More Than a Decade of International Space Station Microbial Sampling in the Environmental Control and Life Support Systems

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Microbial monitoring has taken place in major units belonging to the Environmental Control and Life Support Systems (ECLSS) of the International Space Station (ISS). For at least a decade multiple modules including US and RU segments were sampled. In the US ECLSS, water reclamation and air systems combine their microbial contents downstream where biofilm incidents have been recorded. Understanding the microbial contents of segments provides a longitudinal perspective to microbiome changes in the system that contribute to this issue. The US ECLSS have been consistently sampled, especially in the Potable Water Bus (PWB) where bacteria from upstream sources have been observed.

I. Nomenclature

CFU = Colony Forming Unit
ECLSS = Environmental Control and Life Support System
EFA = External Filter Assembly
HHP = Human Health and Performance
ISS = International Space Station
JSC = Johnson Space Center
MCD = Microbial Check Valve
MF Bed = Multifiltration Bed
MORD = Medical Operations Requirements Document
NASA = National Aeronautics and Space Administration

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ORU = Orbital Replacement Unit
PWB = Potable Water Bus
PWD = Potable Water Dispenser
QD = Quick Disconnect
R2A = Reasoner's 2 Agar
SDA = Sabouraud Dextrose Agar
UD = Urine Distillate
UPA = Urine Processor Assembly
WPA = Water Processor Assembly
WRS = Water Recovery System
WW = Wastewater

II. Introduction

The International Space Station (ISS) hosts a diverse group of microbial organisms. After at least a decade of operations in ISS, a resident group of microbes has been established on surfaces such as tables, walls, and panels[1]. The changing astronaut crews have provided changes to the existing microorganism communities - microbiome[2,3]. It is understood that the crew has an impact on the cabin microbiome, which may extend to the microbiome in the life support systems. However, the microbes present at the inner surfaces of this and other space systems are hard to study due to the limitations of microbial monitoring instrumentation, due to inaccessible surfaces, and constraints in crew time.

The Water Processor Assembly (WPA) is the part of the life support systems at the ISS that cleans process water from urine and cabin humidity condensate that is then turned into drinking water (**Figure 1**). The ISS WPA has been monitored for the presence of microbes for more than a decade and parts of it have been a target of several biofouling studies. The inner surfaces of the WPA are a contained environment in which pressure and flow changes may affect performance. Therefore, it is challenging to sustain real-time monitoring of changes in microbial presence and of individual bacterial genomic changes. Thus such real-time monitoring has never been performed. However, microbial monitoring in the Water Recovery System (WRS) via classical microbiological methods has been an instrumental task when determining potential system failures or existing risks to astronaut health. Classical methods are dependent on mission schedules, sample return, and availability of consumables.

In this paper we first summarize the microbial monitoring efforts in the the US ISS life support systems during the last decade and then review the known and potential sources of microbial contamination in it. This would provide an overview of the different ways the sections of the life support systems were sampled and monitored for microbial presence. These monitoring efforts throughout the years have provided a large amount of data and we analyze these data to determine the longitudinal overview of present microorganisms in the monitored sections of the life support system.

We hope that these analyses will help the efforts by the National Aeronautics and Space Administration (NASA) at the NASA Johnson Space Center (JSC) and the Human Health and Performance Directorate to develop streamlined methods of microbial monitoring for future missions. Due to the severity and distance of the planned missions to the Moon and Mars, future missions will require different microbial motinoring methods – with more automation and diminished handling of microbial samples. The advantages of the improved microbial monitoring will allow to track microbial changes in the life support systems before biofilm formation or progression of unwanted microbes through the system will lead to the system operation failures. The mitigation of biofilm is of special importance to advance the current life support systems into the anticipated Mars habitation standards.

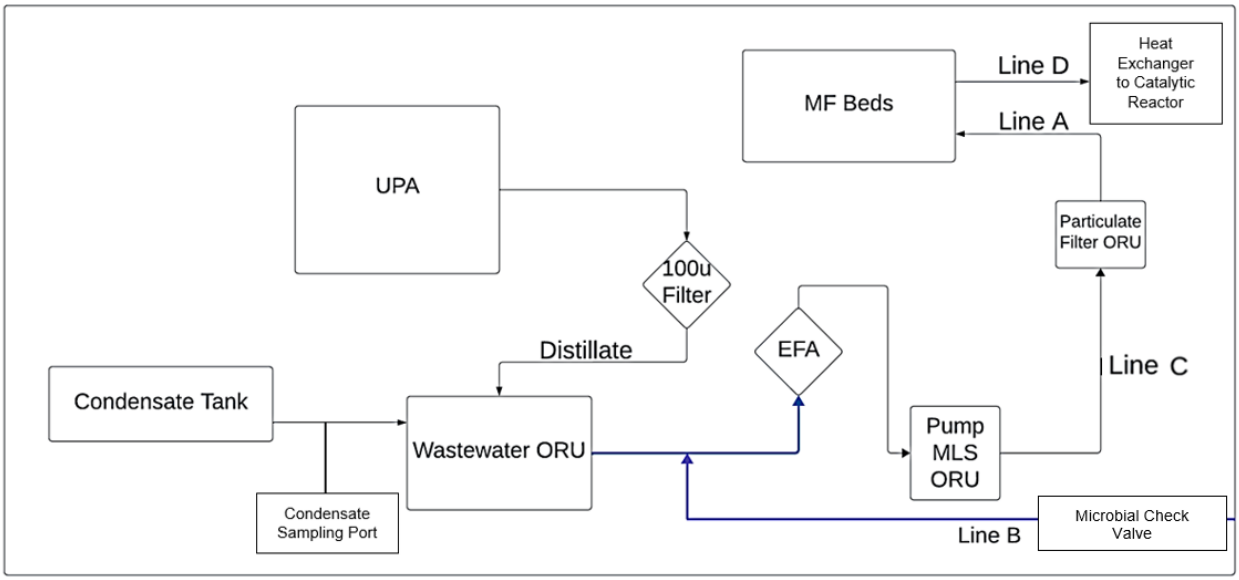

Figure 1. Simplified schematic of relevant process lines in the WPA.

