STALEY, BRYAN FLEET. Environmental and Spatial Factors Affecting Microbial Ecology and Metabolic Activity during the Initiation of Methanogenesis in Solid Waste. (Under the direction of Morton A. Barlaz and Francis L. de los Reyes III.)

Anaerobic decomposition of organic matter occurs in both natural (e.g., soil, peat bogs, digestive tracts) and engineered (e.g. landfills, anaerobic digesters) ecosystems. The primary end-products of anaerobic decomposition are methane (CH$_4$) and carbon dioxide (CO$_2$). Upon landfilling, rapidly degradable materials within the refuse anaerobically decompose resulting in an accumulation of volatile fatty acids (VFAs) and a commensurate drop in pH to a minimum ranging between 5.5 and 6. These low pH, high carboxylic acid conditions have been shown as inhibitory to methanogenic Archaea in analogous ecosystems such as peat and the rumen. In contrast to these findings, methanogenesis initiation occurs under these conditions, indicating the mechanism by which methane production begins in refuse is poorly understood.

There are two theories for how methane production initiates in landfills. One is that methanogenic Archaea (i.e. methanogens) tolerant to the low pH, high VFA conditions consume acids until the bulk pH is suitable for the establishment of methanogens that grow under pH-neutral conditions. The second theory is that spatially isolated areas of neutral pH exist while bulk pH is acidic and these localized regions of neutral pH act as initiation centers for methanogenesis. The goal of this study was to test these two theories and validate their importance relative to methanogenesis initiation in refuse.

To evaluate methanogen acid tolerance in decomposing refuse, three liquid inocula were derived: (1) refuse just entering active decomposition, (2) well-decomposed
refuse and, (3) peat. Under high VFA concentrations, results showed methanogenesis initiation occurred at pH minima of 6.25, 5.75 and 5 for actively decomposing refuse, well-decomposed refuse and peat, respectively. The hydrogenotrophic *Methanoculleus* genus facilitated methane initiation in actively decomposing refuse (pH 6.25) while *Methanosarcina* triggered methane production in well-decompose refuse (pH 5.75). In peat, methanogenesis was facilitated by an uncultured *Methanosarcinales*. This is the first study to fully characterize methanogens responsible for methane initiation under low pH, high VFA conditions and suggests acid tolerance (pH 5 – 6.25) is relatively common provided sufficient acclimation time. However, methane production rates at lower pH were found to be 3 to 6 fold lower than those at neutral pH.

To evaluate the spatial influences on methanogenesis initiation, fresh refuse was placed into triplicate laboratory scale reactors, decomposed to the anaerobic acid phase, and destructively sampled when methanogenesis initiated. Large differences were observed spatially in refuse pH, moisture content and VFA concentration. No pH neutral niches were observed in reactors prior to methanogenesis. RNA clone library results showed most bacterial activity was attributed to the *Clostridiales* order. Methanogenic *Archaea* activity at low pH was catalyzed by *Methanosarcina barkeri*. After methanogenesis, pH neutral conditions developed in high moisture content areas containing substantial populations of *M. barkeri*. These areas expanded with increasing methane production, forming a unified reaction front that advanced into low pH areas. In the absence of pH neutral niches, this study suggests methanogens tolerant to low pH, such as *M. barkeri*, are required to overcome the low pH, high VFA conditions typically present during the anaerobic acid phase of refuse decomposition.
Environmental and Spatial Factors Affecting Microbial Ecology and Metabolic Activity during the Initiation of Methanogenesis in Solid Waste

by
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A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Civil Engineering, Raleigh, North Carolina, 2009

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DEDICATION

I dedicate this work to Ann-Margaret for her unconditional love and patience, and to my son, Isaac, who receives my unconditional love and patience.
BIOGRAPHY

Bryan Staley received his Bachelor of Science degree in Biological and Agricultural Engineering from North Carolina State University in 1994. He received his Master of Science degree in Biosystems Engineering, with an Environmental Engineering minor, from the University of Tennessee in 2000. Bryan began his consulting career in 1996 with Agri-Waste Technology, Inc. (Raleigh, NC) where he was a project manager. After receiving his professional engineering license in 2000, Bryan was briefly Vice President and Engineering Manager for a small consulting firm prior to becoming a project manager at Duane K. Stewart and Associates, a land development engineering firm in Durham, NC. His consulting work ranged from the design of large-scale livestock waste treatment systems and irrigation systems to land development engineering, where he designed sites for commercial, residential and institutional uses. In mid-2003, Bryan left consulting and joined Dr. Morton Barlaz and Dr. Francis de los Reyes III at North Carolina State University to work on his doctoral degree in environmental engineering.
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Chapter 1.

METHANOGENESIS INITIATION: BACKGROUND AND JUSTIFICATION OF RESEARCH

Introduction and research objectives

Anaerobic decomposition of organic matter occurs in both natural (e.g., soil, peat bogs, digestive tracts) and engineered (e.g. landfills, anaerobic digesters) ecosystems. The primary end-products of anaerobic decomposition are methane (CH$_4$) and carbon dioxide (CO$_2$). Both of these gases contribute to global warming and CH$_4$ has been implicated as being 25 times more potent as a greenhouse gas compared to CO$_2$ (IPCC, 2007). It is estimated that landfills are a major anthropogenic source of methane emissions, releasing roughly 25 Tg CH$_4$/yr to the atmosphere (Khalil, 1999). Biogenic organic carbon not ultimately released as CO$_2$ or CH$_4$ in landfills is sequestered at an estimated rate of 119 Tg C/year (Barlaz, 1998). Both CH$_4$ formation and carbon sequestration are microbially mediated via a series of complex processes. Using recently developed molecular techniques, studies have identified the landfill microbial community to the genera and species level (Huang et al., 2002; Chen et al., 2003a; Chen et al., 2003b; Huang et al., 2003; Uz et al., 2003; Huang et al., 2004; Huang et al., 2005), but have yet to correlate community structure with functional characteristics of refuse degradative processes (e.g., CH$_4$ formation, production/consumption of metabolic intermediates, pH, etc.).

Decomposition of organic matter in various anaerobic ecosystems (e.g., landfills, soil, sediments, peat, rumen, termite gut, etc) progresses through distinct phases, occurring...
both temporally and spatially (Barlaz et al., 1989a; Yao et al., 1999). In refuse, these phases have been defined as aerobic, acid, accelerated methane and well-decomposed or decelerating methane (Barlaz et al., 1989a). Of these, the acid phase is brought about by rapid fermentation and hydrolysis reactions that typically cause an accumulation of carboxylic acids, thereby driving down ecosystem pH. Typically, refuse pH minima in the acid phase range between 5.5 and 6 (Barlaz et al., 1989a; Mormile et al., 1996). Over time, accumulated acids are consumed and the pH increases to neutral. It is widely accepted that under these conditions methanogenic *Archaea* are the primary microorganisms that consume carboxylic acids or their metabolites, but the mechanisms by which this occurs are poorly characterized. One difficulty is that most methanogens isolated in pure culture have an optimal pH range between 6.5 and 7.8 (Ferry, 1993) and the optimal pH for methane production in refuse has been reported as 6.8 to 7.2 (Kasali et al., 1988). If true for all methanogens, one would expect methanogenesis to be absent or at least severely inhibited when pH is well below optimal. However, previous research in refuse (Barlaz et al., 1992), peat (Cadillo-Quiroz et al., 2006) and anaerobic bioreactors (Ueno et al., 2006) has shown methanogenesis initiation can occur when the bulk ecosystem pH is around 5.75, 4 and 6, respectively. This leads to the question of how is methanogenesis initiated.

There are currently two hypotheses on how methanogenesis is initiated in landfills. The first is that acid-tolerant or acidophilic methanogens consume acids until the bulk pH is amenable for the establishment of pH-neutral methanogens. Thus far, one study has identified a methanogen isolate that exhibited some growth at pH 5 (Ladapo and Barlaz, 1997), suggesting that the potential exists for acid-tolerant methanogens in refuse. The
second hypothesis is that localized areas of neutral pH exist while bulk pH is acidic. It is presumed that these zones act as methanogenesis initiation centers and expand as acids are consumed (Martin et al., 2003). Currently, the existence of localized pH neutral areas in refuse has been implicated via modeling (Vavilin and Angelidaki, 2005). However, the experimental work performed to validate this model examined the impact of refuse mixing on methanogenesis initiation and did not measure discrete spatial characteristics. Thus, the presence, size and abundance of any pH-neutral zones have not been definitively proven.

The overall objective of this research is to evaluate causal factors contributing to methanogenesis initiation during refuse decomposition. Based on the hypothesized mechanisms initiating CH₄ formation, specific goals of this research are to: 1) determine the role of acid-tolerant methanogens in facilitating the transition from the acid phase to the accelerated CH₄ phase during refuse decomposition, 2) evaluate the importance of localized pH-neutral areas, and 3) correlate microbial metabolic activity to environmental conditions in a particular phase of decomposition.

The remainder of this chapter discusses previous research performed related to the mechanisms affecting methanogenesis initiation such as acid tolerance, dependence on pH, and spatial factors. Subsequent chapters contain the following manuscripts submitted for publication in peer-reviewed journals:

Chapter 2. Processing heterogeneous materials prior to DNA extraction is essential for minimizing bias: a comparison of methods using decomposed refuse

Chapter 3. Comparison of Bacteria and Archaea communities in mixed refuse, individual refuse components and leachate
Mechanisms affecting methanogenesis initiation

Two potential hypotheses for the initiation of methanogenesis have been proposed. The primary variables in both hypotheses, acid-tolerant methanogens and pH, appear to be inextricably linked to many other factors, such as: syntrophic interactions, population size, substrate availability, and initial or start-up conditions, among others. In addition, variables affecting acid-tolerant methanogens and pH can also be dependent on one another, emphasizing the complexity of methanogenesis initiation. For example, volatile fatty acid (VFA) concentration impacts pH which affects methanogen activity which, in turn, directs VFA consumption.

Although these intricate associations are expected, a literature review of every interrelated variable would represent an extensive body of literature. While the coverage of this review primarily focuses on studies directly relevant to acid-tolerant methanogens and spatial variations in methanogenesis, a number of secondary variables described in previous research have been identified as important. Therefore, background information presented here incorporates research related to substrate availability, reactor start-up conditions, population size and syntrophic interactions.
Acid-tolerant methanogens. Very few ecosystems have been evaluated for the presence of acid-tolerant methanogens, and northern peat bogs are by far the most studied since they are typically characterized by low pH conditions (Horn et al., 2003). Numerous studies have indicated methane production in peat at a pH <5 (Williams and Crawford, 1985; Bergman et al., 1998; Brauer et al., 2004; Kotsyurbenko et al., 2004; Juottonen et al., 2005). This research has led to the identification of a number of acid-tolerant methanogens belonging to either *Methanobacteriaceae* (Williams and Crawford, 1985; Horn et al., 2003; Sizova et al., 2003), or *Methanomicrobiales* (Horn et al., 2003; Sizova et al., 2003). Recently an acidophilic methanogen from the *Methanomicrobiales* order was isolated from a New York, USA peat bog (Brauer et al., 2006). Optimal CH$_4$ production from this species occurred at a pH of 4.8-5.0, which is lower than any previously described methanogen.

All acid-tolerant methanogens identified thus far have been hydrogenotrophic, most likely because the low acetate concentrations seen in peat do not promote robust populations of acetoclastic methanogens. In a study by Kotsyurbenko et al (2004), acidic peat soils that produced CH$_4$ optimally around a pH of 5 were incubated for 90 days at different pHs. After incubation, optimal CH$_4$ production shifted to a more neutral pH range (pH = 6-7) indicating that acid-tolerant and neutral-pH methanogens appear to co-exist but that the dominant group is selected by pH. Research on peat indicates that high acetate concentrations (>1 - 5 mM) tend to inhibit methanogenesis at low pH (Brauer et al., 2004). This is in agreement with studies on the bovine rumen, which found that acetate in high enough concentrations can prove inhibitory to methanogens when pH is low (Russell,
Hydrogenotrophic methanogens typically dominate in both peat (see above) and rumen ecosystems (VanKessel and Russell, 1996).

In refuse, both H₂/CO₂ methanogens (*Methanomicobiales*) and acetate utilizing methanogens (*Methanosarcina/Methanosaeta*) dominate, with acetate utilizers tending to be more active in younger refuse (Chen et al., 2003a; Chen et al., 2003b; Huang et al., 2003; Mori et al., 2003). At a pH of less than 6, acetate concentrations in refuse ranged between 120 – 135 mM in a laboratory-scale simulation (Barlaz et al., 1989b). Assuming no methane generation, this suggests that methanogens in refuse may not experience the toxic effects reported under high acetate concentrations in peat and rumen ecosystems.

One study in refuse (Ladapo and Barlaz, 1997) found an isolate that produced measurable CH₄ at a pH of 5 and an acetate concentration of 20 mM. The experiment was conducted in a liquid inoculum, thus implicating the existence of isolates that can tolerate both low pH and high acetate levels.

Similarly, methane composition from an anaerobic digester treating solid waste was 60% methane at pH 6 and an acetic acid concentration of 84.8 mM (Griffin et al., 1998). This experiment was performed under mesophilic conditions and the data noted refers to day 12 from reactor start-up. The same research group observed that high acetate concentrations (135.7 mM) did not affect *Methanobacteriaceae* whereas *Methanosarcina* and *Methanosaeta* species were inhibited (McMahon et al., 2004). Another anaerobic digester study isolated a hydrogenotrophic acid-tolerant methanogen, *Methanobrevibacter acididurans*, that was able to produce CH₄ at a pH of 5; however, optimal production occurred at a pH of 6 (Savant et al., 2002). Interestingly, a subsequent study by Savant and
Ranade (2004) found that biochemical methane potential assays of acidogenic (pH = 5.6, acetate = 122.2 mM) and methanogenic (pH = 6.2, acetate = 41.6 mM) digester slurries bioaugmented with *Methanobrevibacter acididurans* enhanced methane production by 12.2% and 7.5%, respectively. This study, in particular, provides the most definitive proof of a methanogen able to grow at low pH and high acetate concentrations. The evidence from these studies and research on peat confirms the existence of acid-tolerant methanogens; however, their role in the transition from acidic to neutral pH in refuse is still unclear.

*pH as a Controlling Variable in Methanogenesis Initiation.* The inhibitory effect of low pH on methanogenesis in landfills and anaerobic digesters has been well documented (Griffin et al., 1998). Other work implies that high acetate concentrations inhibit methanogenesis under some conditions (see above); however, these results may indicate that high VFAs act as a selection mechanism for certain microbial populations. For example, James et al. (1998) operated chemostat reactors treating landfill leachate where butyrate and valerate were the sole carbon sources prior to an acetate spike. Limiting available substrates in this manner likely diminished the acetoclastic methanogen population since hydrogenotrophic methanogens would have been selected (due to butyrate/valerate oxidization to H\(_2\)/CO\(_2\) by syntrophic bacteria). Thus, methanogenesis in this situation would be very sensitive to acetate fluctuations which may explain the lack of methane production when acetate was added. Furthermore, Barlaz et al. (1989b) found relatively high rates of methane production from refuse at neutral pH and high carboxylic
acid concentrations. These VFA concentrations were almost as high as measured in the acid phase of decomposition, suggesting that VFAs do not inhibit CH$_4$ production. Another study in an anaerobic digester treating solid waste found that methane formation was not inhibited by high VFA levels and production increased with increasing VFAs (Ohmura et al., 2003).

However, the selection of specific microbial populations by VFA concentration has been observed (McMahon et al., 2001; Karakashev et al., 2005; Conklin et al., 2006), and this can inhibit or delay the onset of CH$_4$ formation if substrate conditions abruptly change (Hori et al., 2006). Indeed, many issues related to process instability or inhibited CH$_4$ generation during start-up or initiation can be attributed to low pH and/or selection effects due to VFA accumulation (Gujer and Zehnder, 1983; Griffin et al., 1998). The importance of pH as a controlling variable for methanogenesis initiation has been noted (Dearman et al., 2006); however, VFA levels are obviously linked to pH since they contribute to pH reduction and select for microbial sub-populations.

Spatial variation and microniches in refuse. Barlaz et al. (1992) found no differences between trophic level (fermentative, acetogenic, methanogenic) microbial populations in laboratory-scale reactors degrading refuse operated with and without leachate recirculation. Despite this, CH$_4$ production in recirculated reactors was higher and was attributed to a more uniform distribution of substrate and microorganisms. Mathematical modeling suggests that as decomposition proceeds methanogen populations may become more abundant in discrete areas (Martin, 2001; Vavilin and Angelidaki, 2005). If leachate
recirculation makes substrate more widely available across a reactor, a more uniformly distributed population would be expected compared to reactors without leachate recirculation which, in contrast, would exhibit greater non-uniformity. Differential methanogen populations have been attributed to localized areas of high substrate variability, or ‘hotspots’, in various types of soils (Wachinger et al., 2000). The differential CH$_4$ production observed by Barlaz et al. (1992) implies that, despite similar population sizes at the trophic level, substrate availability may induce spatial differences that affect the extent of degradation. In addition, methanogen populations in undegraded refuse and individual refuse components appear to be highly variable (Qian and Barlaz, 1996). This suggests that initial population differences in undegraded refuse could also cause spatial variations in methanogenesis once landfilled. However, many unknowns exist amongst these experiments, stressing the need for studies designed specifically to address initiation of methanogenesis and to bridge the gap between experimental and modeled results.

Spatial differences in anaerobic digesters. Spatial variation in methane formation is also observed in highly engineered processes such as anaerobic digesters and upflow anaerobic sludge blanket (UASB) reactors. In digesters with high organic loading rates and low mixing conditions, spatial variation has been implicated in the formation of microenvironments that contribute to shorter methanogenesis initiation times (i.e. start-up time) (McMahon et al., 2004). In a UASB process, biomass granules are formed that consist primarily of a bacterial and methanogenic consortia (Macario et al., 1991; Visser et al., 1991). Lens et al. (1993) examined pH and glucose microprofiles in granules where
bulk liquid outside the granule was at a neutral pH. A pH drop from 7 to 5.4 was measured from the surface of the granule to a depth of 200 µm after which pH increased. It was found that most fermentative activity was in the outer 300 µm of the granule while methanogenesis occurred at depths greater than 300 µm. These results confirm that spatial stratification occurs and implies that substrate consumption by the methanogens maintain more neutral pH microniches, even when the surrounding material is acidic.

**Vertical stratification of methanogenesis in peat bogs.** Given the depleted nutritional state of organic matter typically found in peat bogs (Kotsyurbenko et al., 2004), the anaerobic degradation phases observed in refuse are absent. Nevertheless, spatial variation in methane production is observed and typically follows a bell curve distribution with depth. In a study by Galand et al (2002), three peat cores were advanced to a depth of 40 cm and were located 20 cm from one another laterally. Methane production was found to vary by depth, with most CH$_4$ production at higher depths (10-20 cm). A shift in methanogen community structure from *Methanomicrobiales* to *Methanosarcinales* was observed from shallow to deeper layers, respectively. Two to six fold differences in methane production were observed laterally (at a depth of 20 cm). Localized variations in methanogen activity were attributed to substrate differences or competition with other bacteria and similar observations have been noted in other studies (McDonald et al., 1999; Merila et al., 2006). These results are applicable to the proposed research because they suggest that spatial differences, and the factors associated with them, affect methanogen community structure even in a highly degraded material, such as well-decomposed refuse. From this, one might
extrapolate that the larger the shift in environmental conditions (e.g. perturbation) the greater the potential for spatial differences.

*Impact of scale on soil and sediment communities.* A study by Wachinger et al. (2000) sampled soil at the 1 cm scale and found wide changes in methanogenesis with depth. Interestingly, no CH$_4$ production was found at some depths, whereas high CH$_4$ production occurred at depths directly above and below. This finding shows that, in unsaturated conditions, spatial differences in CH$_4$ production do not necessarily follow a gradient induced by diffusive/convective processes, as typically seen in peat (see above) or in sediments (Falz et al., 1999; Inagaki et al., 2004). Wachinger (2000) suggested that spatial variations are due to both the presence fresh organic material and the degree to which the material is accessible. This finding parallels the concept of neutral pH microhabitats in refuse since limited accessibility would be necessary to maintain localized pH conditions suitable for methanogen activity during the acid phase. Roy et al. (1997) found methanogenesis was initiated in rice paddy soils one day after anoxic conditions were established, while in the presence of higher redox potential electron acceptors (NO$_3^-$, SO$_4^{2-}$, Fe$^{3+}$). Methanogenesis typically occurs after higher electron acceptors have been depleted, suggesting that spatial variations may have resulted in microhabitats where higher electron acceptors were absent.

*Effect of Soil Structure/Particle Size.* Both particle size and soil structure have been shown to play an important role in microbial diversity and degradative capabilities. The
degradation of 2,4-dichlorophenoxyacetic acid (2,4-D) was correlated to localized niches of 2,4-D degraders and niche abundance (Pallud et al., 2004). The study found that low abundances of 2,4-D degraders correlated to much larger distances between niches, suggesting that microbial population is not uniform. Mummey et al (2006) found that soil microbial communities in microaggregates (<250 um in size) were enriched in or contained microbial groups absent in macroaggregates (>250 um in size). Results showed that these microniches select for specific microbial lineages within the soil matrix. Mummey et al. (2006) state that “soil structure is a dominant factor controlling microbial diversity and processes”. Other studies have shown similar trends (Grundmann and Debouzie, 2000; Corgie et al., 2006). This implies that: 1) the presence of microbial niches in a more heterogeneous material such as refuse would be expected, and 2) niche formation due to particulate structure could be an important factor affecting degradative processes in solid waste.

In fact, the range in refuse particulate size is arguably much wider and variable than soil (Hull et al., 2005) which suggests that refuse “structure” after placement in a landfill may be a dominant factor affecting microbial community diversity. Many variables determine the structure of refuse in a landfill setting (e.g. waste composition, compaction, inert material content, etc.); however, no studies have been performed assessing how such variables affect microbial community structure. Research has also suggested that microniche formation is both a function of particulate structure and microbially directed processes (Tisdall and Oades, 1982; Oades, 1984; Six et al., 2000). Given this, the disruption of particulate materials in refuse via movement of refuse or leachate recycle
may serve to disrupt relatively stable niche communities which could promote (methane phase) or inhibit (acid phase) decomposition. This idea may explain why extensive lag times are sometimes observed when neutralized leachate is recirculated at a high frequency during the acid phase of solid waste.

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Chapter 2.

PROCESSING HETEROGENEOUS MATERIALS PRIOR TO DNA EXTRACTION IS ESSENTIAL FOR MINIMIZING BIAS: A COMPARISON OF METHODS USING DECOMPOSED REFUSE

ABSTRACT

Landfills represent a unique microbial ecosystem and play a significant role in global biogeochemical processes. However, sample processing and nucleic acid extraction from refuse is challenging due to material heterogeneity. Decomposed refuse was used to evaluate the effect of seven sample processing methods on Bacteria and Archaea community structure using T-RFLP. Bias was assessed using measured richness and by comparing community structure using multi-dimensional scaling (MDS). Results showed bias in microbial community structure was primarily dependent on the processing method used rather than variation induced by refuse heterogeneity. Generally, direct methods were most biased while indirect methods (i.e. blending in buffer followed by hand squeezing through mesh fabric) were least biased. An indirect method using PO$_4$ buffer gave consistently high bacterial and archaeal richness and also resulted in 28 and 34% recovery of *R. albus* and *M. formicicum* spiked into refuse, respectively. However, the highest recovery of less abundant ribotypes was achieved using multiple processing methods. Results suggest differences in measured ribotype diversity from studies of complex ecosystems could be caused by methodological variation rather than true divergence in community structure.
INTRODUCTION

The relationship between community structure and microbial function in landfill environments is important as these ecosystems significantly impact global biogeochemical processes (e.g., greenhouse gas emissions, carbon sequestration). Landfilled refuse is the second largest global source of anthropogenic methane (CH$_4$), which is a potent greenhouse gas (Denman et al., 2007). Refuse decomposition is microbiologically mediated and the diversity and metabolic function of microbial communities act as mechanistic controls on the rate of greenhouse gas emissions, extent of decomposition and carbon sequestration capacity. There is a pressing need for improved technologies to enhance solid waste decomposition. Unfortunately optimization of these technologies is limited by a poor understanding of microbial interactions during refuse degradation which are due, in part, to the heterogeneous and unstructured nature of the wastes.

Compared to culture-based techniques, the study of complex microbial ecosystems using molecular techniques can be advantageous since they allow for analysis of uncultured organisms and offer higher resolution in measuring demographic (number of species) and metabolic (functional) diversity. However, accurate ecological assessments are predicated on extracting nucleic acids that are representative of the microbial community. The importance of DNA extraction and sample processing procedures in assessing microbial diversity has been recognized and it has been suggested that using multiple extraction techniques may give rise to the least biased DNA pool in sediments (Luna et al., 2006). Previous studies on soil and activated sludge have shown that sample processing can impose a bias because microbial community structure in the extracted nucleic acids is different from that in the sample (Keith et al., 2005; Sessitsch et al., 2002).
This suggests that methods used to process samples from complex or heterogeneous materials may be equally or more important than the extraction procedure. Heterogeneous materials can be defined as having variable consistency, particle size, and composition and include organic-rich soils/sediments, humus, leaf litter, manure, rumen/cecum contents, peat, compost and refuse.

The sample mass from which nucleic acids are extracted using commercial kits and many laboratory protocols typically ranges from 0.25 to 1 g with lower and upper limits of roughly 0.1 and 10 g, respectively. This range is governed by a number of factors including available tube size, reagent volume:sample mass ratio and cell lysing efficiency. Such small masses, along with large particle sizes, often preclude direct placement of heterogeneous materials into extraction tubes. For example, particle size in municipal refuse can vary from millimeters to meters depending on the composition and extent of degradation (Hull et al., 2005). Additionally, refuse contains varying fractions of paper, plastic, metals, food scraps and other components (USEPA, 2005), making it extremely heterogeneous from a chemical and structural perspective. Thus, traditional extraction protocols used for soils, sediments, wastewater and other comparatively homogeneous matrices cannot be employed. Rather, heterogeneous materials must be processed prior to extraction to reduce particle size and ensure a reasonable degree of sample homogeneity.

The distinction between the extraction procedure and sample processing is often vague since most extraction protocols employ some form of sample processing. Most sample processing protocols utilize one or more types of physical, chemical or enzymatic methods to make the sample material more amenable for subsequent nucleic acid recovery.
addition, direct methods, where cells are lysed within the sample matrix, have been compared to indirect methods, where cells are first separated from non-cellular debris prior to lysing (6). Recently, the most widely used protocols have been direct methods. The direct method assumes the material placed into extraction tubes is representative of the sampled ecosystem; however, this assumption is tenuous for heterogeneous materials. Thus, indirect methods may prove beneficial for heterogeneous samples since separation from the material matrix may serve to concentrate cells from larger sample masses.

