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An anaerobic hybrid bioreactor for biologically enhanced primary treatment of domestic wastewater under low temperatures

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Abstract

Anaerobic treatment of domestic wastewater is a methane-generating alternative to the current aerobic wastewater treatment paradigm. To explore biologically enhanced primary treatment of domestic wastewater, a pilot-scale hybrid reactor system, consisting of a three-compartment anaerobic baffled reactor (ABR) and an anaerobic fixed film reactor (AFFR), was operated for 720 days under low wastewater temperatures. The ABR-AFFR removed 49% of organics (as chemical oxygen demand, COD) and 72% of suspended solids, exceeding the performance of conventional primary treatment and achieving secondary discharge standards for suspended solids under warmer wastewater temperatures (> 20°C). The ABR-AFFR produced stoichiometric volumes of methane (0.36 L CH_4 per g COD removed), at times exceeding the calculated theoretical maximum methane production from biodegradable organic removal. The mean electrical energy potential of gaseous CH_4 produced by the ABR-AFFR was 0.16 kWh m^{-3} wastewater treated (assuming 32% electrical energy conversion efficiency). Examination of the microbial communities under warm (23°C) and cold (12°C) wastewater temperatures indicates that Euryarchaeota was in higher relative abundance under cold wastewater temperatures and that *Methanosaeta*, an acetate-utilizing methanogen, dominated the methanogenic community. The difference in community structure under varying wastewater temperatures indicates that long-term studies are required before accurate models tying system performance to community structure can be constructed. Results of this study suggest that the ABR-AFFR may be a viable

methane-generating alternative to conventional primary treatment in an anaerobic-aerobic treatment paradigm.

Water Impact Statement

Biologically enhanced primary treatment of wastewater using multiple-compartment anaerobic reactors removes organics and suspended solids beyond conventional primary treatment while generating stoichiometric quantities of methane. *Methanosaeta,* an acetate-utilizing methanogen, dominated the methanogenic community. Energy generated from methane produced during anaerobic primary treatment is sufficient to power activated sludge processes at some wastewater reclamation facilities.

1. Introduction

The current centralized domestic wastewater treatment paradigm centers on aerobic treatment technologies, e.g., conventional activated sludge, which are energy-intensive and require substantial aeration.[1,2] Municipal wastewater treatment accounts for approximately 3% of U.S. electricity consumption, with aeration of activated sludge typically accounting for about one-half of electricity use at wastewater reclamation facilities (WWRFs).[3,4] Anaerobic technologies, which can generate methane-rich biogas from the degradation of organic carbon, are expected to be less energy-intensive than aerobic processes.[3,5] To date, however, full-scale mainstream anaerobic treatment of domestic wastewater has been primarily limited to tropical and subtropical climates with warmer ambient temperatures.[6–9] The single-compartment upflow anaerobic sludge blanket (UASB) is currently the most widely used anaerobic treatment technology;[10,11] however, UASBs can produce varying effluent wastewater quality and often fail to meet established discharge standards in developed nations.[12] Anaerobic technologies have been further limited by the perception that anaerobic treatment is primarily for sludge digestion[13,14] and the notion that low-temperature anaerobic treatment of dilute wastewater will

result in low chemical oxygen demand (COD) removal, low methane production, and high concentrations of dissolved methane in the reactor effluent.[15] Reactor systems such as the anaerobic membrane bioreactor (AnMBR) have demonstrated the ability to achieve discharge standards for organics and total suspended solids (TSS) while producing methane, but currently use more energy than can be recovered in doing so.[16] Further research is required to determine if anaerobic technologies can meet effluent discharge standards while simultaneously producing energy in excess of the energy required to operate treatment processes.[3]

Multiple-compartment baffled reactor configurations, such as the anaerobic baffled reactor (ABR) or similar anaerobic hybrid reactor systems (i.e., reactors that couple two or more anaerobic treatment technologies) have been the subject of study since the first bench-scale ABR was introduced over 30 years ago.[17] The baffled configuration of the ABR directs wastewater through sequential compartments under upflow and downflow conditions such that treated water passes through several sludge beds prior to exiting the reactor system.[18,19] The hydraulic flow pattern allows for sludge to be retained, decoupling the hydraulic retention time (HRT) from the solids retention time (SRT) and allowing time for additional hydrolysis of solids and particulate COD.[20] Biogas produced in the sludge bed, which consists primarily of methane (CH_4) (65-70%) and carbon dioxide (CO_2) (25-30%), allows the sludge to rise and slowly settle, and increases substrate-to-biomass contact time.[14] Other advantages of the ABR include simple design and operation, low energy inputs, and resistance to shock loads of COD and total suspended solids (TSS) from the influent wastewater.[19] Despite the potential advantages of the ABR, pilot-scale demonstrations in colder regions with low wastewater temperatures ranging from 10 to 25°C are limited. The majority of ABR studies have been conducted at bench-scale (i.e., < 25 liters), with synthetic or filtered wastewater, or for periods of time < 1 year.[20] Previously noted disadvantages of the ABR include the requirement to construct shallow reactors to accommodate gas and liquid upflow velocities, and the difficulty to evenly distribute influent wastewater to the sludge bed.[13,19] Further, bench-scale domestic wastewater ABR

studies suggest that organic (i.e., 5-day biochemical oxygen demand, BOD_5) and TSS removal capabilities of the ABR do not achieve effluent discharge standards (e.g., 30 mg L^{-1} for BOD_5 and TSS for the U.S. EPA), thereby limiting current ABR configurations to biologically enhanced primary treatment.[21–25] Anaerobic reactor systems, to include the ABR and the AnMBR, also fail to remove nitrogen and phosphorus, while producing dissolved methane (dCH_4) and hydrogen sulfide (H_2S) – all of which must be addressed prior to widespread implementation.[26]

Microbial community structure, as well as the stoichiometry and kinetics of observed community members, must be characterized to construct models that inform bioreactor design and/or accurately predict performance.[27,28] While studies of microbial communities in multiple-compartment bioreactors such as the ABR do exist, most examine ABRs with waste streams other than raw domestic wastewater or employ techniques other than 16S rRNA gene sequencing that provide a less complete understanding of the microbial community structure (e.g., fluorescent in-situ hybridization, scanning electron microscopy, or gene amplification (polymerase chain reaction) coupled with denaturing gradient gel electrophoresis).[19,29–33] An investigation of the microbial community structure under warm (23°C) and cold (12°C) wastewater temperatures in each baffled reactor compartment is needed to compare differences and determine if further study is required prior to the development of models that accurately predict performance of multiple-compartment sludge bed bioreactors such as the ABR-AFFR.[34]

The purpose of this study was to characterize the long-term performance (720 days) of a pilot-scale multiple-compartment hybrid anaerobic biological reactor consisting of three baffled compartments (i.e., an ABR; 12:1 height-to-diameter ratio) coupled with an anaerobic fixed film reactor (AFFR; 4:1 height-to-diameter ratio) operated under low wastewater temperatures. The large height-to-diameter ratio of the ABR portion of the bioreactor was designed to directly address aforementioned disadvantages and enhance settling of suspended solids. Specific objectives included characterization of: (1) bioreactor performance for removal of organics (i.e.,

COD and BOD_5) and TSS relative to established discharge standards; (2) methane generation over varying wastewater temperatures, with comparison of observed methane production to the theoretical maximum methane generation from the removal of organics (i.e., biodegradable COD); and (3) methanogenic community structure in the anaerobic sludge beds of the ABR at observed warm and cold wastewater temperatures (23˚C and 12˚C).

2. Materials and methods

2.1. Anaerobic reactor configuration

An anaerobic reactor consisting of three equal-sized cylindrical compartments (0.152 m radius and 3.66 m tall) operated as an ABR for 390 days under low wastewater temperatures (9 to 25˚C) in an unheated structure at the Mines Park Wastewater Test Bed in Golden, Colorado (elevation of 1730 meters). A fourth cylindrical compartment (0.152 m radius and 1.22 m tall), which contained media for biofilm growth (i.e., AFFR), was added on day 390, which resulted in a total hydraulic volume of 800 liters; the hybrid reactor system was operated for an additional 330 days (720 days total for the study). Figure S1 depicts a schematic of the ABR-AFFR system. Raw, unheated wastewater from a 250-unit housing complex was first routed to a 2500-gallon holding tank with submerged grinder pump and 2 mm screen. From there, wastewater was routed to a 40-gallon influent feed tank prior to being fed to the reactor system at a rate of 0.5 L min^{-1} (720 L day^{-1}) via a Masterflex L/S digital drive peristaltic pump. Grease was primarily retained in the holding tank and influent solids were slightly reduced in the ABR-AFFR influent feed tank due to settling. The total system hydraulic retention time was 26.7 hours (8 hours for each ABR compartment; 2.7 hours for the AFFR). Wastewater was treated as it flowed sequentially through the sludge bed or fixed film of each reactor compartment. Each compartment contained a downcomer pipe that routed influent wastewater (from the feed or the previous compartment) to the bottom of the compartment beneath the sludge bed. Wastewater

then flowed upward through the sludge bed and into a clarified zone at an upflow velocity of 0.41 m h^{-1}. Wastewater exited each reactor compartment through an effluent pipe located at the top of each compartment, but below the water surface. This hydraulic flow pattern was repeated for each reactor compartment. Each compartment contained a gas-liquid-solid separator above the sludge bed and below the water surface (installed on day 118 of reactor operations). For the AFFR, the gas-liquid-solid separator held media for biofilm growth in the upper half of the reactor compartment. Biogas was allowed to accumulate in the headspace of each reactor compartment for a minimum of five days prior to sampling.

