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Supplementary Information for

Succession of founding microbiota in an anaerobic baffled bioreactor treating low-temperature raw domestic wastewater

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Section S1. Supplementary Methods

1. Analyses for Measured Parameters

Performance measurements collected from each ABR compartment included temperature, pH, total suspended solids (TSS), volatile suspended solids (VSS), chemical oxygen demand (COD), dissolved organic carbon (DOC), 5-day biochemical oxygen demand (BOD_5), alkalinity, volatile fatty acids (VFA) (i.e., acetate, propionate, butyrate, lactate), and biogas production and composition (CH_4 and CO_2). Measurements taken from the influent and effluent of each ABR included nitrogen (NH_3, NO_3^-, and NO_2^-) and phosphorous. Temperature and pH were continuously monitored. Grab samples were taken weekly for tCOD, sCOD, pCOD, TSS, and VSS. Biogas and dCH_4 sampling was conducted weekly when no operational issues were encountered.

Analyses for tCOD, sCOD, pCOD, BOD_5, TSS, VSS, alkalinity, and nitrogen species were conducted according to Standard Methods (APHA, 2005) or approved EPA methods (see table below). In ABR 1, pH values were collected with Broadly James pH ProcessProbes and temperature was monitored and logged with submersible HOBO Temp Pro V2 temperature logger. In ABR 2, pH was measured with Cole-Parmer pH electrodes (100 Ohm Pt RTD, EW-27003-23). Temperature was measured with LabJack EI-1034 probes. Biogas flowrate in ABR 1 was measured using Cole Parmer 0 to 500 SSCM gas flow meters. Biogas flowrate in ABR 2 was measured using an Agilent Digital Flow Meter (Optiflow 520). Biogas composition was determined on a Hewlett Packard 6890 with Agilent 5973 Mass Selective Detector GC-MS with an Agilent 113-3133 GS-Carbonplot capillary column at max temperature of 360˚C, flowrate of 1.2 mL min^{-1}, and helium carrier gas. dCH_4 was analyzed according to the method described in (Pfluger et al., 2011) with minor modification. Specifically, the method described in Pfluger et al. 2011 withdrew exactly 25 mL from sampling ports located on the fluidized bed reactor, which were subsequently injected into a vacuum-degassed and crimp sealed 58-mL serum bottle. Headspace was then equilibrated to ambient pressure by inserting a needed to release the vacuum. In this study, water samples (approximately 25 mL) were withdrawn from an effluent sampling port located at the top of each reactor compartment directly into a 58-mL serum bottle, which was immediately crimp-sealed with a butyl rubber stopper. The bottle was subsequently shaken to allow methane to equilibrate between the gas phase and the aqueous phase.

Organic acids were analyzed on a Shimadzu LC-20AT liquid chromatograph with Agilent Zorbax StableBond 80Å Aq, 4.6 x 150 mm, 3.5 µm HPLC column with 0.01 N H_3PO_4 eluent at 0.6 ml min^{-1} at 22°C. Ions were analyzed on a ThermoFisher Dionex (Thermo Fisher) ICS-900 ion chromatograph with Dionex IonPac AS14A-5 µm RFIC 3x150 mm column with 8.0 mM sodium carbonate and 1.0 mM sodium bicarbonate eluent using method SM4110B. DOC was analyzed using a Shimadzu TOC-L CSH with NTM-L detector via oxidative combustion infrared-analysis (method SM5301B Total Organic Carbon via High-Temperature Combustion) with a high-salinity combustion tube (platinum catalyst, ceramic fiber) and ultra-high purity air as carrier gas.

Standard Methods. The below list contains methods used during this study.

