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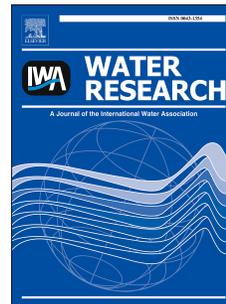
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1 **Anaerobic digester bioaugmentation influences quasi steady state**
2 **performance and microbial community**

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9 **Abstract**

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

25 *Methaospirillum* originating from the bioaugment culture. In conclusion, different
26 anaerobic digester seed biomass can result in different quasi steady state CH₄ 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 CH₄ 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 (H₂) is thermodynamically favorable only when the
54 partial pressure of the generated H₂ remains below 10⁻⁴ atm. Thus, degradation of
55 propionate requires a synergistic relationship between H₂ producing and H₂ consuming
56 microorganisms to maintain low H₂ concentrations (McCarty & Smith 1986). It was
57 shown previous that adding cultures enriched to consume H₂ or propionate to anaerobic

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

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

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

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 CH₄ 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 CH₄/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 O₂/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 NH_4Cl [400]; $\text{MgSO}_4 \cdot 6\text{H}_2\text{O}$ [250]; KCl [400]; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ [120]; $(\text{NH}_4)_2\text{HPO}_4$ [80];
127 $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ [55]; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ [10]; KI [10]; the trace metal salts $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, NH_4VO_3 ,
128 $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{Zn}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O}$, $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, H_3BO_3 , $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$,
129 $\text{NaWO}_4 \cdot 2\text{H}_2\text{O}$, and Na_2SeO_3 [each at 0.5]; yeast extract [100]; NaHCO_3 [6000]; and
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 μm 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 CH₄ per L of digester per day (L CH₄/L_R-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 ± 0.2 , $60\pm 3\%$ biogas methane
267 content, methane yield of 330 ± 16 mLCH₄/g-COD removed and observed biomass yield
268 of 0.08 ± 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, H₂, 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 CO₂ reduction
286 to CH₄ (Stams & Plugge 2009; Rotaru et al. 2014). Addition of an IRB consortium has
287 been shown to increase the methane production rate in anaerobic digesters (Baek et al.
288 2015).

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

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

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

308 order *WCHA1-57* (Figure S5) (Liu & Whitman 2008). Conversion of propionate to
309 methane only becomes thermodynamically favorable through H₂ 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 H₂/CO₂.
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.

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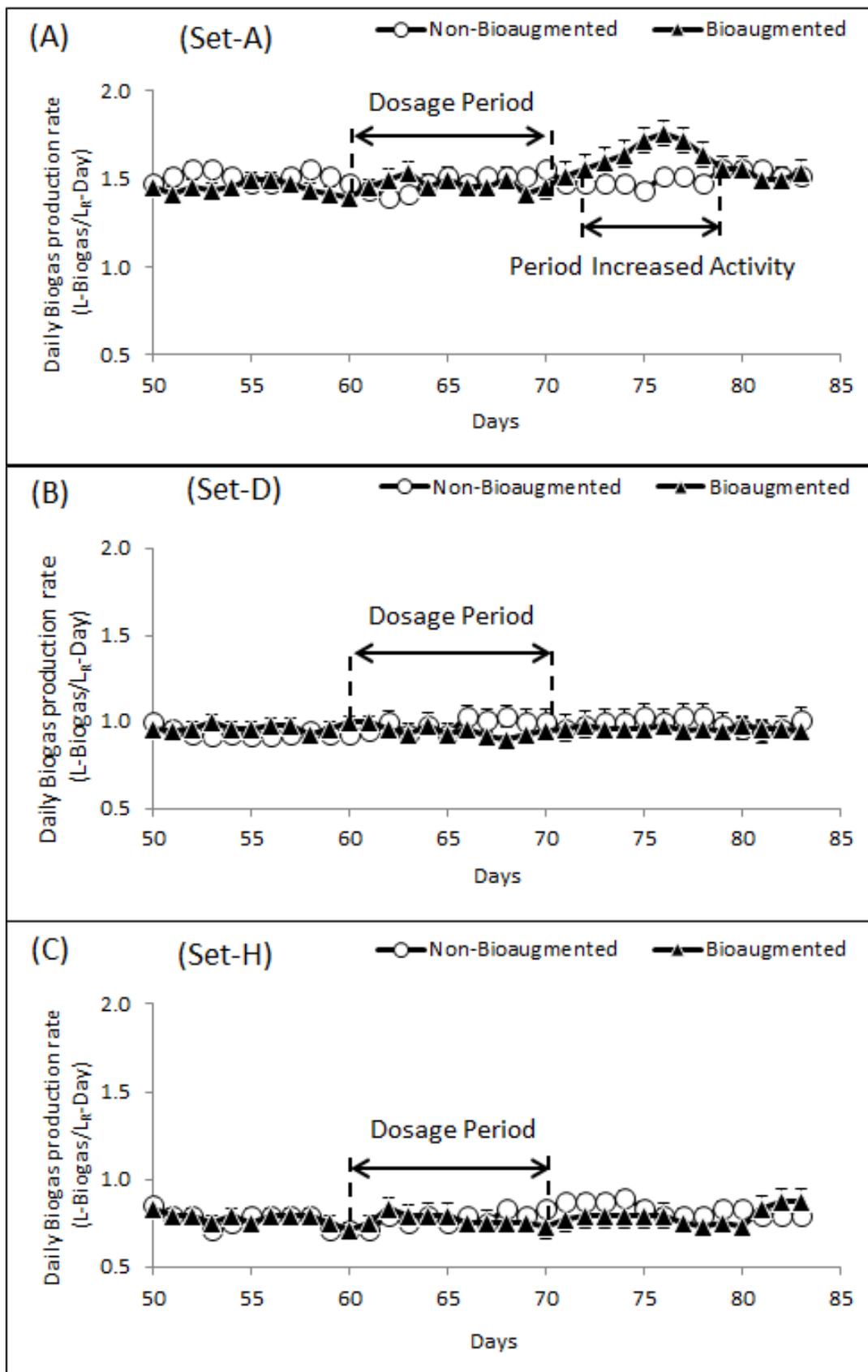
Table 1: Performance parameters of digester groups.

