11-1-2016

Anaerobic Digester Bioaugmentation Influences Quasi Steady State Performance and Microbial Community

Kaushik Venkiteshwaran
Marquette University, kaushik.venkiteshwaran@marquette.edu

K. Milferstedt
INRA

J. Hamelin
Laboratoire de Biotechnologie de l'Environnement

Daniel Zitomer
Marquette University, daniel.zitomer@marquette.edu

Anaerobic Digester Bioaugmentation Influences Quasi Steady State Performance and Microbial Community

K. Venkiteshwaran
Department of Civil, Construction and Environmental Engineering, Marquette University, Milwaukee, WI
K. Milferstedt
INRA, UR0050, Laboratoire de Biotechnologie de l'Environnement, Narbonne F-11100, France
J. Hamelin
, UR0050, Laboratoire de Biotechnologie de l'Environnement, Narbonne F-11100, France
D. H. Zitomer
Department of Civil, Construction and Environmental Engineering, Marquette University, Milwaukee, WI

Water Research, Vol 104 (November 2016): pg. 128-136. DOI.

Abstract: Nine anaerobic digesters, each seeded with biomass from a different source, were operated identically and their quasi steady state function was compared. Subsequently, digesters were bioaugmented with a methanogenic culture previously shown to increase specific methanogenic activity. Before bioaugmentation, different seed biomass resulted in different quasi steady state function, with digesters clustering into three groups distinguished by methane (CH_4) production. Digesters with similar functional performance contained similar archaeal communities based on clustering of Illumina sequence data of the V4—V5 region of the 16S rRNA gene. High CH_4 production correlated with neutral pH and high *Methanosarcina* abundance, whereas low CH_4 production correlated to low pH as well as high *Methanobacterium and DHVEG 6* family abundance. After bioaugmentation, CH_4 production from the high CH_4 producing digesters transiently increased by 11 ± 3% relative to non-bioaugmented controls ($p < 0.05$, $n = 3$), whereas no functional changes were observed for medium and low CH_4 producing digesters that all had pH higher than 6.7. The CH_4 production increase after 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 CH_4 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 CH_4 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, Methanospririllum,* 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, Guiot et al., 2002, Hajji et al., 2000, Cirne et al., 2006, Schauer Gimenez et al., 2010 and 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 and 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 and Smith, 1986, Speece et al., 2006 and 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 and Stuckey, 2014).

Intermediates such as propionate can be biotransformed by a specific consortium of synergistic microorganisms (McCarty and Smith, 1986 and 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 and Smith, 1986). It was shown previous that adding cultures enriched to consume H_2 or propionate to anaerobic digesters can reduce recovery time after organic overload or toxicant exposure (Schauer Gimenez et al., 2010 and Tale et al., 2011). Tale et al. (2015) employed aerotolerant propionate consuming, methanogenic cultures for bioaugmentation. The aerotolerant culture may be commercially beneficial since it can be easily handled and dried in ambient air (Zitomer, 2013). In addition, micro-aerated cultures outperformed a strictly anaerobic culture when used for bioaugmentation, resulting in higher specific methanogenic activity (SMA) against propionate and shorter recovery time after organic overload (Tale et al., 2015).

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

Water Research, Vol 104 (November 2016): pg. 128-136. DOI.

In this work, bioaugmentation using a methanogenic, aerotolerant propionate enrichment culture was investigated as a possible method to improve methane production after quasi-steady operation for anaerobic digesters fed a readily degradable waste. Nine groups of 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 8 g 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 60–70. 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 CH_4 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 CH_4/day) from days 60–80. 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 4 L 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 O_2/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]; $MgSO_4 \cdot 6H_2O$ [250]; KCl [400]; $CaCl_2 \cdot 2H_2O$ [120]; $(NH_4)_2 \cdot HPO_4$ [80]; $FeCl_3 \cdot 6H_2O$ [55]; $CoCl_2 \cdot 6H_2O$ [10]; KI [10]; the trace metal salts $MnCl_2 \cdot 4H_2O$, NH_4VO_3, $CuCl_2 \cdot 2H_2O$, $Zn(C_2H_3O_2)_2 \cdot 2H_2O$, $AlCl_3 \cdot 6H_2O$, $Na_2MoO_4 \cdot 2H_2O$, H_3BO_3, $NiCl_2 \cdot 6H_2O$, $NaWO_4 \cdot 2H_2O$, 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 min 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

DNA was extracted from all bioaugmented and non-bioaugmented digesters (including all replicates, n = 54) on Day 71 using the PowerSoil™ DNA Isolation Sample Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The biomass samples were subjected to bead beating on a vortex (Model 58816-121, VWR International, Radnor, PA, USA) for 10 min. Primer pair 515–532U and 909–928U was used (Wang and Qian, 2009) including their respective linkers, to amplify the V4—V5 region of the 16S rRNA gene over 30 amplification cycles at an annealing temperature of 65 °C. The primer pairs target both archaeal and bacterial 16S rRNA genes. An index sequence was added in the second PCR of 12 cycles, and the resulting products were purified and loaded onto the Illumina MiSeq cartridge for sequencing of paired 300 bp reads following manufacturer's instructions (v3 chemistry). Sequencing and library preparation were performed at the Genotoul Lifescience

Network Genome and Transcriptome Core Facility in Toulouse, France (get.genotoul.fr). A modified version of the standard operation procedure for MiSeq data (Kozich et al., 2013) in Mothur version 1.35.0 (Schloss et al., 2009) was used to assemble forward and reverse sequences and preclustering at 4 differences in nucleotides over the length of the amplicon. Uchime was used for chimera checking (Edgar et al., 2011). Sequences that appear less than three times in the entire data set were removed. Alignment of the 16S rRNA sequences was done using SILVA SSURef NR99, release 119, as provided by Mothur (Schloss et al., 2009). The same database was used in Mothur's classify. seqs () command to assign taxonomic affiliation using a cutoff value of 80%. Custom R scripts were used to perform dual hierarchical clustering (using R command hclust and heatmap) and nonmetric multidimensional scaling (nMDS) (using the default Bray-Curtis index), of anaerobic community sequence data gathered from Illumina sequencing (Carey et al., 2016 and McNamara and 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 (Fig. 1 and Fig. 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 (Fig. 1 and Fig. 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.

Water Research, Vol 104 (November 2016): pg. 128-136. DOI.

