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Bioaugmentation of Overloaded Anaerobic Digesters Restores Function and Archaeal Community

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Abstract: Adding beneficial microorganisms to anaerobic digesters for improved performance (i.e. bioaugmentation) has been shown to decrease recovery time after organic overload or toxicity upset. Compared to strictly anaerobic cultures, adding aerotolerant methanogenic cultures may be more practical since they exhibit higher methanogenic activity and can be easily dried and stored in ambient air for future shipping and use. In this study, anaerobic digesters were bioaugmented with both anaerobic and aerated, methanogenic propionate enrichment cultures after a transient organic overload. Digesters bioaugmented with anaerobic and moderately aerated

cultures recovered 25 and 100 days before non-bioaugmented digesters, respectively. Increased methane production due to bioaugmentation continued a long time, with 50–120% increases 6 to 12 SRTs (60–120 days) after overload. In contrast to the anaerobic enrichment, the aerated enrichments were more effective as bioaugmentation cultures, resulting in faster recovery of upset digester methane and COD removal rates. Sixty days after overload, the bioaugmented digester archaeal community was not shifted, but was restored to one similar to the pre-overload community. In contrast, non-bioaugmented digester archaeal communities before and after overload were significantly different. Organisms most similar to *Methanospirillum hungatei* had higher relative abundance in well-operating, undisturbed and bioaugmented digesters, whereas organisms similar to *Methanolinea tarda* were more abundant in upset, non-bioaugmented digesters. Bioaugmentation is a beneficial approach to increase digester recovery rate after transient organic overload events. Moderately aerated, methanogenic propionate enrichment cultures were more beneficial augments than a strictly anaerobic enrichment.

Keywords: Anaerobic digestion, Bioaugmentation, Organic overload, *Methanospirillum hungatei*

1. Introduction

Bioaugmentation is the practice of adding specialized microorganisms to biological systems for improved performance (Nyer and Bourgeois, 1980, Rittmann and Whiteman, 1994, Hairston et al., 1997, Maier et al., 2000, Deflaun and Steffan, 2002, Mulligan, 2002 and Evans and Furlong, 2003). The approach has been used for hazardous waste remediation as well as aerobic waste treatment, but not in full scale for anaerobic, methanogenic systems. In aerobic wastewater treatment, bioaugmentation has resulted in more reliable nitrification, improved sludge settling, enhanced grease degradation and accelerated transformation of xenobiotic organic contaminants (Rittmann and Whiteman, 1994).

Although bioaugmentation of full-scale anaerobic digesters has not been reported, it has been studied at laboratory scale to increase methane production from animal manure (Angelidaki and Ahring, 2000), distillery wastewater (Savant and Ranade, 2004), lipid-rich wastes (Cirne et al., 2006), sewage sludge mixed with pig manure (Bagi et al., 2007) and cellulose (Nielsen et al., 2007 and Weiss et al., 2010). Furthermore, bioaugmentation has decreased the recovery time of anaerobic digesters stressed by hydrogen sulfide (O'Flaherty et al., 1999 and O'Flaherty and Colleran, 1999) and oxygen (Schauer-

Gimenez et al., 2010). Production of individual bioaugmentation cultures, each enriched to degrade a specific substrate, would be time consuming. It may be more practical to target key, ubiquitous intermediates to improve anaerobic digestion. Acetate and propionate are reasonable targets since chronically elevated concentrations are often observed in anaerobic digesters during periods of low COD removal and low biogas production (Smith and McCarty, 1990). Adding propionate-utilizing enrichment cultures that can convert acetate and propionate to methane may lead to improved digestion.

Previous methanogenic bioaugmentation research involved adding strictly anaerobic cultures. Digesters bioaugmented with anaerobic propionate enrichment cultures after organic overload recovered approximately 25 days before non-bioaugmented digesters (Tale et al., 2011). In addition, benefits of bioaugmentation continued for more than 12 solids retention times (SRTs) after the transient overload. However, adding facultative or aerotolerant methanogenic cultures (i.e., cultures exposed to oxygen that produce measurable amounts of methane) to anaerobic digesters may be more practical and effective. Aerotolerant cultures consistently exhibited higher methanogenic activity before as well as after heat and freeze drying in air and after being held under conditions simulating 20 years of storage subsequent to drying (Bhattad et al., 2010 and Zitomer, 2013). This is beneficial when considering the production of commercial, dried bioaugmentation products; aerotolerant biomass can be easily processed in ambient air and still retain methanogenic activity.

The comprehensive review by Botheju and Bakke (2011) describe beneficial effects of adding limited oxygen during growth of methanogenic biomass. Methanogenic mixed cultures exposed to limited aeration still produce significant amounts of methane (Zitomer, 1995), and some low-aeration biomass exhibited 20% higher specific methanogenic activity (SMA) values than control cultures maintained anaerobically (Zitomer and Shrout, 1998). The higher SMA of aerotolerant cultures may result in a superior outcome when used for bioaugmentation to increase methane production rate.

