The Potential of Water Hyacinth (*Eicchornia crassipes*) from Hartbeespoort dam in Biogas and Soil Ameliorant production, as a Solution to Water Weed Challenges

Report to the
WATER RESEARCH COMMISSION

by

A Roopnarain, R Adeleke, R Makofane, L Obi, M Lubinga

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orders@wrc.org.za or download from www.wrc.org.za

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EXECUTIVE SUMMARY

BACKGROUND

The Hartbeespoort Dam is situated in the North West province of South Africa and is one of the most significant dams in the economic hub of the province and the Crocodile (West) Marico (CWM) Water Management Area (WMA). The dam is primarily utilised for domestic, industrial, agricultural and recreational purposes. The numerous ecosystem services provided by the dam contribute to its economic significance. These services include provisioning (the availability of water for abstraction), supporting services (holiday, commercial and residential) and regulatory (waste assimilation). City dwellers are frequently attracted to the large water body that is situated within a mountainous setting. This has contributed to the increasing importance of the dam as a regional tourist and recreational centre. The socio-economic activities and facilities available at the dam include holiday resorts, conference venues, weekend cottages, golf courses, fishing, boating and water-skiing. However, recreational demand is dwindling due to the deteriorating condition of the dam. This has numerous economic consequences.

Since the 1970s eutrophication has been a major concern in the dam. Eutrophication of the Hartbeespoort Dam has resulted in the growth and proliferation of vast amounts of water hyacinth. The elevated levels of phosphorous in the dam water coupled with the high levels of ambient sunlight provides the ideal environment for this photosynthesising entity. Growth of water hyacinth is common throughout the dam surface. Wind action however results in the concentration of the biomass along the shoreline and in funnelled areas (e.g. at the dam wall which is the biggest concentration point). Hyacinths are of particular concern due to the coverage of the water surface and the restrictions that they place on recreational activities at the dam. Furthermore, these weeds may have direct and indirect negative effects on the ecosystem as they are capable of impacting on bird activities as well as other wildlife.

RATIONALE

Water hyacinth (*Eichhornia crassipes*) is a harmful weed which has one of the highest growth rates of all plants in the world. This fast growing property causes numerous water problems related to navigation, recreation, irrigation and hydropower generation. The complete elimination of water hyacinth from waterways is almost impossible due to the production of hardy seeds by the plant that remain viable for up to 20 years. Furthermore, the complete removal of water hyacinth is questionable due to its indirect role in water treatment. Therefore, control of the plant rather than complete removal is suggested. The continual removal of the plant will result in a sustainable organic supply which makes water hyacinth attractive as a bioenergy crop. Other factors that make it ideal in terms of use as a substrate for bioenergy production include the fact that it is naturally grown and does not compete with arable crop plants for nutrients, space or light. It is also easily degradable and has a low lignin content. Water hyacinth can be used as feed for biogas production via anaerobic digestion (AD). Anaerobic digestion results in the reduction in the volume, mass and toxicity of the input substrate, produces a methane-rich biogas and a nutrient-rich soil ameliorant.

AIMS AND OBJECTIVES

AIM

To optimise biogas production from water hyacinth obtained from Hartbeespoort Dam and to determine the feasibility of the use of the digestate from the AD process as a soil ameliorant.

Objective 1

To determine the suitability of water hyacinth for use as a feedstock in biogas production

Objective 2

To optimize biogas yield using substrate pre-treatment and bioaugmentation

Objective 3

To determine the effect of organic loading rates on microbial communities and biogas production during mono- and co-digestion

Objective 4

To determine the feasibility of the use of digestate as a soil ameliorant

Objective 5

To determine the cost implications of the hyacinth removal system that incorporates anaerobic digestion and to obtain feedback from the community with regard to removal of water hyacinth from Hartbeespoort dam for biogas production.

METHODOLOGY

Experimental setup

For the present study all water hyacinth samples were obtained from the Hartbeespoort Dam (North West Province, South Africa, 25°44′51″S 27°52′1″E) and used as feedstock in both batch and semi-continuous bioreactors.

Batch culture experiments were conducted to optimize biogas yield using substrate pre-treatment and bioaugmentation. Batch cultures were setup in 500 ml Schott® bottles (250 ml working volume) equipped with lids containing rubber septa. For the pre-treatment experiment, methods tested included physical and biological treatments. Water hyacinth was pre-treated as follows; homogenization (H), hand cut (HC), oven dried (OD), sun dried (SD) and hand cut and decomposed at room temperature for 7 days (HCD). For the bioaugmentation experiment, the microorganisms used to augment biomethane yield were obtained from digested water hyacinth (water hyacinth inoculum). All batch experiments ran over approximately 30 days.

Continuous culture experiments were conducted to determine the effect of organic loading rate on microbial communities and biogas production during mono- and co-digestion. Two 20 L anaerobic semi-continuous stirred tank reactors (10 L working volume) were set up. Digester 1 contained hand cut water hyacinth (mono-digestion) while digester 2 contained hand cut water hyacinth and fresh cow dung (co-digestion) mixed at a ratio of 3:1 respectively. Various organic loading rates were tested.

Analytical methods

The organic carbon, total solids (TS), volatile solids (VS) and heavy metal content of water hyacinth was analyzed according to the Standard methods for the examination of Water and Waste water (APHA, 2012). The chemical composition (proteins, fats, carbohydrate, lignin, hemicellulose and cellulose content) of water hyacinth was evaluated using the following AOAC official methods 920.39, 934.01, 930.15, 942.05 and 954.01. Inductively Coupled Plasma-Mass Spectrophotometry (ICP-MS) was used for macronutrient (nitrogen, carbon, phosphorus and potassium) and micronutrient analysis. At the beginning and end of digestion trials, the pH of the substrate and digestate were measured using a pH meter (AD1030). Gas chromatography (SRI 8610C) was used to analyse the composition of biogas and a potentiometer titrator (877 Trino plus) was used to calculate the FOS/TAC ratio.

Microbiological analysis

For microbial community analysis DNA was extracted using the PowerSoil kit (Whitehead Scientific (Pty) Ltd), in accordance with manufacture instructions. Selection of the kit was on the basis of a screening of extraction methods for anaerobic digestion samples. Extracted genomic DNA was quantified using Qubit 2.0 fluorimeter (ThermoFisher, Edenvale, South Africa). Denaturing Gradient Gel Electrophoresis or Next Generation Sequencing (Illumina Miseq) followed to analyse microbial community structure.

Cost benefit & Socio-economic analyses

A cost benefit analysis was conducted to compare the proposed method of water hyacinth control (biogas and soil ameliorant production) to the current method utilised (composting). For the socio-economic analysis a survey was conducted to investigate the societal impact of the utilization of water hyacinth from Hartbeespoort dam in biogas and soil ameliorant production. Data was collected in the form of questionnaires (sample size: 92). Knowledge was disseminated in the form of flyers and verbal communication. The study sites for the activity included two sites frequented by both members of the Hartbeespoort community and visitors from other areas. These sites included the Hartbeespoort Village Mall (25.7318° S, 27.8879° E) and the Harties Aquarium/ French Toast (25.7371° S, 27.9024° E). Selection of the sites was primarily based on the close proximity to the Hartbeespoort Dam (<15 km).

RESULTS AND DISCUSSION

Objective 1 – *To determine the suitability of water hyacinth for use as a feedstock in biogas production*

The suitability of water hyacinth as a feedstock for biogas production was evaluated. The plant was found to contain high carbon content and low nitrogen content. Carbon is used as a source of energy while nitrogen is used for growth of microorganisms during the anaerobic digestion process. The C/N ratio of the plant was found to be low (low C/N ratio is associated with ammonia inhibition). The optimal C/N ratio of approximately 20-30 is required for CH_4 production without ammonia inhibition. Lower C/N ratio of the plant showed that co-digestion may be necessary during AD. The plant contained important micronutrients such as nickel, molybdenum, selenium and tungsten. Some of these metals are termed essential due to their

functions. They are found in the active site of enzymes responsible for the conversion of complex compounds during hydrolysis.

Objective 2 – *To optimize biogas yield using substrate pre-treatment and bioaugmentation*

The ultimate purpose of evaluating different pre-treatment methods of water hyacinth was to select the most efficient method for future biogas trials when using the plant as a feedstock. There was no significant difference in biogas production among all physical pretreatment methods used, thus the selection of pre-treatment methods for further analysis was based on time consumption, water and the performance of the pre-treated sample in the absence of inoculum. Sun dried and oven dried pre-treatment methods were not selected for further analysis because the process of drying removes water from the plant, implying that the addition of more water will be necessary during AD in comparison to when utilizing non-dried feedstock. Homogenized pre-treatment method was also not selected since the homogenized method resulted in process failure in control trials, implying that the process of homogenizing may have destroyed or damaged most of the methanogens within the plant. Hand cut pre-treatment method was selected.

For the bioaugmentation trial, an inoculum was produced from the collected water hyacinth (water hyacinth inoculum). Digestion to produce the inoculum prior to anaerobic digestion of water hyacinth was to harness hydrolytic/acidogenic microorganisms to improve biomethane production. The incorporation of water hyacinth inoculum led to an increased production of biomethane. All the bioaugmented treatments produced more biomethane in comparison to the non-augmented controls. This could be attributed to the presence of hydrolytic/acidogenic microorganisms in the inoculum and the availability of the substrate to the microbes (due to initial pre-treatment – hand cutting). The 100% water hyacinth treatment (control) produced the least amount of biomethane as a result of unavailability of appropriate microorganisms to propel the digestion process. Furthermore, the control with 100% water hyacinth inoculum did not also perform too well, possibly because of shortage of substrates/limited nutrients for microbial activities.

Objective 3 – *To determine the effect of organic loading rates (OLRs) on microbial communities and biogas production during mono- and co-digestion*

The effects of irregular OLRs on biogas production and bacterial and archaeal community structure during the anaerobic digestion of water hyacinth (mono and co-digestion) was investigated. Although irregular OLRs affected the microbial communities in both mono and co-digestion, the prevalence of these effects was greater in co-digestion and can be related to the low biogas production for co-digestion and subsequent process failure. The comparison of the abundance and dominance of bacterial communities between mono and co-digestion showed variation. The substrate composition played a role in the bacterial diversity in both digestion experiments. In addition, it was also observed that bacterial communities are more sensitive to OLRs in comparison to archaeal communities. The abundance of the archaeal community was not influenced by the changes in OLRs however, by the type of products produced during the previous stages, thus indicating the importance of bacterial community in

AD processes. The dominance of *Acinetobacter* and *Pseudomonas* support the reason that the type of substrate (mainly the nutrients presence) mainly contributes to the selection of microbial communities. Co-digestion is usually associated with a wider range of nutrients as compared to mono-digestion. The results from this study showed higher numbers of different bacteria in co-digestion than in mono-digestion. This was supported by the alpha diversity indices calculated. In this case the high numbers of active bacteria in co-digestion negatively affected efficiency of the process which contradicts the general findings that high microbial activity is related improved process efficiency. The results from this study showed that AD of water hyacinth as a mono substrate has the potential to withstand process disturbances caused by irregular OLRs without negatively affecting biogas production.

Objective 4 – *To determine the feasibility of the use of digestate as a soil ameliorant*

Digestate produced in this study had readily available nutrients as well as plant growth promoting microorganisms (PGPM) to enhance crop productivity and this proves its feasibility as a soil ameliorant. The conversion of the nitrogen content of water hyacinth to a form of nitrogen that is available to plants which is ammonium was also observed. Optimum activity of the anaerobic microorganisms has been recorded at pH 6.5-7.5. The *nifH* genes and the *phoD* genes were found in all the treatments including controls. This suggests the presence of PGPMs that possess these genes in the digestate. The *nifH* gene encodes the nitrogenase enzyme which is responsible for the conversion of atmospheric nitrogen to a form of nitrogen that is available to plants. The digestate can therefore be regarded as a nitrogen fixing biofertilizer. The *phoD* genes were also identified in the digestate samples which implies the capacity of the digestate to solubilise insoluble phosphate when applied to the soil and this qualifies the digestate to be a potential phosphate solubilising biofertilizer. Absence of cultivable pathogens in the digestate could be due to their destruction during AD through the generation of metabolic heat as mesophilic AD has been known to destroy pathogenic organisms. This ensures biosafety of the digestate (soil ameliorant) to animals and humans.

Objective 5 – *To determine the cost implications of the hyacinth removal system that incorporates anaerobic digestion and to obtain feedback from the community with regard to removal of water hyacinth from Hartbeespoort dam for biogas production*

As proven by findings of the present study, water hyacinth is a suitable substrate for biogas and soil ameliorant production. However, it is of great importance, from an economical point of view, that a cost-benefit (CBA) analysis be conducted to determine the profitability of the use of water hyacinth as a feed for anaerobic digestion. The profitability will ultimately determine if Dam management adopts the proposed method of water hyacinth control. A CBA of the system that incorporates anaerobic digestion, in comparison to the current method of water hyacinth control (harvesting and composting) was conducted. The findings of the cost-benefit analysis proved that if the proposed project uses over 50000 tons of water hyacinth per annum, approximately R2.6 million will be realised as benefits from biogas and the soil ameliorant while R4 million will be saved for the case of the composting approach being used. This highlights the economic viability of the proposed approach which was primarily due to the

production of two products (biogas and soil ameliorant) as opposed to one product (compost) in the current approach.

A survey was conducted to obtain feedback from the community on the use of water hyacinth for biogas production. Acceptance of the technology, associated infrastructure and products was overwhelming and prove that the respondents are ready for the implementation of the technology at the Hartbeespoort dam. However a wide-scale survey is necessary to further establish acceptance prior to implementation. It is envisaged that the flyers that were distributed would have a wide reaching impact on the community and initiate further knowledge transfer. Some of the respondents such as school-goers remarked that they will share the flyers with teachers and initiate further communication with the ARC with regard to the project. Students also volunteered to assist with removal of the hyacinth from the dam and some respondents were excited about the project due to the prospect of future jobs if the project is initiated.

CONCLUSIONS

The overall aim of the project was to optimise biogas production from water hyacinth obtained from Hartbeespoort Dam and to determine the feasibility of the use of the digestate from the AD process as a soil ameliorant. To achieve this aim, it was necessary to initially verify if the water hyacinth is suitable as a feed for anaerobic digestion (AD). The AD process is microbially driven. The outcome of the AD process is dependent on the activity of the microbial community, while the activity of microbial community is dependent on the availability of certain nutrients. These nutrients are supplied by the selected feedstock. The presence of the macro and micronutrients necessary for AD in water hyacinth makes the plant a suitable feedstock for the process. However, low C/N ratio of the plant proved that co-digestion may be necessary.

The optimisation of the AD process is necessary to ensure maximal biogas/ energy production. This in turn will contribute to the economic viability of the process. Methods tested to optimise AD in the present study were pre-treatment of the hyacinth, bioaugmentation and co-digestion. Physical pre-treatment of the hyacinth resulted in increases in biogas yield and the hand cutting method was selected and utilized in all subsequent experiments. Bioaugmentation using inoculum obtained from water hyacinth also proved to increase biogas yield. Interestingly, unlike initially anticipated, the co-digestion of water hyacinth with cow dung resulted in lower biogas yield than the mono-digestion of water hyacinth. This finding was strongly linked to the microbial communities involved in the AD process. This further motivates the importance of understanding the microbiology of the AD process and conducting microbiological analysis alongside physico-chemical analysis at any biogas plant.

Bacteria and archaea play key roles in the AD process. It is therefore important to analyse these microbial communities during the course of AD. To achieve this, it is necessary to extract DNA from AD samples. Analysis of various DNA extraction methods has shown that the choice of method of DNA extraction influences metagenomic results when analysing AD

samples. Extraction methods that result in optimal DNA extraction from AD samples were identified and utilised during the course of the study. Analysis of microbial communities during AD enabled the identification of biogas producing microorganisms naturally associated with the collected hyacinth. This has major implications for uncontrolled anaerobic digestion of hyacinth in the dam, which would result in biogas release to the atmosphere. This has environmental implications since biogas is composed primarily of methane which is a potent greenhouse gas and further motivates the present study, i.e. removal of the hyacinth from the dam and AD in a controlled environment.

The utilisation of the effluent from the anaerobic digestion process as a soil ameliorant will further aid in improving the economic viability of the technology. The presence of plant available nutrients and plant growth promoting microorganisms in the effluent was observed in this study. The digestate may therefore serve as a promising soil additive. The production of two products (biogas and soil ameliorant) from water hyacinth has significantly aided in ensuring the economic viability of the proposed method of hyacinth control. The widespread acceptance of the technology by the surveyed community is also very beneficial for eventual large scale implementation.

RECOMMENDATIONS FOR FUTURE RESEARCH

Studies on AD mainly focus on methanogenesis (archaea). Although important, bacterial communities also play an important role and without the activity of the bacterial community, methanogens will not have precursors to use in methanogenesis. As seen from the results, the presence of certain bacterial communities may be related to process efficiency or inefficiency. Thus, more understanding of bacterial community during AD is required especially to disturbances by OLRs and type of substrate to allow for the selection of bacteria that can be used as indicators of AD stability. These indicators may be useful in prevention of process failure. Future research to isolate microorganisms from water hyacinth inoculum for bioaugmentation could help attain further improvements in biogas yield. Studies on metabolomics could be conducted to determine the metabolites produced at different stages of AD and how to bioaugment the process with appropriate microorganisms to improve biomethane production.

The technology has thus far been tested at lab-scale. Field trials are necessary to determine feasibility on site. Various digester configurations will need to be tested. Future studies should also focus on the utilisation of digestate for improvement of plant growth and development in the greenhouse and during field trials.

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LIST OF ABBREVIATIONS

1/D	Inverse Simpsons index
ACC	Aminocyclopropane-1-carboxylate
AD	Anaerobic digestion
ALP	alkaline phosphatase
ARC	Agricultural Research Council
C/N	carbon to nitrogen ratio
CCA	Canonical correspondence analysis
CSTR	continuous stirred tank reactor
CTAB	Cetyltrimethylammonium bromide based extraction
DGGE	Denaturing Gradient Gel Electrophoresis
EPI	Meta-G-Nome DNA Isolation Kit – Epicentre
Fo	Functional organisation
H	Homogenised
H’	Shannon Weiner index
HC	Hand cut
HCD	Hand cut and decomposed
HRT	Hydraulic Retention Time
I	Inoculum
IAA	indole-3-acetic acids
ICP-MS	inductively coupled plasma mass spectrophotometry
ISCW	Institute for Soil, Climate and Water
Mag-Aut	MagMAX Total Nucleic Acid Isolation Kit – Automated – Thermo Fisher Scientific
Mag-Man	MagMAX Total Nucleic Acid Isolation Kit – Manual – Thermo Fisher Scientific
MN	NucleoSpin Soil Kit – Macherey-Nagel
OD	Oven dried
OLR	organic loading rate
PGPM	plant growth promoting microorganisms
PL	Pareto-Lorenz
PS	Powersoil DNA Isolation Kit – MO BIO Laboratories
QIA	QIAamp Fast DNA Stool Mini Kit – QIAGEN
SD	Sun dried
TS	Total Solids
VFA	volatile fatty acids
VS	Volatile Solids
WH	water hyacinth
Whinc	water hyacinth inoculum
ZR	ZR Soil Microbe DNA MiniPrep – Zymo Research

1 INTRODUCTION AND OBJECTIVES

1.1 Introduction

Water hyacinth

Water hyacinth, *Eichhornia crassipes* (Mart.) Solms, is an aggressive free floating aquatic plant notoriously known for its rapid reproduction (Malik *et al*., 2007). It belongs to the family pickerelweed (Pontederiaceae). The plant comprises of dark green, thick, glossy round leaves attached to spongy petioles containing air filled sacs that enables the plant to float in water (Sudani *et al*., 2014; Patil *et al*., 2012). Water hyacinth's growth may vary from few centimetres to 1 meter. According to Mitchel (1976), the plant is indigenous to South America but it is currently found in lakes, dams, rivers and swamps in tropical and subtropical countries. Water hyacinth can successfully outcompete other aquatic plants and tolerate a wide range of environmental conditions, but prefers nutrient rich environments. The plant reproduces sexually by seeds and vegetatively by budding and stolen production (Buchanan, 2015). The sexual reproduction is rare and seed germination may occur in a few days or remain dormant for years (Malik *et al*., 2007). Stolen reproduction is associated with the rapid reproduction resulting in the ability of the plant to double its population within a week. The plant's growth is directly correlated to the nutrient concentration in the water bodies especially nitrogen and phosphorus (Heard & Winterton, 2000). In nutrient-rich water bodies the proliferation rate of the plant can cause negative effects, including blocking light penetration for other submerged aquatic plants, decreasing dissolved oxygen thus affecting water quality and preventing water activities (Gupta *et al*., 2012; Villamagna & Murphy, 2010).

Water hyacinth is one of the world's invasive aquatic plant that is extremely difficult to eliminate (Heard & Winterton, 2000). The negative effects mentioned above motivated the development of a number of approaches to manage the proliferation of the plant. These include, the physical removal through harvesting, application of chemicals such as herbicides and the release of biological agents such as weevils. The physical removal approach is seen as the most efficient method that is environmentally friendly as compared to the chemical approach (Wang & Calderon, 2012), while environmental impacts of the biological agents is still in research stage. The problem with the physical method is the cost implications and the removal is usually temporary (Malik, 2007). Alternative approaches for the sustainable control and use of the water hyacinth are currently evaluated. Some common uses of the plant include animal fodder, fish feed, paper and furniture production. The plant is also used for phytoremediation, a process that may include additional benefits of biogas, biofuel, and soil ameliorant production as well as composting (Hendriks & Zeeman, 2009).

Water hyacinth in Hartbeespoort Dam

Eutrophication of the Hartbeespoort Dam has resulted in the growth and proliferation of vast amounts of water hyacinth. The elevated levels of phosphorous in the dam water coupled with the high levels of ambient sunlight provides the ideal environment for this photosynthesising entity. Accumulation of the plant on the water surface is unattractive and its decomposition

results in foul odours. Growth of water hyacinth is common throughout the dam surface. Wind action however results in the concentration of the biomass along the shoreline and in funnelled areas (e.g. at the dam wall which is the biggest concentration point). Hyacinths are of particular concern due to the coverage of the water surface and the restrictions that they place on recreational activities at the dam. Furthermore, these weeds may have direct and indirect negative effects on the ecosystem as they are capable of impacting on bird activities as well as other wildlife (Keto, 2013).

Hyacinth growth in the Hartbeespoort dam has been a nuisance since the mid-1970s. The problem accelerated quickly with approximately 80% of the dam being covered by the hyacinths by the late 1970s. One of the first attempts to eradicate the problem was through a process introduced by the then Department of Water Affairs. Chemical methods were used for hyacinth elimination with detrimental outcomes. It resulted in the destruction of up to 40 km of natural vegetation along the shoreline of the dam and the growth of even more hyacinth. Furthermore, it resulted in the change of the status of the dam from eutrophic to hypertrophic (Mbiza, 2014). A recent attempt at rehabilitating the dam was the Hartbeespoort Dam Integrated Biological Remediation Program, which was also referred to as *Harties Metsi a Me* (My water). This program involved the mechanical retrieval of both water hyacinth and algal soup using hand labour, harvesting tools and equipment. The composting of the harvested debris via vermiculture followed. This compost was used for the rehabilitation of the shoreline and for the construction of floating wetlands. The program also focused on the reconstruction of the food web by the removal of certain fish species (e.g. carp and catfish) and the reversion of the fish population back to the indigenous type (Yellow fish, Tilapia and other small species) (Keto, 2013). However the decade long program has been abruptly terminated due to allegations of corruption, nepotism and infighting. This has resulted in the rapid spread of water hyacinth which is presently covering 30% of the Dam surface (Komorant, 2017). This has huge implications on water quality and activities like boating and fishing.

There is dire need for a solution to the water hyacinth problem in the Hartbeespoort Dam. Water hyacinth are well known weeds and their complete removal from water bodies has proven to be futile. One contributing factor to their persistence is seed control, since seeds can remain viable for up to 20 years (Bhattacharya and Kumar, 2010). Furthermore, they grow rapidly with growth rates reported at approximately 175 kg per 100 square meters per day, under favourable conditions (Rezania *et al*., 2015). This results in hyacinth removal in large water bodies being an ongoing process. This elevated growth rate could be regarded as an advantageous factor if the hyacinth is to be used as a bioenergy crop. Other factors that make it ideal in terms of use as a substrate for bioenergy production include the fact that it is naturally grown and does not compete with arable crop plants for nutrients, space or light, it is also easily degradable and has a low lignin content (Rezania *et al*., 2015).

The harvested hyacinth may be used as feed for biogas production via. Anaerobic digestion (AD). Anaerobic digestion results in the reduction in the volume, mass and toxicity (killing of pathogenic organisms) of the input substrate, produces a methane-rich biogas and a nutrient-rich soil ameliorant (Tafdrup, 1995; Curry and Pillay, 2012; Manyi-Loh *et al*., 2014). The

anaerobic digestion of the harvested hyacinths with municipal wastewater or cow dung would result in the production of bio-methane, which is an environmentally friendly source of energy. This energy may be converted to electricity that can be utilised to power the pump station used for the pumping of thick algal scum from the water surface. This electricity can even be fed into the national grid. The nutrient rich soil ameliorant by-product of the anaerobic digestion process may be used for the rehabilitation of the shoreline or may be utilized as an organic fertilizer by farms in and around the Hartbeespoort area. The hardy hyacinth seeds will be broken down during the course of the anaerobic digestion process. Furthermore, the continual removal of the hyacinths for biogas production will serve the additional purpose of remediating the dam of the large amount of phosphorus (P) since hyacinths remove roughly 60 t of total P (The P taken up by the hyacinth will be removed during the harvesting process; Keto, 2013).

The phytoremediation properties of water hyacinth have been established in multiple studies especially in terms of the removal of nitrogen (N) and P (Jayaweera & Kasturiarachchi, 2004; Mahujchariyawong & Ikeda, 2001). However, the removal of the plant from the water body is of utmost importance if remediation of the water body is to be achieved as plant decay will release the nutrients back into the water. The collection and removal of water hyacinths is an expensive process. The use of the collected biomass for additional purposes, such as the proposed biogas and soil ameliorant production, will completely or partially offset the costs incurred by the collection process. Whilst water hyacinth has been used for compost production in the *Harties Metsi a Me* program this use was on a small-scale and the rate of consumption using this method (composting) is much lower than the plants growth rate. This will result in a surplus in hyacinth which could be landfilled creating environmental problems. The use of the plant as a substrate for biogas production ensures that all of the collected biomass will be continually put to use (Wang & Wan, 2013).

Biogas

Biogas is a clean renewable energy that comprises of methane (50 to 70%), carbon dioxide (30 to 40%) and traces of other gases (Patil *et al*., 2012). Currently biogas production has attracted worldwide attention and it is seen as an alternative source of energy to the current conventional energy sources such as fossil fuels (Rezania *et al.,* 2014). The problem with fossil fuels is the fast depletion rate, high cost and environmental impact, especially the emission of greenhouse gasses (Ganguly *et al*., 2011). Biogas is produced during a biological process called anaerobic digestion (AD), which uses a wide range of organic substrates and benefits the environment through the reduction of the organic biomass and greenhouse gas emission.

Anaerobic Digestion

Anaerobic digestion is a biological technology in which micro-organisms breakdown almost any organic matter under anaerobic conditions to biogas and digestate (Sahito & Brohi, 2013). Anaerobic digestion occurs in four stages that involves the conversion and stabilisation of the organic matter to biogas. The stages include hydrolysis, acidogenesis, acetogenesis and methanogenesis. The products of anaerobic digestion are methane, carbon dioxide, hydrogen, hydrogen sulfide, ammonia, siloxanes and other substances (Molino *et al*., 2013). The

efficiency of AD can be affected by the substrate concentration, time of biodegradation and ammonia concentration (Sahito & Brohi, 2013). The biogas produced through AD has a variety of uses such as direct combustion for cooking and heating or it may be used for the production of electricity and as a transportation fuel (Molino *et al*., 2013).

Role of microorganisms in anaerobic digestion

Each microbial stage (i.e. hydrolysis, acidogenesis, acetogenesis and methanogenesis) has its own group of microorganisms that differ in nutritional and pH requirement (Chourari *et al*., 2005). Anaerobic digestion microbial compositions are influenced by the feedstock type (Ziganshin *et al*., 2013). Microbial consortia breakdown organic matter in a series of steps as mentioned above, therefore malfunctioning of one microbial community at a certain stage may result in imbalances or failure of the process (Guo *et al*., 2014). Chauori *et al*. (2005) explained that methane is produced mainly from acetate and hydrogen/carbon dioxide, so in the absence of methanogens the substrates will accumulate, resulting in pH decrease which will ultimately inhibit the AD process. Bacteria and fungi are known to occur during hydrolysis, acidogenesis and acetogenesis while the last stage is carried out by archaeal consortia (Ziganshin *et al*., 2013). During hydrolysis, complex organics such as cellulose and hemicellulose are broken down by extracellular enzymes secreted by microorganisms. They are broken down to simple organics such as sugars, amino acids and fatty acids (Molino *et al*., 2013; Ziganshin *et al*., 2013). This first stage can be a rate limiting step due to surface area for enzyme digestibility and the presence of high lignin content, thus emphasising the importance of the pre-treatment methods (Molino *et al*., 2013). The second stage, acidogenesis is carried out by acid forming microorganisms, which converts the simple organics to volatile fatty acids (VFA) and the VFAs are converted to acetic acid and hydrogen (H_2) during the third stage, acetogenesis (Sreekrishnan *et al*., 2004). The last stage, methanogenesis, is carried out by archaea which produce the final products of the AD, mixture of methane (CH_4) and carbon dioxide (CO_2) as well as by-products, digestate. Microbial communities from AD have been isolated from substrates such as granular sludge, food waste, sewage sludge, municipal waste but not much from the water hyacinth as feed for biogas production.

Water hyacinth as feed for biogas production

Water hyacinth, *Eichhornia crassipes*, provides a lignocellulosic biomass that can be converted to biogas through anaerobic digestion (AD) (Gao *et al*., 2012). Lignocellulosic biomass consists of three different polymers, cellulose, hemicellulose and lignin (Sanchez, 2009) which are linked together by different bonds (Hendricks & Zeeman, 2009). Cellulose occurs in two forms the crystal cellulose and the non-crystal cellulose (Harmsen *et al*., 2013). Lignocellulose from different plants differ significantly on the variation of cellulose, hemicellulose and lignin content (Harmsen *et al*., 2013) and it was found that cellulose in water hyacinth is much lower when compared to other plants such as wood and straw (Sanchez, 2009). The cellulose and hemicellulose in water hyacinth serve as a source of sugars for AD and are more biodegradable than the lignin (Xie *et al*., 2012). Lignin is a recalcitrant complex polymer responsible for preventing the biodegradation of the cellulose and the hemicellulose (Hendricks & Zeeman, 2009). The direct use of water hyacinth's lignocellulose structure in biogas production is not feasible due to the resistance of the structure to enzyme degradation (Xie *et al*., 2012). The

hydrolysis of cellulose by enzymes can be enhanced through the application of pre-treatment methods available for the disruption of the lignocellulosic structure (Xie at al. 2012) and also co-digestion with other substrates for organic matter variation.

Pre-treatment methods

There are three main pre-treatment methods for the disruption of the lignocellulosic structure to modify the lignin and expose and increase the surface area for cellulose degradation. The first method is physical pre-treatment which includes mechanical (grinding, chopping, homogenising and cutting with scissors), microwaves, ultrasound, steam explosion and liquid hot water (Harmsen *et al*., 2013). The function of mechanical pre-treatments is the reduction of substrate size (between 5 cm to few mm) and to increase the surface area for rapid enzyme digestibility (Harmsen *et al*., 2013). Other physical methods such as ultrasound and microwave involves the cleavage of β-1, 4-glucan bonds increasing the accessible surface area and reducing the crystallinity of cellulose (Lin *et al*., 2015). The second method is the use of chemicals to initiate the disruption of the lignocellulosic structure. Examples include weak or strong acid hydrolysis, alkaline hydrolysis, oxidative hydrolysis and use of ionic liquids. The last method is biological pre-treatment, which involves the use of microorganisms or commercially available enzymes to disrupt the lignocellulosic structure. The purpose of pre-treatment is to open up the cell wall to allow enzymes to breakdown the molecules. Patil *et al*. (2012), evaluated the effects of pre-treatments on biogas yield and it was suggested that dried and chopped water hyacinth resulted in the highest biogas yield. Gao *et al*. (2012), evaluated the effect of ionic liquid pre-treatment and it was found that the crystallinity of the cellulose was decreased and biogas yield was increased. Other studies such as those of Ofoefule *et al*. (2009), combined the physical and biological pre-treatment methods, they sun dried the water hyacinth, chopped the dried matter and soaked it in water for partial decomposition by microorganisms.

These studies suggest that pre-treated water hyacinth alone can be used as feed for AD to produce biogas. However previous studies showed that the use of single organic substrate may result in a number of drawbacks such as improper carbon-nitrogen (C/N) ratio and poor buffering capacity (Wang *et al*., 2014). Kumar (2005) showed that feed from a mixture of water hyacinth and night-soil had improved nitrogen, phosphorus and potassium as compared to feed from water hyacinth alone. The ratio for water hyacinth to night soil was 3:1 and it was suggested that the plant can be used as a major feed for AD with other organic substrates serving as inoculum and to help with buffering capacity. Patil *et al*. (2012) reported that water hyacinth is a good biogas producer but blending it with poultry waste significantly increased biogas yield. While Wang *et al*. (2014), reported that the use of a single substrate may affect the efficiency of AD due to insufficient amount and diversity of organic matter. Pre-treatment and co-digestion are methods that can be used to improve the yield and stability of anaerobic digestion. Pre-treatment increases the rate of hydrolysis, while co-digestion helps through the combination of nutrients to reach an optimum balance for AD or can help to establish the required moisture content and organic diversity that may help in controlling the pH.

Use of effluent as a soil ameliorant

The effluent from anaerobic digesters may be used as a nutrient-rich soil ameliorant. Essentially, all of the nutrients that are input into the digester remain in the sludge after anaerobic digestion (Hans *et al*., 1993). Complex lipids, lignin-like material and steroids also become concentrated during the AD process. It has been reported that these molecules are precursors to humus production which plays a significant role in the short-term soil organic matter turnover (Lorenz *et al*., 2007). Most of the nitrogen contained in the sludge is in the organic form. There are also large amounts of ammonium and much smaller amounts of nitrate (Hans *et al*., 1993). Aerobic sludge and chemical fertilisers, on the other hand, have elevated levels of nitrate and nitrite (Gunnarsson & Petersen, 2007). Ammonium is less likely to leach from the soil.

In a study conducted by Tambone *et al*. (2010) it was deduced that the elevated nutrient content (N, P, K) in the bioavailable form in AD digestate made it a candidate to actually replace inorganic fertilizers. Another study conducted in China showed that the agricultural productivity increased by 30% when using AD digestate as opposed to farmyard manure as a fertiliser (Gunnersson and Stuckey, 1986).

Bio-augmentation strategies

Bio-augmentation refers to the addition of actively growing microbial strains to a particular microbial community in an effort to augment/enhance a particular process or the abilities of the microbial community (Deflaun and Steffan, 2002). Bio-augmentation has been used previously in anaerobic digesters to improve the methane yield obtained from various substrates such as cattle manure, wheat straw and lipid rich waste (Nielsen *et al*., 2007; Cirne *et al*., 2006; Peng *et al*., 2014). However, to our knowledge no previous bio-augmentation studies have been conducted with water hyacinth as an AD substrate. To augment biomethane yields it is necessary to determine the stage in the AD process that is rate limiting and with lignocellulosic substrates the rate limiting stage is generally hydrolysis (Peng *et al*., 2014)

Multiple studies have proven that bio-augmentation improves AD. This includes a study conducted by Peng *et al*. (2014) that demonstrated that bioaugmentation of the AD process with the cellulose degrading anaerobic bacteria *C. cellulolyticum* resulted in an improvement of the biomethane potential of wheat straw. Similarly, Cirne *et al*. (2006) showed that the lipolytic strain, *Clostridium lundense* improved the degradation of long chain fatty acids thereby improving the digestion process and Kovacs *et al*. (2012) showed that *Caldicellulosiruptor saccharolyticus* and *Enterobacter cloacae* intensified biogas production by increasing the abundance of hydrogen producers.

Literature survey of water hyacinth use for biogas production

Minimal studies have been conducted on the use of aquatic plants such as water hyacinth for biogas production. However, the use of water hyacinth as a substrate for biogas production is very promising due to its elevated growth rate which ensures continual substrate availability. Singhal and Rai (2003) conducted a study were water hyacinth and channel grass were used as the substrate for biogas production. They showed that AD of plants used for phytoremediation

produced more biogas than plants grown in deionized water and this was attributed to the changes in C, N and the C/N ratio of the slurry brought about by phytoremediation. A study was also conducted testing solid phase biogas production with garbage and water hyacinth. Here, biodegradative bacteria were inoculated daily to maintain elevated biogas levels. Majority of the methanogenic activity was evident at the lower parts of the bed which enabled weekly feeding without disrupting the AD process (Chanakya *et al*., 1993). Kivaisi and Mtila (1997) used a two stage bioreactor for the AD of water hyacinth inoculated with rumen fluid. Via modifications in the loading rate, solid retention time, dilution rate and connecting a methanogenic reactor they were able to achieve 100% conversion of the VFAs into biogas which was made up of 80% methane. Physical pre-treatment by cutting of the water hyacinth into smaller fragments, varying N content and inoculum volume was tested by Moorhead and Nordstedt (1993). Intermediate particle size (6,4 mm) resulted in the highest biogas yield in batch cultures and biogas production increased with increasing inoculum volumes in plants with elevated N content (day 15). After 60 days the total biogas and methane yields were similar for all treatments regardless of particle size, N content or inoculum volume.

With the present energy crisis, numerous previously overlooked substrates are being explored for bioenergy production. One such substrate is water hyacinth. The use of water hyacinth as a feed for biogas production will enable energy production, environmental sustainability and food security.

1.2 Aim

To optimise biogas production from water hyacinth obtained from Hartbeespoort Dam and to determine the feasibility of the use of the digestate from the AD process as a soil ameliorant

1.3 Objectives

1. To determine the suitability of water hyacinth for use as a feedstock in biogas production
2. To optimize biogas yield using substrate pre-treatment and bioaugmentation
3. To determine the effect of organic loading rates on microbial communities and biogas production during mono- and co-digestion
4. To determine the feasibility of the use of digestate as a soil ameliorant
5. To determine the cost implications of the hyacinth removal system that incorporates anaerobic digestion and to obtain feedback from the community with regard to removal of water hyacinth from Hartbeespoort dam for biogas production

2 METHODOLOGY OPTIMISATION: BIASES DURING DNA EXTRACTION AFFECT BACTERIAL AND ARCHAEAL COMMUNITY PROFILE OF ANAEROBIC DIGESTION SAMPLES

2.1 Literature review

Much interest has been directed to the use of anaerobic digestion (AD) for the conversion of organic waste to bioenergy because this process contributes to waste management, renewable energy production and food security. Microorganisms such as bacteria and archaea play a key role in the AD process, hence, it is of great importance that the microbiology of the AD system be explored (Bergmann *et al*., 2009; Roopnarain & Adeleke, 2017). This can be achieved using culture dependent or culture independent techniques. Culture dependent techniques are notorious for the underestimation of microbial diversity in most environments because only a small percentage of the population is culturable (Amann *et al*., 1995). Currently, culture independent, molecular approaches are frequently exploited for the investigation of community structure and diversity in practically all environments (Theron & Cloete, 2000).

The basis of molecular biodiversity analyses is to obtain a representative nucleic acid extract from the entire microbial community that is under investigation. The quality and representability of the nucleic acid extract is directly influenced by the choice of the extraction method that is used (Carrigg *et al*., 2007). Inefficiencies at various stages in the extraction process could negatively affect the quality of the final extract. Such inefficiencies include incomplete cell lysis, damage of the extracted DNA, DNA sorption to the surface of various particles in the sample, the loss of DNA at different stages in the extraction process and the co-extraction of various enzymatic inhibitors that could interfere with downstream processing of the DNA, e.g. PCR inhibitors (Claassen *et al*., 2013; Miller *et al*., 1999). The efficacy of the DNA extraction process is further influenced by the source of the sample. Samples consisting of a complex microbial matrix and large amounts of inhibitors such as activated sludge and soil contribute to the challenges in DNA extraction (Vanysacker *et al*., 2010).

Commercial DNA extraction kits and laboratory designed protocols are frequently used for the extraction of DNA from environmental samples. Commercial kits are often used because they are designed to optimise DNA yield and ensure the reproducibility of the extraction. These kits are easy to use and require considerably shorter durations for complete extraction in comparison to conventional methods (Herrera & Cockell, 2007). Regardless of the type of method used for DNA extraction, one or more of the following processes are incorporated: chemical lysis, physical disruption and/or enzymatic lysis (Miller *et al*., 1999). These processes should ensure that sufficient amounts of high molecular weight DNA are extracted with minimal inhibitors and the extract should reflect an accurate representation of the total microbial diversity within the sample (Yeates *et al*., 1998). Furthermore, the method of DNA extraction should be efficient and reproducible. In addition, the method should also be applicable to a wide range of sample types and be cost effective (Fahle & Fischer, 2000).

At present, there are no commercial DNA isolation kits that are specifically designed for the extraction of DNA from anaerobic digester samples. Kits that are frequently used for digester samples include soil (Garcia-Peña *et al*., 2011; Xu *et al*., 2014) and stool kits (Kampmann *et al*., 2012; Slana *et al*., 2011). However, digester samples are generally rich in inhibitors such as humic acids, which are a by-product of the AD process (Bergmann *et al*., 2009). Furthermore, a wide range of microorganisms such as bacteria and archaea are evident in digester samples. These microorganisms are integral to the AD process, hence, both bacterial and archaeal communities are frequently analysed when conducting AD studies (Ariesyady *et al*., 2007; Riviere *et al*., 2009; Sundberg *et al*., 2013). One of the major factors influencing the choice of the extraction method used for digester samples is the ability to achieve complete cell disruption of both bacterial and archaeal cells in the sample.

In the present study, various methods of DNA extraction, including commercially available kits and laboratory designed protocols, were tested on samples obtained at different stages of the AD process. The study was aimed at determining the suitability of various DNA extraction methods for AD samples and to determine how the method of extraction impacts on the observed diversity of bacteria and archaea (using DGGE analysis). This will enable the proposal of a single extraction method that facilitates DNA extraction from the majority of or all bacteria and archaea involved in the AD process which would aid significantly in AD microbial ecology studies.

2.2 Methods

Sample collection and dry weight measurements

Samples were collected from the inlet (fresh feed), digester chamber (partially digested feed) and slurry/ outlet (completely digested feed) of a pre-fabricated digester situated in QwaQwa village (Free State province, South Africa). The digester has been working since August 2012 and is fed continuously with a mixture of cow dung and water. The samples were collected in sterile plastic bags and transported on ice, in a cooler bag, to the Agricultural Research Council – Institute for Soil, Climate and Water microbiology laboratory in Pretoria (Gauteng province). Upon arrival, the samples were mixed, aliquoted into sterile centrifuge tubes (50 ml Falcon® tubes) and stored at -20ºC until further analysis.

