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Water Impact Statement

Anaerobic baffled reactors treating raw domestic wastewater stratify microbial processes longitudinally, separating acidogenesis and methanogenesis, allowing microbiota to develop in response to the types and concentrations of organics present. While previous work has focused on operational characteristics and bulk performance of low-temperature ABRs, this study advances understanding of pilot-scale ABRs by evaluating microbial community development and impacts of operational perturbations.

Abstract

Continuously operated pilot- and full-scale anaerobic baffled reactors (ABRs) treating low-temperature raw domestic wastewater are currently few in number but offer significant advantages. As ABRs treating domestic wastewater become more prevalent, engineers and operators face the practical challenge of effectively transferring and seeding anaerobic sludge from existing “donor” ABRs to newly constructed ABRs. Unlike activated sludge, which predominantly consists of relatively fast-growing aerobic heterotrophic bacteria, anaerobic microbiota are slower-growing and the community structure may be impacted by process disturbances during bioreactor start-up. Examining the spatiotemporal development of anaerobic microbiota after transfer can enhance understanding of start-up dynamics in engineered anaerobic bioreactor systems. To understand the impacts of sludge transfer and seeding from an existing ABR operated for 3.5-years treating raw, low-temperature domestic wastewater to a new, similarly configured ABR treating a different raw domestic wastewater stream, influent wastewater and sludge microbiota samples were withdrawn biweekly for 275 days and used to characterize changes to the microbial community structure over time in both ABRs. Results suggest that the donor ABR communities maintained relatively consistent structure over time, but the microbial communities in the receiving bioreactor experienced two apparent successional trajectories post-inoculation. The first trajectory, which lasted for ~120

days, showed increasing divergence between communities in the two ABRs. This trajectory was marked by lower wastewater temperatures (12-14°C, with extreme lows of 8°C) and numerous disturbances to the sludge blankets. A second successional trajectory, observed when wastewater temperatures increased (> 16°C) and disturbances were eliminated, was marked by significant increases in the relative abundance of Euryarchaeota, especially *Methanosaeta* ("*Methanothrix*"), and increasing convergence of microbial communities in complementary donor and receiving bioreactor compartments. Further, the relative abundance of founding microbial community members significantly decreased during the first successional trajectory but significantly increased, or rebounded, during the second successional trajectory. The results of this study indicate that an anaerobic sludge inoculum can be effectively transferred from a long-running ABR treating raw, low-temperature domestic wastewater to a new ABR, and that similar performance can be achieved despite differing environmental conditions and disturbances to the sludge blanket microbial communities during start-up of the new ABR.

1. Introduction

The dominant wastewater treatment paradigm today is aerobic activated sludge, a technology with a negative energy balance due to high electrical energy requirements. Activated sludge requires ~0.6 kWh per m^{-3} of wastewater treated which, in aggregate, accounts for ~3% of U.S. electrical energy demand [refs. 1, 2]. Several other factors have caused critical examination of the wastewater treatment sector in the U.S., to include aging infrastructure, population increases, greenhouse gas production (to which energy-intensive wastewater treatment processes contributes ~1.6% of total emissions), and the recent passing of a $1.2T USD infrastructure bill that has ~$82.5B USD earmarked for improvements in water and wastewater [refs. 3, 4]. Anaerobic bioreactors engineered for direct treatment of domestic wastewater have gained increased attention as an alternative to activated sludge due to their relatively low

energy requirements and the generation of methane-rich biogas [refs. 2, 5-6]. Many of the most promising anaerobic bioreactor configurations are the subject of ongoing bench- and pilot-scale research. Several configurations exist, to include variations of anaerobic membrane bioreactors and anaerobic sludge blanket bioreactors [refs. 5, 7-11].

The anaerobic baffled reactor (ABR) is a multi-compartment sludge blanket bioreactor first introduced by Bachmann et al. (1985) [ref. 12], which is often described as a collection of upflow anaerobic sludge blankets (UASB) reactors coupled together in series [ref. 13]. The degradation of organic substrate longitudinally through successive ABR compartments, coupled with spatial separation of sludge blankets in an ABR, can create functional differentiation of the mixed anaerobic consortia in each successive bioreactor compartment [refs. 14-15]. The ABR has been used for several applications, to include the degradation of food production wastewaters, industrial wastewaters, and domestic wastewater streams [refs 13, 16-17]; however, few studies of pilot-scale ABRs treating low temperature (6 to 24 °C) raw domestic wastewater over long timescales exist [refs 10-11, 18-19]. Of studies examining ABRs treating large volumes of raw domestic wastewater at low temperatures, most analysis centers on ABR operational performance with less attention paid to the development of the anaerobic microbial communities over time and space within the ABR sludge blanket communities, especially immediately after seeding. Transferring seed sludge from functional aerobic activated sludge bioreactors to unseeded bioreactors for start-up purposes is common practice in the wastewater sector today [ref. 20]. A similar approach may be employed for anaerobic sludge blanket bioreactors once such reactors become mainstream. The start-up of a new pilot-scale ABR, therefore, provides opportunity to examine whether anaerobic sludge transferred from separate compartments (i.e., a “founding microbiome”) in an existing “donor” bioreactor to corresponding compartments in a new “receiving” bioreactor retains its original community structure or assembles differently over time.

The ecological concepts of community succession and founder effect may be useful constructs for understanding the spatiotemporal development of an anaerobic microbiome after sludge transfer; however, these phenomena are not well studied in engineered bioreactors [refs. 21-22]. Succession can be defined as the orderly and predictable manner by which communities change over time [ref. 23]. Succession has been studied in several microbial contexts, including stream biofilms, composts, deglaciated soils, and human neonates [refs. 24-29], though community succession remains an active area of research [refs. 30-33]. Two types of succession have been described: primary and secondary. Primary succession occurs when a habitat is colonized for the first time, whereas secondary succession occurs when a previously occupied area is re-colonized following a disturbance event that kills much or all the existing community [ref. 34]. Disturbance events, which can be described as pulse (i.e., short duration) or press (i.e., long duration), can influence the development of a microbiome [ref. 35], possibly modifying the successional trajectory or inducing secondary succession.

Transplanted microbiomes, such as inoculating a new bioreactor treating microbially-rich wastewater, are subject to the founder effect, where a portion of the inoculating microbiome will flourish in the new environment. In microbial ecology, community composition can strongly depend on the order in which species are introduced to an environment [ref. 36]. For example, the lasting composition of the human microbiota can be traced back to maternal transmission during the human birthing process [refs. 37-38]. However, much remains unknown concerning how colonizing taxa influence future states of their respective microbiomes [ref. 39]. Examining the prevalence of an inoculating, or seed, community in later community composition, especially considering different environmental conditions (e.g., varying substrate concentrations, wastewater temperatures, and a different microbial community in the influent wastewater) and

disturbances to the sludge blankets, can inform whether this phenomenon occurs in anaerobic sludge blanket bioreactors.

Recent studies of community succession in anaerobic bioreactor systems have examined high temperature (30 – 65 °C) anaerobic digesters treating wastewater sludge (primary clarifier and waste activated) [refs. 40-41], anaerobic co-digestion treating mixed organic wastes (sludge, food scraps, and fats, oils, and grease) [refs. 42-44], upflow anaerobic sludge blanket bioreactors treating various wastewaters [refs. 45-46], and high-temperature (> 35 °C) bench-scale ABRs treating wastewaters other than raw domestic wastewater [refs. 47-48]. While each provides insight into the spatiotemporal assembly of anaerobic communities, no study has examined the succession of anaerobic microbiota in a pilot-scale ABR treating ambient temperature raw domestic wastewater. To address this gap in literature, this study examined the succession of an anaerobic microbial consortium over 275 days after transfer of anaerobic seed sludge from a donor pilot-scale ABR (ABR-1) with 3.5 years of continuous operation to a similarly configured, new receiving pilot-scale ABR (ABR-2). The two ABRs operated in different locations with distinct domestic wastewater feed, therefore each was exposed to differing substrate composition and microbial communities in the influent wastewater. Consequently, the principal objectives of this work were to examine: (1) spatiotemporal community assembly (i.e., succession) in receiving ABR compartments after seed sludge transfer, to include whether the community became similar or dissimilar to the donor reactor considering exposure to different influent wastewater microbiota, low temperatures, and process-related disturbances; and (2) whether the founding microbiome from the seed sludge persisted over time in each bioreactor compartment. Studying these objectives provides insight on how psychrophilic sludge communities assemble over time in each pilot-scale bioreactor compartment, and whether

sludge in each corresponding donor and receiving bioreactor can achieve similar levels of performance.

