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Identification of Volatile Organic Compound Markers for Bacterial Growth in Spacecraft Wastewater

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Abstract

As humans move beyond low-Earth orbit, where resupply of water will be impractical or impossible, systems that can ensure the provision of safe, clean, recycled water are critical. Current methods of microbial detection for spacecraft water are crew time- and consumable-dependent and would not be possible during periods of dormancy. One potential method for microbial detection that can be performed remotely and without sample preparation is the analysis of headspace to determine if specific volatile organic compounds (VOC) are present. Here, the use of gas chromatography-

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mass spectrometry (GC-MS) to analyze the headspace above cultures of bacteria returned from the International Space Station is discussed. Three species of bacteria: *Ralstonia pickettii, Burkholderia contaminans,* and *Klebsiella aerogenes,* were cultured, and the VOCs emitted by each culture were analyzed through headspace GC-MS analysis. A number of species-specific compounds were detected, but a particular marker, dimethyl disulfide, was present in all species and test conditions. The isolates' genomes were sequenced, and genes encoding for enzymes that could contribute to the production of dimethyl disulfide were identified. This analysis supported the premise that each organism examined was capable of producing this compound as a byproduct of methionine metabolism and suggests that dimethyl disulfide would be a suitable target compound for the detection of process escapes in the area of microbial control in future spacecraft water recovery systems. The results shown here suggest that the use of MS-based techniques for headspace VOC analysis is a promising avenue for the detection of these common spacecraft bacteria in spaceflight wastewater.

1. Introduction

To ensure healthy spacecraft crew and systems, it is essential to provide clean water for drinking, hygiene, oxygen generation, urinal flush water, and payloads. On the International Space Station (ISS), this clean water is provided by the Water Recovery System (WRS), consisting of the Water Processor Assembly (WPA) and Urine Processor Assembly (UPA), which treats wastewater (WW) obtained from a mixture of humidity condensate and urine distillate; other sources, such as make-up water and

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Sabatier product water, may also be included in the wastewater stream.[1] To accomplish this treatment, a number of different processes are used, including multifiltration (MF) beds containing activated charcoal and ion-exchange resin to remove dissolved contaminants, high-temperature catalytic oxidation of organics and microbes, and a standalone ion exchange bed that removes dissolved oxidation products while also providing iodine for microbial control. This water is stored in a product water tank until it is needed by the potable bus, or it can be reprocessed by the WPA. Users of the potable bus include the Potable Water Dispenser (PWD)/Exploration Potable Water Dispenser (xPWD), the Oxygen Generation Assembly (OGA), Extravehicular Mobility Units (EMUs), flush water for the Waste Hygiene Compartment (WHC), and various payloads. This system and the numerous upgrades have been described extensively since their installation on the ISS.[2-6]

As the wastewater fed into the WPA is generally a mixture of humidity condensate and urine distillate, an exact chemical and microbial composition is difficult to define. The chemical composition of the condensate collected by the Common Cabin Air Assembly (CCAA) Condensing Heat Exchangers (CHXs) varies as a result of factors such as the number of crew members, visiting vehicles, and payloads. Efforts have been undertaken to limit the amount of atmospheric contaminants to reduce the potential for dangers to both crew health[7] and the WPA, for which increased resupply of consumables (e.g. MF beds) can result from even relatively low amounts of non-toxic compounds.[8-10] As such, the overall chemical contaminant load of the condensate has dropped markedly over the past half-decade following the installation of Charcoal HEPA

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Integrated Particle Scrubber (CHIPS) filters; these have also maintained low levels of microbes in the ISS atmosphere.[6]

As would be expected, the urine distillate provided by the UPA also varies in composition due to input from different crew members. Additionally, the composition of the distillate may also vary depending on the type of urine being processed. Due to the elevated urinary calcium levels present in spaceflight, the standard urine pretreatment designed for use in the Russian toilet (chromic acid/sulfuric acid)[11] to maintain chemical and microbial control was found to cause precipitation of calcium sulfate at elevated water recovery levels (> 75%).[12] As a result, urine produced in the US Operating Segment (USOS) of the ISS is now treated with a chromic acid/phosphoric acid mixture, which allows for > 85% water recovery from urine.

While the high temperatures of the catalytic reactor and the iodine imparted by the ion exchange beds successfully control microbial growth in the US potable water bus, there is no microbial control in the WPA WW tank. In fact, biofouling was observed within the first year of WPA operations.[13] Efforts to understand the nature of the microbial population in the WW tank have been problematic, largely due to the current lack of insight into the water in real time.[14] While samples of the WW are collected approximately quarterly for return to Earth for analysis, a lack of environmental control and preservation, a varied time between sample collection and analysis, and standard operating procedures that do not provide for the identification of certain species, results in a skewed representation of the actual microbial community;[15] this has been shown to affect insight into the chemical composition of the returned samples, as well.[16] Recent studies have provided further insight into some of the microbial inhabitants of the WW

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DOI: 10.1039/D6AN00060F

that have not been previously identified, confirming that there is a much more diverse microbial community present in the WW tank than previously reported.[17, 18]

With the extension of missions beyond low-Earth orbit, current methods of microbial identification involving the return of archival samples for analysis will not be feasible. The past decade has seen the development and testing of advanced sequencing technology on the ISS, first for surface analysis[19] and then for water.[20] This work provided the first off-Earth identification of microbes collected from spacecraft surfaces.[21] While these techniques have been successful and are currently being refined, they remain crew-time intensive. As NASA prepares for exploration missions that may include periods without crew and/or dormancy, and will likely include some form of water recovery,[22-24] it is important to determine the potential for other non-hands-on, lower-fidelity methods to gain an insight into the presence of microbes in the water system prior to crew reentering a spacecraft or habitat.

A potential avenue for this monitoring lies in the analysis of volatile organic compounds (VOCs). It is well established that microorganisms emit VOCs as a result of their interactions with the environment, signaling, and primary and secondary metabolism.[25, 26] Studies have shown that these VOCs can be used to detect and discriminate between different groups of organisms and provide information about the organism that produced them. Because VOCs are accessible in the headspace above a culture of microbes, they can be more easily analyzed than other indicators that must be extracted and isolated through lengthy sample preparation procedures.[26]

VOCs have been used to successfully detect and differentiate between microbes in a variety of environments. Headspace studies of the VOCs emitted by cultures of

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bacteria in various growth media have shown that it is possible to identify different groups of organisms based on the emitted VOCs.[27, 28] Slingers *et al*.[29] and Ahmed *et al*.[30] applied mass spectrometry techniques to detect VOCs indicative of a bacterial infection in exhaled human breath, while a study by Ratiu *et al*. detected bacterial infections in human tissue samples through VOC analysis with gas chromatography-mass spectrometry (GC-MS).[31] A recent review regarding the detection of urinary tract infections using VOCs describes other potential techniques that could prove useful for microbial detection.[32]

