Bioflocculation of Wastewater Organic Matter at Short Retention Times

Lena Faust

Thesis committee

Promotor

Prof. dr. ir. H.H.M. Rijnaarts

Professor, Chair Environmental Technology

Wageningen University

Co-promotor

Dr. ir. H. Temmink

Assistant professor, Sub-department of Environmental Technology

Wageningen University

Other members

Prof. Dr A.J.M. Stams, Wageningen University

Prof. Dr. I. Smets, University of Leuven, Belgium

Prof. Dr. B.-M. Wilen, Chalmers University of Technology, Sweden

Prof. Dr. D.C. Nijmeijer, University of Twente, The Netherlands

This research was conducted under the auspices of the Graduate School SENSE (Socio-Economic and Natural Sciences of the Environment)

Bioflocculation of Wastewater Organic Matter at Short Retention Times

Lena Faust

Thesis

submitted in fulfillment of the requirements for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus

Prof. Dr M.J. Kropff,

in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

on Wednesday 3rd of December 2014

at 1.30 p.m. in the Aula.

Lena Faust

Bioflocculation of Wastewater Organic Matter at Short Retention Times,

163 pages.

PhD thesis, Wageningen University, Wageningen, NL (2014)

With references, with summaries in English and Dutch

ISBN 978-94-6257-171-6

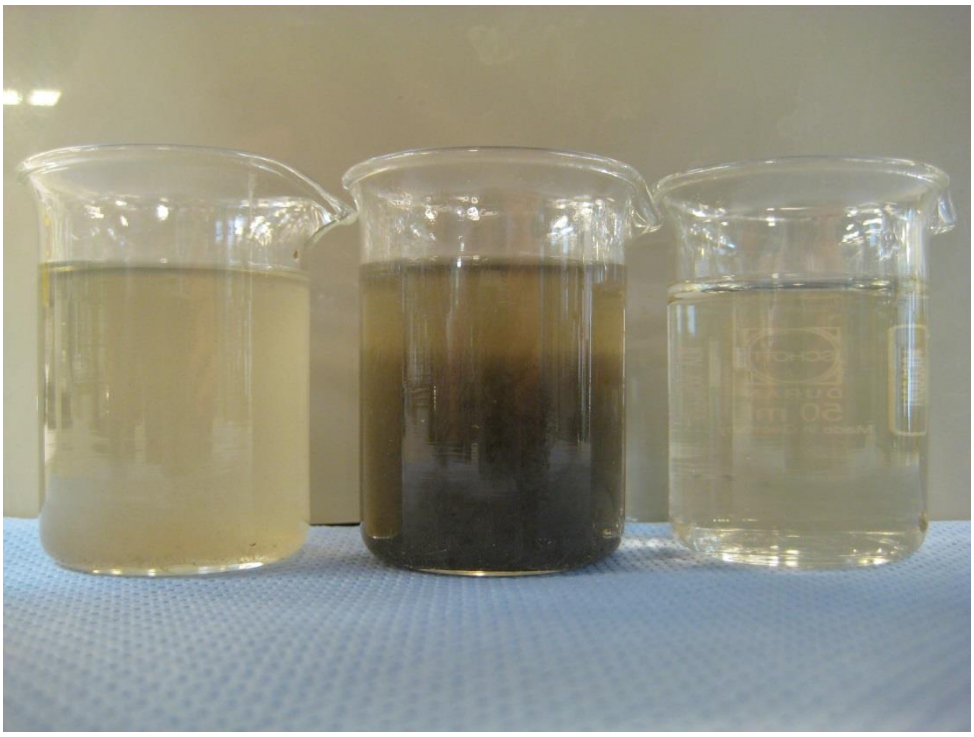
”Da steh ich nun, ich armer Tor,
und bin so klug als wie zuvor.”

Dr. Faust
(in Faust I written by Johann Wolgang von Goethe)

Contents

1

General Introduction



1.1 Wastewater treatment and energy- and chemical recovery

Conventional aerobic wastewater treatment

The aerobic (activated) sludge process has been the most common treatment technology for municipal wastewater during the last decades, mainly because it is robust and provides an effluent quality that meets the discharge guidelines. During this process heterotrophic microorganisms oxidize 50-60% of the (biodegradable) organic pollutants to end products such as CO_2 and water and use oxygen as the (terminal) electron acceptor. This provides the microorganisms with the energy that they need to grow, i.e. to incorporate the other 40-50% of the (biodegradable) organic pollutants into new biomass. The mixture of this biomass and wastewater including organic and inorganic particles usually is referred to as sludge. Under the proper conditions this sludge forms settleable flocs. The sludge and the treated water are separated by sedimentation or (membrane) filtration, after which the treated water can be discharged to surface waters. The separated sludge is partly recycled to the biological treatment process and partly wasted (excess or waste sludge).

The potential chemical energy contained in the wastewater organic pollutants (1.5-1.9 kWh/m^3 of wastewater, (Metcalf and Eddy, 2003)) is however largely destroyed during the activated sludge process, so that this process can hardly be considered a sustainable wastewater treatment technology. Besides, not only the potential chemical energy of the organic pollutants is destroyed, but also energy is consumed for aeration required to provide the microorganisms with oxygen. Typically, 60-70% of the overall energy consumption of a wastewater treatment plant is used for aeration (Zessner *et al*., 2010). Only a minor amount of the potential chemical energy, typically 20-30%, is recovered when the waste sludge is digested anaerobically to produce the energy carrier methane (Müller and Kobel, 2004; Rulkens, 2007).

Anaerobic wastewater treatment

Energy recovery from wastewater could be increased considerably when wastewater is treated anaerobically. Depending on the type of wastewater, about 70% of municipal wastewater organic matter is in principal anaerobically biodegradable and therefore could be recovered as methane (Appels *et al*., 2008). Anaerobic treatment processes, however, are limited to warm (typically > 25 ˚C) and high strength industrial wastewaters (typically > 1000 mg/L of COD) because the anaerobic microorganisms, which convert organic matter to methane, are dependent on higher temperatures (Van Haandel *et al*., 1994). Under (sub-) tropical temperature conditions anaerobic sewage

treatment is feasible, but an extensive aerobic post-treatment to remove remaining COD, nutrients and pathogens would still be required (Aiyuk *et al*., 2006).

The temperature of municipal wastewater under moderate climate conditions is 6-15 °C (Singh and Viraraghavan, 2003). This implies that **direct** anaerobic treatment is not feasible. However, with an appropriate pre-concentration step for wastewater organic matter, anaerobic treatment technology could become suitable because the water volume that needs to be heated to a temperature that is suitable for anaerobic treatment would become much smaller (Verstraete *et al*., 2009). This would not only facilitate energy production (as methane) but also may allow the anaerobic production of other valuable products such as Volatile Fatty Acids (VFAs) (Lee *et al*., 2014).

Up-concentration of wastewater organic matter for improved energy recovery

Several techniques can be used for up-concentration of wastewater organic matter, namely, dissolved air flotation, chemical flocculation and direct membrane filtration. During dissolved air flotation, organic matter is selectively adsorbed at the gas/liquid or gas/solid interface of rising bubbles and removed at the top of the bubble separation reactor (Wang *et al*., 2005). In this manner organic matter is removed from the wastewater without using microbial oxidation and the treatment process is considerably faster compared to conventional wastewater treatment using a settler for separation (Krofta *et al*., 1995). Polymers can be used to enhance dissolved air floatation processes. Alternatively, flocculation of wastewater organic matter can be combined with sedimentation of the flocculated material. Mels *et al*. (2001) showed that 80% of the particulate chemical oxygen demand (COD), a measure of the concentration of organic matter from municipal wastewater, can be removed using high molecular weight polyelectrolytes. However, high polymer costs and the possible inhibitory effect of these polymers on anaerobic digestion processes are major drawbacks. Membrane filtration represents another up-concentration technology for wastewater organic matter. It provides a high recovery efficiency because the majority of the COD is retained and only soluble COD can leave the system with the permeate water. However, severe membrane fouling is observed during direct filtration of municipal wastewater, which results in relatively low fluxes and high operational costs due to high energy consumption and costs for membrane cleaning and replacement (Diamantis *et al*., 2010).

Bioflocculation to up-concentrate wastewater organic matter

A bioflocculation stage prior to membrane filtration can overcome the problem of severe membrane fouling (Diamantis *et al*., 2014; Ivanovic *et al*., 2008). Besides reduced membrane fouling,

mineralization of organic substances is minimized by applying extremely short solids retention times (SRTs). The bioflocculation or contact stage, also called the A-stage of the AB process, was initially successfully applied in the wastewater treatment plant of Krefeld, Germany, in 1997 to handle high strength industrial wastewater with high particle concentrations (Boehnke *et al*., 1997). More recently, Akanyeti *et al*. (2010) and Diamantis *et al*. (2014) proposed to use a bioflocculation stage in combination with membrane filtration. In the following this will be referred to as a high-loaded membrane bioreactor or HL-MBR.

During aerobic bioflocculation, the colloidal and suspended organic matter fraction of the wastewater (around 20-30% of total COD for sewage) is flocculated with the aid of extracellular polymeric substances (EPS), sometimes also referred to as bioflocculants, produced by microorganisms, yielding a concentrated stream of organics (Figure 1.1). Only a minor amount of biodegradable COD (<15%) is utilized for microbial growth and EPS production. Thus, in this manner, costs and other negative effects of chemical polymers used for flocculation are avoided.

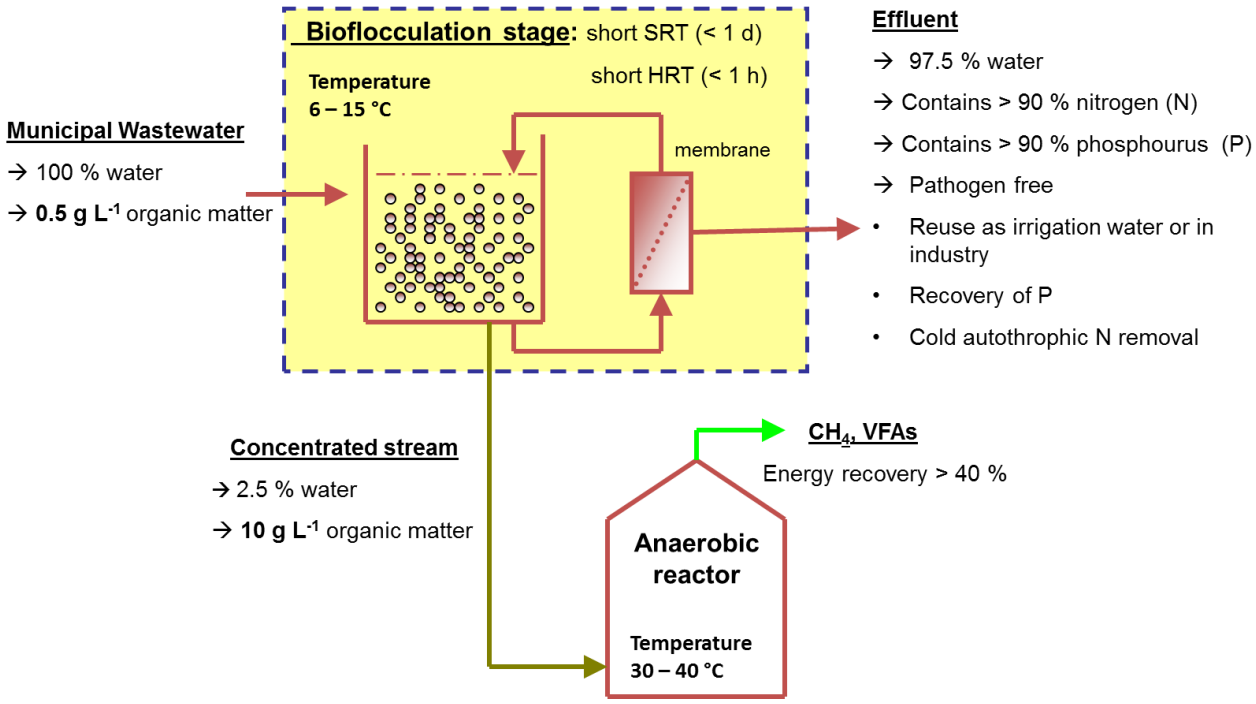


Figure 1.1. *Aerobic bioflocculation in a membrane bioreactor (HL-MBR) combined with anaerobic energy recover as methane or other highly valuable compounds (e.g. VFAs)*

The HL-MBR is characterized by extremely short SRTs (typically ≤ 1 d) to minimize microbial conversion of COD to CO_2 and water. At the same time the SRT should be long enough to allow growth of microorganisms by utilizing some of the soluble COD to be able to produce the EPS that can help to flocculate the colloidal and suspended COD. Also the hydraulic retention time (HRT)

should be sufficiently short (typically ≤1 h) to obtain a high concentration of COD for subsequent anaerobic conversion to methane of VFAs.

Cardoen (2011) compared the overall energy efficiency of municipal wastewater treatment with a HL-MBR operated at 1.2 d SRT and subsequent thermophilic anaerobic digestion of the concentrate to conventional aerobic treatment with digestion of secondary sludge. They found that the treatment using a HL-MBR was, with an overall energy efficiency of 25%, more favorable than the treatment in a conventional system which gave 6% overall energy efficiency. Applying even shorter SRTs may further increase the energy efficiency due to lower mineralization of organic matter. At shorter SRTs, however, the flocculation process may be worsening due to less microbial growth and therewith also less EPS production. Thus, the SRT needs to be chosen based on the balance of a good bioflocculation process and sufficiently low mineralization. Another advantage of applying such short SRTs is that the nutrients phosphorus and nitrogen are hardly removed and will still be present in the effluent. Because this effluent also is pathogen free, it has a high reuse potential as irrigation water. Alternatively, if discharge or industrial reuse of the effluent is anticipated, phosphate and nitrogen should be removed to obey the discharge guidelines for these nutrients and to prevent biofouling, i.e. while further upgrading the water quality with reverse osmosis membranes. The phosphate should be recovered from the effluent as it is becoming a scarce resource (Rockström *et al*., 2009). The nitrogen concentration is too low for an economic recovery and should be removed. Because the carbon to nitrogen ratio of the effluent of the HL-MBRs is very low, a cold autotrophic nitrogen removal process is required, i.e. a combination of partial nitritation and anammox.

1.2 Bioflocculation and the role of extracellular polymeric substances (EPS)

Bioflocculation is the formation of aggregates/flocs from smaller, finely divided particles induced by EPS (bioflocculants) produced by microorganisms (Figure 1.2). Hence, the ability of microorganisms to produce EPS and thus bind to each other and to other particles is fundamental to the bioflocculation process.

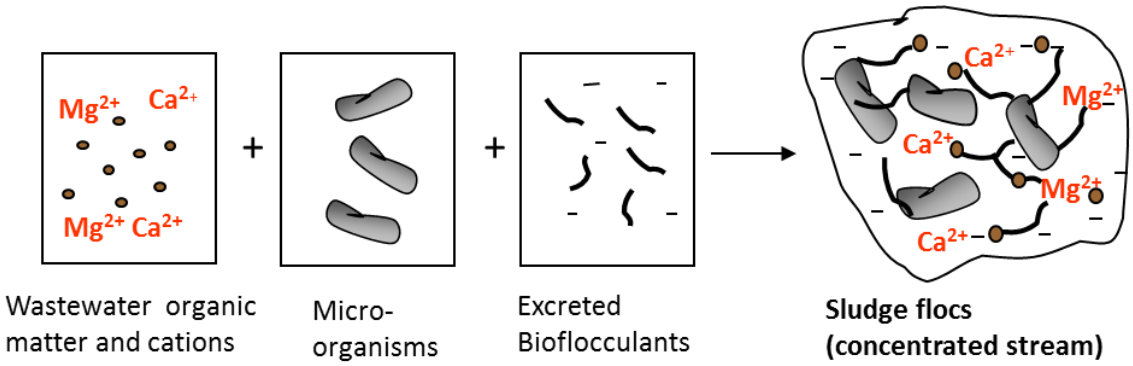


Figure 1.2. *Bioflocculation process of wastewater organic matter particles with bioflocculants excreted by microorganisms*

These EPS act as a glue between microorganisms and/or wastewater particles, due to their highly adhesive and cohesive nature (Fang *et al*., 2000; Wingender *et al*., 1999). At neutral or close to neutral pH, EPS carry a net negative charge, resulting from the presence of acidic functional groups (Sheng and Yu, 2006). Divalent cations such as Ca^{2+} and Mg^{2+}, but also metal ions interact with these negatively charged EPS to form bridges and allow cells and particles to adhere to each other (Bruus *et al*., 1992; Tian *et al*., 2006). This way EPS fill and form the spaces between cells and particles and are responsible for the structural and functional integrity of activated sludge flocs and as a result determine the sludge properties such as flocculation potential, floc strength, settleability and dewaterability (Flemming and Wingender, 2001). However, literature is contradictory about the effect of EPS on these properties. In some studies it was even found that high EPS concentrations have a negative effect on flocculation and on settling and dewatering characteristics of sludge (Liu and Fang, 2003; Wilén *et al*., 2003). Moreover, it was suggested, that the composition of EPS may be equally if not more important than the concentration of EPS (Badireddy *et al*., 2010). The following paragraphs will present an overview about EPS composition and factors impacting their composition and concentrations in wastewater treatment systems.

1.3 Composition of EPS

By definition, EPS are located outside the cell or are attached to the cell wall and may be products of different processes such as: active secretion, cell surface material shedding, cell lysis and adsorption from the environment (Liu and Fang, 2002; Wingender *et al*., 1999). In biofilms EPS can contribute up to 90% of the total dry mass (Flemming and Wingender, 2010). In sludge, EPS may constitute between 50 and 60% of the organic fraction while cell biomass only contributes up to 20% (Frølund *et al*., 1995; Wilén *et al*., 2003). In general, EPS have been described to consist of various organic substances including proteins, carbohydrates, lipids, nucleic acids, humic acids and uronic acids (Dignac *et al*., 1998; Frølund *et al*., 1996; Nielsen *et al*., 1996). However, regarding the specific

composition of EPS in sludge samples conflicting findings are reported in literature. Yet it is generally accepted that proteins are the main constituents of sludge and biofilm related EPS (Bura *et al*., 1998; Liao *et al*., 2001; Urbain *et al*., 1993; Wilén *et al*., 2003). Reasons for these conflicting findings could be: (1) The complexity of the activated sludge process and the large number of process variables involved, such as the type of wastewater, nutrient level, SRT, dissolved oxygen and reactor configuration, all may affect the nature of the sludge and EPS composition. (2) The extraction method strongly affects the quantity and composition of the extracted EPS, and there is no standardized EPS extraction procedure (Sheng *et al*., 2010). (3) Since there is no standard protocol for the characterization of EPS, often only the carbohydrates and protein content is reported. Thus, interpretation and evaluation of the effect of the EPS quantity and composition on treatment processes and sludge properties remains difficult.

1.4 EPS and soluble microbial products (SMP)

Due to the fact authors use different terminology to refer to EPS in activated sludge systems, the interpretation of literature data is often complicated. When defining excreted microbial products, two schools of thinking need to be distinguished as proposed by Laspidou and Rittmann (2002a): the “EPS school” and the “SMP (soluble microbial products) school”. The “EPS school” targets active biomass and EPS, with the latter being further divided into “bound EPS” and “soluble EPS” (Laspidou and Rittmann, 2002a). Other definitions used are “sheath” or “tightly-bound”, and “slime” or “loosely-bound” EPS, depending on their association with the cells and/or sludge. Slime EPS or loosely-bound EPS are loosely attached to the cell or sludge surface and are obtained in the supernatant after centrifugation, while bound EPS are retained in the sludge pellet. Since slime EPS, loosely bound or loosely attached EPS are obtained after centrifugation and bound EPS after extraction from the pellet, their definition is moreover determined by the analytical techniques applied to obtain these EPS. A scheme of tightly bound, loosely bound and soluble EPS is shown in Figure 1.3.

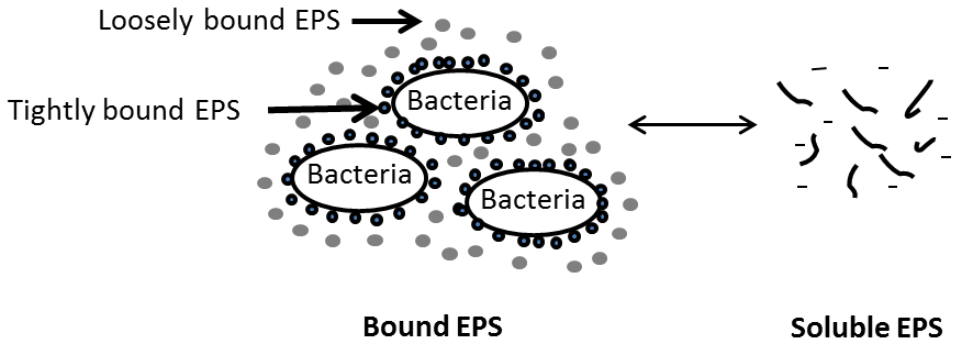


Figure 1.3. *Depiction of different EPS structures (Nielsen and Jahn, 1999)*

The "SMP school" considers active biomass, SMP and inert biomass. SMP are defined as soluble compounds of cellular origin that are released during cell lysis, diffuse through cell membranes, or are produced and excreted for other purposes as described by Laspidou and Rittmann (2002a).

The same authors proposed a unified theory for EPS, SMP, and active and inert biomass to couple and reconcile apparent contradictions (Figure 1.4).

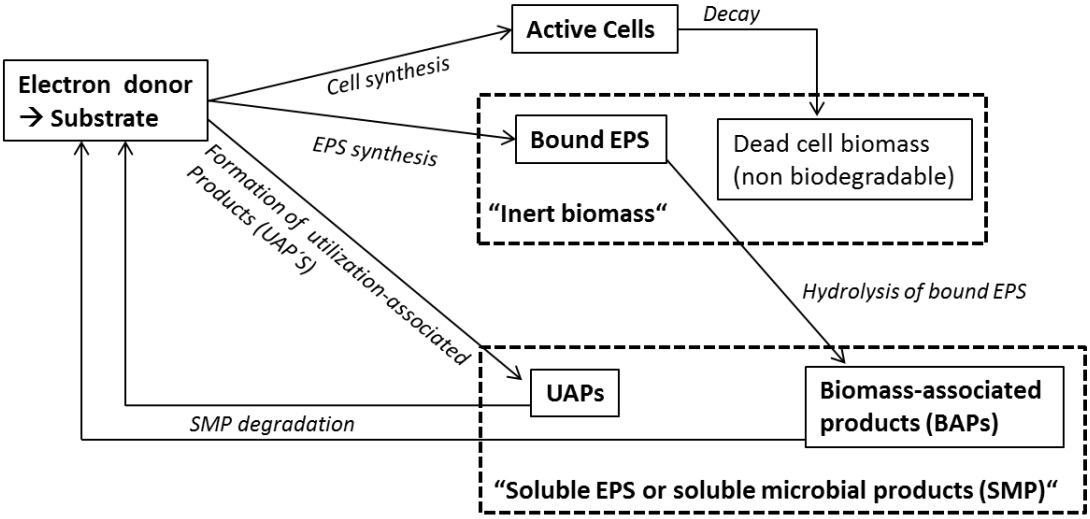


Figure 1.4. *Schematic representation of the unified theory for active biomass, EPS, SMP and inert biomass (adapted from Laspidou and Rittmann, 2002a)*

It proposes that soluble EPS can be substrate utilization-associated products (UAPs) as well as biomass-associated products (BAPs). UAPs are produced as a direct result from substrate utilization, whereas BAP´s are formed from biomass, presumably during decay. Furthermore it was proposed that soluble EPS and SMP are the same. For bound EPS it was suggested that they derive from both, active (living cells with energy demand for maintenance) and inert biomass (residual, non-biodegradable products of endogenous biomass decay). A part of the bound EPS may be hydrolyzed to BAPs. Also soluble EPS (SMP) could be used as electron donor by the biomass. In real systems,

however, it remains difficult to separate EPS production from EPS degradation since these processes occur simultaneously. Based on this unified theory a mathematical model was developed to predict and quantify the relationship between bacteria, EPS and inert biomass and between SMP, original substrate and electron acceptor. It was found that the modeling data captured all trends observed in an experimental data set obtained by Hsieh *et al*. (1994), and could provide an explanation for the observed trends in the activated sludge system (Laspidou and Rittmann, 2002b). In practice, however, it is difficult to distinguish between e.g. UAPs and BAPs. Therefore most researchers reduce the complexity of the EPS system by distinguishing only between soluble and bound EPS. In this thesis we used a defined centrifugation step to discriminate between soluble and bound EPS: EPS that are released from the sludge during the centrifugation or dissolved in the supernatant of the sludge are referred to as supernatant EPS; EPS extracted from the sludge pellet using a defined cation exchange resin procedure are referred to as bound EPS.

1.5 Factors impacting EPS production and concentrations in activated sludge systems

Operational parameters such as SRT, HRT, dissolved oxygen (DO) concentration and shear conditions all affect the production of EPS in terms of concentration and composition. The effect of SRT on EPS production is highly controversial in literature. Many studies showed that EPS concentrations increase with SRT, while others report a lower concentration at longer SRTs. Yet others found that total EPS was independent of SRT, and only a strong effect was observed on individual constituents e.g. on the protein/carbohydrate ratio. These controversies may have been the result of the wide ranges of SRTs that were investigated.

Ng and Hermanowicz (2005) studied total EPS concentrations in a MBR and a conventional activated sludge system at SRTs ranging between 0.25-5 d and found higher EPS concentrations at shorter SRTs. Similarly, Badireddy *et al*. (2010) investigated EPS concentrations during the cultivation of activated sludge samples in batch tests and found an increase in EPS concentration with increasing cultivation time ranging between 1-3 d. Studies investigating longer SRTs in a membrane bioreactor, however, reported a decrease in EPS concentration with increasing SRTs, for a relatively higher range of SRTs, namely from 10-60 d (Masse *et al*., 2006). Similarly, Chen *et al*. (2011) showed that EPS concentration decreased with increasing SRT ranging from 10 to 30 d. Wang *et al*. (2013) on the other hand found, that the concentration of tightly bound EPS remained fairly constant in a sequencing batch reactor, whereas the concentration of loosely bound EPS decreased when SRT was increased from 5 to 20 d. Clearly, the relation between SRT and EPS concentration and composition

requires more research. Especially, in the domain of extremely short SRTs (< 1 d), the relation between EPS and SRT has not yet been studied.

Another operational parameter that affects EPS concentrations is the DO concentration. Under oxygen limited or depleted conditions, activated sludge deflocculates. Generally this is assumed to be caused by a reduced EPS production rate (Nielsen *et al*., 1996; Rasmussen *et al*., 1994; Wilén and Balmér, 1999). Also Starkey and Karr (1984) reported a decrease of EPS production and an increase of EPS hydrolysis as the cause of deteriorating flocculation. DO also may have an impact on the production of each individual constituent of EPS. For example, according to Shin *et al*. (2001) high DO levels resulted in higher carbohydrate content while EPS related proteins remained at a constant level. At low DO levels both concentrations did not significantly change. However, these studies were conducted with sludge from treatment systems operating at much longer SRTs (5-35 d) than applied in HL-MBRs (0.25-1 d). How the DO concentration affects EPS concentrations and herewith bioflocculation at very short SRTs is not known and still needs to be investigated.

Shear forces, mainly caused by (fine bubble) aeration, are also suggested to affect EPS concentrations and distribution, in particular when activated sludge flocs are "weak". Assessment of the floc strength however, remains difficult mainly because no unified method exists to measure this parameter. This explains why contradictory results are reported about the relationship between EPS and shear force (Jarvis *et al*., 2005). Furthermore, all of these shear experiments were performed by changing the air flow rate in the reactors causing the DO concentration to vary simultaneously. Ji and Zhou (2006) showed that the aeration rate in lab scale MBRs did not have an effect on bound and supernatant EPS concentrations, but the degradation of supernatant EPS was enhanced at higher air flow rates and prolonged operation. Menniti *et al*. (2009) reported that low aeration rates (low shear levels) promoted the growth of predatory organisms such as aquatic earthworms and gave an increase in the concentration of bound and supernatant EPS.

Wastewater composition also has a strong effect on EPS production and composition. For example, activated sludge systems fed with wastewaters from the pulp-, petrochemical and textile industry contained lower concentrations of EPS proteins compared to systems that were fed with municipal and winery wastewater (Sponza, 2003). In activated sludge systems operated with synthetic wastewater and either glucose or sodium-acetate it was found that sludge fed with glucose produced more EPS than sludge fed with sodium-acetate (Li and Yang, 2007). A possible explanation for this may be that the different feed waters are likely to influence the microbial population in the reactors and thus leading to the production of different quantities and types of EPS. For example, high-energy substrates, such as carbohydrates are known to promote the production of polysaccharide

EPS (Imai *et al*., 1997; Schmidt and Ahring, 1996; Thaveesri *et al*., 1995), which may be important for a good flocculation and/or granulation process (Thaveesri *et al*. (1995).

1.6 Role of cations in the bioflocculation process

Multivalent cations are known to improve sludge flocculation and settling (Higgins and Novak, 1997; Murthy and Novak, 2001; Sobeck and Higgins, 2002). Several mechanisms were proposed to explain this effect such as double layer interactions (DVLO theory), the divalent cation bridging with EPS (DCB theory) and the alginate theory. The DVLO theory is the classical theory for colloid stability, which describes charged colloidal particles as possessing a double layer of counter ions. This double layer surrounding the particle results in repulsive force between particles depending on the ionic strength of the liquid medium and counter balancing attractive van der Waals forces, and thus inhibits aggregation. As the ionic strength increases, the size of the double layer decreases, which reduces the repulsion between particles, allowing long range as well as short-range attractive forces to promote aggregation/flocculation. This theory is often used to explain better flocculation, dewatering, and settling of activated sludge in the presence of multivalent cations (Higgins and Novak, 1997; Liu *et al*., 2007). Microorganisms and wastewater particles are negatively charged (Bala Subramanian *et al*., 2010; Rijnaarts *et al*., 1999) and thus the increase in ionic strength could promote flocculation. Yet in practice, in complex sludge systems, a distinction between the various particle aggregation and disintegration mechanisms remains difficult.

Certain types of EPS such as alginates are characterized by a highly specific cation bridging activity, with generally one type of cation involved. Because of its unique structure, alginate forms gels only with Ca^{2+} cations (Bruus *et al*., 1992). Several alginate producing microorganisms have been detected in activated sludge and apparently the alginate theory, as a subset of the DCB theory, also may be important for bioflocculation (Sobeck and Higgins, 2002).

It is widely accepted that high concentrations of Na^{+} deteriorate flocculation, settling and dewatering of activated sludge (Biggs *et al*., 2001; Higgins and Novak, 1997). For example, municipal wastewater treatment plants suffer from a poor sludge quality during the winter period when salt is infiltrated into the sewer or when industrial wastewater with a high salt concentration is discharged. One reason for this may be that a sudden increase in monovalent salt concentration causes an exchange of divalent cations (e.g. Ca^{2+} and Mg^{2+}) for sodium in the extracellular polymer matrix, leading to weaker cation bridges with the EPS (Van den Broeck *et al*., 2010). However, high sodium concentration resistant flocculating sludges are also reported in literature, in particular for industrial

wastewater treatment systems (Lefebvre and Moletta, 2006). Thus, in addition to DCB theory based mechanisms, other sludge particle binding mechanisms may also need to be taken into account under specific conditions e.g. high sodium concentrations.

1.7 Outline of this thesis

The objective of this thesis was to study bioflocculation process in HL-MBRs, including the effect of operational parameters and a more detailed investigation of the mechanisms that are involved.

Chapter 2 of this thesis describes the role of EPS on the bioflocculation process in various HL-MBRs, operated at different short SRTs ranging from 0.125-5 d. The objective was to find the optimal SRT for a proper balance between a maximum flocculation efficiency of wastewater particles and a minimum mineralization of wastewater organic matter. Furthermore, the EPS concentrations found in the HL-MBRs were compared to the EPS concentrations of wastewater treatment systems operated at longer SRTs, including e.g. a full scale membrane bioreactor. Membrane fouling experiments were conducted to investigate the effect of SRT and EPS concentrations on the fouling potential of the HL-MBR concentrate.

Chapter 3 presents the findings regarding the effect of two different dissolved oxygen concentrations of 1 mgO_2/L and 4 mgO_2/L on bioflocculation in a HL-MBR. Concentrate characteristics such as EPS concentration, cation concentrations, sludge volume index, particle size distribution and fouling potential were compared.

In **Chapter 4**, the bacterial community in various HL-MBRs operated at different SRTs was characterized. The diversity of the bacterial communities in the (solid) sludge fraction, the supernatant and in the inflow wastewater was analyzed by using a PCR-DGGE approach. Similarities of the bacterial communities were investigated by construction cluster analysis using the Pearson Correlation Coefficient and unweighted pair group method with arithmetic averages (UPGMA). Furthermore 7 clone libraries were constructed to real the role of certain bacterial species in the bioflocculation process. Fluorescence in situ hybridization (FISH) was used to localize different classes of *Proteobacteria* in the sludge flocs.

In **Chapter 5** a systematic approach is described to model bioflocculation in HL-MBR systems. Poorly flocculated peptone fed biomass was used to investigate the effect of various cations and kaolin clay particles on the bioflocculation process of this poorly flocculated biomass. Kaolin clay particles were

used to mimic wastewater particles. Subsequently, the effect of various types of EPS on the flocculation of kaolin clay was investigated. Therefore, EPS were extracted from various sludge sources, such as municipal and industrial wastewater sludges.

Chapter 6 presents an overall discussion about the main findings in this thesis including an outlook and recommendations for further research.

References

Aiyuk, S., Forrez, I., Lieven, D.K., Van Haandel, A., Verstraete, W., 2006. Anaerobic and complementary treatment of domestic sewage in regions with hot climates—A review. Bioresource Technology 97, 2225–2241.

Akanyeti, I., Temmink, H., Remy, M., Zwijnenburg, A., 2010. Feasibility of bioflocculation in a high-loaded membrane bioreactor for improved energy recovery from sewage. Water Science & Technology 61, 1443–1439

Appels, L., Baeyens, J., Degrève, J., Dewil, R., 2008. Principles and potential of the anaerobic digestion of waste-activated sludge. Progress in energy and combustion science 34, 755–781.

Badireddy, A.R., Chellam, S., Gassman, P.L., Engelhard, M.H., Lea, A.S., Rosso, K.M., 2010. Role of extracellular polymeric substances in bioflocculation of activated sludge microorganisms under glucose-controlled conditions. Water research 44, 4505–4516.

Bala Subramanian, S., Yan, S., Tyagi, R., Surampalli, R., 2010. Extracellular polymeric substances (EPS) producing bacterial strains of municipal wastewater sludge: Isolation, molecular identification, EPS characterization and performance for sludge settling and dewatering. Water research 44, 2253–2266.

Biggs, C., Ford, A., Lant, P., 2001. Activated sludge flocculation: direct determination of the effect of calcium ions. Water Science & Technology 43, 75–82.

Boehnke, B., Diering, B., Zuckut, S.W., 1997. Ab process removes organics and nutrients: Adsorption/bio-oxidation combines high food-to-microorganism ratio efficiency with advanced activated sludge operations. Water environment & technology 9, 23–27.

Bruus, J.H., Nielsen, P.H., Keiding, K., 1992. On the stability of activated sludge flocs with implications to dewatering. Water research 26, 1597–1604.

Bura, R., Cheung, M., Liao, B., Finlayson, J., Lee, B., Droppo, I., Leppard, G., Liss, S., 1998. Composition of extracellular polymeric substances in the activated sludge floc matrix. Water Science and Technology 37, 325–333.

Cardoen, D., 2011. Up-concentration techniques for zero-waste water treatment. Master Thesis, Faculty of Bioscience Engineering, Gent University.

Chen, K., Wang, X., Li, X., Qian, J., Xiao, X., 2011. Impacts of sludge retention time on the performance of submerged membrane bioreactor with the addition of calcium ion. Separation and Purification Technology 82, 148–155.

Diamantis, V., Antoniou, I., Athanasoulia, E., Melidis, P., Aivasidis, A., 2010. Recovery of reusable water from sewage using aerated flat-sheet membranes 156, 314–321.

Diamantis, V., Eftaxias, A., Bundervoet, B., Verstraete, W., 2014. Performance of the biosorptive activated sludge (BAS) as pre-treatment to UF for decentralized wastewater reuse. Bioresource technology 156, 314–321.

Dignac, M.-F., Urbain, V., Rybacki, D., Bruchet, A., Snidaro, D., Scribe, P., 1998. Chemical description of extracellular polymers: implication on activated sludge floc structure. Water Science and Technology 38, 45–53.

Fang, H.H., Chan, K.-Y., Xu, L.-C., 2000. Quantification of bacterial adhesion forces using atomic force microscopy (AFM). Journal of microbiological methods 40, 89–97.

Flemming, H., Wingender, J., 2001. Relevance of microbial extracellular polymeric substances (EPSs)-Part I: Structural and ecological aspects. Water Science & Technology 43, 1–8.

Flemming, H.-C., Wingender, J., 2010. The biofilm matrix. Nature Reviews Microbiology 8, 623–633.

Frølund, B., Griebe, T., Nielsen, P., 1995. Enzymatic activity in the activated-sludge floc matrix. Applied microbiology and biotechnology 43, 755–761.

Frølund, B., Palmgren, R., Keiding, K., Nielsen, P.H., 1996. Extraction of extracellular polymers from activated sludge using a cation exchange resin. Water Research 30, 1749–1758.

Higgins, M.J., Novak, J.T., 1997. The effect of cations on the settling and dewatering of activated sludges: laboratory results. Water Environment Research 215–224.

Hsieh, K.M., Murgel, G.A., Lion, L.W., Shuler, M.L., 1994. Interactions of microbial biofilms with toxic trace metals: 1. Observation and modeling of cell growth, attachment, and production of extracellular polymer. Biotechnology and bioengineering 44, 219–231.

Imai, T., Ukita, M., Liu, J., Sekine, M., Nakanishi, H., Fukagawa, M., 1997. Advanced start up of UASB reactors by adding of water absorbing polymer. Water Science and Technology 36, 399–406.

Ivanovic, I., Leiknes, T., Odegaard, H., 2008. Fouling Control by Reduction of Submicron Particles in a BF-MBR with an Integrated Flocculation Zone in the Membrane Reactor. Separation science and technology 43, 1871–1883.

Jarvis, P., Jefferson, B., Gregory, J., Parsons, S.A., 2005. A review of floc strength and breakage. Water Research 39, 3121–3137.

Ji, L., Zhou, J., 2006. Influence of aeration on microbial polymers and membrane fouling in submerged membrane bioreactors. Journal of Membrane Science 276, 168–177.

Krofta, M., Miskovic, D., Burgess, D., 1995. Primary-secondary flotation of three municipal wastewaters: pilot-scale study. Water Science and Technology 31, 295–298.

Laspidou, C.S., Rittmann, B.E., 2002a. A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass. Water Research 36, 2711–2720.