III. Overview of the WPA Microbial Sampling Methods and History of Sampling

Currently, there are different ways that the ISS life support systems are sampled and monitored. In this section we describe those water sampling methods. Such methods result from classical sampling such as plating and colony forming unit (CFU) counts. Identification is performed by 16S rRNA gene and ITS sequencing of individual microbial isolates and therefore this identification has only been available for culturable organisms. Sampling bias has been present when it comes to system sampling of preference, where it is clear that some segments have been sampled more than others through the years.

Monitoring of the Potable Water Dispenser (PWD) is indispensable to maintaining crew health, as the direct component involved in dispensing potable water that should be safe for astronauts to use. As such, the Microbiology Laboratory in JSC has carried out downstream procedures for microbial monitoring of this component for more than a decade. The PWD is sampled in three ways: in-flight for the presence of coliforms, through a Microbial Capture Device (MCD), and through an attachable water bag in the dispensing needle[4].

As stated in the ISS Medical Operations Requirements Documents (ISS MORD)[5] water samples are analyzed and verified to fulfill the water quality standards. Potable water quality is monitored monthly for the presence of coliforms, with the requirement being non-detectable per 100 mL[5]. Quarterly monitoring is performed with the Microbial Capture Device (MCD) to determine the level of bacteria, as well as their identity upon sample return to Earth[5]. Coliform analysis is based a colorimetric indication, and bacterial colonies are visualized and enumerated on the MCD filter via metabolic dye[4]. The microbial acceptability for bacterial levels is < 50 CFU/ mL[5]. Additionally, PWD archive bags are collected simultaneously with the MCD samples and returned for ground-based analysis. Bacteria in these samples are isolated, identified and archived. It is important to note that a true assessment of wastewater within the WPA is not possible. While archive bags are collected, neither the microbial level or identities are a true representation of the community, as the time between sample collection and receipt in the laboratory leads to a few types of bacteria outcompeting others.

Unlike the PWD monitoring with the three main sampling procedures, upstream components of the WPA are less strictly monitored as the water is still not ready for consumption. Similarly other components are analyzed in a less stringent way when it comes to water microbiological analysis. Water bags are used to collect samples in life support system sampling locations, such as the humidity condensate, the multifiltration beds, and the wastewater line, attached to quick disconnects. These water bags are analyzed via R2A medium plating for bacterial contents and Sabouraud Dextrose Agar with Chloramphenicol (SDA) for fungal contents. Cultured isolates are identified via current methods such as 16S rRNA sequencing for bacteria and LSU sequencing in combination with morphological method for fungi. There are no microbiological requirements for wastewater, humidity condensate, or multifiltration bed water. Biofilm-related requirements for detection and analysis also do not exist.

The main sample port for the multifiltration beds is process line D (**Figure 1**), for the wastewater tank it is process line B. The condensate samples originate from the condensate sample port. Meanwhile, urine distillate samples are not generally obtained.The only example of this being 2010 samples obtained via line B after a flush through the wastewater tank. Additionally, hardware under refurbishment and replacement is also sampled as needed[6].

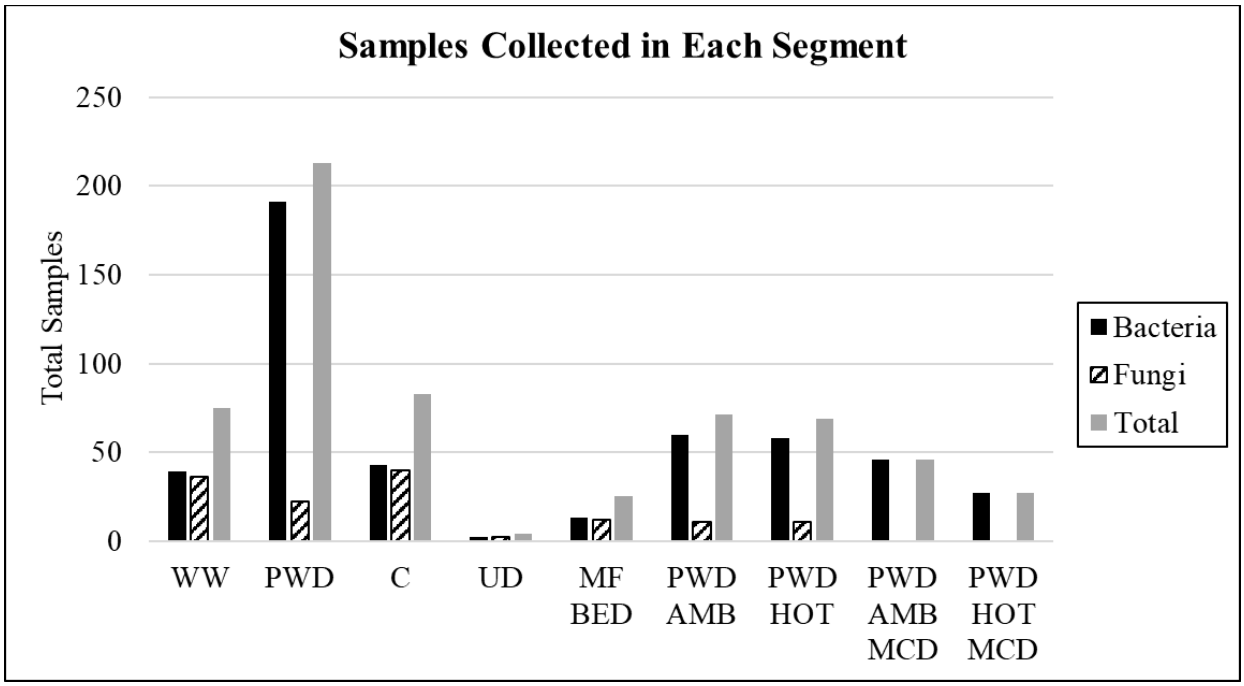

Figure 2. Samples collected from 2009 to 2022, including those with no detected microorganisms, related to each segment. *The segments are labeled as wastewater (WW), potable water dispenser in totality (PWD), humidity condensate (C), urined distillate (UD), Multifiltration Bed (MF BED), the potable water dispenser's ambient water line (PWD AMB), the potable water dispenser's hot water line (PWD HOT), and the MCD samples for both*.

