

Biofilm Mitigation Technology Comparison for Life Support Systems Utilizing the Biofilm Test Stand

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Biofilm accumulation in spacecraft water recovery systems is an ongoing challenge and effective biofilm management and mitigation strategies must be identified as long duration space flight and habitation missions commence. Biofilm produced by bacterial and fungal biomass causes severe clogs, damages equipment, and reduces the processing efficiency of recovery systems. Marshall Space Flight Center founded the Biofilm Lab dedicated to addressing this problem and collaborated with the Center for Biofilm Engineering at Montana State University to assess biofilm mitigation technology specific to exploration wastewater systems. The Biofilm Test Stand was created (as discussed previously in ICES-2024-96) specifically to evaluate new technologies for this use case. Over several phases of testing, the Biofilm Test Stand has evaluated 17 mitigation techniques and technologies for biofilm prevention on a smaller scale with ISS like conditions. This paper will discuss the results from each phase of testing, focusing on technology and technique effectiveness, complexity of the technologies and techniques tested, and the paths forward for mission integration.

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Acronyms and Nomenclature

C	= Celsius
CFU	= Colony forming unit
cm	= Centimeter
DAPI	= 4',6-diamidino-2-phenylindole
DAQ	= Data acquisition system
EC	= Electrical conductivity
ECLSS	= Environmental Control and Life Support Systems
ISS	= International Space Station
LB	= Luria-Bertani Broth
MABR	= Membrane-aerated bioreactor
min	= Minute
mL	= Milliliter
PAW	= Plasma activated water
PLA	= Polylactic acid
ppm	= Parts per million
SDB	= Sabouraud Dextrose Broth
TOC	= Total organic carbon
TRL	= Technology Readiness Level
WPA	= Water Processing Assembly

I. Introduction

THE Biofilm Test Stand was created to advance a variety of biofilm mitigation technologies from low TRL through benchtop testing. There are many ways to prevent biofilm, from traditional biocides, nutrient limitation, antibiofilm coatings, heat shock, and shockwaves, and while all have potential, some are more suited to spaceflight applications. While literature reviews, modeling, and basic tests can give scientists an understanding of what may or may not work, technology demonstration in a representative system is essential to identify successful strategies. The general principles of the Biofilm Test Stand target biofilm mitigation strategies with the overall model based on the International Space Station (ISS) Water Processor Assembly (WPA) wastewater (WW) tank. The WPA completed installation on ISS in 2009 but failed in 2010 due to biofilm growth in the tank and downstream flow occlusion of the Mostly Liquids Separator solenoid valve requiring hardware replacement to resume operation. This failure identified a need for biofilm prevention or mitigation technologies in or around the WPA tank such that future missions do not face similar issues when hardware replacement is less feasible. The Biofilm Test Stand informs biofilm mitigation testing for current and future water recycling systems using bioreactors to represent a scaled-down wastewater storage tank based on the WPA WW tank. Bioreactors are inoculated with ISS WPA isolates and fed synthetic wastewater or ersatz representing the tank influent streams (approximately 50:50 urine distillate and humidity condensate). Since initial buildout of this test stand in 2020, multiple phases of testing have addressed a spectrum of technologies with some variation since early testing while the requirements and physical capabilities of the test stand were being established. Testing in Phase 1 was educational but stopped early due to inoculating the upstream feed tank and clogging of the influent filters as reported previously [1, 2]. For Phase 2, the bioreactor inoculation was moved to the reactors and since Phase 2 the test stand has run smoothly, although some quality-of-life improvements and modifications have been made.

II. Biofilm Test Stand

A. Bioreactors and Setup

The standard test setup for all bioreactors is based on calculations for a scaled down version of the wastewater tank on the ISS, where the biofouling has previously resulted in system failure. The bioreactors are commercially available BioSurface Technology Anaerobic CDC Biofilm Reactors (Bozeman, MT), a modified chemostat or continuous flow stir-tank reactor with eight coupon holder rods holding three 0.5 in diameter coupons each made from target material suspended in the bulk fluid for biofilm growth on a defined surface area with stirring via baffled stir bar. The CDC Biofilm Reactors design has been extensively tested and standardized for EPA and ASTM testing to

grow repeatable biofilms [3, 4, 5] and is used on the test stand with modified operation protocols to model WPA biofilms. The bioreactors vessels are a 1000 mL borosilicate glass beaker with a barbed side arm outlet for effluent drainage with polypropylene coupon rods holding Inconel-718 coupons (BioSurface Technologies), WPA tank material. Each reactor has independent feed lines of 1/8th inch silicone tubing connected to a flow break just prior to tubing connected to the port in the reactor lid. The flow break causes an air break in influent media to prevent back contamination from the reactor to the sterile feed tank. The port into the reactor also drops to the bulk fluid liquid level, acting as a secondary flow break. When upstream technology is tested, the technology is sterilized and operated with flowbreak-inline technology-reactor. For MABR and Duckweed testing that contained microbes, the influent line had a flowbreak, the technology, and then the bioreactor. Effluent lines are 3/8th inch silicone tubing. The test stand can run up to 9 bioreactors at a time (Figure 1), with one being an inoculated but untreated control in which there is no mitigation strategy implemented. An uninoculated control is also used and is continuously fed media to confirm the sterility of ersatz throughout the test. Bioreactors are operated at 25 °C and stirred at 125 RPM.

