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Article

Microbial Community Response to Seasonal Temperature Variation in a Small-Scale Anaerobic Digester

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Abstract: The *Bacterial* and *Archaeal* communities in a 1.14 m^3 ambient temperature anaerobic digester treating dairy cow manure were investigated using terminal restriction fragment length polymorphisms (T-RFLP) and direct sequencing of the cloned polymerase chain reaction (PCR) products. Results indicate shifts in the structure of the both the *Archaeal* and *Bacterial* communities coincided with digester re-inoculation as well as temperature and loading rate changes. Following re-inoculation of the sour digester, the predominant *Archaea* shifted from *Methanobrevibacter* to *Methanosarcina,* which was the most abundant *Archaea* in the inoculum. *Methonosarcina* was replaced by *Methanosaeta* after the resumption of digester loading in the summer of 2010. *Methanosaeta* began to decline in abundance as the digester temperature cooled in the fall of 2010 while

Methanobrevibacter increased in abundance. The microbial community rate of change was variable during the study period, with the most rapid changes occurring after re-inoculation.

Keywords: anaerobic digestion; fixed-dome digester; microbial communities; psychrophilic; T-RFLP

1. Introduction

Anaerobic digestion is the microbial degradation of organic matter in the absence of molecular oxygen and is utilized as a method to manage livestock wastes and generate renewable energy in the form of methane biogas. During anaerobic digestion, complex organic molecules such as carbohydrates, proteins and fats are transformed through a multistep biochemical pathway to produce methane, carbon dioxide and inorganic nutrients [1]. The microbial community responsible for the anaerobic digestion is composed of species from the domains *Bacteria* and *Archaea*. In the first stage of anaerobic digestion, hydrolysis, *Bacteria* convert carbohydrates, lipids and proteins to simple sugars, fatty acids and amino acids. These products are then converted to short chain fatty acids through the process of acidogenesis. These compounds are then further degraded to acetic acid, carbon dioxide (CO_2) and hydrogen (H_2) through the process of acetogenesis. In the final stage of anaerobic digestion, methanogenesis, the *Archaea* utilize these products to produce methane and carbon dioxide [2].

More than 30 million households in tropical and temperate regions of Asia and Latin America use small-scale, ambient temperature fixed-dome and Taiwanese model anaerobic digesters as energy sources [3,4]. In temperate climates these systems typically suffer from reduced biogas yield, which is often accompanied by souring of the digester during winter [5]. A better understanding of the microbial community dynamics during seasonal temperature shifts may offer insight into better management of the digesters during seasonal temperature changes and result in increased energy production. Improving the performance of low-cost and easy to operate digesters such as the fixed-dome may make the benefits of anaerobic digestion available to a larger group of farmers. Many digester designs are not used on farms due to the need for skilled operators and additional labor [6].

Because the majority of previous studies on microbial communities in anaerobic digesters have been completed at constant temperatures in the mesophilic (30–40 °C) and thermophilic (45–60 °C) ranges [5], there is a lack of knowledge about the microbial processes that produce biogas in the psychrophilic (<20 °C) temperature range and how the structure and function of the microbial community changes with temperature [2,5]. There is also insufficient knowledge about the structure and dynamics of *Bacterial* and *Archaeal* communities in anaerobic digesters [7]. The dynamics of community change are also unclear in ambient temperature systems digesting livestock manure. Despite the development of a parameter that quantifies the percent change in a microbial community per unit time [8], it is still not clear how quickly microbial communities respond to changes in temperature and digester operation.

The objective of this study was to determine how the microbial community structure varies with seasonal temperature variation and how these changes impact digester performance. The composition of both the *Bacterial* and *Archaeal* communities in a 1.14 m^3 ambient temperature anaerobic digester

treating dairy cow manure were determined by evaluating terminal restriction fragment length polymorphisms (T-RFLP) and direct sequencing of the cloned polymerase chain reaction (PCR) products.

2. Results and Discussion

2.1. Digester Performance

At the beginning of the study period in April 2010, the digester was in a state of poor performance. With an average internal temperature below 15 °C the digester was only producing about 25 L of biogas per day with an average of 22% methane. The average pH of the digester slurry was 6.2, indicating a sour digester (Table 1). The analysis of total volatile fatty acid (VFA) and alkalinity did not begin until 9 July 2010 and the levels in the sour digester are uncertain. The digester was re-inoculated on 11 May 2010 and loading was resumed on 6 July 2010 at a rate of 1.50 kg VS/m^3day. The organic loading rate (OLR) was increased until a maximum rate of 3.41 kg VS/m^3day was reached on 20 September 2010. The OLR was subsequently reduced until a value of 1.38 kg VS/m^3day was reached on 10 January 2011. The digester was not loaded in February of 2011. The monthly averages of the OLR and hydraulic retention time (HRT) are given in Table 1. The OLR and HRT were changed by either increasing or decreasing the volume and concentration of the manure feedstock that was loaded into the digester.

Table 1. Monthly averages of digester performance data beginning in April 2010.

Month	OLR (kg VS/m^3 day)	HRT (Days)	Gas Prod. (L/day)	CH_4%	Air Temp. (°C)	Digester Temp. (°C)	pH	VFA (mg/L)	Alk. (mg/L)	VFA/Alk	Notes
April	0.31	204	25	22	20.0	14.6	6.2	-	-	-	-
May	-	-	47	47	25.5	19.4	6.5	-	-	-	re-inoculated
June	-	-	411	54	26.5	24.8	7.2	-	-	-	not loaded
July	1.67	61	644	48	26.8	27.0	7.4	1,776	7,320	0.24	-
August	2.80	27	1016	51	26.0	26.9	7.4	2,263	8,184	0.28	-
September	2.95	26	1,172	56	24.5	25.1	7.3	2,477	9,238	0.27	-
October	3.28	27	814	55	20.4	23.2	7.4	2,249	10,550	0.21	-
November	2.87	28	650	50	11.9	18.9	7.4	2,174	9,390	0.23	-
December	1.46	49	194	35	−1.8	11.9	7.3	4,498	8,188	0.55	-
January	1.38	51	79	29	−3.0	8.5	6.9	6,561	6,800	0.99	-
February	-	-	5	-	2.7	5.4	6.8	8,814	5,550	1.59	not loaded