The objective of this study was to develop a sample processing method for municipal refuse for DNA extraction that minimizes bias while ensuring quantity and quality are sufficient for highly sensitive downstream molecular applications such as qPCR. Previous molecular studies evaluating landfill microbiology have relied on aqueous phase (leachate) samples or have extracted DNA without consideration of sample processing or extraction bias (9,10). Seven processing methods, comprising both direct and indirect protocols, were modified from previously published studies and compared using terminal restriction fragment length polymorphism (T-RFLP). Most studies evaluating sample processing and DNA/RNA extraction methods have used yield and extract purity as metrics for assessing effectiveness (Table 1). Only a few have evaluated how a particular method affects community structure using fingerprinting methods (e.g. DGGE, T-RFLP) or clone libraries (1,3,6). Although T-RFLP is not effective at estimating absolute diversity or detecting very rare species, it has been shown to be effective for comparing differences in community structure between treatments (7,8).
MATERIALS & METHODS

Refuse sample preparation and reactor monitoring. Roughly 1.4 m$^3$ of municipal refuse was obtained from a residential area collection vehicle at the Holly Springs (NC) Transfer Station. The refuse was shredded the same day in a slow speed, high torque shredder to a particle size of approximately 2 cm x 6 cm. After shredding, refuse was mixed on a clean plastic sheet using hand tools and then stored at 4°C in plastic trash bags prior to placing a portion of the refuse (~20% by volume) in a 208-L reactor for anaerobic degradation. The reactor was maintained at 37°C and operated with leachate recycle and neutralization to accelerate decomposition, as described previously (Staley et al., 2006). Gas was collected in tedlar gas bags (PMC, Inc; Oak Park, IL) and volume was measured by evacuation into a container of known volume (Sanin et al., 2000). CH$_4$, CO$_2$, O$_2$, and N$_2$ were analyzed using a GOW-MAC 580 gas chromatograph with a thermal conductivity detector (Price et al., 2003). Samples were removed from reactors during the accelerated methane and decelerated methane (also referred to as well-decomposed) phases of decomposition (Barlaz et al., 1989). For accelerated methane phase sampling, refuse was removed when the fraction of CH$_4$ in the biogas reached 50% and while the gas production rate was increasing exponentially (~31 days after reactor initiation). The CH$_4$ production rate at the time of sampling was 0.76 mL d$^{-1}$ dry g refuse$^{-1}$. For the well-decomposed phase, refuse was allowed to decompose for approximately 6 months and the CH$_4$ production rate at the time of sampling was 0.004 mL d$^{-1}$ dry g refuse$^{-1}$. For each phase of decomposition, roughly 2 kg refuse was removed from the reactor and mixed well prior to separation into 50g aliquots, which were then frozen until sample processing.
**Experimental design.** Triplicate 50 g aliquots from the same refuse sample were processed with each method and treated individually. Triplicate DNA extractions were performed for each aliquot, resulting in 9 total DNA extractions per processing method. The variability due to the heterogeneous nature of the refuse material was assessed using T-RFLP for three processing methods FT-D, DG-D and PO₄-C (processing methods are described below). The richness and Bray-Curtis similarities using multi-dimensional scaling (MDS) analysis (see below) were compared for the triplicate aliquots. The results showed that in general, the variation due to refuse heterogeneity was lower than the differences between sample processing methods. Thus, in subsequent comparisons for all methods, equal DNA masses from each of the nine extracts were combined to create a pooled DNA sample for T-RFLP analyses. This pooling served to minimize both sample-to-sample as well as extraction variability for each processing method. Parallel aliquots were used for moisture content analysis.

**Processing methods.** A number of methods identified from the literature review (Table 1) were highly cited but were not incorporated into sample processing protocols for this study. For example, bead beating was a component of the DNA extraction kit used and thus was not considered for sample processing. Differential centrifugation was not selected as an indirect protocol given its potential to select against attached microorganisms (Robe *et al.*, 2003). CTAB is a cationic detergent typically used in DNA extraction to precipitate polysaccharides and proteins and does not aid in either cell detachment or lysis (Mygind *et al.*, 2003). Similarly, PVPP aids humic acid removal but is ineffective for cell lysis (Robe *et al.*, 2003); thus, neither were used. Refuse samples were
subjected to the following direct or indirect sample processing techniques prior to DNA extraction.

**Direct methods**

*Direct phosphate buffer method (PO$_4$-D)* (Barlaz et al., 1989). Each aliquot was combined with 250 mL chilled 23.7 mM PO$_4$ buffer at pH 6.73 (31.87 g Na$_2$HPO$_4$•7H$_2$O L$^{-1}$ and 16.1 g KH$_2$PO$_4$ L$^{-1}$) and homogenized in a sterile Waring blender (Torrington, CT) for 1 min. Then 200 mL of the mixture was transferred immediately after blending to four sterile 50 mL tubes and centrifuged at 3,220 X g for 5 min. In supplemental work, the effect of two centrifuge speeds, 3,220 X g and 16,000 X g (Burgmann et al., 2001) was compared and there were no significant differences (p>0.05) in richness and total sample fluorescence using T-RFLP (data not shown). Supernatant was decanted and pellets were combined into a single tube. Residual in emptied tubes was suspended with deionized (DI) water, combined with the pellet and centrifuged for 5 min. at 3220 X g. The resulting combined pellet was well-mixed by hand using a sterile spatula.

*Freeze-Thaw method (FT-D)* (Miller et al., 1999). Aliquots were subjected to three freeze-thaw cycles, which exclude the initial freezing step noted in the Refuse sample preparation and reactor monitoring section. Each cycle included freezing at -80°C for more than 4 h followed by thawing at 60°C until the aliquot reached 25°C. Each aliquot was then combined with 250 mL chilled PO$_4$ buffer containing 27% sodium dodecyl sulfate (SDS) (20% w/v) and 5.5% lysozyme (50 mg mL$^{-1}$) by volume (Gabor et al., 2003). Aliquots were homogenized in a Waring blender for 1 min and then pelletized as described in the PO$_4$-D method.
**SDS/Lysozyme method (SDS-D)** (Gabor et al., 2003). This method is identical to method FT-D except there were no freeze/thaw cycles prior to SDS and lysozyme addition.

**Dry and grind method (DG-D).** The DG-D method was developed as part of this study. Aliquots were placed in sterile mason jars and dried at 65°C for approximately 48 h. Two layers of absorbent wipes were used to cover the opening while drying and preliminary work indicated no cross-contamination between aliquots during drying. Aliquots were then ground in a wiley mill to pass a 1 mm screen. The wiley mill was wiped with ethanol between aliquots. The dry, ground material was transferred to a 50 mL centrifuge tube and mixed using a sterile spatula.

**Indirect (Cell Separation) Methods**

**Indirect phosphate buffer method (PO₄-C)** (Barlaz et al., 1989). Aliquots were combined with 250 mL chilled PO₄ buffer and homogenized in a Waring blender for 1 min. To separate microbial cells, the blended mixture was poured into a 3.8 L 75-µm nylon paint strainer bag (Trimac Co.) and hand-squeezed. Supernatant was collected in a 1.9 L sterile plastic container, transferred to six sterile 50 mL tubes, centrifuged and combined to generate a single pellet as described in method PO₄-D.

**Indirect methylcellulose method (M-C).** This method is the same as PO₄-C except that the PO₄ buffer included 1% (w/v) methylcellulose and the mixture was allowed to stand for 10 min after blending (Kudo et al., 1987).

**Methylcellulose/Tween 80 method (MT-C).** This method is identical to the M-C method except that 0.1% Tween 80 (Sigma-Aldrich) by volume was also included in the phosphate buffer (Whitehouse et al., 1994).
**DNA quantitation and extraction.** In preliminary work, two DNA quantitation methods were compared: (a) spectrophotometry at 260 nm and (b) fluorometry using SYBR Green I as described (Zipper et al., 2003). Quantification using spectrophotometry gave higher but statistically similar (p>0.05) values compared to fluorometry using SYBR Green I (data not shown), suggesting any impurities carried over during extraction did not translate into significant quantitation error using spectrophotometry. Thus, spectrophotometry was used in the main study for DNA quantitation and to assess purity ($A_{260}/A_{280}$) (Sambrook and Russell, 2001).

The centrifuged pellet (300 mg) or dried, ground material (50 mg) generated by the processing methods were used for DNA extraction, which was performed following the protocol supplied by the extraction kit manufacturer. Initial work was performed to compare two commercial kits: MoBio PowerSoil (Carlsbad, CA) and Epicentre SoilMaster (Madison, WI) and the MoBio kit was selected for use in the main experiment (see Results and Discussion section). Comparisons between the two kits were made by processing aliquots of the well-decomposed refuse sample using the PO$_4$-D and PO$_4$-C methods. For each kit, five replicate extractions were performed on each aliquot of processed material, resulting in 10 extractions per kit.

DNA mass was normalized per dry g of material based on the centrifuged pellet or dried material remaining after processing. For both direct and indirect methods, normalizing per dry g of centrifuged pellet or dried material was computed by dividing total extracted DNA (µg) by the pellet dry mass. For direct methods, this is identical to the DNA mass per dry mass refuse. For indirect methods, DNA mass collected in the
supernatant reflects the total DNA harvested from the 50 g refuse aliquot and the DNA mass per dry g refuse was normalized by taking into account the total mass of pellet generated from hand squeezing as follows:

\[ \text{DNA Mass (\(\mu g\) DNA dry g refuse\(^{-1}\))} = \frac{\left(\text{DNA conc., } \mu g \text{ mL}^{-1}\right) \times \text{Elution vol., mL} \times \text{Total pellet mass, dry g}}{\left(\text{Pellet mass sub – sample used for extraction dry g}\right) \times \text{Refuse aliquot mass, dry g}} \]

Dry masses were calculated using the moisture content. All statistical analyses related to DNA quantitation were performed using a two-tailed student’s t-test assuming unequal variances (Microsoft Excel).

**PCR conditions.** Polymerase chain reaction (PCR) on pooled DNA (from the 9 DNA extractions noted in the Experimental Design section unless noted otherwise) was performed using 25 µL of FailSafe PCR system reaction mix F (Epicentre; Madison, WI), 0.6 µL FailSafe enzyme mix (Epicentre), 0.25 µM of each primer, ~15 ng DNA and sterile pure water added to a total volume of 50 µl. The template mass used was based on preliminary PCR optimizations to achieve approximately equal final masses of amplified product (Blackwood et al., 2003). The primers used were specific for conserved 16S RNA gene sequences targeting the bacterial and archaeal domains, respectively. Bacterial primers used were 8f (5’-AGAGTTTGATCTGCTGCTG) and 1492r (5’-GGTTACCTTGTTACGACTT) (Klappenbach et al., 2000). Archaeal primers used were ARC-8f (5’-TCCGTTGATCCTGCC) and ARC-1492r (5’-GGCTACCTTGTTACGACTT) (Banning et al., 2005). Forward primers for both primer sets were labeled at the 5’ end with 6-FAM for T-RFLP analysis. PCR product
concentrations were computed by loading equal product volumes on a 1% agarose gel and estimating mass using Gel-Pro analyzer software (Media Cybernetics; Silver Spring, MD).

PCR was performed in an Eppendorf thermocycler programmed as follows: initial denaturing step at 94°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1.5 min, and final extension at 72°C for 45 min. Duplicate PCR reactions were performed and subsequently combined prior to quantification.

**T-RFLP analysis.** Amplicons (100 µl) were purified using QIAquick PCR purification (Qiagen, Valencia, CA) per the manufacturer’s protocol. Purified PCR products were quantified by spectrophotometry and approximately 300 ng were digested separately with restriction endonucleases HhaI,MspI and Rsal (New England Biolabs; Ipswich, MA) for Bacteria and HhaI, HaeIII and TaqI (New England Biolabs; Ipswich, MA) for Archaea. The restriction enzymes for Archaea were selected based on previous studies (Banning *et al.*, 2005; Chan *et al.*, 2005). Restriction digestion was performed in 20 µL reactions containing: 1.5 µL restriction enzyme, 2 µL 10X buffer, a volume equivalent to 300 ng PCR product, and DI water. The same enzyme volume was used for all reactions resulting in activities ranging from 15-30 U per reaction depending on the enzyme used. Terminal restriction fragments were analyzed by capillary electrophoresis at the Michigan State University Research Technology Support Facility using a 3130 Genetic Analyzer (Applied Biosystems; Foster City, CA). T-RFLP profiles were analyzed using Genescan™ (Applied Biosystems) and GenescanView software (CRIBI, Italy).
Analysis of T-RFLP profiles. Electropherograms were visually inspected and numerical output from each fragment pattern was exported to Microsoft Excel. To minimize analytical error, comparable loads of PCR product for T-RFLP analysis were verified using the total fluorescence. Fluorescence was verified as being within a factor of 2 compared to that of other samples or was re-analyzed. Terminal restriction fragment (T-RF) areas were standardized to eliminate noise and identify true peaks via an automated algorithm using a standard deviation from the baseline of 3 and a bin size of ±0.5 bp (Abdo et al., 2006). To compare community structure between processing replicates and methods, the number of ribotypes, also referred to as richness (S), and the Bray-Curtis similarity index were computed using Community Analysis Package software 4.0 (Pisces Conservation Ltd).

Enzymes yielding higher numbers of T-RFs offer greater resolution between OTUs. Of the three enzymes used, MspI and HhaI gave the highest resolution for Bacteria and Archaea, respectively, and thus were used to compare treatments (data not shown).

Comparison of methods using MDS analysis of Bray-Curtis Similarities. T-RFLP results from each method were compared using non-metric multi-dimensional scaling using Bray-Curtis similarity. Bray-Curtis similarity was used as it has been shown as an effective metric in comparing microbial communities using T-RFLP (Rees et al., 2004). A datum to compare the performance of individual methods was created by pooling T-RF profiles from all methods, resulting in a compiled set of accumulated T-RFs recovered from all methods. Establishing a datum in this manner is analogous to that used in ecology studies, in which aggregated diversity from multiple capture methods is used to compare performance of a particular individual method (Magurran, 2004). Non-metric
multidimensional scaling was computed from T-RF profiles using Community Analysis Package (Seaby and Henderson, 2007).

**Pure culture spiking and quantitative PCR.** To determine the recovery of processing methods, pure cultures of a cellulolytic bacterium (*Ruminococcus albus* Hungate, ATCC 27210) and methanogenic archaeon (*Methanobacterium formicicum* MF, ATCC 33274) were grown in liquid media as recommended by the supplier (ATCC; Manassas, VA) then spiked into refuse 10 min prior to processing. Spiked and unspiked aliquots were processed using PO$_4$-C, MT-C and DG-D methods and frozen until DNA extraction could be performed. Triplicate aliquots were used per method and triplicate DNA extractions were performed from each aliquot. To determine the volume of live cells to add based on DNA quantity, pure culture DNA was extracted from triplicate 1.8 mL (O.D.$_{600} = 0.87$) culture samples and pooled to establish a ratio between extracted pure culture DNA and inoculum volume (ng DNA/mL inoculum). Then live whole cells were added based on the DNA mass per mL of pure culture inoculum at a pure culture DNA:total DNA ratio of 2.5% ± 0.8%. Spiking ratios of 0.4% to 46% were tested in preliminary work using the same spiking protocol noted above and recovery, measured using qPCR, showed a spiking ratio of 2.5% gave the highest recovery. Pure culture DNA recovery was computed by subtracting the pure culture:total DNA ratio measured in unspiked aliquots from the same ratio measured in spiked aliquots to normalize for DNA native to the refuse that might be targeted with the PCR primers. The result was then divided by the pure culture:total DNA ratio initially spiked into the refuse.
Quantitative PCR was performed using a SYBR Green I assay with the Bio-RAD iQ5 thermocycler (Hercules, CA). Reactions were prepared in 25 µL volumes using 12.5 µL SYBR Green Supermix, 0.25 µM of each primer, 1 µL DNA extract and 10.5 µL sterile pure water. A serial dilution was performed on extracted pure culture DNA and triplicates from each dilution were subjected to qPCR to generate a 7 point standard curve. 16S RNA gene PCR primers were designed using Primer 3 software (Rozen and Skaletsky, 1998) for R. albus (15f: 5’- CACATGCAAGTCGAACGAGCGAAA, 101r: 5’- CGAAAGGCAGATTGCTCACGTGTT) and M. formicicum (246f: 5’- TGCCCAACCAAGCCAGTAATCTGTA, 366r: 5’- ACTTTCGTGCATTGCGAGGTTC). Primer specificity was verified in silico via a BLAST search (NCBI) and in the laboratory via PCR on DNA from R. albus, M. formicicum, and mixed refuse. Primers were optimized for annealing temperature at the concentrations stated above and PCR was performed as follows: initial denaturing step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 45 sec, extension at 72°C for 1 min. qPCR results were analyzed using iQ5 Optical System software (Bio-Rad).

RESULTS AND DISCUSSION

DNA Extraction and Yield. Initial work was performed to ensure the commercial kit used for the main study had the ability to recover measurable DNA yields from decomposed refuse. Two commercial DNA extraction kits were evaluated, MoBio PowerSoil and Epicentre SoilMaster, because they used different approaches to isolate
DNA from environmental samples. DNA yields from the kit containing a bead beating step (MoBio) were significantly greater (p<0.05) and averaged 3.5 times higher than the kit without a bead beating step (Epicentre). DNA yields were also less variable with the MoBio kit (Coefficient of Variation [CV]=13%) compared to the Epicentre kit (CV=91%). This is most likely because a DNA binding column is used with the MoBio kit, whereas the Epicentre kit uses ethanol precipitation to isolate the DNA pellet. It is suspected that ethanol precipitation and the subsequent decanting step introduces more variability. Based on these results, the MoBio PowerSoil kit was used for all DNA extractions in the main study.

DNA yields from all sample processing methods are shown in Figure 1. The M-C and DG-D methods resulted in the highest yields for well-decomposed refuse and were statistically similar (p>0.05). The PO₄-C method had the highest yield for accelerated methane phase refuse and was statistically similar to the DG-D method (p>0.05). DNA yield from well-decomposed refuse had CVs ranging from 11% (SDS-D) to 50% (M-C) with the majority being less than 20%. Accelerated methane phase DNA yields were variable and CVs ranged from 12% (FT-D) to 64% (DG-D). Roughly one-third of the methods had CVs less than 20% for this substrate. While purity ratios (A₂₆₀/A₂₈₀) exhibited some variability between methods (data not shown), DNA from all treatments was successfully PCR amplified.

The DNA yields obtained from indirect methods (PO₄-C, M-C) were comparable to direct methods, which contrasts to previous work (Jacobsen and Rasmussen, 1992; Robe et al., 2003; Tien et al., 1999). It is hypothesized that cell separation via hand-squeezing
serves to consolidate a larger mass of material and does not remove particulates to the same extent as the more common differential centrifugation technique (Faegri et al., 1977; Robe et al., 2003; Steffan et al., 1988; van Elsas et al., 1998). For cell separation methods, roughly 30% (well-decomposed phase) and 8.3% (accelerated CH₄ phase) of the aliquot dry mass was recovered from the hand-squeezed supernatant. This inefficiency in particulate removal appears advantageous and is consistent with previous work which suggests a significant fraction of the cells are likely attached to the particulates (Corinaldesi et al., 2005).

Although different methods provided varying yields, there was no correlation between yield and ribotype richness (R²=0.26), which is similar to other studies (Frostegard et al., 1999; Luna et al., 2006). Despite the inability of T-RFLP to detect less abundant ribotypes (Luna et al., 2006), methods resulting in significantly larger DNA yields should have increased detection of ribotypes at abundances near T-RFLP detection limits if a correlation between yield and richness existed. Rank abundance curves exhibited a ‘long tail’ of less abundant ribotypes (data not shown), which is typical of organism distributions in most ecosystems (Magurran, 2004). This suggests no anomalous distributions of taxa were inherent in the refuse ecosystem that would have skewed a yield-richness correlation. Thus, taxa capture efficiency by the processing method appears to play a larger role than extracted DNA yield in detecting dominant ribotypes.

**Microbial community structure bias.** Most studies evaluating sample processing and DNA/RNA extraction methods have used yield and extract purity as metrics for assessing effectiveness (Forney et al., 2004). Only a few have evaluated how a particular method
affects community structure using molecular fingerprinting methods or clone libraries (Gabor et al., 2003; LaMontagne et al., 2002; Luna et al., 2006; Martin-Laurent et al., 2001; Sessitsch et al., 2002). Differences in measured community structure can be especially important if a particular method is biased against ubiquitous or functionally important microbial taxa. Bias, as defined in this study, refers only to measured ribotype richness (rather than absolute species richness), and Bray-Curtis similarities based on T-RFLP analysis. It is well known that T-RFLP is not effective at estimating absolute diversity or detecting rare species (Hartmann and Widmer, 2008). However, previous work has shown T-RFLP to be very effective in comparing community structure differences between treatments, making its use in this study particularly applicable (Dunbar et al., 2001; Hartmann and Widmer, 2008; Hartmann and Widmer, 2006). Additionally, taxonomic assessment of rare species usually requires libraries consisting of thousands of clones which can be cost prohibitive (Dunbar et al., 2002).

Variation induced by refuse heterogeneity. Variability between triplicate aliquots of well-decomposed refuse processed by the same method was generally lower than the observed differences between sample processing methods FT-D, DG-D and PO₄-C. Two-tailed t-tests (assuming unequal variance) performed using T-RF richness from triplicate aliquots showed differences between processing methods for Bacteria at the 92% confidence level (p<0.08). T-tests between methods for Archaea were different between the PO₄-C/DG-D and PO₄-C/FT-D methods (p<0.13). There was no difference between the DG-D/FT-D methods (p=0.56) but this was because aliquot T-RF richness was similar between methods (data not shown). MDS analysis showed the community structure
between replicates processed by the same method clustered together while the community structure recovered by separate processing methods clustered separately (Fig. 2). For *Archaea*, differences between DG-D replicates were substantially larger than that observed from the PO$_4$-C and FT-D methods (Fig. 2b). However, the PO$_4$-C and FT-D methods clustered together for *Archaea* (Fig. 2b) and the DG-D replicates for *Bacteria* clustered together (Fig. 2a). Additionally, the T-RFLP and qPCR results comparing all methods indicated the DG-D method did not result in similar archaeal community structure relative to other methods (see subsequent sections). Taken together, the results from this experiment highly suggest that differences between replicates were not induced by refuse heterogeneity but, rather, by the DG-D method itself. Collectively, these results show differences between processing methods were not attributed to variation induced by refuse heterogeneity. As a result, equal DNA masses from each of the nine extracts were combined to create a pooled DNA sample for T-RFLP analyses in subsequent comparisons of all 7 sample processing methods. This pooling served to minimize both aliquot to aliquot and extraction variability for each processing method.

*Comparison of sample processing methods.* Generally, T-RFs with the highest capture frequency were most abundant. Capture frequency, as used in this study, refers to the number of processing methods that recovered a particular T-RF, which is analogous to the way in which trapping methods are compared in ecological diversity studies (Magurran, 2004). Roughly 96±5.0% and 91±7.2% of total relative abundance was from T-RFs occurring in 3 or more methods for *Archaea* and *Bacteria*, respectively. For this reason, richness at a capture efficiency ≥ 3 was compared in addition to all T-RFs
recovered by a particular method (Table 2). However, the relative abundance for individual ribotypes occurring in 3 or more methods ranged from ~0.3 to 65%, indicating T-RFs with a high capture frequency were not limited to those with high relative abundances. It should be noted that T-RF abundances were used in this study comparatively and do not necessarily reflect abundances from the native ecosystem, primarily due to limitations in T-RF resolution, extraction efficiency and PCR amplification bias (Dunbar et al., 2001; Hartmann and Widmer, 2008).

Richness from individual processing methods was generally comparable, with the difference averaging ~5 T-RFs between methods at a capture frequency ≥3 (Table 2). Exceptions to this were the DG-D method for *Bacteria* and the MT-C method for *Archaea*, both of which had a richness roughly 10 ribotypes lower than other methods. Trends were similar when all T-RFs were included (capture frequency ≥ 1) but richness was expectedly higher since this reflects capture of less ubiquitous T-RFs (Table 2).

MDS plots show community structure varied between methods (Fig. 3). For *Bacteria*, methods DG-D, SDS-D and FT-D were consistently biased and were not clustered with other sample processing methods in both types of refuse (Fig. 3 a,b). In the well-decomposed refuse, methods PO$_4$-D and MT-C also exhibited bias relative to the pooled T-RF profile for all methods (Fig. 3a). Collectively, this indicates that the least biased methods for *Bacteria* are the PO$_4$-C and M-C methods. MDS plots for *Archaea* show community structure recovered by the DG-D method was substantially different than that of the other methods (Fig. 3 c,d). This resulted in a pooled T-RF profile from all methods that was obviously skewed relative to other methods. Excluding the DG-D, one
can see that the PO₄-C method clustered centrally within the other sample processing methods for the well-decomposed refuse treatment. For accelerated CH₄ refuse, methods clustered with one another with the FT-D method exhibiting a slight bias (Fig. 3b). These results show that relatively unbiased recovery of *Archaea* was achieved with multiple methods (excluding the DG-D and FT-D methods); however, the PO₄-C method appeared to recover an archaeal community structure comparable to or more diverse than other processing methods (Fig. 3 c,d).

**Pure culture spiking and recovery using quantitative PCR.** Recovery of spiked pure cultures was quantified using qPCR since the technique has a detection limit several orders of magnitude lower than T-RFLP based on published T-RFLP detection limits (Luna *et al.*, 2006) and qPCR instrumentation specifications (Bio-Rad, 2003). Variation in measured pure culture abundance was eliminated since cultures were spiked in known quantities and unspiked aliquots were used as controls. Methods PO₄-C, MT-C and DG-D were evaluated because they ranged from most effective to least effective, respectively, based on the T-RFLP comparisons of richness and MDS of Bray-Curtis similarities (Table 2, Fig. 3).

*R. albus* recoveries were higher using PO₄-C compared to MT-C and all methods were statistically different from one another at the 95% confidence level (p<0.05) (Table 3). The DG-D method gave the highest average recoveries of *R. albus*; however, results were highly variable (CV=57%) compared to the PO₄-C (CV=13%) and MT-C (CV=39%) methods. The PO₄-C and DG-D methods gave the highest *M. formicicum* recovery and were statistically similar (p>0.05). However, recoveries using the DG-D method were
again more variable (CV=46%) compared to the PO$_4$-C method (CV=13%). The MT-C method gave significantly lower *M. formicicium* recoveries compared to the PO$_4$-C method.

The PO$_4$-C method, which consistently exhibited low bias relative to other methods (Fig. 3), gave consistent pure culture recoveries and was less variable (Table 3). In contrast, the DG-D method performed reasonably well for *M. formicium* (*Archaea*) but exhibited large variation in recovering *R. albus* (*Bacteria*). This is consistent with the measured richness and MDS plots (Table 2, Fig. 3), which showed the DG-D was consistently biased compared to other methods.

**Recommendations.** This study confirms that observed bias is most likely caused by the sample processing method rather than an analytical artifact, sample variation or other confounding variable. It has been previously reported that recovery of rare taxa are difficult unless countered by a massive sampling effort and that higher richness can be obtained using multiple methods since unique ribotypes are captured by different methods (Luna *et al.*, 2006; Magurran, 2004). This was confirmed here since the richness from all 7 methods combined was ~2 times higher on average ($S = 63\pm3$) than richness from a single method alone ($S = 29\pm7$). Thus, if the goal is to capture less abundant taxa, then multiple processing methods and replicates must be used. Despite the recommendation to employ multiple methods, use of a single protocol still prevails in many microbial ecology studies for reasons of convenience, increased sample throughput, reduced labor and lower cost. Thus, if it is necessary to use a single method, the PO$_4$-C method offers the least bias against more ubiquitous taxa and may provide an acceptable number of less abundant taxa depending on the experimental goal(s). From a practical standpoint, the end material
generated by cell separation (hand squeezing) is less fibrous and results in lower pellet volumes after centrifugation, making the material less difficult to work with compared to direct methods.

