Microbial Diversity and Dynamics during Methane Production from Municipal Solid Waste

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\textbf{A B S T R A C T}

The objectives of this study were to characterize development of bacterial and archaeal populations during biodegradation of municipal solid waste (MSW) and to link specific methanogens to methane generation. Experiments were conducted in three 0.61-m-diameter by 0.90-m-tall laboratory reactors to simulate MSW bioreactor landfills. Pyrosequencing of 16S rRNA genes was used to characterize microbial communities in both leachate and solid waste. Microbial assemblages in effluent leachate were similar between reactors during peak methane generation. Specific groups within the Bacteroidetes and Thermotogae phyla were present in all samples and were particularly abundant during peak methane generation. Microbial communities were not similar in leachate and solid fractions assayed at the end of reactor operation; solid waste contained a more abundant bacterial community of cellulose-degrading organisms (e.g.,\textit{Firmicutes}). Specific methanogen populations were assessed using quantitative polymerase chain reaction. \textit{Methanomicrobiobiales, Methanosarcinaceae,} and \textit{Methanobacteriales} were the predominant methanogens in all reactors, with \textit{Methanomicrobiobiales} consistently the most abundant. Methanogen growth phases coincided with accelerated methane production, and cumulative methane yield increased with increasing total methanogen abundance. The difference in methanogen populations and corresponding methane yield is attributed to different initial cellulose and hemicellulose contents of the MSW. Higher initial cellulose and hemicellulose contents supported growth of larger methanogen populations that resulted in higher methane yield.

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\section{1. Introduction}

Municipal solid waste (MSW) landfills remain the predominant end-state for waste disposal in the US (USEPA, 2010). Operation of a MSW landfill as a bioreactor is an attractive waste management option for in situ treatment and stabilization of solid waste and leachate (Pohland, 1980; Barlaz et al., 1989; Reinhart et al., 2002; Mehta et al., 2002; Benson et al., 2007; Bareither et al., 2010a; Barlaz et al., 2010). Anaerobic decomposition of MSW in landfills yields methane ($\text{CH}_4$) and carbon dioxide ($\text{CO}_2$) as end-products. Methane can be collected and used in on-site biogas-to-energy systems, which adds to the US alternative energy portfolio and decreases methane released to the atmosphere as a greenhouse gas.

Methane production from MSW depends on a consortium of organisms that degrade complex organic molecules in sequential phases referred to as hydrolysis, fermentation, acetogenesis, and methanogenesis (Farquhar and Rovers, 1973; Zehnder, 1978; Barlaz et al., 1989; Zinder, 1993; Pohland and Kim, 1999; Levén et al., 2007). The initial step is hydrolysis of complex polymers (e.g., cellulose, hemicellulose, starch, protein) to lower molecular weight monomers (e.g., sugars, amino acids). Fermentation of these monomers to alcohols, carboxylic acids (e.g., acetate, propionate, butyrate), hydrogen ($\text{H}_2$), and carbon dioxide ($\text{CO}_2$), combined with acetogenesis of $\text{H}_2$ and $\text{CO}_2$ to acetate, yields the substrates and chemical and microbial equilibrium necessary for methanogenesis (Zehnder, 1978). Methane producing \textit{Archaea} (i.e., methanogens) are critical to efficient and balanced waste decomposition as they carry out the terminal step in the anaerobic decomposition process.

Methanogenesis occurs primarily through two pathways: acetotrophic and hydrogenotrophic decomposition (Zinder, 1993). Acetotrophic methanogenesis involves consumption of
acetate, whereas hydrogenotrophic methanogenesis involves consumption of $\text{H}_2$ and $\text{CO}_2$ or formate (Zehnder, 1978; Pavlostathis and Giraldo-Gomez, 1991). Hydrogenotrophic methanogens form syntrophic associations with hydrogen-producing fermentative and acetogenic bacteria, allowing the syntrophic partner to carry out fermentations by removing inhibitory waste products.

Methanogens pertaining to both acetotrophic and hydrogenotrophic decomposition pathways have been identified in MSW landfills (e.g., Huang et al., 2002; Huang et al., 2003; Calli and Cirgin, 2005; Calli et al., 2006; Laloui-Carpentier et al., 2006). Staley et al. (2011a) report that acid-tolerant acetoclastic methanogens may play a crucial role in initiating methanogenesis in MSW and neutralizing pH. Methanogen population dynamics have been studied extensively in other anaerobic systems (e.g., wastewater treatment, anaerobic digesters), yet the environmental, operational, and material factors promoting dominance of acetotrophic vs. hydrogenotrophic methanogenesis are complex and still not well understood (Demirel and Scherer, 2008; Semrau, 2011).

The objectives of this study were to characterize development of microbial communities and quantify methanogen populations during MSW decomposition in laboratory-scale bioreactor landfills. Microbial communities and methanogen population abundances were linked to temporal changes in leachate chemistry and methane generation. Three 188-L laboratory reactors (A, B, and C) containing shredded MSW were operated for 181 d at mesophilic temperatures with leachate recirculation. Leachate chemistry and methanogen populations were evaluated weekly. Decomposed solid waste was exhumed from one reactor to compare microbial communities in the solid waste and leachate fractions. Quantitative polymerase chain reaction (qPCR) targeting 16S rRNA genes was used to assess abundance of specific methanogens in leachate and solid waste. Pyrosequencing of 16S RNA genes was used to describe bacterial community composition.

