The microbial community in fouling membrane bioreactors

distribution and diversity of important bacteria

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THE MICROBIAL COMMUNITY IN FOULING MEMBRANE BIOREACTORS

DISTRIBUTION AND DIVERSITY OF IMPORTANT BACTERIA

BY
ANJA SLOTH ZIEGLER

DISSERTATION SUBMITTED 2017

AALBORG UNIVERSITY
DENMARK

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ENGLISH SUMMARY

The membrane bioreactor (MBR) technology has existed since the 1960's and its popularity in European wastewater treatment plants (WWTPs) has increased over the last years. However, the technology has not yet overwhelmed the market due to some serious drawbacks of which operational costs due to membrane fouling is the major contributor. Optimisation of the MBR process (minimising fouling) has so far largely been focused on improving technical parameters such as retention times, membrane hydrophobicity etc. However, a main problem seems to be the microorganisms colonizing the membrane. Therefore, a better understanding of the microbial community in MBRs and the effect of abundant microbial species on sludge characteristics and membrane fouling might lead to improved operation and widespread use of MBRs in wastewater treatment plants. The aim of this project was to obtain better understanding of the microorganisms in the fouling layer in MBRs using state-of-the-art molecular methods and to find key parameters/factors that cause membrane fouling in order to provide better basis for fouling control in MBR reactors.

The use of microbial community analysis by 16S ribosomal RNA (rRNA) amplicon sequencing has become the method of choice, not only in this PhD project but also for microbiologists all over the world. The analyses are carried out using various different extraction methods, microbial primers, PCR settings etc. All of these affect the outcome of the community analysis. We conducted a comprehensive study on the evaluation and optimisation of DNA extraction from activated sludge using different bead beating intensities, primers and PCR settings. Even minor changes affected the outcome of the analysis. Based on our results we were able to recommend the following approach for 16S rRNA analyses of activated sludge: DNA extraction using the FastDNA® SPIN Kit for Soil with four times the normal bead beating intensity and V1-3 primers.

The microorganisms of activated sludge MBRs either grow in flocs, as dispersed single cells or attached to immobilised surfaces (membranes). Bacteria vary largely in terms of adhesion characteristics, substrate specificity and exopolymer production. This together may determine which bacteria preferentially grow on membrane surfaces. We studied and compared the microbial community composition of bulk sludge and fouling layer from a pilot-scale MBR system set up at and connected to a full-scale conventional wastewater treatment plant in Aalborg, Denmark, using 16S rRNA amplicon sequencing and fluorescence *in situ* hybridization (FISH). Time series were made in order to investigate specific changes in the microbial communities and bioinformatics and multivariate data analysis were used as tools for revealing patterns and correlations within the large datasets. It was shown that the microbial community of the fouling layer was different from the one in the bulk sludge. This difference was most pronounced in the early fouling layer and interestingly, as the fouling layer evolved, the microbial communities became more similar. Furthermore, filamentous Chloroflexi and *Gordonia* were enriched in the fouling layers of MBRs. This indicates that even though some degree of selection/enrichment of bacteria occurs in the fouling layer, the composition of the bulk sludge community will have a larger effect on the mature fouling layer.

Operational parameters and feed characteristics influence sludge properties, likely by determining the species composition of the activated sludge. We followed the changes in sludge properties and microbial community structure after start-up of a pilot-scale MBR using 16S rRNA amplicon sequencing and physico-chemical analysis of sludge samples. Pearson correlation coefficients were calculated in order to correlate changes in the bacterial community with changes in mean floc size, sludge compressibility and sludge dewatering. Both sludge properties and microbial community structure changed after start-up. Correlation analysis indicated that floc size and sludge compressibility were influenced by certain bacterial species, and it was possible to identify some bacteria that promoted good flocs and some that did not. Among good floc formers were the genera *Dechloromonas, Ca*. Accumulibacter and *Nitrospira*, whereas filamentous Chloroflexi caused poor flocs. Control of their presence may be a way to ensure good floc properties and less fouling problems.

In activated sludge, Chloroflexi and other filamentous bacteria take part in the formation of the floc backbone. The presence and abundance of filamentous bacteria are especially important for good floc and settling properties and the growth of some species leads to sludge bulking. To elucidate the filaments responsible for bulking episodes in activated sludge WWTPs we conducted a survey of 20 Danish full-scale WWTPs by using 16S rRNA amplicon sequencing in order to get a complete overview of the diversity and abundance of filamentous bacteria present. Furthermore, the role of important/abundant bacteria on sludge settleability was investigated. The dominant filamentous microorganisms causing problems were *Ca*. Microthrix and some Chloroflexi, primarily *Ca*. Amarilinum. Control of these would improve floc properties and also potential fouling problems.

The overall conclusion of this project is that strong flocs are important for good plant operation in MBR systems since lower degree of flocculation and small flocs contribute to more fouling. This study underlines that the activated sludge bacteria play a significant role in the formation of good flocs. Expanding our knowledge of good/bad floc formers in terms of ecology and physiology, the microbial community within MBR systems may be manipulated for selection of good floc forming bacteria that contribute positively to membrane fouling.

DANSK RESUME

Membran bioreaktor-teknologien (MBR) har eksisteret siden 1960'erne og dens popularitet i spildevandsrensning er steget i de seneste år over hele Europa. Imidlertid er teknologien endnu ikke dominerende på markedet på grund af nogle alvorlige ulemper, hvoraf driftsomkostninger forbundet med tilstopning af membranen, også kaldet fouling, i høj grad spiller en rolle. Optimering af MBR-processen (minimering af fouling) har hidtil været fokuseret på at forbedre de tekniske parametre som slamopholdstider, membranhydrofobicitet, osv. En af hovedårsagerne synes dog at være mikroorganismer der koloniserer membranen. Derfor må en bedre forståelse af disse mikrobielle samfund samt effekten af de mest hyppige arter på slamkarakteristika og fouling kunne føre til forbedret drift og mere udbredt brug af MBR i spildevandsrensning. Formålet med dette projekt var at opnå en bedre forståelse for mikroorganismerne i foulinglaget i MBR ved hjælp af de nyeste molekylære metoder samt at identificere nøgleparametrene/faktorerne, der forårsager fouling af membranen for at kunne skabe bedre grundlag for foulingkontrol i MBR anlæg.

Analyse af mikrobielle samfund med 16S rRNA amplicon sekventering er den foretrukne metode både i dette PhD projekt såvel som for mikrobiologer verden over. Analysen bliver udført ved brug af diverse forskellige metoder til ekstrahering af DNA, mikrobielle primere, PCR indstillinger, osv. Alle de førnævnte påvirker analysens udfald. Vi lavede et omfattende studie med evaluering og optimering af DNA ekstraktion fra aktivt slam ved hjælp af forskellige bead beating intensiteter, primere og PCR indstillinger. Selv mindre ændringer påvirkede udfaldet af analysen. På baggrund af vores resultater anbefales den følgende procedure til 16S rRNA analyse af aktivt slam: DNA ekstraktion ved hjælp af FastDNA® SPIN Kit for Soil med fire gange den normale bead beating intensitet og V1-3 primere.

Mikroorganismerne i aktivt slam i MBR anlæg vokser enten i flokke, som enkelte celler eller fastgjort på overfalder (membraner). Bakterierne varierer meget i form af adhæsionsegenskaberne, substrat specificitet og produktion af exoplymere. Samlet set kan dette muligvis afgøre hvilke bakterier, der fortrinsvis vokser på overfladen af membraner. Vi studerede og sammenlignede de mikrobielle samfunds sammensætning i aktivt slam og foulinglaget fra et pilot MBR system, som var koblet på et konventionelt renseanlæg i Aalborg, Danmark. Dette gjorde vi ved hjælp af 16S rRNA amplicon sekventering og fluorescens *in situ* hybridisering (FISH). Tidsserier blev lavet for at kunne undersøge konkrete ændringer i de mikrobielle samfund og bioinformatik samt multivariat dataanalyse blev brugt som redskaber til at afsløre mønstre og sammenhænge inden for de store datasæt. Det blev påvist, at de mikrobielle samfund i foulinglaget var anderledes end dem fra slammet. Denne forskel var tydeligst i det tidlige foulinglag og som foulinglaget udviklede sig blev de mikrobielle samfund blev mere lig hinanden. Ydermere, viste foulinglaget sig at være beriget med de trådformede arter Chloroflexi og *Gordonia*. Dette indikerer, at selvom der sker en hvis grad af selektion/berigelse af bakterier i foulinglaget, så har sammensætningen af slammet en større effekt på det udviklede foulinglag.

Driftsparametre samt indløbssammensætning har indflydelse på slammets egenskaber, og bestemmer sandsynligvis artssammensætningen i det aktive slam. Vi fulgte ændringerne i slammets egenskaber og de mikrobielle samfunds struktur ved hjælp af 16S rRNA amplicon sekventering samt fysisk-kemiske analyser af slamprøver efter at have opsat et pilot-skala MBR anlæg. Pearson korrelations-koefficienter blev udregnet for at kunne sammenholde ændringer i de bakterielle samfund med ændringer i flokstørrelse, slam kompressibilitet og afvanding. Både slammets egenskaber og strukturen i de mikrobielle samfund ændrede sig efter opstarten. Korrelationsanalysen indikerede, at flokstørrelsen og slammets komprimerings evne blev påvirket af visse bakteriearter, og det var muligt at identificere nogle som fordrede gode flokke og nogle der ikke gjorde. Bakterier fra slægterne *Dechloromonas, Ca*. Accumulibacter and *Nitrospira* dannede gode flokke. Hvorimod Chloroflexi forårsagede dårlige flokke. Kontrol af deres tilstedeværelse kan være en metode til at sikre gode slamegenskaber og færre problemer med fouling.

