a Qubit 1X dsDNA High Sensitivity Kit (ThermoFisher Scientific, Waltham, MA) and Agilent D5000 DNA ScreenTape on the 4200 TapeStation (Agilent Technologies, Santa Clara, CA), respectively.

A second buffer, E2, was tested using three heating conditions. Briefly, extractions were prepared by resuspending a pellet of *S. aureus* and *R. pickettii* in duplicate in either 150 µl of ES or E2. First, E2 was incubated at ambient temperature. Then, E2 was incubated on miniPCR at 65 °C for 6 minutes followed by 98 °C at 2 minutes. Finally, E2 was incubated using the thermal conditions from the standard method. DNA purification and amplification were performed per the ISS method,[5] followed by qualitative and quantitative analysis as described above.

C. PCR Reagents

Three shelf stable versions of PCR reagents, referred to as P1, P2, and P3, were assessed and compared to the swab-to-sequencer standard, PS. Duplicates of S. *aureus* and *R. pickettii* pellets were extracted, purified and amplified using P1, P2, P3 and PS following the standard ISS swab-to-sequencer method.[5] Post amplification purification, was performed using the standard method, followed by qualitative and quantitative analysis as described above. Library preparation and sequencing was performed using the standard method.

Further comparison of amplification buffers included sampling miscellaneous environmental surface samples (Table 1). In doing so, the samples were collected and processed through sequencing as described above with the addition of an amplification DNA control. For this control, the ZymoBIOMICS Microbial Community DNA standard (Zymo Research, Irvinve, CA) was used at a concentration of 0.1 ng/µl.

Table 1. Sample abbreviations of environmental swabs used for PCR reagent testing

Sample Abbreviation	Sample Description
S1	Women's Toilet
S2	Men's Toilet
S3	Restroom Bench
S4	Breakroom
Z	Zymo Community DNA Standard

D. Shelf-Stable Extraction and PCR Reagent Compatibility

In order to ensure shelf-stable enzyme buffer and PCR reagent compatibility, a total of four sequencing runs using both E1 and E2 were prepared using environmental surface swabs and amplified using P2. Briefly, processing consisted of following the standard swab-to-sequencer method with the exception of using SPRIselect beads for DNA purification. After sequencing, these four experiments were compared in terms of taxonomic profiles and statistical metrics to obtain the final extraction method for the full shelf-stable method.

E. Shelf-Stable Comparison to Swab-to-Sequencer

Once a full shelf-stable method was selected, it was directly compared to the ISS swab-to-sequencer method. This was completed through the use of a commercial standard, 10 Strain Even Mix Whole Cell Material (ATCC MSA-2003) (American Type Culture Collection, ATCC, Manassas, VA). Briefly, cells were prepared per the manufacturer's recommendation to approximately 10^4 cells. Pellets were then resuspended in triplicate for the swab-to-sequencer and shelf-stable method, hereafter referred to as the Artemis method, directly using extraction buffers and processed through sequencing as described above. For the Artemis method, extraction buffer E2 and PCR reagent P2 were selected based on data from independent testing. Comparative analysis focused on recovery of organisms in the mock community standard through taxonomic profiling.

F. ISS Standard Swab-to-Sequencer Method

Identification of microorganisms in spaceflight through culture-independent nanopore sequencing follows the standard swab-to-sequencer method, first described by Stahl-Rommel *et al*.[5] This method has been updated to Kit 14 chemistry and R10.4.1 flow cells and will be used as the standard method for comparison of all shelf-stable validations.

Surface sample collection was performed aseptically using Sterile Micro Alpha® Swabs (Texwipe, Kernersville, NC) wetted with NFW. A surface of approximately 100 cm^2 was sampled by rolling and rotating swabs in a multidirectional pattern for approximately 60 seconds. Once sampling was complete, the swab head was placed into a 0.2 mL PCR strip tube (USA Scientific Inc., Ocala, Florida) prepared with 150 µl of QuickExtract DNA Extraction Solution (ES) (LGC BioSearch Technologies, Middlesex, UK), and heated in a miniPCR for 65 °C 30 min and 98 °C for 10 min. DNA purification was performed using Agencourt AMPure XP beads (Beckman Coulter Genomics, Brea, CA) at 1X concentration with modified wash buffer (1.25 M NaCl/5% PEG) and eluted in NFW.

Purified DNA was amplified using LongAmp Hot Start Taq 2X Master Mix (PS) (New England Biolabs (NEB), Ipswich, MA) and primers from ONT's 16S Barcoding kit (16S114.24 Oxford Nanopore Technologies, ONT, Oxford, UK). Amplification used the following thermal conditions on miniPCR: initial denaturation at 95 °C for 60 s, 30 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 30 s, and extension at 65 °C for 120 s, followed by a final extension at 65 °C for 300 s. The amplified DNA underwent an additional 0.6X purification as above prior to eluting in 25 µl of Elution Buffer (EB).