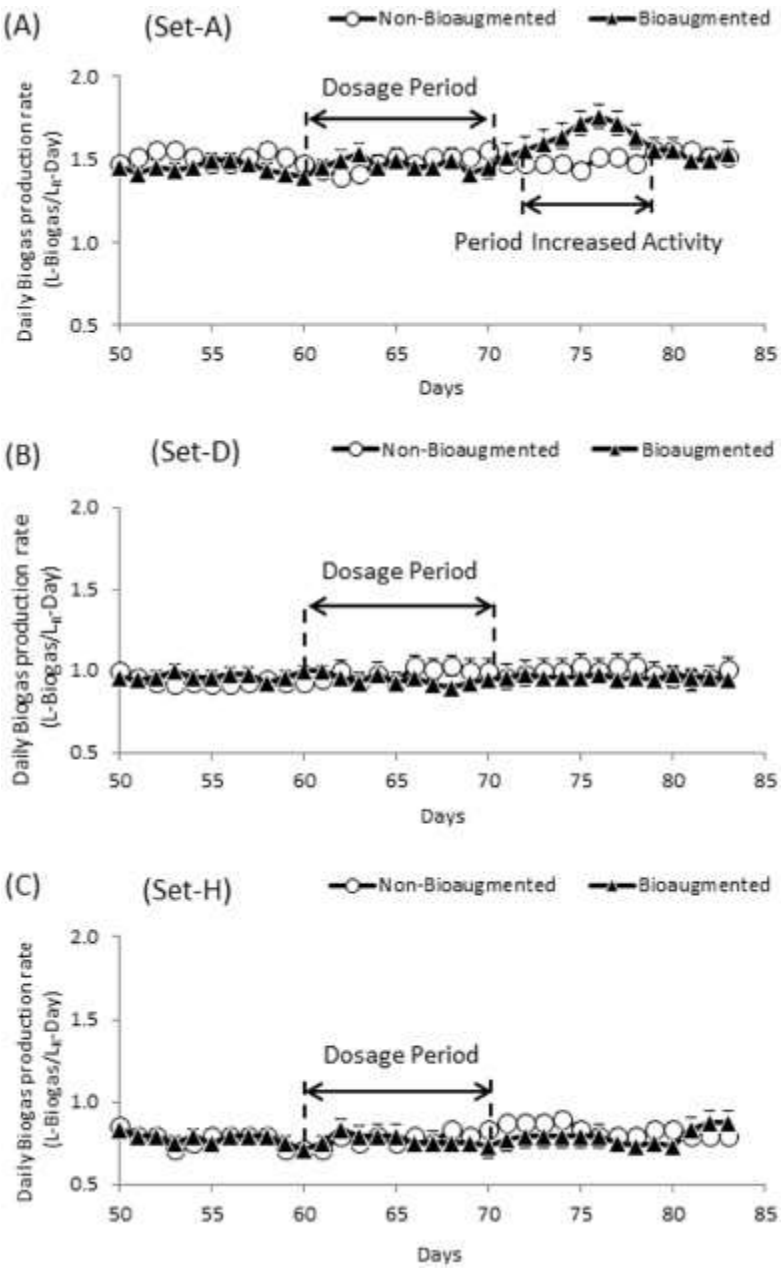


Fig. 1. Typical quasi steady state biogas production. Daily biogas production rate observed from non-bioaugmented and bioaugmented digesters of **(A)** Set-A, **(B)** Set D and **(C)** Set H, respectively. The error bars represent standard deviation among triplicate digesters; some error bars are small 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.

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 CH_4 per L of digester per day (L CH_4/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 (Fig. 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.

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

Water Research, Vol 104 (November 2016): pg. 128-136. DOI.

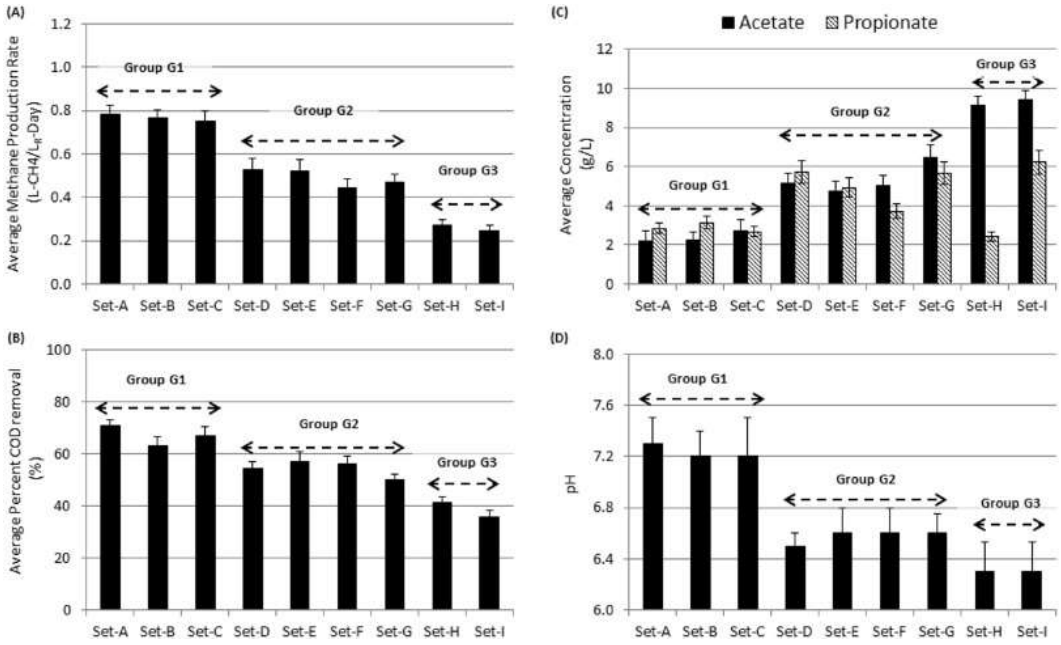


Fig. 2. Digester group functional parameters. **(A)** Average methane production rate (L-CH_4/L_R-day), **(B)** average percent COD removal, **(C)** average effluent acetate and propionate concentration, **(D)** and average effluent pH. Error bars represent standard deviation among triplicates. Based 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).

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 0.1–1% for G3 digesters, respectively. Eight archaeal OTUs represented more than 99% of the archaeal abundance in all non-bioaugmented digesters (Fig. 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) (Fig. S2).

The nine non-bioaugmented digester sets clustered in the same three groups that were identified by functional data (95% confidence interval) (Fig. 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 (Fig. 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 (Fig. S2).

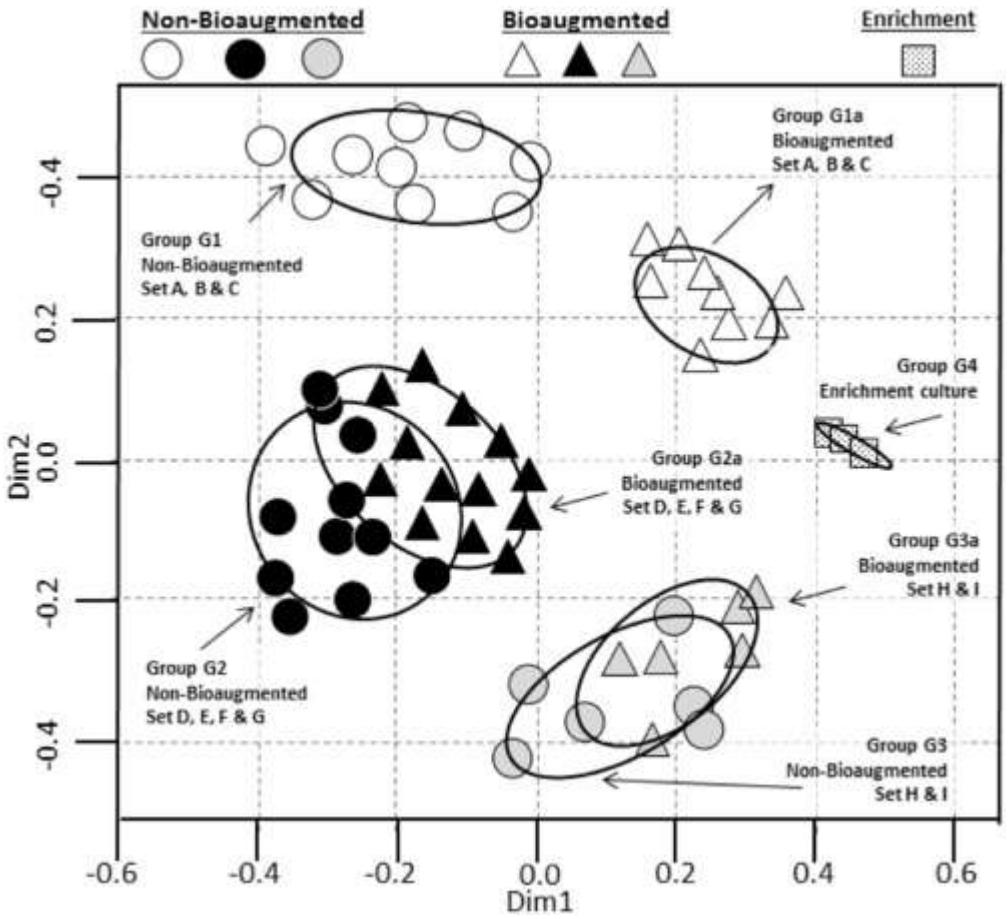


Fig. 3. Archaeal sequence nMDS plot. Non-bioaugmented digesters cluster 1, G1 (○); non-bioaugmented digesters cluster 2, G2 (●); non-bioaugmented digesters cluster 3, G3 (■); bioaugmented digesters cluster 1, G1a (■); bioaugmented digesters cluster 2, G2a (▲); bioaugmented digesters cluster 3, G3a (■); 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.

Methanosarcina and *Methanosaeta* are the only two methanogenic genera known to consume acetate (Liu and 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 and 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 (≤1% of the total sequence) and further by high relative abundance of *DHVEG 6*, which ranged from 60 to 90% in G3 digesters (Fig. 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., 2012, Hugoni et al., 2013 and Grosskopf et al., 1998). Given that *DHVEG 6* 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 (Fig. 4). Bacterial communities in all nine digester sets were dominated by OTUs most similar to fermenters belonging to the phyla *Firmicutes*, *Bacteroidetes* and *Synergistetes* (Fig. 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 (Fig. S3).