Following re-inoculation on 11 May 2010 the digester performance began to improve resulting in increased biogas yield with a higher percentage of methane, as well as increasing pH. When loading resumed on 6 July 2010 the digester was producing an average of 450 L biogas/day that was approximately fifty percent methane. The digester temperature increased by more than 12 °C from April to June, but remained below the average monthly air temperature (Table 1). From July to September of 2010 the ambient air temperature and the digester temperature were similar with a maximum digester temperature of 27.9 °C on 18 August 2010. After resuming loading, the biogas output increased to a monthly average maximum of 1172 L/day in September of 2010. Although the volume of biogas increased during this time, the average percent methane concentration remained

around fifty percent. The digester temperature began to decline from the summer maximum in September 2010 and was followed by a subsequent decrease in the biogas yield. Insulation and burial of the digester resulted in higher monthly average temperatures than the ambient temperature during the second half of the study, but was not sufficient to keep the digester from dropping below 10 °C. The average monthly digester temperature decreased to a low of 5.4 °C in February 2011. By the end of the study period, the digester was producing only 5 L of biogas/day. Biogas percent methane data were not available for February 2011, but based on the previous months average, it was likely around thirty percent of the biogas volume. Beginning in December 2010 there was a rapid increase in both the total volatile fatty acids (VFAs) and VFAs/Alkalinity. These values increased to 8814 mg/L VFAs and 1.59, respectively by the end of the study.

Based on the operational and performance milestones, the study period was divided into four phases: (1) sour digester, (2) post re-inoculation, (3) peak biogas yield and (4) reduced biogas yield. The sour digester phase occurred from April 2010 until re-inoculation on 11 May 2010 and was followed by the post re-inoculation phase. The peak biogas yield phase occurred following the resumption of loading on 6 July 2010 and continued until 1 November 2010 when the average daily biogas production dropped below 800 L/day. The reduced biogas yield phase lasted until the end of the study period on 7 February 2011.

2.2. Relationship of Microbial Community Structure to Digester Phase

Cluster analysis was used to group the *Bacterial* and *Archaeal* community types into four different groups (Figure 1a,b). The most abundant terminal restriction fragments (TRFs) in the microbial community T-RFLP profiles were used to characterize the microbial community structure of each cluster. There were twelve *Bacterial* and seven *Archaeal* TRFs that represented at least 10% of the relative abundance in at least one sample.

2.2.1. Sour Digester

All four of the *Archaeal* TRF profiles from the sour digester showed a high degree of similarity (Figure 1a). The most abundant Archaeal TRF in the sour digester was 786 bp (Figure 2a) and accounted for more than 50% of the relative abundance in the sour digester samples. The vertical lines in Figure 2 separate the different community types as defined by the cluster analyses (Figure 1a,b). The second most abundant *Archaeal* TRF in the sour digester was 791 bp with an average relative abundance of 11%. The 786 bp TRF has been identified as belonging to the genus *Methanobrevibacter* based on similarities to TRF patterns from a virtual digest of the Microbial Community Analysis (MiCA) database and the results of the virtual digest of the clone sequences. The TRF of apparent size 791 bp was identified as *Methanobrevibacter acidiurians*, which has previously been identified in a sour anaerobic digester [9].

Nineteen of the twenty clones from the sour digester sample were genera in the family *Methanobacteriaceae*. The presence of the remaining clone, *Methanosarcina mazei* is supported by the TRF 182 bp, which is characteristic of the family *Methanosarcinaceae*. This fragment accounted for 11.8% of the relative abundance on 11 May 2010. A total of 10 *Archaeal* sequences from the sour digester sample (11 May 2010) were subjected to virtual digestion. The results indicate that there was a

difference in the predicted TRF size and the observed TRF size in the T-RFLP profiles. For example, the TRFs associated with the genus *Methanobrevibacter* were predicted to be in the range of 792 to 798 bp when digested with *Taq*I. The observed TRF size for this genus in the T-RFLP profile was 786 to 792 bp. In general the TRFs predicted from the virtual digest, were in the range of four to ten base pairs larger than what was observed in the T-RFLP profiles. These observations are consistent with the differences in predicted and observed TRF size as reported in the literature [10].

Figure 1. Cluster dendrogram (Ward linkage, squared Euclidean distance) for the dominant *Archaeal* (**a**) and *Bacterial* (**b**) TRFs.

