

MATERIAL COATINGS AND STRATEGIES FOR BIOFILM MITIGATION IN
SPACECRAFT WATER SYSTEMS

by

Madelyn Kelly Mettler

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Chemical Engineering

MONTANA STATE UNIVERSITY
Bozeman, Montana

May 2025

©COPYRIGHT

by

Madelyn Kelly Mettler

2025

All Rights Reserved

DEDICATION

I dedicate this dissertation to myself—for persevering through every moment I doubted I could. This has been a long journey, full of unforgettable highs and lows. But I pipetted, autoclaved, experimented, wrote, proposed, presented, and, finally, celebrated my way to the end. Here's to the version of me that never gave up.

ACKNOWLEDGEMENTS

There are countless people who have helped me during my graduate journey and during my time at the Center for Biofilm Engineering. My advisor, Dr. Brent Peyton, has been an excellent source of research, professional, and gardening advice. Dr. Elizabeth Sandvik has been instrumental in developing my research methods. The entire NASA EPSCoR team provided ideas and support that I could not have completed my research without. The same goes for the Jet Propulsion Laboratory group with whom I began my graduate research. Special thanks to the folks in the Standardized Biofilm Methods Laboratory. My committee members, Dr. Phil Stewart, Dr. Christine Foreman, and Dr. Kathryn Morrissey have been incredible for providing additional research support and feedback.

All my friends and family have been endlessly supportive during these five years. I am especially grateful to my mom, dad, stepmom, and sister for their visits and listening ears which were essential to my sanity. My partner, Matt, came into my life during this journey and he immediately took on the role of being my greatest cheerleader. I truly do not think I would have been able to do it without his love and support. There are not enough “thank-yous” for Haley Ketteler for her guidance, commiseration, laughter, and excitement. Thank you to all the folks in WISE, the GSA, the MBGM (Dr. Jennifer Brown especially) for lifting me up when I was down. A final thank-you to the undergraduate researchers who have helped me along the way, especially Natasha Peterson for her assistance during methods development.

TABLE OF CONTENTS

1. INTRODUCTION	19
Background	19
Coatings for microbial control	21
Dissertation overview:	23
Funding and collaborations:	25
References	27
2. CONSIDERATIONS FOR TESTING ANTI-FOULING COATINGS DESIGNED FOR IMPLEMENTATION INTO EARTH-BASED AND SPACECRAFT WATER SYSTEMS	30
Contribution of Authors and Co-Authors	30
Manuscript Information	31
Abstract	32
Introduction	32
Considerations for experimental evaluation of coatings	36
Consideration 1: Biofilm reactor operation	36
Consideration 2: Microorganism selection	37
Consideration 3: Reinoculation	40
Consideration 4: Coverage of the coating in the test system	42
Consideration 5: Liquid medium selection	44
Consideration 6: Experiment	45
duration & pre-aging of surface	45
Consideration 7: Performance evaluation of the surface	48
Consideration 8: Microgravity	51
Current methods for coating evaluation	58
Summary of the methods used in evaluation	60
of coatings for Earth-based water systems	60
Summary of methods used in evaluation	62
of coatings for spacecraft water systems	62
Conclusions and Recommendations	65
Supplementary material	68
References	68
3. ANTIMICROBIAL COATING EFFICACY FOR PREVENTION OF <i>PSEUDOMONAS AERUGINOSA</i> BIOFILM GROWTH ON ISS WATER SYSTEM MATERIALS	81
Contribution of Authors and Co-Authors	81
Manuscript Information	82
Abstract	83

TABLE OF CONTENTS CONTINUED

Introduction.....	83
Materials and Methods.....	86
Microorganisms Used	86
CDC Bioreactor Preparation	86
Microorganism Preparation for CDC Bioreactor.....	88
Biofilm Growth.....	89
CDC Bioreactor Sampling	89
Statistical Analysis	90
Scanning Electron Microscopy	91
Results.....	92
Biofilm Measurement Using.....	92
Continuous Flow CDC Bioreactor.....	92
SEM Analysis of CDC Bioreactor Materials	95
Discussion	97
Conclusion	101
Supplementary Material.....	102
Funding	104
Acknowledgments.....	104
References.....	105
 4. MULTIMODAL BIOFILM CONTROL STRATEGIES FOR SPACECRAFT WATER SYSTEMS: EVALUATING COATINGS, NUTRIENT REMOVAL, AND BIOCIDES FOR IMPROVED SUSTAINABILITY	 108
Contribution of Authors and Co-Authors	108
Manuscript Information	109
Abstract	110
Introduction.....	110
Methods and materials	113
Organism selection and preparation.....	113
Coupon preparation.....	114
Reactor preparation.....	115
Medium preparation.....	115
Biocide preparation.....	117
Experiment iterations.....	117
Reactor operation.....	118
Sampling	119
Statistical analysis of plate counts	120
Results.....	121
Viable biofilm density: CFU plate counts.....	121
Biomass and biofilm morphology: Confocal image analysis	126

TABLE OF CONTENTS CONTINUED

Discussion	130
Viable biofilm density: CFU plate counts.....	131
Biomass and biofilm morphology: Confocal image analysis	133
Comparison to previous work.....	135
Conclusions.....	137
Acknowledgements	138
Funding	138
Author disclosure statement.....	139
References.....	139
 5. MULTIDOMAIN BIOFILM CONTROL IN A NOVEL SIMULATED MICROGRAVITY REACTOR	 143
Contribution of Authors and Co-Authors	143
Manuscript Information	144
Abstract	145
Introduction.....	145
Methods.....	148
Organism selection and preparation.....	148
Nutrient medium	149
Biocide preparation.....	150
Reactor assembly and preparation	151
Reactor operation.....	153
Sampling	154
FlowCam.....	156
Results.....	156
Biofilm community.....	156
Planktonic community	159
Discussion	162
Biofilm cell density using plate counts	163
Confocal images.....	165
Planktonic plate counts	166
FlowCam.....	166
Conclusion	168
Acknowledgements	169
Funding	169
Author disclosure statement.....	170
References.....	170
 6. CONCLUSION.....	 176
Major conclusions.....	176

TABLE OF CONTENTS CONTINUED

Recommendations for future work	178
References.....	182
APPENDICES	184
APPENDIX A: DATA IN SUPPORT OF CHAPTER 3	185
APPENDIX B: ADDITIONAL METHODS IN SUPPORT OF CHAPTER 3	192
Introduction.....	193
Methods.....	193
Results.....	195
Discussion	199
Data	200
References.....	205
APPENDIX C: PRELIMINARY WORK FOR CHAPTER 4 AND SELECTION OF SHER-LOXANE® 800 COATING	206
Overview.....	207
Methods.....	207
Results and discussion	212
Data	215
Funding	216
Disclosure statement	216
References.....	217
APPENDIX D: DORMANCY EXPERIMENTS	218
Overview.....	219
Methods.....	219
Results and discussion	221
Funding	224
Disclosure statement	224
Data	224
References.....	229
APPENDIX E: DATA IN SUPPORT OF CHAPTER 4	230
APPENDIX F: DATA IN SUPPORT OF CHAPTER 5	249
APPENDIX H: SIMULATED MICROGRAVITY BIOFILM REACTOR OPERATING PROCEDURE	258
Required materials:	259
Reactor overview	260

TABLE OF CONTENTS CONTINUED

Organism preparation:	263
Reactor preparation:	263
Filling and inoculating the reactor:	267
Continuous flow operation.....	270
Periodic flow operation.....	270
Effluent sampling.....	270
Biocide dosing:	271
Coupon sampling:	272
Reactor clean-up:	273
APPENDIX I: ADDITIONAL PUBLICATIONS FROM	
GRADUATE SCHOOL	274
REFERENCES CITED.....	276

LIST OF TABLES

Table	Page
1. Table 1. Biofilm experiments performed under simulated gravity or in space. Modified from Flores et al. (2024). BM: Biofilm morphology; BF=Biofilm formation; EPS= Extracellular polymeric substances; NC =no change with respect to controls; ↑ = increased; ↓ =decreased; — = data not provided, or analysis not done.	52
2. Supplementary Table 1. Summary of experimental methods relating to the considerations presented in the review. (Inserted as image due to size).	68
3. Table 1. Recipe for microbial ersatz MTN featuring final component concentrations. Colors correspond to components that were added together as a concentrated stock solution.....	116
4. Table 2. Selective agar used for microbial enumeration of planktonic and biofilm samples.	120
5. Table 3. P-values for statistical significance of each factor and select combined factors in mixed effects models for the viable cell densities of the accumulated biofilms. Bolded values represent those less than 0.05.	124
6. Table 4. P-values for statistical significance of each fixed factor in mixed effects models for the viable cell densities of the planktonic cells in the reactor fluid. Bolded values are less than 0.05.	126
7. Table 1. Recipe for microbial ersatz (MTN) featuring final concentrations. Colors correspond to components that were added together in concentrated stock solutions.	150
8. Table 2. Selective agar recipes.....	156
9. Table A1. Data used to prepare Figure 2A in Chapter 3.	187
10. Table A2. Data used to prepare Figure 2B in Chapter 3.	189
11. Table A3. Data used to generate Figure 2C in Chapter 3.....	190
12. Table A4. Planktonic cell densities (log ₁₀ CFU/mL) used to generate Figure 2D-E in Chapter 3.....	190

LIST OF TABLES CONTINUED

13. Table A5. Data used to generate Figure 2F in Chapter 3.	190
14. Table A6. Data used to generate Figure 3A-C in Chapter 3. CI= confidence interval. Units for average biofilm density are $\log_{10}\text{CFU}/\text{cm}^2$	191
15. Table A7. Data used to generate Figure 3D-F in Chapter 3. CI= confidence interval. Units for average planktonic cell density are $\log_{10}\text{CFU}/\text{mL}$	191
16. Table B1. <i>R. mucilaginosa</i> single-species biofilm density data used to generate Figure B1.A-B.	201
17. Table B2. Log reduction of <i>R. mucilaginosa</i> single species biofilms on coated vs uncoated coupons used to generate Figure B1.C.	202
18. Table B3. Planktonic cell densities ($\log_{10}\text{CFU}/\text{mL}$) in reactors with <i>R.</i> <i>mucilaginosa</i> single-species biofilms used to generate Figure B1.D-E.....	202
19. Table B4. Log reduction for planktonic cells in reactors with single- species <i>R. mucilaginosa</i> biofilms with coated vs uncoated coupons used to generate Figure B1.F.	203
20. Table B5. Mixed domain biofilm data on uncoated coupons used to generate Figure B2.	203
21. Table B6. Mixed domain biofilm data on coated coupons used to generate Figure B2.	204
22. Table B7. Mixed domain biofilm log reduction data used to generate Figure B3.	204
23. Table C1. Coatings tested on Inconel and Teflon coupons. The AgXX mesh was only available as stainless steel.	208
24. Table C2. Surface roughness quantification values reported by Dektak Stylus Profilometer.....	210
25. Table C3. Various recipes attempted for selective agar*.	212
26. Table C4. Viable biofilm density data used to create Figure C5. Units for each value are $\log_{10}\text{CFU}/\text{cm}^2$	215
27. Table C5. Data used to create Figure C6.	216

LIST OF TABLES CONTINUED

28. Table D1. Data used to generate Figure D2. Average and standard deviation are represented as $\log_{10}\text{CFU}/\text{cm}^2$	225
29. Table D2. Data used to generate Figure D3. Average and standard deviation are represented as $\log_{10}\text{CFU}/\text{mL}$	227
30. Table E1. Data used to generate Figure 3 in Chapter 4. FS= Full strength media, NP= No phosphorus media, AgF= Silver fluoride biocide. Average density and standard deviation reported as $\log_{10}\text{CFU}/\text{cm}^2$	232
31. Table E2. Data used to generate Figure 4 in Chapter 5. FS= Full strength media, NP= No phosphorus media, AgF= Silver fluoride biocide. Average density and standard deviation reported as $\log_{10}\text{CFU}/\text{mL}$	241
32. Table F1. Data used to generate Figure 2 in Chapter 5. Average and standard deviation are presented as $\log_{10}\text{CFU}/\text{cm}^2$	251
33. Table F2. Data used to generate Figure 4 in Chapter 5. Average and standard deviation presented as $\log_{10}\text{CFU}/\text{mL}$	252

LIST OF FIGURES

Figure	Page
1. Figure 1. Biofilms that required part replacement in A) the condensate plumbing at the entrance to the Russian condensate processor and B) the solenoid valve in the Mostly Liquid Separator. Figures adapted from Zea et al., 2018 [15] with original credit from NASA.....	20
2. Figure 2. A schematic representing the general categories of material coatings for use in microbial control.	22
3. Figure 1. Schematic representing important properties of real water systems that should be considered for informed design of laboratory experiments for coating evaluation.	35
4. Figure 2. Summary of the eight studies presented and the methodological approach to evaluating coatings for implementation into water systems. Blank spaces indicate lack of incorporation of a specific experimental approach.	59
5. Figure 1. Center for Disease Control (CDC) bioreactor, material types, uncoated vs. coated materials. (A) Experimental setup consisting of 20 L carboy, peristaltic pump, and CDC Biofilm Reactor atop a stir plate. (B) Close-up of materials loaded into CDC Biofilm Reactor rods, from left to right, materials are stainless steel, Teflon, Inconel, and titanium. (C) Close-up of two titanium materials without coating (left) and with antimicrobial coating (right).	87
6. Figure 2. Tryptic soy broth (TSB)-grown <i>Pseudomonas aeruginosa</i> biofilm accumulation and planktonic cell density. TSB-grown <i>P. aeruginosa</i> biofilm cell densities at 24 and 48 h after start of continuous flow on (A) uncoated and (B) coated materials. (C) Log reduction of TSB-grown biofilm accumulation on materials at 24 and 48 h. Inoculum and planktonic cell densities at 0, 24, and 48 h after start of continuous flow for reactors with (D) uncoated and (E) coated materials. (F) Log reduction of planktonic cells at 24 and 48 h. Error bars represent 95% CI.....	93

LIST OF FIGURES CONTINUED

7. Figure 3. Potato dextrose broth (PDB)-grown *P. aeruginosa* biofilm accumulation and planktonic cell density. PDB-grown *P. aeruginosa* biofilm cell densities at 24 and 48 h after start of continuous flow on (A) uncoated and (B) coated titanium. (C) Log reduction of TSB-grown biofilm accumulation on titanium at 24 and 48 h. Inoculum and planktonic cell densities at 0, 24, and 48 h after start of continuous flow for reactors with (D) uncoated and (E) coated titanium. (F) Log reduction of planktonic cells at 24 and 48 h. Negative log reductions indicate the coating resulted in higher cell densities. Error bars represent 95% CI. 94

8. Figure 4. Scanning electron microscopy (SEM) imaging of *P. aeruginosa* biofilm formation on titanium after 48 h. Images (A,B) represent uncoated and coated titanium that were unprocessed (not exposed to the reactor, bacteria, or SEM preparation steps). Images (C,D) represent uncoated and coated titanium that were not exposed to the reactor or bacteria but went through the SEM preparation steps at the JPL. Images (E,F) represent uncoated and coated titanium that came from a reactor that utilized TSB medium while (G,H) came from a reactor with PDB. Biofilms on uncoated materials hosted numerous cells with small amounts of dried EPS attaching the cells to each other and to the surface..... 96

9. Figure 5. SEM imaging of TSB-grown *P. aeruginosa* biofilm on stainless steel, Teflon, and Inconel after 48 h. Images (A,B) represent uncoated and coated stainless steel; (C,D) represent uncoated and coated Teflon; and (E,F) represent uncoated and coated Inconel. All biofilms were grown in reactors with TSB as nutrient medium. The coated stainless steel demonstrates the presence of coating and coating debris..... 97

10. Supplementary Figure 1A. Scanning electron microscopy (SEM) imaging of stainless steel coupons used for biofilm accumulation. “Unprocessed” refers to coupons that did not go through the SEM preparation process. “Uninoculated” refers to coupons that were not exposed to biology, but did go through the SEM preparation process. 102

11. Supplementary Figure 1B. Scanning electron microscopy (SEM) imaging of Teflon coupons used for biofilm accumulation. “Unprocessed” refers to coupons that did not go through the SEM preparation process. “Uninoculated” refers to coupons that were not exposed to biology, but did go through the SEM preparation process. 103

LIST OF FIGURES CONTINUED

12. Supplementary Figure 1C. Scanning electron microscopy (SEM) imaging of Inconel coupons used for biofilm accumulation. “Unprocessed” refers to coupons that did not go through the SEM preparation process. “Uninoculated” refers to coupons that were not exposed to biology, but did go through the SEM preparation process.	103
13. Figure 1. Coupons for sampling biofilm accumulation in the CDC reactor.....	114
14. Figure 2. Biofilm control methods used in each of the 8 iterations of experiments. The empty section in the upper left represents the base condition of unmitigated biofilm growth. This layout is used throughout the results to present data for each experiment.	118
15. Figure 3. Viable biofilm density for each of the eight iterations of biofilm control combinations. Continuous flow was initiated on day 1 after the sample was taken. The grey dashed line represents the limit of detection, $y=1.69$. Error bars are standard deviation.	122
16. Figure 4. Viable planktonic cell density for each of the eight iterations of biofilm control combinations. Continuous flow began on day 1 after the sample was taken. The grey dashed line represents the limit of detection, $y=1.3$. Error bars represent standard deviation.	125
17. Figure 5. Confocal images of biofilms accumulated on Inconel CDC coupons 7 days after inoculation. Viable bacterial cells are in green, non-viable bacterial cells are in red, and fungal cells are in blue. The fluorescence of the coating can be seen in the three images on the lower right. The scale bar for each image in 30 μm	127
18. Figure 6. Confocal images of biofilms accumulated on Teflon CDC coupons 7 days after inoculation. Viable bacterial cells are in green, non-viable bacterial cells are in red, and fungal cells are in blue. The fluorescence of the coating can be seen in the three images on the lower right. The scale bar for each image in 30 μm	128
19. Figure 7. Side views of biofilms on A) uncoated Inconel, B) coated Inconel, C) uncoated Teflon, and D) coated Teflon grown in full strength medium without biocide dosing. Scale bar in each image is 30 μm	129
20. Figure 1. Simulated microgravity reactor featuring Sher-Loxane® 800 coated polycarbonate walls. For a detailed CAD drawing of the original reactor design, refer to Ketteler et al., 2025 [25].	151

LIST OF FIGURES CONTINUED

21. Figure 2. Viable biofilm density accumulated on coupons 7 days after reactor inoculation. The bars represent the average of 10 coupons across 2 biological replicates (5 coupons per reactor). Error bars represent standard deviation of the same. The grey dashed line represents the limit of detection ($2.08 \log_{10}\text{CFU}/\text{cm}^2$)..... 157
22. Figure 3. Confocal microscopy images of biofilms after 7 days in the SMBR grown A) with no biofilm control methods, B) on Sher-Loxane® 800 coated coupons as the only method of biofilm control, and C) on Sher-Loxane® 800 coated coupons in no-phosphorus medium and daily silver fluoride biocide doses. Viable bacteria are represented in green, non-viable bacteria in red, and fungal biomass in blue/purple/pink depending on the panel. Note: the scale bar in panel A is 30 μm while the scale bars in panels B and C are 20 μm 158
23. Figure 4. Planktonic cell densities of each species in the consortium. The dots are the average of the biological replicates of the reactors and the error bars are the standard deviation between the two biological replicates. The dashed line represents the limit of detection, $1.00 \log_{10}\text{CFU}/\text{mL}$ 160
24. Figure 5. Diameter for every particle detected by the FlowCam, with both replicates plotted together. Note that samples for the experiment without any biofilm control methods (in blue) were imaged using the 4x and 10x flow cells. Larger symbols are used to call attention to the largest particles from each experiment. 161
25. Figure 6. Collages from the sampling day with the largest particles of each experimental iteration captured by the FlowCam. A) Uncoated coupons and walls (first replicate, day 5, 4x magnification) B) Sher-Loxane® 800 coated coupons and walls (second replicate, day 6, 10x magnification), C) Sher-Loxane® 800 coated coupons and walls, no-phosphorus medium, and daily silver fluoride biocide dosing (second replicate, day 7 effluent sample, 10x magnification)..... 162
26. Figure B1. *R. mucilaginosa* single-species biofilm density on uncoated coupons (A), coated coupons (B), and the biofilm log reduction (C). Planktonic cell density for reactors with uncoated coupons (D), coated coupons (E), and the planktonic log reduction (F). Error bars represent 95% confidence intervals..... 196

LIST OF FIGURES CONTINUED

27. Figure B2. Biofilm densities on uncoated and coated coupons for mixed domain biofilm on four substratum materials. Error bars represent 95% confidence intervals.	197
28. Figure B3. Biofilm log reductions of mixed domain biofilm on uncoated versus coated coupons.....	198
29. Figure B4. Mixed domain biofilm on a coated Teflon coupon after 48 h of continuous flow. The yeast and bacteria can be seen closely associated with and attached to one another.	199
30. Figure C1. The coupons and the coatings tested in the CDC reactor. The coupons on the left are Inconel and those on the right are Teflon. The AgXX mesh is stainless steel.	208
31. Figure C2. Topographic heat maps of Inconel coupons.	209
32. Figure C3. Topographic heat maps of Teflon coupons.	210
33. Figure C4. CDC reactor with microscopy slide rods for AgXX evaluation.....	211
34. Figure C5. Total viable biofilm density, excluding <i>C. metallidurans</i> counts due to uncertainty of counts associated with isolation issues on selective agar.	213
35. Figure C6. Relative abundance of species in the viable biofilm of uncoated and Sher-Loxane® 800 coated Teflon coupons. <i>C. metallidurans</i> counts are included in this figure, but their validity is uncertain.....	214
36. Figure C7. Images of biofilm on uncoated Teflon coupons after 7 days of continuous flow. A) Confocal scanning laser microscopy image with the fungal hyphae in blue, viable bacteria in green, and non-viable bacteria in red. B) Field emission scanning electron microscopy image.....	215
37. Figure D1. Timeline of dormancy experiments with inoculation occurring on Day 0. Triangles represent days of planktonic and biofilm sampling. No additional planktonic samples were taken to reduce contamination risk.....	220

LIST OF FIGURES CONTINUED

38. Figure D2. Viable biofilm accumulation during dormancy experiments. Continuous flow and biocide dosing started on Day 1 after the sample. A potable water flush and then initiation of batch conditions took place on Day 7 after the sample to simulate the start of dormancy. After the Day 35 sample, continuous flow and biocide dosing were reinitiated. Colored bars at the top of the graphs correspond to experimental phases presented in Figure D1.....	221
39. Figure D3. Viable planktonic cell density during dormancy experiments. Continuous flow and biocide dosing started on Day 1 after the sample. A potable water flush and then initiation of batch conditions took place on Day 7 after the sample to simulate the start of dormancy. After the Day 35 sample, continuous flow and biocide dosing were reinitiated. Colored bars at the top of the graphs correspond to experimental phases presented in Figure D1.....	222
40. Figure G1. Overview of reactor setup on frame. The reactor in this image features polycarbonate tubing that has been coated with an opaque white coating.	260
41. Figure G2. Flow break in influent tubing.	261
42. Figure G3. Effluent tubing from reactor with spot for oxygen measurements and a y-connector for effluent sampling.	262
43. Figure G4. In-line luer injection port used for biocide dosing. Positioned several inches upstream of influent oxygen sensor spot.	263
44. Figure G5. Disassembled and mostly assembled coupon holder.	264
45. Figure G6. Autoclave tray with reactor parts foiled for sterilization. Before autoclaving, a sheet of foil should be placed over the entire tray.	266
46. Figure G7. Vertical positioning of SMBR in a ring stand for second phase of filling.	269

ABSTRACT

Biofilms are diverse microbial communities that can be attached to surfaces or present in liquid as floating aggregates. Previously, biofilms in the International Space Station (ISS) water recovery system have caused clogs that required part replacement. As spaceflight missions become longer and travel farther from Earth, it will no longer be feasible for biofilm-related issues to be treated with part replacement. To improve spaceflight sustainability and permanence of humans in space, water recovery systems in future spacecraft must be more robust to biofilms and their related issues. The goal of the research in this dissertation is to explore methods of biofilm control for implementation in spacecraft water systems. This was achieved by examining multiple material coatings for their ability to reduce viable biofilm accumulation in normal gravity benchtop experiments. Once it was determined that material coatings offer promise for reducing biofilm accumulation at normal gravity and with a single-species biofilm, additional methods of biofilm control— nutrient limitation and biocide dosing— were incorporated. As the research progressed, the experiments became more accurate to modeling the water recovery system of the ISS by incorporating a consortium of four direct isolates from the system, a medium designed to simulate the fluid present in the wastewater tank, and eventually, simulated microgravity. The coating tested most thoroughly effectively delayed biofilm accumulation for at least 3 days in normal gravity experiments, and its effect was bolstered by the removal of phosphorus (a key nutrient) from the growth medium and daily dosing of silver fluoride biocide. These combined strategies resulted in a 99.9999% reduction in viable biofilm accumulation compared to the experiments without methods of biofilm control. Following the normal gravity experiments, the biofilm control methods were evaluated in a novel simulated microgravity biofilm reactor. With all three methods of control implemented in simulated microgravity, no viable biofilm was recovered from the surfaces nor were viable biofilm aggregates recovered from the bulk fluid in the reactor. These results show that simultaneously using three methods of biofilm control may be promising for managing microbial growth in future spacecraft water recycle systems.

INTRODUCTION

Background

Biofilms are complex communities of microorganisms, frequently associated with surfaces [1, 2]. They are diverse and can consist of bacteria, fungi, protists, algae, and archaea as single-species or as multi-species and multi-domain communities [3]. Many survival advantages are conferred to the microorganisms living in a biofilm morphology including increased resistance to environmental stressors like starvation, desiccation, osmotic lysis, and temperature [4-7]. The organisms within a biofilm are often much more resistant to biocides and antimicrobials like antibiotics [8]. Biofilms are composed of microorganisms and extracellular polymeric substances (EPS), which hold the community together and aid in attachment to surfaces [9, 10]. The EPS consists of nucleic acids, lipids, proteins, and polysaccharides produced by the microorganisms within the biofilm. The sticky nature of the EPS and the advantages that living in a community confer, make biofilms and the cells within them incredibly difficult to mitigate, remove, and kill.

The issues biofilms can cause in spacecraft have been known to astronauts and government space agencies since the MIR space station [11], with microbial tracking taking place on spacecraft as early as the Apollo era [12, 13]. Prior to the inclusion of a water recycle system in the International Space Station (ISS), spacecraft were launched with enough bagged water to last an entire mission or between resupplies. Recently the ISS water recovery system became capable of recovering 98% of the water used on the station [14]. However, biofilms have continued to cause issues with several processes on the ISS, including the water recovery system. In 2009, significant biofilm accumulation in the solenoid valve at the inlet of the mostly liquid

separator and a valve upstream of Russian condensate processor resulted in near total occlusion, requiring part replacement for proper functioning of the water systems (Figure 1) [15, 16]. The water system has been continually updated and redesigned to improve water recovery, water quality, and to address biofilms, with some solutions, such as a 300 μm mesh screen filter, being short-lived for their detrimental enhancement of biofilms [17].

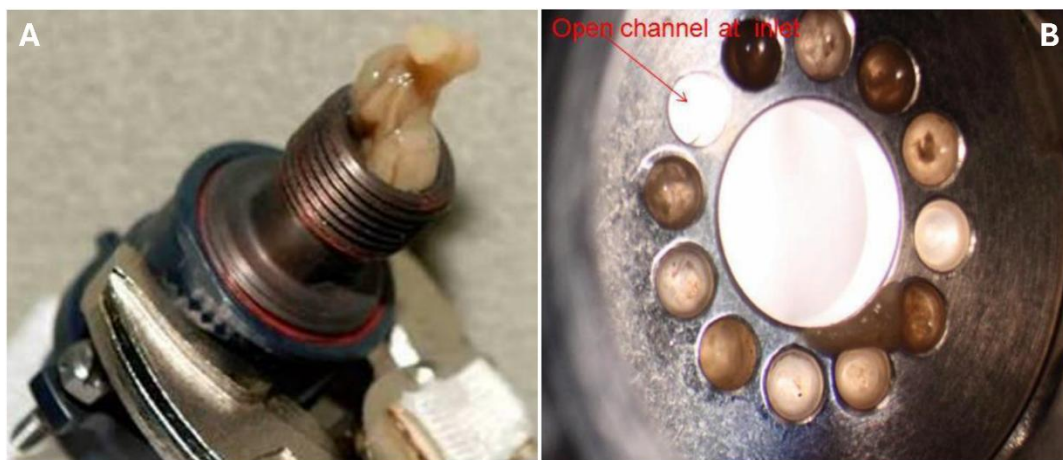


Figure 1. Biofilms that required part replacement in A) the condensate plumbing at the entrance to the Russian condensate processor and B) the solenoid valve in the Mostly Liquid Separator. Figures adapted from Zea et al., 2018 [15] with original credit from NASA.

As the space community anticipates the launch of the Lunar Gateway in 2028 followed by the decommission and de-orbiting of the ISS in 2030, plans must be made for resupply missions to take longer and for space stations to be more independent from Earth than the ISS. For this reason and to increase the sustainability of spaceflight and human presence in space, improvements must be made to water recovery systems in spacecraft such as better control of biofilms. Many suggestions have been made for methods of controlling biofilms in spacecraft water systems [18]. In 2019, the Center for Biofilm Engineering hosted a workshop with multiple biofilm researchers and representatives from the National Aeronautics and Space

Administration (NASA) discussing potential biofilm control options. Many options were explored more in depth in a literature review published in 2020 after that workshop. Several of the ideas explored include the use of biocides, coatings, ionizing radiation (like ultraviolet light), predatory microorganisms, chemical removal of nutrients, heat shock treatments, and mechanical disaggregation via sonication [19]. This dissertation largely investigates the potential of material coatings to control biofilm accumulation in spacecraft water systems alone and in combination with other control methods.

Coatings for microbial control

Coatings aimed at microbial control generally fall into two categories: antimicrobial/biocidal and antifouling/biostatic. Many literature reviews have been published that discuss the various types of coatings, their mechanisms of action, and how to synthesize the coatings, with several referenced here [20-23]. There is little consensus in the field regarding definitions of the terms antimicrobial and antifouling—some researchers use them interchangeably while some researchers use terms like “active” and “passive,” respectively. This can make it difficult to synthesize data and discuss coatings in general. Here, antimicrobial coatings are those that actively seek to damage, inhibit, or kill microbial cells that attach to the coated surface, while antifouling coatings seek to prevent cells from attaching to or settling on the coating typically by altering the surface energy of a surface. The terms and definitions presented above are used throughout this dissertation. Figure 2 shows general mechanisms of action of both antimicrobial and antifouling coatings.

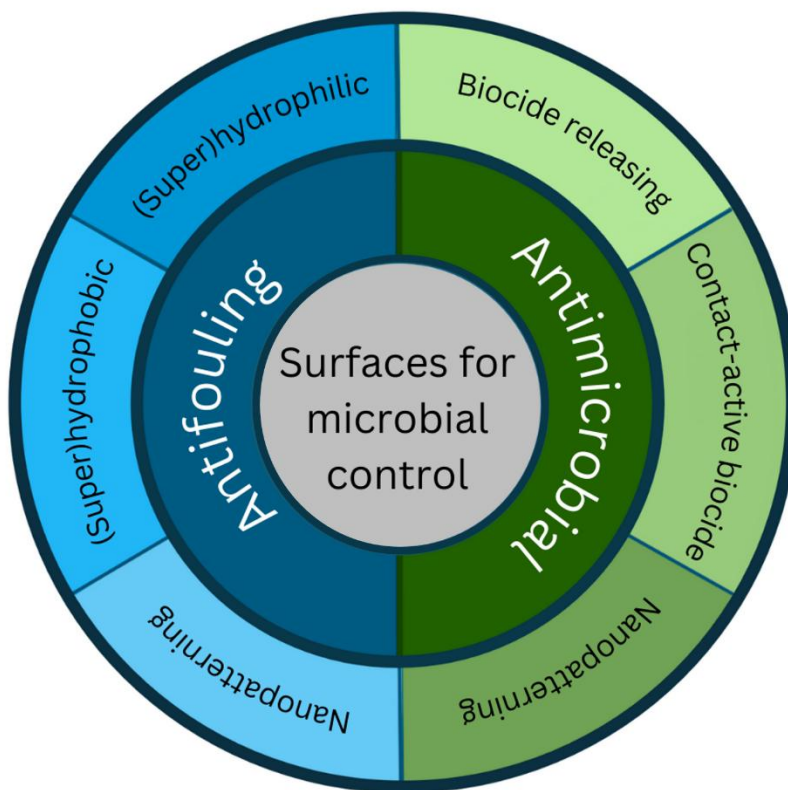


Figure 2. A schematic representing the general categories of material coatings for use in microbial control.

There are several ways antimicrobial coating mechanisms can be achieved, including incorporation of a biocide within the surface which leaches out into the environment to kill cells, biocides that require direct contact with cells, and surface nanopatterning of lances/spikes to lyse cells. A variety of biocides have been incorporated into coatings, from metal oxides like copper and silver, to enzymes and peptides, to antibiotics [24]. One concern regarding antimicrobial coatings is that the active agent can become depleted, leading to an ineffective coating. Recharging antimicrobial coatings is one way to extend their lifetime [25], but this approach is not possible for all chemistries and application environments. Antifouling mechanisms that have been tried include (super)hydrophobic coatings that repel water and microbes (low surface

energy) [26, 27], (super)hydrophilic coatings that strongly attract water to the surface to act as a barrier to microbial colonization [28], and surfaces with nanopatterning, often to mimic naturally resistant materials like shark skin [29] or lotus leaves [30]. It is important to note that nanopatterning falls under both antifouling and antimicrobial mechanisms, which is another artifact of the inconsistencies and lack of consensus with the definition and differences between the two approaches. If the patterning acts to trap and lyse cells, it falls under the definition of antimicrobial used in this dissertation, while nanopatterning which repels cells or prevents attachment would fall under the antifouling definition provided here. Both antimicrobial and antifouling coatings were explored in this dissertation.

Dissertation overview:

Chapter 2 is a literature review entitled “Considerations for testing anti-fouling coatings designed for implementation into Earth-based and spacecraft water systems,” published in *Biofouling* [31]. In the review, eight considerations and recommendations are presented for key components of experimental design when investigating material coatings for biofilm control. Following that discussion, eight studies of coatings for biofilm control in water systems are presented. The methods used within each study are evaluated and a discussion is presented about how the methods may or may not accurately represent coating performance in water systems. This chapter serves as a roadmap for the research and decisions made throughout the remainder of the dissertation.

Chapter 3 is a published manuscript that presents proof-of-concept findings that show promise for using material coatings to control biofilm accumulation in spacecraft water systems. The title of the manuscript is “Antimicrobial Coating Efficacy for Prevention of *Pseudomonas*

aeruginosa Biofilm Growth on ISS Water System Materials,” and published in *Frontiers Microbiology* [32]. The methods used in this chapter are quite general, using a common microbiological nutrient medium, tryptic soy broth, a model biofilm organism, *P. aeruginosa*, and investigating biofilm control on short, 72 h time scales. The results from this chapter indicated that continuing to investigate coatings for biofilm control in spacecraft water systems was a worthwhile endeavor.

Chapter 4 presents the results of a study that greatly improved upon methods used in Chapter 3 to advance the applicability of the experimental results to the ISS wastewater tank. The manuscript is titled “Multimodal Biofilm Control Strategies for Spacecraft Water Systems: Evaluating Coatings, Nutrient Removal, and Biocides for Improved Sustainability,” and was submitted on February 14, 2025 to *Gravitational and Space Research*. The wastewater tank in the ISS was determined to be the target area for biofilm accumulation and the goal for mitigation by researchers at Marshall Space Flight Center. Research presented in this chapter features many of the suggestions and considerations from Chapter 2, including a relevant multidomain consortium of microorganisms, a relevant medium designed specifically to mimic the ISS wastewater tank, performance evaluation that includes viable biofilm density and biofilm morphology, and a longer time scale of 7 days. This chapter also includes two additional methods of biofilm control tested alone, and in combination with coatings, an approach not yet reported in the literature.

Chapter 5 builds on Chapter 4 using system relevant organisms and biofilm control methods, but with a major improvement to modeling the ISS wastewater tank: use of a novel simulated microgravity bioreactor. This is another consideration and suggestion presented in

Chapter 2. Results are presented for a) unmitigated biofilm growth (uncoated surfaces, full strength medium, and no biocide dose), b) biofilm control via a coating as the sole method, and c) biofilm control via the same three strategies used in Chapter 4. Microbial activity was tracked in the reactor using daily planktonic sampling for viable plate counts and particle size to understand clogging potential. Biofilm was sampled at the end of the experiment and evaluated with microscopy techniques and viable plate counts.

Lastly, Chapter 6 presents key conclusions drawn from the three major studies presented as well as recommendations for future research.

Many appendices are included to facilitate interpretation of the data and tell the whole story of the research conducted, including experiments that were not publishable. Appendix A provides data from Chapter 3. Appendix B includes work that investigated the coating from Chapter 3 against a yeast and a mixed domain biofilm. Appendix C explains the preliminary work from Chapter 4, which led to the selection of the coating used in Chapters 4 and 5. Appendix D presents experiments that investigated the coating and biocide dosing as potential biofilm control strategies in spacecraft dormancy conditions. Appendix E gives the data from Chapter 4. Appendices F and G provide the data used for figures in Chapter 5 and the operating procedure for the simulated microgravity reactor, respectively. Lastly, Appendix H lists citations from additional publications from graduate school not related to this dissertation.

Funding and collaborations:

There were many funding sources that supported the research presented in this dissertation. Funding for research, tuition, and/or stipend was provided by the Jet Propulsion Laboratory, Montana Space Grant Consortium, NASA EPSCoR, and the Raymond and Erin

Schultz Fellowship. Funding for conference travel was supported by Montana State University Professional Advancement Grants, American Society for Gravitational and Space Research student travel awards, and the International Space Education Board.

Research collaborators included researchers from the Jet Propulsion Laboratory Planetary Protection group and researchers within the Center for Biofilm Engineering who collaborated on research related to the NASA EPSCoR funding.

References

1. Donlan RM. Biofilms: microbial life on surfaces. *Emerg Infect Dis.* 2002;8(9):881-90; doi: 10.3201/eid0809.020063.
2. Flemming H-C, Wuertz S. Bacteria and archaea on Earth and their abundance in biofilms. *Nature Reviews Microbiology.* 2019;17(4):247-60; doi: 10.1038/s41579-019-0158-9.
3. Penesyan A, Paulsen IT, Kjelleberg S, Gillings MR. Three faces of biofilms: a microbial lifestyle, a nascent multicellular organism, and an incubator for diversity. *npj Biofilms Microbomes.* 2021;7(1):80; doi: 10.1038/s41522-021-00251-2.
4. Characklis WG, Cooksey KE. Biofilms and Microbial Fouling. In: Laskin AI, editor. *Advances in Applied Microbiology.* Academic Press; 1983. p. 93-138.
5. Esbelin J, Santos T, Hébraud M. Desiccation: An environmental and food industry stress that bacteria commonly face. *Food Microbiology.* 2018;69:82-8; doi: <https://doi.org/10.1016/j.fm.2017.07.017>.
6. Parrilli E, Tedesco P, Fondi M, Tutino ML, Lo Giudice A, de Pascale D, et al. The art of adapting to extreme environments: The model system *Pseudoalteromonas*. *Physics of Life Reviews.* 2021;36:137-61; doi: <https://doi.org/10.1016/j.plrev.2019.04.003>.
7. Yin W, Wang Y, Liu L, He J. Biofilms: The Microbial "Protective Clothing" in Extreme Environments. *Int J Mol Sci.* 2019;20(14); doi: 10.3390/ijms20143423.
8. Donlan RM. Biofilm Formation: A Clinically Relevant Microbiological Process. *Clinical Infectious Diseases.* 2001;33(8):1387-92; doi: 10.1086/322972.
9. Flemming H-C, Neu TR, Wozniak DJ. The EPS Matrix: The “House of Biofilm Cells”. *Journal of Bacteriology.* 2007;189(22):7945-7; doi: 10.1128/jb.00858-07.
10. Flemming H-C, Wingender J. The biofilm matrix. *Nature Reviews Microbiology.* 2010;8(9):623-33; doi: 10.1038/nrmicro2415.
11. Novikova ND. Review of the Knowledge of Microbial Contamination of the Russian Manned Spacecraft. *Microbial Ecology.* 2004;47(2):127-32; doi: 10.1007/s00248-003-1055-2.
12. Puleo JR, Fields ND, Moore B, Graves RC. Microbial contamination associated with the Apollo 6 spacecraft during final assembly and testing. *Space life sciences.* 1970;2(1):48-56; doi: 10.1007/BF00928955.

13. Puleo JR, Oxborrow GS, Fields ND, Herring CM, Smith LS. Microbiological profiles of four Apollo spacecraft. *Appl Microbiol.* 1973;26(6):838-45; doi: 10.1128/am.26.6.838-845.1973.
14. Gaskill ML. NASA Achieves Water Recovery Milestone on International Space Station. 2023.
15. Zea L, Nisar Z, Rubin P, Cortesão M, Luo J, McBride SA, et al. Design of a spaceflight biofilm experiment. *Acta Astronautica.* 2018;148:294-300; doi: <https://doi.org/10.1016/j.actaastro.2018.04.039>.
16. Carter DL: Status of the Regenerative ECLSS Water Recovery System. In: International Conference of Environmental Systems. Barcelona, Spain; 2010.
17. Weir N, Wilson M, Yoets A, Molina T, Bruce R, Carter L: Microbiological characterization of the international space station water processor assembly external filter assembly S/N 01. In: 42nd international conference on environmental systems. 2012: 3595.
18. Zea L, McLean RJC, Rook TA, Angle G, Carter DL, Delegard A, et al. Potential biofilm control strategies for extended spaceflight missions. *Biofilm.* 2020;2:100026; doi: <https://doi.org/10.1016/j.biofilm.2020.100026>.
19. Velez Y-A, Carter D, Nur M, Angle G: Developing Methods for Biofilm Control in Microgravity for a Water Recovery System. In.: 2020 International Conference on Environmental Systems; 2020.
20. Song B, Zhang E, Han X, Zhu H, Shi Y, Cao Z. Engineering and Application Perspectives on Designing an Antimicrobial Surface. *ACS Appl Mater Interfaces.* 2020;12(19):21330-41; doi: 10.1021/acsami.9b19992.
21. Mahanta U, Khandelwal M, Deshpande AS. Antimicrobial surfaces: a review of synthetic approaches, applicability and outlook. *J Mater Sci.* 2021;56(32):17915-41; doi: 10.1007/s10853-021-06404-0.
22. Balaure PC, Grumezescu AM. Recent Advances in Surface Nanoengineering for Biofilm Prevention and Control. Part I: Molecular Basis of Biofilm Recalcitrance. Passive Anti-Biofouling Nanocoatings. *Nanomaterials.* 2020;10(6):1230.
23. Balaure PC, Grumezescu AM. Recent Advances in Surface Nanoengineering for Biofilm Prevention and Control. Part II: Active, Combined Active and Passive, and Smart Bacteria-Responsive Antibiofilm Nanocoatings. *Nanomaterials.* 2020;10(8):1527.

24. Cloutier M, Mantovani D, Rosei F. Antibacterial Coatings: Challenges, Perspectives, and Opportunities. *Trends in Biotechnology*. 2015;33(11):637-52; doi: 10.1016/j.tibtech.2015.09.002.
25. Demir B, Taylor A, Broughton RM, Huang TS, Bozack MJ, Worley SD. N-halamine surface coating for mitigation of biofilm and microbial contamination in water systems for space travel. *Biofilm*. 2022;4:100076; doi: <https://doi.org/10.1016/j.bioflm.2022.100076>.
26. Li Sip YY, Jacobs A, Morales A, Sun M, Roberson LB, Hummerick ME, et al. Slippery lubricant-infused silica nanoparticulate film processing for anti-biofouling applications. *J Appl Biomater Funct Mater*. 2023;21:22808000231184688; doi: 10.1177/22808000231184688.
27. Flores P, McBride SA, Galazka JM, Varanasi KK, Zea L. Biofilm formation of *Pseudomonas aeruginosa* in spaceflight is minimized on lubricant impregnated surfaces. *NPJ Microgravity*. 2023;9(1):66; doi: 10.1038/s41526-023-00316-w.
28. Cheng G, Zhang Z, Chen S, Bryers JD, Jiang S. Inhibition of bacterial adhesion and biofilm formation on zwitterionic surfaces. *Biomaterials*. 2007;28(29):4192-9; doi: <https://doi.org/10.1016/j.biomaterials.2007.05.041>.
29. Dundar Arisoy F, Kolewe KW, Homyak B, Kurtz IS, Schiffman JD, Watkins JJ. Bioinspired Photocatalytic Shark-Skin Surfaces with Antibacterial and Antifouling Activity via Nanoimprint Lithography. *ACS Applied Materials & Interfaces*. 2018;10(23):20055-63; doi: 10.1021/acsami.8b05066.
30. Jiang R, Hao L, Song L, Tian L, Fan Y, Zhao J, et al. Lotus-leaf-inspired hierarchical structured surface with non-fouling and mechanical bactericidal performances. *Chemical Engineering Journal*. 2020;398:125609; doi: <https://doi.org/10.1016/j.cej.2020.125609>.
31. Mettler MK, J. E-OE, M. GD, and Peyton BM. Considerations for testing anti-fouling coatings designed for implementation into Earth-based and spacecraft water systems. *Biofouling*. 2025:1-19; doi: 10.1080/08927014.2025.2479692.
32. Mettler MK, Parker CW, Venkateswaran K, Peyton BM. Antimicrobial Coating Efficacy for Prevention of *Pseudomonas aeruginosa* Biofilm Growth on ISS Water System Materials. *Front Microbiol*. 2022;13; doi: 10.3389/fmicb.2022.874236.

CHAPTER TWO

CONSIDERATIONS FOR TESTING ANTI-FOULING
COATINGS DESIGNED FOR IMPLEMENTATION INTO
EARTH-BASED AND SPACECRAFT WATER SYSTEMS

Contribution of Authors and Co-Authors

Manuscript in Chapter 2

Author: Madelyn K. Mettler

Contributions: Determined literature review concept, created figures, and drafted manuscript.

Co-Author: Erika J. Espinosa-Ortiz

Contributions: Drafted simulated microgravity section and edited manuscript.

Co-Author: Darla M. Goeres

Contributions: Determined organization of manuscript, drafted experiment duration and performance evaluation sections, and edited manuscript.

Co-Author: Brent M. Peyton

Contributions: Determined literature review concept and edited manuscript.

Manuscript Information

Madelyn K. Mettler, Erika J. Espinosa-Ortiz, Darla M. Goeres, and Brent M. Peyton

Biofouling

Status of Manuscript:

- ☐ Prepared for submission to a peer-reviewed journal
- ☐ Officially submitted to a peer-reviewed journal
- ☐ Accepted by a peer-reviewed journal
- ☒ Published in a peer-reviewed journal

Taylor & Francis

10.1080/08927014.2025.2479692

Abstract

Biofilms are common in water systems and can lead to mechanical failure or illness of water system users. Methods for evaluating anti-fouling coatings have largely been informed by the medical industry and have not been tailored to industrial or spacecraft water systems. The goal of the paper is to help guide researchers in designing experiments to evaluate coatings that accurately represent the system under investigation. This review identified eight experimental design considerations when evaluating coatings in water systems: biofilm reactor operation, microorganism selection, reinoculation, coating surface area, liquid medium, experiment duration, coating performance evaluation, and the use of microgravity. The impact of each decision made within each of these considerations is presented. Further, the methods featured in eight studies investigating coatings for Earth-based or spacecraft water systems are discussed. This review serves to guide researchers toward improved experimental design to enable successful technology transfer from the lab bench to Earth and beyond.

Introduction

Biofilm related mechanical failures such as cooling tower biofouling [1], occlusion of pipes and irrigation systems [2, 3], and microbially induced corrosion (MIC) of pipes in industrial and residential water systems are common [4]. These failures often require repair or replacement of the damaged parts, leading to high operation costs. A 2019 study estimated the global market of biofilms in water distribution systems to be \$90.4 billion [5]. Unfortunately, the estimate did not specify between the cost of damage caused by biofilms and the cost associated

with preventing these issues. Further, biofouling of wastewater systems, drinking water distribution systems (DWDS), and industrial water systems can lead to health issues via consumption of fouled water or exposure to fouled water (such as aerosols produced by showers and flushing toilets), as has been observed in hospitals and residential homes [6, 7]. Lockdowns during the COVID-19 pandemic brought new concerns about biofouling in corporate water systems, especially concerning *Legionella pneumophila* [8]. Additionally, the amoeba *Naegleria fowleri* has been listed on the Environmental Protection Agency's (EPA) contaminant candidate list 5 and causes yearly fatalities from primary amoebic meningoencephalitis [9-13]. Biofilms in water systems are known to act as reservoirs for *N. fowleri* and other pathogenic free-living amoeba [14]. Controlling biofilms and therefore reducing reservoirs for pathogens in DWDS, cooling towers, and other industrial water systems could reduce pathogenic risks to human health [6].

The issues caused by biofilms in water systems are not limited to Earth-based settings. Several mechanical failures have resulted from biofilm growth in the International Space Station (ISS) water system [15, 16]. Despite some successful applications of coatings for biofilm and microbial control on Earth, their application in spacecraft has been limited. Many of the coatings and surface modifications that have been tested were for high-touch surfaces such as the bathroom door and walls of the ISS [17-20]. Less attention has been given to coatings for biofouling control in spacecraft water systems. With the impending retirement of the ISS, the National Aeronautics and Space Administration (NASA) is partnering with other government space agencies and private industry to maintain permanent presence in space with the Lunar Gateway. The station will orbit the moon and is planned to cycle through periods of uncrewed

dormancy [22]. Biofilm control methods such as coatings must be evaluated and incorporated to ensure the most effective water system design for Gateway and future spacecraft that may eventually take humans to Mars. Functional water systems are critical to mission success, and coatings could play a key role in biofilm control.

It is difficult to accurately mimic real-world conditions in the laboratory, especially for space travel applications, which is why designing the “perfect experiment” to test a coating is unlikely. However, researchers must strive to accurately replicate key aspects of a technology’s final implementation conditions. This seems to have been largely disregarded in testing coatings for water systems as many of the methods, organisms, metrics, and timelines appear to be driven by the historical use of coatings in the medical device industry. When thinking about spacecraft water systems, the ISS informs what conditions may exist in future systems. There are vast changes in chemistry, temperature, oxygen availability, materials, and other factors within the ISS water recovery system [23]. It is important to consider that one coating will not fit all when it comes to biofilm control. Material coatings that are effective in DWDS may not be effective in wastewater systems. The methods for testing coatings for implementation into DWDS and wastewater systems should account for their intrinsic differences.

In the first half of this review, eight important factors to consider when evaluating coatings and surface modifications for application in Earth-based and spacecraft water systems are presented (Figure 1). These considerations include biofilm reactor operation, microorganism selection, reinoculation, coating coverage, medium selection, experiment duration/pre-aging of surfaces, performance evaluation, and the use of (simulated) microgravity. It is difficult to prioritize their importance, so these considerations are presented randomly. It is not the goal of

this review to discuss the state of anti-biofilm coating technologies. Thorough reviews discussing many types of coatings and how well they are suited for various applications are available [22-25], including several specifically focused on coatings for use in spacecraft [26, 27].

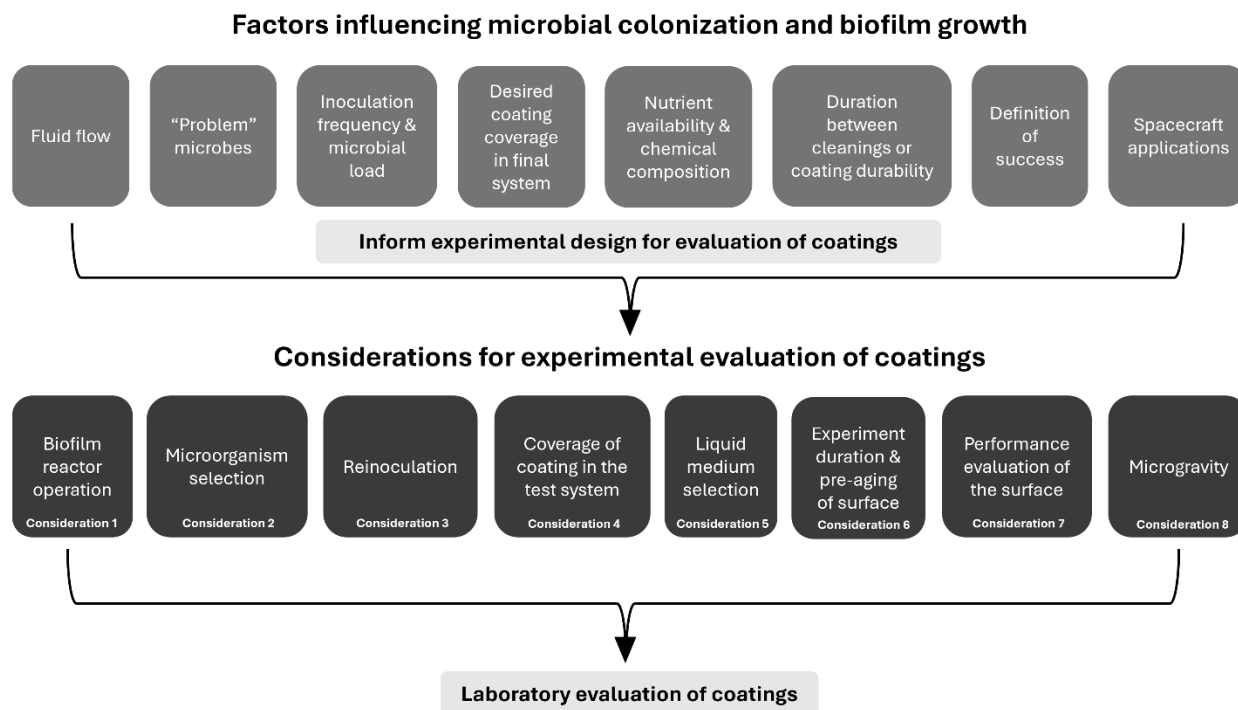


Figure 1. Schematic representing important properties of real water systems that should be considered for informed design of laboratory experiments for coating evaluation.

Despite the vast literature focused on the use of coatings for biofilm control, to the best of the authors' knowledge, only a few studies specifically list water systems as the desired application for coatings. The second part of this review details the variety of methods that have been used for evaluating coatings for biofilm control in wastewater systems, DWDS, and water devices with several studies focusing on Earth-based implementation and several focusing on spacecraft applications. The advantages and drawbacks of approaches used so far are presented and methods to improve evaluation of anti-biofilm coatings for anthropogenic water systems are

suggested. The goal of this document is to guide researchers through the experimental design process for the most accurate evaluation of coatings for use in industrial, residential, wastewater, and spacecraft water systems.

Considerations for experimental evaluation of coatings

Consideration 1: Biofilm reactor operation

Nearly all submerged environments exist in one of four conditions: always submerged without refreshing the liquid (batch), always submerged with constant exchange of fresh liquid with spent liquid (continuous flow), always submerged with cyclical batch and continuous flow periods (semi-batch), or cyclical submersion with the spent liquid leaving the system for a duration of time known as dryout before new liquid enters (flush and fill). In terms of microbial control, knowing which of these operation options is most representative of the final implementation target is key for accuracy of laboratory tests.

True batch conditions are unlikely to exist in water systems, and if they do, it is doubtful that coatings are the best option for microbial control. A batch process implies that any microbes in the system are only introduced at the start when the vessel is filled and grow in the vessel from the initial inoculum. In this case, a biocide or antibiotic dose would likely be sufficient for killing the inoculum and preventing further growth in the system. When batch tests are used to evaluate coatings that will be implemented in continuous flow, semi-batch, or flush and fill systems, their performance can be greatly misrepresented. For coatings that leach biocides as a mechanism of action, the biocide can leach into the supernatant and kill or inhibit the initial microbial inoculum. This can easily yield “promising” results of low or no viable biofilm, or planktonic cells detected. However, in real-world applications if the water system is not truly a batch

system, the leachate can be washed away with little residual effects at the coating surface, resulting in an ineffective coating when new liquid and microorganisms are introduced to the system.

Continuous flow, semi-batch, or flush and fill test methods likely better mimic the real-world systems the experiment is attempting to replicate and should be used in coating evaluation. The biofilm reactor system (and its inherent fluid dynamics) chosen for evaluating coatings must consider the fluid flow in the target system as this parameter could be highly influential on biofilm formation [30-32] and therefore coating success or failure.

Consideration 2: Microorganism selection

Microbiologists often utilize model species for focused and fundamental scientific studies. However, model organisms are probably not the most relevant for applied studies of coating efficacy. *Pseudomonas aeruginosa* has long been used as a model organism by biofilm researchers [28-31] and continues to be used for coating research in medical and water system applications [32-39]. This organism has and will likely continue to function as a baseline for comparing results between studies; however, the range of organisms studied should also be expanded to create more complex systems. Researchers investigating coatings for use on medical devices often use organisms belonging to the ESKAPEE group (*Escherichia faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, *Enterobacter* spp. and *E. coli*) and several of these organisms have been carried over for research in other coating applications, including those discussed in this review [40-42]. However, these organisms may not be the most relevant for water systems, depending on the ultimate reason for controlling biofilm. Additionally, it is imperative to test surfaces against multiple organisms of

varying morphology and physiology. Several researchers across different industries (and some included in this review) have tested coatings against one Gram-positive bacterium and one Gram-negative bacterium in their experiments, which is an improvement over a single model organism [43-45]. The composition of cell walls is vastly different between these organisms, and based on the coating's mechanism of action this can result in a successful or failed coating [51]. Furthermore, fungi are prevalent in water system biofilms [52], but they have not been addressed in any of the coating research reviewed here for spacecraft or Earth-based applications. During 10 years of sampling the ISS wastewater tank, portable water bus, and condensate, fungal species have been isolated multiple times. These organisms are significant, warrant further exploration [53], and should be included in studies on biofilm control in spacecraft water systems.

Ideally, multiple organisms should be tested in consortium in case beneficial relationships amongst the microbes overcome a coating's biofilm control mechanism. It is not feasible to laboratory test every single organism that may exist in the real-world system, but a selection of multiple relevant organisms is key. Namely, researchers should consider additional microbial-associated issues caused by potential biofilms in their system and include microorganisms such as metal reducers, oligotrophs, and extremophiles pertinent to the water system. Using microbial isolates from the target system is a method that researchers can use to address several of these concerns.

The organisms on the ISS are largely reported based on culturing of liquid samples followed by sequencing techniques. Some of the commonly isolated genera in the ISS water system (potable and wastewater) are *Ralstonia*, *Burkholderia*, *Cupriavidus*, *Sphingomonas*, *Cryptococcus*, *Curvibacter*, *Mesorhizobium*, *Methylobacterium*, and *Chryseobacterium* [53, 54].

Due to the nature of the water system on the ISS, the isolates have all come from bulk fluid, so the correct composition of the biofilms is unknown. The main concerns with biofilms in Earth-based water systems are their ability to damage water system materials and their potential for harboring human pathogens. These concerns indicate that coating efficacy should be tested against relevant microorganisms that could enhance biofilm formation or pathogenicity. A comprehensive list of waterborne pathogens and “key microbes” that could be targeted for their role in biofilm formation are listed in Simões and Simões (2013) [55].

It is reasonable for coatings and other biofilm mitigation strategies to be screened against one organism (likely *P. aeruginosa*) before experiments are expanded to include more organisms. These organisms should include those that contribute the most cell density/biomass and those that could contribute to other microbial issues such as corrosion. Screening tests of multiple coating formulations may be used to determine the best coating composition with which to move forward. However, it must be taken into consideration that a coating which does not work against a single organism could perform well against other target organisms. Alternatively, it is highly likely that a coating that works well against a pure culture may have limited control against a mixed culture, multi-domain (fungal and bacterial) biofilm. Starting with a simpler experiment before expanding into a more relevant test is an approach that applies to many considerations presented in this review.

Overall, it is important to test additional relevant combinations of organisms, and not stop after promising results are obtained from a single species experiment. One strategy that could be employed is to change experimental parameters until the tested coating or biofilm mitigation

method fails. Ultimately, it should be a goal to select microorganisms most relevant to the target system for evaluating the ability of a coating to reduce biofilm accumulation.

Consideration 3: Reinoculation

In addition to the biofilm reactor operation and microorganism selection, the method and frequency of experiment inoculation may have a significant impact on biofilm formation [49] and therefore observed anti-biofilm coating efficacy. The density of inoculum can also affect biofilm formation and morphology [57-59]. In laboratory experiments evaluating coating efficacy against biofilms in water systems, researchers often inoculate reactors with high cell densities, typically ranging from 10^4 to 10^8 colony-forming units (CFUs) per milliliter [38-41, 60]. These elevated densities are commonly used to accelerate biofilm development and to simulate worst-case scenarios, as is done in multiple American Society for Testing and Materials (ASTM) standard methods [61-63]. However, such densities are considerably higher than those typically found in actual drinking water systems. For instance, biofilms developed under chlorinated surface water supply exhibited lower cell densities compared to those resulting from treated groundwater [64]. This discrepancy suggests that laboratory conditions may not accurately reflect the natural biofilm dynamics present in water systems, especially DWDS which have lower microbial loads. Consequently, bench experiments conducted at these elevated cell densities may not fully represent the conditions encountered in real-world scenarios. Therefore, while these high-density inoculations are useful for assessing the maximum potential of coating materials, they may not provide a precise evaluation of their performance under typical operational conditions.

In the ISS, the water system is reinoculated with all condensed humidity and every time the toilet is used. In Earth-based water systems, reinoculation is even more frequent from air exchange, rain, flushing of toilets, and water release from a treatment plant, etc. This inoculation frequency is rarely reproduced in the laboratory and may be key for evaluating biofilm control coatings in continuous flow, semi-batch, and flush and fill experiments. As described earlier, coatings are not the best approach to control biofilms in true batch conditions and therefore batch experiments should not be used for coating evaluation.

For continuous flow systems, the initial inoculum can be washed out if the organisms are not given time to attach to the surface and establish in the system or if the dilution rate is faster than the specific growth rate of the organism [59, 65, 66]. Additionally, with leaching coatings, continuous flow can quickly wash away the inhibitory mechanism of the coating, and after the disappearance of the coating, secondary inoculations may lead to biofilm formation. Microbial washout is less of a concern with semi-batch and flush and fill systems, assuming the first continuous flow cycle or flush occurs after a period of a few hours. Washout concerns are addressed in the ASTM standard methods for the Center for Disease Control (CDC) biofilm reactor and the drip flow reactor which both feature a (no flow) batch phase of 24 hours and 6 hours, respectively, prior to starting continuous flow [61, 62]. In addition to operating in a temporary batch mode, reinoculation is another method of addressing cell washout.

An additional consideration to note is that in essentially every apparatus used to test coatings, there is uncoated surface area present where biofilms can accumulate. Biofilm detachment from uncoated surfaces can serve as unintentional reinoculation to further challenge coated surfaces. This is especially true in continuous flow systems where fresh nutrients are

introduced, thus giving newly detached biofilm (and planktonic cells) the best chance at colonizing coated surfaces. While reinoculation from uncoated surfaces in the test apparatus can be beneficial, it is likely that the final coating implementation in a water system will feature close to 100% surface coverage by the coating. It is important to consider the impact that the coated surface area can have on the coating performance.

Since reinoculation is a frequent and natural occurrence in Earth-based and spacecraft water systems, it should be included in experiments and involve the same relevant microbes used in the initial inoculation. These may include oligotrophs, isolates from the target system, and secondary organisms that could promote biofilm formation [61]. The incorporation of reinoculation will continuously challenge the coated surface as would occur in the target system and could greatly alter results compared to experiments with a single inoculation event where the initial inoculum died, leading to convincing results of an effective coating.

Consideration 4: Coverage of the coating in the test system

In all the papers discussed later in this review, none tested the coating as the only surface exposed to liquid medium and microorganisms during evaluation of biofilm control. The coating is often evaluated on a small piece of test material in a larger apparatus of uncoated material. While it is desirable to test the coatings with repeated inoculations, it would be best to do so in a controlled manner and not from uncoated surfaces within the test apparatus. The difficulty in testing a coating with it being the only surface exposed to liquid medium is the effort and cost associated with coating an entire test apparatus. Material and coating compatibility problems may arise, especially with material modifications such as nanopatterning rather than paint-like coatings. These issues could make it impossible to coat an apparatus like a 6-well plate or CDC

reactor, adding time and cost to the experimental design and testing process. However, again, taking the percentage of coated surface into consideration will likely save far more money and time when it comes to the real-world application.

A closely related consideration is the coated surface area to liquid volume ratio or how much coated surface area is used to treat a unit volume of media and cell culture. In a CDC reactor, the liquid media used is about 325 mL being exposed to at most 97.3 cm² of coated coupons, for a A/V ratio of 0.3 cm² /mL [39], while in the JIS Z 2801/ISO 22196 standard method there is only about 200 µL of cell culture exposed to a coated surface area of about 16 cm² for a drastically different A/V ratio of 80 cm² /mL [52, 53], approximately 266 times greater surface area per liquid volume than the CDC reactor. These two examples highlight the vast differences in biofilm evaluation methods. Biocide efficacy depends on the concentration of the active and contact time. Testing a leaching coating in a small volume of diluent results in a high leachate concentration, whereas using a large volume of diluent will dilute the leachate, thereby impacting the measured efficacy.

Both the coating surface area coverage and the surface area to volume ratio must be considered in the experimental system and should be implemented in similar ratios as would exist in the target system. With higher coated to uncoated surface area ratios, there are fewer uncoated surfaces that can act as reinoculation points for the system. Further, it is quite likely that some currently used experimental systems have much higher surface area to volume ratios than the eventual target system which could greatly sway results to indicate a better coating performance in the laboratory than would occur otherwise.

Consideration 5: Liquid medium selection

Just as it is unreasonable for human prosthetics researchers to use simulated wastewater to test coatings on pacemakers, it is unreasonable for researchers studying coatings for water systems to use human serum for biofilm tests. As discussed by Mettler et al., it is possible for coatings to perform well in one medium and poorly in another [39]. While it was a good approach to test the coating in two different media (tryptic soy broth and potato dextrose broth) the coating tested was meant for implementation into the ISS water system. It would have been more appropriate to test the coatings in simulated ISS wastewater or simulated potable water. The medium for testing coatings can be crucial, especially as the implemented coating environment becomes more “extreme,” which can include particularly harsh chemical challenges, high salinity, high nutrient availability, and non-neutral pH. These aspects can be detrimental to coatings while potentially benefiting microorganisms in a biofilm. Further, in mixed culture applications, these extreme conditions may select for extremophiles. In general, limited previous results suggest that the liquid medium should closely represent the composition of the target system for best coating implementation.

Common choices are high-nutrient, general purpose media such as Luria-Bertani (LB) broth and tryptic soy broth (TSB) [32, 34, 35, 37, 52, 54]. These media can act to simulate nutrient-rich wastewater and they will accelerate biofilm accumulation compared to lower nutrient media such as potable water. The acceleration of biofilm accumulation is an attractive attribute for their use, but these media fail to represent some of the intricacies of specific nutrient concentrations and pH values in target water systems. The relevance (and nutrient density) of the selected medium is closely correlated with the rate at which biofilm accumulates and therefore the required duration of experiments.

With all the considerations presented, researchers should strive to make selections that are most relevant to the target system. This will ensure coatings are more accurately evaluated. Traditional media may not be the most appropriate choice, and their use could hinder or improve coating performance, misrepresenting the coating efficacy. Fluctuations in water quality, nutrient availability, chemical composition, etc. that could exist in the target system also suggest that it may be best practice to test coatings in several media formulations. Researchers investigating biofilms in spacecraft water systems may be interested in a medium called microbial ersatz was developed by Marshall Space Flight Center and the Center for Biofilm Engineering based on “worst case scenario” nutrient and chemical concentrations in the ISS wastewater tank [69]. The reported carbon concentrations in the wastewater that informed the design of microbial ersatz were 18.9 mg/L total inorganic carbon and 43.9 mg/L total organic carbon. Synthetic potable water recipes are also available for Earth-based systems [70, 71] and tap/drinking water can be sterilized for use as media, though this can result in issues with standardization. To evaluate closeness to true drinking water, researchers should look to regulations for their location regarding water quality standards. In the United States, researchers can refer to the EPA guidelines, but should note that regulations are presented on a per-chemical basis, and not by total organic carbon [72].

Consideration 6: Experiment

duration & pre-aging of surface

Coatings are often positioned as long-term, low-maintenance methods of biofilm control. In practice, it is unlikely the coating will be reapplied to pipes, water storage tanks, valves, devices, etc., such that coatings and antimicrobial surfaces will likely be in place for the lifetime

of the equipment, especially when used in spacecraft. With time, coatings can degrade [73], conditioning films of extracellular products, dead microbes, or nonbiological deposits can form [74], potentially conferring “chemical properties on the surface of the substratum that may completely mask the properties of the underlying substratum itself” [75], and mechanical damage to a coating can allow access of water and microbes to the substratum [76, 77]. However, when researchers conduct benchtop testing to determine the efficacy of a coating intended for long term use, the experiments often last a few days, to a week at most [37-42, 68]. Field scale studies that monitor a surface coating over its lifetime are rare and costly, and while it is feasible to collect and assess samples of the coated surface once the equipment is decommissioned, this is often not done.

