SEPTIC TANK MICROBIAL COMMUNITIES

AN INVESTIGATION OF POTENTIAL RELATIONSHIPS BETWEEN SEPTIC TANK MICROBIAL COMMUNITIES AND SYSTEM DESIGN AND PERFORMANCE

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Lay Abstract

Septic systems (anaerobic digesters) are extensively used for on-site wastewater treatment. We evaluated the use of next-generation DNA sequencing to (1) assess the variability of septic system microbial communities and (2) to investigate relationships between communities and septic system type/performance. Microbial communities within septic systems were determined to be heterogeneous. Analyses also indicated that communities were highly variable between septic systems. Despite this variability, specific system types exhibited distinct microbial profiles. System performance was positively correlated with the abundance of hydrogen-producing bacteria. These results demonstrate the potential of next-generation DNA sequencing as a new tool to augment traditional wastewater analyses.

Abstract

Septic tanks are utilized by many households across North America for wastewater treatment. Despite the economic and environmental importance of septic tanks, there has been limited innovation in septic tank design and research on the microbial communities responsible for wastewater treatment within these systems. InnerTube systems are septic tanks that employ a novel design to reduce solid accumulation in comparison to conventional septic tanks. For this project, 16S metabarcoding was employed to characterize conventional and InnerTube septic tank microbial communities and evaluate relationships between community composition, system design, and treatment efficacy. Wastewater was sampled along the length of InnerTubes to determine patterns of microbial succession and how they may impact InnerTube function. Wastewater was separated into liquid and solid fractions to identify differentially abundant taxa in each fraction. Populations of methylotrophic methanogens increased with distance from the InnerTube inlet. Solid communities were differentially more abundant in methanogens than liquid communities. Higher rates of solid degradation in InnerTubes may be due to longitudinal stratification of substrates and functionally distinct communities and the activity of methanogenic biomass. Septic tanks throughout Ontario were also surveyed to evaluate the effect of system design (conventional vs. InnerTube) and operational flow (single-pass vs. recirculation) on microbial community composition and to identify taxa correlated with chemical oxygen demand (COD) reduction. Single-pass InnerTube communities were more abundant in *Pseudomonas* which was attributed increased availability of long-chain fatty acid substrates. Recirculating conventional communities were more abundant in *Arcobacter* and *Desulfomicrobium* which was attributed to greater resistance to oxidative stress. *Desulfovibrio* and *Brevundimonas* were positively correlated with COD reduction. These putative hydrogen producers may facilitate greater COD reduction by forming syntrophic relationships with

hydrogenotrophic methanogens. The findings of this project may be used to develop bioaugmentation inoculum, system designs, or operational strategies to optimize septic tank performance.

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List of Acronyms

ACE – Abundance-based Coverage Estimator

AD – Anaerobic Digester

ASV – Assigned Sequence Variant

BNQ – Bureau de Normalisation du Québec

BRC1 – Bacterial Rice Cluster 1

CAP – Constrained Analysis of Principal Coordinates

CBOD – Carbonaceous Biological Oxygen Demand

COD – Chemical oxygen demand

EMP – Earth Microbiome Project

HVR – Hypervariable Region

LCFA – Long-Chain Fatty Acid

MASSTC – Massachusetts Alternative Septic System Test Center

NMDS – Non-Metric Dimensional Scaling

OTU – Operational Taxonomic Unit

PERMANOVA – Permutational Analysis of Variance

PFR – Plug Flow Reactor

RC – Recirculating Conventional

RI – Recirculating InnerTube

SBS – Sequencing by Synthesis

SC – Single-Pass Conventional

SGS – Second-Generation Sequencing

SI – Single-Pass InnerTube

VFA – Volatile Fatty Acid

VST – Variance Stabilizing Transformation

WBS – Waterloo Biofilter Systems Inc.

Chapter 1: Review of Wastewater Treatment and Sequencing Technologies

1.1 Environmental Impacts of Sewage and Wastewater Treatment

The release of organic waste and nutrients from wastewater treatment facilities has negatively impacted North American freshwater ecosystems (Watson *et al.*, 2016; Holeton *et al.*, 2011). Organic wastes create carbonaceous biological oxygen demand (CBOD) because of their potential to be aerobically catabolized by microbes (Penn *et al.*, 2009). Continuous release of high CBOD wastewater results in hypoxic conditions that are fatal for aquatic biota (McCallum *et al.*, 2016; Jenny *et al.*, 2014). Nutrients, mainly nitrogen and phosphorus, have a positive effect on aquatic algal growth (Smith and Schindler, 2009). Excessive nutrient loading due to inadequate wastewater treatment causes harmful algal blooms (HABs) in coastal and freshwater environments (Lapointe *et al.*, 2015; Watson *et al.*, 2016). HABs release biotoxins that are potentially lethal to fish and invertebrates and can cause a wide range of illnesses in humans (Anderson 2009; Hilborn *et al.*, 2014). Furthermore, bacterial decomposition of these blooms produces hypoxic conditions (Watson *et al.*, 2016).

Contamination from centralized municipal wastewater treatment plants has been curtailed by improvements in treatment technology and enforcement of effluent quality regulations (Holeton *et al.*, 2011). However, millions of households across Canada and the US are not connected to centralized systems and rely on septic tanks to treat their residential wastewater (Perks and Johnson, 2008; Diaz-Valbuena *et al*., 2011). Septic tank technology has not changed substantially in the last century (Butler and Payne, 1995; Jowett *et al*., 2017) and septic tank effluent is poorly regulated. Unsurprisingly, discharge of inadequately treated waste from septic tanks (septic tank failure) is a major source of surface and groundwater contamination (Schaider *et al.*, 2017). However, septic tanks are likely to remain the most prevalent choice for on-site wastewater treatment technology due to their low operating costs (Chan *et al*., 2009). Therefore,

innovation is required to address the issue of ongoing septic tank pollution. A promising area of research that has been extensively explored in engineered systems, but not septic tanks, is the manipulation of communities of bacteria and archaea (microbes) that are responsible for waste treatment. However, to evaluate the potential for innovation in this area we must first obtain a more comprehensive understanding of the microbial communities within septic tanks.

1.2 Domestic Wastewater

Septic tanks (Fig. 1) are on-site wastewater treatment systems that also function as primary sewage receivers and solid settling tanks (Gill *et al.*, 2009). Domestic wastewater is divided into two streams, greywater and blackwater, which account for approximately 70% and 30% of daily household effluent volume, respectively (Friedler *et al.*, 2013). Organic waste and inorganic compounds within greywater and blackwater provide microbes with the carbon substrates and nutrients required for growth.

Greywater consists of effluent from household activities such as bathing, dishwashing, and laundry and blackwater consists of urine, feces, and toilet paper (Friedler *et al.*, 2013). Greywater contributes to organic carbon, nitrogen, phosphorous, copper, and zinc loadings due to food waste, grease, soaps, and detergents and metallic piping (Larsen and Maurer, 2011). Urine in blackwater is the primary source of nitrogenous compounds in septic tank effluent (Larsen and Maurer, 2011). In addition, urine contains relatively high concentrations of Na^+, Cl^-, and K^+ (Kirchmann and Pettersson, 1995). Human feces are comprised of approximately 75% water and 25% solids by mass, although these proportions can change with diet (Rose *et al.*, 2015). The solid fraction of feces is comprised of inorganic compounds, lipids, proteins, polysaccharides, and bacterial biomass (Rose *et al.*, 2015). Feces are the primary source of septic tank microbial input and solid settled sludge (Rose *et al.*, 2015). The proportions of all components in fecal solids are listed in Table 1.

1.3 Microbial Ecology of Anaerobic Digestion

Septic tanks are sealed vessels in which oxygen is quickly depleted. Therefore, treatment of domestic wastewater occurs primarily through anaerobic digestion (Diaz-Valbuena *et al*., 2011). Engineered systems, such as septic tanks, that employ anaerobic digestion are referred to as anaerobic digesters (ADs). Degradation of organic waste in ADs is carried out in 4 steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Wirth *et al.*, 2012). These processes are dependent on the coexistence of functionally diverse microbial taxa. In ADs, the metabolic products of many taxa become the growth substrates for progressively fewer taxa (Fig. 2) (Campanaro *et al.*, 2016).

During hydrolysis, extracellular bacterial enzymes break down complex lipids, proteins, and polysaccharides into fatty acids, amino acids, and simple sugars, respectively (Wirth *et al.*, 2012; Lyberatos and Skiadas, 1999). These exoenzymes are primarily produced by members of the bacterial phyla Firmicutes and Bacteroidetes (Azman *et al.*, 2015; Tang *et al.*, 2005). Acidogenesis refers to the production of volatile fatty acids (VFAs; i.e. butyric acid, propionic acid) and alcohols from the fermentation of the products of hydrolysis (Bengtsson *et al.*, 2008). The most prevalent acidogenic bacterial phyla are: Firmicutes, Bacteroidetes, Proteobacteria, Synergistetes, and Chloroflexi (Rui *et al.*, 2015; Campanaro *et al.*, 2016; Guo *et al.*, 2015; McIlroy *et al.*, 2017). Acetogenesis, or acetate formation, can occur heterotrophically or autotrophically. During heterotrophic acetogenesis, bacteria catabolize VFAs to generate adenosine triphosphate (ATP) and produce acetate, hydrogen gas (H_2), and carbon dioxide (CO_2) as by-products (Chalima *et al*., 2017). Autotrophic acetogenesis occurs through a series of biochemical reactions, called the Wood-Ljungdahl pathway, in which bacteria convert CO_2 and H_2 into acetate (Ragsdale and Pierce, 2008). Most autotrophic acetogenic bacteria in ADs belong to the phylum Firmicutes (Rui *et al.*, 2015; Campanaro *et al.*, 2016).

Extensive research of methanogenesis has been done to develop waste-derived methane (CH_4) as an alternative energy source (De Vrieze and Verstraete, 2016). Methanogenesis is carried out by acetoclastic, hydrogenotrophic, and methylotrophic archaea. These archaea are collectively referred to as methanogens and belong to the phyla Euryarchaeota, Bathyarchaeota, and Verstraetearchaeota (Berghuis *et al.*, 2019). CO_2, H_2, methyl compounds, and acetate produced during acidogenesis and acetogenesis are substrates for methanogenesis. Acetoclastic methanogens convert acetate into CH_4 and CO_2, hydrogenotrophic methanogens utilize H_2 or format (HCO_2^-) to reduce CO_2 to form CH_4, and methylotrophic methanogens convert methanol, methylamine, and simple halogenated organics into CH_4 (Liu and Whitman, 2008). CH_4 formation in each pathway is coupled to ATP production (Thauer 1998; Kaster *et al.*, 2011). Methanogens increase the thermodynamic favourability of the ATP-generating reactions of other microbes by consuming their metabolic end-products (Morris *et al*., 2013). As a result, acidogenic and acetogenic bacteria form mutually beneficial relationship with methanogens. Formation and maintenance of these cross-feeding relationships is referred to as syntrophy (Morris *et al*., 2013).

1.4 Microbial Ecology of Nitrogen Reduction

The primary function of septic tanks is to break down organic waste and decrease oxygen demand loading into the surrounding environment. When coupled with aerobic treatment systems, anaerobic microbial processes can also reduce effluent nitrogen levels (Jaafari *et al.*, 2017). Anaerobic digestion of proteins and other organic nitrogen compounds releases ammonia (NH_3) (Tang *et al.*, 2004). During aerobic treatment, NH_3 is converted to nitrate (NO_3^-) through nitrification (Awolusi *et al.*, 2015). In recirculating systems, aerobic treatment effluent containing NO_3^- is returned to the septic tank. Under anaerobic conditions, heterotrophic bacteria utilize NO_3^- as an electron acceptor to produce ATP in a process called denitrification (Shoda,

2017). This includes multiple enzyme-catalyzed reactions in which NO_3^- is sequentially reduced to nitrite (NO_2^-), nitric oxide (NO^-), nitrous oxide (N_2O), and finally nitrogen (N_2) which escapes wastewater as a gas (Shoda, 2017). Denitrifying bacteria primarily belong to the phylum Proteobacteria (Campanaro *et al.*, 2016; Lu *et al.*, 2014).

Another potential biochemical pathway for anaerobic nitrogen reduction in recirculating systems is the anammox process. In this process, NH_4 is oxidized by NO_3^- (Robertson *et al.*, 2012) or NO_2^- to produce N_2 (Kuenen, 2008). Energy created from this process is utilized by autotrophic bacteria to fix carbon for cellular growth (Kuenen, 2008). Members of the bacterial phylum Planctomycetes are capable of anammox metabolism (Jin *et al.*, 2012).

1.5 Industrial Applications of Microbial DNA Analysis and Bioaugmentation

Bioaugmentation refers to the enhancement of waste degradation through the addition of specific microbes (Tale *et al.*, 2015). By studying microbial communities within ADs, researchers can identify microbial species or community compositions that are more proficient at carbon and nutrient reduction. This data can then be used to manufacture inoculum for bioaugmentation (Tale *et al.*, 2015). Sun *et al.* (2016) bioaugmented bioreactors with slurries containing *Clostridium cellulolyticum* which resulted in greater rates of cellulose and straw degradation. Furthermore, lab-scale activated sludge reactors inoculated with *Rhodococcus sp*. YYL, *Bacillus cereus* MLY1, and *Bacillus aquimaris* MLY2 were able to degrade tetrahydrofuran, whereas control reactors containing only activated sludge could not (Yao *et al.*, 2013). Interestingly, the addition of the *Rhodococcus* sp., a tetrahydrofuran-degrader, alone did not increase tetrahydrofuran removal (Yao *et al.*, 2013), which indicates the combined metabolism of the 3 bacterial species is required for tetrahydrofuran degradation. In addition, acetate catabolism and methane production in failing bioethanol waste and manure digesters are improved by inoculation with acetoclastic consortium (Town and Dumonceaux, 2016).

Microbial community surveys can also be used to improve septic tank design by identifying

operating parameters and design modifications that promote the growth of functionally relevant microbes (Venkiteshwaran *et al.*, 2015). In a comparison of two triplicate sets of lab-scale expanded granular sludge bed reactors with differing dimensions, narrower, longer reactors showed greater abundances of Firmicutes and species richness than their wider counterparts (Connelly *et al.*, 2017). The microbial communities in the narrower, longer reactors were associated with greater oxygen demand reduction and methane production (Connelly *et al.*, 2017).

1.6 Second Generation Sequencing

Characterization of complex microbial communities is currently accomplished by culture-independent DNA sequence analysis using high-throughput second-generation sequencing (SGS) (Morgan and Huttenhower, 2012). In comparison with culture-dependent techniques, SGS is a relatively unbiased method for the identification and proportional quantification of functionally relevant, rare, and unculturable taxa (Su *et al.*, 2012; Cocolin *et al.*, 2013). Illumina sequencing platforms have been widely adopted as the sequence platforms of choice (Schirmer *et al.*, 2016) for these applications due to lower per-base costs and error-rates and more reads output in comparison to other SGS platforms (Liu *et al.*, 2012). The following is a summary of the sequencing-by-synthesis (SBS) process utilized in Illumina sequencing technology (Illumina, 2019).

During SBS, DNA is channeled through flow cells embedded with oligonucleotides complementary to adapter sequences attached to the DNA to be sequenced. After DNA is bound to the flow cell, copies are generated through bridge amplification, a process similar to PCR. Copies of DNA localized to an area of the flow cell are called clusters. DNA clusters are

sequenced by the addition of fluorescently labelled nucleotides with reversible terminators. With the addition of each nucleotide, fluorescent signals unique to each base are detected for every cluster. The number cycles of addition and detection are designated by the read length. Illumina platforms can sequence DNA molecules from both ends to produce forward and reverse reads. If there is an overlapping region between the forward and reverse read, a contiguous DNA sequence can be assembled (Illumina, 2019).

1.7 16S Ribosomal RNA Gene Sequencing

16S rRNA gene (16S) amplicon sequencing is the de facto standard for microbial metabarcoding (Yang *et al.*, 2016) and is utilized in microbiome studies in systems ranging from the *Caenorhabditis elegans* gut (Zhang *et al.*, 2017) to the Amazon River (Tessler *et al.*, 2017). 16S rRNA is a ubiquitous and highly conserved subunit of bacterial and archaeal ribosomes that facilitates transcript binding and translation (Li *et al.*, 2012). Bacterial and archaeal phylogeny is based on levels of similarity between full-length (~1540 bp)16S sequences (Kim *et al.*, 2011). Within 16S, there are nine (V1 – V9) hypervariable regions (Fig. 3) (HVRs); gene fragments with relatively diverse nucleotide compositions that can distinguish microbes at the species level (Barb *et al.*, 2016).

HVRs are flanked by conserved sequences that allow them to be targeted and amplified through polymerase chain reaction (PCR) using universal primers that capture a broad range of taxa (Chakravorty *et al.*, 2007). PCR amplification allows for the isolation of 16S genes from complex mixtures of DNA. In addition, PCR can be used to attach adapter sequences that facilitate binding to sequencing machines and index sequences that identify the sample of origin for each amplicon. By convention, 16S primers are named according to their corresponding nucleotide positions in *E. coli* 16S (Chakravorty *et al.*, 2007) and their replication direction with respect to the 5’ to 3’ direction of the sense strand denoted by ‘f’ and ‘r’ for forward and reverse,

respectively. For example, 341f/785r is a primer pair that spans the V3 – V4 regions (Klindworth *et al.*, 2013).

Taxonomic profiles consisting of the bacterial and archaeal taxa within a sample and their relative abundances are constructed from the analysis of HVRs. Microbial groups are identified based on HVR composition and the number reads attributed to each group are used to calculate the relative abundance those organisms in a sample. HVR-based taxonomic assignment is reliable down to the genus level (Bokulich *et al.*, 2018). However, taxonomic assignment accuracy substantially decreases at the species level (Bokulich *et al.*, 2018). Species assignment is only recommended for HVR sequences that match exactly with reference database sequences (Edgar, 2018).

1.8 Hypervariable Region and Primer Choice

Illumina platforms are currently unable to sequence the entire length of 16S in a single read. Therefore, researchers amplify and analyze shorter DNA fragments (Yang *et al.*, 2016) that may include sequence elements of up to 3 HVRs. However, some taxa may be difficult to resolve based on a given 16S region, therefore, microbial community profiles can differ depending on the HVR(s) selected (Walters *et al.*, 2016). There is a lack of consensus regarding which HVRs are the most reliable for taxonomic profiling (Fouhy *et al.*, 2016). Ideally, studies without *a priori* targets should employ 16S regions that capture the greatest degree of diversity and obtain accurate estimates of relative abundance. Table 2 shows examples of HVRs used in literature studies of different systems.

In addition to the HVR regions targeted, primers can differ in based on sequence composition. 16S sequencing results obtained from targeting the same HVR with different primers can vary due to primer bias; greater primer binding affinity for sequences that have fewer base mismatches. An *in-silico* evaluation of 16S primers determined that a V4 primer

(515f/806r) provided both bacterial and archaeal coverage (Klindworth *et al.*, 2013). Allowing for one base mismatch greatly increases primer taxonomic coverage *in silico* (Klindworth *et al.*, 2013; Parada *et al.*, 2016). However, experimental primer testing has shown that even single base mismatches can bias sequencing results against specific taxa (Apprill *et al.*, 2015). Substituting bases prone to mismatch with degenerate bases can reduce primer bias (Apprill *et al.*, 2015).

1.9 V4 Amplicon Sequencing for Septic Tank Microbial Community Profiling

AD studies have utilized other HVRs such as V1 – V3 (27f/ 519r), V3 – V4 (349f/806r) (Razaviarani and Buchanan, 2014), V6 – V8 (926f/1392r) (Vanwonterghem *et al.*, 2014), and V5 – V6 (807f/1050r) (De Vrieze *et al.*, 2016). Primers that include multiple HVRs accurately characterize mock communities and provide greater taxa resolution due to the greater amount of sequencing information compared to single HVR primers (Parada *et al.*, 2016). However, longer sequences have shorter overlaps between paired ends which may lead to the loss of reads during sequence quality filtering (Whelan and Surette, 2017). Base calls at read termini tend to have lower quality than preceding base calls. Quality-filtering programs trim lower quality bases and discard paired reads that cannot be assembled with a threshold overlap (Masella *et al.*, 2012). Sequencing longer 16S fragments may increase the risk of read loss due to lack of sequence overlap.

Despite the lack of consensus regarding HVR and primer choice for microbial taxonomic profiling, informed decisions can be made based on in silico and empirical testing of primer pairs. Consistency in ongoing microbiome studies to increases the likelihood that observed community differences are a result of system environment and biological activity opposed to changes in the methodology (Fouhy *et al.*, 2016; Tremblay *et al.*, 2015). Many studies utilize the 515f/806r V4 primer published by the Earth Microbiome Project (EMP) (Thompson *et al.*,

2017). The EMP is a collaboration of hundreds of researchers to catalogue the Earth's bacterial and archaeal communities (Thompson *et al.*, 2017). The EMP primer pair has been designed to include degenerate bases to reduce primer bias and broaden taxonomic coverage (Caporaso *et al.*, 2011; Apprill *et al.*, 2015). Similar 515f/806r V4 primer pairs have been used to characterize microbial communities in human stool (Halfvarson *et al.*, 2017; Jovel *et al.*, 2016) and urine (Kramer *et al.*, 2018) which are major components of septic tank influent. Furthermore, 515f/806r V4 primer pairs have been validated in silico (Mizrahi-Man *et al.*, 2013; Klindworth *et al.*, 2013) and experimentally.

1.10 16S Sequence Data Processing Methods

16S amplicon sequence data are outputted in text format with quality scores which indicate the probability of error for each base call (Bokulich *et al.*, 2013). Reads are grouped into files according to their sample of origin based on index sequences in a process called demultiplexing. Before taxonomic assignment, sample sequence reads are trimmed to remove adapter sequences (Bolger *et al.*, 2014) and low-quality bases (Joshi and Fass, 2011) to prevent spurious taxonomic assignment. Paired-end reads can be merged if they meet a threshold for sequence overlap (Massella *et al.*, 2012). In addition, reads should be processed to identify and remove chimeric sequences that can be mistakenly attributed to unique taxa (Edgar *et al.*, 2011). Sequences are then taxonomically classified using bioinformatic tools that align sample sequences with sequences in 16S reference databases (Bokulich *et al.*, 2018). The primary reference databases used in 16S microbiome studies are Greengenes, RDP, SILVA and NCBI (Balvociute and Huson, 2017).

A common method for sequence annotation is to first cluster sequences at a level of 97% similarity into operational taxonomic units (OTUs) and assign taxonomy to these OTUs by comparing representative sequences with sequences in reference databases (Bokulich *et al.*,

2013). Whelan and Surette (2017) compared multiple clustering programs and determined that some algorithms produced substantially erroneous results when characterizing human-sourced mock microbial communities. Most notably, for certain mock communities, DNACLUST overestimates diversity by nearly 4000% and UPARSE underestimates diversity by greater than 300%. Although no single clustering program outperformed all others for every mock community, AbundantOTU+ coupled with the GreenGenes 2011 database provided the best overall performance (Whelan and Surette, 2017).

Errors associated with clustering algorithms can be circumvented with DADA2, an alternative amplicon processing program that uses Illumina error-modelling to resolve sequence reads (Callahan *et al.*, 2016). Rather than OTUs, DADA2 produces assigned sequence variants (ASVs). Whereas OTUs may change with the sequences included in an analysis (Westcott and Schloss, 2015), ASVs are characterized on a sequence-to-sequence basis using sequencing error profiles and are thus more stable (Callahan *et al.*, 2017). Therefore, between-study comparisons of ASVs are more robust than those of OTUs because they are less dependent on study-specific data processing parameters. In benchmarking tests, DADA2 outperforms other established OTU sequence clustering programs when characterizing mock, mouse fecal and human vaginal communities (Callahan *et al.*, 2016). DADA2 provides greater resolution for the identification of sequence variants and has lower output residual error rates, fewer false positives, and more correct taxonomic assignments than UPARSE, MED, UCLUST and mothur (Callahan *et al.*, 2016). ASV stability and greater taxonomic assignment accuracy make DADA2 a strong candidate for the sequence processing program of choice for future 16S microbiome studies.

1.11 Effect of Sequencing Depth on Observed Community Composition

The total number of 16S sequence reads (sequencing depth) may differ by several orders of magnitude between samples. This complicates statistical analyses because variation in read

counts can lead to differences between sample communities due to sequencing depth rather than biology (Weiss *et al.* 2017). Furthermore, the number of taxa, particularly rare species, detected increases with sequencing depth (Thompson *et al.*, 2017), which leads to greater estimates of diversity (McMurdie and Holmes, 2014). A common practice to account for sequencing depth differences is to rarefy sample counts (Weiss *et al.*, 2017) by randomly removing sequence reads without replacement until all samples reach a chosen sequencing depth (Caporaso *et al.*, 2011). Sequencing depths should be chosen to balance the number of samples kept with level diversity captured. In some cases, it is advisable to discard samples with lower read counts because these samples may contain higher proportions of contaminating sequences (Salter *et al.*, 2014).

An alternative method to removing counts to normalize read counts is to apply a variance stabilizing transformation (VST) to count data (McMurdie and Holmes, 2014). Proponents of VST argue that rarefying data discards statistically relevant data and that VST offers more statistical power when differentiating sample groups (McMurdie and Holmes 2014). However, opponents of VST argue that VST fails to address library size effects and leads to higher false discovery rates than rarefying (Weiss *et al.*, 2017). Furthermore, they argue that rarefied community data clusters more accurately according to biological origin than VST community data (Weiss *et al.*, 2017).

1.12 Limitations of 16S Analyses for Functional Inferences of Anaerobic Digester Communities

16S sequencing is used identify microbial taxa and determine the composition and relative abundance profiles of microbial communities. It cannot be used to verify the presence of genes of interest or evaluate microbial metabolic activity. This information can be obtained with metagenomic or transcriptomic analyses, respectively. However, 16S sequencing is used to infer the metabolic potential of microbial communities based on phylogenetic relatedness of assigned

taxa to reference taxa with known functions (Langille *et al*., 2013). Although related taxa often share phenotypes, functional inferences based on taxonomic assignment can be incomplete or misleading (Langille *et al*., 2013; Inkpen *et al*., 2017) because the reliability of these inferences varies substantially with taxa and scale of taxonomic resolution. For example, although H_2 production can be attributed to the genera *Brevundimonas* and *Desulfovibrio* (Danial *et al*., 2015; Li *et al.*, 2011), H_2-production is inconsistent at the species level, and even the strain level, for *Brevundimonas* (Danial *et al*., 2015). In contrast, most species of *Desulfovibrio* possess genes involved in H_2 production (Li *et al.*, 2011). Microbes can also acquire functional genes through horizontal gene transfer (Sheth et al., 2016) which is not readily discernible through analysis of phylogenetic relationships. Therefore, inferences of metabolic potential based on taxonomy should be evaluated on a case-by-case basis.