Laspidou, C.S., Rittmann, B.E., 2002b. Non-steady state modeling of extracellular polymeric substances, soluble microbial products, and active and inert biomass. Water Research 36, 1983–1992.

Lee, W.S., Chua, A.S.M., Yeoh, H.K., Ngoh, G.C., 2014. A review of the production and applications of waste-derived volatile fatty acids. Chemical Engineering Journal 235, 83–99.

Lefebvre, O., Moletta, R., 2006. Treatment of organic pollution in industrial saline wastewater: a literature review. Water Research 40, 3671–3682.

Li, X., Yang, S., 2007. Influence of loosely bound extracellular polymeric substances (EPS) on the flocculation, sedimentation and dewaterability of activated sludge. Water Research 41, 1022–1030.

Liao, B., Allen, D., Droppo, I., Leppard, G., Liss, S., 2001. Surface properties of sludge and their role in bioflocculation and settleability. Water Research 35, 339–350.

Liu, H., Fang, H.H., 2002. Extraction of extracellular polymeric substances (EPS) of sludges. Journal of Biotechnology 95, 249–256.

Liu, X.-M., Sheng, G.-P., Yu, H.-Q., 2007. DLVO approach to the flocculability of a photosynthetic H2-producing bacterium, Rhodopseudomonas acidophila. Environmental science & technology 41, 4620–4625.

Liu, Y., Fang, H.H., 2003. Influences of extracellular polymeric substances (EPS) on flocculation, settling, and dewatering of activated sludge. Critical Reviews in Environmental Science and Technology 33, 237–273.

Masse, A., Spérandio, M., Cabassud, C., 2006. Comparison of sludge characteristics and performance of a submerged membrane bioreactor and an activated sludge process at high solids retention time. Water Research 40, 2405–2415.

Mels, A., Rulkens, W., van der Meer, A., Nieuwenhuijzen, A., Klapwijk, A., 2001. Flotation with polyelectrolytes as a first step of a more sustainable wastewater treatment system. Water Science & Technology 43, 83–90.

Menniti, A., Kang, S., Elimelech, M., Morgenroth, E., 2009. Influence of shear on the production of extracellular polymeric substances in membrane bioreactors. Water research 43, 4305–4315.

Metcalf, I., Eddy, H., 2003. Wastewater engineering; treatment and reuse. McGraw-Hill New York.

Müller, E., Kobel, B., 2004. Energetische Bestandsaufnahme an Kläranlagen in Nordrhein-Westfalen mit 30 Millionen Einwohnerwerten. Korrespondenz Abwasser 51, 625–631.

Murthy, S.N., Novak, J.T., 2001. Influence of cations on activated-sludge effluent quality. Water environment research 73, 30–36.

Ng, H.Y., Hermanowicz, S.W., 2005. Membrane bioreactor operation at short solids retention times: performance and biomass characteristics. Water Research 39, 981–992.

Nielsen, P.H., Frølund, B., Keiding, K., 1996. Changes in the composition of extracellular polymeric substances in activated sludge during anaerobic storage. Applied microbiology and biotechnology 44, 823–830.

Nielsen, P.H., Jahn, A., 1999. Extraction of EPS. In: Microbial Extracellular Polymeric Substances. Springer, pp. 49–72.

Rasmussen, H., Bruus, J.H., Keiding, K., Nielsen, P.H., 1994. Observations on dewaterability and physical, chemical and microbiological changes in anaerobically stored activated sludge from a nutrient removal plant. Water Research 28, 417–425.

Rijnaarts, H.H., Norde, W., Lyklema, J., Zehnder, A.J., 1999. DLVO and steric contributions to bacterial deposition in media of different ionic strengths. Colloids and Surfaces B: Biointerfaces 14, 179–195.

Rockström, J., Steffen, W., Noone, K., Persson, Å., Chapin, F.S., Lambin, E.F., Lenton, T.M., Scheffer, M., Folke, C., Schellnhuber, H.J., others, 2009. A safe operating space for humanity. Nature 461, 472–475.

Rulkens, W., 2007. Sewage sludge as a biomass resource for the production of energy: overview and assessment of the various options†. Energy & Fuels 22, 9–15.

Schmidt, J.E., Ahring, B.K., 1996. Granular sludge formation in upflow anaerobic sludge blanket (UASB) reactors. Biotechnology and Bioengineering 49, 229–246.

Sheng, G.-P., Yu, H.-Q., 2006. Characterization of extracellular polymeric substances of aerobic and anaerobic sludge using three-dimensional excitation and emission matrix fluorescence spectroscopy. Water Research 40, 1233–1239.

Sheng, G.-P., Yu, H.-Q., Li, X.-Y., 2010. Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review. Biotechnology Advances 28, 882–894.

Shin, H., Kang, S., Nam, S., 2001. Effect of carbohydrate and protein in the EPS on sludge settlingcharacteristics. Water Science & Technology 43, 193–196.

Singh, K., Viraraghavan, T., 2003. Impact of temperature on performance, microbiological, and hydrodynamic aspects of UASB reactors treating municipal wastewater. Water Science & Technology 48, 211–217.

Sobeck, D.C., Higgins, M.J., 2002. Examination of three theories for mechanisms of cation-induced bioflocculation. Water Research 36, 527–538.

Sponza, D.T., 2003. Investigation of extracellular polymer substances (EPS) and physicochemical properties of different activated sludge flocs under steady-state conditions. Enzyme and Microbial Technology 32, 375–385.

Starkey, J., Karr, P., 1984. Effect of low dissolved oxygen concentration on effluent turbidity. Journal (Water Pollution Control Federation) 7, 837–843.

Thaveesri, J., Daffonchio, D., Liessens, B., Verstraete, W., 1995. Different types of sludge granules in UASB reactors treating acidified wastewaters. Antonie van Leeuwenhoek 68, 329–337.

Tian, Y., Zheng, L., Sun, D., 2006. Functions and behaviors of activated sludge extracellular polymeric substances (EPS): a promising environmental interest. Journal of Environmental Sciences 18, 420–427.

Urbain, V., Block, J., Manem, J., 1993. Bioflocculation in activated sludge: an analytic approach. Water Research 27, 829–838.

Van den Broeck, R., Van Dierdonck, J., Caerts, B., Bisson, I., Kregersman, B., Nijskens, P., Dotremont, C., Van Impe, J., Smets, I., 2010. The impact of deflocculation-reflocculation on fouling in membrane bioreactors. Separation and Purification Technology 71, 279–284.

Van Haandel, A.C., Lettinga, G., others, 1994. Anaerobic sewage treatment: a practical guide for regions with a hot climate. John Wiley & Sons.

Verstraete, W., Van de Caveye, P., Diamantis, V., 2009. Maximum use of resources present in domestic "used water". Bioresource technology 100, 5537–5545.

Wang, H., Deng, H., Ma, L., Ge, L., 2013. Influence of operating conditions on extracellular polymeric substances and surface properties of sludge flocs. Carbohydrate polymers 92, 510–515.

Wang, L.K., Fahey, E.M., Wu, Z., 2005. Dissolved air flotation. In: Physicochemical Treatment Processes. Springer, 431–500.

Wilén, B.-M., Balmér, P., 1999. The effect of dissolved oxygen concentration on the structure, size and size distribution of activated sludge flocs. Water Research 33, 391–400.

Wilén, B.-M., Jin, B., Lant, P., 2003. The influence of key chemical constituents in activated sludge on surface and flocculating properties. Water research 37, 2127–2139.

Wingender, J., Neu, T.R., Flemming, H.-C., 1999. Microbial extracellular polymeric substances: characterization, structure, and function. Springer.

Zessner, M., Lampert, C., Kroiss, H., Lindtner, S., 2010. Cost comparison of wastewater treatment in Danubian countries. Water science & Technology 62, 223–230.

2

High loaded MBRs for organic matter recovery from sewage: Effect of solid retention time on bioflocculation and on the role of EPS

Abstract

High loaded MBRs (HL-MBR) can concentrate sewage organic matter by aerobic bioflocculation for subsequent anaerobic conversion to methane or volatile fatty acids. In the range of very short solid retention times (SRT), the effect of SRT on bioflocculation and EPS production in HL-MBR was investigated. This short SRT range was selected to find an optimum SRT maximizing recovery of organics by aerobic bioflocculation and minimizing losses of organics by aerobic mineralization. Bioflocculation was studied in five HL-MBRs operated at SRTs of 0.125, 0.25, 0.5, 1 and 5 d. The extent of flocculation, defined as the fraction of suspended COD in the concentrate, increased from 59% at an SRT of 0.125 d to 98% at an SRT of 5 d. The loss of sewage organic matter by biological oxidation was 1, 2, 4, 11 and 32% at SRT of 0.125-5 d. An SRT of 0.5-1 d gave best combination of bioflocculation and organic matter recovery. Bound extracellular polymeric substances (EPS) concentrations, in particular EPS-protein concentrations, increased when the SRT was prolonged from 0.125 to 1 d. This suggests that these EPS-proteins govern the bioflocculation process. A redistribution took place from free (supernatant) EPS to bound (floc associated) EPS when the SRT was prolonged from 0.125 to 1 d, further supporting the fact that the EPS play a dominant role in the flocculation process. Membrane fouling was most severe at the shortest SRTs of 0.125 d. No positive correlation was detected between the concentration of free EPS and membrane fouling, but the concentration of submicron (45-450 nm) particles proved to be a good indicator for this fouling.

This chapter has been published as:

Faust, L., Temmink, H., Zwijnenburg, A., Kemperman, A., Rijnaarts, H., 2014. High loaded MBRs for organic matter recovery from sewage: Effect of solids retention time on bioflocculation and on the role of extracellular polymers. Water research 56, 258–266.

2.1 Introduction

Currently, there is increased interest in improving the sustainability of municipal wastewater treatment processes. In particular, aerobic mineralization of sewage organic matter should be avoided because this not only destroys the energy contained in this organic matter (1.5-1.9 kWh/m^3 of wastewater), but also requires energy intensive aeration (0.3-0.5 kWh/m^3 of wastewater). A much better approach would be to concentrate and recover particulate and colloidal organic matter for subsequent anaerobic methane production or the production of volatile fatty acids and other chemicals (Agler *et al*. 2011).

Such a concentration step can be achieved by aerobic bioflocculation. This process, in which microorganisms excrete polymers that flocculate particulate and colloidal organic matter, is also important for a good separation of sludge and treated wastewater in settlers or by membrane filtration in membrane bioreactors (MBRs). To concentrate organic matter from diluted wastewaters, Akanyeti *et al*. (2010) and Hernández Leal *et al*. (2010) proposed to use a high loaded MBR (HL-MBR). The effluent from such a HL-MBR still contains most of the nutrients phosphorus and nitrogen, is free from pathogens, and could be re-used, for instance as irrigation water. Sutton *et al*. (2011) estimated that the energy requirements for municipal wastewater treatment in this manner could be reduced considerably compared to conventional MBR treatment.

When sewage organic matter is concentrated in a HL-MBR, an optimum must be found between maximum bioflocculation of organic matter and a minimum with respect to its aerobic mineralization. Such an optimum can be found at extremely short sludge retention times (SRTs) to prevent too much mineralization in combination with extremely short hydraulic retention times (HRTs) to obtain high concentrations of organic matter in the concentrate. This study presents new data regarding this optimum, and helps elucidating underlying bioflocculation mechanisms.

Bioflocculation of wastewater particles and bacteria results from the synthesis and secretion of extracellular polymeric substances (EPS) by microorganisms (Salehizadeh & Shojaosadati 2001). Due to their adhesive nature, EPS form an interconnecting matrix (Fang & Jia 1996) between microorganisms and particles. Multivalent cations further enforce this bridging nature of EPS. EPS can constitute up to 60% of the organic fraction in activated sludge, while cell biomass generally only accounts for 2 to 20% (Frølund *et al*. 1995; Wilén *et al*. 2003). They are composed of a wide variety of organic substances, but mainly consist of proteins, carbohydrates, lipids, nucleic acids, humic acids and uronic acids (Dignac *et al*. 1998; Frølund *et al*. 1996; Nielsen *et al*. 1996). Table 2.1 gives the composition and concentration of EPS in activated sludge samples from different municipal wastewater treatment plants operated at a wide range of SRTs (3-35 d). In all cases the EPS were

extracted with a cation exchange resin (CER). The data show a wide range of concentrations and composition for extracted EPS. Overall protein content varied between 17.6 and 510 mg/gVSS and the polysaccharide content varied between 6.8 and 48 mg/gVSS.

Table 2.1. *EPS composition after CER extraction from municipal wastewater sludge (mg/gVSS).*

SRT (d)	Poly-saccharides	Proteins	Humus-like	Uronic acids	DNA	Reference
13	12.7	17.6	16.4	1.2	0.14	Liu & Fang (2002)
n.a.	15.7	97.1	-	-	-	Wuertz *et al*. (2001)
n.a.	12.7	162	-	4.5	11.2	Finlayson *et al*. (1998)
n.a.	37.0-37.7	90-127	-	-	-	Jorand *et al*. (1998)
7-9	48	243	126	-	-	Frølund *et al*. (1996)
30-35	40	212	101	3.9	16	Nielsen *et al*. (1996)
30-35	-	410-510	130-205		-	Frølund *et al*. (1995)
36	6.8-8.8	64-75	-	3.2- 3.3	-	Frølund *et al*. (1994)
3-12	19.2-21.8	83-92	-	-	-	Rudd *et al*. (1984)

The EPS content is determined by the balance between microbial production and subsequent degradation. It is suggested that EPS production is a result of bacterial response to changing environmental factors, including changes in substrate concentration and stress conditions induced by shear and/or predation (Bossier & Verstraete 1996). Under near starving conditions, EPS can help to trap nutrients from the bulk water and in this way microbial aggregates experience higher nutrient concentrations than dispersed cells (Flemming & Wingender 2010). Adverse environmental conditions may as well result in a higher EPS production due to switching on of EPS production genes. Also it is suggested that EPS production is closely related to microbial growth- and substrate consumption rates (Laspidou & Rittmann 2002). However, the relationship between EPS production and substrate consumption- and growth rates is subject of significant controversy, and the precise nature of this relationship may very well be dependent on the individual species of microorganism that are involved. Degradation of EPS is assumed to especially occur at high cell densities as these have been shown to induce the production and release of lyases that can degrade EPS (Davies *et al*. 1998). The lysis of other EPS fractions than proteins may also be responsible for the higher protein concentrations found in older biofilms when compared to younger biofilms (Nielsen *et al*. 1997).

Literature is controversial with regard to the effect of operational parameters such as SRT on EPS production. Some studies indicated higher EPS concentration at longer SRTs (Badireddy *et al*. 2010; Ng & Hermanowicz 2005), while others observed lower concentrations at longer SRTs (Ahmed *et al*.

2007; Masse *et al*. 2006). In some studies the total EPS concentration was found to be independent of the SRT, although a strong effect was observed on the composition of the EPS, i.e. the fraction of proteins and carbohydrates (Liao *et al*. 2001; Morgan *et al*. 1990).

In the present study, the effect of extremely short SRTs (0.125-5 d) on bioflocculation was investigated in laboratory scale HL-MBRs, using real sewage water. The objective was to identify an optimum between satisfactory bioflocculation and a sufficiently low degree of mineralization, allowing a maximum recovery of sewage organic matter. In addition, EPS concentrations in these HL-MBRs and in a number of reference systems operated at similar and longer SRTs were measured to quantify the EPS concentration and composition as function of SRT. Finally, membrane fouling in the HL-MBRs was studied to identify the effect of SRT and varying EPS concentration on membrane performance.

2.2 Material and Methods

2.2.1 HL-MBR and other wastewater treatment systems

Five laboratory scale HL-MBRs, with an effective volume of 2.6 L, were operated in parallel. The reactors were operated at identical HRTs of 0.7 h, but varying SRTs of 0.125 d, 0.25 d, 0.5 d, 1 d and 5 d. The reactors were operated at room temperature with municipal wastewater which was screened (5 mm) before entering the HL-MBRs. The dissolved oxygen concentration in the reactors was above 2 mg/L. The reactor design has been described previously in more detail by Akanyeti *et al*. (2010) and Hernández Leal *et al*. (2010). In each reactor two submerged flat sheet membranes (Kubota Corporation) made from chlorinated polyethylene were used, each with a surface area of 0.124 m^2 and a nominal pore size of 0.2 µm. The permeate pumps (Masterflex L/S, Cole-Parmer) were operated in cycles with 15 minutes permeate extraction followed by 5 minutes of relaxation. This resulted in a net flux of 15 L/m^2/h. The gross flux while pumping was 20 L/m^2/h.

A minimum of three wastewater-, HL-MBR concentrate- and HL-MBR permeate samples were taken separately, after the HL-MBR reactors had been operated for a period of at least three times their SRT. In addition, sludge samples were collected from a pilot scale MBR operated at an SRT of 50 d (Remy *et al*. 2009), a full scale MBR in Heenvliet, The Netherlands operated at a SRT of 30 d and from the A-stage from a full scale AB treatment plant in Dokhaven, the Netherlands operated at a SRT of 0.3 d. All these systems treated municipal wastewater.

2.2.2 Chemical analyses and oxygen uptake rate

COD was measured using Dr. Lange test kits (LCK, Hach Lange), heated in a thermostat (HT 200S, Hach Lange) to the desired temperature and analyzed in a spectrophotometer (DR 3900 VIS spectral photometer, wavelength range 320-750 nm). For fractionation the sludge samples was first paper filtered (Whatman Black Ribbon 589/1, 12–25 µm) and subsequently membrane filtered (Cronus PTFE syringe filter, nominal pore size of 0.45 µm). The difference between the total COD and the paper filtered COD will be referred to as suspended COD, the difference between paper and membrane filtrate as colloidal COD and the membrane filtrate as soluble COD.

Analysis of Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) were carried out according to the Standard Methods for the Examination of Water and Wastewater (APHA. 1976).

EPS were extracted from the sludge using a cation exchange resin (DOWEX Marathon C, Fluka 91973, Sigma-Aldrich, Na^+ form, 20-50 mesh size) as described in Frølund *et al*. (1996). For this purpose a sludge sample containing 0.5 g dry weight was centrifuged at 12 000 g at 4˚C (J-26 XP, Avanti) for 10 minutes. The sludge pellet was washed twice with Phosphate Buffer Saline (PBS) solution (0.328 g/L $Na_3PO_4 \cdot 12H_2O$ (Boom Laboratorium); 0.552 g/L $NaH_2PO_4 \cdot H_2O$ (VWR BDH Polabo); 0.526 g/L NaCl (VWR BDH Polabo); 0.0746 g/L KCl (Boom Laboratorium) at pH 7. Afterwards the pellet was homogenized using a multivortex shaking plate (Multi Reax, Heidolph) at 1200 rpm. For extraction of EPS the sludge was added to flasks containing 35 g resin and extracted in PBS for 2 h at 800 rpm (MR Hei-Max L, Heidolph). After the centrifugation step at 12 000 g (described above) the supernatants were collected to determine supernatant EPS concentrations.

Polysaccharides were measured according to the method described by Dubois *et al*. (1956) with glucose as standards. Proteins were determined using the Microplate procedure of the Pierce BCA (bicinchoninic acid) Protein Assay Kit (Thermo Scientific). Bovine serum albumin (BSA) protein was used for standard preparation. Dilutions were prepared with PBS. The Microplate containing the standards and samples was incubated for 30 min at 35 °C (Snijders Scientific). Optical density was measured at 570 nm in a spectrophotometer (Victor^3 1420 Multilabel Counter, Perkin Elmer).

Oxygen uptake rates (OUR) were determined according to standard methods Standard Methods for the Examination of Water and Wastewater (APHA. 1976) (2710 B). 100 mL of sludge sample was withdrawn from the reactor, to completely fill a flask and aerated to a dissolved oxygen concentration of 6 mgO_2/L after which oxygen depletion was measured (Hach, HQ 40d). From the OUR and VSS the specific oxygen uptake rate (SOUR) was calculated.

Concentrations of submicron particles (25 nm-450 nm) were measured in sludge supernatant, obtained after 30 minutes of settling. Before analysis the supernatant was filtered with a 0.45 µm filter (Cronus PTFE syringe filter). Nanoparticle Tracking Analysis (NTA) was performed using a NanoSight NS 500 instrument (NanoSight, Amesbury, UK), equipped with a conventional optical microscope and a Marlin charged coupled device (CCD) camera, and a sample unit (NS 500) with a laser light source. The capturing settings (shutter and gain) and analyzing settings were adjusted manually.

2.2.3 Membrane fouling experiments

Membrane fouling tests were carried out using stirred dead end filtration cells with a volume of 400 mL. Membrane circles (44 cm^2) were cut from commercially available Kubota plates and placed at the bottom of the test cell. Afterwards 100 mL of sludge was added to the cells. Each filtration test was carried out with a fresh and Milli-Q rinsed membrane. The test cells were stirred at 700 rpm (MR Hei-Max L, Heidolph) to minimize concentration polarization effects. The test cells were operated at a TMP of 1 bar. The filtrate was collect in beakers which were placed on a balance (PL 3001-S, Mettler Toledo). The balances were connected to a laptop for data recording (LabVIEW, National Instruments). Hydraulic resistance was calculated as follows:

$$R = \frac{TMP}{\eta J}$$

in which R is the total hydraulic resistance (m^{-1}), TMP is the transmembrane pressure (Pa), η is the dynamic viscosity of the permeate (Pa·s, and J is the flux ($m^3m^{-2}s^{-1}$).

Because the dynamic viscosity of the permeate is influenced by temperature, it was corrected for the temperature using the following equation:

$$\eta = 0.497(T + 42.5)^{-1.5}$$

where T is the temperature (°C) of the permeate.

2.3. Results

2.3.1 HL-MBR performance

Table 2.2 gives the characteristics of the screened wastewater that was fed to the five HL-MBR systems. The average contributions of suspended, colloidal and soluble COD to the total COD concentration of 614 ± 13 mg/L were 59, 24 and 17%, respectively.

Table 2.2. *Wastewater characteristics.*

characteristic	unit	value	COD fraction (%)
total COD	mg/L	614 ± 13	100
suspended COD	mg/L	363 ± 14	59
colloidal COD	mg/L	145 ± 9	24
soluble COD	mg/L	106 ± 1	17
TSS	g/L	0.25	-

Figure 2.1 plots the solids concentrations (TSS and VSS) of the HL-MBR concentrates as a function of the SRT. The "theoretical" TSS concentration that would result from accumulation of wastewater solids, i.e. the concentration assuming that no solids solubilization and/or degradation would take place, is also plotted. This theoretical TSS concentration was calculated by multiplying the TSS concentration of the wastewater of 0.25 g/L by the SRT/HRT ratio.

Figure 2.1 shows that between a SRT of 0.125-0.5 d measured TSS concentrations of 1.6-4 g/L were almost equal to this "theoretical" TSS concentration. From this it can be concluded that at these extremely short SRTs solids degradation was very limited or even absent. At SRTs above 0.5 d the difference between the "theoretical" and measured TSS concentrations became bigger, showing that solids degradation started to take place. At an SRT of 1 d still 80% of the wastewater TSS could be diverted to the concentrate, at a SRT of 5 d this already was reduced to only 30%. Solids degradation was accompanied by a decrease of the VSS/TSS ratio from 0.92 to 0.95 between an SRT of 0.125-0.5 d to 0.84 at an SRT of 1 d and 0.74 at an SRT of 5 d. This decrease in the VSS/TSS ratio can be explained by increasing microbial activity and associated uptake of inorganic substances such as ammonium, phosphate, calcium and magnesium.

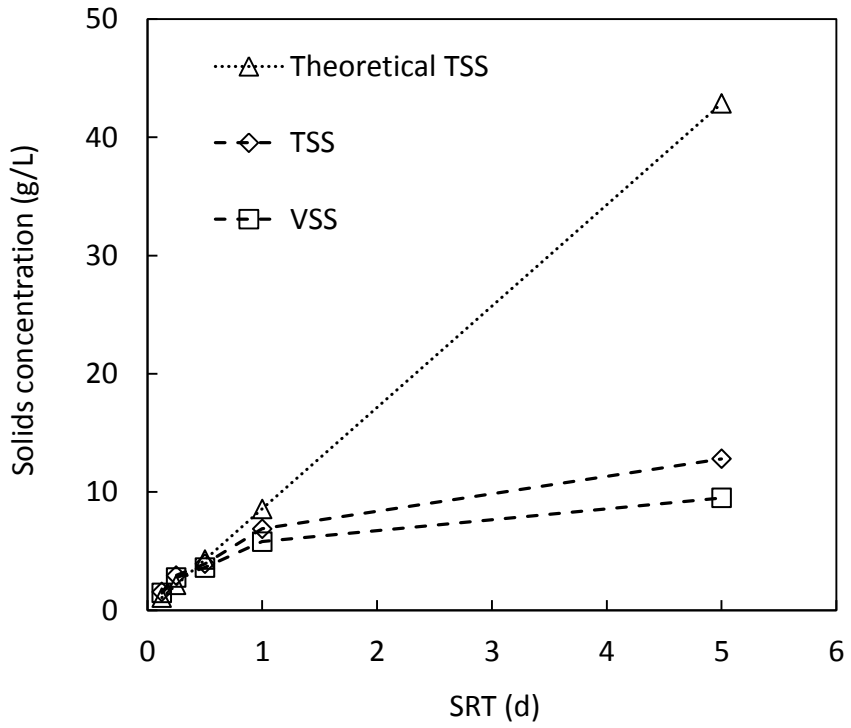


Figure 2.1. *TSS and VSS concentration in MBRs operated at respective short SRTs and theoretical TSS concentrations calculated assuming total membrane retention and no solubilisation of solids.*

Average (soluble) COD concentrations in the permeate of the HL-MBRs were 71, 74, 61, 61 and 43 mg/L at an SRT of 0.125, 0.25, 0.5, 1 and 5 d, respectively. For all SRTs this corresponds to a COD load distributed to the permeate below 10% of the wastewater COD load. Consistent COD mass balances over the reactors could not be made, because of the COD losses by the removal of the fouling layers on the membrane surface. To better quantify COD losses by microbial conversion and mineralization, separate sludge OUR measurements were conducted.

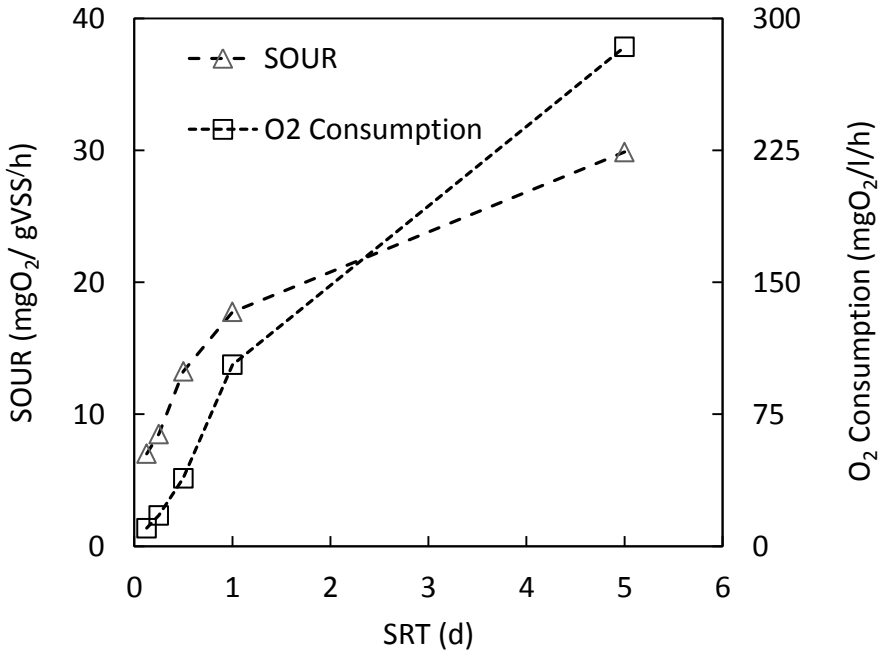


***Figure 2.2**. Oxygen consumption and specific oxygen uptake rates of the sludge from HL-MBRs operated at different short SRTs.*

Figure 2.2 shows OUR values for sludge obtained at the different SRTs from the five HL-MBR systems, together with the SOUR which was calculated from these. In all HL-MBRs, even at a SRT of 5 d, analyses of ammonium concentrations showed that nitrification was absent. Therefore, oxygen consumption was caused only by COD oxidation. A very low but significant oxygen consumption could be observed even at the shortest SRT of 0.125 d (OUR of 10.2 mgO_2/g VSS/h and SOUR of 7.0 mgO_2/g VSS/h). Most likely, this was caused by low numbers of (fast growing) microorganisms that utilized soluble and easily biodegradable COD supplied with the wastewater. The OUR and the SOUR rapidly increased with the SRT up to a SRT of 1 d. Above this SRT a more gradual increase was observed towards an OUR of 284 mgO_2/g VSS/h and a SOUR of 30 mgO_2/g VSS/h at a SRT of 5 d. The SOUR-SRT relation found is in agreement with what can be expected from Monod type microbial growth and associated substrate consumption. Theoretically, oxygen consumption corresponds with COD mineralization. From this, it was estimated that 1, 2, 4, 11 and 32% of the wastewater COD load was oxidized at an increasing SRT from 0.125, 0.25, 0.5, 1 to 5 d, respectively.

2.3.2 Bioflocculation efficiency

Figure 2.3 shows the suspended, colloidal and soluble COD fractions in the wastewater and in the concentrate of the HL-MBR reactors operated at different SRTs. Total COD concentrations in the

concentrate were 1155 ± 5, 2400 ± 17 , 6030 ± 55, 11415 ± 158 and 12600 ± 160 mg/L at an SRT of 0.125, 0.25, 0.5, 1 and 5 d, respectively.

The results show that at longer SRTs the fractions of colloidal and dissolved COD in the concentrate decreased while the fraction of suspended COD increased. An increase in the suspended COD fraction together with a decreased in the colloidal COD fraction indicates bioflocculation.

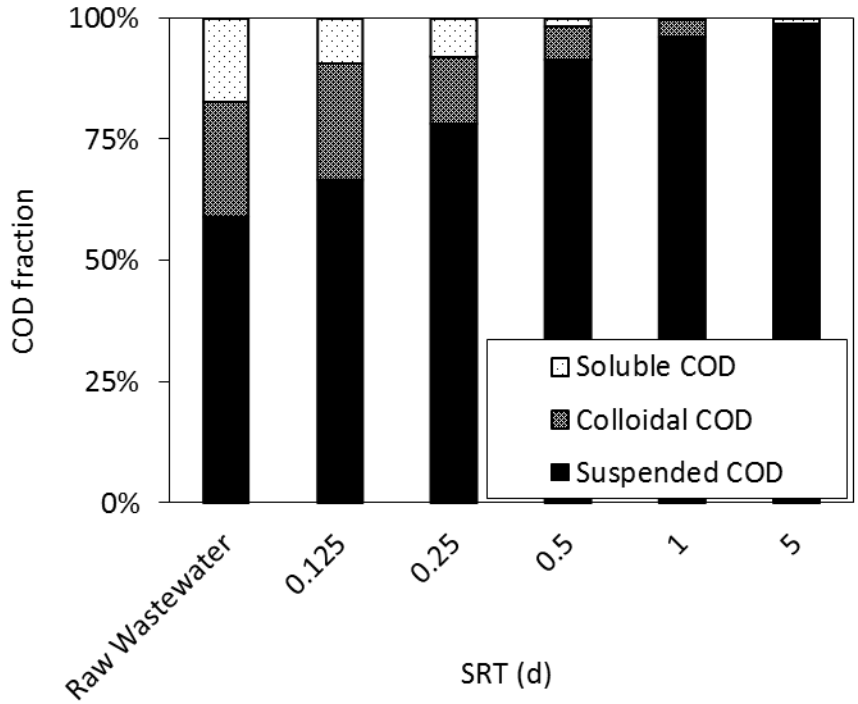


Figure 2.3. *COD fractions in the wastewater and in HL-MBR concentrate at different SRTs.*

Figure 2.3 demonstrates that longer SRTs promote flocculation. The suspended COD fraction increased from 59% in the wastewater (Table 2.2) to 67% at a SRT of 0.125 d, 78% at a SRT of 0.25 d and to more than 90% at a SRT of 0.5 d and longer. Improved flocculation at longer SRTs is further demonstrated by comparing the colloidal COD concentrations in the concentrates as a function of the SRT. At the shortest SRT of 0.125 d this concentration was 50% of the theoretical maximum, i.e. the concentration when all colloids in the influent are retained as free colloids in the concentrate. At an SRT of 1 d this already was below 10% and at a SRT of 5 d even less than 1%, which means that at longer SRTs the colloids became increasingly entrapped in the sludge matrix.

2.3.3 EPS concentration and composition

EPS, extracted from the five HL-MBR concentrates and from activated sludge samples from three selected municipal wastewater treatment systems, were measured as proteins and polysaccharides. Figure 2.4 shows their concentrations. In addition, protein and polysaccharide concentrations in the wastewater of the HL-MBR systems were measured to determine their maximum possible contribution to EPS proteins and polysaccharides in the concentrates of these reactors. The average concentration in the wastewater for EPS-proteins was 0.14 mg/L and for EPS-polysaccharides 0.07 mg/L. From this, it was estimated that EPS-proteins and EPS-polysaccharides originating from the wastewater only made up a maximum of 0.5% (at a SRT of 0.125 d) to 5% (at a SRT of 5 d) of the total EPS in the HL-MBR reactors. This shows that most of the EPS in these reactors were a result of microbial activity.

Figure 2.4 shows that protein-EPS in all MBRs were found at higher concentrations than polysaccharide-EPS, which is consistent with the literature data of Table 2.1. As the SRT of the HL-MBRs was prolonged from 0.125 d to 1 d, the total concentration of bound EPS increased, mainly caused by an increase of the protein concentration from 40 to 95 mg/gVSS. Probably this is related to an increasing microbial activity at longer SRTs and associated higher EPS-proteins production. The EPS-polysaccharides concentration only increased from 15 to 22 mg/gVSS. At SRTs above 1 d the opposite trend was observed. Bound EPS concentrations became significantly lower at longer SRTs, mainly caused by a decrease of the EPS-proteins concentration. Possibly, this decrease is a result of biodegradation of EPS-proteins, which became (relatively) more important at longer SRTs. Conversely, a clear trend could not be detected for EPS-polysaccharides. However, at SRTs beyond 30 days, relatively low EPS concentrations were found, possibly also due to biodegradation or by a lower production.

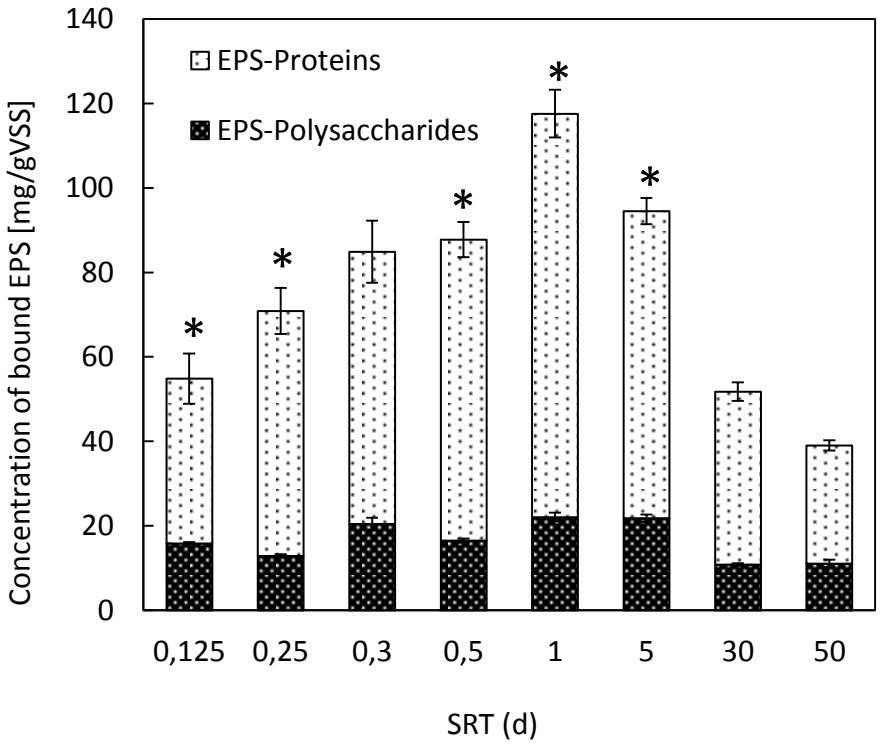


Figure 2.4. *Sludge bound EPS-Polysaccharide and EPS-Protein concentration in five HL-MBRs (*) and in A-stage sludge (SRT = 0.3 d), sludge from a pilot scale MBR (30 d) and a full scale MBR (50 d).*

In the HL-MBRs also supernatant EPS concentrations were measured. Figure 2.5 shows the distribution between these supernatant EPS and bound EPS as a function of the SRT. Interestingly, between SRTs of 0.125 and 1 d a strong shift in distribution took place with an increasing fraction of bound EPS (from 34 to 78% of the total EPS) and a decreasing fraction of supernatant EPS (from 66 to 22% of the total EPS). Above a SRT of 1 d this effect was less significant. Although more EPS were produced at longer SRTs (Figure 2.4), a higher fraction of these EPS apparently ended up in the sludge matrix. Possibly, this is caused by the higher solids concentrations at longer SRTs, providing more surface area for the EPS to attach to.

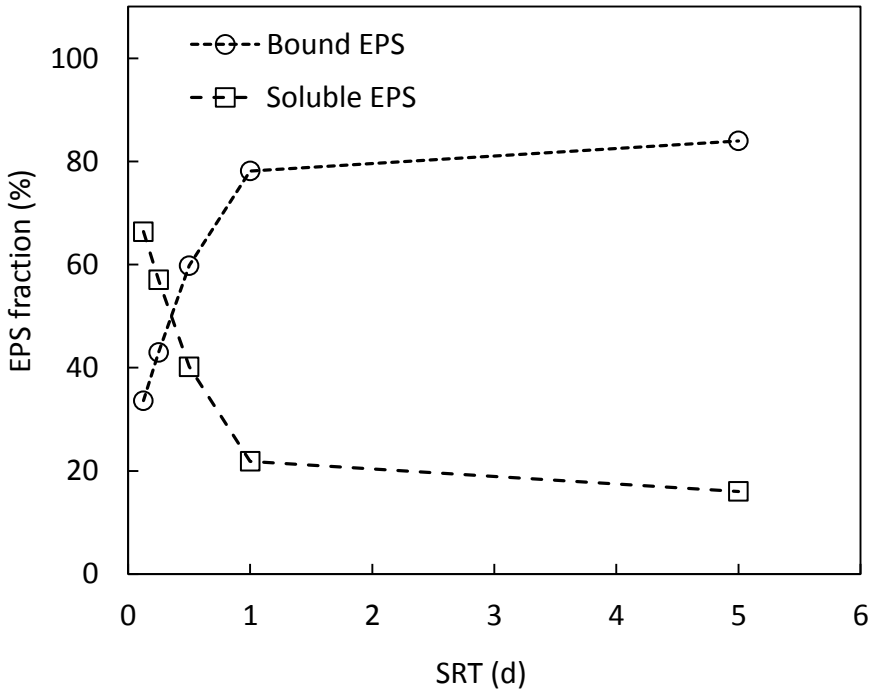


Figure 2.5. *Distribution between bound and supernatant EPS in HL-MBRs operated at different SRTs.*

2.3.4 Membrane fouling

A well performing bioflocculation process is important to avoid severe membrane fouling in MBR systems (Van den Broeck *et al*. 2010). Therefore, the impact of the HL-MBR sludges on membrane fouling was further examined in batch fouling tests. Figure 2.6 shows membrane resistances as a function of permeated water volume. The sludge sample from the HL-MBR operated at a SRT of 0.125 d, i.e. with the poorest bioflocculation performance, resulted in the highest membrane resistance, even though the sludge concentration in this HL-MBR was much lower than in the other HL-MBRs (Figure 2.1). Sludge samples taken from HL-MBRs operated at longer SRTs gave decreasing resistances although this effect of the SRT seemed to become less important.