These results show the sample processing method used can have a substantial effect on the number of ribotypes captured and the microbial community structure observed. This implies that differences between microbial ecology studies could be attributed, in whole or in part, to methodological differences rather than environmental selection mechanisms and suggests caution must be used when interpreting microbial ecology data sets where different methods were used to process samples.

ACKNOWLEDGEMENTS

This work was funded by Waste Management, Inc. We would like to thank Dr. Jose Barcena and the N.C. State University Biotechnology Training and Education Center for use of the real-time PCR machine, Dr. Stefan Franzen (N.C. State University Chemistry Department) for use of the fluorometer and Dr. Kenneth Pollock (N.C. State Zoology Department) for his kind review of the approach used to measure bias. Bryan Staley’s work was partially funded through a scholarship from the Environmental Research & Education Foundation and a fellowship from N.C. Beautiful.

REFERENCES


Figure 1. Effect of sample processing methods on DNA yield. Abbreviations: PO$_4$-D, direct phosphate buffer method; FT-D, direct freeze-thaw method; SDS-D, direct sodium dodecyl sulfate method; DG-D, direct dry and grind method; PO$_4$-C, cell separation phosphate buffer method; M-C, cell separation methylcellulose method; MT-C, cell separation methylcellulose/Tween 80 method.
Figure 2. MDS using Bray-Curtis similarity for triplicate aliquots of sample processing methods FT-D, DG-D and PO₄-C for (a) *Bacteria* and (b) *Archaea* using well-decomposed refuse. Inset for *Archaea* shows differences between methods PO₄-C and FT-D not evident at original scale. Analysis indicates relative impact of refuse heterogeneity on differences between sample processing methods.
Figure 3. MDS using Bray-Curtis similarity for sample processing methods for Bacteria in (a) well-decomposed and (b) accelerated CH$_4$ phase refuse and for Archaea in (c) well-decomposed and (d) accelerated CH$_4$ phase refuse. The ‘All Methods’ treatment refers to the aggregate T-RF profile compiled from all sample processing methods. Abbreviations shown are given the Methods section.
MDS - Axes 1 vs Axes 2 - 2D Model - ARDIApr09_forCCA
Rotated, Bray-Curtis

Axis 1
Axis 2

All Methods

PO4=D
FT=D
SOS=D

DG=D
PO4=C
MC=C
MT=C

2D Stress = 0.172498

MDS - Axes 1 vs Axes 2 - 2D Model - ARDIApr09_forCCA
Rotated, Bray-Curtis

Axis 1
Axis 2

All Methods

PO4=D
FT=D
SOS=D

DG=D
PO4=C
MC=C
MT=C

2D Stress = 0.0096615
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ENZYMATIC METHODS

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² Number of times the method was used in 51 studies. In cases where the method was used multiple times in a single study, each method was counted once per study to avoid artificially inflating the method’s frequency of use.

²² Performed by either hand squeezing or sieving.

²³ Centrifugation procedure denotes differential centrifugation was performed for cell separation.

²⁴ For clarity, numbers in this table are designated for cited references as follows: 1 (Cullen and Hirsch, 1998), 2 (Duarte et al., 1998), 3 (Kuske et al., 1998), 4 (Reilly and Attwood, 1998), 5 (Burgmann et al., 2001), 6 (Martin-Laurent et al., 2001), 7 (Niemi et al., 2001), 8 (Stach et al., 2001), 9 (LaMontagne et al., 2002), 10 (Lehman and O’Connell, 2002), 11 (Sessitsch et al., 2002), 12 (Anderson and Lebepe-Mazur, 2003), 13 (Chen et al., 2003), 14 (Chen et al., 2003), 15 (Costa and de Oliveira, 2003), 16 (Fus et al., 2003), 17 (Gabor et al., 2003), 18 (Uz et al., 2003), 19 (Webster et al., 2003), 20 (Grubb and Dehority, 1976), 21 (Miller et al., 1986), 22 (Craig et al., 1987), 23 (Leadle et al., 1987), 24 (Leadle and Butine, 1987), 25 (Olubobokun et al., 1987), 26 (Barsuhn et al., 1988), 27 (Barlaz et al., 1989), 28 (Whitehouse et al., 1994), 29 (Martin-Orue et al., 1998), 30 (Yanagita et al., 2000), 31 (Courtois et al., 2001), 32 (Bockelmann et al., 2003), 33 (Ranilla and Carro, 2003), 34 (Frostegard et al., 1999), 35 (Tien et al., 1999), 36 (Luna et al., 2006), 37 (Wikstrom et al., 1996), 38 (Zhou et al., 1996), 39 (Miller et al., 1999), 40 (Volossiouk et al., 1995), 41 (Krause et al., 2001), 42 (Ranjard et al., 1998), 43 (Sharma et al., 2003), 44 (Cheng et al., 1991), 45 (Huang et al., 2003), 46 (Huang et al., 2004), 47 (Fortin et al., 2004), 48 (Minato and Suto, 1978), 49 (Kudo et al., 1987), 50 (Rasmussen et al., 1989), 51 (Minato and Suto, 1981).

Abbreviations: SDS, sodium dodecyl sulfate; CTAB, cetyltrimethylammonium bromide; PVPP, polyvinyl polypyrrolidone.
Table 2. Effect of sample processing method on bacterial and archaeal richness at capture frequencies (F) of \( \geq 1 \) and \( \geq 3 \)^a.

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<td>F ( \geq 3 )</td>
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<td>All Methods</td>
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^a Bacteria and Archaea data shown for MspI and HhaI restriction enzymes, respectively. Abbreviations are defined in the Methods section. Capture frequency refers to the total number of methods that recovered a particular T-RF.
Table 3. Recovery of pure cultures spiked into refuse and processed with selected methods measured using qPCR\textsuperscript{a}.

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<td>Initial Spike (%)</td>
<td>Recovered\textsuperscript{c} (%)</td>
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<td>\textit{Ruminococcus albus}</td>
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<td>PO\textsubscript{4}-C</td>
<td>3.7 (0.2)</td>
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<td>DG-D</td>
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<td>\textit{Methanobacterium formicicum}</td>
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<td>PO\textsubscript{4}-C</td>
<td>1.9 (0.1)</td>
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<tr>
<td>DG-D</td>
<td>2.2 (0.4)</td>
<td>1.0 (0.6)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Data presented are averages and standard deviation is given in parentheses. Whole cells were spiked into refuse at a level corresponding to a known DNA mass per volume of pure culture. Abbreviations shown are defined in Figure 1 and the Methods section.

\textsuperscript{b}Total DNA is inclusive of the pure culture DNA added to the sample.

\textsuperscript{c}Recovered values were corrected for \textit{R. albus} or \textit{M. formicicum} DNA existing in the sample prior to spiking. Different superscripts indicate that, for a particular pure culture, methods were statistically different (p<0.05).
Chapter 3.

COMPARISON OF BACTERIA AND ARCHAEA COMMUNITIES IN MIXED REFUSE, INDIVIDUAL REFUSE COMPONENTS AND LEACHATE

(Formatted for Submission to Microbial Ecology)

Abstract

Refuse decomposition in landfills is a microbially mediated process that occurs primarily under anaerobic conditions. Due to limited moisture conditions, hydraulic transport as a means of cellular translocation within the landfill appears limited, especially during the initial stages of decomposition. Thus, microbial communities within the incoming refuse serve as a primary source of facultative and obligate anaerobic microbes that initiate refuse decomposition. Fresh residential refuse was collected at five times throughout the year and microbial communities in these samples were compared to those in individual refuse components and decomposed refuse. In addition, Bacteria and Archaea in decomposed refuse solids were compared to the liquid fraction (leachate). Results showed that microbial DNA was 2 to 20 times lower in fresh refuse compared to decomposed refuse. Also in fresh refuse, archaeal DNA, which includes methanogens, was <0.5% of qPCR quantified prokaryotic DNA, suggesting that fresh refuse is not a substantial source of methanogens in newly buried refuse. Roughly 33% of bacterial clone sequences in fresh refuse were represented by facultative anaerobes and were comprised of the Carnobacterium, Proteus, Enterobacter and Serratia genera. Bacterial community
structure in fresh refuse using TRFLP was highly correlated ($r^2=0.91$) to seasonal
differences in ambient air temperature. Compared to other individual waste components,
food waste shared the highest number of operational taxonomic units with residential
refuse and likely plays a substantial role in shaping microbial demographics in solid waste.
Microbial communities in the solid and leachate phases were different, indicating that both
matrices must be considered when characterizing microbial diversity within the landfill.

Introduction

Landfills are a primary means of solid waste disposal globally and, in the U.S., roughly
55% of solid waste generated is disposed of in this manner (USEPA, 2007). In addition,
landfills are a significant sink for sequestered carbon (Barlaz, 1998) and the second largest
source of anthropogenic methane globally (Denman et al., 2007). Despite their importance
as an anaerobic ecosystem, little is known about the sources of microbial inputs to landfills
and how microbial demographics from separate waste streams may impact community
structure and the extent of refuse degradation.

Microbial community compositions in the incoming waste streams are important since
microbial transport within the landfill appears limited, particularly soon after burial. Most
conventional landfills in developed countries are designed to minimize moisture
infiltration (e.g. rainfall, runoff, etc.) and have an initial moisture content of 15 to 20%.
Refuse is typically compacted to a density of roughly 800 kg m$^{-3}$ during initial placement
in the landfill and recently placed, well-compacted waste can have a hydraulic conductivity
ranging from $5.4 \times 10^{-6}$ to $6.1 \times 10^{-5}$ cm s$^{-1}$ which restricts even distribution of infiltration
(Jain et al., 2006). In addition, preferential flow paths can be present that divert moisture away from large volumes of waste. It is widely accepted that non-airborne transport of microbes is largely facilitated by macroscopic and microscopic liquid and/or particulate movement (Gammack et al., 1992; McLoed et al., 2008). However, the non-uniform, limited moisture condition and restrictive hydraulic conductivity in a landfill likely impede microbial translocation within the compacted refuse. Thus, bacterial and archaeal populations in ‘fresh’ refuse discarded to a conventional landfill may be largely responsible for degradative activity within a spatially localized area.

Conditions during refuse collection are aerobic, whereas conditions within the landfill are largely anaerobic, suggesting that microbes contributing to decomposition must either be facultative or be able to survive aerobic conditions during collection and transport to the landfill. This is important since methanogenic *Archaea* are critical to the anaerobic food web and are obligate anaerobes. Putative sources of methanogens in disposed waste include pet/human feces, wastewater treatment plant sludge and soil. It is surmised that similarities between fresh refuse and anaerobically decomposed refuse would include the microbial populations retained after oxic conditions dissipate, and exclude obligate aerobes or facultative microbes unable to compete in a landfill setting.

The first objective of this study was to compare bacterial and archaeal communities in fresh residential refuse to the dominant biodegradable waste components (i.e. food waste, grass, branches, leaves, paper) and to degraded refuse. The second objective was to compare microbial compositions in refuse collected at different times. Since hydraulic transport of microbes within a conventional landfill may be limited and/or non-uniform, it
is useful to assess if microbial populations transported by the liquid fraction (leachate) are similar to those in the solids. Thus, a third objective was to compare the microbial community in decomposed solids to the leachate fraction of solid waste. Results from this study will help determine if specific waste components carry microbes suited to anaerobic conditions and provide information to better understand the initiation of anaerobic refuse decomposition.

Materials and Methods

Sample Collection

Refuse samples were taken five times over a 26-month period to evaluate seasonal differences in microbial community composition. Each refuse sample was obtained from a residential area waste collection vehicle at the Holly Springs (NC) Transfer Station. The refuse was shredded the same day in a slow speed, high torque shredder to a particle size of approximately 2 cm x 6 cm. After shredding, refuse was mixed on a clean plastic sheet using hand tools and ~6000 cm$^3$ sub-samples were obtained for analysis.

Food waste, branches, grass, leaves, newspaper and office paper were sampled individually. Food waste was collected from a residential kitchen over several weeks. Grass was collected immediately after cutting from a residential lawn. Dead branches and fallen leaves were collected from a landscaped residential/commercial area. Newspaper and office paper were collected from recycling bins on the NC State University campus. Branches were cut into 6 cm long fragments. Newspaper and office paper were cut 2 cm x
6 cm strips. All refuse and individual component samples were frozen at -20°C until samples could be processed.

To generate decomposed refuse, waste collected on 1/5/2006 was placed in a 208-L reactor for anaerobic degradation. The reactor was maintained at 37°C and operated with leachate recycle and neutralization to accelerate decomposition, as described previously (Staley et al., 2006). Gas was collected in tedlar gas bags (PMC, Inc; Oak Park, IL) and volume was measured by evacuation into a container of known volume (Sanin et al., 2000). CH$_4$, CO$_2$, O$_2$, and N$_2$ were analyzed using a GOW-MAC 580 gas chromatograph with a thermal conductivity detector (Price et al., 2003). Samples were removed from reactors during the accelerated methane and decelerated methane (also referred to as well-decomposed) phases of decomposition (Barlaz et al., 1989). For accelerated methane phase sampling, refuse was removed when CH$_4$ in the biogas reached 50% and while the gas production rate was increasing exponentially (~31 days after reactor initiation). The CH$_4$ production rate at the time of sampling was 0.76 mL d$^{-1}$ dry g refuse$^{-1}$. For the well-decomposed phase, refuse was degraded for 6 months and the CH$_4$ production rate at the time of sampling was 0.004 mL d$^{-1}$ dry g refuse$^{-1}$.

To compare microbial communities in the solid fraction of refuse with leachate, fresh refuse collected on 11/18/2006 was anaerobically decomposed as described above except that a 10-L reactor was used and 2L of methanogenic leachate from decomposing refuse was added. Refuse in the reactor was mixed by shaking and inverting every 2-3 days during the course of decomposition. Solid and leachate samples were removed simultaneously at separate times representing four distinct phases decomposition: acid,
accelerating CH₄, decelerating CH₄, stabilized (Barlaz et al, 1989). The term ‘stabilized’ as used here refers to material sampled at the end of reactor operation when no gas production had occurred for 20 days but reactor headspace conditions were still anaerobic. Leachate samples were collected from a sampling port near the reactor bottom as described previously (Price et al., 2003). For each sample, roughly 50 g decomposed refuse and 30 mL leachate was removed from the reactor and mixed prior to freezing.

Sample Processing and DNA Extraction
Waste samples were thawed to ~4°C and then combined with 250 mL chilled 23.7 mM PO₄ buffer at pH 6.73 (31.87 g Na₂HPO₄•7H₂O L⁻¹ and 16.1 g KH₂PO₄ L⁻¹) and homogenized in a sterile Waring blender (Torrington, CT) for 1 min. The blended mixture was poured into a 3.8 L 75-µm nylon paint strainer bag (Trima Co.) and hand-squeezed. Supernatant was collected in a 1.9 L sterile plastic container, transferred to sterile 50 mL tubes and centrifuged at 3,220 X g for 5 min. Supernatant was decanted and pellets were combined into a single tube. Residual in emptied tubes was suspended with sterile deionized (DI) water, combined with the pellet and centrifuged for 5 min. at 3220 X g. The resulting combined pellet was well-mixed by hand using a sterile spatula and used for DNA extraction. DNA was extracted using the MoBio PowerSoil kit (Carlsbad, CA) following the manufacturer’s protocol. Extracted DNA was quantified via spectrophotometry using a NanoDrop 1000 (Thermo Scientific; Wilmington, DE) with a lower detection limit of 2 ng DNA µL⁻¹.
PCR Conditions

Polymerase chain reaction (PCR) was performed using 25 µL of FailSafe PCR system reaction mix F (Epicentre; Madison, WI), 0.6 µL FailSafe enzyme mix (Epicentre), 0.25 µM of each primer, ~5 ng DNA and sterile pure water added to a total volume of 50 µl. The primers used for T-RFLP and cloning were 8f/1492r (Klappenbach et al., 2000) and 109f/915r (Miller and Wolin, 1986) for Bacteria and Archaea, respectively, and targeted the 16S rRNA gene. Forward primers used for T-RFLP were labeled at the 5’ end with 6-carboxy-fluorescein (6-FAM) for T-RFLP analysis. PCR products were run on a 1% agarose gel to verify amplification.

PCR was performed in an Eppendorf thermocycler programmed as follows: initial denaturing step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1.5 min, and final extension at 72°C for 10 min. Duplicate PCR reactions were pooled and DNA was purified using a Wizard SV Gel and PCR Clean-Up kit (Promega, Madison, WI).

Clone Library of 16S rRNA Gene

Bacterial and archaeal clone libraries were constructed using DNA extracted from the 11/18/2006 undecomposed mixed refuse sample. The clone library was generated by inserting PCR products into the pCR 4-TOPO vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA), in accordance with the manufacturer’s protocol.

Colony PCR was performed by picking transformants using a sterile plastic pipette tip and placing them into a 96-well plate containing a PCR cocktail of 25 µL of FailSafe PCR
system reaction mix F (Epicentre; Madison, WI), 0.6 µL FailSafe enzyme mix (Epicentre), 0.25 µM of each primer and sterile pure water added to a total volume of 50 µl. The forward primer used for colony PCR was the T3 primer (5’-ATTAACCCTCACTAAAGGGA) and was specific to the vector. The reverse primer used was specific to the insert (1492r for Bacteria, 915r for Archaea). PCR conditions for colony PCR were: a lysis step at 95°C for 10 min, an initial denaturing step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1.5 min, and final extension at 72°C for 10 min. Plates containing amplified inserts were submitted to the NC State University Genome Sciences Laboratory (Raleigh, NC) for purification and sequencing.

T-RFLP analysis
Purified PCR products were quantified by spectrophotometry and approximately 360 ng were digested with the restriction endonuclease AluI (New England Biolabs; Ipswich, MA). Restriction digestion was performed for 4 hours using 20 µL reactions containing: 10 U restriction enzyme, 2 µL 10X buffer, 360 ng PCR product, and DI water. Terminal restriction fragments were analyzed by capillary electrophoresis either at the Michigan State University Research Technology Support Facility (Bacteria) or at the NC State University Genome Sciences Laboratory (Archaea). T-RFLP profiles were analyzed using PeakScanner software (Applied Biosystems) and numerical output from each fragment pattern was exported to Microsoft Excel. Terminal restriction fragment (T-RF) areas were standardized using an automated algorithm to eliminate noise and identify true peaks.
(Abdo et al., 2006). The following parameters were used as recommended by the algorithm developers: a minimum peak height of 15 RFU, standard deviation from the baseline of 3 and a bin size of ±1 bp. Phylogeny was inferred by comparing measured T-RF lengths to those from in silico digests of clone sequences from the same decomposed MSW used to compare solids and leachate.

Statistical and Phylogenetic Analyses
Principal component analysis of microbial community structure was performed using Community Analysis Package version 4.0 (Pisces Conservation Ltd, Lymington UK). The Jaccard index and T-RF richness were computed using Species Diversity and Richness version 4.0 (Pisces Conservation Ltd, Lymington UK). Phylogenetic and molecular evolutionary analyses of 16S rRNA gene sequences were conducted using MEGA version 4 (Tamura et al., 2007). Sequences were aligned using the ClustalW algorithm and phylogenetic trees were constructed using the neighbor-joining method. Bootstrapping was performed (500 replicates) to evaluate tree reliability. Sequences from related species and similar environmental clones were obtained from GenBank (www.ncbi.nlm.nih.gov) and included in alignments.

Quantitative PCR
Standards for quantitative PCR (qPCR) were generated by growing pure cultures of a bacterium (Ruminococcus albus Hungate, ATCC 27210) and a methanogenic archaeon (Methanobacterium formicicum MF, ATCC 33274) in liquid media as recommended by
the supplier (ATCC; Manassas, VA). DNA was extracted from each culture using a MoBio PowerSoil kit following the manufacturer’s protocol and quantified by spectrophotometry. A serial dilution was performed on extracted pure culture DNA and duplicates from each dilution were used to generate a 7 point standard curve. Based on this standard curve, the lower quantitation limit for both *Bacteria* and *Archaea* DNA was 100 femtograms.

qPCR was performed using a SYBR Green I assay with the Bio-RAD iQ5 thermocycler (Hercules, CA). Reactions were prepared in 25 µL volumes using 12.5 µL SYBR Green Supermix, 0.25 µM of each primer, 2 µL DNA extract and 9.5 µL sterile pure water. 16S RNA gene qPCR primers to quantify bacterial (338f/805r) and archaeal (787f/1059r) targets were from Yu et al. (2005). PCR was performed as follows: initial denaturing step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 45 sec, extension at 72°C for 1 min. qPCR results were analyzed using iQ5 Optical System software (Bio-Rad).

Chemical Analyses

Moisture content was measured by drying to a constant weight at 105°C. pH was measured using a glass combination electrode (Fisher Scientific; Pittsburgh, PA) by suspending solids in 10 mL DI water and mixing or directly for leachate. Cellulose, hemicellulose, lignin and volatile solids analyzed as described previously (Mehta et al., 2002). Volatile fatty acid (VFA) concentrations were measured from the leachate or liquid suspension from the solids using solid phase microextraction (SPME) followed by GC-MS.
as described previously (Sadri et al., 2007). Lower quantitation limits for acetic, propionic, i-butyric, butyric, i-valeric and valeric acids were 21, 20, 2, 19, 2 and 5 mg L$^{-1}$, respectively.

**Nucelotide Sequence Accession Numbers**

Bacterial sequences obtained in this study were deposited in the GenBank database under accession numbers GQ443086 to GQ443106.

**Results**

**Chemical Analyses**

Moisture content, pH, cellulose, hemicellulose and lignin for fresh refuse, individual waste components and decomposed solids and leachate are given in Table 1. The fresh refuse moisture content and pH averaged 37±8% and 7.0±0.5, respectively. The moisture content varied widely amongst the individual waste components, ranging from 2.1% in office paper to 82.4% in food waste. pH was also variable and ranged from 4.3 in leaves to 9.9 in office paper. The elevated pH in office paper is likely attributable to the presence of calcium carbonate that is routinely used as a filler. Given the addition of leachate, the moisture content for decomposed refuse was expectedly higher, 64% in the accelerated CH$_4$ phase and 83% for well-decomposed refuse.

Cellulose and hemicellulose are the major biodegradable components of refuse while lignin is essentially recalcitrant under anaerobic conditions and limits access to cellulose and hemicellulose (Barlaz et al., 1990; Colberg, 1990). The cellulose, hemicellulose and
lignin content were reasonably consistent in fresh refuse and averaged 47±5%, 10±1% and 14±2%, respectively. For individual components, leaves had the lowest cellulose content (15.7%) but the highest lignin (41.1%). Conversely, office paper was highest in cellulose (70.7%) but lowest in lignin (0.9%). The low lignin content of office paper is because it is manufactured from chemical pulp in which the lignin is removed. Decomposed refuse exhibited decreasing cellulose content and lignin enrichment as decomposition advanced from the accelerated CH₄ to well-decomposed phase (Table 1).

With the exception of food waste, VFAs were low in fresh refuse and individual components compared to decomposed accelerated CH₄ refuse (Table 2). This is expected given most fresh waste materials experience aerobic conditions while acid production is primarily a function of anaerobic fermentation. Acetic acid concentrations were typically highest followed by butyric and propionic acids which was similar to the trend observed by Barlaz et al. (1989).

In comparing the solid fraction of refuse with leachate, pH was comparable in the acid and stabilized phases of decomposition (Table 1). During the accelerated and decelerated CH₄ phases, the pH in the solids was 0.5 and 1.5 units lower compared to the leachate. For comparison, VFAs in solids and leachate were compared by computing the total mass for each acid for the entire reactor. Results showed VFA masses were similar with the exception of acetic acid in the acid phase, which was roughly 1.7 times higher in the solids versus leachate (data not shown).
Fresh MSW 16S Clone Library

Both bacterial and archaeal clone libraries were constructed from the November 18 fresh refuse sample. Phylogenetic affiliations from bacterial sequences retrieved are shown in Figure 1. The majority of sequences were from the genus *Pseudomonas* (24%) followed by *Xanthomonas* (10%), *Enterobacter* (10%) and *Psychrobacter* (10%). Bacterial sequences identified fell into 8 major taxa (by family) with roughly 14% of sequences having no known taxonomic affiliation (Figure 2). By phylum, roughly 68% of sequences were from *Gammaproteobacteria* followed by *Firmicutes* (10%), *Actinobacteria* (5%) and *Bacteriodetes* (5%). No archaeal sequences were found and further analysis using qPCR was conducted to identify the extent and variability of *Archaea* in fresh refuse.

Total DNA Recovery and qPCR of *Bacteria* and *Archaea*

Total DNA recovered from fresh refuse ranged from 111 to 493 ng per dry g refuse (Table 3). DNA in office paper and newspaper was near or below quantitation limits. DNA from well-decomposed refuse was roughly twice that of accelerated CH$_4$ phase refuse which was 2 to 8 times higher than fresh refuse. Bacterial DNA content in individual waste components was highest in leaves and food waste and <0.4 ng per dry g refuse in office paper and newspaper. In fresh refuse, bacterial DNA content ranged from 3 to more than 259 ng per dry g refuse.

Archaeal DNA in all fresh refuse materials was less than 0.2 ng per dry g refuse (Table 3) and was less than 0.5% of the combined *Bacteria* and *Archaea* DNA mass.
However, archaeal DNA made up 38% and 67% prokaryotic DNA in accelerated CH$_4$ and well-decomposed refuse, respectively.

MSW and Individual Refuse Components T-RFLP

T-RFLP results for *Bacteria* and *Archaea* were compared using principal component analysis (PCA), the Jaccard index and T-RF richness. PCA analysis for bacterial community structure showed the fresh refuse samples clustered together (Figure 3). Roughly 55$\pm$18% of terminal restriction fragments (T-RFs) were shared amongst fresh refuse samples. Food waste and, to a lesser extent, decomposed refuse in the accelerated CH$_4$ phase also clustered with fresh refuse (Figure 3). Despite this clustering, Jaccard similarity was relatively low (0.33$\pm$0.10) between fresh refuse samples.

Food had the highest percentage of bacterial T-RFs in common with fresh refuse samples (50$\pm$12%) as compared to grass (26$\pm$3%), branches (15$\pm$3%) or leaves (26$\pm$5%). Branches and leaves had markedly different community structure compared to other fresh materials (i.e. refuse, food, grass) (Figure 3). Data also showed that the bacterial community in fresh refuse was not similar to decomposed refuse. Fresh refuse T-RFs in common with decomposed refuse decreased as refuse became more degraded with 40$\pm$7% being shared in accelerated CH$_4$ and only 28$\pm$6% shared in well-decomposed refuse.

T-RFLP results for *Archaea* showed significant variation in community structure with the exception of decomposed accelerated CH$_4$ and well-decomposed MSW, which clustered together (data not shown). The fraction of T-RFs in common between fresh and decomposed samples was approximately 20%, on the other hand; 64% of T-RFs were
shared between accelerated CH\textsubscript{4} and well-decomposed refuse. The August 14 fresh refuse sample exhibited a weak correlation with the decomposed MSW samples. Interestingly, the August 14 refuse sample also had the highest \textit{Archaea} concentration of the fresh refuse materials (Table 3).

Microbial Community Demographics in MSW Solids versus Leachate

T-RFLP statistics comparing MSW solids versus leachate are shown in Table 4. Percent differences in bacterial richness (number of T-RFs) between solids and leachate in the acid phase, accelerated CH\textsubscript{4} phase and stabilized refuse were 3\%, 30\% and 12\%, respectively (data for the decelerated CH\textsubscript{4} phase is not shown due to methodological issues). Except for the acid phase, bacterial richness was higher in the solids. A larger disparity was observed for \textit{Archaea}, which had percent differences of 35\%, 47\%, 47\%, 69\% for acid, accelerated CH\textsubscript{4}, decelerated CH\textsubscript{4} and stabilized refuse, respectively. Archaeal T-RF richness was higher in the leachate except during the acid phase (Table 4).