An R10.4.1 flow cell was loaded into a Mk1D MinION, and the total pore availability was determined using a flow cell check. The flow cell was primed by adding 200 µl of the priming mix (prepared as recommended by the manufacturer) twice to the priming port. Using an ONT SQK-16114.24 kit for 16S rRNA gene sequencing, a rapid adapter solution was prepared by combining 1.5 µl Rapid Adapter (RA) to 3.5 µl Adapter buffer (ADB), followed by a second dilution taking 2 µl from the solution prepared above and 3 µl of ADB. 22 µl of DNA library was added to

the 5 µl RA dilution and incubated for five minutes. The sample was then added to 80 µl Sequencing Buffer (SB) and 55 µl of Library Solution (LIS). The final 150 µl library was loaded into the priming port following Stahl-Rommel *et al*.[5] and sequenced.

G. Nanopore Sequencing Data Analysis

Libraries were sequenced using MinKNOW v25.09.18 (Oxford Nanopore Technologies, UK) with a run duration limited to 6 hours and barcode trimming enabled. Reads were basecalled and demultiplexed with MinKNOW Dorado v7.11.2 using the super accurate basecalling algorithm. Raw and processed reads were assessed for sequencing quality and summary statistics using Seqkit v2.10.0. Quality and length filtering was performed using Chopper v0.10.0, retaining bacterial reads between 1350 and 1650 base pairs with a minimum quality score of 20. Filtered reads were aligned using Minimap2 v2.28 with secondary alignments disabled, a minimum alignment score ratio of 0.9, and a maximum fragment length of 50,000. Alignments for environmental samples were performed against the RefSeq complete bacterial 16S database (BioProject: PRJNA33175; downloaded May 5, 2025). The ZymoBIOMICS Oral Microbiome Standard (D6332) 16S rRNA gene sequences were obtained from the manufacturer and used in place of the RefSeq 16S database for benchmarking and taxonomic accuracy assessment. The 10 Strain Even Mix Whole Cell Material (ATCC MSA-2003) (American Type Culture Collection, ATCC, Manassas, VA) 16S rRNA gene sequences were obtained from the manufacturer and used in place of the RefSeq 16S database for benchmarking and taxonomic accuracy assessment. Samtools was used to filter alignments by excluding unmapped and supplementary reads. Alignment identity metrics were calculated using aln_stats.py (https://github.com/microbemarsh/aln_stats), and reads were filtered to retain alignments with greater than 97% alignment identity and greater than 97% read identity. Relative species abundances were calculated, and operational taxonomic unit tables were generated in R v4.5.1 following removal of taxa representing less than 0.5% relative abundance. Figures were generated using ggplot2 v4.0.1, and ecological diversity analyses were performed with vegan v2.7.2. Diversity was evaluated using the Shannon-Wiener index and Bray-Curtis dissimilarity. Analyses were performed in R (v4.5.1) using the ggplot2 package (v4.0.1) for visualization and the vegan package (v2.7.2) for ecological diversity calculations.

III. Results and Discussion

A. DNA Extraction Buffer Optimization

A series of shelf-stable extraction buffers were evaluated to identify conditions that maximized DNA yield and amplification performance when compared to the standard ISS swab-to-sequencer extraction buffer (ES). Initial optimization focused on extraction buffer E1 containing increasing concentrations of Proteinase K (0.1–5 mg/mL). Optimization of Proteinase K concentration, at the time of buffer preparation for extraction, was required due to its long-term instability in solution. E1 is a proprietary formulation that closely resembles the composition of the established swab-to-sequencer extraction buffer; as such, additional conditions such as thermal profiles were not assessed, as they were thoroughly investigated previously. Comparison of E1 to ES demonstrated that E1 supplemented with 0.5 mg/mL of Proteinase K provided the most consistent amplification across all control organisms tested (Figure 2A). While amplification of the 16S rRNA gene was observed at higher Proteinase K concentrations (2 and 5 mg/mL), sensitivity was reduced relative to 0.5 mg/mL (Figure 2A). Specifically, higher Proteinase K concentrations appeared to enhance lysis efficiency for *R. pickettii*, yielding higher-quality genomic DNA and robust amplification in some duplicates; however, amplification failure was also observed for *S. aureus* under these conditions, suggesting partial degradation of genomic DNA at elevated Proteinase K concentrations (Figure 2A). Overall, E1 containing 0.5 mg/mL Proteinase K demonstrated improved sensitivity across all control bacteria when compared to ES and was selected for forward work.