To determine dry weight, frozen samples were thawed at 4ºC and pre-weighed aliquots of the samples were incubated at 105ºC for 24 hours. The dried samples were weighed and standard curves were constructed showing the correlation between wet and dry weight measurements. These standard curves were used to determine dry weight from wet weight measurements.

DNA extraction

Samples were thawed at 4ºC and centrifuged at 16,000 x g for 10 minutes for the collection of solids and microorganisms. The supernatant was discarded and the pellet was used as the substrate for various DNA extraction methods. Eight methods of DNA extraction were tested in this study (Table 2.1): One laboratory-based extraction method (CTAB method) and seven commercially available kits were evaluated. The selection of commercial kits used was based on popularity, cost, availability, novelty, variations in methods of cell lysis and variations in the format of DNA purification. All the methods listed in Table 2.1 were used to extract DNA from samples obtained from the inlet, digester chamber and the outlet of a working digester. The extractions were conducted in triplicate for each sample to determine the reproducibility of the various methods.

Table 2.1: Methods of DNA extraction evaluated in the study

Kit/extraction method name	**Abbreviation**	**Recommended source material**	**Method of substrate homogenisation & cell lysis**	**Format of DNA purification**	**Approximate duration of extraction process (9 samples) (hr)**	**Weight of starting material (mg)**
ZR Soil Microbe DNA MiniPrep – Zymo Research	ZR	Soil samples	Bead beating & cell lysis buffer	Spin column filter based	4	150
QIAamp Fast DNA Stool Mini Kit – QIAGEN	QIA	Stool samples	Cell lysis buffer & heat	Spin column filter based	3.5	220
NucleoSpin Soil Kit – Macherey-Nagel	MN	Soil, sludge and sediment samples	Bead beating & cell lysis buffer	Spin column filter based	4.5	500
MagMAX Total Nucleic Acid Isolation Kit – Manual – Thermo Fisher Scientific	Mag-Man	Broad range – biological and environmental samples	Bead beating & cell lysis buffer	Paramagnetic bead based – manual	5	300
MagMAX Total Nucleic Acid Isolation Kit – Automated – Thermo Fisher Scientific	Mag-Aut	Broad range – biological and environmental samples	Bead beating & cell lysis buffer	Paramagnetic bead based – automated	2.5	300
Powersoil DNA Isolation Kit – MO BIO Laboratories	PS	Soil, compost, sediment and manure	Bead beating & cell lysis buffer	Spin column filter based	3.5	250
Meta-G-Nome DNA Isolation Kit – Epicentre	EPI	Water or soil samples	Cell lysis buffer	Solution based	11	1000
Cetyltrimethylammonium bromide based extraction (Minas *et al*., 2011)	CTAB	Rumen fluid, plant and bacterial pure cultures	Bead beating & cell lysis buffer	Solution based	26	250

For all kits tested, the amount of starting material was determined by the protocols available in the kit. The maximum quantity was used in each instance (e.g. if the kit required 0.5-1 g, 1 g of the sample was used as the starting material; see Table 2.1). The kit extractions were conducted as per manufacturer instructions with minor amendments. For instance, for the ZR

kit, a standard benchtop vortex (MX-S; Dragonlab) was used instead of a bead beater. For the QIA kit, a lysis temperature of 95ºC was used instead of 70ºC. For the MN kit, buffer SL1 was used instead of buffer solution SL2, 75 µl Enhancer SX was used instead of 150 µl and 50 µl elution buffer was used. For the EPI kit, sterile cheesecloth was used instead of miracloth. The CTAB extraction was conducted as described in Minas *et al*. (2011) with minor deviations. The standard method in 2.0 ml microcentrifuge tubes was used. For the CTAB method, DNA was extracted from 250 mg samples. Cells were lysed by vortexing the material in microcentrifuge tubes containing 2 mm glass beads with 900 µl CTAB lysis buffer.

All the extraction methods tested were direct methods (i.e. cells were lysed directly within the sample) with the exception of the EPI kit which was an indirect method of DNA extraction (i.e. cells are removed from the samples prior to cell lysis and DNA extraction) (Delmont *et al*., 2011).

Quality and quantity of extracted DNA

DNA yield was measured using two methods: Nanodrop (Nanodrop One, Thermoscientific, USA) and the Qubit fluorimeter (Invitrogen, USA, using the Qubit® dsDNA HS Assay Kit). DNA yield measurements were normalised based on the dry weight of the respective samples. DNA purity was determined spectrophotometrically using a Nanodrop (Nanodrop One, Thermoscientific, USA). Protein contamination was measured using the ratio of absorbances at 260 and 280 nm. A ratio between 1.8 and 2.2 was indicative of no protein contamination (Weiss *et al*., 2007). The ratio of absorbances 260 and 230 nm was used to determine contamination by aromatic compounds, phenols and carbohydrates (Roh *et al*., 2006). Ratios between 1.5 and 1.8 were taken as an indication of DNA without aromatic compound contamination (Weiss *et al*., 2007). The integrity of the DNA extracts was evaluated by gel electrophoresis on a 1% agarose gel (w/v) stained with ethidium bromide and run in 1 x TAE buffer at 100 V.

PCR

PCR amplification of the 16S rDNA with the universal bacterial primer set 341F-GC and 907R (Table 2) was carried out (Muyzer *et al*., 1993). Methanogenic archaeal DNA was first amplified using the primer set for methanogenic archaea, i.e. 0357F and 0915aR. The resulting PCR products were re-amplified using primer set 0357F-GC and 0691R (Table 2.2; Ikenaga *et al*., 2004; Watanabe *et al*., 2004).

Table 2.2: Primers used in the study

Primer	Sequence (5'- 3')	Annealing temp (°C)
341F-GC	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGCCTAC GGGAGGCAGCAG	65-55*
907R	CCGTCAATTCCTTTGAGTTT	65-55*
0357F	CCCTACGGGGCGCAGCAG	69
0915aR	GTGCTCCCCCGCCAATTCCT	69
0357F-GC	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCCTAC GGGGCGCAGCAG	57
0691R	GGATTACARGATTTCAC	57

*These primers were used in a touchdown protocol where the annealing temperature decreased from 65 to 55ºC in 20 cycles.

DGGE

Denaturing Gradient Gel Electrophoresis (DGGE) was used to establish microbial community profiles of the DNA samples obtained using the various extraction methods. For DGGE analysis, triplicate DNA extracts from each kit and respective samples (inlet, digester or slurry) were pooled. DGGE was performed as described by Muyzer *et al*. (1993) with slight variations. The DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA) was used. Amplicon separation proceeded on an 8% (wt/vol) polyacrylamide gel (40% acrylamide/bis solution, 37.5:1) using denaturing gradient ranges of 40-60% for bacterial samples and 25-60% for archaeal samples. The 100% denaturant consisted of 40% formamide and 7 M urea. Glycerol (2%) was added to the gel to increase gel flexibility. To enable gel comparisons, a mixture of 5ul of PCR amplicons from 4 pure isolates was used as a marker. Electrophoresis was performed at 200 V for 15 minutes, then at 100 V for 16 hours. The 0.5 X TAE buffer was maintained at 60ºC throughout the run. Gels were stained with GelRed and photographed using a UV transilluminator (GelDoc XR; Bio-Rad Laboratories, Hercules, CA). Bands were excised from the gels and DNA was eluted overnight in 10 µl sterile distilled water. Bacterial DNA was re-amplified with the primer pair 341F and 907R and archaeal DNA with 0357F and 0691R. The resulting PCR amplicons were sequenced. Sequences were inspected and edited using BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/bioedit/bioedit) and identified using NCBI Blast and EzTaxon (http://www.ezbiocloud.net/eztaxon).

Images of the DGGE gels were analysed using the Image Lab software (Bio-Rad). Each DGGE gel is composed of numerous lanes that were loaded with individual samples. These samples were separated into several bands of varying intensities. The software detects each band and

calculates the relative contribution of the individual band to the overall signal in the lane of interest. The resultant data were used to calculate two widely used diversity indices, viz. Shannon-Wiener (*H*') and Simpsons indices (*D*) using the following formulae:

$$H' = -\sum_{i=1}^{i=n} p_i \, ln(p_i)$$

$$D = \sum_{i=1}^{i=n} {p_i}^2$$

where n is the total number of bands in the lane/community and p_i is the relative abundance/intensity of the i^{th} band in the lane/community (Magurran, 1988). To ensure that the Simpsons index increases with increasing diversity, 1/D was used instead of the original formulation.

2.3 Results and Discussion

DNA yield and purity

Maximal DNA yield and purity is important when selecting a DNA isolation procedure. Differences in DNA yield from the same sample, using different DNA extraction methods, could indicate variations in efficacy of cellular lysis. This could imply that only the cells most sensitive to the lytic protocol have been lysed which skews community analysis data. Purity of the extract is important for downstream molecular techniques required for community analysis such as PCR (Krsek & Wellington, 1999).

All DNA extraction methods that were tested were successful in extracting DNA from samples obtained from various stages in the AD process. However, the DNA yield varied between extraction methods (Fig. 2.1A & B). For most extraction methods tested, a negative correlation existed between the DNA yield and the weight of the starting material used. This corroborated the results obtained by Ariefdjohan *et al.* (2010) when extracting DNA from human faecal samples. Elevated DNA yield obtained with smaller sample weights may be attributed to increased contact between the sample, lysis buffer and beads. The CTAB method resulted in the largest DNA yield and the EPI kit resulted in the smallest DNA yield (Fig. 2.1A & B). The low DNA yield obtained using the EPI kit is justifiable because this kit is an indirect method of DNA extraction. Similar results have been reported in previous studies where it was concluded that DNA yield is greater with direct extractions in comparison to indirect extractions (Delmont *et al*., 2011; Leff *et al*., 1995).

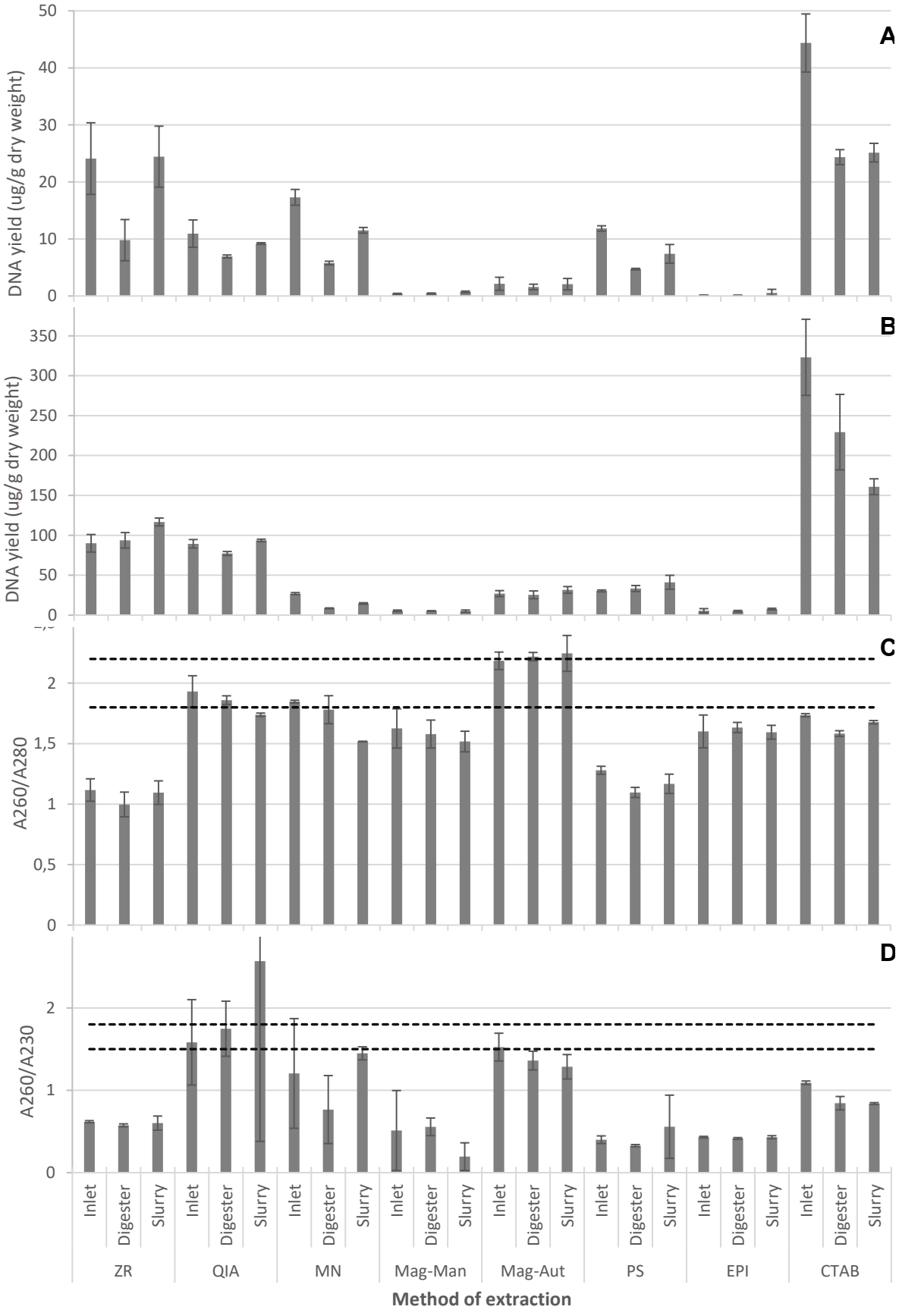
A
DNA yield (ug/g dry weight)
50
40
30
20
10
0
B
DNA yield (ug/g dry weight)
350
300
250
200
150
100
50
0
C
A260/A280
2
1,5
1
0,5
0
D
A260/A230
2
1
0
Inlet
Digester
Slurry
ZR
QIA
MN
Mag-Man
Mag-Aut
PS
EPI
CTAB
Method of extraction

Fig. 2.1 (Previous page): Yield and quality of DNA extracted from samples obtained from the inlet, digester and slurry using various methods of DNA extraction. (**A**) DNA quantified with Qubit. (**B**) DNA quantified with NanoDrop. (**C**) DNA quality determined by A260/A280 ratio. (**D**) DNA quality determined by A260/A230 ratio. Area between perforated lines in C & D is indicative of pure DNA, i.e. DNA with no protein contamination in C and DNA with no contamination by aromatic compounds, phenols and carbohydrates in D. Error bars represent standard deviation (n=3).

The minute DNA yield obtained in this study when using the EPI kit could also be a function of the limitations of the kit when using cow dung samples at various stages of the AD process. Even though the recommended source material included soil (Table 2.1), which is quite granular, significant clogging of the filters was observed for all samples used. This could have resulted in loss of DNA because of potential exclusion of certain microbes prior to the second stage of the extraction process (i.e. DNA extraction from filters containing cells). Furthermore, the clogging of filters contributed to the extended duration of the extraction process (Table 2.1). Clogging of the filters might have been avoided if Miracloth was used for the efficient removal of large particulates in the initial filtration as opposed to cheesecloth. Unlike with cheesecloth, Miracloth has uniform pore sizes therefore enabling adequate filtration (Endres *et al*., 2003). In the present study, sterile cheesecloth was used since it was mentioned as an option in the EPI protocol.

Clogging of filters was also experienced when using the ZR kit, which utilises solid phase nucleic acid extraction. This method of DNA extraction consists of four key steps: cellular lysis, adsorption of nucleic acids, washing and final elution of pure DNA (Kojima & Ozawa, 2002; Shaw *et al*., 2009). Clogging of the filters resulted in reduced adsorption of nucleic acids to the filter material because all of the lysate was unable to pass through. This resulted in the inconsistency of the kit as evidenced by the large standard deviation between DNA yield replicates (Fig. 2.1A). Reproducibility of DNA extraction is very important when selecting a DNA extraction method (Tan & Yiap, 2009). As with the EPI kit, the recommended source material for the ZR kit is soil samples (Table 2.1). This implies that the kit should be well suited for granular material like cow dung.

All direct methods of DNA extraction resulted in greater DNA yields in comparison to the paramagnetic bead based extractions (Mag-Man & Mag-Aut) (Fig. 2.1A). Similar results have been reported elsewhere when comparing DNA yields obtained using magnetic bead extractions versus organic extractions (Kishore *et al*., 2006; Montpetit *et al*., 2005). The general consensus was that magnetic based extractions were sub-optimal with low yield and degraded samples as is the case with certain forensic samples (Kishore *et al*., 2006). However, the samples used in the present study were expected to contain large amounts of intact DNA. The low DNA yield obtained in the present study, when using paramagnetic bead based technology, is corroborated by the study of Brownlow *et al*. (2012). They reported that the DNA yield obtained using automated paramagnetic technology was significantly lower than that obtained when using automated and manual spin column, silica based technology, regardless of the amount of DNA in the sample material (Brownlow *et al*., 2012).

The reduced DNA yield when using paramagnetic DNA extraction in the present study may be due to the type of samples used. However, the MagMax kit should efficiently extract DNA from a broad range of sample types including manure (environmental samples; Table 2.1). Reduced yield may also be due to non-specific adhesion of the DNA to the walls of the extraction tubes. The paramagnetic method resulted in extended periods when the extracted DNA is in direct contact with the tube whereas other methods such as the filter based techniques involve the entrapment of DNA on filters. Furthermore, low DNA yield may also be due to the incomplete release of DNA from the magnetic beads, thus preventing complete elution. This is verified by the present work where manual and automated DNA extraction were tested using a single paramagnetic kit (Mag-Man and Mag-Aut respectively; Table 2.1). The DNA yield obtained using the automated system was up to six times greater than that obtained from the same samples using manual extraction (Fig. 2.1A). For both manual and automated paramagnetic DNA isolation, the agitation of the magnetic beads in specific reagents is required for DNA binding, washing and final elution. This is advantageous as it prevents problems usually associated with other kits, e.g. filter clogging (Fang *et al*., 2007). However, insufficient agitation may result in reduced DNA yields. Agitation in the automated system is achieved via the up and down movement of magnetic rods, whereas the manual method (Mag-Man) is agitated by low speed shaking on an orbital shaker. The speed has to be minimal to avoid spillage which could result in cross-contamination of samples in the processing plate. Hence, agitation was limited when conducting manual paramagnetic DNA extraction. Limited agitation probably contributed to incomplete release of DNA from the magnetic beads and the resultant lower DNA yield when using Mag-Man extraction.

The results showed a general trend in the yield of DNA obtained from the various samples. The same samples from the inlet, digester and slurry were used for all extraction methods to enable a clear comparison between kits. The Qubit data showed that the DNA yield, using all methods of extraction, was largest in the inlet, intermediate in the slurry and smallest in the digester (Fig. 2.1A). However, such clear trends were not evident when using the Nanodrop for DNA quantification (Fig. 2.1B). Furthermore, the DNA yield was highly overestimated (on average approximately 10 times; Fig. A2.1) when using the Nanodrop in comparison to the Qubit for quantification (cf. Fig. 2.1A & B). Similar results were observed in previous studies when comparing data obtained using the Qubit and Nanodrop (Guo & Zhang, 2013; Sironen *et al*., 2008). Qubit quantification is fluorescence based whereas quantification using the Nanodrop is based on UV absorbance. Elevated yield measurements obtained when using the Nanodrop is due to co-extracted impurities in the eluted DNA contributing to the DNA yield measurements (Guo & Zhang, 2013). However, the Nanodrop measurements were overestimated to a larger degree in the digester samples than in the inlet and slurry samples with majority of the extraction methods (Fig. A2.1). This indirectly implies that the digester extracts contain more impurities, which is not surprising considering that the AD process results in the production of substances such as humic acids. This further highlights the need for DNA extraction methods that suit each stage of the process since impurities and inhibitors are present in varying amounts in the inlet, digester chamber and slurry.

Such co-extracted impurities are undesirable because they may negatively influence downstream applications such as PCR. The Nanodrop is advantageous in that it may be used to determine the approximate level of DNA contamination. This is achieved via the analysis of ratios of absorbances at 260/280 nm and at 260/230 nm which represent protein and aromatic compound (e.g. humic acid) contamination respectively (Roh *et al*., 2006; Weiss *et al*., 2007). Protein contamination was mostly evident in DNA samples extracted with the ZR and PS kits, whereas aromatic compound contamination was evident in all samples with the possible exception of the QIA and Mag-Aut kits (Fig. 2.1C & D). Protein and aromatic compound contamination did not affect PCR for all DNA extracts with the exception of the CTAB method (Fig. A2.2). Furthermore, upon visual inspection the only DNA extract that was not clear was the one that did not amplify using PCR, i.e. the CTAB extract. However, PCR was successful upon dilution of the CTAB extract. Dilution resulted in the reduction in the concentration of contaminants and DNA. However, the dilution of the DNA did not negatively affect the PCR process because large amounts of DNA were obtained using the CTAB method (Fig. 2.1A).

DNA integrity

Cell lysis is the first and fundamental step of DNA extraction methods. For all extraction methods tested, the cells were lysed using mechanical, chemical, heat or combinations of various methods (Table 2.1). Mechanical methods of DNA extraction such as bead beating have been attempted to increase DNA yield via the improved lysis of bacterial cells. However, mechanical lysis may also result in the shearing/fragmenting of genomic DNA (Wintzingerode *et al*., 1997).

In the present study, the indirect method of DNA extraction (EPI) resulted in minimal or no DNA shear (Fig. 2.2). These results corroborate the observations of Roh *et al*. (2006) who showed that DNA shear was more prevalent when using direct extractions as opposed to indirect extractions. Only one direct DNA extraction method resulted in limited/no DNA shear in the present study, namely the paramagnetic extraction method (Mag-Man & Mag-Aut) (Fig. 2.2). All other direct methods, including mechanical, heat and chemical based methods, resulted in a significant amount of DNA shear (as indicated by the smear on the gel in Fig. 2.2). The integrity of the DNA was lowest in the QIA samples where all the extracted DNA was fragmented into between 0.1 and 0.3 kb fragments (the only method with no high molecular weight fragments at all). Interestingly, no bead beating was conducted when using the QIA kit. Cells were lysed using chemical means and heat (Table 2.1). Due to the proprietary nature of the reagents of the kit, we are unable to deduce the reason for the elevated level of DNA shear, but other investigators have also noted that slow cell disruption such as the addition of lysis solution with no additional physical disruption may lead to DNA degradation (Chaudhary *et al*., 2011). The integrity of the DNA obtained using the CTAB method was also low. Unlike with the QIA kit, the CTAB method yielded a portion of high molecular weight DNA but the DNA extract was highly fragmented with fragments that were even smaller than 0.1 kb in size (Fig. 2.2). Fragmented nucleic acids are not ideal because they may contribute to the formation

of chimeric PCR products and are also sources of artefacts in PCR amplification or reverse transcription (Liesack *et al*., 1991; Wintzingerode *et al*., 1997).

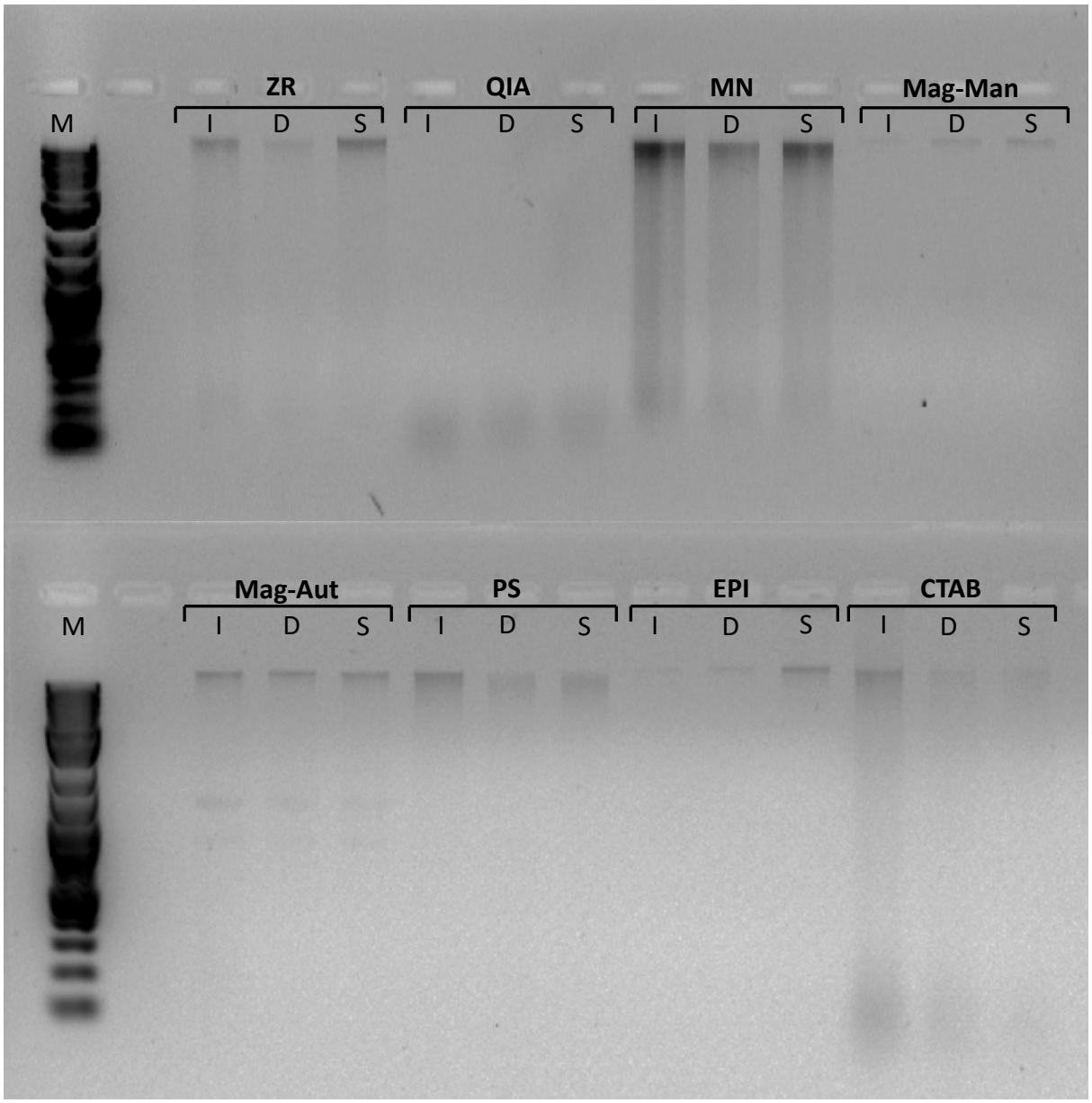

Fig. 2.2: DNA isolated from inlet, digester and slurry using various DNA extraction methods. M = DNA ladder; I = Inlet; D = Digester; S = Slurry; ZR = ZR Soil Microbe DNA MiniPrep – Zymo Research; QIA = QIAamp Fast DNA Stool Mini Kit – QIAGEN; MN = NucleoSpin Soil Kit – Macherey-Nagel; Mag-Man = MagMAX Total Nucleic Acid Isolation Kit – Manual – Thermo Fisher Scientific; Mag-Aut = MagMAX Total Nucleic Acid Isolation Kit – Automated – Thermo Fisher Scientific; PS = Powersoil DNA Isolation Kit – MO BIO Laboratories; EPI = Meta-G-Nome DNA Isolation Kit – Epicentre; CTAB = CTAB based extraction.

Species richness

Theoretically, all DNA extracts from the same source, i.e. inlet, digester chamber or slurry, should have identical species composition (DGGE profiles) because the respective samples originated from the same location. However, visual inspection of the bacterial and archaeal DGGE profiles shows that this was not evident. Some methods of extraction resulted in vastly different or wider community profiles than others (Fig. 2.3). The QIA and CTAB methods of extraction were the least effective at extracting DNA from bacterial species (as demonstrated by the limited banding pattern in Fig. 2.3A) whereas the QIA, MAG-Man, EPI and CTAB extraction methods did not successfully extract DNA from all archaeal species (Fig. 2.3B).

The present study proves that some methods of extraction work optimally for bacterial DNA extraction from AD samples but are not as efficient for archaeal DNA extraction (e.g. EPI; Table 2.3) and vice versa (e.g. ZR; Table 2.3). Whilst other methods of extraction worked poorly for both bacterial and archaeal DNA extraction (e.g. QIA & CTAB; Fig. 2.3). Extraction methods also varied in efficacy based on the stage of AD at which the samples were collected. The highest Shannon Weiner (H') and inverse Simpsons indices (1/D) were observed when using the EPI kit for bacterial DNA extraction from digester and slurry samples. However, the EPI kit was not as efficient when extracting bacterial DNA from the inlet sample (Table 2.3). Inlet, digester and slurry samples vary in the degree of digestion where the inlet samples are the least digested (most granular) and the slurry samples are the most digested (least granular). Granular samples need to be homogenised sufficiently to remove all adhering cells that may be hidden in various crevices of the sample, thus enabling complete downstream DNA extraction, whilst less granular samples require a lower degree of homogenisation for sufficient cell removal and subsequent cell lysis. The EPI kit incorporated a solvent based method of substrate homogenisation (Table 2.1). This method worked optimally for the digester and slurry samples but did not offer adequate homogenisation of the more granular inlet samples. The highest H' and 1/D indices were observed when using the MN kit for inlet, bacterial DNA extraction (Table 2.3). The MN kit incorporated bead beating and cell lysis buffer for sample homogenisation and cell lysis. Bead beating enables the even infiltration of the lysis buffer to the entire sample, regardless of its granularity/consistency, while chemical lysis methods alone may contribute to biases in extraction from granular substrates because the spatial access to all target organisms may be limited (Salonen *et al*., 2010).

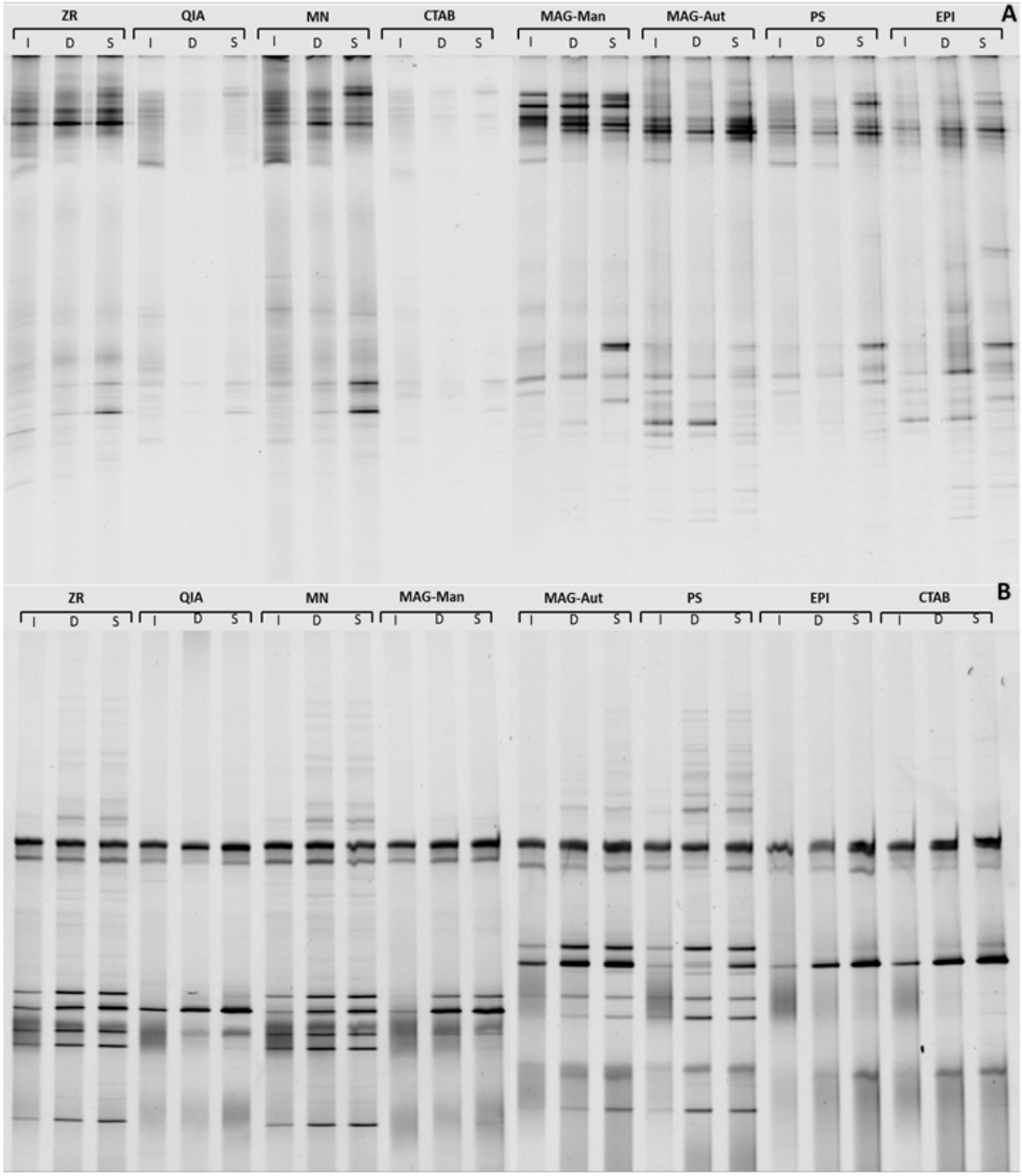

Fig. 2.3: DGGE banding patterns of (A) bacterial & (B) archaeal DNA isolated from samples obtained from the inlet, digester and slurry using various DNA extraction methods. I = Inlet; D = Digester; S = Slurry; ZR = ZR Soil Microbe DNA MiniPrep – Zymo Research; QIA = QIAamp Fast DNA Stool Mini Kit – QIAGEN; MN = NucleoSpin Soil Kit – Macherey-Nagel; Mag-Man = MagMAX Total Nucleic Acid Isolation Kit – Manual – Thermo Fisher Scientific; Mag-Aut = MagMAX Total Nucleic Acid Isolation Kit – Automated – Thermo Fisher Scientific; PS = Powersoil DNA Isolation Kit – MO BIO Laboratories; EPI = Meta-G-Nome DNA Isolation Kit – Epicentre; CTAB = CTAB based extraction.

Table 2.3: Bacterial and archaeal diversity indices obtained when extracting DNA using various extraction methods

	BACTERIA						**ARCHAEA**					
	Inlet		*Digester*		*Slurry*		*Inlet*		*Digester*		*Slurry*	
KIT	**H'**	**1/D**	**H'**	**1/D**	**H'**	**1/D**	**H'**	**1/D**	**H'**	**1/D**	**H'**	**1/D**
ZR	2.3	7.6	2.1	5.6	2.2	6.6	2.4	9.1	2.6	10.5	2.6	11.3
QIA	2.5	10.3	1.4	3.7	2.0	6.2	1.8	5.0	2.0	6.4	1.9	5.4
MN	2.7	11.1	2.4	7.9	2.4	8.6	2.4	9.0	2.6	12.1	2.8	13.0
Mag-Man	2.1	6.1	2.3	7.3	2.2	6.7	1.7	6.0	2.2	7.6	2.1	5.9
Mag-Aut	2.6	10.1	2.2	6.1	2.3	7.1	2.2	8.1	2.0	6.2	2.3	7.6
PS	2.5	10.8	2.3	7.7	2.4	8.2	1.6	3.6	2.6	10.3	2.6	10.6
EPI	2.6	10.3	2.7	10.2	2.6	9.3	1.7	4.4	2.1	7.3	2.1	6.8
CTAB	2.5	11.0	1.8	5.4	2.0	6.1	2.2	7.1	1.9	4.9	2.1	6.4

Unlike with the bacterial DNA extracts, the EPI kit yielded poor banding patterns (Fig. 2.3) and H' and 1/D indices (Table 2.3) for archaeal extracts from all sources (I, D & S). This may be attributed to the differences between the cell surface structure of archaeal and bacterial cells. Bacteria are covered by a peptidoglycan layer whereas the archaeal surface structure is divided into various groups ranging from S-layers to methanochondroitin (König, 1988; Kubota *et al*., 2008). Based on the surface covering, some archaeal cells may be more resistant to lysis than others. The EPI kit incorporated proteinase K and a lysozyme solution for cell lysis. These reagents worked optimally for bacterial DNA extraction but showed limited efficacy on hard to lyse archaeal cells (Fig. 2.3; Table 2.3). Furthermore, the lack of a mechanical lysis stage may have also contributed to the limited observed archaeal diversity. This is corroborated by a study conducted by Salonen *et al*. (2010) where they deduced that mechanical cell disruption was more effective than enzymatic means for the extraction of archaeal DNA. In the present study, the MN extraction method resulted in the highest H' and 1/D indices for archaeal extracts (Table 2.3). The MN kit incorporates mechanical and chemical lysis. Furthermore, a proprietary 'enhancer solution' is also included in the kit. This solution, in combination with the lysis buffer, ensures that the highest possible DNA yield is obtained from the sample.

A better understanding of the link between cellular surface structure and lytic requirement is necessary to enable optimisation of methods of DNA extraction from bacteria and archaea. Hence, bands were isolated from the DGGE gels and sequenced. For the bacterial DGGE profiles, bands that were not well represented in all lanes were excised. These bands represent hard to lyse bacteria. Interestingly, all of the excised bands were gram-positive bacteria (Table A2.1). Gram-positive bacteria are harder to lyse than gram-negative bacteria due to varying cell structures. Gram-positive and gram-negative bacteria have cell walls containing

peptidoglycan but gram-positive cells differ in that the thickness, quantity, length distribution and degree of crosslinking of the peptidoglycan is more extensive than in gram-negative cells (Fig. A2.3) (Cabeen & Jacobs-Wagner, 2005; Mahalanabis *et al*., 2009). The ideal method of DNA extraction should ensure that all cells are equally lysed, including hard to lyse gram-positive and archaeal cells. Some extraction methods such as the MN kit and the Mag-Aut kit were more successful at extracting DNA from gram-positive bacteria than others (e.g. QIA, CTAB; Table A2.1). These MN and Mag-Aut extraction methods incorporated bead beating in the lytic protocol which implies that mechanical methods of cell lysis are effective for some hard to lyse gram-positive bacterial cells.

For the archaeal DGGE profiles, bands were randomly excised. These bands represent a variety of archaeal species, i.e. both hard and easy to lyse cells. Archaea possess cell walls of various types including protein surface layers (S-layer), pseudomurein, methanochondroitin, sheath layers and combinations of the various polymers and the S-layer (Albers & Meyer, 2011; Fig. A2.3). The unusual cell wall structure renders certain archaeal cells resistant to lytic protocols that work well for bacterial cells (Jarrell *et al*., 1991). This was evident in the present study (see EPI kit in Fig.2.2). Interestingly, the sequence data shows that species possessing an S-layer were not lysed using all methods of extraction whereas the species lacking the S-layer were lysed using all extraction methods, albeit at varying levels (Table A2.1). Methods that effectively lysed most S-layer containing cells included the ZR, MN, PS and Mag-Aut methods. The Mag-Man method also successfully lysed some S-layer containing cells, although to a lower degree than the Mag-Aut method (Table A2.1). This may be as a consequence of the rapid agitation achieved by automated DNA extraction in comparison to manual extraction. The ZR, MN and PS methods of DNA extraction are spin column filter based methods that incorporate bead beating and cell lysis buffer for cell lysis (Table 2.1), whereas the Mag-Man and Mag-Aut methods are paramagnetic bead based methods that also incorporate bead beating and cell lysis buffer (Table 2.1). The common variable that may have resulted in adequate archaeal cell lysis may have been the incorporation of bead beating and lysis buffer. The CTAB method also incorporated bead beating in the lysis step, however, the CTAB and EPI methods are solution based (Table 2.1). Both solution based methods yielded poor results for archaeal DNA extraction from cells surrounded by an S-layer.

Bacteria and archaea play integral roles in the anaerobic digestion process. Hence, both are frequently explored when conducting microbial community analyses of biogas reactors (Ariesyady *et al*., 2007; Riviere *et al*., 2009; Sundberg *et al*., 2013). Ideally, a single DNA extraction method should be used for DNA sequestration from both bacteria and archaea since this will minimise costs and time. Of the extraction methods tested the QIA kit generally performed the poorest in terms of both bacterial and archaeal species diversity analyses (Table 3). This may be a function of the reduced integrity of the initial DNA extract (Fig. 2.2) and the reliance on chemical and heat lysis instead of mechanical lysis when using the QIA kit, preventing DNA extraction from hard to lyse cells (Table 2.1 & 2.2). The limited diversity obtained when using the QIA kit in the present study is consistent with what has been previously reported by Claassen *et al*. (2013) and Ariefdjohan *et al*. (2010). The extraction

method that yielded optimal results (H' and 1/D) for both bacterial and archaeal community profiling was the MN kit (Table 2.3).

It may be concluded that all DNA extraction methods tested were successful at extracting DNA from AD samples. However, the yield of extracted DNA varied between methods. The microbial diversity was significantly influenced by the choice of DNA extraction method used. However, there was no correlation between DNA yield and diversity. Maximal species diversity and richness in all samples was achieved when using a spin-column filter-based kit that incorporated mechanical and chemical lysis (MN kit). This study proves that it is important to take the method of extraction into consideration when comparing microbial communities obtained by different researchers and laboratories. This study was also the basis for the selection of the DNA extraction methods that were utilized during the course of all metagenomic analyses that was conducted during the anaerobic digestion of water hyacinth in the project.

3 SUITABILITY OF WATER HYACINTH FOR USE AS FEEDSTOCK IN BIOGAS PRODUCTION

3.1 Literature review

Water hyacinth

South Africa is one of the countries that suffer from the invasion of water hyacinth in many of its aquatic ecosystems. This is because South Africa has the most eutrophic aquatic ecosystems in the world (Coetzee & Hill, 2012). Eutrophication refers to a high level of nutrients in water bodies, and in South Africa this problem can be related to the adopted 1 mg/l of phosphorus standard for all water treatment by the Department of Water Affairs (DWAF). Dams also, create favourable growth conditions for water hyacinth, due to the slow-moving water (Coetzee & Hill, 2012). Dams such as Hartbeespoort (North West province), Roodeplaat (Gauteng province), Kleinfontein (Benoni, Gauteng) and rivers such as the Vaal (Gauteng province) are examples of water bodies with water hyacinth invasion in South Africa.

Literature suggests that the significance of the invasion highly depends on the trophic status of the water body (Coetzee & Hill, 2012). Therefore, the eutrophic state of the water serves as the main reason for the high proliferation rate of water hyacinth as compared to water under the oligotrophic (low nutrients) and mesotrophic state (intermediate level of nutrients). A study by Heard & Winterton (2000) measured the growth of water hyacinth using high and medium nutrient concentrations (0.4-1.6 mg/l nitrogen and 0.025-1 mg/l phosphorus). Their results showed that at high nutrient concentration the plant multiplied quickly, increasing the biomass. Thus, supporting the reason that nutrient-rich water bodies increase the chance of water hyacinth invasion.