Jaccard similarity between solids and leachate, averaged for all decomposition phases, was 0.34±0.10 for \textit{Bacteria} and 0.37±0.07 for \textit{Archaea}, indicating community structure was different in both domains (Table 4). PCA analysis gave similar results and showed microbial communities in solids and leachate did not cluster together (data not shown). However, archaeal communities in the solid fraction tended to cluster together and were more similar to one another than those in leachate. No obvious trends were observed for \textit{Bacteria}. 
Relative abundances of individual bacterial T-RFs were variable and no trends were apparent. For Archaea, the two most dominant T-RFs had lengths of 430 bp and 471 bp and represented 85±7% of the sample relative abundance, except for acid phase solids which was 24% (Table 4). In silico digests of clone sequences from this reactor (data not shown) indicate these T-RFs, 430 bp and 471 bp, are from the Methanomicrobiales and Methanosarcinales orders, respectively. Relative abundances for the 430 bp T-RF were 3 to 50 times higher in leachate in all but the acid phase. On the other hand, the 471 bp T-RF had relative abundances that were 1.4 to 14 times lower in leachate compared to the solids, except in the acid phase which had relative abundances of 23% and 85% in solids and leachate, respectively.

Total DNA recovered and qPCR results comparing solids and leachate are shown in Table 5. Comparing DNA concentration between a liquid and a solid can be difficult since one is based on liquid volume and the other is based on mass. Thus, DNA in the solid and leachate phases was compared by multiplying the DNA concentration by the entire dry mass or liquid volume in the reactor to obtain a total DNA mass in solids and leachate, respectively. Recovered DNA tended to be higher from solids versus leachate in all decomposition phases. Relative masses of bacterial DNA were roughly the same in solids versus leachate for all phases of decomposition. On the other hand, the majority of archaeal DNA in the acid phase was from leachate rather than solids. This was opposite that of other phases which had 27 to 85 times more archaeal DNA in the solid fraction of MSW. Generally, most DNA recovered from leachate tended to be from bacterial sources.
while DNA from the solid fraction was split between *Bacteria* and *Archaea*, except for acid phase solids, which was comprised largely of bacterial DNA (Table 5).

**Discussion**

Comparison of Microbial Communities in Refuse

This study is the first to compare bacterial and archaeal communities in fresh refuse, individual waste components and to evaluate differences in the solid fraction of refuse versus leachate. Microbial communities in refuse transported to and placed in a landfill represent the initial populations present and no doubt play a role in subsequent refuse degradation given the limited potential for microbial translocation during the initial stages of decomposition. While chemical analyses of individual components showed wide variation in moisture content, pH, cellulose and lignin, these parameters were more consistent in mixed fresh refuse. Despite greater consistency in mixed refuse, moisture content was relatively low in fresh mixed refuse (31 to 52%), although not as low as the 20% measured in previous work (Mehta et al., 2002). No correlation was observed between moisture content and DNA recovered from *Bacteria* ($r^2=0.10$) or *Archaea* ($r^2=0.24$) in fresh refuse and individual waste components. However, it is well known that low moisture conditions limit microbial activity once refuse has been landfilled (Barlaz et al., 1990). In the absence of additional moisture inputs, the low moisture content in fresh refuse materials (Table 1), coupled with low microbial DNA concentrations (Table 3), likely influence the initial stages of refuse decomposition in the form of extended lag times.
to CH$_4$ initiation and low gas production rates. This has implications for successful CH$_4$
capture and subsequent utilization for energy.

Of particular importance were the very low concentrations of Archaea DNA measured
in individual refuse components using qPCR (Table 3). Methanogenic Archaea are critical
to the anaerobic decomposition process and, in the absence of significant methanogen
populations, volatile fatty acids will accumulate and the resulting lower pH will inhibit
further decomposition. However, previous work using culture-based techniques has
suggested that significant methanogen populations can be present in fresh refuse (Barlaz et
al., 1989). Interestingly, the August 14 fresh mixed refuse sample, which had the highest
archaeal DNA, was also visually confirmed as having a noticeably higher diaper content
compared to the other mixed samples collected. This suggests that methanogen content in
fresh refuse is highly variable and strongly correlated to MSW composition, especially to
individual components most likely to be from anaerobic sources (e.g. human and pet
feces). Thus, these results show mixed refuse is an inconsistent source of methanogens in
newly landfilled refuse.

Although bacterial community structure between fresh refuse samples clustered
together in the PCA analysis (Figure 3), overall Jaccard similarity was low (0.33±0.10).
However, when compared to grass, branches and leaves the average Jaccard similarity was
0.14±0.04, which shows that even though refuse samples were different from one another,
they were more dissimilar to bacterial communities in these individual refuse components.
Of individual components, food waste had the most T-RFs in common with fresh mixed
refuse (50±12%) suggesting food waste content is one of the primary contributors to
bacterial community composition in mixed refuse. The similarity between bacterial communities in branches and leaves is likely related to their originating from similar wooded ecosystems and unique composition. For example, the lignin content in branches and leaves was 1.5 to 40 times higher than that in the other individual refuse components.

When compared to decomposing refuse, 40±7% of shared T-RFs in fresh refuse persisted under anaerobic conditions in the accelerated CH$_4$ phase. By the time refuse was well-decomposed, only 28±6% of T-RFs in the fresh refuse remained, thus; the fraction of microbes in fresh refuse that persevere under anaerobic conditions is roughly one-third of the initial population. This value is consistent with clone library results (November 18 sample) indicating roughly 33% of sequenced clones were closely related to the *Carnobacterium*, *Proteus*, *Enterobacter* and *Serratia* genera. These genera are facultatively anaerobic fermenters and would likely remain viable after refuse is landfilled.

**Fresh Refuse Collected at Different Times**

Fresh refuse samples were collected at five different times throughout the year spanning all four seasons. Differences in *Bacteria* T-RF richness can be explained in part by differences in composition, which was weakly correlated to cellulose content ($r^2=0.53$). Monthly temperatures of 9°C (Jan. 2006), 19°C (May 2006), 24°C (Jun. 2005), 29°C (Aug. 2007) and 11°C (Nov. 2006) were obtained from the Southeast Regional Climate Center (www.sercc.com). Bacterial richness was highly correlated to seasonal temperature during the months refuse was sampled ($r^2=0.88$), suggesting that ambient air temperature, and/or waste composition that correlates with season (e.g. yard waste) is a dominant factor(s)
shaping microbial community structure in fresh refuse prior to landfilled. Other parameters (i.e. lignin, pH, moisture content) had little apparent effect on the number of bacterial T-RFs in refuse ($r^2<0.14$). No trends were observed for *Archaea* based on collection time, an expected result given their presence in fresh MSW varies.

Solids versus Leachate

Results showed a divergence between microbial demographics in MSW solids versus leachate. In addition to low Jaccard similarity, bacterial T-RFs that were shared between solids and leachate exhibited variation in relative abundance between the two matrices. This suggests that distinct groups of *Bacteria* populate solids or leachate preferentially, an observation verified by Burrell et al. (2004) who showed that cellulolytic bacteria attached to solid particulates while other bacteria that consumed primarily dissolved substrates were less likely to attach. qPCR results showed that bacteria DNA content was similar between solids and leachate except in the decelerating CH$_4$ phase, which had two times more DNA in the solids. A plausible explanation is that this phase of decomposition preferentially selected for bacterial populations better adapted to the solids phase (i.e. cellulolytic bacteria). One might also argue the discrepancy could be attributed to sampling variability, but previous work in our lab has shown sample to sample variation of recovered DNA from the same well-mixed reactor typically ranges from 27% to 36% depending on the state of refuse decomposition (data not shown), which does not explain the difference measured here.
Except for acid phase solids, over 85% of archaeal relative abundance was from two T-RFs representing the *Methanomicrobiales* (430 bp) and *Methanosarcinales* (471 bp) orders. During active methane production, relative abundances for the 430 bp T-RF were markedly higher in leachate versus solids while the opposite was true for the 471 bp T-RF (Table 4). The *Methanomicrobiales* order is strictly hydrogenotrophic and must be in close association with hydrogen producing bacteria for interspecies hydrogen transfer; on the other hand, *Methanosarcinales* can utilize a wider array of substrates including acetate (Kendall and Boone, 2006). It is well known that syntrophic bacteria metabolize fatty acids and many species are known to be motile, suggesting they are less likely to preferentially attach to particulate matter (McInerney et al., 2008). Therefore, we surmise the higher abundances of putative hydrogenotrophic *Methanomicrobiales* in leachate are likely due to a larger fraction of syntrophs in the leachate. However, the fraction of archaeal DNA in the solids was many times higher than in leachate (Table 5). This shows that methanogen populations were substantially higher in the solid fraction of MSW and that these methanogens were from the order *Methanosarcinales*.

These results show that selection pressures can preferentially stratify microbial groups into solids or leachate. Thus, the assessment of microbial community structure using either MSW solids or leachate alone does not fully characterize microbial community structure in decomposing refuse and both must be considered. This has implications for field-scale monitoring since most prior assessments of landfill microbiology in have been made using leachate.
Acknowledgement

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References


Figure 1 Phylogenetic tree of representative bacterial sequences recovered from fresh MSW (November 18 sample) using the neighbor-joining method (Jukes-Cantor algorithm). Bootstrap values shown are based on 500 replicates.
Figure 2 Representative bacterial sequences categorized by family from fresh refuse (November 18 sample).
Figure 3  T-RFLP PCA correlation of bacterial communities.  Dates shown are for fresh refuse.  T-RFLP was not performed on office paper and newspaper due to low DNA recovery.
### Table 1: Moisture content, pH, cellulose and lignin for MSW and individual components

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<th></th>
<th>Moisture Content&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>pH</th>
<th>Cellulose&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>Hemicellulose&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>Lignin&lt;sup&gt;b&lt;/sup&gt; (%)</th>
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</thead>
<tbody>
<tr>
<td><strong>Fresh mixed refuse</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>January 5, 2006</td>
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<td>6.7</td>
<td>47.1</td>
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<td>13.7</td>
</tr>
<tr>
<td>May 5, 2006</td>
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<td>6.8</td>
<td>50.2</td>
<td>11.5</td>
<td>16.7</td>
</tr>
<tr>
<td>June 24, 2005</td>
<td>33.3</td>
<td>7.1</td>
<td>41.4</td>
<td>9.4</td>
<td>14.4</td>
</tr>
<tr>
<td>August 14, 2007</td>
<td>51.8</td>
<td>6.7</td>
<td>42.8</td>
<td>9.6</td>
<td>11.6</td>
</tr>
<tr>
<td>November 18, 2006</td>
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<td>7.9</td>
<td>53.3</td>
<td>9.6</td>
<td>14.0</td>
</tr>
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<td></td>
<td></td>
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<td>Office paper</td>
<td>2.1</td>
<td>9.9</td>
<td>70.7</td>
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<td>Newspaper</td>
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<td>5.0</td>
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<td>9.5</td>
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<td>Leaves</td>
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<td>15.7</td>
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<td>41.1</td>
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<tr>
<td>Branches</td>
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<td>Grass</td>
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<td>5.8</td>
<td>19.5</td>
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<td><strong>Decomposed mixed refuse</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Accelerated CH&lt;sub&gt;4&lt;/sub&gt; phase</td>
<td>63.9</td>
<td>6.2</td>
<td>44.4</td>
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<td>24.7</td>
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<tr>
<td>Well-decomposed phase</td>
<td>83.1</td>
<td>8.5</td>
<td>6.9</td>
<td>3.4</td>
<td>53.4</td>
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<td><strong>MSW Solids versus Leachate</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Acid solids</td>
<td>76.8</td>
<td>5.8</td>
<td>41.4</td>
<td>9.8</td>
<td>16.0</td>
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<tr>
<td>Acid leachate</td>
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<tr>
<td>Accelerated CH&lt;sub&gt;4&lt;/sub&gt; solids</td>
<td>74.2</td>
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<td>22.8</td>
<td>6.9</td>
<td>25.9</td>
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<tr>
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<tr>
<td>Decelerated CH&lt;sub&gt;4&lt;/sub&gt; solids</td>
<td>75.6</td>
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<td>14.0</td>
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<td>31.7</td>
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<tr>
<td>Decelerated CH&lt;sub&gt;4&lt;/sub&gt; leachate</td>
<td>8.0</td>
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<tr>
<td>Stabilized solids</td>
<td>80.4</td>
<td>8.4</td>
<td>11.9</td>
<td>4.5</td>
<td>27.4</td>
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<tr>
<td>Stabilized leachate</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Mass water divided by the mass of wet refuse.

<sup>b</sup> % on a per dry g refuse basis
<table>
<thead>
<tr>
<th>Table 2</th>
<th>Volatile fatty acid concentrations for MSW and individual components (mg acid per dry g refuse)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetic</td>
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<tr>
<td>Fresh mixed refuse</td>
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</tr>
<tr>
<td>January 5, 2006</td>
<td>0.75</td>
</tr>
<tr>
<td>May 5, 2006</td>
<td>1.83</td>
</tr>
<tr>
<td>June 24, 2005</td>
<td>0.76</td>
</tr>
<tr>
<td>August 14, 2007</td>
<td>1.19</td>
</tr>
<tr>
<td>November 18, 2006</td>
<td>BQL</td>
</tr>
<tr>
<td>Individual fresh refuse components</td>
<td></td>
</tr>
<tr>
<td>Office paper</td>
<td>BQL</td>
</tr>
<tr>
<td>Newspaper</td>
<td>BQL</td>
</tr>
<tr>
<td>Food waste</td>
<td>5.65</td>
</tr>
<tr>
<td>Leaves</td>
<td>BQL</td>
</tr>
<tr>
<td>Branches</td>
<td>BQL</td>
</tr>
<tr>
<td>Grass</td>
<td>0.77</td>
</tr>
<tr>
<td>Decomposed mixed refuse</td>
<td></td>
</tr>
<tr>
<td>Accelerated CH$_4$ phase</td>
<td>10.71</td>
</tr>
<tr>
<td>Well-decomposed phase</td>
<td>0.57</td>
</tr>
</tbody>
</table>

$^a$ BQL, below quantitation limits.
| Table 3  DNA recovery and qPCR quantitation of *Bacteria* and *Archaea* (ng DNA per dry g refuse)*\(^{a}\) |
|----------------------------------|-----------------|----------------|-----------------|-----------------|-----------------|
|                                  | DNA recovered\(^{b}\) | Bacteria DNA\(^{c}\) | Bacteria\(^{d}\) % | Archaea DNA\(^{c}\) | Archaea\(^{d}\) % |
| Fresh mixed refuse               |                 |                 |                 |                 |                 |
| January 5                        | 142 (10)        | 5.4 (0.5)       | 99.9            | 0.005 (0.002)   | <0.1            |
| May 5                            | 127 (9)         | 3.0 (3.6)       | 99.9            | 0.003 (0.0004)  | <0.1            |
| June 24                          | 111 (19)        | 14.2 (0.9)      | 99.9            | 0.02 (0.004)    | <0.1            |
| August 14                        | 493 (93)        | >259\(^{e}\)    | 99.9            | 0.29 (0.15)     | <0.1            |
| November 18                      | 234 (27)        | 25.5 (7.1)      | 99.9            | 0.01 (0.003)    | <0.1            |
| Individual refuse components     |                 |                 |                 |                 |                 |
| Office paper                     | BQL             | 0.38 (0.15)     | NC              | BQL             | NC              |
| Newspaper                        | 46 (3)          | 0.01 (0.001)    | NC              | BQL             | NC              |
| Food waste                       | 656 (97)        | 116 (4)         | 99.9            | 0.10 (0.02)     | <0.1            |
| Leaves                           | 2,215 (220)     | 2,019 (643)     | 99.9            | 0.27 (0.03)     | <0.1            |
| Branches                         | 240 (66)        | 43.9 (2.9)      | 99.9            | 0.05 (0.01)     | <0.1            |
| Grass                            | 184 (53)        | 17.7 (2.0)      | 99.6            | 0.08 (0.01)     | 0.4             |
| Decomposed mixed refuse          |                 |                 |                 |                 |                 |
| Accelerated CH\(_{4}\) phase    | 1,030 (156)     | 586 (12)        | 61.3            | 370 (29)        | 38.7            |
| Well-decomposed phase            | 2,338 (291)     | 617 (135)       | 33.4            | 1,232 (134)     | 66.6            |

\(^{a}\) Standard deviation is given in parentheses. Abbreviations: BQL, below quantitation limits; NC, not computed.

\(^{b}\) Measured via spectrophotometry.

\(^{c}\) Quantified with qPCR.

\(^{d}\) Percentage is qPCR quantified DNA mass of the domain (*Bacteria* or *Archaea*) divided by the sum of Bacteria and Archaea DNA.

\(^{e}\) Value represents upper limit of qPCR standard curve.
Table 4  T-RFLP comparison of microbial communities in MSW solids versus leachate.

<table>
<thead>
<tr>
<th></th>
<th>Acid Solids</th>
<th>Leachate</th>
<th>Accelerated CH&lt;sub&gt;4&lt;/sub&gt; Solids</th>
<th>Leachate</th>
<th>Decelerated CH&lt;sub&gt;4&lt;/sub&gt; Solids</th>
<th>Leachate</th>
<th>Stabilized Solids</th>
<th>Leachate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria&lt;sup&gt;a&lt;/sup&gt;</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Richness (# of T-RFs)</td>
<td>30</td>
<td>31</td>
<td>47</td>
<td>33</td>
<td>NC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NC</td>
<td>43</td>
<td>38</td>
</tr>
<tr>
<td>% of T-RFs in common &lt;sup&gt;c&lt;/sup&gt;</td>
<td>36</td>
<td>58</td>
<td>NC</td>
<td>NC</td>
<td>57</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Jaccard similarity</td>
<td>0.22</td>
<td>0.40</td>
<td>NC</td>
<td>0.40</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Archaea</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Richness (# of T-RFs)</td>
<td>23</td>
<td>15</td>
<td>17</td>
<td>25</td>
<td>15</td>
<td>22</td>
<td>16</td>
<td>27</td>
</tr>
<tr>
<td>% of T-RFs in common&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47</td>
<td>52</td>
<td>65</td>
<td>51</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Jaccard similarity</td>
<td>0.31</td>
<td>0.36</td>
<td>0.48</td>
<td>0.34</td>
<td></td>
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<tr>
<td>Relative Abundance of dominant T-RFs (%)</td>
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<tr>
<td>430 bp&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>&lt;0.3</td>
<td>1</td>
<td>14</td>
<td>49</td>
<td>21</td>
<td>71</td>
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<tr>
<td>471 bp&lt;sup&gt;d&lt;/sup&gt;</td>
<td>23</td>
<td>85</td>
<td>90</td>
<td>64</td>
<td>90</td>
<td>29</td>
<td>72</td>
<td>5</td>
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</tbody>
</table>

<sup>a</sup> Dominant T-RFs are not shown since no obvious trends were observed.

<sup>b</sup> NC, not computed.

<sup>c</sup> Computed by dividing the # of T-RFs in common into the average of the solids and leachate richness.

<sup>d</sup> Inferred phylogeny for 430 bp and 471 bp T-RFs is *Methanomicrobiales* and *Methanosarcinales*, respectively.
<table>
<thead>
<tr>
<th></th>
<th>Total DNA recovered(^a)</th>
<th>Bacteria DNA(^c)</th>
<th>Bacteria(^d) %</th>
<th>Archaea DNA(^c)</th>
<th>Archaea(^d) %</th>
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</thead>
<tbody>
<tr>
<td>Acid solids</td>
<td>579 (107)</td>
<td>130 (19)</td>
<td>98.9</td>
<td>1.4 (0.4)</td>
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<tr>
<td>Acid leachate</td>
<td>226 (13)</td>
<td>96 (11)</td>
<td>73.4</td>
<td>35 (7)</td>
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<tr>
<td>Accelerated CH(_4) solids</td>
<td>451 (138)</td>
<td>110 (13)</td>
<td>50.7</td>
<td>107 (51)</td>
<td>49.3</td>
</tr>
<tr>
<td>Accelerated CH(_4) leachate</td>
<td>216 (34)</td>
<td>170 (33)</td>
<td>98.4</td>
<td>2.8 (0.9)</td>
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<tr>
<td>Decelerated CH(_4) solids</td>
<td>304 (31)</td>
<td>64 (6)</td>
<td>51.7</td>
<td>60 (18)</td>
<td>48.3</td>
</tr>
<tr>
<td>Decelerated CH(_4) leachate</td>
<td>190 (33)</td>
<td>36 (7)</td>
<td>98.0</td>
<td>0.7 (0.02)</td>
<td>2.0</td>
</tr>
<tr>
<td>Stabilized solids</td>
<td>425 (102)</td>
<td>245 (26)</td>
<td>45.5</td>
<td>293 (61)</td>
<td>54.5</td>
</tr>
<tr>
<td>Stabilized leachate</td>
<td>276 (61)</td>
<td>244(^c)</td>
<td>95.4</td>
<td>11(^c)</td>
<td>4.6</td>
</tr>
</tbody>
</table>

\(^a\) Unless noted otherwise, units are µg DNA in entire reactor (based on 885.3 dry g solids and 2 L leachate at initiation). Standard deviation is given in parentheses.
\(^b\) Measured via spectrophotometry.
\(^c\) Quantified with qPCR.
\(^d\) Percentage is qPCR quantified DNA mass of the domain (Bacteria or Archaea) divided by the sum of Bacteria and Archaea DNA.
\(^e\) No standard deviation computed since only duplicates were available.
Chapter 4.

MICROBIAL ECOLOGICAL SUCCESSION DURING MUNICIPAL SOLID WASTE DECOMPOSITION

*(FORMATTED FOR SUBMISSION TO ENVIRONMENTAL MICROBIOLOGY)*

Summary

The decomposition of landfilled refuse proceeds through distinct phases, each defined by varying environmental factors such as volatile fatty acid concentration, pH and substrate quality. The succession of microbial communities in response to these changing conditions was monitored in a laboratory-scale simulated landfill to minimize measurement difficulties experienced at field scale. 16S rRNA gene sequences retrieved at separate stages of decomposition showed significant succession in both *Bacteria* and methanogenic *Archaea*. The fraction of bacterial sequences that were unknown increased from 34% initially to 92% as decomposition proceeded, indicating a large majority of *Bacteria* in landfilled refuse are uncharacterized. Roughly 60% of archaeal sequences retrieved under low pH, high acetate conditions were strictly hydrogenotrophic (*Methanomicrobiales, Methanobacteriales*) and this changed to ~6% once neutral pH conditions developed, with these species being replaced by members of the genus *Methanosarcina*. Correspondence analysis showed bacterial populations shifts were attributed to carboxylic acid concentration and solids hydrolysis, while archaeal populations were affected more by pH. T-RFLP analysis showed specific taxonomic
groups responded differently and exhibited unique responses during decomposition, suggesting that species composition and abundance within *Bacteria* and *Archaea* is highly dynamic. This study shows landfill microbial demographics are highly variable across both spatial and temporal transects.

**Introduction**

Since Winogradsky first showed microbial populations stratify based on the availability of electron acceptors, the selection of specific microbial groups has been widely considered dependent on available substrates (Waksman, 1946). In addition to substrate levels, environmental parameters such as pH, temperature, and moisture content play crucial roles in shaping microbial community structure. In landfills, which contain an abundance of degradable material in the form of municipal refuse, a distinct progression occurs during decomposition whereby the oxygen entrained in refuse at burial is rapidly consumed. Next, prior to the onset of methanogenesis, volatile fatty acids (VFAs) accumulate and the pH decreases. After the initiation of methane (CH$_4$) production, VFAs are rapidly consumed, pH returns to neutral and biogas production is high. During this time, the rate-limiting step is controlled largely by the metabolic activity of methanogenic *Archaea*. Once most rapidly degradable VFAs have been consumed, biogas production is governed primarily by the hydrolysis of cellulosic materials. This decomposition process has been described in a series of four distinct phases: aerobic, anaerobic acid (low pH/high VFAs), accelerated CH$_4$ (neutral pH, high VFAs) and decelerated CH$_4$ phases (neutral pH, low VFAs) (Barlaz et al, 1989).
Most studies evaluating landfill microbiology have used leachate, that is, the liquid collected at the bottom of the landfill (Huang et al., 2002; Huang et al., 2005; Mori et al., 2003; Calli et al., 2006). Using landfill leachate, a number of novel gene sequences have been reported (Lalouï-Carpentier et al., 2006; McDonald et al., 2008), suggesting that landfills harbor a unique ecosystem. The drawback to the use of leachate is that microbial populations are not representative of those contained within the waste material (Staley et al., submitted). Thus, the assessment of microbial community structure may be skewed towards members that are planktonic, easily detached into the liquid percolating through the waste material or better able to compete in a predominantly liquid environment.

Some studies have characterized specific sub-populations rather than the entire community and have focused on members of the genus Clostridium (Van Dyke and McCarthy, 2002), cellulolytic Bacteria (Westlake et al., 1995; Pourcher et al., 2001) and methanogens (Uz et al., 2003; Luton et al., 2002). Other studies have sampled from the solid fraction of waste, typically using refuse excavated at different depths within a landfill (Chen et al., 2003a; Chen et al., 2003b). Because refuse is placed in landfills with the most recent layer at the top, refuse depth is a general indicator of waste age (Hull et al., 2005). However, field scale sampling does not allow a concise assessment of age or an accurate determination of the decomposition phase with depth. Thus, microbial community shifts that occur at each phase of decomposition have not been well characterized aside from a single study that showed functional groups (i.e. cellulolytics, acetogens and methanogens) tended to increase in number as refuse decomposition proceeded (Barlaz et al., 1989).
In this study, we used a laboratory-scale simulated landfill to decompose fresh refuse. This approach did not have the limitations of field scale sampling and allowed tracking of microbial demographic shifts during each phase of refuse decomposition. Due to the smaller scale, the measured gas composition, gas production rate, pH and VFA concentrations could be more accurately quantified over time. Terminal restriction fragment length polymorphism (T-RFLP) was used in conjunction with bacterial and archaeal 16S rRNA gene clone libraries. Differences in community structure were tracked during decomposition and correlated to varying environmental parameters using canonical correspondence analysis (CCA).

Results

*Methane, pH, volatile fatty acids, moisture content, volatile solids and complex carbohydrates*

Refuse (885 dry g) was decomposed for roughly 168 days and resulted in a cumulative methane volume of 59.3 L at STP. By day 41, only 1.3% of total methane yield had occurred, indicating a lag until the initiation of methane production. During this time, pH averaged 5.66±0.36, except for the initial aerobic phase during days 0 to 5, and the acetic acid concentration was nearly 2.5 times higher than any other time during decomposition (Fig. 1). Roughly 10 days later (day 51), methane production was an order of magnitude higher, nearly 10.2% of cumulative yield and pH had increased to 6.6. By day 57, pH was neutral or above and remained so for the rest of the experiment. Roughly 75% of the cumulative methane yield occurred over a 40 day period (days 41 to 82) after
methanogenesis initiation and was commensurate with the consumption of the majority of acetic and butyric acids (>95%). The major phases of refuse decomposition, based on criteria established by Barlaz et al. (1989), are indicated in Fig. 1.