1.13 Current Project

Septic tanks are the most widely utilized on-site wastewater treatment systems in North America (Diaz-Valbuena *et al*., 2011). When these systems fail, they release nutrients that are harmful for local watersheds and pathogens that may negatively impact human health. Despite the economic and environmental importance of septic tanks, there is limited research on the microbes within these systems which are responsible for wastewater treatment. The overall goal of this project was to gain a better understanding of septic tank microbial communities which may facilitate the development of designs and operational parameters that improve system performance. To this end, we characterized septic tank microbial communities using 16S sequencing to identify relationships between septic tank microbial communities, system design, and treatment efficacy.

In the following chapters, we explore relationships between microbial communities and the heterogeneous environment of modified septic tanks to explain trends in system performance.

Furthermore, we quantify differences between microbial communities from 4 septic tank types to determine the effect of system design on community composition, identify microbial taxa associated with increased waste degradation, and assess variability within and between system types. The results of this project will improve our understanding of septic tank processes responsible for waste degradation and identify taxa and system parameters that may be used in future bioaugmentation strategies and septic tank designs.

Table 1: Fecal Solid Fraction Composition (Rose *et al.*, 2015) (Section 1.2)

Fecal Component	Proportion by Weight (%)
Inorganic Compounds	7.5 - 16
Lipids	2 - 15
Proteins	2 - 25
Polysaccharides	25
Bacterial Biomass	25 - 54

Table 2: Hypervariable Regions Analyzed in Studies of the Human Microbiome, Anaerobic Digesters, and the Environment (Section 1.8)

Hypervariable Regions	Sample Types	Primer Pairs	Literature References
V1 – V2	Human Oral Cavity, Activated Sludge	27f-338r, 8f/357r	Szafrankski *et al.*, 2015; Guo *et al.*, 2013
V1 – V3	Human Skin, Human Gut	27f/534r, 27f/519r	Meisel *et al.*, 2016; Walker *et al.*, 2015
V3	Human Gut, Soil	341f/518r; 338f/533r	Lau *et al.*, 2016; Bartram *et al.*, 2011
V3 – V4	Freshwater, Human Gut	341f/805r, 341f/785r	Klindworth *et al.*, 2013; Parulekar *et al.*, 2017
V4	Freshwater, Soil	515f/806r, 515f/808r	Tessler *et al.*, 2017; Whitman *et al.*, 2018
V4 – V5	Anaerobic Digester	515f/909r, 563F/926R	Kuroda *et al.*, 2016; Rui *et al.*, 2015
V5 – V6	Freshwater, Anaerobic Digester	807f/1050r	Brown *et al.*, 2017; De Vrieze *et al.*, 2016
V6 – V8	Anaerobic Digester, Honey Bee Gut	926f/1392r, 969f/1406r	Vanwonterghem *et al.*, 2014; Anderson *et al.*, 2017

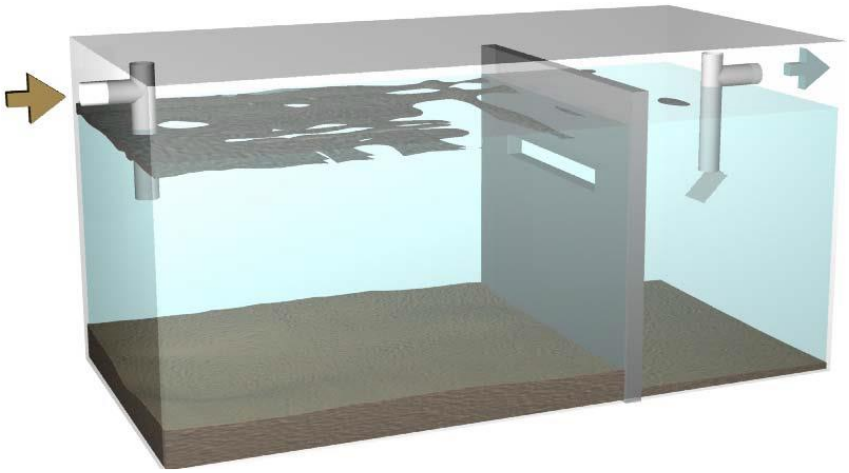

Figure 1: Flow through a conventional septic tank (WBS, 2009). The yellow arrow indicates the influent entry point and the light blue arrow indicates the effluent exit point. Scum and settled solids are shown at the top and bottom of the septic tank, respectively (Section 1.2).

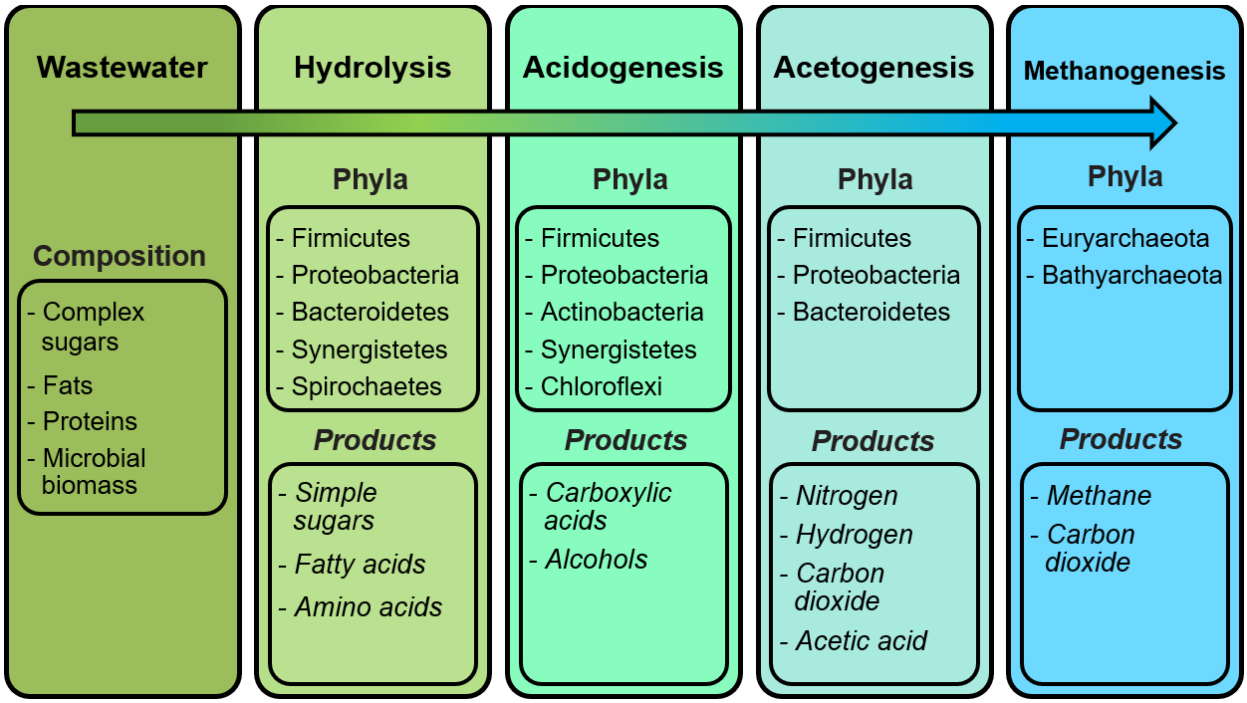

Figure 2: Microbial phyla responsible for anaerobic wastewater treatment processes – hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Decomposition of organic substances occurs in a stepwise fashion in which the metabolites of one group of microbes become the substrates of the next (Section 1.3).

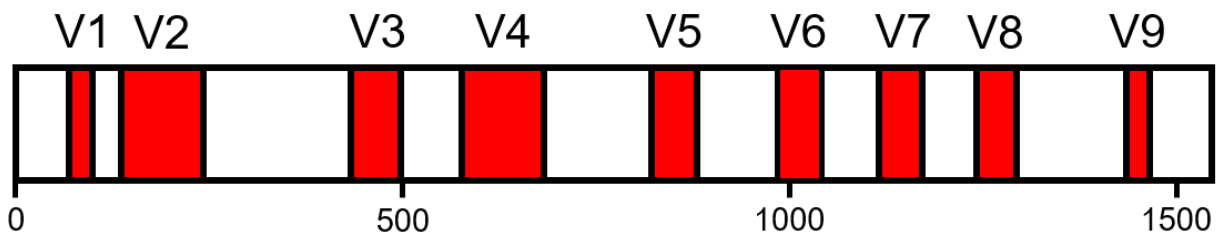

Figure 3: Distribution of V1 – V9 hypervariable regions (HVRs) along the Escherichia coli 16S sense strand. HVRs are shown in red, with widths to scale; white regions represent conserved regions and numbered tick marks indicate nucleotide position (Section 1.7).

Chapter 2: Investigation of Plug Flow Reactor Properties and Settled Solid Microbial Communities in a Novel Septic Tank Design

2.1.1 Septic Tank Design and Failure

Septic tank designs have not changed substantially since their invention over a hundred years ago (Withers *et al.*, 2014). Septic tanks are underground vessels; made of polyethylene, steel, or concrete; that consist of one or more compartments. Tanks with multiple compartments tend to produce higher quality effluent than single compartment systems because they allow sludge settling in upstream compartments prior to the outlet (Gray, 1995; Butler and Payne, 1995). A standard septic tank for a household of 4 has an approximate volume of 3000 L (Gray, 1995). Septic tanks receive sewage directly from a household or mixed influent from several households. The duration for which wastewater remains in the septic tank, or hydraulic retention time (HRT), depends on tank size and water use rates. Generally, HRT range from 1 – 2 days (Gray, 1995). After treatment, effluent from a septic tank is discharged to a drainfield or downstream aerobic treatment systems.

The primary function of septic tanks is to break down organic matter while downstream processes reduce effluent nutrient and pathogen levels (Withers *et al.*, 2014). From top to bottom, wastewater in septic tanks is divided into three layers: a solid surface scum layer, intermediate mixed liquor zone, and a layer of settled solid sludge (Butler and Payne, 1995). The scum layer is comprised of low-density organic compounds and buoyant moulds that grow in the airspace between wastewater and the septic tank ceiling (Diaz-Valbuena *et al.*, 2011; Lay *et al.*, 2005). The mixed liquor zone is comprised of wastewater liquid, dissolved nutrients, and suspended solids. Biomass solids in the mixed liquor zone and the bottom layer of the septic tank contain microbes responsible for anaerobic waste degradation (Wang *et al.*, 2011; Kuroda *et al.*, 2016).

Digestion of solids is often outpaced by solid deposition which results in the accumulation of settled solids at the bottom of a septic tank. Excessive accumulation of solids can lead to septic tank failure – inadequate treatment of sewage and release of CBOD, nutrients, and pathogens into the surrounding environment (Ahmed and Katouli, 2005). As solids accumulate in a septic tank, the available volume for incoming sewage decreases which leads to decreased solid settling and treatment times, increased effluent solids, drainfield clogging, and surface runoff of untreated sewage (Withers *et al.*, 2014; Garcia *et al.*, 2013). The development of a thick scum layer can also clog systems and lead to treatment failure (Dickey *et al.*, 1979). Septic tanks require periodic pump-outs (mechanical removal of solids) to prevent scum and sludge build-up and failure. However, pump-outs often do not occur because of an owner's inexperience or reluctance due to associated costs (Withers *et al.*, 2014). Therefore, a septic tank that minimizes solid and scum accumulation would greatly benefit homeowners and watersheds.

2.1.2 Improvements on Septic Tank Design

Waterloo Biofilter Systems Inc. (WBS) is a company based in Ontario that has designed and maintained on-site wastewater treatment systems since 1993 (WBS, 2019). WBS manufactures conventional septic tanks and modified septic tanks that incorporate a corrugated plastic tube, called an InnerTube, at the inlet of a septic tank that curves back on itself towards the bottom of the vessel (Fig. 4). In pilot-studies and commercial installations, InnerTube septic tanks have been shown to accumulate less scum and solid sludge than conventional septic tanks (Jowett *et al.*, 2017). In an 8-month comparison of an InnerTube septic tank and a conventional septic tank receiving the same influent, the conventional septic tank required two pump-outs due to solid build-up while the InnerTube septic did not require any pump-outs (Jowett *et al.*, 2017). Furthermore, InnerTube septic tank effluent contained an average of 30.1% less total suspended solids than the conventional septic tank throughout the trial (Jowett *et al.*, 2017). Decreased

scum and sludge production was attributed to the elimination of air spaces, increased sewage and treatment biomass contact, and warmer temperatures within the narrower diameter of the InnerTube (Jowett *et al.*, 2017).

2.1.3 Anaerobic Digestion in Plug Flow Reactors

The InnerTube design results in wastewater flow that is similar to the process flow found in plug-flow reactors (PFRs) (Fig. 4). In an ideal PFR, substrates are consumed by microbial processes as they flow through the reactor (Rittman and McCarty, 2001) and waste undergoes radial mixing but no longitudinal mixing (Pal, 2017). Therefore, substrates travel along the length of the tube in discrete segments (Fig. 4, 'A → B'), or 'plugs', that are chemically distinct from proximal and distal segments (Rittman and McCarty, 2001). PFRs maximize waste degradation rates and microbial growth by increasing the substrate concentrations to which microbes are exposed (Han and Levenspiel, 1988). In fully mixed reactors, such as continuously stirred tank reactors, substrate concentrations are reduced when influent is mixed with the substrate-depleted liquid already present in the reactor (Pal, 2017).

2.1.4 Spatial Variation in Plug Flow Reactor Microbial Communities

In the theoretical model of a plug flow AD, a gradient of chemical environments exists along the length of the PFR (Donoso-Bravo *et al.*, 2018). This gradient develops as organic matter in each plug independently progresses through the stages of anaerobic digestion (Li *et al.*, 2014). For example, plugs near the inlet contain more complex polysaccharides, lipids, and proteins that have yet to be broken down through hydrolysis, whereas distal plugs contain greater concentrations of simple carbon compounds such acetate that may be utilized for methanogenesis. The structure of microbial communities is dependent on substrate composition and availability (De Vrieze *et al.*, 2016; Gao *et al.*, 2019). Therefore, stratification of chemical

environments is expected to coincide with differences in microbial communities within an ideal PFR (Li *et al.*, 2014).

In agreement with the ideal PFR model, longitudinal stratification has been observed in operating PFRs. In a study of a lab-scale plug flow reactor treating pineapple waste, acidogenic activity decreased from inlet to outlet while methanogenic activity increased (Namsree *et al.*, 2012). Decreasing microbial diversity along the length of a swine manure PFR was attributed to a decrease in substrate diversity (Roy *et al.*, 2009). Acidogenic and acetogenic bacteria were enriched at the midpoint of a PFR processing cow manure in comparison with digester feed communities (Dong *et al*., 2019). However, PFRs may deviate from the ideal model. Digestate communities and chemical compositions within another cow manure PFR were homogeneous despite 5-day differences in retention time (Li *et al*., 2014).

2.1.5 Functional Potential of Plug Flow Reactor Solids

PFRs accumulate solids (Masse *et al.*, 2013) which creates a two-phase system (liquid and solid) with potentially distinct communities and different waste-degrading capacities in each phase. Anaerobic microbes form aggregates that settle out from liquid mixtures (Kalyuzhnyi *et al.*, 2006). These aggregates, or granules, are micro-ecosystems that contain functionally coordinated bacteria and archaea capable of mineralizing wastewater organics (Kuroda *et al.*, 2016). Upflow anaerobic sludge blanket (UASB) reactors (Fig. 5) are the primary example of anaerobic granule utilization in wastewater treatment. In these systems, wastewater is pumped upwards through a layer of granular biomass that has a greater settling velocity than the upward velocity of the wastewater (Kalyuzhnyi *et al.*, 2006). Organic compounds are catabolized as wastewater flows through a ‘blanket’ of granules (Kalyuzhnyi *et al.*, 2006). Granule formation has not been well-characterized in PFRs. However, PFR biomass retention can be beneficial for wastewater treatment (Donoso-Bravo *et al.*, 2018). Similar to the sludge blanket in UASBs, the

settled solids in PFRs may contain granules that facilitate organic degradation as wastewater flows through these systems.

2.1.6 Chapter 2 Objectives

The overall objective for this chapter was to investigate microbial communities that may carry out metabolic process that explain the improved performance observed in InnerTube systems in comparison to conventional septic tanks. The first objective for Chapter 2 was to determine if microbial community compositions along the length InnerTube systems support a PFR model for InnerTube operation. Under ideal PFR conditions, microbial communities vary throughout a system in accordance with changes in wastewater chemical composition (Rittman and McCarty, 2001). To accomplish the first objective, microbial communities were sampled at consistent intervals along the length of InnerTube systems and characterized using 16S sequencing. Uniform microbial community composition along the length of the InnerTubes would indicate longitudinal mixing or homogeneous substrate availability, whereas a gradient of community compositions would indicate that the InnerTube acts as a PFR.

The second objective of this chapter was to determine if settled solids within InnerTube systems contain taxonomically and potentially functionally distinct microbial communities. With this goal, we characterized differences between microbial communities within the mixed liquor (liquid) and settled solid (solid) phases of InnerTube wastewater. The presence of taxonomically similar communities or communities with similar putative metabolic capacity in liquid and solid phases would indicate that settled solids have a redundant role in wastewater treatment in InnerTube systems. In contrast, differences in community composition and putative function would indicate that settled solids have the potential to play a distinct role in InnerTube septic tank performance.

2.2 Methods

2.2.1 Site Descriptions

The first phase of the project examined septic tanks at two government-administered standardized testing facilities in Canada and the USA. These facilities conduct on site audits of industry proprietary processes and follow up in field audits of company installations. In addition to regulatory analyses, these facilities may also be used by companies for their own specific studies (as was done in this project). Samples were taken from WBS InnerTube septic tanks at the Massachusetts Alternative Septic System Test Center (MASSTC) located on the Otis Air National Guard Base in Bourne, Massachusetts, USA and a Bureau de Normalisation du Québec (BNQ) wastewater treatment testing facility in Québec City, Québec, CA. The system at BNQ comprised of two InnerTube septic tanks, BNQ-A and BNQ-B, that received the same influent. Influent for the MASSTC InnerTube septic tank consisted of sewage from base personnel housing and military facilities (Jowett *et al.*, 2017). Influent for the BNQ InnerTube septic tanks consisted of communal residential sewage (Jowett *et al.*, 2017). Table 3 shows the system specifications of each InnerTube septic tank.

2.2.2 Sampling and Sample Pre-treatment

The overall workflow for sample and data processing of Chapter 2 is shown in Fig. 6. MASSTC and BNQ sampling was performed by WBS technical personnel. All sampling was done in duplicate unless otherwise indicated (Appendix I, Table S1A and Table S1C). MASSTC sampling was conducted on November 13, 2017. Samples were taken at 1-foot intervals along the length of the InnerTube using a vacuum pump. BNQ samples were collected on December 5, 2017 using similar methods as those employed for the MASSTC system. However, vacuum draw samples were taken from the two InnerTube septic tanks at 2-foot intervals rather than 1-foot intervals. Refer to Appendix I for more detailed sampling information. Table S1A and Table S1C

display sample identification, sampling locations, and collection techniques. Table S1B and Table S1D outline the progression of wastewater through MASSTC and BNQ systems, respectively.

All samples were transported without refrigeration and were received at McMaster University 2 - 3 days after collection and processed immediately. At McMaster University, samples were first mixed by inverting the bottles and then allowed to settle for 30 minutes (Dick and Vesilind, 1969; Schmidt and Ahring, 1996; Kuroda *et al.*, 2016). Liquid portions of samples were then poured into sterile 500 mL collection bottles, leaving approximately 1 cm of liquid above the solid slurry layer. Decanted samples were designated as liquid samples. The slurries remaining in the original 500 ml bottles were designated as solid samples (Fig. 7). Samples that did not partition into liquid and solid phases were designated as liquid samples. One set of duplicates was sent to the Centre for Advancement of Water and Wastewater Treatment Technologies (CAWT) in Lindsay, ON for chemical testing and the other was further processed at McMaster University for DNA sequencing and CBOD measurements. A PCR blank consisting of ddH_2O was used to evaluate lab contamination.

2.2.3 Chemical Analysis

Samples were sent to CAWT on ice for chemical analysis. Determinations of suspended solids (TSS), total Kjeldahl nitrogen (TKN), and ammonia (NH_3) were performed for liquid samples. Determinations for volatile solids (VS) and total solids (TS) were performed for solid samples. CAWT protocols are described in their CALA Directory of Laboratories listing (member number: 3628). The carbonaceous biological oxygen demand (CBOD) of liquid samples and 2 solid samples were measured at McMaster University. Protocol for CBOD determination was adapted from Method 5210b in Standard Methods for the Examination of

Water and Wastewater, 22nd ed. (APHA *et al.*, 2012). Dissolved oxygen measurements were taken with a YSI Pro-BOD Probe and YSI Professional Plus multimeter (Xylem Inc.)

2.2.4 DNA Extraction

To concentrate microbes for DNA extraction, all samples were vacuum filtered through sterile 0.22 µm cellulose filters (Brown *et al.*, 2015; Mohiuddin *et al.*, 2019). Sample volumes were added until filters no longer passed liquid. Sample filters were transferred to microfuge tubes and stored at -20°C. Each filter was transferred to screw-cap stock tubes containing 0.25 µl of 0.1 mm zirconium beads (Bag *et al.*, 2016).

Microbial DNA was extracted from sample filters using the Norgen Biotek Soil DNA Isolation Plus Kit (Norgen Biotek Corp.). Cell lysis buffer was added to the stock tubes which were then mechanically agitated at a high frequency (bead beating) for 120 s (3 intervals of 40 s). Stock tubes were centrifuged and the supernatant containing microbial cell contents were pipetted into sterile microfuge tubes. Refer to manufacturer instructions for centrifugation speeds. Protein was precipitated by adding acidic precipitation solution and samples were once again centrifuged to isolate the liquid fraction. Humic acid contamination was removed using an extraction additive followed by centrifugation and supernatant isolation. Samples were then filtered by centrifugation through DNA-binding spin columns. Columns were washed twice with ethanol solution and DNA was eluted from each column by centrifugation with extraction buffer. Extracted DNA was stored at -20°C until PCR amplification. DNA was quantified with a NanoDrop2000 (Thermo Fisher Scientific).

2.2.5 16S PCR Amplification

A two-stage PCR protocol was used to amplify the V4 hypervariable region of the bacterial and archaeal 16S gene (Herbold *et al.*, 2015). The 1st stage of PCR targeted and amplified the 16S hypervariable region and the 2nd stage of PCR attached DNA adapters and

indices required for Illumina sequencing and dual-index sample multiplexing (Kozich *et al.*, 2013), respectively. Indices were adopted from the Nextera XT Index Kit v2 (Illumina, Inc.). PCR was performed using a T100 Thermocycler (Bio-Rad Laboratories, Inc.). Reagent and protocol information is listed in Tables 4, 5, and 6. PCR input DNA was normalized to 15 ng/µl, based on NanoDrop 2000 (Thermo Fisher Scientific) concentrations, by diluting DNA with double-distilled water (ddH_2O).

2.2.6 Amplicon Isolation and PCR Clean-up by Gel Extraction

PCR products (16S amplicons) were visualized using gel electrophoresis to confirm successful reactions and correct fragment sizes (Fig. 8). Once 2^{nd} Stage PCRs were confirmed, 5 µl of each PCR product was pooled for clean-up by gel extraction. Gel extraction of target bands was performed using the Axygen Gel Documentation System (Corning Inc.) and Nucleospin Gel and PCR Clean-up kit (Macherey-Nagel GmbH). Gel extraction clean-up of pooled amplicons was performed in triplicate. For each gel extraction, 25 µl of pooled amplicon was ran on 1.8% agarose gels. Gels were illuminated with 302 nm UV light. Target bands were excised and immersed in guanidinium thiocyanate solution and dissolved at 50^oC.

Dissolved gel solutions were filtered through DNA-binding columns by centrifugation. Refer to manufacturer instructions for centrifugation speeds. Columns were washed 3 times with ethanol solution and then, while still held in a microfuge tube and with column lids open, dried at 70^oC for 1 min. using a heat block. DNA was eluted from each column with buffer solution. Replicate gel extractions were then pooled for sequencing. DNA quality was verified using the NanoDrop 2000 (Thermo Fisher Scientific). Amplicons were sequenced at the McMaster Farncombe Institute Genomics Facility with a MiSeq platform (Illumina, Inc.) using a 2 x 300 bp paired-end sequencing configuration.

2.2.7 Sequence Data Processing and Taxonomic Assignment

Dual-indexed sequence data were demultiplexed by sequencing facility staff prior to upload to the Illumina repository. Adapter trimming was performed using cutadapt-1.2.1 (Martin, 2011). The DADA2 R package was used for sequence quality trimming and filtering, merging, error modelling, sequence variant assignment, and taxonomic assignment with the SILVA 132 SSU reference dataset (Callahan, 2016). To remove redundant sequence assignments and potentially spurious sequences, we agglomerated sequences that shared taxonomic assignments at their lowest level and removed OTUs for which counts totalled to less than 0.005% of total counts (Bokulich *et al.*, 2013), respectively, using the phyloseq R package (McMurdie and Holmes, 2013). Sample read counts were rarefied to 17,129 reads (Weiss *et al.*, 2017). Samples that did not meet this rarefying threshold were removed from further analyses.

2.2.8 Statistical Analyses

Sample rarefaction curves were generated using the rarecurve function in the vegan R package (Oksanen *et al.*, 2019). Relative abundance bar graphs of bacterial and archaeal phyla were constructed using phyloseq. To simplify visualization of community compositions, phyla that accounted for less than 0.5% abundance were agglomerated and shown in black. Shannon and ACE alpha diversity indices were calculated using phyloseq. 10 maximum-likelihood phylogenetic trees of MASSTC and BNQ assigned sequence variants (ASVs) were constructed using RAxML v8.0 (Stamatakis, 2014). Starting trees for the maximum-likelihood trees were generated using maximum parsimony and nucleotide substitution was modelled using a generalised time reversible model with a gamma distribution of substitution rate heterogeneity (Arenas, 2015). The tree determined to be the best by RAxML was chosen as the reference phylogenetic tree for Chapter 2 ASVs. The reference tree was used with rarefied sample count data to generate generalized Unifrac distances.