A second shelf-stable extraction buffer (E2) was subsequently evaluated, and determination of the optimal thermal conditions relative to ES was central. The proprietary formulation of E2 provided a ready-to-use product and did not require the addition of any enzymatic components prior to extraction. However, thermal condition optimization was required in order to better characterize the intrinsic enzymatic components and ensure maximum enzymatic inactivation following lysis, providing downstream DNA stability. Using the standard swab-to-sequencer thermal protocol (65 °C for 30 min followed by 98 °C for 10 min), ES yielded successful amplification of the targeted 16S rRNA amplicon (Figure 2B). In contrast, alternative E2 conditions, including ambient temperature extraction and shortened thermal incubation, resulted in limited or no detectable amplification (Figure 2B). However, when E2 was used with the standard thermal protocol, amplification performance was comparable to that of ES (Figure 2B). Based

on these results, E2 combined with the standard thermal conditions, 65 °C for 30 minutes followed by 98 °C for 10 minutes, was selected for subsequent experiments.

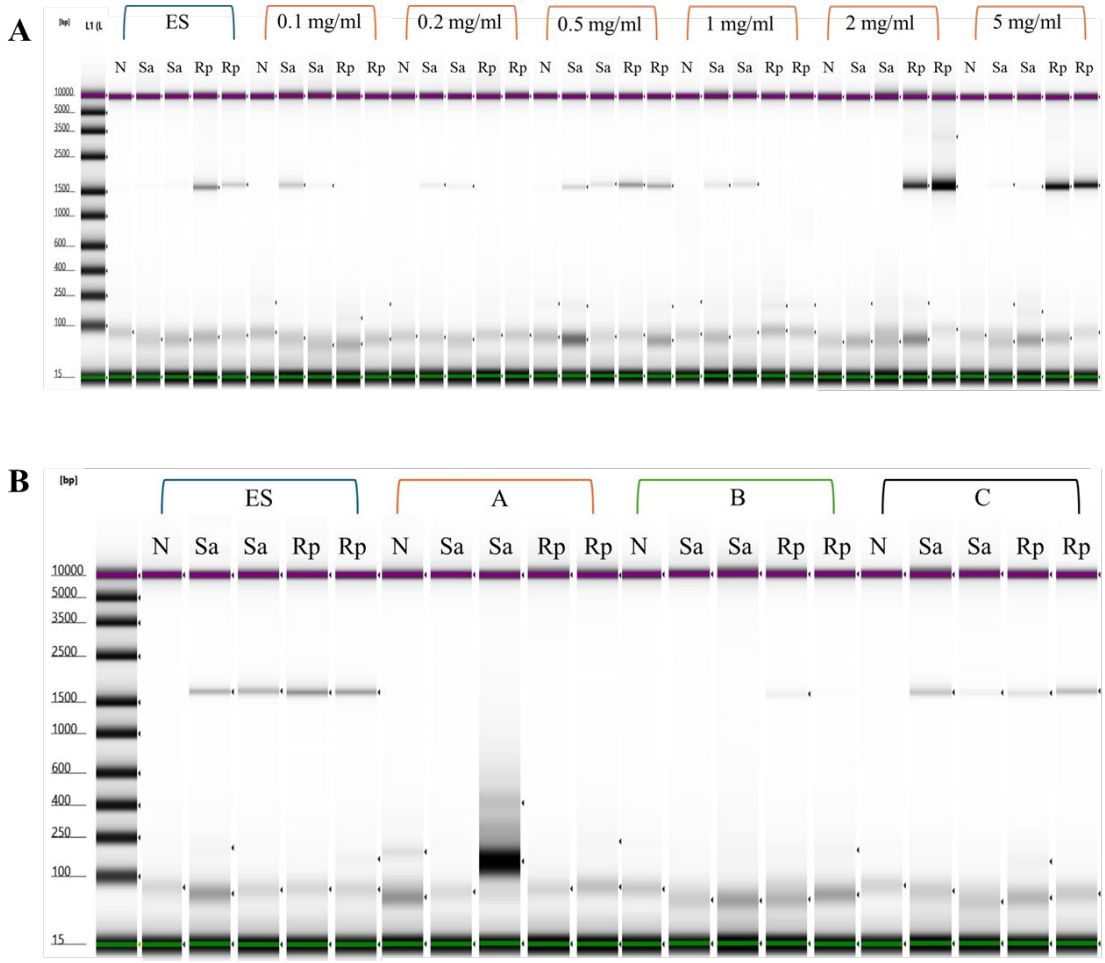


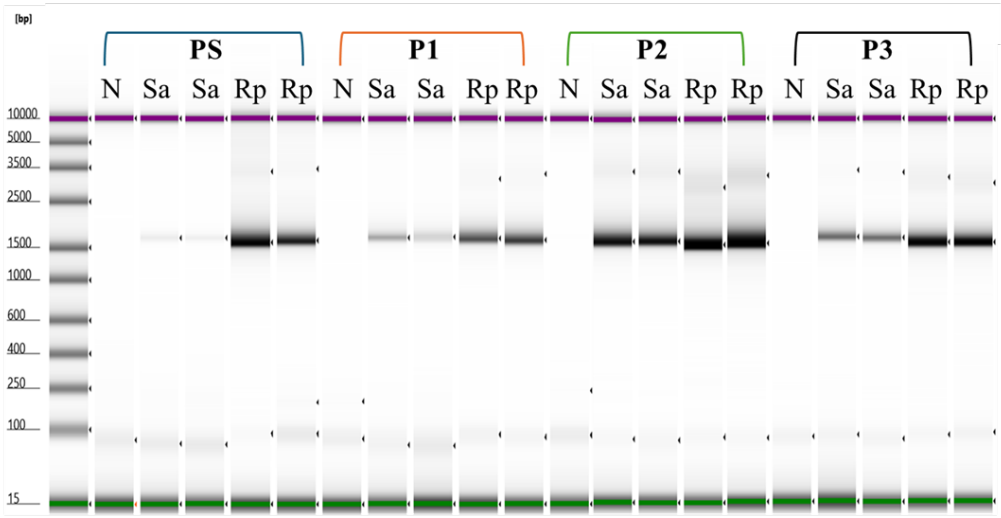
Figure 2. Comparison of shelf-stable DNA extraction buffers. A) Amplicon bands at ~1500 bp following extraction of *S. aureus* (Sa), *R. pickettii* (Rp), or a negative (N) with E1 plus the addition of various concentrations of Proteinase K (0.1, 0.2, 0.5, 1, 2, and 5 