IV. Analysis of Potential Sources of Microbial Contaminants in the WPA

Biofilm has been found in grounded equipment in the past, indicating proliferation of microbial organisms in the system[7], specifically associated to the Wastewater Orbital Replacement Unit (ORU). Many organisms have been isolated from the PWD, as previously explained by Maryatt and Smith in 2017[10], indicating some breakthrough bacterial spread in the PWB. This points to the loss of iodination effectiveness in the potable water, as described in their research. Dead legs have been proclaimed an issue in other parts of the system[16], but have not been explored in the PWD. The lowered performance may have allowed bacterial organisms to make their way to the dispenser, such as *Ralstonia pickettii* and *Burkholderia multivorans*[4]. However, these organisms have made their way to the PWD from various potential sources through the system. This section gives a brief overview of the major processes that aim to eliminate microbial contamination. For each of the steps it discusses known or possible sources of microbial flow or contamination with bacterial and fungal organisms that were found over the years in the ISS US segment's water system via microbiological monitoring and then archived by the JSC Microbiology Laboratory.

A. Urine Distillate Stream

It has been previously pointed to the existence of *Burkholderia* spp. and *Ralstonia* spp. in the upstream locations of the WPA where urea has already been removed, and water has been separated and is stored in the system[1,8]. The initial presence of these bacteria could be related to the environment in which systems are manufactured, entrance from the urinal, or from the water collected in air systems, called humidity condensate. It was stated in 2000 by the

National Research Council[9] that likely contaminants of the water reclamation system would be constituents of human urine, humidity condensate, and wash water. For other areas of the system, data is unpublished or partially published, as in Zea, 2020[1]. Furthermore, novel species have been found within the ISS[10], and some bacteria remain unidentified[4]. Academic consensus for predominant bacteria in ISS surfaces and water varies.

Urine contents in the ISS WRS are treated with an oxidizer and an inorganic acid to stabilize chemical and microbial constituents, which was still an undetermined process in the 2000s. The Russian and US segments both use chromium trioxide as an oxidizer but vary with respect to the inorganic acid[11]. The Russian segment utilizes sulfuric acid, while the US segment utilizes phosphoric acid due to necessitated efforts to mitigate calcium sulfate precipitation. The inorganic acid lowers the pH, while also strengthening the chromium trioxide oxidizer by providing protons for the oxidation reaction[12]. Chromium trioxide reacts with the inorganic acid, along with other urine contents, to form hexavalent chromium. Additionally, urine undergoes a harsh distillation process that exposes the stream to very high temperatures[13].

B. Humidty Condensate

Alternatively, humidity condensate is collected by the heat exchangers and contain all contaminants belonging to the cabin air that survive the temperature of 4-10°C. There is a possibility that cabin air and moisture have had direct contact with surfaces and crew, rich sources of microorganisms. These microbial sources influence waste streams. Other in-line sources of microbes are the decommissioned Sabatier system and the Russian cabin streams. These are less often discussed in literature associated with the WPA microbes.

C. Contamination During System Assembly

Fabrication, assembly, and integration of each WPA ORU serves to ensure the unit not only meets operating parameters, but also adheres to the corresponding cleanliness standards. The degree to which a particular unit is cleaned depends upon its location in the flow path and its specific purpose in the system. Initial fabrication of the individual components is conducted in an open environment without stringent cleanliness standards. From this point forward, however, each of the units undergoes rigorous cleaning procedures unique to the particular Orbital Replacement Unit (ORU). For some units, this involves meeting specific particulate limitations. For others, sterilization is required in addition to particulate constraints. At a fully integrated ORU level, each unit is required to meet a specific particle standard which designates the specific particulate restriction.

Particulate standards serve to regulate cleanliness by identifying the maximum allowable quantity of particles of a specific size. This level is achieved by cleaning the individual pieces to meet this corresponding standard- usually Level 200 or Level 300. Note that certain assemblies are cleaned to Level 200A or Level 300A, which means there is a Non-volatile Residue constraint. Subsequent to cleaning, these parts are assembled into the ORU configuration in a cleanroom which meets or exceeds the requisite 200 or 300 level. Upon integration, each unit is tested to make sure the particle constraints are being met. This involves flushing the ORU with an approved verification fluid and analyzing the sample at its outlet. The effluent sample is filtered, dried, and examined at a microscopic level. If required, the sample is also analyzed for non-volatile residues.

Once the particle standard has been met and verified, clean quick-disconnect (QD) caps are installed as a means of maintaining the resultant cleanliness level. For units at the beginning of the water processing cycle, particulate standards are maintained, and no further cleaning is necessary. This includes the Wastewater ORU, Pump Separator ORU, Particulate Filter ORU, and Sensor ORU. The next series of ORUs are those which contain various types of resins. Each of these units is not only maintained at a certain particulate level but is also subjected to gamma radiation. Irradiating the ORU serves to eradicate microbes from the resin and therefore sterilize the system prior to operation. This process is utilized for the Multifiltration Bed ORU and Ion Exchange Bed ORU. The Microbial Check Valve ORU contains resin but does not undergo irradiation because it houses an iodinating media that serves as a biocide. Finally, units at the end of the water processing cycle must undergo high heat sterilization. These ORUs are maintained at a specific particulate level and are also sterilized in an autoclave. These cleanliness standards encompass the Catalytic Reactor ORU, Gas Separator ORU, Reactor Health Sensor ORU, Water Storage ORU, and Water Delivery ORU. It is essential to sterilize these ORUs because they will be in the path of potable water flow or will serve as storage for potable water.

In conclusion, although NASA has stringent cleanliness requirements for other assemblies such as robotics and search of life missions, these cleanroom and sterile techniques did not extend to the WRS in the past.

V. Longitudinal Analysis of Available Archived Microbial Monitoring Data

Using the archived data on microbial monitoring at ISS water systems, we were able to establish timeline of viable microorganis appearing in different segments.

A. Urine Distillate

According to the data stored by the JSC laboratory, two water samples were obtained from Line B pertaining to the urine distillate. The samples were obtained in March 31 and April 11, 2010, then received on April 22, 2010 in Houston, TX. Such samples were taken from Line B after the urine distillate was flushed through the Wastewater tank. As such the sample may have contained contaminants leftover from the wastewater. In 2010, the organisms found were identified as *Burkholderia multivorans*. Despite there being analysis for fungal species, no fungal isolates were detected.