2. Materials and Methods

2.1. Reactor operation and performance monitoring

A pilot-scale ABR (ABR-1) consisting of four equal-sized rectangular compartments (2.7:1 height-to-width) with a total system hydraulic volume of 870 liters was in operation for 3.5 years at the initiation of this study. Complete description of ABR-1 is found in Hahn & Figueroa (2015) and Pfluger et al. (2018) [refs. 10, 11]. ABR-1 provided anaerobic sludge seed to inoculate a second pilot-scale ABR (ABR-2) consisting of three equal-sized cylindrical compartments (12:1 height-to-diameter) with a total system hydraulic volume of 720 liters. Complete description of ABR-2 is found in Pfluger et al. (2018) and Pfluger et al. (2020) [refs. 15,49]. Figure S1 provides reactor schematics and further description of each ABR. Anaerobic seed sludge was transferred from ABR-1 to corresponding compartments in ABR-2 in a manner designed to preserve reactor-level community structure and promote longitudinal degradation of organics upon start-up. Specifically, ABR-2 Compartment 1 (C1), Compartment 2 (C2), and Compartment 3 (C3) were inoculated with sludge from ABR-1 C1, C2, and Compartment 4 (C4), respectively. For inoculation, 5.6-liters of sludge from ABR-1 compartments were added to approximately 100 liters of raw domestic wastewater (16°C) in corresponding ABR-2 compartments and allowed to acclimate for a period of 48 hours prior to start-up and introduction of fresh influent wastewater (i.e., start of the study period).

The ABRs were geographically separated and subjected to different environmental conditions (e.g., influent wastewater microbial communities, wastewater temperatures, etc.). ABR-1 was

fed raw wastewater (12 to 23°C) at a rate of 1,728 liters d^{-1} (hydraulic retention time (HRT) of 12 hours). The HRT of ABR-1 was adjusted from 12 to 24 hours 101 days into this study to enhance treatment performance. ABR-2 was fed raw wastewater (11 to 24°C) at a constant rate of 720 liters d^{-1} (HRT of 24 hours). Wastewater flowed sequentially through the sludge blankets in each successive reactor compartment. pH did not vary substantially throughout the study, ranging between 6.5 and 7.2 for both ABRs. 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) (acetate, propionate, butyrate, lactate), and biogas production and composition (CH_4 and CO_2). Nitrogen (NH_3, NO_2^-, and NO_3^-) and phosphorus were measured in the influent and effluent of each ABR. Further description of each analysis is found in Supplementary Information Section 1.

Uncontrolled variables during this study included seasonal changes in wastewater temperature, variations in organic substrate loading, and variations in influent wastewater chemistry and suspended solids. Several unmanaged performance variations were observed during the study period, including: (1) accumulation of solids in ABR-2 C1 at low temperatures immediately following sludge inoculation and reactor start-up; (2) bulk sludge transfer from ABR-2 C1 to C2 due to biogas accumulation and subsequent lifting of the sludge from the bottom of the ABR C1; and (3) a valve failure in ABR-2 C1 on day 241 of the study, which led to a loss of approximately 70% of the sludge volume in the reactor compartment. Table S1 summarizes each unmanaged performance variation by date of study.

2.2. Sludge sampling, DNA extraction, and 16S rRNA gene sequencing

Beyond the seed sludge, biological sludge samples from the influent wastewater and each compartment of both ABRs were removed every 14 days for the first 275-days of ABR-2's operation. Sludge samples were removed with a Sludge Judge C09247WA Sampler System from the center of each compartment's sludge bed. Samples from the influent wastewater of ABR-1 were not initially preserved; to determine if the microbial community in ABR-1's influent was consistent over time, samples were preserved after the initial study period (between days 420 and 510) for comparison. Samples were transported on ice and biomass pellets were preserved at -20°C until DNA extraction. Genomic DNA was extracted from 2.0 mL of anaerobic sludge using the DNeasy PowerLyzer PowerSoil DNA extraction kit (Qiagen, Inc., Germantown, MD, USA) according to the manufacturer's protocol and stored at -80°C. DNA was extracted from biological replicates from each sampled location to verify consistency of community composition at each sampling point. DNA was quantified using a Qubit Fluorometer and a Qubit dsDNA High Sensitivity Assay Kit. Excluding ABR-1 influent samples, rRNA gene amplification was performed using 1 ng of DNA following the dual-index barcoded sequencing strategy described in Kozich et al. (2013) [ref. 50] with DNA sequencing performed at the BioFrontiers Institute. ABR-1 influent samples were obtained later in the study, amplified following a two-step amplification strategy [ref. 51], and sequenced at the Duke University School of Medicine. All sequences were generated using Illumina MiSeq 2x250 V2 reagents. Sequences can be accessed from GenBank BioProject ID PRJNA715505.

2.3. Amplicon sequence processing and quality control

rRNA sequences (henceforth called 'amplicon sequence variants', or ASVs) [ref. 52] were processed using DADA2 [ref. 53] and Phyloseq [ref. 54] in R version 3.4.2. Key processing steps include sequence trimming to Q>30, error rate estimation using seed=100, dereplication, paired-end read merging, and chimera removal. Taxonomy was assigned using Silva database

version 128; nomenclature used throughout this study is consistent with that database version. Data for biological replicates were merged using a custom R script. A phylogenetic tree was created from unique ASVs using the Quantitative Insights Into Microbial Ecology (QIIME2) plug-in q2-fragment-insertion [ref. 55] and a Silva version 128 reference database. The tree was imported into R using qiime2R [ref. 56].

2.4. Statistical methods and analyses

Excluding alpha diversity measurements and founding microbiome member identification, analyses were generated from phyloseq objects where the ASV table was first subset into ASVs seen a minimum of two times in two or more samples, then normalized by bootstrap rarefaction. For this normalization, the phyoseq object was rarefied 100 times (with replacement) to a sequencing depth of 7900 reads per sample (6300 for bacteria-only analyses) and the ASV values averaged. Rarefaction was used for normalization to maintain a linear relationship between taxa abundance and ordination results. Phylum-level overviews of the communities were generated using the R packages ggplot [ref. 57] and phylosmith [ref. 58].

To better understand the relationships between ABR-1 and ABR-2 communities by compartment, DNA sequencing results were subset by reactor compartment, then UPGMA dendrograms from weighted UniFrac distance matrices were generated in R using phangorn [ref. 59] and ggtree [ref. 60]. Principal coordinate analysis (PCoA) was performed in Phyloseq using weighted UniFrac [ref. 61] as the distance metric, and the statistical relevance of different groupings was evaluated using PERMANOVA and beta-dispersion. The top two principal coordinates (PCOs) were each compared to community composition (relative consortium percentages at taxonomy levels phylum through genus, respectively) to identify linear relationships between taxa and PCOs. Alpha diversity metrics were determined on non-trimmed ASV data using the R packages Breakaway [ref. 62] and MetagMisc [ref. 63] for multiple

rarefaction. Values were compared by compartment using the Wilcoxon rank sum tests with Bonferroni correction (base R).