mg/mL) as compared to the standard (ES). B) Amplicon bands at ~1500 bp following extraction of *S. aureus*, *R. pickettii*, or a negative (N) with E2 with varied thermal profiles as compared to the standard (ES). A refers to room temperature incubation, B assessed 65 °C for 6 min followed by 98 °C for 2 min, and C evaluated 65 °C for 30 min followed by 98 °C for 10 min. Amplification was achieved using the ISS swab-to-sequencer PCR reagent which requires cold stowage.

B. PCR Reagent Selection

Three shelf-stable PCR reagents (P1, P2, and P3) were evaluated against the standard PCR reagent (PS) to identify an optimal amplification product compatible with the swab-to-sequencer workflow. Initial validation using *S. aureus* and *R. pickettii* control cells demonstrated that all three shelf-stable reagents produced detectable target amplicons (Figure 3A). Quantitative analysis revealed increased DNA yields for P1, P2, and P3 relative to PS (Figure 3B). Despite comparable yields, amplification bias was observed between reagents. P1 and P3 exhibited increased amplification bias toward Gram-negative organisms, similar to that observed with PS (Figure 3). In contrast, P2

demonstrated reduced bias and more balanced amplification between *S. aureus* and *R. pickettii* (Figure 3A). Based on these observations, P2 was selected for further shelf stability and compatibility testing.

A



B

Sample PS	ng/µl
Sa	1.33
Sa	1.29
Rp	18.5
Rp	12.7

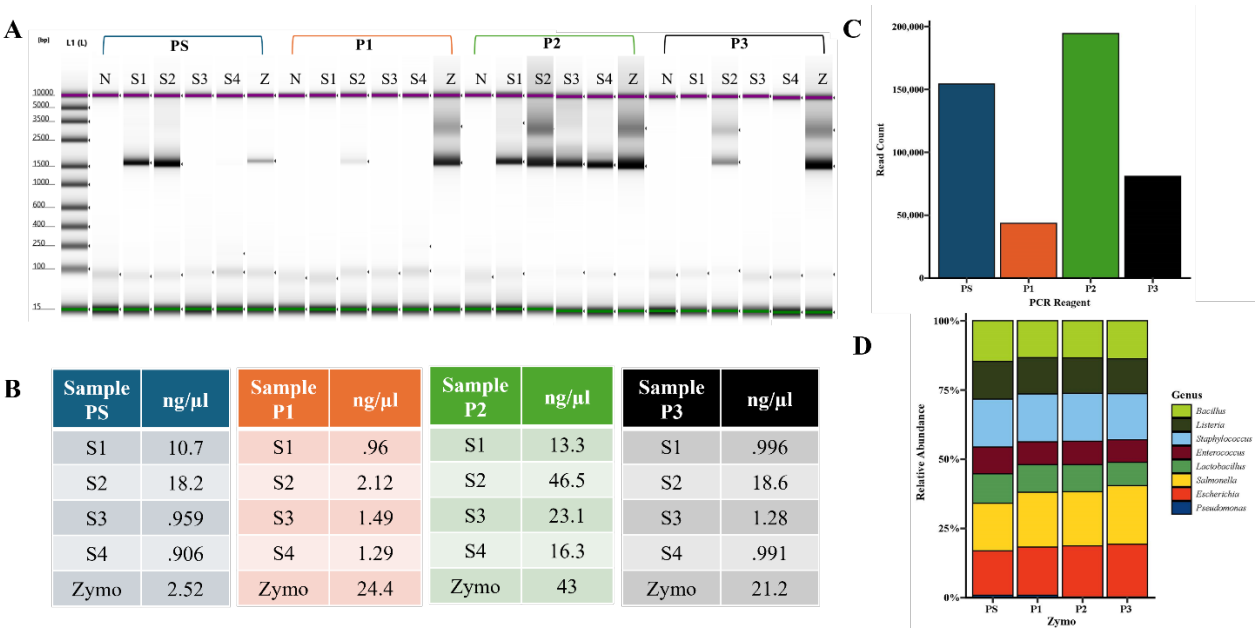
Sample P1	ng/µl
Sa	4.51
Sa	2.61
Rp	9.37
Rp	11.5