I aktivt slam er Chloroflexi og andre trådformede bakterier en del af rygraden i flokkene. Tilstedeværelsen og antallet af trådformede bakterier er især vigtig for at have gode slamflokke og dermed gode bundfældningsegenskaber. For at belyse de filamenter, der er ansvarlige for slamflugt i rensningsanlæg har vi taget prøver fra 20 danske anlæg og ved hjælp af 16S rRNA amplicon sekventering forsøgt at skabe et komplet overblik over diversiteten og antallet af trådformede bakterier som var til stede. Desuden blev indflydelsen af vigtige bakterier for slammets bundfældningsevne undersøgt. De dominerende trådformede mikroorganismer, som forårsagede problemer var *Ca*. Microthrix samt nogle Chloroflexi, primært *Ca.* Amarilinum. Kontrol over disse bakterier kan muligvis forbedre flokegenskaberne samt mindske fouling i MBR.

Den overordnede konklusion på dette projekt er, at stærke flokke er vigtige for en god drift af MBR anlæg. Jo ringere grad af flokkulering og tlstedeværelsen af små flokke i MBR anlæg jo mere fouling. Dette studie understreger, at bakterier i aktivt slam spiller en afgørende rolle i dannelsen af gode flokke. Men en større viden om disse gode/dårlige flokdannere, med henblik på økologi og fysiologi, kan vi manipulere de mikrobielle samfund i MBR anlæg for selektering af gode flokdannende bakterier som bidrager positivt til fouling af membraner.

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Paper 2:

Dynamics of the Fouling Layer Microbial Community in a Membrane Bioreactor. Ziegler, AS, McIlroy, SJ, Larsen, P, Albertsen, M, Hansen, AA, Heinen, N, Nielsen, PH. PLOS ONE (2016) 11(7): doi: 10.1371/journal.pone.0158811

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CHAPTER 1. INTRODUCTION

Human life style has greatly affected our planet and environment, and today we are met with some of the major environmental issues that are effects of human activity, such as climate changes, pollution, environmental degradation and resource depletion (Figure 1). However, over the years our awareness of humans' effect on the environment has grown and the urge to act on it as well.

Every year, municipal and industrial applications produce large amounts of wastewater containing a number of unwanted entities, such as organic and inorganic contaminants, bacteria and viruses (of which some are pathogens), and toxic compounds. If not cleaned properly prior to re-introduction into nature, the wastewater will become a threat to both the environment and the public health, with increasing probability of oxygen-depletion and epidemics (Seviour *et al.* 2003; Daigger *et al.* 2005; Le-Clech 2010). Treating water for reuse is an important part of water conservation efforts and in some regions of the world it makes great economic sense. As world populations grow, sludge production in wastewater treatment plants is accumulating. Because of the high cost of treating and disposing of sludge, recovery of materials or energy and/or reduction of the amount of sludge produced is of high interest (Wang *et al.* 2013). Today, wastewater is considered a resource with a high potential of reuse, and treated wastewater, also called reclaimed water, can be used for several purposes, e.g. agricultural purposes, landscaping, irrigation and recharging groundwater aquifers. Furthermore, recovery and reuse of phosphorus from wastewater can help restore the broken phosphorus cycle (Egle *et al.* 2016).

Figure 1: *Effect of human activities in the biophysical environment. Climate changes, environmental degradation, resource depletion and pollution. (Walsh et al. 2014; Bartram et al. 2014; Millenium Ecosystem Assessment 2005; NRDC 2017)*

Stricter environmental regulations, the need of wastewater recycling and the permanent improvement of the technology has led to a high growth of implementation of membrane bioreactors (MBRs) for wastewater treatment (WWT). The MBR technology provides a compact treatment system that is capable of producing high-quality effluent fit for re-

introduction into nature as well as reducing the volume of sludge produced during wastewater treatment. Thus, MBRs offer a real solution for more sustainable approaches to urban water management in developed and developing countries. (Stephenson *et al.* 2000; Daigger *et al.* 2005; Kraume and Drews 2010; Judd 2011)

1.1. MEMBRANE BIOREACTORS IN WASTEWATER TREATMENT

The MBR technology is a combination of the activated sludge process with membrane separation replacing the settling tank (Figure 2). MBRs can be designed, operated and configured in a range of ways, relating to (i) process configuration (sidestream operated membrane and immersed membrane), (ii) membrane separation process (reverse osmosis, nanofiltration, ultrafiltration and microfiltration), (ii) membrane configuration (flat sheet, hollow fibre and multitubular), (iv) membrane process operation (flux or pressure driven) and (iv) biotreatment process (aerobic, anerobic and anoxic zones). MBRs allow for exceptional versatility in the design of new plants or the retrofitting of existing wastewater-treatment facilities, because membranes can be added in modules. Today, the most commercially significant membrane bioreactor configuration is the immersed membrane (Hardt et al. 1970; Judd 2011; Kraume and Drews 2010).

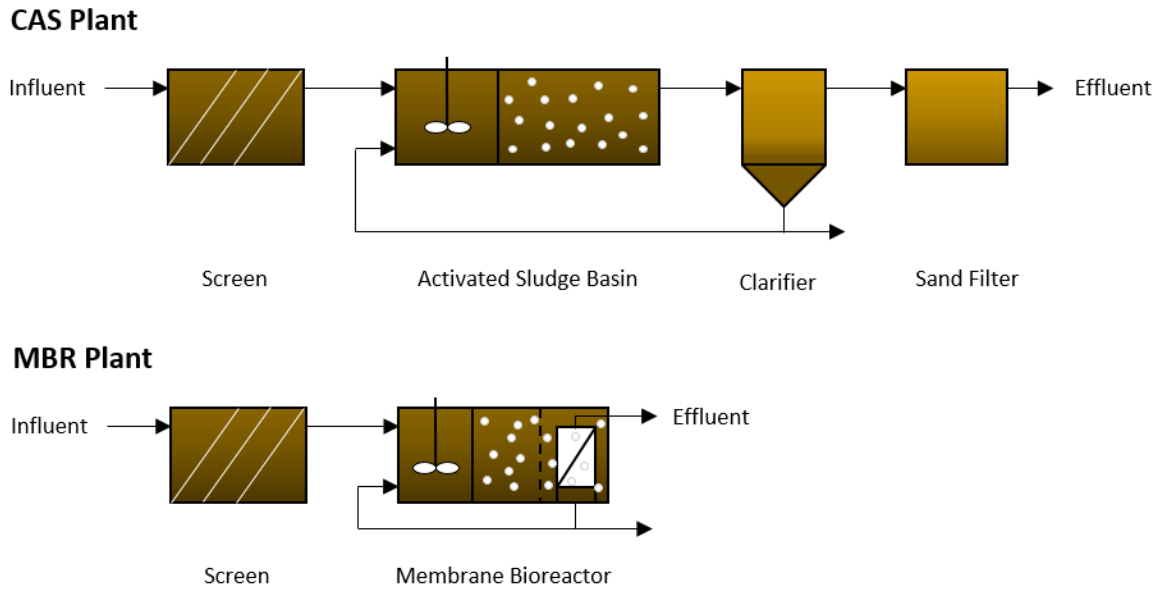


Figure 2: *Conventional activated sludge plant (CAS) and membrane bioreactor plant (MBR).*

MBR technology offers a range of advantages compared to the conventional WWT process. Microorganisms in the activated sludge carry out the biological processes where nutrients are removed from the wastewater and the membrane serves as a filter, rejecting the solid materials (i.e. bacterial flocs) and hence reducing the carry-over of unsettled material to the effluent.

Retention of solid particles results in improved biological treatment due to higher concentrations of mixed-liquor suspended solids (MLSS)[1] and slow-growing microorganisms which take up organic nutrients, such as nitrogen and phosphorus. This makes higher metabolic rates and better nutrient removal possible in MBRs compared to conventional activated sludge processes (CAS). MBRs are usually operated under longer sludge retention time (SRT) and at higher MLSS concentrations this generates a low food to microorganism (F/M) ratio. At low F/M ratio, microorganism will use energy from substrate uptake on cell maintenance instead of growth, hence reducing the sludge volume (Van Loosdrecht and Henze, 1999). High MLSS concentrations reduce the necessary tank size, despite relatively long solids retention times (SRTs). Furthermore, MBRs have a higher tolerance to filamentous bacteria and foaming, which cause serious operational problems in CAS plants (Daigger *et al.* 2005; Le-Clech *et al.* 2006; Kraume and Drews 2010; Judd 2011).

Despite the many advantages of the MBR technology, there is one big obstacle for wider application of MBRs, which is membrane fouling (see section 1.2). Membrane fouling makes MBRs operationally more complex than CAS systems and energy costs are still higher for MBR plants due to the need of membrane cleaning. The energy demand for minimizing fouling has become the main contribution to the overall operating costs and it has been estimated that membrane aeration accounts for up to 60-70% of the total energy costs (Kraume and Drews 2010; Judd 2011).

1.2. MEMBRANE FOULING

Membrane fouling is defined as coverage of the membrane surface by accumulating and/or adsorbing deposits and can be thought of as adding an additional resistance to filtration in the form of an increase in the membrane resistance. Many studies have been performed to unravel the mechanisms by which material deposits end up fouling the membrane and hence impairing the permeate filtration (e.g. Drews 2010; Meng *et al.* 2009; Ahmed *et al.* 2007).

In MBRs, inorganic and organic compounds foul the membranes, reducing their permeability and filtration performance. This lead to higher energy consumption, increased need for chemical cleaning, and shorter membrane lifetime. Activated sludge contains a range of potential foulants, such as microbial flocs, single cells, microbial metabolic products in the form of free and/or bound extracellular polymeric substances (EPS) and soluble microbial products (SMP). These may all contribute to biofouling, and will in the following be addressed as just fouling. EPS, and most significantly SMP, in both free form as well as bound to/in flocs have been argued to be important foulants, and the concentrations of these compounds should be limited to avoid severe fouling of the membranes (Chang and Lee 1998; Trussell *et al.* 2006; Drews 2010; Liang *et al.* 2007; Meng *et al.* 2009; Ahmed *et al.* 2007; Hong *et al.* 2014). Other studies indicate that MLSS levels (Lousada-Ferreira *et al.* 2010), floc size (Wisniewski and Grasmick 1998), number of filamentous bacteria (Meng *et al.* 2006) and concentration of

[1] MLSS concentration for MBRs is 10-20 g/L, whereas 3-5 g/L is typical for traditional activated sludge (Le-Clech *et al.* 2006).

polyvalent cations (Arabi and Nakhla 2009; van den Broeck *et al.* 2011) are important for the fouling propensity and performance of MBRs. In the case of MLSS and number of filamentous bacteria, there is some inconsistency in literature whether higher MLSS concentrations and higher abundances of filaments tend to increase fouling or not (Fane *et al.* 1981; Lousada-Ferreira *et al.* 2010; Meng *et al.* 2006).

