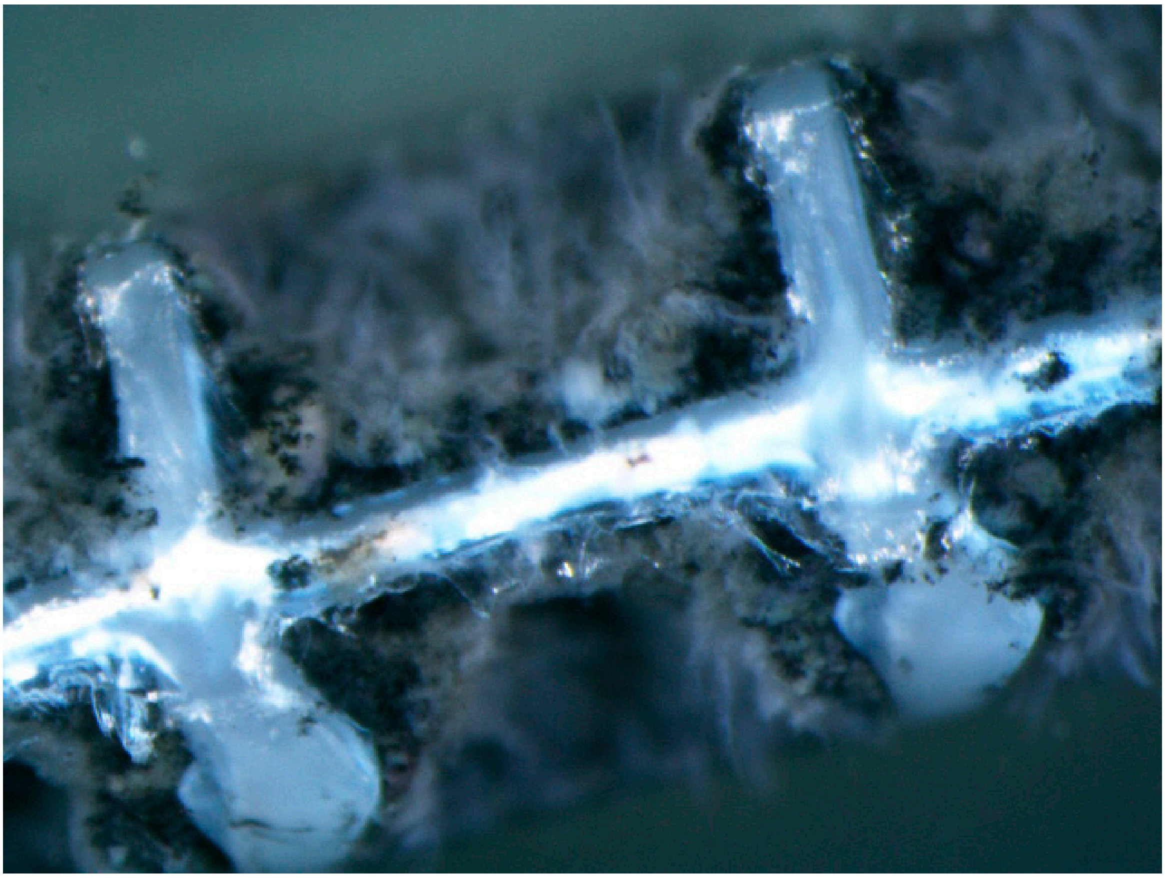
Effect of biofilm thickness & carrier type on the performance of anaerobic moving bed biofilm reactors (AnMBBRs)



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Department of Chemical Engineering
Master Thesis 2020

Lund University

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by

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Picture on front page: Cross-section of an AnoxK™ Z-1000 carrier with biofilm. Picture by Astrid Hermansson.

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Lund, June 2020

Astrid Hermansson

Summary

Anaerobic wastewater treatment for removal of organic matter has gained interest as a complement to conventional aerobic wastewater treatment because of its capacity to treat wastewaters at a high loading rate and its ability to produce energy in the form of biogas. The anaerobic moving bed biofilm reactor (AnMBBR) is an anaerobic wastewater treatment process that has shown effective for the treatment of industrial wastewaters. It consists of a reactor with a freely moving support material ("carriers") on which a biofilm grows. The microorganisms in the biofilm can effectively remove organic material from the wastewater whilst producing biogas which can be used as an energy source. As the AnMBBR is a biofilm process, nutrients, products, etc. must be transported through the biofilm. The thickness of the biofilm may, therefore, impact the process.

The objectives of this study were (1) to evaluate the effect of the biofilm thickness and carrier type on the performance (in terms of soluble organic removal, "sCOD removal", and biogas production) of AnMBBRs and (2) to assess the effect of the different carriers on biofilm development and accumulation. Three continuous lab-scale AnMBBRs were operated in this study, fed with an industrial wastewater from food processing. The reactors were filled with two different carrier types allowing for different maximal biofilm thickness. Reactor "R200" was filled with AnoxK™ Z-200 carriers (a commercial carrier made of recycled HDPE with a maximum biofilm thickness of 200 μm), "R1000" was filled with AnoxK™ Z-1000 carriers (a prototype made of virgin HDPE with a maximum biofilm thickness of 1000 μm) and reactor "RMIX" was filled with a mix of the two carriers.

The results from this study suggested that the biofilm thickness and type of carrier influence the performance of AnMBBRs. R200 showed a low performance with a fluctuating sCOD removal not exceeding 73% and a methane content in the biogas of 63 ± 2%. The fluctuating sCOD removal together with high and fluctuating levels of volatile fatty acids (VFAs) indicated process imbalance with regard to the microbial community in this reactor, possibly as a result of more exposed microorganisms in the thinner biofilm or that the amount of biomass was simply not enough in this reactor. The results from R1000 and RMIX were comparable to other studies made on AnMBBRs treating industrial wastewaters from food processing. Both reactors had high sCOD removal efficiencies of over 90% and a methane content in the biogas of 69 ± 2% for R1000 and 68 ± 2% for RMIX.

Furthermore, the rate of biofilm accumulation was considerably higher for the Z-1000 carriers, as compared to the Z-200 carriers, possibly as a result of the biofilm being less exposed to hydraulic shear in the deeper Z-1000 carriers. At the end of this study, R200 had a low amount of biofilm (5.8 ± 1.1 g VS/m^2 PSA) but a high specific removal rate (3.7 g sCOD removed/g VS/day) while R1000 had a higher amount of biofilm (54.2 ± 12.8 g VS/ m^2 PSA) but a lower specific removal rate (0.64 g sCOD removed/g VS/day). This suggests that the biofilm on the Z-200 carriers was more active or specialized compared to the biofilm on the Z-1000 carriers.

Although this study made interesting findings with regard to biofilm thickness and carrier type, future studies should focus on characterising the microbial community of the biofilm as well as to characterise the surface properties of the carrier material. This to gain deeper understanding concerning how the microbial composition depends on the biofilm thickness and the carrier type, and how this may influence the performance of the AnMBBR.

Key words: Anaerobic, wastewater treatment, AnMBBR, MBBR, biofilm thickness, carrier, organic removal

Sammanfattning

Anaerob avloppsrening för organisk avskiljning har erhållit ökat intresse som ett komplement till konventionell aerob avloppsvattenrening. Detta på grund av dess kapacitet att behandla avloppsvatten med en hög belastningsgrad och dess förmåga att producera energi i form av biogas. Den så kallade *Anaerobic Moving Bed Biofilm Beactor* (AnMBBR) är en avloppsreningsprocess som visat sig vara effektiv för behandling av industriellt avloppsvatten. Den består av en reaktor med ett material (”bärare”), som rör sig fritt i reaktorn, på vilket en biofilm växer. Mikroorganismerna i biofilmen avlägsnar organiskt material från avloppsvattnet, samtidigt som de producerar energi i form av biogas. Eftersom AnMBBR är en biofilmsprocess måste näringsämnen, produkter etc. transporteras genom biofilmen. Tjockleken på biofilmen kan därför påverka processen.

Målen med denna studie var (1) att utvärdera effekten av biofilmtjocklek och bärartyp med avseende på reaktorprestanda (med hänsyn till avlägsnande av lösligt organiskt material, ”sCOD”, och biogasproduktion) i AnMBBR:er och (2) att bedöma effekten av olika bärare med avseende på biofilmutveckling och -ackumulering. Tre kontinuerliga AnMBBR:er i laboratorieskala användes med ett industriellt avloppsvatten från livsmedelsindustrin som substrat. Reaktorerna var fyllda med två olika bärartyper vilka möjliggjorde olika maximal biofilmtjocklek. Reaktor “R200” fylldes med AnoxK^{TM} Z-200-bärare (en kommersiell bärare tillverkad av återvunnen HDPE med en maximal biofilmtjocklek på 200 μm), ”R1000” fylldes med AnoxKTM Z-1000-bärare (en prototyp tillverkad av ny HDPE med en maximal biofilmtjocklek på 1000 μm) och "RMIX" fylldes med en blandning av de två olika bärartyperna.

Resultaten från denna studie antydde att biofilmtjockleken och typen av bärare påverkar AnMBBR:ens prestanda. R200 visade en låg prestanda med ett fluktuerande avlägsnande av sCOD som inte översteg 73 %, och ett metaninnehåll i biogasen på 63 ± 2 %. Det fluktuerande sCOD-avlägsnandet tillsammans med höga och fluktuerande nivåer av flyktiga fettsyror (VFA) indikerade processobalans med avseende på det mikrobiella samhället, eventuellt till följd av mer exponerade mikroorganismer i den tunnare biofilmen, eller att mängden biomassa inte var tillräcklig i denna reaktor. Resultaten från R1000 och RMIX var jämförbara med tidigare studier gjorda på AnMBBR:er som behandlade industriellt avloppsvatten från livsmedelsindustrin. Båda reaktorerna hade hög sCOD-avskiljning på över 90 %, och ett metaninnehåll i biogasen på 69 ± 2 % för R1000 och 68 ± 2 % för RMIX.

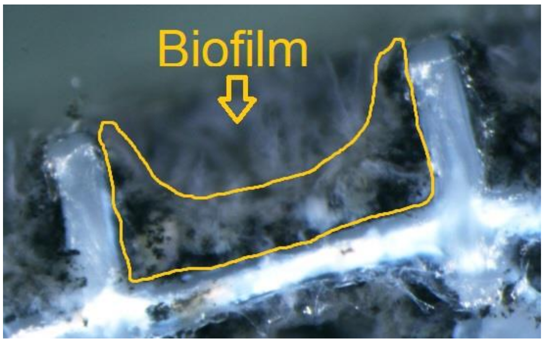
Ackumuleringen av biofilm var avsevärt snabbare för Z-1000-bärarna, jämfört med Z-200-bärarna, möjligen som resultat av att biofilmen var mindre utsatt för hydraulisk skjuvning i de djupare Z-1000-bärarna. I slutet av denna studie hade bärarna i R200 en låg mängd biofilm (5,8 ± 1,1 g VS/m^2 PSA), men en hög specifik avskiljningsgrad (3,7 g sCOD avskilt/g VS/dag) medan bärarna i R1000 hade en högre mängd biofilm (54,2 ± 12,8 g VS/m^2 PSA) men en lägre specifik avskiljningsgrad (0,64 g sCOD avskilt/g VS/dag). Detta tyder på att biofilmen på Z-200-bärarna var mer aktiv eller specialiserad jämfört med biofilmen på Z-1000-bärarna.

Även om denna studie gjorde intressanta upptäckter med avseende på biofilmtjocklek och bärartyp, bör framtida studier fokusera på att även karakterisera biofilmens mikrobiella samhälle samt att karakterisera ytan på bärarmaterialet. Detta för att få djupare förståelse för hur den mikrobiella sammansättningen beror på biofilmtjockleken och bärartypen, och hur detta kan påverka AnMBBR:ers prestanda.

Nyckelord: Anaerob, avloppsvattenrening, AnMBBR, MBBR, biofilmtjocklek, bärare, organisk avskiljning

Populärvetenskaplig sammanfattning

Att rena avloppsvatten med mikroorganismer i en biofilm – Insikt i hur tjockleken på biofilmen påverkar reningsprocessen



Biofilmer finns på ytor överallt runtomkring oss: på våra tänder (plack), på våta ytor i t ex. badrummet och på stenar i vattendrag. Biofilmer är ytterst tunna (vanligtvis mindre än 1 mm) och består av aggregat av mikroorganismer, såsom bakterier, vilka omges av en skyddande hinna. Dessa aggregat bildar ett "supersamhälle" av mikroorganismer som gör det möjligt för dem att lättare samarbeta med varandra och utbyta näringsämnen m.m. I vissa fall kan biofilmer vara av ondo men i andra fall kan de vara till nytta för oss och naturen. Biofilmer kan nämligen användas för att rena avloppsvatten! Om detta görs i en syrefri miljö produceras dessutom biogas vilket kan användas som bränsle.

I livsmedelsindustrin och liknande industrier används ofta vatten för framställning av produkter. I och med denna användning produceras stora mängder avloppsvatten som är rikt på organiska föreningar såsom kolhydrater, fett och proteiner. Om dessa näringsämnen kommer ut i naturen kan detta leda till negativa konsekvenser för både miljö och människa, såsom övergödning och fiskdöd. Avloppsvatten rikt på organiska föreningar måste alltså effektivt renas för att kunna släppas ut i naturen eller för att återanvändas. En teknik, utvecklad av AnoxKaldnes AB, som har visat sig effektivt för att rena avloppsvatten från organiska föreningar är den så kallade "Anaerobic Moving Bed Biofilm Reactor" (förkortat AnMBBR). "Anaerobic" står för att processen görs utan syre medan "Moving Bed Biofilm" syftar till att tekniken använder sig av biofilm som växer på ett material (så kallade "bärare") som rör sig fritt i reaktorn.

Näringsämnen m.m., som är nödvändiga för att mikroorganismerna ska kunna växa och vara aktiva, måste kunna penetrera hela biofilmen. Detta betyder i sin tur att biofilmens tjocklek kan ha betydelse för reningsprocessen. Syftet men denna studie var därför att undersöka hur tjockleken av biofilmen påverkar reningsprocessen. Detta gjordes genom att driva tre stycken reaktorer i ett labbskaleförsök. Dessa reaktorer var fyllda med två olika typer av bärare, en som tillåter en maximal biofilmstjocklek på 200 mikrometer ("Z-200" bärare) och en som tillåter en maximal biofilmstjocklek på 1000 mikrometer ("Z-1000" bärare). De två bärartyperna hade dessutom vissa skillnader i form och material.

Studien visade att den AnMBBR som var fylld med Z-200-bärare hade en sämre prestanda jämfört med den AnMBBR som var fylld med Z-1000-bärare, eftersom avlägsnandet av organiskt material var lägre i denna reaktor och mer fluktuerande. Detta misstänks bland annat kunna bero på att den tunna biofilmen på Z-200-bärarna är mer utsatt för förändringar i sin omgivning (såsom förändringar i näringsinnehåll och sammansättning av avloppsvattnet), men även att det var en obalans mellan de samarbetande mikroorganismerna i biofilmen. Dessutom var både mängden biofilm mindre och ackumulationen av biofilm avsevärt långsammare på dessa Z-200-bärare, vilket troligen beror på att ytan på dessa bärare var mindre skyddad och mikroorganismerna därför har svårare att etablera sig. Alternativt skulle detta även kunna bero på skillnader i materialet på bärarna vilket skulle kunna påverka hur mikroorganismerna interagerar med materialet och hur "lätt" de har för att fästa och bilda biofilm på ytan. Vad som dock var intressant var att, trots att Z-200-bärarna hade en mindre mängd biofilm, så avlägsnade de mer organiskt material per gram biofilm. Detta visar på att mikroorganismerna var mer aktiva på Z-200-bärarna jämfört med Z-1000-bärarna, vilket skulle kunna vara en konsekvens av den tunnare biofilmen som gör det möjligt för näringsämnen att penetrera hela biofilmens tjocklek.

