Study on the microbial community shift during co-fermentation of substrates

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DECLARATION

I declare that the dissertation submitted by me for the degree Magister Scientiae in Environmental Studies at the North-West University (Potchefstroom Campus), Potchefstroom, North West, South Africa, is my own independent work and has not previously been submitted by me at another university.

Signed in Potchefstroom, South Africa

L Bothma
Date: 24/02/2020

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ABSTRACT

Some of the core issues faced today in developed and developing countries are global energy protection and better utilisation of natural resources. Anaerobic digestion is currently one of the options available that may assist in this regard and research has been done on a wide variety of substrates for anaerobic digestion. Several phases can be observed during a typical batch setup for anaerobic digestion, with the lag phase being of particular interest as the microbial communities present typically adapts to substrate during this phase. The lag phase is also typically one of the longest phases observed in batch reactor setups. Pig slurry is readily available and poses a threat to environmental, including over-fertilisation and eutrophication. The aim of the current study was to determine whether back inoculation will reduce the observed lag time before biogas production occurs in batch reactors using pig slurry as the primary substrate. Batch reactors were used in this study, during which back inoculation was done and sample were taken at key intervals. Samples taken from the batch reactors were subjected to physical and chemical analysis, as well as molecular analysis of the microbial communities present during the sample times using metabarcoding on the MiSeq next generation sequencing (NGS) platform. From the NGS data community composition and predicted metabolic activities were derived. Low biogas yields were observed in this study and could be attributed to factors such as imbalanced nutrient levels and ratios between the main functional groups. Methanogenesis was not optimal as the abundance of methane producing microorganism was inhibited The major phyla observed in the microbiomes in the current study included Firmicutes, Bacteroidetes, Proteobacteria, Synergistetes, Euryarchaeota, Chloroflexi, Actinobacteria and Atribacteria, and the major families were *Clostridiaceae 1*, *Synergistaceae*, *Ruminococcaceae*, *Rikenellaceae*, *Marinilabiaceae*, *Porphyromonadaceae*, *Erysipelotrichiaceae* and *Methanosarcinaceae*. These are typically phyla and families associated with the AD processes. The predicted metabolic activities indicated the highest metabolic activity to be unknown, followed by ammonia oxidation. These issues primarily relate to the small size of the bench-top reactors and the state of the seeding sludge. The back inoculation was successful in reducing the observed lag phase, even though the C:N ratio was not optimal.

Keywords: Biogas, pig slurry, microbial community, anaerobic digestion, microbial community shift

ABBREVIATIONS

AD	Anaerobic digestion
COD	Chemical oxygen demand
CSTR(s)	Continuously stirred tank reactor(s)
g	Gram
HRT	Hydraulic retention time
MC	Microcrystalline cellulose
mg/l	Milligram per litre
ml	Millilitre
OLR	Organic loading rate
PCR	Polymerase chain reaction
TS	Total solids
VS	Volatile solids

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CHAPTER 1 – INTRODUCTION

1.1 Introduction

Most of the core issues facing many of today's developed and developing countries are global energy protection and better utilisation of natural resources (Okudoh *et al.,* 2014). The method of biogas generation through the anaerobic fermentation of organic materials is an increasingly popular technology (Dahunsi *et al.,* 2019). Since John Fry introduced biogas engineering in 1957, approximately 700 digester facilities have been completed in South Africa (Mutungwasi *et al.,* 2018). South Africa has since then witnessed minimal development of the biogas industry. The reasons for this shortcoming include the low cost of other sources of electricity, such as fossil fuels, limited subsidies or government incentives to support biogas technology and the lack of local biogas technology (Mutungwasi *et al.,* 2018).

Wang *et al. (*2017) found that current literature shows that it is possible to use a number of substrates for anaerobic digestion. Studies showed that substrates could be a key factor influencing the structure of the microbial community, leading to variations in methane production and system stability (Wang *et al.,* 2017).

Unrestricted disposal of large quantities of food waste has become a significant problem, as it causes severe environmental pollution. However, food waste is an appropriate substrate for anaerobic digestion due to its good biodegradability and high water content (Girotto *et al.,* 2015). It is estimated that approximately 33.3% of food produced for human consumption worldwide are lost or wasted through the food supply chain. Food waste is a non-productive use of scarce assets (land, water and fertiliser) that leads to environmental degradation (Girotto *et al.,* 2015).

Agricultural slurry is very nutritious and should be viewed as a resource rather than a waste. Anaerobic digestion is one of the most promising methods of manure disposal, as it could not only treat manure, but also create biogas (Hu *et al.,* 2019). Animal waste is typically a good anaerobic digestion feedstock (Wang *et al.,* 2017). Since 1970, South Africa's cattle population has risen from 6 to 14 million (Jaja *et al.,* 2018). Agricultural census 2011 shows that 86% of the population maintain an average of one to 10 cattle, while 12.9% maintain an average of 11 to 100 cattle (Jaja *et al.,* 2018). Cattle manure appears to be a widely used substrate for biogas plants, especially in intensive farming countries (Bedoic *et al.,* 2019). While anaerobic digestion of cow manure may yield approximately 63% of the biogas, ruminant manure in general and cattle manure in particular can be used to boost the fermentation stage of anaerobic digestion, as it can provide the required methanogenic bacteria (Caruso *et al.,* 2019).

In 2016, in a form of pig slurry/manure, 21 415 tons of nitrogen were applied to fields in South Africa (Food and Agriculture Organization of the United Nations, 2018). Globally, methane generated through intestinal fermentation and in the process of processing and handling livestock manure in the agricultural sector is the second largest source of greenhouse gas emissions (Shin *et al.,* 2019). With the increasing production of livestock manure, the related environmental issues such as soil/water pollution and odorous gas emissions are attracting significant global attention (Shin *et al.,* 2019).

Piggery manure disposal is correlated with potential impacts on air and water quality (Do *et al.,* 2003). Manure is usually stored in concrete- or steel-lined bins, pits or lagoon systems. The effluent in storage is anaerobic and releases malodorants that can become hazardous (Shin *et al.,* 2019). An estimated 1 710 tons of undiluted waste are generated per year by a typical piggery containing approximately 100 sows (Lutge & Standish, 2013). It means approximately 4.7 tons of manure per day, capable of producing 141.4 m^3 of biogas per day, where 1 m^3 of biogas will generate 2.03 kW of energy. The 100 sow piggery can therefore produce 287.042 kW of electricity per day. By producing biogas, the power potential of pig slurry can be used to meet the power requirements of the farm (Lutge & Standish, 2013).

Due to increased nutrient content, the use of animal excretions as organic fertilisers is very common in South Africa. There is a risk of nutrient over-fertilisation, especially in areas with larger amounts of excrement (Umweltbundesamt, 2014). The effect of over-fertilisation is a surplus of reactive nitrogen in the soil. This can contribute to nitrate contamination of the groundwater by leaching and consequently eutrophication of streams, lakes and the sea. An excessive supply of phosphorus from farmland can also cause eutrophication (Umweltbundesamt, 2014). Therefore, other sustainable methods of handling the organic waste is required i.e. anaerobic digestion, in other to curtail pollution.

The main goal of anaerobic digestion (AD) of primary sludge is to convert carbonaceous material into CH_4 and CO_2. Digestion products therefore include gases, stable sludge solids that are dewatered and disposed of, and sludge liquor, which is further treated (Roman *et al.,* 2006). Co-digestion can also increase the production of biogas by reducing nutrient deficiencies, providing microbial inoculums and reducing process inhibition by modifying the determinants that can inhibit the system, such as pH (McDonald *et al.,* 2008).

Complex microbial communities are responsible for the production of biogas, including bacteria, archaea, protozoa and fungi that can adapt to anaerobic parameters of digestion (Murto *et al.,* 2004). Each of the four stages of anaerobic digestion contains various microbial dominant communities (Repinc *et al.,* 2018). Four steps result in the production of biogas; these steps are hydrolysis, acidogenesis, acetogenesis/dehydrogenation and methanogenesis (Shane *et al.,* 2017). The microbial communities are constantly interacting, but their complex associations and functioning are not yet well known (Repinc *et al.,* 2018). The microbial community's organisational structure and agitation are linked to the anaerobic digestion reactor’s effectiveness, as the microbial community tends to replace, migrate and die (Repinc *et al.,* 2018).

The regulatory power of the core microbiota for AD quality remains poorly understood (Tao *et al.,* 2020). Occasionally, there are imbalances between various microbial species, such as the issue of faster hydrolysis/acidogenesis and slower methanogenesis, resulting in lower anaerobic digestion efficiency. There is therefore an urgent need to investigate the composition and role of microbial communities during anaerobic sludge digestion in order to further improve the production of methane (Feng *et al.,* 2019).

1.2 Aim and objectives

The aim of the current study was to determine whether back-inoculation will reduce the observed lag time before biogas production occurs in batch reactors.

Specific objectives identified for this aim include:

(i) Set-up of bench-top reactors using a series of single substrate combinations

(ii) Inoculating a second set of reactors with inoculum from a set of previous inoculums at the phase of maximum biogas production and evaluating biogas production

(iii) Determine the microbial community composition during start-up (lag phase), during optimal biogas production (middle) and the end phase; and

(iv) Evaluate the effect of back-inoculation with regard to biogas production and microbial community composition.

CHAPTER 2 – LITERATURE REVIEW

The key issues facing many of the world's developed and developing nations today are mainly future energy protection and better use of natural resources (Okudoh *et al.,* 2014). The method of generating biogas through the anaerobic fermentation of organic matter is a technology that is increasingly gaining popularity (Dahunsi *et al.,* 2019). This increase in popularity is due to its capacity to provide relief from two of the issues that have been experienced from day to day in the course of living: (i) the problem of acquiring energy in sufficient quantities for cooking, heating, lighting and machine running purposes, and (ii) the problem of adequate waste disposal in such a way as not to cause harm to man or damage to the environment (Dahunsi *et al.,* 2019). Energy sources can be broadly categorised into two main categories, i.e. non-renewable and renewable energy sources. Examples of non-renewable energy sources include fossil-based fuels such as coal and oil, while renewable energy sources include solar, wind, wave and biogas (Dahunsi *et al.,* 2019; Okonkwo *et al.,* 2018). Since the introduction of biogas engineering in the country in 1957 by John Fry, approximately 700 digester installations have been completed in South Africa (Mutungwasi *et al.,* 2018). John Fry built the first biogas digester in South Africa on a pig farm in 1957. The substrate used was manure from pigs. Electricity was generated on the farm in 1958 from the biogas produced to power pumps. (Mutungwasi *et al.,* 2018). South Africa has since then witnessed limited development of the biogas industry. The reasons for this limitation include the low cost of electricity from other sources, such as fossil fuels, no subsidies or government incentives to support biogas technology and the lack of local biogas technology (Mutungwasi *et al.,* 2018). In South Africa it is also currently not possible to feed renewable electricity back into the electrical grind.

2.1 Waste streams

A survey of literature conducted by Wang *et al. (*2017), shows that a variety of substrates can be used for anaerobic digestion. These can either be dingle substrate digestion or co-substrate digestion. Refer to Table 2-1 for a list as well as theoretical biogas yield potentials. Studies have shown that substrates could be a key factor affecting the microbial community structure, resulting in differences in methane production and system stability (Wang *et al.,* 2017).

2.1.1 Agricultural

Agricultural slurry is very nutritious and should be considered a resource instead of a waste. Anaerobic digestion (AD) is one of the most promising methods to treat manure because it could not only treat manure, but also produce biogas (Hu *et al.,* 2019). Animal waste is usually a good raw material for anaerobic digestion (Wang *et al.,* 2017).

2.1.2 Animal material

The cattle population of South Africa has risen from 6 million to 14 million since 1970 (Jaja *et al.,* 2018). Agricultural census 2011 reveals that 86% of the population keep one to 10 cattle on average, while 12.9% keep 11 to 100 cattle on average (Jaja *et al.,* 2018). Cattle manure seems to be a widely used substrate for biogas plants, particularly in intensive farming nations. It includes significant nutrient and pathogens concentrations that can cause soil and groundwater contamination (Bedoic *et al.,* 2019). Households and smallholder farms in rural communities manage livestock species under extensive low-input farming systems characterised by poor housing, low-quality scavenging food sources and minimal veterinary interventions (Mathole *et al.,* 2017). Livestock species are maintained with limited biosecurity as mixed flocks. The process of low input production and minimal biosecurity steps exposes different species of animals to different pathogens (Mathole *et al.,* 2017). Such animals are raised mainly for purposes of food security and provide cheap and readily available meat, eggs and milk to households (Mathole *et al.,* 2017).

Although anaerobic digestion of cow manure can yield approximately 63% of the biogas, ruminant manure in overall and cattle manure in specific can be used to enhance the fermentation stage of anaerobic digestion, as it can provide the methanogenic bacteria needed (Caruso *et al.,* 2019). The organic matter in the manure of horses is 30 to 47%. Cow manure and horse dung have a large percentage of lignocellulosic organic material that is slowly degradable, and the composition of the manure is linked to nutrition, bedding material used, and the frequency of stall cleaning (Caruso *et al.,* 2019). Studies on horse dung as a biogas substrate concentrate on solid state anaerobic digestion owing to elevated total solids and fibrous content, making this dung not ideally suitable for continuous slurry-based biogas reactors (Caruso *et al.,* 2019). Poultry manure has a strong potential organic substrate for treatment, but the elevated nitrogen content in comparison to manure from other farm animals makes it problematical for an anaerobic digestion. (Caruso *et al.,* 2019).

Slurry comprises primarily of water and dissolved organic substances, nutrient and minerals (Sommer *et al.,* 2013). There are many variables related to the precise composition of slurry, which is why literature information can vary a great deal (Lindemayer *et al.,* 2009). Some of the largest variables are animal feeding, as well as the farm operation to keep the livestock and wash the stables (Lindemayer *et al.,* 2009). Nutrients such as phosphorous is regarded to be the primary cause of freshwater eutrophication in livestock manure, and nearly half of civil complaints about odour issues are linked to livestock manure (Shin *et al.,* 2019).

On the one side, intensive livestock farming is positive from a financial point of view, but on the other side, it has adverse environmental impacts due to the high energy and water demands as well as increased use of pharmaceutical products. In addition, there are significant quantities of animal excretions and gas emissions to be captured and treated. These excretions are frequently used as farm fertilisers for agricultural areas and contain elevated quantities of nitrogen elements such as nitrates, which, if applied incorrectly, can damage the environment by eutrophication of water bodies (Umweltbundesamt, 2018a, 2018b). In 2016, 21 415 tons of nitrogen were applied to the fields in South Africa in the form of pig slurry/manure (Food and Agriculture Organization of the United Nations, 2018). Globally, methane produced through intestinal fermentation and in the process of storage and subsequent management of livestock manure in the agricultural sector is the second largest source of greenhouse gas emissions (Shin *et al.,* 2019). The associated environmental issues such as soil/water pollution and odorous gas emissions are receiving important attention globally with the growing generation of livestock manure (Shin *et al.,* 2019).

2.1.3 Pig slurry

The storage of piggery manure is associated with potential effects on air and water quality (Do *et al.,* 2003). Manure is usually stored in concrete or steel lined bins, pits or lagoon systems. The effluent becomes anaerobic in storage and emits malodours that can become problematic (Shin *et al.,* 2019). According to Lutge and Standish (2013), it is estimated that a regular piggery containing approximately 100 sows and produces an estimated 1 710 tons of undiluted waste per annum. This means approximately 4.7 tons of manure per day, which can produce 141.4 m^3 biogas per day, where 1 m^3 of biogas can produce 2.03 kW of electricity. The 100 sow piggery can therefore produce 287.042 kW of electricity per day. By producing biogas, pig slurry's power potential can be used to meet the farm's power requirements. Using this technique, it is possible to include slurry from all piggery units. The yields of methane ranged from 153.4 to 210.4 mL/gVS for pig slurry anaerobic digestion (Liang *et al.,* 2020). Pig manure is still widely used as the sole feedstock for most farms to produce biogas in China (Duan *et al.,* 2019). Sub-Saharan Africa's pig production is growing rapidly and as such, CH_4 emissions from pig production are increasing significantly (Ngwabie *et al.,* 2018). Commercial, communal and pig growers in South Africa produce 18.5 kgCH_4/pig/year, 0.41 kgCH_4/pig/year and 14.13 kgCH_4/pig/year, respectively (Ngwabie *et al.,* 2018).

2.1.4 Abattoir waste

In large parts of South Africa, stock farming is the most feasible agricultural practice and the meat industry is one of the most important agricultural sub-sectors (Harding *et al.,* 2017; Russo & von Blottnitz, 2017). Slaughtering and processing of meat are multi-stage processes where waste and effluents with different characteristics are created at each stage (Allie *et al.,* 2003). Untreated slaughterhouse waste poses a high risk of pollution when it enters a municipal wastewater treatment system due to the biological and chemical oxygen demand (BOD and COD). This phenomenon is even complicated when the untreated waste stream reaches a river or catchment directly (Russo & von Blottnitz, 2017). Slaughterhouse wastewater comprises a wide variety and quantity of pollutants, primarily distinguished by a complex mixture of protein products, lipids and fibres (Allie *et al.,* 2003). Different techniques, including anaerobic digestion, ammonium sulphate precipitation and microfiltration, can be used in the treatment of effluents from abattoirs (Allie *et al.,* 2003). Research studies conducted by Latifi *et al. (*2017) in this field have shown that slaughter industry waste such as sludge generated in abattoir wastewater treatment plants as well as livestock and poultry slaughterhouse residues contain high amounts of fat, protein and organic matter, making them an acceptable option for biogas production. Co-digestion of slaughterhouse waste in conjunction with other waste is known as one of the preferred methods for solving this problem and thereby increasing the production rate of biogas (Latifi *et al.,* 2017). Many studies have shown that co-digestion of slaughterhouse waste with other organic materials such as fruit and vegetable waste, organic fraction of municipal solid waste, medical and food waste, and sewage treatment plant sludge produces improved biogas and methane yields, as well as anaerobic digestion conditions (Latifi *et al.,* 2017). Nonetheless, the cost of transportation of the co-substrate from the origin point to the AD plant is still important to consider (Panigrahi & Dubey, 2019).

2.1.5 Plant materials

Apart from animal material, plant material is also used for anaerobic digestion and biogas production. The biodegradation of different kinds of lignocellulosic biomass relies on the chemical structure, mainly on the proportion of cellulose, hemicellulose, lignin and C:N presented in different organic substrates (Bedoic *et al.,* 2019). Because of the polyvalent, multiplex chemical structure, polyphenolic compounds such as humic acid are inhibitory compounds yielding from plant material substrates during anaerobic digestion. It can impact the chemistry of the microbial ecosystem and their impacts seem to be based on the bioreactor and feedstock used (Repinc *et al.,* 2018). Trace metals in anaerobic digestion interact with polyphenolic compounds, thus impacting the degree of trace metal accessibility for microbial activity. The accessibility and/or ideal quantity of nutrients can impact anaerobic fermentation activity, growth of microorganisms and the microbial community structure (Repinc *et al.,* 2018). Residue grass is part of a lignocellulosic biomass group and could be used profitably for more sustainable bioenergy manufacturing in biorefineries (Bedoic *et al.,* 2019).

2.1.6 Microalgae and other non-foodstuff sources

Microalgae are common photosynthetic microorganisms that inhabit several environments, and their oxygen photosynthesis is believed to be responsible for the formation of the present oxygen-rich earth atmosphere that supports life (Nagarajan *et al.,* 2019). Microalgae, including photosynthetic bacteria (often known as blue green algae), may be prokaryotic, or eukaryotic in nature. The structures of micro-algal cells are simple, and they grow by fixing atmospheric carbon dioxide into organic biomass powered by light energy, making them the primary ecosystem producers (Nagarajan *et al.,* 2019). Microalgae biorefineries have caught the attention of academia and industry to produce biofuels and high-value goods. Implementing a step of anaerobic digestion can improve resource recovery from microalgae and its residues (Solé-Bundó *et al.,* 2019). Additional benefits of using anaerobic digestion to treat microalgae and microalgae residues are the accumulation of nutrients (N and P) and the availability of carbon dioxide that can be reused for cultivation of microalgae (Solé-Bundó *et al.,* 2019). Residues of microalgae and its residues are typically characterised by low yield of methane (150 - 300 $mLCH_4/gVS$) compared to other substrates such as sewage sludge (200 - 350 $mLCH_4/gVS$), animal manure (200 - 400 $mLCH_4/gVS$) and food waste (400 - 550 $mLCH_4/gVS$) (Solé-Bundó *et al.,* 2019).

2.1.7 Food waste

The unrestricted disposal of large amounts of food waste has become a major problem as it causes intense pollution of the environment. Approximately 33.3% of food generated worldwide for human consumption are estimated to be lost or wasted through the food supply chain. Food waste is a non-productive use of scarce assets (land, water and fertiliser), leading to degradation of the environment. Global hunger is becoming an increasing global issue, paradoxically so is wastage of food, consumers in the United Kingdom throw away 31% of the food that they buy. Reasons given for these wastages are mostly that the food was left unused or was left over during preparation or cooking (Girotto *et al.,* 2015). The developed world uses up precious land and resources when producing more food than is going to be consumed; resources that otherwise could have been used to feed the poor. These big amounts of food wastes end up on landfill sites worldwide, and contribute to the environmental issues, which included greenhouse gas emissions. Consumer food wastage is just one component of the bigger food waste issue. There are various stages of food wastage in the food supply chain, which include during food storage, transportation of these foods, processing, at retailers and in the kitchens of households and restaurants. Infrastructure that is failing or lacking can cause food loss or spoilage (Girotto *et al.,* 2015). The amount of food losses throughout the food supply chain is estimated at 50% of all food that is produced for human consumption; these include pre- and post-consumer food waste. This makes it clear that food production and utilisation processes are not highly efficient. A big amount of these food wastages is still fit for human consumption. If it had been better managed, 61% of food wasted by households in the United Kingdom could have still been eaten (avoidable food waste); 20% can be classified as 'possibly avoidable' and only 19% as unavoidable (inedible) (Girotto *et al.,* 2015). A vast amount of food is going wasted, much of which could potentially feed the almost 1 billion people worldwide (13% of the global population) classified as undernourished, thereby aggravating problems of hunger and food insecurity, particularly in poorer countries. The challenge of feeding the global hunger can be met by reducing the amount of food wasted after production. In addition to the social implications and cost associated with edible food being wasted, inedible food disposal represents the loss of a potentially valuable resource that can be utilised as an input to other processes such as animal feed, composting or the production of biogas. The disposal of food waste to landfill sites is also a significant contributor to greenhouse gas emissions as well as leachate production. Garden waste and food waste make up the biggest proportion of organic waste disposed of a landfill site (Girotto *et al.,* 2015; Nahman *et al.,* 2012). Complex elements and organic material characterise food waste. There are several kinds of food waste, including fruit and vegetable waste, food waste from households and restaurants, brewery waste and milk waste (Pramanik *et al.,* 2019). Food waste, however, is a suitable substrate that can be processed with anaerobic digestion (AD) due to its good biodegradability and high water content (Girotto *et al.,* 2015).

2.1.8 Biomass for anaerobic digestion – the Southern African context

The Polokwane Declaration was drafted during the first South African Waste Summit in 2001 and set goals for the reduction of waste generation and waste disposal. These included a reduction to 50% for waste generation and 25% for waste disposal by 2012, with a full zero waste plan envisaged by 2020. Currently, none of the municipalities can achieve this goal, though various solutions are being investigated/implemented at pilot-scale level (Trois & Simelane, 2010).

Biomass is responsible for 70 to 95% of energy requirements in Sub-Saharan Africa (Shane *et al.,* 2017). These biomasses include municipal solid waste (MSW), manure and sludge from wastewater treatment plants. In South Africa, pig manure, along with chicken and cow manure, are some of the organic materials available in abundance (Rapatsa & Moyo, 2013). Significant contributions to reach renewable energy targets have been made by the agricultural sectors of other countries in the production of biogas (Lutge & Standish, 2013).

Currently in South Africa, untreated sludge is banned from all landfill sites, thereby creating a disposal problem. Therefore, alternative uses for sludge must be looked at, such as fertiliser and alternative fuel sources. The presence of pollutants will depend on what the sludge can be utilised for, as some can be the source of inhibitions. Another possible problem is that untreated sludge consists of 85 to 95% water (Bratina *et al.,* 2016).

Sewage sludge is the ‘waste’ produced by wastewater treatment plants, both domestic and industrial. This is a heterogeneous substance and is made up of a variety of constituents. Sewage sludge consists of six groups:

1. Non-toxic organic material
2. Nitrogen- and phosphorous components
3. Toxic organic and inorganic material
4. Pathogens and other microorganisms
5. Inorganic material (calcium, magnesium etc.)
6. Water (Huang *et al.,* 2014)

Sewage sludge is receiving increased research focus because of the risk to the environment. The fact that sewage sludge is rich in volatile matter makes it a potential resource for biogas production (Huang *et al.,* 2014). The high organic content of sewage sludge makes it degradable. This is also the reason why sludge should undergo chemical and hygienic stabilisation before it can be disposed of at landfill sites or used for agricultures applications. Methane fermentation is one of the possible methods for stabilising and sanitation of sludge (Sosnowski *et al.,* 2003).

There is a long tradition of treating sewage sludge anaerobically at WWTP to reduce the volume of sludge, but the process has not been focused on optimal biogas production. In this study, local waste was identified, including what the current handling of these waste types is, as well as whether there is a potential for biogas production. Biogas has many uses as well as can be a solution to many problems. Sludge volume reduction is a big problem as sludge cannot just be stored, as plants are running out of space to build storage dams. The sludge has potential for biogas production, but currently it is used on land applications, for composting or is incinerated. Sludge disposal/treatment depends on (i) physiogeographical, technical and economic factors, and (ii) ethic factors (values and priorities) related to the acceptability of specific practices/technologies.

Table 2-1 indicates average biogas yields from different substrates used, with most used as single substrates, while some as co-substrates. For agricultural residues, maize is the substrate that has the highest potential biogas yield as well as the substrate most studied. With regards to manure, cow manure has the highest biogas yield potential. Egg waste has the highest potential biogas yield in the food waste category. Some of the substrates in Table 2-1 was discussed in detail in the previous sections.

Table 2-1: Average biogas yields in literature

Category	Substrate	Biogas yield ($m^3kg^{-1}VS$)	Reference
Agricultural residues	Rice straw	0.55-0.62	(Okudoh *et al.,* 2014)
	Wheat straw	0.188	(Okudoh *et al.,* 2014)
	Maize straw	0.4-1.0	(Okudoh *et al.,* 2014)
	Grass	0.28-0.55	(Okudoh *et al.,* 2014)
	Fodder beet	0.278	(Okudoh *et al.,* 2014)
	Sugar beet	0.44	(Okudoh *et al.,* 2014)
	Coffee pulp	0.30-0.45	(Okudoh *et al.,* 2014)

	Corn stalk	0.35-0.48	(Okudoh *et al.,* 2014)
	Cassava peels (residues)	0.661 (0.132)	(Roopnarain & Adeleke, 2017; Okudoh *et al.,* 2014)
Abattoir waste	Sheep blood, stomach content and manure	650 ml	(Roopnarain & Adeleke, 2017)
Manure	Pig	0.27-0.45	(Caruso *et al.,* 2019; Roopnarain & Adeleke, 2017; Okudoh *et al.,* 2014)
	Poultry	0.3-0.8	(Caruso *et al.,* 2019; Roopnarain & Adeleke, 2017; Okudoh *et al.,* 2014)
	Horse	0.4-0.6	(Caruso *et al.,* 2019; Okudoh *et al.,* 2014)
	Cow	0.6-0.8	(Caruso *et al.,* 2019; Roopnarain & Adeleke, 2017; Okudoh *et al.,* 2014)
	Rabbit	37 dm^3/TMS	(Caruso *et al.,* 2019; Roopnarain & Adeleke, 2017)
	Goat	31 dm^3/TMS	(Caruso *et al.,* 2019; Roopnarain & Adeleke, 2017)
	Sheep	0.572 ÷ 1.468 Nm^3/kg VS	(Caruso *et al.,* 2019)
Food waste	Vegetable waste	0.4	(Okudoh *et al.,* 2014)
	Kitchen/restaurant wastes	0.506-0.65 (CH_4)	(Okudoh *et al.,* 2014)
	Leftover food	0.2-0.5	(Okudoh *et al.,* 2014)
	Egg waste	0.97-0.98	(Okudoh *et al.,* 2014)
	Cereals	0.4-0.9	(Okudoh *et al.,* 2014)
	Banana	88 000 cm^3	(Roopnarain & Adeleke, 2017)
	Plantain	2 409 cm^3	(Roopnarain & Adeleke, 2017)
Aquatic plants or seaweed	Algae	0.38-0.55	(Okudoh *et al.,* 2014)
	Salvinia	0.155	(Okudoh *et al.,* 2014)
	Water hyacinth	0.2-0.3	(Roopnarain & Adeleke,

			2017; Okudoh *et al.,* 2014)
	Caboma	0.221	(Okudoh *et al.,* 2014)

2.2 Waste stream implications on water and water sources

Nitrogen-containing fertilisers are widely used in modern farming, but the level of application in most areas exceeds crop demand, with negative short- and long-term effects (Albornoz, 2016). There is a risk of over-fertilisation with nutrients, particularly in areas with larger amounts of excrement produced (Albornoz, 2016; Umweltbundesamt, 2014). A surplus of reactive nitrogen in the soil is the consequence of over-fertilisation (Innes, 2013). This can cause groundwater nitrate pollution by leaching, and thereby the eutrophication of rivers, lakes and the sea (Albornoz, 2016; Umweltbundesamt, 2014; Innes, 2013). Eutrophication can also be caused by an excessive supply of phosphorus from farmland. Eutrophication of water bodies enhances the formation of algae, impairing the lighting circumstances at reduced aquatic concentrations, and therefore the photosynthesizing capacity of other crops (Albornoz, 2016). This leads to biodiversity reduction. Nitrate in the soil can turn into nitrite, which is a hazardous substance for human health. Nitrogen surplus in soils can result in a change in crop and trees growth (e.g. excessive length and soft growth, spongy shoots, cells and tissues), making them more sensitive to heat and frost, crop pests, and bacterial and fungal diseases. Their storage property also reduces, which can cause profit reductions together. Field fertilisation can affect the quality of the groundwater and can trigger additional environmental issues (Umweltbundesamt, 2017; Albornoz, 2016). Anaerobic digestion of these substrates can be a solution to prevent possible pollution of water and water sources.

2.3 Anaerobic digestion (AD)

The main goal of AD of primary sludge is to convert carbonaceous material into CH_4 and CO_2. Therefore, digestion products include gases, stabilised sludge solids that are dewatered and disposed of, and sludge liquor that is treated further (Roman *et al.,* 2006).

There are advantages in using mixed waste streams for co-digestion, as solids can be mixed with liquid waste, to adjust moisture contents also to produce a more pumpable waste stream. Co-digestion can also increase biogas yields by reducing nutrient deficiencies, supplying microbial inoculums as well as reduction in the inhibition of the process by changing of determinants that can inhibit the process such as pH (McDonald *et al.,* 2008).

Free ammonia found in pig manure often inhibits the anaerobic process and can result in poor methane yield; this is one of the benefits of using pig manure as a co-substrate for anaerobic digestion (Li *et al.,* 2017). For co-digestion, the co-substances' sources, transport, and supply stability should be considered to maintain the biogas plant on a long-term basis (Duan *et al.,* 2019). After the process of AD is finished, a digestate is produced that can be used as fertiliser due to the high nutrient contents (N, P and K) (Scaglia *et al.,* 2014).

2.4 Biogas

In the absence of oxygen, organic material in digested by microbes (anaerobic digestion) and biogas is produced (Shane *et al.,* 2017). The metabolisation of fermentation products, like organic acids, by non-methanogenic microbes, produces hydrogen. Hydrogen is then utilised by hydrogenotrophic methanogens to reduce CO_2 to CH_4 (Yang *et al.,* 2015). Complex microbial communities steer the biogas production process; these communities include bacteria, archaea, protozoa and fungi, which can adapt to anaerobic digestion parameters (Repinc *et al.,* 2018). The community structure of these organisms depends on the following conditions:

2.4.1 Temperature

Microbes associated with AD are easily affected by temperature changes. These changes influence the generation of hydrogen and methane as well as the decay (decomposition) of organic matter. A temperature reduction will lead to reduction in yields, as the result of reduction in Volatile fatty acids (VFA) generation rate, concentration of ammonia, rate at which the substrate is utilised, slower metabolic rate of microbes and higher "start-up" times (Repinc *et al.,* 2018; Mao *et al.,* 2015).

2.4.2 pH

The digestive process as well as products are directly influenced by the pH, with the optimal pH being between 6.8 and 7.4. pH has a significant influence on the microbial growth rates (Repinc *et al.,* 2018; Mao *et al.,* 2015). Moa *et al.* (2015) found that the predominant microbial community at pH 6.0 is *Clostridium butyricum.* Furthermore, *Propionibacterium* dominates at pH 8.0.

2.4.3 C:N ratio

Digestion substrate nutrient levels are emulated by the C:N ratio, and therefore digestion setups are easily affected by C:N ratio. When ideal C:N ratio is maintained, it will prevent ammonia inhibition, whereas a greater C:N ratio leads to decreased protein solubilisation, which, in turn, leads to decreased total ammonia nitrogen and fatty acid concentrations (Mao *et al.,* 2015).

2.4.4 Organic loading rate (OLR)

Organic loading rate (OLR) refers to the volatile solid volume supplied to the system. A greater OLR will only escalate biogas production to a degree but can also interfere with the stability and the productivity of the digestion process (Repinc *et al.,* 2018; Mao *et al.,* 2015). These interferences will lead to bacterial inhibitions.

2.4.5 Hydraulic retention time (HRT)

Under mesophilic conditions, the ideal retention time to treat waste would be 15 to 30 days. Substrate composition and OLR are factors on which HRT depends. Volatile fatty acids build-up can occur at lower HRT; with greater HRT, poor utilisation of digester components can occur (Mao *et al.,* 2015).