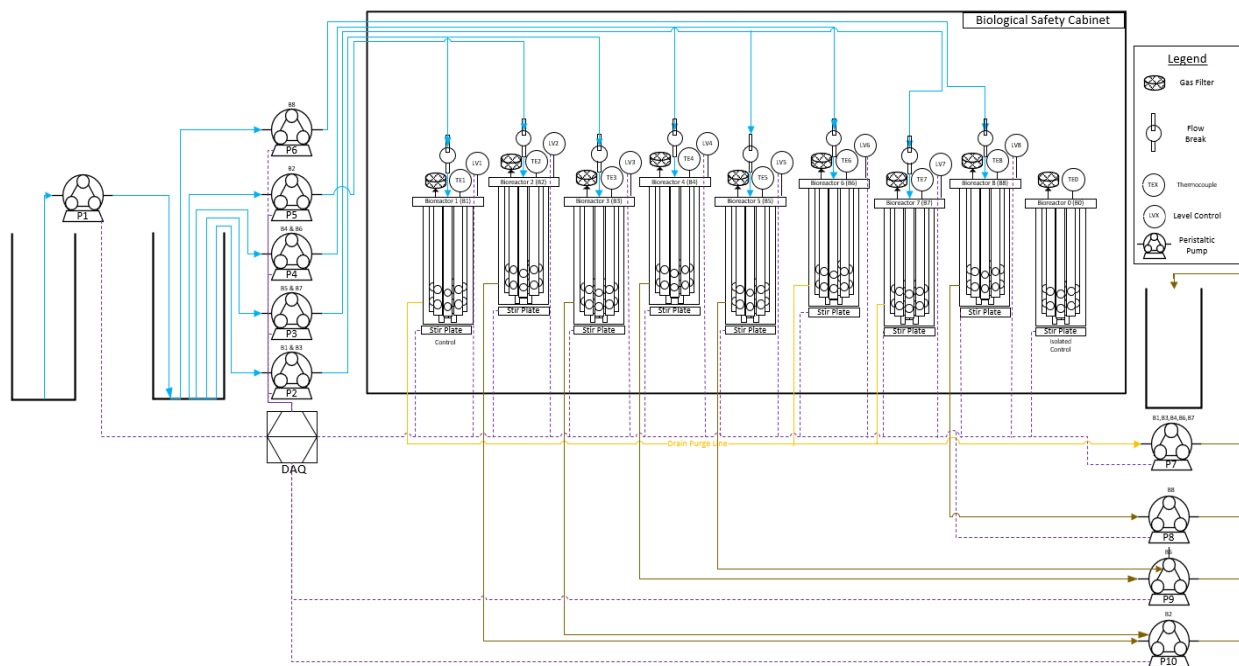


Figure 1. Schematic of the biofilm test stand. Different conditions are tested in each bioreactor.

B. Synthetic Wastewater Feed

The ersatz for testing was Microbial Ersatz [6], a formulation designed on WPA WW tank influents, or a 2022 updated recipe, Microbial Ersatz Minor/Trace nutrients [7] [8]. The ersatz is sterilized in stainless steel tanks, then filter sterilized ersatz concentrates are mixed by the chemistry lab and quality tested for conductivity, TOC, and pH. Quality targets are conductivity between 153 and 187 $\mu\text{mho/cm}$, TOC between 81 and 99 ppm, and pH within 5.0 to 5.5. Any ersatz tank that does not meet these standards is scrapped. The sterile influent ersatz flows at 0.1 mL/min by peristaltic pumps on each feed line from a 20 L tank that supplies all bioreactors. The wetted volume of the bioreactors is approximately 650 mL, and the outlet flow rate is 0.8 mL/min, governed by float switches which activate outlet peristaltic pumps [1]. This provides a residence time of about 3.5 days, similar to the batch processing time of the WPA on the ISS. In early configurations of the test stand, a common drain manifold was utilized, but for later testing campaigns the drain lines were modified to include isolated drains.

C. Inoculation

The assembled reactors and associated tubing are sterilized by autoclaving before each experiment. Inoculum is prepared by overnight culturing of individual organisms in LB for the bacteria and SDB for the fungal strains. At the beginning of the test, the reactors are filled with ersatz and each bioreactor is inoculated through the lid ports to a bulk fluid 10^6 CFU/mL mixture of five bacteria (*Burkholderia contaminans*, *Cupriavidus metallidurans*, *Methylobacterium organophilum*, *Pseudomonas aeruginosa*, *Ralstonia insidiosa*) and three fungi (*Lecythophora mutabilis*, *Aureobasidium pullulans*, *Exophiala spinifera*), and sits for 24 hours with stirring in batch allowing microbes to grow

and establish before continuous flow commences. The reactors are additionally supplemented by weekly inoculations at 10^3 CFU/mL to mimic the continual microbial influx from WPA WW tank influent streams. It should be noted that this inoculation scheme was adopted after Phase 1 testing. In Phase 1, a feed tank (Tank 1) was fed to a secondary tank (Tank 2) that was inoculated prior to pumping to the reactors but filters between the tanks clogged after only 5 days and uninoculated organisms contaminated the feed carboy through the foiled carboy top. There is a natural intuition to inoculate upstream to model systems with a continuous microbial load. However, in biofilm research and reactor systems, this approach is not taken. In addition to practical concerns like biofilm clogging small aperture, high surface area influent tubing (on the Biofilm Test Stand it is approximately 20 ft), from a biofilm engineering perspective, the system boundary for a biofilm reactor is typically set around the reactor volume and the reactor influents/effluents. Inoculating the feed carboy moves the system boundary and mass balance to include the carboy, tubing, and reactor, with the carboy now acting as a bioreactor with tubing modeled as a plug-flow reactor before it reaches the target CDC reactor. This typically means all or most nutrients are consumed upstream and the CDC reactor is fed spent media. For this reason, it is recommended to inoculate the reactor and maintain a sterile feed such that the feed media and target nutrient load is introduced to the organisms in the vessel from which microbial counts will be taken. The recurrent inoculations on the Biofilm Test Stand is a modified version of this inoculation scheme.

D. Sampling

Microbial sampling is performed every 3 weeks in the form of viable counts from bulk water (viable planktonic) and sampling the inner face of three coupons (viable biofilm) which are diluted and plated on R2A and SDA agar using standard methods [4, 5, 3]. Biofilms are also stained with DAPI for microscopy to visualize biofilm and assess biofilm surface coverage. Water chemistry is also monitored with basic water chemistry for TOC, conductivity, and pH and in later phases IC analysis for cation and anions. The tests are concluded at the end of a 9-week period. This test duration was chosen as it allows for a full picture of how a technology performs over time, but by 9 weeks, the outlet tubing of the unmitigated control begins to clog with biofilm for some bioreactors.

This paper will detail results from the testing that has been performed to date (Table 1). As previously mentioned, Phase 1 testing stopped early and those results are not included here. Phase 2 included various elevated temperature treatments and surface coatings. Phase 3 included a MABR, early versions of an in-house duckweed prototype, and a nutrient filter, along with a high inoculation bioreactor and aerated control. Phase 3.5 tested a more advanced in-house duckweed growth unit. Phase 4 tested a redesigned nutrient filter with three downselected resins for biofilm mitigation. Additional testing on using plasma activated water occurred after Phase 4. Some phases had additional tests of proprietary technologies from external partners, which are not discussed in this paper for confidentiality.

II. Phase Two

Table 1. Summary of testing phases on the Biofilm Test Stand. All phases used BioSurface CDC Biofilm Reactors with a target runtime of 9 weeks. MTN=Microbial Ersatz Minor/Trace Nutrients. Methods: VC=viable counts of bulk fluid and biofilm, DS=DAPI staining/microscopy, WC=basic water chemistry (TOC, conductivity, pH), DO=dissolved oxygen, IC=ion chromatography for anion/cations.