Previously, in January 2021, two flex lines from the Urine Processor Assembly (UPA) system were returned for refurbishment purposes, which provided the opportunity of evaluating this section of the WRS for the first time. *Burkholderia* sp. dominated these samples along with *Paraburkholderia* sp. and *Leifsonia* sp[5]. These genera were also recorded in previous archive returned samples.[4]. The isolation of an unidentified Gram-positive had not been previously reported from the UPA, which made this a significant finding. Noticeably, *Fusarium* sp. was also recovered from UPA flex lines, which had not been previously isolated from archive or in-flight samples. Genomic analysis of the bacteria isolated revealed chromate resistance and biofilm formation potential, which differentiated these organisms from those previously isolated.[5]

B. Humidity Condensate

The humidity condensate data spanned multiple years and bacterial and fungal isolation was performed 43 and 40 times in that span, from 2009 to 2022. In the span of time a majority of bacteria identified belonged to the genus *Ralstonia* (38%). Encompassing more than half of the bacterial samples (88%), were *Cupriavidus* spp. (28%), *Ralstonia* spp. (38%), and the unidentified Gram-negative rods (22%) comprised the total of those samples. In the same span of time the genus *Wautersia* was renamed as *Cupriavidus* and as such is included in total *Cupriavidus* counts. The majority of *Cupriavidus* species were *C. metallidurans* (56%) and *C. basilensis* (44%). *Ralstonia* spp. were listed as *R. insidiosa* (81%) and *R. picketti* (19%). Other counts for bacteria present in the humidity condensate were found as in **Figure 3**. As no identity is provided for the Gram-negative rod, there is no clear distinction of whether these samples belonged to the same genus or species. Fungal isolates were only detected in 2009 and 2010, *Aureobasidium* (1), *Acremonium* (1), and *Hyphomycetes* (1).

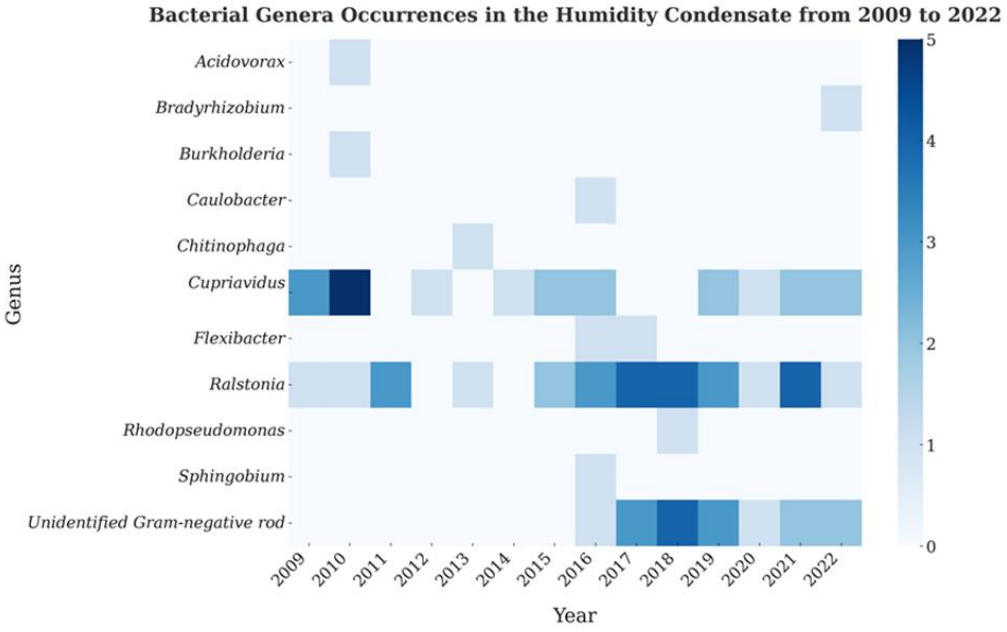

Figure 3. Heatmap of identified bacteria in the humidity condensate samples. *The white-blue color indicates 0 identified bacteria, while darkest blue indicate the highest number of identified bacteria of the same genus, in this case 5.*

C. Wastewater Tank

The wastewater samples acquired from line B have a majority of *Ralstonia*, *Cupriavidus*, and *Burkholderia* (**Figure 4**). There were in total 75 samples corresponding to this section, 39 of them belonging to bacterial sampling methods, and 36 to fungal sampling. The unidentified Gram-negative rods were found present in this section as well. In contrast, *Curvibacter*, *Microbacterium*, *Novosphingobium*, and *Sphingomonas* were present in the wastewater but not in the condensate. However, *Bradyrhizobium*, *Caulobacter*, *Chitinophaga*, and *Rhodopseudomonas* are present in the condensate but not in the wastewater. The *Ralstonia* present belonged to the *R. picketti* (30%) and *R. insidiosa* (70%) species, whereas the *Cupriavidus* belonged to *C. metallidurans* (56%) and *C. basilensis* (44%). *Burkholderia* in the wastewater were identified as *B. multivorans* (59%) and "no species" (41%). Six different fungal genera were detected in the wastewater, five out of ten identified isolates being *Lecythophora*. *Lecythophora* has also been found before as part of biofilm studies in the External Filter Assembly (EFA).