Sample ordination was performed using non-metric dimensional scaling (NMDS) and generalized Unifrac distances with phyloseq. Generalized Unifrac distances were used for all ordinations and beta diversity measurements. Sample ordination and multivariate t-distribution data ellipses were plotted using ggplot2 (Wickham, 2016). Samples were divided into 4 groups: MASSTC liquid, MASSTC solid, BNQ liquid, and BNQ solid. Permutational analysis of variance (PERMANOVA) was used to test for significant differences between sample groups. PERMANOVA was carried out with the adonis function of vegan. Post-hoc pairwise PERMANOVA tests were carried out with the pairwise.Adonis function (Arbizu, 2019). Multivariate dispersion was assessed with the betadisper and permutest functions from vegan (Oksanen *et al.*, 2019).

Constrained analysis of principal coordinates (CAP) was performed using phyloseq. Distance from the inlet of the InnerTube (cm) and sample composition (liquid or solid) were included as environmental variables in the CAP analysis. The varpart function in vegan was used to determine the adjusted R^2 values for variation explained (Oksanen *et al.*, 2019). The SPIEC-EASI (Kurtz *et al.*, 2015) implementation of SparCC (Friedman and Alm, 2012) was used to identify significant correlations between ASVs and ranked distance from the inlet of the InnerTube. Only ASV correlations with $r > |0.04|$ and $p < 0.01$ (Liu *et al.*, 2019) were selected for consideration.

Differential abundance analysis of liquid and solid microbial communities was performed with the DESeq function in the DESeq2 package in R with Wald significance tests and a parametric fitting of dispersions to mean intensity (McMurdie and Holmes, 2014). Only ASVs that comprised at least 1% of total reads of rarefied samples and adjusted p-values of less than 0.05 were displayed in the differential abundance plot. 1% abundance cut-off have been applied

at the phylum, sample, and genus levels for microbiome analysis (Caruso *et al.*, 2019; Zamanzadeh *et al*., 2016; Zhang *et al*., 2019b). This cut-off was chosen for this project as an unbiased method to increase the probability that differences discussed were of functional significance and to minimize the number of false positives in differential abundance analysis (Hawinkel *et al*., 2017). The prevalence of each differentially abundant ASV was plotted and merged with the differential plot using the ggplot2 and cowplot R packages, respectively. Prevalence was calculated as the number of InnerTube samples in which the ASV was present divided by the total number of InnerTube samples. Prevalence was also partitioned by site. MASSTC samples comprised of 28 of 53 InnerTube samples and BNQ samples comprised of 25 of 53 InnerTube samples.

2.3 Results

2.3.1 16S Sequencing Descriptive Statistics

Sequencing statistics were calculated to evaluate sequencing depth and overall quality of sequencing. A total number of 13,799,673 paired-end reads were obtained with a mean of 160,461 ± 109,825 reads per sample. After quality filtering, merging, and chimera removal, a total of 12,199,998 reads remained with a mean of 141,860 ± 96,549 reads per sample. Sequence reads were comprised of 13,297 assigned sequence variants. 44 ASVs (~0.008% of total reads) attributed to the Kingdom Eukaryota or unassigned at the Kingdom level were removed from the dataset. After agglomeration of ASVs with the same taxonomic assignment, 1492 ASVs remained. Sample communities were rarefied to 17,129 reads to match the number of reads obtained from sample B4BL (Fig. 9). 4 samples were removed from further analyses as a result. A subset of 53 samples consisting only of communities from the InnerTubes of the MASSTC and BNQ systems were considered for the following analyses.

2.3.2 Chemical Analyses

Chemical analyses of each sample were performed to evaluate environmental heterogeneity within the InnerTubes. Ammonia concentrations showed a significantly strong positive correlation with distance from the inlet of the InnerTube (Fig.10) (Pearson correlation; $r = 0.51$; $p = 0.0047$). Total suspended solids, carbonaceous biological oxygen demand, and total Kjeldahl nitrogen were not significantly correlated with distance along the InnerTube (Pearson correlation; $p > 0.05$).

2.3.3 Relative Abundance of Microbial Phyla

Relative abundances of microbial phyla were determined as a qualitative analysis of InnerTube community composition. MASSTC InnerTube microbial communities were comprised of 37 identified phyla. 20 of those phyla had proportions greater than 0.5% of total

reads (Fig. 11). Phyla with the greatest median proportions in all MASSTC samples were Proteobacteria (26.6%, 23.8% – 33.4%), Firmicutes (23.7%, 18.6% – 28.0%), and Bacteroidetes (15.0%, 10.9% – 17.4%). Notably, the median proportion of Epsilonbacteraeota was greater in MASSTC liquid samples (17.0%, 6.8% – 21.6%) than in MASSTC solid samples (0.3%, 0.2% – 0.9%). Solid samples contained greater median proportions of Actinobacteria (6.1%, 5.1 – 6.8%) and Chloroflexi (3.3%, 2.6% – 3.5%) than liquid samples which contained 2.4% (0.8% – 2.8%) Actinobacteria and 0.5% (0.3% – 0.6%) Chloroflexi. The median proportion of Euryarchaeota across all MASSTC samples was 6.4% (2.9% – 9.0%). The median proportion of unknown bacteria phyla across all MASSTC samples was 0.3% (0.2% – 0.4%).

BNQ microbial communities were comprised of 33 identified phyla. 12 of those phyla had proportions greater than 0.5% of total reads (Fig. 12). Phyla with the greatest median proportions in all BNQ samples were Proteobacteria (41.5%, 33.5% – 44.9%), Firmicutes (28.6%, 24.1% – 36.4%), Fusobacteria (11.9%, 7.6% – 15.8%), and Bacteroidetes (7.6%, 2.5% – 13.0%). The median proportion of Euryarchaeota across all BNQ samples was 0.2% (0.1% – 0.6%). BNQ samples did not contain unassigned bacterial or archaeal sequences greater than 0.5% at the phylum level.

2.3.4 MASSTC and BNQ Community Alpha Diversity

Sample alpha diversity was calculated to evaluate changes in community structure along the length of the InnerTubes (Fig. 13). Alpha diversity for MASSTC and BNQ sample communities were not significantly correlated with distance from the inlet of the Innertube when evaluated with Shannon indices (Spearman correlation; r_s = 0.08; p = 0.5693) and abundance-based coverage estimator (ACE) indices (Spearman correlation; r_s = -0.11; p = 0.4216). Alpha diversity was significantly greater in solid sample communities than liquid sample communities

when evaluated with Shannon indices (Wilcoxon rank-sum; W = 135; $p = 0.00006$) and ACE indices (Wilcoxon rank-sum; W = 203; $p = 0.006$).

2.3.5 MASSTC and BNQ Sample Community Ordination

NMDS ordination of sample communities was used to evaluate sample community associations and clustering. PERMANOVA was used to compare sample groups and calculate the statistical significance of sample clustering. Communities significantly separated into 4 groups based on generalized Unifrac distances ($p = 0.0001$). These groups were defined by site and composition: MASSTC solid, MASSTC liquid, and BNQ solid and liquid communities (PERMANOVA; $R^2 = 0.476$, $p = 0.0001$) (Fig. 14). Multivariate dispersion was calculated to assess the validity of PERMANOVA tests (Table 7). Multivariate dispersion deviated significantly from homogeneity (PERMANOVA; $p = 0.001$). Permutational pair-wise comparisons of homogeneity of variances showed that MASSTC solids had significantly less dispersion than the MASSTC liquid, BNQ solid, and BNQ liquid groups (PERMANOVA; $p < 0.05$).

2.3.6 Constrained Analysis of Principal Coordinates

Constrained analysis of principal coordinates (CAP) was used to visualize the distribution of microbial communities along environmental gradients (Fig. 15). System location (MASSTC or BNQ) explained a significant proportion (32.3%) of inter-sample microbiota variation (PERMANOVA; pseudo-$F = 31.3$; $p = 0.0001$). Sample community variation (Fig. 15) was significantly correlated with distance (cm) from the inlet of the InnerTube which explained 2.8% of variation (PERMANOVA; pseudo-$F = 5.01$; $p = 0.0003$) and sample composition which explained 6.7% of variation (PERMANOVA; pseudo-$F = 7.83$; $p = 0.0002$).

2.3.7 SparCC Correlation of Taxa with Distance from the Inlet of the InnerTube

SparCC correlation analyses of ASV abundances and distance from the inlet of the InnerTube (cm) were performed to identify taxa that explain differences in community composition along the length of InnerTubes. 5 ASVs were robustly correlated with ($r > 0.4$; $p < 0.01$) distance from the InnerTube inlet. 4 ASVs were positively correlated with distance from the InnerTube inlet and 1 ASV was negatively correlated with distance from the InnerTube inlet. 4 ASVs were assigned at the genus level and 1 ASV was assigned to the phylum level (Table 8). The potential roles in anaerobic digestion of positively correlated taxa were hydrolysis (2 taxa), acidogenesis and acetogenesis (1 taxon), and methanogenesis (1 taxon). The potential waste treatment role of the taxon negatively correlated with distance from the InnerTube inlet was hydrolysis and acidogenesis.

2.3.8 Differential Abundance Analysis of Liquid and Solid Communities

DESeq2 differential abundance analysis was used to identify the ASVs associated with the significant difference observed between liquid and solid communities. 13 differentially abundant ASVs that each sum to greater than 1% of total reads were identified using DESeq2 differential abundance analysis (Fig. 16). There were 7 significantly differentially abundant ASVs in liquid samples and 6 significantly differentially abundant ASVs solid samples (Adj. $p < 0.05$). Log_2-fold changes ranged from ~0.606 to ~4.84. Liquid and solid community types both contained 2 differentially abundant ASVs assigned to the Bacteroidetes phylum and 1 ASV assigned to the Firmicutes phylum. 1 ASV assigned to the phylum Epsilonbacteraeota was differentially abundant in the liquid phase. 1 Fusobacteria ASV and 1 Euryarchaeota ASV were differentially abundant in solid communities. All differentially abundant ASVs were present in all MASSTC communities. Except for ASV8, differentially abundant ASVs were present in

greater than 75% of total BNQ communities. Taxonomic assignments for differentially abundant ASVs and potential roles in anaerobic digestion for these ASVs are shown in Table 9.

11 ASVs were assigned at the genus level and 2 ASVs were assigned to the family level (Table 9). The potential waste treatment roles of the liquid community taxa were hydrolysis and acidogenesis (4 taxa) and acidogenesis (3 taxa). The potential waste treatment roles of solid community taxa were acidogenesis (4 taxa), acidogenesis and acetogenesis (1 taxon), and hydrogenotrophic methanogenesis (1 taxon) (Table 9).

2.4 Discussion

2.4.1 Microbial Succession along the Length of Septic Tank InnerTubes

The overall objective of this chapter was to characterize changes in microbial community composition and metabolic potential that could explain lesser solid accumulation rates in InnerTube systems in comparison to conventional septic tanks. To accomplish this objective, wastewater was sampled at consistent intervals along the length of InnerTube systems and separated into liquid and solid phases. Taxonomic profiles of liquid and solid communities were then constructed using 16S sequencing. It was hypothesized that InnerTubes maximize organic matter degradation by acting as PFRs and develop UASB-like microbial granules that facilitate waste treatment.

Microbial community composition was significantly correlated with distance from the inlet of the InnerTubes (distance). The abundances of 4 taxa were significantly positively correlated with distance: Rikenellaceae_U29-B03, BRC1, *Clostridium*, and *Methanomassiliicoccus*. *Dysgonomonas* was significantly negatively correlated with distance. The candidate phylum BRC1 and genus *Dysgonomonas* are involved in the hydrolysis complex organic substrates (Larsbrink *et al*., 2014; Kadnikov *et al*., 2018), the uncultured bacterium U29-B03 of the Rikenellaceae family is associated with hydrolysis and acidogenesis (Graf, 2014 ; Han *et al*., 2017), *Clostridium spp*. carry out acidogenesis and acetogenesis during anaerobic digestion (Steer *et al.*, 2001; Sun *et al.*, 2018), and the genus *Methanomassiliicoccus* is comprised of methylotrophic methanogens (Borrel *et al*., 2014).These microbial taxa inhabit the human gut (with the exception of BRC1) and anaerobic digesters (Table 8).

The physiology of a BRC1 bacterium was recently inferred from a draft genome assembled from metagenomic DNA extracted from a thermal aquifer (Kadnikov *et al*., 2018). The BRC1 genome featured many glycoside hydrolase genes which suggests that this bacterium

is involved in the degradation of complex organic substrates (Kanikov *et al*., 2018). *Dysgonomonas* are associated with high-fiber diets and complex polysaccharide decomposition (Larsbrink *et al*., 2014). Rikenellaceae outcompete other bacteria when grown on mucin, a heavily glycosylated protein secreted by epithelial cells (Gendler and Spicer, 1995), but not when grown on simple sugars (Graf, 2014). In food waste ADs, Rikenellaceae have been linked to acidogenesis from the fermentation of waste with high total solids content (Han *et al*., 2017). *Clostridium* bacteria are part of the phylum Firmicutes which is often the dominant phylum in anaerobic digesters (Chen *et al*., 2016). *Clostridium* isolated from human feces produce VFAs and alcohols from sugars and starch (Steer *et al*., 2001). *Clostridium spp*. have been found to generate H_2 and form syntrophic relationships with methanogens (Hung *et al*., 2011). *Methanomassiliicoccus spp*. are methanogens that require externally supplied H_2 and simple methylated compounds (i.e. methanol) to produce ATP and methane (Vanwonterghem *et al*., 2016).

Complete anaerobic digestion of complex organic compounds of occurs in 4 steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. However, the overall decomposition of waste occurs in stages in which more readily degradable compounds, such as VFAs, are digested first (Staley *et al*., 2018). In later stages, waste degradation rates are governed by hydrolytic reactions as complex compounds make up the bulk of the remaining substrate (Staley *et al*., 2018). Correlations between taxa and distance suggest that InnerTube wastewater proceeds through these stages of degradation and that InnerTubes undergo longitudinal stratification of microbial communities and substrates similar to a PFR.

Hydrolytic taxa such as BRC1 and Rikenellaceae increase in abundance along the length which may indicate an increase in the proportion of complex substrates due to the depletion of

VFAs (Staley *et al*., 2018). Furthermore, the positive correlation between distance and *Methanomassiliicoccus* could be explained by the accumulation of single-carbon compounds, which are not widely utilized by microbes, as wastewater flows through InnerTubes (Meyer *et al*., 2018). Increased *Clostridium* abundance may be the result of syntrophic relationships with growing methylotrophic populations (Bizukojc *et al*., 2010). NH_3 concentrations were significantly positively correlated with distance which also indicates longitudinal stratification of InnerTube chemical environments. Similarities between PFRs and InnerTubes suggest that InnerTubes reduce solid accumulation by maximizing the co-localization of substrates and microbial communities capable of degrading them. However, the negative correlation between distance and the hydrolytic genus, *Dysgonomonas*, contradicts the hypothesis of increasing utilization of complex substrates with distance. Further characterization of the InnerTube chemical environment and greater resolution of microbial activity and taxa are required to comprehensively evaluate microbial processes in InnerTube septic tanks.

2.4.2 Inferred Metabolic Potential of Microbial Communities in Solid and Liquid Fractions

InnerTube wastewater liquid and solid fractions were inhabited by significantly different communities ($p < 0.05$). Microbial taxa that were differentially more abundant in the liquid fraction are associated with hydrolysis, acidogenesis, and acetogenesis in anaerobic digesters and the human gut (Table 9). *Prevotella spp*., *Bacteroides spp.*, and Lachnospiraceae play roles in the hydrolysis of starches and plant fibers (Matsui *et al*., 2000; Wexler and Goodman, 2017; Biddle *et al*., 2013). *Acinetobacter spp.* specialize in lipid degradation (Doughari *et al*., 2011). *Arcobacter spp*. and *Subdoligranulum spp*. are acidogenic bacteria that catabolize amino acids and simple carbohydrates, respectively (Rahji *et al*., 2016; Holmstrøm *et al*., 2004). *Acidaminococcus spp.* ferment glutamate to produce acetate and H_2 (Jumas-Bilak *et al*., 2007). The hydrolytic activity *Prevotella spp*., *Bacteroides spp.*, Lachnospiraceae, and *Acinetobacter*

may explain their exclusion from microbial granules which rely on aggregation of extracellular polysaccharides and proteins to maintain integrity (Dolfing *et al*., 1985; Molobela *et al*., 2010). The greater abundance of *Arcobacter* and *Subdoligranulum* in the liquid fraction indicate that these genera are metabolically independent. This is further supported by their preferred substrates which can also be the product of abiotic hydrolysis reactions.

Solid fraction communities were more abundant in taxa associated with acidogenesis, acetogenesis, and methanogenesis. Apart from *Paludibacter*, all differentially abundant taxa in solid samples are found in anaerobic digesters and human microbiomes (Table 9). The most functionally distinct in InnerTube solids were *Methanobacterium* and *Trichococcus*. *Methanobacterium* was the only methanogenic genus identified through differential abundance analysis. *Methanobacterium* is comprised of archaea that rely on bacteria to provide H_2 or formate for methanogenesis and ATP-generation (Maus *et al*., 2013). The transfer of H_2 or formate from acetogenic bacteria to hydrogenotrophic methanogens is correlated to the proximity of these organisms to one another (Shen *et al*., 2013). Therefore, hydrogenotrophic methanogens are often found in solids where intercellular distances are relatively small (Shen *et al*., 2013). *Trichococcus* cells also form thick extracellular matrices (Parshina *et al*., 2019) which indicate an ability to form biofilms and sludge aggregates (Liu *et al*., 2010). *Trichococcus spp*. also produce hydrogen and formate from VFAs (Parshina *et al*., 2019). The co-occurrence and physiology of *Methanobacterium* and *Trichococcus* suggests that these microbes associate in syntrophic relationships and play a role in solid formation. The formation of these acetogenic and methanogenic aggregates could explain higher waste degradation rates and reduced solid accumulation in InnerTube septic tanks.

In comparison to *Methanobacterium* and *Trichococcus*, the potential performance-enhancing roles of the solid-fraction taxa Christensenallaceae, Eubacteriaceae, *Leptotrichia*, and *Paludibacter* are less readily discernible. These taxa are acidogenic and may supply *Trichococcus* and other H_2-producing bacteria with VFAs. *Leptotrichia* form coaggregates in the human microbiome which may explain their presence in InnerTube solids (Kriebel *et al*., 2018). Members of Christensenallaceae often co-occur with Methanobacteriaceae (Goodrich *et al*., 2014), the family to which *Methanobacterium* belongs, but there is currently insufficient information regarding Christensenallaceae physiology to hypothesize potential syntrophic relationships.

Co-culturing experiments (Faust et al., 2012) and fluorescent in-situ hybridization analyses (Thurnheer et al., 2004) are required to confirm the syntrophic relationship between Methanobacterium and Trichococcus and identify potential roles of the other differentially abundant solid-phase microbes. However, the metabolic potential of differentially abundant microbes in revealed by 16S analyses suggest that, like high-rate ADs (Kuroda et al., 2016), InnerTubes develop microbial consortia in settled aggregates which facilitate complete anaerobic digestion.

Changes in microbial community compositions due to the lack of sample preservation during transport may have had confounding effects on the relationships observed between microbial communities and distance along the InnerTube and differences between liquid and solid fraction microbial communities. For example, relative abundances of Actinobacteria increase and relative abundances Firmicutes decrease in fecal samples stored at room temperature for 72 hours (Choo *et al*., 2015). Future studies of septic tank communities should explore methods of sample preservation such as sample additives or on-site freezing.

Table 3: MASSTC and BNQ InnerTube Septic Tank System Specifications (Section 2.2.1)

System	Volume (L)	Flow Rate (L/d)	Hydraulic Residence Time (d)	Months Operated
MASSTC	5700	1200	4.75	48
BNQ-A	3800	1600	2.375	36
BNQ-B	2800	1600	1.75	36

Table 4: PCR Reagent Volume per Reaction (Section 2.2.5)

Reagent	Volume (µl)
Double-distilled Water	13.0
Invitrogen 10x PCR Reaction Buffer	2.5
New England Biolab dNTP Solution (10 mM)	0.5
Bovine Serum Albumin (10 mg/ml)	1.0
V3, V4, or Index Forward Primer (1 µM)	2.5
V3, V4, or Index Reverse Primer (1 µM)	2.5
Invitrogen *Taq* Polymerase Recombinant (5 U/ µl)	0.25
Invitrogen $MgCl_2$ (50 mM)	0.75
Sample DNA Template	2.0

Table 5: PCR Temperature Programming (Section 2.2.5)

PCR Stage	Step	Duration
16S V4 Amplification (1^{st} Stage)	1. 95ºC	3 min.
	2. 95ºC	30 s
	3. 50ºC	1 min.
	4. 72ºC	1 min.
	5. Repeat steps 2 – 4 twenty-four times.	
	6. 72ºC	10 min.
	7. 4ºC	Indefinite hold
Indexing PCR (2^{nd} Stage)	Steps 1 – 7 same as above except for 4 repeats of steps 2 – 4. For	N/A

Table 6: Primers Used in this Project (Section 2.2.5)

Primer Name	Sequence
1st Stage V4 Forward Primer (515f) (Thompson *et al.*, 2017)	ACACTCTTTCCCTACACGACGCTCTTCC-GATCTNNNNGTGYCAGCMGCCGCGGTAA
1st Stage V4 Reverse Primer (806r) (Thompson *et al.*, 2017)	GTGACTGGAGTTCAGACGTGTGCTCTT-CCGATCTGGACTACNVGGGTWTCTAAT
1st Stage V3 Forward Primer (341f) (Bartram *et al.*, 2011)	ACACTCTTTCCCTACACGACGCTCTTCC-GATCTNNNNCCTACGGGAGGCAGCAG
1st Stage V3 Reverse Primer (518r) (Bartram *et al.*, 2011)	GTGACTGGAGTTCAGACGTGTGCTC-TTCCGATCTATTACCGCGGCTGCTGG
2nd Stage Forward Indexing Primer	AATGATACGGCGACCACCGAGATCTACACXXXXXXXX-ACACTCTTTCCCTACACGACGCTCTTCCGATCT[1]
2nd Stage Reverse Indexing Primer	CAAGCAGAAGACGGCATACGAGATXXXXXXXX-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

[1]: 'X' characters denote Illumina barcode sequences.

Table 7: Multivariate Dispersion Based on Generalized Unifrac Distances (Section 2.3.5)

Sample Group	Multivariate Dispersion	Significance of Deviation from Homogeneity (*p*)
MASSTC Liquid	0.294	0.001
MASSTC Solid	0.192	0.001[1]
BNQ Liquid	0.2853	0.001
BNQ Solid	0.2612	0.001

[1]: Overall significance of deviation from homogeneity for all groups was due to the relatively low multivariate dispersion of the MASSTC sample group.

Table 8: Potential Roles in Anaerobic Digestion of Taxa Correlated with Distance (Section 2.3.7)

Taxa Positively Correlated with Distance			
ASV	Taxonomic Assignment[1]	Potential Roles	Literature Cited
600	p_BRC1[2]	Hydrolysis	Kadnikov *et al*., 2018
101	g_Rikenellaceae_U29-B03	Hydrolysis	Graf, 2014; Baldwin *et al*., 2015
335	g_Clostridium *sensu_stricto*_12	Acidogenesis, Acetogenesis	Steer *et al*., 2001; Sun *et al*., 2018
38	g_ Methanomassiliicoccus	Methanogenesis (Methylotrophic/ Hydrogenotrophic)	Borrel *et al*., 2014
Taxa Negatively Correlated with Distance			
128	g_Dysgonomonas	Hydrolysis/Acidogenesis	Larsbrink *et al*., 2014

[1]: 1: Each taxa name is preceded by letter representing the level of taxonomic assignment which are as follows:

- g_ - genus
- p_ - phylum

[2]: Not well characterized in anaerobic environments or human microbiomes

Table 9: Potential Roles in Anaerobic Digestion of Differentially Abundant Liquid and Solid ASVs (Section 2.3.8)

Differentially Abundant Liquid ASVs			
ASV	Taxonomic Assignment[1]	Potential Roles	Literature Cited
30	g_Prevotella_9	Hydrolysis	Kovatcheva-Datchary *et al.*, 2015
59	g_Bacteroides	Hydrolysis	Wexler and Goodman, 2017
6	g_Acinetobacter	Hydrolysis	Doughari *et al*., 2011
142	f_Lachnospiraceae	Hydrolysis	Biddle *et al.*, 2013
17	g_Subdoligranulum	Acidogenesis	Holmstrøm and Lawson, 2004
1	g_Arcobacter	Acidogenesis	Supaphol *et al.*, 2011
38	g_Acidaminococcus	Acidogenesis/Acetogenesis	Jumas-Bilak *et al.*, 2007
Differentially Abundant Solid ASVs			
22	g_Paludibacter[2]	Acidogenesis	Gronow *et al.*, 2011; Qiu *et al*., 2017
3	g_Leptotrichia	Acidogenesis	Eribe and Olsen, 2017
8	f_Eubacteriaceae	Acidogenesis	Poeker *et al.*, 2017
31	g_Christensenellaceae_R-7_group	Acidogenesis	Si *et al.*, 2016
16	g_Trichococcus	Acidogenesis/Acetogenesis	Li *et al*., 2016; Debowski *et al*., 2014
51	g_Methanobacterium	Methanogenesis (Hydrogenotrophic)	Koskinen *et al.*, 2017

[1]: Each taxa name is preceded by letter representing the level of taxonomic assignment which are as follows:

- g_ - genus
- f_ - family

[2]: Not described in samples from anaerobic digesters or the human gut

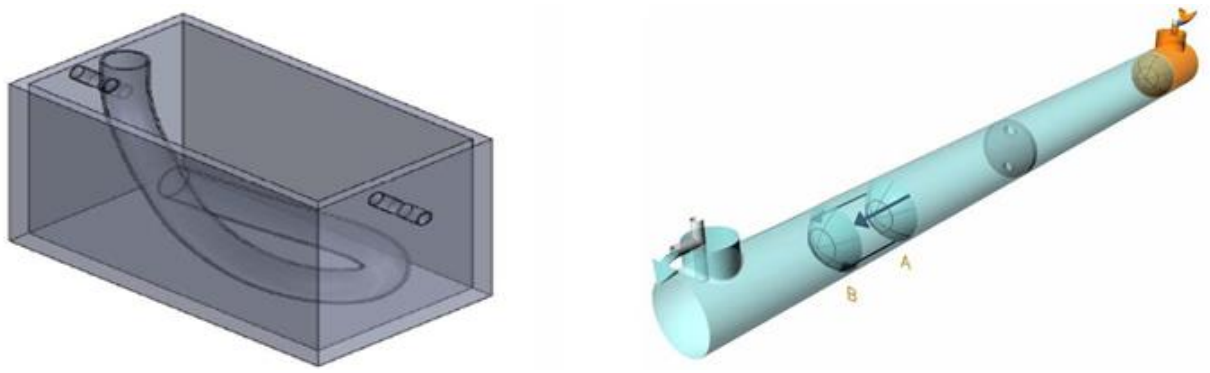

Figure 4: An InnerTube septic tank system (left) and a plug flow reactor (right). The arrows in the plug flow reactor diagram indicate the direction of flow and 'plug' movement (Jowett, 2017) (Section 2.1.2, 2.1.3).

