Characterizing the Impact of Low Shear Modeled Microgravity on Population Dynamics, Biofilm Formation and Silver Susceptibility of Microbial Consortia Isolated from International Space Station Potable Water

by

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ABSTRACT

Understanding how microorganisms adapt and respond to the microgravity environment of spaceflight is important for the function and integrity of onboard life support systems, astronaut health and mission success. Microbial contamination of spacecraft Environmental Life Support Systems (ECLSS), including the potable water system, are well documented and have caused major disruption to spaceflight missions. The potable water system on the International Space Station (ISS) uses recycled wastewater purified by multiple processes so it is safe for astronaut consumption and personal hygiene. However, despite stringent antimicrobial treatments, multiple bacterial species and biofilms have been recovered from this potable water system. This finding raises concern for crew health risks, vehicle operations and ECLSS system integrity during exploration missions. These concerns are further heightened given that 1) potential pathogens have been isolated from the ISS potable water system, 2) the immune response of astronauts is blunted during spaceflight, 3) spaceflight induces unexpected alterations in microbial responses, including growth and biofilm formation, antimicrobial resistance, stress responses, and virulence, and 4) different microbial phenotypes are often observed between reductionistic pure cultures as compared to more complex multispecies co-cultures, the latter of which are more representative of natural environmental conditions. To advance the understanding of the impact of microgravity on microbial responses that could negatively impact spacecraft ECLSS systems and crew health, this study characterized a range of phenotypic profiles in both pure and co-cultures of bacterial isolates collected from the ISS potable water system between 2009 and 2014. Microbial responses profiled included population dynamics, resistance to silver, biofilm formation, and *in vitro* colonization of intestinal epithelial cells. Growth characteristics and antibiotic sensitivities for bacterial strains were evaluated to develop selective and/or differential media that allow for isolation of a pure culture from co-cultures, which was critical for the success of this study. Bacterial co-culture experiments were performed using dynamic Rotating Wall Vessel (RWV) bioreactors under spaceflight analogue (Low Shear Modeled Microgravity/LSMMG) and control conditions. These experiments indicated changes in fluid shear have minimal impact on strain recovery. The antimicrobial efficacy of silver on both sessile co-cultures, grown on 316L stainless

steel coupons, and planktonic co-cultures showed that silver did not uniformly reduce the recovery of all strains; however, it had a stronger antimicrobial effect on biofilm cultures than planktonic cultures. The impact of silver on the ability of RWV cultured planktonic and biofilm bacterial co-cultures to colonize human intestinal epithelial cells showed that, those strains which were impacted by silver treatment, often increased adherence to the monolayer. Results from these studies provide insight into the dynamics of polymicrobial community interactions, biofilm formation and survival mechanisms of ISS potable water isolates, with potential application for future design of ECLSS systems for sustainable human space exploration.

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CHAPTER 1

INTRODUCTION

Aboard the International Space Station (ISS), a limited water supply serves all drinking, personal hygiene and environmental control needs. Thus, a high fidelity purification system must be in place to recycle the water supply and reduce microbial burden and other contaminants to meet potable water requirements. The potable water Environmental Control Life Support System (ECLSS) for American astronauts onboard the ISS was developed and rigorously tested at the NASA Johnson Space Center to ensure that contamination - both chemical and biological - could be effectively reduced and/or removed (**1-4**). The water purification component of this ECLSS system currently utilizes iodine as a chemical disinfectant; yet despite extreme temperatures, minimal nutrient availability and microgravity, bacteria and biofilms have been isolated from the water supply since 2009 (**3, 4**).

Bacterial contamination in potable water on the ISS can be detrimental to both the crew and the integrity and operation of ECLSS systems. Studies have shown that astronaut immune function is compromised during spaceflight, which suggests a potential higher risk for infection (**5-9**). While the exact cause(s) for the observed immune deficiencies in astronauts is not completely understood, the increased likelihood of susceptibility to microbial infection calls for a high fidelity water purification system. Concern for crew health is further amplified as known pathogenic species have been recovered from the ISS potable water system (**2-4, 10, 11**). Because of the unique nature of the microgravity environment of spaceflight and unexpected microbial responses observed in in this environment (**12-16**), it is possible that microorganisms which are not normally pathogenic on Earth could pose a health threat to the crew. For example, spaceflight has been shown to increase the virulence of the bacterial pathogen *Salmonella* Typhimurium, as well as alter pathogenesis-related phenotypes, induce biofilm formation, and globally reprogram gene expression in this organism (**14**). In addition, other pathogens including *Pseudomonas aeruginosa* and *Candida albicans* have exhibited global changes in their gene expression, biofilm formation and stress responses in response to spaceflight culture (**12-16**). Culture of these same

pathogens under spaceflight analogue conditions in the RWV bioreactor has been able to mimic several of these responses as well (**12-15**).

The combination of decreased immune function in the crew (**8, 9, 17-24**) and altered microbial virulence, pathogenesis related stress responses, biofilm formation, and antimicrobial resistance during spaceflight emphasizes the potential risks to crew health and integrity of spacecraft ECLSS systems. A better understanding of the microorganisms in the ISS potable water system, their population dynamics, and the characteristics which enable them to survive this harsh environment (*e.g.,* antimicrobial resistance, biofilm formation, etc.) is essential to help mitigate these risks.

In addition to understanding the mechanisms and relationships which bacterial populations use to survive in the ISS potable water system, a safe and efficient decontamination treatment must be designed for future water supply systems. Silver ions show promising results as safe and effective antimicrobial agents (**25-27**). However, to ensure that silver is a viable treatment, the environment in which antimicrobial efficacy tests are performed should replicate the spaceflight environment (co-culture populations, fluid shear, etc.) as closely as possible.

One major environmental factor to consider in the lab when trying to mimic the spaceflight environment is microgravity. Gravitational forces impact the movement of every object. The gravity on Earth (1xG) is different to the reduced gravity on the ISS, thus the indirect effect of the force imposed on the bacteria on the ISS is different to that on Earth. To model aspects of the microgravity environment on the ISS, bacterial strains can be grown in RWV bioreactors**.** RWV bioreactor technology, developed at the NASA Johnson Space Center (**28**), provides an optimized suspension culture in the low shear modeled microgravity (LSMMG) orientation. This technology can be used to investigate the effect of LSMMG on cell populations. Within the LSMMG oriented RWV bioreactor, cells are suspended under an average fluid shear of less than 0.01 dyne/cm^2 (**29, 30**). This system can also be used to compare the control orientation, which shows the impact of earth's gravity (1xg) to the LSMMG condition by changing the physical orientation of the bioreactor. These two orientations provide a unique system to model cellular responses on Earth versus those in space.

This study aimed to understand the population dynamics, biofilm formation and antimicrobial resistance of pure and mixed species co-cultures of bacteria isolated from the potable water supply of the ISS. With this information, the efficacy of silver as an antimicrobial treatment against the specific bacterial contamination in the ISS potable water system can be gauged. In order to enhance the relevance of this research to the spaceflight condition, experiments were done using the RWV bioreactor.

Table 1: ISS Potable Water Isolates

Year	Species
2009	*Ralstonia pickettii*
	Sphingomonas sanguinis
	Cupriavidus metallidurans
2012	*Burkholderia cepacia complex*
	Ralstonia pickettii
	Ralstonia insidiosa
	Sphingomonas sanguinis
	Methylobacterium species
2014	*Burkholderia cepacia complex*
	Ralstonia picketti
	Ralstonia insidosa
	Bradyrhizobium species
	Mesorhizobium species
	Staphylococcus epidermidis

ISS Water System. The Environmental Control and Life Support System is responsible for temperature and humidity control, atmosphere revitalization, supplementary oxygen production, and fire suppression, as well as recycling wastewater aboard the ISS to create a sustainable source of potable water for crew members (**2, 31**). The potable water system component of the ECLSS has the capacity to recycle approximately 70-80% of the water needed to maintain a healthy environment for the crew (**32-36**). The remaining 30% of the water lost during recycling is compensated by sending potable water to the ISS and then transferring this water into the potable water system to replenish the volume lost. The potable water system was initially expected to have a 93% recovery efficiency; however, urine brine and solid precipitates decrease the overall recovery (**3, 4, 36**). Improvements to the potable water recovery have significance beyond the ISS. Future manned mission to space will require safe and sustainable housing. Thus, maintaining the integrity of space flight potable water systems for an extended period of time can support the mission success.

ISS Bacterial Strains. After careful evaluation of data collected from ISS potable water microbial isolate testing from 2009 through 2014**,**14 bacterial strains were selected as representative test groups for this study. The 14 bacterial isolates analyzed in this study were collected in three different years and were all known biofilm formers. The 2009 bacterial isolates

selected were *Ralstonia pickettii*, *Sphingomonas sanguinis*, and *Cupriavidus metallidurans*. The 2012 bacterial isolates selected were *Burkholderia cepacia* complex (Bcc.), *Ralstonia pickettii*, *Ralstonia insidiosa*, *Sphingomonas sanguinis* and *Methylobacterium* species. The 2014 bacterial isolates selected were *Burkholderia cepacia* complex (Bcc.), *Ralstonia pickettii*, *Ralstonia insidiosa*, *Bradyrhizobium* species, *Mesorhizobium* species and *Staphylococcus epidermidis* (**Table 1**).

The *Ralstonia* genus - found in all three test group years - are commonly found both environmentally (in soil and water sources) and clinically (**37**). This genus of Proteobacteria was reclassified from *Pseudomonas*. These species are Gram-negative, aerobic, non-fermenting, oligotrophic rods. The *Ralstonia* species in this study, *R. pickettii* and *R. insidiosa*, have been identified to have very similar phenotypes, are common nosocomial infections - primarily in cystic fibrosis patients – and are frequently found in highly purified water (**38**). The ability for these strains to develop biofilms (**39**) and prosper in low nutrient environments (**40**) makes the management of contamination difficult. While the biodegradative abilities (**41**) of these strains has potential waste management applications, their presence in the ISS potable water system raises concern for materials and system corrosion. *Cupriavidus metallidurans*, formerly classified as *Ralstonia metallidurans*, is predominantly found in water supplies. This strain thrives in high metal concentrated environments and has channels able to transport cobalt, zinc, cadmium, manganese, nickel and iron (**42**). A key concern with this species is corrosion of metal materials in the water system.

Similar to the *Ralstonia* species, *Burkholderia* strains occupy an extremely wide range of niches. *Burkholderia* are found in both fresh and saltwater sources, soil, and in the rhizosphere of plants and animal respiratory tracts (**43**). *Burkholderia* species have both commensal and pathogenic properties that are not easily differentiated by strain (**44**). Because *Burkholderia* strains have the ability to metabolize many carbon sources, the genus has biotechnology potential in bioremediation and crop protection (**45**). Species of this genus are Gram-negative, aerobic, nitrogen-fixing, straight rods (**46**). The key interest in this species as a contaminant in drinking water is the potential for opportunistic infection.

The two strains of *S. sanguinis* are Gram-negative, aerobic, non-fermenting rods. Members of the *Sphingomonas* family have been found in minimal carbon-source containing water supplies. While other species are only able to survive in natural mineral water in starvation conditions, *Sphingomonas* adapts to these conditions suitably (**46**). While some members of the *Sphingomonas* genus have pathogenic potential, little evidence has been found to label *S. sanguinis* as a pathogen (**47**).

The *Methylobacterium* species, is the only strain in this study that uses methanol and formaldehyde as preferred carbon sources. This microbe is known to be a fastidious, spore-forming, Gram-negative genus which has been identified as an opportunistic pathogen. This environmental species is found primarily in surface level soil and sewage (**48**). However, some members of the genus have been isolated from several anatomical sites including blood, peritoneal fluid and ascitic fluid (**48**).

Both the *Bradyrhizobium* and *Mesorhizobium* isolates are nitrogen-fixing, Gram-negative environmental bacteria (**49, 50**). Both genera have been found to have a symbiotic relationship with legumes and other plant types (**49, 50**). Neither strain is a major concern as a clinical pathogen.

The final isolate, *S. epidermidis*, is the only Gram-positive strain in the study. This isolate is a facultative anaerobe commonly found on human skin and mucosal surfaces (**51**). Recently, *S. epidermidis* has become the most common source of medical implant infections (**51, 52**). The species has increased biofilm formation abilities in anaerobic conditions (**53**). As a biofilm-forming contaminant associated with the ISS potable water supply, this strain raises health concerns. However, as *S. epidermidis* is not commonly found in purified water, it is hypothesized that this organism was recovered as an ECLSS contaminant by human collection error.

Co-culture Biofilm Cooperation and Competition. Within the ECLSS water purification system, biofilm-forming bacterial strains grow in co-culture. Bacteria form biofilms as a survival mechanism in harsh environments. Biofilms are complex networks of multispecies microorganisms which are capable of adapting in response to the surrounding microenvironment. Biofilm organization and interactions promote multispecies population resistance to

antimicrobials, nutrient limitations and extreme environmental stresses (**54-58**). Furthermore, polymicrobial biofilms have been shown to increase the virulence of some pathogens within the population (**59**). In water purification systems, bacterial contamination may persist, in part, through enhanced tolerance to hostile conditions due to the strains' ability to form biofilms. The LSMMG conditions have been previously shown to increase bacterial culture's propensity to form biofilms **(16, 60-64**). To understand the full impact of biofilm networks on bacterial persistence within the ISS potable water system, the influence of microgravity and antimicrobials must be determined for both biofilm and planktonic populations. It should be noted that, when referring to a culture sample in this study, the term "biofilm" is used to describe either a RWV-surface or stainless steel coupon adherent biofilm. The term "planktonic," in this study, refers to bacterial samples of broth culture and might include self-aggregated bacterial biofilms found in the liquid culture.

Biofilms threaten the mechanical integrity of materials and operational systems on the ISS by potentially resulting in microbially influenced corrosion (**32**). Studies have shown that bacteria in biofilms are more capable of metal corrosion than their planktonic counterparts (**65-67**). As with antibiotic resistance and pathogenic potential, horizontal transfer genetic interactions between multispecies bacterial consortia increase the likelihood of corrosive ability to spread throughout the polymicrobial community (**68**). One bacterial species of concern regarding corrosive damage to spacecraft ECLSS systems is *C. metallidurans*. As this organism has the ability to degrade heavy metals, the risk of structural damage to ECLSS systems could increase when this species is part of the polymicrobial community (**42)**.

Since isolates for this study were selected from three separate years, ISS bacterial cocultures grouped by year of isolation were treated as individual data sets rather than coculturing all 14 strains simultaneously. This decision was based on evidence (unpublished data, not shown in this thesis) that, even when the species from this study were kept consistent, interaction of strains in the same isolation year differ from the interactions of strains from differing isolation years (**69**). While, in the isolation year co-cultures, each species likely contributes to the overall survival of the polymicrobial population, the impact of co-culture on individual strain

recovery, biofilm formation, silver susceptibility and ability to colonize epithelial monolayers was of key interest in this study.

Antimicrobial Silver. The current antimicrobial treatment in the potable water system on the U.S. side of the ISS is iodinated resin. Despite the wide usage of iodine as a disinfectant, there is evidence that several species, including *Pseudomonas* and *Burkholderia*, are able to develop resistance to the treatment (**70**). Furthermore, with multiple biofilm forming species in the contaminating populations, the potential failure of an antimicrobial is escalated.

For the ISS and future long-term space missions, silver nanoparticles have been proposed as an antimicrobial in water purification systems (**71**). These particles range from 1 to 100 nm and readily infiltrate bacterial cell membranes (**72, 73**). Within the cell, silver nanoparticles accumulate and inhibit signal transduction by interfering with bacterial DNA. Additionally, silver nanoparticles interact with respiratory enzymes to release reactive oxygen species and induce apoptosis. (**72, 73**). A major concern over the use of silver in ECLSS water purification systems is the presence of *C. metallidurans* and *Ralstonia* strains which have been shown to persist in heavy metal environments (**74**).

Rotating Wall Vessel (RWV) Bioreactors. In order to understand polymicrobial dynamics and antimicrobial resistance in the potable water system aboard the ISS, bacteria need to be studied either in the true microgravity of spaceflight or in ground based spaceflight analogue conditions. Replicating the fluid shear that bacteria experience on the ISS requires specialized technology that can mimic aspects of this environment RWV bioreactors, make mirroring the low fluid shear conditions of the ISS possible on Earth.

The RWV bioreactor, developed at the NASA Johnson Space Center (**75, 76**), is a laboratory tool which provides low-shear, low-turbulence cell suspension culture that is capable of modeling aspects of spaceflight (**Figure 1**) (**12, 13, 63, 64, 77-79**) The RWV bioreactor consists of a hollow cylindrical disk which is completely filled with fluid and mounted on to a rotating motor. A gas permeable membrane at the back of the bioreactor vessel allows for aeration of cultures. When culturing bacteria in the RWV, the rotation speed is commonly maintained at ~25 rotations

per minute r.p.m (**80**). A gas permeable membrane at the back of the bioreactor vessel allows for aeration of cultures.