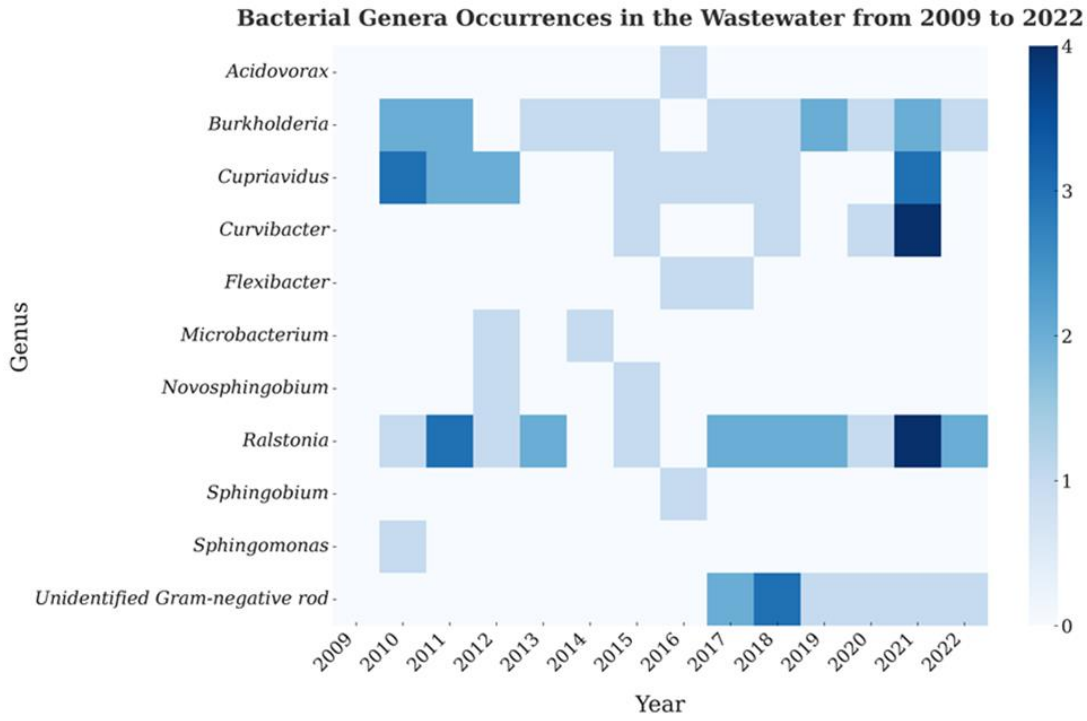

Figure 4. Heatmap of identified bacteria in the wastewater samples. *The white-blue color indicates 0 identified bacteria, while darkest blue indicate the highest number of identified bacteria of the same genus, in this case 4.*

D. Multifiltration Bed

There were around 25 samples associated with the Multifiltration Bed including 13 isolated for bacterial organisms and 12 for fungi. The bacterial genera associated with this area encompass 38% *Burkholderia* and 38% *Ralstonia*. While *Burkholderia* and *Cupriavidus* are a part of the organisms found in these samples, the only other reported genera were *Curvibacter* (10%) and *Cupriavidus* (5%). In this segment, there were also unidentified Gram-negative rod (10%) as shown in **Figure 5**. Fungal isolates were not detected in these samples.

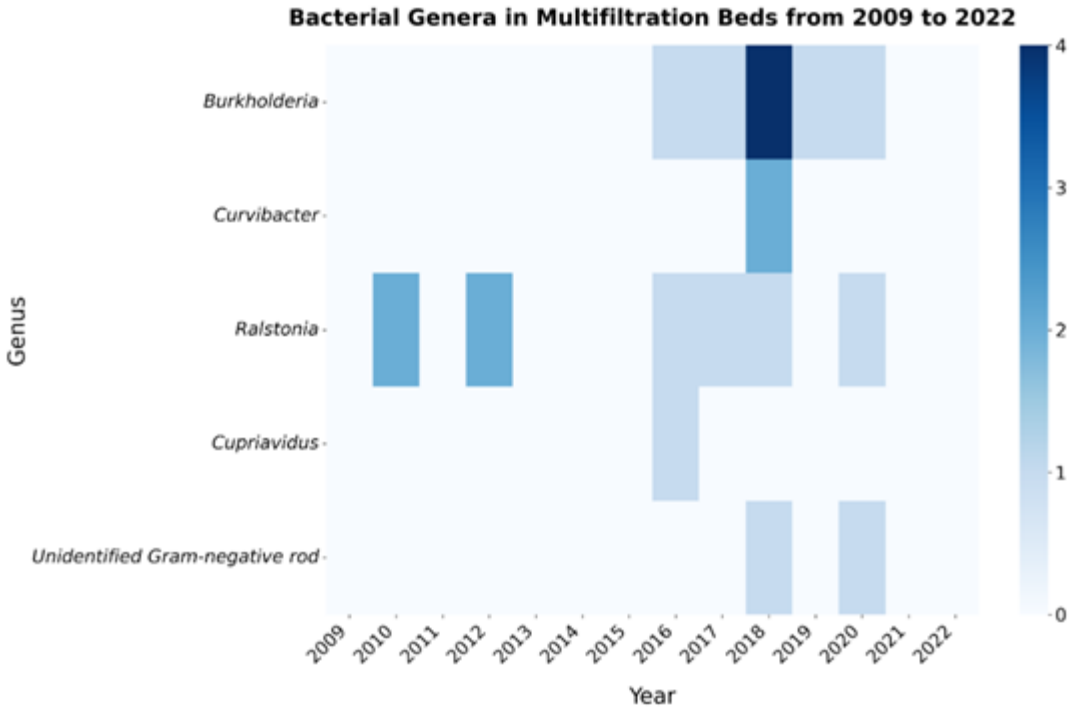

Figure 5. Heatmap of identified bacteria in the Multifiltration Bed samples. *The white-blue color indicates 0 identified bacteria, while darkest blue indicate the highest number of identified bacteria of the same genus, in this case 8.*

E. PWD Sampling and Temperature Differences

The PWD samples have been published before by Maryatt and Smith in 2017[4]. Updated to the year 2022, there were 191 bacterial samples in total with 73 of those belonging to MCD analysis, the others to water bag analysis. In both groups there were analysis for hot and ambient water lines as described in **Figure 6**, the bacterial genera associated with water bag analysis of PWD ambient water were *Ralstonia* (56%) and *Burkholderia* (29%). This line also contained some unidentified Gram-negative rods, however water bag samples for the "hot" PWD line were also *Ralstonia* and *Burkholderia* in lower presence and no unidentified Gram-negative rod. In fact, *Burkholderia* and *Ralstonia* only appeared once in 2009 and 2010. Additionally, the PWD hot line was sampled 58 times via water bags, the ambient water line sampled 60 times, meaning both water conditions were sampled at similar total amount. The PWD ambient line was sampled via MCD less than the water bags, 46 times. However, there are more reported genera associated to the ambient MCD samples, a total of 14, whereas the ambient water bags had 8 reported genera (**Figure 7**). The ambient MCD bacterial genera were reportedly 22% *Burkholderia* and 28% *Ralstonia*. Both *Burkholderia* and *Ralstonia* did not appear in the hot MCD samples.