In this investigation, the VOC profiles of three bacterial species previously observed in ISS water were studied: *Klebsiella aerogenes, Burkholderia contaminans,* and *Ralstonia pickettii.*[33-35] *Burkholderia* spp. and *Ralstonia* spp. are both Gram-negative, rod-shaped bacteria that have been regularly isolated in spacecraft water since 2009. *Burkholderia* has reportedly been the most abundant genus in ISS wastewater while *Ralstonia* has been shown to be the most pervasive genus throughout the entire ISS WPA.[36] Therefore, volatiles produced and emitted by *Burkholderia* spp. and *Ralstonia* spp. could serve as effective indicators of microbial contamination. *K. aerogenes* has not been detected in spacecraft water since the early days of the ISS. However, it was included in this study because it is a coliform bacterium that can serve as an indicator of contaminated water as well as providing further evidence for common bacterial VOCs.[37]

Growth of each isolate was analyzed in both Reasoner's 2A media (R2A), a minimal liquid growth medium, and ersatz wastewater, a simulant for ISS wastewater. Culture headspaces were analyzed for VOCs using a portable GC-MS planned for use

View Article Online
DOI: 10.1039/D6AN00060F

as part of NASA's Moon-to-Mars program, and the detected compounds were identified. These analyses reveal that not only are microbial VOCs detectable in spacecraft-mimicking environments, but they also revealed a VOC that can serve as a potential indicator of bacterial contamination in spacecraft wastewater. Based on these results, the MS-based VOC analysis technique explored in this work shows promise in aiding in the detection of bacterial contamination.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

Three bacteria isolated from the ISS were utilized as model microorganisms in this investigation. *Ralstonia pickettii* was recovered from an archive bag collected from the PWD onboard the ISS on May 6th, 2019, and returned to Earth on Soyuz 57. The archive bag, delivered to the NASA Johnson Space Center (JSC) Microbiology Laboratory, was processed and isolates archived following the JSC Laboratory's standard operating procedures (SOP).[19, 21, 34] *Klebsiella aerogenes* was isolated from the air of the Node 2 module on a tryptic soy agar (TSA) plate collected May 30th, 2019.[38] The media plate was also returned on Soyuz 57 and delivered to the JSC Microbiology Laboratory, where isolation, identification, and archiving followed the lab's SOPs. *Burkholderia contaminans* was isolated from a sample taken from a flex line hose from the ISS UPA that had been returned to Earth for refurbishment, details of which have been described previously.[34] Bacterial cultures were initiated through inoculation of R2A (Hardy Diagnostics, Santa Maria, CA) with a loop from a -80 °C glycerol stock followed by incubation at 35 °C for 48 hours.

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DOI: 10.1039/D6AN00060F

From the 48-hour R2A plate described above, a 1 µL loop was used to collect an individual bacterial colony that was then added to 10 mL R2A for *R. pickettii* or tryptic soy broth (TSB, Hardy Diagnostics) for *B. contaminans* and *K. aerogenes.* The liquid inoculum was incubated at 35 °C with shaking at 150 rpm for 17 hours. Following incubation, bacterial cells were collected by centrifugation at 4,000 rpm for 5 minutes. The supernatant was discarded, and the cells were washed by resuspension in 10 mL of Butterfield's buffer (Weber Scientific, Hamiliton, NJ) followed again by centrifugation at the same conditions to collect the final cell pellet. Again, the supernatant was removed, and the cells were resuspended in 10 mL Butterfield's buffer. The optical density at 600 nm (OD600) was recorded and adjusted to OD600 at 0.43, which corresponded to approximately 1 × 10^9 colony forming units per milliliter (CFU/mL) for *R. pickettii*, *B. contaminans,* and *K. aerogenes*. Bacterial counts were verified using serial dilution and plating on fresh R2A plates incubated at 35 °C for 24 hours. For all liquid cultures, the absolute CFU/mL was determined.

2.2. Bacterial growth conditions for volatile organic compound analysis

R2A (DSMZ Medium 830) and an ersatz wastewater mimicking that found on the ISS[39] served as the bacterial growth media for all analyses. R2A is a reduced nutrient medium primarily used for the cultivation of water organisms, whereas the NASA synthetic ersatz wastewater more accurately represents the conditions bacteria would experience within the ISS wastewater tank. The composition of the NASA ersatz wastewater is provided in **Table 1**.

Table 1. Characteristics of the ersatz media

View Article Online
DOI: 10.1039/D6AN00060F

Physical Characteristics		
Conductivity	150	µMho/cm
pH	4.97	pH units
Inorganics/Metals		
Nitrate (as N)	1.50	mg/L
Phosphate (as P)	0.20	mg/L
Sulfate	0.90	mg/L
Ammonium (as N)	18.10	mg/L
Calcium	0.68	mg/L
Magnesium	0.33	mg/L
Potassium	0.94	mg/L
Sodium	0.48	mg/L
Aluminum	1	µg/L
Boron	4	µg/L
Chromium	1	µg/L
Iron	5	µg/L
Manganese	1	µg/L
Nickel	108	µg/L
Zinc	424	µg/L
Total Inorganic Carbon	6,860	µg/L
Organics		
Total Organic Carbon	73,900	µg/L

View Article Online
DOI: 10.1039/D6AN00060F

Acetate	33,300	µg/L
Acetone	20,400	µg/L
Benzaldehyde	12	µg/L
Benzothiazole	136	µg/L
Benzyl alcohol	3,080	µg/L
Diethylphthalate	1,440	µg/L
N,N-Dimethylformamide	180	µg/L
Dodecamethylcyclohexasiloxane	11	µg/L
Ethanol	31,700	µg/L
Formate	35,000	µg/L
Lactate	4,160	µg/L
Methanol	16,900	µg/L
Methyl sulfone	29	µg/L
Trimethylsilanol	480	µg/L
1-Methyl-2-pyrrolidinone	130	µg/L
1-Propanol	686	µg/L
1,2-Propanediol	9,210	µg/L
2-(2-Butoxyethoxy)ethanol	890	µg/L
2-Ethoxyethanol	96	µg/L
2-Ethyl-1-hexanol	85	µg/L
2-Propanol	802	µg/L
4-Ethylmorpholine	570	µg/L

186

View Article Online
DOI: 10.1039/D6AN00060F

A 100 µL aliquot containing ~10^4 CFU of washed, diluted, and prepared cells as described above was inoculated into a 1 L Erlenmeyer flask containing either 100 mL of R2A or ersatz wastewater media. Blank control flasks, lacking bacteria and containing only the liquid media, were also prepared. All flasks were capped with a sterile rubber stopper and placed on a shaker at 35 °C at 150 rpm. Sampling occurred at various time points as described.

