Microbial community structures in family anaerobic digesters reveal potentially differing waste conversion pathways

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Abstract

Family-scale rural digesters are widely implemented in Nepal for waste management, resource recovery, and environmental stewardship for distributed communities. However, there is little documentation on the microbial community structures in real-world family farm digesters. This work compared microbial community structures in four family digesters to a near-by municipal digester. Included in the family digesters was a high-altitude family digester located on Mt. Everest in Mosi, Nepal (2,634 m elevation). Differences in the community structures included the prevalence in family digesters of Bathyarchaeota MGC-6. MCG-6 is an archaeal population putatively involved in autotrophic acetate generation and conversion of cellulose to sugars. Additionally, Rikenellaceae DMER64, a population thought to degrade sugars, was more prevalent in the family digesters. The ratio of *Methanothrix* to hydrogenotrophic methanogens was higher in the family digesters. Additionally, the dominant species of syntrophic hydrogen-producing bacteria differed. *Syntrophobacter* and *Syntrophomonas* species, documented for their critical roles in waste activated sludge digesters, were not detected. In conclusion, observed

differences in microbial community composition suggested a capacity to support different substrate conversion pathways and a major role of Archaea beyond methanogenesis among the studied digesters.

Key Words

anaerobic digestion, Bathyarchaeota, *Methanothrix*, *Methanosaeta*, Woesearchaeota, Nepal

Introduction

Efforts are underway world-wide to increase access to decentralized anaerobic digestion for populations with limited sanitation and energy options. Lab and field studies have demonstrated that small-scale digesters produce sufficient biogas for cooking and degrade >90% of feedstock chemical oxygen demand (COD) (Lou et al., 2012, Rajendran et al., 2012). Work to date has focused on parameters influencing biogas production, organic carbon degradation, and biokinetics (Kinyua and Stuart, 2022). For household-scale digesters at high altitude, extreme ambient temperature fluctuations, feedstock limitations (Alvarez and Liden, 2008, Ferrer et al., 2011), low ambient pressure (Wei et al., 2014) and difficulty accessing repairs (Lohani et al., 2022) have been identified as added challenges. While decreased internal digester pressure has been associated with increased methane yield (Utami et al., 2021), comparisons have only been tested at low pressures equivalent to ~9000 m elevation (i.e. the elevation the peak of Mt. Everest). All of these variables influence the microbiome of the digesters.

Anaerobic digestion uses a community of microbial populations that – through multiple biological steps – converts proteins, fats, and carbohydrates into methane gas and carbon dioxide (McCarty and Smith, 1986)(Tchobanoglous et al., 2014). Digester upsets are often associated with disruption in the highly interconnected microbial community network (Narihiro and Sekiguchi, 2007). Anaerobic digester microbial communities are inhibited by sudden changes to loadings (Vandenburgh and Ellis, 2002), toxicity from long-chain fatty acids (LCFA)(Hanaki et al., 1981), missing nutrients (Kim et al., 2002), oxygen shock (Conklin et al., 2007), floc disruption such as by overmixing (Stroot et al., 2001),

insufficient solids retention time (SRT), and elevated ammonia (Angenent et al., 2002). However, the systems can adapt when changes occur more gradually (Gough et al., 2013), and shifts in the composition of the microbial community are implicated in these system adaptations (Ziels et al., 2015, Ziels et al., 2017, Bovio et al., 2019). Yet, while studies have established a picture of the microbiome in municipal anaerobic digesters (Nelson et al., 2011), comparisons to family digesters have been less frequent.

Several studies have considered the influence of feedstock composition on family digester microbiomes. For example, a high-protein feedstock (rapeseed) has been documented to enrich for acid-tolerant *Lactobacillus* species and hydrogen-producing Synergistetes while sheep manure enriched for protein and acetate-degrading (hydrogen-producing) *Peptoniphilus* (Liu et al., 2022). Pre-adapted inoculum can improve performance both for temperature (Zhu and Jha, 2013) and for higher ammonia concentrations associated with undiluted human excreta (Colon et al., 2015). Among the expected adaptation to higher ammonia is a shift from *Methanothrix*-associated acetoclastic activity toward *Methanosarcina* and hydrogenotrophic activity associated with *Methanobacterium* (Cai et al., 2022), though this shift might also be related to temperature and pH (Han et al., 2021). Less well understood is how the microbiome develops in uncontrolled (non-experimental) settings associated with every-day use of family-scale digesters.

Expanding understanding of the Archaea (Baker et al., 2020) has potential to reveal new roles in anaerobic digestion microbiomes. Archaea are predominately recognized in anaerobic digestion for their function in the final methane production step (Xu et al., 2021). Yet, recent advancement in the understanding of archaeal metabolic and taxonomic diversity is rapidly expanding, as has been previously described (Adam et al., 2017), and continues to advance at a rapid pace (Berghuis et al., 2019, Rinke et al., 2021, Sheridan et al., 2022). For example, genetic markers within the Bathyarchaeota suggest a broad role for this phylum in anaerobic carbon cycling (Borrel et al., 2016, Adam et al., 2022). In most Bathyarchaeota genomes, the genes for the Wood-Ljungdahl pathway are more prevalent, while a few

contain the genes for hydrogenotrophic methanogenesis (Zhou et al., 2018). Zhou et al (2018) have suggested that the presence of the Wood-Ljungdahl pathway in the absence of genes for methanogenesis supports a capability for homoacetogenesis. Lazar et al (2016) has suggested that the presence of genes within the some Bathyarcaheota for the reduced ferrodoxin required for homoacetogenesis (e.g. [NiFe] hydrogenase and heterodisulfide reductase (HdrABC)) supported a role for acetogenesis for this group. In addition to genes for the Wood-Ljungdahl pathway, the MCG-6 group of the Bathyarchaeota have genetic markers for hydrolysis of plant cellulose materials while both MCG-1 and MCG-6 contain genes for simple sugar processing (Lazar et al., 2016) and the MCG-8 subgroup has been suggested to have lignin-degrading capabilities (Yu et al., 2018). Another group with emerging understanding is the Woesearchaeota. The Woesearchaeota have a broader habitat distribution with consistently low detection levels (i.e. <1% of recovered sequences) (Liu et al., 2018). These continuing discoveries about the Bathyarchaeota have potential for re-defining the classical role of Archaea within anaerobic digestion.