Ideally, the duration of a benchtop experiment is long enough to provide convincing evidence that the coating will perform satisfactorily in a field scale study. The benchtop design should consider if the coated surface will be used in conjunction with other control measures. In this case, the time frame is based upon the time between clean-in-place strategies, not the length of time that the equipment will be in use. Another strategy is to continue the efficacy test until the surface “fails,” which generally is interpreted to mean that the measurement of interest, for instance total number of viable microbes or total protein accumulation, is statistically equivalent for the coated and uncoated control surface.

Surfaces/coatings can be pre-aged prior to their experimental use to perhaps better represent a relevant test duration. Pre-aging may consist of preparing the coating on the surface weeks to months in advance of the benchtop test [78], storing the prepared surfaces in sterile testing medium, exposing the surface to temperature and humidity cycling, radiation exposure,

pH cycling, etc. This can help researchers conduct shorter experiments while capturing some of the realities of long-term implementation. Another approach is developing an accelerated test design such as those used in the cosmetic industry to test preservatives, or to pair efficacy testing with durability testing that artificially wears the surface in between efficacy tests [79, 80]. In this case, the coated surface is exposed to “cleaning” using a device such as an abrasion tester, also known as a Gardner scrubber [79]. Similar to durability testing with a Gardner scrubber, another approach is to artificially scratch the coated surface before the efficacy testing.

The surfaces and materials of most relevance for the ISS water system include stainless steel 316, Inconel 718, titanium, and Teflon [81]. For Earth-based systems, some examples of relevant plumbing materials are stainless steel, polypropylene, PVC-C, PVC-U, PVC-P, mild steel, cement-based materials, cast iron, copper, latex, PE-Xc, PE-Xb, and PE-100 [82-85]. Pre-aging of the surfaces should take place after the coating is applied. For systems where coatings may be applied retroactively, the substratum should be pre-aged in the laboratory prior to coating application. Pre-aging approaches such as exposure to pH cycling, physical abrasion, and temperature cycling could be of use. Temperatures vary along water distribution processes on Earth due to weather, depth of pipes, and the use of water heaters in the dispensing process [86]. Typically, drinking water temperatures at the tap (on cold) are less than 25 °C which falls in line with World Health Organization guidelines [87-89]. Experiments investigating biofilm growth in water systems should likely take place near or just below 25 °C. Lower temperatures could be used, but microbial growth will likely be inhibited. On the ISS, the potable water dispenser (PWD) can dispense water at ambient temperatures (18-51 °C) or hot temperatures (66-93 °C), with most water purification procedures occurring at ambient temperatures [90]. Cycling

between the high and low ends of the temperature spectrum could be beneficial in evaluating coating performance for applications with large temperature swings.

Another concern with biofouling is material degradation such as corrosion because of unchecked biofilm accumulation. Many coatings have been developed for corrosion protection [91, 92] and the use of non-corrosive materials such as PVC has increased in water systems. A goal for some material coatings is to protect against microbially induced corrosion (MIC). Some pre-aging of the coating would likely provide more realistic results, and microbial testing should include factors known to increase MIC such as intentionally varying oxygen availability [93], addition of metal oxides to the reactor/medium, and introduction of corrosive metabolites such as organic acids or sulfur species [94].

The duration of experiments is a key consideration, especially due to the exponential growth capabilities of biofilms and potentially limited coating lifetimes. Overall, reported coating evaluation experiments have all been of such short duration that the exponential growth potential may have been cut short. It is imperative that experiments are conducted on more appropriate timescales, which are typically greater than 24-72 h.

Consideration 7: Performance evaluation of the surface

Antimicrobial coating technologies designed for biofilms may be assessed by three criteria: kill of viable cells, prevention of initial surface attachment of microorganisms or reduction of biomass. The criteria and experimental data used to meet the criteria must support informed decision making that reduces risk. Successful control of biofilms and microorganisms is often evaluated based on reduction in viable cell attachment and growth, as assessed by viable plate counts. This metric makes sense in applications of antibiotics, biocides, and coatings in

high-touch areas like hospital door handles. However, a significant log reduction in viable microorganisms in a water system does not fully capture other important aspects of the biofouling problems such as microbial corrosion [95] and clogging potential of dead biomass [15]. Limitations exist with viable plate counts due to potential presence of viable but not culturable cells and injured cells that are able to recover [96]. Additionally, viable plate counts do not assess removal of biomass, which live or dead, can detach from a coated surface and move farther downstream in the water system to cause clogging elsewhere. These issues are especially relevant in spacecraft water systems where tube diameters are typically smaller than Earth-based systems, the water system is less accessible for regular maintenance and repair, and there are not high-shear fluid dynamics leading to regular detachment of small biofilm aggregates. Since biofilms consist of microbes, extracellular polymeric substances (EPS), and nonbiological deposits, reduction in viable cells does not capture the clogging potential of biofilms and biomass in water systems. Alternatives to viable plate counts for quantifying biofilm presence include crystal violet assays and microscopy techniques [97]. Depending on the approach, these techniques can allow for assessment of removal and an understanding of the non-viable cells present in a biofilm (using specific stains). However, with microscopy techniques, there are questions regarding the capture of a representative field of view, providing a quantitative assessment, and standardizing interpretation of results [98]. Other methods for quantifying biofilm presence involve measuring representative indicators such as proteins, total organic carbon, ATP, and carbohydrates [99]; however, no single method is considered ideal for analysis.

Set guidelines exist for water quality that can be used as targets for successful biofilm control, but they too are limited. NASA's guidelines for potable water state that during flight, the

maximum allowable density of bacteria is 50 CFU/mL with no detectable coliforms in 100 mL of water [100, 101]. This standard is based on viable plate counts, as is common. An important note is that no guideline exists regarding fungal presence in water samples. Since clogging, which can be unrelated to viable bacteria, and presence of harmful organisms (including fungus) is a concern for NASA, this guideline is not comprehensive. The EPA follows a similar approach for its microorganism contaminant guidelines, stating “zero” as the minimum contaminant level goal for all organisms specifically listed out (*Cryptosporidium*, *Giardia lamblia*, *Legionella*, and total coliforms), presumably based on viable plate counts [72]. The EPA’s only additional microbial contamination metric is based on turbidity, which assesses the presence of any contaminant that could add cloudiness to water, including dirt. The limited scope of metrics used by guiding agencies means that researchers must create their own metrics that better capture the system they are modeling and the associated goals of implementing a biofilm control strategy.

A combination of approaches to assess biofilm presence should be used. For example, biomass and viable cell reductions would serve as a more relevant performance standard than either metric alone since together they capture the clogging capability of the biofilm present (biomass reduction) and the capability of viable cell detachment and downstream inoculation (viable cell reduction). For Earth-based water systems, large pipe diameters reduce clogging concerns because of biofilm, so main goals of biofilm control are public health and illness reduction. The performance metrics of coatings for implementation in these systems may be more related to reduction of biofilm formed by pathogens rather than reduction of biofilm as a whole. Without well planned performance metrics, the results of biofilm control experiments cannot be appropriately interpreted.

Consideration 8: Microgravity

Unlike Earth-based water systems, testing of coatings destined for spacecraft water systems should consider the effects of microgravity, as microgravity may influence microbial growth, biofilm formation, and thus, coating efficacy.

The effects of microgravity (real or simulated) on biofilm-forming microorganisms are poorly understood and tend to be controversial. Various studies have reported mixed effects of microgravity on microbial growth rates, EPS production, cell aggregation, virulence, and gene expression, which can ultimately alter biofilm growth and development [32, 59-72].

Microgravity effects on biofilm growth vary depending on factors such as substratum surface, type of microorganism, and exposure to real vs simulated gravity, with no definitive trends over time (Table 1).

Table 1. Biofilm experiments performed under simulated gravity or in space. Modified from Flores et al. (2024). BM: Biofilm morphology; BF=Biofilm formation; EPS= Extracellular polymeric substances; NC =no change with respect to controls; ↑ = increased; ↓ =decreased; — = data not provided, or analysis not done.

Microbial species	Material surface	Growth medium	Experiment duration	Microgravity effect on biofilm	Simulated gravity (SG) or in Space (S)	Reference
<i>Burkholderia cepacia</i> (Isolated from Shuttle water system)	Stainless steel	Water Tryptic soy broth	6 d	BF=↑; BM=NC BF=↓; BM=NC	S	Pyle et al., 1999 [110]
<i>P. aeruginosa</i> PAO-1	Polycarbonate membrane	R2A	1 d, 8 d	BF=NC; BM=NC	S	McLean et al., 2001 [109]
<i>P. aeruginosa</i> PA14 (WT, ΔmotABCD, ΔpilB)	Cellulose membrane	Modified artificial urine media	3 d	BF= ↑; BM=Column-and-canopy	S	Kim et al., 2013 [114]
<i>P. aeruginosa</i> PA14	SS316 pSS316 LIS	Luria-Bertani with potassium nitrate	1d, 2d, 3d	BF=↓; BM=NC BF=↓; BM=NC BF=↓; BM=NC	S	Flores et al., 2023 [32]

Table 1. Continued.

Microbial species	Material surface	Growth medium	Experiment duration	Microgravity effect on biofilm	Simulated gravity (SG) or in Space (S)	Reference
<i>P. rubens</i> ATCC® 28089	Stainless Steel 316	Potato dextrose broth	10 d, 15d, 20 d	BF=NC*; BM=NC	S	Hupka et al., 2023 [111]
	Aluminum alloy			BF=NC; BM=NC		
	Titanium Alloy			BF=NC*; BM=NC		
	Carbon Fiber			BF=NC; BM=NC		
	Quartz			BF=NC; BM=NC		
	Silicone			BF=NC; BM=NC		
	Nanograss			*BF=↑ at t=10 or 15d, but this effect was not maintained over time.		
<i>P. aeruginosa</i> PA14	Silicone Silicone DLIP Cellulose	Modified artificial urine media	1d, 2d, 3d	BF=NC; BM=NC BF=NC; BM=NC BF=NC; BM=NC	S	Flores et al., 2024 [113]

Table 1. Continued.

Microbial species	Material surface	Growth medium	Experiment duration	Microgravity effect on biofilm	Simulated gravity (SG) or in Space (S)	Reference
<i>E.coli</i> AMS6 and AMS150	Glass beads	Luria-Bertani broth	1 d	BF=↑; BM=- Strains under microgravity were more resistant to stressors and antibiotics (e.g., penicillin and chloramphenicol)	SG	Lynch et al., 2006 [112]
<i>K.pneumoniae</i> ATCC BAA-1705	--	Luria-Bertani broth	2 weeks	BF=↑; BM=-	SG	Wang et al., 2016 [72]
<i>Bacillus cereus</i> (isolated from a spacecraft assembly, integration and test center)	5A06 aluminum alloy	simplified humidity simulated condensate solutions of space station	5 d	Cell aggregation =↑; BF=↑, EPS production =↑	SG	Feng et al., 2024 [108]

A major challenge in studying biofilm formation under microgravity is the difficulty in replicating (and reproducing) experiments due to the high cost and limited availability of flight opportunities. Even when researchers can complete replications or reproduce each other's experiments, the conditions are rarely perfectly mimicked. Further, these reproduced studies have occasionally reported different results. For instance, an early study by Kim et al. reported a unique 'column-and canopy' biofilm morphology when growing *P. aeruginosa* PA14 in shuttle flight experiments using modified artificial urine media [114]. A more recent study by Flores et al., [113] attempted to reproduce the same experiment as Kim et al. [114], with the same microorganism and similar experimental factors, except for a slightly lower inoculum density. The unique biofilm morphology previously reported (Kim et al. [114]) was not observed by Flores et al. [113]. In a rare instance of experiment replication, Kim et al. reported consistent results from duplicate experiments on shuttle flights STS-132 and STS-135 [114]. The experiments by Flores et al. (on the ISS) were not replicated during a second flight, which is often the case for ISS experiments [113]. Despite Flores et al.'s [113] attempt at reproducing Kim et al.'s [114] experiments, the vehicles were different, and the cancellation of the shuttle program means that Kim et al.'s [114] experiments will likely never be perfectly reproduced. It is difficult to determine if the variations in results between Kim et al. [114] and Flores et al. [113] were due to the different inoculum densities or any number of differences between the use of a shuttle and the ISS for microgravity.

Effects of microgravity on biofilm development also vary depending on microbial species and type of material used as the substratum. Hupka et al. evaluated the effect of microgravity on fungal biofilm growth of *Penicillium rubens* on different material surfaces for up to 20 days on

the ISS [111]. Opposite to what was observed previously with bacteria (Kim et al. [114]), biofilms formed by *P. rubens* under microgravity did not show any morphological differences compared to biofilms grown at normal gravity. However, biofilm formation decreased or increased under microgravity depending on the substratum material and incubation time, further complicating the effects of microgravity. More confounding results came from reports by Flores et al. that stated *P. aeruginosa* biofilms grown on lubricant-infused surfaces had “virtually the same transcriptome” in microgravity as they did at normal gravity (1 differentially expressed gene; DEG) while biofilms grown on stainless steel had larger transcriptome differences between normal gravity and microgravity (5 DEG) [116].

Given the constraints of performing space flight experiments (cost, time, effort, not accessible to many researchers), most microgravity-related biofilm research is performed using devices that can simulate microgravity conditions on ground. These apparatuses commonly include 2D- and 3D- clinostats [75-77], rotating wall vessel bioreactors [120], and random positioning machines [121]. While useful for simulating microgravity, these devices present several limitations [80, 81]:

- i. These devices cannot fully replicate true microgravity, which may impact biofilm behavior. Variations in shear forces and fluid dynamics compared to actual microgravity can influence how biofilms develop and function.
- ii. Simulated environments are typically smaller and less complex than real space conditions, which could limit the biological relevance of the findings.

- iii. Not many commercially available devices exist that can simulate microgravity; thus, these bioreactors are typically custom-made/designed to the specific experiment, making it difficult to standardize their use.

The varying results of biofilm morphology between normal gravity and simulated microgravity mirrors those between normal gravity and spaceflight microgravity. Wang et al. reported that under simulated microgravity conditions, *K. pneumoniae* biofilm formation was denser in simulated microgravity conditions compared to normal gravity; this was attributed to increased expression of cellulose and fimbriae [72]. In another study, Feng et al. observed increased EPS production and cell aggregation by *Bacillus cereus* under simulated microgravity, which caused the thinning of films of aluminum alloy, suggesting that microgravity could potentially increase the risk of microbiologically influenced corrosion in water recovery systems in spacecrafts [108].

As there are no definitive trends between biofilm growth in true or simulated microgravity and those grown at normal gravity, it is difficult to determine the best approach for evaluating coatings in spacecraft water systems. Ideally, coatings for use in spacecraft would be evaluated in true microgravity before implementation. However, as will be discussed, the one biofilm experiment evaluating coatings for the ISS water system occurred in batch conditions and with many experimental constraints set by NASA [32]. Simulated microgravity devices may offer a bit of a workaround to evaluate coatings for spacecraft water systems, but these systems are also largely operated in batch conditions and feature the limitations listed above. It is the purpose of space research to push through these limitations and create innovative solutions, which is

required for the advancement of biofilm control in spacecraft water systems. As more experiments are conducted, the importance and relevance of (simulated) microgravity tests will become apparent. In the meantime, it would be best practice to include some simulated microgravity experiments in addition to normal gravity tests for evaluation of coatings for spacecraft water systems.

Current methods for coating evaluation

All studies evaluated in the second half of this review used different coatings in their experiments. These eight studies were chosen as they were the only peer-reviewed publications found in the literature that specifically investigated coatings for implementation into various types of water systems (DWDS, wastewater systems, spacecraft water systems, etc.). Many other peer-reviewed studies evaluating coatings simply list water systems as a potential use case in addition to many other cases (such as high touch surfaces or industrial food processes). The mechanisms of action of these coatings are not discussed in this review as the goal here is to evaluate and provide suggestions for improved coating test methods, rather than evaluate specific coating technologies. Also not included in this review are any test methods or coatings designed for application in marine, river, or lake systems, though the considerations presented extend to those environments as well.

Figure 2 summarizes the eight known studies examining anti-fouling coatings for implementation into water systems and their methodological approaches to coating testing. Four of the studies examined coatings for use in spacecraft water systems while the other four evaluated coatings for use in Earth-based water systems. As is seen in Figure 2, three of the studies evaluating coatings for use in spacecraft took place at normal gravity. There are a

plurality of experiments evaluating biofilms in spacecraft that have taken place in real or simulated microgravity (Table 1), but they are not included in this discussion as they did not evaluate anti-fouling coatings for use in spacecraft water systems. Additional experimental details for the eight evaluated studies can be found in Supplemental Table 1.

Consideration Reference	Biofilm reactor operation	Microbe selection	Reinoculation of system	Coating coverage	Liquid medium	Experiment duration & pre-aging of surface	Performance evaluation of surface	Microgravity
Flores et al. (2023) "LIS for spacecraft tested on ISS" [37]				6%		Up to 72 h		
Demir et al. (2022) "N-halamine for spacecraft" [38]				48%		Up to 72 h		
Mettler et al. (2022) "Quat coating for spacecraft" [39]				7-14%		Up to 72 h		
Li Sip et al. (2023) "LIS for spacecraft" [40]				4.3-10.8%		Up to 48 h	 	
Sojka et al. (2023) "Nanogel coating for DWDS" [41]				22%	 	Up to 24 h	 	N/A
Samuel et al. (2022) "LCA-Zn-Cn coating for water devices" [60]				33-50%	 	Up to 30 days 	 	N/A
Filice et al. (2022) "Nexar™ smart coating" [68]				10%		Up to 4 days		N/A
Si et al. (2015) "Norspermidine coating" [42]				No info provided		Up to 6 days		N/A

	Operation featured flow or fresh medium introduction		High-nutrient, broad spectrum medium		Quantification of biofilm with microscopy techniques
	Coating tested against multiple organisms		Medium designed to mimic water system		Quantification of biofilm with viable plate counts
	Experiment featured controlled reinoculation		Pre-aging of coating prior to biological experiments		Coating tested in microgravity

Figure 2. Summary of the eight studies presented and the methodological approach to evaluating coatings for implementation into water systems. Blank spaces indicate lack of incorporation of a specific experimental approach.

Summary of the methods used in evaluation of coatings for Earth-based water systems

Generally, studies for testing coatings for implementation into Earth-based water systems were more relevant to the target system than experiments for spacecraft water systems. This could be a result of greater familiarity with and access to water systems on Earth.

All but one study for coatings in Earth-based water systems tested the coatings against multiple organisms. Si et al., [37] was the only group to test their norspermidine-hosting polyelectrolyte coating against a mixed culture of organisms. They included activated sludge from a municipal wastewater treatment plant in Beijing, China in some of their experiments and used various pure bacterial cultures (Gram-positive and Gram-negative) in others. In one experimental iteration, Si et al. observed the coating reduced biomass of the mixed sludge biofilm better than some single species cultures and worse than one of the single-species cultures [42]. These results present both possibilities of overestimating and underestimating coating performance when only a single culture is used to evaluate coatings. Further, the most responsive to the coating was the single-species *P. aeruginosa* biofilm. As this tends to be a common model organism for biofilm researchers, this result reinforces the very real possibility of overstating coating performance if it is tested against only one organism. Sojka et al. [41] also chose relevant organisms for their studies of a nanogel coating for use in DWDS. The group used four different organisms (as single cultures): *Sphingomonas* Sph5 and Sph10, *P. aeruginosa* (DSM 50071), and *P. extremorientalis* (DSM 15824). As *Pseudomonas* and *Sphingomonas* can be found frequently in water systems, and *Sphingomonas* are especially concerning for microbially induced corrosion, these were excellent choices [32]. Both genera are Gram-negative bacteria, which limits the diversity of the tested organisms, but Gram-negative are the dominant bacterial morphology in

water systems [124]. The nanogel coating reduced attachment of viable biofilm by 1-1.5 log after 4 h in a flow chamber for all four organisms. In the 24 h batch tests, the Sph10 was less susceptible to the coating when measuring surface coverage by the biofilm compared to the other three species tested. The only group to test their coating against a single organism was Filice et al. in their investigation for an antibiofilm smart coating, termed Nexar™ for its ability to inhibit *L. pneumophila* biofilm accumulation [54]. *L. pneumophila* is a common pathogenic bacterium found in water systems, is of special concern for water systems in hospitals due to the compromised immune status of many patients [54, 83, 84], and serves as a relevant microbial choice for water systems.

One of the most encouraging aspects of the studies for Earth-based water systems was the longer duration of tests, with only one group reporting a 24 h experiment. Samuel et al. included tests up to 30 days for their investigation of a lithocholic acid, zinc pyrithione, and cinnamaldehyde containing coating termed LCA-Zn-Cn [60]. The 30-day experiments were batch tests in an incubator where desiccation and starvation could have impacted the results of no viable cells detected after 5 days, but the inclusion of a month-long evaluation is a step in the right direction. An improvement to this experiment would have been regular addition of fresh media and fresh inoculum to address concerns about desiccation and starvation. These inclusions would have also created some sort of flow regime, like flush and fill, which would likely more accurately represent real water systems. Further, Samuel et al.'s study was the only one reviewed that pre-aged their coated surface before one of the tests [60]. Aging consisted of flowing 50 L of tap water (pH 7.2) through a coated silicone hose at 1.32 and 1.99 m/s. The coated silicone was then evaluated for its ability to control biofilm accumulation using the JIS Z 2801/ISO 22196

standard method [67], with no viable *E. coli* cells detected, resulting in a 100% reduction. This marks an encouraging development with pre-aging surfaces, but with only 50 L of water used, it does not capture many of the realities of long-term coating use.

Researchers investigating coatings for Earth-based water systems have taken many of the considerations presented in this review into account while designing their experiments. Generally, the studies conducted have featured promising decisions for several of the considerations presented. With the relative ease of these experiments compared to those investigating coatings for use in spacecraft, perhaps the next large step for studying coatings in Earth-based water systems is field-scale studies. Results from such experiments would also provide useful data and lessons learned for research into coatings for spacecraft water systems, as the two areas of research are closely related. Careful experimental design and communication of results from each field will greatly advance the overarching goal of developing coatings for biofilm control in all water systems.

Summary of methods used in evaluation of coatings for spacecraft water systems

The methods used by researchers for evaluating coatings in spacecraft water systems featured many similarities to earth-based assessments. *P. aeruginosa* was used by all groups in their experiments despite never being isolated from the ISS wastewater tank, portable water bus, or condensate in 10 years of sampling [53]. In a publication explaining the design of their spaceflight experiment, Flores et al. [85] reported using *P. aeruginosa* in an attempt to replicate the column-and-canopy biofilm morphology reported by Kim et al. [114]. Demir et al. [38], Mettler et al. [39], and Li Sip et al. [40] did not share their justification for selecting *P.*

aeruginosa, but it was likely chosen for its ease of use and general relevance to the field of biofilm research. The results reported by Si et al. showed *P. aeruginosa* being most susceptible to their coating [37], which leads to questions regarding an improved perceived performance of the coatings tested by all researchers for spacecraft implementation. As Li Sip et al. also tested lubricant-infused surfaces (LIS) against *E. coli* and *S. aureus*, they have a broader understanding of their coating's performance [35]. However, those organisms were only tested against the coating in preliminary batch tests, not continuous flow biofilm tests which prevents direct comparisons, especially since *P. aeruginosa* was not used in the preliminary batch tests.

An encouraging step by Demir et al. for evaluating an N-halamine coating was inclusion of controlled reinoculation into experiments when the phosphate buffered saline (PBS) covering the coated coupon was removed and replaced daily with fresh inoculum [38]. Even with daily reinoculation, there were no viable cells detected on coated samples at the end of the 72 h experiment. These are the same results reported for their initial experiment which featured the same timeline without reinoculation/PBS replacement. The use of reinoculation is promising, but since the PBS provided no nutrients, it is not representative of ISS water systems. As the only research group to use controlled reinoculation, Demir et al. should still be recognized for that aspect of their experimental design.

Two groups, Mettler et al. [39] and Li Sip et al. [35] used the ASTM standard method for growing biofilms in a CDC reactor [61]. Similarities between the experiments (organism, media, reactor, and timeline) allow for more direct comparisons to be made between the two. The groups differed in that Li Sip et al. [40] used additional batch screening tests against *E. coli* and *S. aureus* (both tests with slightly different media) while Mettler et al. [39] used two very

different media in experiments investigating a quaternary ammonium containing coating. Li Sip et al. found that their LIS coating was effective against all organisms and in slightly different media [40]. In contrast, Mettler et al. found that performance of the quaternary ammonium containing coating was greatly reduced in potato dextrose broth compared to TSB [39]. These results could mean that coating efficacy is robust to slight variations in water quality but not to large changes in composition. Tests must be done to investigate this possibility for each coating developed for water system implementation.

All researchers examining coatings for spacecraft water systems featured a timeline of 72 h or less, which is quite short. If implemented, coatings are likely to be in place for the lifetime of a spacecraft vehicle, which for the ISS has been 24 years and counting. While a 24-year long experiment is not reasonable, a timeline on the order of weeks to months would be ideal to help bridge that gap. With only 72 h and no pre-aging completed, it is impossible to extrapolate results past a few additional days. It would be incredibly irresponsible to extend these results to weeks or months in the ISS.

Lastly, Flores et al. was the only group to incorporate microgravity in testing an LIS coating [32]. Flight experiments are prohibitively expensive and bureaucratic, so it is not expected that every astrobiology-related researcher will have access to the ISS or other commercial spacecraft for experiments. However, incorporation of some method of simulated microgravity by the other research groups could help answer questions regarding coating performance in space. The study by Flores et al. [37] also served as an attempt to reproduce previous results obtained by Kim et al. [114]. These factors greatly influenced experimental design due to NASA restrictions and precedent set by Kim et al. [114]. The biofilm reactor (a

group-activated pack of fluid processing assemblies), organism (*P. aeruginosa* UCBPP-PA 14), media (LB with potassium nitrate), and experiment duration were selected by Flores et al. [37, 127] to reproduce the results from Kim et al. [114]. Despite these restrictions, Flores et al. was the only group to include transcriptomics in their experiments [37]. This approach may help researchers understand genetic responses of the biofilm to the coating (and microgravity) which could play a key role for designing more effective coatings.

Ultimately, as understanding of spacecraft water systems and how to better replicate system conditions improves, so will experiments for biofilm control. In the meantime, researchers must strive to incorporate known aspects of spacecraft water systems into their experiments. Easy improvements include incorporating some flow regime into experiments rather than batch tests, using a consortium of relevant microorganisms (a list of which can be found in Zea et al. 2020 [53]), simulated wastewater (one example of a recipe can be found in Velez-Justiniano et al. [69]), increased duration of experiments past 72 h, and reinoculation of the system during experiments.

Conclusions and Recommendations

Coatings and surface modifications continue to be a promising area of research for controlling biofilm growth in many applications. As the field expands and gets closer to implementation of coatings into Earth-based and spacecraft water systems, researchers must consider evaluation methods more carefully because the risk of a failed coating can be costly in terms of safety and human health. Laboratory performance of a coating (or biocide or antibiotic for that matter) relies heavily on the testing methods used. In the interest of accurate testing and improved technology transfer success, researchers must carefully consider their final

implementation of the coating being tested. The considerations presented here aim to guide researchers in designing experiments that effectively challenge coatings and surface modifications for controlling biofilm in water systems here on Earth and in future spacecraft.

Key considerations to make while designing experiments include deciding what reactor system to use (batch, continuous flow, semi-batch, or flush and fill), the microorganisms tested, reinoculation of the system, the coating coverage in the system, liquid medium selection, duration of the experiment, pre-aging of the coated surface, and, for spacecraft applications, use of (simulated) microgravity. The laboratory test design should include characteristics most accurate to water system dynamics such as continuous flow, semi-batch, or flush and fill reactors, organisms (multiple species and domains) isolated from the target water system, reinoculation of fresh organisms into the experiment, high coating coverage in the reactor, a medium that mimics the water in the system, experiment durations of weeks to months, and relevant performance evaluation criteria. To reduce the time commitment of some experiments, higher nutrient medium can be used to encourage rapid biofilm growth and pre-aging of the coated surface can be completed. However, these experiments should be in tandem with experiments that more accurately mimic the target water system. Ideally, experiments for implementation of coatings into spacecraft water systems would occur in true microgravity (in space) but that is immensely cost-prohibitive. Simulated microgravity experiments are encouraged though they frequently require batch conditions. Once again, these should be done in tandem with continuous flow, semi-batch, or flush and fill normal gravity experiments.

The goal of this review is to remind researchers that the methods used in their experiments directly impact the results. Further, the considerations and suggestions presented

aim to elicit more thoughtful experimental designs to advance the field of coatings for biofilm and biofouling control in water systems. Many authors have presented encouraging results so far which will likely lead to an expansion of this field of research and a transition away from experiments highly influenced by the medical device industry. It is possible that many of the studies discussed here were preliminary screening tests to be built on in a more relevant manner in future experiments. This is a natural progression of research as long as the preliminary results from screening tests are not over-extrapolated to make conclusions regarding coating performance outside the laboratory. To reduce over-extrapolation of results, careful experimental design and accurate representation of water systems is necessary. This will benefit biofilm researchers, material scientists, coatings developers, and eventually the end user of Earth-based and spacecraft water systems.

Supplementary material

Supplementary Table 1. Summary of experimental methods relating to the considerations presented in the review. (Inserted as image due to size).

Reference	Type of coating tested in study	Desired coating implementation	Reactor type	Organism(s) used	Approx. % of coated and wetted surface area	Approx. volume of liquid media exposed to wetted surface area	Approx. coated surface area to volume ratio (cm ² /mL)	Media used	Duration of biofilm tests	Evaluation method	Stains used for microscopy-based evaluation
Flores et al (2023)	Lubricant impregnated surface	Spacecraft wastewater system	Batch	<i>P. aeruginosa</i> UCBPP-PA14	6%	3.25 mL	0.31	LB w/ potassium nitrate	Up to 72 h	Biofilm biomass, thickness, surface area coverage, morphology, differential gene expression	Propidium iodide and FilmTracer FM 1-43 Green Biofilm
Demir et al (2022)	N-halamine coating	Spacecraft water system	Flush and fill	<i>P. aeruginosa</i> ATCC 27853	48%	4 mL	3.28	PBS	Up to 72 h	CFU/sample	N/A
Mettler et al (2022)	Quaternary-ammonium containing coating	Spacecraft water system	Batch and continuous flow	<i>P. aeruginosa</i> PA14 (from Luis Zea)	14% (first sampling) 7% (second sampling)	325 mL	0.30	TSB PDB	Up to 72 h	CFU/cm ² and SEM for morphology	N/A
Li Sip et al (2023)	Lubricant-infused surface coating	Spacecraft water system	Batch and continuous flow	<i>P. aeruginosa</i> 10145 <i>S. aureus</i> MRSAS USA300 <i>E. coli</i> BL21	10.8% (batch in 12-well plate) 4.3% (batch in CDC) 5.3% (continuous flow CDC)	3 mL (batch in 12-well plate) 500 mL (batch phase in CDC) 325 mL (continuous flow in CDC)	0.33 (batch in 12-well plate) 0.03 (batch in CDC) 0.05 (continuous flow in CDC)	TSB TSB w/ NaCl or M9	Up to 48 h	CFU/cm ² and surface area coverage	Crystal violet
Sojka et al (2023)	Nanogel coating	Earth-based DWDS	Batch and continuous flow	<i>S. Sph5</i> <i>S. Sph 10</i> <i>P. aeruginosa</i> DSM50071 <i>P. extremorientalis</i> DSM 15824	22% (batch experiment) No info for continuous flow	6 mL (batch experiment) No info for continuous flow	1.04 (batch) No info for continuous flow	Synthetic tap water RZA	4 h continuous flow 24 h batch	Surface area coverage, CFU, Aggregates/cm ²	DAPI
Samuel et al (2022)	Lithocholic acid, zinc pyrrithione, and cinnamaldehyde (LCA-Zn-Cn) coating	Earth-based water devices	Batch and continuous flow	<i>E. coli</i> <i>E. faecalis</i>	33% (continuous flow) 50% (batch)	No info for continuous flow 200 µL (batch)	No info for continuous flow 80 (batch)	LB Lake Michigan water	24 h	CFU/sample and visual/SEM evaluation for morphology	N/A
Filice et al (2022)	Nexar [™] smart coating	Earth-based water systems	Flush and fill	<i>L. pneumophila</i>	10%	0.7 mL	0.71	TSB	Up to 4 days	Visual analysis of microscopy images	LIVE/DEAD
Si et al (2015)	Norspermidine-containing coating	Earth-based water systems	Batch	<i>B. subtilis</i> ATCC 19385 <i>P. aeruginosa</i> ATCC-25330 <i>E. coli</i> ATCC PTA-1798 Mixed culture from activated sludge	No info provided	30 mL	No info provided	LB	Up to 6 days	Relative biomass of biofilm	Crystal violet

References

1. Di Pippo F, Di Gregorio L, Congestri R, Tandoi V, Rossetti S. Biofilm growth and control in cooling water industrial systems. *FEMS Microbiology Ecology*. 2018;94(5); doi: 10.1093/femsec/fiy044.
2. Li Y, Song P, Pei Y, Feng J. Effects of lateral flushing on emitter clogging and biofilm components in drip irrigation systems with reclaimed water. *Irrigation Science*. 2015;33(3):235-45; doi: 10.1007/s00271-015-0462-8.
3. Japhet N, Tarchitzky J, Chen Y. Effectiveness of hydrogen peroxide treatments in preventing biofilm clogging in drip irrigation systems applying treated wastewater. *Biofouling*. 2022;38(6):575-92.

4. Little BJ, Blackwood DJ, Hinks J, Lauro FM, Marsili E, Okamoto A, et al. Microbially influenced corrosion—Any progress? *Corrosion Science*. 2020;170:108641; doi: <https://doi.org/10.1016/j.corsci.2020.108641>.
5. Cámara M, Green W, MacPhee CE, Rakowska PD, Raval R, Richardson MC, et al. Economic significance of biofilms: a multidisciplinary and cross-sectoral challenge. *NPJ Biofilms Microbiomes*. 2022;8(1):42; doi: 10.1038/s41522-022-00306-y.
6. Wingender J, Flemming H-C. Biofilms in drinking water and their role as reservoir for pathogens. *Int J Hyg Environ Health*. 2011;214(6):417-23; doi: <https://doi.org/10.1016/j.ijheh.2011.05.009>.
7. Walker J, Moore G. *Pseudomonas aeruginosa* in hospital water systems: biofilms, guidelines, and practicalities. *Journal of Hospital Infection*. 2015;89(4):324-7; doi: <https://doi.org/10.1016/j.jhin.2014.11.019>.
8. Horberry M: After Coronavirus, Office Workers Might Face Unexpected Health Threats. In: *The New York Times*. 2020.
9. Miller HC, Morgan MJ, Wylie JT, Kaksonen AH, Sutton D, Braun K, et al. Elimination of *Naegleria fowleri* from bulk water and biofilm in an operational drinking water distribution system. *Water research*. 2017;110:15-26; doi: <https://doi.org/10.1016/j.watres.2016.11.061>.
10. Puzon GJ, Miller HC, Malinowski N, Walsh T, Morgan MJ. *Naegleria fowleri* in drinking water distribution systems. *Current Opinion in Environmental Science & Health*. 2020;16:22-7; doi: <https://doi.org/10.1016/j.coesh.2020.02.003>.
11. Gharpure R, Gleason M, Salah Z, Blackstock AJ, Hess-Homeier D, Yoder JS, et al. Geographic Range of Recreational Water-Associated Primary Amebic Meningoencephalitis, United States, 1978-2018. *Emerg Infect Dis*. 2021;27(1):271-4; doi: 10.3201/eid2701.202119.
12. Ward L, Sherchan SP. Surveillance of *Naegleria fowleri* in Louisiana's public water systems. *J Water Health*. 2023;21(11):1627-31; doi: 10.2166/wh.2023.040.
13. Environmental Protection Agency. Contaminant Candidate List (CCL) and Regulatory Determination. <https://www.epa.gov/ccl/ccl-5-microbial-contaminants> (2023). Accessed.
14. Goudot S, Mathieu L, Herbelin P, Soreau S, Jorand FPA. Growth dynamic of biofilm-associated *Naegleria fowleri* in freshwater on various materials. *Front Microbiol*. 2024;15; doi: 10.3389/fmicb.2024.1369665.

15. Carter DL: Status of the Regenerative ECLSS Water Recovery System. In: International Conference of Environmental Systems. Barcelona, Spain; 2010.
16. Zea L, Nisar Z, Rubin P, Cortesão M, Luo J, McBride SA, et al. Design of a spaceflight biofilm experiment. *Acta Astronautica*. 2018;148:294-300; doi: <https://doi.org/10.1016/j.actaastro.2018.04.039>.
17. Sobisch L-Y, Rogowski KM, Fuchs J, Schmieder W, Vaishampayan A, Oles P, et al. Biofilm Forming Antibiotic Resistant Gram-Positive Pathogens Isolated From Surfaces on the International Space Station. *Front Microbiol*. 2019;10; doi: 10.3389/fmicb.2019.00543.
18. Lemelle L, Campagnolo L, Mottin E, Le Tourneau D, Garre E, Marcoux P, et al. Towards a passive limitation of particle surface contamination in the Columbus module (ISS) during the MATISS experiment of the Proxima Mission. *NPJ Microgravity*. 2020;6(1):29; doi: 10.1038/s41526-020-00120-w.
19. Lemelle L, Mottin E, Le Tourneau D, Rouquette S, Campagnolo L, Thévenot C, et al. Surface contamination rates at different spatial scales in the Columbus module (ISS) during the MATISS campaigns. *Frontiers in Astronomy and Space Sciences*. 2023;10; doi: 10.3389/fspas.2023.1229022.
20. Lemelle L, Rouquette S, Mottin E, Le Tourneau D, Marcoux PR, Thévenot C, et al. Passive limitation of surface contamination by perFluoroDecylTrichloroSilane coatings in the ISS during the MATISS experiments. *NPJ Microgravity*. 2022;8(1):31; doi: 10.1038/s41526-022-00218-3.
21. Shea A, Harvey K, Keeley A, Johnson H, Hansen N, McCormack R, et al. Payload Design and Evaluation of Staphylococcus epidermidis Adhesion to Nonfouling Polyampholyte Coatings Onboard the International Space Station. *Molecules*. 2025;30(4):836.
22. National Aeronautics and Space Administration. Gateway Frequently Asked Questions. <https://www.nasa.gov/gateway-frequently-asked-questions/#:~:text=Unlike%20the%20ISS%2C%20which%20is,Gateway%20will%20not%20be> (2025). Accessed.
23. Williamson J, Wilson JP, Robinson K, Luong H: Status of ISS Water Management and Recovery. In: 52nd International Conference on Environmental Systems. Calgary, Canada; 2023.

24. Balaure PC, Grumezescu AM. Recent Advances in Surface Nanoengineering for Biofilm Prevention and Control. Part I: Molecular Basis of Biofilm Recalcitrance. Passive Anti-Biofouling Nanocoatings. *Nanomaterials*. 2020;10(6):1230.
25. Balaure PC, Grumezescu AM. Recent Advances in Surface Nanoengineering for Biofilm Prevention and Control. Part II: Active, Combined Active and Passive, and Smart Bacteria-Responsive Antibiofilm Nanocoatings. *Nanomaterials*. 2020;10(8):1527.
26. Song B, Zhang E, Han X, Zhu H, Shi Y, Cao Z. Engineering and Application Perspectives on Designing an Antimicrobial Surface. *ACS Appl Mater Interfaces*. 2020;12(19):21330-41; doi: 10.1021/acsami.9b19992.
27. Mahanta U, Khandelwal M, Deshpande AS. Antimicrobial surfaces: a review of synthetic approaches, applicability and outlook. *J Mater Sci*. 2021;56(32):17915-41; doi: 10.1007/s10853-021-06404-0.
28. Wang M, Duday D, Scolan E, Perbal S, Prato M, Lasseur C, et al. Antimicrobial Surfaces for Applications on Confined Inhabited Space Stations. *Advanced Materials Interfaces*. 2021;8(13):2100118; doi: <https://doi.org/10.1002/admi.202100118>.
29. Paton S, Moore G, Campagnolo L, Pottage T. Antimicrobial surfaces for use on inhabited space craft: A review. *Life Sciences in Space Research*. 2020;26:125-31; doi: <https://doi.org/10.1016/j.lssr.2020.05.004>.
30. Manuel CM, Nunes OC, Melo LF. Dynamics of drinking water biofilm in flow/non-flow conditions. *Water research*. 2007;41(3):551-62; doi: <https://doi.org/10.1016/j.watres.2006.11.007>.
31. Tsagkari E, Connelly S, Liu Z, McBride A, Sloan WT. The role of shear dynamics in biofilm formation. *npj Biofilms Microbomes*. 2022;8(1):33; doi: 10.1038/s41522-022-00300-4.
32. Wang S, Zhu H, Zheng G, Dong F, Liu C. Dynamic Changes in Biofilm Structures under Dynamic Flow Conditions. *Applied and Environmental Microbiology*. 2022;88(22):e01072-22; doi: 10.1128/aem.01072-22.
33. Peyton BM, Characklis WG. A statistical analysis of the effect of substrate utilization and shear stress on the kinetics of biofilm detachment. *Biotechnology and Bioengineering*. 1993;41(7):728-35; doi: 10.1002/bit.260410707.
34. Mah T-F, Pitts B, Pellock B, Walker GC, Stewart PS, O'Toole GA. A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nature*. 2003;426(6964):306-10; doi: 10.1038/nature02122.

35. O'Toole GA, Kolter R. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Molecular Microbiology*. 1998;30(2):295-304; doi: <https://doi.org/10.1046/j.1365-2958.1998.01062.x>.
36. Parsek MR, Peter Greenberg E. [3] Quorum sensing signals in development of *Pseudomonas aeruginosa* biofilms. In: *Methods in Enzymology*. Academic Press; 1999. p. 43-55.
37. Flores P, McBride SA, Galazka JM, Varanasi KK, Zea L. Biofilm formation of *Pseudomonas aeruginosa* in spaceflight is minimized on lubricant impregnated surfaces. *NPJ Microgravity*. 2023;9(1):66; doi: 10.1038/s41526-023-00316-w.
38. Demir B, Taylor A, Broughton RM, Huang TS, Bozack MJ, Worley SD. N-halamine surface coating for mitigation of biofilm and microbial contamination in water systems for space travel. *Biofilm*. 2022;4:100076; doi: <https://doi.org/10.1016/j.biofilm.2022.100076>.
39. Mettler MK, Parker CW, Venkateswaran K, Peyton BM. Antimicrobial Coating Efficacy for Prevention of *Pseudomonas aeruginosa* Biofilm Growth on ISS Water System Materials. *Front Microbiol*. 2022;13; doi: 10.3389/fmicb.2022.874236.
40. Li Sip YY, Jacobs A, Morales A, Sun M, Roberson LB, Hummerick ME, et al. Slippery lubricant-infused silica nanoparticulate film processing for anti-biofouling applications. *J Appl Biomater Funct Mater*. 2023;21:22808000231184688; doi: 10.1177/22808000231184688.
41. Sójka O, Keskin D, van der Mei HC, van Rijn P, Gagliano MC. Nanogel-based coating as an alternative strategy for biofilm control in drinking water distribution systems. *Biofouling*. 2023;39(2):121-34; doi: 10.1080/08927014.2023.2190023.
42. Si X, Quan X, Wu Y. A small-molecule norspermidine and norspermidine-hosting polyelectrolyte coatings inhibit biofilm formation by multi-species wastewater culture. *Appl Microbiol Biotechnol*. 2015;99(24):10861-70; doi: 10.1007/s00253-015-6943-0.
43. Yin R, Cheng J, Wang J, Li P, Lin J. Treatment of *Pseudomonas aeruginosa* infectious biofilms: Challenges and strategies. *Front Microbiol*. 2022;13:955286; doi: 10.3389/fmicb.2022.955286.
44. Zou Y, Liu C, Zhang H, Wu Y, Lin Y, Cheng J, et al. Three lines of defense: A multifunctional coating with anti-adhesion, bacteria-killing and anti-quorum sensing properties for preventing biofilm formation of *Pseudomonas aeruginosa*. *Acta Biomaterialia*. 2022;151:254-63; doi: <https://doi.org/10.1016/j.actbio.2022.08.008>.

45. Bouhrour N, Nibbering PH, Bendali F. Medical Device-Associated Biofilm Infections and Multidrug-Resistant Pathogens. *Pathogens*. 2024. doi: 10.3390/pathogens13050393.
46. Mulani MS, Kamble EE, Kumkar SN, Tawre MS, Pardesi KR. Emerging Strategies to Combat ESKAPE Pathogens in the Era of Antimicrobial Resistance: A Review. *Front Microbiol*. 2019;10; doi: 10.3389/fmicb.2019.00539.
47. Chen M, Yu Q, Sun H. Novel Strategies for the Prevention and Treatment of Biofilm Related Infections. *International Journal of Molecular Sciences*. 2013;14(9):18488-501.
48. Nejadnik MR, van der Mei HC, Norde W, Busscher HJ. Bacterial adhesion and growth on a polymer brush-coating. *Biomaterials*. 2008;29(30):4117-21; doi: 10.1016/j.biomaterials.2008.07.014.
49. Kanematsu H, Oizumi A, Sato T, Kamijo T, Honma S, Barry DM, et al. Biofilm Formation of a Polymer Brush Coating with Ionic Liquids Compared to a Polymer Brush Coating with a Non-Ionic Liquid. *Coatings*. 2018;8(11):398.
50. Friedlander A, Nir S, Reches M, Shemesh M. Preventing Biofilm Formation by Dairy-Associated Bacteria Using Peptide-Coated Surfaces. *Front Microbiol*. 2019;10; doi: 10.3389/fmicb.2019.01405.
51. Silhavy TJ, Kahne D, Walker S. The bacterial cell envelope. *Cold Spring Harb Perspect Biol*. 2010;2(5):a000414; doi: 10.1101/cshperspect.a000414.
52. Afonso TB, Simões LC, Lima N. Occurrence of filamentous fungi in drinking water: their role on fungal-bacterial biofilm formation. *Research in Microbiology*. 2021;172(1):103791; doi: <https://doi.org/10.1016/j.resmic.2020.11.002>.
53. Zea L, McLean RJC, Rook TA, Angle G, Carter DL, Delegard A, et al. Potential biofilm control strategies for extended spaceflight missions. *Biofilm*. 2020;2:100026; doi: <https://doi.org/10.1016/j.bioflm.2020.100026>.
54. Justiniano Y-AV, Ledford M-E, Castro-Wallace SL, Nguyen HN, Li W, Irwin T, et al.: More Than a Decade of International Space Station Microbial Sampling in the Environmental Control and Life Support Systems. In: 53rd International Conference on Environmental Systems. 2024.
55. Simoes LC, Simões M. Biofilms in drinking water: problems and solutions. *Rsc Advances*. 2013;3(8):2520-33.
56. Kragh KN, Alhede M, Rybtke M, Stavnsberg C, Jensen P, Tolker-Nielsen T, et al. The Inoculation Method Could Impact the Outcome of Microbiological Experiments. *Appl Environ Microbiol*. 2018;84(5); doi: 10.1128/aem.02264-17.

57. Lichtenberg M, Kvich L, Larsen Sara Louise B, Jakobsen Tim H, Bjarnsholt T. Inoculum Concentration Influences *Pseudomonas aeruginosa* Phenotype and Biofilm Architecture. *Microbiol Spectr*. 2022;10(6):e03131-22; doi: 10.1128/spectrum.03131-22.
58. Ghanbari A, Dehghany J, Schwebs T, Müsken M, Häussler S, Meyer-Hermann M. Inoculation density and nutrient level determine the formation of mushroom-shaped structures in *Pseudomonas aeruginosa* biofilms. *Scientific Reports*. 2016;6(1):32097; doi: 10.1038/srep32097.
59. Kroukamp O, Dumitrache RG, Wolfaardt GM. Pronounced effect of the nature of the inoculum on biofilm development in flow systems. *Appl Environ Microbiol*. 2010;76(18):6025-31; doi: 10.1128/aem.00070-10.
60. Samuel MS, Moghaddam ST, Shang M, Niu J. A Flexible Anti-Biofilm Hygiene Coating for Water Devices. *ACS Appl Bio Mater*. 2022;5(8):3991-8; doi: 10.1021/acsabm.2c00538.
61. International A: ASTM E3161-18, Standard Practice for Preparing a *Pseudomonas aeruginosa* or *Staphylococcus aureus* Biofilm using the CDC Biofilm Reactor. In. West Conshohoken, PA; 2018.
62. International A: ASTM E2647-20, Standard Test Method for Quantification of *Pseudomonas aeruginosa* Biofilm Grown Using Drip Flow Biofilm Reactor with Low Shear and Continuous Flow. In., vol. ASTM E2647-20; 2020.
63. International A: ASTM E2799-22, Standard Test Method for Testing Disinfectant Efficacy against *Pseudomonas aeruginosa* Biofilm using the MBEC Assay. In.; 2023.
64. Waegenaar F, García-Timmermans C, Van Landuyt J, De Gussemme B, Boon N. Impact of operational conditions on drinking water biofilm dynamics and coliform invasion potential. *Appl Environ Microbiol*. 2024;90(5):e0004224; doi: 10.1128/aem.00042-24.
65. Hung C-H, Chang Y-T, Chang Y-J. Roles of microorganisms other than *Clostridium* and *Enterobacter* in anaerobic fermentative biohydrogen production systems – A review. *Bioresource Technology*. 2011;102(18):8437-44; doi: <https://doi.org/10.1016/j.biortech.2011.02.084>.
66. Carrillo-Reyes J, Cortés-Carmona MA, Bárcenas-Ruiz CD, Razo-Flores E. Cell wash-out enrichment increases the stability and performance of biohydrogen producing packed-bed reactors and the community transition along the operation time. *Renewable Energy*. 2016;97:266-73; doi: <https://doi.org/10.1016/j.renene.2016.05.082>.
67. International Standards Organization: ISO 22196:2011, Measurement of antibacterial activity on plastics and other non-porous surfaces. In., vol. 22196:2011; 2011: 15.

68. Filice S, Sciuto EL, Scalese S, Faro G, Libertino S, Corso D, et al. Innovative Antibiofilm Smart Surface against *Legionella* for Water Systems. *Microorganisms*. 2022;10(5); doi: 10.3390/microorganisms10050870.
69. Velez Justiniano Y-A, Carter D, Sandvik E, Stewart P, Goeres D, Sturman P, et al.: Biofilm Management in a Microgravity Water Recovery System. In: 50th International Conference on Environmental Systems. 2021.
70. Choi Y-S, Shim J-J, Kim J-G. Effects of Cr, Cu, Ni and Ca on the corrosion behavior of low carbon steel in synthetic tap water. *Journal of Alloys and Compounds*. 2005;391(1):162-9; doi: <https://doi.org/10.1016/j.jallcom.2004.07.081>.
71. Lee DY, Kim WC, Kim JG. Effect of nitrite concentration on the corrosion behaviour of carbon steel pipelines in synthetic tap water. *Corrosion Science*. 2012;64:105-14; doi: <https://doi.org/10.1016/j.corsci.2012.07.005>.
72. Environmental Protection Agency: National Primary Drinking Water Regulations. In.; 2024.
73. Goodband SJ, Armstrong S, Kusumaatmaja H, Voitchovsky K. Effect of Ageing on the Structure and Properties of Model Liquid-Infused Surfaces. *Langmuir*. 2020;36(13):3461-70; doi: 10.1021/acs.langmuir.0c00059.
74. Banerjee P, Singh M, Sharma V. Biofilm formation: a comprehensive review. *Int J Pharm Res Health Sci*. 2015;3(2):556-60.
75. Donlan RM. Biofilm Formation: A Clinically Relevant Microbiological Process. *Clinical Infectious Diseases*. 2001;33(8):1387-92; doi: 10.1086/322972.
76. Croll SG. Surface roughness profile and its effect on coating adhesion and corrosion protection: A review. *Progress in Organic Coatings*. 2020;148:105847; doi: <https://doi.org/10.1016/j.porgcoat.2020.105847>.
77. Wan H, Song D, Li X, Zhang D, Gao J, Du C. Failure Mechanisms of the Coating/Metal Interface in Waterborne Coatings: The Effect of Bonding. *Materials*. 2017;10(4):397.
78. Miller LA, Buckingham-Meyer K, Goeres DM. Simulated aging of draught beer line tubing increases biofilm contamination. *International Journal of Food Microbiology*. 2024;415:110630; doi: <https://doi.org/10.1016/j.ijfoodmicro.2024.110630>.
79. Environmental Protection Agency: Interim Method for Evaluating the Efficacy of Antimicrobial Surface Coatings. In.; 2020.

80. Yablonski JI, Mancuso SE. Preservative efficacy testing: accelerating the process. *Cosmetics and toiletries*. 2007;122(10):51.
81. Callahan MR, Adam NM, Roberts MS, Garland JL, Sager JC, Pickering KD. Assessment of silver based disinfection technology for CEV and future US spacecraft. *SAE Transactions*. 2007:481-91.
82. Rogers J, Dowsett AB, Dennis PJ, Lee JV, Keevil CW. Influence of Plumbing Materials on Biofilm Formation and Growth of *Legionella pneumophila* in Potable Water Systems. *Applied and Environmental Microbiology*. 1994;60(6):1842-51; doi: 10.1128/aem.60.6.1842-1851.1994.
83. Learbuch KLG, Smidt H, van der Wielen PWJJ. Influence of pipe materials on the microbial community in unchlorinated drinking water and biofilm. *Water research*. 2021;194:116922; doi: <https://doi.org/10.1016/j.watres.2021.116922>.
84. Ren H, Wang W, Liu Y, Liu S, Lou L, Cheng D, et al. Pyrosequencing analysis of bacterial communities in biofilms from different pipe materials in a city drinking water distribution system of East China. *Appl Microbiol Biotechnol*. 2015;99(24):10713-24; doi: 10.1007/s00253-015-6885-6.
85. Liu S, Gunawan C, Barraud N, Rice SA, Harry EJ, Amal R. Understanding, Monitoring, and Controlling Biofilm Growth in Drinking Water Distribution Systems. *Environmental Science & Technology*. 2016;50(17):8954-76; doi: 10.1021/acs.est.6b00835.
86. Agudelo-Vera C, Avvedimento S, Boxall J, Creaco E, de Kater H, Di Nardo A, et al. Drinking Water Temperature around the Globe: Understanding, Policies, Challenges and Opportunities. *Water*. 2020;12(4):1049.
87. Cincotta C, Blokker M, Bragalli C, Kapelan Z. Spatial Analysis of Water Temperature in a Drinking Water Distribution System for Climate Change Adaptation. *Engineering Proceedings*. 2024;69(1):127.
88. World Health Organization. Guidelines for drinking-water quality. *WHO chronicle*. 2011;38(4):104-8.
89. Zlatanovic L, Moerman A, van der Hoek JP, Vreeburg J, Blokker M. Development and validation of a drinking water temperature model in domestic drinking water supply systems. *Urban Water Journal*. 2017;14(10):1031-7; doi: 10.1080/1573062X.2017.1325501.
90. Maryatt B, Smith M: Microbial growth control in the international space station potable water dispenser. In.: 47th International Conference on Environmental Systems; 2017.

91. Montemor MF. Functional and smart coatings for corrosion protection: A review of recent advances. *Surface and Coatings Technology*. 2014;258:17-37; doi: <https://doi.org/10.1016/j.surfcoat.2014.06.031>.
92. Lyon SB, Bingham R, Mills DJ. Advances in corrosion protection by organic coatings: What we know and what we would like to know. *Progress in Organic Coatings*. 2017;102:2-7; doi: <https://doi.org/10.1016/j.porgcoat.2016.04.030>.
93. Lee J, Ray R, Lemieux E, Falster A, Little B. An Evaluation of Carbon Steel Corrosion under Stagnant Seawater Conditions. *Biofouling*. 2004;20(4-5):237-47; doi: 10.1080/08927010400013274.
94. Knisz J, Eckert R, Gieg LM, Koerdts A, Lee JS, Silva ER, et al. Microbiologically influenced corrosion-more than just microorganisms. *FEMS Microbiol Rev*. 2023;47(5); doi: 10.1093/femsre/fuad041.
95. Characklis WG, Cooksey KE. Biofilms and Microbial Fouling. In: Laskin AI, editor. *Advances in Applied Microbiology*. Academic Press; 1983. p. 93-138.
96. Pinto D, Santos MA, Chambel L. Thirty years of viable but nonculturable state research: Unsolved molecular mechanisms. *Critical Reviews in Microbiology*. 2015;41(1):61-76; doi: 10.3109/1040841X.2013.794127.
97. Redfern J, Cunliffe AJ, Goeres DM, Azevedo NF, Verran J. Critical analysis of methods to determine growth, control and analysis of biofilms for potential non-submerged antibiofilm surfaces and coatings. *Biofilm*. 2024;7:100187; doi: <https://doi.org/10.1016/j.biofilm.2024.100187>.
98. Parker AE, Christen JA, Lorenz L, Smith H. Optimal surface estimation and thresholding of confocal microscope images of biofilms using Beer's Law. *Journal of Microbiological Methods*. 2020;174:105943; doi: <https://doi.org/10.1016/j.mimet.2020.105943>.
99. Haas B, James S, Parker AE, Gagnon M-C, Goulet N, Labrie P. Comparison of quantification methods for an endoscope lumen biofilm model. *Biofilm*. 2023;6:100163; doi: <https://doi.org/10.1016/j.biofilm.2023.100163>.
100. Pierson DL, Botkin DJ, Bruce RJ, Castro VA, Smith MJ, Oubre CM, et al.: Microbial monitoring of the international space station. In: 8th International Workshop on Space Microbiology. 2013.
101. Williamson J, Kayatin M, Nguyen HN, Hudson EK, Wallace WT, Williams S, et al.: Overview of the International Space Station's Water and Cabin Air Quality: A Five-Year Status. In: 53rd International Conference on Environmental Systems (ICES). International Conference on Environmental Systems; 2024.

102. Klaus D, Simske S, Todd P, Stodieck L. Investigation of space flight effects on *Escherichia coli* and a proposed model of underlying physical mechanisms. *Microbiology*. 1997;143(2):449-55; doi: <https://doi.org/10.1099/00221287-143-2-449>.
103. Crabbé A, Schurr MJ, Monsieurs P, Morici L, Schurr J, Wilson JW, et al. Transcriptional and Proteomic Responses of *Pseudomonas aeruginosa* PAO1 to Spaceflight Conditions Involve Hfq Regulation and Reveal a Role for Oxygen. *Applied and Environmental Microbiology*. 2011;77(4):1221-30; doi: 10.1128/aem.01582-10.
104. Nickerson CA, Ott CM, Wilson JW, Ramamurthy R, Pierson DL. Microbial responses to microgravity and other low-shear environments. *Microbiol Mol Biol Rev*. 2004;68(2):345-61; doi: 10.1128/MMBR.68.2.345-361.2004.
105. Wilson JW, Ott CM, Bentrup KHZ, Ramamurthy R, Quick L, Porwollik S, et al. Space flight alters bacterial gene expression and virulence and reveals a role for global regulator Hfq. *Proc Natl Acad Sci U S A*. 2007;104(41):16299-304; doi: 10.1073/pnas.0707155104.
106. Rosenzweig JA, Abogunde O, Thomas K, Lawal A, Nguyen YU, Sodipe A, et al. Spaceflight and modeled microgravity effects on microbial growth and virulence. *Appl Microbiol Biotechnol*. 2010;85(4):885-91; doi: 10.1007/s00253-009-2237-8.
107. Searles SC, Woolley CM, Petersen RA, Hyman LE, Nielsen-Priess SM. Modeled Microgravity Increases Filamentation, Biofilm Formation, Phenotypic Switching, and Antimicrobial Resistance in *Candida albicans*. *Astrobiology*. 2011;11.
108. Feng Y, Chen J, Liu Q, Chen N, Zou S, Yao Q, et al. Low-shear modeled microgravity accelerated the degradation of passive films of aluminium alloy by *Bacillus cereus* through increased extracellular polymeric substances. *Applied Surface Science*. 2024;655:159618; doi: <https://doi.org/10.1016/j.apsusc.2024.159618>.
109. McLean RJC, Cassanto JM, Barnes MB, Koo JH. Bacterial biofilm formation under microgravity conditions. *FEMS Microbiology Letters*. 2001;195(2):115-9; doi: 10.1111/j.1574-6968.2001.tb10507.x.
110. Pyle B, McFeters G. Bacterial Growth on surfaces and in suspensions. Biorack on Spacehab- Biological experiments on three Shuttle-to-Mir missions(A 00-30676 07-51), Noordwijk, Netherlands, European Space Agency, 1999. 1999:95-9.
111. Hupka M, Kedia R, Schauer R, Shepard B, Granados-Presa M, Vande Hei M, et al. Morphology of *Penicillium rubens* Biofilms Formed in Space. *Life (Basel)*. 2023;13(4); doi: 10.3390/life13041001.

112. Lynch SV, Mukundakrishnan K, Benoit MR, Ayyaswamy PS, Matin A. *Escherichia coli* biofilms formed under low-shear modeled microgravity in a ground-based system. *Appl Environ Microbiol.* 2006;72(12):7701-10; doi: 10.1128/aem.01294-06.
113. Flores P, Luo J, Mueller DW, Muecklich F, Zea L. Space biofilms - An overview of the morphology of *Pseudomonas aeruginosa* biofilms grown on silicone and cellulose membranes on board the international space station. *Biofilm.* 2024;7:100182; doi: 10.1016/j.biofilm.2024.100182.
114. Kim W, Tengra FK, Young Z, Shong J, Marchand N, Chan HK, et al. Spaceflight Promotes Biofilm Formation by *Pseudomonas aeruginosa*. *PLOS.* 2013; doi: 10.1371/journal.pone.0062437.
115. Wang H, Yan Y, Rong D, Wang J, Wang H, Liu Z, et al. Increased biofilm formation ability in *Klebsiella pneumoniae* after short-term exposure to a simulated microgravity environment. *Microbiologyopen.* 2016;5(5):793-801; doi: 10.1002/mbo3.370.
116. Flores P, Luo J, Mueller DW, Muecklich F, Zea L: Space Biofilms- An Overview of the Morphology of *Pseudomonas aeruginosa* Biofilms Grown on Silicone and Cellulose Membranes on board the International Space Station. In: International Astronautical Congress. Paris, France: International Astronautical Federation; 2022.
117. Hoson T, Kamisaka S, Masuda Y, Yamashita M, Buchen B. Evaluation of the three-dimensional clinostat as a simulator of weightlessness. *Planta.* 1997;203(1):S187-S97; doi: 10.1007/PL00008108.
118. Herranz R, Anken R, Boonstra J, Braun M, Christianen PC, de Geest M, et al. Ground-based facilities for simulation of microgravity: organism-specific recommendations for their use, and recommended terminology. *Astrobiology.* 2013;13(1):1-17; doi: 10.1089/ast.2012.0876.
119. Klaus DM. Clinostats and bioreactors. *Gravit Space Biol Bull.* 2001;14(2):55-64.
120. Hammond TG, Hammond JM. Optimized suspension culture: the rotating-wall vessel. *American Journal of Physiology-Renal Physiology.* 2001;281(1):F12-F25; doi: 10.1152/ajprenal.2001.281.1.F12.
121. Van Loon JJ. Some history and use of the random positioning machine, RPM, in gravity related research. *Advances in Space research.* 2007;39(7):1161-5.
122. Nishimura Y. Technology using simulated microgravity. *Regenerative Therapy.* 2023;24:318-23; doi: <https://doi.org/10.1016/j.reth.2023.08.001>.

123. An R, Lee JA. CAMDLES: CFD-DEM Simulation of Microbial Communities in Spaceflight and Artificial Microgravity. *Life (Basel)*. 2022;12(5); doi: 10.3390/life12050660.
124. Vaz-Moreira I, Nunes OC, Manaia CM. Ubiquitous and persistent Proteobacteria and other Gram-negative bacteria in drinking water. *Science of The Total Environment*. 2017;586:1141-9; doi: <https://doi.org/10.1016/j.scitotenv.2017.02.104>.
125. Lin YE, Stout JE, Victor LY. Controlling Legionella in hospital drinking water: an evidence-based review of disinfection methods. *Infection Control & Hospital Epidemiology*. 2011;32(2):166-73.
126. Kool JL, Bergmire-Sweat D, Butler JC, Brown EW, Peabody DJ, Massi DS, et al. Hospital characteristics associated with colonization of water systems by *Legionella* and risk of nosocomial legionnaires' disease: a cohort study of 15 hospitals. *Infection Control & Hospital Epidemiology*. 1999;20(12):798-805.
127. Flores P, Schauer R, McBride SA, Luo J, Hoehn C, Doraisingam S, et al. Preparation for and performance of a *Pseudomonas aeruginosa* biofilm experiment on board the International Space Station. *Acta Astronautica*. 2022;199:386-400; doi: <https://doi.org/10.1016/j.actaastro.2022.07.015>.

CHAPTER THREE

ANTIMICROBIAL COATING EFFICACY FOR PREVENTION
OF *PSEUDOMONAS AERUGINOSA* BIOFILM GROWTH ON
ISS WATER SYSTEM MATERIALS

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

Author: Madelyn K. Mettler

Contributions: Conducted CDC bioreactor assays, performed microscopy, generated figures, and drafted the manuscript.

Author: Ceth W. Parker

Contributions: Procured and prepared materials (with and without coating), performed microscopy, and drafted the manuscript.

Co-Author: Kasthuri Venkateswaran

Contributions: Designed study, evaluated and discussed results, and helped write manuscript.

Co-Author: Brent M. Peyton

Contributions: Designed study, evaluated and discussed results, and helped write manuscript.

Manuscript Information

Madelyn K. Mettler, Ceth W. Parker, Kasthuri Venkateswaran, and Brent M. Peyton

Frontiers in Microbiology

Status of Manuscript:

- ☐ Prepared for submission to a peer-reviewed journal
- ☐ Officially submitted to a peer-reviewed journal
- ☐ Accepted by a peer-reviewed journal
- ☒ Published in a peer-reviewed journal

Frontiers

Antimicrobials, Resistance, and Chemotherapy Volume 13

10.3389/fmicb.2022.874236

Abstract

Biofilms can lead to biofouling, microbially induced corrosion, physical impediment and eventual loss in function of water systems, and other engineered systems. The remoteness and closed environment of the International Space Station (ISS) make it vulnerable to unchecked biofilm growth; thus, biofilm mitigation strategies are crucial for current ISS operation and future long duration and deep-space crewed missions. In this study, a space flown bacterial strain of *Pseudomonas aeruginosa* (PA14) was used as a model organism for its ability to form biofilms. Additionally, a novel antimicrobial coating's ability to reduce biofilm accumulation on stainless steel, Teflon, titanium, and Inconel (all used in the ISS water treatment and handling systems) was analyzed. Coated materials demonstrated reductions of *P. aeruginosa* biofilm across all materials when tested in a continuous flow system with tryptic soy broth medium. However, the coating lost efficacy in potato dextrose broth medium. These findings were corroborated via scanning electron microscopy. This study illustrates the fundamental importance of using multiple approaches to test antibiofilm strategies, as well as the specificity in which conditions such strategies can be implemented.

Introduction

Biofilms are complex interconnected communities of surface attached biological materials containing the majority of microorganisms on Earth (Wingender and Flemming, 2011). Biofilms confer many advantages to their constituent microorganisms over planktonic cells, including increased resistance to antimicrobials and environmental stresses, easier metabolite interchange (Santos et al., 2018), and increased genetic material exchange via horizontal gene

and plasmid transfer (Madsen et al., 2012). These characteristics impart survival advantages to biofilm communities making their control more difficult than planktonic microorganisms. Along with medical implant rejection, catheter infections, and tooth decay (Aparna and Yadav, 2008), biofilms negatively impact society through biofouling (Bixler and Bhushan, 2012). Biofouling is the colonization of any solid material by microorganisms that increases the corrosion rate, fluid drag, and subsequent system contamination (Characklis and Cooksey, 1983) with many built environments susceptible to biofouling (Gule et al., 2016). Bacteria and other microorganisms can increase the rate and extent of corrosion, causing microbial-induced corrosion (MIC; Kitsutani et al., 1970). This MIC of metal surfaces results from interactions between susceptible surfaces and microbial products including acids, volatile compounds, and electrons (Beech and Sunner, 2004). Biofilms, biofouling, and MIC have a large economic impact, with estimated global cost above \$1 billion annually (Rajala et al., 2015; Little et al., 2020).