Test	Method Used
Total Suspended Solids	Standard Method 2540.D
Volatile Suspended Solids	Standard Method 2540.E
Chemical Oxygen Demand	Standard Method 5220.D using HACH Method 8000 TNT 822
Biochemical Oxygen Demand	Standard Method 5210.B
Alkalinity	Hach Method 20239 TNTplus 870 (EPA compliant)
Ammonia	Hach Method 10205 HR TNTplus 832 (EPA compliant)
Total Nitrogen	Hach Method 10242 TNTplus s-TKN (EPA compliant)
Nitrate	Hach Method 10206 TNTplus 835 (EPA compliant)
Nitrite	Standard Method 4500-NO_2^- using HACH 10237 TNTplus 840

2. Reactor Descriptions

ABR-1 was located in the unheated headworks of the Plum Creek Water Reclamation Authority (PCWRA) in Castle Rock, CO (elevation = 1,830 meters). PCWRA is a 6.44 MGD wastewater treatment facility located along the Front Range of the Rocky Mountains. Raw wastewater fed to the ABR first entered the unheated PCWRA headworks and was routed through a grinder sump pump and 8 mm screen. The de-gritted and screened wastewater was then routed to a continuously mixed 910-liter feed tank with a maximum detention time of 15 min. Influent wastewater was pumped to ABR-1's first compartment via a Watson Marlow peristaltic pump at a rate of 1.2 L min^{-1} (1,738 L d^{-1}) for the first 1,357 days of operation. After that, the pump rate was reduced to 0.6 L min^{-1} (869 L d^{-1}). Each ABR-1 compartment was constructed with PVC sheets reinforced with angle iron frames. ABR-1 was originally seeded with granular sludge from a mesophilic upflow anaerobic sludge blanket (UASB) receiving brewery waste.

ABR-2 was located in an unheated barn at the Mines Park Wastewater Test Bed in Golden, CO (elevation = 1,730 meters). The Mines Park Wastewater Test Bed treated domestic wastewater from a 250-unit housing complex. Influent wastewater was first routed through a holding tank with a grinder pump prior to being pumped to the ABR-AFFR influent holding tank. The raw, unheated wastewater was fed to the ABR-AFFR reactor system at a rate of 0.5 L min^{-1} via a Masterflex L/S digital drive peristaltic pump. Each compartment was constructed with 12" diameter PVC pipe N40. The large height-to-diameter ratio (12:1) was selected to enhance solids settling.