2. Materials and methods

2.1. Laboratory reactors

A schematic of a laboratory reactor used in this study is shown in Fig. 1. The reactors consisted of sealed 0.6-m-diameter by 0.9-m-tall stainless steel tanks. The initial waste specimens contained 115 kg of dry MSW and were compacted to initial waste volumes of approximately 188 L (0.188 m$^3$). Gravel was placed at the base of the tank for leachate collection and on top of the waste specimen to distribute recirculated leachate. A fine-mesh aluminum screen was placed between the waste and drainage gravel to prevent clogging of the effluent port. Leachate was collected in a sealed, inert plastic bag to minimize exposure to oxygen, and was recirculated via perforated PVC pipes installed in the distribution gravel. Biogas generated during waste decomposition passed through an outlet port in the reactor lid and was collected in 40-L flexible gasbags (SKC Inc., Eight Four, PA).

Waste temperature was maintained between approximately 30–40°C using two 300-mm × 600-mm flexible silicone rubber heaters affixed to the sides of the reactors (Fig. 1). Type-T thermocouples (Omega Engineering, Inc., Stamford, CT) were used to monitor waste temperatures in the drainage gravel, distribution gravel, and at four vertically staggered locations in the MSW (Fig. 1). Temperatures were recorded and controlled with a Campbell Scientific CR23X Micrologger (Campbell Scientific Inc., Logan, Utah) connected with an AM25T thermocouple multiplexer.

2.2. Municipal solid waste

Municipal solid waste was collected from Deer Track Park Landfill (Watertown, WI) during construction of a field-scale lysimeter experiment to assess bioreactor performance (Bareither et al., 2012). MSW had been in-place approximately 3–4 months at the time of sampling and contained the following composition on a dry mass basis: 19.0% paper/cardboard, 17.0% gravel/ceramics/inserts, 7.8% wood, 5.2% flexible plastic, 5.6% metal, 5.6% rigid plastic, 2.9% textile, 2.8% miscellaneous, 2.9% glass, 1.1% food waste, 0.3% yard waste, and 29.8% soil-like material (i.e., material passing a 4.75-mm sieve).

The initial waste composition was the same in all three reactors. MSW constituents with particle diameters greater than 25 mm were shredded and passed through a 25-mm screen prior to placement in the laboratory reactors. Shredded MSW was blended thoroughly by hand to achieve homogenized waste specimens for all three reactors. Waste specimens were hydrated to a dry weight water content of 28% and compacted in five lifts to an average total unit weight of 7.7 kN/m$^3$.

Solid waste chemical characteristics, including cellulose (C), hemicellulose (H), and lignin (L) contents; volatile solids (VS); and biochemical methane potential (BMP); were measured on the initial MSW used to fill the laboratory reactors as well as on MSW exhumed from the reactors following experimentation. A summary of these chemical characteristics is in Table 1. A description of the methods used to determine chemical characteristics of the wastes is in Bareither et al. (2010b). The C and H contents, [(C + H)/L], and BMP all decreased for the exhumed wastes relative to the initial waste, indicating that MSW in the reactors decomposed relative to the initial MSW. Further discussion on the implications of waste composition variability on reactor performance and microbial community development is presented subsequently.

2.3. Leachate management

Leachate used for dosing the reactors was collected from Deer Track Park Landfill (Watertown, WI). A 10-L dose was added on Day 41 and on Day 43 of reactor operation to inoculate the waste with an active anaerobic microbial community. Subsequent leachate doses were executed in 1-L volumes applied every 1–2 d throughout the duration of the experiment. Effluent leachate was consistently recirculated in the reactors, with additional fresh leachate added if needed to achieve 1 L. A sufficient volume of
fresh leachate was initially collected from Deer Track Park Landfill such that the incombustibles and all subsequent leachate additions for the three reactors were from the same leachate source. Fresh leachate as well as excess leachate generated from the reactors (i.e., >1 L) was stored in zero-head-space containers at 4 °C to minimize biological activity.

Leachate samples were collected weekly and measured for pH, oxidation–reduction potential (ORP), and chemical oxygen demand (COD). pH and ORP were measured on a Fisher Scientific (Waltham, MA) AR60 with Microelectrodes, Inc. probes (Bedford, NH; MI-410 for pH and MI-800/410 for ORP). Chemical oxygen demand was measured using a HACH (Loveland, CO) 0–1500 mg/L COD test kit. Standard COD curves were created with potassium hydrogen phthalate solution. All absorbance measurements were completed with a Spectronic® 20 Genesys™ spectrophotometer (Sigma–Aldrich Co., St. Louis, MO) at 600 nm wavelength.

2.4. Gas production and chemistry

Gas volume was measured via water displacement in calibrated 50-L (±0.5 L) or 10-L (±0.1 L) containers submerged in acidified water (pH 3.0). Samples for composition analysis were extracted from gas collection bags and stored in evacuated glass vials prior to analysis. Gas composition was assessed with a Shimadzu Gas Chromatograph (GC-2014) equipped with flame ionization and thermal conductivity detectors. Percentages of hydrogen (H₂), nitrogen (N₂), oxygen (O₂), carbon dioxide (CO₂), and methane (CH₄) were calculated with respect to standard gases (Scott Specialty Gases, Plumsteadville, PA; AGA Gas, Inc., Maumee, OH).