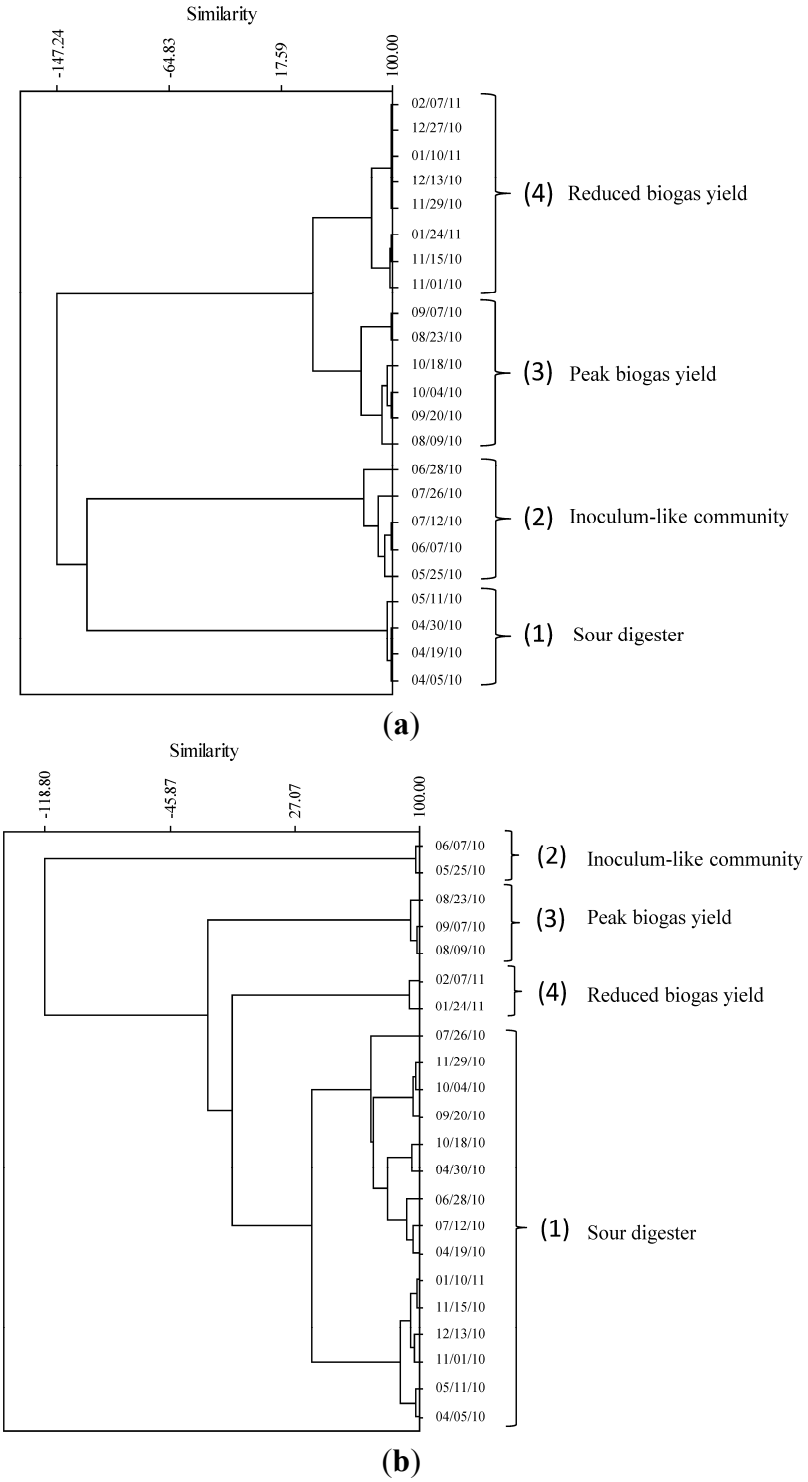
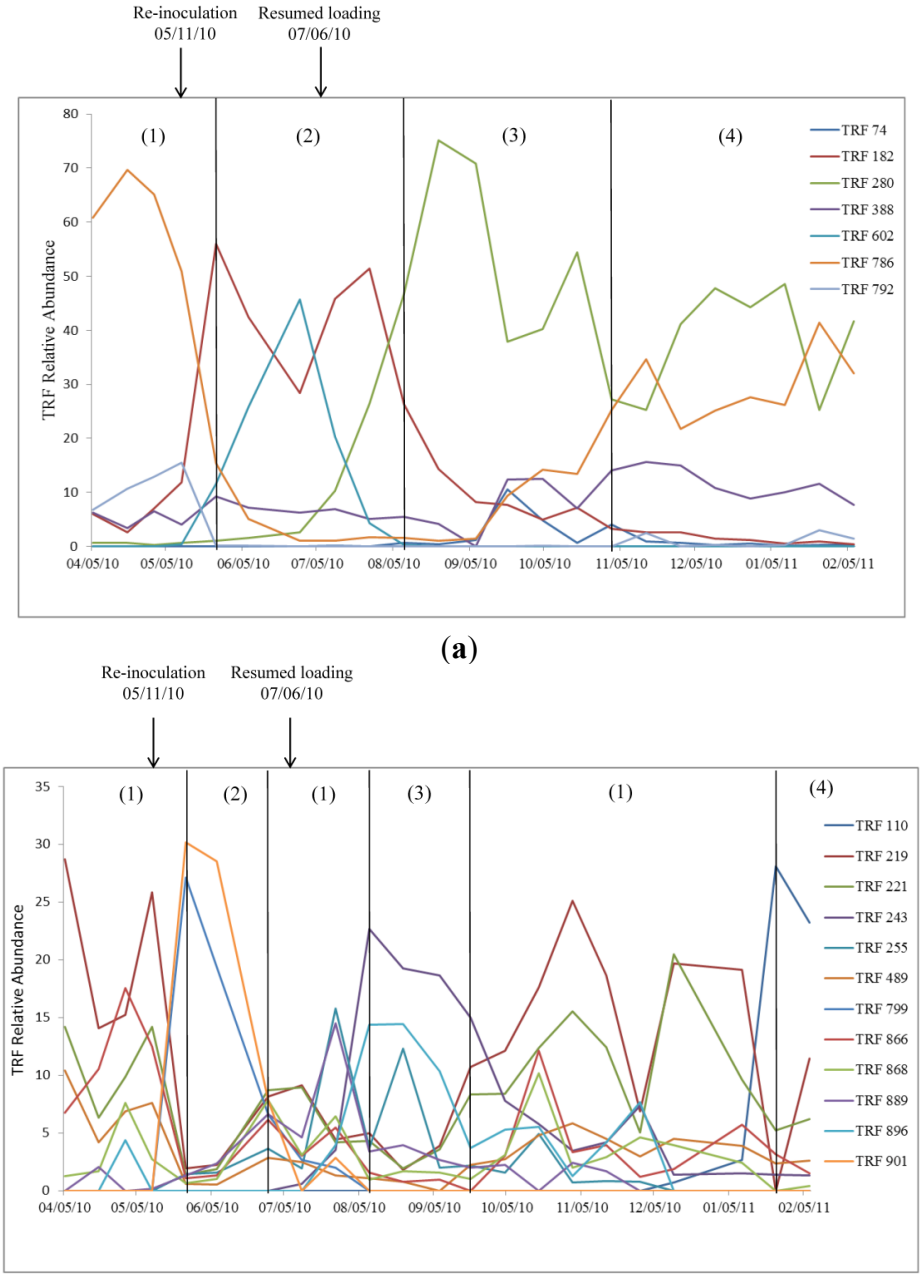


Figure 2. Seasonal variation of the relative abundance of the dominant (**a**) *Archaeal* and (**b**) *Bacterial* TRFs.



