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Kaushik Venkiteshwaran, Kim Milferstedt, Jérôme Hamelin, D. H. Zitomer. Anaerobic digester bioaugmentation influences quasi steady state performance and microbial community. *Water Research*, 2016, 104, pp.128-136. 10.1016/j.watres.2016.08.012 . hal-01608636

**HAL Id: hal-01608636**

**<https://hal.science/hal-01608636v1>**

Submitted on 27 May 2020

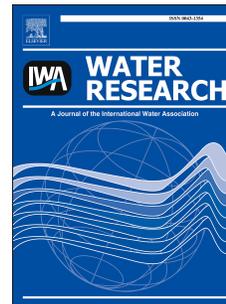
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# Accepted Manuscript

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PII: S0043-1354(16)30612-1

DOI: [10.1016/j.watres.2016.08.012](https://doi.org/10.1016/j.watres.2016.08.012)

Reference: WR 12283

To appear in: *Water Research*

Received Date: 28 March 2016

Revised Date: 3 August 2016

Accepted Date: 4 August 2016

Please cite this article as: Venkiteshwaran, K., Milferstedt, K., Hamelin, J., Zitomer, D.H., Anaerobic digester bioaugmentation influences quasi steady state performance and microbial community, *Water Research* (2016), doi: 10.1016/j.watres.2016.08.012.

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Comment citer ce document :

Venkiteshwaran, K. (Auteur de correspondance), Milferstedt, K., Hamelin, J., Zitomer, D. H. (2016). Anaerobic digester bioaugmentation influences quasi steady state performance and microbial community. *Water Research*, 104, 128-136. DOI : 10.1016/j.watres.2016.08.012

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 (CH<sub>4</sub>) 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 CH<sub>4</sub> production  
18 correlated with neutral pH and high *Methanosarcina* abundance, whereas low CH<sub>4</sub>  
19 production correlated to low pH as well as high *Methanobacterium* and *DHVEG 6* family  
20 abundance. After bioaugmentation, CH<sub>4</sub> production from the high CH<sub>4</sub>-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 CH<sub>4</sub> producing  
23 digesters that all had pH higher than 6.7. The CH<sub>4</sub> production increase after  
24 bioaugmentation was correlated to increased relative abundance of *Methanosaeta* and

25 *Methaospirillum* originating from the bioaugment culture. In conclusion, different  
26 anaerobic digester seed biomass can result in different quasi steady state CH<sub>4</sub> production,  
27 SCOD removal, pH and effluent VFA concentration in the timeframe studied. The  
28 bioaugmentation employed can result in a period of increased methane production. Future  
29 research should address extending the period of increased CH<sub>4</sub> production by employing  
30 pH and VFA control concomitant with bioaugmentation, developing improved  
31 bioaugments, or employing a membrane bioreactor to retain the bioaugment.

32 **Keywords**

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

## 35 **1. Introduction**

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

51 Intermediates such as propionate can be biotransformed by a specific consortium of  
52 synergistic microorganisms (McCarty & Smith 1986; Speece et al. 2006). Bioconversion  
53 of propionate to acetate and hydrogen (H<sub>2</sub>) is thermodynamically favorable only when the  
54 partial pressure of the generated H<sub>2</sub> remains below 10<sup>-4</sup> atm. Thus, degradation of  
55 propionate requires a synergistic relationship between H<sub>2</sub> producing and H<sub>2</sub> consuming  
56 microorganisms to maintain low H<sub>2</sub> concentrations (McCarty & Smith 1986). It was  
57 shown previous that adding cultures enriched to consume H<sub>2</sub> 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 (Venkiteswaran 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

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

## 82 **2. Material and Methods**

### 83 **2.1 Anaerobic digesters**

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

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

103 enrichment culture. Functional parameters including effluent soluble COD (SCOD) and  
104 volatile fatty acids (VFA) concentrations as well as biogas CH<sub>4</sub> concentration were  
105 monitored between days 60 and 80. Digester biomass samples were collected on day 71  
106 for amplicon sequencing.

107 Cumulative methane volume produced was calculated by summing the daily methane  
108 production volumes (ml CH<sub>4</sub>/day) from days 60 to 80. Biomass production rate was  
109 calculated as the product of VSS concentration (mg VSS/L) and effluent flow (L/day).  
110 Observed biomass yield was calculated as the quotient of biomass production rate and  
111 COD added to the digester per day (mg COD/day).