Moisture content was relatively consistent and averaged 76±3% (Table 1). Volatile solids decreased from 84% to 53% during decomposition. Cellulose followed a similar trend and decreased from 53% to 12% with the majority being consumed by day 105. After this, cellulose content was relatively consistent for the duration of the experiment. Conversely, as cellulose and hemicellulose decomposed, lignin was enriched from 14% to 27% (Table 1). This decrease in the cellulose plus hemicellulose to lignin ratio ([C+H]/L), represents a decrease in substrate quality as lignin limits microbial access to cellulose and hemicellulose (Colberg, 1988).

**Bacteria and Archaea 16S rRNA gene clone libraries**

Bacterial sequences retrieved were different between the anaerobic acid, accelerated CH$_4$ and decelerated CH$_4$ phases of decomposition. Roughly 58% of acid phase sequences were from the *Firmicutes* phylum and these were succeeded by *Proteobacteria* (40%) in the accelerated CH$_4$ phase (Table 2). Roughly 34% of bacterial sequences in the acid phases were unknown and not associated with a particular phyla and this increased to 49% and 92% in the accelerated CH$_4$ and decelerated CH$_4$ phases, respectively. Retrieved sequences were compared to known species from GenBank (Fig. 2), and most unclassified sequences in GenBank clustered with those retrieved from decomposing solid waste, leachate or anaerobic digesters treating manure wastes.
All archaeal sequences retrieved were euryarchaeotes and most were from known methanogens, although a small fraction coincided with uncultured sequences obtained from solid waste (Fig. 3). Roughly 57% of archaeal sequences retrieved from the anaerobic acid phase were strictly hydrogenotrophic and this decreased to 6% and 8% in the accelerated CH$_4$ and decelerated CH$_4$ phases, respectively, with the latter two phases being dominated by members of the *Methanosarcina* genus (Table 3). Within the *Methanosarcinales* order, less than 1% of archaeal sequences in the acid phase were related to *Methanosaeta* and no sequences from this genus were detected in subsequent phases.

*Correlating microbial demographics to decomposition phase/environmental parameters*

Canonical correspondence analysis (CCA) showed successive shifts in bacterial community structure over the course of decomposition (Fig. 4A). Bacterial community structure on day 34 (acid phase), day 54 (early accelerated CH$_4$ phase) and day 68 (late accelerated CH$_4$ phase) was correlated with acetic, butyric and propionic acid concentrations, respectively. These respective times are also when each VFA peaked during decomposition (Fig. 1). Note that pH was excluded from the CCA analysis since it was correlated with acetic acid concentration ($r^2 = 0.86$). A large community shift was observed from day 82 to 98, which was commensurate with the onset of a decreasing CH$_4$ production rate and the consumption of the majority of VFA accumulation (>98%). The pH during this period increased from 7.6 to 8.0. A second substantial shift in bacterial community demographics occurred from day 139 to 168 (Fig. 4A) and coincided with a
greater than 100-fold drop in CO$_2$ and CH$_4$, relative to the peak production rate, to less than 1 mL d$^{-1}$. VFA concentrations were negligible or below quantitation limits during this time and the pH increased from 7.8 to 8.4. Bacteria demographics became less dependent on VFA concentrations as they approached zero (around day 98) during the decelerated CH$_4$ phase, with the community composition on day 82 reflecting the transitional period between the accelerated CH$_4$ and decelerated CH$_4$ phases (Fig. 4A). The absence of a relationship between community composition and VFA concentration is consistent with the fact the VFA concentrations were low and stable after day 80.

Archaea community structure was stable during decomposition compared to Bacteria (Fig. 4B). Large changes to archaeal composition were only observed during the shift from the anaerobic acid to accelerated CH$_4$ phases (day 34 to 54), which was associated with a pH increase from 5.51 to 7.03, and again late in the decelerated CH$_4$ phase (day 139 to 168) that was commensurate with a cessation of methane production. Archaeal demographics were much less dependent on VFA concentration except during the anaerobic acid phase (day 34), which was correlated with acetic acid.

Shifts in individual T-RFs during decomposition

T-RFLP results showed the relative abundance of individual terminal restriction fragments (T-RFs) was correlated to the phase of decomposition but how they responded differed. For example, bacterial T-RFs with lengths of 69 bp and 475 bp peaked during the anaerobic acid phase and together comprised 52% of total relative abundance (Fig. 5). Bacterial T-RFs with lengths of 250 bp and 233 bp exhibited similar trends but peaked in
the accelerated CH₄ (22% relative abundance) and decelerated CH₄ (24% relative abundance) phases, respectively. Other T-RFs exhibited a bi-modal response (236 bp) or generally increased (243 bp) in abundance during decomposition (Fig. 5). No trends were observed based on bacterial T-RF richness, which averaged 36±7 during decomposition.

Archaeal T-RFs with lengths of 336 bp, 352 bp and 473 bp represented 77% of total relative abundance in the anaerobic acid phase (Fig. 6). In subsequent decomposition phases the 473 bp T-RF remained highly dominant and represented 87% of the relative abundance, on average. *In silico* digests of clone sequences indicated the 473 bp T-RF is associated with the *Methanosarcinales* order and, based on the clone library results, were specifically from the genus *Methanosarcina*. The 336 and 352 bp T-RF lengths were identified as being from the hydrogenotrophic *Methanobacteriales* order while the 430 bp T-RF, which exhibited an increase in abundance late in the decelerated CH₄ phase (day 168), represented the *Methanomicrobiales* order. Archaeal T-RF richness was 15±4 and variations observed during the course of decomposition exhibited no apparent trends.

**Discussion**

The gas, leachate and solids composition data from the laboratory-scale landfill showed that refuse decomposition was similar to previous descriptions (Barlaz et al, 1989). Cellulose degradation began soon after decomposition was initiated and was not affected by changes in pH or VFA level except for a slight increase the in rate of cellulose loss during the accelerated CH₄ phase after pH became neutral (data not shown). Lignin was
enriched in the remaining solids during decomposition, suggesting a reduction in substrate quality.

CCA showed that most variation in bacterial diversity during the anaerobic acid phase was attributable to acetic acid concentration and subsequently butyric and propionic acids in the early and late portions of the accelerated CH₄ phase, respectively. In the decelerated CH₄ phase, it can be inferred that community structure shifts were likely due to solids hydrolysis (i.e. cellulose and hemicellulose decomposition), given that other controlling variables such as VFA concentrations were near zero and pH was above neutral (mean = 7.9) (Fig. 1). This shows Bacteria community composition is largely affected by the type of substrate and its availability. The shift from Firmicutes to Pseudomonas (Table 2) was unexpected since Pseudomonas species are more widely known as aerobic or facultative aerobic bacteria. However, this phylum is very diverse and not as well characterized as other phyla. Anaerobic Pseudomonas have been identified during anaerobic waste treatment (Rincon et al., 2006; Atuanya and Aigbirior, 2002). A large proportion of unclassified bacteria in the accelerated CH₄ phase were closely related to members of the Firmicutes phylum, suggesting this phylum retained a dominant role in degradation but shifted to a largely uncultured species composition that was similar to that found in other anaerobically decomposed refuse (Li et al., unpublished) (Fig.2).

Changes in archaeal community structure occurred primarily during the onset and cessation of methane production. The shift during methanogenesis initiation can be attributed to high acetate levels and the low pH that results (Fig. 4B). Of particular interest is that the dominant methanogens (Methanobacteriales, Methanomicrobiales) under low
pH conditions were hydrogenotrophic (Table 3). During the anaerobic acid phase, acetic acid levels were high (~31 mg dry g\(^{-1}\)) and known aceticlastic/hydrogenotrophic methanogen clones (\textit{Methanosarcina} sp.) comprised 40% of the clone library compared to 56% for hydrogenotrophic methanogens (Table 3). On the other hand, \textit{Methanosarcina} were dominant (>85%) in subsequent phases. Previous work under low pH conditions in peat bogs has shown hydrogenotrophic methanogens were dominant (Cadillo-Quiroz et al., 2006; Kotsyurbenko et al., 2007). Acetate levels in peat bogs are very low (<5 µM) (Kotsyurbenko et al., 2004) which is over four orders of magnitude lower than in actively decomposing refuse. Thus, the finding that the hydrogenotrophic pathway appears dominant in refuse under low pH and high acetate conditions is significant in that the pH, rather than the acetate concentration appears to be exerting the most selective pressure. Since \textit{Methanosarcina} can use both acetate and hydrogen, the extent to which the hydrogenotrophic pathway is used may be substantially higher than found in this study since the assessment of pathways was done strictly by identification of hydrogenotrophic genera via clone libraries. In addition, the large presence of \textit{Methanosarcina} found after the anaerobic acid phase is consistent with previous work that has suggested this species may be more tolerant of high VFA levels compared to other acetoclastic methanogens (Vavilin et al., 2008), which likely explains the lack of \textit{Methanosaeta} found in our study. However, one would expect \textit{Methanosaeta} to become more dominant after VFAs were depleted. We surmise the reason \textit{Methanosaeta} were not detected here may be because reactor operation was halted before sufficient time elapsed to allow significant growth to occur.
This study shows that microbial ecological succession is substantial during refuse decomposition. T-RFLP analysis showed specific taxonomic units within the microbial community exhibited unique and individual responses based on their affinity for available substrates or changing environmental conditions (Figs. 5 and 6). While functional group populations may generally increase, as indicated by culture-based methods (Barlaz et al., 1989), the greater resolution offered by molecular techniques shows that species composition and abundance within these groups is highly dynamic. Since the stage of decomposition in a landfill varies, this suggests that landfill microbial demographics are highly variable across both spatial and temporal transects and will be affected by localized pH and substrate availability regimes. This study is the first to show substantial ecological succession occurs in decomposing refuse and suggests the selection of certain microbial groups may be possible by implementing specific landfill operating strategies with the goal to enhance refuse decomposition and improve the sustainability of solid waste disposal.

**Experimental procedures**

*Reactor operation and sample collection*

Fresh municipal refuse was obtained from a residential area waste collection vehicle at the Holly Springs (NC) Transfer Station. The refuse was shredded the same day in a slow speed, high torque shredder to a particle size of approximately 2 cm x 6 cm. After shredding, refuse was mixed on a clean plastic sheet using hand tools. The refuse was anaerobically decomposed by combining 1,284 wet g MSW (at 31% moisture content) with 2 L methanogenic leachate in a 10-L reactor that was maintained at 37°C. The reactor
was monitored until the methane production rate was <1% of the maximum rate measured, which occurred at ~148 days of operation. Leachate was re-distributed within the reactor every 2-3 days by shaking and inverting the sealed reactor. Gas was collected in tedlar gas bags (PMC, Inc; Oak Park, IL) and volume was measured by evacuation into a container of known volume (Sanin et al., 2000). CH₄, CO₂, O₂, and N₂ were analyzed using a GOW-MAC 580 gas chromatograph with a thermal conductivity detector (Price et al., 2003). Decomposed refuse samples (~50 wet g) were retrieved routinely during reactor operation. The reactor was well mixed prior to sampling and samples were placed into plastic bags using sterile tongs then frozen at -20°C until processing. Leachate (~5 mL) was collected separately and used to measure pH and volatile fatty acid levels.

Sample processing and DNA extraction

Processing for DNA extraction followed the procedure that was found to be the most appropriate and exhibited the least bias for refuse (Staley et al., submitted). Refuse samples were combined with 250 mL chilled 23.7 mM PO₄ buffer at pH 6.73 (31.87 g Na₂HPO₄•7H₂O L⁻¹ and 16.1 g KH₂PO₄ L⁻¹) and homogenized in a sterile Waring blender (Torrington, CT) for 1 min. The blended mixture was poured into a 3.8 L 75-µm nylon paint strainer bag (Trimaco., Durham, NC) and hand-squeezed. Supernatant was collected in a 1.9 L sterile plastic container, transferred to sterile 50 mL tubes and centrifuged at 3,220 X g for 5 min. Supernatant was decanted and pellets were combined into a single tube. Residual in emptied tubes was suspended with sterile deionized (DI) water, combined with the pellet and centrifuged for 5 min. at 3220 X g. The resulting combined
pellet was well-mixed by hand using a sterile spatula then used for DNA extraction. DNA was extracted using the MoBio PowerSoil kit (Carlsbad, CA) following the manufacturer’s protocol. Extracted DNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific; Wilmington, DE).

**PCR conditions**

Polymerase chain reaction (PCR) was performed using 25 µL of FailSafe PCR system reaction mix F (Epicentre; Madison, WI), 0.6 µL FailSafe enzyme mix (Epicentre), 0.25 µM of each primer, ~5 ng DNA and sterile pure water added to a total volume of 50 µl. The primers used for T-RFLP and cloning were 8f/1492r (Klappenbach *et al.*, 2000) and 109f/915r [Miller and Wolin, 1986] for *Bacteria* and *Archaea*, respectively, and targeted the 16S rRNA gene. Forward primers used for T-RFLP were labeled at the 5′ end with 6-carboxy-fluorescein (6-FAM) for T-RFLP analysis. PCR products were run on a 1% agarose gel to verify amplification.

PCR was performed in an Eppendorf thermocycler programmed as follows: initial denaturing step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1.5 min, and final extension at 72°C for 10 min. Duplicate PCR reactions were pooled and DNA was purified using a Wizard SV Gel and PCR Clean-Up kit (Promega, Madison, WI).
Clone library of 16S rRNA gene

Bacterial and archaeal clone libraries were constructed using DNA extracted from samples retrieved on Days 34, 68, and 139 of reactor operation as these times corresponded to different phases of decomposition. The clone library was generated by inserting PCR products into the pCR 4-TOPO vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA), in accordance with the manufacturers protocol.

Colony PCR was performed by picking transformants using a sterile plastic pipette tip and placing them into a 96-well plate containing a PCR cocktail of 25 µL of FailSafe PCR system reaction mix F (Epicentre; Madison, WI), 0.6 µL FailSafe enzyme mix (Epicentre), 0.25 µM of each primer and sterile pure water added to a total volume of 50 µl. The forward primer used for colony PCR was the T3 primer (5’-ATTACCCTCACTAAAGGG) and was specific to the vector. The reverse primer used was specific to the insert (1492r for Bacteria, 915r for Archaea). PCR conditions for colony PCR were: a lysis step at 95°C for 10 min, an initial denaturing step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1.5 min, and final extension at 72°C for 10 min. Plates containing amplified inserts were submitted to the NC State University Genome Sciences Laboratory (Raleigh, NC) for purification and sequencing. A total of 234 and 145 successful clone sequences were obtained for Bacteria and Archaea, respectively.
**T-RFLP and Statistical analyses**

Purified PCR products were quantified by spectrophotometry and approximately 360 ng were digested separately with restriction endonuclease AluI (New England Biolabs; Ipswich, MA) for *Bacteria* and *Archaea*. Restriction digestion was performed for 4 hours using 20 µL reactions containing: 10 U restriction enzyme, 2 µL 10X buffer, 360 ng PCR product, and DI water. Terminal restriction fragments were analyzed by capillary electrophoresis at the NC State University Genome Sciences Laboratory.

T-RFLP profiles were analyzed using PeakScanner software (Applied Biosystems, Foster City, CA) and numerical output from each fragment pattern was exported to Microsoft Excel. Terminal restriction fragment (T-RF) areas were standardized using an automated algorithm to eliminate noise and identify true peaks (Abdo *et al.*, 2006). The following parameters were used as recommended by the algorithm developers: a minimum peak height of 15 RFU, standard deviation from the baseline of 3 and a bin size of ±1 bp. Phylogeny was inferred by comparing measured T-RF lengths to those from *in silico* digests of clone sequences obtained from this study. CCA was performed using Environmental Community Analysis version 2.1 (Pisces Conservation Ltd, Lymington, UK).

**Phylogenetic analysis**

Phylogenetic and molecular evolutionary analyses of 16S rRNA gene sequences were conducted using MEGA version 4 (Tamura *et al.*, 2007). Sequences were aligned using the ClustalW algorithm and phylogenetic trees were constructed using the neighbor-joining
method. Bootstrapping was performed to evaluate tree reliability (replications=500).

Sequences from related species and similar environmental clones were obtained from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and included in alignments. Sequences from this study were compared to those in the GenBank database using BLAST searches and matches sharing 97% homology were considered identical species. Sequences from this study were submitted to GenBank under accession numbers GQ453462 to GQ453573 for Bacteria and GQ453574 to GQ453590 for Archaea.

Chemical analyses

Moisture content was measured by drying to constant weight at 105°C. Cellulose, hemicellulose, lignin and volatile solids were analyzed as described previously [Mehta et al., 2002]. Volatile fatty acid (VFA) concentrations were measured from the leachate or liquid suspension from the solids using solid phase microextraction (SPME) followed by GC-MS as described previously (Sadri, 2007).

Acknowledgements

Waste Management, Inc. provided funding for this research. Bryan Staley was partially supported by a Francois Feissinger scholarship from the Environmental Research and Education Foundation.
References


Figure 1. Methane production rate, pH and volatile fatty acid concentrations during refuse decomposition in a laboratory-scale simulated landfill. Approximate phase of decomposition, as defined by Barlaz et al. (1989), is shown at top of graph. Methane rate is based on a total initial dry refuse mass of 885.3 g.
**Figure 2.** Phylogenetic tree of representative *Bacteria* 16S rRNA gene sequences recovered from decomposing MSW using the neighbor-joining method (Jukes Cantor). Bootstrap values shown are based on 500 replicates.
Figure 3. Phylogenetic tree of representative *Archaea* 16S rRNA gene sequences recovered from decomposing MSW using the neighbor-joining method (Jukes-Cantor). Bootstrap values shown are based on 500 replicates. Percentages shown in parentheses are the relative abundance of sequenced clones for each phase of decomposition.
(A) Bacteria

(B) Archaea

Figure 4. Canonical correspondence analysis based on T-RFLP of (A) Bacteria and (B) Archaea.
Figure 5. Relative abundance of representative dominant bacterial T-RFs during refuse decomposition.
Figure 6. Relative abundance of representative dominant archaeal T-RFs during refuse decomposition. T-RFs from Day 0 are from the methanogenic leachate used to seed the reactor. Note scale for 473 bp T-RF is on secondary y-axis. Phylogeny shown is inferred based on in silico digests of 16S clone sequences retrieved on days 34, 68 and 139.
Table 1. Cumulative methane, moisture content, volatile solids, cellulose, hemicellulose and lignin content during refuse decomposition.

<table>
<thead>
<tr>
<th>Days from Start</th>
<th>Cumulative CH$_4$ (L)</th>
<th>Moisture Content$^a$</th>
<th>Volatile Solids$^b$</th>
<th>Cellulose$^b$</th>
<th>Hemicellulose$^b$</th>
<th>Lignin$^b$</th>
<th>(C+ H)/L$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>73.0</td>
<td>83.6</td>
<td>53.3</td>
<td>9.6</td>
<td>14.0</td>
<td>4.5</td>
</tr>
<tr>
<td>34</td>
<td>1.7</td>
<td>76.8</td>
<td>78.1</td>
<td>41.4</td>
<td>9.8</td>
<td>16.0</td>
<td>3.2</td>
</tr>
<tr>
<td>54</td>
<td>8.2</td>
<td>74.3</td>
<td>75.2</td>
<td>31.1</td>
<td>8.7</td>
<td>26.1</td>
<td>1.5</td>
</tr>
<tr>
<td>68</td>
<td>25.1</td>
<td>74.4</td>
<td>74.5</td>
<td>37.9</td>
<td>8.1</td>
<td>20.0</td>
<td>2.3</td>
</tr>
<tr>
<td>82</td>
<td>43.3</td>
<td>72.9</td>
<td>67.4</td>
<td>21.9</td>
<td>5.7</td>
<td>30.4</td>
<td>0.9</td>
</tr>
<tr>
<td>98</td>
<td>51.9</td>
<td>74.2</td>
<td>61.8</td>
<td>22.8</td>
<td>6.9</td>
<td>25.9</td>
<td>1.2</td>
</tr>
<tr>
<td>105</td>
<td>54.8</td>
<td>77.9</td>
<td>62.6</td>
<td>16.5</td>
<td>5.1</td>
<td>25.8</td>
<td>0.8</td>
</tr>
<tr>
<td>126</td>
<td>58.8</td>
<td>75.6</td>
<td>64.4</td>
<td>14.0</td>
<td>4.9</td>
<td>31.7</td>
<td>0.6</td>
</tr>
<tr>
<td>139</td>
<td>59.2</td>
<td>80.2</td>
<td>52.0</td>
<td>17.0</td>
<td>6.3</td>
<td>24.1</td>
<td>1.0</td>
</tr>
<tr>
<td>168</td>
<td>59.3</td>
<td>80.4</td>
<td>53.3</td>
<td>11.9</td>
<td>4.5</td>
<td>27.4</td>
<td>0.6</td>
</tr>
</tbody>
</table>

$^a$ % on a wet basis.

$^b$ % of dry weight.

$^c$ Ratio of cellulose plus hemicellulose divided by lignin.
Table 2. Distribution (% sequences) of Bacteria between major phases of refuse decomposition and associated T-RF length\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Closest Relative Phylum/Genus</th>
<th>Anaerobic Acid (Day 34)</th>
<th>Accelerated CH\textsubscript{4} (Day 68)</th>
<th>Decelerated CH\textsubscript{4} (Day 139)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Firmicutes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus</td>
<td>58</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Brevibacillus</td>
<td>ND</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>Clostridium</td>
<td>42</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Leuconostoc</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Pullulanibacillus</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Sporanaerobacter</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Sporolactobacillus</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Proteobacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcaligenes</td>
<td>3</td>
<td>6</td>
<td>ND</td>
</tr>
<tr>
<td>Enterobacter</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Erwinia</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Hydrogenophaga</td>
<td>ND</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>Proteus</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>ND</td>
<td>33</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Actinobacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucobacter</td>
<td>ND</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>Rhodococcus</td>
<td>ND</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Unclassified</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>100</td>
<td>99\textsuperscript{b}</td>
<td>100</td>
</tr>
</tbody>
</table>

\textsuperscript{a} ND, not detected.

\textsuperscript{b} Does not total 100% due to rounding.
Table 3. Distribution (% sequences) of *Archaea* between major phases of refuse decomposition and associated T-RF length\(^a\).

<table>
<thead>
<tr>
<th>Order/Closest Relative</th>
<th>Anaerobic Acid (Day 34)</th>
<th>Accelerated CH(_4) (Day 68)</th>
<th>Decelerated CH(_4) (Day 139)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Methanosarcinales</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Methanosarcina barkeri</em></td>
<td>36</td>
<td>94</td>
<td>ND</td>
</tr>
<tr>
<td><em>Methanosarcina mazeii</em></td>
<td>1</td>
<td>ND</td>
<td>85</td>
</tr>
<tr>
<td>Other <em>Methanosarcinales</em></td>
<td>3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>Methanobacteriales</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Methanobacterium sp.</em></td>
<td>12</td>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td><em>Methanobacteriaceae clone</em>(^b)</td>
<td>44</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>Methanomicrobiales</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Methanofollis</em></td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td><em>Methanoculleus</em></td>
<td>ND</td>
<td>ND</td>
<td>4</td>
</tr>
<tr>
<td>Unclassified</td>
<td>3</td>
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<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
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\(^a\) ND, not detected.
\(^b\) Accession number, AB236069.
Chapter 5.

IDENTIFICATION OF METABOLICALLY ACTIVE METHANOGENS IN REFUSE AND PEAT DERIVED INOCULA UNDER LOW pH AND HIGH VOLATILE FATTY ACID CONDITIONS

(FORMATTED FOR SUBMISSION TO APPLIED AND ENVIRONMENTAL MICROBIOLOGY)

ABSTRACT

Methanogenesis initiation in decomposing refuse occurs under high volatile fatty acid (VFA) conditions at low pH, typically between 5.5 and 6.25. Although these conditions are generally considered inhibitory, one theory for initiation of refuse methanogenesis is that some methanogenic Archaea are acid tolerant and consume VFAs until methanogens more suited to pH-neutral conditions are established. Three liquid inocula were derived to assess methanogen acid tolerance: (1) refuse just entering active decomposition (low pH, high VFAs), (2) well-decomposed refuse (neutral pH, low VFAs) and, (3) peat. Peat was used since acid tolerant methanogens have been identified in this material. Individual treatments within each inoculum were maintained at four different pH levels. Under high VFA concentrations, results showed methanogenesis initiation occurred at pH minima of 6.25, 5.75 and 5 for actively decomposing refuse, well-decomposed refuse and peat, respectively. However, at these pH levels, extended lag times of ~180 d and ~200 d were required to reach methanogenesis in actively decomposing refuse and peat inocula,
respectively. The metabolically active methanogen community structure was characterized by targeting the 16S RNA using clone libraries and T-RFLP. The hydrogenotrophic *Methanoculleus* genus facilitated methane initiation in actively decomposing refuse (pH 6.25) while *Methanosarcina* triggered methane production in well-decompose refuse (pH 5.75). In peat, methanogenesis was facilitated by an uncultured *Methanosarcinales*. This is the first study to fully characterize methanogens responsible for methane initiation under low pH, high VFA conditions and suggests acid tolerance (pH 5 – 6.25) is relatively common provided sufficient acclimation time. However, methane production rates at lower pH were found to be 3 to 6 fold lower than those at neutral pH.

**INTRODUCTION**

The decomposition of organic matter in various anaerobic ecosystems (e.g. landfills, sediments, peat, rumen) progresses through distinct phases, occurring both temporally and spatially (3, 33). In refuse, these phases have been defined as aerobic, anaerobic acid, accelerated methane and decelerating methane, and are used to describe the conversion of cellulose and hemicellulose, the principal degradable components of refuse, to methane and carbon dioxide (3). In the aerobic phase (A), O$_2$ is consumed rapidly. All of the trophic groups required for refuse methanogenesis are present in fresh refuse (cellulolytics, fatty acid degraders and methanogens). In the anaerobic acid phase (B), volatile fatty acids (VFAs) accumulate and the pH decreases because of an imbalance between fermentative, and acetogenic and methanogenic activity. There is limited cellulose and hemicellulose decomposition in phase B. The methanogen population begins to increase and CH$_4$ is first
detected. In the accelerated CH$_4$ production phase (C), the CH$_4$ production rate increases to some maximum value; carboxylic acid concentrations decrease; the pH increases; there is little solids hydrolysis; and the populations of cellulolytic, VFA-degrading and methanogenic bacteria increase. The accumulated carboxylic acids are the principal substrates supporting CH$_4$ production in phase C. In the decelerated CH$_4$ production phase (phase D), the CH$_4$ production rate decreases, the VFA degrading population increases, VFAs are depleted and there is an increase in the rate of cellulose plus hemicellulose hydrolysis. While acid utilization limits CH$_4$ production in phases B and C, solids hydrolysis limits CH$_4$ production in phase D.

Typically, the pH minimum in phase B ranges between 5.5 and 6.25 (3, 22). It is widely accepted that the transition from phase B to C is facilitated by methanogenic *Archaea*, who are the primary microorganisms that consume carboxylic acids or their metabolites. However, the mechanisms by which this occurs under low pH, high acid conditions are poorly characterized. One difficulty is that most methanogens isolated in pure culture have an optimal pH range between 6.5 and 7.8 (11) and the optimal pH for methane production in refuse has been reported as 6.8 to 7.2 (17). If true for all methanogens, one would expect methanogenesis to be absent or at least severely inhibited when the pH is well below optimal. However, previous research in refuse (2), peat (9) and anaerobic bioreactors (31) has shown methanogenesis initiation can occur when the bulk ecosystem pH is around 5.5, 4 and 6, respectively.