Membrane fouling can be either due to passive transport of material from the feed suspension to the membrane or due to microbial growth on the surface of the membrane. The mechanisms of membrane fouling is very complex as the different compounds in activated sludge will interact with each other and the membrane in different ways, making it difficult to identify the foulants. The three main fouling mechanisms are presented in Figure 3, and involve (i) cake formation, (ii) pore blocking and (iii) biofilm formation (Stephenson *et al.* 2000; Judd 2011).

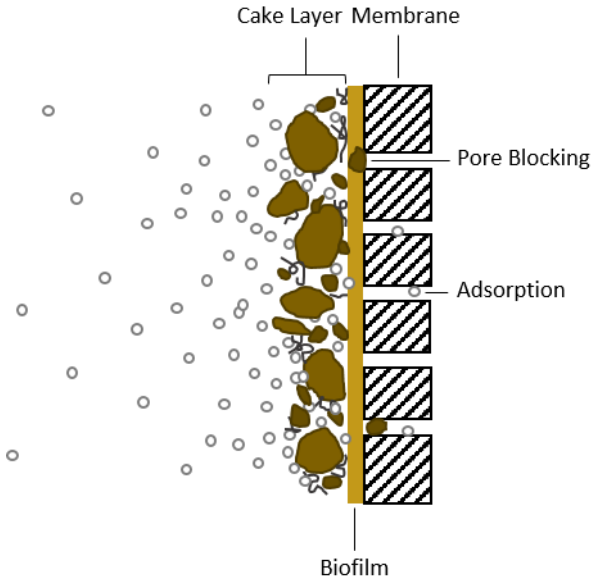


Figure 3: *Fouling mechanisms. (i) cake formation, (ii) pore blocking and adsorption and (iii) biofilm formation.*

Cake formation relates to material with a size larger than the pore size, such as bacterial cells and flocs. They accumulate due to convective transport towards the membrane surface (Le-Clech *et al.* 2006) where they also grow and metabolize. According to Lee *et al.* (2001) and Christensen *et al.* (unpublished), cake layer is considered the major contributor to membrane fouling in MBRs treating wastewater. The formation and behaviour of the cake layer is affected by the flux, crossflow, particle size, and electrostatic interactions (particle-particle as well as particle-membrane) (Le-Clech *et al.* 2006; Ripperger and Altmann 2002). Pore blocking relates to materials with a size comparable to or smaller than the membrane pores and arises when single bacterial cells and colloidal materials deposit in the pore openings completely blocking the pores. This reduces the effective membrane area as well as the permeate flow through the membrane (Le-Clech *et al.* 2006; Meng *et al.* 2009). The term “biofilm” is generally thought of as microorganisms embedded in a self-produced EPS matrix (Flemming and Wingender

2010) and the formation is proposed to occur though several mechanisms involving attachment of SMP onto the membrane, attachment of bacteria and formation of a complex matrix of EPS. The formation of a biofilm does not appear immediately after filtration and is more likely to influence the long-term operation of MBRs (Judd 2011). However, in literature, SMP and single cells are stated to be components that contribute the most to fouling in both short- and long-term operations (Wisniewski and Grasmick 1998; Wang *et al.* 2008; Christensen *et al.*, unpublished).

In general, fouling is considered as either removable, irremovable or irreversible (Meng *et al.* 2009; Le-Clech 2010). Removable fouling is caused by loosely attached foulants, such as flocs and suspended solids, and can be eliminated by physical cleaning (e.g. backwashing, relaxation or aeration). Irremovable fouling is caused by pore blocking and stronger attached foulants and requires chemical cleaning to be eliminated. Irreversible fouling is caused by microorganisms that produce EPS adhering to the membrane and cannot be removed by any means and therefore calls for membrane replacement (Meng *et al.* 2009; Le-Clech 2010). Figure 4 illustrates the concepts of the different types of fouling.

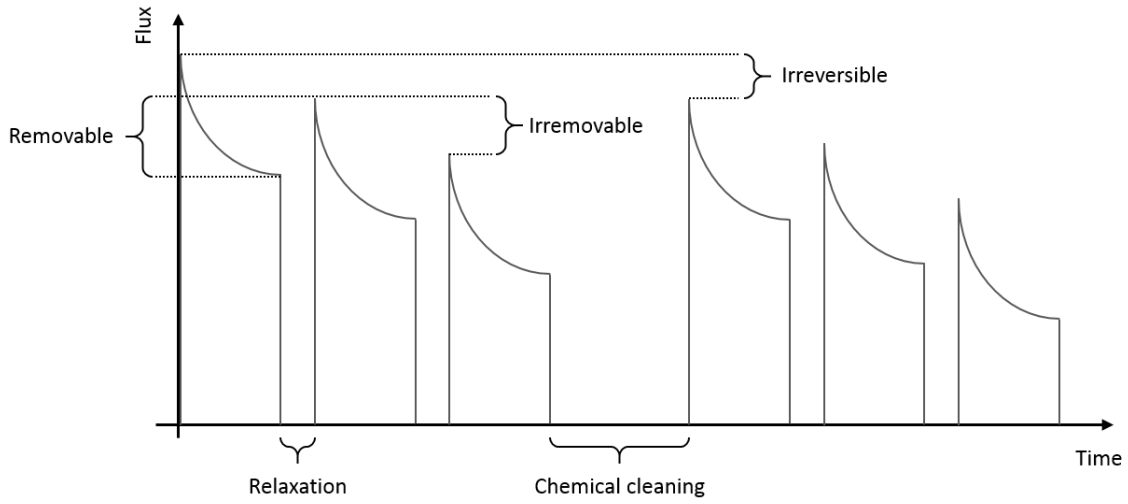


Figure 4: *The concepts of the different types of fouling.*

The cake formation mechanism is considered reversible in short-term operations, however, on a long-term basis, the formed cake layers are unable to be removed by physical cleaning (Meng *et al.* 2009). In submerged MBRs, the mechanism of pore blocking is often considered irreversible because the soluble and colloidal material adhering to the pore walls cannot be removed during relaxation or by air scouring (Meng *et al.* 2009). When describing filtration, the accumulated, irremovable layer on the membrane is caused by biofilm formation.

Several studies have been focusing on the fouling layer in MBRs treating wastewater. However, there seem to be an inconsistency in the published literature regarding the definition of fouling layer. In this thesis, the fouling layer will be defined as consisting of (i) a gel/biofilm and (ii) a

cake layer. The cake layer is defined as the layer that can be washed off (reversible fouling) whereas the biofilm is more tightly bound to the membrane (irreversible fouling). However, the distinction between the two layers (cake layer and biofilm) is not always simple/clear, and hence, the three terms are often used interchangeably.

1.2.1. FOULING AFFECTING FACTORS AND STRATEGIES TO MINIMIZE FOULING

The causes of fouling have received much interest and many studies and reviews have been carried out within this area (e.g. Chang *et al.* 2002; Le-Clech *et al.* 2006; Meng *et al.* 2009). However, due to various ways of operating MBRs as well as the variations in sludge composition, no consensus has been reached on what is the cause of membrane fouling. Many factors have been proposed to potentially influence membrane fouling. In general, they can be divided into three major groups:

- **Membrane characteristics:** pore size and distribution, porosity, roughness and hydrophobicity/hydrophilicity of the membrane.
- **Operating conditions:** aeration, sludge retention time (SRT), hydraulic retention time (HRT), food-to-microorganisms (F/M) ratio and constant transmembrane pressure (TMP) or flux operation.
- **Sludge or biomass characteristics:** MLSS concentration, EPS and SMP concentrations, particle size, viscosity, temperature and particle surface charge.

The specific factors influencing membrane fouling in MBRs and their interrelations are summarised in Figure 5. As illustrated, many of these factors are largely interrelated. For example, the rate at which sludge is withdrawn controls the SRT, which then determines the bacterial speciation and the concentration of biomass (MLSS). The MLSS concentration then impacts physical properties such as viscosity and oxygen transfer rate (OTR) which is a marker for bioactivity.

