Lancaster Environmental Centre

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OPTIMISATION OF SMALL SCALE ANAEROBIC DIGESTION TECHNOLOGY

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Submitted for the degree of Doctor of Philosophy

October, 2015

This project was supported by the Centre for Global Eco-Innovation and is part financed by the European Regional Development Fund.

Centre for Global Eco-Innovation

Declaration

I hereby declare that the body of work presented in this thesis is my own original work and no part of it has been submitted in substantially the same form for the award of a higher degree elsewhere.

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Abstract

The recent advances in anaerobic digestion (AD) technology and changes in government policies have contributed to the gradual increase in the establishment of on-site small-scale anaerobic digesters in developed regions, particularly in Europe. However, these advances have not completely eradicated some of the challenges with operating AD system. The project is aimed at investigating the potential of optimizing small-scale AD through high solid digestion (HSAD) and reduction of substrate induced inhibition (SII). The study of different inocula, changes to environmental conditions, adsorption of inhibitors and reactor modification was explored. To investigate these possibilities, an onsite mono-substrate such as citrus fruit waste (CFW) with an average dry matter of 16% was used as the substrate, biochar material (rice husk, coconut shell and wood biochar) were used as adsorbent while an operating temperature from 35 - 55 ^{0}C were also investigated. Limonene is an inhibitory compound and a constituent of CFW, this was used as the inhibitor, a compartmentalized anaerobic reactor (CAR) was designed to improve HSAD while selected inocula from digested sewage sludge, compost and landfill leachate and their mixture were used as an inoculant. In the first study, the acclimation rate of different inocula to increasing concentration of limonene compound was investigated and the mixed inocula recorded the highest recovery rate and methane yield with a value of 544 ± 21 ml CH_4. The mixed inocula benefited from the synergistic effect of using a broader microbial community to mitigate limonene inhibition. This was followed up with the biochar study on AD of CFW and the result showed that microbial lag phase reduced by 50% which was attributed to sorption of limonene compound and biofilm formation on the biochar material. The study on AD of CFW at a different operating temperature of 35-55 ^{0}C showed that the higher temperature of 45 and 55 ^{0}C

outperformed the other incubation with no detectable microbial lag phase. Finally, the optimization option for HSAD was investigated using a CAR and compared against the conventional continuous stirred tank reactor and a 34%, 43.3%, 48.5% and 79.9% higher cumulative methane production for organic loading rates of 1.42, 2.85, 4.00 and 5.00 $gVSL^{-1}$ day^{-1}, respectively was achieved. This performance was attributed to the lower compartment of the CAR which facilitated leachate treatment and distribution. The result showed that limonene a constituent of CFW and an example of SII can be counteracted by (i) inoculating with a mixture of inocula (ii) addition of biochar (iii) operation at high temperature of 45 and 55 ^{0}C and (iv) the single stage compartmentalized reactor improved HSAD and reduced limonene suppression.

Acknowledgements

I express my heartfelt gratitude and sincere appreciation to my supervisors, Prof Kirk T. Semple and Dr Hong Li, for their immense guidance, and their deep knowledge of science, which contributed to this project. I admire their friendship, understanding, and for the confidence and support, especially during the tough periods of my Ph.D. research. Thank you, Kirk, for your support in securing additional funding and other career building opportunities in the course of this project, I am very grateful.

The project is funded by the Centre for Global Eco-Innovation and is partly financed by the European Regional Development Fund. It brings together the expertise, resources and global contacts of Lancaster University and the University of Liverpool, together with international commercialisation consultancy Inventya Ltd. I acknowledge the dedication of the CGE and business development team towards the success of this project. My gratitude goes to Stopford Energy and Environment for giving me the opportunity to work with them on this project and their unflinching support.

Now, special appreciation to my parents Dr and Mrs Fagbohungbe, and my lovely wife Gbemisola for your kindness, goodness and support throughout the course of this program.

To the almighty God, my source, sustenance, strength, stronghold and salvation, thank you for everything.

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Abbreviation

AD	Anaerobic digestion
AF	Anaerobic filter
BTA	Biotechnische AbFallverwertung
CAR	Compartmentalized anaerobic reactor
CFW	Citrus fruit waste
CH_4	Methane
CL	Compost leachate
CO_2	Carbon dioxide
COD	Chemical oxygen demand
CEC	Cation exchange capacity
CSB	Coconut shell biochar
CSTR	Continuous stirred tank reactor
DAQ	Data acquisition
ECD	Electrochemical detector
FID	Flame ionization detector
GC	Gas chromatography
HPLC	High-performance liquid chromatography
HRT	Hydraulic retention time
HSAD	High solid anaerobic digestion
HSAR	High solid anaerobic reactor
IC	Ion chromatography
ICP-OES	Inductively coupled plasma atomic emission spectroscopy
LCFA	Long chain fatty acids
LL	landfill leachate
LSAD	Low solid anaerobic digestion
ML	Mixed leachate

OLR	Organic loading rate
RHB	Rice husks biochar
RPM	Revolution per minute
SCOD	Soluble chemical oxygen demand
SEBAC	Sequential batch anaerobic composting
SEM	Scanning electron microscope
SII	Substrate-induced inhibition
SIR:	Substrate to inoculum ratio
SL	Digested sewage sludge leachate
SMP	Sequential methane potential
SSAD	Small-scale anaerobic digester
SUBBER	Supper blue box recycling
TPAD	Temperature phased anaerobic digestion
TS	Total solid
TVFA	Total volatile fatty acid
UASB	Up flow anaerobic sludge blanket
UASS	Up flow anaerobic solid state reactor
UV	Ultra violet
VFA	Volatile fatty acid
VS	Volatile solid
WB	Wood biochar

Thesis format – List of papers

This thesis is based on the following papers, which will be referred to in the text by their roman numeral:

I. Fagbohungbe, M. O., Ian, C.D., Herbert, B. M. J., Li, H., Hurst, L., & Semple, K. T: High solid anaerobic digestion: operational challenges and possibilities. ***Environmental Technology & Innovatio****n – published*

II. Fagbohungbe, M. O., Li, H., Herbert, B. M. J., Hurst, L., & Semple, K. T: Evaluating the recovery rate of different inocula to increasing concentration of D-limonene**.** ***Submitted to Biomass and bioenergy***

III. Fagbohungbe, M. O., Herbert, B. M. J., Li, H., Hurst, L., & Semple, K. T: The role of biochar in optimising anaerobic digestion process. ***Bioresource Technology- Accepted***

IV. Fagbohungbe, M. O., Herbert, B. M. J., Li, H., Hurst, L., & Semple, K. T: Impact of biochar on the anaerobic digestion of citrus peel waste. ***Submitted to Bioresource Technology***

V. Fagbohungbe, M. O., Herbert, B. M. J., Li, H., Hurst, L., & Semple, K. T: High solid anaerobic digestion of citrus peel waste**. *Submitted to Biomass and Bioenergy***

VI. Fagbohungbe, M. O., Herbert, B. M. J., Li, H., Ibeto, C.N, & Semple, K. T: An integrated single phase anaerobic reactor for high solid anaerobic digestion of citrus peel waste. ***For submission to Bioresource Technology.***

1. Introduction

1.1.Brief history of anaerobic digestion

Anaerobic digestion (AD) is a microbial mediated biochemical breakdown of complex organic material into simpler forms in the absence of oxygen into ammonia (NH_3) carbon dioxide (CO_2), hydrogen (H_2), hydrogen sulphide (H_2S) and methane (CH_4), known as biogas. This technology was said to have been discovered as far back as the 10^{th} Century B.C by the Persians and Assyrians who used the combustible gas to heat water (He, 2010). In 1764, Benjamin Franklin light the surface of a muddy lake in New Jersey and this was published by Joseph Priestly in England as inflammable air (Tietjen, 1975). Following this discovery, Dalton identified the chemical composition of this inflammable air to be CH_4 in 1804 and consequently Gayon became the first scientist to record the first experimental study where he fermented manure at 35 ^{0}C, produced 100 l CH_4 l^{-1} of biogas (Tietjen, 1975).The success of this experiment led "Le Figaro" to demonstrate the applicability of this technology by illuminating the street of Paris in France with the methane gas produced during the AD of horse manure (Tietjen, 1975).

The 19^{th} Century could be described as one of the crucial moment for AD technology. AD technology transited from laboratory study to field applications, small projects such as sewage treatment using simple air-tight chamber in France and wastewater treatment using a septic tank in Exeter, England were initiated (Gijzen, 2002; McCarty, 2001). At this stage, the anaerobic digesters were much smaller and the risk of failure was much higher. Following this, the 'Imhoff' anaerobic digester was invented by the Germans in the early 20^{th} Century to treat sewage sludge. In 1920, this technology was scaled up to a larger size anaerobic digester in order to feed biogas to the public national gas grid whilst treating sewage sludge (Bond & Templeton, 2011). The performance of the large-scale anaerobic digesters led to the establishment of the first

large agricultural biogas plant by the Germans in 1950 (Bond & Templeton, 2011). The transition of small-scale anaerobic digestion (SSAD) into larger systems had already started, but there were some limitations with the technology, particularly low biogas yield and longer doubling times of the microorganisms (Gijzen, 2002). These limitations contributed to the slow pace in the application of AD technology. However, after the energy crises of 1970, the application of AD gradually gained momentum because it could serve as an alternative source of energy. For the developed countries, more attention was given to the establishment of large-scale anaerobic digestion (LSAD) while the application of SSAD continued to grow in developing countries. The Chinese government facilitated the installation of over 7 million small-scale anaerobic digesters in the 1980s and reports show that presently, about 30 million people use biogas to cook and light their homes in China (Babel et al., 2009; Chen et al., 2010; Ng et al., 2013). Likewise, about 3 million Indian families were also beneficiaries of government subsidised small-scale biogas plants in 2007 (Bond & Templeton, 2011). The reason for these differences could be ascribed to the application of the technology. In most developed countries, the technology has been commercialised while in developing countries the technology is mainly for domestic use. However, if the SSAD is optimised it can serve as an onsite waste treatment and energy production plant for farms, as well as small and medium scale business. At the moment, SSAD is qualified for financial incentives in Europe, this is a good driver for the development of the technology. The Energy Act (2008) provides incentives in the form of feed-in tariff (FIT) and renewable heat incentives (RHI) for SSAD based on their electricity and heating output. The widespread application of AD technology could play a decisive role in the on-going campaign against climate change, with Europe and Asia, having the largest market share of anaerobic digesters.

1.2. Process and operational conditions

1.2.1. Anaerobic digestion process

The microbiology of the AD process is complex because it involves different consortia of microorganisms at each stage of the digestion process. AD can be divided into four stages; hydrolysis, acidogenesis, acetogenesis and methanogenesis (Madsen et al., 2011). A schematic representing the various stages in AD is given in Figure 2. Hydrolysis breaks down the complex materials into soluble monomers while acidogenesis proceeds with the degradation of monomers such as sugars, amino acids and long chain fatty acids into organic acids, H_2, CO_2, alcohol and ammonia. Then in acetogenesis, these metabolites such as organic acid and alcohol are converted into hydrogen, acetate and carbon dioxide. Finally, in methanogenesis, the products are converted in methane and carbon dioxide.

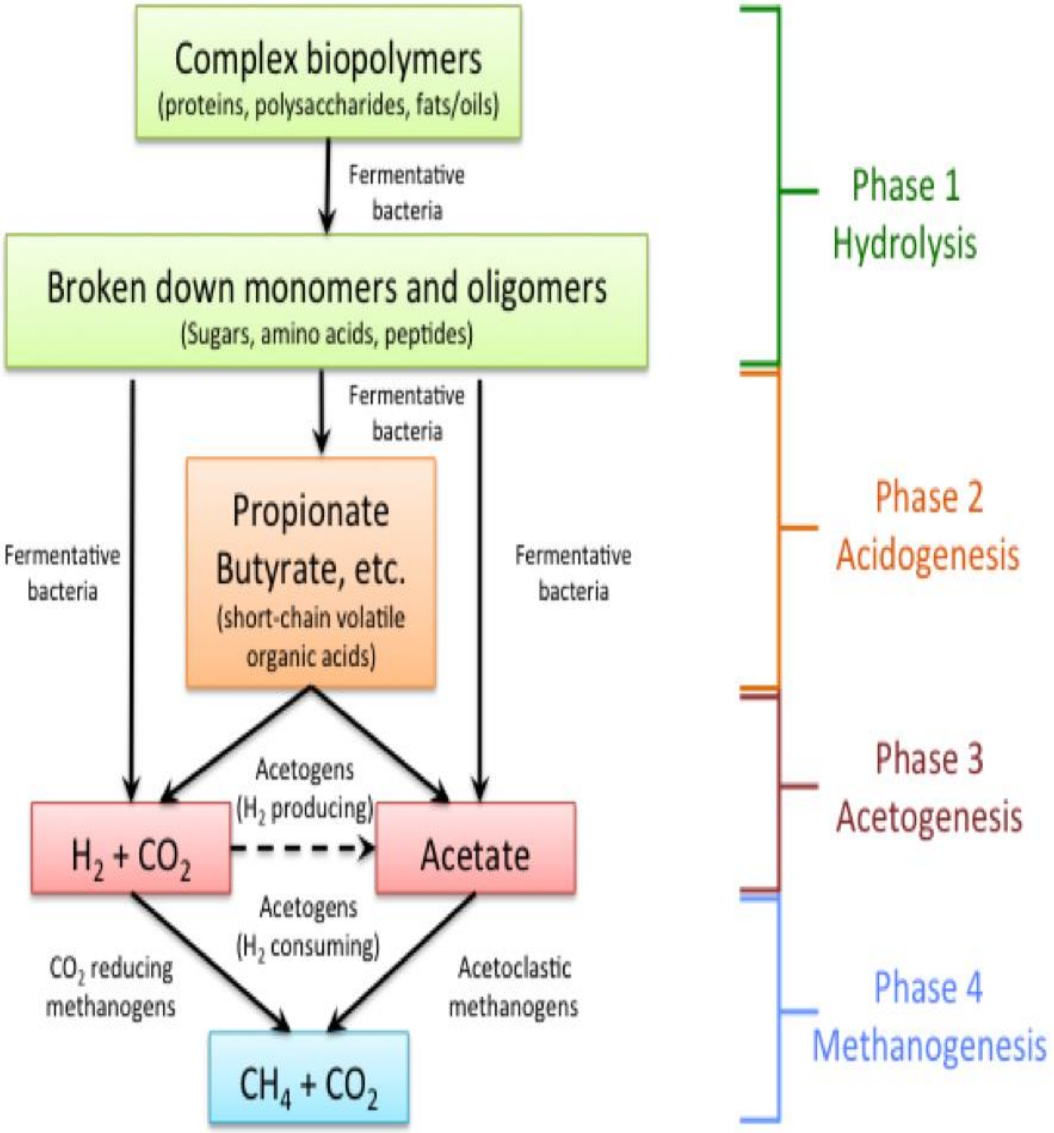

Figure 1-Schematic of the four main pathways of anaerobic digestion processes for organic substrate modified from (van Haandel & Van Der Lubbe, 2007).

1.2.1.1.Hydrolysis

Hydrolysis is a first-order reaction and the first step in AD because it involves the conversion of high molecular weight substrate into soluble products by enzymatic reactions (Batstone et al., 2002). For instance, protein, fat and carbohydrate are broken down by proteases, lipases and cellulases into amino acids, fatty acid and simple sugars, respectively (Stryer, 1995). Once the complex substrates have been solubilised, they become readily available for the subsequent group of microorganisms within the

consortia. Hydrolysis has been described as a rate-limiting step because the rate of microbial activities at this stage of AD is the slowest. According to Hill and Root (2014) when the overall sequence of a reaction is dependent on the slowest reaction, that reaction is termed the rate-limiting step. The effectiveness of the hydrolysis stage plays an important role in determining the overall methane yield from the organic substrate, although controlling factors such as operating temperature, pH, substrate to inoculum ratio (SIR), mixing and particle size also accounts for variation in methane yield and process performance (Chynoweth, 1987; Gunaseelan, 1997).

As mentioned earlier, hydrolysis is a first order reaction but the effect of lower SIR on the hydrolysis of an organic substrate undermines this assertion (Eastman & Ferguson, 1981). This is because lower SIR increases methane yield compare to higher SIRs. For instance, Li et al. (2014) achieved higher methane yield when SIR was lower for AD of algae, this indicates that first order kinetics is not applicable in all cases as this study shows that hydrolysis depends on the microbial biomass. Nonetheless, some other authors have emphasised that first-order kinetics can only be applied to the hydrolytic process; (I) if the surface of the substrate is the rate limiting factor and biodegradability does not interfere and (II) if the rate of hydrolysis increases with the concentration of the extracellular enzymes and accessibility to adsorption sites (Gavala et al., 2003; Sanders et al., 2003; South et al., 1995). The hydrolysis stage of AD process is yet to be well defined (Gavala et al., 2003).

1.2.1.2.Acidogenesis

This is the second stage in AD where soluble substrates are degraded into organic acids (such as acetic acid, butyric, propionic acid), CO_2, H_2 and other organics like alcohol

and lactic acid (Madsen et al., 2011). The concentration of volatile fatty acid (VFAs) and hydrogen provide important information about the stability of the AD process. According to Voolapalli and Stuckey (2001) increase in the accumulation of VFA can result in the acidification of the AD system, particularly when the buffering capacity if poor. Low pH inactivates the enzymatic activities of the methanogens thus inhibiting methane production and increasing the partial pressure of hydrogen (Lyberatos & Skiadas, 1999). Acetic acid is usually the highest VFA product, but cases where propionic and butyric acid concentration suddenly becomes higher than acetic acid concentration indicates a shift in the dominating acidogenic, metabolic pathways and inhibition (Vanvelsen, 1979; Wang et al., 1999). Acidogens have been described has the most robust microbial community within the consortia of microorganisms in the AD system. They have the shortest doubling time, makeup about 90% of the microbial community in the AD system and are not rate limiting (Cohen et al., 1980; Mosey, 1983; Zeikus, 1980).

1.2.1.3.Acetogenesis

The acetogenic bacteria are a diverse group of bacteria able to convert a range of substrates including organic acids, alcohols, aromatic compounds, H_2 and CO_2 into acetate. The fixation of H_2 and CO_2 is carried out by a sub-group obligate anaerobic acetogens, called homoacetogens (Kusel & Drake, 1994). Two moles of CO_2 are reduced to one mole of acetate, although some acetogens are able to reverse acetate into H_2 and CO_2 (Zinder & Koch, 1984). The fixation of H_2 and CO_2 reduces the hydrogen partial pressure and increase acetate concentration (Kusel & Drake, 1994).

1.2.1.4.Methanogenesis

This is the final stage in AD; where intermediate products such as H_2, CO_2 and acetate are converted to methane. A total of 70% of the methane produced during methanogenesis is from the acetoclastic methanogens while the litotrophic methanogens account for the remaining 30% (Madsen et al., 2011). Like the homoacetogens, the activities of the litotrophic methanogens contributes to the reduction in hydrogen partial pressure. Methanogens are sensitive to decreases in pH and substrate induced inhibitors; they are considered as rate limiting microorganisms (Chen et al., 2008; Huang et al., 2003).

1.2.2. Anaerobic digestion operational conditions

1.2.2.1.Hydrogen partial pressure

Hydrogen gas is produced during acidogenesis and it diffuses rapidly through the bacterial membrane so that the hydrogen partial pressure of inside and outside the bacterial cell is balanced. However in the event of higher partial pressure, the production of hydrogen and other volatile fatty acids will be inhibited (Costello et al., 1991). The uptake of hydrogen during AD can only be achieved in the presence of CO_2 to produce either CH_4 or acetate (Shanmugam et al., 2014). Hydrogenotrophic archaea can bind CO_2 with H_2 and convert them into CH_4 (Luo et al., 2012). This group of archaea bacteria belong to the orders, *Methanococoales, Methanomicrobials, Methanobacteriales and Methanosarcinales* (Karakashev et al., 2005). Whereas the fixation of CO_2 with H_2 into acetate requires the activities of the homoacetogens (Park et al., 2005). Table 1 shows the Gibbs free energy for the hydrogen users and their metabolic products.

Table 1- Hydrogen, carbon dioxide and sulphate reaction and their Gibbs free energy (- ΔG^0 kJ mol) (Bredwell et al., 1999; Conrad & Klose, 1999; Luo et al., 2012)

H_2, CO_2 & SO_4^{2-} reaction	Gibbs free energy (- ΔG^0 kJ MOL)
$4H_2 + CO_2 = CH_4 + 2H_2O$	135.6
$4H_2 + 2CO_2 = CH_3COOH + 2H_2O$	105
$3H_2 + SO_4^{2-} = H_2S + 4H_2O$	154

1.2.2.2.Temperature

Temperature is an important parameter to consider during AD because it influences the rate of microbial degradation, settling of solid fractions, the rate of mass transfer between hydrolysis and methanogenesis and the selection of different microbial strains (Metcalf & Eddy, Inc., 2003.; Stronach et al., 1986). The performance of AD have been reported to be most effective at operating temperatures between 30 - 55 ^{o}C and if enough adaptation time is allowed, methane production rates can be similar (Mata-Alvarez, 2003). The operating temperature for AD has been divided mainly into mesophilic and thermophilic conditions. The mesophilic operating temperature (30 – 45 ^{0}C) has been reported to select for the most robust, diverse and tolerable microbial community (Biey et al., 2003). On the other hand, thermophilic operating conditions (50 – 70 ^{0}C) are energy intensive but they reduce the hydraulic retention time, in addition to increasing the rate of methane production and pathogen reduction (Zaher et al., 2009). Furthermore, the operating temperature should be selected based on the type of organic substrate because the performance of some organic substrate is temperature dependent. For example Martín et al. (2010), showed that biodegradability of the limonene-containing

substrates was relatively better at a thermophilic temperature of 55 ^{0}C. On the other hand, the mesophilic operation is more favourable for an organic substrate with high protein content because ammonia is less prevalent at this temperature (Rajagopal et al., 2013). Angelidaki and Ahring (1994) reported an increase of ammonia concentration from 350 to 700 mg/l when the operating temperature was increased from 40 to 64 ^{0}C.

1.2.2.3.Alkalinity

In ideal conditions hydrogen and acetic acid are instantaneously utilized by the methanogens and converted into methane which results in low VFA accumulation, usually between 0.5 – 2.0 mmol dm^{-3}, high bicarbonate alkalinity and a neutral pH (Van Haandel & Lettinga, 1994). However, under unfavourable conditions like high organic loading rate (OLR) and substrate induced inhibition (SII) the activity of the acetogenic and methanogenic microbial community is inhibited and the accumulation of VFA increases, thus causing a decrease in the pH of the system. The extent of pH drop depends on the bicarbonate alkalinity concentration of the system; this is the proton accepting capacity of the water system (Loewenthal et al., 1991). (i.e $CO_3{}^{2-} + 2H_2O \leftrightharpoons HCO_3{}^{3-} + H_2O \rightleftharpoons H_2CO_3 + 2OH^-$ and $H_2C0_3 + 2H_2O \leftrightharpoons HCO_3{}^{3-} + H_3O^+ + H_2O \rightleftharpoons CO_3{}^{2-} + 2H_2O$). The routine measurement of bicarbonate alkalinity is essential because in a well-buffered system using pH measurement alone is not reliable because high VFA is required to have been formed before a detectable decrease in pH can be noticed. Reducing organic loading or direct addition of a strong base or carbonate salts can be used to restore the buffering capacity of a failing AD process. Buffering in AD is not solely a contribution of bicarbonates. Other weak acids such as phosphate,

sulphide and ammonium can enhance the alkalinity of the system (Lahav & Morgan, 2004).

1.2.2.4.pH and VFAs

pH is an important parameter for monitoring AD but as earlier mentioned, it does not respond to an immediate increase in the accumulation of organic acids. Kasali et al. (1988) stated that methanogenesis occurs optimally at pH 6.8-7.2. Although there are indications that some methanogens can survive at a pH 3.8 - 4.7 in acidic peat (Kotsyurbenko et al., 2007). Leu et al. (2011) also recorded a higher methane production at pH 8, which favoured mostly the *Methanococcus* species due to their tolerance for high salinity. A larger community of methanogenic bacteria are neutrophilic hence reducing the pH from 7.2 to 5.0 could inhibit methanogenesis. At lower pH, the acetate-utilizing methanogens are inhibited as they are unable to convert acetic acid into CH_4 and this further decreases the pH of the medium (Fukuzaki et al., 1990).

VFA result from the activities of acidogenic and acetogenic microorganisms and they include acetic, propionic, butyric, isobutyric, valeric, isovaleric and caporic acid. VFA monitoring, particularly butyric, propionic and alcohol can be used to detect imbalances in the AD process (Ahring et al., 1995). High VFA accumulation is synonymous with high OLR and poor buffering capacity of the system. There are indications that the methanogens can adapt to high concentrations of acetate. This was demonstrated by Lins et al. (2012) who showed that methanogens can adapt to 150 mM of acetate which was achieved through stepwise adaptation for over 5 weeks. Like alkalinity, VFA measurement is an important parameter for monitoring AD process.

1.3. Small-scale anaerobic digestion

According to Bishop and Shumway (2009), the 1970's saw the accession of SSAD in America, Asia and Africa. In the USA, this approach was initiated because 90% of the livestock farmers owned less than 250 cows. However 60% of the SSAD failed due to the poor economic viability of the system (Bishop & Shumway, 2009). Klavon et al. (2013) equally identify poor economic viability as a major threat to the operation of SSAD in developed countries partly because of the imbalances between the capital, operational cost, and revenue generated. However in the 21st century, new energy policies have been put forward by some developed countries in Europe to allow SSAD receive incentives in the form of Feed-in tariff (FIT) (Diaz-Rainey & Ashton, 2008; Zglobisz et al., 2010). This new development has contributed to the growth of onsite SSAD in farms and in small and medium scale industries. SSAD in developing countries like China and India has continued to enjoy government backing since the 1970's and these amongst other factors have contributed to the widespread of SSAD in these regions (Chen et al., 2010; He, 2010). There is a huge disparity between developed and developing nations with regards to application of SSAD. The developing countries focus on domestic applications for SSAD technology while the developed countries are more concerned about both the viability of the system in a competitive energy market. However, both share the concern for robust and well developed SSAD. This is partly because of the advantages of operating a SSAD system, these are: (i) increased onsite organic waste management, (ii) reduced investment and capital cost, (iii) system mobility, (iv) smaller space requirement and (v) contribution to reducing global warming and climate change.

SSAD can be classified based on size, the quantity of feedstock input (tonnes per annum) and the amount of energy produced (Chen et al., 2010). There are three main types of SSAD systems; fixed dome, floating drum and plug flow/tubular digester and they are mostly located in developing countries, particularly India and China. The new generation SSAD are more or less like the large scale systems except that they are smaller in size.

1.3.1. Fixed Dome Digester

The fixed dome digester has been operated in China since the early 20th Century and according to He (2010), 6-10 m^3 dome shaped SSAD have been in use in China since the 1970s. This technology was developed in the 19th Century, but its application is not limited to China. Reports have shown that the application of fixed dome digester span to other developing countries like India, Ghana and Kenya (Akinbami et al., 2001; Bond & Templeton, 2011; Chen et al., 2010; Nzila et al., 2012). The fixed dome digester (Fig 2) comprises of a closed, dome-shaped digester with an immovable gas holder, feedstock inlet and digestate outlet. It is usually built under the ground (Akinbami et al., 2001; Nzila et al., 2012). The inner wall of the digester is made impermeable by using cement, clay, and concrete as building materials. According to Chen et al. (2010), the high maintenance cost and low net efficiency led to the emergence of the glass fibre reinforced plastic (GRP) system in the year 2000. The GRP, (a modified dome system) was identified as having a lower maintenance cost and ease of movement.

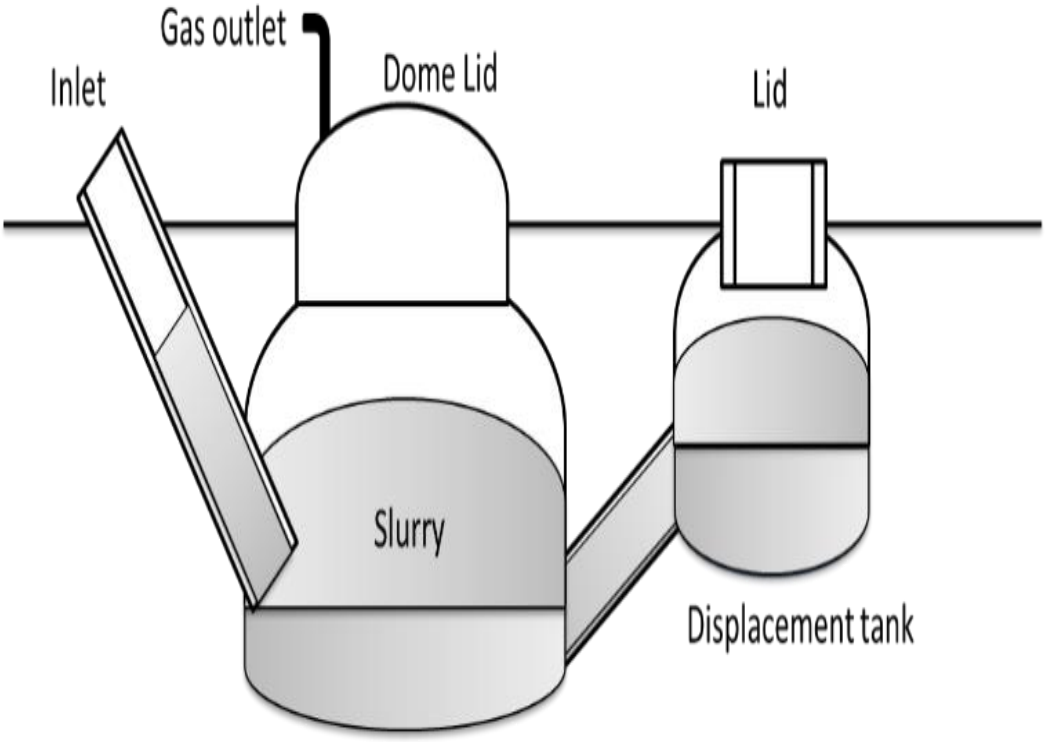

Figure 2-Fixed dome biogas digester

1.3.2. Floating Drum Digesters

The floating drum digester is an underground installed dome-shaped digester consisting of a cylindrical movable gasholder as shown in Fig. 3 and is, predominately found in India (Nagamani & Ramasamy, 1999). The system includes a gasholder, which lifts upwards as the addition of gas increases signifying the availability of biogas. Similar to fixed dome digesters, floating drum digesters are made from concrete, clay and cement with the steel gas holder usually coated with bitumen to decrease corrosion (Nzila et al., 2012). The major challenges of this floating drum system are the high cost of the steel gas holder, high cost of maintenance (de-rusting and painting) and a high potential for diffusion of oxygen through the sides of the gas holder into the slurry (Nzila et al., 2012).

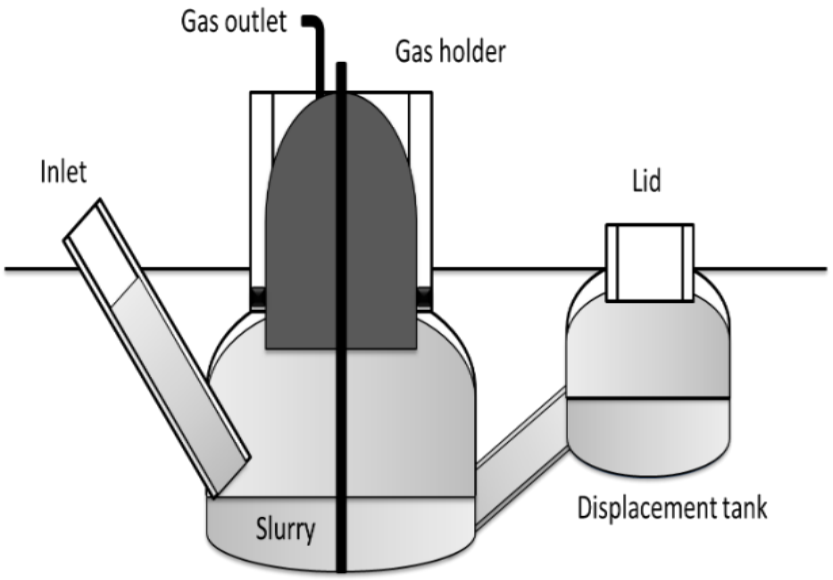

Figure 3-Floating drum biogas digester

1.3.3. Plug Flow Digesters

The tubular digester or plug flow digester (Fig. 4.) is made from high-density polyethylene plastic fitted out with inlet and outlet units (Lansing et al., 2008). The upper section of the digester serves as the gasholder and often, loads are placed on the tubular bag to increase the pressure flow of gas. The assessment of many researchers has shown that, though the investment cost of plug flow digesters has been found to be low (Ferrer et al., 2011), the operational life is relatively short. According to Nzila et al. (2012), this is not economically vi able. The short lifespan can be attributed to the delicate state of the PVC material used in the construction, making it liable to damages resulting from forceful mechanical contact and extreme temperature (Nzila et al., 2012).

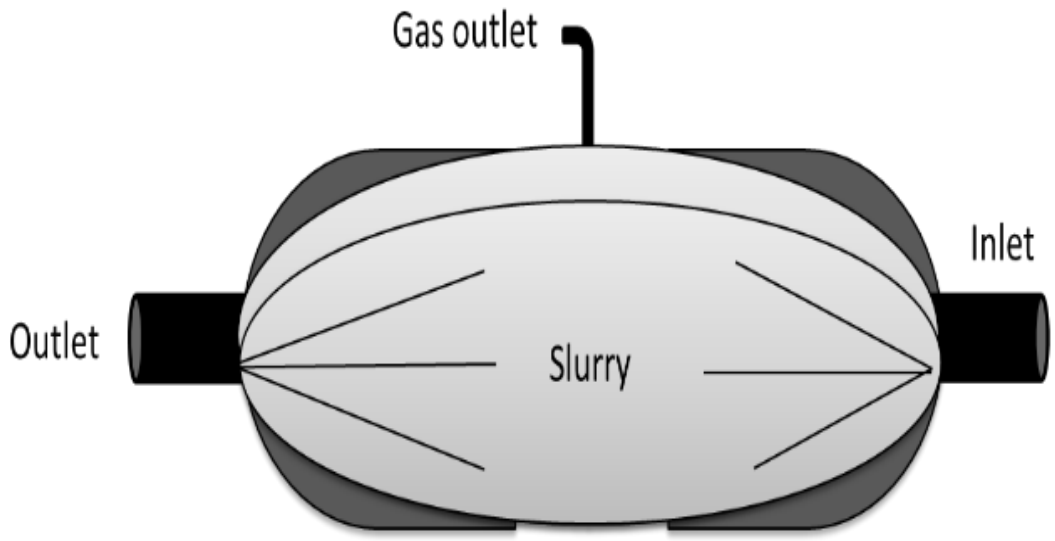

Figure 4-Plug flow biogas digester

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2. Aim and objectives

The optimization of SSAD will improve process performance and increase investment from small farm holders, communities, small and medium scale enterprises. Therefore, adopting HSAD and reducing SII will contribute to the viability of the technology and its sustainability in the marketplace. As mentioned earlier HSAD will improve digestate management, reduce reactor size and increase OLR but methane production is relatively low. In order to increase methane production, researchers have investigated and recommended two-stage high solid and high rate reactors. However, currently, statistics shows that 90% of commercially operated AD plants are single stage systems. This thesis demonstrates the development of a single stage system to combine both high solid and high rate reactor as an alternative to two stage HSAD system.

Furthermore, SII is expected to be higher in SSAD because of their low capacity and that they are frequently used to treat onsite waste materials which are most likely to be mono-substrate. The risk of SII has been identified to be higher in mono-substrate when compared multiple substrate AD otherwise called co-digestion. Recent studies have shown that thermophilic temperature, adsorption (zeolite, bentonite and activated carbon) and acclimation of microbial cells can be used to improve the performance of AD during SII from some organic substrates. However no studies have been carried out on the following; (i) the effect of biochar in reducing inhibition during AD (ii) the acclimation rate of different inocula source to SII and (iii) the effect of different operating temperature on AD during SII.

This project is aimed at investigating the potential of optimizing small-scale anaerobic digestion through high solid digestion and substrate induced inhibition. The study of different inocula, changes in environmental conditions, adsorption of inhibitors and

reactor modification and integration have been explored. In this study, five different experiments were conducted using either a batch, sequential and semi-continuous AD system to investigate and evaluate the following research objectives.

2.1. Objectives

- To select the most tolerant inoculum source by studying the acclimation rate of different inocula to potential inhibitors using a sequential batch test

- To investigate the effect of adding carbonaceous material, such as biochar to the anaerobic digestion process in order to reduced limonene suppression using a batch test

- To determine the most appropriate operating temperature for mitigating limonene suppression using both batch and semi-continuous test

- To access and compare the effect of reactor configuration; compartmentalized anaerobic reactor and continuous stirred tank reactor on HSAD of citrus fruit waste using a semi- continuous test.

3. Paper I

High solid anaerobic digestion: operational challenges and possibilities

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Abstract

The process of high solid anaerobic digestions (HSAD) was developed to reduce water usage, increase organic loading rate (OLR), reduce nutrient loss in digestate and avoid or at least decrease the dewatering of digestate. However, the operation of HSAD is currently constrained by low rates and extents of methane production and high operational costs. Several published investigations have been conducted to study the effects of inhibition, temperature, moisture, and reactor design on the efficiency of HSAD. However, low moisture content of the feedstock and poor mixing, which are required for the dilution distribution and diffusion of metabolites, have been reported to be the major causes of low methane yield in HSAD. In order to optimize the operation of HSAD, technological integration has to be considered, especially thermo–mesophilic digestion, co-digestion, mixing and integration of two or more reactors. This paper provides a critical review of recent research on HSAD while focusing on how these studies can be integrated to improve HSAD

Keywords: Anaerobic digestion Dewatering Digester design Methane output Moisture distribution

1. Introduction

The earliest application of anaerobic digestion (AD) is thought to have commenced in 19th Century, using low solid anaerobic digestion (LSAD) systems (He, 2010; Mccarty, 2001). More recently, AD has gradually become an increasingly acceptable technology for the treatment of biodegradable organic wastes (De Baere, 2000). Between 1995 and 2010, nearly 150–200 large-scale plants were established across Europe with a capacity increase of 6 000,000 tonnes of biomass feedstock annually; 50% of these plants were developed for high solid anaerobic digestion (HSAD) (De Baere et al., 2010). HSAD is a solid state operational system with low water content; this type of AD is called a semi-dry or dry system (Table 1). Recently, HSAD has been demonstrated using various AD technologies, these include the silo shaped Dranco digester and the cylindrical Valorga digester system (Li et al., 2011). The key reasons for the development of HSAD are practical, in that there is low water usage and the digester size is typically smaller than that of the other systems (as summarized in Table 1) (Garcia-Bernet et al., 2011). Apart from reducing water usage, the technology has been reported to increase organic loading rate (OLR), avoid or reduce digestate dewatering and reduce heating requirements; however, methane recovery is lower and volatile solid removal is less than 50% (Dong et al., 2010; Nagao et al., 2012). Another major concern with HSAD relates to the pumping and digestate handling devices, which add to the cost of the technology (Vandevivere et al., 2003). The difficulty in pumping the feedstock's is influenced by the total solid (TS) content; in extreme cases, e.g. 30%–40% total solid (TS) pumping and mixing would require sophisticated equipment (Vandevivere et al., 2003). Mixing is essential during AD because it reduces sedimentation and increases contact between the microorganisms and organic fractions (Karim et al., 2005a, b). For HSAD this could be achieved without the use of internal mixing devices, however leachate recirculation

has been reported to improve mixing during HSAD. The potential in leachate and biogas recirculation has been explored and noted as an option for increasing contact and reducing sedimentation (Nkemka and Murto, 2013; Sponza and Ağdağ, 2004). Nevertheless, amidst the challenges of HSAD the benefits of high quality digestate otherwise called bio-fertilizer, higher volume of treatable waste per digester size, low water usage, and avoidable cost of digestate dewatering could encourage further research for higher methane production. The potential for HSAD is high and so are the challenges, this review will critically and comparatively look at how research has approached these challenges such as low moisture, poor mixing as well as highlighting other benefits such low dewatering, high OLR and higher digestate quality.

2. High solid anaerobic digesters

The recovery of methane through the decomposition of organic compounds by anaerobic microorganisms is usually stimulated using an enclosed system devoid of oxygen. This enclosed system is called an anaerobic digester which varies in design depending on the characteristics of the substrate and the type of the AD process. Anaerobic digestion can be categorized based on the total solid content of the feedstock as wet otherwise called LSAD, semi-dry and dry. Both semi-dry and dry AD processes have been categorized as HSAD. According to Abbassi-Guendouz et al. (2012), the HSAD process can be grouped semi-HSAD (treating 10%–20% total solid) and HSAD (treating > 20% total solid). With regard to HSAD, there are only a few digesters which have been designed and commercialized to achieve methane production and feedstock reduction. It could be said that these digesters are modified from the existing LSAD digesters to operate for batch or continuous flow (Li et al., 2011). According to Vandevivere et al. (2003) HSAD systems can be categorized into a single or multi stage operations. These two categories

of HSAD differ primarily in cost and the OLR. The single-stage digesters are less expensive to operate but the OLR is limiting when compared to a multi-stage operation. On the other hand, multistage AD combines two or more reactors in order to increase process performance and this have been achieved either by aerobic–anaerobic or anaerobic–anaerobic digestion (Vandevivere et al., 2003). The latter separates the initial hydrolysis from methanogenesis and this relatively increases the OLR and methane production.

2.1.Single-stage HSAD systems

In Europe, about 90% of the installed AD plants are from a single stage handling system and as mentioned earlier, 50% of these anaerobic digesters are operated using HSAD (De Baere et al., 2010). Valorga, Dranco and Kompogas are examples continuous systems while the German rectangular is a batch system; the total solid content of the digesters are kept between 20% and 40%.

2.1.1. The Valorga system

Valorga is a cylindrical vertical digester with a horizontal plug flow system. The mid-section of the reactors is demarcated with a vertical wall, which extends across two-thirds of the reactors diameter (Fig. 1). The vertical wall enhances circular flow of organic fraction for a wider coverage of the digester internal surface area (Li et al., 2011). Inlet and outlet valves for inflow and outflow of material are located at the base of the digester. An additional feature of the Valorga system is the internal nozzles at the base of the digester. The nozzles allow high pressure flow of the biogas through the viscous content of the digester. This forceful recirculation of biogas avoids separation

of phases within the reactor and also increases the distribution of fermentative intermediates to microbial cells. However, this technology requires large quantities of energy to pressurize the biogas, and there is a high tendency for the nozzles to be clogged with organic materials. Valorga systems have been operated at 25% and 35% of TS (Li et al., 2011).

2.1.2. The Dranco system

The Dranco system is vertical with a silo shaped base which lacks internal mixing mechanisms (Fig. 2). Unlike the Valorga system which contains nozzles and internal vertical walls, the Dranco digester simply integrates a mixing unit into the process line prior to introducing the feedstock to the AD system. This mixing blends fresh substrate and recycled digestate at a ratio of 1:6 before injection into the digester (Martin et al., 2003). This approach avoids additional energy input and clogging of biogas nozzles, but lacks intermittent mixing. Another consideration is the limited amount of fresh feedstock added to the Dranco system when compared to other HSAD systems. The volume occupied by the recycled digestate limits the amount of fresh feedstock added to the system and eventually the efficiency of the system. The technology operates at TS of 40%, 5% higher than the Valorga (De Baere, 2008).

2.1.3. The Kompogas system

The Kompogas system which originated in Switzerland in the 1980s is a modification of the low solid horizontal plug flow system but with a slowly rotating internal axial mixer (Fig. 3). The mixing keeps dense solids in suspension; increases contact between microorganisms and organic substrate and degases the digestate prior to removal (Li et

al., 2011). The digester is operated between 23% and 28% TS with recycled digestate being mixed with fresh substrate. This is similar to the mixing in Dranco process and reduces the amount of fresh substrate fed into the digester. The principle of adding recycled digestate to the feedstock serves to retain an active microbial community within the reactor.

2.1.4. The rectangular batch digester

An exception to the continuous high solid anaerobic digester is the German garbage type rectangular batch digester, which operates at 40% TS (Fig. 4) (Li et al., 2011). The system is similar to the landfill bioreactor system, in which substrates overlap with a leachate recirculation system (Berge et al., 2009). This approach combines recirculation of leachate within the system and mixture of recycled digestate and fresh substrate prior to AD. Because the German garbage system lacks mixing, substrates and microbial inoculum are distributed unequally; this might cause uneven biodegradation of organic material. This batch digester requires emptying and reseeding, this approach interrupts methane production, making its commercial application of limited value. The batch system has been proven to be the cheapest process in AD but on a commercial scale it requires further research and development.

2.2. Multi-stage HSAD systems

There are relatively few commercially operated multi-stage AD systems because of the high cost of construction, operation and maintenance (Vandevivere et al., 2003). However, research into multi-stage HSAD has continued to grow as there are some examples of successful multi-stage applications in countries, such as Germany, Japan

and Canada. These include continuous systems such as Biotechnische Abfallverwertung (BTA), Linde-KCA, Super Blue Box Recycling (SUBBOR), as well as the batch system, sequential batch anaerobic composting (SEBAC) system.

2.2.1. Biotechnische Abfallverwertung (BTA) system

The BTA multi-stage system was developed to treat municipal waste material (MSW). The system utilizes a pulper and hydrocyclone for solid or liquid separation after which the solid fraction is mixed with pre-treated leachate and then pumped into a hydrolysis tank (Fig. 5). The liquid fractions from the hydrocyclone and the hydrolysis chamber are pumped into the methanogenesis tank for methane production. Data from a Canadian installation showed that VS reduction was between 40% and 45%, indicating poor degradation of the MSW (Chavez-Vazquez and Bagley, 2002; Williams and Davis, 2005).

2.2.2. Linde-KCA system

The Linde-KCA system is operated as a two-stage process incorporating aerobic and anaerobic digestion in separate tanks (Williams and Davis, 2005). The anaerobic digester is a plug flow reactor with axle mixers to increase homogenization (Fig. 6). This system is able to treat feedstock between 15% and 40% TS content. Wastewater companies often pre-treat sludge aerobically prior to AD, making it expensive to operate (Curtis, 2010). As a result, aerobic pre-treatment can reduce the energy value of the feedstock depending on the duration of aeration.

2.2.3. Super blue box recycling (SUBBOR) system

The SUBBOR technology applies steam pre-treatment of feedstock before the AD process (Fig. 7). This is an improvement over the Linde-KCA system because the retention time is shorter and the energy value of the feedstock is not affected. Steam explosion is used to breakdown the complex structure of the feedstock and the technology can be used for the pre-treatment of lignocellulose material (Brodeur et al., 2011). The feedstock is exposed to the 55–63 bar of steam which results in the formation of a paste-like material, thereby enhancing microbial fermentation (Vogt et al., 2002). However, steam explosion requires additional energy input potentially making the process less sustainable for small-scale AD operators.

2.2.4. Biopercolat system

This is a two-stage system with aerobic pre-treatment as the first stage. Unlike the Linde-KCA technology, the aerobic stage is partially aerated as processed water, which is re-circulated continuously and axle rotation homogenizes the feedstock. After 2–3 days of mixing, the separated liquid fraction is fed into the AD (Fig. 8). As mentioned earlier, aerobic pre-treatment can compromise the energy value of the feedstock and eventually reduce methane output.

2.2.5. Sequential batch anaerobic composting (SEBAC) system

The SEBAC system was developed to minimize the constraint of mixing and handling high solid feedstocks (Chynoweth et al., 1991). Feedstocks are introduced into the system sequentially and leachate from the mature reactor is sprayed and recycled continuously until methane production stabilizes in the new batch reactor. The reactor

is then switched to internal recirculation until methane production slowly reduces as the batch matures (Fig. 9). This cycle is repeated for new feedstocks. A major challenge with the operation of SEBAC system is the long period of start-up and stabilization. Studies have shown that the start-up time have been reduced from 250 to 110 d which is still relatively long for commercial operators (Fdéz-Guëlfo et al. (2010). As a result, the SEBAC system is still undergoing research and development.

3. Factors affecting methane production within HSAD

In HSAD, the low water content of the feedstock is mainly responsible for poor distribution of fermentative intermediates such as volatile fatty acids (VFAs) and eventually low methane output (Nagao et al., 2012). This is because water aids the diffusion of these VFAs, particularly acetic acid to the microbial cells, which results in methane production. Another major factor influencing methane production during HSAD is toxicity resulting from the presence of higher concentrations of compounds, such as ammonia, fatty acids, D-limonene and furfurals. These compounds have been reported to be mostly inhibitory to methanogenic bacteria. However, HSAD holds promise for higher methane recovery, although the challenges facing the technology represent a major factor in allowing the proliferation of the technology as a viable approach to AD. The role of VFAs and putative inhibitors in influencing methane production during HSAD will now be considered within this review.

3.1. Fatty acids

Short chain fatty acids, otherwise known as volatile fatty acids (VFAs), and long chain fatty acids (LCFAs) are produced during the acidogenesis and acetogenesis stage in the AD of organic substrates (Madsen et al., 2011).

3.1.1. Volatile fatty acids (VFAs)

Methane is a valuable product arising from the microbial decomposition of organic substrates in the absence of oxygen. One third of the methane produced from AD is strongly dependent on the availability of intermediate metabolites, such VFAs (Buyukkamaci and Filibeli, 2004). The nature of hydrogen and acetate utilization in HSAD suggests that methane gas is produced by the two main groups of methanogens (Zahedi et al., 2013b). The methanogens can be divided into two main groups based on their substrate utilizing capabilities: (i) hydrogen utilizing methanogens are capable of converting hydrogen and carbon dioxide into methane (hydrogentrophic methanogens), and (ii) acetate utilizing methanogens are able to convert acetate into methane (acetotrophic methanogens). In the event of high concentrations of VFAs, it has been reported that this will increase the acidity of the AD system resulting in the inhibition of methanogenesis. As VFAs, particularly acetic acid, are important intermediates within the AD process, their availability in HSAD is not always directly linked with the amount of methane produced. HSAD systems are known to produce lower amounts of methane even at higher organic loading rates (OLR) between 7 and 15 gVS m3 /day (Dong et al., 2010; Nagao et al., 2012). These intermediates could be trapped within the solid fractions of the organic material and because of low water content, diffusion will be reduced (Dong et al., 2010; Nagao et al., 2012). It is expected that high concentrations of VFAs in HSAD will result in an AD system failure, as is the case of LSAD. However,

HSAD may be more stable even at high VFA concentrations because of the poor dissolution of the organic compounds and limited accessibility to methanogenic microorganisms (Dong et al., 2010; Nagao et al., 2012). In an attempt to increase the availability of VFAs to the methanogenic microbial populations, leachate recirculation has been evaluated (Sponza and Ağdağ, 2004). This is a form of mixing in HSAD and considered to be the most economical. In a non-recirculating system, the leachate typically collects at the base of the reactor, which results in variations in the water content throughout the substrate within the reactor. When leachate is recirculated, the water content becomes more homogeneously distributed enhancing VFA availability and resulting in formation of methane. According to Sponza and Ağdağ (2004), the recirculation of leachate reduces hydraulic retention time and increase methane production.

