Microbial consortia dynamics during thermophilic and mesophilic anaerobic digestion of faecal sludge

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ABSTRACT

Globally, water scarcity and sanitation have for a long time been of great concern; therefore, water needs to be preserved, but the demands for safe and adequate water are rapidly increasing. Although sanitation in urban areas of South Africa is of the highest standard, some populations such as rural villages lack adequate sanitation and thus use pit latrines as their primary source. This leads to problematic situations due to pit latrines filling up faster than expected, leading to overflowing or leaching of faecal sludge into water systems. Anaerobic digestion is one of the methods that can be used to treat faecal sludge and holds many beneficial properties, such as pathogen reduction and energy production, which can be used as an alternative energy source and for composting. The microbial consortia taking part in these processes consist of hydrolytic, acidogenic, acetogenic and methanogenic microbes. The study aimed to determine the biogas yield and methane production during two anaerobic digestion systems (mesophilic and thermophilic) and the microbial dynamics in such systems. Bench-top batch reactors were used in this study, during which samples were taken at selective times for mesophilic and thermophilic conditions. In order to determine the gas yield and methane production, the BIOGAS5000 analyser was used. Samples collected from these systems were then subjected to physico-chemical and molecular analyses. Microbial communities during selected times were determined using the MiSeq next generation sequencing (NGS) platform. The data obtained from NGS were analysed through Qiime2® and used to determine the microbial community and metabolic pathways. The major phyla observed in mesophilic conditions included Firmicutes, Chloroflexi, Bacteroidetes, Actinobacteria, Euryarchaeota, Synergistetes and Proteobacteria. While thermophilic conditions were almost similar but dominated by Firmicutes, Proteobacteria, Actinobacteria, Chloroflexi, Synergistetes, Thermotogae, and Bacteroidetes. Biogas production in mesophilic conditions with higher yields than that of thermophilic conditions was observed; this could have been due to the nutrient imbalance (ammonium inhibition). Methane yields were observed in both conditions and shown a lower methane yield compared to other studies, due to the inhibition of methanogens; although methane production was low in both temperature conditions mesophilic conditions compared to thermophilic conditions still produced more Biogas- and methane yields during anaerobic digestion. Thermophilic conditions increase free ammonia in an anaerobic system, which is toxic to methanogens, and therefore, are more likely to inhibit methanogenesis. The predicted metabolic pathway in these systems was unknown and ammonia oxidation and dehalogenase were identified as the leading predicted pathways. Pre-treatment of Sludge to remove high ammonium in wastewater would result in better gas production.

Keywords: Faecal sludge, anaerobic digestion, microbial consortia, sanitation, mesophilic, thermophilic.

ABBREVIATIONS

AD	Anaerobic Digestion
COD	Chemical Oxygen Demand
CSTR	Continually Stirred Tank Reactors
DWA	Department of Water
DWAF	Department of Water and Forestry
DWS	Department of Water and Sanitation
FA	Free Ammonia
IQR	Inter-Quantile Ranges
ISR	Inoculum to Substrate Ratio
MCC	Microcrystal Cellulose
Mg/l	Milligram per Litre
NGS	Next Generation Sequencing
OLR	Organic Loading Rate
OTU	Operational taxonomic unit
PCR	Polymerase Chain Reaction
RLE	Relative Log Expression
SAO	Syntrophic Acetate Oxidation
SAOB	Syntrophic Acetate-Oxidising Bacteria
SRB	Sulphate reducing Bacteria
SSA	Sub-Saharan Africa
TS	Total Solids
UVFA	Un-Ionised Volatile Fatty Acids
VFA	Volatile Fatty Acids
VIP	Ventilated Improved Pit Latrines
VS	Volatile Solids
WWTP	WasteWater Treatment Plant
eDNA	Environmental Deoxyribonucleic Acid

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CHAPTER 1 INTRODUCTION

1.1 Water and sanitation

According to the then South African Department of Water Affairs and Forestry (now Department of Water and Sanitation (DWS)) (DWAF, 2003:5), “Water is life; sanitation is dignity”. This slogan is used as the main encouragement for global coverage of both water and sanitation. These two factors are key elements and play an intricate role in improving public health. Additionally, improving the quality of water and sanitation leads directly to an increase in health standards in urban and rural communities; it is thus essential to keep improving these two primary elements. According to King (2004), water is one of the few essentials required for the well-being, growth and sustainability of humanity and biodiversity. Unfortunately, in South Africa, water is frequently used in excess of the normal sustainable levels for living. In the past, due to the critical state of water affairs in South Africa two acts where implemented: the Water Service Act (No. 108 of 1997) which “guarantees the right to a basic amount of drinking water and sanitation services, which obliges all governmental levels to ensure water quality and preservation of water is met in all levels” (Walter et al., 2011), and the National Water Act (No. 36 of 1998) which “provides the legislative framework for the management of water as a national resource, an improved integration of groundwater and surface water as well as a better water quality and quantity management. The new law identifies sustainability and equity as central principles…”. Figure 1-1 shows the different water management areas in South Africa across the provinces.

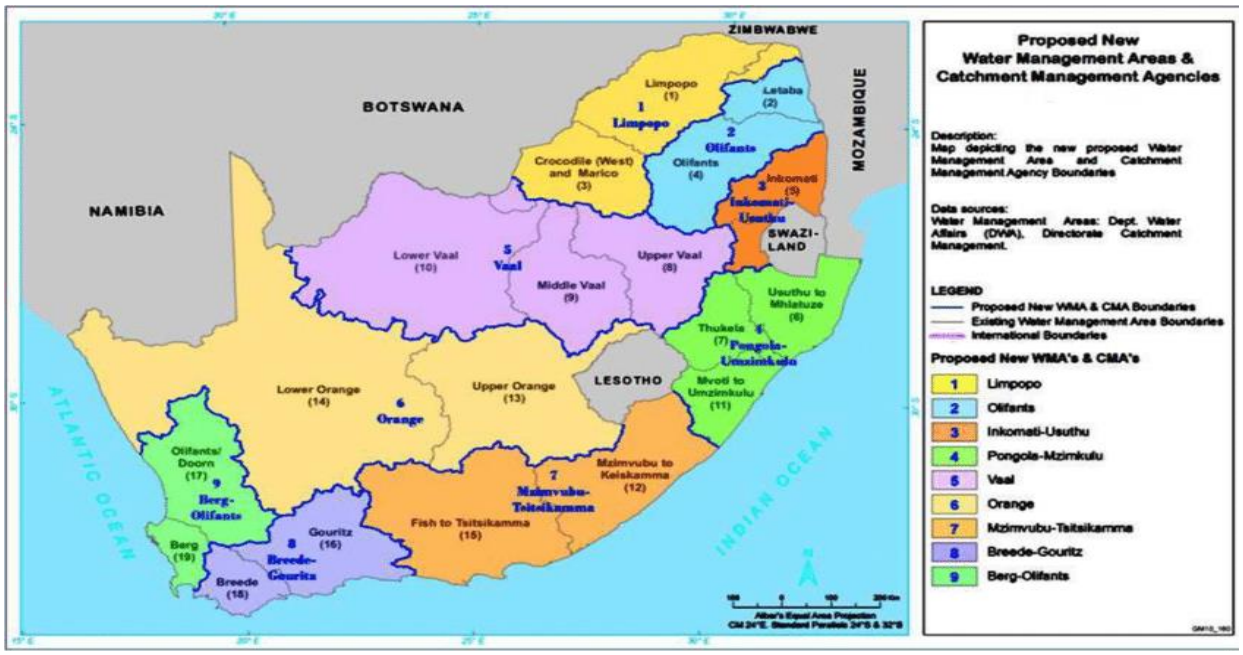

Figure 1-1: Water management areas of South Africa (Source: Abraham & Wisemen (2013). Modelling of Surface Runoff and Infiltration in the Vaal River Water Management Area Using GIS Based RINSPE Hydrologic Model)

Cooper and colleagues (2014) found that despite the implementation of these water acts, 10% of South Africans still lack the basic right to clean and safe water and do not have access to off- or on-site fresh tap water. Mentioned by Wassung (2010) & Ololade (2018), 98% of water reservoirs in South Africa, according to the Water Wheel (2009:29), are already in use or have already been used, which is of great concern, as this leaves little flexibility for future water security and economic development. South Africa is ranked as the 30th driest country in the world, making water preservation, treatment and management of critical importance (Bwapwa, 2018). This is an increasingly difficult task due to a growing demand for water, and therefore, wastewater needs to be treated efficiently and at an acceptable level. Stated by Mulamattathil *et al.* (2015), the quality of water is often used as an indicator of socio-economic conditions, and therefore, the current standard of water quality in South Africa highlights the current issues faced by the country with regard to water pollution – which is a problem increasing at an alarming rate. Many different elements contribute to pollution in South Africa, namely wastewater runoff, inorganic and organic agricultural runoff, industrial runoff and many others, causing health-associated problems in humanity and biodiversity.

Water and sanitation in urban areas of South Africa are generally of the highest standards, but communities in informal settlements still lack adequate sanitation and even water supply facilities, primarily due to the migration of South African citizens to informal settlements. The lack of sanitation is of grave concern; even though pit latrines were built in these communities as a lasting resort, the maintenance of the facilities are at the moment inefficient in most of these communities. The most common maintenance problems that occur include leaching of faecal material through cracks, overflow of pit latrines, and some are safety hazard with broken or open concrete blocks. This increases the possibility of people falling into these pits (Hoossein *et al*., 2016). The census done in 2011 and community survey in 2016 by SA Stats have reported, the number of people using proper sanitation in South Africa has increased from 2011 to 2016(Figure 1-2), which leads one to believe that the sanitation problem has decreased. But Bakare *et al.* (2012) have found in studies that although the number of proper sanitation facilities has increased and the number of pit latrines in use has decreased, the number of people still using pit latrines has increased. Review done by Lategan *et al.,* 2020 on backyard rentals have found that the number of people using pit latrines has increased, due to overcrowded properties, which can lead to inferior hygiene practices.

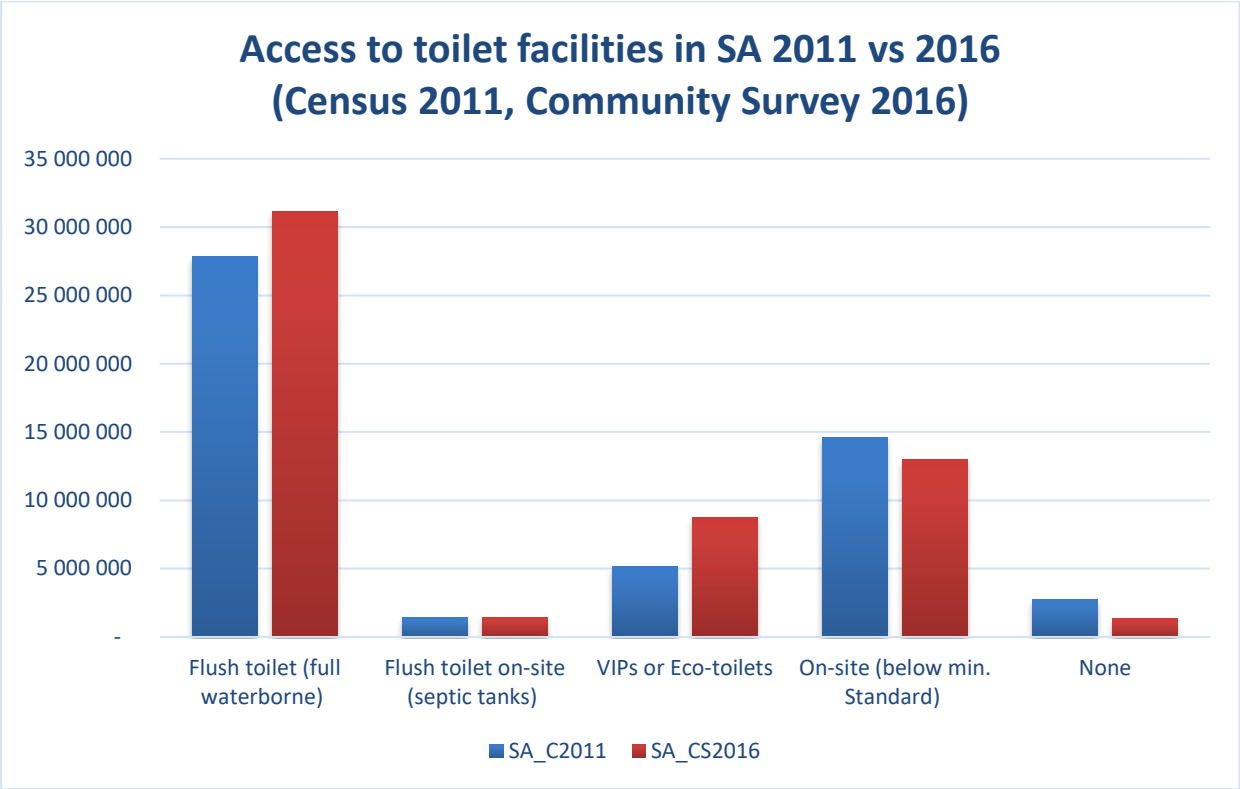

Figure 1-2: Census comparison of sanitation practices between 2011 and 2016 (Source: Adapted from Statistics South Africa Stats SA. 2016. Community survey 2016: statistical release. http://cs2016.statssa.gov.za/wp-content/uploads/2016/06/NEW-CS-2016-Statistical-release-v2_3.pdf.)

In many of these rural communities, groundwater is one of the main water sources; the poor maintenance of pit latrines jeopardises this supply of water due to contamination by faecal sludge as a result of leakage from pit latrines. Thus, proper maintenance of these pit latrines is of vital importance (Mpenyana-Monyatsi *et al.,* 2012). By supplying vacuum trucks and alternative treatments, this problem can be solved or decreased. One solution to this problem includes anaerobic digestion (AD), which is discussed in Section 1.3, as it was one of the key focus points of the study.

1.2 Wastewater treatment in South Africa

According to Wassung (2010), South Africa is facing three very real consequential management problems, namely water, energy and sanitation (waste): Water – its scarcity and susceptibility to pollution, energy – people use coal as their main heat source, and lastly, sanitation such as unconventional pit latrines – which increases transmission of pathogenic microorganism. The pit latrines filling up leaves people to make new but unconventional pit latrines which leads to environmental contamination of faecal material into the water through underground water sources but before addressing this problem, wastewater treatment plants across South Africa needs to maintain a healthy green drop states before deploying vacuum trucks to collected faecal sludge from overflown pit latrines. An assessment done by the government: The Green Drop report

(DWA, 2013), states that South Africa has approximately 824 wastewater treatment systems across 152 municipalities, thus concluding a nationwide capacity of 6.5 billion litres of wastewater that can be treated per day. In 2013, 5.12 billion litres of wastewater were received at these wastewater treatment plants (WWTPs), leaving only 22% capacity for the future of these 824 WWTPs. The Green Drop report's executive summary states that a total of 248 WWTPs were assessed in 2013 and concluded that they were in a critical condition, therefore constant maintenance needed to be applied. Additionally, of these 824 WWTPs, 161 treatment plants were listed as being in urgent need of maintenance. Approximately only 7 % of the treatment plants were of a level where they could receive Green Drop status, thus causing great concern regarding future water preservation and sustainability.

1.3 Anaerobic digestion (AD) as alternative management source

According to Still *et al.* (2009), sanitation includes both on-site and off-site treatment options. Globally, AD has received a substantial amount of attention and is rapidly making its way to the market (Weiland, 2010). The use of faecal sludge as a substrate for various processes holds great promise, especially due to its wide range of uses, including the management of water and energy. According to Kleerebezem *et al.* (2015), AD can be defined as the conversion of organic compounds into biogas that can be used for energy, biofuel and gas. AD is an efficient, eco-friendly option that can be used to reduce organic bio-hazardous waste such as the organic waste molecules generated during activated sludge treatment. In addition to this, the by-products formed during the AD processes, such as gas, can also be used for biofuel or heat generation (Beschkov, 2017). Additionally, substrates generated after AD can also be used for fertilisers in agricultural practices (Möller 2012).

1.4 Microbial consortia in faecal sludge

Microbes in faecal sludge are one of the most important elements for the degradation of organic and inorganic substrates at a WWTP. Specific microorganisms are required for the biodegradation of organic contaminants present in sewage sludge (Dhall *et al.,* 2012) In many instances, one organism will not suffice, and thus a mixture of microorganisms is needed that has a cumulative effect on the increasing biomass activity, growth efficiency and enzyme production for this process to continue. The microbes taking part in these processes need one another to survive and to overcome the feedback regulation and catabolic repression, as products that are produced from one organism group act as a substrate for another (Hassan *et al.,* 2020). According to Gaikwad (2014), naturally occurring microbes can be described as the workers of the wastewater treatment process, consisting of fungi, bacteria, protozoa, rotifers, methanogens and many others. These organisms thrive on the complex compounds present in faecal sludge. Organisms commonly found in faecal sludge include species such as *Pseudomonas* spp., *Actinomycetes* spp., *Bacillus*

spp., *Streptomyces* spp., *Staphylococcus* spp. and *Methanogen* spp. Even though they have degrading properties and play an essential role in wastewater treatment processes, some microbes can also be pathogenic and causes diseases such as diarrhoea, cholera, typhoid, hepatitis, polio, cryptosporidiosis, ascariasis and schistosomiasis (Carr & Strauss 2001). Thus, they may hold great human health risks; in 2017, across the world in low-middle income countries 827 000 people die due to inadequate water, sanitation and hygiene yearly and is believed that inadequate sanitation is the main cause in 432 000 deaths of these cases. (Prüss-Ustün *et al.,* 2019; WHO 2018h).

1.5 Problem statement

Globally, sanitation is of great concern, particularly in low- and middle-income countries (WHO 2019b) with an ever-increasing demand for safe and adequate water, thus driving the need for water preservation. The result of poor sanitation in these rural areas leads to an increase in human health risks and needs immediate attention, especially surrounding the emptying of pit latrines commonly used in these rural areas (Orner *et al.,* 2019). This leads to the challenge of finding appropriate and safe ways to dispose of the waste found in these pit latrines. The use of vacuum trucks is a good representative of pit latrine waste collection and is frequently practised in and around the Potchefstroom area.

This waste can be used for AD processes, which is a technology that will have a positive impact on waste management, agriculture and energy sources. It is thus important to understand the full extent of the AD process, including the different microbes present in faecal sludge. Regarding the literature, no studies on the microbial consortia and how it changes during the AD stages of faecal sludge have been done on the system available in the North West Province of South Africa. This would provide valuable data/knowledge on the efficiency of faecal sludge in anaerobic digesters, the microbes present and the quantity of these microbes in the different stages of the biogas production process. Pit latrines are still the primary mechanism for sanitation in most rural areas; however, this technology is still problematic. One primary concern associated with pit latrines is under-servicing, which then results in overflowing or leaching from these pit latrines into the local water systems (DWS, 2002). Faecal sludge management is one of the lacking factors playing a role in this situation, and by implementing AD, this problem could be resolved, but more data is urgently required before any action can take place. AD can be used for electricity and composting purposes and is thus important to understand the microbial consortia dynamics present in faecal sludge. This study allows one to get a better understanding of the microbial consortia dynamics present in each stage as well as their main function and allows one to determine the biogas potential.

1.6 Aims and Objectives

The aim of the study was to evaluate mesophilic and thermophilic AD of faecal sludge in terms of gas yield and associated microbial consortia dynamics during the different stages of the AD processes.

To reach the aim the following objectives were identified:

- Analyse the physico-chemical parameters of faecal sludge;
- Determine the levels of gas yield produced during AD of the mesophilic and thermophilic reactors;
- Determine the community dynamics in AD of faecal sludge;
- Monitor microbial consortia present in different stages of AD; and
- Identify the different microbial consortia present by targeting functional genes and using specific primers.

CHAPTER 2 LITERATURE REVIEW

2.1 The water situation in South Africa

“Water is life; sanitation is dignity” is the well-known slogan of the DWS (Masindi & Dunker, 2016). In 2015, According to Degebasa *et al.,* (2018) stats that that approximately 1.5 to 2.2 million people die annually from diarrheal diseases and related diseases that are transferred through water bodies and unsanitary hygiene practices (poor sanitation) (Degebasa *et al.,* 2018; Yaya *et al*., 2018). Debatably amongst all environmental resources, water is one of the most important and sought-after resources for all living organisms (Kılıç, 2020). Nonetheless, the preservation of water has proven to be difficult, especially in developing countries. In recent days, pollution of water bodies is not an uncommon sight. In South Africa, water has proven to be especially difficult to both supply and preserve; therefore, systems or guidelines to maintain the water supply is of critical importance. According to an overview investigated by Soyapi (2017) on water rights in South Africa, everyone has the fundamental right to safe and adequate water, but as specified by Donnenfeld *et al.* (2018), water availability is being exceeded past its sustainable levels and alternative methods of water supply need to be implemented. Demand for safe and drinkable water in rural areas is increasing at an exponential rate, leading to numerous problems such as water scarcity, increased water pollution and water born health risks (Walter *et al.* 2011).

Water services are defined by three attributes that contribute to reliable water services: (1) access, (2) availability and (3) portability. Reliability of water supply is usually defined as the proportion of time it uses to function to its prescribed level (Rietveld *et al.* 2009; Teklehaimanot *et al*., 2014). Reliable services should deliver high-quality water to communities in informal settlements. According to DWS (2017), the department is mandated by the National Water Act (No. 36 of 1998) to ensure South Africa’s water resources are protected, used, developed, conserved and managed so that it is controlled and sustained in an equitable manner. The supply of good quality water in sufficient quantities is important for economic growth. Visualised in Figure 2-1, water that contributes to economic growth includes agriculture, which uses 60% of the available resources, livestock uses 2.5% and municipal/domestic utilises another 27%. Municipal/domestic use is divided into two sections: urban resources that use 27% and the other 3% is for rural areas. Furthermore, power generation uses 4.3%, mining 3%, afforestation 3% and industrial uses approximately 3% (Schreiner *et al.,* 2018; DWA, 2013, Mwendera & Atyosi, 2018). Therefore, the essential use of water and water preservation must be managed in the most adequate way possible.

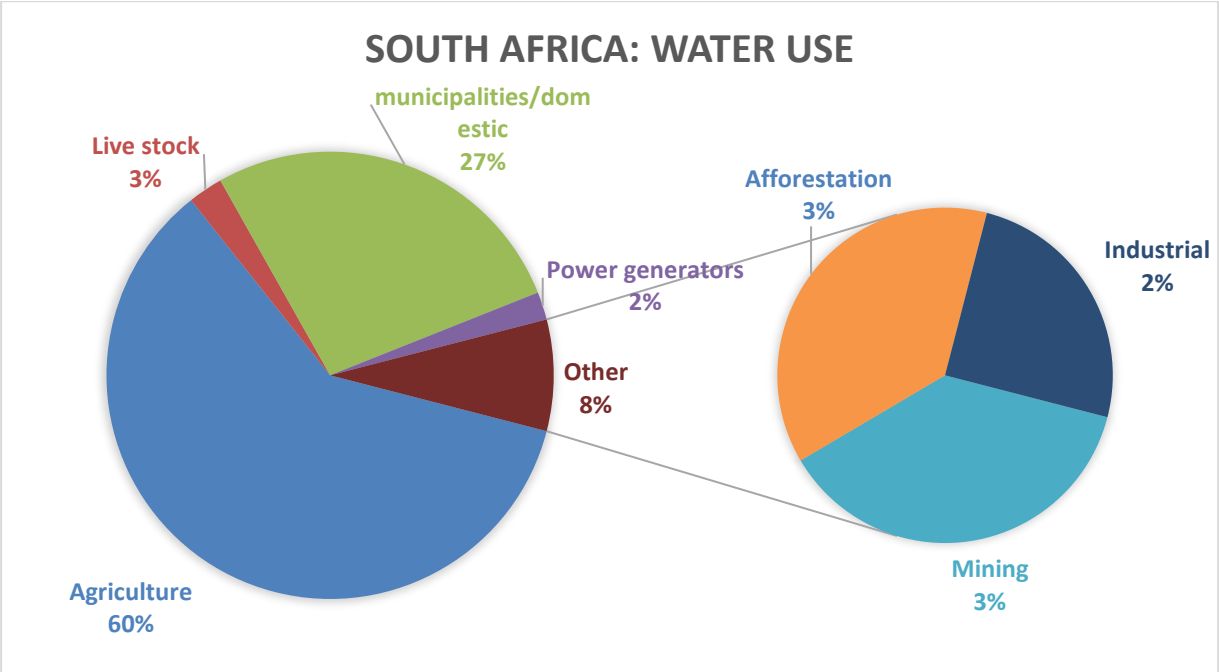

Figure 2-1: Water uses in South Africa (Mwendera & Atyosi, 2018). A Review of Water Storage for Socio-Economic Development in South Africa. Journal of Water Resource and Protection.)

It is estimated that six million people in South Africa are still living in areas with an unreliable source of drinking water, therefore increasing their vulnerability to health-related risks (Mpenyana-Monyatsi *et al*., 2012). In a study done by Hoossein *et al.* (2016), it was concluded that the migration of South Africans to informal urban settlement was caused by over-population in urban areas; as a result, many people moved to rural areas or created areas that lack proper facilities for adequate water and sanitation services. According to a survey done in 2016 by Stats SA, only 44.4% of people had access to adequate water inside their dwelling, whereas 30% had taps on their property. In this survey, it was also noted that only 60.6% of South Africans had access to flush toilets, and 2.2% still use bucket systems (Stats SA, 2016). While improvements are being made towards adequate sanitation facilities needed for basic human necessities, a lack of proper disposal of faecal sludge in rural areas is still seen as a problem in most parts of the country and could consequently lead to the increase of inferior hygiene conditions that would result in a severe safety hazard in these communities. Problems that these communities also encounter other than faecal sludge disposal/removal are improper water supply, water - and groundwater pollution/contamination. Therefore, along with waste management, improved water management and supply are required. Various factors contribute to water pollution, such as inorganic- and organic-agriculture water runoff, industrial waste and water-soluble radioactive substances that are released into these water systems daily (Mulamattathil *et al.,* 2015). This is mainly due to the risk issue, specifically population growth, causing domestic and industrial sewage to increase, leaving WWTPs overloaded, therefore causing water purification systems to function at substantial pressure, resulting in inadequate purification of water (Teklehaimanot *et al*., 2014).

As found by the DWS (2002), groundwater is the main source of fresh water in South Africa for people living in rural communities. Groundwater is a vital resource for domestic, industrial and agriculture purposes that contribute between 13% and 15% of the water usage in South Africa – many of which occurs in rural settlements. Estimated 400 communities use groundwater as their main water source, but the lack of water purifications systems in these communities is still of major concern as it increases various health risks (Mpenyana-Monyatsi *et al.,* 2012). Due to inadequate water supply alternative water source are being used. Water from these sources is contaminated with pathogenic organisms and is mostly untreated before being used by people in these settlements (Gwimbi, *et al.,* 2019).

2.2 Sanitation

In 1994, the population of South Africa consisted of 40 million people, of which 15.2 million lacked basic water supply, and 20.5 million lacked basic sanitation (DHS, 2019). Although sanitation has improved over the years, some people still lack adequate sanitation, which includes water-borne flush toilets and even ventilated improved pit latrines (VIP). According to DWS (2012), delivery of enough water and sanitation according to historic data was mostly provided to middle- and upper-class sections of the municipality and town. Therefore, pit latrines and the bucket method were provided to communities in rural areas but proven to be insufficient, because pit latrines were filling up faster than was expected (Nakagiri *et al.,*2015). This ultimately led to the leaching of faecal sludge content into water born systems (Nakagiri *et al.,*2015). After the newly elected government the African National Congress (ANC) came into power, they immediately acknowledged the lack of sanitation and water service around the country and strived to provide a more sustainable way of living. Although strides have been made in addressing sanitation in most of the country, some communities still live with pit latrines as their primary source of sanitation (DWS, 2002; WHO, 2008)

According to DWS (1994), every person in South Africa has the fundamental right to a clean environment which includes not only proper water but also high-quality sanitation services. The minimum sanitation standards have been provided by the national government (DWS, 2001). These standards are broadly defined and enable authorities to implement good sanitation technology. The choices of technology consist of various sanitation options: dry-sanitation, low-flush and full water-borne sanitation. These systems are seen by the government as the optimum choice for sanitation systems, due to better hygiene, improved human health risks and low impact on the environment (Crouse *et al.*, 2006). This is also supported by Graham & Polizzotto (2013), who state "...that improved sanitary measures include water toilets that flush into sewage septic systems that separate excreta from any human's contact therefore minimizing contamination of pathogens and lowering the mortality rate associated with a lack of sanitation"

Unfortunately, South Africa is still experiencing a high rate of fatalities when it comes to diarrheal illnesses associated with poor sanitation (Chola *et al.,* 2015); and this rate is estimated to grow in the years to come if no changes are implemented especially in communities that shares sanitation facilities (Nguyen *et al.,* 2021). At this stage, pit latrines are used and filling up exponentially leading to the rebuild of new but unconventional pit latrines. These pit latrines with no proper removal systems in place leads to health-associated risks such as diarrheal diseases. Diarrheal illness and mortality can be reduced by giving people access to improved sanitary facilities, given that these systems are installed, applied, and maintained correctly (Albonico *et al*., 2008; Graham & Polizzotto 2013). These pit latrines contain different viruses and bacteria, especially enteric bacteria, most of which are non-hazardous, but several of these organisms can potentially be hazardous to humans under the right circumstances. Therefore, improving this situation is of essential importance.

2.2.1 **Pit latrines**

In sub-Saharan Africa (SSA), sanitation access of the urban population is still in the form of pit latrines and is estimated to be roughly 198 - 200 million people (Cross & Coombes, 2020; Nakagiri *et al.,* 2015). Although the percentage of pit latrines has gone down from 2007 to 2015 (65% to 52.7%) the number of people still using pit latrines has increased (Nakagiri *et al.,* 2015). Furthermore, approximately 36 million people have adopted VIP pit latrines as a form of sanitation, and this number is expected to rise if proper strategy is implemented (Nakagiri *et al.,* 2015). Studies conducted on pit latrines across Africa found that essential maintenance services of emptying pit latrines are lacking (studies seen in Table 2-1); all these studies support that a proper sanitation strategy must be implemented. In Figure 2-2, the percentage of urban populations still using pit latrines is shown, illustrating the high percentage of people living with pit latrines as their source of sanitation, including various unconventional pit latrines. Many types of pit latrines exist in the different countries, but most have adapted by using standard improved pit latrines. In 2007, a survey was done on the conditions and type of pit latrines used in SSA, which found that 14% of these pit latrines were in proper working condition; this increased substantially over time to approximately 63% in 2015(Banerjee *et al.* 2008; Nakagiri *et al.,* 2015). According to Bakare *et al.* (2012), pit latrines in South Africa were reported to be full and overflowing into the environment. Although these situations were of great concern, and still are, strides are being made to improve these conditions in South Africa. The municipality of Durban alone reported that by 2011, 35,000 accessible pit latrines had been emptied (Macleod 2011). However, certain areas in South Africa are inaccessible, and alternate solutions must be explored (Still *et al.,* 2012).

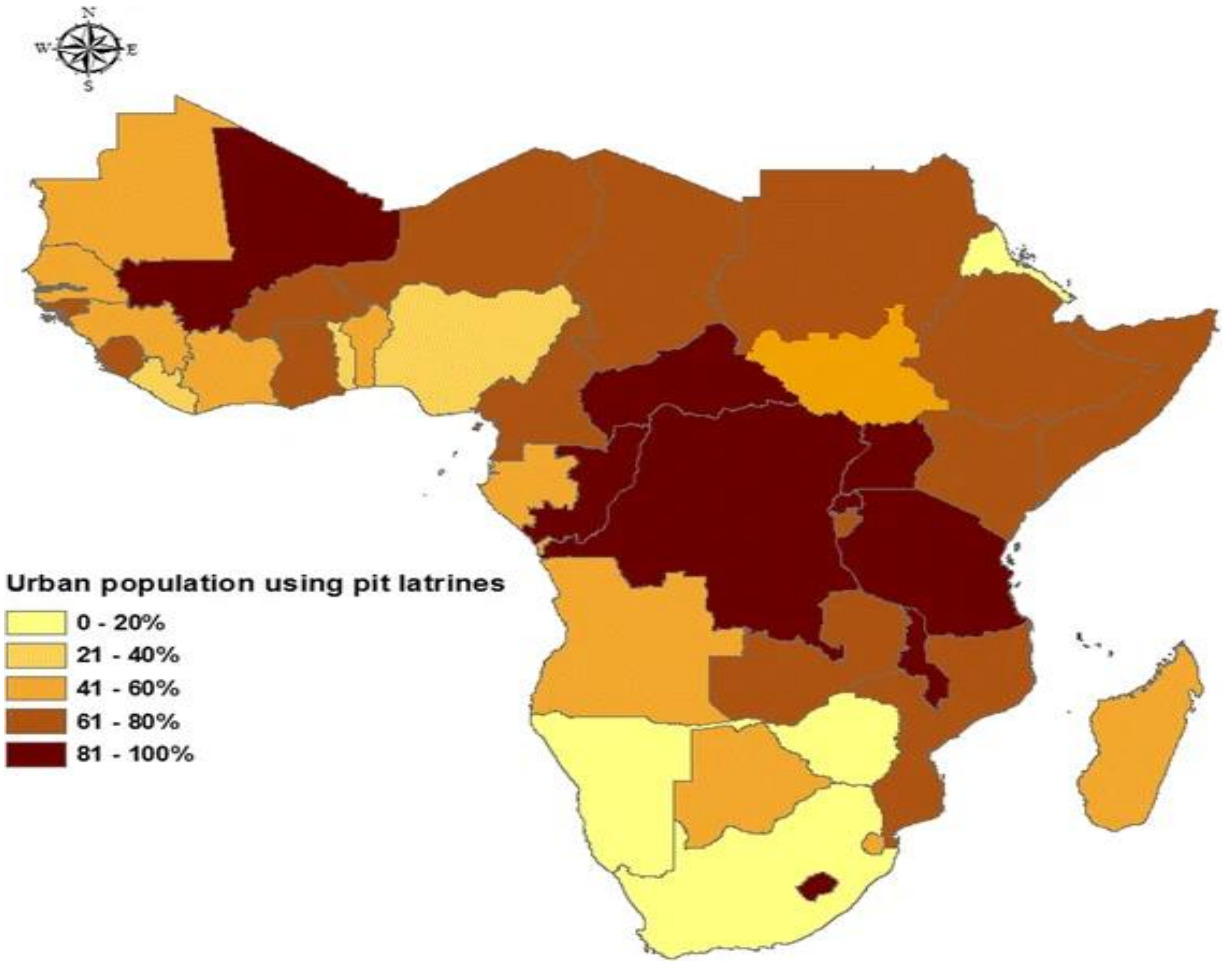

Figure 2-2: Percentage of SSA urban countries still using pit latrines as their primary source of sanitation (Source Nakagiri, A., Niwagaba, C.B., Nyenje, P.M. *et al.* 2015. Are pit latrines in urban areas of Sub-Saharan Africa performing? A review of usage, filling, insects and odour nuisances. *BMC Public Health* 16, 120

According to the Orner *et* al., (2019) & WHO (2018) improved sanitary pit latrines consist of a pit, rectangular or square, dug into the ground with walls and a floor normally constructed with concrete slabs. This reduces the contact of faecal sludge with the environment and the people using the latrines, whereas unimproved/unconventional pit latrines are without a platform or with a platform that is broken. Vacuum trucks are needed to transport waste from pit latrines to wastewater treatment plants, but due to accessibility and cost of vacuum trucks, many people are left without any emptying mechanisms (Mikhael *et al.,* 2018). Vacuum truck is normally a motorised vehicle that is equipped with a vacuum pump and tank; and this pump is used for emptying pit latrines, sewers and septic tanks. These trucks have the capacity of 4-6 m^3 to store and transport faecal sludge from point A (pit latrines) to point B (WWTP), but due to their cost ranging from 20,000 to 100,000 USD and its accessibility to the pit latrines, it is proven nearly impossible to empty them in unreachable areas (Brikker &, Bredero 2003).

Alternative methods need to be implemented for the well-being of the people in these situations and environment. Immunocompromised immune systems, one of the main issues in communities with poor sanitation, is increasing, which leads to more diarrheal and unsanitary related deaths. Improper health care management can have negative ramifications on communities, especially those infected with HIV/AIDS and tuberculosis. These people are more susceptible to being infected with diseases such as gastrointestinal tract illnesses and hepatitis A, B and C. Globally, over 5.2 million people die annually from these diseases, especially in rural communities, caused by inadequate health management (Hangulu & Akintola, 2017). In a study done by Nakagiri *et al*. (2015), it was found that pit latrines, once full, were covered and new pit latrines were dug nearby. This caused a major concern for safety around these pits, due to pit latrines being built through unconventional methods. Therefore, it is proposed that these pit latrines no longer serve as a sanitation option. A system approach to this crisis is currently being adapted and includes a multi-step process, whereas VIP are implemented but with transport and treatment guidelines. VIPs are incorporated to support this chain-strategy, but the cost involved is the main suppressor of full implementation of this strategy. Currently, the focus of this strategy is on the implementation and reduction of costs.

2.3 Sludge treatment technologies

Currently, the conversion of faecal sludge to valuable by-products without any flies, odour and transmission of diseases is a challenging task. Faecal sludge treatment in developing countries mainly depends on the characteristics of faecal sludge used and their reuse potential, which varies significantly within a community and between communities from different locations where water content and storage period is a key influencing factor (Koné *et al.,* 2010) Therefore, faecal sludge characteristics determine the type of faecal sludge treatment that is necessary to obtain useful by-products. In developing countries, faecal sludge treatment depends on the method used for sludge management, such as septic tanks and twin pit latrines. Faecal sludge from pit latrines and septic tanks is generally collected every two to three years and is biochemically more stable due to the long storage period, which decreases the organic concentration (Singh *et al*., 2017). Faecal sludge from septic tanks and pit latrines that are collected more frequently is more biochemically unstable due to the high amounts of organic's still present. Developing countries face several challenges relating to faecal sludge treatment, as it explicitly differs from wastewater treatment because of faecal sludge being a more complex matter in terms of organics and the number of pathogens present (Klingel *et al.,* 2002*)*. Treatment options in rural communities should thus be selected based on the location and faecal characteristics (Singh *et al*., 2017). Table 2-1 describes the different treatment options and uses for faecal sludge.

Table 2-1: Different treatment options for the implementation of faecal sludge

Technology	Description	Source
AD toilets	These toilets are a treatment technology that generally relies on the principle of an anaerobic environment which uses faecal sludge as substrate for biogas production. Biogas from these toilets is caught up in tanks and can be used for conventional uses, such as gas for food purposes and for heating or electricity. These toilets can be used in a community as a cost-effective method.	Forbis-Stokes *et al.,* 2016; Katukiza *et al*., 2012; Reid, *et al.,* 2014 ; Sibisi and Green, 2005
Composting	Composting relies on the principle of aerobic digestion of matter to composting material. This is also done to remove harmful chemicals and pathogens. Excreta from humans can be used as a good source of organic material for agriculture due to the high levels of nitrogen, which generally makes up 70-80% of the faecal content. However, phosphorus is also evenly distributed in faecal sludge, but before use for agricultural purposes, a secondary treatment has to be done, due to the high amounts of pathogenic microbes still present. Some of the well-known techniques which cleanse and convert organic wastes into valued produce are composting, vermicomposting, shallow trenches, anaerobic digester and solar drying. The process normally consists of using the microbes to digest this matter into usable agricultural compounds. After the microbes have converted the matter, temperatures exceeding 50°C from the respiration of the microbes sanitise the compounds.	Gajurel *et al.,* 2003; Vinnerås, 2007; Winker *et al.,*2009).
Solar drying	Solar drying is a direct or indirect implementation of radiation. Many solar drying systems exist, namely solar box dryers, solar cabinet dryer, solar tunnel dryer and solar-biomass hybrid cabinet dryer. Direct solar drying consists of the removal of moisture from samples by directly implementing solar radiation. indirect solar drying operates by capturing the heat of the sun through a solar collector, which leads ambient airflow through the solar collectors and therefore heats the air. After that, the heated air moves through the drying chamber, which then removes moisture. Solar drying is very beneficial in terms of pathogen removal and reduction of substrate mass and volume	Mustayen *et al*., 2014;
Vermicomposting	Vermicomposting is the decomposite of waste by using various species of worms. Vermicomposting of sewage can be done and has been successfully demonstrated by Schaik *et al.* (2016) to reduce biohazardous waste. The casting of earthworms can plausibly lower pathogenic levels in substrata as it limits the nutrients, increases intestinal enzyme action and secretion of coelomic fluids that contains antibacterial properties. As stated by Panikkar *et al.,* (2004) vermicomposting can achieve safe pathogen killing levels due to the remaining pathogens competing for the remaining nutrients however future investigations on this subject need to be done to understand the exact mechanisms involved for the reduction of pathogens during municipal and industrial organic waste.	Lazcano *et al.,* 2008 ; Yang, *et al.,* 2014 ; Panikkar *et al.,* 2004 ; Swati & Hait 2018; Zomorodian *et al.,* 2016

2.4 Anaerobic digestion process

AD is a biochemical process that helps with biodegrading biomass with the use of bacteria and archaea (Voicu *et al.* 2015). The process is divided into four metabolic stages: 1) hydrolysis, 2) acidogenesis, 3) acetogenesis, and 4) methanogenesis (Jiang *et al*., 2018). This process consists mainly of multi-biological processes, to convert organic carbon to mainly carbon dioxide and methane (Refer to

Figure 2-3).

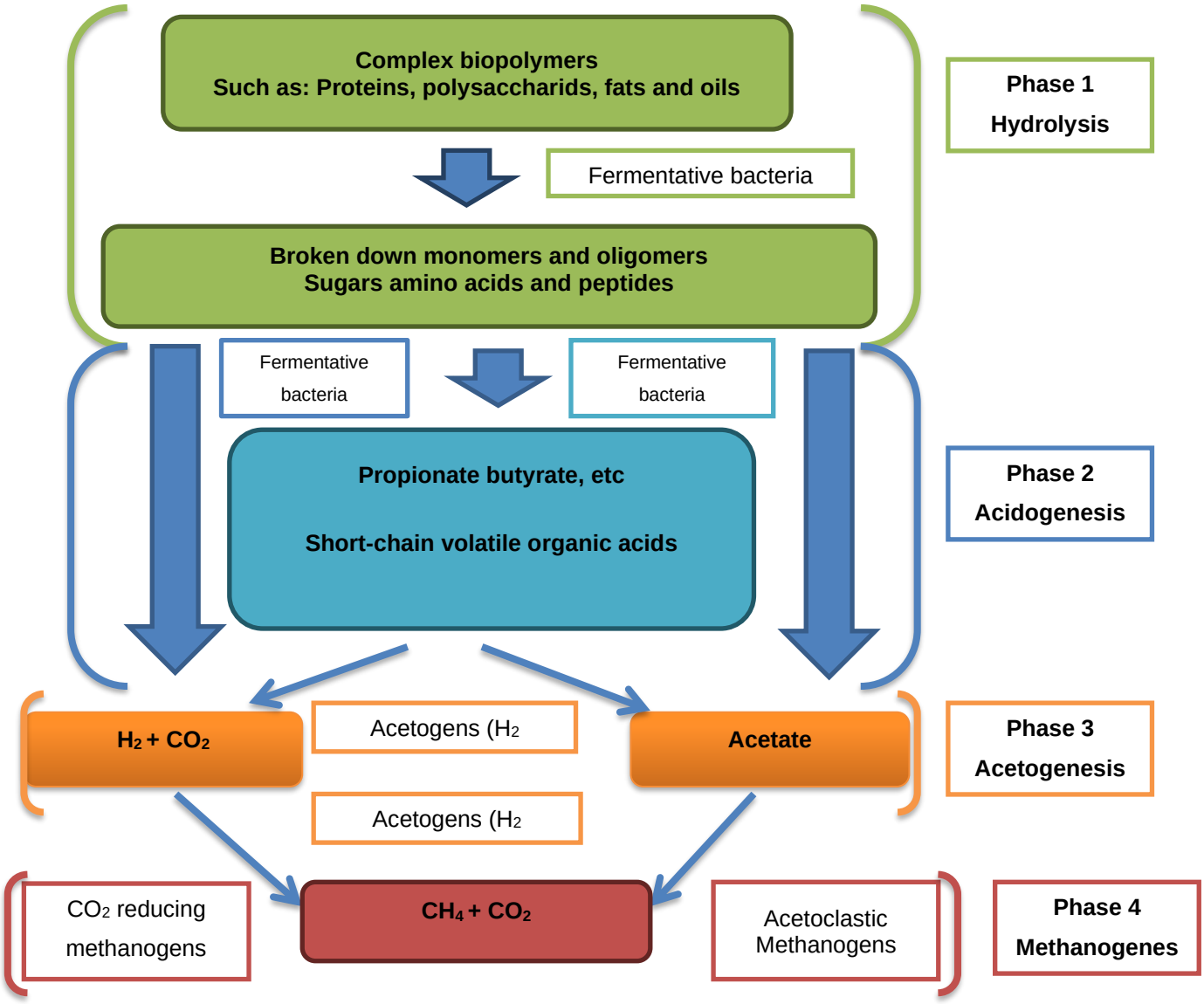

Figure 2-3: AD process illustrating the different anaerobic stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Adapted from: Ramaraj & Dussadee, (2015). Biological Purification Processes for Biogas Using Algae Cultures: A Review. *International Journal of Precision Engineering and Manufacturing-Green Technology*. 4. 20-32.)