Section S2. Supplemental Figures

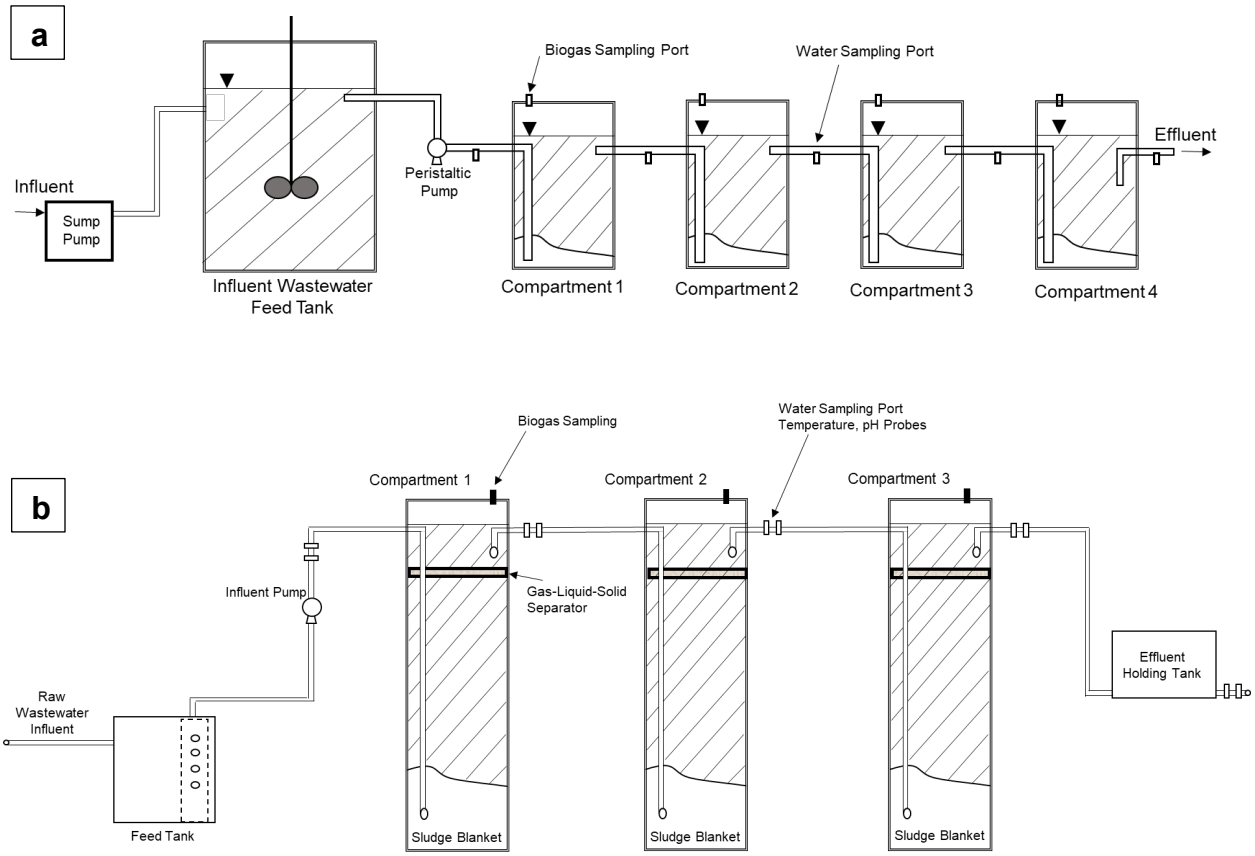

Figure S1. Schematic of pilot-scale multiple-compartment anaerobic reactor systems for the treatment of raw domestic wastewater. (a) ABR-1 located at the Plum Creek Water Reclamation Authority in Castle Rock, CO (elevation = 1830 meters). (b) ABR-2 (ABR-AFFR) located at Mines Park Wastewater Test Bed in Golden, CO (elevation = 1730 meters).