2.5. Microbial analysis

2.5.1. DNA extraction

Leachate effluent samples were collected weekly from each reactor and analyzed individually (i.e., samples from separate reactors were not pooled). Samples were filtered on 0.2-µm pore filters (Pall Gelman, Ann Arbor, MI) and frozen at −20 °C until DNA extraction. DNA was extracted using a PowerSoil DNA extraction kit (Mo-Bio, Carlsbad, CA) following manufacturer’s instructions. However, the initial vortexing step was replaced by beadbeating in a BioSpec (Bio Spec Products Inc., Bartlesville, OK) beadbeater for 3.5 min. DNA concentrations were assessed on a Thermo Scientific ND-1000 (Thermo Fisher Scientific, Waltham, MA) spectrophotometer using absorbance at 260 nm.

Municipal solid waste samples (50 g wet mass) were exhumed from Reactor A at the end of reactor operation (Day 181) at the four locations shown in Fig. 1 for 16S rDNA gene tag pyrosequencing. Each solid waste sample was blended for 60 s in a Waring industrial blender (Waring Laboratory & Science, Torrington, CT) with chilled phosphate buffer (pH 6.7) following the protocol in Staley et al. (2011b). Blended materials were hand squeezed through a paint strainer bag to remove large solids. The strained liquid was filtered on a 0.2-µm pore filter and DNA was extracted similar to the leachate samples. DNA extractions from the four solid waste samples representing the end-state analysis for Reactor A (Fig. 1) were pooled for pyrosequencing.

2.5.2. Quantitative polymerase chain reaction

DNA samples were screened for the presence of Archaea and specific methanogens (orders: Methanomicrobiales, Methanobacteriales, Methanococcales, and Methanosaetales, and families: Methanococcaceae and Methanosetaecae) using endpoint polymerase chain reaction (PCR) and the primers and PCR conditions reported by Yu et al. (2006). Visual examination on ethidium-bromide stained 1% agarose gels was used to confirm presence of the amplified product. Quantitative 5’-exonuclease PCR (qPCR) standards were constructed via cloning endpoint PCR products selected from samples showing positive bands using the TOPO-TA vector (Invitrogen™, Life Technologies Co., Grand Island, NY) following the manufacturer’s instructions. Cloned products used to construct qPCR standard curves were sequenced and compared to the NCBI nucleotide database using BLAST to confirm appropriate phylogeny. DNA concentration was assessed using the NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA). Copy numbers of standards were calculated from the DNA concentration using the known length of the vector and insert.

Quantitative 5’-exonuclease PCR was used to determine copy numbers of methanogen 16S ribosomal DNA following methods in Yu et al. (2006). qPCR was performed on an iCycler (Bio-Rad Laboratories, Inc., Hercules, CA) with initial denaturation for 15 min at 94 °C, followed by 45 × 1 min cycles that included 30 s denaturing at 94 °C and 30 s annealing/elongation at 60 °C. Reactions included 12.5 µl of Bullseye Hot Start Taq 2X mix (MidSci, St. Louis, MO), 0.5 units additional Bullseye Hot Start Taq Polymerase (final concentration 3 U/25 µl), 1 µl of 25-mM MgCl₂ (final concentration 3.5 mM), 500-nM (final concentration) of forward and reverse primer, and 200-nM (final concentration) of dual labeled probe. No more than 5 ng of DNA was used as template.

All qPCR reactions had efficiencies between 90% and 110% and standard curves were linear with a coefficient of determination ($R^2 > 0.99$). Fluorescence cycle threshold values were determined automatically with the iCycler software using the maximum slope method and were between 25 and 50 fluorescence units. Standard curves were run in duplicate for each qPCR assay, using 10-fold dilutions of cloned standard DNA. The starting concentration of target was calculated from the number of copies in the qPCR reaction multiplied by the dilution factor of the PCR, and divided by the volume of leachate filtered (or in the case of solids, mass processed) for DNA extraction. Standard deviations of methanogen abundances were determined by performing triplicate reactions.
2.6. 16S rRNA gene tag pyrosequencing

454 pyrosequencing was conducted on the V6 hypervariable region of the 16S rRNA gene to assess bacterial community composition. PCR was carried out using the barcoded primers of Hamady et al. (2008) and 454 data was processed using recommendations of Huse et al. (2007). Sequences were analyzed with mothur v. 1.27.0 (Schloss et al., 2009) using the standard “Schloss SOP”. Briefly, flowgrams were quality-controlled and de-noised with the commands trim.flows, shhh.flows, trim.seqs; aligned against the Silva reference database using align.seqs; and chimera-checked with the command chimera.uchime. The final sequence dataset was classified using the Silva taxonomy distributed with mothur, using the command classify.seqs. Operational taxonomic units (OTUs) were identified for the 0.03 distance level following construction of a distance matrix using the commands dist.seqs and cluster. Alpha diversity estimates were conducted using the rarefaction.single and summary.single commands. Comparison of community composition was conducted using dist.shared and the Bray Curtis dissimilarity index (\(D_{BC}\)), which is calculated using the following equation:

\[
D_{BC} = 1 - \frac{\sum \min(S_{AB}, S_{BA})}{\sum S_{AB} + \sum S_{BA}}
\]

where \(S_{AB}\) is the number of individuals in the ith OTU of community A and \(S_{BA}\) is the number of individuals in the ith OTU of community B. Effluent leachate samples from all reactors at peak methane generation as well as leachate and solid waste samples from Reactor A at the end-state of the test were analyzed. These samples were selected to compare microbial community composition between (1) leachates representative of different peak methane productions, (2) leachate and solid fractions at similar states of MSW decomposition, and (3) leachate at peak methane production and end-state decomposition.