While biofilms are ubiquitous on Earth (Flemming and Wuertz, 2019), their reach extends into space in microgravity environments of human-made spacecraft. The decommissioned Mir space station was heavily contaminated with biofilms (Klintworth et al., 1999; Kim et al., 2013), and biofouling aboard the International Space Station (ISS) resulted in obstruction of the water processing assembly (Carter, 2009). The microgravity environment can lead to increased production of extracellular polymeric substances, virulence, antimicrobial resistance, shorter lag and longer exponential phases of microorganisms, and biofilms (Klaus et al., 1997; Nickerson et al., 2004; Lynch et al., 2006; Mauclaire and Egli, 2010; Rosenzweig et al., 2010; Searles et al., 2011). The traditional cleaning methods of disassembly, harsh chemicals,

purging water supplies, and/or replacing compromised hardware to mitigate biofilm growth are incredibly difficult (and sometimes impossible) in a confined spacecraft in microgravity. These issues will become more exacerbated as the National Aeronautics and Space Administration (NASA) begins longer missions to send humans to the Moon and Mars, making resupply and replacement parts more costly and harder to execute. Engineers have begun to design and construct the Lunar Gateway and next-generation Lunar landers; thus, it is critical that antibiofilm technologies be tested and successfully incorporated into deep- space habitats.

NASA has ongoing interest in mitigating biofilm growth to improve the viability of long-term space missions. This project sought to evaluate the four materials most used in the ISS water treatment system (stainless steel, Teflon, titanium, and Inconel) and thus most at risk for detrimental biofilm accumulation (Callahan et al., 2007). These materials are also being considered as primary components for constructing NASA's Lunar Gateway water treatment and processing systems. The materials are also common in other parts of the craft. The objectives of this study were to characterize and quantify biofilm formation using a model bacterium in a continuous flow system using the Center for Disease Control (CDC) bioreactor and test the efficacy of a biocidal coating to prevent or reduce biofilm accumulation. The coating is a patented organosilane, surface penetrating formula. In the presence of hydroxyl groups at the surface of glass, minerals, or metals (e.g., aluminum and steel), silanols will form a stable Si bond. This is the key chemistry that allows silanes to function as valuable surface-treating/protecting or "coupling" agents. Four materials used in the ISS were challenged with the known biofilm forming model organism, *Pseudomonas aeruginosa* (previously space flown strain PA14). *P. aeruginosa* has been very well studied in biofilm systems and is a good

benchmark for comparison to other research (Klintworth et al., 1999; Kim et al., 2013). This study will be useful for developing countermeasures to safeguard current and future spacecraft and their inhabitants.

Materials and Methods

Microorganisms Used

Pure culture of reference strain *P. aeruginosa* PA14 was obtained from Luis Zea at the BioServe Space Technologies (University of Colorado Boulder, United States). This bacterium is a Gram- negative, motile, and facultative aerobe. *P. aeruginosa* PA14 is a strain that has been previously flown to and cultured on the ISS in other experiments (Kim et al., 2013). Separate vials of stock cultures were maintained in glycerol at -80°C to ensure a reproducible inoculum throughout the experiment.

CDC Bioreactor Preparation

The experiments were conducted in CDC biofilm reactors (BioSurface Technologies, Bozeman, MT) in a continuous flow system (Figure 1A) The ISS materials evaluated were purchased from a commercial vendor (BioSurface Technologies) and consisted of 12.7 mm diameter cylindrical coupons of 316 L stainless steel, Teflon, Inconel 718 (a nickel-chromium alloy), and Ti-6Al4V titanium (Figure 1B). The ISS materials were purchased and precision cleaned at the Jet Propulsion Laboratory (JPL) following NASA standard practices developed for cleaning spacecraft components as previously described in Venkateswaran et al. (2004). Briefly, cleaning with a water-based detergent was followed by solvent immersion with ultrasonic agitation, prior to application of antibiofilm coating, and/or use in the CDC bioreactor. Once

precision cleaned, the materials were sent to a commercial vendor for application of the antimicrobial coating (Figure 1C).

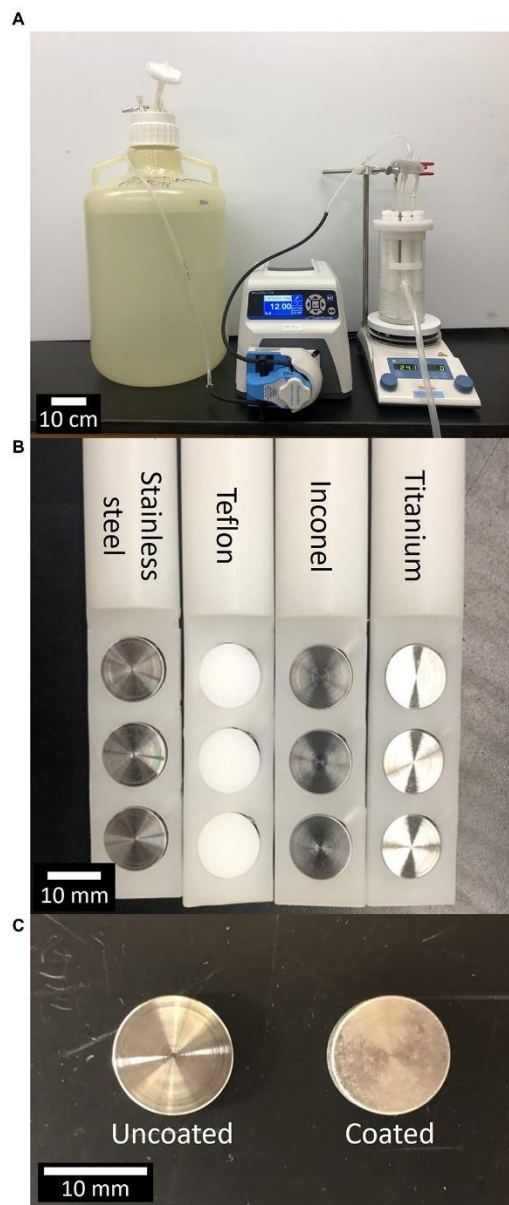


Figure 1. Center for Disease Control (CDC) bioreactor, material types, uncoated vs. coated materials. (A) Experimental setup consisting of 20 L carboy, peristaltic pump, and CDC Biofilm Reactor atop a stir plate. (B) Close-up of materials loaded into CDC Biofilm Reactor rods, from left to right, materials are stainless steel, Teflon, Inconel, and titanium. (C) Close-up of two titanium materials without coating (left) and with antimicrobial coating (right).

The CDC reactor, including the rods without ISS materials, was assembled and autoclaved empty (121°C and 20 PSI for 20 min) to ensure sterility. In a biosafety cabinet, the ISS materials were aseptically loaded into rods which were then submerged in isopropanol (IPA, 99%) for approximately 30 s, allowed to air dry for an additional 30 s, and replaced in the reactor. One trial run was performed using coated materials without isopropanol sterilization to ensure the sterilization process did not affect the efficacy of the coating. It was found that the materials sterilized by submersion in IPA fostered less microbial accumulation than coated materials that were not sterilized prior to use in the reactor (data not shown). All four materials were evaluated in triplicate simultaneously in the same reactor with each rod holding three coupons of the same material type. When uncoated and coated materials were initially tested together, the uncoated materials showed a reduction in cell density compared to uncoated materials tested alone. Further, coated materials tested in the same reactor with uncoated materials experienced an increase in cell density compared to coated materials tested alone (data not shown). Therefore, the remaining experiments (data presented in this paper) were completed with uncoated and coated materials tested in separate CDC bioreactors.

Microorganism Preparation for CDC Bioreactor

P. aeruginosa was grown on tryptic soy agar (TSA) and incubated for 24 ± 2 h at 37°C to isolate individual colonies. Two colonies were used to inoculate a flask containing 100 ml of 300 mg/L TSB or 100 ml of 24 g/L PDB. The *P. aeruginosa* inoculum flasks were placed in an incubator-shaker at 150 rpm and 37°C for 24 ± 2 h and the colony forming units (CFU) were counted (TSB: $2.71 \times 10^8 \pm 1.75 \times 10^8$ CFU/ml, PDB: $1.01 \times 10^9 \pm 6.43 \times 10^8$ CFU/ml,

average $\pm$ SD). To inoculate the CDC reactor, 1 ml of this inoculum was aseptically introduced to approximately 320 ml of sterile liquid medium such that initial cell counts in the reactor were approximately two orders of magnitude less than the cell counts of the inoculum flasks.

Biofilm Growth

The method for operating the CDC biofilm reactors and growing the biofilms was based on the ASTM Standard Practice E31610-18 for preparing a *P. aeruginosa* or *Staphylococcus aureus* biofilm using the CDC Biofilm Reactor (ASTM International, 2018). Sterile liquid medium consisting of 300mg/L TSB or 24 g/L PDB was added to the sterile reactor, and as described above, 1ml of inoculum was introduced through the inoculation port on the CDC reactor lid. The reactor was placed on a stir plate at 125rpm and operated in batch mode with no flow for 24 ± 2 h at room temperature. The reactors were operated in batch mode to allow cells to attach to the surfaces of the reactor and test materials. At $t = 0$ h, a continuous flow of fresh sterile medium of 100mg/L TSB or 600mg/L PDB was introduced to the reactor at a flow rate to create a liquid residence time of 30 min. The volumetric flow rate of the influent medium was sufficiently high (dilution rate greater than maximum specific growth rate) to wash planktonic cells out of the reactor before they could replicate, while providing fresh medium to the attached biofilm cells. Across all three reactors used, flow rate was set to 10.67 ± 0.12 ml/min (average $\pm$ SD).

CDC Bioreactor Sampling

Planktonic cells were sampled from the reactor liquid at the start of continuous flow ($t = 0$ h) and at various sampling time points. Approximately 5 ml of reactor broth (bulk fluid) was removed with a serological pipette from an empty reactor rod slot. Due to the hydromechanics of

continuously stirred tank reactors such as this one, the effluent media concentrations and cell densities are the same as in the bulk fluid. Planktonic cell samples were vortex mixed for 30 s prior to serial dilution and measured as CFU on TSA. Plates were incubated at 37°C for 24 ± 2 h before counting CFU.

For biofilm measurements, rods were removed at 24 ± 2 h and 48 ± 2 h after the start of continuous flow, gently rinsed in 30 ml of 0.01 M sterile phosphate buffered saline (PBS; 0.01 M PO_4 ; 0.137 M NaCl; and 0.0027 M KCl, adjusted to pH 7.4 at 25°C) to remove loosely attached cells, and test materials were placed into 50 ml sterile conical vials containing 10 ml of sterile PBS. The removed rods were replaced with sterile “dummy” rods to avoid altering the fluid dynamics of the reactor. Biofilm was disaggregated via an alternating vortex and sonication series of 30 s intervals beginning and ending with vortex mixing for a total of 2 min and 30 s. Disaggregated biofilm was serially diluted in PBS and plated on TSA for enumeration.

Statistical Analysis

Statistical analysis of biofilm and planktonic cell counts from the CDC biofilm reactor sampling was carried out using MiniTab 19.2 (MiniTab, LLC). Statistical significance was determined via mixed effects ANOVA to determine the effects of the sampling time, experiment number (replicate), and material type on log reduction. The biofilm and planktonic cells were enumerated *via* CFU. The CFUs were converted to log densities for statistical evaluation. The ability of the coating and materials to reduce biofilm growth was evaluated on the basis of log reductions. To calculate the log reduction, the log density of the treatment experiment (coating) was subtracted from the log density of the control experiment. A negative log reduction indicates

the coating resulted in increased CFUs. Similar calculations were made for planktonic cell growth.

Scanning Electron Microscopy

The materials tested were preserved using 4% paraformaldehyde (PFA) prepared in 0.1 M PBS for scanning electron microscopy (Danko et al., 2020). Following the rinse step after removal from the reactor, coupons were fully immersed in a chilled 4% PFA and incubated at 4°C for 10 min and then, PFA was removed. The test material was rinsed thrice with chilled 0.1 M PBS and incubated at 4°C for 10 min between each rinse. After the third rinse, the vial with test material was filled completely with 0.1 M PBS and stored at 4°C until evaluated for scanning electron microscopy (SEM).

Biofilms grown in TSB and uninoculated coupons were imaged at the JPL. Fixed test coupons were transferred into a 24 well uncoated plate (Corning Inc., Corning, NY, United States) and dehydrated in IPA series with increasing IPA% as follows: 50%, 70%, 80%, 90%, 95%, and 100%, with the final 100% IPA replaced three times for 10 min each. Dehydrated test materials were stored at 4°C. The samples were then dried in a Tousimis Automegasamdri 915B critical point dryer (Rockville, MD, United States). Samples were adhered with carbon tape to SEM (Danko et al., 2020) stubs (Ted Pella Inc., Redding, CA, United States) and sputter coated using a Gold–Palladium target with an Anatech Hummer (Sparks, NV, United States) sputterer. A FEI Quanta 200F microscope (Thermo Fisher, Waltham, MA, United States) was used to capture SEM micrographs.

Unprocessed coupons and PDB biofilms were imaged at the Center for Biofilm Engineering (CBE) with a Zeiss SUPRA 55VP field emission SEM (Jena, Germany). Coupons

with biofilm were critical point dried using a Tousimis SAMDRI- 795. Prior to drying, the fixed coupons went through the same dehydration series as described above; however, ethanol was used rather than IPA due to the requirements by the CBE critical point dryer. The Teflon surfaces were sputter coated using an Iridium target with a Pella 108 Carbon Coater (Ted Pella Inc., Redding, CA, United States) prior to imaging.

Results

Biofilm Measurement Using

Continuous Flow CDC Bioreactor

Tryptic soy broth (TSB)-grown biofilm and planktonic cell densities of *P. aeruginosa* for uncoated materials, coated materials, and log reductions are presented in Figure 2. The coating significantly reduced biofilm accumulation of *P. aeruginosa* (99.999%–99.9999%) over 24 and 48 h on all four materials as compared to uncoated materials. Uncoated and coated Teflon allowed the most *P. aeruginosa* biofilm accumulation at 24 and 48 h. Uncoated titanium and coated Inconel fostered the least accumulation. In TSB, the coating performed similarly well across all four materials and at each time point. Reactor broth was sampled at the start of continuous flow and at later time points to measure planktonic cells (Figures 2D–F). Coated materials in the reactor with TSB significantly decreased *P. aeruginosa* planktonic cell density throughout the experiment. The data in Figures 2D–F show the cell density at the time flow to the reactor was turned on (0 h) and after 24 and 48 h of operation as well as the density of the initial *P. aeruginosa* inoculum. The biocidal property of the coating had a strong negative effect on the *P. aeruginosa* planktonic cell numbers even at the start of flow to the reactor (Figure 2E).

After 24 or 48 h of operation with coated materials, *P. aeruginosa* reactors revealed reduction of 99.9%–99.99% in planktonic colony counts when compared to uncoated materials.

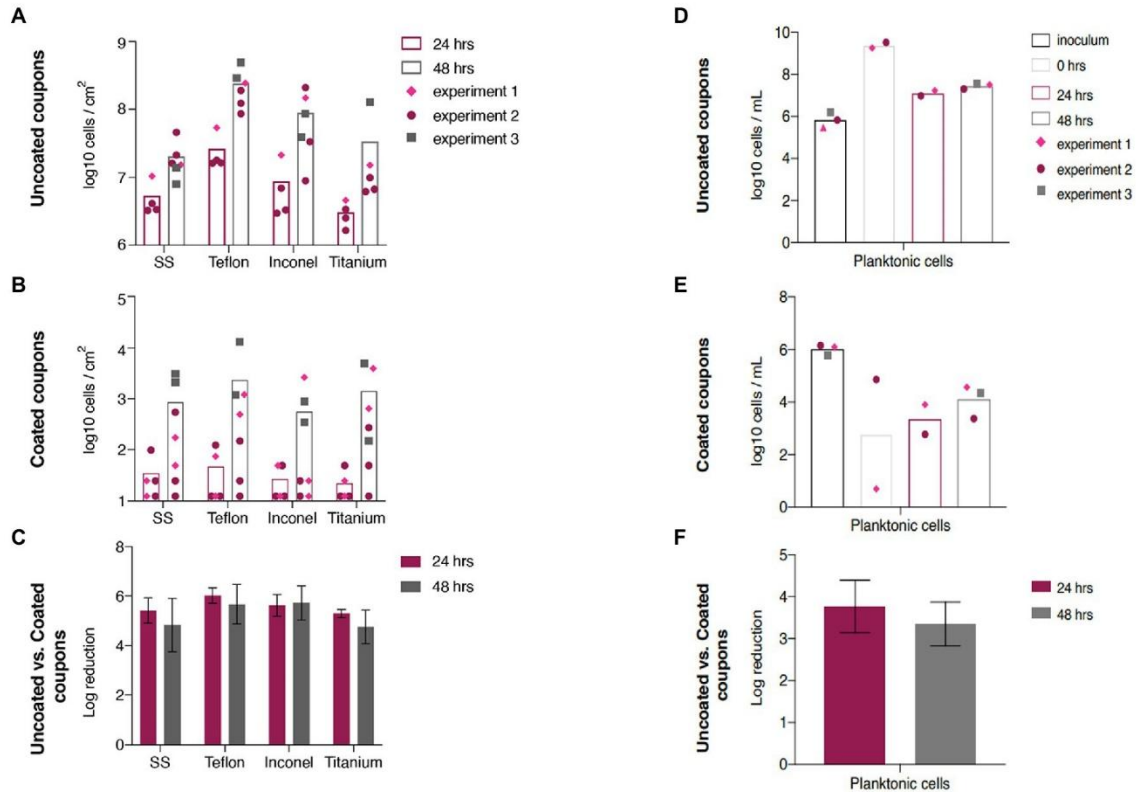


Figure 2. Tryptic soy broth (TSB)-grown *Pseudomonas aeruginosa* biofilm accumulation and planktonic cell density. TSB-grown *P. aeruginosa* biofilm cell densities at 24 and 48 h after start of continuous flow on (A) uncoated and (B) coated materials. (C) Log reduction of TSB-grown biofilm accumulation on materials at 24 and 48 h. Inoculum and planktonic cell densities at 0, 24, and 48 h after start of continuous flow for reactors with (D) uncoated and (E) coated materials. (F) Log reduction of planktonic cells at 24 and 48 h. Error bars represent 95% CI.

The potato dextrose broth (PDB)-grown biofilm and planktonic cell densities for uncoated Titanium, coated Titanium, and log reductions are presented in Figures 3A–C. In this system, the coating does not reduce biofilm accumulation. The log reduction after 24 h was -0.87 , indicating greater biofilm accumulation on the coated titanium than on the uncoated titanium. The log reduction was less negative after 48 h (-0.45) but still indicated increased

accumulation on the coated titanium. This trend was mirrored by the planktonic cell density (Figures 3D–F) in the reactor broth at 24 and 48 h after start of continuous flow where log reductions were -0.63 and -0.17 , respectively.

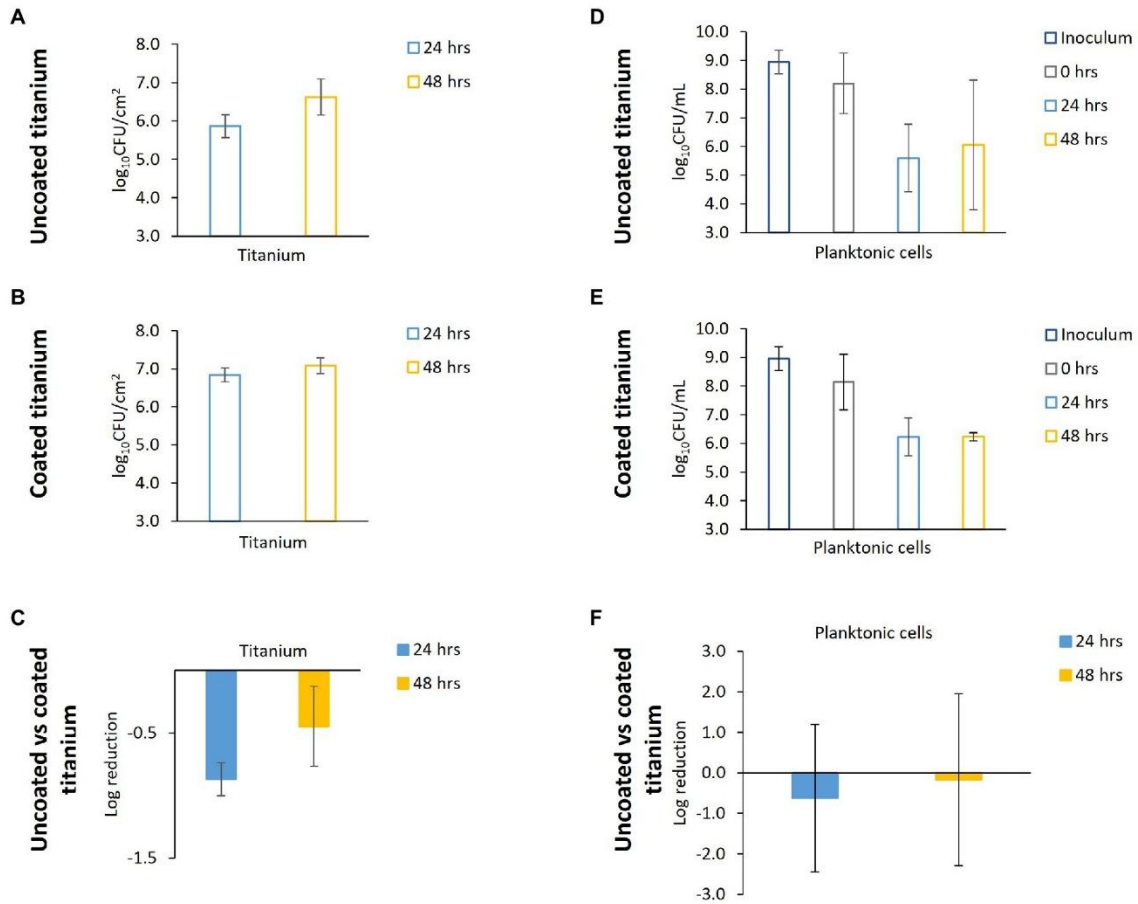


Figure 3. Potato dextrose broth (PDB)-grown *P. aeruginosa* biofilm accumulation and planktonic cell density. PDB-grown *P. aeruginosa* biofilm cell densities at 24 and 48 h after start of continuous flow on (A) uncoated and (B) coated titanium. (C) Log reduction of TSB-grown biofilm accumulation on titanium at 24 and 48 h. Inoculum and planktonic cell densities at 0, 24, and 48 h after start of continuous flow for reactors with (D) uncoated and (E) coated titanium. (F) Log reduction of planktonic cells at 24 and 48 h. Negative log reductions indicate the coating resulted in higher cell densities. Error bars represent 95% CI.

SEM Analysis of CDC Bioreactor Materials

SEM images of coated and uncoated coupons were taken throughout the experiments, including unexposed coupons direct from manufacturing, uninoculated coupons exposed to the SEM preservation and preparation process, and those exposed to *P. aeruginosa*. SEM images of Titanium coupons are presented in Figure 4. Stainless steel, Teflon, and Inconel SEM images are in Figure 5. Coupons not exposed to the SEM preservation and preparation process were imaged to determine potential effects of the preparation process on the surfaces (Figures 4A,B; Supplementary Figures 1A1–2, B1–2, C1–2). Images of uninoculated materials present smooth surfaces relatively free of debris and deformity (Figures 4C,D; Supplementary Figures 1A3–4, B3–4, C3–4), though it appears the coating detached in some areas (Supplementary Figure 1A4). Striations and concentric rings were observed showing evidence of the manufacturing process.

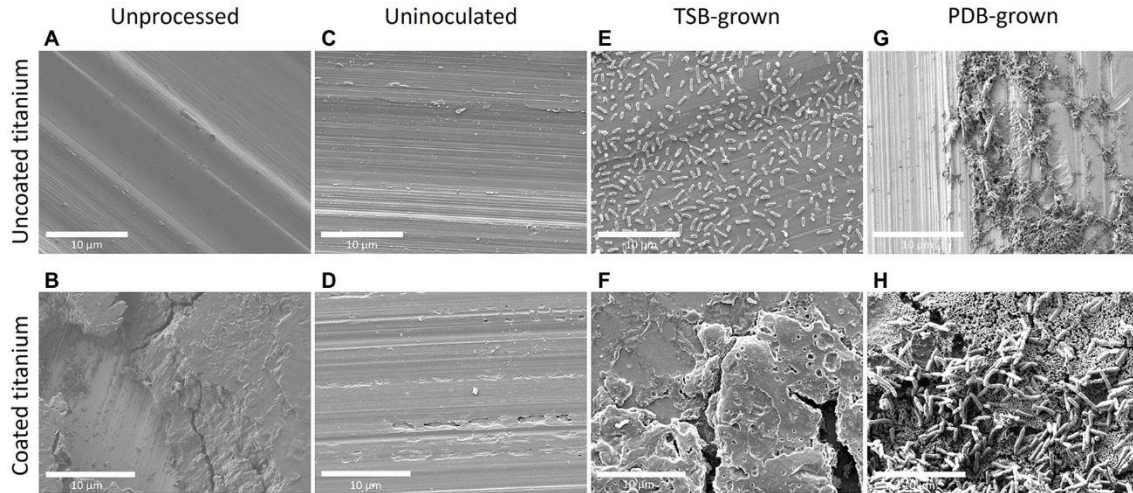


Figure 4. Scanning electron microscopy (SEM) imaging of *P. aeruginosa* biofilm formation on titanium after 48 h. Images (A,B) represent uncoated and coated titanium that were unprocessed (not exposed to the reactor, bacteria, or SEM preparation steps). Images (C,D) represent uncoated and coated titanium that were not exposed to the reactor or bacteria but went through the SEM preparation steps at the JPL. Images (E,F) represent uncoated and coated titanium that came from a reactor that utilized TSB medium while (G,H) came from a reactor with PDB. Biofilms on uncoated materials hosted numerous cells with small amounts of dried EPS attaching the cells to each other and to the surface.

SEM analysis of CDC bioreactor *P. aeruginosa* grown in TSB for 48 h demonstrated production of extracellular polymeric substances (EPS) and the formation of cell agglomerations on uncoated surfaces. This biofilm morphology was observed across all uncoated materials (Figures 4E, 5C,E) except for stainless steel, which had a much lower cell density (Figure 5A). Coated materials had substantially decreased cell abundance across all material types, with only a few isolated attached cells identified (Figures 4F, 5B,D,F).

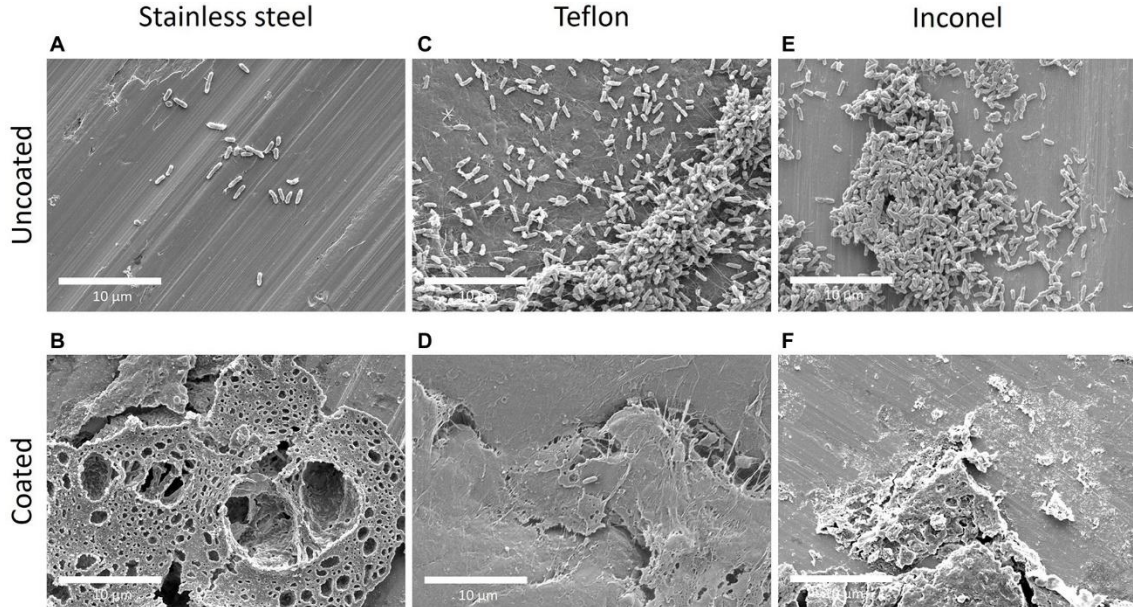


Figure 5. SEM imaging of TSB-grown *P. aeruginosa* biofilm on stainless steel, Teflon, and Inconel after 48 h. Images (A,B) represent uncoated and coated stainless steel; (C,D) represent uncoated and coated Teflon; and (E,F) represent uncoated and coated Inconel. All biofilms were grown in reactors with TSB as nutrient medium. The coated stainless steel demonstrates the presence of coating and coating debris.

SEM images of biofilms grown in PDB for 48 h present similar characteristics to the TSB-grown biofilms. However, there were large biofilm agglomerations present on coated surfaces as well as uncoated surfaces. The titanium is clearly visible on the uncoated coupon in addition to cells clumped together (Figure 4G). On the coated coupon, the titanium is not visible but there is evidence of the coating cracking and lifting off of the surface beneath the attached cells (Figure 4H).

Discussion

Along with testing a variety of materials simultaneously, CDC bioreactors grow biofilms in a high shear environment to allow for robust biofilm growth. The coating selected here previously showed high antimicrobial efficacy in hospital settings (Perez et al., 2015; Fitton et

al., 2017) and exhibited grade 1 toxicity; indicating the coating is below the grade 2 cytotoxic agent threshold, and thus is safe for human contact (Baxa et al., 2011). Since this is a proprietary commercial product, the composition is unfortunately unavailable.

The Environmental Protection Agency details performance test guidelines for antimicrobials of varying types and applications (Environmental Protection Agency, 2015). These guidelines include a 3-log reduction and served as a reference point to evaluate coating performance in tests presented here. The present study included direct microorganism contact with surfaces for 48–72 h (including 24 h spent in batch phase) that still exhibited a 5–6 log reduction of TSB-grown *P. aeruginosa* biofilm on coated surfaces compared to uncoated controls on all four ISS materials (Figure 2C). The mixed effects revealed by the ANOVA show that the sampling time, experiment number (replicate), and material type had no effect on the log reduction. The log reductions from 24 to 48 h were not significantly different. There was no influence from the experiment number (replicate) on the log reduction. Lastly, the log reductions from material to material were similar, meaning the coating performed similarly across all materials. As the material did not affect the efficacy of the coating, only one material was chosen for the PDB tests. Titanium was chosen as there was the greatest abundance of coated and uncoated coupons remaining from previous experiments. Unlike the TSB-grown biofilms, the PDB-grown *P. aeruginosa* biofilm accumulation was not reduced by the antimicrobial coating (Figure 3C). PDB is essentially made by boiling potatoes, dehydrating the leached nutrients, and supplementing with dextrose. It is possible that the starches found in potatoes interact with the coating in a detrimental manner. The starches could be forming a protective layer on top of the coating, preventing the biocidal action (“carbon- chain lances”) from inhibiting biofilm

accumulation in PDB as seen by the negative log reductions in Figure 3C. It is possible that *P. aeruginosa* forms more resistant biofilms in PDB compared to TSB and thus, the coating did not reduce biofilm accumulation. TSB and PDB have different pH (7.1–7.5 and –5.1; respectively), and although not specifically addressed by the vendor as being an issue, pH may alter the effectiveness of the surface coating’s “carbon-chain lances.” Additionally, it has been demonstrated that changes in pH can alter biofilm forming ability (Jones et al., 2015). We used the TSB and PDB growth media at their native pH as to avoid the addition of buffering salts, which may have compounded the issue by interacting with the surface coatings and causing differential biocidal activity. A minor limitation of this study is that we did not perform a full test matrix across all pH and media types to characterize the changes within the biofilms on uncoated and coated surfaces; however, this approach would have been outside the scope of this paper, and our results that the antimicrobial surface coating is as drastically impacted by media type (regardless of pH) are a significant finding.

Extended tests are needed to determine long-term antimicrobial coating efficacy in TSB. In addition, influence of inoculum cell density on antimicrobial coating effectiveness should be tested. Starting cell densities tested here were 10^5 – 10^6 CFU/ml. Coatings have been shown to reduce biofilm accumulation on many surfaces; however, often once a number of microbes attach to a surface, the coating quickly becomes ineffective due to establishment of a conditioning layer (Zea et al., 2020). This may help explain the increase in *P. aeruginosa* CFU/cm² observed on coated materials from 24 to 48 h. The SEM results demonstrate a similar pattern of biofilm abundance across material types and coatings as CFU analysis for both TSB and PDB-grown biofilms (Figures 4, 5).

The coating had very different effects on viable *P. aeruginosa* biofilms grown in TSB and PDB; largely decreasing *P. aeruginosa* CFU in TSB with little effect on CFU in PDB. The antimicrobial coating is a charged substance that imparts a positive charge to the substratum surface (ISS materials) via a nitrogen compound which attracts negatively charged microbes. The biocidal coating then works by penetrating and damaging cell envelopes with positively charged nitrogen on a carbon- chain “lance.” This penetration can disrupt cellular proton motive force and rupture cell membranes leading to lysis and death (Murray et al., 2017). *P. aeruginosa* is a Gram- negative bacterium and its cell envelope consists of an inner and outer phospholipid bilayer separated by a thin peptidoglycan layer measuring 20–30 nm in total thickness (Salton and Kim, 1996). In the TSB system, the coating was likely uninhibited, thus resulting in penetration of the cell membrane and consequent biocidal effects from the coating. However, in the PDB system, it is possible that the starch from the potatoes created a protective carpet that the bacteria were able to attach to without being lanced by the coating’s carbon chains.

Although CDC bioreactors create conditions that mimic biofilm inducing environments, they cannot produce an identical spaceflight environment including microgravity, increased radiation, and CO₂ concentration (Huang et al., 2018). They additionally cannot model spaceflight environments not fully submerged in flowing water where biofilm formation might still be of concern. Such limitations can be addressed with future experiments using different methods to grow biofilms, including simulated microgravity reactors and intermittent submersion of test materials (filling and draining of reactors). Studies such as this one aim to gain an understanding of how antibiofilm regimens work in simplified normal gravity systems to then move promising regimens into more complicated systems that closely mimic the conditions

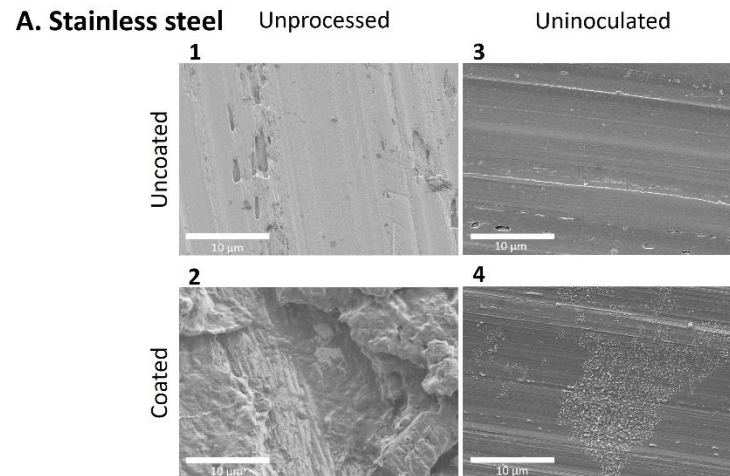
on the ISS. The development of suitable antimicrobial coatings in combination with human-friendly biocidal compounds is warranted. When designing antibiofilm regimens, it is important to assess biofilm formation in various environments. This study provides critical insight into the importance of testing an antibiofilm strategy in differing media as they could alter the efficacy of the strategy.

Conclusion

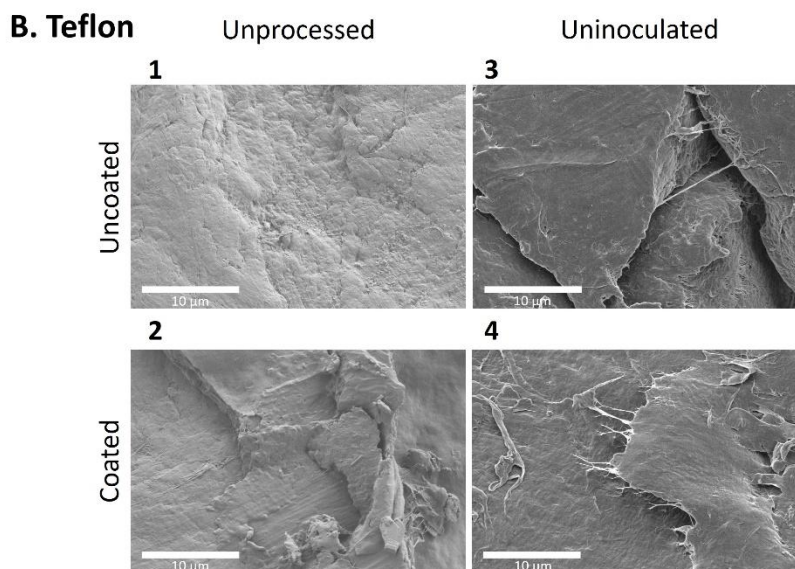
While the coating is promising for reducing *P. aeruginosa* biofilm accumulation in TSB, experiments in PDB revealed an ineffective coating. It is hypothesized that the starch from PDB interfered with the coating, rendering it ineffective. Additionally, these experiments included high nutrient medium designed to grow robust biofilms on short time scales. As demonstrated in this study, it is possible that the experiments could yield different results using media more similar in chemical composition and nutrient availability to that found on the ISS. Systems like those where the coating would be implemented should be tested. Moreover, the combination of surface coated materials (as shown here) along with non-toxic biocides should be tested. While surface coatings are not the sole solution to reducing biofilm growth in long-term spaceflight, coatings will likely be an integral part of the eventual solution to mitigate biofilm accumulation in current and future crewed spacecraft.

Supplementary Material

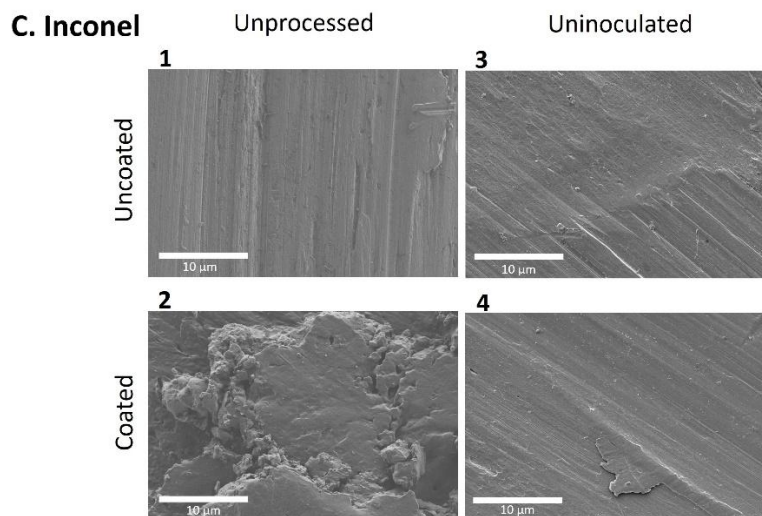
Supplemental Figure 1



Supplementary Figure 1A. Scanning electron microscopy (SEM) imaging of stainless steel coupons used for biofilm accumulation. “Unprocessed” refers to coupons that did not go through the SEM preparation process. “Uninoculated” refers to coupons that were not exposed to biology, but did go through the SEM preparation process.



Supplementary Figure 1B. Scanning electron microscopy (SEM) imaging of Teflon coupons used for biofilm accumulation. “Unprocessed” refers to coupons that did not go through the SEM preparation process. “Uninoculated” refers to coupons that were not exposed to biology, but did go through the SEM preparation process.



Supplementary Figure 1C. Scanning electron microscopy (SEM) imaging of Inconel coupons used for biofilm accumulation. “Unprocessed” refers to coupons that did not go through the SEM preparation process. “Uninoculated” refers to coupons that were not exposed to biology, but did go through the SEM preparation process.

Funding

This research was supported by the ISS Advanced Exploration System Life Support System program funded to JPL and supported post-doctoral fellowship to CWP. The funders had no role in study design, data collection and interpretation, the writing of the manuscript, or the decision to submit the work for publication. No funding was obtained from the company that developed and provided the coating. MSU support for a graduate fellowship to MKM was provided by JPL under subcontract #1635633.

Acknowledgments

Part of the research described in this publication was carried out at the JPL, California Institute of Technology, under a contract with NASA. Additional thanks to Adriana Blachowicz for generating Figure 2. We also thank Natasha Peterson for her help with biofilm sampling. We gratefully acknowledge the critical support and infrastructure provided for this work by The Kavli Nanoscience Institute at Caltech. We thank Luis Zea from BioServe Space Technologies (University of Colorado Boulder, United States) for graciously supplying us with cultures of *P. aeruginosa* PA14.

References

- Aparna, M. S., and Yadav, S. (2008). Biofilms: microbes and disease. *Braz. J. Infect. Dis.* 12, 526–530. doi: 10.1590/S1413-86702008000600016
- ASTM International (2018). ASTM E3161-18, Standard Practice for Preparing a *Pseudomonas aeruginosa* or *Staphylococcus aureus* Biofilm Using the CDC Biofilm Reactor. West Conshohocken, PA.
- Baxa, D., Shetron-Rama, L., Golembieski, M., Golembieski, M., Jain, S., Gordon, M., et al. (2011). In vitro evaluation of a novel process for reducing bacterial contamination of environmental surfaces. *Am. J. Infect. Control* 39, 483–487. doi: 10.1016/j.ajic.2010.10.015
- Beech, I. B., and Sunner, J. (2004). Biocorrosion: towards understanding interactions between biofilms and metals. *Curr. Opin. Biotechnol.* 15, 181–186. doi: 10.1016/j.copbio.2004.05.001
- Bixler, G. D., and Bhushan, B. (2012). Biofouling: lessons from nature. *Philos. Trans. R. Soc. A Math. Phys. Eng. Sci.* 370, 2381–2417. doi: 10.1098/rsta.2011.0502
- Callahan, M. R., Adam, N. M., Roberts, M. S., Garland, J. L., Sager, J. C., and Pickering, K. D. (2007). Assessment of silver based disinfection technology for CEV and future US spacecraft. *SAE Trans.* 116, 481–491. doi: 10.4271/2007-01-3258
- Carter, D. L. (2009). “Status of the Regenerative ECLSS Water Recovery System,” SAE Technical Paper 2009-01-2352.
- Characklis, W. G., and Cooksey, K. E. (1983). Biofilms and microbial fouling. *Adv. Appl. Microbiol.* 29, 93–138. doi: 10.1016/S0065-2164(08)70355-1
- Danko, D., Bezdan, D., Afshinnkoo, E., Ahsanuddin, S., Bhattacharya, C., Butler, D. J., et al. (2020). Global genetic cartography of urban metagenomes and anti-microbial resistance. *bioRxiv* [Preprint]. doi: 10.1101/724526
- Environmental Protection Agency (2015). Protocol for Residual Self-Sanitizing Activity of Dried Chemical Residues on Hard, Non-porous Surfaces. Available at: https://www.epa.gov/sites/default/files/2015-09/documents/cloroxpcol_final.pdf
- Fitton, K., Barber, K., Karamon, A., Zuehlke, N., Atwell, S., and Enright, S. (2017). Long acting water-stable organosilane and its sustained effect on reducing microbial load in the intensive care unit. *Am. J. Infect. Control* 45:S27. doi: 10.1016/j.ajic.2017.04.048
- Flemming, H.-C., and Wuertz, S. (2019). Bacteria and archaea on earth and their abundance in biofilms. *Nat. Rev. Microbiol.* 17, 247–260. doi: 10.1038/s41579-019-0158-9

- Gule, N. P., Begum, N. M., and Klumperman, B. (2016). Advances in biofouling mitigation: a review. *Crit. Rev. Environ. Sci. Technol.* 46, 535–555. doi: 10.1080/10643389.2015.1114444
- Huang, B., Li, D.-G., Huang, Y., and Liu, C.-T. (2018). Effects of spaceflight and simulated microgravity on microbial growth and secondary metabolism. *Mil. Med. Res.* 5, 1–14. doi: 10.1186/s40779-018-0162-9
- Jones, E. M., Cochrane, C. A., and Percival, S. L. (2015). The effect of pH on the extracellular matrix and biofilms. *Adv. Wound Care* 4, 431–439. doi: 10.1089/wound.2014.0538
- Kim, W., Tengra, F. K., Young, Z., Shong, J., Marchand, N., Chan, H. K., et al. (2013). Spaceflight promotes biofilm formation by *Pseudomonas aeruginosa*. *PLoS One* 8, e62437. doi: 10.1371/journal.pone.0062437
- Kitsutani, S., Mura, M., and Nagahisa, M. (1970). Catalase formation in *Rhodotorula mucilaginosa* in relation to the effect of glucose and antibiotics. *Plant Cell Physiol.* 11, 551–558.
- Klaus, D., Simske, S., Todd, P., and Stodieck, L. (1997). Investigation of space flight effects on *Escherichia coli* and a proposed model of underlying physical mechanisms. *Microbiology* 143, 449–455. doi: 10.1099/00221287-143-2-449
- Klintworth, R., Reher, H., Viktorov, A., and Bohle, D. (1999). Biological induced corrosion of materials II: new test methods and experiences from mir station. *Acta Astronaut.* 44, 569–578. doi: 10.1016/S0094-5765(99)00069-7
- Little, B. J., Blackwood, D. J., Hinks, J., Lauro, F. M., Marsili, E., Okamoto, A., et al. (2020). Microbially influenced corrosion: any progress? *Corros. Sci.* 170:108641. doi: 10.1016/j.corsci.2020.108641
- Lynch, S. V., Mukundakrishnan, K., Benoit, M. R., Ayyaswamy, P. S., and Matin, A. (2006). *Escherichia coli* biofilms formed under low-shear modeled microgravity in a ground-based system. *Appl. Environ. Microbiol.* 72, 7701–7710. doi: 10.1128/aem.01294-06
- Madsen, J. S., Burmølle, M., Hansen, L. H., and Sørensen, S. J. (2012). The interconnection between biofilm formation and horizontal gene transfer. *FEMS Immunol. Med. Microbiol.* 65, 183–195. doi: 10.1111/j.1574-695X.2012.00960.x
- Mauclaire, L., and Egli, M. (2010). Effect of simulated microgravity on growth and production of exopolymeric substances of *Micrococcus luteus* space and earth isolates. *FEMS Immunol. Med. Microbiol.* 59, 350–356. doi: 10.1111/j.1574-695X.2010.00683.x

- Murray, J., Muruko, T., Gill, C. I. R., Kearney, M. P., Farren, D., Scott, M. G., et al. (2017). Evaluation of bactericidal and anti-biofilm properties of a novel surface-active organosilane biocide against healthcare associated pathogens and *Pseudomonas aeruginosa* biofilm. PLoS One 12:e0182624. doi: 10.1371/journal.pone.0182624
- Nickerson, C. A., Ott, C. M., Wilson, J. W., Ramamurthy, R., and Pierson, D. L. (2004). Microbial responses to microgravity and other low-shear environments. Microbiol. Mol. Biol. Rev. 68, 345–361. doi: 10.1128/MMBR. 68.2.345-361.2004
- Perez, V., Mena, K. D., Watson, H. N., Prater, R. B., and McIntyre, J. L. (2015). Evaluation and quantitative microbial risk assessment of a unique antimicrobial agent for hospital surface treatment. Am. J. Infect. Control 43, 1201–1207. doi: 10.1016/j.ajic.2015.06.013
- Rajala, P., Carpen, L., Vepsäläinen, M., Raulio, M., Sohlberg, E., and Bomberg, M. (2015). Microbially induced corrosion of carbon steel in deep groundwater environment. Front. Microbiol. 6:647. doi: 10.3389/fmicb.2015.00647
- Rosenzweig, J. A., Abogunde, O., Thomas, K., Lawal, A., Nguyen, Y. U., Sodipe, A., et al. (2010). Spaceflight and modeled microgravity effects on microbial growth and virulence. Appl. Microbiol. Biotechnol. 85, 885–891. doi: 10.1007/s00253-009-2237-8
- Salton, M., and Kim, K. (1996). “Structure,” in Medical Microbiology. 4th Edn. ed. S. Baron (Glaveston, TX: University of Texas Medical Branch at Galveston)
- Santos, A. L. S. D., Galdino, A. C. M., Mello, T. P., Ramos, L. S., Branquinha, M. H., Bolognese, A. M., et al. (2018). What are the advantages of living in a community? A microbial biofilm perspective! Mem. Inst. Oswaldo Cruz 113:e180212. doi: 10.1590/0074-02760180212
- Searles, S. C., Woolley, C. M., Petersen, R. A., Hyman, L. E., and Nielsen-Priess, S. M. (2011). Modeled microgravity increases filamentation, biofilm formation, phenotypic switching, and antimicrobial resistance in *Candida albicans*. Astrobiology 11, 825–836. doi: 10.1089/ast.2011.0664
- Venkateswaran, K., Chung, S., Allton, J., and Kern, R. (2004). Evaluation of various cleaning methods to remove bacillus spores from spacecraft hardware materials. Astrobiology 4, 377–390. doi: 10.1089/ast.2004.4.377
- Wingender, J., and Flemming, H. C. (2011). Biofilms in drinking water and their role as reservoir for pathogens. Int. J. Hyg. Environ. Health 214, 417–423. doi: 10.1016/j.ijheh.2011.05.009
- Zea, L., McLean, R. J. C., Rook, T. A., Angle, G., Carter, D. L., Delegard, A., et al. (2020). Potential biofilm control strategies for extended spaceflight missions. Biofilms 2:100026. doi: 10.1016/j.biofilm.2020.100026

CHAPTER FOUR

MULTIMODAL BIOFILM CONTROL STRATEGIES FOR
SPACECRAFT WATER SYSTEMS: EVALUATING COATINGS,
NUTRIENT REMOVAL, AND BIOCIDES FOR IMPROVED
SUSTAINABILITY

Contribution of Authors and Co-Authors

Manuscript in Chapter 4

Author: Madelyn K. Mettler

Contributions: Designed study, conducted CDC bioreactor assays, performed microscopy, generated figures, completed statistical analysis, and drafted the manuscript.

Co-Author: Brent M. Peyton

Contributions: Assisted with study design, evaluated and discussed results, and edited manuscript.

Manuscript Information

Madelyn K. Mettler and Brent M. Peyton

Status of Manuscript:

- ☐ Prepared for submission to a peer-reviewed journal
- X Officially submitted to a peer-reviewed journal
- ☐ Accepted by a peer-reviewed journal
- ☐ Published in a peer-reviewed journal

February 14, 2025

Abstract

Functional water recovery systems will be a limiting factor for future spaceflight missions traveling beyond low Earth orbit. Previously, microbial communities known as biofilms have clogged valves in the International Space Station (ISS) water recovery system. To prevent part replacement, ensure safe water for astronauts, and enhance sustainability of long-term spaceflight, improved methods of biofilm control are needed. In this study, three approaches were evaluated for their ability to control biofilm alone and in combination. The methods include the use of Sher-Loxane® 800, a commercially available coating, exclusion of phosphorus from the growth medium, and regular dosing with a silver fluoride biocide. These strategies have all been proposed for biofilm control in spacecraft but have not been evaluated together. Further, this research uniquely features a defined multidomain consortium of organisms (a fungus and three bacteria) frequently isolated from the ISS water system. Results indicate that the presence of the coating is a key factor in reducing biofilm accumulation, and that combining all three biofilm control methods reduces accumulation of viable biofilm to just above the limit of detection after 7 days of growth. This study presents the potential for controlling biofilm with multiple methods for spacecraft and Earth-based water systems.

Introduction

In space, as on Earth, water is necessary for human life. To achieve sustainable spaceflight and human presence in space, water must be recycled from urine, sweat, and humidity in the spacecraft. The systems responsible for recycling this water are especially vulnerable to damage and failure due to biofilm accumulation (Carter, 2010; Zea et al., 2020;

Zea et al., 2018). As space exploration moves into a new era of commercial space stations and a government station to orbit the moon (Gateway), special considerations must be made regarding the design of water recycling systems and their susceptibility to biofilm-related issues.

Biofilms are microbial communities that are often associated with surfaces. They can be comprised of a multitude of organisms, extracellular polymeric substances (EPS), and environmental debris such as dirt (Donlan, 2002; Flemming et al., 2007; Wingender & Flemming, 2011). The biofilm morphology confers many advantages to microorganisms such as increased antimicrobial resistance, opportunities for horizontal gene transfer, and protection from environmental stresses like biocides, osmotic lysis, desiccation, UV radiation, and starvation (Elasri & Miller, 1999; Esbelin et al., 2018; Mah & O'Toole, 2001; Roberson & Firestone, 1992; Yin et al., 2019). These properties make biofilms particularly difficult to mitigate, especially in areas that are difficult to manually and mechanically clean, like would be found in a spacecraft.

Many strategies have been proposed for controlling biofilm in spacecraft, and specifically in spacecraft water systems including coated surfaces, predatory organisms, biocides, mechanical disaggregation, UV sterilization, thermal shock treatments, and nutrient removal (Azim et al., 2020; Velez Justiniano et al., 2020; Zea et al., 2020). Other researchers have investigated the use of coatings for biofilm control in spacecraft water systems (Demir et al., 2022; Flores et al., 2023; Li Sip et al., 2023; Mettler et al., 2022), but to our knowledge, none have explored the effects of coatings in combination with other biofilm control strategies. The experiments presented in this paper explore the use of a surface coating, biocide addition, and key nutrient removal, in combination and as individual methods to control biofilms composed of organisms from the ISS water system. The coating tested was Sher-Loxane® 800, a commercially available

coating from The Sherwin-Williams Company and it was tested in combination with silver fluoride dosing (biocide) and removal of phosphorus from the nutrient medium. Further, the research presented here uniquely features a multidomain consortium of organisms, all isolated from the International Space Station (ISS) water system. The previously published studies all used a singular organism, *Pseudomonas aeruginosa*, which in 10 years of sampling, has never been isolated from the ISS wastewater tank, potable water bus, or condensate (Zea et al., 2020).

The ISS serves as the test bed for future spacecraft and space stations. As such, all the methods used in this paper were developed with the National Aeronautics and Space Administration (NASA) portion of the ISS water system and recovery protocols in mind. While these experiments were designed to represent biofilms in the ISS, the results have broad implications for biofilm control on Earth as well. Researchers investigating biofilm control in Earth-based water systems have also explored coatings for biofilm reduction (Filice et al., 2022; Samuel et al., 2022; Si et al., 2015; Sójka et al., 2023). However, the published studies did not investigate the combined effects of coatings and biocides (or nutrient limitation) for biofilm control, despite the frequent use of biocides for water treatment and distribution (Hwang et al., 2012; Munavalli & Kumar, 2003; Pan et al., 2023). The research presented here suggests that incorporation of multiple methods of biofilm control leads to promising reduction of biofilm in an analogue to the ISS wastewater tank, which could be reproduced for Earth-based water systems.

Methods and materials

Organism selection and preparation

The organisms used in the experiments are direct and frequent isolates from the ISS water system (Zea et al., 2020). Three bacteria and one fungus were used in co-culture for all experiments conducted and include the bacteria *Burkholderia contaminans*, *Methylobacterium organophilum*, and *Ralstonia insidiosa*, and a filamentous fungus, *Coniochaeta mutabilis* (formerly *Lecythophora mutabilis*), see Supplementary Table 1 for internal and external strain identifiers. Genomes for the strains of *B. contaminans* and *M. organophilum* used here have been published (Velez Justiniano et al., 2023). The bacteria were provided by Boeing and the fungus was provided by Marshall Space Flight Center (MSFC). Stocks of the organisms were stored at -80 C in a 50/50 mixture of glycerol and cell culture in their corresponding nutrient broth. Streak plates of each organism were prepared on R2A agar (BD Difco) to ensure culture purity. To prepare the inoculum, individual colonies were used to inoculate flasks of sterile full-strength tryptic soy broth (TSB; BD Difco) for bacteria or Sabouraud dextrose broth (SDB; BD Difco) for fungus. Inoculum flasks consisted of 25 mL of sterile nutrient broth in a slip-capped 125 mL baffled flask and were placed in a shaker-incubator at 30 C and 225 rpm. *M. organophilum* and *C. mutabilis* were incubated for approx. 48 h while *B. contaminans* and *R. insidiosa* were incubated for approx. 24 h. Once dense cultures were achieved, they were washed via three several steps of centrifugation (8,000 x g) and resuspension in fresh sterile 0.4% NaCl solution. After excess nutrient broth was washed away, the optical density of each culture was measured at 600 nm. The density was adjusted so addition of 100 μ L of each culture to the Center for Disease

Control (CDC) reactor (BioSurface Technologies, Bozeman, MT) (International, 2018) resulted in initial densities of 10^4 CFU/mL for each of the four organisms.

Coupon preparation

Inconel® 718 (a nickel-chromium alloy) and Teflon™ (polytetrafluoroethylene) cylindrical coupons (12.7 mm diameter, 3.8 mm thickness) were procured (BioSurface Technologies). Prior to coating or use in the reactor, coupons were washed by soaking and sonication in soapy water for approx. 5 min followed by a rinse step and additional sonication of approx. 5 min in Milli-Q water (resistivity $18.2 \text{ M}\Omega \cdot \text{cm}$). The coating tested was provided by The Sherwin-Williams Company and is a commercially available product known as Sher-Loxane® 800. The coating was applied with a paintbrush such that a homogenous thickness of coating was present on the flat surface of the coupon (Figure 1). After at least 48 h of drying at room temperature, the coupon was flipped over so the other flat surface could be coated. The round edges of the coupons were not coated, but the painted coating dripped over the side in some areas. No additional wash step was completed after coating.

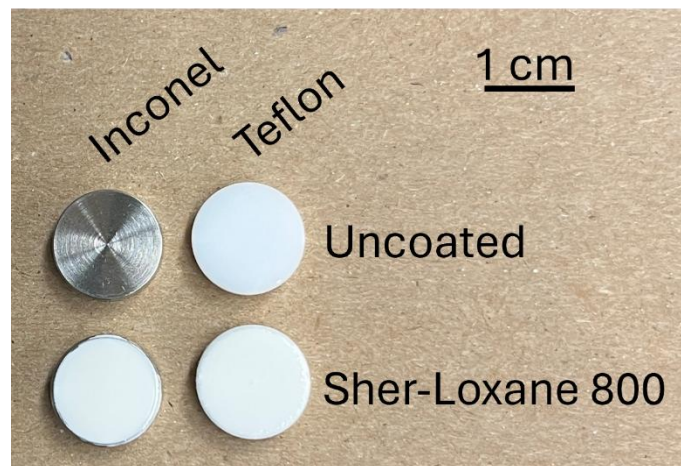


Figure 1. Coupons for sampling biofilm accumulation in the CDC reactor.

Reactor preparation

Center for Disease Control (CDC) biofilm reactors (BioSurface Technologies) were assembled and sterilized empty in an autoclave at 121 C and 21 PSI for at least 30 minutes. Uncoated coupons were loaded into the reactor before autoclaving. For experiments that utilized coated coupons, the reactor was autoclaved without coupons. After autoclaving, coated coupons were loaded and sterilized using the same method outlined previously (Mettler et al., 2022). In summary, the reactor was placed in a Labconco class II biosafety cabinet, coupon holder rods were removed, coated coupons were loaded, and then rods were submerged in 99% isopropanol for at least 30 s before air drying and replacement into the reactor.

Medium preparation

The medium used in the experiments was developed by researchers at the CBE in collaboration with MSFC (Table 1). An early version of the recipe was published in Velez-Justiniano et al. 2021 (Velez Justiniano et al., 2021). Slight modifications were made to the formulation, including the addition of minor/trace nutrients (MTN), removal of KCl, and reducing KI concentrations (Sandvik et al., 2022). The new version of the medium is termed microbial ersatz MTN. To prepare the medium, Milli-Q water was autoclaved for 3 h in 20 L glass carboys. After autoclaving, filter sterilized stock solutions were added to the carboy. A 10 mL aliquot of medium was removed from the carboy with a sterile serological pipette for measuring pH. The pH was adjusted on the aliquot to 5.55 ± 0.05 with hydrochloric acid (1 M) or sodium hydroxide (1 M) and scaled to adjust the medium in the carboy. Sterile HCl or NaOH was added to the carboy and the pH was checked again. The iterations tested in the experiments here include a full-strength version of microbial ersatz MTN and a no phosphorus version which

excluded the phosphate stock. The no phosphorus medium was prepared in carboys that had not held full strength medium, or if they had, they were acid washed with 10% HCl and rinsed thoroughly with Milli-Q water.

Table 1. Recipe for microbial ersatz MTN featuring final component concentrations. Colors correspond to components that were added together as a concentrated stock solution.

Component	Concentration		Component	Concentration	
Propylene Glycol	8.971	μL/L	Benzyl Alcohol	6.075	μL/L
Ethanol	44.946	μL/L	Diethylphthalate	2.01	μL/L
Acetone	25.799	μL/L	Trimethyl Silanol	0.635	μL/L
2-(2-butoxyethoxy) Ethanol	2.356	μL/L	Benzothiazole	0.21	μL/L
N, N-Dimethylformamide	1.486	μL/L	2-Ethyl-1-Hexanol	0.48	μL/L
2-Ethoxyethanol	1.303	μL/L	Decamethylcyclopentasiloxane	1.03	μL/L
1-Methyl-2-Pyrrolidinone	0.782	μL/L	Dodecamethylcyclohexasiloxane	10	μL/L
2-Propanol	0.896	μL/L	Octamethylcyclotetrasiloxane	1.02	μL/L
1-Propanol	0.876	μL/L	Dimethoxydimethylsilane	35.1	μL/L
4-Ethylmorpholine	2.265	μL/L	Calcium Sulfate	1.885	mg/L
Formic Acid	27.489	μL/L	Dimethyl Sulfone	0.205	mg/L
Lactic Acid	8.55	μL/L	Hexamethylcyclotrisiloxane	1.02	mg/L
Benzoic Acid	3.015	mg/L	Monobasic Potassium phosphate	0.835	mg/L
Caprolactam	1.111	mg/L	Magnesium chloride hexahydrate	0.397	mg/L
Urea	1.899	mg/L	Manganese chloride tetrahydrate	0.319	mg/L
Zinc (II) Acetate dihydrate	1.425	mg/L	Ferric chloride	0.129	mg/L
Nickel (II) Acetate tetrahydrate	0.518	mg/L	Boric acid	0.268	mg/L
Acetic Acid	32.088	μL/L	Cobalt chloride hexahydrate	0.004	mg/L
Ammonium Bicarbonate	101.457	mg/L	Sodium molybdate dihydrate	0.016	mg/L
Sodium Fluoride	0.88	mg/L			
Potassium Iodide	0.021	mg/L			

Biocide preparation

Silver (I) fluoride (99+%, Thermo Scientific) was used in experiments featuring biocide dosing. The biocide was prepared at a concentration of 216.66 mg/L so that when it was added to the reactor, it was present at a concentration of 2 ppm. The silver (I) fluoride was added to Milli-Q water and filter sterilized into a sterile container with tubing connected to the reactor.

Experiment iterations

The CDC reactor experiments were conducted to evaluate control strategies alone and in combination with one or two other control strategies. Three replicates of each experiment were completed. Figure 2 presents a schematic to visualize the iterations of each experiment. The format of Figure 2 is carried through the data and microscopy figures that present the results of the experiments.

No biocide	No biocide	Silver fluoride	Silver fluoride	
Full strength medium	No phosphorus medium	Full strength medium	No phosphorus medium	
				Uncoated
				Sher-Loxane 800

Key: Sher-Loxane 800 coating No phosphorous medium Silver fluoride biocide

Figure 2. Biofilm control methods used in each of the 8 iterations of experiments. The empty section in the upper left represents the base condition of unmitigated biofilm growth. This layout is used throughout the results to present data for each experiment.

Reactor operation

Sterile reactors loaded with coupons were filled to the effluent port with fresh, sterile medium (approx. 325mL). For inoculation, 100 μ L of each washed culture was added to the reactor and the stir bar was set to 125 ± 10 rpm. The experiments took place at room temperature and with ambient oxygen concentrations. About 5-10 min after inoculation, a sample of bulk fluid was taken from the reactors to validate the cell concentrations at the start of the experiments.

After 24 h batch operation, continuous flow was initiated. Fresh, sterile medium was pumped (Masterflex L/S, Cole Parmer) into the reactor at 2 mL/min, creating a residence time of about 162.5 min. The medium used for continuous flow was the same as that used for the batch

phase operation. For reactors receiving biocide, the first dose corresponded with the initiation of continuous flow, and silver fluoride was dosed every 24 h at 3 mL/min for 1 min.

Sampling

The first sampling point took place at the completion of 24 h batch conditions, just prior to the start of continuous flow. Sampling consisted of removing a reactor rod and gently dipping it into 30 mL of sterile 1M phosphate buffered saline (PBS). After rinsing, the coupons were removed and put into sterile 50 mL Falcon tubes containing 10 mL sterile PBS. The biofilm was removed from the coupon surface and disaggregated via a vortex mixing and sonication series of 30 s each for a total of 2 min 30 s (vortex, sonicate, vortex, sonicate, vortex). Planktonic samples were taken with a sterile serological pipette from the open port on the CDC reactor where the rod was removed. The rod was then replaced with a sterile dummy rod to maintain similar fluid dynamics in the reactor. Bulk reactor fluid was also sampled 6 h after the biocide dose in experiments that received silver fluoride to monitor effects of the silver fluoride dose on planktonic populations. Planktonic samples were vortex mixed for 30 s to disaggregate potential biofilm aggregates. Disaggregated biofilm and planktonic samples were plated on selective agar for enumeration (see Table 2 for the agar used). Agar plates were allowed to dry at room temperature before placing them into a 30 C incubator. Enumeration took place after the formation of visible colonies, typically after 2 days for general counts, *B. contaminans*, and *R. insidiosa*, and 4-5 days for *C. mutabilis* and *M. organophilum*.

Table 2. Selective agar used for microbial enumeration of planktonic and biofilm samples.

Organism enumerated	Agar
<i>B. contaminans</i>	<i>Pseudomonas</i> isolation agar
<i>C. mutabilis</i>	Sabouraud dextrose agar, pH lowered to 3
<i>M. organophilum</i>	<i>Methylobacterium</i> agar (Atlas, 2010) with amphotericin B (7.5 µg/mL)
<i>R. insidiosa</i>	R2A with chloramphenicol (20 µg/mL) and amphotericin B (7.5 µg/mL)
No isolation, general counts	R2A

During day 7 sampling, coupons were stained for confocal analysis. After gently rinsing in PBS, coupons were placed into sterile 24-well plates. The well was then flooded with freshly prepared LIVE/DEAD™ BacLight™ stain and incubated in the dark for 15 min at room temperature. The stain was removed from the well and the coupons were washed three times with filter-sterilized Milli-Q water. Next, the well was flooded with a 50/50 solution of sterile 1X PBS and Calcofluor White and incubated in the dark for 10 min at room temperature. After the incubation period, the stain was removed, and the coupons were washed with filter-sterilized Milli-Q water three times. The wells were then flooded with a solution of 4% paraformaldehyde and 2.5% glutaraldehyde. The 24-well plate was covered with a foiled lid and wrapped several times with parafilm. The wrapped plate was then placed into a closed plastic bag and stored at 4 C until confocal microscopic imaging on a Leica DM6 Multiphoton Upright Confocal Microscope.

Statistical analysis of plate counts

The effects of Sher-Loxane® 800, phosphorus removal, and silver fluoride dosing were evaluated on a species-by-species basis. To investigate the effects on the entire biofilm and

planktonic community, the viable plate counts on the non-selective R2A plates were used. Mixed effects ANOVA models and statistical comparisons were generated using MiniTab (version 21.1).

Results

Viable biofilm density: CFU plate counts

The experiments featuring unmitigated biofilm growth (no coating, full strength medium, and no biocide; Figure 3A) fostered the highest viable biofilm density of all the experiments. *R. insidiosa* was present at a viable density of approx. $8.4 \log_{10}\text{CFU}/\text{cm}^2$ on Teflon coupons and $8 \log_{10}\text{CFU}/\text{cm}^2$ on Teflon coupons by day 7 and made up the majority of the biofilm. *B. contaminans* was present at the next highest density of $6.8 \log_{10}\text{CFU}/\text{cm}^2$ on Teflon and $6 \log_{10}\text{CFU}/\text{cm}^2$ on Inconel. *C. mutabilis* and *M. organophilum* did not contribute very much to the viable cell density of the biofilm and were only present at densities ranging from 3-4 $\log_{10}\text{CFU}/\text{cm}^2$ on Inconel and Teflon. All species in the biofilm appear to reach steady state by 3 days after inoculation.