For archaeal community dynamics over time, DNA sequences from biological replicates were combined, bootstrap rarefied (100 trials at 7900 sequences), then merged at the genus-level. Community composition is relative to the entire data set (i.e., missing portions of bar plots in Figure 5 represent bacteria). Relative abundance was determined by comparing the number of sequences assigned to specific taxa (e.g., genus) to the total number of sequences. Taxonomic assignments were performed with 'assignTaxonomy' in DADA2 using the Silva database version 128 as the training set. The function 'assignTaxonomy' uses the naïve Bayesian classifier method of Wang et al. (2007) [ref. 64] to assign taxonomy across multiple ranks (e.g., kingdom to genus). "Rare genera combined" depicted in bar charts are the sum of individual genera; each represents less than 1% of a sample.

An ABR-2 "founder" (i.e., member of the founding microbiome) was defined in the following manner: (1) a C1 founder ASV must be present in the C1 seed sample and observed at least once in additional C1 time course samples thereafter; (2) a C2 founder ASV must be present in C1 or C2 seed sample and observed at least once in additional C2 time course samples thereafter; (3) a C3 founder ASV must be present in C1, C2, or C3 seed sample and observed at least once in additional C3 time course samples thereafter. ABR-2 "founder" ASVs were identified by isolating ABR-2 sequences, merging biological sequence replicates, and discounting (i.e., removing) influent wastewater community members from the data set. The founding microbiome was determined from non-normalized, untrimmed ASV abundance data. Reproducible bioinformatics workflows can be found at https://github.com/Coupled-Hybrid-Anaerobic-Reactor/Founding-Microbiome.

3. Results

3.1. Wastewater chemistry and comparative reactor performance

Water quality characteristics over the 275-day study period are given in Tables 1 and S2. Wastewater temperatures at both ABR locations were consistently within 2-4 °C of each other (Figure S2). Over the first 92 days of this study, ABR-1 had greater absolute removal of tCOD, particulate COD (pCOD), and TSS relative to ABR-2. After inoculation, the weekly mean wastewater temperature in ABR-2 was 12-14 °C, with daily temperatures as low as 8 °C, which likely decreased microbial activity (i.e., a post-inoculation lag period) and resulted in low COD and TSS removal, as well as raw solids accumulation in ABR-2 C1. After 100 days, however, mean wastewater temperatures in ABR-2 increased to > 16 °C and COD and TSS removal increased to levels comparable to ABR-1.

Biogas accumulation in the sludge/solids mixture in ABR-2 C1 induced periodic lifting of the sludge bed, which disrupted microbial communities and caused unmanaged transfer of sludge from ABR-2 C1 to C2. The installation of gas-liquid-solids separators on day 112 reduced the biogas-induced pulse disturbances (Table S1; Figure S2). After 118 days, effluent tCOD and TSS concentrations were consistent (i.e., maintained low standard deviations) for both ABRs despite highly variable influent wastewater concentrations, suggesting process stability. Despite disturbances, removal of tCOD, pCOD, and TSS was greatest in C1 of each ABR, indicating that hydrolysis and solids removal were dominant functions. ABR-2 generated sCOD during the first 200 days of operation, but began to remove sCOD thereafter, suggesting that the rate of hydrolysis of pCOD was initially greater than the utilization rate of sCOD. VFA concentrations increased from the influent to the effluent of each ABR (Table 1); however, acetate, the most prevalent VFA, was generated in the middle compartments of each ABR and removed in the terminal compartment, suggesting increased activity of acetate-utilizing metabolisms (e.g.,

acetoclastic methanogenesis). Though methane measurements were initially limited in ABR-2 due to disturbances and associated maintenance, methane production increased in both reactors throughout the study due to increasing temperatures and methanogenic activity. The percentage of methane in the biogas increased longitudinally in successive compartments for both ABRs.

3.2. Community assembly over time and space in reactor compartments

Figure 1 depicts phylum-level stream graphs showing % read abundance by ABR-2 location over time and space, highlighting the increase in relative abundance of Euryarchaeota, all predicted to be methanogenic, during system maturation. Figure S3 shows stream graphs for ABR-1 for comparison. Influent wastewater communities were dominated by a small number of phyla, with ABR-1 influent having 4 phyla averaging 99.6% of all sequences, and ABR-2 having 5 phyla averaging 97.1% of all sequences. While not as homogeneous over time, ABR-1 and ABR-2 compartment samples were dominated by eight phyla each, representing on average between 82.7% and 93.9% of sequences per compartment. The extent to which the top phyla dominate samples decreases over time as space, suggesting an increase in alpha diversity (discussed later).

[Figure 1]

Beta diversity was assessed using weighted UniFrac distances, where the within-location (e.g., ABR-1 influent vs. ABR-1 influent and ABR-2 influent vs. ABR-2 influent) weighted UniFrac distance average were 0.08 (SD=0.03) and 0.12 (SD=0.03), respectively, while the across-location (e.g., ABR-1 influent vs. ABR-2 influent) distances averaged 0.23 (SD=0.02). These results indicate that location (ABR-1 vs. ABR-2) had a larger influence on influent community differences than a within-location time course (Welch two-sample t-test p-value < 2.2e-16).

These results are supported by dendrogram-based separation of ABR-1 and ABR-2 communities. Samples for the influent wastewater clustered by geographical location but were distinct from each other, indicating that community members in ABR-1 influent were more like each other than they were ABR-2 influent community members. Further, influent microbiomes from each location were consistent over time, yet distinct from each other at lower-level taxonomies (pairwise PERMANOVA analysis; Table S3). This result suggests that sludge in ABR-1 and ABR-2 compartments was exposed to distinct influent wastewater communities. Despite differences, the most prevalent phyla observed in each ABR's influent were consistent with communities observed in studies of other influent microbiomes, though with different relative abundance [refs. 65-66].

[Figure 2]

Dendrograms also show the immediate change in community structure after seeding (Figure 2). As expected, the seed sludge for each ABR-2 compartment was most like other samples from ABR-1 compartments, but ABR-2 communities became immediately dissimilar to the seed sludge upon exposure to ABR-2's influent wastewater. Over the study period, however, the microbial communities in each ABR-2 compartment became increasingly like the community in the corresponding ABR-1 compartment. In C2, the microbial communities became so similar that the final four sampling points overlap.

Weighted UniFrac community distance matrices were further analyzed via principal coordinate analysis (PCoA). Figure 3 shows that 74.5% of variance is explained by PCOs 1 and 2. Figure 3 also reveals an initial divergence of the microbial communities in each ABR-2 compartment from the sludge seed along PCO axis 2. The greatest distance, indicating the maximum dissimilarity from respective seed samples, occurred on days 119, 91, and 105 for ABR-2 compartments 1,

2, and 3, respectively. This point of greatest dissimilarity was followed by subsequent migration over time along PCO axis 1, indicating increasing similarity between microbial communities in corresponding ABR-1 and ABR-2 compartments (i.e., ABR-1 C1 became more like ABR-2 C1, etc.). Additional PERMANOVA analysis indicated statistically significant *p*-values for pairwise distances between all ABR-1 compartments and all ABR-2 compartments (excluding C1 compared to C2) (Table S3).

The period of initial divergence in similarity of ABR-2 communities relative to the seed was marked by low wastewater temperatures (12-14 °C) and periods of biogas-induced sludge lifting events that disturbed the microbial community structure in ABR-2 C1 and C2. The installation of a gas-liquid-solid separator in ABR-2 C1 on day 112 of the study, coupled with increasing wastewater temperatures (16-24 °C), corresponds with the subsequent migration along PCO axis 1. From days 119 to 273 of the study, no solids accumulation and fewer biogas-induced disturbances were observed, likely fostering the aforementioned increase in community similarity. The operational performance of ABR-2 also improved after 118 days of study, as indicated by increased COD and TSS removal and a transition from generating to removing sCOD (Table 1).