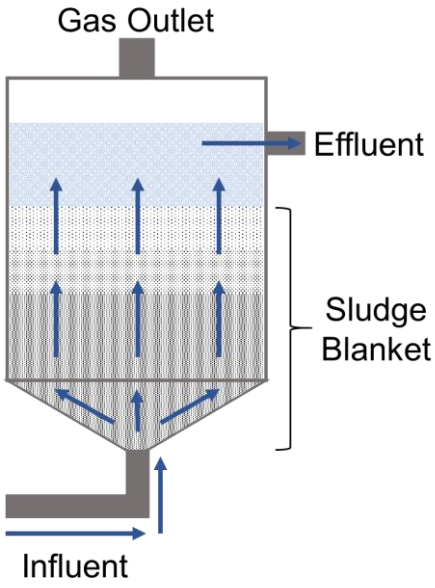

Figure 5: Upflow anaerobic sludge blanket (UASB) reactor. The sludge blanket is comprised of granules containing microbial communities capable of anaerobic digestion. Arrows indicate the direction of wastewater flow (Section 2.1.5).

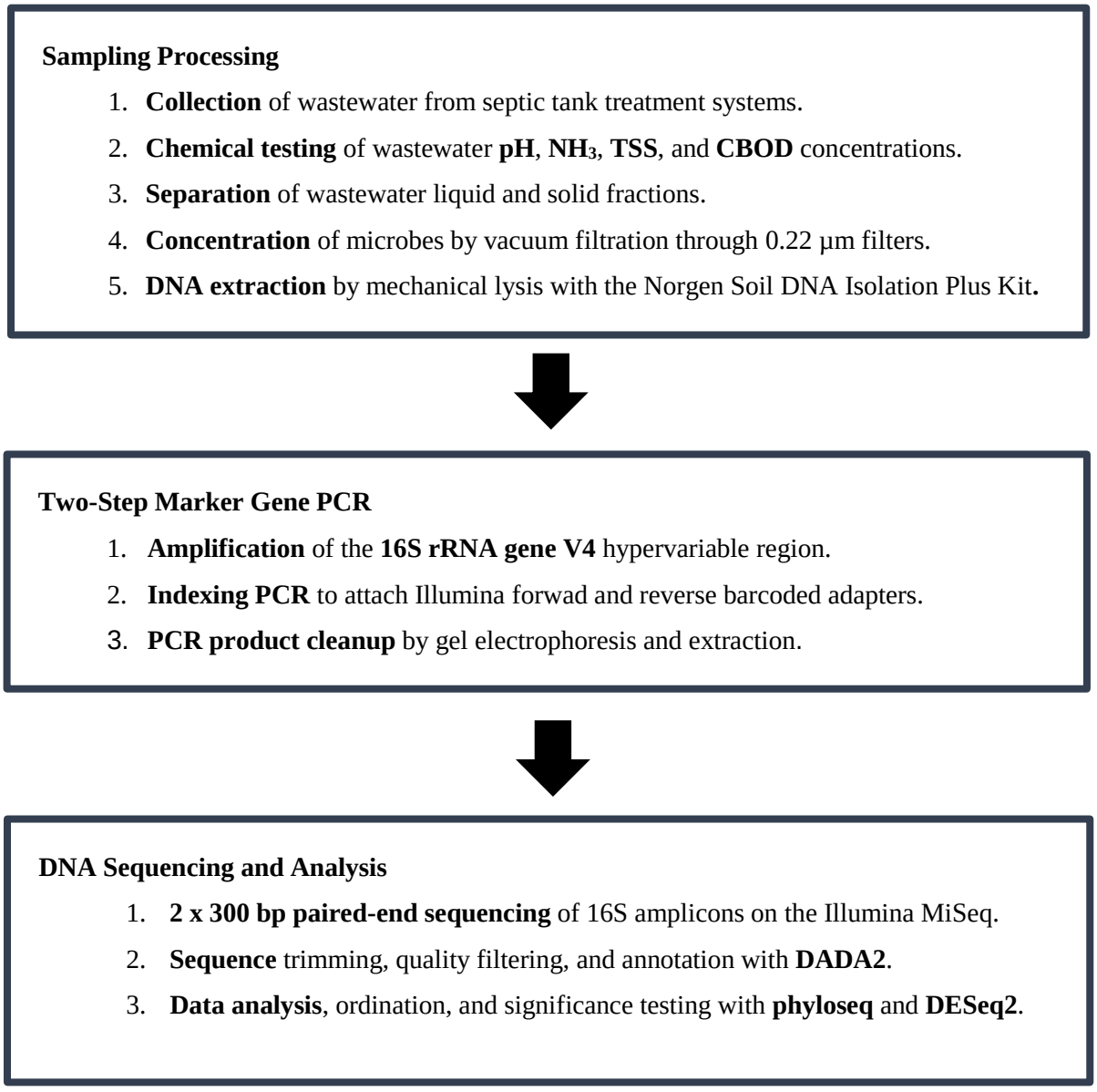

Figure 6: Overview of Chapter 2 methods employed in obtaining and processing 16S data (Section 2.2.2).

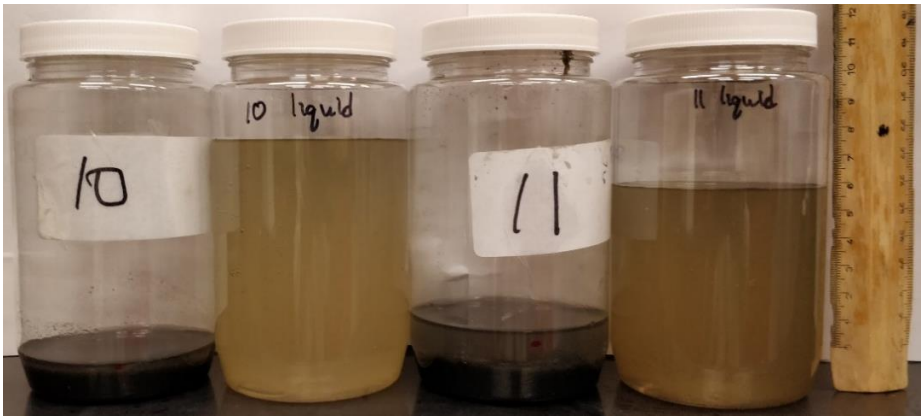

Figure 7: Solid and liquid samples in 500 ml bottles separated after 30 min. of settling (Section 2.2.2).

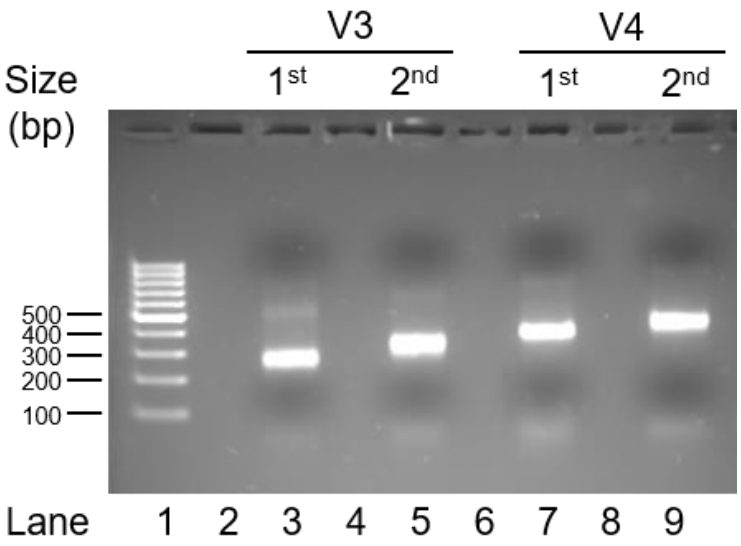

Figure 8: 1st and 2nd Stage V3 and V4 amplicons separated on a 1.8% agarose gel (Section 2.2.6).

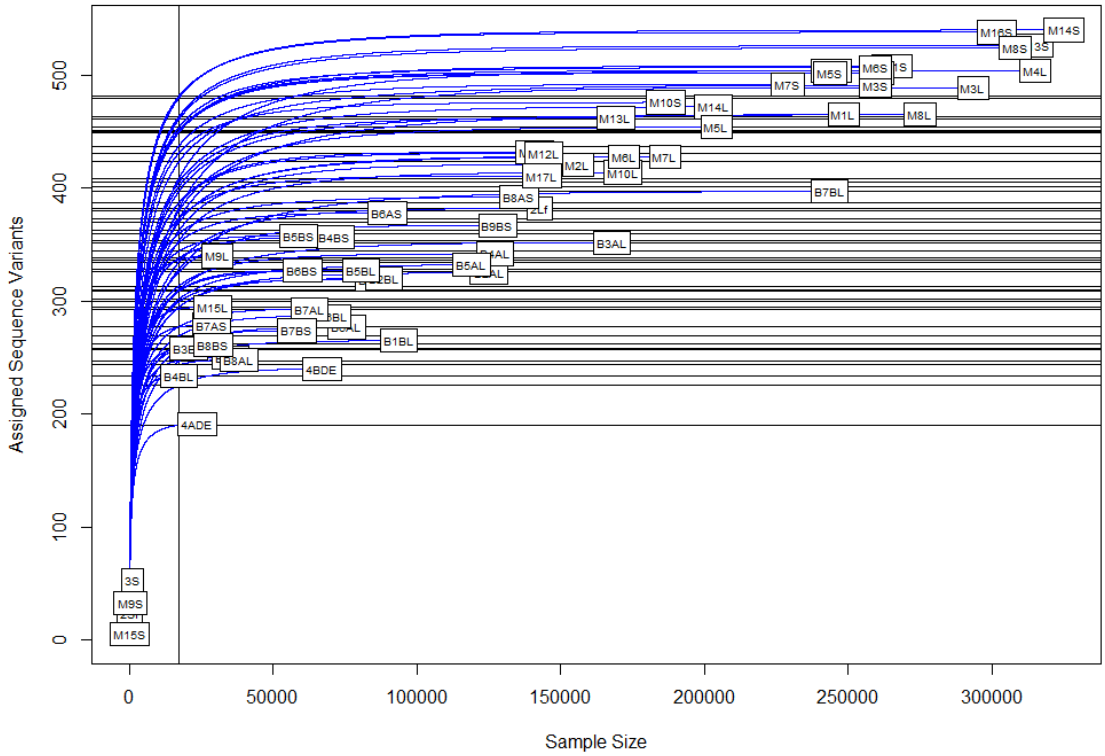

Figure 9: Rarefaction curves for MASSTC and BNQ communities. Sample identifiers are shown in boxes at the terminus of each sample curve. The vertical line in the plot denotes the level to which samples were rarefied (17,129 reads) (Section 2.3.1).

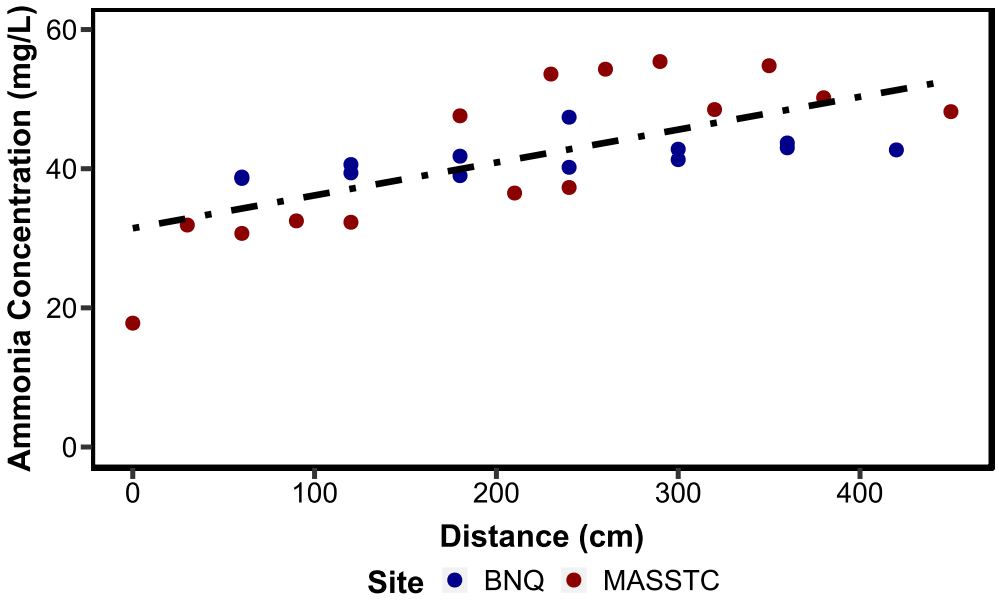

Figure 10: Ammonia concentrations (mg/L) versus distance from the inlet of the InnerTube (cm) at the MASSTC and BNQ testing facilities (Section 2.3.2).

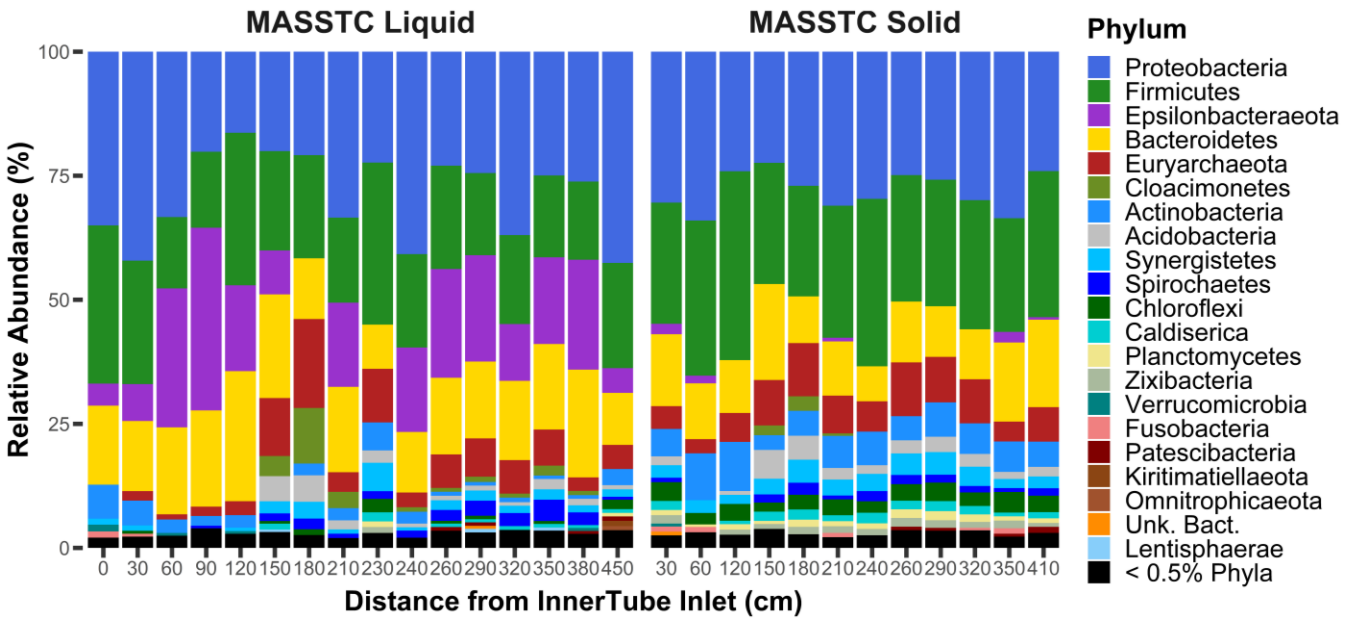

Figure 11: Relative abundances of bacterial and archaeal phyla in MASSTC communities. To simplify visualization of community compositions, phyla that individually comprised less than 0.5% of total counts were agglomerated and are shown in black. Sequences that were assigned to the Kingdom Bacteria but not a phylum were grouped and classified as Unknown Bacteria (Unk. Bact.) (Section 2.3.3).

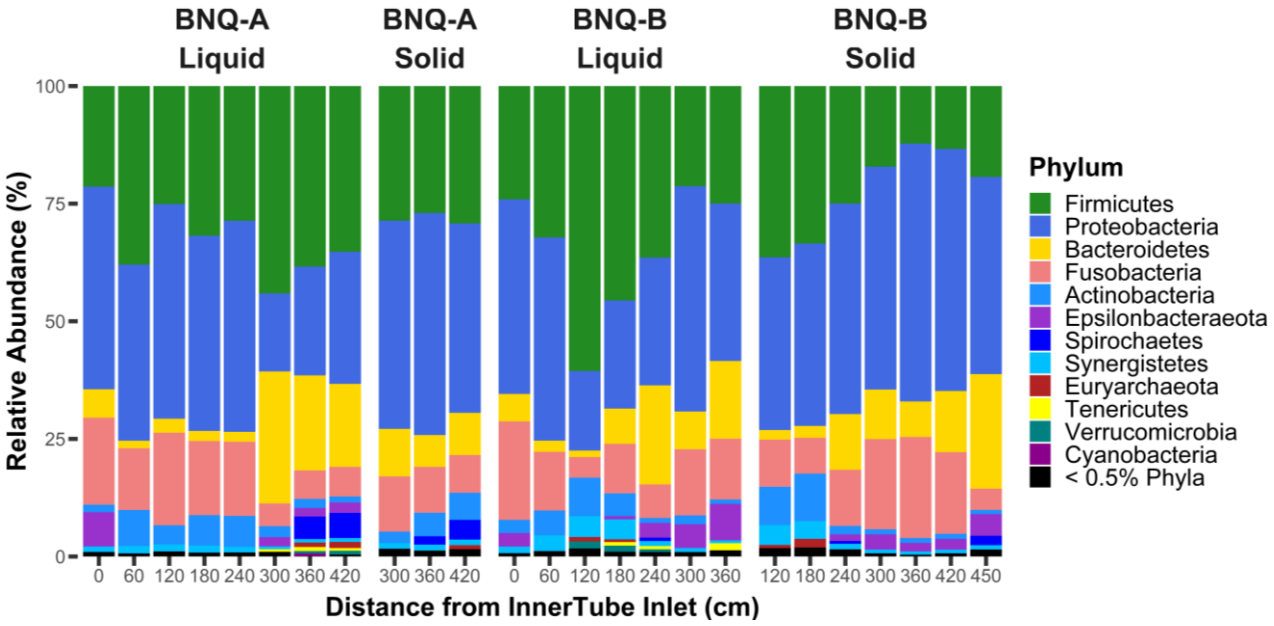

Figure 12: Relative abundances of bacterial and archaeal phyla in BNQ communities. To simplify visualization of community compositions, phyla that individually comprised less than 0.5% of total counts were agglomerated and are shown in black. Sequences that were assigned to the Kingdom Bacteria but not a phylum were classified as Unknown Bacteria (Unk. Bact.) (Section 2.3.3).

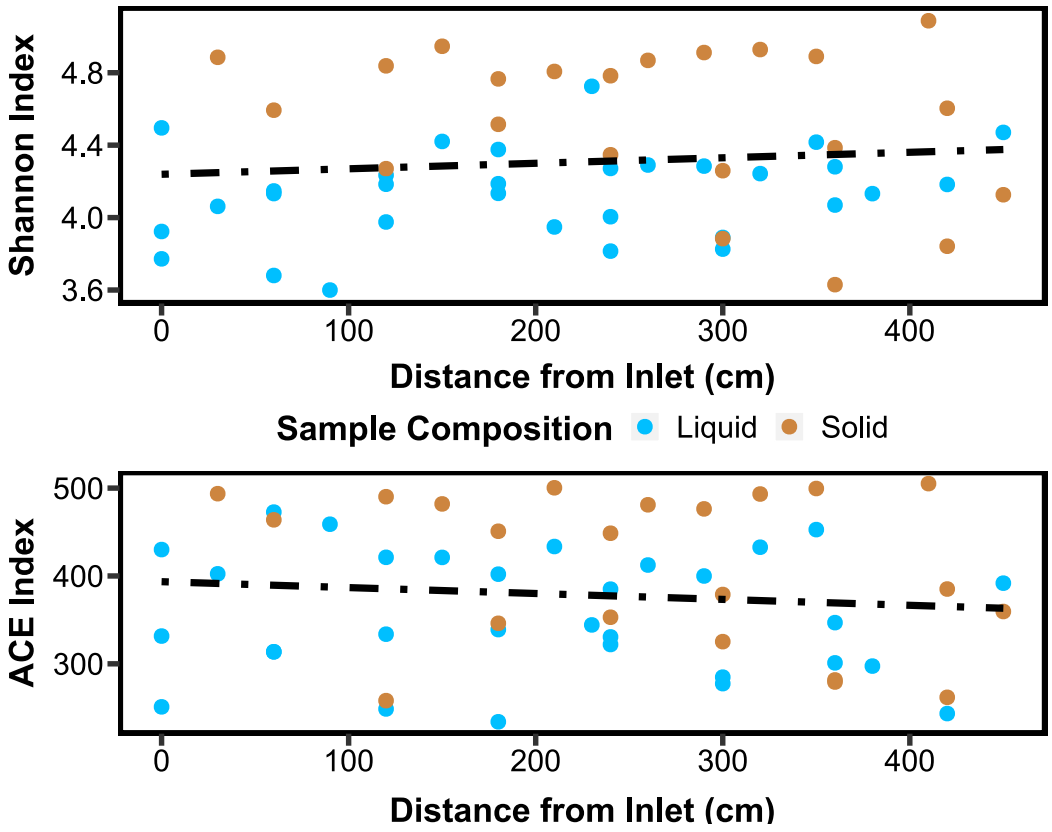

Figure 13: Distribution of Shannon (top) and ACE (bottom) diversity indices versus sample distance from the inlet of MASSTC and BNQ InnerTubes (Section 2.3.4).

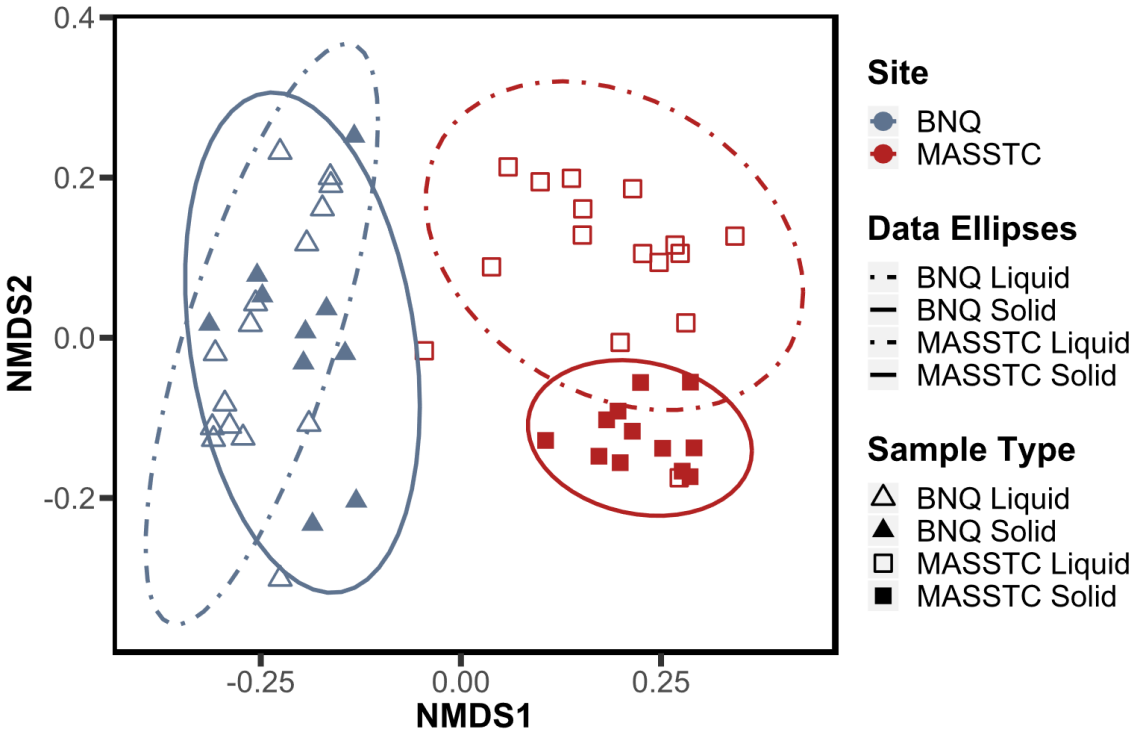

Figure 14: Non-metric multidimensional scaling (NMDS) ordination of MASSTC and BNQ liquid and solid communities (Stress = 0.116). Ordination of sample microbial communities was based on generalized Unifrac distances. Data ellipses illustrate multivariate t-distributions around the center of each community grouping (Section 2.3.5).