2.4.6 Interactions between parameters

Furthermore, the kinetics of the bacterial and archaeal microbial communities, trace metal status and polyphenolic compounds add to the variations in the efficiency of the process. Operational parameters are also important for the performance of anaerobic digestion systems besides chemical parameters, with two of the most important being organic loading rate (OLR) and hydraulic retention time (HRT). In addition, it is crucial to find the right balance between these two parameters to maximise process efficiency. The optimum operating conditions are usually related to the feedstock material characteristics and should therefore be determined for each individual case, with single substrates in mono-digestion systems. Continuous research on this subject is therefore important, especially when considering the high variation within each geographical area in the type and composition of possible substrates (Pellera & Gidarakos, 2017).

2.4.7 Advantages and disadvantages of biogas

There is an immediate need to address society's current issues without having any long-term negative impact that could become a critical issue for future generations to overcome (Alayi *et al*., 2016). Biomass energy technologies, such as biogas production, have the potential to supplement or supplant the use of fossil fuels for energy production making it attractive as a clean energy source (Paolini *et al., 2018;* Shane *et al.,* 2017; Alayi *et al*., 2016; McDonald *et al.,* 2008). Further advantages of biogas energy include benefits such as sustainable management of waste streams created by human activities (e.g. faecal sludge) and finally reduction in greenhouse gas emissions (Paolini *et al., 2018;* Shane *et al.,* 2017). From an ecological vantage point, biogas has the technology to reduce deforestation (where firewood is a primary energy source) (Alayi *et al*., 2016), improvement in soil fertility (when digestate is applied as a fertiliser (Paolini *et al., 2018;* Alayi *et al*., 2016) as well as a reduction in water and soil pollution. The energy available in biogas can also be channelled to heat and electricity production. The fact that biogas production can serve as a mechanism to manage waste as well as generate a valuable resource (such as heat and electricity) (Alayi *et al*., 2016) has distinct economic benefits (Alayi *et al*., 2016) and may also serve as a route to improve people's living conditions (McDonald *et al.,* 2008). Eventually, biogas can be converted to biomethane, used effectively as a fuel for cars, grids or injected into national natural gas (Paolini *et al., 2018;* Alayi *et al*., 2016).

However, despite the advantages mentioned, anaerobic digestion for biogas production does have some limitations. These include a high retention time requirement, limited methane production and lower yields during the anaerobic digestion of dry solid materials due to lower rates of hydrolysis (Pei *et al.,* 2016).

Biogas has a calorific value of 15-24 MJ/m^3, average gross electricity production of 2.07 kWh per m^3 and average heat production of 2.67 kWh (Shane *et al.,* 2017). This means that this is a potential source of energy. Despite national efforts to increase communities' adoption of biogas, biodigesters are being abandoned and their adoption among local African communities seems slow (Surroop *et al.,* 2019).

2.5 Microbial community succession during biogas production

There are many environmental benefits of anaerobic digestion. They include a source of renewable energy, nutrient recycling as well as the reduction of waste volumes (Murto *et al.,* 2004; Van Lier *et al.,* 2001; Ghosh *et al.,* 1975). Interest in anaerobic digestion is increasing, but further investigation of the effects and variation of inputs into reactors, how the stabilisation is influenced by the composition of waste, is needed (Murto *et al.,* 2004).

Complex microbial communities steer the biogas production process. These communities include bacteria, archaea, protozoa and fungi, which can adapt to anaerobic digestion parameters. Each of the four steps of anaerobic digestion contains different dominant microbial communities (Repinc *et al.,* 2018). Four steps result in the production of biogas, these steps are (Shane *et al.,* 2017):

2.5.1 Hydrolysis

Extracellular enzymes hydrolyse the substrate to produce sugars, amino acids and long chain fatty acids (Ezebuiro & Körner, 2017). The hydrolysis rate of substrates needs to be optimised during AD. This will then maximise the production of methane. The hydroxyl (OH^-) group from a water molecule (H_2O) initiates hydrolysis by attacking the substrate. Important factors involved in hydrolysis include substrate composition, pH, hydrolytic enzyme (hydrolases) concentration and enzyme-substrate interactions. Hydrolases are extracellular enzymes released by microbes during the AD process and include lipases, glucosidases and proteases, and result in the production of organic acids (Ezebuiro & Körner, 2017). Microbes involved in hydrolysis include the order Halanaerobium, Clostridiales and Bacteroidales and the genus Acetivibrio (Yang *et al.,* 2017).

2.5.2 Acidogenesis

Alcohols and organic acids are produced from the fermentation of sugars and amino acids (Ezebuiro & Körner, 2017). The acidogenic process is carried out by the Clostridia class and the Bacteroidaceae family (Yang *et al.,* 2017).

2.5.3 Acetogenesis/dehydrogenation

Carbon dioxide, hydrogen and acetic acid are produced by obligated hydrogen producing acetogens (Ezebuiro & Körner, 2017). Acetate is formed from H_2 and CO_2 through the acetyl-CoA pathway, by acetogenic bacteria, which are obligated anaerobic microbes. This group of microbes represents a phylogenetically diverse group. Due to the physiological and phylogenetic diversity of this bacteriological group, it is difficult to distinguish their diverse role in reactors (Lui *et al.,* 2017). The dominant acetogenic bacteria are genus Clostridium, Treponema, Eubacterium, Thermoanaerobacter, Moorella, Methanosaeta and Porphyromonadaceas (Yang *et al.,* 2017).

2.5.4 Methanogenesis

Methane and carbon dioxide (main components of biogas) are produced by hydrogenotrophs and aceticlastic microbes (Ezebuiro & Körner, 2017) and contribute to the chemical oxygen demand (COD) removal (Li *et al.,* 2016). Archaea comprises a group of single-celled microbes. The formation of CH_4 from CO_2 and H_2, formate, methanol, methylamines and/or acetate, is performed by methanogenic archaea, which are a phylogenetically diverse group of strictly anaerobic Euryarchaeota with an energy metabolism that is restricted to the formation of CH_4. The bulk of the methanogens found in anaerobic digesters is said to be Methanococcales, Methanobacteriales, Methanomicrobiales and Methanosaeta sp. (Zahedi *et al.,* 2016).

2.5.5 Interactions between the various communities

The microbial communities are constantly interacting. Their complicated associations and functioning are not well known yet. The organisational structure and agitation of the microbial community are linked to the effectiveness of the anaerobic digestion reactor as the microbial community tends to replacement, migration and dying (Repinc *et al.,* 2018). Microorganisms in the environment are widespread and are the primary drivers in the process of anaerobic digestion (Mao *et al.,* 2019). Overall anaerobic digestion efficiency depends on the roles and interactions of anaerobic microbes (Liang *et al.,* 2020). However, the core microbiota's governing power for AD quality is still poorly understood (Tao *et al.,* 2020). Occasionally, imbalances occur between different microbial species, such as the issue of faster hydrolysis/acidogenesis and slower methanogenesis, resulting in lower anaerobic digestion efficiency. Therefore, in order to further improve methane production, there is an urgent need to investigate the composition and role of microbial communities during anaerobic sludge digestion (Feng *et al.,* 2019).

2.6 Methods to investigate communities

2.6.1 Culture-dependent vs culture-independent methods

Traditional culture-dependent analyses are well known to be inadequate to characterise the whole prokaryotic and eukaryotic microbiota (Pezzolla *et al.,* 2015). Culture-dependent methods consist of isolating and culturing microorganisms based on morphological, biochemical or genetic characteristics before the identification of the organisms (Jany & Barbier, 2008). Results indicate that not only does culturing pick a small fraction of the micro-organisms that can be detected using culture-independent methods, but many of the most abundant micro-organisms identified using culture-independent methods are also not captured using plate culturing techniques (Stefani *et al.,* 2015).

Culture-independent methods provide more information on the structure of microbial populations (Jany & Barbier, 2008). These techniques have been created to study microbial communities from different environments since the implementation of molecular techniques. Over the past 20 years, several techniques have been developed to study environmental microorganisms directly based on the direct amplification and analysis of the small subunit ribosomal RNA gene. These techniques include denaturing/temperature gradient gel, polymorphism of single-strand conformation, polymorphism of fragment length limitation, polymorphism of terminal fragment length restriction fragment, and quantitative polymerase chain reaction (PCR) (Su *et al.,* 2012). Additionally, molecular techniques based on non-PCR, such as microarray and fluorescence in situ hybridisation (FISH), were also implemented. Several innovative research areas such as metagenomics, metatranscriptomics, metaproteomics, and single-cell genomics have been developed in recent years, mainly driven by the development and implementation of next-generation sequencing methods. In the areas of microbial ecology and environmental microbiology, several single-cell-based techniques such as Raman microspectroscopy and nano-scale secondary ion mass spectrometry are also continuously being used. The implementation of these techniques has revolutionised microbiology by enabling researchers to analyse in situ natural microbial communities, including their genes, transcripts, proteins and metabolites, directly, and how their interactions affect their patterns of production (Su *et al.,* 2012).

In recent years, the use of molecular techniques to detect, identify and characterise microorganisms has attracted considerable attention and these techniques are currently considered an indispensable tool for the accurate description of microbial community

(Iacumin *et al.,* 2009). Community-level studies are increasingly focusing on culture-independent techniques based on direct DNA (or RNA) analysis without any culturing steps. These techniques are based on protocols during which total DNA (or RNA) is directly extracted from the substrate (Iacumin *et al.,* 2009; Jany & Barbier, 2008). Most of these methods use the amplification of total DNA by polymerase chain reaction (PCR). The PCR amplicons of different species are discriminated against by using gel or capillary separation or by hybridising specific samples (Jany & Barbier, 2008). In addition, the characterisation of DNA extracted straight from soil by means of high-throughput molecular methods (next-generation sequencing, i.e. NGS techniques) is proving increasingly useful in providing better in-depth data on the entire prokaryotic and eukaryotic microbiotic structure (Pezzolla *et al.,* 2015). Culture-independent techniques typically strive to collect DNA from the whole community.

2.6.2 PCR

Polymerase chain response (PCR) has become a common diagnostic and research technique. Several studies demonstrate that the elevated sensitivity and specificity of the PCR method make it possible to detect, identify and quantify microorganisms as an accurate, effective and quick method (Shahi *et al.,* 2018). The PCR is an in vitro amplification of specific nucleic acid sequences. Conventional culture methods have unique benefits, though with some constraints that include the need to maintain bacterial viability, identification of small amounts of microorganisms, conditions of transportation, labour intensity, the need for specialist staff, an extended period before results and strict sampling methods. The non-motile microorganisms cannot be identified by other microbiological studies such as dark field microscopy. Polymerase chain reaction (PCR) is the best choice among molecular techniques to overcome the aforementioned limitations and is able to identify even one copy of the DNA targets (Shahi *et al.,* 2018).

2.6.3 Metabarcoding (NGS)

DNA metabarcoding is a mixture of high-throughput amplicon-based sequencing (HTS) and DNA taxonomy (Serrana *et al.,* 2019; Hebert *et al.,* 2003). High-throughput amplicon sequencing can simultaneously process large numbers of individuals, making it faster and cheaper than conventional Sanger sequencing (Thudi *et al.,* 2012; Hebert *et al.,* 2003). Following a thorough read processing phase, most metabarcoding pipelines perform taxonomic tasks by comparing clustered reads or taxonomic operational units (OTUs) with reference sequence databases such as GenBank (Benson *et al.,* 2004) and the Life Data System Barcode (BOLD) (Hebert *et al.,* 2003). Metabarcoding offers cost-effective and faster evaluations with a more extensive and verifiable taxonomic identification less dependent on taxonomic knowledge. Previous studies have evaluated the capacity of DNA metabarcoding to identify parallel to morphology-based identifying macroinvertebrate societies. Metabarcoding of DNA offers wider taxonomic coverage and finer strength of resolution. With this benefit, metabarcoding of DNA can provide greater discriminatory authority in identifying environmental factors that affect the structure of the society compared to traditional techniques (Hebert *et al.,* 2003). Due to the high sequencing depth, it is possible with the newly developed sequencing technologies to identify both abundant and small populations in the microbial community. The Ion Torrent PGM (Life Technologies) was launched in early 2011 with the highest performance compared to 454 GS Junior (Roche) and Miseq (Illumina), making sequencing cost-effective and time-saving. In some studies, Ion Torrent PGM has been used to analyse the microbial population composition of environmental samples. With Ion Torrent PGM’s high sequencing depth, possible bacterial pathogens can also be identified in biogas reactors (Luo & Angelidaki, 2014). Miseq is currently dominating this field of microbiology.

2.7 Concluding remarks

In summary, research into understanding the anaerobic digestion and biogas production makes it possible to use waste resources as potential energy sources. Pig slurry is usually stored in containers and applied to agricultural land, creating water pollution problems. Disposal routes for sludge exist in South Africa, while the only disposal route for pig slurry in South Africa, currently, is land application. Using molecular techniques, the microbial community structure as well as community shift during various phases of biogas production can be studied. This can also give a picture of what is taking place in seeding sludge, pig slurry and combinations of pig slurry and seeding sludge and how microbes are influencing biogas production. Single substrate fermentation was used in order to understand what was happening in the reactors, instead of influencing microbial communities with additional substrates, in order to first understand the microbial communities as is and how each contributes to biogas production as well as community shifts. In order to do co-fermentation, feedstock needs to be reliable and available at constant times. Advanced molecular methods such as NGS are available to study these fermentation processes. The advantage of using these methods are cost-effective and faster evaluations with a more extensive and verifiable taxonomic identification less dependent on taxonomic knowledge.

CHAPTER 3 – MATERIALS AND METHODS

3.1 Sampling

Pig slurry was collected from local pig farmers, transported and stored in plastic buckets. Interferences, such as larger pieces as well as grains were removed, and the samples were homogenised. Subsamples were taken for physio-chemical analyses as well as molecular analyses.

3.2 Characterisation of feedstock

Samples were analysed at an Analytical Laboratory for chemistry and heavy metals. (See the table of results in the Results chapter.) Physio-chemical analyses conducted included: heavy metals with an ICP-MS, PO_4, NH_4, chemical oxygen demand (COD), total and volatile solids (TS & VS) and pH. The molecular analyses included: DNA extraction and MiSeq. Methods used by the laboratory are available in the Appendix B.

3.3 Experimental bioreactor benchtop set-up

The most important part when choosing materials is that all instrumentation should be gastight. For all parts that were in contact with the biogas atmosphere, glass was the material of choice. Benchtop fermentation was used for the co-digestion experiments. Once the benchtop setup was complete, the setup was tested to ensure that it is gastight. Leak testing can be done using nitrogen. Samples that were collected were characterised. The feeds were incubated under mesophilic conditions. Benchtop fermenters (Figures 3-1 and 3-2) were used for the biogas production. This works on a gas displacement method. Gas that was produced in the reactors displaced the confining liquid into cylinders, and therefore the volume of gas produced can be measured. After the gas production volume was read, the gas was captured in a gasbag for analysis using a gas analyser. A Biogas 500 gas analyser was used (VDI (Verein Deutscher Ingenieure) 2006).

Seeding sludge from a municipal wastewater treatment works was collected and used, as this was untreated digested sludge. Before the seeding sludge was used, it was be kept at the test temperature for a week, so that the gas production of the sludge was reduced through a hunger phase.

The following needs to be taken into consideration when determining the weight of substrate and seeding sludge to be used in the fermentation batch: The substrate should not weigh more than the seeding sludge. Equation 3-1 for determining reactor loading:

$$\frac{Organic\ dry\ matter_{substrate}}{Organic\ dry\ matter_{seeding\ sludge}} = \leq 0.5 \tag{3-1}$$

A reference sample is recommended to check the process of the seeding sludge, a sample with a known biogas potential. A potential reference sample is Microcrystalline Cellulose (MCC). Experimental runs were all done in triplicate. Before the fermentation reactors were sealed, the gas phase was flushed with nitrogen to remove the remainder of oxygen as this would have a negative effect on the biogas yield due to aerobic digestion.

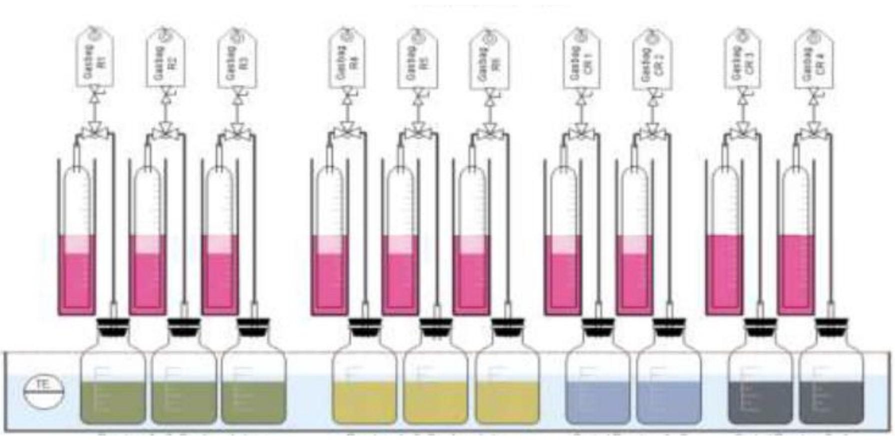


Figure 3-1: Diagrammatical illustration of experimental setup of benchtop batch reactors (VDI (Verein Deutscher Ingenieure) 2006).

Figure 3-2: Experimental setup of bench top batch fermentation

Gas production readings were recorded twice a day. For the first phase of the setup, six samples of each substrate were used. After 15 days, three of the six reactors’ gas production was recorded, then taken off. These three were put together for a composite sample and used for the next phase setup. Composite samples were used as a seeding sludge for the setup with the next batch of the same substrate. Figure 3-3 shows a schematic diagram of how back inoculation was done.

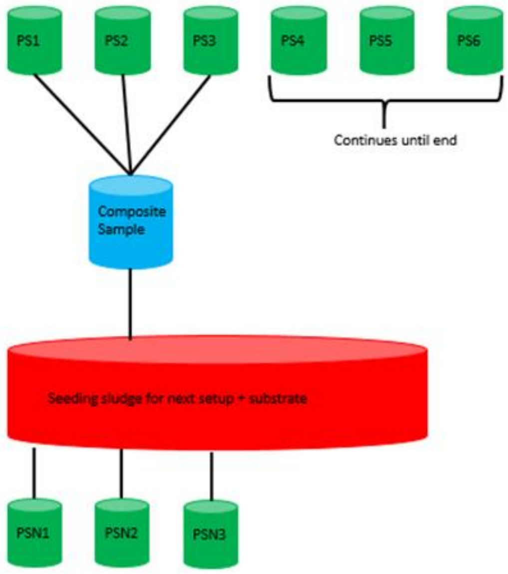


Figure 3- 3: Schematic diagram of back inoculation

3.4 Microbial community characterisation

3.4.1 DNA extraction and characterisation

Total genomic DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., California, USA). Some changes were made to the protocol in order to extract DNA from pig slurry. Changes were as follows: Step 1, PowerBead was removed from the PowerBead Tube, 2ml sample was added and centrifuged for 2 minutes at 10 000rpms, another 2ml sample was added and centrifuged. PowerBead was added to the tube and vortexed as protocol states further. Between steps 3 and 4, an additional step was added; in this step, the tube was incubated for 15 minutes at 70°C before continuing with protocol. The quality of DNA extracted was verified by spectrophotometric (NanoDrop, Thermo Fisher Scientific, US) analysis as well as agarose gel electrophoresis.

3.4.2 Amplification of 16S rRNA

Total DNA was amplified as recommended by Illumina. Locus-specific primers 341F and 805R (Klindworth *et al.,* 2013) were used, targeting the 16S rRNA gene, attached to Illumina specific adapters for amplification:

- Forward: 5’-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3’ and

- Reverse: 5’-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTAC HVGGGTATCTAATCC-3’

All PCRs were performed in a C1000TM thermal cycler (Bio-Rad, USA).

Briefly, the workflow for library preparing involved: a first stage 'amplicon PCR' targeted at amplifying the area of concern by using 12.5 ng of genomic DNA, 2x KAPA HiFi HotStart ReadyMix and 5 µM of the above-mentioned primers. The PCR was performed at a final volume of 25 µl by denaturing at 95 °C for 3 min, followed by 25 cycles of 95 °C at 30 sec, 55 °C at 30 sec and 72 °C at 30 sec. A final elongation step of 72 °C was included for 5 minutes. Using Agencourt AMPure XP beads (Beckman Coulter Genomics, California, USA), amplicons were then subjected to a 'PCR clean-up' phase. After cleaning, index PCR attaching dual indexes (Nextera XT Index Kit) was performed 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 thermoclycing conditions were 95°C for 3 minutes followed by 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and a final elongation step of 72°C for 5 minutes. The index PCR products underwent a second step of 'PCR clean-up'. The partial 16S rRNA libraries were then quantified using a Qubit fluorometer (Qubit 3.0, Life Technologies, Malaysia), standardised, pooled to a final 20 pM concentrations, and denatured in 0.2 N NaOH. The pooled library was diluted to a final concentration of 6 pM, spiked with 10% PhiX control and heat denatured for 2 min before loading samples on the reagent cartridge of MiSeq V3 (Illumina, San Diego, CA, USA). After completing a paired end of 2x300 bp reads sequencing run on the Illumina MiSeq, de-multiplexing and secondary reading analyses were performed using MiSeq reporter software (Illumina, San Diego, CA, USA).

3.4.3 Library preparation for next generation sequencing (NGS)

The 16S rRNA amplicons were sequenced on an Illumina Miseq sequencer according to the MiSeq 16S library preparation guide (Illumina n.d.).

3.4.4 Processing of NGS data

The demultiplexed reads from the next generation sequencing were processed though the QIIME2 pipeline (Guerrini *et al.,* 2019). The quality of the reads was evaluated using demux. After adjusting the parameters based on the quality control, and dada2 was used to assemble the forward and reverse reads. Thereafter, the assembled reads were classified into operational taxonomical units (OTUs) using the feature classifier from Quantitative Insights into Microbial Ecology 2 (QIIME2) software (Guerrini *et al.,* 2019). For taxonomical assignment, the processed sequences were aligned against the SILVANGS rRNA database (SILVA 132 release) (Quast *et al.,* 2012). The generated OTU count table was summarised in QIIIME2. Alpha and beta diversity was done using MicrobiomeAnalyst (Dhariwal *et al.,* 2017). The statistically significant bacteria graphs were done using STAMP (Parks *et al.,* 2017). Metageneassist was used to do taxonomic to phenotype mapping (Arndt *et al.,* 2012). MicrobiomeAnalyst was used to do abundance between various stages (Dhariwal *et al.,* 2017).

3.5 Statistical analysis

Where applicable, descriptive statistics (minimum, maximum, average and standard deviation) were calculated using Microsoft Excel 2016.

CHAPTER 4 – RESULTS

4.1 Characterisation of sludge

The substrates were analysed for organic elements as well as heavy metals. Figures 4-1 and 4-2 show organic material analysed in the substrates used in the reactors for the various setups. In Figure 4-1, the pig slurry contained 10.93 mg/l SO_4 and 23.09 mg/l NO_3 respectively, the seeding sludge used contained 21.14 mg/l SO_4 and 44.09 mg/l NO_3 respectively, while the new pig slurry contained 56.34 mg/l SO_4 and 0.49 mg/l NO_3 respectively.

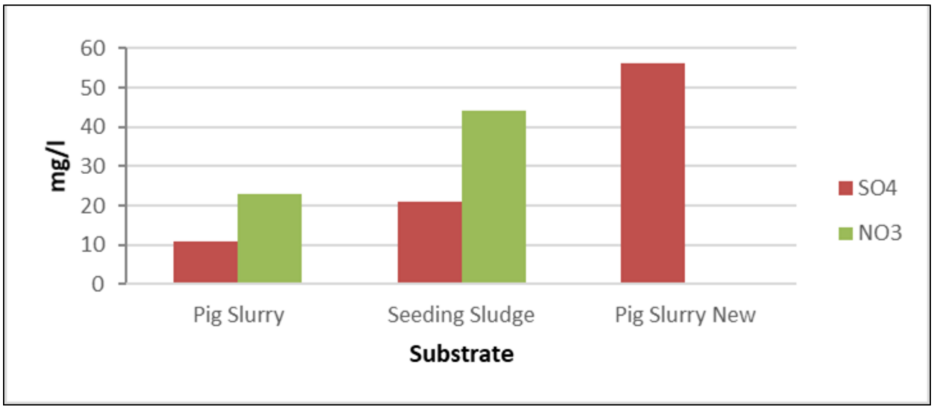


Figure 4- 1: Nutrients of substrates used in reactor runs

According to Figure 4-2, pig slurry contained 153.52 mg/l PO_4 and 1226.32 mg/l NH_4 and 359.74 mg/l Cl, respectively, the seeding sludge used contained 10.55 mg/l PO_4 and 1021.65 mg/l NH_4 and 1580.43 mg/l Cl, respectively, while the new pig slurry contained 330.21 mg/l PO_4 and 1580.43 mg/l NH_4 and 439.12 mg/l Cl, respectively.

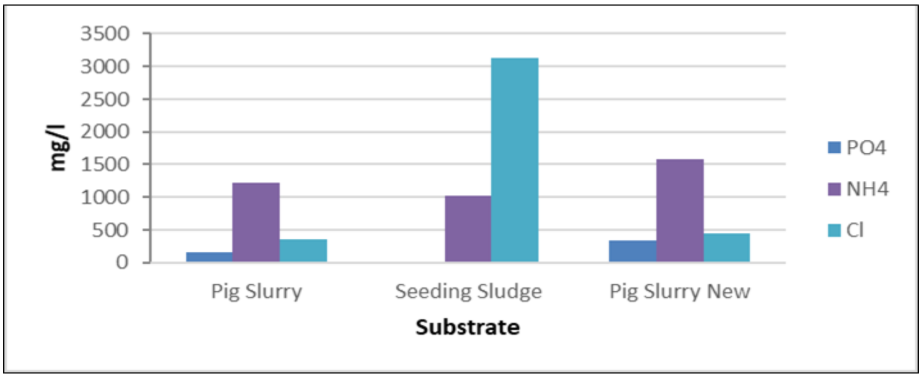


Figure 4- 2: Nutrients of substrates used in reactor runs

Table 4-1 shows heavy metals of substrates used in the reactor setup. There were no values that were high enough to inhibit biogas production. A full list of heavy metal results is available in the Appendix B as well as a list of heavy metals that influence AD in Appendix E.

Table 4-1: Summary of heavy metal content of the substrates used in this study

	Pig slurry (mg/l)	Seeding sludge (mg/l)	Pig slurry new (mg/l)	Biogas inhibitory concentration (mg/l)
Fe	0.32	62.86	0.51	20 000
Ni	0.086	0.19	0.05	100
Cu	0.63	0.26	0.51	500
Zn	0.71	11.48	0.37	50
Cd	0.01	0.002	0.001	1.2

4.2 Biogas yield

Sample designation
Samples were abbreviated as follows:
XXX_Stage

Table 4-2: Abbreviations for samples

Abbreviation	XXX
P	Pig slurry
S	Seeding sludge
B	Back inoculated from previous run
MC	Microcrystalline control

	Stage
Mid	Middle
End	End of reactor run
Start	Start of reactor run

Table 4-3: Explanation of sample names and stages

ABBREVIATION	**MEANING**	**STAGE**
MC_END	Microcrystalline control	END
MC_START	Microcrystalline control	BEGINING
P_END	Pig slurry	END
PS_MID	Pig slurry and seeding sludge	MIDDLE
P_START	Pig slurry	BEGINING
PB_END	Pig slurry back inoculated from previous run	END
PB_MID	Pig slurry back inoculated from previous run	MIDDLE
PS_END	Pig slurry and seeding sludge	END
PS_START	Pig slurry and seeding sludge	BEGINING
PSB_END	Pig slurry and seeding sludge back inoculated from previous run	END
PSB_MID	Pig slurry and seeding sludge back inoculated from previous run	MIDDLE
S_END	Seeding sludge	END
S_MID	Seeding sludge	MIDDLE
S_START	Seeding sludge	BEGINING
SB_END	Seeding sludge back inoculated from previous run	END

Cumulative graphs were made of the three different seeding sludge samples: S_START_Cumulative is the seeding sludge that was stopped and used to back inoculate to form SB_Cumulative as shown in Figure 4-3. Results designated as S_Cumulative ran the whole 30 days as a control that biogas production was taking place. After 45 days, S_Cumulative produced 600 ml of biogas. After 15 days, S_START_Cumulative was removed to produce SB_Cumulative but produced 276 ml biogas. SB_Cumalative ran for the rest of the time with S_Cumulative (30 days) and produced 118 ml.

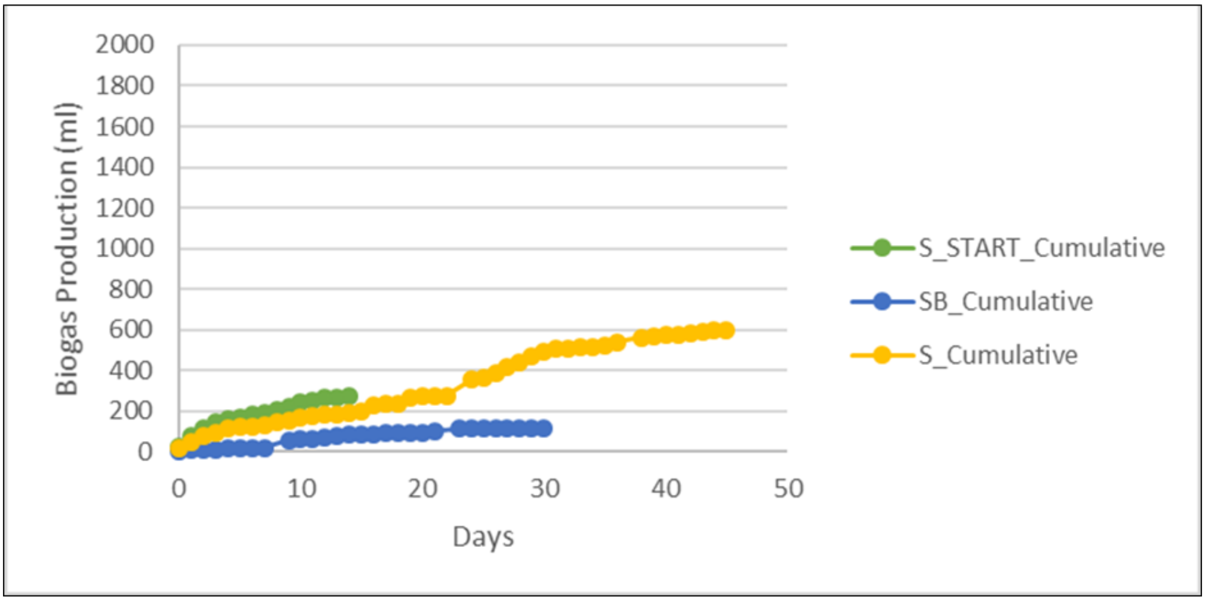


Figure 4-3: Cumulative biogas production from seeding sludge

Cumulative graphs were made of the three different pig slurry samples: P_START_Cumulative is the pig slurry sample that was stopped and used to back inoculate to form PB_Cumulative as shown in Figure 4-4. P_Cumulative ran the whole 30 days as a control that biogas production was taking place. After 45 days, P_Cumulative produced 1 806 ml of biogas. After 15 days, P_START_Cumulative was removed to produce PB_Cumulative but produced 1 507 ml biogas. PB_Cumalative ran for the rest of the time with P_Cumulative (30 days) and produced 806 ml.

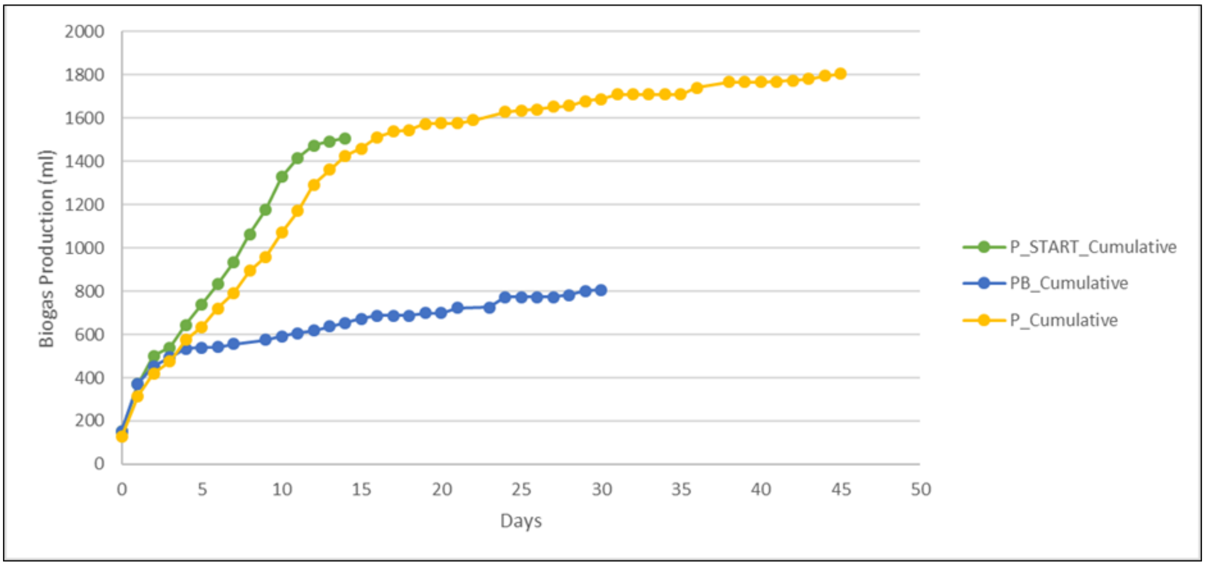


Figure 4- 4: Cumulative biogas production from pig slurry

Cumulative graphs were made of the three different pig slurry and seeding sludge samples: PS_START_Cumulative is the pig slurry sample that was stopped and used to back inoculate to form PSB_Cumulative as shown in Figure 4-5. PS_Cumulative ran the whole 30 days as a control that biogas production was taking place. After 45 days, PS_Cumulative produced 1 052 ml of biogas. After 15 days, PS_START_Cumulative was removed to produce PSB_Cumulative but produced 540 ml biogas. PSB_Cumalative ran for the rest of the time with PS_Cumulative (30 days) and produced 791 ml.