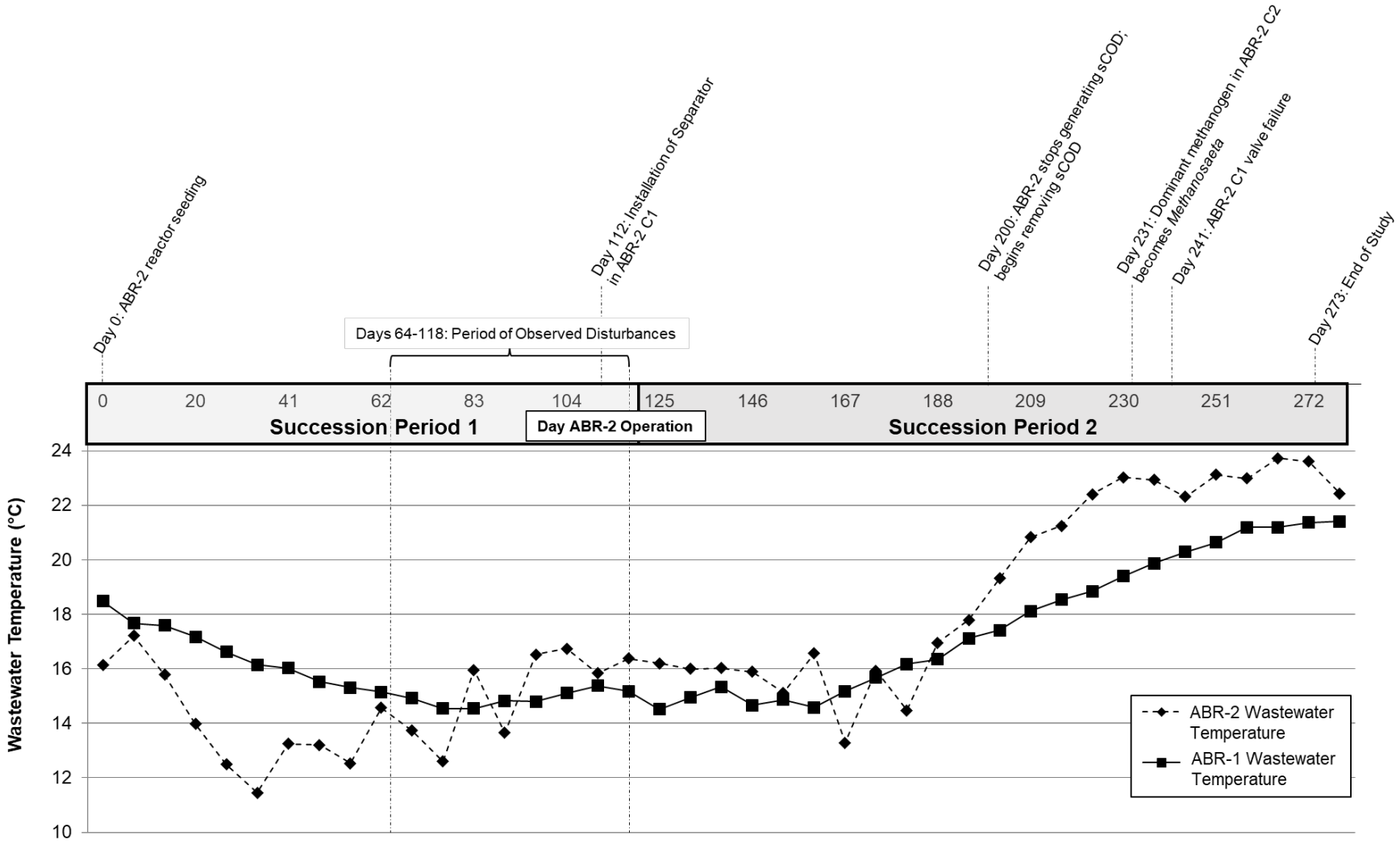

Figure S2. Disturbances, significant events, and wastewater temperature over time.