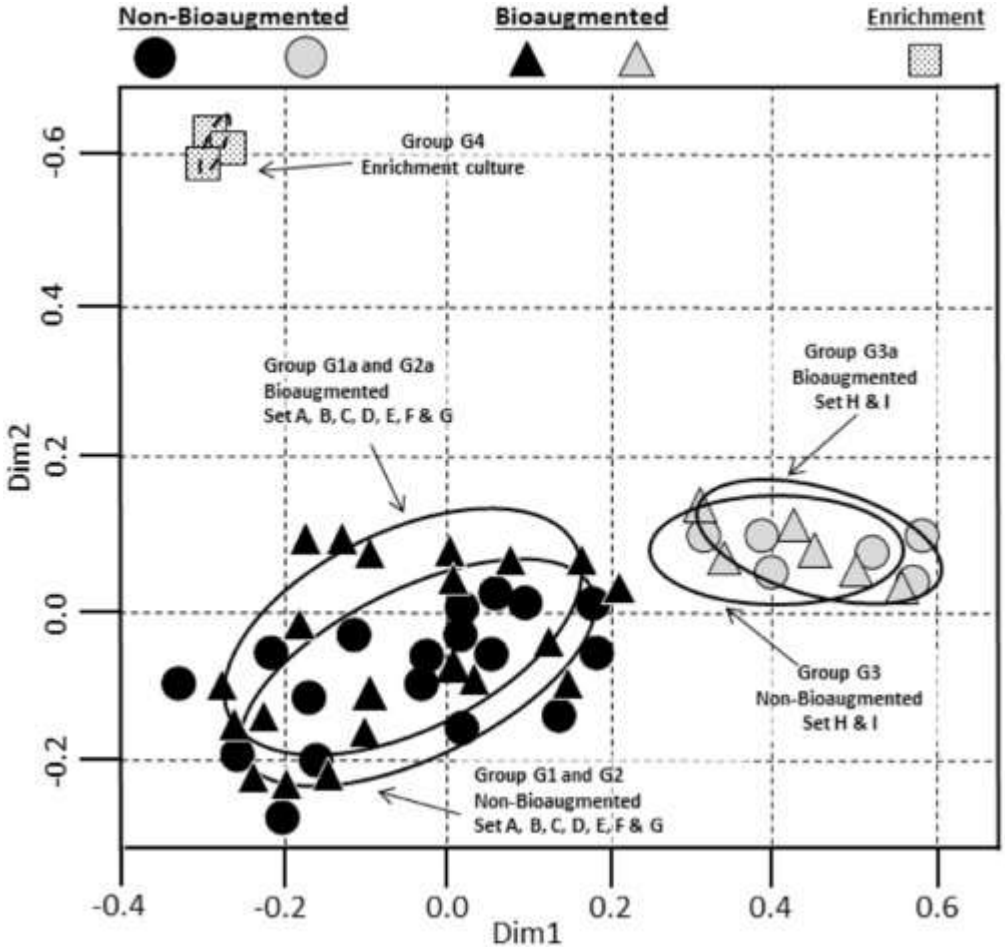


Fig. 4. Bacterial sequence nMDS plot. Non-bioaugmented digesters cluster 1, G1 and G2 (●); non-bioaugmented digesters cluster 2, G3 (■); enrichment culture G4 (■); bioaugmented digesters cluster 1, G1a and G2a (▲); and bioaugmented digester

Water Research, Vol 104 (November 2016): pg. 128-136. DOI.

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.

3.4. Enrichment culture

The enrichment culture functional parameters were stable between days 60–80, with 99% SCOD removal, no detectable VFAs, pH of 7.5 ± 0.2, 60 ± 3% biogas methane content, methane yield of 330 ± 16 $mLCH_4$/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 Fig. 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) (Fig. S4). *Thermovirga* is currently represented by a single member species *Thermovirgalienii*, which is a moderately thermophillic, 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., 2016). 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 and Lee et al., 2015). Iron-reducing bacteria (IRB) are commonly observed in anaerobic systems and can utilize acetate, H_2, 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, transfer electron directly to their methanogenic partner, which can facilitate CO_2 reduction to CH_4 (Stams and Plugge, 2009 and Rotaru et al., 2014). Addition of an IRB consortium has been shown to increase the methane production rate in anaerobic digesters (Baek et al., 2016).

Given that the enrichment culture was fed calcium propionate, it was expected that bacteria associated with syntrophic propionate

degradation would be abundant. Of the known bacterial genera with members capable of degrading propionate, *Desufobulbus, Smithella*, and *Syntrophobacter* were observed with a combined relative abundance of 9% (De Bok et al., 2001, De Bok et al., 2004 and Stams and Plugge, 2009), with *Desulfobulbus* contributing 7% (Fig. S4).

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

Apart from acetoclastic methanogens, the enrichment culture archaeal composition consisted of OTUs most similar to known hydrogenotrophic methanogens including *Methanospirillum, Methanobacterium, Methanolinea* and an unclassified genus in the order *WCHA1-57* (Fig. S5) (Liu and Whitman, 2008). Conversion of propionate to methane only becomes thermodynamically favorable through H_2 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 and 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 and 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 and 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 H_2/CO_2. 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 (Fig. 3) and 29 bacterial (Fig. 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 (Fig. 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 ± 3% after bioaugmentation, with increases of 9 ± 1%, 12 ± 2% and 13 ± 2%, respectively, compared to non-bioaugmented controls (Fig. 5).

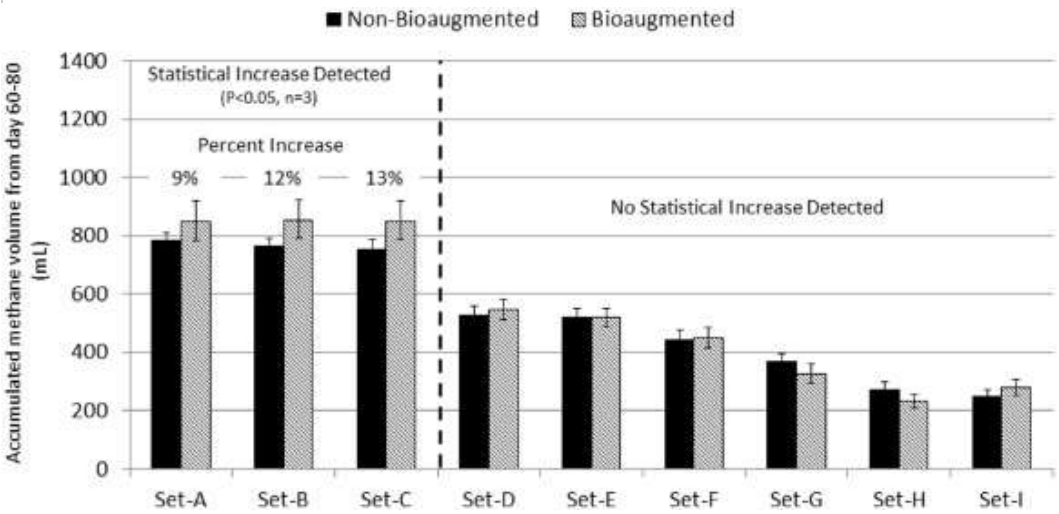


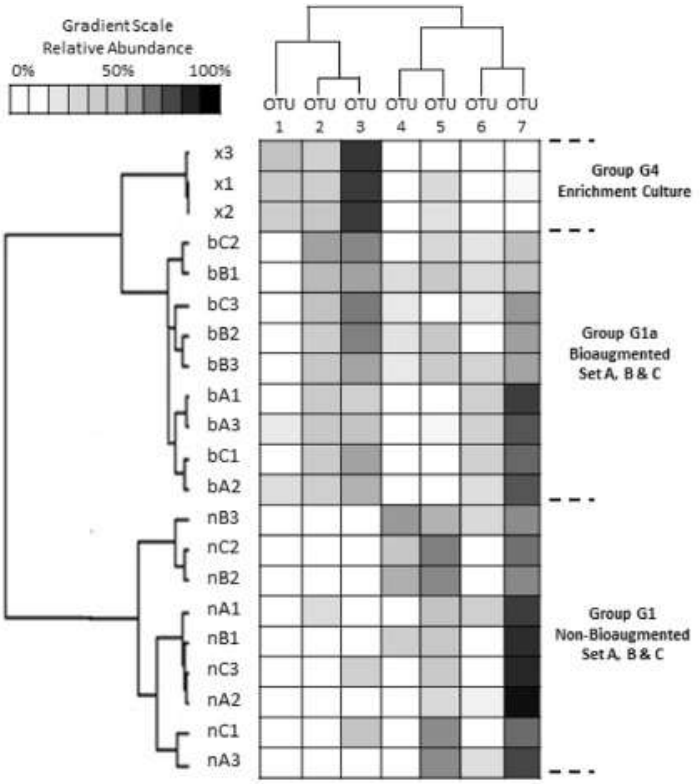
Fig. 5. Cumulative CH_4 produced between day 60–80. Error bars represent standard deviation among triplicates. Bioaugmented digesters of Sets A, B and C showed statistically higher cumulative CH_4 production ($p < 0.05$, $n = 3$).