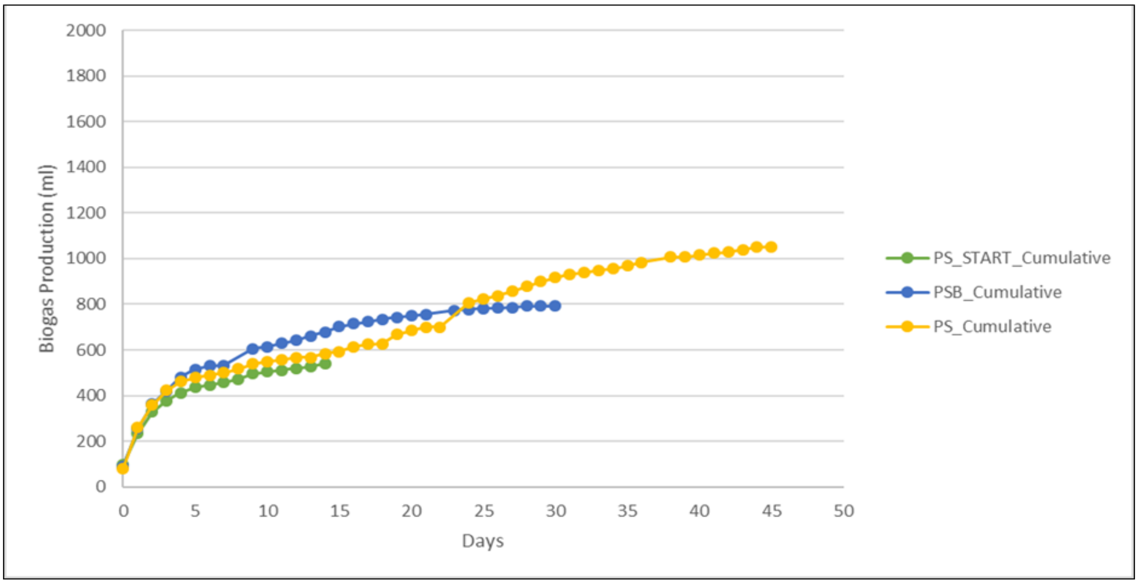


Figure 4-5: Cumulative biogas production from pig slurry and seeding sludge

Cumulative graphs were made of the three different MC samples: MC ran the whole duration of the experimental setup (45 days) and produced 4 210 ml biogas as seen in Figure 4-6

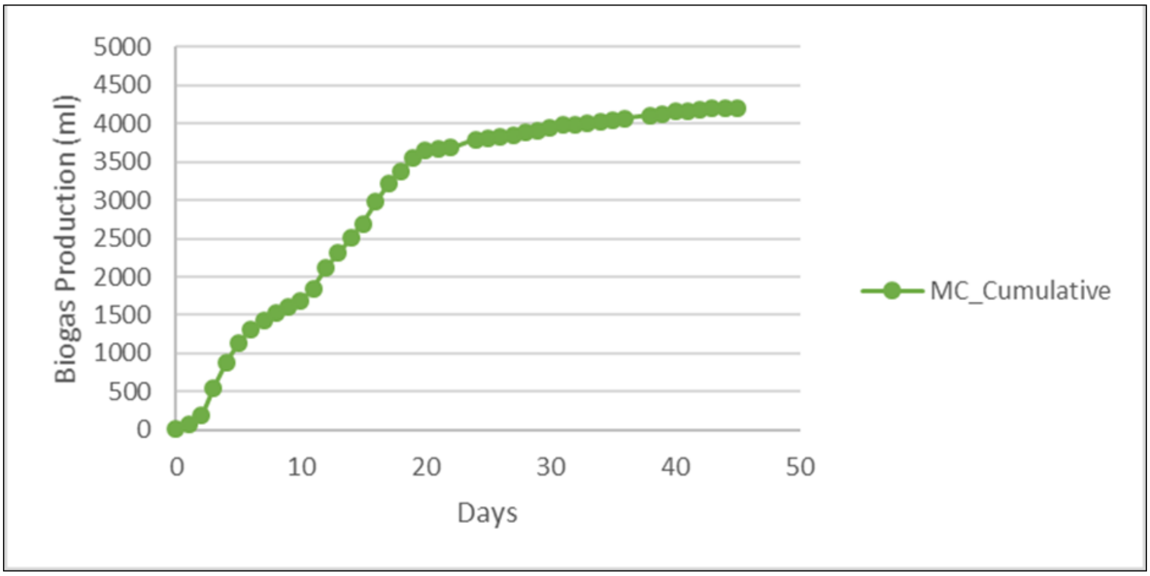


Figure 4- 6: Cumulative biogas production from MC

4.3 Molecular results

Figure 4-7 shows DNA bands before indexing and Figure 4-8 is after indexing, showing that indexes have been added successfully. Table 4-4 contains the absorbance results done with NanoDrop. These results indicate good quality DNA, RNA and nucleic acid, with values being in acceptable purity range. For these DNA extractions, the quality of the DNA can be observed from the 260/280 ratio of ~1.8 indicates "pure" DNA, while ~2.0 indicates "pure" RNA. Purity of nucleic acid is indicated by the 260/230 ratio of 2.0 to 2.2.

Table 4-4: NanoDrop spectrophotometer absorbance results

	NG/µL	A260	A280	260/280	260/230	340 RAW
MINIMUM	11.670	0.233	0.123	1.400	0.500	0.001
AVERAGE	39.864	0.797	0.498	1.651	0.966	0.180
MAXIMUM	72.870	1.457	0.924	1.960	1.620	0.479

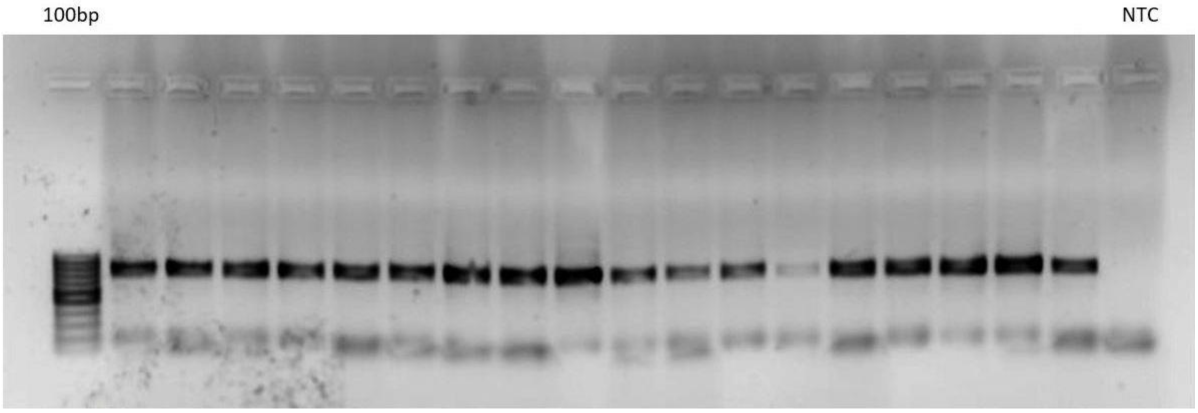


Figure 4-7: Agarose gel before indexing

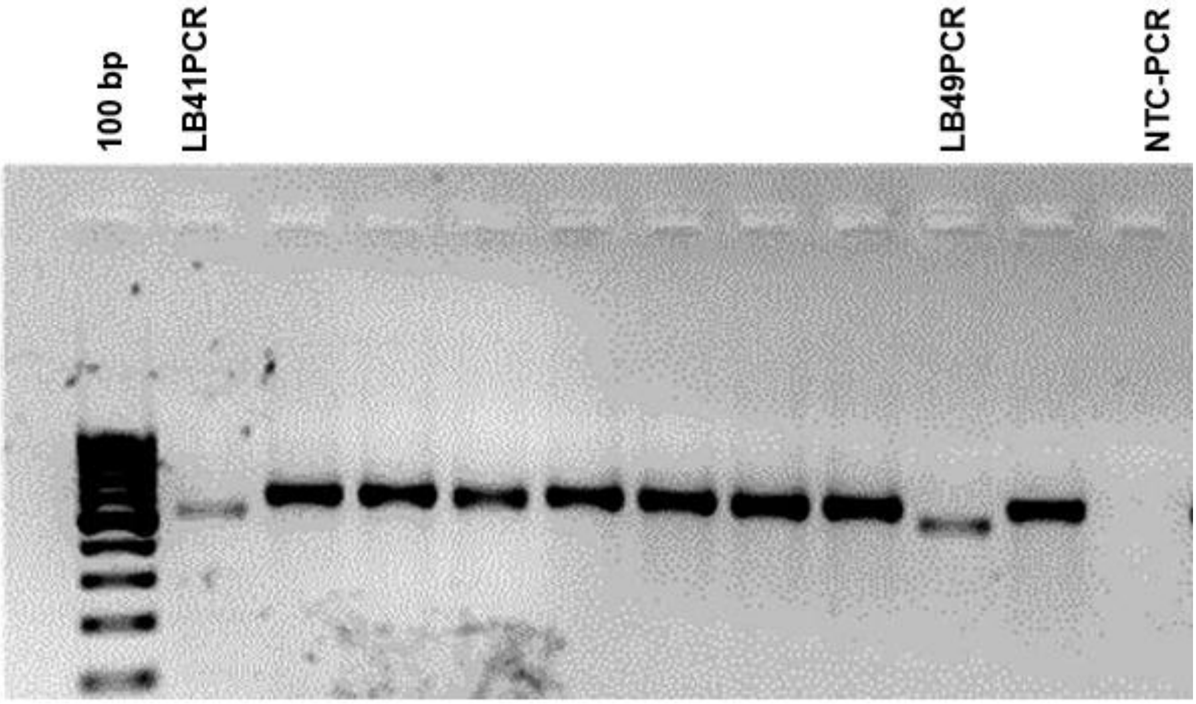


Figure 4-8: Agarose gel after indexing

4.4 NGS data

4.4.1 Microbial communities at Domain level

The domain communities in all the samples were identified and are displayed in Figure 4-9.

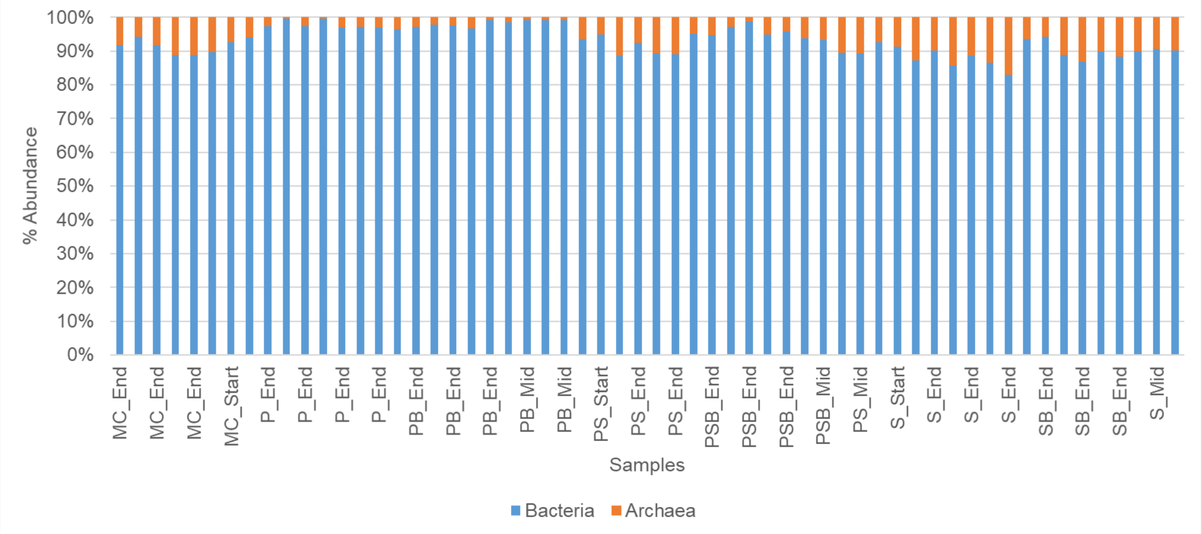


Figure 4- 9: Microbial community of the reactors at domain level

Figure 4-9 indicates that the bacteria domain completely dominated the sample with very little archaea. MC samples contained more archaea than what the pig slurry samples had. Pig slurry has almost no archaea. MC, PS and seed samples all contain seeding sludge as well as low levels of archaea. Pig slurry (P) samples had no seeding sludge and contain little to no archaea.

4.4.2 Microbial communities at Phylum level

The microbial communities at Phylum level were identified for all the samples used in this setup and is displayed in Figure 4-10.

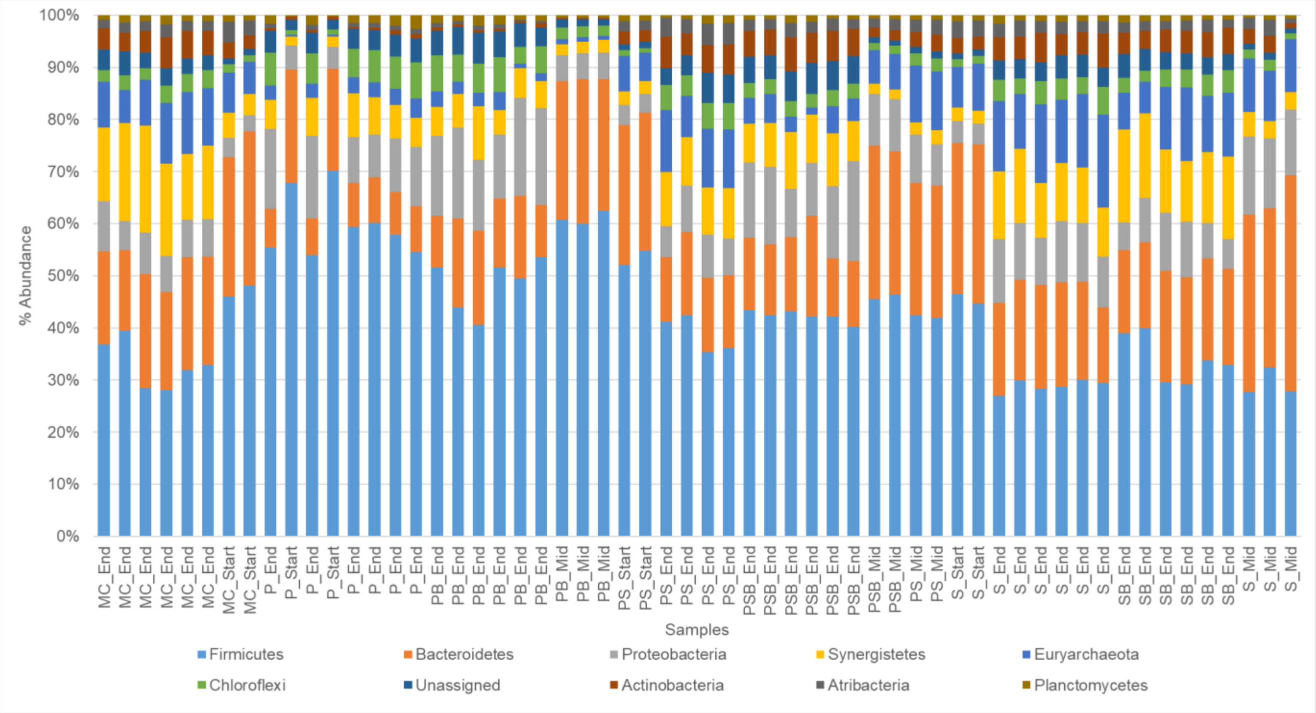


Figure 4-10: Microbial community of the reactors at Phylum level

Figure 4-10 shows the overview of all the samples at Phylum level. The most prominent phyla present at this level seem to be Firmicutes, Bacteroidetes, Proteobacteria and Synergistetes. In the following figures, the phylum level graphs are split up according to the samples used in the setup, to provide a clearer picture of what was happening in specific sample substrates. Firmicutes were dominant in all samples, even though percentages vary between samples. A shift in the microbial community can be observed, as start-, end- and mid-samples differ in each stage.

The phylum level graphs were split up to only indicate the same substrates on one graph; Figure 4-11 indicates MC reactors at Phylum level

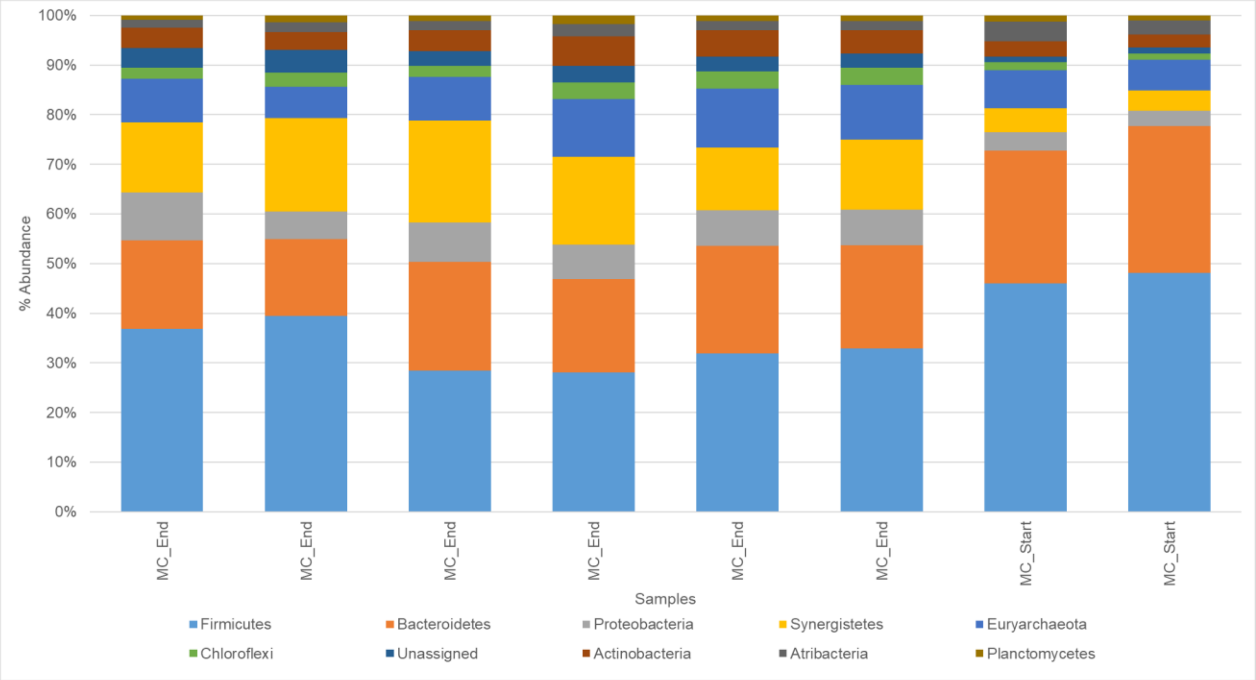


Figure 4- 11: Microbial communities in MC reactors at Phylum level

Figure 4-11 shows MC samples at Phylum level. MC_End had less than 40% Firmicutes, approximately 20% Bacteroidetes and approximately 15% Synergistetes, while MC_Start contains more the 40% Firmicutes, approximately 30% Bacteroidetes and approximately 10% Euryarchaeota. MC_Start had more Firmicutes and Bacteroidetes than MC_End samples, while MC_End has more Synergistetes and Proteobacteria than MC_Start. Some MC_End samples contain more Euryarchaeota than other MC_End samples as well as all MC_Start samples.

The same as above was done with Figure 4-12, which indicates P reactors at Phylum level.

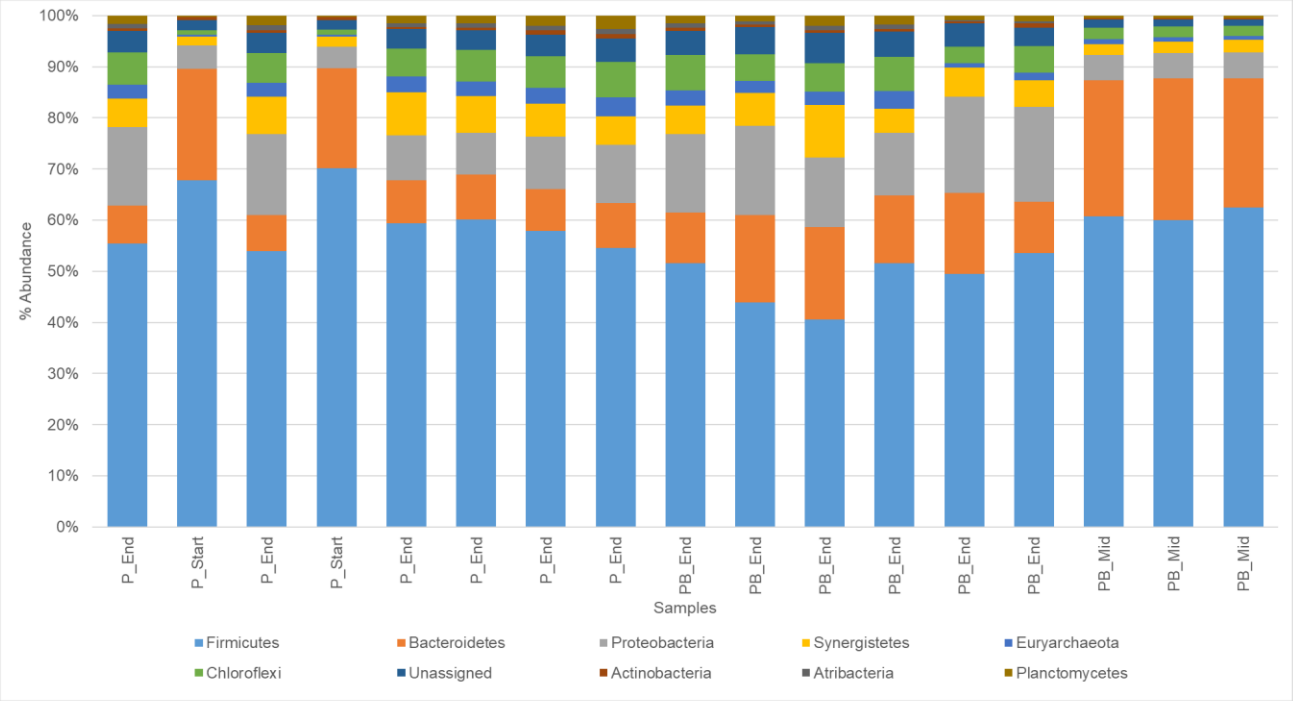


Figure 4-12: Microbial communities in P reactors at Phylum level

Figure 4-12 shows pig slurry samples at different stages at Phylum level. P_End was the pig slurry at the end of the setup, where Firmicutes were dominant at 55%, 15% Proteobacteria and both Bacteroidetes and Synergistetes both at about 10%. P_Start was pig slurry at the start of the setup. Firmicutes was dominant in this stage with 70% dominance, Bacteroidetes at 20% and Proteobacteria making up less than 5% of the organisms at the start of this setup. PB_End is the sample at the end of the back-inoculation setup. Firmicutes was present at this stage of the setup at 50%, while Proteobacteria made up less than 20% and Bacteroidetes less than 15%. PB_Mid is the sample at the middle stage, meaning that it is the back inoculated sample at its start stage, but at the middle of the setup, containing P_End and pig slurry. Firmicutes was present at 60%, Bacteroidetes at less than 30% and Proteobacteria at less than 5%. P_Start contained more Firmicutes than P_End, PS_End and PB_Mid, with PB_Mid containing less than P_Start. P_Start and PB_Mid also contained more Bacteroidetes than the other P samples. All end samples (P_End and PB_End) contained more Proteobacteria, Synergistetes and Chloroflexi than the other samples.

Figure 4-13 shows the PS (pig slurry and seeding sludge) samples at Phylum level at different stages.

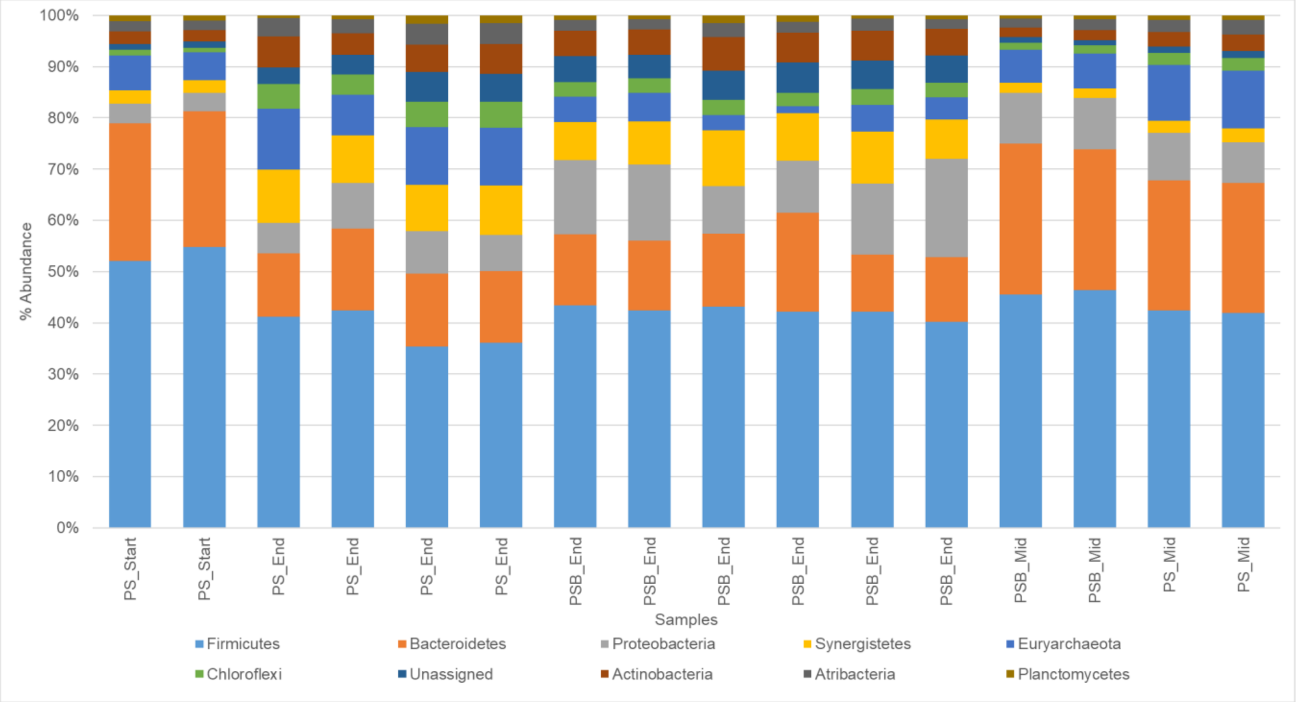


Figure 4-13: Microbial communities in PS reactors at Phylum level

PS_Start was pig slurry and seeding sludge at the start of the setup. Firmicutes was 50% dominant at this stage of the setup, while Bacteroidetes was at 25% and less than 5% was Proteobacteria. PS_End is the pig slurry at the end of the setup, where Firmicutes was only 40% dominant with Bacteroidetes at 15% and Synergistetes at 10%. PSB_End was the sample at the end of the back-inoculation setup. Firmicutes was present at 40%, Bacteroidetes at 15% as well as Proteobacteria at 15%. PSB_Mid is the sample at the middle stage, meaning that it is the back inoculated sample at its start stage, but at the middle of the setup, containing PS_End and pig slurry. Again, Firmicutes was dominant at 45%, Bacteroidetes at 30% and Proteobacteria at 10%. PS_Start contains the most Firmicutes, while PS_Start and PS_Mid also contains the most Bacteroidetes. PS_End and PSB_End contained the most Synergistetes. PSB_End contained more Proteobacteria than the other pig slurry samples. PS_Mid and PS_End contained the most Euryarchaeota. PS_End and PSB_Mid contained the most Chloroflexi, with the other samples containing almost no Chloroflexi. PS_End and PSB_End contain Actinobacteria and Atribacteria, while other pig slurry samples contain little to none of the mentioned organisms.

Figure 4-14 indicates the seeding sludge samples at Phylum level at different stages.

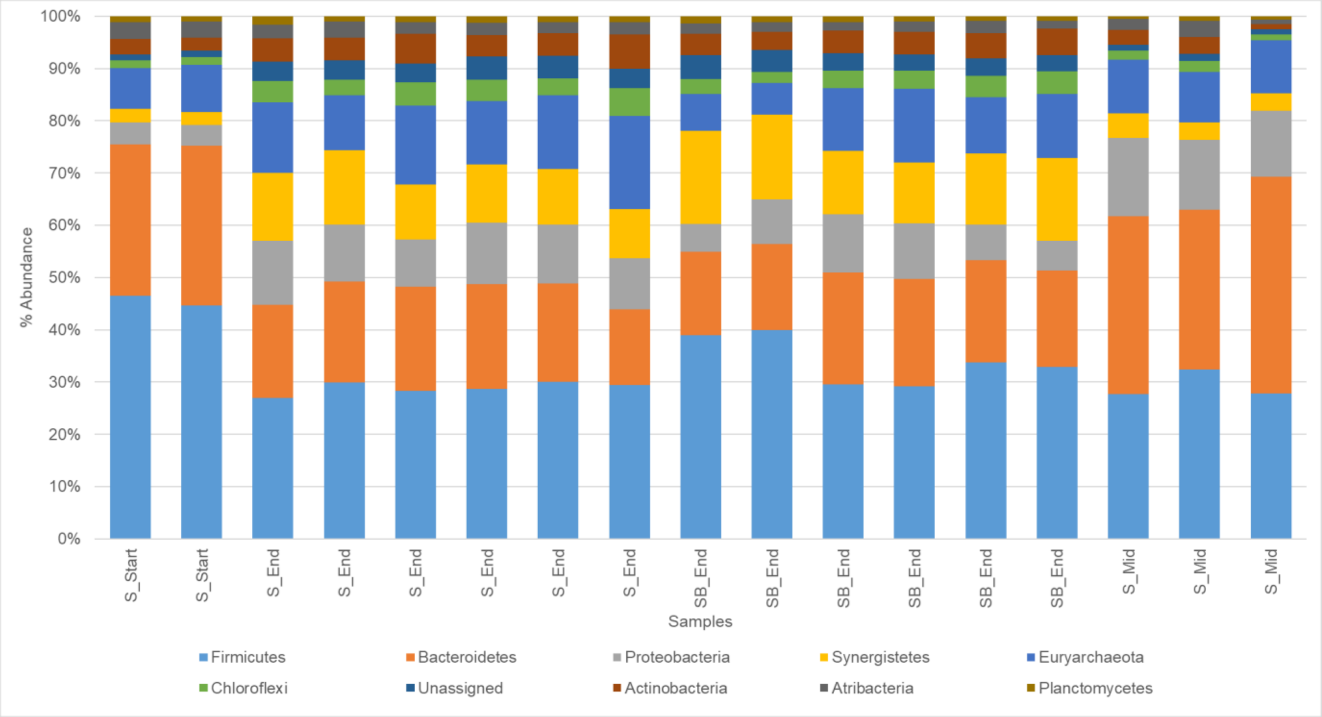


Figure 4-14: Microbial communities in S reactors at Phylum level

S_Start was pig slurry and seeding sludge at the start of the setup. Firmicutes made up 45%, Bacteroidetes 30% and less than 5% Proteobacteria and Synergistetes, respectively. S_End is the pig slurry at the end of the setup, where Firmicutes dominated at 30%, Bacteroidetes at 20%, while Proteobacteria and Synergistetes made up 15%, respectively. SB_End is the sample at the end of the back-inoculation setup. At this stage of the experiment, Firmicutes was present at 35%, Bacteroidetes at 20% and Proteobacteria and Synergistetes at 10%. S_Mid is the sample at in the middle of the setup, S_Mid was used to create the back inoculated sample. Firmicutes was present at 30%, Bacteroidetes at 30% and Proteobacteria at 15%. S_Start contained more Firmicutes than most of the other samples, while S_Start and S_Mid contained the most Bacteroidetes. Synergistetes was present in most samples, except S_Start and S_Mid contained less than the other samples. Proteobacteria was present in all the seeding sludge samples but was present at much lower numbers in S_Start. Euryarchaeota was present in all the samples, with S_End containing the most. Chloroflexi was present in most of the seeding samples, while S_Mid and S_Start did not contain as much as the rest of the seeding sludge samples.

4.4.3 Microbial communities at Class level

Figure 4-15 shows the overview of all the samples at Class level.

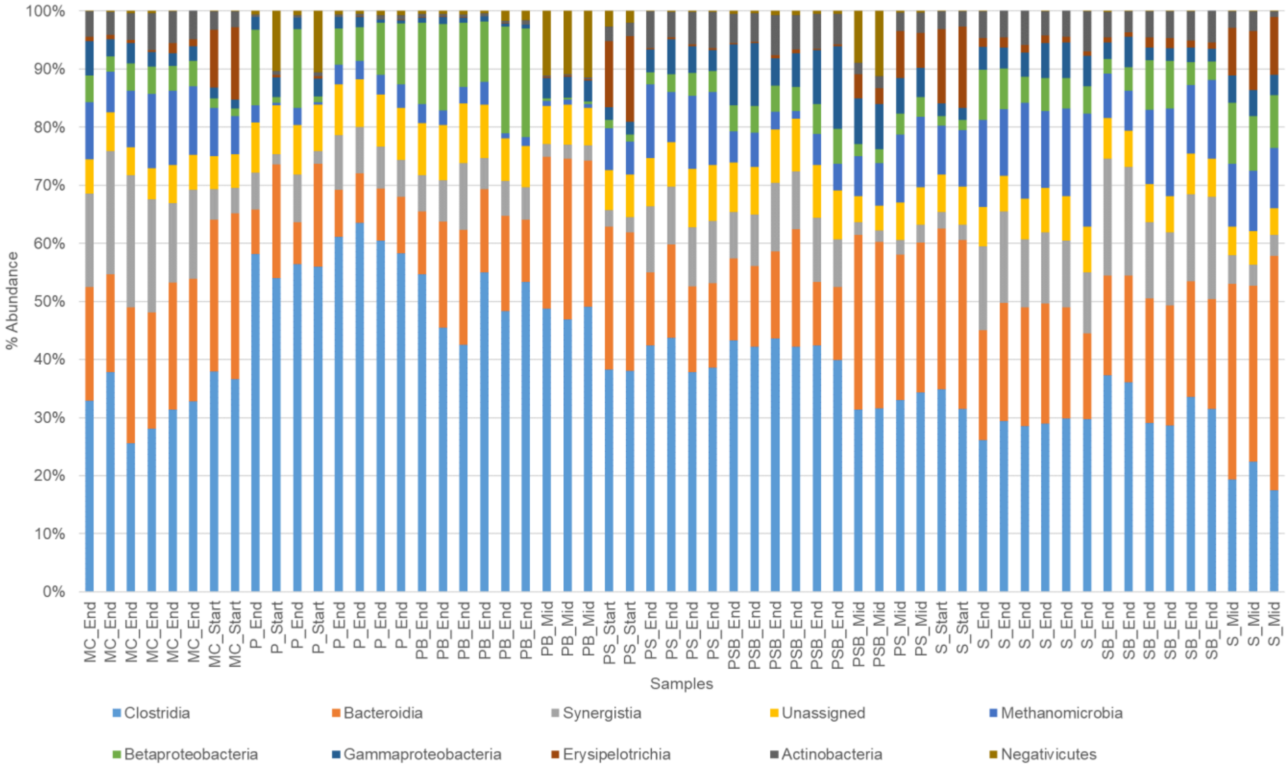


Figure 4- 15: Microbial community of the reactors at Class level

The most prominent organisms present at this level were Clostridia, Bacteroidia, Synergistia, Methanomicrobia and Unassigned. Clostridia was dominant in most of the sample at different percentages, especially P_End samples where Clostridia was present at high percentages. PB_Mid, P_Start and PSB_Mid were the only samples that contain more Negativicutes than all the other samples. In the following figures, the class level graphs are split up according to the samples used in the setup, to get a clearer picture of what was happening in specific sample substrates.

