



Nanopore Sequencing-Based Microbiome Profiling of the Water Recovery System Onboard the International Space Station

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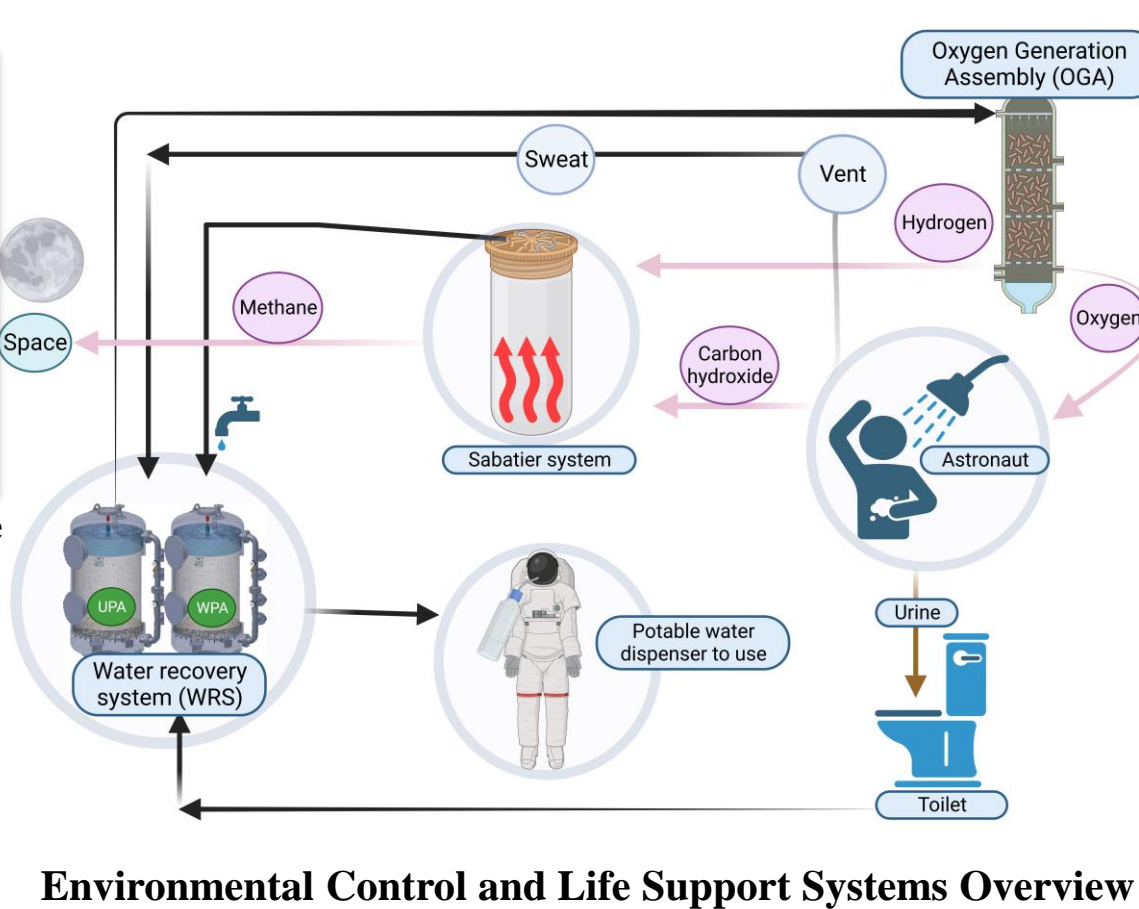
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Introduction

- A distinctive microbial community has inhabited the International Space Station (ISS) Water Recovery System (WRS) for over 14 years and has experienced the stressors associated with the microgravity environment.
- The WRS consists of the Water Processor Assembly (WPA) and the Urine Processor Assembly (UPA).
- The WRS generates potable water from urine distillate, humidity condensate, Sabatier product water, and the occasional off-loading of ground-supplied water.
- The reservoir for these products, the wastewater tank, do not have a means of microbial control.