3.1.2. Long chain fatty acid (LCFA)

LCFAs are produced during the biological breakdown of lipid-containing substrates. Lipid is converted to LCFAs and glycerol during anaerobic hydrolysis while LCFAs (oleate, stearate, and palmitate) are converted into hydrogen and acetate through the β-oxidation pathway (Weng and Jeris, 1976). However, LCFAs have been reported to inhibit methanogenesis by distorting the electron transport system in the cellular membranes of the microorganisms (Hanaki et al., 1981; Rinzema et al., 1994). Sousa et al. (2013) reported a maximum tolerance concentration of 1 mM of LCFAs for methanogens.

3.2.Temperature

The interactions between microorganisms and organic substrates are dependent on temperature. Microorganisms can be divided into two main groups: mesophilic and thermophilic microflora (FernándezRodríguez et al., 2013). Thermophilic microorganisms are known to be active at temperatures of 50–60 °C while mesophilic micoorgansms are active at 35- 38 °C; the elevated temperatures (50–60 °C) has been reported to increase rate of methane production and reduce hydraulic retention time (HRT) (Fernández-Rodríguez et al., 2013; Hidaka et al., 2013). The impact of thermophilic operating temperatures is not only limited to increasing the metabolic activities of microorganisms, but also enhances the solubilization of organic substrates. For example, Battistoni (1997) reported that higher temperatures increase the solubility and viscosity of organic substrates during AD. This can be explained by the frequent evaporation and condensation of water within the AD system. The rate of water evaporation increase as temperature increases and, as it condenses, the water molecules flow through the wall of the digester into the system (Vieira da Silva et al., 2013). In addition, owing to the low water content in HSAD system, higher temperatures have been reported to decrease the viscosity between the substrate particles, thereby increasing diffusion of organic substrates to microbial cells (Battistoni, 1997; Bollon et al., 2013). This is because at higher temperature the time of contact between the molecules of a fluid decreases because of increased velocity of the discrete molecules. The drawback of operating AD under thermophilic conditions is the high energy demand as well as the lower diversity of robust methanogens needed for consistent methane production (Biey et al., 2003). Although, there are reports of AD failure under thermophilic temperature, these cases are not directly associated with the operating temperatures, but rather the high OLR and imbalances in the carbon to nitrogen ratio

(Hidaka et al., 2013; Lianhua et al., 2010). For example, Fernández-Rodríguez et al. (2013) demonstrated that HRT was reduced by 50% under thermophilic HSAD for municipal solid waste with 20% TS. On the other hand, the application of mesophilic temperature is more extensive when compared to thermophilic AD systems. This is because mesophilic temperatures enhance the diversity of methanogenic microorganisms, however, the rate of methane production is not as fast as thermophilic AD. The combination of thermophilic and mesophilic temperature in a two stage AD process could provide a better option for optimizing HSAD, since the methanogens are more robust at mesophilic temperature (Biey et al., 2003).

3.3.Inhibition

The interaction between different groups of microorganisms, organic substrates, and operating parameters can influence the stability and performance of AD and the production of methane. In a situation where this interaction fails, it causes increases in the concentration of fermentative intermediate products, which may impact on the AD of the organic substrate (Chen et al., 2008). Fermentative intermediates, such as VFAs, inorganic nitrogen and long chain fatty acids (LCFAs), are as a result of the microbial interaction with organic substrates. Inorganic nitrogen and LCFAs are specific to protein and lipid rich substrates, respectively, and can be toxic at higher concentrations (Koster and Lettinga, 1988; Rinzema et al., 1994). On the other hand, compounds like D-limonene, furfural and phenolic compounds, which are constituents of the organic substrates and are not produced through microbial interactions, are highly inhibitory (Mizuki et al., 1990). Toxicity is not only associated with LSAD, but it is expected to be more prevalent in HSAD owing to the higher OLR and lower moisture content (Vandevivere et al., 2003). However, the high solid content in HSAD may enhance the

sorption of inhibitory substances, thus reducing the mobility and bioavailability of inhibitory compounds and thereby reducing the negative effect. For example, Achak et al. (2009) reported that banana peel adsorbed phenolic compounds from olive mill waters, indicating that some agricultural substrates may have some level of adsorbtive properties. Likewise, because of the impact on chemical mobility in HSAD, the inhibitory compounds can be unevenly distributed and accumulate forming 'hotspots' in the AD environment, particularly in the absence of mixing (Martin et al., 2003; Vavilin et al., 2003). These are some of the reasons why inhibition in HSAD could go unnoticed, although this requires further study. The source and role of different inhibitors and their influences on HSAD will now be considered within this review.

3.3.1. Ammonia

Protein is a source of two principal forms of inorganic nitrogen namely free ammonia (NH_3) and ammonium (NH_4). Low concentrations of ammonia are essential for microbial growth; however at higher concentration (2000–10 000 mg/l) it becomes inhibitory (Koster and Lettinga, 1988). The occurrence of NH_3 and NH_4 during anaerobic digestion depends on the operating temperature and pH of the system (Anthonisen et al., 1976). It has been reported that NH_3 is more toxic than NH_4 because of its ability to penetrate the cell membranes causing proton imbalances and sometimes interfering with the metabolic enzymes, thus inhibiting degradation of VFA (Gallert and Winter, 1997; Sung and Liu, 2003). Co-digestion provides the most economical solution to NH_3 inhibition because it thrives on the synergy between two or more substrates. Organic substrates high in carbon content are often co-digested with protein-rich substrates to balance the C/N ratio. For example, Yangin-Gomec and Ozturk (2013) a reported 1.2 fold increase in methane production when maize silage was codigested with

chicken and cattle manure. The maize silage was able to balance the carbon to nitrogen ratio thus reducing the possibility NH_3 toxicity. Another way to reduce NH_3 inhibition is through the ability of methanogens to acclimate; reports have shown that methane production becomes stable after the methanogens have adapted to NH_3 toxicity (Koster and Lettinga, 1988).

3.3.2. D-limonene and furanic compounds

D-limonene and furanic compounds have been reported to inhibit methanogenesis, although they are not fermentative intermediates of the AD process. D-Limonene, a colourless, aqueous, cyclic terpene, commonly found in citrus fruits, particularly citrus peel, makes up to 50%–60% of processed fruit waste (Wilkins et al., 2007). The D-limonene compound has been reported to be bactericidal. Mizuki et al. (1990) studied the mesophilic digestion of Citrus unshu peel and observed inhibition of methane production at OLR above 2.0 g/l/day. Similarly, previous research by Lane (1983) observed inhibition by the production of benzoic, phenylacetic and phenylpropionic acids during mesophilic AD of citrus waste. Although higher methane production have been observed in thermophilic systems, inhibition was noticed at loading rates above 4 kg COD/m3 (Martín et al., 2010). The mechanisms of damage to microbial cells are similar to other hydrocarbons since D-limonene is a liquid hydrocarbon (Ruiz and Flotats, 2014). D-limonene is hydrophobic but because the bacterial cell produces surface active compounds these increases the dissolution of D-limonene (Sikkema et al., 1995) in the AD system. The dissolution of D-limonene enhances its diffusion into the microbial cell, thereby increasing cell membrane permeability causing cell leakage and lysis (Burt, 2004). However, the compound can be extracted prior to AD using steam distillation or solvent extraction (Martín et al., 2013; Srilatha et al., 1995). These

methods are energy intensive, however, the application of co-digestion or pre-treatment with fungi has been found to be effective at reducing limonene inhibition (Martín et al., 2013; Srilatha et al., 1995). Furanic compounds, such as furfurals and 5-hydroxyl methyl furfural (5-HMF) originate from the dehydration of hemicellulose (Ramos, 2003). Hendriks and Zeeman (2009) pointed out that these hemicellulosic monomers are inhibitory to anaerobic bacteria. The furanic compounds induce cell lysis by damaging the DNA and inhibiting enzymes involved in glycolytic pathway (Palmqvist and Hahn-Hagerdal, 2000). According to Barakat et al. (2012), AD is stable when the concentration of furans is ≤1 g/l but subsequent increase in concentration to 2 g/l and 3 g/l for furfurals and 5-HMF, respectively, resulted in lower methane production (Badshah, 2012).

4. Optimizing HSAD through technological integration

In recent years, there have been major advances in AD, some of which can be attributed to the integration of technologies to counteract specific challenges. For instance, pre-treatment of lignocellulose feedstock, formerly for ethanol production has recently become a useful method for the solubilization of lignocellulose feedstock prior to AD (Hendriks and Zeeman, 2009). Other technologies, such as co-digestion, reactor integrations and thermo–mesophilic digestion, have been developed relatively recently to improve larger scale AD operations. In line with this thinking, attempts have been made to integrate different technologies to optimize the HSAD (Table 1). For example, co-digestion is increasingly used where mono-digestion has been found to be unsuitable for maintaining AD stability. Furthermore, two stage AD is gradually more used where single stage AD has been found to be less efficient. This section of the paper will focus on how co-digestion of substrates, digester configurations and temperature phased AD can improve HSAD.

4.1. Co-digestion

Co-digestion is an example of a gradual transition from individual organic substrates to an AD process that allows the mixing and digestion of more than one type organic material. The advantage in this approach is that it allows the deficiencies of a single organic substrate to be supplemented by additional sources, thereby improving the performance of the AD process (Angelidaki and Ellegaard, 2003). For instance, Kim and Oh (2011), reported over 80% reduction in volatile solid content of organic substrate when paper and food wastes were co-digested at 40% TS. Considering that food wastes are noted for high content of protein and paper wastes on the other hand contains high content of carbon, blending these two waste streams can balance the carbon to nitrogen ratio, thus reducing possible inhibition from NH3 toxicity (Zhang et al., 2011). Co-digestion is beneficial to all forms of AD; other benefits, such as additional nutrients and robust microbial populations, have been reported in literature (Navaneethan et al., 2011). Previously, Zhang et al.(2012) classified organic substrates based on nutrient and energy value, nevertheless this classification can be modified using three categories (i) energy (ii) nutrient and (iii) methanogens. For instance, the co-digestion of piggery effluent and food waste combines high nutrient and energy rich substrates, respectively (Zhang et al., 2011). Equally, the co-digestion of cattle manure, food waste and sewage sludge combines methanogens, energy and nutrient rich substrates, respectively (Angelidaki and Ellegaard, 2003; Navaneethan et al., 2011). According to Maranon et al. (2012), co-digested cattle manure, food waste and sewage sludge at mesophilic temperature (36 °C) recorded a maximum methane production of 603 l CH_4 $kgVS^{-1}$ added. From the available studies, it appears that co-digestion can be used to achieve better HSAD performance especially when the organic substrates is a mixture of waste streams high in energy, nutrients and methanogens. A major factor to consider when employing co-digestion in

HSAD is the mixing ratios of the organic substrates (Sosnowski et al., 2003). The organic substrate mixing ratios are essential for ensuring a balance between the microbial population, nutrients and organic carbon. Maranon et al. (2012) reported that the anaerobic co-digestion of cattle manure, food waste and sewage sludge was conducted using two different mixing ratios. The mixing ratios used in the experiment were (i) 70:10:20 and (ii) 70:20:10 for cattle manure, food waste and sewage sludge, respectively. The mixing ratios 70:20:10 produced 22% more methane. Experimental trials are often required to determine the most suitable mixing ratios during co-digestion. Co-digestion is an economic approach to reducing potential inhibitions and improving AD, but the question of co-substrate availability and accessibility can be an issue.

4.2. Mixing technologies

AD requires continuous contact between the microorganisms and the organic substrate for rapid methane production. Karim et al. (2005a) reported that mixing becomes very important when the TS of the feedstock exceeds 5%. Although, OLR, methane production and solid retention time were described as the three major factors for designing a continuous high solid anaerobic digester. (Karthikeyan and Visvanathan, 2013), these factors can easily be influenced by the extent of contact between the microbial cells and the feedstock. Mixing of substrates and microbial cells during AD is an effective way of increasing contact and can be achieved either by (i) installing a mechanical internal mixing device in the digester; (ii) by recirculating digested liquor, or (iii) pumping biogas produced through the system (Karim et al., 2005a). However, internal mixing could be energy intensive because of the viscosity of the organic substrate, even though, internal mixing devices are comparatively more effective than biogas recirculation; as observed for HSAD at 15% TS (Karim et al., 2005b).

4.2.1. Mechanical mixing

This form of mixing involves attaching a baffle within the digester, which can be operated either manually or electrically. Anaerobic digesters, such as the continuous stirred-tank (CSTR), are often built with an internal mixing device to increase interaction between microbial cells and organic molecules. The CSTR has been the most widely used for continuous homogenizations in anaerobic digesters. Approximately 50% of full-scale AD plants operating in Europe have adopted the single stage CSTR system (De Baere et al., 2010). Mechanical mixers, such as the CSTR or Kompogas technology, are not only useful for increasing microbe to substrate interaction, they also reduce froth formation and stratification within the AD system. Nonetheless, there are indications that this approach of mixing is not suitable for feedstock with high protein content because high ammonia concentrations can enhance ammonia toxicity (Pommier et al., 2007). Although internal mixers are not extensively used in HSAD, the Kompogas system is noted for its slowly rotating internal axial mixer. Alternatively, the Dranco and Valorgas systems have external mixing devices to homogenize the feedstock before injection into the digester. The lack of internal mixers in HSAD may be attributed to the sophistication and cost requirement (Fruteau de Laclos et al., 1997).

4.2.2. Fluid mixing through recirculation

Fluid mixing offers another option for increasing the contact between the microbial cells and the organic substrates. This can be achieved by using the liquor and/or the biogas to increase mixing within the digester (Fruteau de Laclos et al., 1997; Shahriari et al., 2012). Fluid recirculation within the reactor has been considered an alternative to the CSTR system. Biogas recirculation is commonly used by the Valorgas digester; as stated earlier, there is a nozzle at the base of the reactor to allow the up-flow of biogas under

high pressure (Fruteau de Laclos et al., 1997). This design improves the blending of the material in the digester (Koster and Lettinga, 1984). Another approach to mixing is when the leachate is recirculated within the AD reactor. Unlike CSTR and gas recirculation systems that mix through agitation, leachate recirculation depends on the diffusion of liquid through the pores around the microorganisms and feedstock mixtures (Bolzonella et al., 2003). As the water content of the leachate is high, this increases the mobility and bioavailability of organic substrate to anaerobic microorganisms for faster hydrolysis of the organic substrates (Bolzonella et al., 2003). A good example of this is the Germandesigned rectangular batch HSAD (Li et al., 2011). In this system, leachate drips downwards under gravity to the base of the digester which is later recirculated to the upper layer of the organic substrate. This sequence causes organic molecules and nutrients to be mixed with the microbial cells (Pommier et al., 2007; Sponza and Ağdağ, 2004). However, the downside to the application of leachate recirculation is that it typically does not prevent stratification during AD.

4.2.2.1. Liquid recirculation

Leachate recirculation avoids the additional energy requirements for mechanical stirring or biogas pressurized flow; instead the process depends on diffusion through the pores of the viscous material (Bollon et al., 2013). In this process, the leachate settles at the base of the reactor and causes an uneven distribution of moisture within the reactor but during recirculation water is evenly retained in each stratum, thus enhancing HSAD. Percolation of leachate is a regular occurrence in HSAD, but this often depends on the physical properties of the feedstock and the TS content (Sponza and Ağdağ, 2004; Bollon et al., 2013). For example, feedstocks with high TS content have low viscosity because of low diffusion co-efficient (Battistoni, 1997; Bollon et al., 2013). This further

reduces the interstitial spaces within the feedstock and eventual slows down the percolation of leachate. Miscible feedstock such as animal manure, secondary sewage sludge is highly viscous at moisture content above 25% and it is expected that water percolation will be less than when compared to hydrophobic organic material. For instance, lignocellulose substrates tend to be hydrophobic because of the polymerized outer surface, which may increase the interstitial space between each particle, irrespective of its TS thus enhancing percolation rate of leachate. There are a few challenges with regards liquid recirculation in HSAD. Leachate recirculation can also be detrimental to methanogenesis, particularly when inhibitory compounds, such as ammonium and chloride are present (Sponza and Ağdağ, 2004; Chen et al., 2008). According to Shahriari et al. (2012), complete leachate recirculation can be inhibitory to methanogens, particularly when food waste is solely used. Similarly, Sponza and Ağdağ (2004) reported abundant ammonium during leachate recirculation of municipal solid waste, which potentially decreases methanogenesis. In an attempt to reduce toxicity from remixing digestate with fresh feedstock, the Dranco and Kompogas technologies pre-treat or recycle the digestate to reduce organic fractions (Li et al., 2011). Although, the details on how the digestate was pre-treated were not mentioned, is assumed that composting was used since the technology is relevant for stabilizing organic waste material (Bustamante et al., 2012). Apart from monitoring ammonium and chloride toxicity during leachate recirculation, a highly viscous material is essential to promote faster percolation of liquor for recirculation.

4.3. Single and multi-stage AD systems

Over 95% of commercial AD plants are operated as a single stage system, principally because two stage systems are more expensive to run (Lissens et al., 2001). The two-

stage systems have been reported to be more efficient because it allows the separation of the acid and methane producers, thereby reducing the impacts of pH fluctuations and potential fermentative inhibitors (Demirel and Yenigün, 2002). According to Llabrés-Luengo and Mata-Alvarez (1988), two-stage stabilization of feedstock would be the most suitable configuration for HSAD. Unlike a single stage system, the two-stage AD system can incorporate two different operating temperatures. In a study involving the performance of five different reactor configurations for AD of substrates, the report showed that the two-stage system out-performed single-stage digestion with higher COD removal (Azbar et al., 2001). With regard to HSAD, the choice of digester in a two-stage system must incorporate the necessary solutions needed to enhance methane production. In recent years, several studies have been carried out on multi-stage AD with various digesters including two or more CSTRs, CSTR and high rate digesters (HRD), particularly anaerobic filter and up-flow anaerobic sludge blanket (UASB) system (Table 2). Unlike other high rate digesters, the UASB system has been extensively integrated with other digesters owing to higher efficiency, flexibility and simplicity of operation (Chong et al., 2012). For example, the integration of leachate bed and the UASB system for the HSAD of blue mussel and reed was investigated (Nkemka and Murto, 2013). The leachate bed enhances accumulation of leachate to the base of the digester, which invariably will be pre-treated by pumping it through the USAB digester before reintroducing it into the leachate bed system. The leachate bed is similar to the German garbage type rectangular batch digesters in which the solid and liquid phases are demarcated by perforated layer (Sponza and Ağdağ, 2004; Pohl et al., 2012). This perforated surface allows moisture to trickle to the base of the reactor for easy collection and recirculation, particularly within the HSAD system (Macias-Corral et al., 2008). Similarly, the combination of an up-flow solid state and anaerobic filtration has been

reported to optimize the AD of wheat straw, thereby increasing the methane output by 36% (Pohl et al., 2012). Reactor integration, particularly solid phase and high rate reactors enhance leachate pre-treatment prior to recirculation, but do not necessarily provide an outright solution for substrate induced inhibition. However, the adaptive potential of agglomerated microbial cells in all high rate reactors may survive better and continue to metabolize under unfavourable conditions (Chen et al., 2008; Francois et al., 2007). Despite the major advances in improving HSAD through multi-stage systems, most operators would prefer a single-stage AD system because of the additional operational and maintenance costs (Lissens et al., 2001).

4.4. Temperature-phased anaerobic digestion

Temperature-phased anaerobic digestion (TPAD) combines more than one operating temperature for the anaerobic digestion of organic substrate. The term thermo–mesophilic digestion is otherwise grouped under TPAD. The technology simply incorporates the advantages of thermophilic and mesophilic conditions into an AD process (Song et al., 2004). However, the combination of thermo–mesophilic conditions in AD may be a better option for HSAD, but this approach can only be successfully carried out in a multi-stage AD system (Song et al., 2004). The combination of thermophilic and mesophilic conditions have also been reported to operate at high organic loading rates, particularly with shock-loading of substrates. Ge et al. (2011) reported that when two-stage digesters were used, higher volatile solid reduction (34%–48%) was observed for thermo–mesophilic TPAD, 11%–30% higher than meso–mesophilic TPAD. According to Ge et al. (2011), the thermophilic stage of hydrolysis was 27% more effective than the mesophilic hydrolysis stage. This is similar to the results obtained by Roberts et al. (1999), where higher amount of methane were

recovered from a two-stage thermo–mesophilic AD system. The application of thermo–mesophilic TPAD is not only limited to optimization of methane output; there are also reports that this can lead to great reductions in pathogenic organisms. Currently, the application of mesophilic, thermophilic and TPAD have been able to achieve pathogen inactivation (Fu et al., 2014). However, recent reports have shown that pathogen reduction is higher in thermophilic AD systems. In a report by Astals et al. (2012), the thermophilic AD of sewage sludge recorded a greater pathogen reduction than the mesophilic AD. Similarly, Riau et al. (2012) recorded greater reductions in pathogens when thermo–mesophilic TPAD was operated at sewage sludge to inoculum ratio of 0.25. With regards to HSAD, owing to the high OLR and low moisture content, the abundance of pathogens in the digestate could be relatively higher if the AD process is limited to mesophilic temperatures. However, there are indications that thermophilic AD only alters the culturable state of the pathogenic microorganisms rather than killing them, thereby increasing the potential for cell reactivation under favourable conditions (Fu et al., 2014). The proliferation of pathogens is a major problem with organic substrates; however, this challenge could be minimized if a pre-treatment or post-treatment stage is included in the operational processes.

5. High solid anaerobic digestate

As stated earlier, recent reports have suggested that HSAD containing >20% TS will produce lower methane (Dong et al., 2010; Nagao et al., 2012). Consequently, many studies have been conducted to optimize methane production from HSAD (Benbelkacem et al., 2013; Cho et al., 2013a,b; Fernández-Rodríguez et al., 2013; Li et al., 2014; Liang et al., 2014a,b; Zahedi et al., 2013a,b; Zhu and Jha, 2013). However, the HSAD system provides a better option for a cost effective digestate handling

operation and a nutrient-rich digestate material. This section of the paper will be focusing on how HSAD can improve digestate handling and increase its nutrient content.

5.1. Nutrient content

The residual organic material from AD process is called digestate and it contains nutrients, which are beneficial to agriculture as a nutrient source and/or soil conditioner. According to Alburquerque et al. (2012), the addition of digestate to the soil will increase the immediate availability of nutrients for microbial and plant uptake. Digestate application to land is currently considered to be the most effective route for maintaining nutrient recycling, particularly in developing countries (Tambone et al., 2010). However, the amount of nutrient availability per gram of digestate is often compromised depending on the TS content of the organic waste. It is expected that the addition of water will dilute the available nutrient content and subsequent dewatering may reduce the concentration of the residual nutrients in the solid fraction (Table 3). A report by Vaneeckhaute et al. (2013) shows that more nutrients are contained in a digestate liquid fraction and in the event of dewatering most of this nutrient could be lost. Table 3 shows that more nutrients can be retained in the digestate if it is not dewatered. Apart from dilution of nutrients in digestate, applications of digestate to water-logged farmland have been reported to contribute to leaching, runoff and eutrophication of watercourses (Mangwandi et al., 2013). On the other hand, the HSAD digestate is more compact providing surface area for nutrient adsorption and gradual release of nutrients into the soil. In order to reduce the mobility of nutrients, wastewater companies usually thicken digestates by adding polymers and other thickening agent (Mangwandi et al., 2013; Watanabe and Tanaka, 1999). These chemical amendments are often administered before dewatering to increase the solid content of the digestate to approximately 15%–

25%. Nutrient management is essential for maximizing digestate utilization on land. HSAD provides a better option for nutrient retention owing to the dryness of the digestate. This is because agricultural material, particularly fibre can also serve as an adsorbent (Achak et al., 2009). Other approaches such as application of adsorbents have been reported to improve nutrient uptake and retention from wet digestates, but this approach often increases the cost (Estevez et al., 2014).

5.2. Digestate handling and transport

The handling and transport of digestate is a potentially costly component of running an AD plant (Vandevivere et al., 2003). At the moment, particularly in the UK, digestate is not sold; as a result, operators have to bear the cost for managing and transporting this material to spread on farmland. However, the cost incurred to manage digestate is dependent on the treatment applications (Table 4). There are different digestate-handling methods but the choice of treatment will vary depending on the moisture content of the digestate. Digestate from HSAD has low water content and the use of open-air or solar drying may be suitable, with the operational costs being comparatively lower than other digestate treatment options (Table 4). Unlike HSAD, digestate from LSAD contains more water and, in most cases, drum or belt drying is often used in combination with physicochemical treatments to remove water (Mihoubi, 2004; Watanabe and Tanaka, 1999). The LSAD digestate can also be treated with either open-air or solar drying, but this will take longer because of the higher water content. Furthermore, the emissions of N_2O, NH_3, NO_3^- and CH_4 gas, all of which contribute to acidification of rain water and global warming, constitute a great concern during longer periods of open-air or solar drying (Rehl and Müller, 2011). The emissions of N_2O and NH_3 have been reported to be mostly associated with both the liquid fraction of the digestate and selected drying

options (Amon et al., 2006). According to Rehl and Müller (2011), 85% of the available ammonia was emitted to air in all existing drying methods. Although the emission NH_3 and N2O gases is a major factor of the protein-rich organic substrates, the percentage of dryness in the organic substrates will influence the rate of emission (Amon et al., 2006; Rehl and Müller, 2011). In some cases, the digestate storage container is often closed, thereby reducing the emission of NH_3 and N_2O gases to the atmosphere. Another cost incurred during digestate management is that of transportation. In the UK, digestate are usually spread on farmland at a particular time of the year and, for most on-farm AD systems, transportation costs are minimal. However, unlike the HSAD system, LSAD digestate requires support media to enhance dewatering, which further increases the quantity of material to be transported. AD systems will always incur additional costs for digestate transportation but the cost of transporting HSAD digestate is relatively lower.

6. Conclusion

The operation of HSAD offers a better option for reducing water usage and enhancing digestate handling. This approach to AD will be most suitable in regions with shortage of freshwater and high demand for organic fertilizer. In addition, the application of decentralized small-scale anaerobic digestion in homes, small and medium scale business could be further achieved using HSAD because it reduces or avoids dewatering and effluent handling. However, the technology is faced with challenges of limited methane production when compared with LSAD. More research is required to explore the potential in thermo–mesophilic digestion, co-digestion, multi stage digestion, particularly combination of high rate reactors, and high solid digesters for higher methane production.

Acknowledgements

This work was supported by the Centre for Global Eco-innovation (X02646PR) and Stopford Energy and Environment.

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Table

Table 1: Comparison of LSAD and HSAD processes ((adapted from (Vandevivere et al., 2003)).

Parameter	LSAD	HSAD
Total solid	< 10 %	10-40 %
Operational mode	Single and multi-stage AD	Single and multi-stage AD
HRT	Low	High
Phase separation	High	Low
Feeding regime	Semi and continuous	Batch, sequential batch, semi and continuous
Biogas production	High moisture, High biogas production	Low, moisture, low biogas production
Volatile solid reduction	50 – 70 %	< 40 %
Substrate loading rate	< 7 gVS m3/day	7-15 gVS m3/day
Inhibition	More dispersion and diffusion	Less dispersion and high adsorption into organic material
Mixing device	Internal mixing device, liquor and biogas recirculation	Leachate and biogas recirculation, biogas and partial mixing
Heating requirement	High heating is required due to larger volume	low heating is required due to smaller volume
Operational problem	Pumping equipment is less sophisticated due to high moisture	Sophisticated pumping equipment is required
Substrate	Not suitable for hydrophobic substrates like the lignocellulosic materials	Most suitable for hydrophobic substrates
Digestate handling	Dewatering is required	Dewatering is minimal
Digestate quality	Less stable but nutrient content is high	More stable with low nutrient continent

Table 2: The Performance of different mode of HSAD processes

Substrate	Configuration	Reactor	Mixing device	Temperature	CH_4 Yield	References
OFMSW	Single	Batch	Mechanical stirring	Thermophilc (55^0C)	0.4-0.49 L/L.d	(Forster-Carneiro et al., 2008)
OFMSW	Single	CSTR	Mechanical stirring	Mesophilic (35 ^{0}C)	1.324 L/L.d	(Fdez-Gueelfo et al., 2010)
Food waste and paper waste	Single	CSTR	Mechanical stirring	Mesophilic (35 ^{0}C)	0.25 m^3gCOD added	(Kim and Oh, 2011)
Meat and bone meal	Single	Co-digestion	Mechanical stirring	Mesophilic (35 ^{0}C)	351-381 ml g TVS	(Wu et al., 2009)
OFMSW	Double	CSTR	Mechanical stirring	Thermophilic (55^0C)	5.4 ± 0.3 lCH4/l/d	(Zahedi et al. 2013b)
Blue mussel and reed	Double	Leach bed UASB	Leachate recirculation	Mesophilic (35 ^{0}C)	0.33 m^3/kg VS	(Nkemka and Murto, 2013)
Food waste	Single	Leachate bed	Leachate recirculation	Mesophilic	-	(Sponza and Ağdağ, 2004)
Wheat straw	Double	UASS AF	Leachate recirculation	Thermophilic (55^0C) and mesophilic (35 ^{0}C)	-	(Pohl et al., 2012)
Food waste and Livestock waste	Single	CSTR	Mechanical stirring	Mesophilic (35 ^{0}C)	0.26 m^3gCOD added	(Kim and Oh, 2011)
Thin silage and poultry litter	Single	CSTR	Mechanical stirring	Thermophilic (55 ^{0}C)	-	(Sharma et al., 2013)
Sewage sludge	Double	CSTR	Mechanical stirring	Themo-mesophilic (TPAD)	424-467ml gVS	(Song et al., 2004)
Foodwaste	Single	CSTR tubular reactor	Mechanical stirring	Mesophilic (35 ^{0}C)	2.51 ± 0.17 m^3/m^3/d	(Cho et al., 2013)

Table 3: Physiochemical characterization of pig slurry, mixture of solid and liquid fraction of digestate (A) and liquid fraction of digestate (B) ((adapted from (Vaneeckhaute et al., 2013)).

Parameter	A	B
Dry matter (%)	6.2 ± 0.1	2.5 ± 0.1
Organic carbon (%)	38 ± 0.1	25 ± 0.1
Total nitrogen (g kg^{-1})	4.7 ± 0.0	3.6 ± 0.0
NH_4-N (g kg^{-1})	3.1 ± 0.1	2.8 ± 0.0
Mineral Nitrogen (%)	66 ± 0.0	77 ± 0.0
Total phosphorus (g kg^{-1})	0.9 ± 0.1	0.27 ± 0.0
K_2O (g kg^{-1})	2.6 ± 0.1	3.5 ± 0.0
Ca (g kg^{-1})	1.3 ± 0.3	0.11 ± 0.0
Mg (g kg^{-1})	0.34 ± 0.04	0.016 ± 0.00
S (g kg^{-1})	0.4 ± 0.0	0.11 ± 0.0
Na (g kg^{-1})	2.0 ± 0.0	3.1 ± 0.0
Cl (mg kg^{-1})	2.7 ± 0.0	2.9 ± 0.0

Table 4: Several treatment option of anaerobic digestion digestate (adapted from (Rehl and Müller, 2011))

Treatment	Application	Cost	References
Chemical	Disinfecting digestate liquid fraction using coagulant	Sequential	(Liang et al., 2014)
Physical	Disinfecting digestate liquid fraction using micro filtration, reverse osmosis and ion-exchanger.	Initial and maintenance	(Hoibye et al., 2008)
Drum dryer	Pelletizing solid fraction though drying	Initial and maintenance	(Vaxelaire et al., 1999) (Vaxelaire et al., 2000)
Air dying	Evaporation of moisture from digestate		(Amlinger et al., 2008)
Solar drying	Heat drying of digestate to remove 65% of moisture	Initial cost	(Vaxelaire et al., 2000) (Bux et al., 2002)
Belt dryer	Drying and mixing fresh digestate with dry digestate to increase TS to 20% before pelletizing.	Initial and maintenance	(Mihoubi, 2004; Mihoubi et al., 2003)

Figures captions

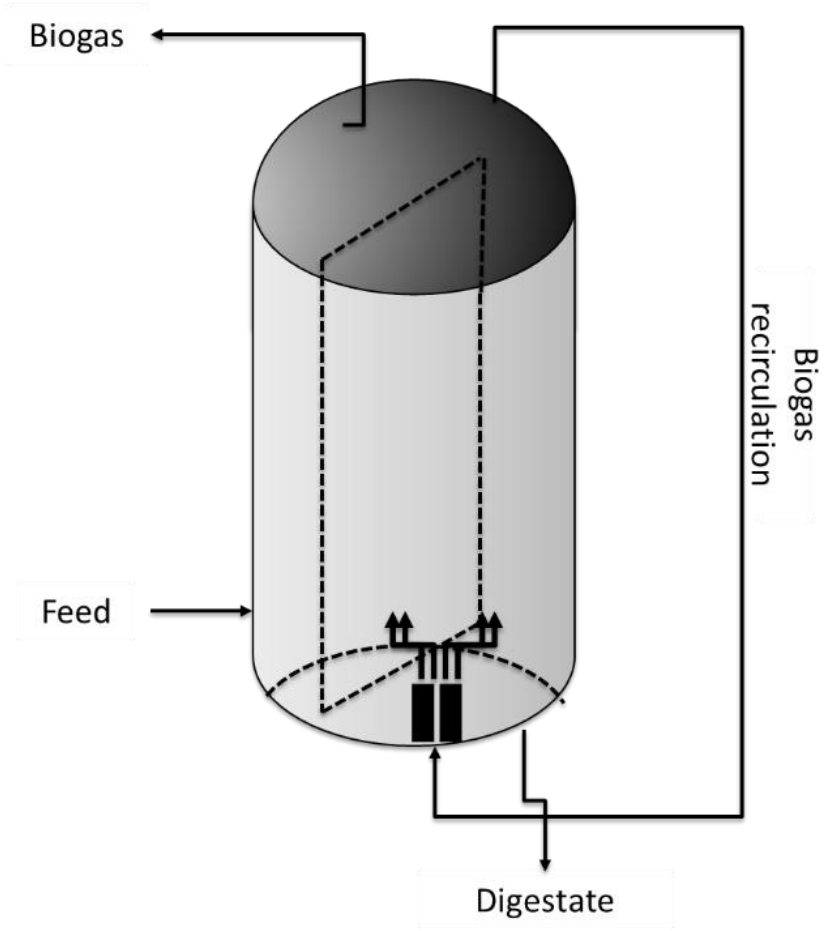

Fig. 1.

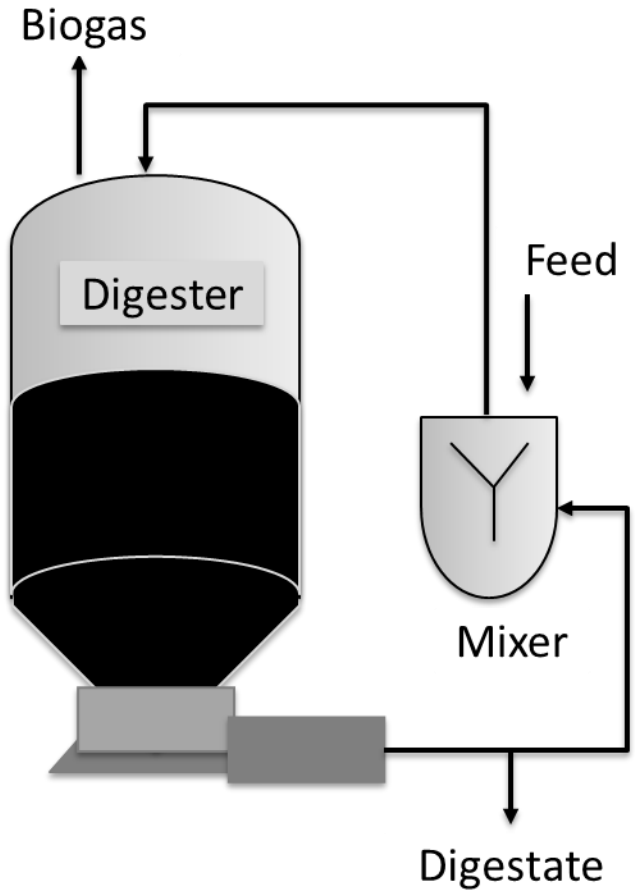

Fig. 2.

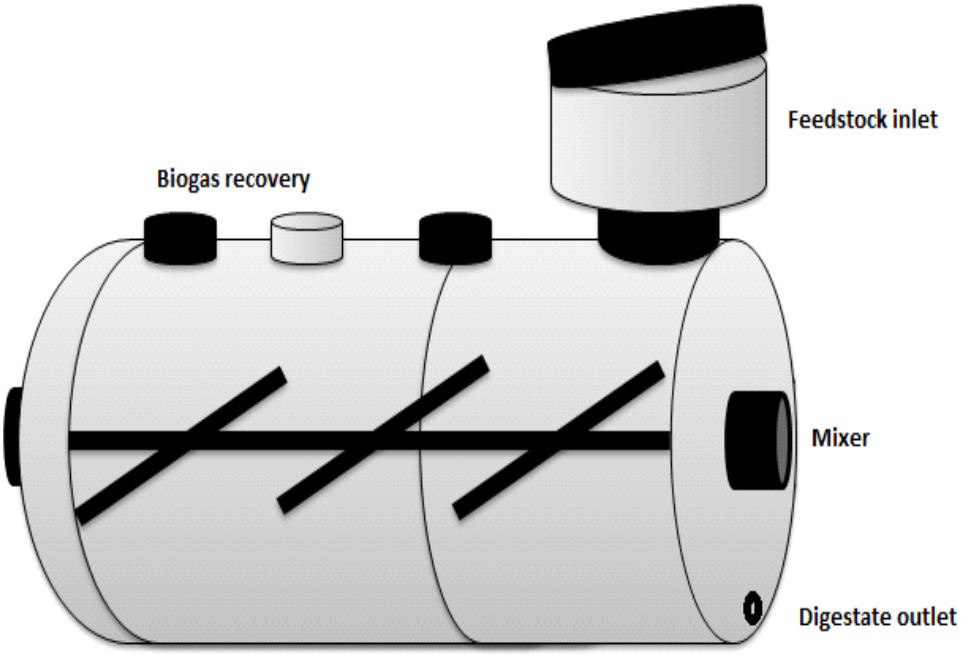

Fig. 3.

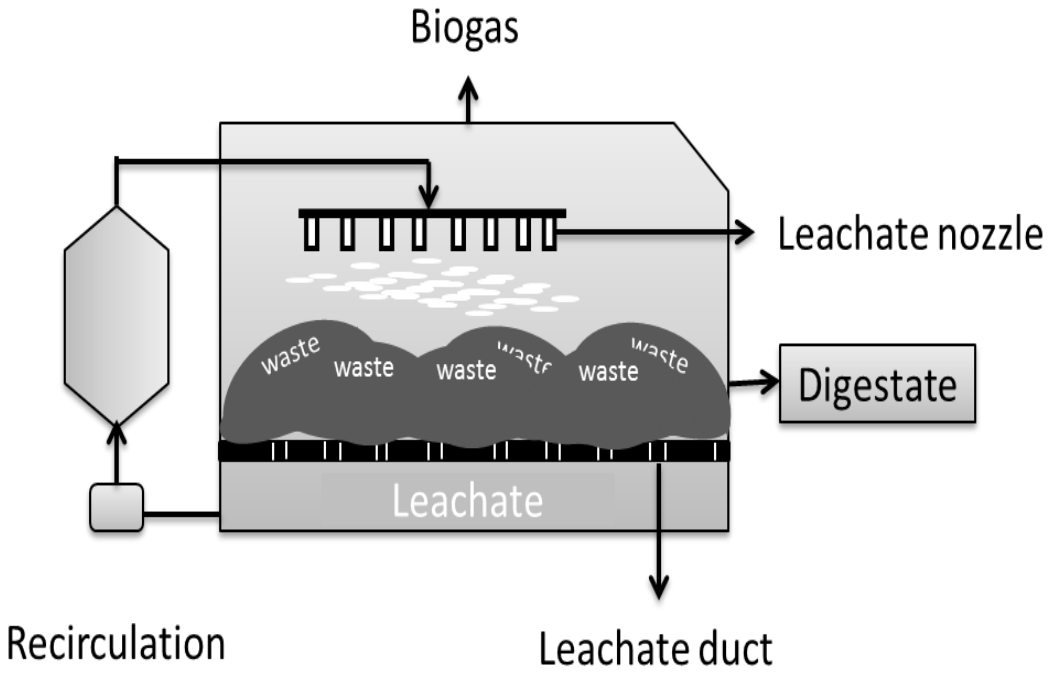
Biogas
Leachate nozzle
waste
waste
waste
waste
Digestate
Leachate
Recirculation
Leachate duct

Fig. 4.

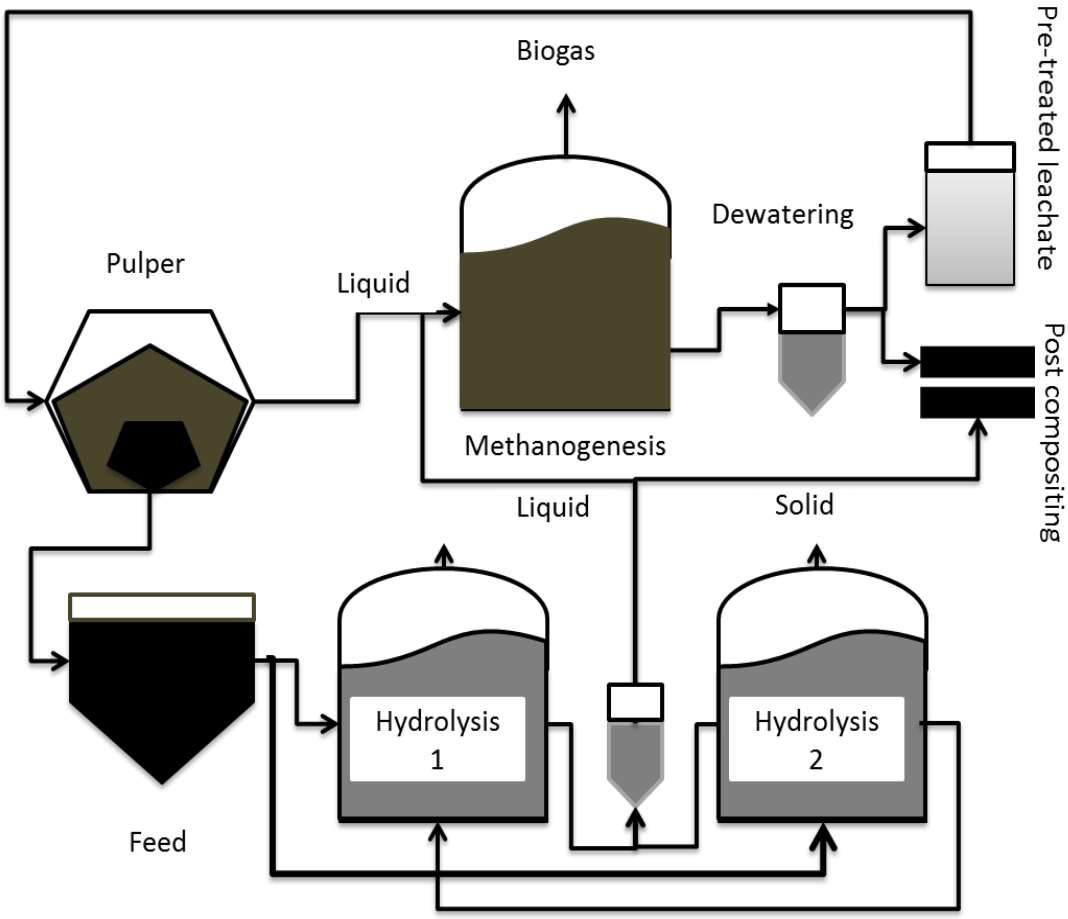
Biogas
Pre-treated leachate
Dewatering
Pulper
Liquid
Post compositing
Methanogenesis
Liquid
Solid
Hydrolysis
1
Hydrolysis
2
Feed

Fig. 5.

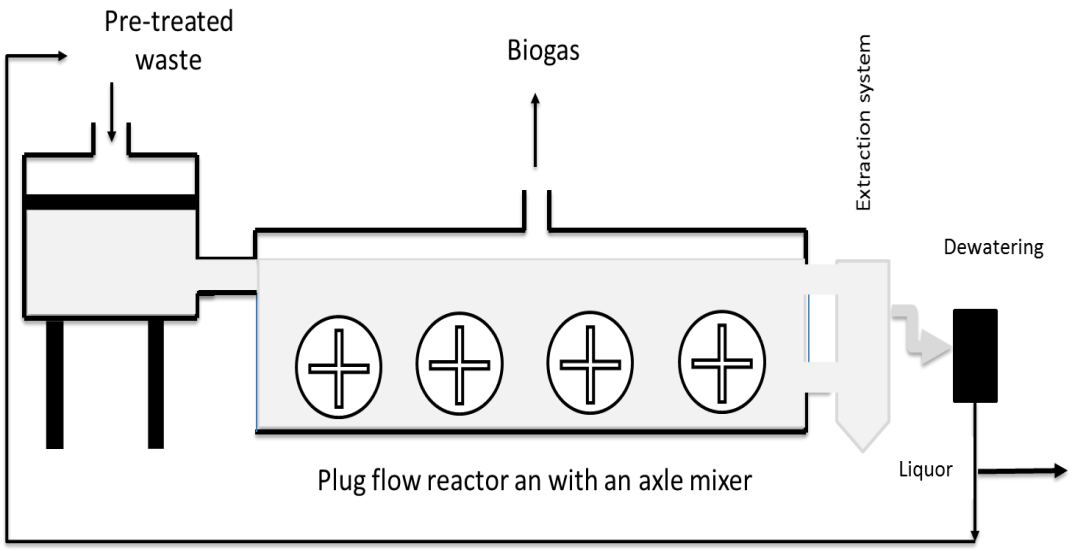
Pre-treated
waste
Biogas
Extraction system
Dewatering
Liquor
Plug flow reactor an with an axle mixer

Fig. 6.

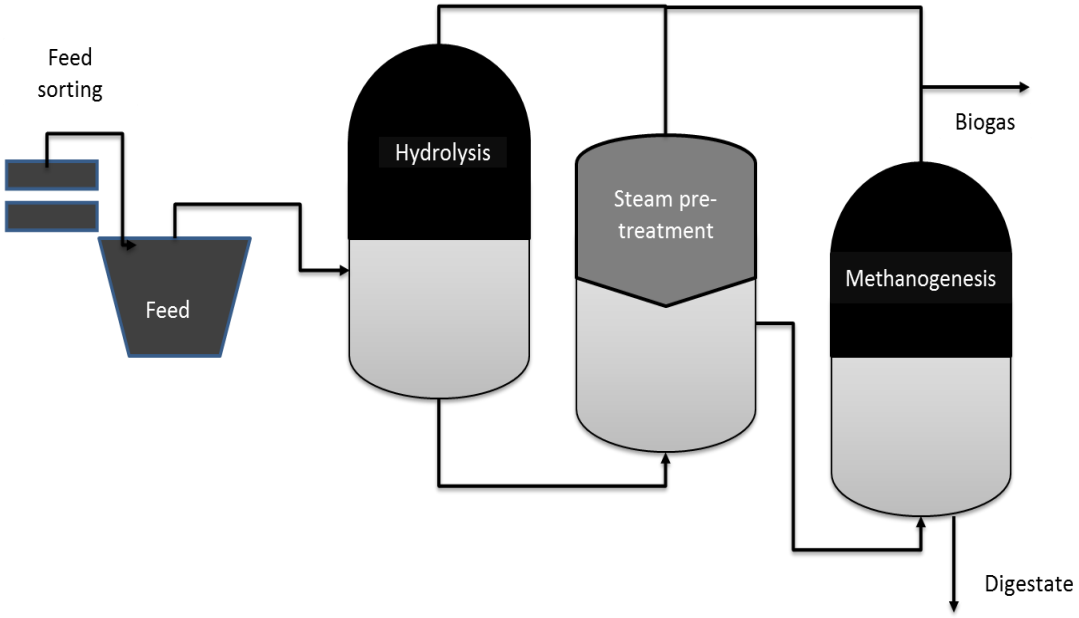
Feed
sorting
Feed
Hydrolysis
Steam pre-
treatment
Biogas
Methanogenesis
Digestate

Fig.7.

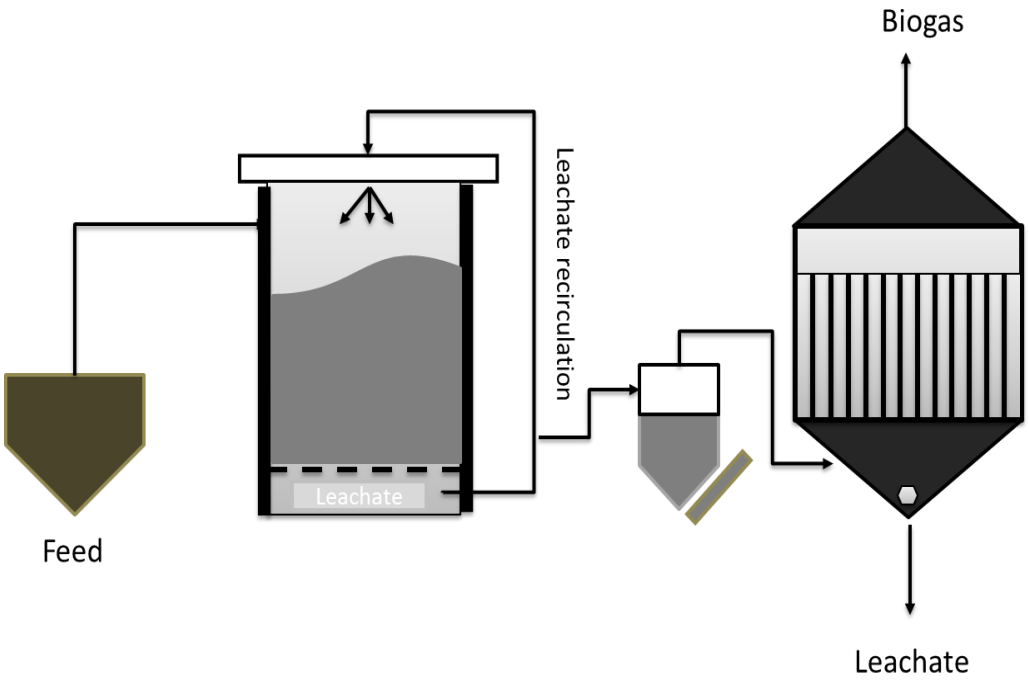

Fig.8.

