Hollow Fiber Membrane Bioreactor Systems for Wastewater Processing: Effects of Environmental Stresses Including Dormancy Cycling and Antibiotic Dosing

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Membrane-aerated biofilm reactors (MABRs) have been studied for a number of years as an alternate approach for treating wastewater streams during space exploration. While the technology provides a promising pre-treatment for lowering organic carbon and nitrogen content without the need for harsh stabilization chemicals, several challenges must be addressed before adoption of the technology in future missions. One challenge is the transportation of bioreactors containing intact, active biofilms as a means for rapid start-up on the International Space Station or beyond. Similarly, there could be a need for placing these biological systems into a dormant state for extended periods when the system is not in use, along with the ability for rapid restart. Previous studies indicated that there was little influence of storage condition (4 or 25°C, with or without bulk fluid) on recovery of bioreactors with immature biofilms (48 days old), but that an extensive recovery time was required (20+ days). Bioreactors with fully established biofilms (13 months) were able to recover from a 7-month dormancy within 4 days (~1 residence). Further dormancy and recovery testing is presented here that examines the role of biofilm age on recovery requirements, repeated dormancy cycle capabilities, and effects of long-duration dormancy cycles (8-9 months) on HFMB systems. Another challenge that must be addressed is the possibility of antibiotics entering the wastewater stream. Currently, for most laboratory tests of biological water processors, donors providing urine may not contribute to the study when taking antibiotics because the effects on the system are yet uncharacterized. A simulated urinary tract infection event, where an opportunistic, pathogenic organism, *E. coli*, was introduced to the HFMBs followed by dosing with an antibiotic, ciprofloxacin, was completed to study the effect of the antibiotic on reactor performance and to also examine the development of antibiotic-resistant communities within the system.

Nomenclature

AOB = Ammonia-oxidizing bacteria
BF = with bulk fluid
BWP = Biological water processor

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DNRA = Dissimilatory nitrate reduction to ammonium
DO = Dissolved oxygen
GFP = Green fluorescent protein
HPLC = High-performance liquid chromatography
ISS = International Space Station
KSC = Kennedy Space Center
MABR = Membrane-aerated biofilm reactor
NBF = without bulk fluid
NOB = Nitrite-oxidizing bacteria
ppm = Parts per million
ssMABR = Sub-scale membrane-aerated biofilm reactor
TN = Total nitrogen
TOC = Total organic carbon
UTI = Urinary tract infection

I. Introduction

A.Dormancy-Recovery Cycling in Biological Water Processors (BWPs)

While terrestrial establishment of biofilms and operation of biological water processors (BWPs) has been examined for many years with batch reactors, membrane-aerated reactors, and similar systems, unique challenges are presented when exploring their use in microgravity[1, 2]. These challenges include transport of the system to its destination, whether that be the International Space Station (ISS) or beyond, as well as procedures for storing the system for various durations of nonuse (e.g., if the ISS or a beyond Earth habitat was to be unmanned for a period of time) with rapid recovery when operations are restarted. Few studies have examined an intentional stop, store, and start operating regime as proposed; these perturbations could introduce stresses to the microbial communities essential for wastewater processing. However, numerous studies, some using small-scale biological reactors, have examined the impact of chemical waste streams, changes in feed composition, and potential starvation conditions due to industrial process shut downs and less than optimal environmental conditions on wastewater treatment plants; the impact on microbial communities involved in conversions of carbon and nitrogen compounds has also been explored[3-6]. Strategies to restore a functioning microbial community to a wastewater treatment process can be utilized in smaller-scale systems. Examples include re-inoculation of a system with viable bacteria, selection for certain groups of microbes by change in feed composition, or mitigation of the impact of detrimental conditions on the microbial communities. Biological nitrogen removal depends on the establishment and maintenance of slower growing ammonia and nitrite oxidizing bacteria (AOB/NOB) with concomitant denitrification accomplished by faster growing organisms. In addition, rapidly growing carbon oxidizing bacteria present in the system compete for resources such as oxygen. Membrane-aerated biofilm reactors (MABRs) provide a solution for partitioning resources for these diverse groups of microorganisms encouraging the establishment of nitrifying bacterial biofilms on the surface of the membrane where oxygen is concentrated. Bacteria in biofilms are less susceptible to chemical and environmental stresses than planktonic populations and have the ability to recover to pre-stress metabolic activities and growth when more optimal conditions are restored[4-6].

Throughout 2014, Kennedy Space Center (KSC) explored several dormancy processes for established bioreactors to determine optimal storage and recovery conditions. This work focused on complete isolation of the microbial community from an operational standpoint (no recycle flow, no feed, no gas flow, etc.). Two major considerations were tested: 1) storage temperature and 2) storage with or without the reactor bulk fluid. The first consideration was tested from a microbial integrity and power consumption standpoint; both ambient (25°C) and cold (4°C) storage conditions were studied. While room temperature would be optimal for low power consumption (no need for cooling), cold storage was also tested to determine if better microbial recovery could be obtained. The second consideration was explored, again, for microbial integrity as well as plausible real-world scenarios of how terrestrially-established bioreactors could be transported to space and stored for periods of time between operations. Established biofilms were stored without the reactor bulk fluid to simulate transport of established biofilms into space, while other biofilms were stored with the reactor bulk fluid to simulate the most simplistic storage condition to initiate in preparation for an extended period of nonuse in a habitat (i.e., simply turning off power, pumps, feed, etc., without the need for extended storage preparations). Detailed results from these studies can be found in previous reports and publications[7]. Briefly, KSC's 1-L MABR 30-day dormancy studies demonstrated that there did not appear to be a dormancy condition (with/without bulk fluid, 4°C/25°C) that was better at preserving reactor performance metrics. While all reactors in the study (reactors 11-14) recovered and even experienced improved performance, the recovery period was

not satisfactory (20+ days). Several factors may have contributed to such a long recovery period. First, the biofilms in these studies were not mature (48 days of reactor operation prior to dormancy initiation), and may not have been robust enough to handle full wastewater feed directly after a four-week dormancy period. Second, the amount of nitrifying and denitrifying communities may not have been well enough established in the biofilms to provide adequate conversion rates. A long duration dormancy experiment with a 1.8-L sub-scale MABR (ssMABR), containing fully established and more mature biofilms (13 months old), showed the system was able to recover from a 7-month dormancy period to steady state operation within 4 days (~1 residence cycle). Both the maturity of the biofilms and the addition of a "buffer" bulk fluid to dilute the introduction of the full wastewater feed likely helped to speed up the recovery period.

Further examination of dormancy-recovery cycles was continued after the aforementioned initial testing to expound relationships between biofilm maturity, dormancy duration, and dormancy condition with the ability to rapidly recover systems to acceptable conversion metrics. Three additional dormancy-recovery experiments have been completed to date focusing on longer duration cycles of 8-9 months, under varied storage conditions. The 1-L and ssMABR systems utilized in the 2014 studies were put into a second dormancy cycle to also examine the ability for repeated cycling of dormancy and operational states.

B. Pathogenic and Antibiotic Dosing of BWPs

In current bioreactor testing at KSC, urine donor requirements restrict donations if the individual is currently taking, or has taken within the last week, any antibiotics which could irreversibly damage the microbial community. If such systems were ever implemented for future long-duration missions, a firm understanding of the effects of antibiotics on these biological systems is pertinent to the success of the technology. While similar microbial communities in typical wastewater treatment plants come into contact with wastewater streams containing antibiotics and their metabolites on a regular basis, the overall size and design of the treatment plants are not easily translated to a smaller MABR system. To test a relevant scenario, a simulated urinary tract infection (UTI) was carried out where pathogenic organisms where introduced to an MABR followed by treatment with an antibiotic.

To simulate bacteria excreted in urine due to a UTI, *Escherichia coli* was chosen for these experiments since it is the causative agent for 85% of such infections. The beginning of the UTI event was initiated by inoculation of a reactor with the *E. coli* followed by antibiotic dosing three days later. The presence of $>10^3$ bacteria per mL of urine collected for testing constitutes a positive diagnosis for a UTI[8]; the inoculum used in these studies was adjusted to 10^4 bacteria per mL to fall within the mid-range of concentrations reported to be present in UTI cultures. *E. coli* strain K12, with a Green Fluorescent Protein (GFP) marker, was utilized to enable tracking of the bacterium in the reactor effluent over the course of the experiment. The bacteria was added to the urine component of the waste stream, which comprises about 15.5% of the total influent, for the first four days of the experiment. It was assumed urine would be cleared of the bacterium after 72 hours of treatment as has been shown in clinical studies.