In this study, bioaugmentation of anaerobic digesters with methanogenic, aerotolerant cultures enriched for propionate

degradation was investigated as a method to reduce recovery time following a transient organic overload. Results of bioaugmentation with aerotolerant and anaerobic cultures were compared. Differences in microbial community structure due to bioaugmentation were also determined.

2. Materials and methods

2.1. Enrichment cultures

Biomass samples from 14, full-scale anaerobic digesters were assayed for SMA against propionate (Tale et al., 2011). The highest SMA value was observed for biomass from an upflow anaerobic sludge blanket (UASB) digester treating brewery wastewater (City Brewery, LaCrosse, Wisconsin). Because of its high activity, this biomass was subsequently enriched by feeding propionate under four conditions that differed based on air addition rate, with individual oxygen loading rates of 0, 25, 125 and 225 mg O₂ per L of reactor per day (mg O₂/L-day). These oxygen loadings were equivalent to 0, 10, 50 and 90% of the COD organic loading rate (OLR), and cultures were designated as Enrichment 0, Enrichment 10, Enrichment 50 and Enrichment 90 (E0, E10, E50 and E90), respectively. Each Culture was maintained in triplicate.

Enrichments were maintained in 750 mL serum bottles containing 150 mL of culture. Every day, 10 mL was removed via plastic syringe and replaced with an equal volume of medium to maintain an SRT and hydraulic residence time (HRT) of 15 days. All enrichments received 0.17 g propionate/L-day (0.25 g COD/L-day) in basal nutrient medium. Before feeding, the volume of biogas in each enrichment was measured at 35 °C and atmospheric pressure by inserting a needle and glass syringe with wetted glass barrel through the serum bottle septa. The excess gas was then released, wasting and feeding were completed, an appropriate volume of air was added using a syringe, and the bottles were placed on a shaker (150 rpm) in a temperature-controlled room at 35 ± 3 °C. The culture volumetric air doses were 0, 14, 68 and 124 mL air/L-day (35 °C, 1 atm), respectively. These enrichment cultures were subsequently used to bioaugment organically overloaded digesters.

2.2. Anaerobic digesters

Anaerobic digesters were 160 mL serum bottles containing 50 mL of active volume incubated on a shaker table in a temperature-controlled room at 35 ± 2 °C. Seed biomass was taken from a laboratory-scale anaerobic, methanogenic system fed non-fat dry milk for over three years and originally seeded with biomass from a municipal anaerobic digester stabilizing primary sludge (South Shore Water Reclamation Facility, Oak Creek, Wisconsin, USA). Digesters were operated at a 10 day SRT and HRT by removing 5 mL of digester contents every day and adding an equal volume of feed. Volatile suspended solids concentration of 4.5 ± 0.1 g/L was maintained in the digesters. Wasting and feeding were performed by inserting a needle with a plastic syringe through serum bottle septa. Biogas production was measured daily by inserting a needle with a glass syringe and wetted glass barrel through serum bottle septa, and excess biogas was wasted to the atmosphere or used for biogas methane content analysis.

Digesters were fed synthetic industrial waste composed of non-fat dry milk in basal medium at an OLR of 2.7 g COD/L-day for the first 30 days (i.e., 3 SRTs). Subsequently, an organic overload of non-fat-dry milk (OLR = 32 g COD/L-day) was given for one day to all the digesters except the undisturbed controls (UCD). Following the organic overload, bioaugmented digesters were provided with a daily dose of enrichment culture (1.7 mL/day equivalent to 70 mg VSS/L-day). The bioaugment volatile suspended solids (VSS) dose was 1.5% per day of the digester VSS mass before bioaugmentation. During bioaugmentation the total daily volume fed (synthetic wastewater plus bioaugment or autoclaved augment) was kept at 5 mL/day to maintain a 10 day SRT and the synthetic wastewater OLR remained at 2.7 g COD/L-day.

Test Digester 0 (TD0) refers to the digester set that was bioaugmented with E0, whereas TD10, TD50 and TD90 refer to the digester sets that were bioaugmented with E10, E50 and E90, respectively. Non-bioaugmented, control digesters were also maintained, and were provided with 1.7 mL/day of an autoclaved version of each enrichment culture. This addition of autoclaved

biomass was estimated to increase the feed COD by a negligible amount (<3.6%). Non-bioaugmented Control Digester 0 (CD0) received autoclaved E0, whereas CD10, CD50 and CD90 refer to non-bioaugmented control digesters that received autoclaved versions of E10, E50 and E90, respectively.