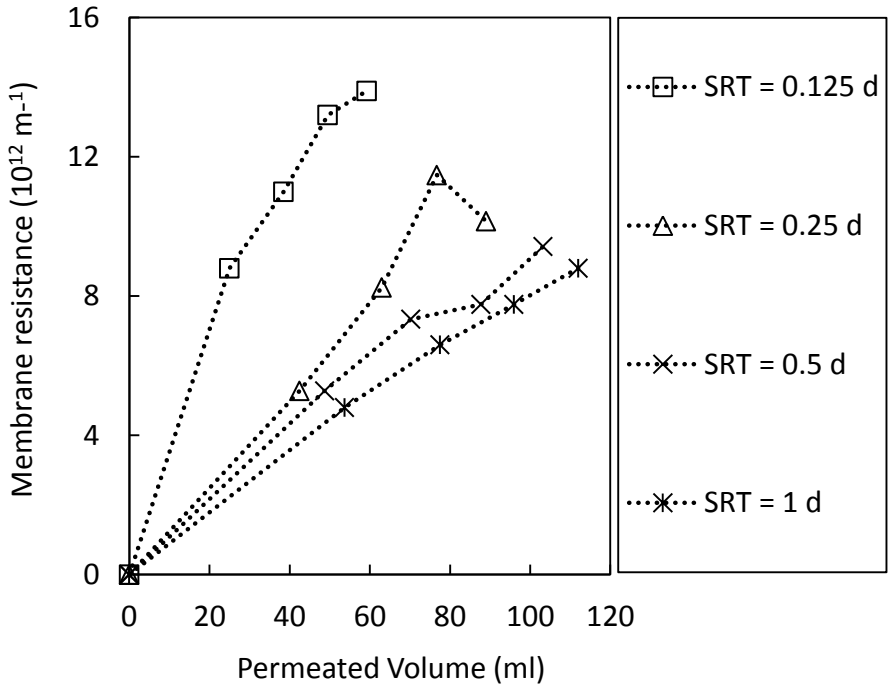


***Figure 2.6**. Membrane resistances as a function of filtration time for sludge of HL-MBRs operated at different SRTs.*

Often soluble and colloidal EPS are mentioned as the most important membrane foulants (Rosenberger *et al*. 2006; Defrance *et al*. 2000). Table 2.3 gives EPS concentrations in the supernatant of the sludges that were produced by the HL-MBRs. Whereas the membrane resistance was lower for longer SRTs, such a correlation with supernatant EPS concentrations could not be detected. In contrast, the concentration of submicron (25-450 nm) particles (Table 2.3) decreased from 9.2×10^7 particles/ml to only 0.02×10^7 particles/mL when the SRT was prolonged from 0.125 d to 1 d. The concentration of submicron particles therefore proves to be a better indicator of membrane fouling than the supernatant EPS concentration. It is appreciated however that this does not exclude the possibility that also supernatant EPS play a role in membrane fouling as they also can be present in the submicron particle range.

***Table 2.3**. Submicron particle and total supernatant EPS concentrations (EPS-proteins + EPS-polysaccharides) in the concentrates of HL-MBRs operated at different SRTs*

SRT	supernatant EPS concentration (mg/L)	submicron concentration (10^7 particles/mL)
0.125 d	158	9.17 ± 0.90
0.25 d	260	0.61 ± 0.08
0.5 d	206	0.49 ± 0.18
1 d	191	0.02 0.01

2.4 Discussion

2.4.1 Recovery of wastewater organics

The main objective of this study was to identify the SRT at which an optimum recovery could be achieved of wastewater organics with a HL-MBR, i.e. a SRT at which a well performing bioflocculation is combined with a minimum loss of wastewater organics by microbial oxidation of these organic. The results showed that such an optimum was achieved at a SRT between 0.5 and 1 d, with a concentrate which contained a suspended COD fraction of 92-96% (Figure 2.3) and a limited, estimated COD loss by microbial oxidation of 4-10% (Figure 2.2). At shorter SRTs even less COD was lost by mineralization, but the fraction of suspended COD also was significantly lower. SRTs longer than 1 d did result in higher fractions of suspended COD but mineralization losses became unacceptably high. Similar findings were made by Akanyeti *et al*. (2010) who studied bioflocculation in HL-MBRs and showed extensive flocculation of organic matter at very short SRTs but also found increased mineralization as SRT was prolonged. In their study they reported that 77-93% of total COD was suspended COD in HL-MBRs operated at 0.25-1 d and mineralization increased from 27% to 54%. Also they state that mineralization could be overestimated due to considerably amounts of COD which were removed from the membrane and not included in the estimation of mineralization values.

Although at a SRT between 0.5 and 1 d losses of COD by mineralization were very low and recovery of wastewater organics was very high, the COD concentration of 6.0-11.4 g/L is likely to be too low for economic conversion of the organic matter to methane gas or for their fermentation to i.e. volatile fatty acids. Lee *et al*. (2014) reviewed VFA production from several waste streams. In this study organic content reported for waste activated sludge and primary sludge used to produce VFAs varied between 5470 mgTOC/L and 22 838 mgCOD/L. It is expected that higher COD concentrations

can easily be achieved if shorter HRTs than 0.7 h are applied than in our experiments. However, the effect that this may have on the efficiency of sludge-water separation, either by a membrane or by settling, still needs to be assessed.

2.4.2 EPS concentration

The extracted EPS concentrations in this study (Figure 2.4) were comparable to values reported by i.e. Rudd *et al*. (1984) at similar SRTs, but were very different from concentrations reported by others such as Frølund *et al*. (1995), Nielsen *et al*. (1996) and Liu & Fang (2002) (Table 2.1). Apparently, it is difficult to compare EPS concentrations in the sludge of different treatment systems, even when they all are operated with municipal wastewater. This may be due to different substrate compositions of the municipal wastewater or to differences in environmental conditions and reactor configurations.

The experiments with the HL-MBRs showed that the SRT has a strong effect on the concentration of bound EPS, and in particular on the EPS-protein concentration (Figure 2.4). The increase of the EPS concentration that was observed between a SRT of 0.125 and 0.5 d probably was caused by more microbial activity at longer SRTs (Laspidou & Rittmann 2002; Ng & Hermanowicz 2005; Sheng *et al*. 2010). However, above a SRT of 1 d the EPS-proteins concentration decreased, either because of lower production rate or by an enhanced degradation rate. According to Witzig *et al*. (2002) a lower production rate can be caused by the reduced availability of easily biodegradable substrate at longer SRTs. Enhanced degradation of EPS at longer SRTs can be explained by a longer contact time between bacteria and these biopolymers (Masse *et al*. 2006). This is also supported by the fact that EPS can serve as a food source for bacteria, especially at lower loading rates (Obayashi & Gaudy Jr 1973). Finally, in general, the diversity of bacteria species increases at longer SRTs (Duan *et al*. 2009) and this also can stimulate EPS degradation as more species will be able to utilize the EPS as their food source.

2.4.3 EPS composition and bioflocculation

The SRT of the HL-MBRs only had little effect on the concentration of EPS-polysaccharides but a large effect on the EPS-protein concentration (Figure 2.4). A similar phenomenon was observed by Li & Yang (2007) and Wang *et al*. (2012) who reported higher concentrations of tightly and loosely bound proteins at increasing SRTs, while total EPS concentrations were independent from the SRT. Nielsen *et al*. (1997) described that the relative protein content of the biofilm in a biofilter increased with increasing biofilm age, most likely due to preferential hydrolysis of other fractions such as polysaccharides.

Proteins are more likely to be involved in electrostatic bounds with multivalent ions such as calcium and magnesium because of the high content of amino acids and thus may play a larger role in the bioflocculation process than polysaccharides (Laspidou & Rittmann 2002). This is also supported by Wilén *et al*. (2003), who showed a good correlation between the concentration of extracted proteins and the flocculation ability of activated sludge. Thus, the higher concentrations of proteins at prolonged SRTs very well may be responsible for the higher extent of flocculation. To our knowledge, little is known about the flocculation ability of specific single proteins and polysaccharides. For further studies it may therefore be important to characterize EPS proteins and EPS polysaccharides on a molecular basis. This would also give more detailed insight in the mechanisms involved in the bioflocculation process. Once EPS related proteins and polysaccharides are better characterized, it may be possible to draw conclusions about the relationship between their molecule structure and their bioflocculation ability.

2.4.4 Distribution of bound and free EPS

It was shown that between SRTs of 0.125 and 1 d a shift took place from supernatant EPS to bound EPS (Figure 2.5). This may have been caused by several mechanisms. At prolonged SRTs the particle concentration in the reactor increases, which provides more binding sites for free EPS. Also, multivalent cations such as calcium, iron and aluminum, which are naturally present in wastewater, could govern this process. Polymer-polymer interactions may also take place during floc formation, e.g. electrostatic bonding between oppositely charged functional groups in the EPS proteins and polysaccharides. In their model Tielen *et al*. (2013) showed the interaction between a positively charged amino acid chain of proteins with the negatively charged uronic acids of a polysaccharide (alginate). However, these mechanisms may be more important for biofilm formation than for floc formation, since in this study proteins were the major component of EPS.

2.4.5 Membrane fouling

A number of authors described a direct relationship between the level of soluble (<0.2 µm) and/or colloidal EPS (<12-25 µm) and membrane fouling in MBRs (Menniti & Morgenroth 2010; Rosenberger *et al*. 2006; Trussell *et al*. 2006). Other studies suggested that colloids and submicron particles (0.05-100 µm) largely (25-50%) determine the fouling potential of sludge (Bouhabila *et al*. 2001; Defrance *et al*. 2000; Wisniewski & Grasmick 1998). Also in this study a correlation was found between the concentration of submicron particles (25-450 nm) and membrane fouling. At longer SRTs bioflocculation improved (Figure 2.3) and this was accompanied by lower submicron particle concentrations (Table 2.3) and less severe membrane fouling (Figure 2.6). Submicron particles in the 25-450 nm range can easily approach the membrane surface at lower SRT, and subsequently cause

internal fouling and exponential progressive pore blocking of the Kubota membranes with a pore size in the range of 200-500 nm. This is followed by other fouling mechanisms such as gel-layer formation. Also in literature it was reported that improved bioflocculation gave less membrane fouling (Ivanovic *et al*. 2008).

In another study performed by Kappel *et al*. (2014) an MBR was operated with the same municipal wastewater and the same membranes, only at a longer SRT of 16 d compared to SRTs of 0.125-5 d in the present study. Sludge samples taken from the MBR operated at a SRT of 16 d gave approximately the same filterability as the sludge from the HL-MBR operated at a SRT of 0.5 d (Figure 2.6). Still, the fouling in the HL-MBR was much more severe, which can be explained by the higher (net) flux of 15 $L/m^2/h$ compared to a flux of 6.5 $L/m^2/h$ applied in the 16 d SRT MBR. This fouling is a bottleneck for the application of HL-MBR systems and can only be solved by installing more membrane surface area. Operation at longer HRTs is not an option, as this would result in COD concentrations which are too low for an economically feasible anaerobic conversion to methane or chemicals such as volatile fatty acids.

2.5 Conclusions

Bioflocculation in HL- MBRs was investigated at different SRTs with the objective to identify an optimum SRT that allows a maximum recovery of sewage organic matter. It was found that: The extent of organic matter flocculation increased from 59% at a SRT of 0.125 d to 98% at a SRT of 5 d. At a SRT of 0.5-1 d more than 90% of the COD in the concentrate was present in the form of suspended matter and the loss of sewage COD by microbial oxidation was limited to 4-10%, suggesting this SRT range as the best choice for recovery of sewage organics. At longer SRT of 5 d mineralization was too high (32%). When the SRT was prolonged from 0.125 d to 1 d the concentration of bound EPS increased, whereas it decreased at even longer SRTs. Together with an 63% increase in bound EPS concentration when SRT was prolonged from 0.125 d to 1 d the extent of flocculation increased by 30%, showing a strong impact of the EPS concentration on the bioflocculation process. Shorter SRTs result in more membrane fouling, which was caused by poor bioflocculation and was associated with high submicron particle concentrations.

Acknowledgement

This work was performed in the cooperation framework of Wetsus, centre of excellence for sustainable water technology (www.wetsus.nl). Wetsus is co-funded by the Dutch Ministry of Economic Affairs and Ministry of Infrastructure and Environment, the European Union Regional Development Fund, the Province of Fryslân and the Northern Netherlands Provinces. The authors like to thank the participants of the research theme "Membrane processes and operation for wastewater treatment and reuse" for the fruitful discussions and their financial support.

References

Agler, M., Wrenn, B., Zinder, S., Angenent, L., 2011. Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform. Trends in biotechnology 29, 70–78.

Ahmed, Z., Cho, J., Lim, B.-R., Song, K.-G., Ahn, K.-H., 2007. Effects of sludge retention time on membrane fouling and microbial community structure in a membrane bioreactor. Journal of Membrane Science 287, 211–218.

Akanyeti, I., Temmink, H., Remy, M., Zwijnenburg, A., 2010. Feasibility of bioflocculation in a high-loaded membrane bioreactor for improved energy recovery from sewage. Water science and technology 61, 1433–1439.

APHA., 1976. Standard Methods for the Examination of Water and Wastewater 14ed. APHA American Public Health Association.

Badireddy, A.R., Chellam, S., Gassman, P.L., Engelhard, M.H., Lea, A.S., Rosso, K.M., 2010. Role of extracellular polymeric substances in bioflocculation of activated sludge microorganisms under glucose-controlled conditions. Water research 44, 4505–4516.

Bossier, P., Verstraete, W., 1996. Triggers for microbial aggregation in activated sludge? Applied Microbiology and Biotechnology 45, 1–6.

Bouhabila, E.H., Ben Aim, R., Buisson, H., 2001. Fouling characterisation in membrane bioreactors. Separation and Purification Technology 22, 123–132.

Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J., Greenberg, E., 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280, 295–298.

Defrance, L., Jaffrin, M.Y., Gupta, B., Paullier, P., Geaugey, V., 2000. Contribution of various constituents of activated sludge to membrane bioreactor fouling. Bioresource Technology 73, 105–112.

Dignac, M.-F., Urbain, V., Rybacki, D., Bruchet, A., Snidaro, D., Scribe, P., 1998. Chemical description of extracellular polymers: implication on activated sludge floc structure. Water Science and Technology 38, 45–53.

Duan, L., Moreno-Andrade, I., Huang, C., Xia, S., Hermanowicz, S.W., 2009. Effects of short solids retention time on microbial community in a membrane bioreactor. Bioresource technology 100, 3489–3496.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P. t, Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Analytical chemistry 28, 350–356.

Fang, H.H., Jia, X., 1996. Extraction of extracellular polymer from anaerobic sludges. Biotechnology techniques 10, 803–808.

Finlayson, J., Liao, B., Droppo, I., Leppard, G., Liss, S., 1998. The relationship between the structure of activated sludge flocs and the sorption of hydrophobic pollutants. Water Science and Technology 37, 353–357.

Flemming, H.-C., Wingender, J., 2010. The biofilm matrix. Nature Reviews Microbiology 8, 623–633.

Frølund, B., Keiding, K., Nielsen, P., 1994. A comparative study of biopolymers from a conventional and an advanced activated sludge treatment plant. Water Science \& Technology 29, 137–141.

Frølund, B., Griebe, T., Nielsen, P., 1995. Enzymatic activity in the activated-sludge floc matrix. Applied microbiology and biotechnology 43, 755–761.

Frølund, B., Palmgren, R., Keiding, K., Nielsen, P.H., 1996. Extraction of extracellular polymers from activated sludge using a cation exchange resin. Water Research 30, 1749–1758.

Hernández Leal, L., Temmink, H., Zeeman, G., Buisman, C., 2010. Bioflocculation of grey water for improved energy recovery within decentralized sanitation concepts. Bioresource technology 101, 9065–9070.

Ivanovic, I., Leiknes, T., Odegaard, H., 2008. Fouling Control by Reduction of Submicron Particles in a BF-MBR with an Integrated Flocculation Zone in the Membrane Reactor. Separation science and technology 43, 1871–1883.

Jorand, F., Boue-Bigne, F., Block, J., Urbain, V., 1998. Hydrophobic/hydrophilic properties of activated sludge exopolymeric substances. Water science and technology 37, 307–315.

Kappel, C., Kemperman, A., Temmink, H., Zwijnenburg, A., Rijnaarts, H., Nijmeijer, K., 2014. Impacts of NF concentrate recirculation on membrane performance in an integrated MBR and NF membrane process for wastewater treatment. Journal of Membrane Science 453, 359–368.

Laspidou, C.S., Rittmann, B.E., 2002. A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass. Water Research 36, 2711–2720.

Lee, W.S., Chua, A.S.M., Yeoh, H.K., Ngoh, G.C., 2014. A review of the production and applications of waste-derived volatile fatty acids. Chemical Engineering Journal 235, 83–99.

Li, X., Yang, S., 2007. Influence of loosely bound extracellular polymeric substances (EPS) on the flocculation, sedimentation and dewaterability of activated sludge. Water Research 41, 1022–1030.

Liao, B., Allen, D., Droppo, I., Leppard, G., Liss, S., 2001. Surface properties of sludge and their role in bioflocculation and settleability. Water Research 35, 339–350.

Liu, H., Fang, H.H., 2002. Extraction of extracellular polymeric substances (EPS) of sludges. Journal of Biotechnology 95, 249–256.

Masse, A., Spérandio, M., Cabassud, C., 2006. Comparison of sludge characteristics and performance of a submerged membrane bioreactor and an activated sludge process at high solids retention time. Water Research 40, 2405–2415.

Menniti, A., Morgenroth, E., 2010. The influence of aeration intensity on predation and EPS production in membrane bioreactors. Water research 44, 2541–2553.

Morgan, J., Forster, C., Evison, L., 1990. A comparative study of the nature of biopolymers extracted from anaerobic and activated sludges. Water Research 24, 743–750.

Ng, H.Y., Hermanowicz, S.W., 2005. Membrane bioreactor operation at short solids retention times: performance and biomass characteristics. Water Research 39, 981–992.

Nielsen, P.H., Frølund, B., Keiding, K., 1996. Changes in the composition of extracellular polymeric substances in activated sludge during anaerobic storage. Applied microbiology and biotechnology 44, 823–830.

Nielsen, P.H., Jahn, A., Palmgren, R., 1997. Conceptual model for production and composition of exopolymers in biofilms. Water Science and Technology 36, 11–19.

Obayashi, A.W., Gaudy Jr, A.F., 1973. Aerobic digestion of extracellular microbial polysaccharides. Journal (Water Pollution Control Federation) 1584–1594.

Remy, M., van der Marel, P., Zwijnenburg, A., Rulkens, W., Temmink, H., 2009. Low dose powdered activated carbon addition at high sludge retention times to reduce fouling in membrane bioreactors. Water research 43, 345–350.

Rosenberger, S., Laabs, C., Lesjean, B., Gnirss, R., Amy, G., Jekel, M., Schrotter, J.-C., 2006. Impact of colloidal and soluble organic material on membrane performance in membrane bioreactors for municipal wastewater treatment. Water Research 40, 710–720.

Rudd, T., Sterritt, R., Lester, J., 1984. Complexation of heavy metals by extracellular polymers in the activated sludge process. Journal (Water Pollution Control Federation) 1260–1268.

Salehizadeh, H., Shojaosadati, S., 2001. Extracellular biopolymeric flocculants: recent trends and biotechnological importance. Biotechnology advances 19, 371–385.

Sheng, G.-P., Yu, H.-Q., Li, X.-Y., 2010. Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review. Biotechnology Advances 28, 882–894.

Sutton, P., Melcer, H., Schraa, O., Togna, A., 2011. Treating municipal wastewater with the goal of resource recovery. Water Science & Technology 63, 25–31.

Tielen, P., Kuhn, H., Rosenau, F., Jaeger, K.-E., Flemming, H.-C., Wingender, J., 2013. Interaction between extracellular lipase LipA and the polysaccharide alginate of Pseudomonas aeruginosa. BMC microbiology 13, 1–12.

Trussell, R.S., Merlo, R.P., Hermanowicz, S.W., Jenkins, D., 2006. The effect of organic loading on process performance and membrane fouling in a submerged membrane bioreactor treating municipal wastewater. Water Research 40, 2675–2683.

Wang, H., Deng, H., Ma, L., Ge, L., 2013. Influence of operating conditions on extracellular polymeric substances and surface properties of sludge flocs. Carbohydrate polymers 92, 510–515.

Van den Broeck, R., Van Dierdonck, J., Caerts, B., Bisson, I., Kregersman, B., Nijskens, P., Dotremont, C., Van Impe, J., Smets, I., 2010. The impact of deflocculation-reflocculation on fouling in membrane bioreactors. Separation and Purification Technology 71, 279–284.

Wilén, B.-M., Jin, B., Lant, P., 2003. The influence of key chemical constituents in activated sludge on surface and flocculating properties. Water research 37, 2127–2139.

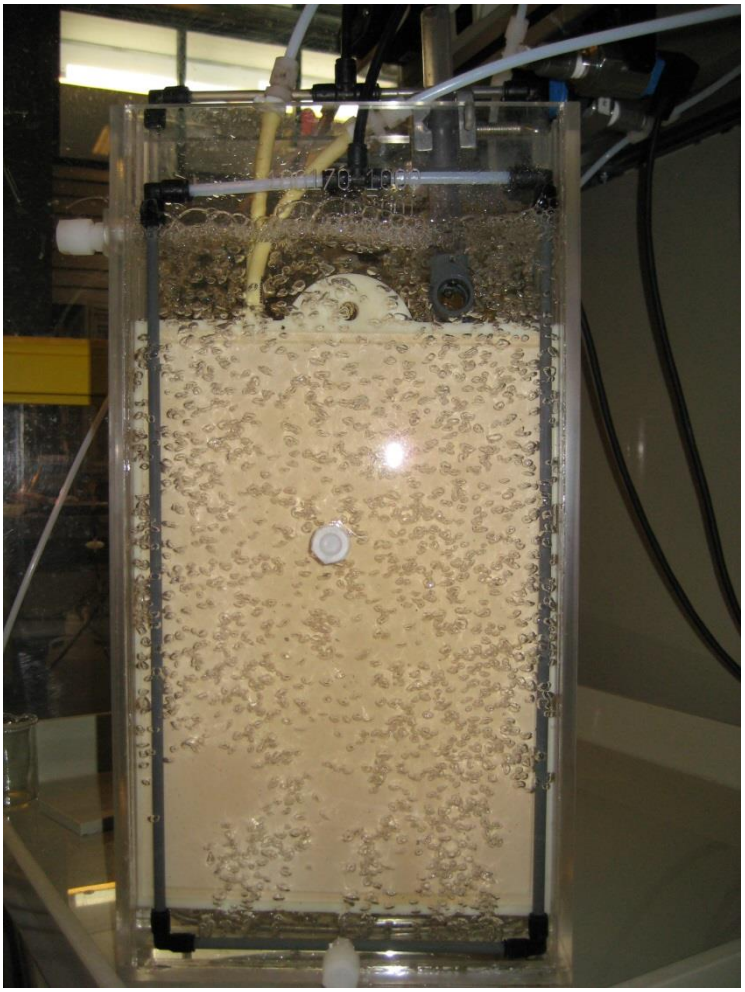
Wisniewski, C., Grasmick, A., 1998. Floc size distribution in a membrane bioreactor and consequences for membrane fouling. Colloids and Surfaces A: Physicochemical and Engineering Aspects 138, 403–411.

Witzig, R., Manz, W., Rosenberger, S., Krüger, U., Kraume, M., Szewzyk, U., 2002. Microbiological aspects of a bioreactor with submerged membranes for aerobic treatment of municipal wastewater. Water Research 36, 394–402.

Wuertz, S., Spaeth, R., Hinderberger, A., Griebe, T., Flemming, H., Wilderer, P., 2001. A new method for extraction of extracellular polymeric substances frombiofilms and activated sludge suitable for direct quantification ofsorbed metals. Water Science & Technology 43, 25–31.

3

Effect of Dissolved Oxygen Concentration on the Bioflocculation Process in High Loaded MBRs



Abstract

High-loaded membrane bioreactors (HL-MBRs), i.e. MBRs which are operated at extremely short sludge and hydraulic retention times, can be applied to flocculate and concentrate sewage organic matter. The concentrated organics can be used for energy recovery, or for the production of more valuable organic chemicals. Little is known about the effect of the dissolved oxygen concentration (DO) on this bioflocculation process. To examine this effect, two HL-MBRs were operated, respectively at a low (1 mg/L) and a higher (4 mg/L) DO. The higher DO resulted in a better flocculation efficiency, i.e. 92% of the colloidal COD in the sewage flocculated compared to 69% at the lower DO. The difference was attributed to a higher microbial production of extracellular polymeric substances at a DO of 4 mg/L and to more multivalent cations (calcium, iron and aluminum) being distributed to the floc matrix. In addition, the HL-MBR that was operated at a DO of 4 mg/L gave a bigger mean floc size, a lower supernatant turbidity, better settleability and better membrane filterability than the HL-MBR that was operated at a DO of 1 mg/L.

This chapter has been has been published as:

L. Faust, H. Temmink, A. Zwijnenburg, A.J.B. Kemperman, H.H.M. Rijnaarts, 2014. Effect of dissolved oxygen concentration on the bioflocculation process in high loaded MBRs, Water Research 66, 199-207.

3.1 Introduction

The organic compounds in municipal wastewater typically represent a chemical energy content of 1.9 kWh per m^3 (McCarty *et al*., 2011).Generally, this municipal wastewater is treated by activated sludge processes. These processes not only consume a considerable amount of energy for aeration (0.3-0.7 kWh per m^3 of wastewater according to (Metcalf and Eddy, 2003), but also mineralize the organic compounds and thus destroy their chemical energy. Recent interest in improving the sustainability of municipal wastewater treatment systems has provided the impetus for new process designs, addressing issues such as maximizing water and energy recovery and producing inorganic and organic fertilizers (Verstraete and Vlaeminck, 2011). In this context sewage organic matter should not be aerobically mineralized, but converted into energy carriers such as methane in anaerobic reactors (Sutton *et al*., 2011), electricity in microbial fuel cells or into more valuable organic compounds such as bioplastics (Freguia *et al*., 2010; Lee *et al*., 2014).

Because municipal wastewater is characterized by a low temperature, low organic strength and a high fraction of suspended and colloidal organic matter, a direct production of energy or more useful organic compounds is not feasible without an appropriate pre-concentration step. Such a step can be accomplished with inorganic coagulants and/or synthetic organic polymers (Mels *et al*., 2001). However, high coagulant and flocculant costs, and their suspected inhibition of anaerobic conversion processes are serious disadvantages. In addition, side products from synthetic polymers can be toxic to humans (Salehizadeh and Shojaosadati, 2001). Also direct micro- or ultrafiltration of municipal wastewater is possible. However, these membrane processes suffer from severe membrane fouling with associated high energy consumption (Al-Malack and Anderson, 1997). A better option would be bioflocculation of the sewage organic suspended and colloidal matter, followed by settling or membrane filtration (Akanyeti *et al*., 2010; Faust *et al*., 2014; Hernández Leal *et al*., 2010). In particular the combination of bioflocculation and membrane filtration could be attractive because this not only concentrates the organic matter, but also produces a nutrient containing and particle free effluent, which can be used as irrigation water (Ravazzini *et al*., 2005).

During bioflocculation microorganisms partly consume soluble biodegradable pollutants and excrete polymers that induce flocculation of colloidal and suspended wastewater particles. Because in this manner smaller particles aggregate into bigger particles, membrane fouling is considerably reduced compared to direct membrane filtration (Ivanovic *et al*., 2008). When operated at very short sludge retention times (SRT, typically 0.1-0.5 d), in combination with very short hydraulic retention times (HRT, typically below 1 h), high concentrations of organic matter can be produced while (aerobic) mineralization of organic matter can be minimized to less than 10% (Faust *et al*., 2014).

The dissolved oxygen concentration (DO) is an important operational parameter for bioflocculation. It has an effect on the flocculation process itself, the efficiency of particle separation (membrane fouling or settleability) and on energy consumption. Studies towards the effect of DO on bioflocculation were mostly conducted by submitting activated sludge samples from reactors operated at long SRTs to short events of oxygen shortage. Generally, this resulted in an increase of the concentrations of biopolymers and ions in the bulk water (Zhang and Allen, 2008) and in smaller floc sizes (Guo *et al*., 2009). Several mechanisms have been proposed that can explain this deflocculation behavior at low DO. A reduced aerobic activity at low DO may result in a slower production or faster anaerobic degradation of the extracellular polymeric substances (EPS) that are needed to accomplish bioflocculation (Rasmussen *et al*., 1994; Starkey and Karr, 1984; Wilén *et al*., 2000). Also microbial reduction of Fe(III) to Fe(II) at low DO levels was proposed as an important mechanism causing deflocculation because Fe(III) yields stronger cation bridges between EPS than Fe(II) (Caccavo *et al*., 1996; Rasmussen and Nielsen, 1996). Other studies mainly focused on a negative role of filamentous bacteria that start to grow excessively at low DO levels on the bioflocculation process (Wilén and Balmér, 1999).

Previous experiments with high loaded membrane bioreactors (HL-MBR), in which the combination of bioflocculation and membrane filtration was investigated, all were performed at relatively high DO concentrations (> 4 mg/L) (Faust *et al*., 2014). In the present study, operation of a HL-MBR at a high DO concentration of 4 mg/L was compared to operation at a low DO concentration of 1 mg/L with respect to flocculation behavior, sludge settleability and sludge filterability. Relatively high DO concentration of 4 mg/L were chosen to avoid the development of anaerobic zones in the sludge flocs, because those anaerobic zones could inhibit the bioflocculation process as explained above. A DO concentration of 1 mg/L was chosen, because it was assumed to not yet be limiting for aerobic growth and may open the possibility to operated HL-MBRs with reduced energy requirements, compared to e.g. the operation at DO of 2 mg/L as commonly done in full scale wastewater treatment plants. To explain mechanistic differences in flocculation behavior also EPS concentrations and relevant cation concentrations were measured. To avoid differences in shear, both reactor systems were operated at the same gas flow rate, but with different mixtures of oxygen and nitrogen gas.

3.2 Material and Methods

3.2.1 Lab scale MBR set-up and sampling procedure

Two lab-scale HL-MBRs were operated in parallel. Details about the reactor design can be found in Akanyeti *et al*. (2010) and Hernández Leal *et al*. (2010). The reactors were operated at a SRT of 0.5 d and a HRT of 1 h. In each reactor two submerged chlorinated polyethylene flat sheet membranes (Kubota Corporation) were used, each with an effective surface area of 0.124 m^2 and a nominal pore size of 0.2 µm. The permeate pumps (Masterflex L/S, Cole-Parmer) were operated in cycles with 15 minutes of permeate extraction followed by 5 minutes of relaxation, giving a net flux of 10.5 $L/m^2/h$. Pressurized air and a mixture of pressurized air and N_2 were used for mixing and aeration. In this manner the desired DO concentrations could be maintained at the same gas flow rates. Two reactors were operated: one at low DO (LDO) concentration of 1 mg/L and one at a high DO (HDO) at a concentration of 4 mg/L. The reactors were operated at room temperature using screened (5 mm) municipal wastewater as the influent. Three experimental runs were conducted, each consisting of 14 d reactor operation with real wastewater. In the first two runs, chemical oxygen demand (COD) and total and volatile suspended solids (TSS, VSS) data were collected at the end of the experimental period (day 13 and day 14), to document the flocculation process and biomass concentration in the reactors. After confirming that the LDO reactor always showed lower flocculation efficiency (based on COD data, see table 3.1) at similar biomass concentrations, compared to the HDO reactor, a third experimental run was conducted measuring additional parameters such as EPS concentration, cation concentration, particle size distribution (PSD), supernatant turbidity, sludge volume index (SVI), oxygen uptake rate (OUR) to investigate the mechanisms responsible for the observed lower flocculation efficiency in the LDO reactor. The third experimental run also consisted of 14 d. Samples for COD (triplicates), TSS, VSS and supernatant turbidity (duplicates) and cation analysis were taken at day 12. Samples for EPS extraction (duplicates) and particle size distribution measurement (triplicates) were taken at day 13. SVI, OUR and fouling analysis was conducted at day 14. All samples were analyzed directly after they were taken. The sampling period was kept as short as 3 days to avoid interference of changing wastewater compositions.

3.2.2 Chemical analyses

COD was measured using Dr. Lange test kits (LCK, Hach Lange), heated in a thermostat (HT 200S, Hach Lange) to the desired temperature and analyzed in a spectrophotometer (DR 3900 VIS spectral photometer, wavelength range 320-750 nm). For fractionation sludge (concentrate) samples were subsequently paper filtered (Whatman Black Ribbon 589/1, 12–25 µm) and membrane filtered

(Cronus PTFE syringe filter, nominal pore size of 0.45 µm). The difference between total COD and paper filtered COD will be referred to as suspended COD, the difference between paper and membrane filtrate COD as colloidal COD and the membrane filtrate COD as soluble COD.

Total suspended solids (TSS), volatile suspended solids (VSS) and sludge volume index (SVI) were all determined according to standard methods 2540 D, 2540 E and 2710 D (APHA., 1976).

Supernatant EPS were obtained after centrifugation of 30 mL sample at 12, 000 g for 10 minutes. From the obtained pellets after centrifugation bound EPS were extracted using a cation exchange resin (DOWEX Marathon C, Fluka 91973, Sigma-Aldrich, Na^+ form, 20-50 mesh size) as described in Faust *et al*. (2014).

Polysaccharides were measured according to the method described by Dubois *et al*. (1956) with glucose as standards. Proteins were determined using the Microplate procedure of the Pierce BCA (bicinchoninic acid) Protein Assay Kit (Thermo Scientific). Bovine serum albumin (BSA) protein was used for standard preparation. Dilutions were prepared with phosphate buffer saline (PBS). The Microplate containing the standards and samples was incubated for 30 minutes at 35 °C (Snijders Scientific).

Oxygen uptake rate (OUR) in the HL-MBR reactors was determined according to the standard method 2710 B. Sludge (concentrate) sample was withdrawn from the reactor to fill up a 100 mL flask and aerated to a DO concentration of 6 mg/L (Hach, HQ 40d), after which oxygen depletion was measured in time (Hach, HQ 40d). Specific oxygen uptake rates (SOUR) were calculated as milligram of oxygen consumed per gram of VSS per hour. From COD mass balances and oxygen consumption rate, the fractions of oxidized COD were calculated.

For the analysis of cations, concentrate pellets of known weight were collected after centrifugation. Together with 10 mL of HNO_3 acid at 65% (VWR BDH Prolabo) these pellets were added to a Teflon vessel and subjected to digestion at 180 °C and high pressure for 45 minutes (Ethos One, Milestone). After digestion the samples were diluted to a final acid concentration of 1% HNO_3 and ions were measured using inductively coupled plasmaoptical emission spectroscopy (ICP-OES,Perkin Elmer, type Optima 5300 DV).

3.2.3 Physical analyses

Turbidity of concentrate supernatant, collected after 30 minutes of settling, was determined in Nephelometric Turbidity Units (NTU) in a turbidity meter (2100N IS, Hach) calibrated in the range from 0.1 NTU to 1000 NTU.

Diluted concentrate samples were measured in a particle size and shape analyzer (DIPA 2000 Particle Analyzer, Donner Technologies). Samples were added to a 2 mL cuvette with magnetic stirrer and laser channel measurements were carried out in triplicate using the time of transition method at 95% confidence level. Results are reported as number based particle size distribution.

3.2.4 Membrane fouling experiments

Membrane fouling tests were carried out using stirred dead end filtration cells with a volume of 400 mL. Membrane samples (44 cm^2), cut from commercially available Kubota chlorinated *polyethylene* plates, were placed in the test cell and rinsed with MilliQ-water prior to sample filtration. Each filtration test was carried out with a new and Milli-Q rinsed membrane. The test cells were stirred at 700 rpm (MR Hei-Max L, Heidolph) to minimize concentration polarization effects and operated at TMP of 1 bar. The filtrate was collect in beakers which were placed on a balance (PL 3001-S, Mettler Toledo) connected to a laptop were data was recorded (LabVIEW, National Instruments). Fouling tests were carried out with the concentrate and with the separated supernatant. For supernatant separation, concentrate was allowed to settle for 30 minutes in a graduated cylinder and afterwards supernatant samples were taken. Hydraulic resistance was calculated as follows:

$$R = \frac{TMP}{\eta J}$$

in which R is the total hydraulic resistance (m^{-1}), TMP is the trans membrane pressure (Pa), η is the dynamic viscosity of the permeate (Pa s) and J is the flux ($m^3/m^2/s$). The dynamic viscosity of the permeate was corrected for the temperature using the following equation:

$$\eta = 0.497(T + 42.5)^{-1.5}$$

where T is the temperature (°C) of the sludge mixture or the permeate.

3.3. Results

3.3.1 Wastewater and HL-MBR concentrate and permeate characteristics

Average total, suspended, colloidal and soluble COD concentrations of the wastewater respectively were 515 ± 0 mg/L, 311 ± 1 mg/L (60% of total COD), 116 ± 4 mg/L (23% of total COD) and 88 ± 1 mg/L (17% of total COD) mg/L. Table 3.1 gives the most important characteristics of the concentrate produced by the LDO and HDO reactor, their permeate and of the COD removal efficiency.

Table 3.1. HL-MBR concentrates and permeate characteristics.