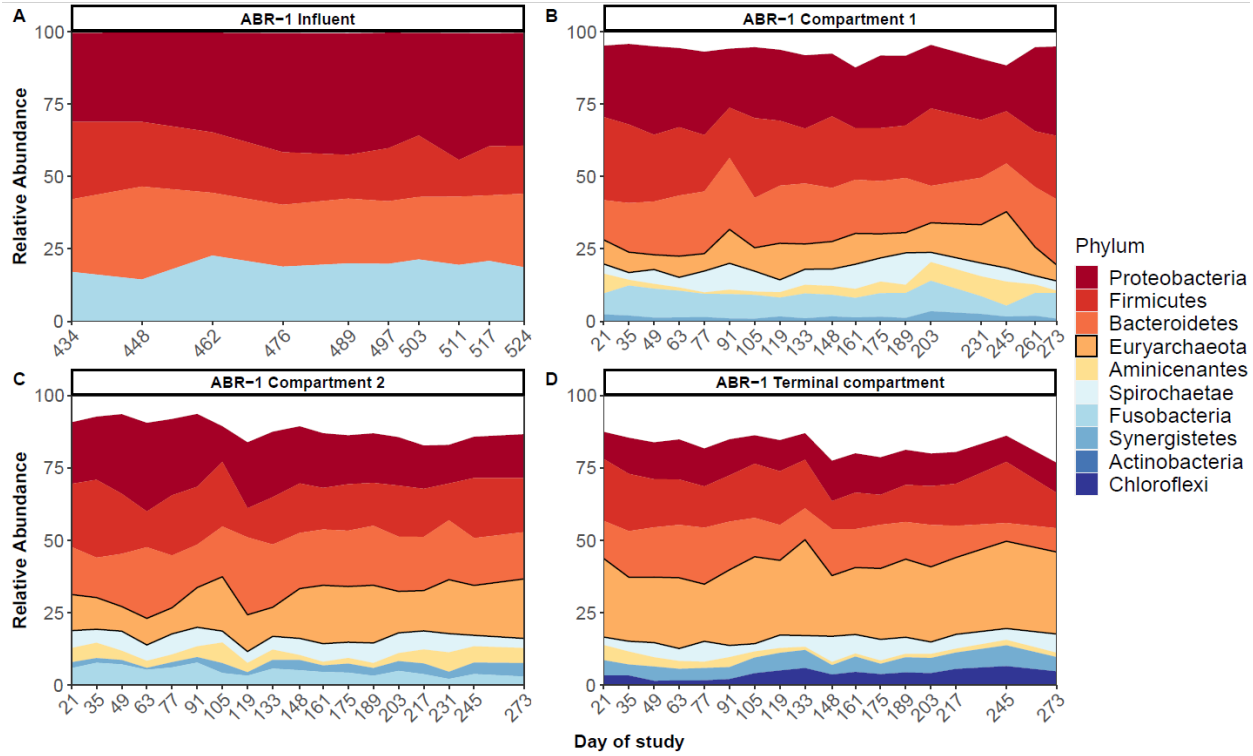

Figure S3. Phylum-level stream graphs for ABR-1 compartments over time

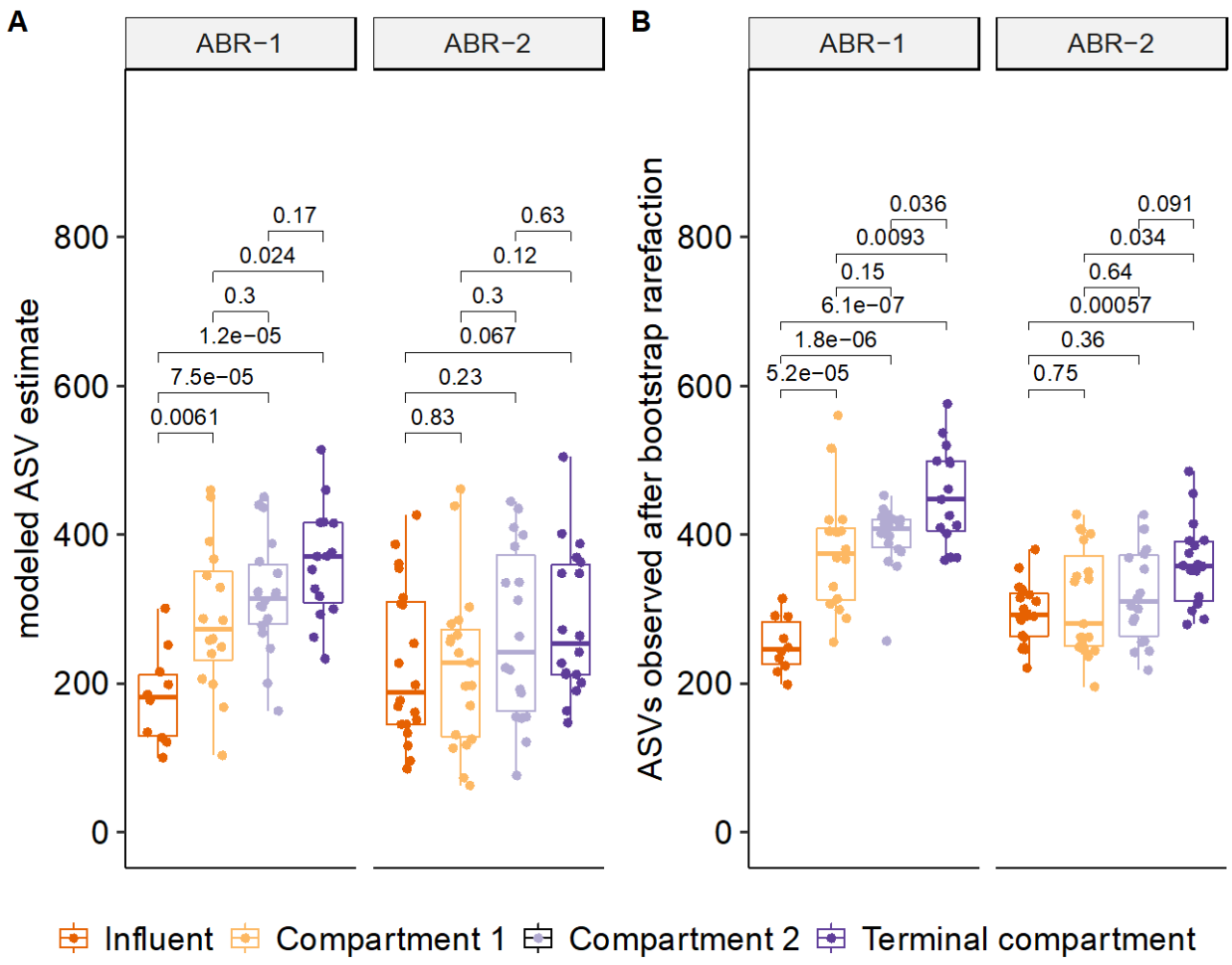