The four *Bacterial* TRF profiles from the sour digester also clustered together (Figure 1b) but communities with similar structure appeared at subsequent points during the study. The dominant *Bacterial* TRFs in the sour digester and similar communities were 219 and 221 bp (Figure 2b). Due to the greater diversity of *Bacterial* TRFs and clones, the relationship between these two was not as obvious as with the *Archaea*. The dominant clones on 11 May 2010 were *Clostridium difficile* and *Clostridium beijerinckii* while the dominant TRFs were 219 and 221 bp. The virtual digest of the MiCA database did identify a number of *Clostridia* TRFs in this range although the two species of clones were represented by TRFs 225 and 235, respectively. There was less agreement between the predicted and observed TRF length for the *Bacteria*. The TRFs of observed size 219 and 221 that are

dominant in the sour digester are likely representative of *Clostridium beijerinckii* NCIMB 8052 and *Clostridium difficile* M68. The *Bacteria* clones do not cluster into groups as distinct as the *Archaea*. The clones from the sour digester are associated primarily with *Clostridium difficile* (T) ATCC 9689. The largest number of clones was from the phylum *Firmicutes*, accounting for 78% of the clones. The *Firmicutes* clones were predominately representative of the Class *Clostridia*. The most abundant clone in the 11 May 2010 sample was *Clostridium difficile* Bl1 accounting for 50% of the clones. This strain was also the most commonly identified clone on 7 February 2011.

2.2.2. Post Re-Inoculation

Measureable changes occurred in both the *Archaeal* and *Bacterial* community structure with the introduction of 379 L of inoculum on 11 May 2010. The digester was sampled just prior to loading the inoculum and again 14 days later. The *Archaeal* profiles for both of these samples are highly dissimilar (Figure 1a) indicating a shift in the microbial community structure. Because the temperature increased by approximately 5 °C during the two weeks following re-inoculation, it is not possible to differentiate between the impact of temperature and re-inoculation on the community structure. However, the pattern of TRFs in the samples does indicate that taxa present in the digester following re-inoculation were introduced by the inoculum and not present in the system the previous week. The *Archaeal* community remained similar to the inoculum community until after the resumption of loading on 6 July 2010.

The dominant *Archaeal* allele changed from 768 to 182 bp in the first sample following re-inoculation. The 182 bp TRF was detected in the inoculum at approximately 28% of the relative abundance. It was also detected in digester samples prior to re-inoculation at 7% of the relative abundance. The results of the virtual digest of the clones and comparison with the MiCA database identified TRF 182 bp as characteristic of the genus *Methanosarcina*. TRF 182 accounts for 56% of the relative abundance on 25 May 2010 and 90% of the clones from this sample were *Methanosarcina barkeri.*

There was also another *Archaea* TRF of 602 bp that was detected in the inoculum, but none of the prior digester samples. This TRF was subsequently detected in the next two digester samples before no longer being detected. The virtual digest of the MiCA database produced many fragments in the range of 604–612 many of which are associated with the phylum *Crenarchaeota*. These Archaea have been detected in anaerobic digesters [11] and it is possible that the TRF of observed size 602 bp was associated with members of this phylum.

Measurable changes in the *Bacterial* community also occurred following re-inouculation. The *Bacterial* profiles before and after re-inoculation are highly dissimilar (Figure 1b) indicating a shift in the microbial community structure. The rapid rise and disappearance of *Bacterial* TRFs of 799 and 901 bp occurred prior to the resumption of digester loading (Figure 2b). These TRFs were also detected in the inoculum and were likely introduced from this source. The large peak at 799 bp in the T-RFLP profile from 25 May 2012 (Figure 1b) is likely the same species, *Clostridium* sp. BNL1000 that produced a predicted TRF of 808 bp by the virtual digestion of the clones. The *Bacterial* community only retained the structure of the inoculum for about one month after re-inoculation as compared to two months for the *Archaea*. The samples analyzed prior to the resumption of loading showed that the *Bacterial* community briefly returned to a structure similar to the sour digester.

2.2.3. Peak Biogas Yield

A second shift in the *Archaeal* community structure occurred between 26 July 2010 and 9 August 2010 during a period of relatively constant digester temperature. The average digester temperature was within 1 °C of 27 °C for the month preceding the shift and for two additional weeks afterwards. Following the resumption of loading on 6 July 2010, the dominant *Archaeal* TRF began to change from 182 to 280 bp and by 23 August 2010 TRF 280 accounts for approximately 75% of the relative abundance (Figure 2a). Seventeen of the eighteen clones from the 7 September 2010 library were identified as *Methanosaeta concilli*, which supports the identification of TRF 280 (71% RA) as belonging to the family *Methanosaetaceae*.

Methanosaetaceae have a competitive advantage in digester systems that are stable and operating efficiently [12]. The period of time that *Methanosaetaceae* dominated the relative abundance in the digester corresponds to the maximum summer biogas production and methane concentration. The presence of a greater abundance of *Methanosarcinacea* following re-inoculation and prior to the resumption of loading could be due to the fact that members of this family are favored in digesters with acetate levels above stable operating conditions [13]. Although there are no VFA data from the digester at the beginning of the study period, it is likely that VFA concentrations were elevated in April and May due to the low pH of the digester (pH = 6.06 on 30 April 2010). The first VFA data was 1176 mg/L on 9 July 2010 and it is possible that the VFA levels had been reduced by the utilization by *Methanosarcinacea* during May and June. This reduction in VFAs could have created the conditions necessary for the rapid grow of *Methanosaetaceae* that occurred after loading had resumed.

A corresponding shift in the *Bacterial* community also occurred after the resumption of digester loading. However, unlike the *Archaea*, the *Bacterial* community no longer had the same structure as the inoculum prior to the community shift. The structure had already changed to one that the cluster analyses grouped together with the sour digester communities from the start of the study. The TRFs at 219, 221, 866 and 868 bp only increased to about ten percent of the relative abundance as compared to abundances between fifteen and thirty percent of the relative abundance before re-inoculation. A second shift in the *Bacterial* community structure occurred between 26 July 2010 and 9 August 2010 during a period of relatively constant digester temperature. The two most abundant *Bacterial* TRFs change to 243 and 897 bp. The next change in the dominant *Bacterial* TRFs occurred during September of 2010. This shift coincided with the drop in digester temperature from the summer maximum of 27.7 °C. This period of time is also when the digester loading rate was increased to 3.0 kg VS/m^3day. The TRF pattern during this time is very similar to the period of time prior to the re-inoculation. The observed TRFs of 896 did not correspond to any of the predicted TRFs from the virtual digest of the cloned sequences.