2.3. Propionate activity tests

SMA against propionate was determined after 580 days of enrichment culture maintenance using a standard protocol (Sorensen and Ahring, 1993). Assays were conducted in 160 mL glass serum bottles. Both test and control assays were run in triplicate using 25 mL of diluted biomass having less than 2 g VSS/L in basal media. Test assays were supplied with 3 g/L of propionate in the form of calcium propionate. Control assays contained no substrate and the biogas generated accounted for endogenous methane production. Headspace was flushed with a nitrogen and carbon dioxide gas mixture (7:3 v:v N₂:CO₂). Bottles were incubated at 35 ± 2 °C and continuously shaken at 150 rpm using a gyratory shaker-incubator. Gas production was monitored every 12 h using a syringe displacement method over a period of 30 days. After each volume measurement, the biogas was reinjected into the assay bottle. At the end of the test, biogas methane concentration was measured. SMA was calculated by measuring the maximum methane production rate divided by total VSS mass in the bottles. The methane produced due to endogenous decay of the sludge was determined using the control assays. Methane produced by the control assays was subtracted from the methane produced by the test assays to determine the methane produced by propionic acid degradation. Biomass VSS concentration was measured before and after the test using standard methods (APHA et al., 1998); the average of initial and final VSS concentrations was used to calculate SMA.

2.4. Basal nutrient medium

Basal nutrient medium, as described by Speece (2008), contained the following [mg/L]: NH₄Cl [400]; MgSO₄·6H₂O [250]; KCl [400]; CaCl₂·2H₂O [120]; (NH₄)₂HPO₄ [80]; FeCl₃·6H₂O [55]; CoCl₂·6H₂O [10]; KI [10]; the trace metal salts MnCl₂·4H₂O, NH₄VO₃,

$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{Zn}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O}$, $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, H_3BO_3 , $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{NaWO}_4 \cdot 2\text{H}_2\text{O}$, and Na_2SeO_3) [each at 0.5]; yeast extract [100]; NaHCO_3 [5000]; and resazurin [1].

2.5. Analytical methods

Digester effluent pH was measured using a glass electrode as described in standard methods (APHA et al., 1998). Soluble effluent COD (SCOD) was measured by filtering the sample through a 0.45 μm pore size membrane filter and determining the filtrate COD by standard methods (APHA et al., 1998). Biogas methane concentration was measured by gas chromatography using a thermal conductivity detector and either GOW-MAC or Agilent instruments by standard methods (APHA et al., 1998). Effluent volatile fatty acid (VFA) concentrations in UCD, TD0 and CD0 were measured by gas chromatography (GC System 7890A, Agilent Technologies, Irving, TX, USA) using a flame ionization detector; VFAs in other digesters were not measured. Two-sample Student's *t*-test with unequal variance was used to compare results of bioaugmented and non-bioaugmented digester SCOD concentrations and methane production rates.

2.6. Microbial community analyses

Microbial community structure was evaluated for the enrichment culture receiving 25 mg $\text{O}_2/\text{L-day}$ (E10), the test digester bioaugmented with E10 (TD10), the control digester augmented with an autoclaved version of E10 (CD10), and the undisturbed control digester (UCD). Effluent samples from digesters were collected on day 120 (6 SRTs following the organic overload) for DNA extraction.

Clone libraries were constructed for the *16S rRNA* gene of archaea. DNA was extracted using a commercial kit according to the manufacturer's instructions with the alternative lysis method (PowerSoil™ DNA Isolation Sample Kit, MoBio Laboratories, Inc., Carlsbad, CA). DNA was amplified by polymerase chain reaction (PCR) in a thermal cycler (PTC-200 DNA Engine Cycler, Bio-Rad Laboratories, Hercules, CA) using 50 μL of a commercial master mix (EconoTaq® PLUS 2× Master Mix, Lucigen Corporation, Middleton, WI). One μL per reaction of either forward or reverse primer solution (0.1 μM) was

added to PCR tubes with nuclease-free H₂O to make a 100 µL reaction volume. The archaea primers used for amplification of the *16S rRNA* gene were A571 (5'-GCT TAA AGS RIC CGT AGC -3') and UA1406R (5'-ACG GGC GGT GWG TRC AA -3') (Baker et al., 2003).

PCR products were cloned using a commercial kit according to the manufacturer's instructions (TOPO TA Cloning® Kit, Invitrogen, Carlsbad, CA). Transformants containing plasmids with amplified product were screened via blue/white selection (Sambrook and Russell, 2001). Light-colored colonies were picked and DNA was PCR amplified using PucF (5'-GGA ATT GTG AGC GGA TAA CA- 3') and PucR (5'- GGC GAT TAA GTT GGG TAA CG - 3') primers. Individual clone samples were processed using a clean-up kit (UltraClean™ PCR Clean-up™ Kit, MoBio Laboratories, Carlsbad, CA) and the purified products were sequenced at the University of Chicago Cancer Research Center DNA Sequencing Facility using universal forward and reverse primers and a capillary automated DNA sequencer (Applied Biosystems, Foster City, CA).