[Figure 3]

3.3. Increasing % relative abundance of Euryarchaeota over time and space

By relating the relative proportion of different taxa within samples to the principal coordinate values shown in Figure 3, a linear relationship between Euryarchaeota and PCO axis 1 was discovered (Figure 4). The increasing relative abundance of Euryarchaeota over time influenced community similarity between corresponding ABR compartments (e.g., ABR-1 C2 and ABR-2 C2) and the terminal compartments, yet not to the exclusion of temporospatial relationships

established for bacteria, as seen in a bacteria-only PCoA (Figure S5). Deltaproteobacteria, which includes sulfate-reducing bacteria known to compete with methanogens for substrates such as acetate [refs. 67-68], also increased longitudinally through ABR-2, as did Synergistetes, which have can have a syntrophic relationship with methanogens [ref. 69].

[Figure 4]

Figure 5 further explores ABR-1 and ABR-2 Euryarchaeota by depicting the genus of methanogens in each ABR by compartment over time. The relative abundance of Euryarchaeota, which included only putative methanogenic archaea, increased longitudinally through each ABR, with the terminal compartments containing the highest abundance. While the relative abundance of Euryarchaeota in each ABR-1 compartment stayed consistent throughout the study, the relative abundance in ABR-2 C1 and C2 initially decreased through day 77 of the study. However, when biogas-induced disturbances ceased and the temperature increased, the relative abundance of Euryarchaeota increased. Further, *Methanobrevibacter*, a hydrogenotrophic methanogen, initially dominated the methanogenic community in both ABR-2 C1 and C2; however, *Methanosaeta* (heterotypic synonym *Methanothrix*), an acetoclastic methanogen, increased in abundance after day 217 of the study. Unlike ABR-2 C1 and C2, ABR-2's terminal compartment was dominated by *Methanosaeta* immediately following sludge seeding. Euryarchaeota comprised 17% of the microbial community in ABR-2's terminal compartment at the end of the study period, with *Methanosaeta* comprising 10% of the total community. *Methanosaeta* dominated ABR-1's methanogenic community in all compartments tested throughout the study period.

[Figure 5]

3.4. Persistence of the founding microbiota

Founding members, defined as those that were present in the seed sludge and never observed in the influent wastewater, initially deceased in each ABR-2 compartment, but increased later in the study period (Figure 6A). Specifically, 90 ASVs from ABR-2 C1's seed sludge that were not detected in the influent wastewater persisted through the study period. More founder ASVs were observed in ABR-2 C2 (111 ASVs) and ABR-2 C3 (217 ASVs). For C1's persisting founding members, the relative abundance decreased from 12.4% post-inoculation to a low of 0.3% on day 133 of the study. After day 133, however, the relative abundance of persisting founders in C1 increased to 10.9% of ASVs on day 273. Similarly, for C2, the relative abundance of persisting founding members decreased from 8.9% post-inoculation to 0.3% (observed on days 35 and 119) but increased to 20.1% by day 273. The relative abundance of persisting founding members in C3 initially declined from 42.4% post-inoculation to 5.5% on day 21; however, the relative abundance of founders rebounded quickly and remained higher than in C1 and C2 throughout the study period.

[Figure 6]

Persisting members of each compartment's founding microbiota by phyla are depicted in Figure 6B and are tabulated in Table S4. In ABR-2 C1, persisting founders were principally from the phyla Firmicutes, Bacteroidetes, and Proteobacteria. In C2, persisting founders were from Firmicutes, Spirochaetae, and Bacteroidetes. In C3, persisting founders were from Firmicutes, Acidobacteria, and Bacteroidetes. Euryarchaeota were observed to be persisting founders in each ABR-2 compartment, with relative abundance of ASVs increasing longitudinally through the ABR (i.e., C1 = 6.1%, C2 = 11.0%, and C3 = 12.1%).

4. Discussion

4.1. Changes in community similarity suggest two successional trajectories occurred in ABR-2 after seeding

Initial colonization in natural systems is principally driven by dispersal rates and abundance of available organisms [ref. 70]. Even in similar environments, variations in colonizing community structure can create significantly different successional trajectories [ref. 34]. Post colonization, succession of community members can be impacted by numerous influences, including substrate availability, disturbances, and environmental factors, e.g., temperature [refs. 20,21,70]. In engineered microbial systems, such as aerobic activated sludge treatment, seed sludge may be transferred between an existing donor and a receiving bioreactor with the purpose of maintaining similar community structure and function [ref. 20]. Therefore, due to the similar influent wastewater chemistry at both ABR locations, and the similar configuration of the ABRs, we initially hypothesized that ABR-2 would immediately follow a successional trajectory that would create a community structure like that of ABR-1. Such deterministic community assembly has been previously suggested for both aerobic and anaerobic wastewater treatment bioreactors [refs. 20-21]. However, a period of dissimilarity initially occurred in ABR-2 (Days 0 through ~118) as the community of each compartment became more like the influent wastewater at ABR-2 than their corresponding ABR-1 compartment (Figure 3).

Several factors may have influenced initial community succession in ABR, to include the transfer and dilution of seed sludge into low-temperature wastewater, and disturbances caused by biogas-induced lifting of the sludge/solids mixture in ABR-2 C1. While efforts were made to maintain the integrity of the sludge blanket, ABR-1 communities were disturbed during transfer and immediately diluted in ABR-2 wastewater (dilution ratio of approximately 20 liters wastewater to 1-liter sludge) upon inoculation. While the sludge beds settled for 48-hours prior to the introduction of fresh influent wastewater, methanogens, as obligate anaerobes, were

invariably impacted by the inoculation process and subsequently decreased in relative abundance during the first successional period (Figure 5). Further, at lower temperatures, accumulation of solids has been observed in the sludge beds of anaerobic reactor systems due to decreased hydrolytic activity [refs. 71-72]. Despite depressed microbial activity, sufficient biogas accumulated within the sludge/solids mixture to lift portions of the sludge bed in ABR-2 C1, disrupting microbial activity on 12 occasions (Table S1; Figure S2). Most of the floated sludge was removed from the reactor manually, but an unknown quantity was transferred to ABR-2 C2, shifting microbial communities there. These stressors may have inhibited microbial activity in ABR-2, accounting for lower COD and TSS removal efficiencies over the first 118 days of study (Table 1).

Despite sludge bed disturbances, members of the influent wastewater's core microbiome, which were consistent over time, did not become members of ABR-2 C1's core microbiome. The failure of influent wastewater community members to take advantage of any open niche caused by disturbances is suggestive of a "priority effect", where early colonizers from the sludge seed have a greater impact on community reassembly post-disturbance [ref. 35]. In plant ecology, secondary succession can be initiated by a catastrophic disturbance event that causes significant loss of the existing community [ref. 34]. In this case, disturbances and environmental conditions (e.g., low wastewater temperatures) do not appear to have initiated a secondary succession event in ABR-2. Instead, the cessation of disturbances, coupled with increasing wastewater temperatures, allowed for more stable reactor operations and initiation of a second successional trajectory (i.e., migration along PCO Axis 1 of Figure 3). Interestingly, the valve failure in ABR-2 C1 on day 241, which caused the loss of approximately 70% (by volume) of the sludge bed, caused no degradation in operational performance, as indicated by COD and TSS removal efficiency, and had little impact on the community structure (Figures 1 and 2).