The increased methane production in G1 digestersafter 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 (Fig. 1, Fig. S1A and B). 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 (Fig. 1, Fig. S1A and B).

The archaeal communities in the bioaugmented digesters were grouped into three distinct clusters based on archaeal sequences (Fig. 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 (Fig. 3). In contrast, however, the G1 bioaugmented digesters showed a statistical improvement in methane production and the archaeal community changed significantly after bioaugmentation (Fig. 3). After bioaugmentation, the archaeal community of G1 digesters became more similar to that of the enrichment culture (G4).

The community structure shift in G1 digesters after bioaugmentation was primarily caused by the increased abundance of

two archaeal genera: *Methanosaeta* and *Methanospirillum* (Fig. 6), which are in high abundance in the enrichment culture (Fig. S5). In contrast, the relative abundance values of *Methanosaeta* and *Methanospirillum* were very low (<1%) in G2 and below detection in the G3 bioaugmented digesters, respectively. The bacterial community compositions of the bioaugmented digesters did not show any significant shift after bioaugmentation (Fig. 4). Sequences related to the two most abundant bacterial genera observed in the enrichment culture, unclassified *Spirochaeata* and *Thermovirga*, which were not detected in the non-bioaugmented digesters, were detected in all the bioaugmented digesters, but their relative abundance remained below 1% after bioaugmentation.



OTU	Class	Order	Family	Genus
1	Thermoplasmata	WCHA1-57	unclassified	unclassified
2	Methanomicrobia	Methanomicrobiales	Methanospirillaceae	Methanospirillum
3	Methanomicrobia	Methanosarcinales	Methanosaetaceae	Methanosaeta
4	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanofollis
5	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium
6	Thermoplasmata	Thermoplasmatales	Thermoplasmatales Incertae Sedis	Candidatus Methanomethylophilus
7	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcina

Fig. 6. Dual hierarchal 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 ×1, ×2 and ×3 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.

The resulting increase in methane production observed in G1 digesters from bioaugmentation was associated with a shift in the archaeal community structure. Increased relative abundance of the genera *Methanosaeta* and *Methaospirillum* was observed in digesters with improvement in the methane production rate. The relative abundance of *Methanosaeta* and *Methanospirillum* increased from below detection in the non-bioaugmented digesters to 10–40% and 10–30%, respectively, in the bioaugmented G1 digesters (Fig. 6). However, it is important to note that the methane production rate increase lasted only 7–11 days in the bioaugmented G1 digesters. This could be due to washout of *Methanosaeta* and *Methanospirillum* once bioaugmentation ceased. It 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 *Methanospiririllum* (i.e., *Methanospirillum hungatii*), are sensitive to low pH and high acid or propionate concentrations (Liu and Whitman, 2008 and Barredo and 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 *Methanospiririllum* 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.

Acknowledgements

The authors thank the following individuals for their laboratory contributions: Mike Dollhopf (Water Quality Center, Marquette University, Milwaukee USA) and Dr. Jean-Philippe Steyer (Laboratory of Environmental Biotechnology, Narbonne, France).

References

American Public Health Association (APHA) et al., 1998. American Public Health Association (APHA), American Water Works Association (AWWA), Water Environment Federation (WEF), *et al. Standard Methods for the Examination of Water and Wastewater*. (twentieth ed.) (1998)

Baek et al., 2016. G. Baek, J. Kim, S.G. Shin, C. Lee. Bioaugmentation of anaerobic sludge digestion with iron-reducing bacteria: process and microbial responses to variations in hydraulic retention time. *Appl. Microbiol. Biotechnol.,* 100 (2) (2016), pp. 927–937 http://dx.doi.org/10.1007/s00253-015-7018-y

Barredo and Evison, 1991. M.S. Barredo, L.M. Evison. Effect of propionate toxicity on methanogen-enriched sludge, Methanobrevibacter smithii, and Methanospirillum hungatii at different pH values. *Appl. Environ. Microbiol.,* 57 (6) (1991), pp. 1764–1769

Carey et al., 2016. D.E. Carey, D.H. Zitomer, K.R. Hristova, A.D. Kappell, P.J. McNamara. Triclocarban influences antibiotic resistance and alters anaerobic digester microbial community structure. *Environ. Sci. Technol.,* 50 (1) (2016), pp. 126–134 http://dx.doi.org/10.1021/acs.est.5b03080

Casamayor et al., 2013. E.O. Casamayor, X. Triadó-Margarit, C. Castañeda. Microbial biodiversity in saline shallow lakes of the Monegros Desert, Spain. *FEMS Microbiol. Ecol.,* 85 (3) (2013), pp. 503–518 http://dx.doi.org/10.1111/1574-6941.12139

Chouari et al., 2005. R. Chouari, D. Le Paslier, P. Daegelen, P. Ginestet, J. Weissenbach, A. Sghir. Novel predominant archaeal and bacterial groups revealed by molecular analysis of an anaerobic sludge digester. *Environ. Microbiol.,* 7 (8) (2005), pp. 1104–1115 http://dx.doi.org/10.1111/j.1462-2920.2005.00795.x

Cirne et al., 2006. D.G. Cirne, L. Björnsson, M.M. Alves, B. Mattiasson. *Effects of Bioaugmentation by an Anaerobic Lipolytic Bacterium on Anaerobic Digestion of Lipid-rich Waste*. 81 (2006), pp. 1745–1752 http://dx.doi.org/10.1002/jctb.1597 (11)

Dahle, 2006. H. Dahle. Thermovirga lienii gen. nov., sp. nov., a novel moderately thermophilic, anaerobic, amino-acid-degrading bacterium isolated from a North Sea oil well. *Int. J. Syst. Evol. Microbiol.,* 56 (7) (2006), pp. 1539–1545 http://dx.doi.org/10.1099/ijs.0.63894-0

De Bok et al., 2004. F.A. De Bok, C.M. Plugge, A.J.M. Stams. Interspecies electron transfer in methanogenic propionate degrading consortia. *Water Res.,* 38 (6) (2004), pp. 1368–1375 http://dx.doi.org/10.1128/AEM.67.4.1800-1804.2001

De Bok et al., 2001. F.A. De Bok, A.J.M. Stams, C. Dijkema, D.R. Boone. Pathway of propionate oxidation by a syntrophic culture of Smithella propionica and Methanospirillum hungatei. *Appl. Environ. Microbiol.,* 67 (4) (2001), pp. 1800–1804 http://dx.doi.org/10.1128/AEM.67.4.1800-1804.2001

Edgar et al., 2011. R.C. Edgar, B.J. Haas, J.C. Clemente, C. Quince, R. Knight. UCHIME improves sensitivity and speed of chimera detection. *Bioinforma. Oxf. Engl.,* 27 (16) (2011), pp. 2194–2200 .http://dx.doi.org/10.1093/bioinformatics/btr381

Grosskopf et al., 1998. R. Grosskopf, P.H. Janssen, W. Liesack. Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. *Appl. Environ. Microbiol.,* 64 (3) (1998), pp. 960–969 http://aem.asm.org/content/64/3/960.full.pdf+html