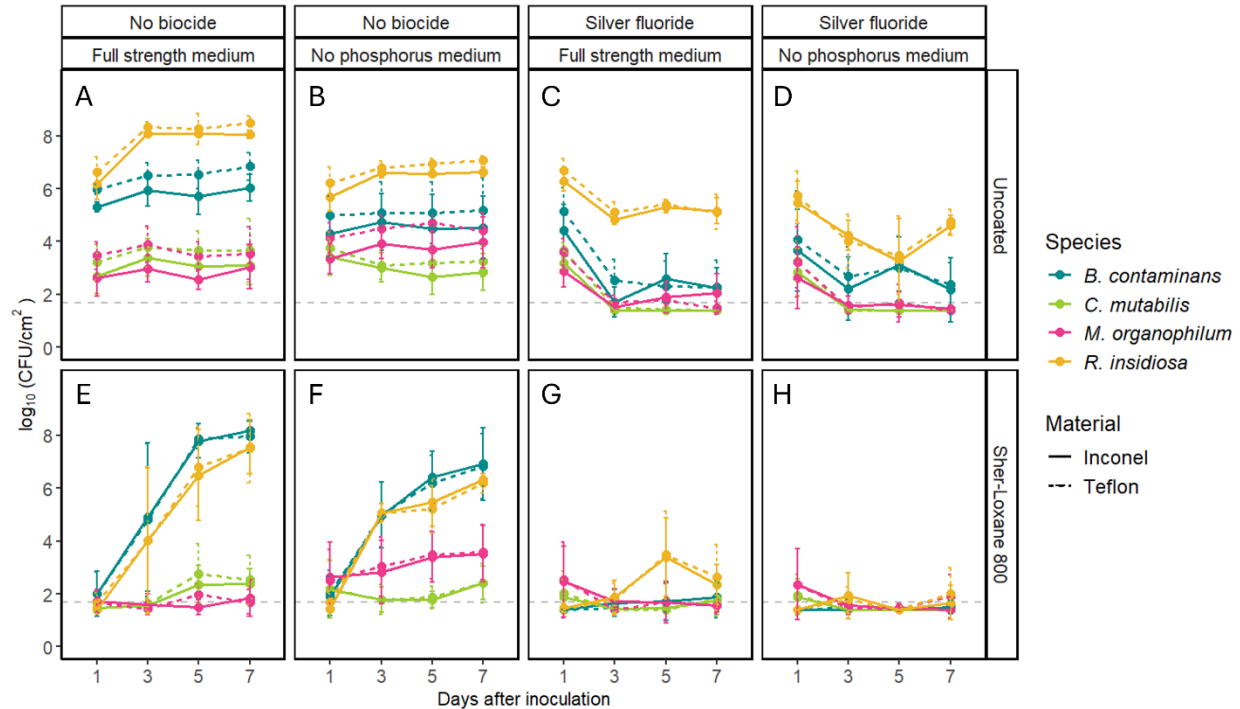


Figure 3. Viable biofilm density for each of the eight iterations of biofilm control combinations. Continuous flow was initiated on day 1 after the sample was taken. The grey dashed line represents the limit of detection, $y=1.69$. Error bars are standard deviation.

In experiments featuring medium without phosphorus as the only method of biofilm control (Figure 3B), the general trend is a reduction in viable biofilm density by 0.5-1 $\log_{10}$ CFU/cm². The experiments with silver fluoride dosing as the sole method of biofilm control (Figure 3C) featured a reduction in viable biofilm density from day 1 to day 3 (biocide was dosed on day 1 immediately after sampling). *R. insidiosa* still made up the majority of the biofilm, with a final viable density of about 5.2 $\log_{10}$ CFU.cm². *B. contaminans*, *C. mutabilis*, and *M. organophilum* were all present in the day 7 biofilm near the limit of detection, ranging from 1.3-2.3 $\log_{10}$ CFU/cm².

The experiments on uncoated coupons with nutrient limitation and biocide as the biofilm control methods (Figure 3D) featured a reduction of about 0.5 $\log_{10}$ CFM.cm² in *R. insidiosa*

viable density compared to experiments with just silver dosing (figure 3C). *B. contaminans* density remained about the same compared to experiments with just silver dosing, while *C. mutabilis* and *M. organophilum* density dropped to below the limit of detection by day 7.

The coated coupons effectively delayed viable biofilm accumulation by several orders of magnitude for at least 3 days after inoculation (Figure 3E vs 3A). This delay presented an excellent opportunity for additional control methods to further suppress biofilm accumulation. The final viable biofilm density of the biofilm was similar to the unmitigated case, reaching approx. $8 \log_{10}\text{CFU}/\text{cm}^2$ for *B. contaminans* and $7.5 \log_{10}\text{CFU}/\text{cm}^2$ (a total cell density of approx. $8.5 \log_{10}\text{CFU}/\text{cm}^2$). An unexpected outcome in the reactors with coated coupons was a change in the composition of the biofilm. The biofilms on uncoated coupons were always dominated by *R. insidiosa* while the biofilms on coated coupons were mainly dominated by *B. contaminans* in reactors not dosed with biocide. The density of *C. mutabilis* and *M. organophilum* in this experiment was also several orders of magnitude lower than in the unmitigated case (Figure 3A).

Mirroring the comparison between Figure 3A and 3B, the inclusion of nutrient limitation as a biofilm mitigation method on coated coupons (Figure 3F) reduced viable biofilm accumulation by approx. one order of magnitude compared to biofilms with the coating as the only method of biofilm control (Figure 3E).

The greatest reduction in biofilm accumulation occurred when silver fluoride dosing was incorporated as a biofilm control method in addition to coating (Figure 3G) and in addition to nutrient limitation and coating (Figure 3H). In these experiments, the biofilm density is significantly reduced on day 1 compared to the unmitigated case (Figure 3A) and the density is

generally highest on day 1. Throughout the experiments, the biofilm density remained low, aside from *R. insidiosa* on day 5 in the experiment with coating and silver fluoride (Fig 3G). By day 7, both experiments feature viable biofilm densities at least 5 orders of magnitude lower than the unmitigated case. However, the experiment featuring all three methods of biofilm control still outperformed the coating and silver dose approach, resulting in a biofilm density of about 1.5 $\log_{10}\text{CFU}/\text{cm}^2$.

Mixed effects statistical analysis was completed to investigate the resulting viable biofilm density in each treatment method more definitively. The models used the experiment replicate as a random factor, and medium, biocide, coating, material, and day all as fixed factors. The p-values associated with all individual factors and several combinations of factors are presented in Table 3, see Supplementary Table 2 for all remaining p-values. MiniTab version 21.1 reports p-values out to only 16 decimals, so p-values represented by 0.00E-16 are reported by MiniTab as such.

Table 3. P-values for statistical significance of each factor and select combined factors in mixed effects models for the viable cell densities of the accumulated biofilms. Bolded values represent those less than 0.05.

Factor	<i>B. contaminans</i>	<i>C. mutabilis</i>	<i>M. organophilum</i>	<i>R. insidiosa</i>	R2A (whole biofilm)
Medium	5.15E-09	5.56E-02	1.00E-16	0.00E+00	0.00E+00
Biocide	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
Material	2.69E-02	2.72E-04	1.81E-04	4.97E-02	9.93E-01
Coating	3.10E-15	0.00E+00	0.00E+00	0.00E+00	0.00E+00
Day	0.00E+00	0.00E+00	5.05E-08	0.00E+00	0.00E+00
Medium*Biocide	7.09E-07	5.45E-01	0.00E+00	7.44E-01	5.72E-01
Medium*Coating	1.31E-01	5.15E-01	1.02E-02	1.88E-05	4.92E-04
Biocide*Coating	1.02E-12	0.00E+00	1.00E-16	2.18E-04	3.80E-15
Medium*Biocide*Coating	2.74E-02	1.76E-01	6.91E-02	1.75E-01	5.52E-04

In addition to biofilm measurements, bulk fluid samples from the reactors were taken to monitor the reactor planktonic populations (Figure 4). The planktonic populations generally mirror the trends observed in the viable biofilm accumulation (Figure 3) in all reactors aside from those treated with silver fluoride. In the reactors receiving biocide, the planktonic population was significantly reduced and there was a cyclical suppression and rebound of viable planktonic (detached biofilm) cells.

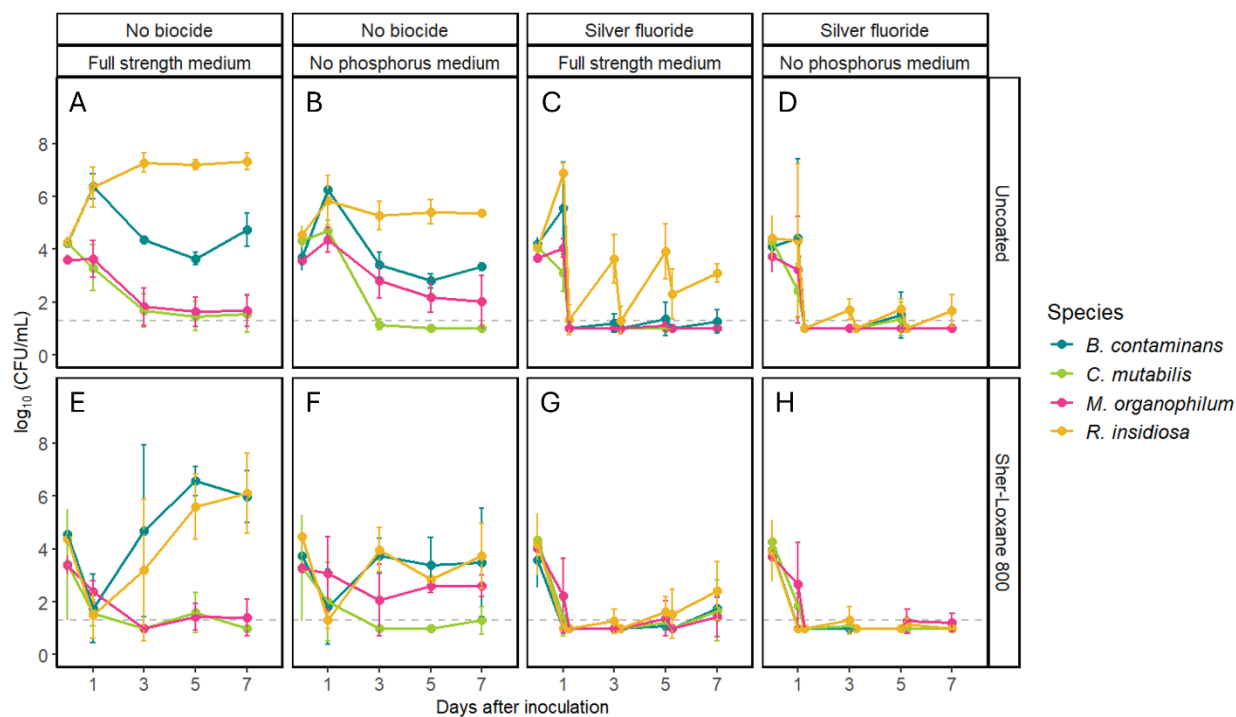


Figure 4. Viable planktonic cell density for each of the eight iterations of biofilm control combinations. Continuous flow began on day 1 after the sample was taken. The grey dashed line represents the limit of detection, $y=1.3$. Error bars represent standard deviation.

Mixed effects statistical analysis was completed to investigate the resulting viable planktonic cell density in each treatment method more definitively. The models used the experiment replicate as a random factor, and medium, biocide, coating, and day all as fixed

factors. The material type was not included as a factor in planktonic analysis because both Teflon and Inconel were present in the same reactor. The p-values associated with all individual factors are presented in Table 4, see Supplementary Table 3 for all remaining p-values. MiniTab version 21.1 reports p-values out to only 16 decimals, so p-values represented by 0.00E-16 are reported by MiniTab as such.

Table 4. P-values for statistical significance of each fixed factor in mixed effects models for the viable cell densities of the planktonic cells in the reactor fluid. Bolded values are less than 0.05.

Factor	<i>B. contaminans</i>	<i>C. mutabilis</i>	<i>M. organophilum</i>	<i>R. insidiosa</i>	R2A counts (total planktonic)
Medium	1.12E-03	9.23E-01	1.33E-03	2.55E-04	4.88E-03
Biocide	0.00E+00	1.10E-02	6.46E-05	1.00E-16	1.00E-16
Coating	2.83E-01	4.18E-03	4.64E-02	1.94E-09	8.63E-04
Day	2.60E-10	0.00E+00	0.00E+00	1.89E-07	7.48E-07

Biomass and biofilm morphology: Confocal image analysis

Confocal microscopy images were generated for biofilms that had accumulated on the coupons after 7 days in the reactors. Figures 5 and 6 present these images for Inconel and Teflon surfaces, respectively. The greatest reduction in biofilm accumulation can be seen when comparing panels C and D to G and H in Figures 5 and 6. Panel E in each figure presents biofilms on Sher-Loxane® 800 coating grown in full strength medium without biocide dosing. The biofilms appear much denser than those grown on uncoated coupons (panel A), however some of the fluorescence in the Sher-Loxane® 800 images is due to the coating itself. The coating became highly fluorescent in the wavelengths used to image the biofilm (excitation wavelengths of 405 nm (blue), 483 nm (green) and 568 nm (red)). The microbial cells present on

the coating have more intense fluorescence than the coating, so the cells remain distinguishable from the surface.

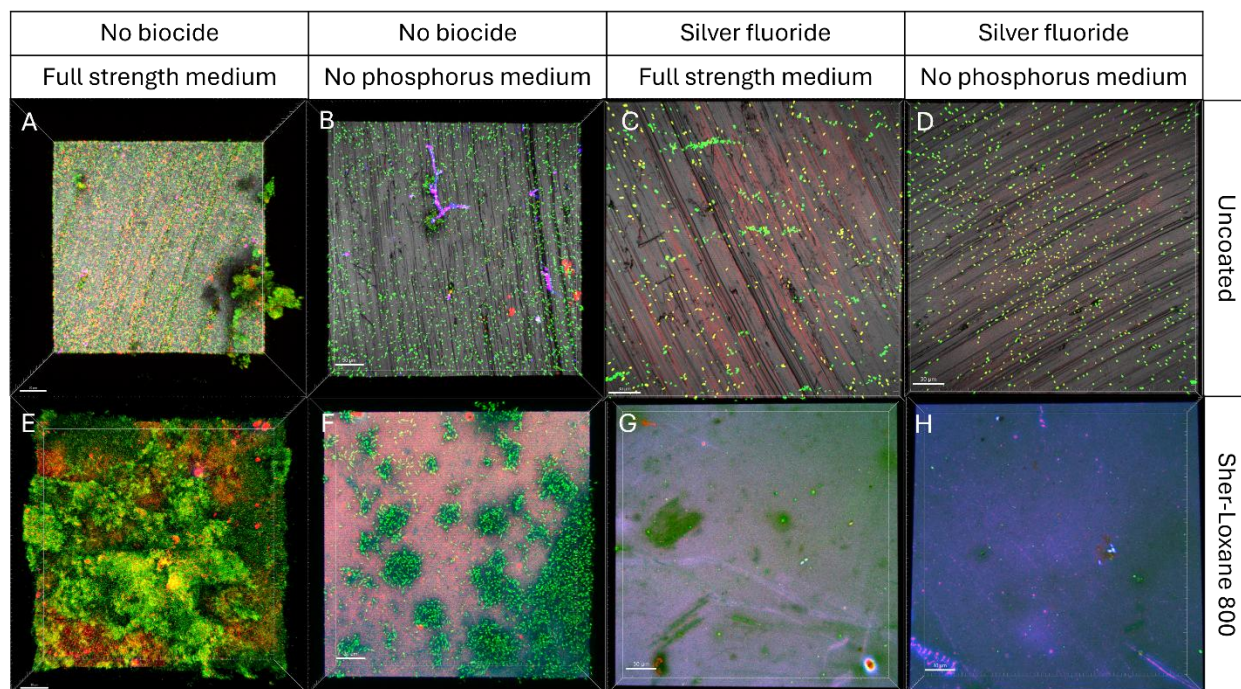


Figure 5. Confocal images of biofilms accumulated on Inconel CDC coupons 7 days after inoculation. Viable bacterial cells are in green, non-viable bacterial cells are in red, and fungal cells are in blue. The fluorescence of the coating can be seen in the three images on the lower right. The scale bar for each image in 30 μm .

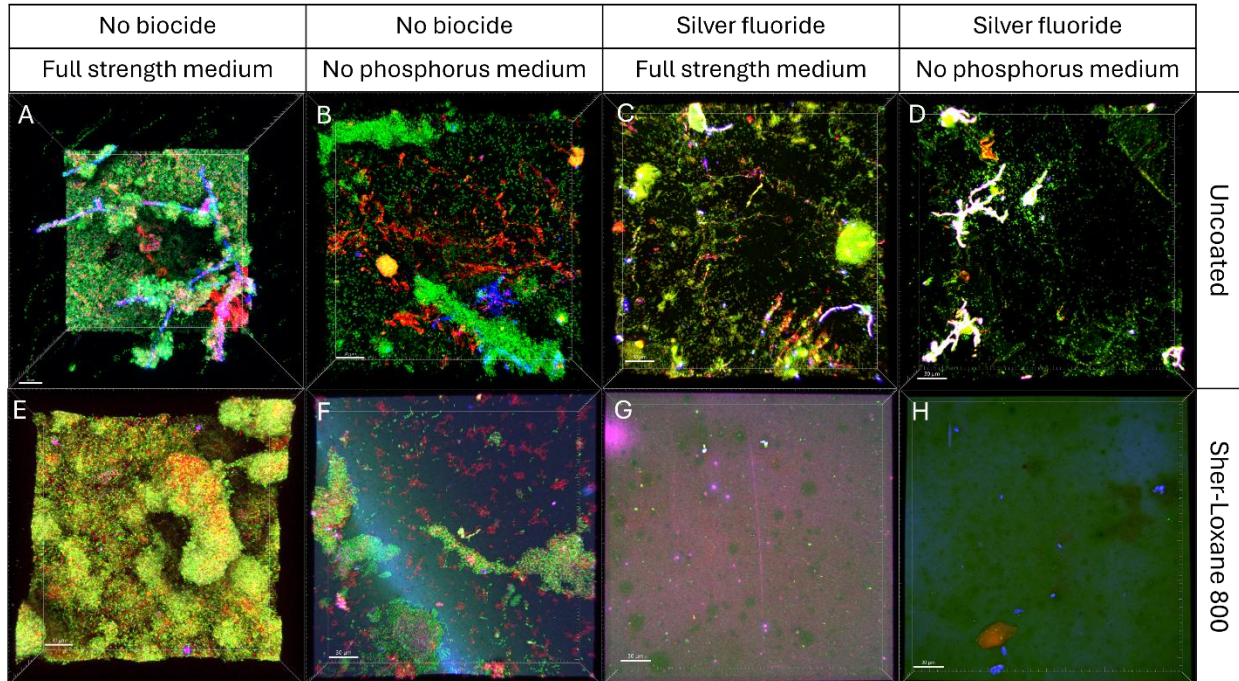


Figure 6. Confocal images of biofilms accumulated on Teflon CDC coupons 7 days after inoculation. Viable bacterial cells are in green, non-viable bacterial cells are in red, and fungal cells are in blue. The fluorescence of the coating can be seen in the three images on the lower right. The scale bar for each image in 30 μm .

Side views are provided for biofilms grown in full strength medium without biocide dosing to provide additional context regarding biofilm structure that may be lost in the 3D projections (Figure 7). It can be seen that the biofilms on the uncoated coupons (panels A and C) have a much larger maximum thickness than those on the Sher-Loxane® 800 coated coupons (panels B and D).

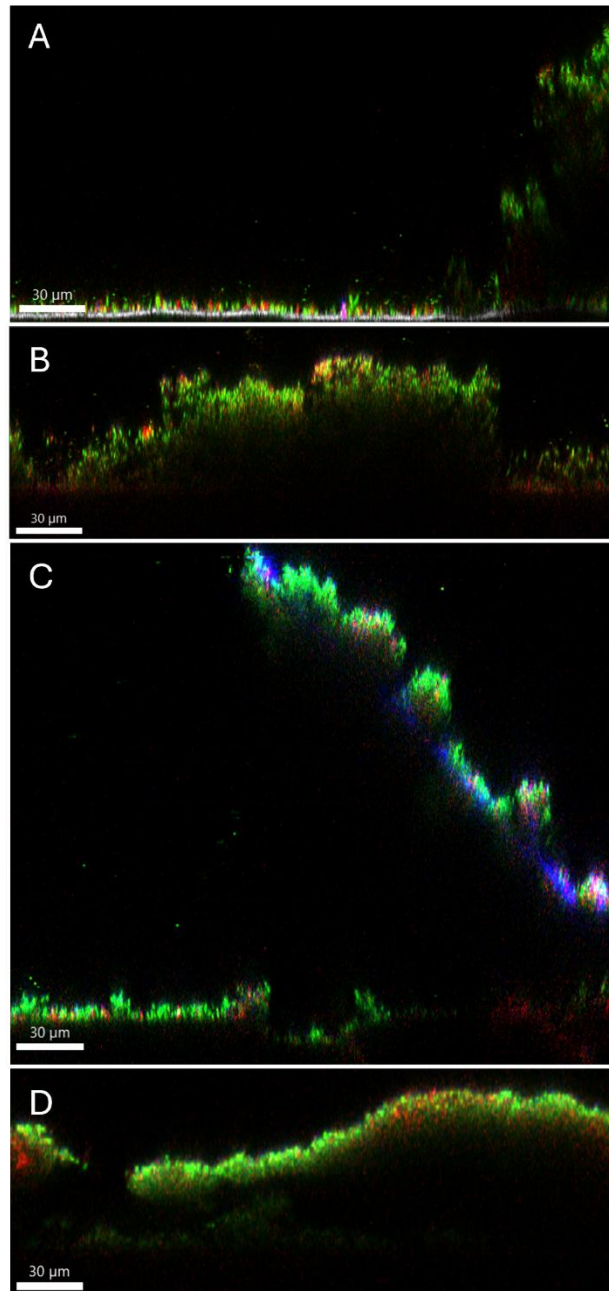


Figure 7. Side views of biofilms on A) uncoated Inconel, B) coated Inconel, C) uncoated Teflon, and D) coated Teflon grown in full strength medium without biocide dosing. Scale bar in each image is 30 μm .

Discussion

Coatings have previously been investigated for biofilm control for use in spacecraft water systems by Mettler et al. (Mettler et al., 2022), Flores et al. (Flores et al., 2023), Demir et al. (Demir et al., 2022), and Li Sip et al. (Li Sip et al., 2023). These previous investigations, while limited in scope and relevance to the ISS water system, confirmed that coatings can decrease unwanted biofilm accumulation. The results presented in this paper build on previously published results (Mettler et al., 2022) with key methodology changes to improve model accuracy to conditions in the ISS wastewater tank. Here, the use of a multi-organism consortia, including a fungus, is a characteristic that has not been addressed in previous research using coatings for biofilm control in spacecraft water systems. Further, these experiments aim to improve accuracy of modeling the ISS water system by utilizing the ersatz MTN medium designed to mimic nutrient availability and chemical composition of the wastewater tank that may lead to a biofouling event (Sandvik et al., 2022). These changes are more realistic and significantly improve upon the use of tryptic soy broth and potato dextrose broth tested in previous experiments (Mettler et al., 2022). One characteristic that remained constant between the early experiments and the ones described here is the use of materials found in the ISS water system (Callahan et al., 2007), though this time the materials were reduced to only Teflon and Inconel (the material that composes the wastewater tank). The final improvement made to the experiments is the extension of the duration from 3 days to 7 days. Within that time frame, the biofilms within the CDC reactor generally seem to achieve steady state.

The coating tested in these experiments was Sherwin-Williams Sher-Loxane® 800. It is an “epoxy siloxane hybrid designed for corrosion control and weatherability.” There are no specific

agents added to the coating to make it antimicrobial or biocidal. It was chosen in these experiments based on its performance in preliminary screening with multiple antimicrobial coatings (data not shown) and as a general antifouling coating with the basic mechanism of action of reducing microbial adhesion.

The iterations of the experiments tested include biofilms grown in reactors fed medium with or without phosphorus, reactors dosed with silver fluoride or not receiving a biocide, and reactors featuring Sher-Loxane® 800 coated or uncoated coupons. These factors were all tested individually and in combination to determine the effect each control method –biocide dosing, nutrient limitation, and use of coatings—have on the viable density and morphology of the biofilm and planktonic cells.

Viable biofilm density: CFU plate counts

Phosphorus was identified as a major limiting nutrient during the development of the microbial ersatz MTN medium (Sandvik et al., 2022) and has been reported as a limiting nutrient in the ISS water processing assembly (Diaz et al., 2021). As can be seen in Figure 3, generally, the removal of phosphorus tended to reduce viable biofilm accumulation by a half a logarithm to a full order of magnitude. The greatest reductions due to phosphorus removal were in full strength medium without biocide dosing, where the uncoated coupons fostered viable biofilm to a total cell density of about $8\text{--}8.5 \log_{10}\text{CFU}/\text{cm}^2$ whereas in reactors without phosphorus, the density dropped to about $6.5\text{--}7 \log_{10}\text{CFU}/\text{cm}^2$. The density of planktonic cells in the reactor also tended to drop by about one order of magnitude with the removal of phosphorus in reactors not receiving biocide dose.

The daily dosing of silver fluoride to the reactors effectively suppressed additional biofilm accumulation during the continuous flow phase. Aside from one timepoint (day 5 for a reactor with coated coupons, full strength medium, and silver dose), the viable biofilm accumulation in reactors receiving silver fluoride was highest after 1 day of batch phase in the CDC reactor, which was before biocide dosing began. As soon as the regular biocide dosing began, viable biofilm accumulation was reduced by at least half an order of magnitude throughout the remainder of the 7 days. Planktonic cell density in the reactor had a cyclical response to the silver fluoride. In reactors featuring biocide dose, additional planktonic samples were taken 6 h after the dose which almost always showed reduction in planktonic cell density below the limit of detection of $1.3 \log_{10}\text{CFU/mL}$. The planktonic cell numbers rebounded by the next sampling point, which was likely due to biofilm detachment. The greatest reduction in planktonic cell density was 1.5-2 orders of magnitude and was seen in reactors with uncoated coupons receiving silver fluoride biocide. In the reactors receiving biocide dose, the planktonic community was almost entirely composed of *R. insidiosa* and its response to biocide dosing was highly significant ($p\text{-value} = 1.00\text{E-}16$). In the ISS water system, reducing planktonic cell density is a key goal in addition to reducing biofilm density because planktonic cells (and/or detached biofilm clumps) can cause clogging issues downstream in the water recovery process. Also, planktonic cells can serve to inoculate surfaces within the wastewater tank (represented by the CDC reactor) or further downstream, leading to additional biofilm formation.

Combining the coating, a daily silver fluoride dosing, and phosphorus removal resulted in the lowest viable biofilm accumulation, ultimately leading to a biofilm total cell density of $1.74 \log_{10}\text{CFU/cm}^2$ (Inconel) and $1.77 \log_{10}\text{CFU/cm}^2$ (Teflon) at 7 days, which is just above the limit

of detection of $1.69 \log_{10}\text{CFU}/\text{cm}^2$. None of the control methods alone came anywhere near this level of biofilm reduction. Interestingly, the best combination of two methods that gave a good level of biofilm reduction was the Sher-Loxane® 800 coating and silver biocide dose, which resulted in a total cell density of $3.24 \log_{10}\text{CFU}/\text{cm}^2$ (Inconel) and $3.07 \log_{10}\text{CFU}/\text{cm}^2$ (Teflon), nearly 1.5 logs higher than when all three control strategies were combined. This, along with the results from the mixed effects model (p-values listed in Table 2), indicates that the presence of the coating and the biocide dose were the most important factors for biofilm reduction; however, the inclusion of the coating had the single greatest effect on viable biofilm accumulation. Testing all three biofilm control methods alone and in combination with each other was important to show that under the conditions tested good biofilm control will almost certainly not be achieved by one method alone.

Biomass and biofilm morphology: Confocal image analysis

In spacecraft water systems, the goals of biofilm control extend beyond logarithmic reduction of viable microbial cells. The accumulation of the biofilm and biofilm detachment (even dead cells) contributes to the potential clogging of valves and pipes. As such, it is important to evaluate the biofilm thickness and morphology in addition to the viable cells within. To investigate the biomass and overall clogging potential of the biofilms in various states of control, confocal imaging provides important insight.

Figures 5 and 6 feature representative confocal images of the biofilms after 7 days in CDC reactors for each of the 8 iterations of experiments conducted. The uncoated Inconel coupons (Figure 5) seemed to foster thinner, near monolayer, biofilms with fewer large aggregates than the uncoated Teflon coupons (Figure 6), which aligns with the viable biofilm

density data (Figure 3). However, when the coating was utilized, there were minimal differences in biofilm morphology (or density) between Inconel and Teflon, which is also supported by the CFU data (Figure 3). In Figure 7 the differences in thickness for all the biofilms grown in full strength microbial ersatz MTN and without biocide dosing is shown. This figure was generated by finding the x-slice of the confocal images in Figures 5 and 6 with the tallest segment of biofilm. Despite the general monolayer morphology of the biofilm on uncoated Inconel (Figure 7A), the clump of biofilm in the image results in a biofilm with a larger maximum thickness than the biofilms on Sher-Loxane® 800 coated coupons. These tall clumps of biofilm are concerning for their potential for sloughing off and clogging pipes. The clogging potential is even more drastic with the uncoated Teflon biofilm (Figure 7C).

A key morphological difference between biofilms on the uncoated and coated coupons was the presence and absence of fungal hyphae, respectively. On the uncoated coupons, especially on Teflon, fungi were present in the hyphal (filamentous) morphology. The hyphae add significant height to the biofilm, sometimes upwards of 200 μm as can be seen in Figure 7C and add additional surface area for bacterial colonization. Both live and dead bacteria (green and red, respectively) appear to have colonized the fungal hypha (blue) in Figure 7C. The increased height further enhances clogging potential in small diameter pipes and valves like those found in spacecraft water systems. The additional surface area provided by the hyphae for bacterial colonization means that any treatment present at the substratum (such as a coating) would not be effective against bacteria colonizing the hyphae. For this reason, it is especially encouraging that this coating fostered minimal fungal cell attachment, and that the fungal cells present were in the conidial morphology (Figures 5 and 6, lower right corner).

Comparison to previous work

Each of the approaches for biofilm control evaluated in this research have been explored individually by other researchers for spacecraft applications. Four peer-reviewed studies on coatings for use in spacecraft water systems have been published (Demir et al., 2022; Flores et al., 2023; Li Sip et al., 2023; Mettler et al., 2022). All four studies featured *Pseudomonas aeruginosa*, an organism that has not been isolated from the ISS wastewater tank, potable water dispenser, or condensate. The media used in the tests varied between researchers, but a simulated wastewater was not employed by any of them. One group used Luria Broth with potassium nitrate as an artificial urine medium (Flores et al., 2023), while the others used TSB (Li Sip et al., 2023; Mettler et al., 2022), PDB (Mettler et al., 2022), or PBS (Demir et al., 2022). All the studies reported promising results, but tests only lasted 72 h or less.

One published study evaluated numerical models of nutrient limitation in the ISS water processing assembly (WPA). The researchers evaluated the effects of carbon and phosphorus limitation to understand biofilm accumulation in the WPA during typical operation and potential dormancy conditions (Diaz et al., 2021). The models also investigated the effects oxygen availability would have on open and closed systems. Though the study discusses bacterial metabolism, no specific organisms were listed as the basis of the model and fungal microorganisms were not included, which as reported in the results here, contribute greatly to biomass and biofilm thickness. While the study was an interesting mathematical evaluation, no biological tests were completed to support the reported results.

Several studies examining biocide use for biofilm reduction in spacecraft water distribution systems have been published. A long-term study (3 years) found that iodine dosing showed promising reduction of biofilm as long as concentrations were maintained between 1-3

mg/L (Schultz et al., 1992). The researchers used stainless steel, a relevant material (Callahan et al., 2007), and used non-sterile Milli-Q water as the medium and inoculum. They frequently isolated organisms in the resulting biofilm included *Pseudomonas cepacia* (now known as *Burkholderia cepacia*), *Pseudomonas pickettii*, *Acinetobacter sp.*, *Bacillus sphaericus*, and a *Methylobacterium sp.*, which is very similar to the defined consortium used in the research presented here. The same group published an additional study evaluating iodine and ozone for biofilm kill and removal in spacecraft water systems (Schultz et al., 1994). Their results showed that 25 mg/L and 250 mg/L iodine, and 2.3 mg/L ozone effectively kill attached biofilm but do not remove biofilm. The biocides were evaluated as a treatment method on established biofilm rather than as a maintenance method with regular dosing.

One paper describes a combination of nutrient limitation and biocide dosing for evaluation of dormancy conditions in spacecraft water systems (Beitle et al., 2024). The consortium used in the experiments was very similar to the one used here, but with the addition of *Cupriavidus metallidurans*, another frequent isolate of the ISS wastewater tank (Zea et al., 2020). The biocide used was silver fluoride and the target nutrient for removal was phosphorus. The experiments also feature a “synthetic wastewater,” but the specific composition is not listed. This study is an exciting synthesis of two methods of biofilm control, but unfortunately it only describes the experimental setup and to the best of our knowledge, the results have not been published.

Promising results have been reported in areas of research associated with all three biofilm control methods examined here: coatings, nutrient limitation, and biocide dosing for control or treatment. More recent studies have reported the research of combining nutrient limitation and

biocide dosing, though results have not been published (Beitle et al., 2024). That study also used a relevant consortium of organisms and a synthetic wastewater, improving relevance to the ISS water system. The results from that study, when published, may corroborate results from this study that show multiple approaches of biofilm control are necessary for proper suppression of biomass and biofilm accumulation.

Similar approaches that were used in this research could be used to evaluate biofilm control in Earth-based water systems. It is likely that using organisms relevant to drinking water distribution systems, industrial water systems, or wastewater systems and the use of a medium relevant to the target system could result in similar biofilm reduction when multiple control strategies are combined. The role of space research is often to pave the way for related application on Earth, as may be the case here.

Conclusions

The presented experiments represent the first time, to our knowledge, a multidomain biofilm has been used in evaluating coatings as a control method in spacecraft water systems. It also represents the first time a relevant medium and consortium were used, and an experiment duration longer than 72 h. Further, this is the first time that additional biofilm control methods were tested in combination with coatings for biofilm control in water systems. The results indicate that with these organisms and conditions, a combination of control methods was necessary for effective biofilm mitigation. Each control method— Sher-Loxane® 800 coating, phosphorus removal, and silver fluoride dosing—had individual effects on reducing biofilm accumulation, but the presence of the coated surface was the key to reducing accumulated biofilm to the limit of detection.

While this research was framed within the context of the ISS water recovery system, and more specifically the wastewater tank, the results may extend to water systems on Earth as well. The combined effects of multiple biofilm control methods are under-investigated despite the general understanding of the difficulty of controlling biofilm accumulation.

Building upon this research further, it will be important to extend the experiments past 7 days and to incorporate reinoculation of the organisms at regular intervals during the experiment. Further improvements include the incorporation of simulated microgravity as the goal of these biofilm control methods is to reduce biofilm associated risks in spacecraft water systems.

Acknowledgements

We would like to thank Elizabeth Sandvik for her immense contribution to laying the groundwork for the methods used in these experiments, namely organism selection and designing the medium, and her assistance with confocal imaging. We would also like to thank Albert Parker for his involvement in the statistical analysis of the data presented in this paper. We would like to thank researchers at Marshall Spaceflight Center for their assistance in designing the nutrient medium.

Funding

Funding was provided by NASA through EPSCoR cooperative agreement 80NSSC21M0331.

Author disclosure statement

The authors jointly disclose commercial associations with The Sherwin-Williams Company, which has been an industrial affiliate of the Center for Biofilm Engineering since 2009. Brent Peyton participates in collaborative research with The Sherwin-Williams Company on a project funded by the Department of Defense. Madelyn Mettler and Brent Peyton have not received direct funding from The Sherwin-Williams Company.

References

- Atlas, R. M. (2010). *Handbook of Microbiological Media, Fourth Edition*. Taylor & Francis. <https://books.google.com/books?id=Pj5FAQAAIAAJ>
- Azim, N., Diaz, A., Li, W., Calle, L. M., Irwin, T., & Callahan, M. (2020). *Literature Review of Disinfection Techniques for Water Treatment* International Conference on Environmental Systems,
- Beitle, E., McCormick, M., McCall, M., Sandvik, E. L., Justiniano, Y.-A. V., & Williamson, J. (2024). Ground Test Validations for Uncrewed Dormancy Preparation and Recovery. 53rd International Conference on Environmental Systems (ICES),
- Callahan, M. R., Adam, N. M., Roberts, M. S., Garland, J. L., Sager, J. C., & Pickering, K. D. (2007). Assessment of silver based disinfection technology for CEV and future US spacecraft. *SAE Transactions*, 481-491.
- Carter, D. L. (2010). Status of the Regenerative ECLSS Water Recovery System. International Conference of Environmental Systems, Barcelona, Spain.
- Demir, B., Taylor, A., Broughton, R. M., Huang, T. S., Bozack, M. J., & Worley, S. D. (2022). N-halamine surface coating for mitigation of biofilm and microbial contamination in water systems for space travel. *Biofilm*, 4, 100076. <https://doi.org/https://doi.org/10.1016/j.biofilm.2022.100076>
- Diaz, A., Li, W., Irwin, T., & Calle, L. (2021). An Updated Modeling Study on Nutrient Deprivation as a Biofilm Mitigation Strategy for Long Term Space Missions.
- Donlan, R. M. (2002). Biofilms: microbial life on surfaces. *Emerging infectious diseases*, 8(9), 881-890. <https://doi.org/10.3201/eid0809.020063>

- Elasri, M. O., & Miller, R. V. (1999). Study of the response of a biofilm bacterial community to UV radiation. *Applied and Environmental Microbiology*, 65(5), 2025-2031.
- Esbelin, J., Santos, T., & Hébraud, M. (2018). Desiccation: An environmental and food industry stress that bacteria commonly face. *Food Microbiology*, 69, 82-88.
<https://doi.org/https://doi.org/10.1016/j.fm.2017.07.017>
- Filice, S., Sciuto, E. L., Scalese, S., Faro, G., Libertino, S., Corso, D., Timpanaro, R. M., Laganà, P., & Coniglio, M. A. (2022). Innovative Antibiofilm Smart Surface against Legionella for Water Systems. *Microorganisms*, 10(5).
<https://doi.org/10.3390/microorganisms10050870>
- Flemming, H.-C., Neu, T. R., & Wozniak, D. J. (2007). The EPS Matrix: The “House of Biofilm Cells”. *Journal of Bacteriology*, 189(22), 7945-7947. <https://doi.org/10.1128/jb.00858-07>
- Flores, P., McBride, S. A., Galazka, J. M., Varanasi, K. K., & Zea, L. (2023). Biofilm formation of *Pseudomonas aeruginosa* in spaceflight is minimized on lubricant impregnated surfaces. *NPJ microgravity*, 9(1), 66. <https://doi.org/10.1038/s41526-023-00316-w>
- Hwang, C., Ling, F., Andersen Gary, L., LeChevallier Mark, W., & Liu, W.-T. (2012). Microbial Community Dynamics of an Urban Drinking Water Distribution System Subjected to Phases of Chloramination and Chlorination Treatments. *Applied and Environmental Microbiology*, 78(22), 7856-7865. <https://doi.org/10.1128/AEM.01892-12>
- International, A. (2018). ASTM E3161-18, Standard Practice for Preparing a *Pseudomonas aeruginosa* or *Staphylococcus aureus* Biofilm using the CDC Biofilm Reactor. In. West Conshohoken, PA.
- Li Sip, Y. Y., Jacobs, A., Morales, A., Sun, M., Roberson, L. B., Hummerick, M. E., Roy, H., Kik, P., & Zhai, L. (2023). Slippery lubricant-infused silica nanoparticulate film processing for anti-biofouling applications. *J Appl Biomater Funct Mater*, 21, 22808000231184688. <https://doi.org/10.1177/22808000231184688>
- Mah, T.-F. C., & O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology*, 9(1), 34-39.
[https://doi.org/https://doi.org/10.1016/S0966-842X\(00\)01913-2](https://doi.org/https://doi.org/10.1016/S0966-842X(00)01913-2)
- Mettler, M. K., Parker, C. W., Venkateswaran, K., & Peyton, B. M. (2022). Antimicrobial Coating Efficacy for Prevention of *Pseudomonas aeruginosa* Biofilm Growth on ISS Water System Materials [Original Research]. *Frontiers in microbiology*, 13.
<https://doi.org/10.3389/fmicb.2022.874236>

- Munavalli, G. R., & Kumar, M. S. M. (2003). Optimal Scheduling of Multiple Chlorine Sources in Water Distribution Systems. *Journal of Water Resources Planning and Management*, 129(6), 493-504. [https://doi.org/doi:10.1061/\(ASCE\)0733-9496\(2003\)129:6\(493\)](https://doi.org/doi:10.1061/(ASCE)0733-9496(2003)129:6(493))
- Pan, R., Zhang, T.-Y., He, H., Zheng, Z.-X., Dong, Z.-Y., Zhao, H.-X., Xu, M.-Y., Luo, Z.-N., Hu, C.-Y., Tang, Y.-L., El-Din, M. G., & Xu, B. (2023). Mixed chlorine/chloramines in disinfected water and drinking water distribution systems (DWDSs): A critical review. *Water Res*, 247, 120736. <https://doi.org/https://doi.org/10.1016/j.watres.2023.120736>
- Roberson, E. B., & Firestone, M. K. (1992). Relationship between Desiccation and Exopolysaccharide Production in a Soil *Pseudomonas* sp. *Applied and Environmental Microbiology*, 58(4), 1284-1291. <https://doi.org/doi:10.1128/aem.58.4.1284-1291.1992>
- Samuel, M. S., Moghaddam, S. T., Shang, M., & Niu, J. (2022). A Flexible Anti-Biofilm Hygiene Coating for Water Devices. *ACS Appl Bio Mater*, 5(8), 3991-3998. <https://doi.org/10.1021/acsabm.2c00538>
- Sandvik, E., Stewart, P., Goeres, D., & Sturman, P. (2022). Center for Biofilm Engineering Recommendations for Addition of Trace Nutrients to Microbial Ersatz. In J. Williamson & Y. A. Velez Justiniano (Eds.).
- Schultz, J. R., Flanagan, D. T., Bruce, R. J., Huls, M. H., Pierson, D. L., & Sauer, R. L. (1994). Evaluation of methods for remediating biofilms in spacecraft potable water systems. *SAE Transactions*, 939-949.
- Schultz, J. R., Flanagan, D. T., Bruce, R. J., Mudgett, P. D., Carr, S. E., Rutz, J. A., Huls, M. H., Sauer, R. L., & Pierson, D. L. (1992). Biofilm formation and control in a simulated spacecraft water system: three year results. *SAE Transactions*, 1032-1044.
- Si, X., Quan, X., & Wu, Y. (2015). A small-molecule norspermidine and norspermidine-hosting polyelectrolyte coatings inhibit biofilm formation by multi-species wastewater culture. *Applied microbiology and biotechnology*, 99(24), 10861-10870. <https://doi.org/10.1007/s00253-015-6943-0>
- Sójka, O., Keskin, D., van der Mei, H. C., van Rijn, P., & Gagliano, M. C. (2023). Nanogel-based coating as an alternative strategy for biofilm control in drinking water distribution systems. *Biofouling*, 39(2), 121-134. <https://doi.org/10.1080/08927014.2023.2190023>
- Velez Justiniano, Y.-A., Carter, D., Sandvik, E., Stewart, P., Goeres, D., Sturman, P., Li, W., Johnson, A., & Cioanta, I. (2021). *Biofilm Management in a Microgravity Water Recovery System* 50th International Conference on Environmental Systems,

- Velez Justiniano, Y.-A., Lim Chae, H., Dunlap Darren, S., & Sysoeva Tatyana, A. (2023). Genome Sequences of Three Common Bacterial Isolates from Wastewater from the Water Processor Assembly at the International Space Station. *Microbiology Resource Announcements*, 12(1), e01189-01122. <https://doi.org/10.1128/mra.01189-22>
- Velez Justiniano, Y. A., Carter, L., Nur, M., & Angle, G. (2020). Developing Methods for Biofilm Control in Microgravity for a Water Recovery System. International Conference on Environmental Systems, Virtual.
- Wingender, J., & Flemming, H.-C. (2011). Biofilms in drinking water and their role as reservoir for pathogens. *International Journal of Hygiene and Environmental Health*, 214(6), 417-423. <https://doi.org/https://doi.org/10.1016/j.ijheh.2011.05.009>
- Yin, W., Wang, Y., Liu, L., & He, J. (2019). Biofilms: The Microbial "Protective Clothing" in Extreme Environments. *Int J Mol Sci*, 20(14). <https://doi.org/10.3390/ijms20143423>
- Zea, L., McLean, R. J. C., Rook, T. A., Angle, G., Carter, D. L., Delegard, A., Denvir, A., Gerlach, R., Gorti, S., McIlwaine, D., Nur, M., Peyton, B. M., Stewart, P. S., Sturman, P., & Velez Justiniano, Y. A. (2020). Potential biofilm control strategies for extended spaceflight missions. *Biofilm*, 2, 100026. <https://doi.org/https://doi.org/10.1016/j.biofilm.2020.100026>
- Zea, L., Nisar, Z., Rubin, P., Cortesão, M., Luo, J., McBride, S. A., Moeller, R., Klaus, D., Müller, D., Varanasi, K. K., Muecklich, F., & Stodieck, L. (2018). Design of a spaceflight biofilm experiment. *Acta Astronautica*, 148, 294-300. <https://doi.org/https://doi.org/10.1016/j.actaastro.2018.04.039>

CHAPTER FIVE

MULTIDOMAIN BIOFILM CONTROL IN A NOVEL SIMULATED MICROGRAVITY REACTOR

Contribution of Authors and Co-Authors

Manuscript in Chapter 5

Author: Madelyn K. Mettler

Contributions: Designed study, conducted simulated microgravity biofilm reactor assays, performed microscopy, generated figures, completed statistical analysis, and drafted the manuscript.

Co-Author: Brent M. Peyton

Contributions: Assisted with study design, evaluated and discussed results, and edited manuscript.

Manuscript Information

Madelyn K. Mettler and Brent M. Peyton

Status of Manuscript:

☒ Prepared for submission to a peer-reviewed journal

☐ Officially submitted to a peer-reviewed journal

☐ Accepted by a peer-reviewed journal

☐ Published in a peer-reviewed journal

Abstract

Issues caused by unwanted microbial accumulation in spacecraft water systems must be addressed for successful spaceflight missions traveling much farther from earth than the International Space Station (ISS). Biofilms have previously caused clogs in the ISS water recovery system which required part replacement. The study presented here builds on previous research to control biofilms in the ISS water system by evaluating three methods of biofilm control for their efficacy in a simulated microgravity biofilm reactor. This research aimed to model the ISS by using four direct isolates from the water recovery system, a medium designed to mimic the chemical composition of ISS wastewater, and simulated microgravity. Surface-associated biofilm formation and microbial aggregates in the bulk fluid were evaluated as they both pose concerns for clogging in spacecraft water systems. By the end of the 7 day experiment, no viable biofilm was detected on the surfaces in the reactor with an antifouling coating, no-phosphorus medium, and daily silver fluoride biocide dosing. This research marks a large step in accurately modeling the ISS water recovery system and exploring options for biofilm control in future spacecraft water systems.

Introduction

The Artemis generation of space exploration will require spacecraft that are more independent from Earth than the International Space Station (ISS) has been for the last 20 years. Resupply to the ISS can take place in a matter of hours, while Gateway, phase four of the Artemis missions, will facilitate the farthest human space missions ever completed (1,000 times farther from Earth than the ISS) [1, 2]. Gateway will be uninhabited for long periods of time,

with astronauts only present in the station for approximately three months at a time [1]. This presents a unique challenge for advanced life support systems which must remain functional during long periods of dormancy and restart properly before astronauts return.

Previously, the Environmental Control and Life Support Systems (ECLSS) of the ISS have experienced issues with biofilms that have required part replacement and new methods of microbial control [3-7]. Biofilms are communities of microorganisms that often confer advantages to cells (e.g., antimicrobial resistance) and protection from environmental stresses like desiccation, starvation, biocides, UV radiation, and osmotic lysis [8-16]. The cells within a biofilm are embedded in a sticky matrix known as extracellular polymeric substances (EPS) which are mostly composed of polysaccharides, but also contain nucleic acids, lipids, and proteins. The EPS helps confer many of the survival advantages to the biofilm and can act as a layer of protection to the cells within [10, 11]. These properties make biofilms difficult to mitigate. Challenges associated with biofilm removal and control are exacerbated in cramped environments that make physical cleaning difficult, such as those found in spacecraft life support systems.

Great improvements have been made to ECLSS on the ISS over its lifetime [4, 17-20]. But there will be unique challenges associated with maintaining functional and clean water systems during Gateway's long dormancy periods [21, 22]. The study presented here aims to improve on previously completed studies using three methods of biofilm control for spacecraft water systems: a surface coating, phosphorus limitation, and silver fluoride biocide. The critical improvement in these updated experiments is the use of a novel simulated microgravity biofilm reactor [23] rather than normal gravity biofilm evaluation (as discussed in Chapter 4). The

incorporation of simulated microgravity marks a large step toward accurate modeling of spacecraft water systems. The organisms [22], nutrient medium [24], and surface materials [25] used were all informed by what is present in the ISS, since it is assumed that the life support systems in Gateway will be heavily informed by and modeled after the ISS.

The simulated microgravity biofilm reactor (SMBR) was developed by researchers at the Center for Biofilm Engineering and Department of Mechanical and Industrial Engineering at Montana State University (MSU). The SMBR is a rotating wall vessel that features coupons on the reactor walls which provide a removable physical surface for biofilm evaluation [23]. This is a key design feature for assessing material coatings as a method of biofilm control. The reactor provides a non-unidirectional, low-shear environment, even at the reactor walls, with computational fluid dynamics models showing maximum fluid shear on the wall coupons of 0.17 mPa [23], well below the accepted shear of 1 mPa allowable for SMG [26-28]. Further, this reactor offers increased flexibility over some other reactors on the market, namely the option of operation in batch or continuous flow mode. As shown in this study, these unique features make the MSU SMBR an attractive option for evaluating biofilm control methods for spacecraft applications. The study presented here, builds on previous multi-domain biofilm research (Chapter 4 and [21]) and for the first time, evaluates the combined impacts of a surface coating, nutrient limitation, and biocide addition on biofilm accumulation in a simulated microgravity reactor.

Methods

Organism selection and preparation

The organisms used in the experiments are all frequently isolated from the ISS water system [22]. The three bacterial species used were direct ISS isolates provided by Boeing: *Burkholderia contaminans* (wastewater tank isolate), *Methylobacterium organophilum* (oxygen generation assembly isolate), and *Ralstonia insidiosa* (wastewater tank isolate). The fungus used, *Coniochaeta mutabilis* (formerly *Lecythophora mutabilis*), is a direct wastewater tank isolate provided by Marshall Space Flight Center. These four organisms were used as a consortium in all the experiments. The organisms were stored as frozen stocks in a 50/50 (v/v) mixture of glycerol and cell culture in their corresponding medium (tryptic soy broth for bacteria and Sabouraud dextrose broth for fungus). Streak plates were prepared on R2A agar (BD Difco) to ensure culture purity, and one colony was used to inoculate sterile 125 mL baffled flasks containing 30 g/L tryptic soy broth (for bacteria) or 30 g/L Sabouraud dextrose broth (for the fungus). The flasks were placed in a shaking incubator at 225 rpm and 30 °C. *M. organophilum* and *C. mutabilis* cultures were started 48 h prior to reactor inoculation and *B. contaminans* and *R. insidiosa* cultures were started 24 h prior to reactor inoculation. After incubation, the cultures were washed via three centrifugation steps (3 min at 8,000 xg) and resuspension in 0.85% NaCl. The optical density of each culture was adjusted such that when 100 µL of washed cells were added to the reactor, the initial reactor concentration of each organism was approximately 10⁴ CFU/mL.

Nutrient medium

The nutrient medium used was microbial ersatz minor/trace nutrients (MTN), a medium designed to mimic the nutrient concentration and chemical composition of the ISS wastewater tank. The medium recipe is listed in Table 1 (Chapter 4), and is a slightly modified version of a previously published recipe [24, 29]. The medium was prepared in a 13L glass carboy that had been washed with 10% HCl and thoroughly rinsed with Milli-Q water (18.2 M Ω ·cm).

To prepare the medium, 7 L of Milli-Q water was sterilized in the carboy by autoclaving for at least 90 minutes (121 °C, 21 PSI). After the autoclave cycle, concentrated nutrient stock solutions were added, and the volume was brought to 8 L with sterile Milli-Q water. The pH of a 10 mL aliquot was checked and adjusted to 5.55 ± 0.05 with 1 M HCl or 1 M NaOH. The volume of acid/base was then scaled, and sterile solutions were added to the carboy. The pH of a new 10 mL aliquot was then validated. For experiments without phosphorus, the monobasic potassium phosphate stock was not added to the medium.

Table 1. Recipe for microbial ersatz (MTN) featuring final concentrations. Colors correspond to components that were added together in concentrated stock solutions.

Component	Concentration		Component	Concentration	
Propylene Glycol	8.971	μL/L	Benzyl Alcohol	6.075	μL/L
Ethanol	44.946	μL/L	Diethylphthalate	2.01	μL/L
Acetone	25.799	μL/L	Trimethyl Silanol	0.635	μL/L
2-(2-butoxyethoxy) Ethanol	2.356	μL/L	Benzothiazole	0.21	μL/L
N, N- Dimethylformamide	1.486	μL/L	2-Ethyl-1-Hexanol	0.48	μL/L
2-Ethoxyethanol	1.303	μL/L	Decamethylcyclopentasiloxane	1.03	μL/L
1-Methyl-2- Pyrrolidinone	0.782	μL/L	Dodecamethylcyclohexasiloxane	10	μL/L
2-Propanol	0.896	μL/L	Octamethylcyclotetrasiloxane	1.02	μL/L
1-Propanol	0.876	μL/L	Dimethoxydimethylsilane	35.1	μL/L
4-Ethylmorpholine	2.265	μL/L	Calcium Sulfate	1.885	mg/L
Formic Acid	27.489	μL/L	Dimethyl Sulfone	0.205	mg/L
Lactic Acid	8.55	μL/L	Hexamethylcyclotrisiloxane	1.02	mg/L
Benzoic Acid	3.015	mg/L	Monobasic Potassium phosphate	0.835	mg/L
Caprolactam	1.111	mg/L	Magnesium chloride hexahydrate	0.397	mg/L
Urea	1.899	mg/L	Manganese chloride tetrahydrate	0.319	mg/L
Zinc (II) Acetate dihydrate	1.425	mg/L	Ferric chloride	0.129	mg/L
Nickel (II) Acetate tetrahydrate	0.518	mg/L	Boric acid	0.268	mg/L
Acetic Acid	32.088	μL/L	Cobalt chloride hexahydrate	0.004	mg/L
Ammonium Bicarbonate	101.457	mg/L	Sodium molybdate dihydrate	0.016	mg/L
Sodium Fluoride	0.88	mg/L			
Potassium Iodide	0.021	mg/L			

Biocide preparation

For experiments that received a biocide dose, silver (I) fluoride (99+%, Thermo Scientific) was prepared at a concentration of 521 mg/L such that when it was introduced to the reactor, it was present at a concentration of 2 ppm. The silver (I) fluoride was dissolved in Milli-Q water and filter sterilized into a sterile 50 mL conical vial.

Reactor assembly and preparation

The reactor was a rotating wall vessel custom built by researchers at the MSU Center for Biofilm Engineering (Figure 1). Details of the reactor, fluid modeling, and preliminary batch biofilm studies were recently published in Ketteler et al., 2025 [23]. Slight changes to the reactor were made prior to the experiments presented here, including a coupon ring with 8 slots for disk coupons featuring recesses flush with the walls of the reactor, and the elimination of coupons suspended down the center axis. The rest of the reactor remained the same, including the use of polycarbonate tubing for the walls.

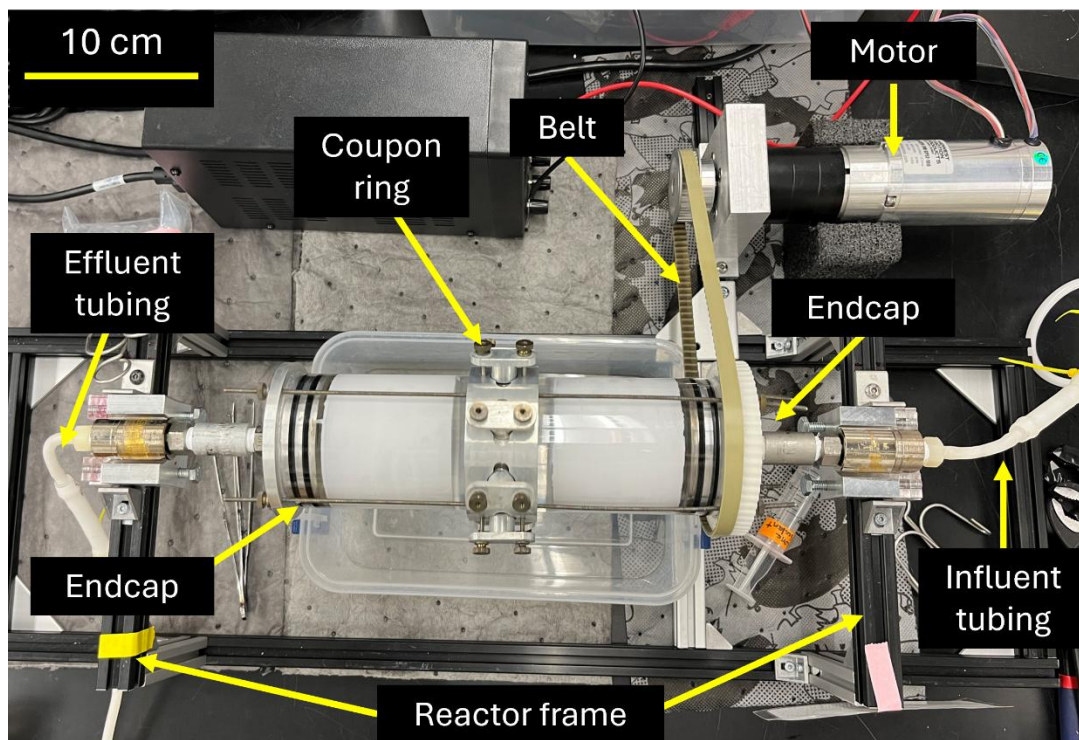


Figure 1. Simulated microgravity reactor featuring Sher-Loxane® 800 coated polycarbonate walls. For a detailed CAD drawing of the original reactor design, refer to Ketteler et al., 2025 [25].

The disk-shaped biofilm coupons (BioSurface Technologies) used in these experiments were 316 stainless steel, a common material in spacecraft water systems [25]. The coupons were designed for use in Center for Disease Control (CDC) biofilm reactors [30] and are 12.7 mm in diameter and 1.8 mm in thickness. Upon receiving the coupons, they were washed by submersion and sonication in soapy water, rinsed with Milli-Q water, and sonicated again in Milli-Q water. Some coupons were left uncoated, and some were coated with Sherwin-Williams Sher-Loxane® 800 coating. The coating applied with a paintbrush such that a visually homogenous layer was present. Only one side of the coupons needed to be coated, as only one side of the coupons was exposed to the bulk fluid in the reactor.

For experiments featuring coated coupons, the polycarbonate tubing (reactor wall) was also coated with the Sher-Loxane® 800 using a paintbrush and rolling the tubing to evenly coat the walls. Tape was used to ensure the ends were not coated to maintain the tolerances in the endcaps and to allow some visibility into the reactor at the end caps and coupon ring during operation.

Prior to experiments, the uncoated reactor was fully assembled and sterilized in an autoclave at 121 °C and 21 PSI. When the coating was used, the polycarbonate tubing and stainless steel coupons were not autoclaved and were instead sterilized with 99% isopropanol and the reactor was assembled in a biosafety cabinet (Labconco, Class II cabinet). The influent nutrient medium and effluent waste was pumped with size 16 C-Flex tubing (MasterFlex #06424-16), selected to maximize flexibility while minimizing gas diffusion and bubbles.

Reactor operation

The reactor was connected via C-Flex tubing to the influent carboy containing sterile ersatz MTN medium and the effluent waste carboy. The reactor was filled to the halfway mark while in the horizontal position on the reactor stand. The pump (Masterflex L/S) was then paused, a coupon holder slot was opened, and 100 μ L of each washed cell culture was added to the reactor. The coupon was replaced, and the reactor was positioned vertically on a ring stand (filling from the bottom to remove air). The pumping was resumed, and the reactor was filled with medium.

After filling with medium so no bubbles were present, the reactor was placed in the horizontal position on the frame and rotation was initiated (24.8 RPM). The delay between inoculation and starting rotation was about 15 min. The reactor remained rotating during the entire duration of the experiments. Cyclical batch and continuous flow phases were implemented to model the filling of the ISS wastewater tank (batch) and the emptying for subsequent processing (continuous flow). The cycle featured 21 h of batch operation followed by 3 h of continuous flow at 4.83 mL/min (a residence time of approximately 160 min). This cycle was continued for 7 days of operation with final sampling taking place about 20 min into the final continuous flow phase.

For experiments that included biocide dosing, 3 mL of sterile silver (I) fluoride was injected through an inline luer injection port. The membrane was sterilized with a 70% ethanol preparation wipe and the biocide was injected with a needle and syringe. Biocide dosing took place with approximately 20 min remaining in continuous flow cycle.

Three iterations of experiments were conducted. The first featured no biofilm control methods. The second iteration focused on coupons and reactor walls coated with Sher-Loxane®

800, with the coating covering about 64% of the reactor interior. The last iteration of the experiment included three previously investigated biofilm control methods: Sher-Loxane® 800 coating on coupons and walls (also 64% surface area coverage), nutrient medium without phosphorus (nutrient limitation), and a daily biocide dose of silver (I) fluoride (similar to the methods used in Chapter 4). Duplicates of all three experiments were completed.

Sampling

About 20 min into the continuous flow phase each day, an effluent planktonic sample was taken via a sampling port in the effluent tubing (a syringe connected to a luer lock in a y-split). A volume of 4.4 mL effluent sample was added to 600 µL of 50% glutaraldehyde so that the final concentration of glutaraldehyde was 6% and another aliquot was diluted and plated for viable counts. Prior to diluting for viable counts, the sample was vortex mixed briefly to break up any large aggregates of cells. The sample fixed in glutaraldehyde was not vortexed and was later examined on a FlowCam (Yokogawa) to determine presence and morphology of suspended biofilm aggregates.

After 7 days the reactor was sampled for biofilm. About 1 hour prior to sampling, the gradual slowing of the rotation began. Continuous flow was initiated for the last 20 min of slowed reactor rotation to allow collection of an effluent sample. After the sample was taken, the pump and reactor rotation were stopped entirely. The effluent tubing was clamped, and a coupon was removed with flame-sterilized tweezers. The first coupon removed was stained for confocal microscopy and a bulk planktonic sample was removed from the reactor with a serological pipette. The coupon was placed biofilm side up in a 24 well plate and stained initially with BacLight LIVE/DEAD stain (Thermo Fisher Scientific) and incubated in the dark for 15 min.

After the incubation period, the stain was removed, and the coupon was gently rinsed 3 times with filter sterilized Milli-Q water. Then the coupon was stained with a 50/50 solution of Calcofluor White (MilliporeSigma) and phosphate buffered saline (PBS) and incubated in the dark for 10 min. The stain was pipetted off, and the coupon was rinsed three times and submerged in 4% paraformaldehyde and 2.5% glutaraldehyde fixative solution until confocal microscopy. An unused coupon was placed into the slot and the coupon holder was reassembled.

The reactor was rotated so a new coupon was at the uppermost slot. The coupon holder was disassembled, and the coupon was removed with flame-sterilized tweezers. The coupon was placed biofilm side up in a sterile petri dish. Sterile tweezers were used to hold the coupon stable, and the biofilm was scraped off with a sterile rayon swab. The swab tip was cut off with flame-sterilized scissors and placed into a 50 mL sterile conical vial containing 10 mL phosphate buffered saline (PBS) and 10 μ L Tween 20. The scraped coupon was then placed back into the reactor and the process was repeated until 5 coupons were sampled for viable plate counts. The biofilm on the rayon swabs was disaggregated via vortex mixing and sonication in an alternating series of 30 second intervals for a total of 2.5 min. The disaggregated biofilm was serially diluted in sterile PBS and plated for viable plate counts.

Selective agar was used to enumerate the species individually and samples were also plated on plain R2A agar for total counts. See Table 2 for agar recipes. After drying, agar plates were placed in a 30 °C incubator until visible colonies formed, typically 2 days for *B. contaminans*, *R. insidiosa*, and general R2A plates, and 4 days for *C. mutabilis* and *M. organophilum*.

Table 2. Selective agar recipes.

Organism selected for	Agar type
<i>B. contaminans</i>	<i>Pseudomonas</i> isolation agar
<i>C. mutabilis</i>	Sabouraud dextrose agar, pH lowered to 3
<i>M. organophilum</i>	<i>Methylobacterium</i> agar [31] with Amphotericin B (7.5 µg/mL)
<i>R. insidiosa</i>	R2A with Amphotericin B (7.5 µg/mL) and chloramphenicol (20 µg/mL)

FlowCam

The planktonic samples for FlowCam analysis were fixed in glutaraldehyde (final concentration 6%) and stored at 4°C until image analysis. Samples for each day and experimental replicates were processed (n=48 total samples). Samples from the experiment with uncoated coupons, full strength medium, and no biocide dosing were analyzed using the 4x and 10x flow cells. Prior to imaging these samples on the 10x flow cell, they were filtered through a sterile 100 µm nylon mesh. The samples from the other two experimental iterations (with Sher-Loxane® 800 coated walls as the only biofilm control method and Sher-Loxane® 800 coated walls, no-phosphorus medium, and silver biocide dosing) were imaged on the 10x flow cell without a prefiltering step. The samples were imaged using context files with 72% efficiency. Particle diameters are presented as area-based diameters, meaning the FlowCam software calculated the area of the particle, converted it to a circle, and determined diameter from that.

Results

Biofilm community

Biofilm sampling took place on the final day of the experiment, 7 days after the reactor was inoculated. In the SMBR with no biofilm control methods applied (Figure 2A), the total

viable biofilm density was approximately $6.5 \log_{10}\text{CFU}/\text{cm}^2$, dominated by *R. insidiosa*. The SMBR with Sher-Loxane® 800 coated walls and coupons (Figure 2B) featured a similar viable cell density to that with uncoated coupons (Figure 2A), with *B. contaminans* and *R. insidiosa* present at similar densities in all four tests. The reactor with coated walls/coupons, medium without phosphorus, and daily silver fluoride doses did not support a viable biofilm that could be detected with plate counts (Figure 2C; limit of detection $2.08 \log_{10}\text{CFU}/\text{cm}^2$).

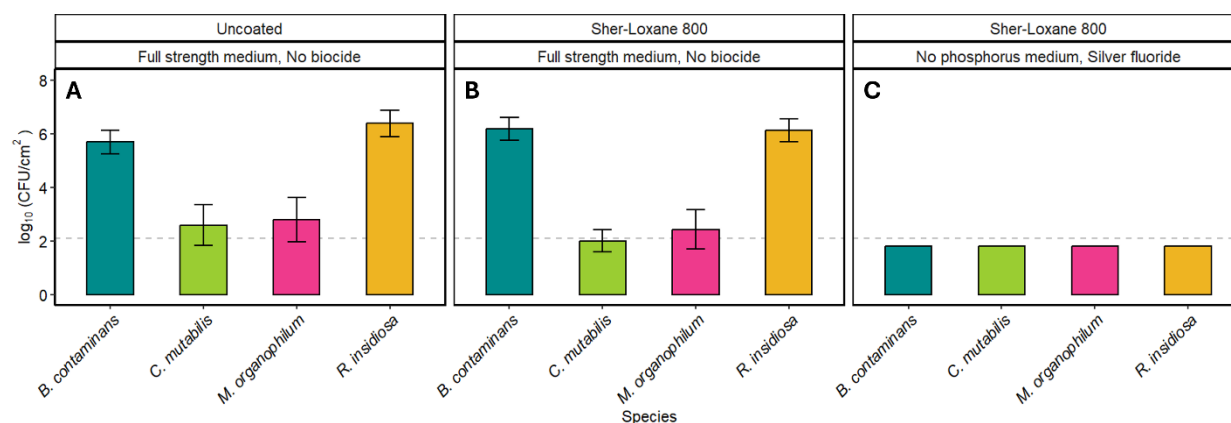


Figure 2. Viable biofilm density accumulated on coupons 7 days after reactor inoculation. The bars represent the average of 10 coupons across 2 biological replicates (5 coupons per reactor). Error bars represent standard deviation of the same. The grey dashed line represents the limit of detection ($2.08 \log_{10}\text{CFU}/\text{cm}^2$).

In the second biological replicate of the experiment with three methods of biofilm control, the limit of detection was lowered to $1.1 \log_{10}\text{CFU}/\text{cm}^2$ by spread plating 1 mL of sample on one set of agar plates and 100 μL on another, instead of only plating 100 μL of sample as was done for the first replicate. No viable colonies were detected for any of the coupons with either limit of detection.

