Considerations for the Establishment of an Anaerobic Biofilm in an AnMBBR

APPLIED MICROBIOLOGY | FACULTY OF ENGINEERING | LUND UNIVERSITY
EMMA MÅNSSON | MASTER OF SCIENCE THESIS 2020

Considerations for the Establishment of an Anaerobic Biofilm in an AnMBBR

A Master's Thesis Project
By: Emma Månsson
January 2020

LUND
UNIVERSITY

Department: Applied microbiology (TMB)
Supervisor: Catherine Paul
Examiner: Ed van Niel
External Supervisors: Fernando Morgan and Susanne Jacobsson

Acknowledgements

I would like to thank my supervisor Catherine Paul for always looking after my mental wellbeing and emphasising that I should always take care of myself. Somehow, our "quick meetings" often turned into 2-hour coffees from which I always walked away happy and relaxed. I truly enjoyed working with you this semester.

Special thanks to my supervisors Fernando Morgan and Susanne Jacobsson at AnoxKaldnes for giving me the opportunity of working with you on this project. You gave me the chance to explore a completely new subject, and I've learned so many things by working with you. Furthermore, I'd like to thank everyone at Veolia Water Technologies for being open and friendly. I've had a great time during my stay with you.

I would also like to thank my family for always being supportive through my many ups and downs during my studies. Thank you for always listening.

Finally, I would like to thank all the friends I've met during my studies here at LTH, both through school and extracurricular activities. No one named, no one forgotten, but do know this: every single one of you that I've met during these five years have had a great impact on my life and I couldn't have done this without you.

Lund, January 2020

Emma Månsson

Abstract

Treatment of wastewater is crucial for removing any compounds which can have a negative impact on human health and nature before the water is reused or released into nature. Interest in anaerobic treatment of wastewater has increased as it is a good complement to aerobic treatment, and it produces energy in form of methane.

The aim of this project was to investigate how different operational parameters influenced the initial establishment and development of biofilm in an anaerobic Moving Bed Biofilm Reactor (AnMBBR). Three lab-scale reactors were loaded with different types of carriers, then started and operated in parallel for 86 days. The reactors were subjected to the same operating conditions as the temperature, organic loading rate (OLR) and hydraulic retention time (HRT) were varied. The wastewater originated from a food factory and varied in content depending on the fluctuation in the factory's daily production.

The results indicate that process stability was not reached by the end of this project. Temperatures in the higher spectrum of the mesophilic range and higher OLR indicated a higher promotion of biofilm establishment. A re-inoculation with a new inoculum increased the microbial activity and seemed to help with the biofilm formation. Carrier design also seemed to influence the biofilm establishment as the carriers which had a larger protected surface area showed signs of biofilm formation earlier. Further evaluation of these parameters is necessary to develop a greater understanding for establishment and development of biofilm in an AnMBBR.

Key words: Anaerobic, MBBR, biofilm, carrier, methane

Sammanfattning

Behandling av avloppsvatten för att avlägsna ämnen som kan ha en negativ påverkan på den mänskliga hälsan och naturen är mycket viktigt innan vattnet återanvänds eller släpps ut i naturen. Intresset för anaerob vattenrening har ökat eftersom det är ett bra komplement till aeroba processer, samt för att det producerar energi i form av metangas.

Målet med detta projekt var att undersöka hur olika driftsparametrar påverkade den initiala etableringen samt utvecklingen av biofilm i en anaerob Moving Bed Biofilm Reactor (AnMBBR). Tre labb-skaliga reaktorer laddades med bärare av olika slag, varefter de sedan startades och kördes i 86 dagar. Reaktorerna utsattes för samma driftsvillkor och temperaturen, belastningen och den hydrauliska retentions tiden (HRT) varierades. Avloppsvattnet kom från en matproducerande fabrik och dess styrka varierade beroende på fabrikens dagliga produktion.

Resultaten indikerade att reaktorerna inte var stabila vid slutet av detta projekt. Temperaturer i den högre delen av det mesofila temperaturintervallet och en högre belastning indikerar ett bättre främjande av biofilmsetablering. En nyare ymp ökade den mikrobiella aktiviteten och verkade underlätta biofilmsformationen. Bärarens design verkade också påverka etableringen av biofilm då bäraren med en större skyddad area visade tecken på biofilmsformation tidigare. Fortsatt utvärdering av dessa parametrar är nödvändigt för att utveckla en djupare förståelse för etablering och utveckling av biofilm i an AnMBBR.

Nyckelord: Anaerob, MBBR, biofilm, bärare, metan

Populärvetenskaplig sammanfattning

Hur man använder anaerob biofilm för rening av avloppsvatten

I dagens samhälle spolar vi ut mängder av olika näringsrika och giftiga ämnen i våra avloppsvatten. Därför är det viktigt att rena detta vatten och minska halten av organiska material innan vi släpper ut det i vattendrag i naturen. Om vattnet inte renas finns det en risk att flertal problem kan uppstå, så som algblomning och fiskdöd.

Detta projekt har undersökt möjligheten att minska halten av organiskt material i avloppsvatten med hjälp av mikroorganismer i en anaerob process, alltså en process utan tillgång på syre. Typen av process som använts är Moving Bed Biofilm Reactor (MBBR)-processen. En MBBR utnyttjar bärare, vilket är små diskar som mikroorganismerna kan växa på i form av biofilm i skyddade hålor, vilka underlättar odlingen av organismerna. Dessutom kan man ha ett kontinuerligt genomflöde av avloppsvatten samtidigt som man på ett enkelt sätt kan hålla kvar biofilmen i reaktorn.

Anaeroba processer kan vara ganska svåra att starta upp och få stabila då mikroorganismerna är kräsna med sina levnadsvillkor. Saker som kan påverka är bland annat temperaturen, mängden organiskt material i avloppsvattnet och uppehållstiden i reaktorn. Trots att anaeroba processer kan vara lite krångliga är de väldigt intressanta eftersom man med hjälp av dem kan producera biogas från organiskt material, vilket sedan kan användas till bland annat bilbränsle.

Under en fyramånadersperiod startades och kördes tre anaeroba MBBR processer på labbskala. De utsattes för samma förhållanden men laddades med olika typer av bärare. Under perioden som reaktorerna kördes hann de aldrig nå stabilitet, det vill säga de hann inte utveckla en *stabil* biofilm med självständig nedbrytning av organiskt material. Dock hann flera olika parametrar utvärderas för att man skulle kunna hitta de optimala förhållandena för odling av biofilm.

Bland annat visar resultaten att en högre temperatur gynnar biofilmens bildande, och en kombination av en högre halt organiskt material i avloppsvattnet och en kortare uppehållstid, alltså tid som avloppsvattnet befinner sig i reaktorn verkar sporra organismerna till en snabbare tillväxt. Bärarnas design verkar också påverka odlingen av mikroorganismerna, eftersom den bärare som hade en större skyddad area för biofilmen att växa på påvisade en snabbare bildning av biofilm.

Resultaten för detta projekt kommer hjälpa till att bidra till en större förståelse för hur en anaerob MBBR process kan startas upp på ett effektivt sätt, och hur olika designer på bärarna påverkar odlingen av biofilm. Även om vidare utredning kommer att krävas för att till fullo förstå de optimala förhållandena för bildande av biofilm, ger resultaten i detta projekt en bra indikation för hur olika parametrar påverkar processen.

List of abbreviations and symbols

AD	Anaerobic Digestion
AnMBBR	Anaerobic Moving Bed Biofilm Reactor
ASP	Activated Sludge Process
COD	Chemical Oxygen Demand
HRT	Hydraulic Retention Time
MBBR	Moving Bed Biofilm Reactor
OLR	Organic Loading Rate
VFA	Volatile Fatty Acid
TSS	Total Suspended Solids
VSS	Volatile Suspended Solids

Table of Contents

1. Introduction

Human activities unavoidably result in the production of wastewater (Henze et al., 2008). If left untreated and allowed to accumulate, wastewater can result in several complex problems, e.g. the production of malodorous gases by the decomposition of organic material (Tchobanoglous et al., 2014). Moreover, wastewater often contains pathogens, nutrients and toxic compounds which could negatively impact both the environment and the human health. Therefore, treatment of wastewater is necessary to remove these compounds so that the water can be either reused or released to nature (Tchobanoglous and Burton, 1991).

Anaerobic digestion is a process which enables the production of sustainable biogas by the treatment of wastewater (Tchobanoglous et al., 2014). Biogas is a renewable energy source which consists mainly of methane and carbon dioxide, which could be reused as e.g. car fuel (Westman et al., 2016), electricity generation and heating (Jonstrup et al., 2011). The anaerobic moving biofilm bed reactor (AnMBBR) is a type of anaerobic digester which utilizes carriers to retain biomass in the reactor in form of biofilm (Henze et al., 2008). The effectivity of this process has previously been studied for the treatment of many kinds of food wastewater, such as for winery (Chai et al., 2014; Rajinikanth et al., 2009), dairy (Wang et al., 2009; Rajinikanth et al., 2009), brewery (di Biase et al., 2018) and fruit canning wastewater (Rajinikanth et al., 2009). The technique has also been evaluated for treatment of other types of wastewater, e.g. oil-contaminated wastewater (Morgan-Sagastume et al., 2019).

The establishment and development of anaerobic biofilm in an AnMBBR and the challenges this may present has not been largely reflected upon. Therefore, this project will focus on the aspect of starting up three lab-scale AnMBBRs with different carrier designs, while using wastewater from a food factory as substrate to evaluate the different operational challenges which may arise.

1.1 Aim

The aim of this project was to investigate how different operational parameters influenced the initial establishment and development of an anaerobic biofilm in an AnMBBR. The questions which this report will aim to answer are:

- What are the optimal conditions for biofilm formation during start-up of an AnMBBR?
- How sensitive is the AnMBBR to changes in operational parameters during start-up?
- What impact does the carrier design have on biofilm growth in an AnMBBR start-up?

2. Background

2.1 Anaerobic Digestion

Anaerobic digestion is a fermentation process which degrades organic material present in wastewater and produces biogas, which is primarily composed of methane, CH_4, and carbon dioxide, CO_2. These processes often occur when there is organic material available and there is a low redox potential, meaning there are no electron acceptors such as oxygen present (Henze et al., 2008). Therefore, during fermentation the organic substrates act both as the electron donors and acceptors (Tchobanoglous et al., 2014). The pathway of anaerobic degradation and conversion of organic matter is a complex process with various microorganisms and multiple steps in series and parallel. Ultimately these reactions result in the formation of CH_4, CO_2 and new cell material, with ammonium (NH_3), hydrogen sulphide (H_2S) and water (H_2O) as side products. The anaerobic pathway for degradation of organic compounds can be divided into four successive stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Henze et al., 2008). An overview of the reaction scheme during anaerobic digestion can be seen in Figure 1.