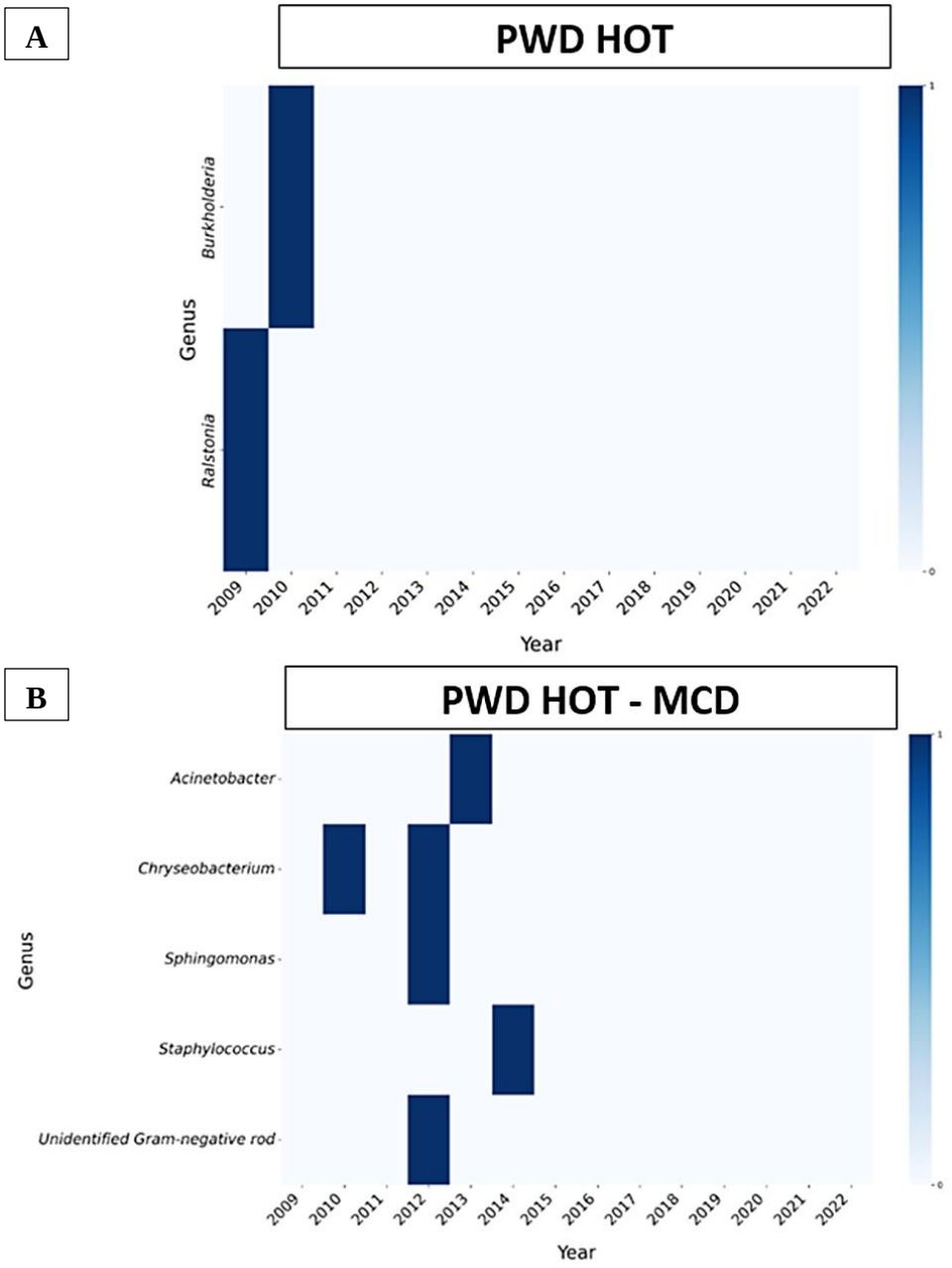

Figure 6. Heatmap of identified bacteria in the PWD hot water line sampled via water bags (A) and hot water line sampled via the MCD (B). *The white-blue color indicates 0 identified bacteria, while darkest blue indicate the highest number of identified bacteria of the same genus, in this case 1.*

