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1 Anaerobic digester bioaugmentation influences quasi steady state 2 performance and microbial community

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9 **Abstract**

10 Nine anaerobic digesters, each seeded with biomass from a different source, were
11 operated identically and their quasi steady state function was compared. Subsequently,
12 digesters were bioaugmented with a methanogenic culture previously shown to increase
13 specific methanogenic activity. Before bioaugmentation, different seed biomass resulted
14 in different quasi steady state function, with digesters clustering into three groups
15 distinguished by methane (CH₄) production. Digesters with similar functional
16 performance contained similar archaeal communities based on clustering of Illumina
17 sequence data of the V4-V5 region of the 16S rRNA gene. High CH₄ production
18 correlated with neutral pH and high *Methanosarcina* abundance, whereas low CH₄
19 production correlated to low pH as well as high *Methanobacterium* and *DHVEG 6* family
20 abundance. After bioaugmentation, CH₄ production from the high CH₄-producing
21 digesters transiently increased by 11±3% relative to non-bioaugmented controls (p <0.05,
22 n=3), whereas no functional changes were observed for medium and low CH₄ producing
23 digesters that all had pH higher than 6.7. The CH₄ production increase after
24 bioaugmentation was correlated to increased relative abundance of *Methanosaeta* and

Methaospirillum originating from the bioaugment culture. In conclusion, different anaerobic digester seed biomass can result in different quasi steady state CH₄ production, SCOD removal, pH and effluent VFA concentration in the timeframe studied. The bioaugmentation employed can result in a period of increased methane production. Future research should address extending the period of increased CH₄ production by employing pH and VFA control concomitant with bioaugmentation, developing improved bioaugments, or employing a membrane bioreactor to retain the bioaugment.

Keywords

Digester efficiency; *Methanobacterium*, *Methanosaeta*, *Methanosarcina*, *Methanospirillum*; Next generation sequencing

1. Introduction

Adding beneficial microorganisms to anaerobic digesters (i.e. bioaugmentation) has been shown to increase degradation rates of specific organics and reduce upset digester recovery time (Guiot et al. 2000; Hajji et al. 2000; Guiot et al. 2002; Cirne et al. 2006; Schauer-Gimenez et al. 2010; Tale et al. 2011). Anaerobic digester bioaugmentation may be more widely applicable if a culture was enriched to target a key, ubiquitous intermediate in existing anaerobic processes. The existing anaerobic processes typically treat readily degradable substrates, such as food production and dairy wastewater. When treating readily degradable substrates, one ubiquitous and potentially problematic intermediate is propionate (Schauer-Gimenez et al. 2010; Tale et al. 2015). Propionate accumulation is often an indicator of process imbalance in anaerobic digesters which can be caused by organic overload, nutrient deficiency, toxicant exposure or other factors (McCarty & Smith 1986; Speece et al. 2006; Ma et al. 2009). The subsequent recovery time of upset digesters depends on the abundance of microorganisms that can biotransform an intermediate (i.e. propionate) or inhibitory compound into less harmful products (Herrero & Stuckey 2014).

Intermediates such as propionate can be biotransformed by a specific consortium of synergistic microorganisms (McCarty & Smith 1986; Speece et al. 2006). Bioconversion of propionate to acetate and hydrogen (H_2) is thermodynamically favorable only when the partial pressure of the generated H_2 remains below 10^{-4} atm. Thus, degradation of propionate requires a synergistic relationship between H_2 producing and H_2 consuming microorganisms to maintain low H_2 concentrations (McCarty & Smith 1986). It was shown previous that adding cultures enriched to consume H_2 or propionate to anaerobic

58 digesters can reduce recovery time after organic overload or toxicant exposure (Schauer-
59 Gimenez et al. 2010; Tale et al. 2011). Tale et al., (2015) employed aerotolerant
60 propionate consuming, methanogenic cultures for bioaugmentation. The aerotolerant
61 culture may be commercially beneficial since it can be easily handled and dried in
62 ambient air (Zitomer 2013). In addition, micro-aerated cultures outperformed a strictly
63 anaerobic culture when used for bioaugmentation, resulting in higher specific
64 methanogenic activity (SMA) against propionate and shorter recovery time after organic
65 overload (Tale et al. 2015).