A “priority effect” may be complimented by the concept of environmental filtering, which would suggest that influent wastewater microbiota would likely be unable to take advantage of any open niches caused by environmental conditions and disturbances [refs. 73-74]. Conversely, bench-scale anaerobic bioreactor studies suggest influent wastewater microbiota strongly influences the anaerobic community structure [ref. 75]. However, the ABRs used in this study are significantly larger and house more anaerobic sludge than many other studies examining anaerobic bioreactor community structure over time and space. Influent microbiota were observed to have little impact on the anaerobic microbial community in a pilot-scale gas sparging anaerobic membrane bioreactor (AnMBR) treating domestic wastewater [ref. 76], consistent with our findings.

4.2. Increasing % relative abundance of Euryarchaeota drove community similarity

Community similarity in ABR-2 after day 118 of the study was principally driven by increasing percent relative abundance of Euryarchaeota, especially *Methanosaeta* (Figures 4 and 5). The lower relative abundance at earlier sampling points in ABR-2 is suggestive of depressed methanogenic activity, especially during the first 118 days of the study when biogas-induced pulse disturbances and low wastewater temperatures were observed. The transfer of seed sludge from ABR-1 to ABR-2 and subsequent dilution with influent wastewater prior to reactor start-up also likely affected the viability of methanogens, which are obligate anaerobes [ref. 77], and further inhibited growth during the first successional trajectory. As temperatures increased and disturbances were reduced (days 118 to 273), the relative abundance of methanogens increased, and their composition changed. Specifically, a shift from a methanogenic community dominated by *Methanobrevibacter* to a more diverse community dominated by *Methanosaeta* occurred around day 217, which corresponds a wastewater temperature above 22°C and high concentrations of available acetate, a substrate for *Methanosaeta*.

The results suggest that hydrogenotrophic methanogens, e.g., *Methanobrevibacter*, dominated the methanogenic community during initial succession, but a shift to acetoclastic methanogens, i.e., *Methanosaeta*, began to occur during the second successional trajectory (after day 118) when disturbances subsided and temperatures increased. Low wastewater temperatures likely facilitated the initial dominance of *Methanobrevibacter*; thermodynamically, hydrogen is a more favorable substrate than acetate at lower temperatures [72]. Hydrogenotrophic methanogenesis has been shown to be the dominant pathway in anaerobic bioreactors treating low-temperature domestic wastewater [75]. Only in ABR-2 C1 did *Methanobrevibacter* still dominate the methanogenic community at the end of study, though the proportion of *Methanosaeta* in ABR-2 C1 increased during the last 70 days of the study (Figure 5). The dominance of *Methanosaeta* in all other compartments, including ABR-1 C1, suggests that *Methanosaeta* may come to dominate ABR-2 C1's methanogenic community over time.

At consistent low concentrations of acetate in wastewater sludge, *Methanosaeta* species have been observed to dominate the methanogenic community [ref. 14, 78]. Specifically, *Methanosaeta* has a high substrate affinity and will outcompete other methanogens, to include other acetate-utilizing methanogens (e.g., *Methanosarcina*), at low acetate concentrations – a result that has been observed in AnMBRs, UASBs, and ABRs [refs. 79-82]. The baffled configuration of the ABR allows for increased acetogenesis and acetate production in the middle compartments. Lower acetate concentrations in the influent wastewater may provide an opportunity for hydrogenotrophic methanogens (e.g., *Methanobrevibacter*) to outcompete *Methanosaeta* in the first ABR compartment. This result has been reported in bench-scale ABRs treating synthetic wastewater [ref. 14]. However, the increased acetate production in the middle compartments facilitates dominance of acetoclastic methanogens in middle and latter ABR compartments. As levels of hydrolysis and acetogenesis increase at higher temperatures or with a more stable anaerobic microbial consortium in an ABR, a shift to domination by acetoclastic

methanogens will likely occur, even in the first ABR compartment (as evidenced in ABR-1's methanogenic community). The methanogenic community structure in ABR-2 became more like corresponding donor ABR-1 compartments over time, further suggesting that the baffled reactor configuration can help determine community structure. A similar successional trajectory for methanogens may be observed in similar anaerobic multiple-compartment reactors treating domestic wastewater, especially if acetoclastic methanogens are prevalent in the seed sludge, although the initial successional trajectory may be more direct if the reactor is seeded under warmer wastewater temperatures and with no disturbances.

4.3. A founding microbiome persists in each ABR-2 compartment over time

In this study, each ABR-2 compartment received a unique seed sludge from a corresponding compartment in ABR-1, similar to microbiota transplantation or transmission during maternal birthing events. An initial loss of unique community members from the founding microbiome was evident in each ABR-2 compartment; however, founding members that persisted through the initial successional trajectory (days 0 to 118), which included periods of biogas-induced pulse disturbances, subsequently increased in relative abundance under more stable reactor conditions and higher wastewater temperatures (Figure 6). This result suggests that founding community members played an important legacy role and persisted throughout the development of a stable microbial community, despite disturbances post-inoculation. The relatively large percentage of founders that remained in ABR-2 C3 despite disturbances also suggests that the baffled configuration of the bioreactor likely protected the microbial communities in ABR-2 C3 from biogas-induced pulse disturbance events that occurred in ABR-2 C1 and transferred sludge to ABR-2 C2. Results further suggest that stochastic influences such as unmanaged performance variations may only negatively impact the founding community during the timeframe in which the variations occur. More stable reactor operations may facilitate the

persistence or proliferation of the founding microbial communities, thereby increasing similarity in community structure between the receiving and donor reactors.

Conclusion

The results of this study suggest that a transfer of anaerobic sludge inoculum from a long-running ABR treating raw low-temperature domestic wastewater to a new ABR can result in similar levels of performance despite differing environmental conditions and numerous disturbances to the community after inoculation. Low wastewater temperatures, different substrate concentrations, different influent microbial communities, and sludge blanket disturbances caused an initial divergence in community similarity between donor and receiver bioreactors during an initial successional trajectory (between days 0 and 118); however, the communities became similar once disturbances were controlled and temperatures warmed (between days 119 and 275). Community similarity during the second successional trajectory was driven by abundance of methanogenic archaea, specifically *Methanosaeta*. Further, prevalence of the founding microbiota increased during the second successional trajectory. Last, this study suggests that anaerobic sludge may follow a deterministic trajectory post transfer, such that future operators of full-scale ABRs may be able to transfer sludge between like bioreactor compartments using a similar approach as activated sludge wastewater treatment operators commonly use today.

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Competing Interests

The authors declare no conflicts of interest.