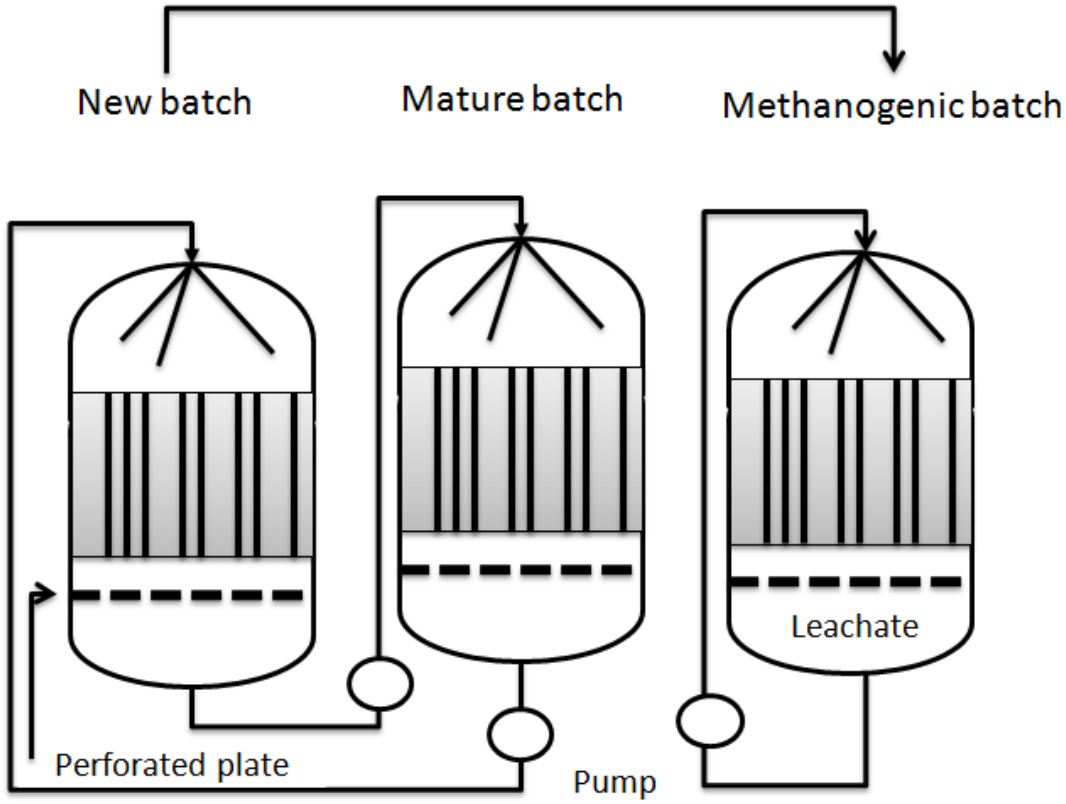
New batch
Mature batch
Methanogenic batch
Leachate
Perforated plate
Pump

Fig 9.

4. Paper II

The role of biochar in optimizing anaerobic digestion processes

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Abstract

Biochar, like most other adsorbents, is a carbonaceous material, which is formed from the combustion of plant materials, in low-zero oxygen conditions and results in a material, which has the capacity to sorb chemicals onto its surfaces. Currently, research is being carried out to investigate the relevance of biochar in improving the soil ecosystem, digestate quality and most recently the anaerobic digestion process. Anaerobic digestion (AD) of organic substrates provides both a sustainable source of energy and a digestate with the potential to enhance plant growth and soil health. In order to ensure that these benefits are realised, the anaerobic digestion system must be optimised for process stability and high nutrient retention capacity in the digestate produced. Substrate-induced inhibition is a major issue, which can disrupt the stable functioning of the AD system reducing microbial breakdown of the organic waste and formation of methane, which in turn reduces energy output. Likewise, the spreading of digestate on land can often result in nutrient loss, surface runoff and leaching. This review will examine substrate inhibition and their impact on anaerobic digestion, nutrient leaching and their environmental implications, the properties and functionality of biochar material in counteracting these challenges'.

Keywords: biochar; inhibition; nutrient leaching; digestate; anaerobic digestion

1. Introduction

The number of anaerobic digestion (AD) systems has increased rapidly because of various factors including financial incentives for renewable energy facilities, governmental policies on climate change, landfill and an increasing energy need (Zglobisz et al., 2010; Klavon et al., 2013). Currently, in Europe and Asia, there are over 30 million large and small-scale anaerobic digesters for both commercial and domestic applications (Chen et al., 2010; De Baere, 2010; Donoso-Bravo et al., 2011; Ferrer et al., 2011). AD is the stepwise breakdown of an organic substrate by a consortium of mutually dependent groups of microorganisms (Fig 1). If the correct conditions are maintained, the AD process will be stable with high energy recovery (Dechrugsa et al., 2013). However, the technology still faces two major challenges: (i) operational instability and (ii) the quality of the digestate produced.

Organic substrate selection plays an important role in the stability of an AD system as some feedstocks can have inhibitory effects on AD processes. Substrate-induced inhibition (SII) in AD can occur when the constituent fraction(s) or metabolic intermediate product(s) from organic substrates inhibit microbial activity. These forms of inhibition have been reported for organic substrates containing high amounts of protein, lipids, limonene, furans, metals, pesticides, antibiotics and other organic compounds (El-Gohary et al., 1986; Palmqvist & Hahn-Hagerdal, 2000; Lallai et al., 2002; Wilkins et al., 2007; Alvarez et al., 2010; Sousa et al., 2013; Yangin-Gomec & Ozturk, 2013). Individual feedstocks such as livestock manure, abattoir wastewater, citrus peel waste, fat and oil, are often avoided because they can result in SII either through the direct addition of inhibitory compounds, such as limonene, or indirectly through the production of inhibitory intermediates, such as ammonium and hydrogen sulphide from protein (Fig 2). In most cases, AD operators prefer co-digestion of two

or more substrates in order to reduce possible inhibition that might result from the treatment of individual feed-stocks (Jung et al., 2000; Wilson et al., 2008; González-Fernández et al., 2013; Cheng & Zhong, 2014). Microbial adaptation and co-digestion with two or more substrates are commonly used to reduce inhibition (El-Mashad & Zhang, 2010; Zhang & Jahng, 2012). During microbial adaptation, the inhibitor can be transformed into metabolites with a similar or lower level of toxicity while the application of co-digestion reduces the concentration of the inhibitor by increasing the ratio of the co-substrate (Athanasoulia et al., 2014). However, an alternative approach to reducing inhibition in AD is to remove or reduce the mobility/bioavailability of the inhibitors without affecting with the AD process.

Another major concern with AD is how to retain the nutritive value of the digestate before and after application to land (Mihoubi, 2004; Mangwandi et al., 2013). In most cases, digestate has a high moisture content and in an attempt to reduce this, phase separating equipment is utilised. According to Vaneeckhaute et al. (2013), 43% of the total nitrogen (N) and 25% of the total phosphorus (P) will be lost if the liquid fraction of pig slurry digestate is separated. Further nutrient and metal losses can occur during and after the spreading of the digestate on farmland via transfer to the surrounding watercourses or to the atmosphere. The volatilization of ammonium, leading to ammonia emission, and the leaching of heavy metal as diffuse pollution, are examples of losses that have a negative impact on the environment and crops (Svoboda et al., 2013; Page et al., 2014). Nutrient recovery from digestate has been considered as an option to reduce the nutrient loss from the digestate. However, this approach might reduce the economic value of the digestate (Verstraete et al., 2009; Batstone et al.,2015).

A better approach may be to focus on increasing the nutrient retention capacity of the digestate material. There is a growing interest in the use of biochar in AD to both

increase the recovery rate of the process during SII and decrease the nutrient loss before and after land application (Mumme et al., 2014; Dicke et al., 2015). This will potentially increase the operation of mono-substrate AD, which is often used by single substrate onsite AD operators, increase nutrient availability during digestate application to land and reduce the environmental implications of diffuse pollution and nutrient leaching. This review examines substrate-induced inhibition and its impact on anaerobic digestion, nutrient leaching and its environmental implications, and the properties and functionality of biochar material in counteracting these challenges.

2. The Challenges with anaerobic digestion of organic susbtrate

AD is the breakdown of complex organic material under anoxic conditions by a consortia of microorganisms via a multistep process (Fig 1) (Chen et al., 2008). The microorganisms that drive AD are divided into two groups: (i) acid producers (acidogens and acetogens) and (ii) methane producers (methanogens). These two groups of microorganisms differ physiologically and have different growth rates and sensitivities to operational conditions (Ruiz & Flotats, 2014). The inability to maintain a population balance between these two groups of microorganisms often results in AD process failure. If conditions such as temperature, hydrogen partial pressure, pH and organic loading rate are favourable for both microbial populations, the AD process should be stable (Rudolfs & Amberg, 1952).

In addition to the controls exerted by the operating conditions, the stability of the AD system can also be disrupted if metabolic intermediates of a substrate are inhibitory to microbial activity (Palmqvist & Hahn-Hagerdal, 2000; Wilkins et al., 2007; Sousa et al., 2013; Yangin-Gomec & Ozturk, 2013). This form of instability is substrate-induced and is called substrate-induced inhibition (SII). According to Ruiz

and Flotats (2014), a chemical or metabolite can be termed inhibitory when it causes a shift in microbial population or inhibits microbial activity. There is a wide variety of biodegradable organic materials that have been classified as inhibitory to microbial growth, particularly at higher concentrations (Fig 2 and Table 1). SII can be classified into two categories, direct and indirect sources of inhibition. Direct inhibitors are those that are supplied directly from substrates in the feedstock whilst indirect inhibitors are metabolic intermediates produced during the AD process (Fig 2). The following sections (2.1 and 2.2) describe the types of direct and indirect inhibitors commonly associated with AD and the mechanisms by which inhibition occurs.

2.1. Direct inhibition

As mentioned earlier, direct inhibition in AD results from a constituent of the organic substrate; this implies that the compound is readily available to the microbial cells, thus increasing the risk of AD process failure. The indirect inhibitors are not released until after hydrolysis-acidogenesis and thus they do not pose an immediate threat to the AD process. Figure 2 presents examples of direct SII, their effects and counteracting measures. Example of direct inhibitors include limonene from citrus peel, furans hydrolysate from the chemical pre-treatment of lignocellulose materials, azo-dye from textile production, antibiotics and pesticides. Limonene occurs naturally in citrus peel and reports show that the compound can inhibit the AD process at concentrations of 65-88 g l^{-1} (Mizuki et al., 1990). Even after the extraction of limonene prior to AD, studies have shown that inhibition of the AD process occurred, particularly when the organic loading rate (OLR) was increased from 3.67-5.10 gVS l^{-1} d^{-1} (Martin et al., 2010; Wikandari et al., 2015). In addition, the co-digestion of orange peel and sewage sludge (70:30) resulted in a methane yield of 0.165 l gVS^{-1}_{added} and the accumulation of volatile

fatty acids when the OLR was above 1.6 gVS l^{-1} d^{-1} (Serrano et al., 2014). Likewise, furans (furfural, hydroxymethylfurfural (5-HMF)) are produced during the dehydration of pentose- and hexose-sugars locked within the lignin structure (Barakat et al., 2012). These are metabolites from the hydrolysis of lignin but because they are not produced because of the microbial interaction, they are considered to be directly inhibitory. There are indications that the furans are not inhibitory and can be utilised for methane production at a concentration of less than 25 mM (Rivard & Grohmann, 1991; Belay et al., 1997). According to Barakat et al. (2012), the 5-HMF is more inhibitory than the furfural compound because, after incubation of 1 g l^{-1} of the compounds with 2 g l^{-1} of xylose separately, methane values of 533 and 583 ml/g were recorded, respectively. Similarly, Monlau et al. (2013) observed that the AD process was severely inhibited at 5-HMF concentration, which was above 6 g l^{-1}. Other direct inhibitors are antibiotics and pesticides, which are present in industrial and pharmaceutical wastewater (Lin, 1990; Ji et al., 2013). Antibiotics such as amoxicillin (160 mg l^{-1}), trihydrate (60 mg l^{-1}), oxytetracycline (120 mg l^{-1}) and thiamphenicol (80 mg l^{-1}) have been used to treat pigs and reports show partial inhibition to AD (Lallai et al., 2002). Ji et al. (2013) showed acute toxicity of four antibiotics in the order amoxicillin (399 mg l^{-1}), lincomycin (432 mg l^{-1}), kanamycin (511 mg l^{-1}) and ciprofloxacin (563 mg l^{-1}). A noticeable trend common to all direct inhibitors is the similarities in the mechanisms of inhibition. These compounds inhibit the growth of microbial cells as follows: (i) diffusing through the cell membrane; (ii) increasing the surface area of the cell membrane, and (iii) causing leakage of the contents of the microbial cell (Sikkema et al., 1995; Griffin et al., 1999; Fisher & Phillips, 2008).

2.2. Indirect inhibition

Indirect inhibition is displayed when metabolic intermediates are produced at high concentrations during the AD thereby inhibiting microbial activity. They have been reported to suppress microbial activity and reduce methane production. Figure 2 presents examples of indirect SII, their effects and counteracting measures. Metabolic intermediate products are generally produced during the AD process and they depend on the constituent of the substrate (Figure 1). Metabolic products such as acetic acid, hydrogen and carbon dioxide are essential to the AD process and are used to produce methane (Madsen et al., 2011). However, intermediates such as long chain fatty acid, ammonia (NH_3) and ammonium (NH_4^+) are examples of indirect inhibitors. Researchers have shown that free ammonia is more toxic than ammonium nitrogen because of its ability to penetrate the cell membrane (Gallert & Winter, 1997; Sung & Liu, 2003). According to Zeshan et al. (2012), an increase in the C/N ratio of the feedstock can minimise the possible effect of high protein feedstock because the addition of carbon will reduce the concentration of nitrogen rich material and also provide alternative metabolic routes thereby reducing the production of NH_4^+. They recorded a 30% reduction in the NH_3 content of the digestate and 50-73% surplus energy when the C/N ratio of the feedstock was increased to 32. Yangin-Gomec and Ozturk (2013) achieved a 1.2 fold increase in the methane yield when maize silage was co-digested with chicken and cattle manure to suppress ammonia toxicity. As mentioned earlier, protein is essential for microbial growth but at a high concentration, it will increase the possibility of ammonia toxicity. Ammonia is beneficial to the growth of anaerobic bacteria as long as it does not exceed a certain concentration that can be toxic to methangenic activity (Angelidaki and Ahring, 1994). Similarly, a substrate high in lipid produces a higher concentration of long-chain fatty acids (LCFAs) and glycerol during hydrolysis. LCFAs (e.g. oleate, stearate and palmitate) can be converted into hydrogen and acetate through

the β-oxidation pathway (Alves et al., 2009). According to Sousa et al. (2013), methanogens can be inhibited by LCFAs at concentrations between of 0.3 and 1 mM. Like LCFAs, the mechanisms of suppression of microbial activity during indirect inhibition are similar (i) diffusing through the cell membrane; (ii) inhibiting methane producing enzymes, and (iii) causing proton imbalance and potassium deficiency (Rinzema et al., 1994; Gallert & Winter, 1997; Chen et al., 2008; Rajagopal et al., 2013; Zonta et al., 2013).

2.3. Acclimation of microbial cells to inhibition

The mechanisms of direct and indirect inhibition are not similar; a general model illustrating the various mechanisms of attack (cell membrane disorder, interference with fermentative pathway and intracellular swelling/leakage) of the microbial cell is represented in Figure 3. SII cannot be avoided during the operation of AD systems, but to some extent the ability of microorganisms to adapt to unfavourable conditions can alleviate the effects of SII. Acclimation is the adaptation of microbial populations to changes in conditions and can be achieved in different ways: (i) synthesis of specific enzymes which were absent prior to exposure to the inhibiting condition; (ii) emergence of new metabolic capabilities/pathway, and (iii) modification of the surface layer of the microbial cell membrane (Liebert et al., 1991; Ruiz & Flotats, 2014). An example of modification of the surface layer of a cell membrane was observed during the exposure of microbial cells to a high dose of limonene; this resulted in increases in the concentration of unsaturated fatty acids in the cell membrane (Ruiz & Flotats, 2014). Another example has been reported where methanogens were exposed to 2 g l^{-1} of ammonia and, following a subsequent increase in the concentration of ammonia to 11 g l^{-1}, no inhibition was recorded (Koster & Lettinga, 1988; Borja et al., 1996a). This

implies that the microbial cells were able adapt to the unfavourable conditions and further suggests that AD operators should only inoculate their plant with inoculum from an active AD system using a similar substrate. Quintero et al. (2012) showed that the hydrolysis of lignocellulose was more efficient when the feedstock was inoculated with microflora from cattle rumens rather than pig manure. Likewise, Van Velsen (1979) showed that the microbial community in the pig manure inoculum acclimated to 2.4 g l^{-1} of NH_4^+ while the digested sewage sludge acclimation rate was limited to 1.8 g l^{-1} of NH_4^+.

3. Nutrient loss and environmental pollution

In order to keep up with the increasing demand for food production, soil fertility is maintained by adding fertilizers (Qin et al., 2015). The spreading of anaerobic digestate and compost material on farmland has increased and has become a method of complimenting or replacing synthetic fertilizer usage. In addition, this is driven by changes in agricultural practices and policies that focus on reducing climate change and improving soil quality (Qin et al., 2015; Stoate, 2009; Riding et al., 2015). Anaerobic digestate is rich in minerals, biomass, nitrogen, phosphorus and carbon which are essential for maintaining the soil ecosystem and sustaining increased plant growth (Montemurro et al., 2010; Tambone et al., 2010). In a study carried out by Alburquerque et al. (2012), the effect of digestate on horticulture crop production showed that the application of digestate provided a short term source of phosphorus and nitrogen and the microbial biomass and enzyme activities were relatively higher than the non-amended soil. Despite the benefits of utilizing digestate, the risk of atmospheric and water pollution following the application of digestate to land are high (Tiwary et al., 2015). This problem is particular to digestate because of the fast release of nutrients,

which is often beyond the utilization rate of the plants and soil microorganisms, thus making leaching and nutrient loss unavoidable. Unlike the digestate, the nutrient content of the inorganic fertilizer is slowly released into the environment, thus reducing the possibility of leaching in relation to organic fertilizers (Basso & Ritchie, 2005; Kim et al., 2014). Digestates with high concentrations of inorganic N are of particular concern due to the high potential for volatilization of NH_3 (Fernandes et al., 2012). Reports have shown that N losses are also significant during the processing of digestate with up to 85% of the NH_4^+ content emitted as NH_3 gas (ApSimon et al., 1987; Rehl and Müller, 2011). NH_3 is recognised as a major contributor to nitrous oxide (N_2O) production, a biological process carried out by ammonia-oxidizing bacteria (Law et al., 2013). The N_2O is formed as an intermediate product between nitrification and de-nitrification. The microorganisms first convert NH_3 into hydroxylamine (NH_2OH), then into nitrite (NO_2^-) and finally into N_2O. N_2O is an important atmospheric gas but at high concentrations it contributes to the formation of acid rain and thinning of the ozone layer (Badr & Probert, 1993). Tiwary et al. (2015) reported that the emission of NH_3 may be reduced by 85% if the digestate is introduced into the subsoil but the emission of N_2O is inevitable and it was found to be 2% higher than the other assays because of the contribution of the subsurface denitrifying microorganisms. Another route for nutrient loss from digestate applied to soil is diffuse pollution. Nutrient leaching from the digestate can result in the transfer of N and P to water bodies causing eutrophication (Anthonisen et al., 1976; Soaresa et al., 2011). Eutrophication itself is a process whereby an ecosystem is transformed through nutrient enrichment from an external source (Conley et al., 2009). Following the increase in nutrients, the growth of certain organisms such as algae, photosynthetic and heterotrophic bacteria increases, thus raising demand for resources which were present during the influx of the external

enrichment resources (O'Sullivan, 1995). Accelerated eutrophication of aquatic ecosystems owing to nitrogen and phosphorus enrichment has been reported to have a negative impact on the aquatic life. Firstly, light penetration into the littoral zone is limited thus inhibiting the growth of plant and predators that depend on light for survival; dissolved inorganic carbon is depleted and the alkalinity of the water increases (Lansing et al., 2008). Secondly, after depletion of the nutrients, the algal boom dies and microbial decomposition of the algal biomass depletes the dissolved oxygen, thus creating an anoxic or dead zone (Nagamani & Ramasamy, 1999). In addition, the proliferation of pathogens such as *Ribeiroia ondatrae,* which infects birds, snails and amphibian lava causing limb deformation has also been reported in the literature (Johnson et al., 2007). Apart from nutrients, digestate may also contain metals, particularly heavy metals (Ni, Zn, Cu, Pb, Cr, Cd, and Hg) in varying concentrations (Demirel et al., 2013). Digested sewage sludge is an example of feedstock with high heavy metal concentrations; this places a limitation on its land application (Wang et al., 2005). In Guangzhou, China, the concentrations of heavy metals in wet sludge samples were 4567±143, 81.2±2.8, 148±6, 121±4, 785±32 and 5.99±0.18 $mg \cdot kg^{-1}$ DM for Cu, Pb, Ni, Cr, Zn and Cd, respectively (Liu & Sun, 2013). Comparing these values with the PAS 110 upper limit standards, which were set at 200, 200, 50, 100, 400 and 1.5 $mg \cdot kg^{-1}{}_{DM}$, only the concentrations of Pb and Zn were below the standard thresholds. German sewage sludge recorded 202, 5, 131, 349, 53 and 1446 mg kg^{-1} DM for Pb, Cd, Cr, Cu, Ni, Zn and only copper and nickel were below the standard thresholds (Benckiser & Simarmata, 1994). Amongst the prevalent heavy metals in sewage sludge, Cr, Ni, Cd and Pb have been considered as the most toxic elements in the environment (Lei et al., 2010). When applied to farmland, high levels of these metals in soil can lead to phytotoxicity, which ultimately ends up in the human diet through crop uptake (Islam

et al., 2014). The ingestion of heavy metals is associated with health risks and reports show that countries like Bangladesh have high levels of Pb and As in their cereals and pulses (Islam et al., 2014). However, in developed countries, such as the UK, PAS 110 sets a threshold standard for heavy metal concentration in digestate and for operators who cannot meet this standard the digestate resource cannot be spread on farmland.

4. Optimizing the AD process: the use of adsorbent

As mentioned earlier, inhibition in AD has been counteracted with numerous approaches ranging from the acclimation of bacterial cells, adopting thermophilic operating conditions and reducing the concentration of the inhibitors either by dilution or co-digestion with other substrates (Table 1). These approaches do not remove the inhibitor from the process, which can result in accumulation of the inhibitor and the eventual destabilization of the AD system. It is beneficial to look for methods that remove, reduce the mobility or bioavailability the inhibitor within the digestion process (Chen et al., 2008). An example of a technique that can be used to remove potential inhibitors is the steam distillation of citrus peel to remove limonene prior to AD (Martin et al., 2010). Air stripping and chemical precipitation have also been used to remove NH_3 and toxic heavy metals, respectively (Chen et al., 2008). There is the possibility that carbonaceous sorbents could also be used to remove contaminants or toxic compounds. This approach is currently employed by industries involved in food, beverage and textile production and by water companies (Borja et al., 1996b; Palatsi et al., 2012). The use of adsorbents such as bentonite, activated carbon and zeolites in AD has been investigated and the removal of inhibitors has been observed (Angelidaki & Ahring, 1992; Milan et al., 2003; Bertin et al., 2004; Mumme et al., 2014). Adsorbents are chemically inert materials with adhesive properties that cause the accumulation of

atoms, ions or molecules on their surface. This is a surface based interaction between a solid and a fluid; the rate of sorption depends on the adsorbent (the material used as the adsorbing phase) and the adsorbate (the material being adsorbed). There are different types of adsorbent with a variety of applications; some are synthetic whilst others are made from agricultural residues or modified plant and animal material (Angelidaki & Ahring, 1992; Milan et al., 2003; Bertin et al., 2004; Mumme et al., 2014). Biochar is an example of adsorbent made from agricultural residues and because it relatively cheaper to adsorbents like activated carbon, zeolite, and its application is gradually increasing. The subsequent subheading will be focusing on different adsorption mechanisms of the biochar material.

4.1. Mechanisms of biochar adsorption

Adsorption is a dynamic process where the adsorbate associates with the surface of the adsorbent until an equilibrium state is achieved. The process of adsorption can be achieved by (i) adsorbate settling on the surface of the adsorbent (physical adsorption), (ii) adsorbate forming layers on the surface of the adsorbent (surface precipitation and complexation), (iii) condensation of the adsorbate into the pores of the adsorbent (pore filling), hydrogen bonding, electrostatic attraction, ion exchange and hydrophobic effect (Pignatello, 2011). This process occurs in stages: the clean zone (no adsorption), the mass transfer zone (adsorption in progress) and the exhausted zone (equilibrium), (De Ridder, 2012). Furthermore, the saturated and clean zones will increase and decrease respectively but the mass transfer zone will remain unchanged unless the concentration of the adsorbate is increased. When the material is passed through the adsorbent, it associates with the first section of the adsorbent before moving to another section. This trend continues until the adsorbent is nearly saturated; the near saturation point is called

the breakthrough point (Moreno-Castilla, 2004). This is the equilibrium state of the adsorbent. Figure 4 represents the breakthrough curve in a fixed bed column reactor; the adsorption rate is plotted against time. The adsorbate associates with the adsorbent until the available sites are unable to hold additional adsorbate and consequently, the concentration of the adsorbate on the outer surface of the adsorbent (D) begins to rise rapidly, At this point, the so called "breakthrough point" has been reached. However, the breakthrough point time varies with different adsorbates and is influenced by various operating parameters. The relationship between the adsorbate, adsorbent and operating parameters is usually described through isotherms, as a function of concentration, temperature and other parameters. According to Allen et al. (2004), the equilibrium state or breaking point during adsorption is dependent on the solute concentration, temperature and other factors. The modified Freundlich, Langmuir and Redlich-Peterson model is often used to describe the adsorption isotherm of an adsorbent (Table 2).

Figure 5 shows the mechanisms of adsorption of organic and metal adsorbates. For metals adsorption largely occurs through electrostatic attraction, ion-exchange and precipitation onto the surface of the adsorbent. For organic molecules, important mechanisms are hydrophobic interactions and hydrogen bonding (Figure 5). Another mode of adsorption that is common for organic compounds is the van de Waals force of attraction. This form of adsorption is induced by the surface chemistry of the biochar. Brennan et al. (2001) showed evidence of different functional groups such as nitro, chloro, hydroxyl, amine, carbonyl, and carboxylic on the surface of biochar. This form of sorption can be described as the electron donor-acceptor mechanism (Mattson et al., 1969). The uneven distribution of electrons between the adsorbent functional group and the organic compound creates an electron donor-acceptor relationship. However, for

complex organic compounds with substituent groups (nitro- and chloro-) the electron density of the interaction between the compound and the adsorbent is greatly reduced and this increases the electrostatic interaction between them (Cozzi et al., 1993). This is because the substituent group in the compound is a stronger electron acceptor (Dubinin, 1960; Liu et al., 2010).

4.2. Controls on biochar adsorption processes

The factors that influence the performance of adsorbent during adsorption have been extensively reported in literature. These parameters include the contact time, operating temperature, adsorbent and adsorbate dosage, particle size and pore distribution, surface chemistry and pH (Li et al., 2014; Hadi et al., 2015; Yargicoglu et al., 2015).

4.2.1. Structure and pore size

Adsorbent materials contain pores of various sizes, which have been categorised into micropores mesopores and macropores. Based on the size of the various pores, the sorption rate of the adsorbate is expected to increase in this order: macropores > mesopores > micropores, although this also depends on the size of the adsorbate (Zabaniotou et al., 2008). Biochar material has been reported to have an abundance of micropores, which have a high capacity for adsorbate and water uptake (Zabaniotou et al., 2008). As mentioned earlier, the size of the adsorbate also has some effect on the rate of sorption (Duku et al., 2011). For example, if the size of the adsorbate is relatively large or there are fewer sites for diffusion, this might be affected by steric hinderance (Liu et al., 2010). Further, large adsorbate size can cause exclusion or blockage of smaller sorption sites (Duku et al., 2011). Studies have shown that smaller particle sizes

reduce the mass transfer limitation and increase the van der Waal or electrostatic force for penetration of the adsorbate inside the adsorbent (Daifullah & Girgis, 1998).

4.2.2. Surface chemistry and pH

The functional groups on the surface of the biochar will influence the adsorption rate. For instance, biochars derived from sewage sludge and poultry manure have higher amounts of nitrogen and sulphur functional groups than woody biomass materials (Koutcheiko et al., 2007). Brennan et al. (2001) reported the presence of different functional groups on the surface and pores of biochar, including hydroxyl, amine and carboxylic groups. The surface chemistry of a carbonaceous sorbent can change, particularly when it is immersed in water; these changes are attributed to the chemical characteristics of the adsorbent (functional groups or ionic compound present in water) and the pH of the solution (Moreno-Castilla, 2004). As illustrated in Figure 6, at higher pHs, phenolic and carboxylic groups release protons and obtain a negative charge, while at low pH basic functional group, such as amine, take up a proton and obtain a positive charge (Schwarzenbach et al., 2005). This implies that the adsorption behaviour of asorbent is a function of the pH of the medium. Changes in the pH can have significant impacts on the ability of a material to adsorb certain compounds. For example, soluble mercury species can be easily adsorbed at higher pHs, whereas lowering the pH increases the solubility of mercuric ions (Eligwe et al., 1999). Changes in pH may also result in reductions in the electrostatic force between the adsorbate ions and the adsorbent (Rao et al., 2009).

4.2.3. Hydrophobicity

The presence and number of O- and N-containing functional groups determine the hydrophobic nature of biochars. Biochars with less O- and N-containing functional groups are typically less hydrophobic (Moreno-Castilla, 2004). Hydrophobic interactions are believed to contribute to the sorption of insoluble adsorbates (Moreno-Castilla, 2004). In aqueous solutions, the adsorbate with the least solubility has the tendency to be adsorbed and retained in the pore of the adsorbent. According to Li et al. (2003), removal of adsorbates, such as 2-propanol, is higher with β-zeolite than dealuminated β-zeolite because the latter is less hydrophobic. Equally, Li et al. (2002) showed that hydrophobic activated carbon is more effective in the removal of relatively polar methyl tertiary-butyl ether (MTBE) and relatively nonpolar trichloroethene (TCE). The hydrophilic adsorbents are less effective because of the sorption of water, which in turn reduces the available sites for the adsorbate-adsorbent interaction (Li et al., 2002).

4.3. Mechanisms of desorption or regeneration

Adsorbents are useful for separation applications, especially in the purification of wastewater and gaseous compounds. However, the progressive accumulation of adsorbate on the surface of the adsorbent will reduce its sorption capacity until the breakthrough point and finally equilibrium (Salvador et al., 2015). However, the regeneration of the adsorbent gives it an economical advantage over other separation methods and numerous regeneration methods have been developed (Lu et al., 2011; Martin & Ng, 1987; Salvador et al., 2015). Regeneration pathways involve the removal of the adsorbate from the adsorbent. These have been demonstrated using chemical

reagents, water, hot gases, ozone, superficial fluid, electric current and microorganisms (Salvador et al., 2015).

In AD the application of water in regeneration is not efficient because water is not a good solvent of organic material and in the process of regeneration the water is polluted with the contaminant. Chemical regeneration employs the use of reagents such as NaOH to remove contaminants, or to change the pH of the adsorbent so that non-reactive chemicals like aniline and dye can be desorbed (Leng & Pinto, 1996). However, chemical agents are expensive and the chances of environmental pollution are often high. Supercritical fluid regeneration employs a combination of pressurised CO_2 and water at 125-250 bar to desorb benzene, naphthalene and phenol from activated carbon (Chihara et al., 1997; Tan & Liou, 1989). However, this approach is energy intensive. Another approach called ozone (O_3) regeneration employs the O_3 in direct oxidation of the contaminant. The hydroxyl and oxygen radicals are very reactive and able to oxidize the contaminant. There are indications of moderate efficiency of 80-90% when O_3 is used because some of the oxidative product might block the pore sites (Alvárez' et al., 2009). Unlike the other regeneration methods mentioned earlier, the biological approach is the most economical and environmentally friendly because it employs the activities of microorganisms in the regeneration of the adsorbent. For instance, the biological activated carbon added to activate sludge in wastewater treatment improves the simultaneous sorption and biological degradation of the contaminant under aerated conditions (Xiaojian et al., 1991). Another approach to the microbial regeneration of an adsorbent is the inoculation of an exhausted adsorbent with microorganisms. This approach has been reported to be less effective because of the eventual blockage of the pore entrance by colonies of microorganisms. (Hutchinson & Robinson, 1990; Toh et al., 2013). Perhaps the application of water solvent as a backwash can be used to

supplement the microbial regeneration of exhausted adsorbent. Considering that the level of contamination from SII is relatively lower and less recalcitrant when compared to wastewater industries, biological regeneration could be easily achieved but this needs to be optimized with solvent backwash.

5. The role of biochar in anaerobic digestion

5.1. Biochar

Biochar is a soil additive produced from the thermal degradation of organic material in the presence of little or no amount of oxygen, a process known as pyrolysis (Shafizadeh, 1982). During pyrolysis the volatilization of the organic matter increases, the pore sizes enlarge and the structure of the biomass is rearranged (Lua et al. (2004). Factors such as biomass retention time, the properties of the biomass and the operational parameters (temperature, pressure and retention time) can influence the final structure of the biochar (Lua & Guo, 2000). Novakov (1984) describes biochar (or black carbon) as "combustion produced black particulate carbon having graphitic microstructure". Biochar is a carbonaceous, porous and carbon stable material but it is distinctly different because it is produced at a lower temperature (< 700 ^{0}C) without any form of activation (Schulz & Glaser, 2012). This makes the surface area of the biochar less efficient than that of the activated carbon but in terms of production cost, biochar is cheaper (Lehmann & Joseph, 2012). Biochar material is attracting attention as a means of improving plant growth and cleaning contaminated water and land (Tan et al., 2015). Apart from the direct benefits of plant growth and the cleaning-up of polluted ecosystems, biochar can serve as carbon storage, thus contributing to the mitigation of climate change (Montanarella & Lugato, 2013). Biochar material is stable and like other carbon capture technologies it can ensure long-term storage of carbon and reduced CO_2

emission (Woolf et al., 2010). The use of biochar as an adsorbent in AD has not been fully investigated as yet, but there is potential for it to have a positive impact both on the operational stability of the AD process and the quality of the digestate produced (Mumme et al., 2014). The continuous addition of biochar during AD can be suggested to reduce SII and increase process stability in three ways: (i) through the sorption of inhibitors, (ii) by increasing the buffering capacity of the system, and (iii) through immobilization of bacterial cells. In addition, the application of biochar can be extended to the improvement of digestate quality. The addition of biochar to digestate can contribute to nutrient retention, increase the carbon to nitrogen ratio and reduce nutrient leaching after land application of the digestate mixture (Figure 7).

5.2. Adsorption of inhibitors

Inhibitors, such as LCFA, ammonia, limonene, heavy metals and phenols, are either degraded or transformed into other metabolites and these metabolites can be as inhibitory as their precursors (Duetz et al., 2003). There is the opportunity for microbial acclimation to inhibitory compounds, but for most commercial operators there are cost implications of waiting for the whole consortia of cells to acclimate. The application of an adsorbent such as biochar creates an alternative route for removing and reducing the effect of SII during AD. This is because there are indications that biochar can sorb heavy metals and other organic compounds like pesticides, furfural and limonene (Kılıç et al., 2013; Taha et al., 2014; Hale et al., 2015). According to Komnitsas et al. (2015), 10 g l^{-1} biochar produced after pyrolysis at 550 ^{o}C was able to remove 15 mg l^{-1} of Cu and Pb with almost 100% removal efficiency. Likewise, biochar has been shown to sorb organic compounds. For instance, in the amendment of polycyclic aromatic hydrocarbons in sewage sludge, when compared to the expensive activated carbon

material, biochar does not have a greater effect with regard to sorption (Oleszczuk et al., 2012). Taghizadeh-Toosi et al. (2012) showed that biochar can adsorb NH_4^+ and remain stable in ambient air but on exposure to the soil the NH_4^+ is made bioavailable for plant uptake. In addition, a recent report by Chen et al. (2015) showed that biochar can also be deployed to contaminated fields because of its affinity for polycyclic aromatic hydrocarbons. The sorption capacity of biochar with different organic and inorganic materials has been extensively reported in the literature but with regard to most inhibitory compounds during AD it has not been well documented (Mohan et al., 2014). This may be attributed to the uncertainty surrounding the addition of biochar to AD systems. Adsorbents like biochar are not selective during sorption; hence, there is the possibility that some of the nutrients or useful metabolites will be adsorbed during the AD process (Mumme et al., 2014). This may not pose a major issue as a proportion of the material trapped within the pores of the adsorbent can be metabolised by the microorganisms attached to the adsorbent surface. In order to avoid nutrient or metabolite fouling of the biochar pores, the organic substrate can be pre-treated with the biochar before AD. However, this approach might limit the benefits of applying biochar with regard to the removal of only direct forms of SII.

5.3. Increasing buffering capacity

Alkalinity is a measure of the reactor's liquid capacity to neutralise acids, i.e. absorb hydrogen ions without a significant pH change. Alkalinity is produced in AD through the degradation of some feedstocks and alkalinity is lost through the production and accumulation of VFAs. The accumulation of acid is an expected occurrence during AD, but in the event of high organic overloading and microbial inhibition, the accumulation of VFA can reduce the buffering capacity of the system (Chen et al., 2008; Rétfalvi et

al., 2011). Nonetheless, the buffering capacity of an AD process can be increased or maintained by adding some alkali compounds or by controlling the OLR (Ward et al., 2008). A biochar material can be alkaline depending on the biomass source (Gul et al., 2015). Yuan et al. (2011) showed that the alkalinity of a biochar increases with an increase in the pyrolysis operating temperature. The application of biochar for the purpose of increasing the buffering capacity is not well known, but this could be recognised as one of the benefits of adding biochar to the AD process. For instance, most operators usually add lime to the AD system to combat acidification. However, the continuous addition of alkaline biochar could increase the buffering capacity of the system (Cao et al., 2012; Zhang et al., 2014). A study by Luo et al. (2015), which compared biochar and non-biochar incubation using glucose as a substrate, showed that the biochar containing incubation increased the methane yield by 86.6% and reduced acidification.

5.4. Immobilization of microbial cell

Immobilization refers to the colonization of microbial cells on the surface of a solid material. The conventional methods for the immobilization of microbial cells are the use of entrapments such as gels, and physical adsorption to a solid surface, but this approach is limiting because of poor mass transfer (Hori et al., 2015). The discovery of naturally occurring immobilized cells called biofilms has received more attention because it allows the colonization of microbial cells on polymerised surfaces (Cheng et al., 2010). The immobilization of microbial communities in AD is important, particularly for the methanogens because it facilitates electron transfer between interspecies (Lü et al., 2014). One of the benefits of cell immobilization is to reduce biomass washout, an occurrence that is common with wastewater treatment. Anaerobic

digesters such as fixed and fluidised beds have been designed with support media to increase and retain the growth of microbial cells (Fernandez et al., 2007). Another advantage of using an immobilized cell is the acclimation rate of the microbial cell during SII (Chen et al., 2008; Montalvo et al., 2012). Sawayama et al. (2004) compared dispersed and immobilised cells, and observed that the biomass and methane production rate of the immobilised cells were higher even at an ammonium concentration of less than 6000 mg/l. Furthermore, immobilization of microbial cells has also been reported to reduce the distance between syntrophic bacteria and methanogens. It has been reported that a distance of less than 1 µm is essential for the oxidation of volatile fatty acids and hydrogen production (Stams, 1994; Schink, 1997). Cell immobilization is often achieved when a bacterial cell is able to attach or grow on a support material. Support materials such as zeolite, clay, activated carbon and other plastic materials have been used to support microbial attachment and growth (Borja et al., 1993; Sawayama et al., 2004; Chauhan & Ogram, 2005; Bertin et al., 2010). The macropores aid the attachment of bacterial cells (Laine et al., 1991). Although, the application of biochar for cell immobilization is not as extensive as most other adsorbents, there is an indication that the macropores enhance the attachment of bacterial cells (Watanabe & Tanaka, 1999). Luo et al. (2015) observed the colonization of *Methanosarina* on biochar material during the AD of glucose solution and when compared to the non-biochar study, methane production was higher by 86.6%.

5.5. ***Nutrient retention***

The management of digestate is attracting attention currently because it contains useful amounts of micronutrients, ammonium, phosphate, metal and organic material, hence making it a good soil conditioner (Sapp et al., 2015). Using a circular economic

approach where food waste is returned to land as a resource reduces the dependency on inorganic fertilizer, improves the soil ecosystem and provides an alternative source of phosphorous, which is currently limited (Hendrix, 2012; Zeshan & Visvanathan, 2014). Depending on the characteristics of the organic substrate and the stability of the AD process, the nutrient content of the digestate will vary. However, as mentioned earlier, a major problem with spreading digestate on land is leaching as this causes diffuse pollution to watercourses or the emission of residual CH_4 and NH_3 gas into the environment (Menardo et al., 2011). In order to reduce diffuse pollution resulting from digestate application to land, the C/N ratio of the digestate must be adjusted and the season of application must be considered (Zeshan & Visvanathan, 2014). However, these approaches are not solely effective because of the slow rate of microbial processes in soil thus extending the chances for nutrient loss from applied digestate via leaching or changes in the soil conditions (Alburquerque et al., 2012). The addition of biochar during or after AD can potentially improve nutrient retention and reduce leaching of digestate nutrient.

Studies examining the interactions between biochar and digestate have shown that the addition of biochar to digestate before land application increases the retention period of the nutrients for plant and bacterial uptake (Marchetti & Castelli, 2013; Eykelbosh et al., 2014). Biochar material was found to allow the sorption of organic matter and inorganic nutrients (Lehmann & Joseph, 2012). The surface of biochar is complex with pores containing metallic and organic compounds; this property is essential in the sorption behaviour of biochar. Research has shown that biochar can adsorb organic substrates, phosphate, nitrate, nitrite, ammonium, metals and carbon dioxide (Bagreev et al., 2001). According to Koukouzas et al. (2007) some biochar material may contain metal oxides (MgO, CrO, CaO and Fe_2O_3) on its surface or pores

and this induces the adsorption of NH_4^+, thus reducing leaching and diffuse pollution (DeLuca et al., 2006). Le Leuch and Bandosz (2007) showed that the sorption of ammonium by biochar immobilizes the ammonium concentration in soil thus reducing the volatilization of ammonium to ammonia under alkaline conditions and during temperature changes within the soil. DeLuca et al. (2006), observed that ammonification reduction was higher in soil containing biochar and this would only have been possible due to the slow release of the ammonium compound. The advantage of this behaviour to the soil is that it immobilizes organic nitrogen within the pores and reduces nutrient loss during leaching thus making nutrients readily available over a longer term. An additional environmental benefit of nutrient sorption by biochar is the potential to mitigate the microbial production of N_2O following digestate application. Dicke et al. (2015) studied the effect of biochar material and digestate on N_2O fluxes under field conditions and showed that the addition of biochar reduced N_2O emissions, although the emission of N_2O was mostly influenced by temperature and precipitation. It could be argued that the higher specific surface area of the activated carbon is better than the biochar material thus making it a more reliable resource for microbial cell immobilization and the sorption of contaminants (Wang & Han, 2012). However, because biochar is cheaper to make there is no need to recover the material after the AD process and this will increase the value of the digestate.

6. Conclusions.

The application of biochar has the potential to improve AD process by counteracting SII, improve digestate quality through nutrient retention, contributing to the buffering capacity of the system and create a surface area for the colonization of microbial cell. Comparatively, these functions can be achieved by another adsorbent like activated carbon with higher efficiency. However, the production of biochar is cost effective hence AD operators can afford to use the material without any need for recovery and this will further encourage the spreading of biochar and digestate on land. Biochar was not primarily designed for AD, hence future research in the interaction between biochar and AD microbes, buffering capacity of biochar during AD and sorption effect of biochar material on the AD using a continuous-fed digestion process should be investigated.

Acknowledgements

This work was supported by the Centre for Global Eco-innovation (X02646PR) and Stopford Energy and Environment.

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Tables

Table 1: Inhibitors, their functions, effects and exiting counteracting methods

Inhibitor	Function	Inhibition	Counteracting methods
Heavy metals (Cu^{2+}, Zn^{2+}, Cr^{3+}, Cd, Ni, Pb^{4+}& Hg^{2+})	Part of essential enzymes and drives anaerobic enzymatic reactions Formation of complexes to form unspecific complex compounds (Nies, 1999)	The order of inhibition to the acetogens (Cu>Zn>Cr>Cd>Ni>Pb) and methanogens (Cd>Cu>Cr>Zn>Pb>Ni) (Lin, 1993)	Production of hydrogen sulphide to precipitate as metal sulphide (Gadd & Griffiths, 1977) Co-digestion with another substrate (Pahl et al., 2008) Retention of metal on the cell wall (Jankowska et al., 2006) Lowering permeability of the cell membrane (Jankowska et al., 2006)
Light metals (Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, and Al^{3+})	Required for microbial growth Enhances bacterial cell immobilization (Ca) (Thiele et al., 1990; van Langerak et al., 1998)Formation of adenosine phosphate (ADH) (Na^{+})(Dimroth & Thomer, 1989)	Restrict production of double cells (Mg^{2+}) Neutralize cell membrane potential (K^{+})(Jarrell et al., 1987) Inhibit acetoclastic methanogens (Na^{+}) Precipitation of carbonates and phosphates thus destabilizing the buffering system (Ca^{2+}) (van Langerak et al., 1998) Competition with adsorption of other metals (Al^{3+}) (Cabirol et al., 2003)	Acclimation of bacterial cell (Chen et al., 2008) Na^{+}, Mg^{2+} and NH_4^{+} mitigate potassium toxicity(Chen et al., 2008)
Volatile fatty acids (VFAs)	Methane production	Reduces pH at high concentration Acidity of the system	pH adjustment Reduce organic loading rate
Long-chain fatty acids (LCFAs)	-	Distorting the electron transport system in the cell membrane of the bacterial cell(Hanaki et al., 1981) Suspension of bacterial cell Contributes to foaming during interaction with filamentous microorganisms in an anaerobic condition(Ganidi et al., 2009)	Acclimation of bacterial cell(Rinzema et al., 1994) Co-digestion with lipid-free substrate
Limonene	-	Increases permeability of cell membrane and causes leakage of cell content (Burt, 2004)	Acclimation of bacterial cell Removal of essential oil Thermophilic operation

			Co-digestion with crude glycerol (Mizuki et al., 1990; Martin et al., 2010; Martín et al., 2013)
Lignocellulose hydrolysate	-	Inhibition of anaerobic digestion process (Furfural > 5-HMF>phenol) Damage of DNA Distortion of the glycolytic pathway (Palmqvist & Hahn-Hagerdal, 2000).	Acclimation of the bacterial cell (Palmqvist & Hahn-Hagerdal, 2000).
Sulfide $SO_4^{2-} + 4H_2 = H_2S + 4H_2O + 2OH^-$ $SO_4^{2-} + CH_3COOH = H_2S + 2HCO_3^-$	co-enzyme production, ferredoxin and other compounds(Khan & Trottier, 1978).	Compete with acetate users for acetate utilization Corrosion of pipes and engine Inhibition of methanogens Khan and Trottier (1978)	Acclimation of the bacterial cell Removal of sulphide (Song et al., 2001)
Inorganic nitrogen $NH_4^+ + OH^- \leftrightharpoons HCO_3^- + H_2O$ $CO_2^+ + H_2O + OH^- = HCO_3^- + H_2O$	Availability of nitrogen as nutrient (Liu & Sung, 2002)	Proton imbalance (NH_3-N) Inhibit methane producing enzymes (NH_4-N) Methane production will be inhibited Accumulation of VFAs	Bacterial cell immobilization (Sasaki et al., 2011) Acclimation of bacterial cell (Chen et al., 2008) pH adjustment (Angelidaki & Ahring, 1993) Addition of trace element (Banks et al., 2012) Dilution of feedstock (Kelleher et al., 2002) Adjustment of the C/N ratio (Resch et al., 2011)
Chlorophenols and Halogenated aliphatic	Reduction of pathogens	Interference with cellular energy transduction Disruptions of proton gradient through the cell membrane (Chen et al., 2008) Methanogens are greatly inhibited (Chen et al., 2014)	Removal of contaminant using activated carbon (Liu et al., 2010)
Pesticides and antibiotic	-	Inhibition of protein and cell Wall Synthesis Alteration of Cell Membranes Antimetabolite Activity(Neu, 1984)Inhibits methanogens (Alvarez et al., 2010; El-Gohary et al., 1986)	Removal of contaminant using biochar (Yao et al., 2013)

Table 2: Equilibrium model principles, limitation, equations, slope and intercept (Allen et al., 2004; Bhatt et al., 2012)

Model	Principle/limitations	Equation, slope and intercept
Langmuir Isotherm	Strong attraction between adsorbent and adsorbate	$q_e = \frac{Q_m K_L C_e}{1+K_L C_e}$ (Nonlinear)
	Specific sites number for solute adsorption	$\frac{C_e}{q_e} = \frac{1}{Q_m} C_e + \frac{1}{Q_m k_L}$ (Linear)
	Monolayer adsorption and homogenous sorption	Slope $= \frac{1}{Q_m}$
	Although the model ignores adsorbate/adsorbate interactions and does not account for adsorbent with rough surfaces (multiple sites)	Intercept$= \frac{1}{K_L Q_m}$
Modified Freundlich	Adsorption rate is directly proportional to concentration	$\frac{q_e}{C_e} = K_{mf} C_e^{\left(\frac{1}{n_{mf}}-1\right)}$ (Nonlinear)
	Adsorption rate is independent of concentration at high pressure	$In\left(\frac{C_e}{q_e}\right) = \text{In}\left(K_{mf}\right) + \left(\frac{1}{n_{mf}} - 1\right) In(C_e)$ (Linear)
	Although adsorption varies with concentration until the saturation point is reached	Slope $= \frac{1}{n_{mf}} - 1$
		Intercept $= InK_{mf}$
Redlich–Peterson	An hybrid of Langmuir Isotherm and Modified Freundlich model	$q_e = \frac{K_{R\,P}\, C_e}{1+\alpha\, C^{\beta}{}_e}$ (Nonlinear)
		$\text{In}\left[K_{R\,P}\ \ \frac{C_e}{q_e} - 1\right] = \beta\ \text{In}\ (C_e) + In(\alpha)$ (Linear)
		Slope $= \beta$
		Intercept$= In(\alpha)$

Tempkin isotherm	Heat of adsorbing adsorbate to adsorbent will reduce linearly with coverage	$q_e = \frac{RT}{B}\ In(AC_e)$	(Nonlinear)
	Suitable for Intermediate range of ionic concentration	$B = \frac{RT}{B}$	(Linear)
Toth isotherm	The adsorption energy across the sites of the adsorbent is lower than the maximum adsorption energy	$q_e = \frac{K_T\ C_e}{[a_T + \alpha\ C^t{}_e]^{\frac{1}{t}}}$	(Nonlinear)

Ce = concentration at equilibrium (mg L^{-1}); q_e = equilibrium adsorption capacity (mg g^{-1}); K_L = Langmuir adsorption constant (L mg^{-1}); Q_m = maximum adsorption capacity (mg g^{-1}); K_F = Freundlich constant (L g^{-1}); ^nF = heterogeneity factor of adsorption sites (dimensionless); K_{MF} = modified Freundlich constant (L g^{-1}); ^nMF = heterogeneity factor of the adsorbent sites (dimensionless); K_{RP} = constant that is varied to maximize the linear correlation coefficient (r2), (L g^{-1}); α = constant (mg L^{-1}); β = Redlich–Peterson exponent (dimensionless); KT= Toth isotherm constant (mg g^{-1}); t = Toth isotherm exponent; A = Tempkin isotherm constant; B = Tempkin isotherm energy constant (J mol^{-1}); R = Gas constant (J mol^{-1} K^{-1}); T= Temperature (K); n = Number of isotherm parameters; p = Number of data points, B = heat of adsorption; T = temperature (^{0}C); R = gas constant; t = time (hr)