## 112 2.2 Enrichment culture for bioaugmentation

113 A moderately aerated, propionate-utilizing, mixed methanogenic enrichment culture  
114 developed by Tale et al. (2011) was employed for bioaugmentation. The original seed  
115 biomass for the enrichment culture was from an upflow anaerobic sludge blanket (UASB)  
116 reactor treating brewery wastewater that exhibited a high methanogenic activity (Tale et  
117 al. 2011). When previously used for bioaugmentation, this aerotolerant mixed microbial  
118 culture reduced the recovery time of transiently organically overloaded digesters (Tale et  
119 al. 2015). The enrichment was maintained in two completely mixed vessels with a  
120 volume of four liters at 35 °C at a 15 day HRT and fed 0.17 g propionate/L-day as  
121 calcium propionate with basal nutrient media. Immediately after feeding, ambient air was  
122 added directly into the headspace of the vessel at a volume equivalent to 25 mg O<sub>2</sub>/L-day  
123 or 10% of the OLR to provide a micro-aerated environment.

## 124 2.3 Basal nutrient media

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

#### 131 2.4 Analytical methods

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

#### 147 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),

171 of anaerobic community sequence data gathered from Illumina sequencing (Carey et al.  
172 2016; McNamara & Krzmarzick 2013).

### 173 **3. Results and Discussion**

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

#### 186 **3.1 Non-bioaugmented digester function**

187 Although operated identically, the digester sets did not achieve identical operational  
188 values. For example, the quasi steady state methane production rates ranged from 0.3 to  
189 0.8 L CH<sub>4</sub> per L of digester per day (L CH<sub>4</sub>/L<sub>R</sub>-day) (Table 1). Each non-bioaugmented  
190 digester set was classified into one of three distinct groups based on statistically similar  
191 methane production rate, SCOD removal, pH and effluent acetate concentration (p value  
192 <0.05, n=3) (Table 1). Group 1 (G1) (sets A, B and C) contained the best performing  
193 digesters with the highest methane production rate, highest SCOD removal rate, highest

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

### 204 3.2 Non-bioaugmented digester archaeal community

205 A total of 32 archaeal OTUs, based on 97% similarity, were identified among all the  
206 digester samples analyzed. The relative abundance of archaeal sequences varied from 1 to  
207 4% for G1, G2 and 0.1 to 1% for G3 digesters, respectively. Eight archaeal OTUs  
208 represented more than 99% of the archaeal abundance in all non-bioaugmented digesters  
209 (Figure S2). These eight OTUs were most similar to the genera *Methanofollis*,  
210 *Methanosarcina*, *Methanospirillum*, *Methanosaeta*, *Methanobacterium*, *Candidatus*  
211 *Methanomethylophilus* and two unclassified genera in the order *WCHA1-57* and the  
212 family *Deep Sea Hydrothermal Vent Grp 6 (DHVEG 6)*, based on the SILVA SSURef  
213 NR99 v119 reference database from Mothur (Schloss et al. 2009) (Figure S2).

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

217 digester communities were distinguished from G2 and G3 communities by high relative  
218 abundance of *Methanosarcina*, which ranged from 60 to 95% of archaea in G1 digesters  
219 (Figure S2). *Methanobacterium* dominated non-bioaugmented G2 digesters, with a  
220 relative abundance that ranged from 80 to 99% of the archaeal diversity. The G3  
221 digesters were distinguished by high relative abundance of the *DHVEG 6* family, with  
222 *Methanobacterium* also observed in high relative abundance (Figure S2).  
223 *Methanosarcina* and *Methanosaeta* are the only two methanogenic genera known to  
224 consume acetate (Liu & Whitman 2008). High relative abundance of *Methanosarcina* in  
225 the high-performing G1 digesters correlated to higher methane production and lower  
226 effluent acetate concentration. This is ostensibly because the *Methanosarcina* maximum  
227 specific substrate utilization rate is higher than that of *Methanosaeta* (Liu & Whitman  
228 2008). Therefore, the presence of *Methanosarcina* in digesters with moderate to high  
229 VFA concentrations, such as the ones of this study, may be beneficial to maintain more  
230 rapid bioconversion of acetate to methane. Compared to G1 digesters, *Methanosarcina*  
231 relative abundance in the lesser performing, non-bioaugmented G2 digesters was lower,  
232 ranging from 0.5 to 18%. In addition, *Methanosarcina* was undetectable in the poorest  
233 performing, non-bioaugmented G3 digesters which had the highest acid concentrations  
234 and lowest pH.