66 Despite some success, anaerobic digester bioaugmentation is still at a nascent stage. A
67 comprehensive review published by Herrero & Stuckey (2014) reported either transient
68 improvement in performance or a complete failure of bioaugmentation to improve
69 anaerobic digestion, but no instances of long-term improvement. Therefore, it is still
70 questionable whether or not adding a limited quantity of externally cultured
71 microorganisms can increase long-term methane production (Herrero & Stuckey 2014).
72 Microbial community analysis has often been employed to understand the relationship
73 between microorganisms and digester function (Venkiteshwaran et al. 2016). However
74 changes in digester microbial communities after bioaugmentation have not been
75 extensively studied.

76 In this work, bioaugmentation using a methanogenic, aerotolerant propionate enrichment
77 culture was investigated as a possible method to improve methane production after quasi-
78 steady operation for anaerobic digesters fed a readily degradable waste. Nine groups of
79 anaerobic digesters were seeded with different starting biomass to obtain different

microbial communities and digesters were then bioaugmented and monitored for changes in function and microbial community using high throughput Illumina sequencing.

2. Material and Methods

2.1 Anaerobic digesters

Biomass samples were obtained from nine full-scale municipal anaerobic digesters in different US states; Delaware (Set-D), Florida (Set-B), Michigan (Set-I), Mississippi (Set-F), New Jersey (Set-H), Ohio (Set-E), South Dakota (Set-G), West Virginia (Set-C) and Wyoming (Set-A) to obtain a variety of microbial communities. With the exception of a thermophilic digester in Michigan, all other biomass samples were from mesophilic digesters. All digesters were continuous stirred-tank reactors stabilizing municipal wastewater sludge with solids retention times between 15 and 30 days. The Florida digester was also fed food waste as a co-digestate.

Each biomass sample was used to seed two sub-sets (bioaugmented and non-bioaugmented) of triplicate, 160-mL lab-scale digesters with 50-mL working volume and biomass concentration of 8g volatile solids (VS)/L. Digesters were operated at a 10-day HRT and fed synthetic wastewater (non fat-dry milk) and basal nutrient media at an organic loading rate (OLR) of 3 g COD/L-day. The digesters were operated for 60 days until they attained quasi-steady state operation during which the digester daily biogas production coefficient of variation was less than 20%. The bioaugmented digesters received a daily dose of the enrichment culture from day 60 to 70. The daily dose was equivalent to 1 % of the digester biomass total adenosine triphosphate (tATP) mass (this was equivalent to 1.5-2 % of the digester VS mass). Simultaneously, the non-bioaugmented digesters received a COD equivalent dose of inactivated (autoclaved)

enrichment culture. Functional parameters including effluent soluble COD (SCOD) and volatile fatty acids (VFA) concentrations as well as biogas CH₄ concentration were monitored between days 60 and 80. Digester biomass samples were collected on day 71 for amplicon sequencing.

Cumulative methane volume produced was calculated by summing the daily methane production volumes (ml CH₄/day) from days 60 to 80. Biomass production rate was calculated as the product of VSS concentration (mg VSS/L) and effluent flow (L/day). Observed biomass yield was calculated as the quotient of biomass production rate and COD added to the digester per day (mg COD/day).

2.2 Enrichment culture for bioaugmentation

A moderately aerated, propionate-utilizing, mixed methanogenic enrichment culture developed by Tale et al. (2011) was employed for bioaugmentation. The original seed biomass for the enrichment culture was from an upflow anaerobic sludge blanket (UASB) reactor treating brewery wastewater that exhibited a high methanogenic activity (Tale et al. 2011). When previously used for bioaugmentation, this aerotolerant mixed microbial culture reduced the recovery time of transiently organically overloaded digesters (Tale et al. 2015). The enrichment was maintained in two completely mixed vessels with a volume of four liters at 35 °C at a 15 day HRT and fed 0.17 g propionate/L-day as calcium propionate with basal nutrient media. Immediately after feeding, ambient air was added directly into the headspace of the vessel at a volume equivalent to 25 mg O₂/L-day or 10% of the OLR to provide a micro-aerated environment.

2.3 Basal nutrient media

Basal nutrient media, as described by Speece (2008), contained the following [mg/L]:
 NH_4Cl [400]; $\text{MgSO}_4 \cdot 6\text{H}_2\text{O}$ [250]; KCl [400]; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ [120]; $(\text{NH}_4)_2\text{HPO}_4$ [80];
 $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ [55]; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ [10]; KI [10]; the trace metal salts $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, NH_4VO_3 ,
 $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{Zn}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O}$, $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, H_3BO_3 , $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$,
 $\text{NaWO}_4 \cdot 2\text{H}_2\text{O}$, and Na_2SeO_3 [each at 0.5]; yeast extract [100]; NaHCO_3 [6000]; and
 resazurin [1].