Fluid shear is the force that a liquid exerts on the surface of a cell and is known to regulate numerous bacterial responses, including gene expression, stress responses, adherence, biofilm formation and virulence (**21, 30, 75, 77, 81**). Because the RWV bioreactor is completely filled with liquid, the media and cells rotate as a solid body. Within the RWV, constant fluid shear results in mass distribution of nutrients, cell by-products and the bacterial population. In normal Earth gravity fluid shear conditions (1xG), cells sediments to the bottom of the RWV bioreactor. Conversely, the LSMMG conditions results in cells being maintained in suspension (**75**). Comparing these two conditions side-by-side allows for a better understanding of the impact of fluid shear on bacterial growth, biofilm formation and potential pathogenicity.

Figure 1:

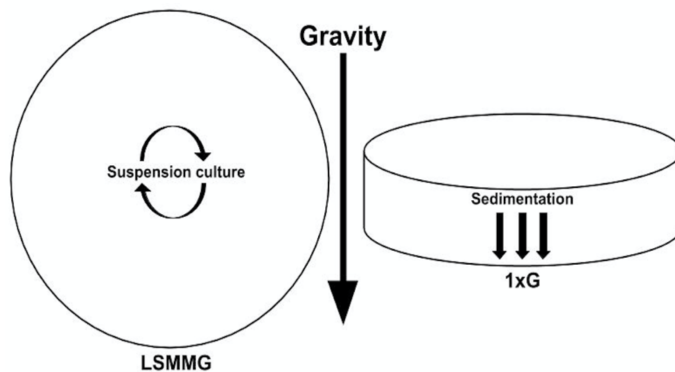


Figure 1: Rotating Wall Vessel Bioreactor. RWV experiments for this study were run with two separate bioreactors, each in a different orientation. One bioreactor was in the LSMMG orientation and the other in the control (1xG) orientation. Both bioreactor orientations were operated at 25 r.p.m. In the LSMMG orientation, there is no mass sedimentation caused by gravity and cells are cultured in a low fluid shear environment that simulates that encountered by cells grown in liquid culture in spaceflight. Conversely, in the control orientation, cells can sediment at the bottom of the RWV and putative higher fluid shear levels are encountered.

The need for ground-based spaceflight analogue culture systems that can mimic aspects of cellular responses to spaceflight is furthered by evidence that microgravity can alter microbial gene expression, stress responses, biofilm formation, pathogenesis related stress responses and virulence in ways that are not observed using traditional experimental culture approaches on Earth (**12, 13, 30, 63, 64, 75, 77-79, 80, 82-86**). In both pathogenic and nonpathogenic microorganisms, LSMMG-correlated gene expression changes have been hypothesized to alter cell size, nutritional preference, antibiotic susceptibility and the likelihood for biofilm formation (**87,**

88). Repeated studies showed enhanced virulence of *S.* Typhimurium strain (χ3339) in spaceflight culture compared to Earth-based cultures (**12, 13, 84**). While LSMMG culture of this same pathogen mimicked the increased virulence observed in spaceflight (**77, 78**). However, LSMMG conditions do not always increase the virulence of bacterial strains, as shown with a different *S.* Typhimurium strain (D23580) which decreased in virulence in response to LSMMG culture conditions compared to control orientation (**80, 89**). Thus, to best evaluate the risk that the ECLSS contamination poses on the ISS crew and mechanical system, experiments need to be performed with relevant ISS microgravity.

Experimental Approach. The goal of this study was to analyze the growth of polymicrobial communities from ISS potable water isolates when simultaneously co-cultured in spaceflight analogue RWV bioreactors by examining i) biofilm formation on biotic and abiotic surfaces, ii) antimicrobial susceptibility, and iii) population dynamics. To address this goal, the experimental design was divided into three aims: 1) the development of selective and/or differential media for each isolate for the purpose of extracting a specific strain from co-culture, 2) the analysis of the effects of co-culture and physiological fluid shear on the growth of each individual strain within isolation year group populations, and 3) the determination of the viability of silver as an antimicrobial for the ISS potable water source based on sample strains' reaction.

The first aim of this research involved an in-depth analysis of the antimicrobial and reagent susceptibility and resistance of each ISS bacterial strain to antibiotics/reagents and the metabolic capabilities of the strains. These experiments were run for the purpose of developing a selective and/or differential media that allowed for the isolation or identification of an individual strain from a co-culture of other bacteria from the same year. This was necessary to allow identification and selection of a single strain during co-culture of all strains isolated in the same year. For the purpose of this research, an ideal media was defined as one that isolated the desired strain from all other strains isolated in the same year without decreasing its recovery. The growth rate and optimal growth conditions for each strain were gauged individually.

The second aim of this study used the media developed in Aim one to determine the population dynamics of bacteria from isolation year group co-cultures. Experiments performed in this aim were intended to establish how each strain responded to the co-culture environment and compare the polymicrobial dynamics in the LSMMG and control orientations of the RWV bioreactor.

The final aim of this study determined the antimicrobial efficacy of silver on bacterial populations. Aim three evaluated the combined impact of silver and low fluid shear culture on both planktonic and biofilm populations. In addition to general population recovery, the impact of these two variables on planktonic and biofilm populations' pathogenicity was assessed. To support the quantitative data collected in this aim, biofilm cultures were imaged using confocal microscopy to visualize the impact of silver and fluid shear on biofilm structure.

CHAPTER 2

METHODOLOGY

ISS Bacterial Strains. Of the original sixteen bacterial ISS potable water isolates selected to analyze in this study, fourteen strains were chosen for further experimentation and the development of selective media. One strain was eliminated as it was found to be a species duplicate isolated from the same year and the other was separated out for independent research. Bacterial isolates were provided by NASA Johnson Space Center (Houston, TX) to the Nickerson Laboratory at the Biodesign Institute at Arizona State University. Bacterial species' identities were confirmed by NASA prior to shipment. All strains were grown in Reasoner's 2A (R2A) liquid medium in sterile polypropylene snap cap tubes at ambient temperature (~24 ^{o}C) and shaken at 130 r.p.m until the strains reach stationary phase, as determined by growth curves.

Nonselective Growth Media. R2A medium was designated as the nonselective growth medium for this experiment. R2A is a permissive growth medium designed to assist in high colony retrieval for species that grow in low nutrient conditions, such as potable water (**90, 91**). R2A contains casein acid hydrolysate (0.5 g/L), yeast extract (0.5 g/L), proteose peptone (0.5 g/L), dextrose (0.5 g/L), soluble starch (0.5 g/L), dipotassium phosphate (0.3 g/L), sodium pyruvate (0.3 g/L), casein peptone (0.25 g/L), peptic digest of animal tissue (0.25 g/L) and magnesium sulfate (0.024 g/L). Once prepared, the media has a final pH of 7.2 when dissolved in deionized water. In both liquid and plate preparations, 3.15 g/L of R2A was used. Plates were prepared with 1.3% rather than 1.5% agar concentration to increase colony size.

Antibiotic Sensitivity Testing. To develop selective and/or differential media, the resistance and susceptibility of each isolate was examined. For this purpose, 19 antibiotics and three reagents were tested in various concentrations. The antibiotics included penicillins (ampicillin, carbenicillin), cephalosporins (ceftriaxone), fluoroquinolones (levofloxacin, nalidixic acid), aminoglycosides (streptomycin, hygromycin b, gentamicin, kanamycin), glycopeptides/ lipoglycopeptides (vancomycin), macrolides/lincosamides/ streptogramins (azithromycin), tetracyclines (tetracycline), and others (chloramphenicol, cycloheximide, polymyxin b, rifampicin, spectinomycin, trimethoprim, bacitracin). To thoroughly test the susceptibility and resistance of

each bacterial strain, the antibiotics covered a wide range of effector mechanisms including cell wall synthesis inhibitors (penicillins, cephalosporins, vancomycin, bacitracin), protein synthesis inhibitors (aminoglycosides, tetracyclines, macrolides, chloramphenicol, streptogramins, spectinomycin, cyclohexamide), DNA synthesis inhibitors (fluoroquinolones), RNA synthesis inhibitors (rifampicin) and folic acid synthesis inhibitors (trimethoprim). The natural antibiotic resistance of each species was noted for comparison to the discovered resistances and susceptibilities.

The three selective and/or differential reagents used in this study were Congo red, crystal violet and sodium chloride. Crystal violet has antifungal, antibiotic, anthelminthic and antimitotic properties. By dissipating the action potential on the cell surface, crystal violet increases permeability, inhibiting respiration and inducing cell death in susceptible bacteria. Congo red is a benzidine-based anionic diazo dye which binds to amyloid proteins and allows discrimination between bacteria based on biofilm production. Finally, the addition of sodium chloride to media increases the osmolarity. Due to the osmotic effect, increased sodium chloride concentrations in media can cause hypertonic conditions that can kill some bacteria. Halophiles survive high saline concentrations by either accumulating salt within the cell or by excluding it from the cytoplasm (**92**). While none of the strains in this study are halophilic, *S. epidermidis* is haloduric and thus can survive in high NaCl concentrations (**93**).

Development of Selective and/or Differential Media. To develop media, 19 antibiotics and three selective/differential reagents (Congo red, crystal violet and sodium chloride) were tested for each of the 14 strains in different concentrations for the purpose of identifying susceptibilities and resistances. For each antibiotic and reagent, between 1-8 different concentrations were tested to establish the optimum media concentrations. The initial concentrations were selected based on available product manufacturer information as well as previously published protocols. Additional concentrations were later added as necessary to specifically target individual strains. Each antibiotic or reagent plate was prepared using R2A as the base medium. After the R2A medium was autoclaved, it was cooled to ~ 50 ^{o}C before the antibiotic or reagent was added. Once thoroughly mixed, plates were poured with 25-30 mL of

medium and allowed set at room temperature before being moved to 4 °C storage. Plates were standardly used within a week of preparation. For media testing, 5 mL R2A broth cultures were inoculated for each strain. The inoculation of colonies from quadrant streaked R2A plates took place 48 hours (hr.) prior to the experiment time for all cultures except *Bradyrhizobium* spp., *Mesorhizobium* spp., *Methylobacterium* spp., and *S. epidermidis*. For *Bradyrhizobium* spp. and *Mesorhizobium* spp., inoculations were made 96 hr. prior to the experiment while *Methylobacterium* spp. and *S. epidermidis* cultures were inoculated 36 hr. prior to the experiment. In order to have all strains at stationary phase at the time of experimentation, these four strains were different incubation times compared to the other 10 strains. The broth cultures were grown shaking at 130 r.p.m and ambient temperature to allow each strain to reach stationary phase by the time of the experiment. For each strain, eight serial dilutions (10^{-2} to 10^{-9}) were prepared in 96 well-plates using R2A broth. Five microliters of each dilution were then plated onto the different selective/differential media and incubated in a humidified incubator at 28.5 °C. This temperature was selected as a midpoint between body temperature (37 °C) and room temperature to allow the diverse collection of species in this study to grow in co-culture conditions. Plates were analyzed at 24, 72, 120 and 168 hr. post inoculation to monitor growth of both slow- and fast-growing strains.

In addition to testing the custom media above, previously established selective/differential media were reviewed (**42, 94**). These media were first evaluated using established recipes, without additional antibiotics or reagents. Tests were performed as described above. For *C. metallidurans*, Tris-buffered mineral medium was tested due to its high heavy metal salt concentrations. For *Burkholderia cepacia* complex, *Burkholderia cepacia* Selective Agar (BCSA) medium was evaluated due to its wide usage for high recovery of this organism. The BCSA recipe was additionally tested with adjustments to the sucrose and lactose concentrations for optimization of colony morphology differentiation. A sucrose-containing BCSA medium was ultimately developed containing casein peptone (10 g/L), yeast extract (1.5 g/L), sodium chloride (5 g/L), sucrose (10 g/L), 1.7% agar and phenol red (0.6 mg/mL). For *Methylobacterium* spp., a methanol based medium in which methanol acts as the sole carbon source (1-2%), was

evaluated selectivity. Finally, for *S. epidermidis*, mannitol salt agar (MSA) was evaluated due to the media's high salt concentration and the halotolerance of *S. epidermidis* compared to other 2014 strains.

By combining some of the previously developed selective medium recipes with new combinations of antibiotics and reagents, selective and/or differential media recipes were developed for each strain based on selective and/or differential success within the isolation year group.

Media Selectivity Testing. *Inoculation:* To establish the selective and/or differential success of a particular medium, every strain was grown, serially diluted and plated as described above for each media type. In addition to testing pure cultures of the individual species, a mix of isolates from the same year was prepared at a 1:1 ratio (CFU/mL). Cultures were thoroughly resuspended prior to mixing and before dilutions of the mixed culture were made. The primary focus of these tests was the selective inhibition of all but the intended strain from the year. Plates were analyzed at 24, 36, 48, 72, 120 and 168 hr. post inoculation.

Growth Curves: In order to evaluate the recovery of each species on its respective medium, growth curves were performed for each strain using both its specific medium and on R2A simultaneously. Quadrant streaked R2A plates were prepared and incubated at 28.5 ˚C till colonies were discernable. Colonies were inoculated into 5 mL of R2A media. After mixing thoroughly, each inoculated culture was used to make serial dilutions. Additionally, 100 µL was transferred to a cuvette and an OD600 was read for each species. Based on the OD600 reading, 2-4 of the serial dilutions were plated in triplicate on both the selective medium and R2A. The plates were incubated as described above until countable colonies grew. The process was repeated for all strains at 0, 12, 24, 36, 48, 60, 72, and 96 hours post inoculation with additional time points for all strains—except *Burkholderia* spp. and *Mesorhizobium* spp.—at 4, 8, and 16 hours post inoculation. For the slower growing ISS strains, *Burkholderia* spp. and *Mesorhizobium* spp., the growth curve was extended to 120 hours post inoculation to ensure the full growth cycle was monitored. Between time points, the broth cultures were incubated on a 130 r.p.m shake table at ambient temperature.

RWV Bioreactor Preparation. For all experiments using RWV bioreactors, 55 mL Synthecon disposable bioreactors were prepared. The cleaning protocol (patent in preparation) was developed at Texas State University by Dr. Robert McLean. This protocol allowed for the preparation of RWV bioreactors for culturing biofilm forming bacteria.

Co-culture Growth in the RWV. For all co-culture experiments, each set of bacteria isolated from the same year were evaluated as separate groups. For each group, quadrant streaked R2A plates were prepared for each strain individually 96 hr. prior to the start of experiment (with the exception of *Bradyrhizobium* spp. and *Mesorhizobium* spp. which were started 192 hours prior to the experiment). Streak plates were incubated at ~28.5 ^{o}C at 48 hr. prior to the start of the experiment (96 hr. for *Bradyrhizobium* spp. and *Mesorhizobium* spp., to accommodate slower growth rate of these two strains). Five mL R2A broth cultures were inoculated for each strain and placed on a shaker (130 r.p.m) at ambient temperature. After cultures reached stationary phase, a 1:1 mixture (CFU/mL) was prepared using all ISS isolates from the same year. This mixture was then diluted to 0.5% into R2A media, loaded into two RWV bioreactors positioned in the LSMMG and control orientations and rotated at 25 rotations per minute (r.p.m.) under ambient conditions. Using this inoculum, all strains from each isolation year could successfully be recovered from co-culture when grown in test tubes.

Since these co-cultures formed biofilms, the entire reactor was harvested to enumerate the strains at each time point. To efficiently remove the entire culture (including adherent biofilm) from the reactors, 25 mL culture was first removed from the RWV and then the bioreactor was then resealed and held on a vortex for 10 seconds. Once re-suspended, the remaining culture in the RWV was then removed, combined with the previously removed aliquot and then homogenized by vortexing. Cultures were serially diluted in R2A media and plated on the selective and/or differential media specifically developed for the strain and its isolation year. Plates were incubated at ~28.5 ^{o}C for the predetermined incubation time for each media. This process was repeated for each group of bacteria (i.e., isolated in different years) at 12, 24, 36, 48, 60, 72 and 96 hours. The triplicated data were combined to create a co-culture growth curve for both the LSMMG and control conditions.

