



## Characterization of Fast Anaerobic Digestion in a Novel Reactor Design with Immobilized Biofilms

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TECHNICAL UNIVERSITY OF DENMARK

DOCTORAL THESIS

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# Characterization of Fast Anaerobic Digestion in a Novel Reactor Design with Immobilized Biofilms

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*A thesis submitted in fulfillment of the requirements  
for the PhD degree*

*in the*

Department of  
Chemical and Biochemical Engineering

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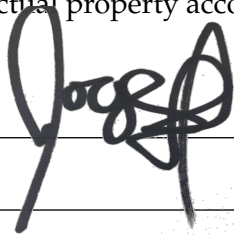
## Declaration of Authorship

I, Jorge Enrique GONZÁLEZ LONDOÑO, declare that this thesis titled, “Characterization of Fast Anaerobic Digestion in a Novel Reactor Design with Immobilized Biofilms” and the work presented in it are my own. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University.
- Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated.
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- I have acknowledged all main sources of help. I have made clear exactly what was done by others and what I have contributed myself.
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## English Abstract

Anaerobic Digestion (AD) has become a prioritized technology for energy production from diverse waste streams from industry and agriculture, especially from enzymatically hydrolyzed Organic Fraction of Municipal Solid Waste (OFMSW). Through this practice, it is possible to produce a gas mixture of methane and carbon dioxide that is popularly referred to as biogas. This product can then be used to sustain different energy intensive activities through electricity or heat production. Despite a lot of research in the field, the reactor designs available cannot handle substrates with high organic loads and particulate material. In this project, we characterize the Fast Anaerobic Digester (FAD), a novel reactor design that could potentially improve the digestion process in particular of complex biomass. Amongst the main feature of the FAD there is a particular compartmentalization and immobilization of active microorganisms in fixed biofilm carrier. Within the scope and hypotheses of the thesis, the most crucial factors during the degradation of enzymatically liquefied OFMSW have been characterized in laboratory and pilot scale, the main microbial populations involved in the AD conversion have been unraveled using high throughput DNA sequencing and the influence of high concentrations of cat-ions have been examined.

The performance of the FAD reactors studied in the project has revealed that the reactor design did deliver significant improvements in efficiency and high degradation of COD at low HRTs. The compartmentalization design provided improved retention of the convertible material, while the flow regimes inside the reactor secured improved contact between the digesting media and the immobilized microorganisms. During more than 6 months in operation, the FAD reactor expressed more than 70% of the methane potential in the substrate up to an OLR of  $20.8 \text{ gCOD} \cdot \text{l}_{\text{reactor}}^{-1} \cdot \text{day}^{-1}$  at HRTs lower than 5 days. The yields between  $0.2$  and  $0.3 \text{ l}_{\text{CH}_4} \cdot \text{gCOD}^{-1}$  and productivities of  $2\text{--}6 \text{ l}_{\text{CH}_4} \cdot \text{l}_{\text{reactor}}^{-1} \cdot \text{day}^{-1}$  achieved below 10 days HRT were higher than any other anaerobic digestion data reported in literature for OFMSW.

Despite hypothesizing that there would be a selective pressure to form distinct populations in response to substrate composition and compartmentalization, it was found that there were dense and diverse microbial communities in the biofilm layers and no compartment specialization. Remarkably, it was shown that the methanogenic archaeal populations are less prone to changes in composition compared to the bacterial communities regardless of whether they are in the biofilm or in the digesting media. During mesophilic conversion, *Methanosarcina spp.* and *Methanoculleus spp.* constitutes almost two thirds of the total methanogenic population. Increasing the temperature to the thermophilic range shifted these groups to be dominated by *Methanoculleus thermophilus* and other known thermophiles. In regard to the bacterial populations, unclassified *Cloacamonas* and unclassified *Bacteroidetes* were the most abundant until unclassified *Firmicutes* and even *Clostridia* increased in relative abundance when the temperature regime shifted.

In a separate study on the effects of the inoculum composition on the microbial diversity in AD of enzymatically liquefied OFMSW, it was possible to show that regardless of the seed material, the archaeal populations quickly establish to constitute between 30% to 50% of the total populations. In addition, the data obtained strongly indicates that the enzymatic treatment to generate the liquefied OFMSW enhances the AD by circumventing the rate limiting hydrolysis step in the overall biomethanation process.

A limitation of a fast anaerobic conversion process is determined by a high level

of cat-ions in the digesting media. The inhibitory effects of Na and Mg were systematically assessed in batch reactors to determine the repercussion on the methanogenesis. It was possible to show that these cat-ions retarded methane production in a dose dependent manner using ethanol and methanol as pure substrates. Within the substrate levels examined, it was found that the methane yields in cat-ion inhibited reactors is highly dependent on the organic load as well as the inhibitor concentration. However, the initial methane production rates mainly dependent on the cat-ion concentration. Addition of EDTA or crown ethers were able to abolish the inhibitory effects, indicating that cat-ion inhibition is a reversible process that could potentially be controlled during fast anaerobic digestion processes.

## Danish Abstract

Metanisering er en prioriteret teknologi til energiproduktion fra diverse affaldsstrømme fra industri og landbrug, og specielt fra enzymatisk hydrolyseret organisk fraktion af husholdningsaffald (Engelsk: Organic Fraction of Municipal Solid Waste (OFMSW)). Gennem denne process er det muligt at producere en gasblanding af metan og kuldioxid som ofte referes til som biogas. Dette produkt kan blive brugt til at udføre forskellige energiintensive aktiviteter via elektricitets- eller varmeproduktion. På trods af meget forskning på området kan de tilgængelige reaktordesign ikke håndtere substrater med højt indhold af organisk og partikulært materiale. I dette projekt karakteriserer vi "Fast Anaerobic Digester" (FAD), som er et nyt reaktordesign som potentielt kunne forbedre metaniseringsprocessen, specielt for komplekse biomasser. En af de primære karakteristika ved FAD er at der er en rumdeling i reaktoren og immobilisering af aktive mikroorganismer i en fikseret biofilmsbærer. Indholdet og hypoteserne i denne afhandling omhandler de mest kritiske faktorer under nedbrydelse af enzymatisk behandlet OFMSW i laboratorie- og pilotskala, optrævler den primære mikrobielle population involveret i metaniseringsprocessen via "high throughput" DNA- sekvensering og undersøger effekten af høje koncentrationer af kationer.

Ydeevnen af FAD-reaktorerne, som blev undersøgt i dette projekt, viste at reaktordesignet leverer betydelig forbedring i effektivitet og høj grad af nedbrydelse af COD (forkortelse for "Chemical Oxygen Demand") ved lav hydraulisk retentionstid (engelsk: Hydraulic Retention Time (HRT)). Det opdelte design leverer forbedret retentionstid af the nedbrydelige materiale, mens gennemstrømningsregimet inde i reaktoren sørger for forbedret kontakt mellem fordøjelsesmediet og de immobiliserede mikroorganismer. Gennem mere end 6 måneders drift udløste FAD-reaktoren mere end 70% af metanpotentialet fra substratet med op til en organisk belastningsrate (engelsk: Organic Loading Rate (OLR)) på  $20.8 \text{ g}_{\text{COD}} \cdot \text{l}_{\text{reactor}}^{-1} \cdot \text{day}^{-1}$  ved HRT'er lavere end 5 dage.

På trods af hypotesen om at der ville være selektiv pres til at danne specifikke populationer som respons til substratkompositionen og rumdelingen i reaktoren blev det erfaret at der var kompakt og forskelligartet mikrobielle populationer i biofilmlagene og ingen specialisering i rummene. Det blev desuden vist, at den metanogene arkæopopulation, sammenlignet med den bakterielle population, er mindre følsom over for ændringer i substratkomposition uanset om de var i biofilmen eller i fordøjelsesmediet. Under mesofil konvertering består den metanogene population af to tredjedele *Methanosarcina spp.* og *Methanoculleus spp.* Ved at øge temperaturen til at ligge i termofilområdet blev disse grupper i stedet domineret af *Methanoculleus thermophilus* og andre kendte termofile. Den bakterielle population var domineret af uklassificerede *Cloacamonas* og *Bacteroidetes* indtil temperaturregimet skiftede så uklassificerede *Firmicutes* og tilmed *Clostridia* steg i tilstedeværelse.

I et andet studie angående effekten af inokulumkomposition på den mikrobielle diversitet i metanisering af enzymatisk behandlet OFMSW blev det vist, at uafhængigt af opstartsmaterialet etablerede arkæopopulationen sig hurtigt til at udgøre 30% til 50% af den totale population. Data indikerede meget, at enzymatisk behandling for at generere flydende OFMSW forbedrer metanisering sproccessen ved at omgå det ratebegrænsende hydrolysetrin i den samlede biometaniseringsprocess.

En af begrænsningerne for en hurtig metaniseringsprocess er høje koncentrationer af kationer i fordøjelsesmediet. Den inhibitoriske effekt af Na og Mg blev systematisk undersøgt i batch-reaktorer for at undersøge konsekvenserne på metaniseringen. Det var muligt at vise at disse kationer hæmmede produktionen på en doseafhængig måde ved brug af ætanol og metanol som rene substrater. Ved de substratniveauer som blev undersøgt blev det erfaret at metanudbyttet er meget afhængigt af den organiske belastning såvel som inhibitor-koncentrationen. Den indledende metanproduktionsrate afhænger primært af kation koncentrationen. Tilsættelse af EDTA eller kroneethere ophævede den inhibitoriske effekt, hvilket indikerede at kation inhibering er en reversibel process som potentielt kan kontrolleres under metaniseringsprocesser.



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This thesis builds on the following studies:

- Paper 1. Fast anaerobic digestion of complex substrates via immobilized biofilms in a novel compartmentalized reactor design. Jorge Enrique Gonzalez Londoño, Bjarne Uller, Hanne R. Sørensen, Anne S Meyer. (Londoño et al., 2018)
- Paper 2. Microbial diversity of a high performance fixed biofilm biogas reactor. Jorge Enrique Gonzalez Londoño, Mikael Lenz Strube, Dorothee L. Kurz, Bjarne Uller, Anne S Meyer. (manuscript).
- Paper 3. Rapid microbial diversity adaptation during anaerobic digestion of the organic fraction of municipal solid waste after enzymatic pretreatment Jorge Enrique Gonzalez Londoño, Mikael Lenz Strube, Panagiotis Charachalios, Ines L. Caetano, Bjarne Uller, Hanne R. Sørensen, Anne S Meyer (manuscript).
- Effect of cat-ions on Anaerobic Digestion. Jorge Enrique Gonzalez. (Chapter 10).

I have also contributed to the following scientific outputs which are not included in the PhD thesis:

- Patent: Methods and bioreactors for microbial digestion using immobilized biofilm (Uller, 2016).
- Patent: Methods and bioreactors for microbial digestion using immobilized biofilms(Uller, Londono, and Lardon, 2017).
- Feasibility study of Fixed Film Fixed Filter reactor for anaerobic treatment of liquefied municipal solid waste. *Proc. 15rd World Conference on Anaerobic Digestion (IWA, Beijing, China, October 2017) 2017.*





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# List of Abbreviations

<b>ABR</b>	Anaerobic Baffled Reactor
<b>AD</b>	Anaerobic Digestion
<b>AF</b>	Anaerobic Filter
<b>AMPTS</b>	Automatic Methane Potential Test System
<b>BMP</b>	Biomethane Potential
<b>COD</b>	Chemical Oxygen Demand
<b>CSTR</b>	Continuous Stirred Tank Reactor
<b>DNA</b>	Deoxyribonucleic Acid
<b>EDTA</b>	Ethylenediaminetetraacetic acid
<b>FAD</b>	Fast Anaerobic Digester
<b>HRT</b>	Hydraulic Retention Time
<b>ISR</b>	Inoculum to Substrate Ratio
<b>LCFA</b>	Long Chain Fatty Acids
<b>MSW</b>	Municipal Solid Waste
<b>OFMSW</b>	Organic Fraction of Municipal Solid Waste
<b>OLR</b>	Organic Loading Rate
<b>RNA</b>	Ribonucleic Acid
<b>sCOD</b>	Soluble Chemical Oxygen Demand
<b>SRT</b>	Solid Retention Time
<b>SMA</b>	Specific Methanogenic Activity
<b>TAN</b>	Total Ammoniacal Nitrogen
<b>TS</b>	Total Solids
<b>TSS</b>	Total Suspended Solids
<b>TSVS</b>	Total Suspended Volatile Solids
<b>UASB</b>	Upflow Anaerobic Sludge Blanket
<b>VFA</b>	Volatile Fatty Acids
<b>VS</b>	Volatile Solids
<b>VSS</b>	Volatile Suspended Solids



## Chapter 1

# Preface

As the light of human development, research and innovation has accompanied the expansion of modern societies. For instance, the increase in the global population during the last decades has been the motivation for many scientific advances. Tangible examples of this interrelationship are improvements in novel agricultural systems or the enhancement of animal breeding practices, which aim to support larger numbers of people through lower resource utilization and improvement in crucial factors like land usage and water consumption. A similar relationship can be perceived in the bioenergy sector. The higher energy requirements linked to the necessity to feed more people can be perceived as an intensification of productive systems, which creates demand for alternatives to alleviate the current dependency on fossil fuels. If the results make it possible to guarantee our future generations with the resources to sustain their development while providing enough wisdom to maintain that trend, then it will be possible to justify human development as such.

The motivation for this thesis is development of the field of **Anaerobic Digestion**, based on the strong conviction that a contribution in this area can have a positive impact in our society. The term "anaerobic digestion" proper describes degradation processes that occur in oxygen depleted environments. In the context of this study, the term Anaerobic Digestion refers to all the processes and technologies that occur under such conditions (anoxic) within the framework of the degradation of organic compounds to produce a methane rich biogas. In contrast to many other research subjects, the product per se of the degradation processes cannot be improved. However, there are many factors, such as yields, the methane ratio in the obtained biogas or the velocity of degradation, which can be enhanced.

The project "Characterization of Fast Anaerobic Digestion in a Novel Reactor Design with Immobilized Biofilms" has been conceived as an attempt to develop a novel biogas reactor system that overcomes many of challenges encountered within the field of anaerobic digestion. During this research, a novel reactor design denominated Fast Anaerobic Digester (FAD) was designed, constructed and utilized to produce biomethane. Most of the experiments in this study are based on the anaerobic digestion of a the unique substrate, "Renescence Bioliquid", which results from the enzymatic liquefaction of the organic fraction of Municipal Solid Waste (OFMSW). . Renescence<sup>1</sup> is a novel process developed by Ørsted in Denmark, which for the first time allows the recovery of energy from non-sorted Municipal Solid Wastes without compromising recycling rates.

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<sup>1</sup><https://orsted.com/en/Our-business/Bioenergy-and-Thermal-Power/Renescence>



## Chapter 2

# Introduction

### 1

#### Structure of the Thesis

This thesis is divided into two main parts. The first part comprises from Chapter 2 through to Chapter 6. This part includes the objective statement of this project and a comprehensive overview of the main phases of anaerobic digestion, a description of the main factors that influence biogas production from organic matter with emphasis on the treatment of municipal solid waste. The first section also includes a detailed description of the Fast Anaerobic Digester (FAD) in the context of reactor design and the laboratory procedures used during the experimental work. The second part, from Chapter 7 through to Chapter 11, presents and seeks to explain the engineering aspects and scientific phenomena behind the results obtained throughout the development and characterization of the novel AD reactor. Each section of this part includes a discussion and main findings. Lastly, in Chapter 12, there is a general conclusion that sums up the different lessons and includes the publications generated during this study.

#### Hypothesis

Different hypothesis have been formulated in the context of this project:

- Specialization of the microorganisms occurs in the different compartments within the biofilm of the FAD reactor.
- There are differences between the microbial communities in the biofilm and those free-floating in the digesting media.
- It is possible to significantly improve AD performance on heterogeneous substrates by reactor design involving fixed immobilized biofilms and compartmentalization
- The initial inocula have an influence on the microbiome of the AD process.
- The concentration of cat-ions has an influence on the rates and yields of the AD process,

#### Objectives

The main objectives of this project are:

- To determine the most important factors in an anaerobic digestion reactor process.

- To discuss the rate-limiting mechanism in anaerobic digestion and cation inhibition.
- To optimize the reactor performance on heterogeneous substrates through reactor design.
- To investigate the microbial diversity on optimal operational conditions.
- To propose mechanisms to improve the process and to prevent inhibition by cations.

## 2.1 Background

During the last decades, societal and economic growth has been supported by an increased utilization of fossil fuels. The dependence on non-renewable resources has become so strong that current governments are searching for alternative practices and technologies that could potentially reduce reliance on fossil fuels and prevent further environmental consequences. The Paris Agreement, which has involved most of the countries globally, has promoted the reduction of greenhouse emissions to keep global warming under 2 degrees C (*United Nations*). At the same time, the European Commission has framed a detailed road-map that intends to reduce the above mentioned emissions to 80 percent below 1990 levels. It is recognized that this goal is achievable only if important milestones are accomplished in different sectors of society.

Anaerobic digestion has been proposed as a robust and flexible practice with great potential to alleviate dependence on fossil fuels. During anaerobic digestion, the organic fraction of many different types of biomass undergo microbial degradation in order to produce a gaseous mixture mainly consisting of methane and carbon dioxide (CO<sub>2</sub>), denominated as biogas. The utilization of biomass for bioenergy production poses an advantage over fossil fuels due to the atmospheric carbon entrapment that occurs when plants grow, which results in lower net greenhouse emissions. The biomass source for biogas production is generally more flexible than for other biofuels, and can be derived from many different sources, including both agricultural and industrial streams, and is especially attractive for treatment of waste products.

The resulting biogas produced through AD also has a broad range of versatile applications. The gas can be used directly in a combustion engine to produce electricity, it can be stored as raw biogas for heating, for example in housing, or can potentially be upgraded into pure methane to support many of the societal energy requirements otherwise requiring use of natural gas, as in modern practices. Furthermore, there has been recent interest in developing biogas driven solutions to improve the integration of renewable energy sources like wind and solar into the grid. The flexibility of gas engines permits fast electricity production from stored biogas, which would be able to balance the grid in moments of demand surge.

Anaerobic digestion is not only a biological method to produce a high energy carrier. The results are also greatly beneficial for degradation and stabilization of organic material in a controlled manner. The reduction of organic matter from different sources leads to a production of nutrient rich digestate, sometimes referred to as sludge, that can be applied in arable land as fertilizer. Nutrient recovery brings the possibility of additional environmental advantages, especially mitigation of resource depletion in arable land. Important elements, such as nutrients and phosphorus, can be re-introduced into agriculture after the biogas production process.

### 2.1.1 The Anaerobic Digestion Process

A key approach for understanding the anaerobic digestion processes is by dissecting the degradation reactions into four phases, namely hydrolysis, acidogenesis, acetogenesis and methanogenesis (Han et al., 2016). During these phases, biomass components are oxidized from large macro-molecules into smaller intermediates, until these are finally converted into biogas. The absence of oxygen is commonly considered a prerequisite, since the lack of this strong electron acceptor induces the utilization of alternative microbial mechanisms through electron transfer chains (Peces et al., 2018, Herrmann et al., 2018). Lack of light or other inorganic electron acceptors which could be nitrates, iron, manganese or sulfates are also a prerequisite for anaerobic processes (Hattori, 2008). These conditions make biogas production a slower degradation process than its counterpart, thereby limiting the microbial biomass growth and resulting in higher energy intermediates that can be converted into biofuel (Regueira et al., 2018). A comprehensive illustration of the major steps in anaerobic digestion is shown in Fig. 2.1.

#### Hydrolysis

Hydrolysis is the first step of anaerobic digestion. In this phase, large biopolymers (fats, proteins and carbohydrates) are broken down into their soluble monomers (Herrmann et al., 2018). There is a vast variety of microorganisms involved in this process, which includes many phyla from the eubacteria. Although many hydrolytic microorganism have been identified and isolated in anaerobic digesters, their roles in the process are yet to be properly clarified. The presence of particular microbial consortia in anaerobic digesters has frequently been described to be dependent of the original inocula of the bioreactor (Liu et al., 2017). Amongst the most abundant hydrolytic bacteria retrieved in AD reactors are members of the phylum *Firmicutes* and *Bacteroidetes*. Constituents of the phyla *Fibrobacter* and *Thermotogae* are also commonly reported (Bengelsdorf et al., 2013). Furthermore, many members of the fungal and protist kingdoms are also known to be useful in this initial degradation of biomass in anaerobic digesters; however, their clear role remains still to be studied in depth (Matsubayashi et al., 2017, Goux et al., 2016). It has been hypothesized that fungal species contribute anaerobic digestion with fibrolytic potential (Kazda, Langer, and Bengelsdorf, 2014).

Hydrolysis of large biopolymers is possible following secretion of enzymes from hydrolytic bacteria into the digester media (Herrmann et al., 2018). Hence, the hydrolytic ability of the microbial communities inside the anaerobic digesters plays a very important role in dissolving the biomass into its monomer components. Hydrolysis of biomass also depends greatly on the nature of the substrate that is converted into biogas, due to the structural recalcitrance of many streams digested in AD reactors. Depending on the type of biomass treated, the hydrolysis can become the most important factor to optimize in a biogas process. In fact, many researchers have reported this stage to be the rate limiting step in biomethanation of complex organic substances (Ariunbaatar et al., 2014). Alternatively, when less complex substrates are digested and the hydrolysis rates are not an influential factor, methanogenesis is considered to be the limiting step because acidogenic reactions rates are typically higher.

Many strategies have been suggested in order to accelerate the hydrolysis step (Ariunbaatar et al., 2014). Amongst the most relevant is to perform a pretreatment of the biomass prior to the anaerobic digestion. Many possible pretreatments schemes



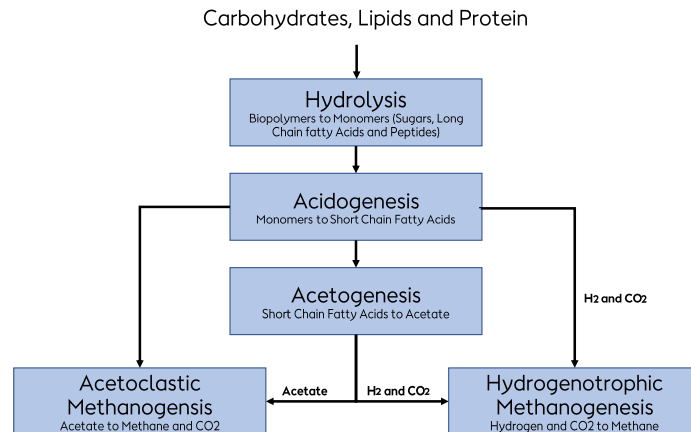


FIGURE 2.1: Phases of Anaerobic Digestion.

have been studied, which have led to faster and more stable anaerobic digestion; these strategies have been pursued through mechanical, chemical or biological processes (Hendriks and Zeeman, 2009, Nasir, Ghazi, and Omar, 2012). The main goal of the pretreatment is to modify the structure of treated biomass to make it prone to the enzymatic cleavage that consequently favours solubilization of the monomeric compounds and microbial consumption (Zheng et al., 2014). In the case of lignocellulosic biomass, there is a rigid structure, due mainly to the carbohydrate polymers embedded in a complex matrix with lignin and pectins, which requires modifications in order to ease accessibility. In addition to any pretreatment type, the optimization of process parameters, such as the hydrodynamic regime of the reactor in which the AD takes place, have also been successful for decreasing the required processing time by enhancing the hydrolysis phase (Puri, Heaven, and Banks, 2013). Researchers in this field are developing new technologies for mixing, process monitoring, surveillance and designing novel reactor configurations (Azman, 2016).

In the context of this thesis, in-depth knowledge of the occurrences during the hydrolytic phase is of great relevance because the main substrate used to study and optimize the FAD reactor is Renaissance bioliquid. This particular substrate is the result of enzymatic liquefaction of the organic fraction of municipal solids waste (OFMSW). Many publications have been produced to decipher the advantages of supplementing industrially produced enzymes in order to improve the AD process (Parmar, Singh, and Ward, 2001 Puri, Heaven, and Banks, 2013 Wagner et al., 2010). For instance, Parawira, 2012 has explained that assisting the anaerobic digestion of OFMSW with enzymatic treatment has remarkable effects when compared to other traditional substrates such as activated sewage sludge. The enzymes in this case were found to accelerate the hydrolysis phase, reduce the amount of volatile suspended solids (VSS) in the process and reduce the viscosity of the digesting media (Ye et al., 2018). A later section of this thesis is dedicated to describing the Renaissance process in the context of the current study.

## Acidogenesis

Acidogenesis is the second step of anaerobic digestion and consists of converting the hydrolysed monomers into short chain organic acids ( $C_1$ - $C_5$ ), alcohols,  $CO_2$  and  $H_2$ . These metabolic reactions are carried out by microorganisms termed acidogens, which are usually characterized by fast growth, short duplication times that can be as low as 0.5 hours, and high conversion rates (Herrmann et al., 2018). For this reason, acidogenesis is not considered to be a bottleneck in the biomethanation process. However, an unbalanced acidogenesis may lead to problems in the process due to acidification of the reactor media in cases where the subsequent phases are not able to utilize the generated products.

To a high degree, most common biomass transformations proceed through a glycolytic process, since cellulose is the most abundant biopolymer in nature (Regueira et al., 2018). Most of the degradation products derived from glucose are converted into pyruvate through glycolysis (the Embden-Meyerhof-Parnas EMP pathway), which results in the conversion of 1 mole glucose into two moles of pyruvate. Other acidogens may utilize alternative metabolic routes for producing pyruvate through the Entner Doudoroff (ED) pathway. There is, however, a major energetic distinction between these routes in that the EMP yields twice the amount of ATP in comparison to the ED. The products and intermediates of the fermentation are diverse and energy-rich compounds that undergo different metabolic transformations until they are converted into methane. Even though many of the pathways have been characterized, the environmental conditions that regulate the products of mixed microbial consortia like those found in anaerobic digesters are less understood (Angelidaki et al., 2011). A wide range of bacteria, mostly low GC Grampositive and particularly the genus *Clostridium*, are commonly found in digesters (Ramsay and Pullammanappallil, 2001; Cai et al., 2016). These diffuse microorganisms are known to be involved in the fermentation of some of the main metabolic products (Cai et al., 2016). From a general perspective, *Clostridium*-type fermentations are acidogenic reactions that can take place through degradative steps that yield  $H_2$  (Sikora and Detman, 2017). There are other fermentation routes that do not yield  $H_2$ , such as ethanol and homolactic acid fermentation.

The directionality that determines the fermentation products during acidogenesis depends on the most dominant microbial consortia present in the reactor, on the chemical and physical conditions in the digesting media, and on the type of substrate that is degraded. Monosaccharides undergoing EMP or ED can be either converted into  $C_3$  organic acids (lactate and propionate) or to products with even numbers of carbon atoms (acetate, ethanol and butyrate) following a decarboxylation of pyruvate to acetyl-CoA (Regueira et al., 2018). Many of these conversions are governed by the partial pressure of  $H_2$  in the system and the conversion that allows the most advantage to be gained from the energy of the anaerobic reactions proceeding through the most advantageous thermodynamic pathway (Herrmann et al., 2018). Regarding the degradation and fermentation of protein, in the hydrolysis step there must be enzymatic cleavage after the secretion of proteases. The resulting amino acids are degraded through two main ways (Ramsay and Pullammanappallil, 2001): (1) Through the so-called Stickland reaction where pairs of amino acids are degraded in pairs since one acts as electron donor and the other as electron acceptor; (2) Through an uncoupling oxidation reaction that requires low partial pressure of  $H_2$  and is suitable only in energy favorable conditions, that occur when the degradation is accompanied by hydrogen-consuming microorganism.

Glycerol and long chain fatty acids are the products from the hydrolysis of lipids.

Acidogenesis from glycerol can be achieved through a reductive pathway that produces 1,3-propanediol or through an oxidative pathway that enters the same degradation route than carbohydrates following its conversion to phosphoenolopyruvate (Sikora and Detman, 2017). The fatty acids are degraded to acetate and  $H_2$  in a catabolic process called beta oxidation because the beta carbon of the fatty acid is oxidized to a carbonyl group.

### Acetogenesis

The last remaining steps of anaerobic digestion, acetogenesis and methanogenesis, are very closely related. Up to this point, the hydrolyzed compounds have been oxidized into shorter organic acids. As mentioned previously, these reactions may be accompanied by the production of  $H_2$  and  $CO_2$ , depending on the microbial metabolism present in the anaerobic digesters. The process also depends on the nature of the substrate that is converted. The acetogenic phase refers to the production of acetate from all the products of the previous steps. Acetate can either evolve from the non-gaseous compounds that have been converted during the acidogenic phase, or alternatively from reduction of  $CO_2$  by hydrogen-utilizing acetogens (Detman et al., 2018). These different conversions in anaerobic digesters are bounded to a syntrophic cooperation of acetogenic bacteria with the methanogenic *Archaea* (Sikora and Detman, 2017). The ability of acetogenic bacteria to convert many of the metabolites produced during degradation of organic materials bridges the anaerobic food web because this ability permits the transformation of biomass to methane. This is probably the reason of why acetogenic bacteria are ubiquitous bacteria, present in many different anoxic environments. For instance, such bacteria have been retrieved in samples from human intestines, different soil environments and marine sediments (Schuchmann, 2014).

The oxidation of the reduced compounds that have been catalyzed in the acidogenic phase into acetate is thermodynamically unfavorable (Hattori, 2008). The microorganisms that produce acetate from these compounds are forced to cooperate with methanogenic *Archaea* in order to overcome the thermodynamic barrier and gain energy from the process. Within this collaboration, an interspecies electron exchange occurs between the  $H_2$  producers and  $H_2$  consumers. In these reactions,  $CO_2$  and the protons act as the main electron acceptors (Shen et al., 2016). In order for these reactions to proceed, the methanogenic *Archaea* must consume the formed  $H_2$  in order to maintain a low  $H_2$  partial pressure (typically below  $10^{-5}$  atm) which then makes the overall reaction exergonic. In essence, the interspecies electron transfer joins the abilities of acetogenic bacteria to produce acetate,  $CO_2$  and  $H_2$  to their syntrophic partners' ability to consume these compounds to produce methane. Some of the most important metabolisms involved in acetogenesis are summarized in Figure 2.2 and Table 2.1.

As mentioned earlier, the processes that allows syntrophically growing acetate producers to gain energy in close to a thermodynamic equilibrium from non-gaseous metabolites during anaerobic digestion are dependent on interspecies electron transfer. Currently three types of interspecies electron transfer are recognized (Dang et al., 2017). (1) Indirect electron exchange. This exchange results when bacteria and their syntrophic methanogen exchange electrons while  $H_2$  and formate serve as carriers (Stams and Plugge, 2009 and Dang et al., 2017). Over the last decade, many studies have been dedicated to elucidating this type of mechanism, the results of which are crucial for energy conservation in the oxidation–reduction reactions required to circumvent thermodynamic barriers (Peters et al., 2016). The so-called flavin-based

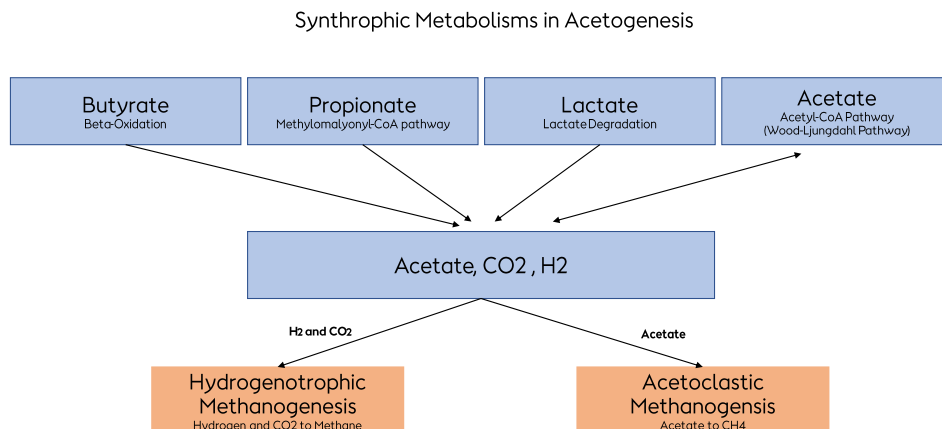


FIGURE 2.2: Synthrophic metabolims in Acetogenesis.

electron bifurcation has been proposed as the main mechanism of energy coupling in anaerobic metabolisms (Detman et al., 2018, Buckel and Thauer, 2013). This reaction is possible due to bifurcating enzyme complexes containing multiple electron transfer centers that include both iron–sulfur (FeS) clusters and flavins. (2) Direct electron exchange from the acetogenic bacteria to the methanogen through electrical connections. This type of interspecies electron transfer, unlike the previous one, does not require an electron shuttle but instead relies on outer membrane cytochromes, electrically conductive pili, or the formation of multispecies aggregates (Shen et al., 2016). Members of *Geobacter* species and *Shewanella* species have been characterized to possess this kind of mechanism when detected in waste water treatment plants. The proximity of the microbial cells is crucial in the two types of interspecies electron transfer, and in particular when the anaerobic cell conglomerates are organized in biofilms. (3) The third type of interspecies electron transfer is mediated by conductive materials (Dang et al., 2017). Hydrophobic materials such as granular activated carbon, biochar, carbon cloth, graphite, magnetite or humic substances can serve as electron shuttles during these processes. In all the different relationships where interspecies electron transfer by syntrophic communities occurs, the physical distance between the acetogenic syntroph and the hydrogen scavenging *Archaea* has an impact on the conversion rates and on the growth rate of the microorganisms involved (Angelidaki et al., 2011).

The production of acetate can also result from  $\text{CO}_2$  and  $\text{H}_2$  when low partial pressure of  $\text{H}_2$  cannot be maintained within syntrophic cooperation. Acetate can be evolved by acetogens through a pathway termed the Wood-Ljungdahl pathway (also named the acetyl-CoA pathway). This process is basically the reductive synthesis of acetyl-CoA from  $\text{CO}_2$ , a mechanism where  $\text{CO}_2$  is assimilated into producing cell carbon, and is the opposite to the conversion performed by acetate oxidizing syntrophs (Drake, Kusel, and Matthies, 2013). The bacterial microorganisms capable of producing acetate through this pathway are autotrophs; they are able to gain energy from the oxidation of a large variety of substrates such as carbohydrates, but also from inorganic  $\text{H}_2$  and  $\text{CO}_2$  when reducing  $\text{CO}_2$  into acetate. The acetogens

TABLE 2.1: Reactions and energetics in Acetogenesis and Methanogenesis. Adapted from Hattori, 2008.

Conversion	Reaction	Products	$\Delta G^{\circ}$ (kJ/mol)
(1) Aceticlastic methanogenesis	$\text{CH}_3\text{COO}^- + \text{H}_2\text{O}$	$\text{CH}_4 + \text{HCO}^-$	-31.0
(2) Syntrophic acetate oxidation	$\text{CH}_3\text{COO}^- + 4\text{H}_2\text{O}$	$2\text{HCO}_3^- + 4\text{H}_2 + \text{H}^+$	+106.6
(3) $\text{H}_2$ consuming methanogenesis	$4\text{H}_2 + \text{HCO}_3^- + \text{H}^+$	$\text{CH}_4 + \text{H}_2\text{O}$	-135.6
(4) sum (2)+(3)	$\text{CH}_3\text{COO}^- + \text{H}_2\text{O}$	$\text{HCO}_3^- + \text{CH}_4$	-31.0
(5) $\text{H}_2$ -consuming acetogenesis	$4\text{H}_2 + 2\text{HCO}_3^- + \text{H}_2$	$\text{CH}_3\text{COO}^- + 4\text{H}_2\text{O}$	-104.6

are widespread and have been found in 23 different bacterial genera, though most frequently they belong to the *Firmicutes* phylum. Members of the genera *Clostridium* and *Acetobacterium* are the most commonly retrieved acetogens (Drake, Kusel, and Matthies, 2013). Certain hydrogenotrophic methanogens (methanogenic *Archaea* that produce methane from  $\text{CO}_2$  and  $\text{H}_2$ ) are also capable of reducing  $\text{CO}_2$  into acetyl-CoA. However, these species preferentially tend to produce methane from the  $\text{CO}_2$  and  $\text{H}_2$  in the anaerobic media since this reaction is more thermodynamically advantageous than to produce acetate.

### Methanogenesis

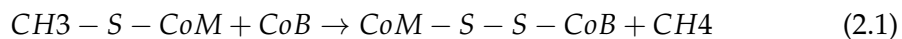
The connection between acetogenesis to methanogenesis in an anaerobic digester depends on the dynamics of the populations over time and on the conditions under which the reactor is operated. The production and consumption of the different metabolites is governed by microbial mechanisms that in stable and functionally systems tends to adapt in time (Carballa, Regueiro, and Lema, 2015). Moreover, it is not yet clear if it may be better to have a diverse microbiome that shifts in time when adapting to environmental changes or to have diverse but well established communities. However, it seems obvious that microbial pathways in an anaerobic digester adapt to the route through which the microorganism can gain the most energy from the degradation process, depending on the microbiome in the digester. Following this line of thought, it is important to consider the most common transformations during acetogenesis (In Figure. 2.2). When butyrate or propionate are the products from the acidogenic phase, the biomethanation process can occur only after conversion by syntrophic acetogenic bacteria. This situation results in production of acetate,  $\text{CO}_2$  and  $\text{H}_2$ , which can then be utilized by *Archaea* through direct acetate conversion into methane (acetogenic methanogenesis) or through hydrogenotrophic methanogenesis (Hattori, 2008). In an analogous manner, the production of methane from lactate can be achieved following lactate oxidation through diverse routes. This conversion may proceed through acetoclastic methanogenesis when lactate is converted to acetate, for example when 2 moles of lactate are oxidized into 3 moles of acetate when bacteria with the metabolic machinery of bacterium *Acetobacterium woodii* are present. Analogously, the methane could be produced from lactate through any of the methanogenic routes when the organic acid is transformed by a mixed metabolism into acetate,  $\text{CO}_2$  and  $\text{H}_2$  (in some cases also propionate) and mixed oxidation of lactate is possible, as in the case of *Desulfobulbus propionicus*. The oxidation of lactate and ethanol into methane is thermodynamically advantageous compared to that of butyrate and propionate because it does not depend on interspecies electron transfer and proceeds in most cases through exergonic reactions (Detman et al., 2018 and Pipyn and Verstraete, 1981). A metagenomic reconstruction of the most



common pathways in anaerobic digestion and some of the key microorganisms involved are illustrated in Cai et al., 2016.

Methanogenesis is the final process of anaerobic digestion in which methane is produced from the most reduced intermediates. Methane production is performed solely by methanogenic *Archaea* species unlike all the other phases of anaerobic digestion. The methane-evolving metabolism is exclusive in these type of organisms which are considered taxonomically different than bacteria. For a long time the methanogenic *Archaea* were exclusively classified in the phylum *Euryarchaeota*. Recent advances in genome-centric genomics, however, have made it possible to distinguish methanogenic genes in archaeal species belonging to the *Bathyarchaeota* (Evans et al., 2015). Most of the methanogens retrieved in anaerobic digesters are, however, still classified in seven main taxonomic orders: *Methanopyrales*, *Methanococcales*, *Methanobacteriales*, *Methanocellales*, *Methanomassiliicoccales*, *Methanomicrobiales* and *Methanosarcinales*. This list has recently been updated through improvements on the ability to track methanogenic genes from mixed microbial DNA samples (Borrel et al., 2013 and Sakai et al., 2011). These members of *Archaea* Kingdom are strict anaerobes that usually coexist in harsh environments such as marine sediments where external electron acceptors such as  $\text{O}_2$ ,  $\text{NO}_3^-$  or  $\text{SO}_4^{2-}$  are limited, and possess different physiological traits than bacteria (Angelidaki et al., 2011). In other environments where the mentioned electron acceptors are present, the methanogens can be outcompeted by aerobic respiration, for thermodynamic reasons (Thauer et al., 2008).

Surprisingly, the methanogenic *Archaea* possess outstanding ecological and phylogenetic diversity despite having limited metabolic heterogeneity (Yunchen and Whitman, 2008). A distinction can be made between three methanogenic pathways based solely on the initial substrate: (1) Acetoclastic methanogenesis, (2) Hydrogenotrophic methanogenesis, (3) Methylotrophic methanogenesis. Independently of the substrate, the most common denominator of all the methanogenic processes is the reaction of methyl-coenzyme M ( $\text{CH}_3\text{-S-CoM}$ ) and coenzyme B to produce heterodisulphide  $\text{CoM-S-S-CoB}$  and methane in a reaction catalyzed by the methanogenic marker enzyme methylcoenzyme M reductase (Mcr), as can be seen in the equation 2.1 and Figure 2.3 (Sikora and Detman, 2017). The analysis of the crystal structure of the Mcr of *Methanothermobacter marburgensis* has revealed the presence of two active sites which each contain a cofactor called F430, which depends on the presence of nickel (Grant et al., 1997).



Acetoclastic methanogenesis refers to the methane production that emanates from acetate as primary substrate. During this type of metabolism, acetoclastic methanogens convert acetate into acetyl-CoA aided by the presence of an acetate kinase and a phosphotransacetylase (Ragsdale and Pierce, 2008, Ferry, 2011). The electrons necessary to reduce  $\text{CH}_3\text{-S-CoM}$  to methane are harvested from the oxidation of the carboxyl group of acetate. Researchers have calculated that approximately 70% of the methane produced during anaerobic digestion derives from the acetoclastic pathway since the theoretical yields of dark fermentation produce two-thirds of acetate during the acetogenesis (Sikora and Detman, 2017). However, the ability to use acetate as substrate can be encountered exclusively in members of the order *Methanosarcinales* and from the genus *Methanosaeta* (Yunchen and Whitman, 2008 and Ferry, 2010). Species from the latter genus are the only known methanogenic

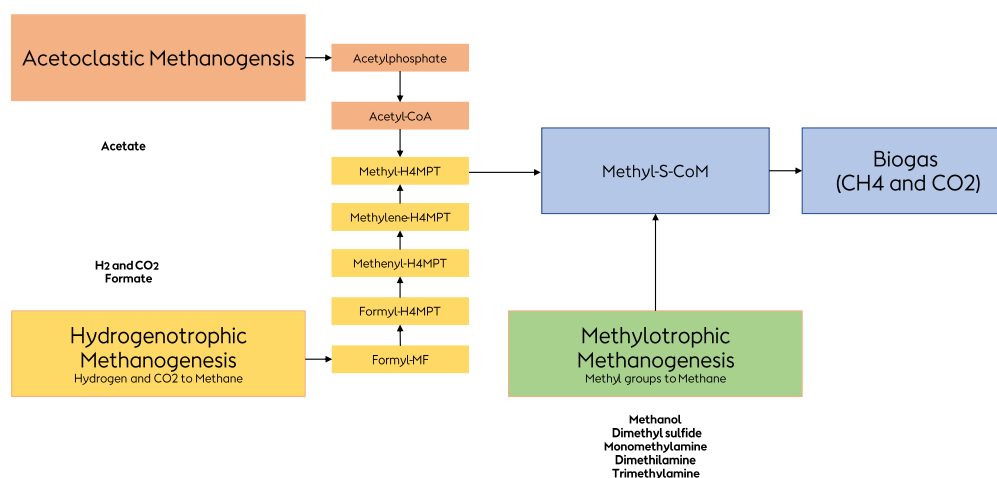


FIGURE 2.3: Methanogenic metabolisms

*Archaea* to depend uniquely from acetoclastic methanogenesis (Ferry, 2010 and Borrel et al., 2013). In contrast, *Methanosarcina* species are known to be the most metabolically versatile methanogenic *Archaea* since the methanogenic genes from the other methane evolving pathways have also been also found in their genomes (Lambie et al., 2015).

The energy yields from the overall methanogenic reactions are shown in Table 2.1. The acetoclastic methanogenesis are shown to yield the lowest net Gibbs Free Energy ( $\Delta G^\circ$ ) when compared to the other methane producing pathways. The low energy efficiency gained from acetate oxidation into methane is an explanation for the slow growth of the methanogens that utilize this route. In fact, several authors have reported acetoclastic methanogenesis to be a limiting reaction in anaerobic digestion due to the slow doubling time of these acetate consuming microorganisms (Michaud et al., 2005). The presence of acetogenic bacteria in anaerobic digesters could represent competition for the acetate in reactors when the environmental conditions for the acetoclastic methanogens are not ideal (Fotidis, A. Karakashev, and Angelidaki, 2014). For instance, members of the genus *Methanosaeta* are predominant in reactors with low ammonia and VFA concentrations, while other type of methanogens prevail in reactors with higher organic strength (Ziganshin et al., 2013 and Perrotta et al., 2017). In general the predominance of the acetoclastic pathway is seen as a positive sign of healthy anaerobic digesters (Pap et al., 2016). The transition from acetate to the hydrogenotrophic pathway is evident when there is an increase in the abundance of syntrophic acetate oxidizing bacteria in the digesting media.

During hydrogenotrophic methanogenesis, the CO<sub>2</sub> is reduced to CH<sub>4</sub> with H<sub>2</sub> as primary electron donor (Welte and Deppenmeier, 2014). In some cases, hydrogenotrophs can also utilize formate or 4-isopropanol as the main electron donor, or utilize the carbon of CO for methane production (Yunchen and Whitman, 2008). During this process, methyl-S-CoM is converted to CH<sub>4</sub> and CO<sub>2</sub> in six steps with formyl-, methenyl-, methylene- and methyl-coenzymes as intermediates as depicted in Figure 2.3 (Borrel et al., 2013). Most of the methanogens possess this type of metabolism, whereas

members of the order *Methanosaeta* are one of the most recognized exceptions. Regarding substrate utilization, most of the methanogenic species are known have a preference for the hydrogenotrophic pathway, probably due to thermodynamic reasons as discussed earlier. In fact, around 75% of the *Archaea* that produce methane utilize  $\text{CH}_4$  and  $\text{H}_2$  in the presence of acetate or methylated compounds (Jabłoński, Rodowicz, and Łukaszewicz, 2015).

A distinction can be made between hydrogenotrophic methanogenesis based on an important physiological difference. Some methanogens, like all the members of the order *Methanosarcinales*, contain cytochromes, which is a feature that allows them to utilize a broad spectrum of substrates. These types of microorganisms couple the first and last steps of  $\text{CO}_2$  reduction through chemiosmotic mechanisms (Thauer et al., 2008). In contrast, the other hydrogenotrophic methanogens, which usually lack cytochromes, couple the energy production through flavin-based electron bifurcation (Costa and Leigh, 2014 and Thauer, 2012). As discussed previously, this energy conservation mechanism is also present in some acetogenic bacteria and was only unraveled as recently as in 2011 by Lie et al., 2012 and termed the Wolfe cycle (Thauer, 2012).

The last type of methanogenesis is possible for species capable of producing methane from the methyl groups of methanol, methylamines and dimethylsulfide, see Figure 2.3 (Ferry, 2010). This type of metabolism is possible when the *Archaea* are able to produce substrate-specific enzymes that allow the transfer of the methyl group from the substrate to the HS-CoM. These methyltransferases contain a UAG codon encoding L-pyrrolysine, which is a unique amino acid retrieved in nature exclusively from methanogenic *Archaea* (Rother and Krzycki, 2010). These reactions are exergonic, and therefore usually proceed in a thermodynamically favored manner when environmental conditions permit (Dong et al., 2017). Members of the order *Methanosarcinales* are known to possess the methylotrophic pathway with the exception of *Methanosaeta spp* (Borrel et al., 2013). Organisms such as *Methanomicrococcus blatticola* and some species of the *Methanobacteriales*, such as *Methanosphaera spp.*, have also been found to possess the enzymatic machinery to utilize methanol for methane production (Sprenger et al., 2000). Even though these last mentioned microorganisms are less common in anaerobic digesters, evidence of their presence has been found in small amounts in the sludge in operational reactors (Guo et al., 2015). A broad review of the mechanisms behind methanogenesis can be found in (Deppenmeier and Müller, 2007).