One potential explanation for methanogenesis initiation in landfills is that methanogens that are tolerant to the low pH consume acids until the bulk pH is suitable for the
establishment of methanogenic *Archaea* that grow under pH-neutral conditions. However, very few ecosystems have been evaluated for the presence of acid-tolerant methanogens to substantiate this theory. Northern peat bogs are by far the most studied since they are typically characterized by low pH conditions (13). Previous studies have indicated methane production in peat occurs at a pH <5 (6, 8, 15, 18, 32). This research has led to the identification of a number of hydrogenotrophic acid-tolerant methanogens belonging to either *Methanobacteriaceae* (13, 28, 32), or *Methanomicrobiales* (13, 28). Recently an acidophilic methanogen from the *Methanomicrobiales* order was isolated from a New York, USA peat bog that produced CH$_4$ at a pH of 4.8-5.0, which is lower than any previously described methanogen (7).

The primary difference between peat and decomposing refuse is that carboxylic acids are orders of magnitude higher in refuse. For example, acetate concentrations in leachate from acid phase refuse range between 25 to 376 mM (3), compared to less than 0.005 mM in peat (18). Research on peat indicates acetate concentrations of 1 to 5 mM inhibit methanogenesis at low pH (8).

Previous research on anaerobic digesters suggests methanogens may not be inhibited under low pH conditions when carboxylic acids are high. For example, a mesophilic anaerobic digester treating solid waste had a gas composition that was 60% CH$_4$ at pH 6 and an acetic acid concentration of 84.8 mM (12). In contrast to the work on peat, research on anaerobic digesters has shown that high acetate concentrations (135.7 mM) do not affect *Methanobacteriaceae* whereas *Methanosarcina* and *Methanosaeta* species can be inhibited (20). Another anaerobic digester study isolated a hydrogenotrophic acid-tolerant
methanogen, *Methanobrevibacter acididurans*, that was able to produce CH$_4$ at pH 5; however, optimal production occurred at a pH of 6 (27). Only one study has identified a putative acid tolerant methanogen isolate in decomposing refuse, which exhibited growth at a pH of 5; however, the identity of this isolate was not determined (19).

The objectives of this study were to: (a) identify the presence and growth potential for methanogens under low pH, high carboxylic acid concentrations in refuse, (b) evaluate these findings as a mechanism contributing to the transition from the anaerobic acid phase (low pH, high acids) to the accelerated CH$_4$ phase (neutral pH, high acids) of refuse decomposition, and (c) compare microbial communities from acid and accelerated methane phase refuse to that of peat, an ecosystem known to harbor low pH tolerant methanogens.

**MATERIALS AND METHODS**

**Experimental Design.** Due to varying particle size and waste composition (14), a liquid inoculum was derived from methanogenically active refuse to minimize the effects of spatial heterogeneity. Inocula from two distinct phases of refuse decomposition, the anaerobic acid and decelerating CH$_4$ (well-decomposed) phases, were generated (see below). Since acid tolerant methanogens have been positively identified from peat bogs, an inoculum was also derived from material collected at the Chicago bog (near Ithaca, NY), which has a pH ~4. Methane production and methanogen diversity have been well characterized from this bog (9, 10), which allow it to be used as an external reference for comparing methanogen community structure against those found in the refuse derived inocula.
Microcosms containing the generated inocula were incubated at pHs of 5.5, 5.75, 6.25 and 7 except for the peat, which was adjusted to pHs of 4, 5, 6 and 7. Acetic, propionic and butyric acids were added as necessary to maintain high VFA concentrations for all inocula commensurate with those in decomposing refuse. High VFA values were 11,800 mg L\(^{-1}\) for acetic acid, 1,300 mg L\(^{-1}\) for propionic acid, and 11,280 mg L\(^{-1}\) for butyric acid. Lower VFA concentrations are typically observed during the decelerated CH\(_4\) (well-decomposed) phase of refuse decomposition, when the pH is \(\geq 7\). Low VFA concentrations are also typical for peat. Thus, additional treatments with low VFAs were established for the peat material and pH 7 microcosms containing refuse derived inocula. Low VFA target concentrations were 370 mg L\(^{-1}\), 185 mg L\(^{-1}\), and 185 mg L\(^{-1}\) for acetic, propionic and butyric acids, respectively. The VFA ranges used in this study were estimated from values typically observed during the anaerobic acid and decelerated CH\(_4\) phases of refuse decomposition (3). pH was adjusted routinely to maintain relatively constant levels and VFAs were added as necessary to ensure concentrations were within typical ranges. Additions were made when analytical results, from weekly (at a minimum) microcosm sampling, indicated adjustments were necessary. Duplicate microcosms were used for all treatments and controls with no pH or VFA adjustments were also set up. Microcosms were incubated between 200 to 225 days for refuse and 300 to 250 days for peat.

Samples were retrieved from microcosms for microbiological analysis based on the methane production rate. Sampling was performed when methanogenesis was observed and the production rate was reasonably consistent. The metabolically active methanogen
community was characterized using clone libraries and terminal restriction fragment length polymorphism targeting the 16S RNA.

**Sample collection and refuse decomposition.** Fresh residential refuse was obtained from the Holly Springs (NC) Transfer Station. The refuse was shredded the same day in a slow speed, high torque shredder to a particle size of approximately 2 cm x 6 cm. After shredding, refuse was mixed on a clean plastic sheet using hand tools, and then stored at 4°C. Fresh refuse (1200 - 1500 g) was combined with 1.5 – 2 L methanogenic leachate from a seed reactor and anaerobically decomposed in 10-L reactors. Leachate was re-circulated to the top of the reactor every 2 to 3 days. Gas was collected in tedlar gas bags (PMC Inc; Oak Park, IL) and volume was measured by evacuation into a container of known volume (26). CH$_4$, CO$_2$, O$_2$, and N$_2$ were analyzed using a GOW-MAC 580 gas chromatograph with a thermal conductivity detector (23).

Two sets of duplicate reactors were operated to develop inocula for subsequent microcosm experiments: (a) anaerobic acid phase, and (b) decelerated CH$_4$ phase. Reactors operated to the anaerobic acid phase were monitored until a pH increase and measurable CH$_4$ production were observed, which occurred on day 33 of reactor operation. The second set of reactors was operated to late in the decelerated CH$_4$ phase, when the pH was neutral and the CH$_4$ rate was less than 3% of the maximum rate measured, which occurred on day 102. The reactor conditions at time they were taken down and used to generate inocula are shown in Table 1.

Peat samples were collected from the Chicago bog (near Ithaca, NY) anaerobically using hand tools at a depth of roughly 10 to 20 cm, placed into sterile glass jars and sealed.
This depth range was used for sampling since it is known to contain the highest populations of the acid-tolerant/philic E2 clade within the Methanomicrobiales order (7, 9). Conditions at the time of sampling are shown in Table 1. Samples were maintained at ~15°C and transported immediately to the laboratory for processing.

**Inoculum Generation.** Reactors were transferred to an anaerobic glove box (Coy Laboratory Products, Coy MI) and the entire reactor contents (solids and leachate) were homogenized in a Waring blender (Torrington, CT) for 1 min. The blended mixture was poured into a 3.8 L 75-µm nylon paint strainer bag (Trimaco; Durham, NC) and hand-squeezed. Supernatant was collected in a 1.9 L sterile plastic container and then transferred in 100 mL aliquots to 160 mL glass bottles to generate microcosms in the anaerobic hood. Peat samples were treated similarly. Duplicate microcosms were generated for all treatments. Bottles were sealed with rubber stoppers and crimped with aluminum crimps. All bottles were incubated at 37°C and constantly mixed using a shaker table at 125 rpm.

**Microcosm monitoring and sampling.** Gas production and pH were monitored every 1 to 2 days. Gas volume was measured using a glass volumetric syringe to measure the overpressure. CH$_4$, CO$_2$, O$_2$, and N$_2$ were analyzed by GC using a thermal conductivity detector. pH was monitored by removing 2 mL from the microcosm, placing it in a sterile plastic bottle that was constantly flushed with a 30% CO$_2$/70%N$_2$ gas mixture (to maintain anaerobic conditions and to minimize pH fluctuation) and inserting a glass combination probe (Fisher Scientific; Pittsburgh PA). Adjustments to pH were made to within 0.1 unit
of the target pH using 1 M or 5 M NaOH or HCl. Samples were then returned to the microcosm.

Samples (2 mL) were periodically removed for measurement of VFA concentrations and for microbiological analysis. Analytical results from VFA analyses were used to adjust concentrations to the target values indicated above. VFA concentrations were measured using solid phase microextraction (SPME) followed by GC-MS as described previously (24). Samples for microbiological analysis were frozen at -20°C until they could be processed. Liquid volumes removed from microcosms were replaced using N\textsubscript{2} gas at the same temperature as the microcosm headspace.

**RNA extraction.** Samples removed from microcosms were thawed to 4°C and centrifuged at 14,000 Xg (Eppendorf, Westbury, NY) for 5 min and the remaining pellet was used for RNA extraction. RNA extraction was performed using a low pH-hot phenol-chloroform protocol (29). Briefly, 0.5 g sample pellet was placed in a sterile 2 mL tube and combined with 0.5 g zirconium beads, 50 µL SDS (20%) and the remaining volume was filled with phenol (pH 5.1). The mixture was subjected to bead beating for 2 min, incubated at 60°C for 10 min, and bead beat for another 2 min. After centrifuging at 5,000 rpm for 5 min, the lysate was removed to a clean tube and centrifuged at 10,000 rpm for 10 min to separate the aqueous and organic phases. The aqueous phase was then removed to a clean tube, combined with an equal volume of 4:1 phenol:chloroform and centrifuged (10,000 rpm) for 10 min. This step was repeated two more times, first with 4:1 phenol:chloroform and then with chloroform. RNA was precipitated using a half volume of 10M NH\textsubscript{4}Ac and 2 volumes of absolute ethanol. After incubating at -20°C for at least 8
h, the mixture was centrifuged for 30 min at 14,000 rpm. The resulting pellet was washed in 80% ethanol, allowed to air dry, and re-suspended in 100 µL sterile, RNase-free water. Extracted RNA was quantified via spectrophotometry using a NanoDrop 1000 (Thermo Scientific; Wilmington, DE) with a lower detection limit of 2 ng DNA µL⁻¹ (25).

**Reverse transcription and PCR conditions.** Extracted total RNA was DNased using the Qiagen RNase-free DNase set (Germantown, MD) following the manufacturer’s protocol. Reverse transcription was performed on total RNA using a Bio-Rad iScript cDNA synthesis kit (Hercules, CA) following the manufacturer’s protocol. Polymerase chain reaction (PCR) was performed on cDNA using 25 µL of FailSafe PCR system reaction mix F (Epicentre; Madison, WI), 0.6 µL FailSafe enzyme mix (Epicentre, Madison, WI), 0.25 µM of each primer, ~2 ng cDNA and sterile pure water added to a total volume of 50 µl. The primers used for archaeal T-RFLP and cloning were 109f /915r (21). Forward primers used for T-RFLP were labeled at the 5′ end with 6-carboxy-fluorescein (6-FAM) for T-RFLP analysis. PCR products were run on a 1% agarose gel to verify amplification. PCR was performed in an Eppendorf thermocycler programmed as follows: initial denaturing step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1.5 min, and final extension at 72°C for 10 min. Duplicate PCR reactions were pooled and cDNA was purified using a Wizard SV Gel and PCR Clean-Up kit (Promega, Madison, WI).

**16S RNA Clone Library.** Archaeal clone libraries were constructed from cDNA by inserting PCR products into the pCR 4-TOPO vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol. Colony
PCR was performed by picking transformants using a sterile plastic pipette tip and placing them into a 96-well plate containing a PCR cocktail of 25 µL of FailSafe PCR system reaction mix F (Epicentre; Madison, WI), 0.6 µL FailSafe enzyme mix (Epicentre), 0.25 µM of each primer and sterile pure water added to a total volume of 50 µl. The forward primer used for colony PCR was the T3 primer (5’-ATTAACCCCTCACTAAAGGGA) and was specific to the vector. The reverse primer used was specific to the insert (915r). PCR conditions for colony PCR were: a lysis step at 95°C for 10 min, an initial denaturing step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1.5 min, and final extension at 72°C for 10 min. Plates containing amplified inserts were submitted to the NC State University Genome Sciences Laboratory (Raleigh, NC) for purification and sequencing.

**T-RFLP and Statistical analyses.** Purified PCR products were quantified by spectrophotometry and approximately 360 ng were digested separately with restriction endonuclease AluI (New England Biolabs; Ipswich, MA) for Bacteria and Archaea. Restriction digestion was performed for 4 h using 20 µL reactions containing: 10 U restriction enzyme, 2 µL 10X buffer, 360 ng PCR product, and DI water. Terminal restriction fragments were analyzed by capillary electrophoresis at the NC State University Genome Sciences Laboratory.

T-RFLP profiles were analyzed using PeakScanner software (Applied Biosystems; Foster City, CA) and numerical output from each fragment pattern was exported to Microsoft Excel. Terminal restriction fragment (T-RF) areas were standardized using an automated algorithm to eliminate noise and identify true peaks (1). The following
parameters were used as recommended by the algorithm developers: a minimum peak height of 15 RFU, standard deviation from the baseline of 3 and a bin size of ±1 bp. Phylogeny was inferred by comparing measured T-RF lengths to those from *in silico* digests of clone sequences obtained from this study. Principal component analysis of microbial community structure was performed using Community Analysis Package version 4.0 (Pisces Conservation Ltd, Lymington UK).

**Phylogenetic analysis.** Phylogenetic and molecular evolutionary analyses of 16S rRNA gene sequences were conducted using MEGA version 4 (Tamura et al., 2007). Sequences were aligned using the ClustalW algorithm and phylogenetic trees were constructed using the neighbor-joining method. Bootstrapping was performed to evaluate tree reliability (replications=500). Sequences from related species and similar environmental clones were obtained from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and included in alignments. Sequences from this study were compared to those in the GenBank database using BLAST searches and matches sharing 97% homology were considered identical species. Sequences from this study were submitted to GenBank under accession numbers GQ453591 to GQ453615.

**RESULTS**

**Microcosm pH and volatile fatty acids.** Measured pH and VFA concentrations during microcosm operation are shown in Table 2. The conditions at the time of sampling for microbiological analysis are also provided. Periodic pH adjustment allowed microcosm target pH values to be maintained relatively well. Generally, static conditions
were more difficult to maintain in treatments producing substantial volumes of methane. Thus, for these treatments, larger standard deviations in pH were observed and a wider range of VFA concentrations occurred despite active monitoring and adjustment. The well-decomposed refuse inoculum was substantially more difficult to manage than the acid phase inocula. This may be due to larger initial methanogen populations since the reactor used to derive this inoculum was operated 3 times longer than the acid phase reactor (102 vs. 33 d).

Methane production. No methane production was measured for either refuse inoculum at pH 5 (Fig. 1). Generally, methane production was higher and methanogenesis was initiated sooner as the treatment pH increased in both refuse and peat derived inocula (Figures 1 and 2). For the acid phase refuse inoculum, CH$_4$ production exhibited an exponential increasing trend between days 45 and 50 of microcosm operation at pH 7 while, a similar trend did not occur at pH 6.25 until roughly days 168 and 191 for microcosm replicates A and B, respectively (Figure 1A). While replicate microcosms at other pHs behaved almost identically, there was a 23 day lag between increases methane production for those at pH 6.25 (Figure 1A), despite similar ranges in VFA concentrations and pH (Table 2). While no exponential increase was observed at a pH of 5.75, CH$_4$ production steadily increased after day 90. However, total cumulative methane was substantially lower compared to the pH 6.25 and pH 7 treatments and was attributed to a methane production rate that was 2 and 3 orders of magnitude below that at pH 6.25 and pH 7, respectively (data not shown). Since VFAs were initially high in the starting acid phase inoculum (Table 1), substantial CH$_4$ production was observed in the pH 7, low VFA
treatment until day 88, after which VFA additions began to maintain low concentration target levels from this point forward.

Methane production from the well-decomposed inoculum was observed at pH 5.75 and higher (Figure 1B). For the pH 7 and pH 6.25 treatments, methanogenesis initiation occurred within 5 to 10 days after VFA additions were made to bring concentrations to their target levels. The apparent lag observed after some CH₄ generation for the pH 7, high VFA treatment (days 10 to 25) occurred because of analytical issues which caused delays in VFA additions. For the pH 5.75 treatment, a lag of ~37 days after the first VFA addition was observed until methanogenesis. However, once CH₄ production began, production rates in the pH 5.75 treatment (129 mL CH₄ L⁻¹ d⁻¹, days 37 to 105) were comparable to those observed at pH 6.25 (132 mL CH₄ L⁻¹ d⁻¹, days 12 to 129). For the pH 7 low VFA treatment, CH₄ production was 20 mL CH₄ L⁻¹ d⁻¹, which was 6 – 20 times lower than that measured from the high VFA treatments at a pH of 5.75 or higher.

The peat inocula maintained under low VFA conditions exhibited similar trends in methanogenesis at pHs of 6 and 7 (Figure 2A). A lag time of ~250 days was observed before methane production began at pH 5 while no methane was observed at pH 4 for the duration of microcosm operation (322 days). Under high VFA conditions, methanogenesis did not occur until 100 and 29 days at pHs of 6 and 7, respectively (Figure 2B). No significant methane was generated at pHs of 4 and 5.

In summary, no methane production was observed at pH 5 and there was less time to methane initiation in the well decomposed inoculum relative to the acid phase inoculum. In addition, methane initiation occurred more quickly and production rates were higher as
pH increased in both refuse and peat derived inocula (Figures 1 and 2). For example, the methane production rate at pH 7 was 3 to 6 times higher compared to a pH around 6 to 6.25.

RNA clone libraries. To compare differences in metabolic activity at different pH, clone libraries targeting the 16S RNA were constructed on those treatments producing CH$_4$ at the lowest pH and at neutral pH. Phylogenetic affiliations of retrieved cDNA sequences are shown in Figure 3. Nearly all of the cDNA sequences retrieved fell into the

*Methanomicrobiales, Methanosarcinales, and Methanobacteriales* orders and remaining sequences were related to unclassified sequences that had no taxonomical classification. Most cDNA sequences (>95%) retrieved from peat and acid phase MSW microcosms were from the *Methanomicrobiales* and *Methanobacteriales* orders, except for the acid phase pH 7 treatment which was dominated by *Methanosarcinales* (63.4%) and *Methanomicrobiales* (36.7%) (Table 3). For peat at pH 5 with low VFAs conditions, most clones were highly related to *Methanobacterium bryantii*; whereas, *Methanoculleus palmaeoli* was dominant at high VFA conditions and pH 6 and 7 (Table 3). Sequences from the well-decomposed MSW microcosms were largely of the *Methanosarcinales* order at pH 5.75 (94.5%) and pH 7 (82.5%); however, species differed within this order based on pH. At pH 5.75, approximately 91.7% of retrieved sequences were closely related to *Methanosarcina barkeri* whereas 80% of sequences at pH 7 were related to *Methanosaeta* sp.
**RNA T-RFLP.** Given the large number of samples, RNA-based T-RFLP was performed to correlate shifts in community metabolism between treatments. Phylogeny was inferred by comparing terminal restriction fragment lengths (T-RFs) to *in silico* fragment lengths generated from cDNA sequences from this study. Generally, relative abundances of T-RFs correlated well to clone abundances and phylogeny could be inferred using a single restriction enzyme (AluI) to the order level. Within the *Methanosarcinales* order, it was possible to differentiate *Methanosarcina* (≈475 bp) from *Methanosaeta* (108 bp) using AluI. Relative abundances of cDNA T-RFs show methanogen demographics varied between inocula and, within a particular inoculum, large shifts in community activity were observed based on pH (Table 4).

For acid phase refuse, methanogen activity at pH 5.75 was dominated by the 342 bp T-RF which correlates to the *Methanobacteriales* order. At a pH of 6.25, methanogen community structure differed between replicate microcosms with replicate A being dominated by *Methanobacteriales* (56.8%) and *Methanomicrobiales* (29.4%) while replicate B consisted primarily of *Methanomicrobiales* (65.4%) (Table 4). It should be noted that at the time of sampling for the pH 6.25 treatment (day 182), cumulative CH$_4$ production was 1.6 times higher in replicate A (0.8 L CH$_4$ L$^{-1}$) compared to replicate B (0.5 L CH$_4$ L$^{-1}$). At pH 7, both high and low VFA treatments shifted to *Methanosarcinales* (>62%).

In well-decomposed refuse, the majority of T-RF relative abundance was attributed to the *Methanosarcinales* order with *Methanosarcina* sp. being dominant at pH 5.75 (~67%), pH 6.25 (50%) and in the pH 7, low VFA treatment (46%) (Table 4). However,
the pH 7, high VFA treatment had a 71% relative abundance of the 105 bp T-RF, which correlates to *Methanosaeta* sp.

For peat, large differences in methanogen demographics were evident based on differences in VFA concentrations and pH. Under low VFA concentrations, dominant methanogens at pH 5 and 6 were affiliated with *Methanobacteriales* (78%) and *Methanomicrobiales* (71%), respectively (Table 4). At pH 7 (low VFA), community structure shifted primarily to Methanobacteriales (38%) and the 653 bp T-RF, which had no identified phylogeny based on the clone libraries performed in this study. Methane production from high VFA peat treatments was only significant at pH 6 and 7, and these treatments were comprised largely of members of the *Methanomicrobiales* order (>85%) (Table 4). At pH 4 and 5 under high VFA conditions, methanogen community structure was primarily *Methanosarcinales* with abundances of 86% and 56%, respectively.

**DISCUSSION**

The microcosms in this study were able to generate methane at pH minima of 6.25, 5.75 and 5 for inocula derived from acid phase refuse, well-decomposed refuse and peat, respectively. As pH decreased, increasing lag times were observed until methanogenesis initiation (Figures 1 and 2). Lower pH treatments had methane production rates 4 to 5 times lower than neutral pH treatments once methanogenesis began and, under high VFA conditions, lag time to methane initiation was substantially affected by pH. Despite lower rates compared to neutral pH treatments, methane production was steady and VFA consumption consistent after methanogenesis began (Figures 1 and 2). This suggests
methanogens tolerant of low pH, high VFA conditions are a plausible mechanism for methanogenesis initiation in refuse.

Differences in methanogen community structure between peat and refuse inocula suggest that acid tolerance in these ecosystems is borne by different methanogen species. At pH 5.75, methanogenesis was carried out primarily by *Methanosarcina barkeri* in well decomposed refuse. In acid phase refuse, significant CH$_4$ production was not observed until pH 6.25 and initiation was mediated by *Methanoculleus palmaeoli*. This suggests that there is functional redundancy in methanogen acid tolerance. However, the time at which methane production began at pH 6.25 was substantially different, being ~180 days for the acid phase inoculum and only ~15 d for well decomposed inoculum at pH 5.75. Similarly, lag to methanogenesis in peat at pH 6 was ~210 days (Figure 2). Previous work on decomposing refuse also found *M. barkeri* was dominant during the acid phase of decomposition (30). This suggests *M. barkeri* exhibits resiliency to low pH, high VFA conditions and is able initiate CH$_4$ production under inhibitory conditions compared to other methanogens identified in this study. This is comparable to pure culture work on M. barkeri which suggests an optimal pH range from 5 to 7 (11).

In all inocula, differences in the active methanogen community structure were observed at different pH values. As other variables were constrained, this shows pH is a primary selection mechanism for individual methanogen species in decomposing refuse under high VFA conditions. In the acid phase refuse inoculum, strictly hydrogenotrophic methanogens dominated when pH was less than neutral in treatments producing CH$_4$. At neutral pH, roughly two-thirds of the relative abundance was *Methanosarcina* sp. with
one-third comprised of hydrogenotrophic methanogens (Tables 3 and 4). Since routine additions of acetate, propionate and butyrate were required to maintain consistent VFA levels, this shows hydrogen production via syntrophic acetate oxidation was a primary mechanism driving methane metabolism when pH was less than neutral. No Methanosaeta were detected in any acid phase treatments which agrees with previous work indicating syntrophic acetate oxidation is a dominant metabolic pathway in the absence of Methanosaeta (16). Since Methanosarcina utilize both acetoclastic and hydrogenotrophic pathways, the relative importance of syntrophic acetate oxidation at neutral pH in the acid phase inoculum was not ascertained.

In the well-decomposed refuse inoculum, members of the Methanosarcinales order were dominant in all treatments producing methane with Methanosaeta sp. being the dominant species in the pH 7, high VFA treatment (Tables 3 and 4). This observation is in contrast to the acid phase refuse inocula, which was comprised of strictly hydrogenotrophic methanogens at lower pH. RNA T-RFLP results showed starting communities in both refuse derived inocula were dominated by Methanosarcinales (Table 4), which indicates dissimilar methanogen community composition in the initial samples is likely not a primary reason for the difference. Previous work has suggested chaotic instability resulting from intrinsic properties within the community contributes to population shifts (4). Subsequent work has attributed the cause of these shifts to interactions between different species that comprise the microbial food web (5) and, given the highly interdependent nature of the this food web under anaerobic conditions, suggests this phenomenon may contribute to the differences observed here.
High and low VFA levels were evaluated in peat since, at low pH, low VFA levels are typical of peat bogs and high VFA are typical in decomposing refuse. Such an evaluation provides a point of external comparison of methanogen communities to those observed in refuse. Generally, the peat inocula under high VFA levels experienced increasing lag times to methanogenesis as pH decreased, similar to the refuse derived inocula. Community structure in the peat starting material was substantially different than that of the starting refuse inocula. T-RFs from the initial peat sample were unaffiliated with any clone sequences recovered in this study while T-RFs from the initial refuse samples were related to *Methanosarcinales*. Low VFA treatments were comprised primarily of hydrogenotrophic methanogens (Table 4) which agree with previous field studies of peat microbial ecology (7, 9). In contrast, high VFA level treatments consisted of >50% *Methanosarcinales* in pH 4 and 5 treatments and were >85% *Methanomicrobiales* in pH 6 and 7 treatments (Table 4). However, the uncultured *Methanosarcinales* recovered from peat (accession no. DQ301879) was not identified in any of the refuse treatments. The original sequence was retrieved from the same peat bog in a prior study (9), suggesting this species may be unique to peat bogs. The identification of *Methanosarcinales*, an order that can utilize acetate and/or hydrogen as substrates, as facilitating methane initiation in peat at pH 5 (and high VFAs) is significant since the only strict hydrogenotrophic methanogens have been attributed as acidophilic in peat bogs thus far (7, 9).

In this study, acid tolerance under high VFA conditions was exhibited by some members of all methanogen orders found (*Methanomicrobiales, Methanobacteriales* and
Methanosarcinales), although extended lag times may be necessary for acclimation and methanogenesis initiation. Previous work evaluating methanogen population dynamics in anaerobic digesters found Methanosarcina and Methanobacteriaceae were dominant during reactor start-up (12), which agrees with this study except that Methanomicrobiales were also present in some treatments. However, most acid tolerant or acidophilic methanogens found in peat are of the Methanomicrobiales order (7). Coupled with this previous work, our study indicates mild to intermediate (pH 5.75 - 6.25) acid tolerance is rather common among methanogens. However, it should be recognized that methanogenesis at lower pH is commensurate with a 3 to 6 fold reduction in production rates.