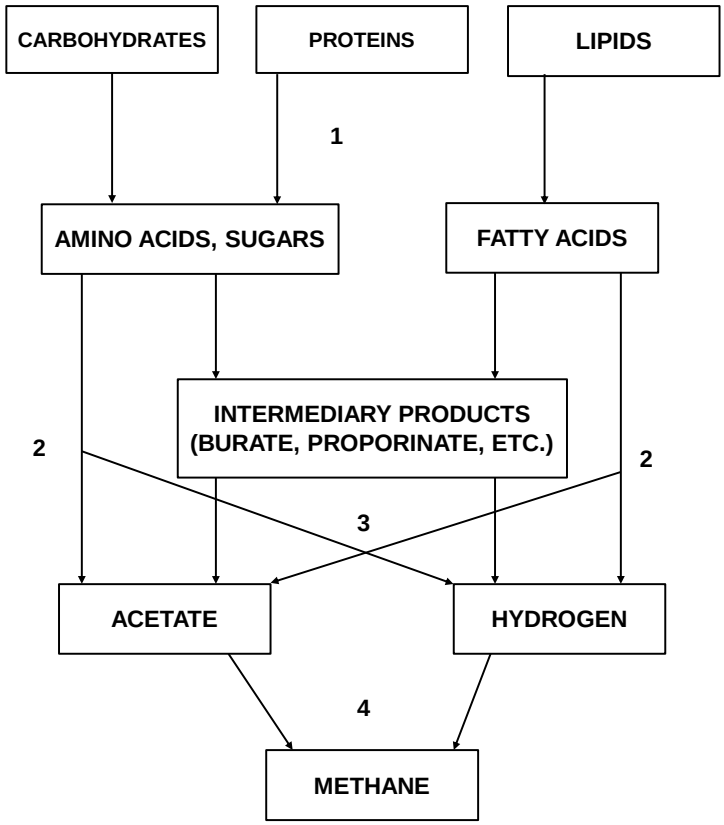


Figure 1: Schematic overview of the anaerobic digestion pathway. Step 1 is the hydrolysis, step 2 the acidogenesis, step 3 the acetogenesis and step 4 the methanogenesis.

2.1.1 Hydrolysis

The first step of anaerobic digestion is performed by extracellular enzymes excreted by fermentative bacteria (Tchobanoglous et al., 2014). These 'exo-enzymes' convert undissolved and complex organic compounds into monomeric and dimeric molecules. Proteins, carbohydrates and lipids are hydrolysed to amino acids, simple sugars and long chain fatty acids (LCFA). These small complexes are then transported into the bacteria across their cell membranes. This extracellular

hydrolysis is generally considered to be the rate-limiting step during anaerobic digestion, as the end-products are substrates for the acidogenesis. This limitation is however commonly not due to the lack of enzymatic activity, but rather the limited accessible surface area and overall structure of the large substrates. Moreover, as enzymes and bacteria are sensitive to both temperatures and temperature fluctuations, the effectiveness of the hydrolysis depends on the temperature of the environment (Henze et al., 2008).

2.1.2 Acidogenesis

During the second step of the anaerobic digestion, the products from the hydrolysis are further processed inside the bacteria by either fermentation or anaerobic oxidation (Henze et al., 2008). *Chloroflexi*, *Firmicutes*, *Spirochaetes*, and *Bacteroidetes* are examples of bacterial genera which have been commonly found in wastewater sludge (Nakano and Zuber, 2004). The fermentation of amino acids, LCFA and sugars results in production of the volatile fatty acids (VFA) acetate, propionate and butyrate, with CO_2 and hydrogen (H_2) as by-products (Tchobanoglous et al., 2014). VFAs and carbonic acid are typically the main products of the digested sugars and proteins, defining the fermentative bacteria as acidifying. Since acidogenesis is the fastest step in the anaerobic digestion, anaerobic reactors can be subjected to souring, i.e. sudden pH drops, when the reactors are overloaded or there is a high concentration of toxic compounds. The drop in pH is countered by the consumption of alkaline compounds which in turn could, if the alkalinity is completely consumed, lead to a higher concentration of VFAs that inhibits the methanogenesis. Thus, a good buffering capacity is vital for anaerobic digestion. Amino acids are generally de-ammonified as they are anaerobically oxidised to VFAs and H_2. The produced H_2 is consumed during de-ammonification of other amino acids, and NH_3 is a product from both the fermentation and oxidation. The ammonia acts as a proton acceptor, producing NH_4^+, increasing the pH and therefore effectively preventing any pH drops (Henze et al., 2008).

2.1.3 Acetogenesis

Acetogenesis is a further fermentation step that produces acetate, CO_2 and H_2 from the propionate and butyrate produced during the acidogenesis. As the conversion of these intermediate products requires energy the concentration of H_2 must be low for the reaction to proceed, as the H_2-concentration is a thermodynamic constraint for the reaction (Tchobanoglous et al., 2014). Therefore, it is vital that hydrogen-consuming methanogens are also present in the reactors to regulate the H_2-level in the environment (Henze et al., 2008).

2.1.4 Methanogenesis

During the final step of the anaerobic digestion, methane is produced by two groups of methanogenic archaea. Aceticlastic methanogens split acetate into CH_4 and CO_2, while hydrogenotrophic methanogens use H_2 and CO_2 to produce CH_4. A higher fraction of lipids in the substrate will result in a larger fraction of methane in the produced gas, as digestion of LCFAs results in the production of acetate, CO_2 and H_2 (Tchobanoglous et al., 2014). Methanogens are anaerobes with a narrow substrate spectrum, resulting in their growth rate being sensitive to the presence of certain substrates. Aceticlastic methanogens are slow-growing with a cell doubling time of several days while the hydrogenotrophic methanogens have a higher maximal growth rate and can double their biomass in a few hours. Due to these characteristics, anaerobic systems are capable of remaining stable even if subjected to fluctuating conditions (Henze et al., 2008). *Methanomicrobiales, Methanobacteriales, Methanococcales,* and *Methanosarcinales* are archaeal genera which have been found present in mesophilic anaerobic digesters (Demirel and Scherer, 2008).

2.2 Anaerobic Treatment of Wastewater

Anaerobic processes are mostly used for treatment of wastewater with high concentrations of organic matter (Tchobanoglous et al., 2014). This is because of their high effectivity in removal of biodegradable organic compounds, while mineralised compounds such as NH_4^+, PO_4^{3-} and S^{2-} are left in the suspension (Henze et al., 2008). Since there is neither a need for nutrient addition nor a net energy consumption, due to the production of biogas, anaerobic wastewater treatment has proven to be a cost-effective alternative to aerobic processes (Tchobanoglous et al., 2014). The production of excess sludge, which is the by-product of residual organic matter from wastewater treatment processes, is considerably lower than in aerobic treatment. Aside from lowering the costs of sludge-disposal, granular anaerobic sludge is reusable as an inoculum, thus maintaining some market value (Henze et al., 2008). Reactor volumes can be reduced since higher volumetric organic loading rates (OLR), which is a measure of the quantity of substrate entering the process per unit of time, can be used for anaerobic reactors (Tchobanoglous et al., 2014). In contrast to aerobic processes, the maximum load for anaerobic processes is only limited by contact between the anaerobic bacteria and active biocatalysts to the wastewater constituents, rather than the rate at which necessary reagents, e.g. oxygen, can be supplied (Henze et al., 2008). Anaerobic processes do have several disadvantages, however, with longer start-up periods and higher sensitivity to toxic compounds. Operational stability and potential odour production require careful consideration for the process to be as effective as possible. With proper characterisation of the wastewater and process design, however, these issues are all avoidable. Proper control of the feed, temperature and pH, helps maintain the optimal balance between the acidogenic VFA-production and the methanogenesis. A more notable problem is the potential need for alkalinity addition to maintain an adequate pH. If the required alkalinity is not present in the influent wastewater, nor produced during the process, it must be supplied. This could have a negative effect on the overall economy of the process. The quality of the anaerobic effluents is lower than aerobic effluents, limiting applications of anaerobic processes to mostly pre-treatment before additional, aerobic treatment (Tchobanoglous et al., 2014).

2.3 Parameters of Interest in Anaerobic Digestion

In order to avoid process instability and achieve a high biogas production it is important to keep a balance between the acidogenesis and methanogenesis. This is because the physiology, nutritional needs, growth kinetics and sensitivity to the environment differ largely between the organisms active in these phases (Adekunle and Okolie, 2015). Some parameters which may affect the microorganisms are described below.

2.3.1 Environmental Parameters

2.3.1.1 Temperature

The optimal efficiency of various microorganisms is achieved at different temperature ranges. The temperature is therefore a crucial parameter for anaerobic digestion, as the microorganisms are highly sensitive to changes in the external temperature. Anaerobic treatment of wastewater is usually operated within two temperature ranges, the mesophilic range (25-40°C) and the thermophilic range (>45°C). The methanogenesis could also be active, at a lower rate, during psychrophilic conditions (<20°C) (Jonstrup et al., 2011). However, the activity of the archaea ceases at temperatures below 15°C (Tchobanoglous et al., 2014). When the temperature increases, the rate of enzyme-catalysed reactions rises, which in turn results in a faster metabolism and growth. Too high temperatures could be damaging for the organisms, and ultimately result in cell death. At lower temperatures the nutrient transport will cease to function properly and the activity in the

cell stops. Thus, microorganisms that are less sensitive to temperature changes may outcompete more sensitive organisms, resulting in a change of the microbial community (Jonstrup et al., 2011).

2.3.1.2 pH and alkalinity

As with temperature, microorganisms have a pH range for optimal activity and can be sensitive to changes. Each type of organism which is active during anaerobic digestion has their own pH optimum. Both the acetogens and methanogens are most active at a neutral pH, while the acidogens are most efficient at a pH of 6. As the methanogenesis is usually the rate-limiting step, and the acidogens are able to function at a neutral pH, an anaerobic process should be kept at a pH close to 7. Since anaerobic processes also produces VFAs, a well-functioning buffer system is necessary (Jonstrup et al., 2011). The alkalinity is the measurement of a reactor's buffering capacity to neutralize such acids, and a high value indicates higher resistance to pH changes (Schnaars, 2012). Carbonic acid, hydrogen sulphide, dihydrogen phosphate and ammonia are examples of compounds which have a strong buffering capacity; therefore the presence of these compounds can contribute to a well-functioning buffer system (Jonstrup et al., 2011).

2.3.1.3 Hydraulic Retention Time and Organic Loading Rate

The hydraulic retention time (HRT) is the average time the suspension is kept in a reactor (Tchobanoglous et al., 2014). It is in direct relation to the volume of the reactor and the substrate flow, see equation 1.

$$HRT = \frac{V_R}{F_S} \qquad \text{(Equation 1)}$$

Where:
HRT= Hydraulic retention time [d]
V_R= Reactor volume [L]
F_S= Substrate flow rate [L/d] (Jonstrup et al., 2011).

As the methanogens have a very slow growth rate, a longer HRT promotes their growth while avoiding washout of active biomass (Rarooq and Ahmad, 2017).

The organic loading rate (OLR) is the amount of organic material added per unit of volume and time to the process (Tchobanoglous et al., 2014). For wastewater it is expressed as kg COD per volume and time unit, therefore directly in relation to HRT, see equation 2.

$$OLR = \frac{C}{HRT} \qquad \text{(Equation 2)}$$

Where:
OLR= Organic loading rate [kg COD/(L*d)]
C= Concentration of organic material [kg COD/L] (Jonstrup et al., 2011).

2.3.1.4 Mixing

Mixing in an anaerobic process is essential for keeping a high rate of digestion (Davis, 2011). Keeping a homogenous mixture is essential for effective substrate delivery, and to avoid formation of foam and temperature gradients in the reactor in order to have a well-balanced system (Abbasi et al., 2012).