Confocal microscopy was used to visualize the biofilm on the coupons after 7 days in the reactors. Representative images from random locations on the coupon surface are presented in

Figure 3. In reactors with no biofilm mitigation strategy (Figure 3A), fungal hyphae were present on the surface (in blue and white) as well as 20-30 μm aggregates of bacteria. The biofilm grown on Sher-Loxane® 800 coupons as the only method of biofilm control had fewer bacterial cell aggregates and very little fungal biomass (Figure 3B). Minimal biomass was detected on the coupons featuring biofilm grown under all three methods of biofilm control (Sher-Loxane® 800 coupons, no phosphorus in the nutrient medium, and daily silver fluoride biocide doses; Figure 3C). Several non-viable bacteria (in red) and a few conidial fungal cells (purple/pink) are apparent in panel 3C. For panels 3B and C, the coating is fluorescent in the same wavelengths used to image the biomass: excitation wavelengths of 405 nm (blue), 483 nm (green) and 568 nm (red), making images appear different.

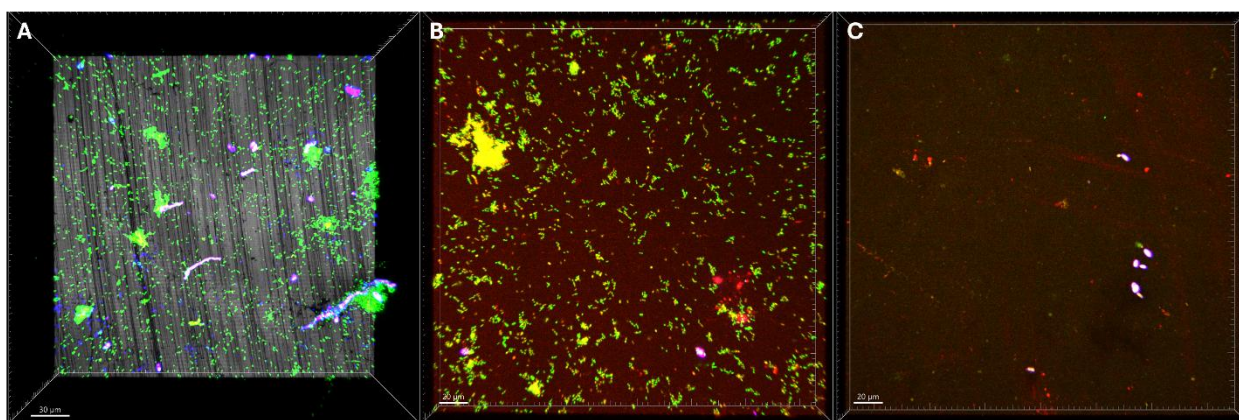


Figure 3. Confocal microscopy images of biofilms after 7 days in the SMBR grown A) with no biofilm control methods, B) on Sher-Loxane® 800 coated coupons as the only method of biofilm control, and C) on Sher-Loxane® 800 coated coupons in no-phosphorus medium and daily silver fluoride biocide doses. Viable bacteria are represented in green, non-viable bacteria in red, and fungal biomass in blue/purple/pink depending on the panel. Note: the scale bar in panel A is 30 μm while the scale bars in panels B and C are 20 μm .

Planktonic community

The planktonic cell concentrations were tracked throughout the 7 days of reactor operation (Figure 4). On the last day of reactor operation, two samples for planktonic cell concentration were taken: one from the reactor effluent tubing (marked with 7) as was the sampling method for days 1-6 and one from the bulk fluid in the reactor during coupon sampling (marked with 7 RB for *reactor bulk fluid*). The two endpoint samples were not statistically different, with the variation between the two being less than $0.5 \log_{10}\text{CFU/mL}$. This indicates that the planktonic samples taken from the effluent tubing days 1-6 are likely representative of the planktonic cells in the reactor itself.

In the reactor with no biofilm control method (Figure 4A), the planktonic community was dominated by *R. insidiosa*, which continued to increase in concentration throughout the duration of the experiment. The other three organisms fell in concentration until about day 4 after inoculation when they reached a steady state. In experiments with the Sher-Loxane® 800 coating as the only method of biofilm control (Figure 4B), the planktonic community was still dominated by *R. insidiosa*, which continuously increased and reached a slightly higher concentration than in the uncoated system. The *M. organophilum* and *C. mutabilis* fell in concentration throughout the coated only experiment, with the *C. mutabilis* eventually dropping below the limit of detection. In the SMBR reactor treated with three methods of biofilm control (Sher-Loxane® 800 coating, medium without phosphorus, and silver fluoride biocide dose; Figure 4C), the planktonic concentration for all organisms was highest one day after inoculation, before the biocide dosing began. The repeated dosing of silver fluoride in combination with the other mitigation methods reduced the planktonic concentration by several orders of magnitude after one day, eventually

resulting in planktonic cell concentrations at or below the limit of detection by the end of the experiment.

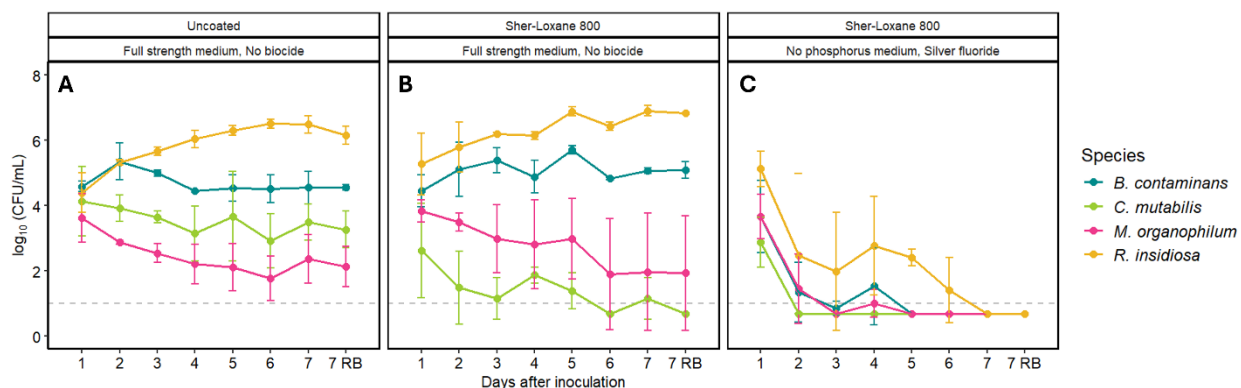


Figure 4. Planktonic cell concentrations of each species in the consortium. The dots are the average of the biological replicates of the reactors and the error bars are the standard deviation between the two biological replicates. The dashed line represents the limit of detection, 1.00 log₁₀CFU/mL.

Again, in the second biological replicate of the experiment with three methods of biofilm control, the limit of detection was lowered, this time to 1 CFU/mL by spread plating 1 mL of sample on one set of agar plates and 100 μ L on another, instead of only plating 100 μ L of sample as was done for the first replicate. No viable colonies were detected for either limit of detection.

The size and shape of biofilm aggregates in the planktonic samples were visualized using a FlowCam. The diameter for every particle detected by the FlowCam in each sample is presented in Figure 5. The particle with the largest diameter from each experiment is emphasized in the figure with a larger symbol. Both replicates are plotted together.

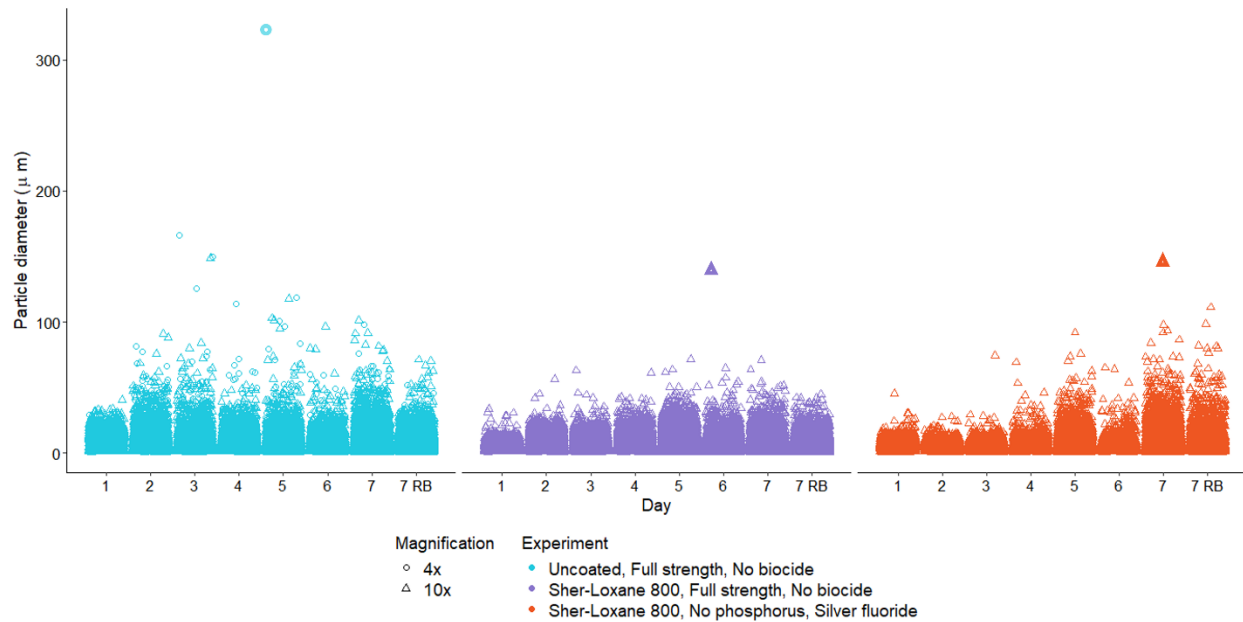


Figure 5. Diameter for every particle detected by the FlowCam, with both replicates plotted together. Note that samples for the experiment without any biofilm control methods (in blue) were imaged using the 4x and 10x flow cells. Larger symbols are used to call attention to the largest particles from each experiment.

The output image collage featuring the particles with the largest diameters from each experimental condition are presented in Figure 6. The collages aid in visualizing the shape of the particles and how they may contribute to clogging in a spacecraft water system. The FlowCam system takes an image of each detected particle and sorts based on diameter, so a capture of the top row (largest diameters detected) of the sample with the largest diameters is presented. The particles in the reactor without treatment (Figure 6A) are dominated by fungal hyphae. The reactors with Sher-loxane® 800 coating (Figure 6B-C) do not have obvious fungal hyphae and instead appear to be dominated by bacterial aggregates. The particles in the reactor featuring all three methods of control are optically much clearer (Figure 6C) than the particles from the reactor with just the coating (Figure 6B).

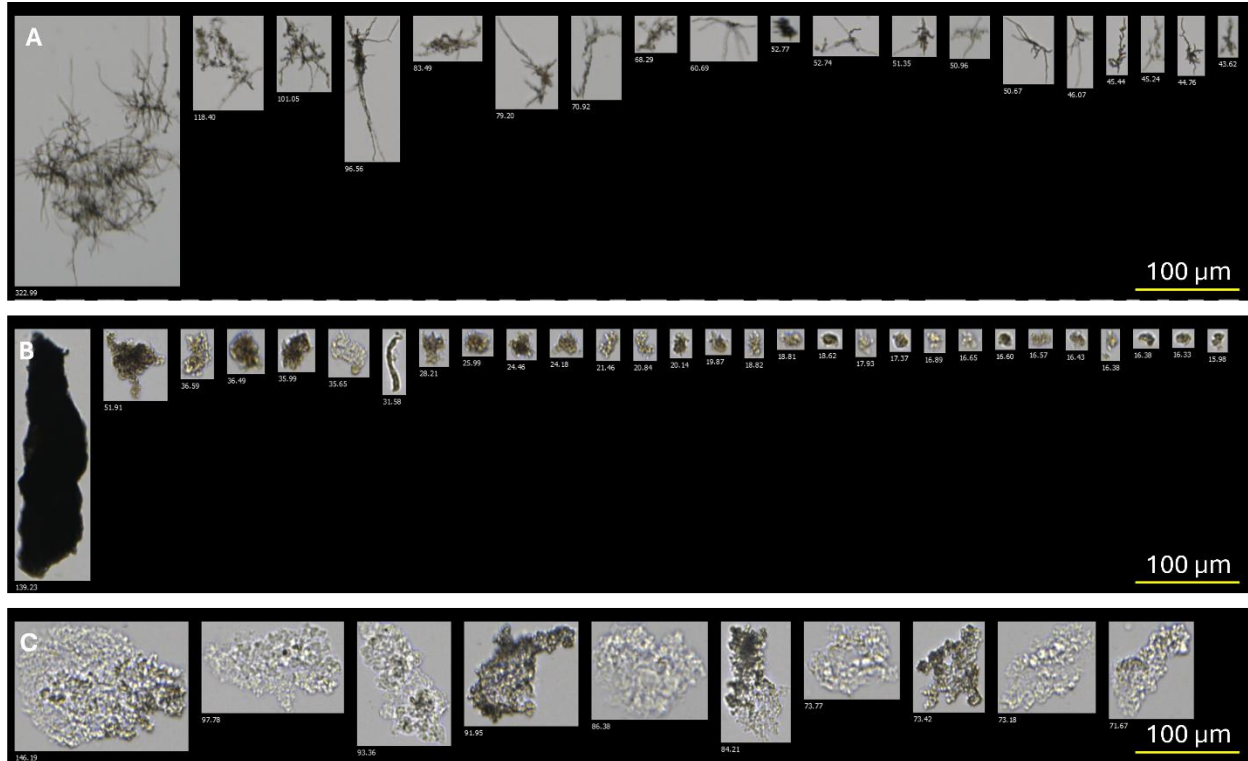


Figure 6. Collages from the sampling day with the largest particles of each experimental iteration captured by the FlowCam. A) Uncoated coupons and walls (first replicate, day 5, 4x magnification) B) Sher-Loxane® 800 coated coupons and walls (second replicate, day 6, 10x magnification) C) Sher-Loxane® 800 coated coupons and walls, no-phosphorus medium, and daily silver fluoride biocide dosing (second replicate, day 7 effluent sample, 10x magnification).

Discussion

While biocides, nutrient limitations and antibiofilm coatings have all been used to control biofilms, surprisingly little research has been published to determine the efficacy of all three control strategies combined. Coatings have long been used for biofilm control on surfaces, largely in the medical device industry and more recently proposed for use in spacecraft [32] for high touch surfaces [33-38] and spacecraft water systems [22, 39-42]. Phosphorus can be a major limiting nutrient for all organisms, including microorganisms, due to its pervasiveness in various molecular processes such as DNA replication, membrane production, and energy production

[43]. In spacecraft, phosphorus may be removed using nutrient filters, resins, or biological methods such as plant growth—all of which would need external validation and incorporation into the water system. Silver has long been used to control microorganisms, whether in textiles [44], material coatings [45, 46], or as a biocide [47]. It has broad antimicrobial effects including interaction with membrane proteins, disruptions of electron transfer and respiration, interactions with DNA and proteins, and generation of reactive oxygen species [48]. Many experiments investigating silver compatibility in spacecraft systems have been conducted [25, 49-55], and NASA has expressed interest in moving away from iodine treatment towards silver treatment for potable water [48]. All three of these microbial control strategies (coatings, phosphorus limitation, and silver biocide) have been investigated for use in spacecraft water systems (Chapter 4 and [21]), but this study represents the first time to our knowledge these approaches have been tested together in an SMG environment. Further, this study marks the first time this novel SMG biofilm reactor was used in experiments longer than 24 h and with intermittent periods of continuous flow. These achievements show promise for greater flexibility of the reactor system and the experiments it can facilitate.

Biofilm cell density using plate counts

When compared to the previously published normal gravity study using many of the same experimental parameters (Chapter 4), the final viable biofilm density achieved in the SMBR was several orders of magnitude lower than in the CDC reactor experiments with the same four organisms, medium, and of the same 7 d experiment duration ($6.5 \log_{10}\text{CFU}/\text{cm}^2$ in the SMBR vs $8.4 \log_{10}\text{CFU}/\text{cm}^2$ in the CDC reactor). The SMBR was operated in alternating batch and continuous flow modes, so the lower biofilm density may be due to oxygen limitation as the

SMBR likely went through periods of lower oxygen concentration or anoxic operation compared to CDC reactors which are very well mixed with a large head space. The low shear stress required for SMG could have also contributed to lower viable biofilm density as fluid shear helps transport substrate and nutrients to the biofilm surface and often tends to increase biofilm accumulation rates [56-58]. Another possibility for lower viable biofilm density in the SMBR is the longer contact time of the biocide. In these experiments, the silver fluoride was added to the SMBR just prior to a 21 h batch phase, giving it sufficient contact time to kill accumulated biofilm, whereas in the CDC reactor (residence time of 160 min), biocide may have been washed out prior to an effective contact time during the sustained continuous flow.

In the SMBR, accumulated biofilm was only sampled 7 days after inoculation because biofilm sampling required the reactor rotation to be stopped, which reintroduces unidirectional gravity into the system, resulting in normal gravity. Additionally, because the fluid in the reactor is assumed to achieve solid body rotation, any stoppages of rotation may lead to a sudden fluid shear force at the reactor walls due to a short interruption of the no-slip boundary, potentially removing accumulated biofilm from the coupons. To maintain SMG and prevent excess shearing events associated with intermittent stopping and starting of rotation, only end point (7 day) biofilm samples were collected from coupon surfaces.

The viable biofilm density of experiments without biofilm mitigation (Figure 2A) was very similar to that of experiments with the Sher-Loxane® 800 coating as the only method of biofilm control (Figure 2B). This is not an unexpected result considering when the Sher-Loxane® 800 coating was used as the only method of biofilm control in CDC reactors, the final viable biofilm density ($8.5 \log_{10}\text{CFU}/\text{cm}^2$) was statistically indistinguishable from the

unmitigated biofilm accumulation ($8.4 \log_{10}\text{CFU}/\text{cm}^2$; Chapter 4). In the normal gravity CDC experiments, it appeared that the function of the coating was to delay biofilm accumulation by several days; however, since only endpoint sampling could occur in the SMBR experiments, it is impossible to know if a similar trend occurred here. For experiments with the coating, with phosphorus eliminated from the nutrient medium, and daily silver fluoride biocide dosing, no viable biofilm was detected in either replicate using plate counts (Figure 2C). Even with another plating technique for the second replicate, which gave biofilm densities at two limits of detection (2.08 and $1.1 \log_{10}\text{CFU}/\text{cm}^2$), still no viable cells were recovered on selective or plain R2A agar plates. This is similar to what was previously reported in CDC reactors where the viable biofilm was present at approximately $1.5\text{--}2 \log_{10}\text{CFU}/\text{cm}^2$ density. This is a very promising result and indicates that these three methods of biofilm control may operate well together in a true microgravity environment like the ISS or Gateway.

Confocal images

The confocal microscopy images show that larger biofilm aggregates formed on uncoated coupons than on Sher-Loxane® 800 coated coupons (Figure 3A vs 3B). Also, fungal hyphae were observed on the uncoated coupons, which provided additional surface area for biofilm accumulation. The fluorescence of the coating itself in Figure 3B makes it difficult to ascertain if there were conidial fungal cells present or if there were portions of the coating fluorescing at the same wavelength as the fungus (405 nm). There was a large decrease in the quantity of biomass present on the Sher-Loxane® 800 coated coupon in the reactor with no phosphorus that was dosed with silver fluoride daily (Figure 3C). In the image, the main source of biomass was from several conidial fungal cells, despite no viable cells (fungal or bacterial) being detected by plate

counts (Figure 2C). All fungal cells were stained by Calcofluor white, whether they were viable or non-viable. So, it is difficult to state if the cells present in Figure 3C were viable but non-culturable [59] or truly non-viable.

Planktonic plate counts

In the SMBR, the daily planktonic samples taken during the continuous flow phase offered a method of tracking microbial activity throughout the experiments (Figure 4). The trends in viable planktonic cell concentration on the final day of reactor operation generally follow the trends in viable biofilm density, except for a few instances. In reactors where the Sher-Loxane® 800 coating was the only method of biofilm control, *R. insidiosa* viable planktonic cell concentration was approximately 2 log₁₀CFU/mL higher than *B. contaminans* (Figure 4B) despite the two species being present in the biofilm at approximately the same density (6 log₁₀CFU/cm²; Figure 2B). This differs from previous observations in CDC reactors where the planktonic community in reactors with Sher-Loxane® 800 coating was dominated by *B. contaminans* (Chapter 4). Additionally, in SMBR experiments with no biofilm control method (Figure 4A), the fungus was present in the planktonic community at a concentration approximately one order of magnitude higher than *M. organophilum* despite *M. organophilum* being present in the biofilm at a slightly higher density than *C. mutabilis* (Figure 4A).

FlowCam

Reduction in biofilm density on surfaces is a key goal of the microbial control methods investigated in this study. Another major goal in reducing microbial contamination in spacecraft water systems is the reduction of biofilm aggregates present in bulk fluid. These aggregates pose clogging threats in the small diameters of spacecraft water system tubes and valves. FlowCam

analysis of planktonic samples allows for an investigation into the morphologies of cellular aggregates in the bulk fluid, which may cause clogging in small diameter tubing, filters, or valves [3, 22]. The reactor without microbial control methods featured particles with the largest diameters throughout the majority of the 7 day experiments (Figure 5), indicating that control methods tested here may have some beneficial impact by decreasing particle sizes. However, the shift that occurred on the last sampling day, where the reactor with all three microbial control methods fostered the largest particles, is concerning. Since the day 7 samples were taken after the rotation was slowed (Day 7) or stopped (Day 7 RB), it is possible that the larger aggregates could have been due to biofilm detaching from the reactor walls or coupons (Figure 5). The lack of viable biofilm cells detected on agar plates (Figure 2C) and lack of significant biomass on the coupons (Figure 3) suggest that non-viable or viable but non-culturable biomass may have detached from the reactor surfaces. However, the plate counts for planktonic concentration (Figure 4C) do not indicate the aggregates imaged by the FlowCam (Figures 5 and 6) were viable. Multiple potential hypotheses for these observations suggest further research may be needed to understand the particles in reactors with coated walls, phosphorus limitation, and silver fluoride dosing.

The particle shapes were observed to be vastly different in reactors with coated and uncoated coupons and walls (Figure 6). Figure 6A shows fungal hyphae appeared to dominate the morphology of the large particles in uncoated reactors while Figure 6B-C shows reactors with Sher-Loxane® 800 coating had denser aggregates dominated by bacteria. Aggregates in reactors with the coating as the sole method of microbial control were optically much darker than aggregates in reactors with the coating, no phosphorus, and silver fluoride dosing. One potential

explanation for these optical differences could be the accumulation of polyphosphates in the phosphate containing medium. To our knowledge, the specific organisms used in these experiments have not been shown to produce polyphosphates; however, other *Burkholderia* and *Ralstonia* species have been shown to produce polyphosphates [60, 61] which often appear as darker areas in light microscope images. Some physical aggregation of phosphorus within bacteria grown with phosphate present could have potentially darkened the observed FlowCam images (Figure 8A-B), which may be why the aggregates of cells grown without phosphorus are optically clearer (Figure 6C). Additionally, biomass particles in experiments with all three methods of microbial control could have remained intact due to the EPS holding cells together despite potential autolysis of cells due to the silver fluoride [48]. The lysed cells could have resulted in optically clearer clumps of biomass. Large particles with fungal hyphal networks could become lodged in tubing, valves, and filters, acting as a net to catch additional particles flowing through. As such, in reactors featuring biofilm control measures, the reduction of fungal hyphae present in the large aggregates is encouraging.

Conclusion

The experiments presented here report, for the first time, three methods of biofilm control used in combination during simulated microgravity tests. This study marks a large step toward accurately modeling the ISS water system on Earth by using direct ISS microbial isolates, a medium designed to mimic ISS wastewater, relevant materials of construction, and now a relevant (micro) gravitational regime. Results indicate that the Sher-Loxane® 800 surface coating, exclusion of phosphorus from the nutrient medium, and daily silver fluoride biocide doses offer promise for significant microbial control in spacecraft water systems. The novel

SMBR was used for the first time outside of its initial design application, with the inclusion of continuous flow operation, a duration 7 times longer than the original study [23], and the use of a mixed domain consortium of microorganisms. Successful observation of microbial growth within the reactor indicates promise for its continued use in other configurations and applications. Future expansion of the study presented here should include reinoculation of the organisms throughout the experiments and an increased duration past 7 days. Additional methods of microbial metabolic activity tracking, such as oxygen measurements, could help build further understanding of critical factors in biofilm formation and control in microgravity systems. Another powerful addition to these experiments would be ground testing in pilot systems and full-scale replicas of the ISS water recovery system.

Acknowledgements

This research could not have been done without the help of Haley Ketteler, Matthew Culp, Stephan Warnat, and Christine Foreman who were all instrumental in the design and optimization of the simulated microgravity reactor. Elizabeth Sandvik was immensely helpful with FlowCam imaging and the design of the nutrient medium and biofilm consortium. An additional thank you to Phil Stewart who has worked to coordinate the EPSCoR effort at the Center for Biofilm Engineering.

Funding

Funding was provided by NASA through EPSCoR cooperative agreement 80NSSC21M0331.

Author disclosure statement

The authors jointly disclose commercial associations with The Sherwin-Williams Company, which has been an industrial affiliate of the Center for Biofilm Engineering since 2009. Brent Peyton participates in collaborative research with The Sherwin-Williams Company on a project funded by the Department of Defense. Madelyn Mettler and Brent Peyton have not received direct funding from The Sherwin-Williams Company.

References

1. National Aeronautics and Space Administration. Gateway Frequently Asked Questions. [https://www.nasa.gov/gateway-frequently-asked-questions/#:~:text=Unlike%20the%20ISS%2C%20which%20is,Gateway%20will%20not%20be%20\(2025\).](https://www.nasa.gov/gateway-frequently-asked-questions/#:~:text=Unlike%20the%20ISS%2C%20which%20is,Gateway%20will%20not%20be%20(2025).) Accessed.
2. European Space Agency. Gateway. https://www.esa.int/Science_Exploration/Human_and_Robotic_Exploration/Exploration/Gateway Accessed.
3. Zea L, Nisar Z, Rubin P, Cortesão M, Luo J, McBride SA, et al. Design of a spaceflight biofilm experiment. *Acta Astronautica*. 2018;148:294-300; doi: <https://doi.org/10.1016/j.actaastro.2018.04.039>.
4. Carter DL: Status of the Regenerative ECLSS Water Recovery System. In: International Conference of Environmental Systems. Barcelona, Spain; 2010.
5. Weir N, Wilson M, Yoets A, Molina T, Bruce R, Carter L: Microbiological characterization of the international space station water processor assembly external filter assembly S/N 01. In: 42nd international conference on environmental systems. 2012: 3595.
6. Bruce RJ, Ott CM, Skuratov VM, Pierson DL. Microbial surveillance of potable water sources of the International Space Station. *SAE transactions*. 2005:283-92.
7. Velez Y-A, Carter D, Nur M, Angle G: Developing Methods for Biofilm Control in Microgravity for a Water Recovery System. In.: 2020 International Conference on Environmental Systems; 2020.

8. Donlan RM. Biofilms: microbial life on surfaces. *Emerg Infect Dis*. 2002;8(9):881-90; doi: 10.3201/eid0809.020063.
9. Donlan RM. Biofilm Formation: A Clinically Relevant Microbiological Process. *Clinical Infectious Diseases*. 2001;33(8):1387-92; doi: 10.1086/322972.
10. Flemming H-C, Neu TR, Wozniak DJ. The EPS Matrix: The “House of Biofilm Cells”. *Journal of Bacteriology*. 2007;189(22):7945-7; doi: 10.1128/jb.00858-07.
11. Flemming H-C, Wingender J. The biofilm matrix. *Nature Reviews Microbiology*. 2010;8(9):623-33; doi: 10.1038/nrmicro2415.
12. Wingender J, Flemming H-C. Biofilms in drinking water and their role as reservoir for pathogens. *Int J Hyg Environ Health*. 2011;214(6):417-23; doi: <https://doi.org/10.1016/j.ijheh.2011.05.009>.
13. Elasri MO, Miller RV. Study of the response of a biofilm bacterial community to UV radiation. *Applied and environmental microbiology*. 1999;65(5):2025-31.
14. Mah T-FC, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology*. 2001;9(1):34-9; doi: [https://doi.org/10.1016/S0966-842X\(00\)01913-2](https://doi.org/10.1016/S0966-842X(00)01913-2).
15. Roberson EB, Firestone MK. Relationship between Desiccation and Exopolysaccharide Production in a Soil *Pseudomonas* sp. *Applied and Environmental Microbiology*. 1992;58(4):1284-91; doi: doi:10.1128/aem.58.4.1284-1291.1992.
16. Yin W, Wang Y, Liu L, He J. Biofilms: The Microbial "Protective Clothing" in Extreme Environments. *Int J Mol Sci*. 2019;20(14); doi: 10.3390/ijms20143423.
17. Pruitt JM, Carter L, Bagdigian RM, Kayatin MJ: Upgrades to the ISS Water Recovery System. In.; 2015.
18. Gaskill ML. NASA Achieves Water Recovery Milestone on International Space Station. 2023.
19. Williamson J, Kayatin M, Nguyen HN, Hudson EK, Wallace WT, Williams S, et al.: Overview of the International Space Station’s Water and Cabin Air Quality: A Five-Year Status. In: 53rd International Conference on Environmental Systems (ICES). International Conference on Environmental Systems; 2024.
20. Williamson J, Luong H, Robinson K, Wilson JP: Status of ISS Water Management and Recovery. In: The 52nd International Conference on Environmental Systems. Calgary, Canada; 2023.

21. Beitle E, McCormick M, McCall M, Sandvik EL, Justiniano Y-AV, Williamson J: Ground Test Validations for Uncrewed Dormancy Preparation and Recovery. In: 53rd International Conference on Environmental Systems (ICES). 2024.
22. Zea L, McLean RJC, Rook TA, Angle G, Carter DL, Delegard A, et al. Potential biofilm control strategies for extended spaceflight missions. *Biofilm*. 2020;2:100026; doi: <https://doi.org/10.1016/j.bioflm.2020.100026>.
23. Ketteler HM, Johnson EL, McGlennen M, Dieser M, Foreman CM, Warnat S. A simulated microgravity biofilm reactor with integrated microfabricated sensors: Advancing biofilm studies in near-space conditions. *Biofilm*. 2025;9:100263; doi: <https://doi.org/10.1016/j.bioflm.2025.100263>.
24. Velez Justiniano Y-A, Carter D, Sandvik E, Stewart P, Goeres D, Sturman P, et al.: Biofilm Management in a Microgravity Water Recovery System. In: 50th International Conference on Environmental Systems. 2021.
25. Callahan MR, Adam NM, Roberts MS, Garland JL, Sager JC, Pickering KD. Assessment of silver based disinfection technology for CEV and future US spacecraft. *SAE Transactions*. 2007:481-91.
26. Nauman Eric A, Ott CM, Sander E, Tucker Don L, Pierson D, Wilson James W, et al. Novel Quantitative Biosystem for Modeling Physiological Fluid Shear Stress on Cells. *Applied and Environmental Microbiology*. 2007;73(3):699-705; doi: 10.1128/AEM.02428-06.
27. Lynch SV, Mukundakrishnan K, Benoit MR, Ayyaswamy PS, Matin A. Escherichia coli biofilms formed under low-shear modeled microgravity in a ground-based system. *Appl Environ Microbiol*. 2006;72(12):7701-10; doi: 10.1128/aem.01294-06.
28. Begley CM, Kleis SJ. The fluid dynamic and shear environment in the NASA/JSC rotating-wall perfused-vessel bioreactor. *Biotechnol Bioeng*. 2000;70(1):32-40; doi: 10.1002/1097-0290(20001005)70:1<32::aid-bit5>3.0.co;2-v.
29. Sandvik E, Stewart P, Goeres D, Sturman P: Center for Biofilm Engineering Recommendations for Addition of Trace Nutrients to Microbial Ersatz. In. Edited by Williamson J, Velez Justiniano YA; 2022.
30. ASTM International: ASTM E3161-18, Standard Practice for Preparing Pseudomonas aeruginosa or Staphylococcus aureus Biofilm using the CDC Biofilm Reactor. In. West Conshohoken, PA; 2018.
31. Atlas RM. Handbook of Microbiological Media, Fourth Edition. Taylor & Francis; 2010.

32. Wang M, Duday D, Scolan E, Perbal S, Prato M, Lasseur C, et al. Antimicrobial Surfaces for Applications on Confined Inhabited Space Stations. *Advanced Materials Interfaces*. 2021;8(13):2100118; doi: <https://doi.org/10.1002/admi.202100118>.
33. Touching Surfaces <https://event.dlr.de/en/ila2022/touching-surfaces/> (2022). Accessed.
34. Lemelle L, Campagnolo L, Mottin E, Le Tourneau D, Garre E, Marcoux P, et al. Towards a passive limitation of particle surface contamination in the Columbus module (ISS) during the MATISS experiment of the Proxima Mission. *NPJ Microgravity*. 2020;6(1):29; doi: 10.1038/s41526-020-00120-w.
35. Lemelle L, Mottin E, Le Tourneau D, Rouquette S, Campagnolo L, Thévenot C, et al. Surface contamination rates at different spatial scales in the Columbus module (ISS) during the MATISS campaigns. *Frontiers in Astronomy and Space Sciences*. 2023;10; doi: 10.3389/fspas.2023.1229022.
36. Lemelle L, Rouquette S, Mottin E, Le Tourneau D, Marcoux PR, Thévenot C, et al. Passive limitation of surface contamination by perFluoroDecylTrichloroSilane coatings in the ISS during the MATISS experiments. *NPJ Microgravity*. 2022;8(1):31; doi: 10.1038/s41526-022-00218-3.
37. Shea A, Harvey K, Keeley A, Johnson H, Hansen N, McCormack R, et al. Payload Design and Evaluation of Staphylococcus epidermidis Adhesion to Nonfouling Polyampholyte Coatings Onboard the International Space Station. *Molecules*. 2025;30(4):836.
38. Sobisch L-Y, Rogowski KM, Fuchs J, Schmieder W, Vaishampayan A, Oles P, et al. Biofilm Forming Antibiotic Resistant Gram-Positive Pathogens Isolated From Surfaces on the International Space Station. *Front Microbiol*. 2019;10; doi: 10.3389/fmicb.2019.00543.
39. Flores P, McBride SA, Galazka JM, Varanasi KK, Zea L. Biofilm formation of *Pseudomonas aeruginosa* in spaceflight is minimized on lubricant impregnated surfaces. *NPJ Microgravity*. 2023;9(1):66; doi: 10.1038/s41526-023-00316-w.
40. Mettler MK, Parker CW, Venkateswaran K, Peyton BM. Antimicrobial Coating Efficacy for Prevention of *Pseudomonas aeruginosa* Biofilm Growth on ISS Water System Materials. *Front Microbiol*. 2022;13; doi: 10.3389/fmicb.2022.874236.
41. Li Sip YY, Jacobs A, Morales A, Sun M, Roberson LB, Hummerick ME, et al. Slippery lubricant-infused silica nanoparticulate film processing for anti-biofouling applications. *J Appl Biomater Funct Mater*. 2023;21:22808000231184688; doi: 10.1177/22808000231184688.

42. Demir B, Taylor A, Broughton RM, Huang TS, Bozack MJ, Worley SD. N-halamine surface coating for mitigation of biofilm and microbial contamination in water systems for space travel. *Biofilm*. 2022;4:100076; doi: <https://doi.org/10.1016/j.bioflm.2022.100076>.
43. Elser JJ. Phosphorus: a limiting nutrient for humanity? *Current Opinion in Biotechnology*. 2012;23(6):833-8; doi: <https://doi.org/10.1016/j.copbio.2012.03.001>.
44. Radetić M. Functionalization of textile materials with silver nanoparticles. *Journal of Materials Science*. 2013;48(1):95-107; doi: 10.1007/s10853-012-6677-7.
45. Schierholz JM, Lucas LJ, Rump A, Pulverer G. Efficacy of silver-coated medical devices. *Journal of Hospital Infection*. 1998;40(4):257-62; doi: [https://doi.org/10.1016/S0195-6701\(98\)90301-2](https://doi.org/10.1016/S0195-6701(98)90301-2).
46. Knetsch MLW, Koole LH. New Strategies in the Development of Antimicrobial Coatings: The Example of Increasing Usage of Silver and Silver Nanoparticles. *Polymers*. 2011;3(1):340-66.
47. Sim W, Barnard RT, Blaskovich MAT, Ziora ZM. Antimicrobial Silver in Medicinal and Consumer Applications: A Patent Review of the Past Decade (2007-2017). *Antibiotics (Basel)*. 2018;7(4); doi: 10.3390/antibiotics7040093.
48. Li W, Calle L, Hanford A, Stambaugh I, Callahan M: Investigation of silver biocide as a disinfection technology for spacecraft—an early literature review. In.: 48th International Conference on Environmental Systems; 2018.
49. Roberts MS, Hummerick ME, Edney SL, Bisbee PA, Callahan MR, Loucks S, et al.: Assessment of silver based disinfection technology for CEV and future US spacecraft: microbial efficacy. In.: SAE Technical Paper; 2007.
50. Wallace W, Castro-Wallace S, Kuo C, Loh L, Hudson E, Gazda D, et al.: Effects of material choice on biocide loss in Orion water storage tanks. In.: 46th International Conference on Environmental Systems; 2016.
51. Wallace WT, Wallace SL, Loh LJ, Kuo CM, Hudson Jr EK, Marlar TJ, et al. Effects of materials surface preparation for use in spacecraft potable water storage tanks. *Acta Astronautica*. 2017;141:30-5.
52. Petala M, Tsiridis V, Darakas E, Mintsouli I, Sotiropoulos S, Kostoglou M, et al.: Silver deposition on wetted materials used in the potable water systems of the International Space Station. In: 46th International Conference on Environmental Systems. 2016: 10-4.

53. Petala M, Tsiridis V, Mintsouli I, Pliatsikas N, Spanos T, Rebeyre P, et al. Silver deposition on stainless steel container surfaces in contact with disinfectant silver aqueous solutions. *Applied Surface Science*. 2017;396:1067-75.
54. Adam NM: Compatibility study of silver biocide in drinking water with candidate metals for the crew exploration vehicle potable water system. In.: SAE Technical Paper; 2009.
55. Beringer DM, Steele JW, Nalette TA: Long-term storage of potable water in metallic vessels. In.: Google Patents; 2014.
56. Manuel CM, Nunes OC, Melo LF. Dynamics of drinking water biofilm in flow/non-flow conditions. *Water research*. 2007;41(3):551-62; doi: <https://doi.org/10.1016/j.watres.2006.11.007>.
57. Tsagkari E, Connelly S, Liu Z, McBride A, Sloan WT. The role of shear dynamics in biofilm formation. *npj Biofilms Microbomes*. 2022;8(1):33; doi: 10.1038/s41522-022-00300-4.
58. Wang S, Zhu H, Zheng G, Dong F, Liu C. Dynamic Changes in Biofilm Structures under Dynamic Flow Conditions. *Applied and Environmental Microbiology*. 2022;88(22):e01072-22; doi: 10.1128/aem.01072-22.
59. Pinto D, Santos MA, Chambel L. Thirty years of viable but nonculturable state research: Unsolved molecular mechanisms. *Critical Reviews in Microbiology*. 2015;41(1):61-76; doi: 10.3109/1040841X.2013.794127.
60. Tumlirsch T, Sznajder A, Jendrossek D. Formation of Polyphosphate by Polyphosphate Kinases and Its Relationship to Poly(3-Hydroxybutyrate) Accumulation in *Ralstonia eutropha* Strain H16. *Applied and Environmental Microbiology*. 2015;81(24):8277-93; doi: 10.1128/AEM.02279-15.
61. Mullan A, Quinn JP, McGrath JW. Enhanced phosphate uptake and polyphosphate accumulation in *Burkholderia cepacia* grown under low pH conditions. *Microb Ecol*. 2002;44(1):69-77; doi: 10.1007/s00248-002-3004-x.

CHAPTER SIX

CONCLUSION

The research presented in this dissertation sought to explore material coatings and other antibiofilm strategies for use in spacecraft water systems to control multidomain biofilms. The use of material coatings in these systems has only recently been explored as new spacecraft and exploration missions are being designed [1-6]. The realities of the International Space Station (ISS) water system were modeled as closely as possible in a laboratory setting: using direct microbial isolates from the ISS water system, a medium composition designed to mimic the nutrient availability of the wastewater on the ISS, materials used in the system, and finally, simulated microgravity (SMG).

Major conclusions

After an initial proof of concept that coatings offer potential for biofilm control for up to 72 h with a model organism (Chapter 3 [4]), the goal of the research became modeling the ISS wastewater system as closely as possible in the laboratory (Chapters 4 and 5).

Testing more than one organism at a time while evaluating coatings for spacecraft water system implementation had not been published prior to this research, let alone testing a consortium consisting of bacteria and a fungus. This research went one step further to improve model accuracy by using direct isolates from the ISS water system. Additionally, coatings were identified as a promising approach for biofilm control, but it became clear that they would not perform long term as an individual solution. To address this, two additional biofilm control methods were incorporated into tests. No other known published research for coatings in

spacecraft water systems has taken this approach. Promising results in Chapter 4 showed synergy between all three methods of biofilm control: Sher-Loxane® 800 coating, no phosphorus in the medium, and silver fluoride biocide.

To better understand how the Sher-Loxane® 800 coating and other biofilm control methods may perform in spacecraft, SMG experiments were completed (Chapter 5). The ability for SMG to accurately represent true microgravity, and the effects of simulated (and true) microgravity on microorganisms are slightly controversial but can still provide meaningful insights. The apparatus used here for SMG tests uniquely incorporated flow, allowing for the introduction of fresh nutrients and the outflow of cells and cellular byproducts. Many other methods/apparatuses used for SMG tests do not include flow and therefore do not represent this key aspect of the ISS water system. Biofilms were grown in the SMG under three conditions a) without use of any control method, b) with the Sher-Loxane® 800 coating alone, and c) incorporation of all three biofilm control methods previously tested. After 7 days, no viable biofilm was recovered from the SMBR with Sher-Loxane® 800, no phosphorus medium, and silver fluoride biocide dosing. Further, confocal laser microscope imaging showed very little microbial accumulation which indicates that the strategies tested not only prevented *viable* biofilm accumulation but also reduced overall biomass at the surface. Interestingly, FlowCam data showed similar results for number of suspended particles in all three reactors, but the reactor with all three biofilm control methods showed significantly reduced viable planktonic cell density compared to the other two reactors.

Recommendations for future work

The research presented in this dissertation, while sizable, does not address every question or concern that was uncovered during experimental analysis. Further work should be done to lengthen the duration of experiments performed here. As was discussed in Chapter 2, if coatings are used in spacecraft, their presence is essentially permanent, and experiments of 7 days do not effectively address questions regarding long-term efficacy of coatings (alone or in combination with other biofilm control methods). Longer experiments are presented in Appendix D; however, these did not include one of the control methods tested in this dissertation (phosphorus removal from the medium). Additional experiments that could be performed are further inspired by Chapter 2 which include repeat inoculation of the reactor system as occurs on the ISS and 100% surface area coverage by the coating (this was improved upon in Chapter 5 compared to Chapter 4, but did not reach 100%).

Regarding the Sher-Loxane® 800 coating itself, additional testing should be done to assess the coating lifetime, durability, and compatibility with biocides and temperature/chemical variations in the ISS. Further, optimal practices for surface preparation and coating applications should be investigated for their potential to improve the coating lifetime. Maintenance of the coating and its efficacy after cleaning, such as mechanical disaggregation of biofilm off the surface, also serves as a route for further research.

The novel simulated microgravity biofilm reactor (SMBR) used in Chapter 5 was developed at MSU [7] and is a non-standard system which, while it attains the main goals of creating SMG in continuous flow with surfaces for biofilm accumulation and evaluation, could be improved for usability. In experiments featuring uncoated walls and coupons, biofilm could

be seen accumulated on the surface of the polycarbonate. However, it was incredibly difficult to gradually slow down the rotation of the reactor. When the rotation was aggressively slowed or stopped, the fluid in the reactor continued with solid-body rotation and the no-slip boundary at the wall was temporarily disrupted. When this occurred, biofilm was observed detaching from the wall and disaggregating into the bulk fluid. It is possible that the biofilm accumulation on the coupons was actually greater than observed by plate counts and confocal microscopy because of mass detachment immediately before sampling. In future design iterations of the SMBR, more precise control of the motor and a programmed method for slowing down rotation gradually would be a great improvement. Additional computational fluid dynamics models may be of use to determine ideal slowing speed to maintain low enough shear to avoid biofilm detachment.

Another main concern surrounding the operation of the SMBR pertains to issues with air leaks through the rotary joints. Air bubbles would occasionally be introduced to the reactor through the influent line via an air leak in the rotary joint. Replacement of the seals within the rotary joint helped address this issue, but exploring different options may improve the longevity of the hardware and reactor operation. The final large improvement that could be made with the SMBR is a method of tracking biofilm accumulation during the experiment. Currently the only way to track microbial activity in the reactor is by sampling the effluent for dissolved oxygen concentration and suspended microbial cell density. In the current SMBR design, rotation must be stopped to sample surfaces for biofilm accumulation, which means only end point samples can be taken. Suggestions for how to incorporate a method of reactor surface sampling during operation are not presented here, but this would greatly improve the knowledge gained by completing experiments in the SMBR.

While the SMBR provides important insight into how the methods of biofilm control may perform in a microgravity environment, there is still more work that could be completed at normal gravity. Marshall Space Flight Center hosts exact replicas of the water recovery system that is present on the ISS. It would be beneficial to understand how the coating, nutrient limitation, and biocide doses could be incorporated into spacecraft hardware, and how well they would continue to control biofilm in an increasingly complicated system. If that is not possible, a different pilot test could be conducted using more complex tanks and tubing networks than is present in a CDC reactor.

Another area of research that would be of interest to explore is the biofilm response to the various control methods used in normal gravity and SMG. Transcriptomics is the investigation of the transcriptome of a cell, which is all the RNA being transcribed from DNA in a cell at the time of sampling [8]. Metatranscriptomics refers to the application of transcriptomics to a community of organisms, as opposed to one species. This approach could highlight genes actively expressed in the biofilm and help elucidate specific mechanisms employed by the cells to survive the anti-biofilm coatings. Understanding survival responses to various control methods may assist with the design of improved coatings and treatment strategies for more effective biofilm prevention in the ISS and future spacecraft water systems. Additionally, beneficial interactions and dependencies between community members could be revealed, providing information for specific species that might be more important to target for improved biofilm control. There are several papers that have investigated transcriptomic responses —and found differential gene expression— on coated surfaces [3, 9], but they have investigated single-species biofilms. Metatranscriptomics of biofilms in the early stages of development would likely best highlight

responses to control strategies, especially coatings, but issues may arise with the low quantity of RNA that might be obtained. Further, whole genomes for each organism are required for metatranscriptomics. Though whole genomes are available for the *Burkholderia contaminans* and *Methylobacterium organophilum* used in this dissertation [10], they are not well-annotated. Whole genomes for *Ralstonia insidiosa* and *Coniochaeta mutabilis* are required before metatranscriptomic analysis can take place. Further, for effective interpretation of the metatranscriptomics, a well-annotated genome is preferred.

As with every set of experiments, more work could be done to improve overall understanding of the problems at hand. However, the research completed and presented in this dissertation is an encouraging step forward towards more effective biofilm control in spacecraft and Earth-based water systems. These solutions may facilitate a more permanent human presence in space and longer missions, helping take humanity back to the Moon and onto Mars. Chapter 2 presented a literature review detailing studies that have explored the use of coatings in Earth-based and spacecraft water systems [11]. The methods used by researchers for coating applications in Earth-based water systems and spacecraft water systems were comparable. The similarities in evaluation hint towards potential similarities in implementation as well. Further research would be required to validate these biofilm control strategies in Earth-based water systems, but there are no results presented within this dissertation that indicate coatings, nutrient limitation, and biocide dosing would only be successful for biofilm control in spacecraft water systems.

References

1. Wang M, Duday D, Scolan E, Perbal S, Prato M, Lasseur C, et al. Antimicrobial Surfaces for Applications on Confined Inhabited Space Stations. *Advanced Materials Interfaces*. 2021;8(13):2100118; doi: <https://doi.org/10.1002/admi.202100118>.
2. Li W, Hummerick M, Khodadad C, Buhrom J, Spencer L, Coutts J, et al.: Biofilm Resistant Coatings for Space Applications. In: 48th International Conference on Environmental Systems. Albuquerque, New Mexico; 2018.
3. Flores P, McBride SA, Galazka JM, Varanasi KK, Zea L. Biofilm formation of *Pseudomonas aeruginosa* in spaceflight is minimized on lubricant impregnated surfaces. *NPJ Microgravity*. 2023;9(1):66; doi: 10.1038/s41526-023-00316-w.
4. Mettler MK, Parker CW, Venkateswaran K, Peyton BM. Antimicrobial Coating Efficacy for Prevention of *Pseudomonas aeruginosa* Biofilm Growth on ISS Water System Materials. *Front Microbiol*. 2022;13; doi: 10.3389/fmicb.2022.874236.
5. Demir B, Taylor A, Broughton RM, Huang TS, Bozack MJ, Worley SD. N-halamine surface coating for mitigation of biofilm and microbial contamination in water systems for space travel. *Biofilm*. 2022;4:100076; doi: <https://doi.org/10.1016/j.bioflm.2022.100076>.
6. Li Sip YY, Jacobs A, Morales A, Sun M, Roberson LB, Hummerick ME, et al. Slippery lubricant-infused silica nanoparticulate film processing for anti-biofouling applications. *J Appl Biomater Funct Mater*. 2023;21:22808000231184688; doi: 10.1177/22808000231184688.
7. Ketteler HM, Johnson EL, McGlennen M, Dieser M, Foreman CM, Warnat S. A simulated microgravity biofilm reactor with integrated microfabricated sensors: Advancing biofilm studies in near-space conditions. *Biofilm*. 2025;9:100263; doi: <https://doi.org/10.1016/j.bioflm.2025.100263>.
8. Lowe R, Shirley N, Bleackley M, Dolan S, Shafee T. Transcriptomics technologies. *PLoS Comput Biol*. 2017;13(5):e1005457-e; doi: 10.1371/journal.pcbi.1005457.
9. Vaishampayan A, Ahmed R, Wagner O, de Jong A, Haag R, Kok J, et al. Transcriptomic analysis of stress response to novel antimicrobial coatings in a clinical MRSA strain. *Materials Science and Engineering: C*. 2021;119:111578; doi: <https://doi.org/10.1016/j.msec.2020.111578>.

10. Castro CL, Velez-Justiniano Y-A, Stahl-Rommel S, Nguyen HN, Almengor A, Dunbar B, et al. Genome Sequences of Bacteria Isolated from the International Space Station Water Systems. *Microbiology Resource Announcements*. 2023;12(7):e00158-23; doi: doi:10.1128/mra.00158-23.
11. Mettler MK, J. E-OE, M. GD, and Peyton BM. Considerations for testing anti-fouling coatings designed for implementation into Earth-based and spacecraft water systems. *Biofouling*. 2025:1-19; doi: 10.1080/08927014.2025.2479692.

APPENDICES

APPENDIX A

DATA IN SUPPORT OF CHAPTER 3

The data tables presented below were used to generate Figure 2 and Figure 3 from Chapter 3.

Mettler MK, Parker CW, Venkateswaran K, Peyton BM. Antimicrobial Coating Efficacy for Prevention of *Pseudomonas aeruginosa* Biofilm Growth on ISS Water System Materials. Front Microbiol. 2022;13; doi: 10.3389/fmicb.2022.874236.

Table A1. Data used to prepare Figure 2A in Chapter 3.

Material	Day	Experiment	Biofilm density ($\log_{10}\text{CFU}/\text{cm}^2$)
Stainless steel	1	1	7.016
Stainless steel	1	2	6.615
Stainless steel	1	2	6.526
Stainless steel	1	2	6.513
Stainless steel	2	1	7.185
Stainless steel	2	2	7.327
Stainless steel	2	2	7.205
Stainless steel	2	2	7.662
Stainless steel	2	3	7.141
Stainless steel	2	3	6.898
Teflon	1	1	7.729
Teflon	1	2	7.205
Teflon	1	2	7.250
Teflon	1	2	7.212
Teflon	2	1	8.388
Teflon	2	2	8.092
Teflon	2	2	7.937
Teflon	2	2	8.279
Teflon	2	3	8.461
Teflon	2	3	8.691
Inconel	1	1	7.327
Inconel	1	2	6.840
Inconel	1	2	6.520
Inconel	1	2	6.472
Inconel	2	1	8.171
Inconel	2	2	7.523
Inconel	2	2	6.949
Inconel	2	2	8.322
Inconel	2	3	7.937
Inconel	2	3	7.591
Titanium	1	1	6.662
Titanium	1	2	6.529
Titanium	1	2	6.219
Titanium	1	2	6.401
Titanium	2	1	7.178
Titanium	2	2	6.824

Table A1. Continued.

Material	Day	Experiment	Biofilm density ($\log_{10}\text{CFU}/\text{cm}^2$)
Titanium	2	2	6.790
Titanium	2	2	6.995
Titanium	2	3	8.109

Table A2. Data used to prepare Figure 2B in Chapter 3.

Material	Day	Experiment	Biofilm density ($\log_{10}\text{CFU}/\text{cm}^2$)
Stainless steel	1	1	1.393
Stainless steel	1	1	1.092
Stainless steel	1	2	1.092
Stainless steel	1	2	1.995
Stainless steel	1	2	1.393
Stainless steel	2	1	2.238
Stainless steel	2	1	1.694
Stainless steel	2	2	1.393
Stainless steel	2	2	1.092
Stainless steel	2	2	2.735
Stainless steel	2	3	3.489
Stainless steel	2	3	3.327
Teflon	1	1	1.870
Teflon	1	1	1.092
Teflon	1	2	2.092
Teflon	1	2	1.092
Teflon	1	2	1.092
Teflon	2	1	2.694
Teflon	2	1	3.083
Teflon	2	2	1.092
Teflon	2	2	1.393
Teflon	2	2	2.171
Teflon	2	3	3.074
Teflon	2	3	4.117
Inconel	1	1	1.092
Inconel	1	1	1.696
Inconel	1	2	1.092
Inconel	1	2	1.694
Inconel	1	2	1.092
Inconel	2	1	3.422
Inconel	2	1	1.393
Inconel	2	2	1.092
Inconel	2	2	1.092
Inconel	2	2	1.393
Inconel	2	3	2.949
Inconel	2	3	2.539
Titanium	1	1	1.393
Titanium	1	1	1.092

Table A2. Continued.

Material	Day	Experiment	Biofilm density (log ₁₀ CFU/cm ²)
Titanium	1	2	1.393
Titanium	1	2	1.092
Titanium	1	2	1.092
Titanium	2	1	3.597
Titanium	2	1	2.808
Titanium	2	2	1.092
Titanium	2	2	1.694
Titanium	2	2	2.434
Titanium	2	3	3.694
Titanium	2	3	2.171

Table A3. Data used to generate Figure 2C in Chapter 3.

Material	Day	Log reduction	95% confidence interval
Stainless steel	1	5.416	0.181
Stainless steel	2	4.830	0.273
Teflon	1	6.023	0.114
Teflon	2	5.677	0.202
Inconel	1	5.626	0.156
Inconel	2	5.730	0.175
Titanium	1	5.306	0.058
Titanium	2	4.761	0.172

Table A4. Planktonic cell densities (log₁₀CFU/mL) used to generate Figure 2D-E in Chapter 3.

Hours	Uncoated			Coated		
	Experiment 1	Experiment 2	Experiment 3	Experiment 1	Experiment 2	Experiment 3
0	9.252	9.512	-	0.699	4.857	-
24	7.230	6.982	-	3.903	2.771	-
48	7.505	7.305	7.544	4.568	3.371	4.362

Table A5. Data used to generate Figure 2F in Chapter 3.

Hour	Average log reduction	95% confidence interval
0	6.604	0.854
24	3.769	0.194
48	3.351	0.131

Table A6. Data used to generate Figure 3A-C in Chapter 3. CI= confidence interval. Units for average biofilm density are $\log_{10}\text{CFU}/\text{cm}^2$.

Hour	Uncoated		Coated		Log reduction	
	Average biofilm density	95% CI	Average biofilm density	95% CI	Average	95% CI
24	5.863	0.298	6.838	0.182	-0.871	0.133
48	6.630	0.472	7.077	0.208	-0.448	0.318

Table A7. Data used to generate Figure 3D-F in Chapter 3. CI= confidence interval. Units for average planktonic cell density are $\log_{10}\text{CFU}/\text{mL}$.

Hour	Uncoated		Coated		Log reduction	
	Average planktonic cell density	95% CI	Average planktonic cell density	95% CI	Average	95% CI
Inoculum	8.952	0.416	8.952	0.416	-	-
0	8.198	1.048	8.138	0.970	-	-
24	5.598	1.172	6.224	0.651	-0.626	1.823
48	6.058	2.263	6.229	0.145	-0.172	2.118

APPENDIX B

ADDITIONAL METHODS IN SUPPORT OF CHAPTER 3

Introduction

In addition to the research presented in Chapter 3, several experiments were completed with a common skin-associated yeast, *Rhodotorula mucilaginosa* (strain IF6SW-F1), grown as a single-species biofilm and in consortium with *P. aeruginosa*. The yeast strain was directly isolated from the International Space Station [1]. The inclusion of a yeast created a multidomain biofilm when grown in consortium with *P. aeruginosa*. Multidomain biofilms are severely understudied, despite their relevance to real-world biofilms. The different morphology of yeast could directly relate to increased or decreased efficacy of surface coatings for biofilm control. Yeast like *R. mucilaginosa* have thicker and more complex cellular envelopes composed of an inner phospholipid bilayer, a cell wall composed of chitin, melanin and the polysaccharide glucan, all surrounded by a heavy capsule [2]. A yeast cell wall can be ~130-180 nm thick [3] without including the inner cellular membrane or the protective outer capsule [2]. These factors can all contribute to increased resistance to surface coatings, especially the one used in these studies which features a carbon-chain lance that may not be able to penetrate and lyse the *R. mucilaginosa* cells.

Methods

The methods presented in Chapter 3 are very similar to those used for the growth of the *R. mucilaginosa* biofilms in CDC reactors. The same materials (stainless steel, Teflon, Inconel, and titanium) were used, and the coated coupons featured the same coating. *R. mucilaginosa* was grown on potato dextrose agar (PDA) and incubated at 25 °C for 48 ± 2 hrs to isolate colonies. Four colonies were used to inoculate a flask containing 100 mL of 24 g/L potato dextrose broth

(PDB). The flask was placed in an incubator-shaker at 150 rpm and 25 °C for 24 ± 2 hrs and attained CFU counts of $6.08 \times 10^7 \pm 3.15 \times 10^7$ CFU/mL.

The overnight *R. mucilaginosa* culture was used to inoculate CDC reactors either in consortium with *P. aeruginosa* (see Chapter 3 for additional details on the organism preparation) or alone for single-species biofilms. When grown in consortium, the nutrient medium was the same as when *R. mucilaginosa* was grown alone. Sterile liquid medium consisting of 24 g/L PDB was added to the sterile reactor, and 1 mL of inoculum from the overnight culture flask(s) was introduced through the inoculation port on the CDC reactor lid. The reactor was placed on a stir plate at 100 rpm and operated in batch mode with no flow for 48 ± 2 h at room temperature to allow cells to attach to the surfaces of the reactor and test materials. At $t=0$ h, a continuous flow of fresh sterile medium of 600 mg/L PDB was introduced to the reactor at a flow rate of 3.2 mL/min creating a liquid residence time of 100 min.

Planktonic samples were removed from the reactors at $t=0$ h (start of continuous flow) and upon biofilm sampling points. To remove planktonic samples, a rod was removed from the reactor and a sterile serological pipette was used to remove approximately 5 mL of bulk fluid from the reactor. The rod was replaced, and the sample was mixed for 30 s using a vortex mixer to disaggregate any biofilm flocs. Samples containing just *R. mucilaginosa* were plated on PDA and those containing both organisms were plated on TSA and PDA. The PDA plates were incubated at room temperature for 2 days while the TSA plates were incubated at 37 °C for 1 day prior to enumeration.

Biofilm sampling followed the same process as described in Chapter 3, with the organisms plated on the corresponding selective agars and incubated accordingly.

Coupons with attached biofilm were also preserved for field-emission scanning electron microscopy (FE SEM). The methods for this process are the same as those presented in Chapter 3.

Results

When grown alone in the CDC reactor, *R. mucilaginosa* reached a viable biofilm density of approximately 5.5-6.4 log₁₀CFU/cm² on uncoated surfaces (Figure B1.A). The viable biofilms on the coated surfaces were similarly dense, which resulted in near zero, or slightly negative logarithmic reductions in biofilm density (Figure B1.B-C). The absolute values of the log reductions are less than 0.5, which is not typically considered statistically significant when discussing biofilm density and is typically within error. The *R. mucilaginosa* reactors containing coated materials showed very little difference in planktonic cell densities from reactors containing uncoated materials. The planktonic cell logarithmic reduction was also a fraction of a log which is within the noise of experimental variation and does not indicate a significant reduction due to the coating (Figure B1.D-F).

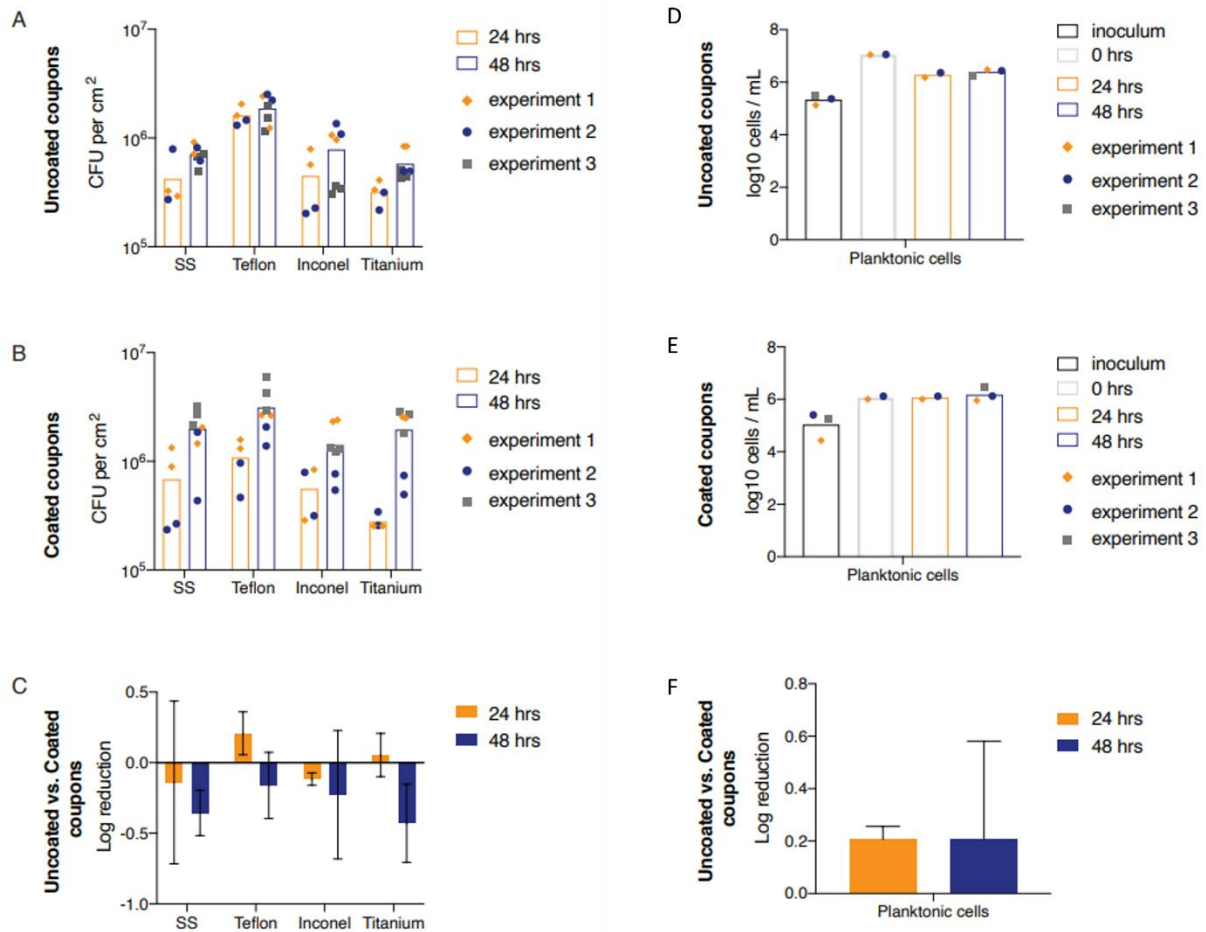


Figure B1. *R. mucilaginosa* single-species biofilm density on uncoated coupons (A), coated coupons (B), and the biofilm log reduction (C). Planktonic cell density for reactors with uncoated coupons (D), coated coupons (E), and the planktonic log reduction (F). Error bars represent 95% confidence intervals.

When grown in consortium, both organisms have lower viable biofilm densities on uncoated coupons than they do grown alone in the same medium (600 mg/L PDB) (Figure B2). This trend continues for biofilms grown on coated coupons, although the biofilm density is slightly higher than for biofilms grown on uncoated coupons (Figure B2). Due to the higher biofilm density on the coated coupons, the log reduction is again negative, although more so than it is for *R. mucilaginosa* alone (Figure B3). Similar trends are present for the planktonic cell

densities (data not shown). *P. aeruginosa* and *R. mucilaginosa* associate with each other very closely in the biofilm, especially on the coated surfaces as seen in FE SEM images (Figure B4).

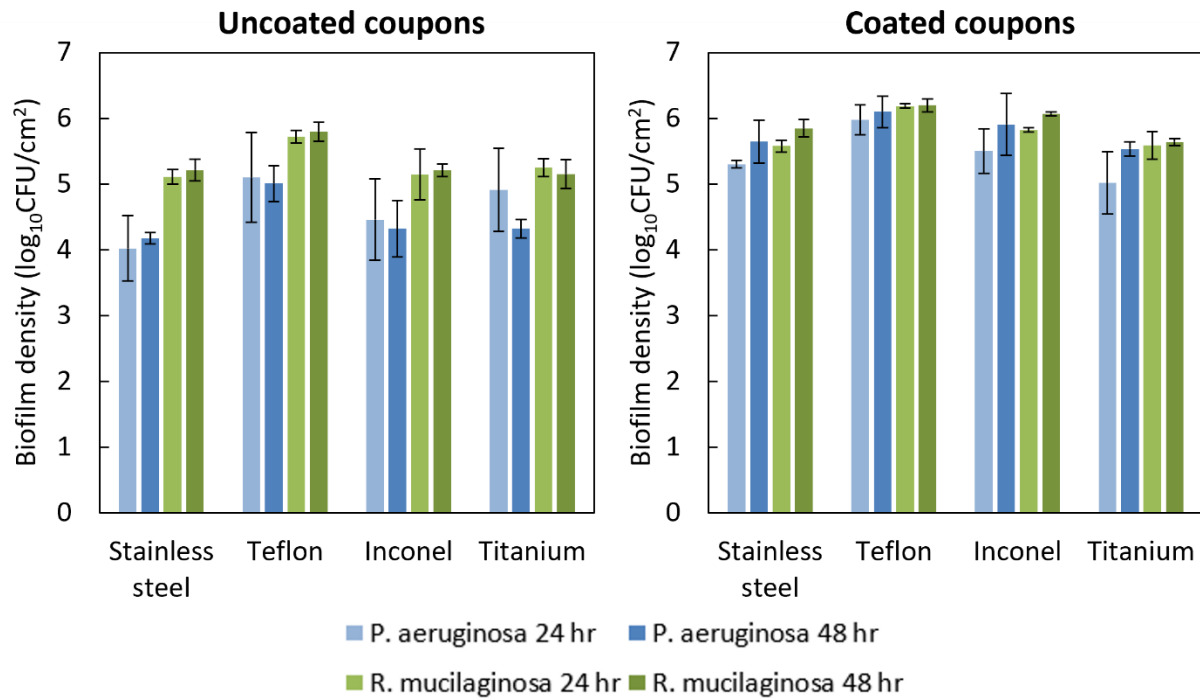


Figure B2. Biofilm densities on uncoated and coated coupons for mixed domain biofilm on four substratum materials. Error bars represent 95% confidence intervals.

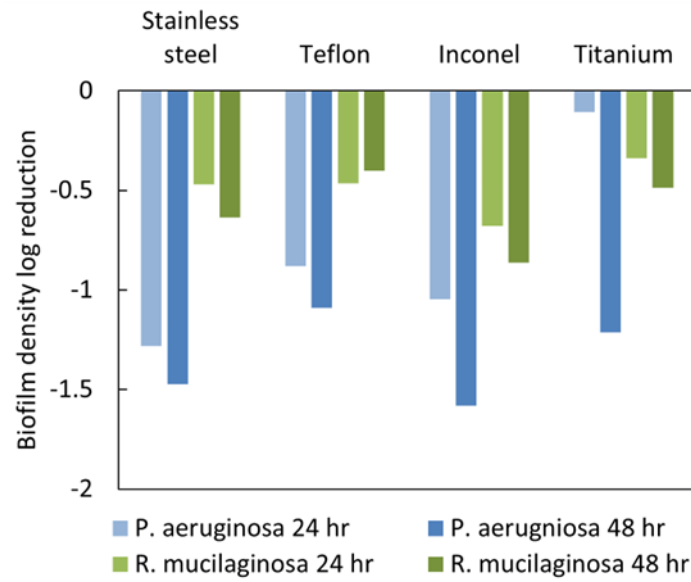


Figure B3. Biofilm log reductions of mixed domain biofilm on uncoated versus coated coupons.