2.2. Data collection and analyses

Measurements collected from the influent wastewater and the effluent of each reactor compartment included temperature, pH, total COD (tCOD), soluble COD (sCOD), particulate COD (pCOD), BOD_5, total suspended solids (TSS), volatile suspended solids (VSS), organic acids (acetate, propionate, butyrate, and lactate), ions (e.g., sulfate and phosphate), hydrogen sulfide, biogas production and composition (CH_4 and CO_2), and dissolved CH_4 (dCH_4). Measurements collected from the influent wastewater and the reactor effluent (either compartment 3 or compartment 4, as appropriate) include dissolved organic carbon (DOC), alkalinity, and nitrogen (total nitrogen, nitrate, nitrite, ammonia). Temperature and pH were continuously monitored. Grab samples were taken twice weekly for tCOD, sCOD, pCOD, TSS, and VSS. Biogas and dCH_4 sampling was conducted weekly. Bimonthly grab samples were taken for DOC, alkalinity, nitrogen, ions, hydrogen sulfide, and organic acids.

Analyses for tCOD, sCOD, pCOD, BOD_5, TSS, VSS, alkalinity, and nitrogen species were conducted according to Standard Methods or approved EPA methods; further detail is provided in supplemental materials section 1.[35] BOD_5 measurements were used to estimate bCOD using the relationship 0.68 bCOD = BOD_5.[14] pH was measured with Cole-Parmer pH electrodes (100 Ohm Pt RTD, EW-27003-23). Temperature was measured with LabJack EI-

1034 probes. 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. Reactor biogas flowrate was measured using an Agilent Digital Flow Meter (Optiflow 520). dCH_4 was analyzed according to the method described in Pfluger et al. (2011) with minor modification (described in supplemental methods section 1.2).[36] 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. Sludge retention time (SRT) was estimated by dividing the total mass of volatile solids in the reactor, as determined from sludge VSS concentration (g L^{-1} sludge) and the observed sludge volume (L), by the mass removal rate of effluent VSS (g d^{-1}), scum removed from the top of each reactor compartment during biological sampling (g d^{-1}), and the sludge removed during biological sampling (g d^{-1}).

Comparisons of the means of two variables were assessed using two-sample t-tests (assuming unequal variances) and the Wilcoxon signed-rank test. Matched pairs t-tests were used to identify reactor compartments for which a significant reduction in the mean of a particular variable (e.g., tCOD, pCOD, TSS, etc.) between compartments was observed and, when appropriate, corresponding 95% confidence intervals (CI) for the mean difference and mean removal were constructed. Linear regressions with varying y-intercept models were fit to assess the impact of temperature on several variables. Boxplots were constructed for

comparison of contaminant removal by individual reactor compartment and identification of statistical outliers. All "±" values presented in this study represent one standard deviation.

2.3. Microbial community structure

As a preliminary investigation into microbial community differences with temperature, biological sludge samples from compartments 1, 2, and 3 were removed with a Sludge Judge C09247WA Sampler System from the center of each compartment's sludge bed on two occasions when high and low wastewater temperatures were observed: (1) day 231 of reactor operation (23°C) and (2) day 395 of reactor operation (12°C). Influent wastewater samples were also preserved on these days. Samples were transported on ice and centrifuged biomass pellets (4000G for 10 min) 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 quantified using a Qubit Fluorometer and a Qubit dsDNA High Sensitivity Assay Kit (Thermo-Fisher, Inc.). DNA samples were amplified using primers 515F (5'GTGCCAGCMGCCGCGGTAA3') and 806R (5'GGACTACHVGGGTWTCTAAT3') following the two-step amplification and barcoding strategy described in Stamps et al. (2016).[37] Illumina MiSeq sequencing targeting the V4 region of bacteria and archaea was performed by the Duke University Center for Genomic and Computational Biology using Illumina 2X250 chemistry. A subset of samples was sequenced in duplicate (but with different barcodes) to evaluate technical consistency. Post sequencing, data were demultiplexed using Sabre (https://github.com/najoshi/sabre) allowing for zero barcode mismatches. rRNA gene sequences (henceforth called 'amplicon sequence variants' or ASVs)[38] were initially analyzed using DADA2 [39] for the following: removal of PCR primer sequences and low quality bases, merging paired end reads, chimera removal, taxonomy assignment using Silva Version 128,[40] and ASV table

construction. Quantitative Insights into Microbial Ecology (QIIME) version 1.9 was used to align and filter ASVs and construct a phylogenetic tree. The ASV table, taxonomy table, metadata, and phylogenetic tree were then imported into Phyloseq,[41] which was used to visualize data. To construct heatmaps, two singleton ASVs were removed, then the ASV table was converted to consortium percentage (i.e., ASV count in a sample divided by the sum of sequences in that sample) and filtered to retain single nucleotide variants representing > 0.1% of a sample's composition. Data were then divided into subsets representing the five most abundant phyla; however, composition values relative to all identified taxa are presented. Ampvis2 [42] and ggplot2 [43] were used to visualize the resultant heatmap. To construct the principal coordinate analysis (PCoA) plot, singleton-free data were normalized using MetagenomeSeq cumulative sum scaling[44] prior to construction of a weighted UniFrac distance matrix [45]. Absolute microorganism abundance was estimated using DNA concentrations, the mass of sludge sampled (g), and relative abundance of ASVs. ASVs can be accessed on GenBank under accession SRP136078 (National Center for Biotechnology Information; see supplemental methods section 1.3). A reproducible bioinformatics workflow is available on GITHUB (see supplemental methods section 1.4).

3. Results and discussion

During the 720-day study period, pH ranged between 6.8 and 7.2. The mean alkalinity concentration was 229 mg $CaCO_3$ L^{-1}, 95% CIs [214, 243]. Wastewater temperatures fluctuated seasonally and weekly averages were observed to vary between 9 and 25°C; however, temperatures as low as 6°C were observed.

3.1. ABR-AFFR approached effluent discharge standards under warmer temperatures

The influent wastewater was considered medium-high strength relative to domestic wastewater characteristics described in Tchobanoglous et al. (2003).[14] Mean concentrations of key performance parameters (tCOD, pCOD, sCOD, BOD_5, and TSS) for the influent and effluent of each reactor compartment are provided in Table 1. For comparison, results are subset into four periods of time based on variations in seasonal wastewater temperatures: Period 1 (days 0-180; mean temperature = 14.88°C, 95% CIs [14.25, 15.52]), Period 2 (days 181-360; mean temperature = 20.97°C, 95% CIs [20.06, 21.88]), Period 3 (days 361-540; mean temperature = 16.51°C, 95% CIs [15.48, 17.55]), Period 4 (days 541-720; mean temperature = 20.50°C, 95% CIs [19.45, 21.54]). Mean removal of tCOD, pCOD, sCOD, and TSS by reactor compartment with 95% confidence intervals are summarized in Table S1.

The mean influent tCOD concentration was 549 mg L^{-1}, 95% CIs [501, 597] over the course of the study, which equates to a mean organic loading rate of 0.55 kg tCOD m^{-3} d^{-1}. System-level tCOD removal (i.e., influent minus effluent tCOD) averaged 208 g tCOD d^{-1}, 95% CIs [174, 241] or 49%, 95% CIs [45, 52]. Effluent tCOD concentrations were consistent throughout the study averaging 209 mg L^{-1}, 95% CIs [193, 224]. tCOD was removed longitudinally through the reactor system; however, tCOD removal in compartment 1 (C1) was significantly greater than removal in any other compartment (Table 1, Table S1) averaging 151 g tCOD d^{-1}, 95% CIs [115, 187]. Variation in observed tCOD removal was evident in C1 due in part to several negative measurements (i.e., measured tCOD concentrations in C1 were higher than influent tCOD concentrations) caused by biogas-induced sludge lifting events, which occurred periodically during the first 118 days of the study, but were negated by installation of a gas-liquid-solid separators. tCOD removal in compartment 2 (C2) was significant throughout the course of the study except for Period 3, while tCOD removal in compartments 3 (C3) and 4 (C4) were significant during the entire study (Table S1). Figure S2, a boxplot, shows mean tCOD removal and outliers by compartment. Observed BOD_5 removal through C3 (i.e., the ABR

portion of the bioreactor) averaged 50%, 95% CIs [43, 57], similar to tCOD removal. The addition of C4 on day 390 increased organics removal by a small, but significant amount. Between days 390 and 720 of the study, system-level organic removal increased from 55%, 95% CIs [47, 63] between C1 and C3 to 62%, 95% CIs [56, 69] between C1 and C4. Based on the observed tCOD-to-BOD_5 ratio of 2.3, the EPA standard for organic concentration of 30 mg L^{-1} BOD_5 is equivalent to 69 mg tCOD L^{-1}. In terms of statistical significance, pCOD removal followed the same trend as tCOD, with the exception that mean removal in C3 was not significant during the first 180 days of the study. A significant amount of sCOD was generated in C1 for the first 150 days of study, then removed thereafter, suggesting that the rate of hydrolysis of pCOD was greater than the utilization rate of sCOD at the beginning of the study when colder wastewater temperatures (12-16°C) and accumulation of solids in C1 were observed (Table S1; Figure S3). While sCOD concentrations decreased longitudinally through the reactor after day 150, statistically significant relationships varied by compartment over time. Only during days 541-720 of the study did all four reactor compartments remove statistically significant concentrations of sCOD.