Denna studie visar på vikten av att undersöka hur biofilmens tjocklek påverkar reningsprocessen, för att kunna utvärdera vilka typer av bärare som lämpar sig bäst för att användas i AnMBBR processen. Detta är av stor vikt för att man i framtiden mer effektivt ska kunna rena olika typer av avloppsvatten. I framtiden skulle det dock även vara av intresse att med hjälp av genteknik identifiera vilka typer av mikroorganismer som biofilmen består av och hur biofilmens tjocklek påverkar detta mikrobiella samhälle.

List of abbreviations

AnMBBR	Anaerobic Moving Bed Biofilm Reactor
AnWWT	Anaerobic Wastewater Treatment
COD	Chemical Oxygen Demand (*mg/L*)
EPS	Extracellular Polymeric Substances
HDPE	High-Density Polyethylene
HRT	Hydraulic Retention Time (*hr or days*)
MBBR	Moving Bed Biofilm Reactor
OLR	Organic Loading rate (*g COD/m^2 carrier/day, or kg COD/m^3/day*)
PSA	Projected Surface Area (*m^2*)
SALR	Surface Area Loading Rate (*g COD/m^2 carrier/day*)
sCOD	Soluble Chemical Oxygen Demand (*mg/L*)
tCOD	Total Chemical Oxygen Demand (*mg/L*)
TS	Total Solids (*mg/carrier*)
TSS	Total Suspended Solids (*mg/L*)
UASB	Upflow Anaerobic Sludge Blanket
VFA	Volatile Fatty Acids (*mg/L*)
VLR	Volumetric Loading Rate (*kg COD/m^3/day*)
VMPR	Volumetric Methane Production Rate (*g CH_4-COD eq./L/day*)
VS	Volatile Solids (*mg/carrier*)
VSS	Volatile Suspended Solids (*mg/L*)

Table of content

1. Introduction

The production and processing of food require large amounts of water, which in turn generates wastewater rich in organic substances such as carbohydrates, proteins, fats and oils. Industrial wastewaters from food processing are characterized by a high content of these organic components and if this wastewater is not treated properly before disposal or reuse it may pose negative environmental effects such as eutrophication of waterbodies and release of environmentally toxic substances. It is therefore of great importance to effectively treat and handle the wastewater.

Anaerobic wastewater treatment (AnWWT) has shown to be effective for the removal of organic matter in high-strength wastewaters. AnWWT has many advantages, including: low energy requirements, energy production in the form of biogas, low sludge production and the ability to treat wastewater at a high organic loading rate (OLR) (Giovanni *et al.*, 2018).

One of the main disadvantages of AnWWT is the slow growth of anaerobic microbial cultures. As a result, very high hydraulic retention times (HRT) are required to avoid wash-out of the microbes if they are grown in suspension. To get around this problem, fixed-growth biofilm systems can be used, where the biomass is retained in the reactor. One such system which has shown to be effective is the anaerobic moving bed biofilm reactor (AnMBBR) (Wang *et al.*, 2009; di Biase *et al.*; 2016, di Biase *et al.*, 2018). In moving bed biofilm reactors (MBBRs), the biomass grows as a biofilm attached to freely moving carriers with a large, protected surface area. The protected biofilm growth allows for low HRT without wash-out occurring. A limiting factor for biofilm process, in contrast to suspended growth processes, is that the substrate and products must be transported through the biofilm by diffusion (The Water Environment Federation, 2010). The thickness of the biofilm could, therefore, impact the process. However, as AnMBBRs is a novel technique that has only been around for a few decades, very few studies have been made on how the thickness affects the process, and up until now it has been difficult to study and control the biofilm thickness. A new type of carrier, the AnoxK^{TM} Z-carrier, recently developed by AnoxKaldnes has made it possible to study the effect of the biofilm thickness in AnMBBRs.

The overall aim of this Master Thesis is to gain more insights into the treatment of industrial wastewaters from food processing with the AnMBBR process, and to investigate the effect of the biofilm thickness and carrier type. These insights will contribute to the optimization of AnMBBRs and the development of more efficient wastewater treatment processes in the future.

1.1. Research problem

The AnMBBR process is a novel technique and the number of studies on AnMBBRs treating industrial wastewaters are therefore limited. Very little is known about how the biofilm thickness affects the process performance in terms of removal of organic matter, biogas production, and resilience to environmental and operational changes of the process. Therefore, the research questions this Master Thesis aims to answer are:

- How does the biofilm thickness affect the organic removal efficiency and biogas production in AnMBBR treating an industrial effluent?
- How does changing environmental and operational conditions affect the resilience of the process depending on the biofilm thickness?

1.2. Hypothesis

It is hypothesised that a thicker biofilm will allow for a more resilient process as the sensitive methanogens are shielded from perturbations and toxins such as pH changes and exposure to oxygen. This in turn will lead to a more efficient and stable process, in terms of organic removal (measured as chemical oxygen demand, COD) and biogas production, when using carriers allowing for a thicker biomass.

1.3. Objective and Approach

The overall objective of this Master Thesis was to investigate the effect of the biofilm thickness and type of carrier used in AnMBBRs treating an industrial wastewater from food processing. The specific objectives were as follows:

1. To evaluate the effect of biofilm thickness and carrier type on the performance (in terms of organic removal and biogas production) of AnMBBRs
2. To assess the effect of different carriers on biofilm development and accumulation.

This was done by operating three lab-scale AnMBBRs and continuously analyse the performance of these reactors. The first reactor contained commercial AnoxK™ Z-200 carriers made from recycled HDPE (allowing for a maximum biofilm thickness of 200 µm), the second with a prototype of AnoxK™ Z-1000 carriers made from virgin HDPE (allowing for a maximum biofilm thickness of 1000 µm) and the third reactor contained a mixture of the two carriers.

Originally, the objective was also to characterise the microbial biomass composition on the carriers and in suspension. This was to be done in order to evaluate how/if the microbial composition differentiated between the two different carriers used and thereby evaluate how/if the biofilm thickness affected the microbial community in the biofilms. The microbial community was to be assessed with 16S rRNA gene sequencing. Due to travel restrictions as a consequence of the corona pandemic, the characterisation of the biomass was not possible. However, this Master Thesis will discuss the possible relations between microbial composition and performance based on the results obtained from analysing the performance of the reactors, combined with existing literature on the subject.

2. Theoretical background and Literature review

2.1. Anaerobic wastewater treatment – Advantages and disadvantages

The biological process behind anaerobic wastewater treatment for the removal of organic matter is anaerobic digestion. Anaerobic digestion is the process in which a consortium of microorganisms (bacteria and archaea) break down organic material without the presence of oxygen. This process is present in natural environments such as lake sediments and in the stomach of ruminant animals but can also be utilized in industrial settings such as wastewater and sludge treatment. The process generally converts the organic material into biogas, mainly composed of methane (CH_4) and carbon dioxide (CO_2), and leaves mineralized compounds such as ammonia (NH_4^+) and phosphate (PO_4^{3-}) in solution (van Lier *et al.*, 2008).

AnWWT processes have been successfully used in the treatment of various wastewaters, especially high-strength industrial wastewaters such as agricultural and food processing effluents. In warmer regions it has also been used for domestic wastewater treatment (Chernicharo, 2007). AnWWT processes may have many advantages over aerobic wastewater treatment processes, depending on the application and technique used. Some of the advantages include: (1) the potential of less energy usage, (2) low sludge production, and (3) the possibility of energy recovery in the form of methane.

1. Aerobic wastewater treatment requires the input of oxygen. For this, blowers are needed which require energy. In an aerobic wastewater treatment plant, the aeration can account for up to 75% of the energy cost (van Lier *et al.*, 2008). When the organic load of the wastewater is high, more oxygen is used, thereby increasing energy consumption. Therefore, anaerobic treatment can be advantageous for wastewater with a high organic content (COD).

2. The low sludge production is associated with the lower growth yield of anaerobic microorganisms since less energy is available for growth when the redox potential is low (absence of oxygen). The biomass production could be 3 to 5 times lower compared to aerobic processes (Chernicharo, 2007). This is technically and economically feasible as the processing and disposal of sludge could be complex and add major costs to the wastewater treatment process.

3. The biogas produced in anaerobic digestion contains a mixture of about 50-70% CH_4 and 30-40% CO_2. The methane has a high energy content and can be used as a fuel or to power electricity. As compared to aerobic processes, where most of the energy is stored in the biomass or lost as process heat, a significant amount of the energy in anaerobic processes can be recovered in the biogas and only a few percentages are stored in the biomass or lost as heat (Sahm, 1984). About 13.5 MJ energy can be produced per kg COD removed (van Lier *et al.*, 2008).

One of the main disadvantages with AnWWT processes is the slow growth of anaerobic microbes, which could make the operation of AnWWT processes challenging and they usually require a long start-up period (Chernicharo, 2007). The complex microbial ecosystem in AnWWT is sensitive to perturbations caused by change in reactor conditions such as substrate composition, temperature, OLR and pH. Other disadvantages include malodourous gases and explosive atmospheres due to the generation of biogas.

2.2. The microbiology of anaerobic digestion

Anaerobic digestion could be considered to contain 4 steps: (1) hydrolysis, (2) acidogenesis, (3) acetogenesis, and (4) methanogenesis. These steps are performed by a consortium of different microorganisms. Step 1-3 are performed by fermentative bacteria and step 4 by methanogenic archaea. The interaction between these groups of microorganisms must be well balanced in order for AnWWT processes to function efficiently. An overview of the anaerobic food web is illustrated in Figure 1. In addition to the bacteria and archaea, protozoans may also play a significant role in anaerobic digestion for the removal of organic matter (Priya *et al.*, 2008). However, the role of protozoans has been less studied, and their exact role is not fully understood yet.

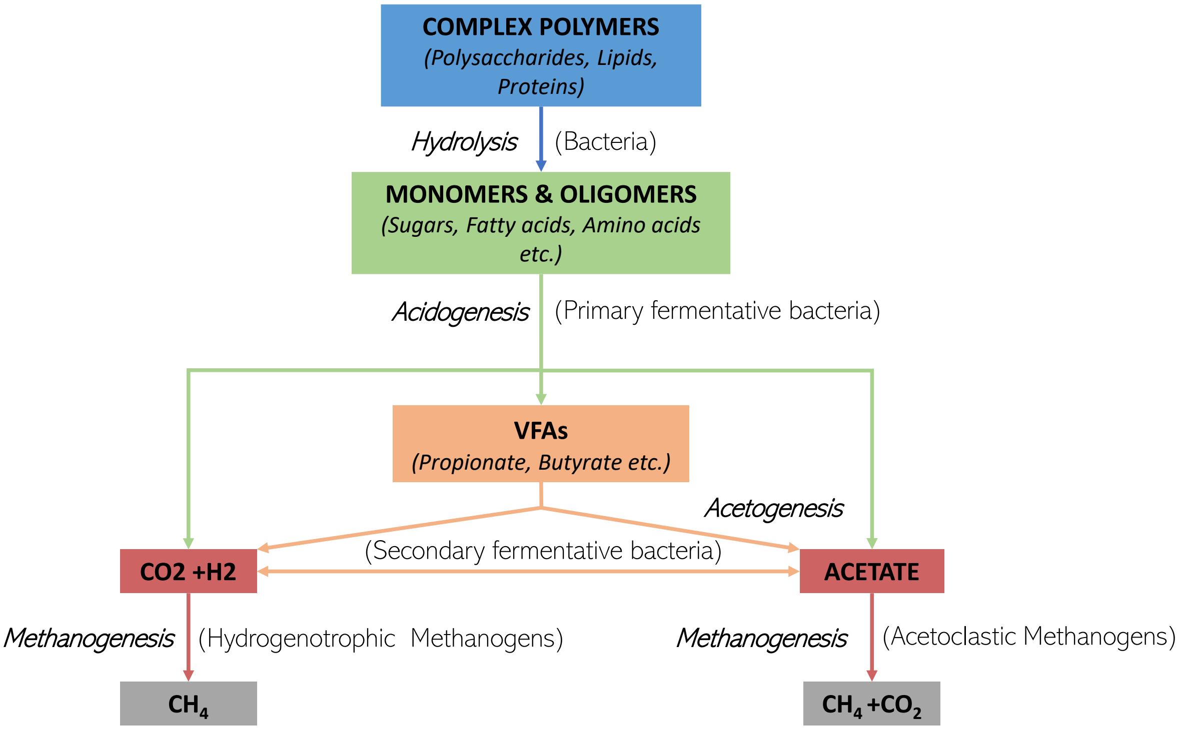


Figure 1. The food web of anaerobic digestion. Inspired by figure in "Methanogenesis" by Stephen H. Zinder (Zinder, 1992).

2.2.1. Hydrolysis – Bacteria

The first step of anaerobic digestion involves the breakdown of complex polymers into simple monomers or oligomers that are small enough to cross the cell membrane. This hydrolysis reaction is performed extracellularly by different bacterial phyla. The bacteria produce extracellular enzymes which break down the polymers into smaller molecules (van Lier *et al.*, 2008). The hydrolysis is generally considered the rate-limiting step of the degradation of solid material. Factors such as temperature, pH, substrate composition and size of particles may affect the rate of the hydrolysis reaction (Chernicharo, 2007).

2.2.2. Acidogenesis – Primary fermentative bacteria

When the large polymers have been hydrolysed into small, soluble compounds, they can be assimilated by the bacteria and used as a carbon and/or energy source. The bacteria that ferments these hydrolysis products are referred to as *primary fermenters* or *acidogens*. The products from the acidogenesis include volatile fatty acids (VFAs, such as acetate, propionate, butyrate and other higher organic acids) as well as alcohols, CO_2, and H_2.

2.2.3. Acetogenesis – Secondary fermentative bacteria

The products from the acidogenesis are further metabolised by a group of organisms referred to as *secondary fermenters* or *acetogens*. In this pathway, the main products are acetate, CO_2 and H_2. The H_2 producing reactions in the acetogenic pathways are not energetically favourable at standard conditions since they are accompanied by a large positive free energy change (a large positive ΔG). However, a low partial pressure of H_2 (generally 10^{-4} atm) makes these reactions energetically favourable (results in a negative ΔG). Hence, these reactions can only occur when coupled with H_2 consuming reactions (van Lier *et al.*, 2008). The secondary fermenters are therefore in obligate syntropy with H_2 consuming microorganisms. In an anaerobic process where these H_2 consuming microorganisms effectively consume the H_2, the acetogens will be active and, hence, acetate will be the main VFA product.

There are also some acetogens that are capable of using H_2 and CO_2 as a substrate to synthesize acetate. These H_2 consuming acetogens, require a H_2 partial pressure of approximately 10^{-3} atm (Zinder, 1992). In a well-functioning AnWWT process, the H_2 partial pressure usually drops below or around 10^{-4} (van Lier *et al.*, 2008). As a consequence, the H_2 consuming acetogens are generally outcompeted by other H_2 consuming organisms (e.g. methanogens) (Zinder, 1992).

2.2.4. Methanogenesis – Methanogenic archaea

In the last step of anaerobic digestion, methanogenic archaea produce CH_4. The methanogens are obligate anaerobes, and most have doubling times of several hours to several days (Zinder, 1992). The low growth rate of the methanogens is associated with the limited energy yield of methanogenesis. The low growth rate of the methanogens is the main reason for the long start-up of AnWWT processes (van Lier *et al.*, 2008). The methanogens are only capable of using a limited number of substrates, these substrates are mainly produced by the fermentative bacteria in AnWWT processes. Most methanogens are capable of reducing CO_2 and H_2 into CH_4 (hydrogenotrophic methanogens). Other substrates include acetate (CH_3COO, acetoclastic methanogens) and simple methylated compounds such as methanol and methylamine (Zinder, 1992). Two of the major reactions carried out by methanogenic archaea are shown in Table 1.

Table 1. Major methanogenesis reactions in AnWWT processes performed by methanogenic archaea. Free energy change at standard conditions (25°C, pH 7) (Zinder, 1992).