Phase	Media	Variations	Duration	Technologies Tested	Analyses	Notes or Challenges
1[1]	Microbial Ersatz	Feed tank inoculation	5d	Heat Shock (65, 72.5, 79.5, 86.4, 93.4°C) Antibiofilm Coatings (brominated, chlorinated)	VC	Testing stopped at 5 days due to clogged influent filters, contaminate feed carboy
2 (2023)	MTN	Modified drain lines, inoculation moved to reactors, carboy sterility controls	9w	Heat Shock (65, 72.5, 79.5, 86.4, 93.4°C) Antibiofilm Coatings (chlorinated)	VC, DS, WC	Elevated CFU counts for a few sample coupons due to dilution issues.
3 (2024)	MTN	Modified drain lines to include better flow	12w	Nutrient Filter (IRN150/Ambersorb 4652) MABR Duckweed Prototype	VC, DS, WC	Weekly inoculation missed at week 5 due to inclement weather.
3.5 (2024)	MTN	Individual drain lines for each technology	9w	Duckweed Growth Unit (two duckweed strains)	VC, DS	Fungal contamination in one duckweed unit after week 3 sample, reactor terminated
4 (2025)	MTN	Same as 3.5	9w	Nutrient Filter (three separate resins)	VC, DS, WC, IC	Week 3 saline contamination

Two categories of technology were examined in Phase 2, temperature treatments, used to rapidly heat and temperature shock the simulated wastewater processing tanks, and a chlorinated antibiofilm coating. Heat shock was anticipated to prevent biofilm by thermal cycling while coatings aim to limit microbial adhesion and reduce biofilm growth.

The temperature shock treatments were evaluated on a cyclical application. The cycle time lasted for 30 minutes every 12 hours. Temperatures varied depending on the bioreactor but stepped up at approximately 7 °C per bioreactor from 65°C to 93.4°C (Bioreactor 1: 65°C, Bioreactor 2: 72.5°C, Bioreactor 3: 79.5°C, Bioreactor 4: 86.4°C, and Bioreactor 5: 93.4°C). Temperature shocks were not tested past 100°C, as the incoming fluid would evaporate. Heated stir plate ramp up time and accuracy to provide heat to the coupons were evaluated and verified prior to testing, work outlined in ICES-2023-90 [9].

The antibiofilm coating for this test was a chlorinated coupon coating (Auburn University). For testing, Inconel 718 coupons were surface modified to allow for coating with a chlorinated compound; surface modification increased chemical adhesion of the chlorinated compound. The functionalized surface with the chlorinated coupon coating was intended to provide a hostile surface to any adhering microbes.

A. Microbial Results

Viable counts for all Phase 2 technologies tested are shown across 9 weeks (Figure 2).

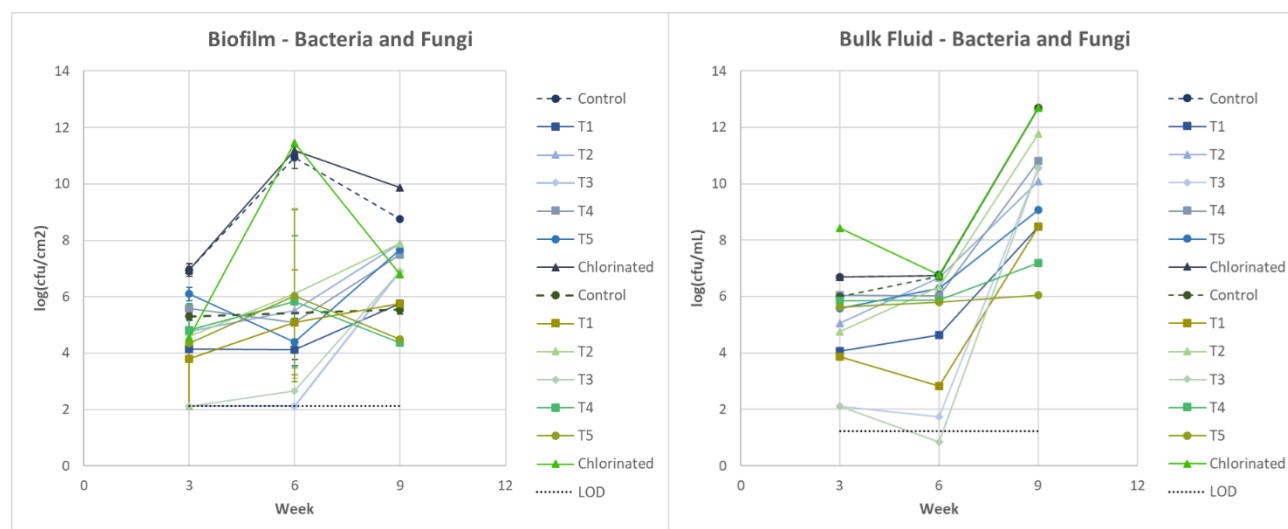


Figure 2. Phase Two biofilm coupon density and bulk water density. *Bacterial results are shown in blue and fungal results are shown in green.*



Figure 3. Clogged drain tubing from all bioreactors (left) and a fully occluded drain tube from bioreactor 3 (right).

The reduced viable biofilm counts from heat shock treatments initially appear positive (Figure 2), with Temperature 3 cycling leading to the largest log reduction of viable bacteria and fungus, however an unanticipated

effect of heat shocking the reactors was transfer of biofilm to the downstream effluent lines (Figure 3). Processing of raw data may have contributed to error in readings for the biofilm counts. All temperature treatments caused clogging in the unheated drains of the bioreactors simulating the WPA tanks. The best treatment per the log reduction, Temperature 3, suffered the worst biofilm formation in the drain line resulting in full flow occlusion (Figure 3, right). The high log reduction in the bioreactors appears to be due to the microbial consortium moving out of the bioreactor that is experiencing heat shocks and into the cooler, non-heated drain line. The microbial consortium then proceeded to set up a robust biofilm in the drain lines, flourishing at an elevated “Goldilocks” temperature, with distance to that zone being dose responsive to the shock temperature. A separate sterile drain line was required for the Temperature 3 reactor for the experiment to continue. Although the temperature shock treatment worked for the wastewater tank, when the entirety of the system is considered, pushing the problem out of the wastewater tank would be more strain on the system versus a mitigation method that eliminates the microbial consortium.

The chlorinated coupon coatings were not effective at mitigating the build-up of biofilm on the Inconel 718 coupons with limited log reductions of bacteria and fungus (Figure 2). The coupon coating surfaces additionally appeared to degrade over the course of the test, due to visible suspended solid chips, which likely affected their ability to stop biofilm.

B. Biofilm Surface Coverage

Below are examples of the DAPI stained coupons. In this phase the images were taken of just a small section of the coupon and used different microscopy settings so can't be directly compared to future phases, but they did act as a baseline visual determination when compared to the log reduction results.