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

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Figure 1: Typical quasi steady state biogas production.

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

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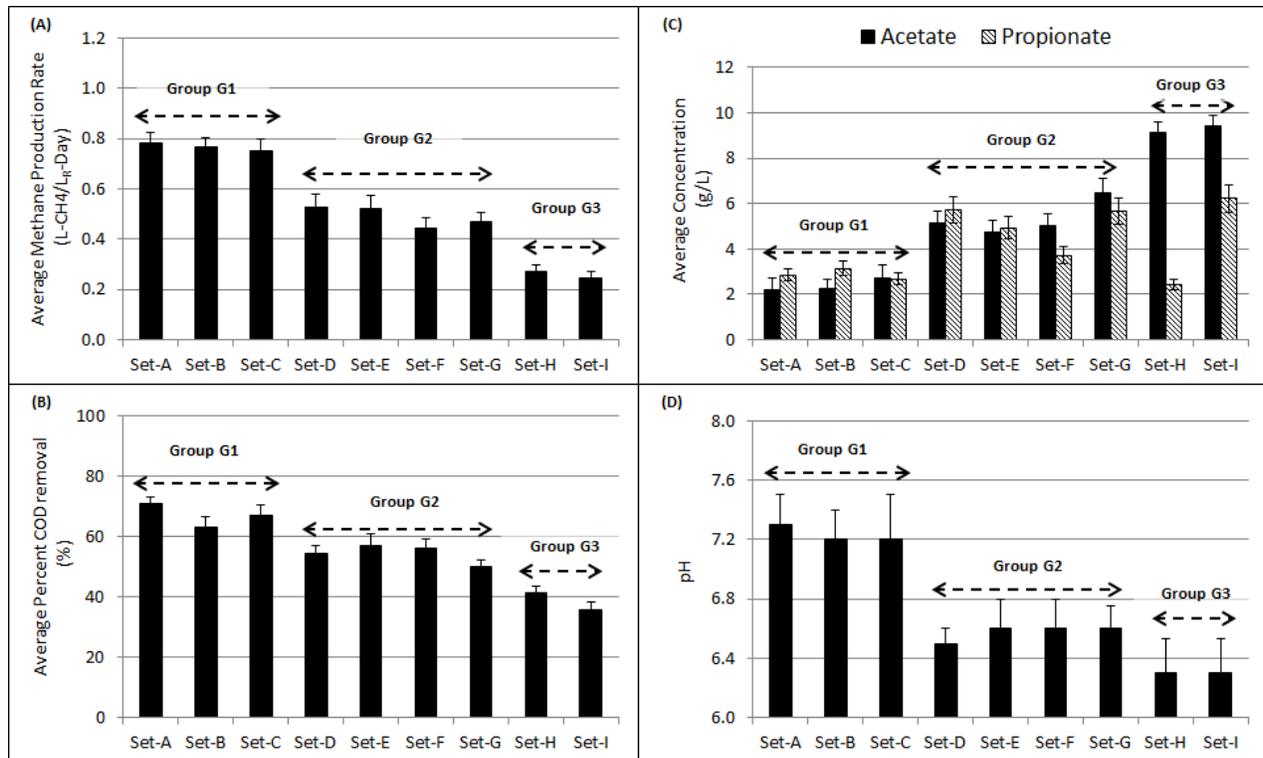


Figure 2: Digester group functional parameters.