Optimization Testing. Prior to silver efficacy testing, the biofilm isolation technique, silver concentration, and bacterial inoculum required optimization. Bacterial biofilms were grown on sterile 316L stainless steel coupons provided by the NASA Johnson Space Center. This is the same material used in the ISS potable water system. For all optimization experiments, a single stainless steel coupon was placed into a snap-cap test tube with 5 mL of 0.5% NaCl solution. The test tube was inoculated with either 0.5%, 1.0% or 5.0% of a 1:1 mixture of strains isolated in the same year. Samples were treated with 0, 100, 200, 300, or 400 parts per billion (ppb) silver fluoride. This range was selected to determine the minimal silver treatment that would impact recovery without rendering data collection impossible. Each sample was grown at ambient temperature on a shaker (130 r.p.m.) for 48 hours. After incubation, each stainless steel coupon was removed from the culture, washed once in sterile 0.5% NaCl and placed into a second test tube containing 2 mL sterile 0.5% NaCl. These tubes were placed into a sonication bath (24 ^{o}C, 40 kHz) for 0, 5, 15, 30, 45 or 120 minutes to dislodge bacteria from the coupon for plating. After sonication, coupons were removed from the tubes and the remaining solution was serially diluted and plated on designated selective media for strains of the same isolation year. Plates were incubated at ~28.5 ^{o}C for the pre-determined incubation time for each media.

Silver Efficacy Testing. As for the previous tests, bacteria isolated from the same year was treated as a unique group. For each strain within a given group, 5 mL of R2A broth was inoculated with colonies from a quadrant streak. Cultures were incubated shaking (130 r.p.m.) at ambient temperature until the cultures reached stationary phase. A mixed culture was then prepared using a 1:1 ratio (CFU/mL) of each strain isolated in the same year. This mixture was diluted to 5% into 300 mL of R2A media. The co-culture was vortexed and divided into two separated flasks - one with no silver treatment (AgF) and the other with 300 ppb silver fluoride.

To load the RWV bioreactors, twelve sterilized 316L stainless steel coupons with an adherent magnet were placed in four RWV bioreactor (three per bioreactor). Using a magnet on the outside of the bioreactor, the coupons were positioned in the center of the upper face of the bioreactor, such that the stainless steel coupon was facing the interior of the bioreactor. If necessary, the external magnets were tapped to the exterior surface of the bioreactor in order to

secure them in place. Once coupons were secured, the 150 mL co-culture without silver was divided into two of the RWV bioreactors. The bioreactors were sealed, bubbles removed and rotated at 25 r.p.m. in the LSMMG or control orientation. This process was repeated for the co-culture containing silver using the remaining two bioreactors. Cultures were incubated at ambient temperature for 48 hours.

After incubation, the external magnets were removed so the internal coupons could be extracted. Each coupon was dipped once in sterile 0.5% NaCl and then transferred to an individual test tube containing 2 mL 0.5% NaCl. Test tubes were sonicated (24 ^{o}C, 40 kHz) for 5 minutes. After sonication, coupons were removed from the tubes and the remaining solution was serially diluted and plated as described above for the strains in each isolation year. To determine the planktonic population, 5 mL of culture was removed from the bioreactor, vortexed, serially diluted and plated as described for the strains in each isolation year.

These experiments were repeated using 0.5% NaCl as the growth media instead of R2A. In these experiments however, the silver dosage was lowered to 100 ppb because 300 ppb silver treatment resulted in inconsistent recovery data.

Monolayer Adherence Studies. Adherence of co-cultures to human colonic epithelial cell monolayers following culture in the RWV was assessed using HT-29 cells (ATCC® HTB-38™). Monolayers were grown to confluency in 24-well plates in GTSF-2 in a 37°C incubator with 10% CO_2. Prior to infection, the GTSF-2 was removed, the monolayers were rinsed with warm HBSS three times and then each well was filled with HBSS to prepare the cell culture for infection.

Bacterial co-cultures of strains isolated in the same year were prepared as detailed in the silver efficacy testing protocol. The only modification was that four coupons were inserted into each bioreactor rather than three. Two of the coupons were used for the adhesion experiment and the remaining two were stained for microscopy. For bioreactor cultures, three samples were prepared for infection - two sonicated biofilm samples from stainless steel coupons and one planktonic broth sample. In each bacterial sample set, the planktonic culture was diluted so that the CFU/mL in the planktonic culture was equalized to that of the biofilm culture.

Each sample was inoculated into two separate wells of the cell culture dish. The samples were incubated at 37 ^{o}C for three hours. After incubation, each well was rinsed three times with warm HBSS. Monolayers were vigorously lysed with 0.1% sodium deoxycholate. Following lysis, samples were serially diluted and plated as described above on specific media for each strain within each isolation year.

Biofilm Staining. Two stainless steel coupons per RWV bioreactor were reserved for staining during the *in vitro* adherence/colonization study. The eight coupons were placed in a glass bottom 12-well plate. The coupons were fixed in 4% paraformaldehyde (PFA) for 20 minutes. The PFA was then removed and the samples were rinsed thrice with cold phosphate-buffered saline (PBS). In a limited-light environment, the coupons were stained with Syto9 green fluorescent stain (10 mg/mL, nucleic acid stain) (**95, 96**) for 15 minutes, Concanavalin A (500 mg/L, β-1,3 and β--1,4 polysaccharide stain) (**97**) for 15 minutes, Ethidium Homodimer-2 (1 mg/mL, dead cell stain) (**98**) for 10 minutes and Calcofluor 5 (0.7 mg/mL, methyl-alpha-D-mannopyranose polysaccharide stain) (**99, 100**) for five minutes. Each well was then filled with cold PBS and imaged with a confocal microscope.

CHAPTER 3

RESULTS AND DISCUSSION

Antibiotic Sensitivity Tests. Antibiotic sensitivity results are summarized in **Figure 2**. Initially, three concentrations were tested for each antibiotic. The middle concentration was determined by reviewing previous research for concentrations which successfully eliminated bacterial growth. From this point, a lower and higher concentration was set, typically at a 10-fold increase and decrease to the middle concentration. Where necessary, additional concentrations were considered to best establish the susceptibility point in a strain. The data from these preliminary experiments was critical for the development of the selective and/or differential media.

Evaluation of Media Selectivity and Recovery. The results of the serial dilution inoculation test are summarized in **Figure 3** and **Figure 4**. Medium efficacy for each strain was evaluated only against other strains isolated in the same year. Once a medium was confirmed as selective for a given strain within each group, growth curves of the pure cultures were performed to assess the quantitative recovery ratio of the bacterium in the selective medium as compared to nonselective R2A (**Figure 5**). Ideally, there would be no difference between the selective media and nonselective R2A at any time point evaluated; however, a media was considered successful if there was no statistically significant difference between the population recovery on the selective media and R2A across a majority of time points in the growth analysis.

Ralstonia pickettii. Each of the three *R. pickettii* strains has a unique selective medium due to both differences in susceptibility/resistance as well as different strains in each year. The 2009 *R. pickettii* medium had the least number of species to select against, thus the media contains only two antibiotics: vancomycin (80 μg/ml) and polymyxin B (700 μg/ml). Vancomycin selected against *S. sanguinis* while polymyxin B was used to eliminate *C. metallidurans*. Initially, the medium included higher concentrations of vancomycin (100 μg/ml) and polymyxin B (800 μg/ml). By reducing the selective agents, the overall recovery was increased without compromising selectivity. Sucrose (1%) increased the nutrient concentration in the medium and acted as a differentiating agent: on medium with 1% sucrose, *C. metallidurans* colonies appeared

pale pink while *R. pickettii* colonies were dark pink. The sucrose caused the medium to have a higher surface tension, thus reducing the spread of a culture drop on the medium (**Figure 4A**). Without spreading the drop, counting colonies became difficult on this medium. Because the 2009 *R. pickettii* medium has high selectivity, plates were incubated for five days to optimize colony size; however countable colonies were present at three days post inoculation.

Growth curve data indicated that this medium had ideal recovery compared to R2A (**Figure 5A**). However, statistical evaluation showed a significant difference in recovery at 8 and 12 hr. post inoculation. The statistical differences in recovery only occur prior to *R. pickettii* reaching stationary phase. Since the remaining eight time points tested in this study did not produce a statistically significant difference between the 2009 *R. pickettii* media and R2A, the media was determined to have successful recovery.

Figure 2:

		Penicillins	**Cephalosporins**	**Fluoroquinolones**	**Aminoglycosides**			**Glycopeptides**	**Tetracyclines**	**Other antibiotics**					**Miscellaneous agents**		
		Carbenicillin (Cb) (100 μ g/mL)	Ceftriaxone (Cro) (5-500 μ g /mL)	Levofloxacin (Lev) (4-400 μ g /mL)	Streptomycin (Str) (10-2000 μ g /mL)	Gentamicin (Gm) (1.5-150 μ g /mL)	Kanamycin (Kan) (5-500 μ g /mL)	Vancomycin (Van) (1-100 μ g /mL)	Tetracycline (Tet) (20 μ g /mL)	Chloramphenicol (C) (3-100 μ g /mL)	Polymyxin B (PB) (10-1200 μ g /mL)	Rifampicin (Rif) (3-120 μ g /mL)	Spectinomycin (Spe) (5-500 μ g /mL)	Trimethoprim (Tmp) (1-100 μ g /mL)	Sodium chloride (0.5-7%)	Crystal Violet (CV) (5-100 μ g /mL)	Congo Red (CR) (100 μ g /mL)
2009	***Ralstonia pickettii***	R	S^{50}	S^{4}	S^{1000}	S^{150}	S^{100}	R	S^{20}	S^{30}	R	S^{30}	S^{250}	S^{3}	S^{5}	S^{50}	R
	Sphingomonas sanguinis	R	S^{500}	S^{4}	R	S^{50}	S^{5}	S^{10}	S^{20}	S^{3}	R	S^{3}	S^{250}	S^{100}	S^{5}	R	R
	Cupriavidus metallidurans	S^{100}	R	S^{40}	S^{1000}	R	R	R	S^{20}	S^{100}	S^{500}	S^{30}	S^{250}	S^{100}	S^{5}	S^{100}	R
2012	***Burkholderia cepacia* complex**	R	R	S^{40}	R	R	S^{200}	R	R	S^{60}	R	S^{30}	R	S^{10}	S^{5}	S^{100}	R
	Ralstonia pickettii	S^{100}	S^{50}	S^{4}	S^{2000}	R	S^{200}	R	S^{20}	S^{50}	R	S^{30}	S^{500}	S^{50}	S^{5}	S^{100}	R
	Ralstonia insidiosa	R	S^{500}	S^{4}	S^{1000}	R	S^{100}	R	R	S^{100}	R	S^{30}	S^{50}	S^{10}	S^{5}	S^{100}	R
	Sphingomonas sanguinis	R	S^{500}	S^{4}	R	S^{50}	S^{25}	S^{10}	S^{20}	S^{3}	R	S^{3}	S^{500}	S^{100}	S^{2}	S^{50}	R
	***Methylobacterium* species**	R	S^{500}	S^{40}	S^{100}	S^{15}	S^{5}	R	S^{20}	S^{20}	R	S^{3}	S^{5}	S^{100}	S^{2}	S^{5}	R
2014	***Burkholderia cepacia* complex**	R	R	S^{40}	S^{1000}	S^{150}	S^{100}	R	R	S^{30}	R	R	S^{500}	S^{100}	S^{5}	S^{100}	R
	Ralstonia pickettii	S^{100}	S^{5}	S^{4}	S^{2000}	R	S^{200}	R	S^{20}	S^{30}	R	S^{30}	S^{500}	S^{50}	S^{5}	S^{100}	R
	Ralstonia insidosa	R	S^{500}	S^{4}	S^{2000}	R	S^{200}	R	R	R	R	S^{30}	S^{50}	S^{10}	S^{5}	S^{100}	R
	***Bradyrhizobium* species**	S^{100}	R	S^{400}	S^{100}	S^{50}	S^{25}	R	R	R	R	R	S^{125}	R	S^{1}	S^{50}	R
	***Mesorhizobium* species**	S^{100}	R	S^{40}	S^{1000}	S^{150}	R	R	R	S^{100}	R	S^{30}	R	R	S^{2}	R	R
	Staphylococcus epidermidis	S^{100}	S^{5}	S^{4}	S^{10}	$S^{1.5}$	S^{5}	S^{10}	R	S^{20}	S^{100}	S^{3}	S^{500}	R	R	S^{5}	R

Figure 2: Overview of Antibiotic and Reagent Sensitivity Tests for Media Development. Only antibiotics that were used in one or more selective and/or differential media are listed in the above table. The concentration ranges tested are shown next to each antibiotic. “S” indicates that the strain became susceptible (no growth detected) at a concentration tested. The concentration (μg/mL) point of susceptibility is indicated by the superscript next to the “S.” Concentrations are given in μg/mL unless, alternatively, percentage is indicated. "R" stands for resistant, meaning no concentration measured produced susceptibility in the strain.

Figure 3:

Strain Media	Media Base	Selective Agent (ug/mL)*	Incubation time	R. pickettii	S. sanguinis	C. metallidurans
2009 *R. pickettii*	R2A	700PB 80Van 1% Sucrose	Day 5	Growth (Selected)	No Growth (Inhibited)	No Growth (Inhibited)
2009 *S. sanguinis*	R2A	40Cro 500Str	Day 5	No Growth (Inhibited)	Growth (Selected)	No Growth (Inhibited)
2009 C. *metallidurans*	R2A	30C 5Cro 30Kan 3Tmp	Day 5	No Growth (Inhibited)	No Growth (Inhibited)	Growth (Selected)

Strain Media	Media Base	Selective Agent (ug/mL)*	Incubation time	B. cepacia	R. pickettii	R. insidiosa	S. sanguinis	Methylobacterium spp.
2012 *B. cepacia*	R2A	250Spe 20Tet 2% NaCl	Day 5	Growth (Selected)	No Growth (Inhibited)	No Growth (Inhibited)	No Growth (Inhibited)	No Growth (Inhibited)
2012 *R. pickettii*	R2A	100C 750Str 50Kan 100Gm	Day 5	No Growth (Inhibited)	Growth (Selected)	No Growth (Inhibited)	No Growth (Inhibited)	No Growth (Inhibited)
2012 *R. insidiosa*	LB	50C 150Gm 100Kan	Day 5	No Growth (Inhibited)	No Growth (Inhibited)	Growth (Selected)	No Growth (Inhibited)	No Growth (Inhibited)
2012 *S. sanguinis*	R2A	40Cro 500Str	Day 5	No Growth (Inhibited)	No Growth (Inhibited)	No Growth (Inhibited)	Growth (Selected)	No Growth (Inhibited)
2012 *Methylobacterium* spp.	Methanol	10Tmp 5Cro 1.5% Methanol	Day 7	No Growth (Inhibited)	Noninterfering growth (differentiating media)	Noninterfering growth (differentiating media)	Noninterfering growth (differentiating media)	Growth (Selected)

Strain Media	Media Base	Selective Agent (ug/mL)*	Incubation time	B. cepacia	R. pickettii	R. insidiosa	Bradyrhizobibum spp.	Mesorhizobium spp.	S. epidermidis
2014 *B. cepacia*	Sucrose	20C 750Str 150Gm 100Kan 100Cb 5Cro	Day 3	Growth (Selected)	No Growth (Inhibited)	No Growth (Inhibited)	No Growth (Inhibited)	No Growth (Inhibited)	No Growth (Inhibited)
2014 *R. pickettii*	Sucrose	100CR 500Str 150Gm 100Kan	Day 2	No Growth (Inhibited)	Growth (Selected)	No Growth (Inhibited)	No Growth (Inhibited)	No Growth (Inhibited)	No Growth (Inhibited)
2014 *R. insidiosa*	LB	50C 150Gm 100Kan 1000Str	Day 5	No Growth (Inhibited)	No Growth (Inhibited)	Growth (Selected)	No Growth (Inhibited)	No Growth (Inhibited)	No Growth (Inhibited)
2014 *Bradyrhizobium* spp.	R2A	100Rif 4Lev 500Cro 100Tmp	Day 7	No Growth (Inhibited)	No Growth (Inhibited)	No Growth (Inhibited)	Growth (Selected)	No Growth (Inhibited)	No Growth (Inhibited)
2014 *Mesorhizobium* spp.	R2A	100CV 200Kan	Day 7	No Growth (Inhibited)	No Growth (Inhibited)	No Growth (Inhibited)	No Growth (Inhibited)	Growth (Selected)	No Growth (Inhibited)
2014 *S. epidermidis*	LB	6% NaCl	Day 7	No Growth (Inhibited)	No Growth (Inhibited)	No Growth (Inhibited)	No Growth (Inhibited)	No Growth (Inhibited)	Growth (Selected)

Growth (Selected) Noninterfering growth (differentiating media) No Growth (Inhibited)

*Media recipe concentrations are measured in ug/mL except those measured in percentage as indicated

Figure 3: Summary of Developed Media Composition and Optimal Timeline for Use. The first column shows the bacterial species targeted by the media. The second column indicates the base media to which antibiotics, reagents and additional nutrient sources were added. The third column summarizes the added components which function as the selective agent(s) of the media. Antibiotics used include carbenicillin (Cb), ceftriaxone (Cro), levofloxacin (Lev), streptomycin (Str), gentamicin (Gm), kanamycin (Kan), vancomycin (Van), tetracycline (Tet), chloramphenicol (C), polymyxin B (PB), rifampicin (Rif), spectinomycin (Spe), and trimethoprim (Tmp). Reagents included in this figure are sodium chloride (NaCl), crystal violet (CV) and congo red (CR). The fourth column shows the time point at which the overall selective ability of the recipe was evaluated. The remaining columns summarize the results of the selectivity tests for the media. Green indicates that bacterial species grew equally well on the developed media as compared to the nonselective R2A control media. Light pink indicates minimal growth which did not interfere with CFU counting. Red indicates no growth.