Two main antibiotics are available to crewmembers during ISS missions: sulfamethoxazole/trimethoprim (brand name, Bactrim) and ciprofloxacin. Both options were researched for excretion of the compound itself, as well as metabolites, from urine; metabolite antimicrobial capacity was also considered. For Bactrim, both compounds, as well as a metabolite, N4-acetylsulfamethoxazole, with high antimicrobial properties, are excreted in urine[9]; to accurately demonstrate the effects on an MABR system, the cost of the metabolite to simulate the concentrations found in urine was prohibitively expensive. While ciprofloxacin has four metabolites, as well as the parent compound, excreted in urine, the metabolites either do not possess antimicrobial properties, or are orders of magnitude lower in activity than the ciprofloxacin itself, eliminating the need of the metabolites in the study[10]. Common treatment for a UTI with ciprofloxacin is an oral dose of 250-500 mg every 12 hours for 7-14 days[11]; for this study, a conservative dose of 250 mg for a 7-day duration was chosen for 1 of 4 crewmembers infected. The average percentage of the dose recovered in urine is 40-50%[12]. After a 250-mg dose, urine concentrations have been seen to exceed 200 μg/mL during the first two hours, and 30 μg/mL 8-to-12 hours after dosing. Urine was dosed with 22 μg/mL (ppm) of ciprofloxacin for an expected concentration in the influent feed of 3.42 μg/ml.

The objectives of this experiment were to 1) determine if the antibiotic persists in the system as shown by breakthrough of the original compound or expected metabolites in the effluent for consideration for downstream treatment for potable water, 2) determine the impact of the introduction of a pathogen and antibiotic on the performance metrics of the reactor, 3) examine the possibility of antibiotic resistance increases in *E. coli* introduced into the system as well as other members of the microbial community, and 4) elucidate if particular types of bacteria are impacted by the antibiotic, especially those integral to the metabolic processes necessary for carbon oxidation and nitrification. In both repetitions of this experiment, a set of our reactors were used with the following experimental setup: a control reactor, a ciprofloxacin-dosed reactor, an *E. coli*-dosed reactor, and an *E. coli*-dosed reactor subsequently treated with ciprofloxacin.

II. Methods

A. Reactor Operations, Storage, and Recovery

A total of eight 1-L MABR systems (reactors 11-18) and a 1.8-L sub-scale MABR system (CR2) were used for all dormancy-recovery studies. An overview of reactor dormancy and recovery parameters is outlined in Table 1. During all dormancy cycles, reactors were completely isolated (i.e., no gas flow, no recirculation, no influent feed) and stored at the specified temperature with or without the bulk fluid within the reactor. Successful recovery was evaluated based on each individual reactor's ability to regain or surpass its pre-dormancy performance metrics rather than a direct comparison between reactors. To recover the reactors stored *with* their bulk fluid, after the dormancy period, full-strength wastewater at either a 3.79- or 5.00-day residence was immediately introduced to the reactors and allowed to slowly dilute into the bulk fluid over time. To recover those stored *without* their bulk fluid, the reactors were filled with a buffer solution consisting of wastewater without urine, and full-strength feed at the desired residence time was immediately introduced; the buffer allowed the biofilms to be gradually introduced to the feed without completely overwhelming the system. Detailed methods are documented in previous work[7] and throughout the results section of this paper.

Table 1: Reactor Dormancy-Recovery Parameters

Reactor	Storage Conditions*	Biofilm Age at First Dormancy	Dormancy Cycle 1	Total Recovery Period	Dormancy Cycle 2
11	4°C, NBF	7 weeks	4 weeks	8 weeks	8 months
12	25°C, NBF				
13	4°C, BF				
14	25°C, BF				
15	4°C, NBF	14 weeks	9 months	4 months	N/A
16	25°C, NBF				
17	4°C, BF				
18	25°C, BF				
CR2**	4°C, NBF 25°C, NBF	13 months	7 months	1.5 months	9 months

* BF: Stored *with* bulk fluid; NBF: Stored *without* bulk fluid.

** Dormancy cycle 1 for CR2 was carried out at 4°C, while dormancy cycle 2 was carried out at 25°C.

B. Pathogenic and Antibiotic Dosing

1-L MABR set 11-14 were used in the first antibiotic dosing experiment with the following scheme: reactor 11 received ciprofloxacin dosing, reactor 12 received inoculation with *E. coli* followed by introduction of ciprofloxacin 3 days later, reactor 13 received inoculation with *E. coli* and no antibiotic dosing, and reactor 14 served as a control /reactor. A duplicate study was performed with 1-L MABR set 15-18 with the following scheme: reactor 15 served as a control reactor, reactor 16 received inoculation with *E. coli* and no antibiotic dosing, reactor 17 received inoculation with *E. coli* followed by introduction of ciprofloxacin 3 days later, and reactor 18 received ciprofloxacin dosing. For reactors receiving the *E. coli* spike, an inoculum adjusted to 10^4 bacterial mL was added to the urine component of the influent waste stream for the first four days of the experiment. On day four, the reactors receiving antibiotic treatment had ciprofloxacin added to the influent waste stream at a concentration of 3.42 µg/mL. Ciprofloxacin treatments continued at this level for seven days. Effluent samples were monitored daily for changes in performance metrics, ciprofloxacin concentration, and for the development of antibiotic resistant strains. Biofilm samples were also collected prior to *E. coli* addition, prior to ciprofloxacin addition, and 14 days after all treatments were ended; these samples were preserved for future microbial community analysis.

C. Chemical Analysis and System Monitoring

Chemical analyses to calculate performance metrics including urea removal, ammonia removal (nitrification/denitrification), and total carbon removal were performed by collecting and analyzing reactor effluent a minimum of three times per week. Further, continuous data monitoring of other system metrics including pH, dissolved oxygen (DO), gas flow rates, system back pressures (liquid and gas) was also attained using Opto 22 software.

1. Quantification of Urea

Urea analysis was completed using an Agilent 1100 HPLC system equipped with a Zorbax HILIC Plus column (4.6 x 100 mm) and Zorbax guard column (4.6 x 12.5 mm). The mobile phase utilized was acetonitrile: 20 mM K_2PO_4 (90:10 v/v) at a flow rate of 1.5 mL min^{-1}; the sample injection volume was 30 µL with detection at 210 nm using a diode array detector. All samples were diluted with acetonitrile so that no more than 10% of the sample volume was water and filtered through a 0.45 µm nylon filter; if required, samples were concentrated under a nitrogen stream prior to analysis.

2. Quantification of Total Organic Carbon & Total Nitrogen

Total Organic Carbon (TOC) samples were collected and analyzed within one week; samples were filtered through 0.2 µm SFCA filters with a GF pre-filter and stored in 4°C until analyzed. The TOC analysis was performed on an OI Analytical Aurora 1030C TOC/TN Analyzer; a 10 to 400 ppm carbon quantification range was utilized.

3. Quantification of Ionic Species

A dual Dionex ICS-2100 system, configured to simultaneously analyze anions and cations, equipped with a conductivity cell (DS6), vacuum degasser, column heater, eluent generator, and self-regenerating suppressor (Dionex ASRA 300, 4 mm and CSRS 300, 4 mm) was used for ion chromatographic analysis of samples using a modified version of EPA Method 300.1. Separation was achieved isocratically on a Dionex IonPac AS18 and IonPac CS12A column (4 x 250 mm) using 32 mM potassium hydroxide and 20 mM methanesulfonic acid, both with a flow rate of 1 mL min^{-1}, column temperature of 30°C, and 5 mL sample volume (through a 25-µL injection loop and Dionex AS-DV autosampler). Samples were filtered through 0.2-µm SFCA filters with a GF pre-filter and stored at 4°C until analyzed.

4. Quantification of Ciprofloxacin

Ciprofloxacin analysis was completed using a Thermo Scientific Accela HPLC system equipped with a Varian Polaris 3 C18-A (2.0 x 100 mm). The mobile phase utilized was acetonitrile: 2% acetic acid in water (14:86 v/v) at a flow rate of 200 µL min^{-1}; the sample injection volume was 5 µL with detection at 280 nm using a photodiode array detector. All samples were filtered through a 0.20-µm nylon filter prior to analysis.

III. Results & Discussion

A.Dormancy-Recovery Cycling Studies

1. 1-L MABR Set 11-14

As previously discussed, 1-L MABRs 11-14 underwent a 30-day dormancy period which demonstrated that the storage condition did not seem to play a role in the recovery capacity of the reactors; further, all of the reactors recovered and even experienced improved performance, though the time it took to reach appreciable conversion metrics was not satisfactory (20+ days). Recovery and monitoring of these systems lasted for approximately 8 weeks to allow for further development of the biofilms before a second dormancy period was initiated (Table 1). This second dormancy cycle mirrored previous conditions and lasted 8 months.