Figure 1 presents monthly mean influent and effluent tCOD concentrations compared to the EPA effluent discharge standard (in terms of tCOD). Influent tCOD concentrations were highly variable during the study period, while variations in effluent tCOD were much lower, suggesting that the ABR-AFFR was resistant to tCOD shock loads. Figure S4 further depicts the low variation in effluent concentrations by displaying all measurements from both C3 and C4 and comparing each to the EPA secondary standard. The ABR-AFFR did not achieve equivalent secondary effluent standards for tCOD; however, effluent tCOD concentrations approached discharge standards under warmer temperatures. Linear regression between effluent tCOD concentrations and wastewater temperature indicates a statistically significant relationship ($R^2 = 0.3925$, $p < 0.001$) (Figure S5). Both increased wastewater temperatures and lower influent tCOD concentrations during the last 180 days of the study likely contribute to the

lower effluent tCOD concentrations depicted in Figure 1. Despite not achieving effluent discharge standards, the ABR-AFFR outperformed conventional primary clarification, which typically removes 25-35% of BOD,[46] and observed organics removal is within the range of larger pilot-scale UASBs operated at wastewater temperatures of 20-30°C.[9,47–50]

Similar to influent tCOD, mean influent TSS concentrations were highly variable, averaging 368 mg L^{-1}, 95% CIs [274, 461] over the course of the study (Figure 2). Mean TSS removal over the course of the study was 230 g d^{-1} (72%); however, system level TSS removal was highly variable due to variable influent TSS (Period 1 = 129 g d^{-1}; Period 2 = 245 g d^{-1}; Period 3 = 394 g d^{-1}; Period 4 = 160 g d^{-1}). Unlike COD, statistically significant concentrations of TSS were removed in each compartment longitudinally through the reactor system for all four time periods examined. C1 removed the most TSS, averaging 207 g d^{-1}, 95% CIs [140, 274]. Similar to tCOD, large variation in mean TSS removal in C1 was observed due to several negative measurements. Figure S6, a boxplot, depicts mean removal of TSS by compartment. VSS comprised 88% of TSS within the reactor system with no difference observed between different reactor compartments. Sludge was not purposefully wasted during the study period to facilitate long-term degradation of pCOD and settled solids. The estimated system SRT was 61 days, 95% CIs [48, 74], or approximately 60 times the HRT. Estimating SRT based on flowrate and VSS concentrations in the effluent, recycle, and bioreactor itself is accurate for suspended growth systems (e.g., activated sludge), but is problematic for sludge blanket or fixed film growth bioreactors where volatile solids accumulate in the sludge or biofilm and may not be wasted or recycled. SRT calculations for suspended growth processes rely on effluent VSS concentrations; however, for bioreactors such as the ABR-AFFR, other factors, such as volatile solids in the sludge blanket or biofilm, should be included or a low (conservative) SRT may be determined. In this study, SRT was weakly correlated with temperature (R^2 = 0.12, p < 0.001) suggesting that other variables impacted SRT more than temperature. For activated sludge systems, the SRT represents the period of time that sludge remains in a bioreactor and varies

312 depending on temperature and the level of treatment required (e.g., BOD removal only, or BOD
313 removal with nitrification). Longer SRTs (i.e., 3 to 18 days) are observed in activated sludge
314 systems when complete nitrification is desired, especially at lower wastewater temperatures.[14]
315 Typical SRTs for the stabilization of waste activated sludge using anaerobic digestion are
316 longer, ranging from 20 to 40 days depending on digester temperature.[14] The SRT for sludge
317 blanket and/or fixed film reactors is likely longer than both activated sludge systems and
318 anaerobic digesters, probably exceeding 60 days (as estimated in this study). However, to
319 accurately calculate SRT for sludge blanket and/or fixed film growth processes, more study of
320 the long-term volatile solids dynamics is required.
321 Under warmer wastewater temperatures, the ABR-AFFR episodically met the EPA
322 secondary discharge standard for TSS despite variable influent concentrations. Figure 2
323 presents monthly mean influent and effluent TSS concentrations compared to the EPA effluent
324 discharge standard. As shown, measured effluent TSS concentrations had lower variability

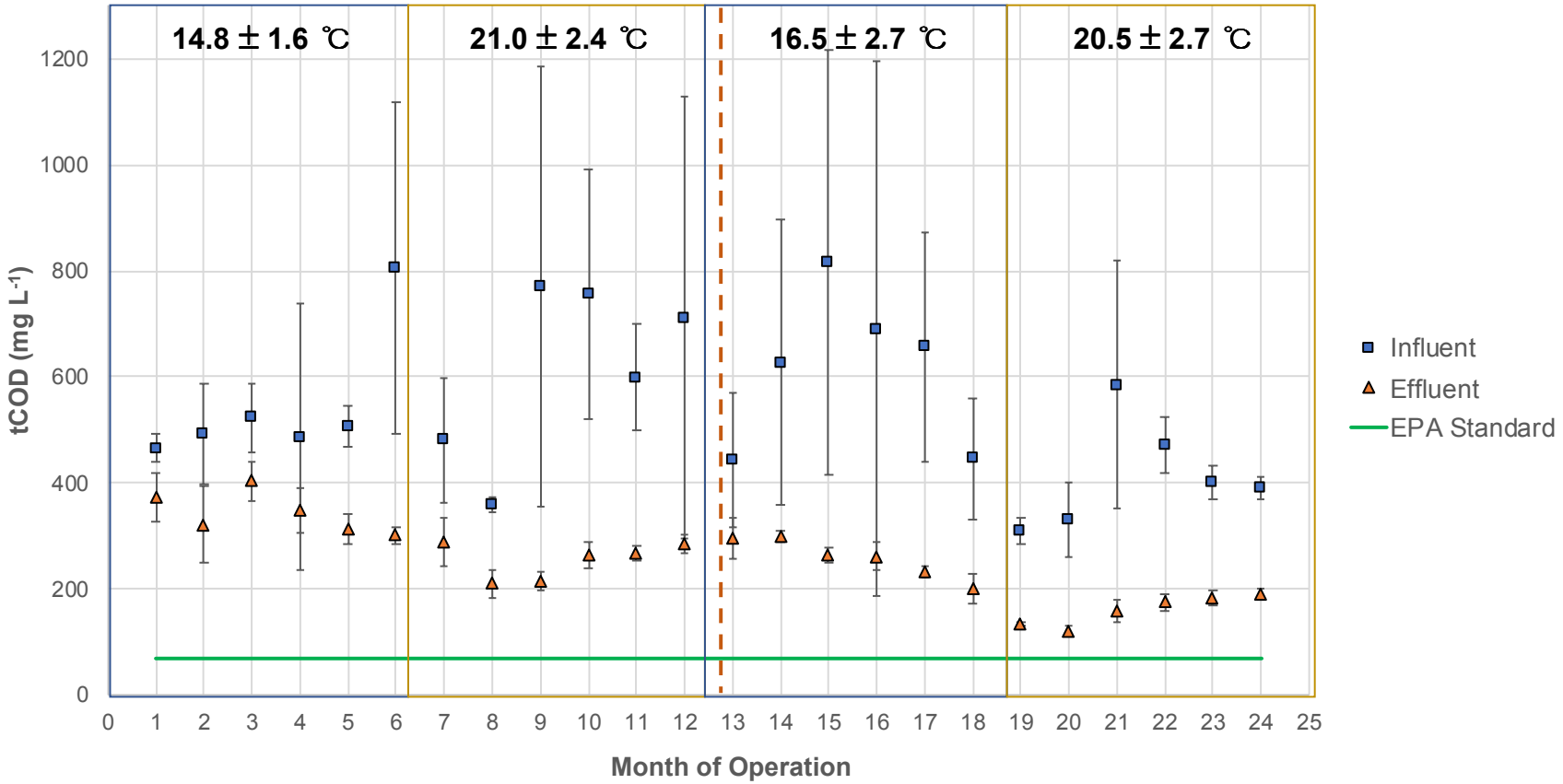

Figure 1. Monthly mean influent and effluent tCOD concentrations with 95% CIs for this bioreactor system compared to the COD-equivalent EPA 30-day secondary discharge standard (69 mg L^{-1}). Influent concentrations were highly variable throughout the study period. The vertical dotted red line represents the addition of C4.

relative to influent concentrations. Figure S7 displays all effluent TSS measurements from C3 and C4 compared to the EPA secondary discharge standard. Linear regression between plotted effluent TSS concentrations and temperature suggests a statistically significant relationship (R^2 = 0.472, p <0.001) (Figure S8). The ABR-AFFR regardless of temperature removed TSS beyond conventional primary clarification, which typically removes 50-65% of TSS in influent wastewater, and is comparable to removal observed with chemically enhanced primary treatment with flocculation and settling (range = 60-90% TSS removal).[46]

Results from this study indicate that follow-on treatment processes are required to remove additional organic carbon and suspended solids, especially under colder temperatures. While enhanced performance could be achieved by heating wastewater to warmer temperatures, substantial energy input would be required (approximately 1.17 kWh for each °C increase per m^3 of wastewater treated),[13] negating the energy generating advantage of the ABR-AFFR. Coupling the ABR-AFFR to an aerobic secondary treatment process, e.g. conventional activated sludge, to remove residual carbon and suspended solids is an approach that could be implemented near-term.