2.7. Bioinformatics for community analysis

Forward and reverse sequences were analyzed using the FinchTV, v 1.4.0 (Geospira Inc., Seattle, WA) and Vector NTI, v 11.0 (Invitrogen Corporation, Carlsbad, CA) software. Contiguous sequences were assembled for each clone using forward and reverse sequences. Vector segments from the contiguous sequences were removed using a tailor-made computer program that searched the UniVec database of the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/VecScreen/UniVec.html>) using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) for cloning vectors. If there was a match in the UniVec database sequences and the submitted query sequence then the program removed vector segments. Chimera detection analysis was performed using Chimera Check, v 2.7 (Cole et al., 2005) and Bellerophon (Huber et al., 2004). Unaligned sequences were submitted to online versions of Chimera Check and Bellerophon tools. For Bellerophon, a window size of 200 was selected and no distance correction method was applied. The identified chimera sequences were removed from the data.

Valid sequences were submitted to the Ribosomal Database Project (RDP) (Cole et al., 2005 and Cole et al., 2007) for RDP seqmatch query. Also, the sequences were aligned using RDP server which used the program Infernal, v 1.0 (Nawrocki et al., 2009) to align *16S rRNA* gene sequences based on their secondary RNA structure. The resulting alignment was downloaded from the RDP server (<http://rdp.cme.msu.edu/>).

Distance matrices were calculated from the alignment using the DNADIST algorithm of the Phylogeny Inference Package (PHYLIP, v 3.68) (Felsenstein, 2005). All distances were calculated by the Kimura "2-parameter" model that assigns different probabilities for the transitions and transversions for a nucleotide base change.

Operational taxonomic units (OTUs) were formed from the distance matrices using the DOTUR program, v 1.53 which assigned sequences to OTUs by the farthest neighbor algorithm (Schloss and Handelsman, 2005). A criterion of 99% sequence similarity was used to form OTUs. DOTUR was also used to plot rarefaction curves and calculate the coverage, richness and diversity values S_{ACE} (Chao and Yang, 1993) and Shannon-Weaver Index (Shannon and Weaver, 1963).

Unique sequences were submitted to the NCBI server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for nucleotide (BLASTN) search to find the closest related microorganisms in the database. Subsequently, DNA sequences of the closest related organisms from the database were collected to use as reference organisms to construct phylogenetic trees. The selected reference sequences and the sample sequences were re-aligned using the RDP server (Cole et al., 2007). Neighbor-joining trees were created using the Phylogeny Inference Package (PHYLIP, v 3.68) (Felsenstein, 2005) and bootstrap analysis was done for 100 replications. Consensus trees generated by bootstrap analysis were visualized using FigTree v1.2.2 software (Rambaut, 2008). Comparisons among different tree structures were done using the TreeClimber software package (Schloss and Handelsman, 2006).

To perform principal components analysis (PCA) on the archaeal clone libraries, all the archaeal clones were combined and the distance matrix for the combined library was calculated using the RDP aligner

and the DNADIST algorithm of the PHYLIP Package (PHYLIP, v 3.68) (Felsenstein, 2005). All the distances were calculated by the Kimura "2-parameter" model. The resulting distance matrix was submitted to the DOTUR program (v. 1.53) and OTUs were formed using a 99% similarity criterion. The contribution of each sample to individual OTU was determined by calculating the ratio of the number of clones present in that OTU from a particular sample and the total number of clones of that OTU. These fractions were used as dimensions to perform PCA using the MATLAB v.7.6 (R2008a) software package which calculated a covariance matrix for the given data. The first two coordinates that represented the highest amount of variation among the data were determined. These two principal components were used to plot the data. The samples were clustered within the two-dimensional space using the farthest neighbor algorithm. Projections of the dimensions that demonstrated the highest contribution to the principal components variation were also projected on the data plots.

3. Results and discussion

3.1. Activity of enrichment cultures

SMA values of enrichment cultures varied over two orders of magnitude and the SMA of culture receiving 25 mg O₂/L-day (E10) was 30% higher than that of the strictly anaerobic culture ($p = 0.11$) (Fig. S1). In contrast, oxygen doses greater than 25 mg O₂/L-day resulted in decreased average SMA, with 225 mg O₂/L-day (E90) resulting in a 90% decrease in SMA compared to the anaerobic culture ($p = 0.015$) (Fig. S1). Low aeration could have caused a shift in the microbial community resulting in higher SMA values. Others have observed higher methanogenic activity for biomass exposed to limited aeration (Zitomer and Shrout, 1998). The higher doses of oxygen (>25 mgO₂/L-day) putatively decreased the SMA of the enrichment culture by producing a higher fraction of aerobic or micro-aerobic biomass or inhibiting the growth of the oxygen-sensitive methanogens. Additional work is required to determine the exact mechanism causing higher SMA values in the 25 mg O₂/L-day enrichment (E10).