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

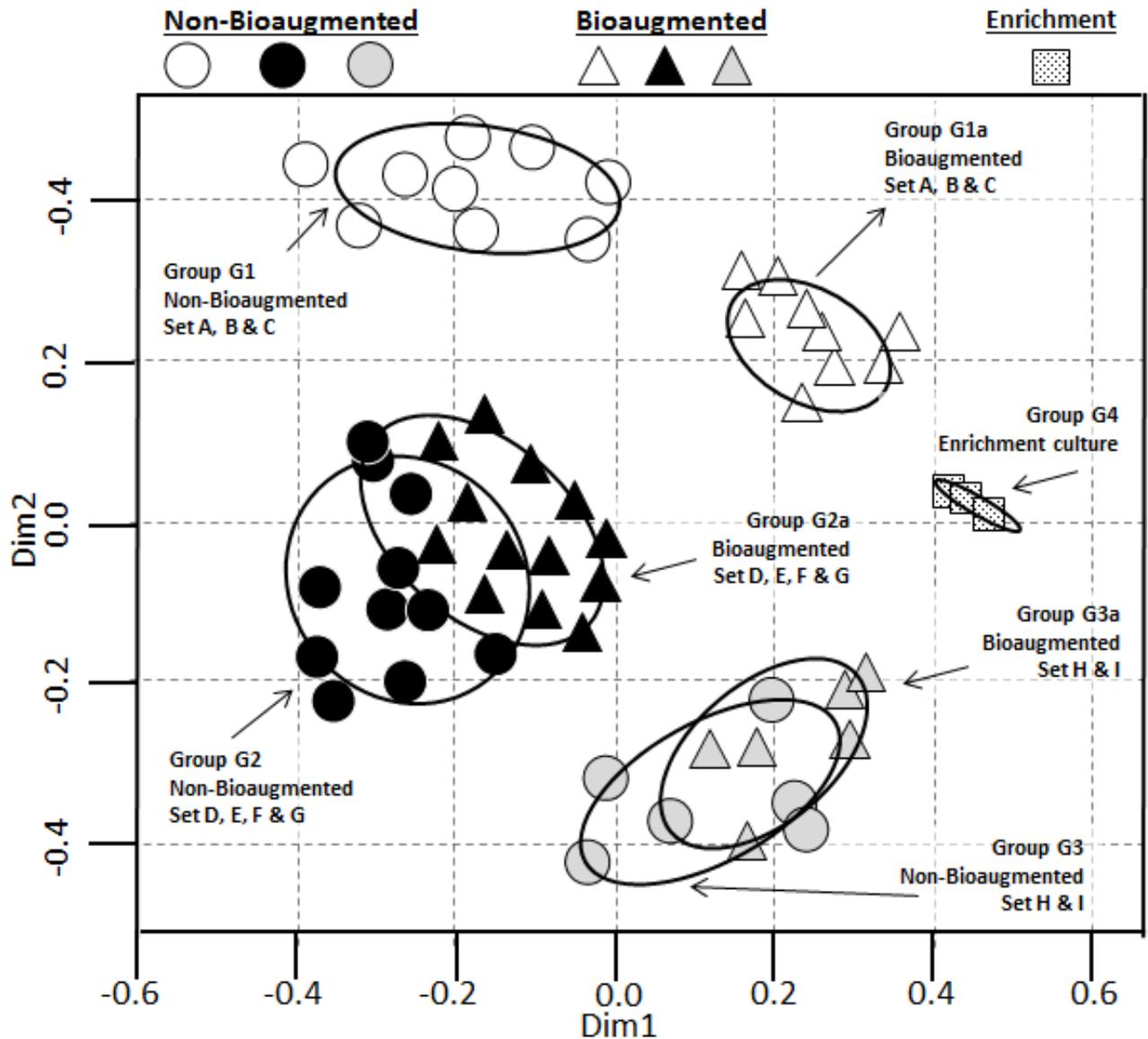


Figure 3: Archaeal sequence nMDS plot.