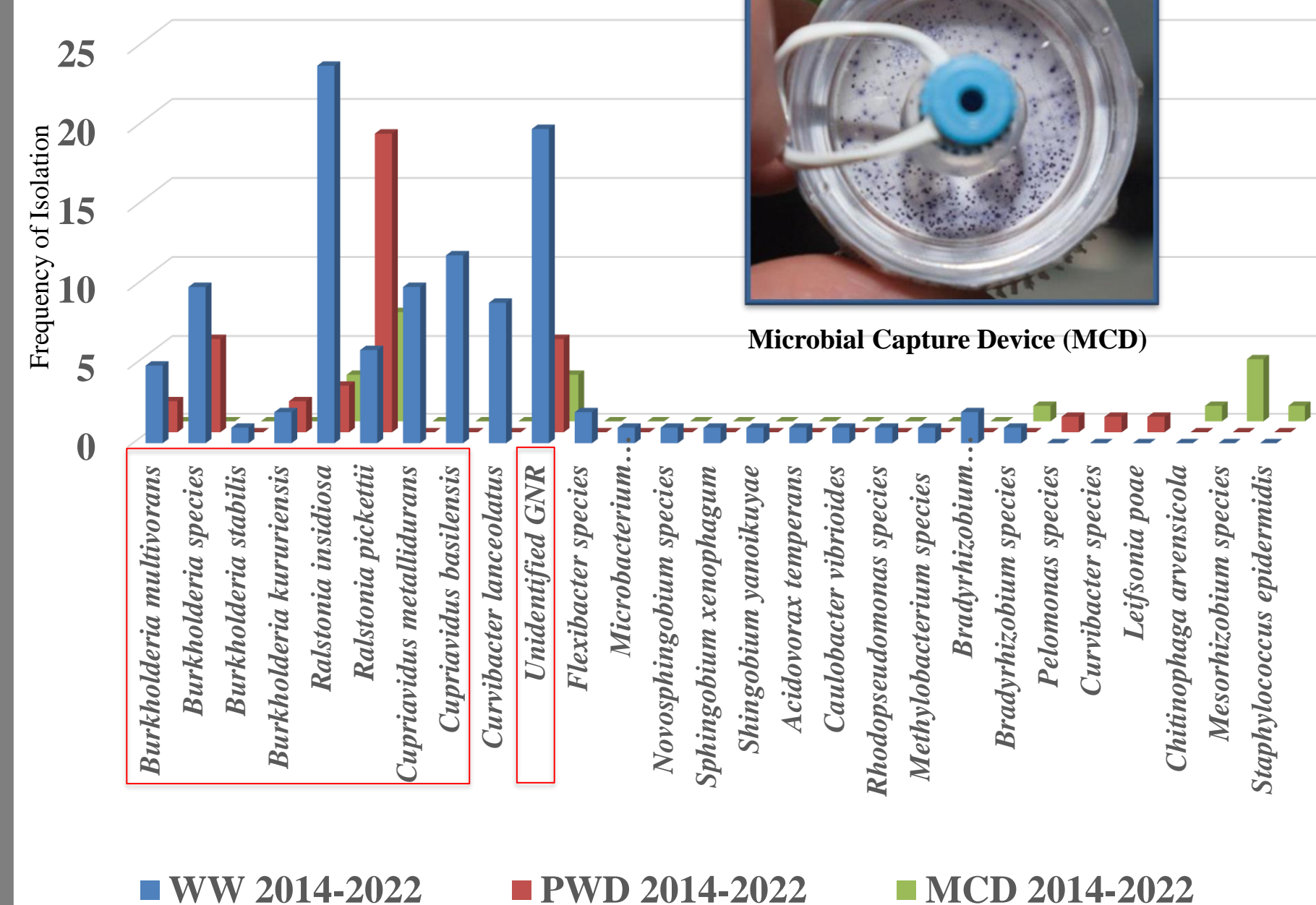
Coliform indicator bag (in-flight) and post-flight analysis bag (returned to ground for analysis)