Figure 4-16 shows MC samples at Class level

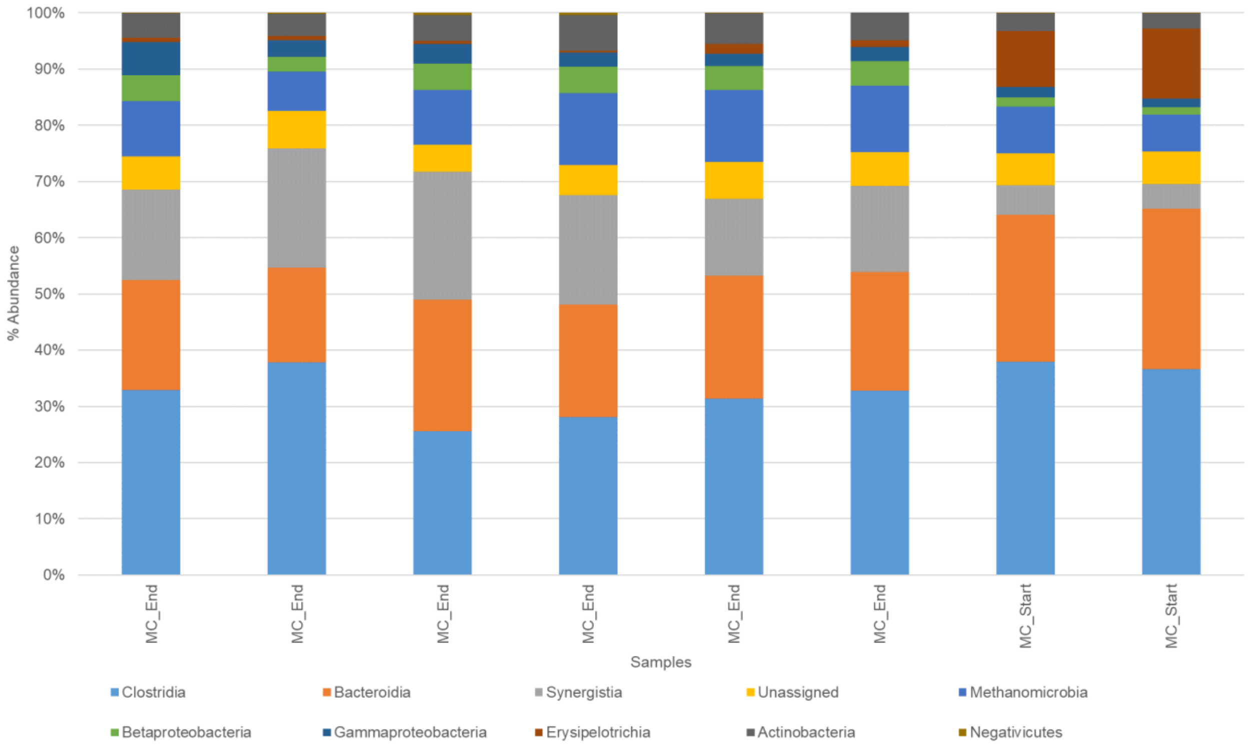


Figure 4-16: Microbial communities in MC reactors at Class level

MC_End showed less than 40% Clostridia, approximately 20% Synergistia and approximately 15% Bacteroidia, while MC_Start contained more than 40% Clostridia approximately 30% Bacteroidia and less than 5% Methanomicrobia and Unassigned respectively. MC_Start contained more Clostridia than MC_End. MC_Start also contained more Bacteroidia than MC_End samples. Synergistia was more dominant in MC_End samples than in MC_Start samples. An unassigned group were present in almost all the samples at similar levels. Methanomicrobia was more dominant in MC_End samples than MC_Start samples. The same with Betaproteobacteria and Gammaproteobacteria that were more dominant in MC_End samples compared to MC_Start samples. Additionally, Erysipelotricia, Actinobacteria were also more dominant in MC_Start samples compared to MC_End samples. Negativicutes was rarely present in the MC samples.

Figure 4-17 depicts pig slurry samples at different stages at Class level.

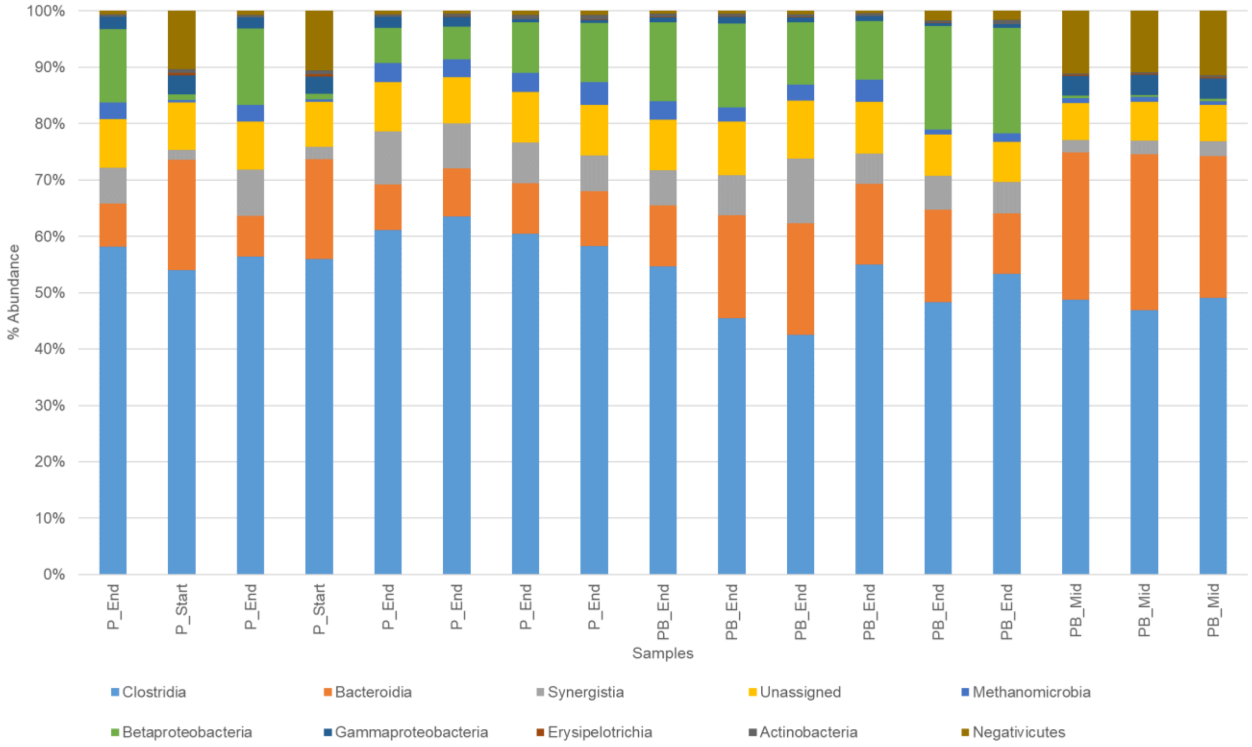


Figure 4-17: Microbial communities in P reactors at Class level

P_End was the pig slurry at the end of the setup. At this stage, Clostridia was dominant at 60%, Bacteroidia at 10% and Synergistia and Unassigned at less than 10% respectively. P_Start is pig slurry at the start of the setup, where Clostridia was dominant at 55%, Bacteroidia less than 10% and Negativicutes at 10%. PB_End was the sample at the end of the back-inoculation setup. Clostridia was dominant at the stage at 55%, Bacteroidia at 15% and Unassigned less than 10%. PB_Mid is the sample at the middle stage, meaning that it is the back inoculated sample at its start stage, but at the middle of the setup, containing P_End and pig slurry. Clostridia was dominant at approximately 55%, Bacteroidia 40% and Negativicutes at 10%. Clostridia was mostly dominant in all the samples, but P_End samples contain the most Clostridia. Bacteroidia was dominant in PB_Mid samples, where these samples contain more Bacteroidia than other pig slurry samples. Synergistia was present at low levels in all the samples but P_End samples contained more Synergistia than the other samples in this setup. Unassigned and Methanomicrobia was at approximately the same level in all the samples. Betaproteobacteria was present in all samples except in P_Start and PB_Mid, where it was rarely present. Gammaproteobacteria levels were similar in all the samples. Erysipelotrichia and Actinobacteria were rarely present in the samples.

Negativicutes was only present in P_Start and PB_Mid samples, while other samples rarely contained any Negativicutes.

Figure 4-18 shows the PS (pig slurry and seeding sludge) samples at Class level at different stages.

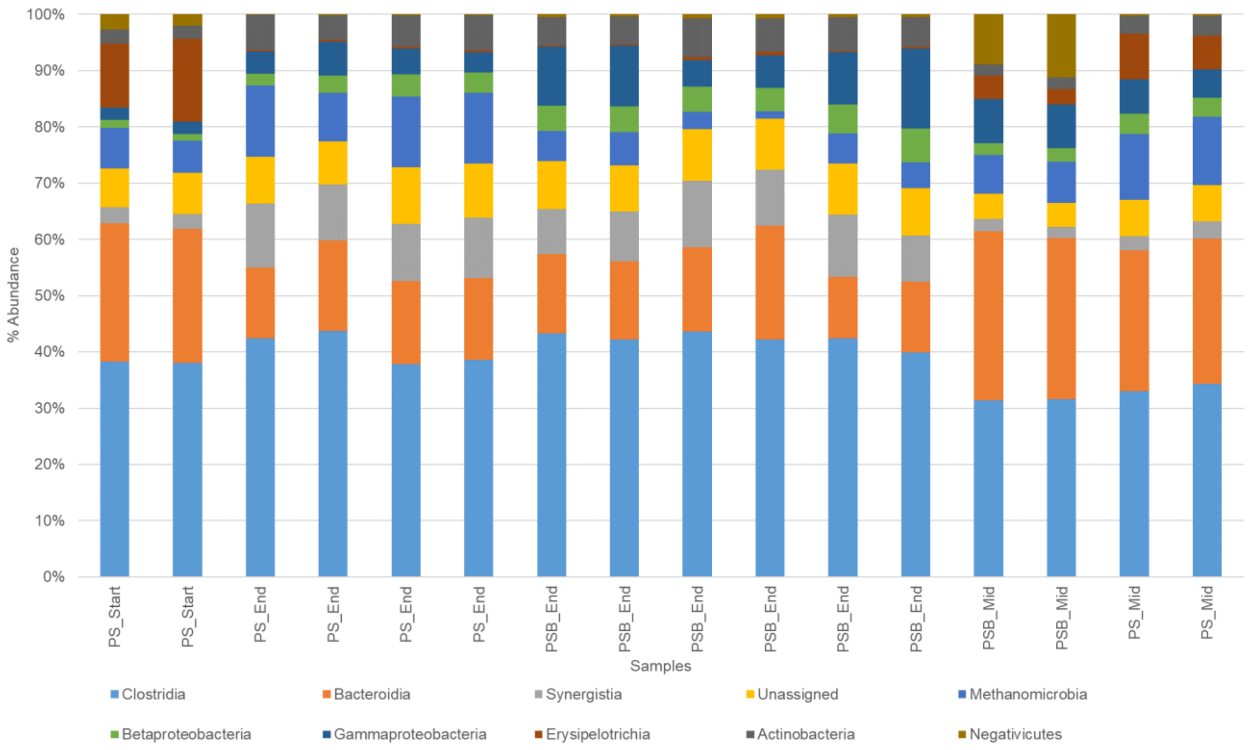


Figure 4-18: Microbial communities in PS reactors at Class level

PS_Start was pig slurry and seeding sludge at the start of the setup, where Clostridia was present at 40%, Bacteroidia at 20% and Unassigned and Methanomicrobia less than 10% each. PS_End was the pig slurry at the end of the setup. Clostridia was dominant at this stage at 40%, Bacteroidia at 10% and Unassigned and Synergistia was present at 5%. PSB_End was the sample at the end of the back-inoculation setup. At this stage, Clostridia was present at 40%, Bacteroidia at 15% and Unassigned and Synergistia was present at 10%. PSB_Mid was the sample at the middle stage, meaning that it was the back inoculated sample at its start stage, but at the middle of the experimental setup, containing PS_End and pig slurry. Clostridia was present at 30%, Bacteroidia at 30% and Negativicutes at 10%. Clostridia was dominant in all the samples, while Bacteroidia was more dominant in the PS_Mid sample than in the other samples. Synergistia was present in all the samples, but levels were the lowest in PS_Start, PS_Mid and PSB_Mid. Unassigned was present in all the samples, with PSB_Mid containing the least. Methanomicrobia was present in all the samples, but PS_End contained the most. Betaproteobacteria was present at low levels in all the samples. Gammaproteobacteria was present at low levels in all the samples, with PSB_End and PSB_Mid containing more than the other samples. Erysipelotrichia was present at low levels in PS_Start, PSB_Mid and PS_Mid samples. Actinobacteria was only present in PS_End and PSB_End samples. Negativicutes was only present in PS_Start and PSB_Mid samples, where the PS_Start Negativicutes rarely present.

Figure 4-19 shows the seeding sludge samples at Class level at different stages.

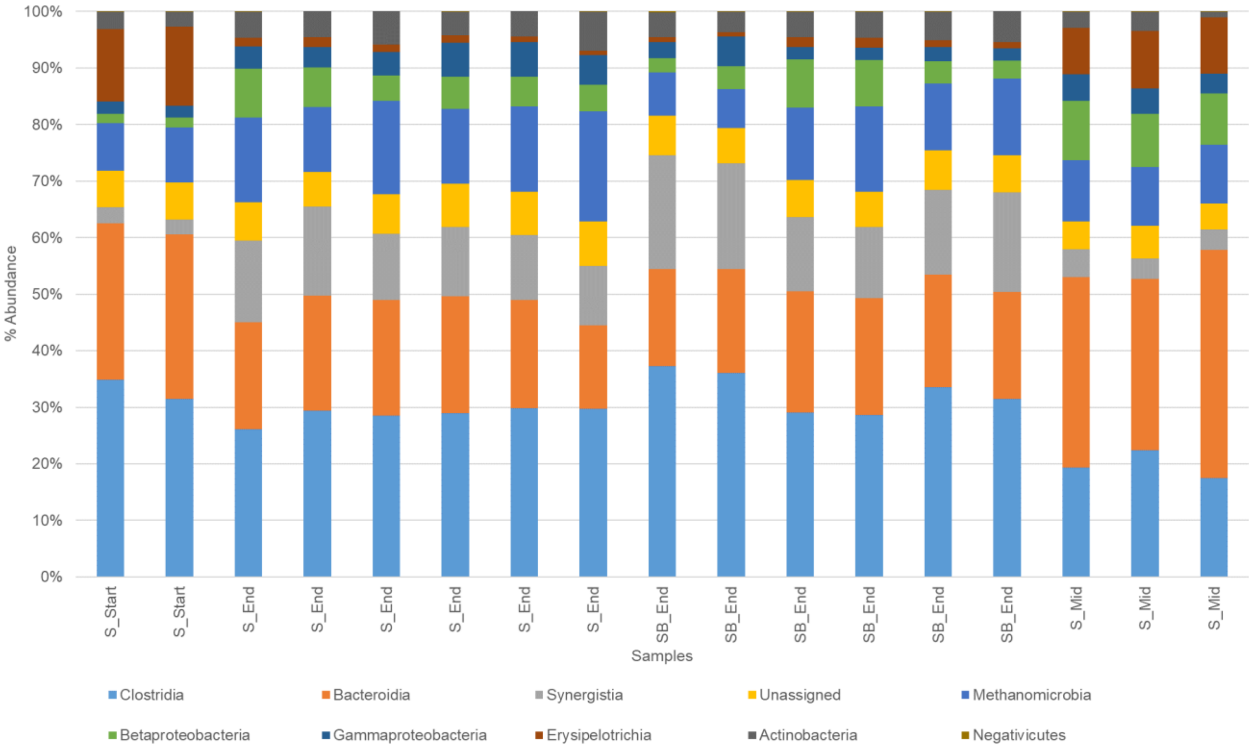


Figure 4-19: Microbial communities in S reactors at Class level

S_Start was pig slurry and seeding sludge at the start of the setup. At this stage, Clostridia and Bacteroidia was present at 30% and Unassigned at 5%. S_End was the pig slurry at the end of the experiment, where Clostridia was present at 30%, Bacteroidia at 20% and Synergistia is present at 10%. SB_End is the sample at the end of the back-inoculation setup. Here, Clostridia was dominant at 35%, Bacteroidia at 20% and Synergistia was present at 10%. S_Mid was the sample taken at the middle of the setup and was used to create the back inoculated sample. In this sample, Bacteroidia was present at 40%, Clostridia at 20% and 10% was Betaproteobacteria and Methanomicrobia respectively. Clostridia was dominant in most samples except for S_Mid and S_Start. Bacteroidia is dominant in S_Mid samples and S_Start samples. Synergistia was present in all the samples, but S_End and SB_End contained more than the other samples. Unassigned was at the same levels in all the samples. Methanomicrobia was present in all samples with S_End and SB_End containing the most. Betaproteobacteria was present in all the samples with S_Mid containing the most. Gammaproteobacteria was present in all samples with S_End containing the most. Erysipelotrichia was present in low levels in all samples but more in S_Start and S_Mid. Actinobacteria was present in all samples at low levels but S_End and SB_End containing the most. Negativicutes was absent from all samples.

4.4.4 Microbial communities at Family level

Figure 4-20 shows the overview of all the samples at Family level.

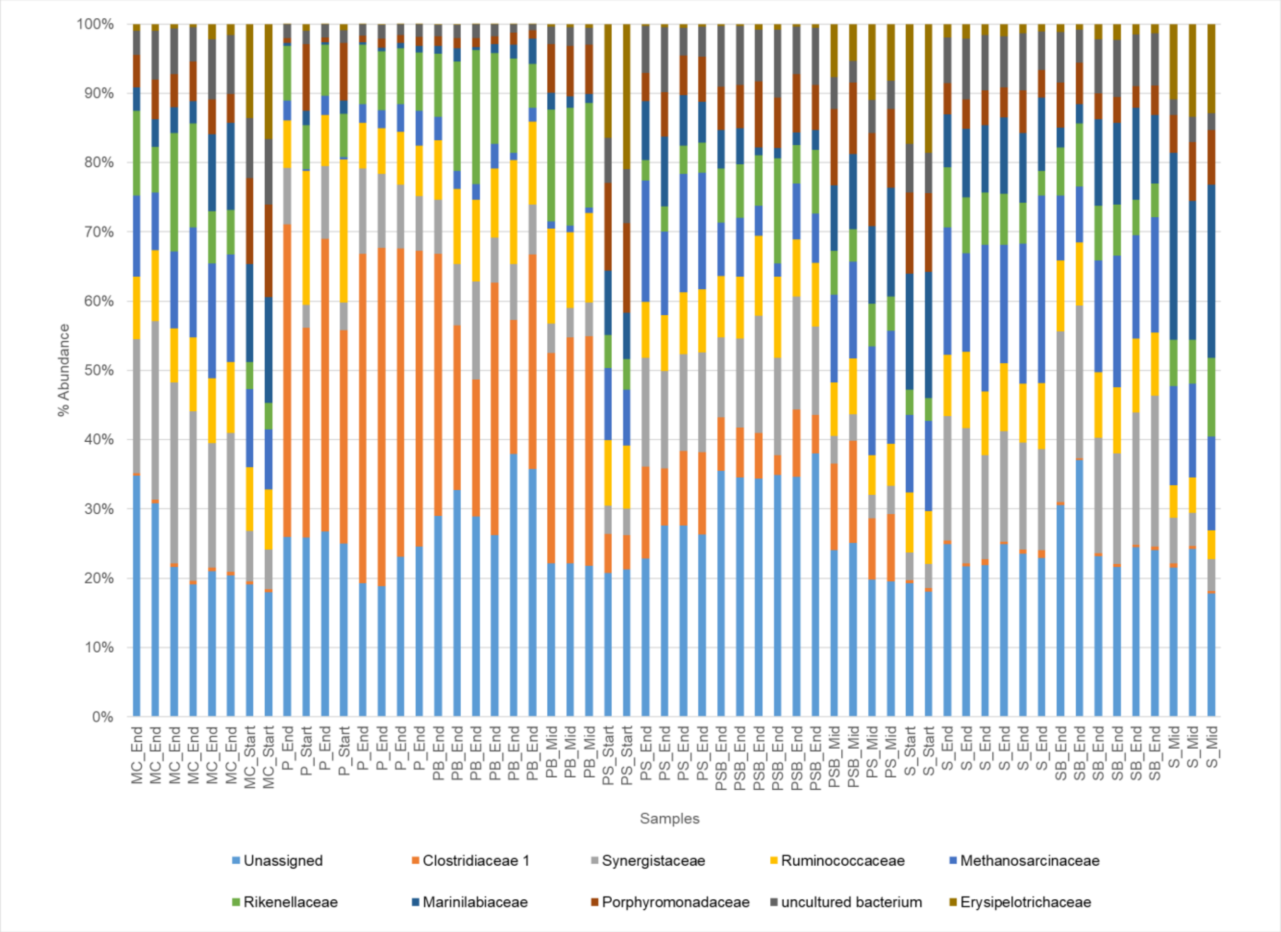


Figure 4-20: Microbial community of the reactors at Family level

The most prominent organisms present at this level were Unassigned, Clostridiaceae 1, Synergistaceae and Methanosarcinaceae. Family resolution gives a clear picture of the changes as changes at this level are more prominent. Family resolution shows the clear differences between the microbial communities of the different samples. In the following figures, the family level graphs are split up according to the samples used in the setup, to get a clearer picture of what was happening in specific sample substrates.

Figure 4-21 shows MC samples at Family level. Once again variations in the various reactors were observed.

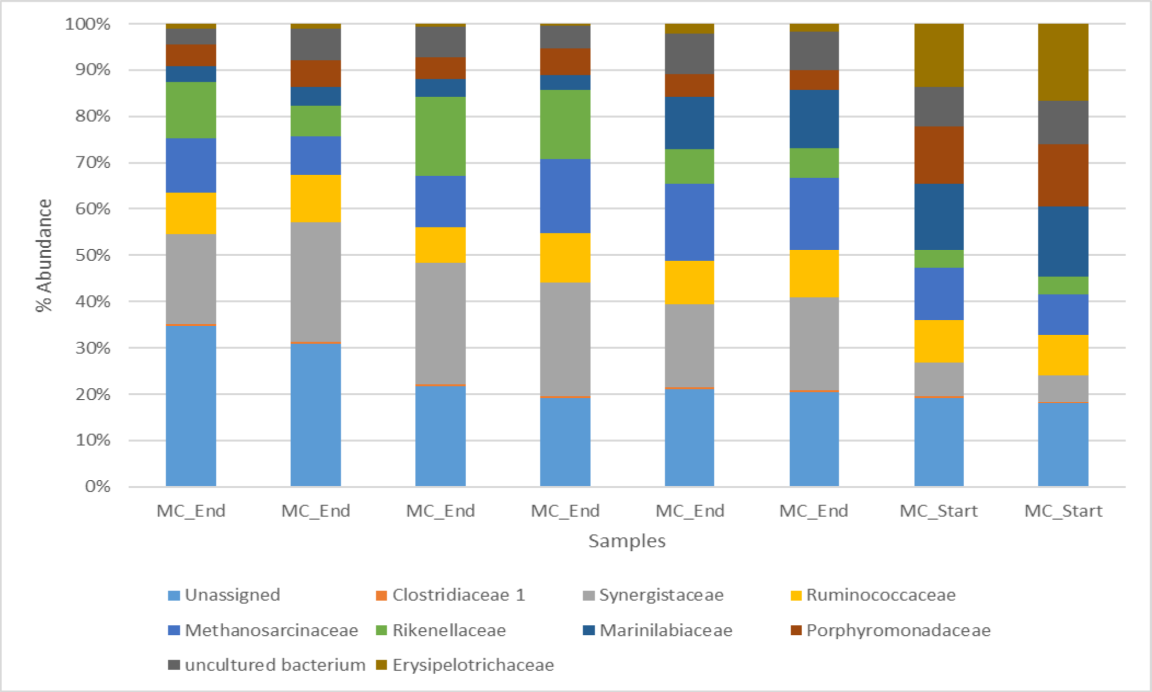


Figure 4-21: Microbial communities in MC reactors at Family level

MC_End had 25% Unassigned, 20% Synergistaceae and 10% Rikenellaceae, Methanosarcinaceae and Ruminococcaceae respectively, while MC_Start contained 20% Unassigned, 10% Methanosarcinaceae, Porphyromonadaceae and Erysipelotrichaceae respectively. Unassigned bacteria were present in all the samples at almost the same levels, except two of the MC_End samples which contained more Unassigned bacteria compared to the rest of the samples. Clostridiaceae 1 was only present in two MC_End samples as well as one of the MC_Start samples, the other samples did not contain Clostridiaceae. All the samples contained Synergistaceae, but MC_Start samples contained less than the MC_End samples. Ruminococcaceae was present in all the samples at more or less the same level. Methanosarcinaceae was present in all the samples at more or less the same levels, with some MC_End samples containing more. Rikenellaceae was present in all the sample, except most MC_End samples which contained more than the other samples. Marinilabiaceae was present in all the samples, but MC_Start and some MC_End contained higher levels than the rest of the MC_End samples. Porphyromonadaceae was present at lower levels in MC_End samples, while MC_Start samples contained more. Uncultured bacterium was higher in MC_Start and two MC_End samples, than in the rest of the MC_End samples. Erysipelotrichaceae was present in all the MC_Start samples but was only present in four of the MC_End samples.

Figure 4-22 indicates the pig slurry samples at Family level at different stages.

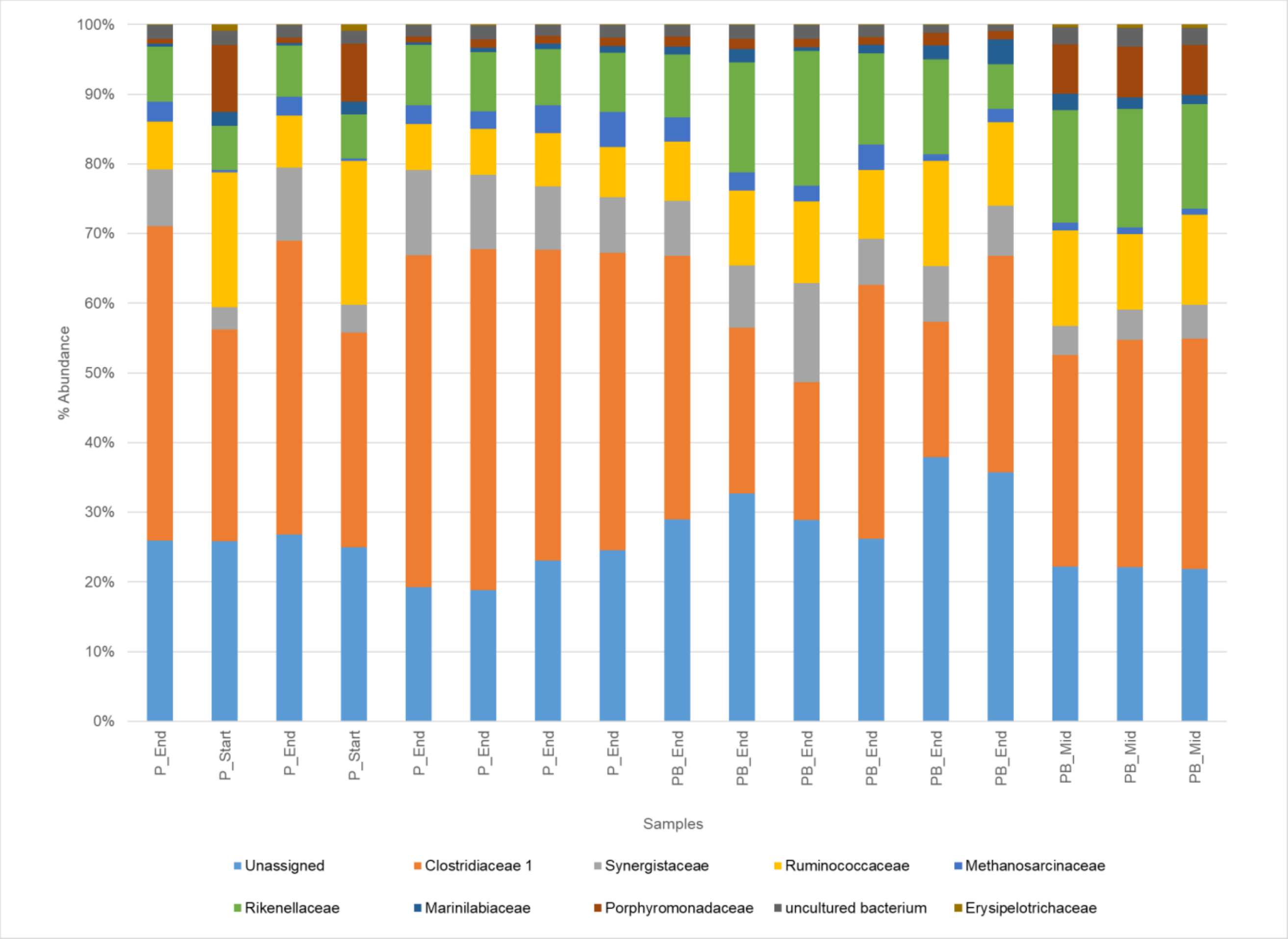


Figure 4-22: Microbial communities in P reactors at Family level

P_End was the pig slurry at the end of the experiment, where Clostridiaceae 1 was present at 50%, Unassigned at 25% and 10% was Synergistaceae, Ruminococcaceae and Rikenellaceae, respectively. P_Start is pig slurry at the start of the setup, here 35% was Clostridiaceae 1, 25% Unassigned and Synergistaceae made up 10%. PB_End was the sample at the end of the back-inoculation setup. At this stage Unassigned made up 30%, Clostridiaceae 1 25% and Rikenellaceae 15%. PB_Mid was the sample at the middle stage, meaning that it was the back inoculated sample at its start stage, but at the middle of the experiment, containing P_End and pig slurry. Clostridiaceae 1 made up 35% of the organisms present, Unassigned 20% and Rikenellaceae 15%. Unassigned bacteria were present in all the samples used for the various experiments. Clostridiaceae 1 was present in all samples and was also dominant in most of the samples. Synergistaceae was present in all samples but P_Start and PC_Mid. In these experiments they were present at low levels. P_Start contained the highest levels of Ruminococcaceae while it was present in all the samples. Methanosarcinaceae was present in all the samples at low levels. Rikenellaceae was present in all samples, with MC_Start containing the least and PB_End and PB_Mid containing the most. Marinilabiaceae was present in low levels in all the samples. Porphyromonadaceae was present in most of the samples, except P_Start and PB_Mid which contained more than the other samples. P_Start and PB_Mid were the only samples where Uncultured bacterium was present at very low levels. Erysipelotrichaceae was not present in any of the samples.

Figure 4-23 indicates the PS (pig slurry and seeding sludge) samples at Family level at different stages.