Recovery of the systems after the long-duration dormancy cycle included introduction of full-strength feed containing urine, flush water, hygiene wastewater, laundry wastewater, and humidity condensate, at a 3.79-day residence period for all reactors. For reactors that contained no bulk fluid, the reactor was filled with fluid containing typical levels of humidity condensate, hygiene wastewater, and laundry wastewater (with no urine) as a buffer solution to assist in easing the microbial community back into operation, as this was seen to assist in fast recovery for the ssMABR CR2 reactor previously. Figure 1 shows average performance metrics for 1-L MABRs 11-14 for both dormancy cycles completed. Reported average values for post-cycle 1 conversion rates are for metrics obtained after 21 days of recovery, as previously discussed. Average values for post-cycle 2 conversion rates remained consistent, and similar to pre-dormancy rates, after 7 days of recovery with minimal further improvement as time progressed. In an attempt to improve reactor conversion rates further, 100% oxygen as a gas feed was introduced into reactors 12 and 14; both of these systems had undergone dormancy at 25°C and appeared to have slightly lower conversion metrics than reactors 11 and 13, having undergone their dormancy cycles at 4°C. Further, on Day 55 of recovery, automated heating of all four reactors to 28°C was initiated. Room temperature for the laboratory remained between 21 and 23°C, with several sharp decreases to as low as 18°C on several occasions due to building maintenance. It is known that AOB perform optimally between 25-30°C; with an increase of 10°C, growth rate of nitrifiers can triple[13]; due to the fickle nature of the room temperature control, localized heating of the reactors to the optimal range for AOB was initiated. An increase in ammonia removal was observed after these changes in operation from Days 55 to 85 (Figure 2) for all reactors; those with 100% oxygen instead of air as a gas feed showed slightly higher removal, though this

difference is likely not statistically significant. Urea hydrolysis and Total Organic Carbon (TOC) removal were already at maximum conversion and were not seen to have any obvious alternations.

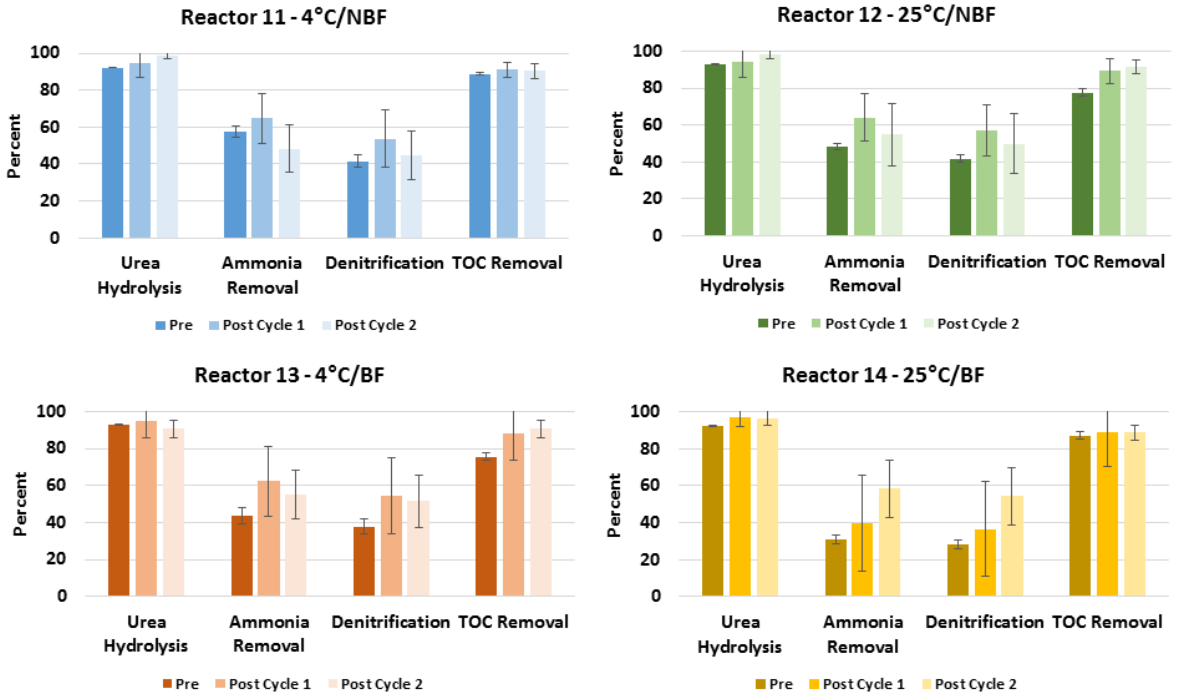

Figure 1: Performance metrics for 1-L MABRs 11-14 prior to any dormancy cycle, after dormancy cycle 1, and after dormancy cycle 2. Error bars represent standard deviation.

Over the course of the recovery of all reactors, 1 M HCl was added to the influent feed in an attempt to lower reactor pH; pH in all reactors ranged from 8.3-8.7, far above the range where nitrifying communities perform optimally. Reactors were slowly weaned off acid dosing after three months of operation, when it appeared that acid did not assist in lowering pH values or have any positive effect on the systems. From Days 87-92, as acid in the feed was decreased to nothing, a sharp decrease in ammonia removal for reactors 11 and 13 (air feed) was observed (Figure 2); only a minor decrease in performance was seen in reactors 12 and 14 (oxygen feed); this sudden change in performance points to a positive effect of acidifying the feed, even if pH values within the reactor were not greatly changed. It took both reactors 11 and 13 nearly two weeks to recover ammonia removal capacity, unlike the other two reactors, recovering in only 2-3 days. After the end of acid dosing, both air-supplied reactors also experienced a slight decrease in urea hydrolysis and TOC removal as well as increase in pH from 8.1-8.3 to 8.8 and remained at this high level throughout the remainder of the study. Oxygen-supplied reactors also saw an increase in pH from 7.5-7.8 to 8.0, followed by a rapid decrease back into the mid-to-low 7 range, with no disruption in urea hydrolysis and TOC removal capacity. By Day 111 (Figure 2), all reactors had increased their ammonia removal capacity to high levels; however, it is apparent that the addition of 100% oxygen to reactors 12 and 14 not only helped the microbial community better handle the elimination of acidified feed more than those receiving only air, but also had allowed both reactors to maximize ammonia removal to ~90% - roughly 15-20% higher removal than reactors 11 and 13. As seen in the metrics reported in Figure 2, while pH has remained high in reactors 11 and 13, conversion rates have not seemed to suffer greatly. It was hypothesized that the NOB communities, while present, may have suffered from repeated dormancy cycles. All reactors demonstrated appreciable ammonia removal, showing AOB activity. There existed a persistence of nitrite (50-175 ppm) in all four reactors, while no buildup of nitrate was seen; this trend shows that NOB communities may have been weakened after the long-duration dormancy cycle. What nitrite was converted to nitrate appears to have been subsequently denitrified by other communities, or nitrite was directly denitrified and never processed by NOB.

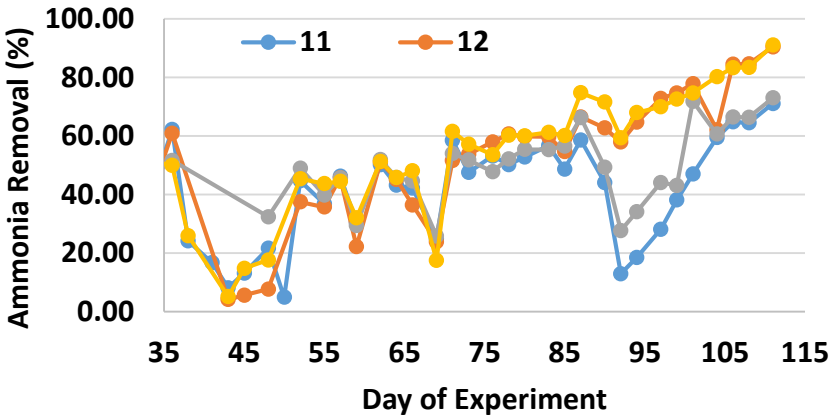

Figure 2: Ammonia removal for 1-L MABRs 11-14 after addition of 100% oxygen gas feed for reactors 12 (Day 43) and 14 (Day 38), temperature control to 28°C for all reactors (Day 55), and end of acid dosing to feed (Days 87-92).