2.4.1 Hydrolysis

Hydrolysis is the first step in the process of AD. During the hydrolysis step, insoluble organic compounds (polymers), such as proteins, carbohydrates and lipids, are hydrolysed into monomers, such as sugars, amino acids and fatty acids, with the help of extracellular enzymes (Ezebuiro & Körner, 2017; Ali-Shah *et al.,* 2014). Hydrolysis is catalysed by hydrolases (proteases, lipases, amylases and lipase) which are produced and excreted as extracellular enzymes by hydrolytic bacteria from genera such as *Bacteriocides, Clostridium, Bifidobacteria, Enterobacterium* and *Streptococcus.* These hydrolytic bacteria, which hydrolyse the substrate, are known as facultative anaerobes. The hydrolysis stage is the rate-limiting step if the substrate contains a low surface-to-volume ratio and the molecules present are large. However, if the substrate contains small molecules and are readily degraded, acetogenesis and methanogenesis will become the rate-limiting step. Substrate, after hydrolysed activity, then become available for cell transport and are available for further degradation by fermenting bacteria in the acidogenesis step. The hydrolysis stage is normally the focus of studies, and by increasing the rate this stage occurs, could maximise methane production (Amani *et al.,*2010; Borja. 2011; Ezebuiro & Körner, 2017).

2.4.2 Acidogenesis

Acidogenesis step is generally known as the fastest step during anaerobic processes (Meegoda *et al.,* 2018; Borja, 2011). In this step, soluble organic molecules that were formed by the hydrolytic bacteria are utilised by fermentative bacteria or anaerobic oxidisers. Clostridia and Bacteroidetes are a major part of this stage (Yangin-Gomec *et al.,* 2017). According to Tezel *et al.,* (2011), microorganisms present in this step are both obligate and facultative anaerobes. Generally, in a stable AD reactor, one main degradation pathway is followed, which results in the production of acetate, carbon dioxide and hydrogen (Schink, 1997; Ali-Shah *et al.,* 2014). Volatile fatty acids (VFA) and alcohol are intermediates that play a small role in this pathway described above; by following this degradation pathway, greater energy yields are available for microorganisms, and these products can directly be utilised by methanogenic microorganisms. Nonetheless, if the concentration of the hydrogen and formate is high, it will force the fermentative bacteria into following another pathway that will produce a more reduced metabolite (Angelidaki *et al*., 2002). Products formed consist of approximately 51% acetate, 19% H_2/CO_2 and reduced products such as alcohols, 31% in lactate and VFA, but this number of products formed depend

on the hydrogen and formate present (Angelidaki *et al*., 2002; Borja, 2011;). Intermediates such as volatile organic fatty acids, alcohols, carbon dioxide, hydrogen and aldehydes are used in the acetogenesis step.

2.4.3 Acetogenesis

Products formed in the acidogenesis stage consist of fatty acids longer than two carbons, alcohols longer than one carbon atom, branched chains and aromatic fatty acids cannot be used by methanogenic organisms and need to undergo further oxidation. These intermediates are broken down into acetate and hydrogen with the help of proton reducing bacteria present in this step, such as *Syntrophomonas* and *Syntrophobacter*, which are syntrophic acetogens (Ali-Shah *et al.,* 2014; Voicu *et al.,* 2015; Weiland, 2010). *Methanobacterium suboxydans* converts pentanoic acid into propionic acid, where *M. propionicum* utilises the propionic acid and converts it into acetic acid. In this step, acetogenic reactions need to be thermodynamically favourable, thus meaning partially low H_2 is essential (Borja, 2011; Schuchmann & Müller, 2016). The products formed in this step are then used as substrate for methanogens in the last step (Angelidaki *et al.,* 2012).

2.4.4 Methanogenesis

The last step in an anaerobic system is where the acetate, H_2 and CO_2 are converted to CH_4 and CO_2 by methanogenic Achaea. These organisms can grow directly on H_2, CO_2, acetate and other one-carbon compounds. Acetate in this step is generally the forerunner for methane production and is responsible for up to 70% of the methane produced; the other 30% originated from H_2 and CO_2 (Ali-Shah *et al.,* 2014; Leng *et al.,* 2018; Angelidaki & Karakashev, 2012). Methanogenic microbes produce the sought-after CH_4 by one of the following two pathways: In one of the pathways only certain heterotrophic methanogens such as *Methanosarcina barkeri, Methanococcus mazei* and *Methanotrix soehngenii* can convert acetate/acetic acid into CH_4 and CO_2 ($CH_3COOH = CH_4 + CO_2$) though the majority of CH_4 production (70%) occurs through this pathway and is the more favourable path (Borja, 2011). The second pathway, known as the acetate oxidation pathway, is more favourable under high temperatures; all autotrophic/hydrogenotrophic methanogens are able to utilise CO_2 and H_2 to produce CH_4 ($CO_2 + 4H_2 = CH_4 + 2H_2O$); however, this pathway contributes less in terms of biogas production (30%).

2.5 Mesophilic versus thermophilic digestion

AD can occur naturally and/or under the following applied conditions (Weiland, 2010). Four common conditions are mostly studied when it comes to biogas yield; this includes; 1) psychrophilic conditions (temperature range from 10-16°C); 2) mesophilic conditions (temperature range from 30-40°C), which are most commonly used in studies of the rumen of

ruminant animals (sheep, pigs, cattle, humans, etc.) due to methanogens that are mostly adapted to these temperatures; 3) thermophilic conditions (temperature range from 50-60°C) and 4) hyperthermophilic conditions (temperature above 60 °C) both thermophilic and hyperthermophilic are used in studies as a promising method to treat concentrated blackwater (Lee, *et al.,* 2008; Sailer *et al.,* 2021; Moerland *et al.,* 2021). The two most used anaerobic digestion conditions commonly operate at either mesophilic or thermophilic as these conditions produces the best results in terms of methane production (Albihn 2009; Kardos *et al.,* 2011). Although both these conditions are widely used for their results, both these conditions have advantages and disadvantages. According to Albihn (2009) material that is collected from mesophilic operations, after AD do not guarantee sanitized material, therefore a sanitizing step is needed to ensure that the material is safe for other utilities such as composting or biofuels. Generally, biogas yields are dependent on the temperature, the type of biological waste in a reactor and have shown that lower biogas yields were produced in reactors outside of these ranges. Therefore, a comparison was done on the Operating and controlling parameters of both these conditions and are illustrated in Table 2-2. This table was modified from Kardos *et al*., 2011 using recently updated articles and are noted in Table 2-2.

Table 2-2: Operating and controlling parameters of mesophilic and thermophilic AD systems

Parameter	***Mesophilic system***	***Thermophilic system***	***Sources***
Optimal temperature (°C)	35 to 40	40 to 55	(Kardos *et al.,* 2011) (Lee, *et al*., 2008*)* (Sailer *et al.,* 2021)
pH	6.5 to 8.0	6.5 to 8.5	(Kasinski, 2020) (Sibiya *et al.,* 2014) (Zaitseva *et al*., 2004)
Max. COD removal (%)	60 to 94	85 to 95	(Hamzah *et al.,* 2019) (Ince, *et al.,* 2017) (Karndos *et al.,* 2019)
BOD removal (%)	45 to 55	55 to 70	(Davey, 2020) (Karndos *et al.,* 2011)
Biogas production (Nm^3/1000kg dry organic material)	920 to 980	950 to1 000	(Davey, 2020) (Karndos *et al.,* 2011)
Methane yield (%)	60 to 70	70 to 85	(Karndos *et al.,* 2011) (Ferrer *et al*., 2011)
Volatile fatty acids (mg CH_3COOH/dM^3)	1 500 to 2 500	3 000 to 4 000	(Karndos *et al.,* 2011)((Franke-Whittle et al., 2014)

Comparing biogas potential between mesophilic and thermophilic conditions is one of the main debatable topics when it comes to AD. Most of the studies on these conditions concluded that thermophilic conditions, despite the disadvantages, are favourable over mesophilic conditions due to the high methane and biogas production they deliver (Kardos *et al.,* 2011; Kasinski, 2020).

AD may also be done with mesophilic or thermophilic settings using two reactor types, according to Facchin *et al.,* (2013) and Verma (2002). Continuously stirred tank reactors (CSTR) and batch reactors are two types of reactors. As part of the CSTR, feed (sludge containing digestible organics and bacteria) is continually added at a rate and volume that corresponds to effluent removal and effluent volume. Thus, the sludge and digestate are continually stirred to ensure effective AD by enabling anaerobes to come into contact with the full volume and surface area of the sludge. When fed daily, a CSTR can be maintained for an indefinite period. Anaerobic batch reactors that run between 25-40 days are the opposite of CSTRs; they are used for loading once-off feedstocks, in a closed anaerobic environment and is however, dependant on the nutrient's availability in the reactors.

2.6 Role of environmental factors in the AD process

Faecal sludges can be characterised by their viscosity, pH, ammonia levels and their chemical oxygen demand (COD)/biological oxygen demand (BOD). However, due to the differentiation in faecal material in pit latrine, because of the amount of people using it and the disposal of household waste in pit latrines it is proven difficult to characterized. Faecal sludge is nearly impossible to characterise or to put in a specific class as mentioned; thus, parameters need to be taken to evaluate if faecal sludge can be used as AD substrate (Nakagiri *et al.,* 2015). The quality of faecal sludge is influenced by the source, density, storage system in place, temperature and moisture. The pH of faecal sludge is an important quality due to the hydrogen-ion concentrations that determine the acidity and alkalinity of the sludge. Faecal sludge is also characterised by total solids (TS), volatile solids (VS), volatile fatty acids (VFA), COD ratio and heavy metals contributing to the biological process in faecal sludge (Bassan *et al.,* 2013a).

2.6.1 Temperature

AD processes can be applied in a wide range of temperatures and can be operational from psychrophilic temperatures ranging around 20°C to extreme thermophilic conditions that are 55°C and above; each of these temperatures sets depends on the substrate application (Kashyap *et al*., 2003; Tassew *et al.,* 2019). However, all of them have their advantages and disadvantages. Increasing the temperature of reactors can have several advantages such as 1) solubility of organic compounds, 2) increases in the rates of chemical and biological reaction, 3) increases in

the diffusivity of soluble substrate, and 4) pathogen removal increases (Gaby *et al.,* 2017; Chow *et al.,* 2020; Moerland *et al.,* 2020). Disadvantages of high temperatures is that they cause decreases in the pKa of ammonia, leading to an increase of free ammonia (FA). This leads to the inhibition of the microorganisms. Thus, thermophilic conditions are more sensitive to inhibitors.

2.6.2 pH

The pH values are of one of most important factors playing a role in AD and can cause a reactor to malfunction. According to Boe *et al.,* (2008), the pH in many of the microbial groups is the same, but in AD, microbial function occurs at different pH values. For methanogens to function, a pH value between 4.5 and 8.5 is needed and to function optimally, a pH value between 5.5 and 8.5 is needed. The pH ranges for fermenting bacteria are quite wide, ranging from 4 to 8.5 and differ in means of optimal pH in respect to the available fermenting products. In an anaerobic digester with a mixed culture, it is suggested that the optimal pH ranges is 6.6 to 7.8. Therefore, it is important when setting up a reactor that the pH values of the substrate are between the values previously mentioned to ensure successful operation (Gómez *et al.,* 2011).

2.6.3 Chemical Oxygen Demand (COD)

COD's is used to determine the amount of organic and inorganic material present, which can indicate whether an anaerobic digestion process will be successful depending on the number of degradable organics available. Changara *et al.,* (2019) estimate that a pit latrine can have COD levels between 30,000 mg/l and 50,000 mg/l, excluding latrines that contain out of the ordinary compounds. However, values of the pit latrines differ based on the location, since these pits are not only used for faecal and urine deposits, but also for food and plastic waste. As per the above-mentioned, the CODs used to determine the amount of inorganic and organic matter differs from pit to pit; which in some cases makes it very difficult to obtain a representative sample (Verma, 2002).

2.6.4 Volatile Fatty Acids (VFA)

In an anaerobic digestion process, VFAs are one of the most important intermediates which is converted by organisms to produce methane and carbon dioxide (Pind *et al*., 2003; Franke-Whittle *et al.,* 2014). An instability in an anaerobic digestion process is well-known to increase the concentration of VFA and therefore widely been suggested as an operational efficiency indicator of an anaerobic digestion system (Boe *et al*., 2008; Poh *et al.,* 2016). It has been found that the un-ionised fraction of VFA leads to methanogenesis inhibition. Un-ionised fatty acids (UVFA) can pass through cell membranes and dissociate, which interferes with cell homeostasis (Russell & Diez-Gonzalez, 1997). The two main parameters, pH and the total VFA concentration must be

considered in order to assess and observe the effect of UVFA in an anaerobic digester. Systems that contain VFA concentration levels above 4000 mg/l and higher can leads to digestion failure (Lee *et al.,* 2015). When the UVFA concentration exceeds the buffering ability of the reactor material, the differential growth of fermentative bacteria and methanogens can cause pH to change, which can lead to disintegration of particles, causing a decrease in methanogens. (Tiwari *et al*., 2006 Fang*,* 2010b; Chen *et al.,* 2014; Russell and Diez-Gonzalez, 1997;). VFA levels can be affected by an offset in temperature, pH or ammonia concentrations. VFA concentration can inhibit or reduce CH_4 yield and lead to an increase of H_2S. (Verma, 2002; Weiland, 2010).

2.6.5 Hydraulic Retention Time (HRT)

The Average period that a particular volume of sludge stays in a digester, is known as the hydraulic retention time. This design parameter affects the economics of a digester and is therefore one of the key factors playing an important role during the AD process. Therefore, a lower amount of sludge, results in a shorter HRT. The HRT in a CSTR is unfixed and is fed daily, which can be stopped at any time. The average HRT on batch reactors, depends mainly on the conditions such as mesophilic or thermophilic. Mesophilic reactors in general have an HRT between 20 to 40 days, in which a thermophilic reactor has an HRT between 10 to 15 days depending on the digester size and substrates. (Verma, 2002)

2.6.6 Organic Loading Rate (OLR)

The OLR is a critical parameter, which is measured by the biological conversion capacity of an AD system and represents the amount of VS supplied into the reactor on a daily basis (Mao *et al.,* 2015). Volatile solids (VS) represent the amount of organic materials that can be digested, whereas the majority of the remaining solids are fixed and are undegradable. The actual organic loading rate is determined by the type of waste used in the digester as the different kind of waste determine the amount of biological activity (Nkuna *et al.,* 2020; Babae & Shayegan, 2011). When feeding the reactor in exceedance of its usual sustainable OLR level, it will produce lower biogas volumes, due to the accumulation of obstructing/inhibiting organics such as VFA, which indicates imminent reactor failure (Verma, 2002).

2.6.7 Volatile Solids (VS)

VS is the fragment of biodegradable organics in a substrate and plays an important role in the total VS of an anaerobic digestion system. The organic fragment is composed out of different readily degradable material, which includes protein, fats, carbohydrates and cellulose. The combustible fraction is lignocellulosic organic matter (paper, cardboard, coarser wood), which is less biodegradable under anaerobic conditions. The inert fraction is metals, stones, glass and

sand that is mostly or completely non-degradable. Both the combustible and inert fraction of VS should ideally be removed to better biogas yield, prevent metabolic complications, wear and tear of system equipment (Mao *et al*., 2015). The expected reduction of volatile solids in an anaerobic digestion process ranges between 20% to 60% depending on the substrate type. Higher amount of VS in substrates can lead to higher methane production depending on the composite and organics materials available (Wei *et al*., 2018)

2.6.8 Inoculum to Substrate Ratio (ISR)

According to Akyol (2020), the inoculum to substrate ratio (ISR) in an anaerobic digestion system is an important operational parament for methane production, which greatly varies depending on the substrate composite. The optimal ISR mainly depends on the inoculum's origin (Ali-Shah, *et al.,* 2014). The Metabolic activity of different inoculum sources will vary (Ali-Shah, *et al.,* 2014) as most anaerobic inoculum contains the same anaerobic microbes required for AD. The best ISR for AD was discovered in previous studies to be two parts inoculum, one part substrate (2:1), while some studies also shown a three parts inoculum and one part substrate (3:1), as it delivered higher amounts of potential methane gas. This will enable an early methanogenic initiation, allowing a rapid exponential growth phase in reactors (Ali-Shah *et al.,* 2014; Silva *et al.,* 2020; Sri Bala Kameswari *et al.,* 2012). Lower ISR than the above-mentioned ratio's, might result in the inhibition of methanogenic activity and an increase in H_2S production. This is due to the accumulation of VFA and would cause a negative effect on the methane yield. (Sri Bala Kameswari *et al.,* 2012).

2.6.9 Hydrogen Content

Hydrogen is produced when VFA is digested and converted into acetate and hydrogen through obligated H-producing acetogenic bacteria (*Acetobacterium woodii* and *Clostridium aceticum)* (Weiland, 2010). Hydrogen content in an AD reactor controls the amount of methane produced. High amount of Hydrogen can become toxic to acetogenic bacteria and disrupts electron flow, as a result these bacteria must form a symbiotic relationship with autotrophic methane bacteria (Ali-Shah *et al*., 2014; Borja & Rincón, 2017). Any increase in H^+ ions will result directly to a reduction in the system's pH value, which may have a negative affected on the microbial community and therefore leads to a disturbance in the AD process.

2.6.10 Macro and micronutrient content: Carbon (C): Nitrogen(N): Phosphate(P): Sulphate(S)

According to Ceron-vivas *et al.,* (2019) on the carbon and nitrogen (C/N) ratio of wastewater, the ratios between 20 and 30 should be sufficient for to microbes to function. In a study done by

Weiland (2010), found that a C:N:P:S ratio of 600:15:5:1 is optimum for desirable biogas formation and methanogen growth (Kuila & Mukhopadhyay, 2020). Digestion systems are sensitive to the C:N ratio. According to Weiland (2010), high C:N ratios provide inadequate N to maintain biomass, which eventually leads to a fast N reduction. Low C:N ratios increases ammonia inhibition risk, which proves to be toxic to methanogenic bacteria. This could lead to carbon being utilised insufficiently and ultimately a lower biogas and methane yield. According to Weiland (2010) and Kuila & Mukhopadhyay, 2020, these micronutrients include iron, selenium, cobalt, tungsten, molybdenum and nickel. These trace elements are important for microbial growth, the function of different metabolic systems and associated pathways. Nickel is especially important, as it is necessary for synthesis of the F_{430} co-factor component of methanogenic bacteria. This co-factor is involved in the synthesis of CH_4 for growth and cobalt is necessary to build up corrinoid factor (Weiland, 2010). Each anaerobic bacterium has its own specific concentration when it comes to the concentration of micronutrients in general. Receiving less or more than usual micronutrients influence the microorganism's growth and function which will either lead to difficulties or improvements and therefore affect the AD process.

2.6.11 Accelerants of biogas production.

According to Mao *et al.* (2015) and Yadvika *et al.* (2004), biogas production can also be increased by exploiting and controlling various parameters in addition to the ones that requires monitoring, as mentioned above. These accelerants include bio-filters, recycling of slurry, manipulation of parameters, greenery biomass, biological- and inorganic additives. According to Weiland (2010), pre-treatment is another accelerant, which constitutes chemical, thermal, enzymatic and/or mechanical processes.

2.7 Molecular methods used to study microbial communities

2.7.1 Polymerase chain reaction

Technology for exponential amplification of a fragment of DNA is polymerase chain reaction (PCR), whereas a single molecule is the limit of its sensitivity rendering PCR a superb qualitative process for the precise identification of rare DNA sequences (Garibyan, *et al.,* 2014*)*. The yield of amplified DNA under proper conditions is proportional to the initial number of target molecules, making it a quantitative analytical instrument as well. PCR radically transformed biological science from the time it was created (Wages, 2005). PCR is a technique used by many scientists for the identification of microorganisms. This technique is a method created by Kary Mullis in the 1980's and is based on DNA polymerases to synthesize new strands of DNA complimentary to the template strand. PCR occurs through a series of events called thermal cycling, a method which

DNA containing solutions is repeatedly heated and cooled, which melts the DNA, whereas the short anneal DNA fragments called primers bind to the target complementary DNA and by using temperature-dependent DNA polymerases such as Taq polymerase, the primer-bound sequences enzymatically replicate (Caetano-Anollés, 2013) from the first time it was used in practise PCR strategies propelled significantly and is widely used for clinical and research for the diagnostics of pathogens, illnesses and microorganisms to study and understand them, which leads to better medical treatment or industrial applications.

This technique needs a primer on which it can add a nucleotide on the 3'OH group and makes it possible to delineate a specific region. (NCBI) Components needed for successful PCR:

1) DNA template targeted gene in the DNA sample. This is then heated up for the double stranded DNA to separate, thus making a single strand from a double strand DNA molecule: and
2) DNA polymerase, which is a type of enzyme that synthesises new strands of DNA that are complimentary to the target sequence. Example of this enzyme are *Taq*DNA polymerases;
3) Primers are short-stranded DNA pieces containing the sequence of the region that must be amplified; and
4) Nucleotide bases A, T, G and C, which are the building blocks for DNA strand. From the first time it was used in practice, PCR strategies propelled significantly and are widely used in clinical research for the diagnosis of pathogens, illnesses and microorganisms to study and understand them – which leads to better medical treatment or industrial applications.

2.7.2 Next generation sequencing

According to Ploski (2016), next generation sequencing (NGS) is defined as "technology allowing one to determine in a single experiment, the sequence of a DNA molecule(s) with total size significantly larger than 1 million base pairs (1millionbp or 1Mb)". This technique is one of the most advanced methods used for the identification of microbial communities (Tan *et al*., 2015). NGS consists of different NGS platforms using different sequencing technology. Behjati and Tarpey (2013) state that it doesn't matter what type of NGS process used, the principle stays the same where millions of small fragments in a sample is sequenced. Bioinformatics analysis is further used to piece together these small fragments' by means of mapping the individual reads and can also be used for the sequencing of an entire genome or for sequencing a specific part of the genome that communities share with one another, such as bacteria and archaea. According to Herzyk (2014), NGS applied to targeted amplicon studies can focus on one or more phylogenetic marker genes. 16SrRNA genes are most widely used in NGS due to their excellent phylogenetic markers and because they consist of nine different regions that are located

throughout the different conserved regions of the genome. NGS can be use with specific primer sets to classify different taxonomic levels of organisms in a sample. (Sambo *et al.,* 2018) Therefore, the resulting amplicon is sequenced by using NGS technology of relatively long reads. Thus, following the initial filtering step, a classification method is used: such as phylogenetic clustering. Reads that are gathered from this technique can be assigned to different taxonomic unites using a ribosomal database (Escobar-Zepeda, *et al., 2018)*.

2.7.3 Alpha and Beta diversity

The 'count' or number of NGS reads assign to each taxon/OTU is utilized as a proxy for the abundant taxon in a sample. After the abundance have been determined through the 16 S-sequencing, diversity indices such as Alpha and beta diversities can be used as an analysing tool for the microbiota dynamics. (Fintello *et al*., 2016). According to Moore, 2013 the total microbial diversity is influence by two factors; Alpha diversity (the average species richness in a sampling site) and beta diversity (The difference in microbial community between different sample sites, changes that occurs in specie richness from one location to another location).

Alpha diversity also known as 'local diversity' may be define as the compositional complexity of a community measure within a location (Fintello *et al.,* 2016). Therefore, the alpha diversity increases when the number/abundance of a specie and evenness increases within a sample (Cameron *et al.,* 2021). Different diversity index metrics such as Simpson, Shannon and Chao1 is used for the alpha diversity calculations. (Willis, 2019b; Lemos et al., 2011).). The Shannon index is a statistical information index and assumes all species are present in the sample and are randomly sampled. The Simpson index although it also determines the total species in a sample, the index give more weight to the dominant species, and a few rare species will not affect the diversity (Lemos et al., 2011). Chao1 is used as a non-parametric index for determining the smallest number of OTU' in a sample (Lemos et al., 2011). Using these metrics changes over time can in a sample can be evaluated to note the change that occurs in the community structure.

Beta diversity compares the abundance of OTUs in different samples and or sites and calculates a value which indicated how similar/dissimilar a community are in these samples (Modin *et al.,* 2020b). As with the Alpha diversity indices mentioned above several Beta diversity indices exists which includes: Jaccard, Bray-Curtis, Morisita-Horn and Sorenson (Wagner *et al.,* 2018). The Jaccard index is a measurement of the similarity between two data sets of different samples which range from zero to one. If the value of the two samples is closer to one, the more similarities samples share with each other whereas the Bray-Curtis index (most wildly used abundance index) measure the dissimilarities between two data sets of different samples (Kiernan, 2014). Sorenson index are similar to the Jaccard Index but place more emphasis on the shared species rather than

the unshared. Morista-Horn index, not used as much as the Bray-Curtis index is one measurement that can be used for Beta diversity; Morista-Horn is a well-known angular overlap metric, which have a single value between zero (No overlap) and one (Perfect overlap) (Sahin Honorine Guiraud et al., 2021).

SECTION SUMMARY:

In Summary, biogas production and the understanding of the microbial dynamics during anaerobic digestion are well-known topics studied globally, which is lacking in South Africa on wastewater treatment plants. There are numerous studies using wastewater resources successfully as a biogas substrate recorded in varies countries. South Africa currently has a major sanitation problem within the rural areas, because of pit latrines filling up faster than was expected as well as the lack of maintenance, causing water contamination and diseases in the surrounding communities. Therefore, alternative sanitation options need to be implemented and could lead to a more positive environmental outcome. One such option is anaerobic digestion, which could provide energy and a promising way to dispose of sludge by composting. Understanding the Anaerobic digestion process and the microbial consortia of South Africa's wastewater will give a clearer picture of the community structure and dynamics of the microorganism's presence during the different phases specifically; hydrolysis, acidogenesis, acetogenesis and methanogenesis. Different parameters can influence the biogas production; therefore, it is important to determine the parameters obtained from pit latrines to optimise anaerobic digestion processes. VFA and Free ammonium are two of influencing factor that could inhibit methanogenesis. By understanding the microbial dynamics and how each community contributes to biogas production, communities can subsequently be identified to ensure optimal reactor conditions to dispose of the waste. For co-digestion to occur with faecal sludge, a reliable and readily available inoculum needs to be provided at a constant time. Two treatment conditions (Mesophilic and thermophilic) can be applied to this alternative treatment option, which plays an integral role in the performance of AD and removal of pathogenic organisms. Anaerobic digestion of faecal sludge can be studied using molecular techniques. NGS, an advanced molecular method was used to study the microbial dynamics in different stages of anaerobic digestion in previous studies as it is a more cost effect and faster technique.

CHAPTER 3 METHODOLOGY

3.1 Sampling

Sampling could only take place if the necessary health precautions and sampling gear needed were implemented; this includes hepatitis vaccination, protective overall-garments, eye protection, thick protective gloves, gum boots and a gas respirator. Sampling took place at the WWTP in Potchefstroom, North West Province and at the biogas plant near Bronkhorstspruit, Gauteng Province. Faecal sludge samples were collected from vacuum trucks (Nigiri vacuum trucks) that contained pure faecal sludge and were used as one of the co-digestion substrates. For the second co-digestion substrate, primary digested sludge (seed) that consisted of cow manure was collected from the biogas plant near Bronkhorstspruit. A total of 20 litres of these co-digesting substrates was collected and transported to North-West university. The sludge was then prepared and characterised as one of two categories: non-viscose and viscose. Substrates were homogenised and 100 ml were then stored in a -20°C for experimental analysis (COD; VS, TS and pH). Mechanics samples for lab analysis were collected and put in 45 ml Falcon tubes, and send to Eco-Analytica for analysis.

3.2 Reactor design of a benchtop set-up

After the sorting phase, bench-top reactors were set up to simulate a closed AD system (Figure 3-1). Numerous materials were needed for this: A temperature-controlled water bath, eighteen 500 ml Thermo-Fisher Scientific glass bottles for samples, eighteen 500 ml Thermo-Fisher Scientific glass bottles for confining liquid, gasbags, eudiometers, confining liquid, office clamps, rubber tubing, Vaseline (petroleum jelly), silicon, water and a nitrogen gas tank. Each reactor was set up as follows: the first eighteen bottles were divided into four substrate categories so samples could be noted in triplicates

1. Seed (cow manure control sample), Obtained from Bronkhorstspruit biogas plant
2. Microcrystal cellulose and seed (MCC) (Reference sample to evaluate the process, of seeding samples usually a sample with a known biogas potential)
3. Faecal sludge obtained from septic trucks
4. Composite (Faecal and cow manure mixture).

Three bottles were filled with MCC and were the control of the biogas production process to solely evaluate the biogas yields of seed; three with seeding sludge; three with faecal sludge and triplicates of three composite samples in which each of the samples contained a substrate ratio of 5:1 where one was the faecal sludge and 5 the seeding sludge. After the 500 ml substrate bottles were assembled, they were placed in a heated water bath and closed with sealed bottle caps containing one glass tubes in the cap, rubber tubing was then used to connect the glass to

a three-way tee plastic tub connector occupying the first outlet, and rubber tubing was then used to connected to the gasbag- Occupying the second outlet of the three-way tee plastic tube connector.

Thereafter, a further eighteen glass bottles were filled with pink-coloured confining liquid consisting of sulphuric acid and were placed outside the water bath to allocated spaces next to the reactors, whereas the caps contained two glass tubes. Following this, rubber tubing was used to connect the one glass outlet of the cap to the allocated three-way tee plastic tube connecting it to the gas bag and substrate bottle. The second outlet of the confining liquid bottle cap was used to connect to a 250 ml cylinder to allow displacement of liquid from the bottle to the cylinder for biogas production. Biogas displacement was noted each day for the duration of the experiment as mentioned above, whereas gas composite was determined by a biogas 5000 analyser (VDI (Verein Deutscher Ingenieure) 2006) on selected timelines.

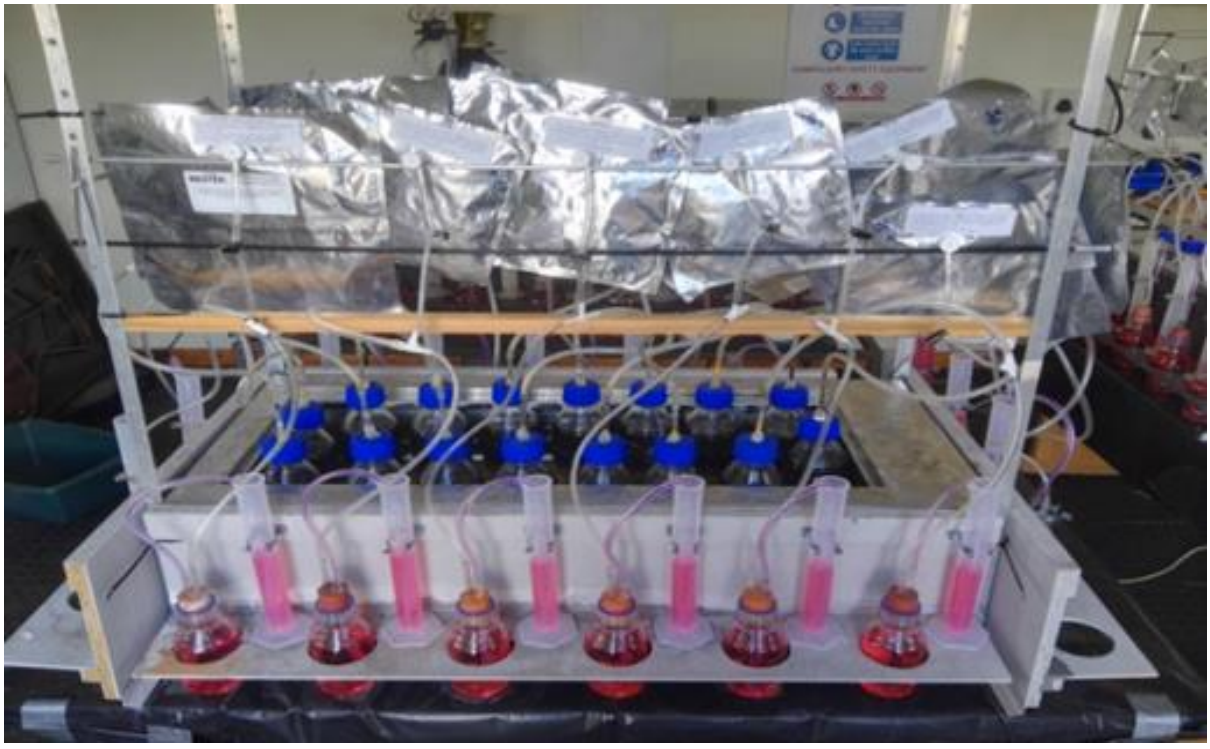

Figure 3-1: Reactor set-up of mesophilic and thermophilic conditions (Photo taken by Senta Berner on a Samsung S4 cell phone)

Water was poured into the water bath and set to preferred temperatures. Both the mesophilic and thermophilic bench-top batch reactors were set up according to the above steps. The mesophilic batch reactors' temperature was set between 35°C and 40°C to ensure mesophilic conditions. The thermophilic batch reactor was set between 55°C and 60°C. Gasbags and batch reactors were flushed with nitrogen (N) to ensure all Oxygen (O_2) still present exit the system. The reactors were then monitored for gas leaks (gas leaks indicator: when the volume of confining liquid in the

cylinder stays the same or when it drops below the value noted). If any gas leaks occurred, Vaseline (petroleum jelly) or silicon was used to seal this system. The system was then left to run for the following preferred times: 15 days for thermophilic and 36 days for mesophilic, depending on the degradation rate of the faecal sludge present at that current time. Clamps were then used to close the connection from the gasbag to the reactor to ensure that the gas displaced the confining liquid into the cylinder where the gas yields per day can be noted.

3.3 Physico-chemical parameters

Measurements were taken of physico-chemical parameters such as pH and temperature at the WWTP with Oakton PCS Test™ 35 waterproof field multi-parameter probe (Thermo-Fisher Scientific, US) in compliance with manufacturer's instructions. Chemical parameters, such as CODs, were measured in mg/l at the laboratory of the North-West University with the HACH DR 2800™ instrument (HACH, Germany). Ammonia (NH_4^+), phosphorus (PO_4^-) percentage carbon (%C) and sulphates (SO_2^-) were sent to the Eco-Analytica Laboratory located on the North-West University's Potchefstroom campus. Volatile suspended solids and total suspended solids were carried out using standard methods for the Examination of Water and Wastewater, 23rd Edition. (Rodger *et al.,* 2017). Physico-chemical parameters for each sample were recorded on Day 0, Day 10, Day 16 and Day 36 for mesophilic conditions and Day 0 (T0), Day 1 (T1), Day 10 (T2) and Day 15 (T3) for thermophilic conditions. After the above-mentioned analysis was done, 100 ml of the sample was taken, frozen to preserve the microbial community present and stored in a -80°C fridge.

3.4 Molecular analysis

3.4.1 DNA extraction

Samples from each reactor were homogenised and kept on ice, 250 mg of each environmental sample was used for DNA extraction with the Mo PowerSoil® DNA isolation kit (Mo Bio, Laboratories, California, USA) and carried out according to the manufacturer instructions given with the DNA isolation kit (See Link below). Minor modifications were done to better adapt the kit to faecal sludge which were as follows: all of the samples were centrifuged at 13,400 rpm due to their wet nature and repeated until the total weight of the pellet was 250 mg, lysis (Solution 1 from kit) was then added, it was then vortexed for 5 seconds and placed into a dry bath for 15 minutes at 70°C to ensure protein degradation due to the high amount of humic acid. Thereafter, normal extraction steps were followed as indicated in the manufacturer's instructions (https://mobio.com/media/wysiwyg/pdfs/protocols/12888.pdf). The quality of DNA extracted was measured through spectrophotometric analysis (Nanodrop, Thermo-Fisher Scientific, US) and agarose gel electrophoresis.

3.4.2 **PCR amplification of 16S rRNA and NGS**

Environmental DNA (eDNA) samples of each triplicate reactor were first pooled before PCR was performed (faecal sludge at the beginning are the same for both conditions and therefore only 32 samples were used to pool and put through NGS for thermophilic and 36 for mesophilic conditions; Table 0-12 contains description of the samples used); 2 µl of the pooled DNA described in the above context was used; the PCR reaction was performed in a 25 µl. This mixture consisted of

1) 12.5 µl Dream taq PCR master mix (2X) (Thermo-FIsher Scientific, US) (DreamTaq DNA Polymerase, 2X DreamTaq buffer, dNTPs and 4 mM $MgCl_2$);
2) 8.5 µl of MiliQ® water;
3) 1 µl of each forward and reverse primers (0.4 µl); and
4) 2 µl of eDNA template.

Primers used were the universal 27F primer (5'- AGA GTT TGA TCM TGG CTC AG- 3') and 1492 R (5'- GG TTA CCT TGT TAC GAC TT- 3') (Inqaba Biotec; SA) and have an amplicon of ~ 1465bp. PCR was done according to Hongoh (2003). The following conditions were programmed into the ICycler thermocycler (Bio-Rad, US): starting with the denaturing step at 94°C for two minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 60 seconds and extension at 72°C for one minute, concluding with extra annealing step at 72°C for five minutes. Amplicons were then exposed to a 'PCR clean-up' process using Agencourt AMPure XP beads (Beckman Coulter Genomics, California, USA). After clean up, an index PCR was performed attaching dual indexes (Nextera XT Index Kit) using 5 µl of amplicon PCR product DNA, 5 µl of Illumina Nextera XT Index Primer 1 (N7xx), 5 µl of Nextera XT Index Primer 2 (S5xx), 25 µl of 2x KAPA HiFi HotStart Ready Mix, and 10 µl of PCR-grade water.The following conditions were used during thermocycling: starting at 95°C for 3 minutes, followed by eight cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds and thereafter a final elongation step of 5 minutes at 75°C. A second phase of 'PCR clean-up' was carried out for the index PCR products. Using a Qubit fluorometer, the partial 16S rRNA libraries were then quantified, standardized, pooled to a final 20 pM concentration, and denatured at 0.2 N NaOH. Prior to the loading of samples on the MiSeq V3 reagent cartridge (Illumina, San Diego, CA, USA)., the pooled library was firstly diluted to a final concentration of 6 pM following a spike for 2-minutes with10% PhiX control and heat denatured. De-multiplexing and secondary reading analyses were conducted using MiSeq reporter software (Illumina, San Diego, CA, USA) after completing a paired end of 2x300 bp reads sequencing run on the Illumina MiSeq.

NGS was performed at the North-West University's Potchefstroom laboratory by using the MiSeq apparatus (Illumina, San Diego, California, USA) with the MiSeq reagent kit (V3 600 cycles PE) (Illumina, San Diego, California, USA). After the NGS was performed, the files were saved as a FASTQ files. MiSeq automatically detected the barcodes and subsequently demultiplexed the clusters passing through the on-board quality checks. In section 3.4.3 NGS DATA processing will be explained.

3.4.3 **NGS Data Processing:**

The QIIME2® pipeline was used to handle a series of demultiplexed reads created from the Next generation sequencer (Guerrini *et al.,* 2019). Demux was then used to analyse the quality of the reads. Following the changes made to the parameters in accordance with the quality control, forward and backward reads were assembled using dada2. Next, the collected reads were further categorized into operational taxonomical units by the classifier features on QIIME2® (Quantitative Insight into Microbial Ecology) software. The Processed sequences obtained were then aligned to the SILVANGS rRNA database for taxonomic assignment (SILVA 132 release) (Quast *et al.* 2012), Using QIIME2® the generated OTU (operational taxonomic unite) count was summarized in an excel table format. Alpha and beta diversity was visualised using the websites METAGENassist® and MicrobiomeAnalyst®. The relative metabolic pathways present in each sample were constructed using METAGENassist® to ensure a pairwise statistical comparison between mesophilic and thermophilic conditions. Alpha and beta diversities were performed in Qiime2® with a sampling depth of 139,217 counts. A wide range of diversity indices was calculated. A control specie such as *Escherichia coli* was used as we know it contains the targeted gene. Seeding sludge was also used as control to evaluate the diversity index of faecal sludge and composite samples. Alpha diversity indexes were calculated using alpha diversity metrics, specifically, observed operational taxonomic unite (OTU), Shannon index, Chao1 and Simpson index and determines the community richness and diversity. These indices were used to determine the statistically significant/differences through the Mann-Whitney/Kruskal-Wallis test. Beta diversity involving the clustering of samples was measured using the Bray-Curtis distance metrics and Jaccard distances, which determines the overlap of community members in all samples. Probability values lower than 0.05 (p-value ≤ 0.05) were considered statistically significant. All statistical analysis performed on sampled data were rarefied and scaled (relative log expression (RLE) Principal coordinate analysis (PCoA) was then performed as an ordination method, based on the Bray-Curtis and Jaccard distances. Thereafter, permutational multivariate analysis of variance (PERMANOVA) was performed to calculate and analyse the statistical significance of these indices.

3.4.4 **Statistical analysis**

Statistical analyses of all parameters regarding the AD process (COD, pH, TS, VS, biogas yield, biogas composition, ammonium, phosphates, nitrates, sulphates and percentage carbon were done in Microsoft Office Excel 2016 and included averages, minimum, maximum and standard error values (representation of triplicates).

CHAPTER 4 RESULTS

4.1 Digester performance

The digester performance is one of the main aspects of AD; thus, certain parameters such as temperature were kept at a set level to determine the effects on the biological processes. Mesophilic and thermophilic bench-top reactors were set up and compared according to the changes in the community dynamics and microbiological parameters. The physico-chemical parameters recorded throughout the study had a direct impact on the microbial dynamics and shift during AD. Three different samples (faecal, seed and composite) were investigated in terms of selected parameters and microbial dynamics. All substrates in the mesophilic and thermophilic conditions had a temperature of 35 ºC (mesophilic) and 55 ºC (thermophilic) to determine which of the conditions are more effective for gas production and what major community role players were present. Microbiological parameters were recorded on Day 0 (T0), 10 (T1), 16 (T2) and 36 (T3) for mesophilic conditions and Day 0 (T0), 1 (T1), 10 (T2), 15 (T3) for thermophilic conditions. Parameters that were recorded during the experimental set-up was phosphates (PO_4), sulphates (SO_4), nitrates (NO_3), ammonium (NH_4), percentage carbon, COD, pH, biogas yield and gas composition. Faecal sludge was documented in an aerobic environment and due to the closed system simulated the gas production analysis could only be done after AD, although nitrogen was inserted to simulate anaerobic conditions, reactors of faecal sludge still shown high levels of oxygen, nonetheless this samples was also evaluated and compared to composite samples as it contains a mixture of seeding sludge and faecal sludge.

4.1.1 Physico-chemical parameters

The levels of COD and pH were recorded throughout the digestion processes. The pH levels of all samples in mesophilic and thermophilic condition varied between 6.74 and 8.4. The highest recorded pH value in mesophilic condition was Seed_T3 (8.01) and the lowest value was in Faecal_T1 (6.90) (See Appendix A:Table 0-1 and

Table 0-2). The pH levels of Samples throughout thermophilic conditions were recorded: the highest in Seed_T1 (8.40) and the lowest in Faecal_T2 (6.74). Overall, the pH remained between the optimal ranges for microbial growth. CODs within all digesters indicated a decrease after AD at both conditions (Appendix A:Table 0-1 and

Table 0-2) During mesophilic conditions, the maximum COD removal rates were recorded in the faecal samples (removing 40% of CODs), followed by seed samples with 22% and then composite samples with 21%. Thermophilic conditions indicated more than 20% COD removal, but removal

rates recorded were most efficient in composite sample with 40%, followed by faecal samples with 26% and lastly, seed samples with 26%.

Levels of TS and VS can be seen in appendix Table 0-3 and Table 0-4. The VS observed in both conditions were recorded as decreasing in all samples except for seed in both these conditions. An increase was observed in seed during mesophilic conditions ranging from 59.55 to 62.56 g/100g, whereas seed samples in thermophilic conditions increased from 61.87 to 62.42 g/100g. The recorded TS in the mesophilic conditions decreased from 1.46 to 1.27 g/100g in faecal samples and were recorded at lower levels compared to seed and composite samples. Lower levels were recorded during the mesophilic condition compared to thermophilic conditions of all samples, which ranged from 1.46 to 0.53 g/100g. All samples were recorded to have a similar trend of that recorded during CODs (a decrease, with few exceptions).