Problems related to water hyacinth invasions

Water hyacinth is characterised by high proliferation rates in nutrient-rich water bodies (Deivasigamani, 2013). This enables the plant to cover water surfaces in short periods of time (Yan *et al*., 2016). The high proliferation rate has become a large threat to socio-economic development. This is because the dense mats of water hyacinth produced causes degradation of water quality and consequently limits water utilisation (Shanab *et al*., 2010). Eutrophication and the absence of natural enemies also motivate the high growth rates (Charudattan *et al*., 1995).

Control methods are available which include physical, chemical and biological. Physical method refer to the direct removal or harvesting of the plant from water surfaces manually or mechanically (Vásquez *et al*., 2015). The second type refers to the use of chemical herbicides for the control of the plant. The implications of using chemical herbicides are costs and apart from controlling the target plant, the chemical further degrades the water quality and inhibits the growth of other aquatic organisms. More so, the environmental effect caused by the

herbicides can last for years. The last method uses natural enemies to reduce the dense mats of the weed below the level of economic damage (Vásquez *et al*., 2015).

Despite the available control options, water hyacinth is still the most problematic water weed in countries of the world affected (Coetzee & Hill, 2012; Heard & Winterton, 2000). In South Africa, the three control methods have been implemented. However, environmental factors such as temperature, trophic state and the size of the aquatic ecosystem prevented the success of the control methods in most of the affected water bodies (Malik, 2007; Moran, 2006; Hill & Olckers, 2001).

Water hyacinth as a resource

The biological characteristics of water hyacinth pose a lot of challenges and opportunities to researchers in countries that have been invaded by them. Yan *et al*. (2016), described the biological characteristics of water hyacinth as 'unique' due to the number of capabilities of the plant. Although the plant has a number of negative environmental effects, research efforts have proven that the characteristics of water hyacinth have the potential to overwrite the problems that the plant causes (Malik, 2007; Okoye *et al*., 2000; Patil *et al*., 2014; Z. Wang & Calderon, 2012; Yan *et al*., 2016). Examples of potential uses include anaerobic digestion of the weed to different products such as biogas, alcohol and bio-fertilizer. It has also been found useful in phytoremediation, production of compost, animal fodder, furniture and ropes (Malik, 2007).

The use of biomass for biogas production has become important due to the environmental benefits. Biogas is produced by an important and environmentally friendly process called anaerobic digestion (AD). This is a complex systematic process in which microbial community in the absence of oxygen breakdown and convert macromolecules into simple compounds that can be converted to biogas (Bryant, 1979). Microbial communities are key parameters in the success of the AD process (Leung & Wang, 2016). Thus, the objective of any AD process is to ensure that microbial communities multiply and function properly for process efficiency (Weiland, 2010). More importantly, their performance depends on the availability of nutrients from the substrate (Bryant, 1979).

Nutrients that are required by microbial communities are divided into macronutrients (nutrients that are required in high concentration) and micronutrients (nutrients required in low concentration). Limitation of these nutrients is known to be one of the causes of process failure (Demirel & Scherer, 2008; Liu *et al*., 2014). Thus, their availability during AD is important. Examples of macronutrients include carbohydrates, fats, proteins, potassium, magnesium and calcium. These have different functions and ensure that the microbial communities are multiplying and active. Nutrients such as potassium are known to have an important physiological function to methanogens. It is used to increase cell wall permeability (Kayhanian & Rich, 1995; Scherer *et al*., 1983). Magnesium is found in high concentrations in methanogens and serves as a cofactor for certain enzymatic reactions (Scherer *et al*., 1983).

Examples of essential micronutrients include iron, nickel, cobalt, molybdenum, selenium, and tungsten (Fermoso *et al*., 2009). Extracellular enzymes carry out the first stage of AD (hydrolysis) and the activity of these enzymes is associated with the presence of some of these micronutrients known to form part of the active site of the enzymes (Wu *et al*., 2015). Other functions include the ability to serve as nutrients binding agents such as phosphatases (Oleszkiewicz and Sharman, 1990). Although these trace elements are important, they function better in low concentration. As an example, a study by Demirel & Scherer (2008) observed that dissolved nickel with a concentration greater than 1 gm^{-3} inhibited the last stage of AD. However, the presence of nickel in the AD process at the required concentration ensures good performance and process stability (Demirel & Scherer, 2008).

Moreover, microbial communities differ from digester to digester and the type of substrate used influence these differences. Substrates differ in the composition of certain compounds, some have less carbon and high nitrogen. According to Ziganshin *et al*. (2011), substrates containing less carbon will have different microbial community as compared to substrates containing high carbon content. According to Carballa *et al*. (2015), the more diverse the microbial community structure is the better the AD performance and this diversity is linked to variation in nutrient composition. Thus, analysis of substrate composition prior to AD is important and allows for improvement where necessary. Improvements can be obtained by the combination of different substrates (co-digestion). Such combinations are even more important if the chemical composition of each substrate is known. Thus, this chapter aims to evaluate the suitability of water hyacinth as feed for biogas production by determining the chemical composition of the plant.

3.2 Methods

Raw material

Fresh water hyacinth plants were harvested from the Hartbeespoort Dam in the North West province, South Africa (25°44′51″S 27°52′1″E). The water hyacinth was manually washed using tap water to remove dirt, and the used water was autoclaved before discarding.

Compositional analysis of water hyacinth

The cleaned plant was separated into three samples, the leaves, petioles and roots to evaluate the compositional differences of various parts of the plant. The three samples were oven dried separately at 105°C for 24 hours. The samples were ground and analysed for macronutrients (nitrogen, carbon, phosphorus and potassium). The plant samples were also subjected to micronutrient analysis. The above analyses were carried out at Agricultural Research Council (ARC) – Institute for Soil, Climate and Water (ISCW) analytical services using inductively coupled plasma mass spectrophotometry (ICP-MS). The whole plant was also evaluated for its chemical composition (proteins, fats, carbohydrate, lignin, hemicellulose and cellulose) at ARC-Irene analytical services using the following AOAC official methods 920.39, 934.01, 930.15, 942.05 and 954.01 (Greenfield & Southgate, 2003; Harris, 1970; Robertson, 1978)

3.3 Results

Physicochemical analysis

Compositional analysis of water hyacinth

Four macronutrients were analysed and the results showed varying compositions (Fig. 3.1). The results showed that water hyacinth had high carbon content in all the different plant parts with the leaves containing the highest carbon content (38%). The nitrogen, phosphorus and potassium were below 5% in all the three plant parts. The C/N ratio for the different parts of the plants are as follows; leaves – 8.4, petioles – 7.6 and roots – 10.7. Overall, the C/N ratio was low.

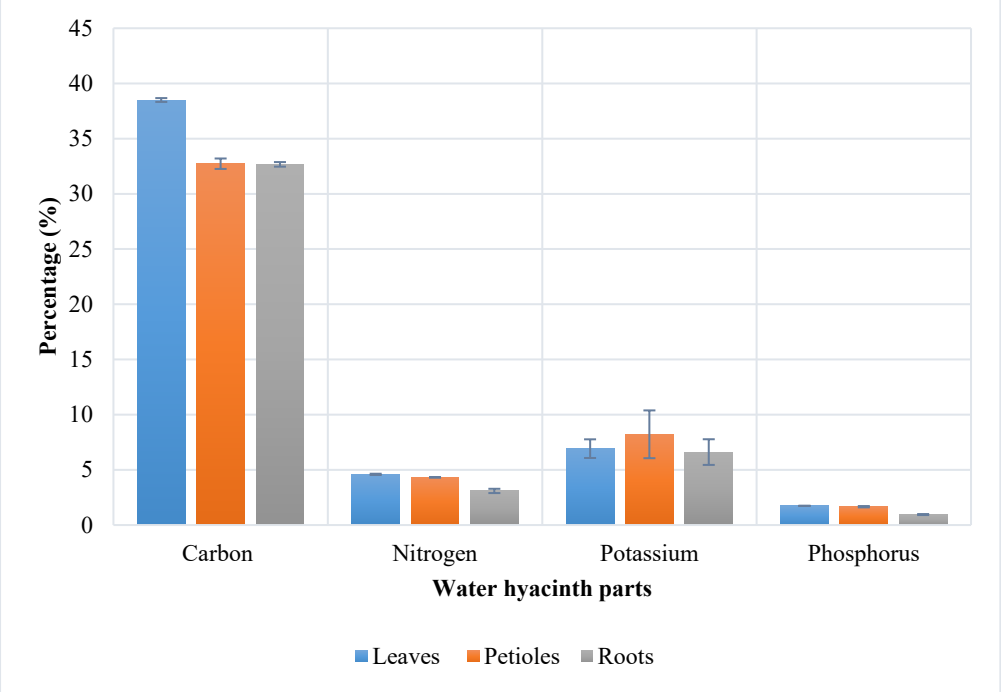

Fig. 3.1: Chemical analysis of different part of the plant, Error bars represent standard deviation (n=3)

Heavy metal scan

The mean concentration of heavy metals from roots, petioles and leaves is represented in Fig. 3.2 A and B. The roots contained more heavy metals in comparison to the petioles and leaves. The plant showed high concentrations of Manganese 4486.5, 372.4, 711.03 mg/Kg for roots, petioles and leaves respectively (not represented in Fig. 3.2 A and B). Other metals that were found in high concentrations include Nickel, Zinc, barium and Titanium (Fig. 3.2 B).

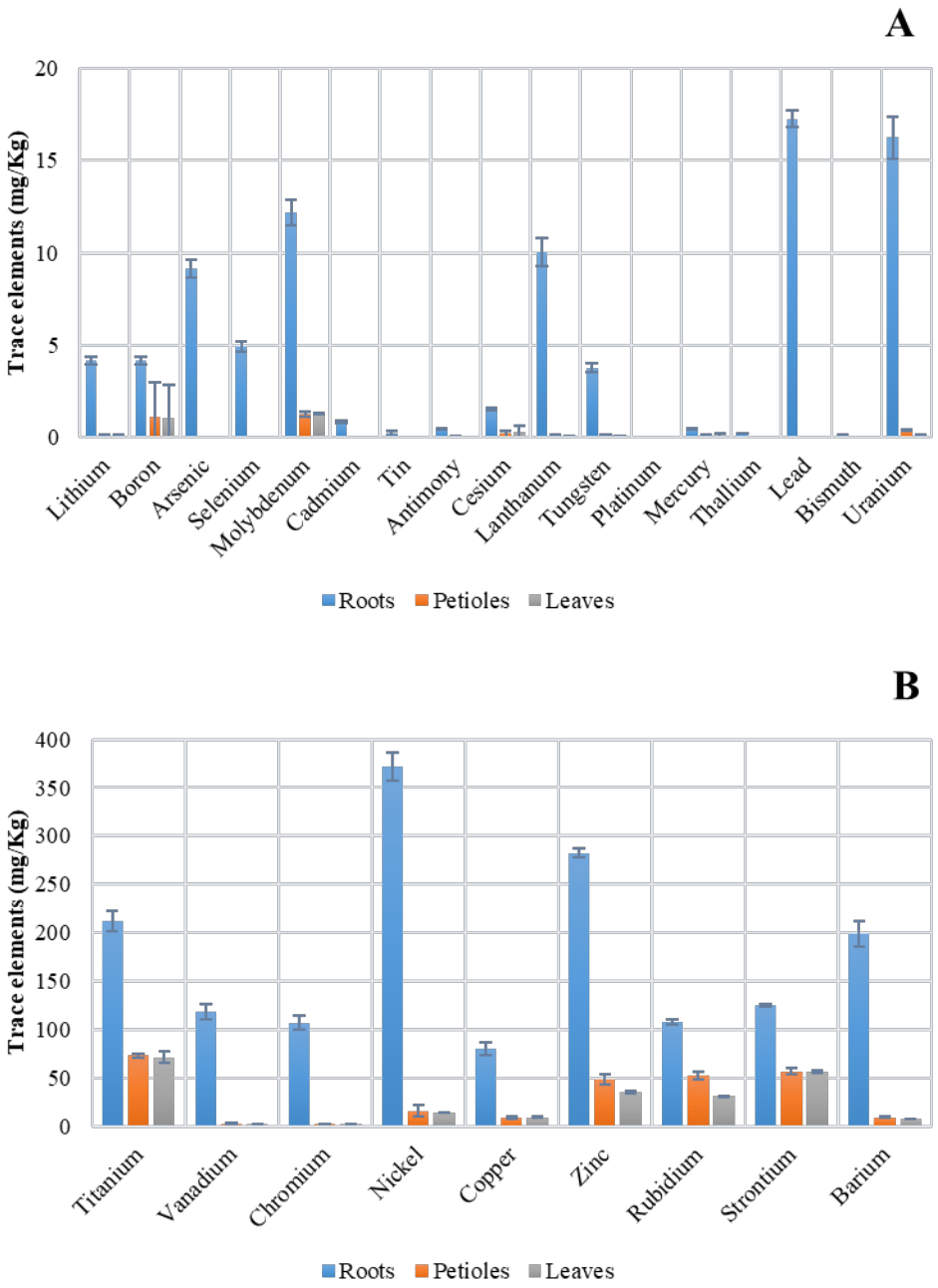

Fig. 3.2: Trace elements from different parts of the plant. **A** – Range of between 0.01-18 mg/Kg and **B** – Range of between 20-400 mg/Kg , Error bars represent standard deviation (n=3)

3.4 Discussion

The suitability of water hyacinth as a feedstock for biogas production was evaluated. The plant was found to contain high carbon content and low nitrogen content (Fig. 3.1). Carbon is used as a source of energy while nitrogen is used for growth (Neubeck *et al.,* 2016; Xie *et al*., 2012). The C/N ratio of the plant was found to be low (low C/N ratio is associated with ammonia inhibition). According to Wang *et al*. (2014) and Yen & Brune (2007) the optimal C/N ratio of

approximately 20-30 is required for CH_4 production without ammonia inhibition. Lower C/N ratio of the plant showed that co-digestion may be necessary during AD.

The amount of potassium and phosphorus obtained from the plant was also low. Potassium is known to increase cell wall permeability and is used mainly by methanogens during AD (Wu *et al*., 2015). In addition, the plant contained important micronutrients such as nickel, molybdenum, selenium and tungsten (Fig. 3.2 A and B). Some of these metals are termed essential due to their functions. They are found in the active site of enzymes responsible for the conversion of complex compounds during hydrolysis (Neubeck *et al.,* 2016). The roots also contained high heavy metal content in comparison to the leaves. High metal content in the roots were expected because the roots are the entry point of the metals before they are transported to other parts of the plant.

In conclusion, the outcome of AD process is dependent on the activity of the microbial community, while the activity of microbial community is dependent on the availability of certain nutrients. Thus, the presence of these macro and micronutrients in water hyacinth makes the plant a suitable feedstock for AD.

4 EFFICIENCY OF DIFFERENT PRE-TREATMENT METHODS IN ENHANCING BIOGAS YIELD

4.1 Literature review

Substrate description

Water hyacinth, like any other lignocellulosic biomass, is composed of different polymers, that is, cellulose, hemicellulose and lignin (Sánchez, 2009) as well as other minor components. These polymers are linked together by different bonds to form a rigid structure, resistant to microbial degradation (Hendriks & Zeeman, 2009). Cellulose is the major component material of the cell wall, representing up to 50% of the support structure (Agbor *et al*., 2011). It is known to exist as an unbranched homopolymer of ß-D-glucopyranose moieties that are connected by ß-(1,4) glycosidic bonds (Agbor *et al*., 2011). It is found in both, crystalline and non-crystalline (amorphous) region as indicated in Fig. 4.1A (Harmsen *et al*., 2010). The amorphous region is composed of movable molecular organisation with large porosity enabling easier accessibility for enzyme adsorption for degradation than the crystalline region (Xie *et al*., 2012).

Hemicellulose represents about 20-30% of the support structure (23-32%) which also serves as a source of sugar during AD. It is a heterogeneous polymer of sugars such as pentose, hexose and acetylated sugars (Agbor *et al*., 2011). Unlike cellulose, hemicellulose occurs in branches of short lateral chains that are easily degraded. Hemicellulose from different plants differ in the sugar composition. For example, agricultural biomass such as grass are composed of xylan while soft wood are composed of glucomannan (Agbor *et al*., 2011).

Lignin differs from cellulose and hemicellulose because it is a rigid and impermeable polymer (Agbor *et al*., 2011), representing around 15-25% of the support structure. Due to its rigid and impermeable structure, it is responsible for preventing microbial degradation of cellulose and the hemicellulose (Kim *et al*., 2016: Hendriks & Zeeman, 2009). In addition, lignin is one polymer of the lignocellulosic biomass that is not degraded during AD. Moreover, the presence of unmodified or reduced lignin during AD inhibits hydrolysis by preventing the accessibly of enzymes to the cellulose and hemicellulose (Xie *et al*., 2012).

Pre-treatment methods

AD occurs in four stages, where complex organic matter is converted to biogas (a mixture of gases mainly methane and carbon dioxide). The stages include, hydrolysis, acidogenesis, acetogenesis and methanogenesis (Leung & Wang, 2016). Hydrolysis is known as the rate limiting stage. In this stage, extracellular enzymes secreted by microorganisms breakdown complex compounds into simple compounds. However, lignocellulosic biomass as indicated in Fig. 4.1 A, presents a number of challenges when it comes to the accessibility of cellulose and hermicellulose due to the protective barrier (lignin) (Carlsson *et al*., 2012). This causes a delay in the hydrolysis stage which consequently affects the whole process

(Carlsson *et al*., 2012). Thus, the disruption or modification of the lignocellulosic structure is required to release and make the sugar available for biodegradation (Fig. 4.1B).

Currently, the focus is on substrate pre-treatment and the possible advantages the methods have to offer. Pre-treatment is a method currently available to improve the solubility and bioavailability of the organic matter to increase the bioavailability of the cellulose and hemicellulose (Alvira *et al*., 2010). Once lignin is modified, the hydrolytic enzymes that carry out the first stage of AD, hydrolysis, are able to easily convert the cellulose and the hemicellulose to fermentable sugars (Harmsen *et al*., 2010). There are three main pre-treatment methods currently used for the disruption of the lignocellulosic structure to modify lignin and the methods are elaborated below.

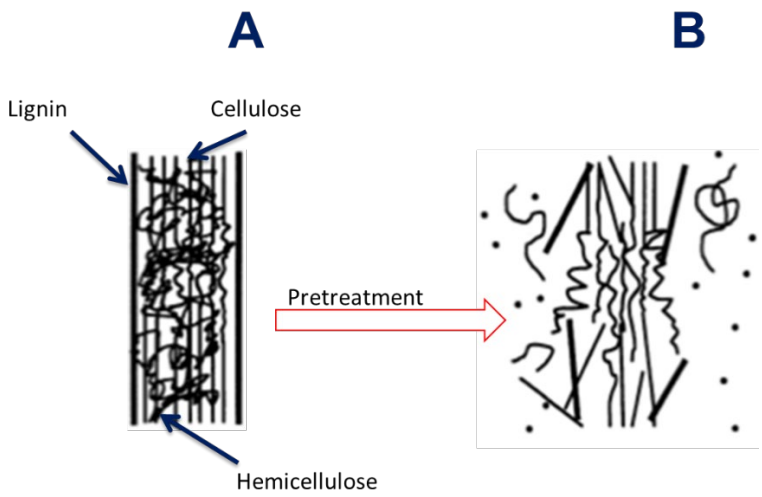

Fig. 4.1: Lignocellulosic structure with the three polymers. (A) and the effect of pre-treatment on lignocellulosic structure (B) (adapted from (Harmsen *et al*., 2010)).

Physical pre-treatment

Physical pre-treatment methods include mechanical (grinding, chipping, homogenising, and milling), microwaves, ultrasound, steam explosion, and liquid hot water (Agbor *et al*., 2011; Harmsen *et al*., 2010). The function of mechanical pre-treatment is the reduction of substrate size (between 5 cm to few mm) and reducing the degree of polymerisation thereby increasing the surface area for rapid enzyme digestibility during hydrolysis (Harmsen *et al*., 2010). Each type varies in the mechanism it uses, for example, chipping reduces biomass size and enhances heat and mass transfer during AD, while grinding and milling are more effective in particle size as well as crystallinity reduction (Agbor *et al*., 2011). Other physical methods such as ultrasound and microwave mechanisms involve the cleavage of β-1, 4-glycosidic bonds increasing the accessible surface area and reducing the crystallinity of cellulose (Lin *et al*., 2015). Energy requirements for mechanical pre-treatment depend on substrate characteristics and the required particle size. For example, woody biomass will require more energy as compared to grass or agricultural waste.

Chemical pre-treatment

The second method is the use of chemicals to initiate the disruption of the lignocellulosic structure. Classification based on pH divides the chemical pre-treatment into acidic, alkaline and neutral (Agbor *et al*., 2011). Examples include weak or strong acid hydrolysis, alkaline hydrolysis, oxidative hydrolysis and the use of ionic liquids (Ariunbaatar *et al*., 2014). Alkali pre-treatment mechanism causes swelling of the biomass and breaks the linkage between the lignin and the carbohydrates, thus increasing the internal surface as well as reduction of the degree of polymerisation (Agbor *et al*., 2011). However, the alkaline pre-treatment is more efficient when used to treat biomass with low lignin content as compared to biomass with high lignin content (Agbor *et al*., 2011). Acidic pre-treatment is not preferred due to its high corrosive ability as well as the fact that after pre-treatment it requires the addition of alkaline to neutralise it. Gao *et al*. (2013) evaluated the effect of ionic liquid pre-treatment and it was found that the crystallinity of cellulose was decreased and biogas yield was increased.

Biological pre-treatment

The third method is biological pre-treatment, which involves the use of microorganisms that produce extracellular enzymes or commercially available enzymes to disrupt the lignocellulosic structure (Ariunbaatar *et al*., 2014). The enzymes used or produced have the ability to biodegrade all the three polymers of the lignocellulosic biomass (Agbor *et al*., 2011). However, using this method on an industrial scale has a number of disadvantages. The microbial decomposition of the substrate requires time (10-14 day), space and careful growth conditions. In addition, the microorganism may utilize some of the carbohydrates during the process (Alvira *et al*., 2010). However, the biological pre-treatment can be used in combination with other pre-treatment methods, like in the study by Ofoefule *et al*. (2009). They combined the physical and biological pre-treatment methods. They sun dried the water hyacinth, chopped the dried matter and soaked it in water for partial decomposition by microorganisms and after digestion, the methane produced was higher.

Combination of various pre-treatment methods

From the three pre-treatments above, it can be seen that each method uses a different mechanism to solubilise substrates. Studies have shown that combining pre-treatment methods with different mechanisms for solubilising substrates assist in substrate solubilisation (Ariunbaatar *et al*., 2014). As mentioned, hydrolysis is the rate-limiting step. Theoretically, the purpose of pre-treatment is to open up the cell wall to allow the hydrolytic enzymes to breakdown the molecules (Demirbas, 2007). However, because of the differences in the type of pre-treatment method used sometimes pre-treatment methods do not give the expected results, which ultimately affects biogas yield. An effective pre-treatment method should have the following advantages (Agbor *et al*., 2011).

- It should preserve and decrystallise the celluloses and depolymerize hemicelluloses
- It should restrict the formation of inhibitors which negatively affects the hydrolysis of carbohydrates
- It should avoid sugar degradation
- It should have low energy input and be cost-effective

However, the effect of pre-treatment depends on substrate characteristics. Substrates contain different properties which react differently when subjected to modification (Carlsson *et al*., 2012). Thus, the selection of the pre-treatment method prior to AD is considered crucial.

Selection of pre-treatment methods for specific substrates

Pre-treatment methods can become one of the additional costs required during the AD process. Therefore, when selecting pre-treatment methods a lot is taken into consideration such as the method's efficiency, energy balance, environmental sustainability, capital, operational and maintenance cost (Ariunbaatar *et al*., 2014). In addition, different substrates will require different pre-treatment methods, where plants with high lignin content will require complicated pre-treatment methods, while plant with low lignin content, such as water hyacinth, will require simple pre-treatment. An important aspect of selecting pre-treatment methods is to ensure that the method does not cause sugar degradation. Therefore, in this chapter, the impact of pre-treatment on water hyacinth composition recovery and ability to enhance methane production (accelerate hydrolysis) were evaluated.

4.2 Methods

Inoculum collection

The inoculum was collected from a running 20 L mesophilic lab-scale anaerobic semi-continuous stirred tank reactor (CSTR) with a working volume of 14 L. The reactor was fed every alternate day with 140 g fresh cow dung with water (1:1). The performance of the digester in which the inoculum was collected was stable with an average methane (CH_4) yield of 1450 L gVS^{-1}.

Impact of pre-treatment on water hyacinth composition

The cleaned water hyacinth was subjected to four physical and one combination of physical and biological pre-treatment methods (Table 4.1). The physical pre-treatment mainly focused on varying the particle size, while a combination of physical and biological focused on size reduction as well as the use of naturally occurring aerobic microorganisms to release the sugars.

Table 4.1: Summary of the five pre-treatments

Samples	Pre-treatment	Condition
Physical pre-treatment		
H	Homogenised using a mortar and pestle	Wet
HC	Chopped using a scissor	Wet
OD	Oven dried at 105°C for 24 hours and powdered	Dry
SD	Sun dried for 7 days and chopped	Partially dry
Combination of physical and biological pre-treatment		
HCD	Chopped and allowed to decompose aerobically for 7 days	Wet

Batch assay

A series of batch anaerobic digestion trials were conducted using lab-scale glass anaerobic digesters with a total volume of 500 ml with 250 ml working volume. The batch digesters were set up as follows: 1 – pre-treated water hyacinth and inoculum, 2 – pre-treated water hyacinth without inoculum and 3 – inoculum only (Table 4.2). AD of the water hyacinth with actively digested cow dung slurry (inoculum) was conducted at a ratio of 3:1 (water hyacinth: inoculum) with total solids of 2%. The pre-treated substrate (HC, H, OD, SD and HCD) with the addition of inoculum will henceforth be referred to as 'treatments' and the pre-treated substrate without inoculum will be referred to as controls (HC control, H control, OD control, SD control and HCD control). All assays were conducted in triplicates. The mesophilic temperature range was chosen because it requires less energy input for heating (Levén *et al*., 2007). Tap water was used to make the volume up to 250 ml. The batch bottles were purged with nitrogen for 3 minutes to create anaerobic conditions and sealed with a lid equipped with rubber septa (Silicone cream/PTFE beige, Hardness 55°, shore A, Thickness 3.2 mm) (Monitoring & control laboratories (PTY) LTD). The digesters were continuously mixed at 130 RPM for substrate and heat distribution during incubation for 35 days.

Evaluation of biogas composition

Gas chromatography (SRI 8610C) was used to analyse the composition of biogas. The instrument is equipped with HayeSep D packed column and thermal conductivity detector. The method information in which the gas chromatograph operated was: Oven had an initial temperature of 50°C held for 4 minutes, initial ramp temperature at 20°C per minute and final temperature at 220°C. The thermal conductivity detector was operated at 155°C with reference flow of 20 ml per minute and make up flow of helium gas at 10 ml/min. Biogas samples were taken using a 5 ml gas tight syringe with Luer lock valve (SGE 10MDR-VLLMA-GT) to obtain the percentage of CH_4 and CO_2 produced. At the beginning and end of digestion trials, the pH of the substrate and digestate were measured using a pH meter (AD1030).

Statistical analysis

The data was subjected to an appropriate analysis of variance (ANOVA). The Shapiro-Wilk's test was performed on the standardized residuals to test for deviations from normality (Shapiro and Wilk, 1965). Student's t-LSDs (Least significant differences) were calculated at a 5% significance level to compare means of significant source effects (Snedecor & Cochran, 1967). The above analysis was performed using Genstat Release 18 and SAS version 9.3 statistical software (SAS, 1999).

4.3 Results

Impact of pre-treatment on water hyacinth composition

The comparison of components recovered after pre-treatment methods is listed in Table 4.2. All the physical pre-treatment methods recovered approximately similar percentages of cellulose and hemicellulose (with the exception of OD for cellulose). Whereas, the HCD pre-treatment method resulted in a lower recovery of cellulose. The lignin recovery was highest from HCD while SD resulted in the lowest recovery.

Table 4.2: Lignocellulosic components of water hyacinth

Components (% dry matter)	Physical pre-treatment				A combination of physical and biological pre-treatment
	H	HC	OD	SD	HCD
Cellulose	20.22	20.36	17.01	21.39	17.10
Hemicellulose	22.47	22.17	28.08	19.97	19.97
Lignin	4.87	7.69	9.57	3.87	10.88

pH

The pH of the treatments before and after AD is represented in Table 4.3. The mean pH was in a range of 7.2-7.6, with HCD having the highest pH value before AD. After AD, the pH of the treatments slightly increased to a range of 7.6-7.7, except for HCD in which the pH remained the same. A different trend was observed with the controls where the comparison of the pH after AD (in a range of 6.2-7.6) to the initial pH (in the range of 7.0-8.2) varied. For some controls, the pH increased while for others the pH decreased. The pH drop was the most pronounced in the H control where the pH decreased from 7.3 to 6.2.

Table 4.3: pH before and anaerobic digestion for the 5 pre-treatments

Pre-treatments	Before AD		After AD	
	Samples	Control	Samples	Control
HC	7.4±0	7.7±0	7.6±0	7.5±0.06
H	7.2±0.06	7.3±0.1	7.6±0.1	6.2±0.9
OD	7.2±0.06	7.0±0.06	7.7±0.1	7.5±0.06
SD	7.2±0.06	7.0±0.1	7.6±0.06	7.4±0.11
HCD	7.6±0.23	8.2±0.07	7.6±0.06	7.6±0
Inoculum	7.6±0.06		7.2±0.15	

*n=3; ± standard deviation

Evaluation of inoculum effect

Cumulative CH_4 for the physical pre-treatment method assays is represented in Fig. 4.2. The CH_4 production for the treatment H, HC, OD and SD started on day 3 and greatly increased until day 17 before stabilising. Treatment H, HC and OD produced the highest cumulative CH_4 of above 0.7 L while SD produced around 0.63 L at day 17. At day 35, SD produced the highest cumulative CH_4 of 0.95 L, while the cumulative CH_4 remained below 0.9 L for the remaining 3 pre-treatment methods. A different trend was observed in the controls, which slowly produced small amounts of CH_4 from day 3 to day 13 and increased slightly until day 21 before stabilising. However, the cumulative CH_4 production from H control only slightly increased around day 29. The controls produced the highest cumulative methane of 0.4 L from the OD control on day 21. At day 35, the highest cumulative CH_4 was 0.5 L from the SD control, while the H control produced the lowest amount of 0.19 L CH_4.

Cumulative CH_4 production for the combination of physical and biological pre-treatment method (HCD) is presented in Fig. 4.3. Cumulative CH_4 production started slowly on day 3 and increased until day 35. Cumulative CH_4 production of 0.35 L at day 17 was obtained and slightly increased to 0.49 L on day 35. HCD control produced 0.25 L of CH_4 showing a similar trend observed in H control from the physical pre-treatment methods.

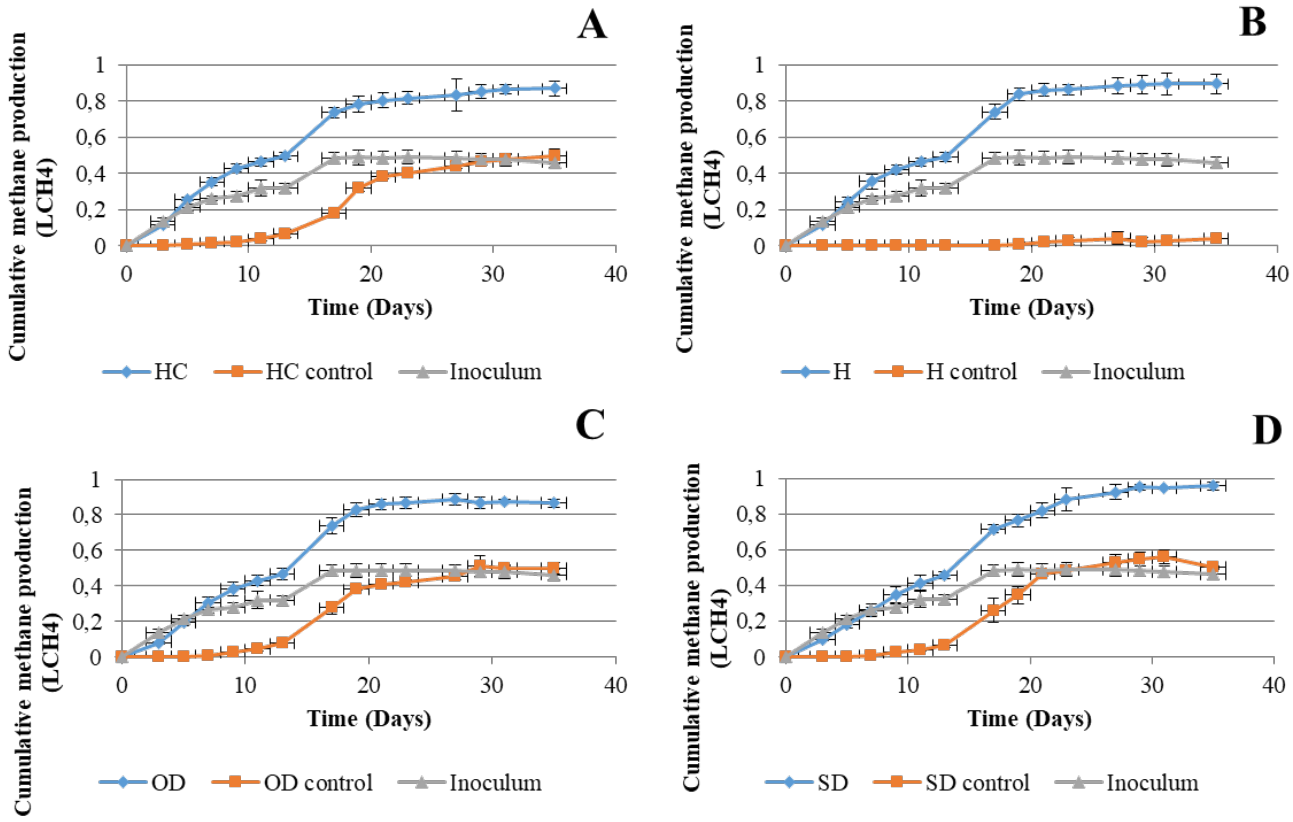

Fig. 4.2: Cumulative CH_4 production for physical pre-treatment methods; A – hand cut, B – homogenised, C –oven dried and D – sun dried, error bars represent standard deviation (n=3)

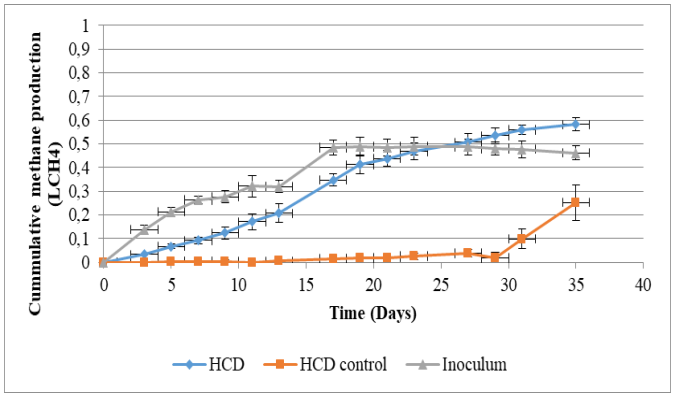

Fig. 4.3: Cumulative CH4 production from a combination of physical and biological pre-treatment method (HCD). Error bars represent standard deviation (n=3)

4.4 Discussion

The ability of simple pre-treatment methods to enhance biogas yield was evaluated. The comparison of the two pre-treatments showed the highest methane content from all physical pre-treatment method. The inability of HCD to enhance methane content is related to how the pre-treatment method was carried out. During the process of biological pre-treatment, the microorganisms consume some of the carbohydrates, thus reducing the amount of carbohydrates that remain after pre-treatment (Agbor *et al*., 2011). This was evidenced when comparing the impact of pre-treatment on composition (HCD resulted in the lowest recovery of biodegradable polymers). Moreover, for water hyacinth, 7 days of decomposition was too long. The elevated duration of decomposition resulted in the consumption of a large amount of carbohydrates.

The treatments and controls of physical pre-treatment were able to produce high CH_4 yield. Thus implying the suitability of the pre-treatment method's ability to make nutrients available for microbial communities. However, the treatments performed better than the controls. This can be linked to the addition of inoculum which contained active microorganisms already adapted to AD process (Xie *et al*., 2012) in the treatments. Thus, allowing rapid CH_4 production unlike in the controls in which the microbial community had to adapt (long lag phase) before CH_4 production increased. However, the presence of microbial communities associated with water hyacinth plant with the ability to produce CH_4 was interesting.

The ultimate purpose of evaluating different pre-treatment methods of water hyacinth was to select the most efficient method for future biogas trials when using the plant as a feedstock. There was no significant difference ($P>0.05$) among all physical pretreatment methods used thus, the selection of pre-treatment methods for further analysis was based on time consumption, water and the performance of the pre-treated sample in the absence of inoculum. SD and OD pre-treatment methods were not selected for further analysis because the process of drying removes water from the plant, implying that the addition of more water will be necessary during AD in comparison to when utilising non-dried feedstock. H pre-treatment method was also not selected. H control was the only control method that resulted in process failure implying that the process of homogenising may have destroyed or damaged most of the methanogens within the plant and the majority of them were unable to recover during AD. HC pre-treatment method was selected for further analysis.

The differences observed in the pH trend between the treatments and the controls emphasize the importance of co-digestion with actively digested cow dung slurry which is known to have qualities such as buffering capacity and a variety of compounds (Mitchell *et al*., 2015). All the treatments' pH increased after AD because the inoculum contained microorganisms that were able to convert protein-rich organic matter into compounds such as carbonate and bicarbonate that neutralised the acid produced during the first three stages of AD (Mitchell *et al*., 2015). This process is called alkalinity and is preferred during AD because it keeps the pH between 7 and 8.

5 METAGENOMIC LINK BETWEEN ENHANCED BIO-METHANE PRODUCTION AND PRE-TREATMENT OF WATER HYACINTH (*EICHHORNIA CRASSIPES*)

5.1 Literature review

Water hyacinth (*Eichhornia crassipes*) is a harmful aquatic weed, which has one of the highest growth rates of all plants in the world. This fast growing property causes numerous ecological problems in infested waterways. While several strategies have been proposed for the elimination or control of this plant, few of these strategies are economically viable and environmentally friendly (Santibañez-Aguilar *et al*., 2013). The utilisation of water hyacinth as a feed for anaerobic digestion (AD) is attractive due to the numerous benefits of the AD process, which include control of the plant, energy production in the form of biogas and organic soil ameliorant production. Water hyacinth has high potential as a feed for biogas production due to its elevated cellulose (20%) and hemicellulose (48%) content, and low lignin content (3.5%) (Patil *et al*., 2011; Sindhu *et al*., 2017). Furthermore, the plant exhibits elevated productivity ensuring availability of a sustainable biomass source for AD. Another advantage of the utilization of water hyacinth as a feed for AD is the capability of the plant to grow in water alleviating the need for competition with food crops for arable land (Sindhu *et al*., 2017).

However, water hyacinth is a lignocellulosic material hence pre-treatment of the plant is necessary to optimise biogas production (Patil *et al*., 2011; Sindhu *et al*., 2017). Pre-treatment involves the breakdown of complex organic material to simpler molecules which are more accessible to the microbial communities involved in the AD process (Patil *et al*., 2011). Hyacinth pre-treatment has been proven to increase biogas yield (Patil *et al*., 2011, Patel *et al*., 1993, Ofoefule *et al*., 2009, Gao *et al*., 2013). Various pre-treatment methods have been exploited including physical, chemical and biological methods (Patil *et al*., 2011, Sindhu *et al*., 2017, Patel *et al*., 1993, Ofoefule *et al*., 2009, Gao *et al*., 2013). Although numerous studies have been conducted on the effect of method of substrate pre-treatment on biogas yield (Patil *et al*., 2011, Patel *et al*., 1993, Ofoefule *et al*., 2009, Gao *et al*., 2013), the metagenomic link between biogas yield and method of pre-treatment has been neglected. Anaerobic digestion is microbial driven hence it is important that more emphasis be placed on the microorganisms involved in the process (Roopnarain *et al*., 2017).

The present study was undertaken to investigate the effect of various methods of hyacinth pre-treatment on microbial (bacterial and archaeal) diversity and concomitant biogas yield.

5.2 Methods

Experimental setup

Water hyacinth obtained from the Hartbeespoort Dam (North West Province, South Africa, 25°44′51″S 27°52′1″E) was used as feedstock in this study. Batch culture experiments were set up in 500 ml Schott® bottles (250 ml working volume) equipped with lids containing rubber septa. The pre-treatment methods tested included physical and biological treatments. Water hyacinth was pre-treated as follows; homogenization (H), hand cut (HC), oven dried (OD), sun

dried (SD) and hand cut and decomposed at room temperature for 7 days (HCD). Inoculum was obtained from a running 20 L mesophilic lab-scale anaerobic continuous stirred tank reactor (CSTR) (14 L working volume). The reactor was fed every alternate day with 140 g fresh cow dung with water (1:1). Inoculum was harvested when the digester performance stabilised with an average bio-methane yield of 1.45 L/kg VS.

The batch AD assay was carried out using the pre-treated hyacinth as feedstock in the presence and absence of the inoculum. Treatments that included inoculum were mixed at a ratio of 3:1 (hyacinth: inoculum). All batch cultures contained 2% total solids (TS) and were conducted in triplicate. The treatments were incubated at 30°C and continuously mixed at 130 RPM for 36 days.

Biogas yield and composition

Biogas compositional analysis was conducted midway through (day 15 – henceforth phrased as 'Mid') and at the end of the experiment (day 35 – henceforth phrased as 'End') using a SRI 8610C gas chromatograph (SRI Instruments, CA, USA) equipped with HayeSep D packed column and thermal conductivity detector (TCD). Biogas yield was determined by measuring the pressure build-up in the headspace of each batch bottle using a digital manometer (MP 210 Kimo® thermo-anemo-manometers, kimo instruments – UK). Biogas production was calculated as described by El-Mashad and Zhang (El-Mashad, 2010).

Microbial analysis

Samples were obtained from the inoculum and each treatment at the Mid- and End time interval for microbial analysis. DNA was extracted using the Powersoil DNA Isolation Kit (MO BIO laboratories) (Roopnarain *et al*., 2017) and quantified using a qubit fluorimeter (Invitrogen, USA, using the Qubit® dsDNA HS Assay Kit). Amplification of bacterial and archaeal gene fragments followed using the primer pairs and amplification profile outlined in Roopnarain *et al*. (2017). Denaturing gradient gel electrophoresis (DGGE) was used to establish community profiles of bacteria and archaea in the respective samples (Roopnarain *et al*., 2017). Resulting gels were stained with GelRed and photographed using a UV transilluminator (GelDoc XR; Bio-Rad Laboratories, Hercules, CA). After digitalisation, bands of interest (dominant or unique) were excised and DNA was eluted overnight in 10 µl sterile distilled water. Resulting DNA was reamplified and sequenced (Roopnarain *et al*., 2017). Sequences were inspected and edited using BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/bioedit/bioedit) and identified using NCBI Blast and EzTaxon (http://www.ezbiocloud.net/eztaxon).