2.2.4. Reduced Biogas Yield

All eight of the *Archaeal* TRF profiles during the reduced biogas yield phase showed a high degree of similarity (Figure 1a). The two most abundant TRFs were 280 and 786 bp that are characteristic of the genera *Methanosaeta* and *Methanobrevibacter* (Figure 2a). The final sample on 7 February 2011 identified the clones *Methanosaeta concilii* and *Methanobrevibacter smithii* in a ratio of 11 to 7.

The drop in temperature may have favored the growth of the colder tolerant species of *Methanobrevibacter* [13]. Differences in the relative amounts of acetoclastic and hydrogenotrophic methanogens can also be influenced by changes in pH and free ammonia. The optimum pH range for acetoclastic methanogens is between 6.6 and 7.3 with strong inhibition below pH 6.2 and free ammonia inhibition above pH 7.4 [14]. In this study, the hyrogenotrophic methanogens of the genus *Methanobrevibacter* dominated the *Archaea* community when the digester pH was 6.06 during the sour digester phase. These *Archaea* also increased in relative abundance during the period of reduced biogas yield when the pH had dropped to 6.75. By 7 February 2011, the percentage of *Methanosaeta concilii* had decreased to 61% of the clones, while *Methanobrevibacter smithii* had increased to 39% of the clones. The increase in the abundance of hydrogenotrophic methanogens due to decreasing temperature was also observed by Bialek *et al.* [15].

Despite the increase in VFA concentration that began in late November, the digester pH remained at least 6.7 during remainder of the study period. This is within the optimum pH range of 6.6 to 7.3 for acetoclastic methanogens [14] and should not have resulted in complete inhibition of *Methanosaeta.* Despite these intermittent increases, the relative abundance of *Methanosaeta* remained below the maximum levels observed when the pH was between 7.2 and 7.4 and the overall trend was decreasing levels of *Methanosaeta*. It is not certain why the levels of *Methanosarcina* remained below 10% of the relative abundance during this time period despite the fact that this genus is favored at higher acetate levels. It is possible that the decreasing temperature prevented the increase in *Methanosarcina* while favoring the increase in the levels of *Methanobrevibacter* (TRF 786).

The eight *Bacterial* community profiles during the period of reduced biogas yield did not cluster together like the *Archaea*. Only the final two *Bacterial* profiles were together in a single cluster. The *Bacterial* community structure in the remaining samples did return to the pre-inoculum community structure during the reduced biogas yield phase. The dominance of *Bacterial* TRFs 219 and 221 bp persisted into the reduced biogas phase. The final change in the *Bacterial* TRF pattern began to occur during December of 2010. A TRF of 110 bp first appears on 13 December 2010 and rapidly increases to account for 28.1% of the relative abundance on the final sample. The increase in TRF 110 bp coincides with an increase in the average monthly VFA concentration from 4498 mg/L in December to 8814 mg/L in February. The observed TRFs of 110 did not correspond to any of the predicted TRFs from the virtual digest of the cloned sequences.

2.3. Relationship between Biogas Yield and Microbial Community Structure

An additional cluster analysis was performed that combined both the dominant *Archaeal* and *Bacterial* TFRs into a single dendrogram. The four clusters contained the same cases as the *Archaeal* dendrogram (Figure 1a). These four clusters were used to assess the relationship between overall community structure and digester performance as measured by biogas yield. The Kurskal–Wallis test of biogas yield *versus* community type indicated that the biogas yield is not equal for all different community types ($H = 16.38$ $DF = 3$ $P = 0.001$). However, the temperature of the digester also influenced the biogas yield. Biogas yield can be similar for two different community types at approximately the same temperature, and the gas production can vary with temperature even though the community structure stays the same. The first example occurred around the *Bacterial* community shift

between 7 and 20 September 2010. The average daily biogas production for the three samples preceding the change was 995 L/day compared to 1053 L/day for the three weeks following the shift in the Bacterial community structure. The average digester temperatures for these three-week periods were 26.6 °C before the community change and 23.7 °C after. The reduction in temperature by approximately 3 °C was sufficient to alter both the *Bacterial* and *Archaeal* community structure but not sufficient to substantially decrease biogas yield. The increase in loading to 3.0 kg VS/$m^3$$day^{-1}$ may have also accounted for the lack of reduction in biogas yield as the temperature dropped. The second example occurred after the 20 September 2010 community shift (Figure 2b) through the remainder of the study period. Although the microbial community structure remained largely stable between 20 September 2010 and 10 January 2011 the biogas output declined to 84 L/day as the temperature dropped to below 10 °C. In this case, the temperature drop influenced the function of the community rather than the structure. Eventually, the bacterial community structure did change over the next month as digester temperatures remained below 10 °C.

2.4. Archaea and Bacteria TRF Richness

For each domain, there were six different enzyme/primer combinations and the samples were analyzed in two separate 96-well plates. One enzyme/primer combination was chosen for each domain to calculate species richness and diversity. For the *Bacteria*, *Bfa*I/11F and for the *Archaea*, *Taq*I/Ar912rt was selected based on superior resolution of the different microbial taxa. There were a total of 163 different *Bacterial* TRFs detected with an average of 44 TRFs per sample. Fifty five percent of the TRFs were ≤1% of the relative abundance while an average of only two TRFs per sample represented > 10% of the relative abundance. There were a total of 206 different *Archaeal* TRFs detected with an average of 88 TRFs per sample. Ninety three percent of the TRFs were ≤1% of the relative abundance while an average of only two TRFs per sample represented >10% of the relative abundance.