Pyrosequencing was successful for effluent leachate samples from Reactors B and C at peak methane flux and on end-state leachate and solid samples from Reactor A; all yielded >2000 sequences following quality control procedures. The leachate sample from Reactor A at peak methane yielded an unusually low number of sequences and was not included in subsequent analysis.

3. Results

3.1. Bioreactor performance

3.1.1. Waste temperature

Temporal trends of weekly average waste temperature for each reactor are shown in Fig. 2. Temperatures plotted in Fig. 2 were averaged from thermocouples installed within MSW specimens (i.e., temperatures measured in the gravel are not included) (Fig. 1). Temperature control issues resulted in waste temperatures in Reactor B varying from 22 to 54 °C between 29 and 60 d. During this period, waste temperatures were more stable in Reactor A (27–34 °C) and Reactor C (27–39 °C). Average waste temperatures for all reactors were relatively constant between 60 and 181 d: 35.9 ± 3.3 °C in Reactor A, 39.2 ± 1.4 °C in Reactor B, and 34.4 ± 2.2 °C in Reactor C. Although waste temperatures and temporal changes differed between reactors (Fig. 2), waste temperatures between 60 and 181 d are representative of mesophilic environments.

3.1.2. Leachate chemistry

Relationships of pH, COD, and ORP measured in the effluent leachate vs. time for all three reactors are shown in Fig. 3. Similar temporal trends are observed in each reactor for all three leachate chemical parameters, and are consistent with previously described leachate chemistry behavior for anaerobic decomposition of MSW (e.g., Barlaz et al., 1989; Pohland and Kim, 1999; Lalou-Carpentier et al., 2006). Stimulation of hydrolytic and fermentative bacteria with the onset of leachate addition (Day 41) produced an accumulation of carboxylic acids, indicated by high COD concentrations and near or below neutral pH (Fig. 3). Subsequently, COD concentrations decreased, pH increased, and ORP approached a minimum (Fig. 3); leachate chemistry trends that are indicative of a transition to active methanogenesis. Following the removal of readily available substrates in the leachate (~80–100 d), COD remained below 10,000 mg/L and pH remained greater than 7.5 in all reactors for the duration of the experiment.

3.1.3. Methane generation

Temporal relationships of methane flow rates and cumulative methane yield for the three reactors are shown in Fig. 4. Methane composition of the biogas was similar in all reactors and averaged between 61% and 64% from 90 d to the end of the experiment, with the balance being CO₂. All three reactors exhibited higher initial methane flow rates characteristic of accelerated methane production phases, followed by decreasing flow rates indicating a transition to a decelerated methane production phases (Barlaz et al., 1989).

Cumulative methane yield, elapsed time for onset of methane production and peak methane flow rate, peak and average methane flow rates, and average waste temperature before and during methane production for each reactor are summarized in Table 2. Although the initial MSW composition was similar between all three reactors, cumulative methane yield ranged from 5.25 to 18.7 L/CH₄/kg-dry-MSW (Table 2, Fig. 4). These differences in cumulative methane yield are attributed to variability in the initial cellulose and hemicellulose compositions between the three reactors (described subsequently). The highest peak and average methane flow rates were measured in Reactor C, which also generated the largest cumulative methane yield (Table 2).

3.1.4. Discussion

The elapsed time to methane production was shortest in Reactor C, with measurable methane production commencing on Day 48, 7 d after the first 10-L leachate dose. Prior to initiation of
methane production, the average waste temperature in Reactor C (Table 2) was near optimal for mesophilic waste decomposition (~38–42 °C; Farquhar and Rovers, 1973; Barlaz et al., 1989) and more stable compared to the other two reactors (Fig. 2). This stable, near optimal mesophilic temperature phase probably contributed to earlier methane generation in Reactor C by allowing rapid establishment of a methanogenic community.

Methane production in Reactors A and B was not observed until Day 75, 34 d after leachate addition began. The longer lag time for onset of methanogenesis in Reactor A was possibly a result of lower waste temperatures (Fig. 2) that retarded activity of microbial communities. Decreases in methanogenic activity and methane generation have been reported with temperature decreases comparable to those measured in Reactor A (Fig. 2) following the initial leachate dose (Hartz et al., 1982; Kettunen and Rintala, 1997). In addition, Kettunen and Rintala (1997) report an increase in the lag time for methane generation from leachate for temperature decreasing below 35 °C. The initial temperature fluctuations in Reactor B (Fig. 2) may have stagnated microbial development during initial leachate recirculation (41–75 d), as temperatures fluctuating between mesophilic and thermophilic ranges have been shown to inhibit waste decomposition (e.g., Pfeffer, 1974). The lag-time for onset of methane production in Reactor B is also supported by lag-times in COD depletion and pH stabilization compared to Reactor C (Fig. 3).