The *R. pickettii* in the 2012 group proved more of a challenge to select for without reducing recovery. Because *B. cepacia* complex and *R. insidiosa* consistently outperformed *R.*

pickettii in antibiotic and reagent tests (**Figure 2**), establishment of a medium that fully inhibited all other strains without reducing recovery required significant testing. Initial media formulations included varying concentrations of chloramphenicol, streptomycin, kanamycin and gentamicin. While it was possible to select for *R. pickettii* over *B. cepacia* complex by only incubating for 1.5-2 days, *B. cepacia* complex was never fully inhibited and slight growth rate discrepancies between trials made determining an exact incubation time difficult (**Figure 5B**).The *R. pickettii* medium was considered to be a success as it selected for only *R. pickettii* (**Figure 4E**) without compromising the overall recovery (**Figure 5B**).

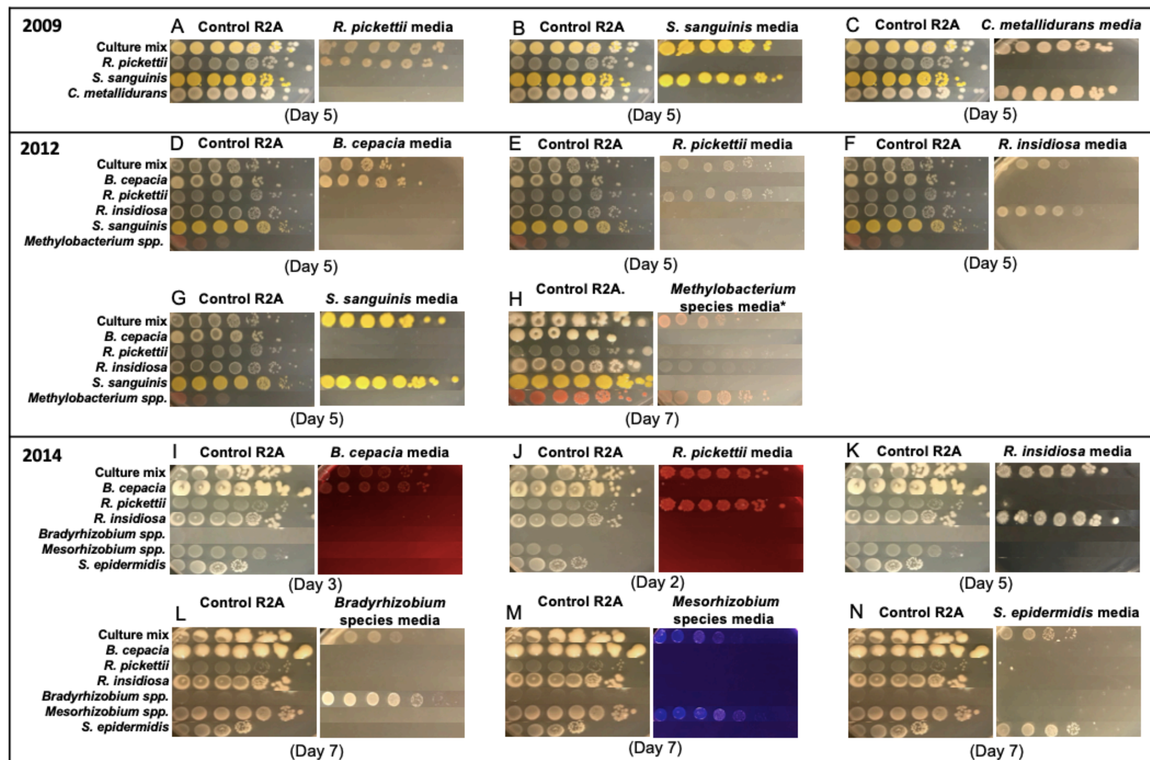
Similar to the 2012 strain, the *R. pickettii* from the 2014 group was difficult to select for without compromising recovery. Because *B. cepacia* and *R. insidiosa* had higher resistance to more antibiotics than *R. pickettii*, the preliminary media compromised recovery for selectivity. The final 2014 *R. pickettii* medium contained Congo red (100 μg/ml), streptomycin (500 μg/ml), gentamicin (150 μg/ml) and kanamycin (100 μg/ml). The medium was prepared using sucrose as a base because its nutrient rich quality increased the survival of the target strain. The inclusion of sucrose prevented the growth of *Burkholderia* spp. (**Figure 4J**). Furthermore, sucrose-containing medium caused more dramatic color changes between *R. pickettii* and *R. insidiosa,* allowing for improved differentiation (**Figure 4J**). The antibiotic combination was successful in inhibiting the growth of *B. cepacia species* and *S. epidermidis*; however, *R. insidiosa* and *Mesorhizobium* spp. still grew if the plates were incubated long enough. To heighten selectivity, this medium can only be incubated for 2 days. At this point, the *R. pickettii* colonies are countable, but *R. insidiosa* and *Mesorhizobium* spp. colonies are not. The growth curves showed no statistical difference between selective medium and control R2A at any time point (**Figure 5C**). At the specified incubation point, the medium was successful because it selected for only *R. pickettii* without conceding recovery.

Ralstonia insidiosa. The selective media for the 2012 and 2014 isolate were almost the same. Both media utilize Luria-Bertani (LB) broth as the nutrient source with the addition of chloramphenicol (50 μg/ml), gentamicin (150 μg/ml) and kanamycin (100 μg/ml) (**Figure 3**). The

2014 *R. insidiosa* media also contains streptomycin (1000 μg/ml). The 2014 media contained streptomycin to restrict the growth of *B. cepacia* complex, which was sensitive to streptomycin at 1000 μg/ml (**Figure 2** and **Figure 4K**). This addition was unnecessary for the 2012 (**Figure 4F**).

For both the 2012 and 2014, none of the time points tested showed a statistically significant difference between the selective and R2A media (**Figure 5B**, **Figure 5C**).

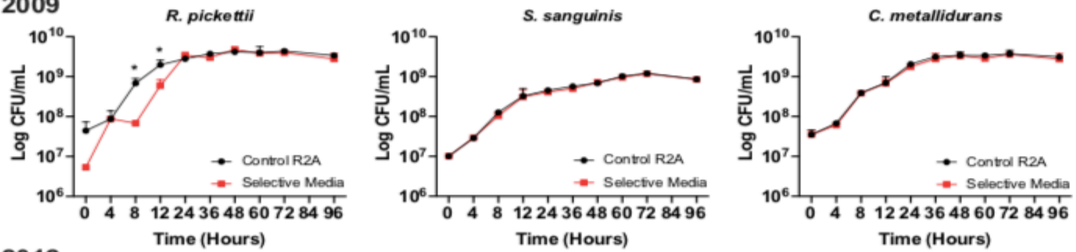
Figure 4:

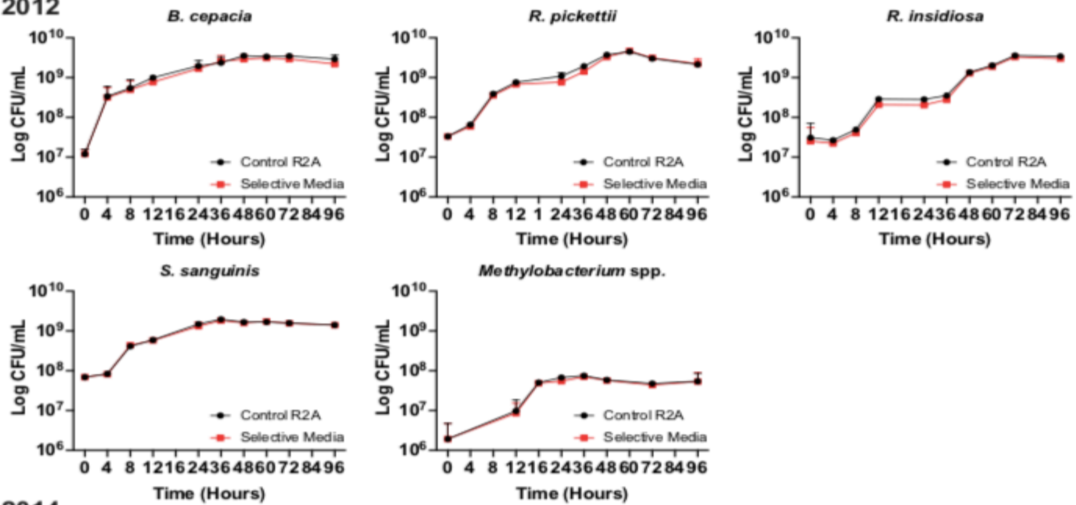


*Differential media

Figure 4: Validation of Selective and/or Differential Media Efficacy. Photographic representation of the data summarized in Figure 2. Each set of bacteria isolated from the same year is separated into different portions of the image. Selective/differential media are shown next to the nonselective R2A control media. For each medium, the growth of bacterial co-cultures (mixed post inoculation) are shown on the top row followed below by the growth of each individual species. The selective medium in panel **M** is purple due to the addition of crystal violet. Selective media in panel **I** and **J** are bright red due to phenol red in the sucrose media. Due to the intense color of the 2014 *R. pickettii* media, modifications were made to the original photo to increase the brightness and decrease the color contrast. The photo edits were made across the entire image and do not alter the integrity of the data; they simply enhance the viewability. All cultures were incubated for the indicated time at ~28.5^{o}C in an incubator.

Figure 5:





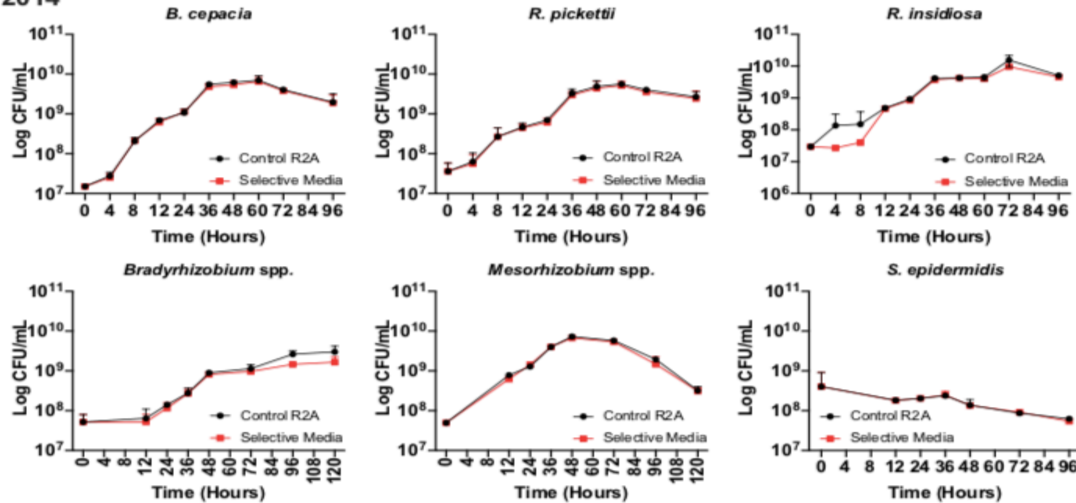


Figure 5: Growth Curves of Individual Strains on Developed Selective Media Relative to Control R2A. (A) 2009 strains **(B)** 2012 strains **(C)** 2014 strains. For each strain, cultures were grown in test tubes which were inoculated from colonies isolated from a quadrant streak. At each time point, an aliquot of the culture was serially diluted and plated on both control R2A plates and the strain's respective selective media. All dilutions were plated in triplicate. Statistics: Each individual data set was assessed for normality using the D'agostino-Pearson test. Statistical significance between data sets, both at each time point and overall, was assessed using unpaired T-tests with Welch's correction (n=3). The only time points with a statistically significant difference between control R2A and the strain's selective media was at 8 and 12 hours for the 2009 *R. pickettii*. * indicates $P \leq 0.05$.

***Cupriavidus metallidurans*.** The *C. metallidurans* medium was initially prepared with Tris-buffered mineral medium as the base to inhibit *R. pickettii* and *S. sanguinis*. The Tris-buffered medium was abandoned because it failed to inhibit *R. pickettii* even with the addition of chloramphenicol (30 μg/mL). The final medium included chloramphenicol (30 μg/mL), ceftriaxone (5 μg/mL), kanamycin (30 μg/mL) and trimethoprim (3 μg/mL). The chloramphenicol and kanamycin were primarily added to inhibit the growth of *S. sanguinis*. Ceftriaxone was included to reduce the growth of *R. pickettii* while different concentrations of trimethoprim were also tested to eliminate *R. pickettii* without reducing the recovery of the target strain. The concentration selected showed ideal selectivity without reducing the recovery of *C. metallidurans.* Because the medium successfully inhibited both *R. pickettii* and *S. sanguinis* (**Figure 3**, **Figure 4C**), the incubation time for experiments on this medium was set at 5 days to optimize the colony size and number of the desired strain.

Recovery ratio data from growth curve experiments showed ideal recovery of *C. metallidurans* across all time points tested (**Figure 5A**). Because there was no statistically significant difference seen at any time point tested, the medium was concluded to be efficacious.

***Burkholderia cepacia* complex**. The 2012 and 2014 *B. cepacia* complex media differ greatly from each other. This is partly due to differing antibiotic susceptibilities and partly due to the selectivity requirements due to their year of isolation. Initially, the 2012 medium - like the 2014 medium - was sucrose-based. However, it was determined that sucrose promoted the growth of 2012 *R. pickettii* even with the addition of antibiotics. When the antibiotics optimized for the selective sucrose medium were instead added to R2A, the 2012 *R. pickettii* was successfully inhibited. The 2012 *B. cepacia species* medium includes spectinomycin (250 μg/mL), tetracycline (20 μg/mL) and sodium chloride (2%) (**Figure 3**). Spectinomycin was added to reduce the growth of 2012 *R. insidiosa* and *Methylobacterium* spp*.* (**Figure 2**). The addition of tetracycline was intended to reduce the growth of *R. pickettii*, *R. insidiosa* and *Methylobacterium* spp. As *S. sanguinis* and *Methylobacterium* spp. fail to grow at sodium chloride concentrations above 2%, sodium chloride was added to the medium (**Figure 2**, **Figure 4D**). By using the R2A as a medium

base, experimental plates could be incubated for a full 5 days based on the medium's selective efficacy.

As previously stated, the 2014 *B. cepacia* complex medium was prepared using a sucrose medium base. This medium, initially intended to suppress the growth of *Burkholderia* species, proved the opposite with this 2014 strain. Utilizing this high nutrient media to promote growth, sufficient antibiotics for the repression of other strains was possible. The 2014 *B. cepacia* complex medium included chloramphenicol (20 μg/mL), streptomycin (750 μg/mL), gentamicin (150 μg/mL), kanamycin (100 μg/mL) carbenicillin (100 μg/mL) and ceftriaxone (5 μg/mL) (**Figure 3**). Because the 2014 isolate group contained the most strains from the greatest number of genuses, more antibiotics were required. Chloramphenicol was added to reduce the growth of *S. epidermidis*, while streptomycin, gentamicin and kanamycin were added to eliminate *Bradyrhizobium* spp. and *S. epidermidis*. At 100 μg/mL, carbenicillin killed *R. pickettii*, *Bradyrhizobium* spp., *Mesorhizobium* spp., and *S. epidermidis*. Ceftriaxone further reduced the growth of *R. pickettii* and *S. epidermidis* (**Figure 2**). However, even with the range of antibiotics added, the medium still had a time limit; the 2014 *B. cepacia* complex medium can only be incubated for 3 days before *R. pickettii* and *R. insidiosa* colonies begin to grow. At day 3, the desired strain has grown sufficiently for counting colonies, but all other strains are repressed (**Figure 4I**).