Figure captions

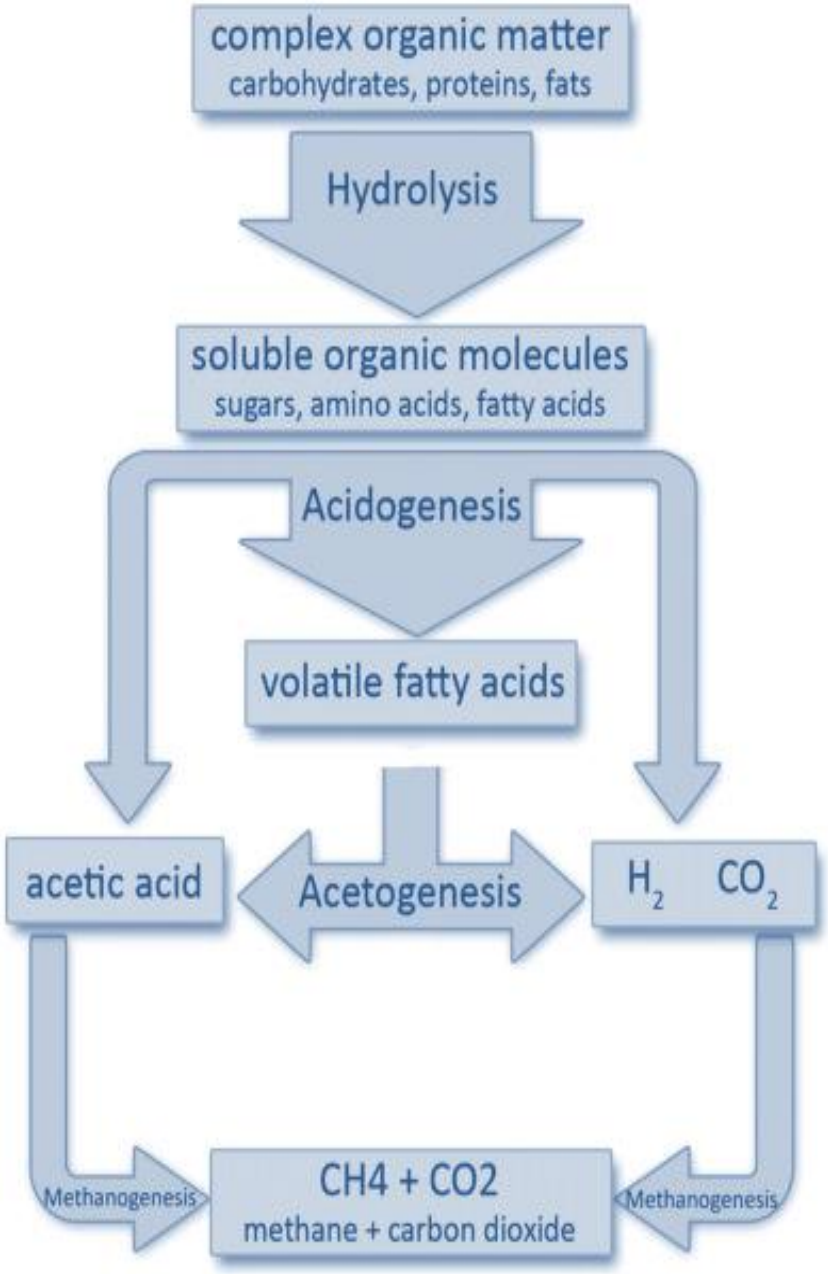
complex organic matter
carbohydrates, proteins, fats
Hydrolysis
soluble organic molecules
sugars, amino acids, fatty acids
Acidogenesis
volatile fatty acids
acetic acid
Acetogenesis
H2 CO2
Methanogenesis
CH4 + CO2
methane + carbon dioxide
Methanogenesis

Figure 1

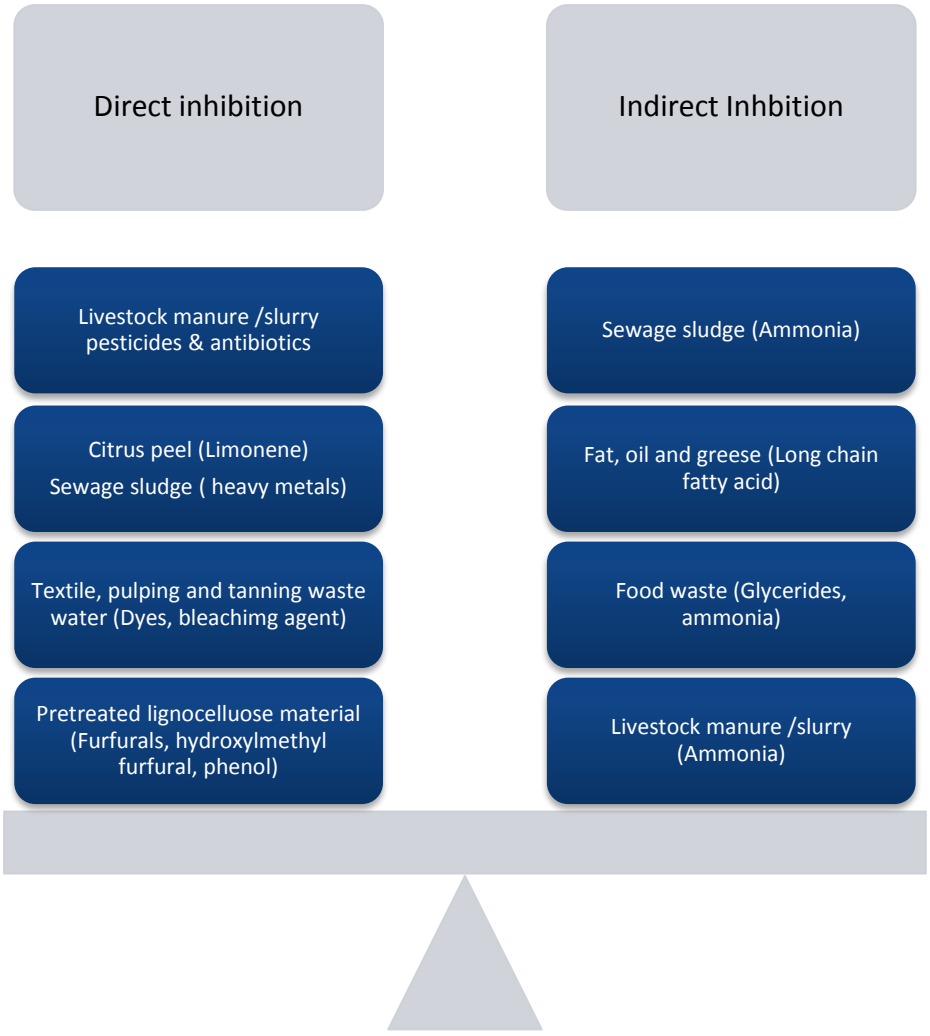
Direct inhibition
Indirect Inhbition
Livestock manure /slurry pesticides & antibiotics
Sewage sludge (Ammonia)
Citrus peel (Limonene)
Sewage sludge (heavy metals)
Fat, oil and greese (Long chain fatty acid)
Textile, pulping and tanning waste water (Dyes, bleachimg agent)
Food waste (Glycerides, ammonia)
Pretreated lignocelluose material (Furfurals, hydroxylmethyl furfural, phenol)
Livestock manure /slurry (Ammonia)

Figure 2

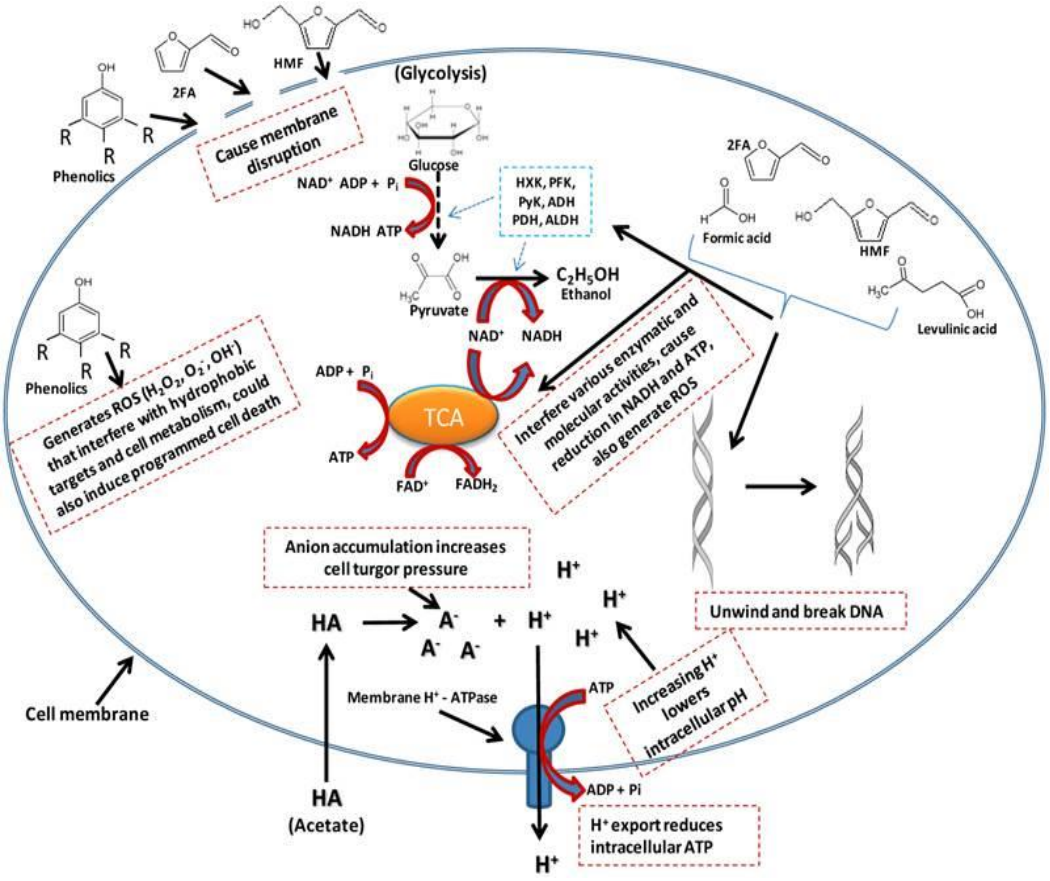
2FA
HMF
(Glycolysis)
Phenolics
Cause membrane disruption
Glucose
NAD+ ADP + Pi
NADH ATP
HXK, PFK, PyK, ADH PDH, ALDH
Pyruvate
C2H5OH
Ethanol
NAD+ NADH
Formic acid
Levulinic acid
Generates ROS (H2O2, O2-, OH-) that interfere with hydrophobic targets and cell metabolism, could also induce programmed cell death
ADP + Pi
TCA
ATP
FAD+ FADH2
Interfere various enzymatic and molecular activities, cause reduction in NADH and ATP, also generate ROS
Unwind and break DNA
Anion accumulation increases cell turgor pressure
H+
HA
A-
Membrane H+ - ATPase
Increasing H+ lowers intracellular pH
Cell membrane
HA
(Acetate)
ADP + Pi
H+ export reduces intracellular ATP

Figure 3

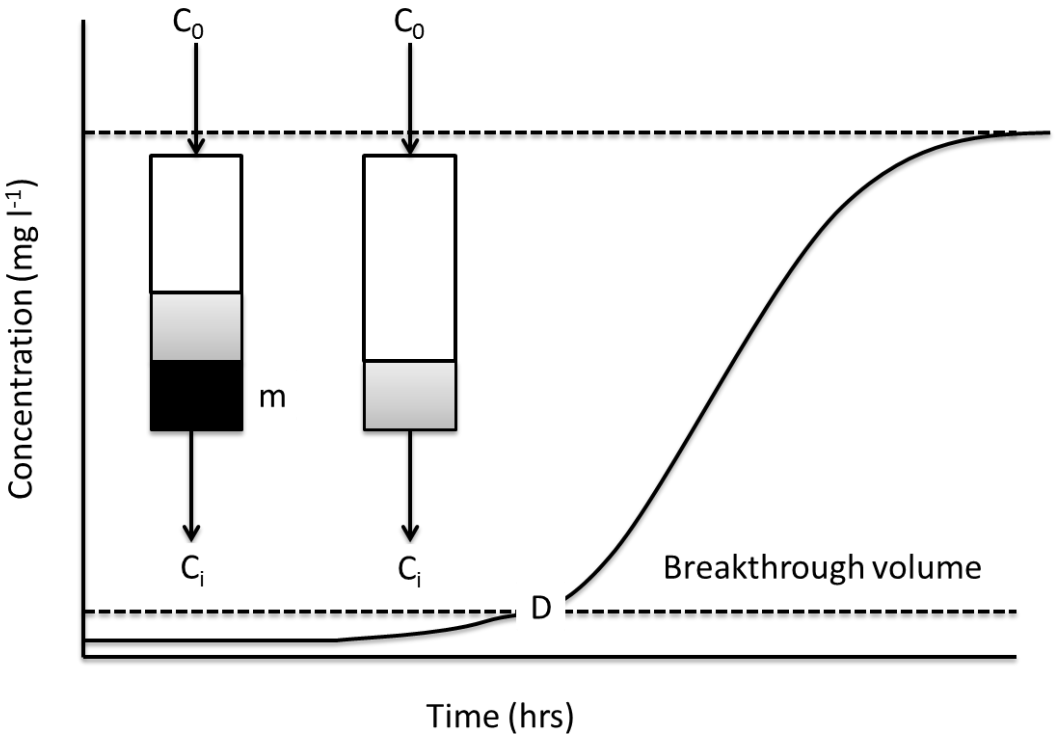
C0
C0
Concentration (mg l-1)
m
Ci
Ci
D
Breakthrough volume
Time (hrs)

Figure 4

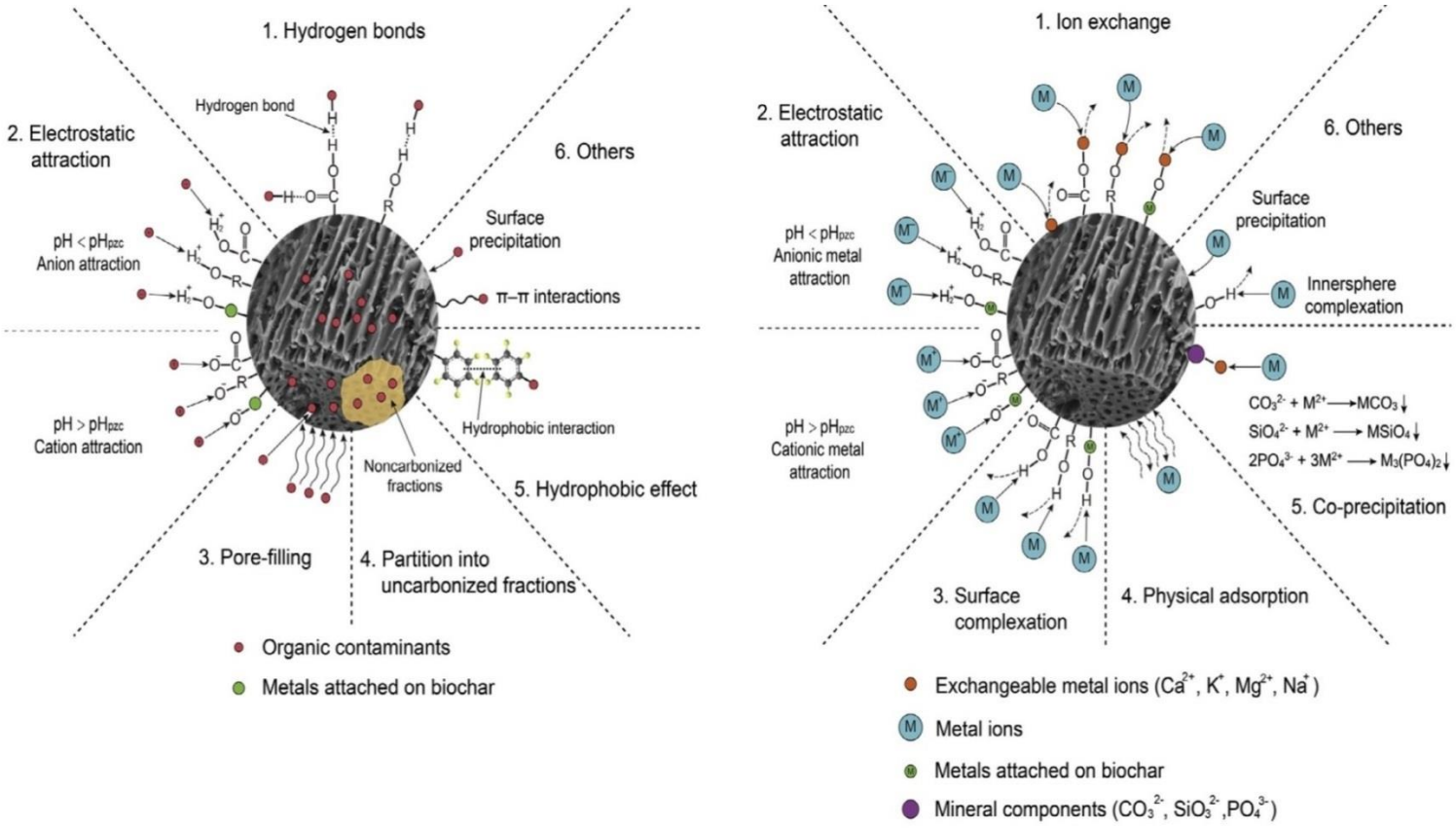
1. Hydrogen bonds
Hydrogen bond
2. Electrostatic attraction
6. Others
pH < pHpzc
Anion attraction
Surface precipitation
π–π interactions
pH > pHpzc
Cation attraction
Hydrophobic interaction
Noncarbonized fractions
5. Hydrophobic effect
3. Pore-filling
4. Partition into uncarbonized fractions
Organic contaminants
Metals attached on biochar
1. Ion exchange
2. Electrostatic attraction
6. Others
pH < pHpzc
Anionic metal attraction
Surface precipitation
Innersphere complexation
pH > pHpzc
Cationic metal attraction
CO3 2- + M2+ → MCO3↓
SiO4 2- + M2+ → MSiO4↓
2PO4 3- + 3M2+ → M3(PO4)2↓
5. Co-precipitation
3. Surface complexation
4. Physical adsorption
Exchangeable metal ions (Ca2+, K+, Mg2+, Na+)
Metal ions
Metals attached on biochar
Mineral components (CO3 2-, SiO3 2-, PO4 3-)

Figure 5

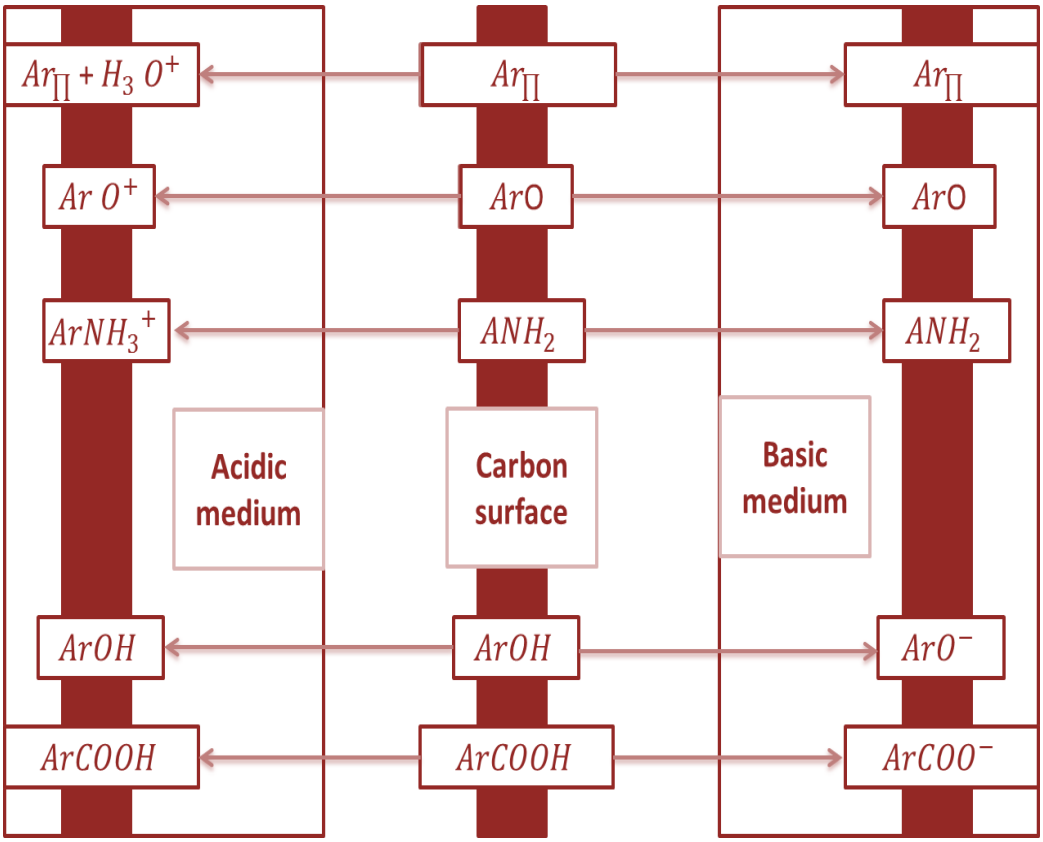
$Ar_{\prod} + H_3\ O^+$
$Ar_{\prod}$
$Ar_{\prod}$
$Ar\ O^+$
ArO
ArO
$ArNH_3{}^+$
ANH_2
ANH_2
Acidic medium
Carbon surface
Basic medium
$ArOH$
$ArOH$
ArO^-
$ArCOOH$
$ArCOOH$
$ArCOO^-$

Figure 6

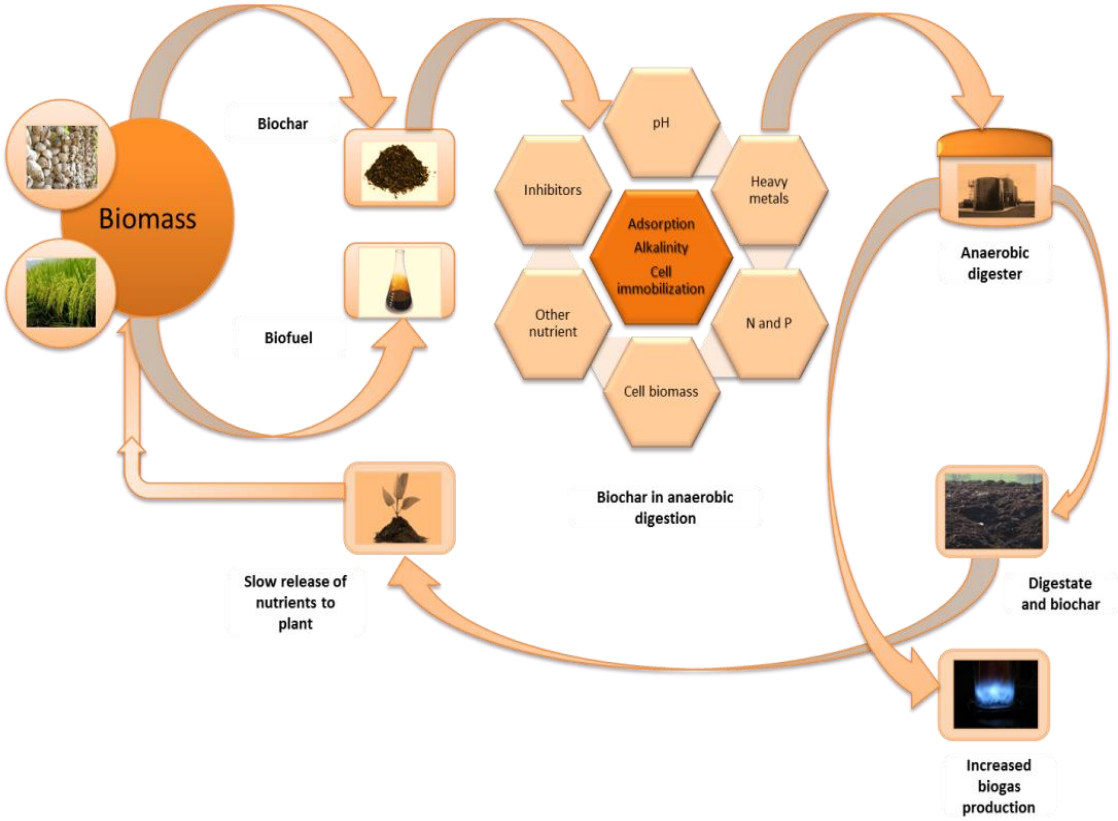
Biomass
Biochar
Biofuel
pH
Inhibitors
Heavy metals
Adsorption
Alkalinity
Cell immobilization
Other nutrient
N and P
Cell biomass
Biochar in anaerobic digestion
Anaerobic digester
Digestate and biochar
Increased biogas production
Slow release of nutrients to plant

Figure 7

5. Paper III

Evaluating the recovery rate of different inocula to increasing concentrations of D-limonene

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Abstract

Due to the varying acclimation rates of different inocula to substrate induced inhibition, the source of inocula prior to starting an anaerobic digestion system has become a central focus for both researcher and operators. In this anaerobic digestion study, the performance of different microbial inocula from digested sewage sludge leachate (SL), landfill leachate (LL), compost leachate (CL) and mixtures of these leachates (ML) exposed to D-limonene were evaluated. All the tests were carried out against a control over a 40-day incubation. The sequential addition of D-limonene and glucose solutions on the extent of methane production were 544 ± 21, 394 ± 2.8, 131 ± 14.9 and 62 ± 13.0 ml CH_4, with a methane conversion efficiency of 52%, 38%, 12.5% and 6% for ML, SL, CL and LL inocula, respectively. The stability of the anaerobic digestion process was significantly inhibited when the D-limonene concentration was increased to 0.25 mg ml^{-1}, resulting in the accumulation of volatile fatty acids and a suspected shift in the acidogenic pathway. The ML inoculum on exposure to D-limonene and cumulative methane production was most adequate of all the inocula used in this study. The findings point to the possibility of enhancing the anaerobic digestion of limonene containing organic substrates by selecting the appropriate inoculum source.

Keywords: limonene inhibition; recovery rate; methane production; anaerobic digestion; inoculum

1. Introduction

Citrus peel is a constituent of food waste and it contains chemicals that may be inhibitory to microbial activity within the anaerobic digestion system (Martin et al., 2010). The outer surface of the citrus peel has a thick lignocellulosic membrane, which contains vesicles filled with essential oils and volatile aromatic compounds (Fisher & Phillips, 2008). Limonene is a key constituent, making up 32 - 98% of the essential oil and is inhibitory to microbial growth (Droby et al., 2008; Badee et al., 2011; Espina et al., 2011). Limonene has been classified as a colourless cyclic terpene hydrocarbon, which exists in two isomeric forms: (i) D-limonene (R-(+) - limonene and (+)-carvene) and (ii) L-limonene (S-(-) - limonene, (-)-carvene) (Figure 1) (Duetz et al., 2003) . D-limonene is more abundant in orange-like citrus fruits while L-limonene is mostly found in lemon turpentine-like citrus fruits.

The precise mechanism of D-limonene inhibition is not fully known, but there are some suggestions in the literature as to how limonene inhibits microbial growth during anaerobic digestion (Ruiz & Flotats, 2014). Due to the nature of D-limonene as a lipophilic hydrocarbon, it is expected that the mechanisms of microbial growth inhibition should be similar to other hydrocarbons (Sikkema et al., 1995). Typically, hydrocarbons are water insoluble compounds, but due to the secretions of emulsifying agents by the microorganisms, the dissolution rates of limonene in the reactor's liquor increases their absorption across the cell membrane (Thomas et al., 1986; Sikkema et al., 1995). The continuous diffusion of hydrocarbons, such as D-limonene, through the cell membrane increases the surface area of the cell and enhances the possibility of cell leakage (Fisher & Phillips, 2008; Griffin et al., 1999; Sikkema et al., 1995). However, some groups of microorganisms have been reported to adapt to a high concentration of limonene by degrading the compound or maintaining a stable fluidity (Di Pasqua et al.,

2006). A constant fluidity is achieved when the microorganisms modify their surface layer by producing more saturated lipids, which can increase the rigidity of the cell (Di Pasqua et al., 2007; Di Pasqua et al., 2006). Nevertheless, anaerobic limonene degraders, such as *Bacillus stearothermophilus* and *Escherichia coli* as well as anaerobic fungi, such as *Penicillium digitatum* and *Penicillium italicum,* are able to transform limonene into metabolites, such as carveol, carvone, limonene-1,2-diol, epoxide, perillyl alcohol and perillaldehyde (Chang & Oriel, 1994; Droby et al., 2008) (Figure 2). Although the effect of these metabolites on anaerobic digestion is not well known, there are indications that they are equally inhibitory to some groups of microorganisms (Kang et al., 1992). In addition, some archaea, such as *Methanosaeta, Methanospirillum* and *Methanoculleus,* have shown some resistance to limonene inhibition (Foss et al., 1998; Glöckner et al., 2010).

Recent studies on the anaerobic digestion of limonene containing substrate have looked at the effect of varying the operating temperature and co-digestion with crude glycerol (Mizuki et al., 1990; Martin et al., 2010; Martín et al., 2013). However, the performance of different inocula at varying concentrations of D-limonene has not been evaluated. Therefore, the aim of this study was to monitor the process performance of different inocula and their mixtures in the presence of increasing concentrations of D-limonene. The process performance was evaluated by measuring the rate and extent of methane production, acidification and other chemical analysis.

2. Materials and Methods

2.1. Materials

2.1.1. Substrate

A glucose solution (Sigma-aldrich, UK) of 0.25mg ml^{-1} was prepared and used as a carbon source for the anaerobic digestion process. The glucose solution was stored in the refrigerator at 4 ^{o}C prior to use and infused sequentially to the experimental setup.

2.1.2. Microbial inocula

Three sources of inocula were used in this study. The inocula are (i) digested sewage sludge leachate (SL) (ii) compost leachate (CL) (iii) landfill leachate (LL) and (iv) SL, CL and LL inocula (ML). The LL inoculum was collected from a newly decommissioned landfill site in Wigan, UK; the SL inoculum was obtained from United Utilities' digested sewage sludge storage tank, Lancaster, UK, and the CL inoculum was collected from a composting barn in Lancaster University, Lancaster, UK. The inocula were sieved with a 250 nm mesh to bring the total solid content to approximately 1.9%, before storing in the cold room at 4^{0}C for 2 d. The characteristics of the inocula are represented in Table 1.

2.1.3. Inhibitor

The inhibitor used for experiment was D-Limonene (Sigma-Aldrich, UK). A stock solution of D-limonene was diluted accordingly with distilled water and well agitated before introducing into the AD units. D-limonene solution (1 ml) was sequentially fed

into the reactor with an increasing concentration of ×0.025, 0.050, 0.1, 0.25, 0.50 and 1.0 mg ml^{-1} over a 40 d incubation.

2.2. Methods

2.2.1. Sequential batch reactor

This experimental study was carried out using a 500 ml glass bottles (Duran bottle, SLS Ltd, UK). The bottles were modified by inserting an amended butyl rubber stopper through the bottle's mouth. The butyl rubber stopper was provided with a stainless steel stirrer opening, a gas and liquor sampling port. The stainless steel stirrer opening allowed the insertion of the stirrer shaft (Figure 3). The bottles were continuously stirred with 12 V DC motors (Lojer component, UK) at 30 rpm and placed in a temperature-controlled water bath at 35 ºC (Fisher-scientific, UK). An acrylic support was attached on the collar of the bottle to hold the DC motor in position (Figure 3).

2.2.2. Experimental design

The sequential methane production (SMP) potentials of the inocula and their mixtures were examined in duplicate for both test and control incubations. Each reactor was inoculated with 200 ml of SL, LL, CL and ML inocula, respectively. A total volume of 400 ml was maintained by adding 200 ml of distilled water to each of the reactors. Each of the reactors was flushed with nitrogen gas for a period of 30 seconds to remove oxygen before tightly inserting the butyl rubber lid. The feeding regime was a sequential batch system of 2-4 d interval depending on the stability of the digestion process and 1 ml of a trace mineral solution (containing per l: 150 mg $FeCl_2.4H_2O$, 70 mg $ZnCl_2$, 100 mg $MnCl_2.4H_2O$, 190 mg $CoCl_2.6H_2O$, 2 mg $CuCl_2.2H2O$, 24 mg $NiCl_2.6H_2O$, 36 mg

$Na_2MoO_4.2H_2O$, 6 mg H_3BO_3, 3 mg Na_2-$SeO_3.5H_2O$ and 4 mg $Na_2WO_4.2H_2O$) were added during the start-up stage of each experiments, modified from (Zhang et al., 2011). The pH of the medium was adjusted to 7.2. A summary of the experimental set up is represented in Table 2.

2.2.3. Analysis

Samples (5 ml) were collected from the digesters every 3 d using syringe and, after the pH was measured, 4 ml of the sample was injected back into the digester. A 1 ml sample was retained and placed in an Eppendorf tube, centrifuged at 10,000 rpm for 10 min before storing the supernatant in the freezer (-20 ^{o}C) for further chemical analysis. Each of samples (1 ml) were tested for soluble chemical oxygen demand. The supernatant was then filtered using 0.45 µm cellulose nitrate membrane filter (Whatman, England) and analysed for volatile fatty acids. All samples were appropriately diluted before chemical analysis.

2.2.3.1. Determination of soluble chemical oxygen demand (SCOD)

SCOD were carried out using the Hach dichromate digestion kits containing potassium dichromate (50%) and sulphuric acid solution (Hach LCK 514) was used to conduct this analysis. The method involved a digestion stage at 150 ^{o}C for 2 h before examining the absorbance of the cuvette using a quantitative Hach spectrophotometer (DR/2800, Hach) with a detection range of 0-2000 mg l^{-1}.

2.2.3.2. Determination of the total solid (TS)

The TS was determined according to standard methods (APHA, 1998). The crucibles were weighed before and after adding the sample. Crucibles containing the samples were then placed in the oven (Memmert, Germany) at 105 ^{o}C for 24 h, after which the crucibles were allowed to cool in a desiccators and the weight was recorded.

2.2.3.3. Determination of volatile fatty acids (VFAs)

VFAs were measured with ion chromatography (IC) (Dionex, ICS-30000, Thermo-Scientific, USA) using a UV index detector and an Aminex HPX-87H column (Bio-Rad, UK). The separation of VFAs during the IC analysis was achieved using a mobile phase of 2.5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1} and a column temperature of 65 ^{o}C. The detector temperature was 40 ^{o}C. The VFA marker mix containing acetic, propionic, iso-butyric, butyric, iso-valeric and valeric acids, each of 1 mg ml^{-1} (Sigma-Aldrich, UK) was used for to calibrate the IC (Oh et al., 2005; de Sá et al., 2011).

2.2.3.4. Methane gas measurement

A 100 ml glass bottle containing 80 ml of 3 M NaOH solution was connected to the sequential batch reactor to fix carbon dioxide. Methane gas production was measured volumetrically using a 10 – 15 ml tip meter. The meters were equipped with switches to a relay electrical pulse, an impulse capturing and data recording device. The methane production was expressed in ml.

2.2.3.5. Determination of pH

The pH reading was monitored with a pH meter (Conrad, Model 100 ATC) and reported to one decimal place.

2.2.3.6. Theoretical methane production

The theoretical methane production was calculated from equation 1 using the elemental composition of the molecular formula of glucose substrate, which is given as $C_aH_bO_cN_dS_e$ where a, b, c, d and e represents values of 6, 12, 6, 1 and 1, respectively (Buswell & Mueller, 1952; Raposo et al., 2011).

$$\text{Theoretical methane (ml)} = \frac{\mathbf{22.4(4a+b-2c-3d-2e)}}{\mathbf{8(12a+b+16+14d+16e)}} \times g\ substrate \quad (1)$$

2.2.3.7. *Efficiency of methane conversion (*$\mathrm{EMC_{CH_4}}$ *)*

The methane conversion efficiency is defined as the percentage of the actual methane divided the theoretical methane. This is expressed in equation 2:

$$\mathbf{EMC_{CH_4}} = \frac{\boldsymbol{Actual\ methane\ (ml)}}{\boldsymbol{Theoretical\ methane\ (ml)}} \times \mathbf{100} \quad (2)$$

2.3. Statistical analysis

Calculation of mean, standard deviation, and standard error were conducted using Microsoft Excel 2010. For methane production (n=2), normality and variance homogeneity were previously verified by the Levene and Shapiro Wilks tests ($P>0.05$), respectively. A Welch's one way Anova test of means ($P<0.05$) was used when methane production was approximately normally distributed with unequal group of variances. The post-hoc test, Games-Howell ($P<0.05$) was selected for multiple comparison due to inhomogeneity of variances. Statistical analyses were performed using a SPSS software, version 22.0.

3. Result and discussion

The impact of D-limonene on the anaerobic digestion process and anaerobic bacteria was evaluated for three different inocula and their mixtures: digested sewage leachate (SL), coimpist leachate (LL) and mixed leachate (ML).

3.1. Methane production

3.1.1. Rate of methane production

The rate of methane production was measured for the different inocula in the presence and absence of D-limonene and the ML and SL inoculum yielded the highest rates of methane production (Figure 4). The inhibitory effect of D-limonene on microbial activity was not instantaneous, but became evident after 24 h; this was probably due to the hydrophobic nature of the compound (Hale et al., 2015). According to Sikkema et al. (1995), the dissolution of hydrophobic compounds in water can be enhanced by emulsifying agents secreted by the bacteria. As presented in Fig. 4, methane production commenced immediately on day 1 of digestion indicating active microbial populations and a balanced interaction between the acetogenic and methanogenic microbial populations (Zhou et al., 2011). The values obtained for the test inocula were 19.0 ± 1.8, 11.3 ± 0.8, 31.3 ± 1.4 and 18.7 ± 5.7 ml CH_4 day $^{-1}$ for each of the ML, SL, LL and CL inocula, respectively (Figure 4). The ML, SL, CL and LL control inocula achieved values of 26.2 ± 0.2, 47.9 ± 4.2, 15.5 ± 0.25 and 29.5 ± 6.5 ml CH_4 day $^{-1}$, respectively (Figure 4). The p-value (>0.05) was not statistically significant for all of the incubations except between the ML and CL control inocula. After 2 days of incubation, there was no methane production for any of the test inocula, even when 0.25 mg ml^{-1} of glucose solution was added on the 4th day of incubation. However, the rate of methane

production was continuous, but not statistically significant (P> 0.05) for the control inocula, particularly for ML and SL, with values ranging from 6.5 ± 0.6 - 26.2 ± 0.2 and 12.0 ± 2.0 – 47.9 ± 4.2 ml CH_4 day $^{-1}$, respectively. Similar to that reported by Martín et al. (2010), D-limonene inhibited the test inocula (ML and SL), as methane production was evident in the control inocula (ML and SL).

After recovery for limonene inhibition on the 5th day of incubation, methane production peaked for all of the test inocula, with values of 65.6 ± 2 .9, 11.3 ± 0.8, 12.4 ± 1.9 and 19.1 ± 6.8 ml CH_4 day $^{-1}$for ML, SL, LL and CL, respectively. At this point, the rate of recovery was highest for the ML inoculum with a value of 65.6 ± 2.9 ml CH_4 day^{-1}. This value was statistically significant (P<0.05) when compared to the other incubation except for the LL test inoculum. The sudden peak in methane production for ML and SL test inocula could be attributed to the acclimation of the anaerobic microbial cells to the initial concentration of limonene. There are indications that some anaerobic microorganisms can adapt to the presence of limonene or transform it into other metabolites (Chang & Oriel, 1994; Droby et al., 2008). Similar behaviour was described by Mizuki et al. (1990), who reported an inhibition to AD when OLR of the citrus peel oil was about 0.065 mg ml^{-1} day^{-1}. Subsequent addition of 0.1 mg ml^{-1} of D-limonene solution on the 9th day of incubation did not inhibit methanogenesis, with methane production continuing in all of the test inocula incubations, except CL and LL. The maximum values of 60.4 ± 8.1 and 34.0 ± 2.6 ml CH_4 day $^{-1}$ were achieved for inocula ML and SL from days 9-14 of incubation, respectively. This disagrees with past reports, which suggest that AD will be severely inhibited if D-limonene concentrations are between 0.065 - 0.088 mg ml^{-1}, although this study did not consider the possibility of microbial acclimation to limonene inhibition (Lane, 1984; Mizuki et al., 1990). According to Chen et al. (2008), after a long period of exposing bacterial cells to

unfavourable conditions, the bacterial community is able to acclimate. This explains the stability in methane production when the limonene dosage was increased to 0.1 mg ml^{-1}, particularly for test ML and SL inocula. However, this was not the case for CL and LL test inocula. The rates of methane production were not measurable from the 10^{th} day of incubation, although the control CL inoculum continued to produce 13.0 ml CH_4 day $^{-1}$ until the 12^{th} day of incubation. This phenomenon could be ascribed to the microbial community present in the CL and LL inocula. There are indications that compost and landfill leachate contains a high population of fungi, which have been reported to transform limonene into other metabolites, but lack the methanogenic population density required to consistently convert volatile fatty acid into methane gas (Ángel Siles López et al., 2010; Neher et al., 2013; Ruiz & Flotats, 2014; Saetang & Babel, 2010). In as much as the build-up of acid could not be prevented (Figure 6), it is suspected that this might have resulted in the repeated failure of the anaerobic digestion process (Zhou et al., 2011). From this point on, the CL and LL inocula produced no measurable amounts of methane gas.

The D-limonene concentration was increased to 0.250 mg ml^{-1} on the 15^{th} day of incubation, and no measurable methane production was recorded. Consequent addition of a glucose solution on the 20^{th} day of incubation resulted in no measurable amount of methane production. The ML and SL control inocula continued to produce methane with maximum values of 32.8 ± 6.8 and 60.4 ± 2.2 ml CH_4 day $^{-1}$, respectively. However, on the 23^{rd} and 24^{th} days of incubation, SL and ML test inocula recovered with methane production values of 11.34 ± 0.8 and 27.6 ± 6.7 2 ml CH_4 day $^{-1}$, respectively. The recovery rate was fastest for the SL inoculum with only 7 days of inhibition, while the ML inoculum was inhibited for 9 days. On days 29 and 36, the test inocula were equally dosed with 0.5 and 1.0 mg ml^{-1} of D-limonene and a similar trend

was observed. The rates of methane production only lasted for 1-2 d for the SL and ML inocula, respectively, while the LL and CL inocula remain inhibited. At this point, the rates of recovery from D-limonene toxicity were fastest for test inocula SL and ML, which suggests higher tolerance to limonene (Figure 4). This trend is similar to the Lins et al. (2012) study on the stepwise adaptation of inocula to an increasing concentration of acetic acid. In this study, the faster recovery of the ML and SL test inocula through methane production suggests higher tolerance to limonene.

3.1.2. Total methane production and conversion efficiency

The production of methane was measured in the presence and absence of D-limonene while the methane conversion efficiency was determined by dividing the actual methane by the theoretical methane production. The findings showed that there was a significant difference between the total amounts of methane produced by all incubations ($P<0.05$). The total amount of methane production by the control incubation was 853 ± 34.0, 983 ± 21.0, 162 ± 19.0 and 62 ± 19.5 ml CH_4 with a methane conversion efficiency of 81.6%, 94.0%, 15.5 and 5.9 for the ML, SL, CL and LL, respectively (Figure 5, Table 3). The SL control inoculum produced the highest amount of methane and had the greatest methane conversion efficiency, which suggests that methanogenesis was most sufficient ($P<0.05$). However, the amounts of methane produced in the D-limonene incubations were 544 ± 21, 394.4 ± 2.8, 131 ± 14.9 and 63.2 ± 2.8 ml CH_4 with a methane conversion efficiency of 52.1%, 37.7% 12.5% and 6% for the ML, SL, CL and LL inocula, respectively (Figure 5, Table 3). The ML and SL test inocula produced the highest amounts of methane and methane conversion efficiency when compared to the other test inocula, which suggests a higher tolerance to limonene inhibition ($P<0.05$). The

result showed that the control incubation produced the highest amounts methane and methane conversion efficiency, except for LL and CL ($P<0.05$). This further suggests that the low total amounts of methane production and methane conversion efficiency of the test incubation, particularly inocula ML and SL, could be attributed to the presence of limonene. In addition, the total methane production and methane conversion efficiency values for the test incubations showed that the ML inoculum delivered the highest value. This may be attributed to the mixture of different inocula that synergistically contributed to the performance of the ML inoculum during limonene suppression (Schink, 2002). As mentioned earlier, the CL and LL inocula may have contained limonene-degrading fungi. It can therefore be concluded that the synergy between the different inocula contributed to the high performance of the ML inoculum.

3.2. VFA concentration and pH

Samples were collected every 2-4 days to measure the VFA concentrations over the incubation period (Figure 6). Data from all of incubations showed varying trends except for the LL and CL inocula, which recorded continual increases in VFA production after 8 days of incubation. Therefore, data from LL and CL inocula were limited to the 0 - 7 day incubations.

All of the inocula showed a low concentration of VFAs in the first 4 days of incubation with values of less than 0.084 mg ml^{-1} except for the CL inoculum, which had an accumulated VFA concentration of 0.40 mg ml^{-1}. This result is consistent with the daily methane measurement, which showed no significant methane production from days 2-4 of incubation (Figure 4). At this point, acidogenic and methanogenic bacteria in the ML and SL test inocula were suspected of being partially inhibited by the addition

of 0.025 mg ml^{-1} of D-limonene, although the accumulation of VFAs in the CL and LL inocula suggested that there was no inhibition of acidogenic bacterial cells (Figure 6). It has previously been suggested that the interaction between an inhibitor and VFA will lead to a process where the operational process will be stable but methane production will be low (Angelidaki & Ahring, 1993; Angelidaki et al., 1993). However, after the addition of 0.050 mg ml^{-1} of D-limonene after 6 days of incubation, the accumulation of VFAs was observed in all of the incubations, except for the SL inoculum, with a value of 0.062 mg ml^{-1}. Similarly, no measurable methane value was recorded for the SL test inoculum on the 6^{th} day, which suggests inhibition of acidogenesis and methanogenesis. The volatile fatty acids are precursors for methane production and are produced during the activities of the acidogenic and acetogenic bacterial cells (Voolapalli & Stuckey, 2001). Nevertheless, the ML, CL and LL inocula recorded total VFA values of 0.996, 1.14, 1.03 mg ml^{-1}, respectively, with 73.5% being acetic acid. This observation is consistent with the results of previous studies, which observed that 70 to 73% of the methane gas is produced from acetic acid production (Mountfort & Asher, 1978; Madsen et al., 2011). Unlike the SL test inoculum, where acidogenesis and methanogenesis were inhibited, the relatively high accumulation of VFAs and low levels of methane gas production suggested that only methanogenesis was inhibited in the ML, LL and CL inocula (Wang et al., 2009).

After 8 days of incubation, the ML and SL incubations showed a reduction in the total VFA and acetic acid concentrations to 0.34 and 0.26 mg ml^{-1}, respectively. Whereas, for the CL and LL incubations, the VFA concentrations had increased to a record high of 2.0 and 2.3 mg ml^{-1} of acetic acid (Figure 6). This was followed by a substantial drop in the pH of the CL and LL inocula from an average of 7.2 to 5.2 ± 0.17 and 5.7 ± 0.22, respectively (Table 3). On the other hand, the SL and ML

incubations showed efficient metabolism of substrate, an indication of a good mass transfer between the acidogenic and methanogenic populations present in the incubations, which indicated a recovery from limonene inhibition. On the 9th day of incubation, 0.1 mg ml^{-1} of D-limonene and 0.25 mg ml^{-1} of glucose solution were added to the test inocula (Figure 5). A slight increase in acetic acid was observed in the ML and SL inocula, which could be attributed to the glucose solution and partial inhibition from the limonene compound. Acetic acid values of 0.56 ± 0.03 and 0.39 ± 0.06 mg ml^{-1} were recorded for the ML and SL test inocula on days 12 and 14, respectively. The slight increase in acetic acid concentration resulted in higher rates of methane production (Fig 4). This may be attributed to the rapid conversion of the glucose metabolites by the acidogenic bacteria or the inhibition of methanogens by limonene. The acidogenic bacteria are fast growers and make up 90% of the microbial community in the inoculum (Cohen et al., 1980; Zeikus, 1980; Mosey, 1983). After 15 days, an additional 0.250 mg ml^{-1} of D-limonene was added to all of the inocula; 3 days later 0.750 ± 058, 0.20 ± 0.014 and 0.61 ± 0.075 of acetic, propionic and butyric acids, respectively, had accumulated in the ML inoculum. Similarly, the SL inoculum accumulated 0.14 ± 0.001 and 0.057 ± 0.01 mg ml^{-1} of propionic and butyric acids, respectively. The production and accumulation of propionic and butyric acids in anaerobic digestion suggests a shift in acidogenic microbial populations, which is an unfavourable pathway for acetogenic bacterial activity because the energy loss is greater (Vanvelsen, 1979; Wang et al., 1999). The shift in the acidogenic pathway was more pronounced in the ML inoculum because low values were recorded for the SL inoculum, signifying that the ML inoculum was more tolerable at this stage. With regard to methanogenesis, no measurable methane production was recorded for ML and SL incubation, signifying that the methanogens were more inhibited at this stage (Fig 4).

After 22 days of incubation, the production and accumulation of butyric and propionic acids was less than 0.09 mg ml^{-1} but acetic acid increased to 0.98 ± 0.04 in the ML inoculum. On the other hand, the SL inoculum accumulated 0.75 ± 0.07 and 0.79 ± 0.072 mg ml^{-1} of propionic and butyric acids, respectively. These figures reduced to 0.18 and 0.05 on the 28^{th} day of incubation. The accumulation of VFAs and no measurable methane production further suggested inhibition of methanogenesis for both the ML and SL incubations. Sequential addition of 0.25 mg ml^{-1} glucose solutions after 24 and 28 days of incubation showed a consistent reduction in the accumulation of acetic acid and steady methane production (Figures 4 and 7). However, the addition of 0.5 and 1.0 mg ml^{-1} of D-limonene on the 29^{th} and 36^{th} days of incubation showed a gradual increase in the accumulation of VFAs for both the SL and ML inocula, suggesting further inhibition of methanogenesis. A maximum acetic acid concentration of 1.36 ± 0.02 and 0.57 ± 0.03 mg ml^{-1} was measured on the 40^{th} day of incubation by ML and SL test inocula, respectively, suggesting a gradual increase in the accumulation of VFAs and inhibition of methanogenesis

4. Conclusion

This study investigated the impact of D-limonene on the anaerobic digestion of glucose by different inocula in a sequential batch system. The addition of D-limonene solution caused no significant inhibition until the concentration was increased to 0.25 mg ml^{-1}. After subjecting all the test inocula to an increasing concentration of limonene, the ML inoculum recorded the highest methane production with a value of 544 ± 21 ml CH_4. The investigation on the acclimation rate of different inocula and their mixtures provided important information on the relevance of inoculum source during inoculation of anaerobic digestion system, particularly operations that use limonene containing substrate as their feedstock. In the future, a molecular study would be required to identify the changes in the microbial community, especially during the inhibitory and acclimation phase.

