Dormancy and Recovery Testing for Biological Wastewater Processors

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Bioreactors, such as aerated membrane type bioreactors have been proposed and studied for a number of years as an alternate approach for treating wastewater streams for space exploration. Several challenges remain before these types of bioreactors can be used in space settings, including transporting the bioreactors with their microbial communities to space, whether that be the International Space Station or beyond, or procedures for safing the systems and placing them into dormant state for later start-up. Little information is available on such operations as it is not common practice for terrestrial systems. This study explored several dormancy processes for established bioreactors to determine optimal storage and recovery conditions. Procedures focused on complete isolation of the microbial communities from an operational standpoint and observing the effects of: 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 room temperature (25°C) and cold (4°C) storage conditions were studied. The second consideration was explored; again, for microbial integrity as well as plausible real-world scenarios of how terrestrially established bioreactors would be transported to microgravity and stored for periods of time between operations. Biofilms were stored without the reactor bulk fluid to simulate transport of established biofilms into microgravity, while biofilms stored with the reactor bulk fluid simulated the most simplistic storage condition to implement operations for extended periods of nonuse. Dormancy condition did not have an influence on recovery in initial studies with immature biofilms (48 days old), however, a lengthy recovery time was required (20+ days). Bioreactors with fully established biofilms (13 months) were able to recover from a 7-month dormancy period to steady state operation within 4 days (~1 residence cycle). Results indicate a need for future testing on biofilm age and health and further exploration of dormancy length.

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Nomenclature

AOB-Ammonia oxidizing bacteria
DI - Deionized
DO – Dissolved Oxygen
FID – Flame Ionization Detector
FISH-Fluorescent in-situ hybridization
GF – Glass Filter
HFMB – Hollow Fiber Membrane Bioreactor
HPLC – High Performance Liquid Chromatography
ISS – International Space Station
JSC – Johnson Space Center
KSC – Kennedy Space Center
ssMABR – Small Scale Membrane Aerated Bioreactor
MS – Mass Spectrometer
MTS – Modular Test Stand
NOB-Nitrite oxidizing bacteria
PBS – Phosphate Buffered Saline
θ Residence Time
TN – Total Nitrogen
TOC – Total Organic Carbon
TTU- Texas Tech University

I. Introduction

A sustainable source of potable water is a critical element of life support systems for manned space missions. Recovery and recycling waste streams to usable water via biological water processors is a possible alternative or addition to current systems used by NASA solely utilizing physical and chemical methods. As part of an integrated water purification system, biological processing as a pretreatment can reduce the load of organic carbon and nitrogen compounds entering P/C systems downstream. A trade study done on the use of biological processors in an integrated system showed that achieving high levels of nitrification can result in significant lower impacts and costs to P/C systems[1]. While terrestrial establishment of biofilms and operation of biological water processors has been examined for many years with batch reactors, membrane aerated reactors, and the similar, unique challenges are presented when exploring their use in microgravity[2,3]. These challenges include transport of the system to its destination, whether that be the International Space Station 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 of that system when operations are restarted. Few studies have been done to examine a intentional stop, store, and start operating regime as proposed which would introduce stresses to the microbial communities essential for waste water processing. However, numerous studies have been done, some using small scale biological reactors, to address the impact of chemical waste streams, changes in feed composition and potential starvation conditions due to industrial process shut downs and less then optimal environmental conditions on waste water treatment plants and the microbial communities involved in conversions of carbon and nitrogen compounds[4,5,6,7]. Strategies to restore a functioning microbial community to a waste water treatment process can be utilized, for example, 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 with concomitant denitrification accomplished by faster growing organisms. In addition, rapidly growing carbon oxidising bacteria present in the system compete for resources such as oxygen. Membrane aerated bioreactors 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 then planktonic populations and have the ability to recover to pre-stress metabolic activities and growth when more optimal conditons are restored[5,6,7]. Studies done by Christenson et.al.[8] documented a 21-day event where a full scale membrane aerated bioreactor was

placed in recycle mode essentially starving the microbes with lowered effluent gas flow rate to maintain a bulk DO concentration of >1 mg/L. After the 21-day starve event, the Counter Diffusion Membrane Aerated Nitrifying Denitrifying Reactor (CoMANDR) was brought back online with full strength feed at a full flow rate and found to recover quickly with only a slight drop in carbon removal efficiency. A similar study[9] using the Texas Tech University's CoMANDR 2.0 reactor system and a 30-day starve period with DO >6 mg/L, showed similar results.