3.2. Recovery of overloaded digesters: substrate

Bioaugmentation of transiently overloaded digesters with the strictly anaerobic enrichment culture was previously found to result in more rapid recovery of methane production and lower SCOD_e (Tale et al., 2011). The purpose of the current investigation was to evaluate the affect of oxygen addition on propionate enrichment cultures, their bioaugmentation ability and changes in microbial community structure during bioaugmentation.

Immediately following the organic overload, the pH of all overloaded digesters dropped to 6.8 ± 0.05 ; however, the average, pH of the digesters was 7 ± 0.08 during the entire study (Fig. S2). All digesters required 40 days (4 SRTs) to attain an initial, quasi steady-state SCOD_e concentration of 290 ± 150 mg/L (Fig. 1). The shock organic overload occurred on Day 57, resulting in elevated SCOD_e (5000 ± 750 mg/L) for all overloaded digesters and a subsequent, 17-SRT recovery period spanning Days 58–220 (Fig. 1). Control digesters CD10, CD50 and CD90 receiving autoclaved augment recovered with a consistent SCOD_e decrease over time, whereas CD0 demonstrated a period of sustained, high SCOD_e from days 90–160 (Fig. 1a). The different SCOD_e patterns over time may be due to differences among the autoclaved cultures added to each CD digester.

Fig. 1. Digester Effluent Soluble COD. a. Anaerobic Enrichments (E0) UCD (◆), TD0 (○) and CD0 (■), b. 10% O₂ Enrichments (E10) UCD (◆), TD10 (○) and CD10 (■), c. 50% O₂ Enrichments (E50) UCD (◆), TD50 (○) and CD50 (■), and d. 90% O₂ Enrichments (E90) UCD (◆), TD90 (○) and CD90 (■). Error bars in Fig. 1a, b represent standard deviations among three replicates.

During the recovery period, SCOD decreased more rapidly in bioaugmented as compared to non-bioaugmented digesters. Lower

SCOD_e due to bioaugmentation became noticeable less than 5 days after organic overload for digesters bioaugmented with 25 mg O₂/L-day enrichment (Fig. 1b), whereas a longer time passed (20–50 days) before differences were observed for digesters bioaugmented with other cultures (Fig. 1).

Effluent acetic, butyric and iso-valeric acid concentrations were significantly lower in undisturbed and bioaugmented digesters (UCD and TD0) compared to nonbioaugmented controls (CD0) ($p < 0.01$) from days 167–200 (Fig. S3); subsequently, all digesters recovered and no significant differences were observed after day 260. As much as 70% of SCOD_e in the non-bioaugmented digesters was due to VFAs (40% from the sum of acetate and propionate alone), whereas less than 30% of the SCOD_e present in the bioaugmented and undisturbed digesters was due to VFAs (Figs. S3 and S4); this demonstrated more complete utilization of VFAs, including acetate and propionate, in overloaded, bioaugmented digesters compared to nonbioaugmented digesters.

All bioaugmented digesters exhibited new quasi steady-state SCOD_e concentrations after the recovery period (Fig. 1). In digesters bioaugmented with low-O₂ cultures (E0 and E10), the new average steady-state SCOD_e concentrations were relatively low (<500 mg/L) and not different from non-overloaded controls. In digesters bioaugmented with high-O₂ cultures (E50 and C90), however, the new steady-state SCOD_e concentrations were elevated (>500 mg/L) and higher than those of non-overloaded controls.

COD removal in digesters bioaugmented with aerated enrichments recovered more rapidly than digesters bioaugmented with the anaerobic enrichment (Fig. 2). The time required after organic overload for SCOD_e concentration to decrease below 1000 mg/L was selected as an endpoint of the COD removal recovery period. Digesters bioaugmented with aerated enrichments recovered 100 days (10 SRTs) before non-bioaugmented digesters, whereas digesters bioaugmented with the anaerobic enrichment recovered only 25 days before non-bioaugmented digesters ($p < 0.03$).

Fig. 2. Time to Attain Low Effluent COD After Overload. Low effluent COD is defined as less than 1 g/L. Error bars represent standard deviation among three replicates. Absence of error bars indicates that no replicates were maintained.

Bioaugmentation benefits were observed a long time after the organic overload event. Even after 6 to 12 SRTs had passed after the overload, bioaugmented digester COD_e was 45–80% lower than that of non-bioaugmented digesters (Fig. S5). After 15 SRTs, bioaugmented and nonbioaugmented digester COD_e concentrations were not statistically different; during the last quasi-steady state period of all digesters (final 30 days of operation), the sum of COD_e, COD equivalent of methane produced and COD of effluent VSS was $90 \pm 5\%$ of the influent COD (see Fig. S6). The bioaugmentation benefits observed are in contrast to the findings of others that bioaugmenting with cultures enriched for propionate and butyrate following an organic overload did not speed up recovery for prolonged periods (Lynch et al., 1987). The reason behind successful and prolonged recovery of shock overloaded digesters may be because, in the current study, the bioaugmentation cultures were supplied on a daily basis, whereas Lynch et al. (1987) supplied bioaugmentation culture only once leading to washout from the bioaugmented digester. The daily dose of bioaugment employed was relatively high, amounting to six 20 kg containers of dried bioaugment microorganisms added to a 2 million L digester. Future research should be performed to determine the minimum bioaugment dose required.