All three bacteria were assessed following growth in R2A at two distinct time points. For a shorter duration assessment, 24 hours of growth was evaluated across six biological replicates for *R. pickettii*, *B. contaminans,* and *K. aerogenes.* To gauge the production of products at a later point, 14 days of growth was investigated in across two biological replicates for all bacteria. For this longer 14-day duration, the spent media was also analyzed. VOCs were assessed at 24 hours and 14 days with the portable GC-MS. At the same point of VOC analysis, a liquid sample was collected from each flask, diluted, plated on fresh R2A, and incubated to determine accurate bacterial levels.

Unlike R2A, ersatz wastewater is not a typical isolation medium for bacteria; therefore, optimization was needed to determine the best VOC sample collection time points. *B. contaminans*, known for faster growth and for having the highest abundance in ISS wastewater, was selected for the optimization. Five flasks containing 100 mL of sterile ersatz wastewater were inoculated with *B. contaminans*. VOC samples were collected and analyzed every 24 hours (one day) for five days.

After the optimization, the experiment was carried out with *B. contaminans*, *R. pickettii*, and *K. aerogenes* in ersatz wastewater. The samples were collected for VOC

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DOI: 10.1039/D6AN00060F

analysis and to determine bacterial levels at four days and seven days. To have comparative growth data at 14 days, two biological replicates were assessed in ersatz for all three bacteria. The flasks were sampled after 14 days for VOC analysis, and liquid samples were collected for bacterial growth analysis. Additionally, spent media was collected after seven days and 14 days to determine the changes in dissolved organic compounds. A full summary of all studied conditions and replicates is provided in **Table 2.**

Table 2. Summary of test conditions and replicates

Isolate	Media	Growth time (days)	Biological Replicates	Technical Replicates/ Biological Replicates
B. contaminans	R2A	1	6	1
		14	2	1
	ersatz	1	1	1
		2	1	1

View Article Online
DOI: 10.1039/D6AN00060F

		3	1	1
		4	3	1
		5	1	1
		7	4	1
		14	2	1
R. pickettii	R2A	1	6	1
		14	2	1
	ersatz	4	2	1
		7	4	1
		14	2	1
K. aerogenes	R2A	1	6	1
		14	2	1
	ersatz	7	2	1
		14	2	1

2.3. Volatile organic compound analysis using a portable GC-MS

At selected time points, following equilibration of the flasks to room temperature (~ 25 °C), the VOCs from the headspace of the sample flasks were collected and measured using a FLIR Griffin G510e portable GC-MS. Each sample was collected for two minutes with a flowrate of 300 mL/min using the instrument sample wand, which was directly connected to the stopper of the sample flask. The sample was collected on an internal sorbent bed built into the instrument to allow for online sampling. The sorbent bed was comprised of 5 mg Carbopack™ B 60/80, 15 mg Carbosieve™ SIII 60/80, and 15 mg Carboxen™ 1018 60/80. The concentrated VOCs were thermally desorbed and then entered the GC column (DB-624, 20 m x 0.18 mm x 0.25 µm) with a 90:10 split. VOCs were identified via a quadrupole mass spectrometer with a 70 eV

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DOI: 10.1039/D6AN00060F

electron impact ionization source. Analysis of the spectra was performed using the on-board NIST 17 (version 2.3) mass spectral library. While the use of analytical standards instead of mass spectral libraries is generally preferred, the focus of this study was the use of planned spaceflight hardware for volatile identification, making the use of the on-board library more suitable. Similarly, while the sorbents used for preconcentration could result in preferential absorption/detection of specific VOCs, the planned usage of this hardware "as is" precludes the usage of different types and amounts of the different sorbents.

2.4. Analysis of spent media following VOC measurement

Media was collected from each organism under the following conditions: (1) after 24 hours of growth in R2A broth and (2) after 7 days of incubation in ersatz wastewater. Following VOC analysis, 80 mL of the culture was centrifuged at 4000 rpm for five minutes. The supernatant was then filtered with a sterile 0.2 µm syringe filter (PTFE membrane, Millex-FG filter unit) to completely remove bacterial cells. The dissolved organic compounds were analyzed with the final filtrate for total organic carbon (TOC), volatile organics, and alcohols. These analyses were performed by the JSC Environmental Chemistry Laboratory using a combination of custom methods (alcohols – direct aqueous injection GC-MS) and modified EPA methods (volatiles GC-MS – EPA 524.2 and TOC – EPA 415.1). The results of these analyses are shown in Table S1 (R2A) and Table S2 (ersatz).

2.5. Genomic DNA extraction

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DOI: 10.1039/D6AN00060F

To obtain genomic DNA (gDNA), bacterial cultures were prepared as described above. Briefly, *B. contaminans* and *R. pickettii* were cultured from glycerol stocks on R2A agar plates for 48 hours at 35 °C. For each organism separately, a 1 µL loop from a single colony was used to inoculate 10 mL of R2A liquid media (DSMZ Medium 830). Following culture at 35 °C with shaking at 150 rpm for 17 hours, cells were collected by centrifugation at 4000 rpm for five minutes. The supernatant was removed, the cell pellet rinsed twice with PBS (1x PBS, Gibco™), and then resuspended in 20 µL of PBS. DNA was extracted according to the manufacturer's protocol for the Nanobind CBB kit for high molecular weight DNA for Gram-negative bacteria (PacBio, Menlo Park, CA). The quality and quantity of the gDNA was evaluated using a BioTek Synergy H1 microplate reader (Agilent, Santa Clara, CA), Qubit 4 Fluorometer (ThermoFisher Scientific, Waltham, MA), and genomic DNA ScreenTape with the 4200 TapeStation platform (Agilent).

2.6. Nanopore sequencing and basecalling

Sequencing libraries were prepared following the manufacturer's protocol for the Rapid Barcoding Kit v14 (SQK-RBK114-24, Oxford Nanopore Technologies, Oxford, UK). Briefly, gDNA from *B. contaminans* and *R. pickettii* was independently fragmented, barcoded, and pooled prior to a final 1:1 AMPure XP bead-based purification (Beckman Coulter, Indianapolis, IN, USA). The quantity and quality of the libraries were assessed using the Qubit 1X dsDNA kit (ThermoFisher Scientific) and genomic DNA ScreenTape with the 4200 TapeStation platform (Agilent). Finally, 1 µL of RAP (ONT) was added to the libraries and incubated at room temperature for five minutes to allow for attachment of the nanopore sequencing adaptors. Prepared libraries were loaded into a R10.4.1 flow

View Article Online
DOI: 10.1039/D6AN00060F

cell seated within a MinION Mk1B (ONT). Sequencing was initiated with MinKNOW software version 24.02.16 (ONT) and conducted for 48 hours. The resulting reads were basecalled with Dorado (version 7.3.11) using the Super High Accuracy model, filtered to contain a Q-score > 10, and output into the fastq_pass folder for downstream analysis. The sequencing data is deposited into National Center for Biotechnology Information (NBIC) under the Bioproject number PRJNA1450556.