Selection of PCR primers, such as F515/R806 (Caporaso et al., 2011) target the methanogenic Archaea that dominate in anaerobic digesters while simultaneously targeting bacterial groups common in anaerobic digestion. However this primer set preferentially targets Archaea in phyla associated with methanogenesis while missing many other archaeal phyla (Petropoulos et al., 2021) that are detected by Archaea-specific primers such as 349F/806R (Bahram et al., 2019). Use of Archaea-specific primers have been reported to detect Bathyarchaeota and other Archaeal phylum in anaerobic digesters (Lam et al., 2020).

The current work was part of a larger effort to establish an anaerobic digester along the Mt. Everest Summit Trail at 5,164 m to treat human waste left by climbers at the Mt. Everest Base Camp. A main question was identifying the appropriate starting material for the base camp digester. The highest elevation functioning digester along the Summit Trail in Nepal is a family digester at Mosi (elevation 2,634 m) (Karki et al., 2015b). The high-altitude digester was installed through efforts by the Biogas

Support Program in Nepal (BSP-Nepal). Anaerobic digestion has been recognized in the region of Kathmandu for its high potential for reaching UN Sustainability Goals for wastewater treatment (Lohani et al., 2021b). For rural regions, the BSP-Nepal program established over 430,000 domestic digesters as of 2019 (Lohani et al., 2021a). They aim to provide economic benefits for rural families due to reduced expenditure on cooking and lighting fuels, as well as environmental benefits such as improved soil fertility by use of bio-slurry, improved local air quality due to cooking with biogas rather than dung or firewood, lower greenhouse gas emissions, and a reduction in deforestation (Dhungana et al., 2022). The motivations mirror the goals in many under-served locations. Lifecycle analysis (LCA) supports environmental and economic benefits from the installation of anaerobic digesters in both moist tropical and dry mountainous regions (Garfi et al., 2019).

This work describes a field evaluation conducted to compare the digester microbiomes in four family digesters to the microbiome of a regional municipal digester. The study begins to fill the informational void about microbiome composition in family digesters in remote locations under real-world operational conditions (i.e. without experimental manipulation). The studied digesters were at farms near Dhulikel (elevation 1380 m) and Mosi (elevation 2634 m). These were all originally established by BSP-Nepal. The preliminary hypothesis was that the microbiomes from the family digesters would be similar to each other, but different from the municipal digester. This hypothesis was based on the similar sources of feedstock and operational conditions among the family digesters compared to the municipal digester (i.e. direct waste inputs including animal wastes rather than human wastes conveyed through a sewer). To evaluate the hypothesis, a high throughput whole community 16S sequencing approach was used similar to approaches described elsewhere (Chen et al., 2022). The approach was updated to include phylogenetic inference to resolve taxonomy for sequences that were poorly identified using common databases. Additionally, implications of the differences in community structure for waste conversion pathways was evaluated. Contrary to expectation, Archaeal community structure in the digester from Mosi was more

similar to the municipal digester than to the other family farm digesters, and differences suggested a potential for differing feedstock conversion pathways.

Methods

Sample collection and processing

Samples were collected in March and April 2015. The three family farms and the municipal plant (locality Khadpu) were all in Dhulikel near Kathmandu, Nepal (~1,400 m elevation; atmospheric pressure ~87 kPa). The average temperature in Kathmandu for April 2015 was 14°C (GMAO, 2015). The high-altitude family farm (2,634 m elevation, atmospheric pressure ~73 kPa) was located near Mosi, Nepal; the location and digester were previously described (Karki et al., 2015b). The Mosi digester is currently the highest elevation digester on the Nepalese side of Mt. Everest. The average temperature near Mosi in March 2015 was 8°C (GMAO, 2015). Digester sludge was transported from Mosi in a 5-gallon container by a porter using a duko basket and namlo strap for the 2 hour trek to the Lukla (Tenzing-Hillary) Airport. From there, the container was transported on a flight to Tribhuvan International Airport (Kathmandu, Nepal; 40 minute flight) and transported to Kathmandu University (~50 minute drive).

The family digesters were fixed dome type Gobar Gas Company (GGC) 2047 designs with a 4 m^3 volume for the sum of slurry and gas storage. GGC 2047 digesters are designed to operate with a 35 day hydraulic retention time (HRT) (Mears and Anderson, 2011) when the digester is the only treatment step. The Khadpu municipal digester had a volume of 150 m^3. It treated raw sewage serving 200 households (no agricultural or industrial wastes) with an average flow of 103 $m^3 \cdot day^{-1}$, a biochemical oxygen demand (BOD) loading of 60 $kg \cdot day^{-1}$ and a 1.5 day HRT. Sewage was processed through a grit removal chamber prior to being fed to the anaerobic digester and effluent from the digester is discharged to a reedbed wetland system for continued treatment.

Hydraulic and waste loadings are not recorded by the families using the single-family digesters. Feedstock at the high-altitude digester was previously described as a mixture of human (i.e. latrine) and animal waste (cow dung) (Karki et al., 2015a). Lohani et al. (2022) previously documented the typical feedstock composition for family digesters in the study region as combined cow-dung and toilet waste for 82% of families they surveyed. In their study, the average reported pH was 7.7 +/- 0.6 (n=108) with range of 6 - 8.7, an average VS of 55.2 +/- 23 mg/g (n=102) with range of 14-113.2 mg/g, and average C:N ratio of 11:1 (n=100) with range of 5.1 - 24.1. The exceptionally low C:N was attributed the households that added large amount of animal urine to the digesters.

DNA Extraction and Sequencing

Short-read whole community DNA 16S sequencing and analysis was used, following approaches commonly used for evaluating community structure at the genus level (Chen et al., 2022). DNA was extracted in April 2015 at Kathmandu University using MO BIO's PowerSoil DNA Isolation Kit (Qiagen; Germantown, Maryland, USA), modified to adapt to available equipment by attaching tubes to a vortexer for the bead-beating step. Samples were preserved with DNALater and transported to the University of Washington, where they were stored at -80°C. Quality control prior to PCR included assessing the A260:A280 ratios of the extracts; the A260 value was used to estimate the concentration of DNA in each sample. Amplification and sequencing was conducted for the 16S rRNA gene using an Illumina MiSeq (2x 300bp, pair ended) at Mr.DNA Lab (Shallowater, Texas, USA) using Archaeal Domain Primers (349F: 5'-GYGCASCAGKCGMGAAW-3', 806R: 5'-GGACTACVSGGGTATCTAAT-3') (Takai and Horikoshi, 2000) and Bacterial Domain Primers (27Fmod: 5'-AGRGTTTGATCMTGGCTCAG-3'(Frank et al., 2008), 519Rmodbio: 5'-GTNTTACNGCGGCKGCTG-3') with a barcode on the forward primers. PCR amplification used the HotStarTaq Plus Master Mix Kit (Qiagen, USA) using the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. After amplification, PCR products were checked in 2% agarose gel. Multiple samples were pooled in equal

proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads. Then the pooled and purified PCR products were used to prepare Illumina DNA library and sequencing was completed using a MiSeq following the manufacturer's guidelines. Sequence data were processed using MR DNA Lab analysis pipeline. Briefly, raw 16S paired reads were joined, barcodes were removed then sequences <150bp in length discarded, as were sequences with ambiguous base calls. At the University of Washington, sequences were clustered into operational taxonomic units (OTUs) based on 98% similarity using usearch 10.0.240 (Edgar, 2010) , similar to 16S community DNA sequence approaches used by Chen et al. (2022).