2.3.1.5 Nutrients

Nutrients, such as nitrogen, phosphorus and trace elements, are required to acquire an efficient biodegradation. The addition of trace elements, e.g. nickel and cobalt, have proven to stimulate

anaerobic processes. However, too high concentrations of nutrients are toxic to the microorganisms (Jonstrup et al., 2011).

2.3.2 Chemical Parameters

2.3.2.1 COD

The chemical oxygen demand (COD) is a measurement of the required amount of oxygen needed to oxidize the equivalent amount of organic compounds in wastewater, thus quantifying the amount of organics in the sample (Tchobanoglous et al., 2014). The COD value includes all the organic compounds which could be degraded, both biochemically by the microorganisms and chemically by a strong chemical oxidant (Jonstrup et al., 2011). Total COD (tCOD) is the measurement of the total amount of organic material, while soluble COD (sCOD) is the quantity of the easily biodegradable soluble organics present in the sample (Tchobanoglous et al., 2014).

2.3.2.2 VFA

The concentration of different volatile fatty acids (VFA) indicates the status of the metabolism of the different microbes in the anaerobic digestion, and accumulation of different types of VFAs indicates which step in the digestion chain is inhibited or overloaded (Jonstrup et al., 2011). All VFAs are converted into acetic acid before being degraded into methane (Wang et al., 2009), and acetic acid, propionic acid and butyric acid are the VFAs which are most commonly produced (Tchobanoglous et al., 2014). Acetic acid is an important intermediate for anaerobic digestion as it is directly related to the end products methane and carbon dioxide (Gorris et al., 1989; Wijekoon et al., 2011). Accumulation of propionic acid indicates a process instability (Ahring et al., 1995; Björnsson et al., 2000; Murto et al., 2004), and some studies have even found that propionic acid should be treated as a toxic compound as some methanogenic archaea show vulnerability to concentrations between 1,000-2,000 mg/L (Wijekoon et al., 2011). However, accumulation of VFAs itself does not cause inhibition of the digestion. It is instead the combination of the accumulated acids and a decreased pH-value which disturbes the process. Thus, if the system is well-buffered, i.e., if the VFAs are kept unprotonated, the reactor will be able to operate at higher VFA levels (Jonstrup et al., 2011). Ahring et al. (1995) therefore suggested that the VFAs should be treated as a monitoring parameter rather than an inhibitor (Ahring et al., 1995).

2.3.2.3 TSS and VSS

Total suspended solids (TSS) is the fraction of total solids (TS) which are retained on a filter in order to separate the suspended solids from the dissolved. The organic segment of this value is the volatile suspended solids (VSS) and can be used as an estimation of the amount of microorganisms and organic material present in the sample (Jonstrup et al., 2011). The ratio of VSS to TSS (VSS/TSS) gives an indication of the amount of suspended organic material, and a greater value could indicate a larger proportion of biomass (Vlyssides et al., 2009).

2.3.2.4 Biogas

Biogas is the final product of anaerobic digestion and an renewable energy source which could be reused for e.g. car fuel (Westman et al., 2016), electricity generation and heating (Jonstrup et al., 2011). A typical composition of biogas is 55-70% methane, 30-40% carbon dioxide together with small amounts of N_2, H_2, H_2S and water vapour (Tchobanoglous et al., 2014). The theoretical potential of methane production is 0.350 NmL produced methane per mg reduced COD (Carlsson and Schnüer, 2011). The amount and methane yield of the produced biogas is mostly governed by the effectivity of the methanogenesis step, which in turn depends on the characteristics of the

organic matter, such as its degradability and composition, and operating conditions (Lee et al., 2017).

2.4 Moving Bed Biofilm Reactors

The Moving Bed Biofilm Reactor (MBBR) is a biological wastewater treatment process where carriers of different materials are used to support biofilm, and thereby retain biomass in the reactor (Henze et al., 2008). A biofilm is a cluster of cells with a defined structure that are capable of communication and exchange of genetic material between the microorganisms (Donlan, 2002). The design of the MBBR originates in the desire to combine the advantageous traits of the activated sludge process (ASP) and biofilm processes, while limiting the disadvantageous characteristics of both. Like the ASP, the MBBR utilizes the whole reactor volume for biomass growth, which is a huge advantage compared to many other biofilm processes. In contrast to the ASP, there is no need for recirculation of biomass, which reduces the process cost (Ødegaard, 2006). This results in a more specialized biofilm, with higher concentrations of relevant microorganisms in the biofilm and the quality of the effluent is less dependent on biomass separation than in an ASP, as the concentration of the suspended biomass can be more than 10 times lower. The MBBR is more compact than the ASP, reducing process costs and is more flexible than other biofilm processes, as operating loads are easily controlled by the amount of carriers and almost any reactor shape is possible (Ødegaard, 1999). In MBBR processes, as in every biofilm process, the diffusion of compounds in and out of the biofilm plays a key role in the effectivity of the digestion and the thickness of the biofilm, i.e. depth of which the substrates can penetrate the biofilm, has a large impact. As the depth of the substrate penetration normally does not exceed 100μm, the ideal MBBR biofilm should be thin and spread evenly across the surface of the carriers. The turbulence in the reactor is consequently important, as it both controls the amount of substrate delivered to the microorganisms and maintains a thin biofilm by shearing forces (Ødegaard, 2006).

2.4.1 Carriers

The carriers used in MBBR processes are designed to allow microorganisms to grow in a sheltered environment inside voids and cavities. The density of the carriers is approximately the same or lower than that of water, enabling the carriers to stay suspended and move throughout the reactor. In aerobic processes this movement is achieved by aeration, and by mechanical mixing in anaerobic and anoxic reactors. As the carriers are transported through the reactor collisions occur, causing abrasions on the exposed areas of the carriers. This results in lower biofilm growth on the outside of the carriers than in the protected area of the voids (Ødegaard, 1999). Since the carriers move through the reactor, the whole reactor volume is utilized for biomass growth, which is hugely advantageous compared to many other biofilm processes. The use of carriers ensures that the working biomass is kept inside the reactor without any need for recycling (Ødegaard, 2006), however, without any biomass circulation, the retention of biomass in the MBBR is limited to that retained as biofilm on the carriers (Henze et al., 2008). The area of biofilm is therefore an important design parameter for MBBR processes, and the effectivity rate of a process is most correctly determined by the effective carrier area (g/m^2d). In order to keep the carrier suspension moving as freely as possible, it is not recommended to exceed a 70% carrier filling degree of the reactor's working volume. (Ødegaard, 2006).

3. Materials and Methods

3.1 Reactor Setup

Three AnMBBRs were started up in parallel and consisted of a glass reactor that had double walls for a water jacket, connected to a recirculating temperature-controlled water bath. The AnMBBRs were gastight with top-mounted mechanical 2-blade mixers and a stirring frequency of 25-35 Hz. Near the surface on each reactor a baffle was mounted to disturb the stagnant top-layer of carriers. Each reactor was connected to a feeding pump (Watson Marlow 520S, Watson Marlow) and the wastewater-substrate was kept refrigerated (3-15°C) while at a continuous feed. The effluent from the AnMBBRs was discharged into a water trap by overflow so that the systems were kept gastight. All AnMBBRs were connected to a gas flow meter (AMPTS II, Bioprocess Control) and had a targeted equivalent surface area of 0.475 m^2. The Anox K™ Z-200 consists of high-density polyethylene (HDPE) of recycled material, are saddle-shaped with a projected surface area of 0.00128 m^2 and cell depth of 200µm, while the Anox K™ Z-1000 prototype consists of virgin HDPE, is flat with an estimated projected surface area of 0.00190 m^2 and a cell depth of 1000µm. 371 clean Anox K™ Z-200 carriers were added to a reactor with a working volume of 4 L (R200) and 250 clean prototypes of Anox K™ Z-1000 carriers were added to the second reactor with a working volume of 3.8 L (R1000). The third reactor had a working volume of 3.8 L (Rmix) and was loaded with a mixture of 186 clean Anox K™ Z-200 carriers and 125 clean prototypes of Anox K™ Z-1000 carriers, thus acting as a reference for the other two reactors as both types of carriers were subjected to the exact same conditions.

The Anox K™ Z-1000 carriers used in this project were a prototype whose projected surface area had been estimated, not determined, prior to the start-up. To get a quick start-up and acclimatisation of the reactors the estimated projected area was used to calculate the amount of carriers needed to correspond to a total projected area of 0.475 m^2 in each reactor. Meanwhile, the projected area of the prototype Anox K™ Z-1000 carriers was determined by measuring and summarizing the projected areas of the cells by stereomicroscopy (Nikon SMZ1270, Nikon Corporation) to 0.00164 m^2. The reactors were then opened on day 20 to correct the number of carriers to correspond to the new total projected area of 0.410 m^2. The corrected amount of Anox K™ Z-200 in R200 was 320 and 160 in Rmix. The amount of Anox K™ Z-1000 prototypes in R1000 and Rmix were kept the same. A summary for each of the reactors after correction can be seen in Table 1 below.

Table 1: Summary of the characteristics for each of the three reactors after modification of carrier amounts.

Reactor:	Working Volume:	Carrier Type:	Number of Carriers:
R200	4.0L	Anox K™ Z-200	320
R1000	3.8L	Anox K™ Z-1000 prototypes	250
Rmix	3.8L	Anox K™ Z-200/Anox K™ Z-1000 prototypes	160/125

The reactors will henceforth be referred to as R200, R1000 and Rmix according to the description in Table 1.

3.2 Characterisation of Wastewater

Wastewater was collected from a food production factory in six batches. Samples of the batches were collected and characterised by following analyses: pH (HQ11D, Hach), VFA (gas chromatography (GC), Clarus 400, Perkin Elmer), tCOD and sCOD (HACH LCK 114/814, Hach), alkalinity, NH_4-N, PO_4-P and SO_4 (spectrophotometry by Gallery Plus, Thermo Fisher Scientific), S_2^- (HACH LCK 635, Hach), TSS (SS-EN 872:2005, 2nd ed.) and VSS (former SS028112, 3d ed.). The samples for VFA, sCOD, alkalinity, NH_4-N, PO_4-P, SO_4 and S_2^- were filtered through a 1.6 µm glass fibre filter before analysed. A 1.6 µm glass fibre filter was used to collect TSS and VSS.

3.3 Start-up of Anaerobic Reactors

The carriers for R200 and Rmix were first wetted in tap water in the reactors by continuous stirring of the mechanical mixers. The carriers for R1000 were wetted in an aerobic reactor using air-bubble mixing, also in tap water, due to their strong hydrophobicity. The reactors were then emptied of water and the carriers for reactor R1000 were returned to the anaerobic reactor. All three reactors were inoculated with 1.5 L of anaerobic granular sludge from Rotneros (Söderhamn, Sweden) that had been stored (+4 °C) for 16 months. The reactors were then filled to their working volume with tap water. All three reactors were started at 25 °C and left to acclimatise for four days before substrate loading began.