Guiot et al., 2002. S.R. Guiot, B. Tartakovsky, M. Lanthier, M.J. Lévesque, M.F. Manuel, R. Beaudet, C.W. Greer, R. Villemur. Strategies for augmenting the pentachlorophenol degradation potential of UASB anaerobic granules. *Water Sci. Technol. A J. Int. Assoc. Water Pollut. Res.,* 45 (10) (2002), pp. 35–41 http://www.ncbi.nlm.nih.gov/pubmed/12188570

Guiot et al., 2000. S.R. Guiot, K. Tawfiki Hájji, F. Lépine. Immobilization strategies for bioaugmentation of anaerobic reactors treating phenolic compounds. *Water Sci. Technol.,* 42 (5) (2000), pp. 245–250 http://wst.iwaponline.com/content/ppiwawst/42/5-6/245.full.pdf

Hajji et al., 2000. K.T. Hajji, F. Lépine, J.G. Bisaillon, R. Beaudet, J. Hawari, S.R. Guiot. Effects of bioaugmentation strategies in UASB reactors with a methanogenic consortium for removal of phenolic compounds. *Biotechnol. Bioeng.,* 67 (4) (2000), pp. 417–423 http://dx.doi.org/10.1002/(SICI)1097-0290(20000220)67:4<417::AID-BIT5>3.0.CO;2-#

Hattori, 2008. S. Hattori. Syntrophic acetate-oxidizing microbes in methanogenic environments. *Microbes Environ./JSME,* 23 (2) (2008), pp. 118–127 http://www.ncbi.nlm.nih.gov/pubmed/21558697

Herrero and Stuckey, 2014. M. Herrero, D.C. Stuckey. Bioaugmentation and its application in wastewater treatment: a review. *Chemosphere*, 44 (2014) http://dx.doi.org/10.1016/j.chemosphere.2014.10.033

Hugoni et al., 2013. M. Hugoni, N. Taib, D. Debroas, I. Domaizon, I. Jouan Dufournel, G. Bronner, I. Salter, H. Agogué, I. Mary, P.E. Galand. Structure of the rare archaeal biosphere and seasonal dynamics of active ecotypes in surface coastal waters. *Proc. Natl. Acad. Sci. U. S. A.,* 110 (15) (2013), pp. 6004–6009 http://dx.doi.org/10.1073/pnas.1216863110

Kim et al., 2014. S.J. Kim, S.J. Park, I.T. Cha, D. Min, J.S. Kim, W.H. Chung, J.C. Chae, C.O. Jeon, S.K. Rhee. Metabolic versatility of toluene-degrading, iron-reducing bacteria in tidal flat sediment, characterized by stable isotope probing-based metagenomic analysis. *Environ. Microbiol.,* 16 (2014), pp. 189–204 http://dx.doi.org/10.1111/1462-2920.12277

Kozich et al., 2013. J.J. Kozich, S.L. Westcott, N.T. Baxter, S.K. Highlander, P.D. Schloss. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl. Environ. Microbiol.,* 79 (17) (2013), pp. 5112–5120 http://dx.doi.org/10.1128/AEM.01043-13

Lee et al., 2013. S.H. Lee, J.H. Park, H.J. Kang, Y.H. Lee, T.J. Lee, H.D. Park. Distribution and abundance of Spirochaetes in full-scale anaerobic digesters. *Bioresour. Technol.,* 145 (2013), pp. 25–32 http://dx.doi.org/10.1016/j.biortech.2013.02.070

Lee et al., 2015. S.H. Lee, J.H. Park, S.H. Kim, B.J. Yu, J.J. Yoon, H.D. Park. Evidence of syntrophic acetate oxidation by Spirochaetes during anaerobic methane production. *Bioresour. Technol.,* 190 (2015), pp. 543–549 http://dx.doi.org/10.1016/j.biortech.2015.02.066

Liu and Whitman, 2008. Y. Liu, W.B. Whitman. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. *Ann. N. Y. Acad. Sci.,* 1125 (2008), pp. 171–189 http://dx.doi.org/10.1196/annals.1419.019

Ma et al., 2009. J. Ma, M. Carballa, P. Van De Caveye, W. Verstraete. Enhanced propionic acid degradation (EPAD) system: proof of principle and feasibility. *Water Res.,* 43 (13) (2009), pp. 3239–3248 http://dx.doi.org/10.1016/j.watres.2009.04.046

McCarty and Smith, 1986. P.L. McCarty, D.P. Smith. Anaerobic wastewater treatment. *Environ. Sci. Technol.,* 20 (12) (1986), pp. 1200–1206 http://dx.doi.org/10.1021/es00154a002

McNamara and Krzmarzick, 2013. P.J. McNamara, M.J. Krzmarzick. Triclosan enriches for Dehalococcoides-like Chloroflexi in anaerobic soil at environmentally relevant concentrations. *FEMS Microbiol. Lett.,* 344 (1) (2013), pp. 48–52 http://dx.doi.org/10.1111/1574-6968.12153

Nunoura et al., 2012. T. Nunoura, Y. Takaki, H. Kazama, M. Hirai, J. Ashi, H. Imachi, H. Kazama. Microbial diversity in deep-sea methane seep sediments presented by SSU rRNA gene tag sequencing. *Microbes Environ.,* 27 (4) (2012), pp. 382–390 http://europepmc.org/articles/pmc4103545

Nunoura et al., 2010. T. Nunoura, H. Oida, M. Nakaseama, A. Kosaka, S.B. Ohkubo, T. Kikuchi, H. Kazama, S. Hosoi Tanabe, K. Nakamura, M. Kinoshita, H. Hirayama, F. Inagaki, U. Tsunogai, J. Ishibashi, K. Takai. Archaeal diversity and distribution along thermal and geochemical gradients in hydrothermal sediments at the Yonaguni Knoll IV hydrothermal field in the Southern Okinawa trough. *Appl. Environ. Microbiol.,* 76 (4) (2010), pp. 1198–1211 http://dx.doi.org/10.1128/AEM.00924-09

Rivière et al., 2009. D. Rivière, V. Desvignes, E. Pelletier, S. Chaussonnerie, S. Guermazi, J. Weissenbach, T. Li, P. Camacho, A. Sghir. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. *ISME J.,* 3 (6) (2009), pp. 700–714 http://dx.doi.org/10.1038/ismej.2009.2

Rotaru et al., 2014. A.E. Rotaru, P.M. Shrestha, F. Liu, M. Shrestha, D. Shrestha, M. Embree, K. Zengler, C. Wardman, K.P. Nevin, D.R. Lovley. A new model for electron flow during anaerobic digestion: direct interspecies electron transfer to Methanosaeta for the reduction of carbon dioxide to methane. *Energy & Environ. Sci.,* 7 (1) (2014), pp. 408–415 http://dx.doi.org/10.1039/c3ee42189a

Schauer Gimenez et al., 2010. A.E. Schauer Gimenez, D.H. Zitomer, J.S. Maki, C.A. Struble. Bioaugmentation for improved recovery of anaerobic digesters after toxicant exposure. *Water Res.,* 44 (12) (2010), pp. 3555–3564 http://dx.doi.org/10.1016/j.watres.2010.03.037

Schloss et al., 2009. P.D. Schloss, S.L. Westcott, T. Ryabin, J.R. Hall, M. Hartmann, E.B. Hollister, R.A. Lesniewski, B.B. Oakley, D.H. Parks, C.J. Robinson, J.W. Sahl, B. Stres, G.G. Thallinger, D.J. Van Horn, C.F. Weber. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.,* 75 (23) (2009), pp. 7537–7541 http://dx.doi.org/10.1128/AEM.01541-09

Speece et al., 2006. R.E. Speece, Saroch Boonyakitsombut, Moonil Kim, Nuri Azbar, Pepi Ursillo. Overview of anaerobic treatment: thermophilic and propionate implications. *Water Environ. Res. A Res. Publ. Water*

Environ. Fed., 78 (5) (2006), pp. 460–473
http://www.ncbi.nlm.nih.gov/pubmed/16752608

Speece, 2008. R.E. Speece. *Anaerobic Biotechnology and Odor/corrosion Control for Municipalities and Industries*. Fields Publishing, Inc, Nashville, TN (2008) ISBN: 1-57843-052-9

Stams and Plugge, 2009. A.J.M. Stams, C.M. Plugge. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. *Nat. Rev. Microbiol.,* 7 (8) (2009), pp. 568–577
http://dx.doi.org/10.1038/nrmicro2166

