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A transient event in anaerobic membrane bioreactor effluent quality influenced the chemistry and microbiology of a downstream phototrophic membrane bioreactor. However, the membrane separation element allowed for recovery of the microbial community once the transient event passed. Understanding failure modes is imperative for future implementation of membrane bioreactors, especially as it relates to remote, resource-limited environments.

Effects of an anaerobic membrane bioreactor upset event on nitrogen speciation and microbial community in a downstream phototrophic membrane bioreactor

Daniella Saetta[a*], Jason A. Fischer[b], Ashley Triana[e], Talon Bullard[c], Alexandra Smith[c], Cory J. Spern[d], Anirudha Dixit[b], Christina L. Khodadad[d], Daniel H. Yeh[c], and Luke B. Roberson[e].

[a]Bennett Aerospace, Inc.,
Kennedy Space Center, FL, 32899, USA

[b]Aetos Systems, Inc.,
Kennedy Space Center, FL, 32899, USA

[c]Department of Civil and Environmental Engineering Department,
University of South Florida,
4202 E. Fowler Ave. ENG 030, Tampa, FL, 33620, USA

[d]Noetic Strategies,
Kennedy Space Center, FL, 32899, USA

[e]National Aeronautics and Space Administration,
Kennedy Space Center, FL, 32899, USA

*** Corresponding Author**
Tel: 1-954-557-6182
E-mail Address: Daniella.saetta@nasa.gov (D. Saetta)

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ABSTRACT

A wastewater treatment architecture with an anaerobic membrane bioreactor and a phototrophic membrane bioreactor was created to close resource loops for use on the Moon and Mars. During an anomaly, an increase of carbon-to-nitrogen ratio led to a shift in the microbial community within PMBR. It is imperative to understand failure modes of the system and the system's ability to respond to perturbations in treatment because of their proposed application in remote, resource-limited locations. During this transient event, the carbon-to-nitrogen ratio in the AnMBR permeate increased from 0.341 to 11.2. Results showed that the microbial community became more diverse during the event and enriched in species related to the remediation of aromatic compounds. The community shift led to conditions required for greater than 90% carbon removal by the PMBR. The inclusion of the PMBR in the treatment architecture increased resilience and robustness for treating high-strength wastewaters in extreme environments.

1. Introduction

Technology development for decentralized wastewater treatment has been increasing as a means for providing access to sanitation in rural, resource-limited, underserved, marginalized, and aging communities. Decentralized wastewater treatment is challenged to its most extreme realms when designed for extraplanetary, partial gravity habitats, such as those planned for the Moon and Mars. The wastewater treatment technology development community has identified anaerobic digestion (AD) as an alternative to conventional activated sludge wastewater treatment, especially in decentralized contexts.[1] When coupled with membrane filtration, AD can be applied to high-strength wastewaters with a small treatment footprint.[2] Anaerobic membrane bioreactors (AnMBRs), which is AD coupled with membrane filtration, allow for a decoupling of solids and hydraulic retention times, which increases treatment rates, chemical oxygen

demand (COD) removal between 76–99%, and total suspended solids (TSS) removal higher than 99%.[2,3] This allows for further reduction in the treatment footprint, reinforcing their applicability for decentralized communities. Moreover, AnMBRs can also produce biogas, composed mainly of carbon dioxide and methane, which can be recovered to offset energy requirements.[4] However, AnMBRs are limited by their inability to remove nutrients and in some cases, such as in municipal wastewater treatment, downstream technologies are proposed for nutrient removal.[5] In AnMBRs, nitrogen and phosphorus, in the form of ammonium and phosphate, are released into the water phase as solids are hydrolyzed.[6] This can be used as an asset in nutrient recovery applications, such as fertigation systems, because the nutrients are released in a more usable form. However, high-ammonium waters have higher rates of nitrogen loss and are not preferable for fertigation systems, as ammonium has a large impact on solution pH when plants release H^+ ions after absorbing NH_4^+ to maintain solution neutrality.[7,8]

Researchers propose the use of algae-based MBRs, also known as phototrophic membrane bioreactors (PMBRs), as a secondary treatment for AnMBR permeates because of their ability to remove excess nitrogen, phosphorus, and COD.[9,10] If seeded with an algal–bacterial consortium, oxygen produced by the algae can be used by nitrifiers during nitrification and the system can act as a nitrate recovery technology.[11,12] Studies have shown that the presence of algae increases the rate of nitrification and ammonia uptake by the microalgae due to the oversaturation of dissolved oxygen.[13] Yet, the algal and bacterial communities need to be balanced to form a symbiotic relationship that does not create conditions that limit the other.

The balance between algae and bacteria can be influenced by the following parameters: (1) influent nitrogen form and concentration, (2) temperature, (3) light, (4) carbon concentration in the influent, (5) pH, (6) oxygen concentration, and (7) hydraulic and solid retention times. [14,15]

Of the seven parameters identified by Gonzalez-Camejo et al., five parameters can be manipulated via the control software and hardware operating the system. The operational software and hardware of a PMBR is not able to control the concentration of carbon or the nitrogen speciation and concentration in its influent, as that is dependent on the operation of the system treating and supplying the influent. Nitrogen speciation in the PMBR influent is independent of the control system and it influences the efficiency of nitrogen removal, as systems have been shown to have higher removal efficiencies when treating 100% NH_4^+ versus 100% NO_3^-.[16] However, while the concentration of ammonia may increase or decrease, the nitrogen speciation was not expected to change from NH_4^+ to NO_3^- at any point of the study, as the conditions for nitrification were not provided in the AnMBR. Conversely, if carbon is added to PMBRs in excess, fast-growing bacteria can flourish and block light penetration required for the algae to perform photosynthesis.[17] There is a gap in the literature surrounding the response of algal–bacterial PMBRs to perturbations in upstream treatments. It is important to understand failure modes and responses to perturbations with AnMBR and PMBR technology, especially because they have been proposed as solutions for low-resource and remote locations, and because the likelihood of perturbations occurring is plausible in real-world applications. In general, the functional stability of bioreactors can be influenced by three important factors: (1) functional diversity within the microbial community, (2) evenness of the community leading to robustness, and (3) population dynamics governed by resistance, resilience, and redundancy of the community when facing perturbations.[18] Research related to failure modes of photobioreactors for application in outer space has identified bacterial competition as a critical failure mode, among other failure modes related to improper illumination, insufficient CO_2, and exposure to elevated radiation.[19] Failure mode analysis is a critical element in future planning of

space exploration, and this type of research can inform stakeholders on a failure mode related to culture robustness during a stress event.

In this research, an architecture with AnMBR followed by a PMBR was designed as bioregenerative life support systems (BLiSS) for planetary habitats on the Moon and Mars.[20] The AnMBR would serve as a carbon removal technology and the PMBR would serve as a nutrient polishing technology, with carbon removal being the primary goal and nutrient recovery for fertigation of crops being a secondary goal. AnMBR and PMBR technologies are suitable for application on the Moon and Mars because they do not depend on chemical inputs, have a small footprint, and high treatment efficiencies.[21] As opposed to terrestrial wastewater treatment systems, nutrients are recovered for reuse in BLiSS architectures, because the nutrient stream is seen as a resource that would otherwise need costly resupply from Earth.[22] Once a BliSS is integrated into the habitation systems, the astronauts need to have a reliable and robust system that will provide its intended services, which may include wastewater treatment, water recovery, and nutrient delivery for fertigation of crops. Additionally, because these systems are designed to treat wastewater in parallel, the latter system should not produce an effluent that diminishes the quality of the primary system, such as increasing the chemical oxygen demand (COD) or total suspended solids (TSS) concentrations in this case. Therefore, the goal of this study was to provide an improved understanding of AnMBR and PMBR operations during off-nominal periods of increased organic carbon loading for an improved understanding of failure modes as they pertain to application in extreme environments. The specific objectives of the research were to (1) describe the transient event that caused off-nominal upstream conditions in the AnMBR permeate, (2) detail the water quality and treatment parameters in the PMBR during operation before and after the transient loading event, (3) identify the microbial communities present in the

mother culture, during, and after the transient event, and (4) discuss reliability and robustness of these systems in terms of their application for Lunar and Martian partial gravity habitats.