To address why processes changed over the course of this study, biofilms were sampled in all reactors for determination of the community profile after reactor inoculation from the KSC inoculum tank, prior to a dormancy cycle, at the beginning of the recovery phase, and after extended recovery when the reactors had reached steady state. Upon completion of a 16s rRNA PCR reaction, which barcoded each sample with a unique identifier, a sequencing library was constructed with equimolar concentrations of each sample and sequenced on the Illumina MiSeq next-generation sequencer. Table 2 highlights the general sequencing results to the genus and species level for reactors 11-14 throughout each phase of the dormancy cycles. "Prehib-1" includes the establishment of biofilm after the initial reactor inoculation while "Posthib" represents the state of the biofilm as the system was taken out of the dormancy cycle to begin recovery. "Stabilize" refers to biofilm samples taken after extended recovery at the end of all dormancy-recovery experiments.

Table 2: Reactors 11-14 Dormancy conditions and General Sequencing data to the genus and species taxonomic categories. The Shannon Index indicates the level of diversity.

Sample	Dormancy Conditions	Total Reads	Total Reads PF	# Genus ID	# Species ID	Shannon Index
011-Prehib-1	4°C/NBF	444,520	391,211	375	514	2.099
011-Posthib-1	4°C/NBF	607,590	556,715	370	497	2.111
011-Prehib-2	4°C/NBF	376,418	339,916	416	567	2.454
011-Posthib-2	4°C/NBF	461,465	405,672	439	575	2.376
011-Stablize	4°C/NBF	645,450	578,725	455	659	2.389
012-Prehib-1	25°C/NBF	704,840	627,258	422	621	2.221
012-Posthib-1	25°C/NBF	422,152	343,090	379	460	1.930
012-Prehib-2	25°C/NBF	596,268	528,619	456	632	2.097
012-Posthib-2	25°C/NBF	403,262	359,351	444	591	2.330
012-Stablize	25°C/NBF	525,039	462,963	494	739	2.692
013-Prehib-1	4°C/BF	512,575	464,212	409	603	2.429
013-Posthib-1	4°C/BF	422,152	384,874	379	508	2.346
013-Prehib-2	4°C/BF	596,974	530,967	438	596	2.288
013-Posthib-2	4°C/BF	402,216	353,757	456	596	2.493
013-Stablize	4°C/BF	612,975	547,970	487	711	2.650

014-Prehib-1	25°C/BF	466,473	409,651	401	510	1.913
014-Posthib-1	25°C/BF	554,761	496,104	427	613	2.293
014-Prehib-2	25°C/BF	436,972	385,399	415	575	1.740
014-Post-2	25°C/BF	31,718	27,240	215	217	2.489
014-Stablize	25°C/BF	643,845	570,418	497	725	2.489

Sequencing results indicated that the inoculum used for the reactors 11-14 had low diversity and was primarily populated with numerous species of *Pseudomonas*. However, after initial biofilm formation, it appeared that the community diversity changed and the *Pseudomonas* was outcompeted and replaced by other species. For example, in Table 2, the number of genus and species, as well as the Shannon Diversity Index, is reported for each biofilm at each stage and each treatment. The first dormancy cycle for reactor 11 was 4°C with no bulk fluid. During the first dormancy period, there was a small loss in the number of species with no significant difference in diversity. After 8 weeks of recovery, the number of species increased along with an increase in diversity. A longer, second dormancy period lasting 8 months was completed with no significant difference in the number of species or diversity indicating that the lower temperature may not have a negative effect upon an established biofilm. After a recovery period, the stabilized reactor showed a significant increase in the number of species. Reactor 13, also stored at 4°C but *with* bulk fluid showed similar trends with a small, initial decrease in the number of species during the first dormancy period, but upon recovery, continued a gradual increase (Table 2). This may be due to the fact that cellular metabolism of the bacterial species present may be slower at the lower temperature and the nutrients available (i.e., carbon) would be sufficient to maintain the biofilm community. This may indicate that bulk fluid may not be required to sufficiently maintain the bioreactor during a dormancy period. A longer dormancy study with replication to confirm this would be required.

Reactors 12 and 14, which were both stored at 25°C, without bulk fluid and with bulk fluid, respectively, presented a different trend with a decrease in the number of species during each dormancy period (Table 2). During the time between cycles, however, both reactors recovered and the species numbers increased indicating that though the higher temperature may have a negative effect on the bioreactors, they were able to recover once placed back into service. Reactor 14, though following the same trend as 12, had a larger loss in the number of species and in diversity during dormancy cycle 2, but again after a period of stabilization, was able to respond and diversity increased from 1.7 to 2.4 (Table 2). Over 700 species in nearly 500 genera were identified near the completion of this dormancy study. Of the most abundant genera present in Bioreactor 14, the Denitrobacter, an aerobic denitrifying bacteria, had the largest decrease during dormancy cycle 2, and remained at the reduced numbers throughout the remainder of the study. Even though the loss of the Denitrobacter may have had some influence on the denitrification process within the bioreactor, other species present, though less abundant were capable of denitrification or dissimilatory nitrate reduction to ammonium (DNRA).

2. 1-L MABR Set 15-18

1-L MABRs 15-18, containing 14-week-old biofilms (twice the age of the biofilms tested in reactors 11-14 for their initial dormancy cycle), underwent a 9-month dormancy cycle under similar conditions (Table 1). Recovery of the systems after the long-duration dormancy cycle included introduction of full-strength feed at a 5.00-day residence period for all reactors in an attempt to for the systems to recover more rapidly to a normal pH range. For reactors that contained no bulk fluid, recovery included the same addition of buffer solution as previously discussed for reactors 11-14. Figure 3 shows average performance metrics for 1-L MABRs 15-18 prior to the dormancy cycle, after 1-week of recovery, and after 2-weeks of recovery. Average conversion rates between one and two weeks remained consistent, and similar to pre-dormancy rates. TOC removal was not included for this study as during the recovery phase, the instrument had encountered errors; as a result, a loss of data was experienced for this metric. TOC analysis capabilities were restored by Day 29 of recovery, where all reactors were seen to maintain TOC removal between 92 and 93%. Extended monitoring of the recovery (67 days) of the four reactors showed that urea hydrolysis, ammonia removal, denitrification, and TOC removal remained consistent or continued to marginally increase. A recovery period of approximately 7 days is much improved over that seen with the 30-day dormancy cycle and 7-week-old biofilms from reactors 11-14; the improvement may be attributed both to the more mature biofilms and higher residence period not overwhelming the microbial community. Based on these results, conclusions based on chemical data analysis alone remain consistent that there is not a dormancy condition set proving to be better than another. These results, however, do indicate that mature, healthy biofilms play a significant role in the ability to hibernate biological water processor systems.

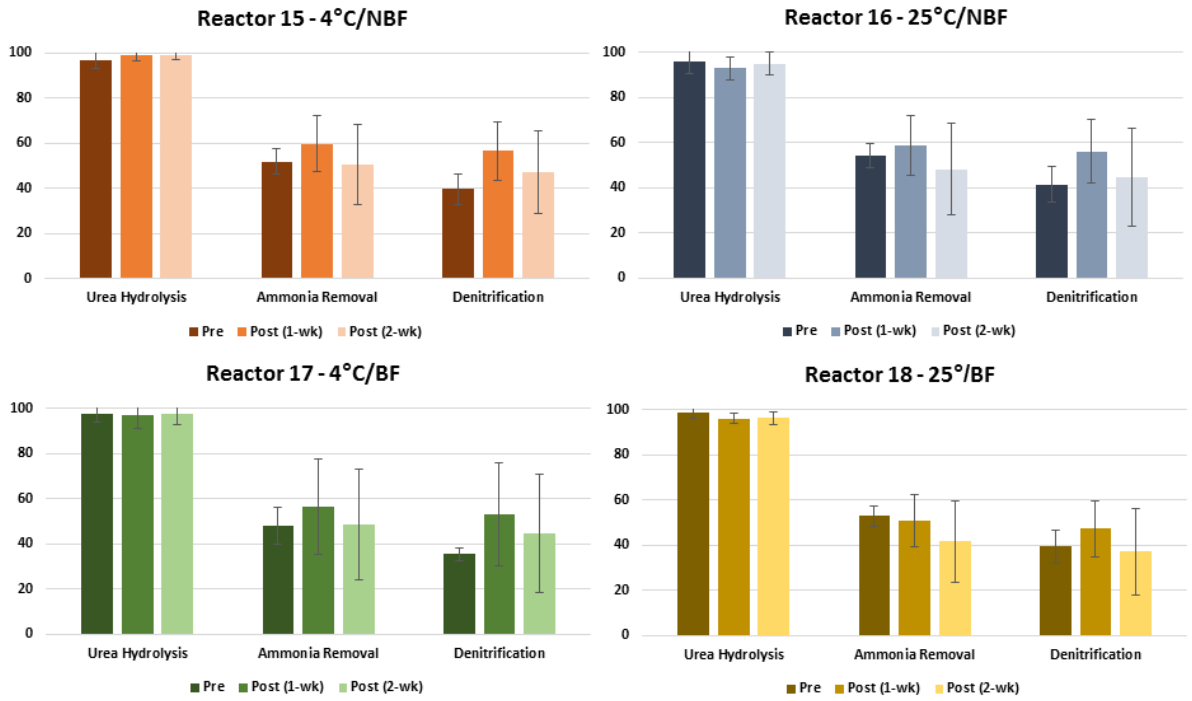

Figure 3: Performance metrics for 1-L MABRs 15-18 prior to any dormancy cycle, 1-week into recovery, and 2-weeks into recovery. Error bars represent standard deviation.