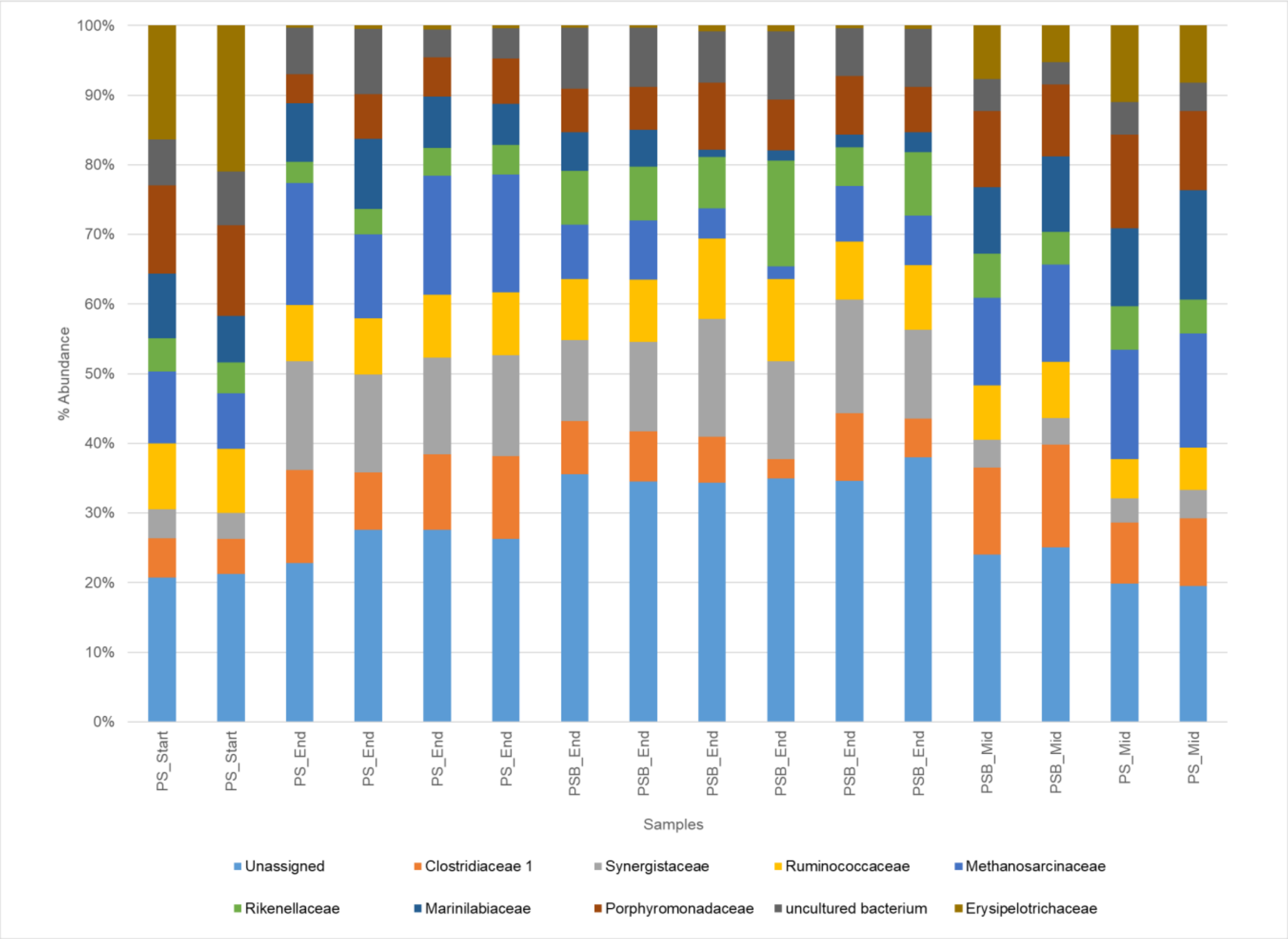


Figure 4-23: Microbial communities in PS reactors at Family level

PS_Start was pig slurry and seeding sludge at the start of the setup, here 20% was Unassigned and Erysipelotrichaceae respectively and 10% Porphyromonadaceae and Methanosarcinaceae respectively. PS_End was the pig slurry at the end of the setup, where 25% was Unassigned, 20% Clostridiaceae 1 and Synergistaceae respectively. PSB_End was the sample at the end of the back-inoculation setup. At this stage, 40% Unassigned, and 10% Synergistaceae, Rikenellaceae and Ruminococcaceae respectively. PSB_Mid was the sample at the middle stage, meaning that it is the back inoculated sample at its start stage, but at the middle of the experimental setup, containing PS_End and pig slurry. Here 20% Unassigned was present, Methanosarcinaceae at 15% and 10% Marinilabiaceae. Unassigned bacteria were present in all the samples with PSB_End containing the most. Clostridiaceae 1 was also present is all the samples while PS_Mid and PS_End contained the most of these bacteria. Synergistaceae was present at low levels in PS_Start and PS_Mid, but PS_End and PSB_End contained higher levels of these organisms. Ruminococcaceae was present in all the samples used for the experimental setup. The levels were low with PS_Mid containing the least amount of these organisms. Methanosarcinaceae was present in all the samples with PS_End and PS_Mid containing the highest levels. Rikenellaceae was present in all the samples at low levels with PSB_Mid containing a bit more. Marinilabiaceae was also present in all the samples but PS_Mid contained the most of these organisms. PS_Start and PS_Mid contained the most Porphyromonadaceae, while being present in all the samples. Uncultured bacteria were present at higher levels in PSB_End samples. Erysipelotrichaceae was present in low levels in PS_End and PSB_End, while PS_Start and PS_Mid contains higher levels of these organisms.

Figure 4-24 shows the seeding sludge samples at Family level at different stages.

Figure 4-24: Microbial communities in S reactors at Family level

S_Start was pig slurry and seeding sludge at the start of the setup, here 20% Unassigned, Marinilabiaceae and Erysipelotrichaceae were present respectively. S_End was the pig slurry at the end of the setup, where Unassigned, Methanosarcinaceae and Synergistaceae were present at 20% each. SB_End was the sample at the end of the back-inoculation setup. At this stage Unassigned was present at 20%, Synergistaceae at 20% and Methanosarcinaceae at 15%. S_Mid was the sample at the middle of the experimental setup. S_Mid was used to create the back inoculated sample, where Unassigned and Marinilabiaceae were present at 20% and Methanosarcinaceae and Erysipelotrichaceae were present at 10%. Unassigned bacteria were present in all the samples, but SB_End contained more than the other samples. Clostridiaceae 1 was rarely present in S_End, two of the SB_End samples and two S_Mid samples, while absent from the other samples. Synergistaceae was present in all the samples used for the experimental setup. All samples contained Ruminococcaceae but S_Mid contains the lowest level. Methanosarcinaceae was present in all the samples but two of the SB_End samples contained the least. Rikenellaceae was present in all the samples at low levels. S_Mid contained the most Marinilabiaceae among all the samples. Porphyromonadaceae was present in all the samples but present in low levels in SB_End. Uncultured bacterium was present in all the samples, with S_End containing the most. Erysipelotrichaceae was present in S_Start and S_Mid samples and present in very low numbers or absent from the rest of the samples.

4.5 Alpha and beta diversity

PigS_Start had the highest Chao1 alpha diversity index among the samples of each reactor as well as the lowest standard deviation (Figure 4-25). Seed_Mid and Seed_Start had the lowest alpha diversity index. Control_End and Pig_End had very similar diversity indexes. Control_Start and PigB_End had similar diversity indices within communities. There were also similarities between diversity index within communities within PigB_Mid and PigS_End samples. Seed_End had a low diversity index.

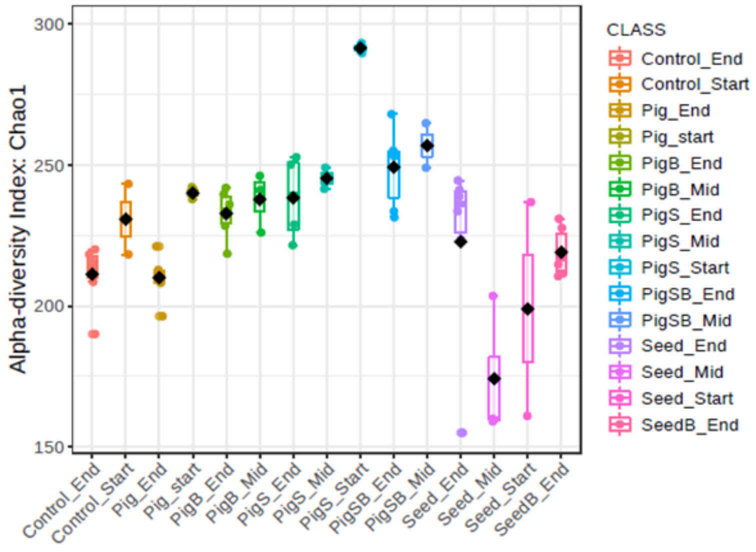


Figure 4-25: MicrobiomeAnalyst derived Chao1 alpha diversity index per sample. Samples showed statistically significant species richness (p<7.8767 e-0.6)

Figure 4-26 shows alpha diversity index between reactor stages. The beginning stage shows the highest diversity index within communities with some outliers, while middle and end stages indicate the same level of diversity index within communities, but the middle stage has the biggest standard deviation.

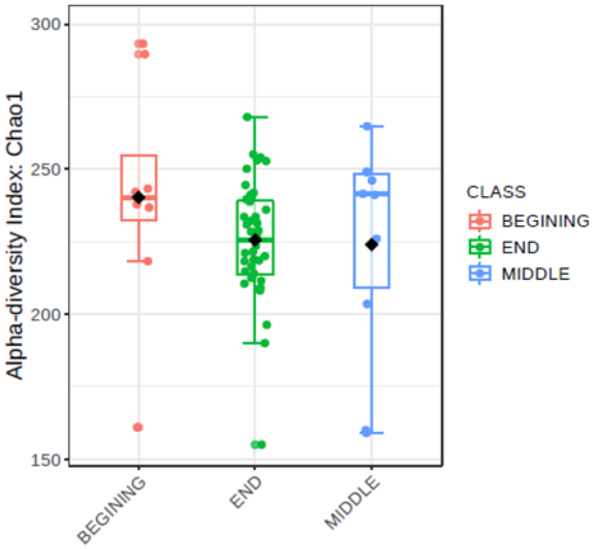


Figure 4-26: MicrobiomeAnalyst derived Chao1 alpha diversity index per reactor stage. Samples did not show statistically significant species richness (p<0.33123)

Beta diversity between communities is shown in Figure 4-27. Samples indicate statistical significance (p<0.001). All seed, pig and PS end samples group together at the bottom of the graph, while mid- and start samples are scattered at the top of the graph.

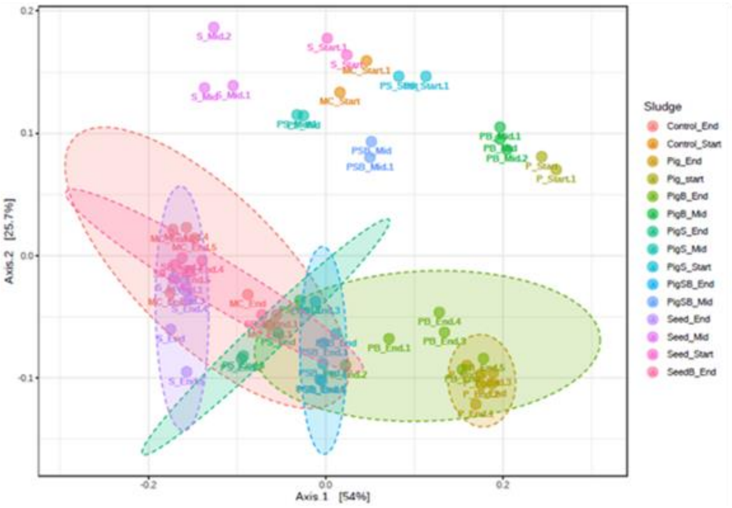


Figure 4-27: MicrobiomeAnalyst derived beta diversity of samples

Figure 4-28 shows the beta diversity of the reactor stages. The samples indicate statistical significance ($p<0.001$). This also indicates a significant difference between the end stage in comparison to beginning and middle stages of the reactors. The beginning stage and middle group together, while the end groups on its own.

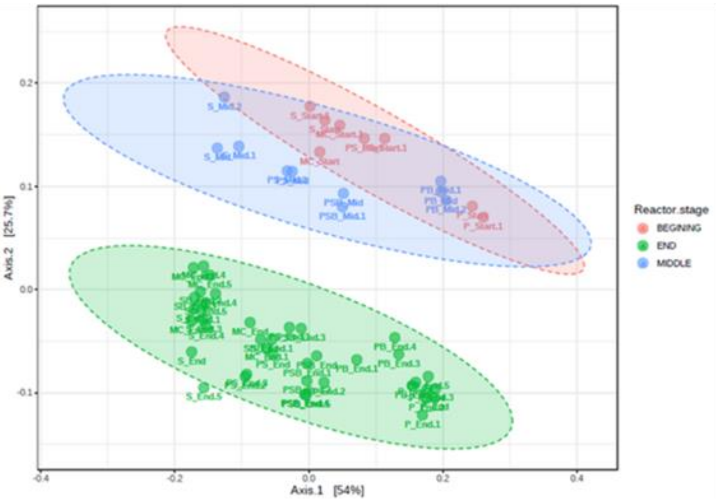


Figure 4- 28: MicrobiomeAnalyst derived beta diversity of reactor stages

4.6 Statistical analysis between stages

Most organisms were the same in the middle and end stages (Figure 4-29). Except for Synergistetes, Bacteroidetes and Actinobacteria. Synergistetes had a larger mean proportion percentage in end stages. Bacteroidetes on the other hand, had a larger mean proportion percentage in the middle stage. While Actinobacteria had a smaller mean proportion percentage in middle stage. Bacteroidetes thus played a significant role in middle stage, whereas Synergistetes and Actinobacteria were more prominent in end stage.

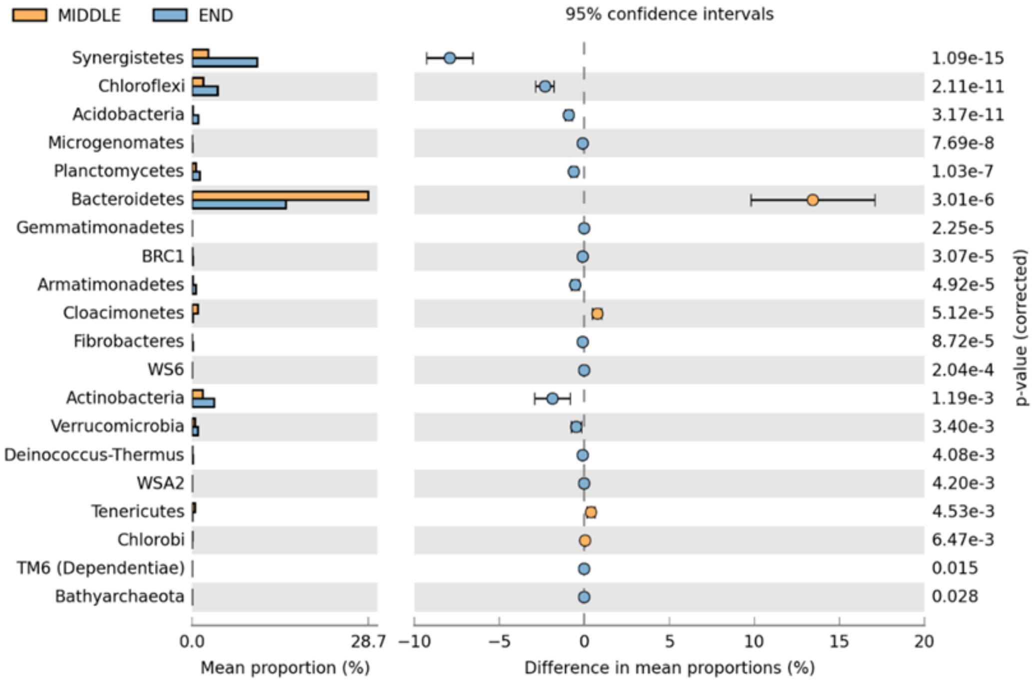


Figure 4-29: Statistically significant bacteria at Phylum level between middle and end stages using STAMP

Figure 4-30 indicates there were no significant differences between most of the organisms present in start and end stages, except for Chloroflexi, Proteobacteria, Synergistetes, Bacteroidetes, Actinobacteria and Firmicutes. Chloroflexi had a greater significance in the end stage of reactors. Proteobacteria also had a greater significance in end stages. As in Figure 4-29, Synergistetes had a greater significance in the end stages. Bacteroidetes had a greater significance in start stages and middle stages as indicated in Figure 4-29. Actinobacteria had a greater significance in end stages, this in also indicated in Figure 4-29. Firmicutes has a greater significance in start stages.

4.7 Taxonomic to phenotype mapping

Figure 4-31 indicates the abundance of phenotypes in the start stages were mostly unknown (55.5%). The rest (44.5%) were made up mostly by known phenotypes. Ammonia oxidisers made up 32% of the overall abundance. While the dehalogenation phenotype had 26.6% abundance in start reactors. Nitrite reducers and Nitrogen oxidizers made up 8.5% and 7.8% respectively of the abundance in start samples. Sulphate reducers and sulphide oxidiser made up 6.9% and 6.1% respectively. Chitin degradation made up 6.2% of start reactors.

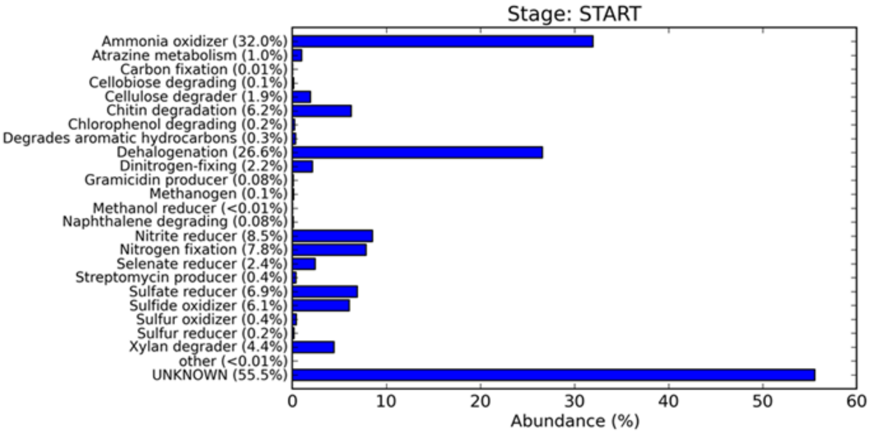


Figure 4-30: Taxonomic to phenotype mapping of start stage using Metageneassist

Figure 4-31 indicates the abundance of phenotype in middle stages. Of these, 55.5% were unknown, whilst ammonia oxidiser made up 36.7% of the phenotype abundance, and dehalogenation 35.3%. Nitrite reducers and Nitrogen fixation were more abundant in middle stages than at start stages, at 13.7% and 8.7% respectively. Sulphate reducers and sulphide oxidisers made up 11.1% and 8.7% abundance in middle reactors, respectively, both more than in start stages. Chitin degradation was abundant at 11.1%, also more than in at the start stages.

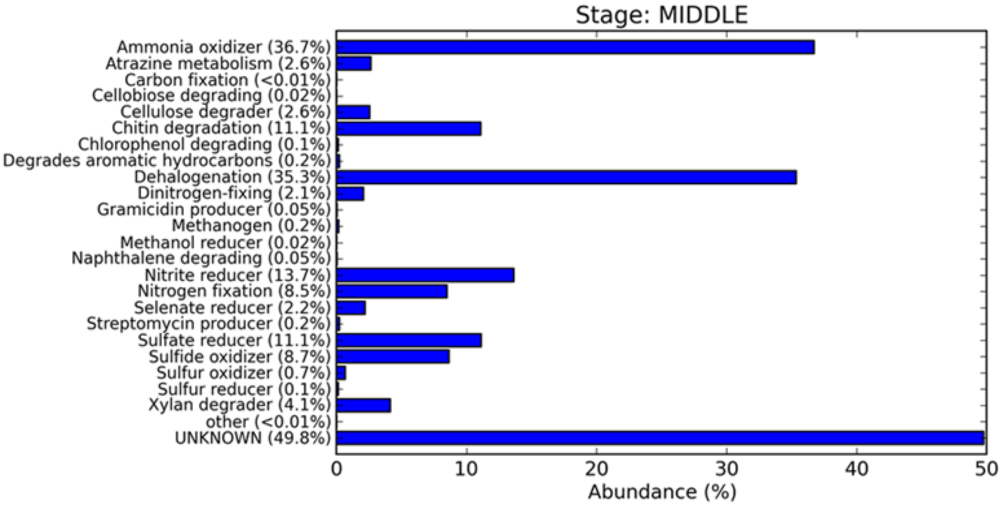


Figure 4-31: Taxonomic to phenotype mapping of middle stages using Metageneassist

The abundance of unknown phenotype in end stages was 60.6%. This was more than at the start and middle stages (Figure 4-30 and 4-31). Ammonia oxidiser was less abundant in the end stage compared to start and middle stages. Dehalogenation made up 28.7%, which is more than at the start stages but less than the middle stages. Nitrite reducers and Nitrogen fixation were each abundant at 11.2%, which is more than the start stages but less than the middle stages. Sulphate reducers and sulphide oxidisers were abundant at 7.2% and 5.4%, respectively, which is more than start stages and less than middle stages. Chitin degradation was abundant at 11.5%, which was more than both the start and middle stages.

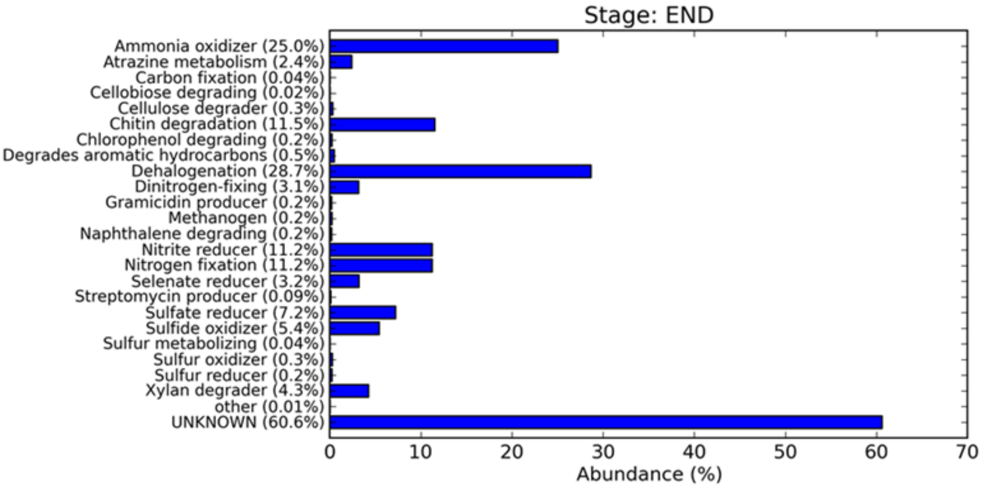


Figure 4-32: Taxonomic to phenotype mapping of end stage using Metageneassist

4.8 Abundance between stages

According to Figure 4-33, the mean abundance of the beginning stages with regard to Firmicutes was more significant than the end and middle stages. Both the transformed and non-transformed abundances show the significance in the beginning in comparison to the middle and end stages. The abundance of Firmicutes in the middle stage was not much lower than the start stage, in comparison to the end stage where Firmicutes was the lowest.

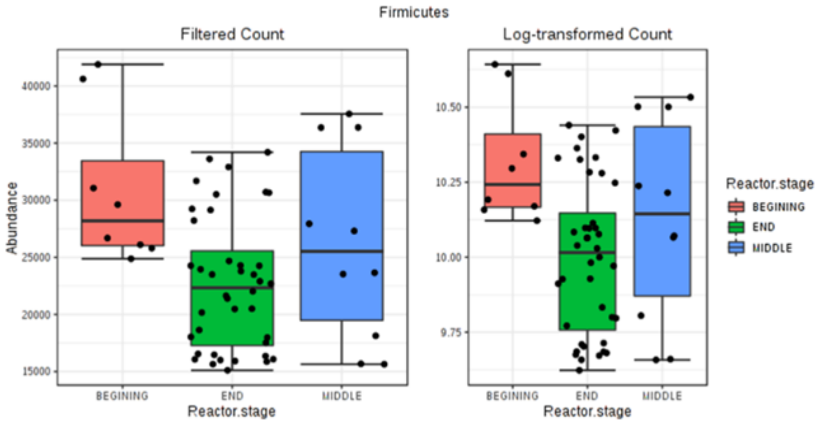


Figure 4-33: Firmicutes abundance between various stages using MicrobiomeAnalyst. Transformed samples (right) and non-transformed samples (left).

The abundance of Bacteroidetes in the different stages is shown in Figure 4-34. This indicates that the mean abundance of the beginning stage was the most significant followed by the middle stage. The end stage has the lowest mean abundance of all three stages. This is depicted in both the transformed and the non-transformed abundance.

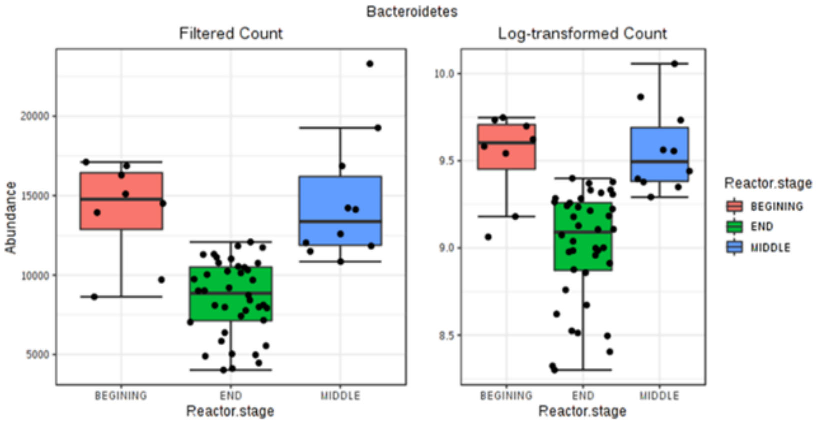


Figure 4-34: Bacteroidetes abundance between various stages using MicrobiomeAnalyst. Transformed samples (right) and non-transformed samples (left).

The abundance of Proteobacteria in the different stages is depicted in Figure 4-35. Both the transformed and non-transformed abundances indicate that there was barely a difference between the abundance of Proteobacteria in the end and middle stages. The beginning stage has the lowest abundance of Proteobacteria.

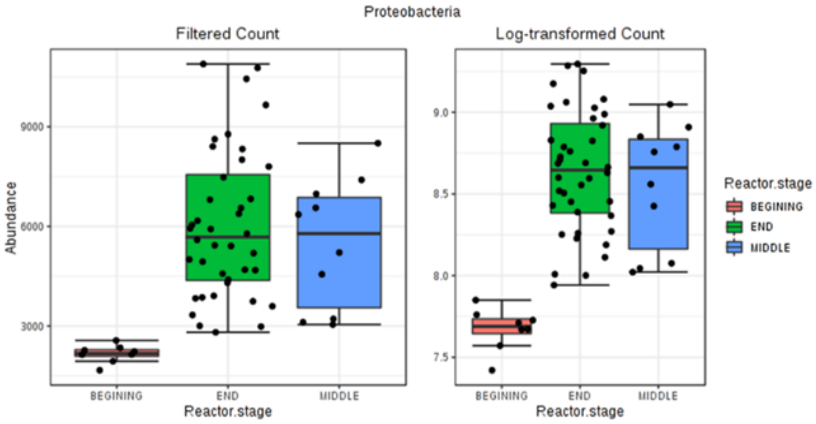


Figure 4-35: Proteobacteria abundance between various stages using MicrobiomeAnalyst. Transformed samples (right) and non-transformed samples (left).

The abundance of Synergistetes in the different stages is depicted in Figure 4-36. The mean abundance of the end stage was the highest of all the stages. The abundance indicates low abundance for the beginning and middle stages.

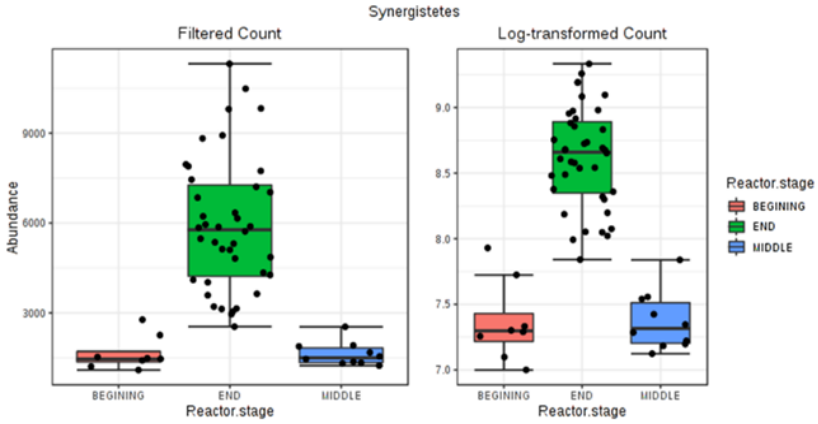


Figure 4-36: Synergistetes abundance between various stages using MicrobiomeAnalyst. Transformed samples (right) and non-transformed samples (left).

Chloroflexi abundance between the different stages is depicted in Figure 4-37. According to the transformed and non-transformed data, Chloroflexi was more abundant in the end stages, then middle stage and the lowest in the beginning stage.

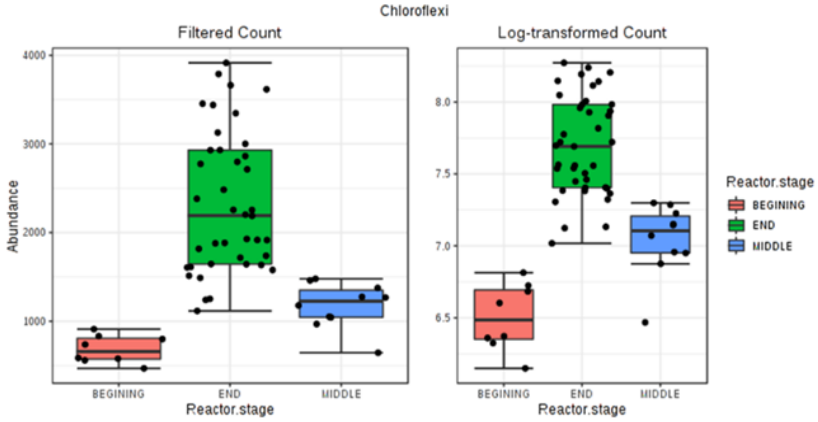


Figure 4-37: Chloroflexi abundance between various stages using MicrobiomeAnalyst. Transformed samples (right) and non-transformed samples (left).

The abundance of Actinobacteria in the different stages is shown in Figure 4-38. The mean abundance for Actinobacteria was higher in the end stages than the beginning stage, and the middle has the lowest abundance of Actinobacteria.

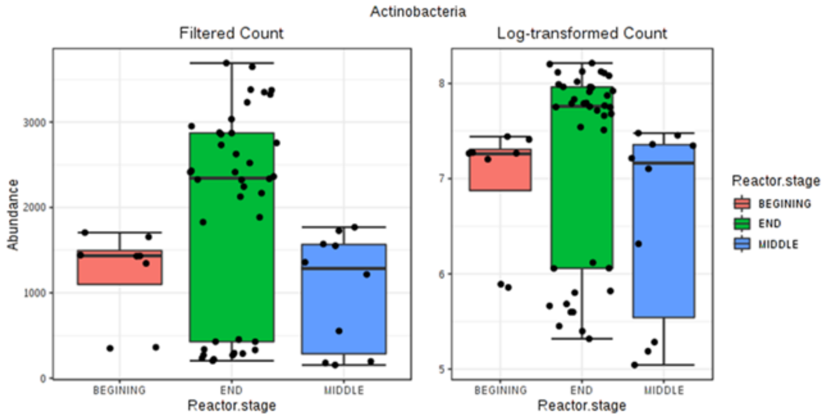


Figure 4-38: Actinobacteria abundance between various stages using MicrobiomeAnalyst. Transformed samples (right) and non-transformed samples (left).

The abundance of Planctomycetes in the different stages is shown in Figure 4-39. The mean abundance for Planctomycetes was higher in the end stages than beginning stage and the middle has the lowest abundance of Planctomycetes.

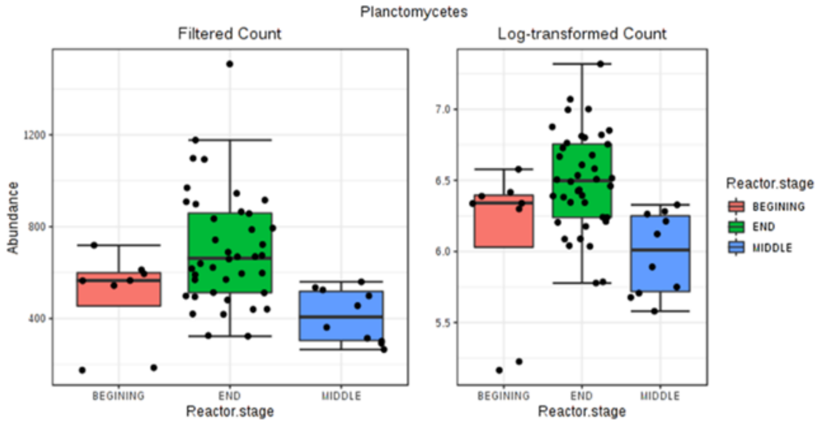


Figure 4-39: Planctomycetes abundance between various stages using MicrobiomeAnalyst. Transformed samples (right) and non-transformed samples (left).

CHAPTER 5 – DISCUSSION

5.1 Substrate characterisation

The C:N ratios for this study were as follows: pig slurry 11:1, seeding sludge 16:1 and the new pig slurry was 9:1. During this study, the C:N ratio was not optimal, thereby leading to low biogas production and possible ammonia inhibition. For the growth of anaerobic microorganisms, macronutrients such as carbon (C), nitrogen (N), phosphorus (P) and sulphur (S) are essential (Gunes *et al.,* 2019). An imbalance in the available nutrients is a major limiting factor for anaerobic digestion (Gunes *et al.,* 2019; Mao *et al.,* 2015). The C:N ratio is typically sub-categorised within anaerobic digestion as the relative amount of carbon and nitrogen that has a direct effect on the production of methane due to its direct relationship to possible inhibition (Gunes *et al.,* 2019). For any type of anaerobic digester, the known optimum C:N ratio is between 20:1 and 30:1 to provide adequate N for bacterial growth as well as to prevent excess (Gunes *et al.,* 2019; Nagy & Wopera, 2012). Substrates with low C:N ratios (15:1 or lower) can also result in lower methane production (Gunes *et al.,* 2019). For the microorganisms, the carbon-to-nitrogen balance is a very important factor. Anaerobic bacteria digest carbon 30 times faster than nitrogen, and therefore the optimal C:N ratio is 20:1 to 30:1 (Nagy & Wopera, 2012). Furthermore, the same process can result in ammonia accumulation through methanogenic activity, leading to a pH>8. Such a pH is toxic to acidogens (Gunes *et al.,* 2019; Nagy & Wopera, 2012). Carbon is typically supplied by the substrate during biological processes and used to strengthen the cell structure of a microorganism (Mao *et al.,* 2015). Protein biosynthesis requires nitrogen as well as sulphur and is vital nutrients for the growth of methanogenic bacteria (Mao *et al.,* 2015). Sulphur is required as a constituent of essential amino acids. The phosphate content, however, is also essential to the metabolism of ATP and ADP energy carriers (Mao *et al.,* 2015).