PO_4, SO_4, NO_3 and NH_4 is reported hereafter.

Table 4-1: Comparison of the macro-elements in AD of mesophilic conditions (pre- and post-AD)

Sample_ID	PO_4 mg/l	PO_4 STDEV	SO_4 mg/l	SO_4 STDEV	NO_3 mg/l	NO_3 STDEV	NH_4 mg/l	NH_4 STDEV
F_T0	166.97	132.30	68.73	60.24	2.44	0.97	66.61	27.51
F_T3	155.31	106.09	62.96	99.42	8.95	10.21	156.95	29.10
S_T0	3.54	2.56	50.17	35.67	25.72	35.26	1276.65	347.82
S_T3	8.70	10.99	25.09	30.22	13.83	11.02	1156.37	108.40
C_T0	1.60	0.93	19.07	12.16	7.06	8.13	1012.23	357.19
C_T3	3.20	3.09	21.38	18.23	14.17	14.93	999.70	290.73

Substrate: F = Faecal, S = Seed, C = Composite. Timeline: T0 = Day 0, T3 = Day 36

Table 4-2: Comparison of the macro-elements in AD of thermophilic conditions (pre- and post-AD)

Sample_ID	PO_4 mg/l	PO_4 STDEV	SO_4 mg/l	SO_4 STDEV	NO_3 mg/l	NO_3 STDEV	NH_4 mg/l	NH_4 STDEV
F_T0	166.97	132.30	68.73	60.24	2.44	0.97	66.61	27.51
F_T3	62.65	68.83	9.24	10.71	4.59	1.21	164.51	58.81
S_T0	5.89	3.80	28.85	22.68	9.02	6.41	1614.85	343.62
S_T3	1.99	0.86	35.81	27.11	18.07	26.61	1418.41	394.42
C_T0	13.35	22.00	21.51	18.98	11.83	10.39	1130.11	419.36
C_T3	1.95	0.78	18.93	12.85	18.76	20.67	1316.30	329.32

Substrate: F = Faecal, S = Seed, C = Composite. Timeline: T0 = Day 0, T3 = Day 16

PO_4 levels during both mesophilic (Table 4-1) and thermophilic (Table 4-2) conditions documented for faecal, seed and composite were recorded between the levels of 1.6 and 166.7 PO_4mg/l (all samples are triplicates and contain standard deviation); thus, variety in different samples can be

observed. Throughout mesophilic conditions of faecal samples, a high level of PO_4 was observed ranging between 155.33 and 166.97 PO_4 mg/l which was much higher than that observed in seed and composition. The PO_4 levels observed during AD of seed (3.54 and 8.70 PO_4 mg/l) and composite samples (1.60 and 3.20 PO_4 mg/l) ranged from 1.6 to 8.7 PO_4 mg/l and both increased after AD, which was observed as the reverse trend recorded in faecal sludge. Samples documented in all thermophilic conditions, faecal, seed and composite samples, ranged from 1.95 to 166.97 mg/l (PO_4), indicating large variance between the different samples. Thermophilic conditions showed similar PO_4 levels trends during the AD process of the different substrate as those in mesophilic conditions. Faecal samples were recorded between 62.65 to 166.97 PO_4 mg/l, which indicate much higher PO_4 levels than those recorded in seed samples (1.99 and 5.89 PO_4 mg/l), and in composite samples (1.95 to 13.35 PO_4 mg/l). All sample types in thermophilic conditions showed a decreased in PO_4. The faecal samples in mesophilic conditions indicate a decline in PO_4 levels during AD compared to seed and comp samples during mesophilic conditions which increased.

Sulphate levels documented in both mesophilic and thermophilic conditions were recorded from 9.24 to 68.73 mg/l in SO_4 as with PO_4, they showed large differences within the different sample and conditions. When observing faecal samples, it was recorded that faecal during thermophilic conditions had a drastic decline in SO_4 than that recorded in mesophilic conditions. Mesophilic conditions of faecal decreased from 68.73 to 62.96 SO_4 mg/l, where thermophilic conditions were recorded a decrease from 68.73 to 9.24 SO_4 mg/l. Seed samples during the AD process were recorded to decrease from 50.17 to 25.09 mg/l SO_4 in mesophilic conditions, whereas Sulphate levels increased from 28.85 to 35.81 mg/l during the AD of thermophilic condition. Composite samples showed similar trends in SO_4 than those observed in seed samples and increased from 19.07 mg/l SO_4 in mesophilic conditions and decreased in thermophilic conditions from 21.51 to18.93 mg/l SO_4. When mesophilic and thermophilic conditions are considered, a substantial decrease in SO_4 in all the samples from time T0 to T3 was observed. It was also observed that more SO_4 was present in faecal samples than that recorded in seed and composite samples in both mesophilic and thermophilic conditions. The mesophilic conditions tended to have higher SO_4 levels still present after digestion had been completed. The thermophilic conditions indicates that the SO_4 levels had a much faster decrease for the faecal sample than for the seed and composite samples.

The recorded values of samples in both conditions in terms of NO_3 indicated an increase after AD was completed except seed during mesophilic conditions. Mesophilic conditions of all samples ranged from 2.44 to 25.72 NO_3 mg/l, which is slightly higher when maximums are compared to thermophilic conditions with a recorded value between 2.44 and 18.76 NO_3 mg/l. The levels of

NO_3 in both mesophilic and thermophilic conditions of seed and composite samples had a higher concentration of NO_3 during the digestion period than that observed in faecal samples. When observing faecal samples, a lower level of NO_3 was indicated during thermophilic conditions than those observed in mesophilic conditions. The mesophilic seed sample observed during NO_4 at T0 recorded value was 25.09 mg/l and decreased to 11.83 mg/l at the end (T3) of AD. Similar trends were documented in composite and faecal samples throughout mesophilic conditions which both increased after anaerobic digestion. The Mesophilic composite samples increased in NO_3 and recorded values from 7.06 NO_4 mg/l to 14.17 NO_4 mg/l. Mesophilic Faecal samples was observed during the AD period with values increasing from 2.44 and 8.95 NO_4 mg/l.
When observing thermophilic conditions. The NO_4 levels in thermophilic conditions showed higher levels of NO_3 in seed samples compared to faecal and composite samples. Faecal samples increased and indicated levels between 2.44 and 4.59 NO_4 mg/l in thermophilic conditions. Thermophilic seed samples were recorded to increase from 9.02 mg/l to 18.07 NO_4 mg/l. Composite samples showed similar trends than those observed in seed and faecal of thermophilic conditions, with an increase recorded from 11.83 mg/l to 18.76 NO_4 mg/l.

Ammonium levels in the mesophilic conditions were slightly lower for faecal samples when compared to thermophilic conditions, ranging from 66 to 156 NH_4 mg/l in mesophilic and 66 to 270 NH_4 mg/l in thermophilic conditions. Indicated in faecal samples of mesophilic conditions, an increase from 66 to 158 NH_4 mg/l between T0 and T3 could be seen, whereas an increase from 66 to 164 NH_4 mg/l occurred in thermophilic conditions. Seeding samples of both mesophilic and thermophilic conditions were higher in ammonium concentrations than those of composition and faecal samples, ranging from 1276 to 1156 NH_4 mg/l for mesophilic and 1614 to 1418 NH_4 mg/l for thermophilic conditions. Levels for composite samples in both mesophilic and thermophilic conditions showed opposite trends, with a decrease from 1012 to 999 NH_4 mg/l at mesophilic conditions and increased from 1130 to 1316 NH_4 mg/l at thermophilic conditions. Overall compared NH_4 in mesophilic and thermophilic conditions, thermophilic recorded higher ammonium levels than that of mesophilic in each sample time of the different substrates.

4.1.2 **Gas production and composition of mesophilic and thermophilic conditions**

Gas yields were noted each day for mesophilic and thermophilic conditions. For mesophilic conditions, a total of 35 days were noted and for thermophilic conditions, a total of 16 days were noted. Gas composite was determined at each sampling time (T1, T2 and T3). T0 had no gas production and gas composite as it was the starting time. The average of the accumulated biogas production is illustrated in this section as it all showed similar trends recorded during gas production and gas composite.

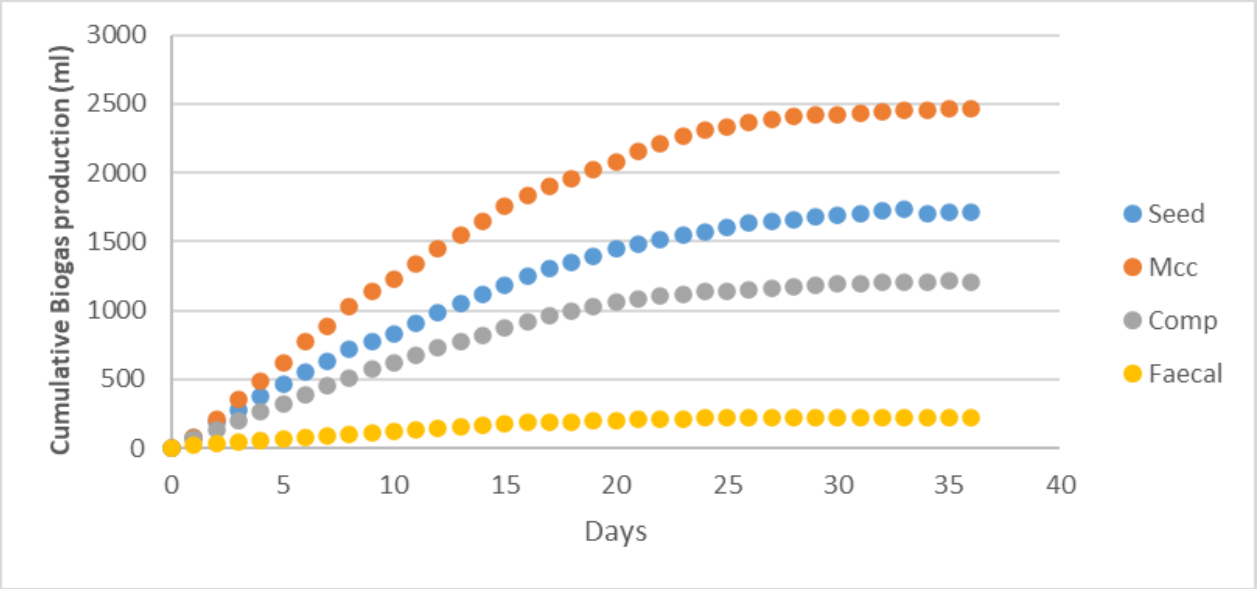

Figure 4-1: Cumulative biogas production during mesophilic conditions during anaerobic digestion. Faecal = yellow, Composite = Grey, microcrystalline cellulose = Orange, Seed = Blue. Cumulative gas production in a period of 35-36 days.

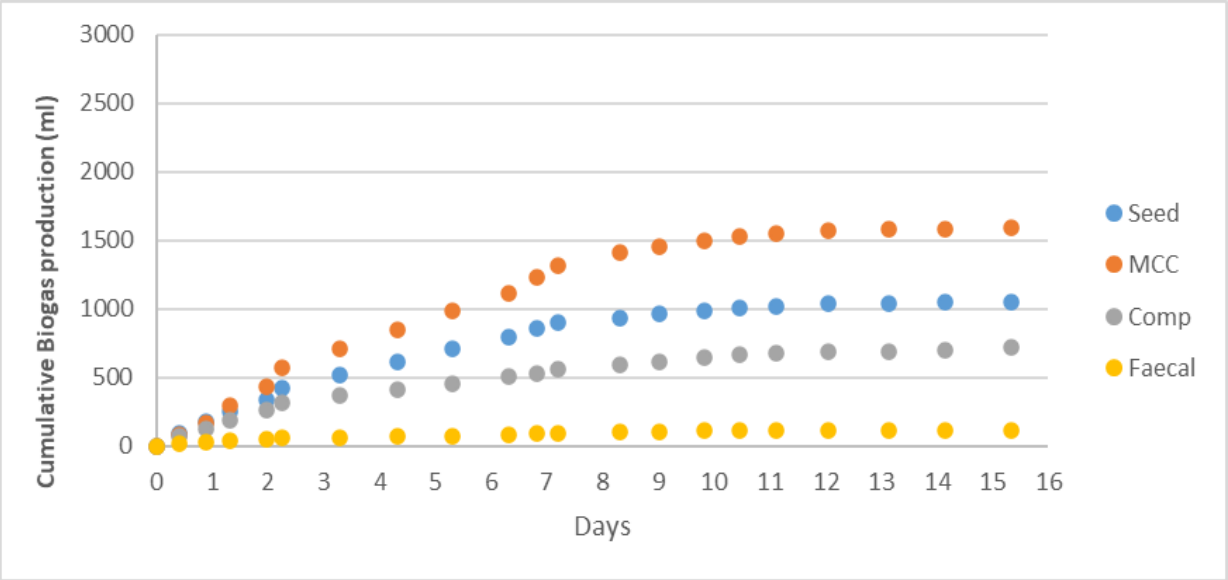

Figure 4-2 : Cumulative biogas production (ml) during thermophilic conditions during anaerobic digestion. Faecal = yellow, Composite = Grey, microcrystalline cellulose = Orange, Seed = Blue. Cumulative gas production in a period of 15-16 days.

An efficient start-up was observed in all conditions during mesophilic and thermophilic conditions with a steady increase in biogas production until a plateau was reached, illustrated in Figure 4-1 and Figure 4-2 Generally, the gas production in all mesophilic conditions showed stability at approximately 16 days, whereas stability in thermophilic conditions was obtained within 10-11 days. In all conditions (mesophilic and thermophilic), it was noted that faecal sludge produced

much less biogas compared to other substrates during both mesophilic and thermophilic digestion. Maximum gas production per day in mesophilic conditions was recorded at ~15 ml during the first 10 days and declined drastically after 14 days. Little to no gas production was recorded from 14 to 35 days for faecal sludge in mesophilic conditions, and in thermophilic conditions between 8 and 15 days, faecal sludge stopped producing gas. The total amount of biogas produced in these conditions are noted in Table 4-3. (Data accumulated Gas yields can be found in appendix B: Table 0-6: **Cumulative biogas yields of all substrates (Seed, MCC, Comp1-3 and Faecal) during mesophilic conditions** Table 0-6 and Table 0-7)

Table 4-3: Total amount of gas produced during AD of mesophilic and thermophilic conditions

Mesophilic	**Total cumulative gas yields**	**Thermophilic**	**Total cumulative gas yields**
MCC	2466 ml	**MCC**	1577 ml
Seed	1719 ml	**Seed**	1053 ml
Composite	1208 ml.	**Composite**	724 ml.
Faecal	220 ml	**Faecal**	120 ml

In Table 4 3, a comparison between the total gas yielded are shown, when considering all samples, MCC (seeding sludge reference sample) of mesophilic and thermophilic conditions produced the highest amount of gas during anaerobic digestion (1577 ml for thermophilic and 2466 ml for mesophilic). Followed by seed samples (1053 ml for thermophilic and 171 ml for Mesophilic) and then composite samples (724 ml for thermophilic conditions and 1208 ml for mesophilic conditions) and as previously mentioned faecal sludge produced the lowest biogas yield during AD (120 for thermophilic and 220 ml for mesophilic).

Table 4-4 and Table 4-5, the documented maximum CH_4 level was recorded in MCC followed by seed, composite and then faecal samples in mesophilic conditions and maximum CH_4 level was recorded in seed, followed by MCC, composite and faecal. In general, when observing both conditions, it was recorded that mesophilic conditions produced more CH_4 than in thermophilic conditions. (Only run1 illustrated, as run two and three can be seen in Appendix C:Table 0-8 and Table 0-9)

Table 4-4: Gas composite of AD during one run in mesophilic conditions

Sample ID	System P[mbar]	Pump time (s)/t(s)	CH_4	CH_4 max [%]	CO_2	CO_2 max [%]	O2	O2 min [%]	H_2S [ppm]	Balance [%]
F_T1	0.01	9	0.00	0.10	0.20	0.20	19.90	19.90	7.00	79.90
F_T2	0.03	12	0.80	0.80	0.10	0.10	19.20	19.20	8.00	79.90
F_T3	0.05	16	0.70	0.70	0.10	0.10	19.70	19.70	9.00	79.50
S_T1	0.04	15	5.50	5.60	3.10	3.10	17.40	17.30	4.00	74.00
S_T2	0.07	21	25.90	26.00	9.70	9.80	16.20	16.20	6.00	48.20
S_T3	0.1	24	17.50	17.60	7.10	7.20	19.70	19.80	6.00	55.60
C_T1	0.2	14	10.13	10.13	3.30	3.30	17.23	17.23	5.67	69.33
C_T2	0.3	15	8.37	8.43	0.50	0.50	16.30	16.30	6.67	74.83
C_T3	0.3	19	17.40	17.47	2.03	2.03	17.03	17.03	8.33	63.53
MCC_T1	0.05	17	15.40	15.40	3.90	3.90	16.50	16.50	5.00	64.20
MCC_T2	0.15	24	17.00	17.10	4.50	4.50	15.30	15.40	3.00	62.90
MCC_T3	0.26	18	17.20	17.20	4.70	4.70	15.40	15.40	1.00	62.70

Substrate: F = Faecal, S = Seed, C = Composite, MCC = Microcrystalline cellulose. Timeline: T1 = Day 10, T2 = Day 16, T3 = Day 36 Full table are available in appendix A Table Max=maximum

Table 4-5: Gas composite of AD during one run in thermophilic conditions

Sample ID	System P[mbar]	Pump time (s)/t(s)	CH_4	CH_4 max [%]	CO_2	CO_2 max [%]	O2	O2 min [%]	H_2S [ppm]	Balance [%]
F_T1	0.02	4	0.00	0.10	0.20	0.20	20.30	20.30	7.00	79.40
F_T2	0.04	15	0.40	0.40	0.10	0.10	20.10	20.10	5.00	79.40
F_T3	0.07	18	0.30	0.30	0.10	0.10	20.20	20.20	3.00	79.40
S_T1	0.06	21	10.10	10.10	2.60	4.30	14.40	14.40	7.00	71.20
S_T2	0.09	32	18.70	18.70	5.30	5.30	14.10	14.10	12.00	61.90
S_T3	0.17	54	21.90	21.90	4.30	2.60	12.10	12.00	16.00	63.50
C_T1	0.05	44	3.73	3.73	0.60	0.60	18.83	18.83	6.67	76.83
C_T2	0.15	38	13.77	13.77	1.00	1.00	18.43	18.43	4.00	66.80
C_T3	0.08	25	15.40	15.43	1.30	1.30	18.90	18.90	10.67	64.37
MCC_T1	0.08	29	11.40	11.60	7.10	7.20	14.30	14.20	4.00	62.40
MCC_T2	0.12	32	9.10	9.10	1.70	1.70	15.32	15.40	9.00	53.50
MCC_T3	0.15	42	27.40	27.40	7.10	7.10	14.30	14.30	7.00	40.60

Substrate: F = Faecal, S = Seed, C = Composite, MCC = Microcrystalline cellulose. Timeline: T1 = Day 10, T2 = Day 16, T3 = Day 36 Full table are available in appendix A Table Max=maximum

The gas composition in mesophilic and thermophilic conditions are presented in Table 4-4 and Table 4-5. The highest levels of CH_4 recorded in mesophilic conditions of seed samples were during sample time T2 with a value of 25.9%, the lowest levels were recorded in faecal T1 with a value of 0% in both conditions. The recorded values of composite and seed samples ranged from 10% to 17.4% (Composite) and 5.5% to 25.9% (Seed) with a slight decrease during T2 of composite sample. Faecal ranged increased from 0% to 0.8%; this substrate recorded lower levels of CH_4 in both mesophilic and thermophilic conditions when the reactors were compared. MCC samples in mesophilic conditions had the 2 largest gas production, which indicated that temperature may have influence these reactors.

The maximum CH_4 recorded in thermophilic conditions was MCC sample and was recorded during sample time T3 with a value of 27.4%. The recorded values of composite and seed samples ranged from 3.7% to 15.4% (Composite) and 10.0% to 21.9% (Seed) with a slight decrease during T2 of seed sample. Faecal ranged between 0% and 0.8%. Composite and faecal samples showed similar trends when decreases and increases were observed in each sample time; seed samples increased in methane yields after T1.

CO_2 for seed, composite and faecal had similar trends than those recorded for CH_4 increasing in AD, whereas the highest recorded value for the composite sample was 1.30 % at sample time T3; seed was 5.4 % at sample time T2, and faecal was recorded at sample time T1 with a value of 0.20%. The lowest value recorded for composite samples was at sampling time T1, though the lowest recorded value for seed was at sample time T1 with a value of 2.4% and for faecal at T3 with a value of 0.1%. When looking at the recorded O_2 values, the maximum O_2 was recorded in both conditions for faecal samples ranging between 19 and 20% stating that the substrate digestion was aerobic and indicated operational error as the reactor was built with resources available at site, but faecal can still be observed when looking at composite samples and the effects its haves on the substrate. The values recorded for seed and composite samples were between 14% and 18% but in each reactor stayed relatively the same; Run 2 and 3 (Appendix C) showed similar trends.

4.2 Molecular analysis

DNA of the different runs was pooled together to determine the microbial communities of each substrate. NGS was done for each sample time (T0, T1, T2 and T3) of each substrate during mesophilic and thermophilic conditions respectively. Different diversity indices were used to provide more information about species representation of communities at each sample time than just the general species richness. This study focused on the Shannon index and Chao1 estimator using MicrobiomeAnalysis and METAGENassist. These statistical parameters were used to describe community dynamics/structure.

4.2.1 Molecular analysis of samples throughout thermophilic and mesophilic conditions

In this study DNA was extracted and put through gel electrophoresis. The maximum DNA concentrations of each condition and substrate are depicted in Table 0-10 and Table 0-11 (see Appendix D) with their 260/280 and 230/260 absorbance ratios. Table 4-6 shows higher DNA concentrations and were recorded in mesophilic seed and composite samples compared to thermophilic. Faecal sample recorded higher DNA concentrations in Thermophilic conditions than Mesophilic conditions.

Table 4-6: Comparison of mesophilic and thermophilic DNA concentrations

	Mesophilic			Thermophilic		
Substrates	**Seed**	**Composite**	**Faecal**	**Seed**	**Composite**	**Faecal**
DNA concentration (ng/µl) Max**.**	54.315	17.123	7.295	41.614	12.058	9.674
DNA concentration (ng/µl) Min.	16.530	7.787	4.674	24.077	4.061	5.454

Min =Minimum, Max= Maximum

A total of 32 samples for thermophilic and 36 samples for mesophilic samples was used which includes triplicates samples, run one, two and three where pooled together and used for NGS. Each sample was pooled together which includes 3 faecal samples, 3 seed samples and 3 composite samples of each timeframe (T0-T3). Faecal sludge starts are the same for both mesophilic and thermophilic thus only triplicates of one was used, table 0-12 contains the full sample description used. The extracted DNA from both mesophilic and thermophilic samples were amplified by PCR assay using 16S-F/16R primer pairs. These primer pairs have been suggested as suitable for microbial community sequencing on the Illumina 16S Metagenomic Sequencing Library Preparation Guide. Amplification bands were obtained for all samples

including faecal, seed and composite throughout both conditions of AD and put through gel electrophoresis. The product was visualised through Bio-Rad's ultraviolet image system with bands present at 450bp (Figure 4-3 and Figure 4-4). All PCR products went through first and second PCR clean-ups and were sequenced using the Illumina sequencing platform.

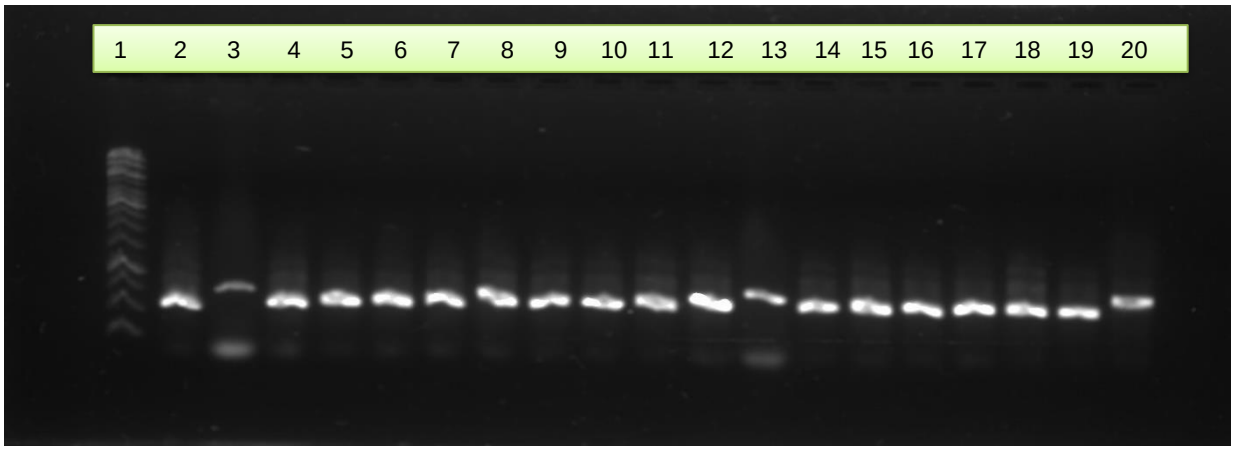

Figure 4-3: Gel electrophoresis image depicting mesophilic and thermophilic samples (faecal, seed, composite) and microbial PCR amplicons at 450bp lines of Run 1.

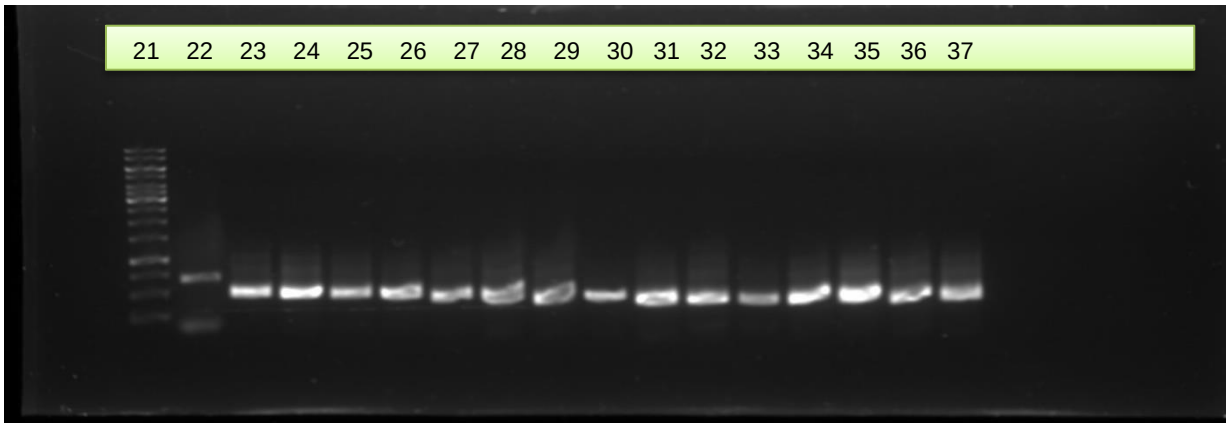

Figure 4-4: Gel electrophoresis image depicting the Mesophilic and thermophilic samples (faecal, seed, composite) and microbial PCR amplicons at 450bp lines of Run1.

PCR before indexing is depicted in Figure 4-3 and Figure 4-4. A 1Kb marker is present in Line 1. Figure 4-3, Line 1 to 17 contained mesophilic samples that were pooled together from Run 1-3. Lines 2 to 5 were faecal samples (T0-T3). Lines 6 to 9 contained seed samples (T0-T3) and Line 10 to 13 were composite (T0-T3). Lines 14 to 28 were thermophilic samples. Lines 14 to 17 depicted faecal samples (T0-T3). Seed samples were present in Lines 18 to 21 (T0-T3), and composite samples were depicted in Lines 22 to 25 (T0-T3). Lines 26 to 37 were samples that were put through PCR assay again (26=FM1; 27= FT2. 28=FT3; 29=ST1; 30=F0 CT1 31=CT2 32=CM1 33=CM2 34=CM 2 35= 36=ST1)

4.3 Diversity index analysis of microbial community

A total of 10,023,635 high-quality paired-end sequence reads were obtained from faecal, seed and composite samples during mesophilic and thermophilic AD. The average count per sample was recorded at 139,217 sequences, whereas the sequences counts were recorded to ranges from 29,968 (min) to 409,093 (max) reads per sample. The total numbers of OTU identified in samples were 1122 across the dataset and had 886 counts lower than 2 counts per OTU. Data were not rarefied nor scaled during analysis but were transformed using the RLE. Low count filters were used and removed a total of 535 low abundance features based on 20% prevalence in samples. Lower abundant filters were also used and removed 36 low variance features based on the inter-quantile ranges (IQR) of 10%. The number of remaining features after data filtering were 315.

4.3.1 Alpha diversity of microbial community associated with mesophilic and thermophilic conditions of the different substrates (faecal, seed and composite)

The determined microbial diversity within a sample was done using the alpha diversity matrices and included indices such as: Observed, Shannon, Simpson and Chao1, as shown in Table 0-11 (Appendix). a total of 5,585,861 high-quality paired-end sequence reads were obtained from faecal, seed and composite samples during mesophilic AD, and for thermophilic samples, a total count of 5,147,329 high-quality paired-end sequence reads was obtained. Throughout mesophilic conditions, the average count per sample was recorded at 143,227 sequences. The sequence counts ranged from 29,968 (min) to 409,059 (max) reads per sample. Thermophilic conditions were recorded at 131,982 average counts per sample and ranged from 37,079 (min) to 287,456 (max). Data were not rarefied nor scaled during analysis and were transformed using the RLE. Low count filters were used and removed a total of 392 low abundance features based on 20% prevalence in samples. Lower abundant filters were also used and removed 38 low variance features based on the IQR of 10%. In table 4-7 all indices of all samples contained a p value below 0.05 which indicates a significant difference in samples when comparing substrate with substrate except for Choa and Observed OTU which was slightly above 0.05 and indicates more similarities within seed material.

The maximum Observed OTU can be seen to be the highest in the F_Meso_T2 and the lowest in S_Meso_T0. As depicted in Figure 4-5 to Figure 4-8. The average number for observed OTUs was recorded between 74.33 ± 39.31 and 138.67 ± 8.15 for the mesophilic condition. In addition, thermophilic conditions varied between 59.00 ± 5.29 and 137.00 ± 6.08, which had a lower OTU value in composite samples compared to that observed in mesophilic conditions. The average number for mesophilic conditions demonstrated in the Shannon index (

Figure 4-6) was between 2.83 ± 0.38 and 3.35 ± 0.33, and for thermophilic conditions, varied between 2.64 ± 0.28 and 3.38 ± 0.23. The average number demonstrated in the Simpson index

(Figure 4-7) was recorded between 0.86 ± 0.07 and 0.93 ± 0.03 for mesophilic conditions, and for thermophilic conditions, between 0.86 ± 0.05 and 0.93 ± 0.03. The average number in Chao1 (Figure 4-8) was recorded between 78.00 ± 45.57 and 140.17 ± 9.54 for mesophilic conditions and between 62.33 ± 1.15 and 138.75 ± 3.07 for thermophilic conditions. The determined indices: Observed, Shannon, Simpson and Chao1 all displayed significant differences within a sample of both conditions and had a p-value of P<0.005. Details are depicted in Table 4-.

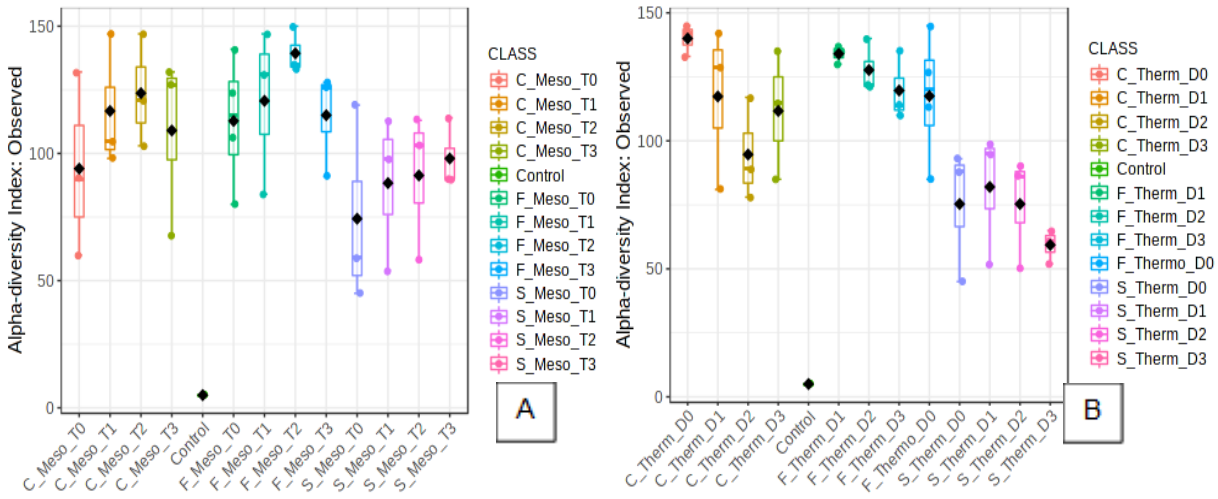

Figure 4-5 A and B: Alpha diversity analysis: Observed OTU of the faecal, seed, and composite content during mesophilic and thermophilic conditions based on the universal 16S rRNA gene at 95% identification. Data represent averages of samples (A = mesophilic and B = thermophilic conditions). Meso: p-value: 0.0047866; [ANOVA] F-value: 3.3488 and thermo: p-value: 2.6889e-06; [ANOVA] F-value: 8.6075

In Figure 4-5 A and B, faecal samples for observed OTUs were recorded between 115.00 ± 20.81 and 138.67 ± 8.15 for mesophilic conditions and between 116.50 ± 24.77 and 132.67 ± 5.86 for thermophilic conditions. Seed samples were between 74.33 ± 39.32 and 96.67 ±15.01 for mesophilic conditions and between 59.00 ± 5.29 and 75.00 ± 20.88 for thermophilic conditions. Composite samples were between 93.67 ± 37.22 and 121.67 ± 21.55 for mesophilic conditions and between 95.00 ± 19.98 and 118.00 ± 31.75 for thermophilic conditions. The Highest Observed diversity index in Mesophilic conditions was observed in F_Meso_T2 and the lowest was observed was in S_Meso_T0. Similarities between C_Meso_T1 and C_Meso_T2 shown similarities in diversity, and can also be observed between S_Meso_T1 and S_Meso_T2. In thermophilic conditions. The highest Observed diverisity index were recorded at C_Therm_T0 and F_Therm_T3 whereas the lowest were observed in S_Therm_T0. Similarities within a community were observed in S_Therm_T0, S_Therm_T1 and S_Therm_T2.

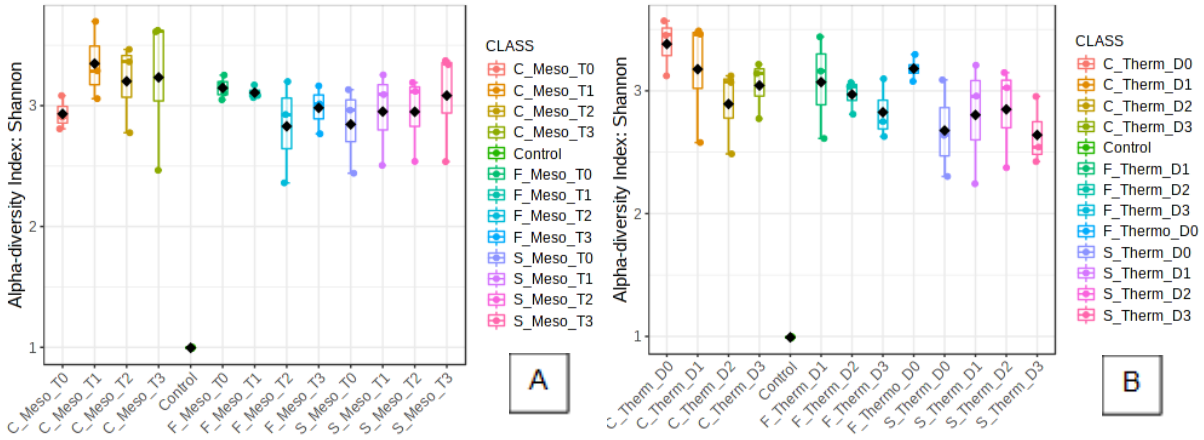

Figure 4-6: Alpha diversity analysis: Shannon index of samples and conditions based on the universal 16S rRNA gene at 95% identification. Data represents averages of samples (A = mesophilic and B = thermophilic conditions). Meso: p-value: 5.8412e-05; [ANOVA] F-value: 6.1053. Thermo: p-value: 1.9068e-05; [ANOVA] F-value: 6.9508

In

Figure 4-6, faecal samples using the Shannon index were recorded between 2.84 ± 0.38 for mesophilic conditions and between 2.83 ± 0.25 and 3.18 ± 0.09 for thermophilic conditions. Seed samples were between 2.83 ± 0.382 and 3.08 ± 0.49 for mesophilic conditions and between 2.64 ± 0.28 and 2.85 ± 0.42 for thermophilic conditions. Composite samples were between 2.94 ± 0.12 and 3.29 ± 0.22 for mesophilic conditions and between 2.89 ± 0.35 and 3.38 ± 0.23 for thermophilic conditions. All samples varied between 2.64 and 3.38. The highes Shannon divirsity value in mesophilic conditions was recorded in C_Meso_T1 and C_Meso_T3 and the lowest was recorded in in F_Meso_T2 and S_Meso_T0. Similarities in diversity was recorded in F_Meso_T2 and S_Meso_T0. In thermophilic and similarities also observed in S_meso_T1 and S_Meso_T2. The Highest Shannon diversity values in thermophilic conditions was recorded at C_Therm_T0 and Lowest was recorded at S_Thermo_T1. Similarities was observed in in S_Termo_T1 and S_Therm_ T2 and can also be observed that similarities in diversity occurred

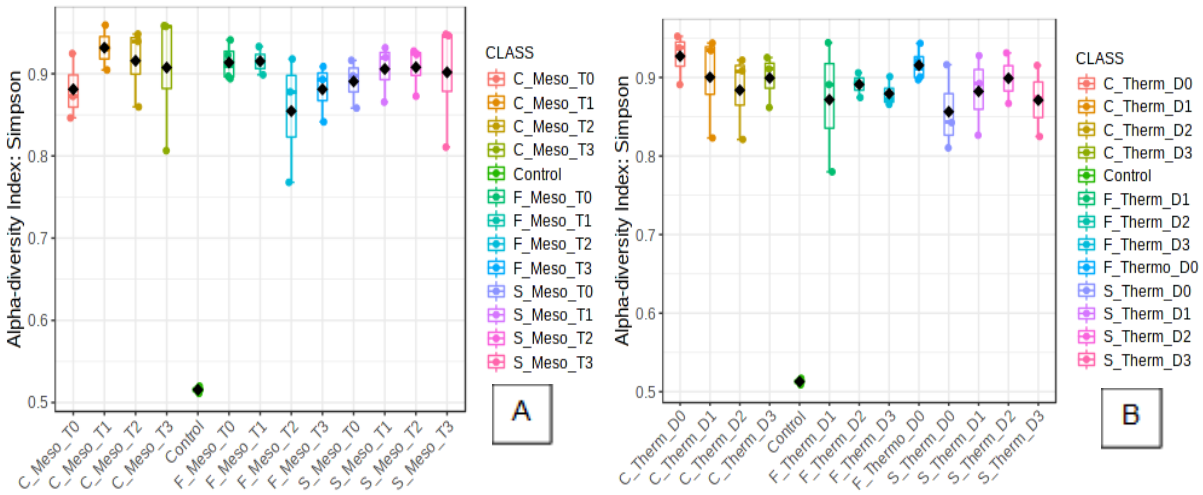

Figure 4-7: Alpha diversity analysis: Simpson index of samples during mesophilic and thermophilic conditions based on the universal 16S rRNA gene at 95% identification. Data represents averages of samples (A = mesophilic and B = thermophilic conditions). Meso: p-value: 3.1883e-07; [ANOVA] F-value: 10.708. Thermo: p-value: 1.4946e-07; [ANOVA] F-value: 11.539

In Figure 4-7, faecal samples in the Simpson index were recorded between 0.86 ± 0.01 for mesophilic conditions and between 0.87 ± 0.05 and 0.89 ± 0.02 for thermophilic conditions. Seed samples were between 0.89 ± 0.03 and 0.91 ± 0.04 for mesophilic conditions and between 0.86 ± 0.05 and 0.90 ± 0.03 for thermophilic conditions. Composite samples were between 0.88 ± 0.04 and 0.93 ± 0.08 for mesophilic conditions and between 0.88 ± 0.05 and 0.92 ± 0.03 for thermophilic conditions. The highest Value observed in the mesophilic Simpson index was in C_thermo_T0 and lowes was obesrved in F_therm_T0. Similarities was observed within a community at S_Meso_T1 and S_Meso_T2. Similarities in diversity in a community was also observed in F_Meso_T0 and F_Meso_T1. The highest Shannon index in thermophilic conditions was obeserved in F_Therm_T1. Similarities was observed in C_Therm_T2 and S_Therm_T1. Similarities was also observed in S_Therm_ T2 and C_Therm_T3

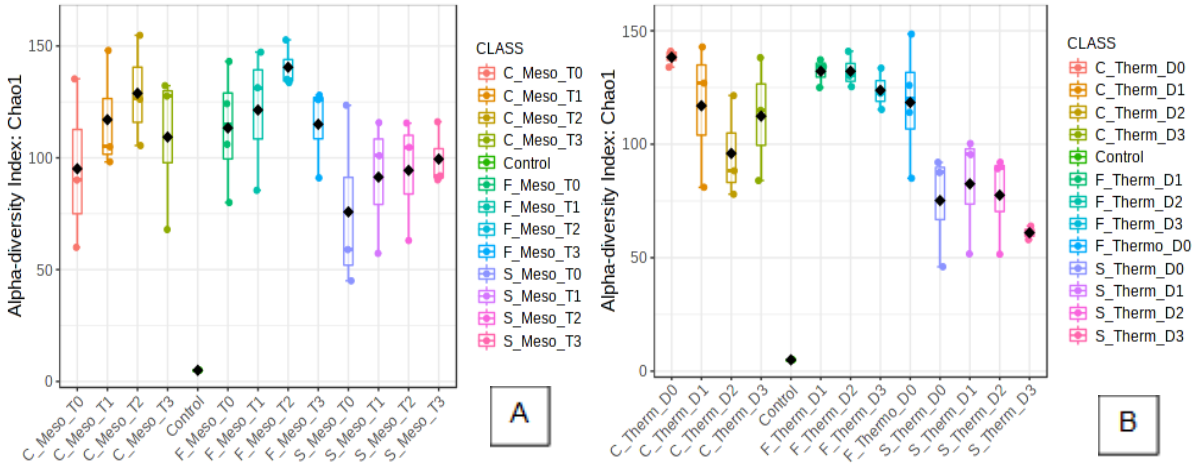

Figure 4-8: Alpha diversity analysis: Chao1 index of the Faecal, seed, and composite content during mesophilic and thermophilic conditions based on the universal 16S rRNA gene at 95% identification. Data represents averages of samples (A = Mesophilic and B = thermophilic conditions). Meso: p-value: 0.0058276; [ANOVA] F-value: 3.2429. Thermo: p-value: 2.7119e-06; [ANOVA] F-value: 8.5997

In Figure 4-8, faecal samples in the Chao1 index were recorded between 114.31 ± 27.15 for mesophilic conditions and between 117.16 ± 25.72 and 133.33 ± 6.35 for thermophilic conditions. Seed samples were between 78.00 ± 45.57 and 100.27 ± 18.17 for mesophilic conditions and between 62.33 ± 1.16 and 82.69 ± 25.72 for thermophilic conditions. Futhermore, composite samples were between 96.17 ± 41.22 and 124.06 ± 23.34 for mesophilic conditions and between 96.90 ± 22.10 and 138.75 ± 3.07 for thermophilic conditions. The highest value recorded at Chao1 index of Mesophilic condition C_Meso_T2 and and F_Meso_T2. The lowest values was recorded at S_Meso_T0. Similarities in diversity was recorded at S_MesoT1 and S_Meso_T2. The highest Shannon index value was recorded in thermophilic conditions at F_Thermo_T3 and the lowest values recored was at S_Therm_T0. Similarities in diversity was observed at S_Therm_T1 and S_Therm_T2. Similarities in diversity was also recored in F_Therm_T0 and F_Therm_T1.