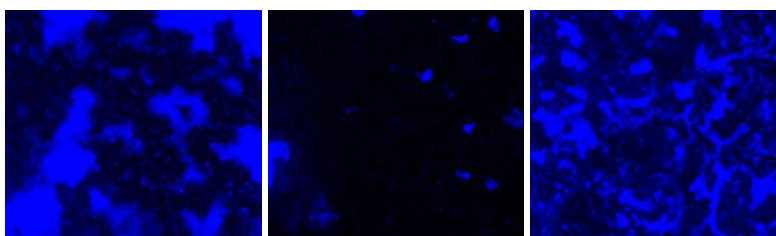


Figure 4. Phase Two DAPI stained Coupons. *Inoculated control (left), temperature 1 (middle), and chlorinated (right) coupons at week 9, taken at 20X zoom and 100% florescence.*

C. Water Chemistry

Water chemistry (TOC, conductivity, and pH) was sampled on a similar cadence as the microbial data, once every three weeks (Table 1). The inoculated untreated control reactor had an approximate 80% reduction in TOC associated with increased pH from the sterile media of 5-5.5 to ~7.5. Reactors with indications of low reductions had intermediate TOC consumption. Values across the 9 weeks was additionally relatively stable suggesting steady state operation at all timepoints.

Table 2. Phase 2 water chemistry.

Bioreactor	TOC (ppm)			Conductivity (µmho/cm)			pH		
	4/5/2023	4/26/2023	5/17/2023	4/5/2023	4/26/2023	5/17/2023	4/5/2023	4/26/2023	5/17/2023
Temp 1	67.6	71.5	35.4	156	180	141	5.6	5.5	7.5
Temp 2	46.3	51.1	29.3	141	148	121	7.5	7.0	8.3
Temp 3	65.5	59.3	29.8	151	150	105	5.8	7.2	8.2
Temp 4	44.9	41.5	36.4	142	135	124	7.1	7.4	7.8
Temp 5	45.3	31.5	27.8	141	131	111	7.5	7.9	8.7
Inoculated Control	16.4	19.2	20.5	131	130	129	7.4	7.7	7.2
Chlorinated	32.4	22.0	14.3	129	130	132	7.2	7.8	7.2
Control	94.5	85.4	91.4	180	180	182	5.2	5.4	5.4

III. Phase 3

Phase 3 technologies all fell under a single concept: nutrient depletion. The idea was to utilize various technologies to reduce or deplete nutrients on the incoming stream of the wastewater tank through physico-chemical or biological

processes, limiting nutrients to the biofilm in the reactor modeling the WPA WW tank. For this test run, three upstream technologies were examined – a resin-based nutrient filter, a Membrane Aerated Bioreactor inoculated with bacteria, and a phytoremediation duckweed prototype.

The physio-chemical approach used resins to target specific chemical compounds. A nutrient depletion canister, built in a similar fashion as the multifiltration beds used on orbit, was used to house two resin types. The design featured a 316 stainless steel canister with a mesh divider at one end, and a spring and floating puck at the other. This compressed the resin to prevent channeling or pocketing. The resin loaded was the same as the current multifiltration beds, 70% Amberlite IRN 150 targeting cations and anions, and 30% Ambersorb 4652 targeting organics. It should be noted that after the experiment concluded, it was discovered that the resins had been temperature treated and dried an unknown number of times during fabrication while optimal loading is of wetted resins and backflow of Ambersorb 4652 to remove air pockets and characterize the resin bed. These factors may impact resin performance.

The Membrane Aerated Bioreactor (MABR) integration has been previously described in ICES-2024-96 [1]. The MABR utilized a purposefully cultivated microbial culture within a bioreactor upstream of the simulated wastewater tank bioreactor to consume carbon and other nutrient sources to limit the downstream biofilm of critical nutrients. An air supply continuously pushed through the center of the MABR forced aeration out into the bacterial growth chamber within the unit, providing aeration to the bacteria. An isolation filter between the technology and MABR was not present.

The final upstream technology used phytoremediation to remove nutrients with a prototype duckweed chamber, with the build being a simple PLA 3D printed tub with overflow skirt, a commercially available growth light, and duckweed sourced from a local aquarium supply store. A more comprehensive discussion of the design and setup is in ICES-2026-410 [10]. The early prototype was integrated into the test stand upstream of the bioreactor, just as the MABR and nutrient depletion canister.

Besides the technologies examined, Phase 3 added two additional untreated control bioreactors. To test the increased aeration of the influent stream for MABR comparison, the first control was an “aerated control” using an uninoculated MABR upstream of the inoculated reactor. The second control was a high inoculum control intended to examine microbial growth in the test bed at 10^6 weekly inoculations throughout the testing regime.

A. Microbial Results:

Bulk fluid and biofilm counts can be found in Figure 5. It should be noted that growth for Phase Three was considerably lower than other test stand phases and fungal counts were below detection by week 9. Based on these results, there was limited but unstable success from two of the technologies, MABR and the Duckweed Early

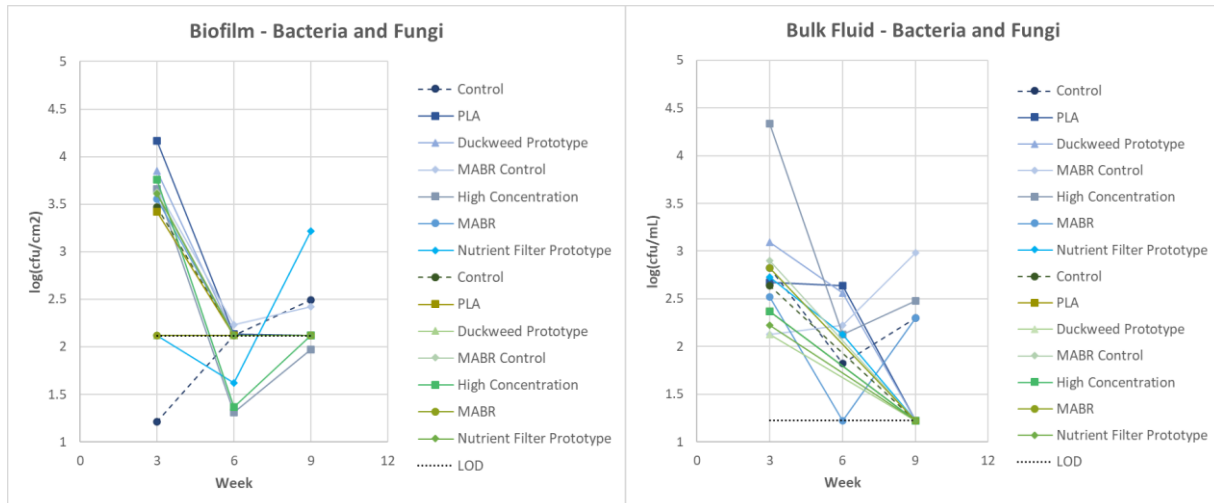


Figure 5. Phase Three biofilm coupon density and bulk water density. *Bacterial results are shown in blue and fungal results are shown in green.*

prototype. However, none of the technologies proved stable throughout the nine weeks, with duckweed having early

success against fungal microbes, and later success against bacterial microbes but issues during other weeks, and MABR similarly having early success against fungal microbes and later success against bacterial microbes but suffering in other weeks. The high concentration mostly showed a slightly higher microbial concentration in weeks three and six for the bacterial and fungal microbes with potentially a small die off of bacterial microbes in week nine and no change in difference between the control in week nine for the fungal microbes. The nutrient canister experienced mostly negative results, with only the first three weeks of bacterial microbial growth experiencing a positive log reduction.