Sample P2	ng/µl
Sa	21.8
Sa	19.1
Rp	32.5
Rp	30.7

Sample P3	ng/µl
Sa	7.97
Sa	7.51
Rp	20.6
Rp	21.7

Figure 3. Comparison of shelf-stable PCR reagents. A) Amplicon bands at ~1500 bp following extraction of *S. aureus* (Sa), *R. pickettii* (Rp), or a negative (N) with P1, P2, or P3 as compared the standard PS. All samples were extracted with the ISS swab-to-sequencer extraction buffer that requires cold stowage. B). Amplicon concentration for *S. aureus* (Sa) and *R. pickettii* (Rp) post-amplification with P1, P2, P3, or the standard PS.

To further validate PCR reagent performance with representative samples, environmental swabs were used to collect samples that were then extracted using the swab-to-sequencer extraction buffer, ES, and amplified using P1, P2, P3, and PS with equal genomic DNA input (Figure 4). Consistent with results obtained using control organisms, P2 generated higher-quality and higher-quantity amplicons compared to PS, P1, and P3 (Figures 4A and 4B). Sequencing of the pooled amplicons revealed that P2 not only produced comparable amplicon quantities, but also generated higher total read counts as compared to PS, enabling enhanced downstream taxonomic assignment (Figure 4C). Moreover, the microbial profiles generated from a commercial mock community DNA standard were highly comparable among P1, P2, P3, and PS (Figure 4D). The lack of a misrepresented microbial profile to that of the swab-to-sequencer method and increased taxonomic resolution of cells and environmental samples, collectively support the selection of P2 as the preferred shelf-stable PCR reagent.



B

Sample PS	ng/µl	Sample P1	ng/µl	Sample P2	ng/µl	Sample P3	ng/µl
S1	10.7	S1	.96	S1	13.3	S1	.996
S2	18.2	S2	2.12	S2	46.5	S2	18.6
S3	.959	S3	1.49	S3	23.1	S3	1.28
S4	.906	S4	1.29	S4	16.3	S4	.991
Zymo	2.52	Zymo	24.4	Zymo	43	Zymo	21.2

Figure 4: Comparison of shelf-stable PCR reagents using environmental swabs. A) Amplicon bands at ~1500 bp following extraction of environmental swabs with P1, P2, P3, or the standard PS. *S* refers to the swab number, while *N* is the negative, and *Z* indicates inclusion of the mock community as a control. All samples were extracted with the ISS swab-to-sequencer extraction buffer that requires cold stowage. B) Amplicon concentration for environmental swabs post-amplification with P1, P2, P3, or the standard PS. C) Total number of sequencing reads associated with P1, P2, P3, or the standard PS. D) Relative abundance of the species in the mock community control following sequencing using P1, P2, P3, or the standard PS. Zymo indicates the mock community control.

C. PCR Stable Buffer Compatibility

Following independent evaluation and optimization of extraction buffers and PCR reagents, compatibility of a combined shelf-stable workflow was evaluated through sequencing-based analysis. While data clearly demonstrated that P2 was the optimal PCR reagent, the data for E1 and E2 were highly parallel. As such, further assessments would include both E1 and E2 in combination with P2. Four nanopore sequencing runs were performed using E1 or E2 in combination with PCR reagent P2, generating a total of 44 comparative samples. Successful sequencing across all runs confirmed that exclusive use of shelf-stable reagents through library preparation did not adversely affect sequencing performance.