From the recovery ratio data, both 2012 and 2014 *B. cepacia* complex media showed no statistically significant difference to nonselective R2A media (**Figure 5B**, **Figure 5C**). Because there was no statistical difference between the selective medium and the non-selective medium across the growth curve, these media was concluded to be a success.

***Sphingomonas sanguinis*.** The same selective medium was successfully developed and used for both 2009 and 2012 strains. The *S. sanguinis* medium was prepared using R2A media containing ceftriaxone (40 μg/ml) and streptomycin (500 μg/ml). Because of the high streptomycin concentration, selectivity was relatively strong. However, the concentration of ceftriaxone had a strong effect on the survival of *S. sanguinis*. After several experiments, the

concentration of ceftriaxone was lowered from 50 μg/ml which proved to increase the strain recovery (**Figure 3**, **Figure 4B**, **Figure 4G**). Initially, carbenicillin was added to further restrict the growth of *R. pickettii* but the combination significantly reduced the recovery of *S. sanguinis.* It was noted that after incubation day four, the colony size and morphology did not change significantly. However for consistency, all plates were incubated for five days.

When growth curves were conducted to determine the recovery ratio between the non-selective R2A media and the *S. sanguinis* selective medium, the selective medium displayed no statistically significant differences at any time point (**Figure 5A**, **Figure 5B**).

***Methylobacterium* species.** *Methylobacterium* spp., being the only strain tested with the capability of using methanol as a primary carbon source, was tested on a methanol base medium. The high vitamin and nutrient concentration promoted growth of *Methylobacterium* spp. while reducing the colony size of other species. Methanol concentrations of 1%, 1.5% and 2% were all tested with the final formulation containing a 1.5% concentration. Trimethoprim (10 μg/mL) and ceftriaxone (5 μg/mL) were also included to reduce the growth of *B. cepacia* complex and *R. insidiosa*. This medium was differential rather than selective (**Figure 3**). The lack of a preferred carbon source decreases the colony size of the other 2012 strains. This combined with the vibrant red coloring of *Methylobacterium* spp. allowed for the differential success of the medium (**Figure 4H**). The medium incubation time was set at seven days to accommodate for the slow growth of the strain. Recovery ratio data showed no statistical difference in population recovery between the two media across the growth curve (**Figure 5B**).

***Bradyrhizobium* species.** *Bradyrhizobium* spp. was found to be resistant to most of the selective reagents tested. R2A was used as the base for this medium as the strain failed to grow on most other media tested. To select against all other strains within the same isolation year, rifampicin (100 μg/mL) was used. Rifampicin was initially added at 120 μg/mL but was eventually lowered to 100 μg/mL because higher concentrations of rifampicin impacted the recovery of *Bradyrhizobium* spp.. Additionally, levofloxacin (4 μg/mL), ceftriaxone (500 μg/mL), and

trimethoprim (100 μg/mL) were added to sufficiently eliminate the growth of other species (**Figure 3** and **Figure 4L**). The strain's slow growth meant that this medium could not be incubated for less than 7 days for full recovery. After reducing the rifampicin concentration to 100 μg/mL, the recovery ratio from the growth curves indicated ideal recovery compared to non-selective R2A (**Figure 5C**).

***Mesorhizobium* species.** The selective medium for *Mesorhizobium* spp. used R2A as the base and included crystal violet (100 μg/mL) and kanamycin (200 μg/mL) as selective agents. All other 2014 strains were susceptible to crystal violet at 100 μg/mL; however, kanamycin was added to further ensure no growth of other strains (**Figure 3**). The crystal violet in the medium causes *Mesorhizobium* spp. colonies to turn dark blue (**Figure 4M**). The differential and selective nature of this medium, combined with the successful recovery across all growth curve time points (**Figure 5C**), indicated that this medium was effective. The medium had a set incubation time of 7 days to ensure full growth of the desired strain.

Staphylococcus epidermidis. *S. epidermidis* is the only haloduric species in this study. Because *S. epidermidis* was able to survive NaCl concentrations of over 6%, sodium chloride was utilized as the primary selective agent in this medium. *B. cepacia species, R. pickettii* and *R. insidiosa* showed growth up to 5% NaCl (**Figure 2**). Initially, the *S. epidermidis* media utilized R2A as the base media and included 6% NaCl. This media was unsuccessful because it had approximately a 20% recovery compared to R2A media across the growth curve time points. Since *S. epidermidis* is a known pathogen, the R2A media was replaced with LB media as the nutrient source. This change produced a media which was both exceedingly selective (**Figure 3**, **Figure 4N**) and had a nonsignificant difference in recovery compared to R2A media (**Figure 5C**).

Impact of Bacterial Co-Culture and Fluid Shear on Individual Population Cell Count. To mimic low fluid shear environment present aboard ISS we grew co-cultures of the potable water isolates recovered for each year in RWV bioreactors oriented in the LSMMG or control position. For each group (2009, 2012 or 2014), an equal number of each bacteria were

combined for a final inoculum concentration of ~10^6 CFU/mL. RWV bioreactor co-cultures were grown for up to 96 hours.

Relative to 1xG controls, LSMMG culture did not appear to largely impact the growth of individual strains within co-cultures for any year of isolation (**Figure 6**, **Figure 7**). It should be noted that every strain, except *Methylobacterium* spp., had between 1 and 3 time points in the growth curve that showed a small but statistically significant difference in strain recovery between the two bioreactor orientations. For the group of bacteria isolated in 2009, each strain had similar bacterial counts and growth pattern when co-cultured in the RWV bioreactor (**Figure 6A**, **Figure 7**). These data suggest the possibility that the strains may have a neutral or symbiotic relationship with one another that allow for the mutually beneficial exchange of bioproducts rather than competition.

Conversely, within the 2012 bacterial isolates, *R. pickettii* and *Methylobacterium* spp. displayed dramatically lower recovery then the other three strains (**Figure 6B**, **Figure 7**). While *R. pickettii* colonies were still recovered (~10^3 CFU/mL) from the co-culture experiment, *Methylobacterium* spp. showed no consistently reliable recovery after inoculation. While *R. pickettii* was less fit than the other three surviving strains, it still persisted in the co-culture environment. As for *Methylobacterium* spp., it is hypothesized that the carbon-based nutrient source in R2A broth allowed the other species to outcompete this strain. It is possible that if the same experiment was performed using purified water or if a greater initial population of *Methylobacterium* spp. were inoculated, this strain might have survived co-culture growth.

All six of the strains in the 2014 group were recovered from co-culture. Similar to the growth curve data for the individual strains (**Figure 5**), *B. cepacia* complex*, R. pickettii, R. insidiosa,* and *S. epidermidis* grew quicker than *Bradyrhizobium* spp. and *Mesorhizobium* spp. (**Figure 6C**, **Figure 7**). However, in pure shaking cultures, *Bradyrhizobium* spp. and *Mesorhizobium* spp. reached significantly higher population density than in co-culture in the RWV bioreactor (**Figure 5, Figure 6C**). This could be because i) nutrition competition within the co-culture environment or ii) the RWV culture environment may provide additional influence on the growth than conventional culture. Since growth curves in the RWV bioreactors were not

Figure 6:

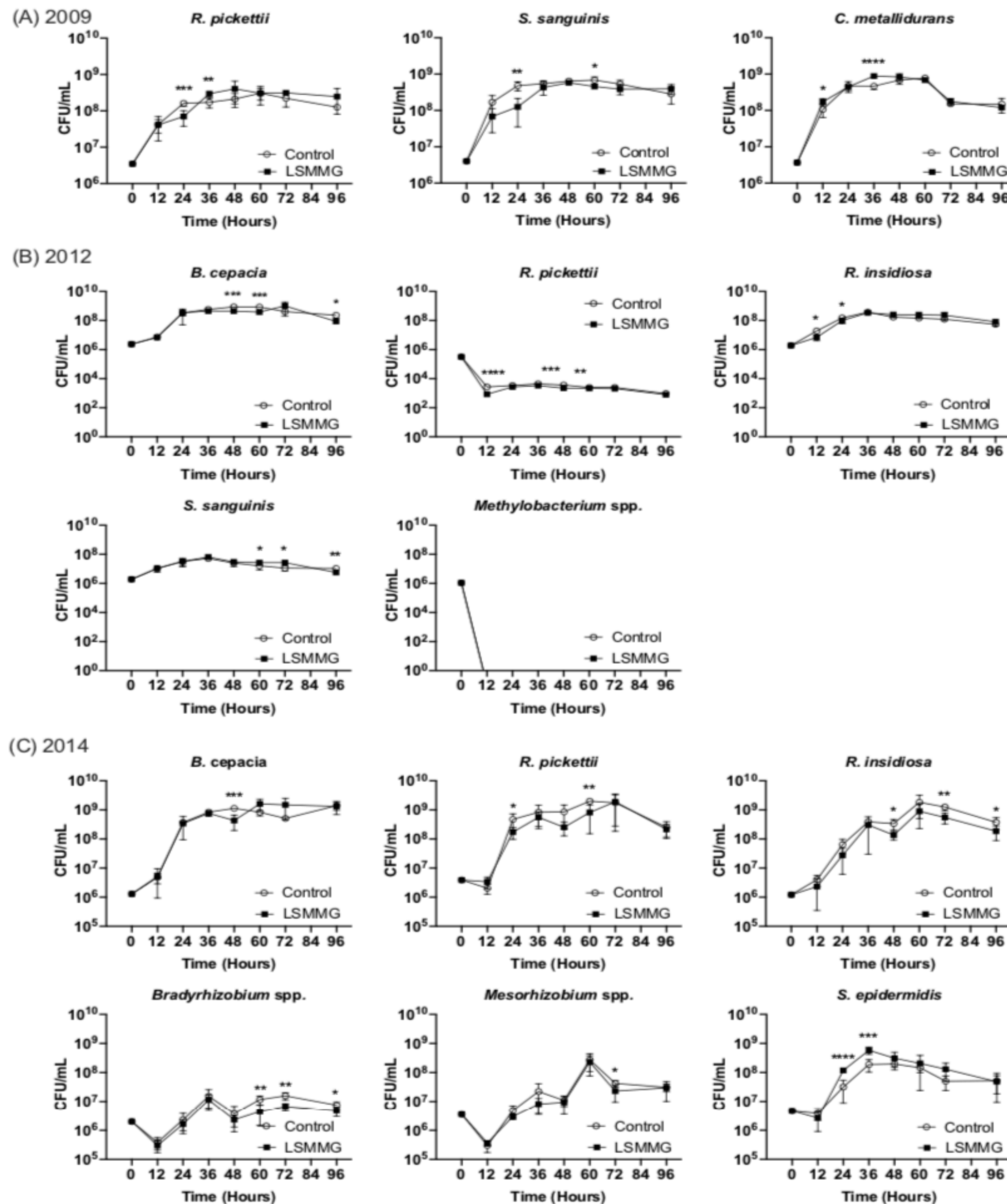
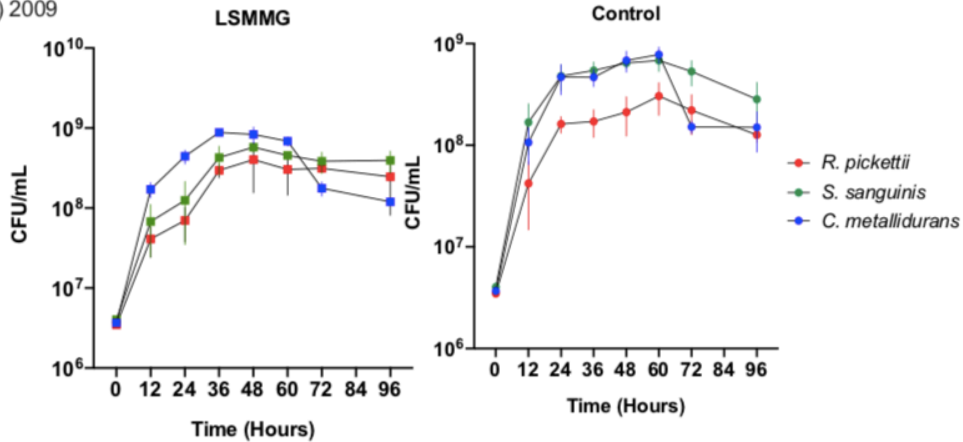


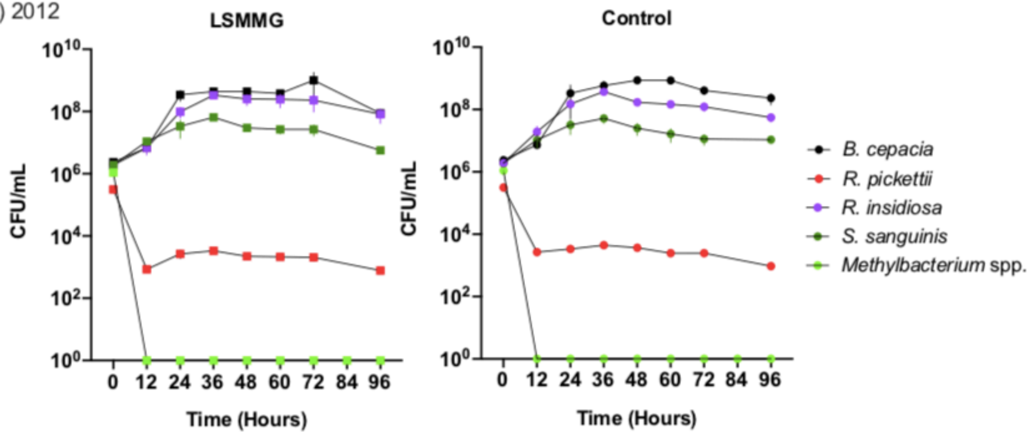
Figure 6: Bacterial Growth Curves of Individual Strains During Co-culture in RWV Bioreactors. For each co-culture group (2009, 2012 or 2014), RWV bioreactors (LSMMG and control 1xG orientation) were inoculated at a 1:1 ratio in R2A media. The bioreactors were incubated at 25 r.p.m. and ambient temperature. At each time point indicated, the cultures were homogenized, serially diluted and plated on selective/differential media. Although the experiment was performed using co-cultures, each individual strain is shown as a separate graph for clarity to assess the impact of fluid shear on the recovered cell counts. Each individual data set was assessed for normality using the D'agostino-Pearson test. Statistical significance between data sets was assessed using unpaired T-tests with Welch's correction (n=2). Statistical significance is indicated by asterisks indicating that P was less than or equal to 0.05 (*), 0.01 (**), 0.001 (***) or 0.0001 (****). Non-statistical significance was left blank instead of being labeled with "ns." **(A)** 2009 strains **(B)** 2012 strains **(C)** 2014 strains.

Figure 7:

(A) 2009



(B) 2012



(C) 2014

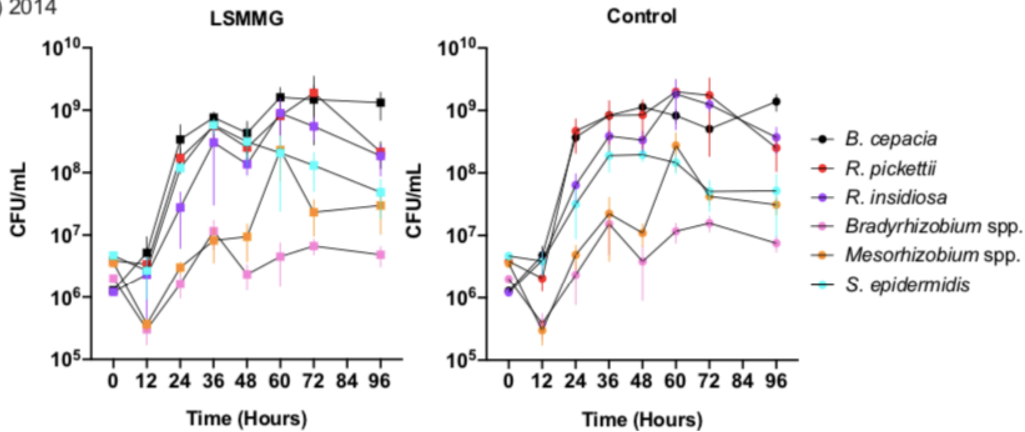


Figure 7: Polymicrobial Population Dynamics in Multispecies Co-cultures. For each set of bacterial strains isolated from the same year, RWV bioreactors (LSMMG and control 1xG orientations) were inoculated with a mixture containing a 1:1 ratio of strains from the same isolation year in R2A media. RWVs were incubated at 25 r.p.m. and ambient temperature. At each time point indicated, the cultures were homogenized, serially diluted and plated on selective/differential media. Each individual data set was assessed for normality using the D'agostino-Pearson test (n=2). **(A)** 2009 **(B)** 2012 **(C)** 2014.

performed using individual strains (i.e., only co-cultures), it was difficult to determine if the lower recovery of these two strains was due to the RWV environment or the co-culture competition.