Initial sequencing was conducted in 2016, with additional archaeal sequencing in 2019 including technical replicate controls as direct between-run comparisons. Archaeal sequence recovery was statistically higher in the first round (*t-test, p=0.00017*), while OTU richness, diversity and evenness did not differ between processing events; supporting comparison of samples processed in different sequencing runs. Sequence data from both runs were processed together.

Community Profiling and Analysis

Community richness (R) was calculated as the number of OTUs detected in a sample (Pielou, 1966, Colwell, 2009). Diversity was quantified using the Shannon diversity index (*H*)

$$H = -\sum_{i=1}^{n} p_i \ln p_i \dots\dots\dots\dots\dots (eq.1)$$

where p_i is the relative abundance of an OTU in a sample, and evenness (*Eh*) (Pielou, 1966, Hill et al., 2003, Colwell, 2009) was quantified as:

$$Eh = \frac{H}{\ln R} \dots\dots\dots\dots\dots (eq.2)$$

Community similarity was evaluated using principal coordinate analysis (PCoA) using the cdmscale function in R(RCoreTeam, 2021). The R coding script is provided in Supplemental Materials. OTU data

was log- transformed as the relative abundance +1 (adding 1 allows processing data sets that include zeros), and a dissimilarity matrix was generated using the vegdist function in the vegan package of R (Oksanen et al., 2022). The argument "points" were exported to EXCEL for PCoA plotting. OTUs contributing to the dissimilarity in the PCoA were identified by comparing the magnitude of the slope of the OTU relative abundance and the graphing coordinate for each PC. OTUs with highest ranks were visualized as overlays on the PCoA plots.

Taxonomic assignments of the OTUs were inferred by phylogenetic analysis using arb software platform with SILVA Version 138.1 (released August 27, 2020). Use of the arb software platform for phylogenetic inference has previously been described (Ludwig et al., 2004, Pruesse et al., 2007). Briefly, sequences were aligned against the pre-aligned arb sequences and added by parsimony to the SILVA Version 138.1 pre-constructed phylogenetic tree. Alignments were checked using the arb sequence editor, and alignment corrections were made when required. As needed, additional sequences were obtained through BLAST (Camacho et al., 2009) using the BLASTn Nucleotide Collection (nr/nt). BLAST sequence results were added to the SILVA tree to resolve ambiguity. This approach for identification was used because greater than 80% of the recovered sequences had poor identity confidence scores using the Ribosomal Database project (RDP) classifier version 2.13 (see Supplemental Information). Functional assignment of taxonomic groups was inferred from the MiDAS Field Guide database (Nierychlo et al., 2020, Dueholm et al., 2022). If the taxonomic groups were not included in MiDAS, primary literature was reviewed for potential functional capabilities.

Data Access

Sequence data was deposited in the National Center for Biotechnology Information (NCBI) Small Read Archive (SRA). Archaeal sequence data are stored as PRJNA772764 (SAMN22416323 through SAMN22416327 and SAMN22416330 through SAMN22416336). Archaeal OTU sequences have been deposited at GenBank under the accession KFUG00000000 (first version: KFUG01000001 through

KFUG01000768). Bacterial sequence data are stored as PRJNA823743 (SAMN27356871 through SAMN27356875). Bacterial OTU sequences have been deposited at GenBank under the accession KFUN00000000 (first version: KFUN01000001 through KFUN01002056).

Results and Discussion

Sequence recovery and ecological indices.

Digester community profile characteristics are summarized in Table 1. Sequences were grouped into a total of 768 and 2078 OTUs for the Archaea and Bacteria, respectively. Archaeal ecological indices were significantly highest in the municipal AD and lowest in the high elevation AD (ANOVA, $p=0.00014$, $p=0.0001$, and $p=0.0017$, for richness, diversity and evenness respectively). Conversely, bacterial indices were not significantly different.

Archaeal community similarity and structure.

Similarity analysis for archaeal OTU profiles is visualized in Figure 1A. Table 2 shows relative detection and identification of the OTUs with influence on the principal coordinates (PC). Family digester samples were 72% similar, on average, and clustered in the PCoA, supporting the original intent of considering these digesters as replicates. Contrary to expectation, the high-altitude family digesters did not cluster with the lower-altitude family digesters. The first PC separated the family digesters from the municipal and high-altitude digesters. The second PC separated the high-altitude digester from the municipal digester. Separation was driven by higher occurrence of OTUs in the family digesters associated with *Methanothrix*, *Methanoculleus*, and Bathyarchaeota MCG-6, OTUs in the high-altitude digester associated with uncharacterized Methanomicrobiales, and OTUs in the municipal digester associated with *Methanolinea*, *Methanothrix*, and *Methanobacterium* (Figure 1A, overlay lines). Two OTUs representing *Methanothrix* (ArcOTU1 and ArcOTU16) acted in opposite directions in the PCoA analysis.