Figure S4. Alpha diversity for ABR-1 and ABR-2 over time.

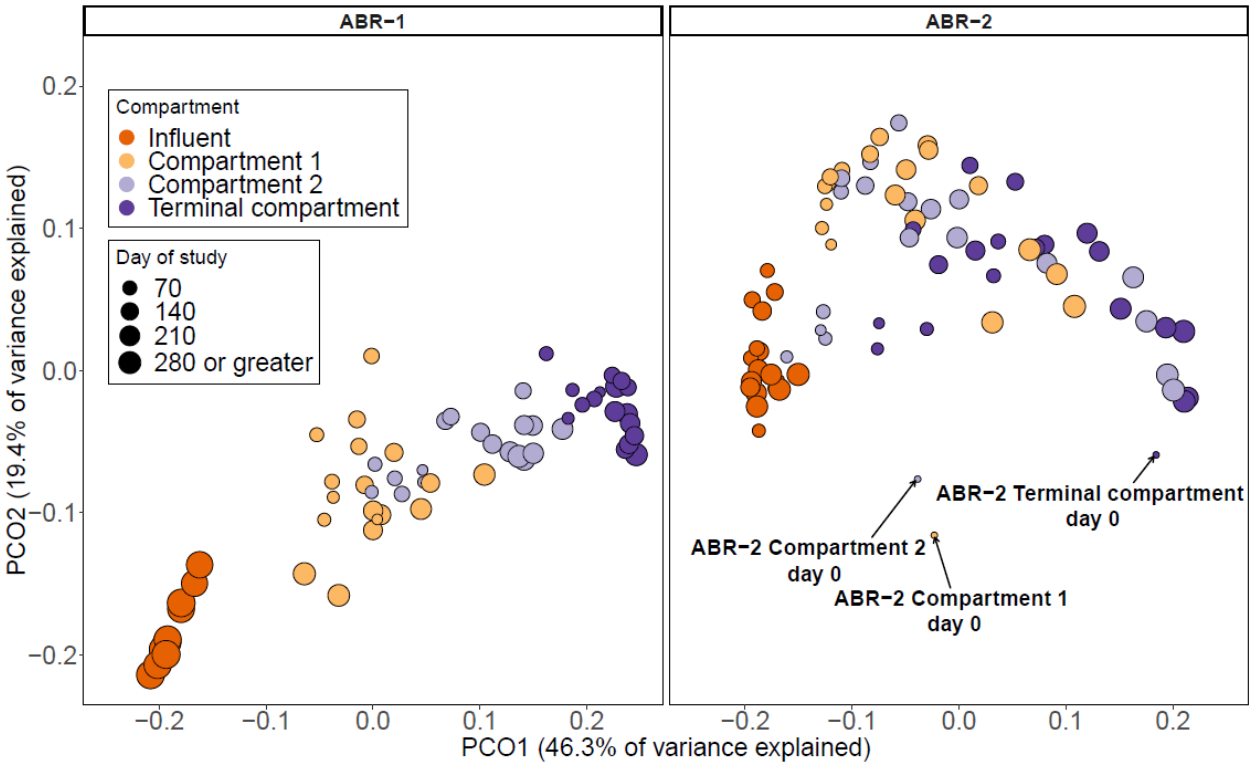

Figure S5. PCoA of weighted UniFrac distance matrices for both ABR-1 and ABR-2 using only bacteria ASVs.