During this study, the phosphorous concentrations varied. The initial pig slurry had a phosphate concentration of 153.52 mg/l, seeding sludge 10.55 mg/l, while the new pig slurry had a concentration of 330.21 mg/l. Phosphorous is a macronutrient that is necessary for bacteria and methanogenic archaea in fairly large quantities and plays a major role in its metabolism (Belostotskiy *et al.,* 2015). In a study conducted by Belostotskiy *et al.* (2015), it was found that phosphate levels between 414 and 465 mg/l improved anaerobic digestion processes in batch reactors; though, this was achieved with low concentrations of ammonia. None of the substrates used for the present study reached the abovementioned phosphate levels. The new pig slurry had the highest phosphate concentration (330.21 mg/l) compared to the initial pig slurry (153.52 mg/l). This might also indicate that the initial pig slurry sample could have been diluted during practices at the farm (wash day) before sampling was done. It is therefore important that sampling regimes for pig slurry that will be used for anaerobic digestion be coordinated with farming practices to avoid the possibility of dilution.

In the present study, the new pig slurry used in the back-inoculation process contained more sulphates (56.34 mg/l) than the initial sample (10.93 mg/l). During biogas production, low levels of H_2S were produced. One of the P_Start replicates produced 7.7 ppm on average and a PS_Start replicate an average of 7 ppm (Appendix D). The rest of the samples produced less than 3 ppm or nothing. It could therefore be concluded that sulphate in the samples used for the study did not play a significant role during the biogas production process. During anaerobic digestion, H_2S is produced by sulphate-reducing bacteria, and these microorganisms have been extensively studied in recent decades using biomolecular techniques (Traversi *et al.,* 2015; Peu *et al.,* 2011). Hydrogen sulphide can also result from the anaerobic reduction of amino acids containing sulphur in addition to this form of production (Peu *et al.,* 2011). The occurrence of sulphate, sulphite or thiosulfate in the reaction mixture leads to a reduction of oxidised sulphuric matter in the digester to different forms of dissolved sulphide (HS^-, S^{2-}) and hydrogen sulphide (H_2S) in the biogas produced (Gunes *et al.,* 2019).

Electron donors include carbonaceous compounds such as methanol glucose, acetate, ethanol, formic acid and sulphite waste, whereas nitrates and nitrites are electron acceptors (Jingura & Kamusoko, 2017). Not all nitrogen in the form of nitrates is converted to ammonia, implying two ways of denitrification, namely (i) assimilating nitrate reduction, where nitrate is converted to ammonia for cell growth, and (ii) dissimilar nitrate reduction or biological denitrification, where nitrate is used as an electron acceptor for the oxidation of organic and inorganic electron donors (Edwards *et al.,* 2017).

In the present study, the average methane yield was 7.8%, this is regarded as poor. The AD process is often associated with poor methane yield resulting from the accumulation of intermediate inhibitory products. Ammonia nitrogen and organic acids are the most common inhibitors among these products (Pan *et al*., 2019; Zhang *et al*., 2019a). Anaerobic digestion is sensitive to high ammonium levels and in particular to high free ammonia concentrations (Peu *et al.,* 2011). During anaerobic digestion, ammonia is considered a potential inhibitor (Wachemo *et al*., 2019; Wu *et al.,* 2016; Wu *et al.,* 2016a; Colón *et al.,* 2015). It has also been suggested that the inhibition is caused by free ammonia in solution rather than ammonium ions and that 150 mg/l of free ammonia nitrogen concentration is completely inhibitory to anaerobic digestion (Wu *et al.,* 2016). Jo et al. (2018) suggest that ammonia concentrations above 100 mg/l could cause significant inhibition of methanogenic activity. On the other hand, Pan *et al.* (2019) reported that ammonia directly inhibits microbial activity at concentrations exceeding 1.7 to 14 g/l. Wachemo *et al.* (2019) reported the ammonia inhibition threshold to be 4 000 mg/l. Methanogens are considered to be the least tolerant and are most likely to stop growth because of ammonia inhibition (Pan *et al*., 2019).

Ammonia inhibition took place in the study, as ammonia concentrations ranged from 1021.65 mg/l to 1580.43 mg/l. Again, there was a difference in ammonia concentration between the initial pig slurry (1226.32 mg/l) and the new pig slurry (1580.43 mg/l) used for the back-inoculation. Ammonia is formed by the biological breakdown of nitrogen, mostly in the form of proteins and urea, in the anaerobic digestion process (Wu *et al*., 2016a). Manure anaerobic digestion (pig manure, cow manure and chicken manure) is often hindered by free ammonia inhibition resulting in poor production of methane (Li *et al.,* 2017). This can also explain why biogas was only produced at low levels. No heavy metals were present at concentrations that can influence the anaerobic digestion process. A list of the heavy metal threshold limits is available in Appendix E.

Even though some ammonia inhibition took place and the carbon source was not optimal, biogas was produced by the different reactors. The back-inoculation process was successful by speeding up the lag phase during biogas production, even though the samples were exposed to oxygen during the back-inoculation. A better way of back inoculating samples needs to be investigated.

5.2 Microbial communities

Major phyla observed in the microbiomes in the current study included Firmicutes, Bacteroidetes, Proteobacteria, Synergistetes, Euryarchaeota, Chloroflexi, Actinobacteria and Atribacteria, and the major families were *Clostridiaceae 1*, *Synergistaceae*, *Ruminococcaceae*, *Rikenellaceae*, *Marinilabiaceae*, *Porphyromonadaceae*, *Erysipelotrichiaceae* and *Methanosarcinaceae*. These are typically phyla and families associated with the AD processes. During such processes, the microbial hydrolyse proteins, fats, carbohydrates and other biodegradable polymers release amino acids, fatty acids and sugars. Hydrolytic bacteria are diverse in phylogenetics, but mostly fall into two phyla, Bacteroidetes and Firmicutes. Then acidogenic bacteria convert amino acids, fatty acids and sugars into ammonia, short-chain fatty acids, carbon dioxide, hydrogen and alcohols. Phyla

Firmicutes, Bacteroidetes, Proteobacteria, Chloroflexi and Actinobacteria are the most common phyla of acidogenic bacteria. Although methanogens can directly use acetate, formate, H_2/CO_2 and methyl compounds, other compounds resulting from acidogenesis such as butyrate, propionate, lactate and ethanol are further biodegraded into acetate, formate and H_2/CO_2 by a group of syntrophic acetogens (Leng *et al*., 2018).

5.2.1 Domain and phylum level

The microbial communities were investigated, and some limitations were encountered. The ratio between bacteria and archaea at domain level indicated that seeding sludge was not optimally or completely adapted. This could possibly be attributed to the pig slurry as a substrate or that the seedings sludge was not fed with the correct substrate in order to accommodate pig slurry as a fermentation substrate. This aspect will need further investigation into substrate-specific seeding sludges. The control sample, however, did produce biogas. It is important to note that although biogas was produced, little or no production of the methane was observed in this study. Various operating conditions and AD processes have a significant impact on the structure of the microbial community in reactors (Liu *et al.,* 2019). These include pH, temperature and organic loading. The growth of various functional microorganisms, including substrate hydrolysing bacteria, acetogenic bacteria and methanogenic archaea, adapts to different environments and/or substrates (Liu *et al.,* 2019). Methanogenic archaea are a phylogenetically diverse group of strictly anaerobic Euryarchaeota with an energy metabolism that is restricted to the formation of CH_4 from CO_2 and H_2, formate, methanol, methylamines and/or acetate (Zahedi *et al.,* 2016). Most methanogens found in anaerobic digesters are considered to be archaea, which include the orders Methanococcales, Methanobacteriales and Methanomicrobiales (Zahedi *et al.,* 2016).

In this study, the most dominant phyla were Firmicutes, Bacteroidetes, Proteobacteria, Synergistetes, Euryarchaeota, Chloroflexi, Actinobacteria and Atribacteria. Each of these organisms played a specific role during biogas production. Most bacteria found in anaerobic digesters belonged to the phyla Firmicutes, Actinobacteria, Proteobacteria and Bacteriodetes (Zahedi *et al.,* 2016). This is in agreement with other studies in literature. Zahedi *et al.* (2016) as well as Maspolim *et al.* (2015) also found Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes to be the most abundant phyla during anaerobic digestion. In a study conducted by Zahedi *et al.* (2016), waste-activated sludge and organic fraction of municipal solid waste were used as co-digestion substrates, using a two-stage thermophilic reactor. Even though thermophilic reactors were used, the dominating phyla identified were similar with this study. Maspolim *et al.* (2015) conducted a study on two municipal sludge digestion systems: single-stage and 2-phase AD configuration, which were operated under various organic loading conditions. In most of the literature surveyed, relatively little or no discussion involves the community compositions at domain and phylum level. Some studies also found WS6 to play a role during anaerobic digestion. These two studies were mostly conducted on food waste (Liang *et al*., 2020; Liu *et al*., 2019). However, no further information was available on the function of these bacteria that were also observed in the present study.

While Niu et al. (2013) found Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria and Synergistetes to be the most dominant phyla during anaerobic digestion, their study was conducted using chicken manure with thermophilic methane fermentation within a wide range of ammonia concentrations. In a study Zhang *et al.* (2019), the aim was to investigate waste-activated sludge as an inoculum for solid-state anaerobic digestion of agricultural waste (livestock manure, crop stalks and vegetable residuals). Firmicutes, Bacteroidetes, Proteobacteria and Chloroflexi were the dominant phyla in studies by Zhang *et al.* (2019).

Firmicutes are known syntrophic bacteria that can break down volatile fatty acids such as butyrate and its metabolites to produce H_2 and can be reduced to produce methane by hydrogenotrophic methanogens (Dai *et al.,* 2017). Syntrophy is a particularly mutual partnership in AD, defined as a thermodynamically interdependent lifestyle in which no partner can function without the other. In order to maintain this cooperative metabolic activity, interspecies electron transfer and the concentrations of electron carriers in the system are critical (Leng *et al*., 2018). Firmicutes and Bacteroidetes are reported in literature to be syntrophic bacteria that secrete specific lytic enzymes, degrade organic material and generate acetic acid during acidogenesis and can be enriched with high concentrations of soluble organic matter (Liu *et al.,* 2019). These bacterial species were also observed in the present study.

Clostridia (phylum Firmicutes) can use cellulose, arabinose, lactose and glucose to produce propionic acid, acetic acid, formic acid and hydrogen at 7.0 pH and moderate anaerobic temperature (Liu *et al.,* 2019). Hydrolysis of complex organics in sludge has been reported to be the rate-limiting step in anaerobic digestion of sludge (Dai *et al.,* 2017). Increases in the relative abundance of Bacteroidetes and Firmicutes can therefore have a positive influence on the hydrolysis and acidification of anaerobic sludge fermentation and potentially increase the yield of methane (Dai *et al.,* 2017).

According to Figure 4-34, Firmicutes only played a significant role during the beginning and middle stages, which confirms that Firmicutes play a role during the hydrolysis and acidogenesis as well as acetogenesis stages of anaerobic digestion. This also confirms that Firmicutes can degrade a wide variety of substrates. The bulk of fermenting bacteria in anaerobic digestion are Firmicutes and Bacteroidetes (Kampmann *et al.,* 2014). The predominance of phylogenetic groups such as Firmicutes, Proteobacteria and Bacteroidetes resulted from their potential to degrade a wide variety of substrates (Zhi & Zhang, 2019) such as cellulose, proteins, pectin and other xenobiotic compounds (Theuerl *et al*., 2018; Fitamo *et al.,* 2017). Reactor efficiency and system performance during anaerobic digestion may be affected by the abundance of Bacteroidetes and Firmicutes (Wachemo *et al*., 2019).

Bacteroidetes are proteolytic bacteria that are involved in protein degradation and are capable of fermenting acetate and NH_3 for amino acids (Lee *et al.,* 2011). The two orders Clostridiales (phylum Firmicutes) and Bacteroidales (phylum Bacteroidetes) often dominate in animal manure and sludge (Solé-Bundó *et al.,* 2019). Phyla Proteobacteria, Chloroflexi and Fibrobacter also often increase the reaction to the introduction of lignocellulosic materials, with some variability depending on the co-digestive content and the prevailing environmental conditions (Solé-Bundó *et al.,* 2019).

In the hydrolysis step of the anaerobic digestion process, Firmicutes, Proteobacteria and Bacteroidetes play an important role during these steps (Zhi & Zhang, 2019). The trends in the present study (Figure 4-35) demonstrate that Bacteroidetes played a crucial role during the beginning and the middle stages and the abundances were relatively higher during these processes. Proteobacteria are the main organisms in the development of sludge hydrolysis and acid production (Yang *et al.,* 2020).

In the present study (Figure 4-36), it was demonstrated that Proteobacteria were a statistically significant part of the microbial consortium during middle and end stages of the AD process. The significance of Proteobacteria during middle stages is due to back inoculation from end stage samples. Proteobacteria are important consumers of glucose and several types of volatile fatty acids involved in the degradation of organic waste (Meng *et al.,* 2017; Yang *et al.,* 2017). Proteobacteria are characterised in anaerobic digesters as the syntrophic, specialised and core community (Nakasaki *et al.,* 2019), which is involved in the degradation of cellulose and proteins and in the syntrophic degradation of organic acids (Ma *et al.,* 2019). Proteobacteria include anaerobic and aerobic bacteria and are involved in anaerobic digestion processes with hydrolysis, acidogenesis and acetogenesis reactions (Díaz *et al.,* 2018).

Figure 4-37 confirms that Synergistetes played the biggest role during the end stages of anaerobic digestion. This can be an indication that *Synergistaceae* and hydrogenotrophic methanogens work in a symbiotic relationship. Synergistetes are known for fermenting organic compounds (carbohydrates, organic acids) and cellulose, sugars and hemicellulose into H_2 and acetate, respectively (Fitamo *et al.,* 2017). It has been proposed that Synergistetes contain bacteria that can conduct syntrophic metabolism in combination with hydrogenotrophic methanogens (Solé-Bundó *et al.,* 2019). Synergistetes are strict anaerobes, fermenting amino acids (Liang *et al.,* 2020). It has been reported that Synergistetes use amino acids and, in turn, provide short-chain fatty acids or act as syntrophic acetate oxidisers (Tao *et al.,* 2020).

5.2.2 Class and family level

In the present study, mainly members of class Clostridia, Synergistia, Bacteroidia, Erysipelotrichia and Methanomicrobia dominated. At family level (Figures 4-15 to 4-19), a different profile emerged. These include the families of *Clostridiaceae 1*, *Synergistaceae*, *Ruminococcaceae*, *Rikenellaceae*, *Marinilabiaceae*, *Porphyromonadaceae*, *Erysipelotrichiaceae* and *Methanosarcinaceae*. Unassigned organisms also played a big role and were the most dominant in some reactors, especially in the control samples (MC). This leaves a big question as to what happened in the reactors.

Clostridia was the most dominant class in all the samples used in this study, at different levels. *Clostridiaceae 1* and *Ruminococcaceae* were identified as the most abundant families from the class Clostridia. Clostridia, including Clostridium and Thermoanaerobacter genera, are a highly polyphyletic class of Firmicutes. They are obligate anaerobes that can produce endospores (Zahedi *et al.,* 2016).

Zahedi *et al.* (2016) found Spirochaetes to be also present in a study conducted on two-phase thermophilic anaerobic co-digestion using waste-activated sludge and an organic fraction of municipal solid waste as substrate. In this study Spirochaetes was not identified in any of the pig slurry or seeding sludge samples. Lee *et al.* (2015) suggest that Spirochaetes are often present in anaerobic digesters, as well as in anaerobic environments that are natural or engineered. Usually, they have a coiled helical structure, and grow chemoheterotrophically. Nevertheless, their ecophysiological roles have remained unclear in anaerobic digesters. Spirochaetes are reported to have metabolic activities, including acetate, ethanol and lactate fermentations from glucose and acetate oxidation (Lee *et al.,* 2015). Lee *et al.* (2015) also estimated that Spirochaetes constituted 1.3 to 30% of the total bacteria used for the treatment of municipal sludge in anaerobic digesters and proposed that cluster II Spirochaetes can syntrophically perform acetate oxidation with hydrogenotrophic methanogenic archaea. In response to the introduction of acetate to an anaerobic batch reactor seeded with anaerobic digester sludge, they observed increased activity of cluster II Spirochaetes, which provided the basis for the hypothesis on syntrophic acetate oxidation. The claim was not supported, however, by the increased activity of hydrogenotrophic methanogenic archaea by cluster II Spirochaetes during acetate degradation (Lee *et al.,* 2015).

Clostridia is reported in literature as a key role player in the production of biogas (Fu *et al.,* 2016) during the acidogenesis stage of anaerobic digestion (Nakasaki *et al., 2019)*. The high cellulolytic activity by most members belonging to the order Clostridiales, which contributed to the breakdown of polysaccharide molecules, and Clostridiales members which can ferment sugar to organic acids (Fu *et al.,* 2016). Fu *et al.* (2016) found that the hydrolysis phase is widely regarded as the rate limiting step during the anaerobic digestion of cellulosic substrates such as corn straw. Microaerobic fermentation resulted in the relative abundance of phylum Firmicutes, class Clostridia and order Clostridiales associated with hydrolysis. This was found in a study by Fu *et al.* (2016). Their study also had an abundance of Clostridia comparable to anaerobic digestion, although the focus of the study was on microaerobic conditions. The greater abundance of order Clostridiales demonstrated the greater ability to break the polysaccharide molecules of substrates and ferment sugar into organic acids, which also resulted in higher hydrolysis under microaerobic conditions (Fu *et al.,* 2016). In a study conducted by Nakasaki *et al.* (2019) in four full-scale anaerobic co-digesters of energy crops, manure and food waste, Clostridiales were observed as the most dominant order.

Clostridiaceae 1 were the most abundant in P (pig slurry) samples, especially in P_End samples. *Clostridiaceae 1* were only present in MC_End samples at very low levels. *Clostridiaceae 1* were only abundant in PSB_End and PSB_Mid samples. This indicates that *Clostridiaceae 1* were transferred from the end sample during back-inoculation, but at the end of the back-inoculated sample, very low levels of *Clostridiaceae 1* were present. *Ruminococcaceae* seem to be persistent in all the reactor configurations and stages as their levels remained relatively stable throughout. *Ruminococcaceae* were another clostridial family commonly found in all bioreactors, particularly in reactors fed with maize straw (Caruso *et al.,* 2019). The function of this group seems to be primarily the cellulolytic digestion of plant fibres based on the production of complex cellulosome enzymes and cellulose adhesion proteins (Caruso *et al.,* 2019; Jo *et al*., 2018).

Rikenellaceae were most abundant in the end samples as well as mid samples. This is to be expected as end samples were used to back-inoculate the samples. The community transfer took place of *Rikenellaceae*. Nakasaki *et al*. (2019) found *Rikenellaceae* to be abundant during the beginning of their experiments with fats, oils and grease and decreased slightly in the later stages of the fermentation process.

Bacteroidia played a major role in the hydrolysis and fermentation of organic materials and in the synthesis of organic acids, CO_2 and H_2 during anaerobic digestion (Díaz *et al.,* 2018; Liu *et al.,* 2019). Bacteroidia are the main sludge degradation bacteria at mesophilic temperatures, which can degrade polysaccharides and other organic matter and can also produce hydrogen, carbon dioxide, fibrous disaccharide, glucose and acetic acid (Yang *et al.,* 2020). The *Rikenellaceae* family ferments carbohydrates and produces hydrogen (Meng *et al.,* 2017). *Marinilabiaceae* produce hydrogen, carbon dioxide and acetate from cysteine, lactate and pyruvate within the Bacteroidia class (Liu *et al.,* 2019). It has been suggested that the *Porphyromonadaceae* family played an important role in the degradation of the accumulated volatile fatty acids (Poirier *et al.,* 2016).

Pig samples did not contain significant amounts of *Marinilabiaceae,* while seeding sludge did contain *Marinilabiaceae*, which were transferred to the PS samples. Seeding sludge contained the most abundance of *Marinilabiaceae*. All start samples contained more *Porphyromonadaceae* than in the other samples. When back-inoculation was done, *Porphyromonadaceae* showed similar relative abundance levels as the start samples, suggesting that *Porphyromonadaceae* play an important role during the first stages of anaerobic digestion.

Synergistaceae were more dominant during end stages of anaerobic digestion in all the samples. Back-inoculated samples only showed increased numbers of *Synergistaceae* towards the end. This shows that *Synergistaceae* and hydrogenotrophic methanogens work in a symbiotic relationship. Synergistia can convert organic acids to acetate and hydrogen (Chen *et al.,* 2017). *Synergistaceae* ferment glucose and organic acids to produce acetate, CO_2 and H_2 when co-cultivated with hydrogenotrophic methanogens (Meng *et al.,* 2017; Vasquez & Nakasaki, 2016). The high abundance of *Synergistaceae* therefore suggests that bacteria of this family can ferment volatile fatty acids to H_2 and CO_2, which can be used by hydrogenotrophic methanogens (Meng *et al.,* 2017). *Synergistaceae* are classified as syntrophic acetate oxidising bacteria (Lv *et al.,* 2019).

Erysipelotrichiaceae were only present in MC_Start, PS_Start, PSB_Mid, PS_Mid, S_Start and S_Mid. P-samples did contain significant amounts of Erysipelotrichiaceae. This was not as expected, as Erysipelotrichiaceae come from pig manure (Han *et al.,* 2011). It seems that the seeding sludge contained Erysipelotrichiaceae and was transferred during the study to the other samples as seeding sludge was added to MC and PS samples. This also indicates that Erysipelotrichiaceae play an important role during the first and second stages on anaerobic digestion, the stages being hydrolysis and acidogenesis. The class Erysipelotrichia is recognised to be frequent in the gut microbiome as they have been isolated from pig manure (Han *et al.,* 2011). Class Erysipelotrichia has hydrolytic properties (Díaz *et al.,* 2018).

MC_End samples contained more *Methanosarcinaceae* than MC_Start, as did P_End, PS_End, PSB_End, S_End and SB_End. This indicates that *Methanosarcinaceae* only plays a role at the end stage (methanogenesis). P samples contained extremely low levels of *Methanosarcinaceae*. Back-inoculation of *Methanosarcinaceae* was successful, as PSB_Mid contained higher levels of *Methanosarcinaceae*, even though *Methanosarcinaceae* levels decreased in PSB_End samples. Back-inoculating S samples also seemed successful as SB_End samples contained more *Methanosarcinaceae*. The most abundant class in the Archaea domain was Methanomicrobia, which used CO_2 as a carbon source during methanogenesis and H_2 as an electron donor, suggesting that the hydrogenotrophic pathway contributed to the formation of CH_4 (Caruso *et al.,* 2019) and belongs to the acetic acid decomposition family (Chen *et al.,* 2017). Achinas *et al.* (2018) found that *Methanosarcinaceae* increased in numbers towards the end of the experiments. As was suggested by Achinas *et al.* (2018), the number of *Methanosarcinaceae* did increase towards the end of the experiments. The types of microorganisms appear to be heavily dependent on substrates, coexisting substrates and operating conditions including temperature, organic loading rate (OLR), hydraulic retention time (HRT) and the type and quantity of seed sludge used for fermentation start-ups (Nakasaki *et al.,* 2019).

5.3 Predicted metabolic activities

In this study, a large number of metabolic activities of microbes could not be predicted, whereas some could not specifically be assigned specific functions. In a study by Theuerl *et al*. (2018) on 36 full-scale anaerobic digesters, there were digesting substrates ranging from manure and crops to biowaste. They also identified clusters that could not be assigned to any known bacteria functions. Nevertheless, unknown species are highly adapted and resistant to these prevailing environmental conditions and occupy a particular habitat that makes them valuable for their microbiomes and functionality (Theuerl *et al*., 2018).

In the present study, most of the ammonia oxidiser organisms belong to the phylum Proteobacteria. These organisms get energy from the oxidation of inorganic nitrogen compounds (Mancinelli, 1996). Ammonia is oxidised to nitrogen gas with nitrite under anaerobic conditions as electron-acceptor and carbon dioxide are used for bacterial growth (Yangin-Gomec *et al*., 2017). This functionality grouping was the most abundant in taxonomic to phenotype mapping (Figures 4-31 to 4-33) and corresponds to Figures 4-10 to 4-14, which demonstrate phylum diversity. During the biogas production study, the gas analyser was not able to do ammonia readings, but taxonomic to phenotype mapping indicates that during all the stages, a large number of bacteria could have been carrying out ammonia oxidation and that low methanogenesis activities were possibly taking place. This was also indicated by the ammonia concentrations before the study was started. During this study, the C:N ratio was not optimal, thereby leading to low biogas production and possible ammonia inhibition. Methanogenic archaea seem particularly susceptible to exposure to ammonia (Ruiz-Sánchez *et al*., 2019). The so-called syntrophic acetate oxidising bacteria (SAOB) can reverse the homoacetogenic process and transform acetate to carbon dioxide (CO_2) and hydrogen (H_2) under such high concentrations of ammonia and/or acetate (Ruiz-Sánchez *et al*., 2019).

In a study by Ros *et al*. (2017), it was reported that high concentrations of ammonia also inhibited the anaerobic digestion process. Their study was also conducted using pig slurry, where biogas production was inhibited as well as methane quality (Ros *et al*., 2017). Similar results were seen in anaerobic digestion of other livestock waste used for feeding substrate for anaerobic digesters as a consequence of the negative effect of ammonia inhibition (Ros *et al*., 2017). Ruiz-Sánchez *et al*. (2019) found that by activating/deactivating different microbial species, biomass from a full-scale anaerobic digester, adapted to relatively high concentrations of ammonia, was able to swiftly switch its metabolic mode from acetotrophic to hydrogenotrophic methanogenesis.

According to Ruiz-Sánchez *et al*. (2018), most of the solutions proposed for ammonia inhibition are based on modifying reactor configurations to control ammonia concentration. Alternative methods, such as enriching methanogenic biomass with ammonia-resistant microbes, have also been created. Adaptation of microbial communities to concentrations of nitrogen that are generally considered toxic was reported under various conditions: discontinuous or pulse ammonia exposure; supplementation with micronutrients; addition of adsorbents such as biofibers and zeolite; and nitrogen dilution by co-digestion. Most of these strategies are costly and time consuming, and the end result are not always satisfactory (Ruiz-Sánchez *et al*., 2018).

Dehalogenation bacteria belong to the phyla Firmicutes (Euzéby, 1997) and Chloroflexi (Löffler *et al.,* 2012) and obtain energy through oxidation of hydrogen and reductive dehalogenation of halogenated organic compounds during anaerobic respiration (Löffler *et al.,* 2012). These organisms were also found in this study. Literature only reports these types of organisms until phylum level. It might be that these organisms have more than one function and that is why it is indicated as also having a dehalogenation function. Nitrite-reducing and nitrogen-oxidiser bacteria are mostly also members of the phylum Proteobacteria (Grunditz & Dalhammar, 2000). Sulphate-reducing microbes are a group of sulphate-reducing bacteria and sulphate-reducing archaea (Muyzer & Stams, 2008), both of which can perform anaerobic respiration using sulphate (SO_4^{2-}) as terminal electron acceptor, reducing it to hydrogen sulphide (H_2S) (Schulze & Mooney, 1993). These belong mostly in the phylum Firmicutes as well as some from Proteobacteria and Euryarchaeota.

Sulphite oxidisers are mostly Proteobacteria, capable of using sulphur, thiosulphate or polythionates as an energy source, making them obligated autotrophs (Boden, 2017). Chitinoclastic is a process where chitin is degraded. This is done by some organisms that fall in the phylum Proteobacteria (Beier & Bertilsson, 2013).

Methanogenesis based on acetate accounts for almost 70% of the total production of methane in anaerobic wastewater treatment. Acetate oxidation in anaerobic digestion is therefore potentially a very important process. The syntrophic relationship between acetate oxidising bacteria and hydrogenotrophic methanogens generates CH_4 from acetate by H_2 and CO_2 intermediates (Leng *et al*., 2018). Methanogens are microorganisms that grow relatively slowly and have a limited range of substrates available. The production rate of CH_4

from methanogenic substrates is affected by various factors, including the microbial community structure and sludge/substrate composition (Leng *et al*., 2018). This is another possible explanation for the low methanogenesis activity in this study; run times might have been too short to allow methanogenic organisms to have sufficient time to grow and produce more methane. Methanogens are generally also the most sensitive to higher ammonia concentrations. Acetoclastic methanogens are recognised as more vulnerable to ammonia than hydrogenotrophic methanogens with comparable and, in some cases, more sensitivity to high levels of ammonia compared to syntrophic acetate-oxidising bacteria in methanogens. Consequently, ammonia concentration may be one of the most important factors affecting the structure of the methanogenic group and may alter the dominant methanogenic pathway in the digester as stated in previous studies (as discussed above) (Jiang *et al*., 2018).

Only one class and one family of methane producing archaea were identified during this study, which can also explain low methanogenesis. Ros *et al*. (2017), who also studied the microbial communities during anaerobic digestion, using pig slurry, identified more orders of methanogenesis archaea; the difference was, these reactors ran for approximately 109 days. Ros *et al*. (2017) sampled reactors on day 13 to identify the community present for stage 1 (hydrolysis). On day 27, samples were taken to identify communities involved in stage 2 (acidogenesis). Acetogenesis (stage 3) communities were identified during sampling on day 44, while methanogenesis (stage 4) was sampled and identified on day 86 (Ros *et al*., 2017). The dominant archaea were identified as Methanosarcina, Methanosaeta and Methanoculleus, which were responsible for approximately 70% of the methane production (Ros *et al*., 2017). Archaeal communities identified by Shin *et al*. (2019) in acidified pig slurry during anaerobic digestion were Methanosarcina, Methanolobus, Methanobrevilbacter, Methanocorpusculum, Methanomassiliicoccus and Methanobacterium. Ruiz-Sánchez *et al*. (2019) also found Methanosarcina, Methanolobus, Methanobrevilbacter, Methanocorpusculum, Methanomassiliicoccus and Methanobacterium to be the abundant archaea during anaerobic digestion of pig slurry and protein-rich agricultural waste.

It seems that this current study might have been too short. This might indicate that during the current study, sampling and back-inoculation were done too soon. It is possible that back-inoculation was done during hydrolysis stage. Another possibility is that the community was not established and stable yet, before back inoculation was done. Back inoculation could have influenced the methanogens' growth as these organisms grow slowly. This is possibly why the predicted metabolic activity of methanogens was very low. In a study conducted by Grohmann *et al*. (2018) on anaerobic digestion of food waste, reactors were analysed after a period of 84 days. Yangin-Gomec *et al*. (2017) studied anaerobic digestion using chicken manure; the reactors ran for a period of 120 days.

Primary and secondary fermenting microbes belong to the Bacteria domain in accordance with taxonomy, while methanogens belong to the Archaea domain. According to the Archaea:Bacteria ratio, low levels of archaea were present in the various samples, which might explain the low biogas yields observed during the current study. The statistical results also indicate that methanogen metabolism in all three stages was less than 0.5%. Mostly, it is not known what was taking place as *unknown* was the most, followed by ammonia oxidation and dehalogenation. The first three stages involve primarily bacteria, and the final stage involves archaea (Cai *et al.,* 2019). In a single-phase system, all AD pathway reaction steps, from hydrolysis to methanogenesis, take place in one reactor operated under optimal methanogens conditions, i.e. neutral pH and long hydraulic retention time (typically more than 20-30 days). It suggests that acidogens, which have unique physiological and growth properties from methanogens, are in such a reactor under suboptimal conditions (Jo *et al*., 2018). The anaerobic digestion process’s stability and efficiency depend on a delicate balance between different microbial communities (Cai *et al.,* 2019).

In summary, macronutrients are essential for growth of anaerobic microbes. An imbalance can be a limiting factor for anaerobic digestion. Each microorganism has a specific role to play during anaerobic digestion. In this study, the most dominant phyla were Firmicutes, Bacteroidetes, Proteobacteria, Synergistetes, Euryarchaeota, Chloroflexi, Actinobacteria and Atribacteria. Most of the microorganisms identified in this study agree with those reported in literature for mesophilic anaerobic digesters. Methanogenesis was not optimal as the abundance of methane producing microorganism was inhibited. Microbial transfer took place when back inoculation was successfully applied.

CHAPTER 6 – CONCLUSION AND RECOMMENDATIONS

The aim of the current study was to determine whether back-inoculation will reduce the observed lag time before biogas production occurs in batch reactors. To achieve this aim, objectives were identified, and these are briefly concluded in section 6.1 to 6.4.

6.1 Set-up of the bench-top reactors

The experimental study was conducted in triplicate and included seeding sludge, pig slurry as well as a pig slurry seeding sludge combination. A control sample with MC was also done. Macronutrients such as carbon, nitrogen, phosphorous and sulphur, the nutrients which are essential for microbial growth were measured, and biogas production was monitored. The initial findings were that an imbalance in nutrients was a limiting factor for anaerobic digestion. The C:N ratio was not optimal for this study. This can also be the reason why low levels of biogas were produced. Elevated ammonia levels led to possible ammonia inhibition, but biogas production still took place at reduced rates. No heavy metal inhibition took place as all heavy metal limits were below the threshold. The set-up of the experiments was therefore successful.

6.2 Inoculating a second set of reactors with inoculum from a set of previous inoculums

A second set of reactors were inoculated from the previous reactors at the phase of maximum biogas production, and biogas production was evaluated. During the back-inoculation process, some samples might have been exposed to oxygen as sample volumes were too small and had to be mixed in order to have enough substrate for the back-inoculation. The results demonstrated that reactors with a volume of 1 litre are not optimal for back-inoculation, chemical analysis and microbiological analysis. However, it was demonstrated that the biogas production increased in the back-inoculated samples.

6.3 Determine the microbial community and evaluate the effect of back-inoculation

The microbial dynamics were demonstrated during the beginning, middle and end stages of anaerobic production. Bacteria domain was completely dominant with little archaea present in all the samples. Most abundant at phylum level were Firmicutes, Bacteroidetes, Proteobacteria and Synergistetes. At class level, the dominant organisms were Clostridia, Bacteroidia, Synergistis, Methanomicrobia and Unassigned bacteria. At family level, differences can be seen clearly between samples. Unassigned organisms played a big role at family level, especially in MC samples. *Ruminococcaceae* were the only organism that seems to be persistent in all the reactor configurations and no significant changes even after back-inoculation.