Leachate chemistry trends for Reactor A suggest that the duration of active methanogenesis was shorter compared to Reactors B and C. pH stabilized to above 7.4 (Fig. 3a), COD decreased to less than 10,000 mg/L (Fig. 3b), and ORP transitioned from negative to positive (Fig. 3c) more rapidly in Reactor A compared to Reactors B and C. These transitions, combined with limited early methane generation, suggest a shorter methanogenic phase occurred in Reactor A. However, the magnitude of COD concentration, which indicates the relative abundance of substrates for methanogenesis, was similar between all three reactors. Thus, while temporal trends in leachate chemistry are indicative of microbial activity contributing to MSW decomposition, the overall magnitude of the different chemical parameters (e.g., COD) do not appear to correlate to methane yield.

3.2. Microbial community and population

3.2.1. Bacteria

16S rRNA gene tag pyrosequencing was conducted to assess community diversity of leachate samples in Reactors B and C at peak methane production, and in Reactor A leachate and solids at the end of the experiment. The total number of high-quality sequences obtained and various measures of community diversity are presented in Table 3. Based on coverage estimates, more than 90% of the predicted diversity was captured in the sequencing effort. Reactor A end-state leachate had markedly higher alpha diversity (as measured by Simpson’s index) than any of the other samples.

The percent of sequences belonging to specific bacterial taxonomic groups are summarized in Tables 4 and S1. Bacteria associated with the phyla Bacteroidetes and Firmicutes constituted greater than 70% of the sequences obtained from leachate in Reactors B and C at peak methane generation. Bacteroidetes and Firmicutes are common in anaerobic environments (e.g., anaerobic digester, rumen), where they ferment complex polysaccharides such as cellulose and starch (e.g., Krause et al., 2008; Jaenicke et al., 2011).
Bacteroidetes-like sequences are also commonly recovered from bioreactor landfill leachate and MSW (e.g., Huang et al., 2004; Cardinali-Rezende et al., 2009).

The extent of similarity in community composition among the four samples was explored using the Bray Curtis metric (Table 5). The assemblages of bacterial operational taxonomic units (OTUs) in the leachate of Reactors B and C at peak methane generation were approximately 70% similar by this metric, and were markedly distinct from the assemblages in Reactor A leachate and solids at the end-state. The most abundant OTU across the whole dataset (comprising 49% of all sequences) was found in all four samples and belonged to the WCHB1-69 group within the Sphingobacteriales (Bacteroidetes phylum). The second most abundant OTU was also found in all samples and was classified in the Petrotoga genus within the Thermotogales (Thermotogae phylum).

Similarity in microbial community composition between Reactors B and C at peak methane generation suggests that temperature fluctuations prior to methanogenesis (Fig. 4) did not influence microbial community composition. Reactor B experienced a pronounced increase in waste temperature prior to leachate addition, followed by fluctuating temperatures before equilibrating at approximately 39 °C and transitioning to methanogenesis (Day

### Table 2

Summary of elapsed times for onset of methane generation and peak methane flow rate, peak and average methane flow rates, cumulative methane yield, and average waste temperature before and during methane generation for each reactor.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Unit</th>
<th>Reactor A</th>
<th>Reactor B</th>
<th>Reactor C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elapsed time to CH₄ generation</td>
<td>d</td>
<td>75</td>
<td>75</td>
<td>48</td>
</tr>
<tr>
<td>Elapsed time to peak CH₄ flow rate</td>
<td>d</td>
<td>91</td>
<td>99</td>
<td>86</td>
</tr>
<tr>
<td>Peak CH₄ flow rate</td>
<td>L-CH₄/kg-dry/d</td>
<td>0.53</td>
<td>0.43</td>
<td>0.69</td>
</tr>
<tr>
<td>Average CH₄ flow rate</td>
<td>L-CH₄/kg-dry/d</td>
<td>0.19</td>
<td>0.21</td>
<td>0.37</td>
</tr>
<tr>
<td>Cumulative CH₄ yield</td>
<td>L-CH₄/kg-dry</td>
<td>5.27</td>
<td>14.25</td>
<td>18.7</td>
</tr>
<tr>
<td>Average waste temperature before CH₄ generation</td>
<td>ºC</td>
<td>29.5 ± 2.3</td>
<td>40.6 ± 7.7</td>
<td>38.2 ± 2.3</td>
</tr>
<tr>
<td>Average waste temperature during CH₄ generation</td>
<td>ºC</td>
<td>32.6 ± 0.8</td>
<td>38.3 ± 0.7</td>
<td>34.6 ± 2.4</td>
</tr>
</tbody>
</table>

### Table 3

Sequencing results and alpha diversity estimates for leachate samples from Reactors B and C at peak methane generation and end-state leachate and solid samples from Reactor A based on 16S rRNA gene tag pyrosequencing.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of sequences</th>
<th>Coverage (%)</th>
<th>OTUs</th>
<th>Alpha diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactor B, peak methane</td>
<td>2170</td>
<td>90</td>
<td>331</td>
<td>5.38</td>
</tr>
<tr>
<td>Reactor C, peak methane</td>
<td>2541</td>
<td>91</td>
<td>353</td>
<td>5.18</td>
</tr>
<tr>
<td>Reactor A, end-state leachate</td>
<td>2032</td>
<td>90</td>
<td>316</td>
<td>21.18</td>
</tr>
<tr>
<td>Reactor A, end-state solids</td>
<td>2542</td>
<td>92</td>
<td>302</td>
<td>9.37</td>
</tr>
</tbody>
</table>

* Number of sequences remaining in the dataset following quality control.
* Estimated Good's coverage based on sequencing effort.
* Number of operational taxonomic units (OTUs) identified at the 97% sequence identity cutoff.
* Alpha diversity as measured by the inverse Simpson's index. Higher values indicate higher diversity. In this case, Reactor A end-state leachate was the most even community.