4.3.2 Microbial community within mesophilic and thermophilic conditions of similar sample types

The faecal, seed and composite samples in both mesophilic and thermophilic conditions were analysed according to sample types. Bray-Curtis and Jaccard were applied using the default setting of beta metric, as mentioned in previous sections, and visualised through MicrobiomeAnalysis. As shown in Table 4-, faecal, seed and composite samples of mesophilic and thermophilic conditions had a p-value <0.001 in all matrixes. The observed OTUs of the substrates had a Pseudo F-value (separated clusters and close-knit) ranging from 2.47 to 5.83, although there was a significant difference demonstrated between the two clusters of each substrate type, the separation of clusters was not far apart between each of the same sample

types. The Shannon index demonstrated a Pseudo F-value ranging from 7.28 to 22.52, whereas faecal indicated the highest separation between the two clusters of the same samples. Furthermore, seed and composite samples showed the lowest significant differences in the substrates. The Simpson index was demonstrated with an F-value ranging between 14.25 to 82.67. The highest F-value at seed samples (82.67), followed by faecal (22.43) and then composite (14.25) with the lowest F-value. Chao1 demonstrated a Pseudo F-value ranging from 2.42 to 21.76. Although a significant difference was observed, faecal samples had the greatest distance between the two clusters with a value of 21.76, whereas seed and composite separation was not far apart, demonstrating low separation of clusters.

Table 4-8: Alpha diversity overview during both mesophilic and thermophilic conditions of the sample type

Alpha Matrices	***Faecal M vs Faecal T***		***Seed M vs Seed T***		***Composite M vs Composite T***	
	F-value	P-value	F-value	P-value	F-value	P-value
Observed OTU	**4.9623**	**0.0019837**	**2.4704**	**0.055719**	**5.8321**	**0.0011305**
Shannon Indice	**22.518**	**0.000000038836**	**7.2789**	**0.00031444**	**8.5531**	**0.00011669**
Simpson Indice	**22.429**	**0.000000040139**	**82.666**	**3.1496e-11**	**14.247**	**3.8329e-06**
Chao1 Indice	**21.76**	**0.0000033615**	**2.419**	**0.059892**	**5.4385**	**0.001657**

M=Mesophilic conditions; T=Thermophilic conditions

4.3.3 Beta diversity of microbial community associated with mesophilic and thermophilic conditions of the different substrates

Mesophilic and thermophilic diversity of the different substrates were calculated by exploiting the default beta diversity metrics of Jaccard and Bray-Curtis distance matric. These distances were

used to determine the variations of the microbial communities and structure of all samples. The PCoA was constructed using data obtained from Qiime2®. This displayed the similarities between different sample substrates and conditions. The two-dimensional PCoA plot was constructed and visualised in MicrobiomeAnalysis. Bray-Curtis and Jaccard distances indicated the community structure in mesophilic (F=7.7041) and thermophilic (F=6.4776) conditions demonstrating a significant difference between samples with a p-value lower than 0.001. When these conditions were observed, it was seen that faecal and seed samples formed distanced clusters (Figure 4-10). The composite sample showed similarities to that found in the faecal and seed samples, as expected. The Jaccard matric displayed diversity differences between samples and conditions (Figure 4-11) and showed similar trends seen with Bray-Curtis with a p-value lower than 0.001 during mesophilic (F = 5.174) and thermophilic (F = 4.4422) conditions. As with Bray-Curtis, similar trends were recorded with the Jaccard matric: Seed and faecal samples also demonstrated two distinct clusters, whereas the composite sample formed part of both these samples. Table 4- shows the overall summary of these two matrices.

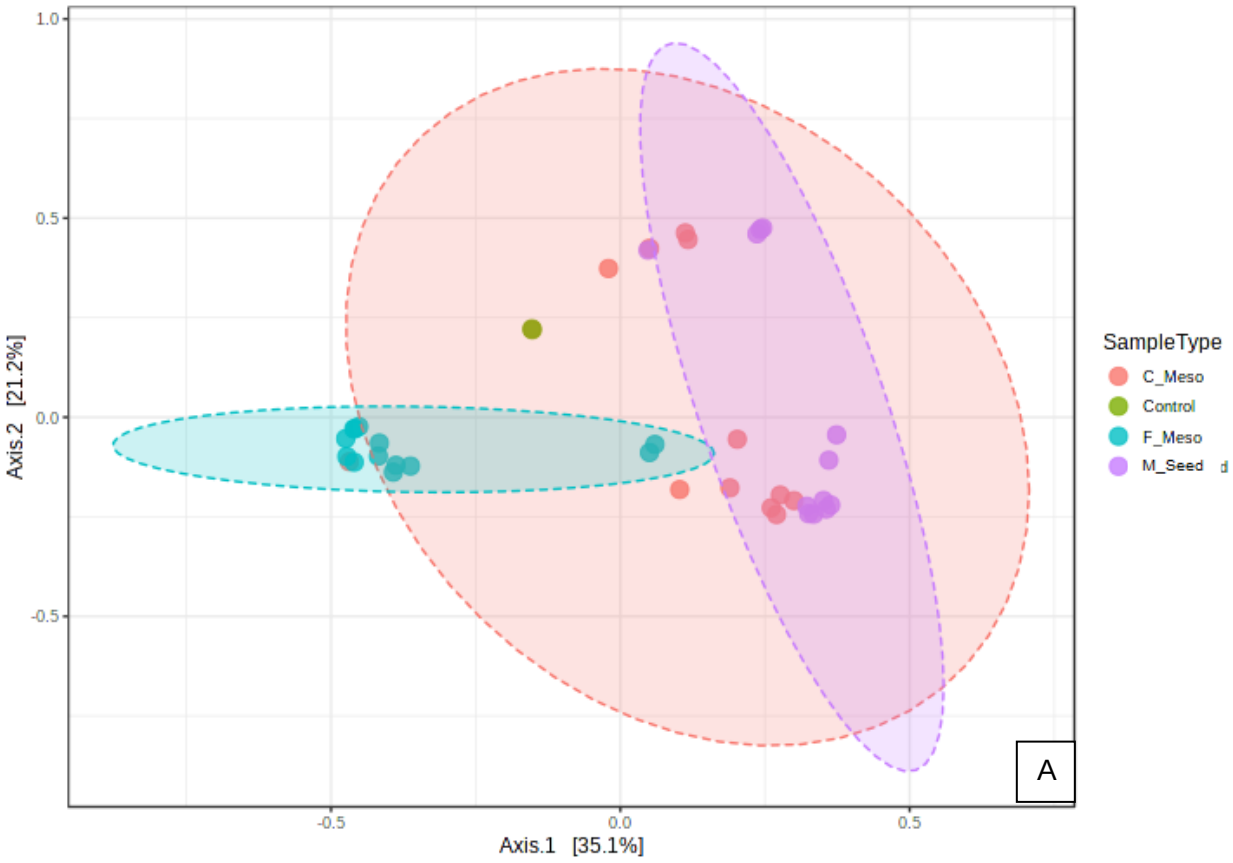

Figure 4-9 A: The PCoA based on Bray-Curtis distance of mesophilic. Percentage variation described with first two principal components. Data represent averages values of pooled data. F=Faecal (green), S=Seed (purple), C=Composite (red). Principal components (PC1 and PC2)

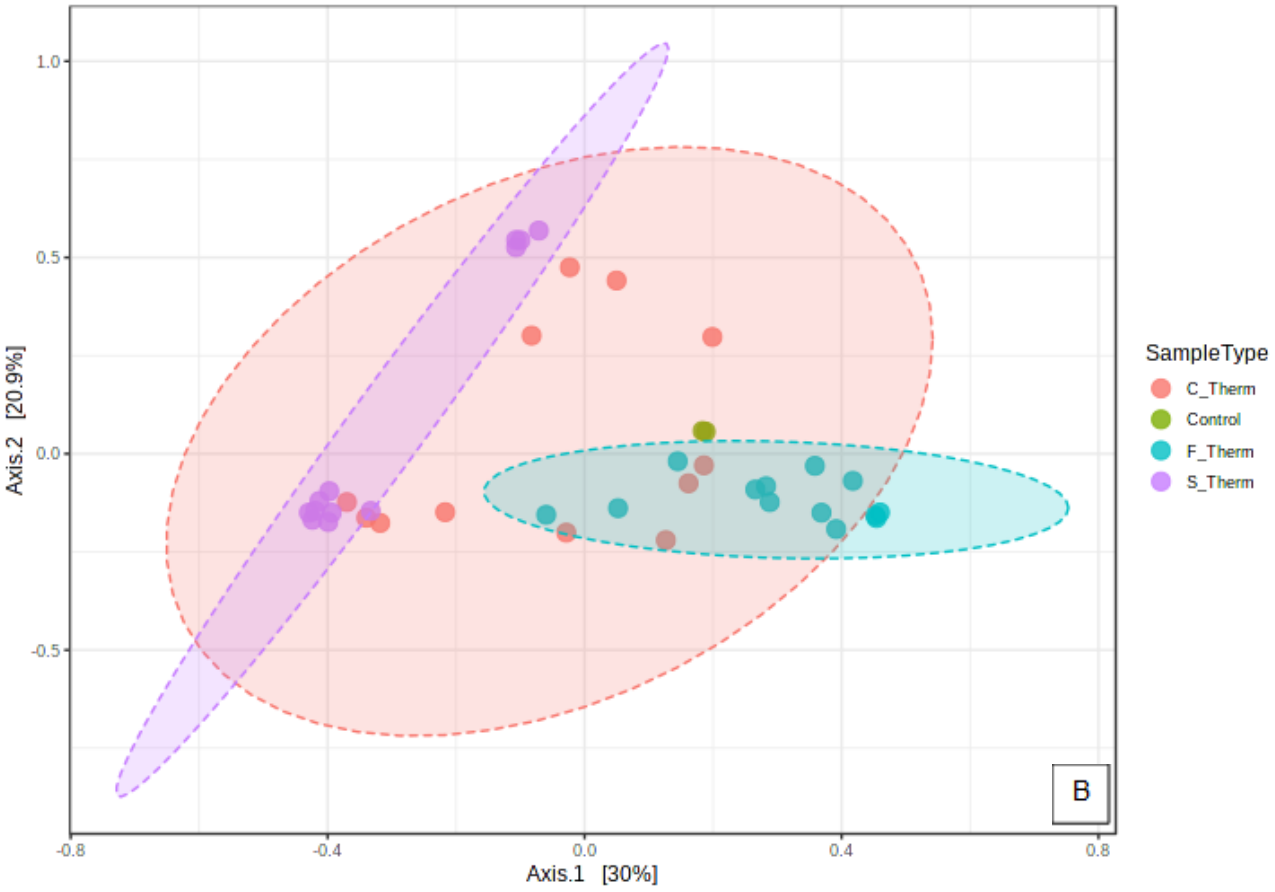

Figure 4-10 B: The PCoA based on Bray-Curtis distance of thermophilic (B). Percentage variation described with first two principal components. Data represent averages values of pooled data. F=Faecal (green), S=Seed (purple), C=Composite (red). Principal components (PC1 and PC2)

Demonstrated in the results of the microbiota composite and structure of different samples, a significant difference among faecal, seed and composite samples was observed within both conditions. Observing the obtained data of a similar sample within different conditions, it was seen that a significant difference in faecal, seed and composite samples was recorded. Although the distances between these two conditions indicated that they were separate, they were to not be far apart observed (Figure 4-11). The Bray-Curtis and Jaccard distances of faecal, seed and composite samples indicated close distances. Faecal was recorded for Bray-Curtis at F: 4.3276 and Jaccard at F: 3.1258. Seed was recorded for Bray-Curtis at F: 5.1609 and Jaccard at F: 3.939. Furthermore, composite was recorded for Bray-Curtis at F: 3.9916 and Jaccard at F: 2.9343. These samples and their conditions were recorded with a p-value lower than 0.001. Table 4- shows an overview of beta diversity.

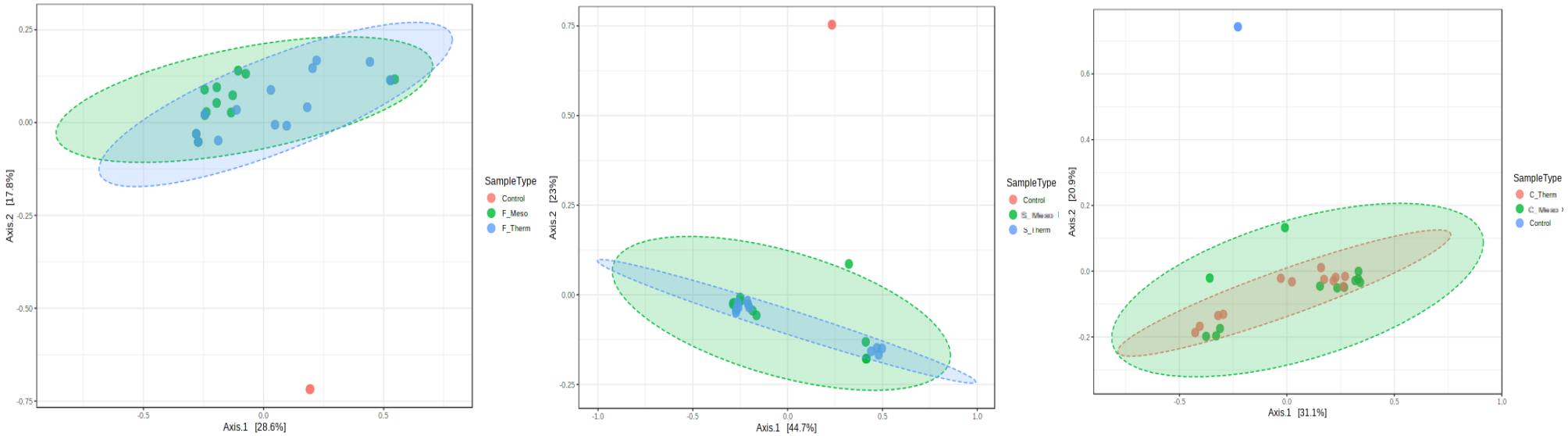

Figure 4-11: Beta diversity of the same sample types within mesophilic and thermophilic conditions. Bray-Curtis illustration of faecal, seed and composite samples. F = Faecal, S = Seed, C = Composite; Meso = Mesophilic, Therm = Thermophilic.

Table 4-9: Overview of beta diversity matrix of mesophilic and thermophilic conditions in all samples

Distance method	***Thermophilic F-value; P-value***		***Mesophilic F-value; P-value***		***FM vs FT F-value; P-value***		***SM vs ST F-value; P-value***		***CM vs CT F-value; P-value***	
Bray-Curtis index	F:6.4776	P < 0.001	F: 7.7041	P < 0.001	F: 4.3276	P < 0.001	F: 5.1609	P < 0.001	F: 3.9916	P < 0.001
Jaccard	F: 4.4422	p < 0.001	F: 5.1741;	p < 0.001	F-: 3.1258	p < 0.001	F: 3.939	p < 0.001	F: 2.9343	p < 0.001

F = Faecal, S = Seed, C = Composite; M = Mesophilic, T = Thermophilic

4.4 Microbial composite and abundance associated with mesophilic and thermophilic conditions of the different samples

The Kingdom that was most dominant in all samples was bacteria ranging between 87 to 99 %, whereas archaea was recorded between 1 to 12 %. The lowest level found in bacteria was recorded in seed mesophilic T2 with a value of 87%, and the highest recorded was in faecal T0 with a percentage of 99% in both mesophilic and thermophilic conditions.

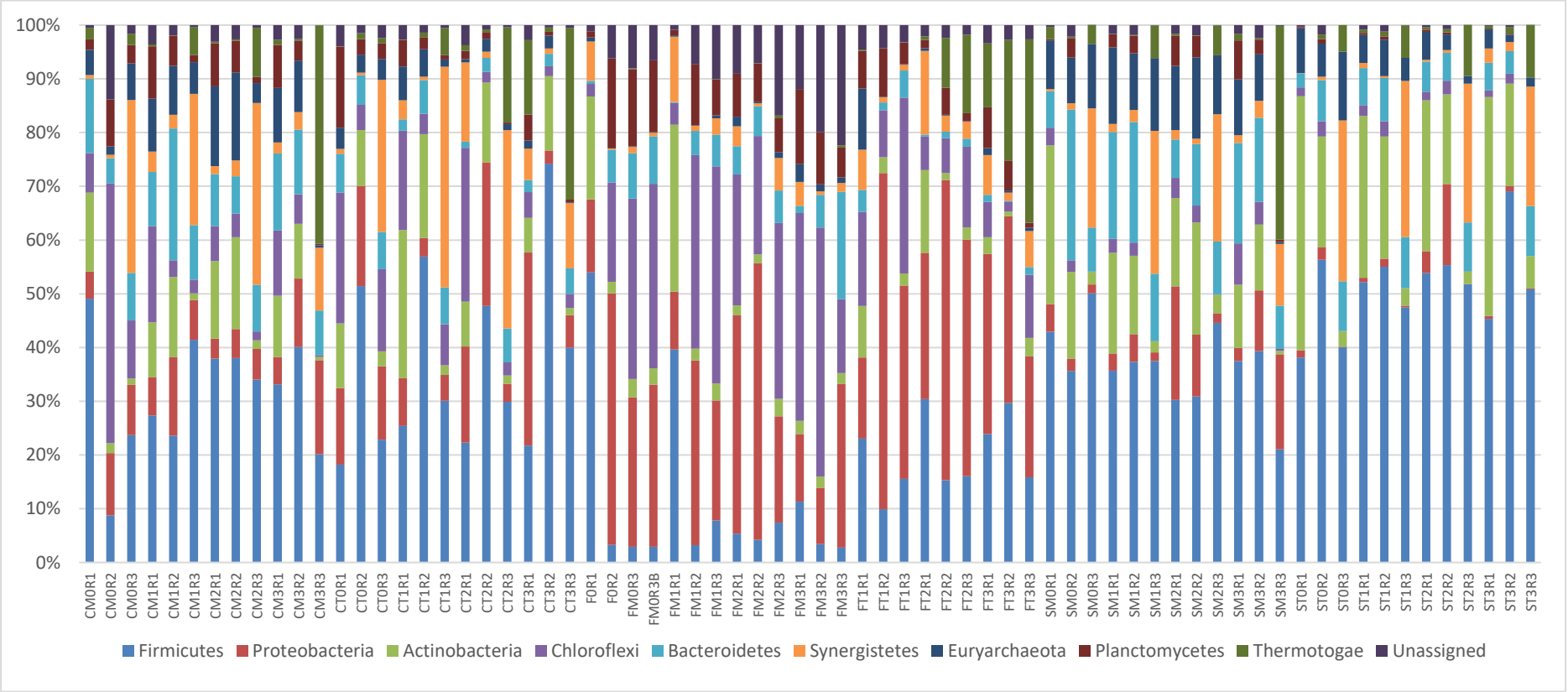

Figure 4-12: Three complete runs of each sample during mesophilic and thermophilic conditions: C = Composite, F = Faecal, S = Seed, and reactor conditions: M = Mesophilic, T = Thermophilic

Illustrated in Figure 4-12 are the three different runs for seed, faecal and composite samples. However, due to the objective of evaluating mesophilic and thermophilic condition in a biogas plant, results have been illustrated as an average, as the main objective was to evaluate a small -scale biogas plant during a full year term.

4.5 The microbial dynamic at phylum level

Exploring the microbial abundance associated with thermophilic and mesophilic conditions and includes faecal, seed and composite content. High throughput Illumina paired-end sequencing was carried out. The recorded results of the taxonomic abundance of all sequenced OTUs at phylum level, which is based on the relative abundance, were visualised in Excel (2016) and are depicted in Figure 4-12 to Figure 4-17. Bacteria accounted for 98.1%, and archaea accounted for 1.9% of the total sequences observed in NGS. A total of 11 phyla were obtained and compared throughout mesophilic and thermophilic conditions.

4.5.1 Faecal sludge samples

The 10 most abundant species documented in faecal sludge samples before mesophilic AD were Proteobacteria (28%), then Chloroflexi (27%), followed by Planctomycetes (13%), Bacteroidetes (7%) and Unassigned (7%). The remaining microbial phyla remained relatively low and included Saccharibacteria (4%), Actinobacteria (3%), Firmicutes (3%), Synergistetes (1%), Armatimonadetes (0.1%) and Thermotogae (0.1%). Proteobacteria recorded the highest abundance during T2 (40%). Chloroflexi decreased during T1 (24%) and T2 (22%) and then increased at T3 (28%). Firmicutes was observed in higher abundance at T1 (16%) than in T0 (3%) and then decreased towards the end of digestion to 4%. Actinobacteria only showed abundance at T2 (11%), which also decreased towards the end. Phyla after mesophilic AD were recorded as follows: Chloroflexi (28%), Unassigned (18%), Proteobacteria (18%), Bacteroidetes (10%), Planctomycetes (7%), Firmicutes (4%), Armatimonadetes (4%), Saccharibacteria (2%) Actinobacteria (2%) and Synergistetes (1%). (Figure 4-13)

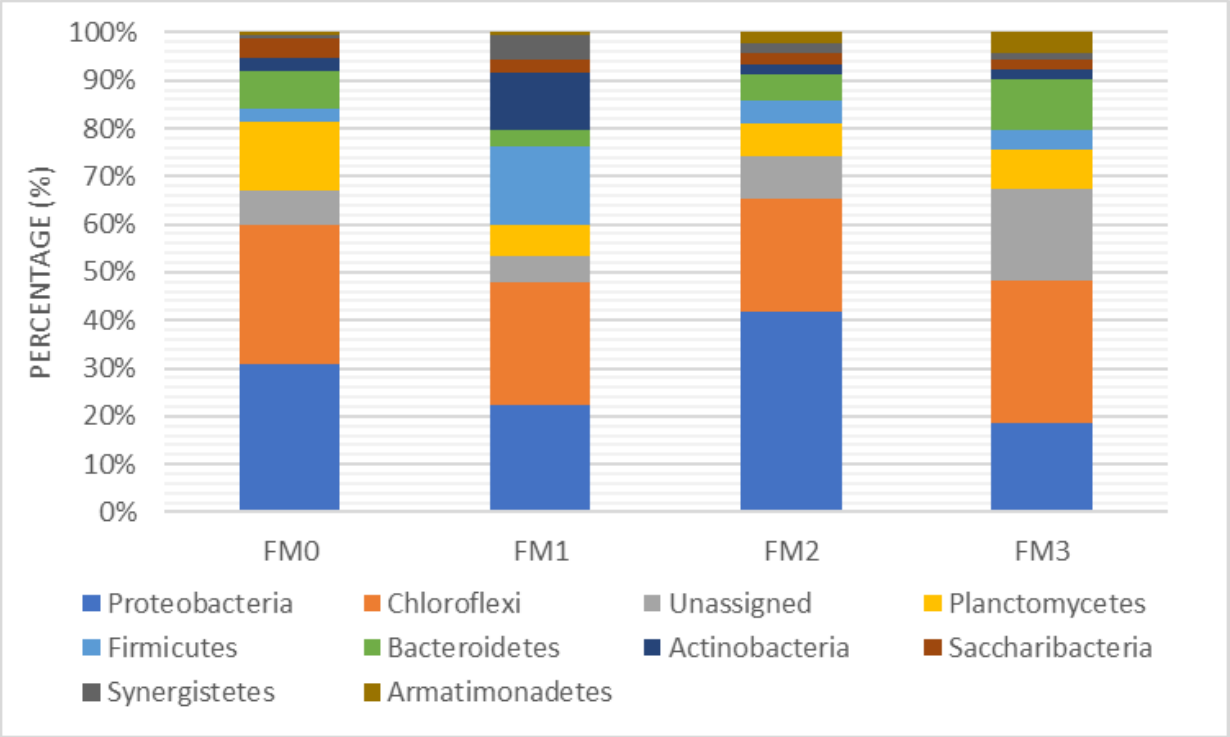

Figure 4-13: Phylum-level classification of faecal community during mesophilic AD. M = Mesophilic, 0 = Day 0, 1 = Day 10, 2 = Day 16 and 3 = Day 36

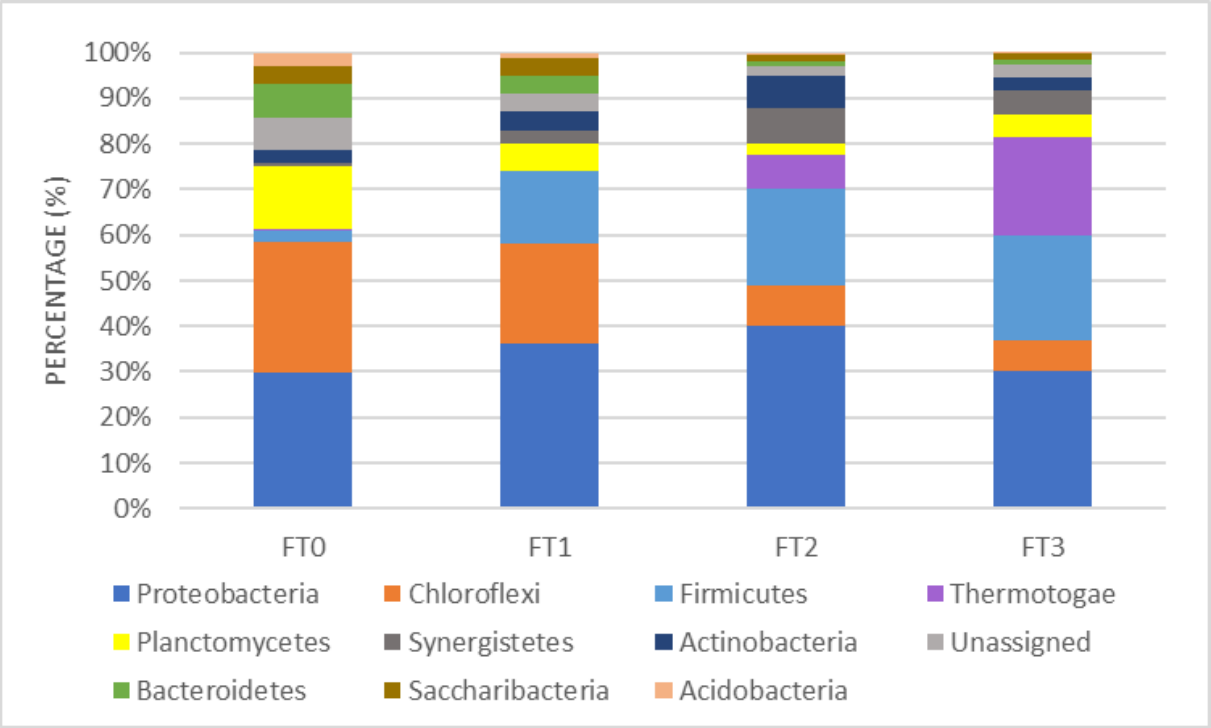

Figure 4-14: Phylum-level classification of faecal community during thermophilic AD. T = Thermophilic, 0 = Day 0, 1 = Day 1, 2 = Day 10 and 3 = Day 16

The 10 most abundant species documented in faecal sludge samples before thermophilic AD were Proteobacteria (28%), then Chloroflexi (26%), followed by Planctomycetes (13%),

Bacteroidetes (7%) and Unassigned (7%) see Figure 4-14. The remaining microbial phyla were relatively low and include Saccharibacteria (4%), Actinobacteria (3%), Firmicutes (3%), Synergistetes (1%), Armatimonadetes (0.1%) and Thermotogae (0.1%). As with mesophilic faecal samples, the highest recording abundance of Proteobacteria was at T3 (40%). An increase in Firmicutes was observed throughout the digestion period, whereas Planctomycetes decreased. Phyla Synergistetes and Actinobacteria became more dominant during T1 and T2 but remained at low levels. Thermotogae began to appear in higher abundance at T2 (7%) and T3 (21%) and were recorded at their highest at T3. Abundant of the phyla after AD was Proteobacteria (29%), Firmicutes (22%), Thermotogae (21%), Chloroflexi (7%), followed by Synergistetes (5%), Planctomycetes (5%), Actinobacteria (2%) Unassigned (3%), Actinobacteria (2%), Saccharibacteria (1%), Bacteroidetes (1%) and Acidobacteria under (1%). Documented in thermophilic AD, the phyla Thermotogae and Firmicutes increased a substantial amount, whereas Chloroflexi decreased by 20% after AD.

4.5.2 Seed sludge samples

A total of 48 phyla were obtained in mesophilic and thermophilic conditions of seed samples. Figure 4-15 and Figure 4-16 depict the 10 most abundant species documented in seed samples before mesophilic and thermophilic AD. The 10 most abundant species in mesophilic conditions were recorded as Firmicutes (39%), Bacteroidetes (20%), Actinobacteria (14%), Euryarchaeota (9%) Synergistetes (6%), Proteobacteria (2%), Actribacteria (2%), Planctomycetes (2%), Chloroflexi (2%) and Thermotogae (1%). During AD, Firmicutes remained dominated in all timelines. Bacteroidetes remained relatively the same in T0 (20%) and T1 (18%) and decreased during T2 (9%). Phyla Euryarchaeota Synergistetes and Atribacteria all increased by 1-2% during T1 and T2 but decreased at T3. Documented phyla after mesophilic AD were recorded as follows: Firmicutes (34%), Bacteroidetes (14%), Proteobacteria (10%), Actinobacteria (9%), Thermotogae (8%), Euryarchaeota (7%), followed by Chloroflexi (4%), Synergistetes (4%), Actribacteria (3%), Planctomycetes (3%), and Unassigned (2%). Increases were recorded in phyla Proteobacteria, Thermotogae, Actribacteria, Chloroflexi, Planctomycetes and Unassigned. Decreases were documented in the phyla Bacteroidetes and Actinobacteria, decreasing by 6% and 5%, respectively.

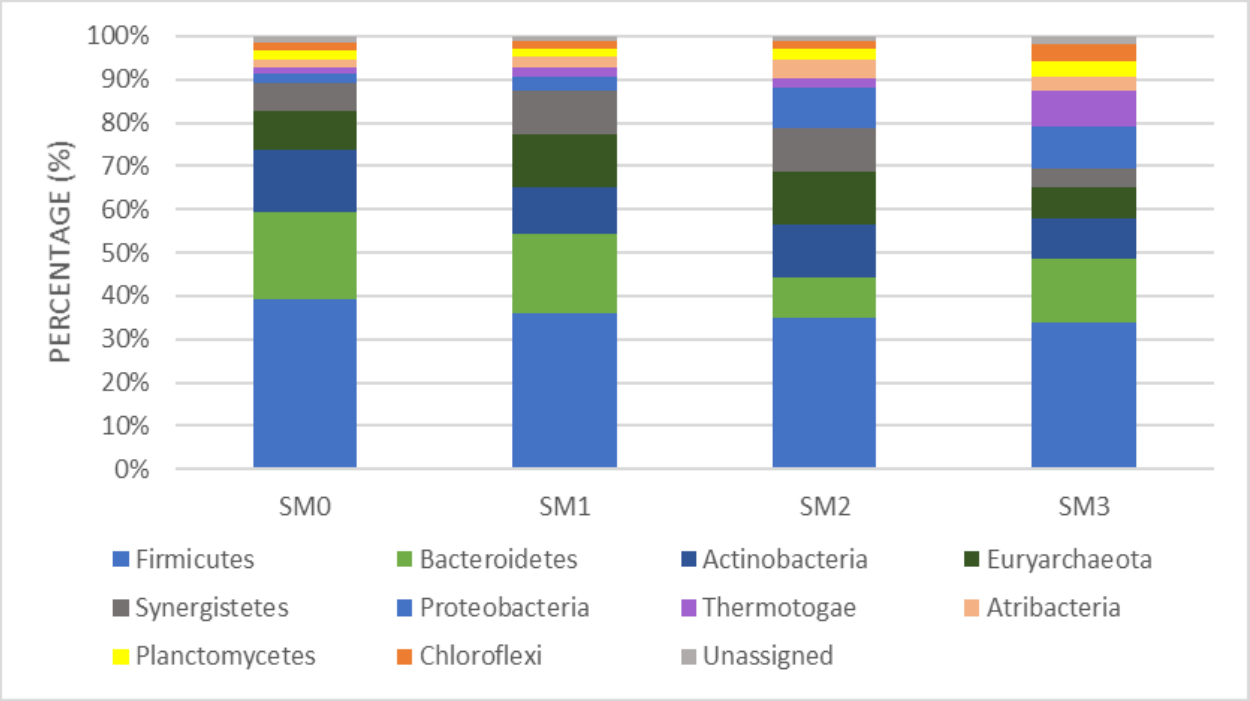

Figure 4-15: Phylum-level classification of seed community during mesophilic AD. M = Mesophilic, 0 = Day 0, 1 = Day 10, 2 = Day 16 and 3 = Day 36

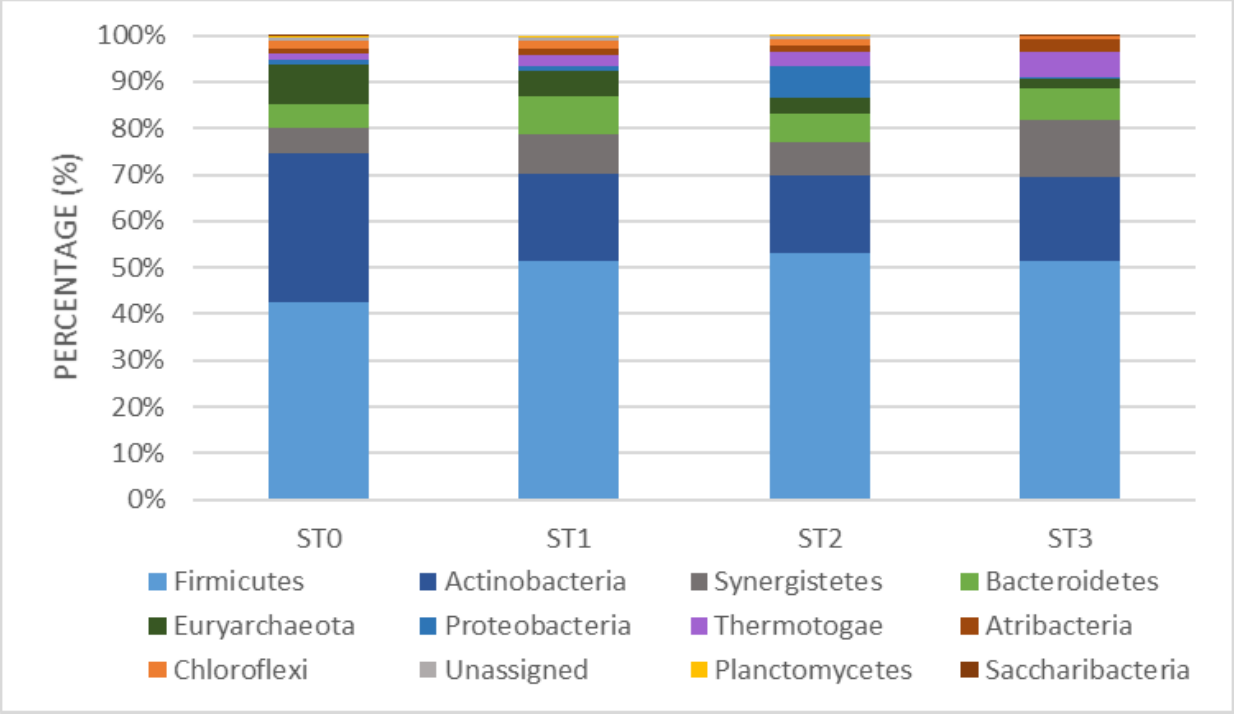

Figure 4-16: Phylum-level classification of seed community during thermophilic AD. T = Thermophilic, 0 = Day 0, 1 = Day 1, 2 = Day 10 and 3 = Day 16

The 10 most abundant phyla documented in thermophilic conditions of seed samples before AD were Firmicutes (42%), Actinobacteria (32%), Euryarchaeota (8%), Synergistetes (5%),

Bacteroidetes (5%), followed by Chloroflexi (2%), Proteobacteria (1%), Thermotogae (1%), Unassigned 1% and Atribacteria 1% As with mesophilic conditions, Firmicutes remained the dominant phyla throughout the digestion period ranging from 42%-53% but were slightly more abundant in thermophilic samples than mesophilic samples. Actinobacteria and Euryarchaeota decreased during AD. Actinobacteria decreased by approximately 15%, while Euryarchaeota only decreased by 6%. Phyla Synergistetes were more dominant during T1 (9%) and T3 (12%). Bacteroidetes increased during T1 (8%), with slight decreases seen at T2 and T3 but were more in abundance compared to the beginning of AD. Thermotogae increased by approximately 1% during each timeline. Proteobacteria increased until T2 (7%) and then decreased below 1% at T3. After Thermophilic AD, the abundant phyla were Firmicutes (52%), Actinobacteria (18%), Synergistetes 12%, followed by Bacteroidetes (7%), Thermotogae (5%), Atribacteria (3%) and Euryarchaeota (2%). The remaining phyla were relatively low, ranging from 1% to 0.1%. and includes phyla included Chloroflexi (1%), Proteobacteria (0.4%) and Unassigned (0.1%). When comparing mesophilic and thermophilic conditions, phyla Firmicutes, Synergistetes and Thermotogae were more dominant in thermophilic conditions, while mesophilic conditions were more dominant in phyla Chloroflexi, Planctomycetes and Bacteroidetes. Proteobacteria retained the same abundance reads in both conditions, with a slightly higher abundance observed in mesophilic conditions.

4.5.3 Composite sludge samples

A total of 48 phyla were obtained in mesophilic and thermophilic conditions of composite samples. Figure 4-17 and Figure 4-18 depict the 11 most abundant phyla documented in composite samples before mesophilic and thermophilic AD. The 11 most abundant species in mesophilic conditions before AD were Chloroflexi (34%), Firmicutes (15%), Proteobacteria (10%), Unassigned (9%), followed by Bacteroidetes (6%), Synergistetes (6%), Planctomycetes (6%), Actinobacteria (3%) and Euryarchaeota (3%). However, the phyla Thermotogae (1%) and Atribacteria (1%) were less dominant amongst the other recorded phyla. The phyla Firmicutes increased by 19%, whereas Chloroflexi decreased by 28%. Bacteroidetes increased in abundance in T1 (13%) and T3(12%), while a decrease was seen in T2 (13–8%). Synergistetes and Euryarchaeota increased from T0 to T2 and decreased at the end seen in T3. Proteobacteria recorded higher levels at T0 (10%) of AD and decreased towards T2(4%). After a sudden increase was observed in T3 (11%), the same Chloroflexi decreased drastically during AD (34-6%) indicating that there was possible competition. A clear community difference was recorded during mesophilic conditions when faecal and seed are compared to composite sample. Faecal contributed most of the Chloroflexi phyla at the beginning, and seed contributed to most of the Firmicutes and Proteobacteria phyla. After 10 days, a community shift was observed similar to that recorded in seed samples. After AD, the dominant phyla were recorded as Firmicutes (34%),

Bacteroides (12%), Proteobacteria (11%), Actinobacteria (9%), followed by Euryarchaeota (8%) and Chloroflexi (6%). The least abundant phyla were recorded as Synergistetes (4%) Planctomycetes (4%), Thermotogae (4%), Atribacteria (4%) and then Unassigned.

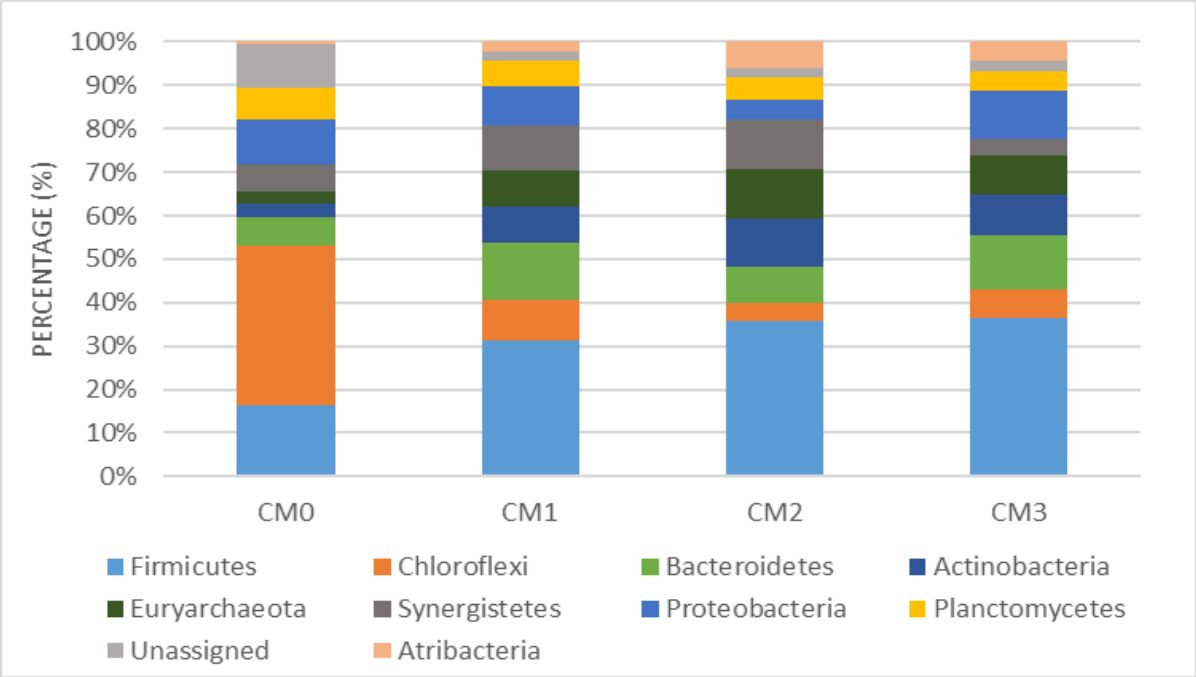

Figure 4-17: Phylum-level classification of community composite samples during mesophilic AD. M = Mesophilic, 0 = Day 0, 1 = Day 10, 2 = Day 16 and 3 = Day 36

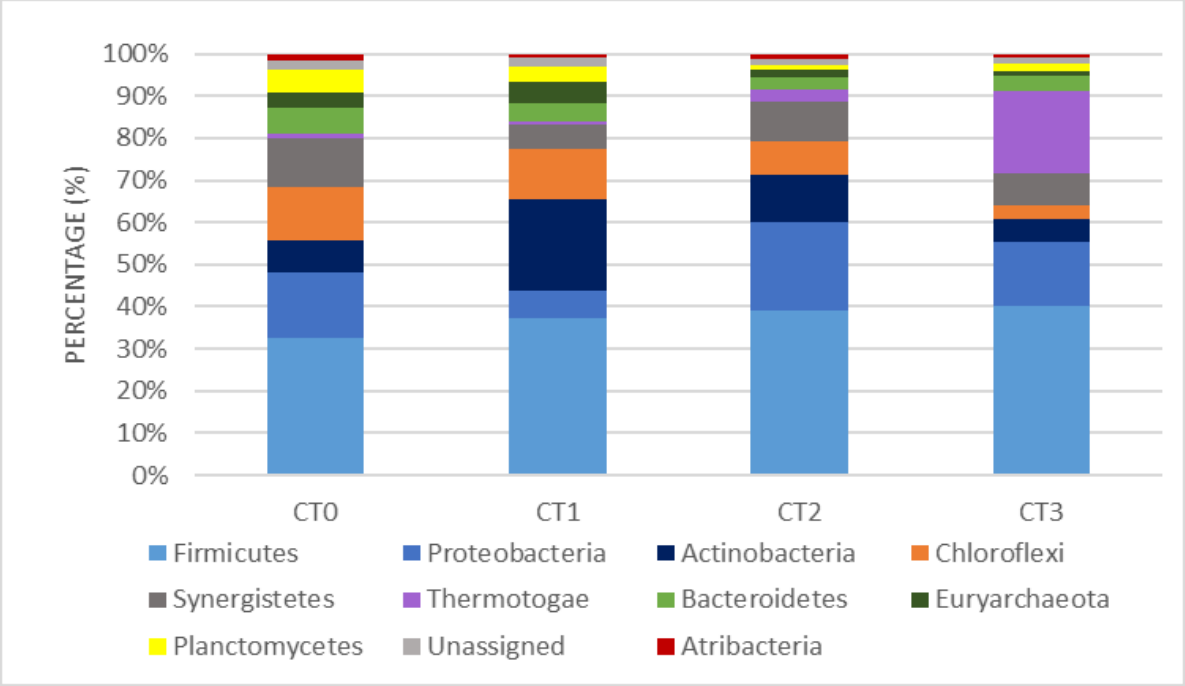

Figure 4-18: Phylum-level classification of community composite samples during thermophilic AD. T = Thermophilic, 0 = Day 0, 1 = Day 1, 2 = Day 10 and 3 = Day 16

The 11 most abundant phyla in thermophilic conditions of Composite samples before AD were Firmicutes (31%), Proteobacteria (15%), Chloroflexi (12%), Synergistetes (11%), Actinobacteria (7%), followed by Bacteroides (6%), Planctomycetes (5%) and Euryarchaeota (4%). The least abundant phyla were Unassigned (2%), Atribacteria (1%) and then Thermotogae (0.9%). Firmicutes were the most abundant phyla during each timeline of thermophilic conditions. This phylum remained at the same level throughout the AD process with increases of 2-5% between timelines. Synergistetes, Bacteroidetes Euryarchaeota and Planctomycetes all decreased towards the end of AD, with slight changes observed in Synergistetes (an increase of 2%) in T1 to T2. Proteobacteria decreased from T0 (15%) to T1 (6%), increased in T2 (21%) and then decreased toward the end of AD to 15%.