2.4 Analytical methods

tATP concentration was analyzed using a commercial kit following the manufacturer
 instructions (BacTiter-Glo, Promega, Madison WI, USA). The inactive culture added to
 non-bioaugmented digesters was placed in an autoclave (Model 3870E, Tuttnauer Co.,
 Hauppauge, NY, USA) at 15 Psi and 121°C for 30 minutes prior to augmentation
 experiments. Daily biogas volume produced was measured by inserting a needle with a
 wetted glass barrel syringe through serum bottle septa. SCOD was measured by filtering
 the sample through a 0.45 μm pore size membrane syringe filter and determining the
 filtrate COD by standard methods (APHA et al., 1998). Biogas methane concentration
 was measured by gas chromatography (GC System 7890A, Agilent Technologies, Irving,
 TX, USA) using a thermal conductivity detector. VFA concentrations were measured by
 gas chromatography (GC System 7890A, Agilent Technologies, Irving, TX, USA) using
 a flame ionization detector. The VS, TSS and VSS analyses were performed by standard
 methods (APHA et al., 1998). Statistical analysis such as two-sample Student's t-test
 with unequal variance and Pearson's coefficient were calculated on Microsoft Excel 2010
 (Version 14.3.2) using built in functions.

2.5 Microbial community analysis

148 DNA was extracted from all bioaugmented and non-bioaugmented digesters (including
149 all replicates, n=54) on Day 71 using the PowerSoil™ DNA Isolation Sample Kit
150 (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's
151 instructions. The biomass samples were subjected to bead beating on a vortex (Model
152 58816-121, VWR International, Radnor, PA, USA) for 10 minutes. Primer pair 515-532U
153 and 909-928U was used (Wang & Qian 2009) including their respective linkers, to
154 amplify the V4-V5 region of the 16S rRNA gene over 30 amplification cycles at an
155 annealing temperature of 65 °C. The primer pairs target both archaeal and bacterial 16S
156 rRNA genes. An index sequence was added in the second PCR of 12 cycles, and the
157 resulting products were purified and loaded onto the Illumina MiSeq cartridge for
158 sequencing of paired 300 bp reads following manufacturer's instructions (v3 chemistry).
159 Sequencing and library preparation were performed at the Genotoul Lifescience Network
160 Genome and Transcriptome Core Facility in Toulouse, France (get.genotoul.fr). A
161 modified version of the standard operation procedure for MiSeq data (Kozich et al. 2013)
162 in Mothur version 1.35.0 (Schloss et al. 2009) was used to assemble forward and reverse
163 sequences and preclustering at 4 differences in nucleotides over the length of the
164 amplicon. Uchime was used for chimera checking (Edgar et al. 2011). Sequences that
165 appear less than three times in the entire data set were removed. Alignment of the 16S
166 rRNA sequences was done using SILVA SSURef NR99, release 119, as provided by
167 Mothur (Schloss et al. 2009). The same database was used in Mothur's classify.seqs()
168 command to assign taxonomic affiliation using a cutoff value of 80%. Custom R scripts
169 were used to perform dual hierarchical clustering (using R command hclust and heatmap)
170 and nonmetric multidimensional scaling (nMDS) (using the default Bray-Curtis index),

of anaerobic community sequence data gathered from Illumina sequencing (Carey et al. 2016; McNamara & Krzmarzick 2013).

3. Results and Discussion

All nine digester sets reach quasi-steady state based on less than 20% coefficient of variation in daily biogas production by day 60 when bioaugmentation was initiated (Figure 1 and S1). Effluent VFA concentrations for all digesters were higher than 2 g/L and methane production was below 70% of the theoretical value assuming all COD was converted to methane. Therefore, residual COD was available and could possibly be removed if system changes occurred. This challenged condition was desired so that bioaugmentation effects could be observed. During the dosage period, autoclaved propionate enrichment culture was added to the non-bioaugmented digesters, whereas live propionate enrichment culture was added to the bioaugmented digesters. Adding inactivated enrichment culture did not result in a statistical change in biogas production rate (Figure 1 and S1). This was expected since the daily COD fed to the digesters from the augments was low and was less than 8% of the total synthetic wastewater COD fed.