3.4 Operation of Anaerobic Reactors

The reactors were operated in three different time periods characterised by the operating temperature, see Table 2. The wastewater was supplied with nutrients by addition of a trace element solution (Vithane, Biothane) and iron by an FeCl solution so that nutrients were in abundance. $NaHCO_3$ was added to the substrate as a buffer to maintain a neutral pH and stable process in the reactors. Substrate loading began on day 5. Inoculum 1 (Rotneros, Söderhamn) was used for inoculation 1 on day 1 (1.5 L) and inoculation 2 on day 22 (200mL). Inoculum 2 (Fiskeby Board AB, Norrköping) a fresh granular sludge, was used for inoculations 3-5 on days 29 (500mL), 48 (200mL) and 65 (200mL). The HRTs was successively lowered over time so the OLR was continuously increased. The measurement of the amount of produced biogas began on day 34. A leakage in R1000 was discovered using a Honeywell ZPFL1 EZ Sense flammable gas detector and corrected on day 69.

Table 2: Characterisation of the three different operating periods by reactor temperature and operation time.

Period	Temperature	Days
1	25°C	1-21
2	34°C	22-48
3	36°C	49-86

3.5 Sampling and Analysis

Liquid samples from all reactors and influents were taken three times a week for 12 weeks and analysed as described in section 3.2. The samples for VFA, sCOD, alkalinity, NH_4-N, PO_4-P, SO_4 and S^{2-} were further filtered through a 0.45 µm syringe filter before analysis. A 1.5 ml duplicate sample of the filtered suspensions was stored at -16°C as a backup. Gas samples from all reactors were taken each week and the compositions were analysed by GC (Clarus 480, Perkin Elmer). Carriers were removed from the reactors on days 48 and 85, and examined using stereomicroscopy (Nikon SMZ1270, Nikon Corporation) for traces of biofilm.

4. Results

4.1 Characterisation of Substrate Wastewater

The collected wastewater was sampled and analysed before being used as final influent, Table 3. Variations in the wastewater content were due to fluctuations in the daily production of the food factory.

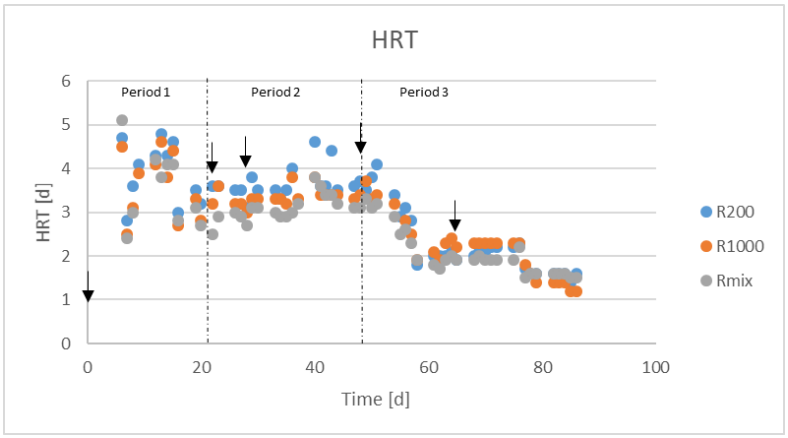
Table 3: Overview of substrate wastewater characteristics separated by batch.

Batch	*1*	*2*	*3*	*4*	*5*	*6*
pH	4.6	4.1	3.9	4.2	3.8	4.7
Alkalinity [mg/L]	31	<25	32	59	44	106
Ca [mg/L]	27	26	24	25	22	22
Mg [mg/L]	5.6	6.6	9.2	8.9	10.7	9.2
SO_4 [mg/L]	<40	12	22	11	27	10
sCOD [mg/L]	700	890	2010	1130	1300	960
tCOD [mg/L]	882	978	2320	1410	2160	1440
NH_4-N [mg/L]	2.03	0.68	9.4	0.04	8.9	4.1
PO_4-P [mg/L]	23	31	12	28	6.0	6.2
TSS [mg/L]	85	39	130	140	220	220
VSS [mg/L]	81	37	120	130	190	200

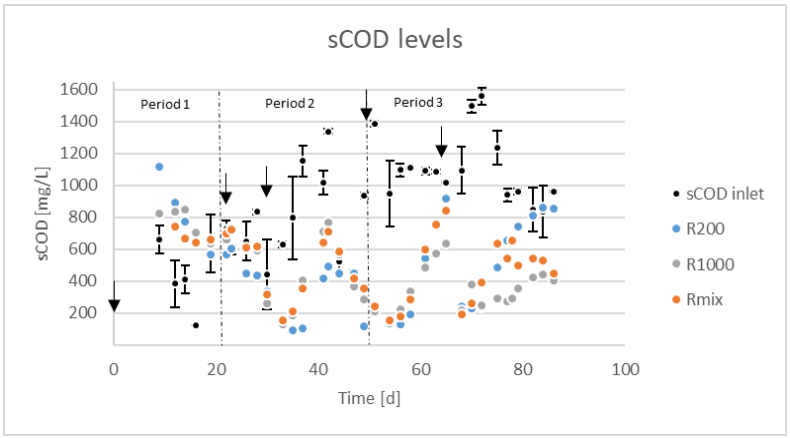
4.2 Reactor Performance at Increasing OLRs

4.2.1 OLR

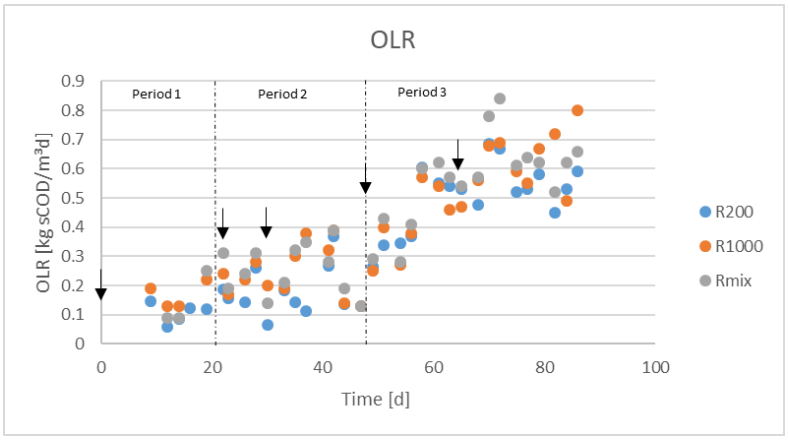
Figure 2 displays how the HRT and mean levels of $sCOD_{influent}$ varied during the 86 days of operation, and how the resulting OLR developed. The HRT decreased over time for each reactor, starting at values of around 4.5 days and ending around 1.4 days. The standard deviation of the $sCOD_{influent}$ fluctuated in scale depending on the levels in each influent, and the mean differentiated depending on the sCOD-concentration in each reactor's substrate. The OLR was gradually increased for all reactors throughout the 86 days of operation, starting at a value of around 0.1 kg $sCOD/m^3d$ and ending approximately at values between 0.6-0.8 kg $sCOD/m^3d$. Some fluctuations of the OLR arose as both the HRT of the reactors and the mean sCOD levels in the inlet unexpectedly varied. These complications could be caused by clogging and wear of the pump tubes, and a fluctuation in fridge temperatures which in turn could have led to possible degradation of organic material in the inlet.



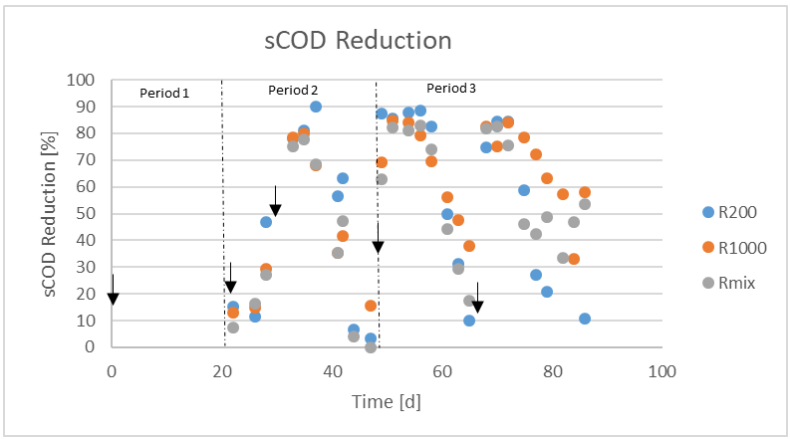
(a)



(b)



(c)



(d)

Figure 2: (a) Hydraulic retention times (HRTs), (b) Mean value and standard deviation of sCOD levels in the influent and the sCOD levels in each reactor, (c) Volumetric organic loading rates (OLR) in the MBBRs during the 86 days of operation, and, (d) sCOD reduction rates in the three anaerobic MBBRs displayed over the three operational periods. The arrows indicate the points where inoculation occurred.

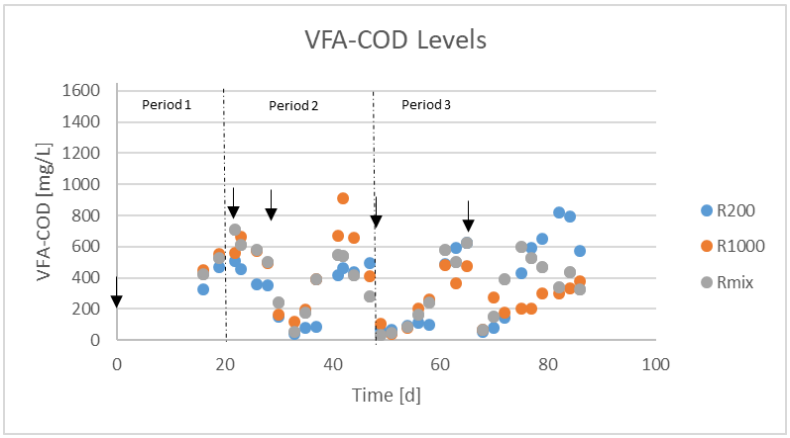
4.2.2 sCOD Levels and Reduction Rates

The sCOD levels in the influent and the reactors were measured to determine the overall presence and activity of microbes in the reactors. During Period 1, when inoculum 1 was used and the temperature was set to 25°C, no sCOD reduction was distinguished and the sCOD levels in the reactors were correspondingly high (Figure 2). The reason for the higher level of sCOD in the reactors compared to the inlet is due to the recent inoculation of the reactors. However, when the temperature was increased in Periods 2 and 3 the reactors began to display reduction rates. After each inoculation, every reactor had a momentary sharp increase of sCOD reductions before decreasing again as the inoculum was washed out. Inoculum 2 (Fiskeby Board AB, Norrköping) had a larger impact on the degree of reduction compared to inoculum 1 (Rotneros, Söderhamn) with a maximum reduction rate of around 90% for all reactors. Inoculum 1 had 47% reduction in R200 and 30% in R1000 and Rmix. After inoculations two through four, R200 had the highest rate of sCOD reduction, while the fifth and final inoculation resulted in that R1000, followed by Rmix, displayed the highest reduction rate. Over time the lowest rate of sCOD reduction in the reactors was increased as the suspended biomass from the inoculations was washed out. This trend implies that some biofilm had begun to form and grow on the carriers between day 48 and 86.