		HL-MBR concentrate	
	units	**LDO (1 mg/L)**	**HDO (4 mg/L)**
SRT	d	0.5	0.5
HRT	h	1	1
DO	mg/L	1	4
Wastewater COD	mg/L	**515** *(312)(505)*[a]	**515** *(312)(505)*[a]
Total COD	mg/L	**5200** *(3676)(3136)*[a]	**5948** *(3855) (5340)*[a]
Suspended COD	mg/L	**4487** *(3523)(2873)*[a]	**5713** *(3774) (5159)*[a]
Colloidal COD	mg/L	**434** *(48) (173)*[a]	**109** *(27)(75)*[a]
Soluble COD	mg/L	**198** *(105)(106)*[a]	**115** *(84)(91)*[a]
TSS	g/L	**3.65** *(3.22)(2.06)*[a]	**3.49** *(3.95)(2.01)*[a]
VSS	g/L	**2.78** *(2.28)(1.22)*[a]	**2.61** *(3.06)(1.17)*[a]
VSS/TSS	%	**76** *(70)(59)*[a]	**75** *(77)(58)*[a]
OUR	$mgO_2/L/h$	37	79
Mineralization*	%	7	15
SVI	mL/g	75	59
Turbidity (supernatant)	NTU	155 ± 15	54 ± 8
Permeate COD	mg/L	59 ± 1	39 ± 1
COD removal	%	88 ± 1	92 ± 1

[a] Numbers in brackets represent data from experimental run 1 and 2 (see section 2.1)

*Calculated from the OUR

The COD mass balance, calculated from wastewater, concentrate and permeate COD concentrations, showed a gap of –5% for the LDO reactor and +3% for the HDO reactor. In particular for the HDO reactor this was considered inaccurate. At least some loss of COD by microbial oxidation should have

taken place and this loss was expected to be higher in the HDO reactor than in the LDO reactor. This was also confirmed by the OUR data, which showed a higher microbial activity in the HDO reactor (79 mg O_2/L/h) than in the LDO reactor (37 mg O_2/L/h). From the OUR data it was calculated that in the LDO reactor 7% of the wastewater COD load was oxidized and in the HDO reactor 15%. Although more COD was oxidized in the HDO reactor, the COD concentration of the HDO concentrate still was higher (5948 ± 12 mg/L) than the COD concentration of the LDO concentrate (5200 ± 83 mg/L). This can be explained by the lower (soluble) COD concentration in HDO permeate of 39 ± 1 mg/L compared to the COD concentration in the LDO permeate of 59 ± 1 mg/L. The COD removal efficiencies that were achieved by the LDO and HDO reactor were 88% and 92%, respectively.

3.3.2 Bioflocculation

Bioflocculation was evaluated based on concentrate COD fractions (Table 3.1 and Figure 3.1), turbidity of the supernatant fraction of these concentrates (Table 3.1) and on particle size distribution (Figure 3.2). The suspended and colloidal COD fractions of the wastewater were 60% and 23%, respectively. In the concentrate these fractions were 86% and 8% in the LDO reactor, and 96% and 2% in the HDO reactor. Based on the mass loads of colloidal COD in the wastewater and concentrate, flocculation efficiencies for colloidal COD were calculated of 69% for the LDO reactor and 92% for the HDO reactor. Thus, in both reactors a considerable amount of colloidal COD was transformed into suspended COD, with the HDO reactor achieving the highest extent of bioflocculation. This last observation was also confirmed by the lower turbidity of the supernatant fraction in the HDO reactor of 54 NTU compared to 155 NTU of the LDO reactor.

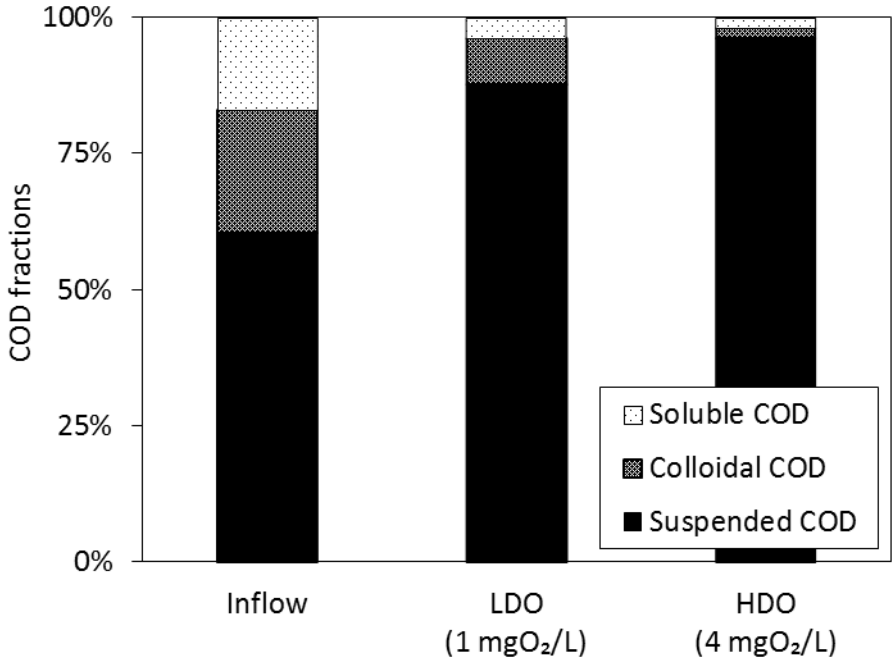


Figure 3.1. *COD fractions of wastewater and reactors operated at different DO concentrations.*

Figure 3.2 shows number based particle size distributions of LDO and HDO concentrate. The average particle concentration in the LDO reactor was 6.0×10^4 mL^{-1}. As expected, in the HDO reactor this concentration was much lower, i.e. 2.7×10^4 mL^{-1}. Compared to the LDO concentrate, the HDO concentrate contained a significantly higher percentage of particles with a size above 8-10 μm. At smaller particle sizes the opposite trend was observed. The mean number based particle size of the HDO concentrate was 8.2 ± 0.9 μm, which is approximately twice the mean particle size of the LDO concentrate of 3.9 ± 0.3 μm. A similar trend towards bigger particles at higher DO levels also was observed by Wilén and Balmér (1999). The mean number based particle size of the supernatant was 2.1 ±0.1 μm for the HDO supernatant and in the LDO supernatant it was with 1.9 ±0.2 μm slightly lower.

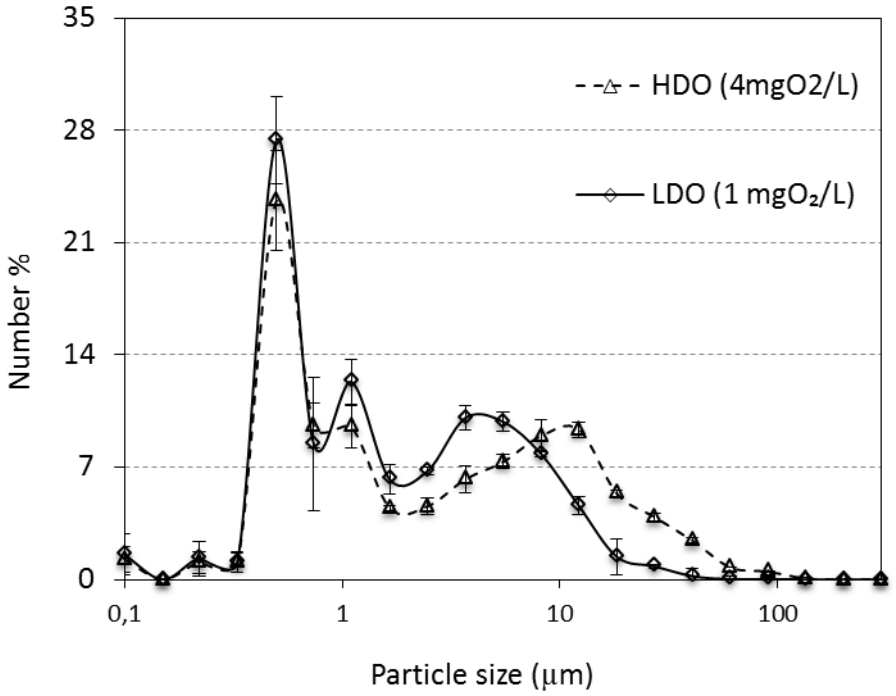


Figure 3.2. *Number based particle size distribution of HL-MBR concentrate of the LDO and HDO reactor.*

3.3.3 EPS and cations

As was mentioned earlier, EPS and multivalent cations both play an important role in bioflocculation. Figure 3.3 shows the concentrations of proteins and polysaccharides that were extracted from the solid fraction of the concentrate. In the following these will be referred to as bound EPS. Figure 3.3 also shows the concentrations of proteins and polysaccharides in the supernatant of the concentrates. In both reactors, 81-84% of the total amount of EPS was present as bound EPS and 16-19% as supernatant EPS. Concentrations in the HDO reactor of bound as well as supernatant EPS (175 mg/gVSS and 87 mg/L) were significantly higher compared to the LDO reactor (122 mg/gVSS and 80 mg/L). All of these EPS concentrations were higher than concentrations found in earlier experiments with the same type of wastewater and at similar SRTs, but fit well into a range of values reported in literature (Faust *et al*., 2014). In both more than 80% of the bound EPS were composed of proteins. The contribution of proteins to the supernatant EPS was considerably lower: 77% of the supernatant EPS in the LDO reactor consisted of proteins and in the HDO reactor this was 61%.

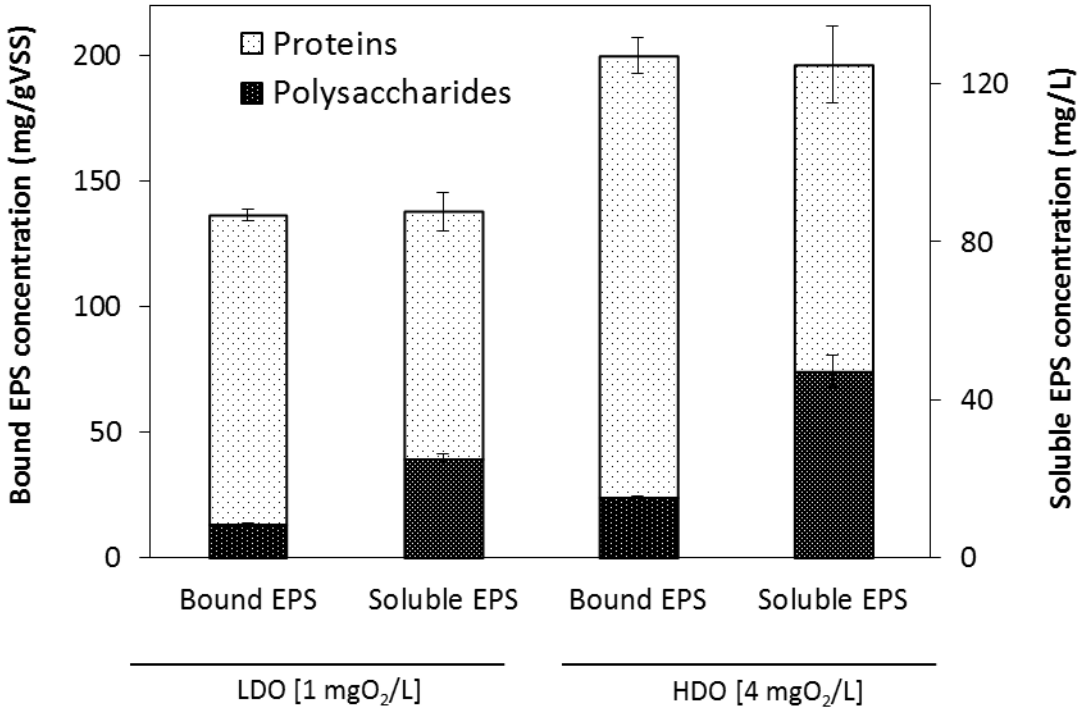


Figure 3.3. *EPS-Proteins and polysaccharide concentrations in the concentrate and the supernatant of HL MBRs operated at different DO concentrations.*

Multivalent cations such as calcium, iron and aluminum form bridges between the negatively charged EPS, and in this manner help to accomplish bioflocculation (Bruus *et al*., 1992; Higgins and Novak, 1997; Park, 2002). Table 3.2 gives solid bound and supernatant concentrations for these cations. In both concentrates, calcium was found at the highest concentrations, followed by iron and aluminum. For all three cations solid bound concentrations were slightly higher in the HDO reactor than in the LDO reactor, while for the supernatant iron and aluminum concentrations the opposite trend was observed. For calcium and iron the measured differences were above the range of the instrumental error of 0.65% for calcium and 0.43% for iron. For aluminum, however, the observed differences were minor and fell into the instrumental error of 0.71%.

For calcium the measured supernatant concentrations were similar in both systems. These observations show that a greater portion of the cations were distributed to the solids in the HDO than in the LDO reactor, which was in line with the higher bound EPS concentration in the HDO reactor. Most likely the cations bridged with EPS and where consequently incorporated into the sludge matrix according to the cation bridging theory explained above.

Table 3.2. *Ca, Fe and Al concentrations in the concentrate and supernatant of the HL-MBR reactors*

	Solid bound concentration mg/g TSS			Supernatant concentration mg/L		
	Ca	Fe	Al	Ca	Fe	Al
LDO	18.8	11.6	3.4	76.1	0.14	0.05
HDO	19.9	13.1	3.6	76.1	0.10	0.04

3.3.4 Settleability and filterability

Both concentrates exhibited a very good settleability, as indicated by their low SVI values of 75 and 59 mL/g TSS for the LDO and HDO concentrate, respectively. However, in particular for the LDO reactor, the supernatant remaining after 30 minutes of settling was very turbid and contained a relatively high concentration of colloidal COD (Table 3.1).

Filterability of the concentrates was examined in dead-end membrane filtration tests. These tests were also carried out with the supernatant fractions of the concentrates obtained after 30 minutes of settling. Figure 3.4 shows the resistances of these samples as a function of the permeated volume. For both reactors, filtration of the supernatant fraction gave a higher resistance than filtration of the whole concentrate. Furthermore, resistances obtained with concentrate and supernatant of the LDO reactor were considerably higher compared to resistances obtained with similar samples taken from the HDO reactor. More excessive fouling by LDO concentrate than by HDO reactor concentrate is also in line with the amount of soluble COD that was rejected by the membranes in these reactor (Table 3.1). In the LDO reactor the difference between soluble COD in the reactor and in the permeate was 198-59 = 139 mg/L (70% rejection), whereas in the HDO reactor this difference only was 115-39 = 76 mg/L (66% rejection).

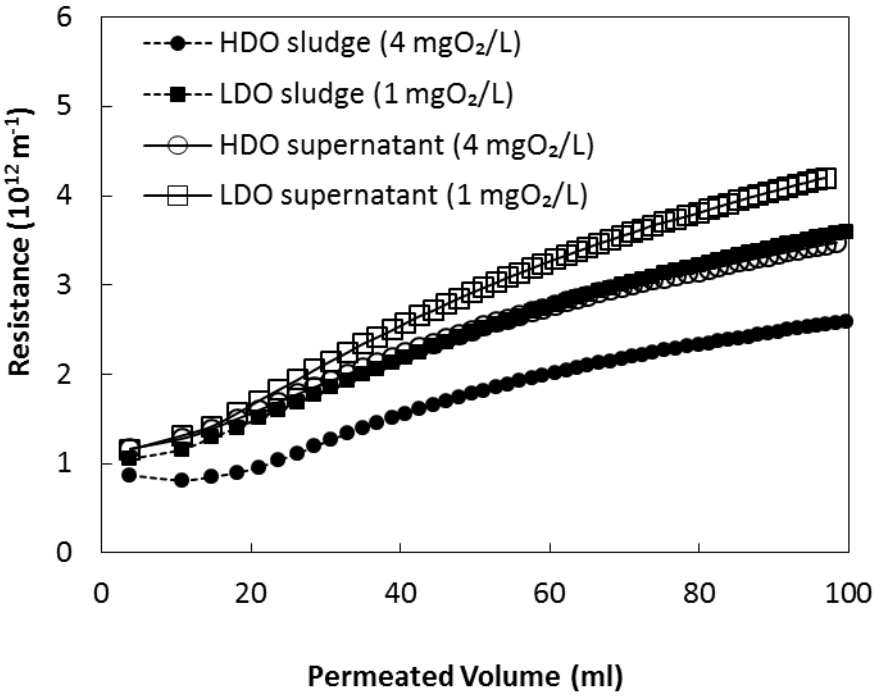


Figure 3.4. *Membrane resistances as a function of permeated volume during dead-end filtration of concentrate and supernatant of the HDO and LDO reactor.*

3.4 Discussion

3.4.1 Bioflocculation in relation to EPS and multivalent cations

It is widely accepted that the functional groups of EPS, such as amino-, carboxyl- and phosphate groups, help to flocculate wastewater particles and maintain floc stability (Eriksson and Alm, 1991; Liao *et al*., 2002). A relationship between EPS and flocculation was also observed by Wilén *et al*. (2003). They studied the flocculation ability of sludge from seven different full-scale wastewater treatment plants and found a positive correlation between flocculation and the amount of EPS-proteins that was extracted from the sludge. In the present study EPS-proteins and polysaccharides were measured in the concentrates produced by two HL-MBR reactors and in the supernatants of the corresponding concentrates. Proteins were the dominant compounds contributing to EPS. However, the contribution of polysaccharides was much higher in the supernatant (25% in the LDO reactor and 37% in the HDO reactor) than in the flocs (approximately 10% in both reactors). This suggests that EPS polysaccharides are more prone to shear and/or have a weaker bioflocculation capability than the EPS proteins. This was also shown by Sheng *et al*. (2006), who observed that mainly

polysaccharides sheared off from a municipal activated sludge after applying turbulent shear by a paddle mixer.

During bioflocculation multivalent cations are responsible for the binding between the negatively charged EPS and (overall) negatively charged wastewater particles. This study showed that enhanced bioflocculation correlated with higher EPS concentrations and more uptake of the multivalent cations calcium, iron and aluminum by the sludge flocs. The consequence for HL-MBRs is that for wastewaters low in concentrations of these cations the concentrate would become weak and dispersed, as was also suggested for conventional activated sludge systems by (Park, 2002). This was also reported by Jin *et al*. (2006), who studied sludge from seven wastewater treatment plants and reported that higher concentrations of cations gave an improved settleability and compressibility. The important role of cations such as calcium also was demonstrated by others, showing that extraction of calcium ions resulted in dispersion of activated sludge flocs (Bruus *et al*., 1992; Sheng *et al*., 2006).

3.4.2 Impact of DO on bioflocculation

Comparing the COD fractions (Figure 3.1), turbidity (Table 3.1) and particle size distribution (Figure 3.2) of the two HL-MBR reactors all showed that a DO of 4 mg/L gave a better bioflocculation than a DO of 1 mg/L. The mean particle size in the HDO reactor was approximately twice as large as in the LDO reactor. Also Guo *et al*. (2009) and Yoon Kim *et al*. (2006) suggested a trend towards bigger floc sizes at high DO levels compared to low DO levels. Wilén and Balmér (1999) reported less compact and more irregularly shaped flocs when low DO levels were applied. This lower compactness was attributed to excessive growth of filamentous bacteria at low DO concentrations. However, even though in both reactors filamentous bacteria were observed, microscopic observations did not reveal a significant difference in the number of filamentous bacteria between the two reactors. Possibly the observations made by Wilén and Balmér (1999) can be explained by differences in shear, caused by different air flow rates to achieve the desired DO levels. In contrast, in the LDO and HDO reactor the gas flow rate and shear conditions were exactly the same. In addition, these reactors were operated at extremely short SRTS, which may have caused a different behavior with respect to growth of filamentous bacteria.

Improved bioflocculation at a DO of 4 mg/L compared to a DO of 1 mg/L correlated with higher EPS concentrations and more multivalent cation concentrations (calcium, iron and aluminum) being distributed to the flocs. This implies that for bioflocculation EPS production and the presence of a sufficient amount of such cations are equally important. Laspidou and Rittmann (2002) suggested that the production of EPS is correlated with the substrate utilization rate. When comparing the OUR

in the HDO and LDO reactors, the OUR of the HDO concentrate was twice as high, indicating that also the substrate utilization rate was higher in the HDO reactor. This is in agreement with more removal of soluble COD by the HDO reactor: the (soluble) COD concentrations in the permeate of this reactor was 115 mg/L compared to 198 mg/L in the permeate of the LDO reactor. Clearly, the higher substrate utilization rate in the HDO reactor was accompanied by a higher (net) production of EPS than in the LDO reactor. Gao *et al*. (2011) studied three different DO levels in a lab scale MBR fed with real municipal wastewater and found similar microbial communities in the sludge of the MBRs operated at a DO of 4 mg/L and a DO of 2 mg/L. At a DO of 0.5 mg/L a less diverse microbial community was found. A reduced substrate utilization, and herewith lower degree of bioflocculation at a DO of 1 mg/L compared to a DO of 4 mg/L, therefore may have been the result of oxygen limitation and/or development of a different microbial population producing less EPS.

3.4.3 Practical implications

An improved bioflocculation at higher DO levels has several implications, in particular with respect to membrane fouling and settleability. In both reactors, the supernatant fraction of the concentrate gave a higher resistance than the concentrate itself. This can only be explained if, at the start of a filtration experiment with concentrate, a layer with a similar composition as the concentrate itself deposits on the surface of the membrane. The presence of relatively large particles results in the formation of a more open fouling structure causing less resistance increase in comparison with the experiments with the supernatant fraction. A higher increase of the resistance with supernatant compared to sludge also was observed by Wisniewski and Grasmick (1998) and Remy (2012). However, others reported similar resistance increases by sludge and supernatant (Kappel *et al*., 2014), or even higher resistance increases caused by sludge than by supernatant of this sludge (e.g.Ognier *et al*. (2002)).

Often fouling in MBR systems is attributed to the presence of (soluble) EPS. However, even though the concentration of supernatant EPS in the HDO reactor was higher than in the LDO reactor (Figure 3.3), the HDO reactor exhibited a lower fouling potential (Figure 3.4). The concentration of sub-micron particles (< 450 nm) was not measured in this study. For the microfiltration membranes with a nominal pore size of 0.2 μm that were applied in this research, Faust *et al*. (2014) and De Temmerman *et al*. (2014) showed that the concentration of these particles, including soluble microbial products due to their macromolecular size of 100 kDa and bigger (Duan *et al*., 2014) and wastewater particles, is a better indicator for membrane fouling than the concentration of supernatant EPS. Therefore, a plausible explanation of the stronger fouling with LDO concentrate and supernatant is that the concentration of sub-micron particles in the LDO reactor was higher than in

the HDO reactor, causing more pore-blocking. This would also mean that higher DO concentrations result into an improved capture ability for nanoparticles compared to low DO concentrations. This is an interesting phenomenon for further study, particularly in relation to the behavior of potentially dangerous nanoparticles in wastewaters.

Settling can be applied as an alternative for membrane filtration. Using a flocculation efficiency for colloidal COD in the LDO reactor of 69% and 92% in the HDO reactor, and assuming all of the remaining colloidal material will leave a settler with the effluent, the colloidal COD concentration would be 40 mg/L in the effluent of the LDO reactor and 10 mg/L in the effluent of the HDO reactor. Together with the higher (soluble) COD concentrations in the permeate of the LDO reactor, this obviously means that it would take more effort to upgrade the effluent from the low DO system for reuse purposes.

From the above it can be concluded that operation at a DO of 4 mg/L results in better bioflocculation than a DO of 1 mg/L and herewith in less severe membrane fouling, a better settleability and an improved effluent quality. However, an important drawback of operation at a higher DO is that more energy will be consumed for aeration. An optimum DO would represent a trade-off between energy consumption for aeration and membrane operation or, in the case a settler is used, between energy consumption needed for aeration and for further upgrading the effluent for reuse applications.

Finally, at a DO of 4 mg/L more iron and aluminum were distributed to the concentrate than at a DO of 1 mg/L and this resulted in lower effluent concentrations of these metals. This suggest that also other metals, including heavy metals, will be removed to a larger extent if a bioflocculation unit is operated at a higher DO.

3.5 Conclusions

In this study the effect of the DO on bioflocculation and concentrate characteristics in HL-MBR systems was operated. The main conclusions were:

- Extensive bioflocculation of wastewater particles took place at a DO concentration in a range of 1-4 mg/L, even at a SRT as short as 0.5 days.
- An HL-MBR can recover more than 85% of the wastewater COD.

- The bioflocculation efficiency at a DO concentration of 4 mg/L (91% on the basis of colloidal COD) was considerably higher than the bioflocculation efficiency at a DO concentration of 1 mg/L (65%).
- An improved bioflocculation was positively correlated to higher concentrations of EPS and to a higher uptake of the multivalent cations calcium, iron and aluminum by the floc matrix.
- The higher extent of bioflocculation at a DO concentration of 4 mg/L was accompanied by a lower membrane fouling potential and better settleability of the concentrate that was produced.
- The effluent quality in terms of permeate COD at a DO concentration of 4 mg/L was better compared to a DO concentration of 1 mg/L.

Acknowledgement

This work was performed in the cooperation framework of Wetsus, centre of excellence for sustainable water technology (www.wetsus.nl). Wetsus is co-funded by the Dutch Ministry of Economic Affairs and Ministry of Infrastructure and Environment, the European Union Regional Development Fund, the Province of Fryslân and the Northern Netherlands Provinces. The authors like to thank the participants of the research theme “Membrane processes and operation for wastewater treatment and reuse” for the fruitful discussions and their financial support.

References

Akanyeti, I., Temmink, H., Remy, M., Zwijnenburg, A., 2010. Feasibility of bioflocculation in a high-loaded membrane bioreactor for improved energy recovery from sewage. Water science and technology 61, 1433–1439.

Al-Malack, M.H., Anderson, G., 1997. Use of crossflow microfiltration in wastewater treatment. Water Research 31, 3064–3072.

APHA., 1976. Standard Methods for the Examination of Water and Wastewater 14ed. APHA American Public Health Association.

Bruus, J.H., Nielsen, P.H., Keiding, K., 1992. On the stability of activated sludge flocs with implications to dewatering. Water research 26, 1597–1604.

Caccavo, F., Frolund, B., Van Ommen, K.F., Nielsen, P.H., 1996. Deflocculation of Activated Sludge by the Dissimilatory Fe(III)-Reducing Bacterium Shewanella alga BrY. Appl. Environ. Microbiol. 62, 1487–90.

De Temmerman, L., Maere, T., Temmink, H., Zwijnenburg, A., Nopens, I., 2014. Salt stress in a membrane bioreactor: dynamics of sludge properties, membrane fouling and remediation through powdered activated carbon dosing. Water Research 63, 112–24.

Duan, L., Song, Y., Yu, H., Xia, S., Hermanowicz, S.W., 2014. The effect of solids retention times on the characterization of extracellular polymeric substances and soluble microbial products in a submerged membrane bioreactor. Bioresource technology 163, 395–398.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P. t, Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Analytical chemistry 28, 350–356.

Eriksson, L., Alm, B., 1991. Study of flocculation mechanisms by observing effects of a complexing agent on activated sludge properties. Water Science & Technology 24, 21–28.

Faust, L., Temmink, H., Zwijnenburg, A., Kemperman, A., Rijnaarts, H., 2014. High loaded MBRs for organic matter recovery from sewage: Effect of solids retention time on bioflocculation and on the role of extracellular polymers. Water research 56, 258–266.

Freguia, S., Teh, E.H., Boon, N., Leung, K.M., Keller, J., Rabaey, K., 2010. Microbial fuel cells operating on mixed fatty acids. Bioresource technology 101, 1233–1238.

Gao, D., Fu, Y., Tao, Y., Li, X., Xing, M., Gao, X., Ren, N., 2011. Linking microbial community structure to membrane biofouling associated with varying dissolved oxygen concentrations. Bioresource technology 102, 5626–5633.

Guo, J., Peng, Y., Wang, S., Zheng, Y., Huang, H., Wang, Z., 2009. Long-term effect of dissolved oxygen on partial nitrification performance and microbial community structure. Bioresource Technology 100, 2796–2802.

Hernández Leal, L., Temmink, H., Zeeman, G., Buisman, C., 2010. Bioflocculation of grey water for improved energy recovery within decentralized sanitation concepts. Bioresource technology 101, 9065–9070.

Higgins, M.J., Novak, J.T., 1997. Characterization of exocellular protein and its role in bioflocculation. Journal of environmental engineering 123, 479–485.

Ivanovic, I., Leiknes, T., \Odegaard, H., 2008. Fouling Control by Reduction of Submicron Particles in a BF-MBR with an Integrated Flocculation Zone in the Membrane Reactor. Separation science and technology 43, 1871–1883.

Jin, Y.-L., Lee, W.-N., Lee, C.-H., Chang, I.-S., Huang, X., Swaminathan, T., 2006. Effect of DO concentration on biofilm structure and membrane filterability in submerged membrane bioreactor. Water research 40, 2829–2836.

Kappel, C., Kemperman, A., Temmink, H., Zwijnenburg, A., Rijnaarts, H., Nijmeijer, K., 2014. Impacts of NF concentrate recirculation on membrane performance in an integrated MBR and NF membrane process for wastewater treatment. Journal of Membrane Science 453, 359–368.

Laspidou, C.S., Rittmann, B.E., 2002. A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass. Water Research 36, 2711–2720.

Lee, W.S., Chua, A.S.M., Yeoh, H.K., Ngoh, G.C., 2014. A review of the production and applications of waste-derived volatile fatty acids. Chemical Engineering Journal 235, 83–99.

Liao, B., Allen, D., Leppard, G., Droppo, I., Liss, S., 2002. Interparticle interactions affecting the stability of sludge flocs. Journal of colloid and interface science 249, 372–380.

McCarty, P.L., Bae, J., Kim, J., 2011. Domestic wastewater treatment as a net energy producer-can this be achieved? Environmental science & technology 45, 7100–7106.

Mels, A., Rulkens, W., van der Meer, A., Nieuwenhuijzen, A., Klapwijk, A., 2001. Flotation with polyelectrolytes as a first step of a more sustainable wastewater treatment system. Water Science & Technology 43, 83–90.

Metcalf, I., Eddy, H., 2003. Wastewater engineering; treatment and reuse. McGraw-Hill New York.

Ognier, S., Wisniewski, C., Grasmick, A., 2002. Influence of macromolecule adsorption during filtration of a membrane bioreactor mixed liquor suspension. Journal of Membrane Science 209, 27–37.

Park, C., 2002. Cations and activated sludge floc structure. Virginia Polytechnic Institute and State University

Rasmussen, H., Bruus, J.H., Keiding, K., Nielsen, P.H., 1994. Observations on dewaterability and physical, chemical and microbiological changes in anaerobically stored activated sludge from a nutrient removal plant. Water Research 28, 417–425.

Rasmussen, H., Nielsen, P.H., 1996. Iron reduction in activated sludge measured with different extraction techniques. Water Research 30, 551–558.

Ravazzini, A., Van Nieuwenhuijzen, A., Van Der Graaf, J., 2005. Direct ultrafiltration of municipal wastewater: comparison between filtration of raw sewage and primary clarifier effluent. Desalination 178, 51–62.

Remy, M.J.J., 2012. Low concentration of powdered activated carbon decreases fouling in membrane bioreactors. Wageningen University.

Salehizadeh, H., Shojaosadati, S., 2001. Extracellular biopolymeric flocculants: recent trends and biotechnological importance. Biotechnology advances 19, 371–385.

Sheng, G.-P., Yu, H.-Q., Li, X.-Y., 2006. Stability of sludge flocs under shear conditions: roles of extracellular polymeric substances (EPS). Biotechnology and bioengineering 93, 1095–1102.

Starkey, J., Karr, P., 1984. Effect of low dissolved oxygen concentration on effluent turbidity. Journal (Water Pollution Control Federation) 837–843.

Sutton, P., Melcer, H., Schraa, O., Togna, A., 2011. Treating municipal wastewater with the goal of resource recovery. Water Science & Technology 63, 25–31.

Verstraete, W., Vlaeminck, S.E., 2011. ZeroWasteWater: short-cycling of wastewater resources for sustainable cities of the future. International Journal of Sustainable Development & World Ecology 18, 253–264.

Wilén, B.-M., Balmér, P., 1999. The effect of dissolved oxygen concentration on the structure, size and size distribution of activated sludge flocs. Water Research 33, 391–400.

Wilén, B.-M., Jin, B., Lant, P., 2003. The influence of key chemical constituents in activated sludge on surface and flocculating properties. Water research 37, 2127–2139.

Wilén, B.-M., Lund Nielsen, J., Keiding, K., Nielsen, P.H., 2000. Influence of microbial activity on the stability of activated sludge flocs. Colloids and Surfaces B: Biointerfaces 18, 145–156.

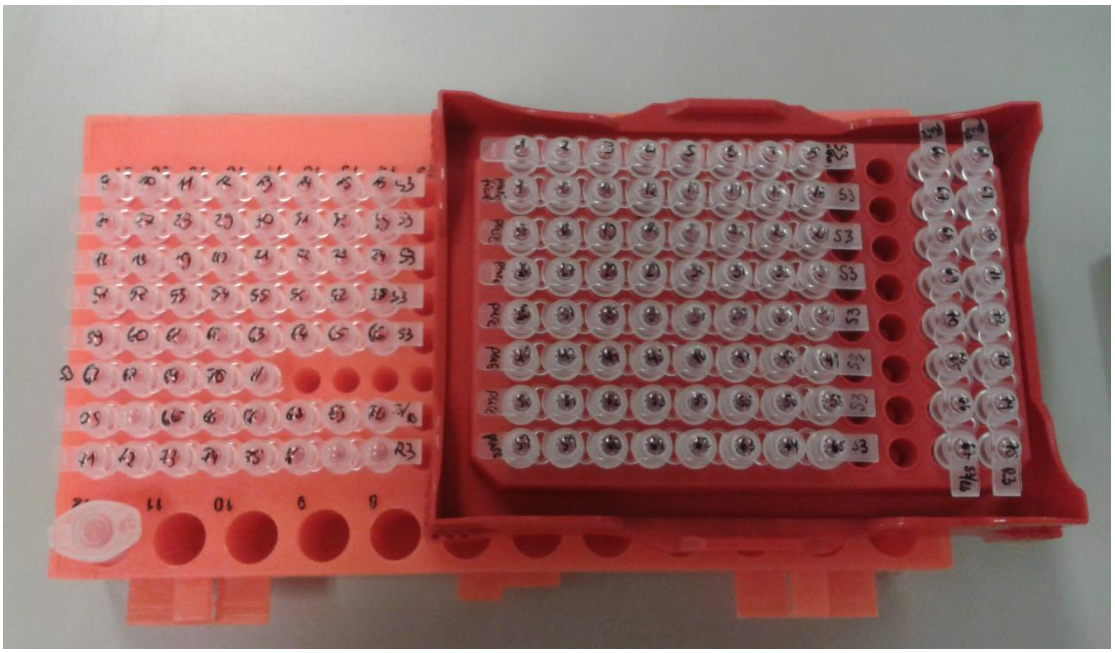
Wisniewski, C., Grasmick, A., 1998. Floc size distribution in a membrane bioreactor and consequences for membrane fouling. Colloids and Surfaces A: Physicochemical and Engineering Aspects 138, 403–411.

Yoon Kim, H., Yeon, K.-M., Lee, C.-H., Lee, S., Swaminathan, T., 2006. Biofilm structure and extracellular polymeric substances in low and high dissolved oxygen membrane bioreactors. Separation science and technology 41, 1213–1230.

Zhang, Y., Allen, D.G., 2008. The effect of short-term dissolved oxygen transients on activated sludge. Water quality research journal of Canada 43, 201–210.

4

Characterization of the Bacterial Community Involved in the Bioflocculation Process of Wastewater Organic Matter in High Loaded MBRs



Abstract

The bacterial diversity of the (solid) sludge fraction, the supernatant and the inflow wastewater was investigated in three high loaded MBRs designed for improved energy and organic resource recovery from sewage. High loaded MBRs were operated at extremely short SRTs of 0.125, 0.5 and 1 d. Bacterial diversity was investigated using PCR-DGGE and clone library analysis. Already at an SRT of 0.125 d a bacterial community developed which was different from the community of the wastewater. Bioflocculation, however, was low at this SRT. The majority of the bacteria, and especially *Arcobacter*, remained planktonic in the supernatant. Upon increasing SRT from 0.125 d to 1 d, bioflocculation was enhanced and the abundance of *Bacteroidetes* in the (solid) sludge fraction increased from 27.5% to 46.4%. Cluster analysis of DGGE profiles furthermore revealed that the bacterial community structure was different between sludge and supernatant. To localize specific bacterial classes in the sludge flocs FISH was carried out with three different bacterial probes. This revealed that *Betaproteobacteria* formed clusters in the sludge flocs whereas *Alphaproteobacteria* and *Gammaproteobacteria* were present as single cells.

A adapted version of this chapter is submitted for publication as:

Faust, L., Szendy, M., Plugge, C.M., van den Brink, P.F.H., Temmink, H., Rijnaarts, H.H.M., 2014. Characterization of the bacterial community involved in the bioflocculation process of wastewater organic matter in high loaded MBRs.

4.1 Introduction

High loaded MBRs (HL-MBR) were proposed to enhance the sustainability of the wastewater treatment process, similar to the A-stage of the AB-process (Akanyeti *et al*., 2010; Hernández Leal *et al*., 2010). In these HL-MBRs bioflocculation was enhanced when higher concentrations of extracellular polymeric substances (EPS), excreted by microorganisms, were present at SRTs of 0.5-1 d, compared to 0.125 d (Faust *et al*., 2014a). Earlier studies have already demonstrated that a minimum SRT is required to achieve a good bioflocculation process, characterized by low concentrations of organic matter in the effluent. Proposed minimum SRTs ranged from 4-9 d (Bisogni Jr and Lawrence, 1971) to more than 10 d (Murthy, 1998). However, in HL-MBRs, good bioflocculation process, characterized by low concentration of colloidal COD and high concentration of suspended COD, took place already at much shorter SRTs (Faust *et al*., 2014a). It was found, that at SRT of 0.5 d already 90% of the total COD was present in the form of suspended COD (> 12-25 um). In MBRs higher numbers of non-flocculated, planktonic bacteria are retained compared to conventional wastewater treatment systems using a settler. Thus, the higher retention of organic substances and bacteria could be a reason that bioflocculation in HL-MBRs works at much lower SRTs. To investigate the role of retained bacteria at short SRTs, characterization of microbial community becomes essential. Until now only a few studies have been conducted regarding the microbial community analysis at short SRTs. The bacterial community in MBRs operated at SRTs 3, 5 and 10 d was investigated and higher diversities were found at longer SRTs with *Betaproteobacteria* being the pre-dominant species in all reactors (Duan *et al*., 2009). Similarly, in MBRs operated at SRTs of 0.5, 1 and 2 d the bacterial community was studies at the initial start-up phase and at steady state conditions (Basaran *et al*., 2013). It was found that different communities develop in the reactors although initial community and loading rates were the same. Both studies were conducted using synthetic wastewater and did not distinguish between the sludge and the supernatant bacterial community. Other studies have taken the challenge to analyze community structures in various activated sludge systems (Ahmed *et al*., 2007; Baek and Pagilla, 2009; Hesham *et al*., 2011; Hu *et al*., 2012; Juretschko *et al*., 2002; Ma *et al*., 2013; Van den Broeck *et al*., 2010; Wan *et al*., 2011; Zhang *et al*., 2011), however, only in a few studies a clear link between the structure of the microbial community and its function, e.g. EPS production for efficient bioflocculation, could be achieved. In another study, the metagenome of the biomass involved in biological phosphorus removal in a wastewater treatment plant was analyzed, to identify genes potentially involved in EPS production (Albertsen *et al*., 2013). Alginate production genes were found and could be assigned to the phylum *Bacteroidetes*.