Throughout FY14, KSC explored several dormancy processes for established bioreactors to determine optimal storage and recovery conditions. This work focuses 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 room temperature (25°C) and cold (4°C) storage conditions were studied. While room temperature would be optimal for low power consumption (no need for a cooling jack, etc.), the cold storage was also tested to determine if better microbial recovery would be obtained. The second consideration was explored; again, for microbial integrity as well as plausible real-world scenarios of how terrestrially established bioreactors would be transported to microgravity and stored for periods of time between operations. Established biofilms were stored without the reactor bulk fluid to simulate transport of established biofilms into microgravity, 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 microgravity (i.e., simply turning off power, pumps, feed, etc. without the need for draining the system).

II. Materials and Methods

A. Reactor Operations, storage and recovery

1. Dormancy Storage Condition Experiments

Four 1L membrane aerated bioreactors (MABRs) were used for a four-week dormancy cycle experiment with varied storage conditions listed in **Table 1**. All 4 reactors were inoculated following a start up procedure utilized by TTU researchers, and operated under the same conditions (retention times, gas flow etc.). Briefly, reactors were inoculated with an actively nitrifying microbial community grown in the wastewater formula used for reactor operations.

Urine feed concentration was dilute (15.5% of full strength feed) and the feed was recirculated until nitrification was evident as indicated by a drop in pH and conversion of ammonia to nitrite/nitrate whereupon full strength feed was initiated. If nitrification was not evident after 5 days, reactors were re-inoculated and monitored. Several re-inoculations were necessary in the start-up of these reactors. In addition, it was necessary to add acid (1M HCl) to the reactor bulk fluid over the course of operations to keep the pH in a range to promote nitrification. Full strength wastewater feed was finally initiated after 34 days of operation. All four reactors were placed into storage after 48 days of operation at approximate steady state. It should be noted that reactors 11-14 were not at operational equivalency at the time of dormancy initiation (Figure 2); due to time constraints, a decision was made to initiate the dormancy cycle regardless of the operational state of each 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 a 3.79-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 same feed and residence time was used and reactors were allowed to slowly fill up the bulk fluid volume over time. These recovery methods were chosen to represent the closest to real-world application scenario for a reactor restart in microgravity or in a beyond-Earth mission application.

Table 1: 1-L MABR Dormancy Storage Conditions

Reactor	Storage Conditions
11	4°C, No Bulk Fluid
12	25°C, No Bulk Fluid
13	4°C, With Bulk Fluid
14	25°C, With Bulk Fluid

2. Long Duration Dormancy Test

A dormancy study using an ssMABR header module with established biofilms from FY13 experiments was also performed. These biofilms were approximately 13 months old and were stored at 4°C with no bulk fluid for a period seven months. Based on results from the 1-L MABR recovery experiments, recovery for this reactor included filling the reactor with a non-urine wastewater stream (using water in place of the urine) to fill the bulk

fluid volume with introduction of a full strength wastewater stream at a 3.79-day residence to dilute into the new bulk fluid over time.

B. 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.

C. Microscopic Analysis of Biofilm

Biofilm samples were removed from fibers in reactors 11-14 on three occasions: when the reactors reached steady state before storage, after the 4-week storage period under the conditions described in, and after continuous operation for 7 weeks post-dormancy. Samples were also collected from ssMABRs used in the long-term dormancy study. To compare biofilm from a stored, dormant reactor to one that had not been stored, samples were collected in triplicate from the top, middle and bottom of fibers (Figure 1)from the reactor "CR2" with established biofilm that had been stored refrigerated for 7 months, and a second ssMABR (R1) after 69 days of continuous operation. For all samples, one set was preserved in a solution of 50% ethanol: 50% phosphate buffered saline (PBS) and stored at -20^{o}C until processing, using the Nitri-Vit® kit (Vermicon, Munich, Germany), for the detection of ammonia and nitrite oxidizing bacteria (AOB and NOB). The Nitri-Vit procedure is a fluorescent in-situ hybridization (FISH) method using specific DNA probes to fluorescently label AOB red and NOB green. During labeling, the samples are fixed on slides and upon completion are viewed using a fluorescent microscope. To analyze the relative quantity of AOB and NOB present, 20 random fields are examined at 1000x magnification and assigned a numeric value based on the amount of cells exhibiting red or green fluorescence. The values range from 1-5, 1 being a few cells present, 5 being excessive cells present. Finally, a numeric average is calculated from these values. Two different filter sets are used to enable visualization of the red and green fluorescent probes so that AOB illuminate red and NOB green.