2.7. Genome assembly

The number of reads, number of bases, N50, and GC content of the raw sequencing reads were calculated using Seqkit stats (version 2.8.0).[40] Sequencing adapters and barcodes were then trimmed using Porechop (version 2.4.0), and the reads containing middle adapters were discarded.[41] The trimmed reads were subset to a read depth of 50X using FiltLong (version 0.2.1),[42] and read QC was again performed using Seqkit. To visualize the reads prior to carrying out genome assembly, FastQC (version 0.12.1)[43] reports were generated and summarized using MultiQC (version 1.25).[44] The trimmed and subset reads were used as input for the *de novo* assembler Flye (version 2.9.4-b1799) with the option –nano-hq to generate a draft long read assembly.[45] The genomes were re-oriented based on the dnaA gene using Circlator (version 1.5.5),[46] and the following flags --assembler canu --threads 32 --merge_min_id 85 --merge_breaklen 1000. The assemblies were then subject to one round of polishing with medaka (version 1.11.3), which was used in conjunction with the r1041_e82_400bps_sup_v4.1.0 model. Assembly completeness and contamination was verified using CheckM2 (version

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DOI: 10.1039/D6AN00060F

1.0.2).[47] The ribosomal RNA genes were predicted with Barrnap (version 0.9),[48] and the 16S rRNA genes underwent a preliminary classification using NCBI blast. The genome statistics of the final assembled data were summarized using Seqkit (version 2.8.0). All genomes were annotated with Bakta (version 1.9.4) with default settings.[49] The pairwise average nucleotide identities (ANI) were computed using skani (version 0.201)[50] against RefSeq assemblies which were downloaded using ncbi-genome-download (version 0.3.3) and the flag --assembly-levels complete.[51] Chromosome and plasmid sequences were characterized by reconstruction, extraction, and typing of plasmids from the draft assemblies using the MOB-recon module of MOB-Suite (version 3.1.9).[52]

3. Results and Discussion

3.1. Analysis of VOCs detected from bacteria cultured in R2A

Figure 1 shows the chromatograms resulting from GC-MS analysis of the headspace collected from bacteria grown for 24 hours in R2A broth, revealing unique profiles for each isolate (library identifications of particular compounds of interest are presented in **Table 3**). Distinct VOCs were present in each isolate's spectra. Isoprene was detected solely in the headspace of *B. contaminans*, while methylsulfonic acid and methylthio(methylthio-methyl)sulfone were exclusive to *R. pickettii*. The chromatogram of *K. aerogenes* was distinguished by a prominent peak of 3-methyl-1-butanol that was absent in the other species. Despite these differences, the compound dimethyl disulfide (DMDS-peak 3) appeared in the spectra collected from all three organisms, though its abundance was higher in *R. pickettii* compared to *K. aerogenes* and *B. contaminans*. As might be expected by the elevated headspace concentrations, DMDS

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DOI: 10.1039/D6AN00060F

was detected in the spent media collected from the *R. pickettii* samples, while 3-methyl-1-butanol was found in the *K. aerogenes* media; isoprene was not detected in the *B. contaminans* media, however (Table S1).

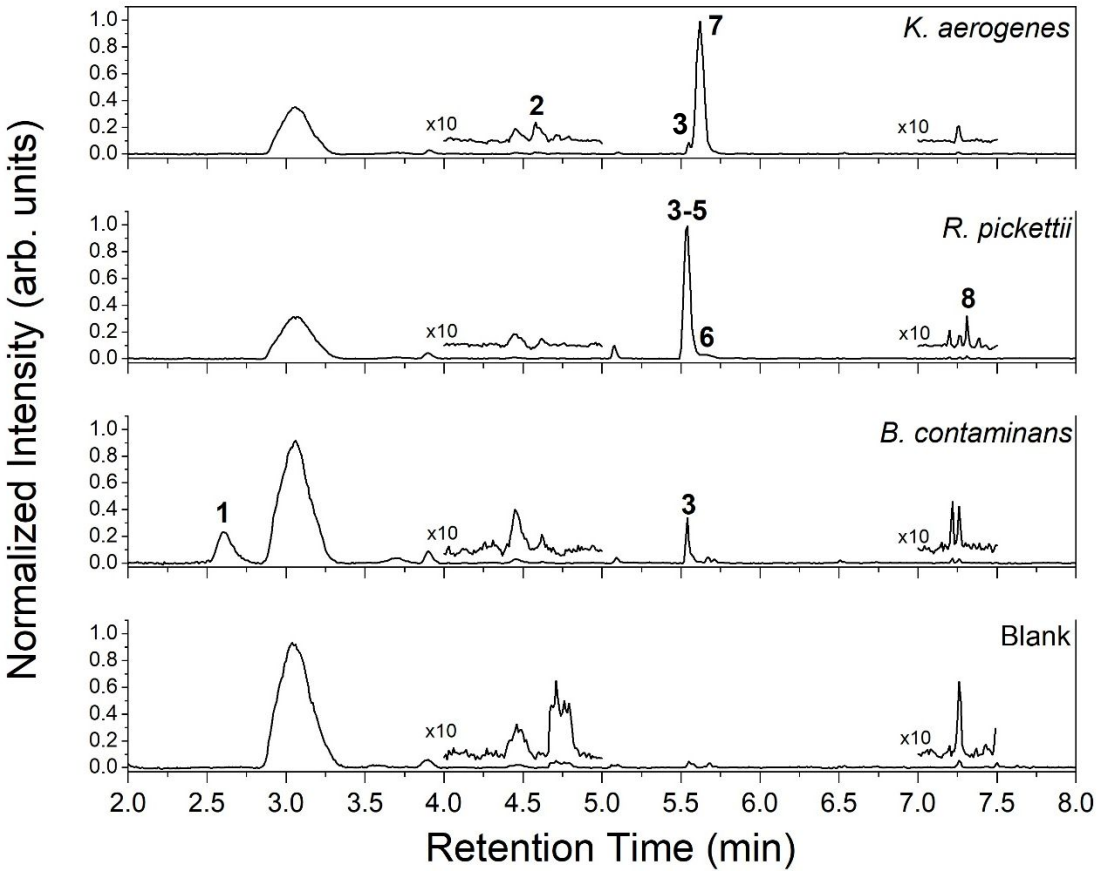

Figure 1. Chromatograms taken of the headspace above B. contaminans, R. pickettii and K. aerogenes cultures grown for 24 hours in R2A broth. Peaks are numbered according to Table 3.