Overall, the growth trend between control (1xG) and LSMMG culture systems for each individual strain were relatively similar, thus it was concluded that fluid shear did not have a distinguishing impact on ISS strain recovery.

Optimization of Stainless Steel Adherent Biofilm Retrieval for RWV Co-culture. The impact of silver disinfectant in RWV culture on biofilm formation and survival of planktonic bacteria was assessed. First, silver concentration, inoculum dose, and protocol for the processing of the biofilms were optimized. The 316L stainless steel was selected due to use in the ECLSS corrugated tubing. The stainless steel coupons were incorporated with the RVW cell culture system to develop biofilm formation on the system material. To determine the number of bacteria in the formed biofilm on the surface of the stainless steel, the biofilm was dissociated from the coupon by using a water bath sonicator. The sonication time was optimized for effective dissociation without killing the bacteria. Sonication times tested included 5, 15, 30, 45, and 120 minutes. The data showed that over 5 min sonication significantly reduced the bacterial recovery (CFU/mL), indicating excessive sonication (> 5 mins) negatively impacted recovery of the microbes (Fig 8).

Effective silver concentration was optimized next, with a testing range of 100 – 400 ppb. Data showed a gradual decrease in the microbial survival exposed to incrementally increased concentrations of silver disinfectant. Yet, no statistically significant difference was seen between 300 and 400 ppb (**Figure 9**). Thus, 300 ppb silver disinfectant, minimal effective concentration, was decided to be used in this study. When cultures were treated with 400 ppb silver, the recovered colonies showed changes in morphology.

In addition, the initial inoculum was determined to optimize the co-culture condition by testing approximately 5×10^3 CFU/mL (0.5% inoculum) to 5×10^4 CFU/mL (5.0% inoculum) per each strain. The aim was to set the inoculum high enough that colony data could still be retrieved when treated with 300 ppb silver. The study resulted indicated that increase bacterial inoculum correlated to increased recovery however there was not a statistically significant difference

between 0.5% and 1.0% inoculum (**Figure 10**). The experimental co-culture inoculum was set at 5% since antimicrobial silver still had an impact on the population recovery and data was consistent and replicable. Additionally, as the 5 x 10^3 CFU/mL of *Methylobacterium* spp. was too low to detect in the co-culture (**Figure 5, Figure 6**) an increase in the initial bacterial inoculum from 0.5% to 5.0%, was thought to aid in the recovery of this strain.

Figure 8:

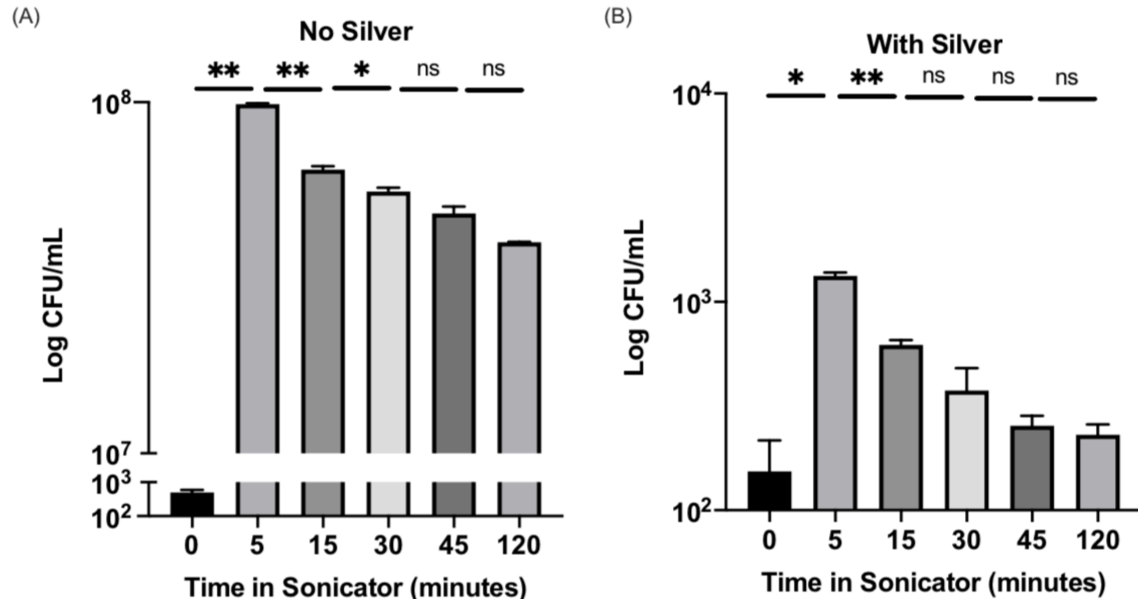


Figure 8: Impact of Sonication Time on Stainless Steel Biofilm Population Recovery. (A) Without silver (0ppb), **(B)** With silver (300 ppb). Using the 2009 bacterial isolates, 12 test tubes containing a sterile stainless steel coupon and 5 mL of 0.5% NaCl were inoculated with 1.0% of a 1:1 co-culture of bacteria isolated from the same year. Half of the samples were treated with 300 ppb silver. The samples were incubated for 48 hours before the coupons were removed, dipped in sterile 0.5% NaCl solution once and then individually placed in 2 mL of 0.5% NaCl solution. Test tubes were sonicated (40 kHz, 24 C) for either 0, 5, 15, 30, 45 or 120 minutes. The 2 mL broth cultures were then plated on nonselective R2A plates. Each individual data set was assessed for normality using the D'agostino-Pearson test. Statistical significance between data sets was assessed using unpaired T-tests with Welch's correction (n=2). Statistical significance was indicated by asterisks indicating that P was less than or equal to 0.05 (*) or 0.01 (**). NS = not significant.

Antimicrobial Efficacy on Planktonic and Biofilm Co-culture. This multivariable experiment compared the impact of LSMMG culture and antimicrobial silver on planktonic and biofilm co-cultures of ISS potable water consortia isolated in three different years. Understanding the impact of fluid shear on the microenvironment is vital to developing an accurate grasp of how the ECLSS bacterial population will respond to alterations to the water system. As previously

shown (**Figure 6**), LSMMG culture did not appear to have a consistent effect on every strain in the co-cultures of bacteria isolated in the same year. In the planktonic cultures of the 2009 group,

Figure 9:

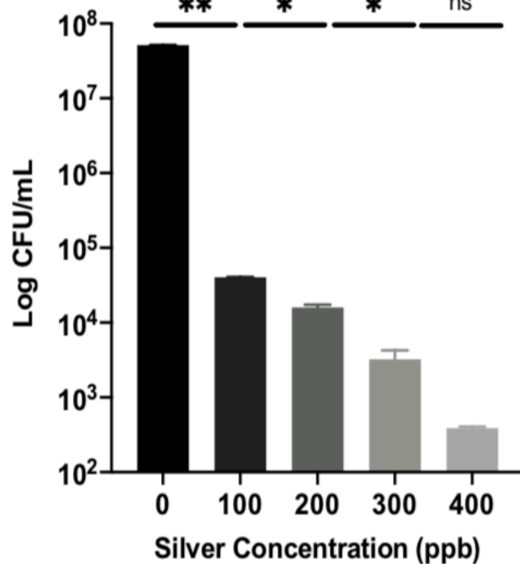


Figure 9: Antimicrobial Efficacy of Silver on Polymicrobial Communities. For the bacterial strains isolated in 2009, 5 test tubes containing a sterile stainless steel coupon and 5 mL of 0.5% NaCl solution were inoculated with 1.0% of a 1:1 co-culture of strains isolated in 2009. Samples were treated with 0, 100, 200, 300 and 400 ppb silver. The samples were incubated for 48 hours before the coupons were removed, dipped in sterile 0.5% NaCl solution once and then individually placed in 2 mL of 0.5% NaCl solution. The test tubes were sonicated (40 kHz, 24 C) for 5 minutes. The 2 mL broth cultures were then plated on nonselective R2A plates. Each individual data set was assessed for normality using the D'agostino-Pearson test. Statistical significance between data sets was assessed using unpaired T-tests with Welch's correction (n=2). Statistical significance was indicated by asterisks indicating that P was less than or equal to 0.05 (*) or 0.01 (**). NS = not significant.

Figure 10:

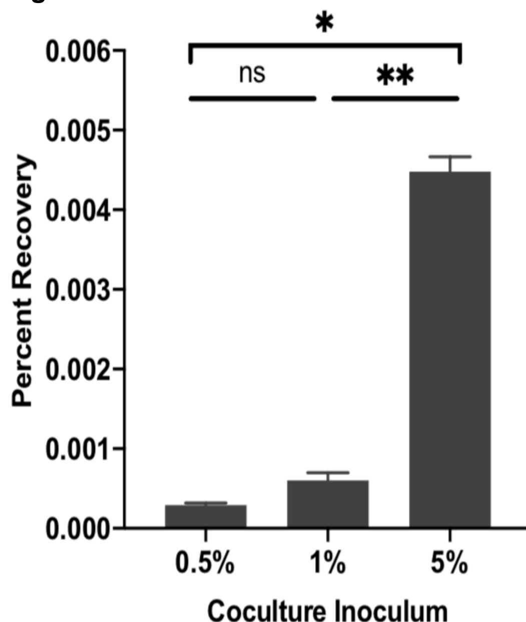


Figure 10: Impact of Initial Inoculum on Polymicrobial Community Survival in The Presence of Silver. For the bacterial strains isolated in 2009, 12 test tubes were prepared with varying 1:1 co-culture inoculum of strains isolated in 2009 (0.5%, 1.0%, or 5.0%) in 0.5% NaCl solution. Each test tube contained a single sterile stainless steel coupon. Samples were treated with 0, 100, 200 or 300 ppb silver. The samples were incubated for 48 hours before the coupons were removed, dipped in sterile 0.5% NaCl solution once and then individually placed in 2 mL of 0.5% NaCl solution. These tubes were sonicated (40 kHz, 24 C) for 5 minutes. The 2 mL broth cultures were then plated on nonselective R2A plates. Each individual data set was assessed for normality using the D'agostino-Pearson test. Statistical significance between data sets was assessed using ratio paired T-tests (n=2). Statistical significance was indicated by asterisks indicating that P was less than or equal to 0.05 (*) or 0.01 NS = not significant.

silver decreased the recovery of each strain in both the LSMMG and control cultures. While the control cultures showed significant differences between all three 2009 isolates tested, no differences were observed for *R. pickettii* and *C. metallidurans* (*S. sanguinis* still displayed a

significant difference in LSMMG between treated and untreated) (**Figure 11A**). This indicates that LSMMG may render *R. pickettii* and *C. metallidurans* more resistant to silver treatment and minimally impact *S. sanguinis*. When analyzing the population counts of the individual 2009 strains in these co-culture studies, the silver treatment appeared to have a greater impact on altering the relative ratio of each strain in the population than fluid shear (**Figure 11B**). Silver treatment of the 2009 strains induced a shift in the population to favor *S. sanguinis* over *C. metallidurans*, while *R. picketti* stays relatively consistent at ~ 5-10% of the total population. This trend is mirrored in both LSMMG and control conditions, further indicating that silver had a more consistently significant impact on population dynamics and individual strain recovery than fluid shear.

The 2012 group showed a similar trend with *S. sanguinis*; Unlike all other strains in the cohort, *S. sanguinis* displayed a statistically significant decrease in recovery when treated with silver in both control and LSMMG conditions (**Figure 11C**). *Methylobacterium* species recovery was negatively impacted by silver treatment but the decrease was only statistically significant in LSMMG conditions. Since this was the first co-culture experiment in which the *Methylobacterium* species was recovered and the only change to the experimental design was the increase in inoculum, the data suggests that increased inoculation strengthened this species chance of survival. The 2012 *B. cepacia* complex*, R, pickettii* and *R. insidiosa* were resistant to silver treatment regardless of culture condition (**Figure 11C**). The population dynamic of the 2012 co-culture swung to favor *B. cepacia* complex over *S. sanguinis* and *R. insidiosa* in both control and LSMMG conditions when the population was change in *B. cepacia* and *R. insidiosa* (**Figure 11C**) were taken into account, the variations in population dynamic was likely solely due to *S. sanguinis*.

In the 2014 group, *B. cepacia* and *Bradyrhizobium* species appeared to be unaffected by silver treatment in both control and LSMMG conditions (**Figure 11E**). Unlike the 2012 strain, 2014 *R. pickettii* showed high recovery and made up a larger proportion of the 2014 co-culture population (**Figure 11F**). In control (1xG) conditions, *R. pickettii* showed susceptibility to

Figure 11:

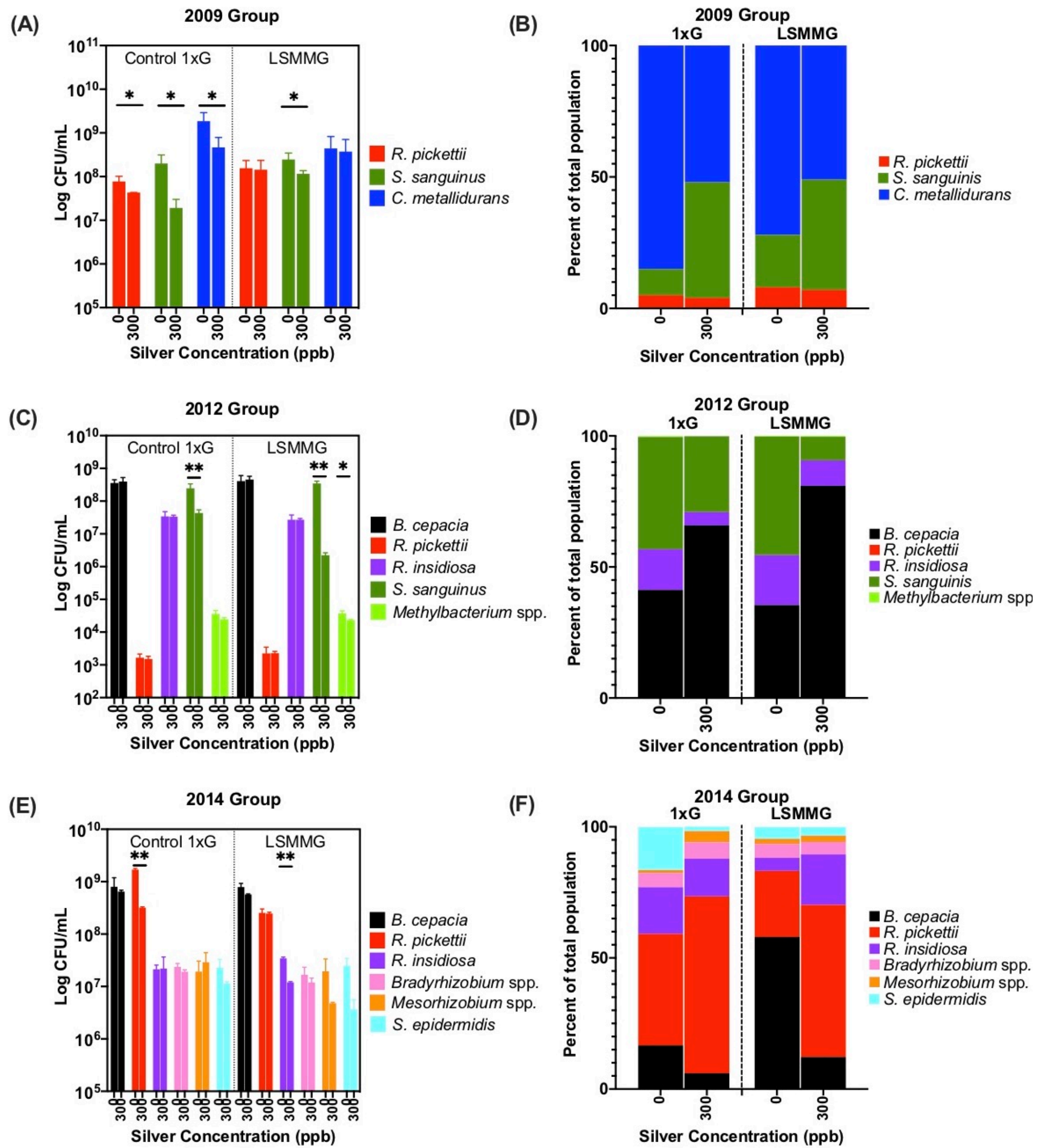


Figure 11: Impact of Silver on Individual Strain Recovery and Population Dynamics of Planktonic Co-cultures in RWV Bioreactors. Co-cultures of bacteria isolated in the same year were grown in RWV bioreactors (LSMMG or control orientation) in R2A media with or without 300 ppb silver at ambient temperature for 48 hours. To determine the planktonic population, broth samples were homogenized, diluted and plated on selective media. Each individual data set (n=5) was assessed for normality using the Shapiro Wilk test. Statistical significance between data sets was assessed using unpaired T-tests with Welch's correction. Statistical significance was indicated by asterisks indicating that P was less than or equal to 0.05 (*) or 0.01 (**).**(A, C, E)** Individual strain recovery in year co-culture **(B, D, F)** Population dynamics in isolation year group co-culture.

silver treatment (**Figure 11E**). However, this same response was not seen in LSMMG conditions. Conversely, the 2014 *R. insidiosa* showed susceptibility to silver in LSMMG conditions but not in control (1xG) conditions. This data could indicate that both the 2014 *R. pickettii* and *R. insidiosa* were more effected by fluid shear than the other strains isolated in the same year. *Mesorhizobium* spp. and *S. epidermidis* both showed a noticeable decrease in recovery when treated with silver in LSMMG conditions compared to the control, however these changes were not statistically significant. The biggest fluctuation in the 2014 co-culture population dynamic, was in the untreated LSMMG sample in which *B. cepacia* was more prevalent than *R. pickettii* (**Figure 11F**).