Tale et al., 2011. V.P. Tale, J.S. Maki, C.A. Struble, D.H. Zitomer. Methanogen community structure-activity relationship and bioaugmentation of overloaded anaerobic digesters. *Water Res.,* 45 (16) (2011), pp. 5249–5256
http://dx.doi.org/10.1016/j.watres.2011.07.035

Tale et al., 2015. V.P. Tale, J.S. Maki, D.H. Zitomer. Bioaugmentation of overloaded anaerobic digesters restores function and archaeal community. *Water Res.,* 70 (2015), pp. 138–147
http://dx.doi.org/10.1016/j.watres.2014.11.037

Venkiteshwaran et al., 2016. K. Venkiteshwaran, B. Bocher, J. Maki, D. Zitomer. Relating anaerobic digestion microbial community and process function. *Microbiol. Insights,* 8 (2) (2016), pp. 37–44
http://dx.doi.org/10.4137/MBI.S33593

Wang and Qian, 2009. Y. Wang, P.-Y. Qian. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. *PloS One,* 4 (10) (2009), p. e7401
http://dx.doi.org/10.1371/journal.pone.0007401

Zitomer, 2013. D. Zitomer. *Preservation of Methanogenic, Hydrogen- Utilizing Microbial Cultures*. (2013) U.S. Patent US 8,557,563 B2, October 15th

Appendix A. Supplementary data

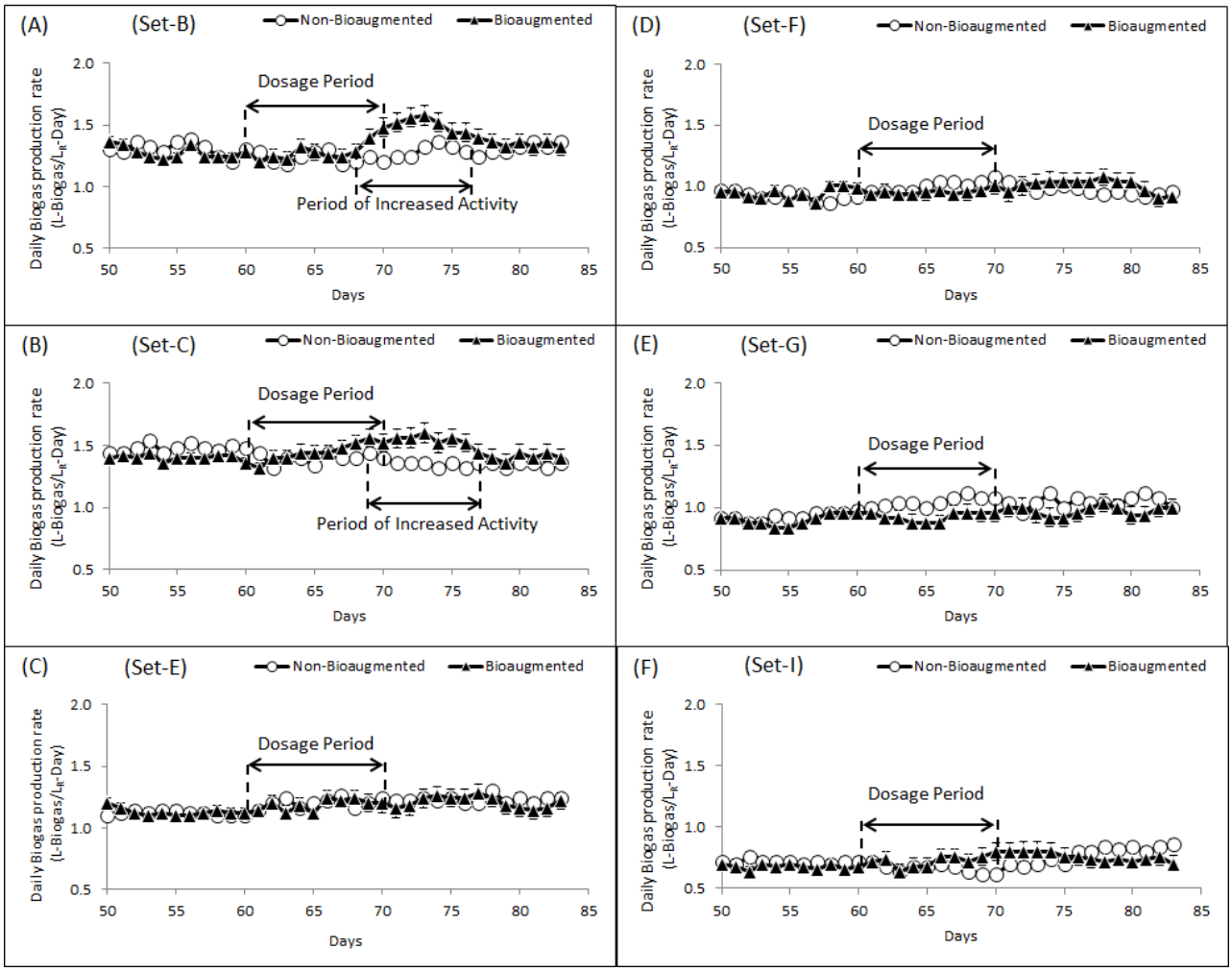
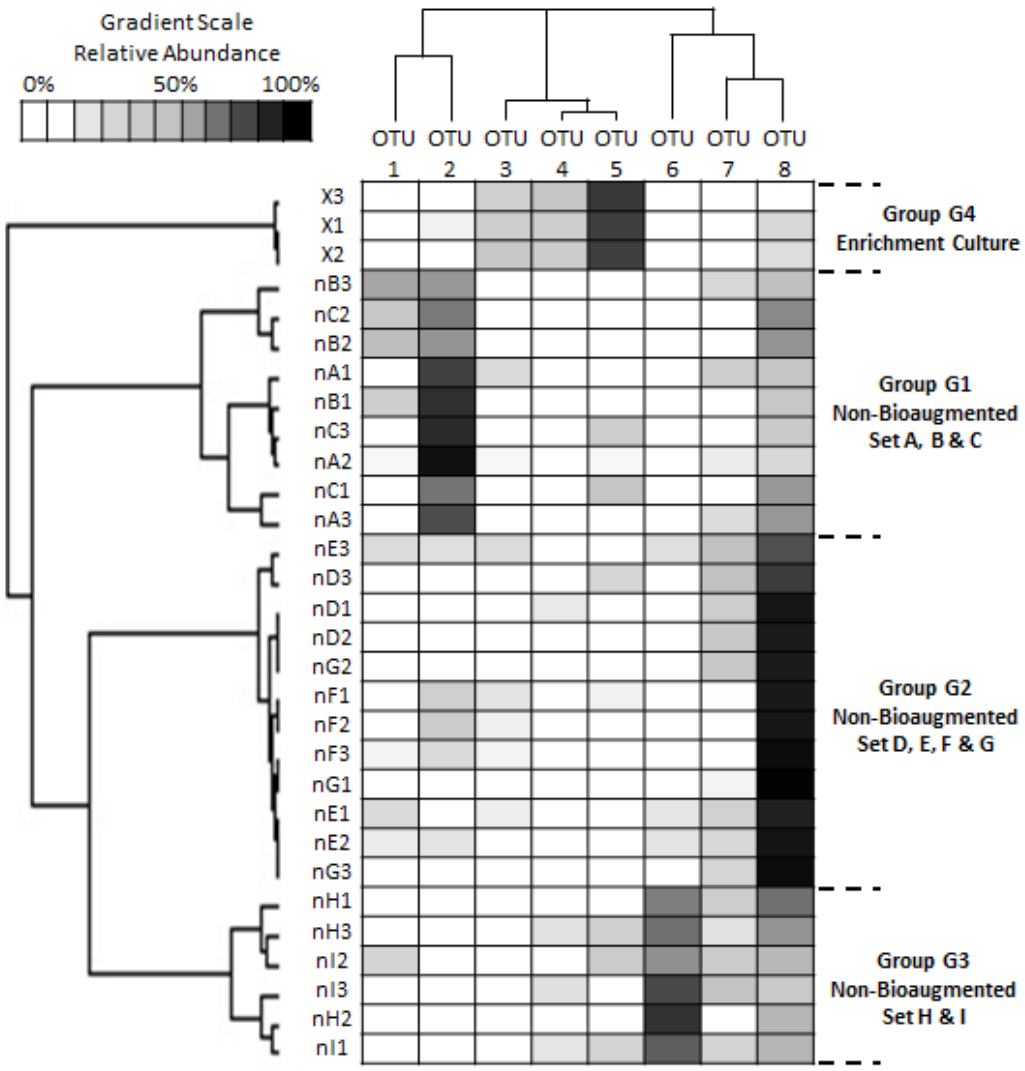