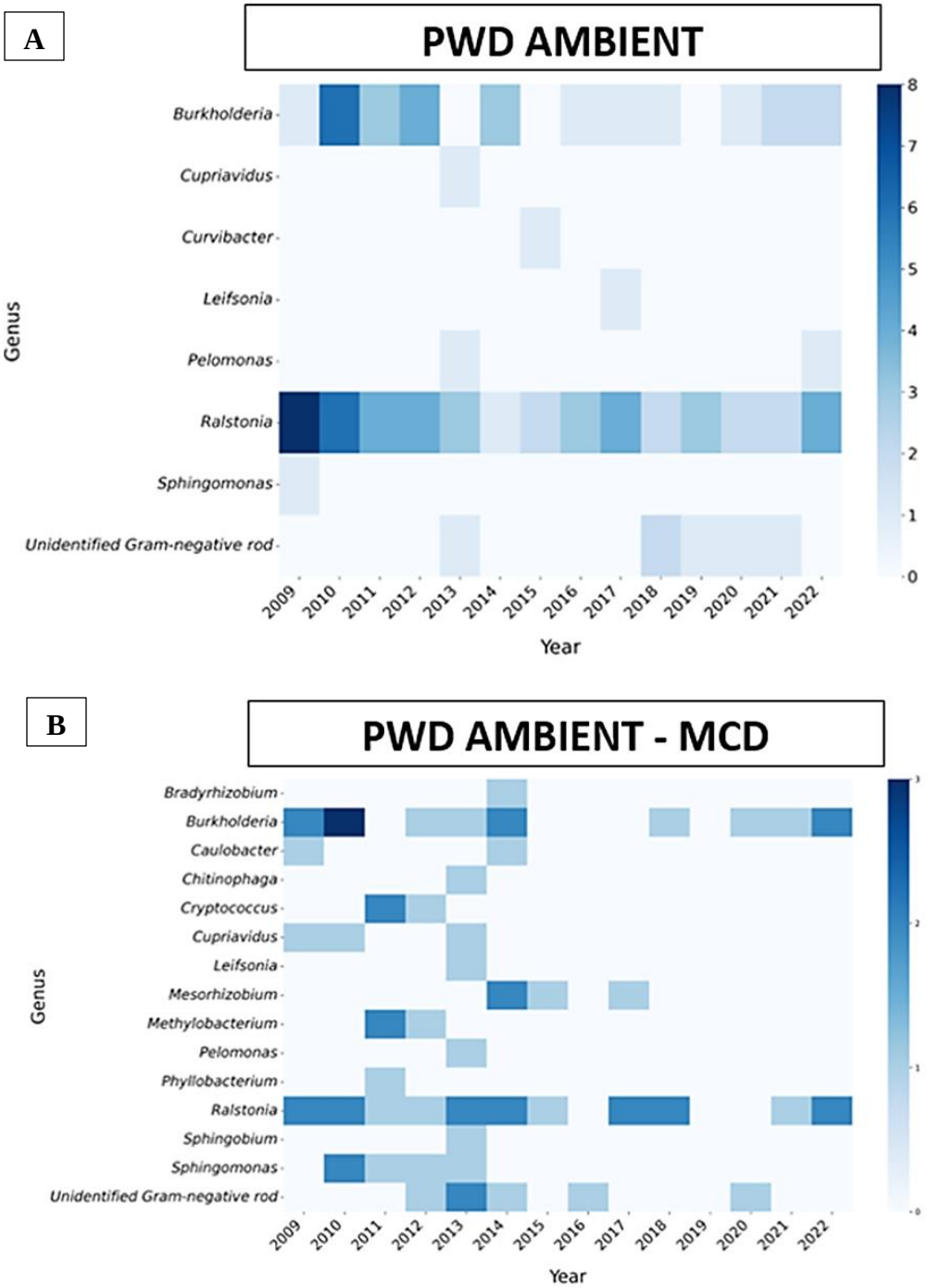

Figure 7. Heatmap of identified bacteria in the PWD ambient water line sampled via water bags (A) and ambient water line sampled via the MCD (B). *The white-blue color indicates 0 identified bacteria, while darkest blue indicate the highest number of identified bacteria of the same genus, in the case of water bags sample it was 8, while MCD samples had 3 identified individuals of the same genus as the highest value.*

F. Adjusted Sampling Frequency

While there are microbial species found in all mentioned segments of the system, samples are collected at different frequencies (**Figure 2**). To represent the normalized amount of bacteria we divided the occurrences of each bacterial genera by the total amount of times bacterial isolation was performed in the given component in the given year. After doing so we compared the raw totals to the normalized totals (**Table 1**). In doing so, we did not identify significant differences between both analysis.

Table 1. Normalized frequency of isolation of all genera in the WRS. *All the isolated organisms were divided by the total number of times bacterial isolation was practice in the given component in the same year. The last column shows the totals for all years, analyzed separately from white to blue.*

Genus	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	Total
MF Beds															
Burkholderia		0.0		0.0				1.0	0.5	1.0	1.0	1.0			0.62
Curvibacter		0.0		0.0				0.0	0.0	0.5	0.0	0.0			0.15
Ralstonia		1.0		1.0				1.0	0.5	0.3	0.0	1.0			0.62
Cupriavidus		0.0		0.0				1.0	0.0	0.0	0.0	0.0			0.08
UGNR		0.0		0.0				0.0	0.0	0.3	0.0	1.0			0.15
WW															
Acidovorax		0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.03
Burkholderia		0.4	0.5	0.0	0.5	1.0	0.5	0.0	0.3	0.3	0.7	1.0	0.4	0.3	0.38
Cupriavidus		0.6	0.5	1.0	0.0	0.0	0.5	0.3	0.3	0.3	0.0	0.0	0.6	0.0	0.36
Curvibacter		0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.3	0.0	1.0	0.8	0.0	0.18
Flexibacter		0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.3	0.0	0.0	0.0	0.0	0.0	0.05
Microbacterium		0.0	0.0	0.5	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.05
Novosphingobium		0.0	0.0	0.5	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.05
Ralstonia		0.2	0.8	0.5	1.0	0.0	0.5	0.0	0.5	0.5	0.7	1.0	0.8	0.7	0.54
Sphingobium		0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.03
Sphingomonas		0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.03
UGNR		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.8	0.3	1.0	0.2	0.3	0.23
Humidity Condensate															
Acidovorax	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.02
Bradyrhizobium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.02
Burkholderia	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.02
Caulobacter	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.02
Chitinophaga	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.02
Cupriavidus	0.8	1.0	0.0	1.0	0.0	1.0	1.0	0.3	0.0	0.0	0.5	0.5	0.5	0.7	0.46
Flexibacter	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.3	0.0	0.0	0.0	0.0	0.0	0.04
Ralstonia	0.3	0.2	0.8	0.0	0.5	0.0	1.0	0.5	1.0	1.0	0.8	0.5	1.0	0.3	0.61
Rhodopseudomonas	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.02
Sphingobium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.02
UGNR	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.8	1.0	0.8	0.5	0.5	0.7	0.35
PWD HOT															
Burkholderia	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.02
Ralstonia	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.02
PWD HOT - MCD															

Acinetobacter		0.0		0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.04
Chryseobacterium		0.5		0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.08
Sphingomonas		0.0		0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.04
Staphylococcus		0.0		0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.04
UGNR		0.0		0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.04
PWD - AMBIENT															
Burkholderia	0.1	0.8	0.6	0.8	0.0	1.0	0.0	0.3	0.2	0.3	0.0	0.5	0.7	0.5	0.42
Cupriavidus	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.02
Curvibacter	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.02
Leifsonia	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.02
Pelomonas	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.03
Ralstonia	1.0	0.8	0.8	0.8	0.8	0.3	0.7	0.8	0.8	0.7	1.0	1.0	0.7	1.0	0.80
Sphingomonas	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.02
UGNR	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.7	0.3	0.5	0.3	0.0	0.10
PWD AMBIENT - MCD															
Bradyrhizobium	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.02
Burkholderia	0.5	0.8	0.0	0.3	0.2	0.3	0.0	0.0	0.0	0.5	0.0	0.5	1.0	1.0	0.33
Caulobacter	0.3	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.05
Chitinophaga	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.02
Cryptococcus	0.0	0.0	0.7	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.07
Cupriavidus	0.3	0.3	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.07
Leifsonia	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.02
Mesorhizobium	0.0	0.0	0.0	0.0	0.0	0.3	0.3	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.09
Methylobacterium	0.0	0.0	0.7	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.07
Pelomonas	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.02
Phyllobacterium	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.02
Ralstonia	0.5	0.5	0.3	0.3	0.4	0.3	0.3	0.0	0.7	1.0	0.0	0.0	1.0	1.0	0.42
Sphingobium	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.02
Sphingomonas	0.0	0.5	0.3	0.3	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.12
UGNR	0.0	0.0	0.0	0.3	0.4	0.2	0.0	0.3	0.0	0.0	0.0	0.5	0.0	0.0	0.14