3.2. Observed methane production approaches the theoretical maximum and varies with wastewater temperatures

Mean observed total CH_4 (i.e., gaseous and dissolved) production by reactor compartment over the entire study period is summarized in Table 2. Figure 3 depicts observed monthly mean total, gaseous, and dissolved methane measurements compared to theoretical CH_4 production from biodegradable COD (bCOD) removal over time. Mean observed system-level CH_4 production was 80 L d^{-1}, 95% CIs [71, 90] with 41%, 95% CIs [37, 45] existing in the dissolved phase. The mean effluent dCH_4 concentration was 35 mg L^{-1}, 95% CIs [30, 41], which is comparable to reported values from other ABRs and UASBs operated under colder conditions (13-25°C).[25,47,51–53] dCH_4 was measured from the effluent of each reactor compartment; however, no

significant difference in dCH_4 concentrations were observed (CI = 35 mg L^{-1}, 95% CIs [28, 41]; C2 = 32 mg L^{-1}, 95% CIs [27, 38]; C3 = 35 mg L^{-1}, 95% CIs [30, 41]; C4 = 37 mg L^{-1}, 95% CIs [31, 44]). The impacts of dCH_4 in the effluent of anaerobic bioreactors are discussed in Section 3.5. Similarly, no statistically significant difference was observed in gaseous CH_4 production between reactor compartments (Table 2), or the percentage of CH_4 in the biogas (C1 = 67%, 95% CIs [65, 69]; C2 = 64%, 95% CIs [62, 65]; C3 = 70%, 95% CIs [69, 71]; C4 = 70%, 95% CIs [69, 71]). Mean methane production in the ABR-AFFR normalized to tCOD removal yielded 0.36 L CH_4 per g tCOD, 95% CIs [0.28, 0.45] removed. The mean methane production is higher than pilot-scale UASB-like reactor systems, which range 0.03 to 0.25 L CH_4 per g tCOD removed but is similar to the four-compartment ABR examined by Hahn & Figueroa (2015) (0.24 L CH_4 per g tCOD). Regression analyses between wastewater temperature and total CH_4 production, gaseous CH_4 production, and dCH_4 production indicated statistically significant relationships for each; however, the total CH_4 production (R^2 = 0.458, p < 0.001) and gaseous CH_4 production (R^2 = 0.440, p <0.001) had stronger relationships with temperature than dCH_4 (R^2 = 0.113, p < 0.001). The relatively weak relationship between effluent dCH_4 and wastewater temperature is likely due to the observation that CH_4 production decreases at lower temperatures while CH_4 solubility simultaneously increases, two phenomena that have offsetting impacts.

tCOD is a measurement of the oxygen demand required to oxidize organic material, including carbohydrates, fats, and proteins found in domestic wastewater. Inorganic material, such as sulfate and iron, can also exert an oxygen demand, which is captured in tCOD measurements. The biodegradable fraction of COD (bCOD) is degraded in anaerobic systems via hydrolysis, acidogenesis, acetogenesis, and methanogenesis, ultimately producing CH_4, a bioenergy end product, and CO_2. At STP, theoretical CH_4 production based on 100% conversion of BOD_L (i.e., ultimate BOD or bCOD) is 0.35 L CH_4 per g of BOD_L removed. This value is modified under temperatures and pressures other than STP. CH_4 production in this

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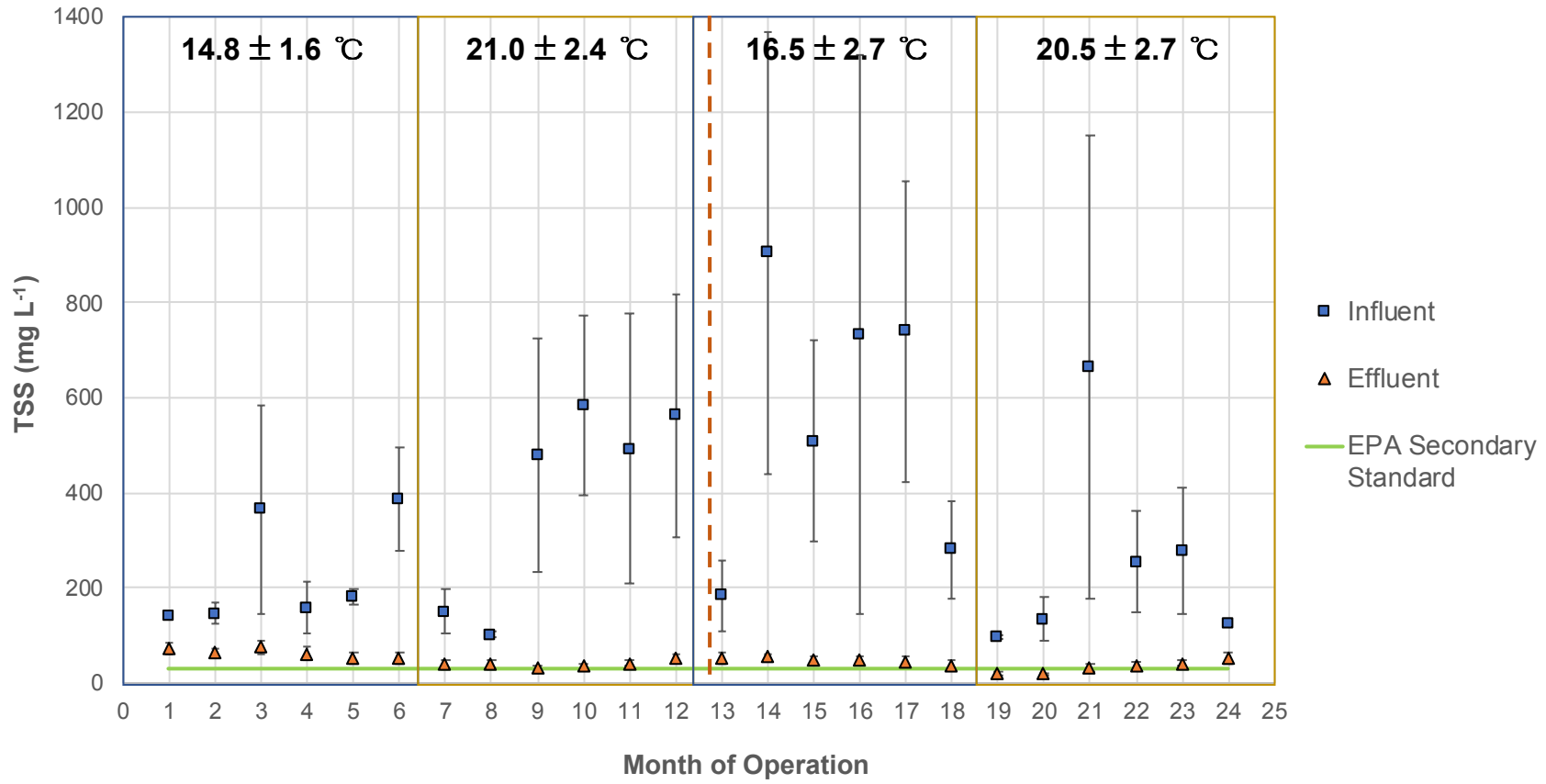

385
386
387
388 **Figure 2.** Monthly mean influent and effluent TSS concentrations with 95% CIs compared to the EPA secondary standard (30 mg L^{-1}). As with
389 tCOD, influent TSS concentrations were highly variable. The vertical dotted red line represents the addition of C4.

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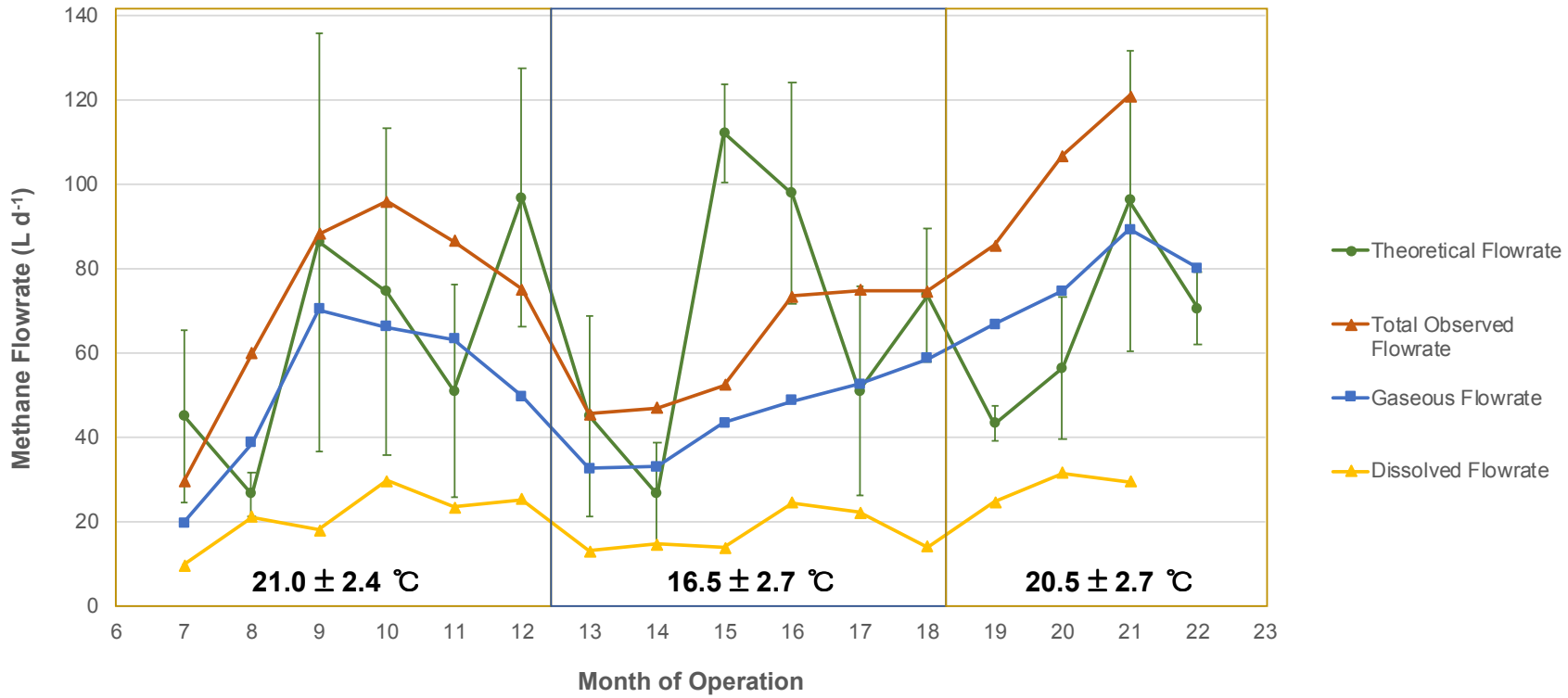