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Figures

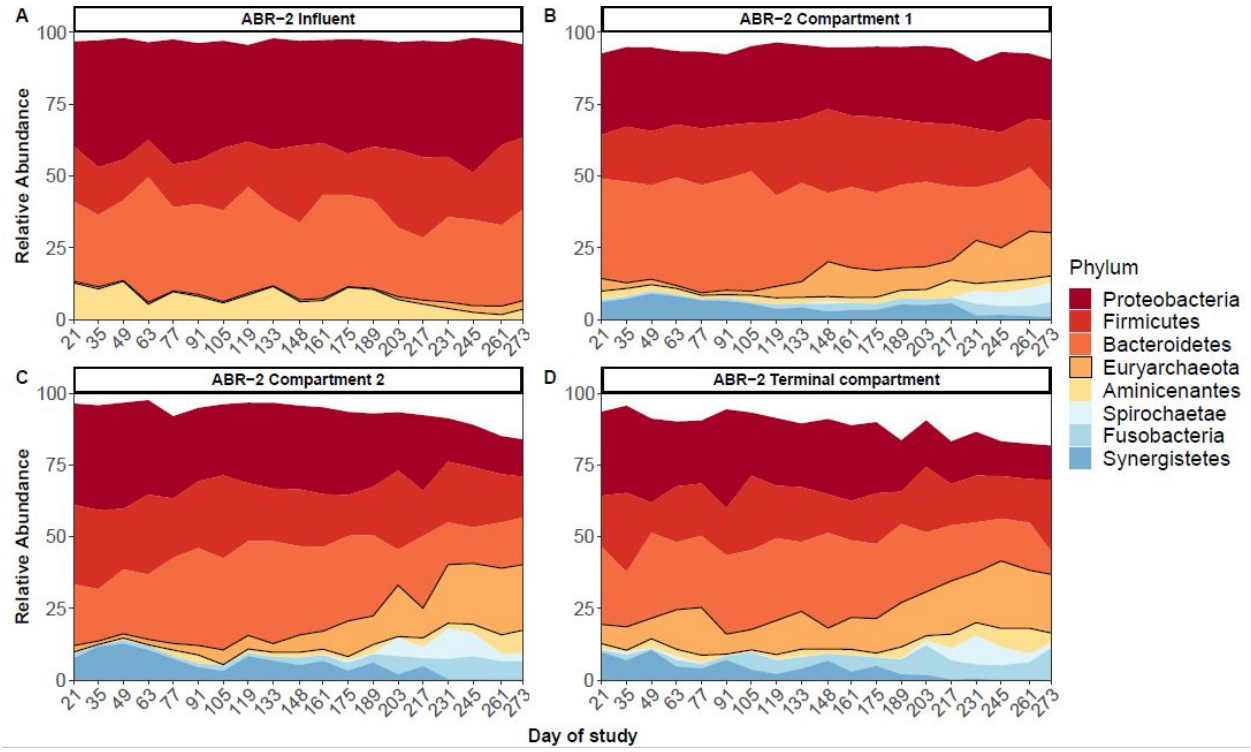

Figure 1. Phyla-level stream graphs for ABR-2, to include influent wastewater (panel A), C1 (B), C2 (C), and C3 (D). Euryarchaeota are shown in orange with bolded black lines. For comparison, Figure S4 shows alpha diversity (ASV richness) by compartment for each ABR.

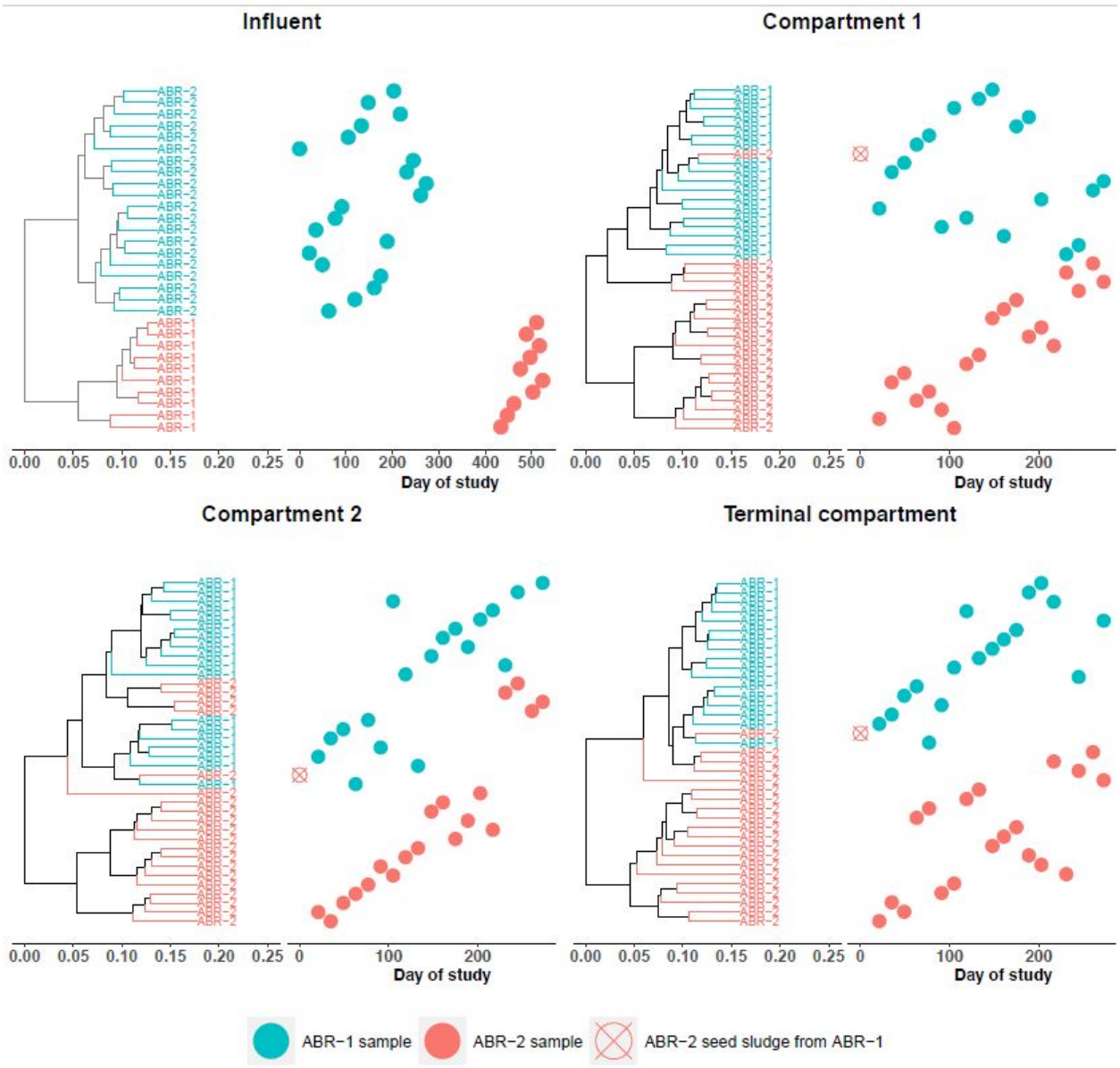

Figure 2. Dendrograms comparing corresponding reactor compartments in ABRs 1 (turquoise colored) and 2 (salmon colored) are depicted on the left side of each panel. The right side of each panel shows the relative dendrogram position of the community in each sample by day of study. ABR-2 seed samples are identified by a red circle with “x” through the center.