3.1 Non-bioaugmented digester function

Although operated identically, the digester sets did not achieve identical operational values. For example, the quasi steady state methane production rates ranged from 0.3 to 0.8 L CH₄ per L of digester per day (L CH₄/L_R-day) (Table 1). Each non-bioaugmented digester set was classified into one of three distinct groups based on statistically similar methane production rate, SCOD removal, pH and effluent acetate concentration (p value <0.05, n=3) (Table 1). Group 1 (G1) (sets A, B and C) contained the best performing digesters with the highest methane production rate, highest SCOD removal rate, highest

pH and lowest effluent acetate concentration; Group 2 (G2) (sets D, E, F and G) showed intermediary performance and Group 3 (G3) (sets H and I) contained the poorest performing digesters with the lowest methane production rate, lowest SCOD removal rate, lowest pH and highest effluent acetate concentration (Figure 2). The functional variation among digester sets can be attributed to the differences in the microbial communities the seed biomass used for each set. Future research is warranted to elucidate quantitative relationships between microbial community descriptors and digester function so that the suitability of various seed biomass samples can be estimated. This would be helpful to identify the most suitable biomass for a given process startup or re-seeding application.

3.2 Non-bioaugmented digester archaeal community

A total of 32 archaeal OTUs, based on 97% similarity, were identified among all the digester samples analyzed. The relative abundance of archaeal sequences varied from 1 to 4% for G1, G2 and 0.1 to 1% for G3 digesters, respectively. Eight archaeal OTUs represented more than 99% of the archaeal abundance in all non-bioaugmented digesters (Figure S2). These eight OTUs were most similar to the genera *Methanofollis*, *Methanosarcina*, *Methanospirillum*, *Methanosaeta*, *Methanobacterium*, *Candidatus Methanomethylophilus* and two unclassified genera in the order *WCHA1-57* and the family *Deep Sea Hydrothermal Vent Grp 6 (DHVEG 6)*, based on the SILVA SSURef NR99 v119 reference database from Mothur (Schloss et al. 2009) (Figure S2).

The nine non-bioaugmented digester sets clustered in the same three groups that were identified by functional data (95% confidence interval) (Figure 3). Digesters with similar functional performance contained similar archaeal communities. Non-bioaugmented G1

digester communities were distinguished from G2 and G3 communities by high relative abundance of *Methanosarcina*, which ranged from 60 to 95% of archaea in G1 digesters (Figure S2). *Methanobacterium* dominated non-bioaugmented G2 digesters, with a relative abundance that ranged from 80 to 99% of the archaeal diversity. The G3 digesters were distinguished by high relative abundance of the *DHVEG 6* family, with *Methanobacterium* also observed in high relative abundance (Figure S2). *Methanosarcina* and *Methanosaeta* are the only two methanogenic genera known to consume acetate (Liu & Whitman 2008). High relative abundance of *Methanosarcina* in the high-performing G1 digesters correlated to higher methane production and lower effluent acetate concentration. This is ostensibly because the *Methanosarcina* maximum specific substrate utilization rate is higher than that of *Methanosaeta* (Liu & Whitman 2008). Therefore, the presence of *Methanosarcina* in digesters with moderate to high VFA concentrations, such as the ones of this study, may be beneficial to maintain more rapid bioconversion of acetate to methane. Compared to G1 digesters, *Methanosarcina* relative abundance in the lesser performing, non-bioaugmented G2 digesters was lower, ranging from 0.5 to 18%. In addition, *Methanosarcina* was undetectable in the poorest performing, non-bioaugmented G3 digesters which had the highest acid concentrations and lowest pH. The poor performing G3 digesters were distinguished from the better performing G1 and G2 digesters by the low archaeal sequence abundance ($\leq 1\%$ of the total sequence) and further by high relative abundance of *DHVEG 6*, which ranged from 60-90% in G3 digesters (Figure S2). *DHVEG 6* have been observed in acidic environments, marine environments, terrestrial soils, hydrothermal sediments, deep sea methane seep

sediments, rice paddy soil and saline lakes (Casamayor et al. 2013; Nunoura et al. 2010; Nunoura et al. 2011; Hugoni et al. 2013; Grosskopf et al. 1998). Given that *DHVEG6* microorganisms have been observed in extreme environmental conditions that typically are not present in a healthy functioning digester, high abundance of *DHVEG 6* in anaerobic digesters ostensibly indicates an upset digester with low pH and low biogas production such as the G3 digesters.