Acknowledgement

This work was supported by the Centre for Global Eco-innovation (X02646PR) and Stopford Energy and Environment Ltd

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Tables

Table 1: Characteristics of inoculums and mixtures of inocula

Assay	Inoculum	Soluble COD (g l^{-1})	Total solid (%)
T1	LL	0.31 ± 0.12	1.90 ± 0.02
T2	CL	0.34 ± 0.02	1.90 ± 0.06
T3	DL	0.49 ± 0.23	1.90 ± 0.10
T4	ML	0.38 ± 0.17	1.90 ± 0.13

Values are expressed in mean and standard error (n=3)

TS and COD were analysed on fresh basis

Table 2: Summary of the experimental set up

Assays	LL (ml)	CL (ml)	SL (ml)	Distilled H_2O (ml)	Working volume (ml)
LL	200	0.00	0.00	200	400
CL	0.00	200	0.00	200	400
SL	0.00	0.00	200	200	400
ML	66.6	66.6	66.6	200	400

Table 3: The actual and theoretical methane productions for different inocula and mixtures of inocula and their final pH (n=2)

Assay	Theoretical CH_4 (ml)	Actual CH_4 (ml)				Final pH
		Control experiment	Conversion efficiency (%)	Test experiment	Conversion efficiency (%)	
ML	1045	853.0 ± 34.0	81.6	544.0 ± 21.0	52.1	7.10 ± 0.11
SL	1045	983.0 ± 21.0	94.0	394.0 ± 2.8	37.7	7.23 ± 0.09
CL	1045	162.0 ± 19.0	15.5	131.0 ± 14.9	12.5	5.24 ± 0.17
LL	1045	62.0 ± 19.5	5.9	63.2 ± 13.0	6.0	5.78 ± 0.22

Figure captions

Figure 1. Structural formula of Limonene

Figure 2. Biodegradation of D-limonene, the product and microorganisms; adapted from (Demyttenaere et al., 2001; Duetz et al., 2003a)

Figure 3. Sequential batch testing reactor; the setup is a combination of an airtight glass reactor, mixing device, CO_2 scrubber and tip meter for gas measurement.

Figure 4. Substrate loading and rates of methane production at increasing concentration of D-limonene for mixed leachate (ML), digested sewage leachate (SL), compost leachate (CL) and landfill leachate (LL). Vertical bars indicate standard error (n=2)

Figure 5. Cumulative methane production for test (glucose and D-limonene) and control (glucose) experiment; mixed leachate (ML), digested sewage leachate (SL), compost leachate (CL) and landfill leachate (LL). Vertical bars indicate standard error (n=2)

Figure 6. The individual volatile fatty acid profiles for test assays; mixed leachate (ML), digested sewage leachate (SL), compost leachate (CL) and landfill leachate (LL). Vertical bars indicate standard error (n=2)

Figures

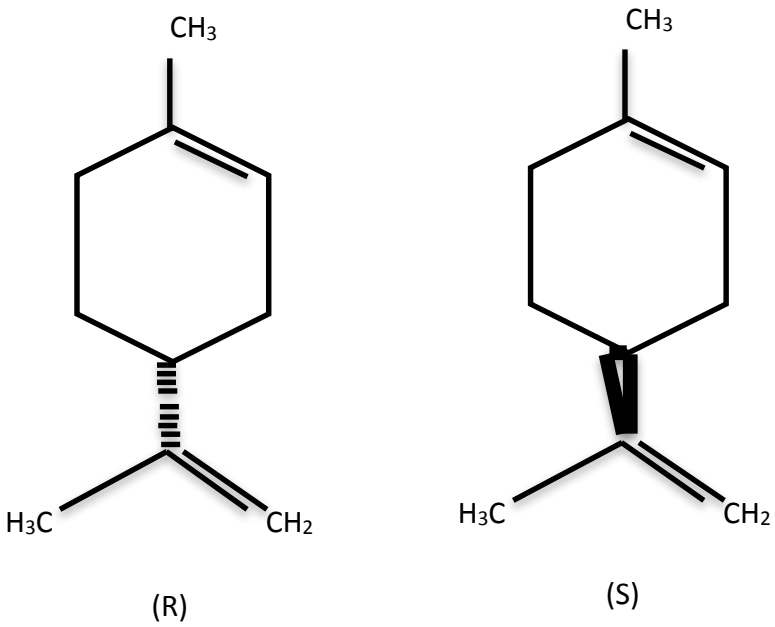

Fig. 1.

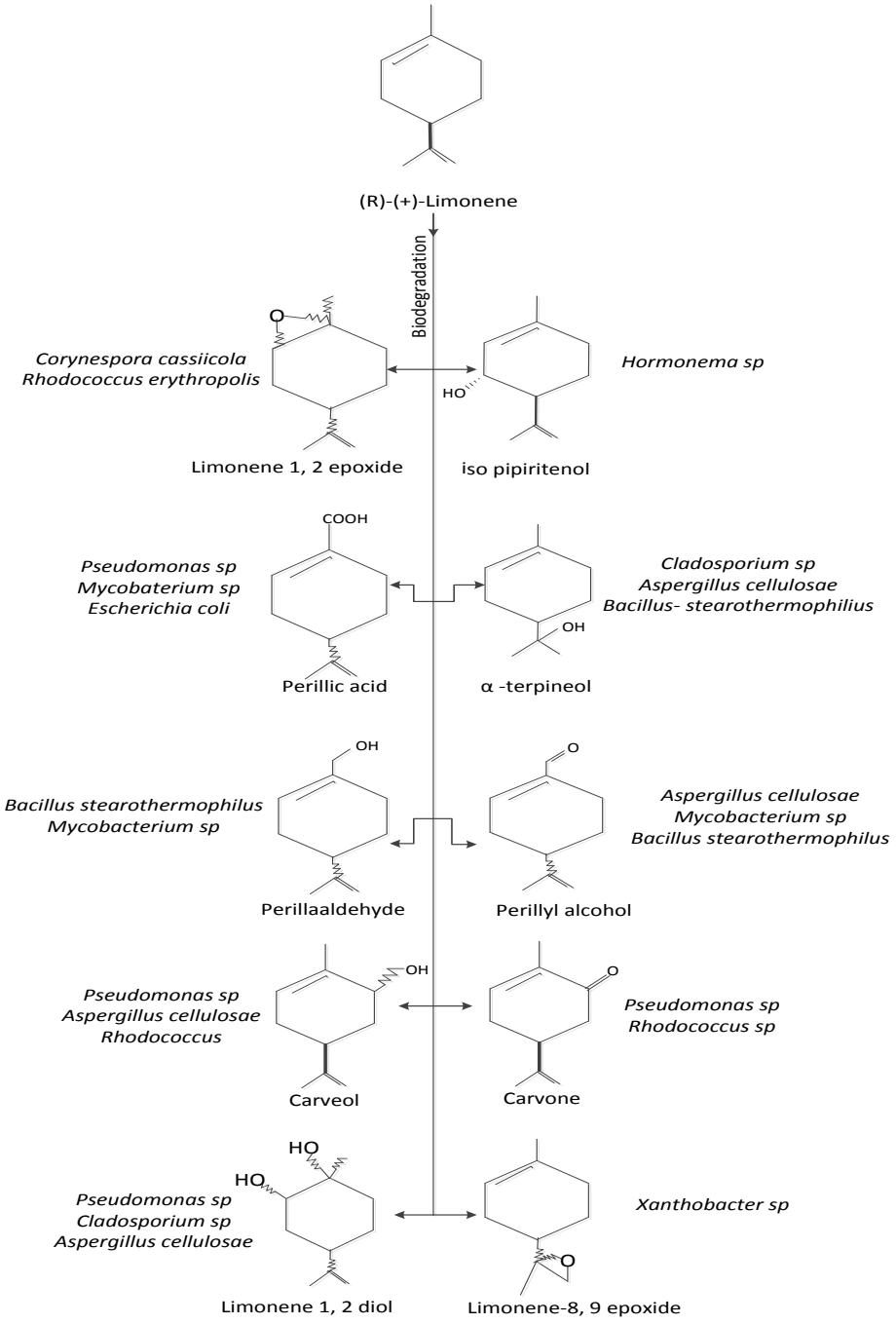
(R)-(+)-Limonene
Biodegradation
Corynespora cassiicola
Rhodococcus erythropolis
Limonene 1, 2 epoxide
Hormonema sp
iso pipiritenol
Pseudomonas sp
Mycobaterium sp
Escherichia coli
Perillic acid
Cladosporium sp
Aspergillus cellulosae
Bacillus- stearothermophilius
α -terpineol
Bacillus stearothermophilus
Mycobacterium sp
Perillaaldehyde
Aspergillus cellulosae
Mycobacterium sp
Bacillus stearothermophilus
Perillyl alcohol
Pseudomonas sp
Aspergillus cellulosae
Rhodococcus
Carveol
Pseudomonas sp
Rhodococcus sp
Carvone
Pseudomonas sp
Cladosporium sp
Aspergillus cellulosae
Limonene 1, 2 diol
Xanthobacter sp
Limonene-8, 9 epoxide

Fig.2.

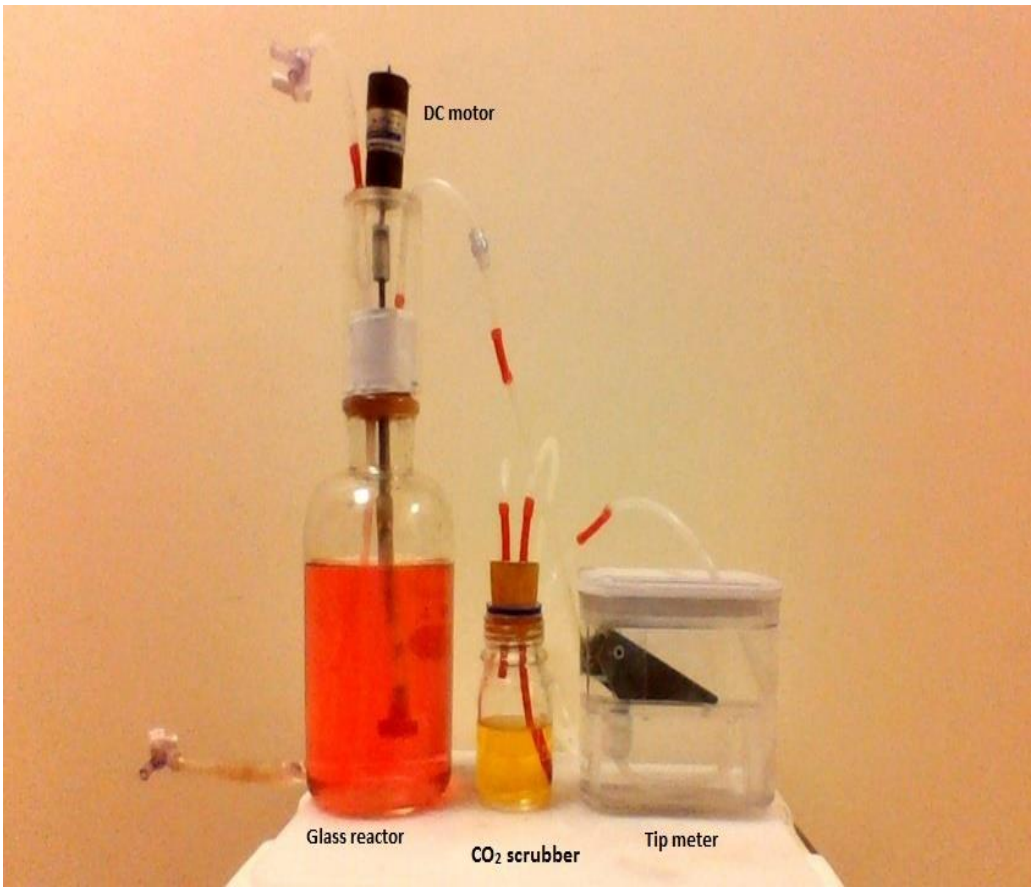

Fig. 3.

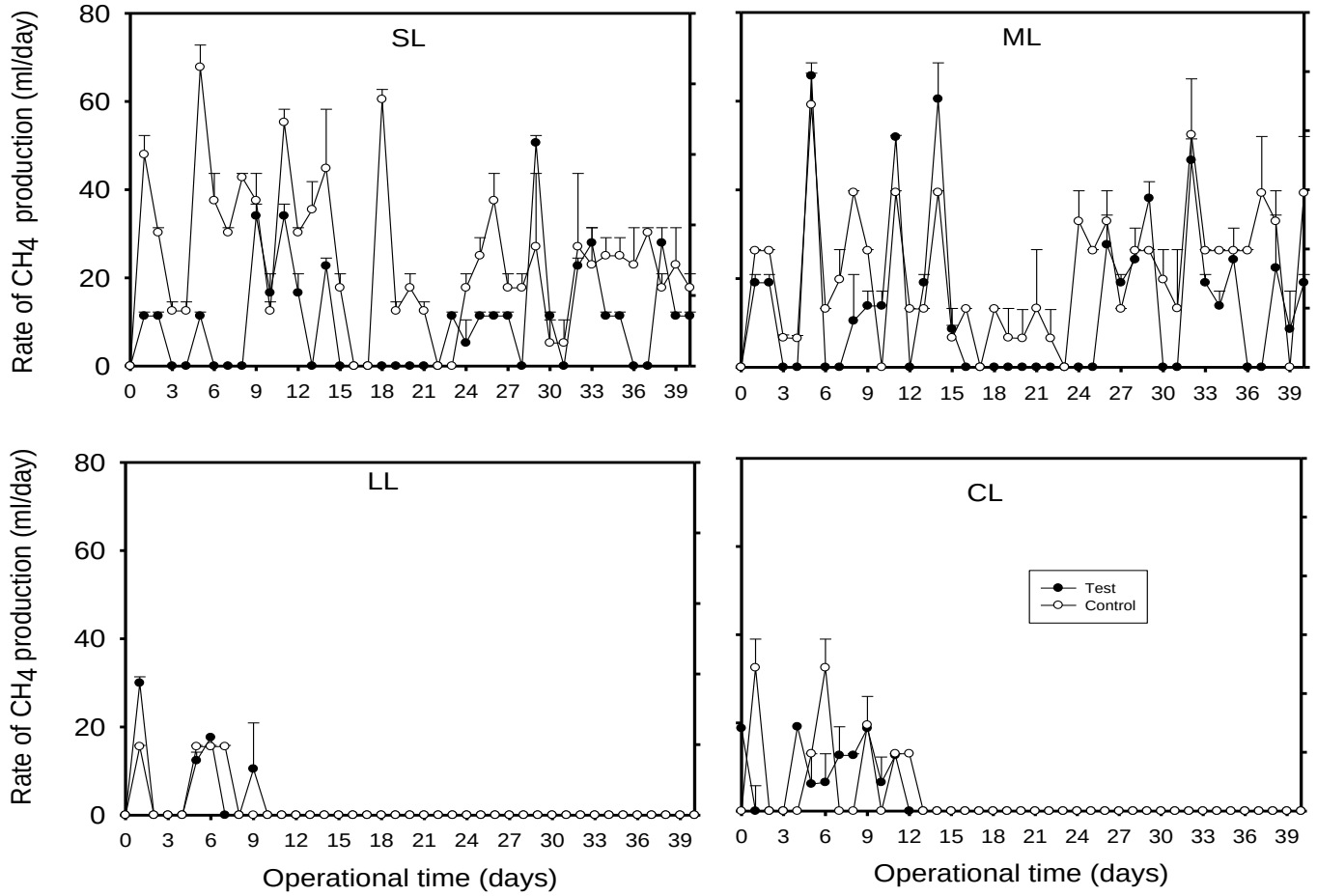
SL
ML
LL
CL
Rate of CH4 production (ml/day)
Rate of CH4 production (ml/day)
Operational time (days)
Operational time (days)
Test
Control
0
20
40
60
80
0 3 6 9 12 15 18 21 24 27 30 33 36 39

Fig. 4.

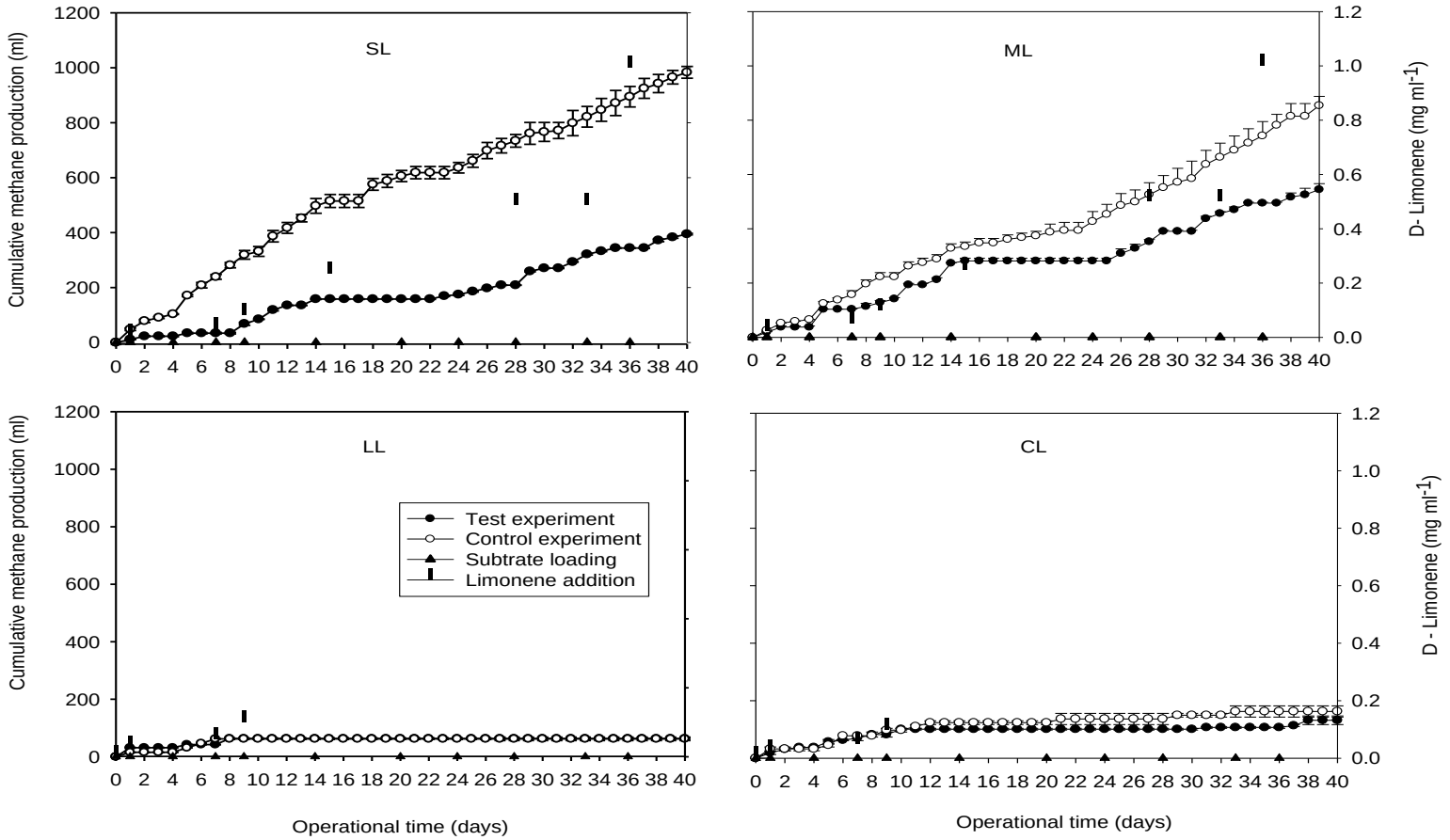
SL
ML
LL
CL
Cumulative methane production (ml)
D- Limonene (mg ml-1)
Test experiment
Control experiment
Subtrate loading
Limonene addition
Operational time (days)

Fig. 5

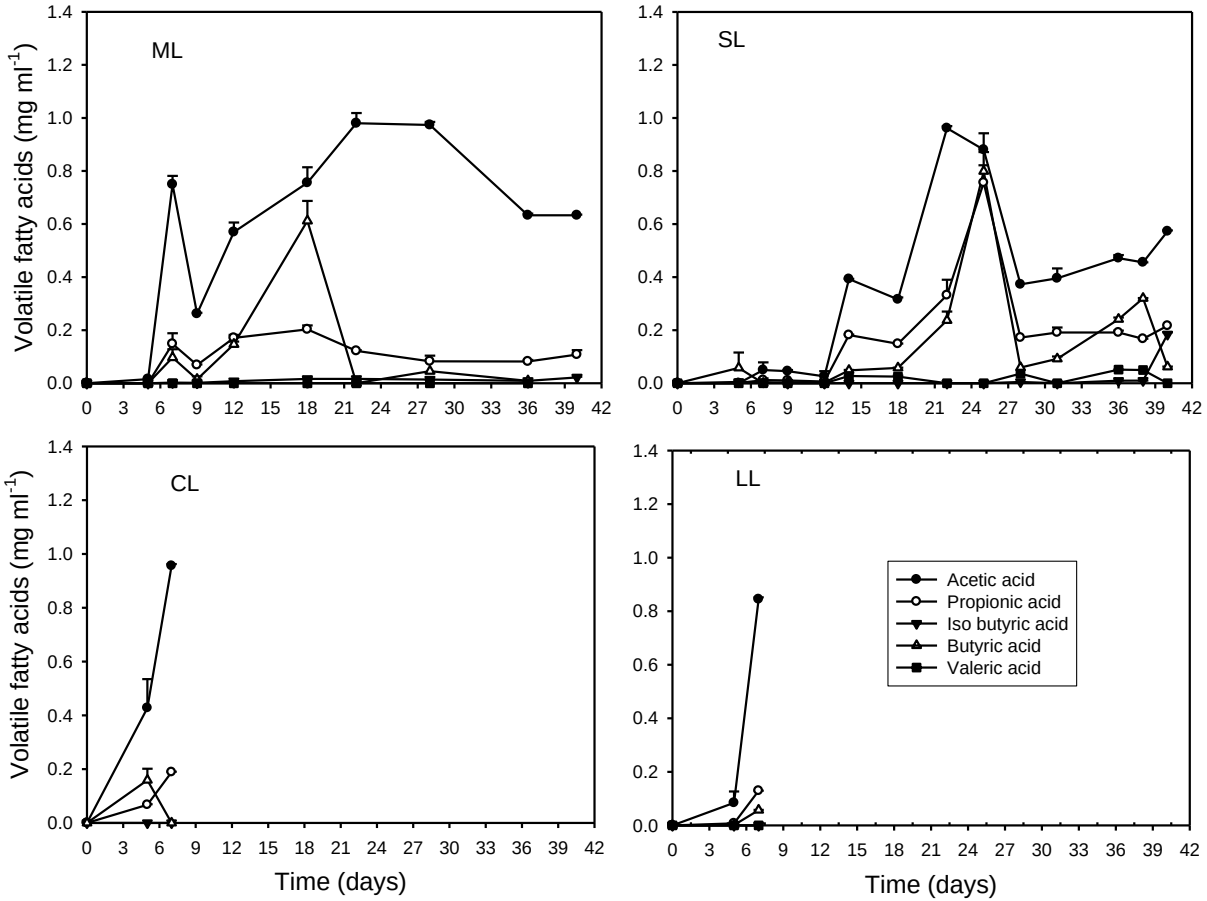
ML
SL
CL
LL
Volatile fatty acids (mg ml-1)
Time (days)
Acetic acid
Propionic acid
Iso butyric acid
Butyric acid
Valeric acid

Fig .6

6. Paper IV

Impact of biochar on the anaerobic digestion of citrus peel waste

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Abstract

In this study, the impact of different types of biochar and biochar ratios on the anaerobic digestion of citrus peel waste was investigated. Citrus peel has an inhibitory effect on anaerobic digestion. The presence of biochar had two effects: a reduction in the length of the lag phase and greater production of methane relative to citrus peel waste only incubations. The microbial lag phases decreased with increase in citrus peel to biochar ratios (2:1<1:1<1:2<1:3), with 2:1 having the longest lag phase of 9.4 days and 1:3, the shortest, with the value of 7.5 days. The cumulative methane production in incubations containing biochar and citrus peel ranged from 163.9 – 185.0 ml CH_4 gVS^{-1}, while citrus peel only produced 165.9 ml CH_4 gVS^{-1}. Examination of the biochar material revealed colonies of putative methanogens. The synergy of D-limonene adsorption and microbial immobilisation by the biochars may contribute to enhanced anaerobic digestion performance.

Keywords: Anaerobic digestion, biochar, citrus peel waste, lag phase, limonene and methane production

1. Introduction

Citrus peel waste is a lignocellulosic material containing fibre and essential oils, of which 32-98% is made up of an akylated aromatic compound called limonene (Droby et al., 2008; Badee et al., 2011; Espina et al., 2011). Limonene is a colourless liquid with a strong smell. Its boiling point is 176 ^{0}C and it is classified as a cyclic terpene. Anaerobic digestion (AD) studies have shown that an organic loading rate of 2-3.5 gVS^{-1} day^{-1} of citrus peel can inhibit microbial activity (Mizuki et al., 1990; Martín et al., 2010; Martín et al., 2013). However, recent findings suggest that co-digestion with other feedstocks or AD at higher operating temperatures is more stable with lower levels of inhibition (Martín et al., 2010; Martín et al., 2013). Although co-digestion provides an economic approach to minimizing the challenges associated with some individual substrates, co-substrate availability and accessibility must be considered (Zhang et al., 2012) Similarly, high operating temperatures (>55 ^{0}C) are seldom used owing to the relatively high operational cost associated with thermophilic operation. Other approaches, such as the acclimation of microorganisms and the removal of putatively toxic chemicals before or during the AD process, have also been explored (Chen et al., 2008; 2009). The existing approaches to counteracting limonene toxicity during AD focus mainly on reducing the concentration and increasing the assimilation time of the bacteria. Nonetheless, the reduction of limonene concentration by physically removing the compound from the AD system is preferable because the resulting metabolites during assimilation are inhibitory (Chang & Oriel, 1994; Foss et al., 1998; Droby et al., 2008; Glöckner et al., 2010; Martín et al., 2010). Steam distillation has been identified as a method for removing up to 70% of limonene from citrus peel waste, but this is equally energy intensive (Martín et al., 2010). Apart from steam distillation and solvent extraction, adsorption has been identified as a method of removing organic

compounds. For example Chen et al. (2008) reported that adsorbents could be effective in reducing potential inhibitors, such as ammonium and long chain fatty acids. Adsorbents, such as zeolites, activated carbon, bentonite and silica gel, have been reported to remove toxic chemicals from the AD process (Angelidaki & Ahring, 1992; Milan et al., 2003; Bertin et al., 2004; Mumme et al., 2014). Although the application of biochar in AD to remove potentially inhibitory chemicals has not been fully investigated, there are indications that biochar can adsorb monoterpene compounds (Hale et al., 2015).

Biochar is produced from plant derived biomass that is subjected to thermal treatment in the partial or total absence of oxygen (Qadeer et al., 1994). The thermal treatment changes the microstructure of the particles to form an aromatic-aliphatic region and a crystalline region (Qadeer et al., 1994), which are made up of different pore sizes based on their internal diameter (ID) micropore (ID <2nm), macropore (ID > 50nm) and mesopore (2 nm < ID < 50 nm) (Laine et al., 1991; Zabaniotou et al., 2008). These pores are responsible for the adsorptive behaviour of biochar for compounds such as phosphate, nitrate, nitrite, ammonium, metals, pesticides and carbon dioxide (Bagreev et al., 2001). The sorption mechanisms of a biochar material are similar to other adsorbents (activated carbon, zeolite and bentonite); ionic and organic compounds are respectively adsorbed using electrostatic and van de Waal forces of attraction (Mattson et al., 1969; Kadirvelu et al., 2001; Zhang et al., 2012). In addition, biochar and other sorbents can offer surfaces that may be colonised by microorganisms (Watanabe et al., 2013). The aim of this study was to evaluate the effect of different types of biochar and biochar ratios on the AD of citrus peel waste by measuring the rate and extent of methane production, observing the morphological images of the biochar material and other chemical analysis.

2. Materials and methods

2.1.Substrate and inoculum

Citrus peel waste was used as the substrate and digested sewage sludge was used as the source of microbial inoculum for this investigation. The digested sewage sludge was supplied by a wastewater treatment facility (United Utilities, Preston, UK). The digested sewage sludge was characterised and it contained 11.0 ± 0.13 % volatile solids (VS), 52.1 ± 0.36% total solids (TS), 27.0 ± 0.23 mg l^{-1} SCOD, 0.71 ± 0.17 mg l^{-1} NH_4-N and pH of 7.26 ± 0.02. Citrus fruits were obtained from a local well-known supermarket in Lancaster, UK. The fruits were washed before squeezing out the juice and weighing both the fibre and the peel. The citrus peel waste was a mixture of 32 g of lemon, 12 g of lime, 53 g of orange, 30 g of tangerine and 65 g of grape peel, which were blended, homogenised and frozen to preserve the citrus peel waste material. The mixing ratio was selected based on the average quantities of different citrus waste generated annually (FAOSTAT, 2013). The characteristics of the substrate are presented in Table 1. Wood biochar (WB), coconut shell biochar (CSB) and rice husk biochar (RHB) were used in this study. The CSB and RHB were sourced from Malaysia, while WDB was obtained in the UK. The biochars were produced through pyrolysis at 450 ^{0}C and their characteristics are presented in Table 1. The biochars were prepared to a particle size of 1.7- 2.0 mm.

2.2.Experimental Design

This study was carried out in a 500 ml Duran bottle capped with a modified rubber stopper containing a gas and liquor sampling port. The gas port was connected to a water displacement electronic tip meter for volumetric gas measurement. Prior to

methane gas measurement, the biogas production was bypassed through a 100 ml Duran bottle containing 3 M NaOH solution to fix CO_2. A stirrer port was also included on the rubber stopper, thus allowing mechanical homogenization using a 12 V DC motor (Lojer component, UK) at 30 rpm. The anaerobic reactor was maintained at 35 ^{0}C in a digital water bath for an incubation period of 30 days. The batch AD of citrus peel waste and biochar was carried out focusing on: (i) citrus peel and biochar types and (ii) citrus peel and biochar ratios. Control incubations included inoculum only, biochar and inoculum, citrus peel and inoculum.

A substrate to inoculum ratio was set between 0.31- 0.33 based on the wet weight of the volatile solid. For the first study involving biochar types, the different biochar materials were combined with the citrus peel at a mixing ratio of 1:1 based on the dry weight of the total solid, while the WB was used in the citrus peel to biochar ratios of 1:3, 1:2, 1:1 and 2:1 based on the dry weight of the total solid. The summary of the experimental plan is shown in Table 2. Incubations were set up in duplicate with a working volume of 300 ml in a 500 ml Duran bottles. The inoculum was incubated at 35 ^{0}C for 2 d to reduce significantly the organic content, after which the components were mixed together with the substrate to commence the experiment. The pH of each of the reactors was adjusted to approximately pH 7, after which nitrogen gas was used to purge the system for 1 min to remove excess O_2 gas. At the end of the incubations, the digestates were separated from the biochar using a 1mm screen for further analysis.

2.3.Chemical analysis

The total solid (TS) and volatile solid (VS) content were analysed by heating the samples in an oven (Memmert, Germany) at 105 ^{0}C and a furnace (Carbonite, Sheffield

UK) at 550 ^{0}C for 24 h, respectively for TS and VS determination (APHA, 1998). The pH reading was monitored with a pH meter (Conrad, Model 100 ATC) after which the samples were centrifuged at 4500 rpm for 15 min and the supernatant were filtered through a cellulose acetate membrane with pore size of 0.45 µm to obtain a soluble fraction. The soluble fractions were used to determine the soluble chemical oxygen demand (SCOD) and the total volatile fatty acid (TVFA). The determination of SCOD (Hach, LCK 514) and TFVA (Hach, LCK 114) was performed using the digestion test kits containing dichromate and diols, respectively. The method involved a simple digestion and the changes in colour were measured using quantitative Hach spectrophotometer (DR/2800). Furthermore, the samples were dried in oven at 60 ^{0}C for elemental and lignocelluloses determination. The elemental determination of carbon, nitrogen, hydrogen, sulphur and oxygen content of the sample was carried out using the ball milled dry sample with an elemental analyser (Vario EL III Elementar) (Carter & Barwick, 2011; Otero et al., 2011). The cellulose, hemicellulose and lignin content of the sample were analysed using the refluxing setup and a fibre detergent concentrate; while the values of the lignocellulose compositions were measured using a gravimetric method (Mertens et al., 2002) (ANKOM, USA). The concentration of limonene was determined using thermal desorption GC–MS with an Ultra-2 capillary column (50 m ×0.22 mm I.D. × 1.05 mm film thickness, 5% phenylmethylsilica, Hewlett Packard; Varian Inc, Palo Alto, CA, USA) for compound separation. The GC oven was initially held at 35 °C for 2 min, heated to 160 °C at 4 °C min^{-1}, then heated at 45 min^{-1} to 300 °C, which was held for 10 min (Vickers et al., 2009). A 5 µl of the sample was injected using a 10 ul syringe into the adsorbent resins, Tenax TA and Carbotrap (SupelcoInc, Bellefonte, PA, USA) as helium gas was continuously flushed through the sampling tubes (Vickers et al., 2009).

2.3.1. Cation exchange capacity (CEC)

This analysis was carried out to measure the capacity of biochar to hold and exchange cations using 1 M solution of sodium and aluminium acetate interchangeably (Huff et al., 2014). The pH of sodium and aluminium acetate was adjusted to 8.2 and 7, respectively. The sodium acetate solution was loaded into a flask containing 4 g of biochar, which displaces the other existing cations on the surface of the biochar. After centrifugation (Rotana Zentrifugen) at 500 rpm for 10 min, the supernatant was discarded. At this point, the negative region of the biochar residue is covered with significant amount of sodium ions. Then the solution of ammonium acetate was added to the biochar to displace the sodium ions. The mixture was then centrifuged, the supernatant was removed and flame photometer (Jenway, UK) was used to measure the amount of displaced sodium ions (Black, 1965). The concentration of the displaced sodium is proportional to the cation exchangeable capacity of the biochar material.

2.3.2. Methylene blue adsorption

The adsorption procedure measures the interaction between the biochar and organic compounds (Huff et al., 2014). A fixed biochar concentration (600 mg l^{-1}) was placed, into a 500 ml Duran bottle containing 200 ml of different concentration of methylene blue (10, 20, 40, 60, 80 and 100 mg l^{-1}). The pH of methylene blue was 6.5. The bottles were placed on a shaker and maintained at room temperature for 2 d after which the solution was filtered using cellulose acetate membrane with a pore size of 0.90 µm. The initial and final concentrations were measured using visible spectrophotometer at a wave length of 665 nm (Yuan et al., 2011; Bhatt et al., 2012). The results were then normalized by subtracting the control values. The sorption of methylene blue to different biochar was enumerated with equation 1 and represented on Figure 1.

$$Q_e = \frac{(C_0 - C_e)V}{M} \quad (1)$$

Where Q_e is the amount of methylene blue sorbed onto the biochar at equilibrium ((mg g^{-1}), C_0: initial concentration of the methylene blue in solution (mg l^{-1}), C_e is the final concentration of the methylene blue in equilibrium (mg l^{-1}), M is the mass of the biochar (g) and V is the volume of the sorbent in solution (ml).

2.3.3. Scanning electron microscopy

The biochar material was separated from the digestate and visualised through a scanning electron microscope (SEM) (PhenomWorld, Netherland). The tissue of the biochar was fixed using 4% glutaraldehyde containing 0.1M phosphate buffer at 4 ^{0}C (Ramage et al., 2002).The biochar samples were later dehydrated with an increasing concentration of ethanol (50, 70, 80 90 and 100%) for an interval of 40 min. The addition of 100% alcohol was repeated twice. The ethanol-treated biochar samples were carefully placed in the desiccators to dry after which the particle were placed on carbon tube for SEM imaging. The images were displayed using the Window Photo Viewer (Figure 6).

2.3.4. Actual methane production

The production of methane was quantified using a calibrated tip meter equipped with electrical impulse sensors and a data logging unit. The actual methane production (ml) was determined by subtracting the methane production from inoculum only, biochar and inoculum only incubations. The total methane production in gVS^{-1}added was

determined by dividing the volume of the methane produced (ml) by the total mass of the initial VS added (gVS).

$$\text{Cumulative methane production} = \frac{ACH_4}{gVS} + \frac{ACH_4}{gVS} + \cdots n \qquad (2)$$

Where A_{CH_4} is the actual volume of methane (ml), VS is the volatile solid content of the citrus peel added (g).

2.3.5. Theoretical methane production

The theoretical methane production was calculated from equation 3, using the elemental composition of the molecular formulae of citrus peel sample, which is given as $C_aH_bO_cN_dS_e$ (Buswell & Mueller, 1952; Raposo et al., 2011). The theoretical methane was expressed as ml g VS^{-1} added.

$$\text{T } CH_4 = \frac{22.4(4a+b-2c-3d-2e)}{8(12a+b+16+14d+16e)} \qquad (3)$$

2.3.6. Efficiency of methane conversion ($\boldsymbol{E_{CH_4}}$)

The methane conversion efficiency is defined as the percentage of the actual methane divided the theoretical methane. This is expressed in equation 4

$$\boldsymbol{E_{CH_4}} = \frac{\boldsymbol{Actual\ methane\ (ml)}}{\boldsymbol{Theoretical\ methane\ (ml)}} \times 100\%$$

2.3.7. Modified Gompertz equation

The cumulative methane outputs for all of the incubations were checked for the alignment to the modified Gompertz equation (Zwietering et al., 1990). The application of the modified Gompertz equation is based on the assumption that methane production is a function of bacterial growth (Zhu et al., 2009). The model has been used to determine lag or acclimation phase in batch growth (Syaichurrozi et al., 2013). The Gompertz equation is given by equation 5

$$F = a * \exp(-\exp(((\frac{r*e}{a}) * (\lambda - t) + 1)) \quad (5)$$

Where F is the cumulative methane production, ml gVS^{-1} at any time t, a is the methane yield potential, ml g VS^{-1}, r is the maximum methane production rate, ml gVS^{-1} day^{-1}, e is the mathematical constant 2.718282, λ is the duration of lag phase and t is the time (days) at which cumulative methane production F is calculated. The kinetic constants of a, R and λ were estimated for each of the assay using non-linear regression with the help of polymath software according to Syaichurrozi et al. (2013).

2.3.8. Statistical analysis

Calculation of mean, standard deviation, and standard error were conducted using Microsoft Excel 2013. Sigma plot software, version 13.0 was used for statistical analysis of data and after passing the Shapiro-Wilk normality test, the one way Anova was implemented to assess the significance of daily methane production (n=2) between biochar types and ratios while the Holm-Sidak method was used for multiple comparison of mean value between groups. The significant test was set at $p<0.05$.

3. Result and Discussion

The result showed that the presence of limonene in the citrus peel waste inhibited methanogenesis but the control incubation that did not contain biochar recorded the longest lag phase. During the first 3 days of test incubation, the impact of limonene on the anaerobic digestion process was not visible as revealed by the production of methane. This could be ascribed to the hydrophobic nature of limonene, which can be enhanced by the emulsifying agent secreted by the bacteria cells to aid adsoption by the microbial cells (Skikkema et al., 1995).

3.1. Impact of biochar on methane production from citrus peel

The first studies involved evaluating the performance of three different types of biochar on anaerobic digestion of citrus peel, over a 30-day incubation period (Figure 2). The rate of methane production started immediately on day 1 of incubation with values of 9.3 ± 0.93, 25.0 ± 0.00, 5.38 ± 0.40 and 4.62-± 0.58 ml CH_4 gVS^{-1} day^{-1} for the CSB, WB, RHB and citrus peel only incubations, respectively (Figure 2). The WB incubation recorded the highest rate of methane production, which was statistically significant ($p<0.05$). At this point, the WB was suspected to have been the most active in mopping up the limonene compound, thereby creating the right condition for immediate break down of organic substrate into various metabolic intermediates (Li et al., 2014). However, after the 1st day, the methane production plateaued thereafter for all of the incubations until after day 3 of incubation, after which there was no measurable rate of methane production. The plateauing of the methane production on the 3rd day of incubation can be attributed to the presence of limonene in the citrus peel organic substrate. Limonene has been reported to severely inhibit the anaerobic digestion

process and methanogenesis (Mizuki et al., 1990; Martín et al., 2010; Martín et al., 2013). On the 8th day of incubation, methane production increased in the CSB and WB incubations with values of 16.6 ± 3.9 and 20.8 ± 0.0 ml CH_4 gVS^{-1} day^{-1}, respectively. A faster recovery rate for CSB and WB suggests that these materials were better for adsorbing limonene. On the other hand, the RHB remained inhibited until the 13th day of incubation, after which 10.4 ± 4.7 ml CH_4 gVS^{-1} day^{-1} was produced. However, significant increases ($p<0.05$) in the rate of methane production were observed from the 17th day of incubation with the RHB having the highest methane production value of 21.5 ± 1.8 ml CH_4 gVS^{-1} day^{-1}. From this point onwards, continuous peaks and increases in the methane production rates were observed for the RHB incubation, while the rate of methane production continued to decline for incubations CSB and WB (Figure 2). The reduction in methane production for incubations CSB and WB suggests that the available organic substrates were being metabolised. The performance of the RHB was not consistent with the methylene blue adsorption test in Figure 1, and this could be ascribe to the non-specificity in the adsorptive behaviour of the biochar could have contributed to early adsorption of the metabolite required for methane production (Bagreev et al., 2001). Li et al. (2013) reported that activated carbon can adsorb soluble metabolites such as acetic acid and possibly hydrogen ion; and biochar is structurally similar to activated carbon. As expected, the citrus peel only incubation had the longest inhibition period, which lasted 14 days (Figure 2). This further supports the notion that the addition of biochar was able to enhance the rate of recovery of the anaerobic bacteria following incubation with the citrus peel. This trend is similar to the result obtained by Watanabe et al. (2013), where showed higher levels of methanogenic activity following the addition of Japanese cedar charcoal to the anaerobic digestion of crude glycerol. This explained by the sorption of limonene to the biochar, thereby reducing the

bioavailable concentration of the compound in the medium (Tada et al., 2005; Palatsi et al., 2012). In addition, Mumme et al. (2014) showed that the addition of biochar to an anaerobic digestion system can reduce the microbial lag phase leading to a faster growth phase. However, the inclusion of different types of biochar in the anaerobic digestion of citrus peel did not increase the total methane production among the biochar treatments and non-biochar treatments, as there was no significant difference in the total methane production ($p>0.05$). Cumulative methane production values of 186.8 ± 5.80, 171.3 ± 0.00, 172.1 ± 2.45 and 165.9 ± 5.35 ml gVS^{-1} added were achieved by the CSB, WB, RHB and citrus peel only incubations, respectively (Table 4). The cumulative methane production were achieved on 19, 19, 23 and 20th day of incubation by the CSB, WB, RHB and citrus peel only incubations, respectively. Similarly, Mumme et al. (2014) measured slightly higher cumulative methane yields for biochar amended incubations during incubations investigating mitigation of ammonia inhibition. The methane yield obtained for the citrus only incubation was similar to the result obtained by Serrano et al. (2014), who recorded 165 ml gVS^{-1} at an OLR of 0.4 to 1.6 gVS L^{-1} day^{-1}.

The second study in this investigation was conducted to evaluate the performance of different ratios of biochar on the anaerobic digestion of citrus peel over a 30-day incubation period (Figure 3). Four different mixing ratios of citrus peel to WB were used in this study, 1:3, 1:2, 1:1 and 2:1, in which the rates and cumulative methane production were measured. The rate of methane production started immediately on the 1st day of incubation with values of 13.02 ± 4.34, 7.6 ± 0.60, 6.97 ± 0.25, and 4.62 ± 0.58 ml CH_4 gVS^{-1} day^{-1} for the incubations 1:3, 1:2, 1:1 and 2:1, respectively (Figure 2). The 2:1 incubation recorded the lowest rate of methane production ($p>0.05$). However, after 3 days of incubation, the rate of methane production plateaued to a non-

measurable rate for all of the incubations except 1:3, which recorded a daily average value of 2.69 ± 1.09 ml CH_4 gVS^{-1} day^{-1} before increasing to 7.37 ± 3.01 ml CH_4 gVS^{-1} day^{-1} on the 9^{th} day of incubation. As mentioned earlier, the limonene content of the citrus peel is thought to have inhibited methanogenesis and reduced the methane production significantly. It was observed that the inhibition of methanogenesis decreased with a decrease in the citrus peel to WB ratio as incubations 1:3, 1:2 and 1:1 were only inhibited for 1, 2 and 3 days, respectively (Figure 3), while the 2:1 incubation was inhibited for 5 days. This further supports the idea that the presence of the WB reduces the bioavailability of the limonene (Mattson et al., 1969). This trend agrees with the phenomenon that incremental increases in sorbent concentration should have a positive effect on the removal of the sorbate (Namasivayam et al., 2001; Gupta et al., 2003). Comparing incubation containing only citrus peel, the rate of inhibition lasted for 7 days as opposed to 1-5 days for incubations containing WB. Furthermore, the addition of WB decreased the time for total methane production by an average of 17 days compared to 20 days by the citrus only incubation. In addition, biochar is thought to have provided a surface on which the microbial cells could colonise (Laine et al., 1991). The immobilisation of microbial cells has been reported to reduce the distance between syntrophic bacteria and methanogens; this increases the oxidation of volatile fatty acids and hydrogen production (Stams, 1994; Schink, 1997). However, the increase in WB ratios did not increase the total methane production among the test incubations, as there was no significant difference in the total methane production ($p > 0.05$). Cumulative methane production values of 184.4, 178.8, 174.2 and 183.9 ml gVS^{-1} added were achieved by incubations 1:3, 1:2, 1:1 and 2:1, respectively (Table 4).

3.2.Kinetics of methane production

Based on the based on the hypothesis that the extent of methane production is directly linked to the growth of methanogenic populations, the modified Gompertz equation was fitted into the cumulative production of methane curve and the values of the parameters obtained (Table 3). The regression obtained was ≥ 0.990 and the modified Gompertz fitted curve for citrus peel, citrus peel to biochar types and ratios is represented in Figures 4. The data show that the addition of biochar resulted in variations in the length of the lag phases, which is linked to the sorbing properties of the biochar and reductions in the bioavailability of limonene thereby increasing the recovery rate of the microbial cells (Hale et al., 2015). Limonene and biochars are hydrophobic and, as a result, van de Waal forces promulgate the sorption of the aromatic compound onto the carbonaceous sorbent (Mattson et al., 1969; Moreno-Castilla, 2004).

3.2.1. Citrus peel to biochar types and ratios

Firstly, the Gompertz equation was fitted into the cumulative production of methane curve for the study on citrus peel and biochar types. As presented in Table 3, based on the Gompertz model (Eq. 5), a lag phase of 6.8, 7.3 and 12.8, 13.4 days was achieved by incubations WB, CSB, RHB and citrus peel only, respectively. The addition of the WB incubation resulted in the shortest lag phase, while the citrus peel only incubation produced the longest lag phase, which was 6.6 and 6.1 days longer than the WB and CSB incubations, respectively. However, among the biochar types, the addition of the RHB to the digestion produced the longest lag phase of 12.8 days, although its sorbtion capacity was the greatest of all of the biochars (Figure 1). This result suggests that the WB was the best biochar material for counteracting the impact of limonene of the citrus peel waste on methanogenesis. This is consistent with the study by Kizito et al. (2015),

which showed that WB was more effective in removing ammonium nitrogen during the anaerobic digestion of piggery slurry. Values of 44.64 ± 0.602 and 39.8 ± 0.54 mg g^{-1} were achieved for WB and RHB, respectively. The long lag phase and non-measurable rates of methane production from the 3^{rd} – 13^{th} days of incubation for RHB suggest limonene suppression.

Similarly, the Gompertz equation was fitted into the cumulative production of methane curve for the study on citrus peel and biochar ratios. As presented in Table 3, the lag phases decreased with an increases in the citrus peel to biochar ratios, but incubations 1:1 and 1:2 exhibited similar lengths of lag phase: 7.5, 8.7, 8.7, 9.4 days were measured in incubations 1:3, 1:2, 1:1 and 2:1, respectively. However, the lag phases measured in the citrus peel incubation was relatively longer than for the biochar ratios, with a value of 13.4 days. These results further suggest that the WB reduced the lag phase of the anaerobic digestion of citrus peel. The data also show that greater amounts of biochar will lower the lag phase of methanogenesis in the presence of citrus peel and associated limonene. This result is consistent with a study by Rao et al. (2009), which reported a 60% increase in mercury removal when the adsorbent was increased 4 fold.

3.3.Process performance

The process performance of the anaerobic digestion study was monitored using the methane conversion efficiency, residual limonene concentration, immobilised cells to the biochar materials and other parameters such as residual VS, TVFA, limonene removal efficiency and pH (Tables 4 and 5).

The methane conversion efficiency ranged from 82 to 95%, the lowest value corresponding to the citrus peel only incubation and 2:1 biochar treatment (Table 4). The value of the total methane conversion efficiency for the citrus peel only incubation was lower than the other incubations except for the 2:1 biochar treatment. Therefore, it can be concluded that the addition of biochar increased methane recovery during the anaerobic digestion of citrus peel waste. However, this parameter is not a good tool for evaluating the performance of the process when comparing the effect of different biochars and ratios. This is because there were no significant differences ($P > 0.05$) in the total methane production. The CSB incubation achieved the highest methane conversion efficiency for the treatment involving the different biochar types, with a value of 93.43% (Table 4). On the other hand, the 2:1 incubation produced the lowest methane conversion efficiency among the treatments involving the biochar ratios, with 82.7%. A high methane conversion efficiency implies that the anaerobic digestion process was effective; therefore, the CSB and incubation 1:3 were the most effective treatments.

During the anaerobic digestion of organic substrates, acidogenic microorganisms form soluble acidic metabolites and other low molecular weight compounds. These acidic metabolites include volatile fatty acids (VFAs) (Parawira et al., 2004). The accumulation of VFAs is an indication of instability during anaerobic digestion, but moderately VFA accumulation suggests good kinetics between acid producers and users (Ahring et al., 1995). In this study, the residual total VFA production was below 145 mg/l for all of the incubations, which reflects good kinetics between the acid producers and users. In addition, the final pH was between 7.3 to 7.5 for all of the incubations although the pH measurement has been reported to be a poor parameter for monitoring process performance during anaerobic digestion (Switzenbaum et al.,

1990). The residual volatile solid is also a good parameter that represents the extent of solubilisation (Raposo et al., 2008). In this current study, the leftover particles of biochar in the digestate interfered with the results from the residual volatile solid analysis.