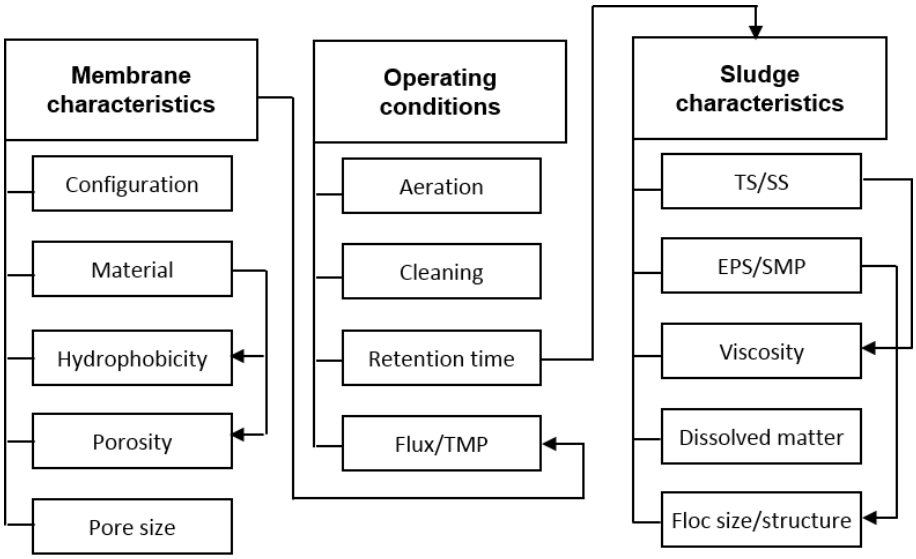


Figure 5: *Various interrelated factors influencing membrane fouling in MBRs. Modified from Chang et al. (2002).*

The process of keeping membrane fouling at a minimum can be costly and often results in system downtime. Therefore, fouling in MBRs has been reviewed several times over the years, summarising novel process configurations, insight into the occurring phenomena and pointing out innovative ways to combat fouling (Le-Clech *et al.* 2006; Meng *et al.* 2009; Kraume and Drews 2010; Meng *et al.* 2017). Much research has been performed to minimise membrane fouling by changing parameters such as membrane hydrophobicity, short solids retention time, carbon sources, etc. (Ahmed *et al.* 2008; Duan *et al.* 2009; Meng *et al.* 2009). Other control strategies include addition of carriers and low flux operation. However, various ways of designing and operating MBRs (type of wastewater, MBR configuration, membrane material and configuration, feed water characteristics), result in different sludge characteristics as well as fouling propensity and behaviour. Hence, the introduction of these more targeted control strategies might not necessarily be used universally. More general control strategies are air scouring of the membrane, backwashing and relaxation. Furthermore, most control strategies for minimising fouling exploit a variety of chemical, mechanical or hydrodynamic means and to a lesser extent include knowledge about the microbiology of the bacteria responsible for the sludge characteristics and fouling behaviour. To aid in the direction of new/better control strategies for membrane fouling, knowledge on the microbial composition and behaviour of the fouling layer is required.

1.3. AIM

The overall aim of this project was to obtain better understanding of the microorganisms in the fouling layer in membrane bioreactors and the key parameters/factors that cause membrane fouling in order to provide better basis for fouling control in MBR reactors.

The specific objectives of the project were:

- To optimise parameters (sampling, DNA extraction, primer choice and PCR settings) influencing the observed community composition in activated sludge when using 16S rRNA gene amplicons.
- To reveal the identity, diversity and dynamics of the microbial population in membrane bioreactors associated with membrane fouling.
- To investigate the effect of microbial species composition on sludge properties in relation to membrane fouling in a MBR started up with new sludge.
- To investigate the diversity and abundance of filamentous bacteria, with focus on Chloroflexi, in Danish WWTPs and their role in floc characteristics.

The work presented here is a research project within the Centre for Design of Microbial Communities in Membrane Bioreactors (EcoDesign MBR), supported by Innovation Fund Denmark and a number of partners (http://www.en.bio.aau.dk/ecodesign). The overall goal of the Centre was to determine the identity, function and interactions of key microorganisms in mixed communities involved in selected environmental processes such as removal of nutrients and micropolutants. This understanding was achieved through studies in membrane bioreactors in collaboration with industrial and public partners.

1.4. SYSTEM FOR STUDYING MEMBRANE FOULING *IN SITU*

For the use in the EcoDesign MBR project, Alfa Laval contributed with a pilot-scale MBR located at the conventional full-scale WWTP at Aalborg West, Denmark. Figure 6 shows a diagram of the pilot-scale MBR and Table 1 presents process data. **Paper 2** describes it in more detail regarding operational parameters.

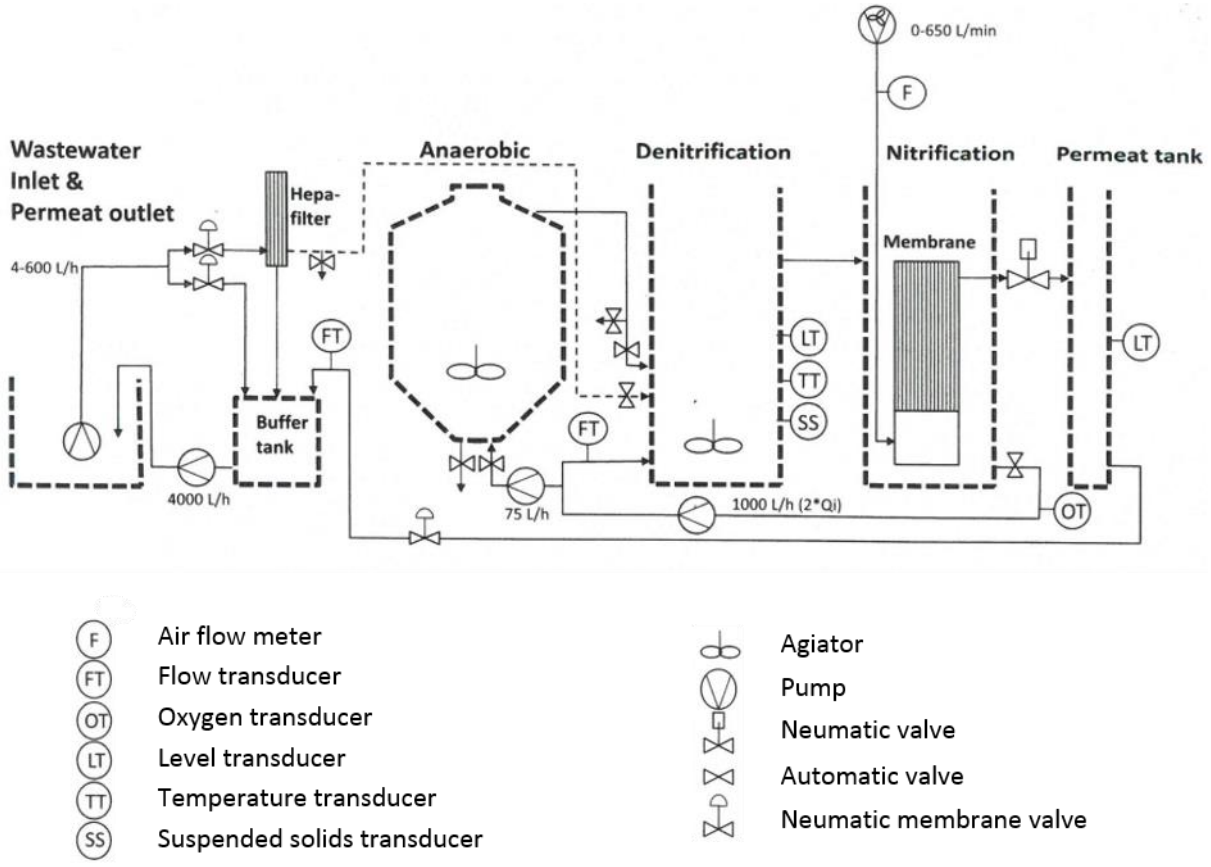


Figure 6: *Pilot-scale MBR used for the in situ studies in this PhD project.*

Pilot scale was chosen because it was possible to mimic a full-scale system better compared to a lab scale reactor. The conditions for the pilot scale MBR were more similar to full scale, especially since it received wastewater from the conventional full-scale plant. Moreover, specific tests or experiments could be conducted due to control possibilities that would not be possible at a full-scale plant, e.g. controlling of flux/TMP and F/M ratio.

The pilot-plant MBR was used in different experiments and in many interdisciplinary projects including this PhD project. It served as a sampling site for MBR activated sludge and fouled flat sheet membranes from a mini-cassette. In this project, membrane fouling was investigated focusing on the role of activated sludge microbial composition and sludge properties. In order to connect the fields of engineering and molecular microbiology, the problem was addressed

by an interdisciplinary approach covering physico-chemical sludge characterisation, microbial ecology studies and applied statistics.

Table 1: *Process data for pilot-scale MBR.*

Permeate production	0.5	m3/h
Aerobic sludge age	15	days
Recirculation flow N/DN	1	m3/h
SS concentration (aim)	8	kg/m3
Design loading	0.05	kg BOD/kg SS * days
Anaerobic recirculation fraction	15	%
Anaerobic HRT	25	h

1.5. CHARACTERISATION OF FOULING LAYERS

For understanding more about parameters determining fouling propensity in membrane bioreactors, examination of physical, chemical and biological properties of the fouling layer on the membrane and identification of major fouling species are key.

1.5.1 Physical characterisation

Physical characterisation relates to the height and compactness of the fouling layers, often described by microscopic investigation, as well as the compressibility and resistance, determined by filtration experiments.

Advanced microscopic methods like scanning electron microscopy (SEM), atomic force microscopy (AFM) and confocal laser scanning microscopy (CLSM) have been used for two-dimensional and three-dimensional visualisation of fouling layers. Investigation by SEM can provide information about surface morphology, indicating the interactions between foulants and the membrane. High resolution SEM images by Fan and Huang (2002) showed a division of the fouling layer in two distinct layers, a gel layer (or biofilm) and a cake layer. AFM is able to show surface roughness and convert it to information about fouling layer compactness (Yu *et al.* 2006). Yu *et al.* (2006) showed that the gel layer was more compact than the cake layer. The same was shown by Chen *et al.* (2006) using CLSM. Fouling layer heights have been estimated to approx. 10-30 µm for the biofilm and up to several mm thick for the cake layer (sludge flocs have an average size of 40-125 µm), see Figure 7 (**Paper 2**; Larsen 2007).