The number of TRFs accounting for at least 1% of the relative abundance was greater for the Bacteria than the Archaea in all samples. These results are consistent with the study by Ziganshin *et al.* [16] who also found a greater number of *Bacterial* TRFs in samples from a mesophylic digester processing distillers dry grains with solubles. However, these authors found almost twice the number of TRFs in both domains than were detected in this study. In another study, Wang *et al.* [17] detected 32 *Bacterial* TRFs and four *Archaeal* TRFs in an anaerobic digester fed with grass silage and cow manure. Despite the differences in the number of TRFs detected the relative number of *Bacterial* and *Archaeal* TRFs is similar in these studies. Another similarity is the fact that the majority of the relative abundance is due to only a few TRFs. This agrees with a previous study where Wang *et al.* [17] found that a TRF of 182 base pairs accounted for approximately 70% to 90% of the relative abundance in all samples. These results demonstrate that microbial communities in anaerobic digesters contain many species of *Bacteria* and *Archaea*, but only a few of them dominate the community profile, and that the dominant members can change depending on variation in digester operation and environmental conditions.

Variation in TRF richness between studies should be interpreted with some caution. It is likely that differences in feedstock, digester temperature and other operational parameters such as loading rate, can influence the diversity of the *Bacterial* and *Archaeal* communities, but there are limitations to the

use of TRF relative abundance as a measure of species richness. For example, there is the assumption that individual TRFs correspond to a particular operational taxonomic unit (OTU). However, a particular TRF may actually be characteristic of more than one different species. The TRF with the apparent size of 104 bp was produced by digestion with the enzyme *Bfa*I. The virtual digest of the cloned sequences showed that this fragment is produced by *Archaea* that are closely related to both *Methanosarcina barkeri* str. Fusaro and *Methanosaeta concilii* GP6. Conversely, two species that have a high percent similarity (±97%) to a known reference strain can produce different sized TRFs when digested with the same restriction enzyme. This was shown by the virtual digest of clones 122-A13 and 122-A18. When digested with *Taq*I, clone 122-A13 produced a fragment of predicted length 187 bp while the predicted fragment length for clone 122-A18 was 286 bp. Other limitations include biases in DNA extraction and PCR amplification which may produce PCR products that have a different relative abundance than the microbial species in the sample [18]. Despite these limitations, the results of this and other studies indicate that the *Bacteria* are generally more diverse than the *Archaea* and that the majority of the relative abundance is accounted for by a small percentage of the species.

2.5. Archaeal and Bacterial Community Rate of Change

The average percent change between samples was 41.6 ± 19.6 for the *Bacteria* and 23.9 ± 15.0 for the *Archaea*. Both the *Bacterial* and *Archaeal* communities showed both a rapid and slow percent change between sampling events. The largest percent change between two samples occurred during the period after re-inoculation for both the *Bacteria* (89.7%) and the *Archaea* (62.8%), and the smallest percent change between two samples occurred during the period of reduced biogas yield for both the *Bacteria* (19.5%) and the *Archaea* (7.5%). The variable rate of community change is in contrast to the results observed by Carballa *et al.* [19] who investigated the rate of change of *Bacterial* communities in pairs of laboratory reactors at mesophylic (34 ± 2 °C) and thermophylic (53 ± 2 °C) temperatures. The standard deviations of the average rate of changes in their study range were consistently lower than what was observed in this study. One likely explanation for this difference is the fact that the digesters studied by Carballa *et al.* [19] were of fixed temperature and did not expose the microbial communities to as variable an environment. Marzorati *et al.* [8] found much lower average rates of change for *Bacterial* communities in three different reactors including a wastewater treatment plant, and two different nitrifying reactors. This may also be due to more consistent temperature ranges during the course of their study. Marzorati *et al.* [8] concluded that these three systems were likely "closed" to the colonization of new species.

3. Experimental Section

3.1. Anaerobic Digester Design

The design of the 1.14 m^3 anaerobic digester in this study was based on the Chinese fixed-dome model [20]. The base of the digester in this study was six feet below the ground surface and additional protection from cold temperature was achieved by locating the digester inside a 9 m by 10 m greenhouse at the Waterman dairy facility on the campus of The Ohio State University in Columbus,

OH, USA (40°0'34" N, 83°2'31" W). Further insulation was provided in the winter by burying the digester under wood chips.

The digester was constructed during the summer of 2009 using a 1.14 m^3 polyethylene tank, as well as two additional plastic tanks for the mixing and compensation tanks. Tiger Foam Insulation™ was sprayed on the outside of the tank to a depth of 2.5 cm to provide additional insulation. 15.2 cm schedule 40 PVC pipe was used to connect the three tanks in series. 15.2 cm Uniseals were used at each pipe-tank connection in order to form a gas tight seal. A rubber seal held in place with two hose clamps replaced the top plate of the digester tank in order to achieve a gas tight seal. The digester was fitted with a gas line connected to a gas meter to record daily biogas production. Between the digester and the gas meter a solenoid valve, set to release at 20.3 cm of pressure, was installed to increase digester mixing. As is typical of fixed-dome digesters, mixing occurred when digester slurry from the compensation tank flowed back into the digester when the pressure was released. Thermocouples were installed at two depths inside the digester tank to measure the temperature of the digester contents. Thermocouples also recorded air and soil temperature inside the greenhouse as well as the ambient outside air temperature.

3.2. Feedstock Source and Digester Operation

The digester was loaded three days per week with fresh manure collected from Jersey cows at the Waterman Dairy Facility. Fresh manure was shoveled from the stall floor and diluted with ambient temperature groundwater prior to loading. The ratio of manure to groundwater varied depending on the desired organic loading rate (OLR). The OLR varied between 0.84 and 2.98 kg VS/m^3day during the study period. Beginning in the winter of 2010 hot water from the milking parlor was used for dilution. This was done in an attempt to increase the temperature of the feedstock prior to loading the digester. A few measurements of the feedstock temperature were made after hot water was used for dilution. Due to the mostly frozen manure during this time the final temperature of the feedstock did not increase significantly. The digester was initially inoculated in October of 2009 with 20 gal (75.7 L) of effluent from a manure digester as well as approximately 100 gal of primary and secondary sludge from the City of Columbus Southerly waste water treatment plant (WWTP). Following a souring of the digester in the winter, the system was re-inoculated in June 2010 with 100 gal (378.5 L) of effluent from the anaerobic digester located at The Ohio Agriculture Research and Development Center (OARDC) in Wooster, OH, USA.