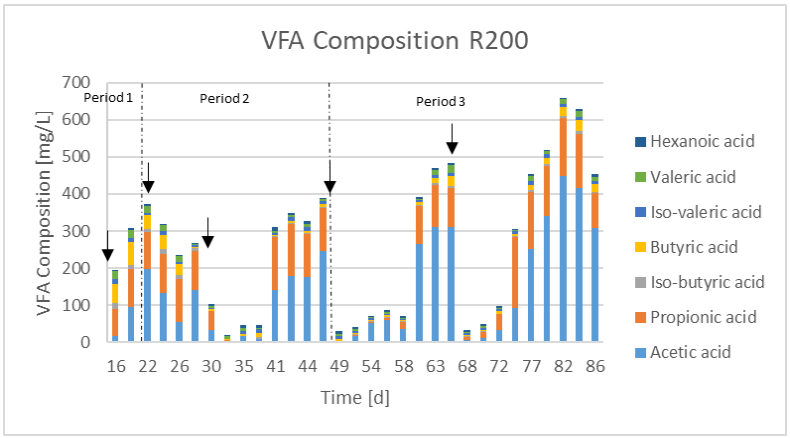
4.2.3 VFA Levels and Composition

The concentration of VFAs, here expressed as VFA-COD equivalents, was measured to investigate the balance of bacteria and archaea in the reactors (Figure 3). After each inoculation the VFA-COD levels in all reactors drop, with a larger decrease after inoculations using inoculum 2. After each inoculation the levels of VFAs were low and stable, before they start increasing as the inoculum was washed out. This indicated that there were no methanogens able to keep up with the conversion of acetic acid to methane in the reactors, resulting in the accumulation of VFAs. The VFA-COD levels during the first 45 days were lower in R200 compared to both R1000 and Rmix. As the operation of the reactors progress the levels in R200 started to increase, resulting in VFA levels that were 1/3 higher compared to R1000 and Rmix. By comparing the VFA-COD levels to the sCOD levels (Figure 3) they showed similar trends as to when there is a microbial activity in the reactors. When the total sCOD levels increased and decreased so did the VFA-COD levels, and the levels were mostly corresponding.

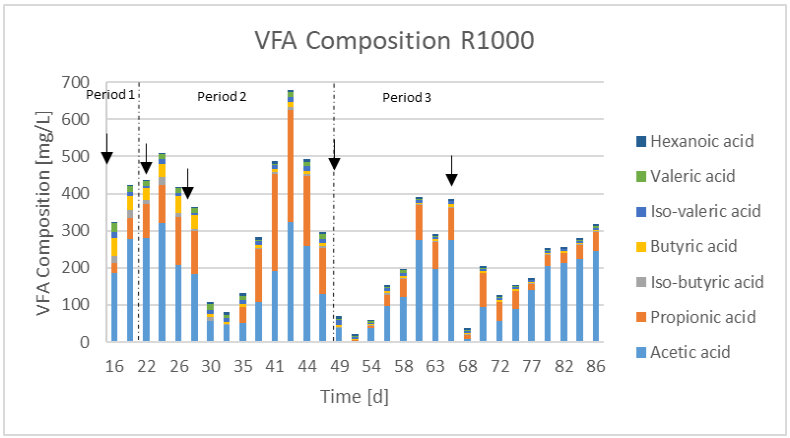
The VFA compositions are an important parameter as it gives information about the degree of hydrolysis and fermentation in the reactors. Acetic and propionic acids were the major products in all three reactors, with negligible levels of other acids. Larger amounts of acetic acid promote biogas production, while high levels of propionic acid indicate an unbalanced anaerobic digestion. During the first 48 days the ratio between propionic and acetic acid were quite high for all reactors. However, after day 48, when the fourth inoculation was performed, the ratio between the two acids decreased. This indicates that the process was beginning to stabilise and the relation between the bacteria and archaea was beginning to balance. This is an indication that some biofilm had begun to grow on the carriers and was slowly developing a stable microbial community.



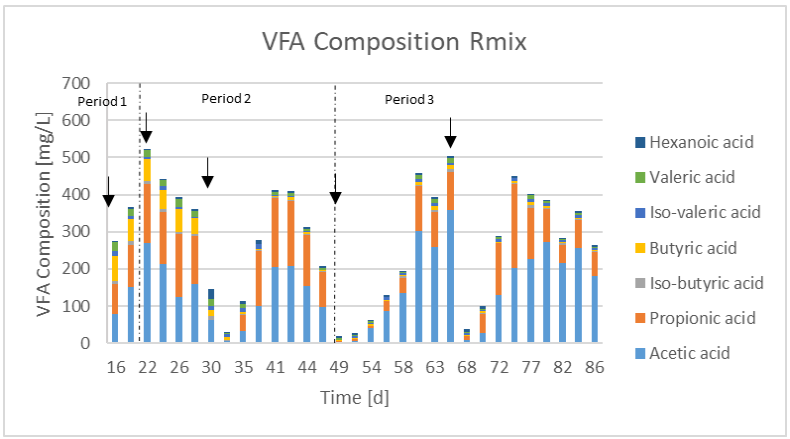
(a)



(b)



(c)



(d)

Figure 3: (a) VFA-COD levels in the reactors, (b) VFA composition in R200, (c) VFA composition in R1000, and, (d) VFA composition in Rmix displayed over the three operational periods. The arrows indicate the points where inoculation occurred.

4.2.4 Biogas Production and Methane Yield

The production of biogas (Figure 4) is directly related to the production of acetic acids by the bacteria and the activity of the methanogens. When the temperature was increased in Periods 2 and 3 the amount of produced biogas also started to increase, with major increases after each inoculation. The trends for R200 and Rmix were similar throughout Periods 2 and 3, with approximately the same amount of produced gas for both reactors. R1000 had a lower biogas production compared to the other reactors, which was believed to be caused by a gas leakage at the lid of the reactor. The leakage was sealed on day 69, resulting in an increase of measured biogas. During the final days of operation, the production of biogas increased, indicating that even though the inoculum had been washed out both bacteria and methanogens were present and active in the reactors. Furthermore, the graph displays a tendency for an increase of the lowest amount of biogas produced in the reactors. These results indicate that microbes were retained in the reactors in form of biofilm. The mean percentage of CH_4 in the produced biogas are 85±5%, 83±4% and 81±4% in R200, R1000 and Rmix respectively.

The production of CH_4 in correlation to the OLR and the amount of reduced sCOD are displayed in Figure 4b-c. The yield of methane did not stabilise for any value of OLR, indicating that none of the reactors had reached process stability. The two values that overshoot for R200 and Rmix for the methane production were caused by the fifth inoculation on day 65. Most of the methane production values for R1000 were low, even as the OLR was increased, and were probably caused by the gas leakage discovered and fixed on day 69. The ratio between the yielded methane production and the amount of sCOD that had been reduced are displayed in Figure 4c. The values for methane production in R1000 were low, which once again could be due to the leakage, and the overshooting values are caused by inoculations. The linear trend lines for each reactor were used to calculate the amount of NmL methane gas produced per mg of sCOD reduced. The intercept of the lines was set to zero, as no methane production is possible without any sCOD reduction. The slopes of the lines were 0.122, 0.074 and 0.154 NmL CH_4/mg reduced sCOD for R200, R1000 and Rmix respectively, which correspond to 34.5%, 21.1% and 44.0% of the theoretical potential of methane production.

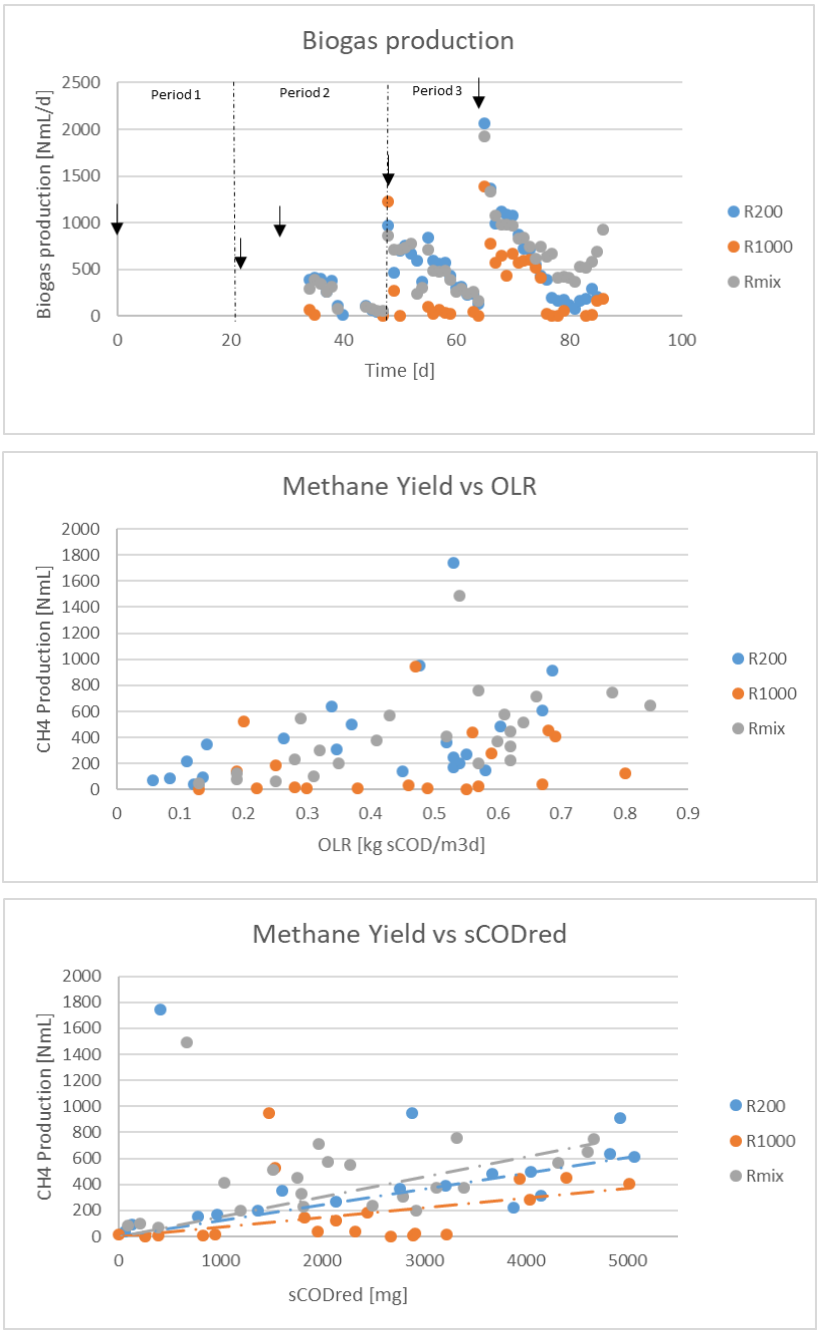


Figure 4: (a) Biogas production over time, (b) Methane yield in relation to OLR , and, (c) Methane yield in relation to reduced sCOD for all three reactors displayed over the three operational periods. The arrows indicate the points where inoculation occurred.