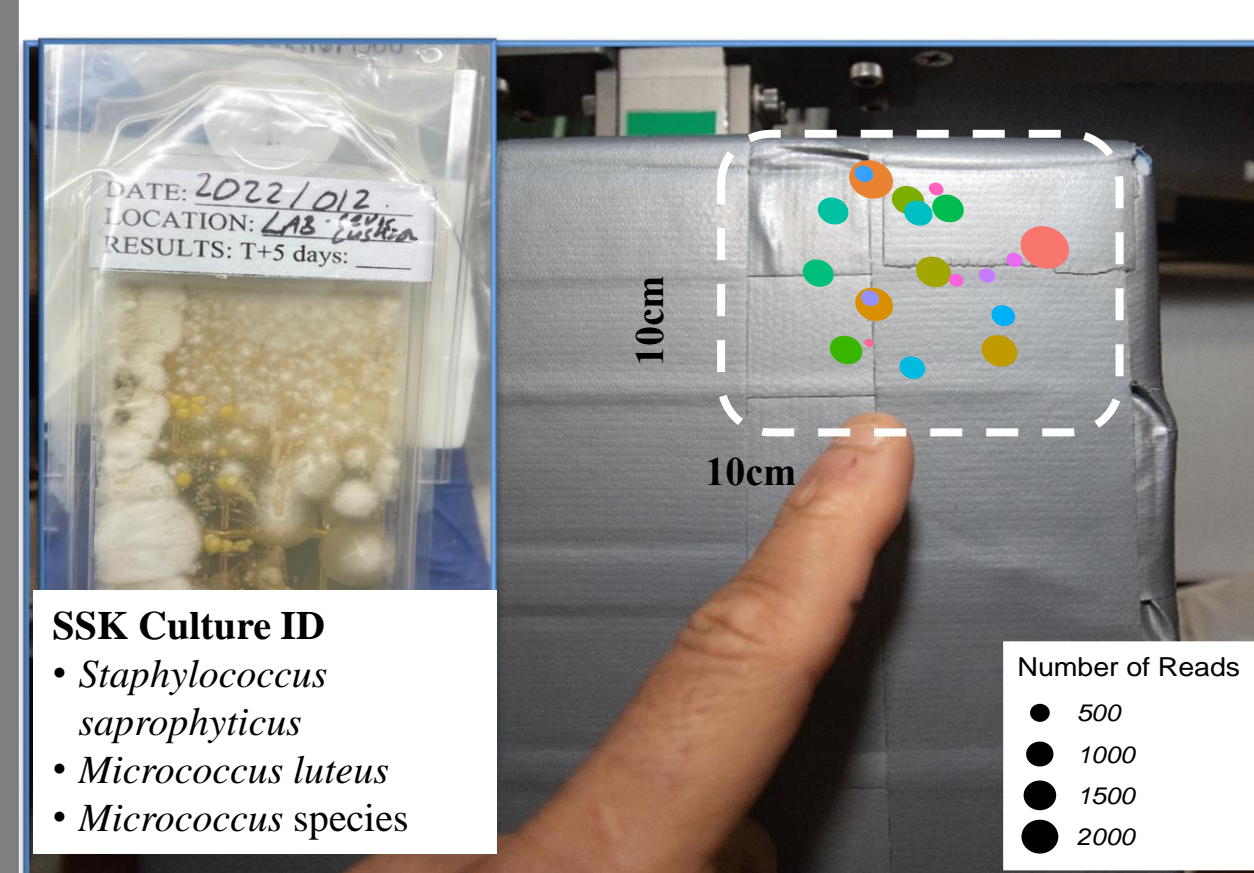
Environmental Control and Life Support Systems Overview

Current *in situ* microbial monitoring is limited to monthly coliform analysis and quarterly culture-based assessments. Additional analysis of potable water, wastewater, and condensate sources are collected into Teflon bags for return followed by ground analysis. The time between sample collection and the return to Earth, as well as the lack of preservation, results in:

- A skewed depiction of the microbiome.
- Routinely observed from these returned water samples are high counts ($10^5 - 10^6$ colony forming units per mL) and three prevailing genera, *Ralstonia*, *Burkholderia* and *Cupriavidus*, as well as a high abundance of unidentified organisms.
- There is likely a more diverse microbiome than observed with the current methods, and a higher diversity of bacteria and fungi has been noted by previous research.