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Figure 1. Cumulative methane production from the (A) acid phase and (B) well-decomposed phase refuse microcosms. Replicate microcosms for the acid phase pH 6.25 microcosms initiated CH$_4$ production at different times and are shown separately. CH$_4$ production was similar in the other treatments and the average is presented. The ‘high’ and ‘low’ designations for the pH 7 microcosms indicate high and low VFA levels, respectively. VFA concentrations are given in Table 2. Note the y-axis scale differs between (A) and (B).
Figure 2. Cumulative methane production from peat microcosms with (A) low and (B) high VFA levels.
Figure 3. Phylogenetic tree of cDNA *Archaea* sequences retrieved from acid phase (A), well-decomposed (WD) and peat (P) inocula at varying pH. Values in parentheses indicate number of clones out of total retrieved per inoculum.
Table 1. Initial conditions of material used to generate inocula for microcosm experiments\textsuperscript{a}.

<table>
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<th>Acid Reactor</th>
<th>Well-decomposed Reactor</th>
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<td>Propionic acid, mg dry g\textsuperscript{-1}(mg L\textsuperscript{-1})</td>
<td>3.0 (1,945)</td>
<td>&lt;0.01 (&lt; 2)</td>
<td>0.3 (2.4)</td>
</tr>
<tr>
<td>Butyric acid, mg dry g\textsuperscript{-1}(mg L\textsuperscript{-1})</td>
<td>15.5 (10,014)</td>
<td>&lt;0.01 (&lt; 2)</td>
<td>0.3 (2.6)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Initial conditions refer to reactor take-down for refuse derived inocula and time of field sampling for peat. Abbreviation: N/A, not applicable/analyzed. VFA concentrations in the leachate (refuse) or porewater (peat). Methane production was normalized to STP.
### Table 2. pH and VFA concentrations during microcosm operation and sampling time for microbiological analysis.a.

<table>
<thead>
<tr>
<th>Treatment/Target pH</th>
<th>Measured pH</th>
<th>Acetic Acid&lt;sup&gt;c&lt;/sup&gt;, mg L&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Propionic Acid&lt;sup&gt;d&lt;/sup&gt;, mg L&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Butyric Acid&lt;sup&gt;e&lt;/sup&gt;, mg L&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Sampling for Microbiological Characterization (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acid Refuse Inoculum&lt;sup&gt;b&lt;/sup&gt;</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 5</td>
<td>5.03 ± 0.05</td>
<td>7,942 – 17,351 (11,399)</td>
<td>1,042 – 2,023 (1,688)</td>
<td>5,857 – 10,189 (6,844)</td>
<td>182</td>
</tr>
<tr>
<td>pH 5.75</td>
<td>5.77 ± 0.04</td>
<td>7,881 – 17,351 (8,952, 10,919)</td>
<td>1,193 – 2,351 (1,309, 1,607)</td>
<td>5,844 – 10,782 (7,391, 7,375)</td>
<td>154, 182</td>
</tr>
<tr>
<td>pH 6.25 A</td>
<td>6.24 ± 0.18</td>
<td>7,807 – 17,570 (9,034)</td>
<td>1,237 – 2,276 (1,780)</td>
<td>7,221 – 12,106 (7,476)</td>
<td>182</td>
</tr>
<tr>
<td>pH 6.25 B</td>
<td>6.21 ± 0.06</td>
<td>8,471 – 18,380 (12,573)</td>
<td>1,211 – 2,352 (2,012)</td>
<td>6,737 – 12,147 (8,364)</td>
<td>182</td>
</tr>
<tr>
<td>pH 7</td>
<td>7.06 ± 0.23</td>
<td>596 – 28,626 (18,222)</td>
<td>1,078 – 2,845 (1,565)</td>
<td>252 – 15,786 (11,342)</td>
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</tr>
<tr>
<td>pH 7 (low VFA)</td>
<td>7.07 ± 0.20</td>
<td>BQL – 431 (115)</td>
<td>BQL – 168 (BQL)</td>
<td>BQL – 235 (14)</td>
<td>116</td>
</tr>
<tr>
<td><strong>Well-decomposed Refuse Inoculum&lt;sup&gt;b&lt;/sup&gt;</strong></td>
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<tr>
<td>pH 5</td>
<td>5.12 ± 0.22</td>
<td>6,371 – 11,305 (10,837)</td>
<td>807 – 1,208 (1,208)</td>
<td>6,875 – 10,879 (8,129)</td>
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<tr>
<td>pH 5.75</td>
<td>5.85 ± 0.29</td>
<td>3,711 – 21,498 (8,932)</td>
<td>877 – 1,242 (990)</td>
<td>5,794 – 10,020 (8,830)</td>
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<tr>
<td>pH 6.25</td>
<td>6.36 ± 0.21</td>
<td>39 – 16,738 (4,329)</td>
<td>280 – 1,534 (1,364)</td>
<td>6,647 – 18,109 (11,117)</td>
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</tr>
<tr>
<td>pH 7</td>
<td>7.24 ± 0.17</td>
<td>BQL – 15,974 (1,157)</td>
<td>BQL – 2,007 (1,202)</td>
<td>BQL – 9,013 (7,844)</td>
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</tr>
<tr>
<td>pH 7 (low VFA)</td>
<td>7.12 ± 0.09</td>
<td>BQL – 71 (40)</td>
<td>BQL – 12 (BQL)</td>
<td>BQL – 142 (41)</td>
<td>78</td>
</tr>
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Table 2. Measured conditions during microcosm operation and sampling time for microbiological analysis (CONTINUED).

<table>
<thead>
<tr>
<th>Treatment/Target pH</th>
<th>Measured pH</th>
<th>Acetic Acid&lt;sup&gt;c&lt;/sup&gt;, mg L&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Propionic Acid&lt;sup&gt;d&lt;/sup&gt;, mg L&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Butyric Acid&lt;sup&gt;e&lt;/sup&gt;, mg L&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Day Sampled for Microbiology</th>
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<tr>
<td>Peat Inoculum</td>
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<tr>
<td>pH 4</td>
<td>4.09 ± 0.13</td>
<td>108 – 598 (252)</td>
<td>BQL – 32 (BQL)</td>
<td>36 – 167 (49)</td>
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</tr>
<tr>
<td>pH 5</td>
<td>5.06 ± 0.21</td>
<td>132 – 274 (165)</td>
<td>BQL – 31 (25)</td>
<td>24 – 63 (29)</td>
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</tr>
<tr>
<td>pH 6</td>
<td>5.98 ± 0.25</td>
<td>BQL – 304 (BQL)</td>
<td>BQL – 808 (BQL)</td>
<td>BQL – 46 (BQL)</td>
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</tr>
<tr>
<td>pH 7</td>
<td>6.93 ± 0.24</td>
<td>BQL – 186 (BQL)</td>
<td>BQL – 42 (42)</td>
<td>BQL – 24 (BQL)</td>
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<tr>
<td>High VFA</td>
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<td></td>
</tr>
<tr>
<td>pH 4</td>
<td>4.16 ± 0.11</td>
<td>6,306 – 11,263 (9,021)</td>
<td>630 – 807 (640)</td>
<td>4,660 – 7,607 (7,562)</td>
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<tr>
<td>pH 5</td>
<td>5.07 ± 0.10</td>
<td>7,602 – 10,839 (9,213)</td>
<td>673 – 859 (742)</td>
<td>4,953 – 7,993 (7,993)</td>
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</tr>
<tr>
<td>pH 6</td>
<td>6.21 ± 0.28</td>
<td>7,301 – 10,441 (9,029)</td>
<td>720 – 918 (899)</td>
<td>4,092 – 8,918 (8,697)</td>
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<tr>
<td>pH 7</td>
<td>7.14 ± 0.29</td>
<td>6,217 – 18,492 (14,744)</td>
<td>161 – 902 (783)</td>
<td>1,120 – 6,173 (3,580)</td>
<td>147</td>
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</tbody>
</table>

<sup>a</sup> VFA value in parentheses is concentration on the day of sampling for microbiological characterization. Two values are given when samples for microbiological characterization were collected at two different times.

<sup>b</sup> Treatments have high VFA levels unless noted otherwise.

<sup>c</sup> Target acetic acid range for high and low VFA treatments are 1,500 to 22,200 mg L<sup>-1</sup> and 0 to 740 mg L<sup>-1</sup>, respectively.

<sup>d</sup> Target propionic acid range for high and low VFA treatments are 0 to 2,600 mg L<sup>-1</sup> and 0 to 370 mg L<sup>-1</sup>, respectively.

<sup>e</sup> Target butyric acid range for high and low VFA treatments are 4,800 to 17,800 mg L<sup>-1</sup> and 0 to 370 mg L<sup>-1</sup>, respectively.
Table 3. Distribution (% sequences) of *Archaea* cDNA from selected peat and refuse derived inocula samples. Associated T-RF lengths from recovered sequences are also indicated.

<table>
<thead>
<tr>
<th>pH</th>
<th>Peat</th>
<th>Acid Phase MSW</th>
<th>Well-decomposed MSW</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>5.75</td>
<td>6.25</td>
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<tr>
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<td>5.75</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VFA level</td>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Days</td>
<td>109</td>
<td>147</td>
<td>147</td>
</tr>
<tr>
<td></td>
<td>154</td>
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<td></td>
<td>78</td>
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</table>

*In Silico* T-RF Length (bp)

<table>
<thead>
<tr>
<th>Order/Species</th>
<th>Peat</th>
<th>Acid Phase MSW</th>
<th>Well-decomposed MSW</th>
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</thead>
<tbody>
<tr>
<td><strong>Methanomicrobiales</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Methanoculleus palmaeoli</em> (Y16382)</td>
<td>17.9</td>
<td>95.7</td>
<td>95.0</td>
</tr>
<tr>
<td><em>Methanoculleus bourgensis</em> (AB065298)</td>
<td>78.9</td>
<td>36.7</td>
<td>436</td>
</tr>
<tr>
<td><em>Methanofollis</em> sp. (&gt;95%)</td>
<td>5.3</td>
<td>2.8</td>
<td>434</td>
</tr>
<tr>
<td><strong>Methanobacteriales</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Methanobacterium bryantii</em> (M59124)</td>
<td>82.1</td>
<td>343</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>95.8</td>
<td>10.5</td>
<td>343</td>
</tr>
<tr>
<td><strong>Methanosarcinales</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Methanosarcina barkeri</em> (M59144)</td>
<td>91.7</td>
<td>475</td>
<td></td>
</tr>
<tr>
<td><em>Methanosarcina mazeii</em> (U20151)</td>
<td>5.0</td>
<td>474</td>
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</tr>
<tr>
<td><em>Methanoaeta</em> sp.</td>
<td>80.0</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td><em>Methanoaeta</em>-like (93%)</td>
<td>2.5</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td><em>Methanosarcinales</em> clone (AB353212)</td>
<td>46.7</td>
<td>2.8</td>
<td>475</td>
</tr>
<tr>
<td>UC <em>Methanosarcinales</em> (DQ301879)</td>
<td>4.3</td>
<td>634</td>
<td></td>
</tr>
<tr>
<td>Unknown <em>Methanosarcinales</em> (&gt;94%)</td>
<td>4.2</td>
<td>16.7</td>
<td>469</td>
</tr>
<tr>
<td>Unknown</td>
<td>4.2</td>
<td>16.7</td>
<td>469</td>
</tr>
</tbody>
</table>
Table 3. Distribution (% sequences) of *Archaea* cDNA from selected peat and refuse derived inocula samples (CONTINUED).

<table>
<thead>
<tr>
<th>pH</th>
<th>Peat</th>
<th>Acid Phase MSW</th>
<th>Well-decomposed MSW</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>6</td>
<td>7</td>
<td>5.75</td>
</tr>
<tr>
<td>6.25</td>
<td>7</td>
<td>5.75</td>
<td>7</td>
</tr>
<tr>
<td>7</td>
<td>5.75</td>
<td>6.25</td>
<td>7</td>
</tr>
<tr>
<td>VFA level</td>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Days(^a)</td>
<td>109</td>
<td>147</td>
<td>147</td>
</tr>
<tr>
<td>147</td>
<td>154</td>
<td>182</td>
<td>116</td>
</tr>
<tr>
<td>154</td>
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<td>116</td>
<td>78</td>
</tr>
<tr>
<td>116</td>
<td>78</td>
<td>78</td>
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</tr>
</tbody>
</table>

**Order/Species**\(^b\)

<table>
<thead>
<tr>
<th>Unclassified</th>
<th>T-RF Length (bp)</th>
<th>In Silico</th>
</tr>
</thead>
<tbody>
<tr>
<td>UC euryarchaeote (EF552187)</td>
<td>2.5</td>
<td>437</td>
</tr>
<tr>
<td>Solid waste clone OTU-A6 (AB428529)</td>
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<td>470</td>
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<tr>
<td>Unknown methanogen Group 1</td>
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<td>434</td>
</tr>
<tr>
<td>Unknown methanogen Group 2</td>
<td>2.5</td>
<td>474</td>
</tr>
</tbody>
</table>

**TOTAL** | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

\(^a\) Days from first VFA addition for well-decomposed phase refuse and days from microcosm initiation for acid phase refuse and peat.

\(^b\) Closest relative with species phylogenetic affiliation based on >97% homology, unless indicated otherwise. Accession numbers or % homology shown in parentheses. Abbreviation: UC, uncultured.
Table 4. Relative abundance of dominant cDNA T-RF lengths measured in inocula samples\textsuperscript{a}.

<table>
<thead>
<tr>
<th>T-RF (bp) Phylogeny\textsuperscript{b}</th>
<th>88</th>
<th>105</th>
<th>141</th>
<th>342</th>
<th>431</th>
<th>470</th>
<th>540</th>
<th>643</th>
<th>653</th>
<th>Total</th>
</tr>
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<tbody>
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</tr>
<tr>
<td>Initial Sample</td>
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<td></td>
<td></td>
<td>93.7</td>
</tr>
<tr>
<td>pH 5</td>
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<td></td>
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<td>24.4</td>
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<td>49.3</td>
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<tr>
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Table 4. Relative abundance of dominant cDNA T-RF lengths measured in inocula samples (CONTINUED).

<table>
<thead>
<tr>
<th>T-RF (bp)</th>
<th>Phylogeny</th>
<th>88</th>
<th>105</th>
<th>141</th>
<th>342</th>
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<th>470</th>
<th>540</th>
<th>643</th>
<th>653</th>
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<td>12.3</td>
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a Dominant T-RFs are those having a relative abundance of >15% in at least one sample. Digested with AluI restriction enzyme.  
c Letters after pH indicate microcosm replicate. No letters indicate results from duplicate microcosms were combined. VFA concentrations are high unless noted otherwise in parentheses.
Chapter 6.

THE EFFECT OF SPATIAL DIFFERENCES IN MICROBIAL ACTIVITY, PH, AND SUBSTRATE LEVELS ON METHANOGENESIS INITIATION IN REFUSE

(FORMATTED FOR SUBMISSION TO APPLIED AND ENVIRONMENTAL MICROBIOLOGY)

ABSTRACT

Methanogenesis initiation in refuse occurs under high volatile fatty acid (VFA), low pH (5.5 to 6.25) conditions, which are generally reported to inhibit methanogenic Archaea. One theory for the initiation of methane production in refuse decomposition is the presence of pH neutral niches within the refuse that act as initiation centers for methanogenesis. Fresh refuse was placed into triplicate laboratory scale reactors, decomposed to the anaerobic acid phase, and destructively sampled when methanogenesis initiation was observed. Bacterial and archaeal activity were evaluated using RNA clone libraries, RNA T-RFLP and RT-qPCR. Eighty-one core samples were taken from vertical and horizontal sections of each reactor. Large differences were observed spatially in refuse pH, moisture content and VFA concentration. No pH neutral niches were observed in reactors prior to methanogenesis. RNA clone library results showed most bacterial activity was attributed to the Clostridiales order. Methanogenic Archaea activity at low pH was catalyzed by Methanosarcina barkeri. After methanogenesis began, pH neutral conditions developed in high moisture content areas containing substantial populations of M. barkeri. These areas
expanded with increasing methane production, forming a unified reaction front that advanced into low pH areas. Measured leachate pH was neutral despite low pH conditions in >50% of the samples, indicating leachate pH is not an accurate indicator of landfill microbial health. In the absence of pH neutral niches, this study suggests methanogens tolerant to low pH, such as *M. barkeri*, are required to overcome the low pH, high VFA conditions typically present during the anaerobic acid phase of refuse decomposition.

**INTRODUCTION**

Landfills are an important anaerobic microbial ecosystem, primarily due to their methane generation potential and carbon sequestration capacity (3, 13). Initially, there is an imbalance between hydrolytic/fermentative activity which results in volatile fatty acid (VFA) production, and VFA consumption by the acetogenic and methanogenic population. Therefore, upon landfilling, rapidly degradable materials within the refuse decompose, resulting in a VFA accumulation and a commensurate drop in pH to a minimum ranging between 5.5 and 6 (4, 17). These low pH, high VFA conditions have been shown to be inhibitory to methanogenic *Archaea* in analogous ecosystems such as peat (5) and the rumen (21, 29). In contrast to these reports, methanogenesis initiation occurs under these conditions in refuse, indicating that the mechanism by which methane production begins in refuse is poorly understood.

One hypothesis is that spatially isolated areas of neutral pH exist while bulk pH is acidic, and these localized regions of neutral pH act as initiation centers for methanogenesis (14). Modeling studies have suggested that as refuse decomposition
proceeds, methanogen populations may become more abundant in these discrete areas (14, 30); however, these studies have not been validated experimentally. In soils, differential methanogen populations have been attributed to localized areas of high substrate variability at the 1 cm scale (31). This shows that unsaturated conditions can induce spatial differences in CH$_4$ production that do not necessarily follow a diffusive/convective gradient as typically observed in peat (9) or sediments (7, 12). Wachinger (2000) suggested that spatial variations are due to both the presence of fresh organic material and the degree to which the material is accessible by microorganisms.

This concept of spatial variation in substrate parallels the concept of neutral pH microhabitats in refuse since limited access or exposure to high VFA levels would be necessary to maintain localized pH conditions suitable for methanogen activity during the initial stages of refuse decomposition where pH is low and VFA concentrations are high. The concept of accessibility is dependent on particle size, matrix structure and material composition. Previous studies on soil found that substrate consumption was correlated to niches of specific microbial populations, the distance between these niches and the size of the niche (6, 11, 18, 19). It was also shown that environmental conditions within the niche were different than in the bulk soil. As a result, these spatially isolated areas enriched for specific populations and it was noted that “soil structure is a dominant factor controlling microbial diversity and processes” (18).

Previous research in soils suggests that: (a) the presence of microbial niches in an unstructured, heterogeneous material such as refuse are likely, and (b) niche formation due to particle size could be an important factor affecting degradative processes in solid waste.
Thus, refuse density and composition after placement in a landfill may be dominant factors in the formation of niches that affect methanogenic activity and microbial community diversity across spatial and temporal transects. The objectives of this study were to determine: (1) if localized areas of neutral pH exist in decomposing refuse and estimate their relative size and abundance, (2) how spatial effects relate to methanogenesis initiation by measuring spatial distributions of microbial population and metabolic activity.

**MATERIALS AND METHODS**

**Experimental design.** To evaluate spatial heterogeneity in decomposing refuse, triplicate reactors were initiated with fresh refuse and methanogenic leachate as an inoculum. Reactors were destructively sampled when there was evidence of methanogenesis initiation including an increase in both the pH of the leachate and methane composition of the biogas. Reactor contents were immediately frozen at -20°C after sampling by carefully placing reactors undisturbed into a freezer. To enhance the rate of decomposition, leachate collected at the bottom of a reactor was recirculated to the top of the reactor every 2 to 3 days. Reactor conditions at the lowest measured leachate pH and at take-down (before freezing and sampling) are shown in Table 1.

Reactors were destructively sampled to assess spatial differences by cutting the frozen reactor into approximately 2 cm thick slices using an industrial grade meat saw (Torrey; Springville, CA), which was sterilized between cuts. The uncut reactor portion was stored at -20°C between cuts to ensure that the refuse contents remained frozen. Reactor slices were also frozen at -20°C after cutting until further sampling.
To obtain core samples, each frozen slice was placed on a sterile Styrofoam surface. Nine samples were removed from each 2 cm thick slice using a 3.2 cm diameter hole saw, which was sterilized between corings. Samples were removed in a radial pattern with a single core in the center, placed into sterile plastic tubes, and used for chemical and microbiological analyses. The exception to this was moisture content, which was measured by quadrant in each slice. Clone libraries were constructed from selected core samples using extracted RNA. Both RNA and DNA were used as target molecules for T-RFLP to represent metabolic activity and taxa presence in the sample, respectively. Quantitative polymerase chain reaction (qPCR) was used to measure total RNA and DNA levels of *Bacteria* and *Archaea* in selected core samples.

**Sample collection and reactor monitoring.** Undecomposed refuse was obtained from a residential area waste collection vehicle at the Holly Springs (NC) Transfer Station. The refuse was shredded the same day in a slow speed, high torque shredder to a particle size of approximately 2 cm x 6 cm. After shredding, refuse was mixed on a clean plastic sheet using hand tools and then stored at 4°C. Fresh refuse (~1,500 g) was combined with methanogenic leachate from a seed reactor and anaerobically decomposed in 10-L reactors. Gas was collected in tedlar gas bags (PMC, Inc; Oak Park, IL) and gas volume was measured by evacuation into a container of known volume (24). CH$_4$, CO$_2$, O$_2$, and N$_2$ were analyzed using a GOW-MAC 580 gas chromatograph with a thermal conductivity detector (20). pH during reactor operation was measured on the leachate immediately after recirculating.
Chemical analyses of core samples. Core samples were analyzed for pH by suspending the solid refuse material (5-10 g) in 10 mL sterile, deionized water and mixing. A 2 mL liquid sample was analyzed for VFAs using solid phase microextraction (SPME) followed by GC-MS as described previously (22).

DNA extraction. DNA was extracted using the Qiagen QIAamp DNA Stool Mini kit and QIAamp DNA Blood Midi kit following a user developed protocol (QA28_Feb02) with some modifications. To adapt the method to refuse, each core sample was combined with 10 mL DI water (as noted above) and homogenized in a Waring blender (Torrington, CT) for 1 min. The mixture was centrifuged at 3,220 X g for 5 min and the supernatant was decanted. Roughly 0.3 g of the resulting pellet was placed into a 2 mL centrifuge tube and re-suspended using 500 mL sterile deionized water. Zirconium beads (0.5 g) were added to the tube and the mixture was bead beat for 1 min at maximum speed. The user-developed protocol was followed exactly for the remainder of the DNA extraction procedure.

RNA extraction. RNA extraction was performed using a low pH, hot phenol-chloroform protocol (25). Briefly, 0.5 g sample material was homogenized as described above, placed in a sterile 2 mL tube and combined with 0.5 g zirconium beads, 500 µL buffer, 50 µL SDS (20%) and the remaining volume was filled with phenol (pH 5.1). The mixture was subjected to bead beating for 2 min, incubated at 60°C for 10 min, and bead beat for another 2 min. After centrifuging at 5,000 rpm for 5 min, the lysate was removed to a clean tube and centrifuged at 10,000 rpm for 10 min so separate the aqueous and organic phases. The aqueous phase was then removed to a clean tube, combined with an
equal volume of 4:1 phenol:chloroform and centrifuged (10,000 rpm) for 10 min. The aqueous phase was removed to a clean tube and previous step was repeated two more times, first using 4:1 phenol:chloroform and then by chloroform only. RNA was precipitated using a half volume of 10M NH₄Ac and 2 volumes of absolute ethanol. After incubating at -20°C for at least 8 h, the mixture was centrifuged for 30 min at 14,000 rpm. The resulting pellet was washed in 80% ethanol, allowed to air dry, and re-suspended in 100 µL sterile, RNase-free water. Extracted RNA was quantified via spectrophotometry using a NanoDrop 1000 (Thermo Scientific; Wilmington, DE) with a lower detection limit of 2 ng DNA µL⁻¹(23).

**Reverse transcription and PCR conditions.** Extracted total RNA was DNased using the Qiagen RNase-free DNase set (Germantown, MD) following the manufacturers protocol. Reverse transcription was performed on total RNA using a Bio-Rad iScript cDNA synthesis kit (Hercules, CA) following the manufacturers’ protocol. Polymerase chain reaction (PCR) was performed on cDNA using 25 µL of FailSafe PCR system reaction mix F (Epicentre; Madison, WI), 0.6 µL FailSafe enzyme mix (Epicentre), 0.25 µM of each primer, ~2 ng cDNA and sterile pure water added to a total volume of 50 µl. The primers used for archaeal T-RFLP and cloning were 109f/915r (16). Forward primers used for T-RFLP were labeled at the 5’ end with 6-carboxy-fluorescein (6-FAM) for T-RFLP analysis. PCR products were run on a 1% agarose gel to verify amplification. PCR was performed in an Eppendorf thermocycler programmed as follows: initial denaturing step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1.5 min, and final extension at 72°C for 10 min.
Duplicate PCR reactions were pooled and cDNA was purified using a Wizard SV Gel and PCR Clean-Up kit (Promega, Madison, WI).

**16S RNA Clone Library.** Archaeal clone libraries were constructed from cDNA by inserting PCR products into the pCR 4-TOPO vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA), in accordance with the manufacturers protocol. Colony PCR was performed by picking transformants using a sterile plastic pipette tip and placing them into a 96-well plate containing a PCR cocktail of 25 µL of FailSafe PCR system reaction mix F (Epicentre; Madison, WI), 0.6 µL FailSafe enzyme mix (Epicentre), 0.25 µM of each primer and sterile pure water added to a total volume of 50 µl. The forward primer used for colony PCR was the T3 primer (5’-ATTAAACCCTCCTAAAGGGA) and was specific to the vector. The reverse primer used was specific to the insert (915r). PCR conditions for colony PCR were: a lysis step at 95°C for 10 min, an initial denaturing step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1.5 min, and final extension at 72°C for 10 min. Plates containing amplified inserts were submitted to the NC State University Genome Sciences Laboratory (Raleigh, NC) for purification and sequencing.

**T-RFLP and Statistical analyses.** Purified PCR products were quantified by spectrophotometry and approximately 360 ng were digested separately with restriction endonuclease AluI (New England Biolabs; Ipswich, MA) for Bacteria and Archaea. Restriction digestion was performed for 4 h using 20 µL reactions containing: 10 U restriction enzyme, 2 µL 10X buffer, 360 ng PCR product, and DI water. Terminal
restriction fragments were analyzed by capillary electrophoresis at the NC State University Genome Sciences Laboratory.

T-RFLP profiles were analyzed using PeakScanner software (Applied Biosystems) and numerical output from each fragment pattern was exported to Microsoft Excel. Terminal restriction fragment (T-RF) areas were standardized using an automated algorithm to eliminate noise and identify true peaks (1). The following parameters were used as recommended by the algorithm developers: a minimum peak height of 15 RFU, standard deviation from the baseline of 3 and a bin size of ±1 bp. Canonical correspondence analysis to correlate environmental parameters with microbial community structure was performed using ECOM software version 4.0 (Pisces Conservation Ltd, Lymington UK).