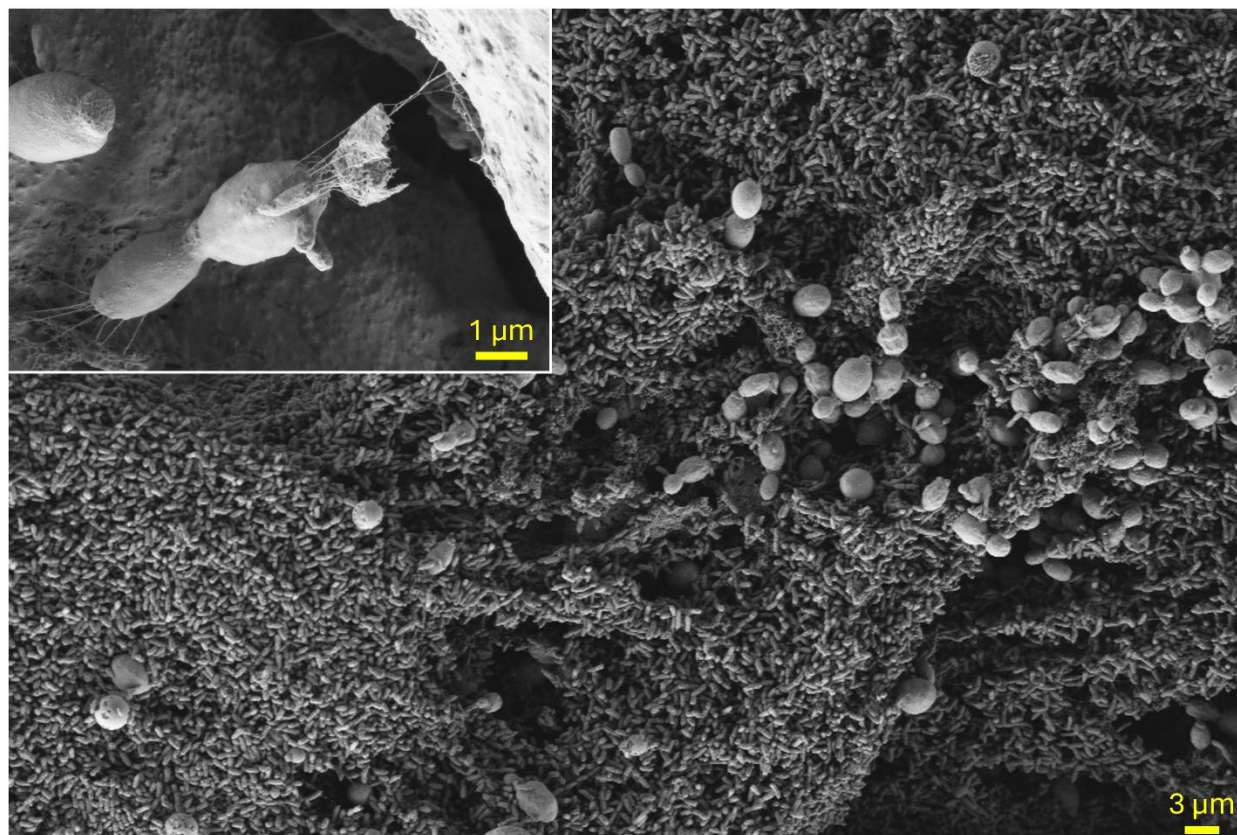


Figure B4. Mixed domain biofilm on a coated Teflon coupon after 48 h of continuous flow. The yeast and bacteria can be seen closely associated with and attached to one another.

Discussion

The growth methods for the mixed domain biofilm followed those used for *R. mucilaginosa* grown as a single-species biofilm because of the difficulty of culturing the yeast. Additional media such as tryptic soy broth (TSB), lower concentration PDB, combinations of both media, various concentrations of yeast extract-peptone-dextrose broth, yeast mold media, Sabouraud dextrose broth (SDB), and these media supplemented with additional peptone, yeast, and/or dextrose were attempted for *R. mucilaginosa* growth, but no robust biofilms or planktonic cultures formed. The research presented in Chapter 3 was presented as such because it could not be determined if the yeast were resistant to the coating tested or if the medium required to grow

the yeast made the coating ineffective. As such, the data presented here in Appendix B were not included in that manuscript.

Data

The data in the tables below were used to generate Figures B1-3 for this Appendix.

Table B1. *R. mucilaginosa* single-species biofilm density data used to generate Figure B1.A-B.

Material	Day	Experiment	Uncoated biofilm density (log ₁₀ CFU/cm ²)	Coated biofilm density (log ₁₀ CFU/cm ²)
Stainless steel	1	1	5.513	5.949
Stainless steel	1	1	5.464	6.125
Stainless steel	1	2	5.434	5.426
Stainless steel	1	2	5.898	5.370
Stainless steel	2	1	5.961	6.312
Stainless steel	2	1	5.855	6.163
Stainless steel	2	2	5.911	5.638
Stainless steel	2	2	5.790	6.268
Stainless steel	2	3	5.824	6.510
Stainless steel	2	3	5.691	6.332
Stainless steel	2	3	5.855	6.430
Teflon	1	1	6.312	6.199
Teflon	1	1	6.205	6.117
Teflon	1	2	6.163	5.984
Teflon	1	2	6.117	5.667
Teflon	2	1	6.384	6.426
Teflon	2	1	6.092	6.422
Teflon	2	2	6.401	6.141
Teflon	2	2	6.347	6.317
Teflon	2	3	6.185	6.773
Teflon	2	3	6.290	6.623
Teflon	2	3	6.065	6.472
Inconel	1	1	5.754	5.898
Inconel	1	1	5.898	5.924
Inconel	1	2	5.306	5.457
Inconel	1	2	5.356	5.500
Inconel	2	1	5.984	6.379
Inconel	2	1	6.026	6.366
Inconel	2	2	6.036	5.884
Inconel	2	2	6.133	5.735
Inconel	2	3	5.529	6.117
Inconel	2	3	5.479	6.125
Inconel	2	3	5.560	6.092
Titanium	1	1	5.523	5.405
Titanium	1	1	5.613	5.405
Titanium	1	2	5.337	5.410

Table B1. Continued.

Material	Day	Experiment	Uncoated biofilm density (log ₁₀ CFU/cm ²)	Coated biofilm density (log ₁₀ CFU/cm ²)
Titanium	1	2	5.500	5.536
Titanium	2	1	5.924	6.397
Titanium	2	1	5.924	6.410
Titanium	2	2	5.694	5.870
Titanium	2	2	5.694	5.694
Titanium	2	3	5.711	6.453
Titanium	2	3	5.641	6.430
Titanium	2	3	5.633	6.262

Table B2. Log reduction of *R. mucilaginosa* single species biofilms on coated vs uncoated coupons used to generate Figure B1.C.

Material	Day	Average log reduction	95% confidence interval
Stainless Steel	1	-0.140	0.179
Stainless Steel	2	-0.355	0.041
Teflon	1	0.208	0.047
Teflon	2	-0.161	0.059
Inconel	1	-0.116	0.014
Inconel	2	-0.227	0.115
Titanium	1	0.054	0.047
Titanium	2	-0.429	0.070

Table B3. Planktonic cell densities (log₁₀CFU/mL) in reactors with *R. mucilaginosa* single-species biofilms used to generate Figure B1.D-E.

Hours	Uncoated			Coated		
	Experiment 1	Experiment 2	Experiment 3	Experiment 1	Experiment 2	Experiment 3
0	7.045	7.053	-	7.079	7.057	-
24	6.179	6.362	-	6.009	6.121	-
48	6.477	6.431	6.241	5.954	6.127	6.447

Table B4. Log reduction for planktonic cells in reactors with single-species *R. mucilaginosa* biofilms with coated vs uncoated coupons used to generate Figure B1.F.

Hour	Average log reduction	95% confidence interval
0	-0.019	0.007
24	0.206	0.016
48	0.207	0.095

Table B5. Mixed domain biofilm data on uncoated coupons used to generate Figure B2.

Organism	Material	24 hours		48 hours	
		Average biofilm density (log ₁₀ CFU/cm ²)	95% confidence interval	Average biofilm density (log ₁₀ CFU/cm ²)	95% confidence interval
<i>P. aeruginosa</i>	Stainless steel	4.022	0.060	4.176	0.328
	Teflon	5.100	0.228	5.007	0.241
	Inconel	4.459	0.338	4.322	0.472
	Titanium	4.910	0.475	4.321	0.108
<i>R. mucilaginosa</i>	Stainless steel	5.108	0.091	5.210	0.131
	Teflon	5.720	0.033	5.797	0.099
	Inconel	5.144	0.032	5.209	0.029
	Titanium	5.248	0.211	5.153	0.051

Table B6. Mixed domain biofilm data on coated coupons used to generate Figure B2.

Organism	Material	24 hours		48 hours	
		Average biofilm density (log ₁₀ CFU/cm ²)	95% confidence interval	Average biofilm density (log ₁₀ CFU/cm ²)	95% confidence interval
<i>P. aeruginosa</i>	Stainless steel	5.302	0.498	5.648	0.084
	Teflon	5.98	0.681	6.097	0.272
	Inconel	5.504	0.619	5.904	0.429
	Titanium	5.016	0.631	5.533	0.137
<i>R. mucilaginosa</i>	Stainless steel	5.577	0.113	5.846	0.165
	Teflon	6.185	0.096	6.199	0.145
	Inconel	5.824	0.387	6.071	0.093
	Titanium	5.585	0.136	5.639	0.217

Table B7. Mixed domain biofilm log reduction data used to generate Figure B3.

Organism	Material	Log reduction	
		24 h	48 h
<i>P. aeruginosa</i>	Stainless steel	-1.280	-1.472
	Teflon	-0.880	-1.089
	Inconel	-1.046	-1.580
	Titanium	-0.107	-1.212
<i>R. mucilaginosa</i>	Stainless steel	-0.469	-0.636
	Teflon	-0.465	-0.402
	Inconel	-0.680	-0.862
	Titanium	-0.338	-0.486

References

1. Daudu, R., et al., *Draft Genome Sequences of Rhodotorula mucilaginosa Strains Isolated from the International Space Station*. Microbiology Resource Announcements. **9**(31): p. e00570-20.
2. Yockey, J., et al., *Cell Envelope Integrity and Capsule Characterization of Rhodotorula mucilaginosa Strains from Clinical and Environmental Sources*. mSphere, 2019. **4**(3).
3. Bzducha-Wróbel, A., S. Błazejak, and K. Tkacz, *Cell wall structure of selected yeast species as a factor of magnesium binding ability*. European Food Research and Technology, 2012. **235**(2): p. 355-366.

APPENDIX C

PRELIMINARY WORK FOR CHAPTER 4 AND SELECTION
OF SHER-LOXANE® 800 COATING

Overview

Prior to the selection of Sher-Loxane® 800 for use in Chapters 4 and 5, there were several additional coatings investigated for their ability to control biofilm accumulation. These initial experiments were conducted using many of the same methods used in Chapter 4 such as the CDC biofilm reactor and Ersatz MTN medium. There were complications with the differentiation between all the organisms in the biofilm consortium which led to the removal of one species. The removal resulted in the consortium used in Chapters 4 and 5.

Methods

The biofilms were grown in a very similar manner to the methods presented in Chapter 4. However, the consortium used in these experiments consisted of the same four organisms used in Chapters 4 and 5, *Burkholderia contaminans*, *Coniochaeta mutabilis*, *Methylobacterium organophilum*, and *Ralstonia insidiosa*, as well as the addition of a fifth microbial isolate from the International Space Station wastewater tank, *Cupriavidus metallidurans*. Additionally, many of the coatings were tested on Teflon and Inconel as was done in Chapter 3, but one of the coatings was only available integrated into a stainless steel mesh from the ae-aqua Filter C2-100 (Feindrahtwerk Adolf Edelhoff, Iserlohn, Germany) and was tested as such. The coatings and their basic mechanisms of action are presented in Table C1. Several of the coatings slightly altered the appearance of the coupons (Figure C1).

Table C1. Coatings tested on Inconel and Teflon coupons. The AgXX mesh was only available as stainless steel.

Coating	Mechanism
Invesil Biocidal	Nanosilver, tantalum, and zirconium nanoparticles
Invesil Biostatic	Nanosilver, tantalum, and zirconium nanoparticles
Next Science coating	Remove metal ions that hold extracellular polymeric substances together, then lyses cells with osmotic imbalance and surfactant
Sher-Loxane® 800	Greatly reduces surface adhesion with high contact angle
AgXX mesh	Reactive oxygen species to damage DNA and proteins

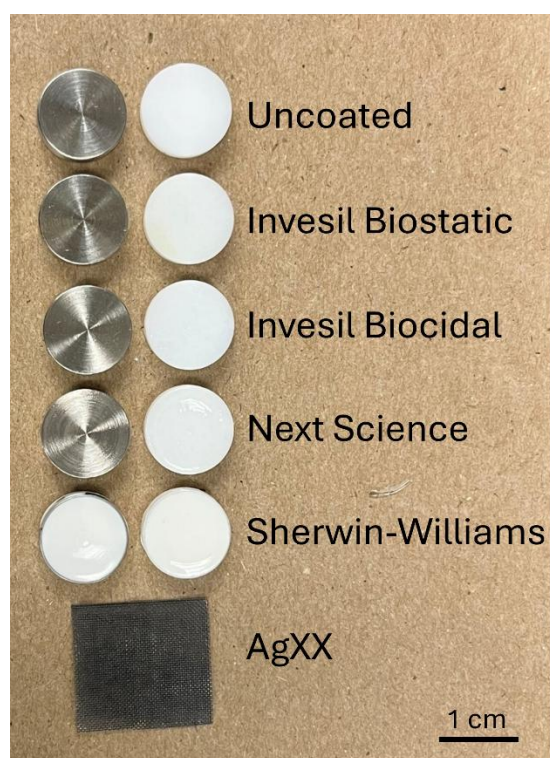


Figure C1. The coupons and the coatings tested in the CDC reactor. The coupons on the left are Inconel and those on the right are Teflon. The AgXX mesh is stainless steel.

The surface topography was measured using a Dektak Stylus Profilometer for each of the coatings on Teflon and Inconel except for the AgXX mesh which was incompatible with the stylus. The topography of the coatings and the uncoated controls are presented in Figures C2 and C3. It was assumed that the coupons were all radially symmetric and representative of the batch,

so the topography of only one coupon was measured. A line map of approximately 6.25 mm by 0.25 mm was created for each coupon. The Teflon coupons generally had larger variations in altitude compared to the Inconel coupons. For both materials, the concentric rings from the machining of the coupons are also visible. The surface roughness values for each coupon and material type are presented in Table C2.

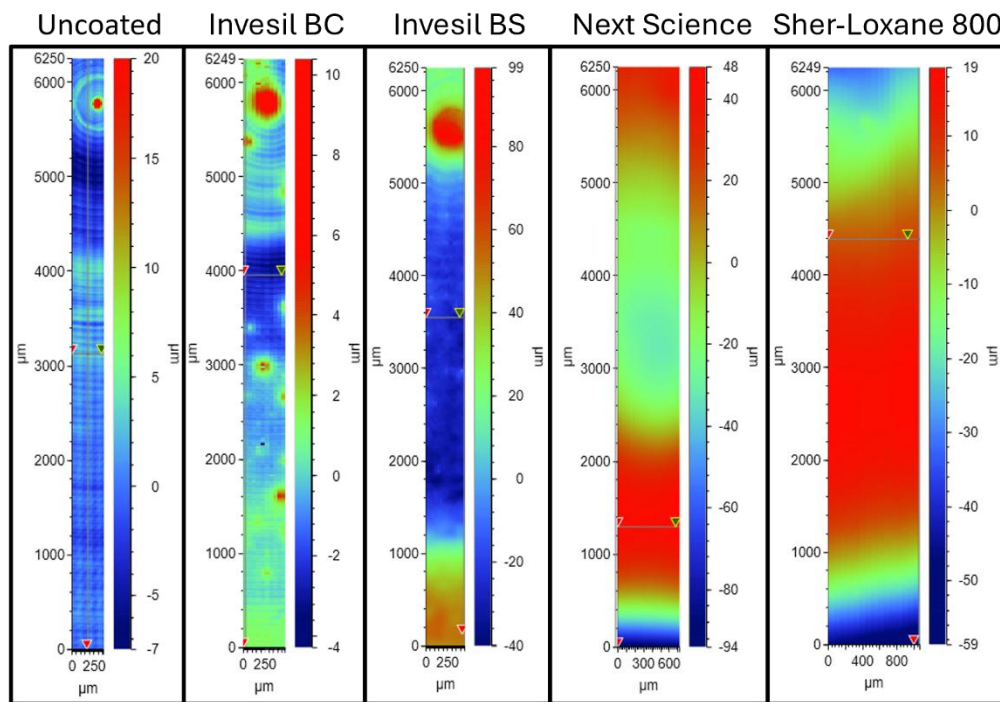


Figure C2. Topographic heat maps of Inconel coupons.

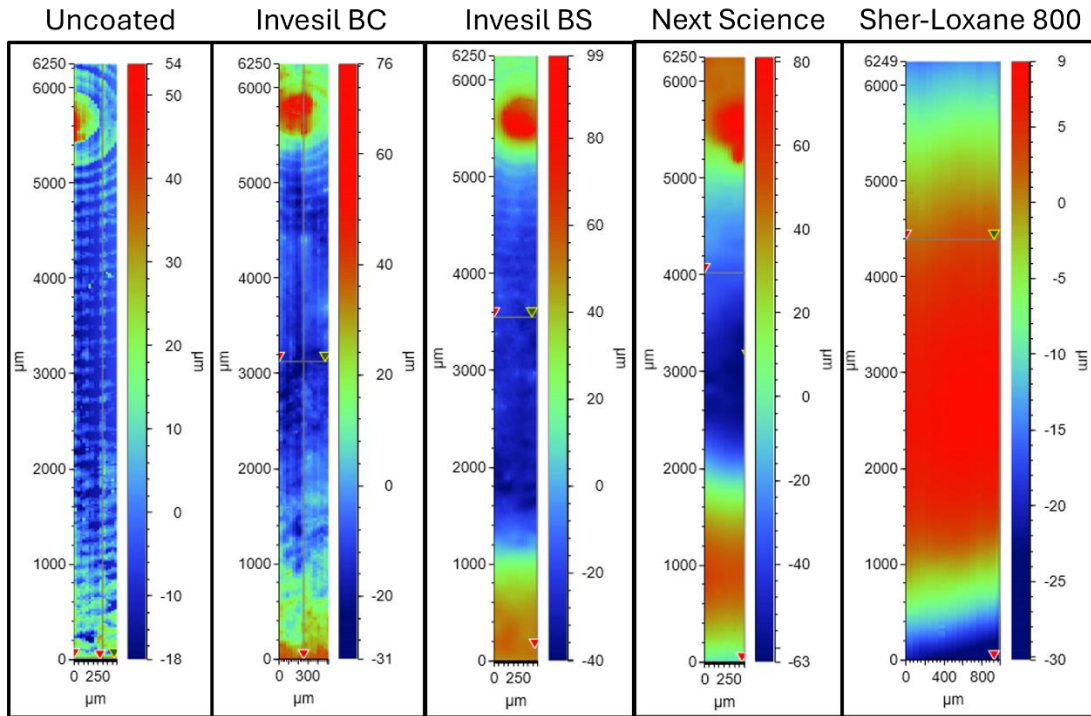


Figure C3. Topographic heat maps of Teflon coupons.

Table C2. Surface roughness quantification values reported by Dektak Stylus Profilometer*.

Coating	Material	Sa	Sq	Sku	Sp	Ssk	Sv	Sz
Uncoated	Teflon	14.59	18.81	3.78	74.62	0.935	-50	124.62
	Inconel	1.72	2.47	12.69	19.58	1.022	-7.46	27.04
Invesil BC	Teflon	12.80	16.02	3.91	76.32	1.13	-31.39	107.71
	Inconel	1.04	1.53	9.61	10.39	1.33	-4.28	14.67
Invesil BS	Teflon	27.72	31.60	2.288	98.89	0.792	-40.22	139.1
	Inconel	1.21	1.56	2.62	4.53	0.01	-3.56	8.09
Next Science	Teflon	37.393	41.653	1.598	81.087	0.061	-63.401	144.487
	Inconel	23.60	27.76	2.660	47.82	-0.180	-94.05	141.9
Sher-Loxane® 800	Teflon	6.80	8.19	3.24	9.32	-0.98	-30.2	39.5
	Inconel	14.66	17.49	2.988	19.24	-0.914	-58.62	77.86

***Key:** Sa= arithmetic average of profile height deviations from the mean line; Sq= root mean square average of profile height deviations from the mean line; Sku= kurtosis of the profile about the mean line; Sp= average maximum peak height above the mean line between single sampling lengths; Ssk= skewness of the profile about the mean line; Sv= average maximum valley depth below the mean line between single sampling lengths; Sz= average maximum peak to valley height of the profile between single sampling lengths.

The methods for isolating the organisms, preparing culture flasks, washing the inoculum, growing the biofilms in the CDC reactors, and sampling were the same as presented in Chapter 4. Teflon and Inconel materials were tested in the same reactor and each reactor had only one coating present (or uncoated coupons). For testing the AgXX mesh, the CDC rods for holding microscopy slides were used instead of the traditional CDC rods that hold the disk-shaped coupons (Figure C4).

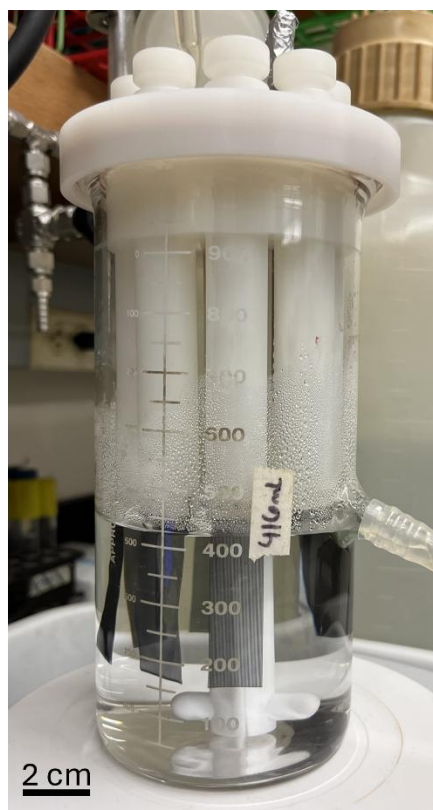


Figure C4. CDC reactor with microscopy slide rods for AgXX evaluation.

The selective agar used for these experiments varied due to issues with isolation. Table C3 presents most of the selective agars tested and the intended organism they were meant to isolate. Additional variations including the listed antibiotics, concentration, and base agars were

tested without success. Near the end of these experiments, the selective agar was honed to the recipes used in Chapters 4 and 5. The issues with the agar and isolation were the impetus for removing *C. metallidurans* from the consortium for Chapters 4 and 5.

Table C3. Various recipes attempted for selective agar*.

<i>B. contaminans</i>	<i>C. metallidurans</i>	<i>C. mutabilis</i>	<i>R. insidiosa</i>
R2A w/ 1X RB, AB, Ca	R2A w/ 1X K, AB	SDA w/ 1X RB, K, Ca	R2A w/ 1X Ca, Ch, AB
R2A w/ 1X RB, 0.75X Ca, AB	R2A w/ 1X K, AB, RB	SDA w/ 1X RB, Ca, 1.5X K	R2A w/ 1X Ca, Ch, AB, G
R2A w/ 2X RB, 1X Ca, AB	R2A w/ 2X K, 1X AB	PDA	R2A w/ 1X RB, Ca, Ch, AB
BCSA w/ 1X RB	R2A w/ 2K K, AB	PDA w/ 1X RB, K, Ca	R2A w/ 1X RB, Ca, Ch, AB, G
R2A w/ 1X RB, AB, 2X Ca	R2A w/ 1X K, AB, G	PDA w/ 2X RB, 1X K, Ca	R2A w/ 2X Ch, 1X Ca, AB, G
PIA	SMA	SDA w/ 2X RB, 1K K, Ca	R2A w/ 2X Ch, Ca, AB
	TSA w/ 2X K, 1X AB	MA w/ 1 X RB	
		Czapek w/ 1X RB	
		SDA pH 3	

***Key:** K=Kanamycin (1X= 30 µg/mL); RB= Rose Bengal (1X= 50 µg/mL); AB= Amphotericin B (1X= 7.5 µg/mL); Ca= Carbenicillin (1X=150 µg/mL); Ch=Chloramphenicol (1X=10 µg/mL); G= Gentamicin (1X= 30 µg/mL); *Burkholderia cepaciae* selective agar= BSCA, *Pseudomonas* isolation agar= PIA, Schatz medium agar= SMA; Tryptic soy agar= TSA; Sabouraud dextrose agar= SDA; Potato dextrose agar= PDA, Malt agar= MA.

Results and discussion

The coatings all resulted in a similar viable biofilm density by 7 days after the start of continuous flow, except for the AgXX which resulted in a cell density about 3 logs higher than the rest (Figure C5). The uncoated coupons fostered a similar biofilm density as most of the coated coupons by day 7. The effect of the coatings is seen largely toward the beginning of the

experiments. Sher-Loxane® 800 and AgXX resulted in the lowest viable biofilm densities after 1 day of continuous flow. After that, AgXX performed the worst out of all coatings tested while Sher-Loxane® 800 continued to have the best performance through 3 days after the start of continuous flow. This delay in biofilm accumulation is why Sher-Loxane® 800 was selected for additional testing. The hypothesis for the research presented in Chapter 4 was that the incorporation of other biofilm control strategies like biocide dosing and nutrient removal would bolster the effects of the coating.

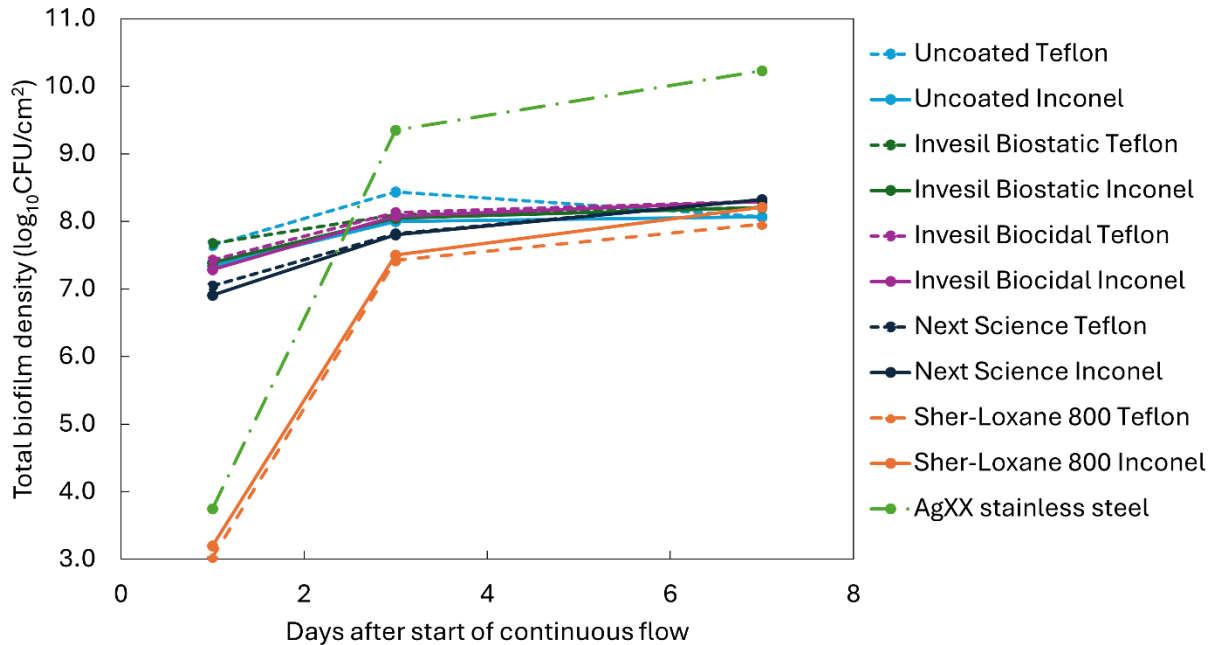


Figure C5. Total viable biofilm density, excluding *C. metallidurans* counts due to uncertainty of counts associated with isolation issues on selective agar.

The biofilm accumulated on the uncoated coupons was largely dominated by *R. insidiosa*, while the biofilm accumulated on the Sher-Loxane® 800 coupons was more dominated by *B. contaminans* (Figure C6). It appears that the fungal hyphae may be creating a scaffold and additional surface area for bacterial attachment (Figure C7). It is known that at least one strain of

B. contaminans produces occidiofungin, an antifungal [1, 2], which could lead to inhibition of *C. mutabilis*, leading to less biomass. Since Sher-Loxane® 800 surfaces tended to foster mainly *B. contaminans*, there could be further benefit from using the coating of less biofilm overall, and significantly less fungal biomass.

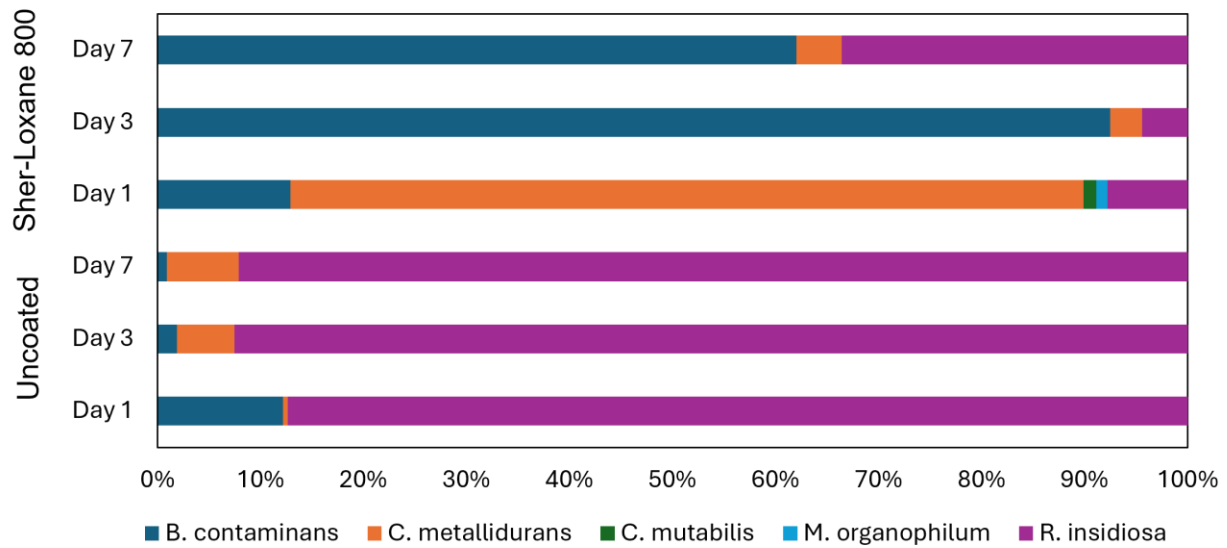


Figure C6. Relative abundance of species in the viable biofilm of uncoated and Sher-Loxane® 800 coated Teflon coupons. *C. metallidurans* counts are included in this figure, but their validity is uncertain.

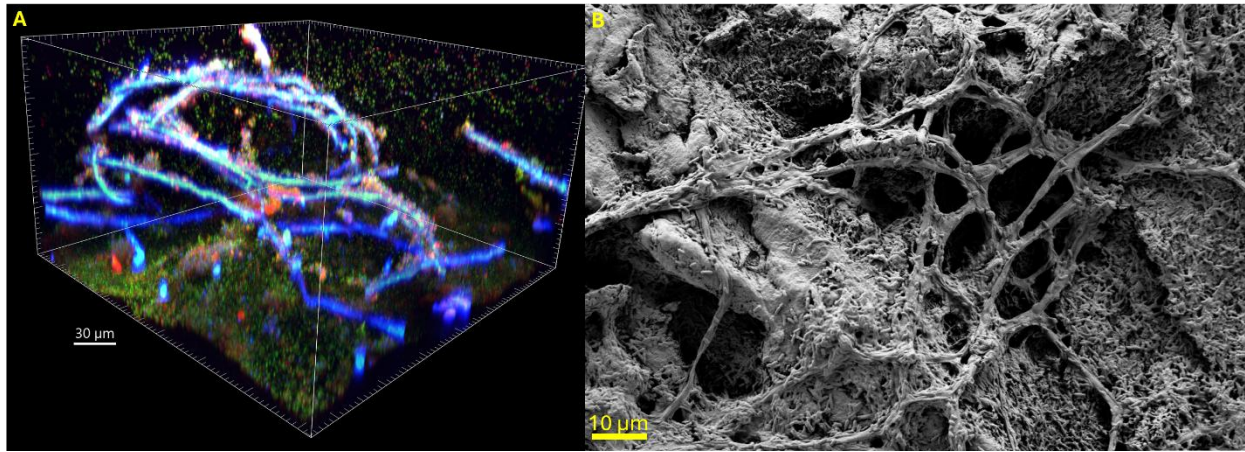


Figure C7. Images of biofilm on uncoated Teflon coupons after 7 days of continuous flow. A) Confocal scanning laser microscopy image with the fungal hyphae in blue, viable bacteria in green, and non-viable bacteria in red. B) Field emission scanning electron microscopy image.

Data

Below are the data used to create Figures C5 and C6.

Table C4. Viable biofilm density data used to create Figure C5. Units for each value are $\log_{10}\text{CFU}/\text{cm}^2$

		Day		
		1	3	7
Uncoated	Teflon	8.042	8.436	8.066
	Inconel	7.342	7.997	8.068
Invesil Biostatic	Teflon	7.676	8.095	8.207
	Inconel	7.390	8.045	8.207
Invesil Biocidal	Teflon	7.429	8.130	8.296
	Inconel	7.285	8.075	8.296
Next Science	Teflon	7.046	7.819	8.322
	Inconel	6.906	7.803	8.325
Sher- Loxane® 800	Teflon	3.032	7.421	7.959
	Inconel	3.198	7.502	8.209
AgXX	Stainless steel	3.750	9.347	10.227

Table C5. Data used to create Figure C6.

	Day	Viable cell density (CFU/cm ²)					Total
		<i>B. contaminans</i>	<i>C. metallidurans</i>	<i>C. mutabilis</i>	<i>M. organophilum</i>	<i>R. insidiosa</i>	
Uncoated	1	5.42E+06	2.05E+05	1.96E+03	2.30E+02	3.88E+07	4.45E+07
	3	5.53E+06	1.61E+07	3.94E+03	2.42E+02	2.67E+08	2.89E+08
	7	1.17E+06	8.69E+06	7.69E+02	3.35E+02	1.15E+08	1.25E+08
Sher-Loxane® 800	1	6.03E+02	3.60E+03	5.70E+01	5.19E+01	3.64E+02	4.68E+03
	3	2.52E+07	8.44E+05	3.11E+01	6.84E+01	1.20E+06	2.72E+07
	7	5.90E+07	4.14E+06	3.12E+01	1.15E+03	3.19E+07	9.51E+07

Funding

Funding was provided by NASA through EPSCoR cooperative agreement 80NSSC21M0331.

Disclosure statement

The coatings tested in this appendix were all provided by the companies, except for the AgXX coating on the stainless steel mesh which was purchased. New coupons were sent to each company to be coated, and the coated coupons were sent back to the Center for Biofilm Engineering, occasionally with postage paid for by the company and sometimes by the research grant. Next Science and The Sherwin-Williams Company are both members of the Center for

Biofilm Engineering industrial associates program. No funds were directly provided for the research by any of the companies tested.

References

1. Gu G, Smith L, Liu A, Lu SE. Genetic and biochemical map for the biosynthesis of occidiofungin, an antifungal produced by *Burkholderia contaminans* strain MS14. Appl Environ Microbiol. 2011;77(17):6189-98; doi: 10.1128/aem.00377-11.
2. Ellis D, Gosai J, Emrick C, Heintz R, Romans L, Gordon D, et al. Occidiofungin's chemical stability and in vitro potency against *Candida* species. Antimicrob Agents Chemother. 2012;56(2):765-9; doi: 10.1128/aac.05231-11.

APPENDIX D

DORMANCY EXPERIMENTS

Overview

The Lunar Gateway is the fourth stage of the National Aeronautics and Space Administration (NASA) Artemis program. Gateway is a space station that will orbit the moon, launching no earlier than 2027 [1]. The much smaller station is only about one fifth the size of the International Space Station (ISS) by volume with room for four astronauts. The station is planned to go through long periods (9 months or longer) of uncrewed dormancy. As such, it is important to understand how various biofilm mitigation strategies may perform during these periods. The experiments presented here explore that question.

Methods

The methods used in these experiments are similar to those used in Chapter 4. The organisms, reactor, coating, and biocide are all the same. For these experiments, only full strength microbial ersatz minor/trace nutrients (MTN) medium was used, and the coupons were stainless steel. Full strength medium was used because of the biofilm control methods explored in this dissertation, the removal of nutrients from a water system would be the most difficult to implement. As such, it was the goal of these experiments to explore biofilm control methods that are more easily incorporated into spacecraft water systems (i.e. coatings and biocide dosing).

The only variation between experiments was the use of uncoated or Sher-Loxane® 800 (Sherwin-Williams) coated coupons. Both reactors were dosed with 216 mg/L silver fluoride at 3 mL/min such that the final concentration in the reactor was 2 ppm. These doses started at the same time as the initiation of continuous flow (24 h after inoculation) and every 24 h during the reactor start-up phase.

On sampling days, planktonic and biofilm samples were taken just prior to the biocide dosing. On day 7 the reactors were sampled, and the spent medium was poured out of the reactors. Sterile Milli-Q water was pumped into the reactors and then was poured out to simulate a rinse with potable water. The reactors were filled once again with sterile Milli-Q water and dosed with 3 mL of silver fluoride. The influent and effluent lines were then clamped, and the reactors were wrapped in foil to block any light. The reactors remained in this condition without stirring for 28 days to simulate spacecraft dormancy.

After the 28 day period, the reactors were sampled. After sampling, the flow of microbial ersatz MTN (2 mL/min), silver biocide dosing, and stirring (125 ± 10 rpm) were reinitiated. The reactors were sampled 2, 7, and 14 days into the regrowth period. Once again, sampling occurred just prior to the biocide dose. See the schematic in Figure D1 for a timeline of the sampling and the experiment.

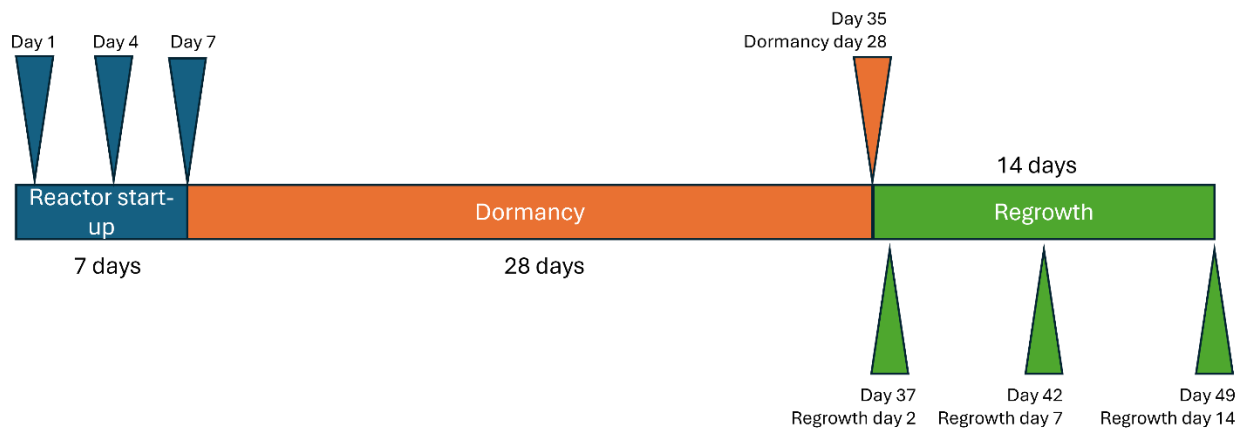


Figure D1. Timeline of dormancy experiments with inoculation occurring on Day 0. Triangles represent days of planktonic and biofilm sampling. No additional planktonic samples were taken to reduce contamination risk.

Results and discussion

The biofilm accumulation in the reactors was higher in the uncoated reactor than the reactor with coated coupons after 7 days of start-up, mirroring the results presented in Chapter 4 (Figure D2). The uncoated coupons continued to foster viable biofilms above the limit of detection throughout the entire experiment. The viable biofilm accumulated on the Sher-Loxane® 800 coated coupons fell below the limit of detection by the end of the dormancy period (28 d). However, as the biofilm quickly rebounded within 2 days of nutrient reintroduction, there were viable cells present in the reactor at the end of dormancy. At the end of the experiment, the coated coupons only fostered about half an order of magnitude fewer viable cells than the uncoated coupons.

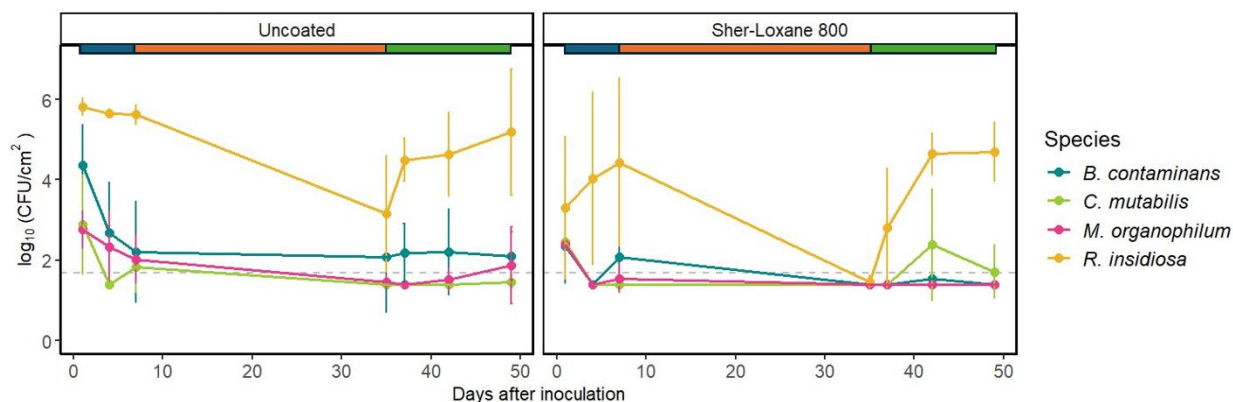


Figure D2. Viable biofilm accumulation during dormancy experiments. Continuous flow and biocide dosing started on Day 1 after the sample. A potable water flush and then initiation of batch conditions took place on Day 7 after the sample to simulate the start of dormancy. After the Day 35 sample, continuous flow and biocide dosing were reinitiated. Colored bars at the top of the graphs correspond to experimental phases presented in Figure D1.

The density of the planktonic cells generally mimics the trends seen in the viable biofilm density (Figure D3).

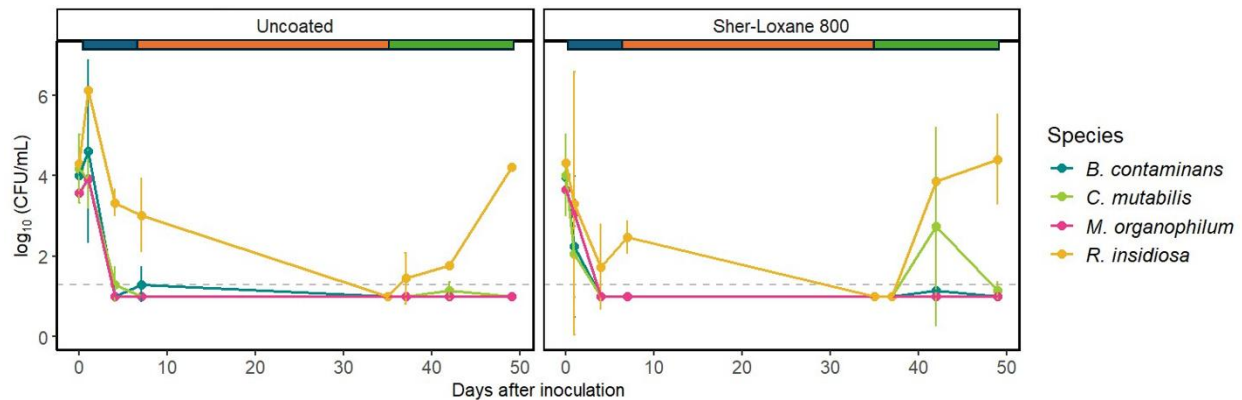


Figure D3. Viable planktonic cell density during dormancy experiments. Continuous flow and biocide dosing started on Day 1 after the sample. A potable water flush and then initiation of batch conditions took place on Day 7 after the sample to simulate the start of dormancy. After the Day 35 sample, continuous flow and biocide dosing were reinitiated. Colored bars at the top of the graphs correspond to experimental phases presented in Figure D1.

These experiments begin to explore the possibilities of biofilm accumulation in dormant spacecraft water systems. However, since the dormancy periods will likely be on a time scale 9 to 30 times the one presented here, it is difficult (and unwise) to extrapolate these results. It is possible that a longer dormancy period would have resulted in lower viable cells, but due to the protective nature of biofilms, it is unlikely that all cells within the biofilm would have been killed.

When examining the results of a 1-month dormancy, the conclusion can be made here that the coating and the biocide dose will not function properly as the only biofilm control methods. The removal of phosphorus from the system (like in Chapter 4) might be key for proper

biofilm control in spacecraft, especially in dormant periods. However, due to time constraints associated with 7-week long tests, more experiments were not feasible.

There are additional factors associated with spacecraft dormancy that were not included in these tests, namely microgravity. Incorporating simulated microgravity tests would be an important consideration for future tests as it simply was not realistic for these experiments. The International Space Station (ISS) is kept at the same ambient temperatures as scientific laboratories tend to be (20-25 °C; comfortable for human existence) but it is unlikely that the temperature in dormant spacecraft will be as closely controlled. Less onboard processes will occur that generate heat, and so dormant spacecraft will probably be colder. These lower temperatures may slow many microbial metabolisms, potentially improving the efficacy of biofilm control methods. The experiments presented here all took place at ambient laboratory temperatures (20-25 °C), so temperature effects were not evaluated. Several more factors regarding dormancy include water system shut down, maintenance, and startup procedures. Tested in these experiments was a simulated potable water flush, where the system is emptied of all wastewater, rinsed with potable water, and filled (stored) with potable water. No maintenance procedures were conducted during the dormancy, but on spacecraft it is possible that additional biocide dosing could occur remotely. The startup condition tested here was simply reintroducing wastewater without emptying the system prior. There are many methods that might be used to further control biofilm accumulation during dormancy via shutdown, maintenance, and startup procedures.

Ultimately, the issue of biofilm control in dormant spacecraft is a new problem that the space community has not faced with the continual presence of humans on the ISS since 2000 [2].

There are many experiments that must be done on Earth and ideally on the ISS to truly understand how to tackle this problem. Water cleanliness and water recycle system operation will continue to be a limiting factor for human space exploration, and solutions to this problem will require a combination of many approaches.

Funding

Funding was provided by NASA through EPSCoR cooperative agreement 80NSSC21M0331.

Disclosure statement

The Sherwin-Williams Company, which provided the coating, has been an industrial affiliate of the Center for Biofilm Engineering since 2009. No direct funding from The Sherwin-Williams Company was provided for this research.

Data

The following data tables were used to generate Figures D2 and D3.

Table D1. Data used to generate Figure D2. Average and standard deviation are represented as $\log_{10}\text{CFU}/\text{cm}^2$. SL refers to the Sher-Loxane® 800 coating.

Species	Modification	Day	Average	Standard deviation
<i>B. contaminans</i>	SL	1	2.342	0.897
<i>B. contaminans</i>	SL	4	1.393	0.000
<i>B. contaminans</i>	SL	7	2.081	0.817
<i>B. contaminans</i>	SL	35	1.393	0.000
<i>B. contaminans</i>	SL	37	1.393	0.000
<i>B. contaminans</i>	SL	42	1.548	0.348
<i>B. contaminans</i>	SL	49	1.393	0.000
<i>B. contaminans</i>	Uncoated	1	4.367	0.999
<i>B. contaminans</i>	Uncoated	4	2.688	1.232
<i>B. contaminans</i>	Uncoated	7	2.201	1.246
<i>B. contaminans</i>	Uncoated	35	2.076	1.366
<i>B. contaminans</i>	Uncoated	37	2.183	0.733
<i>B. contaminans</i>	Uncoated	42	2.205	1.050
<i>B. contaminans</i>	Uncoated	49	2.099	0.595
<i>C. mutabilis</i>	SL	1	2.445	0.679
<i>C. mutabilis</i>	SL	4	1.393	0.000
<i>C. mutabilis</i>	SL	7	1.393	0.000
<i>C. mutabilis</i>	SL	35	1.393	0.000
<i>C. mutabilis</i>	SL	37	1.393	0.000
<i>C. mutabilis</i>	SL	42	2.389	1.379
<i>C. mutabilis</i>	SL	49	1.718	0.651
<i>C. mutabilis</i>	Uncoated	1	2.894	1.225
<i>C. mutabilis</i>	Uncoated	4	1.393	0.000
<i>C. mutabilis</i>	Uncoated	7	1.842	0.634
<i>C. mutabilis</i>	Uncoated	35	1.393	0.000
<i>C. mutabilis</i>	Uncoated	37	1.393	0.000
<i>C. mutabilis</i>	Uncoated	42	1.393	0.000
<i>C. mutabilis</i>	Uncoated	49	1.468	0.151
<i>M. organophilum</i>	SL	1	2.395	0.367
<i>M. organophilum</i>	SL	4	1.393	0.000
<i>M. organophilum</i>	SL	7	1.548	0.348
<i>M. organophilum</i>	SL	35	1.393	0.000
<i>M. organophilum</i>	SL	37	1.393	0.000
<i>M. organophilum</i>	SL	42	1.393	0.000
<i>M. organophilum</i>	SL	49	1.393	0.000
<i>M. organophilum</i>	Uncoated	1	2.768	0.450

Table D1. Continued.

Species	Modification	Day	Average	Standard deviation
<i>M. organophilum</i>	Uncoated	4	2.331	0.873
<i>M. organophilum</i>	Uncoated	7	2.026	0.590
<i>M. organophilum</i>	Uncoated	35	1.468	0.151
<i>M. organophilum</i>	Uncoated	37	1.393	0.000
<i>M. organophilum</i>	Uncoated	42	1.513	0.269
<i>M. organophilum</i>	Uncoated	49	1.868	0.952
<i>R. insidiosa</i>	SL	1	3.309	1.761
<i>R. insidiosa</i>	SL	4	4.032	2.145
<i>R. insidiosa</i>	SL	7	4.431	2.089
<i>R. insidiosa</i>	SL	35	1.468	0.151
<i>R. insidiosa</i>	SL	37	2.801	1.488
<i>R. insidiosa</i>	SL	42	4.645	0.505
<i>R. insidiosa</i>	SL	49	4.700	0.722
<i>R. insidiosa</i>	Uncoated	1	5.814	0.198
<i>R. insidiosa</i>	Uncoated	4	5.642	0.103
<i>R. insidiosa</i>	Uncoated	7	5.618	0.233
<i>R. insidiosa</i>	Uncoated	35	3.151	1.441
<i>R. insidiosa</i>	Uncoated	37	4.491	0.528
<i>R. insidiosa</i>	Uncoated	42	4.634	1.032
<i>R. insidiosa</i>	Uncoated	49	5.184	1.569

Table D2. Data used to generate Figure D3. Average and standard deviation are represented as $\log_{10}$ CFU/mL.

Species	Modification	Day	Average	Standard deviation
<i>B. contaminans</i>	SL	0	3.972	0.039
<i>B. contaminans</i>	SL	1	2.239	1.752
<i>B. contaminans</i>	SL	4	1.000	0.000
<i>B. contaminans</i>	SL	7	1.000	0.000
<i>B. contaminans</i>	SL	35	1.000	0.000
<i>B. contaminans</i>	SL	37	1.000	0.000
<i>B. contaminans</i>	SL	42	1.151	0.213
<i>B. contaminans</i>	SL	49	1.000	0.000
<i>B. contaminans</i>	Uncoated	0	4.017	0.012
<i>B. contaminans</i>	Uncoated	1	4.606	2.260
<i>B. contaminans</i>	Uncoated	4	1.000	0.000
<i>B. contaminans</i>	Uncoated	7	1.301	0.426
<i>B. contaminans</i>	Uncoated	35	1.000	0.000
<i>B. contaminans</i>	Uncoated	37	1.000	0.000
<i>B. contaminans</i>	Uncoated	42	1.000	0.000
<i>B. contaminans</i>	Uncoated	49	1.000	0.000
<i>C. mutabilis</i>	SL	0	4.017	1.012
<i>C. mutabilis</i>	SL	1	2.067	1.083
<i>C. mutabilis</i>	SL	4	1.000	0.000
<i>C. mutabilis</i>	SL	7	1.000	0.000
<i>C. mutabilis</i>	SL	35	1.000	0.000
<i>C. mutabilis</i>	SL	37	1.000	0.000
<i>C. mutabilis</i>	SL	42	2.739	2.459
<i>C. mutabilis</i>	SL	49	1.151	0.213
<i>C. mutabilis</i>	Uncoated	0	4.179	0.847
<i>C. mutabilis</i>	Uncoated	1	3.915	0.707
<i>C. mutabilis</i>	Uncoated	4	1.301	0.426
<i>C. mutabilis</i>	Uncoated	7	1.000	0.000
<i>C. mutabilis</i>	Uncoated	35	1.000	0.000
<i>C. mutabilis</i>	Uncoated	37	1.000	0.000
<i>C. mutabilis</i>	Uncoated	42	1.151	0.213
<i>C. mutabilis</i>	Uncoated	49	1.000	0.000
<i>M. organophilum</i>	SL	0	3.661	0.054
<i>M. organophilum</i>	SL	1	3.067	0.331
<i>M. organophilum</i>	SL	4	1.000	0.000
<i>M. organophilum</i>	SL	7	1.000	0.000

Table D2. Continued.

Species	Modification	Day	Average	Standard deviation
<i>M. organophilum</i>	SL	35	1.000	0.000
<i>M. organophilum</i>	SL	37	1.000	0.000
<i>M. organophilum</i>	SL	42	1.000	0.000
<i>M. organophilum</i>	SL	49	1.000	0.000
<i>M. organophilum</i>	Uncoated	0	3.568	0.017
<i>M. organophilum</i>	Uncoated	1	3.934	0.014
<i>M. organophilum</i>	Uncoated	4	1.000	0.000
<i>M. organophilum</i>	Uncoated	7	1.000	0.000
<i>M. organophilum</i>	Uncoated	35	1.000	0.000
<i>M. organophilum</i>	Uncoated	37	1.000	0.000
<i>M. organophilum</i>	Uncoated	42	1.000	0.000
<i>M. organophilum</i>	Uncoated	49	1.000	0.000
<i>R. insidiosa</i>	SL	0	4.325	0.127
<i>R. insidiosa</i>	SL	1	3.312	3.269
<i>R. insidiosa</i>	SL	4	1.739	1.044
<i>R. insidiosa</i>	SL	7	2.484	0.395
<i>R. insidiosa</i>	SL	35	1.000	0.000
<i>R. insidiosa</i>	SL	37	1.000	0.000
<i>R. insidiosa</i>	SL	42	3.862	0.042
<i>R. insidiosa</i>	SL	49	4.414	1.118
<i>R. insidiosa</i>	Uncoated	0	4.307	0.153
<i>R. insidiosa</i>	Uncoated	1	6.134	0.068
<i>R. insidiosa</i>	Uncoated	4	3.335	0.313
<i>R. insidiosa</i>	Uncoated	7	3.021	0.907
<i>R. insidiosa</i>	Uncoated	35	1.000	0.000
<i>R. insidiosa</i>	Uncoated	37	1.452	0.639
<i>R. insidiosa</i>	Uncoated	42	1.778	0.000
<i>R. insidiosa</i>	Uncoated	49	4.224	4.559

References

1. National Aeronautics and Space Administration. Gateway Frequently Asked Questions. 2025; Available from: <https://www.nasa.gov/gateway-frequently-asked-questions/#:~:text=Unlike%20the%20ISS%2C%20which%20is,Gateway%20will%20not%20be.>
2. National Aeronautics and Space Administration. International Space Station Facts. 2024; Available from: <https://www.nasa.gov/international-space-station/space-station-facts-and-figures/#:~:text=The%20space%20station%20has%20been,station%20during%20a%20crew%20handover.>

APPENDIX E

DATA IN SUPPORT OF CHAPTER 4

The data provided in the following tables were used to generate Figures 3 and 4 in Chapter 4. Additional data for general plate counts labeled “R2A” are also included, though this data was not included in the figures from Chapter 4.

Table E1. Data used to generate Figure 3 in Chapter 4. SL= Sher-Loxane® 800, FS= Full strength media, NP= No phosphorus media, AgF= Silver fluoride biocide. Average density and standard deviation reported as log₁₀CFU/cm².

Species	Material	Modification	Media	Biocide	Day	Average density	Standard deviation
<i>B. contaminans</i>	Inconel	SL	FS	None	1	2.007	0.849
<i>B. contaminans</i>	Inconel	SL	FS	None	3	4.905	2.820
<i>B. contaminans</i>	Inconel	SL	FS	None	5	7.789	0.644
<i>B. contaminans</i>	Inconel	SL	FS	None	7	8.202	0.349
<i>B. contaminans</i>	Inconel	SL	FS	AgF	1	1.393	0.000
<i>B. contaminans</i>	Inconel	SL	FS	AgF	3	1.664	0.506
<i>B. contaminans</i>	Inconel	SL	FS	AgF	5	1.707	0.709
<i>B. contaminans</i>	Inconel	SL	FS	AgF	7	1.879	0.528
<i>B. contaminans</i>	Inconel	SL	NP	None	1	1.918	0.731
<i>B. contaminans</i>	Inconel	SL	NP	None	3	4.983	1.234
<i>B. contaminans</i>	Inconel	SL	NP	None	5	6.422	0.978
<i>B. contaminans</i>	Inconel	SL	NP	None	7	6.920	1.379
<i>B. contaminans</i>	Inconel	SL	NP	AgF	1	1.393	0.000
<i>B. contaminans</i>	Inconel	SL	NP	AgF	3	1.393	0.000
<i>B. contaminans</i>	Inconel	SL	NP	AgF	5	1.393	0.000
<i>B. contaminans</i>	Inconel	SL	NP	AgF	7	1.493	0.246
<i>B. contaminans</i>	Inconel	Uncoated	FS	None	1	5.299	0.184
<i>B. contaminans</i>	Inconel	Uncoated	FS	None	3	5.925	0.588
<i>B. contaminans</i>	Inconel	Uncoated	FS	None	5	5.717	0.689
<i>B. contaminans</i>	Inconel	Uncoated	FS	None	7	6.040	0.512
<i>B. contaminans</i>	Inconel	Uncoated	FS	AgF	1	4.411	0.999
<i>B. contaminans</i>	Inconel	Uncoated	FS	AgF	3	1.706	0.580
<i>B. contaminans</i>	Inconel	Uncoated	FS	AgF	5	2.594	0.932
<i>B. contaminans</i>	Inconel	Uncoated	FS	AgF	7	2.256	0.760
<i>B. contaminans</i>	Inconel	Uncoated	NP	None	1	4.280	0.855
<i>B. contaminans</i>	Inconel	Uncoated	NP	None	3	4.739	1.061
<i>B. contaminans</i>	Inconel	Uncoated	NP	None	5	4.492	1.289
<i>B. contaminans</i>	Inconel	Uncoated	NP	None	7	4.522	1.185
<i>B. contaminans</i>	Inconel	Uncoated	NP	AgF	1	3.659	1.554
<i>B. contaminans</i>	Inconel	Uncoated	NP	AgF	3	2.215	1.201
<i>B. contaminans</i>	Inconel	Uncoated	NP	AgF	5	3.141	1.022
<i>B. contaminans</i>	Inconel	Uncoated	NP	AgF	7	2.172	1.214
<i>B. contaminans</i>	Teflon	SL	FS	None	1	1.990	0.830
<i>B. contaminans</i>	Teflon	SL	FS	None	3	4.798	2.898

Table E1. Continued.

Species	Material	Modification	Media	Biocide	Day	Average density	Standard deviation
<i>B. contaminans</i>	Teflon	SL	FS	None	5	7.883	0.397
<i>B. contaminans</i>	Teflon	SL	FS	None	7	7.970	0.621
<i>B. contaminans</i>	Teflon	SL	FS	AgF	1	1.393	0.000
<i>B. contaminans</i>	Teflon	SL	FS	AgF	3	1.479	0.147
<i>B. contaminans</i>	Teflon	SL	FS	AgF	5	1.436	0.114
<i>B. contaminans</i>	Teflon	SL	FS	AgF	7	1.779	0.694
<i>B. contaminans</i>	Teflon	SL	NP	None	1	2.021	0.873
<i>B. contaminans</i>	Teflon	SL	NP	None	3	4.993	1.223
<i>B. contaminans</i>	Teflon	SL	NP	None	5	6.203	1.049
<i>B. contaminans</i>	Teflon	SL	NP	None	7	6.832	1.225
<i>B. contaminans</i>	Teflon	SL	NP	AgF	1	1.393	0.000
<i>B. contaminans</i>	Teflon	SL	NP	AgF	3	1.490	0.275
<i>B. contaminans</i>	Teflon	SL	NP	AgF	5	1.393	0.000
<i>B. contaminans</i>	Teflon	SL	NP	AgF	7	1.393	0.000
<i>B. contaminans</i>	Teflon	Uncoated	FS	None	1	5.975	0.181
<i>B. contaminans</i>	Teflon	Uncoated	FS	None	3	6.499	0.472
<i>B. contaminans</i>	Teflon	Uncoated	FS	None	5	6.542	0.546
<i>B. contaminans</i>	Teflon	Uncoated	FS	None	7	6.839	0.526
<i>B. contaminans</i>	Teflon	Uncoated	FS	AgF	1	5.155	0.865
<i>B. contaminans</i>	Teflon	Uncoated	FS	AgF	3	2.541	0.783
<i>B. contaminans</i>	Teflon	Uncoated	FS	AgF	5	2.319	0.939
<i>B. contaminans</i>	Teflon	Uncoated	FS	AgF	7	2.276	1.011
<i>B. contaminans</i>	Teflon	Uncoated	NP	None	1	4.987	0.732
<i>B. contaminans</i>	Teflon	Uncoated	NP	None	3	5.092	1.160
<i>B. contaminans</i>	Teflon	Uncoated	NP	None	5	5.078	1.167
<i>B. contaminans</i>	Teflon	Uncoated	NP	None	7	5.171	1.241
<i>B. contaminans</i>	Teflon	Uncoated	NP	AgF	1	4.066	1.831
<i>B. contaminans</i>	Teflon	Uncoated	NP	AgF	3	2.676	1.112
<i>B. contaminans</i>	Teflon	Uncoated	NP	AgF	5	2.992	1.219
<i>B. contaminans</i>	Teflon	Uncoated	NP	AgF	7	2.384	0.814
<i>C. mutabilis</i>	Inconel	SL	FS	None	1	1.468	0.139
<i>C. mutabilis</i>	Inconel	SL	FS	None	3	1.543	0.228
<i>C. mutabilis</i>	Inconel	SL	FS	None	5	2.358	0.707
<i>C. mutabilis</i>	Inconel	SL	FS	None	7	2.369	0.568
<i>C. mutabilis</i>	Inconel	SL	FS	AgF	1	1.887	0.645
<i>C. mutabilis</i>	Inconel	SL	FS	AgF	3	1.436	0.114

Table E1. Continued.

Species	Material	Modification	Media	Biocide	Day	Average density	Standard deviation
<i>C. mutabilis</i>	Inconel	SL	FS	AgF	5	1.479	0.228
<i>C. mutabilis</i>	Inconel	SL	FS	AgF	7	1.739	0.537
<i>C. mutabilis</i>	Inconel	SL	NP	None	1	2.170	1.085
<i>C. mutabilis</i>	Inconel	SL	NP	None	3	1.781	0.465
<i>C. mutabilis</i>	Inconel	SL	NP	None	5	1.769	0.312
<i>C. mutabilis</i>	Inconel	SL	NP	None	7	2.420	0.638
<i>C. mutabilis</i>	Inconel	SL	NP	AgF	1	1.883	0.679
<i>C. mutabilis</i>	Inconel	SL	NP	AgF	3	1.393	0.000
<i>C. mutabilis</i>	Inconel	SL	NP	AgF	5	1.393	0.000
<i>C. mutabilis</i>	Inconel	SL	NP	AgF	7	1.393	0.000
<i>C. mutabilis</i>	Inconel	Uncoated	FS	None	1	2.683	0.665
<i>C. mutabilis</i>	Inconel	Uncoated	FS	None	3	3.384	0.709
<i>C. mutabilis</i>	Inconel	Uncoated	FS	None	5	3.071	0.529
<i>C. mutabilis</i>	Inconel	Uncoated	FS	None	7	3.090	0.723
<i>C. mutabilis</i>	Inconel	Uncoated	FS	AgF	1	3.204	0.231
<i>C. mutabilis</i>	Inconel	Uncoated	FS	AgF	3	1.393	0.000
<i>C. mutabilis</i>	Inconel	Uncoated	FS	AgF	5	1.393	0.000
<i>C. mutabilis</i>	Inconel	Uncoated	FS	AgF	7	1.393	0.000
<i>C. mutabilis</i>	Inconel	Uncoated	NP	None	1	3.400	0.691
<i>C. mutabilis</i>	Inconel	Uncoated	NP	None	3	3.009	0.554
<i>C. mutabilis</i>	Inconel	Uncoated	NP	None	5	2.638	0.638
<i>C. mutabilis</i>	Inconel	Uncoated	NP	None	7	2.839	0.700
<i>C. mutabilis</i>	Inconel	Uncoated	NP	AgF	1	2.827	0.897
<i>C. mutabilis</i>	Inconel	Uncoated	NP	AgF	3	1.436	0.114
<i>C. mutabilis</i>	Inconel	Uncoated	NP	AgF	5	1.393	0.000
<i>C. mutabilis</i>	Inconel	Uncoated	NP	AgF	7	1.393	0.000
<i>C. mutabilis</i>	Teflon	SL	FS	None	1	1.468	0.139
<i>C. mutabilis</i>	Teflon	SL	FS	None	3	1.618	0.213
<i>C. mutabilis</i>	Teflon	SL	FS	None	5	2.774	1.126
<i>C. mutabilis</i>	Teflon	SL	FS	None	7	2.526	0.927
<i>C. mutabilis</i>	Teflon	SL	FS	AgF	1	2.041	0.812
<i>C. mutabilis</i>	Teflon	SL	FS	AgF	3	1.393	0.000
<i>C. mutabilis</i>	Teflon	SL	FS	AgF	5	1.393	0.000
<i>C. mutabilis</i>	Teflon	SL	FS	AgF	7	1.810	0.648
<i>C. mutabilis</i>	Teflon	SL	NP	None	1	2.195	1.112
<i>C. mutabilis</i>	Teflon	SL	NP	None	3	1.790	0.565

Table E1. Continued.