### 2.1.2 Main Parameters in Anaerobic Digestion

#### Organic Loading Rate

The Organic Loading Rate (OLR) is the daily amount of substrate that is fed into a biogas reactor and can be calculated according to equation 2.2 (Herrmann et al., 2018). In this expression,  $c$  is the concentration of organic material, expressed as an amount in percentage of Volatile Solids (VS) or as concentration of Chemical Oxygen Demand (COD) in the substrate. The expression  $Q$  is the daily flow rate and  $V$  is the volume of the reactor. Determination of the concentration of VS is generally performed through gravimetric analysis of the samples. Throughout this work, the VS determination was performed with a modified version of the NREL Laboratory protocol (Sluiter et al., 2008) in which the water evaporation step is done at 60°C instead of the commonly used 105°C. This modification was considered because the substrates used in the experimental phase of this thesis contained a large fraction of VFAs which already evaporate at the boiling point of water. Similar modifications have been used in other studies (Peces, Astals, and Mata-Alvarez, 2014).

The OLR is a main parameter in anaerobic digestion, which represents the amount of organic material that enters the process and thus allows prediction of the expected yields when the conversion rates at certain operational conditions are known. The OLR can also be used to anticipate detrimental effects of acidosis, which occur when a reactor is overloaded with organic material beyond its production capacity. Acidosis or acidification of the digesting media is generally perceived as accumulation of VFAs and pH drop, which results in an unbalance in the kinetics of the different phases of anaerobic digestion (Goux et al., 2015). There is abundant data in literature that can be retrieved on the recommended OLR to run anaerobic digestion processes at different biomasses. In fact, many of the studies conducted in the field of anaerobic digestion evaluate exclusively the effects of OLR for specific biomass conversions (Lerm et al., 2012, Borja, Banks, and Martin, 1995 and Yu et al., 2016). Within the scope of this project, an attempt was made to determine the maximum achievable OLR in the FAD reactor at the point where the organic loads do not compromise the stability of the process.

$$OLR(kg_{VS \text{ or } COD} m^{-3} * d^{-1}) = \frac{c(kg_{VS \text{ or } COD} m^{-3}) * Q(m^3 * d^{-1})}{V_{Reactor}(m^3)} \quad (2.2)$$

#### Hydraulic Retention Time and Organic Loading Rate

The hydraulic retention time (HRT) of the process denotes the time required for the substrate to remain in the reactor until it is discharged. This parameter can be calculated accordingly with equation 2.3, where  $V$  is the working volume of the reactor and  $Q$  represent the daily flow rate. Aside with the OLR, the optimization of the HRT in an anaerobic digester is necessary in order to guarantee an adequate reactor utilization (Herrmann et al., 2018). A high HRT represents a long retention of the substrate in the reactor and therefore sufficient time for the microbial populations to completely digest the substrate, but from a process perspective this is not always optimal. In many situations, coupling the appropriate ORL and HRT provides higher methane yields when there is a balance between the microbial growth and substrate consumption (Kim et al., 2006).

Two different strategies can be employed to modify the ORL and HRT. The first is to increase or decrease the flow rate of the process. When the HRT decreases, there is

an increase of the OLR (and vice-versa), since more substrate is fed per volume of reactor. The second strategy is by dilution through which, for example, it is possible to maintain the same OLR while altering the HRT of the process. This scheme has been adopted during the start-up of a biofilm reactor where low HRT was desired in order to avoid undesirable competition for the substrate by planktonic cells (Escudie et al., 2010). In the previous cited study, it was shown that low HTRs are beneficial for the development of methanogenic populations that attach to inert supports. Furthermore, HRT is a crucial factor determining the retention of microorganisms and unconverted compounds in the digesting media. When the duplication time of the slowest growing microorganisms in the anaerobic digester exceeds the HRT, the desired microbial populations in the reactor are flushed out, which leads to process unbalance. This aspect is explained in more detail further in the present work, since one of the key elements in the development of the Fast Anaerobic Digester is cell immobilization. Another way to avoid flush-out of microorganisms as a consequence of a decreasing the HRT is to decouple the Solid Retention Time (SLR). In this strategy the microbial rich sludge is retained inside the reactor, for example by recirculation.

$$HRT(d) = \frac{V_{\text{Reactor}}(m^3)}{Q(m^3 * d^{-1})} \quad (2.3)$$

## Temperature

The temperature of the process is among the most important factors affecting anaerobic digestion (Levén, Eriksson, and Schnürer, 2007 and Sanchez et al., 2000). In the literature, there is a broadly acceptance of the distinction between two different temperature ranges, namely mesophilic temperature (20°C- 45°C) and thermophilic temperature (45°C- 60°C). The temperature plays an important role since all the biotransformations and metabolic activities that occur during anaerobic digestion are sensitive to changes in temperature. The process temperature thus has an effect on the degradation kinetics, stability and effluent quality, and consequently on the methane yields (Sanchez et al., 2000). Furthermore, dramatic changes in temperature have been found to have large implications in the process, especially in thermophilic systems which appear to be sensitive to even slight fluctuations (Van Lier et al., 1990 and Lier, Sanz Martin, and Lettinga, 1996). The reactors operating at the thermophilic temperature range are also known to perform faster biomethanation than in mesophilic conditions since higher temperatures promote thermodynamically advantageous hydrogenotrophic methanogenesis. In fact, the free energy change ( $\Delta G^\circ$ ) of acetate formation from  $\text{CO}_2$  and  $\text{H}_2$  increases with higher  $\text{H}_2$  concentrations at lower temperatures and results in a pH increase (Thauer et al., 2008). This is probably the main reason for why obligate acetoclastic methanogens are most commonly seen at the mesophilic temperature range.

Many hydrolytic and fermentative microorganisms have been found that are capable of degrading complex organic materials within the entire temperature range and even at pH levels below 7.0, conditions which are not suitable for methanogenic *Archaea* to perform their functions (Chapleur et al., 2016 and De La Rubia et al., 2009). However, it appears that many of the metabolic reactions of the hydrolytic microorganisms occur faster under thermophilic conditions. This is particularly seen in the degradation of lignocellulose rich substrates, which are more prone to enzymatic depolymerization in the presence of cellulytic and xylanolytic microbes, since lignocellulosic structure tends to loosen up at higher temperatures (Lv, Schanbacher,

and Yu, 2010). Similar comments relate to the degradation of biomass-laden substrates, for example from waste water treatment sludges, since it appears that the cellular membrane component is also more vulnerable to lysis in those conditions. However, at the most elevated temperature ranges, many of the microorganisms retrieved in anaerobic digesters exceed their maximum values for growth, and therefore the process can suffer loss of removal capacity when the conditions are not optimal (Ahn and Forster, 2002). This has also been described to affect the diversity of the methanogenic populations, which decreases at the thermophilic range ((Rincón et al., 2010).

Similar statements can be made with regard to the effect of temperature regimes on the production and/or solubilization of compounds that are inhibitory for the different microorganisms in anaerobic digesters. The metabolic utilization of the substrates can be altered by temperature fluctuations because temperature has an effect on the solubility of  $H_2$  (Lv, Schanbacher, and Yu, 2010). With increasing temperature, the microbes of the process are more susceptible to inhibitory compounds such as  $NH_3$  and  $H_2S$ , which tend to accumulate at elevated temperature regimes (Chachkhiani et al., 2004 and Yu, Lee, and Hwang, 2005). Total ammonia (TAN), for example, can be found either in the form of ammonium ion  $NH_4^+$  or as free ammonia  $NH_3$  (FAN), depending on the pH and temperature conditions inside the digesters (Tian et al., 2018). The rates of degradation of complex organic nitrogen and production of ammonia nitrogen are also affected by changes in the temperature, depending on the molecular complexity of the compounds (Sanchez et al., 2000). An increasing concentration of FAN is commonly recognized to be the problematic form of ammonia in anaerobic digestion due to the high permeability of cellular membranes to FAN (Massé, Rajagopal, and Singh, 2014). In the literature, much attention has been given to elucidating the effects of ammonia in the biomethanation processes. Yet there has never been consensus about the real thresholds or levels of inhibitory concentrations in the digesting media. For some time it has been believed that concentrations of  $NH_3$  are inhibitory to the acetoclastic methanogens when these exceed  $1000\text{ mg}\cdot\text{l}^{-1}$ . Nevertheless, Esquivel-Elizondo et al., 2016 have challenged previous results by showing that it is actually possible to maintain acetate consumption even at higher concentrations of  $NH_3$ . However, their study reports that increasing  $NH_3$  levels does affect the substrate degradation pathways, which partially shift towards the  $H_2$  consuming routes. What appears to be more evident is that the temperature regime alongside with the  $NH_3$  concentrations induces strong changes in the long term dynamics of the microorganisms in anaerobic digesters. For instance, a similar study shows that increasing  $NH_3$  concentrations at the mesophilic range partially compromised the population of acetoclastic methanogens, while the amount of syntrophic acetate consumers and their activity increased (Moestedt et al., 2013). Sudden changes in temperature can also influence the performance of the reactor because rapid accumulation of VFAs, especially in the form of propionate and acetate, has been observed when a stable process has been abruptly disturbed (Ahn and Forster, 2002).

From a process perspective, there are many factors that influence the choice of the temperature regime of anaerobic digestion. As described earlier, the temperature may impact in different ways on the microorganisms that thrive in the reactors and may define the metabolic pathways used during degradation of the organic material. However, the effect of temperature on the changes that may be involved during anaerobic processes could be ultimately be determined by the nature of the substrate. For instance, if the feedstock requires high rates of hydrolysis, it may

be preferable to have a higher temperature regime in order to attenuate polymer hydrolysis and solubilization of monomeric compounds. In this case, the first phases of anaerobic degradation could be positively enhanced, at the cost of reducing the stability of acetate-consuming methanogens with acidification of the digesting media as a result. For this reason, many of the existing anaerobic degradation process designs include two stage or multi stage configurations which consist of multiple physically separated digesters with sequential flow amongst them (Lv, Schanbacher, and Yu, 2010). The final purpose of multi stage reactor design is to break up the degradation phases, usually to separate the hydrolysis and acidogenesis steps from the acetogenesis and methanogenesis (Klocke et al., 2008). This strategy can be optimized by selecting the most convenient temperature and flow regimes for each of the reactors within the multi stage configuration. For example, the fermentation reactors could be operated at lower pH values and at lower HRTs than the methanogenic reactors so as to enhance the environmental conditions for hydrolytic bacteria and flush out the slowest growing methanogens and acetogenic bacteria. Despite these clear advantages of multi stage processes, the inclusion of extra digesters can result in dramatic cost increases for biogas plants. It is therefore still preferred to have single stage reactors in order to treat biomasses from several sources. Furthermore, there are other factors that may influence the choice of temperature regime. Higher temperatures are usually advisable to treat the influent of biomass with heavy loads of pathogens. This is usually desired in order to comply with legislation on digestate usage that foresees reduction or absence of undesired microorganisms. However, higher temperatures also result in larger heating demands for the plant owners. The latter also have to take into consideration the effect of process temperature over inhibitory compounds, as in the case of  $\text{NH}_3$ , as discussed previously.



## Chapter 3

# Anaerobic Digestion for Municipal Solid Waste

2

### 3.1 General Overview

Anaerobic digestion of Municipal Solid Waste has gained special attention during the last decades as a feasible alternative for handling the vast amounts of waste produced by household and commercial activities (Hartmann and Ahring, 2006 and Fan et al., 2018). This practice offers the possibility of providing a green source of energy. Moreover, the practice also represents a solution for organic waste disposal in contrast to the traditional landfill practices that have caused many environmental problems for many years. AD of MSW also brings the possibility of environmental protection, including pathogen sanitation, air pollution reduction, production of biofertilizer, and consequently GHG emission reduction, amongst others (Mao et al., 2015).

During the formulation of projects and research regarding anaerobic digestion of MSW, a fundamental problem emerges however. It is not an simple task to compare the data retrieved from the literature or available from commercial plants due to the large variability within waste composition, reactor configuration and operational parameters (Vandevivere, Baere, and Verstraete, 2003). The microbiology of biogas plants is also different, and therefore it is difficult to conclude and generalize about what are the most appropriate parameters or reactor designs to choose at the time of conceptualizing a new process. However, from a researcher's point of view, the most important factors for realizing higher productivity in the biogas plant and as much recovery of materials as possible are: (1) improvement of the stability of the biomethanation process, (2) tuning of the metabolic pathways to obtain the highest methane yields and organic matter degradation, (3) improvement of the rates of the process. This said, all these considerations must aim to decrease the cost of biogas plant and its operation. Therefore in this study, during the characterization and optimization in the Fast Anaerobic Digester (FAD), an important attempt was made to gather all the important aspects of the anaerobic digestion process, and especially to improve the biomethanation of the Renescience bioliquid. A brief description of the Renescience process follows in this chapter.

There are some characteristics that distinguish MSW from other common feedstock used in anaerobic digestion. First, the source of origin is dynamic, and what is usually considered as "municipal garbage" changes geographically and seasonally, therefore it is not possible to predict the organic loads that could arrive in the biogas plant (Burnley, 2007). These variations include undesired materials in the waste

stream, such as garden waste or non-biodegradable materials. For this reason, the anaerobic digestion of MSW streams is commonly performed after a rigorous source sorting that includes the removal of recalcitrant and pollutant materials, such as plastics or other inert solids, which are usually troublesome for the electromechanical devices of the plants. In this study, the MSW streams, which consist mainly of the organic fraction due to some kind of sorting or pretreatment, are termed OFMSW. Second, the heterogeneous origin of the waste makes MSW a complex feedstock on which to cultivate stable communities of hydrolytic and acidogenic microorganisms (Bengelsdorf et al., 2013). The OFMSW is not merely carbohydrates from household waste, but rather a composite mixture of containing lignocellulosic, fats and protein. As a consequence, it is common practice to perform some kind of pretreatment to obtain a less diverse assortment of the waste streams. Finally, another general characteristic of OFMSW is that it has a high moisture and relatively high VS content when compared to other common biogas substrates (Nasir, Ghazi, and Omar, 2012). It is actually possible to obtain methane from most of the fractions present in OFMSW, and thus the organic loads that are introduced in the process must be monitored carefully to avoid overload in the anaerobic digesters.

In different literature sources, the organic fraction of the solids present in the substrate or digesting medium, which is not soluble or can be trapped by filtration, for example microbial cells or fibrous materials, is quantified as Total Suspended Solids (TSS). The amount of the insoluble fibrous fractions in OFMSW streams can be as high as 40% of the total solids (TS) (Cirne, Agbor, and Björnsson, 2008). Due to this substrate characteristic, the anaerobic digestion process relies heavily on the hydrolytic capacity of the microorganisms. On account of this, the bottleneck for degradation of MSW has usually been considered to be the solubilization of materials. Regardless of the mechanical sorting previously mentioned, the OFMSW is also treated in order to increase the methane potential by enhancing the hydrolytic phase (Mata-Alvarez, Macé, and Llabrés, 2000). According to the latter authors, in attempting to model the AD of OFMSW, a distinction should be made between hydrolytic-acidogenic and acetogenic-methanogenic communities. The first group of microorganisms has been found to operate over first-order kinetics and the rates for solubilizing OFMSW depend mainly of the pH and the HRT of the process.

Although most of the full scale biogas plants in Europe that treat OFMSW (>90%) are single stage digesters, there has been recent interest in investigating alternative two-stage or multi stage configurations (Bolzonella et al., 2003). Essentially, researchers have realized that a sound degradation of OFMSW in a single-stage reactor, where all the phases of AD are coordinated, is very complicated due to the complexity of the substrate (Forster-Carneiro, Pérez, and Romero, 2008). In order to exploit the cost efficiency and process simplicity of single-stage digesters, a distinction has been made between dry and wet processes (Hartmann and Ahring, 2006). Admittedly, there is no definite rule to differentiate between dry (high solids) or wet (low solids) AD of OFMSW; as a general observation, however, wet processes perform at dry matter content (TS) above 20% while the ranges for wet processes are below this amount (Figure 3.1). The previous statement is no guarantee that all processes neighboring these ranges can be used to categorize if an AD process is dry or wet, especially because the OFMSW fractions may consist mainly of particulate solids (another denotation for TSS). In the cases where the particulate matter exceeds certain limits, for example 10% TSS of the total dry material, it is not possible to handle the substrate with pumps and thus it could be categorized as dry AD (Hilkiah Igoni et al., 2008). At concentrations of TSS above 8%, it is already difficult to handle slurries with the smaller industrial pumps available.



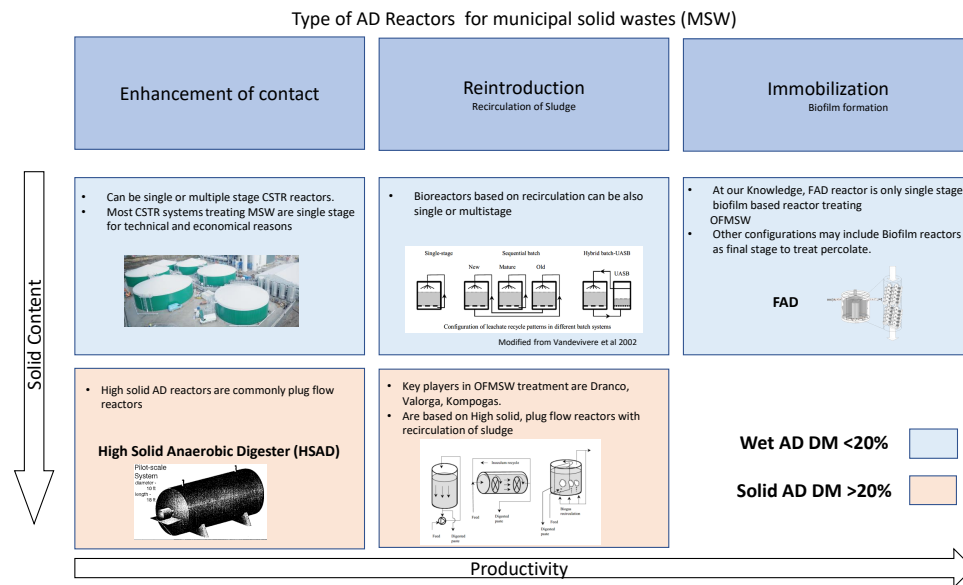


FIGURE 3.1: Type of AD Reactors for treatment of MSW.

From a process perspective, there are important differences between wet and dry AD of OFMSW (Vandevivere, Baere, and Verstraete, 2003). Much information exists based on know-how experience regarding wet AD since it has been commonly implemented for many years. In wet processes, the microbiology present in the digesters is also enhanced because there is dilution of possible inhibitory substances that otherwise be detrimental for the distinct communities at high concentrations. The nutrient exchange and the action of the secreted enzymes are also favored. Additionally, the equipment to handle slurries is cheaper, even though the result can be higher water consumption and heating expenses. However, the maximum OLR that can be effectively treated in a wet AD digester, without comprising the COD removal and yields, has been shown to be in the range of  $9.2 \text{ kg-VS} \cdot \text{m}^{-3} \cdot \text{day}^{-1}$  (Nagao et al., 2012). This means that biomethanation of OFMSW in conventional wet AD process is limited by the organic loads that can be put in the reactor. In cases where the organic loads of the substrate slurry is high, there needs to be a long HRT in the process. By contrast, the treatment of thin OFMSW slurries is limited by the retention of microorganisms and the SLR. In fact, it has been documented that at HRTs below 12 days, there is already a notable decrease in the efficiency of the reactors regardless of the organic load (Hartmann and Ahring, 2006). On the other hand, high solids anaerobic digestion offers the advantage of increased organic load capacity. The configuration of dry MSW digesters does not usually include moving parts such as mixers internally, but instead are based on recirculation of digesting media and the creation of a plug flow. Despite such measures, it is difficult to prevent high concentrations of inhibiting substances, which results in limited dispersion of transient peak concentrations of inhibitors (Vandevivere, Baere, and Verstraete, 2003). In dry processes, the yields expressed in  $\text{l}_{\text{CH}_4} \cdot \text{kg}_{\text{VS}}^{-1}$  or  $\text{l}_{\text{CH}_4} \cdot \text{kg}_{\text{COD}}^{-1}$  are usually higher than those reported for wet processes. Nevertheless, the volumetric productivity of wet process reactors are similar since the dry reactors usually require HRTs higher than 40 days, especially since hydrolysis constitutes an even stronger bottleneck (Fernandez, Pérez, and Romero, 2008). Still, more than 60% of the OFMSW



treated worldwide is based on single stage dry AD plants, estimated in 2013 to handle 3.5 million tonnes of waste from the approximately 6 million tonnes treated via AD technologies (Karthikeyan and Visvanathan, 2013). The most common commercial designs operating in Europe are DRANCO and Kompogas, which operate at the thermophilic temperature range, and Valorga that runs at lower temperatures. A proposed classification of single stage AD reactors for MSW treatment that includes some examples of common commercial technologies is shown in Figure 3.1.

As previously stated, new configurations of two stage or multi stage processes for AD of OFMSW have been attracting attention. Clearly, the main purpose of this deviation from single stage is to physically separate the hydrolysis and fermentation metabolism from the methanogenic phases. It is important to note that multi stage configurations allow the possibility to separate the reactions into dry-wet or wet-wet reactors. In both of these settings, the hydrolytic tank can be optimized to achieve enhanced solubilization. For example, according to the substrate, the hydrolysis can be operated at pH >7 under microaerophilic conditions to enhance microbial propagation of the desirable bacteria possessing faster metabolisms. The subsequent methanogenic reactor is usually a wet digester, since the acetogenic and methanogenic microorganisms evolve methane under conditions of high substrate diffusion and are more prone to inhibitory substances. These methanogenic reactors in two stage or multi stage systems can be conventional CSTRs or high-rate systems based on immobilized microorganisms. The main prerequisite for operating a high rate methanogenic reactor is that the incoming stream from the previous stage must have low concentration of suspended solids because the presence of these particles affects effective granulation and biofilm formation, and hence the stability of the reactor (Nagao et al., 2012 and Wilson, Sharvelle, and De Long, 2016 and Kochany and Lugowski, 2000). Before this study, none of the existing high-rate reactors has been utilized to treat the whole slurry of OFMSW in a single stage configuration, since the existing reactor designs cannot be confronted with high levels of suspended particles (Ye et al., 2018). The different configurations and reactor designs are explained in more detail in the next chapter.

## 3.2 Renescience Technology

Renescience is a state of the art technology for MSW management developed by Ørsted in Denmark. In contrast to other MSW treatment processes, Renescience comprises one or few upstream and downstream steps that allows unsorted MSW to be handled directly to produce a slurry containing the organic fractions of the waste. The diagram of the process is shown in 3.2. The first step consists of mixing MSW with hot water at temperatures in the range of 40 to 70 degrees. This step enhances the opening of plastic bags and initiates pulping of degradable components in the waste. Next is a hydrolysis and fermentation step (liquefaction), where Renescience selected enzymes and optionally selected microorganisms are added to the wet waste materials. In this step, the organic fractions of the MSW are liquefied into a bioliquid, which is an organic material rich slurry that can be utilized for biogas production. During the last step of the process, the non degradable materials, such as plastic and metals, are separated from the liquid and can be sent for treatment at a recycling facility.

Current MSW treatment technologies that are alternatives to landfilling, such as

### How does the Renescience technology work?

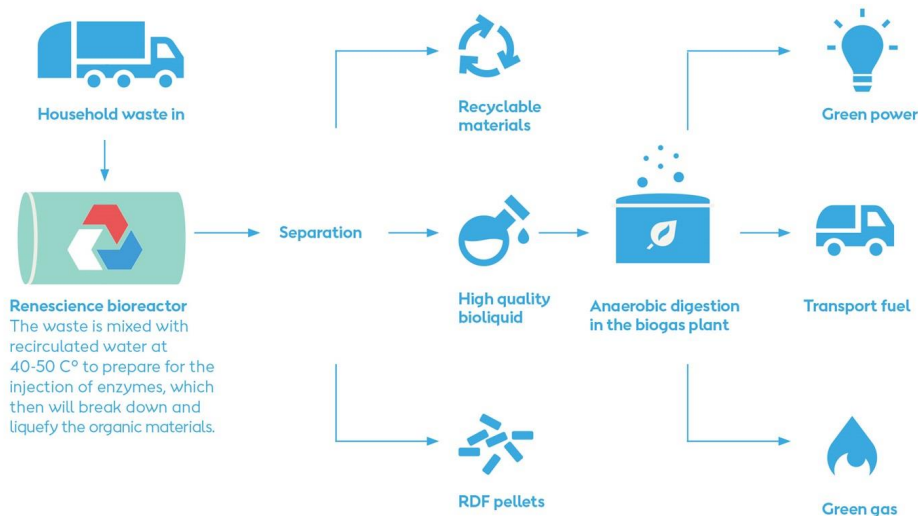


FIGURE 3.2: Diagram of the Renescience Process. Courtesy of Ørsted.

incineration or sort separation combined with anaerobic digestion, are not as efficient in recycling non degradable fractions present in the MSW compared to Renescience (Tonini and Astrup, 2012). The enzymatic process has also been found to give savings in electricity recovery in comparison to other the technologies investigated. Recently, the European Commission has proposed ambitious directives in which the member nations should increase recycling rates from waste as soon as by 2023 to a minimum of 50%. Renescience technology can play an important role as an innovative solution for handling MSW, as well to intensify the AD production from different waste streams.



## Chapter 4

# Reactor Design

3

### 4.1 Development of Reactor Design

Within the field of anaerobic digestion, the design of the digesters has a great impact on the stability and final yields of the decomposition process. In the mid-20th century, rudimentary systems were utilized for the digestion of different substrates under the misconception that settleable solids should be removed from the reactors' media (Tauseef, 2013). This wrong line of thinking led to the most active microbial populations being actively removed from the anaerobic digesters and thus resulted in slow and unstable processes. Advances in reactor design have instead shown the benefits of having large amounts of microorganisms to accelerate decomposition of the biomass. Having more active cells in the process results in a higher metabolic capacity of the digester to convert the degradable organic fractions of biomass into biogas. Long term stability of the reactor can be achieved by maintaining a proper balance that guarantees sustainability of very large populations of the desired microbes inside the reactors.

At this point, a distinction can be drawn between "low-rate" and "high-rate" anaerobic digesters. The main difference between these two types is that in "high-rate" reactors, there is the possibility of either enhancing, reincorporating or retaining microbial rich elements to facilitate the digestion processes. In "low-rate" digesters, by contrast, improving the contact of the treated materials with the microorganisms involved in the process is not taken into account. Some examples of these second type of reactors are rudimentary non-agitated reactors and septic tanks, which are commonly small scale reactors that serve few households (Tauseef, 2013). A diagram summarizing the proposed classification of AD reactors is given in Figure 4.1.

Low rate reactors are usually characterized by lower design complexity and cheaper maintenance and investment costs, but long retention time operation. Low-rate anaerobic digesters commonly require low investments since these can be constructed with materials such as bricks or plastic. These reactors are usually used in batch or in semi-continuous operations, and the substrate is generally introduced based on its availability (rather than on type). The main concern is to maintain a fit organic load in order to avoid process failure. The degradation times in these systems are achieved over long periods, usually between 4 and 8 weeks. There must be a balance of the nutrients introduced in the substrate to maintain sound biodegradability, and undesired components such as sand or woody materials, which could have an impact on the conversion processes, must be avoided.

In high-rate processes, contact between the digesting media and active microbial populations can be enhanced by brisk agitation or internal recirculation. The most widespread reactor type employed for anaerobic digestion, the continuous stirred tank reactor (CSTR), is characterized by intense internal agitation. The mixing in CSTRs leads to the dispersion of the substrate in the digestion volume while maintaining the active biomass, usually found in the form of flocs, in suspension and thus minimizing precipitation. The mixing in the CSTR improves the methane productivity two to three fold in contrast to low rate reactors, and CSTRs are common due to the simple design, possibility of continuous feed-in and scalability. However, the major drawback of CSTRs is flush-out of active microorganisms at high hydrodynamic regimes, similar to what occurs in low rate reactors. For this reason, anaerobic digestion in CSTRs are usually operated at HRTs higher than 10 days and limited by the kinetics of biodegradability of the biomass and the reproduction rate of the slowest growing microorganisms.

The high-rate processes based on reincorporation of microbial rich elements are generally based on recirculation of the sludge, i.e. the fraction of the effluent that contains most of the active microorganisms. Reactor design such as the anaerobic contact (AC) reactor or the anaerobic sequencing batch reactor (ASBR) have a feature which allows prolonged sludge retention time (SRT) compared to the HRT. In these reactor designs it is possible to reintroduce a large proportion of the microorganisms that otherwise would be flushed out of the process. Furthermore, there is extended retention time for degradation of the most recalcitrant organic compounds, for example those present in fibrous rich biomass that requires longer retention inside the reactor. Often, an important drawback of the reactors based on recirculation is the need for an operation unit where the sludge can settle before reintroduction, which results in higher investment costs the need for more space compared with the conventional CSTRs systems. The additional operational unit regularly implies an additional reactor that requires a degasifier. The settleability of the solids can also pose a disadvantage for solid recirculation processes. This is because AD sludge behaves differently depending on the nature of the feedstock digested, and in some cases it is complicated to separate the solids from the effluents as well as the non degradable suspended solids from active biomass.

A great forward step in the evolution of reactor design for anaerobic digesters was taken with the introduction of reactors able to retain dense microorganism communities within the process. The retention is usually performed by taking full advantage of the ability of microbes to form and organize in assemblages encased in a matrix of extracellular polymeric substances (EPS) termed biofilm (Langer et al., 2014). The biofilm formation is a natural process that occurs when a flow containing nutrients is exposed to a fixed conglomerate of well-balanced microbial consortia. These consortia excrete EPS and form layers of cells that remain in proximity to and given rise to the mature biofilm. Biofilm formation does not only allow the maintenance of a one magnitude higher number of microorganisms per reactor volume in contrast to "free-floating" reactors, but also provides higher tolerance to inhibitors and to temperature changes for the microbial communities. Furthermore, the syntrophic metabolic conversions are enhanced in the biofilm due to the proximity of distinct microbial species close together in the biofilm. Some studies have also elucidated the eventual immobilization of secreted enzymes, which favors the hydrolysis and ultimate degradation of organic material. The immobilization of microbes inside the reactors can be achieved by following different strategies, such as granulation, dispersed carriers and stationary carriers. An overview of the different reactor designs based on the immobilization strategy is shown in Figure 4.2.



- Biofilm in granules: Upflow Anaerobic Sludge Blanket  
Expanded Granular Sludge Blanket (EGSB)
- Biofilm in dispersed carriers: Anaerobic fluidized-bed reactor (AFBR)  
Anaerobic expanded-bed reactor (AEBR)  
Plastic carriers, fex. Bioflow 30, flocor
- Biofilm in stationary carriers
  - Randomly oriented: Downflow Stationary Fixed Film (DSFF)
  - Fixed orientation: Cloisonyl  
Fast Anaerobic Digester (FAD)

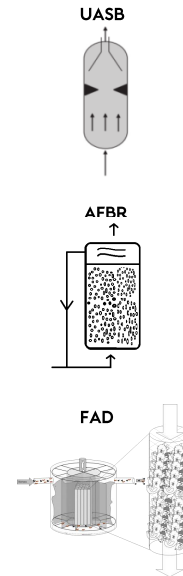


FIGURE 4.2: Type of AD reactors with microbial immobilization in biofilms.

The bacterial immobilization can also be augmented in solid carriers that support maturation of the biofilm (Lettinga et al., 1983). This strategy is of special interest for treating VFA or nitrogen rich substrates like MSW where the natural granulation processes are hindered. There are carriers made of very different materials, morphology and dimensions, which have been used for immobilization of microbial communities for anaerobic digestion processes. Some common examples of supports used in AD are sand particles, activated carbon, basalt and plastics (Lier et al., 2015 and Kennedy and Berg, 1982). The anaerobic digesters based on carrier immobilization, commonly referred as fixed biofilm reactors, can be put in two categories depending on placement of the carriers: fixed and non-fixed carriers. In this case, the term "fixed" refers to the position and not the immobilization. The non-fixed biofilm reactors represent an evolution of the UASB reactors where the microbial biomass remains suspended in the reactors attached to inert supports. The most common designs representing this kind of reactor are the expanded bed reactor (EBR) and the fluidized bed reactor (FBR). The principles of these types of reactors have been exploited for many years in the chemical industry; for example, in cases where solid catalysts are immersed in the reactor and their surface area exploited to improve the yields of the chemical reactions. Within the AD field, there are multiple examples of biofilm reactors with supports randomly immersed in the digesting media. For example, a study has shown excellent performance using open-pore sintered glass beads as supports in an anaerobic fluidized bed reactor (Pérez, Romero, and Sales, 1997). Despite these important results, the non-fixed fixed biofilm reactors are still affected by high concentration of suspended particles in the substrate, in particular those that are inert or slowly degradable. In fact, the bibliographical studies shows that reactors with this design treat only streams with low TSS concentrations.

The other type of biofilm-based anaerobic digesters with carriers have the support material in a fixed orientation. A distinguishable feature is that these reactors have a vertical flow upwards or an upwards fluidization pattern, and commonly occur in the literature as upflow or downflow anaerobic fixed film reactors. For



practical reasons, the supports are usually tubular shaped and are immersed in the media where the flow of the digesting media is directed by means of pumps. It is common for the modular tubular blocks that serve as biofilm carriers to possess a corrugated surface in order to entrap the biomass and prevent washout of the immobilized cells (Hickey et al., 1991). Even though it may not sound intuitive, support materials with high adhesion properties have been observed to perform better than others with higher specific surface area (Thanikal et al., 2007). Construction with porous materials has also been found to enhance the biofilm formation throughout the start-up phase, which is important in order to avoid long start-up times before achieving high performance of the system (Bonastre and Paris, 1988). In order to have a robust system, during the start-up the system should favor the attachment of concentrated syntrophic bacteria and methanogens, positioned in the inner layers of the thin (0.5-3mm) anaerobic biofilm (Austermann-Haun et al., 1994 and Mata-Alvarez and Llabrés, 1988). In fact, some carrier materials have been described that exhibit more active biofilms than others and where observed activity is not related to thickness of the formed biofilm (García-Calderón et al., 1996). The material of choice should preferably be stable with time so as not to interfere with growth of the microorganisms. In some experiments with clay materials as carriers, the support appeared to release compounds inhibitory to methanogens, and therefore low methane production was measured. In other experiments using clay, a faster start-up period was perceived, which was attributed to the inorganic nutrients leachate from the supports, which seems to have enhanced this phase (Hickey et al., 1991).

In contrast to the non-fixed biofilm reactors, the stationary or fixed film systems are able to process substrates that contain high concentrations of suspended particles. Even though there appears to have been few development of these types of systems during the last decade, initial studies to demonstrate the ability of fixed positioned carriers date back to the 1980s in attempts to improve biomethane production from different types of manure and olive mill wastes (Hamdi, Festino, and Aubart, 1992; Mata-Alvarez and Llabrés, 1988; Sánchez et al., 2006; Sánchez et al., 2004). These types of reactors have been shown to cope with larger volumetric or organic loading rates than partially or fully mixed tank reactors (Kennedy and Berg, 1982). Despite the presence of immersed carriers inside the digesting media, there do not appear to be plugging issues due to clogging of the filters until several months or years of operation (Escudie et al., 2010). However, these types of reactor design require a sufficiently strong circulation flow of the media to guarantee maintenance of the biofilm through growth and detachment mechanisms of the EPS layers. The flow should also help in avoiding the formation of scum layers or so-called acid pockets during the anaerobic digestion (Forster and Rockey, 1982). The acid pockets are zones with different concentrations of metabolites or inhibitors that may localize in a certain spot of the reactor when the flow pattern is not regular or strong enough to guarantee the dispersion of the media.

So far there has been no long term study that analyses the complex dynamics of microbial communities that compares the microorganisms present in the biofilm with those in the digesting media. Nor have the effects of drastically changing the temperature range during AD on the microbial diversity of a fixed film reactor been addressed. However, Habouzit et al., 2011 have shown that there are differences in the ratios and diversity of the methanogenic and bacterial populations that depend on the carrier materials with regard to the initial inoculum utilized in the seeding process. In the cited study, the different communities present in the biofilm after the inoculation period were analyzed. The results show that the attachment properties of certain communities are favored depending on the physical and morphological



characteristics of the carrier as described previously. However, common denominator or "fingerprint" of microbial communities were retrieved, which were initially present in the inoculum and attached to the carrier regardless of the material. In the present study, there is an attempt to understand and verify these issues better, with special focus on the microbial populations during anaerobic digestion of MSW in the FAD.

## Chapter 5

# FAD: Fast Anaerobic Digester

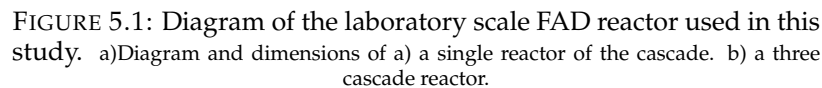
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### 5.1 General Overview

As previously stated, the main objective of this project was to determine the most important factors in an anaerobic digestion reactor process focusing on a novel reactor design: Fast Anaerobic Digester. A major part of the study is based on explaining and understanding the microbial biofilm conversion and reaction phenomena in the novel AD reactor described in Uller, 2016 and Uller, Londono, and Lardon, 2017. Part of the description of the experiments performed is given in these documents. In this thesis, the results are shown in a broader and more systematic manner, with a focus on the bioengineering aspects of the process.

The FAD can be categorized as a high-rate anaerobic digester based on microbial immobilization with the biofilm in fixed (stationary) position according to Figure 4.1 and Figure 4.2. The reactor is designed for degradation of high organic strength substrates through wet anaerobic digestion. The implications and characteristics of wet anaerobic digestion have been described in more detail in Chapter 3. This type of AD generally implies the utilization of pumpable substrates, usually with less than 20% of total solids. However, in contrast to the other reactor types that have biofilms, the FAD design possesses different elements that contribute to solving the issues related to high levels of suspended particles in the substrate. The advantages of this feature of the operation of the FAD are that it should be possible to prolong the SLR of the most recalcitrant suspended solids and also the removal, while in operation, of the most heavy inerts. Furthermore, the reactors possess the advantages of single stage reactor operation, which should address investments and operation costs.

In this work, the performance and operation of the FAD was studied at laboratory scale and then at pilot scale. The configuration of the laboratory scale FAD was three independent cascade reactors containing biofilm carriers. In contrast, the pilot scale system was constructed with all the features of the FAD design in a compartmentalized single reactor that had been designed to take into account the possibility of further scaling-up. In essence, the laboratory scale tests were performed to enable an initial validation of the digestion process of Renaissance bioliquid, taking into consideration yields and performance at different HRTs or organic loading. The laboratory scale test were also used to make a detailed analysis of the microbial diversity that settles in the biofilm and in the digesting media during the start-up period and steady state of the process. This operation would have been difficult to examine at the pilot scale reactor since timely sampling of the biofilm is necessary and the interior of the reactors has to be accessible.



The laboratory scale reactor consisted of a three-reactor cascade of 10 liter stainless steel tanks. The digesting media volume of each reactor was 8 liter (total volume 24l) and the filters holding the biofilm occupied the remaining space. A diagram of the laboratory scale reactor used in this project is shown in Figure 5.1. In this Figure, the dimensions and configuration are also illustrated. The in-feed was supplied by a peristaltic pump that pumps the liquid from a 30 liter agitated tank. Each of the reactors had active recirculation at the top and the bottom of the filters to guarantee mixing in the upper and lower zones of the biofilm carriers. Recirculation also ensured homogeneous distribution of the media that passes through the filters to avoid channeling. Active recirculation in each reactor with downwards directionality maintains the flow of the digesting media through the filters. Nine filters of 40cm length were positioned in each of the tanks. The recirculation was also ensured by peristaltic pumps at the bottom of each reactor, which reintroduced liquid from the top of the reactors. The recirculation rate within each compartment was calculated to be  $43.8 \text{ l} \cdot \text{h}^{-1}$ .

A plug flow reactor can be represented by a cascade of CSTRs, where the cumulative residence time distribution (RTD) of N number of CSTRs resembles more to a plug-flow with increasing N. The RTD test made with methylene blue tracer for the laboratory scale FAD confirmed that the flow patterns do resemble a plug-flow regime and therefore the combination of mixing and recirculation in the reactors are useful to maintain well mixed digesting media in the chosen reactor configuration (Fig. 5.6). Furthermore, the heating jackets of the reactors of the cascade are interconnected to a water bath in order to maintain the same temperature amongst them. All the experiments were conducted in two identical systems of the three reactor cascade in order to demonstrate reproducibility amongst them. They were designated as system 1 and system 2. Fig. 5.2 shows the systems of laboratory scale FAD reactors and the biofilm carriers positioned inside.



FIGURE 5.2: Pictures of the laboratory scale FAD reactors. Left) Picture of the two systems. Right) Evidence of the filters and mixing inside the reactors.

A plug flow reactor can be represented by a cascade of CSTRs, where the cumulative residence time distribution (RTD) of  $N$  number of CSTRs resembles more a plug-flow with increasing  $N$ . The RTD test made with methylene blue tracer for the laboratory scale FAD confirmed that the flow patterns do indeed resemble a plug-flow regime and therefore the combination of mixing and recirculation in the reactors are useful for maintaining well mixed digesting media in the chosen reactor configuration (Figure 5.6). Furthermore, the heating jackets of the reactors of the cascade were interconnected to a water bath to maintain the same temperature amongst them. All the experiments were conducted in two identical systems of the three-reactor cascade in order to demonstrate reproducibility. They were designated as system 1 and system 2. Figure 5.2 shows the systems of laboratory scale FAD reactors and the biofilm carriers positioned inside.

### 5.3 Pilot scale FAD reactor

The pilot scale FAD is a cylindrical single stage compartmentalized reactor. From an engineering perspective, the construction of a cylindrically shaped reactor is preferred since it provides a stronger structure per construction cost with the highest calorimetric energy efficiency. The choice of this shape would also allow retro-feeding existing full scale cylindrical CSTRs with the features that distinguish the FAD design. Therefore this was the most obvious option for the studies at pilot scale. The construction material of the tank was stainless steel, which consisted of a jacket heated reactor of 240 liters of digesting volume, with additional 60 liters of biofilm carrier space. The substrate was pumped into the reactor by means of a peristaltic pump from a 100 liter agitated tank. The digesting media left the reactor after the liquid had passed through all the compartments of the tank. The different elements of the FAD design were as follows:

### Compartmentalization

The interior of the FAD digester is compartmentalized into four equally dimensioned sections (quarters), by means of inner walls of stainless steel. Each of these sections have further internal divisions into four subsections separated by inner baffles within each section, for a total of 16 chambers as shown in Fig. 5.3 and Fig. 5.8. Difference in height amongst the inner baffles allows a preferential flow in both upwards and downwards direction, that starts from the top in the uneven chambers and from the bottom in the even chambers. The flow inside the reactor is facilitated by means of recirculation within each chamber with peristaltic pumps. There are 4 recirculation points that pump the digesting media from the last to the first chamber of each section, in every occasion the liquid is pumped few centimeter beneath the surface of the digesting media. The volume of the digesting media is determined by the height of the uppermost baffles. The recirculation patters within each section and in the whole system is evident from Fig. 5.4. From the schematic in the right, the flow pattern of the pilot scale reactor used in this study is discernible. The larger diagram contains the flow pattern of a further scale up into a 100m<sup>3</sup> tank. In this case, there would be 16 chambers in each of the four sections, for a total of 64 chambers containing the biofilm filters.

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Compartmentalization of anaerobic digestion reactors has been described on different occasions (Yousefzadeh et al., 2017 and Elreedy and Tawfik, 2015). A commonly utilized design is the Anaerobic Baffled Reactor (ABR), which can be found in several waste water treatment plants. The main objective of having such divisions inside the reactors is to prolong the contact of the substrate with the active biomass because the preferential flow allows decoupling of the sedimented solids from the HRT. In the case of the FAD design, the main purpose of compartmentalization was to allow a unidirectional flow through the biofilm carriers and maintain a pumpable flow regime within each of the chambers (Figure 5.8c). The compartmentalization inside the FAD would also allow improved contact of the liquid to the biofilm surface because recirculation within each chambers allows reintroduction of the media into the flow stream in each chamber. Furthermore, this design should also allow specialization of the microorganisms within each of the chambers, because sequential configuration into different compartments should create an organic load gradient amongst them. The effects of reactor design on microbial diversity of the FAD

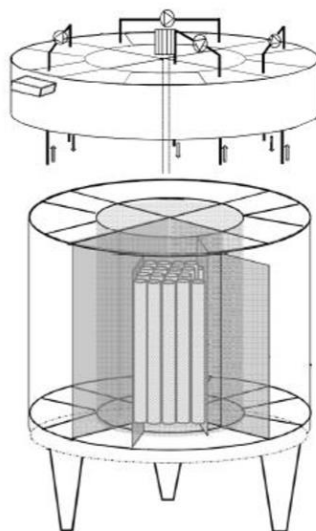


FIGURE 5.3: Compartmentalization pattern of the FAD.

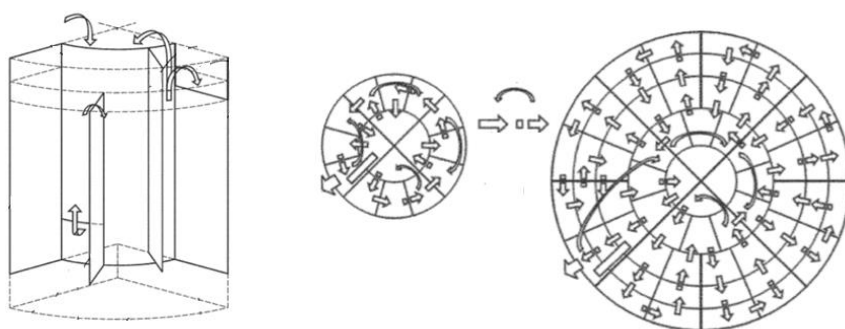


FIGURE 5.4: Flow pattern inside the FAD reactor. Left: Flow pattern within a section. Right: Flow pattern throughout the entire reactor in pilot and full scale.

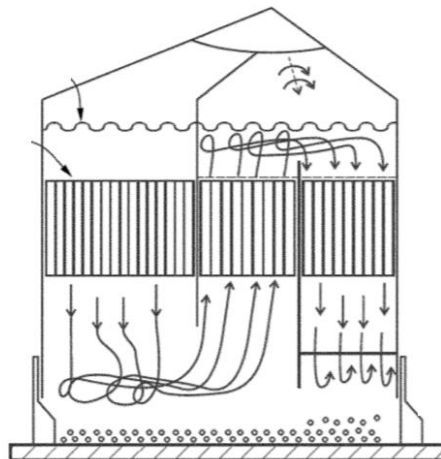


FIGURE 5.5: Mixing zones within the compartments of the FAD.

process is described in 8.

TABLE 5.1: Dimensions and flow recirculation calculations for different scales of FAD reactors.