Samples were grouped into five environmental categories, including the women's restroom (WR), men's restroom (MR), breakroom (B), office area (OF), or other (O), and total sequencing reads were compared between extraction buffers. While both extraction buffers provided ample data, the use of E2 consistently resulted in higher output in terms of the number of sequencing reads generated, supporting improved sequencing depth and taxonomic resolution (Figure 5A). Beyond read number, diversity metrics and taxonomic profiles were assessed to further compare extraction buffer performance. The median number of observed species, or species richness, was similar between E1 and E2 (Figure 5B); however, E2 exhibited a broader distribution of species (Figure 5B). Consistent with these findings, Shannon diversity indices revealed comparable community evenness between extraction methods; these diversity metrics indicate potentially increased detection of low abundance taxa using E2 (Figure 5B). Assessing these same five categories, Bray-Curtis dissimilarity demonstrated that samples clustered primarily by sampling location, as expected microbiologically, rather than based on extraction buffer, indicating minimal buffer-induced distortion of community structure (Figure 5D). Clear separation was observed between the office and breakroom samples relative to men's and women's restroom samples. Additionally, this difference in diversity is highly apparent through taxonomic analysis, where it is clear that office and breakroom samples contain highly abundant genera that are associated with human skin (i.e. *Staphylococcus*) (Figure 6C and D), whereas the men's and women's restroom have higher abundances of organisms typically associated with the gastrointestinal tract (i.e. *Finegoldia* and *Agathobacter*) (Figure 6A and B). Figure 6 depicts the overall similarity in community structure using both methods for identification, suggesting minimal bias when using either E1 or E2 extraction buffer. Collectively, these results demonstrate that

extraction buffer E2 provides increased extraction efficiency, higher sequencing yield, and improved community resolution while maintaining core community composition. Due to these results, a first-step version of the shelf-stable method consists of extraction using E2 followed by amplification using P2.