VI. Discussion

Humans carry microbes as part of their normal and healthy human microbiome. Due to the interaction between humans and habitats it is understood that some of these microbes will become a part of the environmental components. In air systems microbes are said to be influenced by occupancy and ventilation[14,15], in humid environments mold can thrive, and water droplets can carry a multitude of microbial species[16,17]. In water systems and other areas of the ISS, biofilms have been found. While no microbial infections have been a threat to the crew, microbially induced corrosion[1] represents a potential issue in space systems, especially in life support systems for long-term crewed missions to the Moon or Mars. Periods of system dormancy and uncrewed missions containing essential ECLSS systems are a subject of studying as well[18].

Most bacteria found in the ISS water systems are Gram-negative species, which commonly inhabit water sources under normal gravity as well[19]. In the data obtained over time pertaining life support system water samples a variety of bacterial genera are observed to change depending on system segment, while others persist through the entire system. For example, the humidity condensate has a well-established amount of *Ralstonia* and *Cupriavidus* bacteria.

Both are associated with moist environments or water contamination. However, in the wastewater, the genus *Burkholderia* appears to hold a higher population. *Burkholderia*, similarly to *Pseudomonas*, has the ability to form robust biofilms[20,21], however *Pseudomonas* is not found in the US systems. *Burkholderia* proliferation is also found after the Wastewater ORU, where biofilm-induced reduction of water flow has occurred in the past.

However, the reasons why *Burkholderia* is found at higher rates in the wastewater when compared to the humidity condensate are unknown. Higher nutrient conditions and stagnation are two of the conditions differing in this segment[8], as the wastewater tank is the precursor to the process line B, and it is speculated to be the reason for higher biomass associated to this ORU. Additionally, the lower rate of urine distillate line monitoring is not beneficial in determining the success of urine pre-treat in operational systems and other potential causes for *Burkholderia* proliferation in the wastewater.

Alternatively, *Ralstonia* is persistent through the entire water system. It also appeared amongst the two most frequently identified organisms in the scarcely sampled Multifiltration Bed segment, meaning *Ralstonia* may have the most potential to break-through multiple barriers of microbial decontamination.

While the PWD had a high number of isolates, it remains the most monitored segment due to its significance to crew health, thus it would appear that the segment has the most microbial contaminants identified. However, even though we can adjust results to sample frequency, as segments are unevenly monitored this cannot be conluded. Sites such as the urine distillate line coming into the Wastewater ORU are less frequently studied, raising some challenges on whether operational decontamination of the Urine Processor Assembly (UPA) is maintained through the current process. Previously, Nguyen and colleagues[6] have found *Burkholderia*, *Paraburkholderia*, *Leifsonia*, *Fusarium* and *Lecythophora* in UPA flex hoses. Furthermore, they have found predicted hexavelant chrome resistance genes. Overall, there is preference given to systems that directly related to astronaut health and not the overall system risk, as seen in the frequency of samples collected (**Figure 7**).

Evaluation of the microbial profile of each section of the WRS provides greater insight toward preferred mechanisms to control biofouling. The temperature differences in the PWD also influence microbial proliferation, as hot water in the PWD is observed to have less microbial contaminants. Meanwhile, the conditions brought by condensing heat exchangers do not reduce the presence of microbes found in the humidity condensate. Analysis of the archived data concerning RU streams and the air systems could help to define microbial dynamics in the Environmental Control and Life Support Systems (ECLSS). Combining these data will help understand potential effects if temperature conditions in the system and how similar strategies can be used to combat biofouling.

In the past, microbial monitoring in the water systems at ISS was significantly biased due to existing limitations described above, including frequency and location of samples, type of sampling and detection methods, processing times. Sampling methods can also influence results, such as when comparing MCD and water bag samples.

Timeline for sampling is an issue as samples are grounded for analysis. In missions to the Moon or Mars this is not a possibility as such missions could not depend on ground assessments and payload services. Challenges related to astronaut sampling methods and sampling contamination may be an issue in manual water monitoring, as well as the changes in identification methods. Current methods for water monitoring in Earth's water systems have yet to reach the capacity of tracking genomic changes, although monitoring of microbial concentration and identification is carried out periodically[22,23]. Current research in water treatment plants points to the use of genomic methods and in-situ identification as preferred methods for microbial monitoring[24]. Therefore, space mission microbial monitoring should over time diverge from using the Biolog microbial identification to the use of sequencing devices.

VII. Conclusions

Sampling methods should be optimized to capture microbial composition and to fit the type of future missions that are crewed or automated, with and without resupply options. It is clear that methods need to be adjusted to expand monitoring to microorganisms that are different from bacteria and pose risks to human health and hardware functioning. Frequency and protocols for microbial samplings need to be discussed, developed, and implemented. Accumulated data should be organized and stored in a specific way that allow its straightforward search, comparative analysis, and availability in the future studies such as longitudinal analyses. It appears that adding more sampling spots might be beneficial. At this time of change and preparation to the ISS retirement, novel long-term missions at the Earth orbit, Moon habitat and further travel – microbial contamination is a serious risk and should be assessed and monitored expanding planetary protection and astronaut health goals. Microbial monitoring in the space hardware and space human habitat environments become a critical part of the missions and need to be further developed and optimized. In-situ monitoring protocols of water systems should be developed and optimized to assess true changing

microbial profile of built microgravity environment such as ISS. All relevant issues for Moon and Mars missions will have to be addressed in the future by NASA and the Human Health and Performance Directorate[18].

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