4.3 Presence of Biomass in the Reactors

4.3.1 TSS

The composition of the TSS is an important parameter to determine the amount of suspended organic material (VSS) in the reactors, which in turn gives an indication of the amount of suspended biomass. Figure 5 displays the levels of TSS in the reactors, divided into ash and VSS. The mean amount of VSS in the reactors was 89±9%, 92±6% and 98±6% for R200, R1000 and Rmix respectively. Directly after each inoculation the TSS levels increased in the reactors and decreased successively as the inoculum was washed out. An exception to this is seen for R200 after

the inoculation on day 48, as the TSS levels increased between days 49 and 56. By day 63 the level had decreased again. Furthermore, some variations in the amount of TSS could be seen between the reactors despite the same inoculum, amount and technique were used for inoculating all three reactors. This is very distinguishable at day 49, the day after inoculation four, when R200, R1000 and Rmix had TSS levels of 1150 mg/L, 5225 mg/L and 7925mg/L respectively. These two observations could be due to the different shapes and dimensions of the Anox K™ Z-200 carrier and the Anox K™ Z-1000 prototype. The Anox K™ Z-1000 prototype is wider, and flatter compared to the Anox K™ Z-200, which could result in larger shearing forces caused by carrier collisions. Thus, the granular sludge inoculum crumbled quicker and to a higher degree in the reactors with the Anox K™ Z-1000 prototype. The suspended biomass levels increased and decreased faster in R1000 and Rmix as the dispersion of suspended biomass became more homogenous.

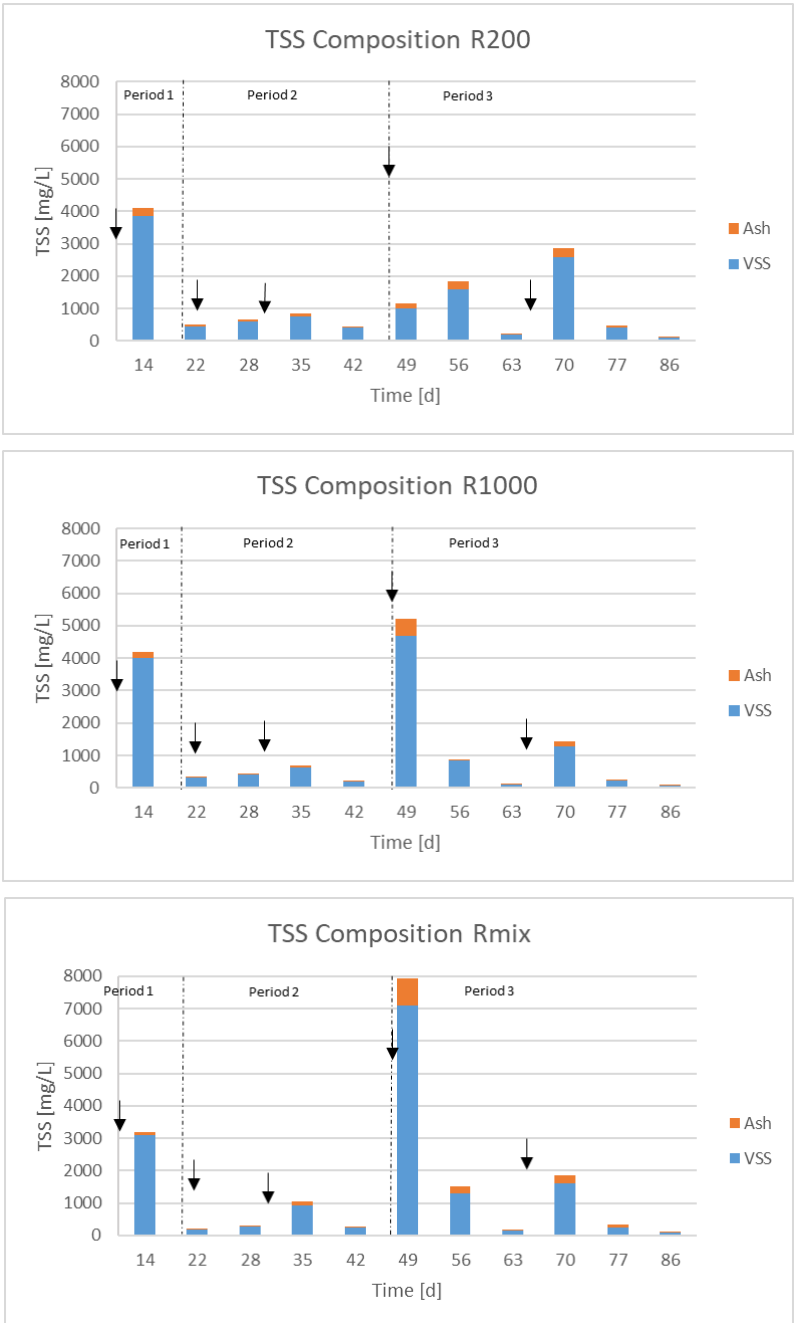


Figure 5: (a) TSS composition in R200, (b) TSS composition in R1000, and, (c) TSS composition in Rmix displayed over the three operational periods. The arrows indicate the points where inoculation occurred.

4.3.2 Carrier Pictures

On days 48 and 85 the reactors were opened, and random samples of carriers were extracted for analysis by stereomicroscopy. Figures 6, 7, 8 and 9 shows the carries of each reactor at the different time points. Both in R200 and Rmix the Anox K™ Z-200 showed no sign of biofilm growth on day 48. However, by day 85 small amounts of biofilm could be distinguished by the walls of the cells. In the spaces where the cell walls were thicker, a higher amount of biofilm could be discerned. These areas provided a more sheltered and protected environment compared to the spaces where the walls were thinner. The Anox K™ Z-1000 prototype carriers in R1000 and Rmix both had small amounts of biofilm in the corners of the cells by day 48. The amount of biofilm then increased by day 85, when the biofilm had somewhat expanded across the walls of the carrier cells. Moreover, larger chunks of biomass granules were caught in some the cells of the Anox K™ Z-1000 prototype carriers, filling them completely (Figure 7b).

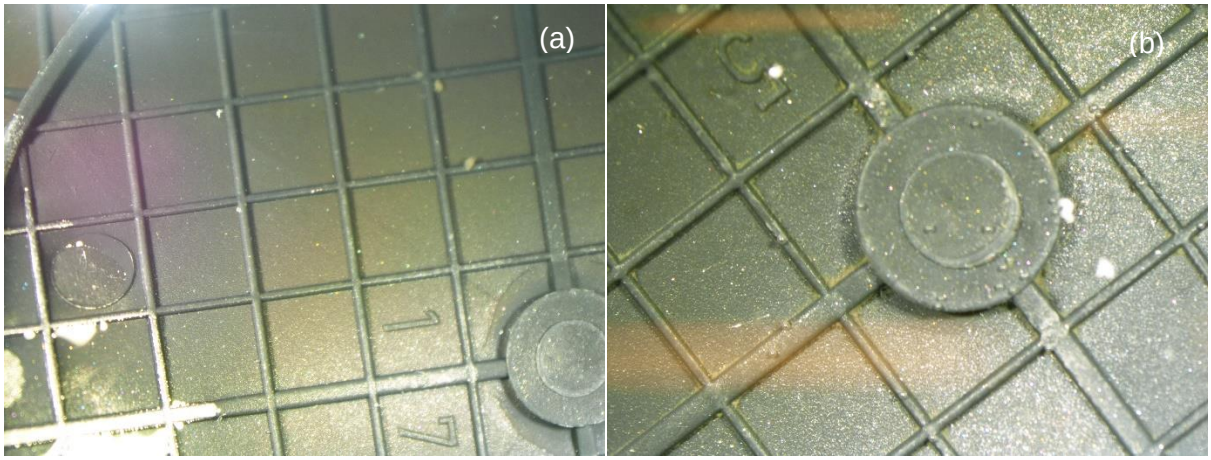


Figure 6: Anox K™ Z-200 carrier from R200 on day (a) 48, and, (b) 85.

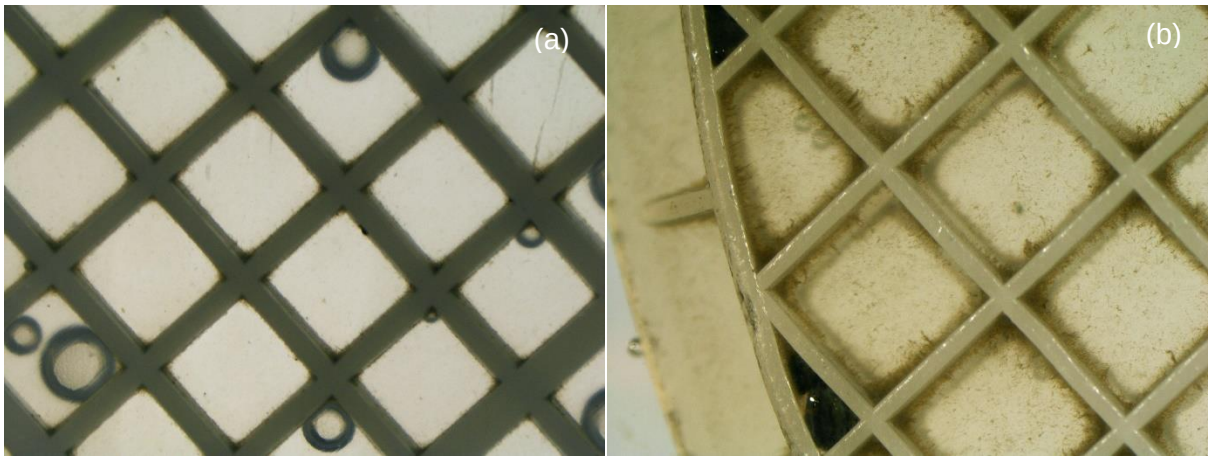


Figure 7: Anox K™ Z-1000 prototype carrier from R1000 on day (a) 48, and, (b) 85.

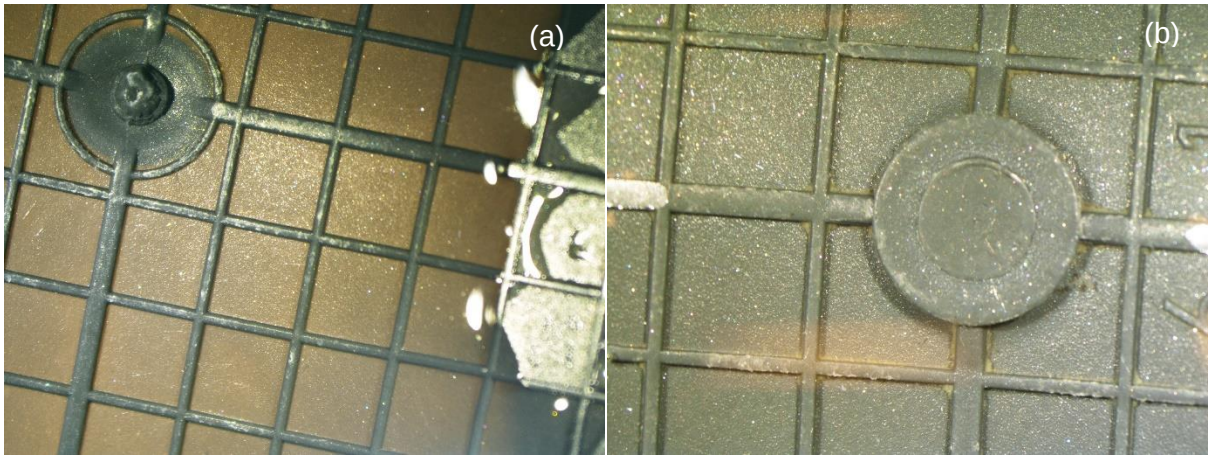


Figure 8: Anox K™ Z-200 carrier from Rmix on day (a) 48, and, (b) 85.