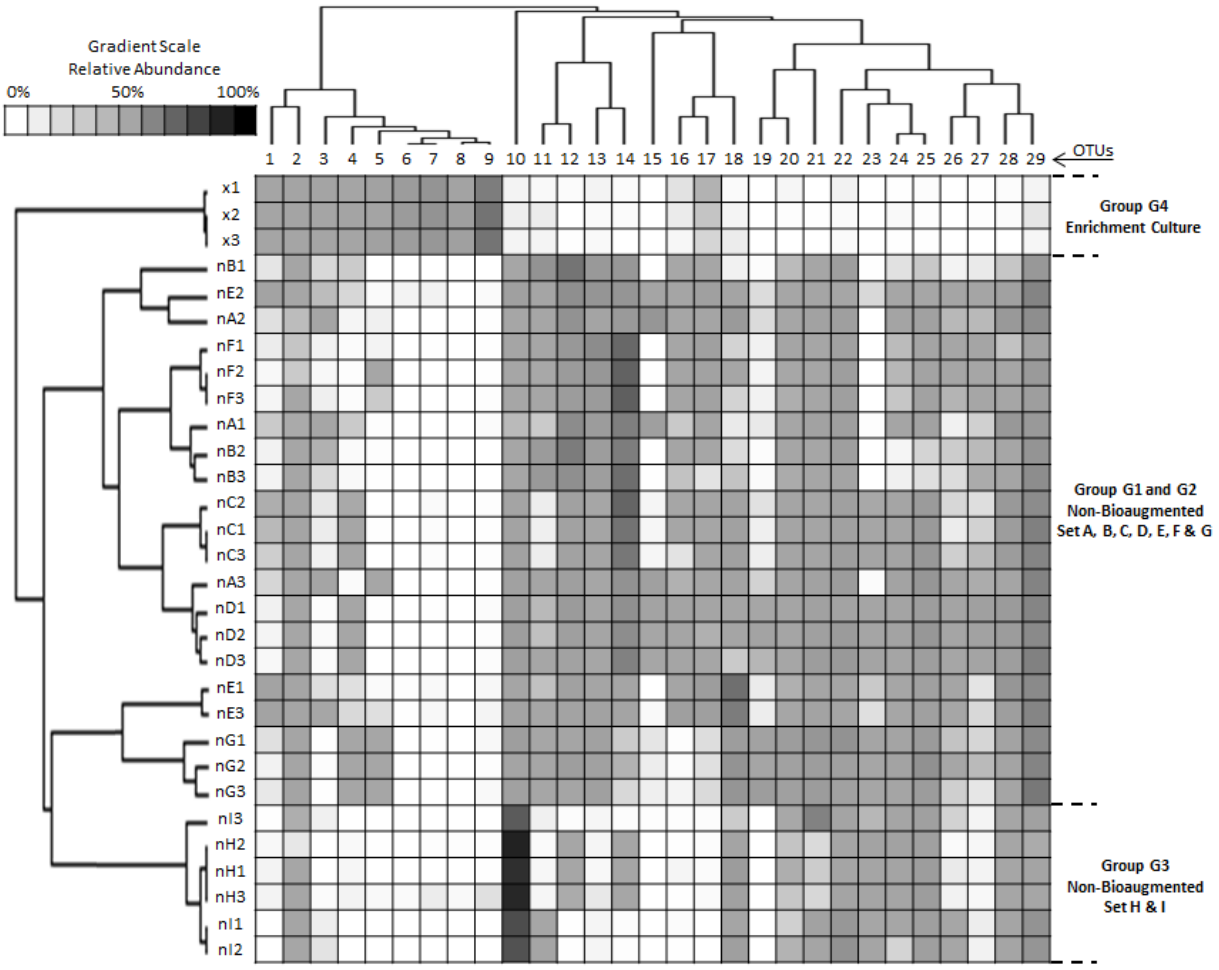
Figure S1: Daily biogas production rate for non-bioaugmented and bioaugmented digester sets B, C, E, F, G and I.

Error bars represent standard deviation among triplicates. The Dosage Period represents the 10-day period during which inactivated and active enrichment cultures were added to the non-bioaugmented and bioaugmented digesters, respectively. The period of increased activity (PIA), as seen in **(A)** Set-B plot and **(B)** Set-C digester plot, represents the days during which the bioaugmented digesters produced statistically higher (p value <0.05, n =3) methane than the non-bioaugmented digesters. PIA was not observed in **(C)** Set-E, **(D)** Set-F, **(E)** Set-G and **(F)** Set-I digesters.



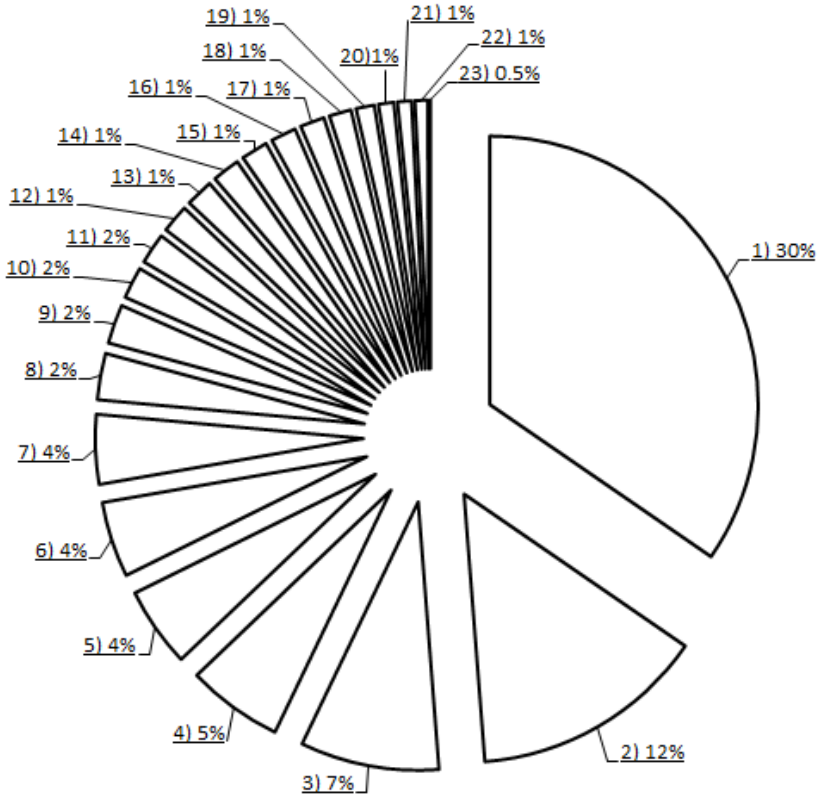
OTU	Class	Order	Family	Genus
1	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanofollis
2	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcina
3	Methanomicrobia	Methanomicrobiales	Methanospirillaceae	Methanospirillum
4	Thermoplasmata	WCHA1-57	unclassified	unclassified
5	Methanomicrobia	Methanosarcinales	Methanosaetaceae	Methanosaeta
6	Halobacteria	Halobacteriales	Deep Sea Hydrothermal Vent Gp 6	unclassified
7	Thermoplasmata	Thermoplasmatales	Thermoplasmatales Incertae Sedis	Candidatus Methanomethylophilus
8	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium

Figure S2: Dual hierarchal clustering of the archaeal communities.
The eight OTUs identified based on 97% similarity, represent >99% of the total archaeal sequences observed in the enrichment culture and G1, G2, G3 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" – the prefix "n" represents "non-bioaugmented, the middle letter "B" represents Set-B and the suffix "1" represent the replicate number. The enrichment culture is dominated by *Methanosaeta* (OTU 5), *Methanospirillum* (OTU 4) and *WCHA1-57* (OTU 3). Set-A, B & C digesters, belonging to group G1 are dominated by *Methanosarcina* (OTU 2). G2 digesters, Set-D, E, F and G, are dominated by *Methanobacterium* (OTU 8). Set H and I are dominated by sequences related to *Methanobacterium* (OTU 8) and *DHVEG6* (OTU 6).