Thermotogae increased slowly during T2 (1-3%) and drastically increased towards the end of T3 (19%). Thermophilic phyla: after AD, the phyla recorded were Firmicutes (40%), Thermotogae (19%), Proteobacteria (15%), Synergistetes (7%), Actinobacteria (5%), Bacteroidetes (3%) and Chloroflexi (3%). The least abundant phyla were Planctomycetes (2%), Unassigned (1%), Euryarchaeota (1%), followed by Atribacteria (1%). When comparing mesophilic and thermophilic conditions, phyla Firmicutes and Actinobacteria were more dominant in thermophilic conditions than in mesophilic conditions. In contrast, mesophilic conditions were more dominant in the phyla Bacteroidetes and Euryarchaeota Compared to the abundant reads found in thermophilic conditions. Firmicutes Proteobacteria, Actinobacteria, Synergistetes and Thermotogae showed more abundance than observed in mesophilic conditions, while Bacteroides, Euryarchaeota and Chloroflexi were more dominant in mesophilic conditions.

4.6 The microbial dynamics at Order level

Microbial diversity, according to order, was documented. A total of 148 orders were identified. Visual representation was done in Excel 2016. Only the 15 most abundant species are represented in this section.

4.6.1 Faecal sludge samples at Order level

The microbiota composite associated with mesophilic and thermophilic conditions of faecal samples at order level was assessed for the microbial dynamics (Figure 4-19 and Figure 4-20). The 15 most abundant orders observed in mesophilic faecal samples before AD occurred was as follows: Caldilineales (17%), Unassigned (16%), Clostridiales (10%), Planctomycetales (10%), Pseudomonadales (5%), followed by Xanthomonadales (4%), Anaerolineales (3%), Rhizobiales (3%), Uncultured Bacterium (3%), Burkholderiales (2%) Synergistales (2%), Micrococcales (2%), *Ambiguous_taxa* (1%) and Corynebacteriales (1%). The least abundant species recorded ranged from 0.9%- 0.0002% and included orders such as Bacteroidales, Lactobacillales and Thermotogales. Unassigned remained dominant during AD, while organisms of the order Caldilineales, Planctomycetales, Planctomycetales and Micrococcales decreased and orders Pseudomonadales, Anaerolineales and Bacteroidales increased during AD. The order Clostridiales increased during T1 (12%) and drastically decreased towards T2(2%) and T3(2%). Pseudomonadales were recorded as the most abundant in T2 (30%) of all the orders during that timeline, while other orders such as Corynebacteriales, Ambiguous_taxa, Micrococcales and others mentioned above remained low in abundance with slight changes. The order levels after AD were recorded as follow: Unassigned (25%), Anaerolineales (21%), Bacteroidales (8%), Planctomycetales (7%), Caldilineales (6%), Clostridiales (3%) followed by Burkholderiales (2%), Synergistales (2%), and Methanosarcinales (1%). Other order-level microbiota was less than 1% and ranged from 0.36–0.0006% and included orders such as Bacillales, Burkholderiales, Pseudomonadales and Petrotogales.

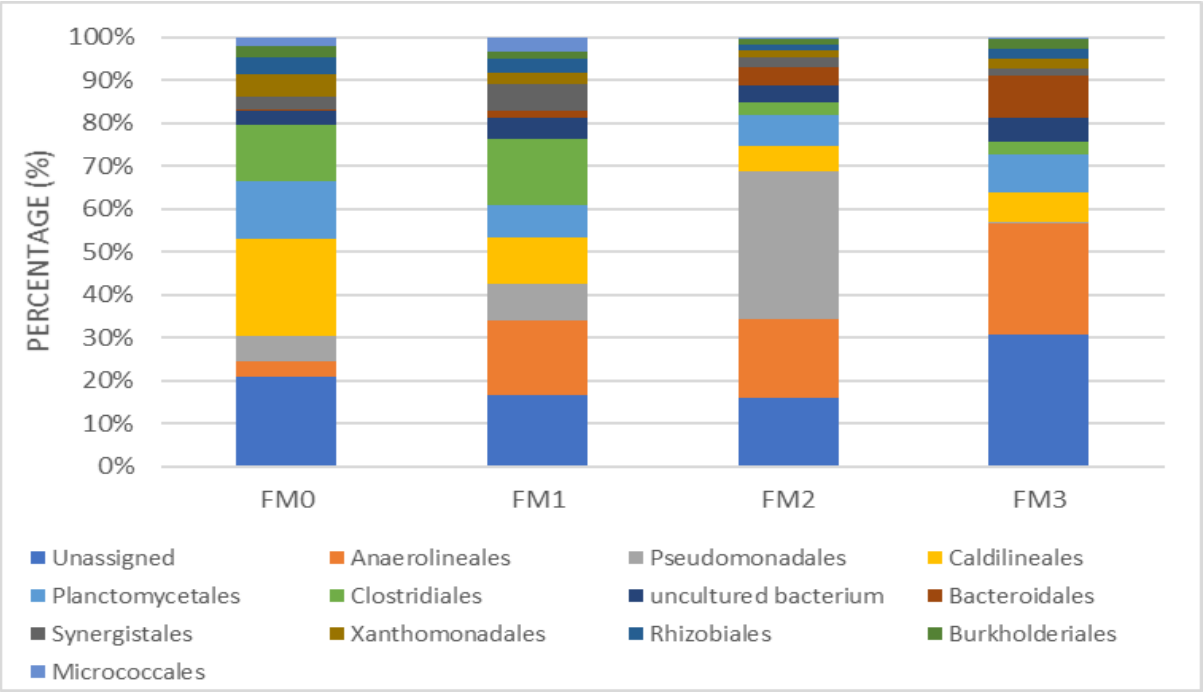

Figure 4-19: Order-level classification of faecal community during mesophilic AD. M = Mesophilic, 0 = Day 0, 1 = Day 10, 2 = Day 16 and 3 = Day 36

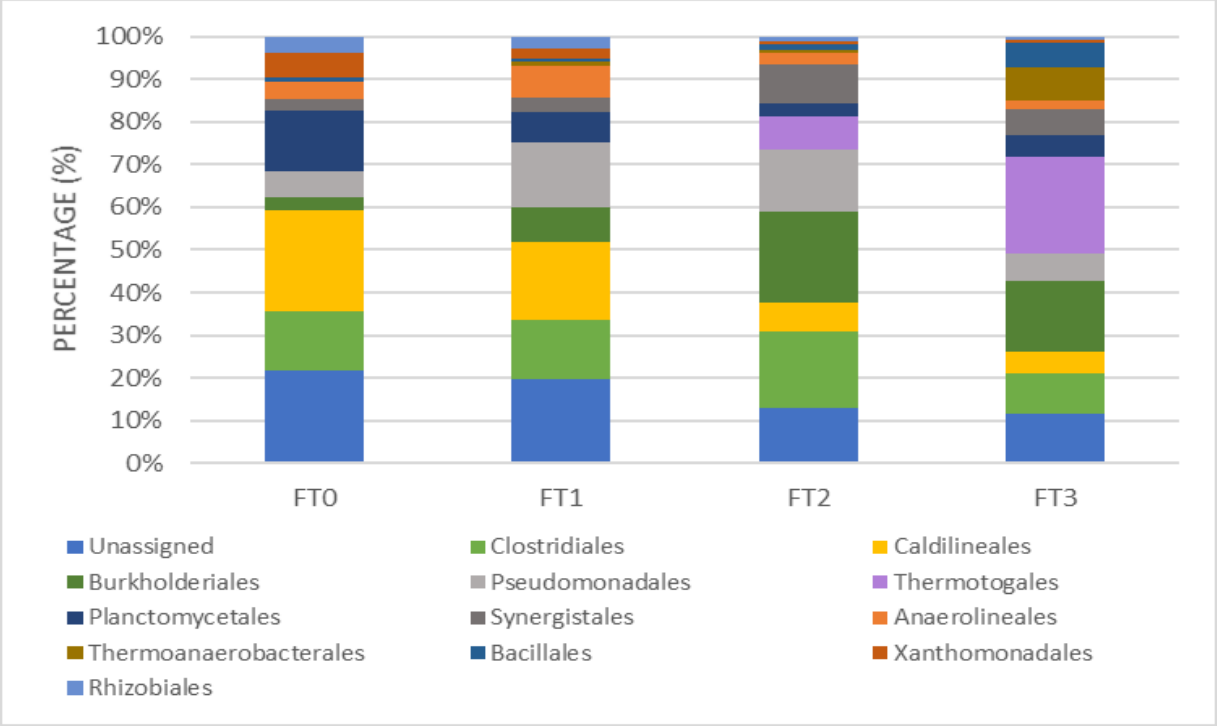

Figure 4-20: Order-level classification of faecal community during thermophilic AD. T = Thermophilic, 0 = Day 0, 1 = Day 1, 2 = Day 10 and 3 = Day 16

The 15 most abundant orders observed in thermophilic faecal samples before AD occurred was as follows: Caldilineales (17%), Unassigned (16%), Clostridiales (10%), Planctomycetales (10%),

Pseudomonadales (5%), followed by Xanthomonadales (4%), Anaerolineales (3%), Rhizobiales (3%), Uncultured Bacterium (3%), Burkholderiales (2%), Synergistales (2%), Micrococcales (2%), *Ambiguous_taxa* (1%) and Corynebacteriales (1%). The least abundant species recorded ranged from 0.9%- 0.0002% and included orders such as Bacteroidales Lactobacillales and Thermotogales. During AD many community shifts occur. The orders Caldilineales, Planctomycetales, Xanthomonadales and Rhizobiales all decreased during AD, while orders Pseudomonadales, Thermotogales and Burkholderiales increased. Thermotogales increased substantially from T2 (6%) to T3 (20%) and became the dominant order during that timeline. Firmicutes increased until T2 (10-14%) and decreased at T3 (8%) of the digestion process; the rest of the orders remained either at the same abundance reads or a small increase/decrease of 1-2% occurred. The order levels recorded after AD were as follows: Thermotogales (20%), Burkholderiales (15%), Unassigned (10%), Clostridiales (8%), Thermoanaerobacterales (7%), Pseudomonadales (6%), Bacillales (5%), Synergistales (5%) followed by Planctomycetales (5%), Caldilineales (5%) and Anaerolineales (2%). The least abundant were documented as Rhizobiales (0.7%) and Xanthomonadales (0.5%). When comparing mesophilic and thermophilic conditions the orders Clostridiales, Caldilineales, Burkholderiales, Pseudomonadales and Thermotogales were dominant in thermophilic conditions. Orders Pseudomonadales, Anaerolineales, Caldilineales Planctomycetales and Clostridiales were recorded as dominant during mesophilic conditions.

4.6.2 Seed sludge samples

The microbiota composite associated with mesophilic and thermophilic conditions of Seed samples at order level were assessed (Figure 4-21 and Figure 4-22). The 15 most abundant orders observed in mesophilic seed samples before AD were as follows: Clostridiales (22%), Bacteroidales (18%), Corynebacteriales (11%), Methanosarcinales (9%), Bacillales (9%), Unassigned (7%), followed by Synergistales (6%), MBA03 (4%), Planctomycetales (2%), Unknown Order (2%) and Micrococcales (2%). The least abundant orders were identified as: Petrotogales (1%), Thermoanaerobacterales (1%), Lactobacillales (0.4%) and Pseudomonadales (0.2%). Clostridiales remained the dominant group throughout AD and remained at the same level of abundance. Bacteroidales were more at the beginning of AD at T1(18%) and T2 (17) and then decreased towards the end of digestion at T3 (12%). Methanosarcinales increased in AD (9-12%), except at T3 (7%) where a decrease was seen. The same was observed in Synergistales, which increased (6-10%) and then decreased at T3. Pseudomonadales increased and then decreased at the end of AD, which showed the same trend as Synergistales and Methanosarcinales. The other orders remained underrepresented with slight changes. The order levels after AD were recorded as follows: Clostridiales (22%), Bacteroidales (12%), Unassigned (12%), Corynebacteriales (8%), Petrotogales (8%), Methanosarcinales (7%), followed by MBA03 (5%),

Pseudomonadales (5%), Synergistales (4%), and Planctomycetales (3%). The least abundant orders recorded were Thermoanaerobacterales (0.9%), Micrococcales (0.8%), Bacillales (0.7%) and Lactobacillales (0.3%).

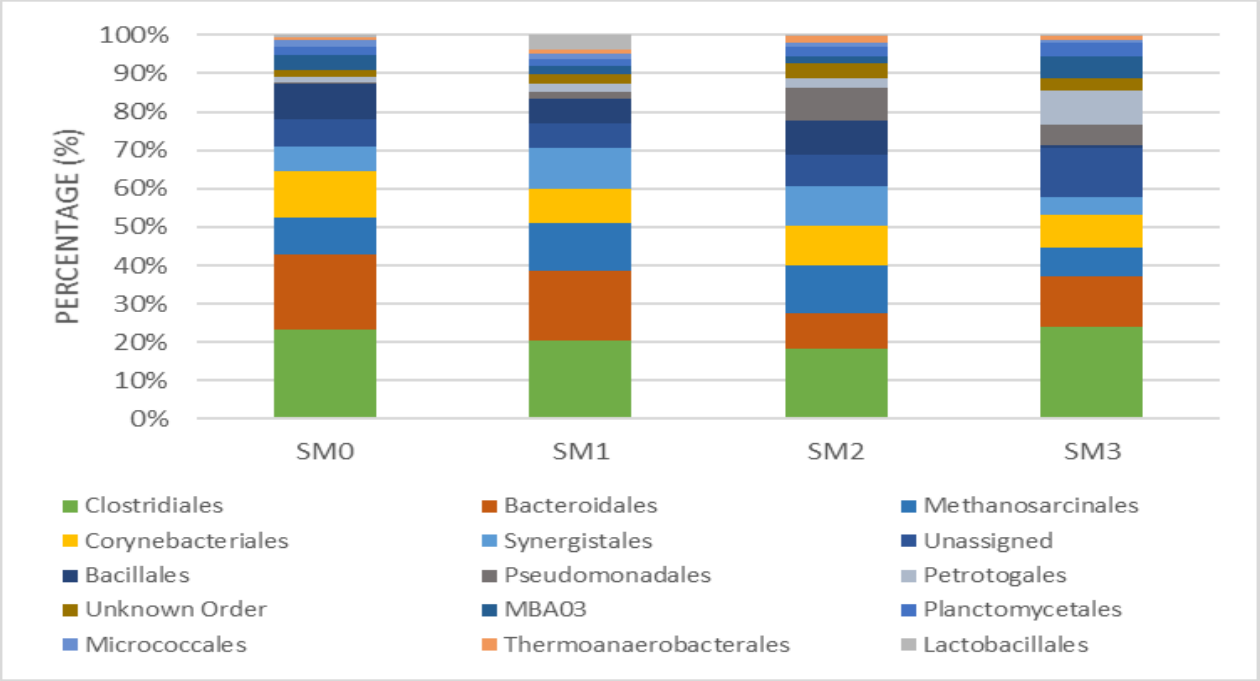

Figure 4-21: Order-level classification of seed community during mesophilic AD. M= Mesophilic, 0 = Day 0, 1 = Day 10, 2 = Day 16 and 3 = Day 36

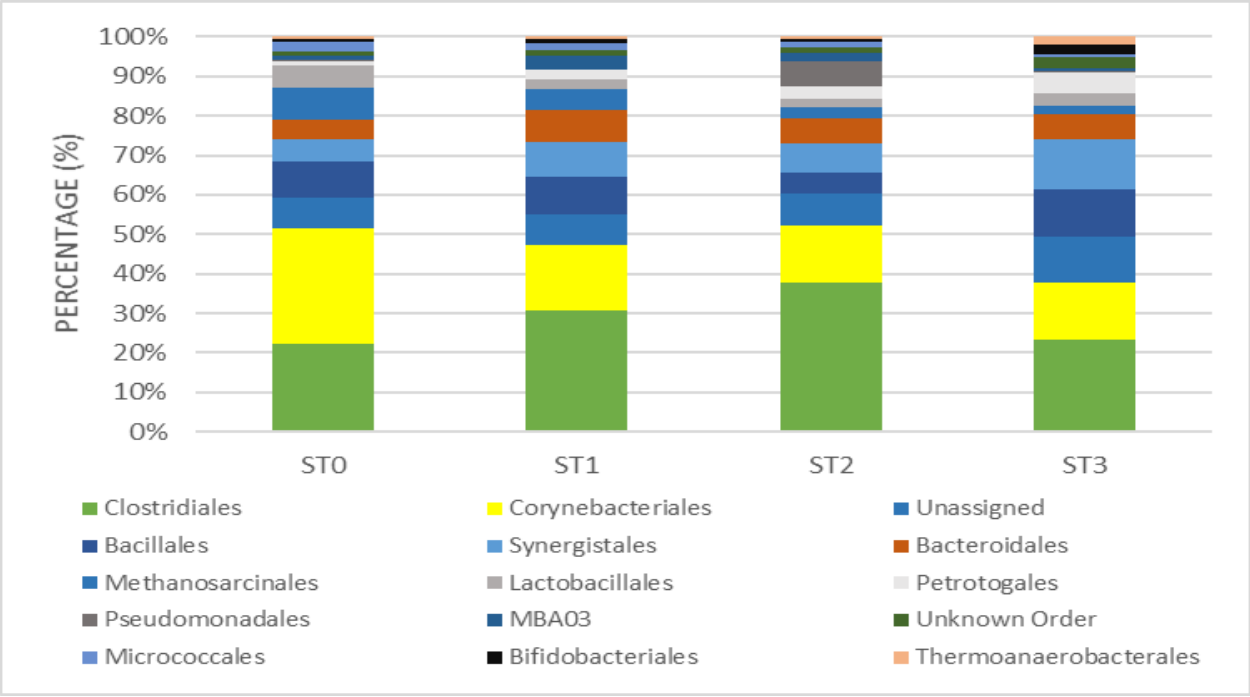

Figure 4-22: Order-level classification of seed community during Thermophilic AD. T = Thermophilic, 0 = Day 0, 1 = Day 1, 2 = Day 10 and 3 = Day 16

The 15 most abundant orders observed in mesophilic seed samples before AD were as follows: Corynebacteriales (28%), Clostridiales (21%), Bacillales (9%), Methanosarcinales (8%), Unassigned (7%), Synergistales (6%), followed by Bacteroidales (5%), Lactobacillales (5%), Micrococcales (2%) and Petrotogales (1%). The least abundant order levels were recorded as Unknown Order (1%), Bifidobacteriales (1%), Thermoanaerobacterales (1%), MBA03 0.9% and Pseudomonadales 0.3%. Clostridiales were observed to increase during AD from T0 (21%) to T2 (36%) and then decreased at the end of T3 (23%); they were mostly dominant throughout the AD process. Corynebacteriales became less dominant during AD (28-14%). Synergistales were more dominant in T3(12%) than Methanosarcinales at T3(2), but with Synergistales, a decrease was seen from T0(8%) to T3(2%). Bacillales, Bacteroidales and Unassigned remained at the same abundance throughout AD, with small increases observed in T3. Petrotogales increased with approximately 1-2% during AD between each timeline. Other orders mentioned above showed very small changes or stayed the same during AD. The order levels recorded after AD were as follows: Clostridiales (23%), Corynebacteriales (14%), Synergistales (12%), Bacillales (12%), Unassigned (11%), followed by Bacteroidales (7%), Petrotogales (5%), while Lactobacillales, Unknown Order and Bifidobacteriales were at 3%. Methanosarcinales and Thermoanaerobacterales were recorded at 2%. The least abundant orders were recorded as Micrococcales (1%), MBA03 (0.8%) and Pseudomonadales (0.04%). When comparing mesophilic and thermophilic seed samples, it was observed that thermophilic conditions were dominant in Clostridiales, Corynebacteriales, Unassigned, Bacillales, Synergistales Bacteroidales and Methanosarcinales, while mesophilic conditions were dominant in Bacteroidales, Methanosarcinales, Corynebacteriales, Synergistales, Unassigned and Bacillales. Many of these groups were dominant at both conditions, but abundance was different. The levels of abundance were depicted from more abundant to less abundant.

4.6.3 Composite sludge samples

The microbiota composite associated with mesophilic and thermophilic conditions of composite samples at order level were assessed (Figure 4-23 and Figure 4-24). The 15 most abundant orders observed in mesophilic composite samples before AD occurred were as follows: Anaerolineales (23%), Unassigned (15%), Caldilineales (9%), Bacillales (6%), Planctomycetales (6%), followed by Clostridiales (6%), Synergistales (6%), Bacteroidales (4%), Uncultured Bacterium (3%), Methanosarcinales (2%) and Corynebacteriales (2%). The least abundant orders were identified as follows: Unknown Order (1%), MBA03 (1%), Pseudomonadales (0.8 %) and Petrotogales (0.6%). As with seed, Clostridiales increased during T1 (17%) and T2 (34%) from the initial start T0(6%) and then decreased in T3(14%). This order became one of the dominant orders during T1 and T2. Methanosarcinales showed a similar trend and was the most abundant for this order during T1(7%) and T2 (16%), which then decreased at T3 (2%). The abundance of

Anaerolineales decreased substantially during AD (23-1%), while Synergistales increased in abundance from T0 (6%) to T2 (16%) and then again decreased at T3 (2%) to below the initial start abundance. Bacteroidales and Corynebacteriales illustrated the same trend observed in Synergistales and Methanosarcinales. MBA03 increased by 3% during AD; Petrotogales showed similar trends but decreased after T2 (4% to 3%). Pseudomonadales increased by 3% after AD but completely decreased after T1 to 0.1% at T2, and then again increased to 3%. Orders Bacillales, Caldilineales, Uncultured Bacterium and Planctomycetales showed a decrease during AD. The order abundance after AD was recorded as follows: Clostridiales (21%), Unassigned (12%), Bacteroidales (9%), followed by Methanosarcinales (8%), Corynebacteriales (8%), MBA03 (5%), Petrotogales (5%), Synergistales (4%), Pseudomonadales (3%), Unknown Order (3%) and Planctomycetales (3%). The least abundant order levels recorded were as follows: Planctomycetales (2%), Anaerolineales (1%), Caldilineales (1%) and Bacillales (0.3%).

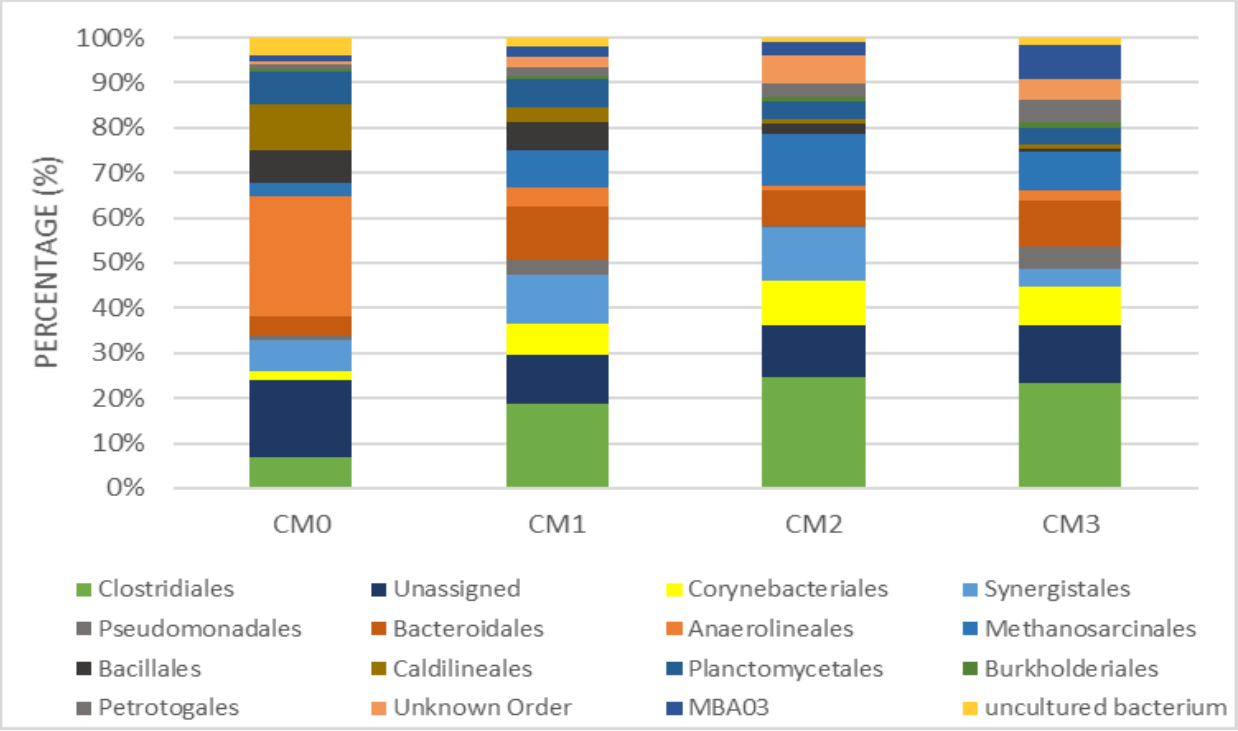

Figure 4-23: Order-level classification of composite community during mesophilic AD. M = Mesophilic, 0 = Day 0, 1 = Day 10, 2 = Day 16 and 3 = Day 36

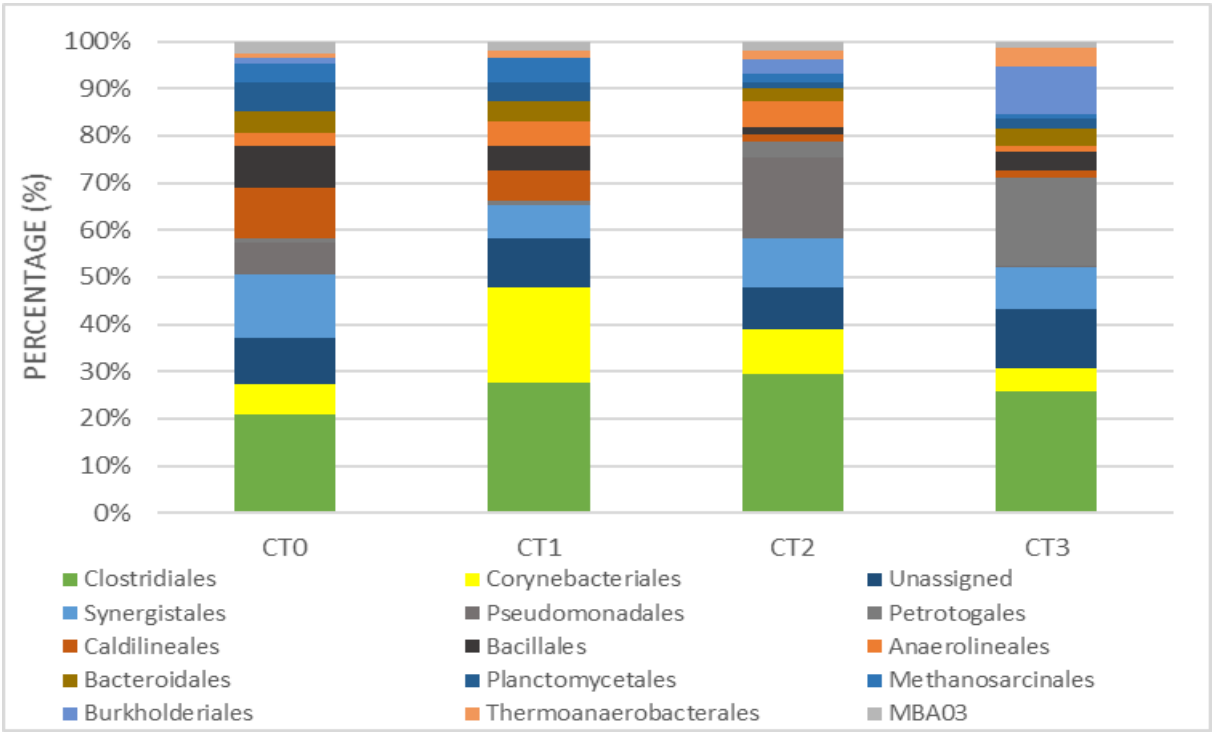

Figure 4-24: Order-level classification of composite community during thermophilic AD.
T = Thermophilic, 0 = Day 0, 1 = Day 1, 2 = Day 10 and 3 = Day 16

The 15 most abundant orders observed in thermophilic Composite samples before AD were as follows: Clostridiales (17%), Synergistales (11%), Caldilineales (9%), Unassigned (8%), Bacillales (7%), followed by Corynebacteriales (5%), Pseudomonadales (5%), Planctomycetales (5%), Bacteroidales (4%), Methanosarcinales (3%), MBA03 (2%) and Anaerolineales (2%). The least abundant order levels were recorded at order Burkholderiales (1%), Thermoanaerobacterales (1%) and Petrotogales (0.8%). The orders Bacteroidales, MBA03, Unassigned and Synergistales remained at same abundance level throughout the AD process, while Caldilineales, Bacillales, Planctomycetales all decreased during AD. Clostridiales remained dominant during the AD proses and increased during T1 (23%) and T2 (26%), followed by a decrease at T3 (22%). Despite the decrease at T3, Clostridiales remained abundant throughout the selected timelines. Methanosarcinales increased at T1 (4%), followed by decrease until the end at T3 (1%). Synergistales decreased at T1 (6%), followed by increase at T2 (9%) where it remained at that abundance with a slight decrease at T3 (8%). Burkholderiales increased at the end of AD at T3 from 1% to 8%. Same trends were observed in Thermoanaerobacterales, where it increased to 4% from 1%at T3. Thermotogales made an appearance only at T3 (4%), whereas it remained underrepresented in T0 to T3.

The order levels recorded after AD were as follows: Clostridiales (22%), Petrotogales (16%), Unassigned (11%), Synergistales (8%), followed by Corynebacteriales (4%), Bacillales (3%), Bacteroidales (3%), Planctomycetales (2%), Caldilineales (2%) and Anaerolineales (1%). The

least abundant order levels were recorded as: Methanosarcinales (0.85%) and Pseudomonadales (0.41%). Orders Clostridiales, Corynebacteriales, Unassigned, Synergistales, Pseudomonadales and Petrotogales were the most dominant in thermophilic conditions, whereas mesophilic conditions were dominated by orders Clostridiales, Unassigned, followed by Synergistales, Methanosarcinales and Bacteroidales.

4.7 The microbial dynamics at Family level

4.7.1 Faecal sludge samples

The microbiota composite associated with mesophilic and thermophilic conditions of faecal samples at family level were assessed (Figure 4-25 and Figure 4-26). The 15 most abundant families of mesophilic faecal sludge samples before AD were as follows: Unassigned (20%), *Caldilineaceae* (18%), *Planctomycetaceae* (11%), Uncultured Bacterium (4%), *Xanthomonadales Incertae Sedis* (3%), *Anaerolineaceae (3%), Peptostreptococcaceae (3%), Pseudomonadaceae (3%), Clostridiaceae 1 (2%) followed by Synergistaceae (2%), Moraxellaceae (2%), Family XI (2%) and Comamonadaceae (2%).* The lowest of the family-level microbiota were *Ambiguous_taxa* (1%) and *Rikenellaceae* (0.1%). *Anaerolineaceae* increased during AD (3-22%); *Moraxellaceae* became the most dominant family at T3 (31%) and increased by 29%. Unassigned remained dominant during T0 (20%), T1 (17%) and T3(28%) and only decreased during T2 (14%) during AD. The families *Caldilineaceae* and *Planctomycetaceae* decreased drastically in abundance. *Synergistaceae*, *Clostridiaceae 1* and *Peptostreptococcaceae* remained similar in T0 and T1 and then decreased toward T3. The abundant family levels after AD in mesophilic conditions were as follows: Unassigned (28%), *Anaerolineaceae* (21%), *Rikenellaceae* (8%), *Planctomycetaceae* (7%), Uncultured Bacterium (7%), *Caldilineaceae* (6%), *Synergistaceae* (1.5%) and *Comamonadaceae* (1.3%). The rest of the selected families were >1% and included family-level microbiota such as *Xanthomonadales Incertae Sedis*, *Peptostreptococcaceae*, *Clostridiaceae 1*, *Pseudomonadaceae* and *Moraxellaceae*.

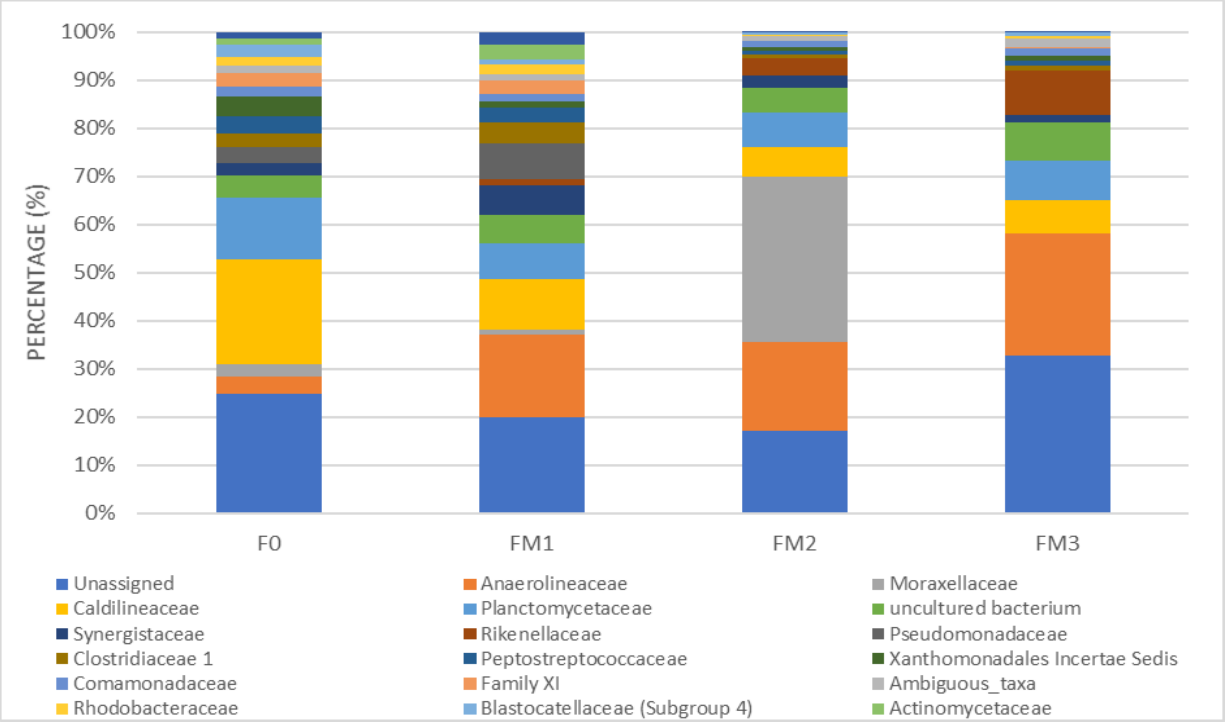

Figure 4-25: Family-level classification of faecal community during mesophilic AD. M = Mesophilic, 0 = Day 0, 1 = Day 10, 2 = Day 16 and 3 = Day 36

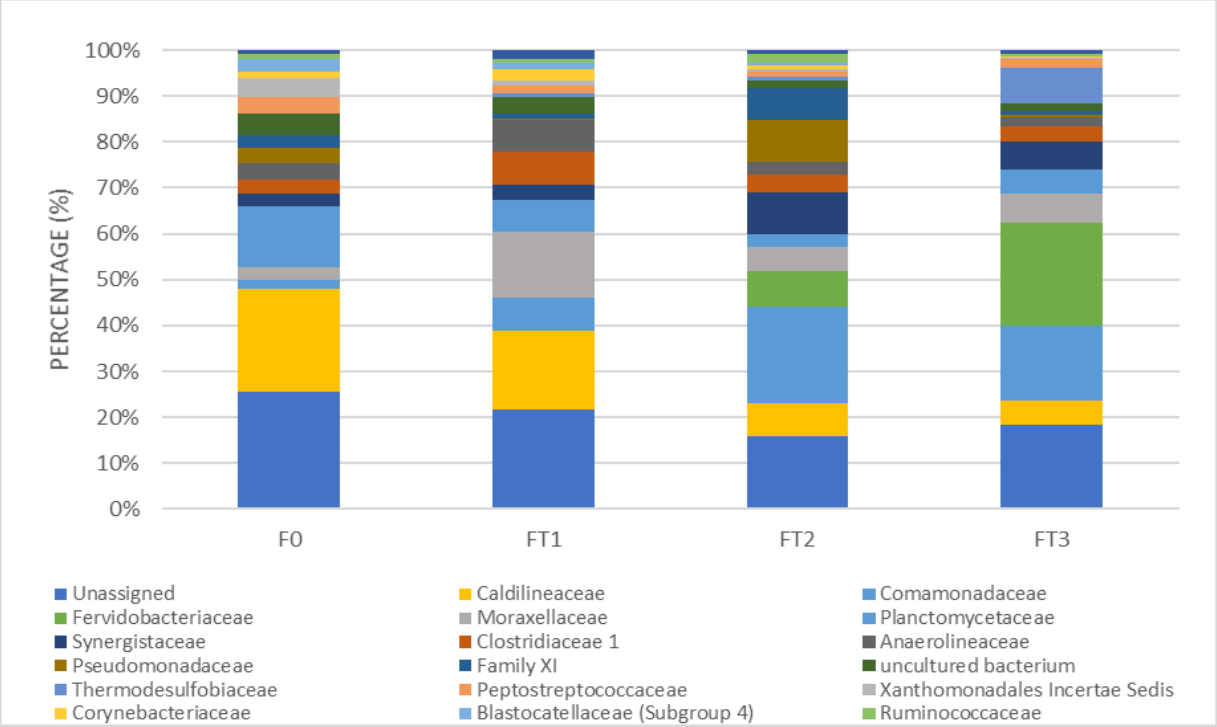

Figure 4-26: Family-level classification of faecal community during thermophilic AD. T = Thermophilic, 0 = Day 0, 1 = Day 1, 2 = Day 10 and 3 = Day 16

The 15 most abundant families found in thermophilic faecal sludge samples were as follows: Unassigned (21%), *Caldilineaceae* (18%), *Planctomycetaceae* (11%), Uncultured Bacterium (4%), *Xanthomonadales Incertae Sedis* (3%), *Anaerolineaceae* (3%), *Peptostreptococcaceae* (3%), *Planctomycetaceae* (5%), *Pseudomonadaceae* (3%), *Clostridiaceae 1* (2%), *Family XI* (2%), *Synergistaceae* (2%), followed by *Moraxellaceae* (2%), Blastocatellaceae (Subgroup 4) (2%), *Comamonadaceae* (2%) and *Corynebacteriaceae* (1%). Other family-level microbiota was lower than 1% and included families such as *Ruminococcaceae*, *Veillonellaceae* and *Fervidobacteriaceae*. Unassigned were dominant in T0 (21%) and T1 (18%), whereas during T2 *Comamonadaceae* became the most dominant family with 19% and *Fervidobacteriaceae* at T3 (20%). *Fervidobacteriaceae* only began to appear in T2 (7%), while it was underrepresented in T0 and T1. *Clostridiaceae 1* increased from T0 (2%) to T1 (6%) and decreased to 3% in T2 and T3.

The most abundant families after AD were as follows: *Fervidobacteriaceae* (20%), Unassigned (16.3%), *Comamonadaceae* (14.42%), *Thermodesulfobiaceae* (6.9%), *Moraxellaceae* 5.5%, *Synergistaceae* 5.4%, *Planctomycetaceae* 4.7%, *Caldilineaceae* 4.6% followed by *Clostridiaceae 1* (2.9%), *Anaerolineaceae* (1.8%), *Peptostreptococcaceae* (1.7%) and Uncultured Bacterium (1.5%). The least abundant family-level microbiota ranged from 0.7 to 0.0098 and included Family levels such as *Family XI*, *Veillonellaceae*, *Ruminococcaceae*, *Pseudomonadaceae*, *Xanthomonadales Incertae Sedis*, Blastocatellaceae (Subgroup 4) and *Corynebacteriaceae*. The dominant families identified in mesophilic conditions were Unassigned, *Anaerolineaceae*, *Moraxellaceae*, *Caldilineaceae* and *Planctomycetaceae*, while thermophilic conditions were dominated by Unassigned, *Caldilineaceae*, *Comamonadaceae*, *Fervidobacteriaceae* and *Moraxellaceae*.

4.7.2 Seed sludge samples

The microbiota composite associated with mesophilic and thermophilic conditions of seed samples at family level were assessed (Figure 4-286 and Figure 4-297). The 16 most abundant families in seed samples of mesophilic conditions before AD were as follows: *Marinilabiaceae* (13%), Unassigned (12%), *Corynebacteriaceae* (11%), *Methanosarcinaceae* (9%), *Ruminococcaceae* (8%), *Bacillaceae* (7%), *Synergistaceae* (6%), *Porphyromonadaceae* (5%), *Caldicoprobacteraceae* (4%), Uncultured Bacterium (4%), followed by *Planctomycetaceae*, Unknown Family at 2% and *Petrotogaceae* (1%). The least assigned abundant families (<1) microbiota was as follows: *Peptostreptococcaceae* (1%), *Clostridiaceae 1*, *Heliobacteriaceae*, *Moraxellaceae* and others. *Methanosarcinaceae* became the abundant family during T1 and T2 (both at 12%) and then decreased at T3 (7%). *Synergistaceae* also increase at T1 and T2 (both at 10%) and decreased towards the end at T3 (4%) – lower than the initial abundance at T0 (6%).

Corynebacteriaceae and *Marinilabiaceae* both decreased during AD from the initial start T0 to T3. *Bacillaceae*, *Porphyromonadaceae*, *Ruminococcaceae* and *Caldicoprobacteraceae* remained at their initial start T0 abundance with slight decreases towards the end T3. *Moraxellaceae* increased significantly from the initial start at T0 (0.02%) towards T2 (7%) and then decreased to 5% at T3. *Petrotogaceae* also increased from 1% at T0 to 8% at the end of AD. The most abundant microbiota composite of mesophilic seed sludge samples after AD was as follows: Unassigned (15%), *Petrotogaceae* 8%, *Corynebacteriaceae* (8%), *Methanosarcinaceae* (7%), *Porphyromonadaceae* (7%), Uncultured Bacterium (6%), *Caldicoprobacteraceae* (6%), *Moraxellaceae* (5%), *Ruminococcaceae* (5%), *Marinilabiaceae* (4%), *Synergistaceae* (4%), followed by *Planctomycetaceae* (3%) and Unknown Family (3%). The least assigned abundant microbiota family includes *Heliobacteriaceae* (2%), *Peptostreptococcaceae* (2%) and others.