Another set was immediately stained with the Molecular Probes BacLight® live/dead kit (Life Technologies, Grand Island NY) to determine the percent of live bacteria present in the biofilm. Live/dead staining was performed on only one set of samples from the 1-L dormancy study after the second storage period. Staining procedures were completed according to manufacturer's protocols. Samples to be stained with the live/dead stains were vortexed vigorously followed by repeated passage through a 1000-ml pipette tip to break up the biofilm and, if necessary, diluted in sterile DI water prior to staining. One mL of stained sample was filtered through a 0.2-µm black filter (Millipore) and after drying, placed on a microscope slide with mounting fluid and a coverslip. Slides were

examined using a Zeiss Axioskope microscope with fluorescent illumination for both the Nitri-Vit® and live/dead stained samples.

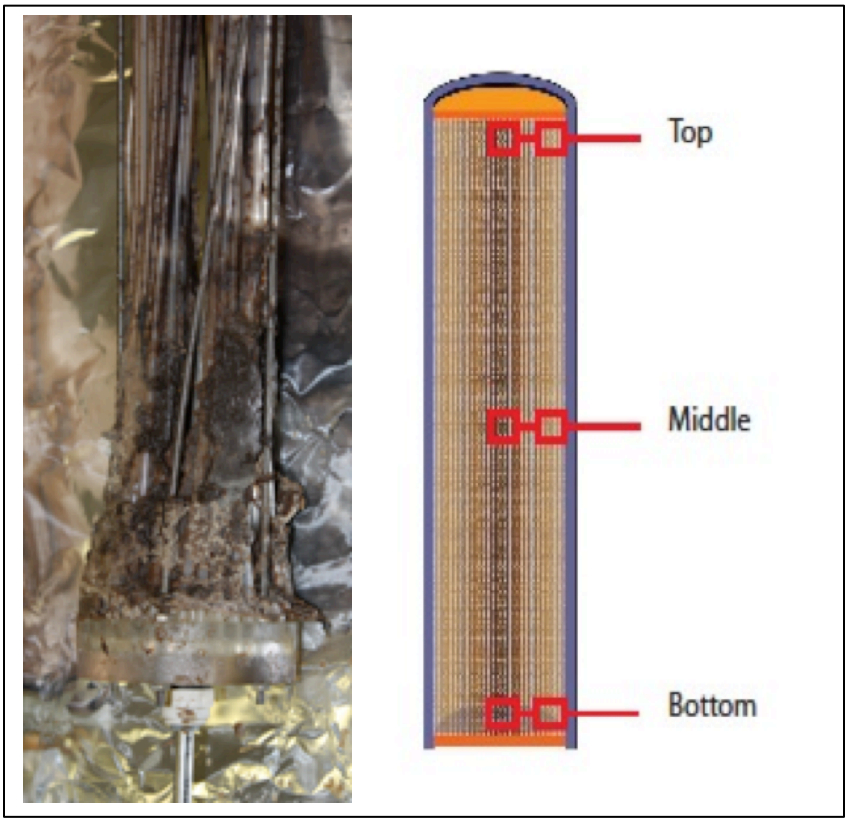

Figure 1: Fiber module "CR2" with 13-month old biofilms after a 7-month dormancy cycle in the absence of bulk fluid at 4°C (left). Diagram of regions of biofilm sampling within MABRs (right).

III. Results

D. Dormancy Storage Condition Tests

1. Bioconversion performance metrics.

As can be seen in Figure 2, all reactors were able to recover after the 28-day dormancy cycle. Further, improved performance was achieved, relative to the pre-dormancy performance, after regaining steady state operation. Average time for recovery, defined as achievement of steady state in each monitored metric, was approximately 21 days and included many pH adjustments using 1 M HCl. Even after repeated adjustments the average pH ranged from 7.75 to 8.56, and never fell below 6.70. Operation in this higher pH range likely caused the lower nitrification and denitrification levels, especially in Reactor 14, which sustained the highest average pH among all four reactors. It is also suspected that the biofilms were not fully mature and may have been overwhelmed during the recovery process when full strength feed was utilized during initiation. Reactors 13 and 14 were stored in the absence of bulk fluid; the immediate filling of the reactor bulk fluid volume with full wastewater may have been too much for the biofilms to handle initially. In reactors 11 and 12, the wastewater stream was slowly introduced into the system as the bulk fluid was exchanged with the wastewater over the first 4 days of operation.