Functional group	Reaction	ΔG° (kJ/mol)
Acetoclastic methanogens	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31
Hydrogenotrophic methanogens	$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$	-131

The methanogens are H_2 consumers and are in a syntrophic relationship with the acetogens in AnWWT. They keep the partial pressure of H_2 at a level which makes the H_2 producing acetogenic reactions energetically favourable (Zinder, 1992).

The predominant methanogens found in mesophilic (20-45 °C) AnWWT belong to the genera *Methanosarcina* and *Methanosaeta*. *Methanosarcina* is capable of using a relatively wide range of substrates including: H_2/CO_2, acetate, methylamines, methanol and formate (van Lier *et al.*, 2008). They have a relatively high growth rate (doubling time of 1-2 days), but a low substrate affinity (Zinder, 1992). *Methanosaeta* is mainly capable of using acetate as a substrate but may also be capable of using the hydrogenotrophic pathway (De Vrieze, 2014) and has a low growth rate (doubling time of 3-7 days) but a high affinity for acetate (Zinder, 1992). Due to the high affinity for acetate, *Methanosaeta* is usually the dominant acetoclastic methanogen at low acetate concentrations not exceeding 100 to 150 mg COD/L (De Vrieze, 2014).

2.2.5. Protozoans

Protozoans are single-celled eucaryotes which mainly feed on bacteria and organic debris. Their role in anaerobic environments are not fully understood but studies suggests protozoans, in particular ciliates, may enhance anaerobic degradation and increase methane production (Priya *et al.*, 2008; Prabhakaran *et al.*, 2016).

2.3. Environmental factors in anaerobic wastewater treatment

The performance of AnWWT processes is mainly influenced by the composition of the wastewater, the pH and the temperature.

2.3.1. Organic content and composition of the wastewater

Anaerobic cultures are able to form methane from a range of different organic materials, including carbohydrates, proteins, lipids, alcohols, and even some petrochemicals and complex aromatic compounds (Sahm, 1984). The organic content in the wastewater is commonly measured as chemical oxygen demand (COD). The COD is the amount of oxygen required to oxidize the organic compounds in the wastewater and is thus an indirect measurement of the concentration of organic compounds. The tCOD is a measurement of the total organics while the sCOD is the fraction of soluble organics, defined as the organic material that pass through a filter with a known pore size (in this study defined as the COD that pass through a 0.45 μm filter). The sCOD generally consists of organic compounds that are easily biodegradable. The type of organic material and its degradability also influences the composition of the biogas (Sahm, 1984).

The amount of biogas produced is related to the organic content in the wastewater. Under normal temperature and pressure (1 atm, 273 K), the theoretical maximum yield of methane is 350 Nml CH_4/g of tCOD degraded (Chernicharo, 2007).

2.3.2. Volatile fatty acids and pH

As mentioned in section 2.2., the interaction between the microbes in the anaerobic food web must be well balanced in order for AnWWT processes to function efficiently. Imbalance of AnWWT processes may eventually lead to reactor souring.

The acidogenesis is the most rapid step of the anaerobic food web (van Lier *et al.*, 2008). Hence, if an anaerobic system is subjected to perturbations, such as a sudden increase in easily degradable organic matter, the acidogens will produce VFAs faster than the methanogens can consume these acids. This will lead to a build-up of acids which could eventually lead to a pH drop. As the H_2-consuming methanogens have a narrow pH range, they will be inhibited, leading to a build-up of H_2. The build-up of H_2 eventually leads to the acetogens being inhibited as the acetogenic reaction is dependent on the low partial pressure of H_2. This leads to a "positive feedback loop", further decreasing the pH and eventually leading to reactor souring (Zinder, 1992).

To prevent reactor souring it is therefore of major importance to have a good buffering system and to closely monitor the pH and VFAs. The VFA levels have shown to be a good indicator of process stability for anaerobic digestion processes (Ahring *et al.*, 1995). The pH of AnWWT processes should not drop below 6.8 (Björnsson *et al.*, 2000). Even small accumulations of VFAs have shown to lead to a decrease in pH for systems with poor buffering capacity (Murto *et al.*, 2004).

2.3.3. Temperature

The microbial activity of AnWWT is sensitive to temperature changes and lower temperatures result in reduced microbial activity. At decreased temperatures, the conversion rate of COD decreases, resulting in a higher fraction of COD in the effluent and consequently leads to a lower biogas production (Van Haandel *et al.*, 2018). A lower temperature also increases the solubility of methane in water, thus leaving a larger fraction of the biogas in solution. This is undesirable as this methane will finally end up in the atmosphere, contributing to the greenhouse effect.

Consequently, AnWWT processes may require heating, unless they are implemented in warmer regions or on wastewater that is already heated. This may contribute to a significant operational cost of the process. Thermophilic conditions usually favours the biogas production but are not favourable from an economic perspective. Usually, the optimal operating temperature of AnWWT processes lies in the higher mesophilic rage, around 35-37°C (Van Haandel *et al.*, 2018). The heat required may be supplied as energy obtained from the methane produced in the process, or from a heat source in another process step. However, for dilute wastewaters (such as domestic wastewaters), heating supplied from the methane may not be economically feasible due to a low volumetric production rate of methane (Chernicharo, 2007).

The cost of reactor heating must be weighed against the benefits of the process. If the heating costs are expected to be high, other systems, such as aerobic activated sludge processes may be more feasible.

2.4. Anaerobic wastewater treatment systems

Conventional anaerobic digestion processes have been used for the treatment of sewage sludge since the 19th century. These conventional anaerobic systems are mainly used for very concentrated effluents (>30,000 mg COD/L) (Sahm, 1984) and are usually not feasible for liquid effluents as they require very high hydraulic retention times (HRT). The development of high-rate systems, however, has enabled anaerobic treatment of more dilute effluents, such as high-strength wastewater from food processing, chemical industry, pulp and paper processing, etc. The high-rate systems are characterized by their ability to treat wastewater at a high volumetric loading rate (VLR), by uncoupling the solid retention time from the HRT. This is achieved by retaining the biomass in the reactor, for example by the formation of highly-settleable sludge or by fixed biofilm growth. As a result, high-rate processes can be made compact with a small footprint.

Some high-rate anaerobic reactors include: (1) anaerobic filter reactors (2) upflow anaerobic sludge blanket reactors and (3) anaerobic moving bed biofilm reactors.

1. Anaerobic filter reactors are comprised of a stationary filter media on which the biofilm grows. The filter media may be of various materials such as plastic, gravel, or pumice. The filter reactors have the advantage of successfully retaining the biomass and therefore allow

for very high VLR. One of the main disadvantages of anaerobic filter reactors is that, due to the stationary filter, they require a relatively large reactor volume compared to other high-rate processes. Filter reactors are also subjected to clogging and may require backwashing (Rajeshwari *et al.*, 2000).

2. The upflow anaerobic sludge blanket (UASB) reactor is one of the most commonly used reactors for anaerobic wastewater treatment and has been successful for the treatment of carbohydrate-rich wastewaters (Daud *et al.*, 2018). In UASB reactors, the wastewater flows upwards from the bottom of the reactor and through an activated sludge blanket. The activated sludge blanket is comprised of large biofilm granules with high density, preventing the granules from being washed out. The granules are formed by the aggregation of microbial flocs. The UASB has the advantage of high organic removal rates, even at high VLR. It can handle loading rates of 10-15 kg COD/m^3/day (The Water Environment Federation, 2010). A disadvantage of UASB reactor is the potential occurrence of degranulation, leading to wash-out of biomass (Macarie *et al.*, 2018).

3. The AnMBBR is comprised of a reactor loaded with freely moving support material, carriers, (usually plastic) on which a biofilm grows. The carriers provide a large surface area available for biofilm growth and thereby enables high organic removal efficiencies. The carriers are suspended in the reactor by mechanical mixers, thereby using the whole reactor volume for wastewater treatment contrary to stationary fixed-film process. The AnMBBR provides a compact treatment system which allows for a high removal capacity at high VLR. It has gained attention over the years as it could be more stable compared to UASB processes and requires less volume compared to stationary fixed-film processes. One disadvantage of the AnMBBR system, compared to the other anaerobic systems mentioned, is that some carriers may be washed out from the system over time, resulting in the potential release of plastics into nature. Furthermore, the AnMBBR may not be capable of treating wastewaters at as high VLRs as for the anaerobic filter. The AnMBBR is further reviewed in the following section (section 2.5).

2.5. The anaerobic moving bed biofilm reactor

The moving bed biofilm reactor (MBBR) is well established for aerobic processes and has been around for about 30 years. In MBBRs, the biomass grows on carriers that are held in suspension in the reactor by aeration (aerobic MBBRs) or mechanical mixing (AnMBBRs). The MBBR is compact, flexible, the amount of sludge produced is reduced, and biomass is retained in the reactor, which reduces the need for solid separation steps. The MBBR process also eliminates the need of sludge recycling (The Water Environment Federation, 2010). Moreover, it has been shown that the MBBR can provide considerably higher removal rates per mg biomass compared to suspended-growth systems, which can be partly attributed to the ability of MBBRs to develop highly specialized microbial communities (The Water Environment Federation, 2010).

The AnMBBR is not as established as the aerobic MBBR but has gained more attention over the last decade for the treatment of industrial wastewaters. It has shown to be effective when treating industrial effluents such as winery wastewater (Chai *et al.*, 2013), brewery wastewaters (di Biase *et al.*, 2018), and oil-contaminated petro-chemical wastewaters (Morgan-Sagastume *et al.*, 2019). The principle behind an AnMBBR is shown in Figure 2.

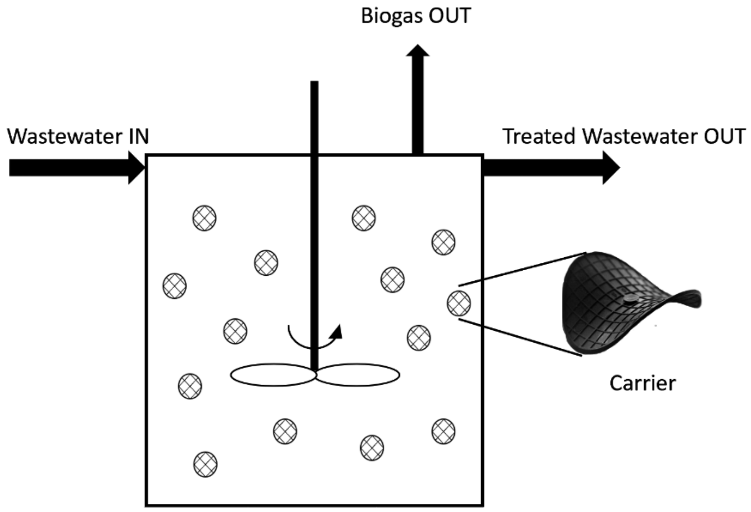


Figure 2. Schematic view of an AnMBBR and an example of a carrier type (the AnoxK^{TM} carrier).

2.5.1. Carrier design and characteristics

The support material on which the biofilm grows, the carriers, are usually made of plastic material. The carrier design should fulfil a number of criteria such as providing enough surface area for biofilm development, have a density slightly lower than that of water to promote suspension, be large enough to be retained in the reactor and it should be affordable (Morgan-Sagastume, 2018). High-density polyethylene (HDPE) is commonly used as it has shown to be durable and provide a good surface for microbial attachment (Morgan-Sagastume, 2018).

Most carrier designs focus on maximizing the protected surface area (the area on which biofilm can grow protected from shear) to maximize biofilm growth, however, the thickness of the biofilm itself may play a crucial role in the performance of MBBRs (Piculell, 2016). The Anox K^{TM} Z is a carrier design that allows for control of the maximum biofilm thickness and thereby allowing the effect of biofilm thickness to be studied. For the Anox K^{TM} Z-carrier, the maximum biofilm thickness is determined by the height of the cell walls in the carrier. Excess biomass will be sloughed off when carriers collide, thereby keeping the maximum biofilm thickness constant.

2.5.2. The biofilm and microbial community of AnMBBRs

Biofilm formation is essential in MBBRs and other biofilm processes. A biofilm is formed when bacteria, archaea, and other microbes attach to a surface. The attachment of the microbes may be initialised due to factors such as surface properties of the material (surface charge, surface free energy, hydrophobicity, surface roughness, etc. (Morgan-Sagastume, 2018)), gravity and chemoattraction (nutrients on the surface of the substrate) (Lappin-Scott *et al.*, 1992). When the first microbes attach, they start to produce a biofilm matrix consisting of extracellular polymeric substances (EPS) in which the microbes become enclosed. The substrate properties of the carrier material may play a major role in the initial attachment of the microbes, whereas the properties of the young biofilm and its EPS components may determine the following development and maturing of the biofilm (Morgan-Sagastume, 2018).

The biofilm has many advantages such as facilitating gene transfer and nutrient transfer between microbes as well as protecting the microbes from toxins. The EPS matrix allows for symbiotic cultures of microbes (such as acetogens and methanogens) to live in close proximity and to exchange metabolites. The biofilm may also allow for microenvironments within the biofilm, which in turn allows for different microbial niches in different layers of the biofilm. A study on the biofilm in UASB reactors treating carbohydrate-rich wastewater showed a layered distribution of microbes. Methanogens (predominantly *Metanosaeta* spp.) were mostly located in the interior, the syntrophic colonies were located in a mid-layer, and the hydrolytic and fermentative bacteria in the outer layer (Fang, 2000). Similar findings have been made in other studies on UASB reactor granules (MacLeod *et al.*, 1990). As of present, no studies on the microbial community layered structure of biofilms in AnMBBRs could be found.

The biofilm characteristics and microbial community in an AnMBBR may be affected by a variety of factors such as the composition of the feed, the composition of the inoculum used, the property of the carrier material, the OLR and the HRT. A study by Habouzit *et al.* (2014) showed that the archaeal community in an AnMBBR during start-up was highly dependent on the support material while the bacterial community was more dependent on the composition of the inoculum used (Habouzit *et al.*, 2014). The HRT has been shown to affect the fraction of suspended biomass and the formation of biofilms in anaerobic reactors. A study by Jensen *et al.* (2019) showed that the biofilm biomass increased with a decreased HRT. It was suggested that the short HRT promoted the growth of acetogens and methanogens able to form biofilms (*Methanosaeta*) due to wash-out of competing planktonic species, which increased the amount of biomass growing as biofilm (Jensen *et al.*, 2019). To promote biofilm formation in AnMBBRs, it may, therefore, be desirable to operate the reactors at a relatively short HRT. However, due to detachment of biomass from the carriers (as a result of carrier collision, bulk liquid shear forces, sloughing of large biomass segments, etc.), some biomass in suspension will always exist in MBBRs, even at very low HRT (Piculell, 2016). The contribution to the COD removal from the suspended fraction should therefore be considered in MBBR processes.

2.5.3. Operational parameters and performance of AnMBBRs

A number of parameters can be used to control and evaluate the performance of AnMBBRs. The most commonly used parameters and their importance are described in this section.

2.5.3.1. Organic loading rate

The organic loading rate (OLR) is an important operational parameter in all wastewater treatment processes. It could be defined as the amount of organic material added per unit volume (volumetric loading rate, VLR) or when applied to biofilm processes, the amount of organic material added per unit area available for biofilm growth (surface area loading rate, SALR). The OLR is thus related to the hydraulic retention time (HRT) and the COD of the wastewater.

By measuring the tCOD and the sCOD in the influent as well as the effluent, the performance in terms of organic removal efficiency can be estimated. It has been shown that AnMBBRs can reach removal rates of above 90% sCOD (Wang *et al.*, 2009; Chai *et al.*, 2013; di Biase *et al.*, 2018).

2.5.3.2. Biogas production and composition

When the microbes in anaerobic processes digest the organic material, they produce biogas. The biogas production of anaerobic processes is, therefore, in direct relation to the COD removal rate and it can therefore be monitored to evaluate the reactor performance in terms of COD removal. Furthermore, it is also of interest to analyse the composition of the biogas. The methane in the biogas has the highest energetic value and, therefore, it is desirable to have a high fraction of methane in the biogas. Moreover, a high fraction of N_2-gas may be an indication that the system is not air-tight.