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392 **Figure 3.** Mean monthly CH_4 flowrate (total, gaseous, and dissolved) compared to theoretical maximum CH_4 production calculated from bCOD
393 removal (no assumed losses). Consistent CH_4 measurements were not taken during the first 180 days due to reactor maintenance issues and are
394 not displayed. Error bars represent 95% CIs for theoretical maximum CH_4 production.

study occurred under lower atmospheric pressure (0.83 atm in Colorado) and variable air temperatures (i.e., 12 to 27°C), which increased the range of theoretical methane production to 0.43-0.47 L CH_4 per g BOD_L removed. According to McCarty et al. (2011), approximately 20% of the biodegradable organic energy potential is lost in the wastewater treatment process and should be accounted for in determining CH_4 generation.[3] The 20% loss of organic energy accounts for anaerobic conversion of higher energy organics (e.g., carbohydrates) to CH_4 (8%), microbial cell synthesis (7%), and inefficiencies in wastewater treatment (5%).[3]

For comparison, theoretical CH_4 production by compartment for the ABR-AFFR using two example scenarios – with and without 20% loss of energy potential – were considered and are shown in Table 2. C1 of the ABR-AFFR removed more bCOD relative to other compartments, and theoretically should have produced the most CH_4; however, observed CH_4 production was evenly distributed between reactor compartments. There are two likely explanations for this observation. First, dCH_4 measurements suggest that migration of dissolved-phase CH_4 occurred as wastewater moved longitudinally through reactor compartments. According to Henry's Law, dCH_4 will partition from wastewater to the bioreactor headspace based on temperature and observed gas-phase CH_4 concentrations,[54] not based the reactor compartment in which the CH_4 was generated. Second, because SRT is decoupled from HRT in the ABR-AFFR, hydrolysis of particulate material and settled solids in the sludge bed of each reactor compartment likely produced CH_4 at a rate independent of measured daily biodegradable organic loading, the value from which theoretical CH_4 is calculated.

As shown in Table 2, observed CH_4 production exceeded theoretical CH_4 generation when losses were considered (i.e., 20% of biodegradable organic energy). High variability was observed in calculating theoretical CH_4 generation is due to large fluctuations in influent organic loading (Table 1). When losses of biodegradable organic energy were not considered, the observed CH_4 production was similar to theoretical CH_4 generation. Losses in biodegradable organic energy are inevitable in wastewater treatment systems; however, results of this study

suggest that the impact of these losses may not be immediately observed in theoretical CH_4 generation calculations from bCOD. As mentioned, no sludge was intentionally wasted from the ABR-AFFR during the study period, which created a scenario for degradation of organic material to include decaying cells and settled solids, over time, with associated CH_4 generation independent of measured bCOD removal. More study is required to accurately model CH_4 generation from immediate bCOD removal (i.e., coupled to HRT) and the generation of CH_4 from the degradation of organic material in the sludge bed (i.e., decoupled from HRT).

The distribution of observed CH_4 production in the ABR-AFFR did not follow trends reported in several other bench and pilot-scale ABR studies,[20,55,56] which reported higher methane flowrates and increased percentage CH_4 in the biogas in later reactor compartments relative to earlier reactor compartments. Hahn & Figueroa (2015) reported that each compartment of a pilot-scale, four-compartment ABR produced at least 20% of the total CH_4; however, gaseous CH_4 flowrate increased from approximately 20 L d^{-1} in the first compartment to approximately 50 L d^{-1} in the last compartment. Additionally, the percent CH_4 in the biogas increased from 55% in the first compartment to 81% in the last compartment.[20] The differing methane production pattern between the four-compartment ABR described in Hahn & Figueroa (2015) and the ABR-AFFR in this study may be attributed to the volume of sludge observed in the compartments of each reactor. In Hahn & Figueroa (2015), sludge volume increased in later compartments over time. In this study, the observed sludge volume was usually greatest in C1, but changed substantially throughout the study due to incidents of sludge bed lifting caused by the accumulation of biogas or mechanical issues (e.g., a valve failure and loss of sludge) (Figure S9). Sludge bed lifting incidents were most commonly observed during the first 118 days following reactor start-up under colder wastewater temperatures. The inclusion of a gas-liquid-solid separators on day 118 of the study reduced observed sludge lifting incidents and likely prevented migration of sludge between compartments.

3.3. The ABR-AFFR is a potentially energy-positive process

Observed CH_4 production varied with changes in wastewater temperatures (Figure 3). WWRFs implementing anaerobic systems such as the ABR-AFFR will need to account for such variations in CH_4 production when conducting facility-level energy balances and assessing grid electricity purchase requirements. Electrical energy conversion efficiencies from combined heat and power (CHP) systems can vary between 5% (low-end for steam turbine) to 63% (high-end for fuel cell). The efficiency in conversion to electrical energy can be increased if a portion of the heat is recovered and converted to electrical energy. For example, fuel cells can increase to 80% effective electrical efficiency if heat is recovered.[57] Assuming a conservative 32% electrical energy conversion efficiency (mid-range for steam turbine, gas turbines, and microturbines) and a CH_4 energy content of 0.222 kWh mol^{-1} (lower heating value),[58] the mean electrical energy potential of the gaseous CH_4 produced by the ABR-AFFR was 0.16 kWh m^{-3}, 95% CIs [0.14, 0.18] of wastewater treated. The electrical energy potential increased to 0.40 kWh m^{-3}, 95% CIs [0.35, 0.44] when a high-end 80% conversion was assumed. Results from this study suggest, however, that electrical energy potential will vary significantly with wastewater temperature (R^2 = 0.477, $p < 0.001$). Assuming 32% conversion efficiency, projected electrical energy potential from gaseous CH_4 was 0.08 kWh m^{-3} at a wastewater temperature of 12°C, whereas electrical energy potential increased to 0.28 kWh m^{-3} at 25°C. The typical energy requirement for activated sludge aeration is between 0.3-0.6 kWh m^{-3} of wastewater treated,[3] suggesting that the ABR-AFFR could produce enough CH_4 at higher wastewater temperatures to power the activated sludge process at some WWRFs with lower aeration requirements, especially with efficient CHP technologies. Further, the enhanced COD removal of the ABR-AFFR relative to conventional primary treatment should reduce activated sludge aeration requirements, thereby decreasing energy requirements. A comparison of the net energy balance of a hypothetical WWTF incorporating anaerobic primary treatment using ABRs with secondary activated sludge

to a conventional activated sludge WWTF (i.e., primary clarification with conventional activated sludge), showed that modeled scenarios incorporating ABRs offset up to 71% of WWTF electricity requirements. Further, net energy balances for scenarios modeled with ABRs were 51% to 193% lower than for conventional activated sludge scenarios.[59]

Electrical energy requirements for conventional primary clarification are approximately 0.008 kWh per m^3 of wastewater treated.[60] By comparison, the ABR-AFFR theoretically requires no energy input if placed within the hydraulic gradient of a WWRF. With no need to purposefully waste or recycle sludge, continuous operation of a pumping system is also not required. All produced CH_4 could be routed to a combined heat and power (CHP) system for production of onsite electricity and heat, making the ABR-AFFR a potentially energy-positive process; however, a more complete analysis incorporating follow-on technologies that treat ABR-AFFR effluents to discharge standards (e.g., the U.S. EPA's secondary effluent standard) is required prior to determining the net energy balance of a complete WWRF incorporating the ABR-AFFR.

The electrical energy generating potential of the ABR-AFFR is greater than reported values for several other anaerobic bioreactors treating low temperature domestic wastewater. Estimates from other studies of anaerobic reactor systems range from 0.04 kWh m^{-3} wastewater treated for a two-stage anaerobic fluidized bed-membrane bioreactor (SAF-MBR) to 0.13 kWh m^{-3} for an expanded granular sludge bed reactor (EGSB) (assuming 32% conversion efficiency; however, estimated energy for fluidization of the sludge bed was not reported).[52,61,62] For all anaerobic reactor systems examined, potential energy generation could be enhanced by the recovery of dCH_4 from the effluent. For the ABR-AFFR, electrical energy recovery potential would increase to 0.12 kWh m^{-3} at 12°C and 0.38 kWh m^{-3} at 25°C if 100% of dCH_4 was captured and converted to electrical energy (assuming 32% conversion efficiency).