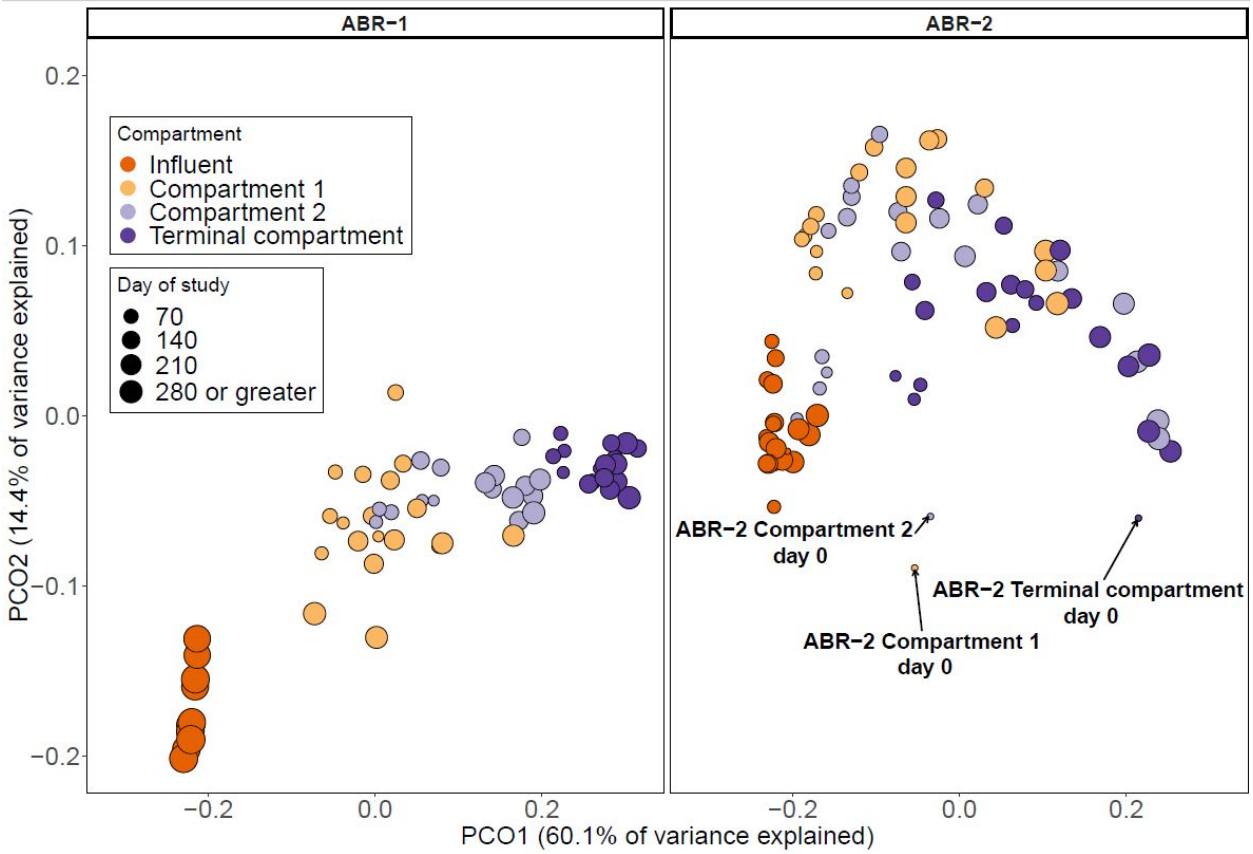

Figure 3. PCoA of weighted UniFrac distance matrices for both ABR-1 and ABR-2. Unique samples are colored by location and sized by date (i.e., larger symbols represent samples taken later in the study period). The ordination was performed on the entire data set (ABR-1 and ABR-2 samples combined), but for visualization purposes the two locations are plotted on separate panels.

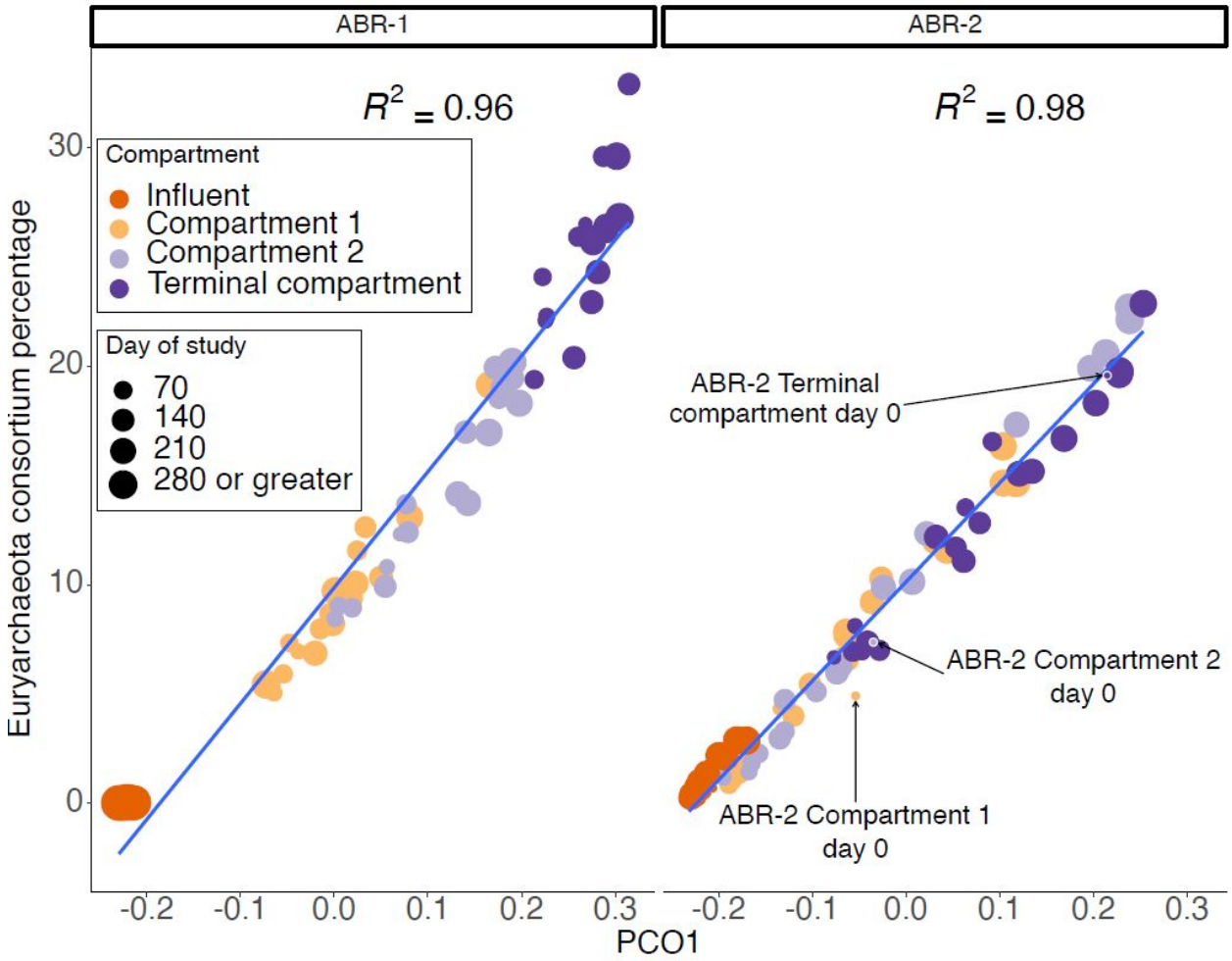

Figure 4. Consortium percentage (ASV percent) of Euryarchaeota plotted against PCO1 of the PCoA for ABRs 1 and 2 (Figure 3). Influent wastewater samples have the lowest ASV percentage, while C2 and the terminal compartment samples taken later in the study have the highest.