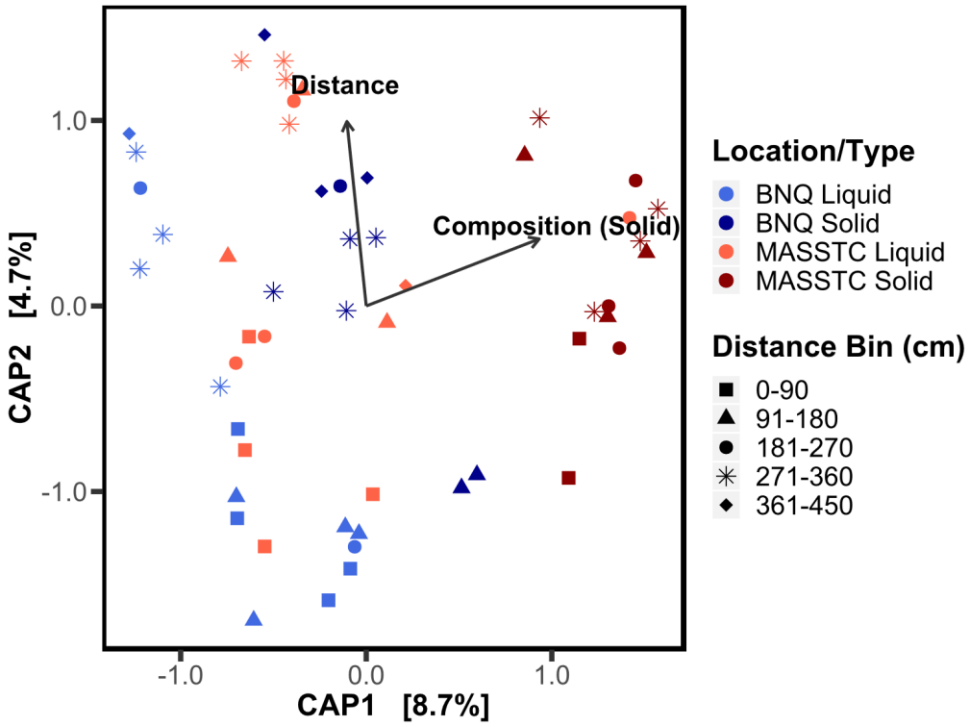

Figure 15: Constrained analysis of principal coordinate of MASSTC and BNQ liquid and solid sample community ordination. Ordination of sample microbial communities was based on generalized Unifrac distances and constrained by distance (cm) from the InnerTube inlet (Distance) and sample composition (solid or liquid). Samples were assigned to distance bins to facilitate the interpretation of the ordination plot. The arrows indicate the direction of increasing value of the named variable (Section 2.3.6).

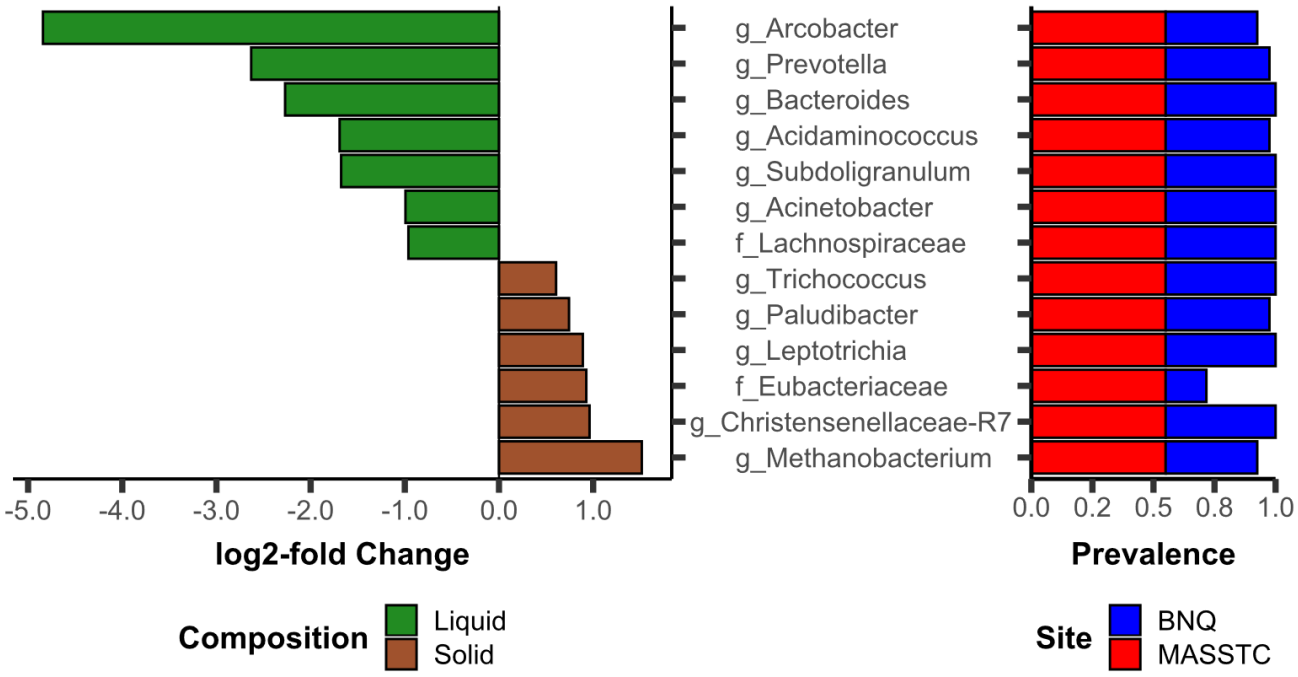

Figure 16: Differential abundance plot for assigned sequence variants (ASVs) in liquid and solid communities (left) and prevalence of these ASVs in MASSTC and BNQ communities (right). The levels of taxonomic assignment were designated by g_ and f_ which denote genus and family, respectively. Only ASVs that comprised of at least 1% of total reads of rarefied samples are displayed in the differential abundance plot. Prevalence was partitioned by site and calculated as the number of InnerTube samples in which the ASV was present divided by the total number of InnerTube samples (Section 2.3.8).

Chapter 3: Evaluating Relationships between Septic Tank Microbial Community Composition, System Design, and Treatment Efficacy

3.1.1 Relationships between Microbial Community Composition and Anaerobic Digester Performance

The primary function of ADs is the removal of influent organic matter through the conversion of complex carbon substrates into carbon dioxide and methane. Differences in AD performance can be explained by differences in community composition. Increased archaeal to bacterial population ratios are correlated with increased methanogenesis (Koo *et al*., 2017). 66% of the variation in methane production by 150 ADs was explained by the abundance of 1 methanogenic, 1 acetogenic, and 8 acidogenic taxa (Venkiteshwaran *et al.*, 2017). Han *et al*. (2016) determined that cellulose-fed ADs inoculated with paper mill and ethanol manufacturing waste removed less COD than ADs inoculated with digested sewage and manure. Furthermore, the addition of microbial taxa that are capable of degrading recalcitrant organic compounds has been shown to improve digester performance (Tale *et al*., 2015; Town and Dumonceaux, 2015).

Deterioration of AD performance is characterized by decreased mineralization of organic waste, accumulation volatile fatty acids (Zhang *et al.*, 2014), and changes in microbial community composition. In ADs processing dairy and poultry waste, loss of methane production and increased VFA concentrations were accompanied by a decrease in Firmicutes abundance and increase in Bacteroidetes abundance (Chen *et al..*, 2016). During process deterioration in a lab-scale food waste AD, the abundance of Bacteroidetes, Firmicutes, Chloroflexi, Spirochaetes, and Synergistetes decreased while the abundance of Actinobacteria and Tenericutes increased (Li *et al*., 2015). Furthermore, declines in methanogenic populations coincide with decreases in methanogenesis and accumulation of VFAs in ADs processing food waste (Williams *et al*., 2013).

3.1.2 Factors Influencing Anaerobic Digester Community Composition

Substrate type and operational temperature determine microbial community composition in ADs (De Vrieze *et al*., 2016). Zhang *et al*. (2014) found that 78 sample communities from digesters processing substrates such as manure and household waste clustered based on substrate type (Zhang *et al.*, 2014). Similarly, microbial communities from 21 ADs formed 3 clusters comprised of sewage sludge digesters, mixed waste digesters, and thermophilic (50 – 55^{o}C) digesters. Even among mixed waste digesters, microbial communities are affected by the proportion of substrate types (Buettner and Noll, 2018).

Microbial communities are also affected by the chemical environment within ADs. Generally, high NH_3 concentrations and temperatures favour hydrogenotrophic methanogens (De Vrieze *et al.*, 2016; Lin *et al.*, 2016). De Vrieze *et al*. (2015) determined that 29 biogas plant AD communities could be separated based on digester NH_3 concentration. In a study of residential mixed-waste ADs, NH_4^+-N levels are positively correlated with the abundance of the hydrogenotrophic genus *Methanoculleus* and negatively correlated with the acetoclastic genus *Methaosaeta* (Rui *et al*., 2015). Apart from methanogens, Tenericutes and Actinobacteria population were positively correlated with NH_4^+-N while Proteobacteria populations were negatively correlated with NH_4^+-N (Rui *et al*., 2015). Changes in pH can also impact AD community structure. Methanogenesis occurs within a pH range 6.5 – 8.5 (Azman, 2015). Acidification of ADs inhibits methanogen activity and growth (Pore *et al*., 2016; Frank-Whittle *et al*., 2014). Similarly, pH increases above 8.5 result in the loss of Euryarchaeota (Chen *et al*., 2017).

In wastewater treatment systems with sequential anaerobic and aerobic stages, aerobic effluent can be recirculated to anaerobic stages for nitrate reduction through denitrification (Jaafari *et al.*, 2017). Differences in community composition between recirculating and non-

recirculating (single-pass) ADs with similar design and substrate type have yet to be extensively explored. Denitrifying genera such as *Nitrospira*, *Thauera*, *Dechloromonas*, and *Ignavibacterium* are prevalent in recirculating anaerobic/anoxic/oxic wastewater treatment systems designed for nitrate reduction (Tian *et al*., 2015). Higher concentrations (> 1.5 g/L) of NO_3 suppress methanogenesis (Sheng *et al.*, 2013) which can negatively impact methanogen populations (Patra and Yu, 2014). Therefore, recirculating septic tanks may contain greater proportions of taxa involved in nitrogen metabolism and lesser proportions of methanogenic archaea than single-pass systems with comparable design.

3.1.3 Chapter 3 Objectives

Millions of households across North America rely on septic tanks to treat their wastewater (Diaz-Valbuena *et al*., 2011; Perks and Johnson, 2008). Failing septic tanks release organic matter and nutrients into groundwater and surface water which are detrimental to aquatic ecosystems (Schaider, 2017; Watson *et al*., 2016). Advances in septic tank technology are required to minimize freshwater contamination. Waterloo has designed a septic tank that decreases solid accumulation and thus the potential for septic tank failure (Jowett *et al*., 2017). Furthermore, WBS on-site wastewater treatment systems can be operated with recirculating flow to increase nutrient removal (Baeza *et al*., 2004). Despite the economic and environmental importance of septic tanks, there is limited research on how design and operational changes affect the microbial communities within septic tanks that carry out waste treatment.

The objectives for this chapter were to: (1) determine the effects of septic tank design and flow type on microbial community composition and (2) identify microbial taxa associated with greater COD reduction. To accomplish these objectives, we characterized microbial communities in conventional and InnerTube septic tanks operating with single-pass or recirculating flow throughout Southern and Central Ontario. Septic tank taxonomic profiles were constructed 16S

sequencing and chemical analyses of samples were performed to assess system performance. The findings of this project may facilitate improvements in septic tank design or operational strategies to maximize septic tank performance and formulate microbial inoculum for bioaugmentation of septic tanks.

3.2 Methods

3.2.1 Sampling Plan

To determine the effects of septic tank design and flow type on microbial community composition, we analyzed 4 septic tank system types which were defined by 4 combinations of septic tank design and flow. The two septic tank design types studied were conventional and InnerTube which were defined by the absence and presence of an InnerTube, respectively. The two operational flow types studied were single-pass and recirculating. Single-pass systems were defined as systems that do not recirculate aerobic biofilter effluent to the septic tank. Recirculating systems were defined as systems that recirculate a portion of aerobic biofilter effluent into the septic tank.

The four system type combinations were: single-pass InnerTube septic tanks (SI), recirculating InnerTube septic tanks (RI), single-pass conventional septic tanks (SC), and recirculating conventional septic tanks (RC). 6 septic tanks from each system type were sampled except for SI for which only 5 septic tanks were sampled (Fig. 17). The number of replicates for each system type was limited by the number of septic tanks scheduled for servicing which determined WBS personnel availability to facilitate site access and sampling.

Septic tank samples were collected by McMaster researchers and WBS employees from installations across Southern and Central Ontario (Fig. 18). All systems were located on residential properties and serviced single households. Each on-site wastewater treatment system was comprised of a septic tank connected to a downstream aerobic biofilter unit. Samples were taken from 3 sampling points along each septic tank to evaluate COD reduction from influent to effluent and to determine if the effect of system type was dependent on sampling point. Sampling points were designated as influent, tank, and effluent (Fig. 19). Each sample was given a unique 4-character alphanumeric code. The first number indicates the site number for the septic tank

type, the following two letters denote system type, and the final number indicates the sampling point. Influent, tank, and effluent were assigned the numbers 1, 2, and 3, respectively. For example, the sample code 1SC1 indicates that the sample was obtained from Site 1 of single-pass conventional septic tanks and that it was taken from the influent sampling point.

3.2.2 Sampling Protocol

Septic tanks were sampled in the order of effluent, tank, and influent sampling points to minimize sample cross-contamination (Fig. 20). For each sampling point, two sterile 500 ml bottles, one NH_3/TKN bottle preloaded with sulfuric acid (H_2SO_4) to provide a final concentration of 0.2% v/v, and one 100 ml COD bottle preloaded with 200 µl of concentrated H_2SO_4 were filled with wastewater. Regardless of sampling apparatus, all wastewater was pooled in the collection vessel (Fig. 21) and mixed before it was dispensed into sampling bottles for storage and transport. Dissolved oxygen (DO), pH, and temperature measurements were taken from the collection vessel for each sampling point. All sampling equipment was rinsed with commercial spring water between sampling points.

Conventional septic tank effluent samples were taken from the effluent holding tank (Fig. 19) by inserting a collection vessel into the effluent holding tank directly beneath the septic tank outlet. Conventional septic tank inlet and tank samples were taken from the inlet and outlet risers, respectively, using the tube sampler (Fig. 20). To obtain samples, the tube sampler was inserted into the vessel, foam sphere end first to the 1 L mark. The rope of the tube sampler was then pulled and secured so that the foam sphere plugged the submerged end of the tube sampler. The contents of the tube sampler were emptied into the collection vessel (Fig. 20) by releasing the foam sphere. Wastewater was repeatedly collected with the tube sampler until approximately 1.5 L was accumulated in the collection vessel.

InnerTube effluent samples were taken from the effluent spray nozzle feeding the biofilter aerobic unit. An effluent sampler or collection vessel (Fig. 20) was used to collect enough effluent from the Biofilter spray nozzle to fill the collection vessel (Fig. 19). For InnerTube inlet and tank samples, the tube sampler was used to collect wastewater from the inlet opening of the InnerTube and the outlet of the InnerTube below the inlet riser (Fig. 19), respectively. A field blank was taken at the end of each sampling day. A field blank was obtained by emptying sterile water from the field blank bottle into a rinsed collection vessel, which was then poured back into the field blank bottle.

After sampling, one of each of the duplicate 500 ml wastewater samples was sent to McMaster University and CAWT. Ammonia/TKN and COD sample bottles were sent to CAWT. All samples were covered with ice packs and shipped in coolers. The maximum holding time between sampling and chemical testing for CAWT samples was 7 days. McMaster samples were stored at -80^{o}C within 24 hours of sampling except for the single-pass InnerTube system samples from Central Ontario which were received up to 6 days after sampling. Samples were kept on ice during this period.

To evaluate the effect of our transport protocol on sample communities we collected 2 sets of triplicate samples from the influent and tank sampling points of a recirculating InnerTube septic tank, for a total of 4 sets. 1 set each of tank and influent samples was stored at 4^{o}C for 1 day and then frozen at -80^{o}C to emulate our standard transportation and storage protocol. For control samples, we extracted DNA from the 2 remaining sets on the same day of sampling. Lab contamination was evaluated by filtering and extracting DNA from 200 ml of ddH_2O.

3.2.3 Chemical Analysis, DNA Extraction, and 16S Amplification and Isolation

Chemical analysis of sample duplicates was conducted as described in section 2.2.3 with the following changes: VS and TS were not measured and pH and chemical oxygen demand

(COD) were measured for all samples. Hydraulic residence time (HRT) in days was calculated by dividing septic tank volume by flow rate. HRT is a measure of how long wastewater stays within a septic tank.

DNA extraction was performed as described in section 2.2.4. 16S amplification was performed as described in section 2.2.5 with the following changes: template volumes were increased to 4 µl and distilled water volumes were decreased to 11 µl for samples with < 10 ng/µl DNA concentrations and index primer annealing temperature (stage 2 PCR) was increased to 52ºC from 50ºC.

Amplicon isolation and PCR clean-up were performed out as outlined in section 2.2.6 with additional steps to standardize amplicon DNA concentrations. 2nd round PCR products were quantified by fluorescence with a Qubit 2.0 fluorimeter. 25.0 ng of each V4 amplicon and 18.3 ng of each V3 amplicon were pooled for gel extraction. V4 and V3 amplicons were pooled separately and gel extractions of pooled amplicons were done in duplicate. V4 and V3 gel extraction products were mixed in a ratio of 5:1, respectively, by mass. This ratio was used to account for the greater length of the V4 products in comparison to V3 products (~430 bp vs. ~320 bp, respectively) and the greater number of samples amplified with V4 primers. Sequence data processing and taxonomic assignment were performed as described in section 2.2.7.

3.2.4 Sequence Processing and Statistical Analyses

Sequence processing and statistical analyses of sequencing data were performed as outlined in sections 2.2.7 and 2.2.8, respectively. In addition, the procrustes function in vegan (Oksanen *et al.*, 2019) was used to evaluate the similarity between influent, tank, and effluent distance matrices and the similarity between community compositions determined using V3 amplicons and V4 amplicons. Furthermore, hierarchical agglomerative clustering was performed with hclust in R (Müllner, 2013).

Tank samples were chosen for ordination analyses and significance tests to avoid pseudoreplication. Variation in tank communities was assumed a priori to best represent the effect of system flow type and design. It was assumed that influent communities would most likely reflect household microbiomes rather than septic tank differences and that effluent filters between the septic tank and aerobic biofilter units may lead to artefactual community results.

3.3 Results

3.3.1 16S Sequencing Descriptive Statistics

Sequencing statistics were calculated to evaluate sequencing depth and overall quality of sequencing. A total number of 12,217,820 paired-end reads were obtained with a mean of 134,262 ± 50,641 reads per sample. After quality filtering, merging, and chimera removal, a total of 10,829,087 reads remained with a mean of 119,001 + 45,868 reads per sample. Sequence reads were comprised of 11,776 assigned sequence variants (ASVs). 16 ASVs (~0.0018% of total reads) attributed to the Kingdom Eukaryota or unassigned at the Kingdom level were removed from the dataset. After agglomeration of ASVs with the same taxonomic assignment, 1584 ASVs remained. Sample communities were rarefied to 73,706 reads to match the number of reads obtained from sample 4SC3. No samples were removed from further analyses (Fig. 21).

3.3.2 Relative Abundance of Microbial Phyla

Relative abundances of microbial phyla were determined as a qualitative analysis of septic tank community structure. Single-pass conventional septic tank microbial communities were comprised of 32 identified phyla. 16 of those phyla had proportions greater than 0.5% of total reads (Fig. 22). Phyla with the greatest median proportions in all single-pass conventional septic tank samples were Firmicutes (22.4%, 15.6% – 84.2%), Proteobacteria (37.8%, 7.7% – 53.9%), Bacteroidetes (10.4%, 1.3% – 26.0%), and Synergistetes (8.6%, 0.8% – 31.7%). Recirculating conventional septic tank microbial communities were comprised of 32 identified phyla. 17 of those phyla had proportions greater than 0.5% of total reads (Fig. 22). Phyla with the greatest median proportions in all single-pass conventional septic tank samples were Firmicutes (28.6%, 12.6% – 67.8%), Proteobacteria (19.7%, 14.0% – 41.4%), Bacteroidetes (17.7%, 1.1% – 28.2%), and Synergistetes (7.4%, 1.1% – 32.3%).

Single-pass InnerTube septic tank microbial communities were comprised of 31 identified phyla. 14 of those phyla had proportions greater than 0.5% of total reads (Fig. 22). Phyla with the greatest median proportions in all single-pass conventional septic tank samples were Firmicutes (39.7%, 4.0% – 83.7%), Proteobacteria (34.8%, 5.3% – 84.4%), Bacteroidetes (5.9%, 0.0% – 27.9%), and Synergistetes (2.5%, 0.1% – 46.0%). Recirculating InnerTube septic tank microbial communities were comprised of 32 identified phyla. 17 of those phyla had proportions greater than 0.5% of total reads (Fig. 21). Phyla with the greatest median proportions in all single-pass conventional septic tank samples were Firmicutes (35.9%, 25.6% – 72.3%), Proteobacteria (22.9%, 12.9% – 45.7%), Bacteroidetes (12.4%, 0.5% – 21.7%), and Synergistetes (5.5%, 0.1% – 22.3%).

3.3.3 Archaeal Abundance

Relative abundance of archaeal orders was determined as a qualitative analysis of septic tank methanogen populations. Median relative abundance of Euryarchaeota across all samples was 0.46% (0 – 3.22%) (Fig. 23). Septic tank Euryarchaeota were comprised of 4 orders. Ranked from greatest to least median relative abundance they were: *Methanomicrobiales* (0.09%, 0 – 2.23%), *Methanobacteriales* (0.12%, 0 – 1.42%), *Methanosarcinales* (0.03%, 0 – 0.64%), and *Methanomassiliicoccales* (0.01%, 0 – 0.28%).

3.3.4 Alpha Diversity

The alpha diversity of septic tank communities was calculated to assess the potential for differences in functional diversity or performance stability between septic tank system types. Mean Shannon indices were not significantly different between system types (Fig. 24) (ANOVA; $F = 1.97$; $p = 0.153$). Mean ACE indices were also not significantly different between system types (Fig. 24) (ANOVA; $F = 2.536$; $p = 0.0873$). The mean Shannon and ACE diversity indices for single-pass conventional septic tanks were 3.5 ± 0.4 and 285.2 ± 36.5, respectively. The

mean Shannon and ACE diversity indices for recirculating conventional septic tanks were 3.8 ± 0.4 and 294.6 ± 50.4, respectively. The mean Shannon and ACE diversity indices for single-pass InnerTube septic tanks were 3.0 ± 1.0 and 215.5 ± 81.4, respectively. The mean Shannon and ACE diversity indices for recirculating InnerTube septic tanks were 3.8 ± 0.6 and 291.9 ± 44.6.

3.3.5 Conventional and InnerTube Septic Tank Hierarchical Agglomerative Clustering

Hierarchical agglomerative clustering based on generalized Unifrac distances (Fig. 25) was used to visualize the grouping septic tank communities of septic tank communities by site. Septic communities significantly differed between sites (PERMANOVA; pseudo-F = 5.583; p = 0.001).

3.3.6 Ordination of Conventional and InnerTube Septic Tank Communities

Variation partitioning analysis was used to validate the choice of tank sample communities for downstream statistical analyses. System type explained similar amounts of variation between influent (18.2%), tank (18.3%), and effluent (17.8%) sample communities. NMDS ordination of sample communities was used to evaluate sample community associations and clustering. NMDS ordination of tank sample communities is shown in Fig. 26. PERMANOVA was used to compare sample groups and calculate the statistical significance of sample clustering. Influent and tank sample communities significantly differed based on system type (PERMANOVA; $p < 0.05$). Pairwise PERMANOVA analyses determined that single-pass and recirculating communities were significantly different (PERMANOVA; Adj. $p < 0.05$).

Procrustes analyses of NMDS ordinations were used to evaluate potential differences in community analyses based on sampling location. Procrustes analyses showed that: influent sample communities were not significantly correlated with tank sample communities (protest; correlation = 0.399; $p > 0.05$) (Fig. 27), influent sample communities were not significantly correlated with effluent sample communities (protest; correlation = 0.320; $p > 0.05$), and tank

sample communities were significantly correlated with effluent sample communities (protest; correlation = 0.901; $p < 0.01$). Ordinations of tank sample communities characterized using V3 and V4 amplicons were compared using Procrustes analysis to validate the results obtained with the V4 amplicon. Ordinations of V3 and V4 tank communities were significantly correlated (protest; correlation = 0.917; $p < 0.01$).

3.3.7 Constrained Analysis of Principal Coordinates

Relationships between microbial community composition and environmental variables were visualized with constrained analysis of principal coordinates (CAP) (Fig. 28). Sample community variation (Fig. 28) was significantly correlated with COD reduction (PERMANOVA; pseudo-F = 2.20; $p = 0.0323$) which explained 6.51% of variation between communities. Temperature, CBOD removal, influent COD levels, pH, flow rate, initial COD levels, HRT and ammonia concentrations were not significantly correlated with community ordination based on generalized Unifrac distances (PERMANOVA; $p > 0.05$). System type also had a significant effect on V3-sequenced tank sample communities (PERMANOVA; pseudo-F = 1.4185; $p = 0.0316$).

3.3.8 Differential Abundance Analysis of Single-pass InnerTube and Recirculating Conventional Septic Tank Communities

DESeq2 differential abundance analysis was used to identify the microbial taxa associated with the significant differences observed between SI and RC communities. 3 differentially abundant genera that each sum to greater than 1% of total reads were identified SI and RC septic tanks using DESeq2 differential abundance analysis (Fig. 29). 1 significantly differentially abundant genus (*Pseudomonas*) was found in SI communities and 2 significantly differentially abundant genera (*Arcobacter and Desulfomicrobium*) were found in RC

communities (Adj. $p < 0.05$). The potential roles in anaerobic digestion of the differentially abundant are shown in Table 10. Log_2-fold changes ranged from 3.46 to 5.94.

3.3.9 SparCC Correlation of Taxa with Distance from the Inlet of the InnerTube

SparCC correlation analyses of ASV abundances and COD reduction were performed to identify taxa that may be responsible for improved septic tank performance. 2 ASVs, *Brevundimonas* and *Desulfovibrio*, were robustly correlated with ($r > 0.4$; $p < 0.01$) COD reduction (Liu *et al.*, 2019). Both ASVs were positively correlated with COD reduction and assigned at the genus level. The potential roles of these ASVs in wastewater treatment were acidogenesis and acetogenesis (1 taxon) and acetogenesis (1 taxon) (Table 11).

3.4 Discussion

3.4.1 Effects of Septic Design and Operational Flow on Community Composition

Septic tank microbial communities throughout Southern and Central Ontario were characterized using 16S sequencing to evaluate the effect of septic design and operational flow on community composition. The two design types and two flow types studied in this chapter were conventional septic tanks and InnerTube septic tanks and single-pass and recirculating flow, respectively. Septic tanks were separated into four system types based on design and flow. The system types were designated as single-pass conventional, recirculating conventional, single-pass InnerTube, and recirculating InnerTube. Single-pass InnerTube septic tank (SI) communities were determined to be significantly different from recirculating conventional septic tanks (RC) ($p < 0.05$).