Moreover, digesters receiving bioaugmentation culture enriched with 25 mg O₂/L-day (E10) demonstrated the greatest decrease in

SCoDe concentration after overload compared to other bioaugmented digesters (Fig. 2 and S4). The reason behind the better performance may be the higher SMA associated with the bioaugmentation culture enriched for 25 mgO₂/L-day which may have led to more rapid metabolism of VFAs in the bioaugmented digesters following the organic overload.

3.3. Overloaded digester recovery: methane production

Digesters required about 40 days (4 SRTs) to reach an average quasi steady-state 32 ± 4 mLCH₄/day methane production rate (Fig. S7). During this period, average biogas methane content in all digesters was $59 \pm 4\%$ (v/v). Following organic overload on Day 57, methane production decreased to 18 ± 13 mLCH₄/day and the average biogas methane content in all the digesters decreased to $16 \pm 11\%$ (v/v). Daily methane production subsequently started to increase, and after about 180 days (i.e. 12 SRTs after organic overload), all digesters attained a new quasi steady-state average methane production value.

Digesters bioaugmented with low-O₂ enrichments (E0 and E10) required 28 and 37 days less to recover biogas production, respectively, compared to non-bioaugmented digesters ($p < 0.01$). The time required to attain 25 mLCH₄/day after overload was considered to be the biogas recovery period (Fig. 3). Digesters bioaugmented with high-O₂ enrichments (E50 and E90) took 32 and 22 days less to recover than non-bioaugmented digesters (Fig. 3).

Fig. 3. Effect of Enrichment Culture O₂ Dose on Biogas Production Rate Recovery Time. Error bars represent standard deviation among three replicates. Absence of error bars indicates that no replicates were maintained.

Increased biogas production due to bioaugmentation continued a long time after the organic overload, with bioaugmentation resulting in a 50–120% increase in methane production rate as long as 6 to 9 SRTs after organic overload (Fig. S8). Even after 12 SRTs, bioaugmentation resulted in a 6–18% increase in methane production rate.

Bioaugmentation with the culture acclimated to 25 mgO₂/L-day showed the highest increase in methane production rate after 6 and 9 SRTs following the organic overload. Moreover, the digesters receiving bioaugmentation culture enriched for 25 mg O₂/L-day exhibited a higher percent increase in methane production up to 6 SRTs after upset. The reason behind the better performance may be the higher SMA associated with the bioaugmentation culture enriched for 25 mgO₂/L-day, which may have led to more rapid VFA metabolism.

3.4. Archaeal diversity

Bioaugmented digesters (TD10) demonstrated lower archaeal clone diversity 6 SRTs after organic overload and recovered more rapidly compared to non-bioaugmented digesters (CD10) (Table 1). As indicated by the S_{ACE} and $H_{Shannon}$ values, the archaeal communities of the enrichment (E10) and non-bioaugmented digester (CD10) were more diverse than those of the undisturbed control (UCD) and

bioaugmented digester (TD10) (Table 1). Therefore, improved functionality due to bioaugmentation was concomitant with a less diverse, more specialized, dominant archaeal community for digesters recently recovered from organic overload. Since E10 was used to bioaugment TD10 on a daily basis, TD10 was expected to have a higher diversity of archaea similar to culture E10, but this was not the case.

Table 1. Archaeal clone libraries parameters.

	Number of good sequences obtained, classified under domain <i>Archaea</i>	Observed number of OTUs	Predicted number of OTUs by S_{ACE}^a	$H_{Shannon}$
UCD 66		5	5 (5–5)	0.76
TD10 68		4	4 (4–4)	0.93
CD10 57		11	17 (12–43)	1.57
E10 55		12	33 (16–127)	1.94

^aValues in the parenthesis indicate 95% confidence interval.

As indicated by Shannon-Weaver Index values, E10 had the highest archaeal clone diversity among the samples tested, followed by CD10, whereas TD10 and UCD had lower diversity (Table 1). For the library sizes, TD10 and UCD clone libraries attained good coverage, whereas E10 and CD10 clone libraries did not. According to S_{ACE} estimates, only 64% of the archaeal clone diversity of sample CD10 was covered, whereas 36% of the clone diversity of sample E10 was covered.

3.5. Composition of archaeal clone libraries

Archaeal clone sequences were most similar to *Methanomicrobiales*, *Methanosarcinales* uncultured *Euryarchaeota* and uncultured *Archaea* (Fig. 4). The closely related sequences were used to construct phylogenetic trees (Figs. S9 through S12).

Fig. 4. Archaeal communities.