Parameter	Lab scale	Pilot scale	FAD 100m <sup>3</sup>	FAD 1000m <sup>3</sup>
Tank r ( m )	0.090	0,370	2,000	5,200
Height ( m )	0,40	0,69	8,00	12,00
Circulation pump flow (L* h <sup>-1</sup> )	43,80	300,00	12000,00	20000,00
Feed flow (L* h <sup>-1</sup> )	0,31	2,10	1380,00	13800,00
Number of Chambers	1,00	16,00	16,00	64,00
Tank cross area (m <sup>2</sup> )	0,025	0,43	12,56	84,91
Tank Vol. ( m <sup>3</sup> )	0,010	0,30	100,48	1018,87
Chamber cross area (m <sup>2</sup> )	0,025	0,027	0,79	1,33
Chamber Volume (m <sup>3</sup> )	0,010	0,019	6,280	15,920
Tank volume exchange ( h )	0,2	1,0	7,5	30,1
Section volume exchange (h)	ND	0,2	1,9	7,5
Chamber reintroduction (1 * h <sup>-1</sup> )	4,3	16,3	2,1	2,1
Tank flow velocity (m * s <sup>-1</sup> )	4,82 * 10 <sup>-4</sup>	1,95* 10 <sup>-4</sup>	2,96* 10 <sup>-4</sup>	1,11* 10 <sup>-4</sup>
Chamber flow velocity (m * s <sup>-1</sup> )	4,82* 10 <sup>-4</sup>	3,12* 10 <sup>-3</sup>	4,73* 10 <sup>-3</sup>	7,08* 10 <sup>-3</sup>
Reynolds number in tubes	11	137	208	311

Substrate filling was accomplished via peristaltic pumps and mixing was ensured by liquid circulation within each of the separate compartments. The recirculation allows dispersion of the digesting media at the top of the chambers into which it is reintroduced and a gentle back-flow mixing in the sedimenting zones at bottom of the baffles where the liquid changes direction from a downwards to an upwards flow (see Figure 5.5) . Different studies have also suggested that the gas bubbles that evolve during biogas production also contribute to maintaining high mixing regimes, especially in high rate systems(Dapelo, Alberini, and Bridgeman, 2015 and Escudié et al., 2005).The recirculation pump flow was set to 300l\*h<sup>-1</sup> to achieve a flow velocity of approximately 3.12 \* 10<sup>-3</sup> m \* s<sup>-1</sup> within each chamber. Differences in the



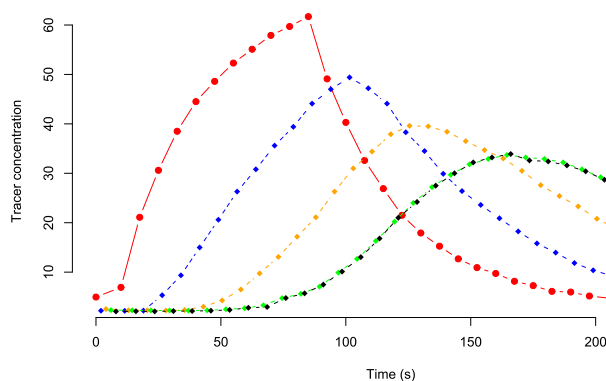


FIGURE 5.6: Residence time distribution curves for the pilot scale reactor.

height and depth of the baffles within each subsection was designed to determine the directionality of the flow. The volume of the digesting media in the reactor was also determined by the height of the uppermost baffles. The effluent leaves the reactor at the same rate as the feed-in through an exit point in the fourth chamber, after the media have been led through the entire system. The low Reynolds number of the flow achieved by the compartmentalization facilitates a plug flow behavior that prevents channeling or plugging in the baffles where the liquid enters into contact with the carriers. In contrast to other stationary fixed film reactors, the dimensions of the chambers permits a scale-up of the systems. The calculations suggest that the available pump capacity from industrial pumps is sufficient to support the flow in a larger reactor of  $1000 \text{ m}^3$  (Table 5.1). Furthermore, the compartmentalization typical of the FAD can potentially be retrofitted to existing CSTR reactors, which can be done by inserting baffles that then hold the biofilm supporting materials.

Before the operation of the pilot scale reactor, the RTD test was performed in order to verify the behavior of the flow pattern, utilizing water and NaCl as tracer. The plots of the measured conductivity over time in the different chambers are shown in Figure 5.6. From this Figure, it is possible to conclude that no back channeling occurs between the compartments since there is a progressive increase in tracer concentration within the different chambers. Furthermore, the concentration of the effluent follows the values measured in the fourth chamber.

### Support material

In the present study, the fixed filters used for the biofilm immobilization were wide-meshed tubular polyethylene carriers of type 150 (Bio-Blok Expo-Net, Hjørring, Denmark). The specific surface, as declared by the manufacturer, was  $150 \text{ m}^2 \cdot \text{m}^{-3}$ . The area of flow in these carriers was approximately 60% without biofilm growth, with a void percentage of 82%. The carriers were cut with an electric saw to match the



length of the baffles and the tube diameter was 55mm. Since the density of the carriers can range from 0.6 to approx.  $0.95 \text{ g}\cdot\text{cm}^{-3}$ , fixation to the baffles was necessary by means of plastic seal rings. In the pilot scale reactor there was space for seven to eight filters in each of the baffles, as shown in Figure 5.8 a and b.

An important characteristic of these types of filters is its threaded structure and the rough surface. As described in Chapter 4, the rough surfaces usually enhance the mechanisms of cellular adhesion and biofilm formation. At the same time, the presence of threads in the tubular carriers increases the superficial area over which the biofilm can form. This feature favors the attachment and detachment dynamics of the biofilm which can develop and can be released from the surface by the act of shear forces. Images of the threaded structure and a colonized surface of the filters used in this study are shown in Figure 5.7.

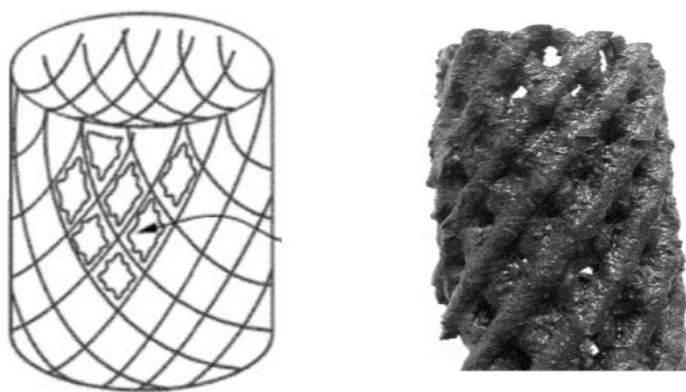


FIGURE 5.7: Biofilm carriers in the FAD. Left: Threaded surface diagram of the carriers. Right: Picture of a colonized carrier.

### Scraper and sedimentation zones

As stated previously, the FAD design should bring the possibility to treat substrates containing high organic substrates that contain high concentration of suspended particles that could be either biodegradable or inert. In both cases, the dimensions of the tubular biofilm carriers should guarantee free passage of the solids through the reactor. The diameter of the holes in the pilot scale FAD reactor is 4cm. This size should be enough to allow passage of the largest particles from conventionally utilized substrates in AD. In the case of Renaissance bioliquid, the largest particulate solids do not exceed 2cm since most of the biodegradable inerts have been dissolved or partially hydrolyzed by the action of the enzymatic pretreatment. The remaining accessible biodegradable suspended solids dissolve when these enter the AD process by the action of the hydrolyzing microorganisms and their secreted enzymes.

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The remaining inert solids, which include smaller fractions of recalcitrant lignocellulosic materials or sand materials, should be kept out of circulation within the chambers of the FAD. Also, the dense microbial biomass should not be allowed to accumulate in the reactor since this may affect mass transfer within the liquid and the biofilm. For these reasons, every compartment is accompanied by a sedimentation zone within each of the baffles. This feature permits the most dense particulate solids to be deposited at the bottom of the reactor and remain excluded from the recirculation flow. In order to avoid accumulation of sedimented materials, the FAD design included a rotary scraper that divides precisely all the sections of the reactor in the lowermost area Figure 5.8d. The presence of a scraper permitted the removal of the suspended solids that become deposited in the sedimentation zones, which could then be evacuated from the reactor through an ejection chamber positioned in the last chamber by means of an eccentric screw pump. The pump was located beneath the reactor and posterior to a deeper sedimentation zone in the fourth compartment where the effluent leaves the reactor. To ensure anaerobic conditions the reactor was sealed with fittings and rubber sealing. Activating the scraper also increased retention of solids that needed to remain for longer time inside the reactor for degradation to occur.

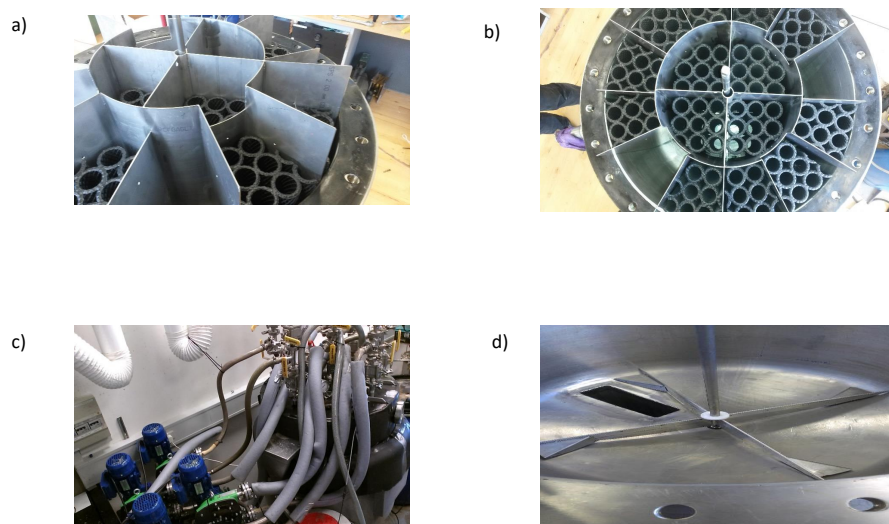


FIGURE 5.8: Pictures of the pilot scale FAD reactors. a) Construction of the inner baffles. b) Top view of the four compartments containing each 4 chambers. c) Connection lines for recirculation of media within each compartment. d) Scraper and sludge ejection chamber.



## Chapter 6

# Materials and methods

5

## 6.1 Analytical methods

### 6.1.1 Determination of solids

#### Total and volatile solids

Total solids and volatile solids were measured by gravimetric determination. This was performed in oven dried crucibles according to modified NREL guidelines (Sluiter et al., 2008). The first drying step was performed at 60°C until further weight loss was not perceived. The reasoning behind this choice is the nature of the substrate, which contains a large fraction of VFAs that would otherwise evaporate at the commonly used temperature of 105°C (Vahlberg, Nordell, and Wiberg, 2013). The incineration of volatile solids was measured after 3 hours in a muffle furnace.

#### Total suspended solids

The total suspended and total volatile suspended solids were also determined gravimetrically after filtration through Whatman Grade 934-AH RTU filters. The weight of these filters is approximately 64 g·m<sup>-2</sup> with 1.5µm of retention. The TSS is the mass that is retained in the filter after drying at 60°C and the TSVS volatilize following incineration.

### 6.1.2 Chemical analysis

#### Chemical Oxygen Demand

Chemical oxygen demand (COD) and soluble COD (sCOD) in the substrate and in the digesting media was quantified using the Hach LCK514 COD and a DR 3900 spectrophotometer (Hach, Düsseldorf, Germany). In this case, the COD was determined by the dichromate method (Dedkov, Elizarova, and Kei, 2000). COD is a measurement of the oxygen necessary to oxidize soluble and particulate organic matter in water and thus is an important parameter for use in anaerobic digestion to determine organic strength of the substrate and of the digestate. The difference between the measured concentrations can be used to determine the degree of degradation under the AD process. The determination of the sCOD was performed in the liquid following centrifugation at 4000rpm for 10 minutes.

### **Volatile fatty acids**

During the experimental phase of this project, the VFAs were determined by two different methods. To monitor the AD process, the VFAs concentrations were measured using the CK365 Organic Acid cuvette tests and a DR 3900 spectrophotometer (Hach, Düsseldorf, Germany). This is a rapid method for determining the concentration of VFA pool in the digesting media expressed in acetate equivalents. For a more precise quantification of the VFAs in the substrate and in the digesting media, the quantification was done using High Performance Liquid Chromatography (HPLC). Before quantification, the samples were centrifuged for 10 minutes at 4000rpm, then diluted to the measuring range with demineralized water, corrected to pH range 2-3 with  $\text{H}_2\text{SO}_4$  and filtered through nylon 0.2  $\mu\text{m}$  filters. The HPLC analysis was conducted under the following conditions: Flow 0,6 ml/min, Eluent was 12mM  $\text{H}_2\text{SO}_4$ , isocratic, oven temperature 60°C, Detector: RID-10A (refractive index detector). The system was a Shimadzu Prominence HPLC (Shimadzu Corporation, Kyoto, Japan) equipped with a Aminex HPX-87H Ion Exclusion Column, 300mm x 7.8mm (from BIO-RAD).

### **Total ammoniacal Nitrogen**

The total ammoniacal Nitrogen ( $\text{tNH}_3$ ) was quantified with Hach Lange kit LCK 304 and DR spectrophotometer (Hack, Germany). The total nitrogen concentration in the sample was determined with the Total Nitrogen Kit LCK338 after diluting the samples 1:40 with demineralized water.

## **6.1.3 Gas analysis**

### **Methane content**

The methane content was determined by Gas Chromatography (GC). The gas samples were collected from the reactors in Tedlar gas bags with a total volume of 4 liters. The GC used was a Mikrolab ML GC 82, with helium as carrier gas, and injection oven and operation temperature of 70°C (Packed glass column 80/100 Porpac Q). Three methane standards were prepared prior to each determination.

### **Biogas flow measurement**

The gas production in the lab scale FAD was measured and monitored through the BioReactor Simulator (BRS) gas flow meter. This is a platform for simulating anaerobic fermentation processes in a continuous mode of operation<sup>1</sup>. The gas production from the pilot scale FAD was monitored with a Macroflow gas meter. Both of the flow measuring systems were acquired through Bioprocess Control AB (Sweden).

### **Biomethane potential**

The total amount of biogas that can be produced from a determined amount of VS or COD from a particular substrate can be determined by the Biomethane Potential Test (BMP). In this procedure, a known amount of substrate was placed in a batch reactor containing an inoculum (AD digestate) containing the necessary microbial consortia for biogas production. The evolution of biogas was measured in time elapsed after

<sup>1</sup><http://bioprocesscontrol.com/products/bioreactor-simulator/>

the substrate is put into the batch reactors. The BPM tests in this work were performed using an Automatic Methane Potential Test System from Bioprocess Control AD (Sweden). This equipment is provided with a CO<sub>2</sub> trap containing 5mM NaOH that permits the recording the evolution of solely the methane from the batch reactor. The volume of the batch reactors was 500ml of which 300ml was digesting media. Another manual BMP methodology for the determination of the effect of cations in AD was performed in glass flasks sealed with rubber following recommendations from Angelidaki et al., 2009. In this case, the evolution of methane was measured by GC under the same conditions as for the methane content. Therefore the determination of the BMP was performed using using two distinct methodologies.

### Cat-ion analysis (ICP OES)

Liquid samples were analyzed for cation concentrations. Samples were taken of the reactor content and centrifuged for 30 min at 4400rpm and then filtered through a 0.45  $\mu$ m filter. The samples were then stored in a freezer until they were sent for analysis of Na<sup>+</sup>, Mg<sup>+</sup>, Ca<sub>2</sub><sup>+</sup>, K<sup>+</sup> and Al<sub>3</sub><sup>+</sup>. The analysis was performed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES).

## 6.1.4 Analysis of microbial diversity

### Microbial diversity in the Fast Anaerobic Digester

As mentioned previously, the microbial diversity in the FAD process was determined in laboratory scale reactors since it was possible to access the biofilm of the reactors to collect colonized filter samples. The DNA from the reactor media was extracted using the powerSoil<sup>®</sup> DNA Isolation Kit, (MO BIO Laboratories, USA) following the manufacturer's protocol. The first steps consisted of sample preparation, followed by cell lysis and inhibitor removal. Next, the DNA was selectively bound to a silica filter, washed and finally eluted with 10 mM Tris buffer. DNA from the biofilm was extracted with the PowerBiofilm<sup>®</sup> DNA Isolation Kit (MO BIO Laboratories, USA). A piece of ca. 0.2 g of the carrier cut-off was introduced directly into the extraction Eppendorf tube. The extraction was performed in the same way as for the liquid reactor media. The concentrations and purity of the samples were evaluated using a NanoDrop 1000 spectrophotometer (Fischer Scientific, Wilmington, MA). Only samples with A260/ A280 ratios > 1.3 were used in further analyses.

PCR amplification of the 16S rRNA gene from DNA extracted from the biofilm and media samples was accomplished using modified universal bacterial primer pairs 341F-785R based on Klindworth et al., 2013 findings and an archaeal specific primer pair 340F-1000R as described in Gantner et al., 2011 (Tab. 6.1). These primer sets target the V3-V4 and V3-V5 hypervariable regions of the rRNA gene, respectively. These include the incorporation of the Nextera XT<sup>®</sup> transposase sequence (Illumina Inc, USA) followed by an additional four random nucleotides N in both forwards and reverse primers. Amplicons were generated in a GeneAmp PCR System 2700 Thermocycler using the Q5<sup>®</sup> Hot Start High-Fidelity DNA polymerase (New England Biolabs Inc, USA). The concentration of the PCR products was adjusted to 1ng\*  $\mu$ L<sup>-1</sup>. The initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30s, 56°C for 40s and 72°C for 1.5 min. Positive (DNA) and negative (distilled water) controls were included in the PCR reactions. The DNA was submitted to Macrogen (Europe) for sequencing on the Illumina MiSeq<sup>TM</sup> platform.



TABLE 6.1: Bacterial and archaeal primers used in this study

Position	Forward	Reverse	Reference
340F-1000R (Archaeal)	5'-CCCTAYGGGG YGCASCAG-3'	5'-GGCCATGCAC YWCYTCTC-3'	Gantner et al., 2011
341F-785R (Bacterial)	5'-CCTACGGGNG GCWGCAG-3'	5'-GACTACHVGG GTATCTAATCC-3'	Klindworth et al., 2013
F341-R907 GC (Bacterial)	5'-CCTACGGGAG GCAGCG-3'	5'-ATTACCGCGG CTGCTGG-3'	Muyzer and Uitierlinden, 1993
F027-R1492 (Universal)	5'-AGAGTTTGAT CMTGGCTCAG-3'	5'-CGGTTACCTT GTTACGACTT-3'	Sun et al., 2016a Sun, Thygesen, and Meyer, 2015
F344-R915R GC (Archaeal)	5'-TCGCGCCTGC TGGICCCCGT-3'	5'-GTGCTCCCCCGC CAATTCCT-3'	Raskin, Rittmann, and Stahl, 1994
F341-805R (Universal)	5'-CCCTAYGGGG YGCASCAG-3'	5'-GACTACNVGGGT ATCTAATCC-3'	Takahashi et al., 2014

### Quantification of Bacteria and Archaeal populations

Denaturing Gradient Gel Electrophoresis (DGGE) was performed to quantify archaeal abundance in the microbial population of the digesting media of the laboratory scale reactors during the steady-state. After extraction of the digestate media described earlier, two different sets of primers were used to amplify the Eubacteria and archaeal 16S rRNA genes. Universal Eubacterial primers targeting V3-V5 region (F341-GC and reverse R907) were used for the total population, and a nested PCR approach using ArchF0025/R1492 and F344/R915-GC was used for the archaea (Tab. 6.1) (Sun et al., 2016a and Raskin, Rittmann, and Stahl, 1994). All PCR reactions were carried out in a Mastercycler (Eppendorf, Hamburg, Germany) and the size of the product was visualized on 1% agarose gel. Comparison of the band intensity between the samples regarding was determined using BioNumerics software v.7.1 (Applied Maths, Saint-Martens Latem, Belgium).

### Microbial diversity of anaerobic digesters seeded with diverse inocula for degrading enzymatic treated OFMSW

The present work also included a study on the effect of the enzymatic treatment of OFMSW on the microbial diversity of AD reactors. In this experiment, two different inocula were used to determine the role of the original microbial communities in the AD reactors, which digest enzymatically treated OFMSW. The resulting microbial structure in two parallel CSTRs was evaluated while treating the Renaissance bioliquid at different HRTs ranging from 20 days down to 10 days. In this experiment, the two different inocula derive from different processes: the first one was from a reactor treating non pretreated OFMSW, and the second was from a reactor treating enzymatically pretreated OFMSW (Chapter 9).

The samples from the two distinct inocula and those from the digesting media of the studied CSTRs were taken after the reactor had processed enzymatically treated OFMSW for at least 1 entire retention. In each case, the DNA was extracted with DNeasy powersoil (QIAGEN, Netherlands). PCR amplification of the 16S rRNA gene was performed using the same primer sets as those utilized for the FAD samples. In contrast to the previous protocol, Q5<sup>®</sup> Hot Start High-Fidelity DNA polymerase 2X (New England Biolabs Inc, USA) was used and the incorporation of the Nextera XT<sup>®</sup> v2 set B of transposase sequences was performed (Illumina Inc, USA). Agarose gel 1% electrophoresis (Biorad PowerPac Basic) was used to confirm the

success of the first amplification, and the resulting DNA was purified with AM-Pure XP and magnetic bead technology. The initial denaturation was performed at 98°C for 3 min followed by 25 cycles of 98°C for 30s, 57°C for 30s and 72°C for 30s. The inclusion of the index was performed under the same conditions for 8 cycles. The resulting DNA concentration was determined by a Qubit3 fluorometer (Invitrogen, USA) and normalized using Mag-Bind EquiPure Library Normalization Kit at 4nM (Omega Biotech, USA). The final concentration of the samples was approximately 5nM. The resulting samples were sequenced on the Illumina MiSeq™ platform.

### Data Analysis and treatment of microbial 16S rRNA gene

The sequences were analyzed using BION-meta software. BION<sup>2</sup> is a supported open-source package for microbial diversity analysis of 16S rRNA (Sequences were treated as in Klitgaard et al., 2017). Briefly, the reads were quality filtered and chimera-checked before classification against the RDP-II database. The number of reads obtained for each barcode was normalized to 100,000 for further data treatment.

Classified sequence counts were normalized to 100.000 before further analysis. Non-metric multidimensional scaling using Bray-Curtis distances was used to visualize multivariate patterns in each of the data-sets of the amplicon data. In order to compare the performance of the two systems, the values of the steady state were averaged and compared. A t-test was used to evaluate statistically significant differences ( $p < 0,05$ )

## 6.2 Methodology

As mentioned previously, the characterization of the performance of the novel fast anaerobic reactors was performed at laboratory and pilot scales. The laboratory scale experiments were mainly done in order to test the ability of the immobilized cell reactor to produce methane at HRTs lower than 10-12 days, which have been described as crucial for AD digestion due to flush-out of the methanogenic populations (Hartmann and Ahring, 2006). The analysis of the microbial dynamics was also performed in laboratory scale experiments because these made it practical to remove the biofilm samples. The laboratory scale experience was moreover used to provide information regarding the crucial maturation phase of the biofilm. In contrast, the pilot scale experiment was designed to test the feasibility of the FAD design and to ensure that the elements integrated into original design are functional. The performance of the pilot scale reactor was compared with a pilot scale CSTR in order to show the differences between the FAD and a conventional AD process.

The study of the effect of cations in AD was included in this project following important observations during the experimental phase. As is described below in the results section, the production of biomethane in the FAD was found to be limited by the pH of the digesting media. In an attempt to recover the process, NaOH and Ca(OH)<sub>2</sub> were added to the substrate in order to increase the pH of the process. The immediate results showed that this remediation strategy was successful for improving the evolution loss of biogas production. However, supplementation with these strong bases resulted in a long term collapse of the process. In order to understand

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<sup>2</sup><https://app.box.com/v/bion>



the consequences of increasing cat-ion concentration on the AD stability, it was decided to perform a targetted study that would elucidate such effects. In this thesis, a new mechanism for recovering cat-ion disturbed processes was discovered.

### 6.2.1 Laboratory Scale Methodology

#### Performance

The laboratory scale setup consisted of two independent systems of a three cascade reactor with filters for immobilization as described in Chapter 5. Both of the systems were fed independently of each other through the peristaltic pumps that connect the first reactors of the cascades with the corresponding feed tanks. Four phases were distinguished during this experiment (Figure 6.1). (1) Inoculation lasted 7 days, after which a seed material derived from a plant in Denmark treating manure and fiber rich residues agricultural straw (Foulum Biogas) was filled in the reactors. During this period, the entire surface of the polyethylene carriers was exposed to this inocula and it was expected that the biofilm initiation process would begin when the process reached 37°C, i.e. as in the mesophilic temperature range. (2) The start-up phase represented a gradual increase of the nominal daily flow rate of substrate, from 0.16L (50 days HRT) until 2L (4 days HRT) was achieved. The HRT values were calculated taking into consideration the volume of the first reactors of the cascade because these reactors receive the entire organic liquid directly from the reservoir. This phase lasted 43 days, from day 7 through to day 50. From day 50 until day 55, the feed-in was halted in order to test the robustness of the biofilm before the transition to the steady state. Gas evolution was measured continuously during this experiment and calculated every 24 hours. Chemical analysis were performed every 2-3 days. (3) During the steady state, there was a constant flow rate of 2L for a total of two entire retentions of the digesting media in the reactors of the cascade. At this point, the HRT was 4 days. (4) During the temperature shift, the process reached 51°C in approximately 4 hours. The flow rate was kept unvaried from that of the steady state in order to evaluate the consequences of this transition.

#### Microbial diversity

Over the last decades, advances in high-throughput sequencing, decrease in sequencing costs and more available databases have enabled the study of the complex ecological communities such as those encountered in Anaerobic Digesters (Caporaso et al., 2012). In this study, the diversity of the microbial populations involved in the FAD reactor process was analyzed in order to categorize the main microorganisms involved in the biomethanation of enzymatically pretreated OFMSW in the biofilm reactor. An important focus has been directed towards understanding the populations that settle in the biofilm in contrast to those that are "free floating" in the digesting media. Furthermore, the effect of the populations originating in the inocula was also studied in order to understand the relevance of the original seed material in the process and the long term repercussions this may have in a AD reactor. In order to investigate the members of the communities, the 16S rRNA gene was surveyed. This gene is a slowly evolved taxonomic marker used to reconstruct phylogenies because it is present in all the bacterial and archaeal ribosomal RNA.

The DNA samples from the reactors of the laboratory scale FAD were retrieved from the digesting media and in the biofilm. Before inoculation, the top of the cylindrical carriers inserted in the reactors were left loose to a depth of approximately 5 cm in to facilitate removal for biofilm DNA extraction. Every 14 days, a tubular

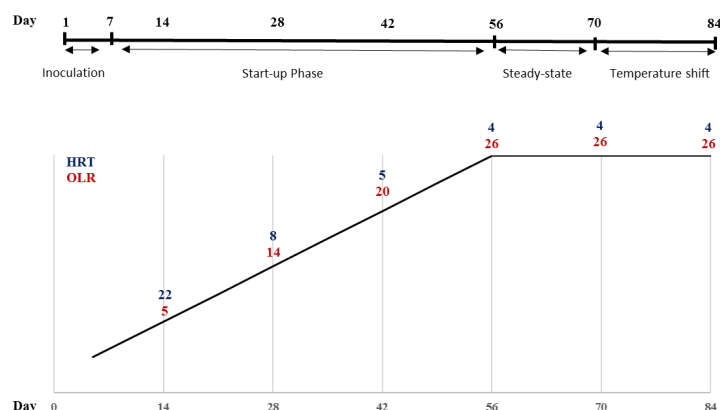


FIGURE 6.1: Experimental phases and timeline of the laboratory scale experiment. The days shown in the timeline are those when the biofilm samples were withdrawn

fragment of the carriers in each reactor was removed from the AD process. The withdrawn carriers were immersed in a saline solution to wash away any possible impurities and media. 1 gram of the carrier was cut off each of the cylinders to be used in the biofilm DNA extraction kit as described in the analytical section. The digesting media was also sampled the same days as the biofilm. Quantification of the ratio between bacterial and archaeal populations using DGGE was performed on the samples in steady state.

### 6.2.2 Pilot scale methodology

The development studies of the FAD reactor at laboratory scale confirmed that it was possible to obtain a rapid development of the biofilm in less than 60 days. In this period, it would be possible to sustain maturation of the anaerobic biofilm within the carriers, and it was decided to achieve a flow rate equivalent to 10 days HRT from an initial of 50 days in a time interval not greater than two months. The AD process was sustained at 50°C. In contrast to the laboratory scale experiment, the VFA concentration in the reactor was monitored carefully during this ramp-up phase to maintain the concentration of VFAs above  $2\text{g}\cdot\text{l}^{-1}$  in the digesting media. This strategy would serve to avoid undesired conversion of  $\text{H}_2$  and  $\text{CO}_2$  into acetate or other VFAs.

Having completed the initiation phase, the flow rate was halted for 3 days and then recommenced at the most recently used flow rate so as to verify biofilm formation. Next, flow rate was gradually increased up to retrieve the highest productivity and yields that the FAD pilot scale could achieve for degrading the enzymatically treated bioliquid. The experiment lasted 211 days from the day of seeding. Once each week, the scraper and the solids ejection pump were used to remove the thick sludge that had been deposited in the deposition areas at the bottom of the reactor. This process was compared in parallel with a conventional CSTR which is a tank of

500L of effective volume and was operated at the same temperature as the FAD. The CSTR reactor was operated at a HRT of 20 days in order to compare the yields between the two types of reactor design. A more detailed explanation of this strategy is explained in Londoño et al., 2018.

### 6.2.3 Effects of inoculum composition on AD of enzymatic treated OFMSW

This experiment was performed to determine the effect of the seed material (inoculum) in an AD process to process enzymatically treated OFMSW in conventional CSTRs. Assessment of the performance and the resulting microbial diversity in the digesting media of the reactors was done by monitoring different physical and chemical parameters throughout the process as well as the resulting microbial communities at different HRTs from 20 days down to 10 days. In order to do so, two parallel 10L CSTRs were fed for at least one entire retention with enzymatically treated bioliquid at HRTs of 20, 16, 13 and 10 days. The first reactor was originally seeded with inoculum derived from the FAD process, which was acclimatized to the enzymatically treated feedstock. The second reactor was inoculated with digestate from a full scale AD plant treating food waste without enzymation and located in March, UK. High-throughput sequencing techniques were utilized to separate out the effects of different organic loadings on the complex microbial communities, as described in the analytical section. In this experiment, the primer sequence from Takahashi et al., 2014 was used to simultaneously analyze the bacterial and archaeal populations using next generation sequencing (Table. 6.1).

### 6.2.4 Effect of cat-ions on AD

In this section the methodology is described that was used to test the influence of cations on anaerobic digestion. In brief, several batch reactor test were used to elucidate the effects of different cat-ion concentrations on final methane production, rates of biomethanation, and degree of inhibition of AD processes in which ethanol and methanol were consumed as the sole substrates.

#### AMPTs and manual BMP measurements

The first part of this study on the effect of cations on anaerobic digestion consisted of setting up a methodology to test the evolution of methane in batch reactors. As mentioned in the analysis section, two different systems were available to perform the activity: manual BMP and the AMPTS. The initial task was to investigate the reproducibility of these two different methodologies. All the experiments were carried out with inoculum taken from the FAD process that was originally seeded with material from Foulum Biogas (Denmark), which then was developed with the enzymatic treated bioliquid. A rigorous characterization of the seed material was performed which included solids and chemical analysis. The concentration of the relevant cations was also investigated.

All the batch reactors were filled with the seed material and left to acclimatize at the process temperature for 2 days. In the case of the manual BMP measurements, the headspace was flushed with N<sub>2</sub> gas to ensure the desired anaerobic conditions. The AMPTS is a semi-open system where the gas produced is free-flowing through the CO<sub>2</sub> trap towards the gas flow meter, and therefore the acclimatization time was sufficient to guarantee buildup of biogas in the headspace. Table. 6.2 summarizes

the operating conditions of the batch systems used in the current work. All the runs were carried out in triplicate.

In order to test the variability of these different test, enzymated OFMSW was fed into the reactors in each of the systems to reach a final concentration of  $5\text{gCOD}\cdot\text{l}^{-1}$ . The reactors were left to run over a period of 20 days during which the gas evolution was recorded.

TABLE 6.2: Operating conditions of the batch systems

Method	Total Volume (mL)	Inoculum Volume (mL)	Run Time (d)	Temperature (°C)	Stirring
AMPTS	600	300	>18	38	continuous
Manual BMP	543.5	200	>18	37	1-2 times per day

### Selection of inhibitor and substrate

Initial testing has revealed that the cations forming the most soluble salts with monovalent charge, that is  $\text{Na}^+$ ,  $\text{K}^+$ , have similar effects on the rates and final yields on batch experiments conducted with ethanol as the substrate. It was preferable to study in depth the effects of Na since this element is the usually the most abundant in biomass compared to the other single valence cations. Furthermore, the high Na concentrations present in MSW and several agricultural wastes have been shown to hinder AD processes.  $\text{NaHCO}_3$  was utilized in all the batch test to study the effects of sodium. For the study on the effects of divalent cations,  $\text{CaCO}_3$  and  $\text{MgSO}_4$  were used. The pure substrates used in this study were ethanol ( $\text{CH}_3\text{CH}_2\text{OH}$ ) and methanol ( $\text{CH}_3\text{OH}$ ). COD calculations were used to determine the final concentration of substrate in the batch test instead of the Inoculum Substrate Ratio (ISR). The reasons of this choice are discussed in the results section.

### Inhibition of cat-ions in AD

Table 6.3 summarizes the experiments performed in this section. The experiments A and B were performed to understand the degree of inhibition caused by varying concentrations of cations. Specifically, ethanol was used as the primary substrate at a concentration of  $7.3\text{ (gCOD}\cdot\text{l}^{-1})$  with in batch tests inhibited with Ca, Na and Mg. Additionally, experiment D investigated the effect of the inhibition when varying the concentration of substrate. Methanol was used as substrate in order to measure only the impact on the methanogenic step. Three different concentrations of Na were tested.

The  $\text{IC}_{50}$  (the half maximal inhibitory concentration measures the potency of a substance in inhibiting a specific biological or biochemical function) for sodium at an methanol concentration of  $7.5\text{ (gCOD}\cdot\text{l}^{-1})$  was calculated using the Modified Gompertz Equation ( Eq. 6.1). This expression has been approved statistically to be sufficient to describe bacterial growth, where  $M$  is the cumulative methane production (ml), at incubation time  $t$ ;  $P$  is the methane production potential (ml);  $R'$  is the methane production rate ( $\text{ml}\cdot\text{day}^{-1}$ );  $\lambda$  = lag phase (day) and  $e = \exp(1) = 2.71828$  (Chen, Han, and Sung, 2003 and Zwietering et al., 1990). The organic concentration was selected since it was within the range of highest methane yields in the current setup.

TABLE 6.3: List of experiments in the cat-ion inhibition research. Concentration of substrate, inhibitors and anti-inhibitor compounds used in the different studies of cat-ion inhibition

Study	Cat-ion	Source	Inhibitor Concentration (g*1 <sup>-1</sup> )	Substrate Concentration (gCOD*1 <sup>-1</sup> )	Anti-inhibitor Concentration (g*1 <sup>-1</sup> )
<b>A</b>	Ca+	CaCO <sub>3</sub>	3-5-7-9	Ethanol 7.3	none
<b>B</b>	Na	NaHCO <sub>3</sub>	5-10-15	Ethanol 7.3	none
	Mg	MgSO <sub>4</sub>	3-6-9	Ethanol 7.3	none
<b>C</b>	Na	NaHCO <sub>3</sub>	5-10-15	Ethanol 7.3	EDTA(2)
	Mg	MgSO <sub>4</sub>	3-6-9	Ethanol 7.3	EDTA (2)
<b>D</b>	Na	NaHCO <sub>3</sub>	0-5-10	Methanol 1.25-2-5-7.5	none
				10-15-30	
<b>E</b>	Na	NaHCO <sub>3</sub>	4	4	18-Crown6 (2-4)

$$M(t) = P * \exp \left\{ -\exp \left[ \left( \frac{R'_{*e}}{P} \right) * (\lambda - t) + 1 \right] \right\} \quad (6.1)$$

### Strategies to alleviate inhibition

The experiments C and E summarized in Table 10.2 examined the possibility of utilizing entrapment and chelation reactions to re-establish the performance of cation inhibited batch reactors. Ethylenediaminetetraacetic acid (EDTA) and 1,4,7,10,13,16-hexaoxacyclooctadecane (18Crown6) were used as ligands of Na. During this experiment, EDTA and 18Crown6 were administered in inhibited batches degrading ethanol and methanol as substrates. Respective controls in the presence and absence of the inhibitors were also performed. Furthermore, the effect on the inocula was also investigated.

## Chapter 7

# Performance of the Fast Anaerobic Digester

6

### 7.1 Overview

In this chapter the performance and operation of the novel FAD reactor is described. The first part describes the experiments done in the laboratory scale reactors that are the two systems of three cascade reactors containing the biofilm carriers for cellular immobilization. For ease of understanding, the set of reactors has been denominated "system 1" and "system2", and the respective reactors within each set are also numbered from 1 to 3 as will become evident in the graphs of this section. We then describe the results of the pilot scale experiences, that is the 240 liter (effective media volume) reactor together with all the elements of the original FAD design. More details about the operation of the laboratory scale reactors are described in Londoño et al., 2018 while for the pilot scale experiments, the results are in the manuscript "Microbial diversity of a high performance fixed biofilm biogas reactor". Both research items can be found in the Chapter 12.

### 7.2 Laboratory scale

Table 7.1 shows the results from chemical and gravimetric analyses of the enzymatically treated OFMSW used in this experiment. From the measured values, it is noticeable that the feedstock slurry had a high organic strength comparable with other biomasses from industrial and agricultural streams commonly used in AD (Al Seadi et al., 2013). The COD/VS ratio was calculated to be 1.56 and could be an indication that there is an important fraction of energy rich VFAs, protein or lipids in its composition. This information is in agreement with the HPLC quantification of compounds in the liquid, which revealed that nearly half the VS can be retrieved as VFAs in a weight basis. From this data it is also evident that there was a considerable proportion of TSS in the liquid and that nearly 68% of this TSS had potential to be converted into biomethane, while the rest was ash and other non-yielding compounds.

The different phases of the experimental phase at laboratory scale are shown in Figure 6.1. During this experiment, special attention was given to the start-up of the systems since it has already been reported that the initiation phase has an effect on the long term performance and operation of a biofilm based reactor (Escudie et al., 2010). The first phase of this experience was the inoculation and lasted 7 days. The purpose of this period within the experimental setup was to allow the inoculum

acclimatize to the imposed environmental conditions while testing the mechanical agitation and recirculation of the reactors. In this short period, it is possible that the microorganisms have already started to build up the biofilm. In fact Cresson et al., 2007 were able to observe the initial signs of cellular attachment within the first 12 hours of inoculum contact with the carriers. The biofilm life cycle has been described to consist of three stages: (1) Initial attachment of single cells to a surface, (2) Maturation of the biofilm into complex micro colonies, and (3) the further dispersal of motile planktonic cells (Langer et al., 2014).

TABLE 7.1: Chemical composition of enzymatically liquefied OFMSW used in laboratory scale FAD experiments.

Component	Measured value
Chemical Oxygen Demand (COD)	103.9 g <sup>*l</sup> <sup>-1</sup>
Soluble Chemical Oxygen Demand (sCOD)	62.9 g <sup>*l</sup> <sup>-1</sup>
Total Solids (TS)	9 %
Volatile Solids (VS)	6.6%
Total suspended Solids (TSS)	4.0 %
Volatile Suspended Solids (VSS)	2.7 %
Volatile Fatty Acids (VFAs)	35 g <sup>*l</sup> <sup>-1</sup>

Quantification of the development of an anaerobic biofilm that grows in AD reactors is not a simple task because the matrix, which comprises exopolymer substances that then hold the microbial cells together on the carrier, can be 5μm thick (Langer et al., 2014)). Different techniques have been developed to quantify and also to observe the position of the different microbial groups within the biofilm layer. There are numerous microscopy techniques that have been shown to be useful for studying biofilms, including environmental scanning electron microscopy, fluorescence microscopy and confocal laser scanning microscopy. For instance, a common trend for studying biofilms from AD processes consists of making use of microbial group specific labeling probes (fluorescence in situ hybridization) that permit quantification and location of targeted microorganisms after immobilization (Wagner et al., 2009). Within the scope of this project, the aim was to develop a methodology for making observations of the developing biofilm. However, it was not possible to do so since the polyethylene carriers that were used to sustain microbial growth have an irregular and threaded surface that complicates the implementation of microscopy techniques, in particular sample preparation.

The inability to carry out microbial observations of the biofilm left us relying on indirect measurements of the biofilm growth. This was done using three different indirect strategies: (1) Gravimetric determination of the biofilm growth, (2) Measurement of DNA concentration retrieved from the biofilm samples, (3) Indirect cellular quantification through measurement of metabolic activity. With the first strategy, we measured the increase of weight of the filters at every sampling time during the experiment. The cylindrical carrier inserts that were placed inside all the reactors of both system 1 and system 2 had been measured before the colonization period and the results are shown in Figure 7.1. With the second strategy, we measured the concentration of the DNA that was harvested from a particular piece of the biofilm carrier. A known amount of carrier was placed in the DNA extraction tubes for every sampling period. The DNA concentration from the digesting media was also quantified. The results of these measurements are illustrated in Figure 7.2. In the



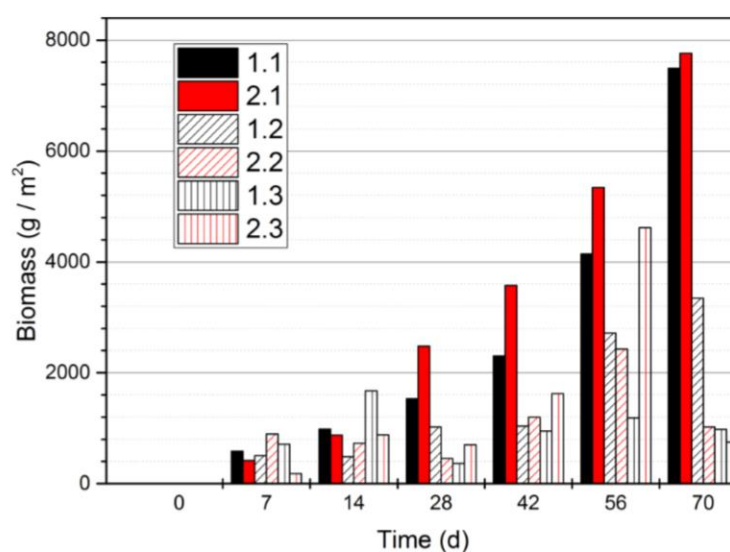


FIGURE 7.1: Gravimetric quantification of biomass formation on the carriers. Kurz, 2016. The first number indicates the system, the second number the reactors in the cascade

third strategy to quantify the biofilm activity, we tried to measure the concentration of ATP in the samples (Dexter et al., 2003). ATP is known to be present in active cells since this organic compound is involved in many metabolic reactions. When ATP reacts with luciferin in the presence of the enzyme luciferase (enzymes produced by fireflies), the catalyzed reaction produces oxyluciferin with the release of light (luminescence). This light can then be quantified in a luminometer to estimate the amount of ATP present in the original sample, and thus is an indirect estimate of the cellular activity of the biofilm samples. Despite the efforts made, it was not possible to obtain reliable data with this technique and the results have been omitted from this work.

From the results presented in Figure 7.1 and Figure 7.2 it is possible to conclude that the biofilm formation already started at an early stage upon inoculation. There was an increase of both biomass and DNA concentration in the biofilm at day 7, which subsequently increased when the systems were systematically fed with liquefied OFMSW. The same remarks apply to the first reactors in both of the systems. This would indicate that comparable processes were happening simultaneously in both of the systems tested and also that biofilm growth as intended was further stimulated by an increase in the organic loading rate.

### Ramp-up

The second phase of this experiment was the ramp-up. The flow rate of the substrate was systematically increased from 50 days down to 4 days HRT calculated on the basis of the volume of the first reactors of the cascades (Fig 6.1). In AD processes are based on cellular immobilization, the organic loading rate must be monitored carefully in order to avoid overloading of the systems, which could harm the synergistic interactions between the bacterial and archaeal populations (Escudie et al., 2010). The organic load should also be high enough to prevent competition from



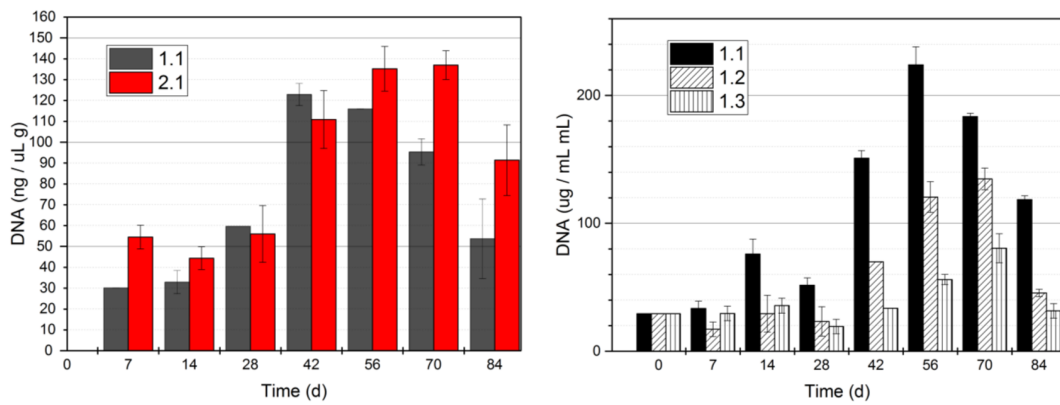


FIGURE 7.2: DNA concentration after the extraction procedure. Kurz, 2016 . Left: DNA from the biofilm in the first reactors of each system. Right: DNA from the digesting media in the three reactors of system 1

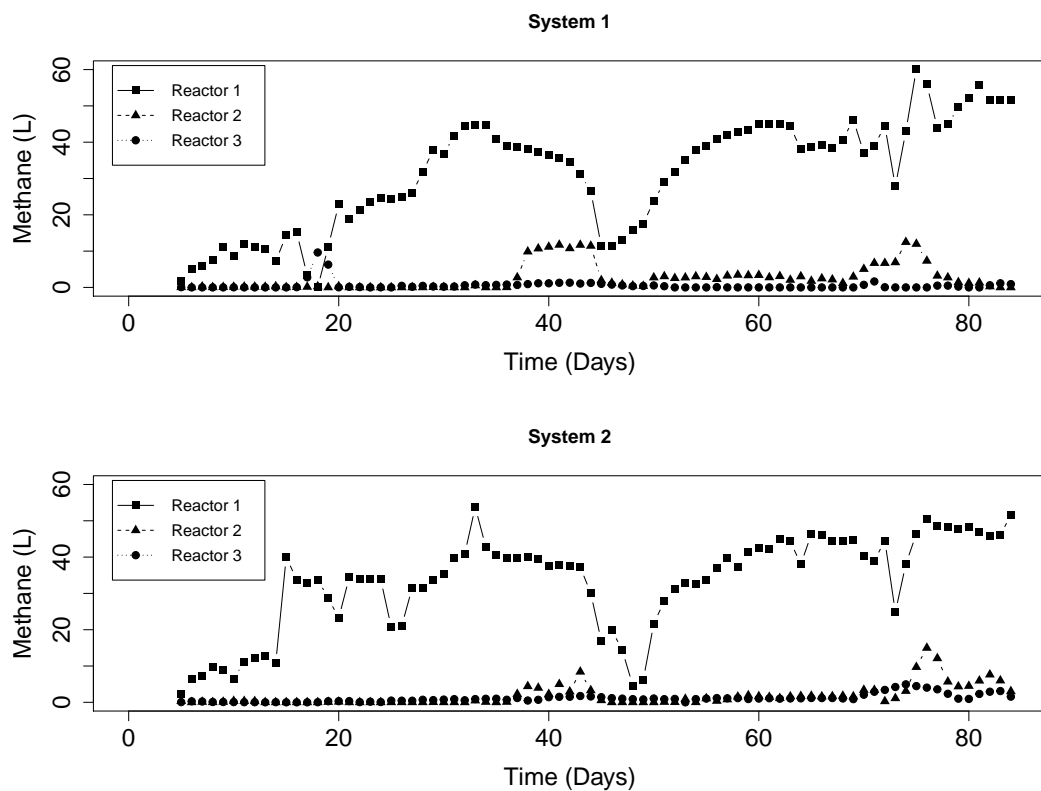


FIGURE 7.3: Methane production in the laboratory scale FAD reactor

planktonic organisms that would then decrease the amount of biogas produced and

lengthen the maturation phase. The desirable time for stimulating the biofilm development should be as short as possible, but still one question remains: How to determine if the biofilm has been established at its maximum productivity? Michaud et al., 2005 suggested a strategy to address this issue, which is simply based on the methane yield as an indication of the metabolic behavior of methanogenic biofilms. When the microbial populations are in a growth phase and utilize some of the energy of the substrate for anabolic purposes, for example in biofilm formation, the balance between the methane produced and the COD converted will be lower than expected. The theoretical yield of obtainable methane per gram of COD used in the reactor is  $0.35 \text{ l} \cdot \text{gCOD}^{-1}$  and is obtained when all the energy from microbial degradation is driven through the methanogenic phase. Hence, quantification of the methane produced per gram COD converted ( $I_{\text{CH}_4} \cdot \text{COD}_{\text{converted}}^{-1}$ ) was used to determine whether the start-up phase was adequate and long enough to promote the biofilm maturation processes. From this balance during the start-up phase, we observed an increase in the methane obtained from 0.27l to 0.33l per gram of COD lost in the digesting media. This behavior is evident from the results of the gas production (Fig 7.3) and the COD concentrations measured in the digesting media during the experiment (Fig 6.1). This methane yield did not increase in the following steady-state phase, which indicates that regardless of the physiological state of the biofilm, the microorganisms use at least 5% of the energy present in the substrate for cellular growth and maintenance.