- To accurately characterize the microbial profile of the wastewater tank, analysis needs to occur at the time of sample collection.
- A method for *in situ* water analysis based on nanopore sequencing was developed.
- This filter-to-sequencer method evolved from previous work that has been validated using the culture-independent, swab-to-sequencer method onboard the ISS during the Biomolecule Extraction and Sequencing Technology (BEST) payload and the Crew Health Care Systems (ChCS) BioMole Facility¹⁻³.



Astronaut Raja Chari performed the swab-to-sequencer method with a sample from the CEVIS back rest. The nominal culture method was also used. All Earth-based identifications from the culture isolates matched the *in-situ* data collected onboard the ISS.

Optimization from Surfaces to Water:

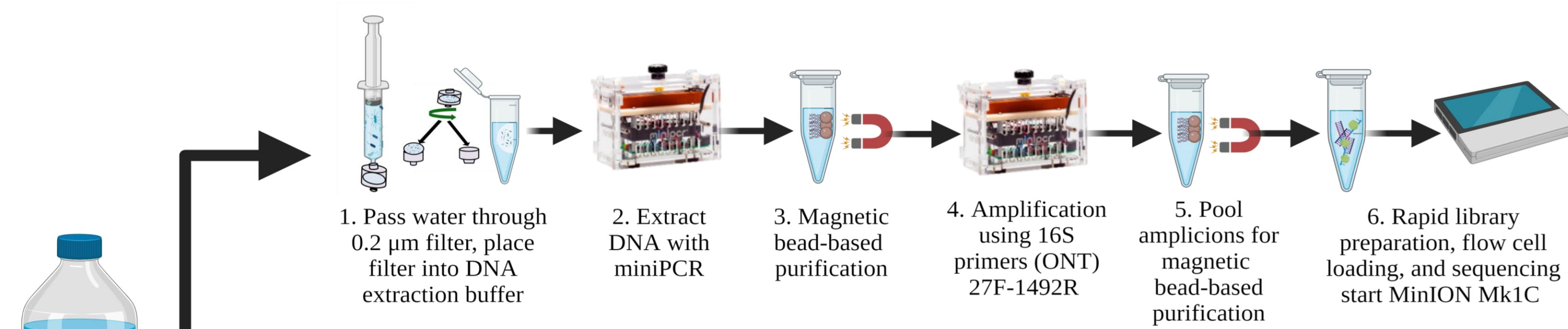
- Filter for microbial capture in place of a swab
- DNA extraction modifications
- Low biomass detection from potable water will require thorough ground validation

Acknowledgements

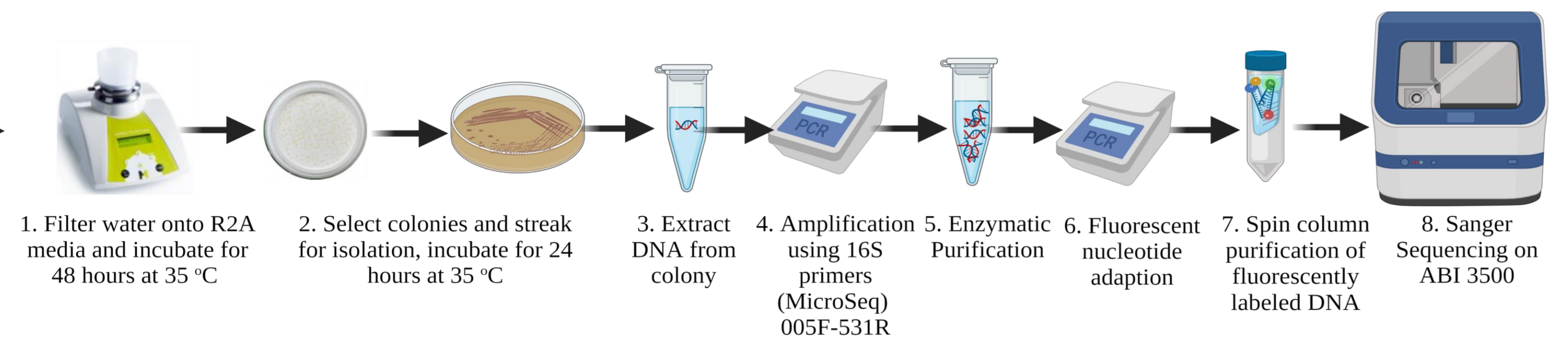
This work was funded by Mars Campaign Office-Life Support Systems (formerly Advanced Exploration Systems and Exploration Capabilities). Additional support was granted by the ISS Vehicle Program Office. We thank W. Schneider, I. Stambaugh, C. Meyer, J. Gensler, M. Everett, and A. Premer, as well as the entire Crew Health Care Systems team.