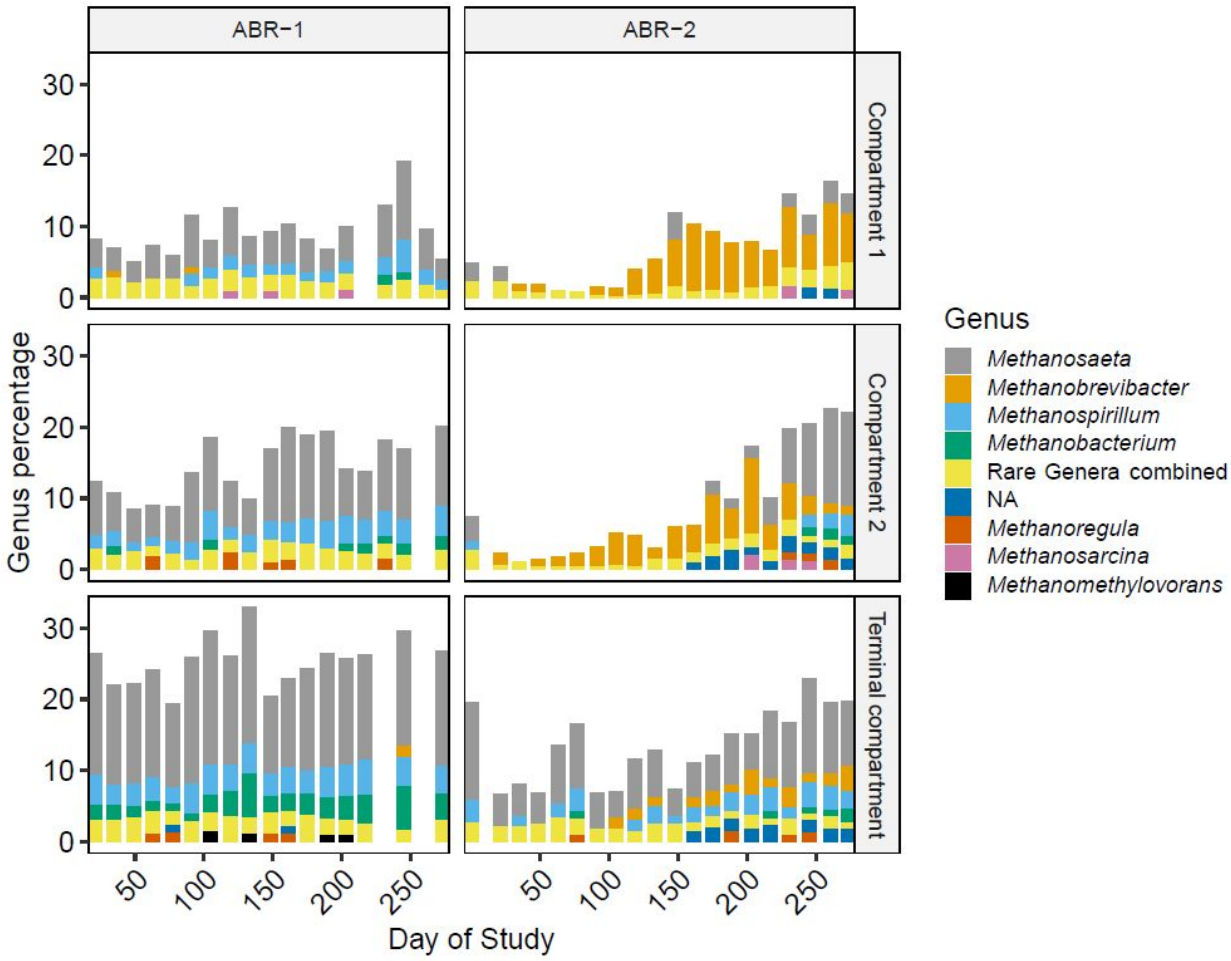

Figure 5. Normalized stacked bars for genus-level comparison of Euryarchaeota in complimentary ABR compartments over time. The x-axis depicts day of study. Data gaps for several ABR sampling points are due to low sequence count.

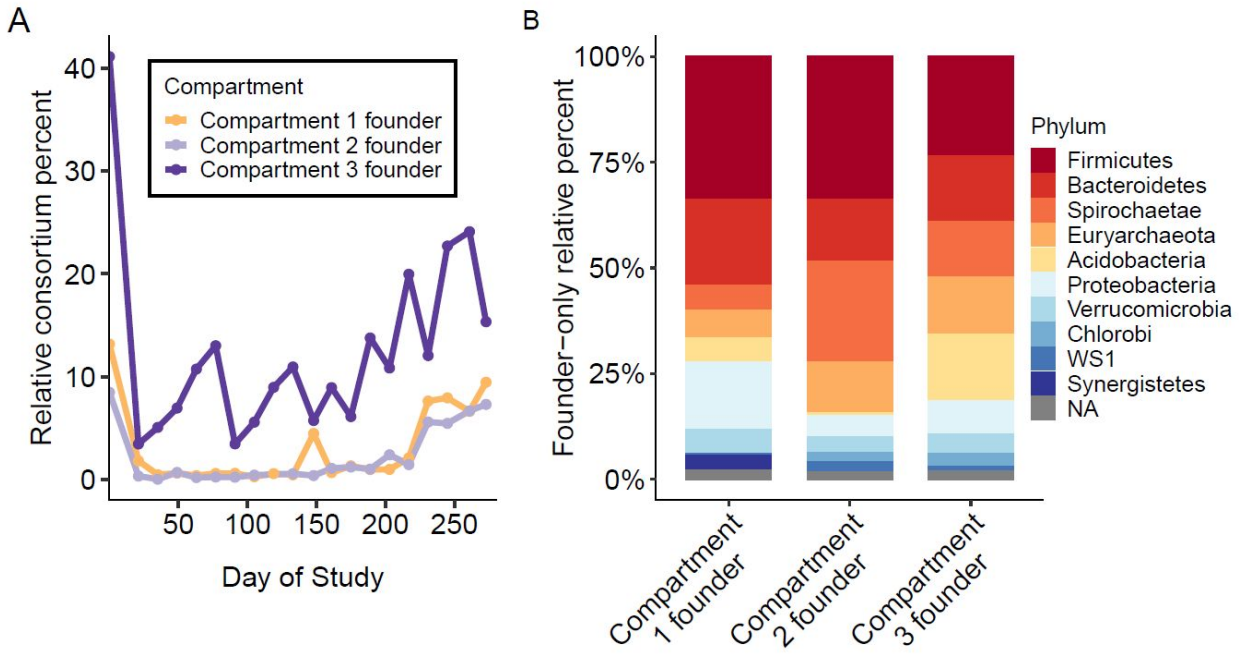

Figure 6. (A) percent relative abundance of founding microbiome over time in each ABR-2 compartment. Founding microbiome prevalence in C1 and C2 increased after reaching a minimum on 133 and 119 days of study, respectively. While the abundance in C3 stayed higher relative to C1 and C2, an increase was observed after day 190 of the study. (B) Phyla-level stacked bar chart of normalized percent relative founder abundance by ABR-2 compartment. The legend order mirrors the bar order from top to bottom.

Table 1. Average ± standard deviation for reactor temperature and analyte concentrations in ABRs 1 and 2. Performance of ABR-1 beyond the 275 days examined in this study can be found in references 10 and 11, while performance data for ABR-2 can be found in reference 49.

Reactor	Timeframe	Temperature (°C)	tCOD (mg L^{-1})		pCOD (mg L^{-1})		sCOD (mg L^{-1})		TSS (mg L^{-1})		VFA (mg COD_{VFA} L^{-1})		Gaseous CH_4 (L day^{-1})
			Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	System Production
ABR-1	Days 0-101	15.0 ± 1.7	810 ± 350	360 ± 40	680 ± 330	190 ± 50	170 ± 30	170 ± 20	370 ± 180	60 ± 30	Not Measured		70 ± 10
	Days 102-275	19.4 ± 2.4	710 ± 120	220 ± 40	570 ± 110	110 ± 30	150 ± 30	110 ± 20	460 ± 210	30 ± 20	30 ± 10	50 ± 40	85 ± 10
ABR-2	Days 0-118	16.0 ± 1.3	490 ± 130	380 ± 60	280 ± 110	180 ± 30	210 ± 30	210 ± 30	210 ± 280	70 ± 10	Not Measured		Not Measured
	Days 119-275	17.4 ± 2.5	590 ± 310	270 ± 50	390 ± 290	90 ± 30	200 ± 40	200 ± 40	270 ± 300	40 ± 10	50 ± 30	60 ± 20	80 ± 50

Notes:
(1) A statistically significant difference ($p < 0.001$ using ANOVA single factor analyses) between influent wastewater was observed between ABRs 1 and 2 for all analytes; however, there was no statistical difference in the temperature of the influent wastewater between ABRs.
(2) The timeframe for ABR-1 is separated into two periods based on the change in system HRT from 12 to 24 hours.
(3) The timeframe for ABR-2 is separated into two periods based on the observed decrease in biogas-induced pulse disturbances and more stable reactor operations.
(4) VFA concentrations were not consistently measured prior to Day 119 of the study due to observed pulse disturbances in ABR-2.
(5) Due to maintenance problems, gaseous CH_4 composition was measured, but biogas flowrates were calculated based on historical data in ABR-1.
(6) Gaseous CH_4 was not measured in ABR-2 prior to Day 119 of the study due to observed pulse disturbances and the resultant inability to collect samples.