B. Examples of DAPI Stained Coupons

Below are examples of the Phase 3 DAPI stained coupons. Settings for DAPI stain imaging were standardized by maintaining focus, distance from coupon, and fluorescence level. Future works will discuss the surface coverage, results and data processing applied to the coupons.

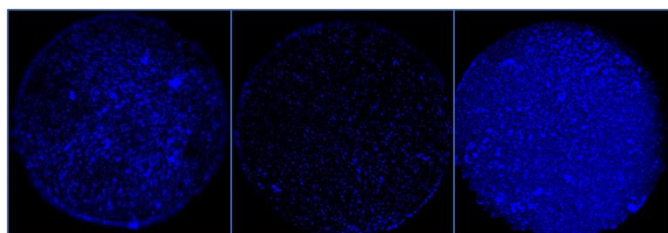


Figure 6. Phase Three DAPI stained Coupons. *Inoculated control (left), MABR (middle), and IRN 150 (right) DAPI stained coupons at week 9 from phase 3 testing.*

C. Water Chemistry

Table 2 shows chemical data taken during the Phase 3 test run. As with Phase 2, only TOC, pH, and conductivity were evaluated. However, the test ran until failure, as opposed to the traditional nine-week test run to push the test stand, chemical sampling data was taken on week one, week three, week six, and week twelve. Additionally, the liquid of the duckweed prototype was also sampled. Results are consistent with reduced nutrients in reactors with high microbial loads. Evidence of cation/anion removal by the nutrient filters can be seen in conductivity measurements while organics removal by TOC is complicated by TOC reduction due to microbial growth.

Table 3. Phase 3 water chemistry.

Bioreactor	TOC (ppm)				Conductivity ($\mu\text{mho/cm}$)				pH			
	1/4/2024	1/25/2024	2/15/2024	3/7/2024	1/4/2024	1/25/2024	2/15/2024	3/7/2024	1/4/2024	1/25/2024	2/15/2024	3/7/2024
Duckweed Early Prototype	14.6	15.7	11.0	11.0	150.2	195.0	156.1	164.2	7.9	7.8	8.3	8.1
Aerated Control	25.0	19.6	14.2	15.0	113.1	129.0	129.0	127.5	7.2	7.6	8.1	7.6
High Concentration	13.0	15.9	10.7	8.5	482.6	161.0	169.9	176.6	6.8	7.6	7.5	8.2
MABR	14.0	12.8	12.5	12.1	103.0	105.0	90.7	100.3	4.7	4.8	5.6	5.4
Innoculated Control	21.4	13.7	15.1	11.1	124.7	133.0	153.2	151.1	7.6	7.6	7.4	8.0
IRN 150/Amborsorb 4652	18.2	18.2	19.0	18.1	7.4	5.7	6.3	6.8	6.2	6.5	6.4	6.6
Clean Control	88.2	85.2	79.6	78.2	176.0	167.6	164.0	168.3	5.0	5.0	5.1	5.0
Duckweed Growth Chamber	17.1	46.7	34.0	12.2	158.9	229.5	165.3	308.2	7.0	6.9	7.3	7.4

IV. Phase 3.5

Phase “3.5” on the Biofilm Test Stand consisted of a mini phase to test an updated duckweed growth unit. For this phase, funding was sourced through a Jacobs Innovation Grant for the testing effort and improved growth chamber design. A more in-depth look at this work can be seen in ICES-2026-410. The updated duckweed unit was still intended as a nutrient depletion technology, but with a refined approach to the hardware. The PLA growth unit was replaced with a stainless steel and borosilicate glass chamber that could be autoclaved for sterility. The grow light was mounted inside the unit along with an integrated level sensor and a glass bubbler for aeration. The float switch plugged into the Biofilm Test Stand system to allow its DAQ to control the level. The aeration line had an inline 0.2 μm filter to maintain sterility of the incoming air that connected into the glass bubbler. Every wetted surface inside the growth unit was sterilized either through autoclaving or a 70% isopropyl alcohol spray.

The duckweed obtained for this test run also represented a more refined approach. The *Lemna minor* sourced from an aquarium store was replaced with a lab grade *Lemna minor* (Blarney 5500) and a *Wolffia arrhiza* (Campania 7678a). Both duckweed samples arrived sterile from Rutgers' research co-op. *Lemna minor* was chosen to replicate the first-round setup, and due to its properties and historical use in spaceflight [11]. *Wolffia arrhiza* was chosen at the time due to it being the selected duckweed crop for the SpaceLabs LLC micro gravity growth unit.

A. Microbial Results:

The graph below illustrates the results for the *Lemna minor* and *Wolffia arrhiza* mitigation. Due to limited resources, only biofilm formation was investigated.

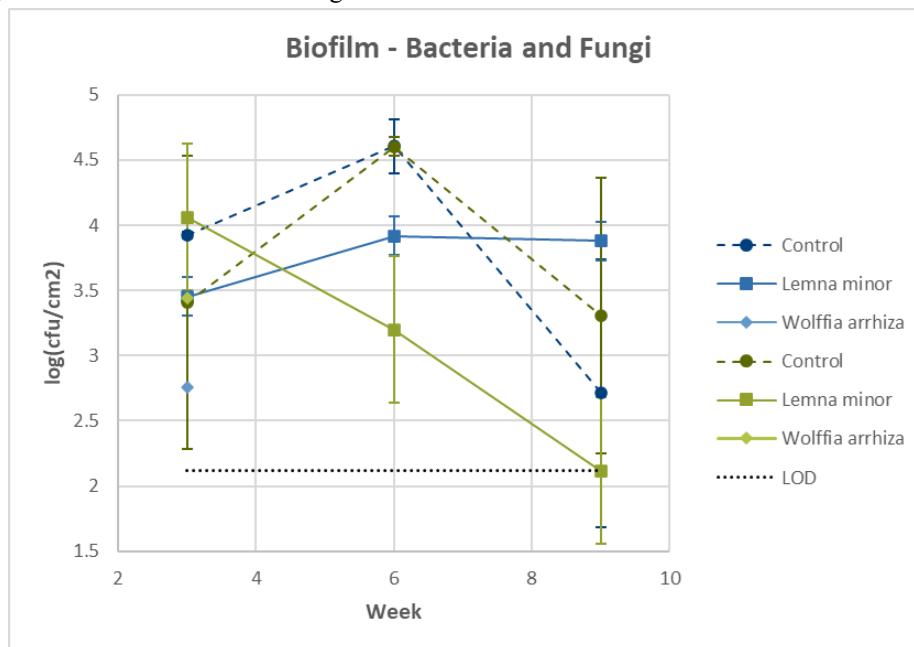


Figure 7. Phase 3.5 biofilm coupon density. Bacterial results are shown in blue and fungal results are shown in green.