Method

A. Culture-Independent Filter-to-Sequencer Protocol



B. Culture-Dependent NASA JSC Microbiology's Standard Protocol



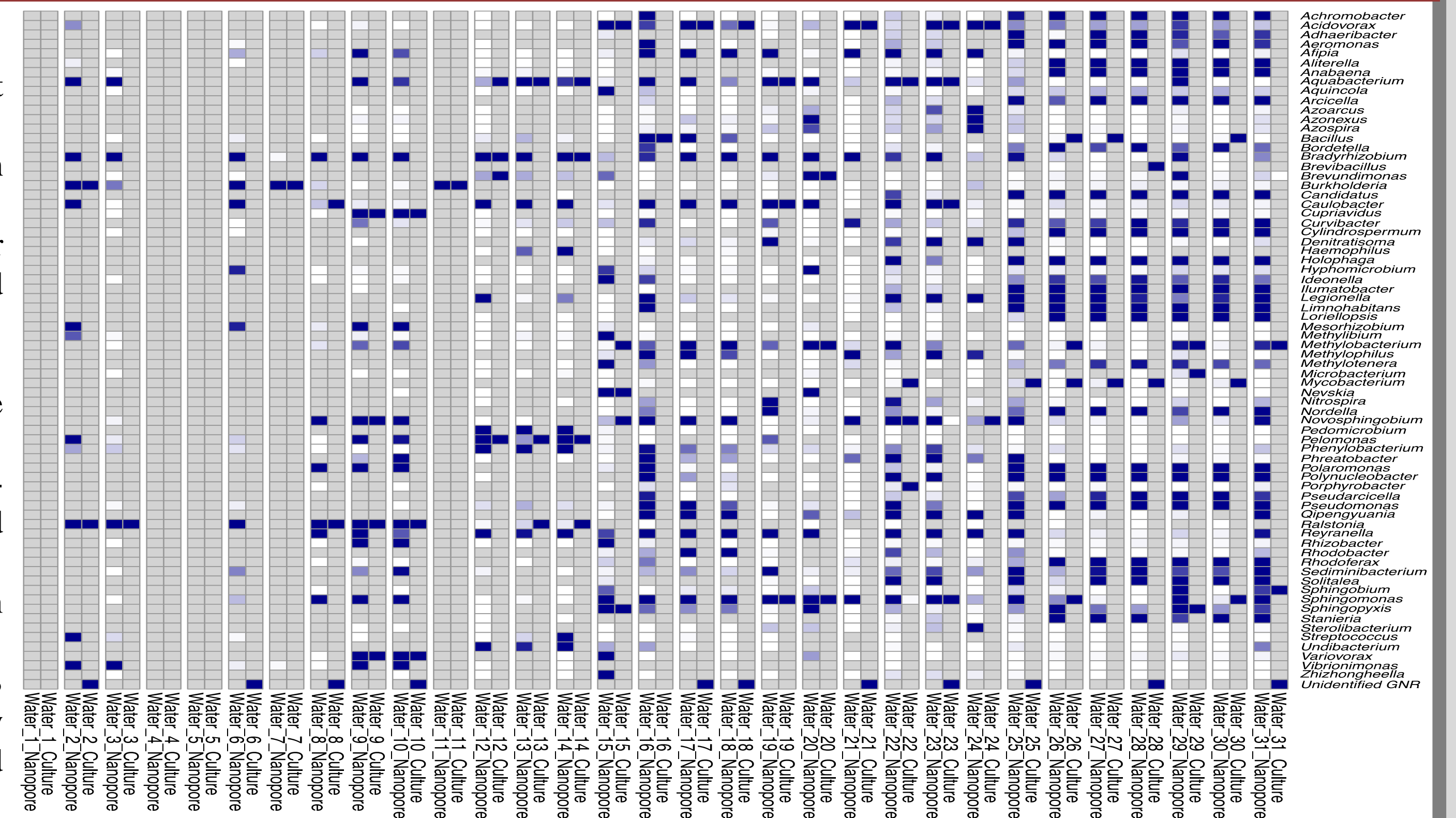
- Water samples were collected from ground water sources, as well as returned ISS water
- Water samples were split to allow for parallel processing:
 - Filter-to-sequencer, culture-independent method
 - Johnson Space Center Microbiology Laboratory's standard operating procedures for Sanger sequencing-based identification from cultures
- Filter-to-sequencer protocol and analysis was modified from Stahl-Rommel *et al.*³
- Basecalling and demultiplexing occurred on the MinION Mk1C using the high accuracy mode
- Reads were mapped to the NCBI Reference Sequence (RefSeq) Database using minimap2 version 2.24-r1122.