Species	Material	Modification	Media	Biocide	Day	Average density	Standard deviation
<i>C. mutabilis</i>	Teflon	SL	NP	None	5	1.866	0.427
<i>C. mutabilis</i>	Teflon	SL	NP	None	7	2.396	0.755
<i>C. mutabilis</i>	Teflon	SL	NP	AgF	1	1.945	0.768
<i>C. mutabilis</i>	Teflon	SL	NP	AgF	3	1.393	0.000
<i>C. mutabilis</i>	Teflon	SL	NP	AgF	5	1.393	0.000
<i>C. mutabilis</i>	Teflon	SL	NP	AgF	7	1.393	0.000
<i>C. mutabilis</i>	Teflon	Uncoated	FS	None	1	3.215	0.684
<i>C. mutabilis</i>	Teflon	Uncoated	FS	None	3	3.788	0.746
<i>C. mutabilis</i>	Teflon	Uncoated	FS	None	5	3.665	0.736
<i>C. mutabilis</i>	Teflon	Uncoated	FS	None	7	3.667	1.200
<i>C. mutabilis</i>	Teflon	Uncoated	FS	AgF	1	3.658	0.286
<i>C. mutabilis</i>	Teflon	Uncoated	FS	AgF	3	1.505	0.224
<i>C. mutabilis</i>	Teflon	Uncoated	FS	AgF	5	1.430	0.106
<i>C. mutabilis</i>	Teflon	Uncoated	FS	AgF	7	1.393	0.000
<i>C. mutabilis</i>	Teflon	Uncoated	NP	None	1	3.759	0.593
<i>C. mutabilis</i>	Teflon	Uncoated	NP	None	3	3.111	0.561
<i>C. mutabilis</i>	Teflon	Uncoated	NP	None	5	3.202	0.429
<i>C. mutabilis</i>	Teflon	Uncoated	NP	None	7	3.257	0.598
<i>C. mutabilis</i>	Teflon	Uncoated	NP	AgF	1	3.259	0.762
<i>C. mutabilis</i>	Teflon	Uncoated	NP	AgF	3	1.393	0.000
<i>C. mutabilis</i>	Teflon	Uncoated	NP	AgF	5	1.393	0.000
<i>C. mutabilis</i>	Teflon	Uncoated	NP	AgF	7	1.393	0.000
<i>M. organophilum</i>	Inconel	SL	FS	None	1	1.716	0.448
<i>M. organophilum</i>	Inconel	SL	FS	None	3	1.603	0.391
<i>M. organophilum</i>	Inconel	SL	FS	None	5	1.490	0.275
<i>M. organophilum</i>	Inconel	SL	FS	None	7	1.838	0.691
<i>M. organophilum</i>	Inconel	SL	FS	AgF	1	2.460	1.335
<i>M. organophilum</i>	Inconel	SL	FS	AgF	3	1.719	0.470
<i>M. organophilum</i>	Inconel	SL	FS	AgF	5	1.690	0.786
<i>M. organophilum</i>	Inconel	SL	FS	AgF	7	1.543	0.252
<i>M. organophilum</i>	Inconel	SL	NP	None	1	2.645	1.311
<i>M. organophilum</i>	Inconel	SL	NP	None	3	2.827	1.205
<i>M. organophilum</i>	Inconel	SL	NP	None	5	3.398	0.944
<i>M. organophilum</i>	Inconel	SL	NP	None	7	3.505	1.077
<i>M. organophilum</i>	Inconel	SL	NP	AgF	1	2.360	1.339
<i>M. organophilum</i>	Inconel	SL	NP	AgF	3	1.555	0.354

Table E1. Continued.

Species	Material	Modification	Media	Biocide	Day	Average density	Standard deviation
<i>M. organophilum</i>	Inconel	SL	NP	AgF	5	1.468	0.139
<i>M. organophilum</i>	Inconel	SL	NP	AgF	7	1.393	0.000
<i>M. organophilum</i>	Inconel	Uncoated	FS	None	1	2.619	0.687
<i>M. organophilum</i>	Inconel	Uncoated	FS	None	3	2.961	0.513
<i>M. organophilum</i>	Inconel	Uncoated	FS	None	5	2.555	0.369
<i>M. organophilum</i>	Inconel	Uncoated	FS	None	7	3.043	0.830
<i>M. organophilum</i>	Inconel	Uncoated	FS	AgF	1	2.868	0.603
<i>M. organophilum</i>	Inconel	Uncoated	FS	AgF	3	1.505	0.156
<i>M. organophilum</i>	Inconel	Uncoated	FS	AgF	5	1.909	0.598
<i>M. organophilum</i>	Inconel	Uncoated	FS	AgF	7	2.050	0.745
<i>M. organophilum</i>	Inconel	Uncoated	NP	None	1	3.351	0.558
<i>M. organophilum</i>	Inconel	Uncoated	NP	None	3	3.922	0.576
<i>M. organophilum</i>	Inconel	Uncoated	NP	None	5	3.702	0.689
<i>M. organophilum</i>	Inconel	Uncoated	NP	None	7	3.966	0.952
<i>M. organophilum</i>	Inconel	Uncoated	NP	AgF	1	2.620	1.175
<i>M. organophilum</i>	Inconel	Uncoated	NP	AgF	3	1.590	0.341
<i>M. organophilum</i>	Inconel	Uncoated	NP	AgF	5	1.615	0.469
<i>M. organophilum</i>	Inconel	Uncoated	NP	AgF	7	1.443	0.123
<i>M. organophilum</i>	Teflon	SL	FS	None	1	1.662	0.427
<i>M. organophilum</i>	Teflon	SL	FS	None	3	1.430	0.106
<i>M. organophilum</i>	Teflon	SL	FS	None	5	1.975	0.384
<i>M. organophilum</i>	Teflon	SL	FS	None	7	1.694	0.466
<i>M. organophilum</i>	Teflon	SL	FS	AgF	1	2.524	1.427
<i>M. organophilum</i>	Teflon	SL	FS	AgF	3	1.393	0.000
<i>M. organophilum</i>	Teflon	SL	FS	AgF	5	1.694	0.673
<i>M. organophilum</i>	Teflon	SL	FS	AgF	7	1.572	0.317
<i>M. organophilum</i>	Teflon	SL	NP	None	1	2.512	1.163
<i>M. organophilum</i>	Teflon	SL	NP	None	3	3.036	1.116
<i>M. organophilum</i>	Teflon	SL	NP	None	5	3.476	0.905
<i>M. organophilum</i>	Teflon	SL	NP	None	7	3.585	1.045
<i>M. organophilum</i>	Teflon	SL	NP	AgF	1	2.356	1.335
<i>M. organophilum</i>	Teflon	SL	NP	AgF	3	1.468	0.139
<i>M. organophilum</i>	Teflon	SL	NP	AgF	5	1.430	0.106
<i>M. organophilum</i>	Teflon	SL	NP	AgF	7	1.920	0.821
<i>M. organophilum</i>	Teflon	Uncoated	FS	None	1	3.465	0.529
<i>M. organophilum</i>	Teflon	Uncoated	FS	None	3	3.877	0.696

Table E1. Continued.

Species	Material	Modification	Media	Biocide	Day	Average density	Standard deviation
<i>M. organophilum</i>	Teflon	Uncoated	FS	None	5	3.427	0.565
<i>M. organophilum</i>	Teflon	Uncoated	FS	None	7	3.549	0.995
<i>M. organophilum</i>	Teflon	Uncoated	FS	AgF	1	3.603	0.507
<i>M. organophilum</i>	Teflon	Uncoated	FS	AgF	3	1.694	0.279
<i>M. organophilum</i>	Teflon	Uncoated	FS	AgF	5	1.762	0.402
<i>M. organophilum</i>	Teflon	Uncoated	FS	AgF	7	1.493	0.246
<i>M. organophilum</i>	Teflon	Uncoated	NP	None	1	4.133	0.573
<i>M. organophilum</i>	Teflon	Uncoated	NP	None	3	4.494	0.531
<i>M. organophilum</i>	Teflon	Uncoated	NP	None	5	4.703	0.359
<i>M. organophilum</i>	Teflon	Uncoated	NP	None	7	4.407	0.687
<i>M. organophilum</i>	Teflon	Uncoated	NP	AgF	1	3.214	1.329
<i>M. organophilum</i>	Teflon	Uncoated	NP	AgF	3	1.522	0.161
<i>M. organophilum</i>	Teflon	Uncoated	NP	AgF	5	1.664	0.719
<i>M. organophilum</i>	Teflon	Uncoated	NP	AgF	7	1.393	0.000
<i>R. insidiosa</i>	Inconel	SL	FS	None	1	1.393	0.000
<i>R. insidiosa</i>	Inconel	SL	FS	None	3	4.008	2.757
<i>R. insidiosa</i>	Inconel	SL	FS	None	5	6.488	1.718
<i>R. insidiosa</i>	Inconel	SL	FS	None	7	7.571	1.017
<i>R. insidiosa</i>	Inconel	SL	FS	AgF	1	1.479	0.147
<i>R. insidiosa</i>	Inconel	SL	FS	AgF	3	1.878	0.634
<i>R. insidiosa</i>	Inconel	SL	FS	AgF	5	3.377	1.484
<i>R. insidiosa</i>	Inconel	SL	FS	AgF	7	2.360	0.758
<i>R. insidiosa</i>	Inconel	SL	NP	None	1	1.430	0.106
<i>R. insidiosa</i>	Inconel	SL	NP	None	3	5.078	0.339
<i>R. insidiosa</i>	Inconel	SL	NP	None	5	5.470	0.896
<i>R. insidiosa</i>	Inconel	SL	NP	None	7	6.340	0.203
<i>R. insidiosa</i>	Inconel	SL	NP	AgF	1	1.393	0.000
<i>R. insidiosa</i>	Inconel	SL	NP	AgF	3	1.924	0.875
<i>R. insidiosa</i>	Inconel	SL	NP	AgF	5	1.393	0.000
<i>R. insidiosa</i>	Inconel	SL	NP	AgF	7	1.656	0.645
<i>R. insidiosa</i>	Inconel	Uncoated	FS	None	1	6.165	0.563
<i>R. insidiosa</i>	Inconel	Uncoated	FS	None	3	8.097	0.151
<i>R. insidiosa</i>	Inconel	Uncoated	FS	None	5	8.071	0.050
<i>R. insidiosa</i>	Inconel	Uncoated	FS	None	7	8.051	0.159
<i>R. insidiosa</i>	Inconel	Uncoated	FS	AgF	1	6.289	0.369
<i>R. insidiosa</i>	Inconel	Uncoated	FS	AgF	3	4.826	0.197

Table E1. Continued.

Species	Material	Modification	Media	Biocide	Day	Average density	Standard deviation
<i>R. insidiosa</i>	Inconel	Uncoated	FS	AgF	5	5.316	0.239
<i>R. insidiosa</i>	Inconel	Uncoated	FS	AgF	7	5.158	0.497
<i>R. insidiosa</i>	Inconel	Uncoated	NP	None	1	5.691	0.542
<i>R. insidiosa</i>	Inconel	Uncoated	NP	None	3	6.598	0.177
<i>R. insidiosa</i>	Inconel	Uncoated	NP	None	5	6.575	0.147
<i>R. insidiosa</i>	Inconel	Uncoated	NP	None	7	6.631	0.203
<i>R. insidiosa</i>	Inconel	Uncoated	NP	AgF	1	5.474	0.812
<i>R. insidiosa</i>	Inconel	Uncoated	NP	AgF	3	4.232	0.562
<i>R. insidiosa</i>	Inconel	Uncoated	NP	AgF	5	3.254	1.619
<i>R. insidiosa</i>	Inconel	Uncoated	NP	AgF	7	4.609	0.384
<i>R. insidiosa</i>	Teflon	SL	FS	None	1	1.640	0.382
<i>R. insidiosa</i>	Teflon	SL	FS	None	3	4.023	2.775
<i>R. insidiosa</i>	Teflon	SL	FS	None	5	6.812	1.494
<i>R. insidiosa</i>	Teflon	SL	FS	None	7	7.524	1.308
<i>R. insidiosa</i>	Teflon	SL	FS	AgF	1	1.436	0.114
<i>R. insidiosa</i>	Teflon	SL	FS	AgF	3	1.790	0.618
<i>R. insidiosa</i>	Teflon	SL	FS	AgF	5	3.478	1.663
<i>R. insidiosa</i>	Teflon	SL	FS	AgF	7	2.620	1.251
<i>R. insidiosa</i>	Teflon	SL	NP	None	1	1.684	0.403
<i>R. insidiosa</i>	Teflon	SL	NP	None	3	5.055	0.356
<i>R. insidiosa</i>	Teflon	SL	NP	None	5	5.213	0.892
<i>R. insidiosa</i>	Teflon	SL	NP	None	7	6.197	0.362
<i>R. insidiosa</i>	Teflon	SL	NP	AgF	1	1.393	0.000
<i>R. insidiosa</i>	Teflon	SL	NP	AgF	3	1.769	0.476
<i>R. insidiosa</i>	Teflon	SL	NP	AgF	5	1.393	0.000
<i>R. insidiosa</i>	Teflon	SL	NP	AgF	7	2.001	0.973
<i>R. insidiosa</i>	Teflon	Uncoated	FS	None	1	6.629	0.561
<i>R. insidiosa</i>	Teflon	Uncoated	FS	None	3	8.342	0.181
<i>R. insidiosa</i>	Teflon	Uncoated	FS	None	5	8.266	0.580
<i>R. insidiosa</i>	Teflon	Uncoated	FS	None	7	8.484	0.258
<i>R. insidiosa</i>	Teflon	Uncoated	FS	AgF	1	6.684	0.456
<i>R. insidiosa</i>	Teflon	Uncoated	FS	AgF	3	5.121	0.370
<i>R. insidiosa</i>	Teflon	Uncoated	FS	AgF	5	5.414	0.175
<i>R. insidiosa</i>	Teflon	Uncoated	FS	AgF	7	5.121	0.658
<i>R. insidiosa</i>	Teflon	Uncoated	NP	None	1	6.207	0.613
<i>R. insidiosa</i>	Teflon	Uncoated	NP	None	3	6.775	0.279

Table E1. Continued.

Species	Material	Modification	Media	Biocide	Day	Average density	Standard deviation
<i>R. insidiosa</i>	Teflon	Uncoated	NP	None	5	6.952	0.175
<i>R. insidiosa</i>	Teflon	Uncoated	NP	None	7	7.069	0.166
<i>R. insidiosa</i>	Teflon	Uncoated	NP	AgF	1	5.733	0.942
<i>R. insidiosa</i>	Teflon	Uncoated	NP	AgF	3	4.013	1.015
<i>R. insidiosa</i>	Teflon	Uncoated	NP	AgF	5	3.461	1.481
<i>R. insidiosa</i>	Teflon	Uncoated	NP	AgF	7	4.760	0.462
R2A	Inconel	SL	FS	None	1	2.112	0.777
R2A	Inconel	SL	FS	None	3	4.072	2.744
R2A	Inconel	SL	FS	None	5	7.939	0.614
R2A	Inconel	SL	FS	None	7	8.514	0.106
R2A	Inconel	SL	FS	AgF	1	2.544	1.438
R2A	Inconel	SL	FS	AgF	3	2.265	0.707
R2A	Inconel	SL	FS	AgF	5	2.629	1.047
R2A	Inconel	SL	FS	AgF	7	3.245	0.767
R2A	Inconel	SL	NP	None	1	2.712	0.842
R2A	Inconel	SL	NP	None	3	5.656	0.919
R2A	Inconel	SL	NP	None	5	6.648	0.583
R2A	Inconel	SL	NP	None	7	7.289	0.864
R2A	Inconel	SL	NP	AgF	1	2.098	0.897
R2A	Inconel	SL	NP	AgF	3	2.069	1.015
R2A	Inconel	SL	NP	AgF	5	1.453	0.135
R2A	Inconel	SL	NP	AgF	7	1.737	0.706
R2A	Inconel	Uncoated	FS	None	1	6.319	0.556
R2A	Inconel	Uncoated	FS	None	3	8.344	0.237
R2A	Inconel	Uncoated	FS	None	5	8.109	0.154
R2A	Inconel	Uncoated	FS	None	7	8.283	0.152
R2A	Inconel	Uncoated	FS	AgF	1	6.378	0.274
R2A	Inconel	Uncoated	FS	AgF	3	4.959	0.217
R2A	Inconel	Uncoated	FS	AgF	5	5.367	0.222
R2A	Inconel	Uncoated	FS	AgF	7	5.323	0.307
R2A	Inconel	Uncoated	NP	None	1	5.953	0.336
R2A	Inconel	Uncoated	NP	None	3	6.680	0.201
R2A	Inconel	Uncoated	NP	None	5	6.644	0.182
R2A	Inconel	Uncoated	NP	None	7	6.761	0.269
R2A	Inconel	Uncoated	NP	AgF	1	5.609	0.841
R2A	Inconel	Uncoated	NP	AgF	3	3.828	0.705

Table E1. Continued.

Species	Material	Modification	Media	Biocide	Day	Average density	Standard deviation
R2A	Inconel	Uncoated	NP	AgF	5	4.674	0.514
R2A	Inconel	Uncoated	NP	AgF	7	4.597	0.417
R2A	Teflon	SL	FS	None	1	2.047	0.904
R2A	Teflon	SL	FS	None	3	4.988	2.616
R2A	Teflon	SL	FS	None	5	8.107	0.488
R2A	Teflon	SL	FS	None	7	8.486	0.210
R2A	Teflon	SL	FS	AgF	1	2.586	1.500
R2A	Teflon	SL	FS	AgF	3	1.954	0.664
R2A	Teflon	SL	FS	AgF	5	2.686	0.918
R2A	Teflon	SL	FS	AgF	7	3.066	0.939
R2A	Teflon	SL	NP	None	1	2.791	0.916
R2A	Teflon	SL	NP	None	3	5.513	0.887
R2A	Teflon	SL	NP	None	5	6.554	0.786
R2A	Teflon	SL	NP	None	7	7.046	0.997
R2A	Teflon	SL	NP	AgF	1	2.321	0.996
R2A	Teflon	SL	NP	AgF	3	1.775	0.462
R2A	Teflon	SL	NP	AgF	5	1.393	0.000
R2A	Teflon	SL	NP	AgF	7	1.773	0.591
R2A	Teflon	Uncoated	FS	None	1	6.842	0.490
R2A	Teflon	Uncoated	FS	None	3	8.465	0.205
R2A	Teflon	Uncoated	FS	None	5	8.141	0.755
R2A	Teflon	Uncoated	FS	None	7	7.884	0.842
R2A	Teflon	Uncoated	FS	AgF	1	6.783	0.369
R2A	Teflon	Uncoated	FS	AgF	3	5.049	0.363
R2A	Teflon	Uncoated	FS	AgF	5	4.934	1.038
R2A	Teflon	Uncoated	FS	AgF	7	4.798	1.291
R2A	Teflon	Uncoated	NP	None	1	6.475	0.368
R2A	Teflon	Uncoated	NP	None	3	6.864	0.196
R2A	Teflon	Uncoated	NP	None	5	6.203	1.448
R2A	Teflon	Uncoated	NP	None	7	6.226	1.491
R2A	Teflon	Uncoated	NP	AgF	1	6.035	1.117
R2A	Teflon	Uncoated	NP	AgF	3	4.395	0.780
R2A	Teflon	Uncoated	NP	AgF	5	4.461	1.084
R2A	Teflon	Uncoated	NP	AgF	7	4.335	0.703

Table E2. Data used to generate Figure 4 in Chapter 5. SL= Sher-Loxane® 800, FS= Full strength media, NP= No phosphorus media, AgF= Silver fluoride biocide. Average density and standard deviation reported as log₁₀CFU/mL.

Species	Modification	Media	Biocide	Day	Average density	Standard deviation
<i>B. contaminans</i>	SL	FS	None	0	4.558	0.426
<i>B. contaminans</i>	SL	FS	None	1	1.749	1.296
<i>B. contaminans</i>	SL	FS	None	3	4.689	3.243
<i>B. contaminans</i>	SL	FS	None	5	6.568	0.550
<i>B. contaminans</i>	SL	FS	None	7	5.980	0.973
<i>B. contaminans</i>	SL	FS	AgF	0	3.581	1.049
<i>B. contaminans</i>	SL	FS	AgF	1	1.000	0.000
<i>B. contaminans</i>	SL	FS	AgF	1.25	1.000	0.000
<i>B. contaminans</i>	SL	FS	AgF	3	1.000	0.000
<i>B. contaminans</i>	SL	FS	AgF	3.25	1.000	0.000
<i>B. contaminans</i>	SL	FS	AgF	5	1.100	0.174
<i>B. contaminans</i>	SL	FS	AgF	5.25	1.000	0.000
<i>B. contaminans</i>	SL	FS	AgF	7	1.761	0.408
<i>B. contaminans</i>	SL	NP	None	0	3.733	0.499
<i>B. contaminans</i>	SL	NP	None	1	1.816	1.413
<i>B. contaminans</i>	SL	NP	None	3	3.728	0.657
<i>B. contaminans</i>	SL	NP	None	5	3.403	1.041
<i>B. contaminans</i>	SL	NP	None	7	3.476	2.053
<i>B. contaminans</i>	SL	NP	AgF	0	3.952	0.383
<i>B. contaminans</i>	SL	NP	AgF	1	1.000	0.000
<i>B. contaminans</i>	SL	NP	AgF	1.25	1.000	0.000
<i>B. contaminans</i>	SL	NP	AgF	3	1.000	0.000
<i>B. contaminans</i>	SL	NP	AgF	3.25	1.000	0.000
<i>B. contaminans</i>	SL	NP	AgF	5	1.000	0.000
<i>B. contaminans</i>	SL	NP	AgF	5.25	1.000	0.000
<i>B. contaminans</i>	SL	NP	AgF	7	1.000	0.000
<i>B. contaminans</i>	Uncoated	FS	None	0	4.236	0.126
<i>B. contaminans</i>	Uncoated	FS	None	1	6.383	0.476
<i>B. contaminans</i>	Uncoated	FS	None	3	4.350	0.113
<i>B. contaminans</i>	Uncoated	FS	None	5	3.642	0.233
<i>B. contaminans</i>	Uncoated	FS	None	7	4.745	0.635
<i>B. contaminans</i>	Uncoated	FS	AgF	0	4.193	0.272
<i>B. contaminans</i>	Uncoated	FS	AgF	1	5.548	1.752
<i>B. contaminans</i>	Uncoated	FS	AgF	1.25	1.000	0.000

Table E2. Continued.

Species	Modification	Media	Biocide	Day	Average density	Standard deviation
<i>B. contaminans</i>	Uncoated	FS	AgF	3	1.201	0.348
<i>B. contaminans</i>	Uncoated	FS	AgF	3.25	1.000	0.000
<i>B. contaminans</i>	Uncoated	FS	AgF	5	1.360	0.623
<i>B. contaminans</i>	Uncoated	FS	AgF	5.25	1.000	0.000
<i>B. contaminans</i>	Uncoated	FS	AgF	7	1.259	0.449
<i>B. contaminans</i>	Uncoated	NP	None	0	3.703	0.511
<i>B. contaminans</i>	Uncoated	NP	None	1	6.250	0.035
<i>B. contaminans</i>	Uncoated	NP	None	3	3.414	0.473
<i>B. contaminans</i>	Uncoated	NP	None	5	2.797	0.275
<i>B. contaminans</i>	Uncoated	NP	None	7	3.345	0.144
<i>B. contaminans</i>	Uncoated	NP	AgF	0	4.097	0.178
<i>B. contaminans</i>	Uncoated	NP	AgF	1	4.415	2.996
<i>B. contaminans</i>	Uncoated	NP	AgF	1.25	1.000	0.000
<i>B. contaminans</i>	Uncoated	NP	AgF	3	1.000	0.000
<i>B. contaminans</i>	Uncoated	NP	AgF	3.25	1.000	0.000
<i>B. contaminans</i>	Uncoated	NP	AgF	5	1.502	0.869
<i>B. contaminans</i>	Uncoated	NP	AgF	5.25	1.000	0.000
<i>B. contaminans</i>	Uncoated	NP	AgF	7	1.000	0.000
<i>C. mutabilis</i>	SL	FS	None	0	3.407	2.087
<i>C. mutabilis</i>	SL	FS	None	1	1.560	0.489
<i>C. mutabilis</i>	SL	FS	None	3	1.000	0.000
<i>C. mutabilis</i>	SL	FS	None	5	1.583	0.764
<i>C. mutabilis</i>	SL	FS	None	7	1.000	0.000
<i>C. mutabilis</i>	SL	FS	AgF	0	4.342	0.288
<i>C. mutabilis</i>	SL	FS	AgF	1	1.382	0.662
<i>C. mutabilis</i>	SL	FS	AgF	1.25	1.000	0.000
<i>C. mutabilis</i>	SL	FS	AgF	3	1.000	0.000
<i>C. mutabilis</i>	SL	FS	AgF	3.25	1.000	0.000
<i>C. mutabilis</i>	SL	FS	AgF	5	1.201	0.174
<i>C. mutabilis</i>	SL	FS	AgF	5.25	1.000	0.000
<i>C. mutabilis</i>	SL	FS	AgF	7	1.667	1.155
<i>C. mutabilis</i>	SL	NP	None	0	3.293	2.007
<i>C. mutabilis</i>	SL	NP	None	1	2.000	1.479
<i>C. mutabilis</i>	SL	NP	None	3	1.000	0.000
<i>C. mutabilis</i>	SL	NP	None	5	1.000	0.000
<i>C. mutabilis</i>	SL	NP	None	7	1.301	0.521

Table E2. Continued.

Species	Modification	Media	Biocide	Day	Average density	Standard deviation
<i>C. mutabilis</i>	SL	NP	AgF	0	4.272	0.220
<i>C. mutabilis</i>	SL	NP	AgF	1	1.842	0.470
<i>C. mutabilis</i>	SL	NP	AgF	1.25	1.000	0.000
<i>C. mutabilis</i>	SL	NP	AgF	3	1.100	0.174
<i>C. mutabilis</i>	SL	NP	AgF	3.25	1.000	0.000
<i>C. mutabilis</i>	SL	NP	AgF	5	1.000	0.000
<i>C. mutabilis</i>	SL	NP	AgF	5.25	1.000	0.000
<i>C. mutabilis</i>	SL	NP	AgF	7	1.000	0.000
<i>C. mutabilis</i>	Uncoated	FS	None	0	4.263	0.234
<i>C. mutabilis</i>	Uncoated	FS	None	1	3.293	0.875
<i>C. mutabilis</i>	Uncoated	FS	None	3	1.678	0.634
<i>C. mutabilis</i>	Uncoated	FS	None	5	1.460	0.557
<i>C. mutabilis</i>	Uncoated	FS	None	7	1.548	0.704
<i>C. mutabilis</i>	Uncoated	FS	AgF	0	4.113	0.043
<i>C. mutabilis</i>	Uncoated	FS	AgF	1	3.101	0.711
<i>C. mutabilis</i>	Uncoated	FS	AgF	1.25	1.000	0.000
<i>C. mutabilis</i>	Uncoated	FS	AgF	3	1.000	0.000
<i>C. mutabilis</i>	Uncoated	FS	AgF	3.25	1.000	0.000
<i>C. mutabilis</i>	Uncoated	FS	AgF	5	1.000	0.000
<i>C. mutabilis</i>	Uncoated	FS	AgF	5.25	1.000	0.000
<i>C. mutabilis</i>	Uncoated	FS	AgF	7	1.000	0.000
<i>C. mutabilis</i>	Uncoated	NP	None	0	4.312	0.234
<i>C. mutabilis</i>	Uncoated	NP	None	1	4.705	0.365
<i>C. mutabilis</i>	Uncoated	NP	None	3	1.151	0.213
<i>C. mutabilis</i>	Uncoated	NP	None	5	1.000	0.000
<i>C. mutabilis</i>	Uncoated	NP	None	7	1.000	0.000
<i>C. mutabilis</i>	Uncoated	NP	AgF	0	4.300	0.200
<i>C. mutabilis</i>	Uncoated	NP	AgF	1	2.428	1.019
<i>C. mutabilis</i>	Uncoated	NP	AgF	1.25	1.000	0.000
<i>C. mutabilis</i>	Uncoated	NP	AgF	3	1.000	0.000
<i>C. mutabilis</i>	Uncoated	NP	AgF	3.25	1.000	0.000
<i>C. mutabilis</i>	Uncoated	NP	AgF	5	1.360	0.623
<i>C. mutabilis</i>	Uncoated	NP	AgF	5.25	1.000	0.000
<i>C. mutabilis</i>	Uncoated	NP	AgF	7	1.000	0.000
<i>M. organophilum</i>	SL	FS	None	0	3.387	0.352
<i>M. organophilum</i>	SL	FS	None	1	2.372	0.413

Table E2. Continued.

Species	Modification	Media	Biocide	Day	Average density	Standard deviation
<i>M. organophilum</i>	SL	FS	None	3	1.000	0.000
<i>M. organophilum</i>	SL	FS	None	5	1.434	0.513
<i>M. organophilum</i>	SL	FS	None	7	1.401	0.695
<i>M. organophilum</i>	SL	FS	AgF	0	4.013	0.587
<i>M. organophilum</i>	SL	FS	AgF	1	2.227	1.417
<i>M. organophilum</i>	SL	FS	AgF	1.25	1.000	0.000
<i>M. organophilum</i>	SL	FS	AgF	3	1.000	0.000
<i>M. organophilum</i>	SL	FS	AgF	3.25	1.000	0.000
<i>M. organophilum</i>	SL	FS	AgF	5	1.382	0.662
<i>M. organophilum</i>	SL	FS	AgF	5.25	1.000	0.000
<i>M. organophilum</i>	SL	FS	AgF	7	1.434	0.751
<i>M. organophilum</i>	SL	NP	None	0	3.277	0.211
<i>M. organophilum</i>	SL	NP	None	1	3.081	1.383
<i>M. organophilum</i>	SL	NP	None	3	2.068	1.362
<i>M. organophilum</i>	SL	NP	None	5	2.609	0.268
<i>M. organophilum</i>	SL	NP	None	7	2.603	0.417
<i>M. organophilum</i>	SL	NP	AgF	0	3.697	0.269
<i>M. organophilum</i>	SL	NP	AgF	1	2.651	1.604
<i>M. organophilum</i>	SL	NP	AgF	1.25	1.000	0.000
<i>M. organophilum</i>	SL	NP	AgF	3	1.301	0.521
<i>M. organophilum</i>	SL	NP	AgF	3.25	1.000	0.000
<i>M. organophilum</i>	SL	NP	AgF	5	1.000	0.000
<i>M. organophilum</i>	SL	NP	AgF	5.25	1.259	0.449
<i>M. organophilum</i>	SL	NP	AgF	7	1.201	0.348
<i>M. organophilum</i>	Uncoated	FS	None	0	3.603	0.196
<i>M. organophilum</i>	Uncoated	FS	None	1	3.621	0.694
<i>M. organophilum</i>	Uncoated	FS	None	3	1.820	0.710
<i>M. organophilum</i>	Uncoated	FS	None	5	1.634	0.552
<i>M. organophilum</i>	Uncoated	FS	None	7	1.683	0.604
<i>M. organophilum</i>	Uncoated	FS	AgF	0	3.668	0.196
<i>M. organophilum</i>	Uncoated	FS	AgF	1	4.034	0.350
<i>M. organophilum</i>	Uncoated	FS	AgF	1.25	1.000	0.000
<i>M. organophilum</i>	Uncoated	FS	AgF	3	1.000	0.000
<i>M. organophilum</i>	Uncoated	FS	AgF	3.25	1.000	0.000
<i>M. organophilum</i>	Uncoated	FS	AgF	5	1.100	0.174
<i>M. organophilum</i>	Uncoated	FS	AgF	5.25	1.000	0.000

Table E2. Continued.

Species	Modification	Media	Biocide	Day	Average density	Standard deviation
<i>M. organophilum</i>	Uncoated	FS	AgF	7	1.000	0.000
<i>M. organophilum</i>	Uncoated	NP	None	0	3.554	0.069
<i>M. organophilum</i>	Uncoated	NP	None	1	4.362	0.476
<i>M. organophilum</i>	Uncoated	NP	None	3	2.799	0.645
<i>M. organophilum</i>	Uncoated	NP	None	5	2.190	0.583
<i>M. organophilum</i>	Uncoated	NP	None	7	2.009	1.001
<i>M. organophilum</i>	Uncoated	NP	AgF	0	3.722	0.586
<i>M. organophilum</i>	Uncoated	NP	AgF	1	3.228	2.018
<i>M. organophilum</i>	Uncoated	NP	AgF	1.25	1.000	0.000
<i>M. organophilum</i>	Uncoated	NP	AgF	3	1.000	0.000
<i>M. organophilum</i>	Uncoated	NP	AgF	3.25	1.000	0.000
<i>M. organophilum</i>	Uncoated	NP	AgF	5	1.000	0.000
<i>M. organophilum</i>	Uncoated	NP	AgF	5.25	1.000	0.000
<i>M. organophilum</i>	Uncoated	NP	AgF	7	1.000	0.000
<i>R. insidiosa</i>	SL	FS	None	0	4.368	0.119
<i>R. insidiosa</i>	SL	FS	None	1	1.510	0.884
<i>R. insidiosa</i>	SL	FS	None	3	3.201	2.687
<i>R. insidiosa</i>	SL	FS	None	5	5.609	1.233
<i>R. insidiosa</i>	SL	FS	None	7	6.106	1.529
<i>R. insidiosa</i>	SL	FS	AgF	0	4.178	1.155
<i>R. insidiosa</i>	SL	FS	AgF	1	1.000	0.000
<i>R. insidiosa</i>	SL	FS	AgF	1.25	1.000	0.000
<i>R. insidiosa</i>	SL	FS	AgF	3	1.259	0.449
<i>R. insidiosa</i>	SL	FS	AgF	3.25	1.000	0.000
<i>R. insidiosa</i>	SL	FS	AgF	5	1.634	0.552
<i>R. insidiosa</i>	SL	FS	AgF	5.25	1.534	0.925
<i>R. insidiosa</i>	SL	FS	AgF	7	2.416	1.115
<i>R. insidiosa</i>	SL	NP	None	0	4.452	0.301
<i>R. insidiosa</i>	SL	NP	None	1	1.301	0.301
<i>R. insidiosa</i>	SL	NP	None	3	3.972	0.827
<i>R. insidiosa</i>	SL	NP	None	5	2.844	0.035
<i>R. insidiosa</i>	SL	NP	None	7	3.740	1.231
<i>R. insidiosa</i>	SL	NP	AgF	0	3.925	1.164
<i>R. insidiosa</i>	SL	NP	AgF	1	1.000	0.000
<i>R. insidiosa</i>	SL	NP	AgF	1.25	1.000	0.000
<i>R. insidiosa</i>	SL	NP	AgF	3	1.301	0.521

Table E2. Continued.

Species	Modification	Media	Biocide	Day	Average density	Standard deviation
<i>R. insidiosa</i>	SL	NP	AgF	3.25	1.000	0.000
<i>R. insidiosa</i>	SL	NP	AgF	5	1.000	0.000
<i>R. insidiosa</i>	SL	NP	AgF	5.25	1.151	0.213
<i>R. insidiosa</i>	SL	NP	AgF	7	1.000	0.000
<i>R. insidiosa</i>	Uncoated	FS	None	0	4.270	0.138
<i>R. insidiosa</i>	Uncoated	FS	None	1	6.354	0.752
<i>R. insidiosa</i>	Uncoated	FS	None	3	7.270	0.358
<i>R. insidiosa</i>	Uncoated	FS	None	5	7.203	0.197
<i>R. insidiosa</i>	Uncoated	FS	None	7	7.335	0.309
<i>R. insidiosa</i>	Uncoated	FS	AgF	0	4.040	0.168
<i>R. insidiosa</i>	Uncoated	FS	AgF	1	6.899	0.366
<i>R. insidiosa</i>	Uncoated	FS	AgF	1.25	1.333	0.577
<i>R. insidiosa</i>	Uncoated	FS	AgF	3	3.640	0.912
<i>R. insidiosa</i>	Uncoated	FS	AgF	3.25	1.301	0.521
<i>R. insidiosa</i>	Uncoated	FS	AgF	5	3.924	1.047
<i>R. insidiosa</i>	Uncoated	FS	AgF	5.25	2.295	0.952
<i>R. insidiosa</i>	Uncoated	FS	AgF	7	3.104	0.345
<i>R. insidiosa</i>	Uncoated	NP	None	0	4.547	0.347
<i>R. insidiosa</i>	Uncoated	NP	None	1	5.846	0.932
<i>R. insidiosa</i>	Uncoated	NP	None	3	5.277	0.545
<i>R. insidiosa</i>	Uncoated	NP	None	5	5.406	0.462
<i>R. insidiosa</i>	Uncoated	NP	None	7	5.379	0.051
<i>R. insidiosa</i>	Uncoated	NP	AgF	0	4.431	0.841
<i>R. insidiosa</i>	Uncoated	NP	AgF	1	4.338	2.896
<i>R. insidiosa</i>	Uncoated	NP	AgF	1.25	1.000	0.000
<i>R. insidiosa</i>	Uncoated	NP	AgF	3	1.719	0.392
<i>R. insidiosa</i>	Uncoated	NP	AgF	3.25	1.000	0.000
<i>R. insidiosa</i>	Uncoated	NP	AgF	5	1.735	0.379
<i>R. insidiosa</i>	Uncoated	NP	AgF	5.25	1.000	0.000
<i>R. insidiosa</i>	Uncoated	NP	AgF	7	1.683	0.604
R2A	SL	FS	None	0	4.762	0.168
R2A	SL	FS	None	1	1.690	0.976
R2A	SL	FS	None	3	4.620	3.175
R2A	SL	FS	None	5	6.920	0.206
R2A	SL	FS	None	7	6.972	0.240
R2A	SL	FS	AgF	0	4.833	0.000

Table E2. Continued.

Species	Modification	Media	Biocide	Day	Average density	Standard deviation
R2A	SL	FS	AgF	1	2.187	1.802
R2A	SL	FS	AgF	1.25	1.100	0.174
R2A	SL	FS	AgF	3	1.301	0.521
R2A	SL	FS	AgF	3.25	1.000	0.000
R2A	SL	FS	AgF	5	2.344	0.368
R2A	SL	FS	AgF	5.25	1.482	0.836
R2A	SL	FS	AgF	7	3.653	0.829
R2A	SL	NP	None	0	4.681	0.000
R2A	SL	NP	None	1	2.793	1.553
R2A	SL	NP	None	3	4.538	0.244
R2A	SL	NP	None	5	4.081	0.127
R2A	SL	NP	None	7	4.740	0.889
R2A	SL	NP	AgF	0	4.643	0.028
R2A	SL	NP	AgF	1	2.088	1.631
R2A	SL	NP	AgF	1.25	1.000	0.000
R2A	SL	NP	AgF	3	1.401	0.695
R2A	SL	NP	AgF	3.25	1.000	0.000
R2A	SL	NP	AgF	5	1.000	0.000
R2A	SL	NP	AgF	5.25	1.540	0.337
R2A	SL	NP	AgF	7	1.301	0.301
R2A	Uncoated	FS	None	0	4.620	0.244
R2A	Uncoated	FS	None	1	6.793	0.255
R2A	Uncoated	FS	None	3	6.824	0.064
R2A	Uncoated	FS	None	5	7.226	0.218
R2A	Uncoated	FS	None	7	7.445	0.310
R2A	Uncoated	FS	AgF	0	4.544	0.018
R2A	Uncoated	FS	AgF	1	7.060	0.713
R2A	Uncoated	FS	AgF	1.25	1.259	0.449
R2A	Uncoated	FS	AgF	3	2.949	0.284
R2A	Uncoated	FS	AgF	3.25	1.401	0.460
R2A	Uncoated	FS	AgF	5	3.946	1.035
R2A	Uncoated	FS	AgF	5.25	1.837	1.198
R2A	Uncoated	FS	AgF	7	3.240	0.300
R2A	Uncoated	NP	None	0	4.698	0.049
R2A	Uncoated	NP	None	1	6.414	0.494
R2A	Uncoated	NP	None	3	5.425	0.520

Table E2. Continued.

Species	Modification	Media	Biocide	Day	Average density	Standard deviation
R2A	Uncoated	NP	None	5	5.371	0.413
R2A	Uncoated	NP	None	7	5.517	0.056
R2A	Uncoated	NP	AgF	0	4.556	0.000
R2A	Uncoated	NP	AgF	1	4.640	3.171
R2A	Uncoated	NP	AgF	1.25	1.000	0.000
R2A	Uncoated	NP	AgF	3	1.949	0.372
R2A	Uncoated	NP	AgF	3.25	1.100	0.174
R2A	Uncoated	NP	AgF	5	2.271	0.500
R2A	Uncoated	NP	AgF	5.25	1.100	0.174
R2A	Uncoated	NP	AgF	7	1.820	0.541

APPENDIX F

DATA IN SUPPORT OF CHAPTER 5

The data presented in this appendix were used to generate Figures 2 and 4 in Chapter 5. Data for R2A plates (general counts) are provided despite not being included in the figures. The data used to generate Figure 5 consists of over 500,000 entries and is included as a supplemental document.

Table F1. Data used to generate Figure 2 in Chapter 5. SL= Sher-Loxane® 800. Average and standard deviation are presented as $\log_{10}\text{CFU}/\text{cm}^2$.

Species	Surface modification	Other experiment details	Average	Standard deviation
<i>B. contaminans</i>	SL	Full strength medium, No biocide	6.184	0.427
<i>B. contaminans</i>	SL	No phosphorus medium, Silver fluoride	1.804	0.000
<i>B. contaminans</i>	Uncoated	Full strength medium, No biocide	5.688	0.432
<i>C. mutabilis</i>	SL	Full strength medium, No biocide	2.004	0.422
<i>C. mutabilis</i>	SL	No phosphorus medium, Silver fluoride	1.804	0.000
<i>C. mutabilis</i>	Uncoated	Full strength medium, No biocide	2.585	0.756
<i>M. organophilum</i>	SL	Full strength medium, No biocide	2.423	0.737
<i>M. organophilum</i>	SL	No phosphorus medium, Silver fluoride	1.804	0.000
<i>M. organophilum</i>	Uncoated	Full strength medium, No biocide	2.803	0.827
<i>R. insidiosa</i>	SL	Full strength medium, No biocide	6.118	0.426
<i>R. insidiosa</i>	SL	No phosphorus medium, Silver fluoride	1.804	0.000
<i>R. insidiosa</i>	Uncoated	Full strength medium, No biocide	6.378	0.487
R2A total cell count	SL	Full strength medium, No biocide	6.502	0.390
R2A total cell count	SL	No phosphorus medium, Silver fluoride	1.804	0.000
R2A total cell count	Uncoated	Full strength medium, No biocide	6.518	0.350

Table F2. Data used to generate Figure 4 in Chapter 5. SL= Sher-Loxane® 800. Average and standard deviation presented as log₁₀CFU/mL.

Species	Day	Surface modification	Other experimental information	Average	Standard deviation
<i>B. contaminans</i>	1	SL	Full strength medium, No biocide	4.450	0.484
<i>B. contaminans</i>	1	SL	No phosphorus medium, Silver fluoride	3.670	1.100
<i>B. contaminans</i>	1	Uncoated	Full strength medium, No biocide	4.588	0.157
<i>B. contaminans</i>	2	SL	Full strength medium, No biocide	5.115	0.826
<i>B. contaminans</i>	2	SL	No phosphorus medium, Silver fluoride	1.349	0.920
<i>B. contaminans</i>	2	Uncoated	Full strength medium, No biocide	5.351	0.561
<i>B. contaminans</i>	3	SL	Full strength medium, No biocide	5.392	0.383
<i>B. contaminans</i>	3	SL	No phosphorus medium, Silver fluoride	0.849	0.213
<i>B. contaminans</i>	3	Uncoated	Full strength medium, No biocide	4.998	0.104
<i>B. contaminans</i>	4	SL	Full strength medium, No biocide	4.886	0.501
<i>B. contaminans</i>	4	SL	No phosphorus medium, Silver fluoride	1.530	1.176
<i>B. contaminans</i>	4	Uncoated	Full strength medium, No biocide	4.462	0.021
<i>B. contaminans</i>	5	SL	Full strength medium, No biocide	5.715	0.129
<i>B. contaminans</i>	5	SL	No phosphorus medium, Silver fluoride	0.699	0.000
<i>B. contaminans</i>	5	Uncoated	Full strength medium, No biocide	4.544	0.408
<i>B. contaminans</i>	6	SL	Full strength medium, No biocide	4.832	0.036
<i>B. contaminans</i>	6	SL	No phosphorus medium, Silver fluoride	0.699	0.000
<i>B. contaminans</i>	6	Uncoated	Full strength medium, No biocide	4.515	0.431
<i>B. contaminans</i>	7	SL	Full strength medium, No biocide	5.071	0.088
<i>B. contaminans</i>	7	SL	No phosphorus medium, Silver fluoride	0.699	0.000

Table F2. Continued.

Species	Day	Surface modification	Other experimental information	Average	Standard deviation
<i>B. contaminans</i>	7	Uncoated	Full strength medium, No biocide	4.554	0.494
<i>B. contaminans</i>	7 RB	SL	Full strength medium, No biocide	5.089	0.262
<i>B. contaminans</i>	7 RB	SL	No phosphorus medium, Silver fluoride	0.699	0.000
<i>B. contaminans</i>	7 RB	Uncoated	Full strength medium, No biocide	4.564	0.084
<i>C. mutabilis</i>	1	SL	Full strength medium, No biocide	2.623	1.443
<i>C. mutabilis</i>	1	SL	No phosphorus medium, Silver fluoride	2.879	0.758
<i>C. mutabilis</i>	1	Uncoated	Full strength medium, No biocide	4.131	1.061
<i>C. mutabilis</i>	2	SL	Full strength medium, No biocide	1.489	1.117
<i>C. mutabilis</i>	2	SL	No phosphorus medium, Silver fluoride	0.699	0.000
<i>C. mutabilis</i>	2	Uncoated	Full strength medium, No biocide	3.924	0.396
<i>C. mutabilis</i>	3	SL	Full strength medium, No biocide	1.151	0.639
<i>C. mutabilis</i>	3	SL	No phosphorus medium, Silver fluoride	0.699	0.000
<i>C. mutabilis</i>	3	Uncoated	Full strength medium, No biocide	3.655	0.174
<i>C. mutabilis</i>	4	SL	Full strength medium, No biocide	1.870	0.242
<i>C. mutabilis</i>	4	SL	No phosphorus medium, Silver fluoride	0.699	0.000
<i>C. mutabilis</i>	4	Uncoated	Full strength medium, No biocide	3.152	0.843
<i>C. mutabilis</i>	5	SL	Full strength medium, No biocide	1.389	0.550
<i>C. mutabilis</i>	5	SL	No phosphorus medium, Silver fluoride	0.699	0.000
<i>C. mutabilis</i>	5	Uncoated	Full strength medium, No biocide	3.671	1.375
<i>C. mutabilis</i>	6	SL	Full strength medium, No biocide	0.699	0.000

Table F2. Continued.

Species	Day	Surface modification	Other experimental information	Average	Standard deviation
<i>C. mutabilis</i>	6	SL	No phosphorus medium, Silver fluoride	0.699	0.000
<i>C. mutabilis</i>	6	Uncoated	Full strength medium, No biocide	2.924	0.822
<i>C. mutabilis</i>	7	SL	Full strength medium, No biocide	1.151	0.639
<i>C. mutabilis</i>	7	SL	No phosphorus medium, Silver fluoride	0.699	0.000
<i>C. mutabilis</i>	7	Uncoated	Full strength medium, No biocide	3.492	0.549
<i>C. mutabilis</i>	7 RB	SL	Full strength medium, No biocide	0.699	0.000
<i>C. mutabilis</i>	7 RB	SL	No phosphorus medium, Silver fluoride	0.699	0.000
<i>C. mutabilis</i>	7 RB	Uncoated	Full strength medium, No biocide	3.266	0.561
<i>M. organophilum</i>	1	SL	Full strength medium, No biocide	3.841	0.337
<i>M. organophilum</i>	1	SL	No phosphorus medium, Silver fluoride	3.673	0.678
<i>M. organophilum</i>	1	Uncoated	Full strength medium, No biocide	3.626	0.744
<i>M. organophilum</i>	2	SL	Full strength medium, No biocide	3.502	0.278
<i>M. organophilum</i>	2	SL	No phosphorus medium, Silver fluoride	1.452	1.064
<i>M. organophilum</i>	2	Uncoated	Full strength medium, No biocide	2.878	0.065
<i>M. organophilum</i>	3	SL	Full strength medium, No biocide	2.994	1.044
<i>M. organophilum</i>	3	SL	No phosphorus medium, Silver fluoride	0.699	0.000
<i>M. organophilum</i>	3	Uncoated	Full strength medium, No biocide	2.545	0.287
<i>M. organophilum</i>	4	SL	Full strength medium, No biocide	2.812	1.367
<i>M. organophilum</i>	4	SL	No phosphorus medium, Silver fluoride	1.000	0.426
<i>M. organophilum</i>	4	Uncoated	Full strength medium, No biocide	2.211	0.612

Table F2. Continued.

Species	Day	Surface modification	Other experimental information	Average	Standard deviation
<i>M. organophilum</i>	5	SL	Full strength medium, No biocide	2.986	1.233
<i>M. organophilum</i>	5	SL	No phosphorus medium, Silver fluoride	0.699	0.000
<i>M. organophilum</i>	5	Uncoated	Full strength medium, No biocide	2.113	0.722
<i>M. organophilum</i>	6	SL	Full strength medium, No biocide	1.903	1.703
<i>M. organophilum</i>	6	SL	No phosphorus medium, Silver fluoride	0.699	0.000
<i>M. organophilum</i>	6	Uncoated	Full strength medium, No biocide	1.778	0.675
<i>M. organophilum</i>	7	SL	Full strength medium, No biocide	1.975	1.804
<i>M. organophilum</i>	7	SL	No phosphorus medium, Silver fluoride	0.699	0.000
<i>M. organophilum</i>	7	Uncoated	Full strength medium, No biocide	2.371	0.744
<i>M. organophilum</i>	7 RB	SL	Full strength medium, No biocide	1.935	1.747
<i>M. organophilum</i>	7 RB	SL	No phosphorus medium, Silver fluoride	0.699	0.000
<i>M. organophilum</i>	7 RB	Uncoated	Full strength medium, No biocide	2.134	0.615
<i>R. insidiosa</i>	1	SL	Full strength medium, No biocide	5.273	0.949
<i>R. insidiosa</i>	1	SL	No phosphorus medium, Silver fluoride	5.127	0.535
<i>R. insidiosa</i>	1	Uncoated	Full strength medium, No biocide	4.392	0.605
<i>R. insidiosa</i>	2	SL	Full strength medium, No biocide	5.801	0.765
<i>R. insidiosa</i>	2	SL	No phosphorus medium, Silver fluoride	2.475	2.511
<i>R. insidiosa</i>	2	Uncoated	Full strength medium, No biocide	5.325	0.078
<i>R. insidiosa</i>	3	SL	Full strength medium, No biocide	6.195	0.043
<i>R. insidiosa</i>	3	SL	No phosphorus medium, Silver fluoride	1.978	1.809

Table F2. Continued.

Species	Day	Surface modification	Other experimental information	Average	Standard deviation
<i>R. insidiosa</i>	3	Uncoated	Full strength medium, No biocide	5.672	0.130
<i>R. insidiosa</i>	4	SL	Full strength medium, No biocide	6.154	0.116
<i>R. insidiosa</i>	4	SL	No phosphorus medium, Silver fluoride	2.766	1.509
<i>R. insidiosa</i>	4	Uncoated	Full strength medium, No biocide	6.039	0.257
<i>R. insidiosa</i>	5	SL	Full strength medium, No biocide	6.887	0.134
<i>R. insidiosa</i>	5	SL	No phosphorus medium, Silver fluoride	2.411	0.255
<i>R. insidiosa</i>	5	Uncoated	Full strength medium, No biocide	6.307	0.153
<i>R. insidiosa</i>	6	SL	Full strength medium, No biocide	6.432	0.126
<i>R. insidiosa</i>	6	SL	No phosphorus medium, Silver fluoride	1.406	1.001
<i>R. insidiosa</i>	6	Uncoated	Full strength medium, No biocide	6.509	0.132
<i>R. insidiosa</i>	7	SL	Full strength medium, No biocide	6.909	0.165
<i>R. insidiosa</i>	7	SL	No phosphorus medium, Silver fluoride	0.699	0.000
<i>R. insidiosa</i>	7	Uncoated	Full strength medium, No biocide	6.491	0.269
<i>R. insidiosa</i>	7 RB	SL	Full strength medium, No biocide	6.845	0.000
<i>R. insidiosa</i>	7 RB	SL	No phosphorus medium, Silver fluoride	0.699	0.000
<i>R. insidiosa</i>	7 RB	Uncoated	Full strength medium, No biocide	6.152	0.280
R2A	1	SL	Full strength medium, No biocide	5.373	0.765
R2A	1	SL	No phosphorus medium, Silver fluoride	5.690	0.013
R2A	1	Uncoated	Full strength medium, No biocide	4.728	0.278
R2A	2	SL	Full strength medium, No biocide	5.863	1.014

Table F2. Continued.

Species	Day	Surface modification	Other experimental information	Average	Standard deviation
R2A	2	SL	No phosphorus medium, Silver fluoride	2.642	2.322
R2A	2	Uncoated	Full strength medium, No biocide	5.505	0.000
R2A	3	SL	Full strength medium, No biocide	6.261	0.044
R2A	3	SL	No phosphorus medium, Silver fluoride	1.976	1.806
R2A	3	Uncoated	Full strength medium, No biocide	5.899	0.131
R2A	4	SL	Full strength medium, No biocide	6.233	0.018
R2A	4	SL	No phosphorus medium, Silver fluoride	2.278	0.819
R2A	4	Uncoated	Full strength medium, No biocide	6.085	0.213
R2A	5	SL	Full strength medium, No biocide	6.859	0.135
R2A	5	SL	No phosphorus medium, Silver fluoride	2.507	0.179
R2A	5	Uncoated	Full strength medium, No biocide	6.320	0.085
R2A	6	SL	Full strength medium, No biocide	6.590	0.047
R2A	6	SL	No phosphorus medium, Silver fluoride	1.910	0.861
R2A	6	Uncoated	Full strength medium, No biocide	6.491	0.269
R2A	7	SL	Full strength medium, No biocide	7.016	0.047
R2A	7	SL	No phosphorus medium, Silver fluoride	1.199	0.707
R2A	7	Uncoated	Full strength medium, No biocide	6.646	0.281
R2A	7 RB	SL	Full strength medium, No biocide	6.930	0.086
R2A	7 RB	SL	No phosphorus medium, Silver fluoride	0.699	0.000
R2A	7 RB	Uncoated	Full strength medium, No biocide	6.250	0.184

APPENDIX G

SIMULATED MICROGRAVITY BIOFILM REACTOR
OPERATING PROCEDURE

Required materials:

- SMBR standard parts: endcaps (with O-rings and polycarbonate reactor walls installed), coupon ring, 2 square shaped O-rings, 8 coupon gaskets, 8 coupon O-rings, 8 CDC coupons, 8 coupon holders, 4 threaded rods, 24 thumb screws (Figure G1)
- C-FLEX tubing from MasterFlex with required connectors (connectors change slightly depending on O₂ measurements)
- At least one flow break in the influent (upstream of O₂ measurements if applicable; Figure G2)
- At least one y-connector in the effluent line (downstream of O₂ measurements if applicable; Figure G3)
- In-line luer injection port, individually sterilized (for operation with biocide dosing; Figure G4)
- Enough 5 mL and 10 mL syringes for number of effluent sampling days
- Reactor frame
- Motor
- Timing belt for motor
- Pump (ideally one that can be easily adjusted to different flow rates)
- Carboy filled with sterile influent media
- Empty carboy for effluent waste
- Sterile petri dishes
- Sterile cotton/ rayon swabs
- Forceps
- Scissors
- Ethanol reservoir, lighter, etc. For flame sterilizing

- Serological pipette (5 mL)
- Extra CDC coupons
- Sample tubes with 10 mL 1X PBS and 10 uL Tween 20

Reactor overview

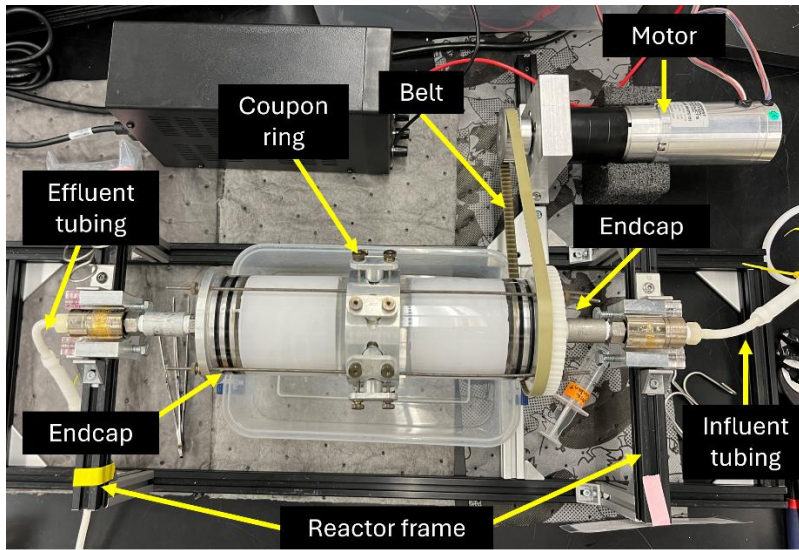


Figure G1. Overview of reactor setup on frame. The reactor in this image features polycarbonate tubing that has been coated with an opaque white coating.

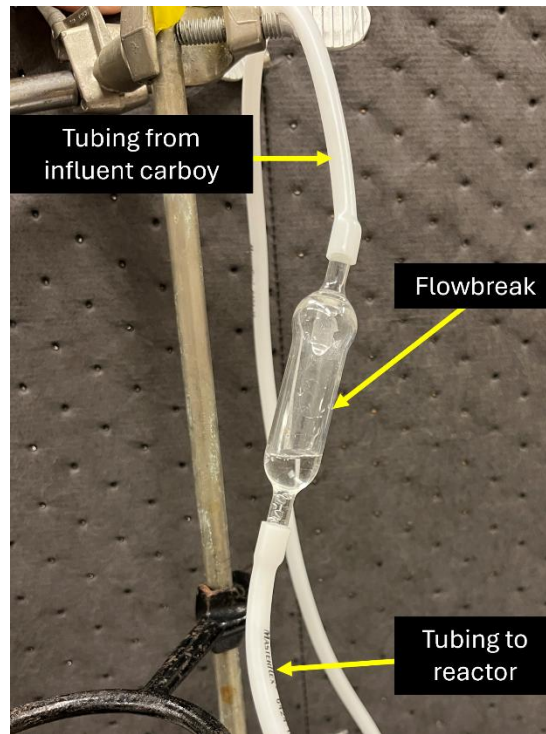


Figure G2. Flow break in influent tubing.

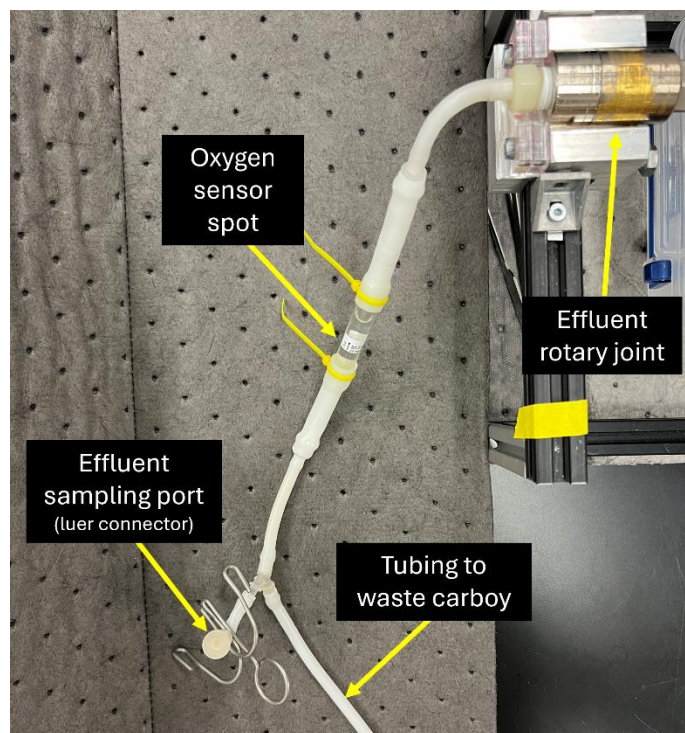


Figure G3. Effluent tubing from reactor with a sensor spot for oxygen measurements and a y-connector for effluent sampling.

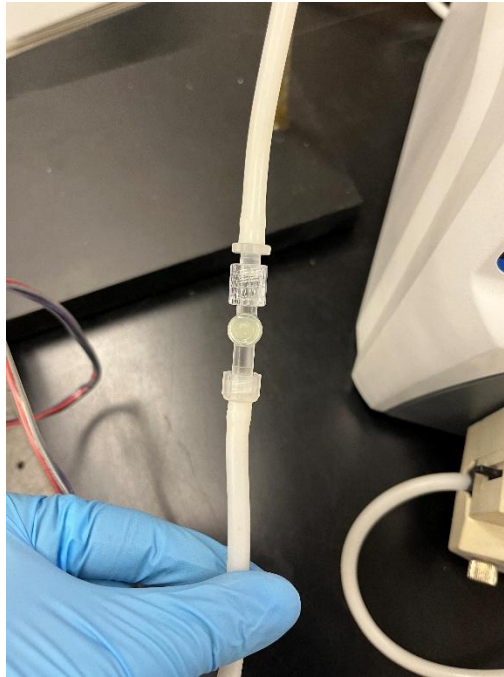


Figure G4. In-line luer injection port used for biocide dosing. Positioned several inches upstream of influent oxygen sensor spot.

Organism preparation:

If using the “Fab 4” or “Fab 5,” follow the same approach that is used for CDC reactors (wash dense cell cultures 3x in 0.4% NaCl; Chapter 4). For OD₆₀₀ target, use 0.083 for bacteria and 2.25 for fungus. 100 µL of each washed culture will be used to inoculate the reactor. The method must be optimized for other organisms.

Reactor preparation:

All parts of the standard reactor setup can be autoclaved.

For the coupon ring, each slot will require 1 coupon gasket (clear silicone ring), 1 coupon (of desired material type), 1 coupon O-ring, 1 coupon holder, and 2 thumb screws (Figure G5). Grab a coupon gasket with a pair of forceps, drag it through a reservoir of water, and place it in

the coupon slot. Using forceps, grab a CDC coupon and center it in the coupon slot. Place a coupon O-ring on top of the coupon. Check to make sure everything is centered and place a coupon holder on top and secure it with 2 thumb screws. Repeat until all the coupon slots have been filled.

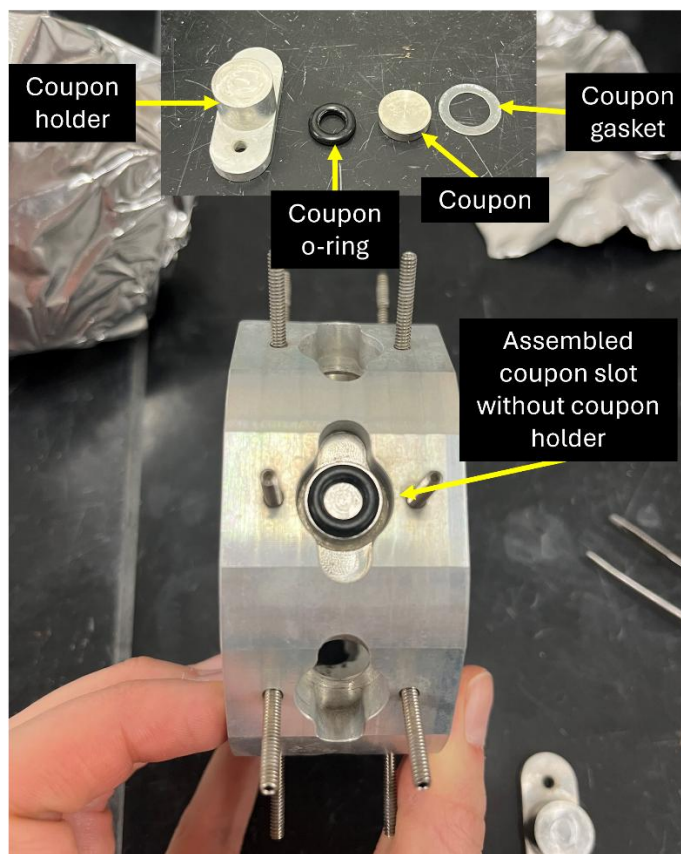


Figure G5. Disassembled and mostly assembled coupon holder.

Prior to autoclaving the reactor, slightly loosen the thumb screws for several of the coupon slots and all the thumb screws on the threaded rods that hold the reactor in place (i.e., the thumb screws on each threaded rod on one end of the reactor). Also be sure to foil any exposed tubing ends.

After removal from the autoclave, allow the reactor to cool. Tighten the thumb screws holding the coupons in place, tighten the thumb screws on the long threaded rods, and tighten the plastic connectors that connect the tubing into the rotatory joint (DO NOT FORGET THIS!). When placing it on the frame, make sure to loop the belt around the reactor before connecting the tubing to the influent carboy. Connect the tubing lines to the corresponding locations (effluent/influent carboys). Ensure that the effluent sampling line (short piece of tubing on effluent y-connector) is clamped. No other tubing should be clamped.

If evaluating coated surfaces, they cannot be autoclaved (coated coupons and coated polycarbonate tubing). The reactor must be autoclaved in a disassembled state. The best way to do this is to have the ends of everything foiled and placed in an autoclave tray together (Figure G6), with the tray foiled over before autoclaving. The coupon ring should be partially assembled with the holder secured into the ports with thumb screws. Place o-rings and gaskets into separate containers with foil covering in an autoclave tray.



Figure G6. Autoclave tray with reactor parts foiled for sterilization. Before autoclaving, a sheet of foil should be placed over the entire tray.

After autoclaving, it is easiest to reassemble everything if the reactor is still warm (about 30-45 min after removal from the autoclave). This must all take place in a biosafety cabinet. Start by sterilizing the polycarbonate with an alcohol prep pad soaked in 99% isopropanol (use tweezers to move it around and wet the entire inner surface of the tube) and then seat the polycarbonate onto the end cap. Repeat for the second endcap. Then connect the endcaps with the coupon ring and secure with threaded rods and thumb screws. Once securely connected, the reactor can be placed on its side for coupon holder assembly. Coupon holder assembly is the same as for other applications, but coupons must be sterilized before placement into the coupon ring. Submersion into 99% isopropanol for 30 s followed by 30 s of air drying should be sufficient for coupon sterilization. The gaskets do not need to be wetted as the remaining alcohol from the coupons can help to wet the gaskets. Once the reactor is assembled, it can be removed

from the biosafety cabinet and placed on the reactor frame where tubing can be attached (see above).

Filling and inoculating the reactor:

Begin pumping media into the reactor with it in the horizontal position (on the frame; Figure G1). The flow rate can be as high as 50 mL/min. Between 35-40 mL/min avoids pressure issues but also gets it done in a reasonable amount of time. As the media goes into the reactor, manually rotate the reactor to check for any leaks coming out of the coupon ring. If there are leaks, rotate the leaky coupon slot to the top position, take apart the holder and ensure everything is properly centered using ethanol/flame-sterilized forceps. If leaks are coming from the influent plastic connector, make sure that it is tight—a wrench may be helpful. If there are leaks coming from anywhere else in the reactor, it is likely assembled improperly and will be difficult to address while maintaining sterility. For this reason, it may be smart to assemble the reactor, fill it partway to check for leaks from the square coupon ring O-rings or the endcaps, then empty the reactor, and autoclave it (only works if all materials can be autoclaved).

Allow the reactor to fill to the halfway mark and pause the pump. Disassemble the top coupon holder and using sterile forceps, place the coupon in a sterile petri dish. Inoculate the reactor through the open coupon slot. Replace the coupon and reassemble the coupon holder. Rotate the reactor to make sure the holder was reassembled properly (no leaks). Then, remove the reactor from the frame and position it upright into a ring stand (Figure G7), ensuring that the influent line is at the bottom and the effluent is at the top so all bubbles can leave the reactor through the effluent. Use a clamp to hold the rotary joint on the influent line for increased stability. Make sure that the flow break is oriented properly and that there are no kinks in the

influent or effluent tubing. Then, resume the pump to continue filling the reactor. Flick the influent tubing several times throughout the filling to dislodge any potential bubbles. Near the end of the filling, remove the reactor from the ring stand and hold it vertically, turn down the speed of the pump, and shake the reactor a bit to dislodge any bubbles held in the effluent end cap. Once satisfied that there are no bubbles, turn off the pump and position the reactor back onto the frame. If bubbles appear, bring it to the vertical position and restart flow while shaking the reactor a bit to dislodge them. If bubbles are small ($\frac{1}{4}$ " or less), they will probably resolve themselves. The goal is to minimize the volume of excess media pumped out of the reactor during this process since the inoculum has already been incorporated. Make sure the reactor is not leaking and has no (or very small) bubbles before beginning rotation. Make sure the belt is positioned on the reactor between the right endcap and the coupon ring. Loop the belt first over the motor and then over the reactor. Begin rotation.



Figure G7. Vertical positioning of SMBR in a ring stand for second phase of filling.

The influent and effluent lines do not need to be clamped during batch operation, but can be done according to preference (the pressure is held by the clamp in the peristaltic pump).

Continuous flow operation

If the influent and effluent lines were clamped, be sure to unclamp them before starting continuous flow. Make sure the effluent waste carboy is positioned on the floor to reduce additional pressure in the system. Maintain the clamp on the effluent sampling tubing and begin continuous flow at the desired flow rate (5.4 mL/min leads to a residence time similar to a flow rate of 2 mL/min in a CDC reactor, but this depends on the length of polycarbonate tubing (4.5”) and volume of the SMBR).

Periodic flow operation

If it is determined that continuous flow is not generating sufficient biofilm, periodic flow can be pursued. Using a 3 h continuous flow phase at 4.16 mL/min (residence time of 160 min) and 21 h batch phase allows introduction of sufficient nutrients into the reactor using the defined consortium and microbial ersatz MTN medium.