Similar to the second recovery cycle for reactors 11-14, 1 M HCl additions were also added to the feed for this set of MABRs in an attempt to better control pH during the initial recovery period. All four reactors were weaned off of acid dosing by Day 32. As seen in Figure 4, the performance metrics were not influenced by the end of acid dosing (Days 32-67), confirming similar findings that acid dosing did not seem to have any effect on performance from the cycle 2 results in reactors 11-14. By Day 40 of recovery, reactor 15 began showing a drop in pH from 8.1 to 7.1 by Day 68, corresponding with increasing ammonia removal and TOC removal seen in Figure 4. On Days 67 and 68 post-recovery, all reactors were moved to a 3.79-day residence and 100% oxygen gas supply, respectively. The residence time was lowered to reach the goal residence period for the systems, while the gas supply was altered based on the positive results seen in reactors 12 and 14, discussed previously. As seen in Figure 4, reactor 15 responded well to the changes, with significant increases in ammonia removal and TOC removal. The increase in ammonia removal for reactor 15 correlates with the appearance of significant levels nitrate in the effluent stream (from ~2ppm to ~300 ppm); with the introduction of oxygen, it appears that the NOB community in the reactor began performing at a higher level, and tolerated the change to a lower residence. Reactors 16-18 did not show similar results, but rather suffered from these changes (note the lower TOC removal in Figure 4 directly after the changes were put in place). It is expected that even with the move to 100% oxygen, the microbial community was not strong enough to overcome the lowered residence period. It should also be noted that reactors 16-18 remained in the pH range of 8.5-8.8, never dropping like reactor 15; while they were able to still possess appreciable levels of removal similar to that seen in recovery cycle 2 for reactors 11-14 above, the systems overall were not robust.

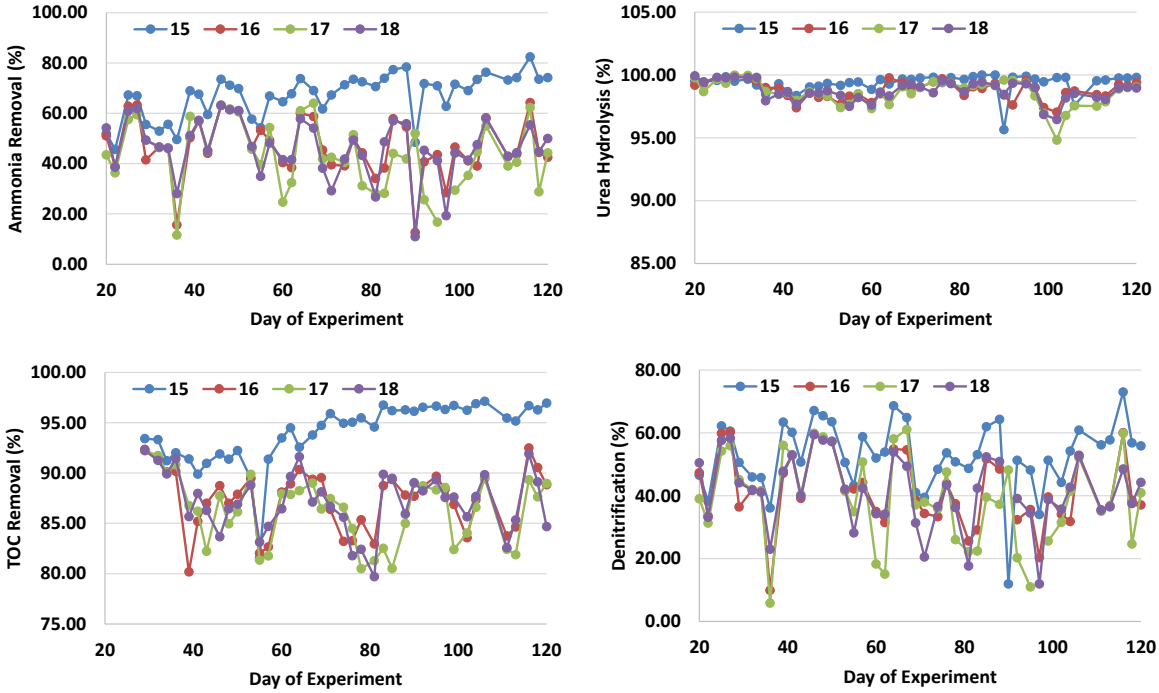

Figure 4: Performance metrics for reactors 15-18 after end of acid dosing to feed (Day 32), move to 3.79-day residence (Day 67), and addition of 100% oxygen gas feed (Day 68).

Similar to the samples taken for reactors 11-14, biofilms were sampled in all reactors for determination of the community profile after reactor inoculation from the KSC inoculum tank, prior to the dormancy cycle, at the beginning of the recovery phase, and after extended recovery. Table 3 highlights the general sequencing results to the genus and species level for reactors 11-14 throughout each phase of the dormancy cycles. "Prehib-1" includes the establishment of biofilm after the initial reactor inoculation while "Posthib" represents the state of the biofilm as the system was taken out of the dormancy cycle to begin recovery. "Stabilize" refers to biofilm samples taken after extended recovery at the end of all dormancy-recovery experiments.

Table 3: Reactors 15-18 Dormancy conditions and General Sequencing data to the genus and species taxonomic categories. The Shannon Index indicates the level of diversity.

Sample	Dormancy Condition	Total Reads	Total Reads PF	# Genus ID	# Species ID	Shannon Index
015-Prehib-1	4°/NBF	737,429	642,820	486	708	2.224
015-Posthib-1	4°/NBF	931,496	823,306	521	778	2.341
015-Stablize	4°/NBF	1,155,954	1,025,990	541	835	2.25
016-Prehib-1	25°/NBF	643,100	545,357	456	656	2.106
016-Posthib-1	25°/NBF	461,875	395,113	422	574	2.002
016-Stablize	25°/NBF	1,081,758	956,885	478	787	2.392
017-Prehib-1	4°/BF	774,731	664,255	470	711	2.181
017-Posthib-1	4°/BF	685,778	599,386	485	698	2.279
017-Stablize	4°/BF	985,919	857,102	499	803	2.34
018-Prehib-1	25°/BF	1,217,062	1,059,494	505	794	2.314
018-Posthib-1	25°/BF	557,559	486,663	479	688	2.422
018-Stablize	25°/BF	1,058,769	929,293	517	824	2.469

As discussed, reactors 15 and 17 were stored at 4°C without bulk fluid and with bulk fluid, respectively. During the dormancy period, reactor 15 (4°C, NBF) maintained the relative number of genus and species at each stage, whereas reactor 17 (4°C, BF) showed an initial decrease during the hibernation, followed by an increase in the number of species as well as diversity during recovery. It is suspected that at 4°C, the metabolic pathways are greatly reduced and that little or no cell division occurs for the majority of the species present. With little metabolic reactions ongoing, the presence of bulk fluid should have minimal influence. The initial decrease in the number of species seen in reactor 17 is similar to the decrease seen in reactors 16 (25ºC, NBF) and reactor 18 (25ºC, BF). Reactors 16 and 18, undergoing a dormancy cycle at approximately 25°C, without or with bulk, showed a loss of 82 and 106 species, respectively. It should be noted, however, that after three months of extended recovery, the number of species increased in both reactors and progressed toward a more stable condition (Table 3). Many of the same genera were apparent and abundant as detected in the reactors 11-14. Denitrobacter was present in the community in all eight reactors and persisted throughout the hibernation evolution. Denitrobacter increased in reactors 15 and 16 (both NBF) but decreased in 17 and 18 (BF). Nitrobacter (NOB) and Nitrosococcus and Nitrosospira (AOB) were also present but in low abundance. Other genera seen in reactors 15-18, but not as abundant or present in 11-14, possessed the capability to denitrify[14, 15]. It is most likely that some or all of these genera were participating in the denitrification process at a low rate.