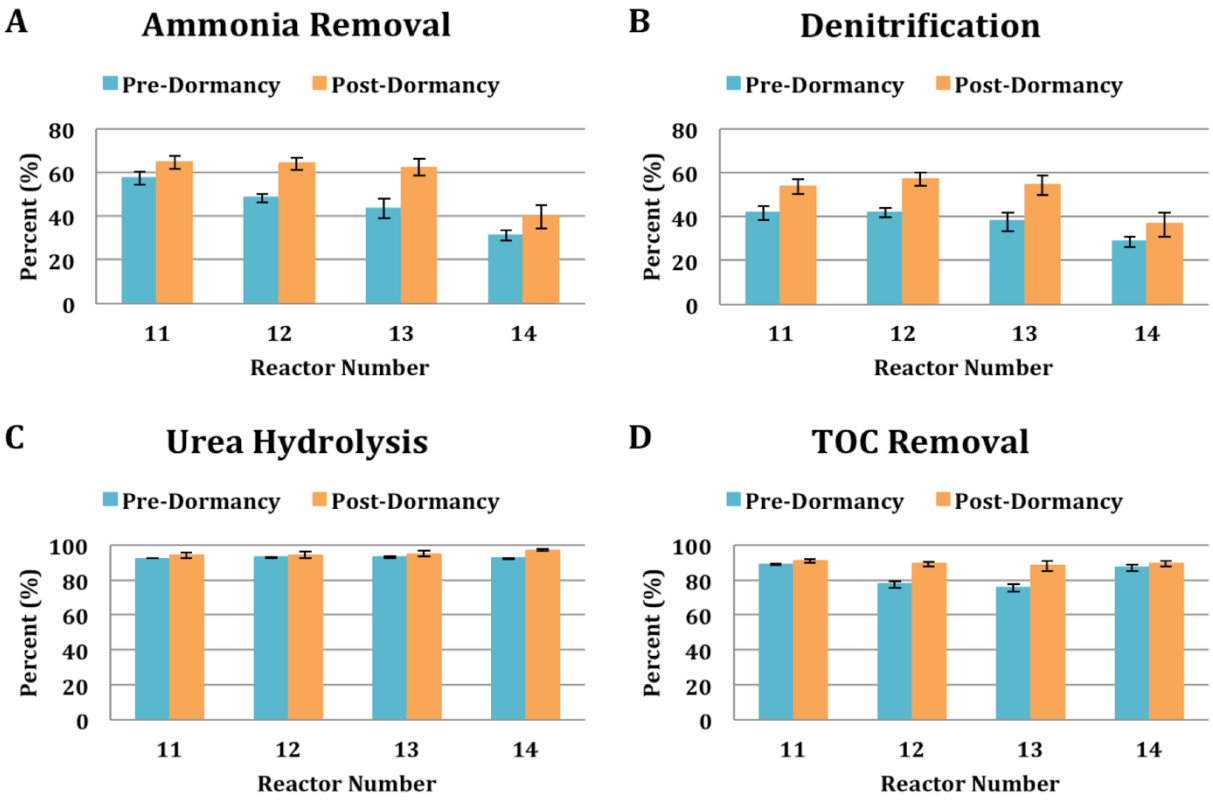

Figure 2: Performance metrics for pre- and post-dormancy of 1-L MABR reactors including A) ammonia removal, B) denitrification, C) urea hydrolysis, and D) TOC removal. Error bars represent standard error.

2. Microscopic Analysis of Biofilm

Viable AOB and NOB were evident in biofilm samples from reactors 11-14 before and after storage, irrespective of storage condition (Figure 3). The relative quantity of active AOB in the biofilm was lower in all reactors post-dormancy however this did not seem to impact the performance since post-dormancy operational metrics exceeded pre-dormancy performance. The amount of AOB decreased 2-3 fold after removal from storage and post-dormancy operation in all the reactors. Overall AOB activity and growth may have been suppressed by sub-optimal environmental conditions, such as high pH, present during post-dormancy operation. Nitrite oxidizing bacteria increased in abundance in biofilms from reactors 11 and 14 and decreased in reactors 12 and 13 during post-dormancy operation (Figure 3). The differences did not correlate with differences in ammonia removal and nitrification between reactor