Gel images were analysed using the Image Lab software (Bio-Rad). The resulting data was used to calculate three widely used diversity indices, viz. Shannon-Wiener, Simpsons (Roopnarain *et al*., 2017) and Evenness indices (Ke *et al*., 2013). The data was also used to construct Pareto-Lorenz distribution curves as previously described (Mertens *et al*., 2005). DGGE gels were normalized and community cluster and ordination analysis was conducted using BioNumerics software (version 7.6, Applied Maths, Ghent, Belgium). Similarities between DGGE fingerprints were shown in dendrograms that were constructed after calculation of the Jaccard correlation coefficient and cluster analysis by Unweighted Pair

Group Method with Arithmetic Averages (UPGMA). Similarities between banding profiles were also analysed by non-metric multidimensional scaling (nMDS).

Statistical procedure

The data was subjected to two-way analysis of variance (ANOVA). Prior to ANOVA, the Shapiro-Wilk's test was performed on the standardized residuals to test for deviations from normality (Shapiro *et al*., 1965). Student's t-LSDs (Least significant differences) were calculated at a 5% significance level to compare means of significant source effects (Snedecor *et al*., 1967). The above analysis was performed using Genstat Release 18.

Canonical Correspondence Analysis (CCA) was carried out to correlate community composition (DGGE patterns) with performance variables (carbon dioxide and methane yield). Band matching tables based on densitometric values (exported from BioNumerics) and biogas measurements were input into XLSTAT (XLSTAT, Belmont, MA, USA) to construct CCA plots. The significance of the relationship between community structure and performance variables was tested by Monte Carlo permutations test (n = 999).

5.3 Results & Discussion

This study investigated the influence of various hyacinth pre-treatment methods on microbial diversity and methane production. Elevated methane yield was evidenced in all pre-treatment methods that included an inoculum in comparison to the treatments lacking the addition of inoculum. However, the HCD method (with and without inoculum) yielded significantly lower methane and carbon dioxide yields than all other treatments ($P < 0.05$) (Fig. 5.1). This method (HCD) involved the decomposition of water hyacinth for seven days with naturally resident microorganisms. Decomposition of substrates result in the breakdown of complex polymers into more readily biodegradable components, which should theoretically increase biogas yield (Zheng *et al*., 2009). The minimal biogas yield observed in this study when using the HCD pre-treatment method may be attributed to the elevated duration of decomposition, which may have resulted in the utilisation of simple sugars by resident microorganisms and/or release of inhibitors.

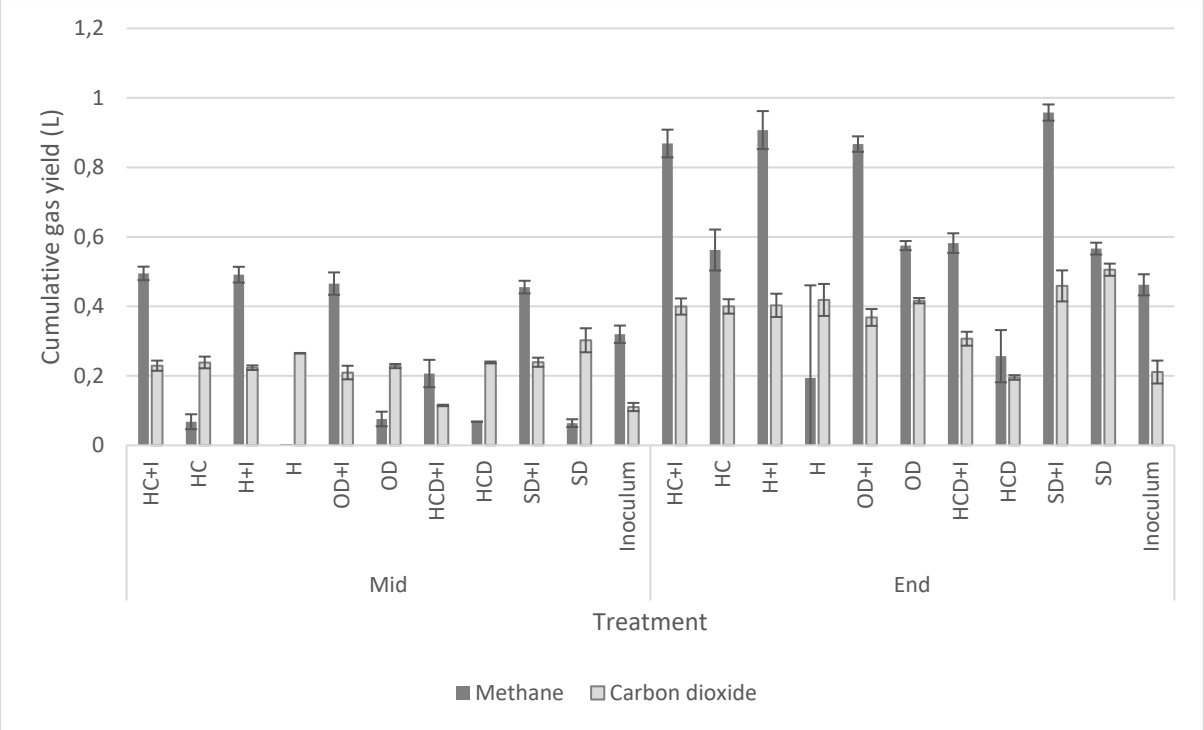

Fig. 5.1: Cumulative biogas production from various substrate pre-treatments (n=3). OD, oven dried; H, homogenised; HCD, hand cut and decomposed; SD, sun dried; HC, hand cut; I, inoculum

The only pre-treatment method that resulted in no methane production during the Mid sampling point was the homogenised hyacinth lacking addition of inoculum. Despite the absence of methane production, carbon dioxide was produced (Fig. 5.1). Methane was produced at the end of the digestion run with the homogenised substrate albeit at a lower level than in the other treatments lacking inoculum (Fig. 5.1). Unlike with the HCD treatment, where both methane and carbon dioxide levels were low (in comparison to all other pre-treatment methods), the H pre-treatment resulted in significantly lower methane levels ($P<0.05$) with no variation in the carbon dioxide levels ($P>0.05$) at the end of the run. This implies that the HCD treatment resulted in complete inhibition of AD (inhibition of all AD stages) whereas the H treatment resulted in partial inhibition (inhibition of one or more AD stages). This could be indicative of either damage of resident methane producing microorganisms during the homogenization process or the release of inhibitory compounds when homogenized water hyacinth was used as a substrate for AD.

Particle size reduction has frequently been used as a strategy to increase biogas yield. The increase in biogas yield induced by substrate size reduction is due to an increase in the accessible surface area of the substrate, decrease in the degree of cellulose polymerization and the reduction in the degree of cellulose crystallinity (Zheng *et al*., 2014). However, AD is a complex process. The reduction/ elimination of one limiting stage may negatively influence other stages. This was evidenced in the H treatment where it is postulated that a reduction in particle size resulted in an increase in hydrolysis and acidogenesis with concomitant increases in volatile fatty acid (VFA) production (decreases in pH – Table A5.1). The accumulation of undissociated fatty acids has been shown to inhibit acetogenic bacteria. Furthermore, elevated

concentrations of acids have been proven to be inhibitory to methanogenic archaea (Brummeler *et al*., 1985).

The distinct influence of homogenization on microbial diversity was verified by the DGGE banding pattern (Fig. 5.2) and hierarchical cluster dendrogram (Fig. 5.3). The microbial diversity evidenced in the H treatment varied considerably from all other treatments without inoculum (Figs. 5.2 and 5.3). Sequencing of isolated bands revealed the absence of *Lentimicrobium saccharophilum* and abundance of *Ruminococcus flavefaciens* in the H treatment (Table A5.2). *L. saccharophilum* was present in large quantities in all other inoculum free treatments tested. One of the primary end products from glucose fermentation by *L. saccharophilum* is acetate (Sun *et al*., 2016). Hence, the species may be key in the acetogenesis stage. The optimal pH for the growth of *L. saccharophilum* is pH 7. Absence of the species in the H treatment may have been induced by reduced pH due to VFA accumulation. *R. flavefaciens* is a rumen bacterium that produces cellulosomes, which exhibit both cellulolytic and hemicellulolytic properties (Cater *et al*., 2015). Proliferation in an AD system may thus improve hydrolysis rates of plant biomass. The abundance of the species in the H treatment in comparison to all other treatments may be due to the particle size of the hyacinth after homogenization. A large surface area to volume ratio potentially facilitated maximal adherence of the species to the substrate. This in turn may have contributed to enhanced hydrolysis, VFA production and eventual inhibition of acetogens and methanogens. Furthermore, the physiological pH range of *R. flavefaciens* is between 6 and 7.1. VFA production and concomitant pH drop in the H treatment may have created the ideal environment for the proliferation of the species. The pH evidenced in the other treatments may have inhibited *R. flavefaciens* growth as indicated by the faint DGGE bands in the respective treatments (Table A5.2).

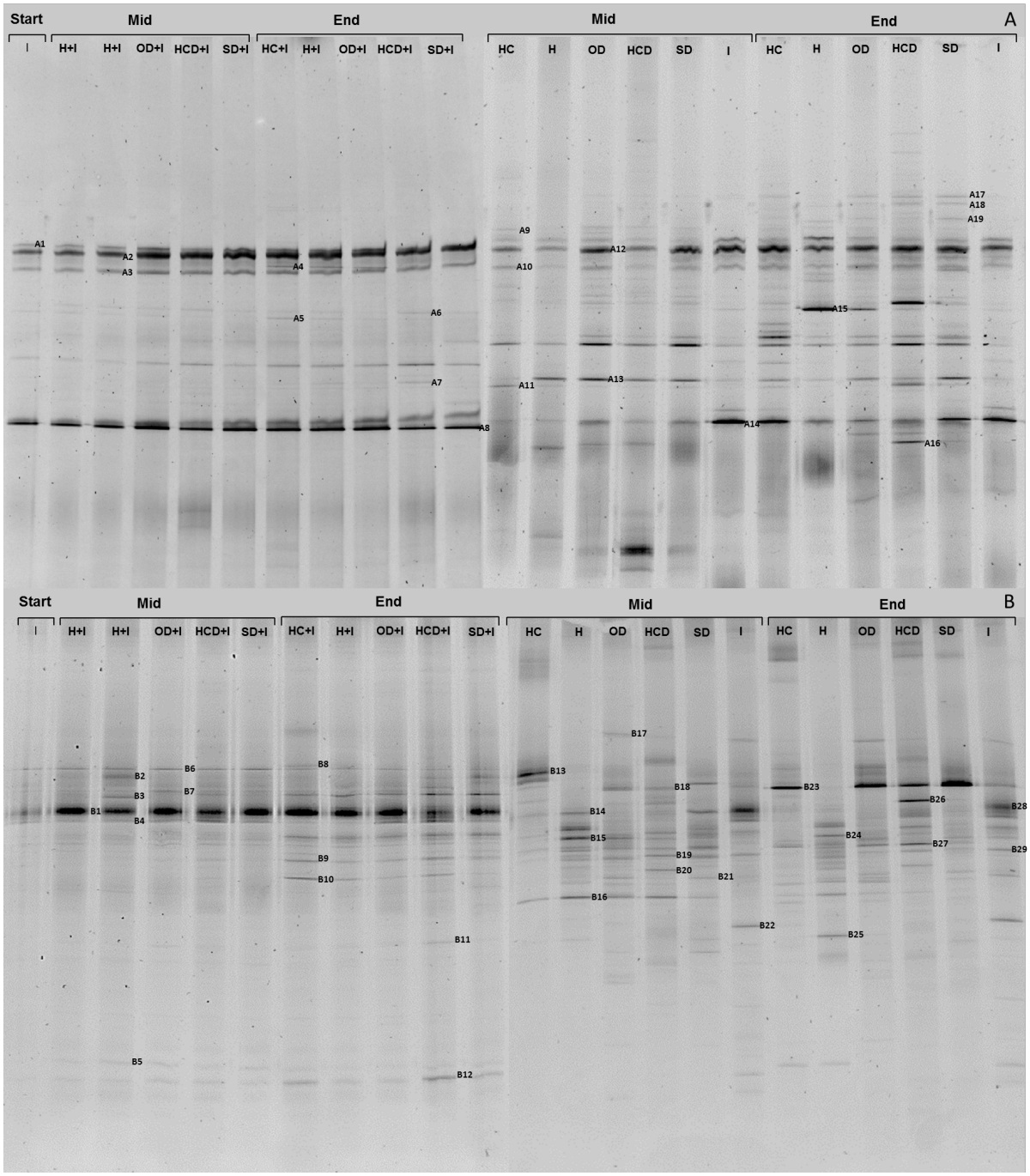

Fig. 5.2: PCR-DGGE image representing (A) archaeal and (B) bacterial communities associated with various methods of substrate pre-treatment. OD, oven dried; H, homogenised; HCD, hand cut and decomposed; SD, sun dried; HC, hand cut; I, inoculum.

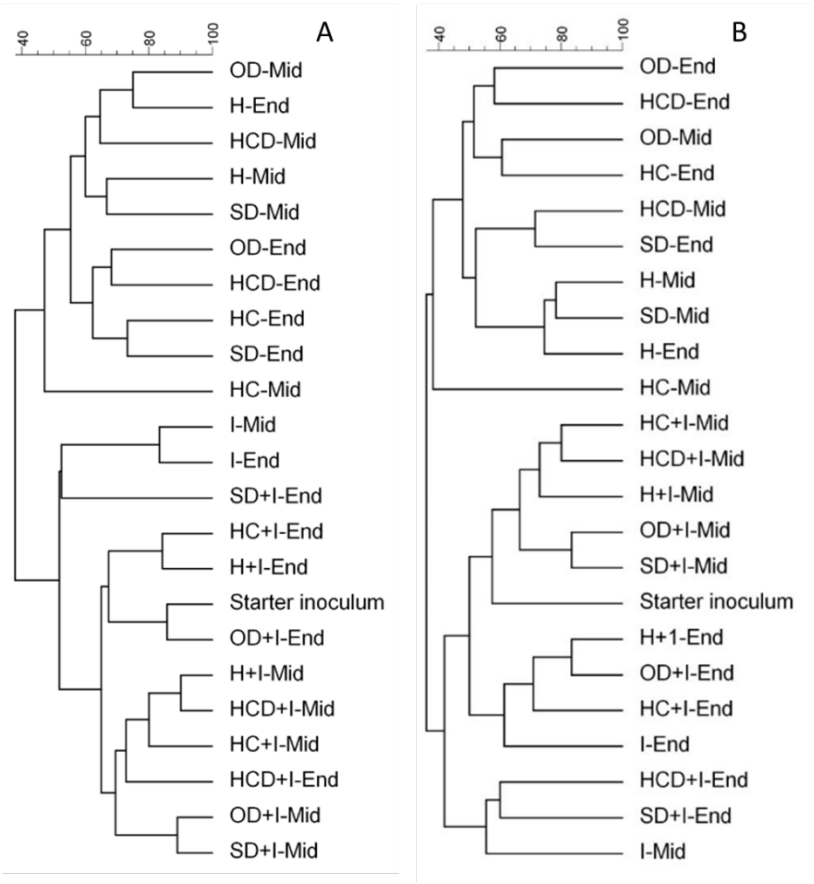

Fig. 5.3: Hierarchical cluster dendrogram (UPGMA) based on DGGE fingerprints of (A) archaeal 16S rRNA amplicons and (B) bacterial 16S rRNA amplicons representing archael and bacterial communities associated with various methods of substrate pre-treatment. OD, oven dried; H, homogenised; HCD, hand cut and decomposed; SD, sun dried; HC, hand cut; I, inoculum

Irrespective of the altered microbial community in the H treatment, methane production was observed at the End sampling point (Fig. 5.1). This may be attributed to the acclimatization of microorganisms to the acidic environment. Environmental stresses may result in the elimination of particular species whereas others may still have the ability to sustain a particular metabolic pathway such as methanogenesis (Zielińska *et al*., 2013). Such acclimated consortia may have applications for the anaerobic digestion of naturally acidic substrates, e.g. fruit and vegetable waste. In fact, it has been proven that sufficiently acclimated low pH consortia resulted in comparable methane production at low and neutral pH (Taconi *et al*., 2008). Therefore, methane yield in the H treatment may have increased to the level of the other treatments without an inoculum had the experiment been maintained for a longer duration.

While variations were observed in microbial populations of treatments lacking an inoculum, the microbial diversity (bacterial and archaeal) for all treatments that included an inoculum were very similar at the respective sampling points (Mid and End) (Figs 5.2 and 5.3). This implies that the addition of an inoculum during the AD process results in microbial community stability. This also reiterates the importance of an inoculum in boosting enzyme activity thereby leading to elevated substrate degradation and biogas yield (Barua *et al*., 2016). Furthermore, the microbial community in the inoculum are adapted to optimize the AD process.

In the present study, the microbial communities in the inoculum seemed to have outcompeted the prevailing bacteria and archaea that were naturally associated with hyacinth (Figs 5.2 and 5.3). This may have contributed to the stability of the H treatment with the addition of an inoculum. The H treatment with an inoculum was potentially less vulnerable to accumulation of VFAs due to the larger population of acetogens and methanogens contained in the inoculum.

Even in the absence of an inoculum, biogas was produced albeit at a lower yield (Fig. 5.1). This indicates that biogas-producing microorganisms were naturally associated with the harvested water hyacinth. Dissimilar community profiles were evidenced in pre-treatments lacking the addition of an inoculum (Fig. 5.1). This suggests that the micro-organisms that were naturally associated with the water hyacinth obtained from the dam were influenced by the method of pre-treatment. Bacterial communities were more susceptible to the method of pre-treatment than archaeal communities as visually depicted in the DGGE banding patterns (Fig. 5.2) and verified in the multi-dimensional scaling ordination plot (Fig. 5.4 – no distinct clustering of the bacterial communities at the Mid and End time points like with the archaea).

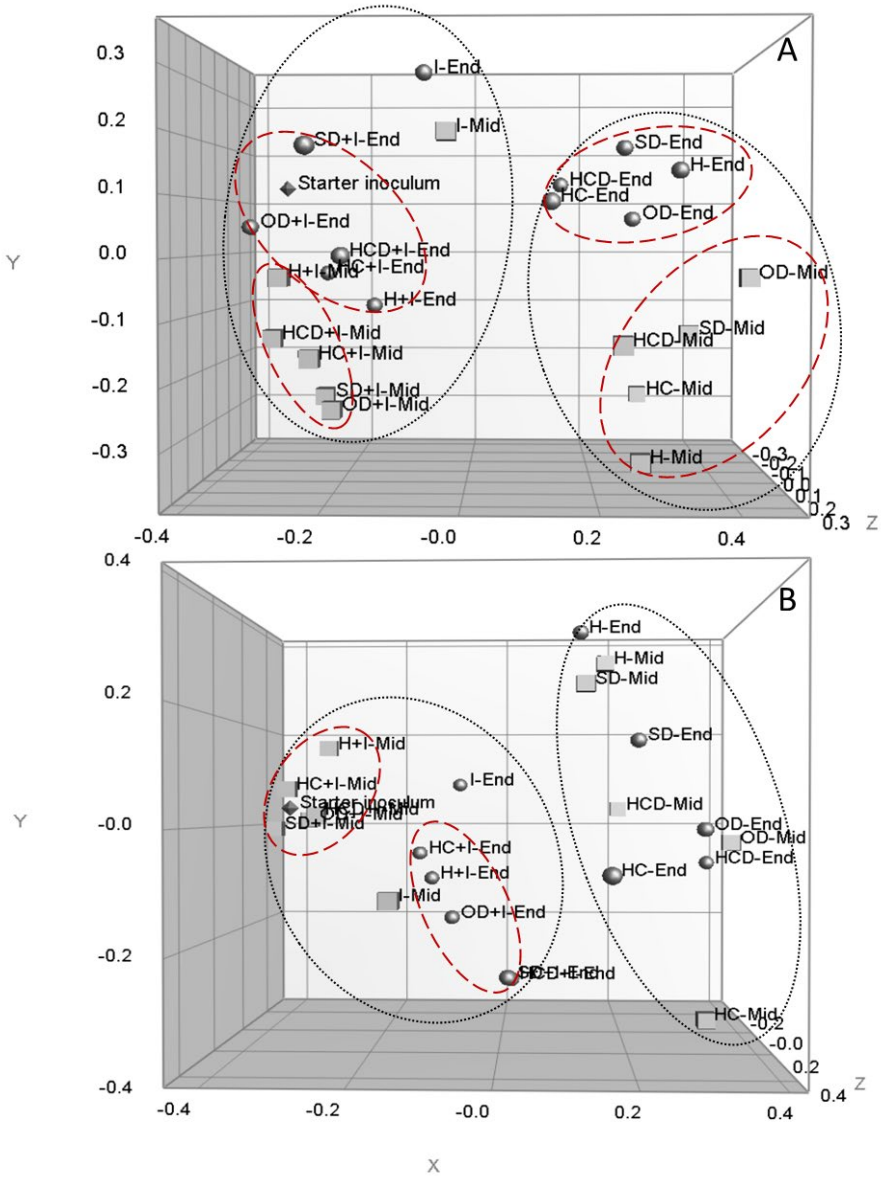

Fig. 5.4: Multi-dimensional scaling ordination plot based on the relative band positions on (A) archaeal and (B) bacterial DGGE profiles. Squares represent profiles obtained mid-way through the trial and circles represent profiles obtained at the end of the trial. OD, oven dried; H, homogenised; HCD, hand cut and decomposed; SD, sun dried; HC, hand cut; I, inoculum

The dominant archaeal bands that were sequenced from all treatments were primarily composed of *Methanosarcina* sp. whereas the dominant bacterial phyla included Bacteriodetes and Firmicutes (Tables A5.2 and A5.3). *Methanosarcina* sp. are robust methanogens that have a high growth rate and are tolerant to various AD stressors such as sudden changes in pH and elevated levels of salt, acetate and ammonium. Furthermore, *Methanosarcina* sp. have the ability to utilise both the hydrogenotrophic and acetoclastic pathways for methane production. Hence, *Methanosarcina* sp. have been identified as key organisms in AD systems (De Vrieze *et al*., 2012). The persistence of *Methanosarcina* sp. even after rigorous pre-treatment methods (e.g. oven drying and homogenization) may be attributed to its large cell size and the ability of the species to grow in clusters (De Vrieze *et al*., 2012). Bacteria belonging to the phylum Bacteriodetes and Firmicutes have been shown to have the capacity to degrade polysaccharides and cellulose (Sun *et al*., 2015). Presence of these bacteria and archaea in AD systems is beneficial due to their contribution to the breakdown of substrates and concomitant biogas production. However, presence in treatments lacking an inoculum implies that the species are naturally found on water hyacinth obtained from the Hartbeespoort dam. This has implications on biomethane production in the dam particularly at lower depths where anaerobic conditions are prevalent. Methane and carbon dioxide emissions from the dam have major implications on the environment. This further motivates the removal of water hyacinth from water ways to ensure that the AD process can occur in a controlled environment and the resulting biogas can be utilized as a source of green energy.

All pre-treatment methods tested that included an inoculum showed lower diversity indices (Shannon-Wiener and inverse Simpsons) than the pre-treatments that lacked an inoculum (Fig. 5.5). Lower microbial diversity (bacterial and archaeal) in the presence of inoculum was expected since the small, AD adapted microbial community in the inoculum outcompeted the large microbial community resident on the hyacinth (Fig. 5.2). The increase in both bacterial and archaeal diversity evidenced at the End time point in all treatments is potentially beneficial to the stability of the AD system. Diversity is often positively related to ecosystem stability. Systems with higher species diversity are generally more likely to resist collapse when exposed to environmental perturbations such as low pH stress in AD systems (Saikaly *et al*., 2005). However, elevated species richness does not imply that the entire community is functional. Pareto-Lorenz (PL) curves were constructed to establish the functional organization of the bacterial and archaeal communities observed from the various treatments.

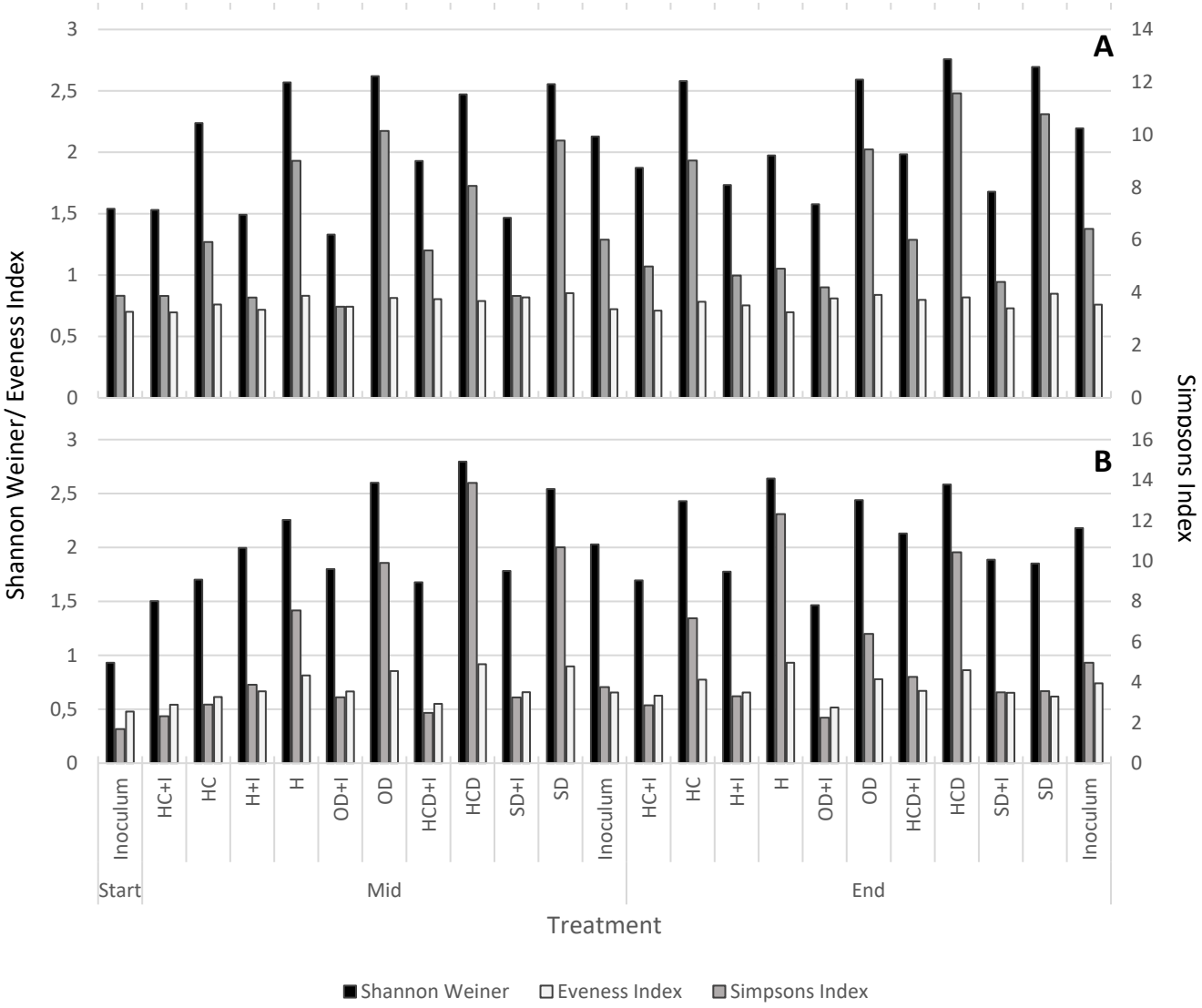

Fig. 5.5: (A) Archael and (B) bacterial diversity indices associated with various methods of substrate pre-treatment. OD, oven dried; H, homogenised; HCD, hand cut and decomposed; SD, sun dried; HC, hand cut; I, inoculum.

Interpretation of the PL curve is based on the degree of deviation of the curve from the theoretical perfect evenness line (45º diagonal, referred to as standard on Figs A5.1 and A5.2). The degree of deviation is directly proportional to the shift in community evenness. The functional organisation (Fo) is determined using the intercept of the PL curve with the vertical 20% x-axis line (see Figs. A5.1 and A5.2). The Fo of bacterial communities ranged from 40 to 80% whereas that of archaeal communities ranged from 37 to 70%. These Fo values represent intermediate to high functional organization and medium to low evenness. Such communities are expected for AD cultures due to the selective pressures that are prevalent during the course of the run that result in the proliferation of particular organisms that can withstand these conditions. Examples of selective pressures include the absence of oxygen and build-up of acids. A general trend that was observed for bacterial communities was larger Fo in treatments lacking an inoculum (approaching 80%) than in samples with an inoculum

(Fig. A5.2). Whilst intermediate Fo is advantageous for community recovery after stress very large Fos can be detrimental to community stability when exposed to stressors. Communities with very high Fo may require longer durations for recovery after periods of stress (Marzorati *et al*., 2008). Such a situation probably occurred in our H treatment that lacked inoculum. The elevated Fo might have influenced the long duration of bacterial community recovery after exposure to low pH. Whereas the same treatment with the addition of an inoculum (intermediate Fo) might have recovered quickly from any low pH stressors. This further emphasizes the importance of an inoculum in AD community stability. Interestingly, unlike with bacterial communities, the Fo of archaeal communities was primarily smaller in treatments lacking inoculum than in treatments with an inoculum (Fig. A5.1). However the archaeal Fo in treatments including inoculum was not as high as 80% therefore duration of archaeal community recovery after stress may not be greatly impacted.

Finally, the combination of molecular and performance data in CCA ordination plots enabled the establishment of the relationship between bacterial and archaeal community structure and biogas production. Canonical correspondence analysis proved that the dynamic changes of microbial populations and biogas production were strongly correlative (Fig. 5.6). Bacterial and archaeal populations associated with cultures with inoculum were more closely associated with methane production than populations obtained from cultures lacking an inoculum (Fig. 5.6). However, as time proceeded considerable successions in bacterial and archaeal populations associated with cultures lacking an inoculum was evidenced. These shifts favoured microorganisms with the ability to produce carbon dioxide and methane (Fig. 5.6).

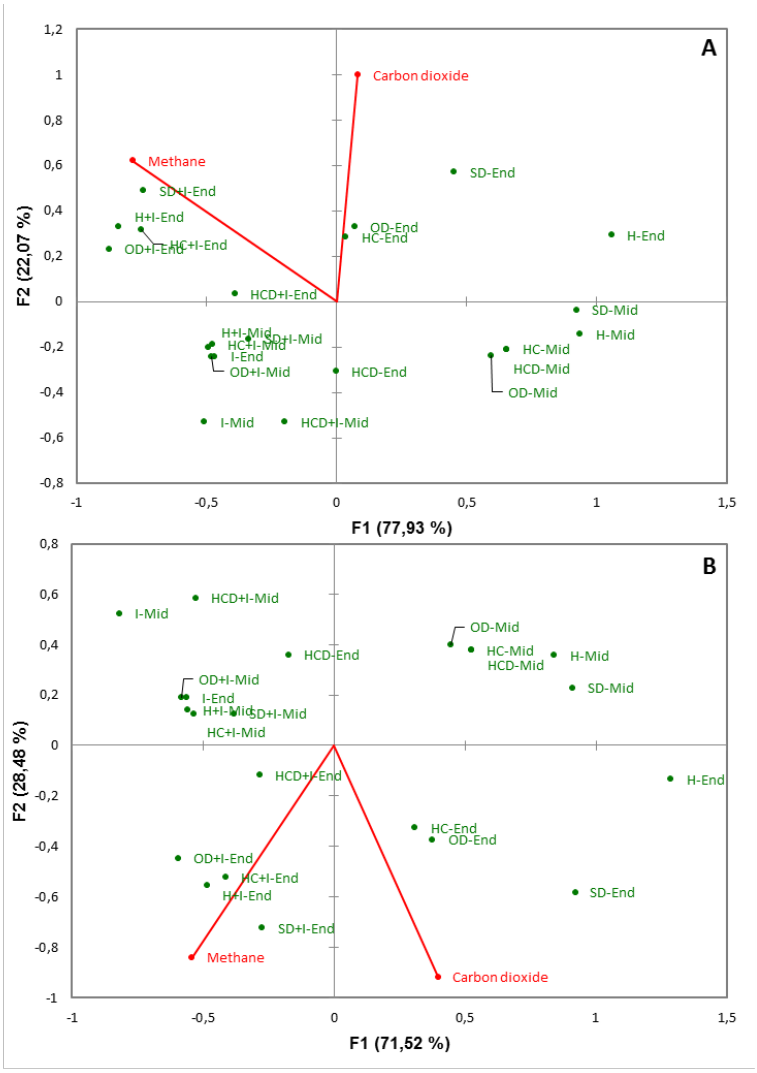

Fig. 5.6: Canonical correspondence analysis (CCA) ordination diagrams showing the correlation between the DGGE bands of archaea (A) and bacteria (B) and the performance variables (carbon dioxide and methane yield).

It may be concluded that there is a direct link between method of water hyacinth pre-treatment, microbial community structure and biogas yield when an established seed culture/ inoculum is not added at the initiation of the anaerobic digestion process. The water hyacinth collected during the course of this study contained indigenous biogas producing microorganisms. Further studies need to be conducted to determine the implications of the use of these indigenous microorganisms as inoculants to augment biogas production from water hyacinth. The presence of biogas producing microorganisms associated with the collected hyacinth needs to be emphasized due to implications on anaerobic digestion in the dam, which would result in biogas release to the atmosphere. Future studies to quantify levels of methane and carbon dioxide released from Hartbeespoort Dam is necessary.

6 EFFECT OF ORGANIC LOADING RATE ON MICROBIAL COMMUNITIES AND BIOGAS PRODUCTION FROM WATER HYACINTH FEEDSTOCK: A CASE OF MONO AND DIGESTION

6.1 Literature review

General operating parameters of anaerobic digestion

As mentioned in the previous chapters, AD is a complex process that is carried out by different microbial communities which require different conditions (Yadvika *et al*., 2004). Therefore, maintaining specific microbial communities in a reactor is essential for optimal biogas production (Weiland, 2010). However, a number of factors that influence or affect microbial activity (Leung & Wang, 2016) can limit the efficiency of AD process. Numerous operational conditions are required for AD process stability (Leung & Wang, 2016). Due to the importance and benefits that the technology has to offer, many studies have been conducted to simplify and optimise the process. The operational conditions such as temperature, pH, C:N ratio, organic loading rate and hydraulic retention time were found to be very important in maintaining process stability (Yadvika *et al*., 2004). These operational parameters need to be held constant throughout the AD process for optimal biogas production. The operational parameters are discussed below.

Temperature

Different temperatures have been used in literature for AD process. These include psychrophilic (10-20˚C) (Sibiya *et al*., 2014), mesophilic (20-40˚C) and thermophilic range (50-60˚C) (Guo *et al*., 2014). The mesophilic temperature with an optimum range of 30-35˚C and thermophilic with an optimum range of 50˚C and higher are commonly used for maximum biogas yield. Microbial communities operate optimally under specific temperatures and this affects the biogas that is produced by the microorganisms in the reactor (Chuang *et al*., 2011).

pH

The pH of the substrate in the digester can be an approximate indication of the state of the AD process but is not suitable as an early indicator of process instability (Brown & Li, 2013). The last stage of AD process is carried out by microbial communities in which their activity is dependent on the pH (Sibiya *et al*., 2014). pH between 6.5-7.5 is considered optimal for AD process (Wang *et al*., 2014). Although each stages of AD is carried out by different microbial communities, these communities work well under the above mentioned pH (Leung & Wang, 2016). The first three stages, known as the acidification stages produce organic acids such as volatile fatty acids (VFA) (Brown & Li, 2013), which are responsible for the decrease in pH while the methanogens work optimally at neutral pH.

Carbon-nitrogen (C/N) ratio

During AD, microbial activities are maintained by the availability of nutrients such as carbon, nitrogen, phosphorus and potassium. Carbon and Nitrogen, however, are considered to be the

most limiting nutrients. Therefore, the C/N ratio is an important indicator for controlling AD and is defined as the mass of carbon to the mass of nitrogen available in the feedstock to be used in the AD process. Carbon serves as a source of energy and nitrogen is used for growth (Leung & Wang, 2016). Therefore, low nitrogen content is associated with slow increasing microbial community and high nitrogen content with fast increasing microbial community (Leung & Wang, 2016). However, breakdown of nitrogen containing compounds results in the production of high levels of ammonia, which must be avoided in AD process.

Improper C/N ratios in AD are usually associated with poor buffering capacity and the possibility of the accumulation of VFAs which will result in pH decrease and eventually the failure of the AD process (Wang *et al*., 2014). Improper C/N ratios are usually caused by the use of a single substrate for anaerobic digestion with high nitrogen content (Leung & Wang, 2016). Therefore, to avoid the production of excess ammonia that might result in process instability, the buffering capacity can be improved by adding feedstocks high in carbon (Rincón *et al*., 2008). This can only be obtained through co-digestion of various feedstock.

The use of different substrates in co-digestion is important since various substrates differ in the carbon and nitrogen content (Wang *et al*., 2014). Kumar (2005) showed that feed from a mixture of water hyacinth and night-soil had improved nitrogen, phosphorus and potassium as compared to feed from water hyacinth alone. Patil *et al*. (2011) reported that water hyacinth is a good biogas producer but blending it with poultry, waste significantly increased biogas yield. Therefore, co-digestion and C/N ratio is very important in process stability and optimum biogas production.

Organic loading rates

In continuous AD, the digesters are fed continuously and organic loading rates (OLR) become important (Rincón *et al*., 2008). OLR is the measure of the quantity of organic matter fed into the digester per unit volume of the digester (Chen *et al*., 2014). Because AD is mainly carried out by microbial communities it is important to take note of the OLR due to the specific organic degradation capacity that the microbial community has (Chen *et al*., 2014). If the OLR is too low, the productivity of biogas will be low and if too high, it will lead to organic overloading. Rincón *et al*. (2008) evaluated the effect of OLR and it was observed that when OLR was increased, it resulted in process instability due to VFAs (up to 6.0 g/L) and the VFAs were assumed toxic to methanogens.

Organic overloading happens when the amount of organics added exceed the degradation capacity of the microbes in the digester (Chen *et al*., 2014). Microbial degradation of organics in AD occurs in a series of steps but the growth rate of acid-forming bacteria is faster than that of methanogens (Chen *et al*., 2016). Organic overloading will result in the increased population of organic acid bacteria, and the production and accumulation of VFAs that cause the pH of the digester to decrease (Rincón *et al*., 2008). Organic overloading (and consequently acidification) may be caused by changes in feedstock mixture and composition and incorrectly measured inputs or increased mixing which suddenly leads to inclusion of unreacted material (e.g. floating layers) into the digestion process.

Hydraulic retention time

Another important parameter is the average time which the feedstock remains in the digester-hydraulic retention time (HRT). The degradation capacity of the microorganism depends on the retention time (Dereli *et al*., 2012). When the digestate (the remaining biomass after anaerobic digestion of organic matter) is removed, active microorganisms are removed (washed out). In a continuous process, if enough HRT is not allowed, it may lead to hydraulic overloading (Dereli *et al*., 2012).This happens when the retention time does not allow enough time for the multiplication of anaerobic microorganisms (especially slow-growing methanogens) and eventually leads to acidification of the digester through the accumulation of VFAs. Therefore, process instability due to the accumulation of VFAs needs to be detected in time to avoid process failure. FOS/TAC ratio is one of the methods used to monitor the stability of the digester and when the ratio is between 0.3-0.4 (equiv. acetic acid/equiv. $CaCO_3$) the process is considered to be in good operating conditions without acidification risk (Rincón *et al*., 2008).

The average time a substrate spends in the digester (to be degraded and converted to biogas) depends on the type of substrate and temperature used. Njogu *et al*. (2015) explained that temperature determines the HRT based on the type of substrate used. For example, the psychrophilic digestion has an estimated HRT of over 100 days, mesophilic has over 20 days while thermophilic has over 8 days (Njogu *et al*., 2015). Substrates can also have an effect on HRT where a biodegradable substrate with low solids content may have short HRT as compared to recalcitrant substrates.

Anaerobic digestion imbalances

AD imbalances arise when VFAs concentration exceeds the buffering capacity of the components in the digester. Characterisation of the feedstock for pH, TS, VS, VFAs, C/N ratio and water content is very important to provide appropriate knowledge about the content of the feedstock before use. These characteristics can also be measured throughout continuous AD. Total solids (TS) is the amount of suspended and dissolved solids and can affect the activity of anaerobic microorganisms. In wet fermentation, which represents the majority of the existing biogas processes, TS content of the feed should not exceed 10%. Yi *et al*. (2014), showed that the substrate TS affects the performance of AD and the change in TS content leads to a change in microbial community structure in the AD system. VFAs are intermediate metabolites that are produced during acidogenesis and are precursors of CH_4. Their reduction in digestate implies that they were converted to CH_4.

General operating parameters of AD provide an overall picture of AD through the identification of process instabilities and avoiding process failure. In addition, microbial communities also provide details related to process stabilities/instabilities. Thus, the identification of the microbial community involved in the process is important. Moreover, certain members of communities may be used as indicator microorganisms in which their presence or absence can be linked to operational conditions and consequently process stability or instability. Therefore,

this chapter reports on how the microbial community structure and composition is influenced by irregular OLR and how biogas production is affected.

6.2 Methods

Inoculum preparation

The inoculum was prepared using a batch reactor operating at a working volume of 3 L. The reactor was initially fed with 10% of fresh cow dung with water (w/v) at a ratio of 1:1. The reactor was incubated at mesophilic temperature for a period of 3 weeks while being continuously mixed at 130 RPM for substrate and heat distribution. The performance of the digester was stable with cumulative CH_4 production of 0.24 L.

Continuous AD assays

The batch section showed that the treatments and the controls were able to produce CH_4, although the controls' CH_4 production was slow at the beginning of the study due to the absence of inoculum. In semi-continuous AD, the ability of the controls to produce CH_4 without process failure was the motivation for evaluating the mono-digestion of the plant in comparison to the co-digestion. In addition, to avoid the long lag phase observed in the controls (the cause of slow CH_4 production at the beginning of the process), inoculum was added.

Evaluation of the effect of organic loading rates on semi-continuous AD

The effect of varied organic loading rates (OLR) on microbial community structure and composition, process performance (biogas production) and stability (FOS/TAC ratio and pH) were evaluated. Two 20 L anaerobic semi-continuous stirred tank reactors (CSTR) operating at a working volume of 10 L were conducted. The two digesters had identical dimensions and configurations. Digester 1 contained hand cut water hyacinth (mono-digestion) while digester 2 contained hand cut water hyacinth and fresh cow dung (co-digestion) mixed at a ratio of 3:1 respectively (Kumar, 2005). Two hundred millilitre of inoculum was used for activation in both digesters and included a start-up period of 17 days (to allow microbial community to increase and produce CH_4). Once microbial community and their function were established, (based on the biogas and CH_4 production) semi-continuous feeding was initiated (collection of 10% digestate and refill with 10% substrate). Semi-continuous feeding with OLR of 1.24 and 1.47 gVS^{-1} for mono and co-digestion respectively once a week was conducted for a period of 21 days (this was referred to as stage 1). Once microbial communities were adapted to the environment, disturbances were initiated where OLR was increased to twice a week this was referred to as stages 2. The microbial community were also allowed to adapt for another 21 days and OLR was changed again (stage 3). In stage 3 the OLR was reduced to once a week, however, the VS were increased to 2.34 and 2.98 gVS^{-1} for mono and co-digestion respectively. The type of AD was wet fermentation, where stage 1 and 2 contained approximately 2% and stage 3 approximately 4% total solids (TS). The calculations of the VS were based on the percentage of TS used (2% and 4%) (The VS in mono-digestion were higher and may be explained by the fact that TS does not contain the same amount of VS (Frigon &

Guiot, 2010)). Biogas production was measured using the gas counter connected to the digesters for digester performance. The biogas content was analysed as explained in batch assay section. Total Biogas production was measured using the gas counter connected to the digesters. CH_4 production in litres was calculated using biogas produced (L) after determining the content of the headspace gas (%) (Duran *et al*., 2006).