3. ssMABR CR2

As seen in Figure 5, the quick recovery of this system was encouraging after a 7-month dormancy cycle at 4°C with no bulk fluid. Extended monitoring of reactor metrics showed a slight decrease in denitrification over time, with all other parameters remaining consistent. With this successful recovery cycle, a second dormancy cycle of 9 months was initiated with storage conditions being altered to 25°C with no bulk fluid to test whether the warmer temperature would compromise microbial integrity over time. Within one week of recovery, urea hydrolysis, ammonia removal, and denitrification levels were comparable to values seen prior to the second dormancy cycle, while TOC removal was nearly 50% reduced (Figure 5). Extended monitoring showed a return of TOC removal capacity within two weeks of recovery. In both recovery phases, the reactor was filled with fluid containing typical levels of humidity condensate, hygiene wastewater, and laundry wastewater (with no urine) as a buffer solution to assist in easing the microbial community back into operation. During the first residence cycle in both recovery phases, small doses of 1 M HCl were added to the feed for pH control. Ammonia and Total Nitrogen (TN) levels are highly dependent on reactor pH; as pH decreased in the reactor, both were seen to also decrease, correlating with increased ammonia removal and denitrification. All three of these metrics are dependent on influent nitrogen values as well. The sharp decrease in these constituents around Day 50 of recovery is due to a weaker batch of feed introduced to the reactor system; feed samples from that period of operation showed ~30% lower TN compared to average values, likely due to a less concentrated batch of urine for that particular feed. When real urine is utilized in the wastewater to be treated, vast differences in nitrogen concentration are not surprising and can cause large momentary changes in reactor performance as a result.

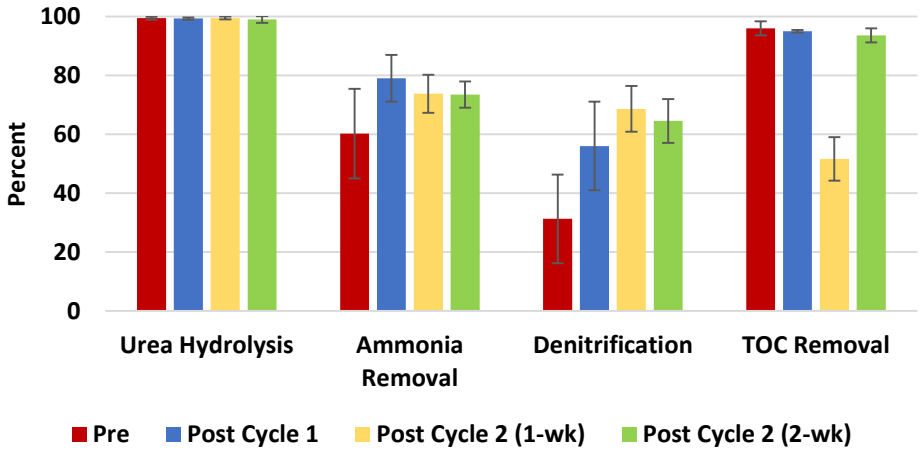

Figure 5: CR2 reactor performance metrics prior to any dormancy cycle, after dormancy cycle 1, and after dormancy cycle 2. Average values for 7-days and 14-days into recovery after dormancy cycle 2 are depicted. Error bars represent standard deviation.

Microbial community analysis for ssMABR CR2 was also completed; the bioreactor was first sampled when the biofilm was well established (13 months) and upon being placed back into operation after the 7-month dormancy cycle described previously. Biofilm samples were taken from the at the top, middle, and bottom areas of the fibers within the reactor, as well as from fibers at the bioreactor core and versus outer portion of the fiber header. Characterization of the genus and species, including diversity derived from sequencing of the 16s rRNA gene can be viewed in Table 4. There was no statistical difference between overall regions (T, M, B, or inner vs. outer) ($p>0.05$). The numbers of species, and hence the diversity, was similar at all locations. This may be due to the fact that the biofilm was well established for 13 months prior to the first hibernation cycle. At the genus taxonomic level, a comparison of the top species occurring in the CR2 bioreactor identifies 24 species that occurring greater than 1% of the total abundance. Of these, the most evident variation occured in the Denitrobacter and Vogesella genera. Both change significantly during the recovery and second dormancy stage ($p<0.05$). In order to determine how each genus functions and influences the function of the bioreactor/film, further studies would be required though characterization of ribonucleic acids (RNA) by transcriptomics. Identifying the species that participate in various metabolic reactions would provide some insight in which long term storage conditions might provide the best survival hibernation and recovery conditions for these and other types of bioreactors.

Table 4: Characterization of ssMABR CR2 to indicate number of Genus and species determined over time during hibernation study. Post 1 samples were taken after dormancy at 4 ºC; Pre2 samples were taken only from the outer region after a recovery period and prior to the second dormancy cycle; Post 2 samples were taken after a dormancy at 25 ºC. No bulk fluid were present. O=outer, I=inner, B=bottom, M=middle, T=top.

Sample ID CR-2	TOTAL Reads	TOTAL Reads PF	# Genus ID	# Species ID	Shannon Diversity Index
IBPost1	197,064	184,815	378	529	2.208
IMPost1	175,581	162,771	382	518	2.020
ITPost1	232,907	215,834	412	563	2.077
OBPost1	226,613	209,480	387	508	2.153
OMPost1	230,353	211,453	391	533	2.199
OTPost1	245,666	227,389	409	550	2.063
OBPre2	216,115	196,170	408	525	2.084
OMPre2	171,065	157,231	360	449	2.078
OTPre2	223,776	204,169	386	475	2.078
IBPost2	231,464	212,416	433	582	2.127
IMPost2	156,085	141,835	372	459	1.954
ITPost2	147,506	134,235	383	509	2.111
OBPost2	211,973	192,598	432	578	2.104
OMPost2	229,587	207,317	414	533	2.041
OTPost2	186,905	171,138	389	493	2.005

B. Antibiotic Studies

4. 1-L MABR Set 11-14

1-L MABR set 11-14 were used in the first antibiotic dosing experiment with the following scheme: reactor 11 received ciprofloxacin dosing, reactor 12 received inoculation with *E. coli* followed by introduction of ciprofloxacin 3 days later, reactor 13 received inoculation with *E. coli* and no antibiotic dosing, and reactor 14 served as a control reactor. The concentration of ciprofloxacin in reactor 11 and 12 feed was 2.97 and 3.07 μg/mL, respectively; the ciprofloxacin persisted in low levels in both dosed reactors for 3 weeks. Reactor 11, which received ciprofloxacin dosing and no *E. coli* shows the initial dose, with a rise in concentration to nearly 2.50 μg/mL starting to decrease

after 5 days, and another slight increase in concentration after the second dose was introduced. Reactor 12, which was inoculated with *E. coli* then treated with the antibiotic showed a much slower rise in ciprofloxacin concentration in the effluent, and maximum values did not reach those seen in reactor 11. A multitude of explanations exist for the effluent concentration differences. Microbial community diversity in the biofilms between the two reactors could vary greatly; while feed bottle concentrations were near equivalent, some of the antibiotic may not have fully dissolved or may have not been well mixed causing a lower amount to enter reactor 12; differences in reactor operation pH and gas feed may also play a role in ciprofloxacin persistence and build up. Biofilm sampling was completed prior to and after the study for sequencing to answer some of these questions.

Table 5 shows the various changes in average urea hydrolysis, ammonia removal, denitrification, and TOC removal for the antibiotic and pathogen matrix detailed above. One-way ANOVA and Sidak's Multiple Comparison Test (95% confidence interval) was used to determine if changes in the averages before and after a treatment were statistically significant. The only reactor that experienced a negative impact in any performance metrics was reactor 11, which received ciprofloxacin dosing only. Both ammonia removal and denitrification were found to be significantly lower after the antibiotic exposure. Both reactors 12 and 14 experienced a significant increase in ammonia removal; it is expected that the systems were continuing to improve after the addition of 100% oxygen as the gas feed rather than as any effect from this study. Reactor 13, which was inoculated with *E. coli* did not show any significant changes in performance, showing that the introduction of a pathogen left untreated had little short-term impact on reactor performance and is not surprising based on the already diverse community present in these biological systems.