Table 3. VOCs detected in R2A culture headspace

View Article Online
DOI: 10.1039/D6AN00060F

	Retention Time	m/z/ compound	*B. contaminans* in R2A	*R. pickettii* in R2A	*K. aerogenes* in R2A
1	2.6	Isoprene	X		
2	4.58	3-methoxy 1-propanamine			X
3	5.52	Dimethyl disulfide	X	X	X
4	5.52	Methylthio(methylthio-methyl)sulfone		X	
5	5.52	Methylsulfonic acid		X	
6	5.65	3-methylbutyl ester pentanoic acid	X	X	
7	5.62	3-methyl 1-butanol			X
8	7.3	Dimethyl trisulfide		X	

With DMDS being present in the mass spectra of all three bacteria according to the mass spectral library, the presence of this volatile was investigated further to confirm its identity. Standard gas mixes of DMDS were prepared to observe the position and change in response with concentration at the primary fragment ion of m/z = 94. As can be seen in **Figure S1**, a peak is present at 8 ppb and grows as the concentration is increased. This peak is centered at a retention time of 5.5 minutes, as seen in **Figure 1**.

Peak areas were obtained for several of the peaks listed in **Table 3** as well as one unidentified peak consistently detected only in the *R. pickettii* chromatograms and one unidentified peak consistently detected only in the *B. contaminans* chromatograms. These unidentified peaks were expected to be useful in clustering the data points obtained from each isolate as each peak was unique to a single isolate's

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DOI: 10.1039/D6AN00060F

chromatogram. Peak areas were compiled into a data set, and principal component analysis was performed.

PCA scores were plotted using PC1 and PC3 in **Figure 2** , which provided the clearest separation among isolates. Distinct clustering was observed, as demonstrated by the 95% confidence ellipses, confirming that the isolates' VOC profiles are highly distinct.

PC1 and PC3 accounted for 55% of the total variance, while PC2 accounted for the remaining 45%. The strong clustering along PC1 and PC3 can be attributed to the corresponding loading patterns. VOCs associated with each isolate exhibited similar loadings on PC1 and PC3, while differing from those associated with other isolates, enabling effective discrimination. However, loadings on PC2 were relatively consistent across most variables, limiting its contribution to isolate separation.

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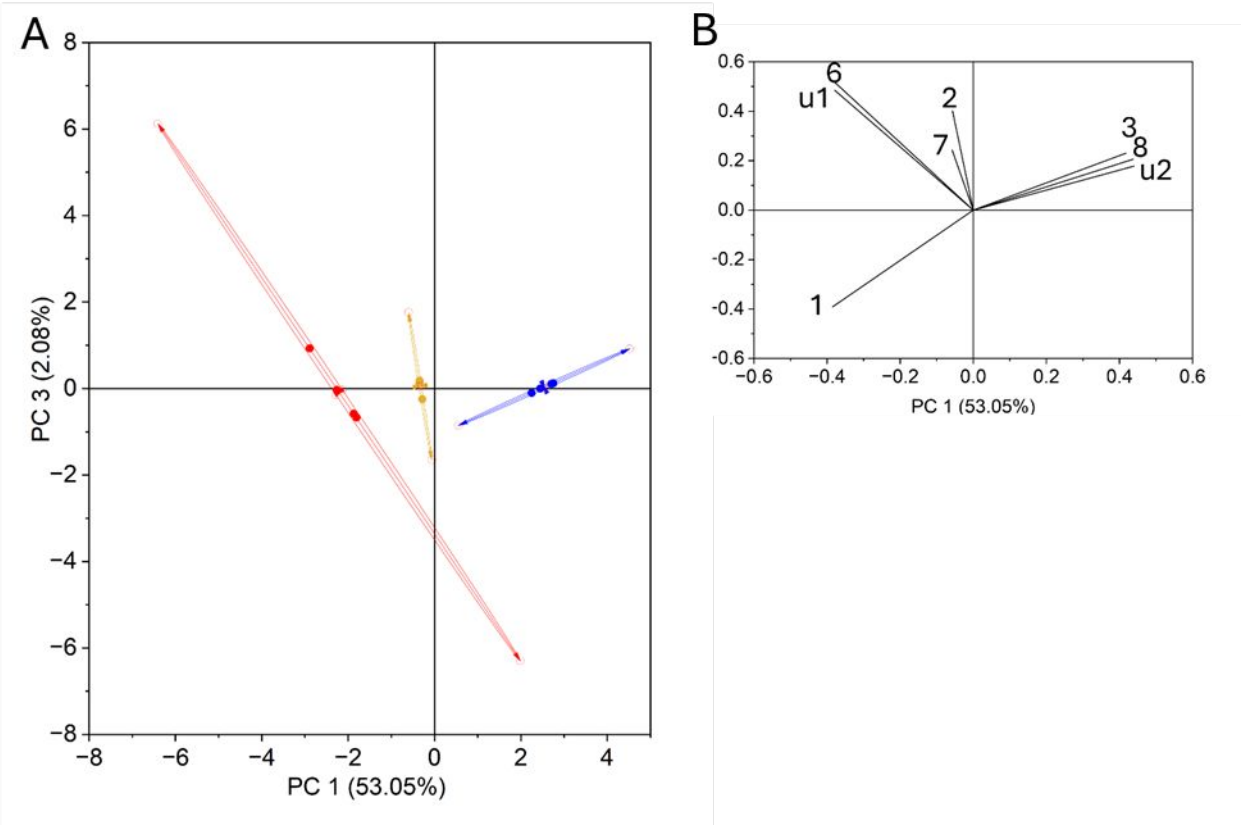

Figure 2. A) Plot of the PCA scores calculated for the R2A dataset. Confidence ellipses were calculated using 2 standard deviations. B) plot of the PCA loadings for each VOC used in the PCA. Peaks are labeled according to ***Table 2.*** *u1 refers to the unidentified peak detected in the B. contaminans headspace, while u2 designates the unidentified peak detected in the R. pickettii samples*

3.2. Analysis of VOCs detected from bacteria cultured in ersatz wastewater

R. pickettii and *B. contaminans* were both found to grow in the ersatz wastewater, and VOC analysis was able to be performed; *K. aerogenes* did not survive in the ersatz wastewater, and volatiles were not detected from the cultures. As can be seen in **Figure 3**, the relative abundances of the marker compounds found in R2A were lower for the cultures grown in ersatz wastewater.