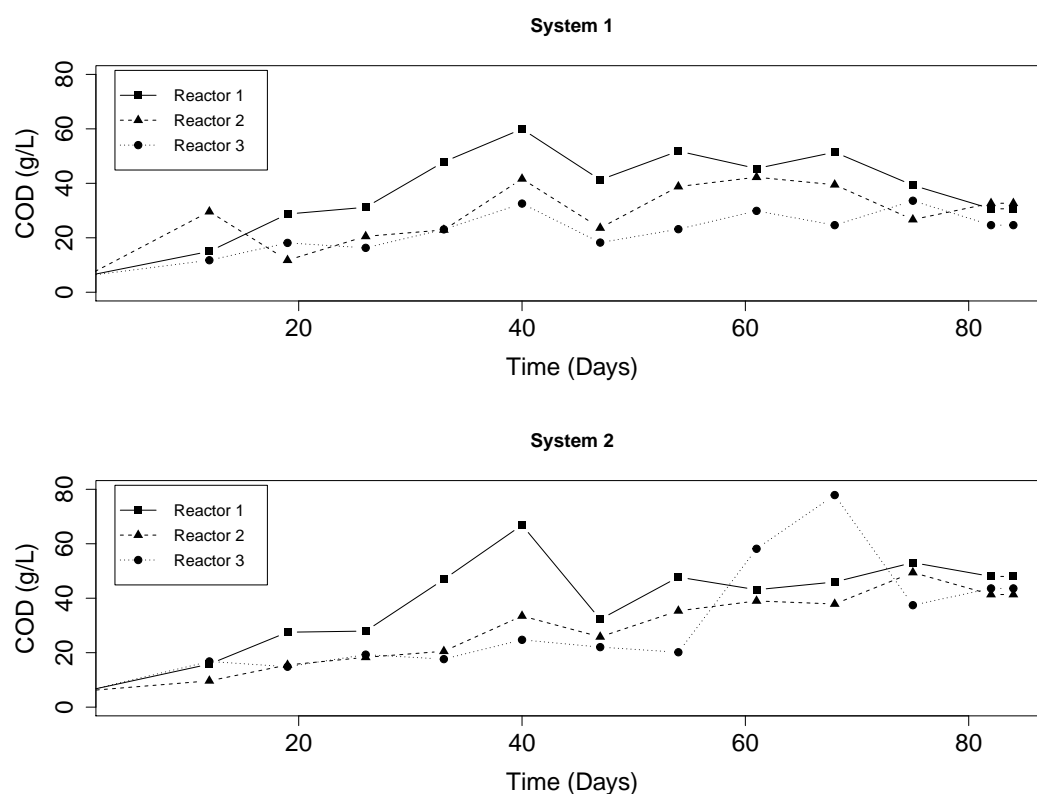


FIGURE 7.4: Concentration of Chemical Oxygen Demand in the laboratory scale FAD reactor

The ramp-up period of this laboratory scale experience lasted 43 days and was

shown to be sufficiently long to promote mature biofilm development (Fig.6.1). The methane production obtained from all the reactors is shown in Figure 7.3. From this graphs it is possible to visualize the rapidly increasing trend of gas production in both of the systems during day 7 through to day 50 of the experiment. It is clear that both of the systems achieved a similar production at the end of the ramp-up. However, there are evident deviations in the production curves. In particular between day 17-20, there was a leak in the first reactor of system 1 that interfereed with measurement of the total biogas produced from this reactor in that period. For this reason, the behavior of the methane production of system 1 seems to deviate from that of other system 2. However, every other parameter that was quantified during this experiment revealed a similar trend between the parallel systems. For example, the pH and TS measurement (Figure 7.5 and Figure 7.6 respectively) demonstrate comparable behavior between the systems fed with an analogous regime.

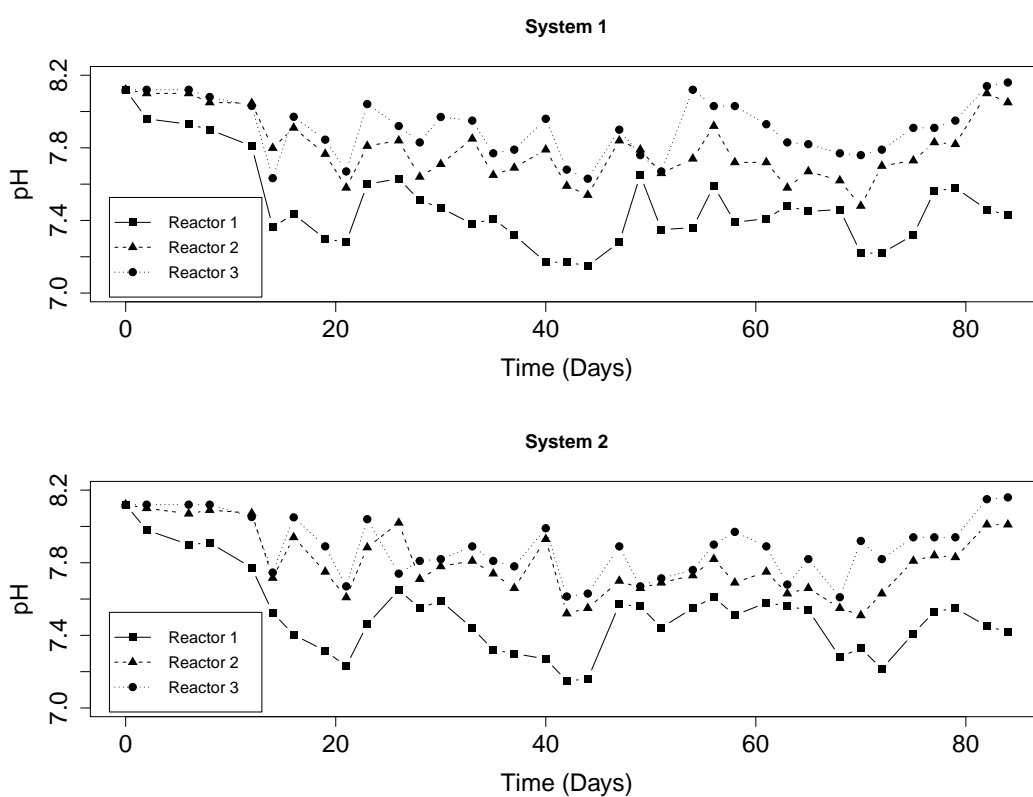


FIGURE 7.5: pH in the laboratory scale FAD

During the ramp-up phase, almost all the biogas production was emitted from the first reactors of the cascades. This is evident in the period when the flow rate was at 10 days HRTs or higher. As early as day 28 of the experiment (8 days HRT), some of the organic potential started to become noticeable in the second reactors of both systems because these reactors started producing nearly 10% of the total methane. This explains why the biomass and the concentrations of DNA in the biofilm in the second reactors of the cascades only increased consistently from this point forwards. However, the amount of organic material that reactors 2 and 3 receive is generally too low to influence the pH and the concentrations of TS and  $\text{NH}_3$ .

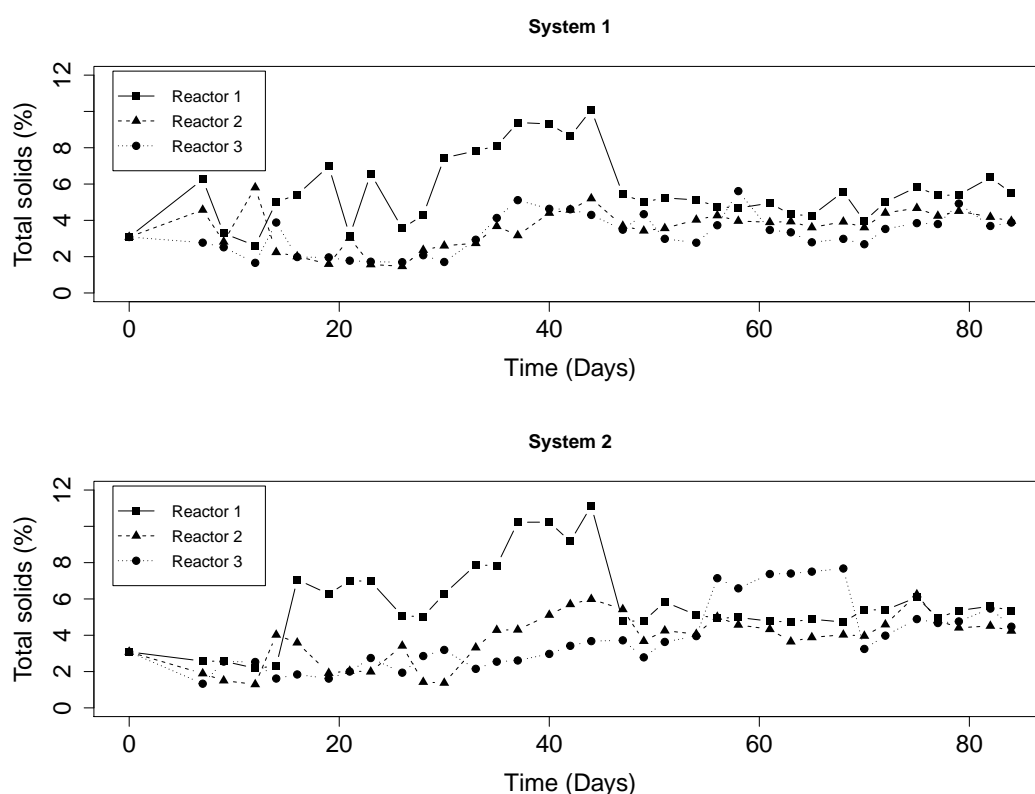


FIGURE 7.6: Concentration of total solids in the laboratory scale FAD

The pH values measured in the reactors during the experiment are shown in Figure 7.5. The first reactors of the cascade have lower pH values than the second and third reactors. The starting inoculum had a pH of 8.12. During the start-up phase, the pH of the reactors decreased steadily until reaching approximately 7.2 in the first reactors. The remaining reactors had stable pH, with values oscillating between 7.6 and 7.9. There is a direct relationship between the concentration of VFA in the reactors and the process pH. At day 50, the feed in was stopped for 5 days in both systems to demonstrate the biofilm formation and to let the reactors consume the remaining VFAs in the digesting media, that had increased dramatically. Quantification of individual VFAs in the HPLC confirmed there was a shift to propionic acid production up to  $8.3 \text{ g} \cdot \text{l}^{-1}$  in reactor 1.1 and  $6.1 \text{ g} \cdot \text{l}^{-1}$  in reactor 2.1. Accumulation of propionic acid is often described as a detrimental process imbalance in anaerobic digesters and a sign of hydrogen accumulation in the reactors (Shah et al., 2014). Since the methanogens are not able to convert rapidly enough the  $\text{H}_2$ ,  $\text{CO}_2$  and acetate, the acetogenic bacteria are not able to redirect this intermediate products that accumulates  $\text{H}_2$  into the methanogenic phase. However, the 5 days of halted feed in were enough to lower the concentration of VFAs in reactors 1.1 and 2.1 to amounts comparable in the other remaining reactors (Fig. 7.7). The total solids also decreased to a comparable level (Fig. 7.6). The flow rate was then re-established at day 56 from 0 to 2 liters per day (HRT 4 days) to commence the steady state phase.

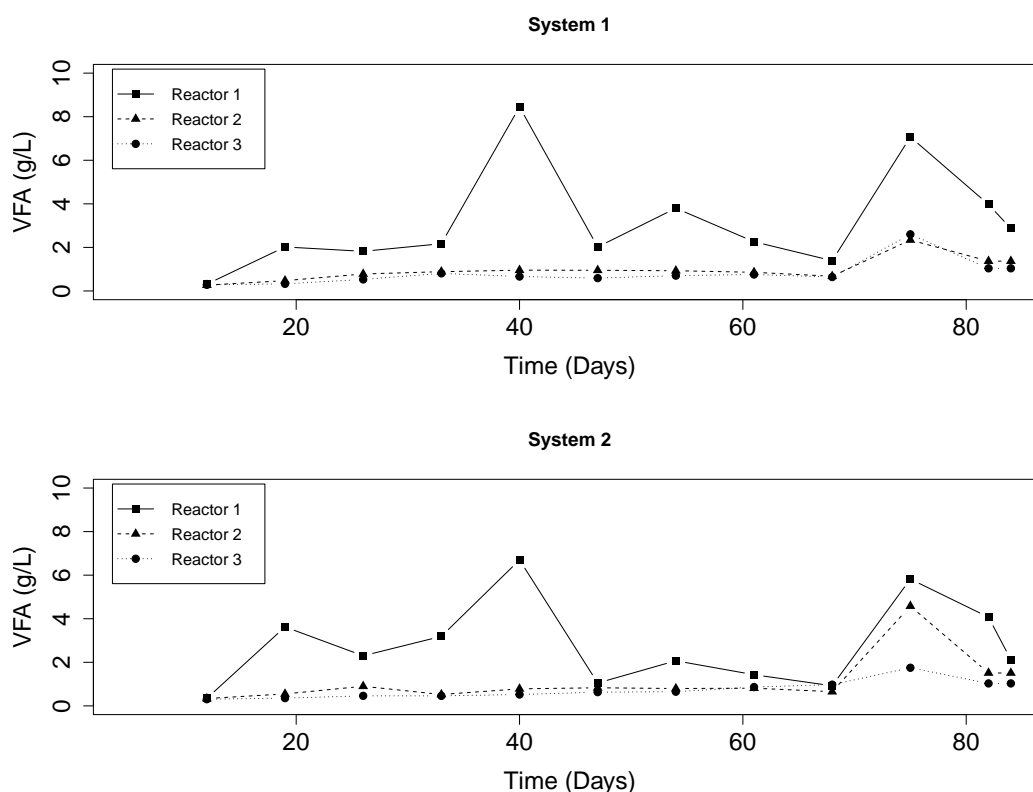


FIGURE 7.7: Concentration of Volatile Fatty Acids in the laboratory scale FAD

### Steady-state

At the steady state phase, it was possible to confirm the robustness of the communities attached to the biofilm. The concentration of solids and VFAs during this period did not exceed  $4\text{g}\cdot\text{l}^{-1}$  in any of the systems. This indicates that the process unbalances observed during the start-phase were overcome after the feed ceased. During the steady state, there was continuous production of biomethane to yield 321.4 and  $348.2\text{ l}\cdot\text{g}^{-1}\text{VS}$  in system 1 and 2, respectively. During the whole experiment, more than 80% of the biogas production occurred in the first reactors of the cascade. Therefore most of the transformation processes occurred in a small fraction of the laboratory systems, and most of the capacity was still unexploited despite the high flow regime. Throughout the whole of the mesophilic phase of this experiment, the total ammoniacal nitrogen was lower in the first reactors of the systems and was measured to average  $530\text{mg}\cdot\text{l}^{-1}$  in system 1 and  $546\text{mg}\cdot\text{l}^{-1}$  in system 2 (Fig. 7.8). The concentrations in reactors 2 and 3 of the cascades exhibited higher concentrations of ammoniacal nitrogen of approximately  $821\text{mg}\cdot\text{l}^{-1}$  and  $830\text{mg}\cdot\text{l}^{-1}$ , respectively. This indicates that under the steady state phase, the rates of nitrogen assimilation from the substrate by the existing microbial populations are slower than the utilization of the organics in the pretreated OFMSW to produce methane. Furthermore, the methane content in the biogas was surprisingly steady at between 59% and 62%.

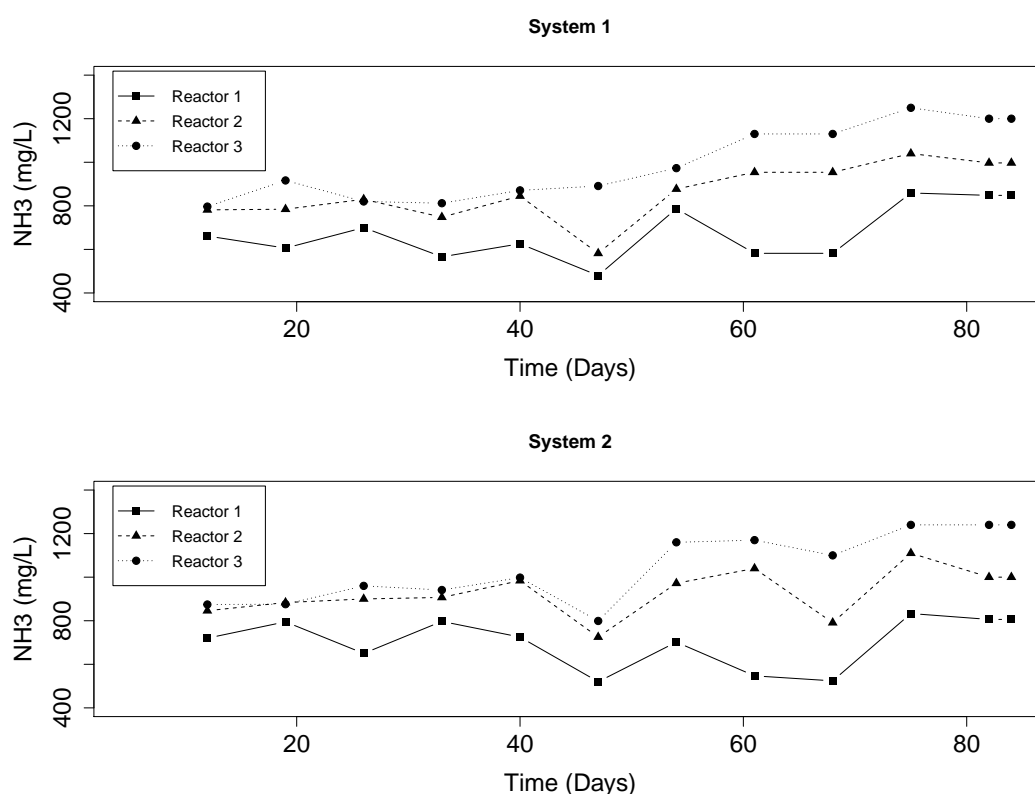


FIGURE 7.8: Concentration of Ammoniacal Nitrogen in the laboratory scale FAD

### Transition to thermophilic AD

In Chapter 2 we have discussed the implications of process temperature on anaerobic digestion processes. It was explained how the reactors usually operate at either mesophilic or thermophilic temperature ranges and also some of the implications that define such a choice from a functional and microbial perspective. The temperature range in an AD context is usually defined by the scope of a process; for example, if the substrate converted is nitrogen rich, the mesophilic range is preferred due to the increased susceptibility of microorganisms at higher temperatures and ammonia inhibition (Sung and Liu, 2003). The effects of rapid fluctuations in temperature have also been studied in detail. Ahn and Forster, 2002 for instance, elucidated the effects of temperature range variations in a digester treating simulated paper-mill wastewater. However, to our knowledge, there are no reported studies that describe the consequences of rapid transitioning within the temperature ranges over a matter of few hours or that describe the long term effects regarding performance and possible changes in microbial structure. For this reason, it was decided to conduct such an experiment and evaluate the possible consequences for the performance and microbial structure of a reactor with mature biofilm

When the steady phase was concluded, there was a rapid increase in process temperature from 37°C to 51°C. This shift lasted approximately 3 hours until the hot water recirculating through the reactor's jackets achieved the thermophilic range. From the measurements, it observed that the dramatic temperature change affected

the production only during the three days that followed the shift. During this period, the VFA concentration increased to nearly  $6.2\text{g}\cdot\text{l}^{-1}$  and  $5.7\text{g}\cdot\text{l}^{-1}$  in reactors 1.1 and 2.1, respectively (Fig. 7.7). The pH slightly dropped in correspondence with the increase in concentration of VFAs. The TS and COD concentrations also increased due to this dramatic shift. Nevertheless, the reactor was able to continue biogas production during the changes in temperature regime. After the third day, the production had already recovered and emitted slightly higher methane than in the previous phase whereas the HRT was the same. In fact, the COD balance revealed a higher conversion, in particular in the first reactors of the cascade, where most of the degradation occurs.

In this study, a fixed film anaerobic reactor processing enzymatically pre-hydrolyzed OFMSW was shown to be able to maintain high productivities even at low HRTs and high OLRs. During the whole experiment, there was low variation between the ratio of biomethane and carbon dioxide despite the steady increase in OLR and the temperature shift. Since this ratio is usually used as an indicator of the process stability of methanogenesis, it can be concluded that the shift in temperature did not alter considerably the degradation of this substrate and that the process can be driven under either mesophilic or thermophilic regime (Mata-Alvarez, Macé, and Llabrés, 2000). The average biomethane yield in the thermophilic phase was calculated to be  $356\text{ l CH}_4\cdot\text{g}^{-1}\text{VS}$  in both systems, which reveals that the production at higher temperature yields from 3% up to 10% more methane than under the mesophilic range. This result can be explained as being due to increased metabolic capacity of microorganisms at  $51^\circ$  and also to higher solubilization of the organic compounds in the digesting media.

### 7.3 Pilot scale

The results of the pilot scale experiments have been described in the publication by Londoño et al., 2018. In this section the most important outcomes of these experiments are summarized. In Table 7.2 the composition analysis of the feedstock is presented, which is similar to that used as feed in the laboratory scale reactors. As explained in Chapter 6, this pilot methodology was the first attempt to characterize the performance of the FAD reactor with its original design, which includes all the different features and in particular the compartmentalization. At the same time, a pilot scale CSTR with 500l of digesting volume was operated with the same substrate at an HRT close to 20 days in order to compare the yields obtainable with these two different technologies.

TABLE 7.2: Chemical composition of enzymatically liquefied OFMSW used in pilot scale FAD experiments.

Component	Measured value
Chemical Oxygen Demand (COD)	$101 \pm 2.6\text{ g}\cdot\text{l}^{-1}$
Soluble Chemical Oxygen Demand (sCOD)	$63 \pm 2.7\text{ g}\cdot\text{l}^{-1}$
Total Solids (TS)	$8.6 \pm 0.07\%$
Volatile Solids (VS)	$6.9 \pm 0.70\%$
Total suspended Solids (TSS)	$3.0 \pm 0.04\%$
Ash	$1.7 \pm 0.09\%$

The strategy of the pilot scale experiments deviated from the laboratory activity. In the previous section, we found that the biofilm can already develop rapidly within the first two months after inoculation. It was decided to simulate this ramp-up process over a similar time span but with a threshold of VFA concentration set at  $2\text{g}\cdot\text{l}^{-1}$ . When the measured values of VFAs in the first compartments of the FAD reactor were close to this boundary, the flow rate was decreased over the next day to avoid propionate accumulation in the digesting media. This strategy explains the irregular trend in the HRT in Figure 7.9. From this graph, it is also possible to observe the evolution of methane production in the two experimental phases, that is from the ramp-up and down to 10 days HRT (approx. day 0-60) and then from a further increment in the organic load from day 60 through to the end of the experiment.

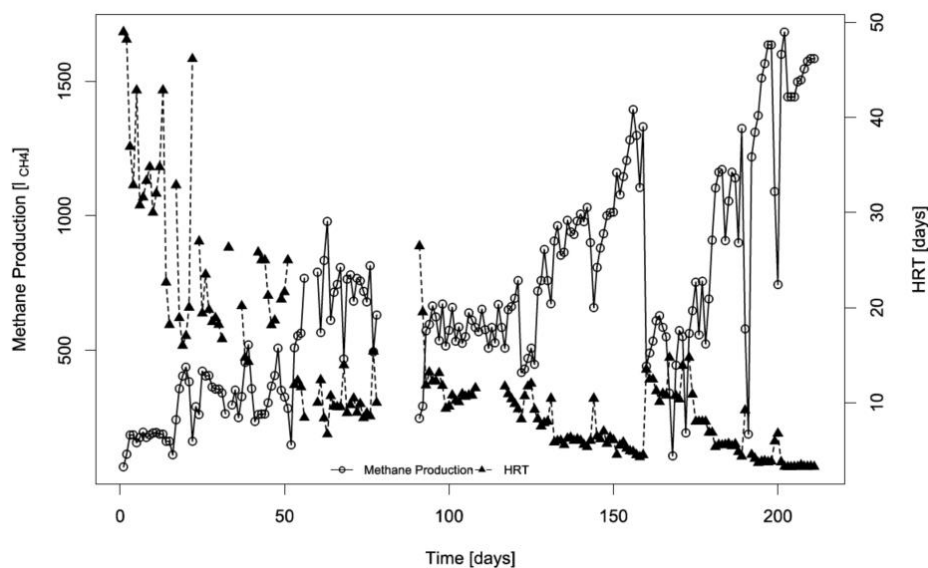


FIGURE 7.9: Methane production and HRT during the pilot scale experiment

In the day 56 of the experiment, the flow rate regime had already achieved 10 days HRT. Within this period, the methane production had followed correspondingly the feed-in as it can be deduced from figure 7.9. Thereafter, the flow was increased to determine the maximum achievable productivity levels of the reactor. Figure 7.10 shows the methane yields and volumetric productivity in every point of the experiment. As expected, it is evident that the yields slightly decrease as a consequence of lower HRTs. Indeed, the organic materials have less time to be degraded and there is flush-out of unconverted potential and microorganisms that are not immobilized. Surprisingly, the yields did not drop considerably in comparison with those recorded at the lowest flow rate regimes. In fact, almost at every point of this experiment, the methane yields were higher than  $0.2\text{l}_{\text{CH}_4}\cdot\text{gCOD}^{-1}$ . Furthermore, the uppermost volumetric productivity recorded in the FAD reactor reached values of  $6\text{-}7\text{ l}_{\text{CH}_4}\cdot\text{l}^{-1}_{\text{reactor}}\cdot\text{day}^{-1}$  at HRTs of 5 days and below. Beyond this point, every increase in the flow rate did not result in a consequential increment in the productivity and there were recorded pH levels of the digesting media close to 7. This possibly hampered the methanogenic performance which then impeded the reactor to produce larger amounts of biogas. In order to counteract such drop in the pH under the methanogenic optimum, different amounts of NaOH and  $\text{Ca}(\text{OH})_2$  were pumped



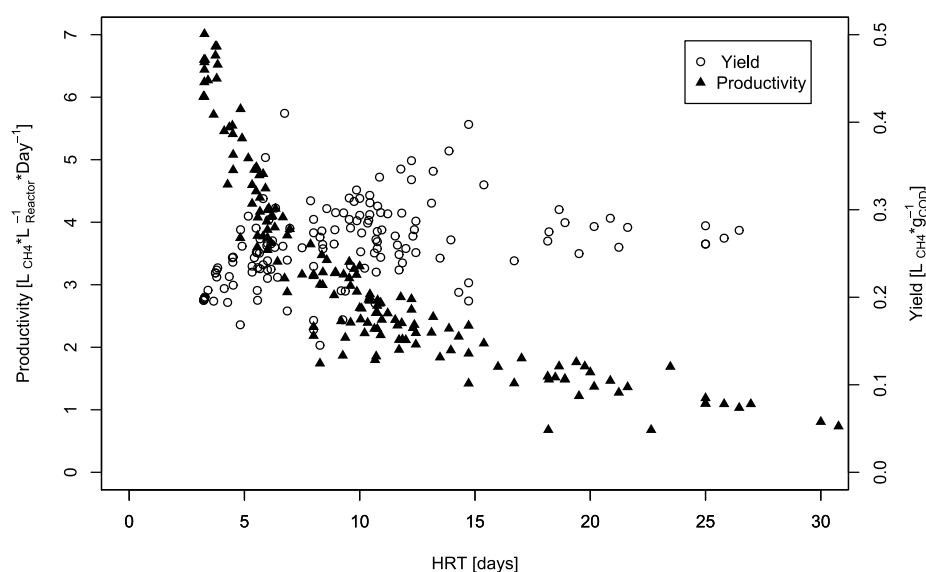


FIGURE 7.10: Methane Yields and Volumetric productivity of the Pilot scale FAD

to the reactors together with the feed. The FAD reactor was able to continue high methane production for more than 2 weeks at HRT below 3 days, as long as the pH was controlled. Unfortunately, from this point on, it was not possible to sustain the process any longer with strong base addition and the concentration of VFAs escalated up to  $13\text{g}\cdot\text{l}^{-1}$ . The reactor was not able to degrade the remaining VFAs in the liquid, even after ceasing the feed-in for few days. Therefore it was decided to empty the FAD and re-inoculate with some of the digestate that has been previously produced at the higher retention times. Replacing the digesting media with high levels of Na and Ca was enough to bring back the performance of the FAD reactor. This experience points out towards two important observations: (1) The biofilm remains active even after short exposure to atmospheric  $\text{O}_2$ , as the dense microorganism in the biofilm were able to rapidly bring back the methane production after the digestate was reintroduced. (2) High levels of cations affect the methanogenic step of AD. For this reason, it was decided to study the influence of cations in the last phase of AD (Chapter 10).

To compare the performance of the FAD and the CSRT, the methane yields of the conventional digester has been measured over a period of 2 months, in operation at an average HRT of 23 days. The inoculum used in the CSTR was a combination of FAD digestate and approximately 50 liters of granular sludge from a waste water treatment plant in the Netherlands. The methane yields over this period in the CSTR and HRTs are shown in figure 7.11. The average biomethane production in the CSTR reactor was calculated to be  $0.246 \pm 0.315 \text{ l}_{\text{CH}_4} \cdot \text{gCOD}^{-1}$ . In turn, the FAD reactor yielded  $0.266 \text{ l}_{\text{CH}_4} \cdot \text{gCOD}^{-1}$  at a comparable flow rate, which indicates that the biofilm containing reactor is able to produce approximately 8% more methane than its counterpart. For the most part, the biogas produced by both of the reactors contained methane concentrations between 59% and 62%. To sum up, there was perceived a higher methane production from the FAD reactor at some of the highest HRTs tested. It is expected that this difference becomes larger at higher flow rates.

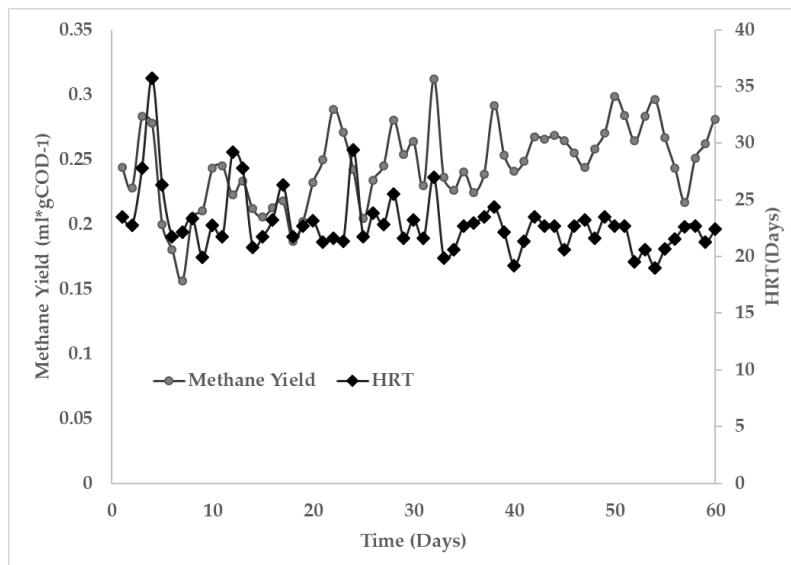


FIGURE 7.11: Methane Yields and Volumetric productivity of the Pilot scale CSTR

## 7.4 Conclusions

In this chapter has been discussed the performance and operation of the FAD reactor. The laboratory scale was an important experiment to learn about the start-up of the system, in particular to improve our knowledge regarding the biofilm formation in the early stages. It was realized that the maturation of a biofilm in an AD process treating liquefied OFMSW can be accomplished in a period shorter than 2 months by gradually increasing the OLR and preventing the accumulation of VFAs. Furthermore, we have seen that the process can be held in both temperature ranges and the obtainable yields may be higher when operated at the thermophilic range. Distinguishably, the temperature shock did not affect the progression of the digestion processes, which could indicate that the biofilm prevents the microbial communities to suffer from environmental stress.

The pilot scale experiment have shown that the different design features of the FAD reactor can be useful to intensify the anaerobic digestion of enzymatically treated of MSW, in particular when a comparison is made with traditional reactor designs. In this experience, we were able to obtain high COD conversion at high organic loading rates of  $20.8 \text{ gCOD} \cdot \text{l}_{\text{reactor}}^{-1} \cdot \text{day}^{-1}$ . This is a remarkable observation since most studies in performed with MSW achieve halve this amount (Hartmann and Ahring, 2006). Furthermore, it was possible to explore the limits of the FAD productivity and to realize that low pH posses a threat towards the methanogenic conversion. Operation of this AD process at pH values close to 7 can lead to acidosis in the digesting media. Even though it was possible to temporarily remediate with addition of strong bases, the induced raise in the concentration of cations can lead towards an even slower conversion process.



## Chapter 8

# Microbial Diversity in Fast Anaerobic Digester

7

### 8.1 Microbial Diversity

The important role that microbial communities play in AD processes has become evident in Chapter 2. To reveal the microbial structure in the reactors doesn't only allow us to understand the possible metabolic pathways that take place during the degradation of a particular substrate, but can become an important tool to monitor and optimize the process, while preventing detrimental unbalances. This is particularly important in the scope of this project since the stationary fixed biofilms have been studied less than the granular immobilizations. The hardest task to unravel the microbial diversity in AD processes is actually the difficulty to isolate single species within the whole communities and this is due to the particular growth requirement of many strict anaerobes. Thus, it has been necessary to implement strategies that do not depend on traditional cell cultivations. A number of cultivation independent techniques have been developed to facilitate this task and can provide with important information like temporal changes in the microbial structure and even to point out main species involved in the conversion processes. To mention some of this techniques, there is denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), real-time PCR and other clone library approaches (Pap et al., 2016). Further advances of the above mentioned techniques make usage of high-throughput genomic sequencing. Nowadays, sequencing strategies consent to study and identify genomes in complex mixed communities (metagenomics), to study their gene expression (metatranscriptomics) or the protein synthesis (metaproteomics). However, a more simplistic approach can be pursued through the 16S rRNA gene profiling, which exploits the highly conserved ribosomal RNA genes. Although evolutionary stable, these sequences present 9 hypervariable regions that can be used to discern between microorganism down to a specie levels (D'Amore et al., 2016). In fact, the analysis of the 16S rRNA gene is used for reconstructing phylogenies within the study of in the evolution of living organisms and for microbial characterization within a community.

An alternative to 16S rRNA profiling is to study nearly complete genomes assembled from shotgun sequenced metagenomes (Albertsen et al., 2013). Even though it is now possible to obtain full genomes from uncultivated species, it still remains a difficult task to assemble them when the samples derive from complex populations, especially since the binning of the obtained sequences is impeded by amplification biases and manipulation of the data can become compute intensive. In the present

work, the microbial analysis relies on the amplicon study of the 16S rRNA gene. Despite the results deriving from this type of analysis is known to be influenced by the impact of primer choice and coverage rate over the distinct hypervariable regions, it poses itself as an important first approach to unravel the dominant microbial player in the FAD process.

The microbial diversity of the laboratory scale reactors has been described in Paper 2, Chapter (11). The samples from both the sources (biofilm and digesting medium) for each of the cascade reactor of system 1 and system 2 were systematically taken during the sampling days (Fig. 6.1). It was possible to obtain enough DNA through a single extraction from every sample before the first amplification. A general remark, the concentration of DNA in the biofilm extracts were generally higher than those extracted from the medium (Figure. 7.2). This confirms that the biofilm samples have more cells than its counterpart.

The microbial diversity of every sample analyzed is shown in Figure 8.1. On top there is the diversity of the bacterial communities present in all the reactors. From this figure, it is possible to notice that most of the microorganisms retrieved in the process are yet to be classified. As mentioned earlier, this is a common problem encountered during the analysis of microbial populations present in AD systems. The difficulty to isolate the anaerobes has made it hard for taxonomists to classify them properly into the lower ranks. However, based on similarities towards other known microorganisms, it was possible to assign them into different microbial groups. In regard to the population of *Archaea*, the most common species present in the reactors were determined. This indicates that the utilization of methanogen specific primers was a useful strategy to categorize the main archaeal species present in the process.

The most abundant bacteria found during the digestion of enzymatically treated OFMSW in the mesophilic range are a core of microorganisms consisting of Uncl. Bacteria, Uncl. *Bacteroidetes*, Uncl. *Clostridia*, *Cloacamonas* and a member of *Porphyromonadaceae* (Fig. 8.1). Most of these are taxonomically undefined, but have been previously retrieved in other anaerobic environments, more in particular in biogas plants. For instance, members of the *Bacteroidetes* have been found to be typical constituents of anaerobic digesters treating lignocellulose rich materials (Sun et al., 2016b). The most abundant methanogens during the mesophilic phases are *Methanoculleus bourgenis*, *Methanoculleus palmolei* and an unclassified *Methanosarcina*. Both the populations of bacteria and methanogens during the mesophilic phase remain stable in time. The populations present in the different reactors of the cascades are also similar. This is an indication that the compartmentalization of the FAD reactor would not induce a specialization of the distinct microbial groups during digestion of enzymatically treated OFMSW. Furthermore, during the sampling days where the HRT of the process was lower (higher OLR), there is a larger component of a known acidogens member of the *Porphyromonadaceae* Family (Hahnke et al., 2015).

The results from Figure 8.1 could indicate there are few differences between the communities present in samples withdrawn from the biofilm and those in the sludge. However, if taken into consideration both the abundance and evenness of the species present in those communities (Shannon Index; Figures 8.2 and 8.2), it is possible to point out some dissimilarities. The bacterial communities in the biofilm are richer than those in the digestion medium. This is perceived in every sample taken during the experiment. In Figure 8.2 there are larger amounts of "other" members of the communities in the biofilm samples. These are low abundant but diverse members of the entire populations. In the contrary, the diversity of methanogenic populations are comparable in every sample taken during the mesophilic range. Particular species of methanogens may take over in certain periods. For example, the *Archaea*

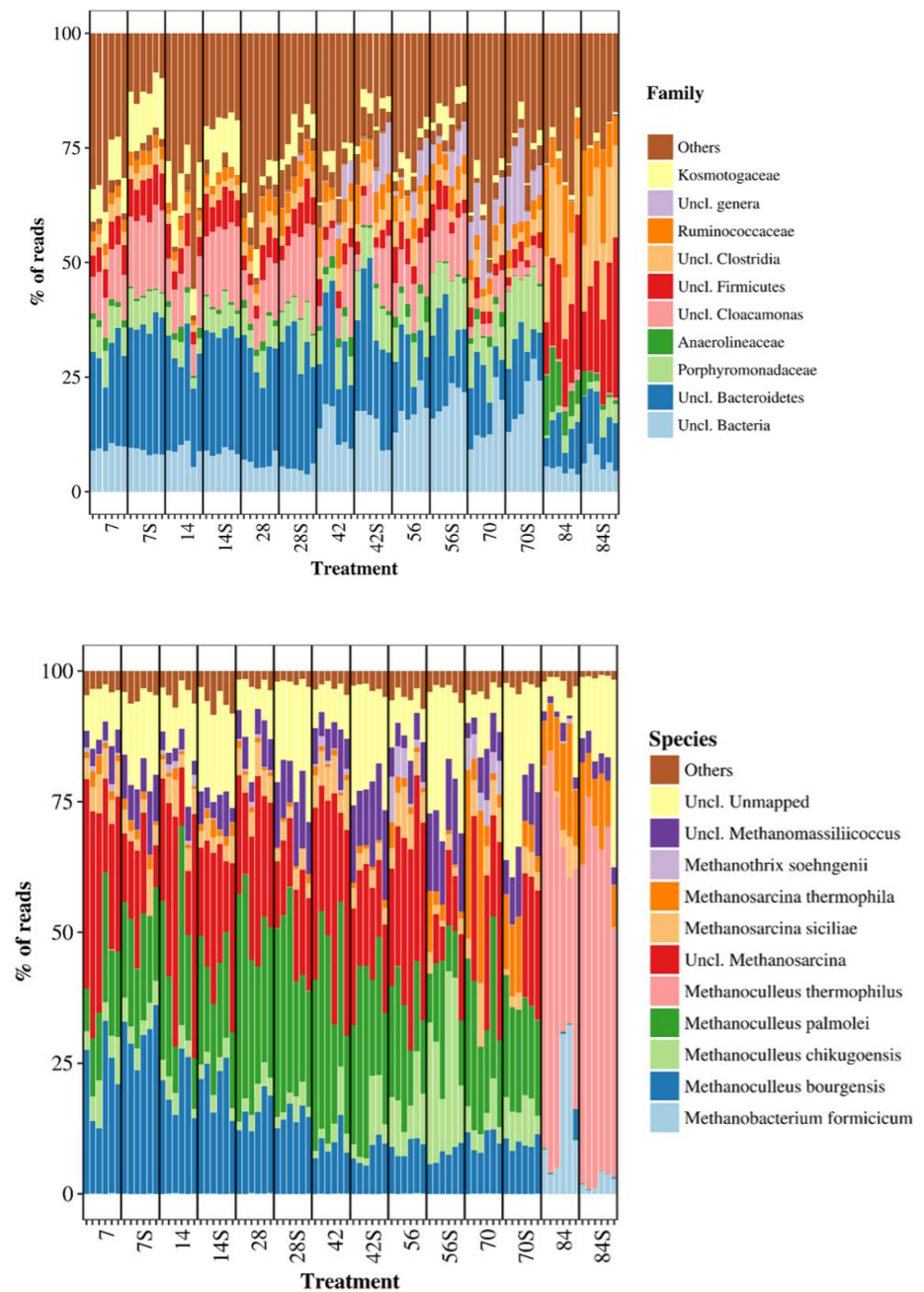


FIGURE 8.1: Microbial diversity over time for biofilm and sludge samples. Top: Bacteria; Bottom: Archaea. Treatment names: The number indicates sampling day; samples without "S" are biofilm samples. Samples with "S" are Sludge samples. For each sampling time, there are 6 bars: from left to right the three reactors in system 1 and thereafter system 2

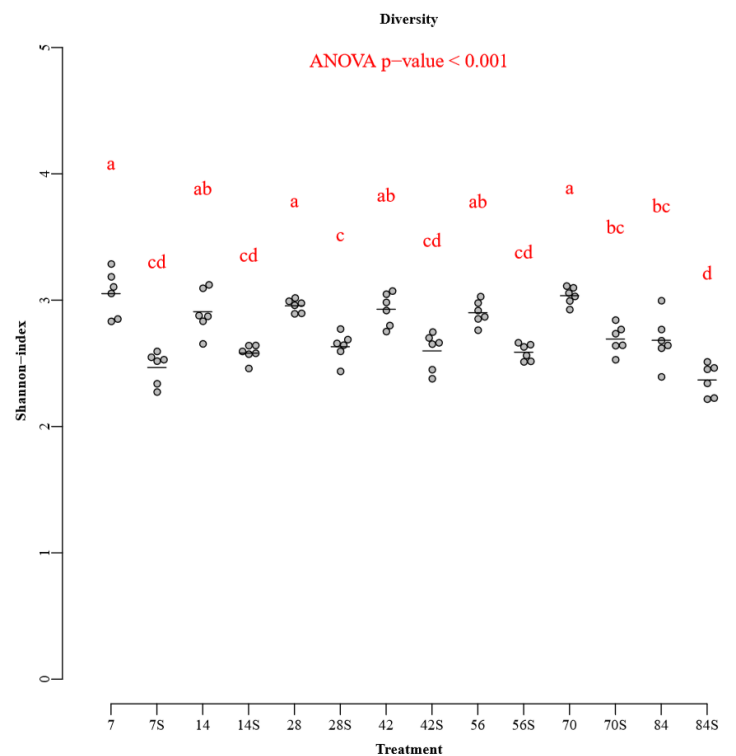


FIGURE 8.2: Shannon Index of Bacterial populations

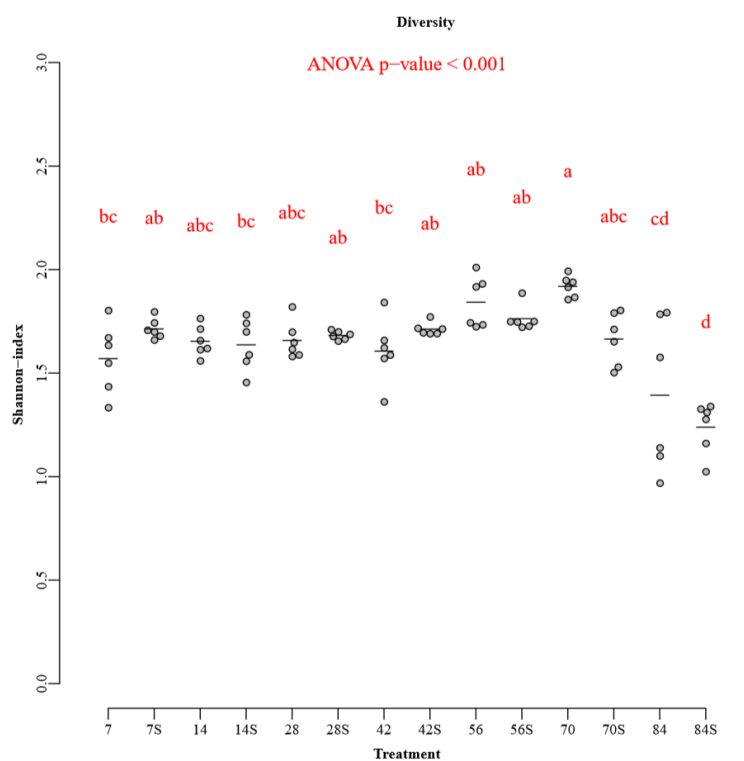


FIGURE 8.3: Shannon Index of and Archaeal populations



*Methanoculleus Chikugoensis* appears to be favored by high organic loads in that temperature range. Despite these differences, the structure of the archaeal communities remains at comparable levels.

### Microbial shift during temperature shift

The largest change in the microbial diversity of both biofilm and sludge samples of all the reactors of the cascades is perceived after temperature transition between the mesophilic to the thermophilic range. Even though this rapid transition did not have a huge impact on the performance of the reactors, the populations of both methanogens and bacteria resulted heavily modified. The clearest visualization of this effect is probably that of Figure 8.4, where it is evident that the microbial populations dramatically change in the samples 84 and 84S. These results are in agreement with those studies that try to elucidate the effects of temperature changes during anaerobic digestion processes. Members of the Families *Clostridia* and *Firmicutes* are the most abundant bacteria during digestion at 51°C. In fact, more than 50% of the operational taxonomic units after the transition have been attributed to these families. Furthermore, all the reactors were colonized by *Methanoculleus thermophiles* and *Methanosarcina thermophila*. In agreement with their given taxonomic names, these species of methanogens have been already retrieved in other thermophilic samples. The presence of *Methanoculleus spp.* indicates that the fixed biofilm reactors produce methane through the hydrogenotrophic pathway when the operation is held at in the thermophilic range. Even though the concentration of ammonia significantly increased during the thermophilic operation, it is not possible to correlate this factor to the microbial specialization.

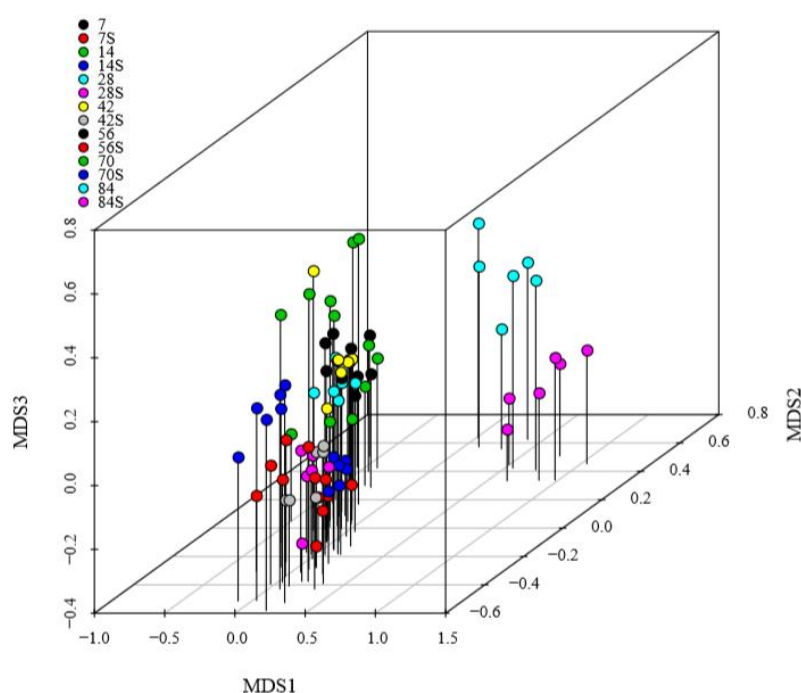


FIGURE 8.4: Three dimensional non-metric multidimensional scaling (3D-NMDS) ordination of all the populations



The results from the DGGE have shown that there is a higher proportion of *Archaea* in the first reactors of the cascade than in the remaining two reactors. This indicates that even though there would not result a specialization of the microorganisms in the different compartments due to substrate composition, the methanogenic *Archaea* can be promoted by high concentrations of organics. In fact, the DGGE results show there is at least twice the amount of *Archaea* in the first reactors of the cascade where the methanogens constitute more than 15% of the total populations. These results should be re-confirmed through other quantifications techniques such as qPCR.