Initial results for both duckweed types were variable. After week three, the *Wolffia arrhiza* experienced what is believed to be an outside fungal infection from contamination and had significant die off, representing a possible challenge to phytoremediation technologies. *Lemna minor* continued to produce positive results, with 1.5-2 LR in bacteria, and variable LR in fungus in biofilm.

B. Examples of DAPI Stained Coupons

Below are examples of the DAPI stained coupons for Phase 3.5. The left coupon is the control, and the right coupon from *Lemna minor* Week 3, both the second coupon. Future work will discuss surface coverage analysis.

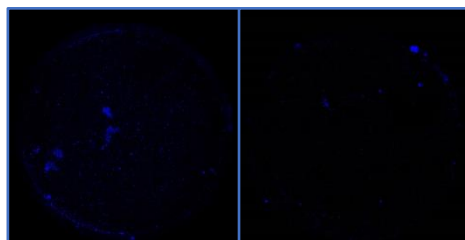


Figure 8. Phase 3.5 DAPI stained Coupons. Inoculated control (left) and *Lemna minor* (right) DAPI stained coupons at week 9 from Phase 3.5 testing.

C. Phase 3.5 Water Chemistry

Water chemistry through Phase 3.5 testing can be found in Table 3. Like the previous duckweed prototype phase, liquid was sampled from the duckweed growth unit. Pond 1 and Pond 2 were the updated growth units for Phase 3.5. *Wolffia* was housed within Pond 1, with the “*Wolffia*” chemical data being the downstream bioreactor. The fungal infection and die off is reflected in sampling stopping after week three. Pond 2 contained *Lemna minor* with the “*Lemna minor*” chemical data being the downstream bioreactor. The duckweed contained in both ponds, along with the bioreactor chemical data reflected a reduction of TOC and a stable pH. Consistent reduction in TOC was observed in the untreated inoculated control reactor and both reactors downstream of phytoremediation units and an increased pH with microbial growth. Phytoremediation units also reduced TOC before the reactor with high reductions in the *Lemna minor* pond.

Table 4. Phase 3.5 water chemistry. Pond 1 is the *Wolffia* growth unit, and Pond 2 is the *Lemna minor* growth unit.

Bioreactor	TOC (ppm)			Conductivity ($\mu\text{mho/cm}$)			pH		
	8/29/2024	9/18/2024	10/10/2024	8/29/2024	9/18/2024	10/10/2024	8/29/2024	9/18/2024	10/10/2024
Clean Control	80.7	79.7	81.5	187.4	188.9	184.0	5.3	5.4	5.3
Inoculated Control	13.4	13.8	13.5	157.0	165.3	148.3	7.7	7.6	7.7
Pond 1	67.4	NA	NA	149.2	NA	NA	6.8	NA	NA
Pond 2	25.7	15.6	15.5	131.4	163.9	147.9	7.9	7.9	8.0
Wolffia	16.5	NA	NA	137.5	NA	NA	7.3	NA	NA
Lemna minor	16.1	12.4	11.4	134.4	176.7	156.3	7.0	7.4	7.5

V. Phase 4

Phase 4 built off the increasing understanding of nutrient limitation to inhibit biofilm growth for this unique waste stream [12]. A previous nutrient limitation screen at CBE found phosphate and magnesium to be growth limiting nutrients and unique targets to limit microbial growth. This testing is detailed in a separate ICES manuscript [13] but will be summarized here. After initial testing of a suite of resins by colleagues at CBE [13], three ion exchange resins were downselected for testing on the Biofilm Test Stand. These resins were IRC 120, BFR 300, and IRN 150 from Dupont. Each resin performs a different function: the IRC 120 removes cations and is meant to target magnesium, BFR 300 removes phosphate, and the IRN 150 removes both cations and anions. Nutrient modeling shows that phosphate (PO_4^-) is the limiting nutrient for biofilm growth in most cases however magnesium significantly changes fungal growth and increased biofouling. For testing, approximately 1.5 L of each resin was loaded into a canister, characterized with backflow, and rinsed with DI water prior to sterilization via autoclaving and integration into the Biofilm Test Stand upstream of reactors. The test stand ran for 9 weeks with no anomalies. Weekly samples from the outlet of each resin canister were tested for their ion content through ion chromatography (IC). This was a new capability for this test stand run as the IC instrument had previously been out of service. Figure 12 shows the results from this phase of testing.



Figure 9. Nutrient canisters integrated into the Biofilm Test Stand. IRC 120 (left), BFR 300 (middle), and IRN 150 (right)

A. Microbial Results

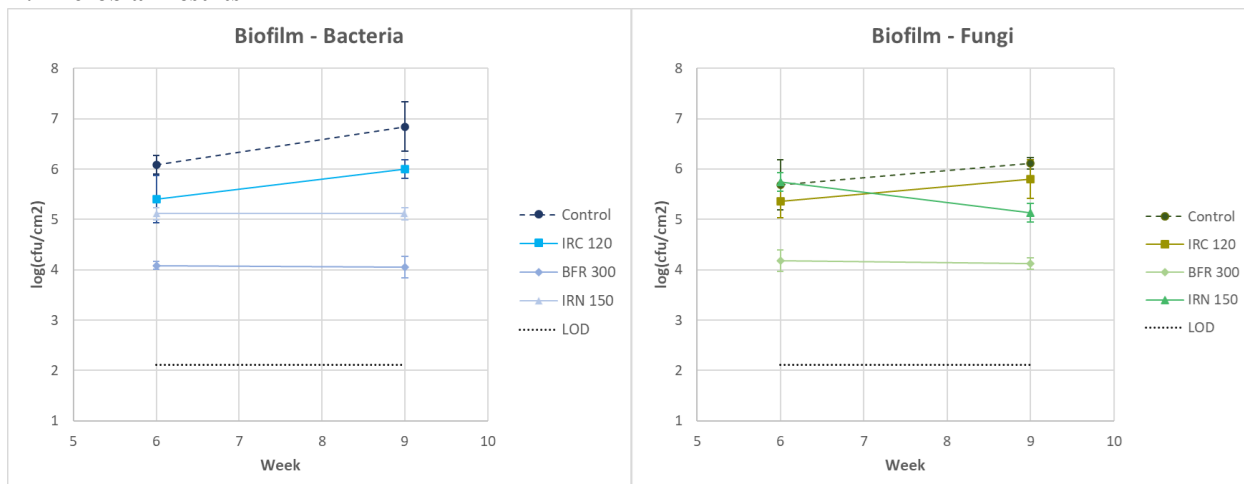


Figure 10. Phase 4 biofilm coupon density. Bacterial results are shown in blue and fungal results are shown in green. Unfortunately, the PBS used for week 3 sample processing was contaminated and therefore the week 3 data is not shown.