2.5.3.3. *Volatile fatty acids, pH and alkalinity*

It is of great importance to constantly monitor the VFAs in AnMBBRs as the VFA levels give an indication of the balance between archaea, acidogenic and acetogenic bacteria in the reactor. Moreover, it is also of interest to analyse the relative fraction of different VFAs in the AnMBBR. It is desirable to have a high fraction of acetate as acetate is the main product from the acetogenesis and the main VFA used by the methanogens. A relative increase in the concentration of other VFAs such as butyrate, isobutyrate and propionate has shown to be signs of process imbalance as this may indicate that acetogenic bacteria are inhibited (Sahm, 1984).

The buffering capacity (alkalinity) and the pH of the process should also be closely monitored in order to avoid reactor imbalance, which could eventually lead to reactor souring. However, studies have suggested that microbes growing in biofilm communities may be somewhat protected against pH changes of the bulk liquid as the biofilm allows for microzonal pH variations (Vroom *et al.*, 1999) and the more sensitive microbes (archaea and syntrophic bacteria) has been shown to grow in the inner layer of the biofilm in UASB reactors (MacLeod *et al.*, 1990; Fang, 2000). Anaerobic biofilm reactors, such as the AnMBBR, may therefore be more resistant to environmental perturbations compared to suspended-growth processes.

2.5.3.4. *Temperature*

As discussed in section 2.3.3., the temperature has great influence on the process and must therefore be monitored closely as a temperature drop may result in a reduced microbial activity.

2.5.3.5. *Suspended Solids*

The suspended solids may be measured as total suspended solids (TSS) and volatile suspended solids (VSS). The TSS is a measurement of the total amount of non-soluble material (organic as well as inorganic), while the VSS is a measurement of the non-soluble organic material in the wastewater. VSS can be used as an indicator of the amount of suspended biomass in the wastewater. The suspended biomass in MBBRs may be a result of detached biomass from the carriers or the growth of planktonic species (especially at longer HRT) and are generally considered to be active biomass contributing to the COD removal (Piculell, 2016). However, if there is a significant amount of particulate COD in the wastewater that is not degraded, this will also contribute to the VSS.

3. Experimental approach

This work included a laboratory study that involved operation and monitoring of three continuous lab-scale AnMBBRs. Two of the reactors were loaded with different carriers, one which allowed for a maximum biofilm thickness of 200 µm and the other 1000 µm. The third reactor was operated as a reference and contained a mixture of the two carriers. The reactors treated an industrial wastewater from a food processing facility and all reactors were subjected to similar operational conditions (temperature, alkalinity, feed composition, HRT, and OLR). When this study started, the reactors had already been operated for 123 days and some biofilm was already established on the carriers. All laboratory work was performed at AnoxKaldnes´ facilities in Lund.

3.1. Carriers

The carriers used in this project were of two different designs, both designed and produced by AnoxKaldnes. The first ones are commercial Anox K™ Z-200 carriers (referred to as "Z-200") (Figure 3 a) with a cell depth of 200 µm and a projected surface area (PSA) of 0.00128 m^2 per carrier. These carriers are saddle-shaped and made of HDPE from recycled material. The second design are a prototype of Anox K™ Z-1000 carriers (referred to as "Z-1000") (Figure 3 b) with a cell depth of 1000 µm and a PSA of 0.00164 m^2 per carrier. The Z-1000 carriers are flat and made of virgin HDPE. The PSA in this study was defined as the summarized floor area of the cells on the carrier (see Figure 3 a).

The difference in cell depth of the two designs would allow for the growth of biofilm of different thicknesses.

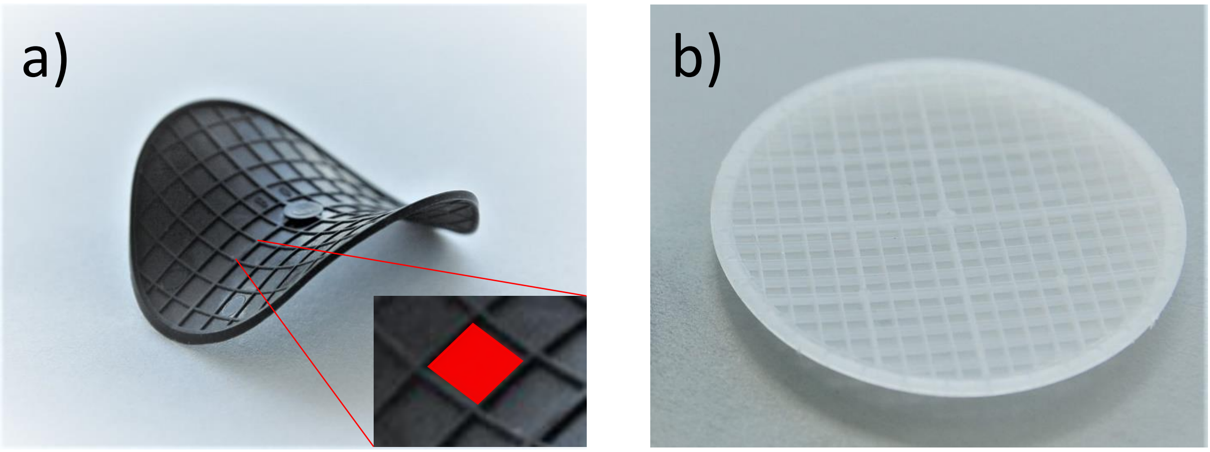


Figure 3. a) AnoxK™ Z-200 carrier (diameter of 3.5 cm) with close-up of the defined "cell floor area". b) prototype of AnoxK™ Z-1000 carrier (diameter of 4.5 cm).

3.2. Reactor setup

All three reactors were initially loaded with carriers so that all had the same *total* PSA. However, as the reactors were of different volumes, it was decided on day 183 that the number of carriers should be corrected so that all reactors had the same PSA *per unit volume of reactor* instead (according to Table 2). The first reactor ("R200") was loaded with Z-200 carriers and the second reactor

("R1000") with Z-1000 carriers. The third reactor ("RMIX") was loaded with a mixture of the two. This reactor was operated as a reference as both types of carriers would be subjected to the same conditions in this reactor.

Table 2. Carrier loading in the three lab-scale reactors used in this study.

Reactor	Working volume (L)	Carrier type	Number of carriers	Total PSA (m^2)	PSA per unit volume (m^2/m^3)
R200	4	Z-200	337 (320)*	0.431 (0.410)*	108 (102)*
R1000	3.8	Z-1000	250	0.410	108
RMIX	3.8	Z-200	160	0.205	
		Z-1000	125	0.205	
		Total	285	0.410	108

* The number was corrected from 320 carriers to 337 carriers on day 183 of operation to achieve the same PSA per unit volume.

The reactors were made of glass, all were gas-tight and were equipped with an external heating jacket, liquid inlet and outlet, liquid sampling outlet, gas outlet and a top-mounted mechanical 2-bladed mixer. In each reactor, a baffle was installed at the liquid surface to prevent a stagnant layer of carriers at the surface. One reactor had a slightly larger working volume of 4 L (R200) compared to the other two which had working volumes of 3.8 L each (R1000 and RMIX).

Each reactor was connected to a feed pump (Watson Marlow 520S, Watson Marlow) which continuously supplied the substrate. The substrate was refrigerated at a temperature between approximately 3-15°C. In order to ensure that the system was gas-tight, the effluent of the reactor was discharged into a water trap by overflow. The gas outlet from all AnMBBRs were connected to a gas flow meter (AMPTS II, Bioprocess control) to monitor the biogas production. The heating jacket was connected to a water-bath with a temperature of 37.5°C to maintain a reactor temperature of approximately 36°C. The reactor set-up is shown in Figure 4.

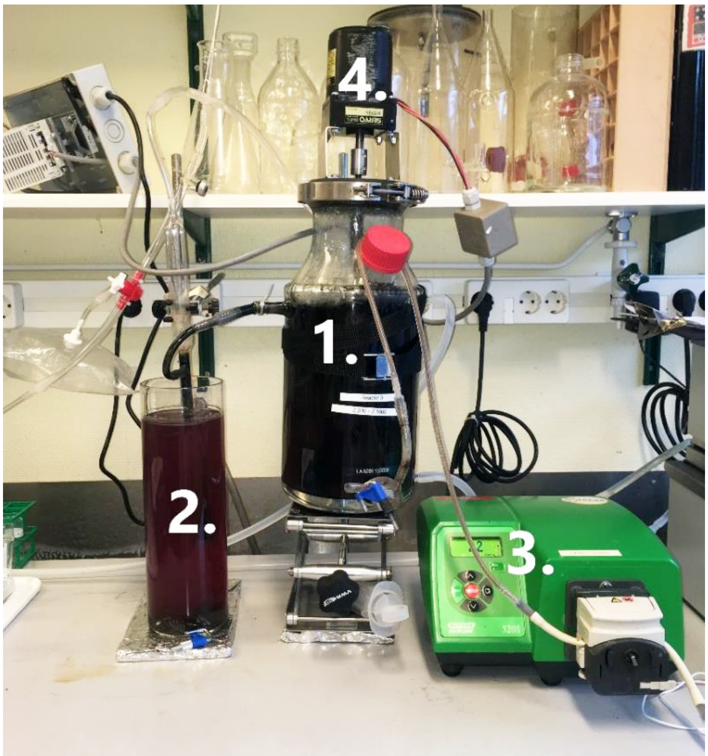


Figure 4. Reactor set-up showing one of the reactors (1) and its effluent trap with overflow (2), feed pump (3) and mechanical stirrer (4).

3.3. Reactor operation

3.3.1. Previous study: Day 1-123

The initial start-up of the reactors (day 1 to day 123 of reactor operation) had been made by another operator and is covered in the work by Emma Månsson (2020). During these days, the reactors had been inoculated 10 times (between day 1 and day 93 of operation) with anaerobic granular sludge and various operational temperatures had been tested (Månsson, 2020). The reactors were otherwise subjected to similar operation conditions during this first start-up period as described in the following section. At the end of this study, some biofilm was already established on the carriers.

3.3.2. This study: Day 124-238

The laboratory study for this Master Thesis was performed for a period of 114 days (day 124 to 238 of reactor operation). The reactors were operated in the mesophilic region, at a temperature of approximately 36°C. The mixers were set to a stirring frequency of 25 Hz for R200 and RMIX and 35 Hz for R1000. The stirring frequency was set to ensure good mixing in all reactors. The frequency was set higher in R1000 since the reactor with Z-1000 carriers alone required a higher frequency to avoid stagnation of the carriers and ensure good mixing.

The OLR had been continuously increased throughout the reactor operation to allow for biofilm establishment (when this study started, at day 124 of reactor operation, there was already some biofilm established on the carriers). The OLR was increased by either increasing the COD of the substrate and/or by decreasing the HRT. During the last period of the study (day 215-238 of operation), the reactors were operated at a constant VLR of 4.13 ± 0.14 kg sCOD/m^3/day.

3.4. Wastewater characteristics

The substrate used in this project was wastewater from a food processing facility. The wastewater had a tCOD concentration of 2367 ± 426 mg tCOD/L and a sCOD (filtered through a 0.45 µm filter) concentration of 1830 ± 282 mg sCOD/L. The wastewater was collected in batches (13 different batches used, approximately one batch used per week) and therefore had daily variations in characteristics. Analysis results from the batches can be found in table A1 in the Appendix A.

A trace element solution (Vithane, Biothane) and a FeCl solution was added to the wastewater to provide micronutrients. $NaHCO_3$ was added as a buffer to maintain a neutral pH and an alkalinity of approximately 3000-5000 mg $CaCO_3$/L. The addition of micronutrients and buffer was based on the tCOD of the wastewater. When needed, the wastewater was diluted with tap-water as to not overload the reactors when the sCOD of the wastewater was high.

3.5. Sampling and Analysis

3.5.1. Reactor influent and effluent

Substrate feed was prepared every, or every second day and the influent flow rate was estimated from measuring how much of the feed had been used in a certain time interval. The gas production of each reactor was monitored daily[1], except weekends. Grab samples of the effluent gas composition was analysed weekly (Gas chromatography, Clarus 450, Perkin Elmer). Grab samples of the liquid influent and liquid effluent were taken approximately 1 time a week for the influent and 3 times a week for the effluent. The liquid influent and liquid effluent samples were analysed according to the parameters given below.

- sCOD and tCOD (HACK LCK 114/814, Hach)
- Alkalinity, NH_4-N and PO_4-P (spectrophotometry by Gallery Plus, Thermo Fisher Scientific)
- pH (HQ11D, Hach)
- Organic acids (HACK LCK 365, Hach)
- VFA composition (Gas chromatography, Clarus 400, Perkin Elmer)
- TSS (SS-EN 872:2005, 2nd ed.)
- VSS (former SS028112, 3rd ed.)

Samples for sCOD, Alkalinity, Organic Acids, VFA, NH_4-N and PO_4-P were filtered through a 0.45 µm filter and VSS and TSS samples were filtered through a 1.6 µm glass fibre filter. The organic acid analysis was done to monitor the VFAs on a regular basis as the analysis of the VFA samples could not be made regularly due to time-consuming analysis method. VFA samples were therefore taken on a weekly basis and frozen for later analysis. The analysis method for organic acids (HACH LCK 365, Hach) may, however, overestimate the VFA levels as it detects other acids than VFAs (etc. lactic acid).

3.5.2. Carriers and biomass

The reactors were opened on day 141, 169 and 232 for carrier sampling. 6 carriers (of each type of carrier from each reactor) were removed for microscopy and biofilm quantification on the carriers, and 2 carriers (of each type of carrier from each reactor) were also frozen for future microbial community analysis. Virgin carriers were added to maintain the same number of carriers in the reactors. To avoid inflow of oxygen, nitrogen gas was continuously added while the reactors were open.

Carriers were photographed in a stereomicroscope (Nikon SMZ1270) to visually estimate the amount of biofilm on the carriers. The carriers were also cut and photographed in the stereomicroscope to visualize the cross-section of the biofilm and visually compare the thicknesses of the biofilm.

Analysis of total solids (TS) and volatile solids (VS) on the carriers were performed in triplicates with biofilm from 2 carriers in each sample (i.e. 6 carriers of each type from each reactor in total), to get a representative sample. The biofilm was scrubbed off the carriers into distilled water and put in a 105°C oven overnight, after which they were weighed. VS was measured by drying in a 550°C oven overnight and weighed.

Light microscopy (Nikon Eclipse Ni) was performed on liquid samples from each reactor on day 238 of operation in order to examine the suspended biomass and identify possible differences in suspended biomass between the reactors.

[1] Between day 187 and 235, the influent flow and the gas production was only monitored approximately 3 times a week due to limited access to the lab as a consequence of the corona pandemic.

4. Results and Discussion

This study was performed during 114 days on three lab-scale AnMBBRs treating an industrial wastewater from food processing. The three reactors were loaded with different carriers. *R200* was loaded with commercial, saddle-shaped Anox K™ Z-200 made from recycled HDPE, *R1000* was loaded with a prototype of Anox K™ Z-1000 which were flat and made from virgin HDPE. *RMIX* was loaded with a mix of the two carriers and operated as a reference. The reactors were operated as described in section 3.3. They had already been operated for 123 days when the study of this Master Thesis started. The results from this first period of reactor start-up are reported in the Master Thesis "*Considerations for the Establishment of an Anaerobic Biofilm in an AnMBBR*" (Månsson, 2020).

The results and discussion from this study (day 124 to 238 of reactor operation) will focus on comparing the reactor performance of R200, R1000 and RMIX in terms of sCOD removal and biogas production and how this is influenced by parameters such as the VLR, HRT, VFA levels and composition, pH, alkalinity, and the biomass in the reactors. It will also focus on the characterisation of the biomass and development of biofilm on the carriers.