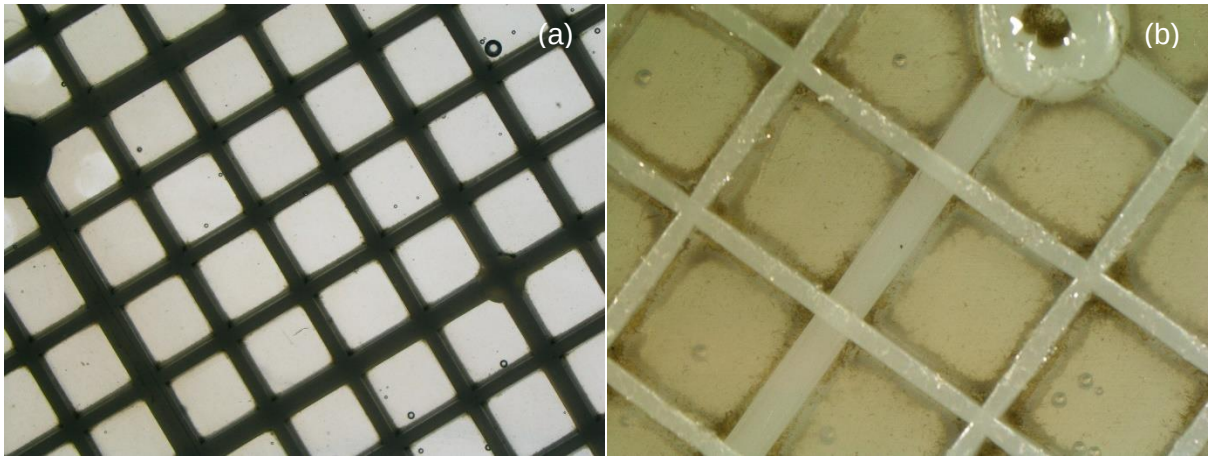


Figure 9: Anox K™ Z-1000 prototype carrier from Rmix on day (a) 48, and, (b) 85

5. Discussion

5.1 Overall Development of the Anaerobic Digestion

By comparing the graphs for the sCOD reduction rates (Figure 2), the VFA levels (Figure 3) and the biogas production (Figure 4) several conjunctions are observed. Each time the reactors were inoculated, the sCOD reduction rates and biogas productions increased simultaneously as the VFA levels dropped. This in correlation to the TSS results (Figure 5), indicates that directly after the inoculations, there were high amounts of suspended biomass in the reactors, resulting in an efficient anaerobic digestion. However, as the suspended biomass from the inoculations was successively washed out, the activity in the reactors also dropped. Period 1 indicates no microbial activity, and Period 2 shows very little activity after the inoculum had been washed out. Though, beginning at the end of Period 2 and throughout in Period 3, the lowest sCOD reduction rates was slowly increased between each inoculation. Similarly, the highest levels of VFAs was successively decreased and the lowest values of the biogas production were gradually raised. Moreover, the ratio of propionic acid in relation to the acetic acid were also decreasing after day 48 in all three reactors (Figure 3). As propionic acid has proven to be more inhibitory to the methanogenesis compared to butyric and acetic acid (Hill et al., 1987; Wang et al., 2009), the accumulation of the acid indicates a process imbalance (Ahring et al., 1995; Björnsson et al., 2000; Murto et al., 2004). The decrease in propionic acids levels therefore indicates that the process is stabilising. These trends in sCOD reduction, biogas production, VFA levels and composition indicate that during the final operating period, which had a temperature of 36°C and an OLR increasing from 0.4 kg sCOD/m^3d to 0.6, 0.65 and 0.8 kg sCOD/m^3d for R200, Rmix and R1000 respectively, small amounts of biomass were retained in the reactors in form of biofilm on the carriers. Sheli and Moletta (2007) found that as the amount of biofilm was increased with the OLR and over time (Sheli and Moletta, 2007), which corresponds to these results. R1000 and Rmix display values that indicate they had grown more biofilm compared to R200, as the results show a higher degree of anaerobic digestion when the inoculum had been washed out. At the end of the operational period the results showed an increase in anaerobic digestion activity for R1000 and Rmix, indicating that the biofilm present in these reactors had begun converting organic material independently of the suspended biomass.

These results are supported by the pictures taken of the carriers on days 48 and 85 (Figures 6-9). In both R1000 and Rmix small amounts of biofilm had begun to grow on the Anox K™ Z-1000 prototype carriers by day 48. On day 85 these biofilms had further developed, and a larger amount could be observed. In contrast, the Anox K™ Z-200 carriers in both R200 and Rmix showed no visible biofilm on day 48. By day 85 small amounts of biofilm had formed in the more sheltered environments of the carrier cells. These visual results indicate that biofilm formed and grew more quickly on the prototype carrier. Chai et al. (2014) showed that carriers with a large specific surface area had a high capability for biomass attachment, which in turn results in the increase of biofilm quantity (Chai et al., 2014). Larger pieces of inoculum sludge granules were also caught in the cells of the Anox K™ Z-1000 prototype carriers, meaning that the resulting activity in R1000 and Rmix could be due to these large collections of biomass. Moreover, the amount of biofilm on the carriers in the reactors weas too small to quantify by the end of the operation period, so the only

measurement method used for quantification of biofilm was visual estimation using stereomicroscopy. As no sequencing of the biofilm was performed, the characteristics of the microbial community in the biofilms was not determined either. The results could however give an indication for when there are bacteria and/or archaea present in the reactors. When there is an accumulation of VFA, the bacteria can convert the larger organic material into smaller compounds while the archaea are not able to further digest the acids into biogas. During the periods when the sCOD reduction rate is high, the VFA levels are low and biogas is produced, there are high concentrations of archaea present in the reactors as the organic material is effectively reduced. Since the methanogens have a much lower growth rate, they are more easily washed out of the reactors than the bacteria (Habouzit et al., 2014). This is distinguishable in the graph for the VFA-COD levels (Figure 3) as the levels drops as soon as the methanogens are present, and then slowly increase as they are washed out while the bacteria remain.

5.2 Influence of Operational Parameters

The three reactors were initially started at 25°C in Period 1 with a low OLR of 0.1 kg sCOD/m^3d and long HRTs of 4.5 days. The OLR was then successively increased by either decreasing the HRT or increasing the sCOD concentration in the substrate. The temperature was increased to first 34°C in Period 2 and 36°C in Period 3. The final HRT in R200 and Rmix reached 1.5 days while R1000 reached 1.2 days, and the corresponding OLR were 0.6, 0.65 and 0.8 kg sCOD/m^3d for R200, Rmix and R1000 respectively. All the reactors showed very little, if any, microbial activity during Period 1. During this period the temperature were kept at 25°C and inoculum 1 was used. Therefore, the combination of the rather low temperature and the older inoculum resulted in a low microbial activity. When the temperature was increased to 34°C, and the OLR successively raised, the activity of the reactors increased. This indicates that the microbes in the reactors prefer higher temperatures and OLRs. This corresponds to the results found by Chai et al. (2014) and Sheli and Moletta (2007), as they conclude that the production of biogas is increased and strongly correlated with the OLR (Chai et al., 2014; Sheli and Moletta, 2007). After the second inoculation with inoculum 1 the activity in the reactors was further increased, with R1000 and Rmix reaching a maximum value of 30% for the sCOD reduction rate while R200 reached almost 50%. The OLR was also slightly increased by 0.1 kg sCOD/m^3d. The combination of the increased temperature and the increased substrate load resulted in a higher activity.

After the third inoculation using inoculum 2, the microbial activity increased in all reactors. Since the temperature was stable at 34°C and the OLR was somewhat fluctuating, the increase in activity could be correlated to the fresh inoculum. In combination with the fourth inoculation the temperature was raised to 36°C and the OLR was slowly increased. The activity was high and stable for 12 days until it successively lowered as the inoculum was washed out. However, the lowest reached sCOD removal rate for all three reactors was higher compared to the previously observed rates, indicating a retention of biomass in the reactors. After the fifth inoculation the microbial activity increased before decreasing again. By the end of the operational time the activity increased somewhat for R1000 and Rmix while it continued to decrease for R200. The lowest rate of sCOD reduction for all reactors had again increased. Therefore, the combination of a raised temperature with higher OLRs and a new inoculum improved the conditions for biofilm formation during the startup of an AnMBBR. However, as Figure 4 shows, the production of biogas in relation to the

OLR had not stabilised for any reactor, nor had either of them reached the theoretical potential of 0.35NmL CH_4 production/mg sCOD reduced (Carlsson and Schnüer, 2011). R200, R100 and Rmix reached 34.5%, 21.1% and 44.0% of the theoretical potential for methane production respectively, indicating that not even half of the potential production were reached. These results indicate that the reactors had yet to reach process stability, which is correlated to the lack of further development of biofilm.

5.3 Influence of Carrier Design

The two different carriers used for this project were very different in design and structure. The Anox K™ Z-200 were saddle-shaped, consisted of recycled HDPE, with a projected surface area of 0.00128m^2 and cell depth of 200µm. The Anox K™ Z-1000 prototype carriers were flat, consisted of virgin HDPE material and had a projected surface area of 0.00164m^2 and cell depth of 1000µm. The three reactors were exposed to the same conditions, except for some variations in OLR, with the carrier type they were loaded with as the major difference. As can be derived from the results, the Anox K™ Z-1000 prototype carriers in both R1000 and Rmix showed signs of a biofilm formation before the Anox K™ Z-200 carriers in Rmix and R200. Since the protected surface area for the Anox K™ Z-1000 prototype carriers is larger than the Anox K™ Z-200 carriers, the prototype offers a larger area for the biofilm to grow on, which has been previously proven favourable (Chai et al., 2014). Furthermore, the prototype's cells were deeper by 800µm compared to the Z-200 carrier, which promotes a quicker initial biofilm formation (Chen et al., 2015; Li et al., 2016). Moreover, the design of the Anox K™ Z-1000 prototype carriers could have caused larger shearing forces as the flat and wide design may increase the number of collisions compared to the saddle-shape of the Anox K™ Z-200 carriers. This causes the inoculum to crumble quicker, which can be seen in the results for TSS (Figure 5). If the granules are disintegrated, the newly freed, suspended biomass could easier access the sheltered areas of the carriers. Another factor which may impact the formation of biofilm are the origin of the material the carriers are composed of. The Anox K™ Z-200 carriers consist of recycled material, while the Anox K™ Z-1000 prototype carriers consist of virgin HDPE. The direct influence of the carrier material on the biofilm formation could not be determined as no defined trends could be observed, however it is something which could be of interest for further analysis.

5.4 Troubleshooting of the Reactor Setup

The reactor setup was continuously examined and evaluated for potential issues. The substrate was kept on a stirrer in a small fridge, so it was continuously stirred and refrigerated. However, the temperatures of the fridges were fluctuating extensively (3-15°C). The stirrers were removed during the final week of operation, and the temperatures in the fridges dropped and stabilised immediately. The fluctuations in temperature were determined to be caused by the heat produced by the stirrers. These fluctuations in turn may have caused some variations of sCOD concentrations in the substrates, even though all the substrates were prepared from the same batch of origin wastewater. Consequently, the OLR deviated between the reactors. Moreover, the inlet pump tubes were over time clogged and worn down, which affected the HRT and OLR. By cleaning and replacing the tubes these issues were resolved. R1000 showed very low values for the biogas production compared to the other two reactors, even after the inoculations. The

interconnections of gas tubes were examined for leakage and sealed using zip ties. Silicone grease was used as lubricant for the packaging between the top of the reactor and the lid. On day 69 a Honeywell ZPFL1 EZ Sense flammable gas detector was used to determine if any methane escaped the reactor. An indication of high methane concentration was given at the lid of the reactor, so the packing at the lid was changed, sealing the leakage and resulting in a more stabilised methane production.