235 The poor performing G3 digesters were distinguished from the better performing G1 and  
236 G2 digesters by the low archaeal sequence abundance ( $\leq 1\%$  of the total sequence) and  
237 further by high relative abundance of *DHVEG 6*, which ranged from 60-90% in G3  
238 digesters (Figure S2). *DHVEG 6* have been observed in acidic environments, marine  
239 environments, terrestrial soils, hydrothermal sediments, deep sea methane seep

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

### 246 3.3 Non-bioaugmented digester bacterial community

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

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

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

#### 264 3.4 Enrichment culture

265 The enrichment culture functional parameters were stable between days 60 to 80, with  
266 99% SCOD removal, no detectable VFAs, pH of  $7.5\pm 0.2$ ,  $60\pm 3\%$  biogas methane  
267 content, methane yield of  $330\pm 16$  mLCH<sub>4</sub>/g-COD removed and observed biomass yield  
268 of  $0.08\pm 0.01$  gVSS/gCOD.

269 More than 700 bacterial OTUs were identified in the enrichment culture based on 97%  
270 similarity. The 25 bacterial OTUs with the highest relative abundance represented  
271 approximately 80% of total bacterial sequences and are shown in Figure S4. The two  
272 most abundant bacterial taxa were most similar to an unclassified genus within  
273 *Spirochaetaceae* (30% of the total bacterial relative abundance) and *Thermovirga* within  
274 *Synergistaceae* (12% of the total bacterial relative abundance) (Figure S4). *Thermovirga*  
275 is currently represented by a single member species *Thermovirgaliinii*, which is a  
276 moderately thermophilic, amino acid degrading fermentative bacterium (Dahle 2006).  
277 Some members of the *Spirochaetaceae* family such as *Treponema* species, are reported to  
278 be abundant in iron-reducing consortia that were used by othersto bioaugmentanaerobic  
279 digesters (Baek et al. 2015). Bacteria related to *Spirochaetes* may be beneficial for the  
280 overall process since some of them have been suggested as syntrophic acetate-oxidizing  
281 bacteria (Hattori 2008; Lee et al. 2013; Lee et al. 2015). Iron-reducing bacteria (IRB) are  
282 commonly observed in anaerobic systems and can utilize acetate, H<sub>2</sub>, ethanol and other  
283 complex substrates and ferric iron as an electron acceptor (Kim et al. 2014). They are  
284 also known to form syntrophic associations and, via interspecies electron transfer,

285 transfer electron directly to their methanogenic partner, which can facilitate CO<sub>2</sub> reduction  
286 to CH<sub>4</sub> (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

308 order *WCHA1-57* (Figure S5) (Liu & Whitman 2008). Conversion of propionate to  
309 methane only becomes thermodynamically favorable through H<sub>2</sub> utilization. Therefore,  
310 the significant presence of hydrogenotrophic methanogens contributing 30-35% of the  
311 total archaeal sequences could have positive functional results. The presence of  
312 microorganisms classified in the genera *Methanospirillum*, *Methanobacterium*,  
313 *Methanolinea* has previously been reported to play an important role in propionate  
314 utilization during digester recovery after organic overload (Tale et al. 2011; Tale et al.  
315 2015; Schauer-Gimenez et al. 2010).

316 The archaeal order *WCHA1-57* was observed at a significant relative abundance (12%) in  
317 the enrichment culture. Although many *WCHA1-57*-related 16S rRNA gene sequences  
318 have been identified in anaerobic digesters (Chouari et al. 2005; Rivière et al. 2009;  
319 Schauer-Gimenez et al. 2010), no reports were found regarding their role in propionate  
320 oxidation or methane production. In some anaerobic digesters treating municipal sewage  
321 sludge, the *WCHA1-57* phylotype population represented one of the predominant  
322 archaeal components, with relative abundance >70% in archaeal clone libraries (Chouari  
323 et al. 2005; Rivière et al. 2009). These observations indicate that *WCHA1-57* archaea  
324 represent a potentially important group in anaerobic digesters. Chouari et al. (2005)  
325 reported the enrichment of *WCHA1-57* phylotypes in cultures fed formate or H<sub>2</sub>/CO<sub>2</sub>.  
326 This indicates that *WCHA1-57* plays a role in reducing hydrogen concentration and,  
327 therefore, aiding in conversion of propionate to methane.