While the impact of antimicrobial silver and fluid shear on planktonic populations has vital importance to ECLSS contamination removal, the planktonic bacteria are one of two population to consider. As all strains in this study are known to form biofilms, the antimicrobial efficiency of silver and the impact of fluid shear on biofilm samples also needed to be determined. The biofilm samples from the 2009 group showed a similar trend to the planktonic samples with one significant difference: *S. sanguinis* in biofilms was not susceptible to antimicrobial silver and was recovered in higher numbers in silver-treated samples (**Figure 12A**). This indicated that removal of this strain from the water purification system could prove more difficult than other strains. Additionally, the decreased recovery of *C. metallidurans* in silver-treated stainless steel biofilms in LSMMG conditions was statistically significant whereas in the planktonic samples, it was not. Despite the increase in *S. sanguinis* in silver-treated conditions, the population dynamic in biofilms favors *C. metallidurans* over *S. sanguinis* more than the planktonic population indicated (**Figure 12B**).

In the 2012 biofilm, silver had a statistically significant impact on *R. insidiosa, S. sanguinis* and *Methylobacterium* species in 1xG conditions (**Figure 12C**). This differed from planktonic cultures were only *S. sanguinis* was significantly impacted (**Figure 11C**). In LSMMG conditions, biofilm-associated *R. insidiosa* was more susceptible to silver than when in planktonic culture. Similar to the 2012 planktonic population dynamic (**Figure 11D**), in the 2012 co-culture biofilms, the population was predominantly *B. cepacia* complex, *S. sanguinis* and *R. insidiosa* (**Figure 12D**). That said, *B. cepacia* complex was more prevalent in the population census in

Figure 12:

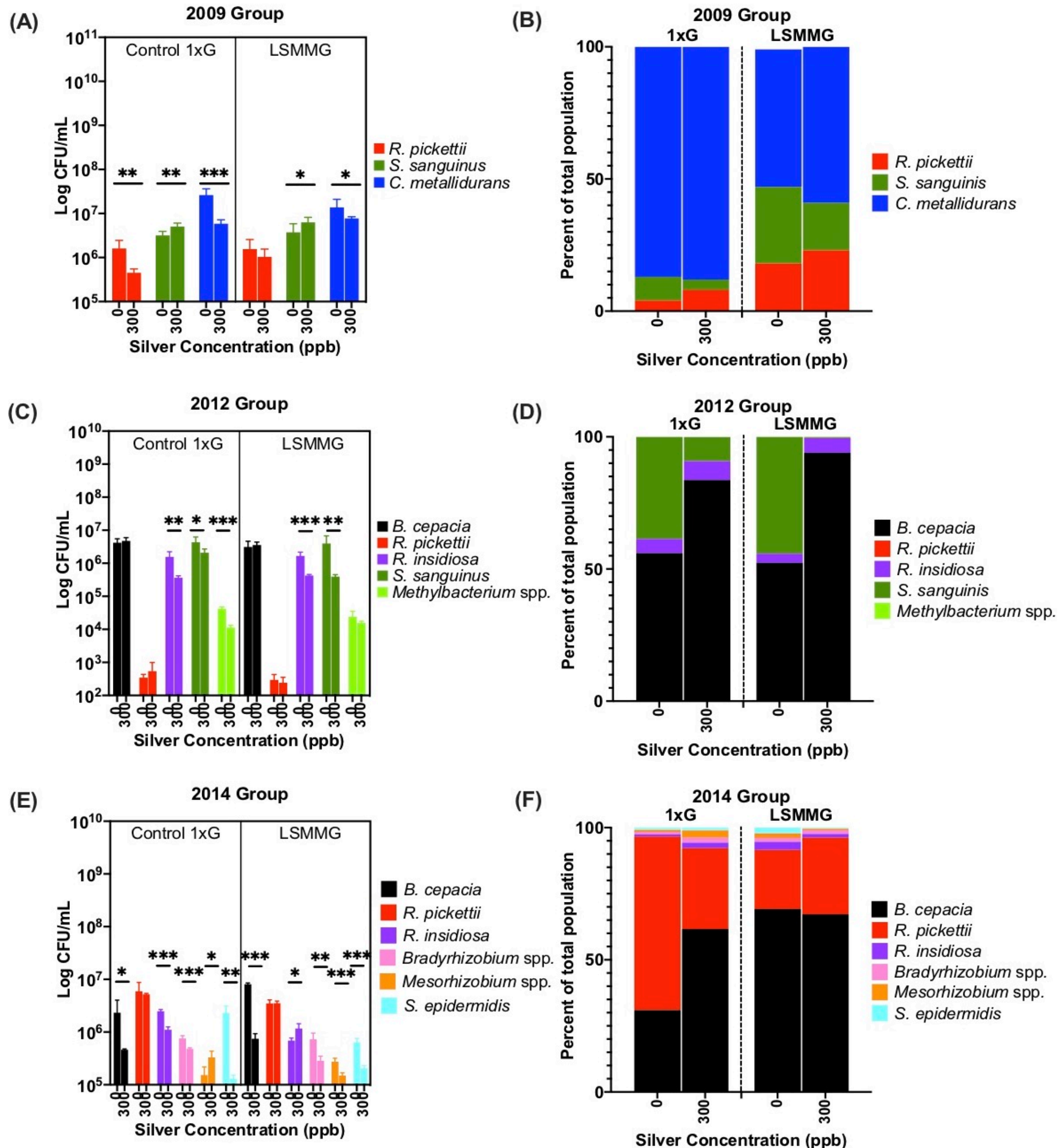


Figure 12: Impact of Silver on Individual Strain Recovery and Population Dynamics of Biofilm Co-cultures in RWV Bioreactors. (A, C, E) Individual strain recovery within co-cultures of each year **(B, D, F)** Population dynamics in co-culture of bacterial strains isolated in the same year. Co-cultures of strains isolated in the same year were grown in RWV bioreactors (LSMMG and control orientation) in R2A media with or without 300 ppb silver at ambient temperature for 48 hours. For the assessment of the biofilm population, stainless steel coupons were removed, dipped in 0.5% NaCl solution, placed in individual test tubes containing 2 mL 0.5% NaCl solution and sonicated (40 kHz, 24 C) for 5 minutes. The 2 mL cultures were then homogenized, diluted and plated on each strain's selective media. Each individual data set (n=10) was assessed for normality using the D' Agostino-Pearson test. Statistical significance between data sets was assessed using unpaired T-tests with Welch's correction. Statistical significance was indicated by asterisks indicating that P was less than or equal to 0.05 (*), 0.01 (**), or 0.001

biofilms than in the planktonic culture—indicating that this species was pivotal to the population's biofilm formation.

The 2014 biofilms showed the most individual strain recovery was statistically impacted when treated with silver (**Figure 12E**). In both LSMMG and control (1xG), the only strain not impacted by silver was *R. pickettii.* This same lack of response was seen in the 2009 and 2012 biofilms in LSMMG conditions (**Figure 12A, Figure 12C, Figure 12E**). This might indicate that *R. pickettii* would be resistant to silver treatment in the water purification system in the ECLSS. In LSMMG biofilms, *R. insidiosa* increased in recovery when the population was treated with silver. However, the same strain was susceptible to silver in planktonic samples. These data possibly indicate that in silver-treated conditions, changes in other strains within the biofilm enabled the survival of *R. insidiosa*. Interestingly, in the 2014 biofilms, *B. cepacia* complex and *R. pickettii* made up at least 90% of the population in every condition (**Figure 12F**). This contrasted the planktonic cultures, where the other strains were more prominent (**Figure 11F**),

Overall, the data indicated that antimicrobial silver had a more consistent impact on the recovery of individual strains than LSMMG culture. Unexpectedly, it appeared that biofilms were affected by the presence of silver more than their planktonic counterparts. These stainless steel coupon adherent biofilms were imaged using confocal microscopy. This data was not included in this study due to the need for additional replications and further analysis.

Influence of Silver on Bacterial Monolayer Adherence Co-culture. All experiments up to this point used strain population growth and cell colony recovery to measure a variable's impact. However, in addition to quantitative population recovery, changes to a bacteria's environment can induce additional cell responses such as alterations in gene expression, biofilm formation, or the ability to adhere to a host cell. This experiment looked at the effect LSMMG or control culture on the ability of bacterial planktonic or biofilm co-cultures to adhere to human intestinal epithelial cells. Studies were done in the presence or absence of silver. Intestinal epithelial cells were selected as the target host cell type for this study, as bacteria consumed by ingestion of potable water would likely reach the GI tract.

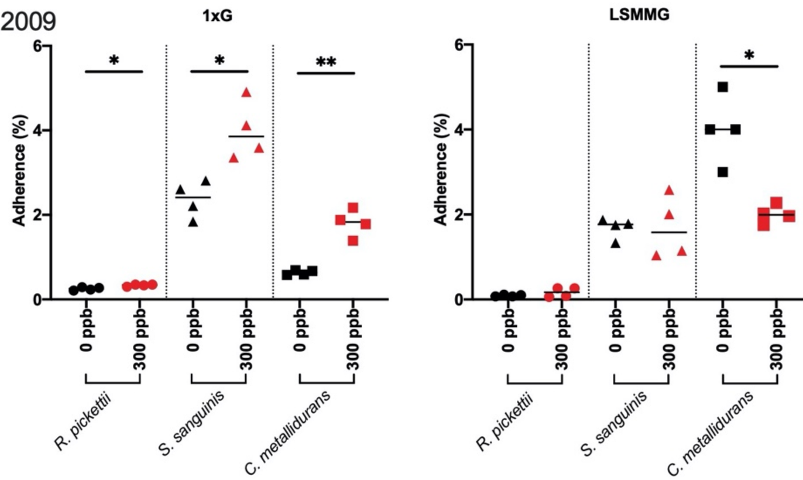
In previous experiments in this study, it was shown that fluid shear did not consistently impact the recovery of each individual strain when grown in the RWV (**Figure 6**). Interestingly, in colonization experiments fluid shear impacted the ability of 2009 planktonic co-cultures to adhere to HT-29 monolayers. In 1xG conditions, all strains displayed a statistically significant increase in adherence when treated with silver (**Figure 13A**). However, LSMMG-grown cultures either showed no change or decreased in adherence following silver treatment. When grown in 1xG conditions, *C. metallidurans* showed an increase in adherence of monolayers in silver treated conditions compared to untreated conditions, while LSMMG grown samples showed a significant decrease in adherence for this strain. None of the 2009 planktonic co-culture samples showed over a 6% adherence to monolayers.

The adherence of some strains in the 2012 group were less impacted by fluid shear and antimicrobial silver treated growth conditions. Neither *B. cepacia* complex nor *R. insidiosa* showed a statistically significant difference in adherence between silver treated and untreated samples in either fluid shear condition (**Figure 13B**). The 2012 *R. pickettii* was not recovered in the colonization study (likely due to its comparatively low recovery in co-culture) (**Figure 7B, Figures 11B, Figure 11C, Figure 12B, Figure 12C**). In both LSMMG and 1xG conditions, only *S. sanguinis* showed a statistically significant difference in adherence between silver treated and untreated conditions. While there was an increase in adherence for both LSMMG and control cultures for *S. sanguinis* following silver treatment, the increase was small for the 1xG cultures (~0.3%, 9.5 fold increase) relative to LSMMG (~ 12%, 842 fold increase). While there were no statistically significant differences in adherence between silver treated and untreated samples, *Methylobacterium* species grown in 1xG showed approximately 15% adherence, compared to LSMMG samples which had approximately a 25% adherence to monolayers. Both the 2012 *S. sanguinis* and *Methylobacterium* species appear to express an increased ability to bind to monolayers in LSMMG conditions compared to 1xG conditions.

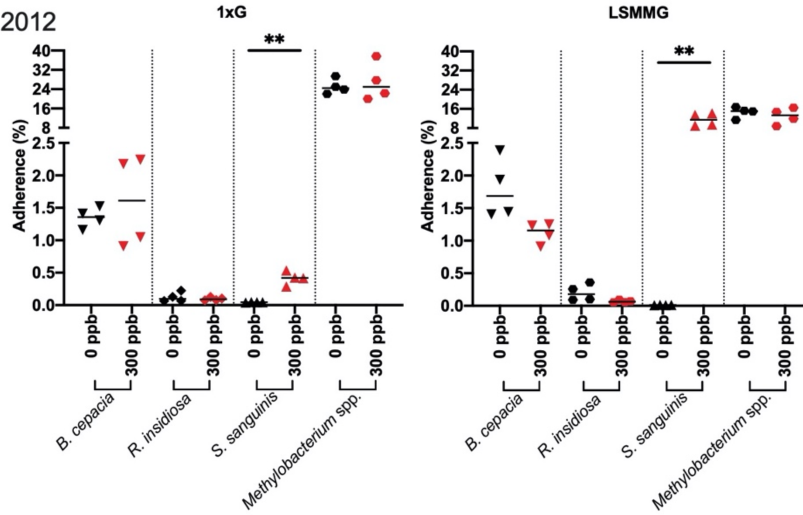
When the 2014 strains were cultured in 1xG conditions, only planktonic *R. picketti* and *Bradyrhizobium* species showed a statistically significant increase in adherence of silver treated samples compared to untreated samples (**Figure 13C**). When the same strains were grown in

Figure 13:

(A) 2009



(B) 2012



(C) 2014

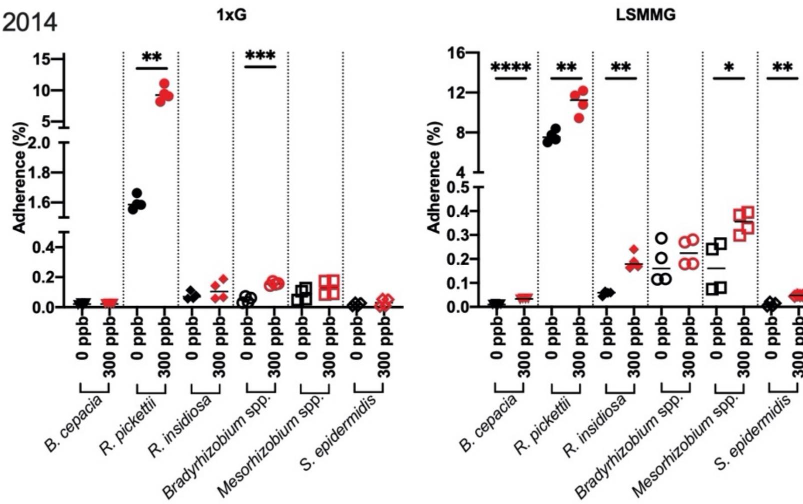


Figure 13. Bacterial Planktonic Co-culture Adhesion of Epithelial Cell Monolayers in the Presence or Absence of Silver. **(A)** 2009 co-culture **(B)** 2012 co-culture **(C)** 2014 co-culture. ISS bacterial co-cultures isolated from the same year were grown in LSMMG or the control orientation in R2A media with/without 300 ppb silver at ambient temperature. At 48 hours post inoculation, HT-29 monolayers were infected with the planktonic broth cultures from the bioreactors. After 3 hours post-infection (h.p.i.), monolayers were washed three times with warm HBSS, lysed, homogenized and plated on selective media. Percent adherence for each strain was calculated by dividing the CFU/mL at 3 h.p.i. by the initial inoculum. No colonies were recovered for the 2012 *R. pickettii* when the 10^1 dilution was plated. Each individual data was set assessed for normality using the Shapiro Wilk test. Statistical significance between data sets was assessed using unpaired T-tests with Welch's correction (n=4). Statistical significance was indicated by asterisks indicating that P was less than or equal to 0.05 (*), 0.01 (**), 0.001 (***) or 0.0001 (****).