Still, knowledge about the differences and the function of sludge and supernatant bacterial community is very limited. Earlier studies in which the sludge and the supernatant bacterial community was investigated, focused on the changes in the bacterial community after shear was applied to the activated sludge (Morgan-Sagastume *et al*., 2008; Wilén *et al*., 2008). Other studies assessing the bacterial communities in different sludge fractions in MBRs aimed at revealing the relationship between sludge-, supernatant- and membrane layer bacterial communities to get more insight into which bacteria would be mostly responsible to cause membrane biofouling (Huang *et al*., 2008; Ma *et al*., 2013; Miura *et al*., 2007; Piasecka *et al*., 2012). However, the relation between bioflocculation efficiency and changes of bacterial communities in the flocculated (solid) sludge fraction and in the supernatant was not investigated yet.

Objectives of this study were to investigate the changes in the bacterial community at different, extremely short SRTs in both the sludge and the supernatant. Furthermore, the relationship between bioflocculation at different SRTs and bacterial community structure was investigated. The bacterial communities in the (solid) sludge fraction, in the supernatant and in the wastewater were characterized by PCR-DGGE approach, followed by construction of clone libraries. Fluorescence in situ hybridization (FISH) was used to visualize community structure of *Proteobacteria* in the sludge flocs.

4.2 Material and Methods

4.2.1 Experimental MBR set-ups and wastewater

Three lab scale high loaded membrane bioreactors (HL-MBR) were operated in parallel at the same HRT of 0.8 h, but varying SRTs of 0.125 d, 0.5 d, and 1 d. The reactor design and operation has been described previously in Faust *et al*. (2014a). In short, HL-MBRs with an effective volume of 2.6 L were operated with real municipal wastewater. The dissolved oxygen concentration was set at ≥ 4 mgO_2/L and shear was kept the same in all reactors by applying the same gas flow rates. Dissolved oxygen concentration of 4 mgO_2/L was shown earlier to be beneficial to the bioflocculation process in HL-MBRs (Faust *et al*., 2014b). Each reactor contained two submerged flat sheet membranes (Kubota Corporation) which were operated at a net flux of 15.0 $L/m^2 \cdot h$. HL-MBRs were operated for at least 3 times their SRTs to assure stable operation before samples were taken.

4.2.2 Sludge and supernatant samples

Mixed liquor samples were withdrawn from each reactor and transferred into a 500 ml graduated cylinder. Samples were allowed to settle for 30 min and afterwards the supernatant was collected and stored at 4°C for further analysis. Sludge samples of the **solid** sludge fraction (in the following called sludge samples) were obtained after the sludge was allowed to settle and after the supernatant was removed. The supernatant and the sludge samples are referred to as described in Table 4.1.

Table 4.1. *Specification of sludge and supernatant samples*

	Sludge samples	Supernatant samples
SRT = 0.125 d	R0.125	S0.125
SRT = 0.5 d	R0.5	S0.5
SRT = 1 d	R1	S1

4.2.3 Fractionation and Chemical oxygen demand (COD)

COD was measured using Dr. Lange test kits (LCK, Hach Lange). COD measurements were carried out immediately after the samples were taken. A detailed description about COD fractionation can be found in Faust *et al*. (2014a). In short, COD was fractionated into suspended COD (> 12-25 µm), colloidal COD (< 12-25 µm) and soluble COD (< 0.45 µm).

4.2.4 Total DNA extraction, amplification and DGGE analysis

For DNA extraction the supernatant samples were centrifuged at 13,000 rpm for 5 min. and afterwards the cell pellet was resuspended in 1 x PBS buffer. Total community genomic DNA was extracted from 0.5 ml of supernatant and 0.5 ml of sludge sample using the Power Biofilm DNA isolation kit (MoBio, Carlsbad, CA, USA). DNA concentration and quality was measured using a NanoDrop® spectrophotometer.

Partial bacterial 16S rRNA genes for DGGE analysis were amplified by PCR using *Go Taq* DNA polymerase (Invitrogen, The Netherlands) with 1401-r (5' -CGG TGT GTA CAA GAC CC- 3') and GC 968-f (including GC clamp) (Nübel *et al*., 1996). Each 25 µl PCR reaction mixture contained 20 ng template DNA, 2.5 µl of 10 x PCR buffer containing 3 mmol/L magnesium chloride (Promega Corp.,USA), 0.2 mmol/L deoxynucleoside triphosphate (DNTP, Invitrogen), 0.2 pmol/L of each primer and 1.25 U Taq Polymerase (Invitrogen, The Netherlands). Reactions were performed in an iCycler (Bio-Rad laboratories BV, Hercules, USA) applying the following program: Initial denaturation at 94° C

for 5 min., followed by 30 cycles of denaturation at 94° C for 1 min., annealing at 56° C for 40 sec., extension at 72° C for 1 min., and final extension was performed at 72° C for 30 min. Amplicons were checked on a 1% (w/v) agarose gel for quantity and size.

Separation of generated amplicons was performed using DGGE (Bio-Rad Laboratories BV, Hercules, USA). Polyacrylamide gels of 8% (w/v) were prepared at a pump speed of 4 ml/min, creating a 30-60% denaturant gradient of formamide and urea (Muyzer *et al*., 1993) Gels were run for 16 hours at 85 V and 60 ^{o}C and stained with silver nitrate (Sanguinetti and Simpson, 1994).

Stained gels were scanned and digitally processed using GelCompare software from BioNumerics (Applied Maths, Belgium). The Pearson Correlation Coefficient and unweighted pair group method with arithmetic averages (UPGMA) was used for cluster analysis.

4.2.5 Cloning, sequencing and phylogenetic analysis

Almost complete 16S rRNA genes were amplified using the primers: Univ1492-r and Bact27-f (Lane, 1991). The PCR reaction mixtures were prepared as described above for DGGE-PCR. Reactions were performed with initial denaturation at 95°C for 2 min., followed by 25 cycles of denaturation at 95° C for 30 sec., annealing at 52° C for 40 sec. and extension at 72° C for 90 sec. The cycles were completed with a final extension step at 72°C for 7 minutes. Obtained amplicons were purified with DNA clean and concentrator 5-Kit (Zymo Research Corp., Irvine, CA, USA). Amplicons were ligated into pGEM-T vector and transformed into competent *E.coli* XL1-Blue (Stratagene, USA) using the pGEM®-T vector cloning kit (Promega Corp. USA). After incubation for 1 h at 37°C, 80 µl of each transformation culture was spread on LB agar plates containing ampicillin (100 µg/ml), IPTG (0.5 mM) and X-Gal (80 µg/ml) and incubated overnight. To enhance the blue color of cells, plates were placed for two hours at 4°C before blue/white screening. Seventy-five white colonies of each sample were picked randomly and transferred to 24 well plates containing LB medium with 100 µg/ml ampicillin and incubated at 37 °C. During colony PCR competent *E. coli* cells were lysed and clone inserts were amplified with the primer SP6-r (5’ -ATT TAG GTG ACA CTA TAG- 3’) and the primer T7-f (5’-AAT ACG ACT CAC TAT AGG- 3’) (Promega Corp., Madison, WI, USA). PCR was performed using the same program as describe above in this section. To verify the size of inserts, PCR products were analyzed by gel electrophoresis using 1.5% (w/v) agarose gels containing 5 µL ethidium bromide. Afterwards, amplicons were purified using the DNA clean and concentrator-5 kit (Zymo Research Corp., Orange, CA, USA). Nearly full length 16S rRNA genes were sequenced by Baseclear (Leiden, the Netherlands) . Chimeric sequences were removed by the online web tool DECIPHER at http://decipher.cee.wisc.edu (Wright *et al*., 2012). Sequence similarities were analyzed using the NCBI BLAST search tool (http://www.ncbi.nlm.nih.gov/BLAST).

4.2.6 Nucleotide sequence accession numbers

Sequences from this study were submitted to the European Nucleotide Institute (ENA) under the accession numbers LK392676-LK393104.

4.2.7 Scanning electron microscopy

Sludge flocs (R0.125, R0.5, R1) were mixed with phosphate buffered saline (PBS) and glutaraldehyde (SIGMA-ALDRICH®, Germany) resulting in a 2.5% (w/v) glutaraldehyde concentration. Fixation was performed over night at 4°C. After fixation samples were centrifuged (4000 rpm., 10 min.) and washed twice with PBS and dehydrated in a graded series of ethanol (30%, 50%, 70%, and 90%, 100%). Dehydration took place for 20 min. in each solution and final dehydration with 100% ethanol was performed twice for 30 min. Afterwards sludge flocs were transferred onto a membrane filter (Isopore membrane filter GBPT, Millipore) using a spoon and dried at 35° C. Filters were glued on a sample holder using adhesive tape and coated with conductive gold alloy. Samples were analyzed with scattered electron microscope JSM-6480LV (JEOL Technics Ltd., Tokyo, Japan) at room temperature and SE detection at 6 kV. Images were digitally recorded and optimized with Image J 1.46 and resized with Adobe Photoshop CS (Adobe Systems Inc., USA).

4.2.8 Fluorescence in situ hybridization

Sludge flocs (R0.125, R0.5, R1) were fixed with 4% (w/v) formaldehyde for 1 h at room temperature. Afterwards samples were centrifuged (4000 rpm, 10 min.) and washed twice with PBS buffer. Single fixed flocs were transferred using a spoon onto microscopic slides and dehydrated in a graded ethanol series (30%, 50%, 70%, and 90%, 100%, 5 min.). Afterwards the samples were allowed to air dry. Specific probes (Ella Biotech GmbH, Germany) targeting *Bacteria*, *Planctomycetes*, *Verrucomicrobia* , *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* can be seen in Table 4.2.

Table 4.2. *Summary of specific probes used for FISH and fluorescent labels*

Probe	Target organism	Probe sequence (5' to 3')	Label
EUB338-I[a]	Most Bacteria	GCTGCCTCCCGTAGGAGT	FAM
EUB338-II[b]	Planctomycetes	GCAGCCACCCGTAGGTGT	FAM
EUB338-III[b]	Verrucomicrobia	GCTGCCACCCGTAGGTGT	FAM
ALF1B[c]	α-Proteobacteria	CGTTCGYTCTGAGCCAG	Cy3
BET42A[c]	β-Proteobacteria	GCCTTCCCACTTCGTTT	Cy5
GAM42A[c]	γ-Proteobacteria	GCCTTCCCACATCGTTT	Cy3
NON338[d]	Negative control	ACTCCTACGGGAGGCAGC	Cy3

a. Amann *et al*. (1990) **b.** Daims *et al*. (1999); **c.** Manz *et al*. (1992); **d.** Wallner *et al*. (1993)

Each specific *Proteobacteria* probe was combined with the EUB-probe mix (containing EUB 338-I, EUB338-II and EUB338III) for dual hybridization.

Hybridization was performed by adding 15 µL hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.5], 0.01% sodium dodecyl sulfate [SDS], 0.5% [v/v] Triton X-100 and either 20%, 30% or 35% Formamide depending on probe) to each well together with two specific probes (25 ng/µL). Incubation was carried out in the dark at 46° C for 1.5 h. After hybridization the slides were washed twice in pre-warmed washing buffer (0.9 M NaCl, 20 mM Tris-HCl, 5 mM EDTA). Subsequently, samples were allowed to air dry and afterwards mounted with Vectashield (Vector Laboratories Inc., USA). Finally samples hybridized with probes with Cy3 and FAM labels were examined using fluorescence microscope BX43 (Olympus, Japan) equipped with filter set for Cy3 and FAM. Samples hybridized with probes with Cy5 and FAM labels were examined using Eclipse E400 (Nikon Instruments, USA) equipped with filter sets for Cy5 and FAM. Images were obtained using a cooled charge-couple device (CCD) camera. Images were process using Image J 1.46 (National Institute of Health, USA) and merged into two channel mode.

4.3 Results

4.3.1 COD fractions

The extent of bioflocculation of wastewater organic matter was examined by measuring COD fractions and comparing them between the reactors and to the wastewater (Figure 4.1). The suspended and colloidal COD fraction in the wastewater was 62% and 23%, respectively. In the

reactors the suspended COD fraction increased to over 90% while the colloidal COD fraction decreased to 1.5% upon prolonged SRT. Using the colloidal COD fraction of the wastewater and in the reactors, flocculation efficiencies were calculated. Flocculation efficiency increased from 49% in R0.125 to 84% in R0.5 and to 94% in R1. This is in line with earlier studies investigating the bioflocculation process in HL-MBRs (Akanyeti *et al*., 2010; Faust *et al*., 2014a). Previous research (Faust *et al*., 2014a) showed that the increase of bioflocculation at longer SRTs was accompanied by higher concentration of sludge bound EPS, and it was concluded that EPS, in particular EPS-proteins govern the bioflocculation process in HL-MBRs.

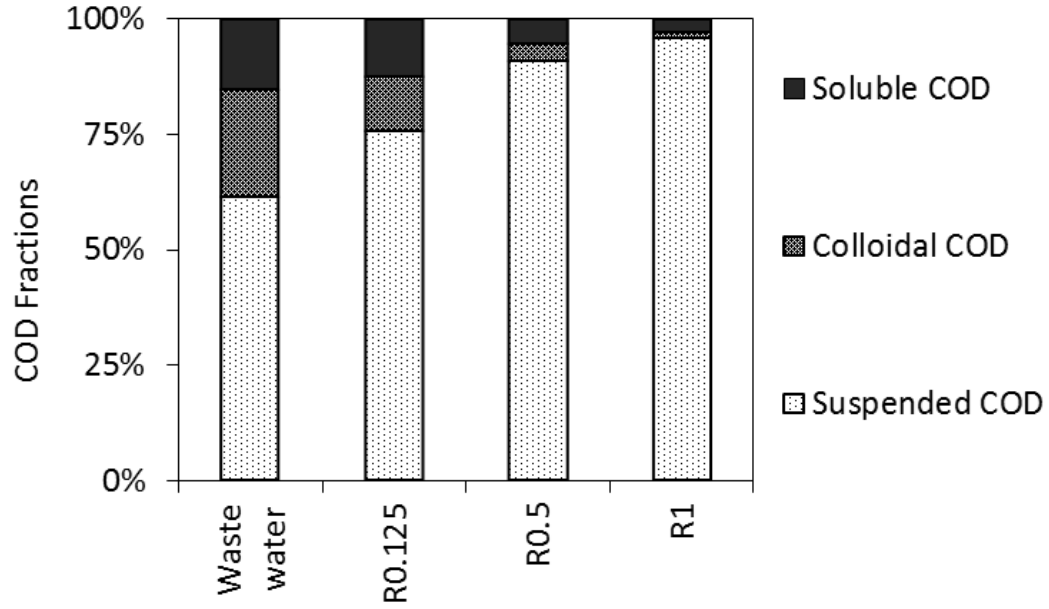


Figure 4.1. *COD fractions in the wastewater and in the HL-MBRs operated at SRT of 0.125 d, 0.5 and 1 d*

4.3.2 Microbial community analysis

4.3.2.1 PCR-DGGE

The DGGE band patterns of wastewater, sludge samples (R0.125-R1), and supernatant samples (S0.125-S1) are shown in Figure 4.2. The sludge samples showed more bands than the supernatant samples, suggesting a larger microbial diversity.

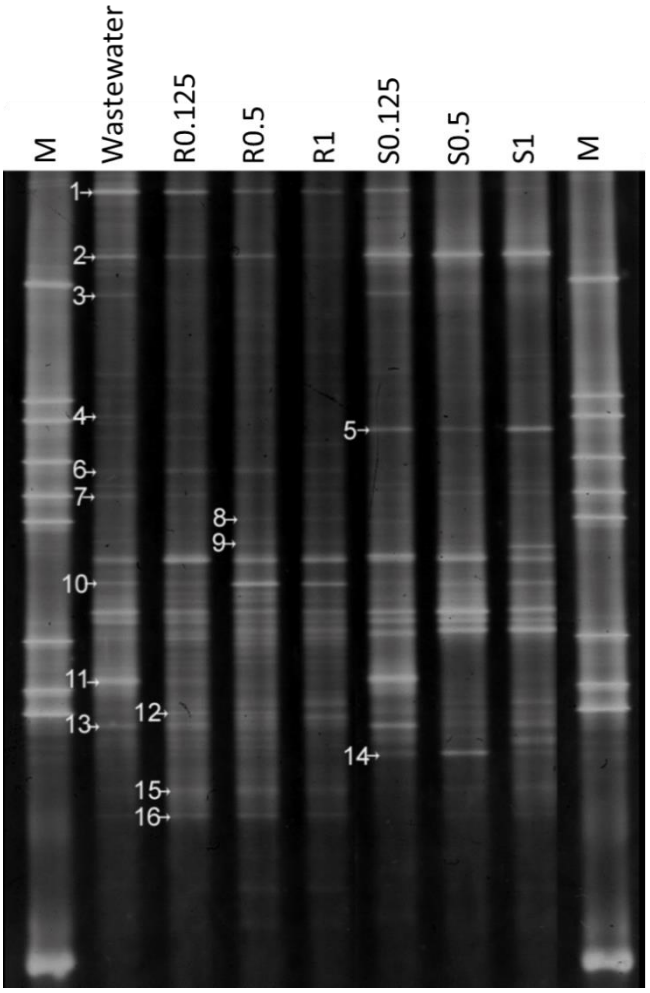


Figure 4.2. *DGGE band patterns of Bacteria 16S rRNA gene amplicons from wastewater, sludge and supernatant samples*

Comparing the sludge and supernatant samples (Figure 4.2), it is also depicted, that some species were only present in the supernatant (e.g. band 5), others only in the sludge (e.g. bands 4 and 6) and again others were present in both the sludge and the supernatant (e.g. band 10).

Comparing the band profiles of the wastewater and the reactors, it can be seen, that in R0.125, the weak bands 12, 15 and 16 appeared which were not visible in the wastewater, showing that already at very short SRT of 0.125 d a distinct microbial community developed. On the other hand, band 4 was present in the wastewater and in R0.125, but was not detected at longer SRTs (R0.5 and R1), suggesting that this species was outcompeted at longer SRTs. Band 9 was found in S1, but not detected in R1, which displays that this species preferred planktonic growth. Also two new bands (bands 15 and 16) were found in S1, which were also found in R0.125, R0.5 and R1 but not in S0.125 and S0.5, illustrating that at longer SRTs in fact some species prefer planktonic growth. Several band intensities became less or bands completely disappeared at longer SRT. For example, the intensity of bands 1 and 2 became very low in R1 compared to the wastewater and R0.125 and R0.5. This could

mean that these species were outcompeted by others with slower growth rates, but better substrate affinity at longer SRTs.

To visualize the relative similarities between the band patterns of wastewater, sludge and supernatant, a dendrogram was constructed, using the Pearson's product moment correlation coefficient (Figure 4.3). Except for S0.125, which band pattern was closely related to the wastewater, the HL-MBR samples could be divided into two groups: sludge samples, R0.125, R0.5 and R1 clustered into one group and supernatant samples (S0.5 and S1) into another group. This shows that the sludge bacterial community was different from the bacterial community in the supernatant. Interestingly, similarities were high between the wastewater and supernatant of the HL-MBR operated at the shortest SRT (S0.125). This means that at the shortest SRT, the majority of the species entering the reactor with the wastewater, were not able to accumulate in the sludge and/or able to form flocs, but remained suspended in the supernatant. At longer SRTs biomass with higher substrate affinity developed.

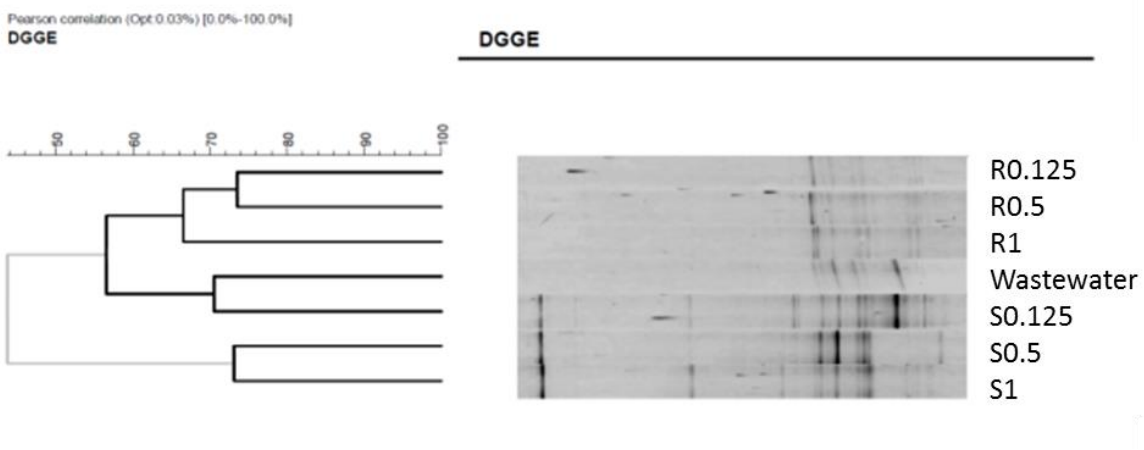


Figure 4.3. *PCR-DGGE fingerprinting of wastewater, sludge and supernatant samples. Dendrogram to the left compares the similarity of PCR-DGGE products, Pearson correlation was chosen with a UPGMA clustering method.*

4.3.2.2 Clone libraries

Seven clone libraries were constructed, including wastewater (69 clones), R0.125 (55 clones), R0.5 (64 clones), R1 (65 clones), S0.125 (64 clones), S0.5 (60 clones) and S1 (52 clones).

The phylogenetic analysis of the sequences obtained from these clone libraries, revealed the dominance of *Proteobacteria* in all samples (wastewater, sludge and supernatant) (Figure 4.4) which

is in line with literature (Zhang *et al*., 2011). Second most abundant in all clone libraries were *Bacteroidetes*, followed by a minor abundance of *Firmicutes*. In the supernatant clone libraries (S0.125, S0.5, S1) the abundance of *Proteobacteria* was higher (86%, 82%, 69%) compared to their abundance in the clone libraries of the sludge (67%, 61%, 50% for R0.125, R0.5 and R1, respectively). As the SRT was prolonged the abundance of *Proteobacteria* decreased in both the sludge samples (from 66% in R0.125 to 50% in R1) and in the supernatant samples (from 85% in S0.125 to 69% in S1). This means that at very short SRTs mainly *Proteobacteria* were able to maintain themselves in the sludge, whereas at longer SRTs also other groups were able to grow.

Bacteroidetes were more abundant in the clone libraries of the sludge (28%, 38% and 47% in R0.125, R0.5, and R1, respectively) compared to the supernatant (12%, 18% and 21% in S0.5, S0.5 and S1, respectively) (Figure 4.4). This could mean that *Bacteroidetes* are more likely to be able to form flocs/aggregates or stick to the already formed flocs. Furthermore, as the SRT was prolonged, the abundance of *Bacteroidetes* increased, from 27.5% in R0.125 to 37.5% in R0.5 and 46.4% in R1, showing that *Bacteriodes* outcompete *Proteobacteria* for the same substrate at longer SRTs.

Firmicutes were found in all samples, except in S0.5. *Firmicutes* were found in much lower abundance than *Proteobacteria* and *Bacteroidetes.* A clear trend between sludge and supernatant samples or regarding prolonged SRT could not be observed for the *Firmicutes*. Other phyla detected were related to *Fusobacteria* and *Synergistetes* (2% each) in the wastewater. In S1, species from the *Verrucomicrobia* and *Actinobacteria* phyla were found with a relative abundance of 2.5% each.

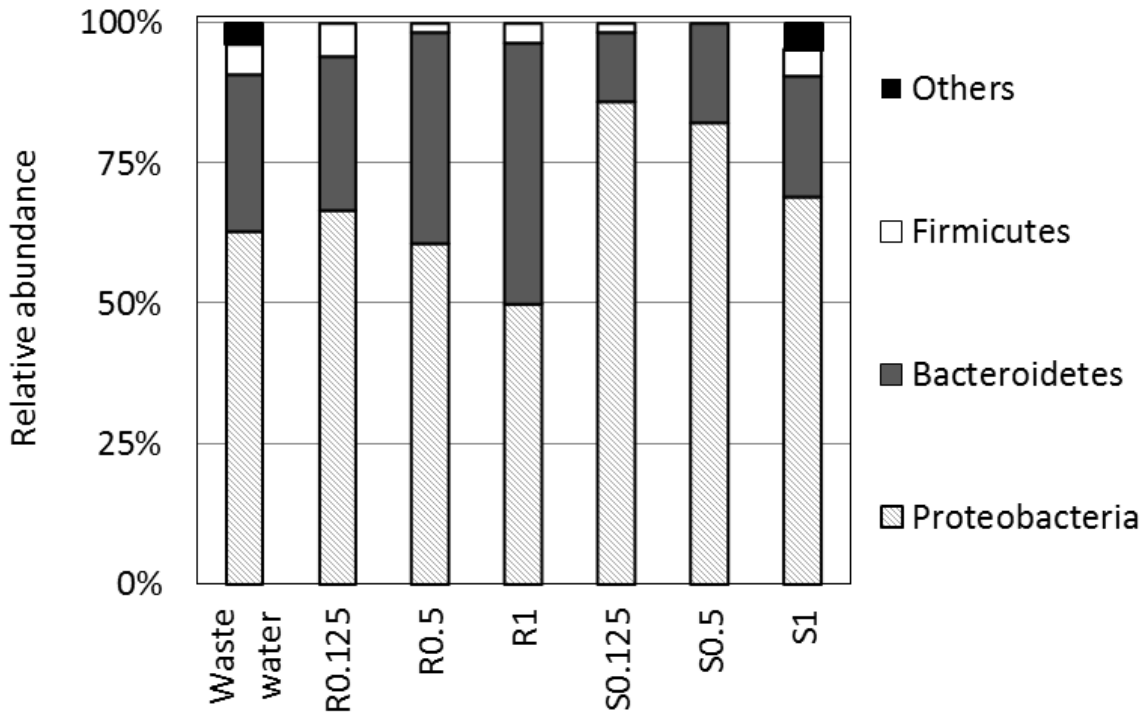


Figure 4.4. *Relative abundance of three main phyla in the 16S rRNA gene clone libraries constructed for wastewater, sludge and supernatant samples of HL-MBRs operated at different SRTs.*

The *Proteobacteria* were further characterized by analyzing the different classes of *Proteobacteria* (Figure 4.5). In all sludge samples and in S0.125, the largest bacterial group was represented by the *Betaproteobacteria* subdivision. In S0.5 and S1 *Epsilonproteobacteria* were most abundant. *Alpha*proteobacteria (present in R0.5, R1 and S1) and *Deltaproteobacteria* (present in R0.125, R1 and S0.125) were present in very low abundance, only. The *Alphaproteobacteria* class constituted most of denitrifying bacteria (Heylen *et al*., 2006), which could explain their low abundance in the HL-MBRs. Similarly, the *Deltaproteobacteria* subdivision in activated sludge was found to be mainly represented by sulfate reducing bacteria (Manz *et al*., 1998) which could explain their low abundances in the HL-MBRs.

The comparison of the classes present at different SRTs, shows, that the abundance of *Betaproteobacteria* in the clone libraries of the sludge samples decreased from 64.7% (R0.125) to 46.4% (R1). The same trend was observed in the clone libraries of the supernatant (from 55.1% to 35.5%), showing that at longer SRTs *Betaproteobacteria* are outcompeted by bacteria with slower growth rates but better substrate affinity. In contrast, the abundance of *Gammaproteobacteria* increased in the clone libraries of the sludge, from 10% in R0.125 to 14.3% in R0.5 and 19.6% in R1, which in turn shows that this class has a better substrate affinity. No clear trend, however, could be observed for the *Gammaproteobacteria* in the supernatant. The clearest trend in sludge and

supernatant samples in relation to SRT was found for the *Epsilonproteobacteria*. The abundance of the *Epsilonproteobacteria* decreased from 12% in R0.125 to 3.6% in R0.5 and disappeared in R1, but increased in the supernatant samples from 28.6% (S0.125), to 48% (S0.5 and S1); this means that at longer SRTs more *Epsilonproteobacteria* remained in the supernatant which could have several reasons: one may be that bacteria were sheared off the sludge because of their lower flocculating ability. In another study similar findings were reported for *Gammaproteobacteria*, which were sheared off from activated sludge samples more easily than other classes of *Proteobacteria* (Wilén *et al*., 2008).

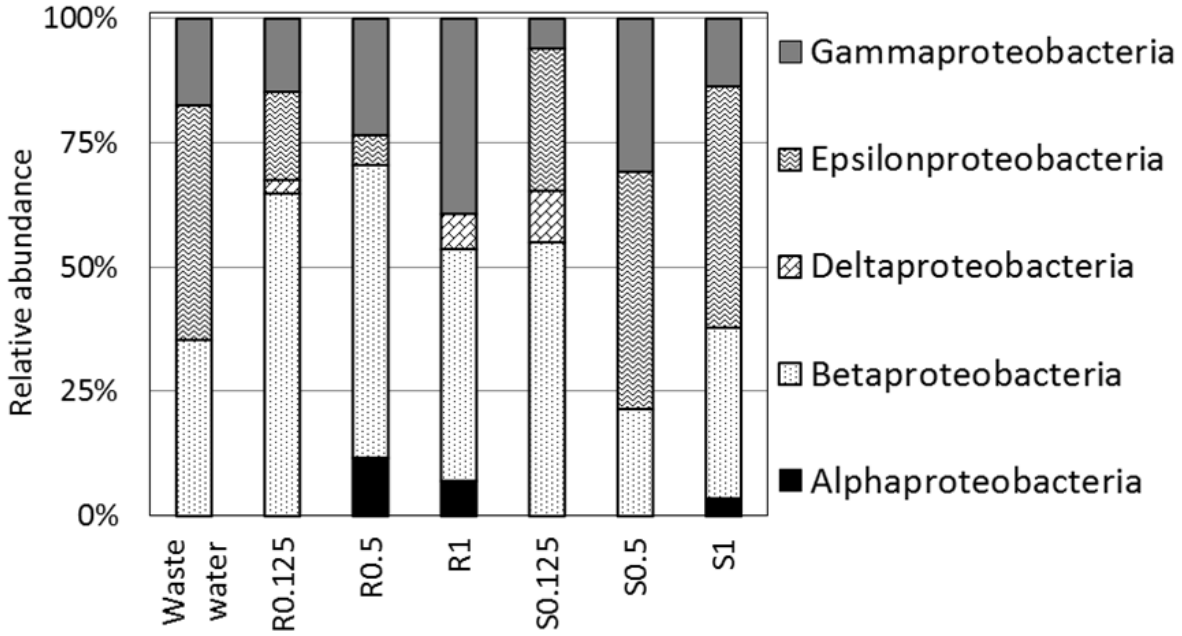


Figure 4.5. *Proteobacteria classes in the 16S rRNA gene clone libraries constructed for the wastewater, sludge and the supernatant samples of HL-MBRs operated at different SRTs.*

Clone library analysis of wastewater, sludge and supernatant samples revealed that the majority of the clones were related to uncultured or unclassified bacteria (287 uncultured clones out of 429 total clones, see Appendix A). A maximum similarity of 97% was used to assign clones to the genus level. Clone libraries depicted a high diversity in all samples (see Appendix A). In the sludge samples 70-75% of the clone only appeared once in the library, in the supernatant samples it was 54-63%. This is in line with the DGGE analysis (Figure 4.2), which also showed more bands in the sludge samples. Because of high diversity and since several clones showed lower maximum similarity than 97%, table 4.3 presents the relative abundance of clones to the level of family.

Table 4.3. *Relative abundance of clones from wastewater, sludge and supernatant samples on family level*

	Relative abundance in clone library (%)						
	I	**R0.125**	**R0.5**	**R1**	**S0.125**	**S0.5**	**S1**
Bacteroidaceae	9	4				3	
Bdellovibrionaceae			3				
Campylobacteraceae	28	7	3		23	33	27
Chitinophagacea		4		15			
Clostridia	3						6
Comamonadaceae		17	11	3	6	16	12
Desulfomicrobiaceae				3			
Flavobacteriaceae		10	18	20	6	3	4
Geobacteraceae					8		
Moraxellaceae	10		5	3		23	8
Porphyromonadaceae	6		3	3		5	
Pseudomonadaceae		4	9	9	5		
Ralstoniaceae	14	4			25		
Rhodocyclaceae		17	11	8	5		
Saprospiraceae				3			4
Shewanellaceae							4
Parabacteroides		4					
Prolixibacter		6	5				
Others (less than 2%)	30	23	32	33	22	17	35

The most abundant family in the wastewater was *Campylobacteraceae* with 29%. *Campylobacteraceae* was also most abundant in all the supernatant samples (S0.125, S0.5 and S1). Lower abundances of *Camplylobacteriaceae* were found in R0.125 (7%) and R0.5 (3%) and they were absent in R1, which clearly shows that *Camplylobacteriaceae* entering the reactors with the wastewater predominantly remained in the supernatant. Within the *Campylobacteracae* family of the *Epsilonproteobacteria* relatives of *Arcobacter* were most abundant in all samples (section 3.2.2; Figure 4.5). It was already shown that the abundance of *Epsilonproteobacteria* was higher in the supernatant than in the sludge, a result from the accumulation of *Arcobacter*. *Ralstoniaceae* was found to be the second most abundant family in the wastewater (16%) and in S0.125 (25%). Similar to the *Campylobacteraceae*, *Ralstoniaceae* were present in low abundance in R0.125 and were absent in the other reactors. Thus, clone library analysis could confirm the conclusions obtained from the DGGE analysis, that at short SRTs, most bacteria do not flocculate but remain planktonic in the supernatant (3.2.1).

In S0.5, members of the family *Moraxellaceae* were found in relatively high abundance of 23%, whereas in R0.5 only relatively low abundance of 5% was observed. As the SRT was prolonged to 1 d the abundances of *Moraxellaceae* decreased in both the sludge and the supernatant samples,

showing that this family was outcompeted at longer SRTs. Identified closest relatives of this family were relatives of *Acinetobacter* in all samples.

Comamonadaceae were present in similar abundance in both sludge samples R0.125 and R0.5 (17% and 11%, respectively) and in the supernatant samples S0.5 and S1 (16% and 12%), meaning that *Comamonadaceae* could grow in the sludge as well as in planktonic form. Members of *Comamonadaceae* identified, included relatives of *Acidovorax , Alicycliphilus, Aquabacterium, Comamonas, Giesbergeria* and *Simplicispira.* Also members of *Flavobacteriaceae* were found in the sludge and in the supernatant, however, higher abundances were detected in the sludge (10% in R0.125, 18% in R0.5 and 20% in R1) compared to the supernatant (6% in S0.125, 3% in S0.5 and 4% in S1). Closest relatives identified in the *Falvobacteriacae* family included relatives of *Cloacibacterium, Flavobacterium* and *Fluviicola.*

In R1 members of *Chitinophagacea* were found in relatively high abundance (15%). The occurrence of more *Chitinophagacea* members at longer SRTs could mean that these bacteria have longer reproduction time. No *Chitinophagacea* were found in the supernatant samples, all *Chitinophagacea* members were found in the sludge. This is interesting considering the higher extent of bioflocculation at longest SRT (R1) (Figure 4.1). Identified members of *Chitinophagacea* were relatives of *Chitinophaga* and *Ferruginibacter*.

4.3.2.3 Microscopic observations (FISH)

To reveal the distribution of three different classes of *Proteobacteria* in the sludge flocs, FISH was carried out with specific proteobacterial probes. *Proteobacteria* were chosen because they were predominantly present as shown in Figure 4.4. Figure 4.6 shows the distribution of *Alpha-*, *Beta-* and *Gammaproteobacteria* in the sludge flocs of R0.125, R0.5 and R1. The probe for all bacteria was labeled with the green fluorescent stain FAM. The specific *Alpha-*, *Beta-* and *Gammaproteobacteria* probes where labeled with the yellow (Cy3) and/or orange/red (Cy5) stain. Dual hybridization was performed since the microscope used were only equipped with two filter sets each. FISH analysis confirmed the low abundance of *Alphaproteobacteria*, which is shown by the low abundance of yellow stained bacteria in all sludge samples (Figure 4.6 A-C). Second most abundant in the floc flos were *Gammaproteobacteria* (4.6 G-I). *Betaproteobacteria* were found to be the dominant class through clone library analysis and this was confirmed by FISH (Figure 4.6 D-F). *Betaproteobacteria* were found to grow in clusters as depicted by the red rectangles. Similar observations were made when the community dynamics in a pilot wastewater treatment plant were studied (Lee *et al*., 2014). They found that mostly *Betaproteobacteria* grew in cluster compared to the growth of *Actinobacteria* and *Alphaproteobacteria*. The other classes were as single cells distributed over the whole sludge

flocs. However *Betaproteobacteria* were found in clusters, these clusters were randomly detected in the sludge flocs, meaning that there was no inner core or outer layer in or around the sludge floc.

In 4.6C and 4.6I single bacteria can be seen (white rectangle), which are not directly connected to each other but seem to be part of the sludge floc. Here, they might be embedded in the matrix of EPS which would keep the bacteria in the floc network.