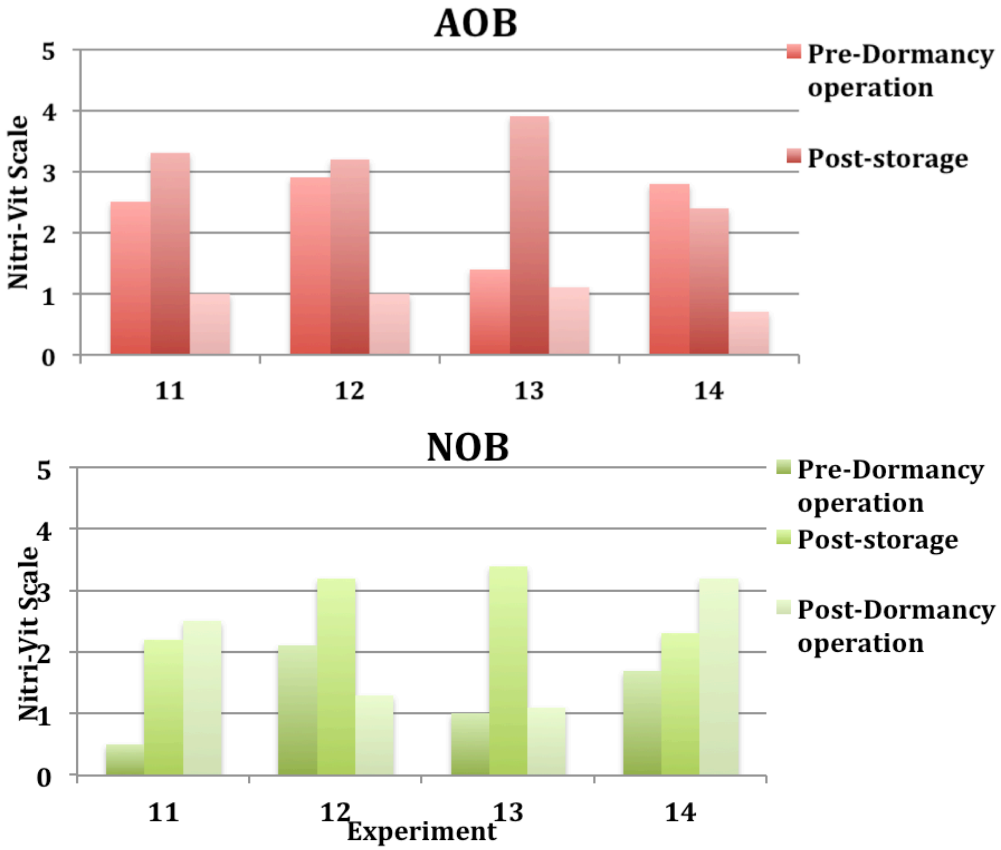

Figure 3: Relative abundance of AOB and NOB in reactors before and after storage and operation.

E. Long Duration Dormancy Test

Prior to entering a dormant state, the FY13-established ssMABR module used in this experiment attained 98-99% urea hydrolysis, 50-60% ammonia removal with low levels of denitrification (~10%), and 95-97% TOC removal during steady state operation at a 4-day residence period using the full wastewater stream (excluding laundry water). Biofilms were approximately 13 months old at the initiation of the dormancy cycle; bulk fluid was drained and the module was stored in the absence of bulk fluid at 4°C, as it was hypothesized that these would be the most successful conditions for reactor dormancy (small scale 1-L studies were not in progress at the time of this action). Based on recovery results for the dry (i.e., no bulk fluid) 1-L MABR studies where it appeared that allowing the bulk fluid volume to fill slowly at the 3.79-day residence pace using full strength wastewater may have overwhelmed the biofilms, a different approach to recovery was executed. Non-urine wastewater (i.e., laundry, hygiene, and humidity condensate with DI water balance) was used to fill the bulk fluid volume. Feeding with full wastewater at a 3.79-day residence was then initiated; this allowed for a slow (several days) introduction of urine to the biofilms in order to decrease shock to the system.

1. Bioconversion performance metrics

Figure 4 outlines nitrogen species trends in the reactor effluent during the recovery phase. By day 4 of recovery, when all initial "buffer" bulk fluid had cycled out of the reactor, ammonia removal was sustained at 85.8% and was largely a result of denitrification (84.1%). Urea hydrolysis and TOC removal were nearly complete at 99.4% and 92.2%, respectively. After 29 days of operation, average performance metrics were as follows: 75.0 ± 3.7% ammonia removal, 62.1 ± 5.1% denitrification, 99.3 ± 0.4% urea hydrolysis, and 95.0 ± 0.5% TOC removal. This demonstrated little to no interruption in reactor performance capability after an extended dormancy period and the

ability for biofilms to be stored without the presence of bulk fluid. This successful dormancy cycle also demonstrates the idea of establishing biofilms terrestrially for possible transport into microgravity.