Community phylum profiles are shown in Figure 2. Numeric values used to generate Figure 2 are presented in Supplemental Materials Table 1. Halobacteriota dominated in all digesters, followed by Thermoplasmatota, Bathyarchaeota, and Euryarchaeota. Prevalence of the Bathyarchaeota significantly differed (ANOVA, p=0.00004) with highest prevalence in the family digesters (Figure 2A), followed by the high-altitude digester (Figure 2B). All Bathyarchaeota were part of group MCG-6. Few studies report Bathyarchaeota in digesters. Some exceptions include a report of Bathyarchaeota 16S signatures in a biogas biofilm (Maus et al., 2018) and in a survey of digester community structures (Lam et al., 2020), though the latter study focused on other populations. Euryarchaeota and Thermoplasmatota were significantly lower in the family digesters (ANOVA, p=0.0002 and p=0.02, respectively). Euryarchaeota was dominated by *Methanobacterium* in the municipal and family digesters, and by *Methanobrevibacter* in the high-altitude digester. All the Euryarchaeota and Thermoplasmatota genera detected were hydrogenotrophic methanogens. Woesearchaeota accounted for 3%, 1.5%, and 0% of the family, high-altitude, and municipal archaeal sequences, respectively. The Woesearchaeota are distinguished by their detection in a broad range of habitats generally at very low (<1%) detection levels(Liu et al., 2018). Micrarchaeota, Iainarchaeota and Aenigmarchaeota were also detected in the family digesters, but not in the other digesters. In anaerobic habitats, the Woesearchaeota have been found to be highly associated with methanogens (Liu et al., 2021). Potential for homoacetogenesis has been suggested for Woesearchaeota in anoxic habitats (Probst et al., 2017, Liu et al., 2018). Though not indicated earlier reviews (Baker et al., 2020), some Woesearchaeota may also harbor genes potentially involved in anoxic nitrate reduction (Liu et al., 2021) and substantial more research is needed to fully elucidate a role for Woesearchaeota in anaerobic digestion.

While Halobacteriota were equally represented in all digesters, the composition of the Halobacteriota differed (Figure 2, smaller divisions). *Methanoculleus, Methanogenium, Methanothrix,* and a group of uncharacterized Methanomicrobiaceae were all more prevalent in the family digesters. A group of uncharacterized Methanomicrobiales was prevalent in the municipal and high-altitude digester and

Methanolinea was prevalent in the municipal digester. *Methanothrix* are obligate acetoclastic methanogens with a high affinity for acetate (i.e. ability to use low concentrations of acetate with a half-saturation constant (K_s) typically estimated as 90 mg/L(Conklin et al., 2006, Tchobanoglous et al., 2014). The other detected Halobacterota were hydrogenotrophic methanogens. Given the high richness of *Methanothrix* detected in the study (i.e. 49 distinct *Methanothrix* OTUs), diversity within the *Methanothrix* was surprisingly low. A single OTU (ArcOTU1) dominated (outer ring of circular insets, Figure 2). Richness among the *Methanothrix* OTU was investigated by phylogenetic tree alignment with known species (Supplemental Materials SM Fig. 4) . The sequences of ArcOTU1 and 20 other OTUs were highly aligned with the sequence of *Methanothrix soehngenii* (demarked as Group 1 in SM Fig 4). ArcOTU16 represented a substantial portion of the *Methanothrix* in the municipal digester samples, and was distantly related to *M. soehngenii* (Group 2 in SM Fig 4). Other *Methanothrix* OTUs were more distantly related to known *Methanothrix* species; through these accounted for less than 0.003% of the archaeal sequences in the municipal and family digesters. This is of particular importance for design considerations since the K_s values vary among isolated strains of the *M. soehngenii* with values as low as 23.4 mg/L reported (Ohtsubo et al., 1992).

Bacterial community similarity and structure.

PCoA separated bacterial communities in the municipal from the family digesters (Figure 1B). Table 2 shows influence of the OTUs to each PC. The family digester bacterial communities formed loose clusters with similarity scores ranging from 32% to 42% compared with 91% for the municipal samples. Separation was associated with higher occurrence of Caldatribacteriota JS1, Rikenellaceae DMER64, Bacteroidetes vadinHA17 and uncultured Anaerolineaceae OTUs in family digesters and higher occurrence of Dehalococcoidia MSBL5, Dehalococcoidia MSBL5, two *Smithella* OTUs in the municipal digester (Figure 1B, overlay lines).

Community phylum and genus profiles are shown in Figure 3. The profiles for the three family digesters are shown separately (Figure 3A-C) to visualize bacterial community variability among these samples. Numeric values used to generate Figure 3 are presented in Supplemental Materials Table 2. Bacteroidota dominated in the family digesters while Chloroflexi and Desulfobacterota dominated in the municipal digester. In the municipal samples, the phyla Chloroflexi, Desulfobacterota, Synergistota, and Acidobacterota were significantly higher compared to the averaged family samples (t-test, p=0.00070, p=0.00072, p=0.00005, and p=0.0013, respectively). Higher occurrence of Chloroflexi and Desulfobacterota in the municipal digester was influenced by Dehalococcoidia MSBL5 and *Smithella* (Figure 3D). Dehalococcoidia MSBL5 is a hydrogen consumer and potential anaerobic organohalide-respirer (Frouin et al., 2018, Yang et al., 2020), *Smithella* species are associated with syntrophic degradation (Juste-Poinapen et al., 2015) of n-alkanes (Tan et al., 2014), long chain fatty acids, butyrate (Qin et al., 2017) and propionate.

In the family digesters, Bacteroidota (28%±3%), Cloacimonadota (10%±2%) and Planctomycetota (3%±1%) were significantly higher. Higher occurrence of Bacteroidota was influenced by Rikenellaceae DMER64 (Figure 3). Rikenellaceae are thought to fermentatively produce VFA and hydrogen (Rachbauer et al., 2017), potentially from glucose based on reports of enrichment in an glucose-fed digester (Wahid et al., 2019). Rikenellaceae DMER64 has also been implicated in direct interspecies electron transfer (DIET) with methanogenic species (Lee et al., 2019). In two of the family digesters (1 and 3) the higher Bacteroidota was also influenced by Bacteroidetes vadinHA17 (proposed Candidatus Aminobacteroidaceae), a putative amino-acid degrader (Mei et al., 2020). Higher occurrence of Cloacimonadota was influenced by *Candidatus Cloacamonas* and Cloacimonadaceae W5. *Candidatus Cloacamonas* is associated with syntrophic amino acid and propionate degradation (Pelletier et al., 2008).

Two of the three family digesters had high relative abundance of Caldatribacteriota JS1. JS1 does not yet have an identified metabolism; genetic analysis of another member of phylum Caldatribacteriota (aka Atribacteria) indicates potential for fermentation of sugars (Katayama et al., 2020).