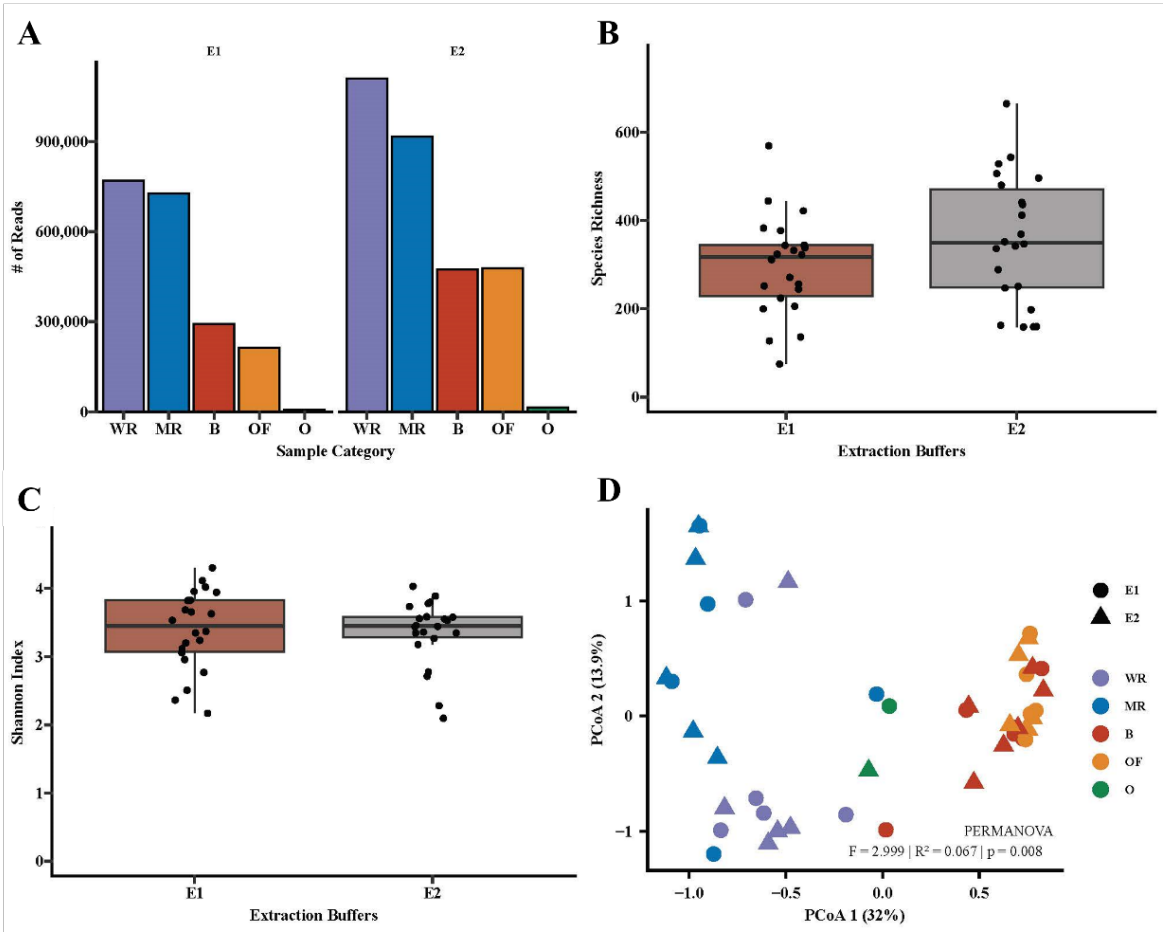


Figure 5: Sequencing and statistical metrics comparison of E1 and E2 extraction buffers with shelf-stable amplification buffer (P2). A) Number of reads generated via sequencing per environmental swab location category: women's restroom (WR), men's restroom (MR), breakroom (B), office area (OF), or other (O). B). Similar species richness based on extraction buffer. C) Shannon diversity indices demonstrating comparable community evenness between extraction methods. D) Bray-Curtis dissimilarity by sample category and extraction buffer used depicting clustering by sampling location.

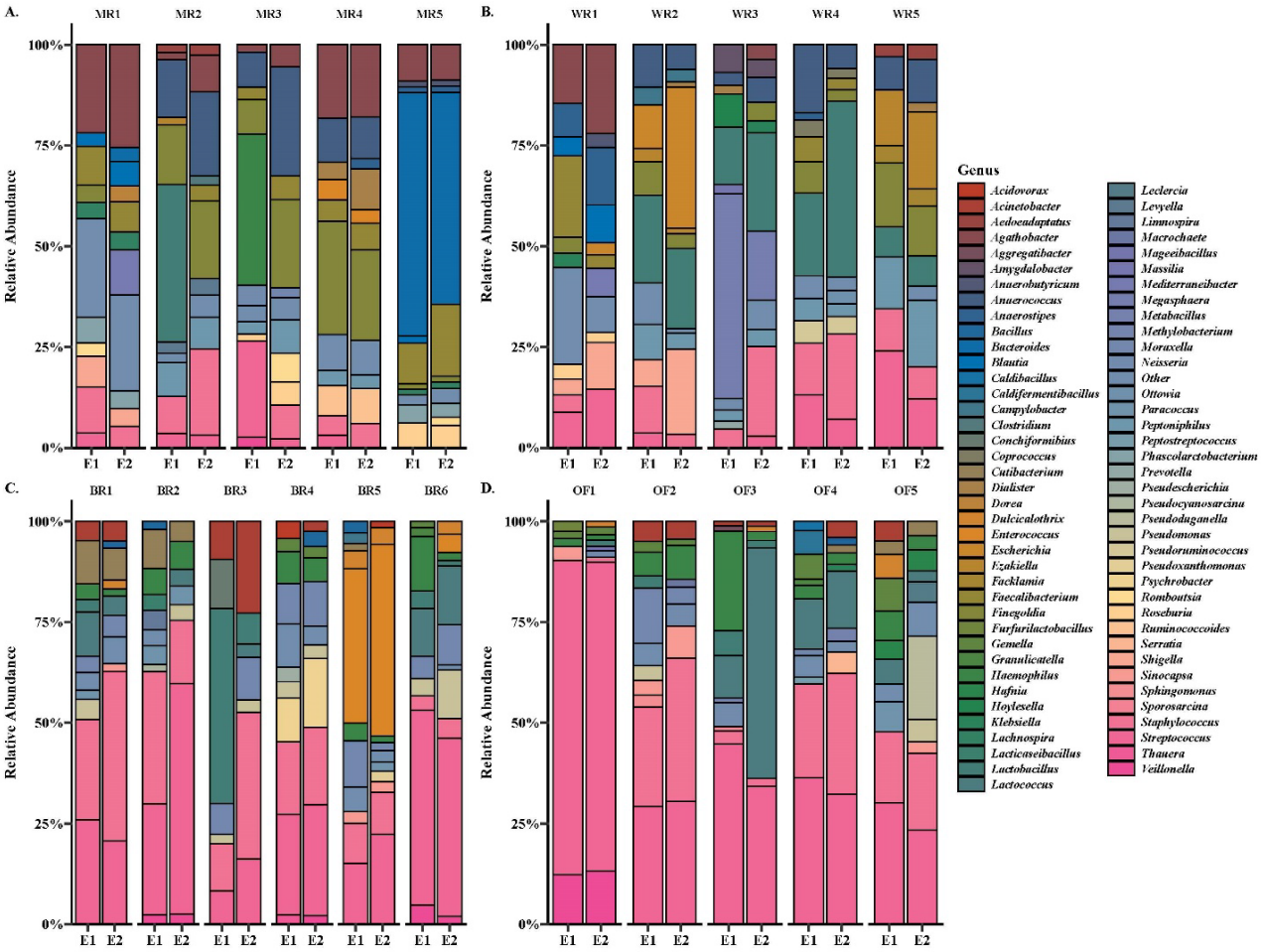


Figure 4. Microbial Profile of the top 10 genus identifications present for each swab sample category. Community profiles comparing E1 and E2 for A) the men's restroom (MR) B) the women's restroom (WR), C) the breakroom (BR), and D) an office area (OF). All samples were sequenced using the shelf-stable P2.