4.1. Reactor operation and performance

4.1.1. COD removal with respect to HRT and VLR

In this study, the COD removal (%) was used as an estimate of the organic removal efficiencies in the reactors. More specifically, the sCOD was used as this accounts for the easily degradable organic matter and most of the COD in the influent was soluble (78.0 ± 6.6%). Both R1000 (Figure 6) and RMIX (Figure 7) reached a stable sCOD removal of over 90%, while the sCOD removal of R200 (Figure 5) fluctuated between 37% and 73% throughout the study. During the last period of constant VLR (4.13 ± 0.14 kg sCOD/m^3/day) the sCOD removal was 57 ± 1 %, 93 ± 2% and 91 ± 1% for R200, R1000 and RMIX respectively. R200 thus showed significantly lower and more fluctuating sCOD removal compared to R1000 and RMIX. The sCOD removal in R1000 and RMIX are comparable to the sCOD removal obtained in similar studies of AnMBBRs treating industrial effluents from food processing (Wang *et al.*, 2009; Chai *et al.*, 2013; di Biase *et al.*, 2016). For example, Wang *et al.* reached sCOD removal efficiencies of 98% at similar VLR, however, at significantly higher HRT (several days).

The VLR and HRT change over the timespan of this study (day 124 to day 238 of reactor operation) is also shown in Figure 5-7. The VLR was increased from approximately 1 to 4 kg sCOD/m^3/day (which corresponded to a SALR between approximately 10 and 40 g sCOD/m^2 PSA/day) while the HRT was decreased from approximately 1 day to 9 hours. R200 appeared to be more sensitive to changes in operational conditions. At day 138 and 152, the water-bath connected to the reactors dropped from 37.5°C to 21.5°C. The effect in all reactors was a sudden decrease in sCOD removal. R200 took a longer time to recover after the temperature drop, compared to R1000 and RMIX which both returned to their previous levels of sCOD removal the day, or a few days after the temperature drop. Increase in VLR as a consequence of decreasing the HRT also appeared to influence the sCOD removal of R200. During periods of constantly decreasing the HRT, the sCOD removal dropped. Whereas, during periods of more stable HRT, the sCOD removal increased. In R1000 and RMIX, no such behaviour could be observed. This suggests that the suspended biomass may contribute to the sCOD removal to a larger extent in R200 compared to the other two reactors. When the HRT is decreased, the suspended biomass is washed out, hence, the sCOD removal drops. However, at constant HRT the suspended biomass has time to acclimatise to the new conditions, hence, resulting in a rise in sCOD removal. It may also be due to the increase in organic load, rather than the HRT, as a sudden increase in load may lead to the acidogenic bacteria producing acids faster than the methanogens can consume them, consequently leading to a lower sCOD removal and process imbalance in R200.

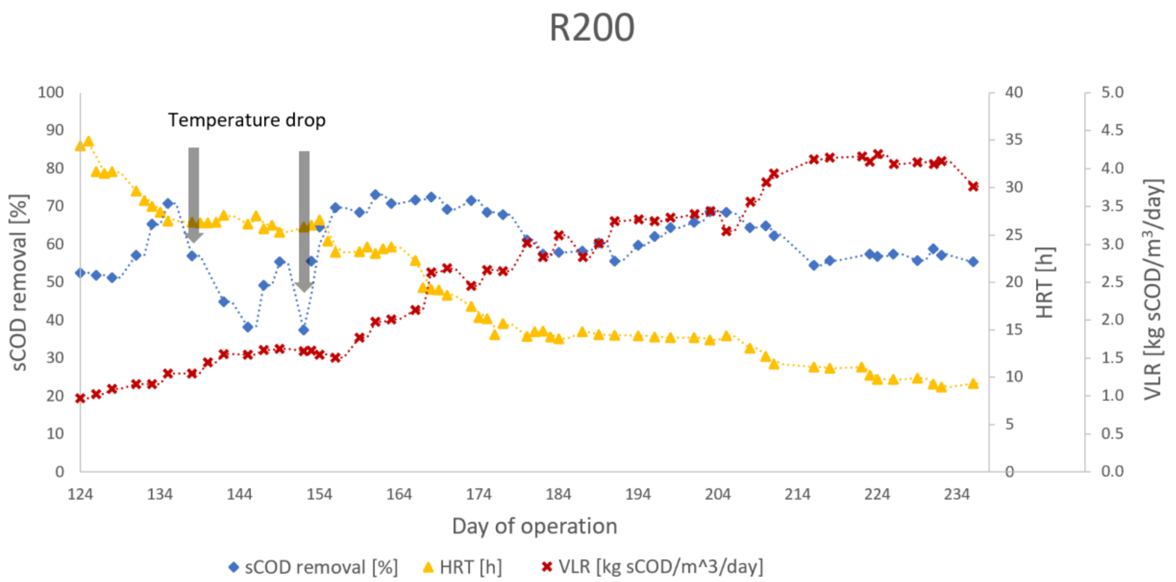


Figure 5. sCOD [%], HRT [h] and VLR [kg sCOD/m3/day] for R200 (containing Z-200 carriers).

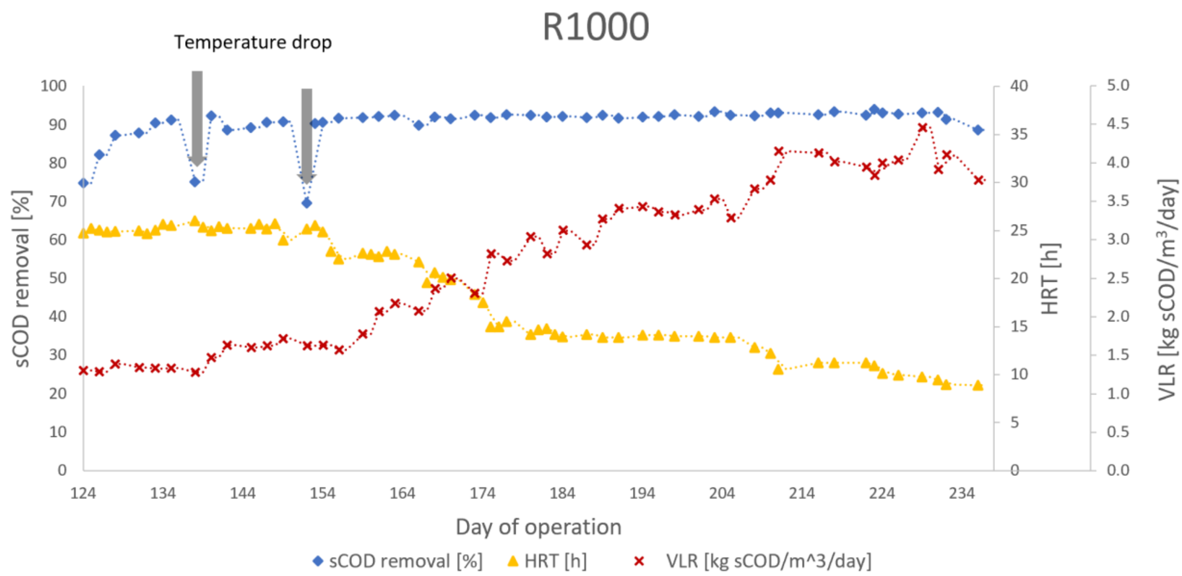


Figure 6. sCOD [%], HRT [h] and VLR [kg sCOD/m3/day] for R1000 (containing Z-1000 carriers).

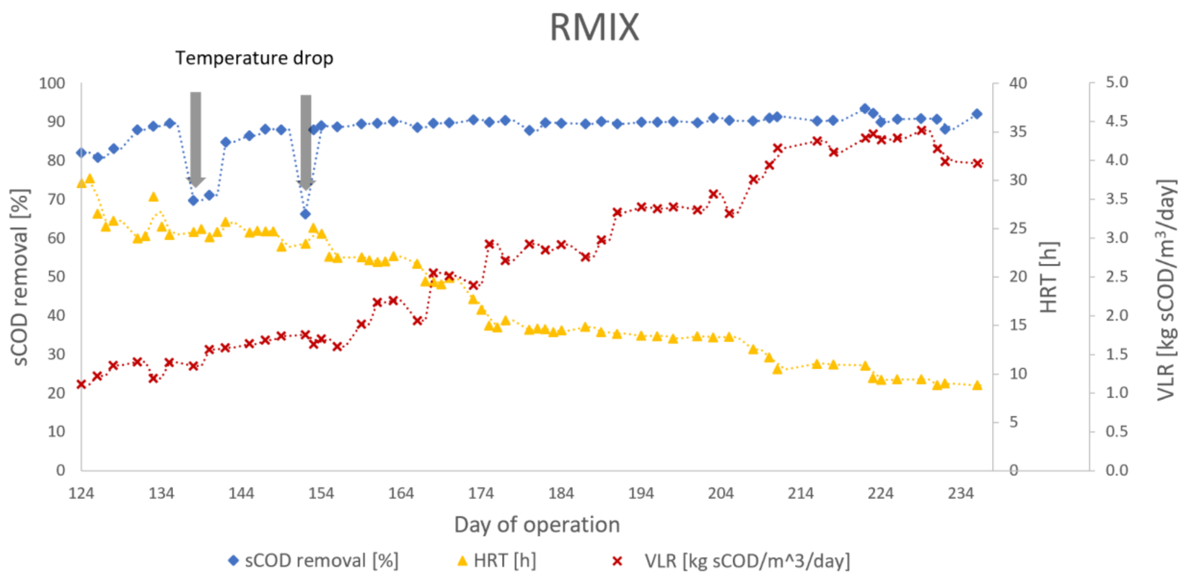


Figure 7. sCOD [%], HRT [h] and VLR [kg sCOD/m3/day] for RMIX (containing Z-200 and Z-1000 carriers).

4.1.2. VFAs, pH and alkalinity

The VFA levels and composition, as well as the pH, was measured as it indicates the balance between bacteria and archaea in the reactor which is related to the process stability. Measurements of organic acids were also made as a complement to the VFA analysis, which were made less frequently (see section 3.5.).

4.1.2.1. VFA levels with respect to sCOD removal and process stability

The organic acid levels in R200 varied between 115 to 829 mg HAc-eq./L (Figure 8). The levels of organic acids were related to the sCOD removal. At high levels of organic acids, the sCOD removal was low and vice versa. The fluctuating organic acid levels indicates process instability of R200. Organic acid levels for R1000 (Figure 9) and RMIX (Figure 10) on the other hand were relatively stable. However, with a drop from around 250 mg/L to below 100 mg/L from day 216 onward. This drop was not observed for the VFA measurements and may therefore be attributed to measurement errors as the organic acids were analysed by a spectrophotometric method (HACH LCK 365, Hach) and during the same period a change in wastewater characteristics was observed (colour changed from pink to beige, which may influence the spectrophotometric readings of the organic acids). At day 138 and 152, the water-bath connected to the reactors dropped from 37.5°C to 21°C. The effect in all reactors was a sudden decrease in sCOD removal accompanied by an increase in organic acids (Figure 8-10).

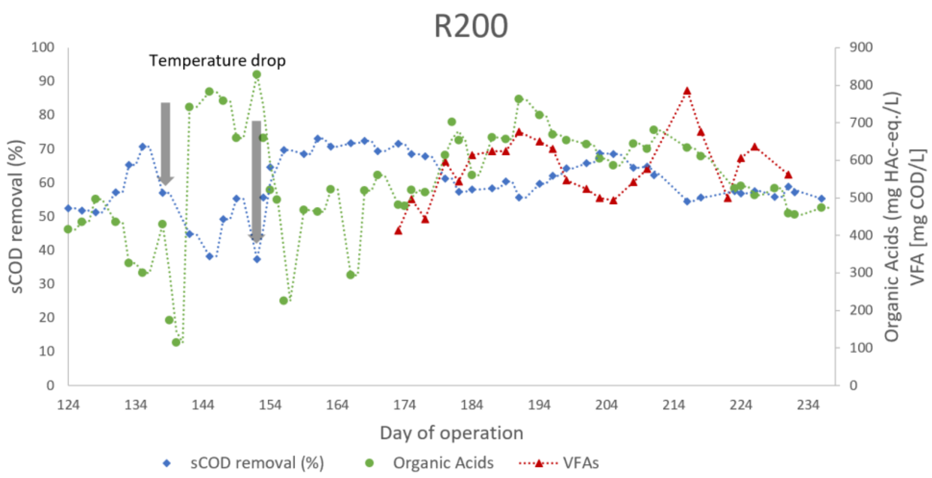


Figure 8. sCOD removal [%], Organic acid [mg HAc-eq./L] and VFA levels [mg COD/L] in R200. Due to complications with the GC apparatus, VFA results could only be obtained between day 173-231 of reactor operation.

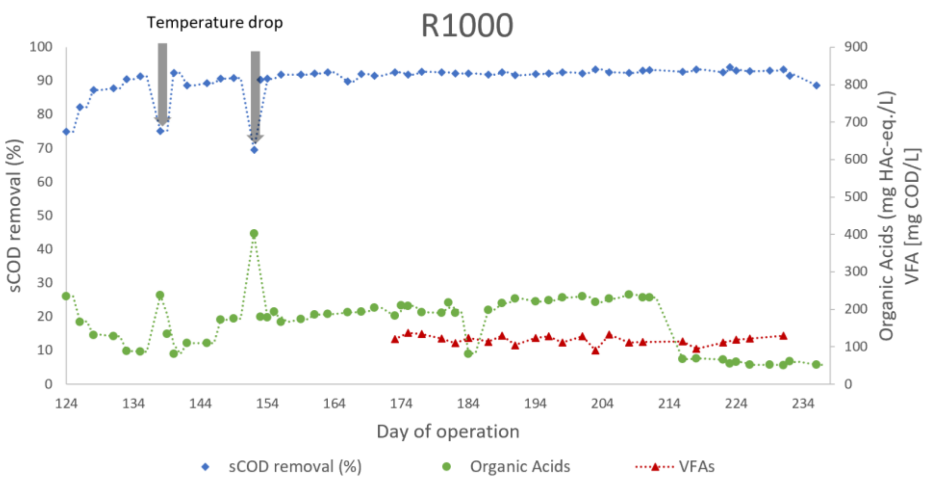


Figure 9. sCOD removal [%], Organic acid [mg HAc-eq./L] and VFA levels [mg COD/L] in R1000. Due to complications with the GC apparatus, VFA results could only be obtained between day 173-231 of reactor operation.

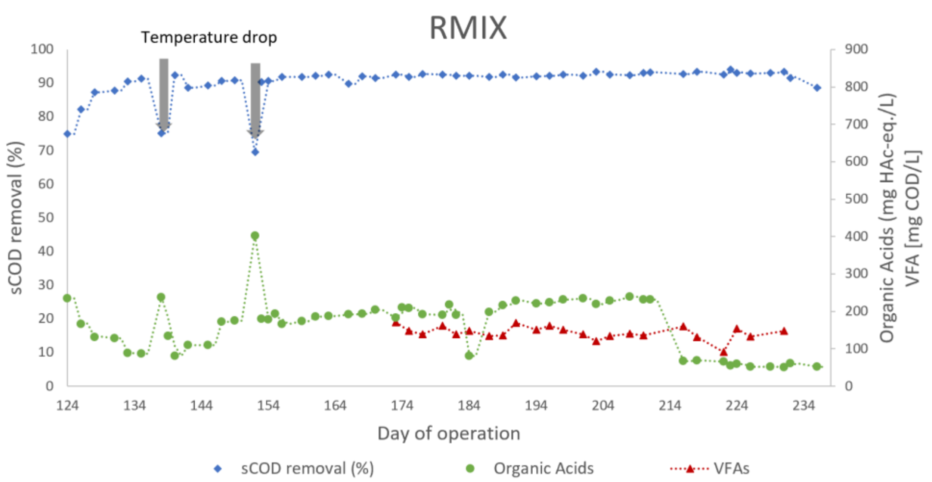


Figure 10. sCOD removal [%], Organic acid [mg HAc-eq./L] and VFA levels [mg COD/L] in RMIX. Due to complications with the GC apparatus, VFA results could only be obtained between day 173-231 of reactor operation.