2. Materials and Methods

2.1. Anaerobic membrane bioreactor

A 54 L AnMBR was designed to reduce chemical oxygen demand and to liberate nutrients in high-strength organic waste with the main goal of closing the water and nutrient cycles on planetary bases (see Figure S1 for reactor schematic).[21,27] The system described in this research was designed to continuously treat the waste produced by 4 crewmembers.[27] The AnMBR consisted of 3 sequential tanks prior to membrane filtration to allow for phased digestion. The buffer tank acted as a flow equalization tank prior to the anaerobic digestion tanks. Reactor tank 1 (R1) and reactor tank 2 (R2) were seeded with anaerobic digestion sludge collected at a local wastewater treatment plant. The contents of R2 were circulated through an PVDF hollow fiber ultrafiltration (UF) membrane (0.199 m^2 membrane surface area), where solids were retained and the nutrient-rich liquid permeate was recovered. The headspace in the BT, R1, and R2 tanks were interconnected and the combined biogas created by the culture was routed to a gas meter that measured the daily biogas production. A lag in biogas production was expected due to the period needed for the sludge to acclimate to a new environment with a lower temperature (room temperature vs. 35°C) and a new wastewater source. During this study, the AnMBR treated 2.5 L/d of a fecal wastewater ersatz called complex organic particulate artificial sewage (COPAS) at a 5% solids concentration.[28] COPAS is an easily reproducible ersatz that uses milled cat food pellets as the source of proteins, carbohydrates, and particulate matter at concentrations similar to those found in fecal matter. COPAS was made by mixing 50 g/L of the milled cat food with tap water to a desired volume. The AnMBR had been used for three runs prior to the study

period described in this paper. A run was defined as the period of operation after each R1 and R2 tank reseed event. The length of time between reseeding events varied but three runs took place in the 487 days prior to this study period.

2.2. Phototrophic membrane bioreactor

A 6 L phototrophic membrane bioreactor (PMBR) was used to treat the AnMBR permeate. The PMBR consisted of a flat-plate photobioreactor with a PVDF hollow fiber UF membrane (0.083 m^2 membrane surface area) for permeate production. The design elements have been described in Saetta et al, 2022.[11] The PMBR was designed to treat 2.5 L/d of AnMBR permeate. The system architecture, including the AnMBR and PMBR, is shown in Figure S1. The AnMBR permeate was stored in a reservoir tank (RT) between the AnMBR and the PMBR. A buffer volume of approximately 15 L was accumulated in the RT to allow the PMBR to continue operation during scheduled or unscheduled maintenance events of the upstream AnMBR. Two level sensors were installed on the RT to allow for communication between the AnMBR and the PMBR. A low-level sensor indicated (to the PMBR) that the liquid level in the RT was below acceptable limits and would, therefore, stop the PMBR feed pump. A high-level sensor indicated (to the AnMBR) that the RT was full and would, therefore, stop the AnMBR from pumping permeate into the RT. This tank had an HRT of about 6 days.

An algal-bacterial culture was used to seed the PMBR. *Chlorella sorokiniana* (UTEX B 2805) was used as the starting microalgae species for the mother culture. The algae culture was initially grown in an ammonia-based growth medium. The recipe of the ammonia-based medium was created based on values described in the literature.[23–26] It had an ammonium (NH_4^+) concentration of 169.6 mg/L and a C/N ratio of 0.16. The medium recipe is provided in Table S1. The mother culture was grown in a 5 L bottle with mixing at 125 RPM, constant temperature

(30°C), and constant light (400 μmol/m²/s) (Heliospectra Elixia, LX601C grow light). Room air was continuously pumped into the bottle at a flow rate of 1 L/min. The bottle was sealed and the air delivery was filtered through a 0.22 μm PTFE filter, but other aseptic methods were not followed when handling the culture. This allowed for a consortium of algae and bacteria to grow in the mother culture. Weekly feeding of the mother culture consisted of removing ¼ of the culture volume and replacing it with an equal volume of the ammonia-based medium. The mother culture was fed in this manner for 1.5 years prior to the beginning of this study period. The PMBR was seeded with a mixture of the mother culture and tap water to a measured optical density of 1 at 680 nm at the beginning of the experiment.

2.3. AnMBR and PMBR Operations

The AnMBR and PMBR were operated continuously during this study. That meant that the AnMBR system was treating 2.5 L/d of COPAS and the PMBR was treating 2.5 L/d of AnMBR permeate during periods of nominal operation. Off-nominal events, such as failures with pump hardware, led to short periods of treatment below 2.5 L/d. However, the system was operating at 2.5 L/d for more than 90% of the study period. The system controller activated the respective influent pumps based on level sensors at the top of the reactor volumes, i.e., the top of BT, R1, R2, and the PMBR reactor tanks. Therefore, the membrane permeate pump flow rate on the PMBR was used to control the flow rate of the entire system because a low level in the PMBR reactor would draw contents from RT and the low level on RT would lead to permeate production in the AnMBR. During nominal operations, 2.5 L/d (membrane flux of 1.21 L/h/m^2) of PMBR permeate was produced. Daily average transmembrane pressure (TMP) for each membrane was calculated using data collected every 30 min.

The nominal HRT for the system and subsystems was calculated via the following equation: HRT = V/Q, where V = volume of the reactor; Q = flow rate. During nominal operations, the combined HRT for the AnMBR and PMBR was approximately 37.2 days. Each tank in the AnMBR had an HRT of 7.2 days, equaling an HRT of 21.6 days for treatment in the AnMBR. The PMBR had an HRT of 2.4 days. The additional days in the HRT for the entire system were attributed to the time spent in the AnMBR permeate tank and the RT. The HRT contributed to the difference in concentrations from tank to tank and from reactor to reactor. As the fluids moved from tank to tank, they were first diluted by the contents in the tank until they had replaced the entire contents of the tank over time. During periods of off-nominal flow rates, such as the transient event described in this paper, the HRT was decreased and more permeate was produced than intended, leading to a shortened treatment time in the R1 and R2 tanks and insufficient time for complete anaerobic digestion to take place. In this study, incomplete anaerobic digestion was defined as periods of less than 90% COD removal and less than 5 L/d of biogas production. Transient events would have impacts on the reactor conditions, such as elevated dissolved concentrations of constituents and elevated solids concentrations.

A full-spectrum LED grow light (Mars Hydro, TSW 2000) was used to illuminate the PMBR surface area with a light intensity of 450 μmol/m²/s. During the first half of the study, the light was delivered in 12 light/dark cycles, with a cycle being a 2-hour light followed by a 2-hour dark period. On day 72, the light cycles were changed to a 3-hour light and 1-hour dark photoperiod for the duration of the study as a strategy to increase the algal population. The PMBR had an illuminated surface area of approximately 1311 cm^2 (203.25 in^2). The temperature in the reactor was controlled at 30°C. Carbon dioxide was introduced in the PMBR at a flow rate of 1 L/min into the bottom half of the reactor and bubbled through the left side of the reactor

before it was released through the CO_2-out port to be recycled through a CO_2 recycle subsystem. In a controlled mixing chamber, the recycled CO_2 from the reactor was mixed with CO_2 from a k-bottle to maintain the CO_2 concentration at 3000 ppm, which is the average concentration of CO_2 in the cabin air of the International Space Station (ISS).[29] This concentration was chosen because the CO_2 for the PMBR would come from the cabin air or a cabin air CO_2 removal technology in the proposed architecture for the Moon and Mars.