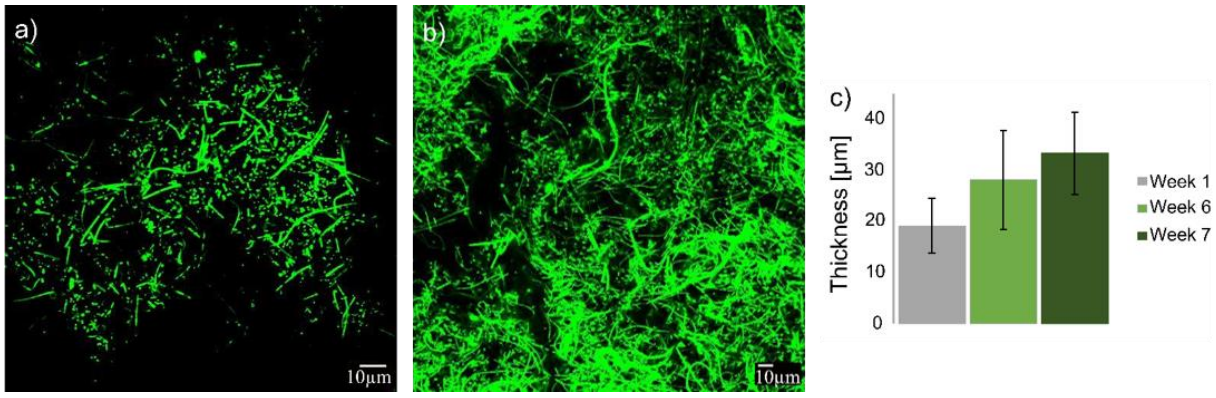


Figure 7: *Microbial cells in the biofilm fouling layer visualised by CLSM and staining with SYTO 9. Picture a) is taken after 1 week of operation and picture b) after 7 weeks. The graph in c) shows the thickness of the biofilm determined by 20 repeated measurements for three diffrent time points. From Paper 2.*

Filtration experiments run as flux/TMP step tests can be used to determine the compressibility and resistance of activated sludge flocs (which shows great resemblance to cake layer). Both compressibility and resistance influence membrane filtration and relies very much on the structure of the sludge flocs/fouling layer (Bugge *et al.* 2013; Jørgensen *et al.* 2017). In **Paper 3**, we showed that the presence of certain bacterial species, especially filamentous Chloroflexi and strong floc-formers like *Dechloromonas, Ca*. Accumulibacter and *Nitrospira* affected sludge compressibility and floc size. *Nitrospira* influenced negatively on sludge compressibility whereas the mean floc size decreased as the abundance of several Chloroflexi increased and the abundance of *Dechloromonas* decreased. Species composition has previously been associated with sludge floc properties (Wilén *et al.* 2000, 2008; Larsen *et al.* 2006; 2008). Sludge with many large flocs generates diffuse fouling layers that are more compressible compared to smaller and more compact flocs that cause more dense fouling layers (Lin *et al.* 2009). Both the cake layer and the gel layer are compressible (Christensen *et al.* 2009; Poorasgari *et al.* 2015). For MBR systems, the compressibility of the fouling layers has a large influence on resistance, hence increasing the pressure (to maintain permeate flux) increases the specific resistance. Cake formation has been shown to give higher filtration resistance and lower process performance (Meng *et al.* 2009), however, at the same time, it may also serve as a protective layer for the membrane limiting irreversible pore blocking (Giraldo and LeChevallier 2007). Lin *et al.* 2009 showed that the filtration performance in terms of filtration resistance was better for sludge with larger flocs (lower resistance) compared to sludge with smaller flocs, i.e. giving lower resistance. Smaller flocs form more compact and less porous fouling cake layers. Hence, forming a thin cake layer with a low resistance can improve MBR performance by limiting irreversible fouling. In more recent studies, activated sludge has been divided into fractions of flocs, colloids and solutes in order to determine the fouling resistance of each component. Christensen *et al.* (unpublished) showed that smaller particles (solutes and colloids), for short term filtration (60 min at different TMPs), gave higher resistance than flocs forming a cake layer. The filtration of sludge maintained a high flux at increasing TMPs as seen in Figure 8.

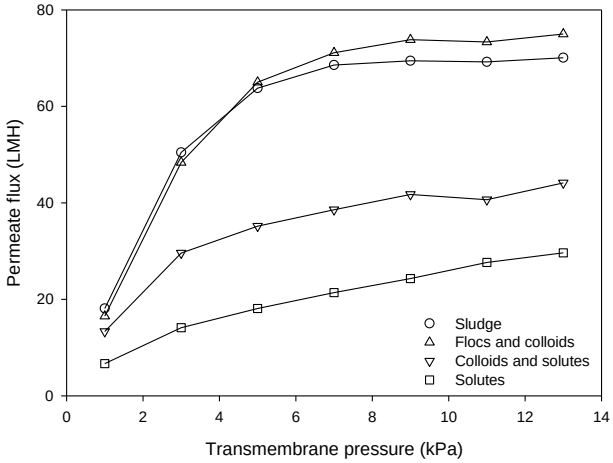


Figure 8: *Cross flow filtration of the different sludge fractions. Christensen et al. (unpublished).*

1.5.2 Chemical characterisation

Chemical characterisation of fouling layers entails determining the chemistry of the different inorganic and organic matters both qualitatively and quantitatively. The many different types of foulants were mentioned in section 1.2. Analysis of the composition of EPS, which constitutes up to 40-60% of the total organic matter in activated sludge (Nielsen 2002), was approached by cationic extraction by Frølund *et al.* (1996). Chromatography-based approaches have also been used for characterising organic matter in activated sludge (Wang and Wu 2009; Ni *et al.* 2009; Al-Halbouni *et al.* 2008). EPS and SMP may be produced and excreted by microorganisms or originate from cell lysis or unmetabolised wastewater components. They are a heterogeneous mixture consisting of polysaccharides, proteins, lipids, nucleic acids, etc. and extraction using different methods provides different results (Frølund *et al.* 1996; Comte *et al.* 2006; 2007). This makes comparison of results from different studies difficult and hence extraction free methods, like microscopic investigation, are preferred. CLSM is a powerful tool for revealing the identity and biovolume of foulants when combined with specific fluorescence probes and image analysis software. Several fluorescence probes have been designed targeting the different membrane foulants making it possible to differentiate between them and show their spatial distribution (Hwang *et al.* 2007) and can be applied simultaneously.

1.5.3 Biological characterisation

Even though the biomass (cells) is estimated to constitute only 10-20 % of the organic fraction in activated sludge (Nielsen 2002; Frølund *et al.* 1996), microorganisms play an important role in the composition, structure and properties of activated sludge flocs and fouling layers. Hence, characterisation of the biological (microbial) fraction is, if not more, then, just as important as the physical and chemical characterisation. However, in many studies it has not been included, partly because it was out of scope and partly because the methods have not been available.

Biological characterisation of fouling layers relates to the identity and activity of microorganisms as well as their spatial distribution. Traditionally, microbial identification classification has been studied by isolating and culturing in laboratories combined with microscopic characterisation. General microscopic characterisation of bacteria includes description of size and form, recognition of different morphotypes of filamentous bacteria and their quantities. Several manuals on microscopic characterisation of activated sludge have been written (Eikelboom 2000; Jenkins *et al.* 1993; Seviour and Nielsen 2010). Even though much of our current knowledge about microorganisms has been obtained this way, these methods suffer from various limitations. Isolation and culturing of bacteria is time consuming and it is assumed that less than 1-15% of the microbial population in activated sludge is culturable (Amann *et al.* 1995), hence the throughput is very low. Morphological characterisation is for many bacteria not possible because their morphologies are too similar. Some filamentous bacteria have very distinct morphologies and may be identified using morphological characterisation (Seviour and Nielsen 2010). However, morphotypes which were previously thought to belong to one particular species have been shown to cover very different bacteria, even from two different phyla (Speirs *et al.* 2015; McIlroy *et al.* 2016).

Within the last 25 years, culture independent methods that can be applied to samples taken directly from the ecosystem has been developed (Wagner *et al.* 1993). Many of these, including core methods like fluorescence in situ hybridization (FISH) and high-throughput amplicon sequencing, rely on the 16S ribosomal RNA (rRNA) genes. In the FISH method, short oligonucleotide probes with a fluorescent tag are used to target the phylogenetic distinct region in the ribosomal RNA of bacteria. This has been used for *in situ* identification in various complex ecosystems including activated sludge and fouling layers (Wagner *et al.* 1993). The FISH procedure is illustrated in Figure 9. Over the years, several methods have been derived from the original FISH method, and are able to provide details of microbial ecophysiology and activity. Examples of such methods are microautoradiography (MAR)-FISH (substrate utilization and activity) (Lee *et al.* 1999), enzyme-labelled fluorescence-FISH (exoenzyme expression) (Nielsen *et al.* 2009), microsphere adhesion to cells-FISH (surface properties) (Nielsen *et al.* 2009), Raman-FISH microspectroscopy (detection of cellular components (Brehm-Stecher and Johnson 2004) and incorporation of labelled substrates (Huang *et al.* 2007)). These microscopy/FISH based techniques are tedious and the design of FISH probes is a challenge in terms of achieving the optimal sensitivity (level of fluorescent signal obtained from hybridized target cells as compared to the background fluorescence of non-targeted cells) and specificity (differentiation between targeted and non-targeted but closely related organisms) and is based on prior knowledge of the target organism. Furthermore, they cannot show the full complexity of the microbial community.