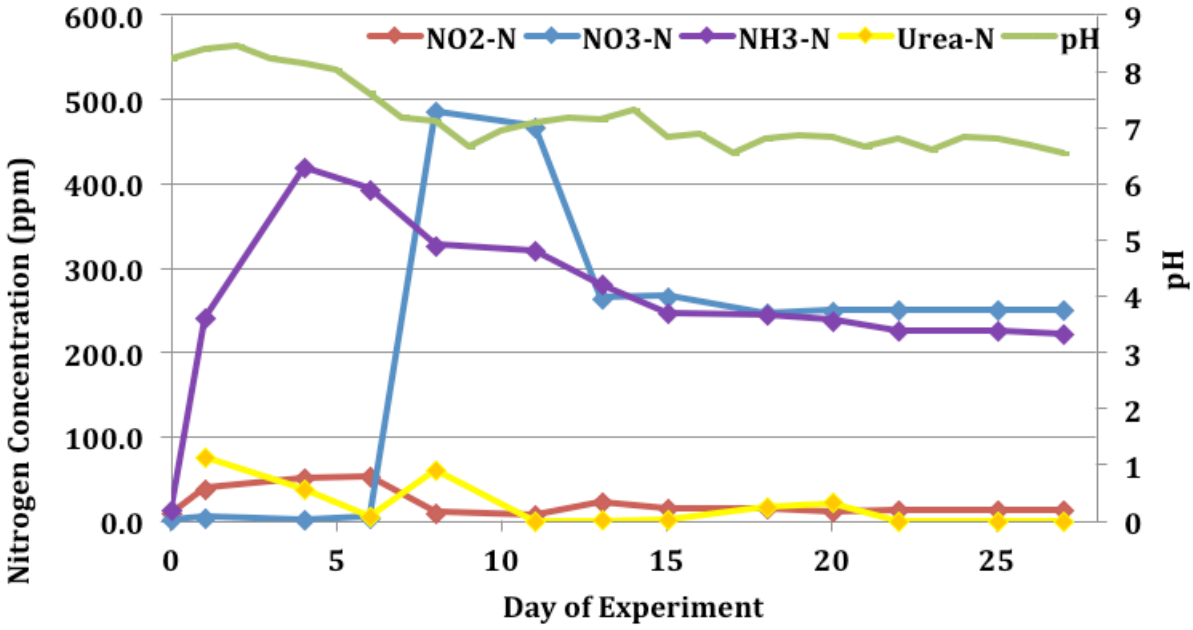

Figure 4: Nitrogen species in ssMABR1-CR2 reactor effluent (post 7-month dormancy)

2. Microscopic analysis.

Microscopy-based analysis of biofilms for viable bacteria and the presence of AOB and NOB showed the presence of all three in the reactor that had been stored for 7 months under refrigeration (CR2). These data were compared to biofilm samples taken from ssMABR1, which had been continuously operating for 69 days and exhibited robust biofilm formation on the fibers. The average percentage of live bacteria in the biofilm from ssMABR1 was between 45 and 65%, considerably higher than the 35-46% from CR2, depending on the sample location. Generally, the biomass at the bottom of the reactors was thicker and, in the case of CR2, moister although there was no significant difference in proportion of live cells spatially in samples collected from CR2. The proportion of live cells from the bottom samples of ssMABR1 was significantly higher ($p=.0017$) than in samples taken from the top portion of the reactor fibers and samples from the bottom of CR2 ($p=.0158$) (**Figure 5**).