Process stability

FOS/TAC ratio and pH

A potentiometer titrator was used to calculate the FOS/TAC ratio and the following formula were pre-programmed in the 877 Titrino plus titrator: TAC = H_2SO_4 – volume added from start to pH 5 in ml x 250 and FOS = (H_2SO_4 – volume added from pH 5 to pH 4.4 in ml x 1.66-0.15) x 500 (Lossie, *et al*., 2008). FOS/TAC ratio was calculated every alternate day. Thirty millilitres of the digestate were centrifuged at 2700 RPM for 20 minutes to remove any coarse components from the digestate. Five millilitres of the supernatant was diluted in 35 ml of distilled water in a beaker. The beaker was placed on the magnetic stirrer, continuously homogenised during the titration process. The titration was conducted by addition of 0.1 N H_2SO_4 until to a pH of five and the volume of acid added was noted (by the equipment), and the titration continues until a pH of 4.4 is reached and the volume of acid added was noted. The pre-programmed formula were used to (automatically) calculate the FOS/TAC ratio. Table 6.1 was used to interpret the FOS/TAC ratio results (Lossie *et al*., 2008) with the exception that digester that uses renewable raw material require FOS/TAC ratio of 0.4 to 0.6 for maximum biogas production. The 877 Titrino plus titrator was also used to measure the pH of the digestate sample before titration.

Table 6.1: Rules for the interpretation of FOS/TAC ratio and actions to be taken

FOS/TAC ratio	Indication	Action to take
>0.6	High excessive biomass input	Stop biomass addition
0.5-0.6	Excessive biomass input	Reduce amount of biomass input
0.4-0.5	Digester is heavily loaded	Monitor digester performance more closely
0.3-0.4	Maximum biogas production	Constant amount of biomass input
0.2-0.3	Biomass input is too low	Slowly increase the amount of biomass input
<0.2	Biomass input is far too low	Rapidly increase the amount of biomass input

Microbial community analysis

Illumina Miseq sequencing

The digestate samples were collected in both digesters as represented in Table 6.2. In stage 1 and 3, retention time of 7 days was allowed before next feeding whereas in stage 2, 4 days retention time was allowed. The DNA was extracted from the collected samples (25 mg each) using DNeasy PowerSoil kit (Whitehead Scientific (Pty) Ltd), in accordance with manufactures instructions. The extracted genomic DNA was quantified using Qubit 2.0 fluorometer

(ThermoFisher, Edenvale, South Africa). The primers 341F (5'-CCTACGGAGGCAGCAG-3') and 805(5'-GACTATHVGGGTATCTAATCC-3') with Illumina overhangs attached to the 5' end of the forward and reverse were used to amplify the hypervariable V3-V4 region of the 16S rRNA gene. The 16S rRNA gene was amplified in a 25 µl reaction containing 12.5 ng DNA template, 5 µM of each primer, 12.5 µl of Tempase HS 2X Master mix (Lasec SA (PTY) LTD, Cape Town). PCR amplification was performed at 95˚C for 3 minutes, 30 cycles of denaturation at 95˚C for 30 seconds, annealing at 55˚C for 30 seconds and extension at 72˚C for 30 seconds; and a final extension at 72˚C for 5 minutes. One percent agarose gel electrophoresis was used to quantify the amplified gene. The prepared samples were submitted to ARC-Biotechnology platform (Pretoria, South Africa), for subsequent processing and sequencing on the Illumina Miseq platform using standard protocols. The sequence reads received were analysed as described by Mashiane *et al*., 2017 using QIIME pipeline.

Table 6.2: Collection of digestate for microbial analysis

Sampling time	**Stage 1**	**Stage 2**	**Stage 3**
Early	3 days after initial feeding (day 20)	3 days after initial feeding (day 41)	3 days after initial feeding (day 62)
Mid	5 days after second feeding (day 29)	2 days after forth feeding (day 50)	5 days after second feeding (day 71)
Late	7 days after third feeding (day 38)	4 days after sixth feeding (day 59)	7 days after third feeding (day 80)

6.3 Results

Process performance and stability

Process performance of the two semi-continuous AD was evaluated by the biogas production and quality of biogas by the methane content, while the stability of the process was monitored by measuring the FOS/TAC ratio and pH. The disturbance to the digesters was simulated by varied OLRs. These parameters were related to the microbial community present at each stage.

Stage 1: In both mono and co-digestion, cumulative biogas production increased slowly from 9 L and 7 L at the beginning of the stage to 16 L and 14 L at the end of the stage for mono and co-digestion respectively (Fig. 6.1). The average absolute biogas production 0.81 L and 0.78 L (Fig. 6.2) for mono and co-digestion respectively was observed. The biogas composition was monitored and a highest of 45.3% and 35% of CH_4 and 41% and 27% of CO_2 was observed in mono and co-digestion respectively. An average of 3.5 L of CH_4 yield (per gVS^{-1}) for mono-digestion as compared to 2.2 CH_4 yield was recorded (Fig. 6.3). The FOS/TAC ratio for mono digestion was high at the beginning of the stage, however, decreased as the stage progressed

(Fig. 6.4). In co-digestion, the FOS/TAC ratio was within the optimum range at the beginning of the stage, however, decreased below optimum range as the stage progressed. In both digesters, the pH fluctuated throughout the stage, with co-digestion slightly higher than mono digestion (Fig. 6.5).

Stage 2: In this stage, differences were observed between the two digesters. Cumulative biogas production greatly increased in mono-digestion (from 17 L in the beginning to 31 L at the end), while in co-digestion it remained within 15.1 L to 15.8 L (Fig. 6.1). A similar trend was observed with the average absolute biogas (Fig. 6.2), wherein mono digestion increased to 1.53 L every second day in comparison to stage 1, while co-digestion drastically reduced to 0.06 L. In mono-digestion, a highest of 44% for CH_4 and CO_2 was observed while in co-digestion decreased as compared to stage 1 to 27% of CH_4 and 28% of CO_2. In mono-digestion, the average CH_4 yield increased to 7.8 L while for co-digestion it slightly increased to 2.5 L (Fig. 6.3). The FOS/TAC ratio remained in the range of 0.4-0.5 for mono digestion and 0.1-0.2 for co-digestion (Fig. 6.4). Also in this stage, the pH fluctuated but slightly higher than in stage 1 although co-digestion was still slightly higher than mono-digestion (Fig. 6.5).

Stage 3: In mono-digestion, cumulative biogas production continued to increase (from 35 L to 52 L) (Fig. 6.1). The biogas content remained approximately similar to that in stage 2 while the CH_4 yield slightly decreased to 7.4 L (Fig. 6.3). Co-digestion resulted in process failure, where the cumulative biogas, absolute biogas and CH_4 continuously decreased until day 77 in which no biogas production was recorded (Fig. 6.1, 6.2 and 6.3). However, 2 days later (day 80) biogas production in low amounts was observed.

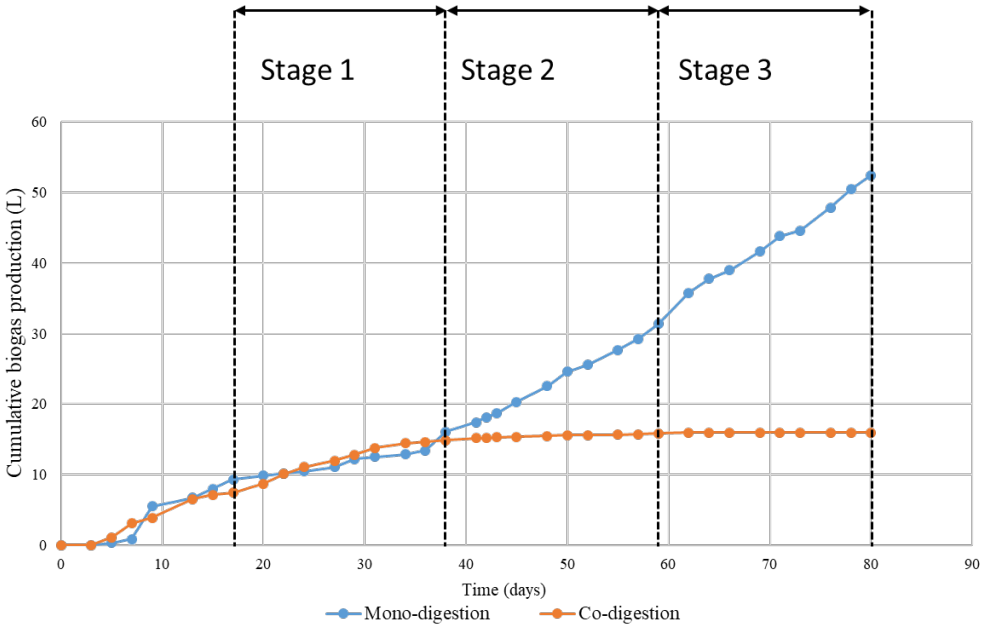

Fig. 6.1: Biogas production. Stage 1 – feeding once a week, stage 2 – feeding twice a week and stage 3 – feeding once a week with increased VS

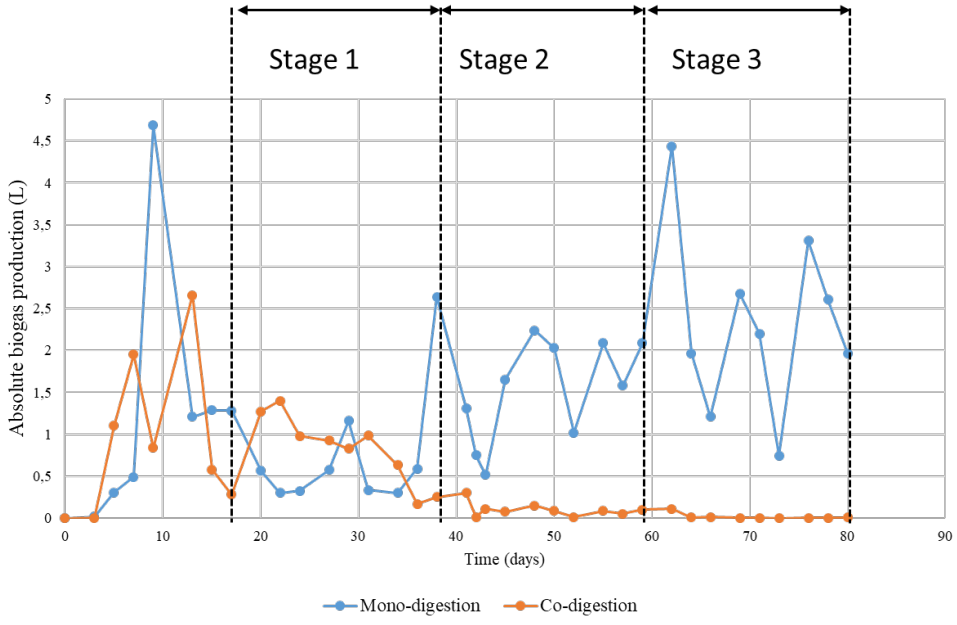

Fig. 6.2: Absolute biogas production from mono and co-digestion. Stage 1 – feeding once a week, stage 2 – feeding twice a week and stage 3 – feeding once a week with increased VS 4% TS.

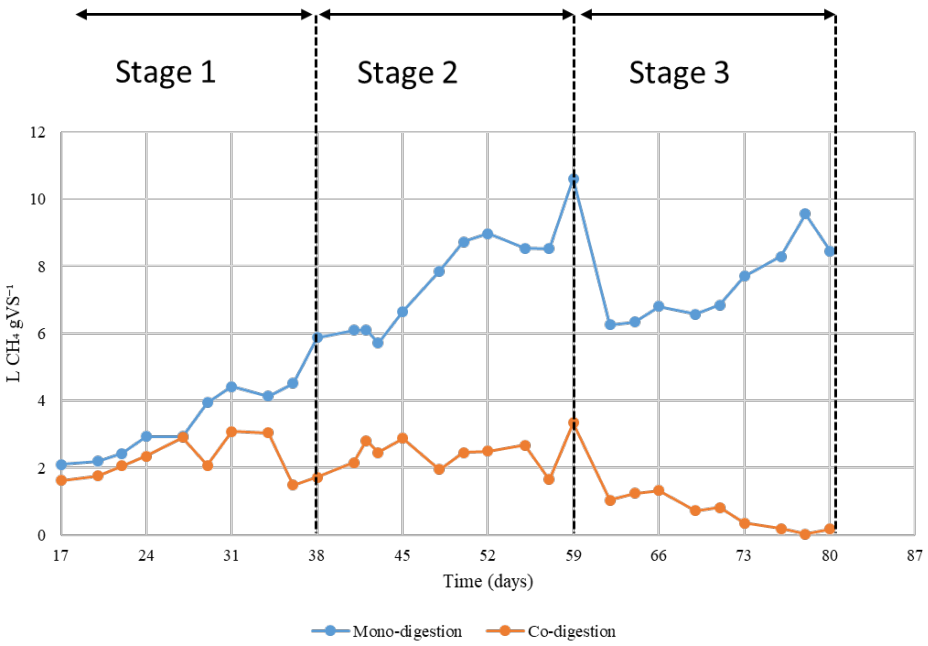

Fig. 6.3: $LCH_4\ gVS^{-1}$ for mono and co-digestion. Stage 1 – feeding once a week, stage 2 – feeding twice a week and stage 3 – feeding once a week with increased VS

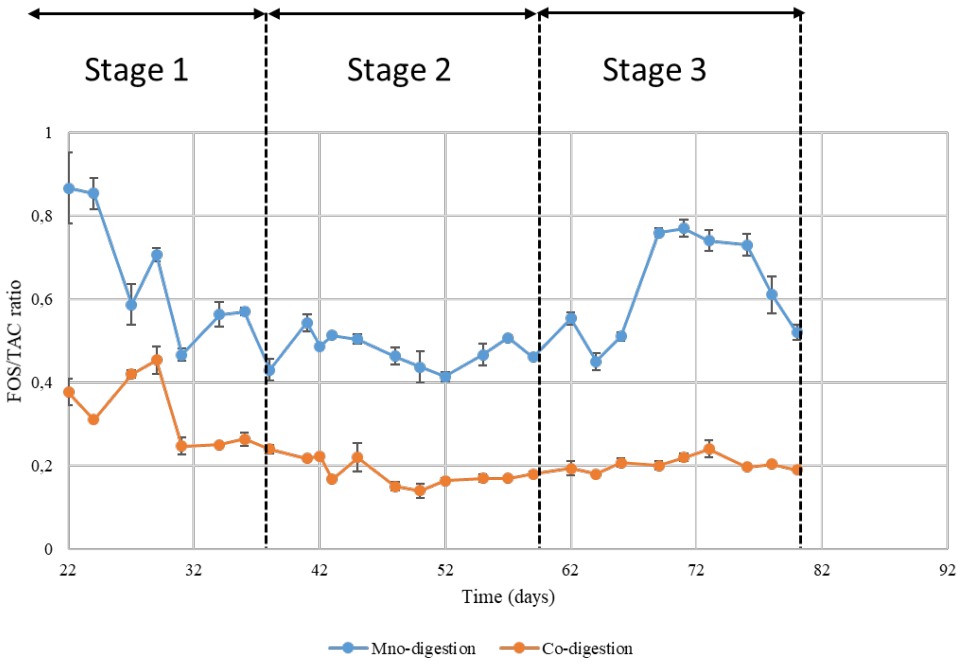

Fig. 6.4: FOS/TAC ratio for mono and co-digestion. Stage 1 – feeding once a week, stage 2 – feeding twice a week and stage 3 – feeding once a week with increased VS

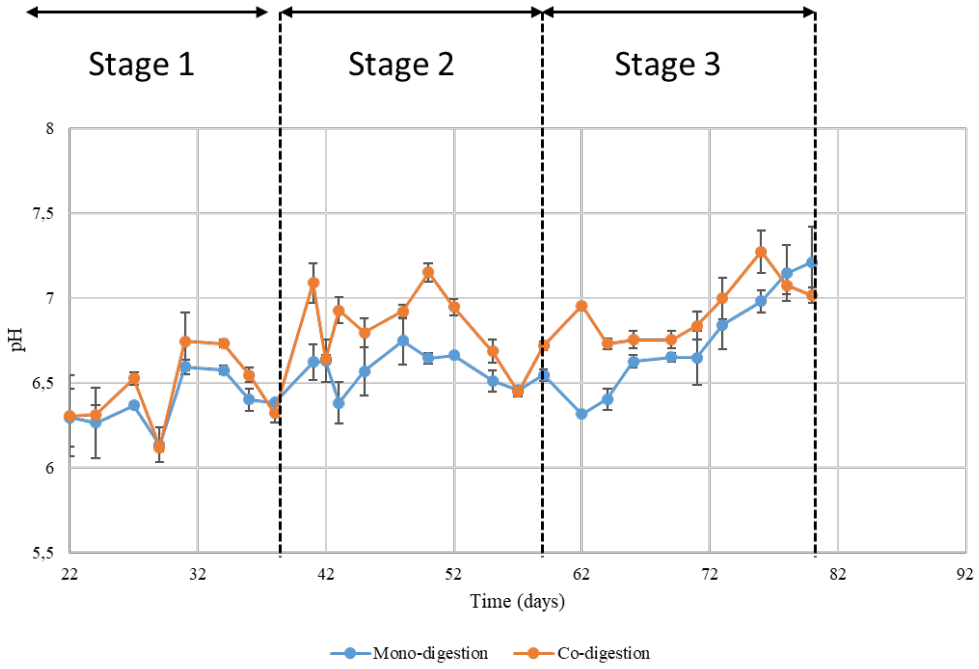

Fig. 6.5: pH of the two digesters. Stage 1 – feeding once a week, stage 2 – feeding twice a week and stage 3 – feeding once a week with increased VS

Diversity of bacteria and archaea

Description of bacterial diversity and shifts in each stage

The relative abundance of bacteria in different stages were analysed at the phylum and genus levels comprising of at least 1% in at least one sample (Fig. 6.6 and 6.7 respectively). Among the bacteria, the phyla Bacteroidetes, Proteobacteria, Firmicutes and Chloroflexi and Parcubacteria were found to be abundant (Campanaro *et al*., 2018; Yi *et al*., 2014). Although dominated by these phyla, each digester had its own characteristic bacterial community composition. The phyla Bacteroidetes was the most dominant in most samples and it was affiliated by genera *Bacteroides, Proteiniphilum, Petrimons, Paludibacter* and *Provatella*.

Early: The genus *Bacteroides* (47.6 and 38.5% respectively) dominated the beginning of stage 1 and 2 in mono-digestion, while *Bacteroides* and *Acinetobacter* dominated stage 3 (21.4% and 20.1%). In co-digestion, this genus was abundant but not dominant (abundance is the prevalence of >1%) at the beginning of stage 2 and 3. The beginning of stage 2 and 3 clearly showed differences in community structure. The beginning of stage 1 was dominated by *Acinetobacter* and *Bacteroides* (36.8 and 33.9% respectively), while stage 2 was dominated by *Bacteroides, Petrimonas,* Bacteroidetes and Firmicutes uncultured bacterium, *Christensenellaceae*, *Paludibacter*, *Proteiniclasticum* and *Proteiniphilum*, (from most abundant to less abundant (18.1-4.1%)) and stage 3 was dominated by *Acinetobacter, Petrimonas*, *Christensenellaceae*, *Proteinclasticum*, (ranging from 31.7 to 4.2%).

Mid: As the stages progressed, more abundant genera became common amongst the stages, these include the genera *Bacteroides, Proteiniphilum, Acinetobacter, Christensenellaceae,*

Petrimonas, Paludibacter and uncultured bacterium from the phyla Bacteroidetes and Chloroflexi, Although differences were observed. In mono-digestion, the mid of stage 1 was mainly dominated by *Bacteroides* (25%) whereas stage 2 and 3 were dominated by *Acinetobacter* (32.4% and 18.4% respectively). High dominant numbers of genera were observed in stage 1 and 3.

In co-digestion, the genus *Pseudomonas, Petrimonas, Acinetobater* and *Christensenellaceae* dominated the beginning of stage 1 (25%, 16.1%, 15.7% and 12.7% respectively), while stage 2 was dominated by *Acinetobacter, Pertimonas and Proteiniphilum*. In addition to genera in stage 2, stage 3 also included genera *Paludibacter* and Chloroflexi uncultured bacterium as dominant genera.

End: At the end of all the stages, commonly shared genera we also observed, however, they varied in their abundance. The genera *Proteiniphilum* and *Paludibacter* were approximately similar in all stages of mono-digestion. *Acinetobacter, Proteiniphilim* and *Paludibacte*r (29.1%, 14.8%, 10.1% respectively) dominated the end of stage 1. *Paludibacter, Proteiniphilum* and *Bacteroides* (19.1%, 16.5% and 14.2% respectively) dominated stage 2 and *Acinetobacter, Paludibacter* and *Bacteroides* (19.8%, 19.7 and 10.3% respectively) dominated stage 3. In co-digestion, the end of stage 1 did not contain the genus *Proteiniphilum* that was observed as abundant in stages 2 and 3 (20 and 29.6% respectively). In addition to *Proteiniphilum*, *Acinetobacter* and *Pseudomonas* (21.3% and 11.75% respectively) dominated stage 1 and *Acinetobacter* and *Petrimonas* (10.9% and 10% respectively) dominated stage 2.

Genera that were distinct from each stage in mono-digestion (between 4-6%) included *Provetella, Enterobacter* and *Ruminococcaceae* from stage 1, *Lachnospiraceae* NK4A136 group, *Ruminiclostridium* and *Erysipelotrichaceae* UCG-004 from stage 2 and *Petrimonas* in stage 3. In co-digestion, *Pseudomonas* was only found in stage 1 mid and end.

Description of archaeal diversity and shifts in each stage

The relative abundance of archaea in different stages was also analysed at the phylum and genus levels (Fig. 6.8 and 6.9 respectively). All the stages of mono and co-digestion were dominated by the phyla Euryarchaeota. The phyla was represented mainly by the genus *Methanobacterium* and *Methanosarchina*, while the genus *Methanobrevibacter* and uncultured *Methanomcrobioles* were less abundant. Similar to results obtained by Barseba (2012).

In mono-digestion, the genera *Methanobacterium* and *Methanosarchina* were abundant in all the stages, with *Methanosarchina* slightly more abundant in most of the samples. However, the end of stage 3 was mainly dominated by *Methanobacterium* (87.1%). It was also observed that in the mid of all the stages the *Methanobacterium* decreased while *Methanosarchina* increased. Moreover, at the end of the stages *Methanobacterium* increased (up to 87% in stage 2) while *Methanosarchina* decreased.

In co-digestion, the genus *Methanosarchina* was more dominant (up to 95%) than *Methanobacterium* in all stages. However, only in the beginning of stage 2 both genera were dominant.

Alpha diversity indices

The alpha diversity indices for bacterial communities fluctuated between the stages (Table 6.3). However, in both mono and co-digestion, stage 2 showed high bacterial diversity and evenness, mostly at the early and mid of some stages for mono-digestion while for co-digestion was mid and end. A decrease in observed OTUs in both digestion at the end of stage 2 and 3 was observed. In addition, for mono digestion the decrease in bacterial diversity was also observed in Shannon and Chao1. Comparison of overall bacterial diversity in mono and co-digestion showed higher diversity in co-digestion.

The alpha diversity indices of archaeal community was much lower than that of bacteria (Table 6.4). The comparison of stage 2 and 3 in mono-digestion to stage 1 showed an increase and decrease in diversity in stage 2 and 3 respectively in the mid and some of the end of stages. Whereas the end of Chao1 was vice versa. In co-digestion, the comparison of stage 2 and 3 to 1 showed a decrease in diversity for Simpson and Shannon at the early and mid of stages. On the contrary, Chao1 and observed OTUs showed an increase in diversity in the early and mid of all stages.

Table 6.3: Alpha diversity indices of bacteria in mono and co-digestion. E – early, M – mid and L – late.

Sample	**Observed OTUs**		**Shannon index**		**Simpson index**		**Chao1**	
Inoculum	973		4.48		34.18		1208.08	
	Mono	**Co**	**Mono**	**Co**	**Mono**	**Co**	**Mono**	**Co**
Stage 1 (E)	1284	1205	3,52	3,29	5,55	6,44	1539,03	1480,77
Stage 2 (E)	1372	1541	3,95	4,33	8,18	23,59	1697,57	2032,02
Stage 3 (E)	1684	1625	4,46	4,08	24,27	13,57	2089,23	2027,57
Stage 1 (M)	1502	1483	4,39	4,16	22,19	16,54	1878,78	2014,43
Stage 2 (M)	1555	1751	3,91	4,30	11,82	14,77	2026.00	2187,50
Stage 3 (M)	1536	1600	4,10	4,29	16,86	20,89	2007,16	2133,27
Stage 1 (L)	1511	1540	4,02	4,35	14,50	23,46	1912,76	1915,11
Stage 2 (L)	1276	1479	4,09	4,55	20,61	30,47	1762,16	1856,40
Stage 3 (L)	1225	1394	3,91	4,16	17,32	18,94	1694,01	1859,41

Table 6.4: Alpha diversity indices of archaea in mono and co-digestion. E – early, M – mid and L – late.

Sample	**Observed OTUs**		**Shannon index**		**Simpson index**		**Chao1**	
Inoculum	30		2.6		8.76		33.00	
	Mono	**Co**	**Mono**	**Co**	**Mono**	**Co**	**Mono**	**Co**
Stage 1 (E)	19	13	1,74	1,75	3,45	3,35	24	13
Stage 2 (E)	21	18	2,02	1,75	4,99	3,62	23	21,33
Stage 3 (E)	19	26	2,01	1,22	5,01	1,88	22,33	35
Stage 1 (M)	19	19	2,14	1,19	5,39	1,81	19,75	22,75
Stage 2 (M)	17	19	2,23	1,17	6,74	1,77	17,33	24,25
Stage 3 (M)	20	23	2,22	1,19	6,59	1,81	20,38	24
Stage 1 (L)	19	19	2,24	1,03	6,95	1,65	26	26
Stage 2 (L)	11	24	1,08	1,39	1,79	2,11	14,33	25,25
Stage 3 (L)	22	16	2,02	1,4	4,17	2,34	23,5	23,5

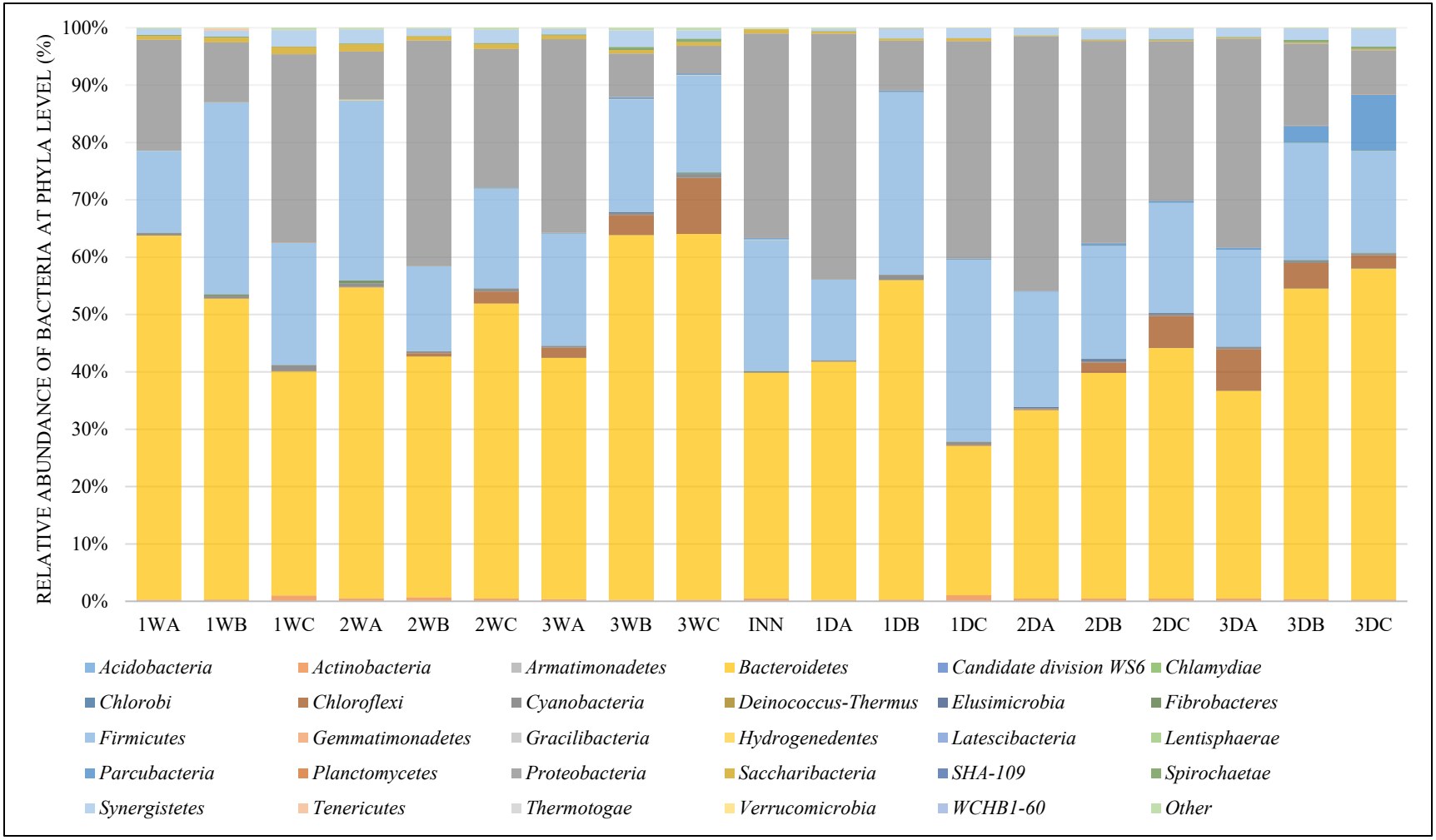

Fig. 6.6: Relative abundance of bacteria at phyla level for mono (W) and co digestion (D). A – Stage 1, B – stage 2 and C –stage 3, while 1 – beginning, 2 – mid and 3 – end of stage. INN – inoculum.

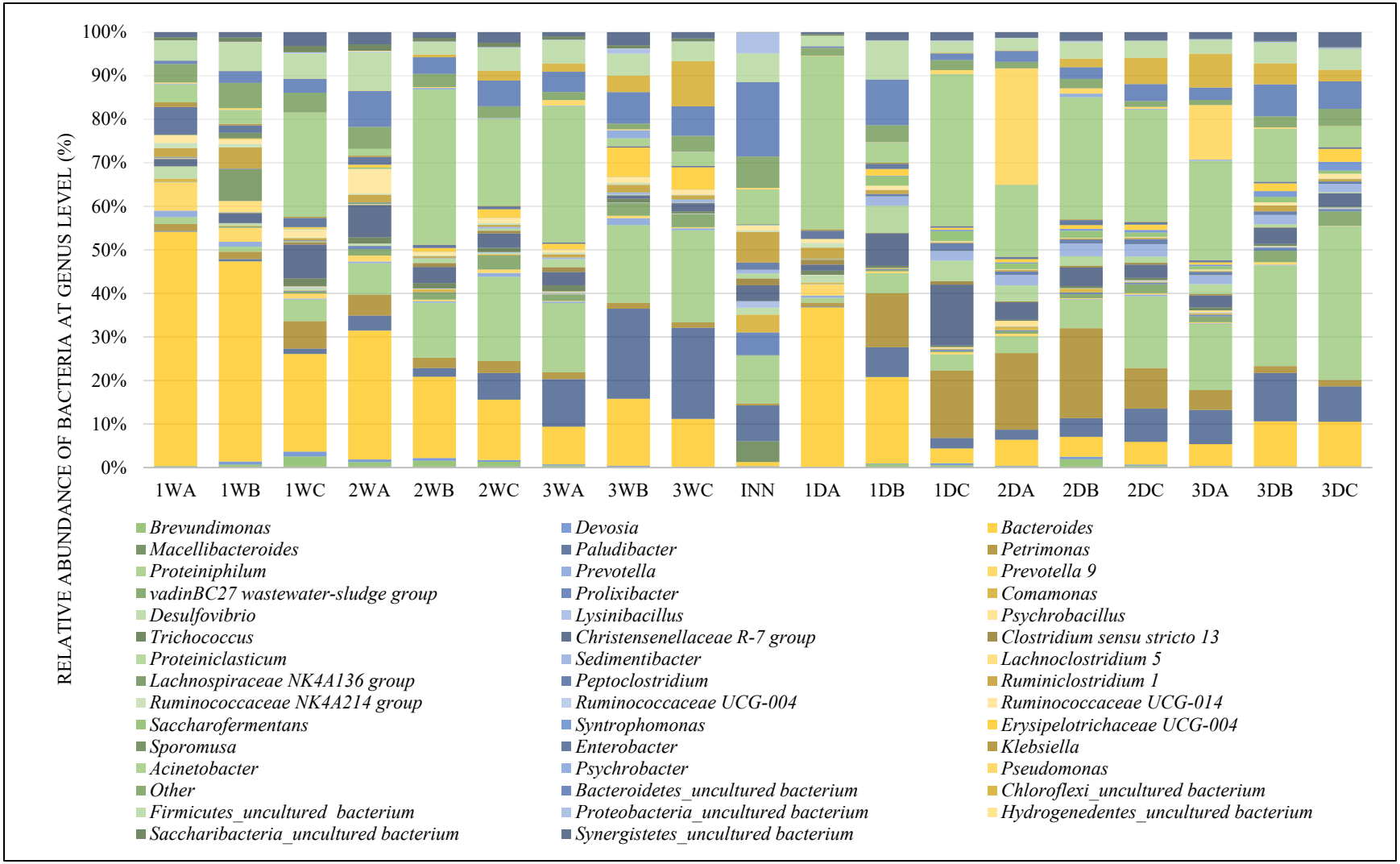

Fig. 6.7: Relative abundance of bacteria at genus level for mono (W) and co digestion (D). A – Stage 1, B – stage 2 and C – stage 3, while 1 – beginning, 2 – mid and 3 – end of stage. INN – inoculum.

Fig.6.8: Relative abundance of archaea at phyla level for mono (W) and co digestion (D). A – Stage 1, B – stage 2 and C – stage 3, while 1 – beginning, 2 – mid and 3 – end of stage. INN – inoculum.

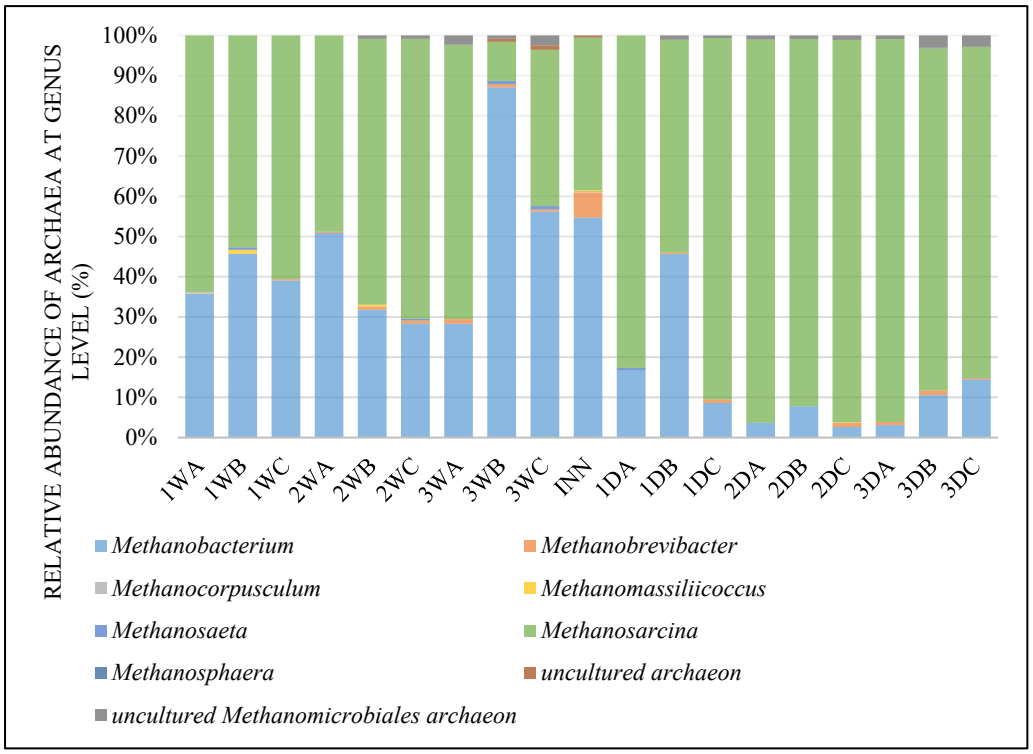

Fig.6.9: Relative abundance of archaea at genus level for mono (W) and co digestion (D). A – Stage 1, B – stage 2 and C – stage 3, while 1 – beginning, 2 – mid and 3 – end of stage. INN – inoculum.

6.4 Discussion

Effects of irregular OLR on process stability, performance and microbial community

In this section of the study, the effects of irregular OLRs on AD of water hyacinth (mono and co-digestion) in biogas production was investigated. Moreover, the effects of irregular OLR on bacterial and archaeal community structure were evaluated using a 16S rRNA gene-based metagenomics approach.

Process stability and performance

The pH of the substrate in the digester reflects the approximate state of the digester and alerts only when the problem is already occurring. The pH obtained in mono and co-digestion remained within the acceptable range for AD although not optimum (optimum range is between 6.8-7.4 (Schloss *et al*., 2009)). In co-digestion, the pH was slightly higher than mono-digestion indicating the buffering capacity of the cow dung. A study by Yi *et al.,* 2014, measured the pH and VFAs as TS were increased. Their results showed acceptable pH range in one of their digesters with high concentration of VFAs was observed implying inefficiency of pH as an appropriate indicator of process stability in substrate with good buffering capacity. Mono-digestion's pH was also within acceptable range emphasising the suitability of the plant in mono-digestion.

Irregular OLRs are known to cause a decrease in biogas production. The difference in biogas production was observed between mono and co-digestion when irregular OLRs were introduced. The biogas production was observed to increase in stage 2 and 3 of mono-digestion. This can be correlated to the FOS/TAC ratio (equiv. acetic acid/equiv. $CaCO_3$) (Rincón *et al*., 2008), which is indicative of process stability in mono-digestion especially in stage 1 and 2, although optimum biogas production with good CH_4 yield was observed in stage 2. Whereas, in stage 3 the high FOS/TAC ratio was indicative of excessive biomass input (>0.6) and it can be linked to a decrease in CH_4 yield due to loading shock (Fig. 6.5), although the CH_4 yield recovered as the microbial community adapted (interpreted using Table 6.1).

In co-digestion, the decrease in biogas production in stage 2 and 3 was also correlated to the FOS/TAC ratio (<0.2), which was mostly below the recommended level. According to Allen *et al*., 2014, a ratio of 0.2-0.4 implied stable condition for co-digestion between seaweed and dairy slurry. In this case, the FOS/TAC ratio was indicative of process instability and this usually happens when the concentration of acetic acid is higher than that of $CACO_3$. Thus, emphasising the importance of FOS/TAC ratio as an early indicator of process instability.

Bacterial community structure and composition

In addition to affecting the biogas production, the result of irregular OLRs also affects the microbial community structure and composition (Regueiro *et al*., 2012). In mono-digestion, the genus *Bacteroides* was dominant at the beginning of each stage and decreased as the stages progressed. The genus *Bacteroide*s is known for its ability to degrade complex plant

polysaccharides such as cellulose (Hatamoto *et al*., 2018; Shah & Williams, 1987), and its high abundance at the beginning of mono-digestion can be explained by the presence of cellulose from water hyacinth (lignocellulosic biomass) (Amez, 2015). According to Shah & Williams, 1987, the capabilities of the genus is related to the nutrients available in the environment, thus implying that the nutrients in mono-digestion favoured their growth. In addition, this happens during the first stage of AD, hydrolysis. The decrease in the dominance of *Bacteroides* during the mid and end shows that the cellulose content has been converted to other compounds. This is the reason why in the mid and end of the stages other groups such as *Proteiniphilum, Acinetobacter, Christensenellaceae* R 7 group*, Petrimonas* and *Paludibacter* increased. Although *Christensenellaceae* R 7 group is known to be involved in both hydrolysis and acetogenesis (Wu *et al*., 2016).

The genus *Proteiniphilum* is a proteolytic bacterium, while *Paludibacter* is a saccharolytic bacterium (S. Chen & Dong, 2005; Ueki *et al*., 2006). And according to Ziganshin *et al*., 2011, these genera are known to produce elevated levels of acetate and propionate. Acetate is known as a product that is produced during acetogenesis (and a precursor for methanogenesis) (Yadvika *et al*., 2004) and explains their dominance at mid and end of mono-digestion. In addition, the decrease of the genus *Bacteroides* was parallel with the increase of *Proteiniphilum* and *Paludibacter* at the end of all stages. The comparison of microbial community structure and composition in all sampling time of all stages showed variation, some genera decreased while others increased. OLRs affected the dominance and abundance of genera in each stage.

In co-digestion, the beginning of each stage was dominated by different genera, although genera such as *Petrimonas, Bacteroides, Proteiniclasticum* were common. The difference in the dominant genera were influenced by the OLRs (Hansen *et al*., 1998; Ho *et al*., 2013; Regueiro *et al*., 2014; Tham, 2012; Zou *et al*., 2014) which favoured certain communities. In comparison to mono-digestion, the bacterial community composition in co-digestion were more different in each stage. Fig. 6.6 shows variation between the genus *Bacteroides* and *Petrimonas* in both digestions. This difference is influenced by the available nutrients in the substrate and according to Ziganshina *et al*., 2015 the diversity of the Bacteroidetes, Proteobacteria, Firmicutes and Chloroflexi phyla is known to be influenced by substrate type and OLRs. In addition, the dominance of the phyla Bacteroidetes in this study can be related to organic overloads and has been reported to be resistance to elevated levels of VFAs concentration (Regueiro *et al*., 2014).

In co-digestion, the mixture of cow dung and water hyacinth resulted in low dominance of *Bacteroides* and an increase in *Petrimonas* in most of the samples. *Petrimonas* is a bacterium that ferments sugar to generate acetate however it can also use nitrate or elemental sulphur as electron acceptors (Grabowski, *et al*., 2005; Nakasaki *et al*., 2009). The increase in *Petrimonas* from the beginning of the stage implies that stages that comes after hydrolysis occurred earlier in co-digestion. Furthermore, the cow dung already contains components that were already or partially degraded as compared to water hyacinth alone. In addition, the mixing of the two substrates resulted in lower cellulose component as compared to mono digestion (explaining the low dominance of *Bacteroides*).