View Article Online
DOI: 10.1039/D6AN00060F

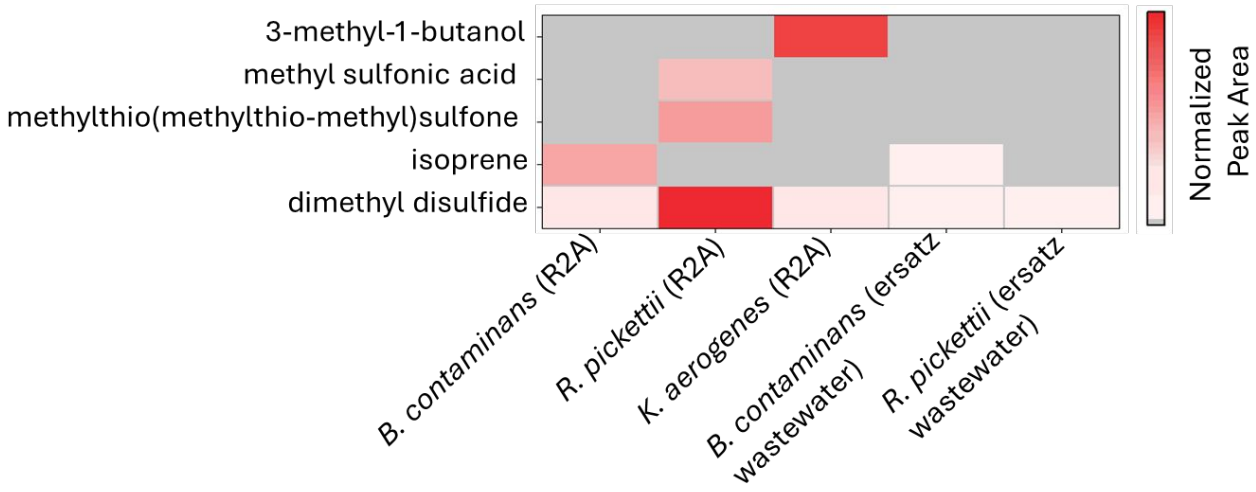

Figure 3. Heatmap showing the VOCs detected in the headspace above each isolate grown in R2A media and in ersatz wastewater. Cell coloration represents the normalized peak area obtained from the data collected on day 14. Gray cells indicate that the compound was not detected for a given isolate.

As can be seen in **Figure 3,** DMDS was again found to be present in the headspace above both *B. contaminans* and *R. pickettii* cultures. In order to understand at what point during culture growth DMDS is first detectable, a provisional quantitative analysis was carried out. A calibration curve was prepared from the DMDS standards. Using this curve, a limit of detection (LOD) of 3.4 ppb was calculated as described in the supplemental information. The concentration of DMDS was calculated for each sample in which it was detected and tracked as a function of growth time. This analysis determined the time required for the indicator to be detected following bacterial contamination. **Figure 4** shows that DMDS first appeared in the headspace spectrum of *B. contaminans* at day four, but it then began to decrease as the culture aged, with the peak area being only slightly above the detection limit by day 14. During this time, the absolute bacterial counts also decreased, though not to the same extent. DMDS only initially appeared in the mass spectra of the *R. pickettii* headspace at day seven of growth, but then, in contrast to *B. contaminans*, the peak continued to increase with

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further bacterial growth. The changes in the DMDS peaks as a function of time are shown in **Figures S3** (*B. contaminans*) and **S4** (*R. pickettii*).

Analysis of the ersatz wastewater following filtration to remove the bacteria was also performed to observe changes in volatile species (**Table S2**). This analysis revealed that the only compounds present were those included in the ersatz wastewater recipe for both *R. pickettii* and *B. contaminans* samples, as would be expected with the overall lower headspace concentrations of DMDS. However, the *B. contaminans* samples appeared to be depleted in alcohols, particularly ethanol. While interesting, this is not necessarily surprising, as *Burkholderia* spp. are known to utilize a variety of carbon sources as nutrients.

Exponential growth of the *B. contaminans* cultures was observed between days one and two post inoculation, and it was determined that DMDS was present in the culture headspace starting on day four. These findings suggest that DMDS could be used to indicate the presence of *B. contaminans* cultured in ersatz wastewater four days after inoculation and two days after population growth. Exponential growth of the *R. pickettii* cultures occurred between days four and seven post-inoculation, though the exact onset is uncertain. DMDS was detected in the culture headspace on day seven, indicating that this VOC becomes detectable within one to two days of growth. The quantification of DMDS served solely to determine at which time points the compound exceeded the detection limit in order to establish a timeline of detection. While DMDS was detected in the headspace of both isolates after growth, its abundance was not proportional to population size. This observation suggests that DMDS can serve as an indicator of the presents of *B. contaminans* and *R. pickettii* rather than a measure of the

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size of their populations. Additionally, the timeframes in which DMDS was detected are suitable for detecting growth in spacecraft wastewater during periods of dormancy, during which no crew will be present for extended periods[58]. Thus, an indicator VOC that provides the ability to detect growth within a week of inoculation is more than sufficient and would allow ample time to develop mitigation options.

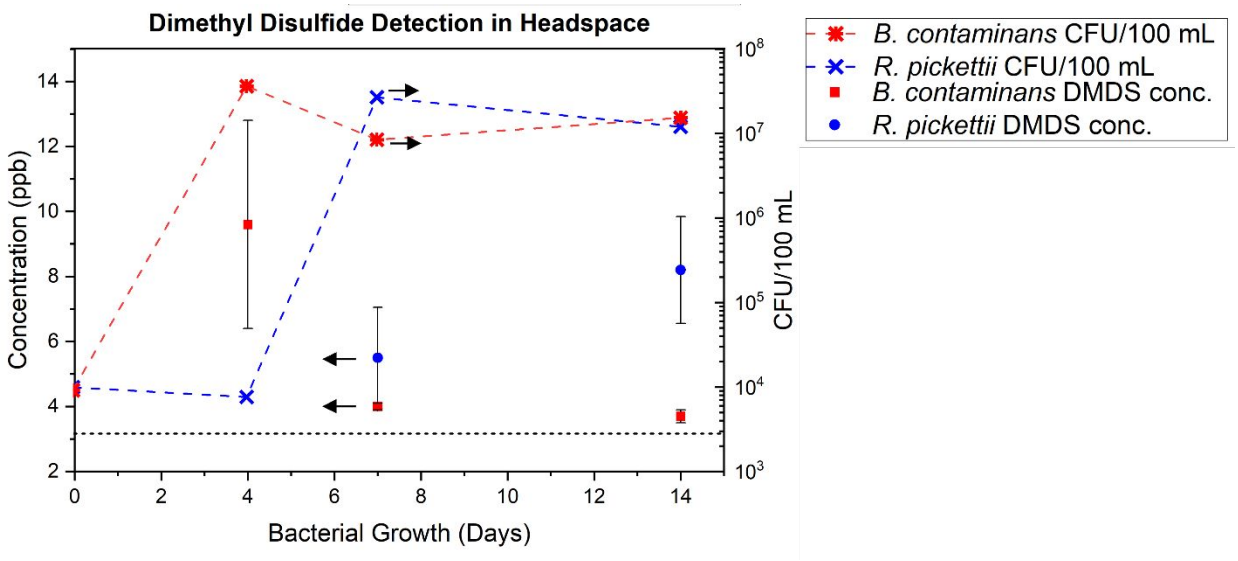

Figure 4. Average concentrations of the dimethyl disulfide plotted as a function of growth time (in days) prior to VOC analysis. The black dotted line represents the detection threshold of three ppb. Average colony counts collected on each day of analysis are plotted with guidelines against the secondary y-axis to show growth of the cultures over time.