Figure made with BioRender

Ground Validation: Potable Water

Results:

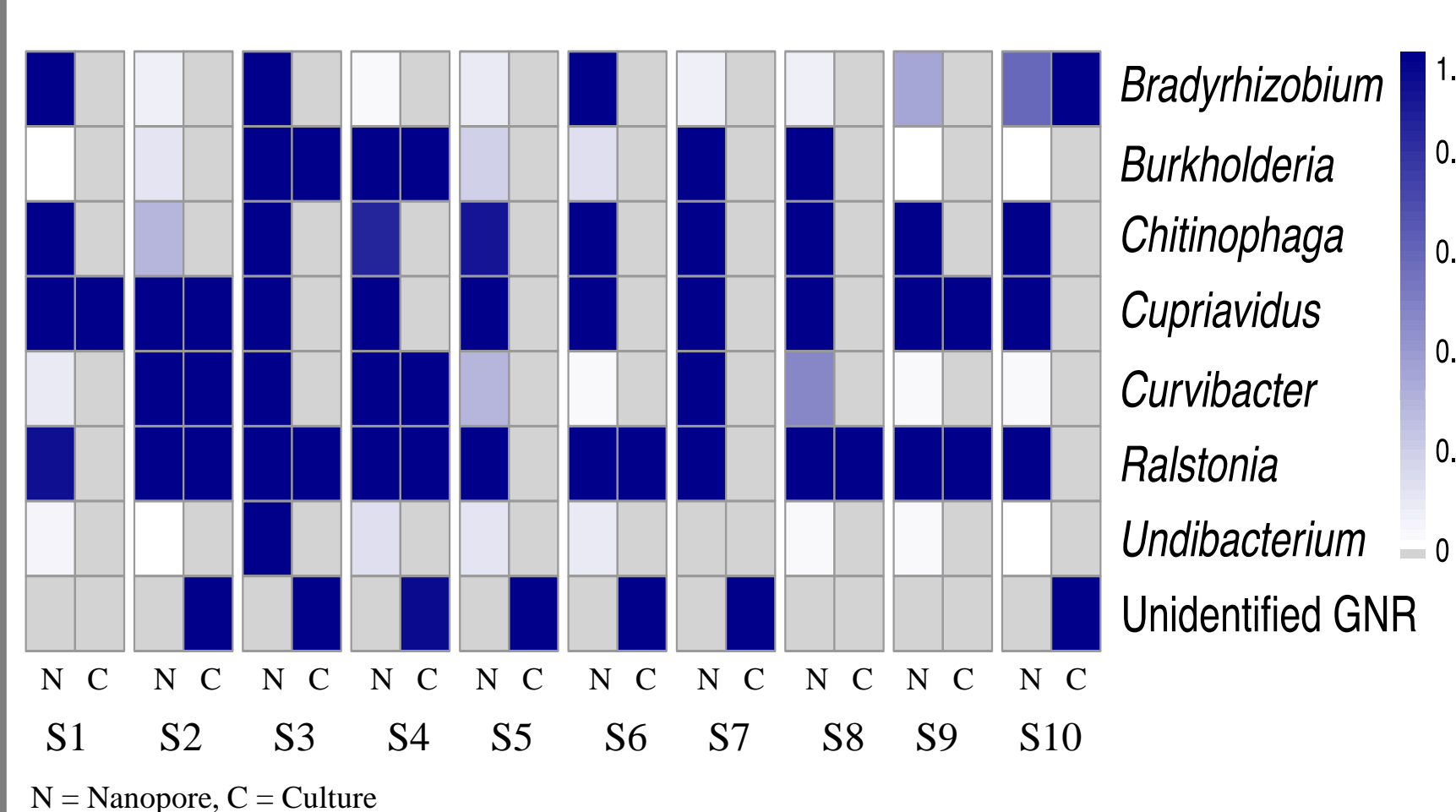
- Negative controls were negative (data not shown)
- Three of the samples were negative for both culture isolates and nanopore reads
- From the 31 independent potable water samples originating from both the ISS and ground sources:
 - 83 culture isolates were obtained
 - Sanger sequencing speciated 60% of the isolates
 - 14% could not be identified beyond Gram-negative rod (denoted as unidentified Gram-negative rod, UGNR)
- A high number of UGNRs is common based on the laboratory's SOP
- Identifications from the returned ISS potable water (samples 1-11) were mostly *Burkholderia* spp., *Ralstonia* spp., and UGNR
- Ground water (samples 12-31) identifications were largely *Mycobacterium* spp., *Acidovorax* spp., *Aquabacterium* spp., *Sphingomonas* spp., *Methylobacterium* spp., and UGNR.
- The filter-to-sequencer nanopore identifications matched to culture isolate identifications at 99% for both genus and species (data not shown) when the UGNRs were removed. As expected, the culture-independent data revealed a wider degree of bacterial diversity.
- In the rare instance where the culture-based method demonstrated the presence of an isolate not noted in the nanopore data, e.g., *Brevibacillus* sp. in sample 28, the NASA risk assessment would not have been altered.
- The data support the ability of this nanopore sequencing-based method to identify a wide diversity of microorganisms beyond traditional culture-based method limitations, which, in turn, facilitates a more advanced microbial risk assessment.