3.3 Non-bioaugmented digester bacterial community

Approximately 1300 bacterial OTUs were identified based on 97% similarity among all the biomass samples analyzed in this study. The 29 OTUs having the highest relative abundance among all the digesters and the enrichment culture were considered for bacterial community analysis. These 29 OTUs contributed 70-85% of the total bacterial sequences in the non-bioaugmented digesters.

The nine digesters bacterial communities formed two clusters, with G1 and G2 non-bioaugmented digesters forming one bacterial cluster and G3 non-bioaugmented digesters in the second bacterial cluster (Figure 4). Bacterial communities in all nine digester sets were dominated by OTUs most similar to fermenters belonging to the phyla *Firmicutes*, *Bacteroidetes* and *Synergistetes* (Figure S3). The most common bacterial OTUs that were observed in the digesters were the genera *Bacteroides*, *Peptostreptococcus*, *Pyramidobacter*, *Aminobacterium*, *Atopobium* and *RC9 Gutgroup*. Non-bioaugmented G1 and G2 digesters were distinguished from G3 digesters by the higher abundance of the genera *Porphyromonas*, *Petrimonas* and unclassified *FamilyXI*, whereas non-bioaugmented G3 digesters were dominated by OTUs most similar to RC9 Gut Group

microorganisms which contributed more than 60% of the total bacterial relative abundance (Figure S3).

3.4 Enrichment culture

The enrichment culture functional parameters were stable between days 60 to 80, with 99% SCOD removal, no detectable VFAs, pH of 7.5 ± 0.2 , $60 \pm 3\%$ biogas methane content, methane yield of 330 ± 16 mLCH₄/g-COD removed and observed biomass yield of 0.08 ± 0.01 gVSS/gCOD.

More than 700 bacterial OTUs were identified in the enrichment culture based on 97% similarity. The 25 bacterial OTUs with the highest relative abundance represented approximately 80% of total bacterial sequences and are shown in Figure S4. The two most abundant bacterial taxa were most similar to an unclassified genus within *Spirochaetaceae* (30% of the total bacterial relative abundance) and *Thermovirga* within *Synergistaceae* (12% of the total bacterial relative abundance) (Figure S4). *Thermovirga* is currently represented by a single member species *Thermovirgaliensis*, which is a moderately thermophilic, amino acid degrading fermentative bacterium (Dahle 2006). Some members of the *Spirochaetaceae* family such as *Treponema* species, are reported to be abundant in iron-reducing consortia that were used by othersto bioaugmentanaerobic digesters (Baek et al. 2015). Bacteria related to *Spirochaetes* may be beneficial for the overall process since some of them have been suggested as syntrophic acetate-oxidizing bacteria (Hattori 2008; Lee et al. 2013; Lee et al. 2015). Iron-reducing bacteria (IRB) are commonly observed in anaerobic systems and can utilize acetate, H₂, ethanol and other complex substrates and ferric iron as an electron acceptor (Kim et al. 2014). They are also known to form syntrophic associations and, via interspecies electron transfer,

285 transfer electron directly to their methanogenic partner, which can facilitate CO₂ reduction
286 to CH₄ (Stams & Plugge 2009; Rotaru et al. 2014). Addition of an IRB consortium has
287 been shown to increase the methane production rate in anaerobic digesters (Baek et al.
288 2015).

289 Given that the enrichment culture was fed calcium propionate, it was expected that
290 bacteria associated with syntrophic propionate degradation would be abundant. Of the
291 known bacterial genera with members capable of degrading propionate, *Desulfobulbus*,
292 *Smithella*, and *Syntrophobacter* were observed with a combined relative abundance of
293 9% (De Bok et al. 2001; De Bok et al. 2004; Stams & Plugge 2009), with *Desulfobulbus*
294 contributing 7% (Figure S4).