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

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

### 333 3.5 Bioaugmentation, digester function and microbial community changes

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

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

347 The archaeal communities in the bioaugmented digesters were grouped into three distinct  
348 clusters based on archaeal sequences (Figure 3). The archaeal community of the  
349 bioaugmented digesters belonging to functional groups G2a and G3a, which did not  
350 improve after bioaugmentation, did not significantly change after bioaugmentation  
351 (Figure 3). In contrast, however, the G1 bioaugmented digesters showed a statistical  
352 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

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

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

#### 392 **4. Conclusion**

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

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

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

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

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

421 **Acknowledgements**

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

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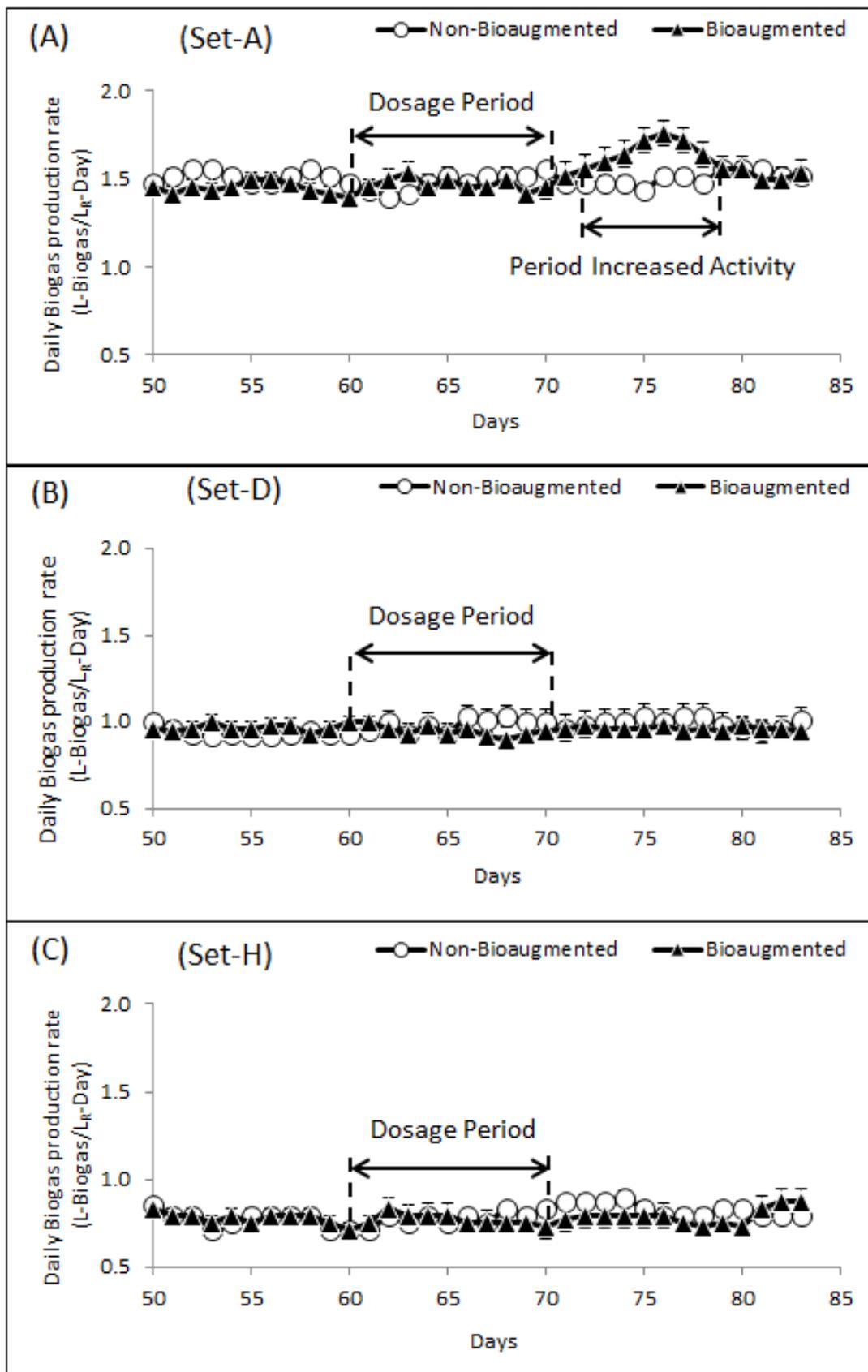
**Table 1:** Performance parameters of digester groups.