2.3.1. AnMBR and PMBR sampling

Daily samples of oxidation-reduction potential (ORP) of R1 and R2 were taken to assess anaerobic performance. Weekly samples were taken from eight locations: (1) the AnMBR Influent tank, (2) the BT, (3) R1, (4) R2, (5) the AnMBR membrane permeate, (6) the reservoir tank (RT), (7) the PMBR reactor contents (PMBR), and (8) the PMBR membrane permeate. About 100 mL were taken at each location. The samples were divided into total and soluble fractions for future analysis. The soluble samples were prepared by centrifuging 50 mL for 15 min at 3400 rpm. The remaining 50 mL were designated as the total fraction (used to measure TSS and COD). The soluble fractions were used to measure soluble COD, dissolved organic carbon (DOC), total nitrogen (TN), total phosphate (TP), and ammonia-nitrogen. Samples were stored at 4°C until analysis took place. All sample analyses were conducted within a week of sample collection and methods are detailed in Section 2.4.

Whole genome sequencing samples were taken for three sample types: (1) the mother culture used to inoculate the reactor, (2) the reactor contents and attached biofilms during the peak in the transient event (day 101), and (3) the reactor contents post transient event (day 161). The biofilm samples were taken from the area around the port where the AnMBR permeate was introduced into the PMBR (called the “Biofilm Feed Corner” sample), the area around the float

switch used for level sensing (called the "Biofilm Float Switch" sample), and the area around the port where the membrane recirculation was reintroduced into the reactor (called the "Biofilm Recirc Corner" sample). Only reactor contents were sampled for the time point after the reactor recovered from the transient event because the thick biofilms were no longer present. The methods used for DNA sequencing and analysis can be found in Saetta et al., 2023.[30] Briefly, whole genome sequencing was performed on DNA samples extracted with Qiagen's DNeasy PowerLyzer PowerSoil Kit. Illumina Miseq was used to sequence samples, Kraken2 was used to analyze the data, and PlusPFP (a pre-built RefSeq database) was used to map archaea, bacteria, viral, human, UniVec_Core, protozoa, fungi, and plant taxonomic information. The alpha diversities (Shannon diversity index) and beta diversities (Bray-Curtis dissimilarity) on the entire dataset were calculated with the Kraken2 tools.

2.4. Analytical methods

The ORP of R1 and R2 was measured using a handheld ORP meter (MW500, Milwaukee Instruments). The optical density (OD) of the PMBR reactor culture was measured with a 1 cm pathlength cuvette on a visible spectrophotometer (Thermo Spectronic Genesys 20) at a wavelength of 680 nm. TSS of the AnMBR reactor contents was measured with the Standard Method 2540 D. Chemical analysis was conducted using Hach Test 'N Tube methods for TN, TP, ammonia-nitrogen (NH_3-N), nitrate-nitrogen (NO_3-N), and nitrite-nitrogen (NO_2-N) using the spectrophotometer mentioned above. DOC was measured using an OI Analytical Aurora 1030 TOC analyzer. COD was measured using Hach COD Digestion Vials in the high range. In the PMBR reactor, dissolved oxygen concentration (optical DO sensor), pH (Pinpoint pH, American Marine Inc.), and CO_2 concentrations (K30 10,000 ppm CO_2 Sensor, CO2Meter.com) were measured continuously and recorded every 5 min by an Opto 22 controller.

2.6. Carbon-to-nitrogen ratio and nitrogen mass balance

The carbon-to-nitrogen ratio in the AnMBR permeate and the RT was calculated using the DOC and TN concentrations in the weekly samples. The concentrations were converted to molar concentrations prior to calculating the ratio. A weekly nitrogen balance was calculated using the weekly concentrations of TN, NH_3-N, NO_3-N, and NO_2-N. The NH_3-N, NO_3-N, and NO_2-N concentrations were converted to molar nitrogen concentrations before being normalized by the molar TN concentration for that weekly sample. The mass balance was calculated for the dissolved and colloidal fraction, due to the presence of the UF membrane. The dissolved fraction was defined as the concentration of the nitrogen species in the membrane permeate samples (dissolved nitrogen that passes through the membrane). The colloidal fraction was defined as the difference between the nitrogen species concentrations in the PMBR reactor samples and the permeate samples (nitrogen colloids rejected by the membrane). For example, the colloidal NO_3-N fraction was calculated as the difference between the NO_3-N in the PMBR permeate sample and the NO_3-N in the PMBR reactor sample. The dissolved and colloidal fractions were calculated for each weekly concentration of the nitrogen species (NH_3-N, NO_3-N, and NO_2-N) and it was normalized by the TN in the influent sample (RT).

3. Results and Discussion

3.1. Description of the AnMBR transient event

The AnMBR, used to treat organic waste, was in nominal operation prior to day 30. Nominal operation was defined as production of a low-carbon, high-nutrient permeate for downstream use while treating 2.5 L/d of the organic simulant wastewater (see Table S2 for average influent concentrations). Specifically, permeate samples were monitored to confirm nominal operation with COD removal above 95%, TSS removal above 90%. Additionally, daily samples for ORP

were taken to ensure that anaerobic conditions were maintained in the reactor tanks and daily biogas production was tracked to ensure that more than 5 L/d were being produced. On the days leading up to day 38, the operators noticed that the permeate production rate was decreasing at the set permeate pump flow rate and TMP was increasing in the AnMBR membrane (see timeline of events in Figure S2; see TMP data in Figure S3). Due to fouling over time, the flow rate of the permeate pump had been slowly increased over its lifetime to maintain the production of 2.5 L/d of permeate. On day 38, a decision was made to install a new membrane module on the AnMBR. The original membrane module was installed 487 days prior to this reactor run and it had reached the end of its life. Upon installing the new membrane module, an operator error was made when the permeate pump flow rate was not reduced to match the state of the new membrane. With the new membrane module and the elevated flow rate, more than 2.5 L/d of permeate was produced, and contents from the feed tank were pushed through the system at a faster rate. The shortened HRT led to an increase in feed rate to the AnMBR, an increase in TSS in the BT and R1, and increased carbon in the permeate due to insufficient treatment time in R1 and R2. The elevated flow rate was kept in operation for 4 days, which delivered the 10 L available in the feed tank into the system and produced permeates from the additional volume available in BT, R1, and R2 before the tanks reached the low-level sensors and the operating software stopped the production of permeate. It is impossible to calculate the actual HRT during this period because the actual flowrate was not known but it could have been as low as 5.4 d if the entire feed tank was introduced in one day (nominal HRT was 21.6 d). After the four days, the flow rate was restored to 2.5 L/d and sampling of the tanks continued to assess the health of the reactor as scheduled.

The transient event had direct (DOC, COD, ORP, daily biogas production, and TP) and indirect (TSS, TN, and TMP) effects on the AnMBR microbiology and chemistry. In terms of anaerobic conditions, between day 0 and day 38, the ORP of R1 and R2 were within ranges expected for anaerobic conditions, with levels below –100 mV in R1 and below –300 mV in R2 (see Figure S4). However, the ORP in R2 increased to –250 mV on day 44 and then slowly decreased back to nominal levels below –300 mV from day 64 until the end of the study. Additionally, the reactor started producing more than 5 L/d of biogas on day 24, with it averaging about 16 L/d for the following 6 days (see Figure S5). Interestingly, the period of production difficulties that led to changing the membrane module can be seen in the biogas data, as the daily biogas production started decreasing on day 30 and reduced to 0 L/d on day 33 and the transient event started on day 38. As seen in Figure 1a, prior to day 38, the AnMBR permeate had an average DOC concentration of 98.3 mg/L, showing that the system was able to reduce the DOC by greater than 95%. A small increase was seen in the total COD (Figure 1b) of the permeate on day 20. However, the COD concentration in the permeate recovered to 123 mg/L total COD in the weekly samples immediately following the increase. On day 42, an increase is seen in the AnMBR permeate DOC and COD, indicating that the transient event had altered the chemistry of the AnMBR and more carbon was being released in the permeate than in previous weeks. At the peak of the transient event (day 56), the permeate concentrations increased to about 3200 mg/L DOC and 9200 mg/L COD. The AnMBR produced 0 L/d of biogas until day 50, and then production started to slowly (between 0–3 L/d). The daily biogas production increased steadily from that moment on, peaking with 30.9 L on day 136, and correlated to the decreasing COD concentrations in the reactor tanks as the effects of transient event passed through the system. The final direct effect of the transient event can be seen in the TP

299 concentration in the permeate samples (see Figure S6). In AnMBRs, phosphate is released into
300 solution as the wastewater solids are hydrolyzed.[5] However, increased pH, alkalinity, and
301 ammonia concentrations within AnMBR systems can lead to precipitation of phosphate-
302 containing minerals, such as struvite ($MgNH_4PO_4 \cdot 6H_2O$), and to an accumulation of phosphate
303 in the sludge.[31,32] Prior to the transient event, the TP in the permeate was about 4x lower than the
304 influent concentration. However, after the transient event, the concentrations of the influent and
305 permeate were within 100 mg/L of each other. This was indicative that decreased HRT did not
306 create the conditions necessary for phosphate precipitation that was seen prior to the transient
307 event.