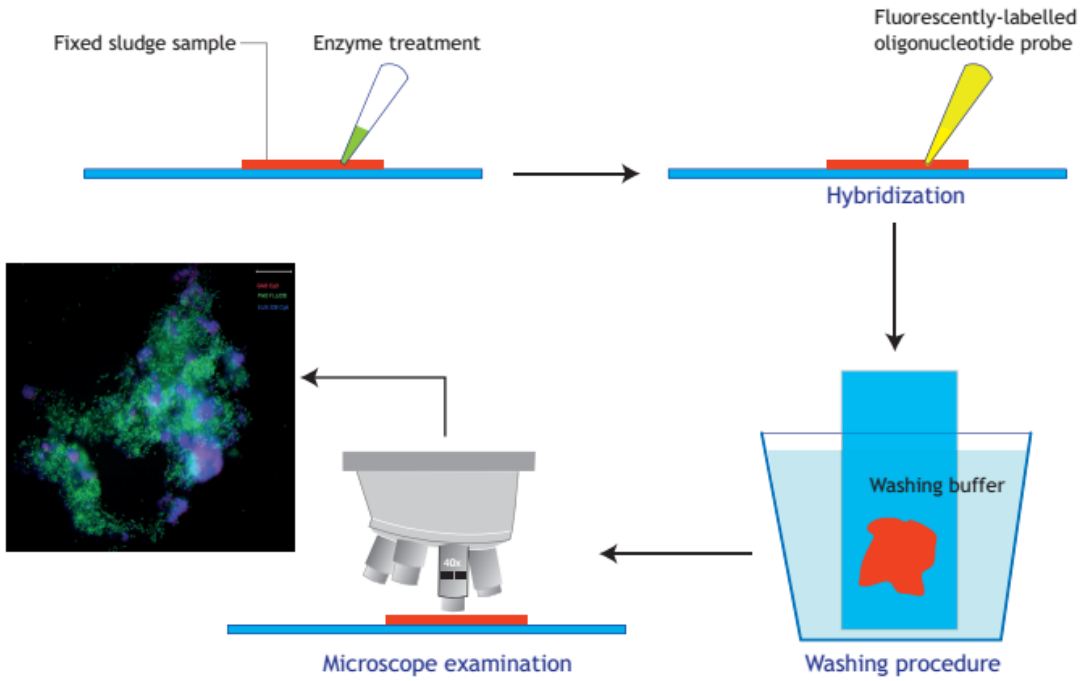


Figure 9: *Illustration of the FISH procedure. From (van Loosdrecht et al. 2016).*

Today, 16S rRNA amplicon sequencing is one of the core tools for studying complex microbial communities in environmental ecosystems. With this technique, it is possible to analyse hundreds of samples, producing millions of sequences from each, only within a matter of days (Caporaso *et al.* 2012). Figure 10 summarises the 16S rRNA amplicon sequencing procedure from DNA extraction to taxonomic and functional classification.

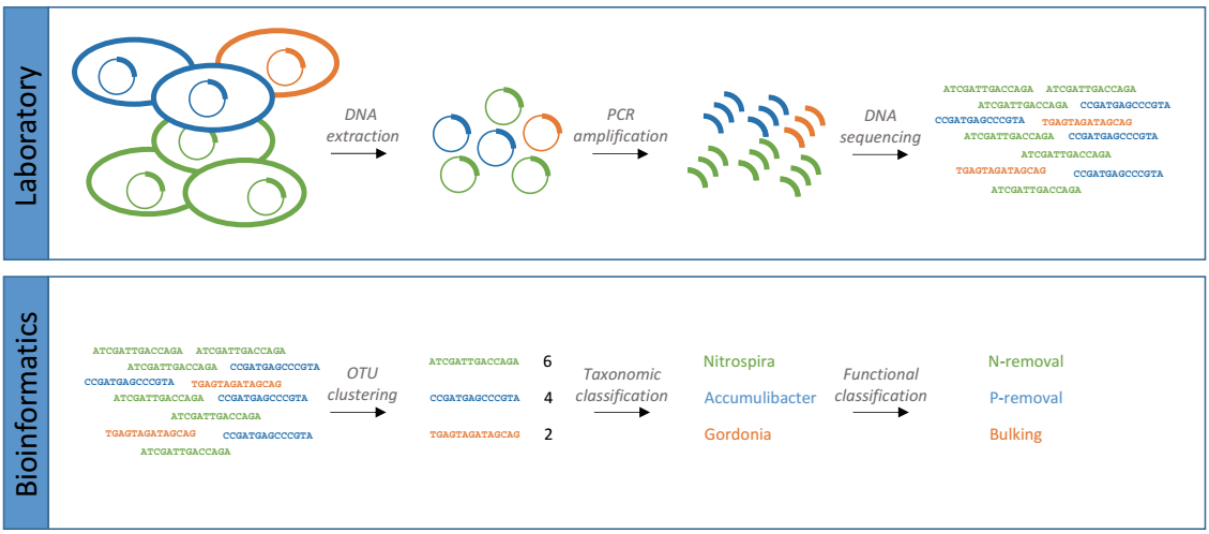


Figure 10: *Conceptual overview of the steps involved in 16S rRNA amplicon sequencing for analysis of microbial communities. From (van Loosdrecht et al. 2016).*

Even though the 16S rRNA amplicon sequencing approach is widespread, is suffers from critical shortcomings. These have been addressed in **Paper 1** where we carried out a comprehensive study on the effect of e.g. DNA extraction method and PCR primers on the observed microbial community structure in activated sludge. Increasing the bead beating intensity for DNA extraction, compared to recommendations in the standard protocol, resulted in dramatic increase in relative abundance of Actinobacteria and Alphaproteobacteria, which are particularly difficult to lyse. Optimising the bead beating step can be a good idea if for example many Gram-positive bacteria are present in order to avoid underrepresentation of these bacteria. Since different primers amplify different regions of the 16S rRNA gene, the observed microbial community structure using the V1-3, V3-4 and V4 primers was very different, and none of them similar to the PCR-free methods. However, the V1-3 primer set was able to cover the same range of phyla as the obtaines sludge metagenome and was best at capturing the phyla Chloroflexi and Actinobacteria. These phyla are of special interest as they contain groups that are important in the activated sludge process, such as *Tetrasphaera* involved in biological P-removal and filamentous Chloroflexi important for floc structure and known for causing bulking (Mielczarek *et al.* 2012).

Proper taxonomic classification is crucial when studying microbial community structures. In taxonomic classification each sequence is compared to reference sequences in a database and inherits the taxonomy of the reference sequence, providing them with a name and biological information, when/if a match. The most applied public databases are Greengenes (McDonald *et al.* 2012), RPD (Cole *et al.* 2014) and SILVA (Quast *et al.* 2013). These are all based on different reference sequences and curated by different experts. Furthermore, some references simply lack taxonomic information (Werner *et al.* 2012). McIlroy *et al.* (2015) has compared the % of classification at various phylogenetic levels for the three most applied databases and the updated and manually curated MiDAS database (Microbial Database for Activated Sludge). This clearly shows the lack of genus level classification in the Greengenes (38%), RDP (53%) and SILVA (49%) databases compared to the MiDAS (91%) database. **Paper 1** and the study by McIlroy *et al.* (2015) states that the quality and quantity of microbial identification is affected by the choice of (i) DNA extraction method, (ii) primers (iii) PCR settings and (iv) taxonomic classifier. On http://www.midasfieldguide.org optimised protocols for microbial community analysis of activated sludge can be found. They are based on recommendation made from the studies in **Paper 1** and McIlroy *et al.* (2015). All of the community analyses in this PhD project was carried out according to these recommendations.

Many aspects of membrane fouling cannot be fully explained by one technique alone. The use of 16S amplicon sequencing and FISH in combination proved to be a strong tool for biological characterization. However, both methods have their limitations and strengths. 16S rRNA amplicon sequencing is a high-throughput method that generates a lot of information, but, the method is disruptive to the fouling layer (the biomass has to be removed from the membrane), and hence FISH should be applied when spatial or in situ information is of interest.

1.6. MICROBIAL COMMUNITIES AND MEMBRANE FOULING

Bacteria are the workhorses of activated sludge. They are involved in degradation of organic matter and removal of nutrients (nitrogen and phosphorus) and pollutants. During these processes, the bacteria produce a wide range of EPS that enables them to clump together, forming aggregates or flocs (Figure 11), and to immobilised surfaces like the membranes in MBRs. The activated sludge microbial community consists of 10^{11}-10^{12} cells g^{-1} wet weight (Frølund *et al.* 1996) comprised of many species. Due to a high complexity and biodiversity within the ecosystem, the majority of the activated sludge bacteria are unknown (Saunders *et al.* 2016). Only a fraction of these have been characterised and assigned a function, e.g. *Nitrospira* involved in ammonia oxidation (Gieseke *et al.* 2005) and *Ca.* Accumulibacter involved in phosphorus removal (Crocetti *et al.* 2000). Besides biological conversion, each of the activated sludge bacteria has their significance on floc characteristics and membrane fouling, e.g. by EPS production (Wilén *et al.* 2008; Larsen *et al.* 2008; Bugge *et al.* 2013; Ziegler *et al.* 2016).

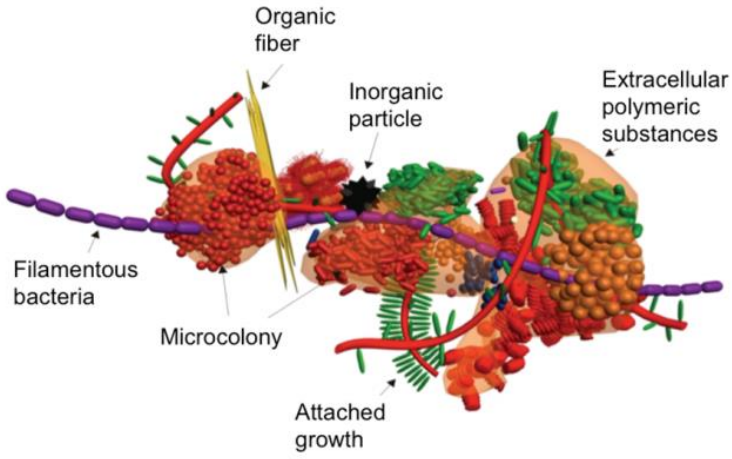


Figure 11: *Activated sludge floc. Microcolonies attach to filamentous bacteria which form the backbone of the floc. EPS contribute to the embedding matrix. (Nielsen et al. 2012)*

For operating MBRs, it is key to obtain information about which bacteria have “good”, “bad” or no impact on membrane fouling/filtration. Expanding our knowledge of these “good” and ”bad” bacteria in terms of ecology and physiology, the microbial community within MBR systems may be manipulated for selection of “good” bacteria that are associated with less membrane fouling. Potentially all bacteria in activated sludge can end up at the membrane, hence basic studies on the microbial community compositions in both the bulk sludge and the fouling layer are key. Many studies have focused on unravelling the significance of bacteria on membrane fouling (Wu *et al.* 2011; Molina-Muñoz *et al.* 2009; Gao *et al.* 2011; Huang *et al.* 2008; Jinhua *et al.* 2006; Miura *et al.* 2007a). The general understanding is that the microbial community of the bulk sludge and the fouling layers are different, with specific bacteria

preferentially growing on the membrane surface environment, e.g. members of the Proteobacteria (Jinhua *et al.* 2006; Miura *et al.* 2007a; Huang *et al.* 2008).