3.3. Sample Collection

The sampling period in this study was from April 2010 to February 2011. Samples for microbial community analysis were collected weekly from the center of the digester tank using a ¾ PVC sampling tube. Samples were collected in 250 mL plastic bottles and immediately placed on ice and stored at −20 °C upon return to the laboratory. Prior to extraction the sample bottles were thawed for approximately 24 h in a 4 °C refrigerator. After mixing the sample bottles by shaking, approximately 0.6 to 0.8 g (400 μL) of digester slurry was transferred to 2.0 mL micro centrifuge tubes using a 1000 μL pipette. The micro centrifuge tubes were frozen at −20 °C until DNA extraction. The pH of each digester sample was measured immediately after collection using a Fisher Scientific pH meter.

3.4. Microbial Community Analysis

3.4.1. Genomic DNA Extraction

Total genomic DNA from each digester sample was extracted in duplicate using the Qiagen Stool DNA MiniKit (Germantown, MD, USA). Approximately 0.5 g of digester sample was extracted in two separate tubes. The MiniKit protocol was altered to include the addition of glass beads (0.1 and 0.5 mm) as well as longer incubation times (10 min) at 70 °C. The glass beads were added prior to vortexing the samples in order to increase the DNA yield in the extracts by physical disruption of the cell wall. The total vortex time was increased to eight minutes, four minutes before and after the water bath lysis. The incubation times were increased in order to give the extraction buffer a longer time to dissolve the cell membrane and increase DNA yield. The presence and quality of DNA in the extracts was confirmed on 1% agarose gels in 0.5 X Tris-borate-EDTA and visualized by ethidium bromide staining. The samples were electrophoresed at 25 V for the first 15 min and then at 50 V for the remainder of the run. A 10 kb DNA size standard (New England Biolabs, Ipswich, MA, USA) was included with each run. The gel images were produced with a ChemiDoc XRS gel imaging system. The quantity of DNA in the extracts was determined using a Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and 10 μL of sample.

3.4.2. Polymerase Chain Reaction

The target sequence in the extracted DNA, the 16S rRNA gene, was amplified by the polymerase chain reaction (PCR) using universal Bacterial primers11F (5'-[VIC]-GTTTGATCMTGGCTCAG-3') [21] and 907R (5'-[FAM]-CCGTCAATTCMTTTRAGTTT-3') [22] and with universal Archaeal primers Ar109f [23] (5'-[VIC]-ACKGCTCAGTAACACGT-3') and Ar912rt (5'-[FAM]-GTCGCTCCCCCG CCAATTCCTTTA-3') [24] in separate reactions. Each 50 μL of PCR reaction mixture contained 1.0 μL DNA extract, which contained approximately 30 ng of genomic DNA. The reaction also contained 25 μL of 2X Phusion Master Mix (New England Biolabs) and 0.4 μM of each primer. The remainder of the reaction was composed of DNA free water. For the *Archaea* reaction, the initial denaturation was at 98 °C for 30 s followed by 35 cycles (denature at 98 °C for 10 s, anneal at 58 °C for 20 s, extend at 72 °C for 24 s and final extension at 72 °C for 5 min) and then held at 4 °C in the thermal cycler until transferred to a −20 °C freezer. The Bacteria thermal cycler program was the same as the *Archaea* except that the annealing temperature was 56 °C. Amplification products were separated on agarose gels and visualized as described above.

Duplicate extracts from each sample were amplified in separate reactions and pooled prior to cleanup for a total volume of 100 μL. Pooled PCR products were purified with the QiaQuick PCR Purification Kit (Qiagen, Germantown, MD, USA) according to the manufacture's protocol. Each sample was eluted with 50 μL of buffer AE. An additional 50 μL of buffer AE was added to the cleanup sample and mixed by pipetting. This was done in order to have sufficient volume for the restriction digestions.

3.4.3. Restriction Digestions

The pooled PCR products were digested separately in 50 µL reactions with *Alu*I, *Bfa*I or *Taq*I restriction endonucleases (New England Biolabs, Ipswich, MA, USA) in 200 µL PCR reaction tubes. For all three enzymes, the reaction mixtures were incubated in a thermal cycler for one hour and then the restriction enzymes were heat inactivated for 20 min. Each digestion included 20 µL of PCR product and 5 µL 10X NEBuffer 4. The *Alu*I digestions contained 1 µL (10 U) of enzyme and 24 µL of DNA free water. The reaction temperature was 37 °C with an inactivation temperature of 65 °C. The *Bfa*I digestions contained 1 µL (5 U) of enzyme and 24 µL of DNA free water. The reaction temperature was 37 °C with an inactivation temperature of 80 °C. The *Taq*I digestion contained 1 µL (20 U) of enzyme, 0.5 µL of bovine serum albumin (0.1 µL/µL) and 23.5 µL of DNA free water. The reaction temperature was 65 °C with an inactivation temperature of 80 °C. Restriction products were visualized on 2% agarose gels as described above. Each gel also was loaded with 3 µL of a 100 bp DNA size standard (New England Biolabs).

3.4.4. T-RFLP Analysis

The terminal restriction fragment (TRF) sizing was performed by The Ohio State University Plant-Microbe Genomics Facility (PMGF) using an Applied Biosystems 3730 DNA Analyzer (Carlsbad, CA, USA) that compares the TRF to the internal size standard LIZ1200. Prior to TRF sizing the restriction products were purified by the PMGF using the AMPure® purification system (Beckman Coulter, Indianapolis, IN, USA). Only those TRFs that generated peaks greater than 50 relative fluorescence units were considered to be operational taxonomic units (OTUs) [25]. The peak area of each OTU was divided by the total peak are of all OTUs in each sample and then multiplied by 100 to calculate the relative abundance of each OTU [25]. The identification of specific microorganisms corresponding to a particular TRF length was aided by using the MiCA database at the University of Idaho [26]. MiCA was used to compare both the *Bacteria* and *Archaea* TRF profiles from all three restriction digestions to TRFs from corresponding *in silico* amplification and digestion of the 16S rRNA sequences in the MiCA database. The RDP (R10, U27) 10,346 Good Quality (>1200) *Archaeal* database was queried allowing 10 mismatches within 15 bases of the 5' end of the primer and returned 9965 records. The RDP (R10, U27) 700,829 Good Quality (>1200) *Bacterial* database was queried allowing 10 mismatches within 15 bases of the 5' end of the primer and returned 699,859 records. Further validation of the dominant TRFs was obtained by comparing the results to the virtual digestions of the cloned PCR products. The virtual digest of these sequences was performed using the NEBCutter V2.0 available on the New England Biolabs website [27].