In addition, it was also observed in co-digestion that the increase in the genus *Petrimonas* was followed by the decrease in *Paludibacter* and vice versa. Similar to mono-digestion, the increase in *Proteinipilum* was also parallel to the increase in *Petrimonas*. It was also observed that the abundance of *Christensenellaceae* R 7 group also increased in samples were *Bacteroides* decreased as compared to mono-digestion. Thus, in co-digestion, both *Bacteroides* and *Christensenellaceae* R 7 group mainly carried out hydrolysis.

Unique to stage 2 co-digestion, the genus *Pseudomonas* was also found to be dominant at the mid and end of stage 2. Mostly, the presence of *Pseudomonas* in AD is usually associated with the Dentrification process. Denitrifying bacteria produce nitrite from nitrate and this can be linked to the dominance of *Petrimonas*, known to use nitrate as an electron acceptor to produce ammonia. In addition, *Acinetobacter* was found to be dominant in mono and co-digestion (more dominant in most samples of co-digestion). According to Sheng Chen *et al*., 2017 and Su *et al*., 2015 *Acinetobacter* and *Pseudomonas* are capable of carrying out denitrification and nitrification (removal of nitrate and ammonia into nitrogen gas).

Water hyacinth used was collected from a Dam that is already in the state of hypertrophication mainly from nitrates and phosphate from agricultural and mining activities. The plant also has phytoremediation ability, thus explains the reason for the presence of these particular organisms (growth encouraged by the presence of preferred nutrients).

Archaeal community structure and composition linked to Bacterial community and analytical methods

The importance and success of AD is mainly attributed to the activity of archaeal community, the producers of CH_4 and other gases. In mono-digestion, *Methanosarchina* and *Methanobacterium* dominated all the stages. Although, *Methanosarchina* was slightly higher in some samples. *Methanobacterium* is a hydrogenotrophic methanogen while *Methanosarchina* is an acetoclastic methanogen (although know to use both the pathways). *Methanobacterium* and *Methanosarcina* are known to be resistant to elevated levels of VFAs (Franke-Whittle *et al*., 2014), however, *Methanosarcina*'s growth increases when elevated levels of VFAs (especially acetate) are detected in the digester (Demirel and Scherer, 2008). The presence of both genera in mono-digestion was related to organic overloading (especially M*ethanosarchina*).

In terms of biogas production, all the stages were able to continuously produce biogas, although the beginning of stage 3 resulted in a decrease in CH_4 yield due to loading shock (Fig. 6.5). The dominance of both genera (approximately similar) in most of the samples collected at different sampling times (Fig. 6.9), imply that archaeal community is not affected by irregular OLRs in comparison to bacteria. Similar results were obtained by Baserba *et al*., 2012. In addition, studies from Carballa *et al*., 2015 showed that methanogenic community composition is influenced by environmental changes such as pH, ammonia and VFAs.

Methanosarchana dominated the co-digestion, indicating that these group played important roles in co-digestion AD. In addition, many studies have reported the genus *Methanosarchina* as the dominant methanogen in AD process regarded as overloaded (high levels of VFAs). Furthermore, *Methanosarchina* genus is known to have high a growth rate in comparison to other methanogens and it can tolerate changes in pH and high concentration of toxic compounds (Demirel & Scherer, 2008).

The dominance of this genus in stage 2 and 3 also put more emphasis on bacteria being sensitive to OLRs and not methanogens. To further explain this, both *Methanosarchina* and *Methanobacterium* dominated the beginning of stage 1 and 2. However, as the stages progressed *Methanobacterium* drastically reduced leaving *Methanosarchina* as the dominant methanogen. This can be explained by the presence of more than two dominant genera involved in the production of acetate, favouring *Methanosarchina*.

In addition, stage 2 and 3 had higher VS as compared to stage 1. Other studies show that increasing TS will result in higher concentrations of VFAs which in turn favour the growth of *Methanosarchina* which uses acetate to produce CH_4 (De Vrieze *et al*., 2012). In addition, it also explains the low FOS/TAC ratio obtained in stage 2 and 3. The FOS/TAC ratio of below 0.2 indicates high production of VFAs (in this context acetate) as well as the need to add biomass (since biomass is been readily used up).

According to Demirel & Scherer, 2008, the genus *Methanoscarchina* is abundant in unstable co-digester with increased levels of acetate. In addition, they also mentioned that the presence of elevated levels of VFAs, ammonia and other toxic compounds favour the growth of *Methanosarchina*. In general, the prevalence of this genus categorises the AD process as being overloaded (Regueiro *et al*., 2012).

Studies that experienced overloaded system and high prevalence of *Methanosarchina* still observed biogas production. However, our results showed that stage 2 and 3 resulted in the drastic decrease in biogas production, so as the quality (based on the CH_4 produced) as compared to mono-digestion.

The presence of *Acinetobacter* and *Pseudomonas* may have contributed to the continuous decrease in biogas production (Sheng Chen *et al*., 2017; Clarens, Bernet, & Delgene, 1998; Su *et al*., 2015). Their presence signals the removal of nitrate and ammonia from the digesters. During denitrification of nitrate to nitrogen gas, the intermediate nitrite is produced. According to Clarens *et al*., 1998, the presence of nitrite (of about 0.18 mM) showed a higher inhibitory ability to methanogenesis. Although strongly affects methanogenesis from H_2 and CO_2 (Balderston & Payne, 1976). Furthermore, the dominance of *Petrimonas* in co-digestion also confirms the presence of nitrite and its conversion to ammonia.

During denitrification, bacteria require electron donors and substrates such as acetate, hydrogen, lactate, and methanol become electron donors (Costa, 2000). In addition, under limited-oxygen condition, CH_4 is used as an electron donor during denitrification (Costa, 2000; Islas-Lima *et al*., 2004; Raghoebarsing *et al*., 2006; Westermann & Ahring, 1987).

The increase and dominance of *Petrimonas* is related to increased availability of nitrate. Implying that in co-digestion, nitrate removal was high, thus the denitrifying bacteria might have turned to CH_4 as an electron donor for the removal of nitrate. In mono-digestion, *Acinetobacter* was dominant either at the beginning, mid or end whereas in co-digestion *Acinetobacter* was dominant in all samples (except the end of stage 3). Thus, implying the co-digestion environment was conducive for such.

It may be concluded that, although the effects of irregular OLRs was observed in both mono and co-digestion, the prevalence was more in co-digestion. The comparison of the abundance and dominance of bacterial community between mono and co-digestion showed variation. The substrate composition played a role in the bacterial diversity in both digestion. In addition, it was also observed that bacterial communities are more sensitive to OLRs in comparison to archaeal community. In the present study, the abundance of the archaeal community was not influenced by the changes in OLRs however, by the type of products produced during the previous stages, thus indicating the importance of bacterial community in AD processes.

The dominance of *Acinetobacter* and *Pseudomonas* support the reason that the type of substrate (mainly the nutrients presence) mainly contributes to the selection of microbial communities. Co-digestion is usually associated with a wide range of nutrients as compared to mono-digestion and the results from this study showed high numbers of different active bacterial communities, this was also supported by the alpha diversity indices calculated.

Studies on AD mainly focus on methanogenesis (archaea). Although important, bacterial communities also play an important role and without the activity of the bacterial community, methanogens will not have precursors to use in methanogenesis. As seen from the results, the presence of certain bacterial communities may be related to the process's efficiency or inefficiency. Thus, more understanding of bacterial community during AD is required especially to disturbances by OLRs and type of substrate to allow for the selection of bacteria that can be used as indicators of the type of methanogenic communities and possible AD inhibitors.

7 BIOAUGMENTATION APPROACH TO INCREASE BIOMETHANE YIELD

7.1 Literature review

Water hyacinth (*Eichhornia crassipes*) is a potential substrate for the generation of biogas and was selected based on the environmental effects of the substrate, its nature (rapid doubling time), the ratio of carbon to nitrogen and the availability of the substrate (Ndimele *et al*., 2011; Divya *et al.,* 2015; Sindhu *et al.,* 2017). The menace of water hyacinth on the aquatic body of the Hartbeespoort dam is an environmental concern and the use of these plants for the production of biogas is an innovative and environmentally friendly technique in managing these weeds (Azman *et al.,* 2015).

Different types of microorganisms are involved in the various stages of AD of organic material. These microorganisms depend on each other for their activities, the product of a step in AD forms a substrate for the next reaction (Nzila, 2017). The success or failure of anaerobic digestion (AD) depends on the metabolic potential of the microorganisms in the digesters. The process of AD to yield biogas has two important rate limiting steps which are the hydrolytic and methanogenic phases but the hydrolytic phase is considered the most important rate limiting step in the production of biogas (Strang *et al*., 2017). Biomethane production could be enhanced during the AD of water hyacinth by hastening the rate limiting step of AD which is the hydrolytic phase. This enhancement can be achieved by the introduction of microorganisms that are capable hydrolyzing the substrates as the hydrolytic enzymes are known to completely degrade cellulose (Strang *et al.,* 2017). The enhancement of this phase of AD as well as other phases by controlling the microorganisms that drive the reactions could assist in increasing the quantity and quality of biogas produced and this enhancement is referred to as bioaugmentation.

Bioaugmentation is the introduction of microorganisms to an AD process in the form of a pure culture or a consortium (Nkemka *et al.,* 2015; Kinet *et al.,* 2015). These microorganisms hasten the AD process and improves biomethane production. Introduction of microorganisms/ bioaugmentation is ideal at the hydrolytic or acidogenic phase as the microorganisms involved in these stages of AD are less sensitive to changes in environmental conditions and are capable of resisting stress (Nzila *et al.,* 2017). Literature have shown increase in biomethane production during AD as a result of bioaugmentation. When a consortium of microorganisms was used to bioaugment a bioreactor comprising of maize and xylan as feedstock, an increase of about 53% in biomethane yield was recorded (Weiss *et al.,* 2010). An investigation by Martin-Ryals *et al*. recorded a 56% increase in biomethane production when bioreactors were bioaugmented (Martin-Ryals *et al.,* 2015). However, there could be some challenges associated with the bioaugmentation process. Such challenges include as accumulation of metabolites like ammonia and volatile fatty acids (VFAs) which could inhibit overall AD (Costa *et al.,* 2012). Ammonia inhibits the hydrogen producing microorganisms while VFAs lower the pH of the system. Furthermore, the presence of anaerobic fungus in the digester could inhibit the growth of methanogens (Nkemka *et al.,* 2015).

7.2 Methods

Sampling

Water hyacinths were harvested by hand from the Hartbeespoort dam, which is located in Madibeng district of the North West province of South Africa and transported in storage boxes to the Microbiology laboratory of the Institute for Soil, Climate and Water – Agricultural Research Council, Arcadia, Pretoria, Gauteng province of South Africa. Samples were cut to small sizes of 2cm x 2cm with a pair of sterile scissors prior to analysis.

Analytical methods

The organic carbon, total solids (TS) and volatile solids (VS) fractions of the water hyacinth and the digestate were analysed according to the Standard methods for the examination of Water and Waste water (APHA, 2012). Ten percent of freshly chopped water hyacinth (wh) was digested anaerobically at a temperature of 30°C using a rotatory incubatory at 120 revolutions per minute (rpm) for 2 weeks to create water hyacinth inoculum (whinc). Freshly chopped water hyacinth (wh) was mixed with the water hyacinth inoculum, whinc in various ratios of wh:whinc1:1, wh:whinc1:2, wh:whinc1:4, wh:whinc4:1 and wh:whinc2:1 in 500 ml screw cap + septum glass bottles. All the mixing ratios had the same TS (2%). Tap water was used to bulk the volume of each of the treatments to 250 ml. Hundred percent water hyacinth, wh:whinc1:0 and 100% water hyacinth inoculum, wh:whinc 0:1 served as different controls. Another water hyacinth treatment was digested without water (wh without water), it was set up to evaluate the ability of water hyacinth to produce biogas when during dry digestion. Treatments were not sparged with nitrogen gas to create an anaerobic environment. These treatments were digested anaerobically as batch cultures for 29 days in triplicates at 30°C and 120 rpm; biomethane production was monitored at intervals using the Gas chromatograph (SR18610C) (GC) (conditions of GC). Biomethane production was monitored at 2 days interval for the first week, 7 days intervals for the second and third week and 3 days interval for the fourth week of AD. Two milliliters aliquots of gas was taken from the headspace of the batch culture bottles and injected into the GC and analysed. Helium gas was used as the carrier gas.

Statistical analysis

Mean differences of the biomethane produced were compared using two-way analysis of variance (ANOVA) on Excel 2013. Students t-LSD (least significance difference) was conducted to determine whether a significant difference existed among the treatments during biomethane production.

7.3 Results

A significant reduction in the organic carbon content, TS and VS fractions of the water hyacinth was observed.

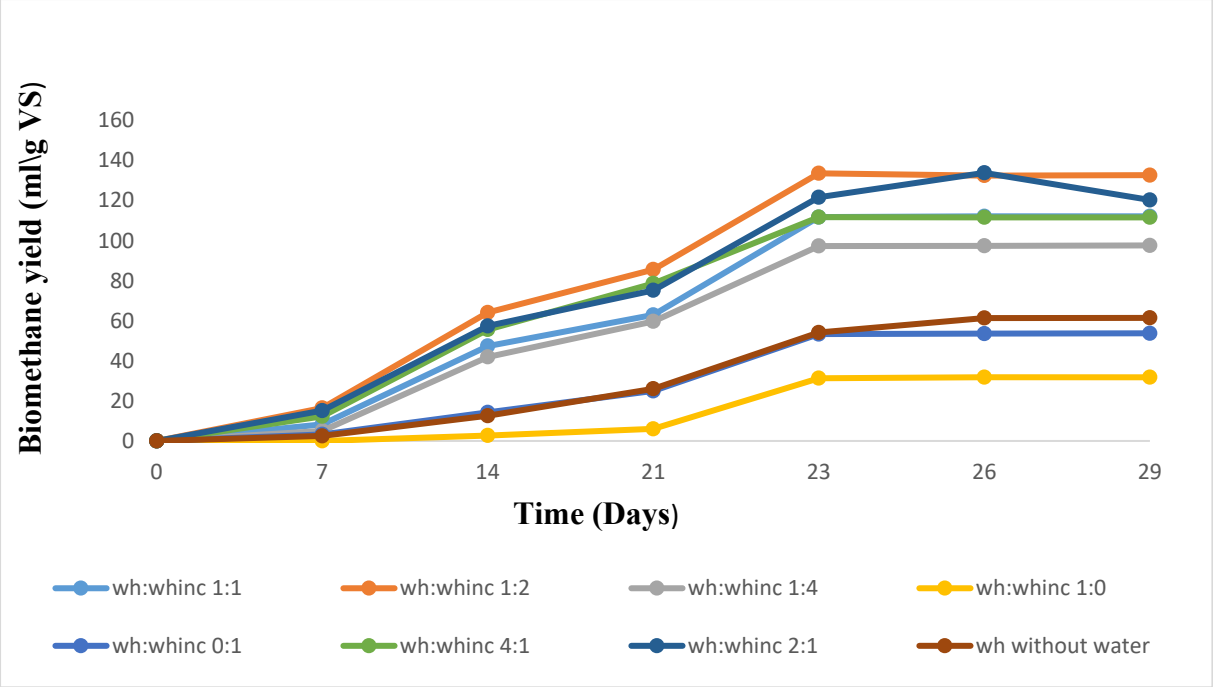

Fig. 7.1: Biomethane yield from anaerobic digestion of water hyacinth with water hyacinth inoculum.

There was delayed production of biogas in the first week of digestion hence the analysis of biogas once a week (Fig. 7.1 and Fig. 7.2). Close monitoring (3 days interval) was done when biogas started building up. During days 14, 21 and 23 of AD, treatment wh:whinc1:2 produced more biogas while treatment wh:whinc4:1 produced more methane. The controls, especially the wh:whinc1:0 which had 100% water hyacinth performed poorly, it had the least quantity of biogas and biomethane (Fig. 7.1 & Fig. 7.2). More biomethane was produced by the treatment with the most water hyacinth and the least water hyacinth inoculum (wh:whinc4:1). According to Fig. 7.1 & Fig. 7.2, the AD of water hyacinth without water also generated biomethane but the treatments with water hyacinth inoculum (which included water) performed better.

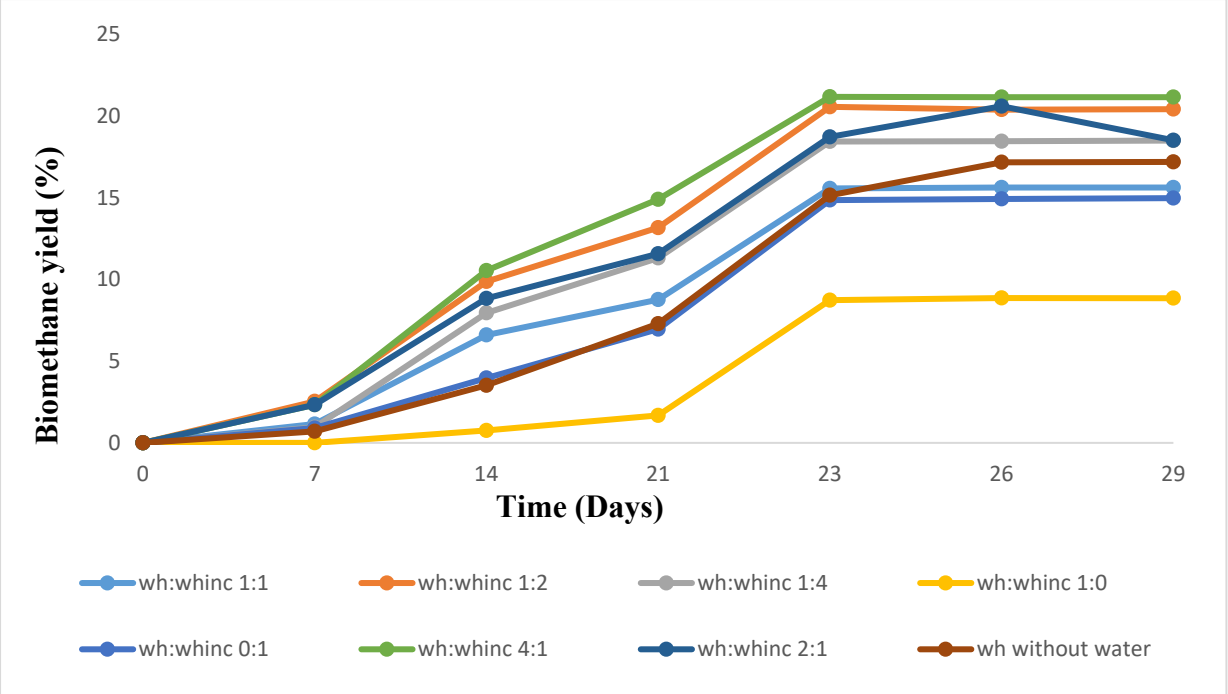

Fig. 7.2: Percentage biomethane yield in biogas from anaerobic digestion of water hyacinth with water hyacinth inoculum.

7.4 Discussion

The environmental hazards caused by water hyacinth at the Hartbeespoort dam have incited the search for a sustainable means of managing the weed. Depletion of fossil fuels and environmental pollution worldwide makes the use of water hyacinth to produce biomethane an attractive, alternative source of energy that is environmentally friendly. Use of agricultural biomass such as water hyacinth to produce biogas is on the rise as water hyacinth has been proven to produce biogas (Simonyan & Fasina, 2013; Fadairo & Fagbenle, 2014; Njogu *et al.,* 2015). Digestion to produce inoculum prior to AD of water hyacinth and water hyacinth inoculum was to harness hydrolytic/acidogenic microorganisms to improve biomethane production (Strang *et al.,* 2017). During AD, biogas production was initially delayed. This could be due to not sparging the treatments with nitrogen gas prior to digestion or that the introduced microorganisms were probably adapting to their new environment. Increasing the digestion time may have had a beneficial impact on the production of biomethane. According to this study, the incorporation of water hyacinth inoculum led to an increased production of biomethane. Inoculum contains different microorganisms that produce biogas and it has been reported that increase in the amount/ratio of inoculum leads to an increased biogas production (Dennis, 2015). All the bioaugmented treatments produced more biomethane as opposed to the 2 controls that contained water hyacinth inoculum and just water hyacinth respectively. This confirms other reports on how the enhancement of AD via bioaugmentation improved biomethane production (Kovács *et al.,* 2013; Ács *et al.,* 2015). This also could be attributed to the presence of hydrolytic/acidogenic microorganisms in the inoculum and the availability of the substrate to the microbes (Strang *et al.,* 2017). The 100% water hyacinth treatment (control) produced the least amount of biomethane as a result of unavailability of appropriate microorganisms to propel the digestion process. Furthermore, the control with 100% water

hyacinth inoculum did not also perform too well possibly because of shortage of substrates/limited nutrients for microbial activities. This corresponds with the study of Corro *et al.* where low nutrient content limited biogas production (Corro *et al.,* 2013).

Moisture is crucial in AD, it encourages the activities of microorganisms as well as the mixing efficiency of the digesters. This confirms the inability of water hyacinth digested without water to produce as much biomethane as the treatments with water. Reduction in the TS and VS contents of the digestate indicates utilisation of organic matter by the microorganisms to produce methane and carbon dioxide. This was due to the metabolic potential of microorganisms and their ability to access suitable nutrients (Hassan *et al*., 2017).

It may be concluded that bioaugmentation of water hyacinth with water hyacinth inoculum led to more biomethane production. This study improved the AD process by maximising the metabolic potentials of the microorganisms that drive the process. Future research to isolate microorganisms from water hyacinth inoculum for bioaugmentation could help attain a more feasible operation. Studies on metabolomics could be conducted to determine the metabolites produced at different stages of AD and how to bioaugment the process with appropriate microorganisms to improve biomethane production.

8 DIGESTATE FEASIBILITY AS A SOIL AMELIORANT

8.1 Literature review

Anaerobic digestion (AD) is a series of biological processes, which involve the breakdown of organic materials by anaerobic microorganisms to produce a mixture of gases (biogas) and digestate (soil ameliorant) (Divya *et al*., 2015). This process is a sustainable waste treatment technology and can be used to stabilise wastes or produce fuels and it can take place in an anaerobic digester or naturally. The digestate which is a by-product of AD is regarded as a potential soil ameliorant as the anaerobes in the digester do not use the inorganic fraction of the substrates during digestion. These organisms also convert some of the organic matter to inorganic compounds that are readily available to plants (Bonten *et al*., 2014). Increases in the use of inorganic fertilizers to improve crop productivity has led to a reduction in soil quality and fertility. These fertilizers have been associated with heavy metal pollution of the environment and leaching of nutrients through agricultural runoffs of inorganic fertilizer (Mukhuba *et al*., 2018). The need for an environmentally friendly, economic and efficient soil additive to improve soil fertility and soil production is of utmost importance hence the use of digestate as a soil ameliorant.

Digestate is also regarded as biofertilizer due to the presence of plant growth promoting microorganisms (PGPM) which when applied to soil/plants enhance the availability of inorganic compounds in the soil. Biofertilizers act through the interaction of plants and microorganisms in the rhizosphere (a region of the soil where microecological zone is in direct vicinity of root nodules) and determines plant health, productivity and soil fertility (Rascovan *et al*., 2016). The microorganisms that enhance plant growth, protect them from abiotic stress and pathogenic attack (diseases) are the plant growth promoting microorganisms (PGPM) (Bais *et al*., 2006; Lakshmaran *et al*., 2014). The PGPM can be used as inoculants to promote plant growth and productivity by increasing the availability of essential plant nutrients such as nitrogen and phosphorus through processes such as the biological nitrogen fixation and phosphate solubilisation (Bello-Akinosho *et al*., 2016). Plant growth promoting microorganisms protect plants from pathogens by producing antibiotics, phytohormones, siderophores and cell wall-lysing enzymes (Hameeda *et al*., 2008; Ji *et al*., 2014). PGPM may also relieve abiotic stress of plants through the activity of the ACC (Aminocyclopropane-1-carboxylate) deaminase, production of siderophores and phytohormones (Ji *et al*., 2014). The exhibition of these plant growth-promoting traits by PGPM assists in the classification of biofertilizer and these traits include:

Biological nitrogen fixation: Nitrogen is an important nutrient for plant growth and productivity. Organisms need nitrogen to produce biomolecules such as proteins and nucleic acids but the major source of natural nitrogen, which is the atmospheric nitrogen, is not available to growing plants (Ahemad & Kibret, 2014). Microorganisms such as bacteria and archaea convert the atmospheric nitrogen to a form that is utilisable by plants which is ammonia

through the action of complex enzymes known as the nitrogenases, which are coded by the gene *nifH* (Dixon & Kahn, 2004; Souza *et al*., 2015).

Phosphate solubilisation: Phosphorus is another vital nutrient for plant growth and productivity as it is essential in metabolic and biochemical pathways of plants such as photosynthesis and biological nitrogen fixation (Richardson & Simpson, 2011; Souza *et al*., 2015). A substantial amount of phosphorus in the soil is not available to plants in nature to perform these necessary functions because it is in an insoluble form, hence the need for soluble forms of phosphorus (Sharma *et al*., 2013). Orthophosphate, which is the available form of phosphorus to plants, is limiting in soil (Fraser *et al*., 2015). Adding phosphorus to plants using chemical fertilizers reduces the effect of the phosphorus as it has been reported that more than 80% of phosphate applied as fertilizers precipitate in the presence of soil metal iron complexes (Qureshi *et al*., 2012; Abbasi *et al*., 2015). Phosphate solubilising microorganisms and plant roots in the soil could improve the solubility of this element thereby increasing its availability to plants (Souza *et al*., 2015). The incorporation of extracellular enzymes such as the phosphatases (alkaline phosphatase – ALP) to soil/plants through microorganisms, aids in the production of orthophosphate which essentially enhances crop production (Zimmerman *et al*., 2013). The *phoD* gene codes for ALP production in bacteria and the presence of these genes signifies the occurrence of phosphate solubilising bacteria in any environment.

Production of indolic compounds: The ability of rhizosphere bacteria to produce indolic compounds is an indication of their capacity to promote plant growth and development as well as protection of plants against pathogenic attack. A report by da Costa *et al.* showed that rhizosphere bacteria produced more indolic compounds than majority of soil bacteria (da Costa *et al*., 2014). These indolic compounds include auxin phytohormones such as indole-3-acetic acids (IAA). IAA is the main auxin in plants and it is responsible for various physiological processes in plants (Ji *et al*., 2014).

Anaerobic digestion (AD) has been proven to use the carbon, hydrogen and oxygen fractions of the substrate/feedstock for biogas production while the vital plant nutrients such as nitrogen, phosphorus and potassium are mostly left in the digestate, which could serve as soil ameliorant (Igboro, 2011). The process of AD also improves the availability of essential nutrients in the digestate than in undigested substrate/feedstock (Alfa *et al*., 2014) and destroys pathogens (Otaraku & Ogedengbe, 2013).

8.2 Methods

Sampling

At the end of the four weeks of AD, digestate samples were collected from batch culture experiments in the previous chapter (Chapter 7) where water hyacinth inoculum was digested with water hyacinth in different proportions. Digestate samples were labelled according to Table 8.1 and were stored at 4°C prior to analysis for physico-chemical and microbial properties.

Table 8.1: Labelling related to feedstock and digestate

Feedstock	Digestate
Wh:whinc1:1	1:1 (Dig)
Wh:whinc1:2	1:2 (Dig)
Wh:whinc1:4	1:4 (Dig)
Wh:whinc2:1	1:2 (Dig)
Wh:whinc4:1	1:4 (Dig)
Wh:whinc1:0	1:0 (Dig)
Wh:whinc0:1	0:1 (Dig)

Analytical methods

Physico-chemical characteristics of the digestate samples such as pH, phosphorus, potassium and ammonium content followed. Heavy metals analysis was done for the water hyacinth and the digestate using the Standard methods for the examination of Water and Wastewater (APHA, 2012). Analysis was done by the analytical laboratory of the Agricultural Research Institute – Institute for Soil, Climate and Water. Samples were also characterized for total solids (TS) and volatile solids (VS) (APHA, 2012).

Molecular characterization

One gram of homogenised sample of the digestate was transferred to 2 ml microfuge tubes and centrifuged for 5 minutes at maximum speed, supernatant was discarded and the pellets were used for the extraction of genomic DNA. DNeasy PowerSoil extraction kit was employed in the extraction of DNA from the digestate following the manufacturer's protocol (Adeleke *et al*., 2010). Qubit 2.0 Fluorometer (a product of Invitrogen, Life Technologies South Africa) was used to quantify filtered DNA extracts and extracts were stored at -20°C for further use (Roopnarain *et al*., 2017). Plant growth promoting abilities were examined in the digestate by targeting the *nifH* genes for nitrogen fixation and the *phoD* genes for phosphate solubilisation using Polymerase Chain Reaction (PCR). The *nifH* genes were targeted with PolF (5'-TGCGAY CCS AAR GCB GAC TC-3') and PolR (5'-ATS GCCATC ATY TCR CCG GA-3') primers (Niu *et al*., 2018). These primers aided the amplification of the *nifH* genes in the microbial isolates. The *phoD* genes were targeted with ALPS-F730 (5' CAGTGGGACGAC CACGAGGT-3') and ALPS-R1101 (5'-GAGGCCGATCGGCATGTCG-3') (Fraser *et al*., 2015).

A 20 µl amplification reaction was prepared in a 0.2 ml tube using the following: 10 µl of One Taq 2x Master mix with standard buffer PCR Master Mix, 1 µl of Forward primer (10 µM), 1 µl of Reverse primer (10 µM), 2 µl of DNA template and 6 µl of sterile distilled water. The preparation for amplification was performed on ice in a 0.2 ml PCR tube and all solutions were placed on ice during the experiment to avoid denaturation of reagents and DNA templates. Sufficient mix (using the above measurements) for the number of reactions was prepared and the mix aliquot was placed in various tubes (i.e. 20 µl per tube). The amplification reaction was preheated to 98°C for 10 seconds in a BIORAD T100^{TM} Thermal Cycler and the cycle was run at 98˚C, 1 seconds; 55°C, 1 minute; 72°C, 15 seconds in 34 cycles and elongated at 72°C, 1 minute. The reaction was held at 4°C until the amplicons were removed from the thermal cycler. One percent agarose gel electrophoresis was used to confirm the size of amplicons. Amplicons were stored at -20°C for further downstream applications. The same amplification and cycling condition were used for the amplification of the *phoD* genes but the annealing temperature was set at 57°C for 1 minute.

Mac Conkey agar medium was used to isolate pathogenic microorganisms (Alfa *et al*., 2014). Fifty-two grams of the medium was weighed into a 1 litre glass bottles and bulked to volume with distilled water. Medium was autoclaved for 15 minutes at 121°C. Medium was allowed to cool to 50°C before dispensing into plates. Hundred microlitre of digestate sample were aseptically spread plated on solidified Mac Conkey agar medium. The plates were incubated at a temperature of 37°C for 24 hours. This medium is known to be a selective medium for cultivable pathogens.

8.3 Results

The pH of the digestate ranged between 6.9 and 8.2, this pH is optimum to anaerobic microorganisms. An increase in the ammonium content of the digestate was observed (Fig. 8.1). However, the nitrogen content of the digestate decreased drastically. There was also a decrease in the phosphorus and potassium content of the digestate (Fig. 8.2 & Fig. 8.3). The decrease in these major nutrients was brought about by AD, these nutrients are essential for optimum growth and productivity in plants.

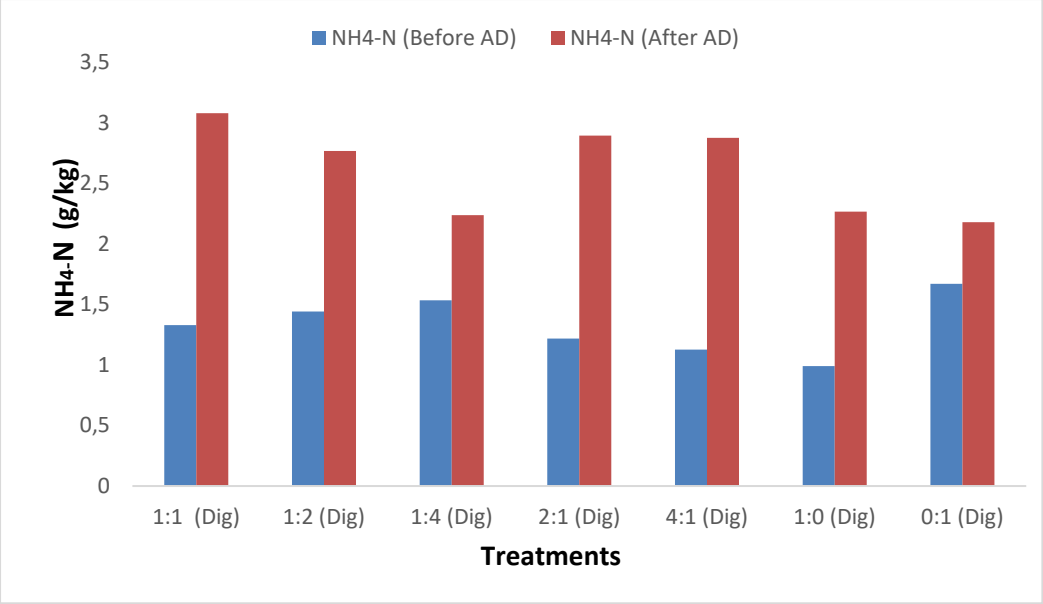

Fig. 8.1: Ammonium nitrogen content of the treatments before and after digestion

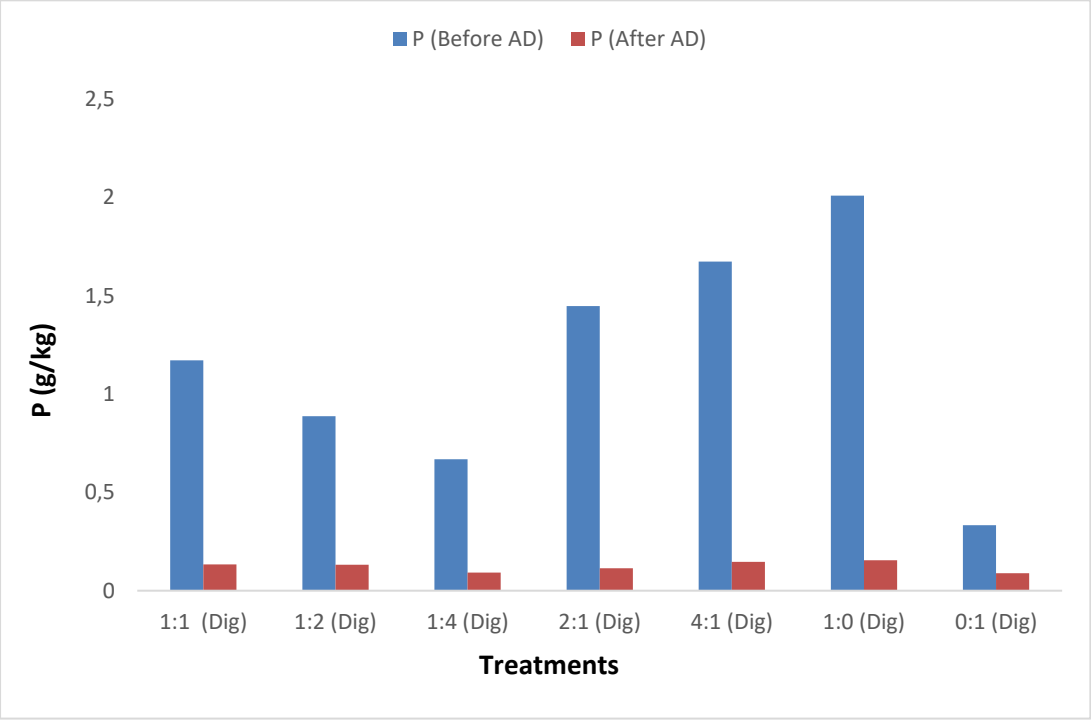

Fig. 8.2: Phosphorus content of the treatments before and after digestion

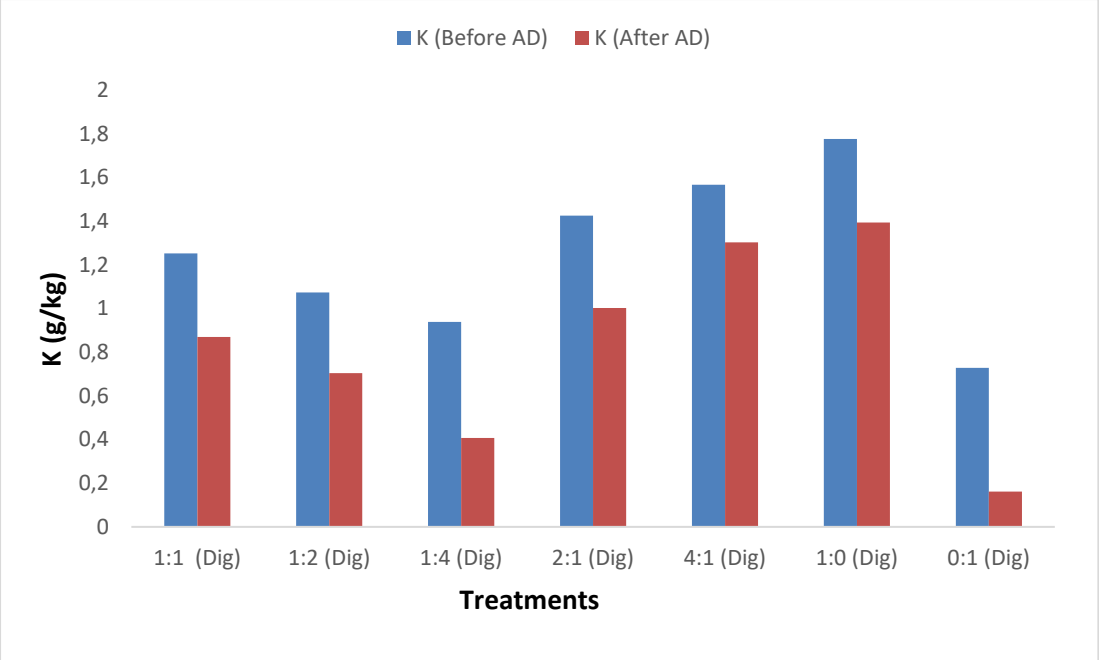

Fig. 8.3: Potassium content of the treatments before and after digestion

Table 8.2: Some of the essential nutrients in the digestate.

Nutrients	**Water hyacinth**	**1:1 (Dig)**	**1:2 (Dig)**	**1:4 (Dig)**	**1:0 (Dig)**	**0:1 (Dig)**	**4:1 (Dig)**	**2:1 (Dig)**
Calcium (mg/kg)	17550	73.11	80.82	69.48	67.43	69.38	72.3	71.44
Magnesium (mg/kg)	6230	32.6	35.19	38.19	30.72	39.74	26.14	34.03
Sodium (mg/kg)	3200	37.12	36.69	43.17	40.43	52.04	39.27	48.24
Iron (mg/kg)	760	15.52	9.2	16.42	24.18	2.81	9.02	5.73
Copper (mg/kg)	7.7	0.36	0.18	0.23	0.22	0.13	0.22	0.12
Manganese (mg/kg)	1.14	4.17	3.64	4.96	2.77	2.57	8.3	4.11
Zinc (mg/kg)	37.65	2.85	1.46	1.07	0.87	3.53	1.89	2.24
Aluminum (mg/kg)	0.422	7.06	4.61	4.52	7.93	2.58	3.33	4.2
Boron (mg/kg)	22.77	1.2	0.76	0.77	0.86	0.64	0.67	0.44

All samples of the digestate contained the essential plant nutrients that are needed for plant growth and productivity. Anaerobic digestion effected a decrease of these nutrients (Table 8.2). Heavy metal analysis showed a substantial decrease of heavy metals in the digestate (Table 8.3). Anaerobic digestion had a positive effect on heavy metals concentrations in the water hyacinth.

Table 8.3: Heavy metal analysis of water hyacinth and digestate

Heavy metals	**Water hyacinth**	**1:1 (Dig)**	**1:2 (Dig)**	**1:4 (Dig)**	**1:0 (Dig)**	**0:1 (Dig)**	**4:1 (Dig)**	**2:1 (Dig)**
Chromium (mg/kg)	7.0	0.07	0.13	0.14	0.20	0.10	0.11	0.06
Nickel (mg/kg)	13.8	0.65	1.45	0.47	0.77	1.53	0.93	0.75
Arsenic (mg/kg)	0.66	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Selenium (mg/kg)	0.51	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Molybdenum (mg/kg)	2.8	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Cadmium (mg/kg)	0.057	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Mercury (mg/kg)	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Lead (mg/kg)	1.32	0.02	0.18	0.02	0.05	0.02	0.13	0.03

The amplification (presence of white bands) of the *nifH* genes (Fig. 8.4) and the *phoD* genes shows their presence in the digestate (Fig. 8.5). These are part of the genes that code the enzymes that catalyse some of the plant growth promoting activities (Bergkemper *et al.,* 2016; Niu *et al.,* 2018).

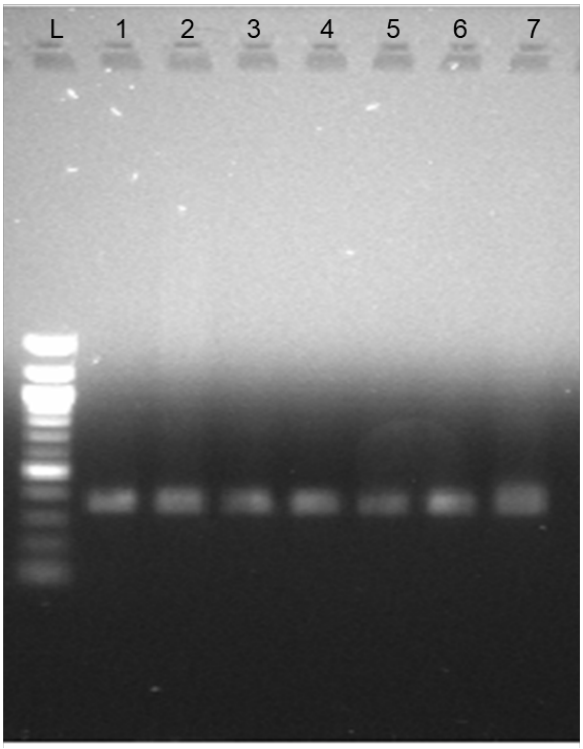

Fig. 8.4: PCR of the nifH gene fragment amplified from the digestate samples. L-100bp DNA ladder; 1, 2, 3, 4, 5, 6 & 7 – Different treatments of digestate samples

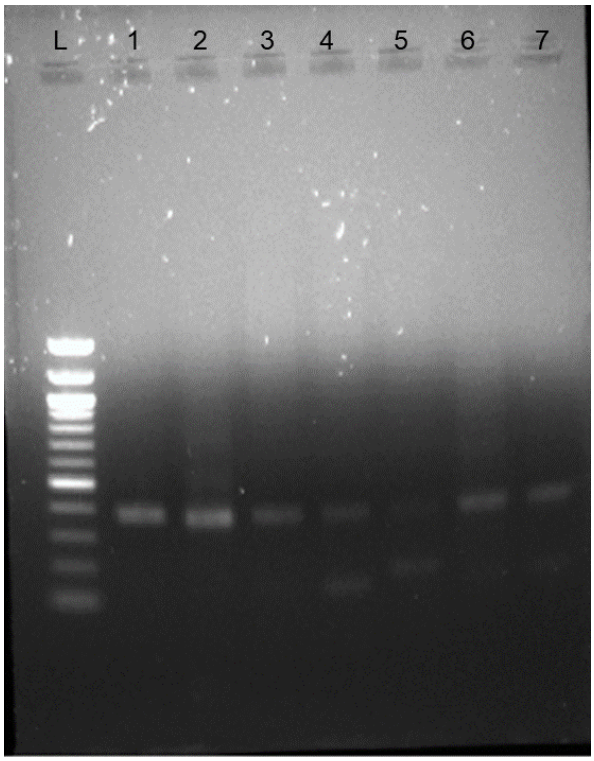

Fig. 8.5: PCR of the phoD gene fragment amplified from the digestate samples. L-100bp DNA ladder; 1, 2, 3, 4, 5, 6 & 7 – Different treatments of digestate samples

No growth was observed on the Mac Conkey agar plates after the incubation period. This indicates the absence of the cultivable pathogenic microorganisms. Significant decrease in the TS and VS content of the digestate was recorded.