4.1.2.2. *VFA composition*

The VFA analysis showed that acetate and propionate contributed to the largest fraction of VFAs measured in the reactors (Figure 11). The high concentration of acetate in R200 suggests an imbalance between acetolactic methanogens (which consume acetate) and acidogenic/acetogenic bacteria (which produce acetate). It may be that the acetolactic methanogens are less active or lower in number in R200 compared to the other reactors. The propionate concentration was also significantly higher in R200 compared to R1000 and RMIX. This may suggest that the acetogenic bacteria were also less active in R200 as the acetogenic bacteria produce acetate from VFAs (such as propionate) produced from the acidogenic bacteria (see Figure 1 in section 2.2, illustrating the anaerobic food web). The acetogens are also in syntropy with the H_2-consuming methanogens (hydrogenotrophic methanogens) as they are dependent on a low partial pressure of H_2, consequently, if the hydrogenotrophic methanogens are less active this would presumably influence the acetogens.

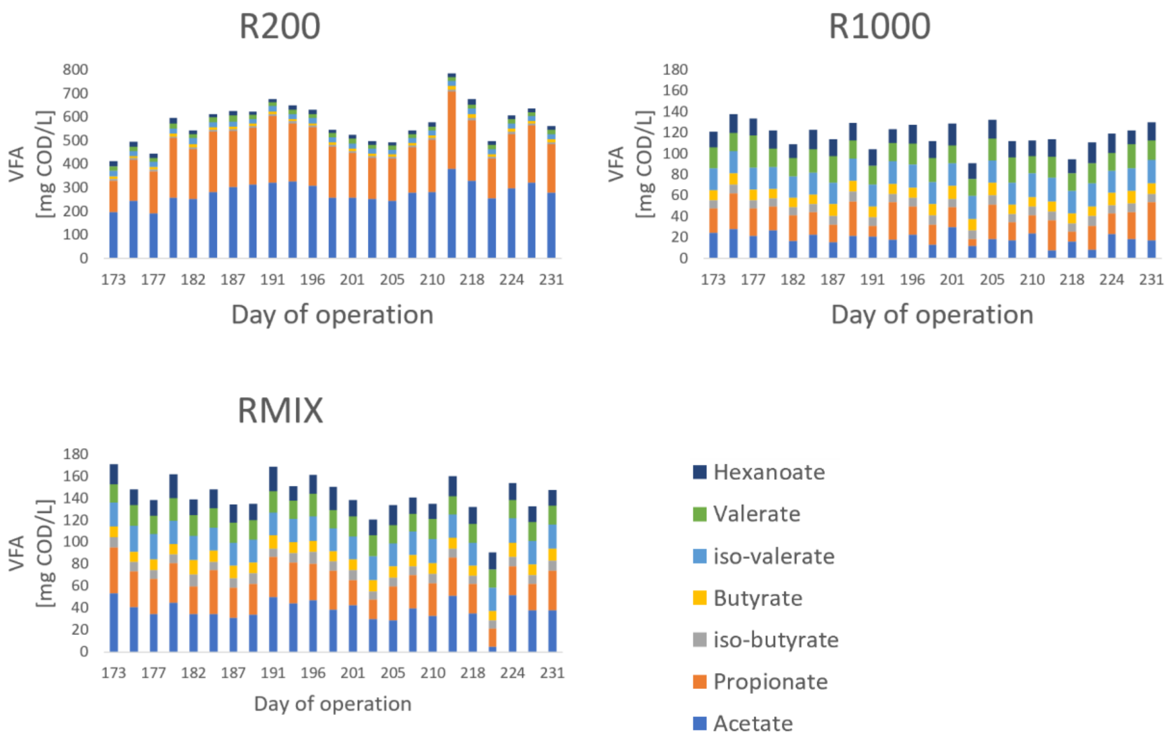


Figure 11. VFA concentrations [mg COD/L] in R200, R1000 and RMIX between day 173 and 231. Note the difference in x-axis interval between the reactors. Due to complications with the GC apparatus, VFA results could only be obtained between day 173-231 of reactor operation.

4.1.2.3. *pH and alkalinity*

The pH in the reactors varied between approximately 7.0 to 7.5, with a consistently lower pH in R200 (Figure 12), which was probably attributed to the higher concentration of organic acids in this reactor as a result of lower methanogenic activity. However, pH did not vary significantly within each reactor with the organic acid concentrations, indicating that the reactors were well buffered.

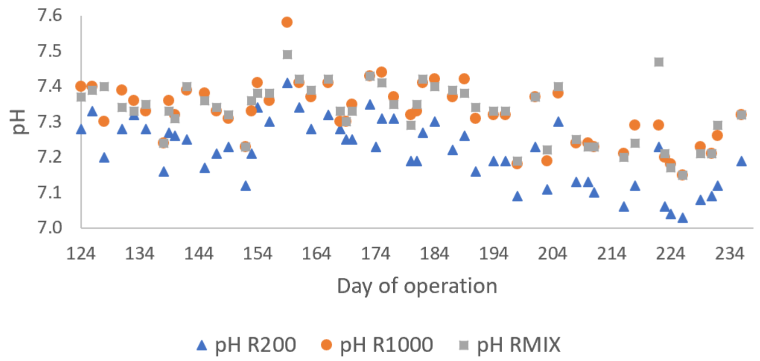


Figure 12. pH of R200, R1000 and RMIX4

4.1.3. Biogas production and composition

The biogas produced in the reactors had a methane content of 63 ± 2% in R200, 69 ± 2% in R1000 and 68 ± 2 % in RMIX (Table 4), with the remainder mainly being carbon dioxide. Wang *et al.* reported similar results with mean methane contents of 66% and 68% (Wang *et al.*, 2009) and di Biase *et al.* reported a methane content varying between 60-70% (di Biase *et al.*, 2016). The lower methane content in R200 compared to R1000 and RMIX may be related to the lack of hydrogenotrophic methanogens (as discussed in section 4.1.2.2.) as this would result in a higher content of hydrogen in the biogas. However, the hydrogen content of the biogas was not measured in this study. During the first period of reactor operation (made by another operator), the methane content was reported to be 85 ± 5% in R200, 83 ± 4% in R1000 and 81 ± 4% in RMIX (Månsson, 2020). The higher methane content during the first period of reactor operation could be due to a switch from a community consisting of more hydrogenotrophic methanogens that consume CO_2 (mainly *Methanosarcina*) to acetoclastic methanogens which consume acetate (mainly *Methanosaeta*). *Methanosarcina* is indeed reported to be the dominant methanogen at an early stage of AnWWT processes, due to its higher growth rate and wider substrate spectrum, while *Methanosaeta* is reported to be dominating later on in the process, at low and stable acetate concentrations, due to its low growth rate and high substrate affinity (van Lier *et al.*, 2008).

The methane yield was 257 ± 32, 243 ± 51 and 289 ± 25 Nml CH_4/ g sCOD reduced for R200, R1000 and RMIX respectively (Table 4). The methane yield was calculated based on the sCOD, as the tCOD measurements were impacted by sedimentation of solids. However, as most of the COD in the feed was sCOD (78.0 ± 6.6%), the results are comparable with the theoretical methane yield, which is based on the tCOD. The results thus showed a somewhat lower methane yield than the maximum theoretical methane yield of 350 Nml CH_4/g tCOD reduced, which could possibly be due to gas leaks or other losses. COD assimilated into biomass may also contribute to the lower methane yield as this will add to sCOD removal without biogas being produced. It should be noted that a gas leak was detected and resolved on day 175 in R1000, which probably has contributed to the lower methane yield of R1000.

Table 4. Methane yield (Nml CH_4/g sCOD reduced) and methane content (%) in the produced gas for the different reactors. Calculations of the methane yield can be found in Appendix C.

Reactor	CH_4 yield (Nml CH_4/g sCOD reduced)	CH_4 content (%)
R200	257 ± 32	63 ± 2
R1000	243 ± 51*	69 ± 2
RMIX	289 ± 25	68 ± 2

* Gas leak detected and solved on day 175.

The fraction of COD assimilated into biomass can be estimated by plotting the volumetric methane production rate (VMPR, g CH_4-COD equivalence/L/day) versus the sCOD removal rate (g sCOD reduced/L/day) over time. The VMPR is calculated based on the assumption that 1 mole of methane is equivalent to 64 grams of COD. The VMPR increased linearly with the sCOD removal at a rate of 66%, 72% and 73% for R200, R1000 and RMIX respectively (Figure 13). The remainder (37%, 28% and 27%) could be considered as biomass synthesis, assuming no gas leak of the system (as a gas leak would result in a lower methane yield per sCOD reduced), or other losses. In a study of an AnMBBR treating milk permeate, Wang *et al.* reported a rate of 89% (Wang *et al.*, 2009). Thus, this may indicate a higher rate of biomass synthesis in this study compared to the one made by Wang *et al.*, 2009. It should be noted that Wang *et al.* (2009) calculated the rate based on tCOD removal while in this study it was based on sCOD removal.

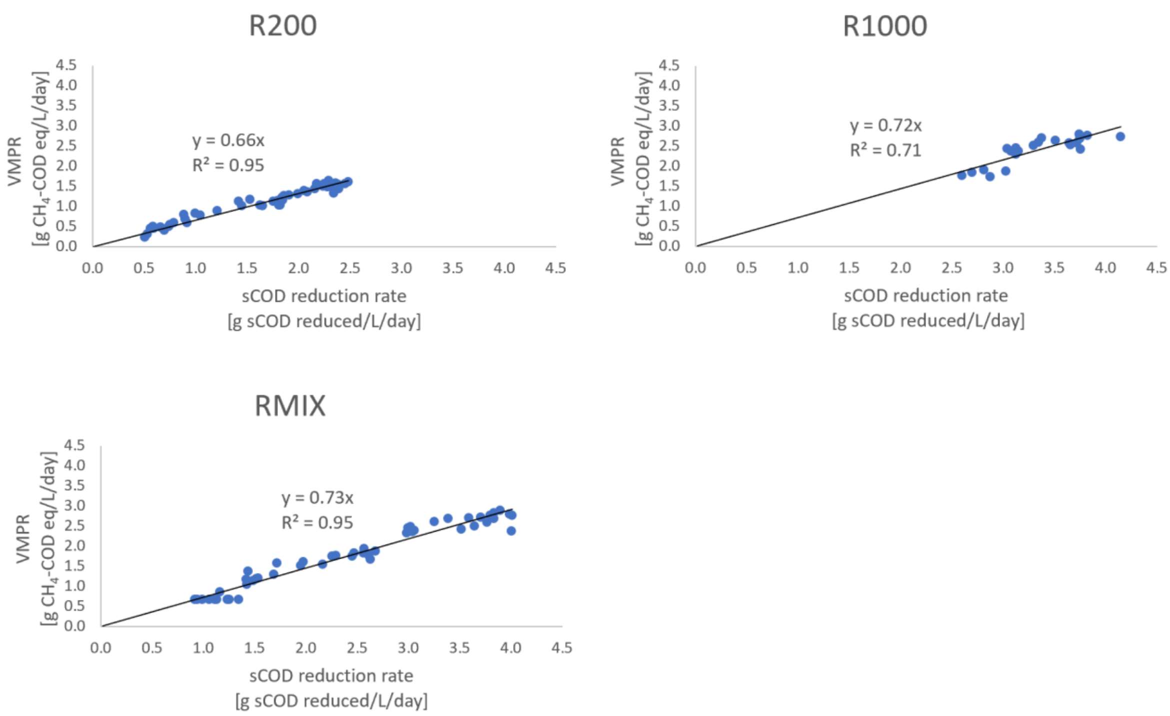


Figure 13. Volumetric methane production rate (VMPR) as a function of sCOD removal rate with linear regression forced through the origin.

4.2. Biomass characterisation

The biofilm development and accumulation on the carriers were analysed by TS and VS measurements of the biomass on the carriers, stereomicroscopy was performed to visually estimate and compare the biofilm on the carriers. This was performed on day 141, 169 and 232 of reactor operation. TSS and VSS analysis was used on as an indicator of the suspended biomass in the reactors. Light microscopy was performed on the suspended biomass on day 238 of reactor operation.

4.2.1. Biofilm development and accumulation from TS and VS analyses

The TS and VS analyses of the biomass attached to the carriers showed that the mass of biofilm (as g VS/m^2 PSA), was substantially higher on the Z-1000 carriers compared to the Z-200 carriers (assuming all VS on the carriers were biofilm) (Figure 14 and Table 5). If the carriers would have reached their maximum biofilm thickness, and the density of the biofilm in Z-200 and Z-1000 was the same, the theoretical mass of biofilm would have been approximately 5 times greater on the Z-1000 carriers compared to the Z-200 carriers (considering the depth of the Z-1000 carriers are 5 times greater than of the Z-200 carriers). However, the result showed that the mass of biofilm was 10-20 times greater on the Z-1000 carriers compared to the Z-200 carriers. This means that either the cells in the Z-200 were not filled with biofilm to the same degree as the Z-1000 carriers, or that the biofilm in Z-1000 was denser, or a combination of the two. Furthermore, Figure 14 and Table 5 show that the amount of biofilm (as g VS/m^2 PSA) was consistently the highest on the Z-1000 carriers in RMIX and consistently the lowest on the Z-200 carriers in the same reactor. This may indicate that the biomass prefers to form biofilm on the Z-1000 carriers rather than on the Z-200 carriers. This since most of the biofilm in the RMIX was attached to the Z-1000 carriers and the Z-200 carriers in RMIX held less biomass compared to the Z-200 carriers in R200. At the last day of sampling (day 232 of operation) the amount of biofilm on the carriers were 5.8 ± 1.1 g VS/m^2 PSA in R200, 54.2 ± 12.8 g VS/m^2 PSA in R1000, 4.2 ± 0.6 g VS/m^2 PSA on Z-200 in RMIX and 92.3 ± 1.9 g VS/m^2 PSA on Z-1000 on RMIX. The high standard deviation of R1000 might be due to one of the carriers originating from the new carriers added on the previous sampling (new carriers were added at all sampling occasions to keep the same number of carriers in the reactors).

The Z-1000 carriers had higher biofilm accumulation rates of an order of magnitude greater than the Z-200 carriers (Table 5). The higher biofilm accumulation rate of the Z-1000 carriers may be due to a more protected environment in the deeper Z-1000 cells causing less shear forces on the biofilm, enabling it to accumulate faster. In contrast, the biofilm in the shallow cells of the Z-200 carrier may be more subjected to shear forces, thereby restricting biofilm accumulation. As the Z-200 carrier was made of recycled HDPE and the Z-1000 carrier from virgin HDPE, it is also possible that differences in surface properties affect the development of the biofilm. Moreover, the difference in shape (saddle-shaped Z-200 and flat Z-1000) may influence hydrodynamics of the reactors and thereby influence biofilm attachment and detachment.

The estimated total amount of biofilm in the reactors at the last carrier sampling (day 232 of operation) were 2.5 g VS/reactor for R200, 22 g VS/reactor for R1000, 0.85 g VS/reactor for Z-200 in RMIX and 19 g VS/reactor for Z-1000 in RMIX (Table 5). This corresponded to a specific removal rate of 3.7 g sCOD/g VS/day for R200, 0.64 g sCOD/g VS/day for R1000 and 0.68 g sCOD/g VS/day for RMIX. The removal rate in relation to the mass of biofilm were thus significantly higher for the R200 reactor, which only contained the Z-200 carriers, compared to the other reactors. This suggests that the biofilm on the Z-200 carriers were more active/specialised compared to the biofilm on the Z-1000 carriers. A higher activity of the biomass in Z-200 may be explained by more dead/inactive biomass in the deeper layer of Z-1000 as the nutrients must be diffused through the biofilm (The Water Environment Federation, 2010). The penetration depth for oxygen in biofilms is said to range between 50-500 μm with similar gradients for other nutrients (Piculell, 2016), thus indicating that the nutrients may not be able to penetrate the deeper layers of the biofilm on the Z-1000 carriers as its maximum biofilm thickness is 1000 μm. The lower specific removal rate in R1000 and RMIX might also be a result of more EPS relative to biomass on the Z-1000 carriers. It should be emphasised that the calculations of the specific removal rate are based on the assumption that the suspended biomass does not contribute to the COD removal. However, in most MBBR systems the suspended biomass may contribute to the COD removal to some extent (Piculell *et al.*, 2014).