5.5 Statistical Analysis

A statistical analysis was considered for this project. However, as none of the reactors reached continuous stability within the operational period, nor developed a sufficient amount of biofilm, a meaningful statistical analysis of the results was not possible.

6. Conclusions

In this project the overall aim was to investigate how different operational parameters influenced the initial biofilm establishment and growth in an AnMBBR. During the 86 days of operation, the reactors had yet to reach operational stability. However, as the temperature and OLR was gradually increased while the HRT was decreased, the activity in the reactors was raised. Furthermore, the fresher inoculate induced a higher activity compared to the older granular sludge, and biofilm began to develop quicker on the Anox K™ Z-1000 prototype carriers.

The optimal conditions for an initial biofilm formation during start-up of an AnMBBR were not determined in this project. However, an insight regarding what conditions may function better compared to others was gained. Higher temperatures and OLRs induced higher microbial activities, which in turn caused a quicker initial biofilm formation. Long HRTs gave the microbes with slower growth rates a chance to start forming biofilm before being washed out, and a fresher inoculum contained more active microbes, increasing the activity of the biomass. Finally, a larger and more sheltered growth area gave the microbes a better protection against turbulence and shearing forces to develop biofilm.

During start-up, an AnMBBR, just like other anaerobic digestion processes, is quite sensitive to changes in operational parameters. Factors like low temperatures, low OLRs and old inoculums all contribute to low microbial activity and a low rate of anaerobic digestion. The results from this project showed that when the temperature and OLR are increased, and a fresher inoculum is used, the activity of the AnMBBR increased. Long HRTs gives the microbes plenty of time to grow, but if a weak substrate is used, the available organic material quickly diminished and caused the microbial growth and activity to decrease.

The impact of the design of the carrier media seems to have a large influence on the biofilm formation during start-up of an AnMBBR. The carrier with the larger protected area and the more sheltered environment seems to have offered a more appealing growth template for the biofilm. Furthermore, the collisions of the larger Anox K™ Z-1000 prototype carriers seem to disintegrate the granules of the inoculum quicker than that of the Anox K™ Z-200 carriers, leading to a larger contact area between inoculum and carrier. These factors suggest the Anox K™ Z-1000 prototype carriers were more suitable for initial biofilm formation in an anaerobic MBBR.

7. Suggestions for Future Work

Although some interesting results were gained in this study, further evaluations will contribute to a greater understanding of the initial establishment and development of biofilm during the start-up of an anaerobic MBBR.

A higher reactor temperature should be used from the beginning as the microbes have proven to be more active at the higher spectrum of the mesophilic range. The OLR should be increased in order to supply the microbes with enough substrate for them to initiate growth and formation of biofilm. However, as excessive amounts of substrate may also inhibit the microbes, the optimal range of OLR should be studied. By keeping the HRT long, the slow growing archaea are given the opportunity to attach and grow on the carriers before the inoculum is washed out. Therefore, the high HRT should be retained.

The inoculum should be fresh in order to have as much initial microbial activity as possible. However, the technique for inoculation should be investigated further. The inoculum could e.g. be prepared as a suspended biomass before inoculation, instead of in the shape of granules, so that the contact area between the biomass and the carriers is increased.

Finally, in regard to the carrier design, further evaluation of which carrier characteristics are more appealing for a biofilm template would be of interest. This could be done by e.g. implementing the changes to the inoculum and inoculations suggested above, in order to assure that the form of the inoculum in each reactor are the same. The impact of the carrier material should also be further investigated to determine whether it has any direct influence on the formation of biofilm.

8. References

1. Abbasi, T., Tauseef, S.M. and Abbasi, S.A. (2012). *Biogas energy*. New York: Springer.

2. Adekunle, K. and Okolie, J. (2015). 'A Review of Biochemical Process of Anaerobic Digestion'. *Advances in Bioscience and Biotechnology*, 06(03), pp.205-212.

3. Ahring, B.K., Sandberg, M. and Angelidaki, I. (1995). 'Volatile fatty acids as indicators of process imbalance in anaerobic digestors'. *Applied Microbiology and Biotechnology,* 43(3), pp. 559–565.

4. Björnsson, L., Murto, M. and Mattiasson, B. (2000). 'Evaluation of parameters for monitoring an anaerobic co-digestion process'. *Applied Microbiology and Biotechnology*, 54(6), pp. 844–849.

5. Carlsson, M. and Schnürer, A. (2011). *Handbok metanpotential, Rapport SGC 237*. Svenskt Gastekniskt Centrum. ISRN: SGC-R-237-SE. [In Swedish]

6. Chai, S., Guo, J., Chai, Y., Cai, J. and Gao, L. (2014). 'Anaerobic treatment of winery wastewater in moving bed biofilm reactors'. *Desalination and Water Treatment*, 52(10-12), pp.1841-1849.

7. Chen, X., Kong, L., Wang, X., Tian, S. & Xiong, Y. (2015). 'Accelerated start-up of moving bed biofilm reactor by using a novel suspended carrier with porous surface'. *Bioprocess and biosystems engineering*, 38(2): 273–285.

8. Davis, M. (2011). *Water and Wastewater Engineering: Design Principles and Practice*. New York: McGraw-Hill Education.

9. Demirel, B. and Scherer, P. (2008). 'The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: A review'. *Reviews in Environmental Science and Bio/Technology*, 7(2), pp.173-190.

10. di Biase, A., Devlin, T., Kowalski, M. and Oleszkiewicz, J. (2018). 'Performance and design considerations for an anaerobic moving bed biofilm reactor treating brewery wastewater: Impact of surface area loading rate and temperature'. *Journal of Environmental Management*, 216, pp.392-398.

11. Donlan, R.M. (2002). 'Biofilms: Microbial Life on Surfaces'. *Emerging Infectious Diseases,* 8(9): pp.881–890.

12. Gorris, L. G. M., van Deursen, J. M. A., van der Drift, C. and Vogels, G. D. (1989). 'Inhibition of propionate degradation by acetate in methanogenic fluidized bed reactors'. *Biotechnology Letters*, 11(1), pp. 61–66.

13. Habouzit, F., Hamelin, J., Santa-Catalina, G., Steyer, J. and Bernet, N. (2014). 'Biofilm development during the start-up period of anaerobic biofilm reactors: the biofilm Archaea community is highly dependent on the support material'. *Microbial Biotechnology*, 7(3), pp.257-264.

14. Henze, M., van Loosdrecht, M., Ekama, G. and Brdjanovic, D. (2008). *Biological Wastewater Treatment*. London: IWA Pub.

15. Hill, D.T., Cobb, S.A. and Bolte, J.P. (1987). 'Using volatile fatty acid relationships to predict anaerobic digester failure'. *Trans. ASAE*, 30(2), pp.496 -501.

16. Jonstrup, M., Murto, M. and Björnsson, L. (2011). *Environmental biotechnology*. KBT080¸course material in environmental biotechnology, Department of Biotechnology, LTH.

17. Morgan-Sagastume, F., Jacobsson, S., Olsson, L., Carlsson, M., Gyllenhammar, M. and Sárvári Horváth, I. (2019). 'Anaerobic treatment of oil-contaminated wastewater with methane production using anaerobic moving bed biofilm reactors'. *Water Research*, 163, pp.1-13.

18. Murto, M., Björnsson, L. and Mattiasson, B. (2004). 'Impact of food industrial waste on anaerobic co-digestion of sewage sludge and pig manure'. *Journal of Environmental Management*, 70(2), pp. 101–107.

19. Nakano, M. M. and Zuber, P. (2004). *Strict and Facultative Anaerobes: Medical and Environmental Aspects.* 1st ed. Boca Raton: CRC Press.
20. Lee, D.J., Jegatheesan, V., Ngo, H.H., Hallenbeck, P.C. and Pandey, A. (2017). *Current Developments in Biotechnology and Bioengineering.* Amsterdam: Elsevier.
21. Rajinikanth, R., Ganesh, R., Escudie, R., Mehrotra, I., Kumar, P., Thanikal, J. and Torrijos, M. (2009). 'High rate anaerobic filter with floating supports for the treatment of effluents from small-scale agro-food industries'. *Desalination and Water Treatment*, 4(1-3), pp.183-190.
22. Rarooq, R. and Ahmad Z. (2017) *Biological Wastewater Treatment and Resource Recovery.* Rijeka: IntechOpen.
23. Schnaars, K. (2012). 'What every operator should know about anaerobic digestion'. *Water Environment and Technology,* 24(12), pp 82-83.
24. Sheli, C. and Moletta, R. (2007). 'Anaerobic treatment of vinasses by a sequentially mixed moving bed biofilm reactor'. *Water Science and Technology*, 56(2), pp.1-7.
25. Tchobanoglous, G. and Burton, F. (1991). *Wastewater Engineering Treatment, Disposal and Reuse.* 3rd ed. New York: McGraw-Hill.
26. Tchobanoglous, G., Burton, F., Stensel, H. and Tsuchihashi, R. (2014). *Wastewater Engineering Treatment and Resource Recovery.* 5th ed. New York: McGraw-Hill Higher Education.
27. Vlyssides, A., Barampouti, E. and Mai, S. (2009). 'Influence of ferrous iron on the granularity of a UASB reactor'. *Chemical Engineering Journal*, 146(1), pp.49-56.
28. Wang, S., Chandrasekhara Rao, N., Qiu, R. and Moletta, R. (2009). 'Performance and kinetic evaluation of anaerobic moving bed biofilm reactor for treating milk permeate from dairy industry'. *Bioresource Technology*, 100(23), pp.5641-5647.
29. Wang, Y., Zhang, Y., Wang, J. and Meng, L. (2009). 'Effects of volatile fatty acid concentrations on methane yield and methanogenic bacteria'. *Biomass and Bioenergy*, 33(5), pp. 848–853.
30. Westman, S., Chandolias, K. and Taherzadeh, M. (2016). 'Syngas Biomethanation in a Semi-Continuous Reverse Membrane Bioreactor (RMBR)'. *Fermentation*, 2(4), p.8.
31. Wijekoon, K.C., Visvanathan, C. and Abeynayaka, A. (2011). 'Effect of organic loading rate on VFA production, organic matter removal and microbial activity of a two-stage thermophilic anaerobic membrane bioreactor'. *Bioresource Technology*, 102(9), pp. 5353–5360.
32. Ødegaard, H. (1999). 'The Moving Bed Biofilm Reactor'. *Igarashi, T., Watanabe, Y., Asano, T. and Tambo, N.: Water Environmental Engineering and Reuse of Water.* Hokkaido Press 1999, p 250-305.
33. Ødegaard, H. (2006). 'Innovations in Wastewater Treatment: The Moving Bed Biofilm Process'. *Water Science and Technology*, 53(9), pp.17-33.