### Table 4

Percent of sequences belonging to specific phyla, orders, and families of Bacteria as determined by 454 tag pyrosequencing of 16S rRNA genes. Data are compiled for leachate samples from Reactors B and C at peak methane generation and end-state leachate and solid samples from Reactor A.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Order</th>
<th>Family</th>
<th>Reactor B peak methane (%)</th>
<th>Reactor C peak methane (%)</th>
<th>Reactor A end-state leachate (%)</th>
<th>Reactor A end-state solids (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroidetes</td>
<td>Bacteroida</td>
<td>Bacteroidales</td>
<td>10.8</td>
<td>9.5</td>
<td>4.0</td>
<td>3.4</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Flavobacteriota</td>
<td>Flavobacteriales</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Sphingobacteriota</td>
<td>Sphingobacteriales</td>
<td>45.8</td>
<td>45.7</td>
<td>18.2</td>
<td>3.5</td>
</tr>
<tr>
<td>Chloroflexi</td>
<td>Anaerolineae</td>
<td>Anaerolineales</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Bacilli</td>
<td>Bacillales</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Bacilli</td>
<td>Lactobacillales</td>
<td>3.6</td>
<td>1.3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
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<td>22.9</td>
<td>9.4</td>
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<tr>
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<td>–</td>
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<td>1.3</td>
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<tr>
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<tr>
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<tr>
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<td>Caulobacteriales</td>
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<tr>
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<tr>
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<td>Thermotogae</td>
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<td>10.1</td>
<td>3.0</td>
<td>30.7</td>
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* Only those phylogenetic groups representing greater than 1% of sequences in any one sample are presented here, and therefore percentages do not add to 100%. Table S1 in the supplementary online material contains a more complete list of taxa identified in the samples.
75). Reactor C experienced decreasing waste temperatures between the onset of leachate addition (Fig. 2) and peak methane generation (Day 86) from approximately 39 to 31 °C. While the fluctuating waste temperatures in Reactor B are believed to have prolonged the onset of methanogenesis by inhibiting growth, variability in waste temperatures between Reactors B and C did not influence the active microbial community composition after temperature stabilization. The similar microbial communities were attributed to similarity in the waste source and leachate inoculum, combined with similar waste temperatures within the mesophilic range during waste decomposition and microbial community development.

Bacterial community compositions determined for the end-state leachate and solid samples in Reactor A were not similar (Tables 4 and 5, and S1). Sequences retrieved from the solid fraction in Reactor A revealed a larger percentage of Clostridia compared to Reactor A leachate as well as both Reactor B and Reactor C leachates (Table 3). Thermotogae, another anaerobic fermentative organism, was also more abundant in the solids, accounting for >31% of the solids sequences (Table S1). The presence of Thermotogae at mesophilic temperatures confirms recent reports of abundant mesophilic members of a genus previously considered to be predominantly hyperthermophilic (Nesbo et al., 2006). Thermotogae ferment complex polysaccharides, including cellulose, and produce hydrogen gas as a byproduct (Conners et al., 2006; Cardinali-Rezende et al., 2009). Thermotogae and Clostridia may have been enriched in the solids fraction due to a preference for growth attached to a solid polysaccharide substrate, such as cellulose.

Proteobacteria accounted for approximately 55% of sequences in the leachate, but less than 3% in solids, for Reactor A at the end-state (Table S1). Members of the Proteobacteria are physiologically very diverse and many can ferment simple polysaccharides or monosaccharides (Krause et al., 2008; Jaenicke et al., 2011). These bacteria were likely enriched in the leachate because they were involved in fermentation of soluble substrates such as simple sugars. The differences between bacterial communities in solids and leachate fractions of MSW agree with recent findings by Staley et al. (2012), and further supports the assessment of both solids and leachate to more broadly capture the bacteria community composition of MSW.

A direct comparison between bacteria community composition in the leachate fraction at peak methane generation and end-state decomposition is not possible due to failure of the pyrosequencing reaction for Reactor A peak methane. However, assuming the bacterial communities in Reactors B and C are representative of peak-methane leachate in Reactor A, the bacterial community shows a shift from a predominantly Bacteroidetes and Firmicutes community during peak methane production (Reactors B and C in Table 4) to a Proteobacteria-dominated community during end-state decomposition (Reactor A in Table 4). This change could represent a depletion of complex polysaccharides, for instance as cellulose is degraded and removed, and a shift towards degradation of less complex breakdown products such as sugar monomers. This progression was previously described by Barlaz et al. (1989), who measured a decrease in cellulolytic bacteria and an increase in acetogenic bacteria during decelerated methane production.