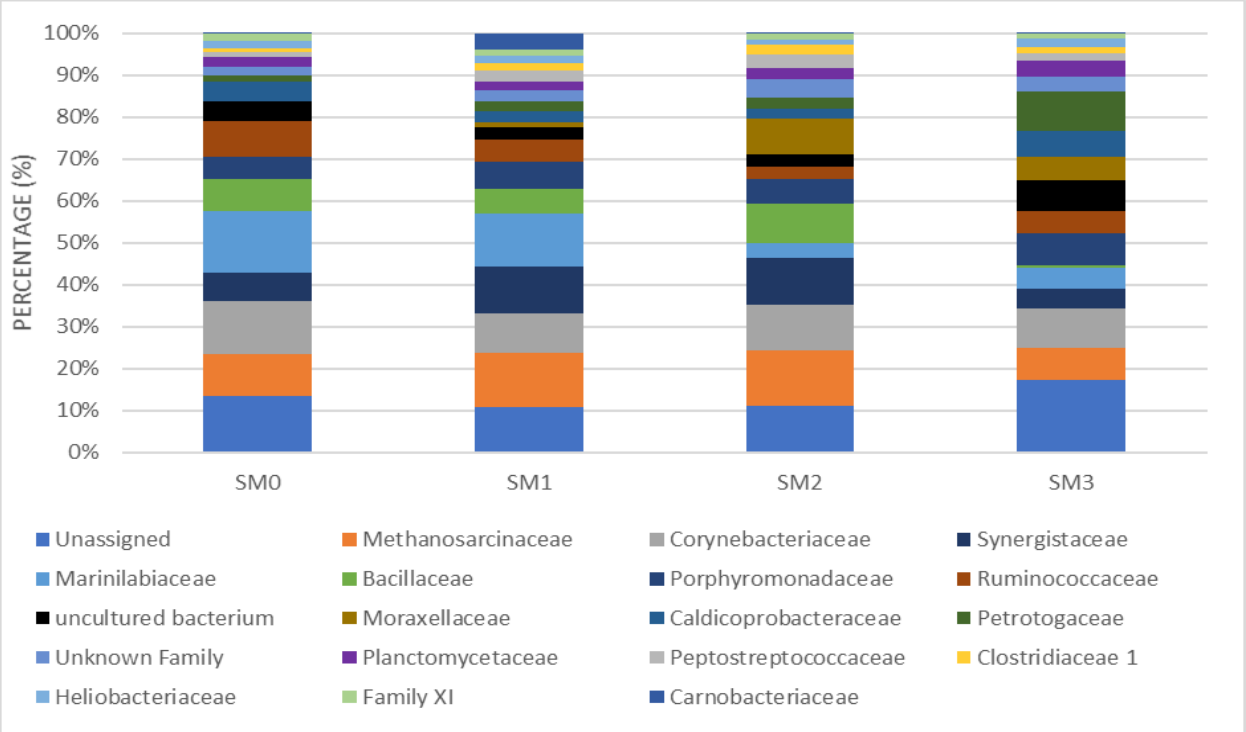

Figure 4-27: Family-level classification of seed community during mesophilic AD. M = Mesophilic, 0 = Day 0, 1 = Day 10, 2 = Day 16 and 3 = Day 36

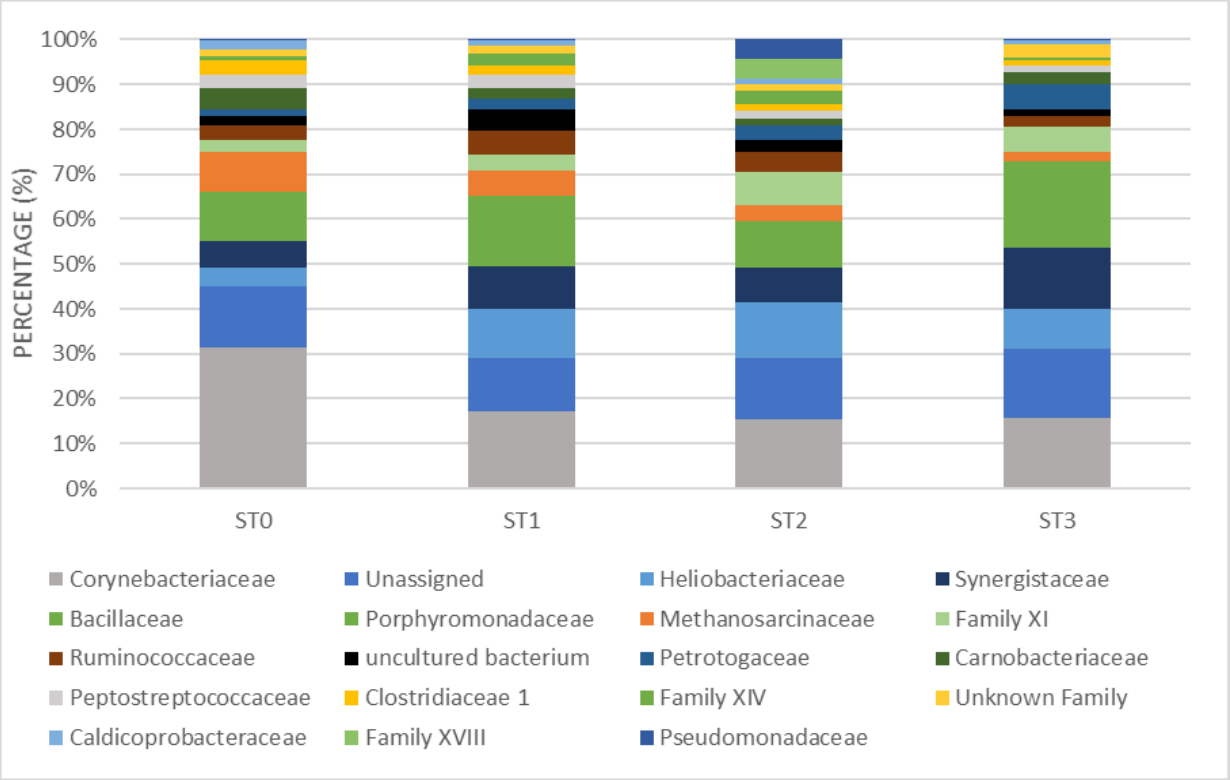

Figure 4-28: Family-level classification of seed community during thermophilic AD. T = Thermophilic, 0 = Day 0, 1 = Day 1, 2 = Day 10 and 3 = Day 16

The microbiota composite associated with the thermophilic seed content before AD at family level was as follows: *Corynebacteriaceae* (28%); Unassigned (12%); *Methanosarcinaceae* (8%); *Bacillaceae* and *Synergistaceae* at 6% while Carnobacteriaceae, *Heliobacteriaceae* and *Porphyromonadaceae* were at 4%; *Clostridiaceae 1* (3%); *Ruminococcaceae*, *Peptostreptococcaceae* and *Family XI* at 3%, which was followed by Uncultured Bacterium and *Caldicoprobacteraceae* at 2%. The least assigned abundant microbiota families recorded were as follows: *Family XI*II, Unknown Family, *Marinilabiaceae* and *Petrotogaceae* were 1 % or less. *Corynebacteriaceae* was the most abundant family during AD of this condition, but a significant decrease was observed during AD (28-14%). *Heliobacteriaceae* increased in T1 (10%) and T (11%), followed by a decrease at T3 (8%). Despite the decrease, this family remained the third-most abundant during AD. *Synergistaceae* increased during AD but was observed at high levels during T1 (9%) and T3(12%). From T1 to T2, a decrease was observed. *Methanosarcinaceae* decreased slowly during AD and was observed at T3 (2%) as the lowest when compared to T0 (8%), T1 (5%) and T2 (3%). *Thermoanaerobacteraceae* was one of the families observed as underrepresented in T0 (0.3%) but increased during AD from 0.3% to 2% at T3. *Bacillaceae* also observed an increase from 6% at T0 to 11% at T3.

The families associated with the thermophilic seed content after AD were as follows: The seed samples content was dominated by the microbiota families *Corynebacteriaceae* (14%), Unassigned (14%), *Synergistaceae* (12%), *Bacillaceae* (12%), *Heliobacteriaceae* (8%), *Porphyromonadaceae* (6%), *Petrotogaceae* (5%), *Family XI* (5%), Unknown Family (3%), followed by Carnobacteriaceae (3%), *Ruminococcaceae* (2%), *Thermoanaerobacteraceae* (2%) and *Methanosarcinaceae* (2%). The least assigned abundant families were Uncultured Bacterium (1.4%), *Peptostreptococcaceae* (1.2%) and *Clostridiaceae 1* (1.1%). Families of Unassigned, *Methanosarcinaceae*, *Corynebacteriaceae*, *Synergistaceae* and *Marinilabiaceae* were most abundant in mesophilic conditions during AD, whereas families of *Corynebacteriaceae*, Unassigned, *Heliobacteriaceae*, *Synergistaceae* and *Bacillaceae* were most abundant in thermophilic conditions during AD.

4.7.3 Composite sludge samples

The microbiota composite associated with the mesophilic composite content before AD at the family level was as follows: *Anaerolineaceae* (24%), Unassigned (17%), *Caldilineaceae* (9%), *Planctomycetaceae* (6%), *Synergistaceae* (6%), Uncultured Bacterium (6%), followed by *Porphyromonadaceae* (2%), *Methanosarcinaceae* (2%), *Bacillaceae* (2%) and *Corynebacteriaceae* (2%). The least assigned abundant microbiota family of the 20 most abundant families included: *Ruminococcaceae* (1%), *Moraxellaceae* (0.7%), *Marinilabiaceae* (0.6%), *Petrotogaceae* (0.6%) *Comamonadaceae* (0.5%), *Clostridiaceae 1* (0.5%) and *Peptostreptococcaceae* (0.5%). *Anaerolineaceae* decreased significantly during AD from 23% to 2% at T3. When AD began, almost all the *Anaerolineaceae* died off. *Synergistaceae* increased during AD and were recorded as the second-most abundant during T1 (10%) and T2 (11%), while a significant decrease was observed in T3. *Methanosarcinaceae* showed similar trends as observed in *Synergistaceae*, whereas the family *Methanosarcinaceae* became the third-most abundant at T1 (10%) and T2 (11%). At T2, *Synergistaceae* and *Methanosarcinaceae* made up 22% of the total abundant families. *Heliobacteriaceae* increased at T1 from 0.3% to 1% and further increased to 3% where it remained at the same level at T3. *Corynebacteriaceae* increased during AD, which became the fourth most abundant family during T2 and second-most abundant family at T3. *Ruminococcaceae* also increased during AD but remained relatively low compared to Unassigned and *Methanosarcinaceae*. The family composite associated with the mesophilic conditions after AD was as follows: Unassigned (15%), Uncultured Bacterium (9%), *Methanosarcinaceae* (8%), *Corynebacteriaceae* (8%), *Porphyromonadaceae* (5%), *Petrotogaceae* (5%), *Ruminococcaceae* (4%), *Synergistaceae* (4%), followed by *Planctomycetaceae* (4%), *Marinilabiaceae* (3%), *Heliobacteriaceae* (3%), *Anaerolineaceae* (2%) and *Peptostreptococcaceae* (2%). The least assigned abundant microbiota family under the 20

most abundant families included: *Clostridiaceae 1* (1%), *Family XI* (1%), *Caldilineaceae* (0.8%), *Moraxellaceae* (0.6%) and *Bacillaceae* (0.4%).

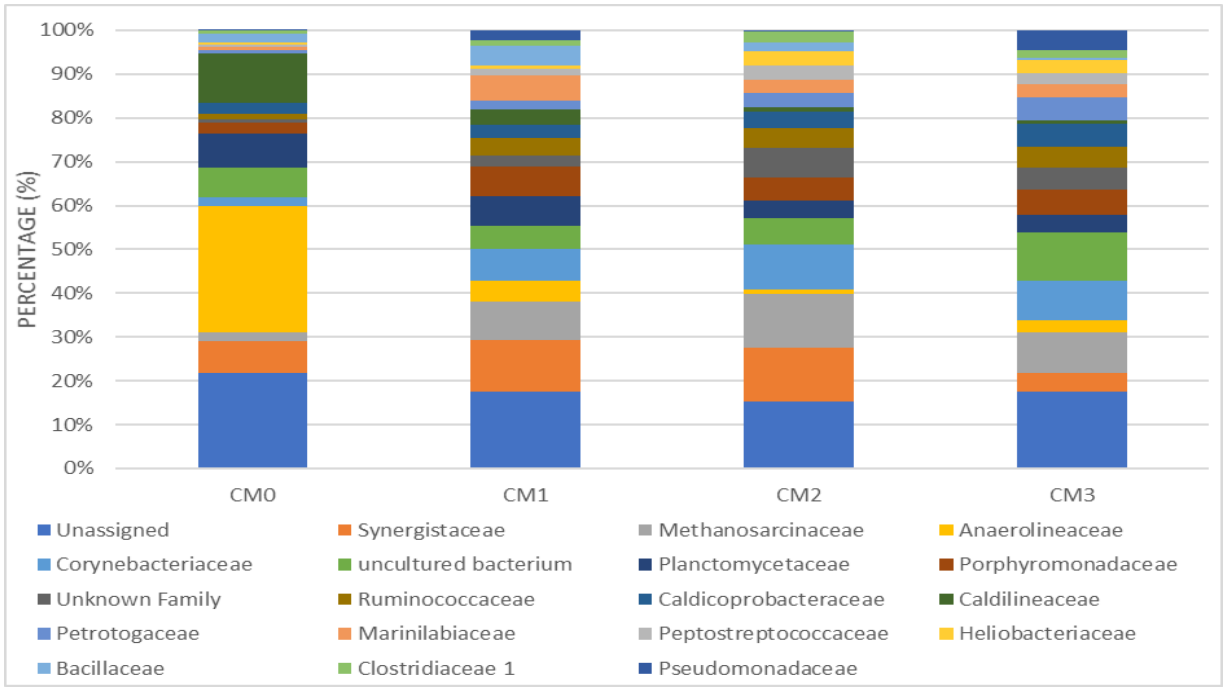

Figure 4-29: Family-level classification of composite community during mesophilic AD. M = Mesophilic, 0 = Day 0, 1 = Day 10, 2 = Day 16 and 3 = Day 36

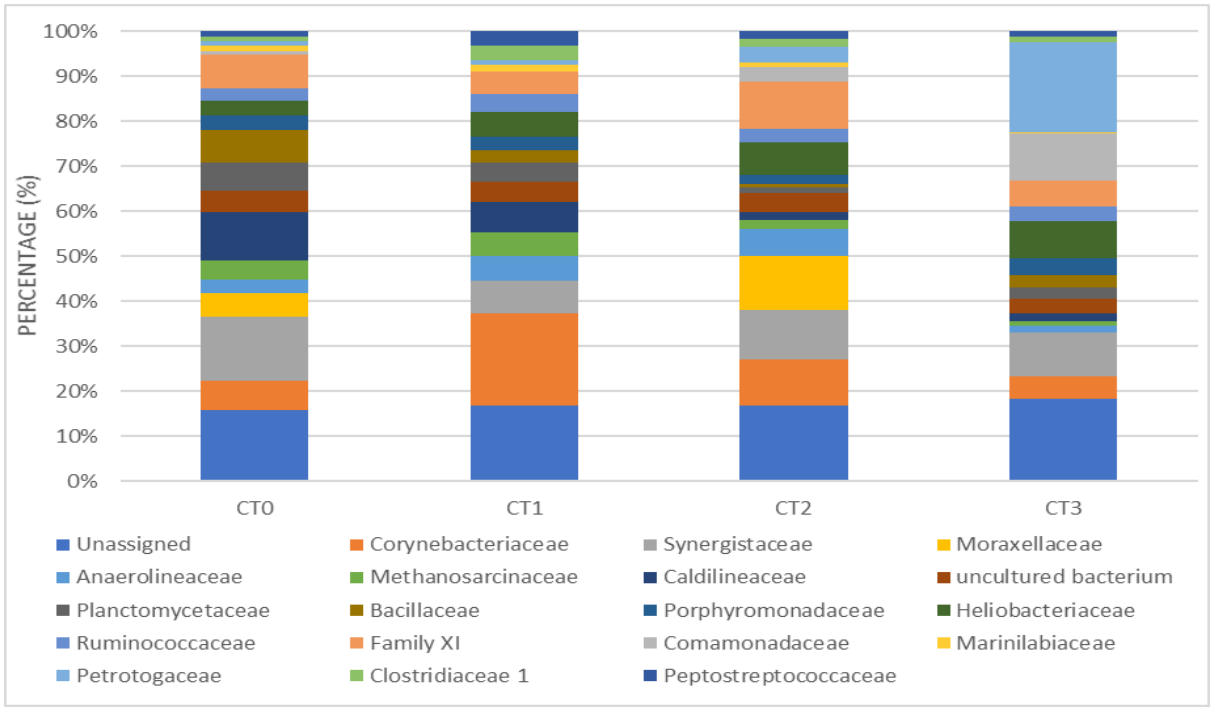

Figure 4-30: Family-level classification of composite community during thermophilic AD. T = Thermophilic, 0 = Day 0, 1 = Day 1, 2 = Day 10 and 3 = Day 16

The microbiota composite associated with the thermophilic composite content before AD at the family level was as follows: Unassigned (13%), *Synergistaceae* (12%), *Caldilineaceae* (9%), *Family XI* (6%), *Bacillaceae* (6%), *Corynebacteriaceae* (5%), *Planctomycetaceae* (5%), *Moraxellaceae* (4%), Uncultured Bacterium (4%), *Methanosarcinaceae* (3%), followed by *Porphyromonadaceae* (3%), *Heliobacteriaceae* (2%), *Anaerolineaceae* (3%), *Ruminococcaceae* (2%) and *Xanthomonadales Incertae Sedis* (2%). The least assigned abundant microbiota family under the 20 most abundant families included: *Pseudomonadaceae* (1.4%), *Comamonadaceae* (1%) and *Petrotogaceae* (1%). *Corynebacteriaceae* were recorded as the most abundant family at T1 (15%) and then decreased to 4% at the end of AD. *Synergistaceae* were recorded to decrease throughout each timeline from 11% before digestion to 7% after digestion. *Methanosarcinaceae* remained at low levels and were recorded at T2 at 5%, which was the highest abundance noted throughout AD of this family.

Moraxellaceae showed different abundance levels than that of *Methanosarcinaceae* throughout AD, which increased to 10% and became the second-most abundant family in T2. *Petrotogaceae* significantly increased at T3 (16%) the level of *Petrotogaceae* remained between remained between 1% and 3% at t T0, T1, and T2. *Comamonadaceae* variety during AD and was recorded the highest at 8% at the end. The family composite associated with the thermophilic composite content after AD was as follows: *Petrotogaceae* (16%), Unassigned (15%), *Comamonadaceae* (9%), *Synergistaceae* (8%), *Heliobacteriaceae* (7%), *Family XI* (5%), *Corynebacteriaceae* (4%), *Fervidobacteriaceae* (4%), followed by *Porphyromonadaceae* (3%), Uncultured Bacterium (3%), *Ruminococcaceae* (3%) and *Bacillaceae* (2%). The least assigned abundant microbiota family under the 15 most abundant families included: *Planctomycetaceae* (2%), *Thermodesulfobiaceae* (2%), *Thermoanaerobacteraceae* (1.8%), *Caldilineaceae* (1.5%), Carnobacteriaceae (1.2%) and *Anaerolineaceae* (1%). Unassigned, *Synergistaceae*, *Methanosarcinaceae*, *Anaerolineaceae* and *Corynebacteriaceae* were the most abundant families during mesophilic AD, while thermophilic conditions during AD were more abundant in families: Unassigned, *Corynebacteriaceae*, *Synergistaceae*, *Moraxellaceae*, and *Family XI*.

4.8 Normalization of collected NGS data.

The metabolic pathways were determined using Qiime2® and visualised as a bar plot that was constructed in METAGENassist. Variables that contained more than 50% zero values were removed and filtered using IQR for both conditions. The mesophilic samples before filtering were recorded at 993 variables in the data set; after filtering, a total of 857 variables were removed and a total of 140 variables were left. The thermophilic samples recorded at 928 variables, of which 795 were removed and, 127 variables were left. These conditions were then put through column-wise normalisation using a generalised Log2 transformation, as shown in Figure 4-31. The

evaluated pathways of samples in mesophilic and thermophilic conditions were visualised using METAGENassist.

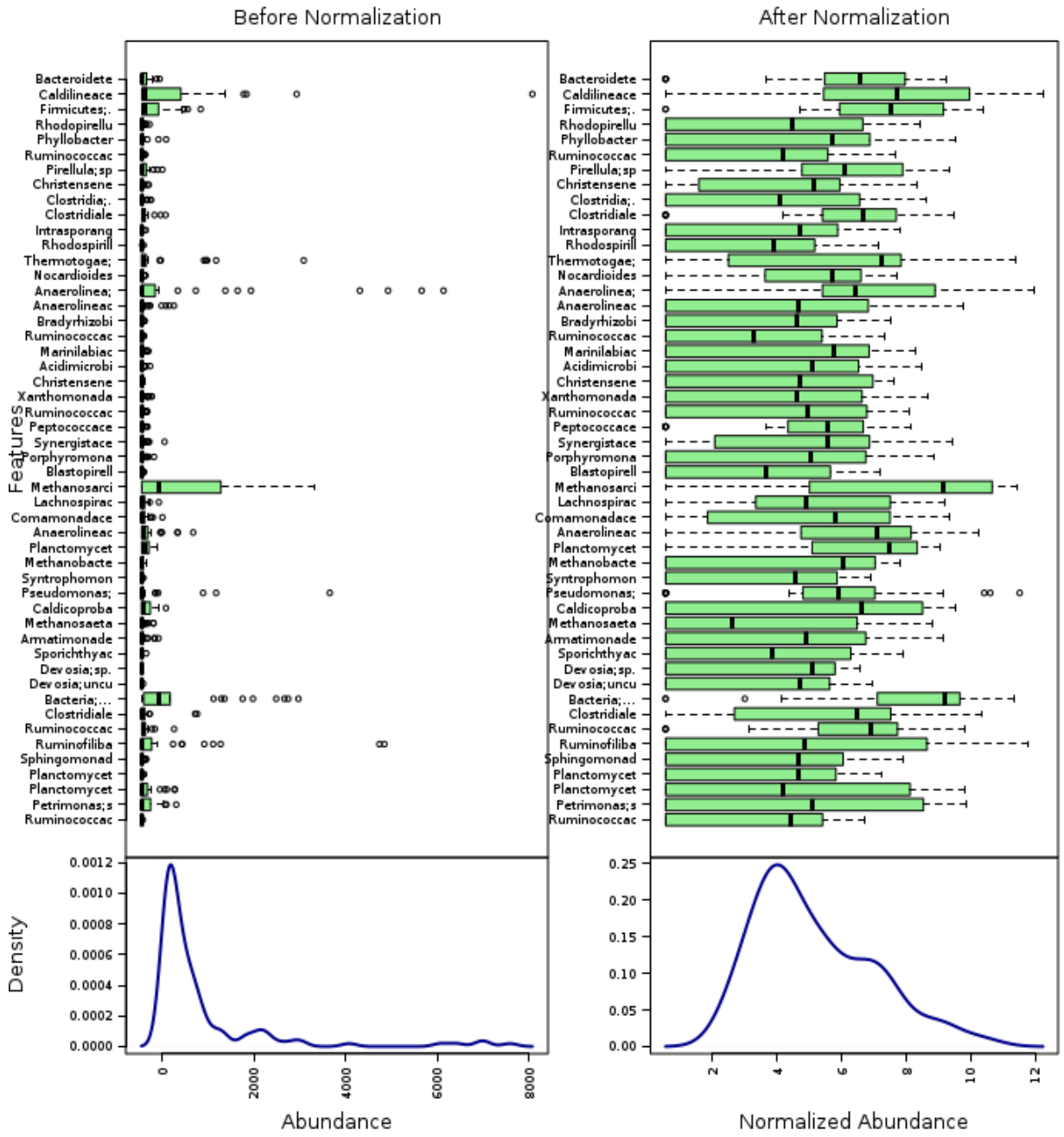

Figure 4-31: Illustration of before and after normalisation of samples in mesophilic and thermophilic samples

4.9 Relative community metabolism within mesophilic and thermophilic conditions of substrates

A total of 23 dominant metabolic pathways were identified within each condition.

1) The five most abundant metabolic pathways in mesophilic faecal samples were identified as ammonia oxidiser (41.9%), dehalogenation (27.6%), nitrite reducer (22.5%), sulphate reducer (22.3%) and sulphide oxidiser (21%). Thermophilic metabolic pathways of faecal samples were recorded with ammonia oxidiser (43%), dehalogenation (26.4%), sulphate reducer (23.9%), xylan degrader (16.9%) and nitrite reducer (16.1%);
2) The five most abundant metabolic pathways in mesophilic seed samples were recorded as ammonia oxidiser (31.1%), dehalogenation (24.9%), nitrogen fixation (11.8%), sulphate reducer (12.1%) and xylan degrader (6.8%). The thermophilic metabolic pathways of seed samples were identified as ammonia oxidiser (34.9%), dehalogenation (25.8%), nitrite reducer (17.3%) and sulphate reducer (10.9%)
3) The five most abundant metabolic pathways in mesophilic composite samples were ammonia oxidiser (29.2%), dehalogenation (21.1%), nitrite reducer (11.3%), sulphate reducer (9.9%) and xylan degrader (7.7%). The thermophilic metabolic pathways of composite samples were ammonia oxidiser (33.3%), dehalogenation (19.4%), nitrite reducer (14.1%), sulphate reducer (12.6%), xylan degrader (8.2%) and chitin degradation (5.5%).

4.9.1 Mesophilic and thermophilic metabolic pathways during Timeline 0 (T0): start stage

Figure 4-32 shows the predicted metabolic pathways during the start (T0) of the mesophilic conditions. The most dominant metabolic pathways were recorded as ammonia oxidisers with a percentage of 45.1%, whereas the second-most abundant were recorded as unknown (44.9%), followed by sulphate reducers (28.7%), nitrate reducers (26.2%), dehalogenation (24.9%), chitin degradation (20.7%), sulphate oxidisers (15.6%) and xylan degrader (14.7%). Sulphur oxidisers made up 13.7% of the metabolic pathways. The rest of the metabolic pathways were lower than 5% and included aromatic hydrocarbons degrading, stores polyhydroxybutyrate, chlorophenol degrading and atrazine metabolism.

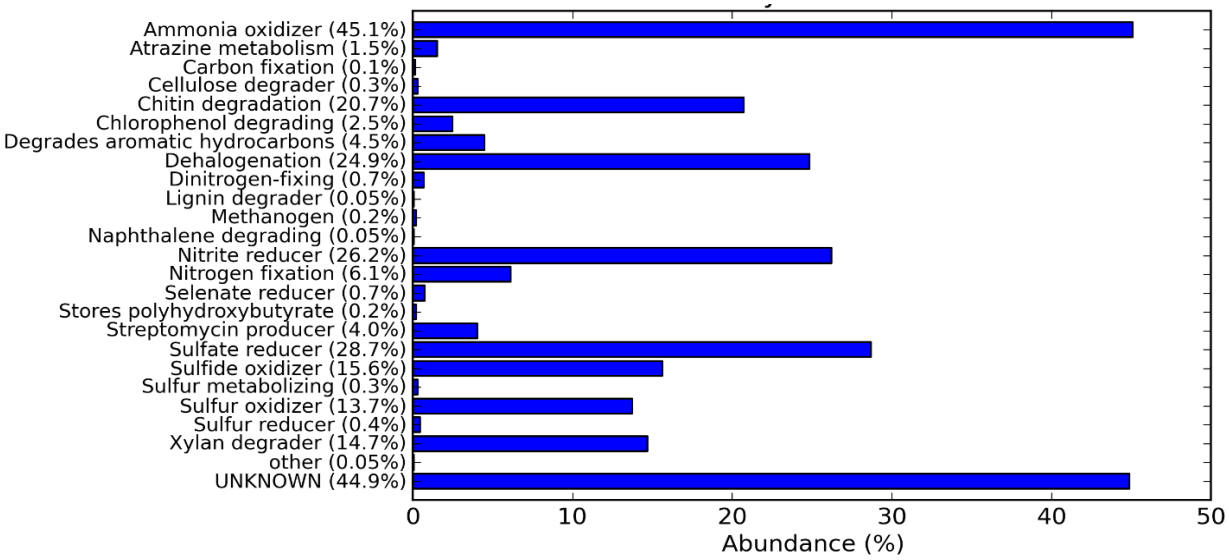

Figure 4-32: Metabolic pathways obtained during start (T0) of mesophilic conditions of samples using METAGENassist

Figure 4-33 shows the metabolic pathways during the start (T0) of thermophilic conditions. The most dominant metabolic pathways were recorded as ammonia oxidisers with a percentage of 46.8%, whereas the second-most abundant were recorded as unknown (43%), followed by sulphate reducers (30%), nitrite reducers (26.3%), dehalogenation (27.6%), chitin degradation (22.4%), xylan degrader (15.9%) and sulphur reducers (14.7%). Sulphite oxidisers made up 13% of the metabolic pathways. The rest of the metabolic pathways were lower than 7% and included pathways such as nitrogen fixation (7%), aromatic hydrocarbons degrading (5.7%), streptomycin production (4%), chlorophenol degrading (3.3%) and selenate reducers (2%).

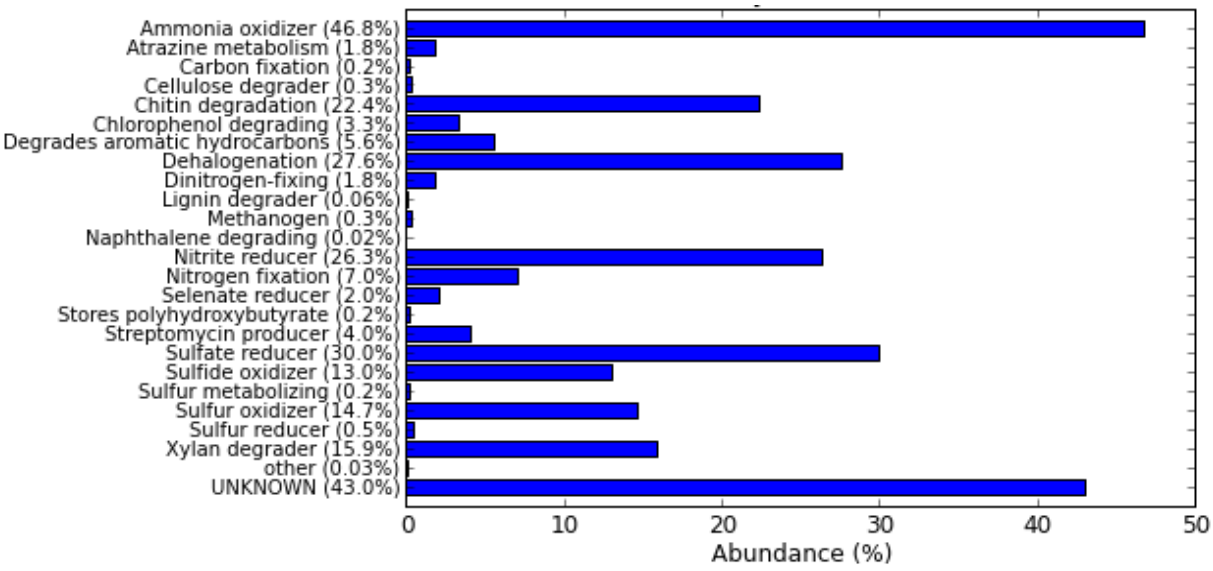

Figure 4-33: Metabolic pathways obtained during start (T0) of thermophilic conditions of samples using METAGENassist.

4.9.2 Mesophilic and thermophilic metabolic pathways during Timeline 1 (T1) :exponential middle stage

Figure 4-34 shows the predicted metabolic pathways during the exponential middle stage (T1) of mesophilic conditions. During this stage, the most abundant metabolic pathways were recorded as unknown (54.8%); the second-most abundant pathways were ammonia oxidisers (30.8%), followed by dehalogenation (23.6%) which was lower than the initial start sample. Nitrite reducers were recorded lower and nitrogen fixation was recorded higher in this stage than observed in the start stage with 13.6% and 9.3%, respectively. Sulphate reducers and sulphite oxidisers were also observed at lower levels than those of the initial start samples and made up 13 % and 12.5% of the predicted pathways, respectively. Chitin degradation (7%), xylan degrader (8.2%), sulphur reducers (0.9%), sulphite oxidisers (0.8%) and chlorophenol degraders (0.3%) were substantially lower than those observed in the start of the AD processes of the metabolic pathways. Atrazine metabolism was recorded at 3%, which was higher than the initial start samples.

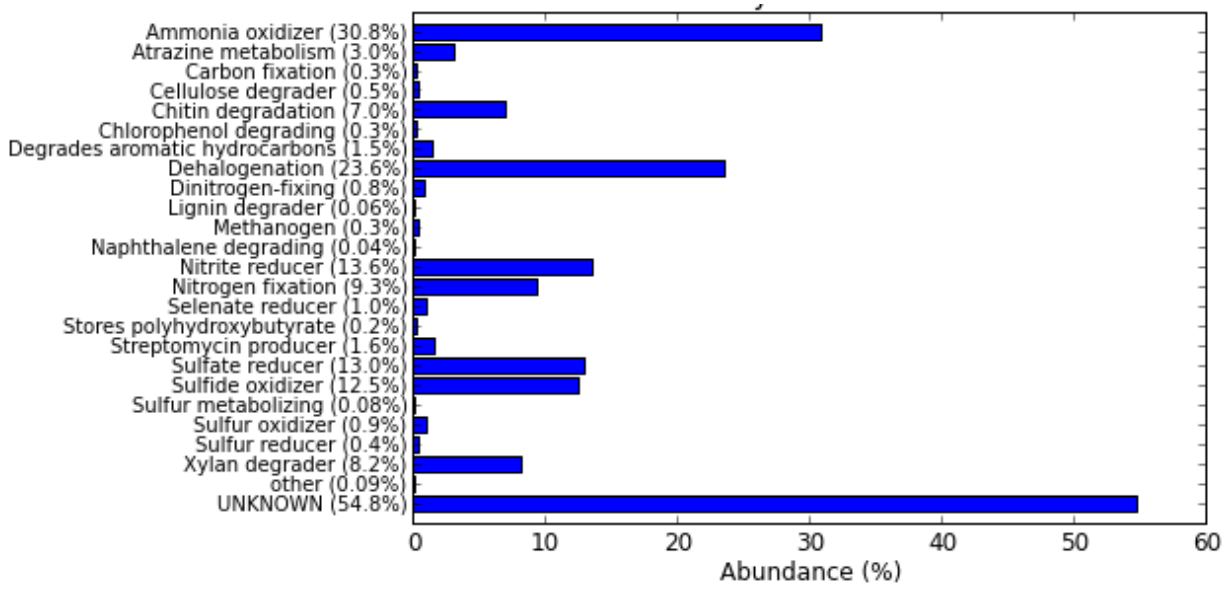

Figure 4-34: Metabolic pathways obtained during the exponential phase (T1) of mesophilic conditions of samples using METAGENassist

Figure 4-35 shows the predicted metabolic pathways during the exponential middle stage (T1) of thermophilic conditions. During this stage, the most abundant metabolic pathways were recorded as unknown (51.8%); the second-most abundant pathways were ammonia oxidisers (35.2%),

followed by dehalogenation (25.2%), which were lower than the initial start sample. Nitrite reducers were recorded lower and nitrogen fixation was recorded as higher in this stage than observed in the start stage, with 17.5% and 10.5%, respectively, and showed the same trends observed in mesophilic conditions at slightly a greater abundance. Sulphate reducers and sulphite oxidisers were also observed at lower levels than those of the initial start samples, which made up 15% and 10.7% of the predicted pathways. Chitin degradation (10%), xylan degrader (12.7%), sulphur reducers (0.5%) and sulphur oxidisers (5.7%) were substantially lower than those observed at the start of the AD processes of the metabolic pathways. However, when looking at the sulphur oxidisers (5.7%) and chlorophenol degraders (5.5%), higher levels were observed in this stage than those in the mesophilic conditions. Chitin degraders were recorded at 10%, which was lower than the initial start samples and higher than those observed in mesophilic conditions.

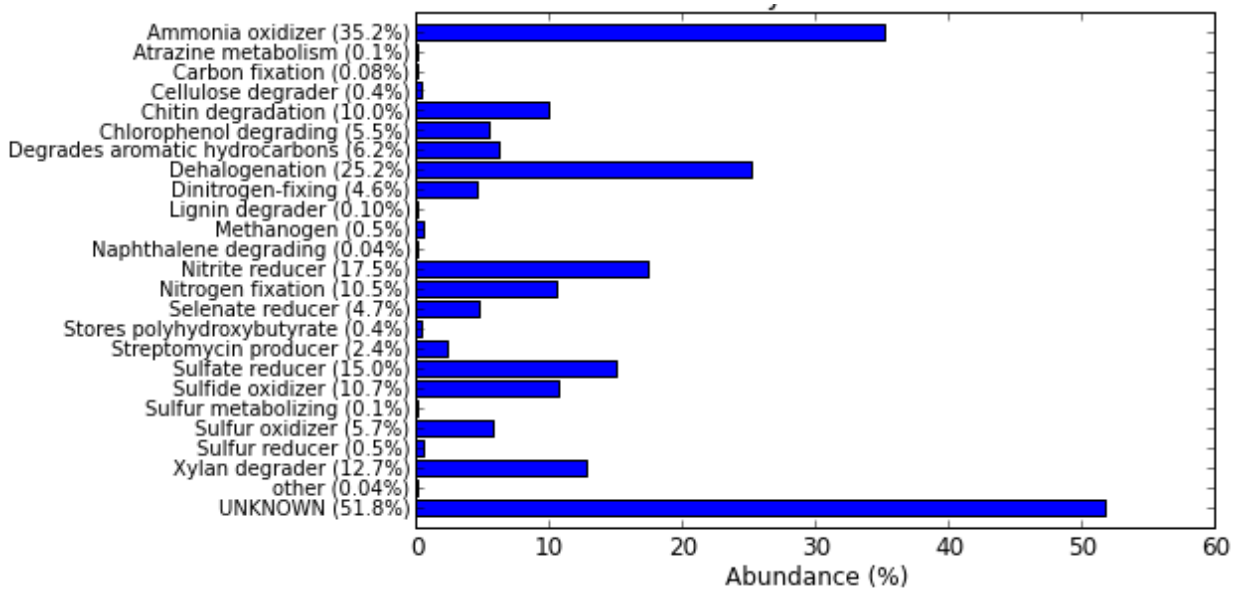

Figure 4-35: Metabolic pathways obtained during the exponential phase (T1) of thermophilic conditions of samples using METAGENassist

4.9.3 Mesophilic and thermophilic metabolic pathways during Timeline 2 (T2) middle stage

Figure 4-36 shows the predicted metabolic pathways during the middle stage (T2) of mesophilic conditions. During this stage, the most abundant metabolic pathways were recorded as unknown (49.5%); the second-most abundant pathways were ammonia oxidisers (35.8%), followed by dehalogenation (30.3%), which was higher than the initial start sample and T1 middle stage. Nitrite reducers were recorded with a lower abundance and nitrogen fixation was recorded with

greater abundance in this stage than observed in the start stage, with 16.2% and 10.5%, respectively, but were greater in abundance for both these pathways than recorded in middle T1. Sulphate reducers were also observed in greater abundance than those of the middle T1, which made up 19%. Sulphite oxidisers were slightly lower than the middle T1 stage with 11.4% of the predicted pathways. Both these previously mentioned pathways were lower than the initial start samples. Chitin degradation (9%), xylan degrader (12.7%), sulphite oxidisers (5.6%) and chlorophenol degraders (5.3.%) were greater in abundance than observed in the middle stage T1 of the AD processes. Sulphur reducer remained relatively the same at 0.3%. When these pathways were compared to start samples, it was recorded in lower abundance. Atrazine metabolism was recorded at 0.3 %, which was lower than the initial start and middle T1 samples.

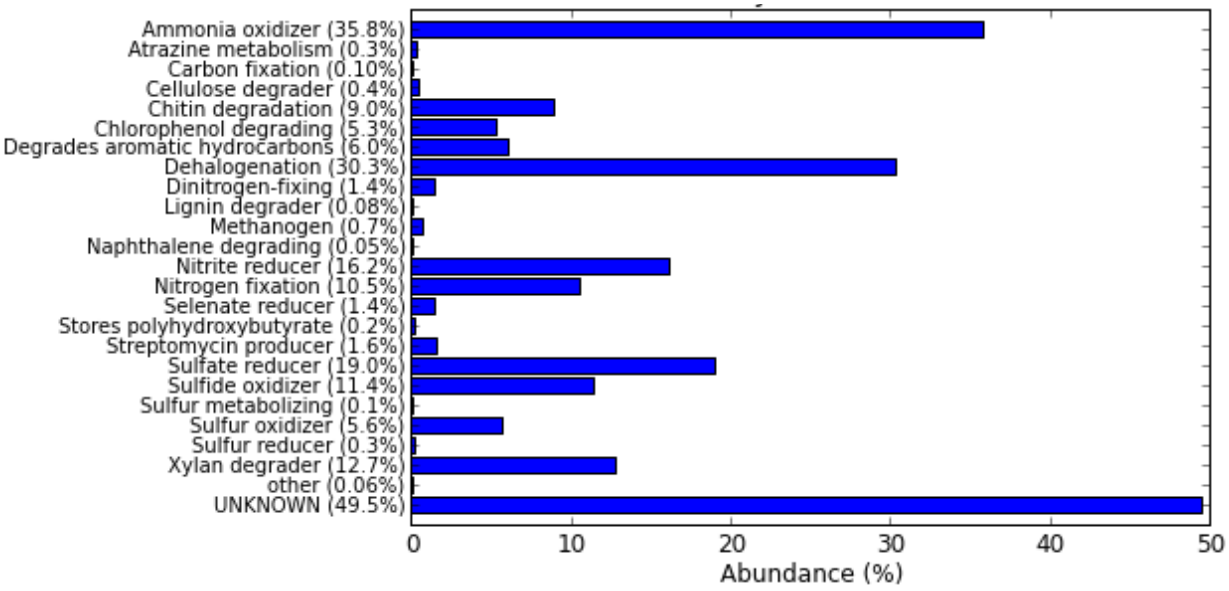

Figure 4-36: Metabolic pathways obtained during the middle stage (T2) of mesophilic conditions of samples using METAGENassist

Figure **4-37** shows the predicted metabolic pathways during the middle stage (T2) of thermophilic conditions. During this stage, the most abundant metabolic pathways were recorded as unknown (53.5%); second-most abundant pathways were ammonia oxidisers (38%), which was higher than the middle stage T1, followed by dehalogenation (24.4%) which was lower than the initial start sample and middle T1 stage. Nitrite reducers were recorded higher and nitrogen fixation was recorded lower in this stage than observed in the middle T1 stage, with 19.2 % and 8.9%, respectively. However, when compared to the start sample, nitrite reducers remained lower than observed at the start, but nitrogen fixation increased. These pathways showed relatively the same

abundance when observing mesophilic conditions. Sulphate reducers were observed at higher levels than those of the middle T1 stage, and sulphite oxidisers were observed in lower abundance and made up 17.7 % and 7.7% of the predicted pathways, respectively. Chitin degradation (8.9%), xylan degrader (5.4%), sulphur reducers (0.09%), and sulphur oxidisers (0.9%) were substantially lower than those observed in the start and middle T1 stages of the AD processes. Atrazine metabolism was recorded at 7.7 %, which was higher than both the initial start and middle T1 samples.

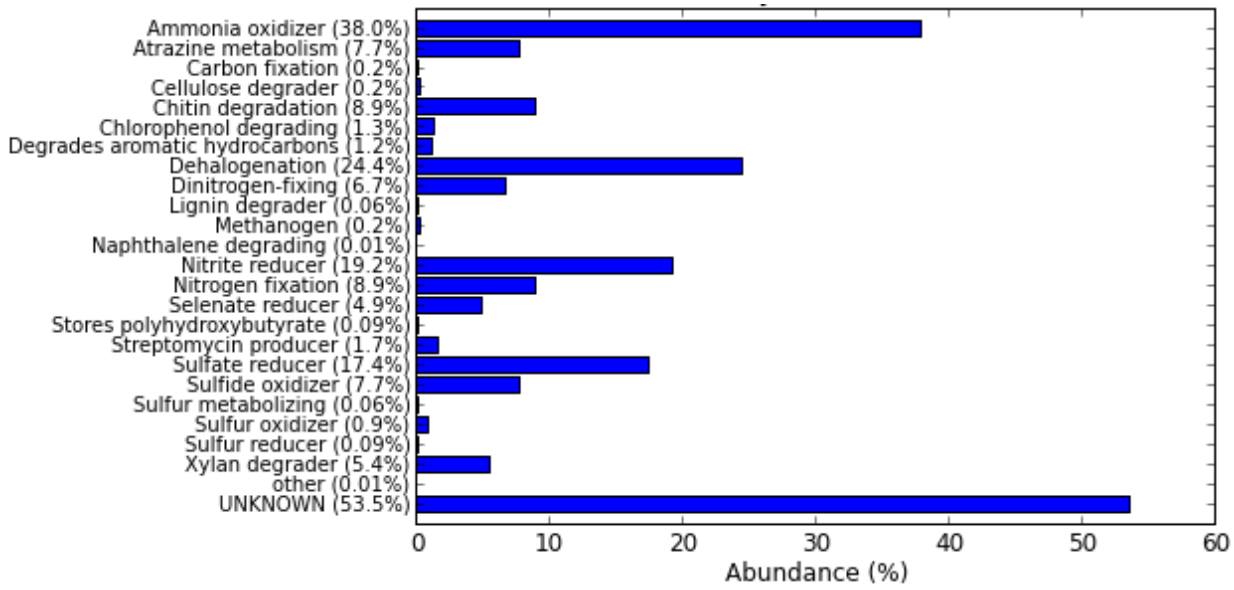

Figure 4-37: Metabolic pathways obtained during the middle stage (T2) of thermophilic conditions of samples using METAGENassist

4.9.4 Mesophilic and thermophilic metabolic pathways during Timeline 3 (T3) end stage

Figure **4-38** shows the predicted metabolic pathways during the end stage (T3) of mesophilic conditions. During this stage, the most abundant metabolic pathways were recorded as unknown (51.8%); the second-most abundant pathways were ammonia oxidisers (36.3%), followed by dehalogenation 19.7(%), which was higher in the initial start sample and lower in both middle stages. Nitrite reducers and nitrogen fixation were recorded at a lower abundance than observed at the middle stage, with 12.4% and 7.1%, respectively. However, nitrogen fixation was higher in abundance when compared to start samples. Sulphate reducers were also observed at a lower abundance (11%) than those of both the middle stages, while sulphite oxidisers were slightly higher than both the middle stages with 13% in the predicted pathways. Both these previously mentioned pathways were lower than the initial start samples. Chitin degradation (2.4%), xylan degrader (6.3%), sulphite oxidisers (0.7%) and chlorophenol degraders (0.3%) were lower in

abundance than observed in the middle stages and start of the AD processes, while sulphur reducer remained relatively the same. Atrazine metabolism was recorded at 1.1 %, which increased again at the end of the AD system.