Group->	G1	G2	G3
Digester Sets->	A, B and C	D, E, F and G	H and I
Methane production rate (L-CH <sub>4</sub> /L <sub>R</sub> -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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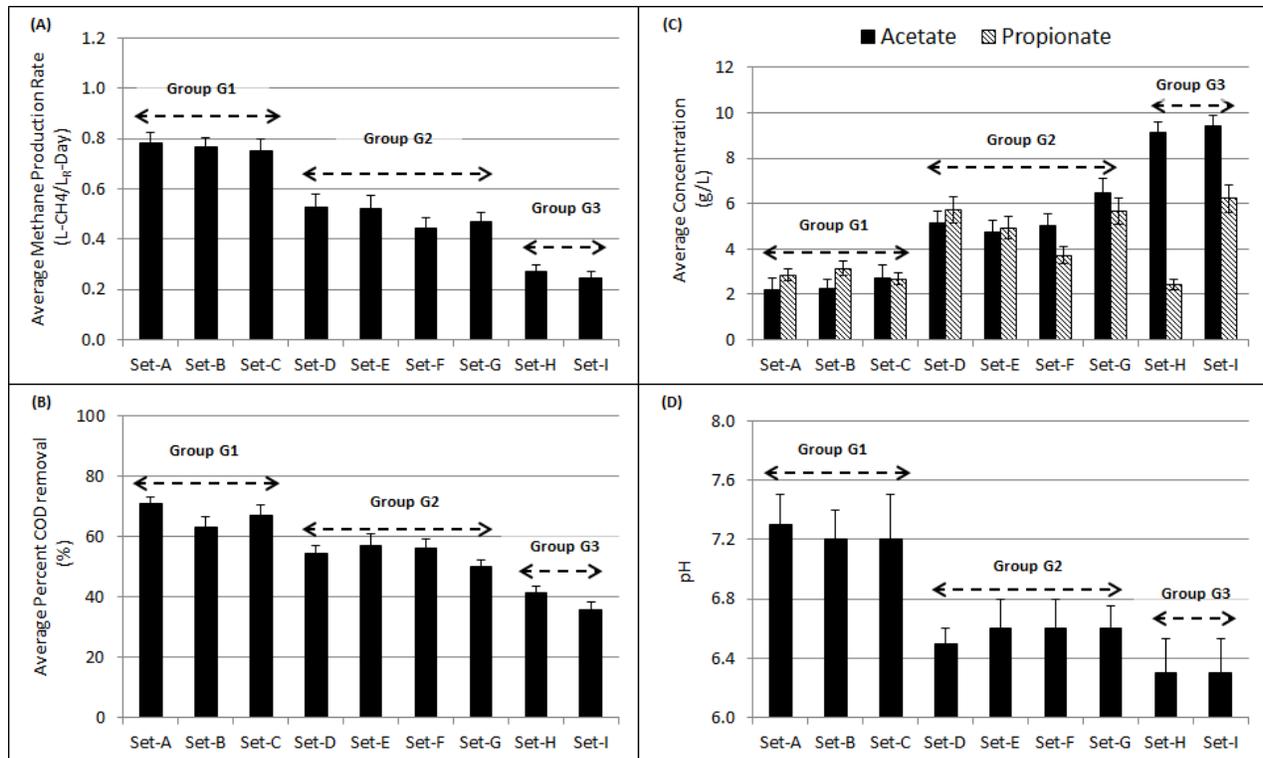
**Figure 1:** Typical quasi steady state biogas production.