Differential community functional potential

In this study, the bacterial and archaeal community profiles were compared between family farm digesters in Nepal and a near-by municipal digester. Many populations in anaerobic digesters are not available for culture-based study and the metabolic potential of many of these populations is only recently emerging. Potentially specialized metabolic groups were identified by their 16S signature in different proportions in the studied digesters. To disentangle inter-connections among the detected digester populations, comparison of the relative occurrence of these groups was investigated to determine if these differences might suggest differing conversion pathways for complex polymers to methane gas. Most sequences recovered were for uncultured and uncharacterized Bacteria and Archaea. In the absence of culture studies, functions can only be inferred based on genomics inference and enrichment reports of closest relatives. Tools identifying the functional potential, such as the MiDAS field guide (Nierychlo et al., 2020, Dueholm et al., 2022), have not yet expanded to include most of the populations identified in the current study (accessed December 23, 2022). For example, MiDAS includes only 23 genera of Archaea associated with only 3 phyla (Euryarchaeota, Halobacteriota, and Thermoplasmatota). MiDAS does not include non-methanogenic Archaea or many of the archaeal phyla detected in this study. This is a common obstacle for microbiome studies of anaerobic digestion. For example, Han et al (2021) noted that dominant syntrophic populations could only be identified at the family taxonomic level. In another case, Liu et al (2022) recorded detection of Bathyarchaeota and DMER64 in their figures, but did not discuss their potential importance. In the current study, uncharacterized populations were included by assigning putative metabolic functions identified in primary literature sources referenced in earlier sections and summarized in Supplemental Materials Table 3.

Figure 4 compares populations with differing occurrence in the study mapped onto the metabolic steps in methanogenesis. This visualization of the step-wise methanogenic process is an established tool for understanding the core processes in anaerobic digestions (McCarty and Smith, 1986). Hydrogenotrophic methanogens were significantly more prevalent in the municipal digesters than in the high-altitude digesters, which in turn were higher than in the family digesters (ANOVA, p=0.0077 and 0.00042, respectively). *Methanothrix* was higher in the family digesters than in the municipal or high-altitude digesters (ANOVA, p=0.006) and was the dominant methanogen in all digesters, representing ~30% of recovered archaeal sequences. This contrasts with other studies where *Methanosarcina* (not detected in the current study) has been reported to be dominant, particularly with high organic loading rates (Kim et al., 2019), or with high total ammonia concentrations characteristic of food waste (Capson-Tojo et al., 2018).

Syntrophic bacteria were significantly higher in the municipal than in family digesters (39%±1% and 20%±1%, respectively; t-test, p=0.0008). Detected syntrophic hydrogen producers included the Cloacimonadaceae and Rikenellaceae (DMER64) in the family digesters, and Smithellaceae (genus *Smithella*). The Syntrophaceae (genus *Syntrophus*), Synergistaceae (genus JGI-0000079-D21) and Syntrophorhabdaceae (genus *Syntrophorhabdus*) made up small fractions of the syntrophic bacteria in the municipal digester and were negligible in the family digesters. Syntrophic bacteria rely on close association with a hydrogen scavenger to make the energetic yield thermodynamically favorable (Conrad, 1999). Syntrophomonas (family Syntrophomonadaceae) and Syntrophobacter (family Syntrophobacteraceae) have emerged as critical butyrate and propionate syntrophic degraders, respectively (Ziels et al., 2015, Mathai et al., 2020). Interestingly, neither of these genera (or their associated families) were detected in the current study. Others have reported an important role of the phylum Synergistetes (aka Synergistota) (Han et al., 2020); these were a minor portion of the syntrophs detected in the current study. Instead, the dominant propionate degraders in the municipal digester and the family digester, respectively were *Smithella* and *Candidatus Cloacamonas* (family Cloacimonadaceae).

382 The metabolic diversity of these predominant syntrophic bacteria is also of note. In addition to volatile
383 fatty acids, *Smithella* can hydrolyze alkanes and Cloacimonadaceae (detected in the family digesters) can
384 degrade amino acids. Additional system analysis would be needed to assess if enrichment of these
385 bacteria was favored by their enhanced metabolic capabilities.
386
387 The lower ratio of hydrogenotrophic to acetoclastic methanogens in the family digesters raised the
388 question of whether hydrogen gas scavenging might be impacted in these family digesters. Hydrogen can
389 be used as an electron donor coupled to sulfate and nitrate reduction. When this occurs, a shift in the
390 microbial community structure has been reported (Lackner et al., 2020). In the current study, none of the
391 bacterial genera contributing to the PCoA separation of communities (Figure 1), nor those observed to
392 differ among reactors (Figure 3) were associated with sulfate or nitrate reduction. Additionally, the lack
393 of bacteria commonly associated with nitrate or sulfate reduction (Supplemental Materials Table 3) would
394 not support a major role for these electron acceptors in the digesters. Homoacetogenesis represents an
395 alternative hydrogen-scavenging process, that might also favor higher prevalence of acetoclastic
396 methanogens. As such, Clostridia has been identified as a linchpin population for hydrogen scavenging
397 through homoacetogenesis (Ryan et al., 2008) particularly under thermophilic operational conditions
398 (Treu et al., 2018). However, Clostridiales detection was low in the study digesters (2% and 3%
399 respectively in the family and municipal digesters), thus, was not a likely explanation in this study.
400 Instead, potential archaeal homoacetogenesis was supported by the high abundance of Bathyarchaeota
401 MCG-6.
402
403 Two steps in Figure 4 putatively associated with non-methanogenic Archaea were higher in the family
404 digesters: cellulose degradation and homoacetogenesis. This contrasts with studies where only the
405 methanogens are profiled instead of all Archaea. Both metabolisms were inferred from recent whole
406 genome analysis for uncultured Bathyarchaeota MCG-6 (Lazar et al., 2016, Zhou et al., 2018). Cellulose
407 degradation was additionally supported by the higher co-occurrence of populations associated with sugar

degradation (the breakdown product of cellulose) in the digesters. For example, the putative sugar degrader Rikenellaceae DMER64 was statistically higher in family digesters than in the municipal digester (t-test, p= 0.006). Another putative sugar degrader, JS1, was detected at high relative abundance in digesters for family 1 and family 3. In addition to genes for cellulose degradation, the MCG-6 have also been reported to have the genetic markers for the Wood-Ljungdahl pathway, suggesting a digester role of homoacetogenesis (Zhou et al., 2018). Potential for homoacetogenesis has also been suggested for Woesearchaeota (Probst et al., 2017, Liu et al., 2018). Homoacetogenesis is the conversion of H_2 and CO_2 to acetate. The Wood-Ljungdahl pathway genes have also been associated with the reverse process (Muller et al., 2013) including syntrophic acetate oxidizing bacteria (SAOB) in the presence of high acetate or organic loading condition (Li et al., 2022, Becker et al., 2023), and often requiring the presence of an electron acceptor such as sulfate (Ragsdale and Pierce, 2008). A role for the reverse Wood-Ljungdahl pathway by MCG-6 would not be supported in the family digesters because the syntrophic partner for SAOB, hydrogenotrophic methanogens, were lower in these digesters and *Methanothrix* was higher indicating a shift toward acetoclastic methanogenesis rather than toward excess hydrogen generation.