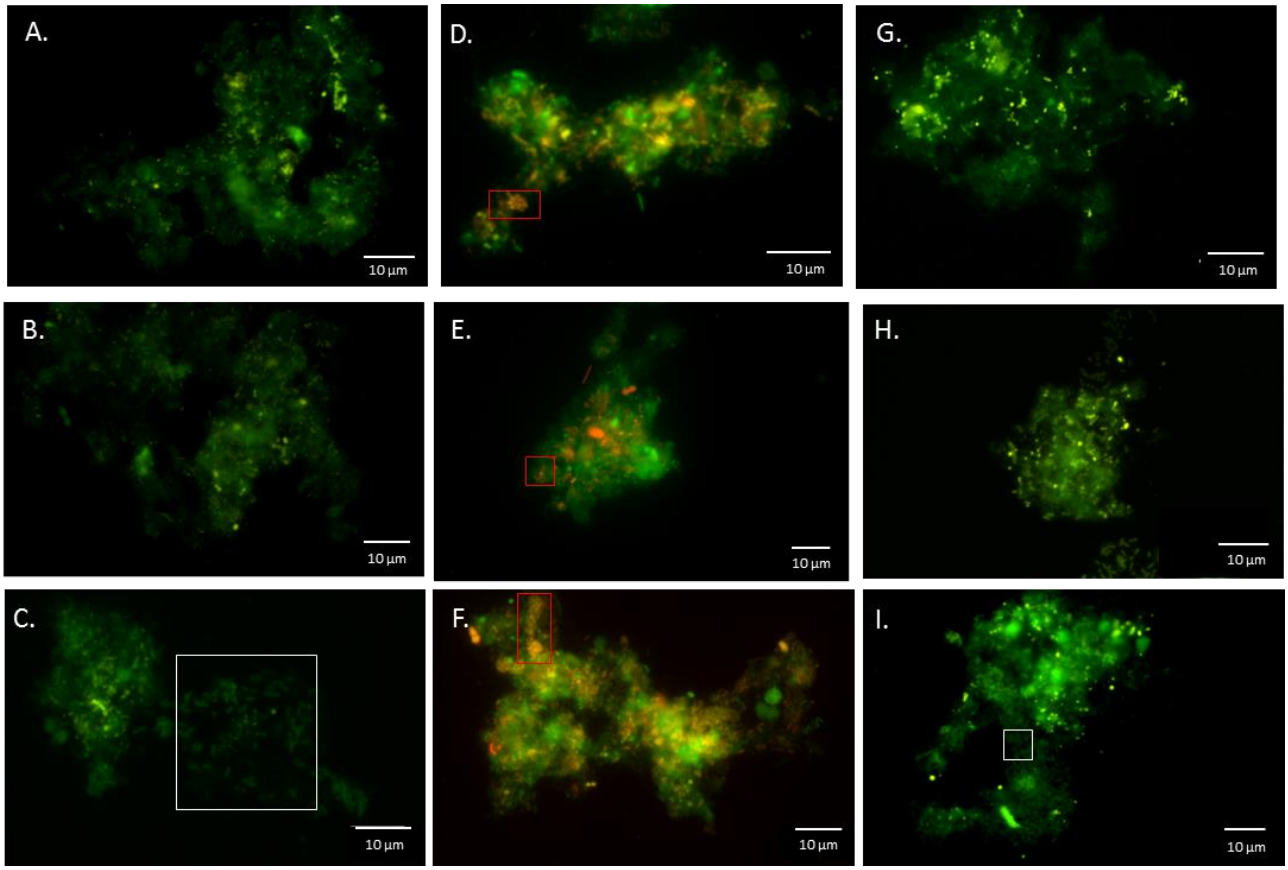


Figure 4.6. *FISH pictures of sludge flocs from reactors R0.125, R0.5 and R1. A, B and C show all bacteria (green) and Alphaproteobacteria (yellow) in R0.125, R0.5 and R1, respectively. D, E and F show all bacteria (green) and Betaproteobacteria (orange/red) in R0.125, R0.5 and R1, respectively. G, H and I show all bacteria (green) and Gammaproteobacteria (yellow) in R0.125, R0.5 and R1, respectively.*

4.4 Discussion

4.4.1 Bioflocculation at different SRTs

When the SRT was prolonged from 0.125 to 1 d, the extent of bioflocculation increased from 49% in R0.125 to 94% in R1 (Figure 4.1). This was in line with earlier studies about HL-MBRs (Akanyeti *et al*., 2010; Faust *et al*., 2014a). Enhanced bioflocculation was positively correlated to the increased

concentration of sludge bound EPS at longer SRTs when 5 HL-MBRs were operated at various SRTs between 0.125 d and 1 d (Faust *et al*., 2014a). EPS concentration increased from 55 mg/gVSS at SRT of 0.125 d to 118 mg/gVSS at SRT of 1d. In Figure 4.3 it was shown, that the supernatant of the HL-MBR operated at shortest SRT of 0.125 d had the highest similarity to the microbial community in the wastewater. This may be linked to the lower extent of bioflocculation and EPS production, meaning that a SRT of 0.125 d was too short to sustain a good flocculating EPS producing microbial community. Instead microorganisms were enriched and remained unflocculated in the supernatant.

4.4.2 Microbial communities at different SRTs

The DGGE band pattern of R0.125 was different from that of the wastewater, demonstrating that already at extremely short SRTs of 0.125 d a distinctive bacterial sludge community developed. Similar findings were reported when DGGE was used to study the bacterial community of MBRs operated with synthetic wastewater and SRTs of 0.5, 1 and 2 d SRT (Basaran *et al*., 2013). In this earlier study the development of different bacterial populations at different SRTs was shown, however, the richness of bacterial species was not necessarily influenced by applying different SRTs, and all communities achieved evenly good COD removal. Other studies investigated the bacterial community dynamics in acetate utilizing sequencing batch reactors at SRTs of 2 and 10 d and found significant differences between the two reactors (Pala-Ozkok *et al*., 2013). At lower SRTs of 2 d a bacterial community developed with faster growth rates, compared to SRT of 10 d under the same acetate feeding conditions. This shows that even though the same substrate is used, different community developed as a result of the different SRTs and thus different growth conditions. Similarly, the present study shows that at longer SRTs *Proteobacteria* were outcompeted by *Bacteroidetes* (Figure 4.4), meaning that within the phylum of *Bacteroidetes* slower growing but more specialized bacteria for the substrate grew.

Bacteroidetes were recently characterized to hold a range of genes involved in EPS and especially alginate production (Albertsen *et al*., 2013). Alginate is known to produce a gel like network in the presence of multivalent ions which are naturally occurring in municipal wastewater (van den Brink *et al*., 2009) and thus could facilitate floc formation. Hence, the higher abundance of *Bacteroidetes* may be associated with the better bioflocculation in HL-MBRs at prolonged SRTs.

Within the *Bacteroidetes* phylum the abundance of *Flavobacteriaceae* increased (Table 4.3). Within the family of *Flavobacteriaceae*, relatives of *Flavobacterium* were present in high abundance especially in the sludge. Members of the genus *Flavobacterium* were already reported to be common in activated sludge (Benedict and Carlson, 1971) and are thought to play a role in the breakdown of complex organic matter (Bernardet *et al*., 1996). *Flavobacterium* was also identified in a lab scale

MBR operated at relatively short SRTs (3-10 d) (Duan *et al*., 2009) and in a biofouling layer on a reverse osmosis membrane (Bereschenko *et al*., 2007). The latter suggests that *Flavobacterium* may be able to produce EPS. EPS production is important for the bioflocculation process and since *Flavobacterium* was found mainly in the sludge, it may be involved in the bioflocculation process in HL-MBRs by producing EPS.

Besides *Flavobacteriaceae, Chitinophagacea* were found in high abundance in R1 including relatives of *Ferruginibacter*. *Ferruginibacter* was reported to be able to degrade EPS (Wang, 2013) and thus could enrich in the sludge, because here bacteria experience more favorable conditions due to higher concentrations and immobilization of EPS. In an earlier study about HL-MBRs it was shown that initially the sludge bound EPS concentrations increased when the SRT was increased, but decreased again when the SRT was further prolonged to 5 d (Faust *et al*., 2014a). As the abundance of *Ferruginibacter* increased at prolonged SRT, those bacteria may be responsible for the degradation of EPS.

4.4.3 Microbial communities in the sludge and in the supernatant

From the DGGE band pattern and cluster analysis (Figure 4.3) it became clear, that microbial populations in the sludge and the supernatant were different. *Bacteroidetes* were found to be more abundant in the sludge than in the supernatant, whereas in the supernatant *Proteobacteria* and especially *Epsilonproteobacteria* were present (Figure 4.4 and 4.5). The high abundance of *Epsilonproteobacteria* was attributed to *Campylobacteraceae* and especially to the high abundance of relatives of *Arcobacter* in the supernatant. *Arcobacter* can be pathogenic to humans and is associated with human enteric diseases (Lehner *et al*., 2005) and other water and food born illnesses (Assanta *et al*., 2002). Also *Arcobacter* was detected in activated sludge samples from full scale municipal wastewater treatment plants (Snaidr *et al*., 1997). Since in the present study *Arcobacter* was clearly associated with the supernatant, the distribution of *Arcobacter* into the environment is very likely, due to the discharge of bacteria from conventional WWTPs. From the receiving waters *Arcobacter* can enter a drinking water production plants and hence *Arcobacter* was found in water distribution pipes (Assanta *et al*., 2002) and from here be infectious to humans. Wastewater treatment in MBRs, however, would represent an advantage in the removal of pathogens like *Arcobacter* from wastewaters, since bacteria are not able to pass the membrane and thus are not discharged into the environment.

Also in the supernatant bacteria were found which were described to produce EPS. In S0.5 for example, *Acinetobacter* was most abundant in the family of *Moraxellaceae*. *Acinetobacter* is known to produce EPS and the use of bioemulsifiers and polysaccharides, polyesters and lipases of

Acinetobacter species in industry was already recognized (Abdel-El-Haleem, 2004; Gutnick *et al*., 1991). Hence, EPS produced by planktonic bacteria may also govern the bioflocculation process by bridging with wastewater particles and/or with already existing sludge flocs. Therefore, floc formation may not only be attributed to bacterial species found in the (solid) sludge fraction. Here it becomes clear that assigning bacteria to their role in the bioflocculation process remains difficult. To investigate which and how bacteria govern the bioflocculation process, experiments may be carried out with pure cultures and EPS production in relation to bioflocculation should be investigated in bioflocculating systems. Furthermore, metatranscriptomics approaches may be used to detect active genes in the bacterial population. This could help to successfully link bacteria to their specific function in the ecosystem.

4.4.4 Implication for HL-MBRs

In practice it was suggested to run HL-MBRs at an SRT of 0.5 d, because at this SRT an optimum balance between flocculation efficiency and mineralization was found (Faust *et al*., 2014a). These finding was supported by the fact that at shorter SRT of 0.125 d most of the bacteria entering the reactor with the wastewater accumulated in the supernatant and thus, flocculation was low. At longer SRTs of e.g. 1 d, the degradation of EPS, which govern the bioflocculation process could be favored, since higher abundances of EPS degrading bacteria were found. Compared to what was suggested to be the minimum SRT for conventional treatment processes using a settler 4-10 d (Bisogni Jr and Lawrence, 1971; Murthy, 1998), minimum SRT in HL-MBRs could be chosen much lower. This could be due to the retention of planktonic bacteria in the supernatant, because as shown, the supernatant also contained bacteria which could produce EPS and hence overall EPS concentrations could be increased. Another advantage of using a membrane for solid separation was described to be the retention of pathogenic bacteria such as *Acrobacter* which helps to avoid the emerging problem of distribution of *Arcobacter* in the environment but also in food and water.

4.5 Conclusion

Bacterial populations in the (solid) sludge fraction and in the supernatant were significantly different. Already at SRT of 0.125 d, a microbial community developed distinct from that in the wastewater influent and from the reactors operated at longer SRTs as shown by DGGE band patterns. However, at this short SRT, the extent of flocculation was low and most of the bacteria entering the reactor with the wastewater influent accumulated in the supernatant. Upon prolonging the SRT the extent of flocculation increased and also the abundance of *Bacteroidetes* in the sludge increased. At longer

SRTs *Flavobacteriaceae* and *Chitinophagacea* were present in high abundance, including relatives of *Flavobacterium* and *Ferruginibacter* which were suggested to be involved in the bioflocculation process by either their production or degradation of EPS. However, also in the supernatant relatives of bacteria known to produce EPS, e.g. *Acinetobacter*, were found which shows that also planktonic bacteria influence the bioflocculation process. Thus, bioflocculation is most likely governed by both, the bacteria in the (solid) sludge fraction and by those in the supernatant. FISH analysis showed that *Betaproteobacteria* were present in clusters, whereas *Alpha-* and *Gammaproteobacteria* were distributed separately over the entire sludge flocs.

Acknowledgement

This work was performed in the cooperation framework of Wetsus, centre of excellence for sustainable water technology (www.wetsus.nl). Wetsus is co-funded by the Dutch Ministry of Economic Affairs and Ministry of Infrastructure and Environment, the European Union Regional Development Fund, the Province of Fryslân and the Northern Netherlands Provinces. The authors like to thank the participants of the research theme “Membrane processes and operation for wastewater treatment and reuse” for the fruitful discussions and their financial support

References

Abdel-El-Haleem, D., 2004. Acinetobacter: environmental and biotechnological applications. African Journal of Biotechnology 2, 71–74.

Ahmed, Z., Cho, J., Lim, B.-R., Song, K.-G., Ahn, K.-H., 2007. Effects of sludge retention time on membrane fouling and microbial community structure in a membrane bioreactor. Journal of Membrane Science 287, 211–218.

Akanyeti, I., Temmink, H., Remy, M., Zwijnenburg, A., 2010. Feasibility of bioflocculation in a high-loaded membrane bioreactor for improved energy recovery from sewage. Water science and technology 61, 1433–1439.

Albertsen, M., Stensballe, A., Nielsen, K.L., Nielsen, P.H., 2013. Digging into the extracellular matrix of a complex microbial community using a combined metagenomic and metaproteomic approach. Water Science & Technology 67, 1650–1656.

Amann, R., Lemmer, H., Wagner, M., 1998. Monitoring the community structure of wastewater treatment plants: a comparison of old and new techniques. FEMS Microbiology Ecology 25, 205–215.

Assanta, M.A., Roy, D., Lemay, M.-J., Montpetit, D., 2002. Attachment of Arcobacter butzleri, a new waterborne pathogen, to water distribution pipe surfaces. Journal of Food Protection 65, 1240–1247.

Baek, S.H., Pagilla, K., 2009. Microbial community structures in conventional activated sludge system and membrane bioreactor (MBR). Biotechnology and Bioprocess Engineering 14, 848–853.

Basaran, S.T., Aysel, M., Kurt, H., Ergal, . I, Akarsubasi, A., Yagci, N., Dogruel, S., Çokgör, E.U., Keskinler, B., Sözen, S., 2013. Kinetic characterization of acetate utilization and response of microbial population in super fast membrane bioreactor. Journal of Membrane Science 455, 392–404

Benedict, R., Carlson, D., 1971. Aerobic heterotrophic bacteria in activated sludge. Water Research 5, 1023–1030.

Bereschenko, L., Stams, A., Heilig, G., Euverink, G., Nederlof, M., Van Loosdrecht, M., 2007. Investigation of microbial communities on reverse osmosis membranes used for process water production. Water Science & Technology 55, 181–190.

Bernardet, J.-F., Segers, P., Vancanneyt, M., Berthe, F., Kersters, K., Vandamme, P., 1996. Cutting a Gordian knot: emended classification and description of the genus Flavobacterium, emended description of the family Flavobacteriaceae, and proposal of Flavobacterium hydatis nom. nov.(basonym, Cytophaga aquatilis Strohl and Tait 1978). International Journal of Systematic Bacteriology 46, 128–148.

Bisogni Jr, J., Lawrence, A.W., 1971. Relationships between biological solids retention time and settling characteristics of activated sludge. Water Research 5, 753–763.

Daims, H., Brühl, A., Amann, R., Schleifer, K., Wagner, M., 1999. The 17 domain-specific probe EUB338 is insufficient for the detection of all bacteria: 18 development and evaluation of a more

comprehensive probe set. Syst. Appl. Microbiol 19, 434–444.

Duan, L., Moreno-Andrade, I., Huang, C., Xia, S., Hermanowicz, S.W., 2009. Effects of short solids retention time on microbial community in a membrane bioreactor. Bioresource technology 100, 3489–3496.

Faust, L., Temmink, H., Zwijnenburg, A., Kemperman, A., Rijnaarts, H., 2014a. High loaded MBRs for organic matter recovery from sewage: Effect of solids retention time on bioflocculation and on the role of extracellular polymers. Water research 56, 258–266.

Faust, L., Temmink, H., Zwijnenburg, A., Kemperman, A., Rijnaarts, H., 2014b. Effect of dissolved oxygen concentration on the flocculation process in HL-MBRs. Water research. Accepted manuscript

Gutnick, D., Allon, R., Levy, C., Petter, R., Minas, W., 1991. Applications of Acinetobacter as an industrial microorganism. In: The Biology of Acinetobacter. Springer, pp. 411–441.

Hernández Leal, L., Temmink, H., Zeeman, G., Buisman, C., 2010. Bioflocculation of grey water for improved energy recovery within decentralized sanitation concepts. Bioresource technology 101, 9065–9070.

Hesham, A.E.-L., Qi, R., Yang, M., 2011. Comparison of bacterial community structures in two systems of a sewage treatment plant using PCR-DGGE analysis. Journal of Environmental Sciences 23, 2049–2054.

Heylen, K., Vanparys, B., Wittebolle, L., Verstraete, W., Boon, N., De Vos, P., 2006. Cultivation of denitrifying bacteria: optimization of isolation conditions and diversity study. Applied and Environmental Microbiology 72, 2637–2643.

Hu, M., Wang, X., Wen, X., Xia, Y., 2012. Microbial community structures in different wastewater treatment plants as revealed by 454-pyrosequencing analysis. Bioresource Technology 117, 72–79.

Huang, L.-N., De Wever, H., Diels, L., 2008. Diverse and distinct bacterial communities induced biofilm fouling in membrane bioreactors operated under different conditions. Environmental science & technology 42, 8360–8366.

Juretschko, S., Loy, A., Lehner, A., Wagner, M., 2002. The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. Systematic and Applied Microbiology 25, 84–99.

Lane, D., 1991. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M. editors. Nucleic acid techniques in bacterial systematics. Chichester: Wiley.

Lee, W.S., Chua, A.S.M., Yeoh, H.K., Ngoh, G.C., 2014. A review of the production and applications of waste-derived volatile fatty acids. Chemical Engineering Journal 235, 83–99.

Lehner, A., Tasara, T., Stephan, R., 2005. Relevant aspects of Arcobacter spp. as potential foodborne pathogen. International journal of food microbiology 102, 127–135.

Ma, J., Wang, Z., Zou, X., Feng, J., Wu, Z., 2013. Microbial communities in an anaerobic dynamic membrane bioreactor (AnDMBR) for municipal wastewater treatment: Comparison of bulk sludge

and cake layer. Process Biochemistry 3, 510–516.

Manz, W., Eisenbrecher, M., Neu, T.R., Szewzyk, U., 1998. Abundance and spatial organization of Gram-negative sulfate-reducing bacteria in activated sludge investigated by in situ probing with specific 16S rRNA targeted oligonucleotides. FEMS Microbiology Ecology 25, 43–61.

Miura, Y., Hiraiwa, M.N., Ito, T., Itonaga, T., Watanabe, Y., Okabe, S., 2007. Bacterial community structures in MBRs treating municipal wastewater: relationship between community stability and reactor performance. Water research 41, 627–637.

Morgan-Sagastume, F., Larsen, P., Nielsen, J.L., Nielsen, P.H., 2008. Characterization of the loosely attached fraction of activated sludge bacteria. Water research 42, 843–854.

Murthy, S.N., 1998. Bioflocculation: implications for activated sludge properties and wastewater treatment. Virginia Polytechnic Institute and State University.

Muyzer, G., De Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and environmental microbiology 59, 695–700.

Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I., Ludwig, W., Backhaus, H., 1996. Sequence heterogeneities of genes encoding 16S rRNAs in Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. Journal of bacteriology 178, 5636–5643.

Pala-Ozkok, I., Rehman, A., Kor-Bicakci, G., Ural, A., Schilhabel, M.B., Ubay-Cokgor, E., Jonas, D., Orhon, D., 2013. Effect of sludge age on population dynamics and acetate utilization kinetics under aerobic conditions. Bioresource Technology 143, 68–75.

Piasecka, A., Souffreau, C., Vandepitte, K., Vanysacker, L., Bilad, R.M., De Bie, T., Hellemans, B., De Meester, L., Yan, X., Declerck, P., others, 2012. Analysis of the microbial community structure in a membrane bioreactor during initial stages of filtration. Biofouling 28, 225–238.

Sanguinetti, C., Simpson, A., 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques 17, 914–921.

Snaidr, J., Amann, R., Huber, I., Ludwig, W., Schleifer, K.-H., 1997. Phylogenetic analysis and in situ identification of bacteria in activated sludge. Applied and Environmental Microbiology 63, 2884–2896.

Van den Brink, P., Zwijnenburg, A., Smith, G., Temmink, H., van Loosdrecht, M., 2009. Effect of free calcium concentration and ionic strength on alginate fouling in cross-flow membrane filtration. Journal of Membrane Science 345, 207–216.

Van den Broeck, R., Van Dierdonck, J., Caerts, B., Bisson, I., Kregersman, B., Nijskens, P., Dotremont, C., Van Impe, J., Smets, I., 2010. The impact of deflocculation-reflocculation on fouling in membrane bioreactors. Separation and Purification Technology 71, 279–284.

Wallner, G., Amann, R., Beisker, W., 1993. Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry 14, 136–143.

Wan, C.-Y., De Wever, H., Diels, L., Thoeye, C., Liang, J.-B., Huang, L.-N., 2011. Biodiversity and population dynamics of microorganisms in a full-scale membrane bioreactor for municipal wastewater treatment. Water research 45, 1129–1138.

Wang, X., 2013. Identification and Cultivation of Exopolysaccharide-Degrading Bacteria in Two Soils. University of Calgary.

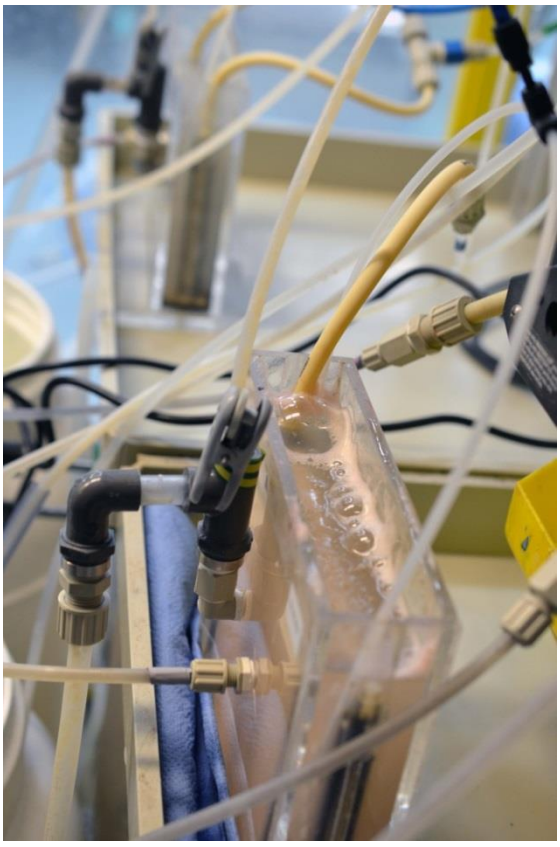
Wilén, B.-M., Onuki, M., Hermansson, M., Lumley, D., Mino, T., 2008. Microbial community structure in activated sludge floc analysed by fluorescence in situ hybridization and its relation to floc stability. water research 42, 2300–2308.

Wright, E.S., Yilmaz, L.S., Noguera, D.R., 2012. DECIPHER, a search-based approach to chimera identification for 16S rRNA sequences. Applied and environmental microbiology 78, 717–725.

Zhang, T., Shao, M.-F., Ye, L., 2011. 454 Pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. The ISME journal 6, 1137–1147

5

Bioflocculation during aerobic wastewater treatment: role of cations and extracellular polymeric substances (EPS)



Abstract

This paper investigates factors affecting the bioflocculation process in wastewater treatment such as the role of different types of cations and the overall characteristics of extracellular polymeric substance (EPS). High loaded membrane bioreactors were fed with bactopeptone synthetic wastewater with very low cation concentration. As a result of the low cation concentration poorly flocculated biomass evolved in the membrane bioreactor. Subsequent jar test experiments showed that the poorly flocculated biomass could be flocculated with cations (Na^+, Ca^{2+}, Al^{3+}). The flocculation efficiency, measured by the decrease in turbidity, increased with the valence of the cations ($Na^+ < Ca^{2+} < Al^{3+}$) which can be explained by the DVLO theory. In a second set of jar tests, the effect of different types of EPS characteristics on the bioflocculation process of model wastewater particles (kaolin clay) was investigated. Different types of EPS were extracted from activated sludge obtained from various wastewater treatment systems (e.g. municipal, industrial and synthetic wastewater). Differences in the EPS were characterized by Fourier Transform Infrared Spectroscopy (FT-IR). In subsequent jar test, the EPS originating from the various sludges yielded distinctively different flocculation activities in the presence of cations. The flocculation activity with EPS and either Ca^{2+} or Al^{3+} was similar, but the extent of flocculation was strongly dependent on the wastewater type from which the EPS originated. In the presence of Na^+ flocculation activities were much lower compared to the presence of Ca^{2+} and Al^{3+}. EPS extracted from municipal wastewater treatment systems did not show flocculation activity with Na^+ whereas EPS extracted from sludge treating industrial and synthetic wastewater showed flocculation activity with Na^+.

This chapter is submitted for publication as:

Faust, L., Temmink, H., Zwijnenburg, A., Rijnaarts, H., 2014. Bioflocculation during aerobic wastewater treatment: Role of cations and extracellular polymeric substances (EPS).

5.1 Introduction

Flocculation of wastewater particles and microbial cells is an important mechanism during biological wastewater treatment, in particular because it has a large impact on settling, membrane separation and other liquid-solid separation processes. Bioflocculation, i.e. flocculation of wastewater particles induced by microbial activity, also is very important in high-loaded membrane bioreactors (HL-MBR) or other high-loaded bioreactors that have the objective to concentrate and recover wastewater organic matter for subsequent anaerobic methane production or fermentation to other valuable organic chemicals e.g. volatile fatty acids (Faust *et al.*, 2014).

Several mechanisms were proposed to be relevant for bioflocculation. These include double layer interactions (DLVO theory), divalent cation bridging (DCB) with microbial extracellular polymeric substances (EPS) and polymer-ion interactions explained by the alginate theory (Sobeck and Higgins, 2002). Generally, wastewater particles and microbial cells carry a negative charge. According to the DVLO theory these particles are surrounded by a double layer of positively charged ions. This causes repulsion between the particles such that the attractive forces induced by van der Waals interaction are counteracted, and particle aggregation is prevented. According to the DCB theory divalent cations such as Ca^{2+} and Mg^{2+} form bridges between negatively charged groups in the EPS and this facilitates aggregation of wastewater microorganisms and wastewater particles. The alginate theory can be considered as a subset of the DCB theory, focusing on alginate (a polysaccharide with repeating mannuronic and guluronic acid units) as the most important EPS causing aggregation.

Role of cations

According to the DLVO theory the addition of cations to a system containing negatively charged wastewater particles and microorganisms reduces the electrostatic repulsion between these particles. This effect is much stronger in the presence of trivalent ions than with di- or monovalent cations. Cations can also adsorb to the polymer matrix (including adsorbed EPS) surrounding these particles. This results in a decrease of the surface charge density and a further reduction of the repulsive forces between the particles (Mikkelsen *et al*., 1996; Zita and Hermansson, 1994). As a result of these effects the particles may approach each other close enough to cause flocculation (Liao *et al*., 2001). Li *et al*. (2012) suggested an extended DVLO theory to describe these effects for activated sludge systems. Several studies have been carried out to elucidate the effect of cations on bioflocculation, but often contradicting results were reported. For example, it is well accepted that high concentrations of Na^{+} prevent flocculation and/or cause deflocculation of activated sludge (De Temmerman *et al*., 2014; Higgins and Novak, 1997; Kara *et al*., 2008; Novak *et al*., 1998). In marine

systems on the other hand biofilms and biological flocs are formed (Salta *et al*., 2013), indicating that bioflocculation is possible at very high Na^{+} concentrations.

Role of EPS

EPS such as proteins and polysaccharides can be present on the surface of microbial cells or adsorb to wastewater particles. Bruus *et al*. (1992) found that half of the Ca^{2+} pool in activated sludge was associated with EPS. This in in line with the DCB theory, in which negatively charged EPS form bridges with multivalent cations such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+} (Higgins and Novak, 1997; Li *et al*., 2012). Also, deflocculation of biological flocs often is accompanied by an increase in free EPS, which shows that these EPS are involved in floc formation. Obviously, the type of EPS plays an important role in bioflocculation. One approach to further elucidate this role, is the isolation of EPS producing microorganisms from biological wastewater treatment systems (Bala Subramanian *et al*., 2010; Deng *et al*., 2003; Gao *et al*., 2006; Salehizadeh *et al*., 2000; Xia *et al*., 2008; Xiong *et al*., 2010). In these studies EPS producing microorganisms were selected by picking mucoid shaped colonies after plate culturing of activated sludge samples. EPS producing pure cultures were cultivated and the EPS was harvested and screened for kaolin clay flocculation activity. Several bacteria, including Bacillus and Vagococcus species, were found to produce EPS with a very high flocculation activity. All of these studies aimed at identifying EPS producing bacteria, which in a later state may be enriched and used to produce commercial bioflocculants. However, these bacteria and the EPS they form may not be predominantly involved in bioflocculation in real world biological wastewater treatment systems. For this purpose the role of the overall EPS pool should be studied rather than the EPS produced by single microorganisms. Besides, the production and characteristics of EPS in wastewater treatment systems is determined by several factors including wastewater characteristics and operational conditions such as sludge retention time (SRT) and dissolved oxygen (DO) concentration (Faust *et al*., 2014; Sheng *et al*., 2010; Sponza, 2003).

This study aims to investigate the factors involved in bioflocculation, including the role of different cations and overall EPS characteristics. For this purpose standardized flocculation experiments were carried out using i) poorly flocculating biomass from a high loaded membrane bioreactor (HL-MBR) fed with synthetic waste water that was low in its cation concentration, ii) extracted and purified EPS from sludges of various biological treatment systems for municipal and synthetic wastewater and iii) EPS extracted from an industrial biological wastewater treatment system treating saline wastewater.

5.2 Material and methods

5.2.1 Effect of cations and particles on poorly flocculating biomass from a HL-MBR

A lab-scale HL-MBR reactor was operated to produce poorly flocculating biomass at low cation concentrations. In separate jar tests, the effect of different cations and of the presence of kaolin clay on floc formation of the poorly flocculated biomass was investigated. Kaolin clay was used to mimic negatively charged wastewater particles (Bala Subramanian *et al*., 2010). The lab-scale HL-MBR with an effective volume of 0.55 L was operated using a submerged Al_2O_3 ceramic membrane plate (ItN, Germany) with a total membrane area of 0.036 m^2 and a nominal pore size of 0.2 µm. The reactor was operated at a SRT of 0.5 d and a HRT of 0.9 h. Permeate was extracted with a Masterflex pump operated in relaxation mode: 15 min. pumping followed by 5 min. relaxation. This resulted in a net flux of 12.7 $L/m^2 \cdot h$. Synthetic wastewater with the cation composition given in table 5.1 was prepared by suspending 300 mg/L Bactopetone in demineralized water. Bactopeptone feed water was chosen because the ion concentrations in this feed water were very low compared to ion concentrations in municipal wastewater. The reactors were inoculated with 250 mL HL-MBR concentrate that treated real municipal wastewater (Faust *et al*., 2014). Biomass samples were taken after the HL-MBR had been operated for at least 3 times the SRT.

Table 5.1. *Cation and nutrient concentration in Bactopeptone feed (Higgins and Novak, 1997).*

constituent	mg/L	mEq/L
Na^+	20.9	0.91
NH_4^+	0.5	0.03
K^+	2.1	0.05
Mg^{2+}	3.8	0.32
Ca^{2+}	13.7	0.68
PO_4^{3-}	2.3	
NO_3^-	15.9	
Total Kjehdal Nitrogen (TKN)	46.5	
Chemical oxygen demand (COD)	300	

Biomass samples from the HL-MBR reactor was used in jar tests to investigate the effect of cations (Na^+, Ca^{2+}, Al^{3+}) and kaolin clay particles on flocculation (Figure 5.1). Biomass was withdrawn from the HL-MBR reactor and 100 mL (44.8 mg dry weight) was poured into four beakers which were placed in

a jar tests flocculation unit (Lovibond, ET 750). Slow mixing (40 rpm) was applied for 2 min. Afterwards cations (NaCl, $CaCl_2$, and $AlCl_3 \cdot H_2O$, Sigma Aldrich) were added to concentrations of 10 mEq/L. This concentration was selected because it is relatively low but in preliminary experiments was found to give a significant reduction of turbidity. After cation addition rapid mixing at 117 rpm was applied for 5 min, followed by slow mixing at 40 rpm for 10 minutes. A blank was prepared which contained biomass without cations. After the flocculation program was completed, the stirrers were stopped and settling was allowed for 10 min. Gently 30 mL samples were taken from the supernatant after which the turbidity was determined immediately as Nephelometric Turbidity Units (NTU) with a turbidity meter (2100N IS, Hach).

Using an identical procedure, the effect of the simultaneous presence of cations and of kaolin particles on flocculation was investigated. Kaolin clay (Sigma-Adrich) was added at a concentration of 0.5 g/L just before the cations were added.

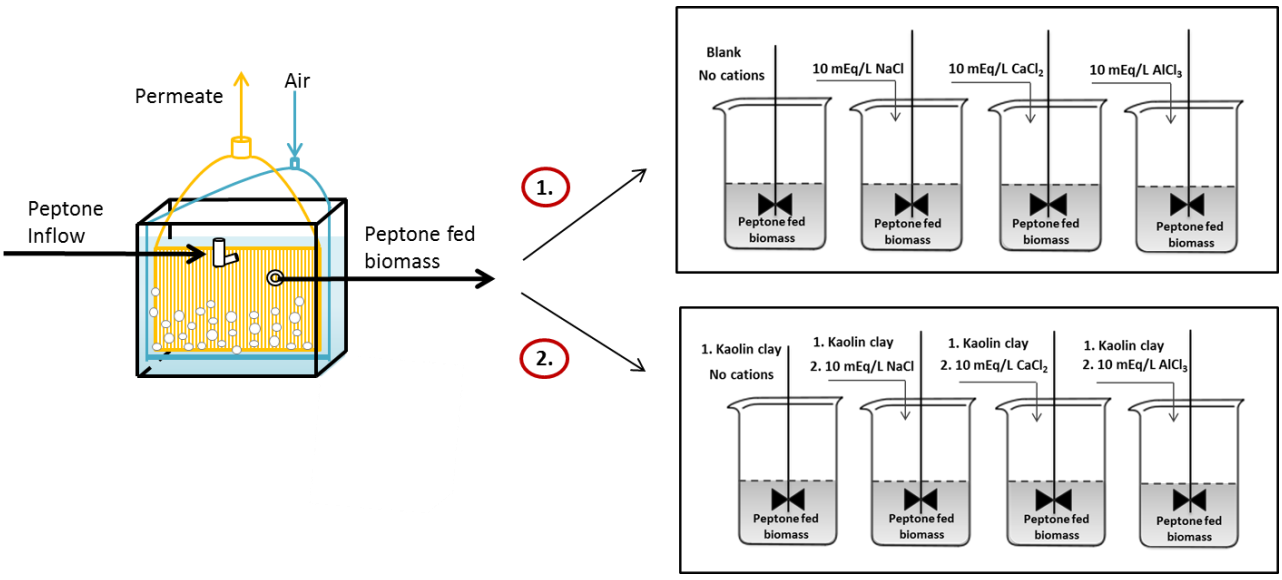


Figure 5.1. *Schematic depiction of HL-MBR and jar tests with biomass from the HL-MBR and (1) various cations and (2) various cations and kaolin clay particles.*

5.2.2 Effect of EPS source and cations on flocculation of particles

EPS were extracted from sludge samples taken from six biological wastewater treatment systems, different in the type of the (main) carbon source in the wastewater, the solids retention time (SRT) and salinity of the wastewater. Table 5.2 gives the most important characteristics of these treatment plants.

***Table 5.2**. Characteristics of the biological wastewater treatment plants that were used for EPS extraction (COD = chemical oxygen demand).*

	Treatment plant	Wastewater	SRT d	Wastewater Na^+ mg/L
1	Full-scale with settler[a]	Municipal	n.a.	100-200
2	Lab-scale MBR[b]	Municipal	16	100-200
3	Lab-scale HL-MBR[c]	Municipal	1	100-200
4	Lab-scale HL-MBR[d]	Acetate (600 mg COD/L)[e]	1	320
5	Lab-scale HL-MBR[d]	Glucose (600 mg COD/L)[e]	1	75
6	Full-scale with settler	Industrial, 50% of COD was glycerin	40-45	~ 4800

[a] Treatment plant of Leeuwarden, The Netherlands.
[b] Details can be found in Kappel *et al*. (2014).
[c] Details can be found in Faust *et al*. (2014).
[d] These HL-MBRs were identical to the one described earlier in Section 2.1.
[e] To promote bioflocculation the monovalent- to divalent cation ratio of the synthetic wastewaters was adjusted to <1 (Higgins and Novak, 1997) by adding $CaCl_2$ (Sigma Aldrich).
[f] Located in Delfzijl, The Netherlands and used to treat wastewater from mixed industrial sources.

The sludge samples were stored at 4°C until EPS extraction was performed within 2 days after sampling according to a procedure described by (Faust *et al*., 2014). After extraction, EPS were dialyzed using tubular dialysis membranes with 12-14 kDa MWCO (Spectra/Por®2) against MilliQ water for 24 h. The purified EPS were subsequently freeze dried in liquid nitrogen and afterwards lyophilized (Modulyo 4K Freeze dryer, Edwards). From the lyophilized powders, EPS solutions were prepared by dissolving the EPS in MilliQ water at a concentration of 1 g/L.

Kaolin clay was used to investigate the potential of the different EPS to flocculate particles in the presence of different cations according to the method proposed by Kurane *et al*. (1986). For this purpose 94 mL of a 5 g/L kaolin clay solution was added to beakers, together with 5 mL of a 10 g/L salt solutions and 1 mL of the 1 g/L EPS solution, giving a total volume of 100 mL. This gave final concentrations in the tests of 4.7 g clay/L, 10 mg/L for EPS and 9-10 mEq/L for the cations, which is similarly low as in the jar tests with poorly flocculated peptone fed biomass described previously. For each cation a blank was prepared containing only kaolin clay and cations and no EPS (Figure 5.2). The jar tests were performed using the same stirring program as described previously. To determine the flocculation activity, settling was allowed for 10 min.

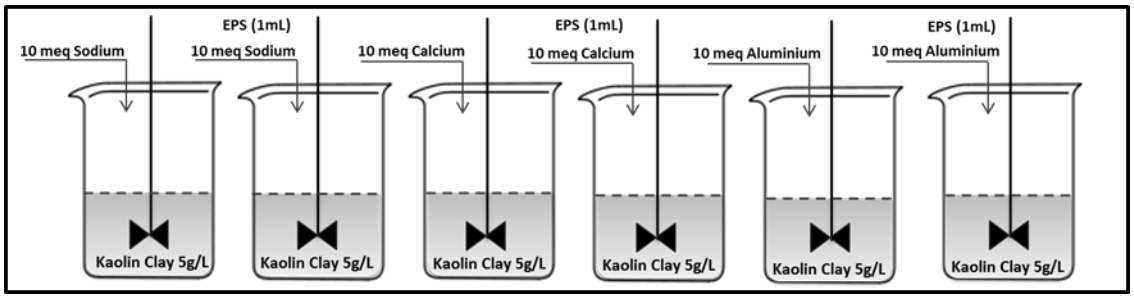


Figure 5.2. *Schematic depiction of jar tests performed with different types of cations and EPS extracted from various sludge sources.*

Afterwards, 2 mL samples were taken from the supernatant and placed in a cuvette to measure the optical density (OD) of the samples and the blank at 660 nm in a spectrophotometer (UV-1650 PC, Shimadzu). The flocculation potential of the EPS was calculated as follows:

$$\text{flocculation potential } (\%) = \frac{\text{OD}_{660,\text{Blank}} - \text{OD}_{660}}{\text{OD}_{660,\text{Blank}}}$$

5.2.3 Analyses

Zeta potential was measured with the NanoSight NS 500 Z-NTA (Nanosight, Amesbury, UK). In this system the zeta potential of nanoparticles in solution can be measured by applying an electric field. The electric field causes motion of the nanoparticles, which is recorded. This electrophoretic velocity is used to calculate the zeta potential of particles. Previous to the zeta potential measurement samples were paper filtered (Whatman Black Ribbon 589/1, 12–25 µm).