Generally, there are two issues with most of these studies. One is that they often apply lab-scale reactors with artificial wastewater and special conditions that are rarely typical for full-scale systems. This makes it difficult to transfer the results to full-scale systems. Another issue is that, these studies have discussed community identity and dynamics at the phylum and class levels. Such observations are of questionable value given the phenotypic diversity encompassed by the higher-level phylogenetic groupings (as mentioned in section 1.5.3), this is especially true for the phylum Chloroflexi that constitutes up to 26% (mean average 23.3%) of the biomass in MBR bulk sludge and 38% (mean average 25.7%) in fouling layer biofilm (**Paper 2**).

Using MiDAS taxonomy and 16S rRNA amplicon sequencing, we investigated the entire MBR community (bulk sludge and biofilm), providing details of the dynamics of most potentially relevant microbes present. Similar to previous studies, we showed that the microbial community of the fouling layer was different from the one in the bulk sludge. This difference was most pronounced in the early fouling layer and, interestingly, as the fouling layer evolved, the microbial communities became more similar (Figure 12).

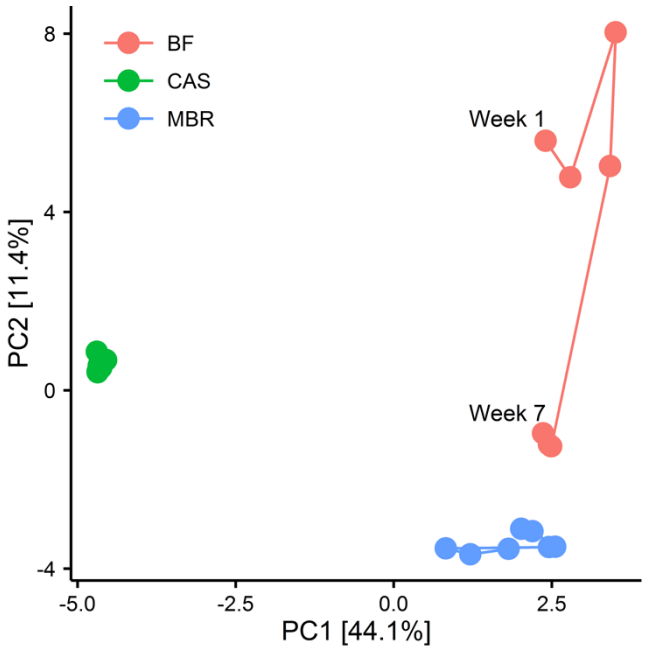


Figure 12: *PCA plot showing overall differences in microbial communities in CAS bulk sludge samples (CAS, green), MBR bulk sludge samples (MBR, blue) and biofilm samples (BF).*

Furthermore, the filamentous Chloroflexi and *Gordonia* were enriched in the fouling layers of MBRs (**Paper 2**). This indicates that even though some degree of selection/enrichment of bacteria occurs in the fouling layer, the composition of the bulk sludge community will have a larger effect on maturing the fouling layer. Based on this, the selection for the "good" bacteria

should be focused more on the composition of dominant species in bulk sludge to improve fouling propensity and not pioneer species as proposed by Zhang *et al.* (2006).

1.7. CHLOROFLEXI IN ACTIVATED SLUDGE AND MEMBRANE FOULING

The findings in **Paper 2** showed that filamentous Chloroflexi constitute a large fraction of the bacteria present in MBR treating wastewater. They were dominant both in MBR bulk sludge as well as in mature biofilm, where they constituted over 23% and 25% of all bacteria present, respectively. Opposite to CAS sludge, Chloroflexi were the most abundant phylum in both types of MBR samples (Figure 13).

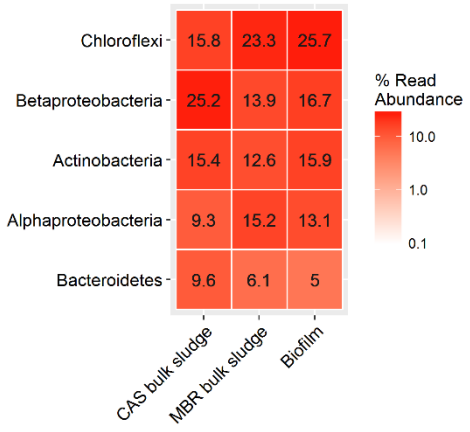


Figure 13: *Heatmap showing the relative abundance of the five most abundant bacterial phyla in CAS bulk sludge, MBR bulk sludge and biofilm. Data from Paper 2.*

In recent studies, the diversity of Chloroflexi species has been elucidated and several novel genera have been identified, including *Ca*. Promineofilum (other names: B45, type 0092) (Speirs *et al.* 2009; McIlroy *et al.* 2016), *Ca*. Amarilinum (type 0092) (Nierychlo *et al.* unpublished), *Ca*. Defluviifilum (type 0803) (Kragelund *et al.* 2011; Speirs *et al.* 2015), *Ca*. Villogracilis (Nierychlo *et al.* unpublished) and *Ca*. Sarcinathrix (type 0914) (Speirs *et al.* 2011; Nierychlo *et al.* unpublished). All these genera are present in activated sludge, both in CAS and MBR systems, as well as in the biofilm (Figure 14).

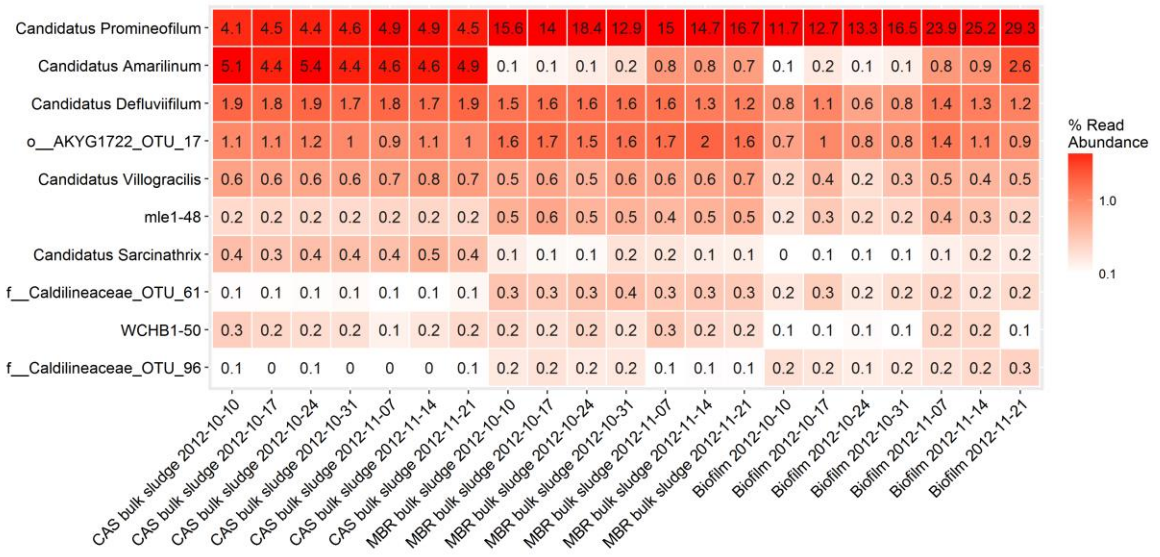


Figure 14: *Heatmap showing the relative abundance of the ten most abundant Chloroflexi genera in CAS bulk sludge, MBR bulk sludge and biofilm. Data from Paper 2.*

Filamentous Chloroflexi play an important role in activated sludge where they contribute to the formation of the structural backbone of flocs (Seviour and Nielsen 2010). Furthermore, operational problems due to sludge bulking has been proposed to be caused by the overgrowth of several filamentous bacteria, including Chloroflexi (Liao *et al.* 2004; Lou and De los Reyes 2005a, 2005b; Martins *et al.* 2004; Vervaeren *et al.* 2005). Interestingly, the findings in **Paper 4** and Nierychlo *et al.* (unpublished) illustrate that the type of filament, especially the type of Chloroflexi present, is of importance as their effect on sludge settleability seem to vary and some are more likely to be the cause of poor sludge settling leading to bulking. Findings from **Paper 2** (Figure YY) show that *Ca.* Amarilinum is abundant in the CAS system, but not in the MBR system. The study in **Paper 2** was carried out in a period where the full scale WWTP Aalborg W experienced problems with poor settling sludge (**Paper 4**). In the MBR, *Ca.* Promineofilum was the dominating Chloroflexi genus.

In **Paper 3**, we showed that the mean sludge floc size decreased immediately after introduction into the MBR system. This drop was accompanied by an increase in the abundance of *Ca.* Promineofilum, but it was not possible to correlate these two coincidences. The findings in **Paper 3** suggest that Ca. Amarilinum is capable of forming larger flocs than Ca. Promineofilum, however, in CAS bulk sludge Ca. Amarilinum was correlated with poor sludge settling (**Paper 4**). A higher concentration of Ca. Amarilinum resulted in less flocculated sludge with poor settleability. This underlines that Ca. Amarilinum is detrimental for good flocculation.

The presence of Chloroflexi does not determine the floc properties alone. Filamentous *Ca.* Microthrix and strong microcolony formers like *Nitrospira*, *Ca.* Accumulibacter and

Dechloromonas also positively affect floc size and strength and are associated with good settling sludge (Bugge *et al.* 2013; **Paper 3**).