295 15 archaeal OTUs were detected in the enrichment culture based on 97% similarity, of
296 which 6 OTUs contributed more than 99% of the total archaeal sequences (Figure S5).
297 Archaeal sequences constituted approximately 5-6% of the total sequences detected in the
298 enrichment culture. The archaeal community was dominated by sequences most similar
299 to *Methanosaeta*, constituting 65% of the total archaeal sequences (Figure S5).
300 *Methanosarcina* constituted only 1.2% of the total archaeal sequences in the enrichment
301 culture (Figure S5). Unlike *Methanosarcina*, *Methanosaeta* have a high substrate affinity
302 and a lower maximum specific substrate utilization rate. Hence, *Methanosaeta* usually
303 dominate over *Methanosarcina* in cultures such as the enrichment culture in this study
304 having acetate concentrations lower than 500 mg/L (Liu & Whitman 2008).

305 Apart from acetoclastic methanogens, the enrichment culture archaeal composition
306 consisted of OTUs most similar to known hydrogenotrophic methanogens including
307 *Methanospirillum*, *Methanobacterium*, *Methanolinea* and an unclassified genus in the

order WCHA1-57 (Figure S5) (Liu & Whitman 2008). Conversion of propionate to methane only becomes thermodynamically favorable through H₂ utilization. Therefore, the significant presence of hydrogenotrophic methanogens contributing 30-35% of the total archaeal sequences could have positive functional results. The presence of microorganisms classified in the genera *Methanospirillum*, *Methanobacterium*, *Methanolinea* has previously been reported to play an important role in propionate utilization during digester recovery after organic overload (Tale et al. 2011; Tale et al. 2015; Schauer-Gimenez et al. 2010).

The archaeal order WCHA1-57 was observed at a significant relative abundance (12%) in the enrichment culture. Although many WCHA1-57-related 16S rRNA gene sequences have been identified in anaerobic digesters (Chouari et al. 2005; Rivière et al. 2009; Schauer-Gimenez et al. 2010), no reports were found regarding their role in propionate oxidation or methane production. In some anaerobic digesters treating municipal sewage sludge, the WCHA1-57 phylotype population represented one of the predominant archaeal components, with relative abundance >70% in archaeal clone libraries (Chouari et al. 2005; Rivière et al. 2009). These observations indicate that WCHA1-57 archaea represent a potentially important group in anaerobic digesters. Chouari et al. (2005) reported the enrichment of WCHA1-57 phylotypes in cultures fed formate or H₂/CO₂. This indicates that WCHA1-57 plays a role in reducing hydrogen concentration and, therefore, aiding in conversion of propionate to methane.

Both bacterial and archaeal enrichment culture communities were distinct from those of the nine digester sets. The nMDS scaling plots based on the top eight archaeal (Figure 3) and 29 bacterial (Figure 4) OTUs, selected based on their relative abundance and

prevalence among all the biomass samples, shows distinct clustering of the enrichment culture separate from the G1, G2 and G3 non-bioaugmented and bioaugmented digesters.

3.5 Bioaugmentation, digester function and microbial community changes

Cumulative methane produced by both non-bioaugmented and bioaugmented digesters between days 60 and 80 were calculated and compared to observe any difference in performance (Figure 5). Only the three G1 digester sets A, B and C showed a statistically significant increase ($P < 0.05$, $n = 3$) in average methane production of $11 \pm 3\%$ after bioaugmentation, with increases of $9 \pm 1\%$, $12 \pm 2\%$ and $13 \pm 2\%$, respectively, compared to non-bioaugmented controls (Figure 5).

The increased methane production in G1 digesters after bioaugmentation was not sustained. The period of increased methane production averaged 9 days, and was 7, 11 and 9 days for sets A, B and C, respectively (Figure 1, S1A and S1B). Also, the increased methane production did not occur immediately after bioaugmentation was initiated. The lag between the dosage period start (Day 60) and the first day of increased methane production for set A, B and C bioaugmented digesters averaged 10 days, and was 12, 8 and 9 days, respectively (Figure 1, S1A and S1B).