Section S3. Supplemental Tables

Table S1. Unmanaged performance variations observed in ABR-2 during this study.

Day of Reactor Operation	Description of Disturbance
64	Approximately 30 liters of sludge / solids from C1 floated. Unknown quantity was transferred to C2. Sludge was reinserted into C1
69	750 mL of sludge was observed floating in C1. Sludge was wasted.
70	Two sludge floating incidents were observed in C1 (700 mL and 2400 mL). Sludge was wasted.
76	300 mL of sludge was observed floating in C1. Sludge was wasted.
78	2000 mL of sludge was observed floating in C1. Sludge was wasted.
80	2500 mL of sludge was observed floating in C1. Sludge was wasted.
83	3650 mL of sludge was observed floating in C1. Sludge was wasted.
86	1800 mL of sludge was observed floating in C1. Sludge was wasted.
92	2000 mL of sludge was observed floating in C1. Sludge was wasted.
97	2000 mL of sludge was observed floating in C1. Sludge was wasted.
104	1400 mL of sludge was observed floating in C1. Sludge was wasted.
108	3400 mL of sludge was observed floating in C1. Sludge was wasted.
240	Approximately 37 liters of sludge was lost in C1 due to a valve failure. Sludge was wasted.

Table S2. Mean concentrations and standard deviations of several performance parameters for the influent wastewater and each reactor compartment during this study.

Variable (mg L^{-1})	Reactor	Influent	C1	C2	C3	C4
Acetate	ABR-1	30 ± 11	47 ± 30	61 ± 43	60 ± 48	48 ± 43
	ABR-2	40 ± 20	64 ± 27	61 ± 24	56 ± 22	N/A
Propionate	ABR-1	4 ± 5	5 ± 2	5 ± 2	5 ± 4	3 ± 4
	ABR-2	9 ± 7	9 ± 9	6 ± 6	6 ± 4	N/A
Sulfate	ABR-1	44 ± 7	21 ± 7	10 ± 6	6 ± 2	6 ± 3
	ABR-2	59 ± 9	31 ± 9	17 ± 8	12 ± 7	N/A
Ammonia	ABR-1	51 ± 5	N/A	N/A	N/A	47 ± 4
	ABR-2	35 ± 4	N/A	N/A	N/A	40 ± 4
Phosphorus	ABR-1	4 ± 1	N/A	N/A	N/A	5 ± 1
	ABR-2	4 ± 1	N/A	N/A	N/A	4 ± 1

Table S3. (A) Pairwise PERMANOVA analysis of influent wastewater and ABR-1 and ABR-2 parallel compartments (e.g., influent ABR-1 vs. influent ABR-2, ABR-1 C1 vs. ABR-2 C1, etc.). Analysis indicates distinct microbial communities over the study period. While PERMANOVA adjusted *p*-values are low, so are beta dispersion *p*-values, indicating the variance in community clusturing is different. This observation is likely attributed to the maturation and adaptation of ABR-2 communities over time.

	comparison	permanova R2	permanova F	permanova p.adj	beta dispersion p.adj
comparison					
Influent	ABR-1 vs. ABR-2	0.58	38.06	0.001	0.015
Compartment 1	ABR-1 vs. ABR-2	0.38	22.21	0.001	0.010
Compartment 2	ABR-1 vs. ABR-2	0.30	15.39	0.001	0.007
Terminal compartment	ABR-1 vs. ABR-2	0.46	30.03	0.001	0.001