Septic tanks are dynamic environments in which microbial communities experience seasonal temperature fluctuations that follow air temperature changes (Viraraghavan, 1976). Moreover, microbes in recirculating systems experience alternating anaerobic and aerobic conditions as they flow from the septic tank to the aerated biofilter and back to the septic tank. Therefore, bacteria that can survive or grow throughout temperature fluctuations and transitions between oxygen regimes are likely to be enriched in recirculating systems in comparison with single-pass systems.

For taxa with greater than 1% abundance across all samples, the bacterial genera *Arcobacter* and *Desulfomicrobium* were significantly more abundant in RC in comparison to SI ($p < 0.05$). *Arcobacter* belong to the phylum Epsilonbacteraeota and grow in a wide range of habitats including plant roots, sewage, and the human gut (Fisher *et al.*, 2014). Human gut *Arcobacter* can survive the transition to environmental waters and their presence is an indicator of fecal contamination (Collado *et al*., 2008). *Arcobacter* are abundant in both anaerobic

(Supaphol *et al*., 2011) and aerobic wastewater treatment systems (Gonzalez-Martinez *et al*., 2016). In addition, Stampi *et al*. (1999) were able to isolate viable *Arcobacter* cells from anaerobic and aerobic stages of a continuous municipal waste water treatment system. The prevalence of *Arcobacter spp*. throughout diverse environments and wastewater oxygen regimes suggests that they are highly adaptable (Fisher *et al*., 2014) which may explain their greater abundance in the dynamic environment of recirculating septic tanks.

The genus *Desulfomicrobium* is part of the phylum Proteobacteria and is comprised of obligate anaerobes that reduce sulfate during respiration (Kushkevych, 2013). Sulfate-reducing bacteria (SRB) produce acetate, hydrogen, and carbon dioxide as byproducts of VFA fermentation (Muyzer and Stams, 2008). As a result, SRB form syntrophic relationships with methanogens (Muyzer and Stams, 2008). *Desulfomicrobium* are strongly correlated with the occurrence of acetotrophic methanogens in full-scale anaerobic digesters treating municipal waste, which indicates syntrophic relationships between the taxa (Zhang *et al*., 2019a). SRB genera, primarily *Desulfovibrio,* were also present in high abundance in SI and the other system types. Therefore, environmental factors that negatively impact SRB such as high metal concentrations (Utgikar *et al*., 2002) were likely not the cause of the differential abundance in *Desulfomicrobium* in RC and SI systems.

SRBs form biofilms that thicken in response to oxidative stress (Mohanakrishnan *et al*., 2011) which provides resistance to oxygen diffusion (Kjeldsen *et al*., 2004). In a study of SRB growth and chemotaxis, *Desulfomicrobium spp*. survival during oxygen exposure was increased when sediment particles were added to the growth media, whereas there was no effect for *Desulfovibrio spp*. (Sass *et al*., 2002). This indicates that *Desulfomicrobium spp*. were able to survive aerobic conditions by forming aggregates with the sediment (Sass *et al*., 2002).

Desulfomicrobium may similarly form biofilms with wastewater particulates which afford them greater survival than other SRB during aerobic treatment in RC systems.

Pseudomonas was significantly more abundant in SI systems in comparison with RC ($p < 0.05$). *Pseudomonas spp*. are facultatively anaerobic Proteobacteria that inhabit the human gut (Markou and Apidianakis, 2014) and anaerobic digesters (Rui *et al.*, 2015). *Pseudomonas* also inhabit wastewater with high concentrations of lipids which has been attributed to their ability to digest long-chain fatty acids (LCFAs) such as oleic acid (Zarzycki-Siek *et al*., 2013; Walter *et al*., 2019). LCFAs are resistant to degradation in anaerobic digesters due to their inhibitory effect on methanogenesis and tendency to cause biomass flotation and washout (Palatsi *et al*., 2012). Adsorption of LCFAs to microbial biomass is required for LCFA hydrolysis and metabolism (Hwu *et al*., 1998). InnerTube septic tanks provide greater surface area for microbial adhesion, biofilm formation, and substrate-microbe contact than conventional septic tanks. Therefore, the differential abundance of *Pseudomonas* between SI and RC may be due to greater adsorption and utilization of LCFAs in SI septic tanks.

The potential explanations for the observed differences between RC and SI systems presented in this discussion posit oxidative stress during recirculation and greater surface area for waste-microbe interactions as mechanisms of community differentiation. However, no significant differences were observed between single-pass recirculating (SC) and recirculating InnerTube (RI) communities ($p > 0.05$). Differences between RC and SI communities may be due to specific synergistic interactions between design and operational flow that do not occur in SC and RI systems. For example, SC systems do not exert the selective pressure of repeated aerobic exposure and lack InnerTubes for substrate adsorption. Therefore, the proposed mechanisms for microbial enrichment and community differentiation do not exist in SC systems.

In RI communities, recirculation and the InnerTube environment may have balancing effects on community composition that constrain the enrichment of any specific taxa. Finally, factors not measured during this project, such as salinity (De Vrieze *et al*., 2016) and antibiotic concentrations (Álvarez *et al.*, 2010), may account for unexplained variation. To fully evaluate the proposed mechanisms and potential sources of variation, future studies should employ controlled temperatures, standardized tank volumes and flow rates, a shared influent source, and a comprehensive assessment of wastewater chemical composition.

3.4.2 Microbial Taxa Significantly Correlated with Improved Septic Tank Performance

COD reduction from influent to effluent showed significant positive correlations (Liu *et al*., 2019) with the abundance of the proteobacterial genera *Desulfovibrio* and *Brevundimonas* ($p < 0.01$). *Desulfovibrio* and *Brevundimonas spp*. include H_2 producers (Danial *et al*., 2015; Meyzer and Stams, 2008) that have been isolated from human specimens (Ryan and Pembroke, 2018; Heberling *et al*., 2013) and anaerobic digesters (Su *et al*., 2014; Smith *et al*., 2015). During anaerobic digestion, H_2 is utilized by hydrogenotrophic methanogens to reduce CO_2, generate ATP, and produce methane as a waste product (Kaster *et al*., 2011). Methanogenesis is the rate-limiting step of the anaerobic digestion of soluble organic compounds (Ma *et al*., 2013). Bassani *et al*. (2015) demonstrated that injecting H_2 into anaerobic digesters can increase the abundance of hydrogenotrophic methanogens and methane production. Therefore, by supplying hydrogenotrophic methanogens with H_2, *Desulfovibrio and Brevundimonas* may increase rates of methanogenesis and COD reduction (Zhao *et al*., 2014).

Desulfovibrio spp. are known to form syntrophic relationships with hydrogenotrophs in which they ferment VFAs and supply H_2 to hydrogenotrophs for methanogenesis (Meyer *et al*., 2013). However, if sulphate is available, *Desulfovibrio spp*. outcompete methanogens for H_2 which they utilize as electron donors for ATP generation (Muyzer and Stams, 2008; Pfennig and

Widdel, 1982). Syntrophic relationships between *Brevundimonas* and methanogens have yet to be characterized, but *Brevundimonas* has been found in conjunction with hydrogenotrophic methanogens in hydrocarbon-degrading communities (Kryachko *et al*., 2012). Positive correlations between COD reduction and *Desulfovibrio* and *Brevundimonas* could also be attributed to their ability to adapt to colder temperatures (< 10^{o}C) and maintain metabolic activity during temperature fluctuations and colder months (Baldwin *et al*., 2016; Aguayo *et al*., 2009).

Bioaugmentation is a viable strategy for AD performance improvement (Town and Dumonceaux, 2015). Using 16S and chemical analyses we have demonstrated that *Desulfovibrio* and *Brevundimonas* are candidates for starter cultures and bioaugmentation strategies in septic tanks. Once again, long-term, standardized bioaugmentation trials are required to evaluate the performance-enhancing capabilities of these bacteria. Furthermore, to design effective inocula, future studies are needed to determine if the activity of *Desulfovibrio* and *Brevundimonas* is dependent on association with specific acidogenic and methanogenic microbes.

3.4.3 Comparison of Septic Tank Community Structure and Domestic Wastewater Anaerobic Digesters

Microbial phyla with the highest median abundances in the septic tanks surveyed were, in descending order, Firmicutes, Proteobacteria, Bacteroidetes, and Synergistetes. These were followed Epsilonbactaerota, Actinobacteria, and Verrucomicrobia in varying order across system types. These results agreed with the composition of comparable anaerobic digesters (Rui *et al*., 2015; Yang *et al*., 2014). The dominant phyla in household anaerobic digesters co-digesting food and domestic sewage included Firmicutes, Bacteroidetes, Synergistetes, Proteobacteria, and Actinobacteria (Rui *et al*., 2015). Firmicutes, Proteobacteria, and Bacteroidetes were also the most abundant phyla in full-scale anaerobic digesters treating sludge from municipal wastewater treatment plants (Yang *et al*., 2014).

The most abundant phyla of the septic tank communities surveyed have the metabolic potential to convert complex organic compounds into precursors for methanogenesis. During anaerobic digestion, Proteobacteria, and Bacteroidetes species play specific roles in hydrolysis, acidogenesis, and acetogenesis (Campanaro *et al*., 2016; Regueiro *et al*., 2012). Synergistetes and Verrucomicrobia are involved in the hydrolysis of polysaccharides and fermentation of the resultant sugars into VFAs (Svartström *et al*., 2017; Geerlings *et al*., 2018). VFA accumulation in anaerobic digesters has also been attributed to acidogenic Actinobacteria (Jang *et al*., 2014). Epsilonbactaerota utilize alternative electron acceptors such as nitrate and sulfide for energy production and facilitate methanogenesis through the production of H_2 (Rajhi *et al*., 2016; Keller *et al*., 2015).

In this study, septic tank Euryarchaeota communities were entirely composed of methanogenic archaea. The median relative abundance of septic tank methanogens (0.5%) was less than those previously reported by studies of household anaerobic digesters (2.172%) (Rui *et al*., 2015) and anaerobic digesters processing sewage sludge (1.22%) (Yang *et al*., 2014). The lesser abundances observed in septic tanks may be due to lower operational temperatures (~9 – 20^{o}C) than in the referenced digesters (18 – 35^{o}C) (Rui *et al*., 2015; Yang *et al*., 2014). Methanogenic activity decreases with temperature (Nie *et al*., 2014) and the growth of hydrogenotrophic methanogens, such as *Methanobrevibacter arboriphilus*, are inhibited below 10^{o}C (Lu *et al*., 2011).

The hydrogenotrophic orders *Methanomicrobiales* and *Methanobacteriales* made up the majority of septic tank methanogens, although acetotrophs and methylotrophs were also present. Hydrogenotrophic methanogens were also the most abundant methanogens in previous studies of anaerobic digesters treating municipal sewage and blackwater (Kim *et al*., 2013; Gao *et al*.,

2019). Hydrogenotrophs are more prevalent in the human gut than acetotrophs (Gaci *et al*., 2014), which may explain their greater abundance in domestic wastewater. Furthermore, the psychrophilic conditions (< 20^{o}C) (McHugh *et al*., 2006) within septic tanks may select for hydrogenotrophs over acetotrophs (Xing *et al*., 2013).

Septic tank microbial community alpha diversities were similar to those reported for anaerobic digesters processing blackwater (Gao *et al*., 2019). In contrast, septic tank communities were less diverse than previously described co-digesters of domestic wastewater and food waste (Rui *et al*., 2015; Zhang *et al*., 2017; Zhang *et al*., 2019b). This is likely due to the greater substrate complexity in mixed waste streams (Chung *et al*., 2018; Regueiro *et al*., 2014). The resistance to perturbation of anaerobic digester processes has been found to be positively correlated with microbial community alpha diversity (De Vrieze *et al*., 2017; Zhang *et al*., 2017). Therefore, co-digestion of household food waste is a potential area for research in septic tank performance improvement. However, prior to this, long-term studies are required to fully assess the stability of septic tank microbial communities and relationships between septic tank performance and alpha diversity.

3.4.4 Sources of Variability in Septic Tank Community Composition

Temperature, NH_3 (De Vrieze *et al*., 2015), pH (Pore *et al*., 2016), initial COD concentrations (Goux *et al*., 2015), and hydraulic retention time (Wei *et al*., 2017) influence microbial community composition. However, these factors did not show significant effects on septic tank community composition in the current study ($p > 0.05$). Potential effects of these factors may have been obscured by variability in influent (Zhang *et al*., 2014). Substrate composition is one of the primary drivers of anaerobic digester community variation (De Vrieze *et al*., 2016). In a meta-analysis of anaerobic digesters processing different substrates such as manure, municipal sludge, and beverage waste, communities grouped by substrate type

irrespective of differences in environmental factors (Zhang *et al*., 2014). Microbial communities in digesters processing pig manure can even differ between batches of manure (Reguiero *et al*., 2014). Although septic tanks only process household wastewater, septic tank influent properties may differ substantially due to variations in household diets which can alter fecal protein, fat, and fiber content (Rose *et al*., 2015). Furthermore, septic tank microbial inputs may differ due to variation in gut microbiomes between household individuals (Davenport *et al*., 2014; Falony *et al*., 2016).

Long-term experiments with standardized influent and system parameters are needed to evaluate the effect of specific environmental variables on septic tank microbial community composition (Zhang *et al*., 2014). However, from this study physicochemical parameters and taxonomic profiles could be utilized to develop predictive models for septic tank performance or microbial community composition. The ADM1 is a formalized generic model for anaerobic digester performance that primarily considers substrate uptake and degradation but also includes indirect measurements of microbial contributions (Batstone *et al*., 2002). This model serves as a basis for further model development and anaerobic digester design (Batstone *et al*., 2002). Wastewater researchers have also developed models to predict microbial growth and community composition (Esser *et al*., 2015). System modelling, which requires further variable analyses, is outside the scope of this pilot study. Future work should evaluate the feasibility, accuracy, and utility of septic tank performance and microbial growth models constructed from 16S sequencing and physicochemical data.

Table 10: Potential Roles in Anaerobic Digestion of Differentially Abundant Genera in Single-Pass and Recirculating Septic Tank Communities (Section 3.3.8)

Differentially Abundant Single-Pass Genera			
ASV	Genus	Potential Roles	Literature Cited
34	*Pseudomonas*	Hydrolysis	Walter *et al.*, 2019; Buettner *et al*., 2019
Differentially Abundant Recirculating Genera			
2	*Arcobacter*	Acidogenesis/Acetogenesis	Supaphol *et al.*, 2011
36	*Desulfomicrobium*	Acetogenesis	Zhang *et al*., 2019a

Table 11: Potential Roles in Anaerobic Digestion of Genera Correlated with COD Reduction (Section 3.3.9)

Genera Positively Correlated with COD Reduction			
ASV	Genus	Potential Roles	Literature Cited
441	*Brevundimonas*	Acidogenesis/Acetogenesis	Su *et al*., 2014; Danial *et al*., 2015
24	*Desulfovibrio*	Acetogenesis	Meyer *et al*., 2013

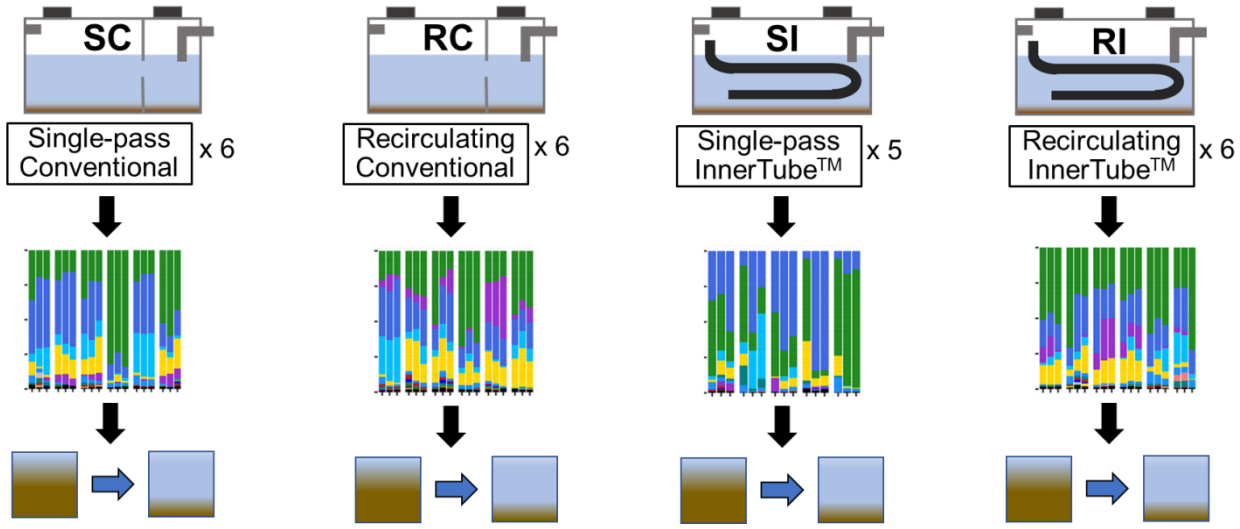

Figure 17: Chapter 3 study design and methods overview (Section 3.2.1). 23 septic tanks from 4 septic system types were sampled from Southern and Central Ontario. DNA was extracted and sequenced and sequence data was processed using the DADA2 16S amplicon pipeline. Statistical relationships between microbial community composition and septic tank performance were analyzed in R.

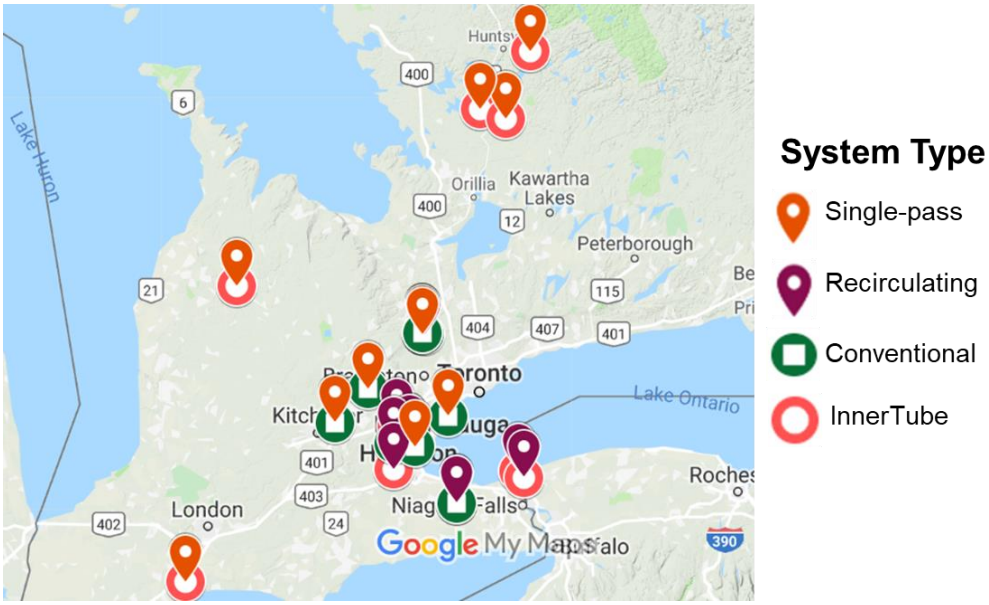

Figure 18: Sampling locations of single-pass and recirculating, conventional and InnerTube, septic tanks in Central and Southern Ontario (Section 3.2.1).

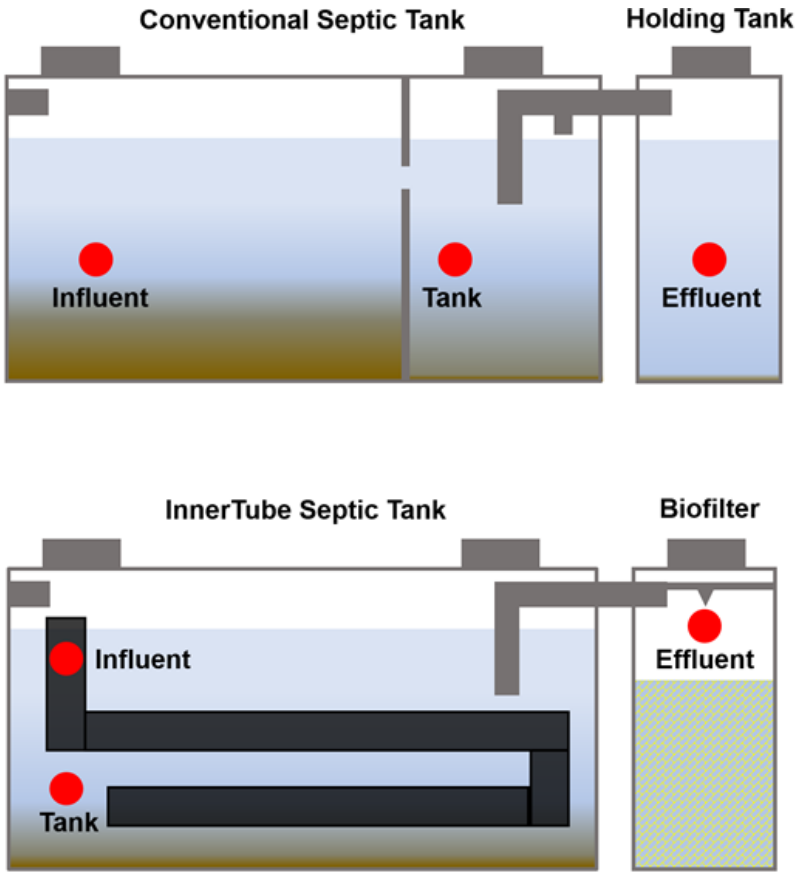

Figure 19: Conventional (top) and InnerTube (bottom) septic tank systems. Effluent, tank, and influent sampling points are shown in red (Section 3.2.1, 3.2.2).

Figure 20: Effluent sampler and collection vessel (top) – Capped tubes with approximate volumes of 0.75 L and 1.25 L affixed to the bottom of ~4 ft. plastic poles with two metallic screws. Tube sampler (bottom) – A ~4 ft. open plastic tube with two open ends. A rope knotted at one end (right) and a hard foam sphere (left) attached to the other runs through the tube sampler. A dual hook is attached to the end of the tube with the rope knot so that the rope can be secured. The green tape designates the 1 L volume mark of the tube sampler (Section 3.2.2).

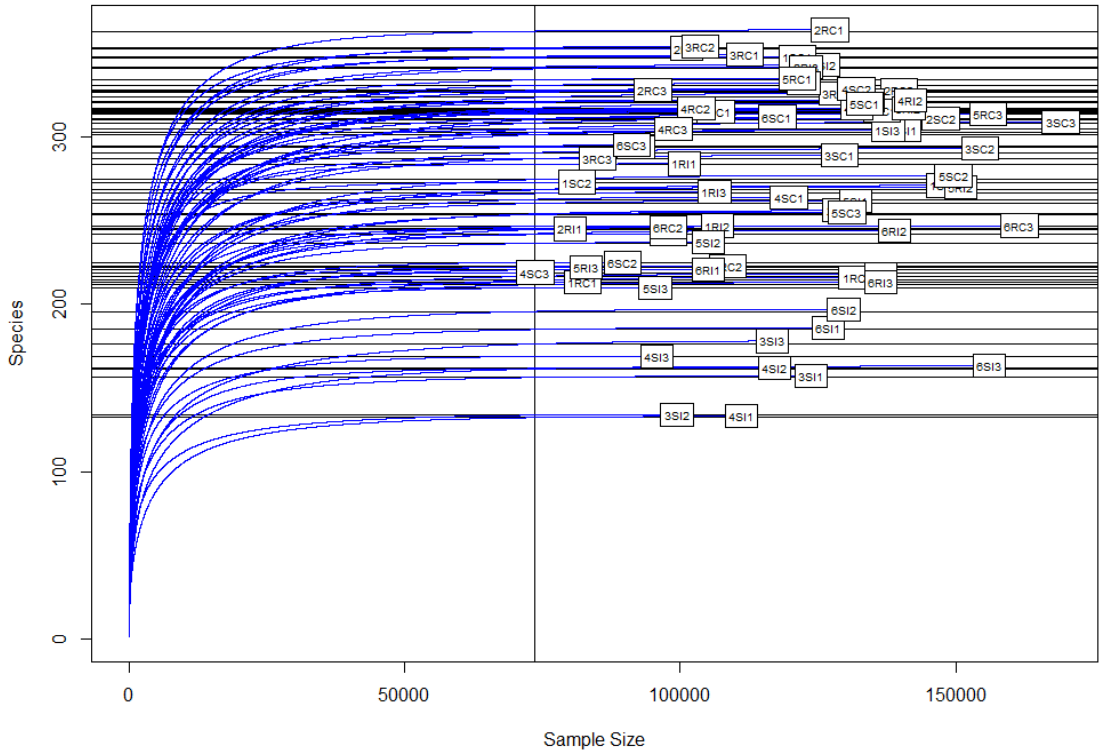

Figure 21: Rarefaction curves for conventional and InnerTube septic tank communities. Sample identifiers are shown in boxes at the terminus of each sample curve. The vertical line in the plot denotes the level sample communities were rarefied (73,706 reads) (Section 3.3.1).