3.3. Full Genome Analysis and Suggested DMDS Biosynthetic Pathway

With the VOC analysis of the culture headspace demonstrating the presence of major sulfur compounds, including DMDS and dimethyl trisulfide (DMTS), long read sequencing was performed for *Burkholderia* and *Ralstonia* to gain insight into their genomic capabilities and to evaluate their ability to potentially produce this compound. Genome metrics are listed in **Table** 4. A 100% ANI match to *B. contaminans* strain UnB1430[50] confirmed the *B. contaminans* identity. Genome assembly revealed an overall genome size of 8,727,029 bp with two chromosomes of 1,529,688 bp and 7,125,637 bp

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and one plasmid at 71,704 bp (**Figure 5**). Annotation described a GC content of 66.26% and a total of 7,786 genes (**Table** 4). Genome assembly and alignment of *Ralstonia* provided a 97.97% ANI to reference *R. pickettii* strain K-288,[50] validating the identity. With a total genome size of 5,930,920 bp, three chromosomes consisting of 107,360 bp, 1,396,269 bp, and 3,612,254 bp and three plasmids with sizes of 364,931 bp, 362,822 bp, and 87,284 bp were described (**Table** 4). Annotation of the *R. pickettii* genome detailed a GC content of 63.26 % and 5,689 genes (**Table** 4). Note that the 107,360 bp contig was classified as chromosome by MOB-suite. Whole-contig sequence identity, chromosomal gene content, coverage, and GC composition consistently supports its classification as a chromosomal region containing integrated mobile genetic elements.

Table 4. Long Read Genome Sequencing Metrics

	B. contaminans	***R. pickettii***
Genome Size (bp)	8,727,029	5,930,920
No. of Chromosomes	2	3
No. of Plasmids	1	3
GC %	66.26	63.26
Total Genes	7786	5689
% Coding Genes	87.1	88.4
No. of rRNAs	18	9
No. of tRNAs	69	58
No. of Pseudogenes	12	33
No. of Hypotheticals	316	574
ANI Match (%)	100	97.97
Best ANI Match NCBI	*Burkholderia contaminans* strain UnB1430	*Ralstonia pickettii* strain K-288

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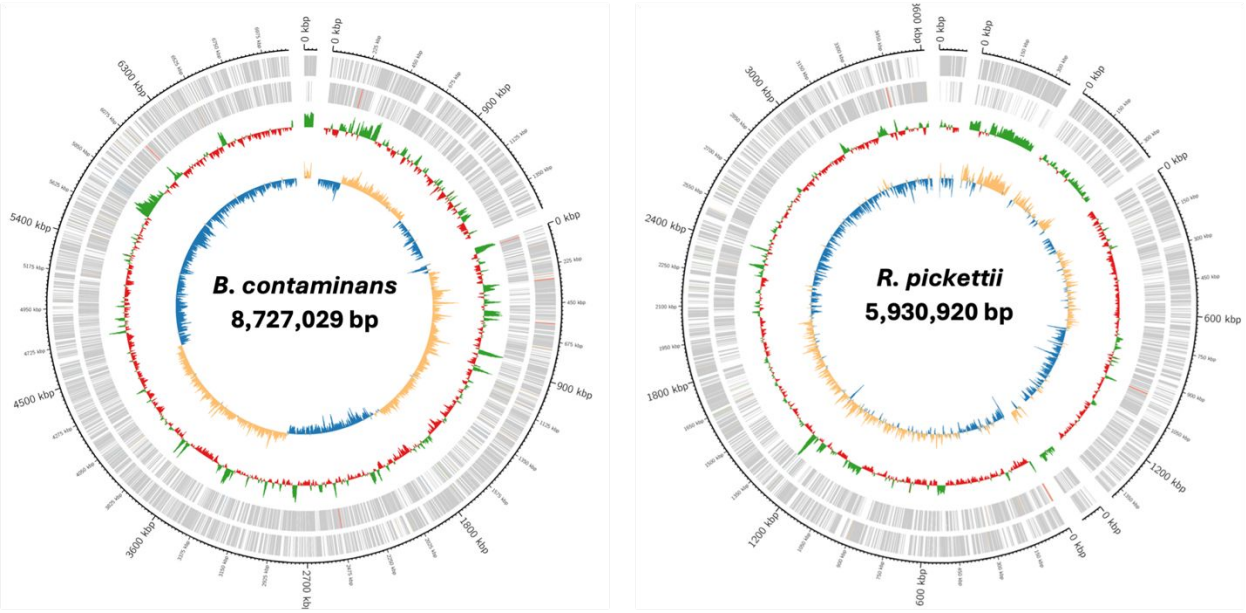

Figure 5. Circular genome maps of B. contaminans and R. pickettii from long read sequencing. The two outermost rings depict protein-coding sequences (CDS) encoded in the forward and reverse strands of genome, respectively. The third ring is the GC content with areas above and below the average shown in green and red, respectively. The innermost ring denotes the GC skew, where the positive is noted in light orange and the negative in blue.

DMDS is a well-established secondary metabolite produced by many bacteria, including *Burkholderia* spp., and is considered to be key in microbial interactions, as it imparts robust antifungal properties.[59-61] As such, the presence of DMDS in the headspace of the *B. contaminans* is not surprising and aligns with previous findings.[61, 62] While bacterial metabolism of sulfur is complex, L-methionine-γ-lyase (aka methionine gamma-lyase), cystathionine-γ-lyase, and cystathionine-β-lyase (aka cysteine-S-conjugate beta-lyase) are known key enzymes that play an essential role in the production of DMDS.[59-61, 63] Moreover, full genome analysis revealed methionine gamma-lyase (EC 4.4.1.11) and cysteine-S-conjugate beta-lyase (EC 4.4.1.13) on chromosome one of *B. contaminans*. These enzymes contribute significantly to cysteine and methionine metabolism, where DMDS is produced either directly or indirectly from *Burkholderia* spp.

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(Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway bdl00270 and bcep00270).[64] Furthermore, methanethiol easily converts into DMDS; this can occur biologically or chemically.[59, 65] With regard to *R. pickettii*, there is no previous published information of its ability to produce DMDS. However, *Ralstonia* is closely related to *Burkholderia,* and *R. pickettii* was previously identified as *B. pickettii.*[66, 67] Based on the close taxonomy and the presence of DMDS in the headspace of *R. pickettii* cultures, its genome was also evaluated for the presence of genes supporting DMDS biosynthesis. Cysteine-S-conjugate beta-lyase (EC 4.4.1.13), a key factor for DMDS production, is located on chromosome two and chromosome three of *R. pickettii*. Additionally, cysteine and methionine metabolism of *R. pickettii* are described in a KEGG pathway (rso00270),[64] which is highly similar to the pathway known to result in the production of DMDS in *Burkholderia* spp. As such, based on the genome analysis of both organisms, the key enzymes involved in cysteine and methionine metabolism supporting DMDS production were reconstructed and simplified into a suggested biosynthetic pathway (**Figure 6**). It is important to note that DMTS was also detected in *R. pickettii*, but not in *B. contaminans*. This does not impact or alter the biosynthetic pathway, as DMTS is also an indirect VOC noted during the conversion of methanethiol to sulfide products. In an aqueous environment, DMDS and DMTS are interchangeable depending on the stage of the reactions.[62]