Analysis of the different stages indicated the different roles that organisms play during each stage. Firmicutes played the biggest role during the start and middle stages of the study, indicating that Firmicutes played a role during hydrolysis, acidogenesis and acetogenesis stages of anaerobic digestion. Firmicutes can degrade a wide variety of substrates. Bacteroidetes played a role during hydrolysis as Bacteroidetes were most significant during start and middle stages of the study. Proteobacteria are significant during middle and end stages of the study, indicating that Proteobacteria contribute towards the hydrolysis, acidogenesis and acetogenesis stages of biogas production. Synergistetes and hydrogenotrophic methanogen work in a symbiotic relationship as Synergistetes play the biggest role in the end stages (methanogenesis stage) of anaerobic digestion.

Taxonomic to phenotype mapping indicated the predicted metabolic activities of the organisms during the experiments. This indicated that a large number of predicted metabolic activities were categorised as unknown. The second highest predicted metabolic activity during the study was ammonia oxidation; this was also indicated by the high ammonia concentrations of ammonia in substrates used in the setup.

According to the Archaea:Bacteria ratio, low levels of archaea were present in the various samples, which might explain the low biogas yields observed during the current study. The statistical results also indicate that methanogen metabolism in all the three stages was less than 0.5%. Mostly, it is not known what was taking place as *unknown* group was the most dominant, followed by ammonia oxidation and dehalogenation. The first three stages involve primarily bacteria, and the final stage involves archaea. The anaerobic digestion process’s stability and efficiency depend on a delicate balance between different microbial communities.

6.4 Recommendations

A series of issues were encountered during this study. These included incomplete adaptation of the sludge to the substrate; lower biogas yields; no apparent methane production; and a non-optimal Archaea:Bacteria ratio. These issues primarily relate to the small size of the bench-top reactors and the state of the seeding sludge. As such, the following recommendations are suggested to potentially resolve these issues.

- Evaluate the Archaea:Bacteria ratio of the seeding sludge with a microcrystalline cellulose test run before progressing to the actual reactor runs, to serve as a method to verify the suitability of the seeding sludge before biogas production experiments progress.
- Investigate whether seeding sludge from biogas plant is suitable for digestion of pig slurry; alternatively, adapt the seeding sludge to the planned feed stock for the experimental set-up. Alternatively, the seeding sludge can be fed with cow manure to establish a methane-producing microbial community.
- The use of CSTRs as well as larger size reactors are highly recommended. This will alleviate several issues experienced during this study. Firstly: oxygen contamination occurred during back-inoculation and had a marked effect on the microbial communities observed during this study. By using CSTRs the system can also be designed in such a manner that no atmospheric oxygen exposure occurs during back-inoculation. The use of CSTRs will also allow sampling to take place without exposure to atmospheric oxygen. The typically larger volumes associated with the operation of CSTRs will also ensure that sufficient volumes are available for the various analyses (both physical-chemical and biological).
- To resolve issues related to the long adaptation (lag) time of anaerobic reactors, another possibility could be to investigate dual-stage reactor system. In the first reactor conditions hydrolysis and acetogenesis will be the focus for study and optimisation. The second reactors will be where methanogenesis will occur. In this type of configuration better resolution might be possible regarding the change in microbial communities in response to variation in substrate during the hydrolysis and acetogenesis stage, while in theory minimally impacting the methanogenic community.
- Lastly during this study there was a paucity of information related to pig slurry availability and volumes for South Africa. This is in stark contrast to the wealth of information available from literature for northern hemisphere countries. The establishment of a database would be of significant value for various institutions in South Africa.

REFERENCES

Achinas, S., Li, Y., Achinas, V. & Euverink, G.J.W. 2018. Influence of sheep manure addition on biogas potential and methanogenic communities during cow dung digestion under mesophilic conditions. *Sustainable Environment Research,* 28(5):240-246.

Alayi, R., Shamel, A., Kasaeian, A., Harasii, H. & Topchlar, M.A. 2016. The role of biogas to sustainable development (aspects environmental, security and economic). *Journal of Chemical and Pharmaceutical Research,* 8(4):112-118.

Albornoz, F. 2015. Crop responses to nitrogen overfertilization: A review. *Scientia Horticulturae,* 205:79-83.

Allie, Z., Jacobs, E. P., Maartens, A. & Swart, P. 2003. Enzymatic cleaning of ultrafiltration membranes fouled by abattoir effluent. *Journal of Membrane Science,* 218:107-116.

Arndt, D., Xia, J., Liu, Y., Zhou, Y., Guo, A.C., Cruz, J.A., Sinelnikov, I.G., Budwill, K., Nesbø, C.L. & Wishart, D.S. 2012. METAGENassist: A comprehensive web server for comparative metagenomics. *Nucleic Acids Research,* 40(W1):W88-W95.

Bedoic, R., Cucel, L., Cosic, B., Krajnc, D., Smoljanic, G., Kravanja, Z., Ljubas, D., Puksec, T. & Duic, N. 2019. Green biomass to biogas - A study on anaerobic digestion of residue grass. *Journal of Cleaner Production,* 213:700-709.

Beier, S. |& Bertilsson, S. 2013. Bacterial chitin degradation-mechanisms and ecophysiological strategies. *Frontiers Microbiology*. 4: 149

Belostotskiy, D.E., Ziganshina, E.E., Siniagina, M., Boulygina, E.A., Miluykov, V.A. & Ziganshin, A.M. 2015. Impact of the substrate loading regime and phosphoric acid supplementation on performance of biogas reactors and microbial community dynamics during anaerobic digestion of chicken wastes. *Bioresource Technology,* 193:42-52.

Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J. & Wheeler, D.L. 2004. GenBank: update. *Nucleic Acids Research,* 32:D23-D26.

Boden, R. 2017. 115 years of sulfur metabolism. *FEMS Microbiology Letters,* 364(6).

Bratina, B., Sorgo, A., Kramberger, J., Ajdnik, U., Zemljic, L.F., Ekart, J. & Safaric, R. 2016. From Municipal/Industrial Wastewater Sludge and FOG to Fertilizer: A Proposal for Economic Sustainable Sludge Management. *Journal of Environmental Management,* 183:1009-1025.

Cai, Y., Hu, K., Zheng, Z., Zhang, Y., Guo, S., Zhao, X., Cui, Z. & Wang, X. 2019. Effects of adding EDTA and Fe^{2+} on the performance of reactor and microbial community structure in two simulated phases of anaerobic digestion. *Bioresource Technology,* 275:183-191.

Caruso, M. C., Braghieri, A., Capece, A., Napolitano, F., Romano, P., Galgano, F., Altieri, G. & Genovese, F. 2019. Recent Updates on the Use of Agro-Food Waste for Biogas Production. *Applied Science,* 9:1-29.

Chen, Y., Lan, S., Wang, L., Dong, S., Zhou, H., Tan, Z. & Li, X. 2017. A review: Driving factors and regulation strategies of microbial community structure and dynamics in wastewater treatment systems. *Chemosphere,* 174:173-182.

Chen, Y., Jiang, X., Xiao, K., Shen, N., Zeng, R.J. & Zhou, Y. 2017. Enhanced volatile fatty acids (VFAs) production in a thermophilic fermenter with stepwise pH increase - Investigation on dissolved organic matter transformation and microbial community shift. *Water Research,* 112:261-268.

Colón, J., Forbis-Stokes, A.A. & Deshusses, M.A. 2015. Anaerobic digestion of undiluted simulant human excreta for sanitation and energy recovery in less-developed countries. *Energy for Sustainable Development,* 29:57-64.

Dai, X., Hu, C., Zhang, D. & Chen, Y. 2017. A new method for the simultaneous enhancement of methane yield and reduction of hydrogen sulfide production in the anaerobic digestion of waste activated sludge. *Bioresource Technology,* 243:914-921.

Dahunsi, O.S., Shoyombo, A. & Fagbiele, O. 2019. “Biofuel development in Sub-Saharan Africa" in Banu, R. (ed) Anaerobic Digestion. Intech Open, pp. 1-14

Dhariwal, A., Chong, J. Habib, S., King, I.L., Agellon, L.B. & Xia, J. 2017. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Research,* 45:W180-W188.

Díaz, A.I., Oulego, P., Collado, S., Laca, A., González, J.M. & Díaz, M. 2018. Impact of anaerobic digestion and centrifugation/decanting processes in bacterial communities fractions. *Journal of Bioscience and Bioengineering,* 126(6):742-749.

Do, Y.S., Schmidt, T.M., Zahn, J.A., Boyd, E.S., de la Mora, A. & DiSpirito, A.A. 2003. Role of Rhodobacter sp. Strain PS9, a Purple Non-Sulfur Photosynthetic Bacterium Isolated from an Anaerobic Swine Waste Lagoon, in Odor Remediation. *Applied and Environmental Microbiology,* 69(3):1710-1720.

Duan, N., Zhang, D., Lin, C., Zhang, Y., Zhao, L., Lui, H. & Liu, Z. 2019. Effect of organic loading rate on anaerobic digestion of pig manure: Methane production, mass flow, reactor scale and heating scenarios. *Journal of Environmental Management,* 231:646-652.

Edwards, W., Leukes, W.D., Bezuidenhout, C.C., Riedel, K.J., Linkov, V.M., Jansen van Rensburg, P.J., Neomagus, H.W.J.P. & Burgess, J. 2017. Dual-stage ceramic membrane bioreactors for the treatment of high-strength industrial wastewaters. *Water Research Commission*. WRC Report No 1371/1/07: 1-94.

Euzéby, J.P. 1997. List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet. *International Journal of Systematic and Evolutionary Microbiology,* 47 (2):590-592.

Ezebuiro, N.C. & Körner, I. 2017. Characterisation of Anaerobic Digestion Substrates Regarding Trace Elements and Determination of the Influence of Trace Elements on the Hydrolysis and Acidification Phases During the Methanisation of a Maize Silage-based Feedstock. *Journal of Environmental Chemical Engineering,* 5(1):341-351.

Feng, S., Hou, S., Huang, X., Fang, Z., Tong, Y. & Yang, H. 2019. Insights into the microbial community structure of anaerobic digestion of municipal solid waste landfill leachate for methane production by adaptive thermophilic granular sludge. *Electronic Journal of Biotechnology,* 39:98-106.

Fitamo, T., Treu, L., Boldrin, A., Sartori, C., Angelidaki, I. & Scheutz, C. 2017. Microbial population dynamics in urban organic waste anaerobic co-digestion with mixed sludge during a change in feedstock composition and different hydraulic retention times. *Water Research,* 118:261-271.

Food and Agriculture Organization of the United Nations (2018) Livestock Manure. Available at: http://www.fao.org/faostat/en/#data/EMN. (Accessed: 29 August 2018).

Fu, S.F., Wang, F., Shi, X.S. & Guo, R.B. 2016. Impacts of microaeration on the anaerobic digestion of corn straw and the microbial community structure. *Chemical Engineering Journal,* 287:523-528.

Ghosh, S, Conrad, J.R. & Klass, D.L. 1975. Anaerobic Acidogenesis of Wastewater Sludge. *Journal of Water Pollution Control Federation,* 47(1):30-45.

Girotto, F., Alibardi, L. & Cossu, R. 2015. Food Waste Generation and Industrial Uses: A Review. *Waste Management,* 45:32-41.

Grohmann, A., Fehrmann, S., Vainshtein, Y., Haag, N.L., Wiese, F., Stevens, P., Naegele, H.J., Oechsner, H., Hartsch, T. & Sohn, K. 2018. Microbiome dynamics and adaptation of expression signatures during methane production failure and process recovery. *Bioresource Technology,* 247:347-356.

Grunditz, C. & Dalhammar, G. 2000. Development of nitrification inhibition assays using pure cultures of Nitrosomonas and Nitrobacter. *Water Research,* 35 (2):433-440.

Guo, Q., Majeed, S., Xu, R., Zhang, K., Kakade, A., Hafeez, F.Y. Mao, C., Liu, P. & Li, X. 2019. Heavy metals interact with the microbial community and affect biogas production in anaerobic digestion: A review. *Journal of Environmental Management,* 240:266-272.

Guerrini, C.J., Botkin, J.R. & McGuire, A.L. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology,* 37:848-857.

Gunes, B., Stokes, J., Davis, P., Connolly, C. & Lawler, J. 2019. Pre-treatments to enhance biogas yield and quality from anaerobic digestion of whiskey distillery and brewery wastes: A review. *Renewable and Sustainable Energy Reviews,* 113:109281.

Han, I., Congeevaram, S., Ki, D.W., Oh, B.T. & Park, J. 2011. Bacterial community analysis of swine manure treated with autothermal thermophilic aerobic digestion. *Applied Microbiology and Biotechnology,* 89(3):835-842.

Harding, G., Courtney, C. & Russo, V. 2017. When geography matters. A location-adjusted blue water footprint of commercial beef in South Africa. *Journal of Cleaner Production,* 151:494-508.

Hebert, P.D.N., Cywinska, A., Ball, S.L. & deWaard, J.R. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society B,* 270(1512):313-312.

Hu, Y., Wu, J., Li, H., Poncin, S., Wang, K. & Zuo, J. 2019. Study of an enhanced dry anaerobic digestion of swine manure: Performance and microbial community property. *Bioresource Technology,* 282:353-360.

Huang, H, Yuan, X., Li, B. & Xiao, Y. 2014. Thermochemical Liquefaction Characteristics of Sewage Sludge in Different Organic Solvents. *Journal of Analytical and Applied Pyrolysis,* 109:176-184.

Iacumin, L., Cecchini, F., Manzano, M., Osualdini, M., Boscolo, D., Orlic, S. & Comi, G. 2009. Description of the microflora of sourdoughs by culture-dependent and culture-independent methods. *Food Microbiology,* 2:128-135.

Innes, R. 2013. Economics of Agricultural Residuals and Overfertilizer: Chemical Fertilizer Use, Livestock Waste, Manure Management, and Environmental Impacts. *Encyclopedia of Energy, Natural Resource, and Environmental Economics,* 2:50-57.

Jaja, I. F., Mushonga, B., Green, E. & Muchenje, V. 2018. Factors responsible for the post-slaughter loss of carcass and offal's in abattoirs in South Africa. *Acta Tropica,* 178:303-310.

Jany, J.L. & Barbier, G. 2008. Culture-independent methods for identifying microbial communities in cheese. *Food Microbiology,* 25(7):893-848.

Jiang, Y., Banks, C., Zhang, Y., Heaven, S. & Longhurst, P. 2018. Quantifying the percentage of methane formation via acetoclastic and syntrophic acetate oxidation pathways in anaerobic digesters. *Waste Management,* 17:749-756.

Jingura, R.M. & Kamusoko, R. 2017. Methods for determination of biomethane potential of feedstocks: a review. *Biofuel Research Journal,* 14: 573-586.

Jo, Y., Kim, J., Hwang, K. & Lee, C. 2018. A comparative study of single- and two-phase anaerobic digestion of food waste under uncontrolled pH conditions. *Waste Management,* 78:509-520.

Kampmann, K., Ratering, S., Geißler-Plaum, R., Schmidt, M., Zerr, W. & Schnell, S. 2014. Changes of the microbial population structure in an overloaded fed-batch biogas reactor digesting maize silage. *Bioresource Technology,* 174:108-117.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. & Glöckner, F.O. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research,* 41(1).

Latifi, P., Karrabi, M. & Danesh, S. 2017. Anaerobic co-digestion of poultry slaughterhouse wastes with sewage sludge in batch-mode bioreactors (effect of inoculum-substrate ratio and total solids). *Renewable and Sustainable Energy Reviews,* 107:288-296.

Lee, I.S., Parameswaran, P. & Rittmann, B.E. 2011. Effects of solids retention time on methanogenesis in anaerobic digestion of thickened mixed sludge. *Bioresource Technology,* 102:10266-10272.

Lee, S.H., Park, J.H., Kim, S.H., Yu, B.J., Yoon, J.J. & Park, H.D. 2015. Evidence of syntrophic acetate oxidation by *Spirochaetes* during anaerobic digestion. *Bioresource Technology,* 190:543-549.

Leng, L., Yang, P., Singh, S., Zhuang, H., Xu, L., Chen, W.H., Dolfing, J., Li, D., Zhang, Y., Zeng, H., Chu, W. & Lee, P.H. 2018. A review on the bioenergetics of anaerobic microbial metabolism close to the thermodynamic limits and its implications for digestion applications. *Bioresource Technology,* 247:1095-1106.

Li, C., Strömberg, S., Lui, G., Nges, I.A. & Liu, J. 2017. Assessment of Regional Biomass as Co-substrate in the Anaerobic Digestion of Chicken Manure: Impact of Co-digestion with

Chicken Processing Waste, Seagrass and Miscanthus. *Biochemical Engineering Journal,* 118:1-10.

Li, Y., Zhang, Y., Liu, Y., Zhoa, Z., Lui, S., Zhoa, H. & Quan, X. 2016. Enhancement of Anaerobic Methanogenesis at a Short Hydraulic Retention Time via Bioelectrochemical Enrichment of Hydrogenotrophic Methanogens. *Bioresource Technology,* 218:505-511.

Liang, Y., Xu, L., Bao, J., Firmin, K.A. & Zong, W. 2020. Attapulgite enhances methane production from anaerobic digestion of pig slurry by changing enzyme activities and microbial community. *Renewable Energy,* 145:222-232.

Lindemayer, H., Propstmeier, G. & Preißlinger, W. 2009: Grundsätze der Schweinefütterung. LfL Tierernährung, Bayerische Landesanstalt für Landwirtschaft, Freising-Weihenstephan.

Liu, Y., Wachemo, A.C., Yuan, H. & Li, X. 2019. Anaerobic digestion performance and microbial community structure of corn stover in three-stage continuously stirred tank reactors. *Bioresource Technology,* 287:1-10.

Löffler, F.E., Yan, J., Ritalahti, K.M., Adrian, L., Edwards, E.A., Konstantinidis, K.T., Müller, J.A., Fullerton, H., Zinder, S.H. & Spormann, A.M. 2012. *Dehalococcoides mccartyi* gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation, belong to a novel bacterial class, *Dehalococcoidia classis* nov., order Dehalococcoidales ord. nov. and family Dehalococcoidaceae fam. nov., within the phylum Chloroflexi. *International Journal of Systematic and Evolutionary Microbiology,* 63(2):625-635.

Lui, H., Shi, J., Zhan, Z., Zhang, L., Fu, B. & Lui, H. 2017. Selective Acetate Production with CO_2 Sequestration Through Acetogen-enriched Sludge Inoculums in Anaerobic Digestion. *Biochemical Engineering Journal,* 121:163-170.

Luo, G. & Angelidaki, I. 2014. Analysis of bacterial communities and bacterial pathogens in a biogas plant by the combination of ethidium monoazide, PCR and Ion Torrent sequencing. *Water Research,* 60:56-163.

Lutge, B. & Standish, B. 2013. Assessing the Potential for Electricity Generation from Animal Waste Biogas on South African Farms. *Agrekon,* 52(2):1-24.

Lv, Z., Chen, Z., Chen, X., Liang, J., Jiang, J. & Loake, G.J. 2019. Effects of various feedstocks on isotope fractionation of biogas and microbial community structure during anaerobic digestion. *Waste Management,* 84:211-219.

Ma, J., Pan, J., Qiu, L., Wang, Q. & Zhang, Z. 2019. Biochar triggering multipath methanogenesis and subdued propionic acid accumulation during semi-continuous anaerobic digestion. *Bioresource Technology,* 293:122-126.

Mancinelli, R.L. 1996. The nature of nitrogen: an overview. *Life Support & Biosphere Science: International Journal of Earth Space,* 3(1-2):17-24.

Mao, C., Feng, Y., Wang, X. & Ren, C. 2015. Review on Research Achievements of Biogas from Anaerobic Digestion. *Renewable and Sustainable Energy Reviews,* 45:540-555.

Mao, C., Wang, Y., Wang, X., Ren, G., Yuan, L. & Feng, Y. 2019. Correlations between microbial community and C:N:P stoichiometry during the anaerobic digestion process. *Energy,* 174:687-695.

Maspolim, Y., Zhou, Y., Guo, C., Xiao, K. & Ng, W.J. 2015. Comparison of single-stage and two-phase anaerobic sludge digestion systems – Performance and microbial community dynamics. *Chemosphere,* 140:54-62.

Mathole, M.A., Muchadeyi, F.C., Mdladla, K., Malatji, D.P., Dzomba, E.F. & Madoroba, E. 2017. Presence, distribution, serotypes and antimicrobial resistance profiles of Salmonella among pigs, chickens and goats in South Africa. *Food Control,* 72:219-224.

McDonald, T., Achari, G. & Aboila, A. 2008. Feasibility of Increased Biogas Production from the Co-digestion of Agricultural, Municipal, and Agro-industrial Wastes in Rural Communities. *Journal of Environmental Engineering and Science,* 7(4):263-273.

Meng, X., Yuan, X., Ren, J., Wang, X., Zhu, W. & Cui, Z. 2017. Methane production and characteristics of the microbial community in a two-stage fixed-bed anaerobic reactor using molasses. *Bioresource Technology,* 241:1050-1059.

Murto, M., Björnsson, L. & Mattiason, B. 2004. Impact of Food Industrial Waste on Anaerobic Co-digestion of Sewage Sludge and Pig Manure. *Journal of Environmental Management,* 70(2):101-107.

Mutungwasi, A., Mukumba, P. & Makaka, G. 2018. Biogas digester types installed in South Africa: A review. *Renewable and Sustainable Energy Reviews,* 81:172-180.

Muyzer, G. & Stams, A.J. 2008. The ecology and biotechnology of sulphate-reducing bacteria. *Nature Reviews Microbiology,* 6:441-454.

Nagarajan, D., Lee, D. & Chang, J. 2019. Integration of anaerobic digestion and microalgal cultivation for digestate bioremediation and biogas upgrading. *Bioresource Technology,* 290.

Nagy, G. & Wopera, A. 2012. Biogas production from pig slurry – feasibility and challenges. *Materials Science and Engineering,* 37/2:65-75.

Nahman, A., De Lange, W., Oelofse, S. & Godfrey, L. 2012. The Cost of Household Food Waste in South Africa. *Waste Management,* 32(11):2147-2153.

Nakasaki, K., Nguyen, K.K., Ballesteros Jr, F.C., Maekawa, T. & Koyama, M. 2019. Characterizing the microbial community involved in anaerobic digestion of lipid-rich wastewater to produce methane gas. *Anaerobe,* 1-7.

Ngwabie, N.M., Chungong, B.N. & Yengong, F.L. 2018. Characterisation of pig manure for methane emission modelling in Sub-Saharan Africa. *Biosystems Engineering,* 170:31-38.

Niu, Q., Qiao, W., Qiang, H. & Li, Y.Y. 2013. Microbial community shifts and biogas conversion computation during steady, inhibited and recovered stages of thermophilic methane fermentation on chicken manure with a wide variation of ammonia. *Bioresource Technology,* 146:223-233.

Okonkwo, U.C., Onokpite, E. & Onokwai, A.O. 2018. Comparative study of the optimal ratio of biogas production from various organic wastes and weeds for digester/restarted digester. *Journal of King Saud University – Engineering Sciences,* 30(2):123-129.

Okudoh, V., Trois, C., Workneh, T. and Schmidt, S. 2014. The potential of cassava biomass and applicable technologies for sustainable biogas production in South Africa: A review. *Renewable and Sustainable Energy Reviews,* 39:1035-1052.

Pan, J., Ma, J., Zhai, L. & Liu, H. 2019. Enhanced methane production and syntrophic connection between microorganisms during semi-continuous anaerobic digestion of chicken manure by adding biochar. *Journal of Cleaner Production,* 240:118-178

Panigrahi, S. & Dubey, B.K. 2019. A critical review on operating parameters and strategies to improve the biogas yield from anaerobic digestion of organic fraction of municipal solid waste. *Renewable Energy,* 143:779-797.

Paolini, V., Petracchini, F., Segreto, M., Tomassetti, L., Naja, N. and Cecinato, A. 2018. Environmental impact of biogas: A short review of current knowledge. *Journal of Environmental Science and Health, Part A,* 53(10):899-906.

Parks, D.H., Tyson, G.W., Hugenholtz, P. & Beiko, R.G. 2017. STAMP: Statistical analysis of taxonomic and functional profiles. *Bioinformatics,* 30(21):3123-3124.

Pei, J., Yao, H., Wang, H., Ren, J. & Yu, X. 2016. Comparison of Ozone and Thermal Hydrolysis Combined with Anaerobic Digestion for Municipal and Pharmaceutical Waste Sludge with Tetracycline Resistance Genes. *Water Research,* 99:122-128.

Pellera, F. & Gidarakos, E. 2017. Anaerobic digestion of solid agroindustrial waste in semi-continuous mode: Evaluation of mono-digestion and co-digestion systems. *Waste Management,* 68:103-119.

Peu, P., Sassi, J.F., Girault, R., Picard, S., Saint-Cast, P., Béline, F. & Dabert, P. 2011. Sulphur fate and anaerobic biodegradation potential during co-digestion of seaweed biomass (*Ulva* sp.) with pig slurry. *Bioresource Technology,* 102:10794-10802.

Pezzolla, D., Marconi, G., Turchetti, B., Zadra, C., Agnelli, A., Veronesi, F., Onofri, A., Benucci, G.M.N., Buzzini, P., Albertini, E. & Gigliotti, G. 2015. Influence of exogenous organic matter on prokaryotic and eukaryotic microbiota in an agricultural soil. A multidisciplinary approach. *Soil Biology & Biochemistry,* 82:9-20.

Poirier, S., Bize, A., Bureau, C., Bouchez, T. & Chapleur, O. 2016. Community shifts within anaerobic digestion microbiota facing phenol inhibition: Towards early warning microbial indicators? *Water Research,* 100:296-305.

Pramanik, S. K., Suja, F.B., Zain, S.M. & Pramanik, B.K. 2019. The anaerobic digestion process of biogas production from food waste: Prospects and constraints. *Bioresource Technology Reports,* 8.

Rapatsa, M.M. & Moyo, N.A.G. 2013. Performance Evaluation of Chicken, Cow and Pig Manure in the Production of Natural Fish Food in Aquadams Stocked with *Oreochromis mossambicus*. *Physics and Chemistry of the Earth,* 66:68-74.

Repinc, S.K., Sket, R., Zavec, D., Mikus, K.V., Fermoso, F.G. & Stres, B. 2018. Full-scale Agricultural Biogas Plant Metal Content and Process Parameters in Relation to Bacterial and Archaeal Microbial Communities over 2.5 Year Span. *Journal of Environmental Management,* 213:566-574.

Roman, H.J., Burgess, J.E. & Pletschke, B.I. 2006. Enzyme treatment to decrease solids and improve digestion of primary sewage sludge. *African Journal of Biotechnology,* 5(10):963-967.

Roopnarain, A. & Adeleke, R. 2017. Current status, hurdles and future prospects of biogas digestion technology in Africa. *Renewable and Sustainable Energy Reviews,* 67:1162-1179.

Ros, M., Filho, J.D.O, Murcia, M.D.P., Bustamante, M.A., Moral, R., Coll, M.D., Santisima-Trinidad, A.B.L. & Pascual, J.A. 2017. Mesophilic anaerobic digestion of pig slurry and fruit and vegetable waste: Dissection of the microbial community structure. *Journal of Cleaner Production,* 156:757-765.

Ruiz-Sánchez, J., Campanaro, S., Guivernau, M., Fernández, B. & Prenafeta-Boldú, F.X. 2018. Effect of ammonia on the active microbiome and metagenome from stable full-scale digesters. *Bioresource Technology,* 250:513-522.

Ruiz-Sánchez, J., Guivernau, M., Fernández, B., Vila, J., Viñas, M., Riau, V. & Prenafeta-Boldú, F.X. 2019. Functional biodiversity and plasticity of methanogenic biomass from a full-scale mesophilic anaerobic digester treating nitrogen-rich agricultural wastes. *Science of the Total Environment,* 649:760-769.

Russo, V. & von Blottnitz, H. 2017. Potentialities of biogas installation in South African meat value chain for environmental impacts reduction. *Journal of Cleaner Production,* 153:465-473.

Scaglia, B., D'Imporzano, G., Garuti, G., Negri, M. & Adani, A. 2014. Sanitation Ability of Anaerobic Digestion Performed at Different temperature on sewage sludge. *Science of the Total Environment,* 466-467:888-897.

Schulze, E.D. & Mooney, H.A. 1993. Biodiversity and ecosystem function. *Springer-Verlag,* 88-90.

Serrana, J.M., Miyake, Y., Gamboa, M. & Watanabe, K. 2019. Comparison of DNA metabarcoding and morphological identification for stream macroinvertebrate biodiversity assessment and monitoring. *Ecological Indicators,* 101:963-972.

Shahi, S., Vahed, S.Z., Fathi, N. & Sharifi, S. 2018. Polymerase chain reaction (PCR)-based methods: Promising molecular tools in dentistry. *International Journal of Biological Macromolecules,* 171:983-992.

Shane, A., Gheewala, S.H. & Kafwembe, Y. 2017. Urban Commercial Biogas Power Plant Model for Zambian Towns. *Renewable Energy,* 103:1-14.

Shin, S. R., Im, S., Mostafa, A., Lee, M.K., Yun, Y.M., Oh, S.E. & Kim, D.H. 2019. Effects of pig slurry acidification on methane emissions during storage and subsequent biogas production. *Water Research,* 152:234-240.

Solé-Bundó, M., Passos, F., Romero-Güiza, M.S., Ferrer, I. & Astals, S. 2019. Co-digestion strategies to enhance microalgae anaerobic digestion: A review. *Renewable and Sustainable Energy Reviews,* 112:471-482.

Sommer, S. G., Christensen, M. L., Schmidt, T. & Jensen, L. S. (eds.) (2013): Animal manure recycling. Treatment and management. Chichester: John Wiley & Sons Ltd.

Sosnowski, P., Wieczorek, A. & Ledakowicz, S. 2003. Anaerobic Co-digestion of Sewage Sludge and Organic Fraction of Municipal Solid Wastes. *Advances in Environmental Research,* 7(3):609- 616.

Stefani, F. O., Bell, T.H., Marchand, C., de la Providencia, I.E., Yassimi, A.E., St-Arnaud, M. & Hijri, M. 2015. Culture-Dependent and -Independent Methods Capture Different Microbial Community Fractions in Hydrocarbon-Contaminated Soils. *PLoS One*.

Su, C., Lei, L., Duan, Y., Zhang, K.Q. & Yang, J. 2012. Culture-independent methods for studying environmental microorganisms: methods, application, and perspective. *Appl Microbiol Biotechnol,* 93(3):993-1003.

Surroop, D., Bundhoo, Z.M.A. & Raghoo, P. 2019. Waste to energy through biogas to improve energy security and to transform Africa’s energy landscape. *Current Opinion in Green and Sustainable Chemistry,* 18:79-83.

Tao, Y., Ersahin, M.E., Ghasimi, D.S.M., Ozgun, H., Wang, H., Zhang, X., Guo, M., Yang, Y., Stuckey, D.C. & Van Lier, J.B. 2020. Biogas productivity of anaerobic digestion process is governed by a core bacterial microbiota. *Chemical Engineering Journal,* 380.

Theuerl, S., Klang, J., Heiermann, M. & De Vrieze, J. 2018. Marker microbiome clusters are determined by operational parameters and specific key taxa combinations in anaerobic digestion. *Bioresource Technology,* 263:128-135

Thudi, M., Li, Y., Jackson, S.A., May, G.D. & Varshney, R.K. 2012. Current state-of-art of sequencing technologies for plant genomics research. *Briefings in Functional Genomics,* 11(1):3-11.

Traversi, D., Romanazzi, V., Degan, R., Lorenzi, E., Carraro, E. & Gilli, G. 2015. Microbial-chemical indicator for anaerobic digester performance assessment in full-scale wastewater treatment plants for biogas production. *Bioresource Technology,* 186:179-191.

Trois, C. & Simelane, O.T. 2010. Implementing Separate Waste Collection and Mechanical Biological Waste Treatment in South Africa: A Comparison with Austria and England. *Waste Management,* 30(8-9):1457-1463.

Umweltbundesamt (ed.) (2018a): Umwelt und Landwirtschaft 2018. Umweltbundesamt, Dessau-Roßlau.

Umweltbundesamt (2018b): Umweltbelastungen der Landwirtschaft. Available at: https://www.umweltbundesamt.de/themen/boden-landwirtschaft/umweltbelastungen-der-landwirtschaft (Accessed: 29 August 2018).

Umweltbundesamt (ed.) (2014): Reaktiver Stickstoff in Deutschland. Ursachen, Wirkungen, Maßnahmen. Fachgebiet II 4.3 – Luftreinhaltung und terrestrische Ökosysteme, Umweltbundesamt, Dessau-Roßlau.

Umweltbundesamt (2017): Stickstoff. Available at: https://www.umweltbundesamt.de/themen/boden-landwirtschaft/ umweltbelastungen-der-landwirtschaft/stickstoff#textpart-1 (Accessed: 29 August 2018).

Van Lier, J. B., Tilche, E., Ahring, B.K., Macarie, H., Moletta, R. & Dohanyos, M.2001. New Perspectives in Anaerobic Digestion. *Water Science and Technology,* 43(1):1-18.

Vasquez, J. & Nakasaki, K. 2016. Effects of shock loading versus stepwise acclimation on microbial consortia during the anaerobic digestion of glycerol. *Biomass and Bioenergy,* 86:129-135.

VDI (Verein Deutscher Ingenieure). 2006. Fermentation of Organic Materials: Characterisation of the Substrate, Sampling, Collection of Material Data, Fermentation Tests. Berlin: *Beuth Verlag,* 4630:1-92.

Wachemo, A.C., Tong, H., Yuan, H., Zou, X., Korai, R.M. & Li, Z. 2019. Continuous dynamics in anaerobic reactor during bioconversion of rice straw: Rate of substance utilization, biomethane production and changes in microbial community structure. *Science of the Total Environment,* 687:1274-1284.

Wang, M., Zhou, J., Yuan, Y., Dai, Y., Li, D., Li, Z. Liu, X. & Yan, Z. 2017. Methane production characteristics and microbial community dynamics of mono-digestion and co-digestion using corn stalk and pig manure. *International Journal of Hydrogen Energy,* 42(8):4893-4901.

Wu (a), L.J., Higashimori, A., Qin, Y., Hojo, T., Kubota, K. & Li, Y.Y. 2016. Upgrading of mesophilic anaerobic digestion of waste activated sludge by thermophilic pre-fermentation and recycle: Process performance and microbial community analysis. *Fuel,* 169:7-14.