As seen in figure 10, the BFR had the highest mean log reduction after the 9-week test with a 3-log reduction in bacteria and 2 log reduction in fungi. While the other two resins did provide some reduction in biofilm growth, they were not as effective as the BFR 300, especially in reducing the fungal portion of the biofilm. A more detailed discussion of these resins is presented in [13] but IRC120 and IRN150 performance may have been impacted by flowrates significantly lower than target flow rates for optimal hydraulics for resin performance.

B. Water Chemistry

Water chemistry was measured for a diversity of ions [13], however, as BFR300 targeting phosphate removal was the strongest performer at reducing microbial growth, dissolved phosphorus as phosphate (PO_4^-) is presented here (Figure 11). The BFR 300 and IRN 150 removed the phosphate from the ersatz below the limit of detection. Despite this removal, BFR300 performance for biofilm mitigation was stronger. IRN150 may also remove inhibitory metals such as zinc and nickel that might enhance growth despite decreased phosphate.

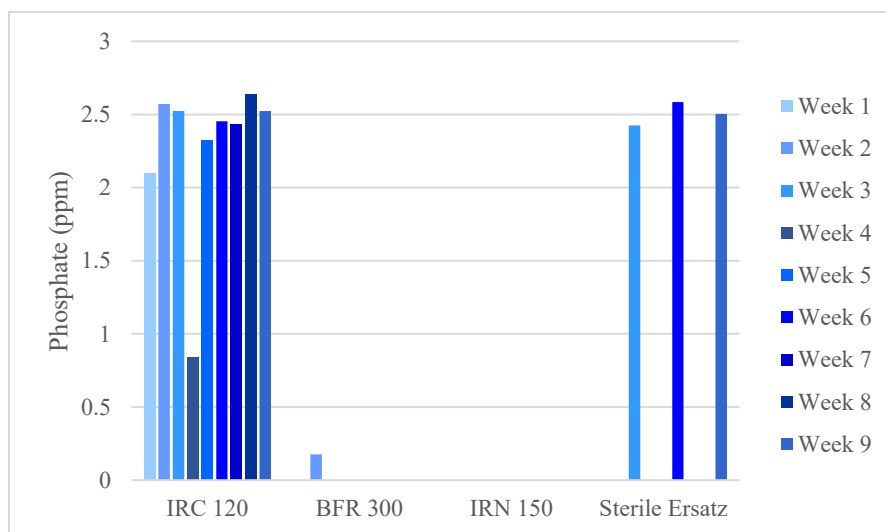


Figure 11. Phosphate levels in the resin bed effluent. Phosphate concentrations in the outlet of each resin canister compared to the sterile ersatz. Limit of detection is 0.10 ppm.

Table 5. Phase 4 water chemistry.

Bioreactor	TOC (ppm)			Conductivity (uS/cm)			pH		
	Week 3	Week 6	Week 9	Week 3	Week 6	Week 9	Week 3	Week 6	Week 9
Control	15.6067	15.7166	18.1443	105.4	165.6	125.5	6.831	7.679	7.499
IRC 120	61.9648	53.9516	51.2365	166.2	157.1	150.2	7.198	6.925	7.427
BFR 300	14.1475	21.2909	28.6723	190.3	188.9	182.5	5.183	5.841	6.18
IRN 150	19.5735	17.5218	18.5868	20.42	5.83	12.365	5.528	5.72	5.7015
Sterile Ersatz	104.7899	93.1012	82.5818	158	165	143.1	5.302	5.26	5.2

C. Examples of DAPI Stained Coupons

Below are examples of DAPI stained coupons from Phase 4 testing. On the left is a coupon from the inoculated control, with the right coupon from BFR 300. These coupons as well as the other Phase 4 coupons will be discussed in future work.

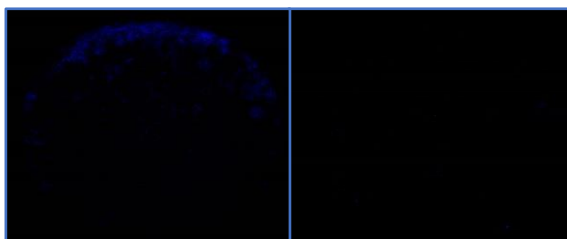


Figure 12. Phase Four DAPI stained Coupons. Inoculated control (left) and BFR 300 (right) DAPI stained coupons at week 9 from Phase 3.5 testing.

VI. Additional Testing

A Marshall Space Flight Center Innovation Fund grant allowed for a test run of plasma activated water (PAW) on the Biofilm Test Stand. When treated with a plasma jet, water becomes more acidic and reactive, which typically inhibits microbial growth, targeting cellular membranes, proteins, and DNA as oxidation sites. In this test, a PAW generator was placed upstream of a bioreactor, treating the ersatz to become “plasma activated.” While confirmed by chemical analysis that the water was indeed more acidic and higher in levels of nitrates and nitrites (plasma byproducts), the treatment bioreactor had more biofilms than the control for much of the test run, by a small and insignificant amount (week 3 mean log reduction = -0.48, week 6 mean log reduction = -0.42, week 9 mean log reduction = 0.09). One notable difference in the control and PAW treatment was the DAPI images, in which the PAW coupons appeared much brighter than the controls. This may be from increased uptake of DAPI in the PAW biofilms due to membrane damage from the reactive species generated in the ersatz, suggesting that perhaps if paired with a biocide, PAW could be an effective treatment. However, on its own, it was not successful. For a more detailed review of this test, see ICES-2026-81 [7].

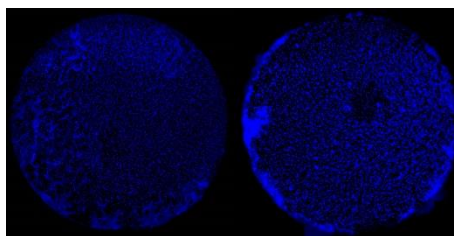


Figure 13. Plasma DAPI stained Coupons. Inoculated control (left) and PAW (right) DAPI stained coupons from week 9 of testing.