Conclusion

This study has documented the community structures via 16S profiles for family-scale anaerobic digesters in remote locations under real-world operational conditions. The results revealed differences between the family digesters and a near-by municipal digester that suggested differing metabolic functional pathways within the communities. Species with putative roles in H_2 production and consumption differed from traditionally described processes. This difference included lower occurrence of hydrogenotrophic methanoges and higher occurrence of Bathyarcheaota MCG-6 (a putative hydrogen-consuming homoacetogen) and Caldatribacteriota JS1 (a potential hydrogen-generating sugar degrader) in the family digesters. *Methanothrix* was additionally higher in the family digesters in Nepal. This result has important implications toward prioritizing anaerobic digester populations for targeted metagenomic studies on

metabolic interactions toward unraveling the complex microbial interactions involved in biogas production. To realize this potential, future work will need to resolve the roles of uncharacterized microbes and examine links among the chemical composition of feedstocks, the resulting microbial enrichments, and the biokinetics of these yet-to-be studied groups.

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Author Contributions

Gough. Conceptualization, Formal Analysis, Writing (drafting and editing), Visualization; Kargol. Data Curation, Formal Analysis, Writing (review and editing); Beck. Data Curation, Formal Analysis; Therrien. Data Curation, Formal Analysis; Dahal. Investigation, Sample Collection, Writing (review and editing); Marsolek., Conceptualization, Sample Collection, DNA Extraction, Investigation, Writing (review and editing).

Author Disclosure

The authors have no conflicts of interest to disclose.

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Figure Legends

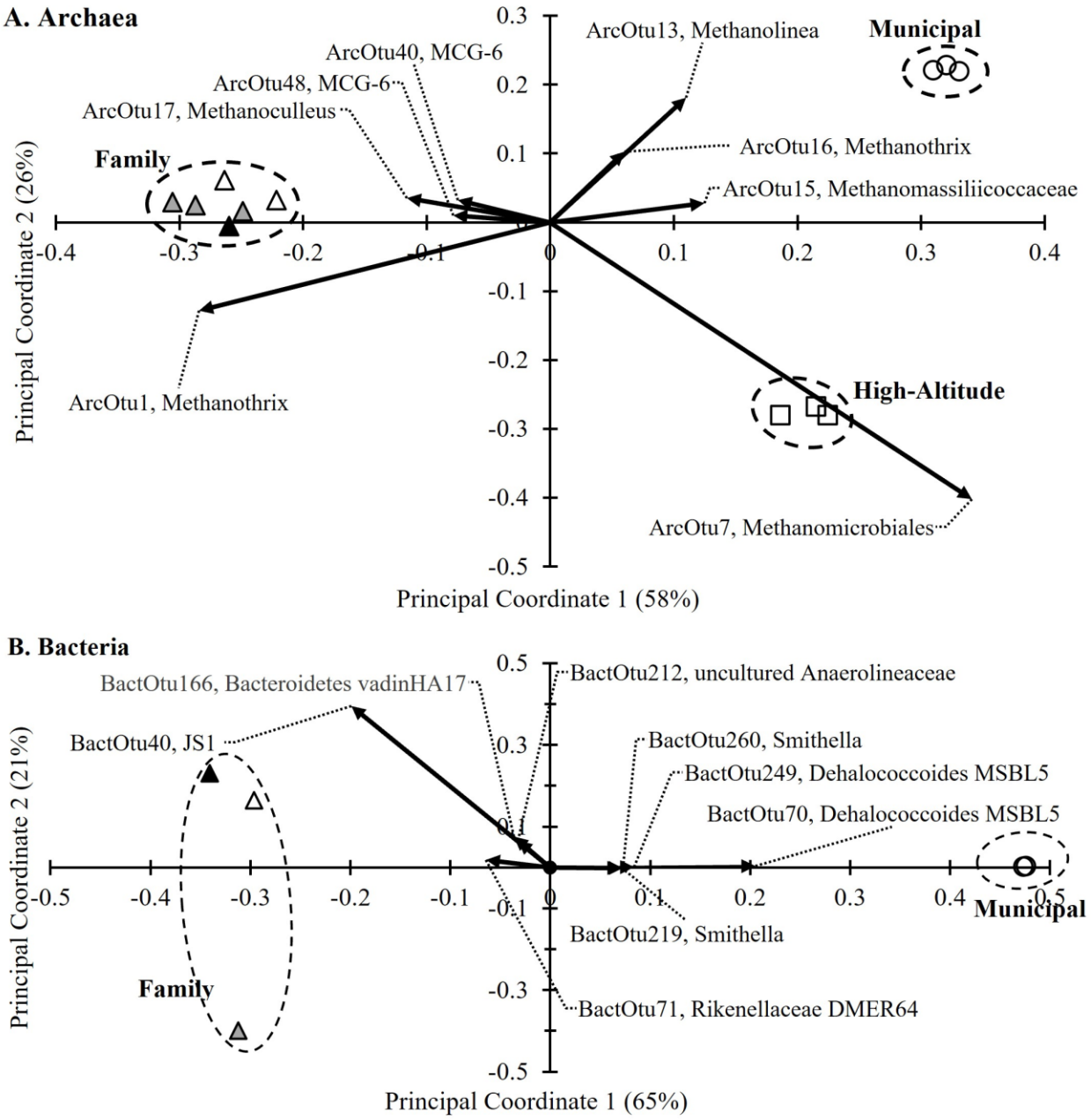
Figure 1. Dissimilarity of Community Structure (Principal Coordinate Analysis, PCoA). Dissimilarities were calculated using relative abundance of all OTU. Marker shape indicate the sample source. Shading differentiates the family digesters (triangle markers). Dashed circles identify the samples from the same source. Note that in Panel B there are two open circle markers that when graphed are so close to each other that they might be mistaken as a single marker. Axis length is scaled to the variance associated with each Principal Component (PC). Arrows show the magnitude and direction of abundance for OTUs with the highest contribution to the PC dissimilarities.

Figure 2. Average community composition of Archaeal OTUs. Area represented by each color shows the relative abundance of detected phyla. Sub-divisions within a color region represent the genus level (or lowest identified taxonomic level when genus is unknown) breakdown within each phylum; names are included for genera occurring at higher than 2.5%. Supplemental Material Table 1 includes names of all genera and their detected levels. Circular graphs show relative occurrence of *Methanothrix* OTUs (outer ring) and clustering of these OTUs into 4 similarity groups (inner ring). Groups were defined by phylogenetic inference as illustrated in Supplemental Materials Figure 4.