Fourier transform infrared spectroscopy (FT-IR) was carried out using a Shimadzu 4800-s ATR-FT-IR spectrometer. Lyophilized EPS were used to record FT-IR spectra at a resolution of 2.0 cm^{-1} and 100 scans.

SEM-EDX analyses were performed to determine the elemental composition of flocs and EPS, using a Scanning Electron Microscope JEOL-6480LV (JEOL Ltd., Tokyo, Japan), and by applying a highly-focused electron beam. Samples were taken from the settled fraction obtained after flocculation in the jar tests and allowed to air dry. Air dried samples were placed on a sample holder and introduced into the sample chamber of the microscope. Samples were analyzed under high vacuum at 10 kV.

For light microscopic analysis, flocs from the settled fraction obtained after flocculation in the jar tests, were transferred with a spoon into small petri dishes and pictures were taken with a Leica MZ 95 stereomicroscope.

5.3 Results and discussion

5.3.1 Effect of cations on poorly flocculated biomass

No obvious floc formation took place in the HL-MBR that was fed with peptone. The biomass did not settle and the concentrate was highly turbid (70-100 NTU, also see blanks in Figure 5.4). To investigate the effect of low concentrations of cations on flocculation of this poorly flocculated biomass, jar tests were conducted with 10 mEq/L of Na^+, Ca^{2+} and Al^{3+}. Figure 5.3 shows that the addition of Na^+ resulted in the formation of a net-like structure of small flocs. The addition of Ca^{2+} and in particular of Al^{3+} resulted in the formation of more compact flocs.

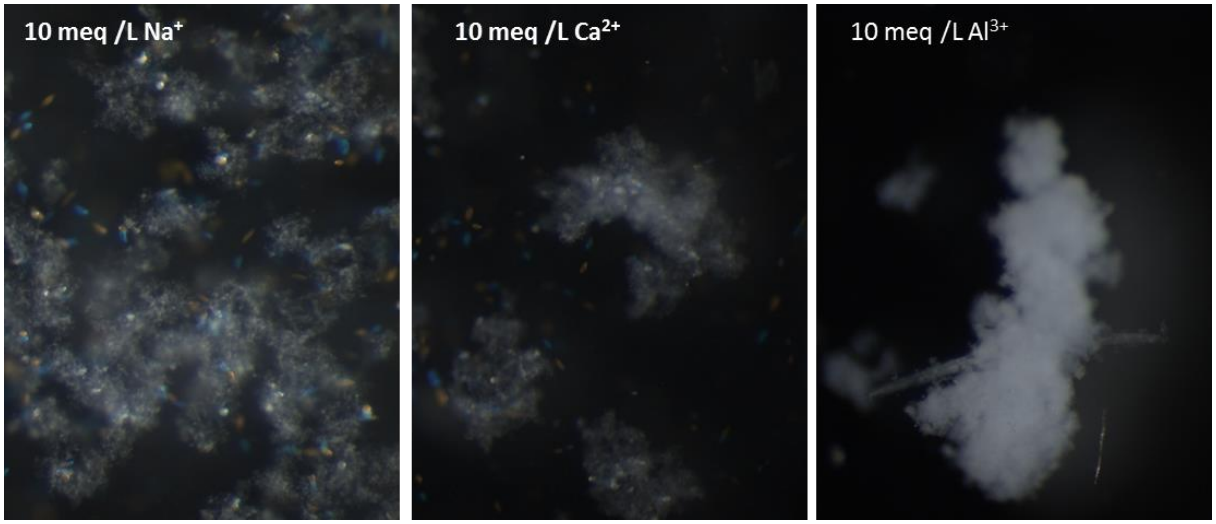


Figure 5.3. *Microscopic images of flocs formed from peptone fed biomass induced by addition of 10 mEq/L of Na^+, Ca^{2+} and Al^3, respectively.*

EDX analyses were performed to verify that the flocs which were formed were of organic origin, and did not consist of inorganic calcium and aluminum precipitates (Table 5.3). Table 5.3 shows the elemental composition of the flocs. Na, Ca and Al were detected only in those flocs formed with the corresponding cation. The high C fraction shows that these flocs predominantly consisted of organic matter.

Table 5.3. *Elemental composition of flocs formed from peptone fed biomass and cations.*

	Element %		
Element	**Na^+ Floc**	**Ca^{2+} Floc**	**Al^{3+} Floc**
C	52	41	40
N	15	16	21
O	20	32	36
Na	6	0	0
Cl	7	7	1
Ca	0	4	0
Al	0	0	2

Figure 5.4 shows supernatant turbidities after addition of the cations. Na^+ only caused minor flocculation, with a reduction of turbidity of 6 NTU compared to the blank (6% reduction). The reduction of turbidity was much stronger with Ca^{2+} (21 NTU units compared to the blank, 24% reduction) and particularly with Al^{3+} (59 NTU units compared to the blank, 84% reduction).

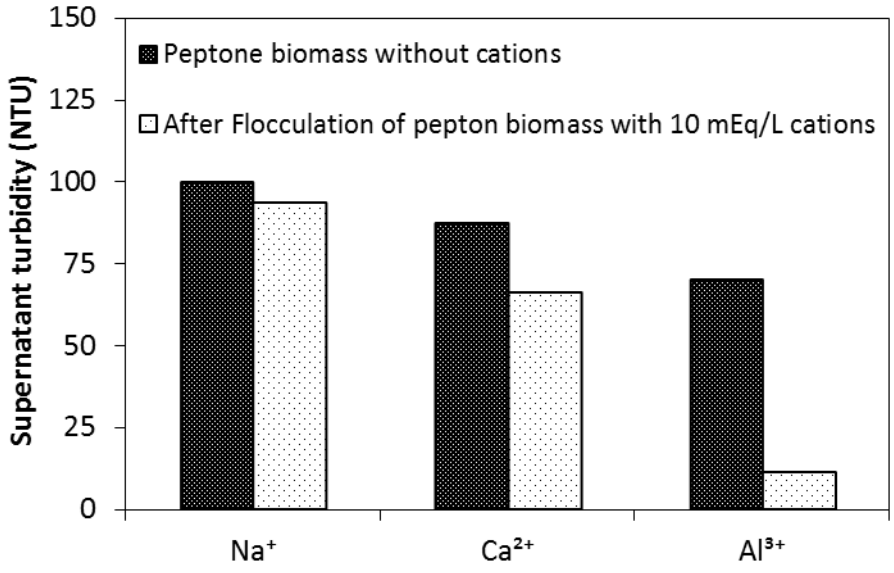


Figure 5.4. *Supernatant turbidity before (blank) and after flocculation of peptone fed biomass with different types of cations.*

Figures 5.3 and 5.4 show that flocculation of the biomass increased in the order $Na^+ < Ca^{2+} < Al^{3+}$, even though these ions were present at the same (charge) concentration of 10 mEq/L. This observation is in full agreement with the DLVO theory, according to which suppression of the electric double layer surrounding charged particles increases with the ionic strength; ions with a higher

valence contribute much more to the ionic strength according to $I = cz^2$ with I the ionic strength, c the concentration of the ion and z the valence of the ion. Thus, Al^{3+} and Ca^{2+} cause a stronger suppression of the double layer than Na^+ such that the particles can approach each other more closely and flocculation becomes more likely. Furthermore the ability of metal ions such as aluminum, to hydrolyze rapidly and to form multi-charged complexes is beneficial for flocculation.

Table 5.4. *Apparent zeta potential ζ_{app} of peptone fed biomass before and after flocculation with Na^+, Ca^{2+} and Al^{3+}.*

Sample	ζ_{app} (mV)
Biomass (blank)	- 21
Biomass + 10 mEq/L Na^+	-4
Biomass + 10 mEq/L Ca^{2+}	-2
Biomass + 10 mEq/L Al^{3+}	+1.9

Flocculation not only occurs due to suppression of the double layer, but also when the surface charge of particles is reduced. The apparent zeta potential (ζ_{app}) of the poorly flocculated biomass was -21 mV (Table 5.4), which is in the range of values for ζ_{app} typically found for microorganisms (Wilson *et al*., 2001). Addition of the cations resulted in less negative (-4 mV for Na^+ and -2 mV for Ca^{2+}) and even a positive (+1.9 mV for Al^{3+}) values for ζ_{app}. With the EPS extraction method used no EPS could be detected in the peptone fed biomass. However, it is known that external cell polymers are present on all bacterial cell surfaces in layers with a thickness varying between a few to several tenth of nm (Lin *et al*., 2013; Rijnaarts *et al*., 1995). It is likely that cations also adsorb into this polymer matrix, thus contributing to charge neutralization of the cell surface. This particularly becomes clear from positive ζ_{app} values when Al^{3+} was added to the biomass, which only can be the result of Al^{3+} accumulating in the cell exterior, counter balancing the net negative charge of that matrix. From other studies it is known that Ca^{2+} and in particular Al^{3+} have a large binding capacity to sludge and thus can have a major contribution to surface charge neutralization (e.g. Li *et al*., 2012).

5.3.2 Fate of particles during biomass flocculation

Figure 5.5 shows microscopic images of the flocs which were formed when poorly flocculated peptone fed biomass was mixed with 0.5 g/L kaolin clay particles and 10 mEq/L of the cations Na^+, Ca^{2+} and Al^{3+}. Similar differences in floc structure with the different cations could be detected as in

the absence of the clay particles (Figure 5.3). As shown by the example SEM image of Figure 5.6, clay particles were found to be incorporated in the floc matrix.

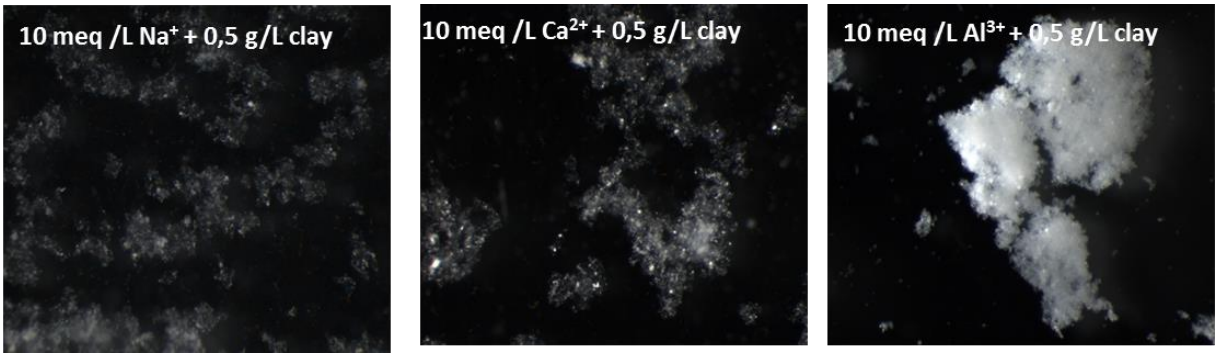


Figure 5.5. *Microscopic images of flocs formed in jar tests with peptone fed biomass after the addition of 10 mEq/L of Na^+, Ca^{2+} and Al^{3+} and 0.5 g/L of kaolin clay particles. Arrows indicate the presence of (shiny) clay particles.*

Figure 5.7 shows that for none of the cations the presence of 0.5 g/L clay particles did have a significant effect on the supernatant turbidity. Since kaolin clay particles remaining in the supernatant would have given a much higher turbidity compared to the turbidity when no clay was added, this implies that all of the clay particles that were added must have ended up in the floc matrix. Probably this can be explained by, amongs others, sweep flocculation that occurred during settling of the biomass (Rijnaarts *et al*., 1995; Yu *et al*., 2009).

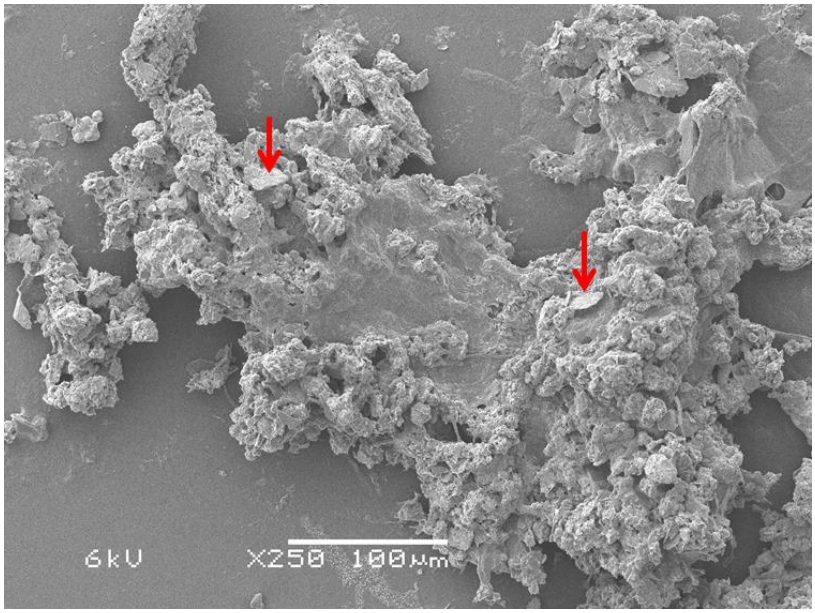


Figure 5.6. *Clay particles (arrow) incorporated in the flocs that were formed with the biomass in the presence of 10 mEq Ca^{2+}/L.*

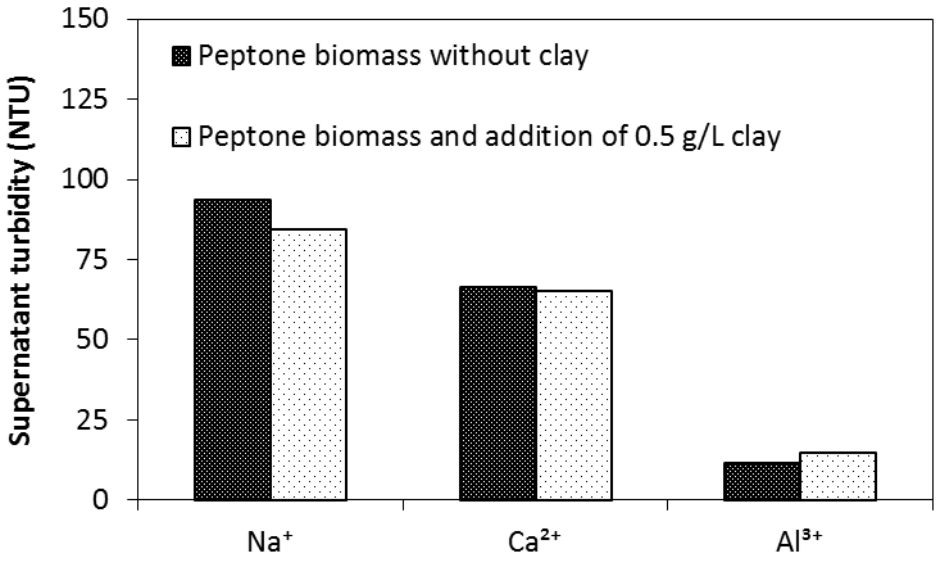


Figure 5.7. *Supernant turbidity after flocculation of peptone fed biomass with various cations and with or without the addition of 0.5 g/L kaolin clay.*

5.3.3 Flocculation activity of EPS extracted from various wastewater treatment systems

In the blank jar tests in which only the cations were and 4.7 g/L of the (negatively charged) clay particles were combined, the flocculation activity was always found to be between 82 and 87% . This can easily be explained by suppression of the electric double layer surrounding the clay particles. Figure 5.8 shows (additional) flocculation activities, i.e. relative to these blanks, when EPS is added to clay particles and cations. This was done for all the EPS that were extracted from the six biological wastewater treatment systems mentioned in Table 5.2. Experiments were performed and dublicates and differences were found to be minor. It should also be noted that due to the extraction procedure Na^+ was the main counter ion in the purified EPS, which was also verified by EDX analyses of the EPS powders (18-23% Na abundance). Mg and Ca were only found at very low abundances between 0.03 and 0.11% and between 0 and 0.04%, respectively. Al was not detected.

In the presence of Ca^{2+} and Al^{3+} all EPS types had a positive, albeit variable effect on flocculation activity (Figure 5.8). A positive effect of EPS on the flocculation potential of kaolin clay particles in the presence of Ca^{2+} was also reported by Bala Subramanian *et al*. (2010). No significant differences could be observed between Ca^{2+} and Al^{3+}. The highest flocculation activities were found with EPS from the acetate fed HL-MBR (60% flocculation activity with Al^{3+}) and with EPS from the full-scale

municipal wastewater treatment plant (38% flocculation with Al^{3+}). The additional flocculation activity caused by EPS in the presence of Na^{+} always was much lower than in the presence of Ca^{2+} and Al^{3+}, and in the case of the three municipal treatment systems even this was negative.

The fact that EPS further enhanced the flocculation activity presumably is caused by the formation of polymer bridges between the clay particles, which were already destabilised by the cations. The observations that (i) Na^{+} in the blank tests gave similar flocculation activities as Ca^{2+} and Al^{3+} and (ii) the (additional) effect of EPS was much stronger in the presence of Ca^{2+} and Al^{3+} than in the presence of Na^{+}, support the DCB theory that divalent cations bind more strongly to the EPS than Na^{+}. Thus, polymer–polymer and polymer–particle bridges are more likely to form in the presence of Ca^{2+} and Al^{3+} than in the presence of Na^{+}. This also is in line with findings that the addition of Na^{+} to activated sludge results in leaching of multivalent cations from the sludge matrix, the release of free biopolymers and deflocculation (Higgins and Novak, 1997; Kara *et al*., 2008; Novak *et al*., 1998; Sobeck and Higgins, 2002).

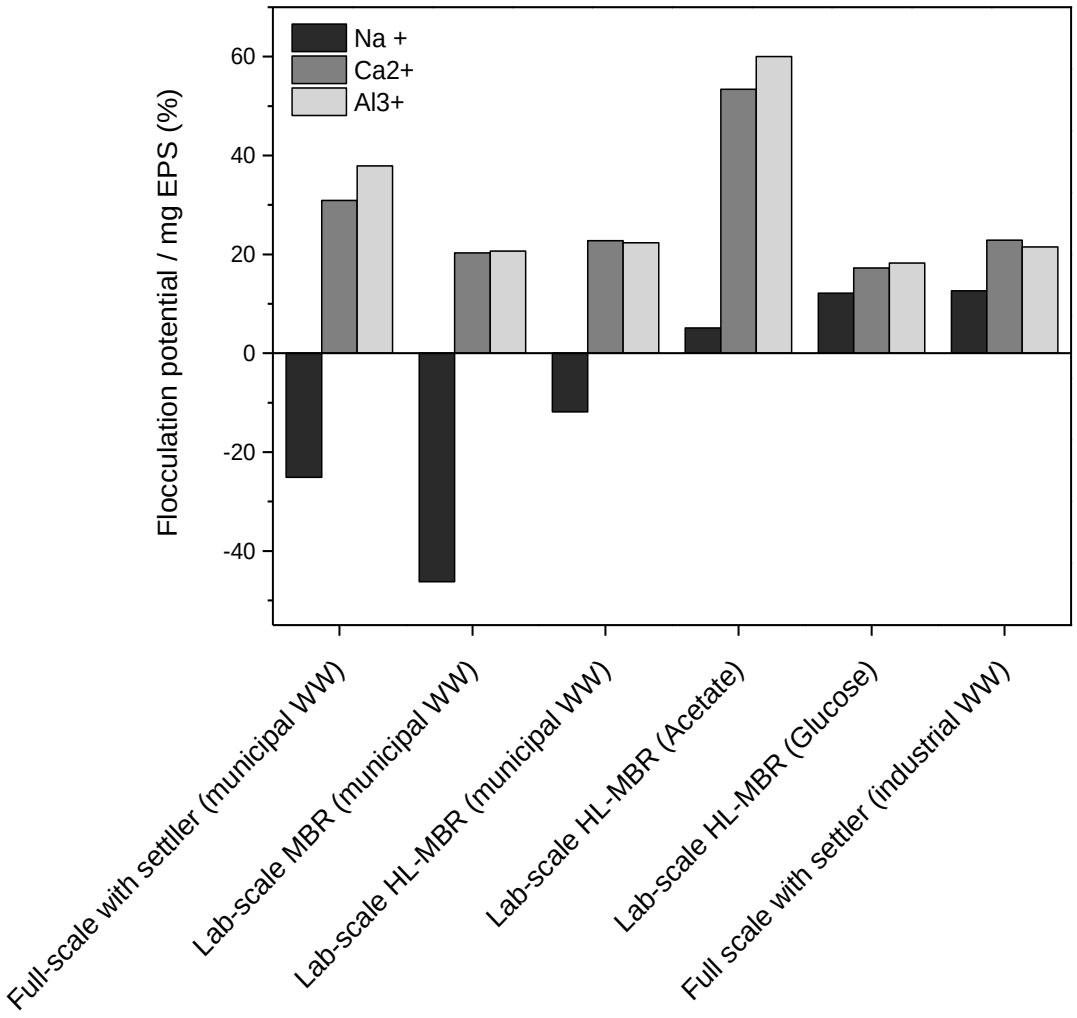


Figure 5.8. Flocculation activity of purified EPS, extracted from sludge samples taken from different wastewater treatment systems (see table 5.2) in the presence of 10 mEq/L Na^+, Ca^{2+} and Al^{3+}.

Interestingly, addition of the EPS from the municipal wastewater treatment systems in the presence of Na^+ had a negative effect on the flocculation process, i.e. the clay particles of the blank clay – Na^+ system became more stabilized due to addition of these EPS. Possibly, the chemical composition, molecular weight and charge of these municipal sludge related EPS is such that they could easily adsorb onto the surface of the clay particles and cause stabilisation of them. In case multivalent ions are present, the same EPS leads to enhanced flocculation of the clay particles. This may be caused by suppression of double layer repulsion, which is stronger for multivalent cations than for mono valent cations, by a cation-bridging between the EPS coated clay particles, or other mechanisms. Obviously,

the type of EPS and its (average) physical-chemical properties are closely related to the type of wastewater that is treated and the operational conditions under which the biomass is cultivated. Although this cannot be further substantiated, a clear difference was observed in the flocculation behaviour between the EPS extracted from the three municipal wastewater treatment systems that had to deal with a complex mixture of organic carbon, and the EPS that was extracted from the three treatment systems that had to deal with less complex wastewater containing an easily biodegradable substrate (acetate, glucose or glycerine). Biodegradabilty of substrates has been reported by Sponza (2003) and Wang *et al*. (2013) to influence EPS production and composition, although they did not link this to flocculation activity of these EPS.

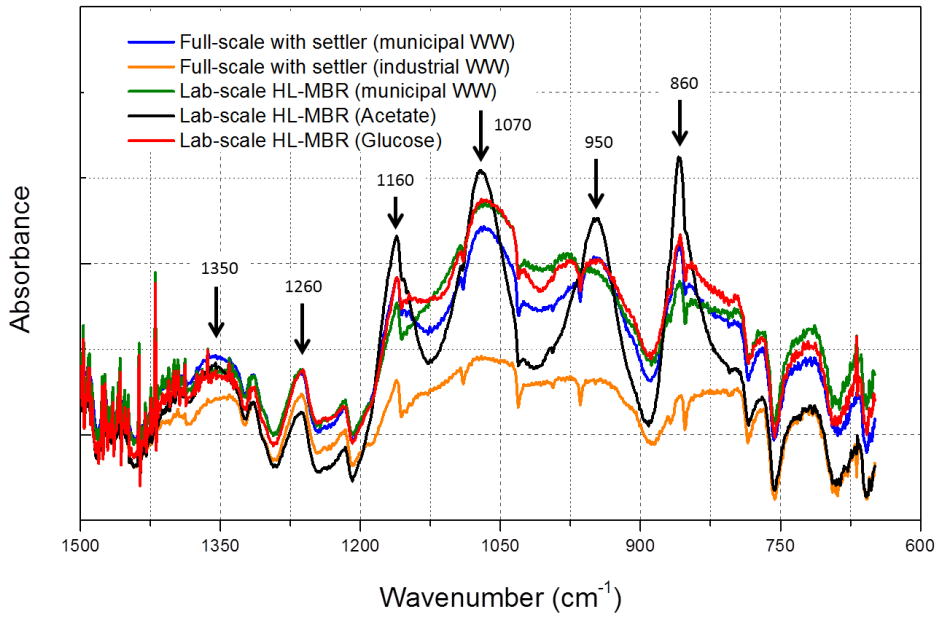


Figure 5.9. *FT-IR spectrum of purified EPS extracted from different wastewater treatment systems.*

FT-IR analyses were carried out to explore differences in the biochemical composition of the EPS types (Figure 5.9). For the EPS extracted from the (municipal) treatment systems, data from the lab-scale MBR is not available. For all types of EPS expected functional groups can be observed in the spectra. At 1350 cm^{-1} C-O and C-H stretches are detected which are associated with amino acids and thus proteins (Badireddy *et al.*, 2010). The band at 1260 cm^{-1} could be assigned to C-N stretches associated with secondary amide III (Badireddy *et al.*, 2010). At 1160 cm^{-1} C-O-C streching vibrations were observed (Comte *et al.*, 2006). At 1070 cm^{-1} ring vibrations are detected of C-O-C and C-O-P which are characteristic for polysaccharides (Badireddy *et al.*, 2010). The band at 950 cm^{-1} can be assigned to O-P-O stretches associated with nulceic acids (Badireddy *et al.*, 2010). Although these FT-IR analyses only give qualitative information, a clear difference can be detected between the

spectrum of the EPS from the industrial wastewater treatment plant and the other spectra, in particular between wavenumbers 1200 and 770 cm^{-1}. Whether this strong deviation in biochemical composition is caused by the different carbon source in the wastewater of this treatment plant (50% of the COD was glycerin) or by the presence of very high Na^+ concentrations in the wastewater (approximately 4800 mg/L) is unclear. Perhaps less significant, but also the spectrum of the EPS extracted from the acetate fed HL-MBR reactor was clearly different from the other spectra.

More research is required to link the conditions under which microorganism produce EPS, the chemical compositions of these EPS, their physical-chemical properties and their flocculation ability in the presence of different cations. All of this information is relevant not only for operation of wastewater treatment systems where sludge-water separation is very important, but also when commercial applications of (extracted) EPS are considered.

5.4 Conclusions

In this study conditions were mimicked in reactors that use bioflocculation as the process to concentrate (municipal) wastewater organic particles. For this purpose jar flocculation tests were performed with poorly flocculated (peptone fed) biomass, low concentrations of the cations Na^+, Ca^{2+} and Al^{3+}, negatively charged kaolin clay particles and low concentrations (10 mg/L) of extracellular polymeric substances (EPS) that were extracted and purified from sludge samples from 6 different biological wastewater treatment plants. The results showed that:

- Adding cations to the poorly flocculating biomass had a positive effect on flocculation of this biomass, increasing in the order $Na^+ < Ca^{2+} < Al^{3+}$. This behavior can be fully explained by the DLVO theory.
- While the biomass flocculated in the presence of the cations, clay particles which were added at a concentration of 0.5 g/L were completely taken up by the biomass aggregates.
- Addition of 10 mg EPS/L to clay particles, which were simultaneously destabilized by adding 10 mEq/L of the cations Ca^{2+} and Al^{3+}, resulted in an additional flocculation activity. The extent of this additional flocculation was similar for Ca^{2+} and Al^{3+}, but was strongly dependent on the wastewater type from which the EPS originated.
- In the presence of 10 mEq/L of Na^+ this additional effect of EPS on kaolin flocculation was much lower than with Ca^{2+} and Al^{3+}. Some EPS types even caused a negative flocculation

activity, indicating destabilization of the clay particles. Remarkably, this only happened with EPS that was extracted from sludge that was sampled from municipal wastewater treatment systems.

- FT-IR analyses indicated a similar biochemical composition of EPS from several municipal wastewater treatment systems and of EPS extracted from a glucose fed reactor. EPS that was harvested from an acetate fed reactor, and in particular EPS from a full-scale plant treating industrial saline wastewater with glycerin as the main carbon source, showed a very different biochemical composition.

Acknowledgements

This work was performed in the cooperation framework of Wetsus, centre of excellence for sustainable water technology (www.wetsus.nl). Wetsus is co-funded by the Dutch Ministry of Economic Affairs and Ministry of Infrastructure and Environment, the European Union Regional Development Fund, the Province of Fryslân and the Northern Netherlands Provinces. The authors like to thank the participants of the research theme “Membrane processes and operation for wastewater treatment and reuse” for the fruitful discussions and their financial support.

The authors also want to thank Perry van der Marel and WLN (Glimmen, The Netherlands) for the supply with industrial wastewater sludge.

References

Badireddy, A.R., Chellam, S., Gassman, P.L., Engelhard, M.H., Lea, A.S., Rosso, K.M., 2010. Role of extracellular polymeric substances in bioflocculation of activated sludge microorganisms under glucose-controlled conditions. Water research 44, 4505–4516.

Bala Subramanian, S., Yan, S., Tyagi, R., Surampalli, R., 2010. Extracellular polymeric substances (EPS) producing bacterial strains of municipal wastewater sludge: Isolation, molecular identification, EPS characterization and performance for sludge settling and dewatering. Water research 44, 2253–2266.

Bruus, J.H., Nielsen, P.H., Keiding, K., 1992. On the stability of activated sludge flocs with implications to dewatering. Water research 26, 1597–1604.

De Temmerman, L., Maere, T., Temmink, H., Zwijnenburg, A., Nopens, I., 2014. Salt stress in a membrane bioreactor: dynamics of sludge properties, membrane fouling and remediation through powdered activated carbon dosing. Water Research 63, 112–124.

Deng, S., Bai, R., Hu, X., Luo, Q., 2003. Characteristics of a bioflocculant produced by Bacillus mucilaginosus and its use in starch wastewater treatment. Applied microbiology and biotechnology 60, 588–593.

Faust, L., Temmink, H., Zwijnenburg, A., Kemperman, A., Rijnaarts, H., 2014. High loaded MBRs for organic matter recovery from sewage: Effect of solids retention time on bioflocculation and on the role of extracellular polymers. Water research 56, 258–266.

Gao, J., Bao, H., Xin, M., Liu, Y., Li, Q., Zhang, Y., 2006. Characterization of a bioflocculant from a newly isolated Vagococcus sp. W31. Journal of Zhejiang University SCIENCE B 7, 186–192.

Higgins, M.J., Novak, J.T., 1997. The effect of cations on the settling and dewatering of activated sludges: laboratory results. Water Environment Research 215–224.

Kappel, C., Kemperman, A., Temmink, H., Zwijnenburg, A., Rijnaarts, H., Nijmeijer, K., 2014. Impacts of NF concentrate recirculation on membrane performance in an integrated MBR and NF membrane process for wastewater treatment. Journal of Membrane Science 453, 359–368.

Kara, F., Gurakan, G., Sanin, F., 2008. Monovalent cations and their influence on activated sludge floc chemistry, structure, and physical characteristics. Biotechnology and bioengineering 100, 231–239.

Kurane, R., Takeda, K., Suzuki, T., 1986. Screening for and Characteristics of Microbial Flocculants (Microbiology & Fermentation Industry). Agricultural and Biological Chemistry 50, 2301–2307.

Li, H., Wen, Y., Cao, A., Huang, J., Zhou, Q., Somasundaran, P., 2012. The influence of additives (Ca2+, Al3+, and Fe3+) on the interaction energy and loosely bound extracellular polymeric substances (EPS) of activated sludge and their flocculation mechanisms. Bioresource technology 114, 188.

Liao, B., Allen, D., Droppo, I., Leppard, G., Liss, S., 2001. Surface properties of sludge and their role in bioflocculation and settleability. Water Research 35, 339–350.

Lin, Y., Sharma, P., van Loosdrecht, M., 2013. The chemical and mechanical differences between alginate-like exopolysaccharides isolated from aerobic flocculent sludge and aerobic granular

sludge. Water research 47, 57–65.

Mikkelsen, L., Gotfredsen, A., Agerbxk, M., Nielsen, P.H., Keiding, K., 1996. Effects of colloidal stability on clarification and dewatering of activated sludge. Water Science and Technology 34, 449–457.

Novak, J.T., Love, N.G., Smith, M.L., Wheeler, E.R., 1998. The effect of cationic salt addition on the settling and dewatering properties of an industrial activated sludge. Water environment research 70, 984–996.

Rijnaarts, H.H., Norde, W., Bouwer, E.J., Lyklema, J., Zehnder, A.J., 1995. Reversibility and mechanism of bacterial adhesion. Colloids and Surfaces B: Biointerfaces 4, 5–22.

Salehizadeh, H., Vossoughi, M., Alemzadeh, I., 2000. Some investigations on bioflocculant producing bacteria. Biochemical engineering journal 5, 39–44.

Salta, M., Wharton, J.A., Blache, Y., Stokes, K.R., Briand, J.-F., 2013. Marine biofilms on artificial surfaces: Structure and dynamics. Environmental microbiology 15, 2879–2893.

Sheng, G.-P., Yu, H.-Q., Li, X.-Y., 2010. Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: a review. Biotechnology Advances 28, 882–894.

Sobeck, D.C., Higgins, M.J., 2002. Examination of three theories for mechanisms of cation-induced bioflocculation. Water Research 36, 527–538.

Sponza, D.T., 2003. Investigation of extracellular polymer substances (EPS) and physicochemical properties of different activated sludge flocs under steady-state conditions. Enzyme and Microbial Technology 32, 375–385.

Wang, H., Deng, H., Ma, L., Ge, L., 2013. The effect of carbon source on extracellular polymeric substances production and its influence on sludge floc properties. Journal of Chemical Technology and Biotechnology 4, 516–521.

Wilson, W.W., Wade, M.M., Holman, S.C., Champlin, F.R., 2001. Status of methods for assessing bacterial cell surface charge properties based on zeta potential measurements. Journal of microbiological methods 43, 153–164.

Xia, S., Zhang, Z., Wang, X., Yang, A., Chen, L., Zhao, J., Leonard, D., Jaffrezic-Renault, N., 2008. Production and characterization of a bioflocculant by Proteus mirabilis TJ-1. Bioresource Technology 99, 6520–6527.

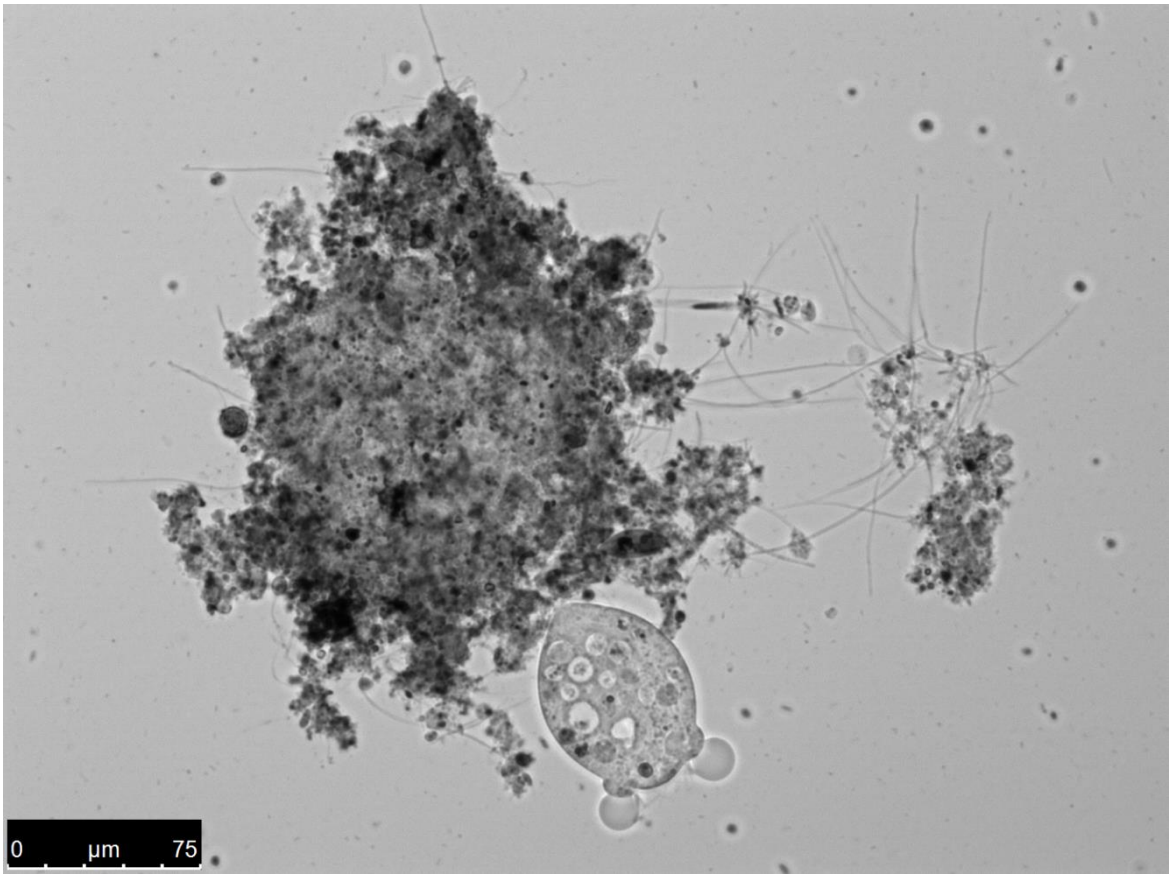
Xiong, Y., Wang, Y., Yu, Y., Li, Q., Wang, H., Chen, R., He, N., 2010. Production and characterization of a novel bioflocculant from Bacillus licheniformis. Applied and environmental microbiology 76, 2778–2782.

Yu, G.-H., He, P.-J., Shao, L.-M., 2009. Characteristics of extracellular polymeric substances (EPS) fractions from excess sludges and their effects on bioflocculability. Bioresource Technology 100, 3193–3198.

Zita, A., Hermansson, M., 1994. Effects of ionic strength on bacterial adhesion and stability of flocs in a wastewater activated sludge system. Applied and environmental microbiology 60, 3041–3048.