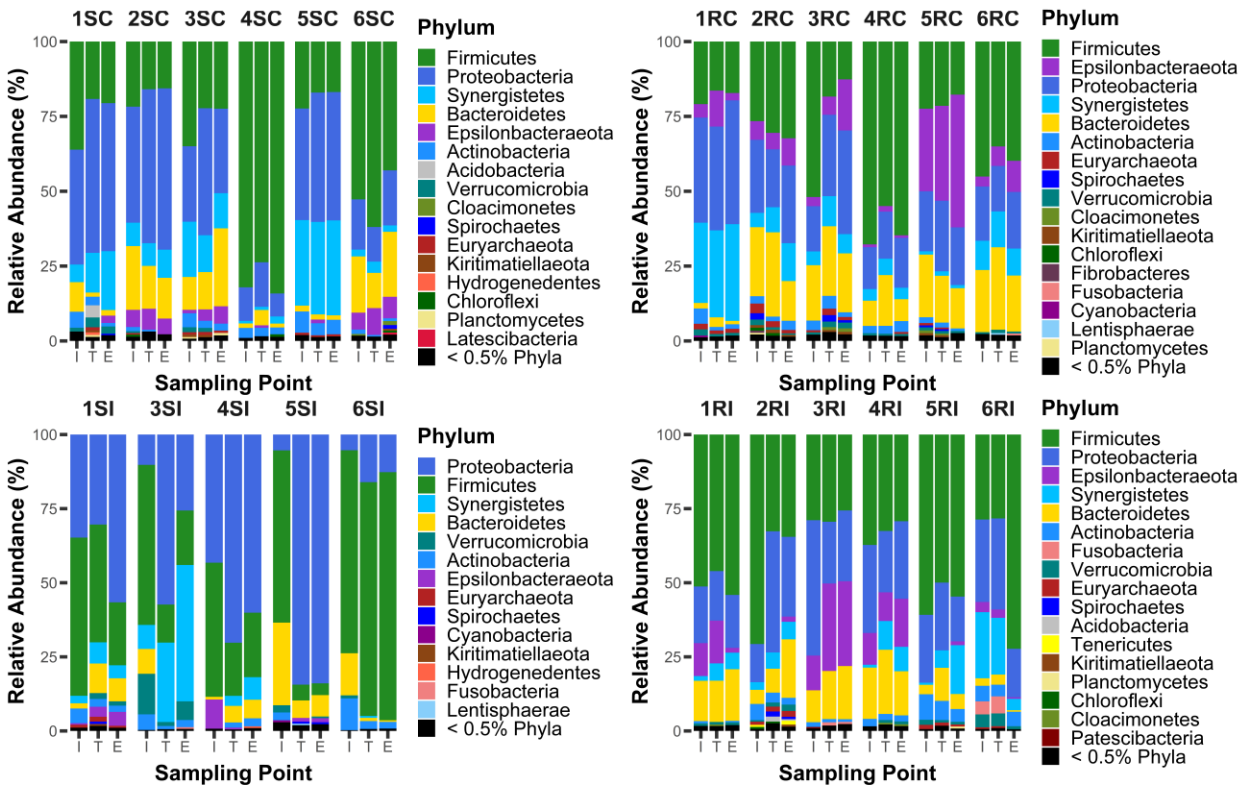

Figure 22: Relative abundances of bacterial and archaeal phyla sampled from the influent, tank, and effluent septic tank microbial communities. To simplify visualization of community compositions, phyla that individually comprised of less than 0.5% of total counts were agglomerated and are shown in black. SC and RC denote single-pass and recirculating conventional septic tanks, respectively. SI and RI denote single-pass and recirculating InnerTube septic tanks, respectively. I, T, and E denote influent, tank, and effluent samples, respectively (Section 3.3.2).

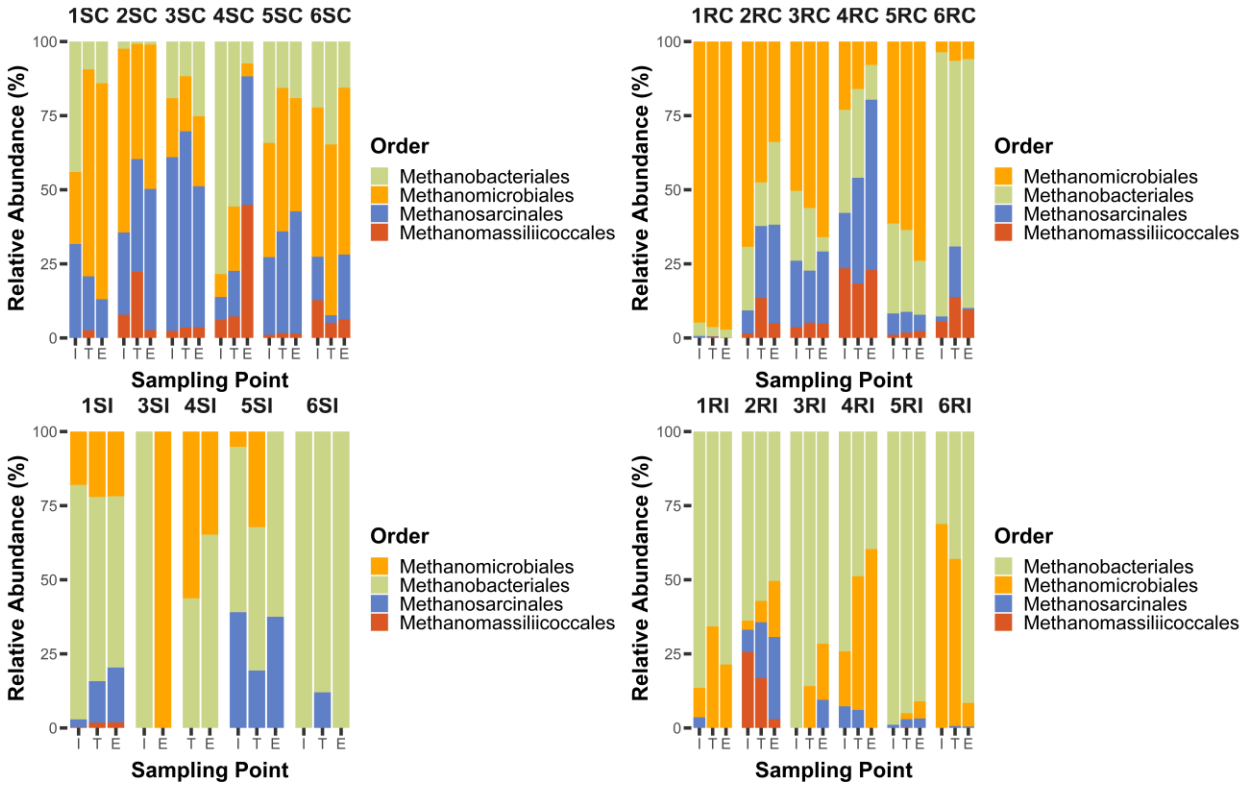

Figure 23: Relative abundances of archaeal orders sampled from the influent, tank, and effluent septic tank microbial communities. SC and RC denote single-pass and recirculating conventional septic tanks, respectively. SI and RI denote single-pass and recirculating InnerTube septic tanks, respectively. I, T, and E denote influent, tank, and effluent samples, respectively. Samples were omitted if no methanogenic orders were detected (Section 3.3.3).

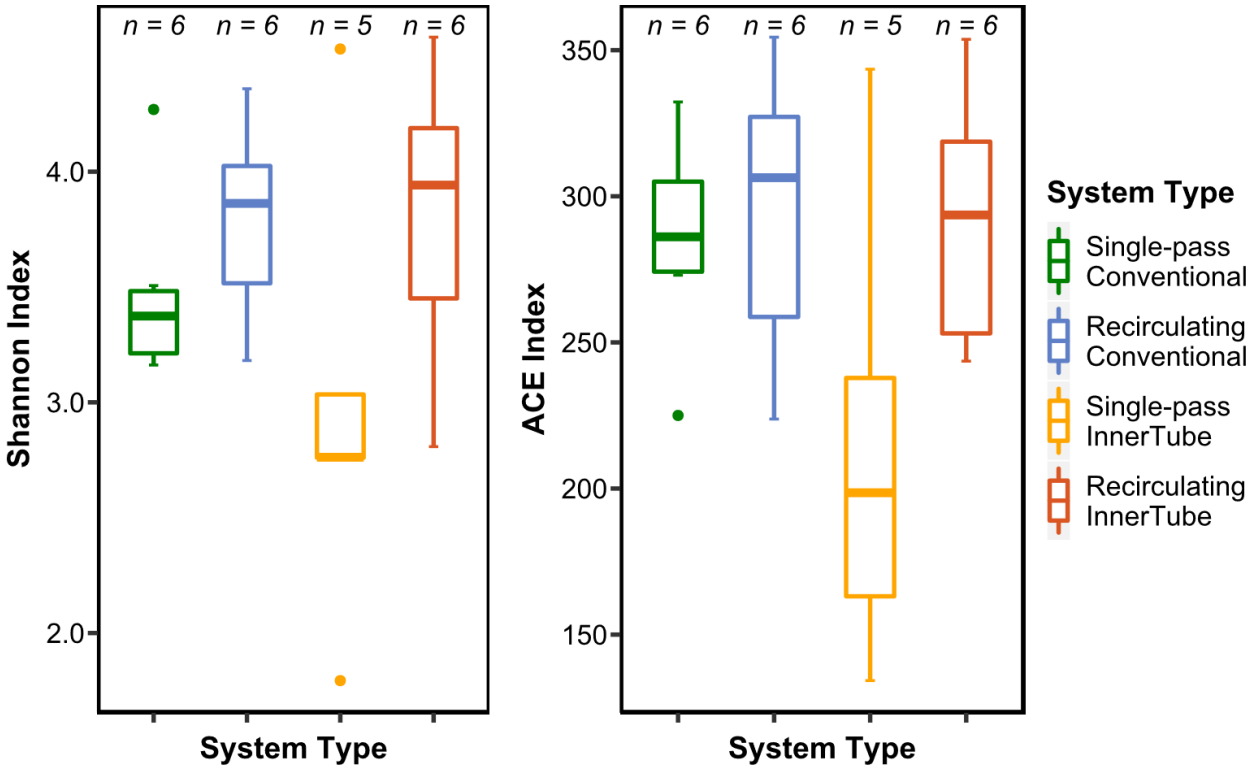

Figure 24: Shannon and ACE diversity index distributions for all system types. Diversity indices were calculated at the ASV level (Section 3.3.4).

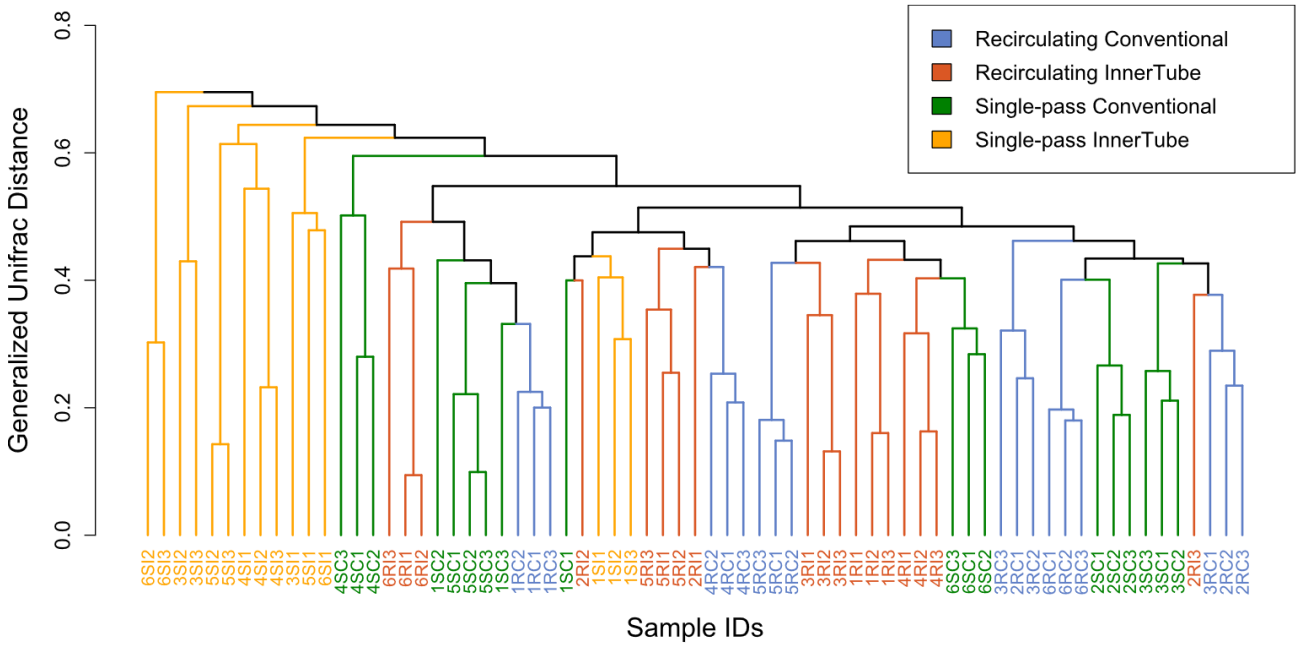

Figure 25: Agglomerative hierarchical clustering of septic tank communities based on generalized Unifrac distances.

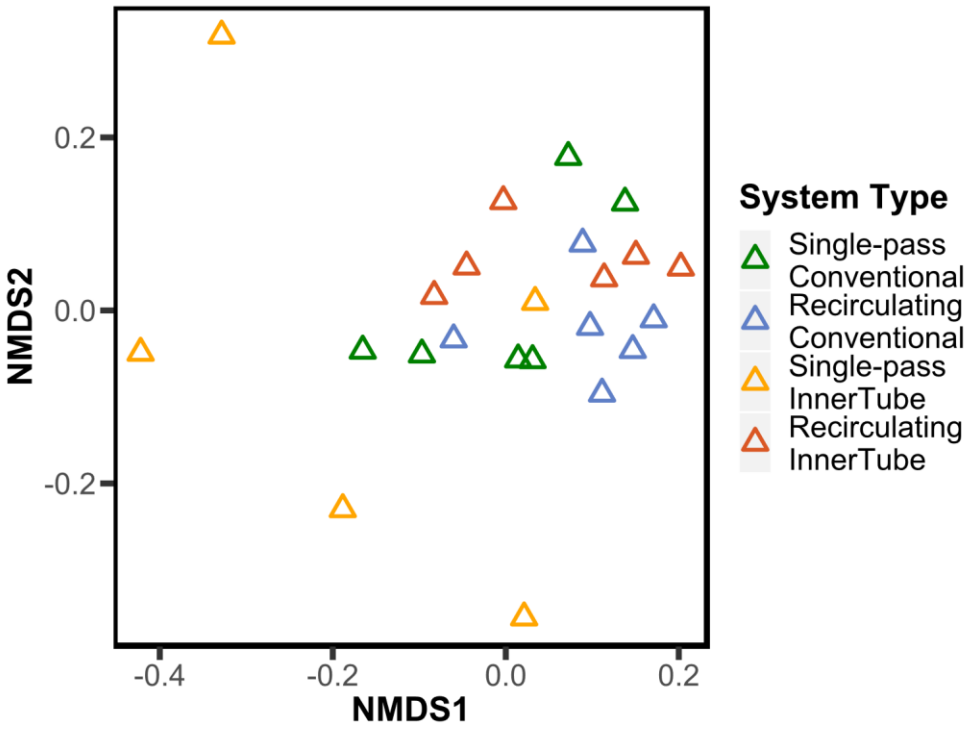

Figure 26: Non-metric multidimensional scaling (NMDS) ordination of tank sample communities (Stress = 0.156). Ordination of microbial communities was based on generalized Unifrac distances (Section 3.3.6).

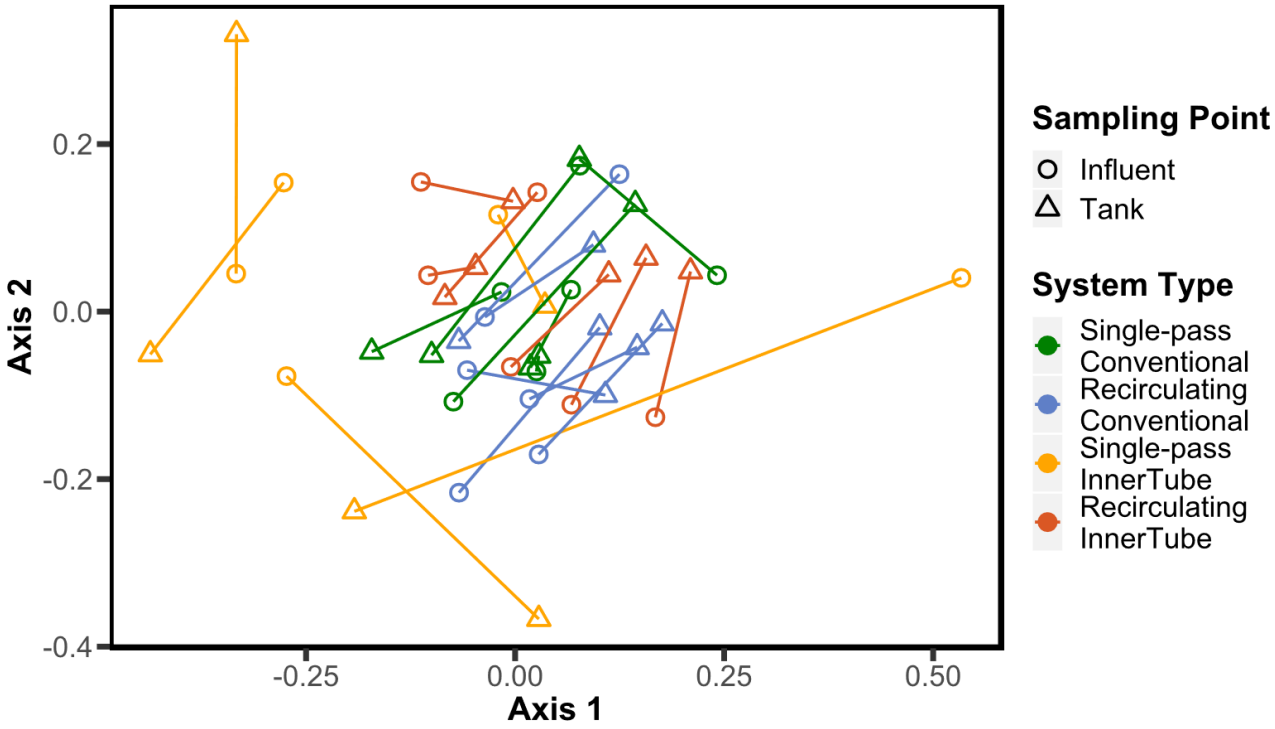

Figure 27: Procrustes rotation plot of influent and tank community ordinations with generalized Unifrac distances. Line segments connect samples from the same system (Section 3.3.6).

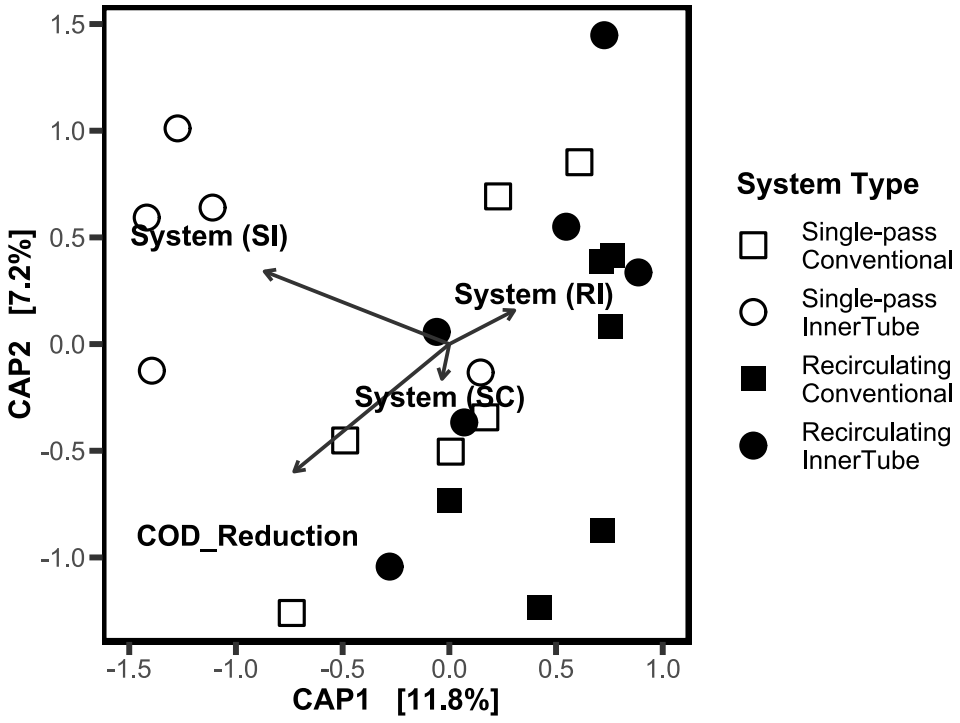

Figure 28: Constrained analysis of principal coordinates of conventional and InnerTube tank sample community ordination. Ordination of sample microbial communities was based on generalized Unifrac distances. The arrows indicate the direction of increasing value of the named variable. The following is a list of variable descriptions: COD_Reduction – proportion of chemical oxygen demand removal between influent and effluent; System – single-pass conventional (SC), recirculating conventional (RC), single-pass InnerTube (SI), and recirculating InnerTube (RI) system types.

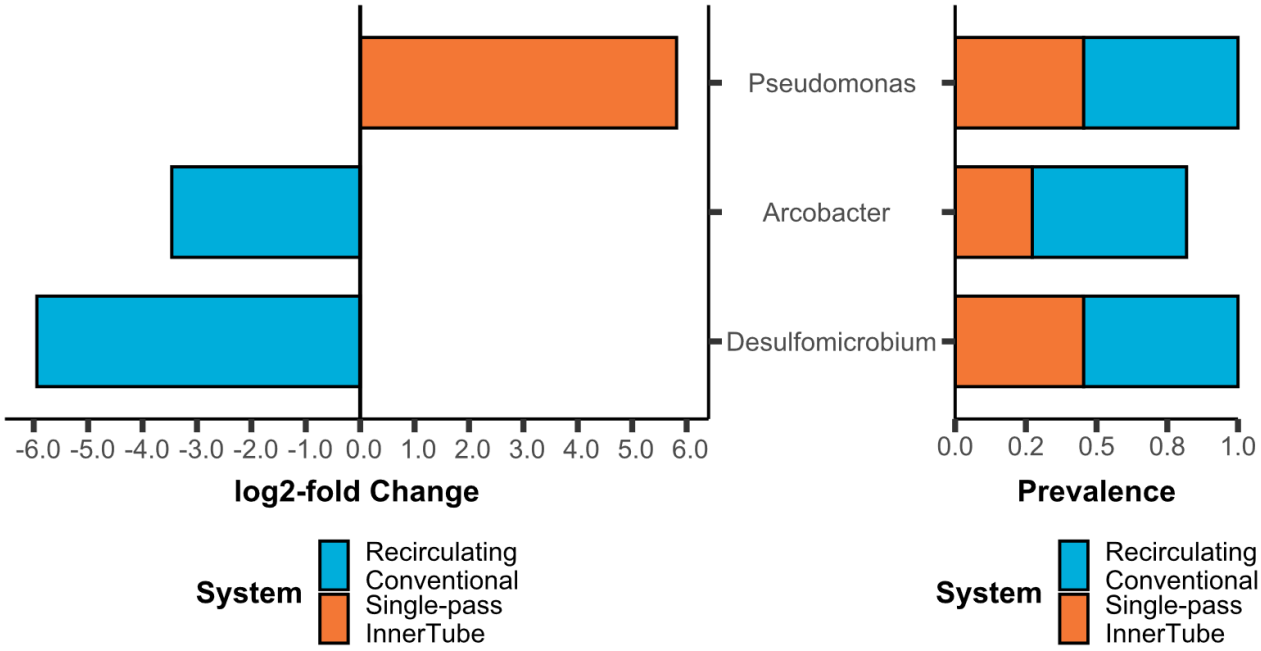

Figure 29: Differential abundance plot of genera in recirculating and single-pass communities. Only genera that comprised of at least 1% of total reads of rarefied samples are displayed (Section 3.3.8).

Chapter 4: General Conclusion

In the first chapter of this project, we utilized 16S sequencing to provide evidence that InnerTube septic tanks act as PFRs. PFRs are designed to maximize microbial growth by separating wastewater into discrete volumes or 'plugs' which maximizes substrate concentrations. Populations of hydrolytic and methylotrophic microbes increased along the length of the InnerTube which suggest an increase in the proportion of complex substrates due to depletion of VFAs and an accumulation of simple degradation products. Furthermore, we demonstrated that InnerTube settled solids were differentially more abundant in microbes with the metabolic potential to carry out methanogenesis and, therefore, improve rates of anaerobic digestion. These system attributes could explain the improved performance and lesser solid accumulation of InnerTube septic tanks in comparison with conventional septic tanks.

In the second chapter of this project, we conducted a 16S survey of 4 types of septic tanks throughout Ontario to characterize septic tank microbial communities and evaluate relationships between community composition, system design, and treatment efficacy. We determined that microbial communities significantly differed between single-pass InnerTube septic tanks and recirculating conventional septic tanks ($p < 0.05$). *Pseudomonas*, a genus of putative lipid-degraders, were differentially more abundant in single-pass InnerTube systems relative to recirculating conventional systems. The greater abundance of *Pseudomonas* in single-pass InnerTube systems may be due to the increased surface area available for long-chain fatty acid adsorption in these systems when compared to conventional systems. *Arcobacter* and *Desulfomicrobium* were differentially more abundant in recirculating conventional systems. *Arcobacter* and *Desulfomicrobium* have been found to thrive in aerobic conditions and be resistant to oxidative stress, respectively, which may explain their enrichment under recirculating flow. *Brevundimonas* and *Desulfovibrio* were significantly positively correlated with COD

reduction in septic tanks ($p < 0.01$). These bacteria are potential H_2-producers and may facilitate greater rates of COD reduction through syntrophic relationships with hydrogenotrophic methanogens.

Future research on septic tank communities should include comprehensive analysis of the substrate composition of wastewater to determine influent effects and evaluate changes in the chemical environment of septic tanks. Long-term surveys are required evaluate the stability of community patterns determined in this project. Furthermore, experiments with standardized systems designs, operational parameters, and influent should be considered to minimize the uncontrolled variability between systems and better characterize the physicochemical and ecological processes responsible for differences between microbial communities across septic tank system types. Co-culturing and fluorescent microscopy experiments could be used to validate the co-occurrence of acetogens and methanogens in InnerTube solids. In addition, transcriptomic studies could be performed to evaluate metabolic activity in the liquid and solid fractions of InnerTubes. In addition, comparisons of microbial communities throughout standardized conventional and InnerTube septic tanks may lead to a greater understanding of the microbial processes behind decreased solid accumulation rates in InnerTube septic tanks. Finally, fungal contributions to anaerobic digestion were not analyzed during this project. Identification of fungi in septic tanks can be accomplished with metabarcoding analyses of 18S or internal transcribed spacers (ITS) and should be included in future work.

Millions of septic tanks are used throughout North America to treat domestic wastewater. Effluent from failing septic tanks is major cause of surface water and groundwater contamination. Therefore, innovation in the field of septic tank engineering is needed to protect aquatic ecosystems and drinking water resources. Cultivation of high-performance microbial

communities offers a promising solution to deficiencies in on-site wastewater treatment. The findings of this project contribute to our understanding of septic tank microbes and may be used to develop bioaugmentation strategies, system designs, or operational parameters to establish microbial communities that improve septic tank performance.

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Appendix I: MASSTC and BNQ Sample Designations and Sampling Information

Table S1: List of Samples, Sampling Equipment Used, and their Locations within the WBS Treatment System at MASSTC, November 13, 2017 (Jowett, pers. comm.)