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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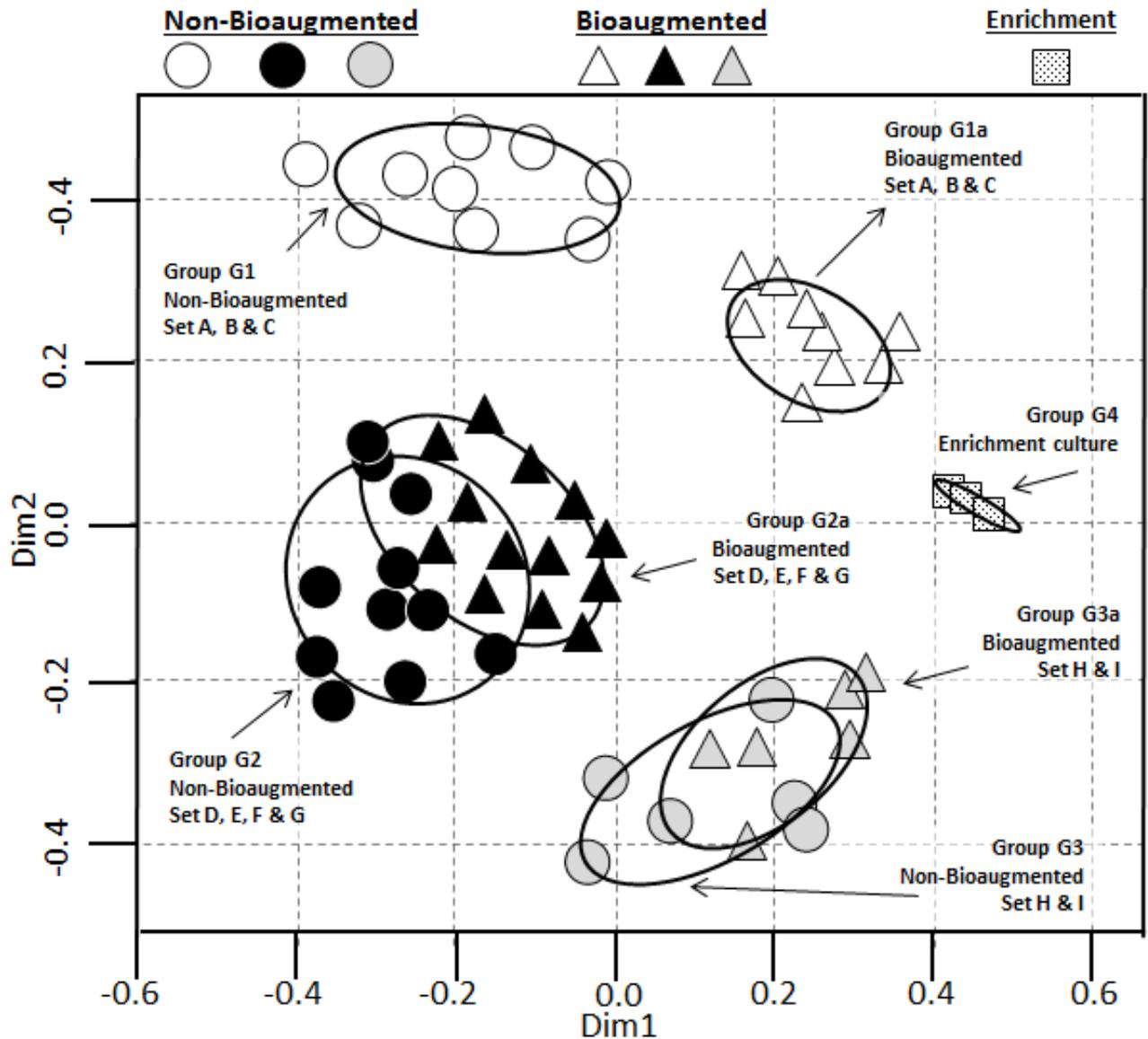