Wu, L.J., Higashimori, A., Qin, Y., Hojo, T., Kubota, K. & Li, Y.Y. 2016. Comparison of hyper-thermophilic–mesophilic two-stage with single-stage mesophilic anaerobic digestion of waste activated sludge: Process performance and microbial community analysis. *Chemical Engineering Journal,* 290:290-301.

Yang, J., Wang, D., Luo, Z., Zeng, W. & Huang, H. 2020. The role of reflux time in a leach bed reactor coupled with a methanogenic reactor for anaerobic digestion of pig manure: Reactor performance and microbial community. *Journal of Cleaner Production,* 242:118367.

Yang, L., Zhang, Y., Xu, Z., Quan, X. & Chen, S. 2015. Enhancement of Sludge Granulation in Anaerobic Acetogenesis by Addition of Nitrate and Microbial Community Analysis. *Biochemical Engineering Journal,* 95:104-111.

Yang, S., Phan, H.V., Bustamante, H. Gou, W., Ngo, H.H. & Nghiem, L.D. 2017. Effects of Shearing on Biogas Production and Microbial Community Structure during Anaerobic Digestion with Recuperative Thickening. *Bioresource Technology,* 234:439-447.

Yangin-Gomec, C., Pekyavas, G., Sapmaz, T., Aydin, S., Ince, B. & Akyol, C. 2017. Microbial monitoring of ammonia removal in a UASB reactor treating pre-digested chicken manure with anaerobic granular inoculum. *Bioresource Technology,* 241:332-339.

Zahedi, S., Solera, R., Micolucci, F., Cavinato, C. & Bolzonella, D. 2016. Changes in Microbial Community during Hydrogen and Methane Production in Two-stage Thermophilic Anaerobic Co-digestion Process from Biowaste. *Waste Management,* 49:40-46.

Zhang, L., Loh, K.C., Lim, J.W. & Zhang, J. 2019a. Bioinformatics analysis of metagenomics data of biogas-producing microbial communities in anaerobic digesters: A review. *Renewable and Sustainable Energy Reviews,* 100:110-126.

Zhang, J., Luo, W., Wang, Y., Li, G., Liu, Y. & Gong, X. 2019. Anaerobic cultivation of waste activated sludge to inoculate solid state anaerobic co-digestion of agricultural wastes: Effects of different cultivated periods. *Bioresource Technology,* 294:122-178.

Zhi, S. & Zhang, K. 2019. Antibiotic residues may stimulate or suppress methane yield and microbial activity during high-solid anaerobic digestion. *Chemical Engineering Journal,* 359:1303-1315.

APPENDIX A: Nextera XT Index Kit

Sample Id	Description		Reactor stage	Sample well	I7 index Id	Index	I5 index Id	Index2
PS6A	Pig_Seed	1	END	4C	N704	TCCTGAGC	S503	TATCCTCT
PS6B	Pig_Seed	2	END	4D	N704	TCCTGAGC	S504	AGAGTAGA
PS5A	Pig_Seed	3	END	4E	N704	TCCTGAGC	S505	GTAAGGAG
PS5B	Pig_Seed	4	END	4F	N704	TCCTGAGC	S506	ACTGCATA
S4B	Seed	5	END	4G	N704	TCCTGAGC	S507	AAGGAGTA
S4A	Seed	6	END	4H	N704	TCCTGAGC	S508	CTAAGCCT
S5B	Seed	7	END	5A	N705	GGACTCCT	S517	GCGTAAGA
S5A	Seed	8	END	5B	N705	GGACTCCT	S502	CTCTCTAT
S6A	Seed	9	END	5C	N705	GGACTCCT	S503	TATCCTCT
S6B	Seed	10	END	5D	N705	GGACTCCT	S504	AGAGTAGA
PN6B	Pig_new	11	END	5E	N705	GGACTCCT	S505	GTAAGGAG
PN6A	Pig_new	12	END	5F	N705	GGACTCCT	S506	ACTGCATA
PN5A	Pig_new	13	END	5G	N705	GGACTCCT	S507	AAGGAGTA
PN5B	Pig_new	14	END	5H	N705	GGACTCCT	S508	CTAAGCCT
PN4A	Pig_new	15	END	6A	N706	TAGGCATG	S517	GCGTAAGA
PN4B	Pig_new	16	END	6B	N706	TAGGCATG	S502	CTCTCTAT
P1B	Pig_slurry	17	END	6C	N706	TAGGCATG	S503	TATCCTCT
P1A	Pig_slurry	18	END	6D	N706	TAGGC	S504	AGAGT

						ATG		AGA
P2A	Pig_slurry	19	END	6E	N706	TAGGCATG	S505	GTAAGGAG
P2B	Pig_slurry	20	END	6F	N706	TAGGCATG	S506	ACTGCATA
P3A	Pig_slurry	21	END	6G	N706	TAGGCATG	S507	AAGGAGTA
P3B	Pig_slurry	22	END	6H	N706	TAGGCATG	S508	CTAAGCCT
PSN1B	Pig_new_seed	23	END	7A	N710	CGAGGCTG	S517	GCGTAAGA
PSN1A	Pig_new_seed	24	END	7B	N710	CGAGGCTG	S502	CTCTCTAT
PSN2B	Pig_new_seed	25	END	7C	N710	CGAGGCTG	S503	TATCCTCT
PSN2A	Pig_new_seed	26	END	7D	N710	CGAGGCTG	S504	AGAGTAGA
PSN3A	Pig_new_seed	27	END	7E	N710	CGAGGCTG	S505	GTAAGGAG
PSN3B	Pig_new_seed	28	END	7F	N710	CGAGGCTG	S506	ACTGCATA
MC1A	Control	29	END	7G	N710	CGAGGCTG	S507	AAGGAGTA
MC1B	Control	30	END	7H	N710	CGAGGCTG	S508	CTAAGCCT
MC2B	Control	31	END	8A	N709	GCTACGCT	S517	GCGTAAGA
MC2A	Control	32	END	8B	N709	GCTACGCT	S502	CTCTCTAT
MC3A	Control	33	END	8C	N709	GCTACGCT	S503	TATCCTCT
MC3B	Control	34	END	8D	N709	GCTACGCT	S504	AGAGTAGA
SN3A	Seed_new	35	END	8E	N709	GCTACGCT	S505	GTAAGGAG
SN3B	Seed_new	36	END	8F	N709	GCTACGCT	S506	ACTGCATA
SN2B	Seed_new	37	END	8G	N709	GCTACGCT	S507	AAGGAGTA
SN2A	Seed_new	38	END	8H	N709	GCTAC	S508	CTAAG

						GCT		CCT
SN1B	Seed_new	39	END	9A	N708	CAGAGAGG	S517	GCGTAAGA
SN1A	Seed_new	40	END	9B	N708	CAGAGAGG	S502	CTCTCTAT
MCC1A	Control	41	BEGINING	1A	N701	TAAGGCGA	S517	GCGTAAGA
MCC2B	Control	42	BEGINING	1B	N701	TAAGGCGA	S502	CTCTCTAT
S1A	Seed	43	BEGINING	1C	N701	TAAGGCGA	S503	TATCCTCT
S1B	Seed	44	BEGINING	1D	N701	TAAGGCGA	S504	AGAGTAGA
PS1A	Pig_Seed	45	BEGINING	1E	N701	TAAGGCGA	S505	GTAAGGAG
PS1B	Pig_Seed	46	BEGINING	1F	N701	TAAGGCGA	S506	ACTGCATA
P1A	Pig_slurry	47	BEGINING	1G	N701	TAAGGCGA	S507	AAGGAGTA
P1B	Pig_slurry	48	BEGINING	1H	N701	TAAGGCGA	S508	CTAAGCCT
PSO1	Pig_old_seed	49	MIDDLE	2A	N702	CGTACTAG	S517	GCGTAAGA
PSO2	Pig_old_seed	50	MIDDLE	2B	N702	CGTACTAG	S502	CTCTCTAT
SO1	Seed_old	51	MIDDLE	2C	N702	CGTACTAG	S503	TATCCTCT
SO2	Seed_old	52	MIDDLE	2D	N702	CGTACTAG	S504	AGAGTAGA
SO3	Seed_old	53	MIDDLE	2E	N702	CGTACTAG	S505	GTAAGGAG
PSNM1	Pig_new_seed	54	MIDDLE	2F	N702	CGTACTAG	S506	ACTGCATA
PSNM2	Pig_new_seed	55	MIDDLE	2G	N702	CGTACTAG	S507	AAGGAGTA
PNM1	Pig_new	56	MIDDLE	2H	N702	CGTACTAG	S508	CTAAGCCT
PNM2	Pig_new	57	MIDDLE	3A	N703	AGGCAGAA	S517	GCGTAAGA
PNM3	Pig_new	58	MIDDLE	3B	N703	AGGCA	S502	CTCTC

				GAA		TAT
LB NTC	No Template Control	3C	N703	AGGCAGAA	S503	TATCCTCT
LB NTC1	No Template Control	9C	N708	CAGAGAGG	S503	TATCCTCT
LB NTC2	No Template Control	9D	N708	CAGAGAGG	S504	AGAGTAGA

APPENDIX B: Full eco-analytical results

Methods used by laboratory

ELEMENT	METHOD / MACHINE
Cations (Exchangeable Cations)	Varian SpectrAA 250 Plus
Anions (1:2 Water Extract)	Metrohm 761 Compact IC
Ammonia (1:2 Water Extract)	Radiometer TTT85 Titrator
LECO %N, %C	LECO Truspec CN
LECO %S	LECO Truspec S
Metals: Be, B, Na, Mg, Al, P, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, Pd, Ag, Cd, Sb, Ba, Pt, Au, Hg, Tl, Pb, Bi, Th and U	ICP-MS

	20/7/2018		**24/8/2018**
SAMPLE:	**Pig Slurry (mg/l)**	**Seeding Sludge (mg/l)**	**Pig Slurry New (mg/l)**
BE 9	0.004344	0.0009505	0.000067
B 11	0.1906	1.588	0.5646
NA 23	310.8	753.5	212.7
MG 24	18.23	76.06	29.98
AL 27	ND	12.17	0.1513
P 31	33.38	36.68	10.06
K 39	989.4	4247	570.5
CA 43	84.98	77.45	28.88
TI 47	0.0671	0.4222	0.02597
V 51	0.01425	0.1396	0.0003925
CR 53	ND	0.06745	ND
MN 55	0.09312	3.785	0.08243
FE 57	0.32	62.86	0.5139
CO 59	0.01587	0.132	0.007826
NI 60	0.08643	0.1856	0.04681
CU 63	0.6331	0.2566	0.508

ZN 66	0.7083	11.48	0.3738
AS 75	0.03199	0.04474	0.0001038
SE 82	0.07947	0.04864	ND
RB 85	0.6676	3.169	0.4285
SR 88	0.2431	0.5559	0.159
MO 95	0.007887	0.09155	0.0035
PD 105	0.00785	0.003583	0.001189
AG 107	0.08522	0.05993	0.00258
CD 111	0.005012	0.001542	0.0006569
SB 121	0.005108	0.004334	0.001863
BA 137	0.0888	0.4458	0.03519
PT 195	0.0000078	ND	ND
AU 197	0.01357	0.001715	ND
HG 202	0.02917	0.008466	0.0191
TL 205	0.005495	0.0003002	ND
PB 208	0.01346	0.01625	0.02378
BI 209	0.004193	0.0006274	0.000274
TH 232	0.004614	0.0106	0.001549
U 238	0.003996	0.004533	0.0005609

	Pig Slurry	**Seeding Sludge**	**Pig Slurry New**
PO_4	153.52	10.55	330.21
SO_4	10.93	21.14	56.34
NO_3	23.09	44.09	0.49
NH_4	1226.32	1021.65	1580.43
CL	359.74	3120.98	439.12

	Pig Slurry	**Seeding Sludge**	**Pig Slurry New**
LECO %N	3.1693	2.4609	5.0553
LECO %C	33.608	38.217	46.863
LECO %S	0.81544	0.78202	0.77703

APPENDIX C: Standard deviations of biogas yields

Seeding sludge

Day	S_START_ Cumulative	Standard Deviation (SD)	SB_Cumulative	Standard Deviation (SD)	S_Cumulative	Standard Deviation (SD)
0	23.6667	3.8944	5.6667	2.9439	16.0000	8.6023
1	77.0000	18.4255	9.0000	6.4807	49.3333	24.1799
2	113.6667	24.7016	14.0000	11.7686	79.3333	27.2886
3	144.0000	29.6057	14.0000	11.7686	97.3333	38.5054
4	164.0000	31.6781	20.3333	19.4722	114.0000	48.6415
5	168.6667	30.6132	22.3333	21.9127	120.6667	53.3916
6	183.3333	31.4192	22.3333	21.9127	127.3333	56.8741
7	193.3333	30.2104	22.3333	21.9127	134.6667	62.1986
8	206.6667	30.0694	56.6667	38.5379	144.6667	68.6780
9	222.3333	34.3099	60.6667	40.3134	153.3333	73.1619
10	243.3333	37.6917	67.3333	44.9017	168.0000	79.4858
11	255.6667	39.4229	73.3333	48.0850	178.6667	81.0720
12	267.3333	38.6286	79.3333	52.3323	187.3333	79.3767
13	269.3333	37.6917	84.3333	53.1099	187.3333	79.3767
14	276.3333	46.1646	86.3333	53.4992	194.6667	77.0108
15			88.6667	53.6299	200.6667	75.9781
16			90.3333	53.8625	228.6667	78.4517
17			91.0000	53.9768	236.0000	85.2174
18			92.3333	54.4855	236.0000	85.2174
19			94.3333	55.1649	263.3333	92.6319
20			102.6667	58.0359	273.3333	95.7636
21			113.3333	63.9153	275.3333	97.6968
22			113.3333	63.9153	275.3333	97.6968
24			113.6667	64.1301	356.0000	104.2785
25			113.6667	64.1301	366.0000	107.0794
26			115.3333	65.2316	386.3333	108.9732
27			116.0000	65.6849	414.0000	108.3351
28			116.3333	65.9141	444.0000	108.5426

29			118.0000	67.0858	473.6667	113.8954
30					493.0000	108.9289
31					510.3333	110.7302
32					510.3333	110.7302
33					513.0000	112.8650
34					516.3333	114.3117
35					522.3333	117.7971
36					539.6667	118.4912
38					563.6667	117.1758
39					568.3333	116.6498
40					573.6667	115.6640
41					579.0000	121.4558
42					582.6667	121.0317
43					593.3333	121.7566
44					595.3333	123.9382
45					600.0000	127.4912

Pig slurry

Day	P_START_Cumulative	SD	PB_Cumulative	SD	P_Cumulative	SD
0	150.0000	18.7083	153.3333	21.6025	127.0000	24.0416
1	370.6667	14.8549	372.0000	27.3130	315.0000	35.3553
2	500.0000	7.0711	454.0000	27.2764	420.0000	42.4264
3	539.3333	31.6333	493.3333	26.7706	474.0000	22.6274
4	644.0000	29.2233	534.6667	15.8955	575.0000	21.2132
5	738.6667	36.3685	538.6667	12.7541	634.0000	8.4853
6	832.6667	39.6316	542.0000	9.2736	721.0000	1.4142
7	935.3333	27.0678	555.3333	17.6824	792.0000	16.9706

8	1064.0000	32.6190	574.6667	27.4348	895.0000	21.2132
9	1178.0000	33.2566	592.0000	21.3542	958.0000	67.8823
10	1327.3333	30.0111	606.6667	21.6025	1070.0000	84.8528
11	1416.6667	28.5774	618.0000	19.6469	1171.0000	100.4092
12	1472.6667	26.4701	636.0000	33.0757	1292.0000	101.8234
13	1493.3333	28.5774	654.6667	40.4063	1361.0000	86.2670
14	1506.6667	31.8852	673.0000	43.5144	1426.0000	65.0538
15			687.6667	48.6638	1459.0000	55.1543
16			687.6667	48.6638	1512.0000	31.1127
17			687.6667	48.6638	1537.0000	24.0416
18			698.3333	55.0379	1546.0000	22.6274
19			698.3333	55.0379	1573.0000	18.3848
20			723.0000	67.5537	1576.0000	22.6274
21			726.3333	64.1106	1576.0000	22.6274
22			772.3333	86.0417	1591.0000	1.4142
24			772.3333	86.0417	1628.0000	25.4558
25			772.3333	86.0417	1635.0000	21.2132
26			772.3333	86.0417	1641.5000	16.2635
27			782.3333	89.4604	1652.0000	2.8284
28			802.3333	94.5154	1657.5000	3.5355
29			805.6667	97.6635	1679.0000	15.5563
30					1688.0000	16.9706
31					1709.0000	29.6985

32					1709.0000	29.6985
33					1709.0000	29.6985
34					1709.5000	28.9914
35					1709.5000	28.9914
36					1739.5000	57.2756
38					1766.5000	47.3762
39					1766.5000	47.3762
40					1766.5000	47.3762
41					1768.5000	44.5477
42					1773.5000	51.6188
43					1780.5000	41.7193
44					1795.5000	62.9325
45					1805.5000	77.0746

Pig slurry and seeding sludge (PS)

Day	PS_START_Cumulative	SD	PSB_Cumulative	SD	PS_Cumulative	SD
0	98.6667	10.8012	89.3333	3.2660	82.0000	5.0990
1	234.0000	79.2591	165.3333	14.1657	259.0000	6.2849
2	328.0000	105.6788	107.3333	9.4163	357.6667	7.4274
3	377.3333	115.1115	58.6667	5.8878	423.0000	8.5732
4	412.0000	116.1981	60.6667	5.3541	461.0000	14.1951
5	434.6667	116.4932	34.0000	6.4807	477.6667	20.9801
6	447.3333	116.6905	14.0000	5.6569	489.0000	26.6177
7	458.0000	115.9051	2.0000	1.4142	500.3333	29.8524
8	472.6667	113.1754	73.3333	17.1075	517.6667	36.6219
9	496.0000	111.4720	8.6667	6.3770	539.6667	37.8968

10	504.0000	115.2128	14.6667	6.5320	550.3333	39.5243
11	511.3333	111.5377	14.6667	6.0139	556.3333	41.9663
12	518.6667	108.2713	18.6667	9.2286	567.0000	42.4323
13	526.0000	104.3360	16.6667	7.7889	567.6667	42.9088
14	540.0000	103.9615	23.3333	3.5590	583.0000	46.3195
15			14.3333	4.1433	591.6667	48.3753
16			7.3333	5.7155	613.0000	46.5242
17			10.6667	4.9666	625.0000	45.2051
18			9.3333	9.0921	627.6667	41.9782
19			7.3333	5.7155	667.6667	38.9893
20			5.3333	4.3205	685.0000	38.7750
21			16.6667	3.5590	699.0000	39.7429
22			4.6667	2.1602	699.0000	39.7429
24			5.3333	0.8165	806.3333	40.8921
25			2.0000	1.4142	822.6667	47.3779
26			0.6667	0.8165	836.6667	53.8393
27			6.3333	2.4833	857.3333	61.6820
28			0.0000	0.0000	877.6667	73.7236
29			0.0000	0.0000	898.6667	83.8640
30					917.6667	91.5023
31					933.0000	90.4765
32					938.3333	88.6604
33					949.6667	91.9384
34					955.0000	93.3381
35					969.0000	95.5406
36					983.0000	95.0368
38					1006.3333	99.4418
39					1007.6667	100.2031
40					1016.3333	103.5793
41					1024.3333	104.9603
42					1029.6667	105.5399
43					1039.6667	110.6737
44					1049.6667	114.7374
45					1052.3333	117.0840

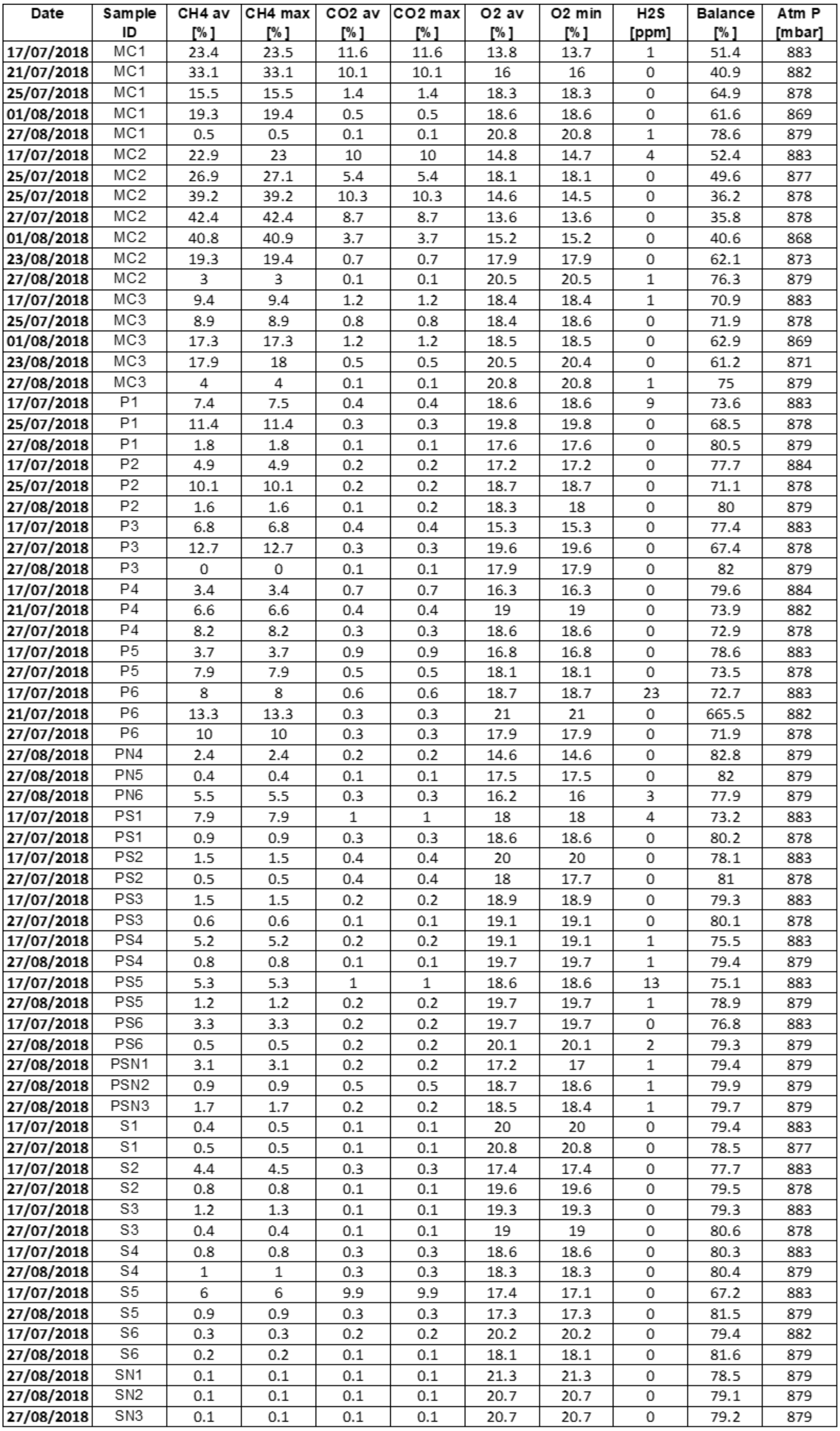
Microcrystalline celluloses

Day	MC_Cumulative	SD
0	13.3333	10.8012
1	80.0000	24.4949
2	190.0000	69.6419
3	553.3333	128.3225
4	876.6667	204.4912
5	1130.0000	327.3377
6	1316.6667	413.6625
7	1423.3333	453.0085
8	1520.0000	508.5764
9	1616.6667	548.3764
10	1676.6667	557.0607
11	1853.3333	564.1070
12	2120.0000	579.9569
13	2310.0000	608.5639
14	2506.6667	623.7922
15	2690.0000	659.4316
16	2976.6667	715.6931
17	3213.3333	764.7658
18	3376.6667	799.2601
19	3546.6667	837.1479
20	3653.3333	833.5866
21	3666.6667	818.5760
22	3690.0000	792.4330
24	3793.3333	747.4735
25	3803.3333	740.4165
26	3826.6667	740.6866
27	3856.6667	729.7031
28	3886.6667	715.5883
29	3913.3333	708.2843
30	3943.3333	701.2251
31	3983.3333	690.3019
32	3993.3333	697.3641

33	4006.6667	704.6394
34	4023.3333	712.1915
35	4046.6667	729.7031
36	4066.6667	729.0519
38	4110.0000	740.1689
39	4123.3333	747.4735
40	4153.3333	775.0591
41	4170.0000	782.5280
42	4176.6667	779.1127
43	4196.6667	790.0738
44	4196.6667	790.0738
45	4210.0000	797.4020

APPENDIX D: Gas production during this study

Table D-1: Gas readings during the bench top study



Date	Sample ID	CH4 av [%]	CH4 max [%]	CO2 av [%]	CO2 max [%]	O2 av [%]	O2 min [%]	H2S [ppm]	Balance [%]	Atm P [mbar]
17/07/2018	MC1	23.4	23.5	11.6	11.6	13.8	13.7	1	51.4	883
21/07/2018	MC1	33.1	33.1	10.1	10.1	16	16	0	40.9	882
25/07/2018	MC1	15.5	15.5	1.4	1.4	18.3	18.3	0	64.9	878
01/08/2018	MC1	19.3	19.4	0.5	0.5	18.6	18.6	0	61.6	869
27/08/2018	MC1	0.5	0.5	0.1	0.1	20.8	20.8	1	78.6	879
17/07/2018	MC2	22.9	23	10	10	14.8	14.7	4	52.4	883
25/07/2018	MC2	26.9	27.1	5.4	5.4	18.1	18.1	0	49.6	877
25/07/2018	MC2	39.2	39.2	10.3	10.3	14.6	14.5	0	36.2	878
27/07/2018	MC2	42.4	42.4	8.7	8.7	13.6	13.6	0	35.8	878
01/08/2018	MC2	40.8	40.9	3.7	3.7	15.2	15.2	0	40.6	868
23/08/2018	MC2	19.3	19.4	0.7	0.7	17.9	17.9	0	62.1	873
27/08/2018	MC2	3	3	0.1	0.1	20.5	20.5	1	76.3	879
17/07/2018	MC3	9.4	9.4	1.2	1.2	18.4	18.4	1	70.9	883
25/07/2018	MC3	8.9	8.9	0.8	0.8	18.4	18.6	0	71.9	878
01/08/2018	MC3	17.3	17.3	1.2	1.2	18.5	18.5	0	62.9	869
23/08/2018	MC3	17.9	18	0.5	0.5	20.5	20.4	0	61.2	871
27/08/2018	MC3	4	4	0.1	0.1	20.8	20.8	1	75	879
17/07/2018	P1	7.4	7.5	0.4	0.4	18.6	18.6	9	73.6	883
25/07/2018	P1	11.4	11.4	0.3	0.3	19.8	19.8	0	68.5	878
27/08/2018	P1	1.8	1.8	0.1	0.1	17.6	17.6	0	80.5	879
17/07/2018	P2	4.9	4.9	0.2	0.2	17.2	17.2	0	77.7	884
25/07/2018	P2	10.1	10.1	0.2	0.2	18.7	18.7	0	71.1	878
27/08/2018	P2	1.6	1.6	0.1	0.2	18.3	18	0	80	879
17/07/2018	P3	6.8	6.8	0.4	0.4	15.3	15.3	0	77.4	883
27/07/2018	P3	12.7	12.7	0.3	0.3	19.6	19.6	0	67.4	878
27/08/2018	P3	0	0	0.1	0.1	17.9	17.9	0	82	879
17/07/2018	P4	3.4	3.4	0.7	0.7	16.3	16.3	0	79.6	884
21/07/2018	P4	6.6	6.6	0.4	0.4	19	19	0	73.9	882
27/07/2018	P4	8.2	8.2	0.3	0.3	18.6	18.6	0	72.9	878
17/07/2018	P5	3.7	3.7	0.9	0.9	16.8	16.8	0	78.6	883
27/07/2018	P5	7.9	7.9	0.5	0.5	18.1	18.1	0	73.5	878
17/07/2018	P6	8	8	0.6	0.6	18.7	18.7	23	72.7	883
21/07/2018	P6	13.3	13.3	0.3	0.3	21	21	0	665.5	882
27/07/2018	P6	10	10	0.3	0.3	17.9	17.9	0	71.9	878
27/08/2018	PN4	2.4	2.4	0.2	0.2	14.6	14.6	0	82.8	879
27/08/2018	PN5	0.4	0.4	0.1	0.1	17.5	17.5	0	82	879
27/08/2018	PN6	5.5	5.5	0.3	0.3	16.2	16	3	77.9	879
17/07/2018	PS1	7.9	7.9	1	1	18	18	4	73.2	883
27/07/2018	PS1	0.9	0.9	0.3	0.3	18.6	18.6	0	80.2	878
17/07/2018	PS2	1.5	1.5	0.4	0.4	20	20	0	78.1	883
27/07/2018	PS2	0.5	0.5	0.4	0.4	18	17.7	0	81	878
17/07/2018	PS3	1.5	1.5	0.2	0.2	18.9	18.9	0	79.3	883
27/07/2018	PS3	0.6	0.6	0.1	0.1	19.1	19.1	0	80.1	878
17/07/2018	PS4	5.2	5.2	0.2	0.2	19.1	19.1	1	75.5	883
27/08/2018	PS4	0.8	0.8	0.1	0.1	19.7	19.7	1	79.4	879
17/07/2018	PS5	5.3	5.3	1	1	18.6	18.6	13	75.1	883
27/08/2018	PS5	1.2	1.2	0.2	0.2	19.7	19.7	1	78.9	879
17/07/2018	PS6	3.3	3.3	0.2	0.2	19.7	19.7	0	76.8	883
27/08/2018	PS6	0.5	0.5	0.2	0.2	20.1	20.1	2	79.3	879
27/08/2018	PSN1	3.1	3.1	0.2	0.2	17.2	17	1	79.4	879
27/08/2018	PSN2	0.9	0.9	0.5	0.5	18.7	18.6	1	79.9	879
27/08/2018	PSN3	1.7	1.7	0.2	0.2	18.5	18.4	1	79.7	879
17/07/2018	S1	0.4	0.5	0.1	0.1	20	20	0	79.4	883
27/07/2018	S1	0.5	0.5	0.1	0.1	20.8	20.8	0	78.5	877
17/07/2018	S2	4.4	4.5	0.3	0.3	17.4	17.4	0	77.7	883
27/07/2018	S2	0.8	0.8	0.1	0.1	19.6	19.6	0	79.5	878
17/07/2018	S3	1.2	1.3	0.1	0.1	19.3	19.3	0	79.3	883
27/07/2018	S3	0.4	0.4	0.1	0.1	19	19	0	80.6	878
17/07/2018	S4	0.8	0.8	0.3	0.3	18.6	18.6	0	80.3	883
27/08/2018	S4	1	1	0.3	0.3	18.3	18.3	0	80.4	879
17/07/2018	S5	6	6	9.9	9.9	17.4	17.1	0	67.2	883
27/08/2018	S5	0.9	0.9	0.3	0.3	17.3	17.3	0	81.5	879
17/07/2018	S6	0.3	0.3	0.2	0.2	20.2	20.2	0	79.4	882
27/08/2018	S6	0.2	0.2	0.1	0.1	18.1	18.1	0	81.6	879
27/08/2018	SN1	0.1	0.1	0.1	0.1	21.3	21.3	0	78.5	879
27/08/2018	SN2	0.1	0.1	0.1	0.1	20.7	20.7	0	79.1	879
27/08/2018	SN3	0.1	0.1	0.1	0.1	20.7	20.7	0	79.2	879

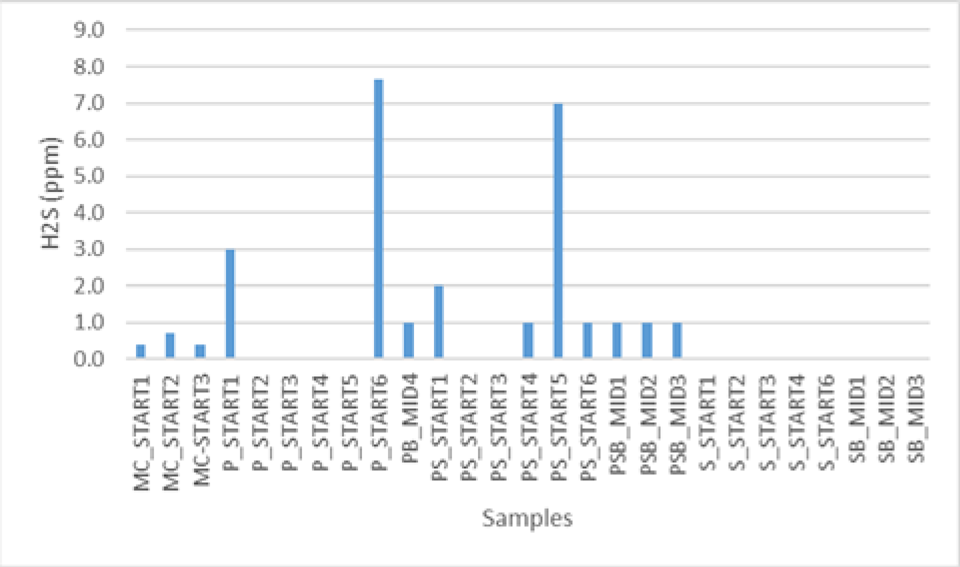


Figure D-1: H_2S production of different samples during setup

APPENDIX E: List of heavy metals that influence AD

Table E: List of heavy metals that influence AD, influencing factors and concentrations (Guo *et al.,* 2019)

Heavy metals	**Influencing factors**	**Biogas yield**		**Methane concentration**	
		Promoting concentration (mg/l)	Inhibitory concentration (mg/l)	Promoting concentration (mg/l)	Inhibitory concentration (mg/l)
Copper	Activity of methanogenesis, activity of cellulase, microbial community and concentration of VFA	0 - 100	500	5	130
Nickel	Activity of cellulase and methanogenesis	0.8 - 50	100	0 - 20	32
Iron	Activity of cellulase	50 - 4000	20 000	0 - 1 000	20 000
Cadmium	Activity of methanogenesis	0.1 – 0.3	1.2	-	1
Zinc	Activity of methanogenesis	5	50	0 - 100	-