8.4 Discussion

Challenges associated with the use of inorganic and organic fertilisers has prompted the use of digestate as a soil ameliorant to improve plant growth and development (Bhardwaj *et al*., 2014). Digestate produced in this study have readily available nutrients as well as PGPMs to enhance crop productivity and this shows its feasibility as a fertilizer (Arati, 2009). The result of this study portrays the conversion of the nitrogen content of water hyacinth to a form of nitrogen that is available to plants which is ammonium (Bonten *et al*., 2014). According to an investigation by Vidya & Girish (2014), water hyacinth has a high concentration of nitrogen and trace elements that are rarely found in chemical fertilizers and these nutrients could enhance the growth and productivity of plant when used as soil conditioner before planting, (Vidya & Girish, 2014; Sindhu *et al*., 2017). It is evident that the nutrients released in the soil, after application of digestate is comparable to or higher than that of organic fertilizers (Möller *et al*., 2012).

Optimum activity of the anaerobic microorganisms has been recorded at pH 6.5-7.5 and this corresponds to the results of this study (Mukhuba *et al*., 2018). The *nifH* genes and the *phoD* genes were found in all the treatments including controls. This suggests the presence of the microorganisms (PGPM) that possess these genes. The *nifH* gene encodes the nitrogenase enzyme which is responsible for the conversion of atmospheric nitrogen to a form of nitrogen that is available to plants (Niu *et al*., 2018). The digestate can therefore be regarded as a nitrogen fixing soil ameliorant. The *phoD* genes were also identified in the digestate samples which implies the capacity of the digestate to solubilise insoluble phosphate when applied to the soil and this qualifies the digestate to be a potential phosphate solubilising biofertilizer (Hassan *et al*., 2017).

Absence of cultivable pathogens in the digestate could be due to their destruction during AD through the generation of metabolic heat as mesophilic AD has been known to destroy pathogenic organisms. This ensures biosafety of the digestate (fertilizer) to animals and humans (Alfa *et al*., 2014, Raimi *et al*., 2017). This study observed a reduction in the solid fractions of the water hyacinth which indicated their degradation by the microorganisms and this led to the production of biomethane (Corro *et al.,* 2013).

Water hyacinth thrives in polluted water and has the ability to absorb heavy metals and high levels of heavy metals in fertiliser is considered harmful to plants as this may affect their growth and productivity (Rai & Singh, 2016). The heavy metals present in the digestate from this study met the required regulations for fertiliser control, the metals were way below the standard for fertilizers according to the fertiliser regulations in South Africa (DAFF, 2012, Mukhuba *et al*., 2018). Heavy metals such as aluminium, arsenic, cadmium and chromium are not needed for plant growth instead are toxic above certain concentrations. However, plants need heavy

metals such as copper, iron, manganese, nickel and zinc for growth and these were identified in the digestate (Table 8.2), this boosts the feasibility of the digestate as a fertilizer (Nanda & Abraham, 2011). Easy penetration of the digestate into the soil after application is advantageous over chemical fertilizers that may possibly form a complex upon application, this justifies the use of digestate from AD of water hyacinth as a soil ameliorant (Lukehurst *et al*., 2010).

It may be concluded that the use of digested water hyacinth as a soil ameliorant is an efficient and environmentally friendly approach to the conventional methods of disposal of the aquatic weeds. It is a promising soil additive as they also possess micro-organisms that can promote plant growth and development.

9 COST BENEFIT ANALYSIS & SOCIO-ECONOMIC IMPACT

9.1 Cost-benefit analysis

As proven by findings of the present study, water hyacinth is a suitable substrate for biogas and soil ameliorant production. However, it is of great importance, from an economical point of view, that a cost-benefit (CBA) analysis be conducted to determine the profitability of the use of water hyacinth as a feed for anaerobic digestion. The profitability will ultimately determine if Dam management adopts the proposed method of water hyacinth control. A CBA of the system that incorporates anaerobic digestion, in comparison to the current method of water hyacinth control (harvesting and composting) was conducted.

Substrate availability: The water hyacinth coverage of Hartbeespoort Dam has recently been reported to be approximately 30% (https://www.environment.co.za/weeds-invaders.../water-hyacinth-crisis-harties.html). Given that the surface area of the dam is 20.62 km² (2 062 ha) (https://kormorant.co.za/17641/the-building-of-hartbeespoort-dam/), 30% coverage implies that approximately 618 ha of the Dam is covered by water hyacinth. It has been reported that the total biomass of water hyacinth can be as much as 420 t fresh weight/ha. Given that the dry weight of water hyacinth is approximately 5-7% of fresh weight, dry weight per hectare is approximately 25 t (Gopal, 1987). Therefore, the dry weight attainable from the water hyacinth presently covering Hartbeespoort Dam (30%) can be estimated at approximately 15450 t. Water hyacinth has an optimal specific growth rate of 0.06 day^{-1} (doubling time = 11.6 days) (Reddy and DeBusk, 1984) hence, the plant is capable of increasing its biomass by approximately 8.5% per day. Due to its elevated growth rate, even if the plant is reduced to 1% of its current cover of the Dam sufficient quantities of hyacinth dry matter will be available to continually feed the proposed digesters, i.e. 0.3% dam cover will result in 154 t of dry weight.

In our analysis, we propose the use of ten small-scale digesters as opposed to one large-scale digester. Multiple, smaller digesters will prevent AD failure from halting the entire process since only the systems that failed will be non-operational. Furthermore, mechanical maintenance of one system will not influence the running of others and in the unlikely event that feedstock levels are low, only a few AD systems can be run at a time. It is assumed that each digester and associated infrastructure costs R100,000 (R1000 000/ 10 digesters) and uses 1 t of dry weight per day (10 t dry weight / day for 10 digesters) of water hyacinth (WH). Following Zheng *et al*. (2008) cited in Wangand Calderon (2012), there are 330 days in a production year. Thus, the annual dry matter weight of WH for the digesters are 3300 t (10 t/day x 330 days). According to the findings in the present research, 730 m^3 of biogas is produced per ton of dry weight of WH, implying that 7300 m^3 (730 m^3 biogas/t x 10 t/day) biogas is produced per day in this case, and annual biogas is 2409000 m^3 (7300 m^3 biogas/ day x 330 production days/yr). According to Banks (2009), the efficiency of biogas to be converted to electricity is 35%. Therefore, the electricity production potential of 1 m^3 of biogas is 2.14 kWh (i.e. its energy potential of 6.1 kWh x 0.35). Given that 1 m^3 biogas = 2.14 kWh (electricity), the biogas yield could produce 5155260 kWh per year (2409000 m^3 x 2.14 kWh), supporting a 588.5 MW power plant (5155260 $kWh.yr^{-1}$ / 8760 $hrs.yr^{-1}$). The government guaranteed electricity price realised for small waste to energy projects under the Renewable

Energy Independent Power Producer Programme is R1.17 / kWh (IPP Office, 2017), therefore the annual revenue from the produced electricity is estimated to be approximately R6 million (5155260 kWh x R1.17/kWh = R6,031,654).

To ascertain the quantity of fresh water hyacinth that is harvested per day, we follow findings of the present study which prove that fresh water hyacinth consists of 5.29% total solid content. Since we know that 3300 t of dry matter are used annually, then fresh WH harvested per year is approximately 62 000 tonnes (62,381.85 t). Harvesting the WH at a rate of R110 per day for 200 people implies that the annual harvesting cost is approximately R7 million (R110 x 200 x 330days = R7,260,000). The shadow price of labour was estimated using the daily rate for non-residing domestic workers. Other costs such as repair and maintenance of the digester, depreciation of the digester over the years, electricity used to run the digester are assumed to surmount to R100 000 (R10000 per annum/digester). Assuming that one hectare (1 ha) of land is used to compost the harvested fresh WH (composting approach), the cost of 1 ha near Hartbeespoort Dam is R3.5 million (Property 24). In this analysis, this is used as the annual cost of using that land to compost the harvested fresh WH. The land required for the AD process is assumed to be 0.5 ha (R1.75 million).

According to our study, 4.3% of fresh WH is organic matter. Assuming that all the sludge (organic matter component) is used for soil ameliorant/ compost, the annual soil ameliorant/ compost produced is 2682 t (62,381.85 t x 4.3%). In terms of nutrient content, WH comprises of 0.04%N, 0.06% P_2O_5 and 0.20% K_2O (ratio of 1:1.5:5) (Jafari, 2010). Given that potassium accounts for the largest nutrient component (67%) the current market price of potassium chloride is used to ascertain the value of the soil ameliorant. According to data by the World bank[1], the average price of potassium chloride between February and April 2018 was slightly above R2500 metric t^{-1}, hence annual value of the produced soil ameliorant/ compost is approximately R6.7 million (2682,42 t x R2500 metric t^{-1} = R6,706,048.88). Although the bio-digesters come in as a fixed cost, it is assumed that its life span is 15 years and that by that time, its salvage value will be zero (Table 9.1).

[1] http://www.indexmundi.com/commodities/?commodity=potassium-chloride&currency=zar

Table 9.1: Amounts and prices of inputs and outputs for the 'composting' and 'biogas & soil ameliorant' scenarios

Item	Amount	Price (R)	Annual amount
	Inputs		
Fresh water Hyacinth	189.04 t day^{-1}		62,381.85 t
Cost of digester	10	1,000,000	R1,000,000
Workers collecting Water Hyacinth (labour)	200 Persons	110 day^{-1}	R 7,260,000
Other costs (electricity, repair and maintenance)		100000	100000
Land	1 ha	3500000	R3,500,000
	Output		
Biogas	7300 m^3 day^{-1} x 2.14 kWh	R1.17 kWh^{-1}	R6,031,654
Soil ameliorant/ Compost	8.129 t day^{-1}	R2500 t^{-1}	R6,706,048.88

Note: t = ton(s), ha = hectare, R =Rand

Results

In the analysis costs and benefits were priced using the market prices as discussed in the previous section. Following Wand and Calderon (2012) a real-term discount rate of 6% was used as the baseline. It was also assumed that even with the introduction, biogas and soil ameliorant technologies as ways of using the WH, there shall be enough quantities of the WH all year round. Thus, if the proposed project uses over 50000 tons of WH per annum, approximately R2.6 million will be realised as benefits from biogas and the soil ameliorant while R4 million will be saved for the case of the composting approach being used (Table 9.2).

Table 9.2: Cost benefit analysis results for the 'composting' and 'biogas & soil ameliorant' scenarios

Component	Composting option (R)	Proposed option (R)
Benefits		
Benefit of Biogas		R6 031 654
Benefit of Soil ameliorant		R6 706 048
Benefit of Compost	R6 706 048	
Sub-Total	**R6 706 048**	**R12 737 702**
Costs		
Harvesting water Hyacinth	R7 260 000	R7 260 000
Fixed cost of digesters		R1 000 000
Land for the composting	R3 500 000	
Land for anaerobic digestion		R1 750 000
Other costs (electricity, repair and maintenance)		100000
Sub-Total	**R10 760 000**	**R10 110 000**
Economic benefit	**-R4 053 952**	**R2 627 702**
Net Present Value (NPV)	**R6 681 654**	

All in all, the project is viable, with an estimated net present value of approximately R6.6 million. Since the scope of the analysis was limited to tangible products, the composting approach seems not to generate any economic benefits. However, it may not be concluded that it is not feasible since there may be other non-tangible benefits accruing as a result of this initiative. In terms of the alternative approach, results reveal that benefits derived from biogas generation are similar to those obtained from the soil ameliorant. In the case of costs, land used for composting accounts for the biggest cost due to the sky rocketing land prices within the areas.

9.2 Socio-economic impact

A survey was conducted to investigate the socio-economic impact of the utilization of water hyacinth from Hartbeespoort dam in biogas and soil ameliorant production. Data was collected in the form of questionnaires (Fig. A9.1). Knowledge was disseminated in the form of flyers (Figs A9.2 & 9.3) and verbal communication. The study sites for the activity included two sites frequented by both, members of the Hartbeespoort community and visitors from other areas. These sites included the Hartbeespoort Village Mall (25.7318° S, 27.8879° E) and the Harties Aquarium/ French Toast (25.7371° S, 27.9024° E). Selection of the sites was primarily based on the close proximity to the Hartbeespoort Dam (<15 km).

Flyers and information transfer in the form of verbal communication was widely disseminated and completion of the questionnaire was optional to all those that were informed about the

project. Ninety two (92) questionnaires were completed. The respondents were evenly spread with regard to gender (Fig. 9.1). Most of the respondents belonged to the 20-29 year age bracket. However, both the 30-39 and 40-49 age brackets were also well represented (Fig. 9.2). The respondents were primarily composed of residents from the area (Hartbeespoort) which was the principal target group. Approximately 30% of the respondents were visitors to the area (Fig. 9.3). Feedback from visitors was valuable to determine influence of the plant on tourism.

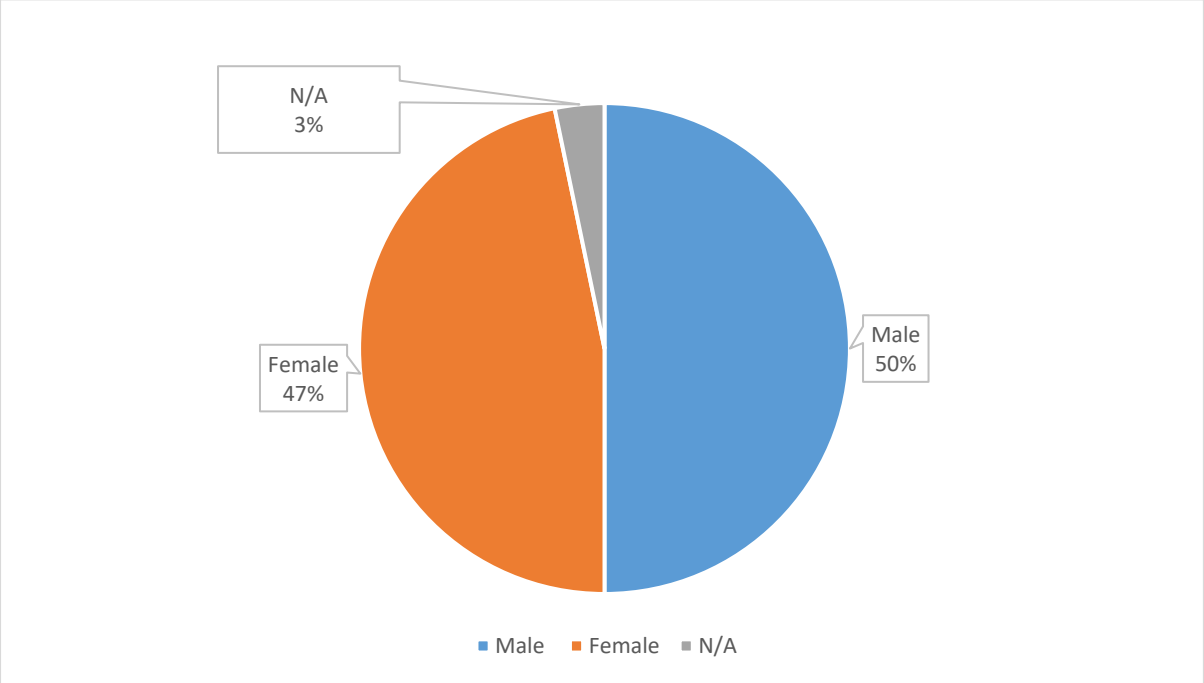

Fig. 9.1: Gender of respondents

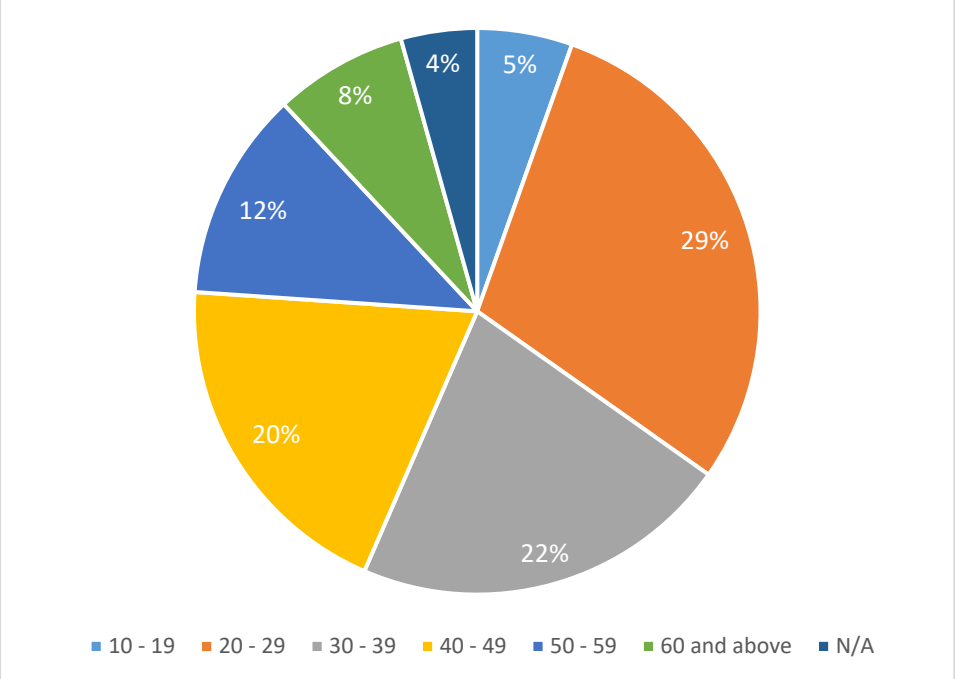

Fig. 9.2: Age of respondents

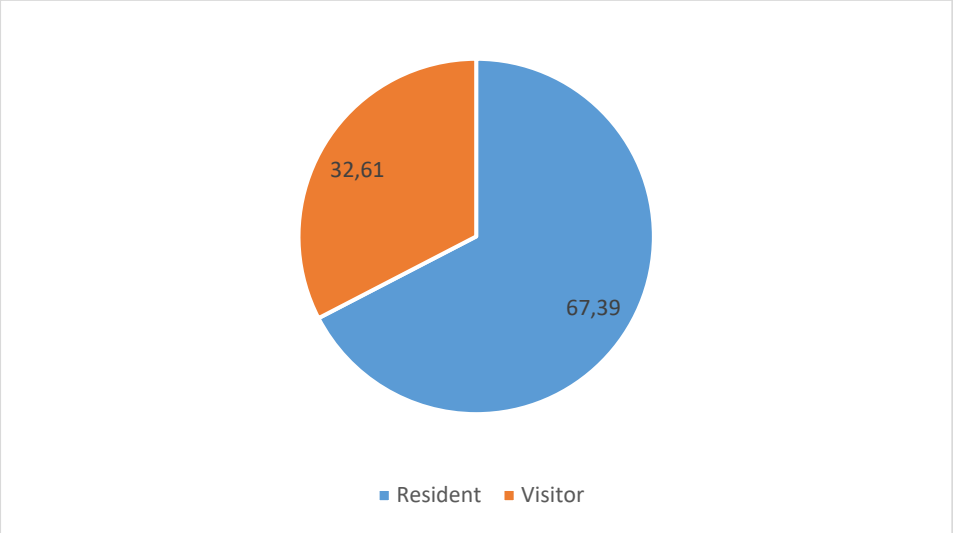

Fig. 9.3: Spread of respondents (visitor/ resident)

The questionnaire consisted of 11 YES/NO questions (Fig. A9.1) that can be divided into 4 categories (Table 9.3), i.e. Location, problem awareness, solution awareness and acceptance.

Table 9.3: Questionnaire outline

No.	Question	Category
1	Do you reside close to the Hartbeespoort Dam?	Location → Visitor/ resident
2	Do you know about water hyacinth?	Problem awareness
3	Does the presence of water hyacinth in the dam negatively affect you?	
4	Are you aware of the methods used to control water hyacinth in the dam?	
5	Do you know the implications of the conventional method used in controlling water hyacinth in the dam?	
6	Do you know about biogas?	Solution awareness
7	Are you aware of the use of biogas as an alternative energy source for cooking?	
9	Do you know that biogas can be produced from water hyacinth?	
8	Would you use biogas to cook at home?	Acceptance
10	Would you be willing to pay more for biogas than LPG? (e.g. 9 kg LPG = R201.00, 9 kg Biogas = R268.00)	
11	Would you accept digesters in your community/near the dam?	

Problem awareness: Overall, a large portion of the respondents (75%) knew about water hyacinth (Fig. 9.4A). Possibly, some of the respondents who answered 'no' to the question 'Do you know about water hyacinth?' did not associate the name 'water hyacinth' with the plant on the surface of the dam. Approximately 60% of the respondents were negatively affected by the presence of water hyacinth in the dam (Fig. 9.4B). This proves that there is a

social need for the removal of the plant from the dam. Even though 75% of the respondents were familiar with the plant being present in the dam, only approximately 45% were aware of methods used to control water hyacinth (Fig. 9.4C). As expected, an even smaller percentage of the respondents were aware of the implications of conventional methods used to control water hyacinth in the dam (Fig. 9.4D).

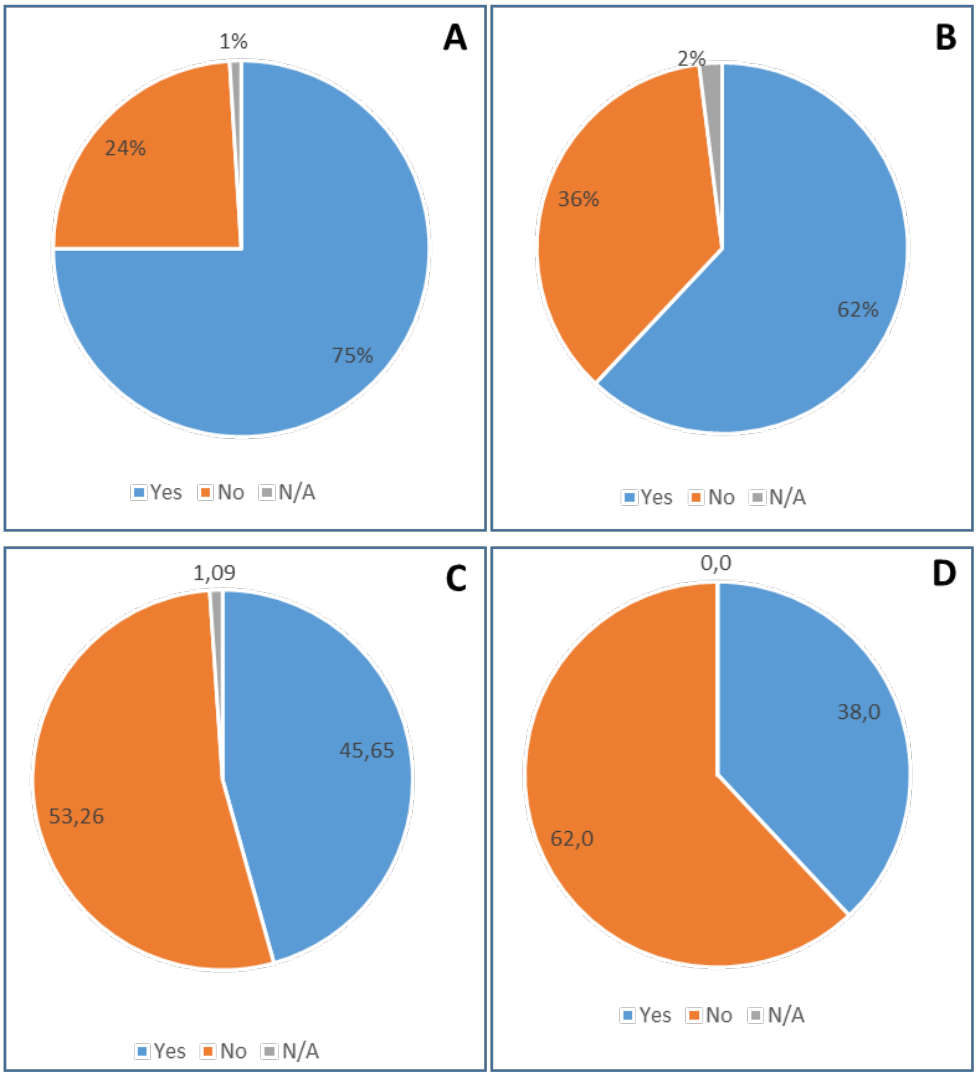

Fig. 9.4: Responses of respondents to (A) question 2, (B) question 3, (C) question 4 and (D) question 5 (problem awareness)

Solution awareness: The next set of questions (6, 7 and 9) were drafted with the aim of establishing the awareness of biogas technology. Furthermore, these questions motivated the respondents to enquire about the technology enabling information transfer. Interestingly, over 50% of the respondents were familiar with biogas (Fig. 9.5A). Approximately, 50% of the respondents were aware of the use of biogas for cooking purposes (Fig. 9.5B). Question 9 interlinked water hyacinth and biogas production. Only approximately 35% of the respondents knew that biogas can be produced from hyacinth (Fig. 9.5C). Many respondents were intrigued by the fact that energy can be produced from a plant and this stimulated conversation and questions.

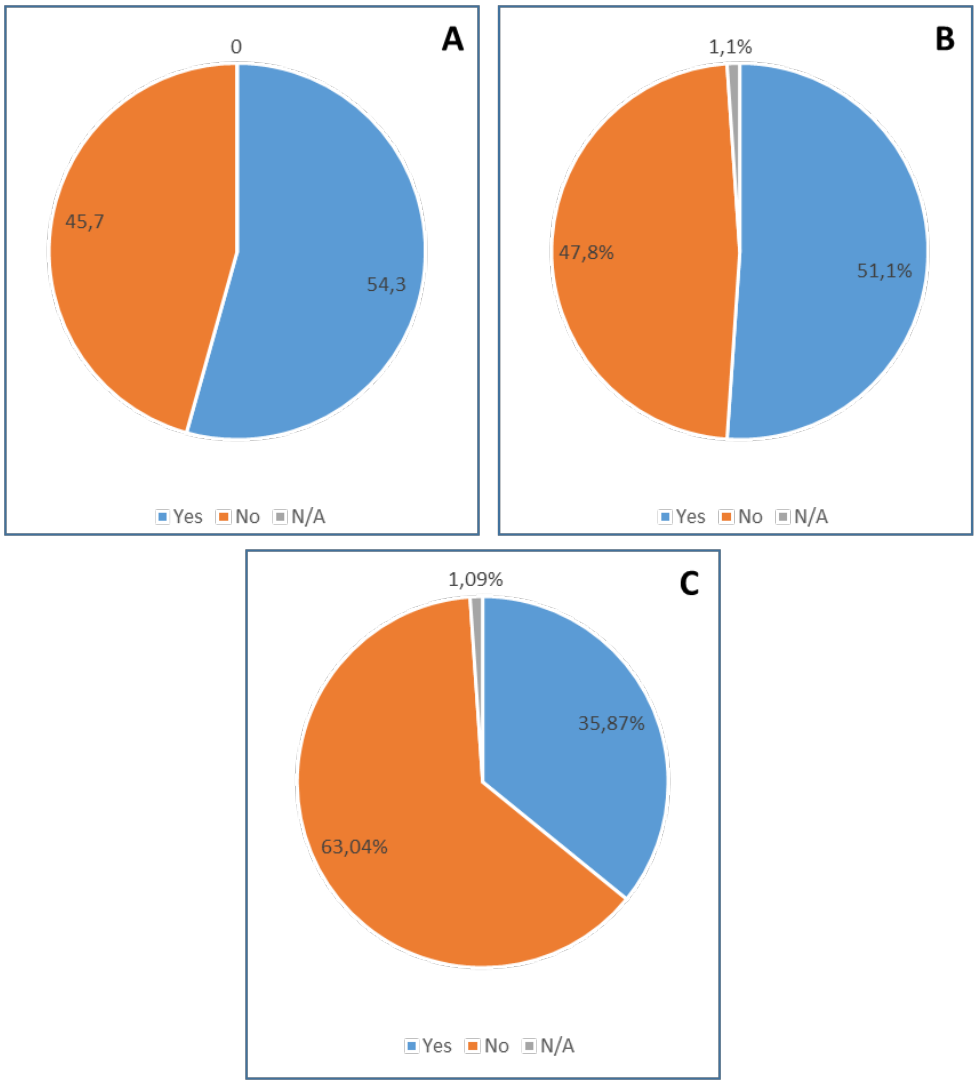

Fig. 9.5: Responses of respondents to (A) question 6, (B) question 7, (C) and (D) question 9 (solution awareness)

Acceptance: Questions 6, 7 and 9 set the scene for the next questions (8, 10 & 11) which were aimed at establishing acceptance of the technology by the respondents. Approximately 70% of the respondents answered 'yes' to 'would you use biogas to cook at home?'. This is positive considering that only 54% were familiar with the technology (Fig. 9.5A). Implying that, even though the technology is new to some of the respondents they are still willing to utilise it. The respondents that replied with 'no' (approx. 30%) may be not keen on using a new technology due to safety implications or overall unacceptance of gas as an energy source (Fig. 9.6A). Interestingly, approximately 60% of the respondents were willing to pay more for biogas (1/3 extra) instead of LPG (Fig. 9.6B). Verbal communication with some of the respondents proved that this answer (in certain instances) was motivated by the fact that biogas is a renewable form of energy. Close to 80% of the respondents would accept biogas digesters next to the dam (Fig. 9.6C). Interestingly, more residents (86%) accepted digesters at the dam than visitors (75%) (Fig. 9.7). This may be attributed to the long term, daily negative effects being

experienced by residents. Residents are therefore, probably more keen on solving the water hyacinth problem at the dam.

Acceptance of the technology, associated infrastructure and products was overwhelming and prove that the respondents are ready for the implementation of the technology at the Hartbeespoort dam. However a wide scale survey is necessary to further establish acceptance prior to implementation. It is envisaged that the flyers that were distributed would have a wide reaching impact on the community and initiate further knowledge transfer. Some of the respondents such as school goers remarked that they will share the flyers with teachers and initiate further communication with the ARC with regard to the project. Students also volunteered to assist with removal of the hyacinth from the dam and some respondents were excited about the project due to the prospect of future jobs if the project is initiated.

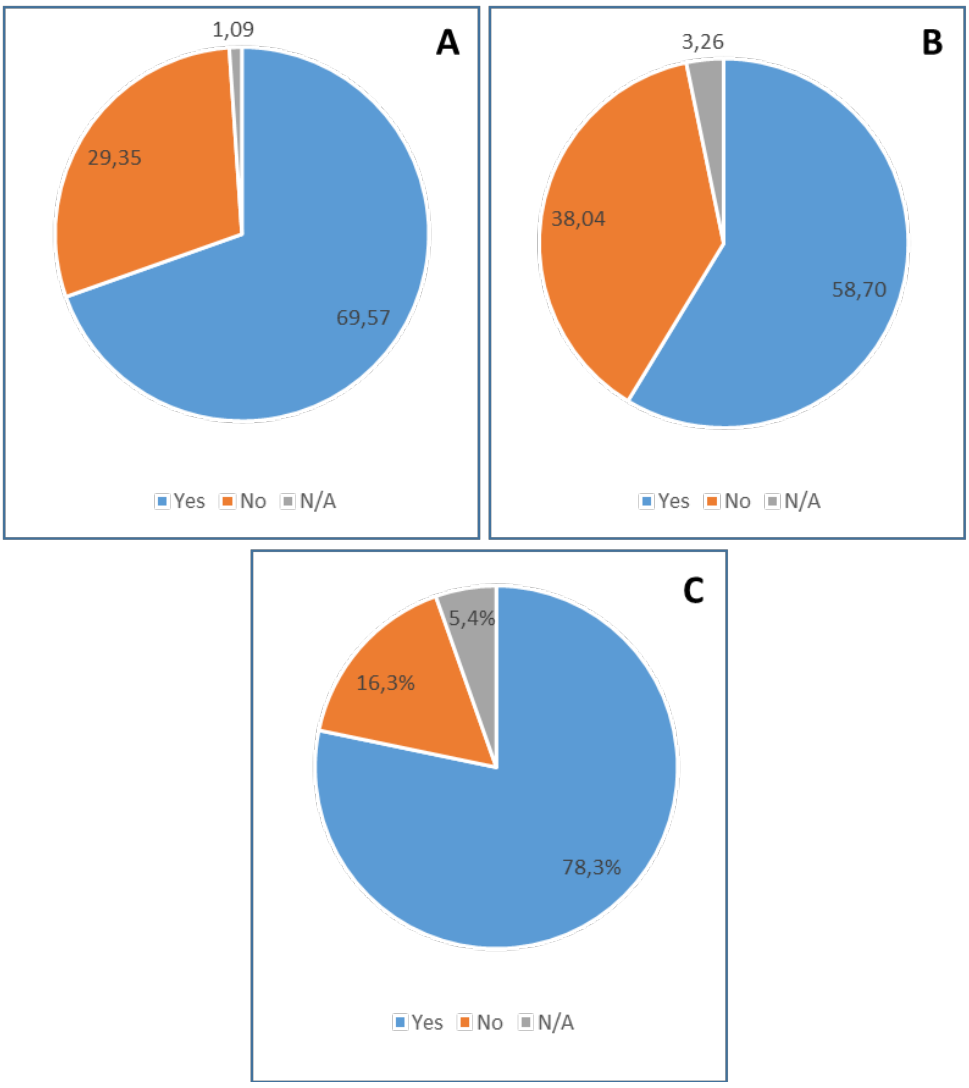

Fig. 9.6: Responses of respondents to (A) question 8, (B) question 10, (C) and (D) question 11 (acceptance)

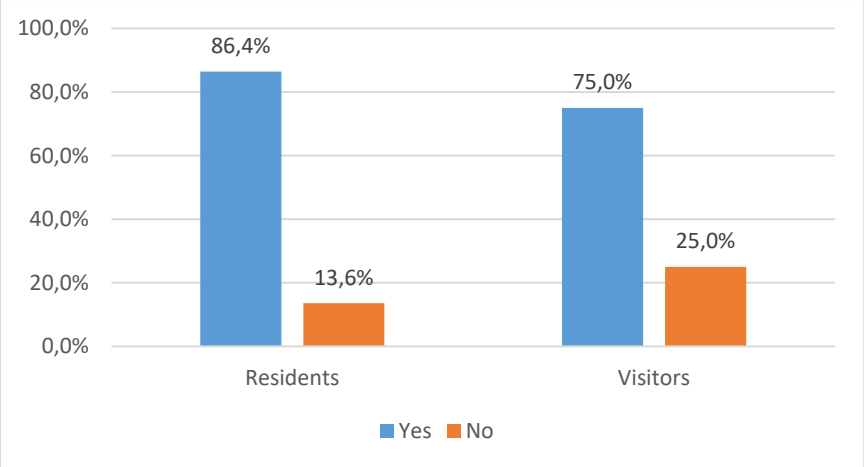

Fig. 9.7: Responses of respondents to question 11 (acceptance of digesters at the dam)

10 CONCLUSIONS & RECOMMENDATIONS

The overall aim of the project was to optimise biogas production from water hyacinth obtained from Hartbeespoort Dam and to determine the feasibility of the use of the digestate from the AD process as a soil ameliorant. To achieve this aim it was necessary to initially verify if the water hyacinth is suitable as a feed for anaerobic digestion (AD). The AD process is microbially driven. The outcome of the AD process is dependent on the activity of the microbial community, while the activity of microbial community is dependent on the availability of certain nutrients. These nutrients are supplied by the selected feedstock. The presence of the macro and micronutrients necessary for AD in water hyacinth makes the plant a suitable feedstock for the process. However, low C/N ratio of the plant proved that co-digestion may be necessary.

The optimisation of the AD process is necessary to ensure maximal biogas/ energy production. This, in turn, will contribute to the economic viability of the process. Methods tested to optimise AD in the present study were pre-treatment of the hyacinth, bioaugmentation and co-digestion. Physical pre-treatment of the hyacinth resulted in increases in biogas yield and the hand-cutting method was selected and utilized in all subsequent experiments. Bioaugmentation using inoculum obtained from water hyacinth also proved to increase biogas yield. Interestingly, unlike initially anticipated, the co-digestion of water hyacinth with cow dung resulted in lower biogas yield than the mono-digestion of water hyacinth. This finding was strongly linked to the microbial communities involved in the AD process. This further motivates the importance of understanding the microbiology of the AD process and conducting microbiological analysis alongside physico-chemical analysis at any biogas plant.

Bacteria and archaea play key roles in the AD process. It is therefore important to analyse these microbial communities during the course of AD. To achieve this, it is necessary to extract DNA from AD samples. Analysis of various DNA extraction methods has shown that the choice of method of DNA extraction influences metagenomic results when analysing AD samples. Extraction methods that result in optimal DNA extraction from AD samples were identified and utilised during the course of the study. Analysis of microbial communities during AD enabled the identification of biogas producing microorganisms naturally associated with the collected hyacinth. This has major implications for uncontrolled anaerobic digestion of hyacinth in the dam which would result in biogas release to the atmosphere. This has environmental implications since biogas is composed primarily of methane which is a potent greenhouse gas and further motivates the present study, i.e. removal of the hyacinth from the dam and AD in a controlled environment.

The utilisation of the effluent from the anaerobic digestion process as a soil ameliorant will further aid in improving the economic viability of the technology. The presence of plant available nutrients and plant growth promoting microorganisms in the effluent was observed in this study. The digestate may therefore serve as a promising soil additive. The production of two products (biogas and soil ameliorant) from water hyacinth has significantly aided in ensuring the economic viability of the proposed method of hyacinth control. The widespread

acceptance of the technology by the surveyed community is also very beneficial for eventual large scale implementation.

Recommendations

Studies on AD mainly focus on methanogenesis (archaea). Although important, bacterial communities also play an important role and without the activity of the bacterial community, methanogens will not have precursors to use in methanogenesis. As seen from the results, the presence of certain bacterial communities may be related to process efficiency or inefficiency. Thus, more understanding of bacterial community during AD is required especially to disturbances by OLRs and type of substrate to allow for the selection of bacteria that can be used as indicators of AD stability. These indicators may be useful in prevention of process failure. Future research to isolate microorganisms from water hyacinth inoculum for bioaugmentation could help attain further improvements in biogas yield. Studies on metabolomics could be conducted to determine the metabolites produced at different stages of AD and how to bioaugment the process with appropriate microorganisms to improve biomethane production.

The technology has thus far been tested at lab-scale. Field trials are necessary to determine feasibility on site. Various digester configurations will need to be tested. Future studies should also focus on the utilisation of digestate for improvement of plant growth and development in the greenhouse and during field trials.

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APPENDIX

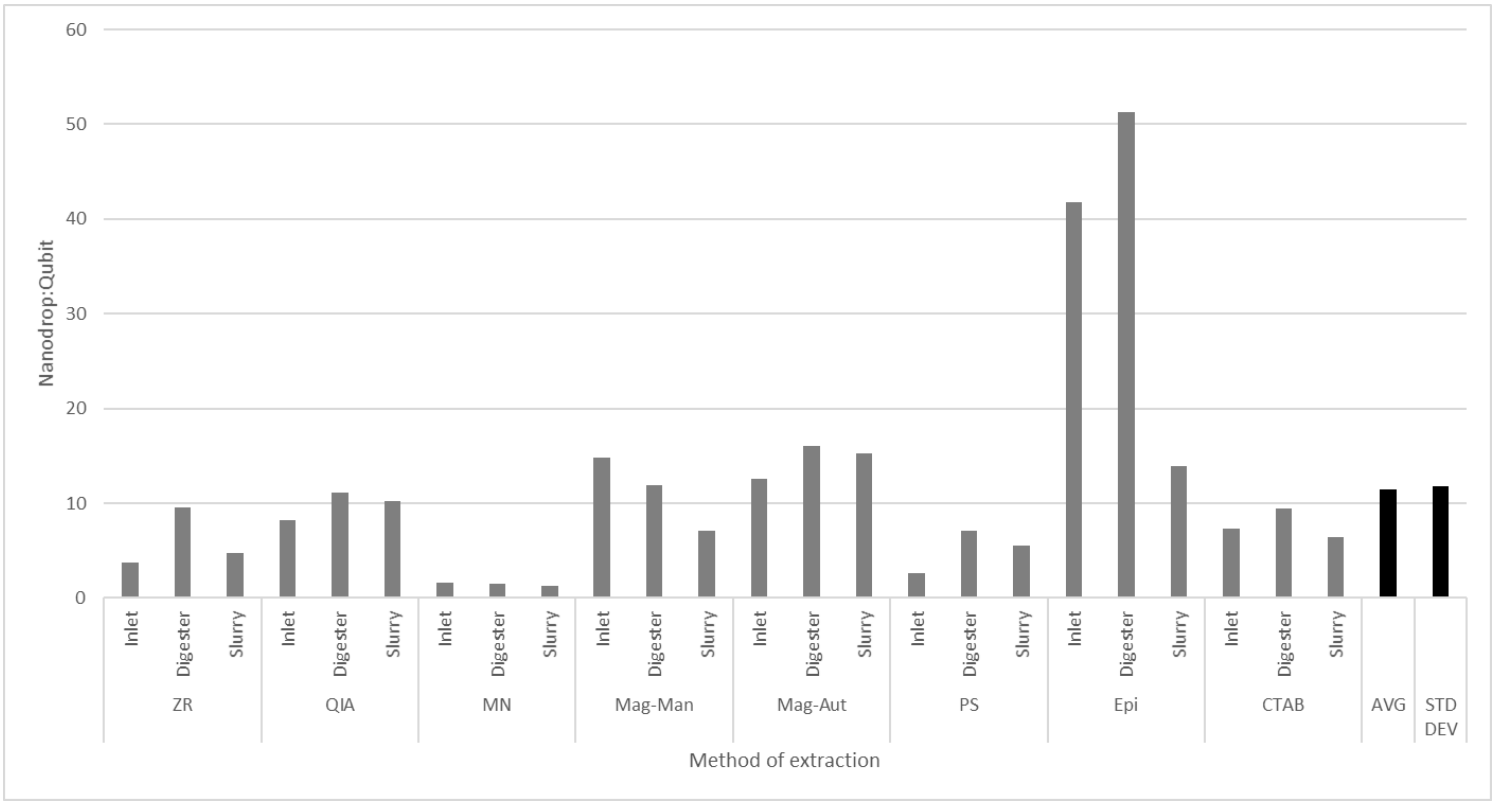

Fig. A2.1 Ratio of Nanodrop and Qubit measurements of DNA extracts from samples obtained from the inlet, digester and slurry using various methods of DNA extraction.