308 Analysis of the TSS concentrations was also used to determine if the reactor tanks had
309 been overloaded with solids as a result of a decrease in HRT. The data show that the permeate
310 TSS concentrations stayed low, as expected for a functioning membrane (Figure 1c). However,
311 the TSS concentration in the BT increased from 35,000 mg/L on day 34 to 110,000 mg/L on day
312 42. That increase in the TSS in BT slowly impacted the TSS in R1, which made its way through
313 the system and impacted the TSS concentrations in R2. The TSS in R1 reached levels above
314 22,000 mg/L on day 84 and the TSS in R2 reached levels above 14,000 mg/L on day 91. The
315 increasing TSS also had an impact on the AnMBR membrane module, with the TMP increasing
316 from about 0.25 bar to about 0.75 bar and fluctuating in that range for the remaining days of the
317 study (Figure S3).

318 The TN concentration followed a similar trend to TSS due to the inherent mechanisms of
319 anaerobic membrane bioreactors. In AnMBR technology, the nutrients trapped in the solid phase
320 are slowly solubilized by the microorganisms in the reactor. This solubilization releases the
321 nutrients into the water phase, which can be recovered in the permeate via membrane filtration.[5]

The TN was expected to increase as the solids were solubilized. As seen in Figure 1d, the average TN concentration in the permeate was 302.9 ± 38.6 mg/L as N between days 27 and 70. After day 70, the concentration increased and peaked at 1165.4 mg/L as N on day 125. The concentration did not increase immediately during the transient event, such as it did with DOC and COD. Rather, it increased slowly over time as the TSS concentrations in R1 and R2 increased. The TN concentrations in the permeate were plotted versus the TSS concentration in R2 and the data had a positive correlation with an R^2 value of 0.872 (see Figure S7), showing the relationship between the increasing solids concentration in R2 and the release of soluble nitrogen into the water phase, which was recovered in the permeate. On day 140, the solids concentration in R2 was too high (>20,000 mg/L) to maintain the integrity of the membrane and the system had to be shut down for complete removal of solids and reseeding for future runs of the system.

3.2. Effects of the transient event on PMBR

As the AnMBR experienced the transient event, the PMBR culture and water chemistry shifted dramatically. It became evident that the increasing carbon had shifted the carbon-to-nitrogen ratio in the RT contents that were fed to the PMBR. Figure 2 shows the carbon-to-nitrogen ratio in the AnMBR permeate and the RT samples before, during, and after the transient event. Prior to the event, the average C/N ratio was 0.62 and 0.33 in the AnMBR permeate and RT samples, respectively, indicating that the AnMBR was removing carbon and releasing nitrogen as expected. A low C/N ratio was desired because research has shown that low C/N ratios allow for higher concentrations of nitrifying bacteria in a mixotrophic system.[13] A low C/N ratio also maintains a lower concentration of heterotrophic bacteria, which have higher growth rates compared to nitrifiers and may cause reduced photosynthesis due to light shading.[13] However, due to the transient event, the C/N ratio in the permeate increased 22-fold from 0.34 to 1.77 from

day 34 to day 42, respectively. The C/N ratio increased steadily for the following 4 weeks of sampling, finally peaking in the RT at 11.2, 34 times higher than the average C/N ratio prior to the transient event. This was due to the dilution rate effect as contents from upstream tanks influenced the concentrations of downstream tanks as a function of HRT. Slowly, the C/N ratio started decreasing after day 70, returning to levels under 1.0 on day 131. The decrease was due to a gradual decrease of carbon and an increase in the TN in the AnMBR permeate, as the system recovered from the transient event and treated the contents in the reactor tanks. The C/N ratio peak in the RT samples was offset by two weeks compared to the C/N ratio in the AnMBR permeate samples (Figure 2). This was indicative of the HRT of the additional tank used to integrate the AnMBR and the PMBR. The peak in the AnMBR permeate samples occurred on day 55 and peaked at 12.1. Without an intermediary tank, such as the RT in our design, the PMBR would have received a higher organic loading during the event and the effects of the increased loading could have been greater than those seen in this study. This is an important future consideration when designing robust systems for downstream treatment of wastewater effluents.

As the C/N ratio increased, due to an increase in DOC concentration in the AnMBR permeate, the reactor responded with a visible shift in the culture. The culture changed in color, from the green color expected in a phototrophic bioreactor to a pale orange color that is more indicative of a bacterial fermenter (see pictures in Figure S8). The change in the culture color was also evident in the optical density (OD, 680 nm) data taken throughout the study period (Figure 3). Prior to the peak in C/N ratio, the OD of the reactor increased as the culture density increased. However, after the peak in C/N ratio, the OD began to drop, indicative of a drop in the chlorophyll production in the reactor. This was true past the point at which the photoperiod was

changed on day 72, which occurred more than thirty days after the overload of solids started the transient event. The OD reached the lowest level on day 94 and then began to increase as the C/N ratio reached levels below 3. The change in the culture also affected the pH and DO concentrations in the reactor, which increased to above pH 8 and decreased to below 1 mg/L during this period (see Figure S9).

The water chemistry in the PMBR reactor and permeate samples also responded to the changes. The AnMBR permeate that is used as the PMBR influent was high in NH_4-N and carbon concentrations. Initially, the goal of the study period was to assess the ability of PMBR to perform nitrification of the AnMBR permeate. The NH_4-N had three removal routes once in the reactor: (1) biomass assimilation, (2) nitrification, and (3) volatilization at high pH. Prior to the event, DOC, TN, NH_3-N, NO_3-N, and NO_2-N were all nominal in the reactor and permeate samples (Figure 4). The variations in the concentrations were due to the nominal start-up period in which nitrification was occurring and biomass grew denser. Then, during the transient event, the nitrate decreased to the background concentrations in the RT, indicating that nitrification was no longer occurring. These data were used to calculate a nitrogen mass balance to determine the fate of nitrogen in the liquid phase in the PMBR (Figure 5). The mass balance was normalized by the influent TN (RT samples). The colloidal and dissolve fractions were calculated for ammonium, nitrate, and nitrite. As seen in Figure 5, there was a large nitrite fraction in the first two weeks of operation. As the operation continued into weeks 3 and 4, the nitrite fraction decreased and the nitrate fraction increased. This was a direct measurement of nitrification taking place. As the system stabilized, in weeks 5 and 6, the largest fraction of nitrogen was in the form of dissolved nitrate, reaching levels up to 75% on day 42. Throughout this period, greater than

80% of the TN in the PMBR influent (RT) was in the form of NH_4-N. Therefore, in the first 6 weeks, ammonia removal was governed by biomass assimilation and nitrification, as expected.