## Chapter 9

# Effects of inoculum composition on AD of enzymatic treated OFMSW

8

### 9.1 Overview

From the characterization of the microbial communities in the FAD reactor it appears that the microorganisms in the biofilm are more diverse and less prone to variations when compared to their "free floating" counterpart. This is particularly relevant since the reactor was able to continue methane production during moments of environmental stress or after dramatic fluctuations in the flow regime. Even though it doesn't seem to be a high level of microbial specialization within the different compartments probably due to high hydrolysis extent of the substrate, it was possible to observe a certain degree of "elasticity" within the communities, that is the ability to rapidly adjust to different conditions or return to a previous state after a perturbation has been made (De Vrieze et al., 2014). Remarkably, it was possible to establish a noticeable trend of the microbial communities that followed nicely variations in the flow rate during the mesophilic phase. It was also observed a rapid reorganization of the communities when the temperature regime was shifted. However, most of the taxonomically different groups relate to the original inoculum before the maturation phase. For this reason, it was decided to study the influence of the original seed material in a AD process during the degradation of enzymatically treated OFMSW.

Functional redundancy or functional equivalence could be understood as the ability of multiple species that constitute diverse taxonomic groups to perform correspondent roles and functions in a determinate ecosystem (Louca, Parfrey, and Doebeli, 2016). It well known that distantly related microbes can often perform similar metabolic functions, which makes it a tough task to interpret the variations within distinct taxonomic groups (Louca et al., 2018). Nonetheless, it was decided to elucidate the anaerobic digestion of enzymatically treated OFMSW starting with two distinct inocula and to quantify the performance of the reactors at different HRTs. This experiment was performed in two CSTR that were inoculated with digestate from the pilot FAD reactor that was degrading enzymatically treated OFMSW (CSTR1) and with inocula from a full scale biogas plant that converts non treated source sorted OFMSW in March, UK (CSTR2). More details about the strategy is described in chapter 6. The two CSTRs were seeded and operated for two entire retentions at each flow rate regime as shown in figure 9.1. The samples for DNA extraction and analysis were taken at the end of each period when the digesting media had been replaced twice. In the moments of transition between HRT, there was a moderate increase of the flow rate in order to minimize ecological drifts.

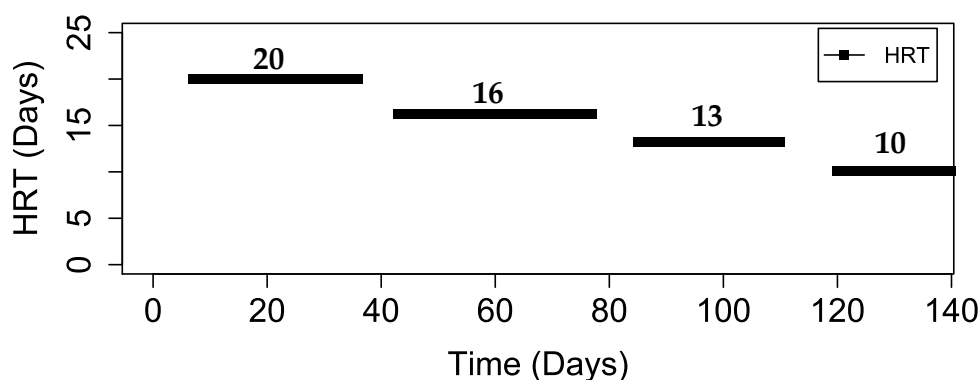


FIGURE 9.1: HRT during AD of enzymatically treated OFMSW in CSTR .

### Enzymatic Treatment of Municipal Solid Wastes

During anaerobic digestion of MSW, the hydrolytic phase is considered to be the rate-limiting step of the conversions. Modeling of these processes have determined that this phase is governed by first order kinetics with respect to the remaining biodegradable particulate substrate (Veeken et al., 2000). These limitations are most likely due to the low processivity of bacterial hydrolytic enzymes to depolymerize biomass, especially in anaerobic environments. Furthermore, the hydrolytic phase depends on different steps (1) enzyme transfer from the bulk aqueous surface to the organic particles (2) adsorption of the enzymes and formation of the enzyme-substrate complexes (3) hydrolytic reactions (4) transfer of the reaction products to the bulk aqueous phase (e.g. cellulose)(5) hydrolysis of the remaining materials (Chatterjee and Mazumder, 2019). The hydrolysis rates are heavily dependent on the population of hydrolytic microorganism. In fact, a correlation has been found between the degradation rate and the composition of the cellulose degrading communities (Liu et al., 2017). In the organic fractions of MSW there is a high portion of lignocellulosic biomass deriving from households or other commercial activities, therefore it is expected that the AD of these materials rely strongly on the performance of these type of communities.

The potential and functional routes of hydrolytic enzymes implementation during the anaerobic degradation of OFMSW has been recently reviewed by Chatterjee and Mazumder, 2019. Their main findings point out that addition of cellulases significantly increase the solubilization of COD and there results an important drop in the pH following the hydrolysis reaction. These observations can be useful to intensify AD from OFMSW since it would be possible to skip many of the rate limitations imposed in the first phases of degradation. Furthermore, the enzymatic treatment of waste is a biological process that promotes separation of organic material such as cardboard and paper from other solid or non degradable fractions (Wagner et al., 2010). From a process perspective, the liquefaction of waste can also facilitate the

use of the degradable fractions since pipes and the equipment would be less prone to problems such as clogging or excessive sedimentation of suspended particles. Furthermore, the impact from a pre-treatment using enzymes prior to AD may also pose environmental advantages in contrast to the commonly employed mechanical and chemical strategies. Hence, this experience can provide useful information regarding the usage of enzymes prior to AD.

### Importance of the inoculum

The initial inoculum is an important factor for a biogas process degrading lignocellulose rich materials such as OFMSW and manure (Liu et al., 2017). Different studies have suggested that the microbial structure of the hydrolytic communities are dependent on the original populations that were present at the beginning in the start-up phase. In particular, members of the *Firmicutes* and *Bacteroidetes* have been found to shape most of the microbial population during these processes since members of these phyla possess broad metabolic abilities, in particular for cellulose degrading enzymes production (Li et al., 2009). In contrast, the study of anaerobic degradation of already hydrolyzed substrates such as distillery effluents or vinasses (ethanol rich) has revealed a lower composition of hydrolytic bacteria within the populations (Pereyra et al., 2010). It is possible that breaking the barriers established during the first phase of AD relieve the selective pressure of hydrolytic populations since the process does not rely as heavily on initial depolymerization of complex materials. In regard to the archaeal populations, it has been shown that the both the populations and abundance of methanogens that constitute the original inocula have deterministic effects over the methane production rates of the process (De Vrieze et al., 2014 and Liu et al., 2017). In this study, the hydrolytic phase is supported by supplementation of hydrolytic enzymes to a stream of OFMSW. The consequential effects over the resulting populations are studied at different HRTs, especially in regards to those deriving from two distinct inocula.

## 9.2 Results and discussion

### Enzymatically treated OFMSW

The chemical composition of the enzymatically liquefied OFMSW used in this experiment is can has been described in table 7.1. A large fraction of the VS can be retrieved as already hydrolyzed and fermented VFAs and a similar composition of the substrate has been already shown in Uller, 2016. The main constituents of the VFA pool are lactic acid and acetic acid, concentrations of  $28.2\text{g}\cdot\text{l}^{-1}$  and  $2.52\text{g}\cdot\text{l}^{-1}$  have been measured respectively. Approximately 30% of the TS in the substrate is non already solubilized material, of which almost a third is non digestible ash. This results show that most of the liquefied OFMSW has already been through the hydrolytic phase. Hence it should not be expected a high pressure on the hydrolytic communities during degradation of this substrate.

### Performance of the CSTRs

The methane production of both CSTRs inoculated with different seed materials is shown figure 9.2. Both reactors were inoculated as intended and there was perceived gas production during the first day of feed in. The level of production during the first 5 days is comparable in both of the reactors, which suggests that both posses active

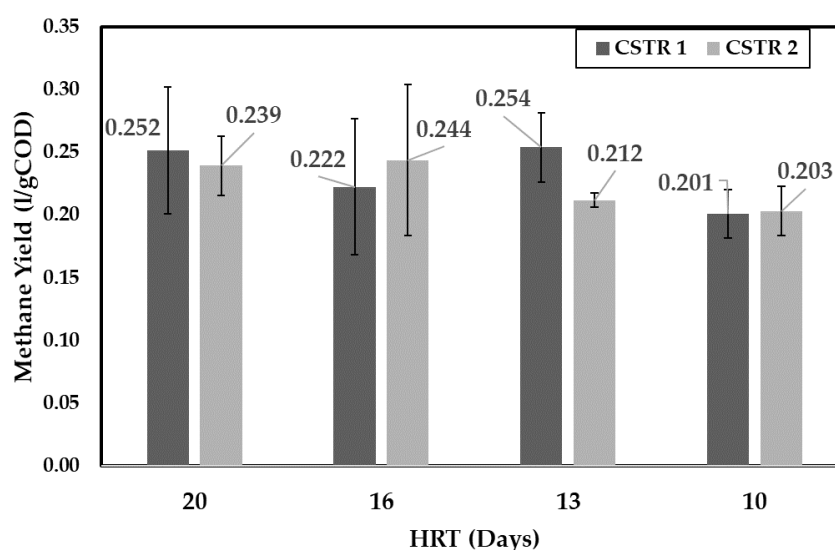


FIGURE 9.2: Methane yields at different HRTs during AD of enzymatically treated OFMSW in CSTR .

methanogenic population ready to convert the substrate into methane. The average yields produced during the first 40 days of experiment (20 days HRT) are shown in the graph. In this period, there appears to be a slightly higher production in CSTR1, which was originally inoculated with digestate that had been already acclimatized to the substrate. It possible that the pre-conditioning to the feed has already specialized the microbial populations for conversion to this substrate which were then ready to carry out the digestion processes, thus less energy would be directed to build up the metabolic functions. In the beginning, there were possibly proportionate amount of microorganisms in both inocula since the VSS concentrations were measured to be similar (Fig. 9.3). In this situation, the concentration of VFAs quantified in both of the CSTRs were different. In the CSTR1, the VFAs did not exceed  $1\text{g}\cdot\text{l}^{-1}$  during the first 40 days of the experiment, while in CSTR2 there was encountered an initial pool of  $2.3\text{g}\cdot\text{l}^{-1}$  which then decreased at the end of this period.

During the digestion at lower HRTs, there seems to be a slight decrease in the methane yields in both CSTRs. However, statistical analysis of the results shows that there is not a significant difference between the productivity of the CSTRs or within their respective yields at HRTs of 20, 16 and 13 days. However, the methane yields in both CSTRs decrease at HRT of 10 days. This observation can be explained with the fact that the microbes have halve the time to achieve complete conversion perceived at an HRT of 20 days and since it is expected to experience flush out of the active populations already at higher flow rate regimes (Hartmann and Ahring, 2006). For the most part, the concentrations of VSS remained unvaried at the higher HRTs but surprisingly increased at the an HRT of 10 days. It is not possible to distinguish if the root of this phenomena if the accumulation of non hydrolyzed compounds or if the number of microorganisms actually increases as a consequence of the accumulation of acids and  $\text{H}_2$  in the digesting media. Generally, in this experience it doesn't seem to be a large effect on eventual performance of the CSTRs in regards to their original seed material.

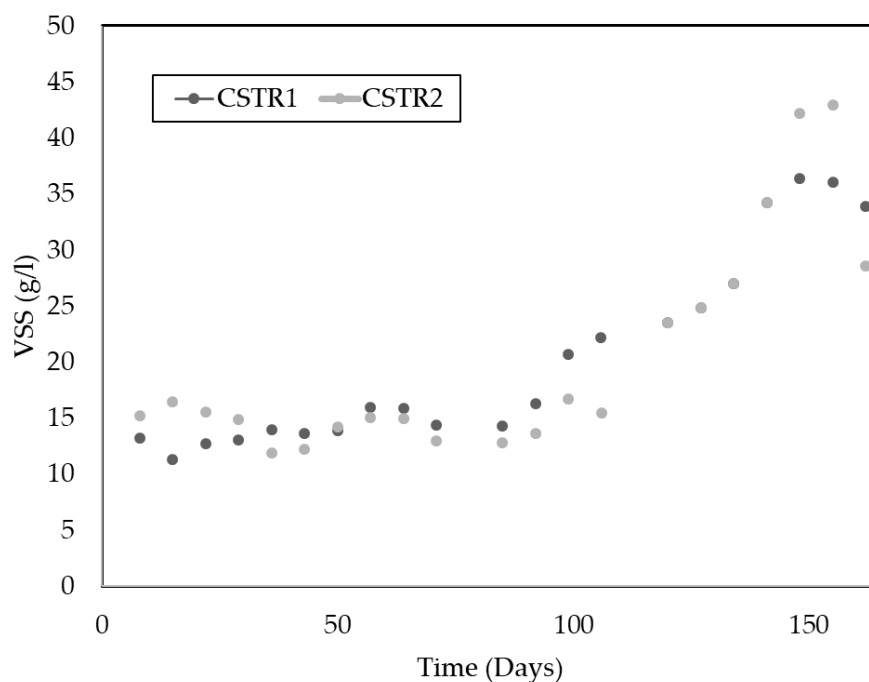


FIGURE 9.3: VSS concentration during AD of enzymatically treated OFMSW in CSTR .

### Microbial diversity

The analysis of the microbial populations in this experience deviates slightly from that in chapter 8. The primer set from Takahashi et al., 2014 were used to target contemporarily the bacterial and archaeal populations. These sequences target as well the V3-V4 hypervariable region of the 16S rRNA gene, which has been previously found very stable in regard to branch length and to have good coverage when compared to nearly full length sequences (Ghyselinck et al., 2013). This strategy consent to make a semiquantitative estimate of the archaeal and bacterial populations in the whole samples.

Figure 9.4 show the changes in the microbial diversity during this experiment in the CSTRs. The bar in the left reveals the composition of the enzymatically treated OFMSW that served as feedstock for the digestion process. It is possible to observe that more than 98% of the microbial population belongs to the *Lactobacillaceae*. These organisms are rod-shaped, gram-positive bacteria with high distribution in commercial commodities due to their ability to produce lactic acid and irreplaceable task in the manufacturing of fermentation products (Novik, Meerovskaya, and Savich, 2017). These results are in agreement with the quantification of VFAs in the substrate. Surprisingly, it is very likely that the enzymatic treatment of OFMSW induces a microbial specialization towards members of this family. This observation is different to that measured from non treated OFMSW, in which the populations have been shown to be more diverse. Members of this family belong to the *Firmicutes* phylum that as discussed in precedence are know to have fast growth rates and are metabolically important during the fermentation of simple sugars.

The results from the microbial characterization of the inocula shows that there exist a high difference between these seed materials. In the CSTR1, the inoculum that derives from the FAD process has an outstanding composition of *Archaea*, which

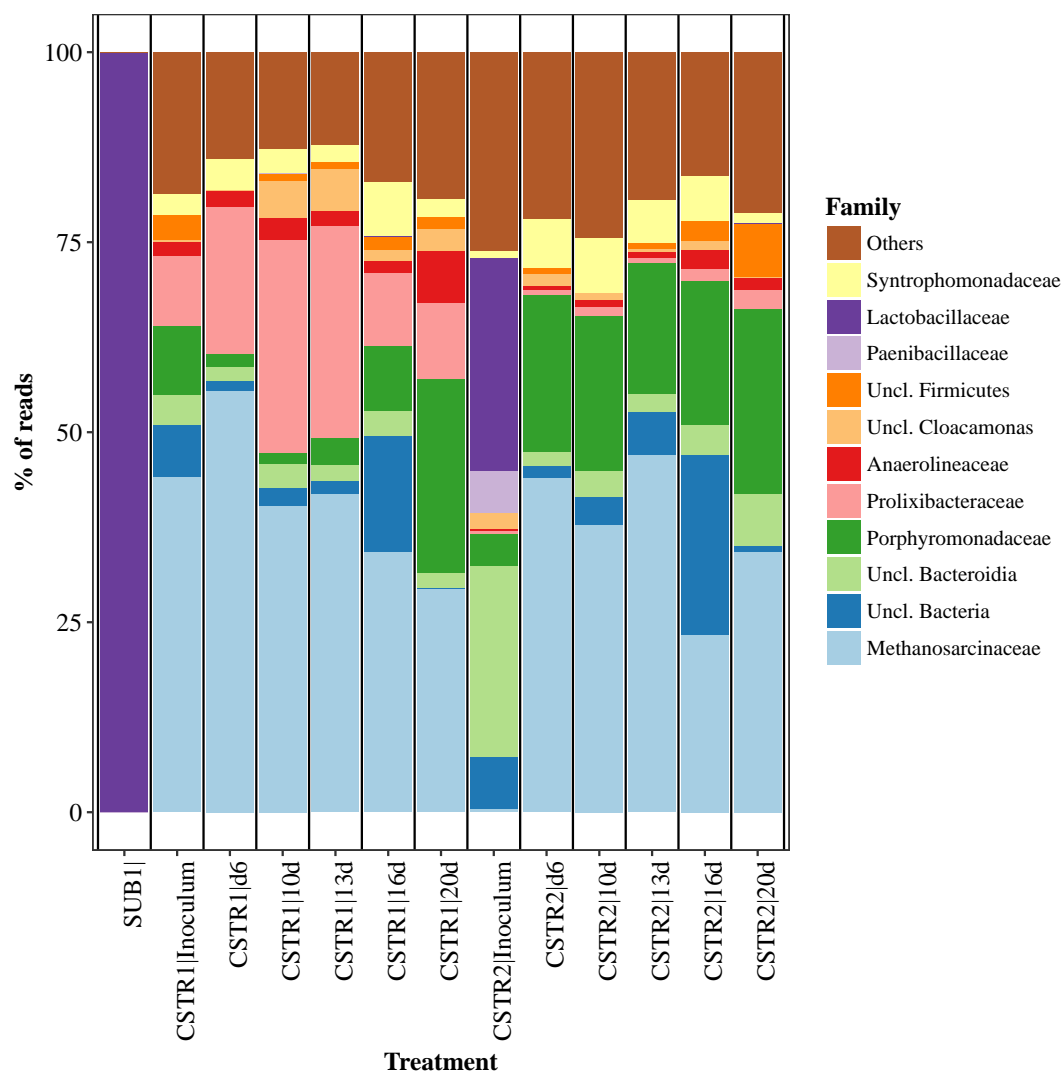


FIGURE 9.4: Microbial diversity over time for CSTR samples and substrate. SUB1: composition of the substrate;  $d$  indicates the HRT where the sample was taken.

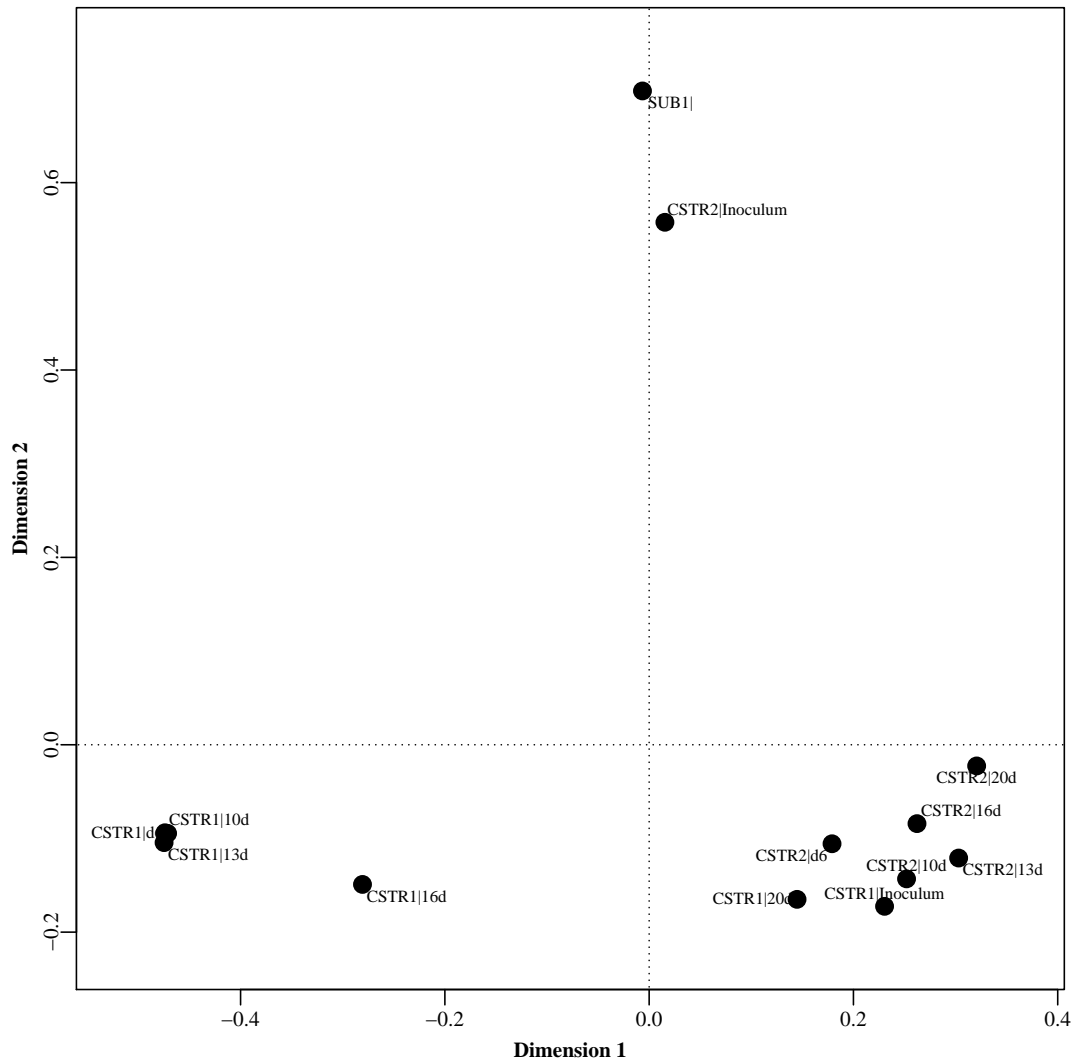


FIGURE 9.5: Non-metric multidimensional scaling ordination diagrams in microbial populations during temporal variations

account for almost 45% of the reads. In turn, the results suggest that the archaeal population in the inoculum from the OFMSW treatment plant represents less than 10% of the microbes in the complex community. Comparably, the methanogens in both of the inocula belong to the family *Methanosarcinaceae*, that are probably the most versatile methanogens retrieved in biogas plants, usually capable to perform methane production through the three methanogenic routes. In the CSTR1, the archaeal populations constitute a meaningful fraction of the entire microbial community, from 32% up to 56% of the total reads. It appears that the amount of *Archaea* slightly increases in parallel with increasing flow rates. In turn, the archaeal populations dramatically increased in the CSTR2 already after the initial two full retentions in the reactors, becoming a major constituent of the total population. There was also a slight tendency to increase when the HRT was decreased, in an analogous manner as it was observed in the CSTR1. Allegedly, the enzymatic treatment of the OFMSW enhances the populations of methanogenic *Archaea* in the reactors.

The characterization of the bacterial populations reveals outstanding differences



amongst the CSTRs. The most evident is the large fraction of members of the *Lactobacillaceae* family present in the inoculum from CSTR2. Approximately 30% of the microbial counts are lactic acid bacteria, which makes this seed material to resemble more the substrate than to the other AD samples (Fig. 9.5). The largest difference observed through the experiment in regard to the bacterial populations during the different HRTs tested prevails in the outlying communities of acidogenic bacteria. In the CSTR1, there is an important population of members of the family *Prolixibacteraceae*, while in the CSTR2 there is a predominant fraction of members of *Porphyromandaceae*. Interestingly, the *Prolixibacteraceae* accounted for approximately 10% of the total population in the inoculum in CSTR1. These microorganisms were present in CSRT2, but almost undetected. Instead, the *Porphyromandaceae* were present in both of the original inocula in both CSTR1 and CSTR2. Their presence accounted for nearly 12% and 8% of the total reads respectively. However, during the anaerobic degradation of the substrate, the populations of these bacteria belonging to separate families specialized differently in the two reactors. The difference in relative abundance of these two microbial groups in the CSTRs is the most solid explanation to explain why the samples have clustered distinctly in figure 9.5. Unfortunately, there hasn't been published yet enough information to confirm their particular roles during the degradation of liquefied OFMSW, even though it seems that members of the family *Prolixibacteraceae* may be involved in direct electron exchange with the methanogenic *Archaea* during hydrogenotrophic methanogenesis (Yin et al., 2018). Furthermore, these type of bacteria have been already identified in AD processes in situations of organic overload (Kampmann et al., 2014). Their ability to withstand and tolerate the high OLR regimes like those in this experiment could be an explanation for their presence.

### 9.3 Conclusions

In this chapter it has been possible to elucidate the effects of the inoculum composition towards the anaerobic digestion of enzymatically treated OFMSW. It was possible to observe that the enzymatic treatment has already a response on the endogenous population of the substrate. The eventual performance of the CSTRs fed with liquefied OFMSW was not affected by the precedence of the seed material. Instead, the enzymatic treatment seems to intensify the AD processes by promoting the methanogenic *Archaea*, even at lower HRT than expected. Furthermore, the ability of both CSTRs to achieve comparable yields regardless of having distinct predominant bacteria confirms again functional redundancy amongst the populations in anaerobic digesters.

## Chapter 10

# Effect of cations on Anaerobic Digestion

9

### 10.1 Background

In the previous experiments, different factors were identified that provide robust microbial communities and sound performance during anaerobic digestion. It appears that the biofilm in the FAD reactor helps to maintain the diversity of the populations of methanogenic *Archaea*, which already results in higher productivity and yields compared to traditional anaerobic digesters at high HRTs above 16 days. This result is seemingly true at different temperature regimes, even though a remarkable microbial shift was observed when a dramatic change was provoked. Moreover, it appears that the plug flow-like behavior brought about by the compartmentalization of the reactor improves the retention of degradable compounds and the microorganisms inside the process and increases the ability of the reactor to produce methane. Despite these advantages, it was also found that the performance of the reactor is at risk when the evolution of methane cannot be maintained, which results in VFA accumulation and eventual failure. For example, when the pH levels inside the reactor drop, the biogas production rate decreases to a point where the feed-in must be stopped in order to recover the process. Even though the addition of the strong bases NaOH and  $\text{Ca}(\text{OH})_2$  were sufficient to maintain temporarily the production by increasing the pH, the accumulation of these cations depressed the process to a point where it could not be recovered even by hindering the organic load. For this reason, it was decided to study the effect of cations on the methanogenic phase of AD. This is done with special focus on  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{Mg}^{2+}$ . Studying the effects of ammonia would certainly also have complement this part of the work as well, but it has been decided to narrow the scope of the present research.

During the anaerobic degradation of organic substances, discrepancies exist in the nutritional needs, growth kinetics and environmental sensitivity amongst the different communities of methanogens and acidogenic bacteria (Chen, 2008). Some of these differences were salient during the operation of the FAD but were also noted in Chapter 8, where the substrate was demonstrated to exert an important effect on the microbial structure of microorganisms despite the original inoculum. In this study, the results show that the enzymatic hydrolysis of OFMSW induces a positive effect on the growth of the above mentioned communities. It is apparent that the use of of enzymes helps to route the hydrolytic and fermentative pathways through lactate production. The result is a more thermodynamically favorable conversion for the remaining phases of AD. For this reason, a prominent methanogenic population

TABLE 10.1: Review of inhibiting concentration ranges of cations for biogas production.

Cation and $\text{NH}_4^+$	Inhibiting concentration range ( $\text{g}\cdot\text{l}^{-1}$ )	Reference
$\text{Na}^+$	IC50=5.6-53	Chen, 2008
	IC50=3-16	Feijoo et al., 1995
	IC50=4.4-26.9	Feijoo et al., 1995
	IC50=7.4	Onodera et al., 2017
	IC50=10.6-22.8	Chen, Han, and Sung, 2003
	Max inhibition 20.15	Zhang et al., 2017
$\text{K}^+$	IC50=29	Mouneimne et al., 2003
	IC50=2.1	Lo et al., 2012
	IC50=5.85	Chen, 2008
$\text{Ca}^{2+}$	IC50=3.3	Lo et al., 2012
	No inhibition at 7	Jackson-Moss, Duncan, and Cooper, 1989
	Moderate inhibition 5-7	Ahn et al., 2006
$\text{Mg}^{2+}$	IC50=1.9	Chen, 2008
$\text{NH}_4^+$	IC50=1.7-14	Sung and Liu, 2003
	IC50=4.5	Lu et al., 2018

was observed in the reactors fed with pretreated wastes. Conversely, in a situation where the OFMSW had not been preconditioned, the substrate showed a more heterogeneous pool of VFAs and compounds, which may be inhibitory to the process. Inhibitory substances have been often found to be the leading cause of AD upset and failure since the former are commonly present in substantial concentrations in wastewaters and sludges (Chen, 2008). Amongst these substances, a wide variety of compounds have been identified as being inhibitory. For example, Long Chain Fatty Acids (LFCAs),  $\text{NH}_4^+$  and cations are the most common substances described as being repressive in AD processes for different reasons (Parawira, 2012). The inhibition processes are usually brought about by negative effects on the steady-state and the methane yields, which can also be observed as an accumulation of VFAs leading to acidosis.

It would be possible to make an extensive list of publications and studies that aim to decipher the effect of cations in AD processes. These can be found as general overviews, for example in Chen, 2008, or as narrower studies that focus on the conversion of particular substrates, as in Zhang et al., 2017 and Tsapekos et al., 2019. Despite these important attempts to explain the effect of cations, five main issues with these studies may be identified. (1) None of the existing research has revealed the mechanisms of inhibition caused by cations. The most common explanation is that the detrimental effects of raised concentrations of cations in AD are due to the resulting changes in osmotic pressure. For example, in an attempt to model sodium inhibition, Hierholtzer and Akunna, 2012 state that high concentrations lead to dehydration of the methanogenic populations, which is an irreversible process that consequently leads the AD process to collapse. To our knowledge, none of the reported studies has actually verified the this argument. (2) The reported values from the inhibition experiments and the retrieved IC50 values differ immensely among the literature sources. Table 10.1 reviews some reported inhibiting concentration ranges of cations for biogas production. From this data, it is clear that the reported values vary considerably, and the ranges do not allow affirmation of the actual inhibiting concentrations of cations. A common argument to explain this phenomenon is long term adaptation of the microbial cells. Even though it would be possible to

have microorganisms adapted to digesting media with high cation concentrations, this explanation is still not convincing to explain such large differences amongst the findings. (3) The third problem with the existing studies of cation inhibition is failure to distinguish between the effects of varying concentrations of inhibitors on distinct microbial groups. It is well recognized that inhibitory cation concentrations affect diverse microbial groups differently. This could be the strongest argument to explain why it becomes an arduous task to obtain a generalized statement about the actual levels of inhibition for AD processes (Lefebvre et al., 2007). However, no general strategy has been presented for studying this aspect in detail. (4) The methodology of current research on inhibition fails to take into account the possible effects of the complementing negative ion when the cations are supplied in the tests. For example, in an attempt to study the effects of sodium on thermophilic methanogens, Chen, Han, and Sung, 2003 utilize NaCl to increase the concentrations. Though this method could be effective for deciphering AD processes with marine substrates, the general effect of increasing salt concentrations cannot be attributed solely to sodium because the concentration of its complementing anion Cl also fluctuates in the digesting media when NaCl is added. Another common mistake that can be discerned in these kinds of experimental setup is that AD processes are supplemented with additional cations as buffers in an attempt to study the effect of a particular cation. Even though many studies describe that supplementation with other cations can have a positive effect on AD processes, no comprehensive study exists that elucidates this phenomena in detail. (5) Lastly, the available research on inhibition seldom considers the concentration of cation species in the inocula used in the toxicity assays, nor the solubility of the cation specie administered in the tests. In the present study, an attempt was made to investigate the most appropriate methodology for the study of cation toxicity tests because it appears from the existing data in literature that the source of  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{Mg}^{2+}$  strongly influences the results.

During the anaerobic treatment of saline wastewater and other substrates, it has been reported that the increased salt concentrations have a greater inhibitory effect on the methanogenic population than on the other microbial communities commonly retrieved in AD. It is apparent that the threshold of cations that methanogens can tolerate is lower than that for other bacterial species. Raising Na concentrations has been shown to dramatically decrease the rates of biomethanation during the degradation of food waste and algal biomass (Cao and Zhao, 2009 and Zhang et al., 2017). In these processes, it was also observed that there are pronounced differences in the thresholds of cations that the different orders of *Archaea* can withstand. For instance, during AD of sargassum, populations of *Methanosaeta* were observed to be already dramatically decreased at Na concentrations of  $4.42\text{g}\cdot\text{l}^{-1}$  while members of the *Methanosarcinales* overpopulated the reactors. Similar behavior in archaeal communities has been observed when the inhibitor studied was  $\text{NH}_4^+$  (Tian et al., 2018). Despite these important findings, it is still not clear if these fluctuations in microbial communities are due to differences in the physiology of the distinct species, which allow them to thrive in higher saline environments or is simply due to metabolic shifts that makes the conversion of the intermediates into methane more favorable through the hydrogenotrophic pathway. In fact, further increase in Na concentrations up to  $20.15\text{g}\cdot\text{l}^{-1}$  increased the occurrence of the strict hydrogenotrophs *Methanoculleus* and *Methanofollis*.

High concentrations of cations have shown negative effects towards natural biofilm

formation and granulation processes, even though it is commonly accepted that already immobilized populations are more resilient to fluctuations in salt concentrations. During the operation of an UASB, increased salinity was observed to hamper biofilm formation and to affect the communities of *Methanosaeta* (Gagliano et al., 2017). It was possible to address this negative effect through the addition of  $\text{CaCO}_3$  in the media. In this situation, the presence of the low-soluble carbonate salt might have contributed to the granulation process and ultimately to microbial development, as the solid particles became a support for immobilization. In fact, it has been shown that the presence of Ca and Mg in the digesting media is necessary to induce aggregation of extracellular polymer substances (Turakhia, Cooksey, and Characklis, 1983). This information is of great relevance in the context of the present work, since it is possible that the carriers could replace the effect of insoluble Ca salts that has been documented. If that is the case, the contribution towards the granulation process or fixed film maturation could serve as another explanation for the improved performance of the FAD in comparison to other processes that lack immobilized microorganisms.

## 10.2 AMPTS and manual BMP measurements

The reproducibility test for the biomethane potential protocols AMPTS and manual BMP was performed by applying the same dose of enzymatically treated OFMSW substrate in the batches and with the same inocula. The results show that the maximum coefficient of variance of the gas production at all time points in the AMPTS system was 2.1%. The gas production in this procedure was recorded using a time resolution of 15min. The results from the manual procedure demonstrated a higher variance of 4.3%. These differences were attributed to the manual injection volume of the gas sample taken from the different batches. However, the final estimated amount of gas produced from the substrate in the AMPTS and the manual BMP method were  $282.1 \pm 1.025 \text{ mlCH}_4 \cdot \text{gCOD}^{-1}$  and  $279.6 \pm 1.73 \text{ mlCH}_4 \cdot \text{gCOD}^{-1}$ , respectively. These results confirm the comparability between the employed methodologies.

Table 10.2 summarizes the characterization investigation of the inoculum used in the experimental phase. From this table it can be seen that the comprehensive concentrations of the studied cations do not exceed  $1.5 \text{ g} \cdot \text{l}^{-1}$ . The highest contribution of the pool in both cases is Na, thus the amount of this cation in the inoculum was taken into account when altering the concentration of the batch tests in the inhibition experiments. Furthermore, from the results in Table 10.2, the calculated Specific Methanogenic Activity (SMA) values with ethanol as substrate show a relative high value when compared to other studies (Cho et al., 2005). The SMA values represent the maximum specific methane production that occurs only when the test is conducted under kinetically saturated conditions. This in turn only takes place when the substrate available for the methanogens is significantly greater than the half the saturation coefficient of the substrate. The high SMA values retrieved in the inoculum can also be explained by the microbial characterization results from Chapter 9, where it was shown that a dense population of *Archaea* are present in the media. Furthermore, the Total Volatile Suspended Solids (VVS) in the inoculum accounted for nearly half of the concentration of the measured VS.

TABLE 10.2: Characterization results of the inoculum used in the cation inhibition tests

Parameter	Unit	BMP	AMPTS
Total Solids (TS)	%	1.43	1.38
Volatile solids (VS)	%	0.83	0.69
pH		8.28	8.22
Chemical Oxygen Demand (COD)	g <sup>*</sup> l <sup>-1</sup>	10.87	13.81
Ammoniacal Nitrogen (NH <sub>4</sub> <sup>+</sup> -N)	mg <sup>*</sup> l <sup>-1</sup>	838	897
Ethanol	g <sup>*</sup> l <sup>-1</sup>	0.64	0.01
Acetic Acid	g <sup>*</sup> l <sup>-1</sup>	0.29	0.31
Other VFAs	g <sup>*</sup> l <sup>-1</sup>	0.47	0.57
Aluminum (Al <sup>3+</sup> ) <sub>aq</sub>	mg/l <sup>-1</sup>	0.13	0.13
Calcium (Ca <sup>2+</sup> ) <sub>aq</sub>	mg <sup>*</sup> l <sup>-1</sup>	36.55	46.87
Potassium (K <sup>+</sup> ) <sub>aq</sub>	mg <sup>*</sup> l <sup>-1</sup>	461.84	542.97
Magnesium (Mg <sup>2+</sup> ) <sub>aq</sub>	mg <sup>*</sup> l <sup>-1</sup>	67.55	71.80
Sodium (Na) <sub>aq</sub>	mg/l <sup>-1</sup>	783.11	905.37
Specific Methanogenic Activity (SMA)	ml CH <sub>4</sub> *gVSS <sup>-1</sup> *d <sup>-1</sup>	2.16	1.95

### 10.3 Selection of inhibitor and substrate

Most of the studies performed to measure the inhibitory effects of sodium utilize NaCl. In the present study, it was preferred to utilize NaHCO<sub>3</sub> in order to vary the concentration in the batch reactors, since this strategy excludes the possible negative effects of the Cl<sup>-</sup> ion. Preliminary test suggested that it was inconvenient to utilize Na<sub>2</sub>CO<sub>3</sub> because this compound affects the carbonate system of the digesting media by increasing the pH considerably and consequently hindering the degradation pathways. The preliminary tests on the effects of Ca using CaCO<sub>3</sub> to vary the concentrations of calcium revealed that there was no effect on the yields or rates of methane production when this almost insoluble salt was used (Fig. 10.1; experiment A in Table. 6.3). In fact, the solubility in water of CaCO<sub>3</sub> is 0.013 g<sup>\*</sup>l<sup>-1</sup> (25 °C). The analysis of the dissolved concentration of divalent cation demonstrated that Mg is more abundant in the inoculum, and thus it was chosen to investigate the effects of Mg instead. Thereafter, MgSO<sub>4</sub> was used to spike the batch test and achieve the desired inhibitor concentration. The solubility of MgSO<sub>4</sub> has been calculated to be 269g<sup>\*</sup>l<sup>-1</sup> in aqueous solutions, thus was expected that most of this compound would be found in ionized form. It remains to be mentioned that the SO<sub>4</sub><sup>2-</sup> anion may also have had an effect on the anaerobic process. However, Isa, Grusenmeyer, and Verstraete, 1986 have documented that no inhibitory effect on methanogenesis resulted from the sulfate species at concentrations of 5g<sup>\*</sup>l<sup>-1</sup>.

Since the present study aimed to assess the effects of cations in relation to the methane producing phase, the most logical approach would be to use acetate as substrate. Indeed, acetate is the intermediate metabolite that connects the different degradation pathways of AD with the methanogenic step. Nevertheless, when acetate was supplemented in the batch tests at the desired organic load, the result was a strong acidification of the digesting media, with the pH already falling to under 6 at concentrations below 4g<sup>\*</sup>l<sup>-1</sup> of acetate. It was therefore decided to use ethanol and methanol as pure substrates to avoid lowering the pH beneath the methane production range.

The alcoholic fermentation of glucose makes it possible to produce ethanol. To



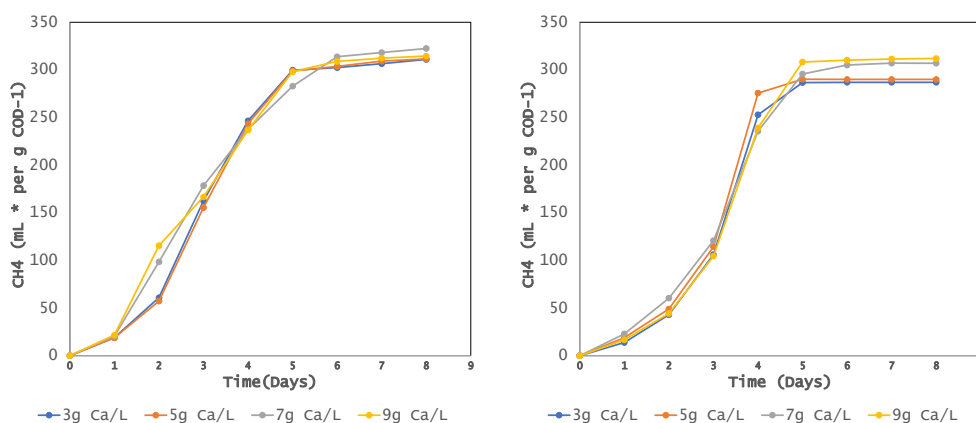
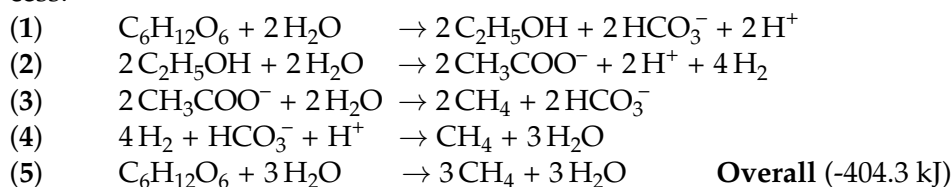


FIGURE 10.1: Effect of different  $\text{CaCO}_3$  ( $\text{g/l}^{-1}$ ) concentrations on the methane yield with ethanol  $7.3(\text{g} \cdot \text{l}^{-1})$  as substrate. Left: AMPTs; Right: manual BMP.

metabolize ethanol into methane, a subsequent conversion into acetate and hydrogen is necessary as can be shown in the conversion of glucose through the AD process:



It is not clear if the methanogens have the capacity to metabolize ethanol directly for biogas production, even though the presence of alcohol dehydrogenases has commonly been found in their enzymatic repertoire (Radianingtyas and Wright, 2003). It is common knowledge that methanogens cannot assimilate carbohydrates or other complex organic substrates, and rely on gluconeogenesis for carbohydrate formation and storage (Bräsen et al., 2014). Hence, conversion of ethanol to methane is most likely to depend on other bacteria to make the conversion into acetate and  $\text{H}_2$ . In turn, methanol can be converted directly into methane through the shortest methanogenic metabolism, as described in Chapter.2 and Fig. 2.3, given that the inoculum comprises archaeal species capable of employing the methylotrophic pathway. It was possible to retrieve these microorganisms during characterization of the microbial diversity of the inoculum used in this experiment. Therefore it is possible to rely on methanol as the sole substrate in the study of cations.

For practical reasons, it was preferred to make the substrate concentration calculations on the basis of COD instead of VS even though it was possible to make a simple conversion between these values since pure substrates were used. The theoretical maximum methane production for any substrate is  $0.35\text{mlCH}_4 \cdot \text{gCOD}^{-1}$  (Prabhudessai, Ganguly, and Mutnuri, 2013). This number serves as a reference to understand the expected methane production from the batch experiments in the

presence or absence of the inhibitors.

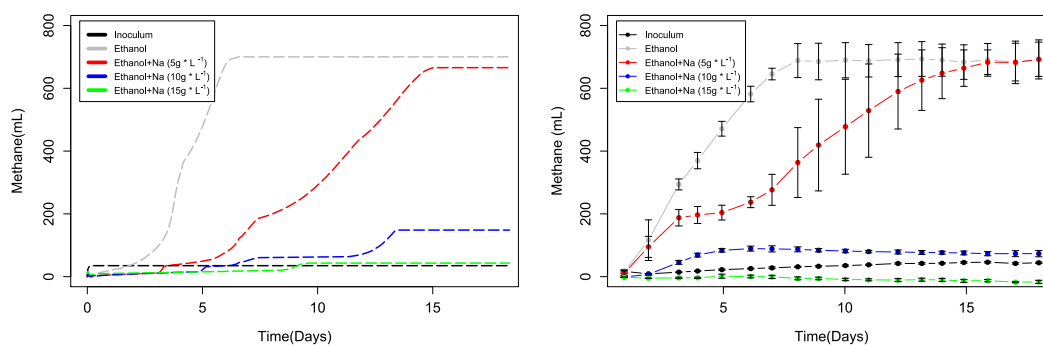


FIGURE 10.2: Effect of different Na concentrations on the methane yield with ethanol  $7.3(\text{gCOD}\cdot\text{l}^{-1})$  as substrate. Left: AMPTS; Right: manual BMP.

## 10.4 Inhibition of cations in AD

The initial toxicity tests on the effects of Na in AD were performed with ethanol as substrate (Fig. 10.2; Experiment B in Table.6.3). From this Figure, it can be seen that there was already a pronounced difference in the rates of production at concentrations of Na of  $5\text{g}\cdot\text{l}^{-1}$  when compared to the uninhibited reactors. At this concentration of inhibitor, a similar amount of methane production was achieved to that in the control, despite the fact that the inhibited reactors took twice the time. In the batch tests with higher Na concentrations, there was a remarkable decrease in the total methane production and rates, in particular those containing  $15\text{g}\cdot\text{l}^{-1}$ . In this case, the reactors produced barely as much gas as the background methane that evolved from the inoculum. This observation was recorded for both the AMPTS and the manual BMP methodologies.

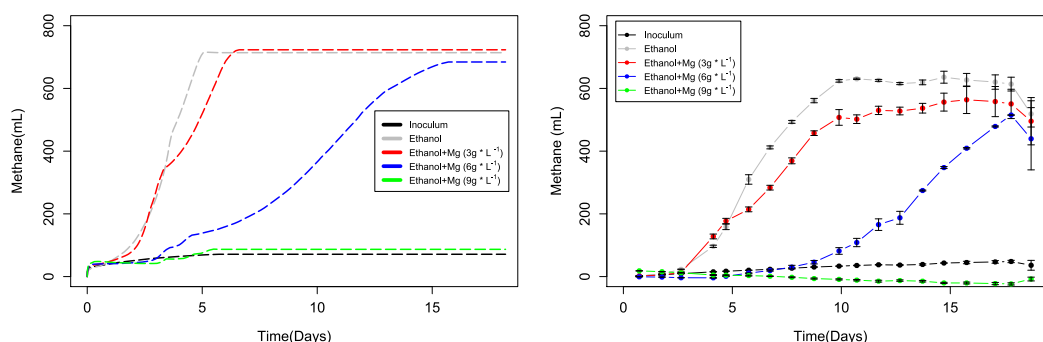


FIGURE 10.3: Effect of different Mg concentrations on the methane yield with ethanol  $7.3(\text{gCOD}\cdot\text{l}^{-1})$  as substrate. Left: AMPTS; Right: manual BMP.