D. Shelf-Stable Artemis Method

To evaluate the shelf-stable Artemis method as compared to the ISS swab-to-sequencer method, a defined microbial mock community standard was evaluated. This standard consists of ten unique species of bacteria in which both methods successfully recovered all of the organisms in the standard (Figure 7). *Escherichia coli* was consistently the most abundant species detected by both methods, while the remaining community was recovered at a similar relative abundance, suggesting minimal bias toward relative abundance estimations using the Artemis method. Although present at low abundance within the mock community, *Deinococcus radiodurans* was present in both datasets, demonstrating that the Artemis method is capable of recovering taxa known for extreme stress tolerance, which are notoriously difficult to lyse. The reproducible detection of this extremophile highlights the sensitivity of the Artemis workflow and supports its potential applicability in future low-biomass or extreme-environment microbial monitoring contexts.

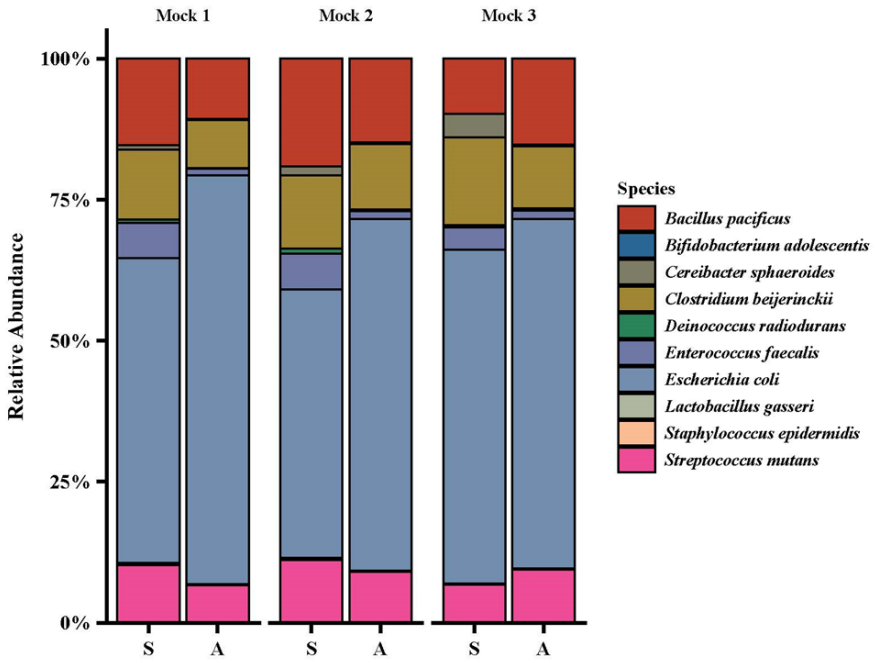


Figure 5. Species-level identification of a mock community standard comparing the Artemis method to the ISS swab-to-sequencer method. Species-level identifications of the ten-strain whole cell standard in triplicate prepared and sequencing with both methods.

IV. Conclusion

Microbial monitoring of crewed spacecraft has provided critical insight into the spacecraft-associated microbial environment and the effectiveness of the ECLS systems and associated remediation strategies.[11, 12] Over the past decade, the implementation of nanopore sequencing in space has further advanced this understanding by enabling culture-independent microbial identification without the need for sample return to Earth. The swab-to-sequencer methodology employed in the BEST payload[5] and BioMole facility[10] represents a significant advancement in onboard microbial monitoring and enables microbial identification beyond low Earth orbit (LEO), with planned application as the MSM for Gateway and as a research payload on Artemis IV. Given the constraints imposed by Artemis missions, specifically the lack of cold stowage, the development of a fully shelf-stable workflow is essential. The work presented in this study represents an initial step toward finalizing such a protocol. While an optimal DNA extraction method and PCR reagent were identified for the Artemis method, additional research and development is underway by ONT to transition proprietary library preparation reagents into shelf-stable formulations.

Upon availability of all required reagents, the proposed method will undergo stringent and extensive validation as compared to the current swab-to-sequencer workflow. While surface sampling remains the primary focus in the short term, future efforts will extend the shelf-stable methodology to additional sample types, including water. As NASA advances human exploration missions through the Artemis Program, in situ sequencing as a core capability will play a critical role in crew health monitoring and toward maintaining vehicle integrity.

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