3.4. Abundance of Euryarchaeota was lower in warm- than cold-weather samples

497 The microbial community structure of the sludge bed in each ABR compartment and the
498 influent wastewater is depicted in Figure 4, which provides a heat map of the most prevalent
499 genera grouped by phyla, wastewater temperature (warm = 23°C, cold = 12°C), and location
500 within the reactor. For the influent wastewater, Proteobacteria, Firmicutes, and Bacteriodetes
501 were the most prevalent phyla in both the warm- and cold-weather samples. The presence of
502 these phyla are consistent with the results of other raw domestic wastewater studies.[63,64]
503 Several genera were observed in high relative abundance in the influent, e.g., *Acinetobacter*,
504 *Acidovorax*, *Arcobacter*, and *Aeromonos*, but decreased within the sludge beds of each
505 compartment regardless of temperature (Figure S10). In C1, the relative abundance of each
506 phylum shown in Figure 4 differed between warm and cold-weather samples. Here relative
507 abundance of Proteobacteria and Firmicutes was greater in the warm weather sample relative
508 to the cold weather sample by approximately 10% and 14% respectively, while that of
509 Bacteroidetes was greater in the cold weather sample by approximately 11%. Euryarchaeota,
510 which consisted solely of methanogens and comprised 7% of the microbial community in C1's
511 warm-weather sample, was approximately 11% in the cold-weather sample. Synergistetes,
512 which can have a symbiotic relationship with Euryarchaeota,[65] was also in lower relative
513 abundance in the warm-weather sample. Similar to C1, in C2 Firmicutes was in greater relative
514 abundance in the warm weather sample, while Bacteroidetes was in greater abundance in the
515 cold weather sample. Euryarchaeota was significantly higher in the cold weather sample than in
516 the warm weather sample in C2 (24% to 9% relative abundance), while Synergistetes was in
517 slightly lower abundance in the cold weather sample. Firmicutes and Bacteroidetes showed
518 similar trends in C3 as in C2. As with C2, Euryarchaeota in C3 was more than threefold greater
519 in relative abundance in the cold weather sample (28%) than in the warm weather sample
520 (12%). In the C3 cold weather sample, Euryarchaeota was the most prevalent phylum.
521 Four methanogen genera were prevalent in the reactor system: *Methanosaeta*,
522 *Methanospirillum*, *Methanobrevibacter*, and an uncultured methanogen from the family

Thermoplasmatales. *Methanosaeta* is an acetoclastic, or acetate-utilizing, methanogen, while both *Methanospirillum* and *Methanobrevibacter* are hydrogenotrophic, or H_2 and CO_2 utilizing methanogens. Figure 4.A. depicts the relative abundance of methanogens by reactor compartment and wastewater temperature, while Figure S11 depicts the estimated absolute abundance of methanogens. Both relative and absolute abundance show the same result: the composition of the methanogen community differed for each reactor compartment and under each temperature condition. In the warm-weather sample, *Methanobrevibacter* was the most prevalent methanogen in C1 (5.4% abundance) but decreased longitudinally through the reactor system (C2 = 2.8% abundance; C3 = 2.7% abundance). In the cold-weather sample, however, *Methanobrevibacter* was the least prevalent methanogen in all reactor compartments. *Methanospirillum* showed an opposite trend, increasing from 0.1% abundance in C1 to 1.8% abundance in C3 in the warm-weather sample, while showing greater prevalence in the cold-weather sample. The uncultured methanogen from the family Thermoplasmatales was in greater relative abundance in each reactor compartment in the cold weather sample relative to the warm weather sample. In the warm-weather sample, *Methanosaeta* increased in abundance longitudinally through the reactor from 1.0% in C1 to 6.5% in C3. Relative abundance of *Methanosaeta* in the cold weather sample of each reactor compartment was four-fold greater relative to the warm weather sample (C1: 1.0% to 5.5%; C2: 4.3% to 19.6%; C3: 6.5% to 23.4%).

The increasing relative abundance of *Methanosaeta* longitudinally through the reactor corresponds with observed acetate concentrations (Table S2). Mean acetate concentrations in the influent wastewater 37 mg L^{-1}, 95% CIs [33, 42] were significantly lower (p-value < 0.05) than C1 46 mg L^{-1}, 95% CIs [41, 53] suggesting that acetogenesis was a dominant function in C1. Acetate concentrations remained consistently high in later reactor compartments with no significant reduction observed (effluent acetate = 47 mg L^{-1}, 95% CIs [41, 52]) suggesting that sufficient acetate was available for acetoclastic methanogenesis to occur. The high effluent

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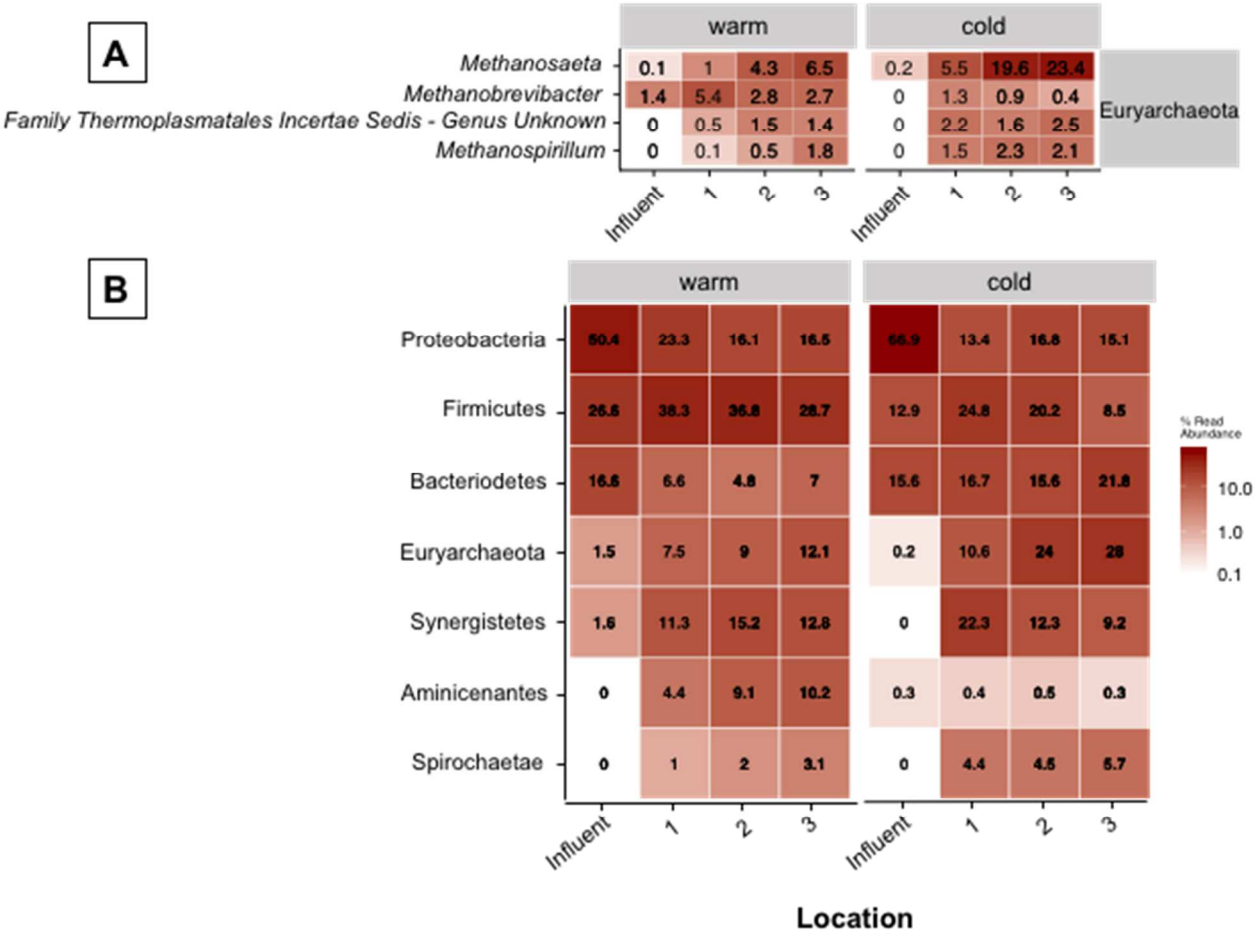


550

551 **Figure 4.** Heat map of the most prevalent microorganisms in the warm- and cold-weather ABR sludge samples. (A) Relative abundance of genera
552 within the phylum Euryarchaeota. (B) The top seven phyla by relative abundance. Organisms are organized within each phylum according to
553 greatest net percent relative abundance observed across all locations and times. The tabulated consortium percentage is relative to the entire
554 consortium. For both (A) and (B), darker red coloration indicates increased relative abundance relative to lighter colors.

555

acetate concentration further suggests that operational modifications can be made to the ABR-AFFR, e.g., the addition of an additional reactor compartment, to enhance acetate removal and increase methane production. Observed total CH_4 production during the eighth month of reactor operation, when the warm weather sample was taken (mean = 60 L d^{-1}), which is higher than observed CH_4 production in the thirteenth month of reactor operation when the cold weather sample was taken (mean = 45 L d^{-1}) despite higher relative abundance of methanogens in the sludge bed. The decrease in CH_4 production under colder weather conditions can likely be attributed to depressed metabolic activity.