VII. Overall comparison

Comparing all results on a single plot allows for direct comparison of each technology. However, some of the more successful technologies in reducing coupon coverage in the bioreactor were unsuccessful in solving the problem.

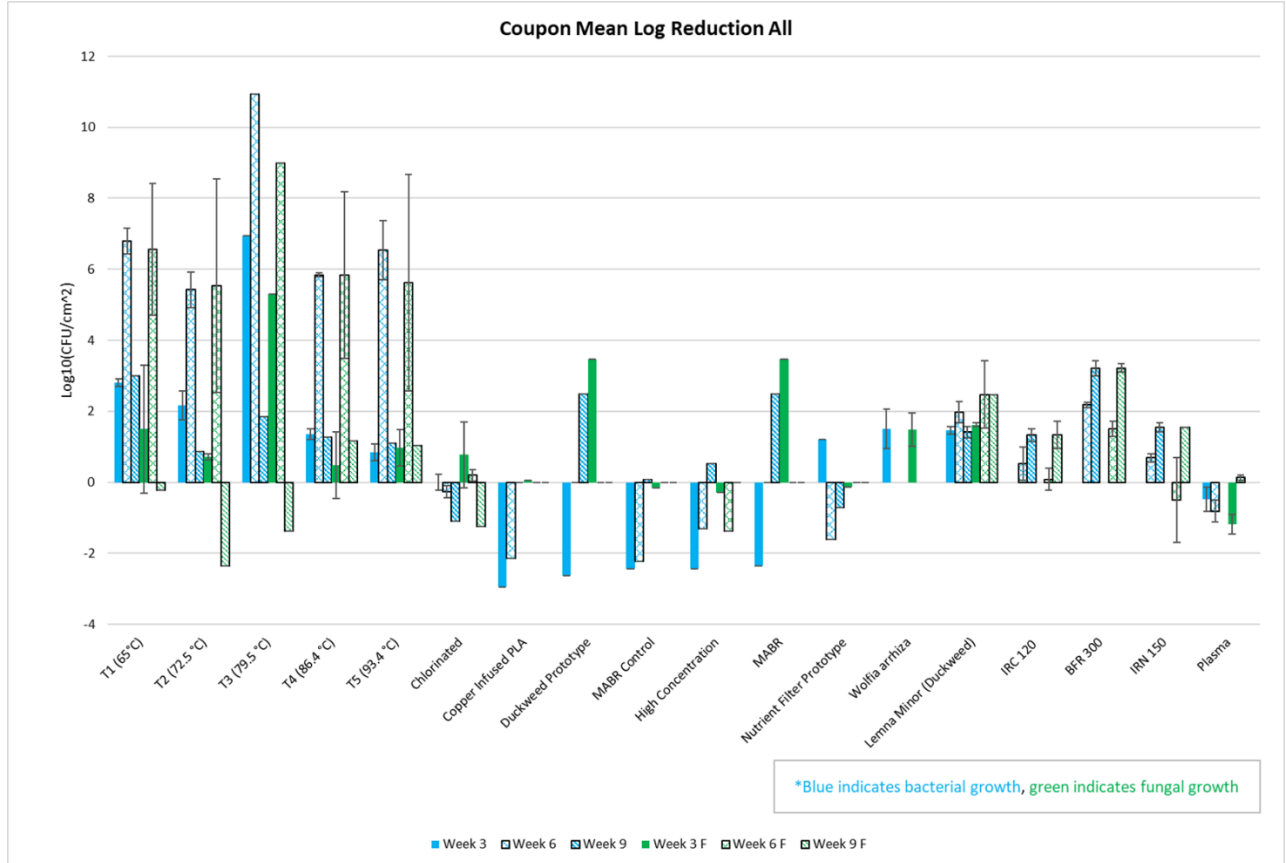


Figure 14. Coupon Mean Log Reduction All Phases. *All phases combined coupon average mean log reduction data.*

After almost three years of biofilm testing, with a combined 341 days of operation on the test stand, the MSFC Biofilm Test Stand has generated abundant data to inform the selection of future biofilm mitigation technologies and techniques. There have been some unexpected results, with duckweed trials outperforming initial expectations and chlorinated coatings promoting growth, instead of reducing it, along with several others. Current data indicates a nutrient limitation strategy appears to be effective in mitigating biofilm formation in the wastewater tank without the negative side effects seen in the elevated temperature treatments. More specifically, a targeted phosphate removal strategy seems very effective.

VIII. Future Work and Full-Scale Testing

Many technologies have been tested on the Biofilm Test Stand, and it is becoming apparent which methods will likely be most successful when implemented into wastewater systems on future missions. While there are still more technologies slated to be tested or improved upon this year, the next step for the most promising candidates is to scale up testing to a flight-like, subsystem-level test. While the Biofilm Test Stand is optimized for testing many technologies quickly, it is no substitute for real system architecture and scale. A full-scale test stand has been designed and is undergoing assembly. It will use 60 L Inconel edge-welded metal bellows wastewater tanks, a pumping schedule resembling the ISS WPA pumping schedule, and materials that are more closely aligned with requirements for spaceflight hardware such as stainless-steel tubing. The test stand will be equipped with water quality sensors and

sample ports to allow for real time data monitoring and frequent chemical and biological sampling. The test stand is designed to be modular and allow for any technology to be tested, although the first run will be with a BFR 300 canister as it showed the best outcome during its Biofilm Test Stand run and is ready in its current configuration for integration into the large-scale test in comparison to a control non mitigated bellows tank.

In addition to building the large-scale bellows test stand, there are 4 technologies running on the Biofilm Test Stand this year. They are an algae photobioreactor, a larger-scale integrated duckweed growth unit modeled after a form factor suited to microgravity, another duckweed growth unit operating with submerged plantlets, and a predatory culture of tardigrades intended to consume biofilm as it is established. While these experiments are all lower TRL, they are all introducing additional species into the system which may make way for future bioregenerative ECLSS developments.

IX. Conclusion

The MSFC Biofilm Test Stand has produced data for many technologies and their effectiveness or lack thereof in stopping biofilm in a closed water recovery system like the WRS aboard the ISS. It has provided a starting point for full-scale testing by assisting in narrowing down the potential technologies ready for integration into such a test. It is a unique platform for testing and has served as a basis for informing biofilm-related decisions here at MSFC and across the agency, as this test stand is the cross-center standard for biofilm testing. The data presented has given the biofilm team insight into what a future wastewater system might need to operate without a biofilm problem.

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