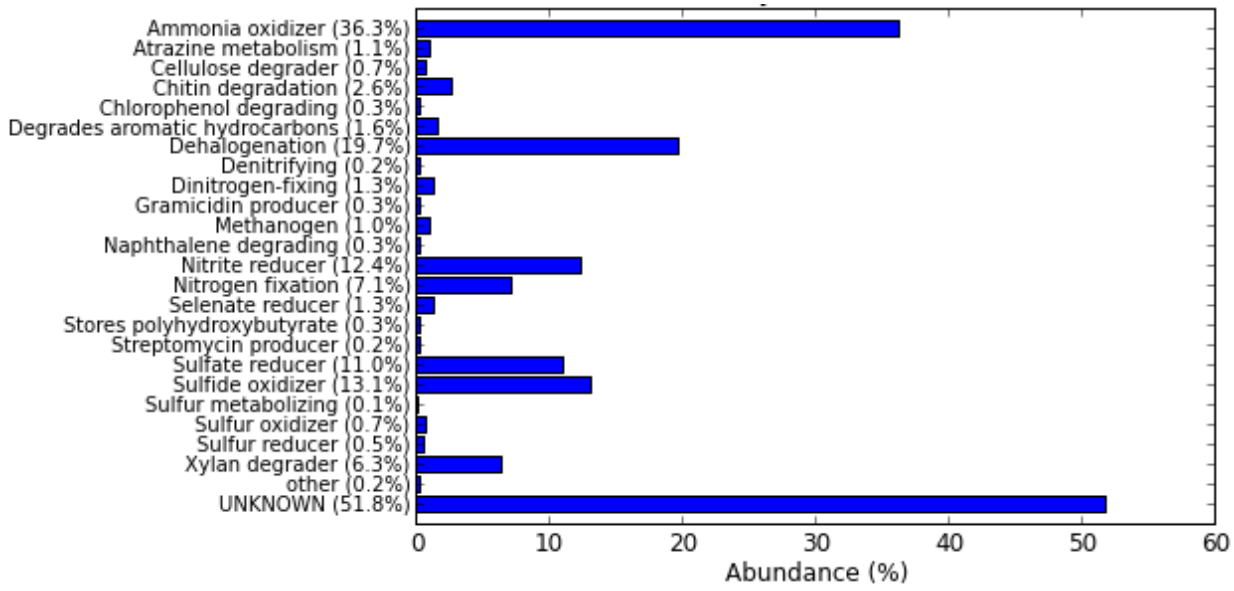

Figure 4-38: Metabolic pathways obtained during the end stage (T3) of mesophilic conditions of samples using METAGENassist

Figure 4-38 shows the predicted metabolic pathways during the end stage (T3) of thermophilic conditions. During this stage, the most abundant metabolic pathways were recorded as unknown (47.9%); the second-most abundant pathways were ammonia oxidisers (39.8%), which was higher than the middle stage T1, followed by dehalogenation (18%), which was lower than the initial start sample and both middle stages.

Nitrite reducers and nitrogen fixation were recorded as lower in the end stage than was observed in both the middle stages with 13.3 % and 8.4%, respectively. However, when compared to the start sample, nitrite reducers remained lower than observed at the start, but nitrogen fixation increased from start samples. These pathways showed relatively the same abundance when observed in mesophilic conditions. Sulphate reducers (13%) were observed at lower levels than those of the middle stages and start stage. Sulphite oxidisers were observed in lower abundance (9.1%) when compared to the middle stages and start stage of the predicted pathways. Chitin degradation (6.3%) and sulphur reducers (0.08%) were substantially lower than observed in the start and middle stages of the AD processes. Atrazine metabolism was recorded at 8.4%, which was higher than both the initial start and middle stages. Xylan degrader (10.5%) and sulphur

oxidisers (2.3%) recorded increased abundance from the T1 middle stage and T2 middle stages but were still recorded with abundance lower than at the start stage.

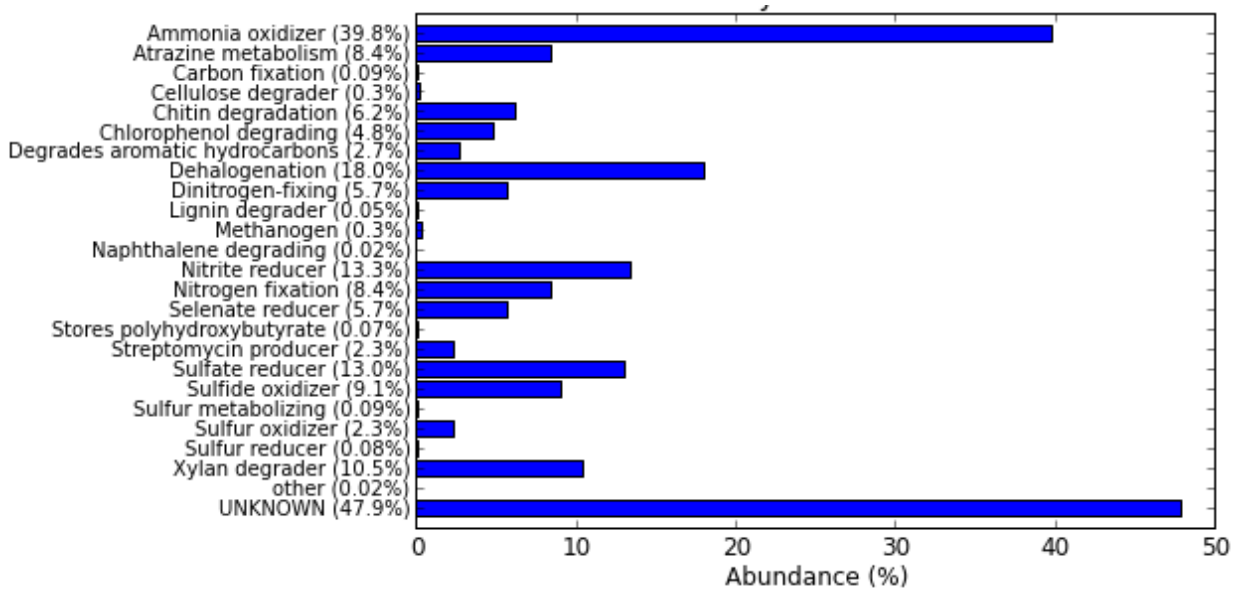

Figure 4-39:Metabolic pathways obtained during the end stage (T3) of thermophilic conditions of samples using METAGENassist

CHAPTER 5 DISCUSSION

Mesophilic and thermophilic AD of faecal sludge and composite sludge (a combination of faecal and seeding sludge) were investigated in this study to determine the performance of bench-top batch reactors and their microbial dynamics. The AD process was characterised by changes in community structure associated with biochemical and physico-chemical changes. In this study, when comparing the composited samples with seed and faecal samples (Figure 4-1 A and B), it was seen that composite samples was seen with a lower gas yield than seed, but higher when compared to faecal samples. Comparing the gas yield in these samples, can also be said that the COD levels in faecal sludge and nutrient content have played a part in the performance of composite samples by lowering the COD concentration. The data in this study are based on faecal sludge and seeding sludge obtained from the WWTP and the biogas plant near Bronkhorstspruit at a given time (as mentioned in Chapter 3) and are based on the system performance throughout a term and can differ from data obtained in other studies. Composite samples are the focus of this section, whereas faecal and seed is used as a reference sample. The mesophilic and thermophilic conditions discussed throughout this section is as follows: bioreactor performance; general trends of physico-chemical properties during AD; methane yield and composite during both thermophilic and mesophilic conditions of faecal, seed and composite samples; an investigation of the microbiota associated with mesophilic and thermophilic conditions during the process of each sample used in AD, and an investigation of the metabolic pathways associated with mesophilic and thermophilic conditions.

5.1 Mesophilic and thermophilic reactor performance based on biogas production and composite during both AD of faecal, seed and composite samples

Mesophilic and thermophilic conditions in this study recorded higher biogas yield in mesophilic conditions than recorded under thermophilic conditions. Faecal sludge shown aerobic conditions throughout the digestion proses and could have been due to the system malfunction due to reactor setup. All runs were flushed with nitrogen to create anaerobic digestion environment. But faecal conditions still proven to contain oxygen, which could be due to human error on the system or the system itself. But evaluating seed and composite samples the effects of faecal sludge in a digestion system can still be observed. According to a study done by Yenigün and Demirel (2013), thermophilic conditions produce a lower biogas yield than mesophilic conditions if ammonium is present in high concentrations. Thus, confirming that the high concentration of ammonium observed in thermophilic conditions (Table 4-1 and Table 4-2), contributed to the lower gas yield seen in Table 4-3, and was one of the main inhibitor factors of AD apart from the low VS/TS. After 10-11 days, biogas production decreased in thermophilic conditions, indicating that the biological process was near completion or was inhibited by other factors such as microbial inhibition,

composition of materials, total solid/volatile solids and temperature effects. According to a review done by Gebreeyessus and Jenicek (2016) on mesophilic and thermophilic conditions of biogas production, it was found that most studies, when comparing these two conditions, mostly focus on the biogas production and speed potential rather than the macro-elements present. In this review by Gebreeyessus and Jenicek (2016), it was also stated that thermophilic AD showed promising results. This current study shown contradictory results, due to mesophilic conditions preforming better than thermophilic conditions. Thermophilic AD did not shown promise in this study seen in Figure 4-2 when compared to mesophilic conditions in Figure 4-1. Though thermophilic conditions indicated promising results in most studies, it is still unclear if the subsequent steps are improved or in some cases, the rate-limiting step (De la Rubia *et al.,* 2012; Ge *et al*., 2011). Unfortunately, little or inconclusive research has been done on the comparison of thermophilic and mesophilic digestion processes based on COD, pH, ammonia, nitrogen, and other macro-elements of faecal sludge in South Africa system. This needs further investigating in future studies with faecal matter as Co-digestion substrate (Gebreeyessus & Jenicek, 2016).

During this study, CODs recorded in composite samples of mesophilic and thermophilic conditions had lower COD values than the seed reference samples of each stage. This is mainly due to the lower levels of CODs found in faecal samples which, when observed in Table 0-1 and Table 0-2 (Appendix A: Parameters during anaerobic digestionAppendix A: Parameters during anaerobic digestion), showed substantially lower COD levels than that of seed samples. According to a statement made by Dobre *et al.* (2014), COD values in a substrate can predict the potential biogas gas production to an extent, and with higher COD levels, higher gas potential can be expected – if the system nutrient, pH and temperature are optimal. Therefore, it can be said that faecal samples lowered the COD concentration (organic and inorganic material) in composite samples, and as a result, lowered the gas yield observed in composite samples when compared to seed samples. It can be alleged that faecal sludge lowered the efficiency of biogas production in composite samples. The CODs found in this study were highly variant in the AD process especially in different substrate types, but a general trend was observed in both mesophilic and thermophilic conditions COD decreased during AD in all substrates, when comparing start with end samples. Though COD levels give an estimated level of organics present in substrates, soluble and insoluble organics also play an important role and determine the number of organics available for normal organism function. TS and VS are other factors influencing biogas reactor performance. The TS and VS in this study were much lower than that observed in the study done by Yi *et al.* (2014): a TS removal of 26.5% after 30 days of food waste. During this study, a TS removal percentage between 8%-15% of the organics was recorded in both mesophilic and thermophilic conditions, supporting the fact that more complex material was present during this study.

The performance of substrates in mesophilic and thermophilic conditions also depend on the pH and macro-elements in a system (Gebreeyessus & Jenicek, 2016). Although the pH illustrated small changes between mesophilic and thermophilic conditions, recorded data showed that the digestate from thermophilic conditions had a higher pH value throughout the AD proses than that observed in mesophilic conditions – which was also previously observed by Labatut *et al.* (2014). In a study conducted by Moset *et al*. (2015), pH values of 8 and higher were recorded in thermophilic conditions of cow manure during AD supporting the high levels found in this study. According to Nagy and Wopera (2012), a pH level of above 8 can have a toxic effect on the acidogens if high amounts of ammonium are present, which leads to the inhibition of such organisms – and can explain the low levels of gas yield and low levels of methane production seen in thermophilic conditions of this current study. During this study, the pH values were recorded between 6 and 8, which is in its preferable ranges for anaerobic conditions. According to Cioabla *et al.* (2012), normal reactor pH range is from 6 to 7.5, but it was also recorded that normal reactor processes can occur in a pH range of between 6 and 8, due to the tolerance many of these organisms have towards the pH in such a reactor. According to a review done on microbial communities and gas production by Möller and Müller (2012), performance is also influenced by macronutrients such as nitrogen, phosphorus, sulphur, ammonium and carbon content present (Gunes *et al.,* 2019; Sheng *et al*., 2013). Insubstantial amounts of these microelements can have a major effect on the AD proses and are thus a limiting factor (Gunes *et al.,* 2019). In this study, nutrients within each reactor condition were analysed for NH_4, PO_4, NO_3, SO_4 and percentage carbon content (carbon percentage in Appendix).

In the present study, phosphorous levels varied between substrates but remained relatively low during AD. During the mesophilic conditions of faecal samples value of 166.97-155.31 mg/l were recorded, whereas seed and composite samples had values of 3.54-8.70 mg/l and 1.6- 3.2 mg/l. Thermophilic conditions recorded values of 155.84-62.65 mg/l in faecal samples and values of 5.89-1.99 and 13.3-1.9 mg/l in seed and composite samples Marco elements such as phosphorous play an essential role in the metabolism of ADP and ATP which, are largely needed by methanogenic organisms to produce methane. Phosphorus is an important building block for bacterial and methanogenic archaea, which helps with growth and is needed in large quantities during AD (Belostotskiy *et al.,* 2015). In a study conducted by Lei *et al*. (2010), data of phosphorus levels between 414 mg/l and 465 mg/l delivered more promising biogas yields during AD than that observed in this study. Stabnikov *et al.* (2003), found that much higher phosphorous concentrations using household waste and activated sludge (with phosphate levels between 971 mg/l and 3500 mg/l) delivers higher gas yields than that under 900 mg/l and above 3500mg/l,

which can also explain the low methane yields observed in this study. None of the substrates in this study achieved the above-mentioned values of Stabnikov *et al.* (2003) or Lei *et al*. (2010).

During the AD process in the mesophilic composite and seed samples, it was recorded that an increase occurs in phosphorous levels. According to Stabnikov *et al.* (2003), this can be due to phosphorus-containing material, when decomposed, releases phosphorus into the environment. Thermophilic conditions showed a decrease in phosphates in all substrates and was due to the rapid decomposing of materials because of the high temperatures, whereas mesophilic conditions decompose material at a much slower rate, phosphorus is a important building block for organisms to form DNA and cell membranes (Cho, 2019). Although faecal sludge (digested aerobically) had more phosphorous present, but a lower COD level were observed, thus contributed to the lower biogas yields in composite samples compared to seed.

Although sulphate levels in this study were relatively low, all recorded a decrease as expected (Montalvo *et al.* 2019). The decrease is due to a shift in pH through microbial activity that changes sulphate into their gaseous (H_2S) or aqua (S^{-2}) form by sulphate reducing bacteria (Traversi *et al*., 2015). In this study, sulphates in composite samples of mesophilic and thermophilic conditions had lower levels than those observed in seed and higher levels than those observed in faecal samples. In the mesophilic composite samples, an initial sulphate level of 19.07 mg/l was recorded, while thermophilic had an initial level of 21.51 mg/l. During AD, low levels of H_2S were produced, which ranged between 5 and 8 ppm for mesophilic conditions and between 6 and 10 ppm for thermophilic conditions. It can therefore be concluded that sulphate did not play a significant role during AD of both conditions. Sulphate reducing bacteria in an anaerobic environment uses sulphate as an electron acceptor during the oxidation of a sulphate substrate, which leads to the production of H_2S (Boshoff *et al.,* 2004; Madden *et al.,* 2014). This can explain why the concentrations of sulphates in the system remained relatively the same. It can also be concluded that sulphate levels had no influence on the methane production, due to sulphate reducing bacteria not being able to reduce hydrogen below a minimum threshold for methanogens to produce methane (Madden *et al.,* 2014).

The ammonium level in seed samples recorded the highest concentration in both mesophilic and thermophilic conditions, followed by composite and then faecal sludge. Though few studies have been done in the North West Province on AD of faecal sludge obtained from septic trucks, data on faecal sludge in South Africa is increasing. According to Yenigün and Demirel (2013), substrate containing <200 mg/l ammonium content is optimal for microbial growth, which provides essential nutrients. However free ammonium nitrogen, exceeding concentrations of 1500 mg/l, and above will inhibit microbial activity, especially organisms such as methanogens. Furthermore, Jo *et al.*

(2018) report that ammonium concentrations above 100mg/l cause significant methanogenic inhibition. Seed and composite samples had values near 1500 mg/l, which confirmed that lower microbial activity was a major factor in methane activity influencing biogas production (Ryue *et al.,* 2019). According to a review by Yenigün and Demirel (2013) on the ammonium inhibitors during AD, ammonia in a reactor is not inhibited by ammonium ions but rather through FA in the system, and total ammonium nitrogen concentrations above 1700-1800 mg/l cause complete reactor failure. In anaerobic digesters, animal manure is commonly used as a substrate, often containing high FA due to its high protein content (Li *et al.,* 2015). It was also stated that FA in thermophilic conditions causes thermophilic conditions to produce lower biogas yield, supporting the low levels of biogas found in this study during thermophilic conditions, thus signifying that it was attributed due to FA inhibition. Higher temperatures increase the concentration of FA present (Chen *et al*., 2008; Jo *et al.*, 2018).

In this study, during the AD of the composite sample, an average of 11.96 % methane was produced during mesophilic conditions and an average of 10.96% was produce during thermophilic conditions. Methane levels achieved in this study was regarded as poor. Anaerobic digester seen at levels of ~65% methane levels are generally viewed as a success (Ros *et al.,* 2017) The poor methane results in this current study can be due to inhibitors present during AD, which can be a combination of elements such as ammonium, sulphide, heavy metalsmand organics. (Chen *et al.,* 2008) Observing the inhibitors during this study of the composite sample, higher levels of ammonium were recorded than that observed in faecal sludge, which explains the low levels of methane yield recorded in both mesophilic and thermophilic digesters, which inhibits methane activity. Both seed and composite samples almost reached the levels of complete failure, as stated by Yenigün and Demirel (2013). This could also explain the low levels of gas production during AD in these two conditions. In a study done by Jo *et al.* (2018), it was found that ammonia levels exceeding 100 mg/l inhibited microbial activity, especially methanogens a microbe highly sensitive to ammonium.

According to Ghyselbrecht *et al*. (2017), nitrates and nitrite were used as electron acceptors in their study, and denitrification that occurred had a strong possibility of reducing the development of anammox bacteria. High levels of nitrates and nitrites can inhibit their own denitrification. When there is no other form of electron donor, these systems begin to use methane as their source of electron donor, which can explain the decrease of methane production during Timeline 2 of the composite samples. In such systems, many electron donors are present and can be acetate, sulphite, formic acids and in some cases, methane (Ghyselbrecht *et al*., 2017; Jingura & Kamusoko, 2017). The course of changes in the concentration of NO_3 levels found in a study done by Sheng *et al.* (2013), concluded that NO_3-N levels between 500-750 mg/l improved the

microbial activity and methane production. In this study, NO_3 levels were observed between 2.44-18.76 mg/l. This is substantially lower than the optimal concentration found in the study done by Sheng *et al.* (2013) on cow manure and can also contribute to the low biogas yields obtained during the study. Ghyselbrecht *et al*. (2017) used nitrite and nitrate during AD and clearly demonstrated in their study that nitrite and nitrate were denitrified during the process which delivered nitrogen gas. Although ammonia inhibition took place, the performance of the reactors can still be used to determine if faecal sludge collected from WWTP can be used for AD, and even compost. The microbial dynamic can also be determined to see if faecal samples contributed towards reactor performance.

5.2 Microbial communities

AD consists of four major stages, namely hydrolysis, acidogenesis, acetogenesis and methanogenesis; each of these stages consist of various types of microbes to produce nutrients for biogas and to be used by methanogens for methane production (Meegoda et al., 2018). During the hydrolysis stage, organisms break down complex polymers such as carbohydrates, fats and proteins into sugars, fatty acids and amino acids and are mostly part of two phyla: Bacteroidetes and Firmicutes (Ali-Shah *et al.,* 2014). Then after these nutrients are produced, acidogenic bacteria such as Firmicutes, Bacteroidetes, Proteobacteria, Chloroflexi and Actinobacteria convert the nutrients produced in the hydrolytic stage into ammonia, carbon dioxide hydrogen, alcohol and short-chained fatty acids. Furthermore, acetogens convert products such as butyrate, propionate, lactate and ethanol to acetate, formate and H_2/CO_2 by the group syntrophic acetogens, whereas methanogens use these products produced during the acidogenesis and acetogenesis stages to form methane gas (Ali-Shah *et al.,* 2014; Voicu *et al.,* 2015; Weiland, 2010). The full description of these stages can be found in Figure 2-3. The major phyla identified in both mesophilic and thermophilic conditions of composite seed and faecal samples are Chloroflexi, Firmicutes, Proteobacteria, Bacteroidetes, Synergistetes, Planctomycetes, Actinobacteria and Euryarchaeota. The major families identified are *Anaerolineaceae, Caldilineaceae, Planctomycetaceae, Synergistaceae, Methanosarcinaceae, Porphyromonadaceae, Ruminococcaceae* and *Clostridiaceae 1.* Most of these phyla and families are associated with the AD process. Composite samples are the focus of this section as they were used to investigate the effects of faecal sludge in an AD system. Whether comparable relationships emerge between mesophilic and thermophilic biogas communities has, however, to be decided (Westerholm & Schnurer, 2019).

5.2.1 Investigating the domain and phylum within mesophilic and thermophilic conditions of composite samples in AD

In this study, the Domain ratio of Archaea to bacteria where investigated in mesophilic and thermophilic conditions which was recorded that archaea were underrepresented in all samples which hold major implication towards methane production although methane was produced by methane producing bacteria, the major methane producing organisms according to literature are from the domain archaea and includes three different types of methanogens such as: acetolactic, hydrogenotrophic and methylotrophic (Venkiteshwaran *et al.,* 2016). The ratio between archaea and bacteria needs future investigating. Observing the ratio between bacterium and archaea (20:1) it can be said that due to seeding sludge not completely adapted or not correctly fed, a lower quantity of archaea was present, although reactor setup was done directly after seed collection another reason could be that the seeding sludge collected at the biogas plant was old. Alternatively, faecal sludge obtained from septic truck was already digested or water down to make extraction of faecal from pit latrines easier and therefore decreasing organic materials for organism to thrive on.

It must also be noted that the ratio between archaea and bacteria can also be influenced by operational conditions such as pH, Temperatures and nutrient presence in pit latrines. Organisms from functional groups such as hydrolytic, acidogenic, acetogenic and methanogenic organisms flourishes in different reactor conditions and therefore achieving these conditions seen in this present study proved difficult throughout the AD proses and an optimal condition for these organisms to thrive in needs to be investigated more in depth when using substrate such as faecal sludge which is known to have an imbalance of nutrients depending on the location, storing mechanism and storage time (age). Methanogenic organisms are particularly sensitive to changes such as pH, temperature and high levels of volatile solids and macro element compounds. Temperatures during AD plays a major role in methanogenic bacterium due to its limited temperature resistance for enzymatic structures (Ali Shah, *et al.,* 2014).

The dominant phyla in this study (Figure 4-11 to Figure 4-17) recorded at mesophilic and thermophilic samples were identified as Chloroflexi, Firmicutes, Proteobacteria, Bacteroidetes, Synergistetes, Planctomycetes, Actinobacteria and Euryarchaeota. Each of these phyla plays a major role in biogas productions. Similar results were found in a study done by Rivière *et al.* (2009), on seven full scale anaerobic digesters selected in France, Germany and Chile that identified AD microbes belonging to the phyla Chloroflexi, Proteobacteria, Bacteroidetes and Firmicutes which is the major bacterial phyla present in anaerobic digesters of sludge. These results and other studies on AD reported similar results. A study conducted by Changara *et al.,* 2019, on mesophilic AD of fresh faecal sludge collected at pit latrines mixed with cow manure,

identified Proteobacteria, Firmicutes Spirochaetes, Bacteroides, Actinobacteria and Planctomycetes as the major phyla groups present. Ozbayram, *et al*. (2018), investigated the microbiota of cow manure during anaerobic digestion and found phyla Firmicutes Bacteroidetes Lentisphaerae, Proteobacteria and Verrucomicrobia as the major groups commonly present, therefore supporting the finding in this study.

Temperatures during AD is one of the most important parameters, which strongly affects the microbial diversity, biogas yields and stability of the process. Thus, it is important for an adaption period when setting up a thermophilic reactor, even a slight change of temperature between 5-10 degrees can have a significant effect on the microbial dynamics which can cause imbalance in fermentation, acetogenesis and methanogenesis (Saha *et al.,* 2020). Thermophilic conditions are normally associated with a decline in microbial diversity and population due to the continues accumulation of volatile fatty acids. Phyla identified in a study done by Zahedi *et al.,* 2016 on the changes of microbial community during hydrogen and methane production in a two stage thermophilic anaerobic co-digestion (Municipal waste and WAS activated sludge) and Li *et al* (2015) on the solid-state anaerobic digestion operated at mesophilic and thermophilic temperatures reported phyla Chloroflexi, Firmicutes and Bacteroidetes to be dominant in a steady-state mesophilic anaerobic condition whereas members of the phyla Thermotogae, Synergistetes and Firmicutes are more dominant in thermophilic conditions. (Saha, *et al.,* 2020).

Firmicutes and Bacteroidetes amongst the bacteria phyla have been suggested to play an important role during the Hydrolytic and acidogenic phase of anaerobic digestion (Sun *et al.,* 2015). In previous studies these organisms have also been shown as important microbes in swine manure and wheat straw (Li *et al.,* 2014). According to Chen *et al.,* (2016) Firmicutes and Bacteroidetes were found to be the two dominant phyla in most anaerobic digestion processes and the abundance of these organisms are typically dependant on to process parameters such as organic loading rate, volatile fatty acids and microelements. The population ratio was suggested by Chen *et al.,* (2016) between Firmicutes and Bacteroidetes as a possible indicator of AD performance and was found to be linked to methane production, organic loading rate and VFA concentration. Firmicutes and Bacteroides are known in an anaerobic digester to be syntrophic bacteria which plays a part in Hydrolysis, acidogenesis and acetogenesis which includes genus such as *Acetobacterium, Clostridiums, Ruminococcus, Eubacterium* and many other such as *Sporobacterium, Lactobacillus* etc (Tezel *et al.,* 2011; Liu et al., 2016; Wachemo et al., 2019). Firmicutes, Bacteroidetes and Proteobacteria are well-known degraders of a variety of compounds (Zhi & Zhang, 2019). Zamanzadeh *et al.* (2016), conducted a study on a laboratory scale digester to see what affect temperature have on the performance of AD in swine manure/cow manure, it was concluded under high FA certain phyla are selected that can tolerate FA. Firmicutes especially *Clostridium sensustricto* was found in their study in high abundance due

to several syntrophic acetate oxidation species belonging to the phyla Firmicutes and can explain the high Abundance of Firmicutes found in our study, and therefore can conclude that Firmicutes, played a major role in AD under high FA concentrations (Li *et al.,* 2015).

In this present study seen in Figure 4-12 to 4-17. Firmicutes played a significant role in all timelines throughout mesophilic conditions with a decreased from 39% to 34%, (Fig4-14) in Seed increase from 15% to 34% (Fig4-16) in composite and 3% to 4% (Fig4-12) in faecal of mesophilic conditions, whereas thermophilic increased from 42% to 52%(Fig4-15) in seed, 31% to 40% (Fig4-17) in composite, and 3% to 22% (Fig4-13) in faecal samples. Demonstrating that Firmicutes played a significant role in digesting a variety of compounds in mesophilic and thermophilic conditions. Firmicutes remained relatively dominant throughout the digestion period supporting the fact that Firmicutes played a major role in hydrolysis, acidogenesis and acetogenesis of both mesophilic and thermophilic conditions (Li *et al.,* 2015). Thus, also supporting the fact that Firmicutes Bacteroidetes and Proteobacteria have the potential to degrade a variety of compounds. Bacteroidetes was also one of the dominant phyla found in this study throughout AD, but as observed by Li *et al* (2014) on the solid-state anaerobic digestion on mesophilic and thermophilic processes, it was seen that in mesophilic conditions Bacteroidetes recorded higher abundance than that of thermophilic conditions. Mesophilic conditions recorded 20% abundance while thermophilic conditions were recorded at 6% explaining the low observed during thermophilic conditions.

Proteobacteria are one of the phyla present during anaerobic digestions due its ability to produce acid. Proteobacteria includes Alpha- Beta- Gamma and Deltaproteobacteria that forms part of the glucose, propionate, butyrate and acetate- utilizing microbial communities (Guo *et al.,* 2015; Ariesyady *et al.,* 2007) Proteobacteria, Firmicutes and Bacteroides represent major bacteria phyla involved in hydrolysis, acidogenesis and acetogenesis stages (Díaz *et al.,* 2018). In this study observed in Figures 4-12 to 4-17 it can be see that Proteobacteria where present in all timelines of mesophilic and thermophilic conditions of all substrates but was most dominant in faecal than in composite and seed but was seen to increase during AD. Observing the mid stages of all samples mesophilic and thermophilic (T1 and T2) and end stage (T3) it can be seen that a slight increase was first observed in the T1 and an increase after that was observed at T2 which relatively stayed similar towards the end of thermophilic conditions suggesting that Acetogenesis occurred at T2 and T3. Mesophilic decreased till T2 and increase again in T3 illustrating that Acetogenesis occurred more in T3 at the end of the stage. Proteobacteria are mostly organisms in digester as syntrophic core communities and are part of the hydrolysis, acidogenesis and acetogenesis stage (Nakasaki *et al.,* 2019). In a study conducted by Sun *et al.* (2016), on the mechanism and effect temperature have on the antibiotic resistance gene during AD of cow

manure, it was found that Proteobacteria decreased during AD in thermophilic conditions, while during mesophilic conditions remained on similar abundance. Inhibition of Syntrophic metabolism are likely found to occur during high concentrations of FA, mainly found in the phyla Proteobacteria, Chloroflexi, Synergistetes and Planctomycetes (Li *et al.,* 2015).

Actinobacteria are known for they role in the fermentation properties in waste activated sludge during anaerobic digestion and may also be part of the organisms group degrading Food waste into VFA (Wan *et al.,* 2018; Li *et al.,* 2016). These organisms from this phylum are normally identified as foam producing bacteria. Mesophilic conditions in this study have shown that lower levels of Actinobacteria were found compared to thermophilic condition. This phylum was observed dominant in the middle stages of AD in both mesophilic and thermophilic condition. Thermophilic conditions shown more abundance and was noted as the 3de most abundant phyla, while mesophilic abundance of this phyla was noted as the 4th most abundant phyla in composite samples (faecal samples shown less abundance compared to seed and composite in both conditions). Results found in this study were contradicted to the results found by Ritari, *et al.* (2012), which conducted a study on the mesophilic and thermophilic microbiota of a stirred tank reactor of biowaste and sewage sludge which observed a lower abundance in thermophilic conditions than in mesophilic conditions respectively. During this study Actinobacteria increased to 2nd most abundant phyla at T0 and decreased to 17.85 at T3 and decreased to the 4th abundant phyla. Seed thermophilic conditions also had the highest abundance compared to the other substrates. Members of this phylum respectively, are less recognized to form part of the biogas producing system and are more affiliated with deceases such as leprosy or diphtheria. The high abundancy of this phylum could be due high amounts of excrement (manure of Cow/livestock), nonetheless these organisms in recent years have become mostly known for decomposing recalcitrant organic matter, especially lignocellulose (Theuerl, *et al.,* 2020).

The phyla Thermotogae is more associated with thermophilic conditions than in mesophilic condition (Lin *et al.,* 2017) Members of this phyla generally degrade polysaccharides to ethanol acetate, CO_2 and H_2, but was also reported to be involved in the degradation of alcohols to CO_2 and H_2 while in a syntrophic association with hydrogen-consuming partners. (Westerholm & Schnurer, 2019). In a study done by Pervin *et al.,* 2013 on the drivers of microbial community composite in mesophilic and thermophilic temperature-phased anaerobic digestion of pre-treatment reactors, recorded a significant increase in Thermotogae phyla during the increases of temperatures, and concluded that Thermotogae are more Abundant at 50 ºC and 60 ºC. While a study by Muas *et al.* (2016), unravelling the microbiome of a thermophilic biogas, found that microbes at thermophilic conditions are mostly dominant in the phylum Firmicutes, Thermotogae and Bacteroidetes. Which supports the high levels of Thermotogae recorded in thermophilic condition, and the low levels present during mesophilic condition. In this study thermophilic

conditions seen in figure 4-12 to 4-17 contained more of the phyla Thermotogae at the end of AD than that of mesophilic conditions of Composite and faecal, whereas seed remained similar in abidance throughout Mesophilic and thermophilic conditions.

Synergistetes occupies numerous anaerobic environments and are known for fermenting carbohydrates, organic acids, cellulose, sugars and hemicellulose into H_2 and acetic acid derivation (Fitamo *et al.,* 2017). It is also known that members of Synergistetes and Chloroflexi can perform syntrophic metabolism with hydrogenotrophic methanogens during AD (Li *et al.,* 2015). In this present study it was seen that that increased during mesophilic conditions at the middle phases (T1 and T2) of the AD process occurred and then decreased during the end samples for Synergistetes. In theomorphic conditions these phyla were present throughout anaerobic digestion. In a study conducted on Lab Scale digester by Zamanzadeh *et al.,* (2016) on food waste and waste sludge, it was found that Synergistetes Chloroflexi, Thermotogae, Firmicutes and Bacteroidetes contributed to the hydrolysis and acidification to sludge substate. Observed in thermophilic conditions Synergistetes at the middle and end (T3) of AD was noted lower than that of mesophilic conditions.

In this study, the phyla Chloroflexi was reported in high abundance at the initial start sample during anaerobic digestion of faecal and composite sample but was underrepresented in seed although metabolic function is still not clear it for a significant part of the phylum Chloroflexi on genus and specie level, it was reported by Speirs, *et al.* (2019), that it played a part in carbohydrate degradation and is an important phyla for providing filamentous platform for the formation in which flocs are formed around organic compounds and feeds and ferment carbohydrates of complex polymeric organics to simple organics for other bacteria growth. However, Chloroflexi was reported in high amount in faecal and Composite samples which, was one of the major phyla during anaerobic digestion. This phylum was more common in plant processing sewage sludge. (Petriglieri *et al.,* 2018; Sun *et al.,* 2016)

As mentioned, and stated by Kirkegaard *et al.,* (2017) in a study conducted on full scale anaerobic digesters of wastewater, phyla such as species in the phyla Chloroflexi, Bacteroides and Firmicutes need to be the target for future research, due to the unknown potential some of the unknown species have on the systems. When observing Figure 4-12 to 4-17 mesophilic conditions, Chloroflexi respectively, decreased substantially within mesophilic conditions suggesting that these organisms only plays a role in the beginning of the composite samples and when observing thermophilic initial levels of Chloroflexi was shown to be lower than that of mesophilic conditions, but in both a decrease during anaerobic digestion occurred supporting that these organisms was the dominant phyla in oxygen and also supporting the that faecal sludge was digested in an aerobic system. In situ studies of the characterisation of the of Chloroflexi in

activated sludge, suggested that they are fermentative facultative anaerobes and may have the potential to survive in the AD environment.

Euryarchaeota, the phylum that the majority methanogens belong to with a couple of exceptions (Tao *et al.,* 2019). The genera *Methanospirillum, Methanoculleus, Methanosarcina* and *Methanomethylovorans* are normally the dominant groups found within Euryarchaeota (Moset *et al.,* 2015). This phylum is one of the main groups investigated during AD, generally the level of Euryarchaeota in a system can predicted the concentration of methane (Lotta *et al.,*2007). In a study conducted by Lotta *et al.,* (2007) on the effects of temperature in a Two methanogenic bioreactor treating organic waste, it was found that in mesophilic conditions had lower abundance of this phylum while Thermophilic respectively illustrated higher abundance supported by Moset *et al.,* (2015). In this study contradictory results were found with much lower abundance observed during thermophilic conditions than there were documented in mesophilic conditions. This could be due to ammonia inhibition, ammonia concentrations in a substrate can significantly influence methane production, especially in high temperature levels, which increase Free ammonia. Free ammonia (FA) can have a toxic effect on Methanogens in levels above 150 mg/l (Yenigün & Demirel, 2013).

5.2.2 Investigating the order and family within mesophilic and thermophilic conditions in AD

In this study, mainly members of the order (Figure 4-18 to Figure 4-23) Clostridiales, Unassigned, Corynebacteriales, Synergistales, Pseudomonadales, Petrotogales, Caldilineales, Bacillales, Anaerolineales, Bacteroidales, Planctomycetales and Methanosarcinales were found in mesophilic and thermophilic conditions. Families identified in mesophilic conditions where *Synergistaceae, Methanosarcinaceae, Anaerolineaceae, Corynebacteriaceae, Planctomycetaceae, Ruminococcaceae, Caldilineaceae, Petrotogaceae,* and *Marinilabiaceae* and in thermophilic conditions families were *Corynebacteriaceae, Synergistales, Moraxellaceae, Family XI, Heliobacteriaceae, Caldilineaceae, Anaerolineaceae, Planctomycetaceae, Ruminococcaceae, Bacillales* and *Methanosarcinaceae* during AD (Figure 4-24 to Figure 4-29). Although Unassigned in both order and families were most dominant in some reactor conditions of faecal, seed and composite, future studies on these organisms are of value and need to be investigated further.

Two abundant orders were identified from the phyla Chloroflexi, namely Anaerolineales and Caldilineales. The order Anaerolineales and family *Anaerolineaceae* are in high concentrations and core order found in sewage sludge (Puig-Castellví *et al.,* 2020), thus explaining the high amounts found in the composite samples. Anaerolineales are identified as part of the

hydrolytic/fermentative bacteria group. The members of this order are known to ferment sugars in AD and have a role in degrading a variety of carbohydrates, including xylan (Ambuchi *et al.,* 2016; Azman *et al.,* 2016). This order and family, respectively, are known to grow together with a hydrogenotrophic partner and could cause a decrease in hydrogenotrophic methanogens if a decrease occurred in Anaerolineales, which could lead to a possible decrease in methanogenic functions (Yamada *et al.,* 2018). The order Anaerolineales has a sole family *Anaerolineaceae* (Yamada *et al.,* 2006). The identified family *Caldilineaceae* from the order Caldilineales is known as a robust population which mainly grow on complex polysaccharides and proteins by secreting exoenzymes (Nielsen *et al.,* 2009; Yoon *et al.,* 2010). High concentration of these organisms is generally due to their high robustness characteristics and slow degradability in waste activated sludge.

In this study, a great abundance of Anaerolineales was found at the beginning of mesophilic AD in composite samples and decreased further during ADs. Thermophilic digestion showed low levels of this family which could be due to the temperature threshold. The temperature range for organisms in this family was reported between 35°C and 50°C, but optimal growth was recorded at 37°C (De Almeida Fernandes *et al.,* 2018; Yamada *et al.,* 2018). This could explain the high levels found at the beginning of the mesophilic conditions of composite and faecal samples. Seeding sludge contained no order members from Anaerolineales during AD, and therefore, it can be said that faecal sludge contributed towards the concentration of Anaerolineales. It can also be said that when the addition of faecal sludge was introduced into an environment of 35°C (conditioned seeding sludge for five days), these organisms increased drastically at the beginning of AD. Thermophilic digestion showed lower levels throughout AD. This can be explained by observing the results found in the study by De Almeida Fernandes *et al.* (2018), on the effects of temperature on microbial diversity and nitrogen removal of an anammox reactor treating anaerobic pre-treated municipal wastewater. The study showed that these organisms were optimal at a growth of 37°C. The low amounts obtained throughout thermophilic conditions of composite and faecal samples were thus due to temperatures being too far from the optimal range. Greater abundance was found in mesophilic conditions than in thermophilic conditions, which could illustrate that the populations of Anaerolineales had a higher interaction with fermentative bacteria and hydrogenotrophic methanogens (Yamada & Sekiguchi, 2009)

Caldilineales was found to be one of the main orders at the beginning of both conditions in composite and faecal samples. Caldilineales were, however, underrepresented in mesophilic conditions of composite and faecal samples with less than 1% after AD. Though thermophilic had a 2% Caldilineales population present, a drastic decreased still occurred. The percentage of Caldilineales still present after AD of thermophilic conditions could be due to the short reactor time of 15-16 days, thus supporting the fact that these organisms have robust characteristics.

Individuals of some *Caldilineaceae* species play a critical part in hydrolysis, fermentation and acetoxylation (García-Ruíz *et al.,* 2019).

The order Clostridiales is one of the major groups in the performance of AD. These organisms are seen as very important microbes during AD as they form part of the hydrolysis, acidogenesis and acetogenesis stages of biogas production. Organisms in this order such as *Clostridium thermocellum* have been very beneficial in the hydrolysis stage of switchgrass and delivered a higher methane production. According to the literature acids (Fu *et al.* 2016)., Clostridiales contains mostly members that can breakdown polysaccharide compounds and ferment sugars to organic acids (Fu *et al.* 2016). When observing the Clostridiales order obtained in this study, families *Clostridiaceae 1, Heliobacteriaceae, Thermoanaerobacteraceae* and *Ruminococcaceae,* whereas *Peptostreptococcaceae* and *Syntrophomonadaceae* were present but underrepresented. These families were identified at different stages during AD of all samples in both conditions. The order Clostridiales from class Clostridia including above-mentioned families is part of the Firmicutes phyla which under harsh conditions can form endospores. The above-mentioned families in this order were present in all conditions during AD. Some of these families were reported as highly versatile organotrophs and form part of the proses by degrading proteins, lips and complex carbohydrates (Walter *et al.,* 2018).

In this study, it was observed that *Ruminoccacaceae* increased during AD of both conditions. It was also observed in lower abundance during both mesophilic and thermophilic conditions but was seen to increase during the middle and end stage of AD. Members of this family are known to degrade cellulose and produce hydrogen as one of the fermentation products (Tian *et al.,* 2014). This family was also reported to produce propionate rather than ethanol. Family *Clostridiacaeae1* were present in very low amounts during AD of both mesophilic and thermophilic digestion. However, in mesophilic conditions, these organisms grew from 1% to 4% during the middle (2%) and end (1%) stages of AD, and in thermophilic digestion, T1 early beginning (3%) stage and middle (1%) stage T2, signifying that these organisms are highly hydrolytic, acidolysis and acetolysis organisms during AD.

In a study conducted by Tian *et al.* (2014) on the impacts of micro aeration on the AD of corn straw and microbial community structure, it was observed that Clostridiales, associated with hydrolysis processes, were most dominant in these reactors. Clostridiales were also observed in high amounts during both mesophilic and thermophilic digestion, illustrating the important role these organisms play during hydrolysis, acidogenesis and acetogenesis. This great abundance observed in these reactors illustrates the important role these organisms have in the ability to degrade polysaccharide molecules of compounds in substrates (Ren *et al.,* 2005).

Heliobacteriaceae are organisms associated with *Thermoanaerobacteraceae* as part of the polymer hydrolysis and syntrophic oxidation of acetate. *Heliobacteriaceae* were mentioned in a report by Noll *et al.* (2010) as a possible candidate for SAO. *Heliobacteriaceae* were present in both mesophilic and thermophilic digestion of seed and composite but were underrepresented in faecal sludge. During thermophilic digestion higher levels of this family were observed than in mesophilic conditions of seed and composite during the middle and end phases. Peng *et al.* (2018) conducted a study to determine the differential temperature effect on the structural and functional organisation of anaerobic food web in rice field soils and found that *Heliobacteriaceae* were more dominated in thermophilic conditions than in mesophilic conditions due to the two activity stages they form part of, namely polymer hydrolysis followed by syntrophic oxidation of acetate.