3.4.Residual limonene

The limonene concentration was measured at the beginning and end of this experimental study. The initial and final concentrations of limonene are presented, respectively, in Tables 1 and 3 and the results show that the concentration of the limonene compound had decreased. The result is similar to the methylene blue adsorption study in Figure 1 as a residual limonene concentration of 19.86, 30.32 and 36.43 mg/l was observed for RHB, WB and CSB, respectively. The RHB achieved the highest sorption efficiency of 86.7%, which was higher than the other biochar materials. For the biochar ratios, the residual content of limonene decreased with an increase in the amount of biochar added to each incubation. The result showed that values of 8.33, 12.00, 15.60 and 29.30 mg/l were achieved by biochar ratios of 1:3, 1:2, 1:1 and 2:1, respectively. The 1:3 incubation achieved the highest removal efficiency of 94.4%, which was higher than the other incubations. However, the control incubation with only citrus peel recorded the second lowest concentration of residual limonene when compared with the biochar ratios and the highest when compared with the biochar types. A value of 10.48 mg/l and a removal efficiency of 92.9% were achieved by the incubation containing only citrus peel. It was expected that the control incubation would contain the highest concentration of residual limonene since no biochar material was added. This phenomenon can only be ascribed to the biodegradation of limonene. There

are indications that the limonene compound can be converted into other metabolites during AD (Di Pasqua et al., 2006; Di Pasqua et al., 2007). The citrus peel only incubation recorded the lowest methane yield and longest microbial lag phase, suggesting that the limonene or putative metabolites are inhibiting methanogenesis.

3.5.Morphology of biochar

Comparative microbial morphology of the different biochars before and after inoculation with digested sewage sludge inoculum and citrus peel waste was conducted with scanning electronic microscopy. Figure 5 shows the SEM images of the biochar materials with microbes. The figures show that the microbes successfully colonized the biochars, with the exception of the CSB. The overall coarseness of the surface of the biochars offer a conducive environment for the colonization and growth of microbial cells and possibly biofilms, as shown in Figure 5. The greatest number and diversity of morphologies were found on the WB. This might be attributed to the abundance of macropores on the surface of the material (Laine et al., 1991). Based on the morphology of the microbial cells on the biochar material, it is suspeced to have been composed of coccobacillus of *Methanosarcina*, short rods of *Methanosaeta* and long rods of *Methanobacterium* – like bacterial cells (Uemura & Harada, 1993). This is similar to the results obtained by Lopez et al. (2014), where corn cob biochar was used as a support for biofilm growth during the anaerobic digestion of grease trap wastewater: archaeal populations such as *Methanobacteriales* and *Methanomicrobia* were identified. However, a more detailed biochemical or molecular biology test would be required to verify this assertion. Strangely, the CSB showed no visible microbial cell attachment; it is possible that the cells were removed during sample preparation. The

presence of colonies on the biochar indicates the immobilization of microbial cells and supports methanogenic activity (Kuo & Shu, 2004).

4. Conclusion

The addition of biochar to the anaerobic digestion of citrus peel waste reduced the microbial lag phase, increased the rate and extent methane production relative to the incubation without biochar. The WB recorded the shortest lag phase while the CSB achieved the highest methane yield when comparing the effect of different biochar on anaerobic digestion of citrus peel. The study also showed that the microbial lag phase increased with increase in biochar ratio, which suggests that WB and CSB material at higher ratios are sufficient to maintain the stability of anaerobic digestion process, especially during substrate-induced inhibition. Now, there are few applications for biochar in anaerobic digestion and this study shows that biochar can be used as a stabilizing agent. However, there is need to investigate the impact of biochar in anaerobic digestion of citrus peel and other substrate-induced inhibition using a continuous test and monitoring the concentration of inhibitor adsorbed and metabolized. This study showed that the addition of biochar would improve anaerobic digestion of citrus peel by adsorbing limonene.

Acknowledgement

This work was supported by the Centre for Global Eco-innovation (X02646PR) and Stopford Energy and Environment Ltd

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Tables

Table 1: Characteristics of different biochar and citrus peel waste material

Parameter	Biochar			Substrate
	Coconut shell	Rice husk	Wood	Citrus peel
TS (% DW)	91.4 ± 0.24	95.0 ± 0.04	93.4 ± 0.24	16.6 ± 0.21
VS (% DW)	99.0 ± 0.49	54.1 ± 0.04	95.3 ± 0.39	97.5 ± 0.26
Carbon (%)	139 ± 7.04	79.4 ± 3.43	68.5 ± 0.00	41.7 ± 0.00
Nitrogen (%)	0.52 ± 0.03	0.53 ± 0.02	0.61 ± 0.00	00.8 ± 0.00
pH	8.3± 0.03	8.4± 0.08	8.67 ± 0.01	5.98 ± 0.00
NH_4-N (mg l^{-1})	0.36	0.37	0.417	0.12
Cellulose (%)	ND	ND	ND	20.45 ± 1.06
Hemicelluloses (%)	ND	ND	ND	6.61 ± 0.79
Lignin (%)	ND	ND	ND	2.29 ± 0.82
CEC (meq 100 g^{-1})	31.1 ± 0.35	43.2 ± 1.49	27.1± 0.82	ND
Limonene (mg/l)	ND	ND	ND	149.5 ± 15.4

Values are expressed in mean and standard error (n=3)
TS, VS, NH_4-N, pH and limonene were analysed based on fresh basis while other parameters were based on dry mass
CEC (Cation exchange capacity)
ND (Not determined)

Table 2: Batch experimental conditions for citrus peel waste, citrus peel with different biochar and ratios

Incubation	g VS	g TS	Incubation	g VS	g TS
WB	2.89	2.95	1:3	10.26	10.70
CSB	2.89	2.95	1:2	6.84	7.16
RHB	2.89	2.95	1:1	3.42	3.59
Citrus peel	2.89	2.95	2:1	1.71	1.79
Digested sludge	8.67	0.00	Digested sludge	11.44	0.00

Table 3: Summary of kinetic data for citrus peel waste, citrus peel with different biochar and ratios after 3 d of incubation

Category	Incubation	CH_4 yield (ml gVS^{-1}added)	Modified Gompertz parameter (model)			R^2
		F	p	r	λ	
Biochar ratios	1:3	154	150	29.0	7.5	0.990
	1:2	148	148	28.4	8.7	0.996
	1:1	154	152	22.5	9.4	0.995
	2:1	121	122	29.0	9.4	0.994
Biochar types	CSB	146	146	26.0	7.3	0.999
	WB	117	116	18.4	6.8	0.993
	RSB	147	150	26.6	12.8	0.999
	Citrus peel	140	156	21.8	13.4	0.992

F is the cumulative methane production, ml gVS^{-1} at any time t, a is the methane yield potential, ml g VS^{-1}, r is the maximum methane production rate, ml gVS^{-1} day^{-1} and λ is the duration of lag phase)

Table 4: Comparison between actual, theoretical methane productions and residual limonene concentration for citrus peel waste, citrus peel with different biochar and ratios

Category	Incubation	CH_4 yield (ml gVS^{-1}added)		CH_4 conversion efficiency (%)	Residual limonene (mg/l)	Removal efficiency (%)
		Theoretical	Actual			
Biochar ratios	1:3	197.40	184.40	93.43	8.33	94.42
	1:2	197.40	168.80	85.34	12.00	91.97
	1:1	197.40	178.20	90.60	15.60	89.56
	2:1	197.40	163.90	82.76	29.30	80.40
Biochar types	CSB	197.40	186.80	94.60	36.43	75.63
	WB	197.40	171.30	86.70	30.32	79.71
	RHB	197.40	172.10	87.10	19.86	86.71
	Citrus peel	197.40	165.90	84.00	10.48	92.98

Table 5: Chemical profile of citrus peel waste, citrus peel with different biochar and ratios after 30 days of incubation

Biochar types	Initial pH	Final pH	Final VS%	Residual TVFA (mg/l)	Biochar ratios	Initial pH	Final pH	Final VS%	Residual TVFA (mg/l)
WB	7.02	7.30 ± 0.05	51.0 ± 0.13	123.50 ± 0.50	1:3	7.01	7.37 ± 0.03	57.8 ± 0.35	86.40 ± 3.00
CSB	7.01	7.36 ± 0.05	53.1 ± 1.46	112.65 ± 16.35	1:2	7.00	7.45 ± 0.02	60.4 ± 0.94	79.70 ± 5.00
RHB	7.00	7.49 ± 0.01	53.3 ± 1.71	143.65 ± 10.35	1:1	7.02	7.36 ± 0.01	61.0 ± 1.50	98.40 ± 7.20
Citrus peel	7.05	7.38 ± 0.04	56.7 ± 0.25	117.80 ± 11.50	2:1	7.00	7.46 ± 0.03	61.0 ± 2.26	110.75 ± 12.25

TVFA (total volatile fatty acid)
Values are expressed in mean and standard error (n=3)

Figure captions

Figure 1. Methylene blue adsorption process as a function of the initial concentration for 48 hrs at 25 ^{0} C. Where Q_e is the amount of methylene blue sorbed onto the biochar at equilibrium ((mg g^{-1}), C_0: initial concentration of the methylene blue in solution (mg l^{-1})

Figure 2. Rates and cumulative methane production in each reactor during the anaerobic digestion of different biochar with citrus peel. (A) CSB + citrus peel. (B)WB + citrus peel. (C) RHB + citrus peel. (D) citrus peel only. Vertical bars indicate standard error (n=2)

Figure 3. Rates and cumulative methane production in each reactor during the anaerobic digestion WDB to citrus peel ratio. . (A) 2:1. (B) 1:1 (C) 1:2. (D) 1:3. Vertical bars indicate standard error (n=2)

Figure 4. Modified Gompertz equation fit and cumulative methane production after 3 d of incubation for different biochar with citrus peel. (A) CSB + citrus peel experiment. (A`) CSB + citrus peel model. (B) WB + citrus peel experiment. (B`) WB + citrus peel model. (C) RHB + citrus peel experiment. (C`) RHB + citrus peel model. (D) citrus peel only experiment. (D`) citrus peel only model, (E) WB to citrus peel ratio 1:3 experiment. (E`) 1:3 model. (F) WB to citrus peel ratio 1:2 experiment. (F`) WB to citrus peel ratio 1:2 model. (G) WB to citrus peel ratio 1:1 experiment. (G`) 1:1 model. (H) WB to citrus peel ratio 2:1 experiment. (H`) 2:1 model.

Figure 5. SEM showing the morphology of the different types of biochar before (D; RHB, E; WB and F CSB; maginfication: 3000x) and after (A; RHB, B; WB and C CSB; magnification: 5000X) inclusion in the anaerobic digestion of citrus peel waste (30 ds incubation time).

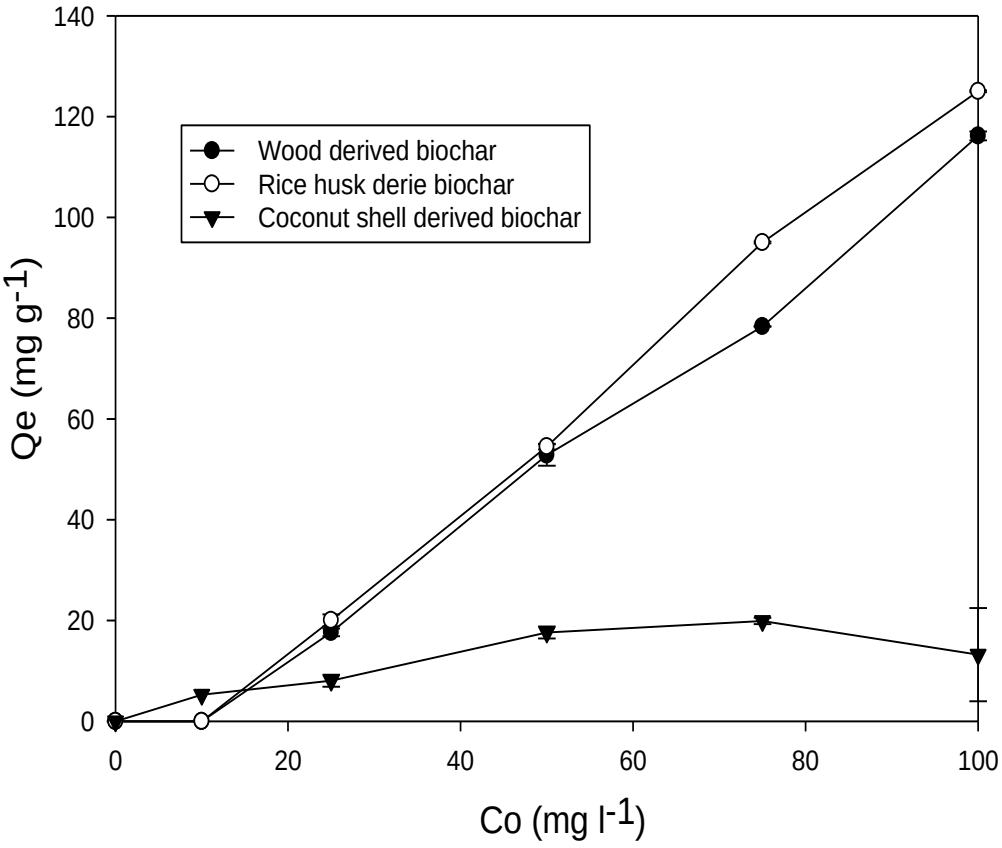
Wood derived biochar
Rice husk derie biochar
Coconut shell derived biochar
Qe (mg g-1)
Co (mg l-1)
0
20
40
60
80
100
120
140

Figure 1

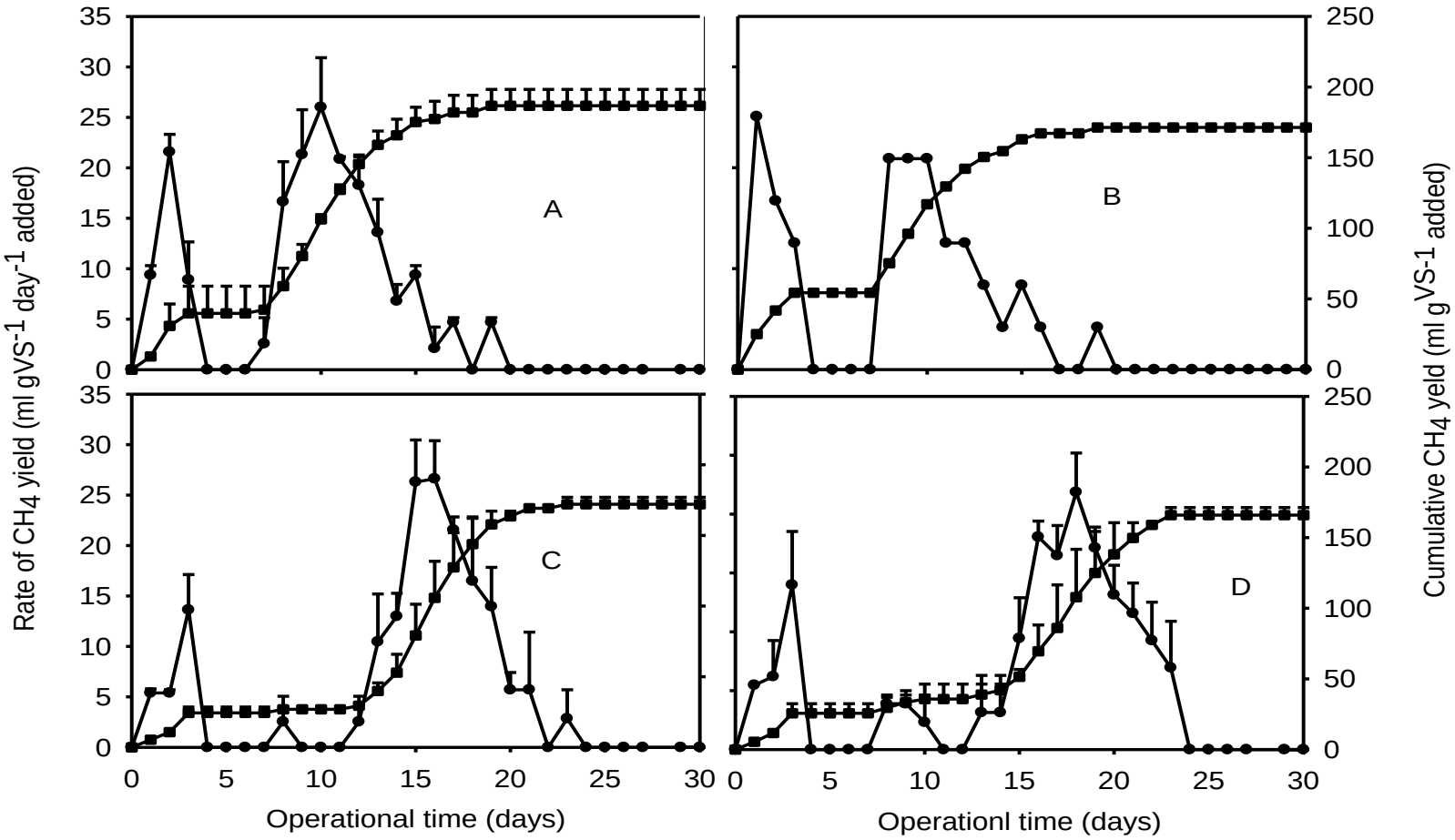
Rate of CH4 yield (ml gVS-1 day-1 added)
Cumulative CH4 yeld (ml gVS-1 added)
Operational time (days)
Operationl time (days)
A
B
C
D
0
5
10
15
20
25
30
35
50
100
150
200
250

Figure 2

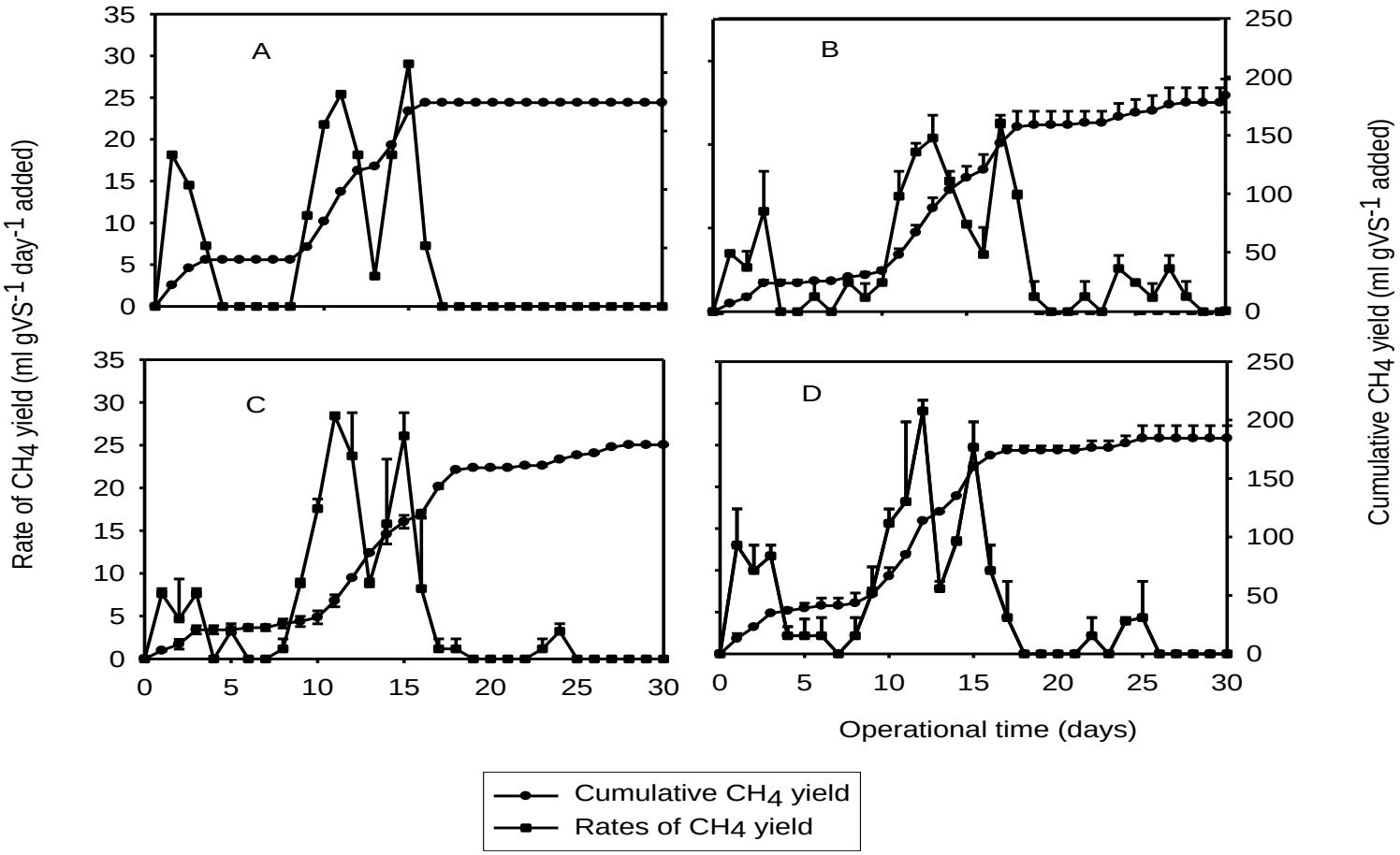
A
B
C
D
Rate of CH4 yield (ml gVS-1 day-1 added)
Cumulative CH4 yield (ml gVS-1 added)
Operational time (days)
Cumulative CH4 yield
Rates of CH4 yield

Figure 3

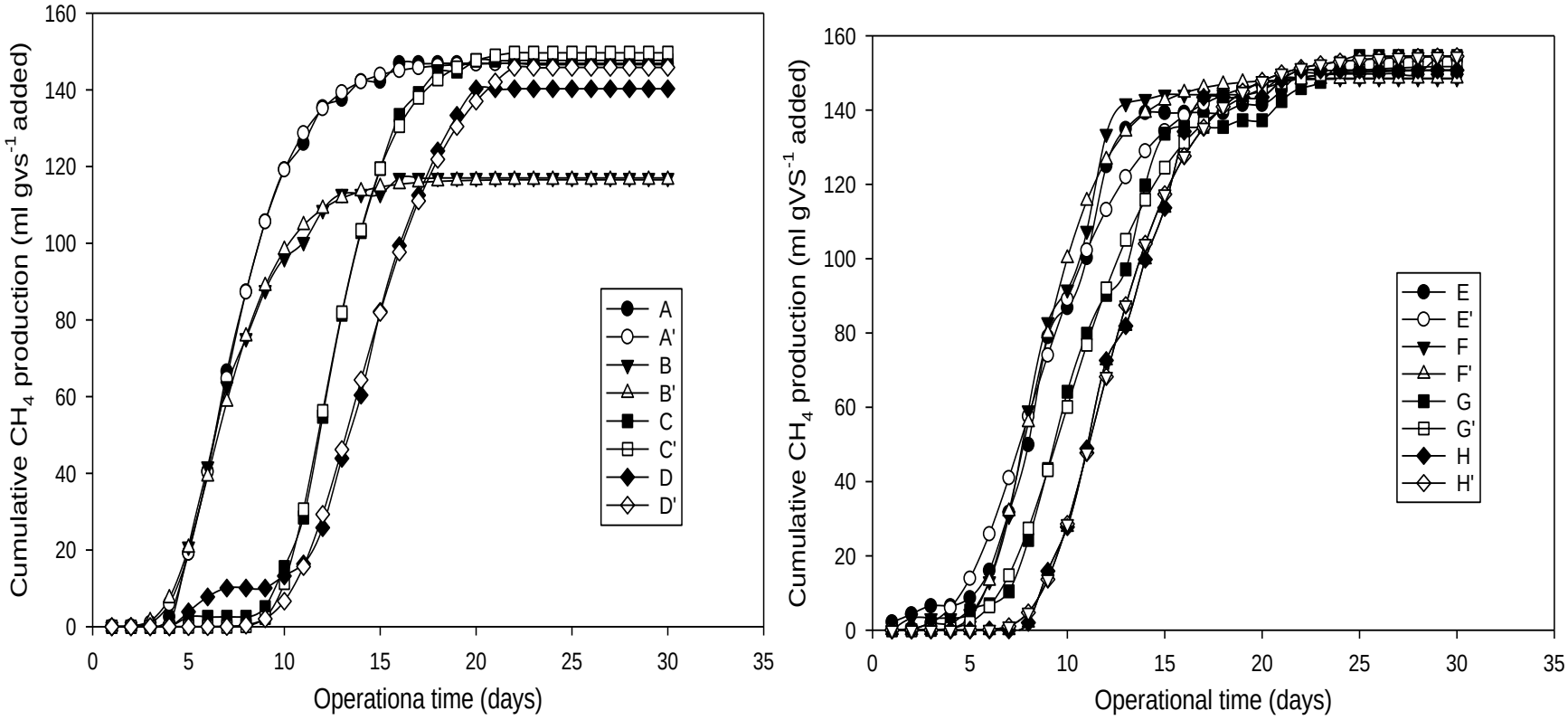
Cumulative CH4 production (ml gvs-1 added)
Operationa time (days)
A
A'
B
B'
C
C'
D
D'
Cumulative CH4 production (ml gVS-1 added)
Operational time (days)
E
E'
F
F'
G
G'
H
H'

Figure 4

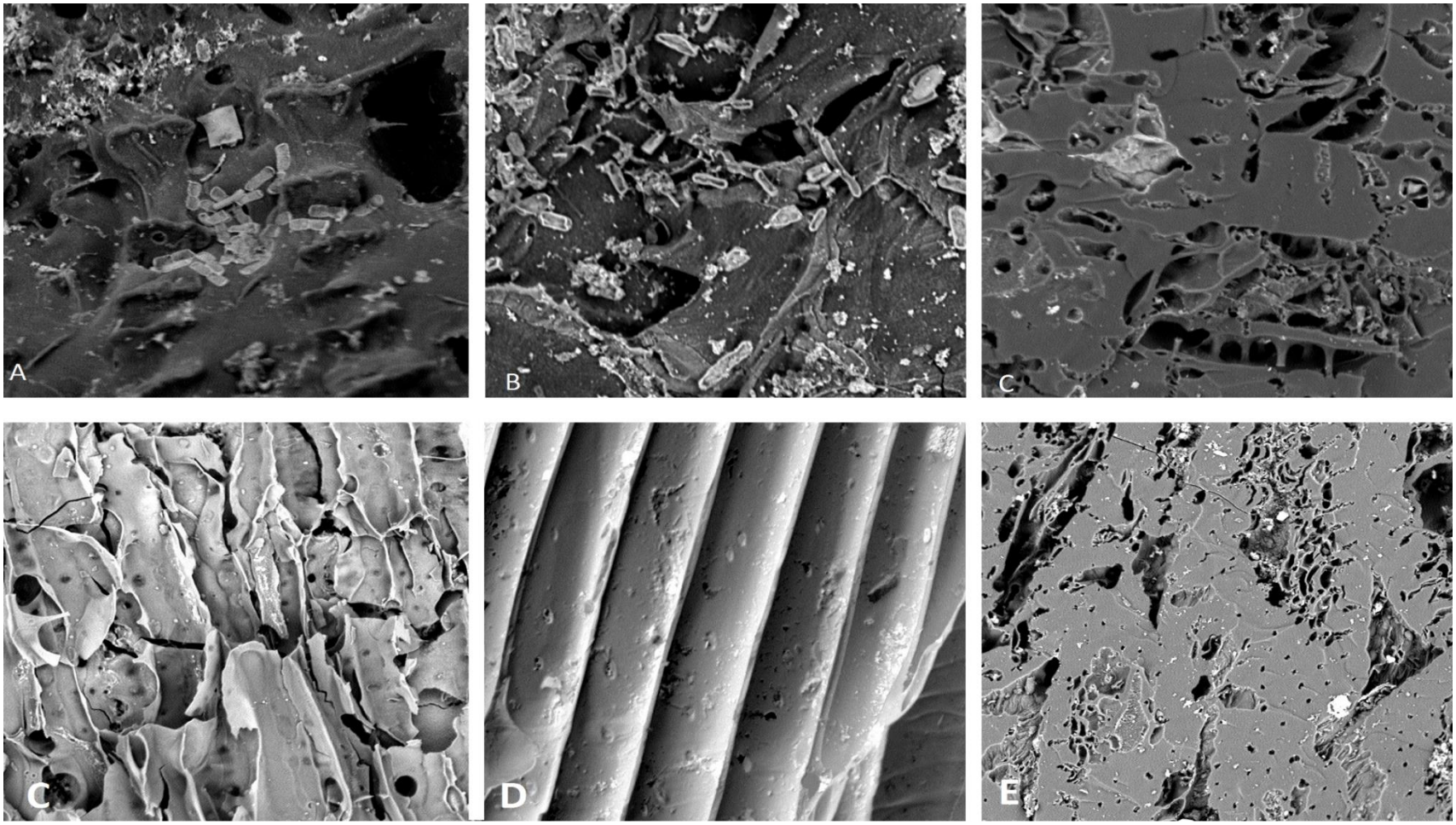

Figure 5

5. Paper V

Comparative performance of mesophilic and thermophilic anaerobic digestion of citrus fruit waste

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Abstract

In this study, the anaerobic digestion of citrus fruit waste was investigated using a batch test system at different operating temperatures (35, 40, 45, 50 and 55 ^{0}C). Semi-continuous anaerobic digestion of citrus fruit waste was also investigated at 35 ^{0}C and 55 ^{0}C with increasing organic loading rates (OLR; 0.71, 1.42, 2.85, 4.00 and 5.00 gVS l^{-1} day^{-1}) and hydraulic retention times of 140, 70, 41, 28 and 22 days, respectively. For the batch test, the addition of 3.42 gVS of citrus fruit waste resulted in 3-7 days of inhibition for incubations at 35, 40 and 50 ^{0}C while incubation 45 and 55 ^{0}C remained uninhibited throughout the experimental study. For the semi-continuous incubations, the rise in organic loading rates reduced methane yields, particularly under mesophilic conditions. Thermophilic conditions resulted in statistically significant ($p<0.05$) higher amounts of methane production at OLRs of 1.46, 4.00 and 5.00 with values of 5.35 ± 0.10 , 2.98 ± 0.05 and 5.61 ± 0.05 lCH_4 gVS^{-1}, respectively. Thus, for the anaerobic digestion of citrus fruit waste, thermophilic conditions (55 ^{0}C) appear sufficient although, this condition requires additional energy for heating. Mesophilic conditions (35 ^{0}C) require less energy but when the OLR was > 2.85 gVS $l^{-1}day^{-1}$, this study showed that limonene suppression was higher and methane output was lower at 35 ^{0}C. These findings show that the operation of the AD process involving CFW is most suitable at higher temperatures of 45 and 55 ^{0}C.

Keywords: citrus fruit waste; limonene; anaerobic digestion; acclimation of microbial cells; thermophilic and mesophilic conditions.

1. Introduction

Anaerobic digestion (AD) is a well-established technology for the treatment of biodegradable organic waste and the generation of sustainable energy. The widespread application of this technology has continued to grow globally with Europe and Asia having the highest numbers of large and small-scale anaerobic digesters**,** respectively **(**Akinbami et al., 2001; Arthur et al., 2011; Bond & Templeton, 2011; IEA Bioenergy, 2014)**.** However, with the consistent increase in the application of this technology, substrate-induced inhibition (SII) needs to be considered before selecting a particular organic substrate (Chen et al., 2008). SII occurs when the constituents or metabolic intermediates from the AD of organic materials inhibit microbial activity, resulting in lower biogas production and potential failure of the AD system. Single-substrate AD, which involves using one type of feedstock, is a major cause of SII. Single-substrates, such as animal fat, citrus residues, slaughterhouse wastewater, textile and pulp residues, are often avoided because they contain chemicals or metabolites that can destabilize AD, leading to a failure in the process. (Pokhrel & Viraraghavan, 2004; Badani et al., 2005; Martín et al., 2010; Yoon et al., 2014; Yalcinkaya & Malina, 2015). However, the occurrence of SII in AD can be directly and indirectly associated with the organic substrate. Direct sources of SII are caused by the constituents of the organic substrates (pesticides, limonene, antibiotics and heavy metals), while indirect sources of SII result from metabolic intermediates (ammonia, sulphide and long chain fatty acid) produced during the AD of organic substrates (Palmqvist & Hahn-Hagerdal, 2000; Georgiou et al., 2004; Wilkins et al., 2007; Chen et al., 2008; Sousa et al., 2013; Xiao et al., 2013; Meyer & Edwards, 2014). Citrus fruit waste (CFW) is an example of a direct source of SII and is both a constituent of food waste and a single waste stream from fruit industries. According to the FAOSTAT (2015) statistics show that approximately 87

million tonnes of citrus fruit were produced globally in 2013. In the UK, the fruit and vegetable sector accounts for 13% of the waste arising from the food industry; this is approximately 1.9 million tonnes of vegetables and 1.1 million tonnes of fruits annually (WRAP, 2012). The UK imports 709 kt of citrus fruit per annum with no home production and reports show that imports of fresh fruits including citrus increased by 10% in 2013 (Defra, 2013). This organic substrate is seldom used in AD; operators are keen to separate citrus fruit material from their waste streams because it contains limonene, which is inhibitory to microbial growth and activity (Wikandari et al., 2015). Previous research on the AD of orange peel at mesophilic (35 ^{0}C) and thermophilic (55 ^{0}C) temperatures showed that organic loading rates (OLRs) of between 5.1 and 5.6 gVS $l^{-1}day^{-1}$ resulted in the failure of the process and this failure was attributed to the limonene present in the orange peel (Kaparaju & Rintala, 2006; Martín et al., 2010; Wikandari et al., 2015).

The most economical approaches for counteracting SII have been through (i) co-digestion with other substrates in order to reduce the concentration of the available inhibitory compound(s), or (ii) acclimation of the microbial cells to the inhibitory compound(s) (El-Mashad & Zhang, 2010; Zhang & Jahng, 2012). For example, the co-digestion of orange peel and crude glycerol reduced the toxicity of limonene and increased the biogas production (Martin et al., 2013). However, co-digestion is only suitable for AD operators who have access to appropriate co-substrates. In the event of low co-substrate availability, a different approach may be required to counteract the SII. An alternative approach is the acclimation of microbial cells to unfavourable conditions; this can be used to counteract SII, but it is time consuming (Palmqvist & Hahn-Hagerdal, 2000; Georgiou et al., 2004; Wilkins et al., 2007; Chen et al., 2008; Sousa et al., 2013; Xiao et al., 2013; Meyer & Edwards, 2014). The acclimation of microbial

cells to SII can be achieved through different physiological changes, such as (i) the synthesis of specific enzymes which are absent prior to the inhibition; (ii) the emergence of new metabolic pathways, and (iii) modification of the surface layer of the cell membrane (Liebert et al., 1991; Ruiz & Flotats, 2014). For example, when microorganisms were exposed to limonene concentrations of between 24 and 192 mg l^{-1} d^{-1}, changes were observed on the surface layer of the microbial cell membranes, with the level of unsaturated fatty acids gradually increasing (Ruiz & Flotats, 2014).

Previous studies on limonene-rich substrates have mainly focused on the AD of orange peel, which only contains D-limonene and makes up only 82% of the amount of citrus fruit produced annually (Duetz et al., 2003; FAOSTAT, 2015). CFW was used in this study because it contains both D and L-limonene, a complete representation of the CFW (orange, lime, lemon and grape). The two aims of this study were to investigate the impact of different operating temperatures (35, 40, 45, 50, and 55 °C) on AD of CFW using a batch test and to explore the stepwise adaptation of the AD process to increasing concentrations of CFW under mesophilic (35 °C) and thermophilic (55 °C) conditions using a semi-continuous digestion system. The ultimate outcome was the establishment of optimal conditions for the viability of CFW as a mono-substrate for AD, thus enabling AD operators to use this waste stream as a potential valuable resource.

2. **Materials and methods**

2.1. Substrate

The CFW was a mixture of orange, grape, lemon and lime in the ratios of 8:4:2:1. The mixing ratio was selected based on the average amount of CFW generated annually (FAOSTAT, 2013). The CFW was sourced from a local grocery store in Lancaster, UK. Firstly, the juice was squeezed after which the peel and the roughages were shredded, homogenised using a commercial blender (particle size of 1-3 mm) and freezed (-20 ^{0}C). Compositional characteristics, such as total solid (TS), volatile solid (VS), pH, lignin, hemicellulose, cellulose, total carbon and nitrogen, were determined (Table 1).

2.2. Inoculum

The inoculum was obtained from an anaerobic digestion treatment plant operated at 37 ^{o}C (Preston, UK). In order to achieve stable microbial populations for mesophilic and thermophilic microorganisms, the inoculum was dosed weekly with 0.25 g l^{-1} of glucose solution and incubated at 35 and 55 ^{o}C for 4 weeks, respectively. A 1 ml trace mineral solution (containing per l: 150 mg $FeCl_2.4H_2O$, 70 mg $ZnCl_2$, 100 mg $MnCl_2.4H_2O$, 190 mg $CoCl_2.6H_2O$, 2 mg $CuCl_2.2H_2O$, 24 mg $NiCl_2.6H_2O$, 36 mg $Na_2MoO_4.2H_2O$, 6 mg H_3BO_3, 3 mg Na_2-$SeO_3.5H_2O$ and 4 mg $Na_2WO_4.2H_2O$) modified from Zhang et al. (2011) was added on 1 d of incubation. At the end of the acclimation period, the inocula were analysed for VS, TS and pH before inoculating the CFW: 55.4 ± 0.21% (DW), 10.3 ± 0.12% and 7.1 ± 0.11 were recorded, respectively, in the mesophilic inoculum while the enriched thermophilic inoculum recorded the values of 52.3 ± 0.05 (DW), 9.8 ± 0.00% and 7.3 ± 0.12, respectively.

2.3.Batch experiments

The batch experimental set up is shown in Figure 1. A series of five batch experiments were performed in duplicate at varying operating temperatures of 35, 40, 45, 50 and 55 ^{o}C to evaluate the effect of temperature on AD of CFW. The ratio of substrate to inoculum was 1:3 (14.87 gVS in 0.5 l Duran bottles) with a working volume of 0.3 l. The reactors were fitted with a modified rubber stoppers containing gas and liquor sampling ports; the reactors were purged with nitrogen for 1 min. The biogas produced was fixed with 3 M NaOH solution to significantly remove the CO_2 before measuring the enriched methane gas volumetrically using a calibrated, electronic tip meter. The bottles were incubated in a water bath and continuously stirred at 30 rpm for 30 d of incubation. The mesophilic inoculum was used to inoculate the incubation set at 35, 40 and 45 ^{0}C, while the thermophilic inoculum was used to inoculate the assays set at 50 and 55 ^{0}C. The conditions of the batch experiment are summarized in Table 2.

2.4.Semi continuous experiments

The semi-continuous experimental schematic is presented in Figure 2. The mesophilic (35 ^{0}C) and thermophilic (55 ^{0}C) AD of CFW was carried out in duplicate using the same reactor - a continuously stirred tank reactor (CSTR) operated over a range of hydraulic retention times (HRTs) and at successive increases in organic loading rates (OLRs) of 0.7, 1.4, 2.8, 4.0 and 5.0 gVS $l^{-1}day^{-1}$. Once a day, the CFW was pumped in the reactor and the effluent removal was carried out. The reactor used for this study had a total capacity of 3 l and a working volume of 1.4 and 1.5 l for the mesophilic and thermophilic incubation, respectively. The reactor was encased with silicone tubing and thermostatically controlled using 5 l water bath and a submersible pump for water recirculation (Zhong ShanJiayu, 150W, 5000 L/H). A peristaltic pump (Watson,

Marlow, UK) was fitted separately on the upper lid and the lower side of the reactor in order to respectively add fresh feedstock and remove effluents. The reactor lid had several ports: central hole for the mixing shaft (30 rpm), a biogas channel, temperature and pH probe (pH, Conrad, Model 100 ATC). The pH was manually adjusted with 3 M Na0H and 1 M H_2SO_4 solution. After inoculating with the appropriate inoculum, the reactors were purged with nitrogen for 1 min to maintain an anaerobic condition. The biogas composition was monitored daily offline using a gas chromatography, volumetric biogas measurement was monitored online using an electronic tip meter. The conditions of the continuous experiment are summarized in Table 3.

2.5.Analytical Methods

The following parameters were determined for both influent and effluent samples: TS, VS, individual fibre content, limonene concentration, pH, elemental carbon, nitrogen, VFAs, individual and volumetric gas measurement. The TS and VS contents were determined according to standard method (APHA, 1998). Fibre analysis was carried using the fibre detergent concentrate (ANKOM, USA), refluxing set-up and a gravimetric device were used to determine the content of lignin, hemicellulose and cellulose. (Mertens et al., 2002). Total carbon and nitrogen were determined according to standard methods using an elemental analyser (Carter & Barwick, 2011) while pH reading was monitored with a pH meter (Conrad, Model 100 ATC) . Biogas volume was measured online using a custom made, electronic volumetric mass flow meter connected through a data acquisition (DAQ) card to a computer and a monitoring program written using LabVIEW software (National Instrument).

Offline biogas compositional analysis was carried out to determine the content of methane and carbon dioxide using gas chromatography (Perkin Elmer Auto system XL

equipped with a Hayesep Q 80 Mesh 6ft × 1/8 inch, 2.0 mm diameter and a dual detector FID and ECD). The injector and detector temperatures were maintained at 60 and 350 °C, respectively. The column temperature was at a gradient of 60 to 250 °C and argon was used as the carrier gas (Perkin Elmer). The Perkin Elmer Auto system XL Gas Chromatography is equipped with an automatic injector, 10 ml of sample gas were pressurised into an exetainer vials before placing it in the injector rack. Each run was carried out in duplicate with a methane and carbon dioxide standard. The VFAs (acetic acid, propionic acid, isobutyric acid, butyric acid and valeric acid) were quantified by ion chromatography (IC) (Dionex, ICS-30000, Thermo-Scientific, USA) using a UV index detector and an Aminex HPX-87H column (Bio-Rad, UK). The separation of VFAs during IC measurement was achieved using a mobile phase of 2.5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1}, a column temperature of 65 ^{o}C and the detector temperature was 40 ^{o}C (Oh et al., 2005; de Sá et al., 2011). The VFA marker mix containing acetic, propionic, iso-butyric, butyric, iso-valeric and valeric acids, each at 1 mg ml^{-1} (Sigma-Aldrich, UK) was used for to calibrate the IC equipment.

The concentration of limonene was determined using thermal desorption GC–MS with an Ultra-2 capillary column (50 m ×0.22 mm I.D. × 1.05 mm by injecting 5 µl of the sample and using a 10 ul syringe into the adsorbent resins, Tenax TA and Carbotrap (SupelcoInc, Bellefonte, PA, USA) as helium gas was continuously flushed through the sampling tubes. Then, samples were desorbed using automated thermal desorption (Turbomatrix ATD; Perkin Elmer, Norwalk, CT, USA) by heating the tubes at 280 °C and focusing the desorbed limonene on a Tenax TA cold trap at -30 °C for 6 min (Vickers et al., 2009). The GC oven was initially held at 35 °C for 2 min, heated to 160 °C at 4 °C min^{-1}, then heated at 45 min^{-1} to 300 °C, which was held for 10 min (Vickers et al., 2009).

*2.6.*Statistical analysis

Mean, standard deviation, and standard error were calculated using Microsoft Excel 2013 edition. For statistical analysis of methane production, the data were Log transformed to pass the Shapiro-Wilk normality test before accessing the two independent sample Welch's t test because of unequal variances and one way Anova Welch's test for the semi-continuous and batch experiment, respectively. Sample size was in duplicate and the SPSS 22.0 edition was used in the analysis as statistical significance was set at $p < 0.05$.

3. Results and discussion

*3.1.*Batch experimental study

The methane production was measured at different operating temperatures over the experimental period (Figure 3). The batch test was carried out at different operating temperatures (35, 40, 45, 50 and 55 ^{0}C) while other parameters, such as the OLR, mixing and SIR, were kept constant. It was observed that methane production was rapid and consistent for all of the incubations for the first 3 - 7 days after which the incubations at 35, 40 and 50 ^{0}C were inhibited for 8, 6 and 23 days, respectively (Figure 4). The early methane production was attributed to active microbial cells present in the inoculum; however, the sudden reduction in methane production may have been caused by the limonene contained within the CFW (Mizuki et al., 1990; Li et al., 2013). On the other hand, the incubations at 45 and 55 ^{0}C showed no inhibition, as methane production remained constant until the organic material was depleted (Figure 3). This further supports the findings that higher temperatures favour the AD of CFW (Martín et al., 2010). This was not the case for the incubation at 50 ^{0}C as there was no measurable methane production from the 11th day of incubation, suggesting inhibition of

methanogenesis. This can be explained by comparing the 50 and 55 ^{0}C incubations. No significant differences ($p>0.05$) in the rates of methane production were observed up to the 7th day of incubation, but after this time point, a decrease was observed in the 55 ^{0}C incubation, which was consistent up to the 12th day of incubation, when it increased, suggesting only a slight inhibition of the AD process. On the other hand, no measurable methane production was observed for the 50 ^{0}C incubation. This might be due to the production of metabolites from limonene biotransformation rather than the limonene itself as the rate of methane production was consistent for more than 6 days. Several studies have shown that monoterpenes, such as limonene, can be transformed into other compounds such as carveol, carvone, limonene-1, 2-diol, epoxide, perillyl alcohol and perillaldehyde (Chang & Oriel, 1994; Droby et al., 2008). However, the differential behaviour observed between the 50 and 55 ^{0}C incubations could be related to the inhibition of the secondary growth of microbial cells; 55 ^{0}C being the optimum temperature for microorganisms to be able to tolerate limonene-derived metabolites. According to Aitkhozhina et al. (1993), the secondary growth of microorganisms after exposure to unfavourable conditions can either be inhibited or stimulated. The secondary growth of microorganisms in the incubation at 50 ^{0}C may have been inhibited. The total methane production values were similar for the incubations at 35, 45 and 55 ^{0}C with values of 0.18 ± 0.01, 0.18 ± 0.10 and 0.19 ± 0.8l l CH_4 gVS^{-1}, respectively while at 40 and 50 ^{0}C were comparatively lower (with values of 0.13 ± 0.01 and 0.12 ± 0.01 l CH_4 gVS^{-1}, respectively (Figure 3). Despite the suppression from the limonene compound present in the CFW, the incubations at 35, 45 and 55 ^{0}C achieved higher methane yields than the incubations at 40 and 50 ^{0}C at the end of day 30. The values were not statistically significant ($p>0.05$) but the 45 and 55 °C incubations were not inhibited, as the methane production was mostly consistent.

*3.2.*Semi-continuous experimental study

3.2.1. Biogas production

Biogas production is a function of the substrate's organic content and digestibility; the daily variations in biogas production are shown in Figure 4. Table 3 shows the variation in the cumulative methane yield for the different OLRs and operating conditions. In this AD study, the OLR was gradually increased from 0.71 to 5.00 gVS $l^{-1}day^{-1}$and the HRT was consequently decreased from 140 to 22 days. The AD of CFW started immediately for both incubations displaying a low methane to carbon dioxide ratio initially.

In the mesophilic incubation, the initial OLR of 0.71 gVS $l^{-1}day^{-1}$ was maintained at an HRT of 140 days. After 5 days of a lower methane to carbon dioxide ratio, which was 20% of the total biogas volume, the rate of methane production increased from a maximum rate of 0.07 ± 0.04 to 0.39 ± 0.02 l CH_4 $gVS^{-1}day^{-1}$. A steady state in methane production was observed from the 5^{th} day of methane production. The rate of methane production ranged from 0.28 ± 0.01 to 0.44 ± 0.02 l CH_4 $gVS^{-1}day^{-1}$ and a cumulative methane production value of 3.85 ± 0.32 l CH_4 $gVS^{-1}$was achieved. Past research has shown that a low methane to carbon dioxide ratio indicates inadequate process performance during AD although the abundance of carbon dioxide during AD is dependent on pH (Hansson et al., 2002; Boe et al., 2010). However, the pH of the system was above 7.0 suggesting that the decrease in methane production was due to process instability, which is common during AD start-up as microbial populations adapt to the operating conditions (Feng et al., 2015). After 17 days of incubation, the HRT was reduced to 70 days and the OLR was increased to 1.42 gVS $l^{-1}day^{-1}$. This resulted in a sudden decrease in the rate of methane production with a value of 0.13 ± 0.01 l CH_4 gVS^{-1} day^{-1}. The methane production later fluctuated between 0.04 ± 0.02 and 0.26 ± 0.03 l CH_4 gVS^{-1} day^{-1}. This phenomenon can be explained based on the poor kinetics

between the acid producers and consumers (Vanvelsen, 1979; Wang et al., 1999; Huang et al., 2003). In this study, the poor kinetics among the different groups of microbial communities could be attributed to the increase in the OLR or the limonene inhibitory effect (Ahring et al., 1995; Martín et al., 2010). At this stage of AD, decreases in methane production were attributed to limonene inhibition because the increase in the OLR did not correspond to higher methane levels of production and the VFA accumulation was less than 0.40 g l^{-1} (Fig 7b). At the end of the second OLR, a cumulative methane production of 3.10 ± 0.13 was achieved (Table 3). Subsequent increases in the OLR to 2.85, 4.00 and 5.00 gVS $l^{-1}day^{-1}$ resulted in further decreases in the rates of methane production. The value ranged from 0.06 ± 0.004 to 0.45 ± 0.05 l CH_4 gVS^{-1} day^{-1}, 0.06 ± 0.01 to 0.13 ± 0.01 l CH_4 gVS^{-1} day^{-1} and 0.08 ± 0.01 to 0.13 ± 0.01 l CH_4 gVS^{-1} day^{-1}, respectively, for the 2.85, 4.00 and 5.00 gVS $l^{-1}day^{-1}$. Cumulative methane production values of 3.18 ± 0.03, 1.65 ± 0.03 and 2.23 ± 0.14 l CH_4 gVS^{-1} were achieved for OLR of 2.85, 4.00 and 5.00 gVS l^{-1} day^{-1}, respectively (Table 3).

Under thermophilic conditions, the daily variations in the compositional biogas production were measured (Figure 4a). Compared to the mesophilic conditions, at an OLR of 0.71 gVS $l^{-1}day^{-1}$, the rates of methane production were only statistically significant ($p<0.05$) on the 6^{th} and 10^{th} days of incubation. The thermophilic condition achieved a methane value of 0.47 ± 0.02 and 0.42 ± 0.02 l CH_4 gVS^{-1} day^{-1} as against 0.11 ± 0.004 and 0.28 ± 0.01 l CH_4 gVS^{-1} day^{-1}, respectively, for the mesophilic conditions. Similarly, the cumulative methane production at an OLR of 0.71 gVS $l^{-1}day^{-1}$ was not statistically significant ($p>0.05$) when compared to the mesophilic conditions; a cumulative value of 4.46 ± 0.35 l CH_4 gVS^{-1} was achieved under the thermophilic conditions (Table 3). At this stage of the investigation, the effect of higher temperature

on the AD of citrus peel waste was not observed possibly because of the low OLR. In the same way, when the OLR was increased to 1.42 gVS $l^{-1}day^{-1}$, the rate of methane production, which ranged from 0.15 ± 0.01 to 0.42 ± 0.05 l CH_4 gVS^{-1} day^{-1} was only statistically significant ($p<0.05$) when compared to the mesophilic values on the 19th and 24th days of incubation. The thermophilic conditions recorded 0.52 ± 0.03 and 0.39 ± 0.02 l CH_4 gVS^{-1} day^{-1} as against 0.23 ± 0.01 and 0.05 ± 0.02 l CH_4 gVS^{-1} day^{-1}, respectively, for the mesophilic conditions. However, a cumulative methane production of 5.35 ± 0.10 l CH_4 gVS^{-1} was achieved by the thermophilic conditions and was statistically significant ($p<0.05$) to the mesophilic conditions (Table 3). This could be as a result of the relatively high operating temperature. It is known that thermophilic temperatures can increase the rates of hydrolysis and methanogenesis and mass transfer of metabolites between the acid producers and consumers (Zaher et al., 2009). This is because thermophilic bacteria are able to utilize organic material that is not easily biodegradable under mesophilic conditions (Converti et al., 1999).