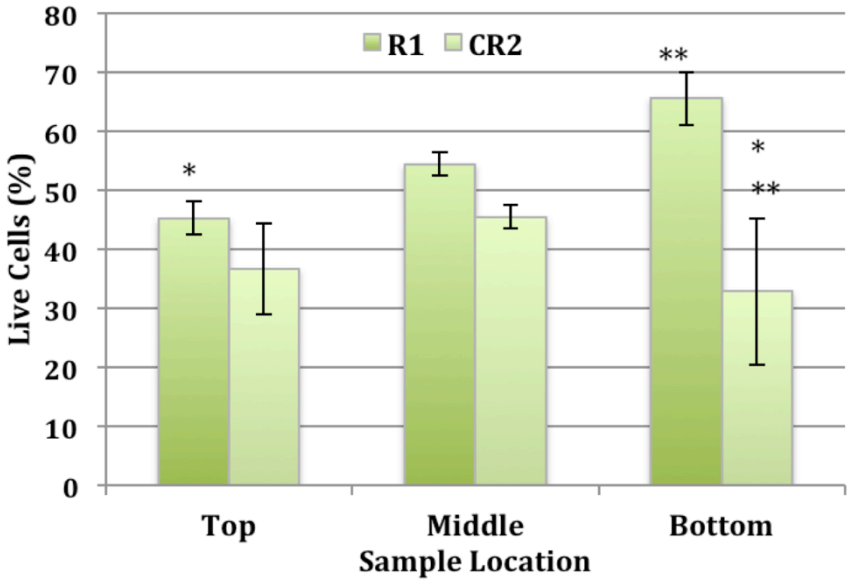

Figure 5: Live cells (%) in biofilm samples collected from CR2 and R1. Like asterisks indicate significant differences. Error bars indicate standard deviation of the mean (n=6).

Images of biofilm illustrating the presence of live bacteria are shown in Figure 6. Viable AOB and NOB were detected using FISH (Nitro-Vit®) in biofilms collected from both the active (R1) and the dormant reactor (CR2). **Figure 7** shows the red and green illumination of AOB and NOB in biofilms due to the labeling with specific DNA probes for those bacteria. Semi-quantitative analysis of the relative abundance of the AOB and NOB bacteria present in the biofilm indicate no real difference between the two reactors (**Figure 8**). This suggests that the impact of storage at 4°C for 7months on the viability and presence of the nitrifying microbial population as a whole may be insignificant for the performance of the reactor after dormancy.

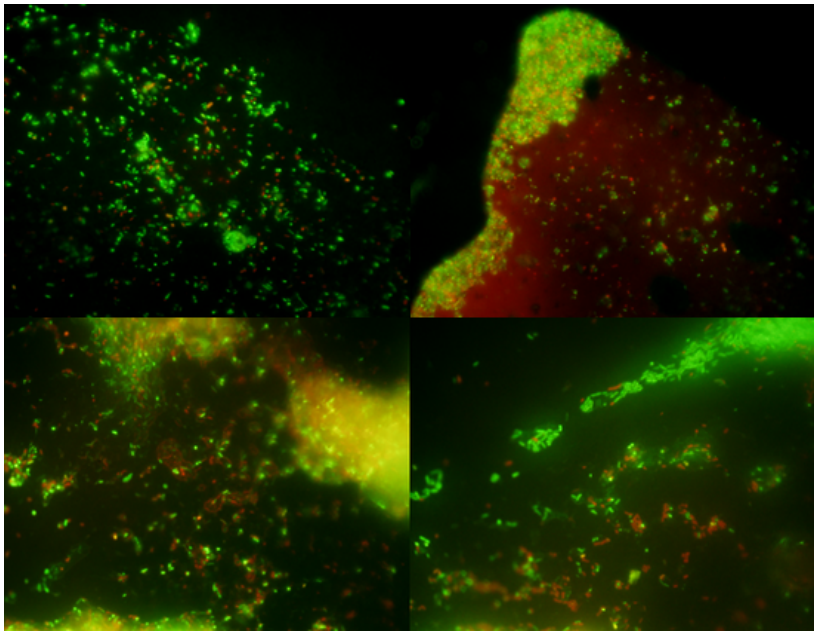

Figure 6: A) Live/dead stains of samples scraped from the surface of silastic fibers after removal from the shell from ssMABR1 for R1 and B) from the surface "CR2" which was hibernated at 4°C for 7 months. Viable bacteria (green) are present in samples from both modules. Shown here are examples of biofilm and micro-colony formation.

These data illustrate the long-term viability of the microbes, including AOB and NOB, within the biofilms of the dormant reactor. This viable community allows for rapid start-up and recovery of operational processes as illustrated in **Figure 4**.