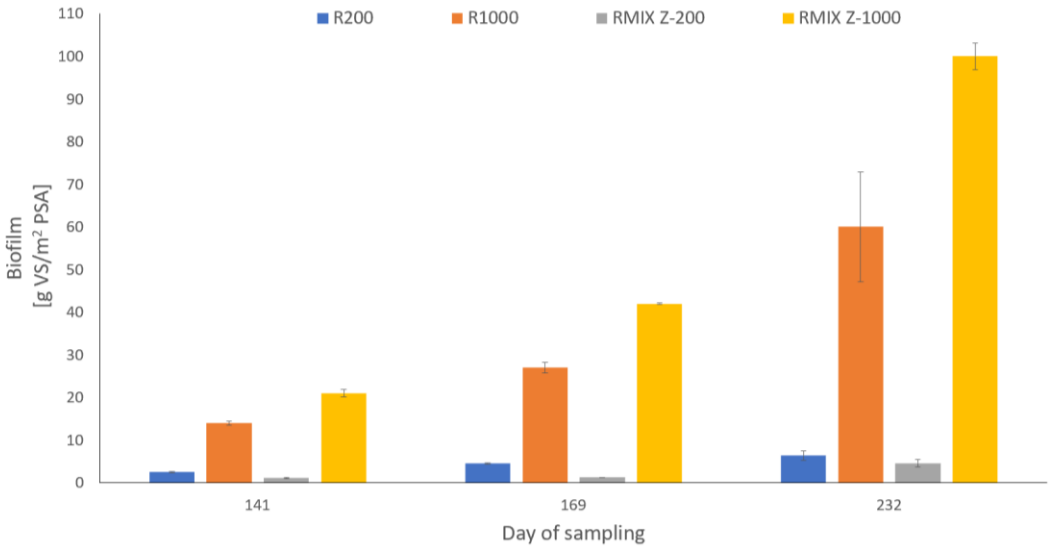


Figure 14. Biofilm development estimated as mg VS/m² PSA. Standard deviation given as error bars.

Table 5. Biofilm results based on TS and VS from carrier samplings at day 141, 169 and 232 (found in Appendix B, Table B1). The specific removal rates are calculated based on the sCOD removal at the time of sampling. Equations can be found in Appendix C.

Day	Reactor	VS/TS	Biofilm [g VS/m^2 PSA]	Total Biofilm [g VS/reactor]	Biofilm accumulation rate [g VS/m^2 PSA/ day]	Specific removal rate [g sCOD/g VS/day]
141	*R200*	0.91	2.3 ± 0.1	0.94	2.0x10^{-2}	3.0
	R1000	0.87	12.8 ± 0.5	5.3	1.1x10^{-1}	1.0
	RMIX Z-200	0.95	1.0 ± 0.1*	0.21	8.3x10^{-3}	1.0
	RMIX Z-1000	0.88	18.8 ± 0.5	3.9	1.7x10^{-1}	
169	*R200*	0.92	4.1 ± 0.1	1.7	7.4x10^{-2}	4.5
	R1000	0.87	24.6 ± 1.2	10	4.7x10^{-1}	0.82
	RMIX Z-200	0.73	1.3**	0.27	5.8x10^{-3}	1.1
	RMIX Z-1000	0.88	38.0 ± 0.1	7.8	7.6x10^{-1}	
232	*R200*	0.82	5.8 ± 1.1	2.5	3.0x10^{-2}	3.7
	R1000	0.86	54.2 ± 12.8	22	5.1x10^{-1}	0.64
	RMIX Z-200	0.81	4.2 ± 0.6	0.85	1.3x10^{-2}	0.68
	RMIX Z-1000	0.88	92.3 ± 1.9	19	2.4x10^{-1}	

* Only two out of three VS samples could be analysed.
** Only one out of three VS samples could be analysed.

4.2.2. Stereomicroscopy of the carriers

The stereopmicroscopy of the carriers confirmed that the amount of biofilm was considerably higher on the Z-1000 carriers compared to the Z-200 carriers. Figures 15-18 shows typical carrier samples from each day of sampling. Comparing Z-1000 carriers in R1000 and RMIX at the last day of sampling, the structure of the biofilm appeared slightly different between the reactors. Z-1000 in RMIX (Figure 18 c)) had patches of light-coloured biofilm while Z-1000 in R1000 (Figure 16 c)) had more evenly distributed light-coloured biofilm. Figure 19 shows the cross-section of the carriers on day 232. The Z-1000 carriers in both R1000 and RMIX appeared to almost have reached the maximum biofilm thickness of 1000 µm (Figure 19 b) and d)), while the Z-200 carriers in R200 and RMIX had not quite reached the maximum biofilm thickness of 200 µm (Figure 19 a) and c)). If the biofilm exhibits a layered structure with sensitive methanogens in the deeper layers, as shown in studies of USAB reactors (MacLeod *et al.*, 1990; Fang, 2000), the thicker biofilm in Z-1000 may be more resistant to perturbations (such as changing VLR, composition of the feed etc) compared to the Z-200.

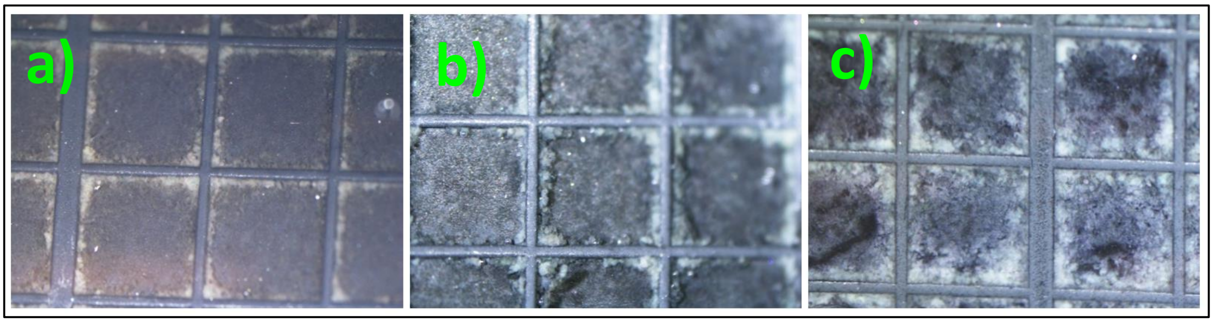


Figure 15. Biofilm on Z-200 carriers in R200. a) day 141. b) day 169 and c) day 232 of operation. Inner dimensions of cells are approximately 2.5x2.5 mm.

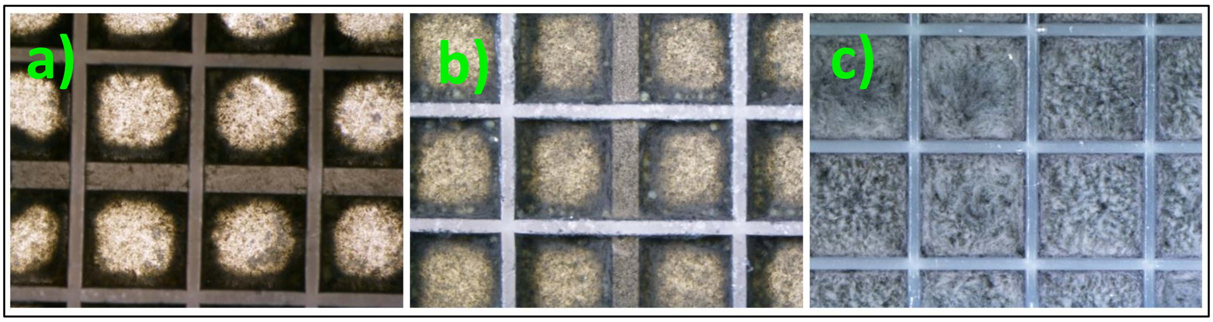


Figure 16. Biofilm on Z-1000 carriers in R1000. a) day 141. b) day 169 and c) day 232 of operation. Inner dimensions of cells are approximately 2x2 mm.

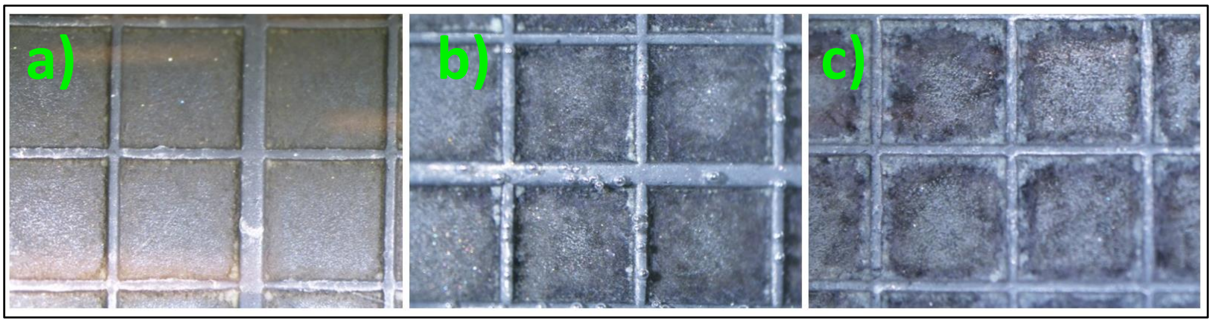


Figure 17. Biofilm on Z-200 carriers in RMIX. a) day 141. b) day 169 and c) day 232 of operation. Inner dimensions of cells are approximately 2.5x2.5 mm.

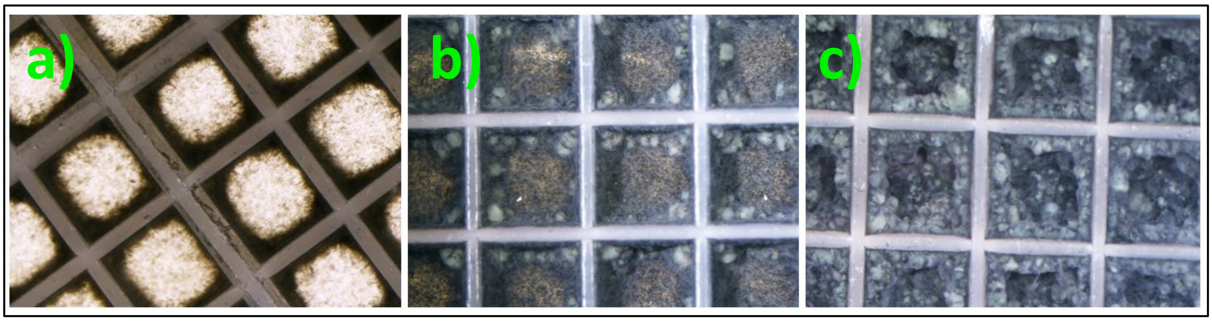


Figure 18. Biofilm on Z-1000 carriers in RMIX. a) day 141. b) day 169 and c) day 232 of operation. Inner dimensions of cells are approximately 2x2 mm.

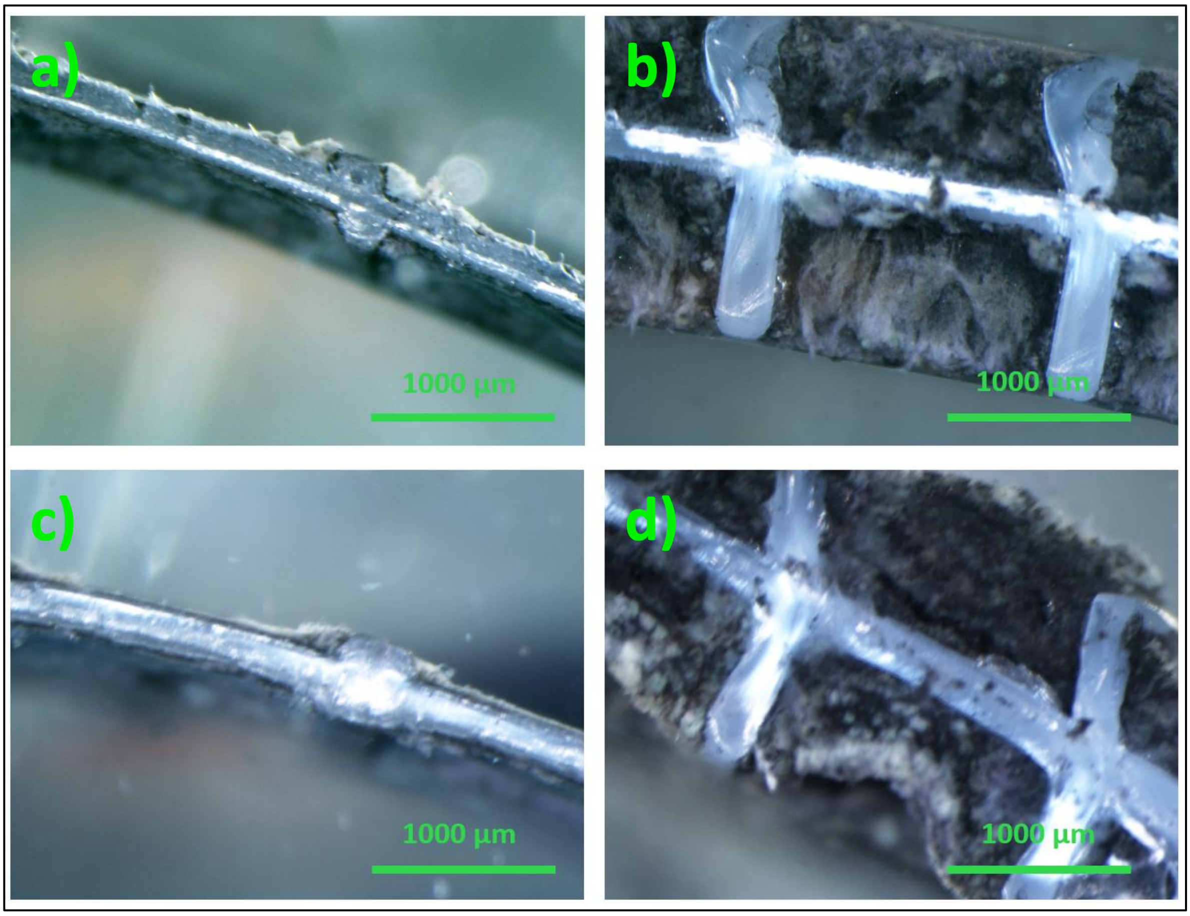


Figure 19. Cross section of carriers with biomass at day 232 of operation. a) Z-200 from R200, b) Z-1000 from R1000, c) Z-200 from RMIX and d) Z-1000 from RMIX.

4.2.3. VSS as an indicator of suspended biomass

The VSS in the reactors during the time of operation were 271 ± 75, 194 ± 59 and 209 ± 74 mg/L in R200, R1000 and RMIX respectively. As the amount of VSS was consistently higher in the R200 reactor (as shown in Figure 20), despite the reactors operating at similar HRT, it could be hypothesized that the amount of suspended biomass was higher, and thus contributed to the COD removal to a larger extent compared to the other two reactors. Which may also be the reason as to why R200 was more sensitive to sudden decreases in HRT, as suspended biomass was being washed out.