As the C/N ratio increased, the mass balance showed that nitrification was inhibited, and the system was not meeting the nitrification goal based on the shift in nitrogen fractions. Beginning in week 7, the nitrogen mass balance began to show decreasing values in the measured nitrogen fractions and an increase in the theoretical fraction. By week 9, more than 90% of the nitrogen mass balance was made up of the theoretical biomass uptake or nitrogen volatilization fraction and it remained at an average of 85% for the next 9 weeks of operation. The ammonia and the TN concentrations in the PMBR permeate during this period were about 25 mg/L as N and 50 mg/L, respectively. The nitrate concentration was low, the dissolved oxygen concentration was below 1 mg/L, and the pH was above pH 8 during this period (see Figure S9), indicating that the TN fraction was either lost to uptake by the culture or by ammonia volatilization. Even though the reactor was no longer nitrifying the ammonia, the concentration in the permeate did not pose a possible risk for NH_4-N toxicity in downstream fertigation applications. After day 126, when the C/N ratio decreased below 1, the measured nitrogen fractions started increasing slowly; a sign that the reactor was recovering from the transient event. A picture taken on day 130 (see Figure S8) showed that the culture had shifted back to predominantly algae compared to the picture taken on day 64.

One parameter that was not negatively influenced throughout the transient event was the PMBR permeate DOC concentration (Figure 6). As the C/N ratio increased, the DOC concentrations in the RT reached 2474 mg/L, almost 30 times the concentration in the weeks before the transient event. The shift in the culture discussed previously contributed to the near-complete removal of the excess carbon load coming from the upstream AnMBR transient event.

Starting on day 42, the DOC removal by the PMBR was 92% on average until the end of the study period. This showed that the PMBR acted as a valuable technology after the carbon washout event of the upstream AnMBR system. The PMBR removed the incoming DOC and continued producing permeate with the low DOC concentration. The average DOC concentration in the PMBR permeate in the samples post-C/N ratio peak was 97.8 mg/L, which was within the range of the DOC concentration for the AnMBR permeate during nominal operation (98.3 ± 9.6 mg/L). During the transient event, the PMBR reacted to the increased DOC concentration and removed the excess carbon to levels that met the AnMBR requirement for carbon removal. The primary goal of the PMBR (i.e., nitrification for fertigation) was not met, but the system met the primary requirements for the AnMBR (i.e., carbon removal). As the C/N ratio recovered, observations indicated that the culture shifted from pale orange to green with decreasing carbon and increasing nitrogen in the AnMBR permeate (see Figure S8). The characterization of the shift in the community structure will be discussed in the following section.

3.3. Characterizing the shift in the PMBR microbial communities associated with the transient event

The microbial community structure of the PMBR was analyzed to confirm the observations that shifts in the culture had occurred during and after the transient event. Samples for biological analyses were taken from (1) the mother culture stock, (2) the reactor during the elevated C/N ratio, and (3) the reactor after the transient event had passed. Samples were not taken prior to the event due to the unforeseen nature of the transient event. The samples represented the microbial communities in that location at the time when the sample was taken. They might not represent the entire microbial community of the reactor. The samples taken after the transient event were taken on the last possible day of operation to fully differentiate it from the sample taken during

436 the peak in the transient event. Figure S10 shows the initial analysis of the DNA sequencing
437 results pertaining to the diversity found in the samples. The Shannon diversity index is a
438 measurement of diversity within each sample. A higher Shannon diversity index indicated that
439 there was more diversity within the sample's community structure. The results showed that the
440 diversity within each sample was highest in the samples taken during the peak in the transient
441 event when compared to the samples taken from the mother culture stock. Alternatively, the
442 Shannon diversity index was similar in the samples after the transient event compared to the
443 diversity in the mother culture. These results indicated that the microbial community of the
444 culture shifted during the transient event and that there was a second shift after the transient
445 event had passed.

446 Beta diversity was used to compare the microbial communities across different samples.
447 More specifically, the principal coordinate analysis (PCoA) of the Bray-Curtis dissimilarities
448 was used to compare the similarities and differences between the samples. Samples that were
449 more similar to each other clustered in the same region. As seen in Figure S10, there were two
450 distinct groupings in the data: (1) the samples taken from the mother culture were clustered
451 separately from the samples taken from the reactor, both during and after the transient event, and
452 (2) the samples taken after the transient event (reactor post) were clustered closer together and
453 away from the samples taken during the transient event (reactor peak and biofilm). These results
454 indicated that there was marked changes in the culture (1) once it began treating AnMBR
455 permeate and (2) while it was experiencing the transient event.

456 Next, the data were analyzed to determine how the community structures changed due to
457 the transient event. A total of 73 phyla were identified in the samples from the bacteria,
458 eukaryote, archaea, and virus domains. Bacterial communities were the first to be analyzed

459 because two bacterial phyla were the two most abundant phyla in the samples: Proteobacteria
460 (80% ± 14%) and Actinobacteria (6.2% ± 1.0%). Figure 7 shows the relative abundance of
461 bacterial genera in the PMBR samples taken from the mother culture, the reactor, and its biofilm
462 during the transient event, and the reactor samples after the transient event had passed. The
463 larger bubbles are indicative of a higher relative abundance within each sample. Only genera
464 with a relative abundance above 1% are shown in the figure. The results confirm that there was a
465 difference in the bacterial community structures based on the transient event. The samples taken
466 during the transient event had a larger number of genera with relative abundances above 1%,
467 with 22 genera identified in the samples compared to 11 and 14 genera in the mother culture and
468 the post-transient events samples, respectively. The communities were then analyzed to
469 determine if the community structure had any connection to the reactor conditions. The most
470 abundant genus in the mother culture was *Sphingopyxis*, a genus containing species known for
471 environmental bioremediation.[33] During the transient event, the most abundant genera were
472 *Hydrogenophaga* and *Thauera*, which contain yellow-tinted, hydrogen-oxidizing species and
473 species that metabolize aromatic compounds in anoxic conditions, respectively.[34,35]
474 *Hydrogenophaga* also have the ability to heterotrophic denitrify nitrate.[35] This correlated with
475 the conditions within the reactor, as the DO decreased with the increase in the microbial
476 overgrowth, the culture turned into a light-orange color, and the decreasing nitrate
477 concentrations. After the transient event, the most abundant genera were *Alicycliphilus*,
478 *Acidovorax*, and *Comamonas*, known for their use in bioremediation and denitrification in the
479 environment.[36–38] These three genera were present in the samples taken during the transient
480 event, but were not present in the mother culture. Also, their relative abundance increased
481 compared to the samples taken during the transient event, indicating enrichment caused by that

the conditions post-transient event. Conversely, *Hydrogenophaga* and *Thauera* decreased in abundance or were not present in abundances higher than 1% in the post-transient event samples. *Mesorhizobium*, *Pseudomonas*, and *Streptomyces* were in similar abundances throughout all samples taken for this study, indicating that they were not affected by the reactor conditions and may contribute to the stability in the culture. Specifically, studies have shown that *Mesorhizobium* have a high tolerance to stresses from temperature, salt, and pH levels.[39] *Pseudomonas* is a large genus that has a wide functional and environmental range in soils and aquatic environments, and its versatility lends itself to applications in bioremediation and biotechnology.[40] *Streptomyces* is a genus of ecologically diverse bacteria that have a large affinity for mutualistic interactions, such as those found to provide resistance to biotic and abiotic stresses in the rhizosphere of plants.[41] Finally, five genera were present in every sample that was taken from the PMBR (during and after the transient event) that were not present in the mother culture. These genera (*Acidovorax*, *Alicycliphilus*, *Comamonas*, *Hydrogenophaga*, and *Variovorax*) could be attributed to the influence of the AnMBR permeate on the PMBR culture, as they were not present in the mother culture. Future research is needed to determine the specific influence of the AnMBR cultures on downstream systems.