Similar to the mesophilic conditions, when the OLR was increased to 2.85, there was a sudden reduction in the rates of methane production, with values ranging from 0.08 ± 0.00 to 0.24 ± 0.002 l CH_4 gVS^{-1} day^{-1}. Compared to the mesophilic conditions, the values remained similar ($p>0.05$) until the last 3 days of operating at this OLR. Methane values of 0.24 ± 0.002, 0.24 ± 0.002 and 0.15 ± 0.001 l CH_4 gVS^{-1} day^{-1} as against 0.17 ± 0.01, 0.17 ± 0.01 and 0.08 ± 0.002 l CH_4 gVS^{-1} day^{-1} were achieved by the thermophilic and mesophilic conditions on the 47th, 48th and 49th days of incubation, respectively. The sudden drop in the rate of methane production for both conditions may have been caused by limonene inhibition, although this is the first observable inhibition under thermophilic incubations. A lower cumulative methane production of 3.12 ± 0.04 l CH_4 gVS^{-1} was achieved at the 2.85 OLR under thermophilic conditions;

there was no statistically significant difference ($p>0.05$) to that of the mesophilic conditions. Unlike the mesophilic conditions, the thermophilic incubations recovered from limonene suppression even when the OLR was increased to 4.00 and 5.00. From the 62nd day of incubation onwards, the thermophilic conditions achieved statistically higher rates and extents of methane ($p<0.05$). A cumulative methane production of 2.98 ± 0.05 and 5.61 ± 0.05 l CH_4 gVS^{-1} was achieved under the thermophilic conditions at OLRs of 4.00 and 5.00, respectively. These findings are similar to those reported by Kaparaju and Rintala (2006), who observed that the AD of orange waste at OLRs between 4.2 and 5.6 gVS $l^{-1}day^{-1}$ produced 0.5 l CH_4 gVS^{-1}.

3.2.2. pH profile

The optimum pH to obtain the maximum biogas yield under anaerobic conditions should be between 6.5 and 7.5 (Liu et al., 2008). In this study, the pH was measured daily for the AD of CFW, under the semi-continuous feeding regime (Figure 6). Usually the pH dropped in the early stages of each experiment because of the rapid hydrolysis of carbohydrates and the accumulation of organic acids. This was also observed by Macias-Corral et al. (2008) during the AD of municipal solid waste, agricultural waste and with dairy cow manure where the pH was between 5.5 and 6.0 in the early stages of their study. The pH changes evaluated in this study ranged from 6.5 to 7.5 for both the mesophilic and thermophilic incubations, indicating a moderate buffering capacity (Figure 6.). These values are in agreement with the previous study by Martín et al. (2010) on the AD of orange peel waste. When the OLR of the mesophilic incubation was increased from 0.70 to 2.85 g VS l^{-1} day^{-1} the pH decreased from an average of 7.3 to 6.7 and this value was maintained for subsequent increases in the OLR (Figure 6b). The fluctuations in pH, after increasing the OLR, could be attributed to the periodic

accumulation of VFAs (Nizami et al., 2009). This explains the sudden decrease in pH after the 41st, 55th and 72nd days of incubation with maximum individual VFA accumulations of 0.30 ± 0.04, 3.23 ± 0.33 and 4.16 ± 0.73 g l^{-1}, respectively (Fig 7b).

The reduction in pH and accumulation of VFAs has been reported as some of the consequences of inhibition. This was observed during the AD of glycerol and orange waste (Kaparaju & Rintala, 2006; Fountoulakis et al., 2010; Serrano et al., 2014). On the other hand, the pH of the thermophilic incubations were generally within the range of 7.54 to 7.56 when the OLR was increased from 2.85 to 4.00 g VS l^{-1} day^{-1} ; this value was maintained when the OLR was subsequently increased (Fig 6a). The pH of the thermophilic incubations stayed above 7.00, indicating higher consumption of VFAs by the methanogens as the maximum individual VFA accumulation was 0.84 ± 0.04 g l^{-1}. This was relatively lower than the mesophilic incubation (Fig 7a). The pH parameter has been reported to be slow in detecting early changes in the pH of the medium but when combined with the VFA analysis it is useful (Switzenbaum et al., 1990).

3.2.3. Changes in VS and VFA profiles

In this study, VS removal was most efficient for the mesophilic incubations between 0.70 -1.42 gVS $l^{-1}day^{-1}$ OLR with an average value of 59.27 ± 0.65 – 61.26 ±1.05% while the VS content for the thermophilic incubation was < 58 % throughout the experimental period (Figure 5). However, when the OLR was between 4.00 and 5.00 gVS $l^{-1}day^{-1}$, the VS content of the mesophilic incubation increased from 62.1 ± 0.85 to 64.8 ± 0.95%, while the thermophilic incubation was between 57.20 ± 1.01 and 58.00 ± 1.71%. The final VS content of the two incubations in the AD process was an average of 61.5% (Fig 5). Under the mesophilic conditions the VS increased with an increase

in OLR, with values of 59.20 ± 0.65, 61.20 ± 1.02, 61.70 ± 1.10, 62.10 ± 0.85 and 64.80 ± 0.95% for 0.70, 1.42, 2.85, 4.00 and 5.00 gVS $l^{-1}day^{-1}$, respectively. Conversely, under the thermophilic conditions, average VS values of 57.10 ± 0.06, 56.80 ± 0.06, 56.30 ± 1.10, 58.00 ± 1.71 and 57.20 ± 1.71% for 0.70, 1.42, 2.85, 4.00 and 5.00 gVS $l^{-1}day^{-1}$ OLR were achieved, respectively. Interestingly, the VS value was low but steady for the thermophilic incubation, thus indicating higher digestion efficiency. According to Cecchi et al. (1991), the AD of municipal solid waste under thermophilic conditions increased the VS removal from 23 to 48%; this confirms the higher methane production relative to mesophilic conditions. The low VS value is confirmed by the high VS input and removal during AD (Aslanzadeh et al., 2014); this was observed under the thermophilic conditions.

The concentration of the individual VFAs for the thermophilic and mesophilic incubations was measured, with acetic, propionic and butyric acids being quantifiable. The concentration of the individual VFAs for both incubations was < 0.45 g l^{-1}at an OLR of between 0.70 and 2.85 gVS l^{-1} day^{-1}. For the mesophilic incubations, maximum individual VFA concentrations of 0.30 ± 0.08, 0.38 ± 0.06, and 0.25 ± 0.06 g l^{-1} of acetic, propionic and butyric acids were measured, respectively. Equally, the thermophilic incubations contained 0.34 ± 0.037, 0.28± 0.22 and 0.46 ± 0.028 g l^{-1} of acetic, propionic and butyric acids, respectively (Figure 7a). The moderately high individual VFA concentrations suggested good kinetics between the acid producers and users (Ahring et al., 1995). At this point, the OLR of between 0.7 and 1.42 g VS l^{-1} day^{-1} of CFW was not inhibitory and did not cause acidification of either the mesophilic or thermophilic incubations. However, a subsequent increase in the OLR to 2.85 gVS l^{-1} day^{-1} after 33 d incubation resulted in lower accumulation of VFAs, which was below 0.45 g l^{-1}, and rates of methane production of 0.09 ± 0.006 and 0.15 ± 0.02 l CH_4 gVS^{-}

^{1} day^{-1} for the thermophilic and mesophilic incubations, respectively (Table 3). This suggests that both the mesophilic and thermophilic incubations were suppressed by the limonene in the CFW, through the inhibition of both the acidogenic and methanogenic activities. Following an increase in the OLR to 4.00 gVS l^{-1} day^{-1} after 56 days of incubation, there was a consistent increase in the accumulation of the individual VFAs. Maximum individual VFA concentrations of 3.23 ± 0.33, 2.77 ± 0.28 and 3.13 ± 0.20 g l^{-1} were measured for acetic, propionic and butyric acids in the mesophilic incubation, respectively (Figure 8a). In contrast, the thermophilic incubation resulted in VFA concentrations of 0.19 ± 0.004, 0.40 ± 0.08, and 0.84 ± 0.04 g l^{-1} of acetic, propionic and butyric acids, respectively. The accumulation of VFAs in the mesophilic incubations suggested inhibition of methanogenesis by limonene. A similar finding was observed by Martín et al. (2010), who reported stronger inhibition of the AD process when the OLR of orange peel was higher than 4.0 gVS l^{-1} day^{-1}. However, after 69 days of incubation, the individual VFA concentrations had reduced, with values ranging from 0.37 ± 0.1 to 1.14 ± 0.06 g l^{-1}, indicating the recovery of methanogenesis from the limonene inhibition under mesophilic conditions (Figure 8b). Finally, the OLR was increased to 5.00 gVS l^{-1} day^{-1} and a similar trend of increases in the VFA concentrations was observed, particularly under the mesophilic conditions. Acetic acid was the dominant VFA with a concentration of 4.16 ± 0.73 g l^{-1}, although after 88 days of incubation, the concentration of acetic acid dropped to 0.90 ± 0.08 g l^{-1}. Propionic and butyric acids were the dominant VFAs under the thermophilic conditions, although the maximum concentrations of propionic and butyric acids at an OLR of 5.00 gVS l^{-1} day^{-1} were 20% and 8% of the value obtained when the OLR was 4.00 gVS l^{-1} day^{-1}, respectively. This is similar to the result obtained by Martín et al. (2010), who recorded a decrease in pH and an increase in the accumulation of propionic and acetic acids (1.30

and 5.00 g l^{-1}, respectively) when the OLR was increased to 5.1 gVS $l^{-1}day^{-1}$. The higher accumulation of propionic and butyric acids over acetic acid suggests a shift in the acidogenic pathway, which could be an indication of slight inhibition of acidogenesis and acetogenesis. Van Velsen (1979) observed that the breakdown of propionic and butyric acids, which were produced as a result of ammonia toxicity, was inhibited because of inhibition of the acetogenic and methanogenic microbial groups. Wang et al. (1999) also reported on higher production of VFAs during AD and showed that free energy is only gained during the breakdown of acetic acid. Ahring et al. (1995) reported that the accumulation of butyric acid is an indication of instability of the AD process because it is not a favourable pathway for the production of methane gas. However, under the thermophilic conditions this effect did not inhibit methanogenesis because the accumulations of VFAs were below 0.8 g l^{-1} as against 3.5 to 4.5 g l^{-1} under the mesophilic conditions.

4. Conclusions

Despite the inhibitory effect of the limonene content of CFW, this study showed that it is possible to anaerobically treat CFW using both mesophilic and thermophilic conditions. The preferred operating condition for CFW under mesophilic condition was less than an OLR of 2.85 gVS l^{-1} day^{-1}. However, for OLRs between 2.85 – 5.00 gVS l^{-1} day^{-1}, thermophilic conditions were preferable. Furthermore, thermophilic conditions achieved 6.7, 49, 55 and 80% greater methane yield at OLR of 0.71, 1.42, 4.00 and 5.00 gVS l^{-1} day^{-1}, respectively than the mesophilic conditions. For further study, the mesophilic condition at 45 ^{0}C should be compared with the thermophilic condition at 55 ^{0}C using a semi-continuous system and changes in the microbial dynamics should be investigated.

Acknowledgement

This work was supported by the Centre for Global Eco-innovation (X02646PR) and Stopford Energy and Environment Ltd

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Tables

Table 1: Chemical composition of citrus fruit waste (n=3 and mean value ± standard error)

Parameter	Citrus fruit waste
TS (%)	16.60 ± 0.21
VS (%)	97.50 ± 0.26
Carbon (%)	41.70 ± 0.22
Nitrogen (%)	0.80 ± 0.21
pH	5.98 ± 0.02
Cellulose (%)	20.45 ± 1.06
Hemicelluloses (%)	6.61 ± 0.79
Lignin (%)	2.29 ± 0.82
Limonene (mg l^{-1})	3.95± 0.33

TS, VS, pH and limonene were analysed based on fresh basis while other parameters were based on dry mass.

Table 2: Batch experimental conditions

Temperature (^{0}C)	Citrus fruit waste		Inoculum	
	g	g VS	g	g VS
35	21.80	3.43	200	11.44
40	21.80	3.43	200	11.44
45	21.80	3.43	200	11.44
50	21.80	3.43	221	11.44
55	21.80	3.43	221	11.44

[a]Digested sewage sludge was taken as inoculum

[b] Assays 35, 40 & 45 were inoculated with the mesophilic inoculum while assay 50 and 55 were inoculated with the enriched thermophilic inoculum

Table 3: Cumulative methane yields (n=2) at different OLR, HRT and operating temperature (mean value ± standard error)

Days	OLR (gVS $l^{-1}day^{-1}$)	HRT (day)	Cumulative methane production (lCH_4 gVS^{-1})	
			Thermophilic	Mesophilic
16	0.71	140	4.46 ± 0.35	3.85 ± 0.32
16	1.42	70	5.35 ± 0.10	3.10 ± 0.13
22	2.85	41	3.12 ± 0.04	3.18 ± 0.29
17	4.00	28	2.98 ± 0.05	1.65 ± 0.03
19	5.00	22	5.61 ± 0.05	2.23 ± 0.14

Figure captions

Figure 1. Batch testing reactor; the setup is a combination of an airtight glass reactor, mixing device, CO_2 scrubber and tip meter for gas measurement.

Figure 2. Scheme of the semi-continuous anaerobic reactor system set up; (a) pH and temperature probes; (b) mixer; (c) influent reservoir; (d) effluent reservoir; (e) pump; (f) volumetric gas tipping meter; (g) gas vent; (h) data acquisition and display and (I) heating

Figure 3. Batch test showing methane production at varying operational temperatures (mean value (n-=2) ± standard error)

Figure 4. Biogas production rates for a semi-continuous test at different operating temperatures (a) thermophilic (55 ^{0}C) operation and (b) mesophilic (35 ^{0}C) conditions (mean value (n-=2) ± standard error)

Figure 5. Volatile solid profiles of semi-continuous test for different operating temperature (a) thermophilic (55 ^{0}C) operation and (b) mesophilic (35 ^{0}C) conditions (mean value (n-=2) ± standard error)

Figure 6. pH profiles of semi-continuous test for different operating temperature (a) thermophilic (55 ^{0}C) operation and (b) mesophilic (35 ^{0}C) conditions (mean value (n-=2) ± standard error)

Figure 7. Individual VFA profiles of semi-continuous test for different operating temperature (a) thermophilic (55 ^{0}C) operation and (b) mesophilic (35 ^{0}C) conditions (mean value (n-=2) ± standard error)

Figures

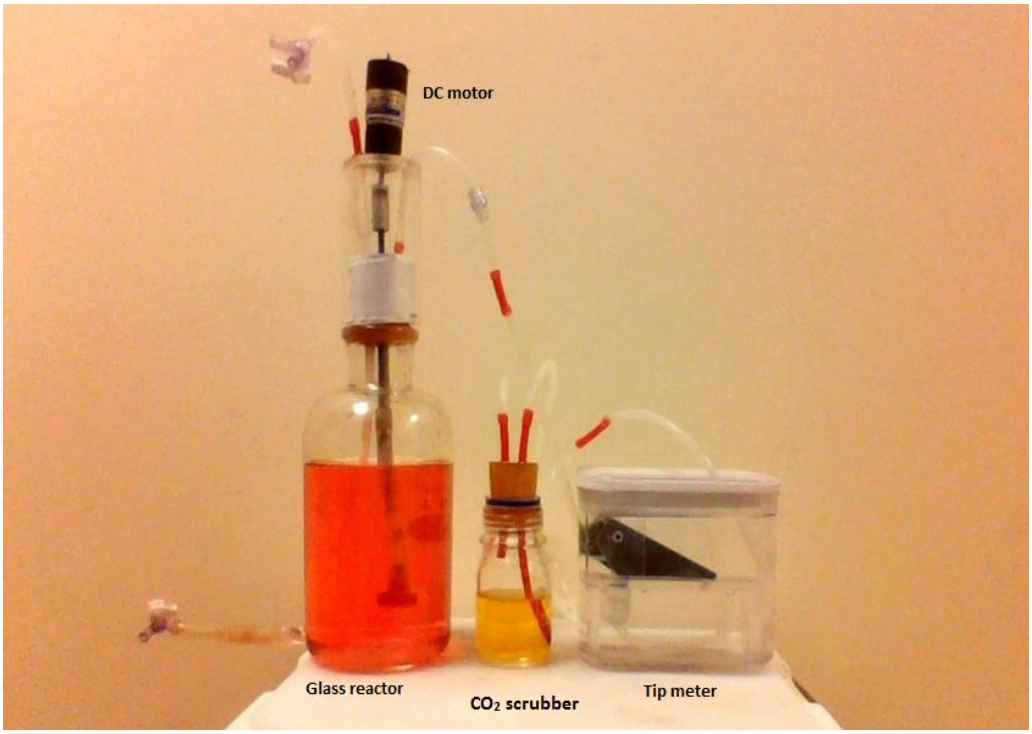


Figure 1

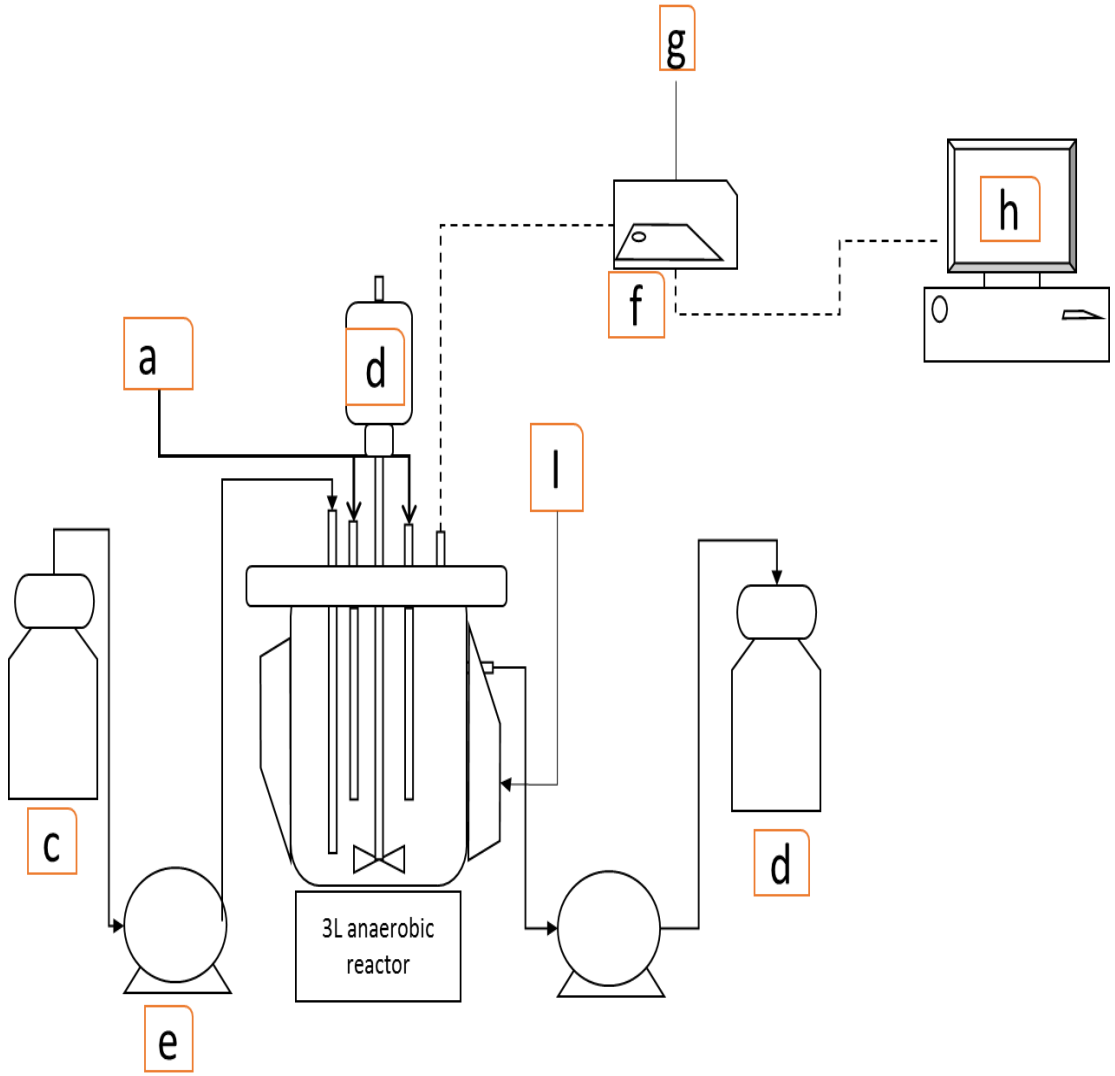


Figure 2

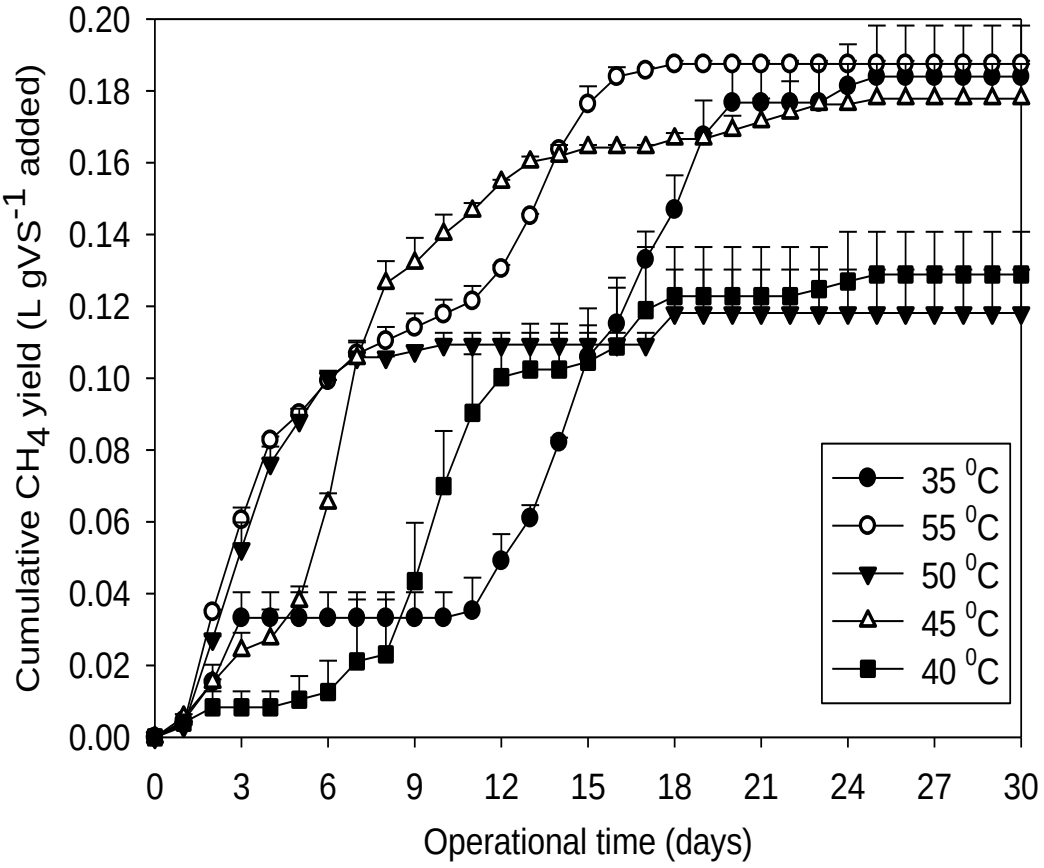
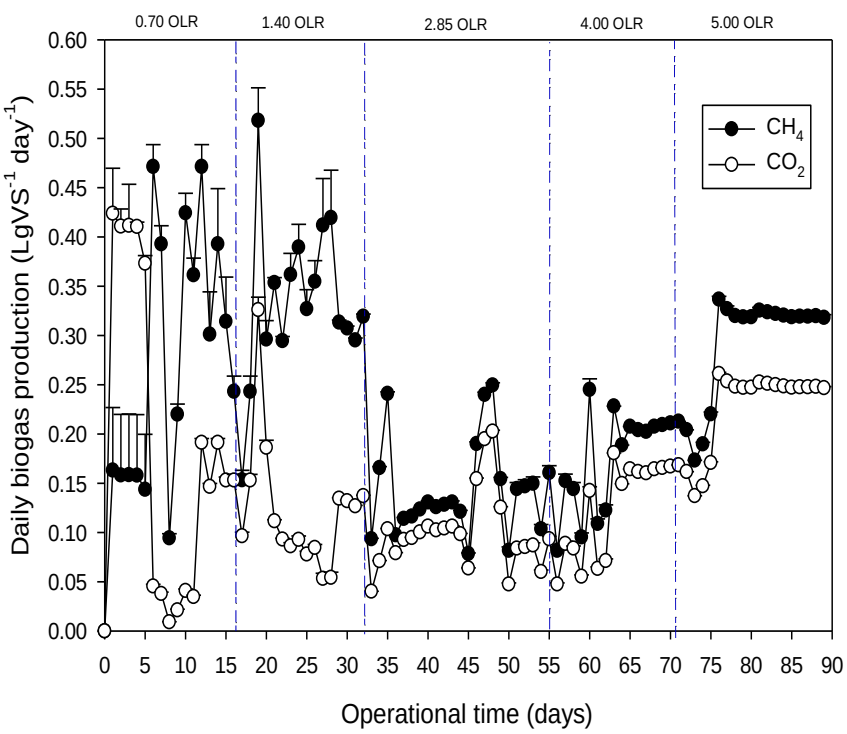
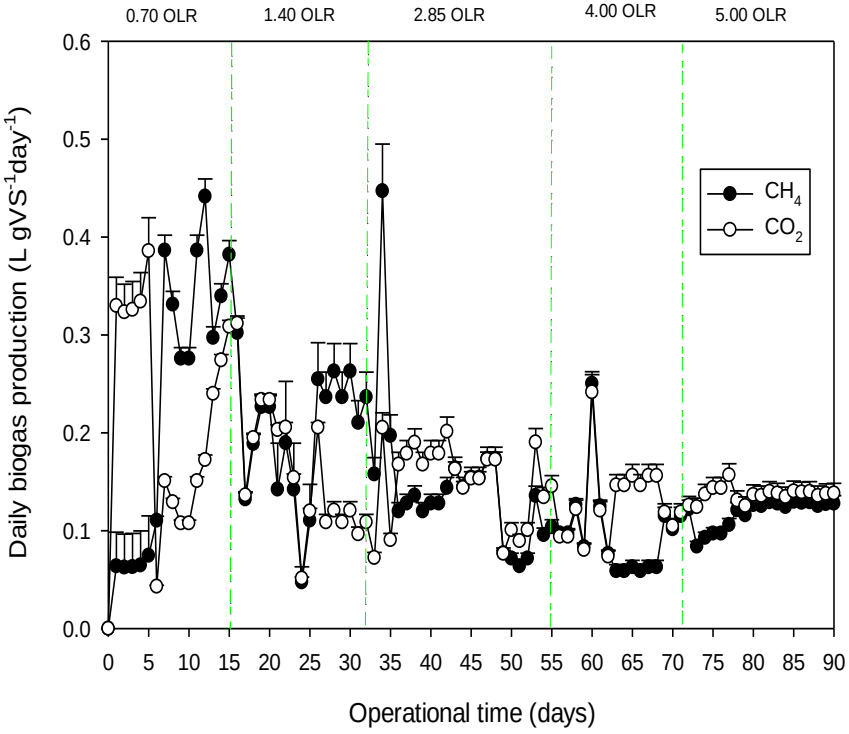

Cumulative CH_4 yield (L gVS^{-1} added)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
3
6
9
12
15
18
21
24
27
30
Operational time (days)
35 ^{0}C
55 ^{0}C
50 ^{0}C
45 ^{0}C
40 ^{0}C

Figure 3


0.70 OLR
1.40 OLR
2.85 OLR
4.00 OLR
5.00 OLR
Daily biogas production (LgVS-1 day-1)
CH4
CO2
Operational time (days)

a


0.70 OLR
1.40 OLR
2.85 OLR
4.00 OLR
5.00 OLR
Daily biogas production (L gVS-1day-1)
CH4
CO2
Operational time (days)

b

Figure 4

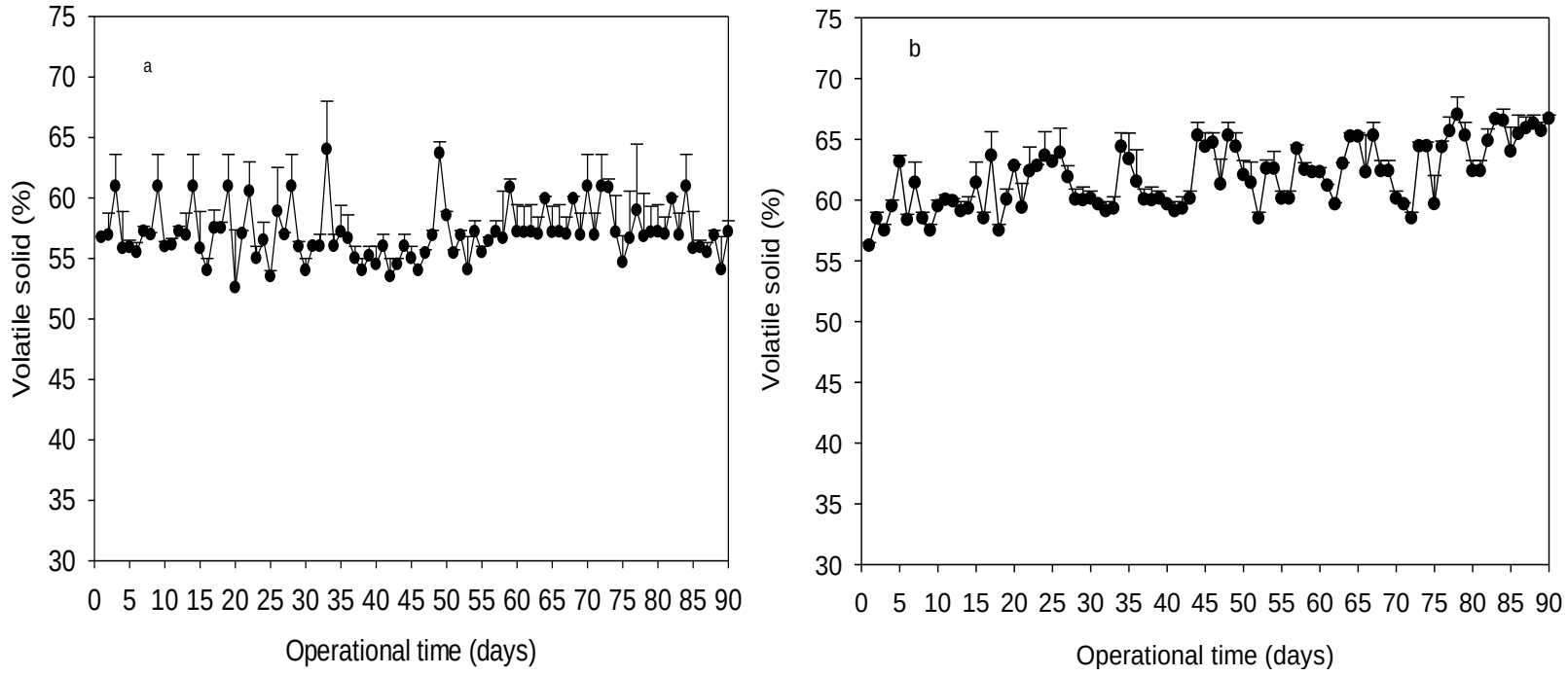

a
b
Volatile solid (%)
Operational time (days)
75
70
65
60
55
50
45
40
35
30
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90

Figure 5

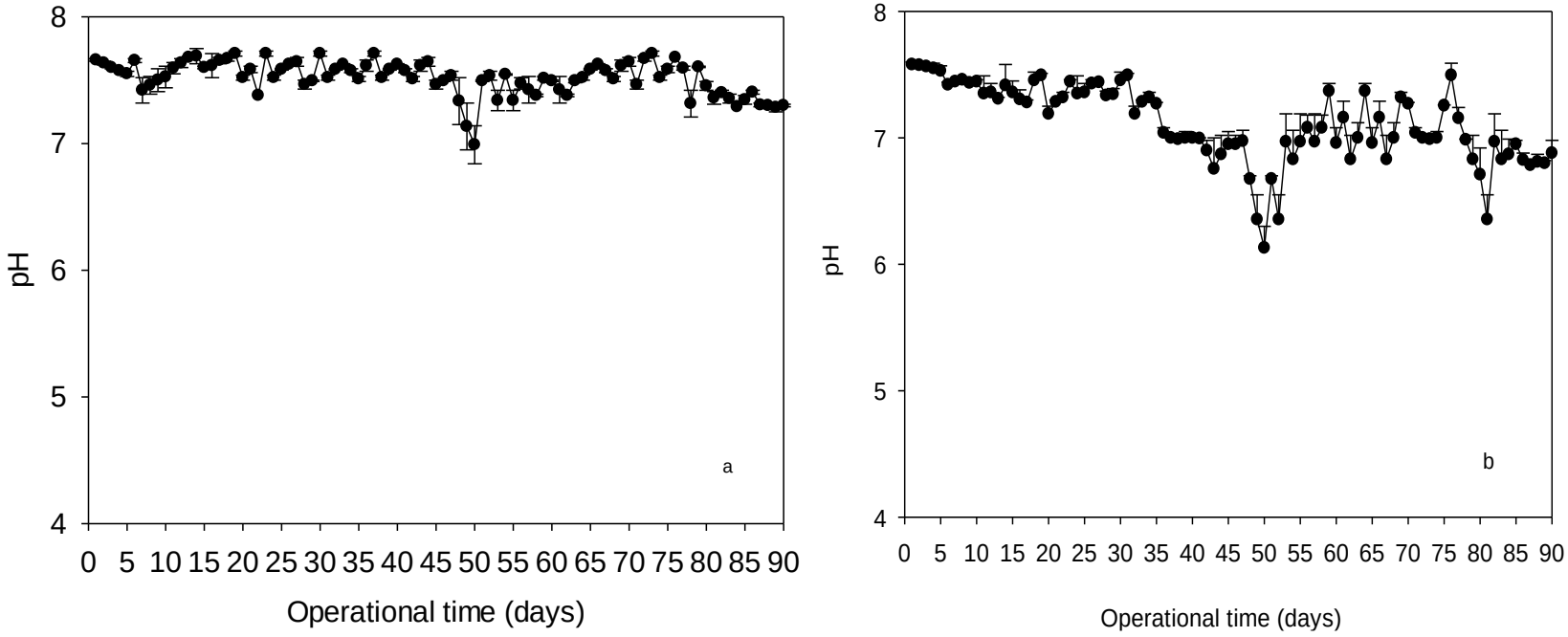

8
7
6
5
4
pH
a
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Operational time (days)
8
7
6
5
4
pH
b
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Operational time (days)

Figure 6

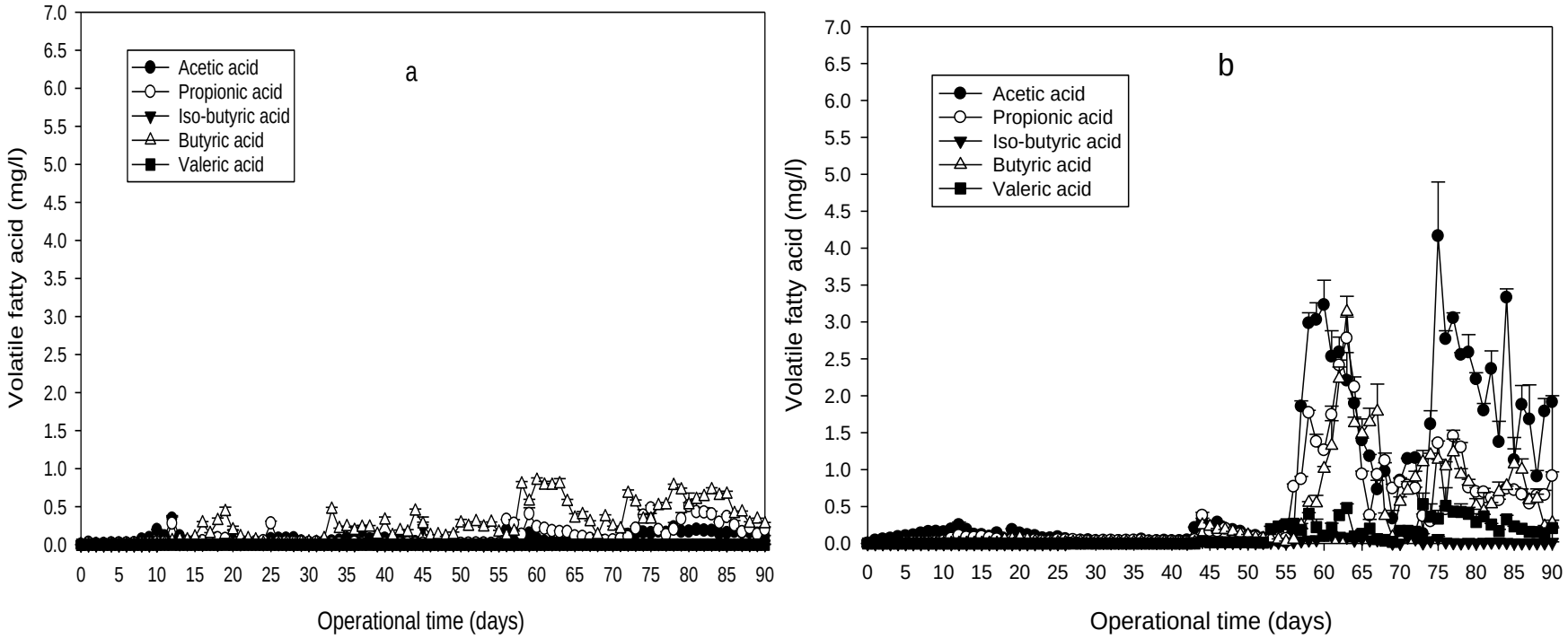

a
b
Acetic acid
Propionic acid
Iso-butyric acid
Butyric acid
Valeric acid
Volatile fatty acid (mg/l)
Operational time (days)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
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Figure 7

2. Paper VI

An integrated single-stage anaerobic reactor for high solid anaerobic digestion of citrus peel waste

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Abstract

A comparative study of the use of compartmentalized anaerobic reactor and continuously stirred tank reactor for treating high solid citrus fruit waste was carried out. The following parameters were measured in the anaerobic reactors: pH, volatile solid, chemical oxygen demand, acidification, methane and carbon dioxide production. Both reactors were operated for 70 days with an increasing organic loading rates of 1.42, 2.85, 4.00 and 5.00 gVS $l^{-1}day^{-1}$ and hydraulic retention time of 70, 41, 28 and 22 days. For improved biological treatment of leachate, the lower chamber of the compartmentalized anaerobic reactor was filled with immobilized cells (granular sludge) and separated by a permeable membrane. During steady state anaerobic digestion of citrus fruit waste the compartmentalized anaerobic reactor achieved 34, 43.3, 48.5 and 79.9% higher cumulative methane production at organic loading rates of 1.42, 2.85, 4.00 and 5.00 gVS $l^{-1}day^{-1}$, respectively whereas, the continuously stirred tank reactor recorded decreases in methane yield as organic loading rate increased. This single-stage compartmentalized anaerobic reactor improved mass transfer, diffusion of leachate and leachate treatment for higher methane yield during high solid anaerobic digestion. It can serve as an alternative to two-stage high solid anaerobic digestion of organic substrate.

Keywords: leachate treatment; diffusion; methane; limonene; biofilm formation

1. Introduction

Anaerobic digestion (AD) is a well-established sustainable technology that is widely used in the management of agricultural and municipal waste (Macias-Corral et al., 2008). This technology could play a decisive role in the on-going campaign against climate change as it provides an alternative route to energy production and soil conditioning (Zeshan & Visvanathan, 2014). As AD becomes more financially viable, there is a need to improve high solid anaerobic digestion (HSAD). HSAD is a solid-state operational system with low water content; this type of AD is also called a semi-dry or dry system. HSAD has been demonstrated using various AD technologies, such as the silo shaped Dranco digester and the cylindrical Valorga digester system (Li et al., 2011). The key reasons for the development of HSAD are the benefits of low water usage and relatively small reactor size (Garcia-Bernet et al., 2011). Apart from reducing water usage, the technology has been reported to increase the organic loading rate, avoid or reduce digestate dewatering and reduce heating requirements. However, methane production is lower and volatile solid removal is less than 50% (Dong et al., 2010; Nagao et al., 2012). In an attempt to optimise HSAD, different reactor configurations have been developed and evaluated, particularly the two stage high solid and high rate reactors (Pohl et al., 2012; Orozco et al., 2013; Pohl et al., 2013; Boske et al., 2014; Shewani et al., 2015).

In recent years, a number of reactor configurations have been developed and modified to increase the process efficiency for HSAD (Mata-Alvarez, 2003; Rapport et al., 2008; Li et al., 2011; Schönberg & Linke, 2012). These reactors operate on the same principles: (i) separation of the acidogenic and methanogenic phases, and (ii) retention of microbial biomass (Chynoweth et al., 2001). However, the new generation of high solid anaerobic reactors (HSAR) are mainly multi-stage anaerobic systems (Nizami &

Murphy, 2010; Pohl et al., 2012; Pohl et al., 2013; Xing et al., 2014; Shewani et al., 2015). Nevertheless, these reactor configurations are beneficial to HSAD because they enhance the distribution of water during leachate recirculation, the diffusion of metabolites and nutrients to bacterial sites, and the methane yield (Bollon et al., 2011; Le Hyaric et al., 2011; Li et al., 2014). The system also separates the acidogenic and methanogenic microbial cells in a two-stage process. Analysis of the existing data has shown that the number of single-stage HSAR is estimated to have increased globally by 71% between 2006 and 2010 because of capital cost (De Baere & Mattheeuws, 2008; De Baere et al., 2010; Nizami & Murphy, 2010). Considering the benefits of the new generation HSAR compared to HSAD, the economic implications could prevent the widespread application of this technology. However, a high solid and high rate reactor can be integrated into a single phase AD system with the upper and lower chambers. This approach avoids the need for two stage reactors, while increasing leachate treatment, retaining methanogenic microbes and increasing the distribution of metabolites between the two chambers.

In this study, a compartmentalized AD system, combining high solid and high rate compartments in a single reactor, was designed to improve HSAD. The aim of this work was to study the impact of a single phase compartmentalized anaerobic reactor as a better option than the current multi stage HSAR. The process performance was accessed through the measurement of pH, volatile solid content, acidification, chemical oxygen demand, volumetric biogas production and compositional biogas analysis. The single phase compartmentalized anaerobic reactor (CAR) was compared with the conventional continuous stirred tank reactor (CSTR).

2. Materials and methods

2.1.Substrate preparation

Citrus fruit was collected from a local grocery store in Lancaster, UK and prepared by pressing out the juice. The citrus fruit residues were diced into smaller pieces before shredding and homogenising using a commercial blender to obtain a particle size of 1-3 mm. A zip plastic bag was used to store the citrus fruit waste (CFW) at -20 ^{0}C. The CFW was a combination of orange, grape, lemon and lime in the ratios of 8:4:2:1. The mixing ratio was selected based on the average quantities of citrus waste generated annually (FAOSTAT, 2013). The CFW was analysed for total solid (TS), volatile solid (VS), pH and limonene concentration. The CFW was later dried at 60 ^{0}C in an oven (Memmert, Germany), before ball milling into granular and powdered form to determine the lignocellulosic and elemental composition, respectively.

2.2.Microbial inocula

Two forms of inocula were used to start up the AD experiment. A granular sludge inoculum normally used for low solid AD and a regular dispersed inoculum. The granular sludge inoculum originated from a mesophilic up-flow anaerobic sludge blanket (UASB) reactor used in treating wastewater from a distillery industry. The dispersed inoculum was obtained from a mesophilic anaerobic digester of sewage sludge at United Utilities, Preston UK. Prior to inoculation both inocula were incubated separately at 35 ^{0}C in an enclosed CSTR for 3 d to remove residual organic material. The granular sludge was analysed for soluble chemical oxygen demand and pH while TS, VS and pH of the dispersed inoculum was determined.

2.3.Experimental setup and procedure

An acrylic material was used to construct the reactor, which was 210 mm in diameter, and was designed to hold several ports for the stirrer shaft, probes, influent, effluent and gas channels. The reactor was wrapped with silicone tubing which was thermostatically controlled using 5 l bath immersed with a submersible pump for water recirculation (Zhong Shan Jiayu, 150W, 5000 L/H). A peristaltic pump (Watson, Marlow, UK) was fitted separately to the upper and base of the reactor in order to remove effluents and add fresh feedstock, respectively. The reactors were continuously stirred at 30 rpm while the pH and temperature were monitored using probes (pH, Conrad, Model 100 ATC). The pH was manually adjusted with 3 M NaOH and 1 M H_2SO_4 solution as operational pH range was set at 6.5–7.8. Each reactor was purged with nitrogen for 1 min prior to incubation to maintain an anaerobic condition and volumetric gas production was measured using an electronic tip meter with data acquisition card (National Instrument) and display system. The conditions for the CSTR and CAR studies were similar. The organic loading rates (OLR) were increased sequential as follow: 1.42, 2.85, 4.00 and 5.00 gVS $l^{-1}d^{-1}$ (Table 3). For sampling and analysis, the liquid phase effluent from the lower compartment of the CAR was collected initially every 2 – 4 d for 18 d of incubation after which it was collected weekly while the effluent from the solid phase was collected daily for both CAR and CSTR.

In this study, a CSTR (1.4 l working volume) and the CAR with an effective working volume of 2.9 l were investigated simultaneously (Fig 1). Working volumes of 1.4 l and 1.5 l were maintained in the upper and lower chambers of the CAR, respectively. The CAR contains upper and lower chambers, with the upper chamber representing a typical high solid system with less water, but as hydrolysis and acidogenesis proceeded, the solid material was solubilised to leachate. The leachate then

trickled downward through a permeable membrane into the lower chamber. The permeable mesh (PVC) covers a diameter of 70 mm with a pore size of 250 µm to allow for the flow of gas, moisture, nutrient, metabolites between the two compartments but retained the granular sludge. The lower chamber was the high rate reactor, which contained granular sludge to rapidly convert the dissolved compounds into organic acid and biogas. The leachate content in the lower chamber extended 10 mm into the upper chamber, thereby increasing the distribution and dissolution of compounds in the high solid substrate. The interaction between the upper (high solid) and lower (high rate) chambers was also facilitated by daily recirculation of nitrogen gas (60 s) into both compartments through the permeable membrane. This helped to increase leachate distribution and reduce permeable membrane fouling.

2.4.Analytical methods

The solid content of samples were examined using standard methods. This required heating the sample to 105 and 550 ^{0}C to determine the TS and VS, respectively (APHA, 1998). Fibre analysis were carried out using the fibre detergent concentrate (ANKOM, USA), refluxing set up and a gravimetric device to determine the content of lignin, hemicellulose and cellulose (Mertens et al., 2002). Elemental carbon and nitrogen were determined according to standard methods using an elemental analyser (Carter & Barwick, 2011). The pH value was monitored using a digital pH meter (Conrad, Model 100 ATC).

For other chemical analysis such as individual volatile fatty acids (VFAs), SCOD and monoterpenes, the sample was centrifuged at 15,000 rpm for 10 min after which the supernatant were filtered through a cellulose acetate membrane with a pore size of 0.45 µm.. The VFAs were quantified by ion chromatography (IC) (Dionex, ICS-30000,

Thermo-Scientific, USA) using a UV index detector and an Aminex HPX-87H column (Bio-Rad, UK). The separation of VFAs during IC measurement was achieved using a mobile phase of 2.5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1} and a column temperature of 65 ^{0}C. The detector temperature was 40 ^{0}C. The VFA marker mix containing acetic, propionic, iso-butyric, butyric, iso-valeric and valeric acids, each of 1 mg ml^{-1} (Sigma-Aldrich, UK) was used to calibrate the IC equipment. Determination of SCOD was performed using Hach dichromate digestion kits containing dichromate and sulphuric acid (Hach, LCK 514). The COD values were measured using a Hach spectrophotometer (DR/2800, LCK 514) with a detection range from 100 to 2000 mg l^{-1}. Volumetric biogas was measured online using a custom made, electronic tip meter connected through a data acquisition (DAQ) card to a computer and a monitoring program written using LabVIEW software (National Instrument). Gas compositional analysis was carried out to measure methane and carbon dioxide content by gas chromatography (GC). A Perkin Elmer Auto system XL equipped with a Hayesep Q 80 Mesh 6ft × 1/8 inch, 2.0 mm diameter and a dual detector (FID) was used. The injector and detector temperatures were maintained at 60 and 350 °C, respectively. The column temperature was set at a gradient of 60 to 250 °C and argon was used as the carrier gas. The Perkin Elmer Auto system XL Gas Chromatography is equipped with an automatic injector, 10 ml of sample gas were pressurised into exetainer vials before placing it in the injector rack. Each run was carried out in duplicate with a methane and carbon dioxide standard. The concentration of limonene was determined using thermal desorption GC–MS according to Vickers et al. (2009). A 5 µl of the sample was injected into the adsorbent resins, containing a carbon trap (SupelcoInc, Bellefonte, PA, USA) as helium gas was continuously flushed through the sampling tubes. The GC oven was

initially held at 35 °C for 2 min, heated to 160 °C at 4 °C min^{-1}, then heated at 45 min^{-1} to 300 °C, which was held for 10 min.

2.5.Statistical analysis

Mean, standard deviation, and standard error were calculated using Microsoft Excel 2013 edition. For statistical analysis of methane production, the data were Log transformed to pass the Shapiro-Wilk normality test before accessing the two independent sample Welch's t test because of unequal variances. Sample size was in duplicate and the SPSS 22.0 edition was used for the analysis and statistical significance was set at $p < 0.05$

3. Results and Discussion

The compartmentalized anaerobic reactor (CAR) configuration achieved a higher rate of methane production, faster recovery during the sequential increases in the organic loading rates (OLR) of citrus fruit waste (CFW) and more tolerance during limonene inhibition. The results of the analysis of CFW for total solid (TS), volatile solid (VS), pH and limonene concentration were 16.6 ± 0.21% (DW), 97.5 ± 0.26% (DW), 5.98 ± 0.12 and 3.95 ± 0.33mg l^{-1}, respectively (Table 1). The granular sludge had a soluble chemical oxygen demand (SCOD) of 0.48 ± 3.31 g l^{-1} and a pH of 7.25 ± 0.12; while the dispersed inoculum source had TS, VS and pH values of 10.3 ± 1.01% (DW), 55.4 ± 1.12% (DW) and 7.1 ± 0.22, respectively.