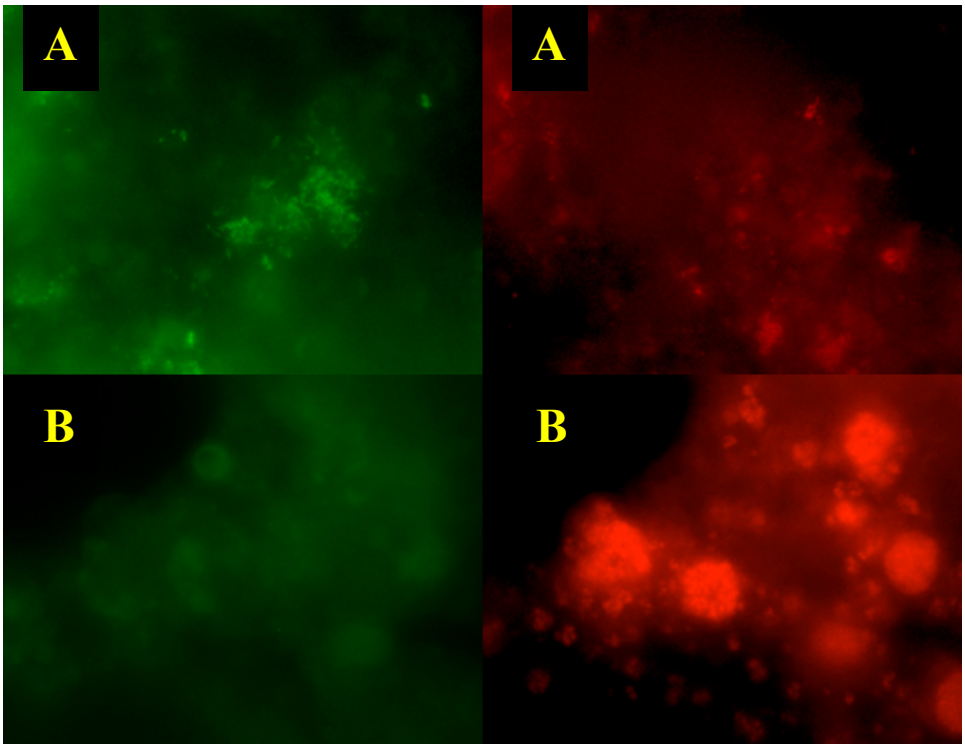

Figure 7: AOB (Red illumination) and NOB (Green illumination) present in biofilm samples from A) R1 (active) and B) R2 (stored). Images show the same section of biofilm from each reactor.

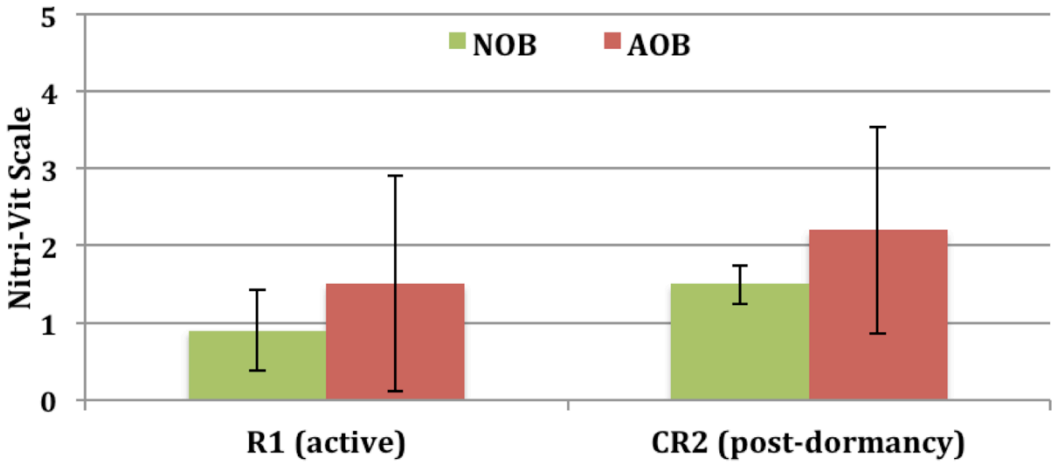

Figure 8: Relative abundance of AOB and NOB present in biofilm collected from R1 and CR2. (Samples are from the same location as images shown in Figure 7-reactor bottom). Error bars indicate standard deviation of the mean (n=3).

IV. Conclusion

Dormancy-recovery cycles are an important feature of bioreactor systems, yet very little is known about the microbial response to such processes in this specific application. In the 1-L MABR studies, it did not appear that any dormancy condition (with/without bulk fluid, 4°C/25°C) was better at preserving reactor performance metrics. While all reactors in the study (reactors 11-14) all recovered and even experienced improved performance, the recovery period of 20+ days is not satisfactory. Several factors may have contributed to such a long recovery period; first, the biofilms in these studies were not well established (48 days of reactor operation), and may not have been robust enough to handle full wastewater feed directly after a four-week dormancy period. Secondly, the amount of nitrifying and denitrifying communities may not have been well enough established in the biofilms to provide adequate conversion rates. In the long duration dormancy experiment, fully established biofilms (13 months old) were 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. Future studies should include further long duration dormancy cycles at room temperature along with studies to correlate of recovery times and biofilm age/initial biofilm health. Regardless, both studies completed were successful in demonstrating that these biological systems can survive periods of nonuse for future microgravity application.

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