3.2.2. Archaea and methanogens

Temporal relationships of methanogen abundance (orders Methanomicrobiales and Methanobacteriales and family Methanosarcinaceae) measured with qPCR on effluent leachate for each reactor are shown in Fig. 5. Methane flow rates are reproduced as weekly averages in Fig. 5 to identify relationships between methane generation and methanogen dynamics. Abundance of all three methanogens (Methanomicrobiales, Methanosarcinaceae, and Methanobacteriales) increased between 48 and 97 d, concurrent with increasing methane flow rates (Fig. 5). Peak methane flow rates approximately coincided with maximum methanogen abundance, and subsequent methanogen abundances remained elevated for the duration of the experiment in all three reactors.
This is consistent with previous work showing elevated methanogen abundance throughout the decelerated methane phase (Barlaz et al., 1989). The order Methanococcales and the family Methanosetaeaceae were not detected by PCR in any leachate sample.

Although the PCR-based approaches to detecting 16S rRNA genes are powerful tools for studying microbial community composition, they do have limitations that should temper interpretation of the results. These approaches are inherently focused on DNA and only provide a proxy for the abundance of different target phylotypes (groups). They do not directly measure activity of the detected organisms or the expression of genes involved in key metabolisms. However, the presence and abundance of specific populations is assumed necessary to drive the measured processes (i.e. hydrolysis, fermentation, volatile fatty acid oxidation, and methanogenesis). In any case, technological advances allowing detection of specific mRNAs and whole transcriptomes may reveal new relationships in future studies.

### 3.3. Methanogen population and methane generation

#### 3.3.1. Implications on methane flow rates

Temporal relationships of methanogen abundance and methane flow rates shown in Fig. 5 indicate that the initial methanogen growth phase coincided with the accelerated methane production phase. Substrate (e.g., acetate) for methane generation is readily abundant in the leachate fraction during accelerated methane production due to initial hydrolysis, fermentation, and acetogenesis of cellulose and hemicellulose in MSW (Barlaz et al., 1989). Following the removal of readily available substrates in the leachate fraction, characterized by the transition from a high rate of COD removal to a low rate of COD removal between 90 and 100 d (Fig. 3b), methane generation rates decrease as waste decomposition transitions to a decelerated methane production phase (Fig. 5).

Relationships between methane flow rate and methanogen abundance during accelerated and decelerated methane generation phases are shown in Fig. 6. During accelerated methane generation, methane flow rate vs. methanogen abundance data from all reactors create a single relationship that indicates higher methane flow rates correspond to higher methanogen abundance. In contrast, there is no correspondence between methane flow rate and methanogen abundance during the decelerated methane production phase (Fig. 6b). In anaerobic decomposition, methanogenesis is rate-limiting when substrates are solubilized (e.g., during accelerated methane production) and hydrolysis is rate-limiting when substrates are complex solid organic matter (Nioke et al., 1985; Pavlostathis and Giraldo-Gomez, 1991; Vavilin et al., 1996). The positive correspondence shown in Fig. 6a suggests methanogen growth drives methane generation when soluble substrates are readily available (e.g., high COD concentration), and that higher methane flow rates are dependent on establishing of a larger methanogen population.

Methane flow rates for all three reactors ranged between approximately 0.01 and 0.3 L-CH₄/kg-dry/d during decelerated methane production. However, methanogen abundance within each reactor remained nearly consistent throughout the decelerated methane production phase, corresponding to the abundances achieved during the growth phase (Fig. 5). The continued detection of high methanogen abundance following peak methane generation suggests that methanogens remain abundant in MSW, but at low metabolic activity after the bulk of readily degradable organic matter have been consumed. Thus, the presence of methanogens may be applicable as an indicator of past or potential future methane production, but is not necessarily a direct indicator of current methanogenic activity.

![Fig. 6. Methane flow rates vs. total methanogen abundance in each reactor during (a) accelerated and (b) decelerated methane production phases. Dashed lines in (a) capture the general trend in the data.](image)

![Fig. 7. Relationship between cumulative methane yield and average methanogen abundance between 97 and 181 d of reactor operation.](image)
3.3.2. Implications on methane yield

The relationship between cumulative methane yield and methanogen abundance for the three reactors is shown in Fig. 7. Average abundance of Methanomicrobiales, Methanosarcinaceae, and Methanobacteria in Fig. 7 are representative of methanogen populations measured on the leachate fraction between 97 and 181 d (i.e., established methanogen population following the accelerated methane generation phase, Fig. 5), and total methanogens were the sum of average individual methanogen abundances for each reactor. Although similar methanogen communities were observed among reactors, higher abundance of Methanomicrobiales and Methanosarcinaceae correlate with higher cumulative methane yield (Fig. 7). The total methanogen abundance for the higher two methane yields (Reactors B and C) is dominated by Clostridia (Fig. 7). This suggests Methanomicrobiales influenced methane generation to a greater extent than Methanosarcinaceae or Methanobacteria, and that the majority of methane was produced via the hydrogenotrophic pathway. The presence of abundant hydrogenotrophic methanogens is in agreement with previous work on MSW and other anaerobic digestion systems (e.g., McMahon et al., 2001; Chen et al., 2003; Krause et al., 2008; Rastogi et al., 2008; Nayak et al., 2009; Jaenicke et al., 2011; Sasaki et al., 2011; Zhu et al., 2011; Staley et al., 2012). Interestingly, Thermotogae and Clostridia, two of the most abundant phyla in the reactors, are both known to produce hydrogen during anaerobic degradation of cellulose and other polysaccharides (Krause et al., 2009; Cardinalli-Rezende et al., 2009; Jaenicke et al., 2011). This suggests that there was likely syntrophic relationships between hydrogen producing bacteria and hydrogen consuming methanogens in the MSW. The Bacteroidetes, on the other hand, are acetogenic bacteria and likely supported the growth of acetotrophic methanogens such as Methanosarcinaceae.