Table 5: Reactor Performance Metrics Pre- and Post-Antibiotic/Pathogen Introduction*

	11: Before	**11: Cipro**	**13: Before**	**13: *E. coli***
Urea Hydrolysis	98.38	97.99	98.76	98.09
Ammonia Removal	52.80	42.04	54.99	49.17
Denitrification	48.93	37.30	50.81	44.70
TOC Removal	84.42	83.78	84.37	86.48
	12: Before	**12: *E. coli* & Cipro**	**14: Before**	**14: Control**
Urea Hydrolysis	98.90	98.02	98.80	98.23
Ammonia Removal	69.48	79.20	72.15	80.81
Denitrification	61.38	69.88	66.10	71.94
TOC Removal	94.11	93.77	93.26	94.17

* Values in red are statistically lower than pre-antibiotic study parameters, while those in green are statistically higher; no text color signifies a statistically insignificant change in the parameter value.

Samples collected for microbial analysis included biofilm samples (in triplicate) from each reactor before the introduction of *E. coli*, before the introduction of ciprofloxacin, and at the conclusion of the study (14 days after *E. coli* inoculation); reactor effluent samples were collected daily. Biofilm samples were stained with Baclight Live/Dead stain to determine the fraction of live cells in the biofilm. Effluent samples were plated on nutrient agar (NA) for heterotrophic plate counts (HPC), NA with ampicillin for *E. coli* with GFP marker detection (fluorescence detected by UV lamp), and NA with ciprofloxacin at concentrations of 2 and 4 μg/mL for detection of antibiotic resistant strains. Bacterial growth from ciprofloxacin plates was inoculated into broth for minimum inhibitory concentration (MIC) testing to confirm bacterial resistance or sensitivity to the antibiotic. The fraction of live cells in the biofilm in reactor 11, treated with Ciprofloxacin, was impacted significantly as illustrated in Figure 6. Interestingly, this effect was not seen in reactor 12, which was also treated with ciprofloxacin.

Bacterial numbers in the reactor effluent, as assessed by plate counts, did not seem to be impacted by the antibiotic treatment with the exception of the presence of *E. coli* in reactor 12. The bacterium was not detected in reactor 12 effluent 3 days after the introduction of ciprofloxacin; the *E. coli* was still detected on the last day of sampling in the reactor 13 which was inoculated with *E. coli* and was not treated with the antibiotic. One could assume that other species of bacteria in the effluent sensitive to the antibiotic were also affected. Figure 6 illustrates effluent sample plate counts over the course of the experiment.

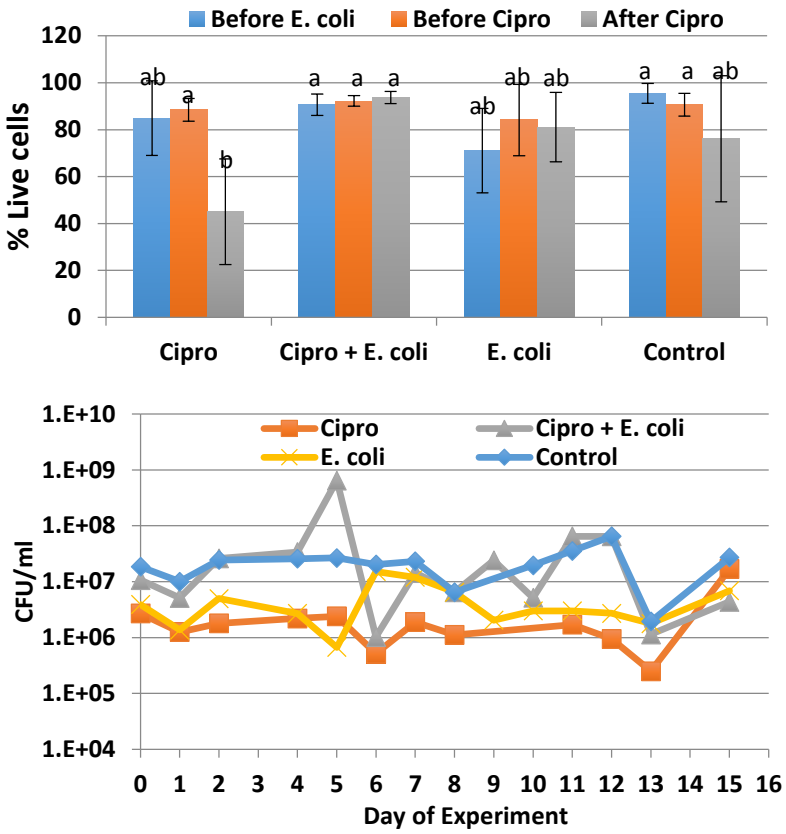

Figure 6: Top: Live bacteria (%) present in biofilm samples collected before and after ciprofloxacin treatment. Different letters (a and b) indicate significant differences (p≤.05 by Students t-test). Bottom: Colony forming units (CFU) per mL of effluent.

Resistant, or intermediately resistant, strains of bacteria from the effluent samples were present in all the reactors at some point during the study. Resistance is defined by growth at ≥ 4 µg/mL and intermediate (strains that could develop resistance) growth at 2 µg/mL. Table 6 shows the development of resistant strains in the reactors. With the introduction of ciprofloxacin in reactor 11, resistance is more prevalent throughout the course of the study. Resistant strains of bacteria can be present naturally in urine and wastewater; with the introduction of ciprofloxacin into the reactors, resistant strains would be those that survive and proliferate while susceptible strains would decline. The strain of *E. coli* introduced into reactor 12 is sensitive to the antibiotic and did not develop resistance as indicated by no growth on media containing ciprofloxacin.

Table 6. Ciprofloxacin sensitivity as determined by broth dilution MIC on bacteria that grew on plates containing the antibiotic.

	Day 0	Day 4	Day 6	Day 8	Day 10	Day 12
Cipro (11)	S	S	R	R	R	R
Cipro + *E. coli* (12)	I	S	S	R	S	S
***E. coli* (13)**	S	S	S	I	R	S
Control (14)	I	S	ND	I	I	S

S=Susceptible, I= Intermediate, R=Resistant. ND = No Data

5. 1-L MABR Set 15-18

A duplicate study was performed with 1-L MABR set 15-18 with the following scheme: reactor 15 served as a control reactor, reactor 16 received inoculation with *E. coli* and no antibiotic dosing, reactor 17 received inoculation with *E. coli* followed by introduction of ciprofloxacin 3 days later, and reactor 18 received ciprofloxacin dosing. The concentration of ciprofloxacin in the feed for reactors 17 and 18 was 3.24 ± 0.32 μg/mL throughout the course of dosing. Similar to that seen in Run 1, the ciprofloxacin concentration level increased during the repeated dosing of the feed, then persisted in low levels in both dosed reactors for approximately 3 weeks.

Table 7 shows the changes in average urea hydrolysis, ammonia removal, denitrification, and TOC removal for the antibiotic and pathogen matrix detailed above. One-way ANOVA and Sidak's Multiple Comparison Test (95% confidence interval) was used to determine if changes in the averages before and after a treatment were statistically significant. All reactors, including the control reactor, experienced a statistically significant decrease in ammonia removal and denitrification. While ammonia removal and denitrification decreased by ~16% and 28%, respectively for the control reactor (15), they decreased by nearly 65% and 70%, respectively, in all treated reactors (16, 17, and 18). The drastic decrease in performance is not expected to be solely due to the introduction of *E. coli* or ciprofloxacin in any of the reactors because all systems experienced a loss in performance. This change in conversion metric is likely due to the inability of microbial community to process the wastewater at a 3.79-day residence (as was seen by lower metrics in the previous dormancy-recovery section) after an extended dormancy cycle. Reactor 15, serving as the control reactor, likely responded to the residence change better than the others since it was already performing at higher conversion rates than the other three reactors. The added stress of antibiotics and possible pathogenic bacteria added to reactors 16-18, may have caused the more dramatic change in metrics, but the data is confounded by the number of alterations made to the systems.