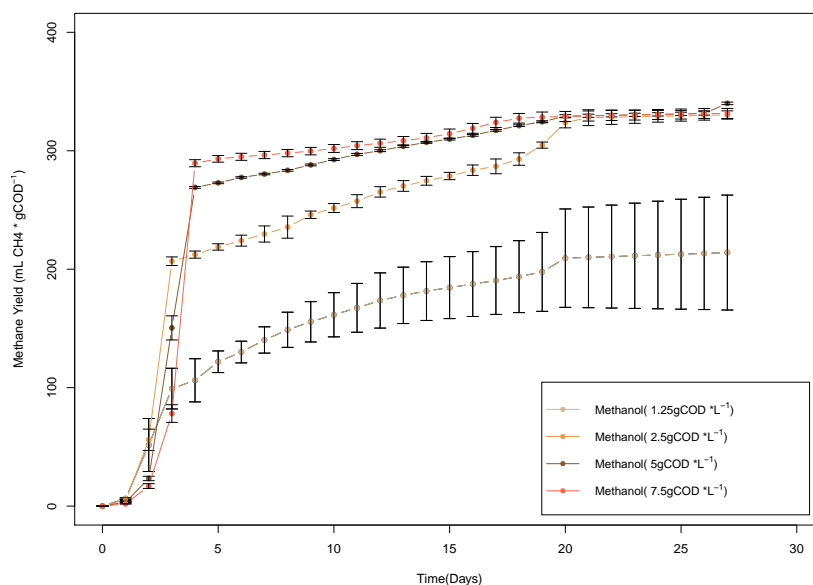


FIGURE 10.4: Yields of methane production from methanol at different organic load. Methanol concentrations ranging from 1.25 to 7.5 gCOD · l<sup>-1</sup>

In relation to the toxicity test with Mg, a similar trend was documented (Fig. 10.3). At the lowest concentration of inhibitor tested (3g · l<sup>-1</sup>), the rates of production were lower than without addition of Mg. Surprisingly, at this concentration, the initial rates of methane production remained the same as in the control and a decline resulted only after the fourth of production. However, at the end of the experiment the expected production levels of methane were achieved, with only 2 days of delay. Meanwhile, the reactors with 6g · l<sup>-1</sup> of Mg produced slightly lower amounts of methane than the control at nearly half the production rate, which was comparable with the reactors that contained 5g · l<sup>-1</sup> of Na. Notably, concentrations of Mg of 9g · l<sup>-1</sup> hampered the methane production for by more than 90%. This observation shows that at this concentration level it is already not possible to sustain the methanogenic metabolism, even though the reactors containing 10g · l<sup>-1</sup> Na produced a small amount of biogas. Data confirm that there is a stronger inhibitory effect of Mg than Na for the last phase of AD. However, the effects of SO<sub>4</sub><sup>-</sup> should not be disregarded, since this could be the cause of the perceived differences between the cations tested. In these experiments, the IC<sub>50</sub> 6.98 g · l<sup>-1</sup> for Na and IC<sub>50</sub> 6.53 g · l<sup>-1</sup> for Mg were calculated.

## 10.5 Effect of substrate concentration in inhibited reactors

To our knowledge, the existing studies on cation inhibition do not consider different concentrations of substrates when the toxicity essays are performed. This is probably due to the lack of a generalized methodology to perform the tests. There is also a high variability in the inoculum used in the different geographical regions, enriched with distinct substrates that originate from very dissimilar microbial communities. In this part of the experimental phase, we tested the effect of different concentrations of Na on the conversion of methanol into methane in reactors with different organic

load ( Experiment **D** in Table. 6.3). The utilization of methanol as the only substrate ensures a focus on methanogenesis and also only on measurements of the ability of archaeal population to produce methane.

Figure 10.4 and figure 10.5 show the methane yields in the experiments without any Na addition. In this situation, the concentration of sodium was approximately  $800 \text{ mg} \cdot \text{l}^{-1}$  from the inoculum itself. With the information from both graphs, it is possible to observe that all the batches except those with a methanol concentration of  $1.25$  and  $30 \text{ gCOD} \cdot \text{l}^{-1}$  yielded similar amounts of methane. Cho et al., 2005 have already suggested that the COD concentration used to calculate yields and SMA in batch tests should be above  $1 \text{ gCOD} \cdot \text{l}^{-1}$ . These initial results show that the commonly accepted organic load that is used should be higher in consideration of the fact that nearly half the possible methane production was lost at the lower organic load of  $1.25 \text{ gCOD} \cdot \text{l}^{-1}$ . With regard to the highest concentration tested ( $30 \text{ gCOD} \cdot \text{l}^{-1}$ ), the production was strongly delayed, probably due to the excessive amounts of methanol in the media. From the graph, it appears that methane yield at  $30 \text{ gCOD} \cdot \text{l}^{-1}$  approaches but does not reach the levels of the other substrate concentrations (Fig. 10.5). For all the intermediate methanol loads, it was possible to obtain nearly  $320\text{--}340 \text{ ml CH}_4 \cdot \text{gCOD}^{-1}$  which is close to the maximum theoretical. This also indicates that only a few fractions of the energy present in the substrate is used for anabolic purposes, for example cellular growth. From Figure 10.5 it is also possible to see that at higher concentrations of methanol it takes the reactors more time to attain the final yield. This evidently happens because it takes more time to convert more substrate in time and thus the substrate amount is not limiting in the experiment.

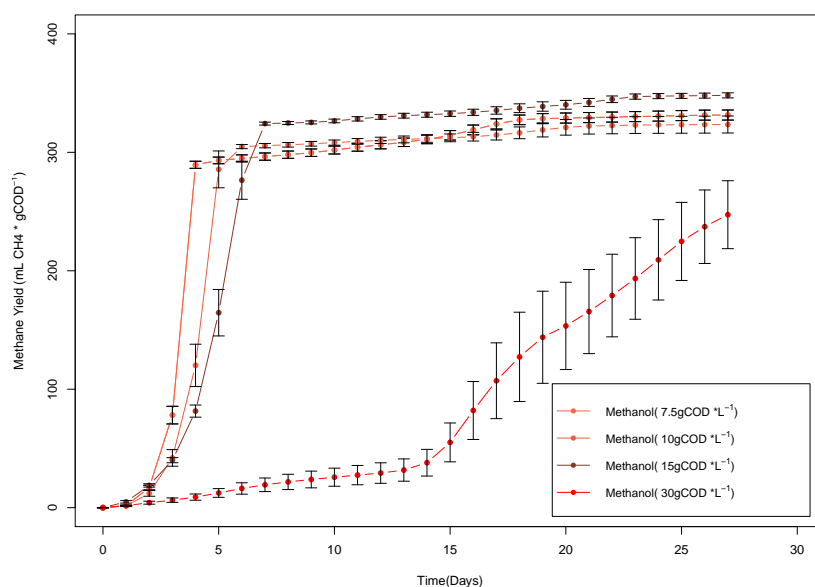


FIGURE 10.5: Yields of methane production from methanol at different organic load. Methanol concentrations ranging from  $7.5$  to  $15 \text{ gCOD} \cdot \text{l}^{-1}$

Figure 10.6 shows the calculated methane yields expressed as obtainable methane volume per gram of COD that was initially added to reactors green color indicates increasing substrate concentration. In contrast to those results from batches without addition of Na, the concentration of methanol does has an effect of the final

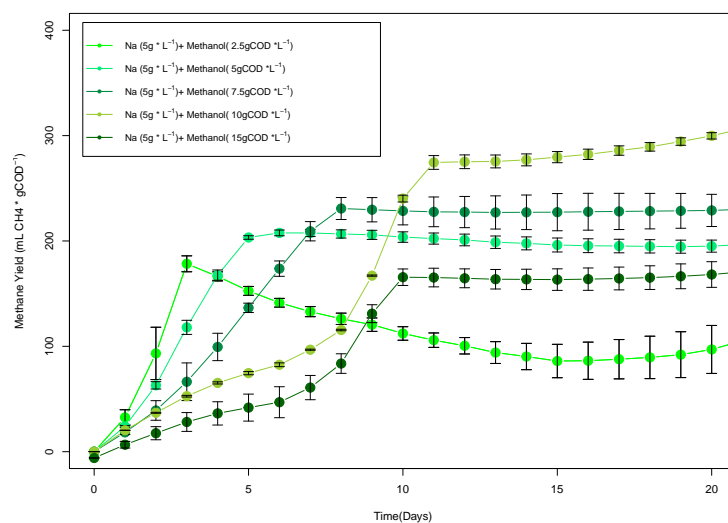


FIGURE 10.6: Yields of methane production from methanol at different organic load with Na concentration of  $5\text{ g}\cdot\text{l}^{-1}$ . Methanol concentrations ranging from 2.5 to  $15\text{ gCOD}\cdot\text{l}^{-1}$

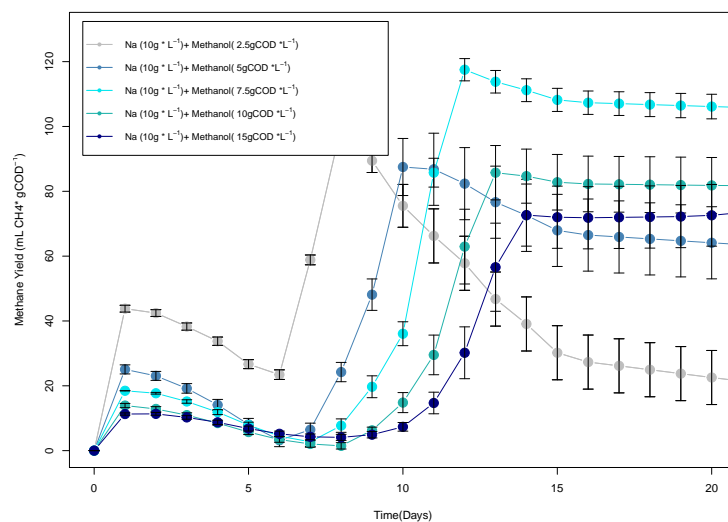


FIGURE 10.7: Yields of methane production from methanol at different organic load with Na concentration of  $10\text{ g}\cdot\text{l}^{-1}$ . Methanol concentrations ranging from 2.5 to  $15\text{ gCOD}\cdot\text{l}^{-1}$

methane yields. The lowest methane yield that was obtained in Figure 10.6, corresponds to the batches containing the lowest substrate concentration ( $2.5\text{ gCOD}\cdot\text{l}^{-1}$ ). The methane yields increase when more amount of methanol is added in the reactors, up to a concentration of  $10\text{ gCOD}\cdot\text{l}^{-1}$ . The reactors with  $15\text{ gCOD}\cdot\text{l}^{-1}$  methanol (highest substrate concentration tested) yielded less methane than those reactors with  $10\text{ gCOD}\cdot\text{l}^{-1}$  methanol. This result is in contrast with the behavior observed in the reactors without inhibition of Na. A possible explanation is that the decrease in capacity to metabolize methanol rapidly enough in the reactors with the highest organic loads causes the substrate to negatively affect the performance of the microbial cells due to stress. It has already been seen from the previous experiments

that there is a limit to the concentration of organic load that can be employed the digesting media before these reactors under perform. Figure 10.6 also shows that the yields of the reactors with the lowest organic load decreases after it reached a peak at around  $200 \text{ ml CH}_4 \cdot \text{gCOD}^{-1}$ . This is a consequence of the correction from the background gas production in the inoculum. After that peak, the production of this reactor ceases, indicating that the concentration of the cation in the media has a dramatic impact on the final methane yields of batch test at the lowest organic concentrations.

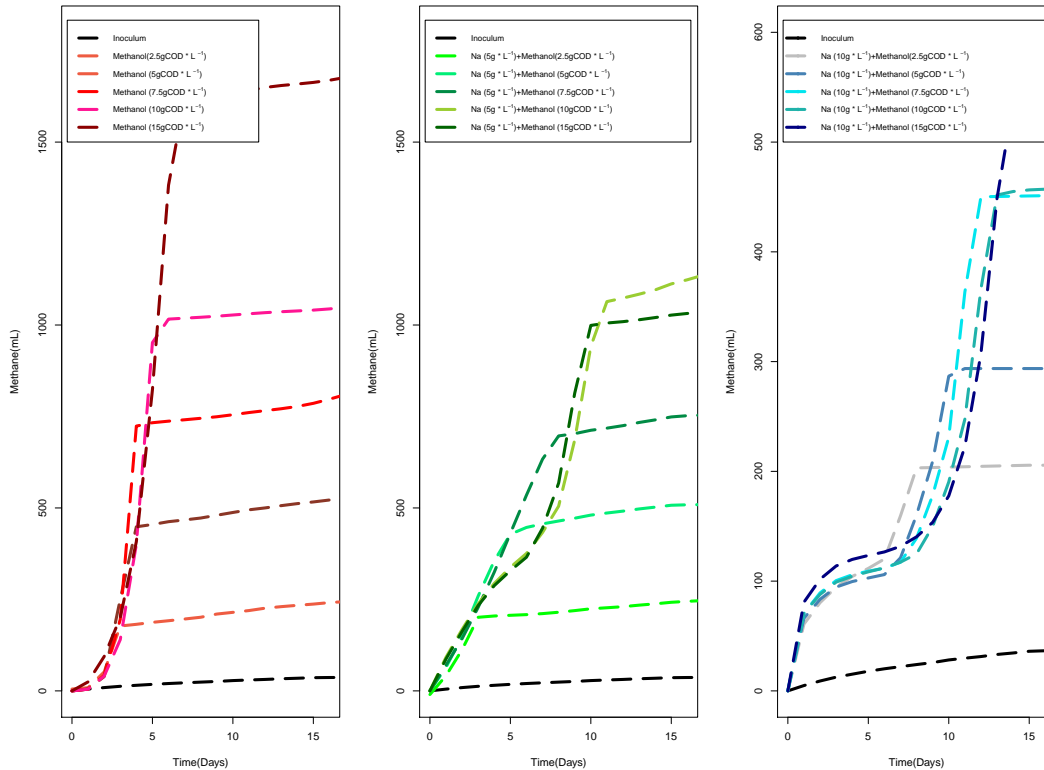


FIGURE 10.8: Methane production from inhibition tests at different organic loads. Methanol concentrations ranging from 2.5 to  $15 \text{ gCOD} \cdot \text{l}^{-1}$  and Na concentrations of 0, 5 and  $10 \text{ g} \cdot \text{l}^{-1}$

It has already been shown that concentrations of  $10 \text{ g} \cdot \text{l}^{-1}$  Na inhibit severely the AD process, particularly since this concentration is above the calculated  $\text{IC}_{50}$  value. Figure 10.7 shows the obtainable yields from the batch tests inhibited with  $10 \text{ g} \cdot \text{l}^{-1}$  of Na with different methanol concentrations. Similar results have been already obtained in Fig 10.6, where it is clear that the methane yields are dependent on the substrate concentration. However, in the batch reactors containing  $10 \text{ g} \cdot \text{l}^{-1}$  Na, a concentration of methanol of  $7.5 \text{ gCOD} \cdot \text{l}^{-1}$  yielded the highest amount of methane per gram of COD instead of  $10 \text{ gCOD} \cdot \text{l}^{-1}$  methanol (as recorded in those batch tests with  $5 \text{ g} \cdot \text{l}^{-1}$  Na). This observation suggests that the ability of methanogens to produce methane at higher substrate concentration is strongly hindered by increasing cation in the medium. It is possible that the substrate becomes an inhibitory substance in the medium when the methanogens are not able to utilize it rapidly enough at a high cation concentration.

Up to this point, it has been shown that the organic load in the toxicity tests is crucial. After an evaluation of data reported from different literature sources that

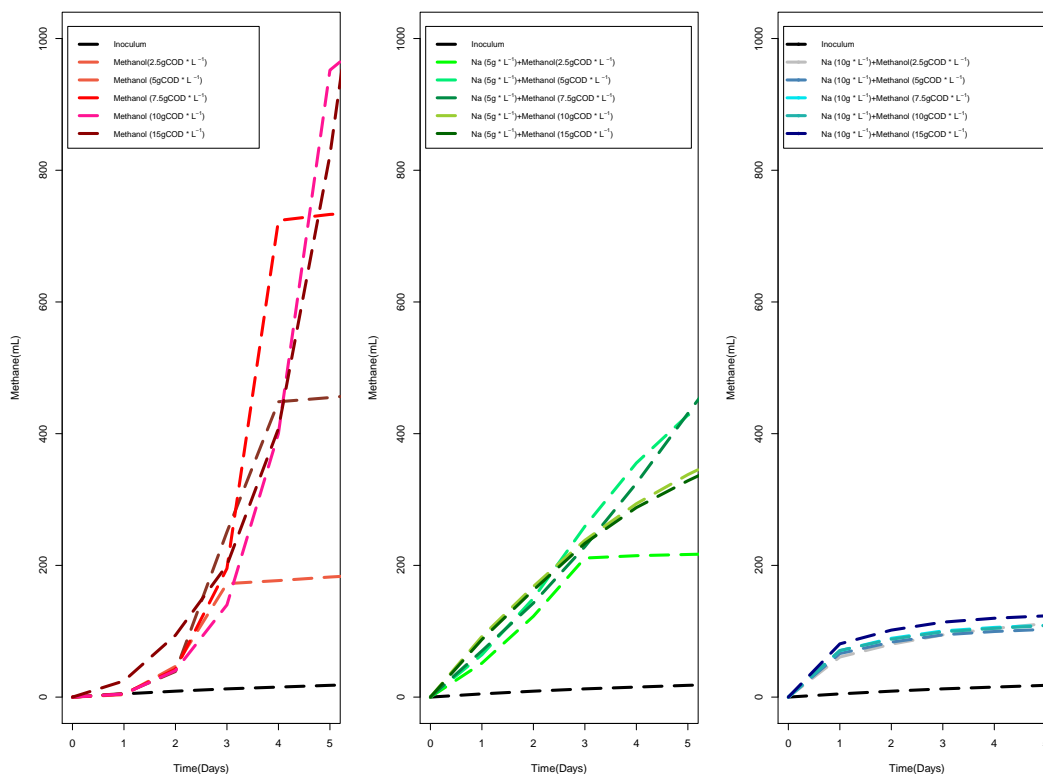


FIGURE 10.9: Initial rates from inhibition tests at different organic loads. Methanol concentrations ranging from 2.5 to 15 gCOD $\cdot$ L $^{-1}$  and Na concentrations of 0, 5 and 10 g $\cdot$ L $^{-1}$

perform these type of experiments, it was possible than notice that none of the authors takes into consideration the effects of substrate concentrations in the batch tests. In fact, none of the studies listed in Table. 10.2 has utilized multiple organic loads in their experimental set-ups. These bibliographical studies take into consideration a single inoculum to substrate ratio without determining previously if there is substrate saturation in the reactors. Furthermore, these studies usually do not focus on a single phase of the biomethanation process, since complex substrates are used during the investigation. The results here obtained suggest that the substrate concentration must be considered during the characterization of the methanogenic phase of the AD process while performing cation inhibition studies.

We have described the effect of the Na concentrations on the methane yields in batch reactors. In figure 10.8 and 10.9 are shown the cumulative methane production and initial rates of production during for this experiment at different substrate concentration for Na concentrations of 0, 5 and 10 g $\cdot$ L $^{-1}$ . From these graphs it is important to notice that the initial rates of production are surprisingly similar for the groups of experiments performed with the same level of inhibitor. This leads to deduce that the cation concentration is a crucial factor that determines and limits the velocity of substrate intake in the methanogenic cells. Increasing the Na concentrations there is evidently slower rate of production no matter the substrate concentration. Moreover, all the production curves reach an inflexion point where the evolution of methane stagnates and only proceeds slowly until the remaining possible substrate consumption is achieved. However, this maximum substrate utilization is not achieved in the experiments with 5 and 10 gCOD $\cdot$ L $^{-1}$  of methanol

since these inhibited batches yielded less methane than the control. Furthermore, it is curious to observe that the production in the reactors with  $10\text{g}\cdot\text{l}^{-1}$  Na proceeds through an apparent two step reaction with two different rates before reaching the inflexion point mentioned earlier. The first conversion step is faster than the second. It is likely that at the methanogens are able to utilize rapidly the substrate at the beginning of the test due to it's availability, but then struggle to consume the rest as a consequence of high cation concentrations.

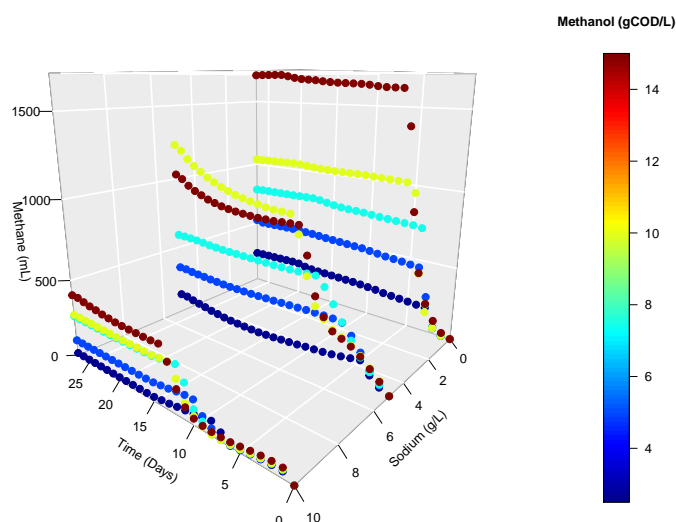


FIGURE 10.10: Effect of Na concentrations at different organic loads on the methane production during the experiment

Figure 10.10 shows the collective results of experiment D in Table. 6.3. There is a strong effect of Na concentration on methane production for all the concentrations of substrate tested. We can also see that the highest amount of methanol does yield the highest amount of methane expected in every batch. This leads us to state once more the importance of taking into consideration the concentration of substrate used in the batch during toxicity tests. This can be observed in particular from the trend of methane production given in the yellow and red dotted lines in Fig. 10.10.

## 10.6 Strategies to alleviate inhibition

Until this point we have intended to describe the consequences of increasing cation concentrations, in particular of Na and Mg, on AD. It was possible to distinguish the effects of the rates and the final yields during the biomethanation of pure substrates like ethanol and methanol. Nevertheless, the results so far have not given us an explanation of the mechanisms behind the inhibition. It is still uncertain if these antagonistic effects are irreversible or if the inhibitory process can otherwise be removed through any strategy. It was therefore decided to attempt to block the inhibition through use of two chemically different compounds that could potentially bind the cations in the digesting media, in the expectation of relieving the toxic effects. The experimental setup of these experiments are summarized in C and E of table. 6.3. The molecules used to counter the toxicity are shown in Figure 10.11.

The first compound to be used was Ethylenediaminetetraacetic acid (EDTA). This is a chelating agent used in many industrial and pharmaceutical processes to bind metals. The stability of this compound to make complexes with divalent cations like calcium and magnesium increases at pH ranges from 5-10. The formation of EDTA and metal complexes allows the metals to remain in solution while also exhibiting less reactivity. The molecule of EDTA also possesses four carboxylic acid groups that contribute to its binding properties and high solubility in aqueous solutions (Fig. 10.11). EDTA was used in this experiment in an attempt to introduce negative charges in digesting media, which could potentially influence the toxic effect of soluble Na and Mg in the batch reactors converting methanol into methane. It was, however, not possible to introduce large amounts of EDTA in the reactors since a rapid acidification of the digesting media was observed. In these experiments  $2\text{g}\cdot\text{l}^{-1}$  of EDTA had been employed, which consequently changed the pH in the inoculum from approximately 8.2 down to 7.247 despite the high buffer capacity. This was the same reason as for not adopting acetate as substrate, because otherwise the experiments with high organic load would have been less likely to succeed.

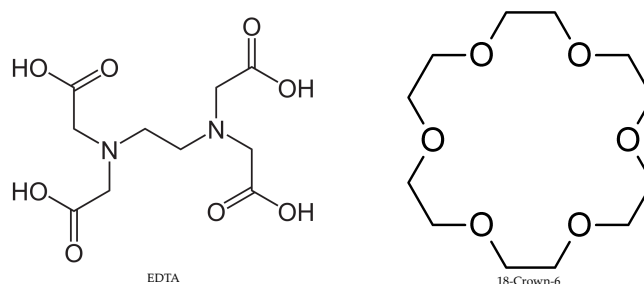


FIGURE 10.11: Chemical structure of EDTA and 18-Crown-6

The second compound used was the crown ether, 18-Crown-6. The first crown ether was chemically synthesized in 1967 by Charles J Pedersen and partly for this contribution he was awarded with the Nobel prize in 1987 (Pedersen, 1967). 18-Crown-6 is one of the molecules with structure specific interactions of high binding selectivity for cations such as K and Na which are stabilized by intramolecular hydrogen bonding (Steed and Junk, 1999, Leszczyński, Nowek, and Wojciechowski, 1981). This crown ether has been also shown to suppress ion pairing in organic protic solvents in the presence of sodium, in particular of sodium and the acetate ion, since the acetic species is a poor nucleophile (Cook, Chauncey, and Liotta, 1974). In this work 18-Crown-6 was used as a cation binding agent in the batch reactors affected by Na inhibition. However, in contrast to EDTA, 18-Crown-6 does not per se influence with the ionic strength of the media.

EDTA was added to the inhibited batch reactors containing  $7.3\text{gCOD}\cdot\text{l}^{-1}$  of ethanol with different concentrations of Na and Mg as indicated in Figure 10.12 and Figure 10.13. The results of this experiment can be compared with those at the beginning of this chapter, where the inhibitory effects of these cations were measured for a fixed organic load. Figure 10.12 shows the methane production curves of the batches at different Na concentration in the presence or absence of EDTA; the colors of the curves were paired to match corresponding concentrations of inhibitor. From this graph it is possible to see that EDTA indeed had a positive effect on methane production for both Na concentrations tested. In the case of Na  $5\text{g}\cdot\text{l}^{-1}$  (red and orange

lines), there was a strong improvement in production velocity and it was possible to achieve the final expected methane production comparison to the control (blue lines). A similar trend was observed with Na concentration of  $10\text{g}\cdot\text{l}^{-1}$  (green and light green), where the rate and the total methane production were also enhanced. However, it was not possible to achieve the same amount of methane as in the control in less than 15 days, even though the same amount was reached in by around day 30 (data not shown). Furthermore, surprisingly, EDTA addition to the uninhibited reactor revealed a faster process than the batch test in the absence of the chelation compound. This effect can be explained in that EDTA would also bind a fraction of the Na already present in the inoculum. Lastly, it can also be observed that EDTA enhanced the background gas production when supplemented in the inoculum alone. All the production curves in Figure 10.12 have been corrected to the background gas production at every point. Therefore it would have been expected that the inoculum plus EDTA (grey) would not have exhibited more methane than the inoculum alone (black). In this experiment approximately 36ml more  $\text{CH}_4$  were measured in the batches with inoculum in the presence of EDTA than without EDTA after 18 days of reaction.

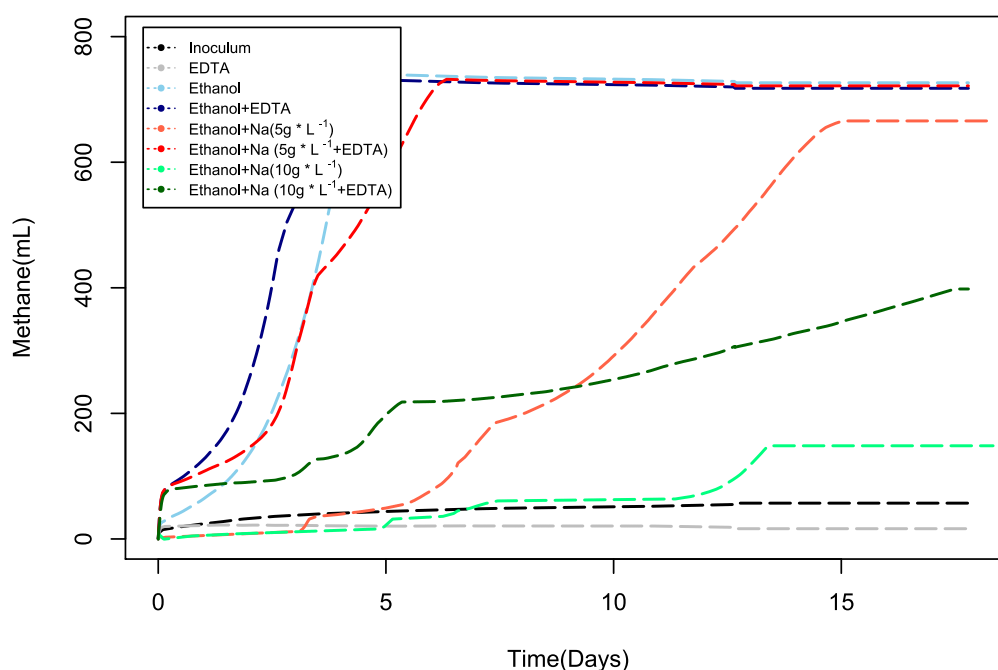


FIGURE 10.12: Effect of EDTA addition on the methane production in the toxicity tests. EDTA concentration of  $2\text{g}\cdot\text{l}^{-1}$ , Ethanol concentration  $7.3\text{gCOD}\cdot\text{l}^{-1}$ .

Interestingly, the HPLC measurement of the total VFAs that remain in the batch test after the AD reactions at completion showed that there were approximately  $400\text{mg}\cdot\text{l}^{-1}$  of acetate equivalents in the reactors with inoculum alone. In contrast, the batches supplemented with EDTA did not register any VFA. This is important



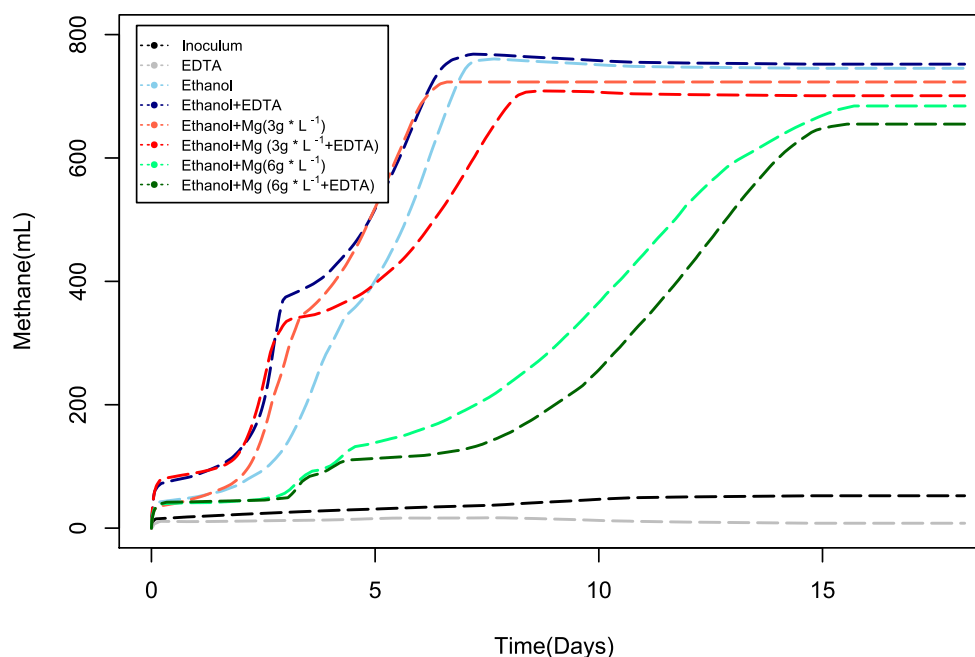


FIGURE 10.13: Effect of EDTA addition on the methane production in the toxicity tests. EDTA concentration of  $2\text{g}^{\ast}\text{l}^{-1}$ , Ethanol concentration  $7.3\text{gCOD}^{\ast}\text{l}^{-1}$ .

information for explaining why anaerobic digesters usually show unconverted concentrations of VFAs, and thus unexpressed potential, as it seems even low concentrations of cations in the digesting media prevent VFA utilization. The experiments with EDTA in the presence of Mg are shown in Figure 10.13. As just mentioned, the effects of EDTA on the rates and total production of methane in this experiment were similar to those recorded with Na. However, in the case of Mg it seems that there was a stronger alleviation of the toxic effects since at concentrations of  $6\text{g}^{\ast}\text{l}^{-1}$  Mg, higher and faster biogas production resulted than with  $5\text{g}^{\ast}\text{l}^{-1}$  Na. This can be explained by comparing the formation constants of metal-EDTA complexes. For EDTA-Na the formation constant is  $10^{1.86}\text{M}^{-1}$  and for Mg  $10^{8.79}\text{M}^{-1}$ <sup>1</sup>. The highest affinity of EDTA towards divalent metal cations such as Mg is due to the stabilization of the molecules through formation of 6 coordination sites. It is possible that this hardly reversible sequestering of Mg, in contrast to just charge-based interaction with Na, makes EDTA a more appropriate candidate to alleviate inhibition from divalent cations.

Is evident that the methane production curves of ethanol and methanol as pure substrates are different from each other. These trends are clearly distinguishable in Figure 10.2 and 10.8, in particular for those production curves without addition of Na. These contrasts are most likely due to the different and more numerous metabolic steps required to transform ethanol, which makes it a slower process to convert this substrate into methane, and also the necessary intervention of different microbial groups to perform the conversion. This difference between the trends

<sup>1</sup>Values reported apply at  $25^{\circ}\text{C}$  and ionic strength of  $0.1\text{M}$

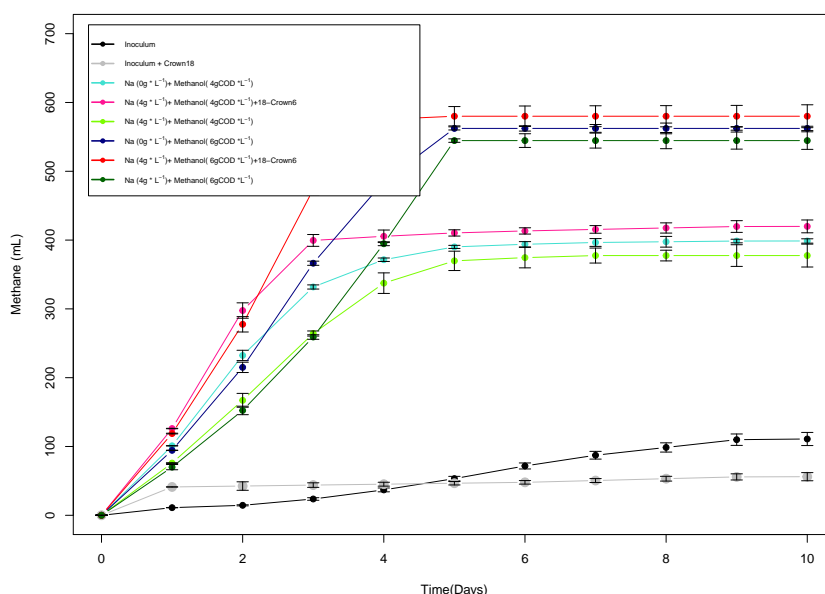


FIGURE 10.14: Effect of 18-Crown-6 addition on the methane production in the toxicity tests with Na. 18-Crown-6 concentration of  $2\text{g}^{\ast}\text{l}^{-1}$ , Methanol concentrations of 4 and  $6\text{gCOD}^{\ast}\text{l}^{-1}$ .

of the production curves of both substrates appears less dissimilar when the cation concentrations are raised. This observation suggests that there is a larger effect of increasing Na concentrations on the biomethanation process than in those happening before. This indicates that there is a higher toxicant effect on the populations of methanogens which hinders their ability to utilize the substrate.

Figures 10.14 and 10.15 show the experiments that depict the effects of 18-Crown-6 towards relieve of cation inhibition. In this experience, different concentrations of substrate were considered (4 and  $6\text{gCOD}^{\ast}\text{l}^{-1}$ ). In Figure 10.14 timely methane production of the batch test is shown in the presence and absence of the crown ether with Na concentrations of  $4\text{g}^{\ast}\text{l}^{-1}$ . These results are comparable to those in Figure 10.2 where the inhibitory effects of sodium were tested. From this figure the crown ether is seen to have a positive effect towards the biomethanation process, like EDTA had. All the batch tests with 18-Crown-6 exhibited a faster rate and methane production than those without, but it was also possible to produce slightly higher amounts of gas than in the absence of the crown ether. This behavior of the AD processes was also observed within the reactors at different concentrations of substrates. Interestingly, the rates of the batch tests containing 18-Crown-6 had the same initial rates of production in comparison to each other, which was higher than in the controls. This information strengthens previous observations that reveal that cation concentrations determine the fate of methanol intake in methanogenic populations. Comparing the effect of 18-Crown-6 and EDTA towards Na, it appears that the crown ether had an improved anti-inhibitory action. This is most likely due to its higher affinity towards Na as in the previous case with EDTA and Mg. Similar observations on the background methane production of the inocula have also been made. However, in presence of the crown ether, it was possible to produce nearly 3-fold more methane than in the inoculum alone. Further tests with varying concentrations of 18-Crown-6 are important to quantify the anti-inhibitory effects, and additional tests with this

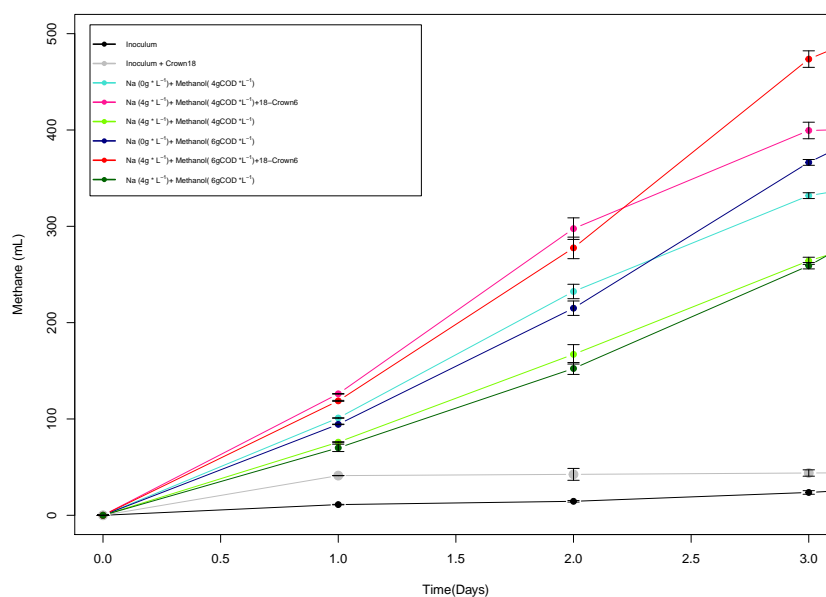


FIGURE 10.15: Effect of 18-Crown-6 addition on the initial rates in the toxicity tests with Na. 18-Crown-6 concentration of  $2\text{g}^{\ast-1}$ , Methanol concentrations of 4 and  $6\text{gCOD}\cdot\text{l}^{-1}$ .

compound should be conducted to reveal more information about the mechanisms behind cation inhibition.

## 10.7 Conclusions

The Biochemical Methane Potential (BMP) is a commonly performed procedure to determine the methane production of an organic substrate that is degraded through AD (Raposo et al., 2011). The BMP has become a routine procedure in many laboratories both for research and industrial purposes, because the methane potential of a specific substrate provides essential information about the extent of biodegradability and expected rates for process calculations. Despite the fact that much effort has been made to optimize methodologies for measurements and the uttermost relevance of these, less effort has been made to standardize the determination procedure of this tests. Consequently, the strategies adopted usually tend to accommodate the purpose of the investigation and have been shown to give misleading estimations. An interlaboratory experience was performed to validate the influence of the inoculum, substrate characteristics and experimental conditions on the BMP results among different laboratories (Raposo et al., 2011). Even though strict guidelines were given for performing the experiments, the results show that the precision of the data reported for the BMP was around 10% and 50-70% for the calculated rates. More than 10 factors influencing the results have been analyzed. However, the cation concentration was not considered amongst the most relevant factors influencing the results. In the present work, we have shown that the concentration of cations, including even in the inoculum, is crucial for determining the rates and final methane production in batch tests. This is the most likely explanation that explains the results obtained by Raposo et al., 2011. Indeed, the ratio between inoculum and substrate can be used to obtain a general methodology to standardize batch tests for

determining the biomethane potential of a specific substrate in a non inhibited batch reactors. However, we have described similar effects for different organic loads. As a future perspective, further tests are recommended to find some more economically feasible anti-inhibitors to be used in commercial AD plants to alleviate the problems of operational AD plants that struggle with high cation concentrations, especially when treating biomass with high bioavailable Na, K, Mg and Ca. Other chelating agents, such as nitrilotriacetic acid (NTA), citrate, tartrate and di-ethylene triamine pentaacetic acid, should be tested because these may be potential candidates to enhance biomethanation from inhibited processes.



## Chapter 11

# Conclusions and Perspectives

10

In this PhD thesis work, it was possible to confirm that via well thought reactor design including fixed immobilized biofilm, internal compartmentalization and the controlled flow regime, obtainable biomethane yields can be significantly increased. In a 240l pilot plant experiment, the methane obtained achieved after the biofilm have been generated was minimum  $700\text{l}\cdot\text{d}^{-1}$  at an HRT of 10 days. During the continuous operation and increasing loadings, it was possible to produce between 0.2 and  $0.3\text{l}_{\text{CH}_4}\cdot\text{g}_{\text{COD}}^{-1}$ , achieving an OLR of a magnitude of  $20\text{g}_{\text{COD}}\cdot\text{l}_{\text{reactor}}^{-1}\cdot\text{d}^{-1}$ . Throughout the conversion processes, the novel FAD reactor did not encounter mechanical or other related issues that are commonly known to affect the AD of complex substrates, particularly those characterized by high organic strength and particulate material.

The study of the microbial diversity in the biofilm and in the digesting media in the FAD reactor has provided important insights about the AD of enzymatically treated OFMSW. Characterization of the populations showed that there doesn't result a microbial specialization of the microorganisms in the different compartments. The communities lying within the biofilm and those in the digesting media are similar at a given operational regime, but can fluctuate when changes are induced, in particular over the organic load and the temperature regime. During these conversion processes, the *Methanosarcina* spp. are the most dominant amongst the methanogenic populations. However, the emergence of *Methanoculleus* spp. in the thermophilic temperature range suggests that methane production proceeds through the hydrogenotrophic pathway, where  $\text{H}_2$  and  $\text{CO}_2$  are consumed. In these conditions it seems that the methane production proceeds faster.

The ability of methanogens to evolve methane has been shown to be conditioned by the pH in the digesting media. Even though it is possible to adjust the alkalinity in the process, the accumulation of cat-ions eventually decrease the rate of methane production that leads facing unbalances and eventual collapse. It has been confirmed that soluble salts inhibit the AD process and the IC50 values for Na and Mg were almost similar and calculated to be  $6.98\text{ g}\cdot\text{l}^{-1}$  and IC50  $6.53\text{ g}\cdot\text{l}^{-1}$  respectively. Although the mechanisms behind cat-ions inhibition are still to be deciphered, the levels of inhibition towards the methanogenic step were systematically assessed. It was described that cat-ions retard the methane production in a dose dependent manner and the resulting yields lean on the inhibitor concentration as well as the organic load of substrate used in the batch reactors. Remarkably, it was possible to revert the inhibitory effects of high cat-ion concentration and reestablish the ability of methanogenic populations to produce biogas. This was done administering compounds that bind the cat-ions such as EDTA and crown ethers. Despite this is not a technically feasible solution for application in large scale biogas plants to deal with

soluble salts, it opens the possibility for future research in the field since now it's known that the inhibition from cat-ion is a reversible process.

The composition of endogenous microorganisms in the inoculum did not have an effect on the long time performance of AD reactors degrading enzymatically treated OFMSW. Instead, the pretreatment of the substrate enhances methanogenic *Archaea* that can establish in the digesters to constitute nearly halve the total population. In this manner, the anaerobic treatment of waste materials deriving from households or commercial activities can be improved considerably by enhancing the rates of methane production since there results less selective pressure on the hydrolytic bacteria.

Despite new technologies such as high throughput DNA sequencing have increased our understanding on the role of the key microbial players and metabolic pathways during the anaerobic conversion processes, there still is a lot of progress to make. In this PhD thesis, the microbial analysis was limited to studying the marker 16S rRNA gene. Even though this approach has proven useful to our purpose, the existing databases are limited to comprise few microbial groups present in aerobic digesters. For future research, it is highly advisable to consider a more complex approach for the analysis of the microbial diversity, for example through whole genomic sequence studies. Furthermore, this PhD project has focused mainly on studying the anaerobic digestion of enzymatic treated waste. Other complex substrates should be included in future projects to reveal possible differences of biogas production process in the novel FAD digester.

## Chapter 12

# Publications

11





## Regular article

## Fast anaerobic digestion of complex substrates via immobilized biofilms in a novel compartmentalized reactor design

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## HIGHLIGHTS

- Compartment reactor design, fixed immobilized biofilms, and controlled flow regime.
- High throughput biogas production on the organic fraction of municipal solids waste.
- Production was minimum  $\sim 700 \text{ L}_{\text{CH}_4} \text{ day}^{-1}$  with Hydraulic Retention Time of 10 days or less.
- Conversion of  $20.8 \text{ g}_{\text{COD}} \text{ L}_{\text{reactor}}^{-1} \text{ day}^{-1}$  at a Hydraulic Retention Time below 5 days.
- Robust reactor performance at fast feed flow rates during 6 months of running.

## ARTICLE INFO

## Keywords:

Fixed biofilm  
Reactor design  
Compartments  
Suspended particles  
Municipal solid waste  
Biogas

## ABSTRACT

Anaerobic digestion of municipal waste is a promising technology for renewable energy production, notably for methane ( $\text{CH}_4$ ) production. Existing reactor designs have limitations that prevent efficient conversion and high throughput, especially of substrates high in suspended solids. Here, we introduce a novel compartmentalized reactor design encompassing controlled feed flow over fixed microbial biofilms for high rate  $\text{CH}_4$  production from enzymatically pre-hydrolyzed municipal solid waste. In a 240 L working volume reactor, the biofilm generation was completed in less than 60 days and  $\text{CH}_4$  production was minimum  $\sim 700 \text{ L day}^{-1}$  in the ensuing months with a Hydraulic Retention Time (HRT) of 10 days or lower. When the organic load was gradually increased, a reactor productivity of  $6 \text{ L}_{\text{CH}_4} \text{ L}_{\text{reactor}}^{-1} \text{ day}^{-1}$  was achieved. At this point, the reactor processed  $20.8 \text{ g}_{\text{COD}} \text{ L}_{\text{reactor}}^{-1} \text{ day}^{-1}$  at a HRT below 5 days, maintaining above 70% of the maximal Chemical Oxygen Demand (COD) conversion rate. Such fast conversion rates and high yields are far beyond what has been reported for other reactor designs, and are a crucial prerequisite for industrial realization of renewable biogas production from municipal solid waste and other organic waste streams.

## 1. Introduction

In the context of a green and circular economy, anaerobic digestion has recently gained renewed interest as a promising process for renewable energy production from different types of organic waste [1,2]. The anaerobic digestion process relies upon microbial consortia to degrade the organics from complex to simple molecules, in turn ultimately producing a methane ( $\text{CH}_4$ ) rich gas [3]. Under anoxic conditions, the microbial digestion involves both bacteria and archaea. First, bacterial communities degrade and convert the organic material into acetate, hydrogen and carbon dioxide, then specialized archaea convert and consume these products during  $\text{CH}_4$  production [4]. Such anaerobic

conversion processes have lower energy requirements than aerobic treatment [5]. The  $\text{CH}_4$  produced, or more precisely, the gas product mixture of  $\text{CH}_4$  and carbon dioxide produced, can be used directly in co-generation of heat and power, or upgraded to almost pure  $\text{CH}_4$  for introduction into a gas distribution grid [6].