3.5. Clone Library Construction and Sequencing

Clone libraries were constructed from samples that were representative of the different *Archaeal* and *Bacterial* community types as defined by the cluster analyses. There were a total of four different community types for each of the two domains resulting in a total of eight libraries. The first sample cloned was collected on 11 May 2010 and was characteristic of the sour digester at the start of the study period. The next sample was collected fourteen days after re-inoculation. The third sample was

collected on 10 September 2010 during the peak biogas yield. The fourth sample was collected at the end of the study period on 7 February 2011 during a period of reduced biogas yield.

The ligation reactions were performed using the Thermo Scientific CloneJet PCR cloning Kit (#K1231) (Waltham, MD, USA) using the blunt-end cloning protocol. The ligation products were used to transform MAX Efficiency® DH5α™ Competent Cells according to the manufactures protocol. Transformed cells were spread on LBAmp agar plates (100 µL/mL) and incubated at 37 °C for 16 h. Individual colonies were inoculated into LBAmp broth (100 µL/mL) and incubated at 37 °C and 225 rpm for 12 h. 1000 µL of each broth culture was transferred to 2.0 mL microcentrifuge tubes and centrifuged for three minutes at 10,000 rpm. Plasmid DNA was extracted from each broth culture using QIAprep Spin Miniprep kit (Qiagen) according to the manufactures protocol. The DNA sequences were determined by The Ohio State University Plant-Microbe Genomics Facility (PMGF) using an Applied Biosystems 3730 DNA Analyzer (Carlsbad, CA, USA).

3.6. Analysis of Biogas and pH

Biogas samples were collected weekly from the gas outlet line with a 0.5 L Tedlar bag (Sigma-Aldrich, St. Louis, MO, USA). The percent methane in the biogas was quantified using gas chromatography with thermal conductivity detection at the Ohio Agriculture Research and Development Center (OARDC) in Wooster, OH, USA. The volume of biogas was recorded during operation of the digester by a biogas meter. The pH of each digester sample was measured immediately after collection using a Fisher Scientific pH meter (Waltham, MD, USA).

3.7. Alkalinity and Total Volatile Fatty Acids

Alkalinity and total volatile fatty acids were determined for each digester sample beginning in July of 2010. For total inorganic carbonate alkalinity (TCA), total fatty acids (TFAs) and total alkalinity, the raw sample was first centrifuged at 7500 rpm for 20 min. Then, the supernatant was diluted with DI water in a 1:3 ratio, and a total of 20 mL were titrated with H_2SO_4 0.1 N to end points of 5.0 and 4.4. The following empirical formulas were used for calculation [28]:

$$\text{TCA (mg } CaCO_3\text{/L)} = (\text{mL } H_2SO_4 - \text{from start to pH 5.0}) \times 250 \quad (1)$$

$$TVFA_S \text{ (mg HAc/L)} = ((\text{mL } H_2SO_4 \text{ from pH 5.0 to pH 4.4} \times 1.66) - 0.15) \times 500 \quad (2)$$

Data from these titrations was used to calculate the VFA to Alkalinity ratio.

3.8. Data Analyses

3.8.1. T-RFLP Data

The species richness (N) was defined as the number of peaks greater than 50 relative fluorescence units. Richness values were also calculated for TRFs representing greater than 1%, 5% and 10% of the relative abundance. Community relatedness dendrograms were produced by cluster analysis (Squared Euclidean distance, Ward linkage) with Minitab 16 Statistical Software (Minitab Inc., State College, PA, USA). Daily biogas production for each sampling day was averaged for each microbial community cluster and the averages compared with the Kuskal-Wallis ANOVA.

The microbial community dynamics parameter (Dy) was estimated using the following formula for percent change [8]:

$$\% \text{ change} = 100 - \% \text{ similarity} \quad (3)$$

The percentage similarity (P) was calculated using the following equation [29]:

$$P = \sum \text{minimum } (p_{1i}, p_{2i}) \quad (4)$$

The rate of change parameter (Dy) was calculated as the average of the percent change values for all samples during the study period.

3.8.2. DNA Sequence Data

The DNA sequences from the *Archaeal* and *Bacterial* clones were compared to the GenBank database using the megaBLAST search tool on the National Center for Biotechnology Information (NCBI) website [30]. The most similar reference was defined as the one with the highest % similarity and the greatest query coverage. The virtual digest of the cloned sequence was performed with NEBCutter V2.0 on the New England BioLabs website [27].

4. Conclusions

Measurable changes in microbial community structure coincided with changes in ambient temperature, re-inoculation and loading rate. Results describe the microbial response to re-inoculation and found the greatest changes in species composition due to re-inoculation. *Methanobrievibacter* was the most abundant methanogen in the sour digester. The inoculum community did not persist in the digester as *Methanosarcina* was replaced by *Methoanosaeta* after resumption of loading. The changes in microbial community composition as well as changes in temperature impacted biogas yield. The results of the study indicate that was not possible to maintain an *Archaeal* community structure indicative of a well functioning digester with this design and operation during the winter months. The increase in the relative abundance of *Methanobrevibacter* after November 2010 indicated that the community structure was beginning to resemble that of the sour digester. The potential for the use of this type of digester in temperate climates is likely limited to the warmer months. However, the use of this type of system eight or nine months of the year can still provide benefits to waste producers that do not have sufficient resources to utilize temperature controlled systems.

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Conflicts of Interest

The authors declare no conflict of interest.

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