Ground Validation: ISS Wastewater

Results:

- Further comparison of the methods was completed for 10 returned ISS wastewater samples.
- Culture identifications from these samples were largely UGNRs and *Ralstonia* spp.
- The filter-to-sequencer identifications matched the culture isolate identifications at 100% to both genus and species (data not shown) when the UGNRs were removed.
- The culture-independent method also identified *Chitinophaga* spp. and *Undibacterium* spp., which were not observed in culture data. Based on whole genome sequencing, it is likely that some of the UGNRs are *Chitinophaga* spp.



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References: (1) Carter, D.L. Status of the Regenerative ECLSS Water Recovery System. Proceedings of the 39th International Conference on Environmental Systems, Savannah, GA (2009) 2009-01-2352. (2) Castro-Wallace et al. Nanopore DNA Sequencing and Genome Assembly on the International Space Station. *Sci Rep* 7, 18022 (2017). (3) Burton et al. Off Earth Identification of Bacterial Populations Using 16S rDNA Nanopore Sequencing. *Genes* 11, 76 (2020). (4) Stahl-Rommel et al. Real-Time Culture-Independent Microbial Profiling Onboard the International Space Station Using Nanopore Sequencing. *Genes* 12, 106 (2021).

Validation Onboard the ISS

Consumable kits and flow cells needed to support *in situ* filter-to-sequencer analysis of the ISS potable water was launched on NG-19 (August 1st, 2023). Initial method validation is underway as part of the ChCS BioMole Facility. Data analysis is in work.



Astronaut Jasmin Moghbeli collected water from the PWD



Water was passed through 0.22 µm filter



DNA was extracted, purified, amplified, and libraries prepped



DNA was sequenced using the MinION 2.0 (MK1C)

Conclusions

- Ground validation of the culture-independent, filter-to-sequencer method demonstrates its ability to provide comparable data to the current culture-based method.
- This method can be completed entirely onboard the ISS to evaluate microbial populations in water systems. Implementation of this protocol moves NASA one step closer to a rapid, in-flight, sample-to-answer microbial profiling capability to enable human exploration of the Moon and Mars.
- BioMole data will result in the first accurate characterization of the microbiome within the Water Recovery System.

All images of the ISS and crew members are credited to NASA.