The reported effect of filamentous bacteria on membrane fouling is ambiguous. Several studies claim that they contribute to the increased fouling (Gil et al 2011; Kim and Jang 2006; Meng et al 2006, 2007; Su et al 2011; Tian et al 2011), while others find negligible effect (Al-Halbouni et al 2008; Li et al 2008; Parada-Albarracín et al 2012), and some even find that membrane filtration was improved due to degradation of dissolved EPS by certain filamentous species (Miura et al 2007b; Wang et al 2010).

Figure 14 (**Paper 2**) show that *Ca.* Promineofilum was enriched in the fouling layer compared to MBR bulk sludge, but in general the same Chloroflexi genera were found in MBR bulk sludge and biofilm. Based on the findings in **Paper 2, 3 & 4** we suggest that *Ca.* Promineofilum may play an important role in membrane fouling, potentially leading to a decrease of floc size that results in higher specific resistance of the fouling layer.

Furthermore, the findings underline that the presence of *Ca.* Amarilinum is detrimental for good flocculation. However, in MBR systems they do not seem to contribute much to membrane fouling as they were not dominating the biofilm community and were significantly less abundant than in CAS sludge. McIlroy *et al.* (2016) showed that Ca. Promineofilum is able to degrade sugars; further physiological characterisation could illuminate the role of this genus in membrane fouling. For CAS systems, *Ca.* Amarilinum could be a subject for further physiological investigation/characterisation in order to develop control strategies in plants struggling with bulking caused by this bacterium.

1.8. CONCLUSIONS

An improved method for community structure analysis using the 16S rRNA amplicon sequencing approach was developed and tested in activated sludge (**Paper 1**). More rigorous physical lysis was implemented and PCR primers targeting the V1-3 region of the 16S rRNA gene were found to best target key bacteria in activated sludge. The 16S rRNA amplicon sequencing approach was already used in a wide range of studies on activated sludge from wastewater treatments plants (CAS and MBR), and used as the standard protocol in the MiDAS fieldguide initiative (www.midasfieldguide.org/).

The identity, diversity and dynamics of the microbial population in a membrane bioreactor associated with membrane fouling were revealed (**Paper 2**). The microbial community structure of MBR bulk sludge and fouling layer in a pilot-scale MBR system treating real wastewater was compared using 16S rRNA amplicon sequencing and fluorescence *in situ* hybridization (FISH). The analysis revealed that, in agreement with other studies, the microbial community in fouling layers was initially different from that in the bulk sludge (**Paper 2**). However, as the fouling layer evolved, the microbial communities became more similar. Filamentous Chloroflexi were highly abundant in the MBR bulk sludge and fouling layer. Comparison with CAS bulk sludge showed that different genera of Chloroflexi may dominate in MBR systems and CAS systems (**Paper 2**). Furthermore, different Chloroflexi genera are potentially involved in floc formation in CAS and MBR activated sludge. *Ca.* Amarilinum was dominant in CAS sludge where it was correlated with floc formation and poor sludge settling (**Paper 4**), but it does not seem to cause problems in MBR in relation to membrane fouling (**Paper 2 & 3**). In the pilot-scale MBR, *Ca.* Promineofilum was associated with smaller mean floc size compared to CAS activated sludge (**Paper 3**) and was even enriched in the biofilm layer on the membrane (**Paper 2**).

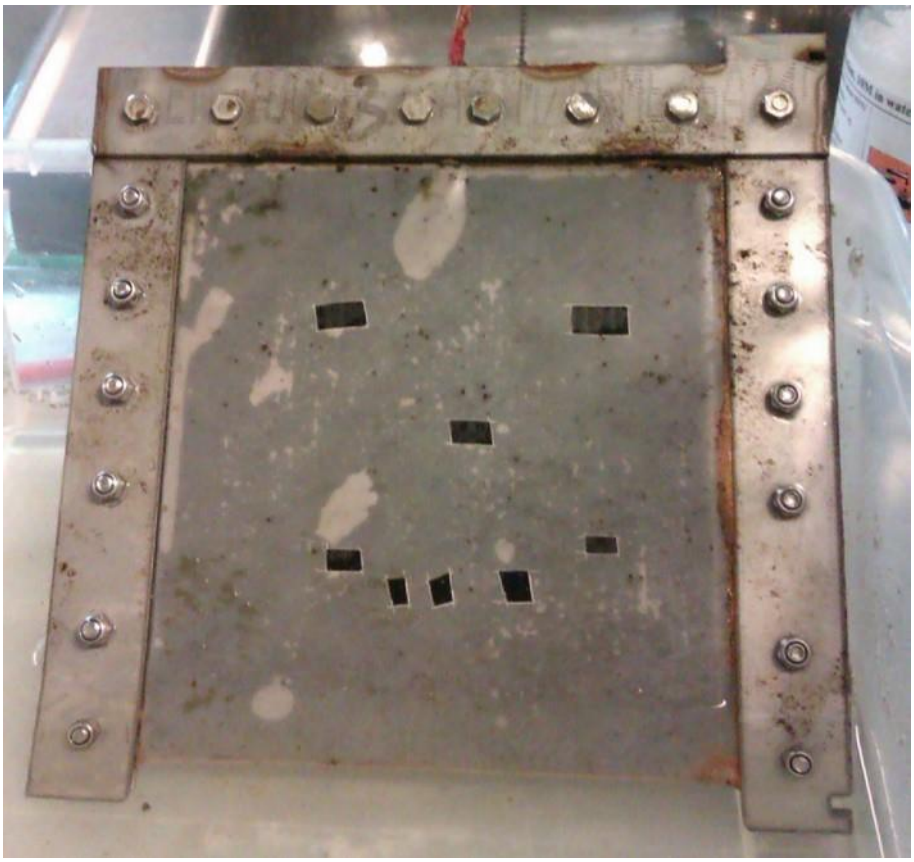
Floc properties (degree of flocculation and size), determined by microbial community composition, have great impact on the nature of the bulk sludge and the fouling layer (**Paper 3**). In a two-year study with several start-up periods of a pilot-scale MBR with new sludge, immediate changes occurred for mean floc size and sludge compressibility. The precesence/absence of Chloroflexi and strong microcolony formers like *Dechloromonas* and *Ca*. Accumulibacter affected (was correlated to) the mean floc size causing smaller flocs. Chloroflexi and *Nitrospira* influenced negatively on sludge compressibility. Using Pearson correlation analysis it was possible to identify bacteria that promoted good flocs and some that did not. Among good floc formers were the genera *Dechloromonas* and *Ca*. Accumulibacter whereas filamentous Chloroflexi caused poor flocs.

The overall conclusion of this project is that strong flocs are important for good plant operation in MBR systems and common activated sludge bacteria play a significant role in the formation of good flocs. Expanding our knowledge of good/bad floc formers among common core species in terms of ecology and physiology, the microbial community within MBR systems may be manipulated for selection of good floc forming bacteria that contribute positively to membrane fouling.

1.9. PERSPECTIVES

This PhD project has contributed with more basic discoveries of bacteria in fouling layers in MBRs, and has given indications of their significance in floc properties and membrane fouling. Similar to CAS plants, the nature of the flocs influence the operational processes in MBR systems, and the big challenge is still how to obtain (and maintain) good, strong flocs. If we want to be able to select for good floc formers and deselect the bad floc formers it is important to understand the ecophysiology of the different Chloroflexi and microcolony formers. From the 16S rRNA amplicon analysis it is possible to describe the microbial community composition of fouling layers and determine the abundant members, but to learn more about the ecophysiology of the species of interest new FISH probes should be designed to visualise and determine their morphology and physiology *in situ*, e.g. by combining FISH with microautoradiography (MAR). With MAR-FISH the eco-physiology of bacteria can be studied and factors controlling their growth elucidated. However, the procedure for MAR-FISH is slow and with the high complexity within the MBR ecosystem, it will be a tedious task to cover all microorganism in fouling layers and MBR bulk sludge. Contrary to the tedious MAR-FISH procedure, the new high-throughput genomic sequencing methods combined with postgenomics might present an alternative in the very near future. The increase in high-throughput long-read area will enable ecosystem specific genome databases within the next five years. This will radically change our ability to study microbial ecosystems. However, the genomes are only the starting point and the challenge will be to develop high-throughput postgenomic methods such as proteomics and transcriptomics to understand the function and interaction of the bacteria in the fouling layers, e.g. by linking species with production of specific EPS.

Development of more targeted control strategies for membrane fouling has been addressed by numerous studies in relation to physical/chemical manipulation of the membrane and operational modifications aimed at the microbial community in MBRs. However, many of these studies have been carried out without prior knowledge on the identity, physiology and ecology of the fouling-causing bacteria. This may likely be the reason why these approaches have not yet been successful enough to keep membrane fouling at an (economically) acceptable level. There is still a need for more interdisciplinary studies combining microbiology and physical prarameters that affect the floc properties. Manipulative studies in lab scale (simplified systems) where such parameters are more easily controllable could show the road ahead. However, it is still important to go to full-scale MBRs to avoid laboratory biases and maintain close collaboration with operators and engineers. The continuous development of the MiDAS database that now includes activated sludge and digester sludge proves to be a strong tool for both plant operators and microbiologists. Even though CAS and MBR sludge are similar, there may still be unidentified species in MBRs that would expand the database and make it very useful for MBRs too.



Happy flat sheet membrane ☺

Pieces of the membrane was cut out and used for visualisation of microorganisms using confocal laser scanning microscopy.

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CHAPTER 2. PAPER 1

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CHAPTER 3. PAPER 2

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CHAPTER 4. PAPER 3

Start-up of a membrane bioreactor – changes in sludge characteristics and microbial community

Ziegler, AS, Bugge, TV, Jørgensen, MK, Larsen, P, Heinen, N, Christensen, ML, Nielsen, PH

(In preparation)

CHAPTER 5. PAPER 4

Survey of Filamentous Bacteria in Full-scale Municipal BNR Danish WWTPs Reveals Novel Bacteria from Phylum Chloroflexi and *Ca.* Microthrix are Main Responsible for Bulking

Nierychlo, M, McIlroy, SJ, **Ziegler, AS**, Kucheryavskiy, SV; Albertsen, M, Nielsen, PH

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