The sequencing results were analyzed for the presence of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) to determine the presence of nitrifiers in the samples (see Figure S11). Of the seven NOB genera identified in the literature,[42] only four were present in the Kraken2 database used for this study and only three were identified in our samples (*Nitrobacter, Candidatus Nitrotoga*, and *Nitrospira*). All three AOB genera were found in our samples: *Nitrosococcus*, *Nitrosospira*, and *Nitrosomonas*. However, the mother culture samples had a higher abundance of the NOB *Nitrobacter* and the reactor samples had a higher abundance

of the AOB *Nitrosomonas*. This could be due to the time between feedings of fresh media for the mother culture compared to the constant introduction of AnMBR permeate in the PMBR system, as HRT has a large influence on the growth rate of AOBs and NOBs.[43] The ratio of AOB to NOB was calculated for each sample to determine whether the samples fell within 2.2–2.7, which is the optimal range for nitrification (see Figure S11).[44] The results show that the ratio in the reactor samples (during the transient event and after the transient event) were all greater than 2.7. The results for the mother culture were all below 2.2. This shows that the AOB content in the reactor was outgrowing the NOBs, leading to accumulation of nitrite.[45] Nitrite spikes were seen in the PMBR throughout this study (Figure 4d). In the mother culture, the results show that the NOBs were more abundant than the AOBs, leading to accumulation of nitrate and a possibility of a nitrite loop as denitrifiers reduce nitrate to nitrite for the NOB.[46,47]

Phyla containing fungi and the cyanobacteria phyla made up a max of 0.8% and 1.14%, respectively, of the phyla present in the samples. The genera-level analysis was done for fungi and cyanobacteria (see Figure S12 and S13) and the most abundant fungi and cyanobacteria were maintained throughout all the samples. Seven fungi genera were found in every sample sequenced: *Purpureocillium, Thermothelomyces, Colletotrichum, Thermothielavioides, Pyricularia, Fusarium*, and *Aspergillus*. The change in the C/N ratio did not seem to influence the most abundant community members in the samples collected during and after the transient event, but it did influence their relative abundance. A recent study showed that the treatment efficiency of a fungal reactor increased with decreasing C/N ratio.[48] Future research is needed to determine the effects of the fungi population in a mixed culture, such as the one used in this study. Three cyanobacteria genera were found in all the samples taken (*Synechococcus, Nostoc*, and *Calothrix*) and one genus was found only in the samples taken from the reactor

(*Gloeobacter*). Cyanobacteria have been identified as organisms that may be found on extra-terrestrial systems because they have a wide variety of adaptations to extreme environments, coming from their long evolutionary history.[49] Specifically, *Gloeobacter* have photosynthetic systems closely related to primitive organisms, are slow growers, and tend to aggregate in mats.[50] Their aggregation to the reactor surfaces may be a reason why they were identified in the reactor samples and not in the mother culture.

3.4. Implications of BLiSS operations on reliability and robustness for Lunar and Martian habitats

This research has shown that PMBR treatment may be a viable post-treatment of AnMBR permeate because its robust community is able to respond to variable water quality in the AnMBR permeate during transient events. The culture adapted to the increased carbon level during the transient event and rebounded to its initial condition when the AnMBR returned to nominal conditions. The use of an algal–bacterial consortium and the physical barrier, i.e., the UF membrane, were key in allowing for the shift to occur. The bacteria responded to increased carbon concentrations, removed the carbon to acceptable levels, and was out-competed by the algae once the incoming carbon levels rebounded to nominal concentrations. Without the physical barrier, the algae could have been washed out, making a shift in the community impossible after the carbon rebounded to background concentrations. The parallel treatment of wastewater with an AnMBR followed by a PMBR increased the resilience of the entire system, as the PMBR responded to an anomaly in treatment in the AnMBR and continued the production of a low-carbon permeate. While the PMBR had its own goal of nitrifying the AnMBR permeate, the PMBR provided the additional benefit of producing a low-carbon effluent while the AnMBR was recovering from the transient event.

Resilience in engineering design, not to be confused with the resilience of the microbial community, is defined as the ability to respond to an adverse event, with a strong emphasis on reliability and restoration.[51] Reliability and restoration are both influenced by the system's robustness (i.e., the ability for a system to keep a failure from affecting downstream processes), its rapidity (i.e., the speed at which the system responds to the failure), and its redundance (i.e., the availability of critical components necessary to respond to the failure).[52] In this case, the resilience of the engineering design is closely tied to the resilience of the microbial community. In microbial communities, resistance and resilience are guided by the communities' response to external stimuli by the response of the individuals, the population's persistence, and community stability.[53] Additionally, the characteristics of the disturbance on the community, such as its intensity, the nature of the disturbance, the time interval between distances, and the order of the disturbance, may influence the time it takes to recover from the disturbance.[54] Proper engineering design and microbial community stability are needed to have a resilient BLiSS architecture. Based on these definitions, the PMBR was able to provide added resilience to the engineering design of treatment architecture because it was reliable during the event and allowed for the AnMBR system to return to nominal conditions. While the system did lose its ability to nitrify the AnMBR permeate, the downstream fertigation system should also include engineering controls to increase robustness. While the design of the fertigation system is out of the scope of this paper, there are many methods for mitigating variable nitrogen speciation including shifting nitrite to nitrate via room air bubbling, inclusion of a biofilter in the nutrient solution recirculation loop, and biochar absorption of ammonia prior to the fertigation system.[55–57] Elevated carbon concentrations would be detrimental to these technologies and having the removal of carbon prior to their implementation is key for hydroponics systems.

Resilience in BLiSS is going to be paramount for a sustained human presence on extraplanetary bases. These systems must be able to respond to anomalies in treatment while still providing the necessary service to the crew. Hence, an approach of designing systems in-series and in-parallel should be explored. Systems designed in-series, such as the AnMBR and the PMBR in this study, are designed to provide resilience within the treatment train. Systems that are designed in-parallel, such as two treatment trains of AnMBRs followed by PMBRs, would be designed to provide reliance to the BLiSS system. If one treatment train were to face an anomaly, the other system could respond and provide the necessary service for the habitat. However, this has major implications for mass and volume requirements, as increasing the mass and volume of a technology makes it less favorable for integration into the final architecture designs due to cost constraints. Future work is needed on how resilience informs trade studies and how designing in-series and in-parallel affects the overall efficiency of the system.

4. Conclusion

AnMBRs have been proposed as solutions for decentralized wastewater treatment in resource-limited, remote locations. However, AnMBRs are limited by their inability to remove nutrients. When paired with algal–bacterial PMBRs, algal photosynthesis provides the necessary dissolved oxygen for bacterial nitrification. In this research, the effects of increasing C/N ratio in the AnMBR permeate on PMBR water quality and microbial communities were shown. The PMBR culture shifted during the transient event, allowing for the continued removal of carbon to meet the requirements of the AnMBR. The shift allowed for the AnMBR to recover from the increased carbon load and the PMBR shifted back to a predominant algal culture after the C/N ratio decreased to below 3. Shotgun metagenomics showed marked differences in the microbial communities during and after the transient event, strengthening the importance of using

membrane technologies to maintain a robust culture of algae and bacteria for the treatment of complex wastewater. This research gives insight into the additional robustness that PMBRs provide to wastewater treatment with AnMBRs. Future research is needed to evaluate the cost and benefits of designing a system with redundancy in terms of mass, volume, and economic costs, especially as it pertains to failure modes and application of the technologies in remote, extreme locations.

Data Availability

Raw data for the DNA sequencing can be found in the NASA Open Science Data Repository with DOI: 10.26030/vmzd-0s72.