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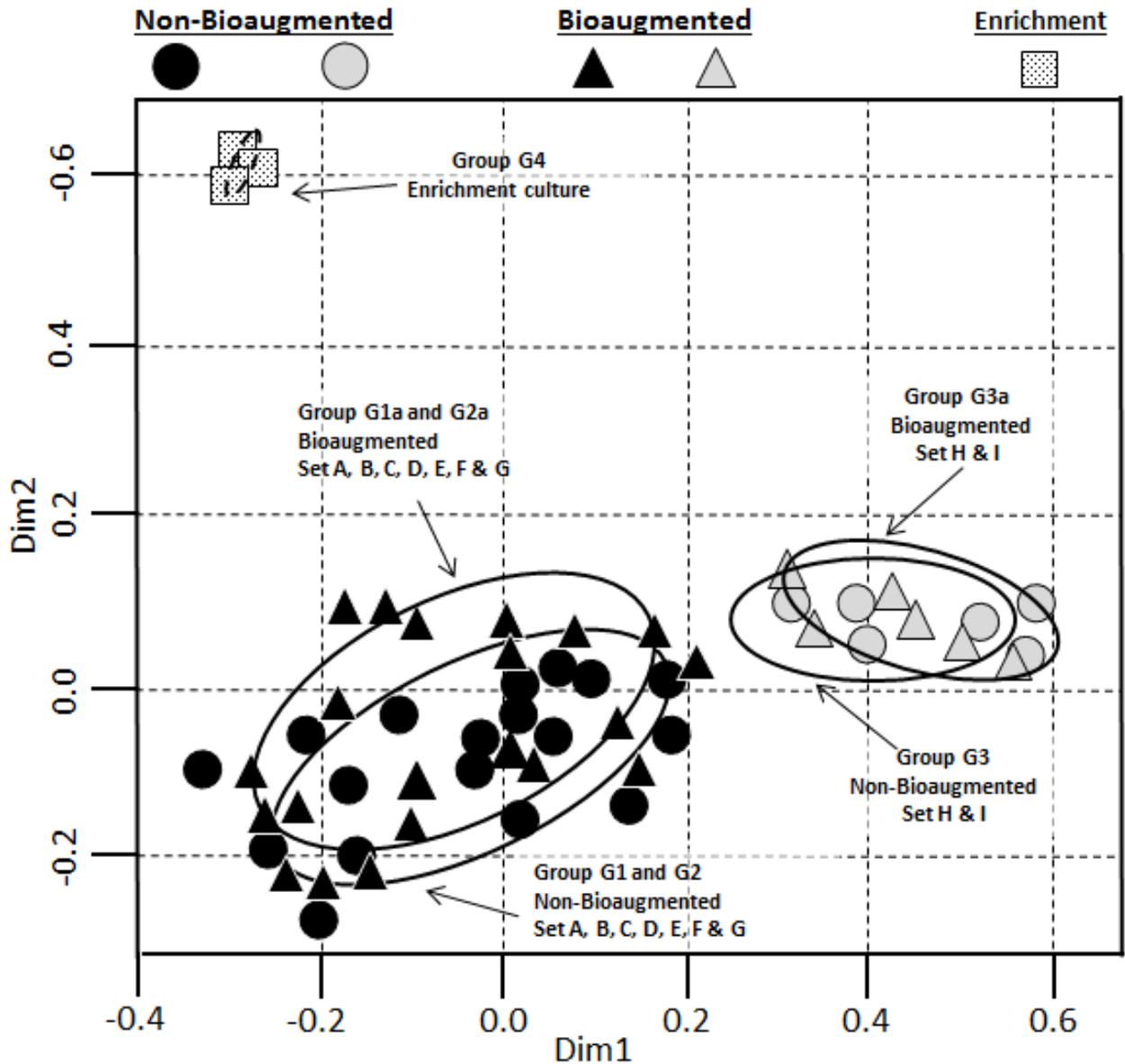
**Figure 2:** Digester group functional parameters.