Clones classified under *Methanomicrobiales* were most abundant, and include the hydrogenotrophic methanogens. A significant majority (>92%) of the *Methanomicrobiales* clones in all samples were most similar to *Methanospirillum hungatei* ($\geq 95\%$ similarity). The high relative abundance of clones similar to *M. hungatei* may be due, in part, to its high growth rate. In this regard, *M. hungatei* has the highest maximum specific growth rate among the hydrogenotrophic methanogens to which clones were most similar (Ferry and Wolfe, 1977). In addition, both TD10 and UCD had higher relative abundance of methanogens most similar to *M. hungatei* than CD10 and UCD. Higher *M. hungatei* abundance may have led to more rapid H_2 metabolism, more complete propionate degradation and,

thus, higher COD removal and faster recovery of TD10 after overload in comparison to CD10.

A lower number of clones in all samples were most similar to *Methanosarcinales* (family *Methanosataceae*), members of which metabolize acetate (Patel and Sprott, 1990 and Fernandez et al., 2000). Since methane production from propionate typically involves conversion of acetate to methane, the detection of organisms classified as *Methanosarcinales* (family *Methanosaetaceae*) in all communities was expected. However, clones similar to *Methanosarcinales* were not detected in TD10 and the rarefaction data (Fig. S13) shows that the archaeal clone library for TD10 covered nearly all the novel OTUs. Acetate-utilizing methanogens that are not yet known may have been included in the TD10 clone library classified under the uncultured *Euryarchaeota* (Fig. 4). Alternatively, acetate oxidation to H₂ and CO₂ may have been the dominant methanogenic pathway in TD10 (Karakashev et al., 2006). Additional research is required to determine the reasons for the possible lack of aceticlastic methanogens in TD10.

Greater than 25% of the archaeal clones from E10 were closely related to *Methanolinea tarda* (AB162774) (sequence similarity > 98%) which shows optimum growth at 50 °C and has a relatively high 4 day doubling time (Imachi et al., 2008).

3.6. Comparison of archaeal phylogenetic trees

Bioaugmentation did not permanently shift community structure, but provided reinforcement after the transient overload to help restore the original community. This coincided with more rapid recovery of methane production following the organic overload. For example, the archaeal communities of TD10 and UCD were not significantly different ($p = 0.55$) based on a pair-wise comparison of phylogenetic trees using TreeClimber software, and both demonstrated low SCOD concentrations after overload. However, the archaeal communities of TD10 and CD10 were significantly different ($p = 0.001$). The archaeal tree of enrichment culture E10 was unique compared to archaeal trees of bioaugmented and non-bioaugmented digesters as well as the undisturbed control ($p = 0.001$).

A plot of the first two principal components also showed that bioaugmentation using E10 helped restore the archaeal community to one more similar to the undisturbed control (UCD) (Fig. 5). For example, TD10 and UCD communities clustered together and were similar, whereas the archaeal communities of TD10 and CD10 did not cluster and were significantly different (Fig. 5). Projection of the original dimensions indicated that organisms related to *M. hungatei* had the highest relative abundance in UCD and TD10, whereas organisms related to *M. tarda* had higher relative abundance in CD10. Both *M. hungatei* and *M. tarda* are hydrogenotrophic methanogens, but *M. tarda* has an optimum temperature of 50 °C and a 4 day doubling time (Imachi et al., 2008), whereas *M. hungatei* is a mesophilic methanogen (35–40 °C optimum) (Yang et al., 1985 and Ferry, 1993) with high maximum specific substrate utilization and growth rates and a 0.7 day doubling time (Ferry and Wolfe, 1977). The higher relative abundance of organisms related to *M. hungatei* apparently resulted in higher COD removal rates of UCD and TD10 digesters.

Fig. 5. Graph of first two principal components for archaeal communities.

4. Conclusions

Methanogenic, propionate enrichment cultures maintained at low aeration (25 mg O₂/L-day) demonstrated higher specific methanogenic activity than the strictly anaerobic propionate enrichment cultures.

Bioaugmentation led to more rapid digester recovery in terms of SCOD removal, methane production and VFA removal during recovery from organic overload. Future research to reduce the bioaugment dose is warranted to help make bioaugmentation more feasible at full scale.

In contrast to the strictly anaerobic enrichment, the aerated methanogenic propionate enrichments were significantly more effective as bioaugmentation cultures, resulting in faster recovery of overloaded digesters.

Bioaugmentation did not permanently shift digester archaeal community structure, but provided reinforcement after the transient overload to help restore the pre-overload archaeal community. Organisms related to *M. hungatei* had the highest relative abundance in the well-operating, undisturbed and bioaugmented digesters after overload. In contrast, the relative abundance of organisms related to *M. tarda* was higher in upset digesters that were not bioaugmented. In this regard, high relative abundance of hydrogenotrophic *M. hungatei* can be advantageous since it has significantly higher maximum substrate utilization and growth rates than *M. tarda*.