Deltaproteobacteria*,* which include sulfate-reducing bacteria (SRB) known to compete with methanogens for resources such as acetate,[66,67] was also in greater relative abundance in each reactor compartment in the cold weather sample relative to the warm weather sample. However, sulfate concentrations, which were relatively high in the influent 73 mg L^{-1}, 95% CIs [66, 80], decreased longitudinally through the reactor system throughout the course of study (effluent sulfate = 8 mg L^{-1}, 95% CIs [6, 11]) (Table S2). The extent of sulfate removal with temperature was statistically significant (i.e., higher levels of sulfate removal were observed at higher wastewater temperatures), again suggesting lower metabolic activity under colder weather conditions.

Several genera identified in the bioreactor, including *Syntrophomonas, Desulfovibrio, Lactivibrio,* and *Aminomonas*, are known to harbor organisms that syntrophically degrade organics and produce hydrogen in partnership with hydrogenotrophic methanogens – a partnership that sustains thermodynamically favorable hydrogen production.[68–71] Relative abundance of these organisms varied with temperature and location; no consistent trend was observed.

Principal coordinate analysis (PCoA) of weighted UniFrac distance matrices was used to examine similarity between microbial communities in each reactor compartment for each temperature condition (Figure 5). As depicted, the influent wastewater community was similar

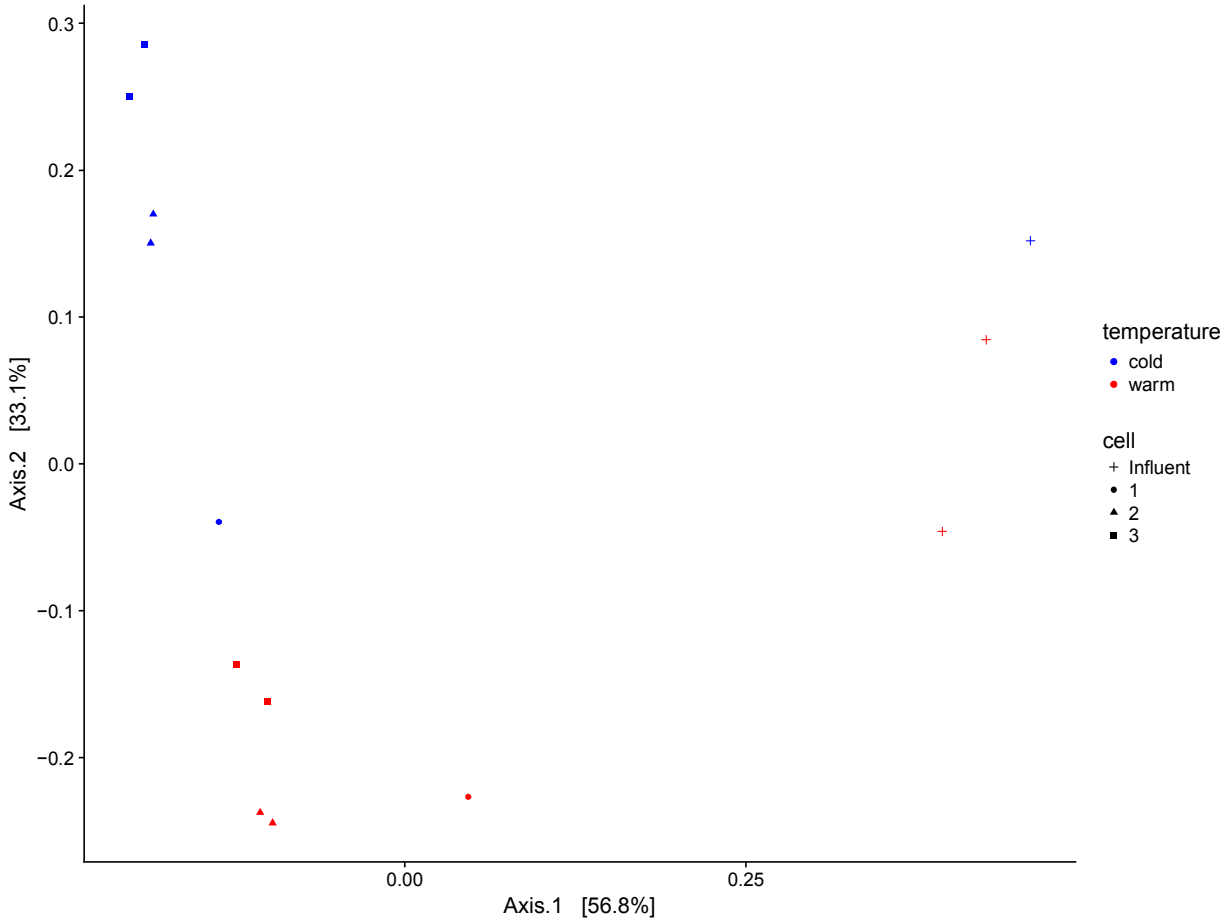
582 under varying temperature conditions, but distinct from the communities in each reactor
583 compartment. The communities in C1, C2, and C3 were relatively similar under warm-weather
584 conditions but were less similar in the cold-weather sampling. The dissimilarity can in part be
585 attributed to the differing relative abundance of methanogens, especially *Methanosaeta*. It is
586 unclear whether the change in temperature or the maturity of sludge (the warm weather sample
587 was taken on day 231 of the study while the cold weather sample was taken on day 395 of the
588 study) facilitated a change in *Methanosaeta* abundance. Study of *mcrA* gene abundance in the
589 pilot-scale four-compartment ABR described in Hahn et al. (2015) identified an increase from
590 the first to second year of operations.[72]

591 These results suggest that a long-term time course study may be required to more fully
592 understand the development of methanogenic community structure over time. Further study is
593 also required to determine whether acetoclastic methanogens (i.e., *Methanosaeta*) will continue
594 to dominate the methanogenic community over time and under varying temperatures.
595 Temperature-driven impacts on syntrophic degradation and hydrogen production must also be
596 characterized. Only after such long-term studies can community structure be tied to reactor
597 performance and accurate models of anaerobic multiple-compartment sludge-bed processes be
598 constructed.

599

600 **3.5. Future work: treatment of anaerobic effluent**

601 Table 3 provides the effluent concentrations of several contaminants that require further
602 treatment prior to discharge into the natural environment. Mean concentrations of contaminants
603 observed in studies of other anaerobic reactors from Delgado Vela et al. (2016) are provided for
604 comparison.[26] Observed concentrations of ammonium, phosphate, sulfide, dCH_4, and sCOD in
605 the ABR-AFFR effluent were within the range of other anaerobic studies. The ABR-AFFR
606 removed little influent nitrogen or phosphorus over the course of the study, an expected result
607 for anaerobic systems. If released to the environment, nitrogen and phosphorus can have



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609 **Figure 5.** PCoA of weighted UniFrac distance matrices for the sludge beds in the ABR portion of the
610 ABR-AFFR and the influent wastewater. Samples are colored by temperature and locations for each point
611 identified. The first two coordinates explain a total of 89.9% of the variance.
612

613

614 substantial eutrophication impacts on downstream ecosystems. Nitrogen and phosphorus
615 removal is currently achieved in aerobic wastewater treatment using several biological or
616 chemical approaches, such as nitrification/denitrification, which converts ammonia to N_2 gas, or
617 chemical phosphorus precipitation using aluminum or iron salts. Partial nitritation coupled with
618 anammox, which requires limited aeration and potentially little or no supplemental carbon
619 addition beyond residual carbon observed in the effluent of the ABR-AFFR, is a promising
620 alternative for anaerobic bioreactors that typically have effluents with low carbon-to-nitrogen
621 ratios.[73,74]

Additionally, effluent dCH_4 represents not just a loss of potential energy but is a potent greenhouse gas, approximately 25 times more impactful than CO_2. A lifecycle analysis conducted by Smith et al. (2014) examining the global warming impacts of an AnMBR found that approximately 75% of global warming impacts were attributed to dCH_4 in the reactor effluent.[16] Several approaches for dCH_4 removal and/or capture from anaerobic effluents have been proposed; however no economically or energetically viable solution has been identified to date.[16] Studies that strip and capture dCH_4 for energy generation, such as membrane degasification, currently use more energy than can theoretically be recovered.[52,75–77] Several biogenic dCH_4 removal solutions have been studied but have not been demonstrated at full scale. Examples include the downflow hanging sponge, which was observed to remove 57 to 88% of dCH_4,[78–80] and a bench-scale microbial fuel cell (MFC) treating synthetic anaerobic effluent (80% methane saturation; dCH_4 concentration not reported) at 20°C that was able remove up to 85% dCH_4 via an aerobic microbial consortium. The MFC relied on a methanotroph cathode biofilm that produced intermediate metabolites, e.g. formate and acetate, which served as substrates for *Geobacter*, a common exoelectrogen, in the anode biofilm, which, when converted to electrical energy, was enough to power the MFC itself.[81] Bioreactors coupling methane-oxidizing microbial communities (i.e., methanotrophs) and microalgae may be a means of removing dCH_4, ammonia, and excess carbon; however, additional treatment for phosphorus would still be required, as would additional energy to process biomass if a follow-on beneficial use is desired, such as biofuel production.[82]

Unfortunately, no single treatment technology is currently able to address all contaminants found in anaerobic effluents. The challenge is to develop a treatment train that removes residual contaminants to discharge levels while simultaneously using less energy than is generated by CH_4 production. In the near-term, the ABR-AFFR, or similar multiple-compartment anaerobic reactor configurations, could replace conventional primary treatment, though global warming impacts from fugitive CH_4 emissions need further study. Future

modifications to the ABR-AFFR to improve system performance include enhancing biomass-substrate contact by increasing the HRT. After further research and optimization, the ABR-AFFR could serve as primary treatment for follow-on partial nitritation coupled with anammox (PN/A) for residual nitrogen and carbon removal, as observed effluent carbon concentrations and carbon-to-nitrogen ratios are within the range of several previous PN/A studies[73,74]; however, follow-on phosphorus removal would still be required prior to discharge. Beyond CH_4 production for heat and energy generation, the physical footprint of the proposed treatment facility would be reduced due to minimal sludge production.