(A) Average methane production rate (L-CH<sub>4</sub>/L<sub>R</sub>-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).



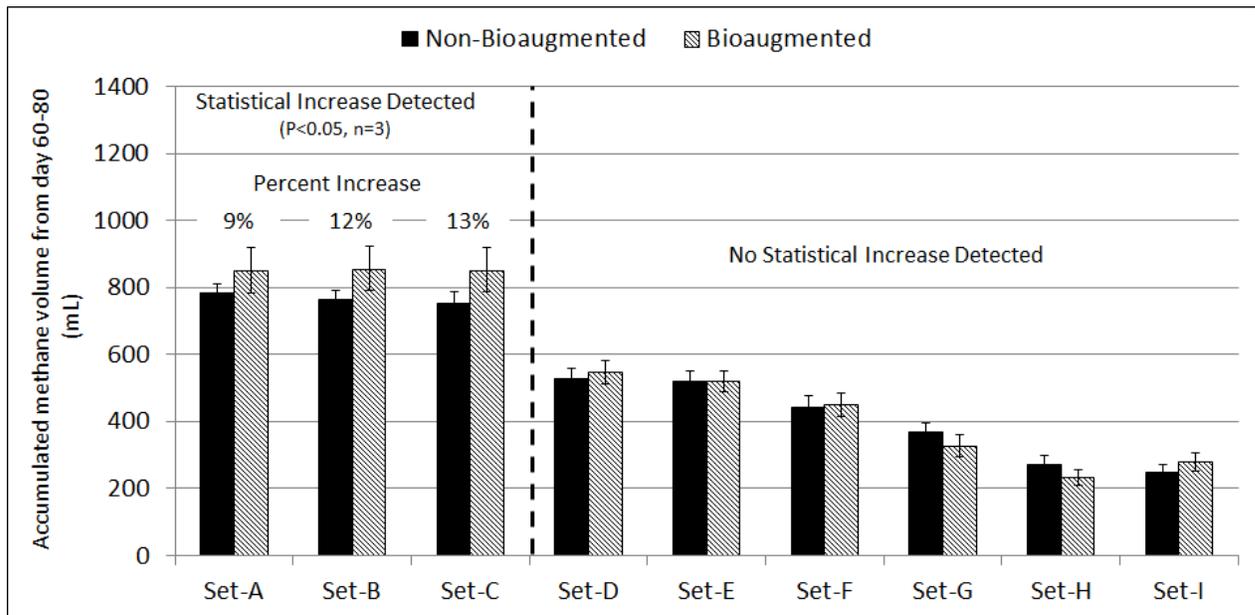
**Figure 3:** Archaeal sequence nMDS plot.

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.



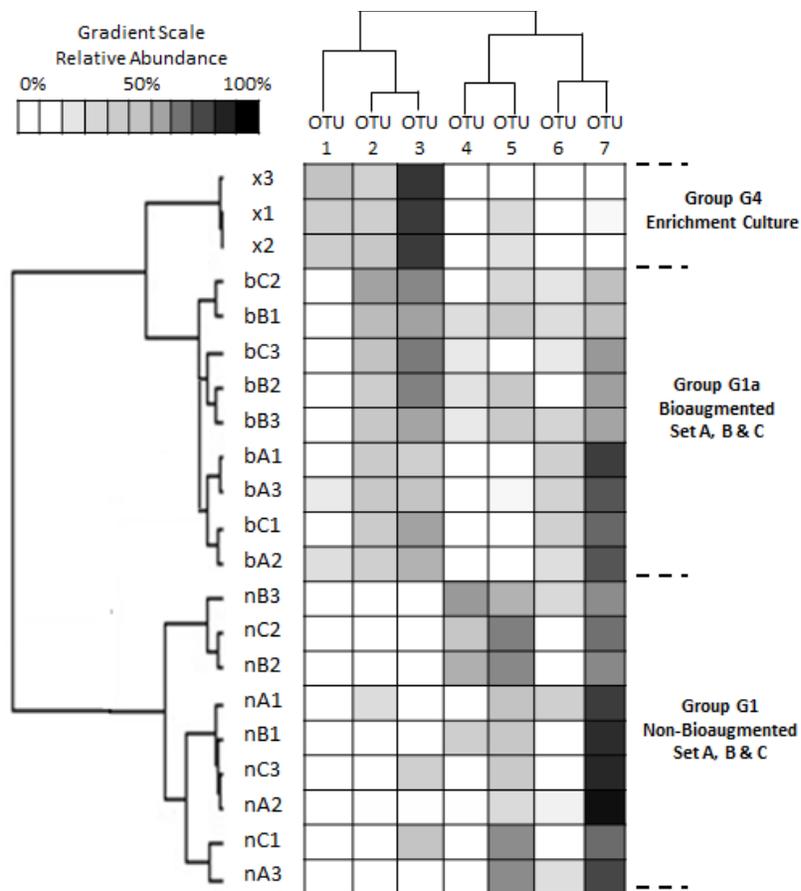
**Figure 4:** Bacterial sequence nMDS plot.

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<sub>4</sub> 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<sub>4</sub> production (p<0.05, n=3).

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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

**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