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Appendix A. Supplementary data

The following is the supplementary data related to this article:
Supplementary Material

Figure S-1. Propionate Activity Versus Oxygen Loading for Enrichment Cultures

Error bars represent standard deviation among three replicates. Error bars smaller than symbols are not visible.

Figure S-2. Digester pH Values

a. Anaerobic Enrichments (E0) UCD (◆), TD0 (○) and CD0 (■), **b. 10% O₂ Enrichments (E10)** UCD (◆), TD10 (○) and CD10 (■), **c. 50% O₂ Enrichments (E50)** UCD (◆), TD50 (○) and CD50 (■), and **d. 90% O₂ Enrichments (E90)** UCD (◆), TD90 (○) and CD90 (■). Error bars in Figures S1a and S1b represent standard deviations among three replicates.

Figure S-3. Effluent Volatile Fatty Acids

Undisturbed control digesters (UCD), anaerobic bioaugmented digesters (TD 0) and non-bioaugmented control digesters (CD 0). (a) Effluent acetic acid concentration (b) Effluent propionic acid concentration (c) Effluent butyric acid concentration (d) Effluent iso-valeric acid concentration.

Figure S-6. Mass Balance During Final Quasi Steady State Operation

Final quasi steady state was during the last 30 days of digester operation. a. Undisturbed Control Digesters, b. Digesters Bioaugmented With Anaerobic Enrichment (E0) , c. Digesters Augmented With Inactivated Anaerobic Enrichment (E0) , d. Digesters Bioaugmented With 10% O₂ Enrichment (E10) , e. Digesters Augmented With Inactivated 10% O₂ Enrichment (E10), f. Digesters Bioaugmented With 50% O₂ Enrichment (E50), g. Digesters Augmented With Inactivated 50% O₂ Enrichment (E50), h. Digesters Bioaugmented With 90% O₂ Enrichment (E90), i. Digesters Augmented With Inactivated 90% O₂ Enrichment (E50)

Figure S-5. Effect of Enrichment Culture O₂ Dose on Percent Effluent Soluble COD Decrease of Bioaugmented Digesters

Figure S-7. Digester Methane Production

a. Anaerobic Enrichments (E0) UCD (◆), TD0 (○) and CD0 (■), b. 10% O₂ Enrichments (E10) UCD (◆), TD10 (○) and CD10 (■), , c. 50% O₂ Enrichments (E50) UCD (◆), TD50 (○) and CD50 (■), and d. 90% O₂ Enrichments (E90) UCD (◆), TD90 (○) and CD90 (■). Error bars in Figures S7a and S7b represent standard deviations among three replicates.

Figure S-8. Effect of Enrichment Culture O₂ Dose on Biogas Production Rate Recovery

Figure S-13. Rarefaction Curves for The Archaeal Clone Libraries

Figure S-9. Phylogenetic Analysis of Culture UCD Archaeal Clone Library. Calculations were based on neighbor-joining algorithm (bootstrap number = 100). Numbers at the node represent bootstrap values. The scale bar represents the number of nucleotide changes per sequence position. The tree was rooted to the organism *Sulfolobus solfataricus* (X03235).

Figure S-10. Phylogenetic Analysis of Culture TD10 Archaeal Clone Library

Calculations were based on neighbor-joining algorithm (bootstrap number = 100). Numbers at the node represent bootstrap values. The scale bar represents the number of nucleotide changes per sequence position. The tree was rooted to the organism *Sulfolobus solfataricus* (X03235).

Figure S-11. Phylogenetic Analysis of Culture CD10 Archaeal Clone Library Calculations were based on neighbor-joining algorithm (bootstrap number = 100). Numbers at the node represent bootstrap values. The scale bar represents the number of nucleotide changes per sequence position. The tree was rooted to the organism *Sulfolobus solfataricus* (X03235).

Figure S-12. Phylogenetic Analysis of Culture E10 Archaeal Clone Library
Calculations were based on neighbor-joining algorithm (bootstrap number = 100). Numbers at the node represent bootstrap values. The scale bar represents the number of nucleotide changes per sequence position. The tree was rooted to the organism *Sulfolobus solfataricus* (X03235).

The numbers at the nodes represent the bootstrap values. The non-bold taxa identify the reference sequences (both cultured and uncultured), followed by their GenBank accession number. The sample sequences are shown in bold-type font and are designated by the name of the culture following the letter “A” for archaea and the OTU number. Only one sequence per OTU is shown and the

number of sequences represented by that particular sequence is mentioned in a bracket at the end of sequence designation. The scale at the bottom of the tree indicates the number of nucleotide changes per sequence position. All the archaeal trees were rooted to the organism *Sulfolobus solfataricus* (X03235). Classification of all the OTUs based on RDP classifier (Wang et al., 2007; Cole et al., 2007) is presented at the right side of the trees.