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

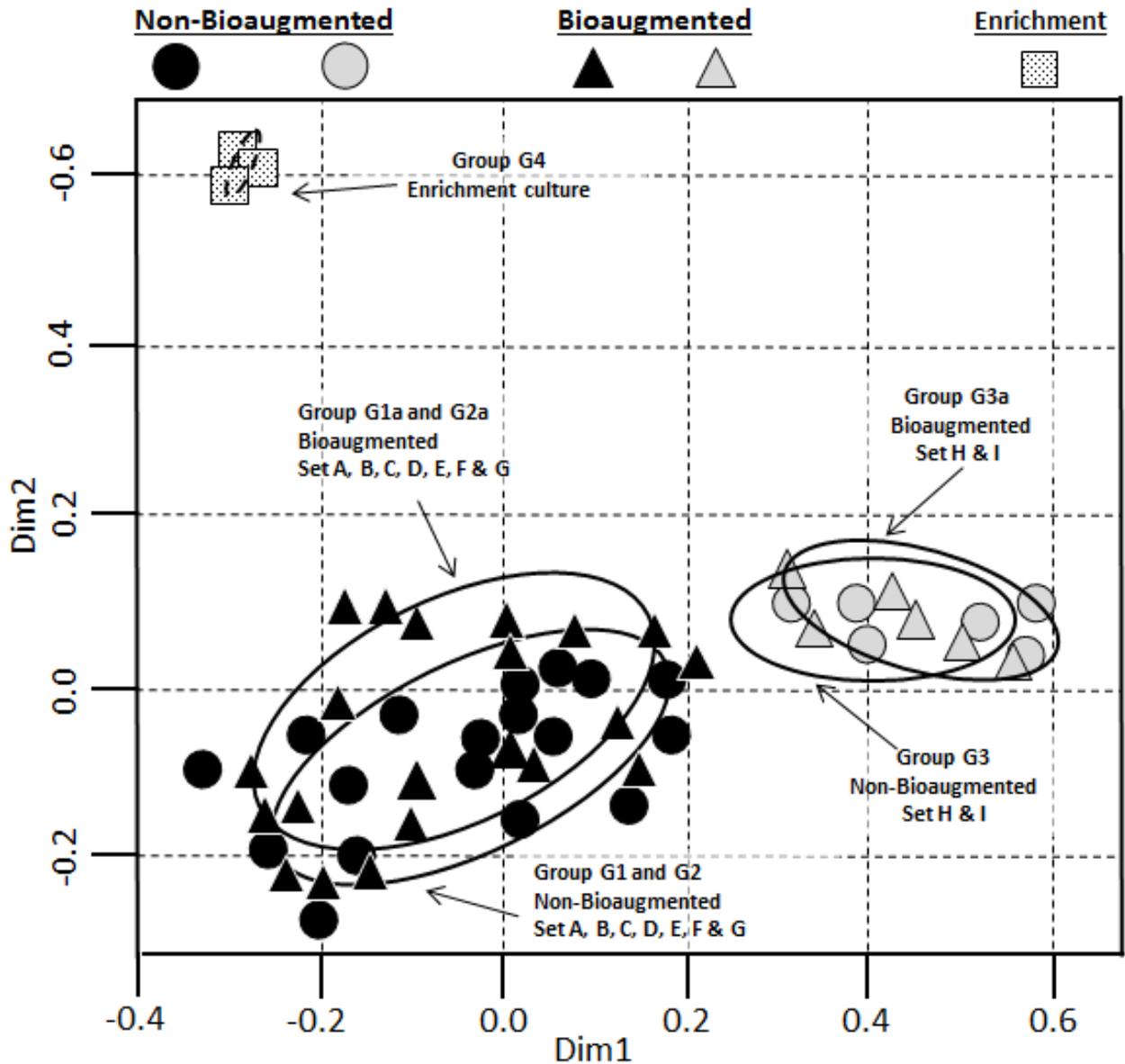


Figure 4: Bacterial sequence nMDS plot.