(B) Within-reactor PERMANOVA analysis of microbial community differences across compartments. In ABR-1, communities form distinct clusters in all compartment vs. compartment comparisons. Same is true for ABR-2 with the exception of C1 vs. C2 (*p*-value = 0.143). Beta dispersion is a measure of community homogenity, with low *p*-values indicating that the different groups being compared to each other have different levels of dispersion. For example, low beta dispersion *p*-values in ABR-1 comparisons where one compartment is compared to the influent wastewater are likely driven by the extremely tight clustering (high homogenity) of the influent samples. Low *p*-values for beta dispersion does not invalidate the PERMANOVA results, but instead drives consideration of differences in community dispersion as an influencing factor. In this study, especially with ABR-2, differences in dispersion are likely driven by start-up effects.

	permanova R2	permanova F	permanova p.adj	beta dispersion p.adj
ABR-1				
Compartment 1 vs. Influent	0.71	64.88	0.001	0.018
Compartment 2 vs. Influent	0.79	99.37	0.001	0.006
Influent vs. Terminal Compartment	0.91	241.39	0.001	0.038
Compartment 1 vs. Compartment 2	0.29	14.02	0.001	0.446
Compartment 1 vs. Terminal Compartment	0.73	90.57	0.001	0.418
Compartment 2 vs. Terminal Compartment	0.53	36.85	0.001	0.080
ABR-2				
Compartment 1 vs. Influent	0.44	29.48	0.002	0.002
Compartment 2 vs. Influent	0.38	23.37	0.002	0.002
Influent vs. Terminal Compartment	0.62	61.70	0.002	0.002
Compartment 1 vs. Compartment 2	0.05	1.95	0.123	0.297
Compartment 1 vs. Terminal Compartment	0.28	14.72	0.002	0.839
Compartment 2 vs. Terminal Compartment	0.13	5.68	0.007	0.310

Table S4. Percent relative abundance of founders in ABR 2 compartments during the study period.

Phylum	Compartment 1 founder (relative %)	Compartment 2 founder (relative %)	Compartment 3 founder (relative %)
Firmicutes	28.59	32.56	20.91
Bacteroidetes	22.13	13.87	13.83
Spirochaetae	3.72	23.72	11.63
Proteobacteria	19.27	4.87	6.83
Euryarchaeota	6.08	10.96	12.05
Acidobacteria	7.12	0.37	14.23
Verrucomicrobia	4.69	3.53	3.77
Chlorobi	0.00	2.18	2.87
NA	1.81	1.18	1.61
Synergistetes	4.15	0.00	0.00
WS1	0.54	2.36	1.05
Chloroflexi	0.48	1.93	1.14
Cloacimonetes	0.29	0.70	1.29
Actinobacteria	0.86	0.29	1.07
Hydrogenedentes	0.00	0.46	0.99
Latescibacteria	0.00	0.00	1.37
Planctomycetes	0.05	0.00	1.13
BRC1	0.00	0.00	1.14
Cyanobacteria	0.00	0.00	1.06
Ignavibacteriae	0.00	0.83	0.20
Omnitrophica	0.00	0.00	0.60
Caldiserica	0.00	0.04	0.41
Lentisphaerae	0.08	0.16	0.10
Armatimonadetes	0.14	0.00	0.19
Nitrospirae	0.00	0.00	0.22
Chlamydiae	0.00	0.00	0.13
RBG-1_(Zixibacteria)	0.00	0.00	0.10
Elusimicrobia	0.00	0.00	0.09
Fibrobacteres	0.00	0.00	0.01
Gracilibacteria	0.00	0.00	0.00

Supplemental Information References

APHA. Standard Methods for the Examination of Water and Wastewater, 21st ed. 2005. American Public Health Association, Washington D.C.

Pfluger A, Wu WM, Pieja AJ, Wan J, Rostkowski KH, Criddle CS. Selection of Type I and Type II methanotrophic proteobacteria in a fluidized bed reactor under non-sterile conditions. Bioresource Technology. 2011; 102(21), 9919–9926. https://doi.org/10.1016/j.biortech.2011.08.054