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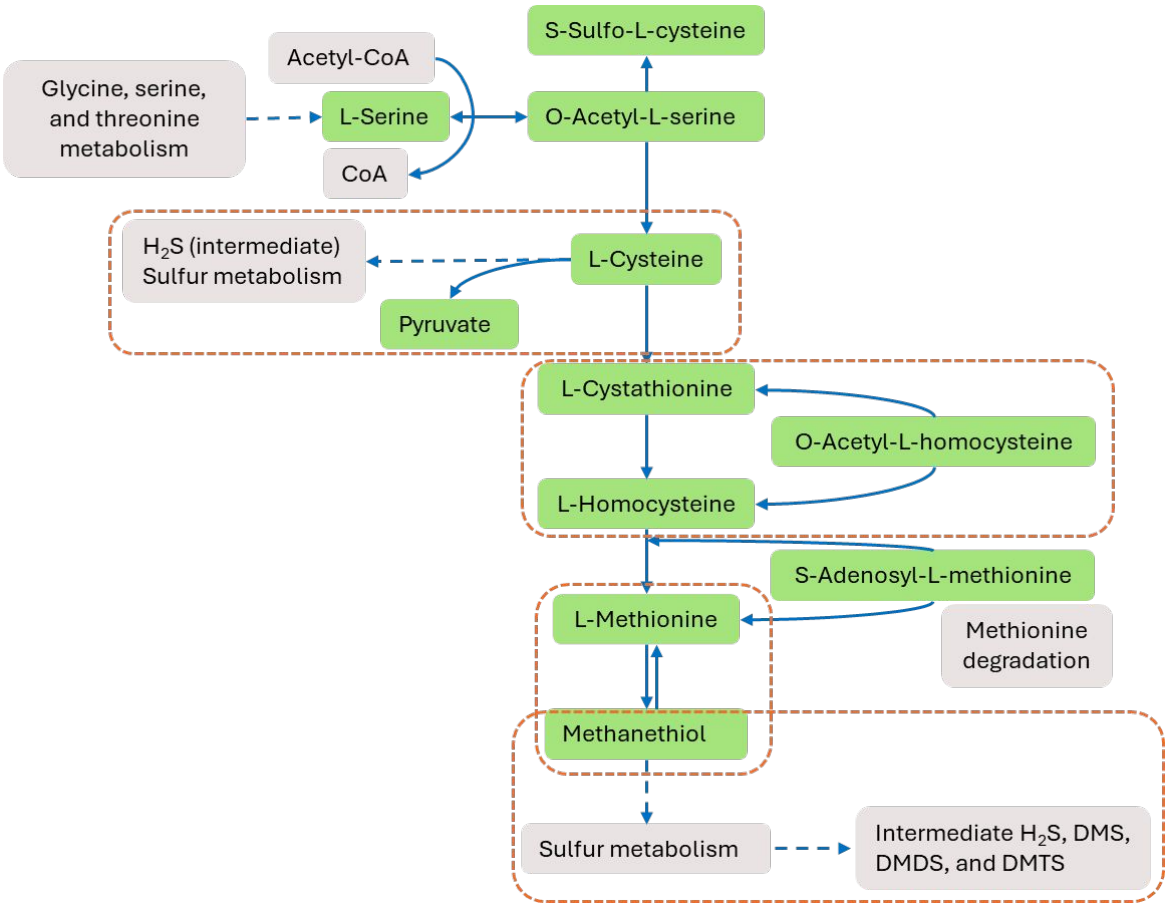

Figure 6: A simplified and suggested cysteine and methionine biosynthetic pathway resulting in DMDS based on the genomes and published KEGG pathways of B. contaminans and R. pickettii. Blue dashed lines depict indirect processes, while orange dashed boxes indicate areas where DMDS production is indicated to occur.

4. Conclusions

This study characterizes the volatile organic compounds (VOCs) emitted by *R. pickettii* and *B. contaminans* when cultured in a spacecraft wastewater simulant. As the most frequently isolated genera from spacecraft water systems, *Ralstonia* and *Burkholderia* exhibit notable resilience in this environment. This was demonstrated by the survival of *R. pickettii* and *B. contaminans* in the ersatz wastewater in contrast to *K. aerogenes* under the same conditions.

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The volatile compound DMDS was detected in the headspace of *R. pickettii* and *B. contaminans* in ersatz wastewater. The isolates' abilities to produce this compound were supported by genetic sequencing. These results allowed for the confident use of DMDS as an indicator of bacterial growth in the experiments described. Given the widespread presence of *Ralstonia* and *Burkholderia* spp. in spacecraft water systems, VOC indicators associated with these organisms are especially valuable in monitoring spacecraft water environments for bacterial growth.

However, further studies of VOC production by bacteria under different conditions and matrices are needed to determine the potential for mass spectrometric monitoring in microbes in other spacecraft environments.Specifically, studies examining additional species of bacteria and co-cultures of bacteria would be beneficial. Previous reports of the microbial make up of spacecraft wastewater have shown that this environment can support complex microbial communities in which multiple species of microbes grow and interact with one another. Further studies that examine the VOCs emitted by a wider range of bacteria as well as microbial communities including multiple microorganisms could provide a deeper insight into other microbial VOCs that could serve as indicators of microbial contamination. The studies carried out in ersatz provide an idea of what bacteria can grow in this environment and what VOCs are detectable when growth occurs based on typical components of the spacecraft wastewater solution. However, it is possible for spacecraft wastewater composition to vary in flight, so a thorough background analysis of system wastewater would be necessary before applying this technique to an actual system. Additionally, while the time frames of DMDS detection were sufficient for the target applications of this work, studies that

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incorporate more frequent monitoring of microbial VOCs could help to determine a more specific window of time in which the cultures are detectable. Finally, a thorough exploration of matrix effects examining how components of the ersatz wastewater solution potentially impact VOC collection and analysis would be useful for analytical validation in this complex environment.

While DMDS has been known to originate from both biotic and abiotic sources, It should be noted, though, that DMDS has not been detected in the ISS atmosphere since routine sampling began, indicating that it has limited abiotic sources onboard. Thus, if detected in an area prone to microbial growth ,a biotic source is most likely.

Overall, these findings present the effective use of mass spectrometry as a sensor for the microorganisms studied. This work establishes DMDS as a promising indicator for the growth of these common spacecraft bacteria in spacecraft water systems and underscore the broader potential of VOC analysis as a practical and effective tool for microbial monitoring in spacecraft environments.

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Raw data was generated at the NASA Johnson Space Center Microbiology and Environmental Chemistry labs. Data that supports the findings of this study are available upon request from the corresponding author, SK.