4. Conclusions

Results of this study suggest that the ABR-AFFR is a viable alternative to conventional primary treatment. Under low wastewater temperatures, the reactor removed organics and suspended solids beyond conventional primary treatment while generating stoichiometric quantities of methane gas. This study also suggests that degradation of particulate material and settled solids in the anaerobic sludge bed over time will produce methane at a rate independent of the calculated theoretical maximum from biodegradable COD removal. The ABR-AFFR is an energy-positive process, which, depending on the CHP technology used, can produce enough electricity to completely power some downstream activated sludge processes. Examination of the methanogenic community structure shows a higher relative abundance, especially of *Methanosaeta*, under cold wastewater temperatures; however more study is needed to create accurate models that tie system performance to abundance of methanogens. While further study is required, the ABR-AFFR could replace conventional primary treatment in an anaerobic-aerobic treatment paradigm near-term.

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677

678 **Conflict of Interest**

679 No conflicts of interest.

680

681 **References**

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Table 1. Mean concentrations and 95% confidence intervals of several key performance parameters for the influent wastewater and each reactor compartment broken into four-time periods based on temperature. Upper and lower 95% CIs are depicted in brackets following mean values.

Period	Period 1	Period 2	Period 3	Period 4
Temperature	Days 0-180	Days 181-360	Days 361-540	Days 541-720
(°C)	**14.88** [14.25, 15.52]	**20.97** [20.06, 21.88]	**16.51** [15.48, 17.55]	**20.50** [19.45, 21.54]

Variable (mg L^{-1})	**Time Period**	**Influent**	**C1**	**C2**	**C3**	**C4**
tCOD	Period 1	548 [464, 631]	405 [366, 444]	365 [344, 386]	351 [329, 372]	N/A
	Period 2	613 [500, 725]	367 [353, 382]	321 [308, 337]	256 [240, 272]	N/A
	Period 3	630 [512, 748]	359 [301, 417]	312 [299, 324]	285 [274, 297]	259 [246, 272]
	Period 4	406 [355, 457]	235 [224, 247]	202 [193, 212]	175 [164, 186]	158 [147, 169]
	Entire Study	549 [499, 599]	341 [391, 363]	300 [286, 314]	267 [252, 281]	203 [187, 220]
pCOD	Period 1	343 [261, 425]	178 [155, 200]	129 [114, 145]	133 [121, 144]	N/A
	Period 2	395 [295, 495]	150 [138, 161]	123 [109, 136]	83 [75, 92]	N/A
	Period 3	398 [288, 508]	168 [113, 223]	121 [112, 129]	106 [99, 112]	90 [83, 96]
	Period 4	224 [184, 263]	111 [100, 123]	86 [78, 95]	68 [58, 78]	59 [48, 70]
	Entire Study	340 [295, 385]	151 [135, 167]	115 [108, 121]	98 [91, 104]	73 [65, 81]
sCOD	Period 1	204 [194, 215]	227 [207, 248]	236 [216, 256]	218 [202, 234]	N/A
	Period 2	217 [201, 234]	218 [207, 228]	200 [189, 210]	173 [162, 183]	N/A
	Period 3	232 [217, 248]	191 [184, 198]	191 [181, 201]	180 [173, 186]	169 [161, 177]
	Period 4	182 [168, 196]	124 [119, 129]	116 [109, 124]	106 [99, 114]	100 [93, 106]
	Entire Study	209 [201, 217]	190 [180, 200]	185 [175, 196]	169 [160, 179]	131 [120, 142]
BOD_5	Period 1[a]	222 [215, 230]	148 [134, 162]	168 [120, 217]	181 [140, 223]	N/A
	Period 2	258 [191, 324]	179 [160, 198]	166 [145, 188]	128 [112, 144]	N/A
	Period 3	287 [171. 403]	131 [122, 141]	122 [110, 134]	111 [101, 121]	91 [82, 101]
	Period 4	165 [126, 205]	89 [75, 103]	70 [60, 80]	61 [51, 71]	51 [42, 59]
	Entire Study	239 [193, 285]	137 [122, 152]	126 [109, 142]	107 [93, 120]	70 [58, 81]
TSS	Period 1	243 [142, 345]	85 [75, 95]	68 [62, 74]	65 [59, 70]	N/A
	Period 2	371 [204, 538]	73 [68, 78]	58 [50, 65]	39 [36, 43]	N/A
	Period 3	598 [324, 873]	93 [84, 103]	71 [67, 75]	59 [56, 62]	49 [46, 53]
	Period 4	254 [92, 416]	84 [75, 93]	52 [48, 57]	37 [33, 41]	31 [27, 36]
	Entire Study	368 [271, 465]	84 [79, 88]	62 [59, 65]	50 [47, 53]	39 [35, 43]

[a] Only two valid data points were gathered for BOD_5 between days 0 and 180 of the study.

Table 2. Comparison of mean theoretical maximum methane generation from the removal of tCOD and observed methane generation (gaseous and dissolved) within each compartment of the ABR-AFFR over the course of study. Mean tCOD and bCOD removal (g d^{-1}) by compartment over the course of study are also displayed. Upper and lower 95% CIs are depicted in brackets following mean values.

Variable	C1	C2	C3	C4	Total System
tCOD removal (g d^{-1})	151 [114, 188]	30 [19, 41]	25 [21, 29]	14 [12, 16]	212 [176, 247]
bCOD removal (g d^{-1})	98 [74, 122]	19 [12, 26]	16 [13, 19]	9 [8, 10]	137 [114, 160]
Theoretical maximum CH_4 production (L d^{-1}) (without 20% energy loss)	56 [44, 67]	12 [8, 16]	8 [7, 9]	4 [4, 5]	76 [67, 90]
Theoretical maximum CH_4 production (L d^{-1}) (with 20% energy loss)	46 [36, 55]	10 [6, 13]	7 [6, 8]	4 [3, 4]	62 [51, 72]
Observed total CH_4 production (L d^{-1})	22 [19, 25]	20 [17, 23]	26 [23, 29]	14 [12, 16]	75 [66, 85]

Table 3. Effluent characteristics from the ABR-AFFR compared to reported effluent characteristics in Delgado Vega et al. (2015) for other anaerobic domestic wastewater treatment systems. Values are expressed as COD equivalents except for ammonia and phosphate. Mean values with one standard deviation are provided for comparison.

		Other anaerobic systems	
Contaminant	**ABR-AFFR**	**Mean**	**Range**
Ammonium (mg N L^{-1})	44 ± 8	36 ± 17	9 – 67
Phosphate (mg P L^{-1})	5 ± 1	6 ± 7	1 – 20
Sulfide (mg COD L^{-1})	17 ± 4	62 ± 83	3 – 184
dCH_4 (mg COD L^{-1})	142 ± 58	91 ± 50	42 – 204
sCOD (mg COD L^{-1})	166 ± 51	99 ± 46	46 – 201

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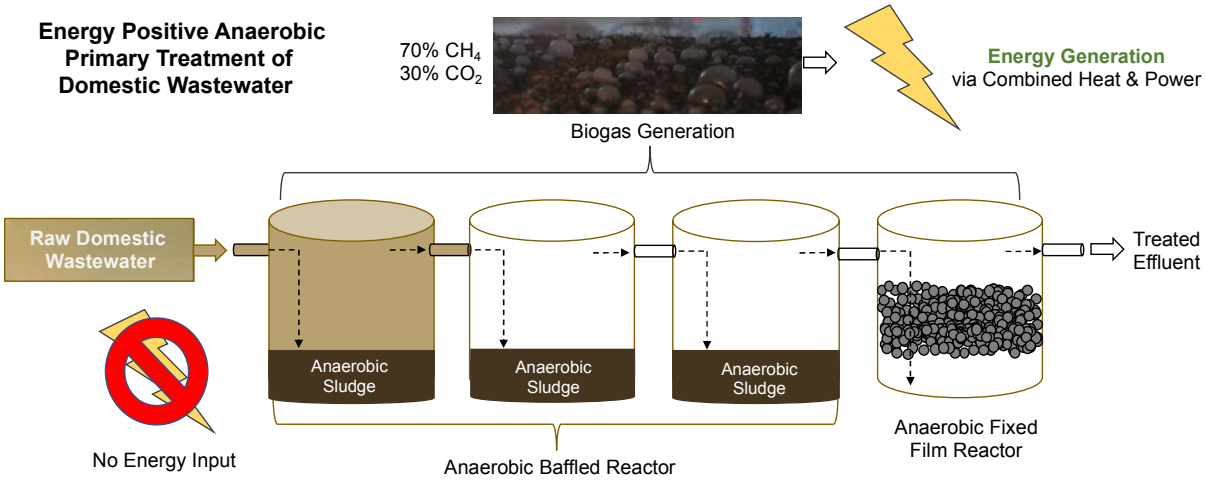

TOC Text: Anaerobic hybrid reactor system for the generation of methane-rich biogas and energy. An energy-positive alternative to conventional primary treatment of raw domestic wastewater.