3.1.Biogas production

The dynamics of daily methane and carbon dioxide production in the CSTR and CAR configurations are shown in Fig 2. An OLR of 1.42 gVS $l^{-1}day^{-1}$ was used to start the experiment and the rates of biogas production peaked in the first 24 h with a lower methane to carbon dioxide ratio for both incubations (Fig 2). Statistical analysis revealed methane production between CSTR and CAR for the first 5 days of incubation was not statistically significant ($P>0.05$). The initial peaks in biogas production suggest active microbial interactions while the subsequent reduction in the methane to carbon dioxide ratio could be ascribed to: (i) cellular respiration (ii) the slow response of carbon dioxide users, and (iii) gradual adaptation of the microbial cells to the operating conditions (Mountfort & Asher, 1978; Gerardi, 2003). After 5 days of incubation, the methane content of the biogas peaked with average values of 54.5% and 69%, respectively, for the CSTR and CAR configurations. At this stage of incubation, the rate of methane production was higher for the CAR configuration, which achieved 0.32 ± 0.01 and 0.35 ± 0.01 l CH_4 $gVS^{-1}day^{-1}$ on days 6 and 7 of incubation. This was significantly ($p<0.05$) higher than the CSTR configuration, which recorded 0.19 ± 0.009 and 0.24 ± 0.01 l CH_4 $gVS^{-1}day^{-1}$, respectively. However, subsequent addition of 1.42 gVS $l^{-1}day^{-1}$ did not result in a significant ($p>0.05$) change in the rate of methane production. At the end of the first OLR, cumulative methane production of 3.46 ± 0.06 and 2.45 ± 0.21 l CH_4 gVS^{-1} was achieved by the CAR and CSTR configurations, respectively (Table 2). The statistical analysis showed that there was no significant difference in the cumulative methane production ($p>0.05$). At this stage the reactor configuration did not greatly influence either the rate or cumulative methane production. The value obtained by the CSTR configuration was within the range

reported by Martín et al. (2010) for the biomethanization of orange peel waste. They obtained a methane yield coefficient of 0.27 – 0.29 l CH_4 gVS^{-1}.

A subsequent increase in the OLR to 2.85 gVS $l^{-1}day^{-1}$ resulted in a sudden drop in the amount of methane gas produced from the two configurations. Methane values ranging from 0.12 ± 0.04 to 0.18 ± 0.02 l CH_4 $gVS^{-1}day^{-1}$ and 0.12 ± 0.008 to 0.30 ± 0.003 l CH_4 $gVS^{-1}day^{-1}$ under a steady state were achieved by the CAR and CSTR configurations, respectively (Fig 2). The decrease in methane production could be due to the presence of limonene, a constituent of the CFW (Mizuki et al., 1990; Li et al., 2013). At the end of the second OLR, the cumulative methane production was 4.02 ± 0.18 and 2.59 ± 0.13 l CH_4 gVS^{-1} for the CAR and CSTR, respectively. Although the rates of methane production were not statistically significant ($p>0.05$) but the cumulative methane production for the CAR configuration was statistically significant ($p<0.05$). The higher cumulative methane production within the CAR configuration was from the contribution of the high rate reactor to the biodegradation of soluble metabolites during leachate diffusion (Boske et al., 2014; Xing et al., 2014). At this point, limonene suppression appeared to be higher for the CSTR configuration as the cumulative methane production was higher for the CAR configuration at an OLR of 2.85 gVS l^{-1} d^{-1} (Table 2).

When the OLR was increased from 2.85 - 4.00 gVS $l^{-1}day^{-1}$, a steady state in the rate of methane production was maintained from 33-44 days of incubation for the CAR configuration. A methane value ranging from 0.20 ± 0.004 to 0.23 ± 0.005 l CH_4 $gVS^{-1}day^{-1}$ was achieved, which was significantly higher than the CSTR ($p<0.05$). The CSTR configuration recorded a methane value ranging from 0.06 ± 0.004 to 0.17 ± 0.006 l CH_4 $gVS^{-1}day^{-1}$. However, for 45- 49 days of incubation, there was a sudden drop in the rate of methane production for both the CAR and CSTR configuration (Fig

2). This is the second inhibition to the CAR configuration and it can be attributed to both limonene toxicity and acidification because this was accompanied by a sudden drop in pH and VS with values of 6.28 ± 0.11 and 68 ± 0.38 %, respectively after 46 days of incubation. The initial reduction in methane yield for both incubations is consistent with Boske et al. (2014) studies, which compared a single stage up-flow anaerobic solid-state reactor (UASS) and a two-stage UASS + anaerobic filter and found that as the OLR increased from 2.5 to 4.5 gVS $l^{-1}day^{-1}$ the methane yield declined rapidly. After the operation of the third OLR, a cumulative methane production of 3.38 ± 0.07 and 2.06 ± 0.03 l CH_4 gVS^{-1} was achieved by the CAR and CSTR configurations; this was statistically significant ($p<0.05$). A further increase in the OLR to 5.00 gVS $l^{-1}day^{-1}$ resulted in methane values ranging from 0.17 ± 0.003 to 0.19 ± 0.019 l CH_4 $gVS^{-1}day^{-1}$ and 0.059 ± 0.005 to 0.08 ± 0.004 lCH_4 $gVS^{-1}day^{-1}$ for the CAR and CSTR configurations, respectively. The rate of methane production was significant ($p<0.05$). From 60 days of incubation, there was an increase in the rate of methane production for both configurations with the CAR having the highest range of methane production (0.22 ± 0.021 to 0.26 ± 0.026 lCH_4 $gVS^{-1}day^{-1}$), although this was not significant ($p>0.05$) when compared to the methane values from the CSTR. The higher recovery observed in the CAR configuration can again be attributed to the integrated high rate reactor in the lower chamber of the CAR. According to Chen et al. (2008), immobilised cells contained within high rate reactors have been reported to reduce inhibition of biogas production and also contribute to the stability of the AD process. This is attributed to the immobilization of the cells and biofilm formation (Davies, 2003). Cumulative methane production values of 4.25 ± 0.26 and 1.82 ± 0.07 l CH_4 $gVS^{-1}day^{-1}$ were observed for the CAR and CSTR configurations at OLR of 5.0 and these were statistically significant ($p<0.05$). The CAR configuration attained higher methane

production during limonene suppression and higher OLR. These results were in accordance with previous findings which showed that the combination of CSTR+UASB outperformed the CSTR only incubation. This was attributed to leachate recirculation and high substrate solubilization (Aslanzadeh et al., 2014). In this study, solubilization was facilitated through gas recirculation and the extension of the leachate 10 mm into the high solid compartment.

3.2.pH and VFA profile

Generally the pH fluctuations are due to the accumulation and conversion of VFAs into methane gas, although this parameter has been considered to be less useful during AD because it only provides a late indication of imbalances or acidification (Switzenbaum et al., 1990). In this study, the measurement of pH was taken daily for both configurations and the result showed that the pH decreased with increasing OLR. Fig. 5 shows the pH values as a function of time for both the CAR and CSTR configurations.

The average pH was 7.00 for both configurations until the OLR was increased to 2.85 gVS $l^{-1}day^{-1}$. This caused the pH to decrease from an average of 7.00 to 6.80 and 6.98 for the CAR and CSTR configurations, respectively, after 20 days of incubation. According to Nizami et al. (2009), a reduction in pH is accompanied by an increase in the accumulation of VFAs, which indicates strong hydrolysis and acidogenesis. However, because the solubilization of substrates was enhanced in the CAR configuration through the integration of a high rate reactor, a higher peak in the VFA concentrations resulted in a lower pH (Veeken & Hamelers, 1999). A subsequent increase in OLR to 4.00 gVS $l^{-1}day^{-1}$ resulted in a further drop in the pH with average values of 6.10 and 6.80 for the CAR and CSTR configurations, respectively, after 34

days of incubation. This is thought to be due to the limonene present in the CFW although only methanogenesis was suspected to be partially inhibited in the CAR configuration as 1.04 ± 0.16 g l^{-1} of acetic acid accumulation and a relatively low rate of methane production (0.20 ± 0.04 l CH_4 $gVS^{-1}day^{-1}$) were observed. This trend is similar to that seen in Martín et al. (2010) study. They recorded a rapid decrease in pH from 7.2 to 6.70 when the OLR was above 3.5 gVS $l^{-1}day^{-1}$. On the other hand, acidogensis, acetogenesis and methanogenesis are thought to be inhibited in the CSTR configuration, as the acetic acid (0.29 ± 0.043 g l^{-1}) and methane production (0.15 ± 0.005 l CH_4 gVS^{-1}) were relatively low. Inhibition in AD is indicated by a decrease in methane production, the accumulation of VFAs and a decrease in pH (Kroeker et al., 1979). The average pH of the CAR configuration remained between 6.30 and 6.80 even when the OLR was increased to 5 gVS $l^{-1}day^{-1,}$ suggesting a good mass transfer between the acidogenic and methanogenic groups (Liu et al., 2008). The retained granular sludges in the high rate reactor facilitated the conversion of organic acid into methane production as the rate of VFA production was almost equal to consumption (Zhou et al., 2011). On the other hand, the CSTR incubation achieved an average pH of 7.00, suggesting a good mass transfer between the acidogenic and methanogenic processes and better buffering capacity (Fig 4b). According to Liu et al. (2008), the optimum pH to obtain the maximum biogas yield under anaerobic conditions should be 6.5-7.5.

The VFAs are the main soluble intermediate precursors for methane production. In this study, the CSTR and CAR configurations accumulated mostly acetic and butyric acids, respectively. As shown in Fig. 6, the main VFAs identified in this study were acetic, propionic, isobutyric, butyric and valeric acids. The individual VFAs were below 0.5 g l^{-1} for both configurations even when the OLR was increased from 1.42 to 2.84 gVS $l^{-1}day^{-1}$. The maximum rate of methane production was 0.31 ± 0.04 and 0.305 ±

0.03 l CH_4 $gVS^{-1}day^{-1}$ for the CAR and CSTR incubations, respectively. After 33 days of incubation, the OLR was increased to 4.00 gVS $l^{-1}day^{-1}$ and a partial increase in the accumulation of VFAs was observed in both configurations. The CAR configuration produced 1.04 ± 0.16 and 0.67 ± 0.11 g l^{-1} acetic and propionic acids, respectively; whereas, the CSTR configuration produced 0.18 ± 0.08 and 0.15 ± 0.04 g l^{-1} acetic and propionic acids, respectively (Fig 5). The accumulation of VFAs in the CAR configuration resulted in a reduction in pH from an average of 7.00 to 6.00, while the CSTR configuration maintained an average pH of 6.80 (Fig 4a). The sudden peak in the VFA concentrations was only sustained until the 35^{th} day of incubation, as the concentration of accumulated acids was less than 0.5 g l^{-1}. This is thought to have been caused by the increases in the OLR and, because methane producers are slow growers, it takes a while to attain the methanogenic biomass required to rapidly utilize VFAs (Nagao et al., 2012). The relationship between VFAs and OLR was observed by De La Rubia et al. (2009) during the AD of sunflower oil cake. They reported that a high OLR was accompanied by high VFA concentrations. Similarly, Serrano et al. (2014) recorded increases in VFA concentrations as a result of higher OLRs during the anaerobic co-digestion of orange peel and sewage sludge. Unlike the CAR, the CSTR configuration recorded lower VFA concentrations, suggesting further inhibition of the acidogenic bacteria. This inhibition was thought to have been caused by the presence of limonene in the CFW material (Mizuki et al., 1990). However, after the 41 days of incubation, accumulation of acetic acid was observed in the CSTR configuration with a concentration of 1.8 ± 0.07 g l^{-1} and methane production of 0.13 ± 0.009 l CH_4 $gVS^{-1}day^{-1}$. At this point, the CSTR configuration showed an improvement in acidogenesis and acetogenesis but the relatively low rate of methane production suggested further inhibition of the methanogenic bacteria. According to Bollon et al. (2011), the rate of

acetate degradation is inversely proportional to the water content of the AD system. In the case of CSTR, the presence of limonene and a lack of leachate distribution might have contributed to the low levels of methane production. In addition, an accumulation of butyric acid was observed at 48 days of incubation with a value of 3.13 ± 0.20 g l^{-1} further suggesting a shift in the acidogenic microbial populations (Vanvelsen, 1979; Wang et al., 1999).

Subsequent increases in the OLR from 4.00 to 5.00 gVS $l^{-1}day^{-1}$ resulted in the accumulation of butyric acid and a reduction in acetic acid in the CAR configuration. While in the CSTR configuration, there was an increase in the acetic acid concentration. The CAR configuration achieved a maximum butyric acid value of 4.24 ± 0.16 g l^{-1} after 68 days of incubation, whilst the CSTR configuration recorded a maximum acetic acid value of 4.16 ± 0.73 g l^{-1} after 63 days. The accumulation of acetic acid by the CSTR configuration indicated the inhibition of methanogenesis, while the accumulation of butyric acid by the CAR configuration suggests a shift in the acidogenic pathway and inhibition of acidogenesis; this could be due to the presence of limonene (Vanvelsen, 1979; Wang et al., 1999). This is consistent with the result obtained by Martín et al. (2010), who reported that at OLRs above 3.67 gVS $l^{-1}day^{-1}$ higher ratio of propionic/acetic acid with values ranging from 1.30 ± 0.15 to 5.00 ± 0.41 g l^{-1} was produced. In this study, the water content contained in the high rate reactor, which extends 10 mm into the upper chamber of the CAR configuration, might have contributed to the uniform distribution of metabolites for enhanced metabolism while the immobilized cells in the lower chamber also increased the conversion of these metabolites into methane gas It has been reported that a distance of less than 1 µm is essential for the oxidation of VFAs and hydrogen production and the proximity between groups of microbial cells was achieved through immobilization of cells (Stams, 1994;

Schink, 1997). In addition, immobilized cells have been shown to out-perform suspended microbial cells, particularly in the presence of inhibitors (Bertin et al., 2004; Park et al., 2012).

3.3. Changes in VS and COD profile

VS measurements were conducted for the solid phase effluent in both configurations, while the COD measurements were specific to the liquid phase samples from the lower chamber of the CAR configuration. Fig. 4 shows the VS contents of the solid phase effluent for both the CSTR and CAR configurations. Generally, the VS fluctuations are due to the relationship between the OLR and the hydraulic retention time (HRT). An increase in the OLR will cause a sudden peak in the VS content and, depending on HRT, the VS content should decrease (Aslanzadeh et al., 2014). In this study, the VS content of the two configurations fluctuated, particularly each time the OLR was increased. The CAR configuration fluctuated from 56 - 70%, 56 - 66% and 56 - 70%, respectively, for OLRs of 1.42, 2.85 and 4.00 gVS $l^{-1}day^{-1}$, while the CSTR configuration fluctuated from 57 - 63%, 59 – 64% and 58 - 65%, respectively (Fig 3). The VS content of the two configurations was relatively low, an indication that hydrolysis was not limiting. Comparatively, there was less variation between the VS content of the two configurations suggesting that the microbial activity for hydrolysis was less inhibited by low moisture and the presence of limonene. Veeken and Hamelers (1999) reported that hydrolysis can occur at relatively low moisture compared to methanogenesis. This is because moisture is needed to transport metabolites to bacterial cells. Also, there are indications that the hydrolysis stage of AD is less susceptible to limonene (Di Pasqua et al., 2006; 2007). However, when the OLR was increased to 5.00 gVS $l^{-1}day^{-1}$, the VS content fluctuation was between 59 and 66% and 57 and 69% for the CSTR and

CAR configurations, respectively. The VS reductions show that the system is able to dissolve particulate matter for further biological degradation

COD removal efficiency is a measurement of organic waste treatment (Razaviarani et al., 2013). The COD from the liquid phase effluent of the CAR configuration was measured to observe the performance of the immobilized cell during leachate treatment. A fluctuation was observed in the COD values, indicating increases in the OLR and the conversion rate of the high rate reactor connected to the upper chamber of the CAR configuration (Fig 6). The high rate reactor contained granular sludge, which was responsible for the treatment of the leachate as it flowed from the high solid reactor (Nizami et al., 2011). The COD value after 1 day of incubation was 0.49 ± 0.08 g l^{-1}. This later decreased to 0.39 ± 0.03 g l^{-1}. Upon increasing the OLR from 1.42 to 2.85 gVS $l^{-1}day^{-1}$, the COD increased to 0.49 ± 0.08 g l^{-1} after 18 days of incubation. However, when the OLR was increased to 5.00 gVS $l^{-1}day^{-1}$, the COD increased to a maximum concentration of 1.26 ± 0.29 g l^{-1} at 63 days of incubation. The COD of the effluent was observed to decrease with HRT, particularly after increasing the OLR. For instance, the COD decreased from 1.06 ± 0.25 to 0.67 ± 0.14 g l^{-1} when the OLR was increased from 1.42 to 2.85 gVS $l^{-1}day^{-1}$. However, subsequent increases in the OLR above 2.85 gVS l^{-1} d^{-1} resulted in a continuous increase in the COD regardless of the HRT. This disagrees with Nizami and Murphy (2011) who reported that an increased COD removal efficiency is attributed to an increased COD concentration. At OLRs of 4.0 and 5.0 gVS $l^{-1}day^{-1}$, the COD concentration increased continuously to a maximum concentration of 1.26 ± 0.29 g l^{-1}. This is an indication that the high rate reactor was treating the leachate as it trickled into the lower chamber through the permeable membrane and the treatment efficiency was highest when the OLR was < 2.85 gVS $l^{-1}day^{-1}$.

4. Conclusion

The CAR configuration was designed with two chambers: the high solid upper compartment for solubilisation of substrate and the high rate lower compartment to accelerate methanogenesis by increasing mass transfer of metabolites through leachate diffusion and also reduce inhibition of methanogenesis though biofilm formation. The CAR was compared with the conventional CSTR configuration using CFW as a mono-substrate. The average VS content was similar for both configurations but the methane yield varied significantly with increase in OLR. The CAR achieved the highest methane yield for every increase in OLR and fastest recovery rate during limonene suppression. The CAR configuration produced 34.0, 43.3, 48.5 and 79.9% higher cumulative methane yield for OLR of 1.42, 2.85, 4.00 and 5.00 gVS $l^{-1}day^{-1}$, respectively thus suggesting that the CAR out-perform the CSTR configuration. However, for subsequent comparative study, the CAR should be compared with a two-stage high solid and high rate reactor configuration.

Acknowledgement

This work was supported by the Centre for Global Eco-innovation (X02646PR) and Stopford Energy and Environment Ltd

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Tables

Table 1: Chemical composition of citrus fruit waste (n=3 and mean value ± standard deviation)

Parameter	Citrus fruit waste
TS (%)	16.6 ± 0.21
VS (%)	97.5 ± 0.26
pH	5.98 ± 0.12
Cellulose (%)	20.45 ± 1.06
Hemicelluloses (%)	6.61 ± 0.79
Lignin (%)	2.29 ± 0.82
Limonene (mg l^{-1})	3.95 ± 0.33

* TS, VS, pH and limonene were analysed based on fresh basis while other parameters were based on dry mass.

Table 2: Cumulative methane yields at different OLR, HRT and reactor configuration (mean value ± standard error (n=2))

Days	OLR (gVS $l^{-1}day^{-1}$)	HRT (day)	Cumulative methane production ($l\ CH_4\ gVS^{-1}$)	
			CAR	CSTR
0-16	1.420	70	3.46 ± 0.06	2.45 ± 0.21
17-32	2.850	41	4.02 ± 0.18	2.59 ± 0.13
33-49	4.000	28	3.38 ± 0.07	2.06 ± 0.03
50-70	5.000	22	4.25 ± 0.26	1.82 ± 0.07

Figure captions

Figure 1. Schematics of the reactor set up (a) pH and temperature probes; (b) mixer; (c) Influent reservoir; (d) Effluent reservoir; (e) pump; (f) volumetric gas tipping meter; (g) gas vent; (h) data acquisition and display; (I) CSTR; (j) CAR; (k) permeable membrane ; (l) granular sludges and (m) temperature control

Figure 2. Biogas production rates for a semi-continuous test for different reactor configuration (a) CAR and (b) CSTR conditions (mean value (n=2) ± standard error)

Figure 3. VS profiles of semi-continuous test for different reactor configuration (a) CAR and (b) CSTR conditions (mean value (n=2) ± standard error)

Figure 4. pH profiles of semi-continuous test for different reactor configuration (A) CAR and (B) CSTR conditions (mean value (n=2) ± standard error)

Figure 5. Individual VFA profiles of semi-continuous test for different reactor configuration (a) CAR and (b) CSTR conditions (mean value (n=2) ± standard error)

Figure 6. Chemical oxygen demand concentration of the low compartment effluent of the CAR conditions (mean value (n=2) ± standard error)

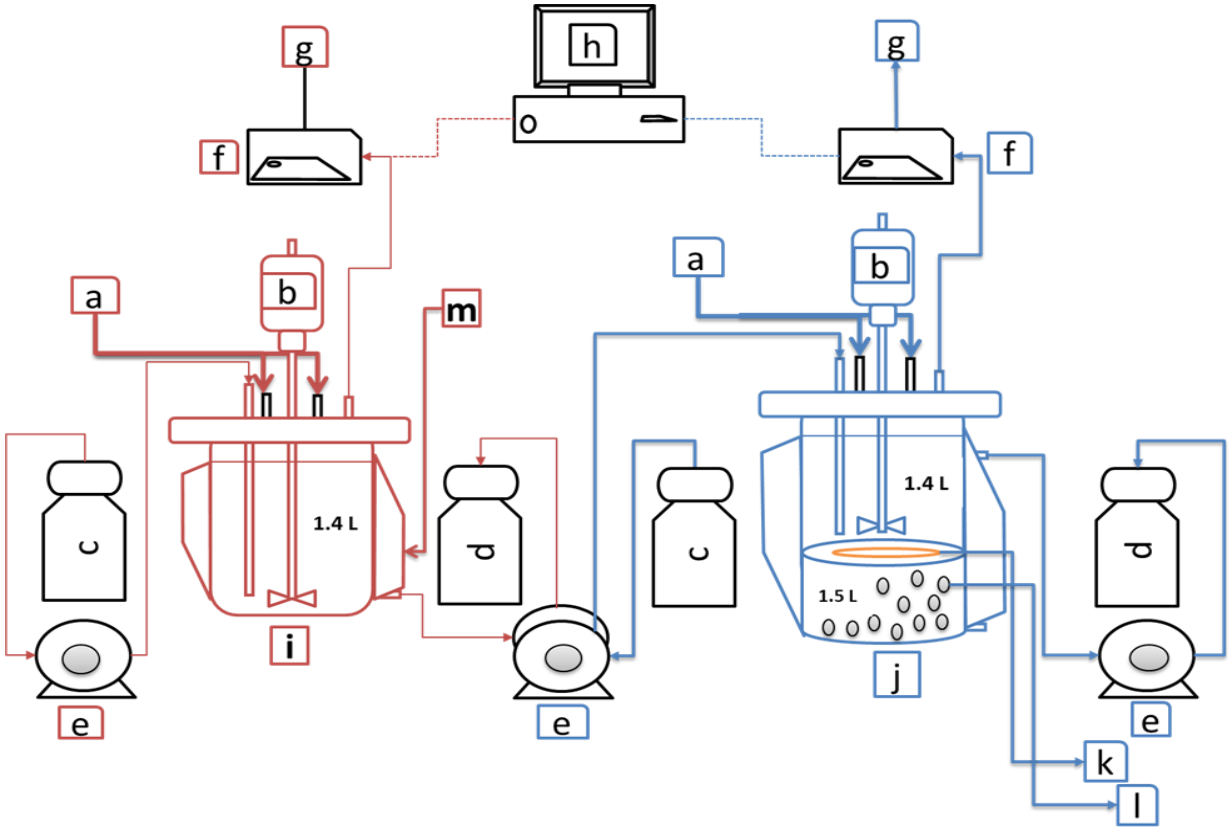

Figure 1

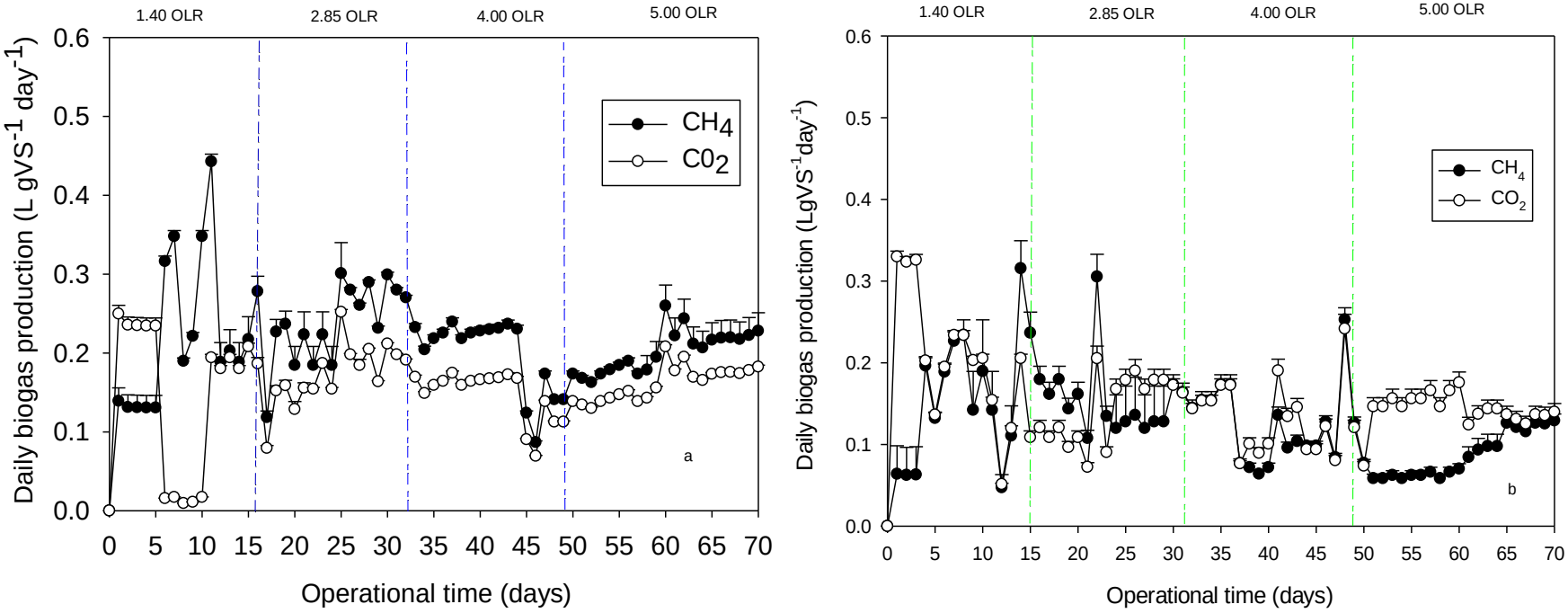
1.40 OLR
2.85 OLR
4.00 OLR
5.00 OLR
Daily biogas production (L gVS-1 day-1)
Operational time (days)
CH4
C02
a
Daily biogas production (LgVS-1day-1)
CH4
CO2
b

Figure 2

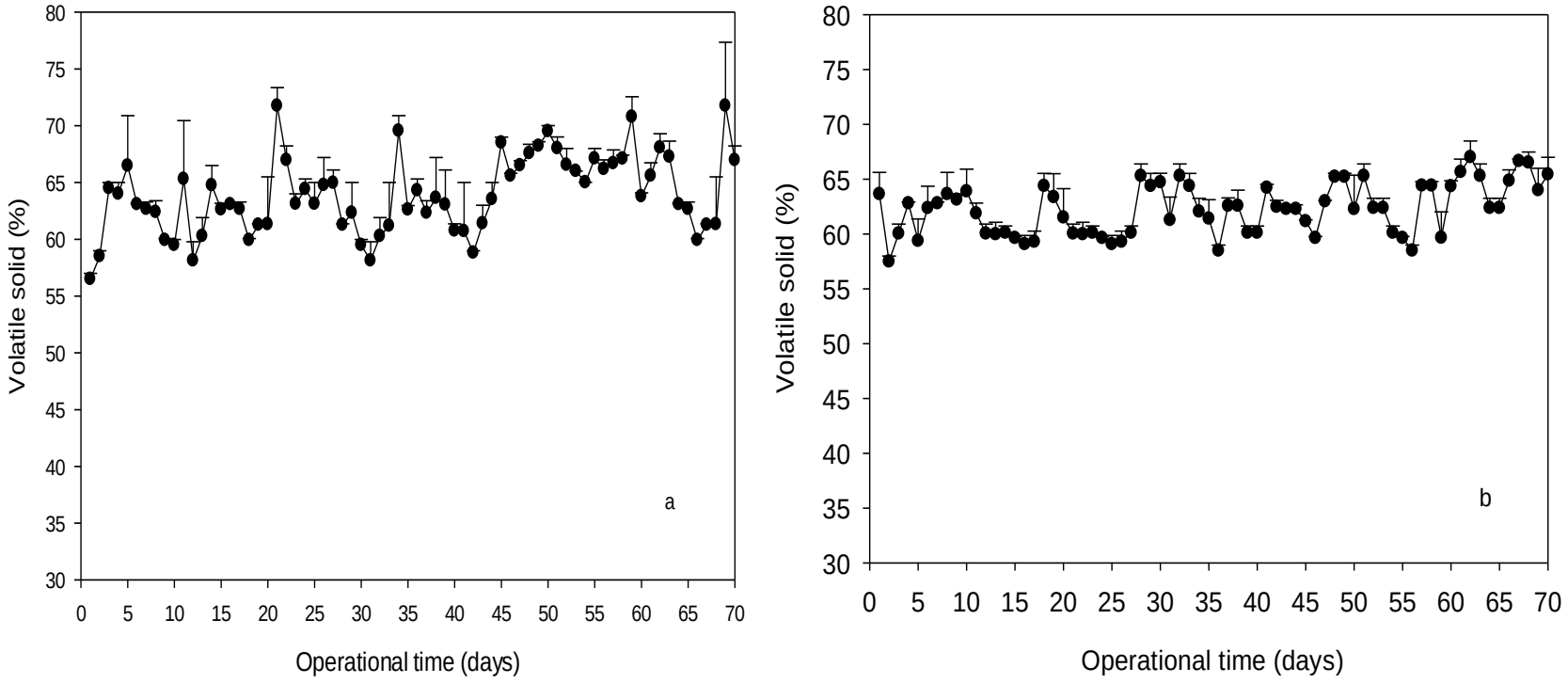

Figure 3

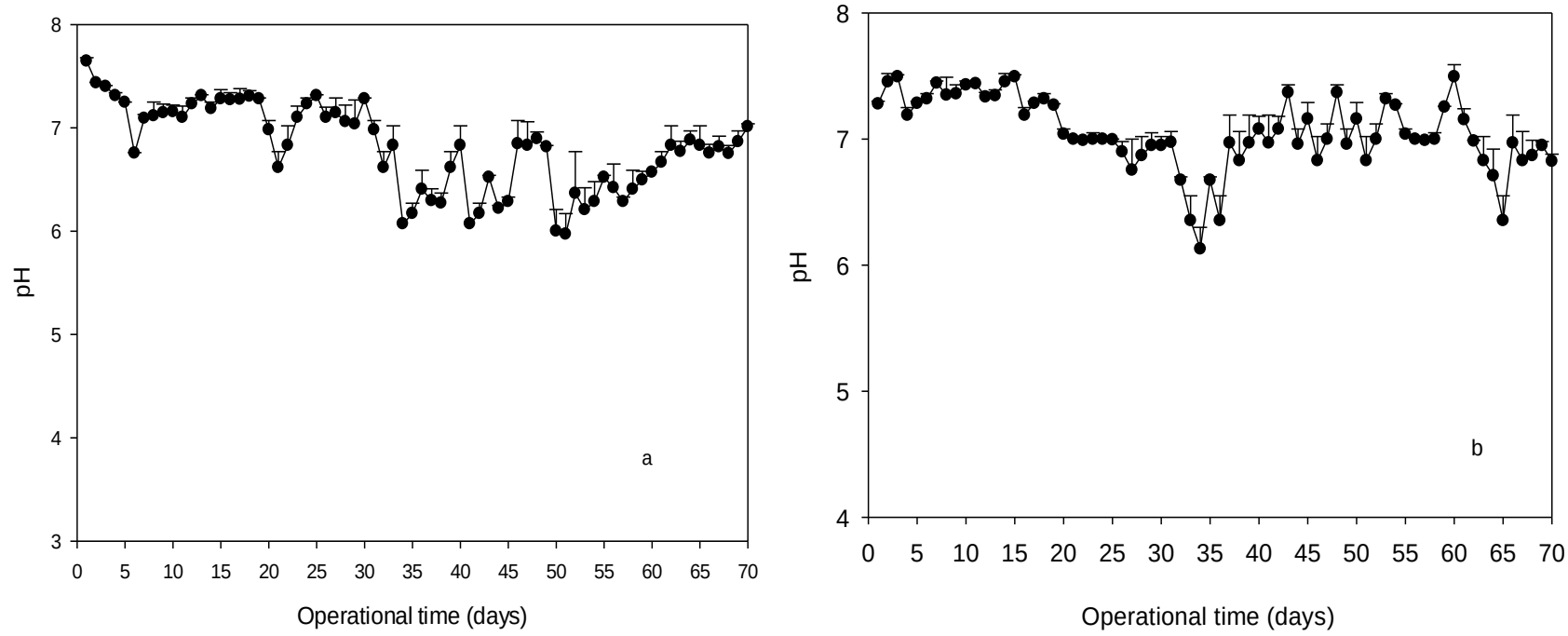

Figure 4

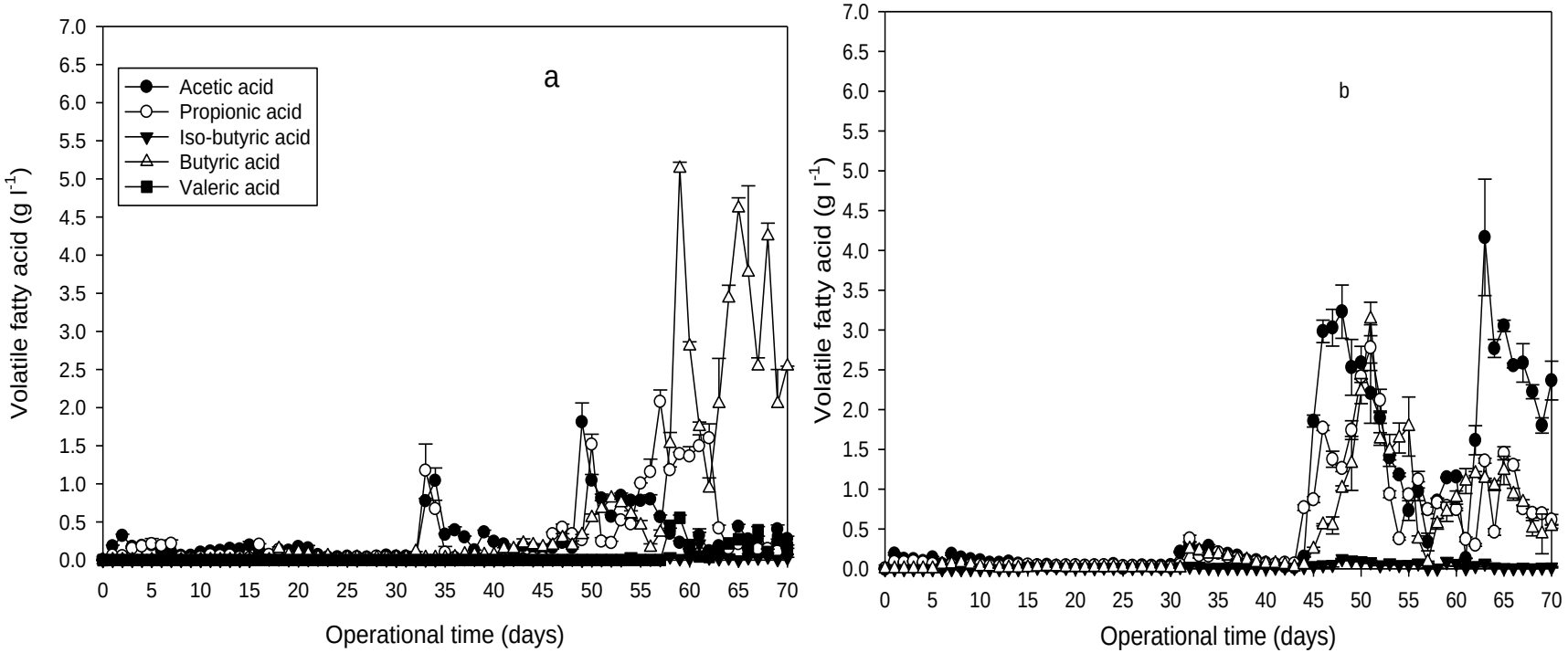
a
b
Acetic acid
Propionic acid
Iso-butyric acid
Butyric acid
Valeric acid
Volatile fatty acid (g l-1)
Operational time (days)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70

Figure 5

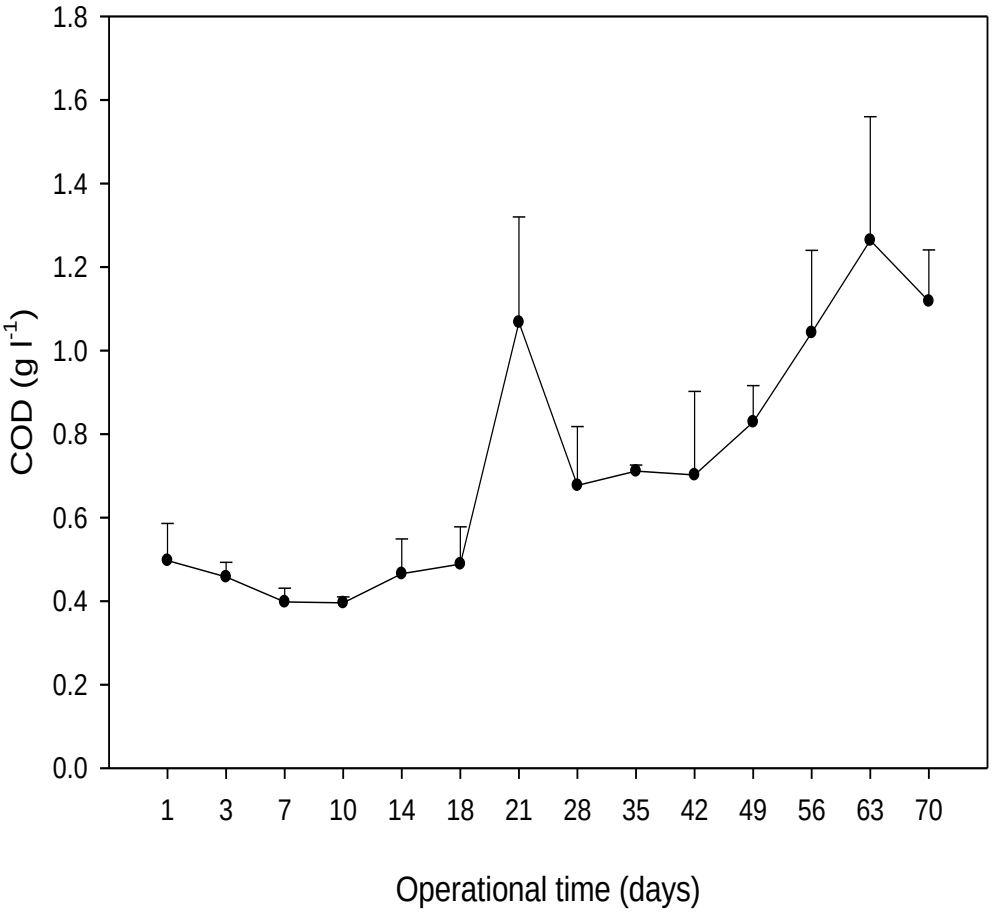
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
COD (g l-1)
1
3
7
10
14
18
21
28
35
42
49
56
63
70
Operational time (days)

Figure 6

3. Précis of results from Papers I-VI

Paper I: In this paper, the applicability and limitation of HSAD were reviewed with specific attention being paid to optimizing methane yield and improving digestate quality. The benefits of HSAD were highlighted, particularly the reduction in water usage, the increase in OLR, the reduced nutrient loss during digestate handling and the decrease in digestate dewatering. Low water, poor mixing and uneven distribution of metabolites were reported to be the major causes of low methane yield. However, this paper evaluated several existing technologies in AD that can be integrated to improve the performance of the system. Technological integrations such as thermo-mesophilic digestion, co-digestion, and mixing and integration of two or more reactors were suggested to be a major contribution to the higher methane yield from HSAD.

Paper II: This review was a follow-up to offer a solution to some of the issues highlighted in Paper I, which were SII and digestate management. In this paper, the relevance of black carbon, particularly biochar in AD was highlighted and evaluated. AD is often faced with inhibition interferences and poor digestate management. These challenges were discussed in the context of several published works focusing on these issues. The adsorbing properties of biochar in reducing or removing potential inhibitors, trapping digestate nutrient to reduce leaching, inducing cell immobilization and contributing to the buffering capacity of the AD system were also evaluated. The potential in applying biochar to AD is high although realising this potential will require significant research.

Paper III: This study was carried out to investigate the acclimation rate of anaerobic bacteria to SII as highlighted in Papers 1 and II. The acclimation rates of different inocula to limonene solution were investigated. The inocula were digested sewage

sludge, landfill leachate, compost leachate and their mixtures. This experiment was carried out at a mesophilic temperature of 35 °C for 40 days using a sequential batch test. The sequential addition of the inhibitors increased from 0.025 - 1.00 mg $ml^{-1}$for limonene solution. The result revealed that the mixed inoculum acclimates faster to limonene solution. A maximum methane yield of 544 ± 21 mlCH_4 was achieved by the mixed inocula (ML).

Paper IV: Following the suggestion in Paper II on the potential for biochar to reduce SII, this study was carried out to investigate the effect of different biochar and biochar ratios on the AD of CFW. In this study, digested sewage sludge was used as the inoculum, based on the findings from Paper III. The result showed that the wood derived biochar outperformed the other biochar material and the CFW to biochar ratio of 1:3 achieved the shortest microbial lag phase of 6.8 days. There were no significant differences in the methane yield amongst the biochar containing incubations; but when compared with the CFW only incubation the methane yield was significantly higher for the incubation with biochar. Colonisation of microbial cells was observed on the surface of the biochar material with the aid of a scanning electron microscope.

Paper V: The effect of a high temperature in mitigating limonene inhibition was discussed in Papers I and II. Previous studies focused on orange fruit waste, which contains mainly D-limonene and two operating temperatures of 35 and 55 ^{0}C. This study focuses on CFW, which is a combination of both D and L-limonene and varying operating temperatures between 35 - 55 °C for 30 days using a batch test. The result showed that higher temperatures of 45 and 55 °C showed no microbial lag phase, but the methane yield was similar. This suggests that the higher temperatures of 45 and 55 °C are sufficient for AD of CFW. Further to this study, a continuous test experiment was carried out in mesophilic (35 °C) and thermophilic conditions (55 °C) for 90 days

at varying OLR of 0.71, 1.42, 2.85, 4.00 and 5.00 gVS $l^{-1}day^{-1}$. The acclimation rate of the two incubations was observed based on the daily and total methane yields for each sequential increase in OLR and the thermophilic temperature performed better.

Paper VI: Reactor modification and integration were one of the suggestions highlighted in Paper I, as an option to optimize the methane yield from HSAD. This was further investigated using a compartmentalized anaerobic reactor (CAR) configuration and compared with the continuously stirred tank reactor (CSTR) system. The CAR configuration is a combination of high solid and high rate reactor separated by a permeable membrane in a single stage operation. The permeable membrane will retain the immobilized cells in the high rate reactor but enhance the diffusion of metabolites and nutrients within the two chambers. This will increase the distribution of metabolites to bacterial sites, increase the mobility of soluble metabolites, reduce the biomass washout and increase acclimation to inhibitors. The study was carried out simultaneously and the result showed that the CAR configuration outperformed the CSTR, particularly when the OLR started to increase from 2.85 to 5.00 gVS $l^{-1}day^{-1}$. This study was carried out for 70 days at a mesophilic temperature of 35 °C.

4. General discussion and conclusion

AD technology could play a decisive role in the ongoing campaign against climate change, with Europe and Asia respectively having the highest numbers of large and small-scale anaerobic digesters. However, as AD is gaining more economic interest there is a need for process optimization, especially through the use of SSAD systems. Currently, there are about 265 AD plants in the United Kingdom and in the last five years the United Kingdom's AD industry has seen 622 % growth outside of the water sector (IEA Bioenergy, 2014). The growth of onsite SSAD with a capacity of 50-250 KWh has been seen to increase in the UK, Germany and Switzerland in recent years (IEA Bioenergy, 2014). SSAD are relatively less expensive and most suitable for onsite organic waste treatment. However, onsite waste streams are often mono-substrates that are either high energy or high nutrient but that also have high potential for inhibition. Mono-substrates such as livestock manure, abattoir wastewater, CFW, fat and oil are often avoided because their constituents or metabolites pose a threat to the performance of the AD system (Chen et al., 2008). In most cases, AD operators prefer co-digestion of two or more substrates to facilitate synergy and counteract possible inhibition that might result from some mono-substrates (Cheng & Zhong, 2014; González-Fernández et al., 2013; Jung et al., 2000; Wilson et al., 2008). This approach discourages mono-substrate AD, particularly when co-substrates are not within reach or not economical for the operator. In this study, CFW, a mono-substrate and often a constituent of food waste material was used as the main feedstock. CFW is a lignocellulose material although the lignin content is relatively low (Palmqvist & Hahn-Hagerdal, 2000; Ramos, 2003). CFW contains an essential oil that is rich in limonene and this compound is inhibitory to microbial growth (Wikandari et al., 2015). The CFW was selected because it is an ideal waste stream for meeting the objectives of this study. The CFW is

an ideal lignocellulose mono-substrate, which contains both D and L limonene compounds and is found in all citrus residues.

With regard to SII, the limonene content of the CFW was investigated. There are existing studies on the effect of orange peel on AD but there are no studies on the specific effect of the limonene compound on AD, particularly using different inocula (Kaparaju & Rintala, 2006; Martín et al., 2013; Martín et al., 2010). Limonene has been reported to be easily degraded by fungi and filamentous bacteria (Demyttenaere et al., 2001; Duetz et al., 2003). On that basis, the following inocula: landfill leachate, compost leachate, digested sewage sludge and a mixture of these were selected and investigated. There are indications that compost and landfill leachate contains a high population of fungi, which have been reported to transform limonene into other metabolites (Ángel Siles López et al., 2010; Neher et al., 2013; Ruiz & Flotats, 2014; Saetang & Babel, 2010). According to Vanvelsen's (1979) study, if an active inoculum is sourced from an unfavourable condition there are higher chances of survival during subsequent exposure to similar conditions. Likewise the inocula used in this study were expected to be robust since they are sourced from very harsh environments. However, the mixed inoculum performed better, perhaps because the combination of the different inocula had a synergistic effect. Aside from acclimation, adsorption and operating temperature were also investigated. The adsorption of contaminant using carbon materials such as bentonite, zeolite and activated carbon has been extensively investigated in both aerobic and anaerobic conditions (Borja et al., 1996; Palatsi et al., 2012). However, the effect of adding biochar to the AD operation has not been investigated. There are indications that biochar can adsorb monoterpenes, particularly limonene, but its impact in AD has not been investigated (Hale et al., 2015). Biochar is produced when plant based biomass is subjected to a high temperature in the absence

or presence of low levels of oxygen (Shafizadeh, 1982). At the moment, the production of the material incurs a moderately high cost but if there are several applications for the material, this will indirectly offset the production cost. In this study, the addition of biochar reduced limonene inhibition during AD. The addition of biochar had the added benefit that it could reduce nutrient leaching during the spreading of the digestate on land (Dicke et al., 2015; Kizito et al., 2015; Vaughn et al.). In addition, there are indications that the adsorption of limonene will reduce the concentration of terpenes in the gas phase. The challenge with injecting the gas grid with food waste derived biogas is the smell of limonene. This compound has a very strong smell, which is able to mask the methyl mercaptan gas added to cooking gas in order to detect gas leaks. The importance of biochar to AD was highlighted and evaluated in paper IV. Further to this study, the effect of higher operating temperatures of 45, 50 and 55 ^{0}C on the AD of CFW was investigated. This was similar to the findings of Martín et al. (2010), as the AD of orange peel was more efficient at a thermophilic temperature of 55 ^{0}C. My findings are similar but more extensive because the higher temperature of 45 ^{0}C was also found to be equally suitable. Most commercial AD operates at a high mesophilic temperature of between 37 and 45 ^{0}C and for operators using food waste as their waste stream, or mono-substrate like CFW, this temperature might reduce limonene inhibition.

HSAD has been investigated and several approaches such as thermo-mesophilic digestion, leachate recirculation, and high solid and high rate reactor integration have been reported to improve methane yield (Fagbohungbe et al., 2015). HSAD has been reported to reduce water usage by 5-20 % depending on the total solid content of the feedstock, enhance digestate handling and reduce reactor size (Garcia-Bernet et al., 2011). On the other hand, the application of HSAD reduces methane production because

of poor transportation of metabolites and nutrients to the bacterial sites (Dong et al., 2010; Nagao et al., 2012). The integration of high solid and high rate reactors in a multi-stage system has been investigated and reported to increase methane yield through the recirculation and redistribution of the leachate (Nizami & Murphy, 2010; Pohl et al., 2013; Pohl et al., 2012; Shewani et al., 2015; Xing et al., 2014). The operation of high solid and high rate reactors requires a two stage/phase anaerobic digestion operation and reports show that single stage AD has been on the increase, principally because the investment cost is lower (De Baere & Mattheeuws, 2008; De Baere, 2010; Nizami & Murphy, 2010). A single stage system that combines high solid and high rate reactors was developed, investigated and reported in paper VII as an alternative to a two stage system. During the steady state anaerobic digestion of CFW the CAR was found to have achieved 34%, 43.3%, 48.5% and 79.9% higher cumulative methane production for OLRs of 1.42, 2.85, 4.00 and 5.00 gVS $L^{-1}day^{-1}$, respectively.

Furthermore, because of the high solid content of the digestate from HSAD, the nutrient content per gram is expected to be high and relatively retained within the solid matrix. HSAD might reduce the operational cost for the dewatering and storage of the digestate since the digestate contains less water. The objectives of this study were met and the solutions offered can be scaled up for industrial applications. As the application of SSAD continues to increase there is a need to deploy solutions that will ensure optimal process performance and higher biogas yield. The result from this study revealed that SII, a common problem with mono-substrate AD can be minimized by selecting a robust inoculum source, extracting the inhibitor with an adsorbent and choosing the appropriate operating condition.

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Recommendation for future research

i. In Paper III, the digested sewage sludge inoculum and the mixed inoculum showed higher rate of acclimation to limonene compound, respectively. For future studies, the microbial population dynamics of the different inocula at varying concentration of the inhibitor should be monitored and compared with the rate of methane production.

ii. In Paper IV, the addition of biochar to CFW recorded higher methane yield and lower microbial lag phase when compared with the incubation without biochar. This was a batch test, for future studies a semi-continuous test over a longer period would be required to comprehensively study the effect of biochar on AD.

iii. In Paper V, the semi-continuous study showed that the thermophilic operation (55 °C) was sufficient for AD of CFW. However, the batch test showed that higher mesophilic operation at 45 °C was not inhibited by the limonene content of the CFW. This can be further investigated using a semi-continuous test to further establish that higher mesophilic temperature is as sufficient as thermophilic temperature during AD of CFW. In addition, the microbial population dynamics at both higher mesophilic and thermophilic temperature should be monitored.