Figure 3 Average composition of Bacterial OTUs. Area represented by each color shows the relative abundance of detected phyla. Sub-divisions within the color region represent the genus level (or lowest identified taxonomic level when genus is unknown) breakdown within each phylum; names are included for genera occurring at higher than 2.5%. Supplemental Material Table 2 includes names of all genera and their detected levels.

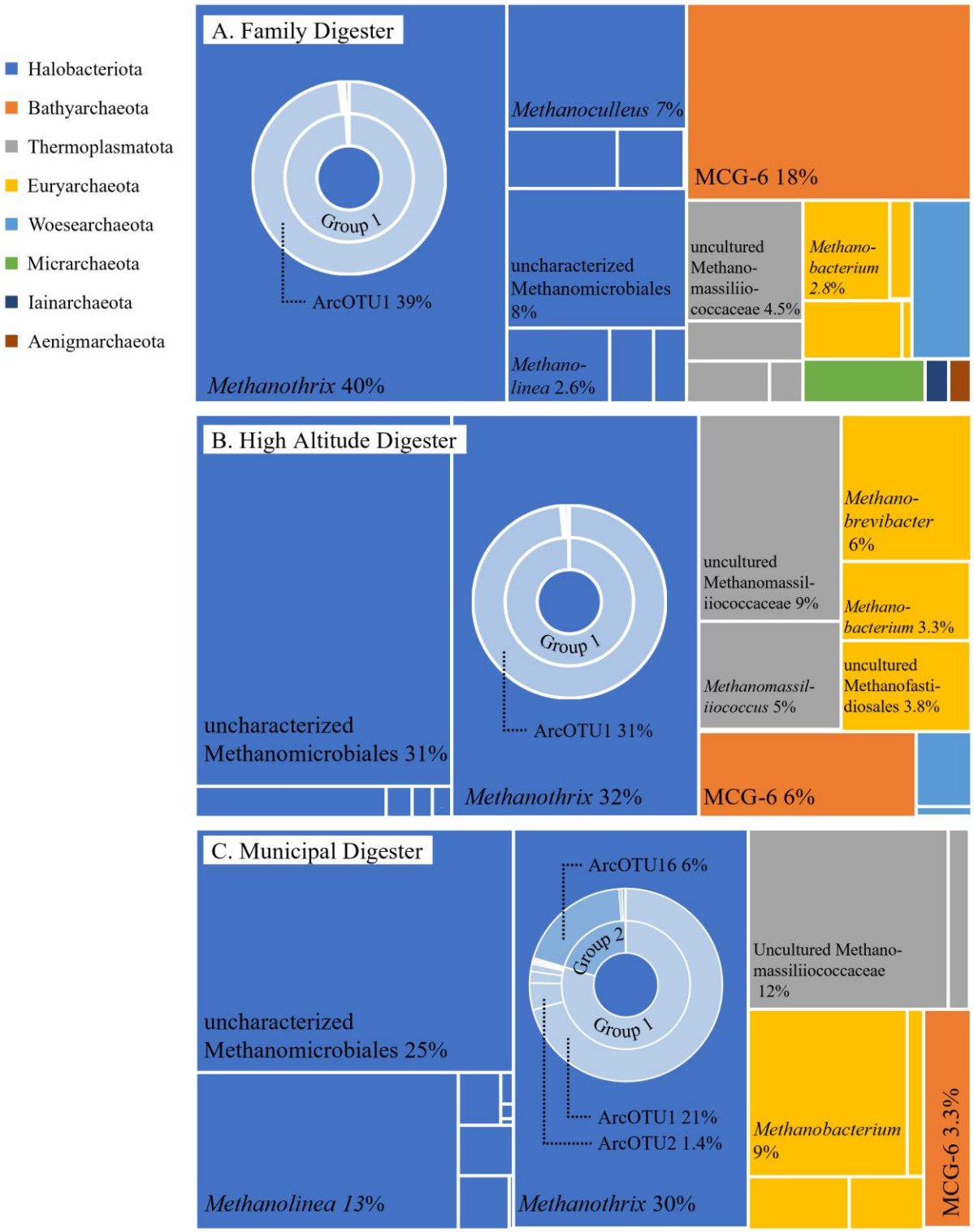
Figure 4. Step-wise methanogenesis in anaerobic digestion with abundance of populations that differed among the studied digesters. The width of the arrows represents the relative abundance of the sum of sequences for the labeled group. Syntrophic hydrogen producers are shown as the sum of Rikenellaceae

DMER64, Caldatribacteriota JS1, Cloacimonadaceae, and *Smithella*. Hydrogenotrophic methanogens in this study were represented as the sum of all detected genera in the phyla Halobacteriota (excluding *Methanothrix*), Thermoplasmatota, and Euryarchaeota; these genera are listed in Supplemental Material Table 1. Archaeal hydrogenotrophic homoacetogens included the sum of Bathyarachaeota MCG-6, Woesearchaeota, and Methanofastidiosales (excluding Candidatus *Methanofastidiosum*). Process steps align with Tchobanoglous G, Stensel HD, Tsuchinhashi R, et al. Section 7-14. Anaerobic Fermentation and Oxidation. In Wastewater Engineering: Treatment and Resource Recovery, 5th Edition. McGraw Hill Education, New York, New York, USA, pp. 655-662;2014.


A. Archaea
ArcOtu13, Methanolinea
Municipal
ArcOtu40, MCG-6
ArcOtu48, MCG-6
ArcOtu17, Methanoculleus
ArcOtu16, Methanothrix
Family
ArcOtu15, Methanomassiliicoccaceae
Principal Coordinate 2 (26%)
ArcOtu1, Methanothrix
High-Altitude
ArcOtu7, Methanomicrobiales
Principal Coordinate 1 (58%)
B. Bacteria
BactOtu166, Bacteroidetes vadinHA17
BactOtu212, uncultured Anaerolineaceae
BactOtu40, JS1
BactOtu260, Smithella
BactOtu249, Dehalococcoides MSBL5
BactOtu70, Dehalococcoides MSBL5
Principal Coordinate 2 (21%)
Municipal
BactOtu219, Smithella
Family
BactOtu71, Rikenellaceae DMER64
Principal Coordinate 1 (65%)