The continuous stirred tank reactor (CSTR) is the most common type of reactor used in anaerobic digestion of heterogeneous biomasses, including e.g. municipal solid waste. In CSTR systems the micro-organisms move freely in the reactor liquid, albeit often as flocs, and most of the degradation of the organics therefore occurs in the liquid phase of the reactor [7]. The disadvantages of these types of systems are evident, particularly at low hydraulic retention times (HRTs), where the

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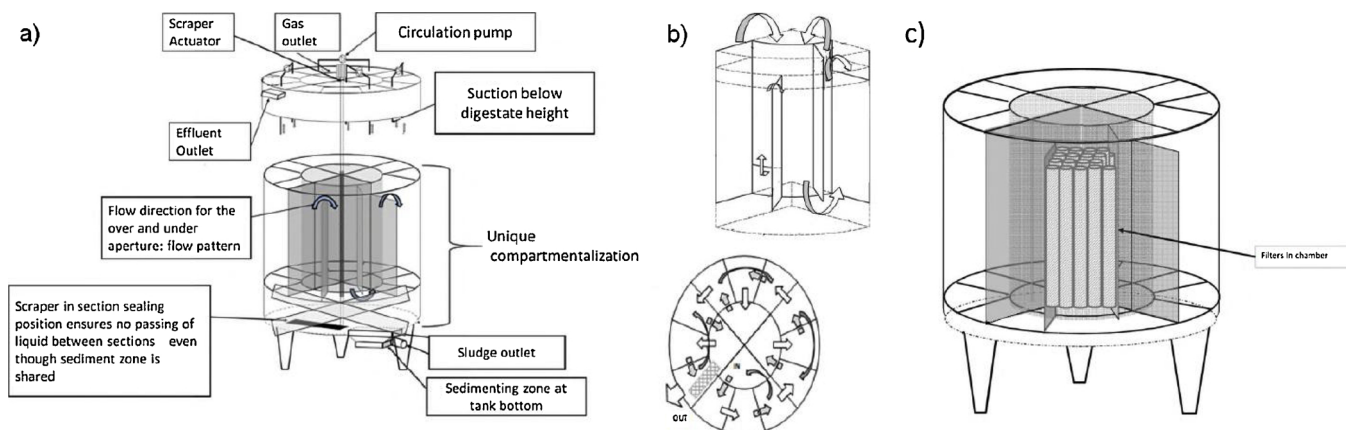


Fig. 1. a) Schematic diagram of the pilot scale FAD reactor. b) Schematic diagram of the flow pattern in the FAD reactor c) Placement of carriers inside the FAD reactor: Top image within a single compartment; bottom image throughout the four compartments in the reactor. Grey arrows indicate underflow; White arrows indicate overflow; Black arrows correspond to pump recirculation.

active biomass is flushed out of the reactor when the HRT becomes lower than the time it takes to regrow the microbial consortium in the reactor. Such digester flush out is often accompanied by acidification of the reactor medium due to buildup of volatile fatty acids and other intermediate products that the methanogenic populations are unable to process sufficiently rapidly [8]. To avoid these limitations, immobilization of the microbes to produce concentrated biofilms that do not flush out has been in focus in the design of anaerobic reactor systems. Immobilization of microbial cells via biofilm formation is based on the natural ability of microorganisms to aggregate and/or attach to solid supports [9]. With immobilization of the microbial cells, anaerobic processes can maintain up to one magnitude higher concentration of microorganisms per reactor volume than reactors having the microbes flowing freely in the reactor liquid [10]. With immobilized cells it is moreover possible to decouple the retention time of the microbes from the overall HRT of the conversion process. Very compact and dense biofilms are presumed to lead to mass transfer limitations that prevent fast nutrients exchange in the bioreactor. However, the available data infer that the microbial biofilm populations and the resulting close physical proximity between the immobilized cells actually do permit fast nutrient exchange within the biofilm, which favors syntrophic interactions of the communities to accomplish degradation of both complex and simple organic substances [11]. The most common example of a classic anaerobic biofilm reactor is the up-flow anaerobic sludge blanket (UASB) reactor. In the UASB reactor design concept, the capacity of microbes to spontaneously organize into free granular aggregates is exploited, but the granular aggregates are kept separate from the liquid effluents of the anaerobic degradation process thus reducing the flush out and maintaining a dense microbial population in the reactor. However, despite significant advances, including different variations of the original UASB design concept [12], the UASB processes are limited to treat thin and homogeneous substrates containing low levels of suspended solids that do not affect the natural granulation of the sludge.

To overcome the disadvantages of reactors based on granulation, various improvements in reactor design have been reported. The improvements mainly concern biofilm based anaerobic digesters that contain inert supports of different types of materials inside the reactor. These reactor designs are categorized as random or fixed depending on whether the biofilm supports are distributed randomly, or organized in stable positions [13]. In the random immobilization type of design, the biofilm carriers are not distributed in a precise pattern inside the reactor, whereas designs with fixed (or stationary) biofilms have the biofilm support materials placed in a precise configuration pattern that allows the feed stream flow to be directed to maximize its contact with the immobilized microbes. Fixed film reactors can withstand higher

concentration of particulate solids with lower risk of clogging than other types of anaerobic digester reactors [14].

A common type of substrate with high concentration of particulate solids and high chemical oxygen demand (COD) is municipal solid waste [15]. This type of substrate mainly consists of waste collected from households, but includes garbage from shops, restaurants, offices, and public institutions [16,17]. This waste is a mixture of food waste, paper, cotton, various types of trimmings, glass, metals, and plastics, and the organic fraction of this waste, abbreviated OFMSW, has recently been estimated to account for almost half (46% by weight) of the total waste stream [16]. This content of easily biodegradable material combined with the option of simultaneously minimizing waste makes municipal solid waste an attractive candidate for energy production through anaerobic digestion. None of the existing high-rate digesters have until now been utilized to process the entire stream (leachate and residual solids) of liquefied OFMSW [15].

With the objective of maximizing the efficiency of biogas production during municipal solid waste conversion, i.e. maximizing  $\text{CH}_4$  yield and minimizing the HRT on as high a COD loading as possible, we here assess the performance of a new anaerobic digester design. This novel reactor design comprises internal compartmentalization, controlled flow pattern within the compartments, and microbial biofilm immobilization on fixed carriers having a defined orientation inside the reactor. We examine the performance in pilot scale of this novel fixed-filter anaerobic digester (FAD) reactor design for direct anaerobic treatment of enzymatically pre-hydrolyzed OFMSW with suspended particles.

## 2. Materials and methods

### 2.1. Reactor design

This study was conducted on a pilot scale jacket heated FAD digester of 240 L (media volume). The reactor is cylindrical in shape and is compartmentalized into four equally dimensioned sections (quarters) by inner walls of stainless steel. Each of the sections have further internal divisions each totaling four subsections separated by baffles (Fig. 1a). Substrate filling and effluent withdrawal are accomplished via peristaltic pumps, and mixing is ensured by liquid circulation within each of the separate compartments. In this study the recirculation pump flow was set to  $300 \text{ L h}^{-1}$  to achieve a flow velocity of approximately  $3.12 \cdot 10^{-3} \text{ m s}^{-1}$  within each chamber. Differences in the height and depth of the baffles within each subsection have been designed to help control the direction of the flow. Additionally, the design includes an open sedimentation zone at the bottom of each compartment of the reactor, enclosed by the positioning of a scraper at the same position as

**Table 1**

Composition of the enzymatically pre-hydrolyzed organic fraction of municipal solid waste (OFMSW) used in this study.

Component	Measured value
COD	101 ± 2.6 g/L
Soluble COD	63 ± 2.7 g/L
Total solids	8.6 ± 0.70 % weight/weight
Volatile Solids (VS)	6.9 ± 0.70 % weight/weight
Suspended solids	3.0 ± 0.04 % weight/weight
Ash	1.7 ± 0.09 % weight/weight

the inner walls dividing the reactor (Fig. 1a). In the present study, the fixed filters used for the biofilm immobilization encompassed 8 wide-meshed tubular polyethylene carriers type 150 (Bio-Blok® Expo-Net, Hjørring, Denmark) that were positioned vertically between the baffles. The fixed filters occupied 20% of the total reactor volume and the specific surface was 150 m<sup>2</sup> m<sup>-3</sup>. The length of the tubular carriers matched the height of the inner walls of the baffles. The reactor was maintained at 50 °C throughout the whole experiment via use of a thermostatically controlled water bath.

During operation the feed was introduced into the first compartment of the reactor and left the reactor after the liquid had been routed through all the sections (Fig. 1b). The suspended particles that precipitated into the bottom space of the compartments were withdrawn from the process after the scraper operation by means of an eccentric screw pump. The pump was located beneath the reactor posterior to a deeper sedimentation zone in the fourth compartment where the effluent leaves the reactor. To ensure anaerobic conditions the reactor was sealed with fittings and rubber sealing. The levels of biogas that evolved during the anaerobic digestion process were monitored by a gas flow device obtained from Bioprocess Control AB (Lund, Sweden).

## 2.2. Composition and analysis of the feed

The FAD reactor was fed with enzymatically pre-hydrolyzed municipal solid waste, OFMSW, supplied by Renescience A/S (Ørsted A/S, Fredericia, Denmark). The initial seed (inoculum) used in this investigation came from Foulum Biogas, a plant in Denmark treating manure and fiber rich residues from agricultural straw. The feed-in was supplied hourly from an agitated storage tank, which was filled every day to avoid undesired degradation. The substrate was stored at 4 °C throughout the experimental period. The Total Solids (TS), Volatile Solids (VS) and ash were analyzed according to the standard protocol from the US National Renewable Energy Laboratory [18]. Suspended solids were also determined gravimetrically after filtration through Whatman Grade 934-AH RTU filters. Chemical oxygen demand (COD) and volatile fatty acids (VFAs) in the digestate were quantified using the Hach LCK514 COD and LCK365 Organic Acid cuvette tests and photometric measurements using a DR 3900 spectrophotometer (Hach, Düsseldorf, Germany).

Gas composition was determined via gas chromatography, (model GC82 Mikrolab Aarhus A/S, Denmark). Quantification of VFAs in the substrate was done by HPLC equipped with an RI detector using a Biorad HPX-87H column (Hercules, CA; USA) at 63 °C with 5 mM H<sub>2</sub>SO<sub>4</sub> as eluent at a flow rate of 0.6 mL min<sup>-1</sup> [19].

## 2.3. Strategy and operation of the FAD pilot scale system

During the development studies of the FAD reactor performed in

laboratory scale, the initiation period was estimated to last approximately 60 days. During this period, the biofilm presumably develops from the initial seed, as it perceives a steady increase of the organic load. The strategy involved commencing the feed rate at an HRT of 50 days, increasing the daily in-take while maintaining a threshold of VFAs below 2 g L<sup>-1</sup> to avoid organic overloading. The biofilm initiation phase was finalized when the system achieved a HRT of 10 days, a point described as critical for microbial flush-out [20]. After completion of the initiation, the feed-in flow was halted and was then re-commenced after 3 days at the most recent flow rate, to verify the biofilm formation and its robustness. Thereafter the organic load was increased steadily until reaching the inflection point of productivity. At the inflection point, an increase of the organic load does not result in an increase of the productivity for the studied process. Following the initiation of the biofilm, the organic load was increased to assess the yields and productivity of this reactor at lower HRTs treating pre-hydrolyzed OFMSW. The experiment lasted 211 days from the seeding day.

## 3. Results

### 3.1. Substrate and inoculum characterization

The pH of the pre-hydrolyzed OFMSW was approximately 4, ranging from pH 3.8–4.6 during the experiment, and the OFMSW was characterized by having a high COD of which ~60% was measured as soluble (Table 1). From the quantification of metabolites in the soluble fraction, 43.2 ± 12.3 g L<sup>-1</sup> were analyzed as VFAs, accounting for approximately 60% (by weight) of the measured VS (Table 1). This level of solubilized VFAs was consistent with the low pH of the feed and most likely due to some metabolism of acidogenic bacteria taking place during the pre-hydrolysis treatment. In the substrate, some larger particles consistent with food residues, although not larger than 3 mm, were also observed. Characterization of the seed material showed that it had pH of 8.1 with 0.41 g L<sup>-1</sup> of VFAs (data not shown).

### 3.2. The initiation period

At the beginning of the experiment, the reactor was inoculated with seed material that was recirculated through all the compartments while achieving the process temperature of 50 °C. Before the first feed-in at the planned feed flow regime, the reactor was fed with substrate pulses of 5 L d<sup>-1</sup> to test the inoculum health and ensure that the reactor system was tight. The first gas production was registered on day 3 after inoculation. From then on, the flow rate of the reactor was increased gradually from a HRT of 50 days (Fig. 2). Previous lab scale experiments had shown that the inoculum was fit and acclimatized to convert a substrate with the characteristics of the pre-hydrolyzed OFMSW, which is consistent with the rapid evolution of gas production. There was no significant fluctuation in the composition of the substrate during the entire experiment (data not shown). The gas production and the COD balance were used as indicators to monitor process stability.

During the initiation period, the process was operated for 56 days until the nominal flow rate of 24 L<sub>substrate</sub> day<sup>-1</sup> was achieved. It was possible to induce a rapid decrease of the HRT (Fig. 2). The VFA pool was maintained below 2 g L<sup>-1</sup> by actively trimming the flow rate, which explains the fluctuations in the HRT recorded over time (Figs. 2 and 4). During the initiation phase, from a HRT of 50 days at day 1 until achieving an HRT of 20 days at day 26 (Fig. 2), the average CH<sub>4</sub> yield was calculated to be 0.266 L<sub>CH<sub>4</sub></sub> g<sub>COD</sub><sup>-1</sup> (Fig. 3). Surprisingly, the methane yield from day 26 through 56 remained at a similar level and was calculated to be 0.273 L<sub>CH<sub>4</sub></sub> g<sub>COD</sub><sup>-1</sup> (Fig. 3). This constant yield level verified that the compartmentalized reactor could perform robustly despite the rapid increase of the substrate flow rate.

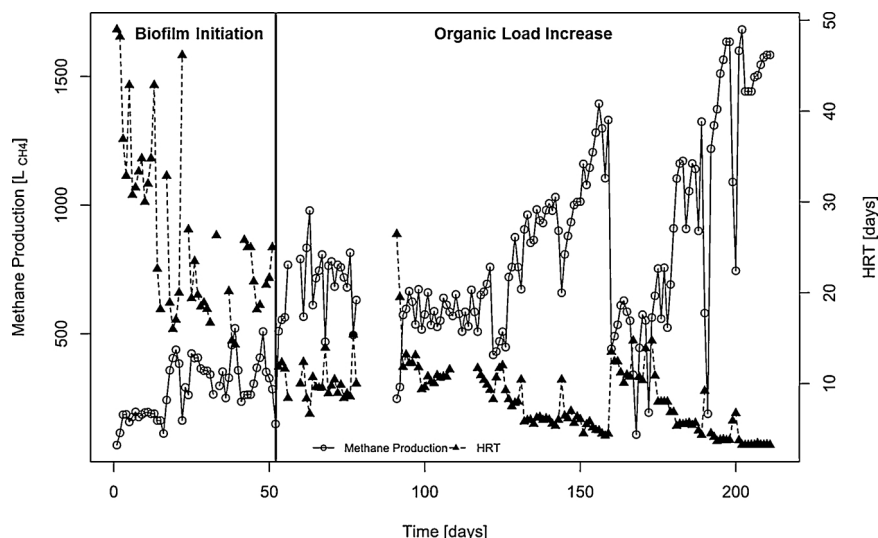


Fig. 2. Evolution of daily methane production (circle symbols, liters ( $L_{CH_4}$ )) and Hydraulic Retention Time (triangle symbols, HRT, days) with time (days).

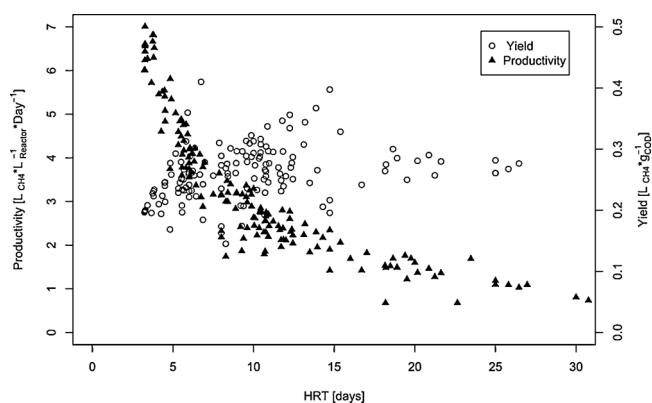


Fig. 3. Methane yields ( $L_{CH_4} g_{COD}^{-1}$ ) and Volumetric Productivity ( $L_{CH_4} L_{reactor}^{-1} day^{-1}$ ) during the experimental phase versus HRT (days).

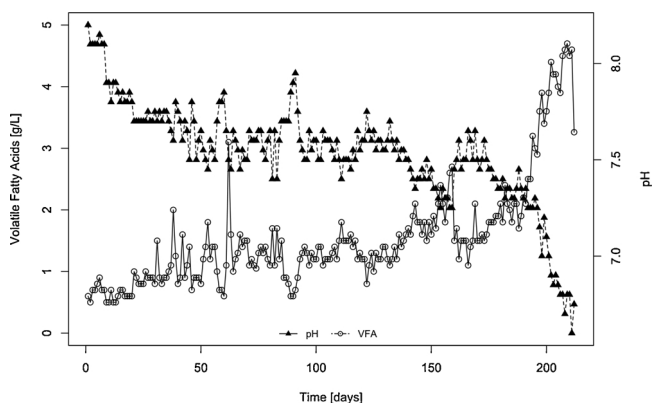


Fig. 4. Evolution of pH and VFA concentration levels ( $g L^{-1}$ ) during the experiment.

### 3.3. Reactor performance

#### 3.3.1. Yields

After reaching a HRT of 10 days at day 56 of the experiment, the feed-in was deliberately halted for 3 days in order to verify the biofilm formation. At this time, the VFA concentration dropped to  $0.8 g L^{-1}$  (Fig. 4). After the feed restoration, the FAD reactor produced the same yield of around  $0.27 L_{CH_4} g_{COD}^{-1}$  as previously registered after 4 days

(day 63). Thereafter,  $CH_4$  production was  $700\text{--}720 L_{CH_4} (\sim 3 L_{CH_4} L_{reactor}^{-1} day^{-1})$  during the 60–80 days ensuing with an average HRT of 10 days (Fig. 2). At this time, the VFA concentration peaked by exceeding  $3.1 g L^{-1}$ , while the pH had dropped to 7.4 (Fig. 4). Within the next days, the VFA pool returned to a level of  $1\text{--}1.5 g L^{-1}$  (Fig. 4), confirming the rapid ability of the reactor to recover its performance. After achieving a HRT of 10 days, the feed flow rate was increased to assess the yields under that regime. After this period the organic load was gradually increased achieving a COD conversion higher than 70% of predictable at an organic loading rate of  $20.8 g_{COD} L_{reactor}^{-1} day^{-1}$ . From the results obtained it was evident that the methane yields ( $L_{CH_4} g_{COD}^{-1}$ ) remained almost constant at lower HRTs (Fig. 3).

#### 3.3.2. Productivity

During both the start-up and the organic load increase phase, the productivity increased steadily with decreasing HRT until a remarkable methane production of almost  $6 L_{CH_4} L_{reactor}^{-1} day^{-1}$  was achieved (Fig. 3). It was possible to maintain this high production regime for 8 days in a row as evident from the  $CH_4$  levels achieved during the last days of the experiment (Fig. 2).

However, when the flow rate was increased further after day 200, the reactor was not able to sustain the high productivity and a rapid decrease of the gas production was observed. Hence, at day 203 of the experiment, the gas production leveled off after the flow rate was increased to  $80 L_{substrate} day^{-1}$ , equivalent to an HRT of 3 days (Fig. 2). The most likely explanation for this productivity limit is acidification of

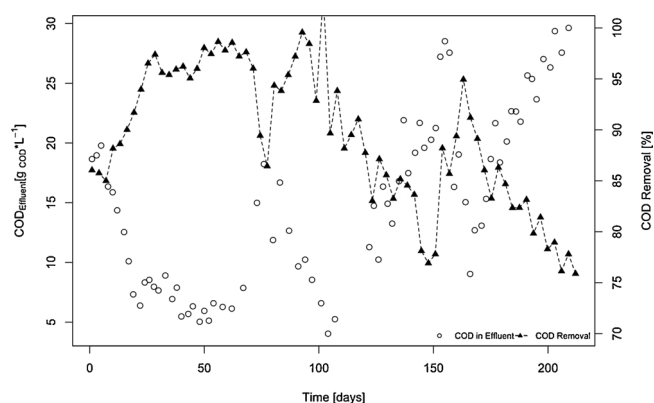


Fig. 5. Progress of COD removal (%) and COD levels in the effluent ( $g_{COD} L^{-1}$ ) during the course of the anaerobic digestion (days).



the reactor media because at this regime, the pH of the reactor media fell to less than pH 7 (Fig. 4) which thus appeared to have a detrimental effect on the methanogenic activity, leading to the process collapse.

It was possible to diagnose the process failure and stop the feed pump at a load of 40 liters.

#### 4. Discussion

During the treatment of complex substrates utilizing fixed film anaerobic digesters, in particular biomass substrates characterized by high concentrations of suspended particles, clogging of the filters is probably the most common problem encountered. The saturation of filters may then lead to further complications such as channeling inside the reactors, accumulation of solids in certain zones of the reactor, and blockage of the recirculation pumps. The FAD reactor features a combination of design elements not described before, that seem to contribute to avoiding this kind of complications. The compartmentalization of the reactor with the introduction of inner baffles permits a controlled flow of the digesting liquid parallel to the surface of the filters where the biofilm develops (Fig. 1b). The digestate leaving the reactor is liquid, and before it leaves the reactor it has passed through all the sections in a directed flow pattern. For this reason, there is an improved retention of unprocessed material before the liquid digestate leaves the process.

The pilot reactor design studied here had four pumps actively recirculating the media through each section. The suction and discharge points of the recirculation pumps were positioned in the upper part of the reactor and the connection points (Fig. 1a). The overhead positioning of these elements may also be favorable with respect to preventing clogging since the heaviest particles evade the flow stream as they remain at the bottom of the reactor. Even though most studies for fixed film reactors deal with thin substrates, the operation of a uni-directional flow can lead to problems with clogging [21]. The active discharge of liquid from the top from each recirculation stream provides for an additional source of mixing in the absence of mechanical agitation. In high-rate anaerobic digestion processes, it is also assumed that the gas bubble dispersion can contribute to the process by promoting local back-mixing. When considering scale-up of the reactor, the dimensioning of the pump capacity can be maintained constant by constructing additional compartments, maintaining similar flow regimes.

In the reactor type investigated here, the compartmentalization design and the flow regime had been carefully designed to foster an improved transport of substances and to maximize the contact between the liquid feed stream and the immobilized cells in the fixed biofilm causing both improved colonization [22] and favorable conversion. The data obtained with the high flow rates of more than  $3 \cdot 10^{-3} \text{ m s}^{-1}$  within each chamber suggest that the intended desirable mass transfer was achieved. Modeling of the nutrient and metabolites flow in the liquid and notably inside the biofilms are required, however, to fully verify the conversion dynamics and to substantiate the theorem that an optimal mass transport of nutrient flow is a determinant factor affecting the performance of the reactor. In this reactor design, it is also possible to recirculate the liquid streams within the four compartments. In standard operation, the liquid recirculates through the baffles of each section and overflows to the next dictated by the flow rate of the feed (Fig. 1b).

The type of carrier medium may significantly affect the attachment and detachment properties of the microbial biofilm [23]. The polyethylene carriers employed in the FAD were selected as support for biofilm growth as this material enhances rapid adhesion of microbial cells. This carrier is moreover characterized by a threaded pattern and a rough surface, described to be important since void materials have been shown to be less prone to clogging since it is easier to remove the excess of solids accumulated by simple process shear forces [24]. Furthermore, the threaded structure of the carrier allowed horizontal dispersion of

the liquid between the filters, therefore increasing the access of nutrients to the biofilm that inevitably forms behind the tubular carriers. At the termination of the experimental phase, the FAD reactor was drained to assess whether there were particles clogging the filters. By visual inspection it was confirmed that all the filters had been exposed to the digesting media without obstruction, since it was possible to see the bottom of the reactor.

The findings that the methane yields ( $\text{L}_{\text{CH}_4} \text{ g}_{\text{COD}}^{-1}$ ) remained almost constant at low HRTs (Fig. 3) were unexpected. A slight drop-in yield was predictable since at those regimes, there may be a flush-out of organic compounds that have not been digested. The ability of the compartmentalized FAD reactor to maintain high COD converting efficiencies (Fig. 5) despite the rapid flow rates, i.e. low HRTs, is remarkable, and the yields attained signifies a distinct performance of the reactor compared to what has been reported by others for single stage digestion of OFMSW [20,25]. Different studies performed on similar substrates indicate that the maximum organic loading rates for anaerobic digestion of this type is in the range of  $10.5\text{--}17 \text{ g}_{\text{COD}} \text{ L}_{\text{reactor}}^{-1} \text{ day}^{-1}$  and the data suggest that higher organic loadings may lead to irreversible acidification [25,26]. In this experiment it was possible to operate at even higher concentrations of organics without compromising COD loss greater than 30%.

In Fig. 5, it can be seen that the COD removal at the highest retention times (from day 10–70 and day 80–100 in the experiment) is larger than 90%. The concentration of COD in the effluent is in correspondence with the data obtained from the gas production and the yields perceived during the experimental phase. At the highest rates, a decrease in the methane yields is observed that parallels the increasing concentration of COD in the media, especially when the in-flow rate is the highest. To our knowledge, this is the first study in which it has been possible to digest the whole slurry of OFMSW in a high rate reactor without any type of previous separation.

Another important aspect of the reactor design employed in the present study is the scraper. By actuation every 5th day, it effectively removed large quantities of the deposited solids. This removal prevented clogging and channeling in the reactor compartments, permitting the digestion media to be recycled efficiently whilst maintaining the high area of contact between the microbial biofilms and the liquid. The solids that were evacuated via the scraper through the eccentric snake pump were predominantly suspended solids having more than 30% ash content. The operation of the scraper at the bottom of the FAD reactor may be optimized to remove or retain certain sedimentable particles from the anaerobic process depending on the nature of the biomass and on the hydrolysis rate of the particulate dry matter at the chosen retention time. If the substrate contains high levels of inert material, such as the substrate used in the present study, it will be convenient to evacuate the sedimented sludge to reduce plugging of the reactor compartments and avoid the decrease of active volume. In the present work, the scraper was operated every 5 days removing 1% by volume of the reactor's volume content estimated to be close to the inert content at an HRT of 10 days.

#### 5. Conclusion

The yields of  $0.2\text{--}0.3 \text{ L}_{\text{CH}_4} \text{ g}_{\text{COD}}^{-1}$  and productivities of  $2\text{--}6 \text{ L}_{\text{CH}_4} \text{ L}_{\text{reactor}}^{-1} \text{ day}^{-1}$  achieved at HRTs below 10 days with the pilot scale FAD reactor fed high COD OFMSW was better than all other anaerobic digestion data reported. The biofilm developed during the first two months of reactor operation. The biofilm could recommence production after 3 days of ceased inflow at an HRT of 10 days, confirming robust attachment of active microorganisms. The compartmentalization and flow regime allowing efficient feed-immobilized biofilm contact plus other distinct reactor design features allowed for high efficiency and lengthy operation without clogging problems.

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## Conflict of interest

All authors declare no conflict of interest. J.E. Gonzalez Londoño and B. Uller are inventors of a patent related to methods and reactor design for anaerobic digestion. Authors J.E. Gonzalez Londoño, B. Uller, and H.R. Sørensen are employed at Ørsted (formerly DONG Energy), a Danish company which develops, constructs, and operates bioenergy plants.

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# Microbial diversity of a high performance fixed biofilm biogas reactor

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## Abstract

Intensification of anaerobic digestion (AD) for bioenergy production is possible by use of high-rate systems such as fixed film biogas reactors. Fixed film reactor systems are generally more productive than traditional Continuous Stirred Tank Reactors (CSTRs) since the microbes are immobilized as dense biofilms. However, knowledge about the microbial diversity and the dynamics of the populations in this type of reactor system is still lacking. In order to assess any possible specialization of the microbial biofilm communities by stage-wise conversion during the digestion process, an examination of the microbial diversity during start-up and steady-state (4 days Hydraulic Retention Time (HRT)) of a 3 reactor cascade of a fixed film biogas system was set up. The substrate was an enzymatically pre-treated Organic Fraction of Municipal Solid Waste (OFMSW). The microbial diversity investigation was based on a 16S rRNA gene analysis of bacteria and archaea and included analytical distinction between the microorganism immobilized in the biofilm and those remaining "free-floating" in the liquid medium. The effect of transition from mesophilic to thermophilic temperature range was also studied. The results confirmed that the fixed film reactor is able to sustain high yields of biogas production at high flow rates and organic loads. The temporal analysis of the microbial diversity showed that the communities in the biofilm and in the liquid medium are essentially similar and no specialization was evident in the three reactors in the cascade. However, the composition of the populations changed in response to changes in HRT and temperature in the process. During mesophilic AD, *Methanoculleus bourgensis* and *Methanoculleus palmolei* were the most dominant methanogenic species in the reactors. At the same time, an unclassified member of the Family Bacteroidetes, which appears to be a syntrophic bacterium, was the most dominant type of bacteria present. After transition to the thermophilic range (51° C) at the end of the run, the populations dramatically shifted in both biofilm and medium. At higher temperatures, *Methanoculleus thermophilus* and unclassified members of *Firmicutes* and *Clostridia* were the most abundant. The results indicate that methane production mainly proceeds via the hydrogenotrophic pathway. This analysis of the microbial diversity provides a new understanding of the dynamics of the microbial populations and AD fixed film reactor resilience in high-rate conversion of enzymatically treated OFMSW.

## Introduction

Anaerobic digestion (also referred to as biomethanation) represents a feasible strategy for producing renewable bioenergy from a variety of waste streams. The bioenergy produced is biogas that can be employed directly for electricity generation or be refined into pure methane. Nowadays, bioenergy production has gained special interest as a replacement for the incommensurate usage of fossil fuels, for example to sustain distinct societal activities such as transportation and heating. The absence of oxygen in the anaerobic digesters stimulates the selection of microorganisms, namely bacteria and archaea, that can degrade organic compounds to form products that are substrates for the methanogenesis accomplished by methanogenic archaea (*Stams and Plugge (2009)*). The degradation begins when hydrolytic bacteria depolymerize the largest carbohydrates, proteins and fats to sugars, amino acids and long chain fatty acids, respectively (*Shah et al. (2014)*). The hydrolysis is driven by the secretion of hydrolytic enzymes to the media, solubilizing organic monomers and dimers (*Weiland (2010)*). This initial break down of the polymers is then followed by acidogenesis, where acidogenic bacteria convert the degraded products into short organic acids. In these reactions, acetate, hydrogen and carbon dioxide is produced and serve as substrate for the methane producing archaea. When longer, and electron-rich organic acids are produced, such as lactate, propionate or butyrate, there must be a conversion into acetate by acidogenic communities before these compounds can be transformed into methane gas *R. Bengelsdorf et al. (2015)*). Because of these complicated reaction dependencies, detailed insight in the microbial diversity and their roles in the anaerobic reactors is an important tool to understand the conversion of organic matter in anaerobic processes.

Process intensification in anaerobic digestion processes has led to the implementation of reactors where the microbial communities are immobilized on suspended or fixed carriers (*Tauseef et al. (2013)*). This immobilization is achieved through microbial biofilm formation. The biofilms form by cellular assemblages that attach to surfaces followed by secretion of extracellular polymeric substances (EPS) (*Langer et al. (2014)*). Microbial immobilization provides many process advantages such as protection of the communities from process imbalances, e.g arising by increased concentrations of toxic compounds. Further, a higher cell density (*Qureshi et al. (2005)*) of microorganisms can be kept in the biofilm layer, which can be one magnitude higher than the cell levels in the liquid phase (*Langer et al. (2014)*). The EPS layer may be a diffusion barrier for nutrients exchange, but provides mechanical support (Sutherland, I. W., 2017). Moreover, the biofilm structure allows retention of enzymes as well as the adhesion of the substrate (*Orell et al. (2013)*). This is of great relevance to high rate processes with high flow rates, since it is possible to retain dense communities inside the reactors, thus avoiding flush-out and decrease in the production efficiency.

Different studies have intended to describe the dynamics and characteristics of the microbial populations in immobilized systems. For instance, *Fernández et al. (2008)* described the initial stages of biofilm formation. The findings showed a constant microbial diversity when the reactor achieved steady state, whereas the diversity fluctuated during the development period, when the biofilm matured. In similar studies, changes in the microbial populations have been monitored in response to varying operational parameters of the anaerobic digestion such as the hydraulic retention time (HRT) and the organic loading rate (OLR) (*Vasquez et al. (2018)*; *Goux et al. (2015)*). In these studies, the microbial dynamics have been found to play an important role, since at shortened HRTs, the fastest growing populations and those attached to the biofilm are established in the reactor. Other operational parameters such as pH, temperature and solute concentrations have also been reported to have large effects on the microbial distribution (*De Vrieze et al. (2015)*).

Anaerobic digesters based on fixed carrier biofilm immobilization depend on the attachment/detachment maintenance of the biofilm. The performance relies upon the initial inoculum and the carrier material employed, but also on the shear forces and the perturbations generated by the flow (*Habouzit et al. (2011)*). To our knowledge, none of the available studies has described the dynamics of microbial diversity taking into consideration the distribution of microorganisms in the digesting media in a biofilm based reactor. However, a relationship between the substrate composition and the populations present in the reactors in anaerobic digestion processes has been described (*Zhang et al. (2014)*). The co-occurrence of bacterial and archaeal families is necessary to carry out the decomposition of organic matter into methane (*De Vrieze et al. (2016)*). In the case of biofilm reactors, this becomes more relevant since the vicinity of the communities facilitates syntrophic interactions between them (*R. Bengelsdorf et al. (2015)*).

Recently, advances in high-throughput sequencing, lower cost and abounding databases have allowed easier analysis of complex microbial communities (*Caporaso et al. (2012)*). Following analysis of ribosomal RNA gene sequences extracted from the complex samples, it is possible to determine the predominant microorganisms involved in specific conditions and stages of the process. This is useful for understanding the role of the different microbial groups, and for identifying the most likely metabolic pathways in play during the conversion of organic matter into methane for a given process and to track possible imbalances (*Goux et al. (2015)*; *Cai et al. (2016)*). In the present study, we examined the performance and microbial diversity in a series of 3 fixed biofilm reactors positioned in a cascade for anaerobic digestion of an enzymatically pre-hydrolyzed OFMSW. We investigated the possible alterations in populations during the start-up phase and at steady state at high organic load, making a distinction between the communities that settle in the biofilm and those dispersed in the media. We also examined the effects on the process caused by rapid shift from a mesophilic to a thermophilic temperature range, and the microbial diversity across the different reactors in the cascade series.



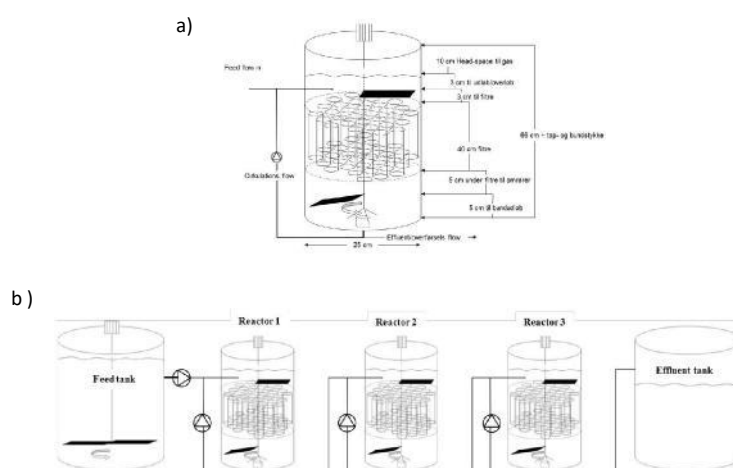
## Materials and Methods

### Reactor Settings

This study was conducted in a cascade of three fixed biofilm reactors with fixed orientation consisting of 10 liter tanks made from stainless steel (8 L media volume). Filters were positioned vertically in each reactor to act as carriers and support the biofilm growth. These carriers are wide-meshed, tubular polyethylene filters, type 150 BIO-BLOK (EXPO-NET Danmark A/S, DK). There were 9 filters of 40cm length in each reactor. An agitator with two rotating blades was placed in the center of the filters, allowing mixing in the volume beneath and over the filters. The digesting media was recirculated in each of the reactors via a peristaltic pump from the top to the bottom of the reactors, at a recirculation rate of  $43.8 \text{ L} \cdot \text{h}^{-1}$ . A plug-flow in the cascade was confirmed with a retention time distribution test using methylene blue. Figure 1 shows the reactors configuration. The substrate, enzymatic pre-hydrolyzed OFMSW was supplied by Renescience A/S, Denmark. This substrate was stored frozen until utilization in a 50L agitated reservoir tank and pumped into the first reactors of the cascade. By volume displacement, the feed flow regime ensures the movement of media within the reactors of the cascade, from the first to the last, until the effluent exits from the process and was stored in a sealed tank. The temperature was controlled through a water bath recirculated through the reactors heating jacket. The experiment was conducted in duplicate in two independent reactor cascades, denominated System 1 and 2. The unique disposition of the reactors is intended to investigate the performance and the microbial diversity of each independent reactor in the cascade during the treatment of the pre-hydrolysed OFMSW.

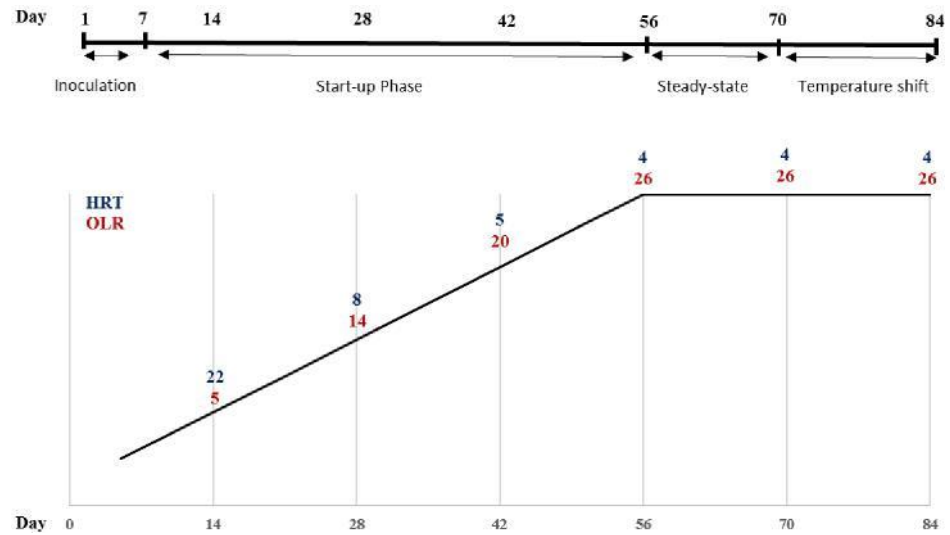
### Reactor Operation

The experimental setup consisted of 4 phases, namely inoculation, ramp-up, steady phase and temperature shift. At the beginning of the experiment, the reactors were seeded with inoculum from Foulum Biogas, a plant in Denmark treating manure and fiber rich residues from agricultural straw. The seeding time was set to 5 days at mesophilic temperature range,  $37^\circ$ . After this period, the substrate flow was increased to stimulate biofilm formation. This ramp-up period took 43 days until a nominal flow of 2 liters was achieved, this corresponds to a HRT of 4 days in the single reactors and 12 days overall on each system. Previous feasibility investigations showed that it is possible to ensure robust biofilm development in less than 2 months. There was a cease in the feed for 5 days to test the biofilm robustness and the flow was reintroduced again at a flow rate of  $2 \text{ L} \cdot \text{day}^{-1}$ . At day 56, the steady state phase period began, and the same flow rate was maintained for 14 days. In this period, more than one entire retention of the digesting media was cleared. The temperature range was then varied from the mesophilic to the thermophilic range at  $51^\circ \text{ C}$ . The temperature transition lasted approx. 4 hours. The systems were fed at the same flow rate as in the steady-state, (4 days HRT in a single reactor), replacing once more the entire digesting medium for at least one retention more.



**Figure 1.** Diagram of the reactors used in this study. a) a single reactor b) The three-reactor cascade system.

The timeline of the experimental phase is shown in figure 2 with the corresponding HRTs and ORLs. The gas evolution from each single reactor was independently measured by a gas flow device (Bioprocess Control, Sweden). The biogas quality was determined via gas chromatography (model ML GC82 from Mikrolab Aarhus A/S with a thermal conductivity detector (TCD) and the carrier gas  $\text{N}_2$  at  $70^\circ \text{ C}$  (packed column 6 ft x 3 mm Porapak Q column).



**Figure 2.** Experimental phases and timeline of the laboratory scale experiment.

The days shown in the timeline are those when the biofilm samples were withdrawn. In blue Hydraulic Retention Time (HRT) and in red Organic Loading Rate (OLR) at the sampling days.

## Sample Analysis

Sampling was performed on the digesting media and to the biofilm attached to the carriers. To facilitate the sampling, small sections of the polyethylene carriers were attached in the top of each cylindrical tube and removed from the reactor and stored immediately after collection in phosphate buffer to avoid dehydration. The liquid media was collected from the recirculation stream of each reactor in the cascade and analyzed for chemical parameters 3 times per week. Total Solids (TS), Volatile Solids (VS) and ashes were determined by gravimetry following NREL guidelines (*Sluiter et al. (2008)*). Total Suspended Solids (TSS) were also determined gravimetrically after filtration through ashless Whatman Grade 934-AH RTU filters. Chemical Oxygen Demand (COD), total ammoniacal Nitrogen (tNH<sub>3</sub>) and VFAs were quantified with Hach LCK514 COD, LCK 304 and LCK365 Organic Acid cuvette test and DR spectrophotometer (Hack, Germany) for monitoring purposes. Quantification of single VFAs in the samples was performed by HPLC using a Biorad HPX-87H column (Hercules, CA; USA), RI detector, 63 °C and 5 mM H<sub>2</sub>SO<sub>4</sub> as eluent, at flow rate of 0.6 ml\* min<sup>-1</sup>. The pH was monitored online in the digesters.

**Table 1.** Chemical composition of enzymatically liquefied OFMSW

Component	Measured value
Chemical Oxygen Demand (COD)	103.9 g*l <sup>-1</sup>
Soluble Chemical Oxygen Demand (sCOD)	62.9 g*l <sup>-1</sup>
Total Solids (TS)	9 %
Volatile Solids (VS)	6.6%
Total suspended Solids (TSS)	4.0 %
Volatile Suspended Solids (VSS)	2.7 %
Volatile Fatty Acids (VFAs)	35 g*l <sup>-1</sup>

## Microbial analysis

Genomic DNA from the reactor's media was extracted using The PowerSoil® DNA Isolation Kit, (MO BIO Laboratories, USA) following the manufacturer's protocol. The first steps consist in samples preparation, followed by cell lysis and inhibitor removal. Thereafter the DNA is selectively bound to a silica filter, washed and finally eluted with 10 mM Tris buffer. DNA from the biofilm was extracted with the PowerBiofilm® DNA Isolation Kit (MO BIO Laboratories, USA). A piece of ca. 0.2 g of the carrier cut-off was introduced directly into the extraction Eppendorf tube. The extraction was performed analogously to the liquid media. The concentrations and purity of the samples were evaluated using NanoDrop 1000 spectrophotometer (Fischer Scientific, Wilmington, MA) Only samples with A260/ A280 ratios > 1.3 were used in further analyses.

## Preparation of 16S RNA gene amplicon libraries and sequencing

PCR amplification of the 16S rRNA gene from DNA extracted from the biofilm and media samples was accomplished using modified universal bacterial primer pairs F-(5'-CCTACGGGNGGCWG- CAG -3') and R-(5'-GACTACHVGGGTATCTAATCC-3') based on *Klindworth et al. (2013)* findings and an Archaea specific primer pair F- (5'-CCCTAYGGGGYGC-ASCAG-3') and R- (5'-GGCCATGCACYWCYTCTC- 3') as described in *Gantner et al. (2011)*. These primer sets target the V3-V4 and V3-V5 hypervariable regions of the rRNA gene, respectively. These include the incorporation of the Nextera XT®transposase sequence (Illumina Inc, USA) followed by an additional four random nucleotides N in both forwards and reverse primers. Amplicons were generated in a GeneAmp PCR System 2700 Thermocycler using the Q5®Hot Start High-Fidelity DNA polymerase (New England Biolabs Inc, USA).

The concentration of the PCR products was adjusted to 1ng µL<sup>-1</sup> before sequencing. The initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30s, 56°C for 40s and 72°C for 1.5 min. Positive (DNA) and negative (distilled water) controls were included in the PCR reactions. The DNA was submitted to Macrogen (Europe) for sequencing on the Illumina MiSeq™ platform. The sequences were analyzed using BION-meta software. BION is a supported open-source package for microbial diversity analysis of 16S rRNA (<https://app.box.com/v/bion/>). Sequences were treated as in *Klitgaard et al. 2017*). Briefly, the reads were quality filtered and chimera-checked before classification against the RDP-II database. The number of reads obtained for each barcode was normalized to 100,000 for further data treatment.

## Quantification of Bacteria and Archaeal populations

Denaturing Gradient Gel Electrophoresis (DGGE) was performed to quantify the archaeal abundance in the microbial population of the digesting media of the reactors of the first system during the steady-state. After extraction of the digesting media described earlier, two primer sets were used, for the assessment. Two different sets of primers were used to amplify the Eubacteria and archaeal 16S rRNA genes. Universal Eubacterial primers targeting V3-V5 region (F341-GC and reverse R907) were used for the total population, and for the archaea was performed a nested PCR approach using ArchF0025/R1517 and F344/R915-GC, respectively (*Sun et al. (2016a)*; *Raskin et al. (1994)*). All PCR reactions were carried out in a Mastercycler (Eppendorf, Hamburg, Germany) and the size of the product was visualized on a 1% agarose gel. Comparison of the band intensity between the samples regarding was determined using BioNumerics software v.7.1 (Applied Maths, Saint-Martens Latem, Belgium).

## Statistical analysis

Classified sequence counts were normalized to 100.000 before further analysis. Non-metric multidimensional scaling using Bray-Curtis distances was used to visualize multivariate patterns in each of the data-sets of the amplicon data. To compare the performance of the two systems, the values of the steady state were averaged and compared. A t-test was used to evaluate statistically significant differences (p<0,05).

## Results and Discussion

### Reactors performance

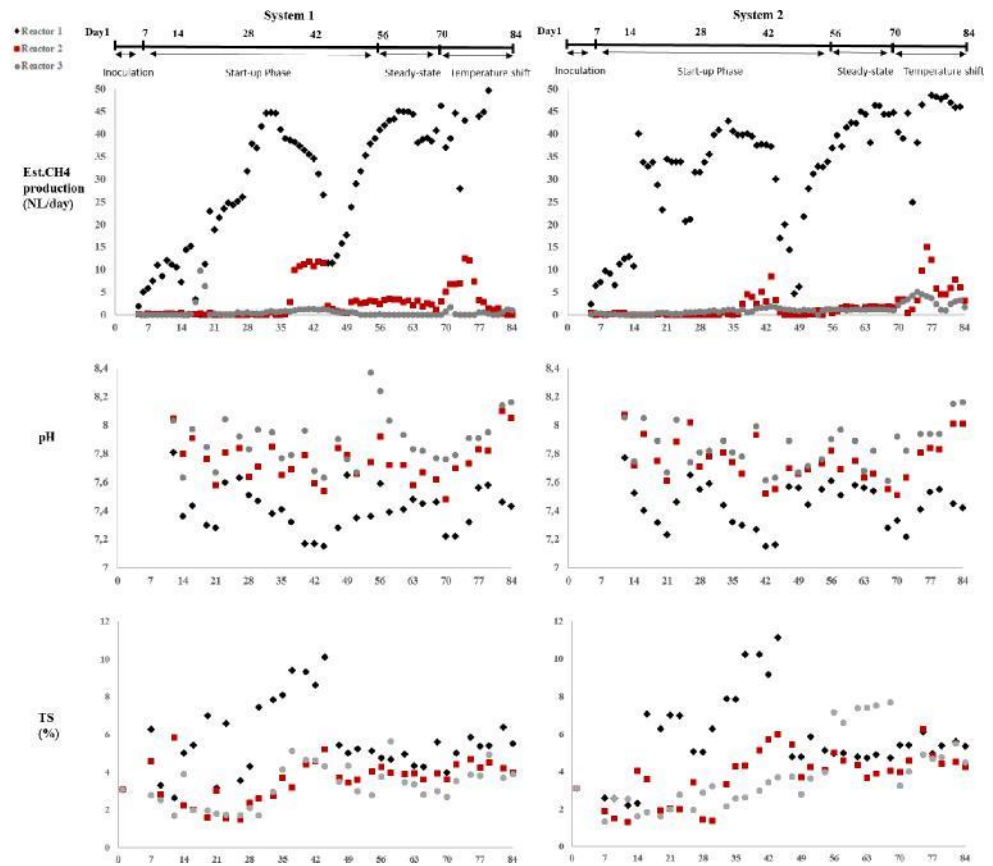
The chemical characteristics are summarized in Table 1. The inoculum was acclimatized to the reactors conditions during the inoculation period that lasted less than a week. Thereafter, the flow rate was increased and it was possible to perceive biomethane production already from the first day of the start-up phase (Figure 3). In this experimental set-up, the HRT was systematically decreased during the process. From the timeline in Figure 2, it is possible to see that already at day 14 of the experiment, the organic loading rate (OLR) is  $5 \text{ g COD} \cdot \text{l}^{-1} \cdot \text{d}^{-1}$ , corresponding to 22 days HRT. From the methane production graph in Figure 3, is perceived that the biogas production follows the flow rate despite the rapid decrease of HRT. However, during this ramp-up period, there was a build-up of total solids and COD (Figure 3 and 4). Both of the cascade systems were able to sustain steady biogas production until approx. day 32, but then the production reached a stagnant point where the productivity started to decrease slightly. This coincided with accumulation of VFAs in the first reactors of the cascade, hence explaining the decrease in methane production. Until this point, there has been exclusively methane production in these first reactors, but from this point on (day 35), some of the organics are passed to the reactors 2 and 3 in the cascade. Therefore, after day 35, there was methane production in reactors 2 and 3, up to  $10 \text{ l} \cdot \text{d}^{-1}$ . As expected, both systems behaved similarly.