Author Contributions

Daniella Saetta: Conceptualization, Investigation, Data Curation, Formal Analysis, Visualization, Writing - Original Draft. **Jason A. Fischer:** Conceptualization, Investigation, Data Curation, Writing - Review & Editing. **Ashley Triana:** Investigation, Writing - Review & Editing. **Talon Bullard:** Conceptualization, Investigation. **Alexandra Smith:** Conceptualization, Writing - Review & Editing. **Cory J. Spern:** Investigation. **Anirudha Dixit:** Data Curation, Formal Analysis. **Christina L. Khodadad:** Formal Analysis, Resources, Writing - Review & Editing. **Daniel H. Yeh:** Conceptualization, Resources, Writing - Review & Editing, Supervision, Funding acquisition. **Luke B. Roberson:** Conceptualization, Resources, Supervision, Writing - Review & Editing, Project administration, Funding acquisition.

Conflict of Interest

Luke Roberson has patent #20230271865 pending to UNITED STATES OF AMERICA AS REPRESENTED BY THE ADMINISTRATIOR OF NASA. Daniel Yeh has patent #11891321 issued to University of South Florida, UNITED STATES OF AMERICA AS REPRESENTED

BY THE ADMINISTRATIOR OF NASA. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Tables and Figures

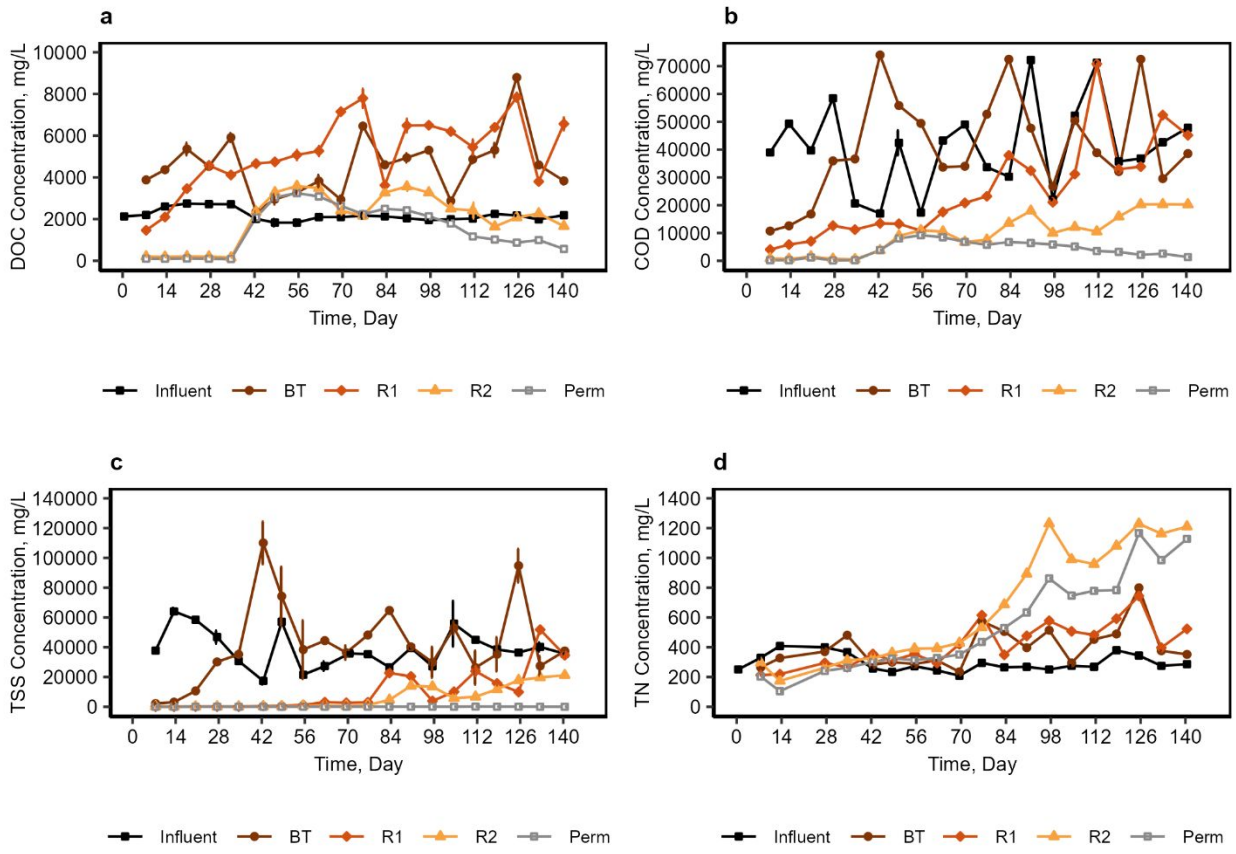
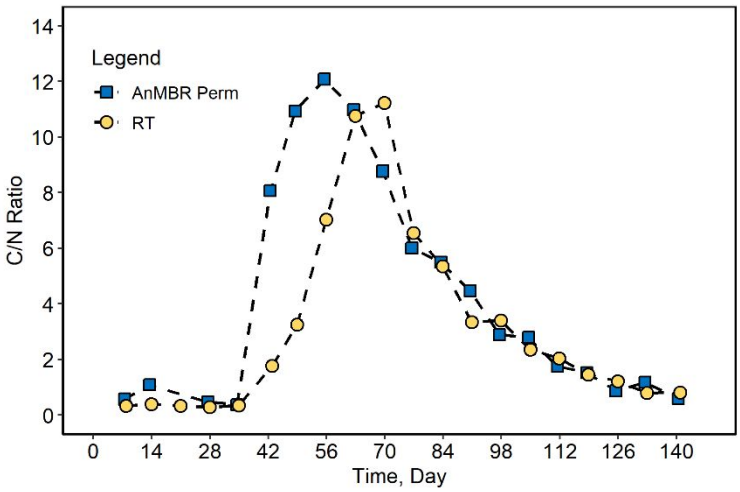


Figure 1 – (a) Dissolved organic carbon (DOC), (b) total chemical oxygen demand (COD), (c) total suspended solids (TSS) concentrations, and (d) TN concentrations in the AnMBR samples. Error bars show the standard deviation in the sample triplicates.



Measure	AnMBR Perm	RT
Average C/N ratio before	0.6	0.3
Day 34	0.4	0.3
Day 42	8.1	1.8
Multiplier 1	22x	5x
Peak C/N ratio	12.1	11.2
Day	55	69
Multiplier 2	19x	34x

803

804 Figure 2 – C/N ratio of the anaerobic membrane bioreactor (AnMBR) permeate and the reservoir
805 tank (RT). Table shows a comparison of the C/N ratio values for the AnMBR perm and the RT
806 samples. Multiplier 1 corresponds to the amount by which the value on Day 42 increased in
807 comparison to the value on Day 34. Multiplier 2 corresponds to the amount by which the value
808 on the peak increased in comparison to the average value before the transient event.