Figure 14:

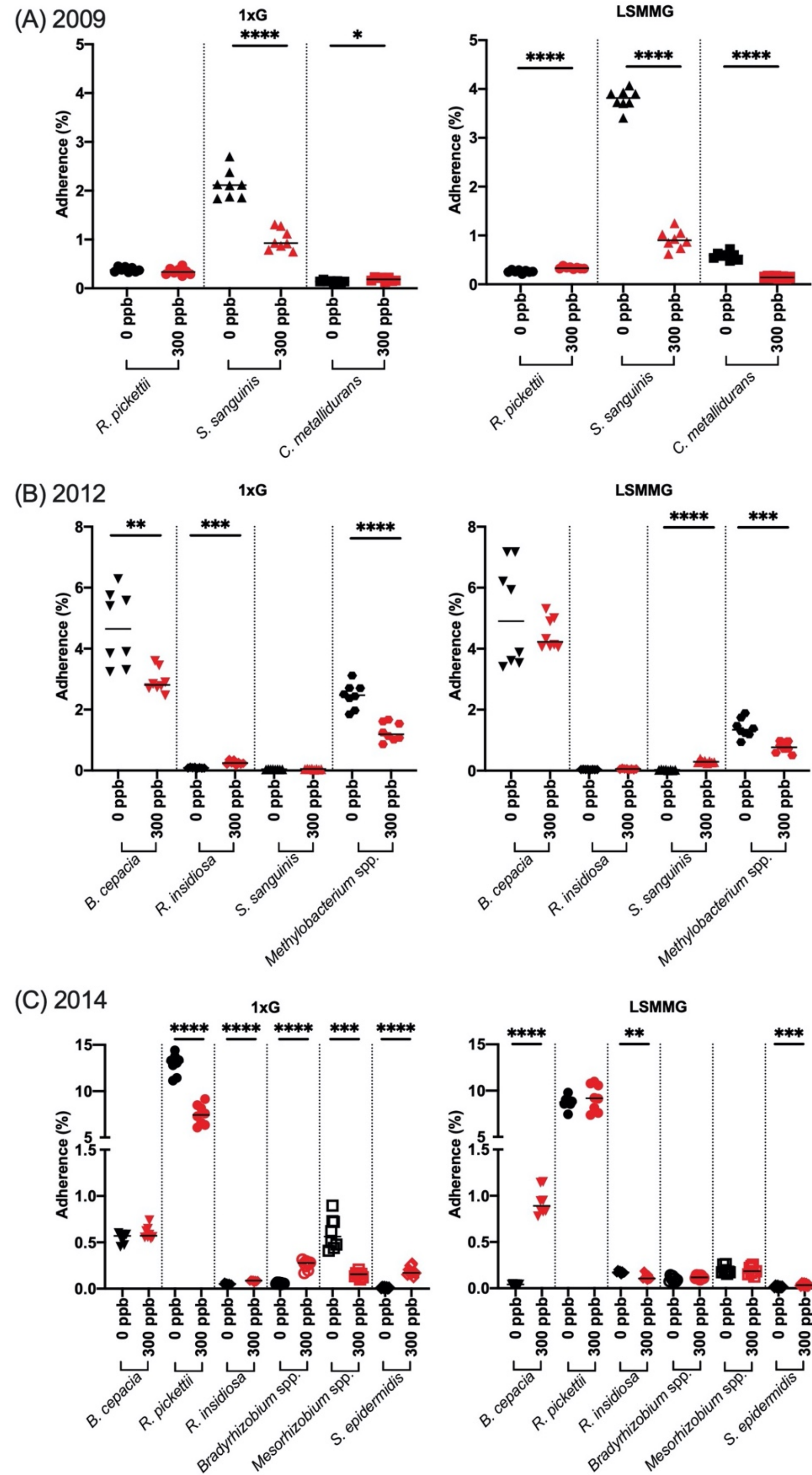


Figure 14. Bacterial Biofilm Co-culture Adhesion to Epithelial Cell Monolayers in the Presence or Absence of Silver. (A) 2009 co-culture **(B)** 2012 co-culture **(C)** 2014 co-culture. ISS bacterial co-cultures isolated from the same year were grown in LSMMG or control bioreactors on stainless steel coupons in R2A media with or without 300 ppb silver at ambient temperature. At 48 hours post inoculation, HT-29 monolayers were infected with the bacteria recovered from coupons following sonication. After 3 h.p.i., monolayers were washed three times with warm HBSS, lysed, homogenized and plated on selective media. Percent adherence for each strain was calculated by dividing the CFU/mL at 3 h.p.i. by the CFU/mL of the initial inoculum. Each individual data set was assessed for normality using the D'Agostino Pearson test. Statistical significance between data sets was assessed using unpaired T-tests with Welch's correction (n=8). Statistical significance was indicated by asterisks indicating that P was less than or equal to 0.05 (*), 0.01 (**), 0.001 (***) or 0.0001 (****).

LSMMG conditions, all strains except *Bradyrhizobium* species exhibited a statistically significant increase in adherence of silver treated compared to untreated samples. In the bacterial group isolated in 2014, *R. pickettii* stood out as it was the only strain in the group to show over a 1% population adherence of the monolayers.

Similar to the antimicrobial efficiency experiment, the infection study was performed with both planktonic and biofilm samples. In the 2009 biofilms grown in the 1xG condition, *S. sanguinis* reversed its trend from the planktonic results (**Figure 13**), showing a decrease of adherence between silver treated and untreated conditions (**Figure 14A**). This *S. sanguinis* adherence pattern was also seen in the LSMMG grown samples. The other two 2009 strains, *R. pickettii* and *C. metallidurans*, produced the same adherence patterns in biofilm cultures as they did in planktonic culture (**Figure 13A**). This data, combined with that in **Figure 11A** and **Figure 12A**, could indicate that the adherence of *S. sanguinis* was more impacted biofilm conditions than either *R. pickettii* or *C. metallidurans.*

The 2012 biofilm adherence trends also differ from their planktonic counterparts. Unlike in planktonic conditions where adherence for *B. cepacia* complex was less than 2.5%, within a biofilm it adhered between 4-5% (**Figure 14B**). Conversely, *Methylobacterium* spp. adhered better to monolayers when grown planktonic co-cultures (15-25% adherence) relative to biofilm-associated co-cultures (1-2% for 1xG biofilms, 0.5-2.5% for LSMMG biofilms).

In the 2014 group, both *R. pickettii* and *Mesorhizobium* species reversed their adherence pattern from planktonic infection data between silver treated and untreated population in 1xG conditions (**Figure 14C**). However, similar to planktonic co-cultures, the biofilm-associated 2014 *R. pickettii* still displayed the highest adherence relative to other strains from the same year. In LSMMG grown biofilms, *R. pickettii* did not show a statistically significant difference in adherence between silver treated and untreated samples.

These data could indicate that treating the ISS potable water biofilms with silver may actually increase the pathogenicity of select bacteria. Further assessment will need to be

performed in order to fully evaluate the risk of using silver as an antimicrobial in the water purification system.

Implementation of Silver in the Potable Water Recovery System. This study used R2A growth media as a control nonselective medium because it is intended to isolate water isolates which survive in minimal nutrient environments. However, R2A media contains multiple nutrient sources and organic compounds. Silver nanoparticles have been shown to interact with organic compounds (**101**), and how these interactions impact the antimicrobial efficiency is currently unknown. To understand the antimicrobial efficiency of silver in an environment with minimal organic compounds, antimicrobial efficiency was also tested using a 0.5% NaCl solution instead of R2A broth. This experiment showed a statistically significant population decrease for the 2009, 2012 and 2014 planktonic co-cultures in both LSMMG and 1xG conditions when treated with 100 ppb silver (**Figure 15A**). The silver dosage was lowered from previous experiments because when 300 ppb silver was used on the bacterial cultures in NaCl, the recovery of each strain was reduced so dramatically that the recovery data was inconsistent. This trend was replicated in biofilm cultures (**Figure 15B**), suggesting that when environmental organic compounds were minimized, silver was more effective.

Although the total bacterial counts in each group decreased following silver treatment, some individual strains in each group actually increased in recovery when treated with silver. In the planktonic co-culture populations, while some strains did not show a statistically significant difference between silver-treated and untreated populations, no individual strain in any of the co-culture groups had a statistically significant increase in recovery. In biofilm co-cultures however, the 2012 *R. insidiosa* and *Methylobacterium* species displayed a statistically significant increase in recovery in LSMMG when treated with silver (data not shown). As both of these species are known biofilm formers and opportunistic pathogens, this could prove a hurtle in the elimination of ISS water purification system contaminants.

Figure 15:

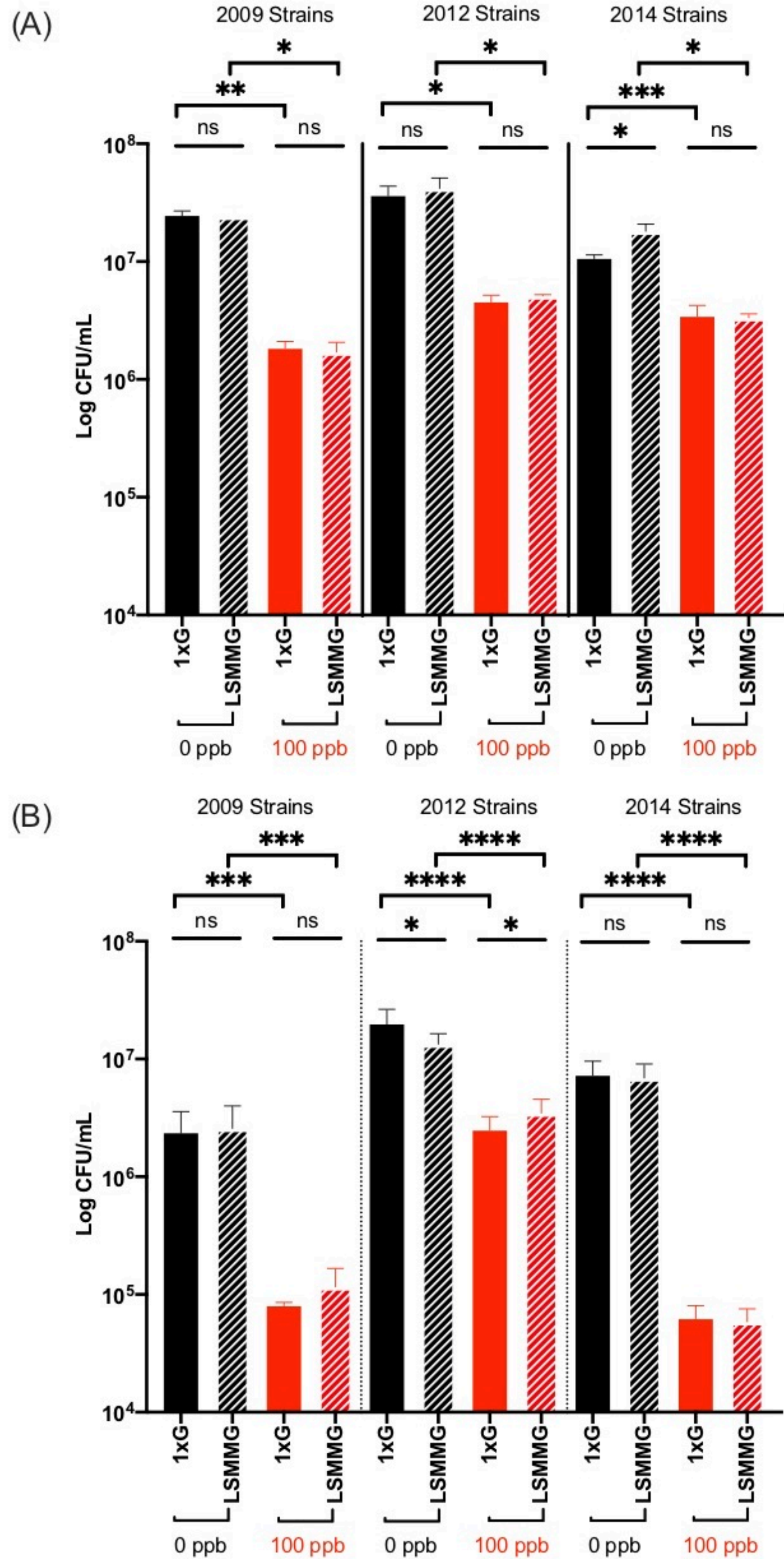


Figure 15: Silver Efficacy Against ISS Bacterial Co-cultures Grouped by Year of Isolation. (A) Planktonic (n=4) **(B)** Biofilm on stainless steel coupon (n=9). Bacterial co-cultures were grown in LSMMG or control bioreactors in 0.5% NaCl with and without 100 ppb silver at ambient temperature. To determine the planktonic population counts at 24 hours post-treatment, cultures were homogenized, serially diluted and plated on selective media. For the assessment of the biofilm population, stainless steel coupons were removed, washed once by dipping in 0.5% NaCl, placed in tubes containing 2 mL 0.5% NaCl and sonicated (40 kHz, 24 C) for 5 minutes. Cultures were then homogenized, serially diluted and plated on selective media. Each individual data was set assessed for normality using the Shapiro Wilk test (for data set **A**) or the D'Agostino Pearson test (for data set **B**). Statistical significance between data sets was assessed using unpaired T-tests with Welch's correction. Statistical significance was indicated by asterisks indicating that P was less than or equal to 0.05 (*), 0.01 (**), 0.001 (***) or 0.0001 (****). Non-statistical significance was indicated with "ns**.**"

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

Water Treatment Implications. While it is clear that microbial contamination of the water purification component of the ISS potable water system poses a serious risk to crew health and ECLSS integrity/function, the complexity of the environment and the polymicrobial dynamics demands careful evaluation. Furthermore, the microgravity environment makes most previous decontamination research that was performed in 1xG conditions a potentially inaccurate representation of water decontamination on the ISS and other future space missions. The low fluid shear environment within the bacterial community's environment combined with the multispecies interactions and other spaceflight factors (spaceflight radiation, multiple contributing water sources, other components of the ECLSS system) makes this contamination uniquely complicated. While silver does function as an efficient antimicrobial under most conditions evaluated in this study, silver did not reduce the population recovery of every strain in R2A growth media. In 0.5% NaCl, silver had a stronger antimicrobial effect than when bacteria were grown in R2A. Because, within the potable water recovery system on the ISS, bacteria are growing in purified water, silver could have a differing level of antimicrobial efficacy due to a unique level or organic material in the water.

Like other antimicrobials, when silver is used as an alternative and/or antimicrobial agent in the ECLSS and other future spaceflight operations, there is a risk that silver could potentially eliminate only the weakest members of each strain leaving resistant bacteria left to flourish. As microbial contamination serves as both a health and materials integrity risk, further research must be done to confirm the viability of this approach before implementation.

Future Research Opportunities. The primary goals of this study were to analyze the growth of polymicrobial communities from ISS potable water isolates when simultaneously co-cultured in the RWV bioreactor by examining i) the potential for these communities to form biofilms, and ii) population dynamics for the selected strains. These goals were accomplished by 1) developing selective and/or differential media for the purpose of isolating each individual strain

from co-culture, 2) analyzing the effects of co-culture and physiological fluid shear on the growth of individual strain within insolation year group populations and 3) determining the viability of silver as an antimicrobial for the ISS potable water system.

To further this research, the antimicrobial efficiency of silver nanoparticles needs to be compared to the current approach of using iodinated resin. This comparative assessment would indicate how silver compares in decontamination and whether better results will be achieved by switching antimicrobial agents. Since silver nanoparticles interacts with organic compounds reducing antimicrobial efficiency, an evaluation of the recycled water system needs to be performed to determine how much organic matter (human waste, dead bacteria, etc.) remains in the water to interact with the silver. This would improve the accuracy of the silver dosage used to treat the system. Moreover, the long-term impacts of silver nanoparticles on both the water purification system and the health of the crew needs to be determined. The in vitro infection experiment in this study could be furthered by also evaluating each strain's ability to invade the host cell in addition to adhering to it, and by optimizing the multidimensional, fluid shear and immune system physiological relevance of the host model.

The water purification system aboard the ISS contains a complex microbial consortium of competitive and cooperative species which are able to survive the constant stress of a microgravity environment. In order to eliminate or reduce contamination risks on the ISS and prevent contamination in future long-term space flight endeavors, microorganisms need to be controlled with the precision only a complete understanding of the environment and its constituents can provide.

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