The archaeal communities in the bioaugmented digesters were grouped into three distinct clusters based on archaeal sequences (Figure 3). The archaeal community of the bioaugmented digesters belonging to functional groups G2a and G3a, which did not improve after bioaugmentation, did not significantly change after bioaugmentation (Figure 3). In contrast, however, the G1 bioaugmented digesters showed a statistical improvement in methane production and the archaeal community changed significantly

353 after bioaugmentation (Figure 3). After bioaugmentation, the archaeal community of G1
354 digesters became more similar to that of the enrichment culture (G4).
355 The community structure shift in G1 digesters after bioaugmentation was primarily
356 caused by the increased abundance of two archaeal genera: *Methanosaeta* and
357 *Methanospirillum* (Figure 6), which are in high abundance in the enrichment culture
358 (Figure S5). In contrast, the relative abundance values of *Methanosaeta* and
359 *Methanospirillum* were very low (<1%) in G2 and below detection in the G3
360 bioaugmented digesters, respectively. The bacterial community compositions of the
361 bioaugmented digesters did not show any significant shift after bioaugmentation (Figure
362 4). Sequences related to the two most abundant bacterial genera observed in the
363 enrichment culture, unclassified *Spirochaeta* and *Thermovirga*, which were not detected
364 in the non-bioaugmented digesters, were detected in all the bioaugmented digesters, but
365 their relative abundance remained below 1% after bioaugmentation.
366 The resulting increase in methane production observed in G1 digesters from
367 bioaugmentation was associated with a shift in the archaeal community structure.
368 Increased relative abundance of the genera *Methanosaeta* and *Methanospirillum* was
369 observed in digesters with improvement in the methane production rate. The relative
370 abundance of *Methanosaeta* and *Methanospirillum* increased from below detection in the
371 non-bioaugmented digesters to 10-40% and 10-30%, respectively, in the bioaugmented
372 G1 digesters (Figure 6). However, it is important to note that the methane production
373 rate increase lasted only 7 to 11 days in the bioaugmented G1 digesters. This could be
374 due to washout of *Methanosaeta* and *Methanospirillum* once bioaugmentation ceased. It
375 may be possible to improve the methane production further by increasing the dose of the

enrichment biomass or extending the duration of the dosage period. In addition microbial carriers, such as alginate beads or granular activated carbon, or membrane bioreactors could be used to retain the bioaugment in the bioreactor.

The enrichment culture used in this study was produced at a pH of 7.5 with no detectable VFAs present. The most abundant methanogens in the enrichment culture, *Methanosaeta* and *Methanospirillum* (i.e., *M. hungatii*), are sensitive to low pH and high acid or propionate concentrations (Liu & Whitman 2008; Barredo & Evison 1991). It is likely that the methane production increase in G1 digesters after bioaugmentation was due to the relatively low VFA concentration and neutral pH, which was conducive for the activity of *Methanosaeta* and *Methanospirillum* added via the bioaugment. In contrast, the low pH, high VFA concentration environment in G2 and G3 digesters may have inhibited the enrichment culture microorganisms. Therefore, the environment the enrichment culture is being added into must be carefully considered and additional steps such as acclimating the augment culture or increasing the digester pH before bioaugmentation may be required to increase methane production and COD removal by bioaugmentation.

4. Conclusion

Different anaerobic digester seed biomass can result in significantly different quasi steady state functional parameters, including methane production rate, SCOD removal, pH and effluent VFA concentration. Therefore, care should be taken to select seed biomass with high activity for digester startup or re-seeding.

Identically operated digesters that contain different archaeal communities can exhibit different functional characteristics during quasi steady state operation. When operating

under challenging conditions, digesters with high methane production rates can be distinguished by high *Methanosarcina* relative abundance. The presence of *Methanosarcina* in digesters with moderate to high VFA concentrations is beneficial to maintain more rapid bioconversion of acetate to methane. In contrast, digesters with low methane production can be distinguished by high abundance of *Methanobacterium* and *DHVEG 6* family organisms. Since *DHVEG6* microorganisms have been found in extreme environments, including deep-sea hydrothermal vents, their high abundance in anaerobic digesters may indicate past or current digester upset (i.e., high VFA concentration and low methane production).

Bioaugmentation with a methanogenic, propionate degrading enrichment culture resulted in a significant increase in methane production when digester pH was neutral or greater. However, methane production did not change after bioaugmenting digesters that had pH values less than neutral. Therefore, when predicting bioaugmentation outcomes, the environment into which an augment culture is added must be carefully considered as well as the composition of the bioaugment itself. Steps such as increasing low digester pH before bioaugmentation may be necessary to improve digester function.

The methane production increase after bioaugmentation was correlated with increased relative abundance of *Methanosaeta* and *Methaospirillum* that were in the bioaugment culture employed. However, the methane production rate increase was only temporary. More research is warranted to develop sustained, steady state improvements via bioaugmentation or bioaugmentation combined with pH adjustment for challenged digesters.

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Table 1: Performance parameters of digester groups.