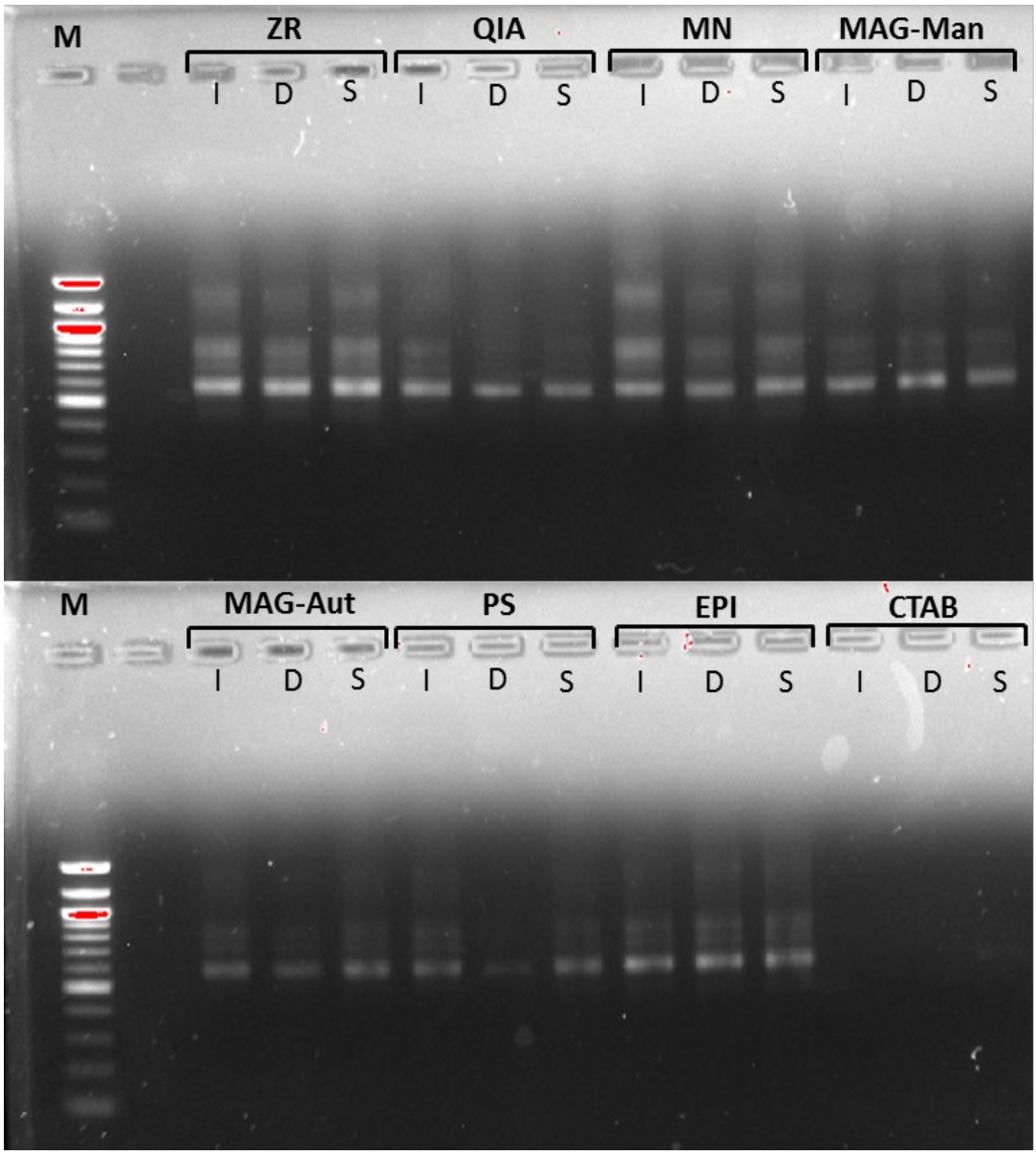

Fig. A2.2 PCR amplicons from samples obtained before, during and after anaerobic digestion using various DNA extraction methods (Primer pair: 341F-907R). M = DNA ladder; I = Inlet; D = Digester; S = Slurry; ZR = ZR Soil Microbe DNA MiniPrep – Zymo Research; QIA = QIAamp Fast DNA Stool Mini Kit – QIAGEN; MN = NucleoSpin Soil Kit – Macherey-Nagel; MAG-Man = MagMAX Total Nucleic Acid Isolation Kit – Manual – Thermo Fisher Scientific; MAG-Aut = MagMAX Total Nucleic Acid Isolation Kit – Automated – Thermo Fisher Scientific; PS = Powersoil DNA Isolation Kit – MO BIO Laboratories; EPI = Meta-G-Nome DNA Isolation Kit – Epicentre; CTAB = CTAB based extraction.

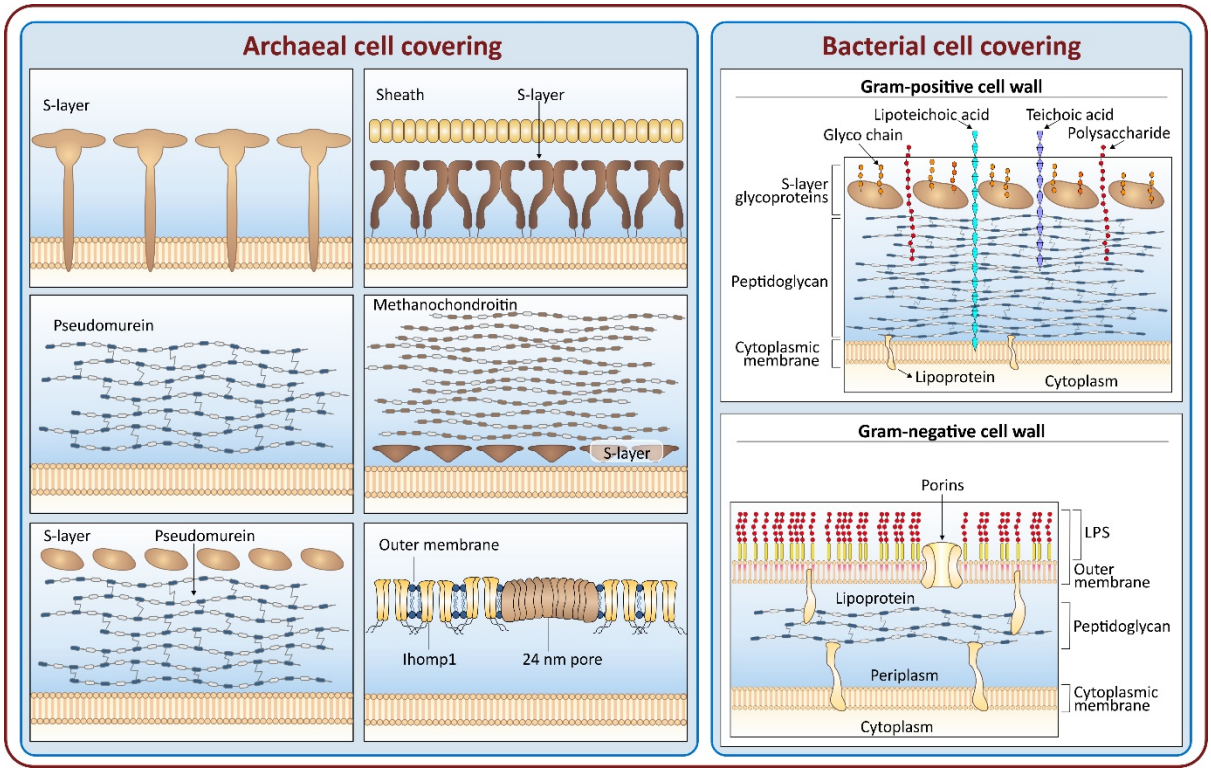

Fig. A2.3 Cell surface structure of archaeal and bacterial cells (adapted from Albers & Meyer, 2011).

Table A2.1 Taxonomic identification of excised bands from DGGE gels of DNA extracted using various methods

Lab ID	Closest relative	Sequence similarity	Cell surface structure*	KIT							
				ZR	QIA	MN	Mag-Man	Mag-Aut	PS	EPI	CTAB
Bacteria											
BJ2	*Macellibacteroides fermentans HQ020488*	97	Gram +, thick peptidoglycan	+	+	++	+++	+	+	+	+
BJ3	*Ruminococcaceae EU466327_s*	98	Gram +, thick peptidoglycan	+	++	+++	+	++	++	+	+
BN1	*Planococcus sp. C2-4c3*	99	Gram +, thick peptidoglycan	++	+	++	+	+++	+	++	+
BO1	*Coriobacteriaceae AB192230_s*	99	Gram +, thick peptidoglycan	+	-	+	+	+	+	+	-
BQ1	*Ruminococcaceae DQ455860_s*	86	Gram +, thick peptidoglycan	+	-	+	+	+	+	+	-
BT1	*Cellulomonas; 4P002473_s*	97	Gram +, thick peptidoglycan	-	-	-	-	-	-	+	-
BU2	*Ruminococcus_g2; KC163015_s*	97	Gram +, thick peptidoglycan	+	-	++	-	-		++	-
Archaea											
AB1	*Methanosarcina barkeri AJ012094*	99	S-layer and Methano-chondroitin	++	-	++	-	-	-	-	-
AB2	*Methanosarcina barkeri str. Wiesmoor CP009526.1*	99	S-layer and Methano-chondroitin	++	-	++	-	+	+	-	-
AC1	*Methanosarcina siciliae NR_104757.1*	99	S-layer and Methano-chondroitin	++	-	+++	-	-	+	-	-
AD1	*Methanocorpusculum AB541926*	94	S-layer	-	++	++	+	+	-	-	-
AH1	*Methanosarcina barkeri AJ012094*	99	S-layer and Methano-chondroitin	++	-	+++	-	++	+++	-	-
AH2	*Methanosarcina horonobensis AB288262*	99	S-layer and Methano-chondroitin	++	-	++	+	+	++	-	-
AH3	*Methanosarcina horonobensis; AB288262*	99	S-layer and Methano-chondroitin S-layer and	+	-	++	-	-	+	-	-

Lab ID	Closest relative	Sequence similarity	Cell surface structure*	KIT							
				ZR	QIA	MN	Mag-Man	Mag-Aut	PS	EPI	CTAB
AH4	*Methanosarcina siciliae NR_104757.1*	99	Methano-chondroitin S-layer and	+	-	+	-	+	+	-	-
AI1	*Methanosarcina horonobensis; AB288262*	99	Methano-chondroitin S-layer and	+	-	++	-	+	++	-	-
AI2	*Methanosarcina horonobensis; AB288262*	99	Methano-chondroitin S-layer and	+	-	+	-	+	+	-	-
AI3	*Methanosarcina horonobensis; AB288262*	99	Methano-chondroitin	+	-	+	-	-	+	-	-
AM1	*Methanobrevibacter ruminantium KP123408.1*	97	Pseudo-murein, hetero-polysaccaride, glutaminylglycan	+++	++	+++	+++	++	++	++	+
AO1	*Methanosarcina barkeri NR_118371.1*	99	S-layer and Methano-chondroitin	++	-	++	+	++	++	-	-
AO2	*Methanosphaerula palustris NR_074167.1*	99	Pseudo-murein, hetero-polysaccaride, glutaminylglycan	+++	+	+++	++	+++	+++	+	+
AQ1	*Methanosarcina horonobensis; AB288262*	99	S-layer and Methano-chondroitin	++	-	++	-	++	++	-	-
AQ2	*Methanosarcina horonobensis; AB288262*	99	S-layer and Methano-chondroitin	+++	+	+++	+	++	+++	-	+
AR1	*Methanosarcina barkeri; AJ012094*	99	S-layer and Methano-chondroitin	+	-	++	-	+	++	-	-
AR2	*Methanosarcina barkeri NR_118371.1*	99	S-layer and Methano-chondroitin	+++	-	+++	+	++	+++	-	+
AR3	*Methanosarcina spelaei; JF812257*	99	S-layer and Methano-chondroitin	+++	-	+++	+	++	+++	-	+
AS1	*Methanosphaerula palustris; CP001338*	99	Pseudo-murein, hetero-polysaccaride, glutaminylglycan	+++	+++	+++	+++	+++	+++	+++	+++

Lab ID	Closest relative	Sequence similarity	Cell surface structure*	**KIT**							
				ZR	QIA	MN	Mag-Man	Mag-Aut	PS	EPI	CTAB
AV1	*Methanobrevibacter olleyae NR_043024.1*	86	Pseudo-murein, hetero-polysaccaride, glutaminylglycan	+++	+++	+++	+++	+++	+++	+++	+++

+ present in small quantities; ++ present in intermediate quantities; +++ present in large quantities;
- absent

*Kersters & Vancanneyt, 2005; Klingl, 2014

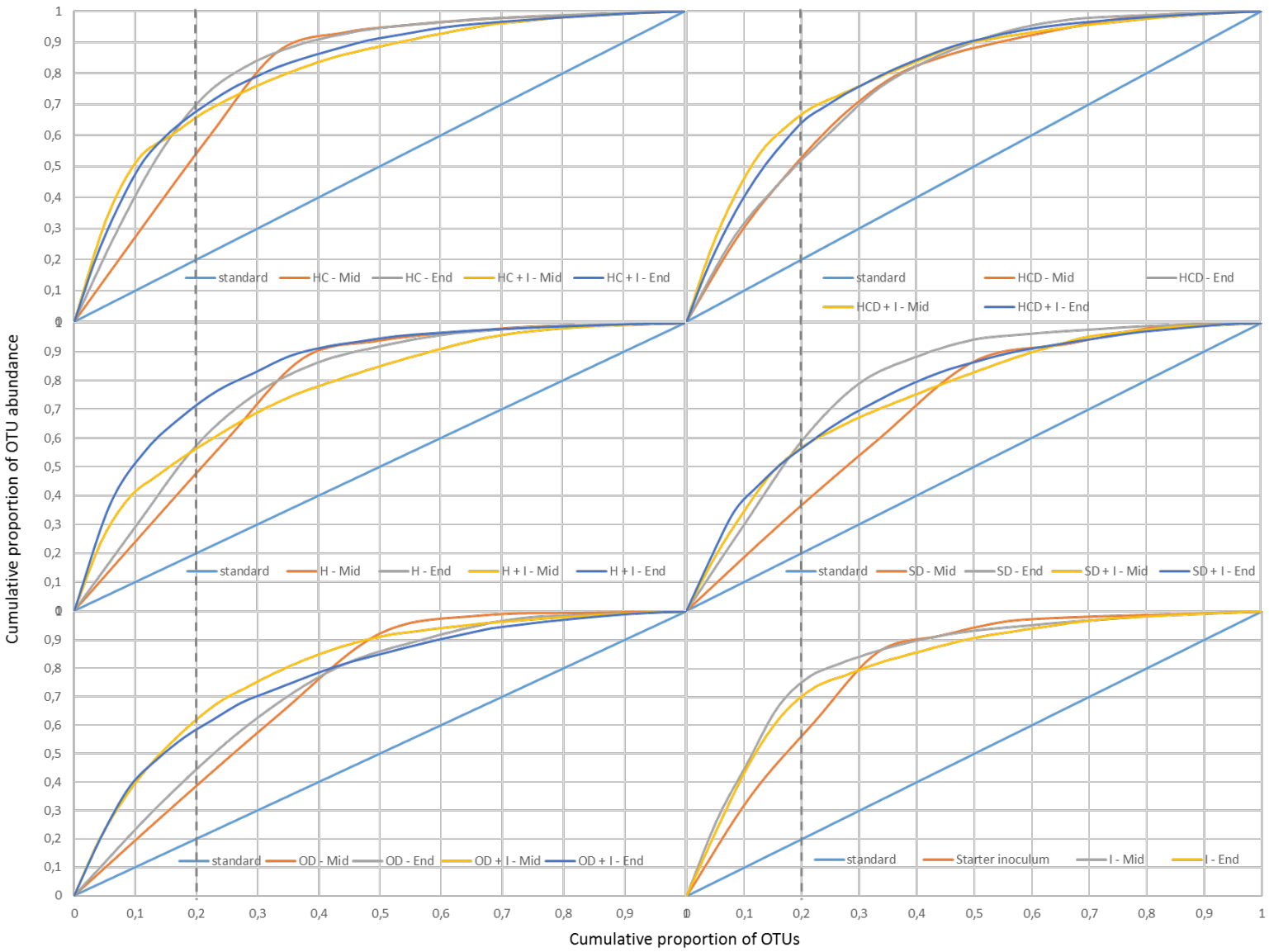

Fig. A5.1: Pareto-Lorenz distribution curves based on the archaeal DGGE profiles associated with various methods of substrate pre-treatment for graphical representation of the archaeal community evenness. Perfect evenness is illustrated as a straight line (standard). A dashed vertical line is plotted to evaluate the range of the Pareto value. OD, oven dried; H, homogenised; HCD, hand cut and decomposed; SD, sun dried; HC, hand cut; I, inoculum.

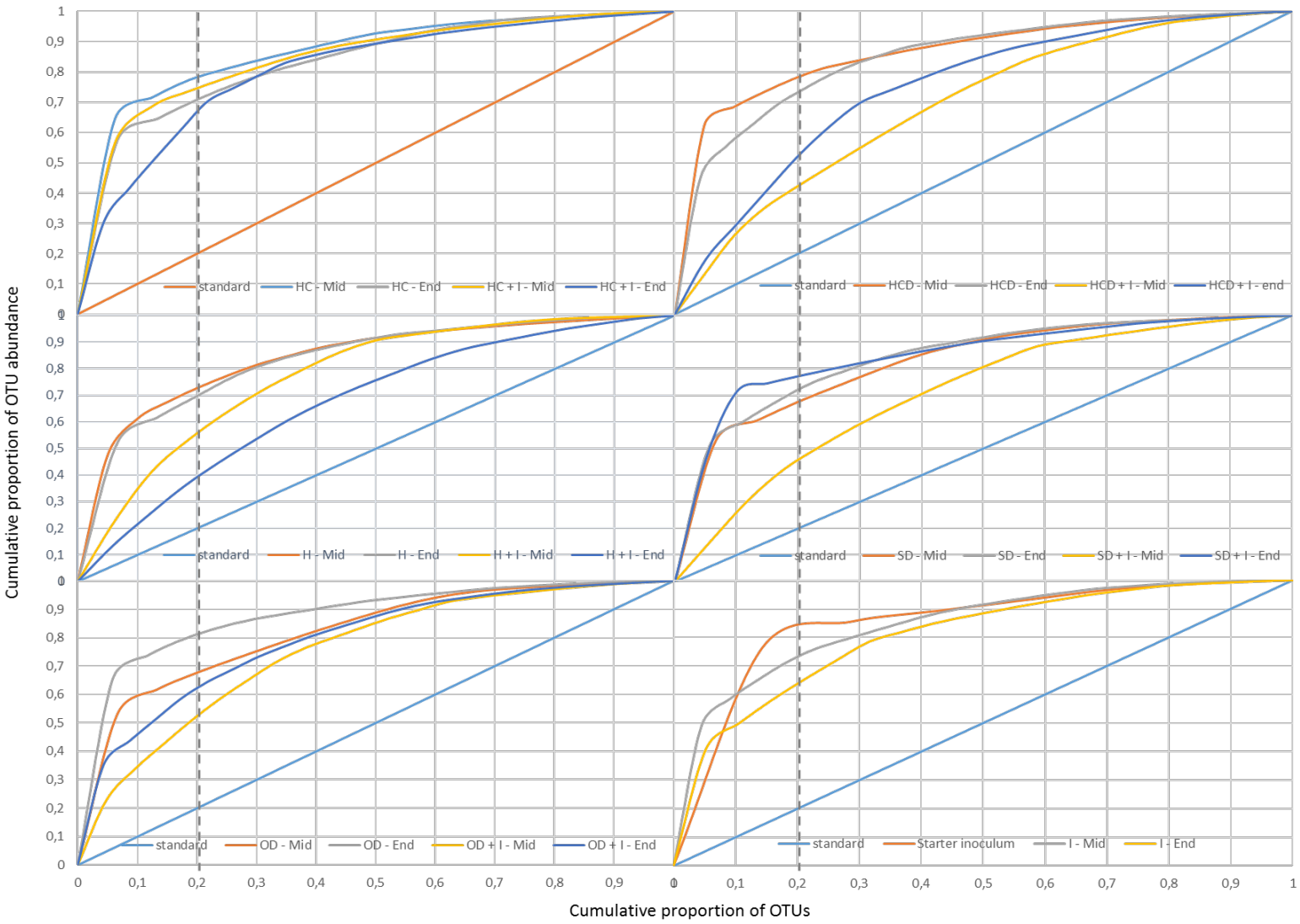

Fig. A5.2: Pareto-Lorenz distribution curves based on the bacterial DGGE profiles associated with various methods of substrate pre-treatment for graphical representation of the bacterial community evenness. Perfect evenness is illustrated as a straight line (standard). A dashed vertical line is plotted to evaluate the range of the Pareto value. OD, oven dried; H, homogenised; HCD, hand cut and decomposed; SD, sun dried; HC, hand cut; I, inoculum.

Table A5.1: pH before and after anaerobic digestion for the five pre-treatment methods

Pre-treatments	Before AD		After AD	
	Samples	Control	Samples	Control
HC	7.4±0	7.7±0	7.6±0	7.5±0.06
H	7.2±0.06	7.3±0.1	7.6±0.1	6.2±0.9
OD	7.2±0.06	7.0±0.06	7.7±0.1	7.5±0.06
SD	7.2±0.06	7.0±0.1	7.6±0.06	7.4±0.11
HCD	7.6±0.23	8.2±0.07	7.6±0.06	7.6±0
Inoculum	7.6±0.06		7.2±0.15	

± Standard deviation (n=3)

Table A5.2: Taxonomic identification of excised major bands obtained from DGGE gels of bacterial communities in AD samples

Band ID	*Closest relative*	Seq sim (%)	Phylum	Accession number	Treatment																						
					I	HC+I-M	H+I-M	OD+I-M	HCD+I-M	SD+I-M	HC+I-E	H+I-E	OD+I-E	HCD+I-E	SD+I-E	HC-M	H-M	OD-M	HCD-M	SD-M	I-M	HC-E	H-E	OD-E	HCD-E	SD-E	I-E
B1	Uncult. *Bacteroides* sp.	100	Bacteroidetes	KC502889	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	++	-	-	-	-	-	++
B2	*Bacteroides paurosaccharo-lyticus*	97	Bacteroidetes	BAJR01000054	-	-	++	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
B3	Uncult. *Bacteroides* sp.	100	Bacteroidetes	KC502889	-	-	++	-	-	-	++	++	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-
B4	Uncult. *Bacteroidetes bacterium*	98	Bacteroidetes	JQ012291	++	++	++	++	++	++	++	++	++	++	++	-	-	-	-	-	+	-	-	-	-	-	+
B5	*Cryptanaero-bacter phenolicus*	93	Firmicutes	NR_025757	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-	+
B6	*Petrimonas mucosa*	98	Bacteroidetes	LT608328	+	++	++	++	++	++	+	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-	+
B7	*Lentimicrobium saccharophilum*	97	Bacteroidetes	DF968182	+	+	+	++	+	+	++	++	++	++	++	+	-	++	++	++	+	+++	-	+++	+++	+++	+
B8	Uncult. *Bacteroides* sp.	94	Bacteroidetes	GU45497	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B9	*Hydrogenophaga temperate*	99	Proteobacteria	AB166886	-	-	-	-	+	-	++	++	++	++	++	-	++	++	++	++	++	-	++	++	++	++	++

Band ID	*Closest relative*	Seq sim (%)	Phylum	Accession number	Treatment																						
					I	HC+I-M	H+I-M	OD+I-M	HCD+I-M	SD+I-M	HC+I-E	H+I-E	OD+I-E	HCD+I-E	SD+I-E	HC-M	H-M	OD-M	HCD-M	SD-M	I-M	HC-E	H-E	OD-E	HCD-E	SD-E	I-E
B10	*Hydrogenophaga palleronii*	96	Proteobacteria	BCTJ01000079	-	-	-	-	+	-	++	++	++	++	+	-	-	-	-	-	++	-	-	-	-	-	++
B11	*Christensenella* sp.	98	Firmicutes	JX223665	-	+	+	+	+	+	+	+	+	++	+	-	-	-	-	-	+	-	-	-	-	-	+
B12	*Sphaerochaeta* sp.	97	Spirochaetes	GQ134383	+	+	+	+	+	+	++	+	+	++	+	-	-	-	-	-	+	-	-	-	-	-	+
B13	*Bacteroides graminisolvens*	100	Bacteroidetes	BAJS01000049	-	-	-	-	-	-	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	-	-	-
B14	*Anaerocolumna cellulosilytica*	95	Firmicutes	AB298755	+	+	+	+	+	+	+	+	+	+	+	+	++	-	+	++	++	-	-	-	-	-	++
B15	*Ruminococcus flavefaciens*	96	Firmicutes	JAEF01000027	-	-	-	-	-	-	+	-	-	+	+	+	+++	+	+	+	-	-	+++	-	+	-	+
B16	*Comamonas jiangduensis*	99	Proteobacteria	JQ941713	-	-	-	-	-	-	-	-	-	-	-	++	+++	++	++	+	-	+	++	+	+	+	-
B17	*Petrimonas sulfuriphila*	100	Bacteroidetes	AY570690	+	++	++	++	++	++	+	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-	+
B18	*Lentimicrobium saccharophilum*	97	Bacteroidetes	DF968182	+	+	+	++	+	+	++	++	++	++	++	+	-	++	++	++	+	+++	-	+++	+++	+++	+
B19	*Hydrogenophaga temperate*	99	Proteobacteria	AB166886	-	-	-	-	+	-	++	++	++	++	++	-	++	++	++	++	++	-	++	++	++	++	++
B20	*Hydrogenophaga electricum*	98	Proteobacteria	AB746948	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-
B21		97	Firmicutes	AB307646	-	-	-	-	-	-	-	-	-	-	-	-	-	++	+	++	-	-	+	+	-	-	-

Band ID	*Closest relative*	Seq sim (%)	Phylum	Accession number	Treatment																						
					I	HC+I-M	H+I-M	OD+I-M	HCD+I-M	SD+I-M	HC+I-E	H+I-E	OD+I-E	HCD+I-E	SD+I-E	HC-M	H-M	OD-M	HCD-M	SD-M	I-M	HC-E	H-E	OD-E	HCD-E	SD-E	I-E
B22	Uncult. *Clostridia bacterium*	99	Proteobacteria	JX843926	-	-	-	-	+	-	++	++	++	++	+	-	-	-	-	-	+	-	-	-	-	+
B23	Uncult. *delta proteobacterium*	97	Bacteroidetes	DF968182	+	+	+	++	+	+	++	++	++	++	++	+	-	++	++	++	+	+++	-	+++	+++	+++	+
B24	*Lentimicrobium saccharophilum*	95	Firmicutes	JN648101	-	-	-	-	-	-	+	-	-	+	+	+	+++	+	+	+	-	-	+++	-	+	-	+
B25	*Caloramator quimbayensis*	95	Firmicutes	JN648101	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+++	-	-	-	-
B26	*Caloramator quimbayensis*	93	Bacteroidetes	ACCH01000108	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-	-	+++	-	-
B27	*Bacteroides cellulosilyticus*	97	Firmicutes	AB221372	-	-	-	-	-	-	-	-	-	-	-	-	++	++	++	++	-	++	+	++	+++	+	-
B28	*Clostridium caenicola*	*100*	*Bacteroidetes*	KC502889	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	++	-	-	-	-	-	++
B29	Uncult. *Bacteroides* sp.	*99*	Proteobacteria	AB166886	-	-	-	-	+	-	++	++	++	++	++	-	++	++	++	++	++	-	++	++	++	++	++
	Hydrogenophaga temperata																										

+ present in small quantities; ++ present in intermediate quantities; +++ present in large quantities; - absent

Table A5.3: Taxonomic identification of excised major bands obtained from DGGE gels of archaeal communities in AD samples

Band ID	Closest relative	Seq sim (%)	Accession number	Treatment																						
				I	HC+I-M	H+I-M	OD+I-M	HCD+I-M	SD+I-M	HC+I-E	H+I-E	OD+I-E	HCD+I-E	SD+I-E	HC-M	H-M	OD-M	HCD-M	SD-M	I-M	HC-E	H-E	OD-E	HCD-E	SD-E	I-E
A1	*Methanosarcina vacuolata*	99	CP009520	++	++	++	+++	++	+++	+++	+++	+++	+++	+	+	+	+	+	+	++	+	++	+	+	+	++
A2	*Methanosarcina vacuolata*	99	CP009520	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	++	+	++	++	+++	++	+++	+++	+++	++
A3	*Methanosarcina vacuolata*	98	CP009520	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	++	+	++	++	++	++	++	++	++	++
A4	*Methanosarcina vacuolata*	95	CP009520	-	-	-	-	-	-	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A5	*Methanosarcina horonobensis*	98	CP009516	+	+	+	+	+	+	++	+	+	+	+	-	-	-	-	-	+	+	+++	++	+	+	+
A6	*Methanosarcina siciliae*	99	CP009506	-	+	+	+	+	+	+	+	+	+	+	+	-	++	+	++	-	+	-	+	+++	+	+
A7	*Methanosarcina siciliae*	99	CP009506	-	-	-	-	-	-	-	+	+	+	+	+	+++	+++	+++	+++	+	++	+	++	++	+++	+
A8	*Methanosarcina vacuolata*	99	CP009520	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	++	+	+	+++	+++	++	++	++	+++	+++
A9	*Methanosarcina mazei*	98	CP009512	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	+	+	+	+	-
A10	*Methanosarcina mazei*	99	CP009512	-	-	-	-	-	-	-	-	-	-	-	++	+	++	+	++	-	++	++	++	++	++	-
A11	*Methanosarcina acetivorans*	99	AE010299	-	-	-	-	-	-	+	+	+	+	+	+++	+	+	+	+	-	++	-	-	+++	+	-

A12	*Methanosarcina horonobensis*	99	CP009516	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	++	+	++	++	+++	++	+++	+++	+++	++
A13	*Methanosarcina siciliae*	99	CP009506	-	-	-	-	-	-	-	+	+	+	+	+	+++	+++	+++	+++	+	++	+	++	++	+++	+
A14	*Methanosarcina vacuolata*	99	CP009520	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	++	+	+	+++	+++	++	++	++	+++	+++
A15	*Methanosarcina siciliae*	99	CP009506	+	+	+	+	+	+	++	+	+	+	+	-	-	-	-	-	+	+	+++	++	+	+	+
A16	*Methanosarcina flavescens*	99	LKAZ01000047	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-	-	+++	-	-
A17	*Methanosarcina acetivorans*	99	AE010299	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	++	-	++	++	++	-
A18	*Methanosarcina horonobensis*	99	CP009516	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	+++	+++	-
A19	*Methanosarcina vacuolata*	99	CP009520	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+++	-

+ present in small quantities; ++ present in intermediate quantities; +++ present in large quantities; - absent

Socio-economic impact of water hyacinth to the surrounding community

Name..... Age:…..... Gender:……..

	YES	NO
1. Do you reside close to the Hartbeespoort Dam?	☐	☐
2. Do you know about water hyacinth?	☐	☐
3. Does the presence of water hyacinth in the dam negatively affect you?	☐	☐
4. Are you aware of the methods used to control water hyacinth in the dam?	☐	☐
5. Do you know the implications of the conventional method used in controlling water hyacinth in the dam?	☐	☐
6. Do you know about biogas?	☐	☐
7. Are you aware of the use of biogas as alternative energy source for cooking?	☐	☐
8. Would you use biogas to cook at home?	☐	☐
9. Do you know that biogas can be produced from water hyacinth?	☐	☐
10. Would you be willing to pay more for biogas than LPG? e.g. 9kg LPG = R201.00; 9kg biogas = R268.00	☐	☐
11. Would you accept digesters in your community/ near the dam?	☐	☐

Fig. A9.1: Questionnaire disseminated to community around Hartbeespoort dam

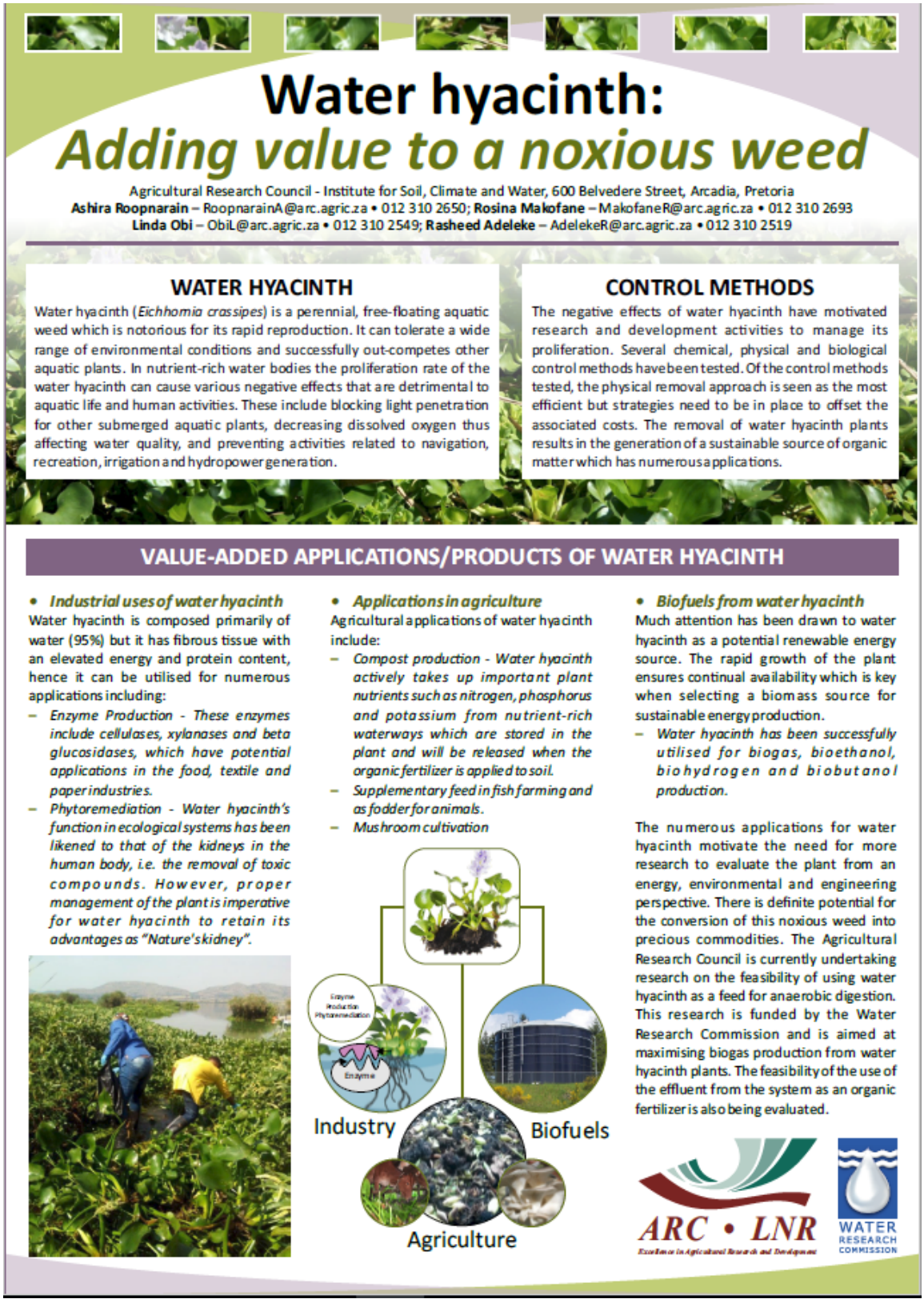

Fig. A9.2: Flyer 1 disseminated to community around Hartbeespoort dam

Fig. A9.3: Flyer 2 disseminated to community around Hartbeespoort dam

Capacity building

Three students and one postdoctoral research fellow were involved in the project.

Rosina Makofane is registered at UNISA for her MSc degree in Environmental Science.

Linda Obi is registered at UNISA for her PhD degree in Environmental Science.

Mashudu Mukhuba is registered at UNISA for her MSc degree in Environmental Science.

Ashira Roopnarain was the postdoctoral research fellow working on the project. She has been employed as a researcher at the ARC.

During the course of the study the project team has attended various workshops including:

- Real-time PCR training hosted by ARC-SCW – Microbiology and Environmental Biotechnology Research Group (MEBRG)
- Next Generation Sequence (NGS) analysis training hosted by ARC-SCW – Microbiology and Environmental Biotechnology Research Group (MEBRG)
- CHPC Introductory programing school: NWU-Potchefstroom

The team presented their work at various conferences including:

- Renewable and Sustainable Energy Postgraduate Symposium, University of Fort Hare. 4-6 September 2016.
- South African Society for Microbiology Conference. 17-20 January 2016, Coastlands, Umhlanga.
- 2nd International Peri-Urban Conference. 26-29 November 2017, Century City Conference Centre, Cape Town
- South African Society for Microbiology Conference. 4-7 April 2018, Misty Hills Hotel and Conference Centre, Muldersdrift.
- Agricultural Research Council – Professional Development Program Conference. ARC. 2016, 2017

Technology transfer

General public

Research findings have been disseminated to the general public through popular articles.

***Popular articles*:**

Roopnarain, A., Mukhuba, M. and Adeleke, R. (2016). Waste to Energy – Microbes as drivers of energy production during anaerobic digestion. Harvest SA. Popular article.

Roopnarain, A., Makofane, R and Adeleke, R. (2018). Water hyacinth: Adding value to a noxious weed. Popular article (under review for publication in Water Wheel) (*Envisaged*)

Community residing close to Hartbeespoort Dam and visitors in the area

A survey was conducted to investigate the socio-economic impact of the utilization of water hyacinth from Hartbeespoort dam in biogas and biofertilizer production. Data was collected in the form of questionnaires. Knowledge was disseminated in the form of flyers (see appendix) and verbal communication. The study sites for the activity included two sites frequented by both, members of the Hartbeespoort community and visitors from other areas. These sites included the Hartbeespoort Village Mall (25.7318° S, 27.8879° E) and the Harties Aquarium/ French Toast (25.7371° S, 27.9024° E). Selection of the sites was primarily based on the close proximity to the Hartbeespoort Dam (<15 km).

Scientific community

Research findings have been disseminated to the scientific community through scientific publications in peer reviewed journals and conference presentations.

***Publications*:**

Roopnarain, A. Adeleke, R. (2017). Current Status, Future Prospects and Hurdles to Biogas Digestion Technology in Africa. Renewable and Sustainable Energy Reviews 67: 1162-1179

Roopnarain, A., Mukhuba, M., Adeleke, R. and Moeletsi, M. (2017) Biases during DNA Extraction Affect Bacterial and Archaeal Community Profile of Anaerobic Digestion Samples. 3 Biotech, 7: 375

Mukhuba, M., Roopnarain, A., Adeleke, R., Moeletsi, M., & Makofane, R. (2018) Comparative assessment of bio-fertiliser quality of cow dung and anaerobic digestion effluent. Cogent Food & Agriculture, 1435019.

Roopnarain, A., Makofane R. and Adeleke R. (2018). Metagenomic link between enhanced bio-methane production and water hyacinth (*Eicchornia crassipes*) pre-treatment (Under review) (*Envisaged*)

Makofane, R., Adeleke R. and Roopnarain A. (2018). Effect of organic loading rate on microbial communities and biogas production from water hyacinth feedstock: A case of mono and co-digestion (In preparation) (*Envisaged*)

***Conference output*:**

Roopnarain, A. Adeleke, R. (2015). Biogas in South Africa. Agricultural Research Council – Professional Development Program Conference. ARC – Central Office, Pretoria. 17-19 October 2015. (Oral presentation)

Mukhuba, M. Adeleke, R. (2015). The effects of microbial composition in the production of biogas from multiple feedstock. Agricultural Research Council – Professional Development Program Conference. ARC – Central Office, Pretoria. 17-19 October 2015. (Poster presentation)

Roopnarain, A. Adeleke, R. (2016). Current Status, Future Prospects and Hurdles to Biogas Digestion Technology in Africa. South African Society for Microbiology Conference. 17-20 January 2016, Coastlands, Umhlanga. (Oral presentation – Keynote address)

Roopnarain, A., Mukhuba, M, Adeleke, R. and Moeletsi, M. (2016). Comparison of DNA extraction methods for the isolation of bacterial and archaeal DNA from various stages in the anaerobic digestion process. Renewable and Sustainable Energy Postgraduate Symposium, University of Fort Hare. 4-6 September 2016. (Poster presentation)

Mukhuba, M., Roopnarain, A., Adeleke, R. and Moeletsi, M. (2016). Potential use of microorganisms isolated from digestate (slurry) for the promotion of plant growth. Renewable and Sustainable Energy Postgraduate Symposium, University of Fort Hare. 4-6 September 2016. (Poster presentation)

Mukhuba, M., Roopnarain, A., Adeleke, R. (2016). Comparison of commercially available DNA extraction kits for the isolation of bacterial and Archaeal DNA from various stages in the anaerobic digestion process. South African Society for Microbiology Conference. 17-20 January 2016, Coastlands, Umhlanga. (Poster presentation)

Makofane, R., Adeleke, M. and Roopnarain, A. (2016). Characterisation and use of water hyacinth for biogas production. Renewable and Sustainable Energy Postgraduate Symposium, University of Fort Hare. 4-6 September 2016. (Oral presentation)

Roopnarain, A., Mukhuba, M, Adeleke, R. and Moeletsi, M. (2016). Method of DNA extraction affects bacterial and archaeal community profile of anaerobic digestion samples. Bioresource Technology for Bioenergy, Bioproducts & Environmental Sustainability – Biorestec, Sitges, Spain. 22-26 October 2016. (Poster presentation)

Roopnarain, A., Makofane, R. and Adeleke, R. (2017). Metagenomic link between enhanced bio-methane production and water hyacinth (*Eicchornia crassipes*) pre-treatment. BBEST 2017, Campos do Jordao, Brazil. (Poster presentation)

Makofane, R., Adeleke, R. and Roopnarain, A. (2017) Comparison of physical and biological pre-treatment methods of water hyacinth in enhancing methane production. Second International Peri-Urban conference Nov 2017. (Poster presentation)

Makofane, R, Adeleke, R. and Roopnarain, A. (2018) Biomethane from water hyacinth (*Eichhornia crassipes*): Effect of mono and co-digestion. SASM conference 4-7 April 2018. (Oral presentation)