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

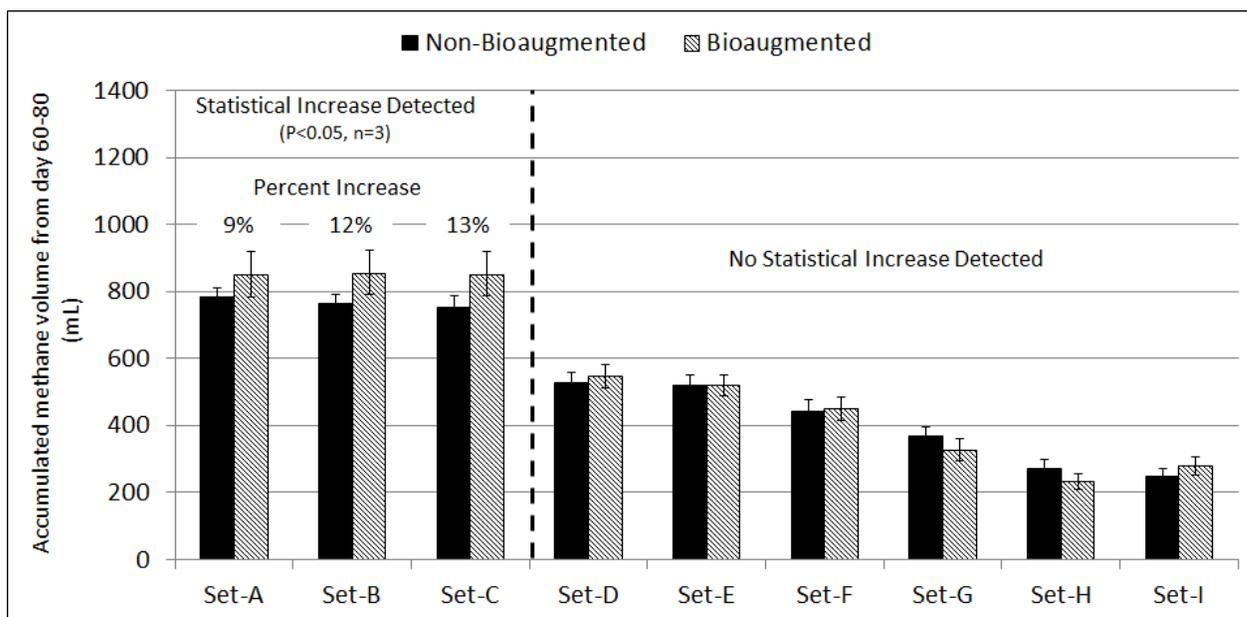
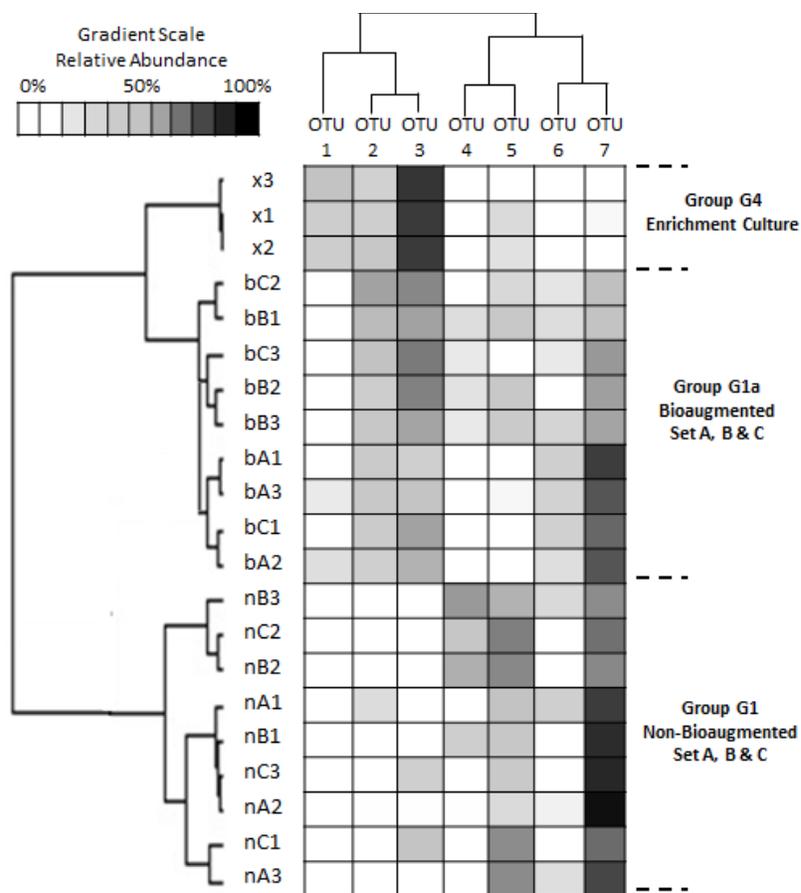


Figure 5: Cumulative CH₄ produced between day 60-80. Error bars represent standard deviation among triplicates. Bioaugmented digesters of Sets A, B and C showed statistically higher cumulative CH₄ production (p<0.05, n=3).

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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