The starting inoculum had a pH of 8.12. The pH values measured in the reactors during the experiment are shown in Figure 3. The first reactors of the cascade have a lower pH value than the second and third. During the start-up phase, the pH of the reactors decreases steadily until achieving approximately 7.2 in the first reactors. The remaining reactors had stable pH, with values oscillating between 7.6 and 7.9. There is a direct relationship between the concentration of VFAs in the reactors and the measured pH values in the reactor media.

At day 45, the feed-in was stopped for 5 days in both systems to verify the biofilm formation and to let the reactors consume the remaining VFAs in the digesting media. Quantification of the individual VFAs by HPLC confirmed there was a shift to propionic acid production up to around  $7 \text{ g} \cdot \text{l}^{-1}$ , meaning that propionate dominated almost all the VFA pool. Accumulation of propionic acid is often described as a detrimental process or imbalance in anaerobic digesters and a sign of hydrogen accumulation in the reactors (*Shah et al. (2014)*). However, at this point in the reactors the remaining organics could be converted into methane within the 5 days of feed cease and VFAs and total solids in the first reactors lowered to concentrations comparable to those in reactors 2 and 3 (Figure 3). The feed flow was then re-established rapidly from 0 to 2 liters per day (HRT 4 days) to commence the steady state phase at day 56.

At the steady state, it was possible to confirm the robustness of the communities attached to the biofilm. The concentration of solids and VFAs during this period were always below  $3 \text{ g} \cdot \text{l}^{-1}$  in all the systems. This indicates that the process unbalances observed during the start-phase had been overcome after the feed ceased in day 45. During the steady state, there was continuous production of biomethane yielding 301.4 and 348.2  $\text{l CH}_4 \cdot \text{g}^{-1} \text{ VS}$  in system 1 and 2 respectively. During the whole experiment, more than 80% of the biogas production occurred in reactor 1 of the cascade, hence most of reactor volume of the reactors 2 and 3 remained unutilized. During all the phases of this experiment, the total ammonia nitrogen was lower in the first reactors of the systems, with values measured averages  $530 \text{ mg} \cdot \text{l}^{-1}$  in system 1 and  $546 \text{ mg} \cdot \text{l}^{-1}$  in system 2. The concentrations in the reactors 2 and 3 of the cascades exhibited higher concentrations, of approx.  $821 \text{ mg} \cdot \text{l}^{-1}$  and  $830 \text{ mg} \cdot \text{l}^{-1}$  respectively. This indicates that at the steady state, the rates of conversion of proteins in the substrate by the existing microbial populations is slower than the assimilation the organics into methane.

Having concluded the steady phase, there was a rapid increase in the process temperature from  $37^\circ\text{C}$  to  $51^\circ\text{C}$ . This shift lasted approximate 3 hours until the hot water recirculating through the reactor's jackets achieved the thermophilic range. From the measurements, it was observed that the dramatic temperature change affected the production only during the first three days immediately following the shift. The VFA concentrations increased, pH slightly dropped, TS and COD concentrations also raised (Figure 3 and 4). In anaerobic digestion, the classic conception is that changing between mesophilic and thermophilic range should be avoided. (*Monayeri Set al. (2013)*) describes that methanogens can be classified accordingly to the



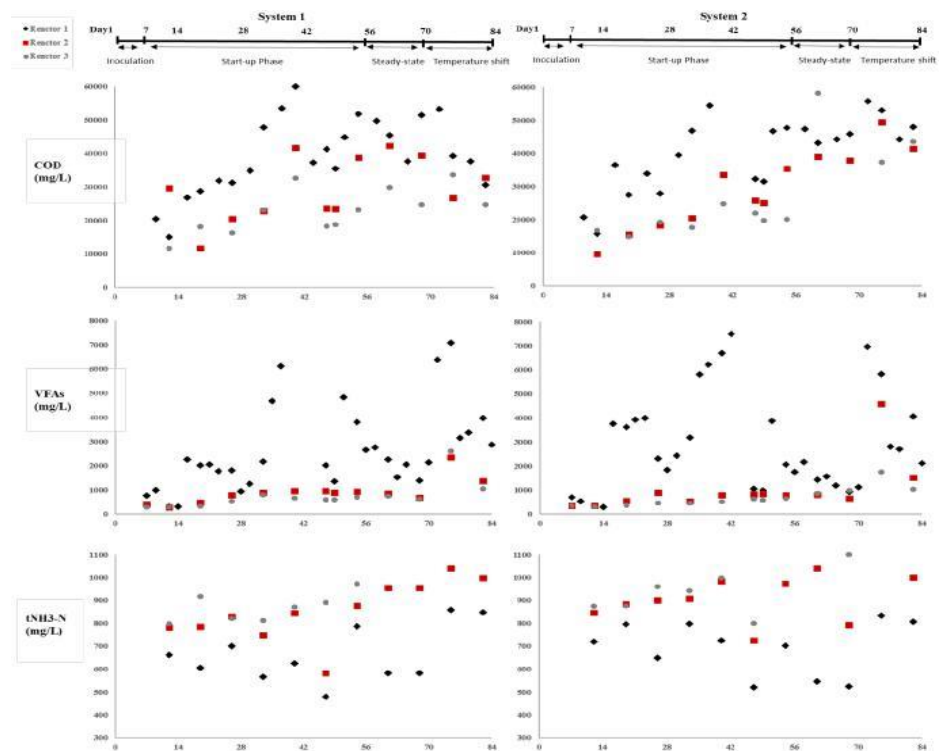
**Figure 3.** Methane production, pH and TS during the experiment. Left: System 1; Right: System 2

temperature range they grow in and it is not possible to sustain rapid fluctuations. In this study, it has been shown that in a fixed film anaerobic reactor processing enzymatically pre-hydrolysed OFMSW, it is possible to maintain high productivity even at low HRTs and high OLRs. During the whole experiment, there was low variation of the biomethane/carbon dioxide ratio in every experimental phase. This ratio usually used as an indicator of process stability of the methanogenesis. During these days, the gas production was also affected, but all the above-mentioned parameters went came back to levels that had been previously measured at the steady state for the remaining days of the temperature shift phase. The average biomethane yields in the thermophilic phase were calculated to be  $356 \text{ l CH}_4 \cdot \text{gVS}^{-1}$ .

## Microbial Diversity

### General aspects

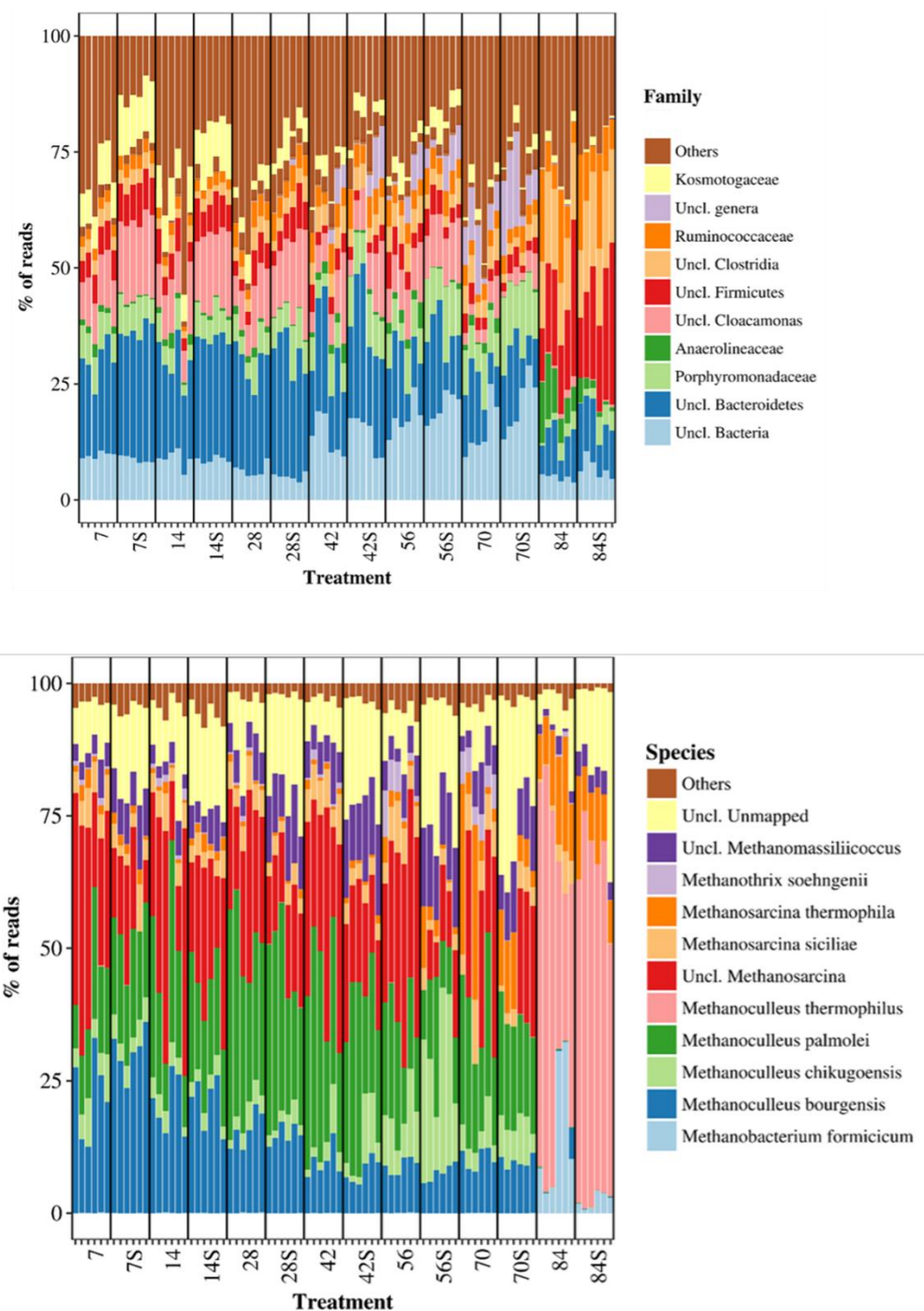
The high-throughput 16S rRNA amplicon sequencing in this study was used to characterize the microbial populations in the fixed biofilm reactor cascade system. As previously described, we sought to demonstrate the variation within the microbial populations that might rise during the experimental phases and/or as a result of specialization of the communities in the different reactor stages. Figure 5 shows the dynamics of the bacterial and archaeal during every phase of the experiment. The bacterial communities have been characterized at the family level since most of the species present in the anaerobic digestion reactors are still to be classified. Anaerobic digesters are specific ecosystems with a high diversity, and they may contain microorganisms which are difficult to cultivate.



**Figure 4.** Concentrations of COD, VFAs and tNH<sub>3</sub> during the experiment. Left: System 1; Right: System 2

The most abundant bacteria found during the digestion of enzymatic treated OFSMW in the mesophilic range are a core of microorganism consisting of Uncl. Bacteria, Uncl. *Bacteroidetes*, Uncl. *Clostridia*, *Cloacamonas* and a member of *Porphyromandaceae* (Figure 5). Most of these are taxonomically undefined, but have been previously retrieved in other anaerobic environments, more in particular in biogas plants. For instance, the members of the *Bacteroidetes* in our reactor have been previously found to be typical constituents of anaerobic digesters treating lignocellulose rich materials (Sun et al. (2016b)). The most abundant methanogens during the mesophilic phases are *Methanoculleus bourgensis*, *Methanoculleus palmolei* and an unclassified *Methanosarcina*. Both the populations of bacteria and methanogens during the mesophilic phase remain stable in time. The populations present in the different reactors of the cascades are also similar. This is an indication that the physical separation of the three reactors in the cascade in the systems does not induce a specialization of the distinct microbial groups during digestion of enzymatically treated OFMSW. Furthermore, during the sampling days where the HRT of the process was lower (higher OLR), there is a larger component of a known acidogens member of the *Porphyromandaceae* Family (Hahnke et al. (2015)).





**Figure 5.** Microbial diversity over time for biofilm and sludge samples. Top: Bacteria; Bottom: Archaea. Treatment names: The number indicates sampling day; samples without S are biofilm samples. Samples with S are Sludge samples. For each sampling time, there are 6 bars: from left to right the three reactors in system 1 and thereafter system 2

Figure 6 depicts the non-metric multidimensional scaling ordination diagrams in microbial populations during temporal variations of this experiment. The calculations have been done based on the Bray-Curtis similarity matrices of the relative abundances in the analysis. From the diagrams, it is possible to see that there is a clear shift in the composition of the microbial populations that corresponds with the increment of the flow rate in the process (lowering of the HRT) as well as with the temperature shift in the end. The duplicate three reactor systems which were independently fed with the same substrate and seeded with the same inoculum shown similar microbial composition of the communities at every time point (Figure 5). This finding indicates that microbial shifts due to changes in the process operation can be consistent and maybe predicable. Furthermore, the data obtained during the feed rate increase, indicates that both *Methanoculleus palmolei* and *Methanoculleus chikugoensis* plays important roles during the syntrophic production of methane, since these species becomes main players in the methane production at highest flow rates. In fact, these species were abundant mainly during the last period of the start-up phase and during the mesophilic steady state. However, despite the fluctuations in the community's populations during the organic loading increase, the abundance and evenness of microbial communities remained at similar levels throughout the mesophilic phase of the experiment, as calculated with the Shannon index. Furthermore, the recurring microbial populations in all the reactors of the cascade are similar for every time sample analysed. This indicates that despite there is an unbalanced organic load on the first reactors in comparison to the subsequent reactors of the cascade, the populations of the whole process fluctuate given a new flow rate regime.

Results from the DGGE analysis (data not shown) have corroborated that there is a higher proportion of *Archaea* in the first reactors of the cascade than in the remaining two reactors. This indicates that even though there does not seem to be a specialization of the microorganisms in the cascade, the methanogenic *Archaea* can be promoted by high concentrations of organics. In fact, the DGGE results show there is at least twice the amount of *Archaea* in the first reactors of the cascade where the methanogens constitute more than 15% of the total populations (data not shown).

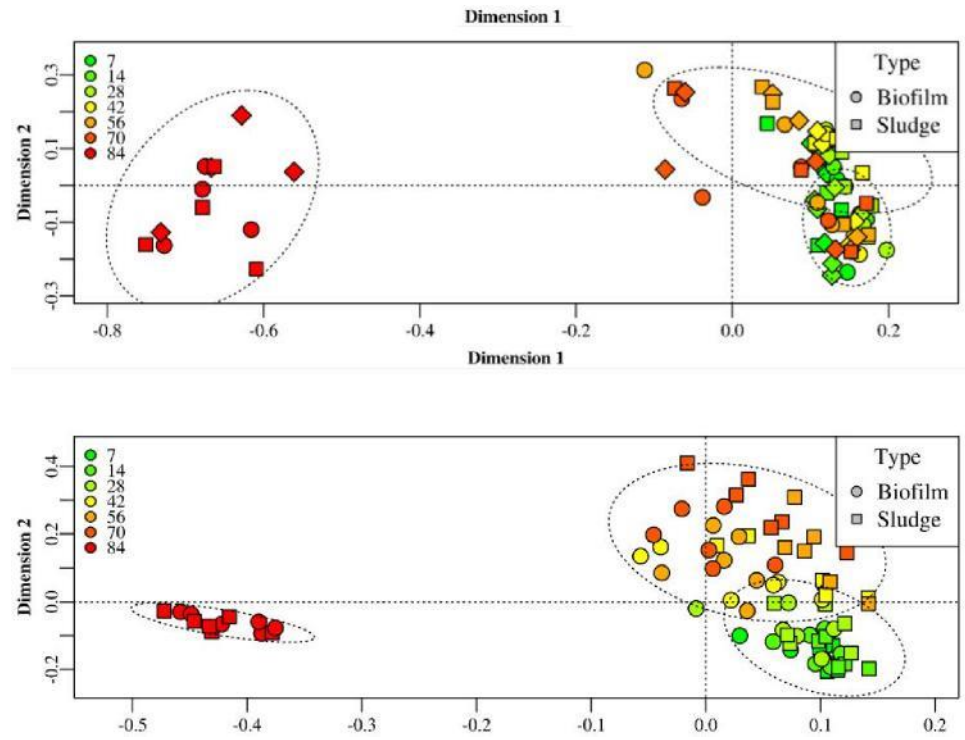
### Comparing Microbial communities in the biofilm and the digesting media

In this study there has been given especial interest to elucidate the differences between the microbial populations in the biofilm of the fixed film anaerobic reactors and those present in the liquid medium. The results of the amplicon analysis show that the populations in the biofilm fluctuate in a similar manner to those present in the liquid. In fact, it is possible to observe from figure 6, that the microbial diversity in the sludge samples resembles the biofilm samples during time variations. This is more evident for the bacterial populations, which cluster closer together for a given HRT than the archaea (Figure 6). Whether there is a continuous exchange between the cells immobilized in the biofilm and those in the liquid, or whether the biofilm settles and then the adaptation is just common for the cells in the liquid medium and in the biofilm remains unclear at present. Despite the similarities, the biofilm samples showed a richer composition at every time point (Shannon Index). This can be explained by the larger number of microbial cells aggregated in the biofilm, which might favor the co-existing of more species as well as their metabolic cooperation.

### Microbial shift during temperature shift

The largest change in the microbial diversity of both biofilm and sludge samples of all the reactors of the cascades is perceived after temperature transition between the mesophilic to the thermophilic range. Even though this rapid transition did not have a huge impact on the performance of the reactors, the populations of both methanogens and bacteria resulted heavily modified. The clearest visualization of this effect is probably that of Figure 6, where it is evident that the microbial populations dramatically change in the samples 84 and 84S. These results are in agreement with those studies that try to elucidate the effects of temperature changes during anaerobic digestion processes. Members of the Families *Clostridia* and *Firmicutes* are the most abundant bacteria during digestion at 51°C. In fact, more than 50% of the operational taxonomic units after the transition have been attributed to these families. Furthermore, all the reactors were colonized by *Methanoculleus thermophiles* and *Methanosarcina thermophila*. In agreement with their given taxonomic names, these species of methanogens have are known thermophiles. The presence of *Methanoculleus spp.* indicates that the fixed biofilm reactors produce methane through the hydrogenotrophic pathway when the operation is held at in the thermophilic range. Even though the concentration of ammonia significantly increased during the thermophilic operation, it is not possible to correlate this factor to the microbial specialization.





**Figure 6.** non-metric multidimensional scaling ordination diagrams in microbial populations during temporal variations. Top: Bacteria; Bottom: Archaea. The color tag is sampling day.

## Conclusions

In this study, we have studied the microbial diversity of a fixed biofilm anaerobic digester during initiation and high performance conversion of enzymatically pre-hydrolysed OFMSW. The findings show that upon inoculation, the biofilm rapidly develops as the flow rate and OLR in the reactors rapidly. From the initiation until the steady state is reached, the biofilm maturation takes place in which stable microbial communities settle and only fluctuate slightly in response to changes in the flow regime. The microbial populations were generally similar for all the biofilm and digesting medium samples. However, the biofilm population remained richer, possibly due to the higher number of microorganisms in the aggregate biofilm environment. Furthermore, rapid fluctuations in the temperature range did not affect the long term process stability and yields. However, a temperature ramp up beyond the mesophilic range implied drastic shifts in the microbial populations, where the species that are able to thrive at higher temperatures colonize both the biofilm and the digesting media. The utilization of DNA sequencing throughout the anaerobic digestion process allowed us to obtain important information about the process and dynamics of the microbial populations during high rate biogas production.

## Acknowledgments

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# Rapid microbial diversity adaptation during anaerobic digestion of the organic fraction of municipal solid waste after enzymatic pretreatment

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## Abstract

Efficient anaerobic digestion (AD) of the Organic Fraction of Municipal Solid Waste (OFMSW) is imperative for the future of waste management. Enzymatic treatment of the OFMSW has recently gained attention since it allows to convert larger fractions present in the waste streams and can potentially reduce the operational costs in full scale biogas plants. Since the original microbial communities are known to be determinant in the future performance of AD reactors, this study is examines the effect of the inoculum composition during the anaerobic treatment of OFMSW at different HRTs. The findings suggest that the enzymatic treatment has already an effect towards the microbial specialization of bacteria present in the substrate. Members of the *Lactobacillaceae* family become predominant and shift the fermentation products towards lactate production. During the AD of the enzymatically treated waste, these are overrun by members of the *Prolixibacteraceae* and *Porphyromonadaceae* families and appear to perform similar functions during the digestion processes. In turn, the enzymatic treatment enhances the methanogenic population of *Methanosarcina spp.*, which become the most predominant microbial group in the reactors. Despite important microbial shifts during the start-up, the original inoculum does not have a long term impact on the performance of the reactors at any given flow regime. Therefore, enzymatic treatment of OFMSW poses itself as an important technology for efficient biogas production.

## Introduction

Management and disposal of municipal solid waste has become of dramatic environmental concern. Increasing populations and the necessity of recovering nutrients and energy has made the traditional landfill practices unsustainable. In many places around the globe, public health, aquifers and air quality have been endangered following the uncontrolled decomposition of the organic material in waste, which has triggered the necessity for alternative solutions of disposal (*Hartmann and Ahring (2006)*). Anaerobic digestion is a feasible technology to convert the biodegradable fraction of municipal solid waste (OFMSW). During anaerobic digestion, the organic materials are converted into biomethane, a storable gas that can be utilized to produce electricity and heat to sustain many societal requirements. Furthermore, this practice can potentially reduce the environmental impacts of waste disposal when compared with landfill or incineration (*Gaby et al. (2017)*).

During Anaerobic digestion, the degrading microorganisms break down the energy rich carbohydrates, proteins and fats present in the waste through complex and cooperative degradation

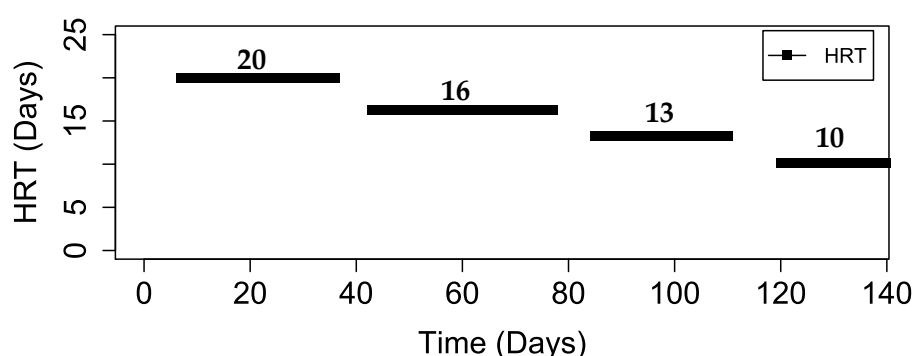
pathways in the absence of inorganic electron acceptors (Stams, A. 2009). It has been well described to frame the steps of anaerobic degradation into four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis. A stable and effective methanation of OFMSW is possible when there is a balance within the physiologically different microbial groups involved in the degradation processes. The hydrolysis has been described to be the rate-limiting phase within anaerobic digestion of OFMSW (Vavilin *et al.* (2004)). This phase is particularly challenging due to the heterogeneous components retrieved in the waste streams, that often results in formation of toxic by-products and accumulation of non-desirable VFAs.

Biomethane production from OFMSW is generally achieved following source separation and may require different chemical/physical pre-treatments steps. Sorting of the waste is performed to avoid recalcitrant and non-biodegradable materials from entering the process (Bernstad *et al.* (2013)). Despite the paper and cardboard fractions represent a large fraction of the OFMSW and could potentially be utilized for biomethane production, these are often excluded from the degradation process due to their slow hydrolysis in anaerobic conditions (Riber *et al.* (2009)). To facilitate the hydrolysis phase, many of the existing AD plants treating OFMSW also incorporate two-stage processes (staged), that separate the hydrolysis and acid formation from the methanogenesis (Ariunbaatar *et al.* (2014); Kinnunen *et al.* (2015)). Addition of more stages in AD provides operational stability and has shown to favour the specialization of the microbial groups within the different reactors (Oliveira and Doelle (2015)). However, staged anaerobic digestion comprises appended operational units to the AD plant that usually implies prolonged retention times and additional costs for the treatment plant.

Enzymatic processing has been evaluated for conditioning of the organic fraction of OFMSW for production of biomethane (Puri *et al.* (2013); Wagner *et al.* (2010)). A similar strategy has also been reported by several authors for degradation of different industrial and agricultural materials (Parawira (2012); Romano *et al.* (2009); Parmar *et al.* (2001)). Supplementation of hydrolytic enzymes has shown to enhance the liquefaction kinetics of the waste, thus reducing the viscosity and the particle size distribution of the mixture. Amongst the advantages of this strategy, the paper and cardboard fractions of OFMSW can be utilized in the biogas reactors, thus enhancing the conversion into methane of larger fractions of the disposed municipal waste streams. Furthermore, the enzymatic treatment could potentially reduce the operation and maintenance cost of commercial biogas plants. The energy necessary for stirring, mixing and mechanically pretreating the waste is reduced since the enzymes solubilize the organics in the process.

The microbial communities of hydrolytic bacteria that are originally present in the inoculum could be crucial during the start-up of AD processes treating lignocellulose rich materials such as OFMSW. These microbial populations have been found determinant during the first HRTs of operation since the rates of degradation depends on their ability to depolymerize larger organic compounds (Liu *et al.* (2017)). Through enzymatic pretreatment of the substrate, it could be possible to promote the first phase of AD by decreasing the selective pressure on the hydrolytic bacteria. There hasn't been performed any study that elucidates the effect on enzymatic liquefaction towards the microbial diversity in AD, in particular with regards to the original populations present in the inoculum.

The present study evaluates the biogas production and microbial diversity of two biogas reactors seeded with diverse inocula digesting enzymatically hydrolyzed OFMSW. The assessment of the performance of the reactors is achieved by monitoring different physical and chemical parameters throughout the process. High-throughput DNA sequencing techniques are utilized to dissect between the eventual microbial communities in operation at different Hydraulic Retention Times (HRT).



**Figure 1.** HRT during AD of enzymatically treated OFMSW in CSTR.

## Materials and Methods

### Municipal Solid Waste

The municipal solid waste substrate used in this study was provided by Rescience A/S, Denmark. The enzymatically treated slurry was prepared by supplementing water and an industrial mixture of hydrolytic enzymes with specificity for various substrates as described in *Wagner et al. (2010)*.

### Reactor Settings

This study was conducted in 2 stainless steel CSTR reactors (10l) seeded with different inocula. In the first reactor (CSTR1), the inoculum derived from Foulum Biogas, a plant in Denmark treating OFMSW and waste water treatment sludge. This inoculum was acclimatized for longer periods with the enzymatically treated waste. The second reactor (CSTR2) was obtained from a full scale biogas plant that converts non treated source sorted OFMSW in March, UK. The feed-in was supplied hourly from an agitated storage tank, filled every day to prevent material loss. Composition of the OFMSW is given in table 1.

The two CSTRs were seeded and operated for two entire retentions at each flow rate regime as shown in figure 1. The samples for DNA extraction and analysis were taken at the end of each period when the digesting media had been replaced twice. In the moments of transition between HRTs, there was a moderate increase of the flow rate in order to minimize ecological drifts.

### Gas Measurements

The gas evolution from each single reactor was independently measured by a gas flow device (Bioprocess Control, Sweden). The biogas quality was determined via gas chromatography (model ML GC82 from Mikrolab Aarhus A/S with a thermal conductivity detector (TCD) and the carrier gas N<sub>2</sub> at 70 °C (packed column 6 ft x 3 mm Porapak Q column).

### Sample Analysis

Sampling was performed on the digesting media. The liquid media was collected from the recirculation stream of each reactor and analysed for chemical parameters 3 times per week. Total Solids (TS), Volatile Solids (VS) and ashes were determined by gravimetry following NREL guidelines (*Sluiter et al. (2008)*). Suspended solids were also determined gravimetrically after filtration through ashless Whatman Grade 934 AH RTU filters. Chemical Oxygen Demand (COD) was quantified with

**Table 1.** Chemical composition of enzymatically liquefied OFMSW.

Component	Measured value
Chemical Oxygen Demand (COD)	103.9 g <sup>*</sup> l <sup>-1</sup>
Soluble Chemical Oxygen Demand (sCOD)	62.9 g <sup>*</sup> l <sup>-1</sup>
Total Solids (TS)	9 %
Volatile Solids (VS)	6.6%
Total suspended Solids (TSS)	4.0 %
Volatile Suspended Solids (VSS)	2.7 %
Volatile Fatty Acids (VFAs)	35 g <sup>*</sup> l <sup>-1</sup>

Hach LCK514 D DR spectrophotometer (Hack, Germany) for monitoring purposes. Quantification of single VFAs in the samples was performed by HPLC using a Biorad HPX-87H column (Hercules, CA; USA), RI detector, 63 °C and 5 mM H<sub>2</sub>SO<sub>4</sub> as eluent, at flow rate of 0.6 ml min<sup>-1</sup>. The pH was monitored online in the digesters.

### Microbial analysis

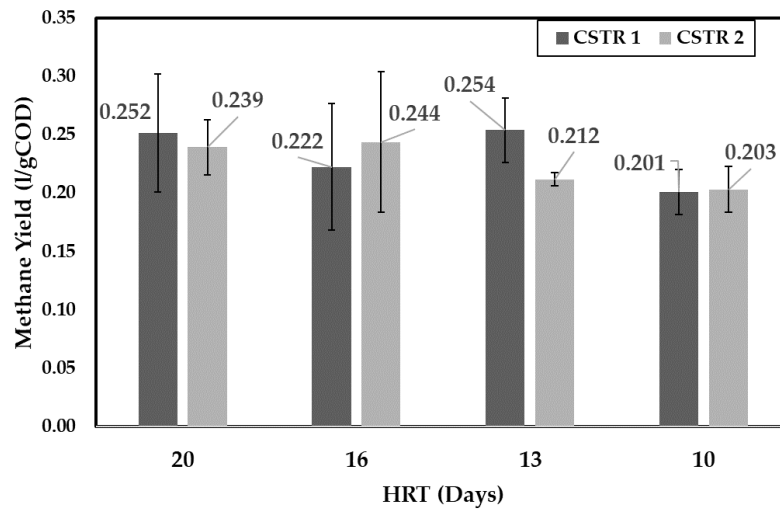
DNA was extracted with DNeasy powersoil (QIAGEN, Netherlands). PCR amplification of the 16S rRNA gene was performed using the modified universal primer pairs F341-805R based on *Takahashi et al. (2014)*. These primer set is based on the V3-V4 hypervariable region of prokaryotic 16S rDNA for the simultaneous detection of *Bacteria* and *Archaea*. Q5®Hot Start High-Fidelity DNA polymerase 2X (New England Biolabs Inc, USA) was used and the incorporation of the Nextera XT®v2 set B of transposase sequence was performed (Illumina Inc, USA). Agarose gel 1% electrophoresis (Biorad PowerPac Basic) was used to confirm the success of the first amplification and the resulting DNA was purified with AMPure XP with magnetic bead technology. The initial denaturation at 98°C for 3 min followed by 25 cycles of 98°C for 30s, 57°C for 30s and 72°C for 30s. The inclusion of the index was performed in the same conditions in 8 cycles. The resulting DNA concentration was determined by a Quibit3 fluorometer (Invitrogen, USA) and normalized using Mag-Bind EquiPure Library Normalization Kit at 4nM (Omega Biotech, USA). The final concentration of the samples was approximately 5nM. The resulting samples were sequenced on the Illumina MiSeq™ platform. The sequences were analyzed using BION-meta software. BION is a supported open-source package for microbial diversity analysis of 16S rRNA (<https://app.box.com/v/bion>) (Sequences were treated as in ). Briefly, the reads were quality filtered and chimera-checked before classification against the RDP-II database. The number of reads obtained for each barcode was normalized to 100,000 for further data treatment.

## Results and Discussions

### Enzymatically treated OFMSW

The chemical composition of the enzymatically liquefied OFMSW used in this experiment is can has been described in table 1. A large fraction of the VS can be retrieved as already hydrolyzed and fermented VFAs. The main constituents of the VFA pool are lactic acid and acetic acid, concentrations of 28.2g<sup>\*</sup>l<sup>-1</sup> and 2.52g<sup>\*</sup>l<sup>-1</sup> have been measured respectively. Approximately 30% of the TS in the substrate is non already solubilized material, of which almost a third is non digestible ash. This results show that most of the liquefied OFMSW has already been through the hydrolytic phase. Hence it should not be expected a high pressure on the hydrolytic communities during degradation of this substrate.





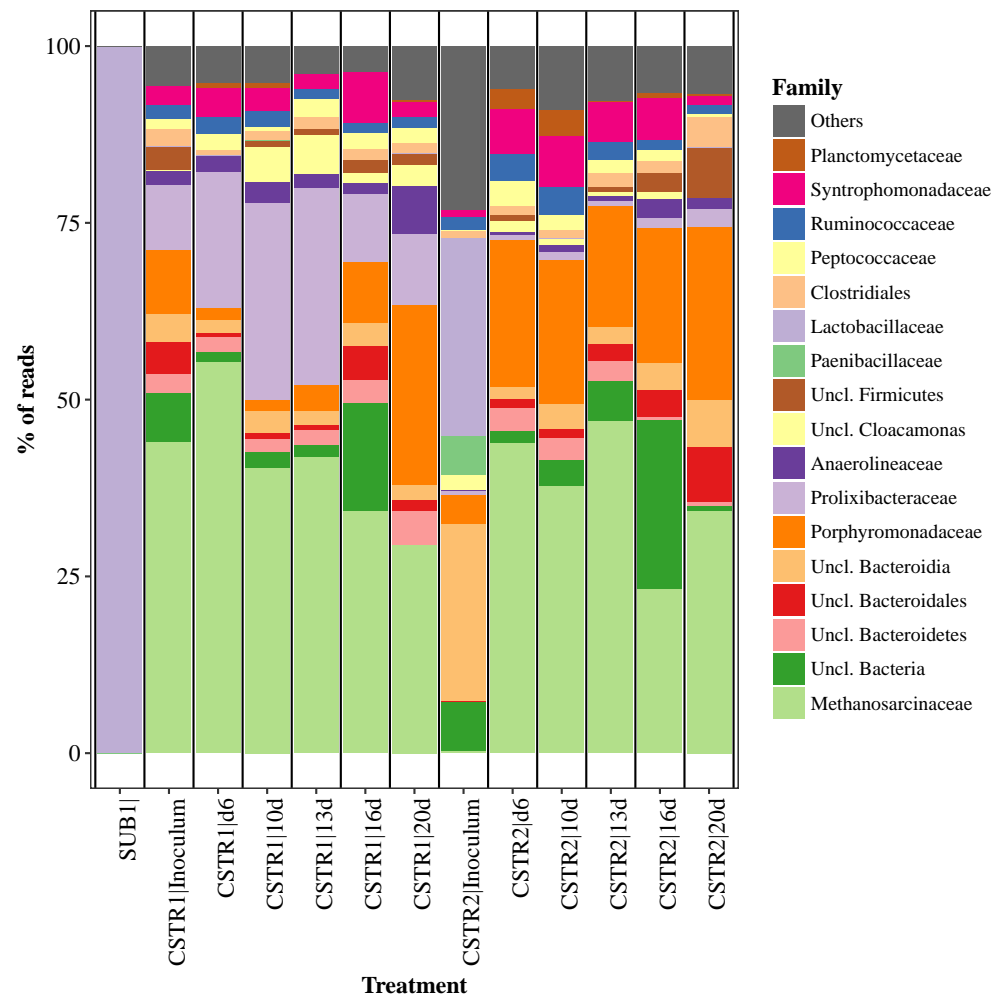
**Figure 2.** Methane yields at different HRTs during AD of enzymatically treated OFMSW in CSTR.

### Performance of the CSTRs

The methane production at the different HRTs of both CSTRs inoculated with different seed materials is shown figure 2. Both reactors were inoculated as intended and there was perceived gas production during the first day of feed in. The level of production during the first 5 days is comparable in both of the reactors, which suggests that both possess actively enough methanogenic populations ready to convert the substrate into methane. The average yields produced during the first 40 days of experiment (20 days HRT) are shown in the graph. In this period, there appears to be a slightly higher production in CSTR1, which was originally inoculated with digestate that had been already acclimatized to the substrate. It is possible that the pre-conditioning to the feed has already specialized the microbial populations for conversion to this substrate which were then ready to carry out the digestion processes, thus less energy would be directed to build up the metabolic functions. In the beginning, there were possibly proportionate amounts of microorganisms in both inocula since the VSS concentrations measured are similar (Fig. 4). However, at the beginning of the experiment, the concentration of VFAs quantified in both of the CSTRs were different. In the CSTR1, the VFAs did not exceed  $1\text{g}\cdot\text{l}^{-1}$  during the first 40 days of the experiment, while in CSTR2 there was encountered an initial pool of  $2.3\text{g}\cdot\text{l}^{-1}$  which then decreased at the end of this period.

During the digestion at lower HRTs, there seems to be a slight decrease in the methane yields in both CSTRs. However, statistical analysis of the results shows that there is not a significant difference between the productivity of the CSTRs or within their respective yields at HRTs of 20, 16 and 13 days. However, the methane yields in both CSTRs decrease at HRT of 10 days. This observation can be explained with the fact that the microbes have half the time to achieve complete conversion perceived at an HRT of 20 days. Flush out of a fraction of the active population and some unconverted organics could be expected already at that flow regime of 10 days HRT (*Hartmann and Ahring (2006)*). For the most part, the concentrations of VSS remained unvaried at the higher HRTs but surprisingly increased at an HRT of 10 days. This information is counterintuitive with the previous observation since the VSS concentrations would decrease in a situation where the microbes are flushed out. However, it is not possible to distinguish if the increment of VSS in the digesting media is due to the accumulation of non hydrolyzed compounds due to loss of production capacity or if the number of microorganisms actually increases as a consequence of the accumulation of acids and hydrogen in the digesting media. A cell counting method should be considered to determine if there actually is biomass formation, which could give an explanation to



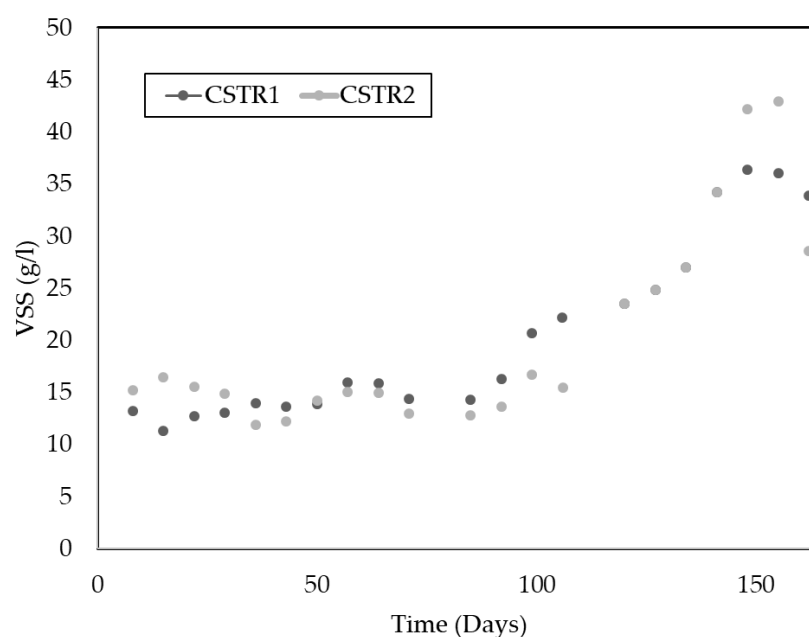


**Figure 3.** Microbial diversity over time for CSTR samples and substrate.  
SUB1: composition of the substrate; *d* indicates the HRT where the sample was taken

the decrease in biogas production at 10 days HRT. In this experience, the original seen material doesn't seem to have an effect on the eventual performance of the CSTRs, not even before the first retention of the media.

### Microbial diversity

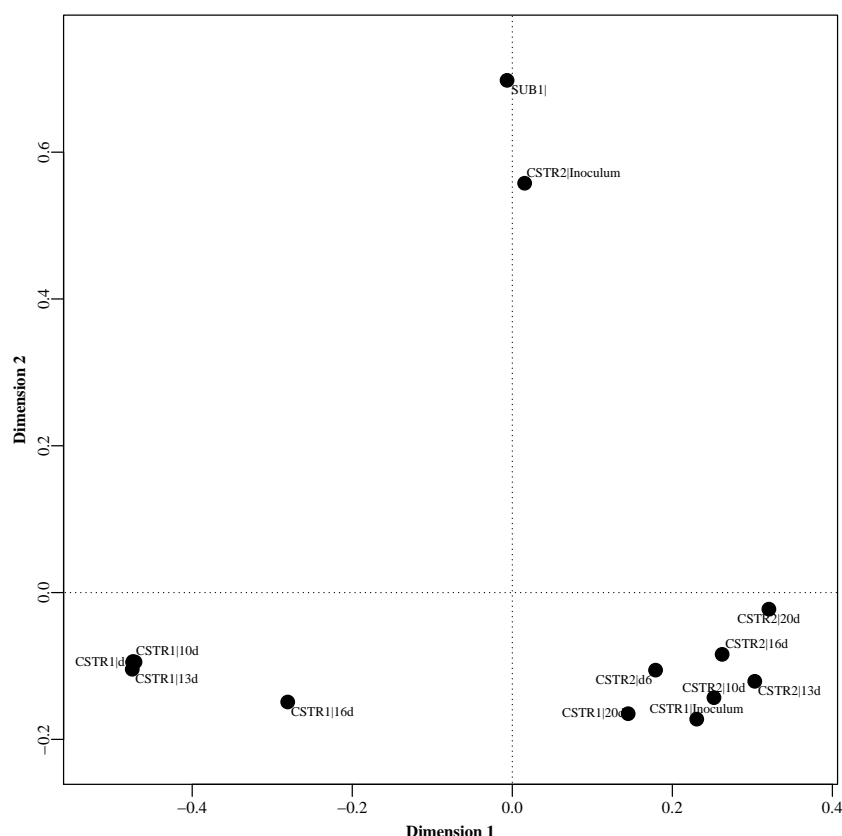
The figure 3 shows the changes in the microbial diversity during this experiment in the CSTRs. The bar in the left reveals the composition of the enzymatically treated OFMSW that served as feedstock for the digestion process. It is possible to observe that more than 98% of the microbial population belongs to the *Lactobacillaceae*. These organisms are rod-shaped, gram-positive bacteria with high distribution in commercial commodities due to their ability to produce lactic acid and irreplaceable task in the manufacturing of fermentation products (citeNovik2017). These results are in agreement with the quantification of VFAs in the substrate. Surprisingly, it is very likely that the enzymatic treatment of OFMSW induces a microbial specialization towards members of this family. This observation is different to that measured from non treated OFMSW, in which the populations have been shown to be more diverse. Members of this family belong to the *Firmicutes* phylum that as discussed in precedence are know to have fast growth rates and are metabolically important during the fermentation of simple sugars.



**Figure 4.** VSS concentration during AD of enzymatically treated OFMSW in CSTR.

The results from the microbial characterization of the inocula shows that there exist a high difference between these seed materials. In the CSTR1, the inoculum that derives from an already acclimatized population towards the substrate and possesses an outstanding composition of *Archaea*, which accounts for almost 45% of the reads. In turn, the results suggest that the archaeal population in the inoculum from the OFMSW treatment plant represents less than 10% of the microbes in the complex community. Comparably, the methanogens in both of the inocula belong to the family *Methanosarcinaceae*, that are probably the most versatile methanogens retrieved in biogas plants, usually capable to perform methane production through the three methanogenic routes. This is also in agreement with the finding reported by *Alcántara-Hernández et al. (2017)*, where members of this family were found to play a role during AD of OFMSW. In the CSTR1, the archaeal populations constitute a meaningful fraction of the entire microbial community, from 32% up to 56% of the total reads. It appears that the amount of *Archaea* in CSTR1 slightly increases with higher flow rates. In turn, the archaeal populations dramatically increased in the CSTR2 already after the initial two full retentions in the reactors. After that, these populations continued to slightly increase as observed in the CSTR1 in operation at lower HRTs. Allegedly, the enzymatic treatment of the OFMSW enhances the populations of methanogenic *Archaea* in the reactors most likely due to lack of selective pressure on the hydrolytic bacteria.

The characterization of the bacterial populations reveal outstanding differences amongst the CSTRs. The most evident is the large fraction of members of the *Lactobacillaceae* family present in the inoculum from CSTR2. Approximately 30% of the microbial counts are lactic acid bacteria, which makes this seed material to resemble more the substrate than to the other AD samples (Fig. 5). The largest difference observed through the experiment in regard to the bacterial populations during the different HRTs tested prevails in the outlying communities of acidogenic bacteria. In the CSTR1, there is an important population of members of the family *Prolixibacteraceae*, while in the CSTR2 there is a predominant fraction of members of *Porphyromonadaceae*. Interestingly, the *Prolixibacteraceae* accounted for approximately 10% of the total population in the inoculum in CSTR1. These microorganisms were present in CSTR2, but almost undetected. Instead, the *Porphyromonadaceae* were present in both of the original inocula in both CSTR1 and CSTR2. Their



**Figure 5.** Non-metric multidimensional scaling ordination diagrams in microbial populations during temporal variations.

presence accounted for nearly 12% and 8% of the total reads respectively. However, during the anaerobic degradation of the substrate, the populations of these bacteria belonging to separate families specialized differently in the two reactors. The difference in relative abundance of these two microbial groups in the CSTRs is the most solid explanation to explain why the samples have clustered distinctly in figure 5. Unfortunately, there hasn't been published yet enough information to confirm their particular roles during the degradation of liquefied OFMSW, even though it seems that members of the family *Prolixibacteraceae* may be involved in direct electron exchange with the methanogenic *Archaea* during hydrogenotrophic methanogenesis (Yin *et al.* (2018)). Furthermore, these type of bacteria have been already identified in AD processes in situations of organic overload (Kampmann *et al.* (2014)). Their ability to withstand and tolerate the high OLR regimes like those in this experiment could be an explanation for their presence.

## Conclusions

In the present work it has been possible to demonstrate that the composition of endogenous microorganisms in the inoculum does not have an effect on the long time performance of AD reactors degrading enzymatically treated OFMSW in the mesophilic temperature range. Alternately, the enzymatic pretreatment of the waste results in a dramatic specialization of the substrate towards members of the *Lactobacillaceae* family, which then turn out almost undetected in the anaerobic reactors. The pretreatment of the substrate enhances the methanogenic *Archaea*, that establish in the digesters to constitute nearly half the total population. In particular those *Methanosarcina spp.* are the most predominant methane producing community. Meanwhile, bacteria belonging to the *Prolixibacteraceae* and *Porphyromonadaceae* appear to have an important role during degradation

of the pretreated waste even though they specialized distinctly amongst the two CSTRs. The comparable yields measured in both systems confirms that different taxonomic groups in complex microbial communities are able to perform similar functions in anaerobic digesters. Even though one inoculum had been previously acclimatized to the substrate, both reactors were able to achieve similar performance at every HRT tested, achieving operation at higher organic loads for AD of OFMSW.

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