Other Clostridiales families included *Peptococcceae* and *Peptostreptococcaceae* are well-known gram-positive anaerobic bacteria that are capable of fermenting protein products (Wang *et al.,* 2020). According to El Houari *et al.* (2020), *Peptococcaceae* are part of the sulphate reducing bacteria during AD. Sulphate reducing bacteria can use sulphate and other oxidised sulphur compounds as their terminal electron acceptor for the oxidation of organic compounds or hydrogen, leading to the production of sulphide. *Peptostreptococcaceae* can ferment saccharides, alcohol and cellulose (Rui *et al.,* 2015). Both *Peptococcaceae* and *Peptostreptococcaceae* were present in composite samples of this current study but at very low amounts. In the mesophilic conditions, these species increased during the middle and end stage. Results obtained in a study done by Langer *et al.* (2019) on nine large-scale anaerobic digesters also obtained a lower level of this family at 41°C than that of mesophilic at 30°C, supporting the low levels found in thermophilic temperatures of this current.

Thermoanaerobacteraceae were one of the families that appeared in thermophilic conditions throughout the middle and end stage, whereas in mesophilic conditions, this family was underrepresented with <1%. This is due to the optimal temperature that these organisms thrive in at 50-60°C. Some members of this family such as *T. phaeum* and *S. schinkii* are identified as syntrophic acetate-oxidising bacteria (SAOB) (Westerholm *et al.,* 2017).

Synergistales is one of the orders that were dominant in the systems of mesophilic and thermophilic conditions of all samples. According to the literature, these organisms have been known to oxidise acetate during AD (García-Lozano *et al.,* 2019) The family *Synergistaceae* was abundant in both conditions during T2 middle stage of the composite sample. These organisms convert organic acids to acetate and hydrogen. This also confirms that these organisms work in a synergetic relationship with hydrogenotrophic methanogens. In a study done by Zhu *et al.* (2020) on an anaerobic vessel ecosystem enriched with H_2 and acetate, these organisms were

the second and third-most abundant family found in the system and thus correlates with the abundance found in this study.

Cellulose hydrolysers include the orders Halanaerobium, Clostridiales and Bacteroidales (Guo *et al.,* 2015; Vanwonterghem *et al.* 2014). These anaerobic cellulitis bacteria, hydrolyse cellulose to soluble sugars. Bacteroidales and Clostridiales are well-known anaerobic microbes in AD of wastewater sludge. In this study Bacteroidales in mesophilic conditions played a significant role in the beginning and middle stage of AD of all composite samples. Although, faecal sludge showed an increase of Bacteroidales during the middle and end stage of AD a lower level of Bacteroidales was found in the faecal samples compared to the composite and seed sample. Seeding sludge contained high abundance of the order Bacteroidales. It can, therefore, be said that the Bacteroidales order in composite samples was mostly contributed by the seeding sludge. Observed in thermophilic digestion, an increase in the beginning T1 and middle stages T2 was seen and then a decrease occurred, which could also explain that this system was nearly completed with digestion at the end. Mesophilic conditions recorded more Bacteroidales than thermophilic conditions. These organisms play an important role in AD, which hydrolyses and ferments organic materials. Family *Porphyromonadaceae* of the order Bacteroidales are one of the important fibre-digesting families, along with *Fervidobacteriaceae* and *Bacteroidaceae* that can enhance AD of lignocellulosic biomass (Yan *et al.* 2012). According to Gao *et al.* (2019), the increase of these families in blackwater-fed reactors could be due to the presence of lignocellulosic matter. In both mesophilic and thermophilic conditions of this current study, Bacteroidales remained relatively consistent in abundance, but it was observed that mesophilic conditions had a higher percentage recorded than in thermophilic conditions. In a study conducted by Moset *et al.* (2015), *Porphyromonadaceae* was higher in mesophilic reactors than in thermophilic reactors, supporting the finding of this study. *Porphyromonadaceae* were the second-most abundant in mesophilic conditions of their study on the AD of cattle manure and was found that these organisms, out-competed others in their optimal temperature range.

Some bacteria in the order Corynebacteriales, belonging to the phylum Actinobacteria, according to the literature are a group of organisms that contributes to the stabilisation of foam formation and increase in foaming samples. These bacteria containing mycolic acid in cell walls and can produce Biosurfactants (He *et al.,* 2017). The order Corynebacteriales contains many species of mycolic acid-containing actinomycetes. These members of this group are highly associated with hydrophobic members of this group (Davenport *et al.,* 2008). These members were implicated in activated sludge as foamers species belonging to the family *Corynebacterium* and are classified as Chemoorganotrophs with fermentative metabolism under facultatively anaerobic conditions (Bernard & Funke, 2015). These organisms have been demonstrated to form and accumulate polyphosphates under phosphate limiting conditions (Klauth *et al.,* 2006). In this study,

Corynebacteriales were identified as the second-most dominant group in thermophilic conditions and eighth-most dominant in mesophilic conditions. When observing the timeline, it can be seen that these members of this order where mostly dominant in T1 and T2 of thermophilic conditions and T2 and T3 of mesophilic conditions, supporting the finding of He *et al.* (2017) that these organisms are part of the fermentation group observed in AD. Corynebacteriales were also observed in seeding sludge as the two most dominant orders found in thermophilic conditions and the most dominant in mesophilic conditions, while faecal sludge of both conditions had underrepresented abundance in their reactors. It can, therefore, safely be said that seeding sludge contributed more to this order in composite reactors.

Methanosarcinaceae were the most dominant archaea family found in both mesophilic and thermophilic digestion. In mesophilic conditions, it was recorded that *Methanosarcinaceae* from the order Methanosarcinales were more dominant during T2 and T3, whereas thermophilic conditions were more dominant in T0 and T1. *Methanosarcinaceae* were the third-most abundant family in mesophilic conditions and only the eighth-most abundant family in thermophilic conditions. Although these members of this order were greater in abundance in mesophilic conditions than in thermophilic conditions, this could be due to the high accumulated free ammonium present in higher temperatures (Jo *et al.*, 2018; Yenigün & Demirel, 2013). These members of the order are well-known acetoclastic microbes, which use CO_2, CO, methylamines and dismethylsulphate as carbon sources, in addition to acetate and H_2 as electron donors (Caruso *et al.,* 2019; Tezel *et al.,* 2011; Whitman, 2008). Acetoclastic methanogens occur only in the order Methanosarcinales. In an environment with high ammonia and organic acid, this family of the class methanogens is found to be the most dominated within AD system, explaining the high levels observed in mesophilic and thermophilic conditions when compared to other methanogenic families. A study done by Karakashev *et al. (*2006) led to a hypothesis that organisms from the family *Methanosarcinacaea* may have the potential to oxidise acetate to H_2 and CO_2 instead of acetate cleavage to methane and then preforms hydrogenotrophic methanogenesis (Angelidaki *et al.,* 2011). In a study done by FitzGerald *et al.* (2015), the methanogen number decreased towards the end due to acetate being the rate-limiting factor, suggesting that acetic acids may be the rate-limiting factor in thermophilic digestion. Although this was not one of the elements looked at in this study, the researcher noted that ammonia in FitzGerald *et al.*'s (2015) study influenced the growth of the family *Methanosarcinacaea,* confirming that high FA was present during thermophilic AD (De Vrieze *et al.,* 2015).

5.3 The metabolic pathways associated with mesophilic and thermophilic conditions

AD is a of a series complex metabolic pathways working in synergy with several groups of microbes which degrades compounds that allow methanogens to produce methane gas and, in

the process, produces safe substrate to use as compost. In this study, there were many unidentified microbes and metabolic pathways, which is not uncommon when observing AD.

In this study, ammonia oxidation was the dominant metabolic pathway throughout mesophilic and thermophilic AD. Illustrated in Table 4-1 and Table 4-2 high concentrations of ammonium were found in both seed and composite samples. This metabolic mechanism is normally carried out by the phylum Proteobacteria and Planctomycetes; the mechanisms used are generally carried out by an anammox reaction (di Domenico *et al.,* 2015; Yangin-Gomec *et al.*, 2018). A community of anammox bacteria is driven by the removal of nitrogen under anaerobic conditions (di Domenico *et al.,* 2015). Many of these organisms in the phylum Proteobacteria and Planctomycetes oxidise ammonia and use nitrite as an electron acceptor in the absence of oxygen to produce nitrogen. In a study done by Jabari *et al.* (2016), Planctomycetes were one of the groups found in wastewater treatment plants. This explains why these organisms are one of the phyla identified in these samples and are known to reduce ammonia under anaerobic conditions. This could explain the abundance found in these anaerobic conditions (Jabari *et al.,* 2016). Proteobacteria carried out most of the ammonia oxidation during both mesophilic and thermophilic digestion. When observing phylum abundance, more Proteobacteria were found in thermophilic conditions than mesophilic conditions throughout AD, supporting that this phylum carried out most of the ammonia oxidation. Figure 4-31 to Figure 4-38 showed that ammonia oxidation was more dominant at the start and decreased a little towards the end but remained relatively stable throughout Timelines T1 to T3 (between 30% and 45%).

When observing Figure 4-31 to Figure 4-38 higher levels of ammonia oxidisers were seen throughout AD of thermophilic conditions than that observed during mesophilic conditions of each stage. This may be due to the higher levels of total ammonia, nitrogen and FA that were present under higher temperatures for these organisms to use (Labatut *et al.,* 2014; Prem *et al.,* 2020). A Study done by Moerland *et al.,* (2021) on free ammonia concentrations during (hyper-)thermophilic anaerobic digestion of wastewater, have observed that FA increases with higher temperatures and can lead to less methane production due to the toxic effects it has on organisms such as methanogens. This is supported by Chen *et al.* (2008). This can also explain the lower levels of biogas and methane yield (Table 4-4 and Table 4-5) found in thermophilic conditions. In a study done by Sung and Liu (2003), the increase in total ammonia nitrogen decreased methane production in thermophilic condition. This is also supported by García and Angenent (2009), who conducted a study on the interaction between temperatures and ammonia on anaerobic sequencing batch reactors of animal waste and found a decrease of methane when temperatures were raised from 25-38°C.

Dehalogenation, also termed in literature as Dehalorespiration, is one of the important anaerobic degradation mechanisms, mainly reductive dehalogenation (Futagami *et al.,* 2008; Löffler *et al.,*

2012; van Eekert and Schraa 2001). There are many dehalogenation mechanisms during AD, but the two most common are hydrogenolysis and hydrolysis and can occur in situ in an anaerobic system (Ghattas *et al.,* 2017). This process is generally complex when it comes to the cleavage of halogen and is dependent on the dehalogenation species present in the anaerobic system. It is now understood that microorganisms can extract halogens from aliphatic compounds by activating enzymes known as dehalogenases (Nielsen *et al.,* 2020). In the literature, mostly the members of the phyla Chloroflexi and Firmicutes are identified as dehalogenation bacteria (Löffler *et al.,* 2012; Praveckova *et al.,* 2016). The temperature changes when comparing mesophilic and thermophilic digestion were noted as small differences that remained relatively stable during the AD process.

Nitrite reducers and nitrogen-fixing are some of the metabolic pathways that were identified in these processes, and members of this pathway are mostly from the phylum Proteobacteria (Müller *&* Strous*.,* 2011). Methanogens are major contributors to nitrogen fixation (Bae *et al.,* 2018). Observing the levels of nitrogen fixation, it was recorded that in both mesophilic and thermophilic conditions the levels remained between 7 and 12% during AD but were observed at their highest during the middle and end stage of both conditions. In a study conducted by Minamisawa *et al.* (2004) on anaerobic nitrogen-fixing consortia, this process normally consists of Clostridia and non-diazotrophic bacteria supporting the abundance of Clostridia found in this study

Sulphate reducing bacteria (SRB) mainly belong to the phylum Firmicutes bacteria, and some have reported members of Proteobacteria and Euryarchaeota are also involved, which use sulphate as a terminal electron acceptor under anaerobic conditions, transforming it into H_2S (El-Houari *et al.,* 2020; Khan *et al.,* 2019). In this study, during AD at the beginning of both conditions, high levels of sulphate reducers occurred and decreased further throughout the AD processes in the absence of oxygen except for faecal samples that proven to be aerobic digestion processes. SRB can inhibit methane production, but is depended on substrate, pH and temperature. Though different temperatures were used, the level of sulphate reducers remained the same concentration during AD of both conditions, and sulphate reducers were present in high levels during AD. According to Lackner *et al.* (2020), the SRB community are well-known organisms that normally outcompete members that use H_2 and propionate under high SO_4 and thermophilic conditions; generally, acetogenic bacteria (Sun *et al.,* 2014). Under lower SO_4 concentration, SRBs rather grow as acetogens not affecting methane production in a system (Chen *et al.* 2008; Lackner *et al.,* (2020). In this study, lower levels of SO_4 were found, but higher predicted metabolism activity of sulphate reducers was recorded throughout. This could indicate the fact that these organisms affected the production of methane during AD, thus also indicates that ammonia with sulphate reducing bacteria was the main inhibitor of both mesophilic and thermophilic digestion. Almost little to no differences in this study were recorded between the

levels of sulphate reducers of both conditions. A study conducted by El Houari *et al.* (2020) on municipal anaerobic sewage sludge digesters, identified sulphate reducing bacteria as *Peptococcaceae, Syntrophaceae, Desulfobulbaceae, Desulfovibrionaceae, Desulfurellaceae.*

Sulphite oxidisers are a group of organisms mostly from the phyla Proteobacteria. These organisms reduce H_2S within an AD system and can be used to reduce the H_2S concentration in a system to promote methane production and efficient system process in a system (Boden, 2017; Fdz.-Polanco *et al.,* 2009). These organisms oxidise H_2S to elemental sulphur and sulphate of thiosulfate in an anaerobic system. In this study, sulphite oxidisers remained relatively at the same level for mesophilic and thermophilic conditions. In a study conducted by Ramos *et al*. (2014), higher temperatures removed more H_2S during the anaerobic system, both mesophilic and thermophilic digestion showed little to no H_2S production during AD, supporting the high levels of SAOB found in this study.

AD seen as effective when methane yield exceeds 70%, which is produced by methanogens 90% of the time (Ros *et al.*, 2017). Methanogenesis is affected by numerous system operations and communities. Hydrogenotrophic methanogens are in a syntrophic relationship with SAOB (Wang *et al.,* 2015) as previously stated and are dependent on acetate producing bacteria in terms of methane production. Acetate oxidation by bacteria is one of the influencing factors that can affect methane production at methanogenesis and is potentially important when it comes to the methane yield (Leng *et al.,* 2018). When observing the domain ratio in this study, the bacteria: archaea ratio (20:1) was lower than expected and could have also contributed towards the methane yield that was observed during both these conditions.

Acetate is the key precursor to the development of methane by two distinct pathways during methanogenesis: the aceticlastic pathway and the SAO pathway (Pampillón-González, 2017). During the first reaction, acetate is divided by aceticlastic methanogens into methane and carbon dioxide during the aceticlastic pathway by species of *Methanosaetaceae* and *Methanosarcinaceae* (Angelidaki *et al.,* 2011). Two descriptive segments distinguish the SAO pathway: firstly, SAOB transforms acetate to hydrogen and carbon dioxide, which includes the species *Methanococcales, Methanobacteriales, Methanocellales* and *Methanopyrales*, and secondly, hydrogenotrophic methanogens use the first segment products, namely hydrogen and carbon dioxide, to generate methane. The resistance of the microorganisms involved in the two methanogenic pathways toward ammonia toxicity is distinct. Although there are many studies available in the methanogenic composite in mesophilic and thermophilic conditions, some of them presented contradictory outcomes. However, information on the impact of the various levels of ammonia on syntrophic cultivation of SAOB and hydrogenotrophic methanogens is still lacking to date (Jiang *et al.,* 2018). In this study, it was observed that the highest abundant methanogenic family identified was *Methanosarcinaceae*, thus confirming that the acetoclastic pathway was

observed rather than the hydrogenotrophic methanogenesis pathway. *Methanosarcinaceae* in mesophilic conditions was observed in great abundance compared to thermophilic conditions. This could be due to ammonia inhibition as previously mentioned. In a study conducted by Jo *et al.* (2018), it was found that concentrations above 100 mg/l decrease methane production, thus supporting the fact that during this study, ammonia inhibition was one of the main inhibitors of methane production. In a study conducted by Prem *et al.* (2020) on the microbial community in mesophilic and thermophilic batch reactors under methanogenic, phenyl acid forming conditions, *Methanosarcina* and *Methanosaeta* species were the most in mesophilic condition. However, in this study, only the *Methanosarcinaceae* family was dominant during AD. Thermophilic conditions of Prem *et al.* (2020) were dominated by genes member of *Methanosarcina, Methanothermobacter* and *Methanoculleus.* However, in this study, only *Methanosarcinaceae* were the dominant group.

Section summary: The microbial dynamics of faecal sludge mixed with cow manure were observed during mesophilic and thermophilic AD to observe the potential faecal sludge has in an AD system combine with Cow manure. Phyla that were most abundant in mesophilic conditions were Firmicutes, Chloroflexi, Bacteroidetes, Actinobacteria, Euryarchaeota and Synergistetes, while in thermophilic conditions, Firmicutes, Actinobacteria, Chloroflexi and Synergistetes were abundant but Proteobacteria, Synergistetes and Thermotogae were more abundant. Studies in the literature agree with these phyla present during these conditions. A nutrient imbalance was observed in these systems, especially ammonia inhibitions, and had more of an effect on thermophilic conditions due to the high FA present during thermophilic conditions, therefore resulting in inhibition of methanogenesis. *Methanosarcinaceae* was the dominant methanogen family in both these conditions, confirming that an acetoclastic pathway was followed in this study. Methane production was not optimal as methanogenesis was inhibited; although inhibition occurred, the effects of and community of faecal sludge could still be determined.

CHAPTER 6 RECOMMENDATIONS AND CONCLUSION

The aim of this study was to evaluate the mesophilic and thermophilic AD of faecal sludge in terms of gas yield and the microbial consortia present during the AD proses, using a combination of approaches such as NGS and network analysis. The study objective was identified and summarised in the following section.

6.1 Reactor performance and set-up

This study was conducted using bench-top reactors with two different temperature baths. Two reactor conditions were used, mesophilic (35°C) and thermophilic (55°C). Each sample was analysed in triplicate using the same seeding sludge from a biogas plant and the same faecal sludge form septic trucks for each condition. A lot of trouble shooting was done in this study for a bench top reactor system and have influenced faecal sludge. Substrate seed and faecal were used as reference samples, while composite samples was the focus of this study, to determine if faecal sludge from WWTPs combined with cow manure (seeding sludge) can be used in a WWTP for energy and secondary treatment. Gas yields and composition of mesophilic and thermophilic conditions were evaluated and compared with macronutrients concentrations which are essential for proper system functions. The study findings in both conditions were substantially affected by the macronutrients, especially ammonia concentrations, inhibiting microbial growth and more so methanogens, but biogas production still took place. Thermophilic conditions increased FA in the systems with high ammonia concentrations, influencing the amount of biogas produced and the percentage of methane. It can thus be concluded that macro elements play an important role during AD of mesophilic and thermophilic conditions. PO_4 present in seeding sludge was another contributor to lower gas production, due to microbial communities using this as building blocks for cell formation. This could also have been a factor influencing biogas production; nonetheless, microbial community dynamics could still be determined despite inhibition taking place.

6.2 Evaluation of microbial communities to determine the effect of temperatures on the biogas system

Microbial dynamic on Mesophilic and thermophilic conditions were identified during four stages: start (T0), exponential middle (T1), stationary middle (T2) and end (T3). The Bacteria domain dominated both mesophilic and thermophilic conditions during each stage, whereas archaea were almost underrepresented. This led to lower methane yield during AD, apart from ammonia inhibition. During hydrolysis, acidogenesis and acetogenesis, bacteria mostly played a major role, while archaea played a major part in methanogenesis. The most abundant phyla in mesophilic conditions were identified as Firmicutes, Chloroflexi, Bacteroidetes, Actinobacteria, Euryarchaeota, Synergistetes and Proteobacteria. Whilst under Thermophilic conditions

Firmicutes, Proteobacteria, Actinobacteria, Chloroflexi, Synergistetes, Thermotogae and Bacteroidetes were dominant. Analysis of the different microbial consortia at phylum level shown that Firmicutes were dominant in both mesophilic and thermophilic conditions during AD, confirming that these organisms play a major part during AD (hydrolysis, acidogenesis and acetogenesis). This also confirmed that Firmicutes are part of a variety of microbial processes. Bacteroidetes were lower in levels at thermophilic conditions and functioned more sufficiently under mesophilic conditions.

Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria played a significant role in the beginning and middle of AD. Therefore, played a role during hydrolysis, acidogenesis and acetogenesis stages of AD. Actinobacteria played a role in the middle and second middle of AD thus playing a role during hydrolysis, acidogenesis and acetogenesis. Higher abundance in Actinobacteria of thermophilic conditions confirms that Actinobacteria played a more dominant role than in thermophilic than mesophilic conditions of hydrolysis, acidogenesis and acetogenesis stage of AD. Chloroflexi played a major role in Composite and faecal samples and was underrepresented in seed samples. Chloroflexi played a role during hydrolysis, acidogenesis and acetogenesis stage of AD in Composite and faecal samples. In the thermophilic conditions phylum such as Thermotogae was observed at a higher level compared to mesophilic conditions, confirming the tolerance to high temperature which, Thermotogae can survive in. Proteobacteria in mesophilic conditions were observed less than in thermophilic conditions and show a that thermophilic conditions were higher in, acidogenesis and acetogenesis activities due to their ability to convert glucose, butyrate, propionate and acetate. Synergistetes were observed in both mesophilic and thermophilic conditions with similarities in abundance – thermophilic only had a slightly greater abundance. A symbiotic relationship between Synergistetes and heterotrophic methanogens played a role in the end stage of AD. Euryarchaeota abundance was seen in middle and end stage of Mesophilic AD and played a significant role in the methanogenesis stage, *Methanosarcinaceae* and made up most of the group but low methane yields was still observed thus confirming that inhibition of some sort occurred (Ammonium).

6.3 Metabolic activity during mesophilic and thermophilic digestion

The predicted metabolic activity during mesophilic and thermophilic digestion was as follow, apart from the large amount of unknown identified metabolic pathways, ammonia oxidation was observed as the main known metabolic pathway during AD of both conditions. Supporting that a high concentration of ammonia was present during this study and could explains the low methanogen activity found during all conditions (Seed, Faecal and composite samples). But further investigation into the ammonia levels is needed to fully support this statement. Dehalogenation was predicted as the second-most abundant metabolic pathway, followed by nitrite and sulphate reduction in both mesophilic and thermophilic conditions. Firmicutes and

Chloroflexi was the main group that preformed dehalogenation (metabolic pathway) (Löffler *et al.,* 2012) and theory should improve methane production thus supporting that the system was inhibited by other factors such as Ammonia. Low methane metabolism was detected throughout anaerobic digestion in this study and only represented 0.3% to 1% and therefore low methane yields where obtained. The only identified family was *Methanosarcinaceae* but was found in low amounts.

6.4 Limitations and Recommendations

This study on the mesophilic and thermophilic anaerobic conditions encountered a series of issues, which include the water bath temperature regulation, substrate type, oxygen contamination, biogas yields, methane production, Archaea: Bacteria ratio and macronutrients concentrations in substrates.

1) Mesophilic and thermophilic conditions can be kept at a constant temperature environment with regulators. Water bath temperature fluctuate during AD when it comes to keeping a constant temperature especially at high temperatures, thus a room with a constant temperature would help keep these water baths at a constant temperature.

2) The Archaea: Bacteria ratio was an issue encountered during AD of these conditions. Evaluating this ratio beforehand of the substrate such as seeding sludge could determine the microbial community needed for optimal growth, by rather using seeding sludge with a higher archaea community.

3) Macronutrients – Determine nutrient composite in seeding and faecal sludge. By doing this, seeding sludge and faecal sludge can be fed with the corrected amount of nutrients to potentially increase the biogas yield and microbial dynamics between stages. Evaluating nutrients before AD can be done in future studies to ensure maximum biogas/methane production.

4) Analysis of heavy metal contamination in a system can explain if there if any other inhibition of methanogens during AD occurred – not only ammonia.

5) Analysis of the Carbon /Nitrogen ratio could lead to better biogas yield and system operation. During this study, only the percentage carbon was determined, not the percentage nitrogen.

6) The use of CSTR to represent a small-scale biogas plant on WWTPs, which would eliminate oxygen contamination experienced during this study. CSTR can also be used to

determine the biogas yield throughout different seasons, especially dry and wet season, which influences the faecal material obtained from a WWTP. The constant feeding of a CSTR can be beneficial when observing heavy metal pollution or macronutrient concentrations, which is of concern in most biogas systems.

7) Substrate selection can be evaluated. This section includes not only seeding sludge (cow manure) but also substrate such as straw materials, chicken manure and household waste which can be evaluated with faecal sludge under thermophilic and mesophilic conditions.

8) Reactor time – Increase reactor time, which could give more time for nutrient removal if imbalanced and give methanogens time to grow. Two-phase reactors could also be established to ensure that only hydrolysis and acetogenesis occurs in Phase 1, which leaves methanogenesis in Phase 2.

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APPENDIX A: PARAMETERS DURING ANAEROBIC DIGESTION

Table 0-1: (A) Comparison of COD and pH in mesophilic conditions

Sample_ID	*COD Ave (mg/l)*	*COD STDEV*	*pH Ave*	*pH STDEV*
Faecal _T0	18093	14800	7.13	0.51
Faecal_T1	15253	12500	6.90	0.23
Faecal_T2	12540	8650	6.91	0.09
Faecal_T3	10853	9380	6.99	0.06
Seed_T0	70653	14600	7.94	0.16
Seed_T1	59733	21100	7.92	0.25
Seed_T2	60893	14400	7.94	0.30
Seed_T3	54473	21300	8.01	0.42
Comp_T0	51824	12300	7.86	0.16
Comp_T1	49564	18000	7.67	0.18
Comp_T2	43960	11800	7.81	0.23
Comp_T3	40873	15400	7.94	0.41
MCC_T0	76467	24758	7.98	0.10
MCC_T1	71300	26887	7.81	0.22
MCC_T2	54060	25492	7.90	0.21
MCC_T3	52120	12624	8.09	0.37

Table 0-2: (B) Comparison of COD and pH in thermophilic conditions

Sample_ID	*COD Ave (mg/l)*	*COD STDEV*	*pH Ave*	*pH STDEV*
Faecal _T0	18093	14800	7.50	0.87
Faecal_T1	14727	8220	7.12	0.28
Faecal_T2	13300	9590	6.74	0.26
Faecal_T3	11667	7290	6.75	0.30
Seed_T0	68193	17400	7.96	0.17
Seed_T1	60347	13600	8.40	0.03
Seed_T2	57667	5680	8.14	0.29
Seed_T3	52200	17200	7.89	0.61
Comp_T0	63267	2491	8.05	0.19
Comp_T1	57578	4834	8.06	0.10
Comp_T2	48307	7146	7.65	0.39
Comp_T3	44922	44922	8.03	0.06
MCC_T0	75740	23921	8.20	0.24
MCC_T1	69713	25831	7.85	0.56
MCC_T2	59373	17716	7.83	0.46
MCC_T3	53967	15042	8.17	0.04

Table 0-3: TS and VS of pre- and post-AD of mesophilic conditions in all samples

SAMPLE_ID	TS [G/100G]	STDEV	VS [G /100GDM]	STDEV	VS [G /100GWETSAMPLE]	STDEV
FM0	1.46	0.53	71.20	4.53	1.02	0.34
FM3	1.27	1.22	53.29	12.40	0.60	0.45
SM0	9.01	5.05	59.55	8.58	4.91	2.08
SM3	6.88	3.00	62.56	4.03	4.36	2.00
CM0	3.59	2.58	64.96	2.29	2.37	1.78
CM3	2.64	1.51	62.09	6.91	1.68	1.11

Table 0-4: TS and VS of pre- and post-AD of thermophilic conditions in all samples

SAMPLE_ID	TS [G/100G]	STDEV	VS [G /100GDM]	STDEV	VS [G /100GWETSAMPLE]	STDEV
FT0	1.46	0.53	71.20	4.53	1.02	0.34
FT3	0.53	0.22	59.04	7.99	0.32	0.18
ST0	7.18	3.95	61.87	4.02	4.28	2.03
ST3	6.82	3.02	62.42	3.54	4.31	2.04
CT0	4.79	1.47	67.63	6.41	3.20	0.88
CT3	4.15	1.55	63.30	1.11	2.63	1.02

Table 0-5: Eco-Analytica results of C%

SAMPLE_ID	% C MESO	STDEV	% C THERMO	STDEV
F 0	36.13	0.48	36.13	0.48
F 1	35.28	2.41	28.42	12.54
F 2	34.40	2.72	34.16	1.59
F3	31.11	1.51	35.29	3.16
S0	31.12	7.70	32.10	1.30
S 1	22.08	9.10	31.59	2.10
S2	32.77	4.03	31.64	0.03
S 3	32.60	1.86	32.60	1.10
C 0	34.29	2.23	31.04	5.32
C1	32.17	2.15	32.01	2.29
C 2	30.59	1.46	32.78	0.33
C 3	33.97	6.81	34.31	2.18

APPENDIX B: BIOGAS YIELDS, STANDARD DEVIATIONS AND GRAPHS.

Table 0-6: Cumulative biogas yields of all substrates (Seed, MCC, Comp1-3 and Faecal) during mesophilic conditions

DAY	SEED		MCC		COMPOSITE		FAECAL	
	Cumulative	Standard deviation	Cumulative	Standard deviation	Cumulative	Standard deviation	Cumulative	Standard deviation
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	72.46	22.42	76.22	27.87	63.30	21.28	22.64	27.38
2	184.37	84.18	211.09	52.79	136.01	42.17	30.09	25.95
3	280.18	104.79	354.08	115.65	196.23	47.71	40.18	23.87
4	370.90	120.16	483.50	171.95	261.98	58.65	52.27	25.43
5	461.91	127.92	620.40	226.81	317.65	55.83	65.54	27.27
6	552.06	124.26	772.99	283.38	385.97	53.41	78.75	27.55
7	633.47	129.34	888.41	308.59	448.34	62.12	91.07	23.74
8	716.38	137.03	1026.74	330.02	511.51	67.68	99.72	21.61
9	770.64	124.82	1140.41	352.91	570.67	86.61	111.10	13.98
10	831.30	136.86	1231.20	367.66	616.19	93.58	118.31	11.41
11	909.48	123.23	1340.16	372.45	673.78	96.47	133.18	15.17
12	984.97	107.23	1445.77	377.17	724.98	102.44	144.26	17.89
13	1046.30	102.44	1548.96	382.77	771.49	116.47	152.79	26.24
14	1118.34	83.42	1645.12	374.90	820.08	131.10	161.23	27.95
15	1186.93	51.28	1753.56	353.59	877.82	153.55	179.31	45.49
16	1247.33	43.25	1835.75	347.98	917.55	170.97	184.55	49.98
17	1303.36	39.19	1898.78	346.95	956.88	188.86	189.31	55.39
18	1348.94	32.11	1960.33	348.71	994.25	202.63	193.31	58.09

19	1398.67	34.80	2020.15	341.48	1033.15	213.39	199.59	64.55
20	1444.57	42.56	2082.43	348.04	1061.94	218.90	202.84	67.52
21	1487.05	42.69	2156.68	361.42	1081.93	227.75	205.59	68.77
22	1514.91	39.78	2210.89	369.05	1100.85	232.96	209.39	73.95
23	1546.22	39.75	2263.46	374.41	1117.97	234.06	212.90	78.67
24	1572.47	41.41	2310.30	380.47	1134.18	236.33	215.91	81.70
25	1603.98	50.09	2337.95	368.06	1144.67	234.17	217.12	81.44
26	1635.05	68.27	2367.57	360.84	1153.42	231.86	219.07	81.56
27	1649.26	64.84	2389.99	357.87	1163.53	232.20	219.33	81.96
28	1662.28	60.07	2405.76	361.34	1173.36	233.05	219.58	81.92
29	1676.94	63.79	2416.73	364.61	1182.21	234.07	219.58	81.92
30	1690.51	72.87	2425.47	364.22	1193.40	237.43	219.82	81.89
31	1705.87	83.53	2437.36	363.77	1199.15	236.92	220.07	81.87
32	1722.17	99.41	2445.64	364.90	1204.37	237.01	220.07	81.87
33	1738.16	117.23	2455.86	362.18	1207.24	238.69	220.07	81.87
34	1700.22	44.71	2460.47	359.64	1208.05	238.23	220.07	81.87
35	1714.04	55.16	2463.52	361.81	1220.18	250.67	220.07	81.87
36	1718.81	57.53	2466.36	365.07	1208.36	238.37	220.31	81.84

Table 0-7: Cumulative biogas yields of all substrates (Seed, MCC, Comp1-3 and Faecal) during thermophilic conditions

Day	SEED		MCC		COMPOSITE			FAECAL	
	Cumulative	Standard deviation	Cumulative	Standard deviation	Cumulative	Standard deviation	Standard deviation	Cumulative	Standard deviation
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.41	90.97	11.63	85.03	50.66	68.19	47.81	57.32	14.86	13.15
0.88	175.19	10.91	172.21	69.98	130.09	65.14	53.90	27.65	15.37
1.32	257.92	17.64	297.04	13.58	190.10	90.98	58.31	40.27	23.19
1.98	343.19	25.82	434.09	39.28	261.14	121.94	36.92	49.64	32.53
2.26	423.30	26.91	575.96	94.85	319.65	142.00	48.96	59.90	46.07
3.29	515.87	53.78	709.13	153.52	374.54	171.61	29.99	63.67	49.24
4.32	612.94	90.55	852.60	227.97	417.71	197.93	31.37	69.84	56.27
5.32	707.58	123.63	983.78	278.07	458.18	223.98	10.48	75.81	58.06
6.32	798.07	166.51	1111.46	335.21	503.48	245.51	13.20	82.01	66.51
6.83	859.69	186.18	1227.94	399.39	533.85	263.58	22.49	91.10	78.32
7.19	899.89	167.90	1318.83	454.90	563.30	275.67	29.40	95.10	82.49
8.32	934.24	167.99	1417.10	526.92	592.65	290.77	41.22	101.99	91.16
9.01	961.22	162.08	1459.33	525.55	619.64	309.19	47.87	106.44	94.93
10.33	986.66	168.70	1493.11	537.40	647.49	323.25	83.55	111.33	97.31
11.46	1002.92	165.43	1529.06	558.35	664.49	333.43	93.02	113.54	97.19
12.13	1019.97	186.12	1553.74	572.61	676.47	340.71	103.64	115.11	96.90
13.042	1037.10	208.24	1567.83	575.51	685.42	347.47	113.85	119.59	90.95
14.13	1041.70	209.50	1578.99	581.66	691.15	351.17	123.56	119.59	90.95
15.15	1083.80	288.09	1577.39	823.01	712.69	383.88	181.92	70.98	48.64

APPENDIX C GAS COMPOSITE.

Table 0-8: Gas Composite during bench top reactor study of mesophilic and thermophilic conditions (Run2).

Date/ Meso	Sample ID	System P [mbar]	Pump time [s] / t [s]	CH_4 avg	CH_4 max [%]	CO_2 avg	CO_2 max [%]	O_2 avg	O_2 min [%]	H_2S [ppm]	Balance [%]
Meso	Seed1	0.04	28	19.5	19.5	8.1	8.1	13.1	13.1	4	59.3
	Seed2	0.07	19	10.2	10.2	5.3	5.3	16.3	16.3	8	68.2
	Seed3	0.10	49	21.3	21.3	9.0	9.1	13.3	13.3	9	56.4
	Mcc1	0.05	17	11.4	11.6	7.1	7.20	14.3	14.2	4	67.2
	Mcc2	0.15	44	16.1	9.10	1.7	1.70	15.3	15.4	9	73.9
	Mcc3	0.26	75	27.4	27.4	7.1	7.20	14.3	14.3	7	51.2
	Comp1	0.14	29	19.2	19.3	3.9	3.9	14.6	14.6	5	62.4
	Comp2	0.31	66	26.4	26.6	7.9	7.9	12.1	12.1	4	53.5
	Comp3	0.49	114	38.2	38.2	10.3	10.3	10.9	10.7	9	40.6
	Faecal	0.01	9	0.1	0.2	0.3	0.40	18.2	18.2	9	81.4
	Faecal	0.03	12	0.2	0.30	0.10	0.10	19.5	19.5	10	80.2
	Faecal	0.05	16	0.5	0.50	0.10	0.10	19.8	19.8	9	79.6
Thermo	**Sample ID**	**System P [mbar]**	**Pump time [s] / t [s]**	**CH_4 avg**	**CH_4 max [%]**	**CO_2 avg**	**CO_2 max [%]**	**O_2 avg**	**O_2 min [%]**	**H_2S [ppm]**	**Balance [%]**
	Seed1	0.06	21	12.4	12.4	0.8	0.8	18.9	18.9	5	67.9
	Seed2	0.09	32	18.7	18.7	9.3	9.3	2.4	2.4	9	69.6
	Seed3	0.17	54	19.7	19.7	8.5	8.5	12.5	12.5	8	59.3
	Mcc1	0.08	29	11.9	11.9	6.1	6.1	14.7	14.7	8	67.3

Mcc2	0.12	32	19.9	20.1	7.4	7.4	13.1	13.1	7	59.4
Mcc3	0.15	42	24.2	24.3	8.5	8.6	12.4	12.1	5	55
Comp1	0.08	32	11.2	11.4	5.9	5.9	12.3	12.4	5	70.3
Comp2	0.09	31	14.3	14.4	8.5	8.6	14.5	14.6	9	62.4
Comp3	0.45	25	13.4	13.4	6.3	6.4	14.7	14.7	17	65.5
Faecal	0.02	42	0.3	0.5	0.2	0.2	20.9	20.9	0	78.6
Faecal	0.04	15	0.4	0.5	0.2	0.2	18.8	18.8	1	80.6
Faecal	0.07	18	0.3	0.3	0.1	0.1	19.2	19.2	2	80.4

Table 0-9: Gas Composite during bench top reactor study of mesophilic and thermophilic conditions (Run3).

Date/ Meso	*Sample ID*	*System P [mbar]*	*Pump time [s] / t [s]*	*CH_4 avg*	*CH_4 max [%]*	*CO_2 avg*	*CO_2 max [%]*	*O_2 avg*	*O_2 min [%]*	*H_2S [ppm]*	*Balance [%]*
Meso	Seed1	0.04	15	21.5	21.6	3.1	3.1	12.4	12.4	9	62.9
	Seed2	0.07	21	11.4	11.5	7.2	7.2	17.2	17.2	5	64.2
	Seed3	0.1	24	31.8	31.8	9.7	9.7	14.1	14.1	5	44.4
	Mcc1	0.05	17	10.6	10.6	5.4	5.4	12.3	12.3	8	71.7
	Mcc2	0.15	44	8.1	8.1	0.7	0.7	18.2	18.2	8	73
	Mcc3	0.26	75	27.4	27.4	7.1	7.2	14.3	14.3	7	51.2
	Comp1	0.03	9	0.7	0.70	1.5	1.5	18.3	18.3	4	79.5
	Comp2	0.09	17	9.0	12.5	0.2	0.2	15.2	15.2	7	72.2
	Comp3	0.12	25	13.0	18.5	2.4	2.4	16.8	16.8	7	62.4
	Faecal	0.01	9	0.2	0.2	0.1	0.1	20.5	20.5	1.0	79.2
	Faecal	0.03	12	0.2	0.2	0.2	0.2	20.2	20.2	7.0	79.4
	Faecal	0.05	16	1.5	1.5	0.1	0.1	20.3	20.3	6.0	78.1

Date/ Thermo	**Sample ID**	**System P [mbar]**	**Pump time [s] / t [s]**	**CH_4 avg**	**CH_4 max [%]**	**CO_2 avg**	**CO_2 max [%]**	**O_2 avg**	**O_2 min [%]**	**H_2S [ppm]**	**Balance [%]**
Thermo	Seed1	0.06	33	13.4	13.4	3.8	3.8	15.3	15.3	8	67.5
	Seed2	0.09	32	17.8	17.8	6.2	6.2	16.1	16.1	7	59.9
	Seed3	0.17	42	19.2	19.2	2.1	2.1	14.5	14.5	14	64.2
	Mcc1	0.08	46	24.9	24.9	2.1	2.1	13.7	13.7	8	59.3
	Mcc2	0.12	38	21.4	21.4	3.4	3.4	14.9	14.9	12	60.3
	Mcc3	0.15	10	0.7	0.7	0.1	0.1	21.2	21.1	7	78
	Comp1	0.00	8	0.8	0.9	0.1	0.2	18.6	18.6	3	80.5
	Comp2	0.00	21	10.3	10.4	0.7	0.7	13.8	13.7	11	75.2
	Comp3	0.00	38	21.9	21.8	2.6	2.6	12	12	14	63.5
	Faecal	0.02	4	0.0	0.0	0.1	0.2	20.3	20.3	6	79.6
	Faecal	0.04	15	0.9	0.9	0.2	0.2	20.3	20.3	4	78.6
	Faecal	0.07	18	1.4	1.4	0.9	0.5	20.4	20.4	2	77.3

APPENDIX D: MOLECULAR

Table 0-10: Mesophilic sample DNA concentrations and spectrophotometer absorbance results.

Sample Name	Nucleic Acid(ng/uL)	A260/A280	A260/A230	A260	A280	Baseline Absorbance
Seed M0	54.315	1.929	1.826	1.086	0.563	0.037
Seed M1	28.041	1.931	1.715	0.561	0.290	0.033
Seed M2	28.357	1.850	1.452	0.567	0.307	0.055
Seed M3	16.530	1.783	0.993	0.331	0.185	0.065
Comp1 M0	11.225	1.761	0.806	0.224	0.149	0.043
Comp1 M1	12.600	1.756	0.879	0.252	0.172	0.040
Comp1 M2	7.787	1.515	0.763	0.156	0.119	0.025
Comp1 M3	9.245	1.432	0.778	0.185	0.126	0.037
Faecal M0	7.295	1.490	0.612	0.146	0.098	0.004
Faecal M1	7.750	1.366	0.798	0.155	0.113	0.022
Faecal M2	5.145	1.224	0.655	0.103	0.084	0.020
Faecal M3	4.674	1.426	0.262	0.093	0.066	-0.005

Table 0-11: Thermophilic sample DNA concentrations and spectrophotometer absorbance results.

Sample Name	Nucleic Acid(ng/uL)	A260/A280	A260/A230	A260	A280	Baseline Absorbance
Seed T0	41.614	1.799	1.902	0.832	0.463	0.064
Seed T1	38.698	1.707	1.226	0.774	0.453	0.044
Seed T2	32.804	1.755	1.624	0.656	0.374	0.041
Seed T3	24.077	1.729	1.233	0.482	0.279	0.252
Comp1 T0	9.857	1.778	0.806	0.197	0.114	0.104
Comp1 T1	12.058	1.643	0.735	0.241	0.148	0.144
Comp1 T2	7.616	1.814	0.676	0.152	0.086	0.079
Comp1 T3	7.648	1.884	0.561	0.153	0.091	0.135
Faecal T0	9.674	1.762	0.835	0.193	0.110	0.087
Faecal T1	9.394	1.755	0.652	0.188	0.107	0.053
Faecal T2	6.925	1.546	0.562	0.139	0.090	0.099
Faecal T3	5.454	1.517	0.538	0.109	0.072	0.063

Table 0-12: Pooled samples for NGS

Samples ID	Timelines	Number of samples pooled	Total
Faecal Mesophilic	T_0 = 3 T_1 = 3 T_2 = 3 T_3 = 3	12	32
Seed Mesophilic	T_1 T_2 T_3	9	
Composite Mesophilic	T_0 = 3 T_1 = 3 T_2 = 3 T_3 = 3	12	
Faecal Thermophilic	T_0 = 3 T_1 = 3 T_2 = 3 T_3 = 3	12	36
Seed Thermophilic	T_0 = 3 T_1 = 3 T_2 = 3 T_3 = 3	12	
Composite Thermophilic	T_0 = 3 T_1 = 3 T_2 = 3 T_3 = 3	12	

Table 0-13 : The total average and range of OTU in mesophilic and Thermophilic conditions.

Sample Conditions	Total OTU counts	Average OTU counts	OTU Range
Mesophilic	5,585,861	143,227	29,968 to 409,059
Thermophilic		131,982	37,079 to 287,456