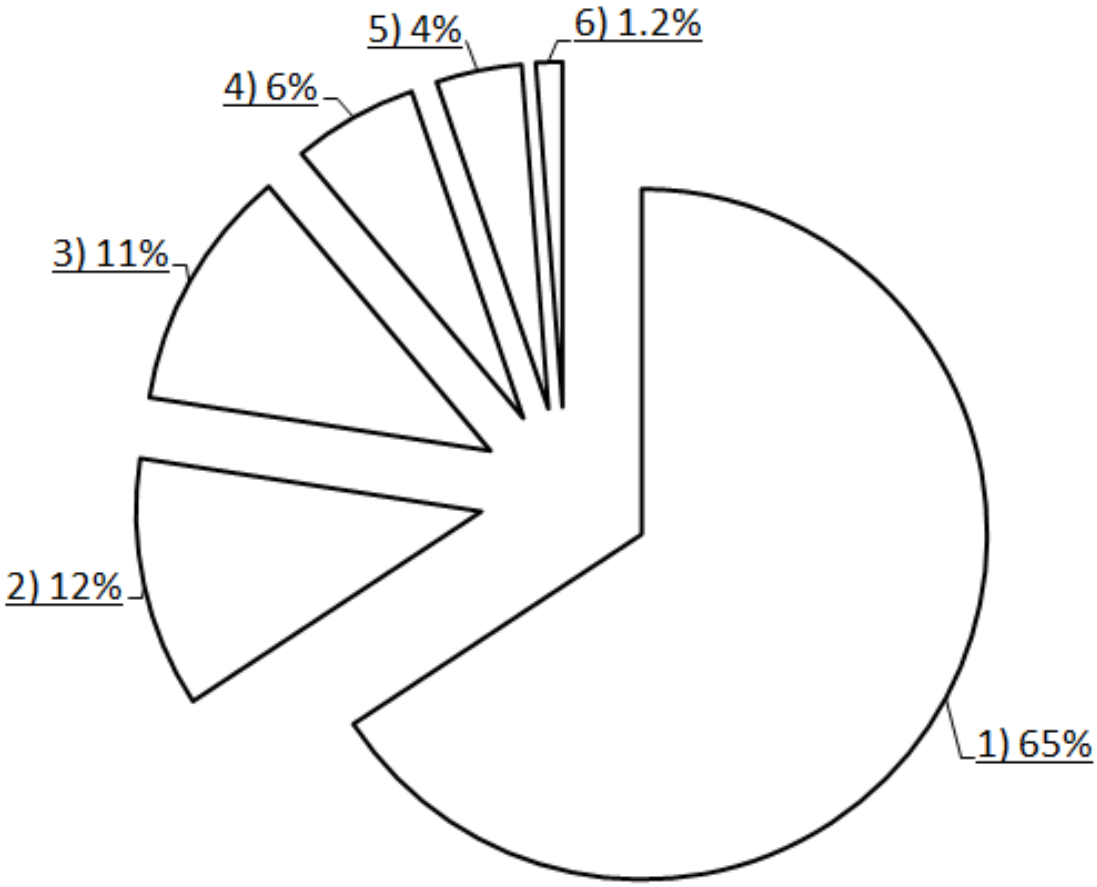
OTU	Phylum	Class	Order	Family	Genus
1	Synergistetes	Synergistia	Synergistales	Synergistaceae	Aminivibrio
2	Spirochaetae	Spirochaetes	Spirochaetales	Spirochaetaceae	Spirochaeta
3	Tenericutes	Mollicutes	NB1-n	unclassified	unclassified
4	Bacteroidetes	vadinHA17	unclassified	unclassified	unclassified
5	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified
6	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobulbaceae	Desulfobulbus
7	Synergistetes	Synergistia	Synergistales	Synergistaceae	Thermovirga
8	Proteobacteria	Deltaproteobacteria	Desulfuromonadales	unclassified	unclassified
9	Spirochaetae	Spirochaetes	Spirochaetales	Spirochaetaceae	unclassified
10	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	RC9 gut group
11	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	vadinBC27 wastewater sludge grp
12	Firmicutes	Clostridia	Clostridiales	Family XI	unclassified
13	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Petrimonas
14	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Porphyromonas
15	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus
16	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter
17	Synergistetes	Synergistia	Synergistales	Synergistaceae	unclassified
18	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	unclassified
19	Firmicutes	Clostridia	Clostridiales	Family_XI	Sedimentibacter
20	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified
21	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Atopobium
22	Synergistetes	Synergistia	Synergistales	Synergistaceae	Aminobacterium
23	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Pseudoramibacter
24	Firmicutes	Clostridia	Clostridiales	Family_XIII	unclassified
25	Synergistetes	Synergistia	Synergistales	Synergistaceae	Pyramidobacter
26	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Fastidiosipila
27	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Trichococcus
28	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus
29	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides

Figure S3: Dual hierarchal clustering of the bacterial communities.
The 29 OTUs detected in all digesters with the highest relative abundance, including the enrichment culture, were considered in the clustering analysis. The 29 OTUs contributed to 70-85% of the total bacterial sequences in all the biomass samples analyzed. 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" – the prefix "n" represents "non-bioaugmented, the middle letter "B" represents Set-B and the suffix "1" represent the replicate number. The enrichment culture has a unique bacterial community structure as compared to the other digesters and clusters separately. Fermenters of the phyla *Firmicutes*, *Bacteroidetes* and *Synergistetes* dominated all non-bioaugmented digesters. G3 digesters (Set-H & I) were uniquely dominated by *RC9 gut group* (OTU 10), contributing 50-60% of the relative abundance.



OTU	Phylum	Class	Order	Family	Genus
1	Spirochaetae	Spirochaetes	Spirochaetales	Spirochaetaceae	unclassified
2	Synergistetes	Synergistia	Synergistales	Synergistaceae	Thermovirga
3	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobulbaceae	Desulfobulbus
4	Proteobacteria	Deltaproteobacteria	Desulfuromonadales	unclassified	unclassified
5	Deferribacteres	Deferribacteres	Deferribacterales	Deferribacteraceae	Denitrovibrio
6	Hyd24-12	unclassified	unclassified	unclassified	unclassified
7	Spirochaetae	Spirochaetes	Spirochaetales	PL-11B10	unclassified
8	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Proteiniphilum
9	Bacteroidetes	vadinHA17	unclassified	unclassified	unclassified
10	Tenericutes	Mollicutes	NB1-n	unclassified	unclassified
11	Synergistetes	Synergistia	Synergistales	Synergistaceae	Aminivibrio
12	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	Smithella
13	Proteobacteria	Gammaproteobacteria	unclassified	unclassified	unclassified
14	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	Syntrophobacter
15	Spirochaetae	Spirochaetes	Spirochaetales	Spirochaetaceae	unclassified
16	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Thauera
17	Candidate division OP8	unclassified	unclassified	unclassified	unclassified
18	Spirochaetae	Spirochaetes	Spirochaetales	Spirochaetaceae	Spirochaeta
19	Chlorobi	Ignavibacteria	Ignavibacteriales	unclassified	unclassified
20	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	unclassified
21	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	WCHB1-69	unclassified
22	Deferribacteres	Deferribacteres	Deferribacterales	SAR406 clade (Marine group A)	unclassified
23	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Defluviimonas
24	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	WCHB1-69	unclassified
25	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Meniscus

Figure S4: Bacterial community composition of the enrichment culture based on percent relative abundance.
The figure includes the 25 bacterial OTUs observed with the highest relative abundance which constituted 80% of the total bacterial sequences.



OTU	Class	Order	Family	Genus
1	Methanomicrobia	Methanosarcinales	Methanosaetaceae	Methanosaeta
2	Thermoplasmata	WCHA1-57	unclassified	unclassified
3	Methanomicrobia	Methanomicrobiales	Methanospirillaceae	Methanospirillum
4	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium
5	Methanomicrobia	Methanomicrobiales	Methanoregulaceae	Methanolinea
6	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcina

Figure S5: Archaeal community composition of the enrichment culture based on percent relative abundance.
The figure includes 6 archaeal OTUs identified based of 97% similarity of the sequences.