Group->	G1	G2	G3
Digester Sets->	A, B and C	D, E, F and G	H and I
Methane production rate (L-CH ₄ /L _R -day at 35°C, 1 atm)	0.77±0.12	0.6±0.04	0.34±0.02
SCOD removal (%)	67±10	55±4	30±4
pH	7.2±0.06	6.6±0.05	6.3±0.0
Acetate (g/L)	2.4±0.6	5.4±1	7.3±2
Propionate (g/L)	2.1±1	4±1	4.3±3

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Figure 1: Typical quasi steady state biogas production.

Daily biogas production rate observed from non-bioaugmented and bioaugmented digesters of (A) Set-A, (B) Set D and (C) Set H, respectively. The error bars represent standard deviation among triplicate digesters; some error bars are small and not visible. The dosage period represents the 10-day period during which inactivated and active enrichment cultures were added to non-bioaugmented and bioaugmented digesters, respectively. The Period of Increased Activity (period when bioaugmented digester methane production was statistically greater than that of non-bioaugmented digesters (p value <0.05 , $n=3$)). PIA was not observed for Set-D (B) and Set-H (C) digester systems.

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Figure 2: Digester group functional parameters.

(A) Average methane production rate (L-CH₄/L_R-day), (B) average percent COD removal, (C) average effluent acetate and propionate concentration, (D) and average effluent pH. Error bars represent standard deviation among triplicates. Based on average functional performance, the nine digester sets were divided into three statistically distinct groups ($p < 0.05$, $n = 3$): digester groups G1 (Sets A, B and C), G2 (Sets D, E, F and G) and G3 (Sets H and I).

Figure 3: Archaeal sequence nMDS plot.

Non-bioaugmented digesters cluster 1, G1 (○); non-bioaugmented digesters cluster 2, G2 (●); non-bioaugmented digesters cluster 3, G3 (◐); bioaugmented digesters cluster 1, G1a (△); bioaugmented digesters cluster 2, G2a (▲); bioaugmented digesters cluster 3, G3a (◑); and enrichment culture G4 (▨). The ellipses represent 95% confidence interval for each cluster. Eight archaeal OTU's, identified based on 97% similarity and representing >99% of the total archaeal sequences in all digesters, including the enrichment culture, were employed for nMDS analysis.

Figure 4: Bacterial sequence nMDS plot.

Non-bioaugmented digesters cluster 1, G1 and G2 (●); non-bioaugmented digesters cluster 2, G3 (○); enrichment culture G4 (▣); bioaugmented digesters cluster 1, G1a and G2a (▲); and bioaugmented digester cluster 2, G3a (△). The ellipses represent 95% confidence interval for each cluster. The 29 OTUs detected in all digesters with the highest relative abundance, including the enrichment culture, were employed for nMDS analysis.

Figure 5: Cumulative CH_4 produced between day 60-80. Error bars represent standard deviation among triplicates. Bioaugmented digesters of Sets A, B and C showed statistically higher cumulative CH_4 production ($p < 0.05$, $n=3$).

Figure 6: Dual hierarchical clustering of the top seven archaeal OTUs observed in the enrichment culture and G1 digesters.

These seven OTU's, based on 97% similarity, represent >99% of the total archaeal abundance in the enrichment culture and G1 digesters. The gradient scale ranges from 0 to 100% relative abundance. Sample names x1, x2 and x3 represent the enrichment culture. The sample names for digesters are denoted as follows: for example "nB1" and bB1 – the prefix "n" and "b" represents "non-bioaugmented" and "bioaugmented", respectively, the middle letter "B" represents Set-B and the suffix "1" represent the replicate number. The enrichment culture is dominated by *Methanosaeta* (OTU 3), *Methanospirillum* (OTU 2) and *WCHA1-57* (OTU 1). The non-bioaugmented digesters are dominated by *Methanosarcina* (OTU 7), followed by *Methanobacterium* (OTU 5). The bioaugmented digester of Set-A, B and C showed a significant abundance of *Methanosaeta* (OTU 3) and *Methanospirillum* (OTU 2) as compared to the non-bioaugmented digesters.

Highlights

- Digesters with similar functional performance have similar methanogenic community structure and vice versa
- Bioaugmentation can improve methane production by shifting the digester's methanogenic community structure
- Washout of microorganisms, low pH and high acid concentrations may decrease the effectiveness of bioaugmentation

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