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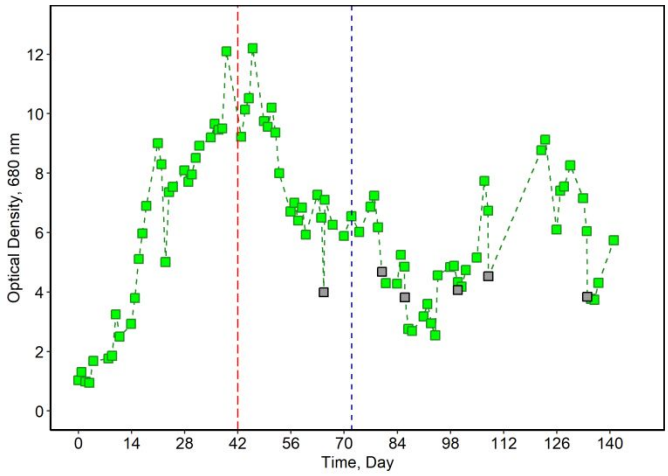
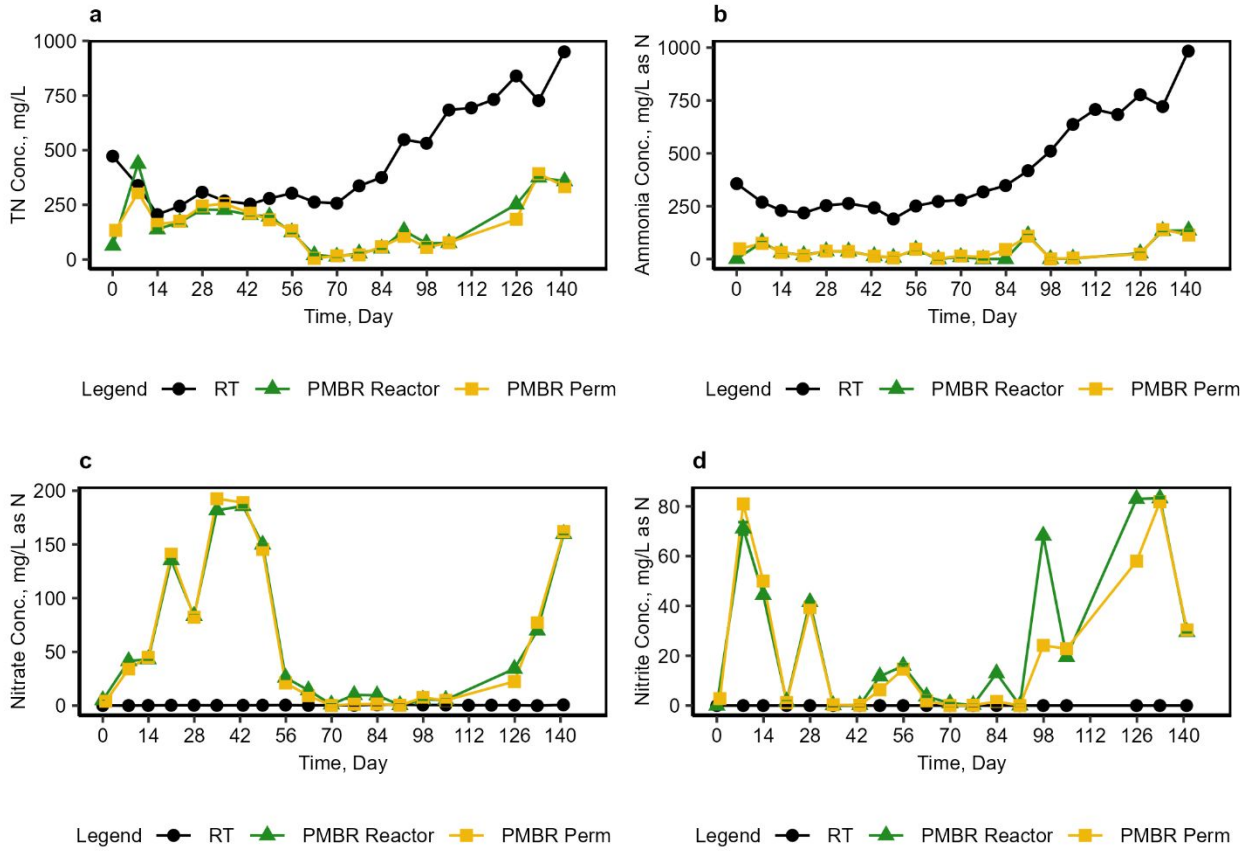


Figure 3 – Optical density (OD) at 680 nm of the phototrophic membrane bioreactor (PMBR) reactor culture. The dark grey squares correspond to the OD measurements taken after harvesting events. The red dashed line represents the peak in C/N ratio and the blue dashed line represents the change in light photoperiods.



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822 Figure 4 – (a) Total nitrogen (TN), (b) ammonia, (c) nitrate, and (d) nitrite in the RT, PMBR

823 reactor, and PMBR perm samples. Error bars show the standard deviation of the sample

824 replicates.

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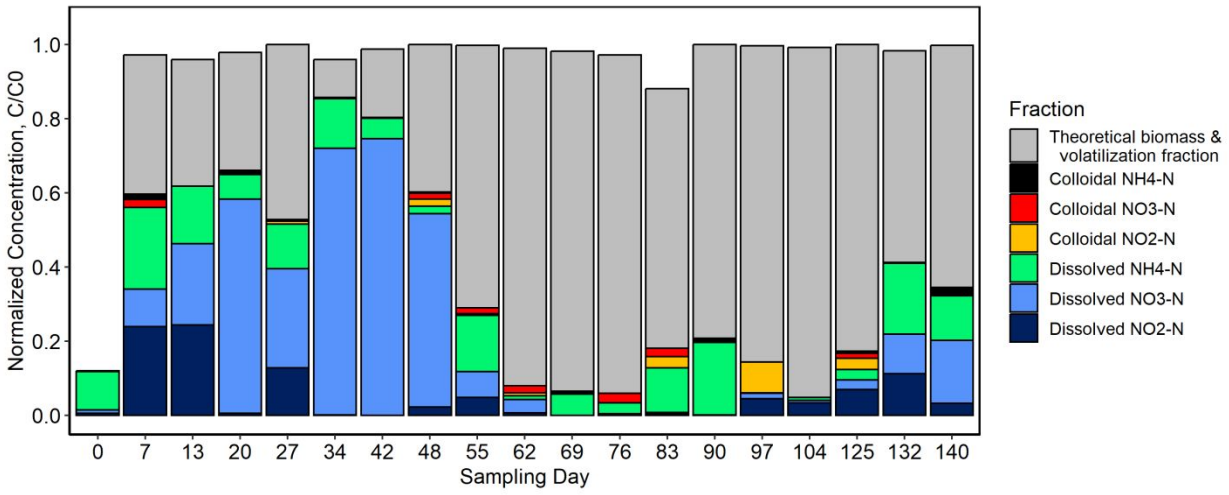
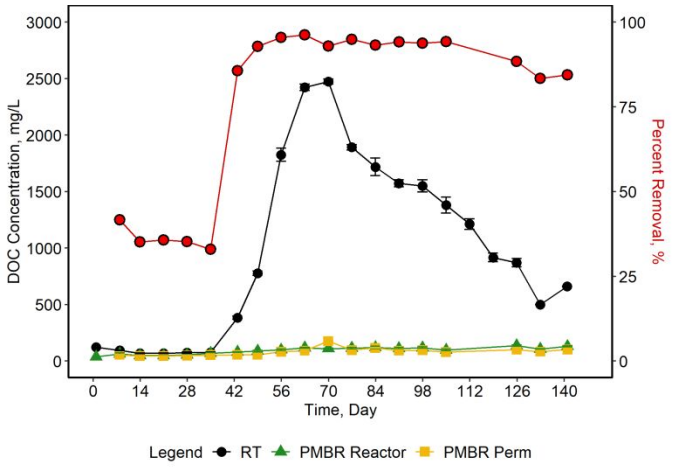


Figure 5 – Nitrogen balance of the phototrophic membrane bioreactor (PMBR). The measured colloidal and dissolved fractions of ammonia (NH_4–N), nitrate (NO_3–N), and nitrite (NO_2–N) are shown. The theoretical biomass and volatilization fraction corresponds to the difference between the sum of the measured fractions and the total nitrogen concentration in the PMBR influent. Days 111 and 118 were not sampled. The concentrations used for this mass balance can be found in the Table S3.



840

841 Figure 6 – Dissolved organic carbon (DOC) concentration in the reservoir tank (RT),
842 phototrophic membrane bioreactor (PMBR) reactor, and PMBR permeate samples. Percent
843 removal of DOC (red line and circles) is shown on the right axis. Error bars show the standard
844 deviation in the analytical duplicates.

845

Figure 7 – Bubble plot illustrating the bacterial genera diversity in the samples taken from the mother culture, reactor biofilm at the peak of the transient event, reactor contents at the peak of the transient event, and reactor contents after the transient event. Size of the bubble corresponds to the relative abundance percentage. Genera with a relative abundance below 1% are not shown in the figure.

Data Availability Statement

Raw data for the DNA sequencing can be found in the NASA Open Science Data Repository with DOI: 10.26030/vmzd-0s72.