Effluent sampling

If effluent planktonic samples are desired, allow flow to continue for ~10-15 minutes and prep a 5 mL luer lock syringe by breaking the seal (pull back on the plunger a bit to hear/feel the seal break). Then place the syringe onto the luer lock connector in the y-split. Move the clamp from this tubing to the tubing just downstream of the y connector. This will force the effluent to flow into the syringe. As the syringe is filling, break the seal on a 10 mL syringe in the same way. When the 5 mL syringe is mostly full, move the clamp back onto the tubing for the effluent sample. Remove the 5 mL syringe and replace it with the empty 10 mL syringe. About 20 minutes into continuous flow, move the clamp back to the tubing connected to the waste carboy

to fill the syringe with the effluent sample. Once the syringe has filled enough, move the clamp back to that tubing, remove the syringe, and replace the cap.

The goal of the 5 mL syringe is to collect any effluent cells/additional biofilm growing in the y-connector. The purpose of waiting until 20 minutes into continuous flow to collect effluent in the 10 mL syringe is to make sure the cells being collected are from the reactor, not just from the effluent tubing/plumbing.

With the effluent sample, some of it can be fixed for FlowCam, and the rest can be diluted and plated for CFU counts.

Biocide dosing:

If biocide dosing is incorporated into the experiment, the biocide should be dosed with enough time remaining in continuous flow so that the biocide reaches the bulk of the reactor before flow is terminated. In reactors with 4" polycarbonate tubing walls, dosing occurred with approx. 20 minutes remaining in continuous flow. Biocide must be prepared to the desired concentration and filter-sterilized. The membrane on the inline luer injection port (Figure 4) must be sterilized with a 70% alcohol preparation wipe and the biocide injected through the membrane with a sterile syringe and needle. Extra biocide should be pulled into the syringe, and bubbles should be carefully expelled from the syringe prior to injection. For doses of 3 mL, taking up 4 mL into the syringe is a good idea so that the whole volume does not need to be injected through the membrane. This is all with the goal of reducing the potential of bubble introduction into the tubing and reactor.

Coupon sampling:

About 1 hour prior to coupon sampling, try to slowly turn down the rotation speed of the motor—this is REALLY hard to do. Ideally it will happen gradually over the course of an hour. About 20 minutes prior to biofilm sampling, start continuous flow again to go through effluent sampling procedure. Turn continuous flow and reactor rotation completely off. Remove the belt from the geared pulley (white part of the reactor), but keep it looped over the motor to help stabilize the reactor. Clamp the effluent line.

The first coupon to be sampled is usually stained for microscopy. Open the coupon holder and, using sterile forceps, remove the coupon and place it into a vessel for staining (sterile 24-well plates work well). Make sure the side of the coupon exposed to the reactor is facing up for staining. Using a serological pipette, pull a bulk fluid sample for planktonic analysis (FlowCam/CFU). Put an extra coupon into the coupon slot and reassemble the coupon holder.

Gently rotate the reactor so a new coupon is at the uppermost spot. Disassemble the coupon assembly and grab the coupon with sterile forceps. Place it in a sterile petri dish with the side exposed to biofilm facing up—a second set of sterile forceps will be helpful. Sometimes the gasket comes along with the coupon, just use one set of forceps to hold the coupon and another to peel off the gasket and put it back into the coupon slot. Take care to do this gently, and without touching the surface of the coupon. Then, use a set of forceps to hold the coupon in one hand and with the other hand, scrape off the biofilm with a sterile cotton/ rayon tipped swab. Swab the entire surface very thoroughly, which may require adjusting where the forceps are holding the coupon. Once the surface has been swabbed, flame sterilize a pair of scissors and cut the tip of the swab into a sample tube containing 10 mL sterile 1 X PBS and 10 uL Tween 20. Discard the

shaft of the swab. Place the scraped coupon back into its slot in the reactor and reassemble the coupon holder.

Repeat the biofilm sampling procedure until the desired number of coupons have been sampled.

Disaggregate the biofilm using a series of vortexing and sonicating for 30 s each, to total 2.5 min (v/s/v/s/v).

Reactor clean-up:

The reactor can be autoclaved with the influent and effluent lines clamped, but C-FLEX tubing will be sealed together/melted by a clamp in the autoclave, so clamp as close to the reactor as possible.

Another option is to remove the clamp from the effluent tubing (keep a clamp on the y-connector for effluent sampling) and remove the tubing from the pump. Drain at least half of the volume of media into the effluent waste carboy. Remove the tubing from the carboys and foil the ends (same with the carboy tubing). Place the reactor into a large autoclave tray. Loosen the screws on several of the coupon holder slots. Autoclave the waste accordingly.

APPENDIX H

ADDITIONAL PUBLICATIONS FROM GRADUATE SCHOOL

- Fernandes-Martins, M. C., Keller, L. M., Munro-Ehrlich, M., Zimlich, K. R., Mettler, M. K., England, A. M., Clare, R., Surya, K., Shock, E. L., Colman, D. R., & Boyd, E. S. (2021). Ecological Dichotomies Arise in Microbial Communities Due to Mixing of Deep Hydrothermal Waters and Atmospheric Gas in a Circumneutral Hot Spring. *Applied and Environmental Microbiology*, 87(23), e01598-01521.
<https://doi.org/doi:10.1128/AEM.01598-21>
- Mettler, M. K., Goemann, H. M., Mueller, R. C., Vanegas, O. A., Lopez, G., Singh, N., Venkateswaran, K., & Peyton, B. M. (2023). Development of Martian saline seep models and their implications for planetary protection. *Biofilm*, 5, 100127.
<https://doi.org/https://doi.org/10.1016/j.bioflm.2023.100127>

REFERENCES CITED

- Adam, N. M. (2009). *Compatibility study of silver biocide in drinking water with candidate metals for the crew exploration vehicle potable water system* (0148-7191).
- Afonso, T. B., Simões, L. C., & Lima, N. (2021). Occurrence of filamentous fungi in drinking water: their role on fungal-bacterial biofilm formation. *Research in Microbiology*, 172(1), 103791. <https://doi.org/https://doi.org/10.1016/j.resmic.2020.11.002>
- Agudelo-Vera, C., Avvedimento, S., Boxall, J., Creaco, E., de Kater, H., Di Nardo, A., Djukic, A., Douterelo, I., Fish, K. E., Iglesias Rey, P. L., Jacimovic, N., Jacobs, H. E., Kapelan, Z., Martinez Solano, J., Montoya Pachongo, C., Piller, O., Quintiliani, C., Ručka, J., Tuhovčák, L., & Blokker, M. (2020). Drinking Water Temperature around the Globe: Understanding, Policies, Challenges and Opportunities. *Water*, 12(4), 1049. <https://www.mdpi.com/2073-4441/12/4/1049>
- An, R., & Lee, J. A. (2022). CAMDLES: CFD-DEM Simulation of Microbial Communities in Spaceflight and Artificial Microgravity. *Life (Basel)*, 12(5). <https://doi.org/10.3390/life12050660>
- Aparna, M. S., & Yadav, S. (2008). Biofilms: microbes and disease. *Brazilian Journal of Infectious Diseases*, 12, 526-530. <https://doi.org/10.1590/S1413-86702008000600016>
- ASTM International. (2018). ASTM E3161-18, Standard Practice for Preparing *Pseudomonas aeruginosa* or *Staphylococcus aureus* Biofilm using the CDC Biofilm Reactor. In. West Conshohoken, PA.
- ASTM International. (2020). ASTM E2647-20, Standard Test Method for Quantification of *Pseudomonas aeruginosa* Biofilm Grown Using Drip Flow Biofilm Reactor with Low Shear and Continuous Flow. In (Vol. ASTM E2647-20).
- ASTM International. (2021). ASTM E3161-21, Standard Practice for Preparing *Pseudomonas aeruginosa* or *Staphylococcus aureus* Biofilm using the CDC Biofilm Reactor. In. West Conshohoken, PA.
- ASTM International. (2023). ASTM E2799-22, Standard Test Method for Testing Disinfectant Efficacy against *Pseudomonas aeruginosa* Biofilm using the MBEC Assay. In.
- Atlas, R. M. (2010). *Handbook of Microbiological Media, Fourth Edition*. Taylor & Francis. <https://books.google.com/books?id=Pj5FAQAAIAAJ>
- Azim, N., Diaz, A., Li, W., Calle, L. M., Irwin, T., & Callahan, M. (2020). *Literature Review of Disinfection Techniques for Water Treatment* International Conference on Environmental Systems,

- Balaure, P. C., & Grumezescu, A. M. (2020a). Recent Advances in Surface Nanoengineering for Biofilm Prevention and Control. Part I: Molecular Basis of Biofilm Recalcitrance. Passive Anti-Biofouling Nanocoatings. *Nanomaterials*, 10(6), 1230. <https://www.mdpi.com/2079-4991/10/6/1230>
- Balaure, P. C., & Grumezescu, A. M. (2020b). Recent Advances in Surface Nanoengineering for Biofilm Prevention and Control. Part II: Active, Combined Active and Passive, and Smart Bacteria-Responsive Antibiofilm Nanocoatings. *Nanomaterials*, 10(8), 1527. <https://www.mdpi.com/2079-4991/10/8/1527>
- Banerjee, P., Singh, M., & Sharma, V. (2015). Biofilm formation: a comprehensive review. *Int J Pharm Res Health Sci*, 3(2), 556-560.
- Baxa, D., Shetron-Rama, L., Golembieski, M., Golembieski, M., Jain, S., Gordon, M., & Zervos, M. (2011). In vitro evaluation of a novel process for reducing bacterial contamination of environmental surfaces. *American Journal of Infection Control*, 39(6), 483-487. <https://doi.org/https://doi.org/10.1016/j.ajic.2010.10.015>
- Beech, I. B., & Sunner, J. (2004). Biocorrosion: towards understanding interactions between biofilms and metals. *Current Opinion in Biotechnology*, 15(3), 181-186. <https://doi.org/https://doi.org/10.1016/j.copbio.2004.05.001>
- Begley, C. M., & Kleis, S. J. (2000). The fluid dynamic and shear environment in the NASA/JSC rotating-wall perfused-vessel bioreactor. *Biotechnol Bioeng*, 70(1), 32-40. [https://doi.org/10.1002/1097-0290\(20001005\)70:1<32::aid-bit5>3.0.co;2-v](https://doi.org/10.1002/1097-0290(20001005)70:1<32::aid-bit5>3.0.co;2-v)
- Beitle, E., McCormick, M., McCall, M., Sandvik, E. L., Justiniano, Y.-A. V., & Williamson, J. (2024). Ground Test Validations for Uncrewed Dormancy Preparation and Recovery. 53rd International Conference on Environmental Systems (ICES),
- Beringer, D. M., Steele, J. W., & Nalette, T. A. (2014). Long-term storage of potable water in metallic vessels. In: Google Patents.
- Bixler, G. D., & Bhushan, B. (2012). Biofouling: lessons from nature. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences*, 370(1967), 2381-2417. <https://doi.org/doi:10.1098/rsta.2011.0502>
- Bouhrour, N., Nibbering, P. H., & Bendali, F. (2024). Medical Device-Associated Biofilm Infections and Multidrug-Resistant Pathogens. *Pathogens*, 13(5).
- Bruce, R. J., Ott, C. M., Skuratov, V. M., & Pierson, D. L. (2005). Microbial surveillance of potable water sources of the International Space Station. *SAE Transactions*, 283-292.
- Bzducha-Wróbel, A., Błażej, S., & Tkacz, K. (2012). Cell wall structure of selected yeast species as a factor of magnesium binding ability. *European Food Research and Technology*, 235(2), 355-366. <https://doi.org/10.1007/s00217-012-1761-4>

- Callahan, M. R., Adam, N. M., Roberts, M. S., Garland, J. L., Sager, J. C., & Pickering, K. D. (2007). Assessment of silver based disinfection technology for CEV and future US spacecraft. *SAE Transactions*, 481-491.
- Cámara, M., Green, W., MacPhee, C. E., Rakowska, P. D., Raval, R., Richardson, M. C., Slater-Jefferies, J., Steventon, K., & Webb, J. S. (2022). Economic significance of biofilms: a multidisciplinary and cross-sectoral challenge. *NPJ Biofilms Microbiomes*, 8(1), 42. <https://doi.org/10.1038/s41522-022-00306-y>
- Carrillo-Reyes, J., Cortés-Carmona, M. A., Bárcenas-Ruiz, C. D., & Razo-Flores, E. (2016). Cell wash-out enrichment increases the stability and performance of biohydrogen producing packed-bed reactors and the community transition along the operation time. *Renewable Energy*, 97, 266-273. <https://doi.org/https://doi.org/10.1016/j.renene.2016.05.082>
- Carter, D. L. (2010). Status of the Regenerative ECLSS Water Recovery System. International Conference of Environmental Systems, Barcelona, Spain.
- Castro, C. L., Velez-Justiniano, Y.-A., Stahl-Rommel, S., Nguyen, H. N., Almengor, A., Dunbar, B., McLean, R. J. C., Sysoeva, T. A., & Castro-Wallace, S. L. (2023). Genome Sequences of Bacteria Isolated from the International Space Station Water Systems. *Microbiology Resource Announcements*, 12(7), e00158-00123. <https://doi.org/doi:10.1128/mra.00158-23>
- Characklis, W. G., & Cooksey, K. E. (1983). Biofilms and Microbial Fouling. In A. I. Laskin (Ed.), *Advances in Applied Microbiology* (Vol. 29, pp. 93-138). Academic Press. [https://doi.org/https://doi.org/10.1016/S0065-2164\(08\)70355-1](https://doi.org/https://doi.org/10.1016/S0065-2164(08)70355-1)
- Chen, M., Yu, Q., & Sun, H. (2013). Novel Strategies for the Prevention and Treatment of Biofilm Related Infections. *International Journal of Molecular Sciences*, 14(9), 18488-18501. <https://www.mdpi.com/1422-0067/14/9/18488>
- Cheng, G., Zhang, Z., Chen, S., Bryers, J. D., & Jiang, S. (2007). Inhibition of bacterial adhesion and biofilm formation on zwitterionic surfaces. *Biomaterials*, 28(29), 4192-4199. <https://doi.org/https://doi.org/10.1016/j.biomaterials.2007.05.041>
- Choi, Y.-S., Shim, J.-J., & Kim, J.-G. (2005). Effects of Cr, Cu, Ni and Ca on the corrosion behavior of low carbon steel in synthetic tap water. *Journal of Alloys and Compounds*, 391(1), 162-169. <https://doi.org/https://doi.org/10.1016/j.jallcom.2004.07.081>
- Cincotta, C., Blokker, M., Bragalli, C., & Kapelan, Z. (2024). Spatial Analysis of Water Temperature in a Drinking Water Distribution System for Climate Change Adaptation. *Engineering Proceedings*, 69(1), 127. <https://www.mdpi.com/2673-4591/69/1/127>
- Cloutier, M., Mantovani, D., & Rosei, F. (2015). Antibacterial Coatings: Challenges, Perspectives, and Opportunities. *Trends in Biotechnology*, 33(11), 637-652. <https://doi.org/10.1016/j.tibtech.2015.09.002>

- Crabbé, A., Schurr, M. J., Monsieurs, P., Morici, L., Schurr, J., Wilson, J. W., Ott, C. M., Tsaprailis, G., Pierson, D. L., Stefanyshyn-Piper, H., & Nickerson, C. A. (2011). Transcriptional and Proteomic Responses of *Pseudomonas aeruginosa* PAO1 to Spaceflight Conditions Involve Hfq Regulation and Reveal a Role for Oxygen. *Applied and Environmental Microbiology*, 77(4), 1221-1230. <https://doi.org/10.1128/aem.01582-10>
- Croll, S. G. (2020). Surface roughness profile and its effect on coating adhesion and corrosion protection: A review. *Progress in Organic Coatings*, 148, 105847. <https://doi.org/https://doi.org/10.1016/j.porgcoat.2020.105847>
- Danko, D., Bezdan, D., Afshinnkoo, E., Ahsanuddin, S., Bhattacharya, C., Butler, D. J., Chng, K. R., De Filippis, F., Hecht, J., & Kahles, A. (2019). Global genetic cartography of urban metagenomes and anti-microbial resistance. *bioRxiv*, 724526.
- Daudu, R., Parker Ceth, W., Singh Nitin, K., Wood Jason, M., Debieu, M., O'Hara Niamh, B., Mason Christopher, E., Venkateswaran, K., & Stajich Jason, E. Draft Genome Sequences of *Rhodotorula mucilaginosa* Strains Isolated from the International Space Station. *Microbiology Resource Announcements*, 9(31), e00570-00520. <https://doi.org/10.1128/MRA.00570-20>
- Demir, B., Taylor, A., Broughton, R. M., Huang, T. S., Bozack, M. J., & Worley, S. D. (2022). N-halamine surface coating for mitigation of biofilm and microbial contamination in water systems for space travel. *Biofilm*, 4, 100076. <https://doi.org/https://doi.org/10.1016/j.biofilm.2022.100076>
- Di Pippo, F., Di Gregorio, L., Congestri, R., Tandoi, V., & Rossetti, S. (2018). Biofilm growth and control in cooling water industrial systems. *FEMS Microbiology Ecology*, 94(5). <https://doi.org/10.1093/femsec/fiy044>
- Diaz, A., Li, W., Irwin, T., & Calle, L. (2021). An Updated Modeling Study on Nutrient Deprivation as a Biofilm Mitigation Strategy for Long Term Space Missions.
- Donlan, R. M. (2001). Biofilm Formation: A Clinically Relevant Microbiological Process. *Clinical Infectious Diseases*, 33(8), 1387-1392. <https://doi.org/10.1086/322972>
- Donlan, R. M. (2002). Biofilms: microbial life on surfaces. *Emerging infectious diseases*, 8(9), 881-890. <https://doi.org/10.3201/eid0809.020063>
- Dundar Arisoy, F., Kolewe, K. W., Homyak, B., Kurtz, I. S., Schiffman, J. D., & Watkins, J. J. (2018). Bioinspired Photocatalytic Shark-Skin Surfaces with Antibacterial and Antifouling Activity via Nanoimprint Lithography. *ACS Applied Materials & Interfaces*, 10(23), 20055-20063. <https://doi.org/10.1021/acsami.8b05066>
- Elasri, M. O., & Miller, R. V. (1999). Study of the response of a biofilm bacterial community to UV radiation. *Applied and Environmental Microbiology*, 65(5), 2025-2031.

- Ellis, D., Gosai, J., Emrick, C., Heintz, R., Romans, L., Gordon, D., Lu, S. E., Austin, F., & Smith, L. (2012). Occidiofungin's chemical stability and in vitro potency against *Candida* species. *Antimicrob Agents Chemother*, 56(2), 765-769.
<https://doi.org/10.1128/aac.05231-11>
- Elser, J. J. (2012). Phosphorus: a limiting nutrient for humanity? *Current Opinion in Biotechnology*, 23(6), 833-838.
<https://doi.org/https://doi.org/10.1016/j.copbio.2012.03.001>
- Environmental Protection Agency. Protocol for Residual Self-Sanitizing Activity of Dried Chemical Residues on Hard, Non-Porous Surfaces.
- Environmental Protection Agency. (2020). Interim Method for Evaluating the Efficacy of Antimicrobial Surface Coatings. In.
- Environmental Protection Agency. (2023). *Contaminant Candidate List (CCL) and Regulatory Determination*. <https://www.epa.gov/ccl/ccl-5-microbial-contaminants>
- Environmental Protection Agency. (2024). National Primary Drinking Water Regulations. In.
- Esbelin, J., Santos, T., & Hébraud, M. (2018). Desiccation: An environmental and food industry stress that bacteria commonly face. *Food Microbiology*, 69, 82-88.
<https://doi.org/https://doi.org/10.1016/j.fm.2017.07.017>
- European Space Agency. *Gateway*.
https://www.esa.int/Science_Exploration/Human_and_Robotic_Exploration/Exploration/Gateway
- Feng, Y., Chen, J., Liu, Q., Chen, N., Zou, S., Yao, Q., Lu, L., & Xiao, K. (2024). Low-shear modeled microgravity accelerated the degradation of passive films of aluminium alloy by *Bacillus cereus* through increased extracellular polymeric substances. *Applied Surface Science*, 655, 159618. <https://doi.org/https://doi.org/10.1016/j.apsusc.2024.159618>
- Fernandes-Martins, M. C., Keller, L. M., Munro-Ehrlich, M., Zimlich, K. R., Mettler, M. K., England, A. M., Clare, R., Surya, K., Shock, E. L., Colman, D. R., & Boyd, E. S. (2021). Ecological Dichotomies Arise in Microbial Communities Due to Mixing of Deep Hydrothermal Waters and Atmospheric Gas in a Circumneutral Hot Spring. *Applied and Environmental Microbiology*, 87(23), e01598-01521.
<https://doi.org/doi:10.1128/AEM.01598-21>
- Filice, S., Sciuto, E. L., Scalese, S., Faro, G., Libertino, S., Corso, D., Timpanaro, R. M., Laganà, P., & Coniglio, M. A. (2022). Innovative Antibiofilm Smart Surface against *Legionella* for Water Systems. *Microorganisms*, 10(5).
<https://doi.org/10.3390/microorganisms10050870>

- Fitton, K., Barber, K., Karamon, A., Zuehlke, N., Atwell, S., & Enright, S. (2017). Long acting water-stable organosilane and its sustained effect on reducing microbial load in the intensive care unit. *American Journal of Infection Control*, 45(6), S27.
- Flemming, H.-C., Neu, T. R., & Wozniak, D. J. (2007). The EPS Matrix: The “House of Biofilm Cells”. *Journal of Bacteriology*, 189(22), 7945-7947. <https://doi.org/10.1128/jb.00858-07>
- Flemming, H.-C., & Wingender, J. (2010). The biofilm matrix. *Nature Reviews Microbiology*, 8(9), 623-633. <https://doi.org/10.1038/nrmicro2415>
- Flemming, H.-C., & Wuertz, S. (2019). Bacteria and archaea on Earth and their abundance in biofilms. *Nature Reviews Microbiology*, 17(4), 247-260. <https://doi.org/10.1038/s41579-019-0158-9>
- Flores, P., Luo, J., Mueller, D. W., Muecklich, F., & Zea, L. (2022). *Space Biofilms- An Overview of the Morphology of Pseudomonas aeruginosa Biofilms Grown on Silicone and Cellulose Membranes on board the International Space Station* International Astronautical Congress, Paris, France.
- Flores, P., Luo, J., Mueller, D. W., Muecklich, F., & Zea, L. (2024). Space biofilms - An overview of the morphology of Pseudomonas aeruginosa biofilms grown on silicone and cellulose membranes on board the international space station. *Biofilm*, 7, 100182. <https://doi.org/10.1016/j.bioflm.2024.100182>
- Flores, P., McBride, S. A., Galazka, J. M., Varanasi, K. K., & Zea, L. (2023). Biofilm formation of Pseudomonas aeruginosa in spaceflight is minimized on lubricant impregnated surfaces. *NPJ microgravity*, 9(1), 66. <https://doi.org/10.1038/s41526-023-00316-w>
- Flores, P., Schauer, R., McBride, S. A., Luo, J., Hoehn, C., Doraisingam, S., Widhalm, D., Chadha, J., Selman, L., Mueller, D. W., Floyd, S., Rupert, M., Gorti, S., Reagan, S., Varanasi, K. K., Koch, C., Meir, J. U., Muecklich, F., Moeller, R., . . . Zea, L. (2022). Preparation for and performance of a Pseudomonas aeruginosa biofilm experiment on board the International Space Station. *Acta Astronautica*, 199, 386-400. <https://doi.org/https://doi.org/10.1016/j.actaastro.2022.07.015>
- Friedlander, A., Nir, S., Reches, M., & Shemesh, M. (2019). Preventing Biofilm Formation by Dairy-Associated Bacteria Using Peptide-Coated Surfaces [Original Research]. *Frontiers in microbiology*, 10. <https://doi.org/10.3389/fmicb.2019.01405>
- Gaskill, M. L. (2023). NASA Achieves Water Recovery Milestone on International Space Station. <https://www.nasa.gov/missions/station/iss-research/nasa-achieves-water-recovery-milestone-on-international-space-station/>

- Ghanbari, A., Dehghany, J., Schwebs, T., Müsken, M., Häussler, S., & Meyer-Hermann, M. (2016). Inoculation density and nutrient level determine the formation of mushroom-shaped structures in *Pseudomonas aeruginosa* biofilms. *Scientific Reports*, 6(1), 32097. <https://doi.org/10.1038/srep32097>
- Gharpure, R., Gleason, M., Salah, Z., Blackstock, A. J., Hess-Homeier, D., Yoder, J. S., Ali, I. K. M., Collier, S. A., & Cope, J. R. (2021). Geographic Range of Recreational Water-Associated Primary Amebic Meningoencephalitis, United States, 1978-2018. *Emerging infectious diseases*, 27(1), 271-274. <https://doi.org/10.3201/eid2701.202119>
- Goodband, S. J., Armstrong, S., Kusumaatmaja, H., & Voitchovsky, K. (2020). Effect of Ageing on the Structure and Properties of Model Liquid-Infused Surfaces. *Langmuir*, 36(13), 3461-3470. <https://doi.org/10.1021/acs.langmuir.0c00059>
- Goudot, S., Mathieu, L., Herbelin, P., Soreau, S., & Jorand, F. P. A. (2024). Growth dynamic of biofilm-associated *Naegleria fowleri* in freshwater on various materials [Original Research]. *Frontiers in microbiology*, 15. <https://doi.org/10.3389/fmicb.2024.1369665>
- Gu, G., Smith, L., Liu, A., & Lu, S. E. (2011). Genetic and biochemical map for the biosynthesis of occidiofungin, an antifungal produced by *Burkholderia contaminans* strain MS14. *Appl Environ Microbiol*, 77(17), 6189-6198. <https://doi.org/10.1128/aem.00377-11>
- Gule, N. P., Begum, N. M., & Klumperman, B. (2016). Advances in biofouling mitigation: A review. *Critical Reviews in Environmental Science and Technology*, 46(6), 535-555. <https://doi.org/10.1080/10643389.2015.1114444>
- Haas, B., James, S., Parker, A. E., Gagnon, M.-C., Goulet, N., & Labrie, P. (2023). Comparison of quantification methods for an endoscope lumen biofilm model. *Biofilm*, 6, 100163. <https://doi.org/https://doi.org/10.1016/j.bioflm.2023.100163>
- Hammond, T. G., & Hammond, J. M. (2001). Optimized suspension culture: the rotating-wall vessel. *American Journal of Physiology-Renal Physiology*, 281(1), F12-F25. <https://doi.org/10.1152/ajprenal.2001.281.1.F12>
- Herranz, R., Anken, R., Boonstra, J., Braun, M., Christianen, P. C., de Geest, M., Hauslage, J., Hilbig, R., Hill, R. J., Lebert, M., Medina, F. J., Vagt, N., Ullrich, O., van Loon, J. J., & Hemmersbach, R. (2013). Ground-based facilities for simulation of microgravity: organism-specific recommendations for their use, and recommended terminology. *Astrobiology*, 13(1), 1-17. <https://doi.org/10.1089/ast.2012.0876>
- Horberry, M. (2020, May 20, 2020). After Coronavirus, Office Workers Might Face Unexpected Health Threats. *The New York Times*. <https://www.nytimes.com/2020/05/20/health/coronavirus-legionnaires-offices.html>

- Hoson, T., Kamisaka, S., Masuda, Y., Yamashita, M., & Buchen, B. (1997). Evaluation of the three-dimensional clinostat as a simulator of weightlessness. *Planta*, 203(1), S187-S197. <https://doi.org/10.1007/PL00008108>
- Huang, B., Li, D.-G., Huang, Y., & Liu, C.-T. (2018). Effects of spaceflight and simulated microgravity on microbial growth and secondary metabolism. *Military Medical Research*, 5(1), 18-18. <https://doi.org/10.1186/s40779-018-0162-9>
- Hung, C.-H., Chang, Y.-T., & Chang, Y.-J. (2011). Roles of microorganisms other than Clostridium and Enterobacter in anaerobic fermentative biohydrogen production systems – A review. *Bioresource Technology*, 102(18), 8437-8444. <https://doi.org/https://doi.org/10.1016/j.biortech.2011.02.084>
- Hupka, M., Kedia, R., Schauer, R., Shepard, B., Granados-Presa, M., Vande Hei, M., Flores, P., & Zea, L. (2023). Morphology of Penicillium rubens Biofilms Formed in Space. *Life (Basel)*, 13(4). <https://doi.org/10.3390/life13041001>
- Hwang, C., Ling, F., Andersen Gary, L., LeChevallier Mark, W., & Liu, W.-T. (2012). Microbial Community Dynamics of an Urban Drinking Water Distribution System Subjected to Phases of Chloramination and Chlorination Treatments. *Applied and Environmental Microbiology*, 78(22), 7856-7865. <https://doi.org/10.1128/AEM.01892-12>
- International Standards Organization. (2011). ISO 22196:2011, Measurement of antibacterial activity on plastics and other non-porous surfaces. In (Vol. 22196:2011, pp. 15).
- Japhet, N., Tarchitzky, J., & Chen, Y. (2022). Effectiveness of hydrogen peroxide treatments in preventing biofilm clogging in drip irrigation systems applying treated wastewater. *Biofouling*, 38(6), 575-592.
- Jiang, R., Hao, L., Song, L., Tian, L., Fan, Y., Zhao, J., Liu, C., Ming, W., & Ren, L. (2020). Lotus-leaf-inspired hierarchical structured surface with non-fouling and mechanical bactericidal performances. *Chemical Engineering Journal*, 398, 125609. <https://doi.org/https://doi.org/10.1016/j.cej.2020.125609>
- Jones, E. M., Cochrane, C. A., & Percival, S. L. (2015). The Effect of pH on the Extracellular Matrix and Biofilms. *Advances in wound care*, 4(7), 431-439. <https://doi.org/10.1089/wound.2014.0538>
- Justiniano, Y.-A. V., Ledford, M.-E., Castro-Wallace, S. L., Nguyen, H. N., Li, W., Irwin, T., & Sysoeva, T. A. (2024). More Than a Decade of International Space Station Microbial Sampling in the Environmental Control and Life Support Systems. 53rd International Conference on Environmental Systems,

- Kanematsu, H., Oizumi, A., Sato, T., Kamijo, T., Honma, S., Barry, D. M., Hirai, N., Ogawa, A., Kogo, T., Kuroda, D., Sano, K., Tsunashima, K., Lee, S.-H., & Lee, M.-H. (2018). Biofilm Formation of a Polymer Brush Coating with Ionic Liquids Compared to a Polymer Brush Coating with a Non-Ionic Liquid. *Coatings*, 8(11), 398. <https://www.mdpi.com/2079-6412/8/11/398>
- Ketteler, H. M., Johnson, E. L., McGlennen, M., Dieser, M., Foreman, C. M., & Warnat, S. (2025). A simulated microgravity biofilm reactor with integrated microfabricated sensors: Advancing biofilm studies in near-space conditions. *Biofilm*, 9, 100263. <https://doi.org/https://doi.org/10.1016/j.bioflm.2025.100263>
- Kim, W., Tengra, F. K., Young, Z., Shong, J., Marchand, N., Chan, H. K., Pangule, R. C., Parra, M., Dordick, J. S., Plawsky, J. L., & Collins, C. H. (2013). Spaceflight Promotes Biofilm Formation by *Pseudomonas aeruginosa*. *PLOS*. <https://doi.org/10.1371/journal.pone.0062437>
- Kitsutani, S., Mura, M., & Nagahisa, M. (1970). Catalase formation in *Rhodotorula mucilaginosa* in relation to the effect of glucose and antibiotics. *Plant and cell physiology*, 11(4), 551-558.
- Klaus, D., Simske, S., Todd, P., & Stodieck, L. (1997). Investigation of space flight effects on *Escherichia coli* and a proposed model of underlying physical mechanisms. *Microbiology*, 143(2), 449-455. <https://doi.org/https://doi.org/10.1099/00221287-143-2-449>
- Klaus, D. M. (2001). Clinostats and bioreactors. *Gravit Space Biol Bull*, 14(2), 55-64.
- Klintworth, R., Reher, H., Viktorov, A., & Bohle, D. J. A. a. (1999). Biological induced corrosion of materials II: new test methods and experiences from MIR station. 44(7-12), 569-578.
- Knetsch, M. L. W., & Koole, L. H. (2011). New Strategies in the Development of Antimicrobial Coatings: The Example of Increasing Usage of Silver and Silver Nanoparticles. *Polymers*, 3(1), 340-366. <https://www.mdpi.com/2073-4360/3/1/340>
- Knisz, J., Eckert, R., Gieg, L. M., Koerd, A., Lee, J. S., Silva, E. R., Skovhus, T. L., An Stepec, B. A., & Wade, S. A. (2023). Microbiologically influenced corrosion-more than just microorganisms. *FEMS Microbiol Rev*, 47(5). <https://doi.org/10.1093/femsre/fuad041>
- Kool, J. L., Bergmire-Sweat, D., Butler, J. C., Brown, E. W., Peabody, D. J., Massi, D. S., Carpenter, J. C., Pruckler, J. M., Benson, R. F., & Fields, B. S. (1999). Hospital characteristics associated with colonization of water systems by *Legionella* and risk of nosocomial legionnaires' disease: a cohort study of 15 hospitals. *Infection Control & Hospital Epidemiology*, 20(12), 798-805.

- Kragh, K. N., Alhede, M., Rybtke, M., Stavnsberg, C., Jensen, P., Tolker-Nielsen, T., Whiteley, M., & Bjarnsholt, T. (2018). The Inoculation Method Could Impact the Outcome of Microbiological Experiments. *Appl Environ Microbiol*, 84(5). <https://doi.org/10.1128/aem.02264-17>
- Kroukamp, O., Dumitrache, R. G., & Wolfaardt, G. M. (2010). Pronounced effect of the nature of the inoculum on biofilm development in flow systems. *Appl Environ Microbiol*, 76(18), 6025-6031. <https://doi.org/10.1128/aem.00070-10>
- Learbuch, K. L. G., Smidt, H., & van der Wielen, P. W. J. J. (2021). Influence of pipe materials on the microbial community in unchlorinated drinking water and biofilm. *Water Res*, 194, 116922. <https://doi.org/https://doi.org/10.1016/j.watres.2021.116922>
- Lee, D. Y., Kim, W. C., & Kim, J. G. (2012). Effect of nitrite concentration on the corrosion behaviour of carbon steel pipelines in synthetic tap water. *Corrosion Science*, 64, 105-114. <https://doi.org/https://doi.org/10.1016/j.corsci.2012.07.005>
- Lee, J., Ray, R., Lemieux, E., Falster, A., & Little, B. (2004). An Evaluation of Carbon Steel Corrosion under Stagnant Seawater Conditions. *Biofouling*, 20(4-5), 237-247. <https://doi.org/10.1080/08927010400013274>
- Lemelle, L., Campagnolo, L., Mottin, E., Le Tourneau, D., Garre, E., Marcoux, P., Thévenot, C., Maillet, A., Barde, S., Teisseire, J., Nonglaton, G., & Place, C. (2020). Towards a passive limitation of particle surface contamination in the Columbus module (ISS) during the MATISS experiment of the Proxima Mission. *NPJ microgravity*, 6(1), 29. <https://doi.org/10.1038/s41526-020-00120-w>
- Lemelle, L., Mottin, E., Le Tourneau, D., Rouquette, S., Campagnolo, L., Thévenot, C., Maillet, A., Barde, S., Garre, E., Teisseire, J., Fontelaye, C., Jousseau, V., Pudda, C., Constantin, O., Marcoux, P., Nonglaton, G., & Place, C. (2023). Surface contamination rates at different spatial scales in the Columbus module (ISS) during the MATISS campaigns [Original Research]. *Frontiers in Astronomy and Space Sciences*, 10. <https://doi.org/10.3389/fspas.2023.1229022>
- Lemelle, L., Rouquette, S., Mottin, E., Le Tourneau, D., Marcoux, P. R., Thévenot, C., Maillet, A., Nonglaton, G., & Place, C. (2022). Passive limitation of surface contamination by perFluoroDecylTrichloroSilane coatings in the ISS during the MATISS experiments. *NPJ microgravity*, 8(1), 31. <https://doi.org/10.1038/s41526-022-00218-3>
- Li Sip, Y. Y., Jacobs, A., Morales, A., Sun, M., Roberson, L. B., Hummerick, M. E., Roy, H., Kik, P., & Zhai, L. (2023). Slippery lubricant-infused silica nanoparticulate film processing for anti-biofouling applications. *J Appl Biomater Funct Mater*, 21, 22808000231184688. <https://doi.org/10.1177/22808000231184688>
- Li, W., Calle, L., Hanford, A., Stambaugh, I., & Callahan, M. (2018). Investigation of silver biocide as a disinfection technology for spacecraft—an early literature review.

- Li, W., Hummerick, M., Khodadad, C., Buhrom, J., Spencer, L., Coutts, J., Roberson, L., Tuteja, A., Mehta, G., Boban, M., & Barden, M. (2018). *Biofilm Resistant Coatings for Space Applications* 48th International Conference on Environmental Systems, Albuquerque, New Mexico.
- Li, Y., Song, P., Pei, Y., & Feng, J. (2015). Effects of lateral flushing on emitter clogging and biofilm components in drip irrigation systems with reclaimed water. *Irrigation Science*, 33(3), 235-245. <https://doi.org/10.1007/s00271-015-0462-8>
- Lichtenberg, M., Kvich, L., Larsen Sara Louise, B., Jakobsen Tim, H., & Bjarnsholt, T. (2022). Inoculum Concentration Influences *Pseudomonas aeruginosa* Phenotype and Biofilm Architecture. *Microbiology spectrum*, 10(6), e03131-03122. <https://doi.org/10.1128/spectrum.03131-22>
- Lin, Y. E., Stout, J. E., & Victor, L. Y. (2011). Controlling *Legionella* in hospital drinking water: an evidence-based review of disinfection methods. *Infection Control & Hospital Epidemiology*, 32(2), 166-173.
- Little, B. J., Blackwood, D. J., Hinks, J., Lauro, F. M., Marsili, E., Okamoto, A., Rice, S. A., Wade, S. A., & Flemming, H. C. (2020). Microbially influenced corrosion—Any progress? *Corrosion Science*, 170, 108641. <https://doi.org/https://doi.org/10.1016/j.corsci.2020.108641>
- Liu, S., Gunawan, C., Barraud, N., Rice, S. A., Harry, E. J., & Amal, R. (2016). Understanding, Monitoring, and Controlling Biofilm Growth in Drinking Water Distribution Systems. *Environmental Science & Technology*, 50(17), 8954-8976. <https://doi.org/10.1021/acs.est.6b00835>
- Lowe, R., Shirley, N., Bleackley, M., Dolan, S., & Shafee, T. (2017). Transcriptomics technologies. *PLoS computational biology*, 13(5), e1005457-e1005457. <https://doi.org/10.1371/journal.pcbi.1005457>
- Lynch, S. V., Mukundakrishnan, K., Benoit, M. R., Ayyaswamy, P. S., & Matin, A. (2006). *Escherichia coli* biofilms formed under low-shear modeled microgravity in a ground-based system. *Appl Environ Microbiol*, 72(12), 7701-7710. <https://doi.org/10.1128/aem.01294-06>
- Lyon, S. B., Bingham, R., & Mills, D. J. (2017). Advances in corrosion protection by organic coatings: What we know and what we would like to know. *Progress in Organic Coatings*, 102, 2-7. <https://doi.org/https://doi.org/10.1016/j.porgcoat.2016.04.030>
- Madsen, J. S., Burmølle, M., Hansen, L. H., & Sørensen, S. J. (2012). The interconnection between biofilm formation and horizontal gene transfer. *FEMS Immunology & Medical Microbiology*, 65(2), 183-195. <https://doi.org/10.1111/j.1574-695X.2012.00960.x>

- Mah, T.-F., Pitts, B., Pellock, B., Walker, G. C., Stewart, P. S., & O'Toole, G. A. (2003). A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nature*, 426(6964), 306-310. <https://doi.org/10.1038/nature02122>
- Mah, T.-F. C., & O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology*, 9(1), 34-39. [https://doi.org/https://doi.org/10.1016/S0966-842X\(00\)01913-2](https://doi.org/https://doi.org/10.1016/S0966-842X(00)01913-2)
- Mahanta, U., Khandelwal, M., & Deshpande, A. S. (2021). Antimicrobial surfaces: a review of synthetic approaches, applicability and outlook. *J Mater Sci*, 56(32), 17915-17941. <https://doi.org/10.1007/s10853-021-06404-0>
- Manuel, C. M., Nunes, O. C., & Melo, L. F. (2007). Dynamics of drinking water biofilm in flow/non-flow conditions. *Water Res*, 41(3), 551-562. <https://doi.org/https://doi.org/10.1016/j.watres.2006.11.007>
- Maryatt, B., & Smith, M. (2017). Microbial growth control in the international space station potable water dispenser.
- Mauclaire, L., & Egli, M. (2010). Effect of simulated microgravity on growth and production of exopolymeric substances of *Micrococcus luteus* space and earth isolates. *FEMS Immunology & Medical Microbiology*, 59(3), 350-356. <https://doi.org/10.1111/j.1574-695X.2010.00683.x>
- McLean, R. J. C., Cassanto, J. M., Barnes, M. B., & Koo, J. H. (2001). Bacterial biofilm formation under microgravity conditions. *FEMS Microbiology Letters*, 195(2), 115-119. <https://doi.org/10.1111/j.1574-6968.2001.tb10507.x>
- Mettler, M. K., Goemann, H. M., Mueller, R. C., Vanegas, O. A., Lopez, G., Singh, N., Venkateswaran, K., & Peyton, B. M. (2023). Development of Martian saline seep models and their implications for planetary protection. *Biofilm*, 5, 100127. <https://doi.org/https://doi.org/10.1016/j.biofilm.2023.100127>
- Mettler, M. K., J., E.-O. E., M., G. D., & and Peyton, B. M. (2025). Considerations for testing anti-fouling coatings designed for implementation into Earth-based and spacecraft water systems. *Biofouling*, 1-19. <https://doi.org/10.1080/08927014.2025.2479692>
- Mettler, M. K., Parker, C. W., Venkateswaran, K., & Peyton, B. M. (2022). Antimicrobial Coating Efficacy for Prevention of *Pseudomonas aeruginosa* Biofilm Growth on ISS Water System Materials [Original Research]. *Frontiers in microbiology*, 13. <https://doi.org/10.3389/fmicb.2022.874236>
- Miller, H. C., Morgan, M. J., Wylie, J. T., Kaksonen, A. H., Sutton, D., Braun, K., & Puzon, G. J. (2017). Elimination of *Naegleria fowleri* from bulk water and biofilm in an operational drinking water distribution system. *Water Res*, 110, 15-26. <https://doi.org/https://doi.org/10.1016/j.watres.2016.11.061>

- Miller, L. A., Buckingham-Meyer, K., & Goeres, D. M. (2024). Simulated aging of draught beer line tubing increases biofilm contamination. *International journal of food microbiology*, 415, 110630. <https://doi.org/https://doi.org/10.1016/j.ijfoodmicro.2024.110630>
- Montemor, M. F. (2014). Functional and smart coatings for corrosion protection: A review of recent advances. *Surface and Coatings Technology*, 258, 17-37. <https://doi.org/https://doi.org/10.1016/j.surfcoat.2014.06.031>
- Mulani, M. S., Kamble, E. E., Kumkar, S. N., Tawre, M. S., & Pardesi, K. R. (2019). Emerging Strategies to Combat ESKAPE Pathogens in the Era of Antimicrobial Resistance: A Review [Review]. *Frontiers in microbiology*, 10. <https://doi.org/10.3389/fmicb.2019.00539>
- Mullan, A., Quinn, J. P., & McGrath, J. W. (2002). Enhanced phosphate uptake and polyphosphate accumulation in *Burkholderia cepacia* grown under low pH conditions. *Microb Ecol*, 44(1), 69-77. <https://doi.org/10.1007/s00248-002-3004-x>
- Munavalli, G. R., & Kumar, M. S. M. (2003). Optimal Scheduling of Multiple Chlorine Sources in Water Distribution Systems. *Journal of Water Resources Planning and Management*, 129(6), 493-504. [https://doi.org/doi:10.1061/\(ASCE\)0733-9496\(2003\)129:6\(493\)](https://doi.org/doi:10.1061/(ASCE)0733-9496(2003)129:6(493))
- Murray, J., Muruko, T., Gill, C. I. R., Kearney, M. P., Farren, D., Scott, M. G., McMullan, G., & Ternan, N. G. (2017). Evaluation of bactericidal and anti-biofilm properties of a novel surface-active organosilane biocide against healthcare associated pathogens and *Pseudomonas aeruginosa* biofilm. *PloS one*, 12(8), e0182624-e0182624. <https://doi.org/10.1371/journal.pone.0182624>
- National Aeronautics and Space Administration. (2024). *International Space Station Facts*. <https://www.nasa.gov/international-space-station/space-station-facts-and-figures/#:~:text=The%20space%20station%20has%20been,station%20during%20a%20crew%20handover.>
- National Aeronautics and Space Administration. (2025). *Gateway Frequently Asked Questions*. <https://www.nasa.gov/gateway-frequently-asked-questions/#:~:text=Unlike%20the%20ISS%2C%20which%20is,Gateway%20will%20not%20be>
- Nauman Eric, A., Ott, C. M., Sander, E., Tucker Don, L., Pierson, D., Wilson James, W., & Nickerson Cheryl, A. (2007). Novel Quantitative Biosystem for Modeling Physiological Fluid Shear Stress on Cells. *Applied and Environmental Microbiology*, 73(3), 699-705. <https://doi.org/10.1128/AEM.02428-06>
- Nejadnik, M. R., van der Mei, H. C., Norde, W., & Busscher, H. J. (2008). Bacterial adhesion and growth on a polymer brush-coating. *Biomaterials*, 29(30), 4117-4121. <https://doi.org/10.1016/j.biomaterials.2008.07.014>

- Nickerson, C. A., Ott, C. M., Wilson, J. W., Ramamurthy, R., & Pierson, D. L. (2004). Microbial responses to microgravity and other low-shear environments. *Microbiology and molecular biology reviews : MMBR*, 68(2), 345-361. <https://doi.org/10.1128/MMBR.68.2.345-361.2004>
- Nishimura, Y. (2023). Technology using simulated microgravity. *Regenerative Therapy*, 24, 318-323. <https://doi.org/https://doi.org/10.1016/j.reth.2023.08.001>
- Novikova, N. D. (2004). Review of the Knowledge of Microbial Contamination of the Russian Manned Spacecraft. *Microbial Ecology*, 47(2), 127-132. <https://doi.org/10.1007/s00248-003-1055-2>
- O'Toole, G. A., & Kolter, R. (1998). Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Molecular Microbiology*, 30(2), 295-304. <https://doi.org/https://doi.org/10.1046/j.1365-2958.1998.01062.x>
- Pan, R., Zhang, T.-Y., He, H., Zheng, Z.-X., Dong, Z.-Y., Zhao, H.-X., Xu, M.-Y., Luo, Z.-N., Hu, C.-Y., Tang, Y.-L., El-Din, M. G., & Xu, B. (2023). Mixed chlorine/chloramines in disinfected water and drinking water distribution systems (DWDSs): A critical review. *Water Res*, 247, 120736. <https://doi.org/https://doi.org/10.1016/j.watres.2023.120736>
- Parker, A. E., Christen, J. A., Lorenz, L., & Smith, H. (2020). Optimal surface estimation and thresholding of confocal microscope images of biofilms using Beer's Law. *Journal of Microbiological Methods*, 174, 105943. <https://doi.org/https://doi.org/10.1016/j.mimet.2020.105943>
- Parrilli, E., Tedesco, P., Fondi, M., Tutino, M. L., Lo Giudice, A., de Pascale, D., & Fani, R. (2021). The art of adapting to extreme environments: The model system *Pseudoalteromonas*. *Physics of Life Reviews*, 36, 137-161. <https://doi.org/https://doi.org/10.1016/j.plrev.2019.04.003>
- Parsek, M. R., & Peter Greenberg, E. (1999). [3] Quorum sensing signals in development of *Pseudomonas aeruginosa* biofilms. In *Methods in Enzymology* (Vol. 310, pp. 43-55). Academic Press. [https://doi.org/https://doi.org/10.1016/S0076-6879\(99\)10005-3](https://doi.org/https://doi.org/10.1016/S0076-6879(99)10005-3)
- Paton, S., Moore, G., Campagnolo, L., & Pottage, T. (2020). Antimicrobial surfaces for use on inhabited space craft: A review. *Life Sciences in Space Research*, 26, 125-131. <https://doi.org/https://doi.org/10.1016/j.lssr.2020.05.004>
- Penesyan, A., Paulsen, I. T., Kjelleberg, S., & Gillings, M. R. (2021). Three faces of biofilms: a microbial lifestyle, a nascent multicellular organism, and an incubator for diversity. *Npj Biofilms and Microbiomes*, 7(1), 80. <https://doi.org/10.1038/s41522-021-00251-2>
- Perez, V., Mena, K. D., Watson, H. N., Prater, R. B., & McIntyre, J. L. (2015). Evaluation and quantitative microbial risk assessment of a unique antimicrobial agent for hospital surface treatment. *American Journal of Infection Control*, 43(11), 1201-1207.

- Petala, M., Tsiridis, V., Darakas, E., Mintsouli, I., Sotiropoulos, S., Kostoglou, M., Karapantsios, T., & Rebeyre, P. (2016). Silver deposition on wetted materials used in the potable water systems of the International Space Station. 46th International Conference on Environmental Systems,
- Petala, M., Tsiridis, V., Mintsouli, I., Pliatsikas, N., Spanos, T., Rebeyre, P., Darakas, E., Patsalas, P., Vourlias, G., & Kostoglou, M. (2017). Silver deposition on stainless steel container surfaces in contact with disinfectant silver aqueous solutions. *Applied Surface Science*, 396, 1067-1075.
- Peyton, B. M., & Characklis, W. G. (1993). A statistical analysis of the effect of substrate utilization and shear stress on the kinetics of biofilm detachment. *Biotechnology and Bioengineering*, 41(7), 728-735. <https://doi.org/10.1002/bit.260410707>
- Pierson, D. L., Botkin, D. J., Bruce, R. J., Castro, V. A., Smith, M. J., Oubre, C. M., & Ott, C. M. (2013). Microbial monitoring of the international space station. 8th International Workshop on Space Microbiology,
- Pinto, D., Santos, M. A., & Chambel, L. (2015). Thirty years of viable but nonculturable state research: Unsolved molecular mechanisms. *Critical Reviews in Microbiology*, 41(1), 61-76. <https://doi.org/10.3109/1040841X.2013.794127>
- Pruitt, J. M., Carter, L., Bagdigian, R. M., & Kayatin, M. J. (2015). Upgrades to the ISS Water Recovery System.
- Puleo, J. R., Fields, N. D., Moore, B., & Graves, R. C. (1970). Microbial contamination associated with the Apollo 6 spacecraft during final assembly and testing. *Space life sciences*, 2(1), 48-56. <https://doi.org/10.1007/BF00928955>
- Puleo, J. R., Oxborrow, G. S., Fields, N. D., Herring, C. M., & Smith, L. S. (1973). Microbiological profiles of four Apollo spacecraft. *Appl Microbiol*, 26(6), 838-845. <https://doi.org/10.1128/am.26.6.838-845.1973>
- Puzon, G. J., Miller, H. C., Malinowski, N., Walsh, T., & Morgan, M. J. (2020). Naegleria fowleri in drinking water distribution systems. *Current Opinion in Environmental Science & Health*, 16, 22-27. <https://doi.org/https://doi.org/10.1016/j.coesh.2020.02.003>
- Pyle, B., & McFeters, G. (1999). Bacterial Growth on surfaces and in suspensions. *Biorack on Spacehab- Biological experiments on three Shuttle-to-Mir missions(A 00-30676 07-51)*, Noordwijk, Netherlands, European Space Agency, 1999, 95-99.
- Radetić, M. (2013). Functionalization of textile materials with silver nanoparticles. *Journal of Materials Science*, 48(1), 95-107. <https://doi.org/10.1007/s10853-012-6677-7>

- Rajala, P., Carpén, L., Vepsäläinen, M., Raulio, M., Sohlberg, E., & Bomberg, M. (2015). Microbially induced corrosion of carbon steel in deep groundwater environment [Original Research]. *Frontiers in microbiology*, 6. <https://doi.org/10.3389/fmicb.2015.00647>
- Redfern, J., Cunliffe, A. J., Goeres, D. M., Azevedo, N. F., & Verran, J. (2024). Critical analysis of methods to determine growth, control and analysis of biofilms for potential non-submerged antibiofilm surfaces and coatings. *Biofilm*, 7, 100187. <https://doi.org/https://doi.org/10.1016/j.biofilm.2024.100187>
- Ren, H., Wang, W., Liu, Y., Liu, S., Lou, L., Cheng, D., He, X., Zhou, X., Qiu, S., Fu, L., Liu, J., & Hu, B. (2015). Pyrosequencing analysis of bacterial communities in biofilms from different pipe materials in a city drinking water distribution system of East China. *Applied microbiology and biotechnology*, 99(24), 10713-10724. <https://doi.org/10.1007/s00253-015-6885-6>
- Roberson, E. B., & Firestone, M. K. (1992). Relationship between Desiccation and Exopolysaccharide Production in a Soil *Pseudomonas* sp. *Applied and Environmental Microbiology*, 58(4), 1284-1291. <https://doi.org/doi:10.1128/aem.58.4.1284-1291.1992>
- Roberts, M. S., Hummerick, M. E., Edney, S. L., Bisbee, P. A., Callahan, M. R., Loucks, S., Pickering, K. D., & Sager, J. C. (2007). *Assessment of silver based disinfection technology for CEV and future US spacecraft: microbial efficacy* (0148-7191).
- Rogers, J., Dowsett, A. B., Dennis, P. J., Lee, J. V., & Keevil, C. W. (1994). Influence of Plumbing Materials on Biofilm Formation and Growth of *Legionella pneumophila* in Potable Water Systems. *Applied and Environmental Microbiology*, 60(6), 1842-1851. <https://doi.org/10.1128/aem.60.6.1842-1851.1994>
- Rosenzweig, J. A., Abogunde, O., Thomas, K., Lawal, A., Nguyen, Y. U., Sodipe, A., & Jegelowo, O. (2010). Spaceflight and modeled microgravity effects on microbial growth and virulence. *Applied microbiology and biotechnology*, 85(4), 885-891. <https://doi.org/10.1007/s00253-009-2237-8>
- Salton, M., & Kim, K. (1996). Structure. In S. Baron (Ed.), *Medical Microbiology* (4th edition ed.). University of Texas Medical Branch at Galveston. <https://www.ncbi.nlm.nih.gov/books/NBK8477/>
- Samuel, M. S., Moghaddam, S. T., Shang, M., & Niu, J. (2022). A Flexible Anti-Biofilm Hygiene Coating for Water Devices. *ACS Appl Bio Mater*, 5(8), 3991-3998. <https://doi.org/10.1021/acsabm.2c00538>
- Sandvik, E., Stewart, P., Goeres, D., & Sturman, P. (2022). Center for Biofilm Engineering Recommendations for Addition of Trace Nutrients to Microbial Ersatz. In J. Williamson & Y. A. Velez Justiniano (Eds.).

- Santos, A. L. S. d., Galdino, A. C. M., Mello, T. P. d., Ramos, L. d. S., Branquinha, M. H., Bolognese, A. M., Columbano Neto, J., & Roudbary, M. (2018). What are the advantages of living in a community? A microbial biofilm perspective! *Memórias do Instituto Oswaldo Cruz*, 113. http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02762018000900200&nrm=iso
- Schierholz, J. M., Lucas, L. J., Rump, A., & Pulverer, G. (1998). Efficacy of silver-coated medical devices. *Journal of Hospital Infection*, 40(4), 257-262. [https://doi.org/https://doi.org/10.1016/S0195-6701\(98\)90301-2](https://doi.org/https://doi.org/10.1016/S0195-6701(98)90301-2)
- Schultz, J. R., Flanagan, D. T., Bruce, R. J., Huls, M. H., Pierson, D. L., & Sauer, R. L. (1994). Evaluation of methods for remediating biofilms in spacecraft potable water systems. *SAE Transactions*, 939-949.
- Schultz, J. R., Flanagan, D. T., Bruce, R. J., Mudgett, P. D., Carr, S. E., Rutz, J. A., Huls, M. H., Sauer, R. L., & Pierson, D. L. (1992). Biofilm formation and control in a simulated spacecraft water system: three year results. *SAE Transactions*, 1032-1044.
- Searles, S. C., Woolley, C. M., Petersen, R. A., Hyman, L. E., & Nielsen-Priess, S. M. (2011). Modeled Microgravity Increases Filamentation, Biofilm Formation, Phenotypic Switching, and Antimicrobial Resistance in *Candida albicans*. *Astrobiology*, 11.
- Shea, A., Harvey, K., Keeley, A., Johnson, H., Hansen, N., McCormack, R., Stelck, K., Lindsay, T., Bryant, A., & Bernards, M. T. (2025). Payload Design and Evaluation of *Staphylococcus epidermidis* Adhesion to Nonfouling Polyampholyte Coatings Onboard the International Space Station. *Molecules*, 30(4), 836. <https://www.mdpi.com/1420-3049/30/4/836>
- Si, X., Quan, X., & Wu, Y. (2015). A small-molecule norspermidine and norspermidine-hosting polyelectrolyte coatings inhibit biofilm formation by multi-species wastewater culture. *Applied microbiology and biotechnology*, 99(24), 10861-10870. <https://doi.org/10.1007/s00253-015-6943-0>
- Silhavy, T. J., Kahne, D., & Walker, S. (2010). The bacterial cell envelope. *Cold Spring Harb Perspect Biol*, 2(5), a000414. <https://doi.org/10.1101/cshperspect.a000414>
- Sim, W., Barnard, R. T., Blaskovich, M. A. T., & Ziora, Z. M. (2018). Antimicrobial Silver in Medicinal and Consumer Applications: A Patent Review of the Past Decade (2007-2017). *Antibiotics (Basel)*, 7(4). <https://doi.org/10.3390/antibiotics7040093>
- Simoës, L. C., & Simões, M. (2013). Biofilms in drinking water: problems and solutions. *Rsc Advances*, 3(8), 2520-2533.

- Sobisch, L.-Y., Rogowski, K. M., Fuchs, J., Schmieder, W., Vaishampayan, A., Oles, P., Novikova, N., & Grohmann, E. (2019). Biofilm Forming Antibiotic Resistant Gram-Positive Pathogens Isolated From Surfaces on the International Space Station [Original Research]. *Frontiers in microbiology*, 10. <https://doi.org/10.3389/fmicb.2019.00543>
- Sójka, O., Keskin, D., van der Mei, H. C., van Rijn, P., & Gagliano, M. C. (2023). Nanogel-based coating as an alternative strategy for biofilm control in drinking water distribution systems. *Biofouling*, 39(2), 121-134. <https://doi.org/10.1080/08927014.2023.2190023>
- Song, B., Zhang, E., Han, X., Zhu, H., Shi, Y., & Cao, Z. (2020). Engineering and Application Perspectives on Designing an Antimicrobial Surface. *ACS Appl Mater Interfaces*, 12(19), 21330-21341. <https://doi.org/10.1021/acsami.9b19992>
- Touching Surfaces* (2022). <https://event.dlr.de/en/ila2022/touching-surfaces/>
- Tsagkari, E., Connelly, S., Liu, Z., McBride, A., & Sloan, W. T. (2022). The role of shear dynamics in biofilm formation. *Npj Biofilms and Microbiomes*, 8(1), 33. <https://doi.org/10.1038/s41522-022-00300-4>
- Tumlirsch, T., Sznajder, A., & Jendrossek, D. (2015). Formation of Polyphosphate by Polyphosphate Kinases and Its Relationship to Poly(3-Hydroxybutyrate) Accumulation in *Ralstonia eutropha* Strain H16. *Applied and Environmental Microbiology*, 81(24), 8277-8293. <https://doi.org/10.1128/AEM.02279-15>
- Vaishampayan, A., Ahmed, R., Wagner, O., de Jong, A., Haag, R., Kok, J., & Grohmann, E. (2021). Transcriptomic analysis of stress response to novel antimicrobial coatings in a clinical MRSA strain. *Materials Science and Engineering: C*, 119, 111578. <https://doi.org/https://doi.org/10.1016/j.msec.2020.111578>
- Van Loon, J. J. (2007). Some history and use of the random positioning machine, RPM, in gravity related research. *Advances in Space Research*, 39(7), 1161-1165.
- Vaz-Moreira, I., Nunes, O. C., & Manaia, C. M. (2017). Ubiquitous and persistent Proteobacteria and other Gram-negative bacteria in drinking water. *Science of The Total Environment*, 586, 1141-1149. <https://doi.org/https://doi.org/10.1016/j.scitotenv.2017.02.104>
- Velez Justiniano, Y.-A., Carter, D., Sandvik, E., Stewart, P., Goeres, D., Sturman, P., Li, W., Johnson, A., & Cioanta, I. (2021). *Biofilm Management in a Microgravity Water Recovery System* 50th International Conference on Environmental Systems,
- Velez Justiniano, Y.-A., Lim Chae, H., Dunlap Darren, S., & Sysoeva Tatyana, A. (2023). Genome Sequences of Three Common Bacterial Isolates from Wastewater from the Water Processor Assembly at the International Space Station. *Microbiology Resource Announcements*, 12(1), e01189-01122. <https://doi.org/10.1128/mra.01189-22>

- Velez Justiniano, Y. A., Carter, L., Nur, M., & Angle, G. (2020). Developing Methods for Biofilm Control in Microgravity for a Water Recovery System. International Conference on Environmental Systems, Virtual.
- Velez, Y.-A., Carter, D., Nur, M., & Angle, G. (2020). Developing Methods for Biofilm Control in Microgravity for a Water Recovery System.
- Venkateswaran, K., Chung, S., Allton, J., & Kern, R. (2004). Evaluation of various cleaning methods to remove *Bacillus* spores from spacecraft hardware materials. *Astrobiology*, 4(3), 377-390.
- Waegenaar, F., García-Timmermans, C., Van Landuyt, J., De Gusseme, B., & Boon, N. (2024). Impact of operational conditions on drinking water biofilm dynamics and coliform invasion potential. *Appl Environ Microbiol*, 90(5), e0004224. <https://doi.org/10.1128/aem.00042-24>
- Walker, J., & Moore, G. (2015). *Pseudomonas aeruginosa* in hospital water systems: biofilms, guidelines, and practicalities. *Journal of Hospital Infection*, 89(4), 324-327. <https://doi.org/https://doi.org/10.1016/j.jhin.2014.11.019>
- Wallace, W., Castro-Wallace, S., Kuo, C., Loh, L., Hudson, E., Gazda, D., & Lewis, J. (2016). Effects of material choice on biocide loss in Orion water storage tanks.
- Wallace, W. T., Wallace, S. L., Loh, L. J., Kuo, C. M., Hudson Jr, E. K., Marlar, T. J., & Gazda, D. B. (2017). Effects of materials surface preparation for use in spacecraft potable water storage tanks. *Acta Astronautica*, 141, 30-35.
- Wan, H., Song, D., Li, X., Zhang, D., Gao, J., & Du, C. (2017). Failure Mechanisms of the Coating/Metal Interface in Waterborne Coatings: The Effect of Bonding. *Materials*, 10(4), 397. <https://www.mdpi.com/1996-1944/10/4/397>
- Wang, H., Yan, Y., Rong, D., Wang, J., Wang, H., Liu, Z., Wang, J., Yang, R., & Han, Y. (2016). Increased biofilm formation ability in *Klebsiella pneumoniae* after short-term exposure to a simulated microgravity environment. *MicrobiologyOpen*, 5(5), 793-801. <https://doi.org/10.1002/mbo3.370>
- Wang, M., Duday, D., Scolan, E., Perbal, S., Prato, M., Lasseur, C., & Hołyńska, M. (2021). Antimicrobial Surfaces for Applications on Confined Inhabited Space Stations. *Advanced Materials Interfaces*, 8(13), 2100118. <https://doi.org/https://doi.org/10.1002/admi.202100118>
- Wang, S., Zhu, H., Zheng, G., Dong, F., & Liu, C. (2022). Dynamic Changes in Biofilm Structures under Dynamic Flow Conditions. *Applied and Environmental Microbiology*, 88(22), e01072-01022. <https://doi.org/10.1128/aem.01072-22>

- Ward, L., & Sherchan, S. P. (2023). Surveillance of *Naegleria fowleri* in Louisiana's public water systems. *J Water Health*, 21(11), 1627-1631. <https://doi.org/10.2166/wh.2023.040>
- Weir, N., Wilson, M., Yoets, A., Molina, T., Bruce, R., & Carter, L. (2012). Microbiological characterization of the international space station water processor assembly external filter assembly S/N 01. 42nd international conference on environmental systems,
- Williamson, J., Kayatin, M., Nguyen, H. N., Hudson, E. K., Wallace, W. T., Williams, S., Castro-Wallace, S., Muirhead, D., & McCool, C. (2024). Overview of the International Space Station's Water and Cabin Air Quality: A Five-Year Status. 53rd International Conference on Environmental Systems (ICES),
- Williamson, J., Luong, H., Robinson, K., & Wilson, J. P. (2023). *Status of ISS Water Management and Recovery* The 52nd International Conference on Environmental Systems, Calgary, Canada. <https://hdl.handle.net/2346/94553>
- Williamson, J., Wilson, J. P., Robinson, K., & Luong, H. (2023). Status of ISS Water Management and Recovery. 52nd International Conference on Environmental Systems, Calgary, Canada.
- Wilson, J. W., Ott, C. M., Bentrup, K. H. Z., Ramamurthy, R., Quick, L., Porwollik, S., Cheng, P., McClelland, M., Tsaprailis, G., Radabaugh, T., Hunt, A., Fernandez, D., Richter, E., Shah, M., Kilcoyne, M., Joshi, L., Neiman-Gonzalez, M., Hing, S., Parra, M., . . . Nickerson, C. A. (2007). Space flight alters bacterial gene expression and virulence and reveals a role for global regulator Hfq [Article]. *Proceedings of the National Academy of Sciences of the United States of America*, 104(41), 16299-16304. <https://doi.org/10.1073/pnas.0707155104>
- Wingender, J., & Flemming, H.-C. (2011). Biofilms in drinking water and their role as reservoir for pathogens. *International Journal of Hygiene and Environmental Health*, 214(6), 417-423. <https://doi.org/https://doi.org/10.1016/j.ijheh.2011.05.009>
- World Health Organization. (2011). Guidelines for drinking-water quality. *WHO chronicle*, 38(4), 104-108.
- Yablonski, J. I., & Mancuso, S. E. (2007). Preservative efficacy testing: accelerating the process. *Cosmetics and toiletries*, 122(10), 51.
- Yin, R., Cheng, J., Wang, J., Li, P., & Lin, J. (2022). Treatment of *Pseudomonas aeruginosa* infectious biofilms: Challenges and strategies. *Frontiers in microbiology*, 13, 955286. <https://doi.org/10.3389/fmicb.2022.955286>
- Yin, W., Wang, Y., Liu, L., & He, J. (2019). Biofilms: The Microbial "Protective Clothing" in Extreme Environments. *Int J Mol Sci*, 20(14). <https://doi.org/10.3390/ijms20143423>

- Yockey, J., Andres, L., Carson, M., Ory, J. J., & Reese, A. J. (2019). Cell Envelope Integrity and Capsule Characterization of *Rhodotorula mucilaginosa* Strains from Clinical and Environmental Sources. *mSphere*, 4(3). <https://doi.org/10.1128/mSphere.00166-19>
- Zea, L., McLean, R. J. C., Rook, T. A., Angle, G., Carter, D. L., Delegard, A., Denvir, A., Gerlach, R., Gorti, S., McIlwaine, D., Nur, M., Peyton, B. M., Stewart, P. S., Sturman, P., & Velez Justiniano, Y. A. (2020). Potential biofilm control strategies for extended spaceflight missions. *Biofilm*, 2, 100026. <https://doi.org/https://doi.org/10.1016/j.biofilm.2020.100026>
- Zea, L., Nisar, Z., Rubin, P., Cortesão, M., Luo, J., McBride, S. A., Moeller, R., Klaus, D., Müller, D., Varanasi, K. K., Muecklich, F., & Stodieck, L. (2018). Design of a spaceflight biofilm experiment. *Acta Astronautica*, 148, 294-300. <https://doi.org/https://doi.org/10.1016/j.actaastro.2018.04.039>
- Zlatanovic, L., Moerman, A., van der Hoek, J. P., Vreeburg, J., & Blokker, M. (2017). Development and validation of a drinking water temperature model in domestic drinking water supply systems. *Urban Water Journal*, 14(10), 1031-1037. <https://doi.org/10.1080/1573062X.2017.1325501>
- Zou, Y., Liu, C., Zhang, H., Wu, Y., Lin, Y., Cheng, J., Lu, K., Li, L., Zhang, Y., Chen, H., & Yu, Q. (2022). Three lines of defense: A multifunctional coating with anti-adhesion, bacteria-killing and anti-quorum sensing properties for preventing biofilm formation of *Pseudomonas aeruginosa*. *Acta Biomaterialia*, 151, 254-263. <https://doi.org/https://doi.org/10.1016/j.actbio.2022.08.008>