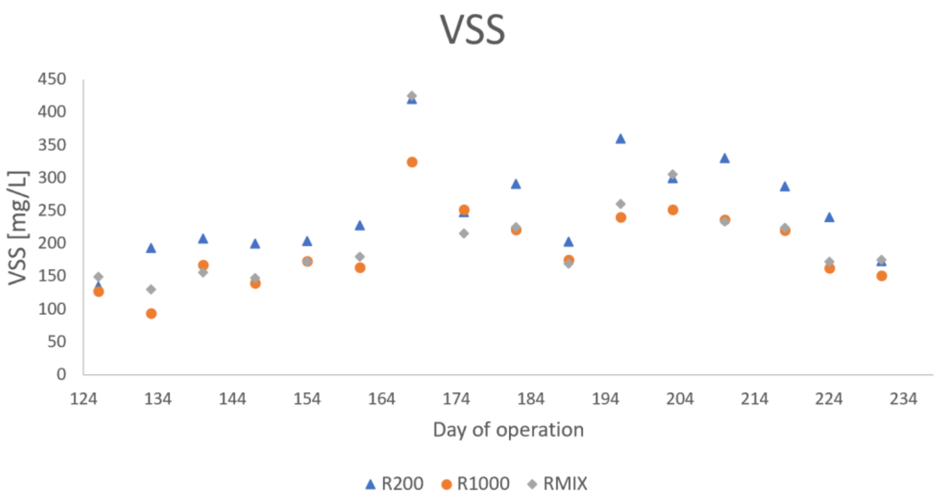


Figure 20. Volatile suspended solids in the reactors over the time of operation.

4.2.4. Light microscopy of the suspended biomass

The light microscopy (Figure 21) of the suspended biomass in the reactors on day 238 of operation showed no noticeable difference in free-swimming microorganisms between the three reactors. Rod-shaped, curved-shaped (spirillum/spirochete) and possibly coccoid-shaped bacteria/archaea were observed in all reactors. What appeared to be free-swimming protozoans (probably flagellates) were also observed in all reactors (Figure 21 d)). Small (<25 µm), medium (25-250 µm) and large (>250 µm) microbial flocs were present in the reactors. Rounded, more dense flocs were observed (Figure 21 a)), and larger irregularly shaped flocs were observed (Figure 21 c)). Some flocs appeared to have oil-like droplets (Figure 21 b)). Piculell *et al.* (2014) found that most of the suspended biomass in a study of aerobic MBBRs came from detached biofilm, it could therefore be expected that the microbial flocs in this study mostly originated from detached biofilm as well. R200 appeared to have smaller, but an overall higher number of microbial flocs compared to R1000 and RMIX which had fewer but larger microbial flocs.

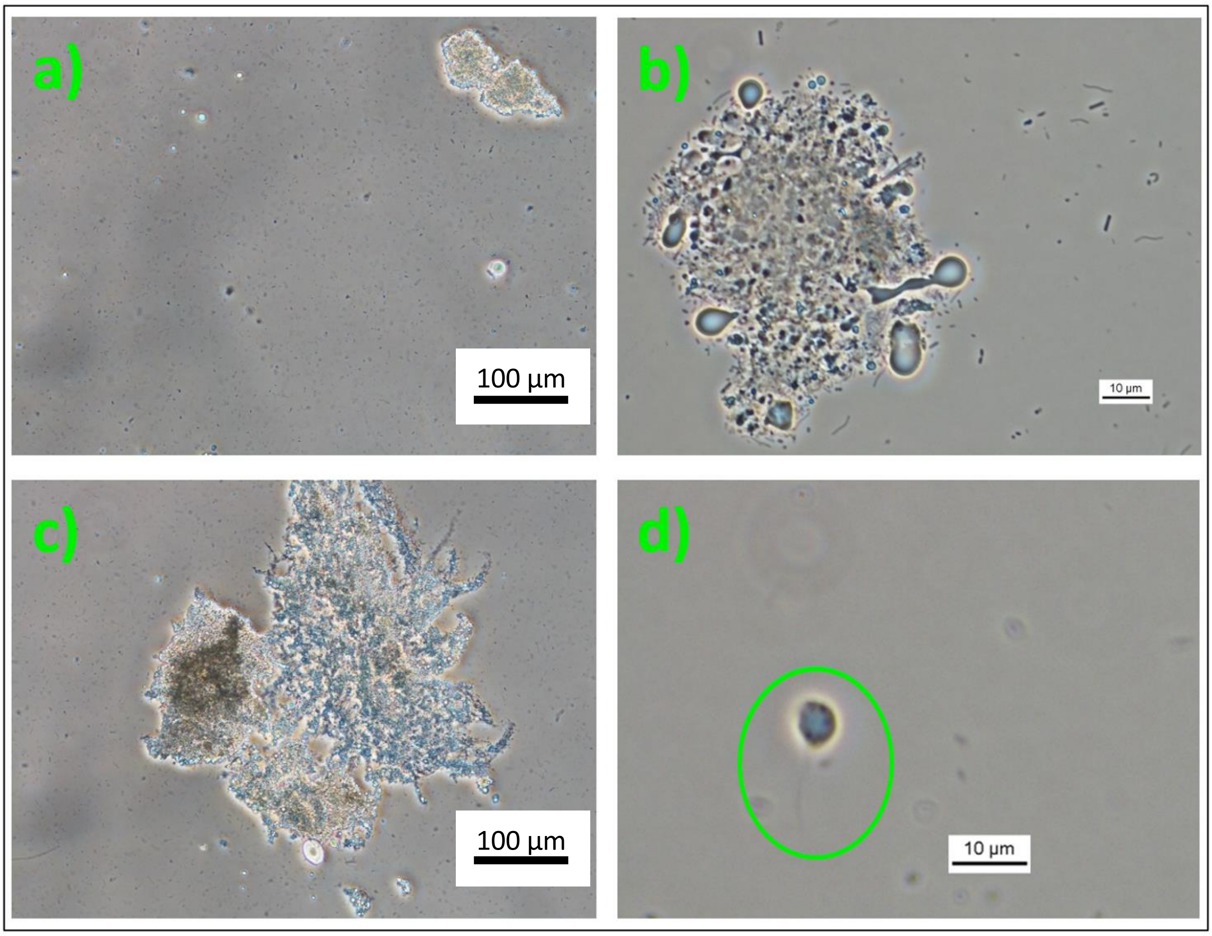


Figure 21. Light microscopy of the suspended biomass in the reactors. a) A medium-sized (25-250 µm) microbial floc in R200 in 200x magnification. b) A medium-sized (25-250 µm) microbial floc in R200 in 1000x magnification, with oil-like droplets. c) A large (>250 µm), irregularly shaped microbial floc in RMIX. d) A protozoan in R1000 in 1000x magnification.

5. Conclusion

The first objective of this study was to evaluate the performance in terms of sCOD removal and biogas production depending on the type of carrier and the biofilm thickness. R200 had a fluctuating sCOD removal not exceeding 73% and a methane content of 63 ± 2%, while R1000 and RMIX reached a stable sCOD removal of above 90% with a methane content of 69 ± 2% (R1000) and 68 ± 2% (RMIX). The results thus clearly showed that the reactor containing only Z-200 carriers (R200) had lower and more fluctuating sCOD removal, as well as a lower methane content of the biogas. The lower and more fluctuating sCOD removal in R200 compared to the reactors which contained Z-1000 carriers (R1000 and RMIX) may be explained by a number of reasons (not listed in order of relevance as this is not possible to pinpoint by this study):

- R200 may contain more suspended biomass which contributes to the COD removal. Changes in HRT will thus influence the sCOD removal to a larger extent in R200 compared to the other reactors.
- There is an imbalance between the methanogens and the bacteria in R200, indicated by the high levels of VFAs, more specifically the high relative levels of acetate and propionate. This imbalance decreases the performance of R200 in terms of sCOD removal and biogas production. This could possibly be confirmed by analysis of the microbial composition in the reactors.
- The amount of biomass in R200 is simply not sufficient (i.e. there is not enough surface for enough biofilm to form) to reach sCOD removal rates of above 90% as for the other two reactors.
- The sensitive methanogens on the Z-200 carriers may be less shielded from perturbations such as changes in VLR, HRT, temperature, and composition of the feed due to a thinner biofilm, leading to more fluctuating sCOD removal and a less resilient process.

Furthermore, the lower methane content of the biogas in R200 is likely related to the imbalance of the syntrophic relationship between the hydrogen-consuming methanogens and the acetogens, resulting in a relatively higher H_2 content of the biogas. However, the H_2 content of the biogas should be measured to confirm this.

The other objective was to assess the effect of the different carriers on biofilm development and accumulation. It was expected that the Z-1000 carriers would hold a greater mass of biofilm than Z-200, as the Z-1000 has a higher maximum biofilm thickness. However, the result showed a significantly greater mass of biofilm (as g VS/m^2 PSA) on the Z-1000 carriers than expected. This may be due to a more dense biofilm in Z-1000 and/or that the biofilm was closer to its maximum biofilm thickness in Z-1000 (as confirmed by the light microscopy). Furthermore, the biofilm may be more protected in the Z-1000 carriers, as the cells of these carriers are deeper, causing less shear on the biofilm. However, even though the Z-1000 held more biofilm, the specific removal rate of the Z-200 carriers was significantly higher. This result indicates that Z-200 carriers allow for a more specialised/active biofilm. This may be due to (not listed in order of relevance):

- A larger fraction of inactive biomass and/or EPS in the thicker biofilm of the Z-1000 carriers, as a result of nutrients not being able to penetrate the deeper layers of the biofilm. In R200, which has a thin biofilm, the nutrients would be available to the whole depth of the biofilm.

- The difference in carrier design and material. This may influence the hydrodynamics in the reactors and the surface properties of the carriers, leading to differences in the microbial community of the biofilm.

In conclusion, this study suggests that the performance of AnMBBRs are largely influenced by the biofilm thickness and that the carrier design and material may influence the biofilm development. The reactor containing Z-1000 carriers showed a significantly higher sCOD removal efficiency while the reactor containing only Z-200 carriers had a significantly higher specific removal rate. It may therefore be preferable to use the design of the Z-200 carrier but instead use carriers allowing for a slightly thicker biofilm, e.g. Z-400 carriers which allow for a maximum biofilm thickness of 400 µm. It may also be possible to use a higher number of these carriers to maximise the surface area available for biofilm formation and thereby achieve better COD removal.

6. Recommendations for future work

One of the original objectives was to assess the microbial community with 16S gene analysis (carrier samples and suspended biomass samples were collected in this study for future analysis of this) to characterise the microbial community and evaluate if/how the microbial community differed depending on the carrier type and biofilm thickness. As this was not possible to perform during this study, and studies on the microbial community in AnMBBRs are few, it would be of interest to analyse the microbial community in future work. Analysis of the microbial community could reveal differences in the bacterial and archaeal community of the biofilm and the suspended biomass and relate this to the difference in performance of the reactors. It would not only be interesting to analyse the microbial community, but also the fraction of active/inactive biomass depending on biofilm thickness to evaluate how nutrients may penetrate the deeper layers of the biofilm.

It was mentioned that the lower methane content of R200 may be the result of a higher fraction of H_2 in the biogas. It would be of interest to measure the H_2 content in the biogas to confirm this.

Furthermore, the carriers in this study were of different design and material (saddle-shaped versus flat and recycled HDPE versus virgin HDPE). Studies have suggested that the archaeal community in anaerobic processes may be dependent on the support material, while the bacterial community may be more dependant on the inoculum used (Habouzit *et al.*, 2014). By characterizing the surface properties (surface charge, water contact angle, surface roughness, etc.) of the carriers together with analysing the microbial community, it could be evaluated if the microbial community varies depending on the surface properties of the carrier material.

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Appendix A: Wastewater characteristics

Table A1. Analysis results from the wastewater used as substrate. Average and standard deviation from 13 batches.

Analysis	Average	Std
pH	3.80	0.17
Alkalinity (mg $CaCO_3$/L)	48.3	35.1
Ca^{2+} (mg/L)	28.8	6.1
Mg^{2+} (mg/L)	9.81	2.83
Hardness (°dH)	6.31	1.15
Cl^- (mg/L)	180	180
SO_4 (mg/L)	22.4	11.4
sCOD (mg/L)	1830	282
tCOD (mg/L)	2367	426
sCOD/tCOD	0.780	0.066
NH_4-N (mg/L)	6.8	3.4
N-tot (mg/L)	46.7	18.4
PO_4-P (mg/L)	11.7	4.5
P-tot (mg/L)	16.5	5.2
TSS (mg/L)	309	121
VSS (mg/L)	260	103
VSS/TSS	0.857	0.24

Appendix B: TS and VS results from carrier samplings

Table B2. TS and VS results from the carrier samplings on day 141, 169 and 232 of reactor operation.

Day	Reactor	TS (mg/carrier)	VS (mg/carrier)
141	*R200*	3.2 ± 0.1	2.9 ± 0.1
	R1000	24.1 ± 1.0	21.1 ± 0.8
	RMIX Z-200	1.4 ± 0.2	1.3 ± 0.2*
	RMIX Z-1000	34.9 ± 0.7	30.9 ± 0.8
169	*R200*	5.7 ± 0.4	5.3 ± 0.1
	R1000	46.3 ± 2.1	40.4 ± 1.9
	RMIX Z-200	2.1 ± 0.2	1.7**
	RMIX Z-1000	70.8 ± 0.6	62.4 ± 0.2
232	*R200*	9.1 ± 1.1	7.5 ± 1.4
	R1000	103.5 ± 22.4	88.9 ± 21.0
	RMIX Z-200	6.6 ± 0.3	4.2 ± 0.8
	RMIX Z-1000	171.7 ± 3.2	151.3 ± 3.1

* Only two out of three samples could be analysed.
** Only one out of three samples could be analysed.

Appendix C: Equations

Equations C1: Methane yield (Table 4)

The methane yield was calculated on a weekly basis, based on the volume of biogas per day produced by the reactors (as normal (N) millilitres per day and the average methane content of the biogas from the reactors. The methane yield given in Table 4 is thus the average from day 124-328 of reactor operation.

$$Methane\ Yield\ R200 = \frac{Q_{biogas}\ [\frac{NmL\ biogas}{day}]*0.63}{sCOD_{red}\ [\frac{g\ sCOD\ reduced}{day}]}$$ (Equation C1.1)

$$Methane\ Yield\ R1000 = \frac{Q_{biogas}\ [\frac{NmL\ biogas}{day}]*0.69}{sCOD_{red}\ [\frac{g\ sCOD\ reduced}{day}]}$$ (Equation C1.1)

$$Methane\ Yield\ RMIX = \frac{Q_{biogas}\ [\frac{NmL\ biogas}{day}]*0.68}{sCOD_{red}\ [\frac{g\ sCOD\ reduced}{day}]}$$ (Equation C1.1)

Where Q_{biogas} is the volumetric flow of biogas and $sCOD_{red}$ is the removal rate of sCOD.

Equations C2: Biofilm development from TS and VS results

The biofilm results in Table 5 are based on the TS and VS results in Table B1.

The mass of biofilm per PSA was based on the PSA for the carriers, 0.00128 m^2 for Z-200 and 0.00164 m^2 for Z-1000, according to:

$$Biofilm\ on\ Z-200\ carriers = \frac{VS_{carrier}\ [g\ VS/carrier]}{0.00128\ [m^2\ PSA/carrier]}$$ (Equation C2.1)

$$Biofilm\ on\ Z-1000\ carriers = \frac{VS_{carrier}\ [g\ VS/carrier]}{0.00164\ [m^2\ PSA/carrier]}$$ (Equation C2.2)

The biofilm accumulation rate was based on Equation C2.1 and C2.2, divided on the number of days between sampling.

The total amount of biofilm in the reactors was based on the amount of biofilm per carriers and the number of carriers at the sampling occation, according to:

$$Total\ biofilm\ = VS\ [g\ VS/carrier] * number\ of\ carriers$$ (Equation C2.3)

The specific removal rate was calculated based on the sCOD removal rate (g sCOD removed/day) and the total amount of biofilm in the reactors, according to:

$$Specific\ removal\ rate = \frac{sCOD_{red}\left[\frac{g\ sCOD\ removed}{day}\right]}{VS_{reactor}\left[\frac{g\ VS}{reactor}\right]} \qquad \text{(Equation C2.4)}$$