**Phylogenetic analysis.** Phylogenetic and molecular evolutionary analyses of 16S rRNA gene sequences were conducted using MEGA version 4 (28). Sequences were aligned using the ClustalW algorithm and phylogenetic trees were constructed using the neighbor-joining method (Jukes-Cantor algorithm). Bootstrapping was performed to evaluate tree reliability (replications=500). Sequences from related species and similar environmental clones were obtained from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and included in alignments. Sequences from this study were compared to those in the GenBank database using BLAST searches and matches sharing 97% homology were considered identical species. Sequences from this study were submitted to GenBank under accession numbers GQ453616 to GQ453667.
RESULTS

Moisture Content. Spatial differences in moisture content were assessed via measurements from 36 samples per reactor, with four samples analyzed from each ~2 cm thick slice (one sample per quadrant). Results showed refuse moisture content profiles were comparable between reactors (Fig. 1). Generally, moisture content was lower near the top of the reactor and increased with depth with an average minimum and maximum of 49% and 88%, respectively. At a particular depth within a reactor, the difference between maximum and minimum moisture content averaged 12%. The higher moisture content at the bottom is logical as leachate accumulated at the bottom for 2 to 3 d between recirculation events.

pH. Large differences in pH were observed between reactors and spatially within a reactor (Fig. 2). Methane composition increased as average solids pH increased for each reactor, indicating a positive linear correlation between pH and methane content ($r^2 > 0.99$). However, differences spatially within a reactor increased as methane content increased. Reactor A, with a methane content of 4.6%, had a relatively consistent pH of 6.04±0.19. In contrast, pH throughout Reactors B ($\text{CH}_4=10.2\%$) and C ($\text{CH}_4=23.1\%$) were more variable and averaged 6.29±0.65 and 6.95±0.80, respectively. Leachate pH measured from Reactor A was 6.0 (Table 1), which was similar to that measured from the solids. For reactor B, leachate pH was 7 and was markedly different than the solids pH. Compared to the solid fraction, leachate pH in reactor C was ~7 but the pH measured in the solids varied, ranging from 6.0 to 8.5.
Large pH differences were observed in Reactors B and C with depth (Fig. 2c,e). pH also varied laterally at the same depth (Fig. 3), with differences between the maximum and minimum pH in excess of 0.75 pH units (Fig. 2c,e). Since moisture content also increased with reactor depth, a correlation between pH and moisture content was observed but was apparent primarily at or above neutral pH (Fig. 2b,d,f) and at or above 70% moisture at which level there would be substantial free water. At pH <7, no identifiable trends were observed between moisture content and pH. Based on the pH profiles observed in each of the three reactors, Reactor B appeared to provide the most demonstrative evidence of methanogenesis initiation and was subjected to further analyses to identify differences in VFA concentration. Selected samples from this reactor, as well as a few samples from Reactors A and C, were analyzed for microbial community structure and activity.

**Volatile Fatty Acids.** Acetic, propionic and butyric acid concentrations for Reactor B are shown in Fig. 4 as a function of reactor depth and moisture content. On average, acetic acid concentrations were highest (7.9±3.0 mg dry g\(^{-1}\)) followed by butyric (5.3±2.4 mg dry g\(^{-1}\)) and propionic acids (2.4±1.7 mg dry g\(^{-1}\)). Trends were similar to pH in that VFA concentrations tended to increase with reactor depth and were relatively variable at specific depths (Fig. 4a,c,e). Higher VFA concentrations were correlated with increasing moisture content (Fig. 4b,d,f), a trend that was more cohesive compared to pH (Fig. 3b,d,f). VFA concentrations increased exponentially once the refuse moisture content reached about 80%.

**16S RNA Clone Libraries.** Bacterial and archaeal 16S cDNA clone libraries were performed on three samples from Reactor B to assess the structure of the active portion of
the microbial community during methanogenesis initiation. Two samples were taken at the same reactor depth (5 cm) while the third was retrieved at ~14 cm from the reactor bottom. The two 5 cm samples had a pH of 6.2 and 6.98 while the pH of the sample at the 14 cm depth was 5.6. All sequences retrieved were from the *Firmicutes, Actinobacteria, α-proteobacteria, β-proteobacteria*, and *Bacteriodetes* phyla (Fig. 5). Roughly 90% of cDNA sequences recovered at pH 5.6 were of the *Clostridiales* order (*Firmicutes* phylum), compared to 62% and 48% of sequences at pH 6.2 and 7, respectively (Table 2). The *Clostridiales* order includes many fermentative and cellulolytic species and most sequences recovered clustered within the genus *Clostridia* (Fig. 5). Members of the *β-proteobacteria* and *Bacteriodetes* phyla were only present at pH 7.

No *Archaea* sequences were retrieved in the pH 5.6 (14 cm depth) sample. At the 5 cm depth, over 80% of archaeal cDNA sequences retrieved from both the pH 6.2 and 7 samples were closely related (>97% homology) to *Methanosarcina barkeri* (Fig. 6). The remaining sequences were from the hydrogenotrophic *Methanobacterales* order. Two of the sequences were not closely related (<97% homology) to known methanogens in the Genbank database.

**Canonical Correspondence Analysis using RNA T-RFLP.** Canonical correspondence analysis (CCA) was performed using 16S RNA T-RFLP and compared against moisture content, pH, reactor depth and acetic acid concentration. Propionic and butyric acids were highly correlated with acetic acid concentration ($r^2 \sim 0.82$); as a result, these variables were excluded from the CCA to avoid multicolinearity. Results showed RNA-based bacterial community structure for most samples was not dependent on a single
measured environmental variable, although a few samples exhibited correlation with increasing pH, moisture content and depth (Fig. 7a). The lack of strong correlation to one environmental parameter is indicative of the broad metabolic diversity of Bacteria over a range of environmental conditions. In contrast, CCA showed archaeal community structure was correlated with increasing pH and moisture content (Fig. 7b). However, samples analyzed from Reactor A (4.6% CH$_4$) showed a dependence on reactor depth rather than pH or moisture content. This is likely due to the fact that pH in this reactor was still acidic (pH 6.04±0.19) and methanogenesis initiation had not yet occurred at the time of sampling.

**qPCR and RT-qPCR.** Bacterial RNA and DNA levels differed by 2 to 67 fold throughout the reactor but the differences were relatively well dispersed across the ranges of reactor depth, pH, moisture content, and VFA concentrations; thus, no identifiable trends were observed (data not shown). This result is consistent with the CCA results (Fig. 7a) and previous work indicating facultative Bacteria that decompose refuse under anaerobic conditions are relatively ubiquitous in the initial waste material at the time of disposal (26). Archaeal DNA levels ranged from 1 x 10$^7$ to 24 x 10$^9$ copies dry g$^{-1}$ and roughly 43% of samples had archaeal DNA <1 x 10$^9$ copies dry g$^{-1}$, most of which were localized to the upper portion of the reactor. This shows that large methanogen populations were not evenly distributed but occurred in spatially isolated areas despite leachate recirculation every two to three days. 16S RNA levels were also variable and ranged from approximately 7 x 10$^9$ to 43 x 10$^{12}$ copies dry g$^{-1}$ in Reactor B (Table 2), but tended to increase from the top of the reactor to the bottom (Fig. 8a). Significant
variability was observed in DNA and RNA copy number at the same depth, indicating substantial differences existed laterally as well as with depth in population size and relative metabolic activity (Table 2, Fig. 8a). Compared to reactor depth, a stronger correlation was observed between RNA level and pH (Fig. 8b). Generally, RNA levels increased with increasing pH and were roughly 20 times higher at pH 8 compared to 5.7. The lowest pH threshold at which substantial RNA copy numbers were detected was 5.7 (8 x 10^{11} copies dry g^{-1}), which was roughly two orders of magnitude greater than the copy number at pH 5.6 (7 x 10^9 copies dry g^{-1}). This data suggest methanogenesis initiation occurred at roughly a pH of 5.7.

**DISCUSSION**

Heterogeneity in refuse composition (Fig. 3) resulted in substantial spatial variability in pH, moisture content, VFA concentration and microbial population (as indicated by DNA copy number) for the *Bacteria* and *Archaea* domains. Despite this, a clear trend was evident between pH and increasing methane content (Fig. 2 a,c,e). It was shown that the initial pH decrease during the acid phase of decomposition appears systemic in that no pH neutral niches were identified prior to methanogenesis initiation in 81 core samples retrieved from Reactor A (4.6% CH_4) (Fig. 2a). The average pH of these core samples was 6.04±0.19. This suggests that either (a) pH neutral niches existed at scales smaller than the 3 cm diameter core sample size used in this study, or (b) no pH neutral niches were present. Evidence from this study suggests that measurement scale was likely not an issue since any pH neutral niches at <3 cm dia. x 2 cm thick would have become detectable as
CH₄ content increased. Thus, areas of pH 7 or greater would have been randomly detected in the upper portions of Reactors B (10.2% CH₄) and C (23.1% CH₄), but this was not the case. Instead, variable pH increases were initially observed near the reactor bottom (Fig. 2c) that unified into a stratified layer of pH neutral or higher conditions with a transitional, acid to neutral pH zone, that advanced towards the top of the reactor as methane production increased (Fig. 2e). This is the first study to measure methanogenesis initiation over spatial transects and provides experimental confirmation that a reaction front catalyzes methane initiation in refuse.

Specifically how this reaction front develops appears dependent on specific methanogen groups. During the acid phase of decomposition, substrate levels are high and the rate-limiting step in methane formation is governed by methanogen activity. RNA-based archaeal clone libraries showed the majority of recovered RNA sequences (82%) came from methanogens closely related to *Methanosarcina barkeri* when pH was acidic (pH ~6.2) (Fig. 6). Previous work in our lab evaluating the acid tolerance of methanogens under conditions similar to the acid phase of refuse decomposition also found *M. barkeri* was metabolically dominant (92% of RNA sequences) and produced measurable methane at a pH of 5.75 (27). These results are supported by the RT-qPCR results in this study, which showed methanogen RNA levels were detected and increased substantially at a minimum pH of ~5.7 (Fig. 8b). Collectively, this work shows methanogenesis initiation is driven by specific methanogen species tolerant to low pH, high VFA conditions, with *M. barkeri* playing an important role. These results agree with pure culture studies on *M. barkeri* which indicated an optimal pH range from 5 to 7, which is the lowest of cultured
*Methanosarcina* species, as well as most methanogens (8). This finding is particularly important since most acidophilic methanogens characterized thus far have been found in ecosystems with comparatively low VFA concentrations (4, 17). Despite this evidence, the presence of pH neutral niches is not ruled out. However, the role of pH neutral areas in facilitating methanogenesis initiation does not appear obligatory provided pH levels throughout the refuse do not become inhibitory. In previous work and the RT-qPCR results of this study, it was shown that pH values below 5.7 can be inhibitory or result in extensive lag times until substantial CH$_4$ production occurs (27). Under such conditions, pH neutral niches would likely be necessary in stimulating methanogenesis to avoid a prolonged period of minimal CH$_4$ production.

The mere presence of *M. barkeri*, or other putative acid tolerant methanogens, does not appear to be the only factor driving the 2 – 3 order of magnitude increase in methane production over a 4 day span (Table 1) as methanogenesis initiated. It is evident from this study that methanogen metabolic activity is dependent on a number of environmental factors such as available substrates and moisture content. In particular, moisture content is important given it correlated with pH (Fig. 2 b,d,f) and VFA concentrations (Fig. 4 b,d,f), with a content of 70% or higher promoting a rapid, active state of methanogenesis in refuse (Fig. 2 d,f).

We speculate that a higher moisture content promotes methanogen transport since they are known to be more planktonic rather than being attached to surfaces (15). Bacterial transport no doubt occurs but given their wide disbursement and ubiquity throughout the refuse material (26), the relative importance of bacterial translocation is likely less
important compared to the methanogens given they used a limited range of substrates and are the rate limiting step during the anaerobic acid phase of decomposition.

The reaction front theory, initially proposed by Martin et al. (2001), assumes that the front advances from a spatially isolated pH neutral area. Evidence from this study suggests that a reaction front begins at an area characterized by a high methanogen population which contains sub-populations of acid tolerant methanogens (i.e. *M. barkeri*) able to metabolize over a wide pH range. The importance of methanogen population under start-up conditions has been noted in previous research during the start-up of anaerobic digesters treating solid waste (10). This study suggests these initiation centers also contain sufficiently high moisture content to promote diffusion of substrates and translocation of methanogens. It is likely the translocation of methanogens during leachate recirculation created higher methanogen populations at the reactor bottom (Table 3), which explains why methanogenesis may have initiated at this location. The concentration of methanogens at the reactor bottom would also explain why leachate pH was neutral in Reactors B and C even when a large portion of the refuse remained relatively acidic (Table 1, Fig. 2 c,e). This result suggests that leachate pH is not an accurate indicator of the state of refuse decomposition, which has implications when evaluating landfill microbial activity at field-scale.

During methanogenesis initiation, the data suggest metabolic activity is pH dependent and substantially lower until pH neutral conditions develop. Interestingly, elevated VFA concentrations were present at depths with the highest pHs (Figs. 2, 4). Although this finding is somewhat counterintuitive, the data suggest neutral pH conditions promote a
higher rate of syntrophy with methanogenic *Archaea* resulting in stimulated methane production and a commensurate increase in fermentative activity. However, previous work has shown bacterial growth rates in mutualistic cocultures with methanogens are faster than methanogen growth rates (2). Due to this and the transitional environmental conditions related to methanogenesis initiation, one would expect activity of the methanogens to lag fermentative *Bacteria* initially until methanogen populations increased to balance VFA consumption with generation. At this time, a substantial drop in VFAs would be expected as methane content increased. The data show this is indeed what occurred. At a pH 7 or higher, acetate concentrations at methane compositions of 10.2% (Reactor B) and 23.1% (Reactor C) averaged 12.1±4.0 mg dry g$^{-1}$ and 5.8±2.5 mg dry g$^{-1}$, respectively. Similarly, propionate and butyric acids were 1.7 and 1.9 times higher when methane content was 10.2% and 23.1%, respectively. Once areas of the reactor are at neutral pH, metabolic activity increased substantially (Fig. 8b), which drove reaction front advancement into low pH areas of the refuse at a faster rate compared to the time necessary to facilitate methanogenesis initiation. This is also supported by evidence from the reactor operation since it took ~10 days for methanogenesis initiation to occur but only 4 days for CH$_4$ composition to increase by a factor of 6 to 15 fold for Reactors B and C, respectively (Table 1).

In summary, differences in environmental parameters, substrate levels, and microbial activity were remarkably different across spatial scales on the order of centimeters in decomposing refuse. Given these differences, leachate does not appear to provide an accurate assessment of landfill microbial health since large portions of refuse can remain
under low pH conditions and contain minimal microbial activity even while leachate pH is neutral (Table 1, Fig. 2c). Methanogenesis initiation is substantially affected by these spatial differences and the presence of methanogen populations that contain acid tolerant species such as \textit{M. barkeri} are critical. In the absence of pH neutral niches, this study suggests such acid tolerant species are required to overcome the low pH, high VFA conditions typically present during the anaerobic acid phase of refuse decomposition.

After methane production begins and a pH neutral area is established within the waste, data suggest a reaction front advances into low pH areas rapidly. This study is the first to experimentally demonstrate the complex interactions between microbial metabolism, environmental parameters and spatial variability in decomposing refuse.

ACKNOWLEDGEMENTS

Waste Management, Inc. provided funding for this research. Bryan Staley was partially supported by a Francois Feissinger scholarship from the Environmental Research and Education Foundation. We thank Fang Xu and David Black for their assistance in analyzing VFAs. We thank Johnsie Hingley for her assistance in sample processing and nucleic acid extraction. We are also grateful to Skip Richardson for providing access to the meat saw for reactor cutting.

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the anoxic sediment of Rotsee (Switzerland). Applied and Environmental Microbiology 65:2402-2408.


26. **Staley, B. F., F. L. de los Reyes, and M. A. Barlaz.** Comparison of Bacteria and Archaea communities in mixed refuse, individual refuse components and leachate, Submitted to Microbial Ecology.

27. **Staley, B. F., J. R. Hingley, F. L. de los Reyes, and M. A. Barlaz.** Identification of metabolically active methanogens in refuse and peat derived inocula under low pH and and high volatile fatty acid conditions.


Figure 1. Moisture content as a function of reactor depth. Values shown are the average of four measurements at each depth.
Figure 2. pH profiles at methane compositions of 4.6% (Reactor A), 10.2% (Reactor B) and 23.1% (Reactor C) as a function of reactor depth (a,c,e) and moisture content (b,d,f). The mean reflects 9 cores at each depth.
4.6% CH₄ (Reactor A)

10.2% CH₄ (Reactor B)
23.1% CH₄ (Reactor C)
Figure 3. Typical reactor slice from Reactor A (10.4 cm from bottom) indicating core sample locations and corresponding pH and moisture content (MC).
Figure 4. Acetic, propionic and butyric acid concentration as a function of reactor depth (a, c, e) and moisture content at 10% CH₄ (b, d, f) (Reactor B).
Figure 5. Phylogenetic tree of representative *Bacteria* sequences recovered from Reactor B (10.2% CH$_4$) at pH values of 5.6 (Sample 861), 6.2 (Sample 828) and 6.98 (Sample 822). See Table 3 for measured environmental conditions of each sample.
Figure 6. Phylogenetic tree of representative *Archaea* cDNA sequences recovered from Reactor B (10.2% CH₄) at pH values of 6.2 (Sample 828) and 6.98 (Sample 822). No archaeal sequences were detected at pH 5.6 (Sample 861). Both samples were obtained 5.1 cm from the reactor bottom. See Table 3 for measured environmental conditions of each sample.
Figure 7. Canonical correspondence analysis for (A) *Bacteria* and (B) *Archaea* using RNA T-RFLP. Samples located further away from the vector origin and along the environmental parameter vector are more correlated to that variable.
Figure 8. RT-qPCR quantitation of archaeal 16S RNA for selected samples as a function of (A) depth and (B) pH.
Table 1. Reactor operating conditions at lowest pH (day 10) and immediately before destructive sampling (day 14).

<table>
<thead>
<tr>
<th></th>
<th>Reactor A</th>
<th>Reactor B</th>
<th>Reactor C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet Refuse Mass (g)</td>
<td>2,745</td>
<td>2,523</td>
<td>2,567</td>
</tr>
<tr>
<td>Moisture Content (%)(^{a})</td>
<td>70.3</td>
<td>70.5</td>
<td>71.4</td>
</tr>
<tr>
<td>Operating Time (d)</td>
<td>10</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>% CH(_4)</td>
<td>0.7</td>
<td>4.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Cumulative CH(_4) (mL dry g(^{-1}))</td>
<td>0.04</td>
<td>0.11</td>
<td>0.07</td>
</tr>
<tr>
<td>CH(_4) Rate (mL d(^{-1}) dry g(^{-1}))</td>
<td>0.003</td>
<td>0.003</td>
<td>0.006</td>
</tr>
<tr>
<td>Leachate pH(^{b})</td>
<td>5.8</td>
<td>6.0</td>
<td>6.3</td>
</tr>
</tbody>
</table>

\(^{a}\) Computed by aggregating 36 discrete measurements taken throughout the entire reactor. 
\(^{b}\) Collected at the bottom of the reactor after recirculation.
Table 2. Distribution (%) of *Bacteria* cDNA sequences in Reactor B at pH values of 5.6, 6.2 and 7

<table>
<thead>
<tr>
<th>Phylum/Order</th>
<th>pH 5.6 (Depth=14 cm)</th>
<th>pH 6.2 (Depth=5cm)</th>
<th>pH 7 (Depth=5cm)</th>
</tr>
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<tbody>
<tr>
<td>Firmicutes</td>
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<td></td>
<td></td>
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<tr>
<td><em>Clostridiales</em></td>
<td>90.3</td>
<td>61.9</td>
<td>47.7</td>
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<tr>
<td>Unclassified Firmicutes</td>
<td>1.6</td>
<td>2.4</td>
<td>13.6</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Actinomycetales</em></td>
<td>3.2</td>
<td>21.4</td>
<td>4.5</td>
</tr>
<tr>
<td><em>Solirubrobacteriales</em></td>
<td>7.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unclassified Actinobacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-proteobacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rhizobiales</em></td>
<td>1.6</td>
<td></td>
<td>13.6</td>
</tr>
<tr>
<td>β-proteobacteria</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Unclassified β-proteobacteria</td>
<td>2.3</td>
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<td></td>
</tr>
<tr>
<td>Bacteriodetes</td>
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<td></td>
<td></td>
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<tr>
<td><em>Sphingobacteriales</em></td>
<td>2.3</td>
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<tr>
<td>Unclassified Bacteriodetes</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Unclassified</td>
<td>3.2</td>
<td>7.1</td>
<td>4.5</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup> Depth indicated is cm from the bottom of the reactor.
Table 3. Environmental conditions and RT-qPCR/qPCR quantitation of archaeal RNA and DNA in selected Reactor B (10.2% CH₄) core samples.

<table>
<thead>
<tr>
<th>Refuse Depth (cm)</th>
<th>pH</th>
<th>Moisture Content (%)</th>
<th>Acetic (units for acids in mg dry g⁻¹)</th>
<th>Propionic</th>
<th>Butyric (copies x 10⁹ dry g⁻¹)</th>
<th>DNA</th>
<th>RNA</th>
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<tr>
<td>21</td>
<td>6.6</td>
<td>72</td>
<td>5.7</td>
<td>1.8</td>
<td>3.4</td>
<td>7.1</td>
<td>2,538</td>
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<tr>
<td></td>
<td>6.6</td>
<td>72</td>
<td>5.7</td>
<td>1.8</td>
<td>3.4</td>
<td>7.1</td>
<td>2,538</td>
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<tr>
<td>18</td>
<td>6.3</td>
<td>43</td>
<td>7.9</td>
<td>1.4</td>
<td>4.8</td>
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<td>0.01</td>
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<td>71</td>
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<td><strong>1.6</strong></td>
<td><strong>4.9</strong></td>
<td><strong>0.02</strong></td>
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<td></td>
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<tr>
<td>5.9</td>
<td>73</td>
<td>6.9</td>
<td>1.6</td>
<td>6.0</td>
<td>1.0</td>
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<td></td>
</tr>
<tr>
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<td>80</td>
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<td><strong>4.5</strong></td>
<td><strong>11.6</strong></td>
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<tr>
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<td>11.6</td>
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<td>6.0</td>
<td>4.4</td>
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<td>11.3</td>
<td>24.1</td>
<td>17,058</td>
<td></td>
</tr>
</tbody>
</table>

ₐ Clone libraries were performed on samples in bold.

₇ From the bottom of the reactor.

ₙ NQ, not quantified.
Chapter 7.

SUMMARY AND RESEARCH IMPLICATIONS

The objective of this research was to correlate microbial activity to environmental conditions in a particular phase of refuse decomposition and evaluate causal factors contributing to methanogenesis initiation. Since molecular methods were used to evaluate the landfill microbiology, a sample processing method was developed to extract nucleic acids from refuse that was minimally biased relative to other extraction methods. One of the primary challenges in developing this method related to the heterogeneous and unstructured nature of solid waste, which made methods used for other ecosystems (i.e. soil, rumen) generally unsuitable. This was one of a few studies to compare the recovered microbial community for several sample processing methods and the first to develop a method specifically for refuse. The method developed here was used extensively in subsequent experiments as part of this research.

The initial thrust of this research was to evaluate microbial community demographics entering landfills from mixed refuse and individual waste components. This work was necessary to gain a better understanding of the microbial ecology prior to methanogenesis initiation and provided a more refined and detailed assessment of the bacterial and archaeal communities in discarded waste components and the role these communities play relative to refuse decomposition. The study showed that methanogen populations, which are critical to the anaerobic food web in landfills, likely come from isolated sources (e.g. food waste) in the incoming refuse and/or decomposing waste below freshly placed refuse. The findings also suggest that refuse decomposition and methane
production may be initiated more rapidly or enhanced if supplemental methanogen populations (e.g. from recirculated leachate, decomposed refuse, biologically active anaerobic biosolids) are introduced into freshly placed waste. Another key finding was that microbial communities in solids and leachate differed, suggesting that assessments of landfill microbiology using only one particular matrix may provide an inaccurate picture of microbial demographics.

Subsequent work was performed to track microbial community shifts through all phases of refuse decomposition. This work was followed by studies evaluating two theories for how methane production initiates in landfills, the (a) acid tolerant methanogen theory and (b) pH neutral niche theory. Collectively, this research provides a cohesive picture of temporal and spatial microbial interactions in decomposing refuse from initial placement of fresh material to a relatively complete state of degradation. Previous culture-based work suggested microbial groups increased in population; however, the work lacked sufficient resolution to explore community structure within these groups. This research was the first to experimentally show that microbial succession is substantial as refuse degrades and that shifts to different genera correlated to changing environmental conditions.

It was found that the *Methanosarcinales* order plays a substantial role in initiating methanogenesis during the anaerobic acid phase. *Methanosarcina barkeri* was dominant under the low pH, high carboxylic acid conditions associated with the anaerobic acid phase of decomposition. Thresholds for acid tolerance under high VFA conditions were established for mixed methanogenic cultures derived from refuse, increasing our
understanding of how methanogenic communities persist and metabolize under less than optimal conditions. Research evaluating spatial effects showed pH neutral niches at the cm scale are not necessary for methanogenesis initiation. It was observed that methanogenesis initiated rapidly in the absence of pH neutral niches but this was facilitated by high moisture conditions and sufficient methanogen populations containing acid-tolerant methanogens (e.g. *M. barkeri*). Once methanogenesis begins, it was demonstrated that spatially isolated areas within the refuse become pH neutral and quickly coalesce into a stratified reaction front that advances into low pH areas of refuse. These studies show that, in the absence of pH neutral niches, acid tolerant methanogens are required for methanogenesis initiation. However, it was also shown that spatial differences create the conditions necessary to promote expansion of isolated methanogenically active areas within the refuse. Thus, this work suggests that acid tolerant *M. barkeri* and small regions of neutral pH create a synergistic effect that facilitates methanogenesis initiation.

Although the focus of this research was on solid waste, it is anticipated that these results can be linked to environmentally important natural systems that are carbon sinks or are sources of methane emissions. Understanding the contribution of spatial factors and acid-tolerant methanogens to microbial interactions provide clues as to how carbon cycling may be affected due to climate change and/or global warming. For example, peat has been suggested as an analogue for older landfills (Bozkurt et al., 2001). A rise in long term global temperatures may result in increased nutrient fluxes into peat bog systems (Shannon and White, 1996; Avery et al., 1999). Such scenarios could result in subsequent selection effects on methanogen community structure or activity (Hines et al., 2001), perhaps also
shifting carbon sequestration dynamics. Population shifts occurring when well-
decomposed refuse is exposed to higher substrate levels may provide clues as to how such
shifts may alter carbon sequestration dynamics and CH$_4$ emissions in peat bogs and
wetland ecosystems.

Efforts in defining sustainable waste management strategies emphasize an
integrated approach involving pre-consumer strategies such as reduction of source
materials during manufacturing and packaging, as well as post-consumer strategies such as
recycling, and refuse decomposition enhancement with improved CH$_4$ recovery. Future
efforts to enhance refuse decomposition may well focus on manipulation of microbial
community structure, based on selection or genetic modification, to maximize the extent
and rate of waste decomposition. This work provides a foundation for subsequent research
on manipulation of microbial community structure to enhance refuse decomposition.

**RECOMMENDATIONS FOR FUTURE WORK**

1) Assess the importance of acetate oxidation to H$_2$/CO$_2$ under varying environmental
conditions including depressed pH and high volatile fatty acid concentrations.

2) Explore the role of homoacetogen activity during methanogenesis initiation and their
contribution to refuse degradation.

3) Evaluate the potential contribution of stable isotope probing in elucidating relevant
metabolic pathways during the acid phase of refuse decomposition and thereafter.
4) Determine the effect on landfill operating strategies (e.g. leachate recirculation, dosing regimes, pre-treatment) on microbial community structure during methanogenesis initiation.

REFERENCES