The relationship between methanogen abundance and initial percent contribution of cellulose (C) plus hemicellulose (H) in each reactor is shown in Fig. 8. The initial C and H for each reactor were back-calculated from cumulative methane yield (Table 2) and state C and H measured on exhumed waste (Table 1). The back-calculated initial C and H (Table 1 and Fig. 8) indicate that the initial available substrate to support microbial growth differed between reactors. These different initial C and H contributions are attributed to differences in the material specific biodegradable constituents comprising the paper and cardboard fraction (e.g., office paper, newsprint, corrugated cardboard), which was the primary biodegradable fraction for MSW used in this study. MSW components comprising the paper/cardboard fraction have varying rates of decomposition and cumulative methane yield (Owens and Chynoweth, 1993; Eleazer et al., 1997; Staley and Barlaz, 2009). Thus, although the initial waste composition was consistent between the three reactors, the chemical signature of the MSW constituents likely varied and contributed to different initial substrate abundance to support microbial growth.

The back-calculated C and H (Table 1) are believed to more adequately represent the initial C and H available in each reactor as compared to the C and H measured on the initial MSW (Table 1). The lack of trends in the C, H, [C + H]/L, VS, and BMP between the three exhumed wastes and initial waste (Table 1) are likely due to inherent heterogeneity in chemical properties of components contained in each biodegradable group (Bareither et al., 2010b). Relative percentages of C and H contributing to a methane yield from a given reactor (Table 2) were based on waste decomposition measured in a field-scale experiment on MSW from the same source and with the same initial composition as used in this study (Bareither et al., 2012). Comparison of initial to final C + H in the field-scale experiment revealed that 93% of the change between initial and final biochemical methane potential was attributed to C + H decomposition (Bareither et al., 2012). This observation is in agreement with Barlaz et al., (1990), who report >90% of methane yield in MSW originates from C and H.

Statistically significant correlations for Methanomicrobiales, Methanosarcinaceae, and total methanogen abundance versus initial C + H are shown in Fig. 8 for trendlines with coefficients of determination \((R^2) > 0.90\). These correlations are evidence that methanogen abundance in MSW is dependent on the available C and H, which are the primary substrates that support microbial growth in MSW. Relationships between cellulose degrading organisms such as Thermotogae or Clostridia, and hydrogenotrophic methanogens, likely provide the means for C + H to indirectly support methanogen growth and activity. Comparisons between environmental factors measured in the MSW (e.g., waste temperature and leachate chemistry) provide insights on the establishment and proliferation of microbial communities. However, availability and type of substrate eventually determined the microbial community that developed in the MSW.

4. Conclusions

Bacterial diversity and methanogen abundance were evaluated in this study in three laboratory reactors simulating bioreactor landfill conditions. Quantitative molecular tools (5′-exonuclease qPCR) and next-generation sequencing (454 pyrosequencing) were used to investigate microbial communities during biodegradation of municipal solid waste (MSW). The following conclusions are drawn from this study:

- Similar microbial communities (bacterial and archaeal) develop during the accelerated methane production phase in MSW at mesophilic temperatures independent of initial temperature fluctuations.
- Temporal transitions in methanogenic activity as well as the duration of methanogenesis can be linked to transitions in
leachate pH, chemical oxygen demand, and oxidation–reduction potential. However, chemical oxygen demand concentration is not an indicator of cumulative methane yield. This may well be because the concentration does not adequately reflect the flux of carbon through the ecosystem.

- Methanogen abundance increased during the accelerated methane phase and higher abundance corresponds to higher methane flow rates; whereas methanogen abundance remained nearly constant during decelerated methane production with abundance similar to levels established during growth. Thus, methanogen abundance likely remains high following accelerated methane production and is not a direct indicator of active methanogenesis.

- Larger cumulative methane yield corresponded to higher abundance of *Methanomicrobiales*, *Methanosarcinaeaceae*, and total methanogens, which correlate with initial percent contribution of cellulose and hemicellulose. Larger initial cellulose and hemicellulose fractions support growth of larger methanogenic populations, via relationships with cellulose-degrading organisms that ultimately contribute to higher cumulative methane yield.

- *Methanomicrobiales* was the most abundant methanogen in this study, suggesting that hydrogenotrophic methanogenesis was probably the predominant pathway for methane generation.

### Acknowledgements

Funding for this project was provided by the University of Wisconsin System Solid Waste Research Program. The authors wish to thank Steven Fong for his laboratory assistance, and Eric Roden and Noah Fierer for assistance with pyrosequencing analysis. This paper is dedicated to the legacy of Professor Robert K. Ham, the University of Wisconsin-Madison. His legacy lives on in vibrant research and educational programs throughout the campus.

### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.wasman.2012.12.013.

### References