Table 7: Reactor Performance Metrics Pre- and Post-Antibiotic/Pathogen Introduction*

	15: Before	15: Control	16: Before	16: *E. coli*
Urea Hydrolysis	99.57	99.94	98.53	97.68
Ammonia Removal	69.68	58.37	47.02	15.20
Denitrification	48.27	34.60	40.69	11.71
TOC Removal	96.02	94.99	86.34	83.31
	17: Before	**17: *E. coli* & Cipro**	**18: Before**	**18: Cipro**
Urea Hydrolysis	95.94	92.37	98.12	96.60
Ammonia Removal	43.78	15.67	48.07	17.28
Denitrification	40.27	11.00	41.67	13.59
TOC Removal	83.92	78.38	85.04	83.36

* Values in red are statistically lower than pre-antibiotic study parameters; no text color signifies a statistically insignificant change in the parameter value.

The methods used for the second run with reactors 15-18 were the same as the first described above. Figure 7 illustrates the percentage of live bacteria in biofilm samples collected before treatment and at the end of the run. The only significant change could be seen in the reactor dosed with *E. coli* and ciprofloxacin with a significant loss of live cells. Biofilm viability in the reactor treated with ciprofloxacin only was not impacted by the antibiotic. This result is different than the first run in that the cipro- and cipro plus *E. coli*-dosed reactors were conversely affected. This effect may be a function of specific types of bacteria present in the individual biofilms and possible antibiotic resistance. Biofilm samples were collected throughout this experiment and have been preserved for future community analysis to better elucidate these findings.

As with the first run, bacterial numbers in the reactor effluent did not seem to be impacted by the antibiotic treatment with the exception of the presence of *E. coli* in the dosed reactors. (Figure 7). The bacterium was not detected in reactor 17 effluent three days after the introduction of ciprofloxacin; *E. coli* was still detected until 14 days after inoculation and without the antibiotic dosing. As with the first run, resistance of effluent bacteria that grew on agar plates containing ciprofloxacin was confirmed by MIC testing as described above. Resistant bacteria were present throughout the second run in all reactors from day 1 (data not shown). This suggests the presence of bacteria resistant to ciprofloxacin in the urine component of the feed stream.

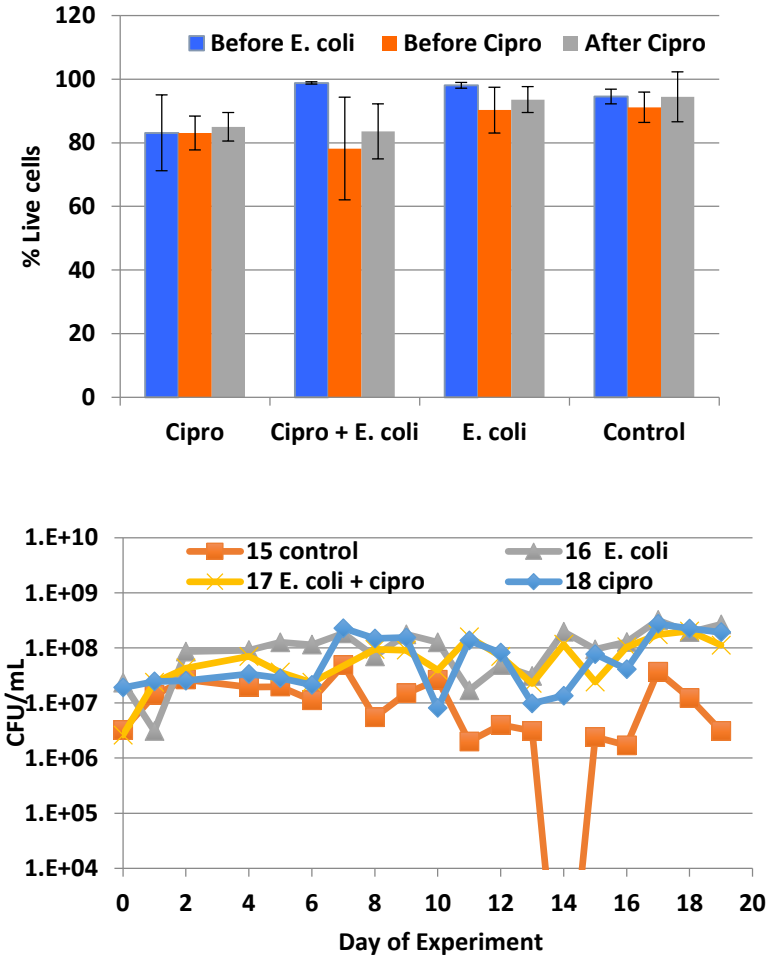

Figure 7: Top: Live bacteria (%) present in biofilm samples collected before and after ciprofloxacin treatment. Significantly less live cells (p≤.05 by Students t-test) were seen in the biofilm treated with Cipro and *E. coli.* Bottom: Colony forming units (CFU) per mL of effluent.

IV. Conclusions

C.Dormancy-Recovery Cycling within Biological Water Processors

With these additional dormancy and recovery cycles, no matter the storage condition tested, all systems were able to recover with appreciable urea, ammonia, and TOC removal capacity within 7 days. Most systems were also seen to continue improving with extended recovery periods (after 2-weeks). An exact methodology for recovering these systems is yet to be fully elucidated; however, several trends can be noted. It is apparent, that, for systems stored without bulk fluid, a buffer solution is required to assist in diluting the reintroduction of the urine-containing waste stream or the biofilms are overwhelmed. For future testing, it is encouraged that acidification is not used, but rather the microbial communities be allowed to develop pH control on their own to limit input requirements for the bioreactor systems. A residence time regime for starting recovery at a higher residence (5-days, 15-18) has not proven any more useful than resuming full feeding at shorter residence periods (3.79-days, 11-14) for recovery. Ensuring the microbial community is not oxygen deprived was also an important factor during recovery stages as seen in reactors 12 and 14; had oxygen been added to reactors 15-18 early on in their recovery, 16-18 may have been able to better handle the move to a lower residence period by not starving the microbial community. Introduction of more oxygen to both MABRs greatly improved performance metrics. Lastly, based on the various biofilm ages tested, it appears that the more mature the biofilm, the more resilient the system becomes for dormancy capability; for instance, 13-month old

biofilms in CR2 have shown excellent recovery and 14-week-old biofilms were demonstrated to recover much faster than 7-week-old biofilms. Further testing with multiple age biofilms could possibly determine a minimum maturation for the system prior to successful, rapid recovery. Examination of the microbial communities in these MABR systems throughout the dormancy-recovery experiments shed light on changes in the community make-up and diversity. From the molecular identification of genus and species, only trends could be seen; and does not elucidate dormancy conditions suitable for long term hibernation of bioreactors. From these trends it appears that a 4°C hibernation temperature may be inferred, but with respect to fluid storage, data does not either method. All bioreactors were able to recover after multiple dormancy cycles; however, it should be kept in mind that after a period of time in bulk fluid and higher (though not optimal) temperatures, nutrient levels would drop and carbon sources could be depleted causing a negative effect on the biological system.

Based on these results, if a recommendation for hibernation of a reactor were to be given without further testing, the following regime would be suggested: 1) use of 8-12-month-old biofilms demonstrating high conversion at desired operating parameters, stored at 4°C with or without bulk fluid; 2) upon recovery, resume full processing of wastewater at desired operating conditions with elevated oxygen levels in gas feed, if possible. This calls for a compromise between up-mass and power consumption for a system launching into microgravity, but saves on power throughout a launch and allows for easy deactivation during periods of non-use in future long duration missions when crew are not present to run the system. As stated, further in-depth examination of these results as well as further studies would assist in finalizing a dormancy-recovery strategy for these biological systems.

D.Effect of Pathogenic Bacteria and Antibiotics

These initial experiments on the effects of antibiotics and pathogens on biological wastewater processors have shed light on effects experienced by the reactors as well as added several questions yet to be answered. Changes in the performance metrics in both studies were confounded by residual effects of operational changes to the reactors after finishing a series of dormancy-recovery experiments (e.g., addition of 100% gas supply causing an increase in performance, lowering of residence time hindering performance, etc.). Due to a tight timeline, new systems were not able to be initiated for these experiments. Regardless, the initial data indicates that the introduction of ciprofloxacin may have a slightly negative effect on a biological water processing system, but not to the extent that the system would be compromised. The *E. coli*, the pathogenic species introduced as the on-set of a urinary tract infection in a crewmember, was eliminated within the reactor after the addition of ciprofloxacin, leaving little time for it to proliferate further. Proliferation of antibiotic-resistant species was also denoted after exposure to the ciprofloxacin; further analysis of this effect on long-term operation is required. Biofilm samples were collected and preserved for Next Generation Sequencing to further examine effects on the microbial community in future work. Future work, past the scope of this project, should also assess various antibiotics, different concentration loading of antibiotics into reactor systems, and effects after multiple dose events over a long duration.

Acknowledgments

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