740

741

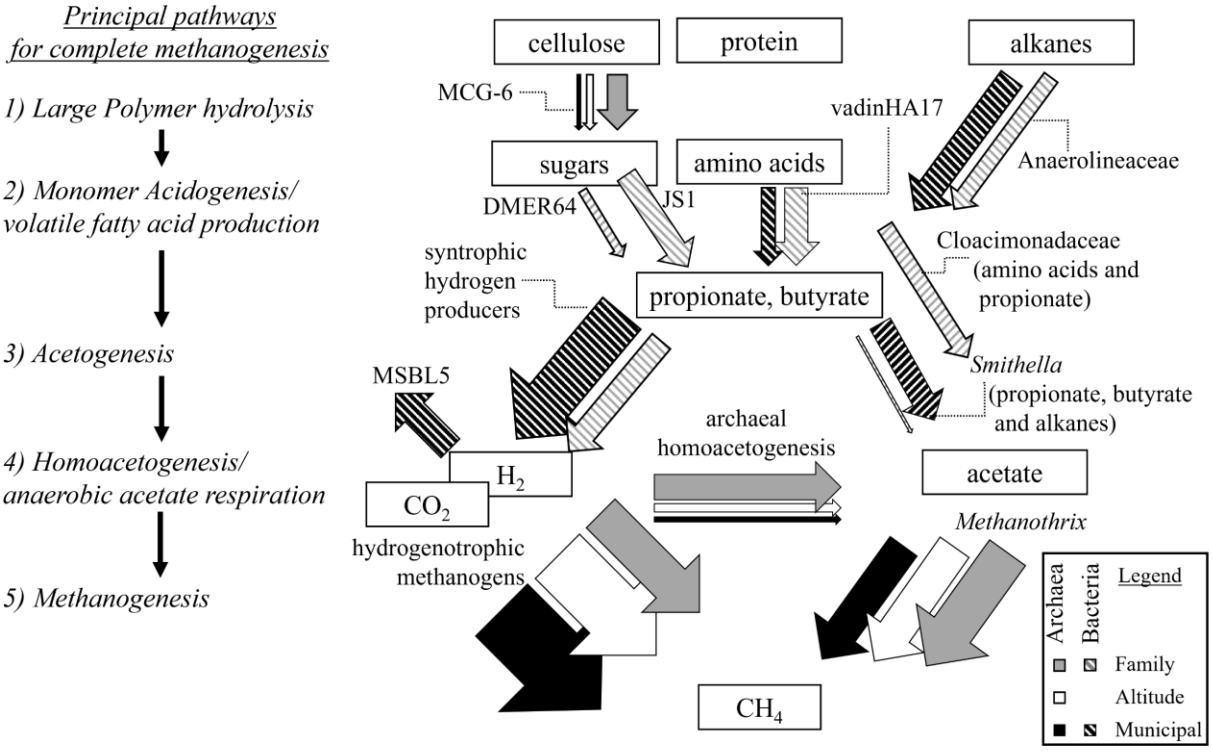

Halobacteriota
Bathyarchaeota
Thermoplasmatota
Euryarchaeota
Woesearchaeota
Micrarchaeota
Iainarchaeota
Aenigmarchaeota
A. Family Digester
Group 1
ArcOTU1 39%
Methanothrix 40%
Methanoculleus 7%
uncharacterized Methanomicrobiales 8%
Methano-linea 2.6%
MCG-6 18%
uncultured Methano-massiliio-coccaceae 4.5%
Methano-bacterium 2.8%
B. High Altitude Digester
uncharacterized Methanomicrobiales 31%
Group 1
ArcOTU1 31%
Methanothrix 32%
uncultured Methanomassil-iiococcaceae 9%
Methanomassil-iiococcus 5%
Methano-brevibacter 6%
Methano-bacterium 3.3%
uncultured Methanofasti-diosales 3.8%
MCG-6 6%
C. Municipal Digester
uncharacterized Methanomicrobiales 25%
Methanolinea 13%
ArcOTU16 6%
Group 2
Group 1
ArcOTU1 21%
ArcOTU2 1.4%
Methanothrix 30%
Uncultured Methano-massiliiococcaceae 12%
Methanobacterium 9%
MCG-6 3.3%

742

743


Bacteroidota
Chloroflexi
Firmicutes
Desulfobacterota
Caldatribacteriota
Cloacimonadota
Proteobacteria
Spirochaetota
Planctomycetota
Synergistota
Armatimonadetes
Acidobacteriota
Verrucomicrobiota
Fibrobacterota
Actinobacteriota
A. Family 1 Digester
Bacteroidetes vadinHA17 13%
Rikenellaceae DMER64 6%
Caldatribacteroita JS1 24%
uncultured Anaerolineaceae 11%
Candidatus Cloacamonas 11%
Phycisphaerae SG8-4 4%
B. Family 2 Digester
Bacteroidetes vadinHA17 9%
Rikenellaceae DMER64 6%
uncultured Dysgonomonadaceae 3%
Bacteroidales SB-5 2%
Saccharofermentans 2.9%
Acholeplasma 2%
Candidatus Cloacamonas 8%
Cloacimonadaceae W5 2.5%
uncultured Anaerolineaceae 8%
Treponema 7%
Fibrobacterales BBMC-4 3.0%
C. Family 3 Digester
Bacteroidetes vadinHA17 19%
Rikenellaceae DMER64 4%
Caldatribacteroita JS1 15%
uncultured Anaerolineaceae 5%
Anaerolineaceae Adurb.Bin120 5%
Saccharofermentans 2.0%
Smithella 2.9%
Candidatus Cloacamonas 8%
uncultured Armatimonadota 6%
Phycisphaerae SG8-4 2.6%
D. Municipal Digester
Dehalococcoidia MSBL5 16%
uncultured Anaerolineaceae 10%
Longilinea 2.5%
Smithella 14%
Syntrophus 2.5%
Syntrophorhabdus 2.6%
Bacteroidetes vadinHA17 8%
Palsa1ibacter 2.5%
Lachnospiracea UCG-004 2.8%
Synergistaceae JGI-0000079-D21 2.9%

745


Principal pathways
for complete methanogenesis
1) Large Polymer hydrolysis
2) Monomer Acidogenesis/
volatile fatty acid production
3) Acetogenesis
4) Homoacetogenesis/
anaerobic acetate respiration
5) Methanogenesis
cellulose
protein
alkanes
MCG-6
vadinHA17
Anaerolineaceae
sugars
amino acids
DMER64
JS1
syntrophic
hydrogen
producers
propionate, butyrate
Cloacimonadaceae
(amino acids and
propionate)
Smithella
(propionate, butyrate
and alkanes)
MSBL5
archaeal
homoacetogenesis
acetate
H2
CO2
Methanothrix
hydrogenotrophic
methanogens
CH4
Legend
Archaea
Bacteria
Family
Altitude
Municipal

746