Sample No.	Sampling Location	No. of 500 mL bottles	No. of TKN bottles	Notes
InnerTube tube sludge samples by vacuum draw (distance from inlet end)				
M2	12” from water level at inlet	2	1	3/8” restriction
M3	24”	2	1	“
M4	36”	2	1	“
M5	48”	2	1	“
M6	60”	2	1	Thick sludge at 60”
M7	72”	2	1	5/8” restriction
M8	84”	2	1	“
M10	96”	2	1	“
InnerTube tube scum at 2” diameter hole drilled through tube at 16” from tube outlet				
M16	Sludge below scum layer	2	1	Sludge judge
InnerTube tube sludge samples by vacuum draw (distance from drilled hole at outlet)				
M15	12” from hole toward middle of tank	2	1	5/8” restriction
M14	24” from hole toward middle of tank	2	1	“
M13	36” from hole toward middle of tank	2	1	“
M12	48” from hole toward middle of tank	2	1	“
M11	72” from hole toward middle of tank	2	1	“
M9	84” from hole toward middle of tank	2	1	“
Raw Sewage taken at sewage trough				
M1L	Raw sewage trough	2	1	Grab sample

Table S2: List of Samples, Sampling Equipment Used, and their Locations within the WBS Treatment System at BNQ, Dec. 5, 2017 (Jowett, pers. comm.)

Sample Grouping	Sample No.	Sampling Location	No. of 500 mL bottles	No. of TKN bottles	Notes
Train 4A InnerTube					
InnerTube™ Outlet A (ITO-A)	B9A	Sludge in inlet area of tank at outlet of InnerTube tube: sludge is 12" thick	2	1	Sludge judge used
Train 4A InnerTube sludge samples by vacuum draw (distance from inlet end)					
InnerTube™ A (IT-A)	B2A	24" from water level at tube inlet	2	1	Vacuum draw
	B3A	48"	2	1	"
	B4A	72"	2	1	"
	B5A	96"	2	1	"
	B6A	120"	2	1	"
	B7A	144"	2	1	"
	B8A	168"	2	1	"
	Other effluent samples (not sludge) taken with labelled bottles as labelled				
Train 4B InnerTube samples by sludge judge					
InnerTube™ Outlet B (ITO-B)	B9B	Sludge in inlet area of tank at outlet of InnerTube tube: sludge sample liquid was pretty clear	2	1	Sludge judge used
Train 4B InnerTube sludge samples by vacuum draw (distance from inlet end)					
InnerTube™ B (IT-B)	B2B	24" from water level at tube inlet	2	1	Vacuum draw
	B3B	48"	2	1	"
	B4B	72"	2	1	"
	B5B	96"	2	1	"
	B6B	120"	2	1	"
	B7B	144"	2	1	"
	B8B	168"	2	1	"

Appendix II: Ontario Septic Tank Survey Sampling Information

Table S3: Single-pass and Recirculating InnerTube (SI and RI) Samples

Sample Code	Region	Sample Type	pH	DO (mg/L)	Temp. (ºC)	Sampling Date
1SI1	Southern Ontario	Inlet	7.69	1.19	22.7	Oct. 29, 2018
1SI2	Southern Ontario	Tank	7.58	0.37	19.5	
1SI3	Southern Ontario	Effluent	7.59	0.35	19.3	
3SI1	Central Ontario	Inlet	6.51	5.10	11.5	Nov. 2, 2018
3SI2	Central Ontario	Tank	7.76	0.79	8.9	
3SI3	Central Ontario	Effluent	7.29	1.36	9.7	
4SI1	Southern Ontario	Inlet	9.40	4.28	7.8	Nov. 5, 2018
4SI2	Southern Ontario	Tank	8.86	1.71	4.2	
4SI3	Southern Ontario	Effluent	8.82	3.51	4.3	
5SI1	Central Ontario	Inlet	5.61	1.10	8.7	Nov. 6, 2018
5SI2	Central Ontario	Tank	6.98	1.94	8.7	
5SI3	Central Ontario	Effluent	6.95	2.35	8.9	
6SI1	Central Ontario	Inlet	6.37	1.10	9.1	Nov. 9, 2018
6SI2	Central Ontario	Tank	6.98	1.94	8.9	
6SI3	Central Ontario	Effluent	6.95	2.35	8.9	
1RI1	Southern Ontario	Inlet	7.42	1.86	16.7	Oct. 23, 2018
1RI2	Southern Ontario	Tank	7.47	1.86	17.1	
1RI3	Southern Ontario	Effluent	7.61	5.58	16.9	
2RI1	Southern Ontario	Inlet	7.27	4.84	19.2	Oct. 23, 2018
2RI2	Southern Ontario	Tank	7.13	1.64	18.9	
2RI3	Southern Ontario	Effluent	7.55	1.27	18.6	
3RI1	Southern Ontario	Inlet	7.53	2.25	14.2	Oct. 23, 2018
3RI2	Southern Ontario	Tank	7.59	0.65	15.4	
3RI3	Southern Ontario	Effluent	7.67	2.93	15	
4RI1	Southern Ontario	Inlet	7.18	1.82	11.8	Nov. 5, 2018
4RI2	Southern Ontario	Tank	7.35	1.68	12.6	
4RI3	Southern Ontario	Effluent	7.58	4.57	12.4	
5RI1	Southern Ontario	Inlet	6.31	0.29	14.1	Nov. 5, 2018
5RI2	Southern Ontario	Tank	6.73	0.29	13.5	
5RI3	Southern Ontario	Effluent	7.03	0.80	12.8	
6RI1	Southern Ontario	Inlet	7.62	0.78	13.4	Nov. 6, 2018
6RI2	Southern Ontario	Tank	7.45	1.18	13.5	
6RI3	Southern Ontario	Effluent	7.56	4.21	13.3	

Table S4: Single-pass and Recirculating Conventional (SC and RC) Samples

Sample Code	Region	Sample Type	pH	DO mg/L	Temp (ºC)	Sampling Date
1SC1	Southern Ontario	Influent	7.22	0.69	18.4	Oct. 22, 2018
1SC2	Southern Ontario	Tank	7.3	0.55	17.3	
1SC3	Southern Ontario	Effluent	7.7	1.39	15.7	
2SC1	Southern Ontario	Influent	7.52	0.55	19.3	Oct. 22, 2018
2SC2	Southern Ontario	Tank	7.51	0.66	18.3	
2SC3	Southern Ontario	Effluent	7.80	1.16	16.8	
3SC1	Southern Ontario	Influent	7.15	0.54	16.1	Oct. 23, 2018
3SC2	Southern Ontario	Tank	7.25	0.56	15.0	
3SC3	Southern Ontario	Effluent	7.62	1.14	13.3	
4SC1	Southern Ontario	Influent	7.42	0.86	8.7	Nov. 5, 2018
4SC2	Southern Ontario	Tank	7.38	0.57	9.6	
4SC3	Southern Ontario	Effluent	7.41	0.89	8.7	
5SC1	Southern Ontario	Influent	7.17	1.42	13.1	Nov. 5, 2018
5SC2	Southern Ontario	Tank	7.19	1.61	12.9	
5SC3	Southern Ontario	Effluent	7.38	2.10	11	
6SC1	Southern Ontario	Influent	7.39	1.83	10.9	Nov. 6, 2018
6SC2	Southern Ontario	Tank	7.36	1.08	10.6	
6SC3	Southern Ontario	Effluent	7.41	0.71	10.5	
1RC1	Southern Ontario	Influent	7.22	1.76	11.6	Nov. 2, 2018
1RC2	Southern Ontario	Tank	7.21	1.34	11.6	
1RC3	Southern Ontario	Effluent	8.25	0.57	10.8	
2RC1	Southern Ontario	Influent	6.72	0.61	16.9	Oct. 16, 2018
2RC2	Southern Ontario	Tank	7.03	0.75	16	
2RC3	Southern Ontario	Effluent	7.38	1.25	15.5	
3RC1	Southern Ontario	Influent	7.09	0.65	17.7	Oct. 16, 2018
3RC2	Southern Ontario	Tank	6.99	0.73	17.1	
3RC3	Southern Ontario	Effluent	7.16	1.95	16.6	
4RC1	Southern Ontario	Influent	7.37	1.23	13.2	Nov. 2, 2018
4RC2	Southern Ontario	Tank	7.42	1.62	12.7	
4RC3	Southern Ontario	Effluent	7.39	3.20	13.2	
5RC1	Southern Ontario	Influent	7.26	0.65	17.0	Oct. 16, 2018
5RC2	Southern Ontario	Tank	7.12	0.70	16.7	
5RC3	Southern Ontario	Effluent	7.38	1.29	16.5	
6RC1	Southern Ontario	Influent	7.11	1.63	11.5	Nov. 2, 2018
6RC2	Southern Ontario	Tank	7.09	1.41	11.1	
6RC3	Southern Ontario	Effluent	7.21	0.42	10.5	

Table S5: Recirculating InnerTube (RI) Quality Control Samples

Sample Code	Region	Sample Type	Notes
4RI1-A	Southern Ontario	Influent	Processed on the day of sampling
4RI1-B	Southern Ontario	Influent	
4RI1-C	Southern Ontario	Influent	
4RI2-A	Southern Ontario	Effluent	Processed on the day of sampling
4RI2-B	Southern Ontario	Effluent	
4RI2-C	Southern Ontario	Effluent	
4RI1-A F	Southern Ontario	Influent	Processed after storage at -80ºC
4RI1-B F	Southern Ontario	Influent	
4RI1-C F	Southern Ontario	Influent	
4RI2-A F	Southern Ontario	Effluent	Processed after storage at -80ºC
4RI2-B F	Southern Ontario	Effluent	
4RI2-C F	Southern Ontario	Effluent	

Appendix III: Conventional Septic Tank Sampling for Chemical and DNA Analysis

Sampling Equipment

Table S6: Sampling Materials

Storage/Transport	Sampling	Cleaning
- ~40 L Styrofoam coolers or hard plastic coolers - Ziploc bags - Frozen gel packs - Packing boxes - Shipping forms - Packing tape	- 500 ml plastic sampling bottles - 500 ml Field blank bottle filled with ddH2O - 500 ml rinse bottle - Labelled 100 ml plastic sampling bottles with sulfuric acid preservative - Labelled 100 ml glass sampling bottles with sulfuric acid preservative - Dissolved oxygen (DO) and pH probe - 100 ml graduated cylinder	- 10 L bottles of commercial spring water - Disposable nitrile gloves - Garbage bags - Hand sanitizer - Paper towels/Rags

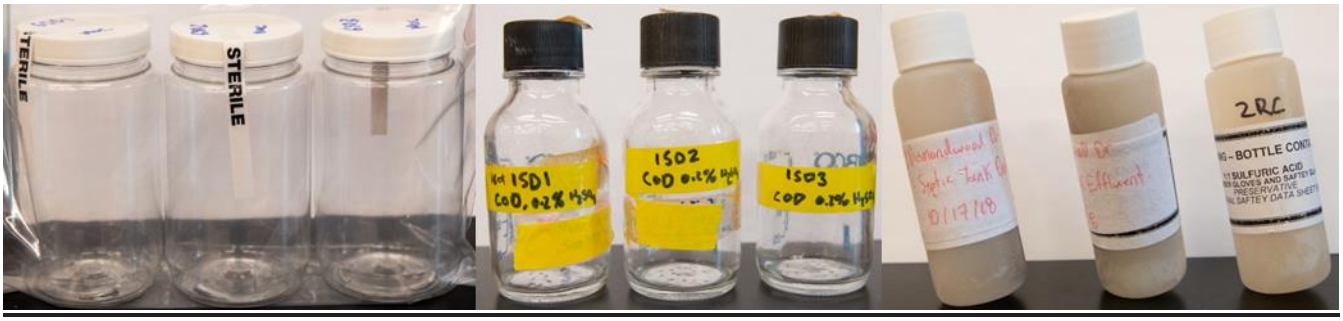

Figure 1: 500 ml (left), 100 ml glass (middle), and 100 ml plastic (right) sampling bottles.

100 ml glass and plastic sampling bottles are preloaded with sulfuric acid preservative.

Sampling Tools

1. Effluent sampler – A capped PVC tube with an approximate volume of 0.75 L affixed to the bottom of a ~4 ft. plastic pole with two metallic screws (Fig. 2).

2. Collection vessel – A capped PVC tube with an approximate volume of 1.25 L affixed to the bottom of a ~4 ft. plastic pole with two metallic screws (Fig. 2).

Figure 2: Effluent sampler (top) and collection vessel (bottom).

3.) Tube sampler – A ~4 ft. open plastic tube with two open ends. A rope knotted at one and a hard foam sphere attached to the other runs through the tube. A dual hook is attached to the end of the tube with the rope knot so that the rope can be secured. When rope is pulled at the knotted end, the foam sphere blocks the other end of the sampler. Tape designates the 1 L volume mark of the sampler (Fig. 3).

Figure 3: Tube sampler with rope, foam sphere (left), and 1 L marking in green tape.

Personal Protective Equipment

All personnel wear disposable nitrile gloves and steel-toe safety boots during sampling activities.

Tool Cleaning

Sampling tools and graduated cylinder are cleaned between sampling points (i.e. effluent, outlet, and inlet) by first rinsing with commercial spring water and then with sample. Any implement used to mix samples should be rinsed with distilled water before each mixing.

Sampling Order

Samples are first collected from the effluent, then outlet, and finally inlet. Moving from the cleanest to most polluted sample minimizes cross contamination.

On-site Chemical Testing

1. At each site, empty and rinse the 500 ml rinse bottle with commercial spring water and then fill it with fresh commercial spring water.

2. After rinsing the probes in the rinse bottle, measure dissolved oxygen (DO) and pH of wastewater in the collection vessel before dividing the liquid among sampling bottles.

Sampling Protocol

Recirculation

Where applicable, please record recirculation valve-openness in approximate percentage (0%, 25%, 50%, or 100%).

Effluent Sampling Procedure

1. Septic tank effluent samples are taken from the septic tank effluent holding tank.

2. Use the effluent sampler to collect enough effluent from the holding tank to fill the collection vessel.

3. Once the collection vessel is full, use a screwdriver or any other appropriate implement to mix the sample in the collection vessel.

4. Measure pH and DO from the collection vessel.

5. Pour liquid from the collection vessel to fill two 500 ml bottles (one each for Fleming College and McMaster University), and one TAN/Ammonia bottle preloaded with sulfuric acid preservative solution.

6. Measure out 100 ml of sample from the collection vessel with a graduated cylinder and transfer the liquid to a 100 ml glass bottle preloaded with sulfuric acid.

Outlet and Inlet Sampling Procedure

1. The outlet and inlet samples are taken from their respective riser access ports.

2. Use the tube sampler to sample the septic tank at the inlet and outlet by inserting the tube sampler into the vessel, foam sphere end first, just beyond the 1 L mark on the tube sampler.

3. Pull the rope and secure it so that the foam sphere plugs the submerged end of the tube sampler.

4. Empty the tube sampler into the collection vessel. Sample repeatedly until greater than 1.5 L of sample is obtained. Once a sufficient volume has been collected, stir the sample.

5. Measure pH and DO from the collection vessel.

6. For both outlet and inlet, pour sample from the collection vessel to fill two 500 ml bottles (one each for Fleming College and McMaster University) and one TAN/Ammonia bottle preloaded with sulfuric acid preservative solution.

7. Measure out 100 ml of sample from the collection vessel with a graduated cylinder and transfer the liquid to a 100 ml glass bottle preloaded with sulfuric acid.

Field Blank Sampling

1. After sampling all sites indicated on the sampling sheet, at the final site, thoroughly rinse the collection vessel with commercial spring water.

2. Empty the field blank bottle into the collect vessel and then pour the water back into the field blank bottle; seal and store.

Sample Storage

1. Separate samples based on destination and then sampling site in Ziploc bags.

2. Place samples in designated styrofoam coolers and cover with ice packs.

3. Leave samples in truck if sampling takes place over more than 24 hours

Sample Storage and Transport

1. Seal coolers with packing tape.

2. Using the information provided, send samples to McMaster University and Fleming College.

Appendix IV: InnerTube Septic Tank Sampling for Chemical and DNA Analysis

Sampling Equipment

Table 1: Sampling Materials

Storage/Transport	Sampling	Cleaning
- ~40 L Styrofoam coolers or hard plastic coolers - Ziploc bags - Frozen gel packs - Packing boxes - Shipping forms - Packing tape	- 500 ml plastic sampling bottles - 500 ml Field blank bottle filled with ddH2O - 500 ml rinse bottle - Labelled 100 ml plastic sampling bottles with sulfuric acid preservative - Labelled 100 ml glass sampling bottles with sulfuric acid preservative - Dissolved oxygen (DO) and pH probe - 100 ml graduated cylinder	- 10 L bottles of commercial spring water - Disposable nitrile gloves - Garbage bags - Hand sanitizer - Paper towels/Rags

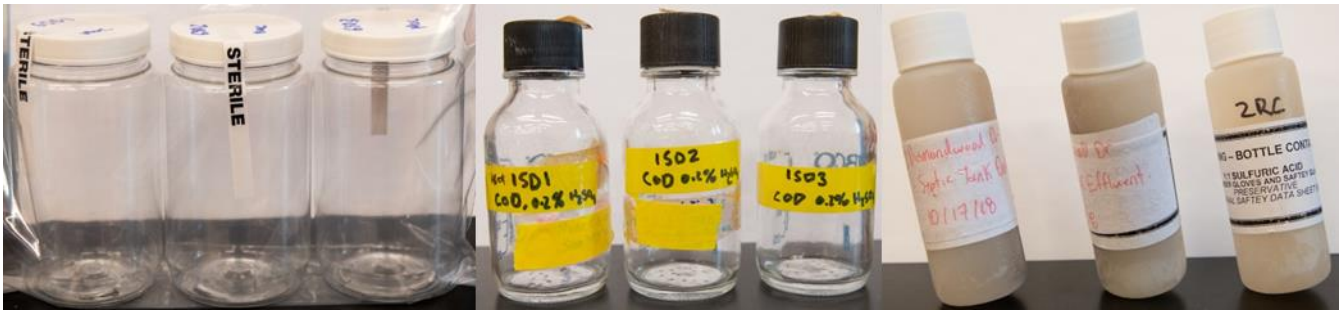

Figure 1: 500 ml (left), 100 ml glass (middle), and 100 ml plastic (right) sampling bottles. 100 ml glass and plastic sampling bottles are preloaded with sulfuric acid preservative.

Sampling Tools

1. Effluent sampler – A capped PVC tube with an approximate volume of 0.75 L affixed to the bottom of a ~4 ft. plastic pole with two metallic screws (Fig. 2).

2. Collection vessel – A capped PVC tube with an approximate volume of 1.25 L affixed to the bottom of a ~4 ft. plastic pole with two metallic screws (Fig. 2).

Figure 2: Effluent sampler (top) and collection vessel (bottom).

3. Tube sampler – A ~4 ft. open plastic tube with two open ends. A rope knotted at one and a hard foam sphere attached to the other runs through the tube. A dual hook is attached to the end of the tube with the rope knot so that the rope can be secured. When rope is pulled at the knotted end, the foam sphere blocks the other end of the sampler. Tape designates the 1 L volume mark of the sampler (Fig. 3).

Figure 3: Tube sampler with rope, foam sphere (left), and 1 L marking in green tape.

Personal Protective Equipment

All personnel wear disposable nitrile gloves and steel-toe safety boots during sampling activities.

Tool Cleaning

Sampling tools and graduated cylinder are cleaned between sampling points (i.e. effluent, outlet, and inlet) by first rinsing with commercial spring water and then with sample. Any implement used to mix samples should be rinsed with distilled water before each mixing.

Sampling Order

Samples are first collected from the effluent, then outlet, and finally inlet. Moving from the cleanest to most polluted sample minimizes cross contamination.

On-site Chemical Testing

1. At each site, empty and rinse the 500 ml rinse bottle with commercial spring water and then fill it with fresh commercial spring water.

2. After rinsing the probes in the rinse bottle, measure dissolved oxygen (DO) and pH of wastewater in the collection vessel before dividing the liquid among sampling bottles.

Sampling Protocol

Recirculation

Where applicable, please record recirculation valve-openness in approximate percentage (0%, 25%, 50%, or 100%).

Effluent Sampling Procedure

1. Digester effluent samples are taken from the Biofilter spray nozzle.

2. Use the collection vessel or effluent sampler (if the clearance below the nozzle is insufficient) to collect enough effluent from the Biofilter spray nozzle to fill the collection vessel.

3. Once the collection vessel is full, use a screwdriver or any other appropriate implement to mix the sample in the collection vessel.

4. Measure pH and DO from the collection vessel.

5. Pour liquid from the collection vessel to fill two 500 ml bottles (one each for Fleming College and McMaster University), and one TAN/Ammonia bottle preloaded with sulfuric acid preservative solution.

6. Measure out 100 ml of sample from the collection vessel with a graduated cylinder and transfer the liquid to a 100 ml glass bottle preloaded with sulfuric acid.

Outlet and Inlet Sampling Procedure

1. The digester outlet sample is ideally taken from the digester tank through the hole in the internal pumping chamber. If the hole is too small and cannot be cut larger, samples can be taken from the InnerTube outlet. Please make note of the sampling point.

2. The inlet sample is taken from inside the inlet of the InnerTube.

3. Use the tube sampler to sample the digester at the inlet and outlet by inserting the tube sampler into the vessel, foam sphere end first, just beyond the 1 L mark (or as far as possible) on the tube sampler.

4. Pull the rope and secure it so that the foam sphere plugs the submerged end of the tube sampler.

5. Empty the tube sampler into the collection vessel. Sample repeatedly until greater than 1.5 L of sample is obtained. Once a sufficient volume has been collected, stir the sample.

6. Measure pH and DO from the collection vessel.

7. For both outlet and inlet, pour sample from the collection vessel to fill two 500 ml bottles (one each for Fleming College and McMaster University) and one TAN/Ammonia bottle preloaded with sulfuric acid preservative solution.

8. Measure out 100 ml of sample from the collection vessel with a graduated cylinder and transfer the liquid to a 100 ml glass bottle preloaded with sulfuric acid.

Field Blank Sampling

1. After sampling all sites indicated on the sampling sheet, at the final site, thoroughly rinse the collection vessel with commercial spring water.

2. Empty the field blank bottle into the collect vessel and then pour the water back into the field blank bottle; seal and store.

Sample Storage

1. Separate samples into Ziploc bags based on destination and then sampling site.

2. Place samples in designated coolers and cover with ice packs.

3. Leave samples in truck if sampling takes place over more than 24 hours.

Transport

1. Seal coolers with packing tape.

2. Using the information provided, send samples to McMaster University and Fleming College.

Appendix V: Septic Tank Wastewater Microbe Isolation by Vacuum Filtration

The following procedure describes the process to isolate microbes from one 500 ml wastewater sample using vacuum filtration.

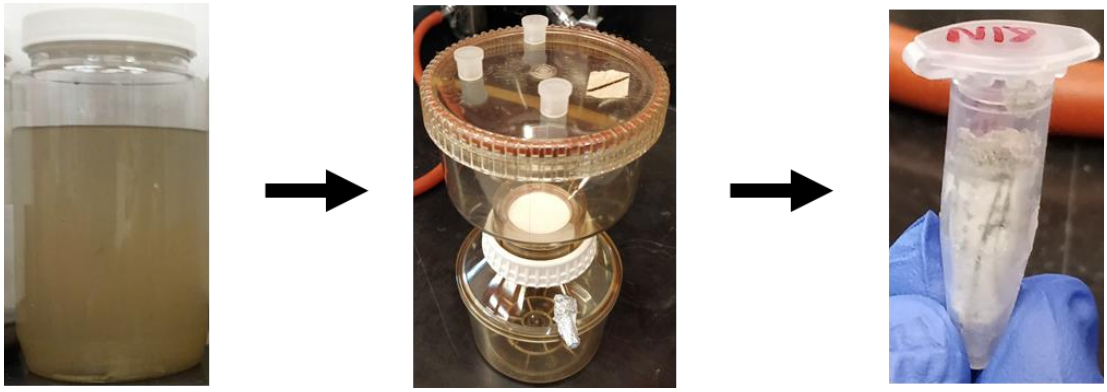

Figure 1: Overiew of wastewater sample filtration for isolation of microbes. Wastewater samples are collected in 500 ml sterile bottles. Samples are filtered through 47 mm 0.22 μm filters in vacuum filtration units. Filters containing microbes are stored in sterile 1.5 ml microcentrifuge tubes and stored at -80°C.

1. Invert the 500 ml sample wastewater 3 times to mix the sample.
2. Proceed to step 5 if wastewater is **not** to be separated in liquid and solid phases.
3. Allow the sample to settle for 30 min.

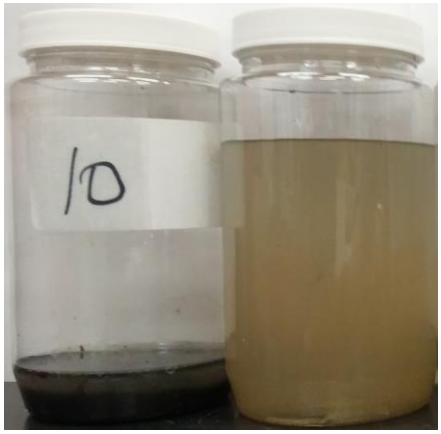

Figure 2: Septic tank wastewater sample separated into solid (left) and liquid (right) fractions.

4. Do not agitate the sample. Using aseptic technique, transfer the liquid fraction to a sterile 500 ml sample bottle using a serological pipette until 1 cm of liquid remains above the solid fraction (Fig. 2). Process both fractions according to the following steps.

5. Using aseptic techniques, assemble an autoclaved vacuum filtration unit with a 0.22 μm sterile cellulose nitrate filter. Use a pair of sterile forceps to place the filter onto the filtration unit. 95% ethanol and a bunsen burner should be used to sterilize forceps after each use.

6. Transfer a 10 ml aliquot of the wastewater sample into the vacuum filtration unit using a serological pipette. This is done for quantification of microbial load or μg DNA/ml after DNA extraction. Turn on the vacuum pump.

7. Repeat step 6 until the filter no longer passes liquid. Smaller aliquots may be added to the vacuum filtration unit as the filtration rate slows.

8. Once the filter has clogged, turn off the vacuum pump.

9. Using 2 pairs of sterile forceps, fold the filter 4 – 5 times and transfer it to a sterile 1.5 ml microcentrifuge tube. Store the microcentrifuge tube containing the filter at -80^{o}C before DNA extraction.