6

General Discussion and Outlook



6.1 Introduction

In this thesis the up-concentration of wastewater organic matter was investigated. This up-concentration is essential for making further processing of the organics to other valuable products feasible. The thesis focusses on using bioflocculation processes induced by extremely short retention times in a membrane bioreactor. During the bioflocculation process, wastewater particles are flocculated with the help of extracellular polymeric substances produced by microorganisms. In contrast to conventional wastewater treatment plants, a high loaded membrane bioreactor (HL-MBR) is operated at extremely short retention times to elicit bioflocculation, and to minimize mineralization of organic matter to CO_2. From the concentrate of HL-MBRs energy or chemicals can be recovered, e.g. as methane or as volatile fatty acids (VFAs), which are building blocks for bioplastics and other valuable organic chemicals. The new knowledge on the up-concentration of wastewater organic matter using HL-MBR systems presented in this thesis concerns the following topics: assessing the organic chemical recovery potential of the system (Chapter 2), resolving crucial operational aspects of the HL MBR system (Chapter 2, Chapter 3), elucidation microbial processes involved in the production of extra cellular polymeric substances (EPS) during bioflocculation (Chapter 4), and characterizing EPS and relating this to the potential for further processing to valuable products and applications (Chapter 5).

6.2 Methane and chemical recovery from HL-MBR concentrate

In chapter 2 it was found that an SRT of 0.5 d was optimal for the operation of HL-MBRs because at this SRT the flocculation efficiency of wastewater colloidal and suspended matter was high, while mineralization of organic matter was low (<15%). The potential to produce energy from the concentrate still needs to be investigated. Preliminary studies by Akanyeti *et al*. (2010) showed that 64% of the concentrate COD could be converted into methane in batch tests, using sludge from a HL-MBR that was operated at SRT of 1 d. In contrast, when secondary sludge from conventional wastewater treatment plants is digested anaerobically, only 20-30% of the sludge organic matter is converted into methane (Rulkens, 2007). The rate of anaerobic conversion of HL-MBR sludge is favored because this sludge contains less bacterial biomass and less complex substances than secondary sludge. Hydrolysis of bacterial biomass and other organic substances determines the anaerobic digestion rate. Thus, the fraction of methane that can be produced from HL-MBR sludge, containing easily biodegradable organic matter, is higher than from secondary sludge.

Because of the low economic value of methane, the production of organic chemicals from waste streams was recently suggested (Agler *et al*., 2011). Thus, instead of methane it may be more useful to produce e.g. VFAs and other valuable organic chemicals. This can be achieved by reducing the SRT in the anaerobic reactor (between 8 and 10 d, Lee *et al*. (2014)) to wash-out methanogens while maintaining a population of VFA producing microorganisms. Too short SRTs on the other hand, may reduce VFA production due to inefficient hydrolysis. In preliminary studies it was found that 30-50% of the HL-MBR concentrate chemical oxygen demand (COD) could be converted to volatile fatty acids at a SRT of 5 d under uncontrolled pH conditions (personal communication). Controlling the pH, e.g. at pH 10, the production of VFA may be increased. Firstly, at this pH chemical sludge hydrolysis will take place which promotes solubilization of particulate and colloidal COD. Secondly, at such a high pH methanogenesis is inhibited, allowing longer SRTs with a higher VFA yield. Furthermore, at higher pH anionic VFAs are produced which might be recovered more easily compared to uncharged VFAs, e.g. by electrochemical separation processes.

However, the organic matter concentration found in the HL-MBR concentrates operated at a SRT of 0.5 d and 1 d and a hydraulic retention time (HRT) of 0.7 h were 6 and 11 g_{COD}/L, respectively (Chapter 2). This would probably be too low for an economic VFA or methane production: the anaerobic reactor would have to treat a relatively large water volume and high heating costs would be needed since anaerobic digestion requires temperatures between 30 and 40 °C. Lee *et al*. (2014) reviewed research about VFA production from secondary and primary sludge and it was shown that (initial) COD concentrations between 15 and 23 g_{COD}/L would be required. Such concentrations could be achieved if the HRT would be further reduced from 0.7 to 0.1 h (also see below).

6.3 Up-concentration of wastewater organic matter using membrane filtration

Several techniques have been proposed to up-concentrate wastewater organic matter. Among these are dissolved air floatation, optionally with the use of polymers to enhance the process, direct chemical flocculation of wastewater organic matter using metal salts or polymers and direct membrane filtration. Direct membrane filtration of raw wastewater, however, results in severe membrane fouling (Diamantis *et al*., 2014). Operating a bioflocculation stage in combination with membrane filtration on the other hand could significantly decrease the membrane fouling potential (Diamantis *et al*., 2014; Ivanovic *et al*., 2008). Membrane fouling experiments as described in chapter 2 and 3, showed that the fouling potential of the sludge from the HL-MBR was comparable to other submerged MBR systems operated with the same wastewater but at longer SRTs i.e. 16 d (Kappel *et al*., 2014). However, operational costs of MBRs still are higher than those of conventional wastewater

treatment plants using a settler for solid-liquid separation, mainly because of their higher energy consumption and membrane costs (Judd, 2008). On the other hand, if a settler would be applied in combination with a bioflocculation stage, the effluent would still contain relatively high concentrations of colloidal matter (approximately 10 mg COD/L, chapter 3). This would present a problem when the water needs to be upgraded for reuse, for instance by nanofiltration or reversed osmosis treatment. The produced permeate of the HL-MBR, on the contrary, is particle and pathogen free, but still contains most of the nitrogen and phosphorus, which would also allow the used as irrigation water.

To achieve even higher concentrations factors necessary for e.g. economically VFA production, the HRT should be further reduced, which in the case of the HL-MBR implies that a higher membrane area in relation to reactor volume would be needed. With available submerged modules this does not seem to be possible, simply because the membrane would not fit in the reactor. Instead, a side-stream membrane module could be used. Using a side stream configuration the bioreactor volume could be kept sufficiently small whereas the amount of membrane area could be increased by external e.g. tubular membrane filters. Tubular membranes also permit good fouling control though e.g. backwashing. However, external membrane modules generally are operated at higher shear conditions, and this may result in weaker concentrate flocs. Possibly, with external tubular membranes, operated according to the air-flush principle, this problem can be avoided (Futselaar *et al*., 2007).

6.4 Microbial population and EPS production

In chapter 4 the bacterial community in three HL-MBR systems operated at various SRTs was characterized. The bacterial community of the concentrate flocs and in the supernatant was significantly different from each other. Both, the supernatant and the (solid) sludge fraction, contained several genera, such as *Acinetobacter,* which are well known for their EPS production. Thus, it was suggested that the bioflocculation process was not only governed by EPS production of bacteria associated with the concentrate flocs, but also by those in the supernatant. However, the presence of genera which are known for EPS production is not sufficient to be sure about their actual EPS production in the specific environment, e.g. the concentrate flocs or the supernatant. To unveil this link between the specific microorganisms that are present and their interaction and function in the given environment, direct DNA or RNA sequencing approaches could be used. These environmental metatranscriptomics approaches recently became available, due to the development of e.g. next generation sequencing techniques. In metatranscriptomics, a direct cDNA cloning is

applied to study RNA transcripts in environmental samples and thereby identifying active genes (Morales and Holben, 2011). Most important in this approach is the reduction in community complexity by focusing only on active gens. Knowing which genes of which bacterial species are active in the environmental samples gives insight into the interaction between microorganisms and the process taking place *in situ*. To study EPS production of microorganisms in the concentrate flocs and in the supernatant through metatranscriptomics approaches, genes which are active during EPS production would need to be studied. However, genes responsible for microbial EPS production are not very well known yet. This is because it is still not precisely understood what triggers microorganisms to produce EPS. In activated sludge systems it is assumed that factors such as substrate concentration, growth rate, chemical and physical stresses and predation could induce microorganisms to produce EPS (Bossier and Verstraete, 1996). Thus, it first need to be investigated which genes would be responsible for EPS production and subsequently metatranscriptomics could be applied to the HL-MBR sludge and supernatant to find out which bacterial species produced EPS.

Another possibility would be to study EPS production of bacteria from the HL-MBR *ex situ* by isolation EPS producing bacteria. Concentrate flocs and also supernatant samples should be used for cultivation. In this way EPS production of supernatant bacteria could be studied in comparison with the EPS production from bacteria associated with the concentrate flocs. Subsequently, the potential of the produced EPS to flocculate wastewater particles can be studied (Bala Subramanian *et al*., 2010). Bacteria which produce EPS with a high flocculation potential should be characterized and the triggers for those bacteria to produce EPS can be investigated in more detail, e.g. by studying the effect of varying nutrient concentrations, shear conditions etc. Knowing the factors which induce bacteria to produce EPS may also allow better control of bioflocculation and even optimize a commercial production of bioflocculants (also see below).

6.5 Characterization of EPS and flocculation potential

EPS that have a high flocculation potential should be characterized with respect to their chemical composition and compared to EPS with a lower flocculation potential. In relation to bioflocculation in real wastewater treatment systems, however, it may be better to study the characteristics and composition of the whole EPS pool present in the sludge, as was described in chapter 5, rather than one type of EPS produced by one bacterial species. This became clear since it was found that various wastewater treatment systems e.g. industrial and municipal wastewater treatment, yielded EPS mixtures with very different bioflocculation potentials. It was also found that these differences in bioflocculation potential were a result of differences in the bio-chemical composition of the EPS,

probably caused by wastewater characteristics such as cation concentration, type of organic substrate and probably also by operational treatment conditions. EPS extracted from these various wastewater treatment systems should be further characterized to find out which properties (molecular weight, hydrophobicity, charge density, affinity for cations, etc.) determine their flocculation potential. For example Lin *et al*. (2013) found that the difference in the chemical structure of alginate-like substances extracted from aerobically grown granules and flocculent sludge growing on the same municipal wastewater, were responsible for the differences in their chelating properties.

Also the total concentration of EPS was found to be important for the bioflocculation process when operating HL-MBRs. In Chapter 2 it was shown that the bound EPS concentration increased from 55 mg/gVSS to 118 mg/gVSS, when the SRT of a HL-MBRs was increased from 0.125 to 1 d SRT. At the same time, the bioflocculation efficiency increased from 59 to over 90%. It was assumed that the higher EPS concentration resulted from higher substrate utilization rates (Laspidou and Rittmann, 2002). However, lower degradation rates of produced EPS also can contribute to higher concentrations of EPS. To find out how much EPS are produced due to substrate utilization and how much EPS are subsequently degraded, ^{14}C labeled substrate could be feed to the reactors and the distribution between ^{14}C-EPS and $^{14}CO_2$ could be investigated (Decho *et al*., 2005). In this way mass balances for EPS production could be established

6.6 Estimation of EPS recovery potential and application of EPS

Extracted EPS may also have a commercial application (see below). The recovery potential of EPS from HL-MBR concentrate was estimated from an average EPS concentration in the HL-MBR concentrate of 135 mg/gVSS (448 mg EPS/L of concentrate; Chapters 2 and 3). For a 100.000 population equivalent HL-MBR, operated at a SRT of 0.5 d and a HRT of 0.7 h, this would yield 360 kg EPS per day, or 131 400 kg per year (Figure 6.1).

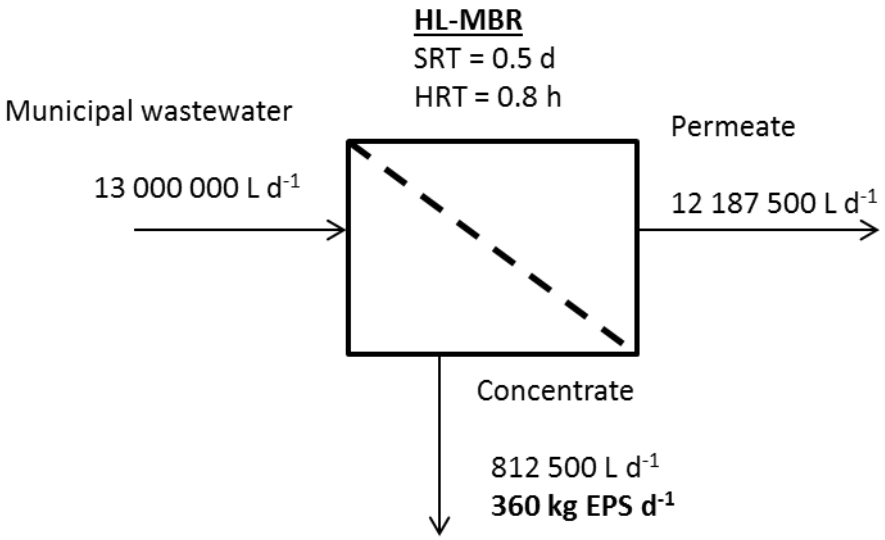


***Figure 6.1**. Daily EPS recovery potential from HL-MBR concentrated when 100 000 PE municipal wastewater is treated.*

However, the extraction of these produced EPS with an ion exchange resin as described in chapter 2 would not be feasible. The procedure requires 0.35 g resin to extract EPS from 0.5 g of TSS. At an estimated yearly concentrate production of 1186 tons TSS per year and resin costs of 50 € per kg resin, the yearly costs would be 4 billion euros, or 260 Euros per kg of EPS. However, the extraction method was not yet optimized for the minimum amount of resin necessary to extract EPS from the sludge. Thus, optimization of the extraction method could decrease costs for the cation exchange resin. Additionally the cation exchange resin should be reused after regeneration with sodium chloride, which would decrease the price for EPS extraction significantly. Alternatively, EPS could be extracted chemically with e.g. EDTA, formaldehyde or alkaline treatment with NaOH. However, the addition of chemicals may alter the EPS structure and properties including their flocculation potential, and may require further cleaning steps to separate EPS and chemicals (Sheng *et al.*, 2010).

Sludge settling and dewatering

Usually sludge settling and dewatering in wastewater treatment plants is done with the aid of metal cations (e.g. Al^{3+}, Fe^{3+}) and cationic – or anionic synthetic polymers (Higgins and Novak, 1997). However, application of these synthetic polymers is expensive and the polymers may further pollute the environment, e.g. through odor release upon their degradation (Chang *et al.*, 2005). Tyagi *et al.* (2012) investigated the costs of polymers used in a wastewater treatment plant in Quebec, Canada in 2002 and found out that 3-7 tons of polymers per ton of dry sludge was used, which resulted in costs of approximately $7.6-10.6 million per year. For wastewater treatment plants treating wastewater

from the starch industry, the consumption of polymers to dewater sludge was even was 28.8 tons/year.

Once the polymers are released into the environment e.g. by using dewatered sludge for agriculture, they can be harmful for soil microorganisms. Acrylamide monomers even are carcinogenic and neurotoxic (Salehizadeh and Shojaosadati, 2001). Thus, bio-based polymers, as for example extracted EPS may be used as alternative flocculants due to their non-toxic characteristics. In chapter 5 it was shown that EPS extracted from an HL-MBR treating municipal wastewater reached a flocculation efficiency of kaolin clay particles of 22% per mg EPS applied. Bala Subramanian *et al*. (2010) studied the EPS production of 25 bacterial strains isolated from municipal wastewater and found the best kaolin clay flocculation efficiencies when slime EPS were used in the flocculation tests, compared to capsular EPS. Based on high flocculation efficiencies, as high as 80%, they selected 6 bacterial strains and conducted sludge settling experiments by using the EPS of the selected bacterial strains to flocculate freshly collected sludge samples. Their ability to settle sludge in comparison with commercially available cation synthetic polymer was studied. It was found that the extracted EPS were similarly effective in sludge settling as the synthetic polymer, even at very low concentrations of 0.3 g/L. Thus, EPS represent a powerful alternative to the use of synthetic polymers; however, more research is needed especially regarding the optimization of EPS extraction and the costs involved.

Other applications

Bacterial surfactants and emulsifiers gained attention due to their biodegradability and possible production from renewable resources. As such it was found, that an exopolysacchride from *Sphingomonas paucimobilis GS1* was more effective in emulsifying several hydrocarbons such as xylene, benzene and also paraffin and olive oil than commercially available gums such as arabic and tragacanth (Ashtaputre and Shah, 1995).

Becerra (2010) conducted a comprehensive study about the possibilities to utilize activated sludge as a source or surface active agents. Firstly an effective alkaline treatment to extract 75% of the sludge organic matter was established. It was found that the alkaline extracts had properties which were comparable to commercial detergents, with as low surface tension as 37 mN/m. Additionally, the extract could be converted into wood adhesives with high adhesive shear strength using glutaraldehyde as a cross linker. In the end a cost estimation was carried out, showing that the production of detergents from sludge could be economical feasible. However, operational, capital and maintenance costs were not included in the costs estimation. The production of alternative

adhesives from EPS was found to be not economically feasible, mainly due to more extensive down streaming and purification processes.

6.7 Conclusion

This thesis focused on studying the up-concentration of wastewater organic matter through bioflocculation induced by microbial EPS production. Further research should be conducted on optimizing the bioflocculation process to make the recovery of energy or other valuable products from the wastewater organic matter feasible, such as increasing the concentration factor for organic matter, by reducing the HRT. Furthermore the production and efficient recovery methods of valuable products such as VFAs produced from the HL-MBR concentrate should be investigated. Because of their good biodegradability and nontoxic properties compared to synthetic polymers, the possibilities of applying EPS as alternative polymers for e.g. sludge dewatering, and the production of other surface active agents from EPS should be further explored. Therefore more research about the characteristics and properties of EPS is required, and it needs to be investigated which EPS characteristics are responsible for either good or bad flocculation properties.

References

Agler, M., Wrenn, B., Zinder, S., Angenent, L., 2011. Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform. Trends in biotechnology 29, 70–78.

Akanyeti, I., Temmink, H., Remy, M., Zwijnenburg, A., 2010. Feasibility of bioflocculation in a high-loaded membrane bioreactor for improved energy recovery from sewage. Water science and technology 61, 1433–1439.

Ashtaputre, A., Shah, A., 1995. Emulsifying property of a viscous exopolysaccharide fromSphingomonas paucimobilis. World Journal of Microbiology and Biotechnology 11, 219–222.

Bala Subramanian, S., Yan, S., Tyagi, R., Surampalli, R., 2010. Extracellular polymeric substances (EPS) producing bacterial strains of municipal wastewater sludge: Isolation, molecular identification, EPS characterization and performance for sludge settling and dewatering. Water research 44, 2253–2266.

Becerra, F.Y.G., 2010. Recovery of surface active material from municipal wastewater activated sludge. University of Toronto

Bossier, P., Verstraete, W., 1996. Triggers for microbial aggregation in activated sludge? Applied Microbiology and Biotechnology 45, 1–6.

Chang, J.-S., Abu-Orf, M., Dentel, S.K., 2005. Alkylamine odors from degradation of flocculant polymers in sludges. Water research 39, 3369–3375.

Comte, S., Guibaud, G., Baudu, M., 2006. Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and EPS complexation properties: Part I. Comparison of the efficiency of eight EPS extraction methods. Enzyme and Microbial Technology 38, 237–245.

Decho, A.W., Visscher, P.T., Reid, R.P., 2005. Production and cycling of natural microbial exopolymers (EPS) within a marine stromatolite. Palaeogeography, Palaeoclimatology, Palaeoecology 219, 71–86.

Diamantis, V., Eftaxias, A., Bundervoet, B., Verstraete, W., 2014. Performance of the biosorptive activated sludge (BAS) as pre-treatment to UF for decentralized wastewater reuse. Bioresource technology 156, 314–321.

Futselaar, H., Schonewille, H., de Vente, D., Broens, L., 2007. NORIT AirLift MBR: side-stream system for municipal waste water treatment. Desalination 204, 1–7.

Higgins, M.J., Novak, J.T., 1997. The effect of cations on the settling and dewatering of activated sludges: laboratory results. Water Environment Research 215–224.

Ivanovic, I., Leiknes, T., \Odegaard, H., 2008. Fouling Control by Reduction of Submicron Particles in a BF-MBR with an Integrated Flocculation Zone in the Membrane Reactor. Separation science and technology 43, 1871–1883.

Judd, S., 2008. The status of membrane bioreactor technology. Trends in biotechnology 26, 109–116.

Kappel, C., Kemperman, A., Temmink, H., Zwijnenburg, A., Rijnaarts, H., Nijmeijer, K., 2014. Impacts of NF concentrate recirculation on membrane performance in an integrated MBR and NF

membrane process for wastewater treatment. Journal of Membrane Science 453, 359–368.

Laspidou, C.S., Rittmann, B.E., 2002. A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass. Water Research 36, 2711–2720.

Lee, W.S., Chua, A.S.M., Yeoh, H.K., Ngoh, G.C., 2014. A review of the production and applications of waste-derived volatile fatty acids. Chemical Engineering Journal 235, 83–99.

Lin, Y., Sharma, P., van Loosdrecht, M., 2013. The chemical and mechanical differences between alginate-like exopolysaccharides isolated from aerobic flocculent sludge and aerobic granular sludge. Water research 47, 57–65.

Morales, S.E., Holben, W.E., 2011. Linking bacterial identities and ecosystem processes: can “omic”analyses be more than the sum of their parts? FEMS microbiology ecology 75, 2–16.

Rulkens, W., 2007. Sewage sludge as a biomass resource for the production of energy: overview and assessment of the various options†. Energy \& Fuels 22, 9–15.

Salehizadeh, H., Shojaosadati, S., 2001. Extracellular biopolymeric flocculants: recent trends and biotechnological importance. Biotechnology advances 19, 371–385.

Sheng, G.-P., Yu, H.-Q., Li, X.-Y., 2010. Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review. Biotechnology Advances 28, 882–894.

Tyagi, R., Surampalli, R., Zhang, T.C., others, 2012. Green Biotechnology for Municipal and Industrial Wastewater Treatment. In: Green Chemistry for Environmental Remediation. Wiley Online Library, pp. 627–660.

Appendix A

Assigment of clones to closest relative (Chapter 4)

Assignment of clones obtained from the **wastewater** to their closest relative						
No. of clones	Closest Relative	Max identity	Accession number	Closest cultured relative	Max identity	Accession number
10	Uncultured bacterium clone SL-119	98-99%	JF497786.1	*Ralstonia solanacearum* strain LMG 2299	91-93%	NR_044040.1
3	*Arcobacter cibarius* strain LMG 21996	99%	NR_042218.1	*Arcobacter cibarius* strain : LMG 21996	99%	NR_042218.1
2	Uncultured *Acinetobacter sp.* clone 3.29m14	99%	JN679102.1	*Acinetobacter johnsonii* strain ATCC 17909	98-99%	NR_044975.1
2	Uncultured *Arcobacter sp*. clone ATB-KS-16910	98-99%	JQ845798.1	*Arcobacter cibarius* strain : LMG 21996	94-97%	NR_042218.1
2	Uncultured *Arcobacter sp.* clone DS081	98-99%	DQ234164.2	*Arcobacter nitrofigilis* strain CI	94-95%	NR_025906.1
2	Uncultured bacterium clone 13	99%	JN256094.1	*Arcobacter cryaerophilus* strain A 169/B	98%	NR_025905.1
2	Uncultured bacterium clone a4	98-99%	HM467984.1	*Proteocatella sphenisci* strain PPP2	97-98%	NR_041885.1
2	Uncultured epsilon proteobacterium BioIuz K34	92-96%	AF324539.1	*Arcobacter cibarius* strain : LMG 21996	91-94%	NR_042218.1
1	*Acinetobacter bouvetii* strain EU40	99%	JF681285.1	*Acinetobacter johnsonii* strain ATCC 17909	98%	NR_044975.1
1	*Acinetobacter johnsonii* strain CAI-1	99%	DQ257425.1	*Acinetobacter johnsonii* strain ATCC 17909	99%	NR_044975.1
1	*Acinetobacter sp.* FY3	98%	JX393018.1	*Acinetobacter gyllenbergii* strain : RUH 422 = NIPH 2150 = ACI 651	98%	NR_042026.1
1	*Arcobacter cloacae* type strain SW28-13T	99%	HE565360.1	*Arcobacter nitrofigilis* strain CI	95%	NR_025906.1
1	*Arcobacter cryaerophilus* culture collection LMG:9865	99%	FR682113.1	*Arcobacter cryaerophilus* strain A 169/B	99%	NR_025905.1
1	*Arcobacter cryaerophilus* LMG:9865	99%	FR682113.1	*Arcobacter cryaerophilus* strain A 169/B	99%	NR_025905.1
1	*Arcobacter cryaerophilus* strain NW94	91%	JF915357.1	*Arcobacter cryaerophilus* strain A 169/B	90%	NR_025905.1
1	*Arcobacter* cryaerophilus strain W2-1	99%	JX392996.1	*Arcobacter cibarius* strain : LMG 21996	98%	NR_042218.1
1	Bacterium enrichment culture clone R4-76B	99%	GU196238.1	*Paludibacter propionicigenes* WB4 strain	96%	NR_074577.1
1	*Bacteroides graminisolvens* JCM 15093	99%	AB547643.1	Bacteroides graminisolvens strain XDT-1	99%	NR_041642.1
1	*Bacteroides sp.* CannelCatfish9	89%	JQ317253.1	*Cloacibacterium normanense* strain :CCUG 46293	88%	NR_042187.1
1	*Comamonas sp.* 20.10 KSS strain 20.10	95%	HE575934.1	*Comamonas denitrificans* strain 123	95%	NR_025080.1
1	Endosymbiont of Sphenophorus levis clone L_D11full	94%	FJ626263.1	*Ralstonia solanacearum* GMI1000 strain GMI1000	94%	NR_074551.1
1	Uncultured *Acinetobacter sp.* clone 248	96%	JN082574.1	*Acinetobacter haemolyticus* strain DSM 6962	93%	NR_026207.1

1	Uncultured anaerobic bacterium clone B-4I 1	94%	AY953245.1	*Clostridium akagii* strain CK58	88%	NR_025352.1
1	Uncultured bacterium clone 6-12W42	92%	KC179073.1	*Sulfurospirillum multivorans* strain K	92%	NR_044868.1
1	Uncultured bacterium clone A15	99%	JN882037.1	*Cloacibacterium normanense* strain :CCUG 46293	99%	NR_042187.1
1	Uncultured bacterium clone BANW563	92%	DQ264531.1	*Acinetobacter junii* strain DSM 6964	90%	NR_026208.1
1	Uncultured bacterium clone BF4-40	99%	HM584357.1	Anaerovorax odorimutans strain NorPut	95%	NR_028911.1
1	Uncultured bacterium clone BST14-13	99%	HQ436927.1	*Arcobacter nitrofigilis* strain CI	92%	NR_025906.1
1	Uncultured bacterium clone BWB0303-02	98%	JN397825.1	*Paludibacter propionicigenes* WB4	92%	NR_074577.1
1	Uncultured bacterium clone C6	99%	EU234245.1	*Trichococcus flocculiformis* strain DSM 2094	99%	NR_042060.1
1	Uncultured bacterium clone CFT112C7	99%	DQ456015.1	*Bacteroides coprophilus* DSM 18228 strain CB42	99%	NR_041461.1
1	Uncultured bacterium clone DP3.1.30	99%	FJ612126.1	*Dysgonomonas mossii* DSM 22836 strain CCUG 43457	83%	NR_025484.1
1	Uncultured bacterium clone EBL1	99%	GU591498.1	*Alistipes shahii* WAL 8301 strain WAL 8301	89%	NR_043319.1
1	Uncultured bacterium clone EMIRGE_OTU_S0.5b2b_1498	94%	JX222306.1	*Cetobacterium somerae* strain WAL 14325	90%	NR_025533.1
1	Uncultured bacterium clone F74	96%	JN379055.1	*Bacteroides fragilis* NCTC 9343 strain ATCC 25285	95%	NR_074784.1
1	Uncultured bacterium clone HAW-RM37-2-B-1600d-D	89%	FN563281.1	*Bacteroides graminisolvens* strain XDT-1	89%	NR_041642.1
1	Uncultured bacterium clone inf28	99%	JN245797.1	*Thermanaerovibrio acidaminovorans* DSM 6589 strain	88%	NR_074520.1
1	Uncultured bacterium clone inf83	99%	JN245828.1	*Bacteroides graminisolvens* strain XDT-1	99%	NR_041642.1
1	Uncultured bacterium clone inf-98	98%	JN245875.1	*Bacteroides graminisolvens* strain XDT-1	97%	NR_041642.1
1	Uncultured bacterium clone LJ4	99%	AY756593.1	*Dechloromonas agitata strain* CKB	98%	NR_024884.1
1	Uncultured bacterium clone MBR0.583-102 16S	89%	AY913837.1	*Paludibacter propionicigenes* WB4 strain WB4	89%	NR_074577.1
1	Uncultured bacterium clone MS041	95%	FR691501.1	*Fusibacter paucivorans* strain SEBR 4211	91%	NR_024886.1
1	Uncultured bacterium clone Q7689-ASSA	99%	JN391604.1	*Paludibacter propionicigenes* WB4 strain WB4	96%	NR_074577.1
1	Uncultured bacterium clone R-7757	92%	FJ879671.1	*Parasutterella excrementihominis* YIT 11859	92%	NR_041667.1
1	Uncultured bacterium clone SINH737	88%	HM128224.1	*Bacteroides graminisolvens* strain XDT-1	90%	NR_041642.1
1	Uncultured bacterium clone X0010	90%	FJ820480.1	*Arcobacter cryaerophilus* strain A 169/B	89%	NR_025905.1
1	Uncultured bacterium clone:BSA2B-17	93%	AB175389.1	*Arcobacter cryaerophilus* strain A 169/B	92%	NR_025905.1
1	Uncultured *Bacteroidetes* bacterium clone 20m-28	90%	GU061287.1	*Flavobacterium aquatile* strain : DSM 1132	85%	NR_042495.1
1	Uncultured *Betaproteobacteria* bacterium clone QEDN11CE04	97%	CU926352.1	*Propionivibrio pelophilus* strain asp 66	95%	NR_024855.1
1	Uncultured *Epsilonproteobacteria* bacterium	90%	CU926906.1	*Arcobacter cryaerophilus* strain A 169/B	89%	NR_025905.1
1	Uncultured organism clone ELU0114-T283-S-NI_000183	99%	HQ788800.1	*Parabacteroides merdae* strain JCM 9497	99%	NR_041343.1

1	Uncultured organism clone ELU0116-T290-S-NI_000254	99%	HQ789473.1	*Bacteroides dorei* DSM 17855 strain JCM 13471	99%	NR_041351.1

Assignment of clones obtained from the **R0.125** to their closest relative						
No of clones	Closest Relative	Max identity	Accession number	Closest cultivated relative	Max identity	Accession number
2	*Arcobacter cryaerophilus* LMG:9865	99%	FR682113.1	*Arcobacter cryaerophilus* strain A 169/B	99%	NR_025905.1
2	Uncultured bacterium clone ACS7	99%	FJ375420.1	*Prolixibacter bellariivorans* strain F2	89%	NR_043273.1
2	Uncultured bacterium clone C53	99%	EU234264.1	*Ferruginibacter lapsinanis* strain HU1-HG42	84%	NR_044589.1
2	Uncultured bacterium clone eff102	99%	JN245771.1	*Flavobacterium johnsoniae* UW101 strain	94%	NR_074455.1
2	Uncultured bacterium clone OTU-4_W	99%	JN981872.1	*Thauera aminoaromatica* strain S2	99%	NR_027211.1
2	Uncultured bacterium clone Q7204-HYSO	99%	JN391943.1	*Zoogloea ramigera* strain 106	98-99%	NR_026130.1
2	Uncultured bacterium clone SL-119	99%	JF497786.1	*Ralstonia solanacearum* strain LMG 2299	92%	NR_044040.1
1	*Acidovorax sp*. JHL-3	99%	KC197035.1	*Acidovorax defluvii* strain BSB411	99%	NR_026506.1
1	*Acinetobacter tjernbergiae* strain DSM 14971	98%	HE651928.1	*Acinetobacter gyllenbergii* strain	97%	NR_042026.1
1	*Arcobacter cryaerophilus* strain W2-1	99%	JX392996.1	*Arcobacter cryaerophilus* strain A 169/B	98%	NR_025905.1
1	Bacterium DP-6 gene	98%	AB596978.1	*Alicycliphilus denitrificans BC* strain BC	98%	NR_074585.1
1	Bacterium rJ12 gene	98%	AB021330.1	*Alicycliphilus denitrificans* BC	97%	NR_074585.1
1	*Beta proteobacterium* Npb-03	98%	JN104394.1	*Leeia oryzae* strain HW7	92%	NR_043684.1
1	*Brevundimonas sp.* 248	93%	DQ825662.1	*Mycoplana bullata* strain IAM 13153	92%	NR_025831.1
1	*Hydrogenophaga pseudoflava* strain: NBRC 102511	99%	AB681845.1	*Hydrogenophaga pseudoflava* strain GA3	99%	NR_028717.1
1	*Macromonas bipunctata* strain IAM 14880	99%	NR_040903.1	*Macromonas bipunctata* strain IAM 14880	99%	NR_040903.1
1	Uncultured *Acidovorax sp.* clone BPS_CK2	99%	HQ857618.1	*Acidovorax temperans* strain PHL	99%	NR_028715.1
1	Uncultured bacterium BA2	97%	AF087043.1	*Fluviicola taffensis* DSM 16823	92%	NR_074547.1
1	Uncultured bacterium clone 7E7	99%	JF826427.1	*Alicycliphilus denitrificans* BC strain BC	97%	NR_074585.1
1	Uncultured bacterium clone A21	99%	HM007533.1	*Zoogloea oryzae* strain A-7	98%	NR_041286.1
1	Uncultured bacterium clone a33	99%	HM468001.1	*Clostridium aldrichii* strain P-1	91%	NR_026099.2
1	Uncultured bacterium clone a-99	99%	JX040392.1	*Marinobacter zhejiangensis* strain CN74	90%	NR_044457.1
1	Uncultured bacterium clone AFZEB_aaj71b12	98%	EU464810.1	*Pseudomonas gessardii* strain CIP 105469	90%	NR_024928.1

1	Uncultured bacterium clone B27	99%	EF655634.1	*Parabacteroides goldsteinii*	92%	NR_043317.1
1	Uncultured bacterium clone B52	92%	FJ660531.1	Trichococcus flocculiformis strain DSM 2094	92%	NR_042060.1
1	Uncultured bacterium clone BF23	99%	DQ327689.1	*Aquabacterium parvum* strain B6	99%	NR_024874.1
1	Uncultured bacterium clone EMIRGE_OTU_s1t2b_2503	97%	JX221908.1	*Pseudomonas umsongensis* strain Ps 3-10	95%	NR_025227.1
1	Uncultured bacterium clone EV818SWSAP20	98%	DQ337065.1	*Aquabacterium commune* strain B8	97%	NR_024875.1
1	Uncultured bacterium clone F-34	99%	HQ132441.1	*Aquabacterium fontiphilum* strain CS-6	95%	NR_044322.1
1	Uncultured bacterium clone FC1_16S_81	98%	EU662490.1	*Arcobacter cibarius* strain : LMG 21996	94%	NR_042218.1
1	Uncultured bacterium clone LJ4	99%	AY756593.1	*Dechloromonas agitata strain* CKB	97%	NR_024884.1
1	Uncultured bacterium clone M0509_43	99%	EU104128.1	*Comamonas odontotermitis* strain Dant 3-8	97%	NR_043859.1
1	Uncultured bacterium clone MS041	99%	FR691501.1	*Fusibacter paucivorans strain* SEBR 4211	94%	NR_024886.1
1	Uncultured bacterium clone RS06101_B24	98%	EU101225.1	*Beggiatoa alba* B18LD strain B18LD	90%	NR_041726.1
1	Uncultured bacterium clone RS-C16	99%	KC541150.1	*Aquabacterium commune* strain B8	97%	NR_024875.1
1	Uncultured bacterium clone SJTU_F_11_34	99%	EF399089.1	*Bacteroides graminisolvens* strain XDT-1	99%	NR_041642.1
1	Uncultured bacterium clone SS-3	98%	AY945894.1	*Bdellovibrio bacteriovorus* strain HD 100	97%	NR_027553.1
1	Uncultured bacterium clone SWB0301-08	99%	JN398132.1	*Dechloromonas hortensis* strain MA-1	99%	NR_042819.1
1	Uncultured bacterium clone W-16S-28	96%	HM445955.1	*Dechloromonas agitata* strain CKB	94%	NR_024884.1
1	Uncultured *Bacteroidetes* bacterium from clone QEDN2BC04	98%	CU926228.1	*Parabacteroides goldsteinii*	92%	NR_043317.1
1	Uncultured *Bacteroidetes* bacterium clone QEDN9BC03	99%	CU926652.1	*Prolixibacter bellariivorans* strain F2	89%	NR_043273.1
1	Uncultured *Cryomorphaceae* bacterium clone G103	99%	GQ891860.1	*Fluviicola taffensis* DSM 16823 strain DSM 16823	90%	NR_074547.1
1	Uncultured *Flavobacterium* sp. clone S117	96%	JN217052.1	*Flavobacterium aquatile* strain : DSM 1132	93%	NR_042495.1
1	Uncultured organism clone ELU0035-T194-S-NIPCRAMgANb_000529	99%	HQ754177.1	*Streptococcus vestibularis* ATCC 49124	99%	NR_042777.1
1	Uncultured organism clone ELU0107-T266-S-NI_000205	99%	HQ786454.1	*Bacteroides cellulosilyticus* DSM 14838 strain : CRE21	99%	NR_042203.1
1	Uncultured organism clone ELU0157-T387-S-NIPCRAMgANb_000062	97%	HQ806210.1	*Cloacibacterium normanense* strain :CCUG 46293	96%	NR_042187.1
1	Uncultured *Zoogloea sp.* clone B13	95%	GQ249371.1	*Zoogloea caeni* strain EMB 43	94%	NR_043795.1
1	Unidentified bacterium DNA	99%	Z93999.1	*Arcobacter cryaerophilus* strain A 169/B	98%	NR_025905.1

Assignment of clones obtained from the **R0.5** to their closest relative						
No. of clones	Closest relative	Max identity	Accession number	Closest cultured relative	Max identity	Accession number
4	Uncultured bacterium BA2	97-98%	AF087043.1	*Fluviicola taffensis* DSM 16823 strain DSM 16823	92-94%	NR_074547.1
3	Uncultured bacterium clone Hyd82	99%	KC189708.1	*Cellvibrio fibrivorans* strain R-4079	99%	NR_025420.1
2	*Arcobacter cryaerophilus* culture collection LMG:9865	99%	FR682113.1	*Arcobacter cryaerophilus* strain A 169/B	99%	NR_025905.1
2	Uncultured bacterium clone 3C002567	96%	EU801307.1	*Micavibrio aeruginosavorus* ARL-13 strain ARL-13	90%	NR_074210.1
2	Uncultured bacterium clone M0509_43	98%	EU104128.1	*Comamonas odontotermitis* strain Dant 3-8	95%	NR_043859.1
2	Uncultured bacterium clone RS-C16	98%	KC541150.1	*Aquabacterium commune* strain B8	97%	NR_024875.1
2	Uncultured *Flavobacterium sp.* clone SB97	99%	JQ723659.1	*Flavobacterium terrae* strain R0.5A1-13	94%	NR_044096.1
2	Uncultured *Flavobacterium sp.* clone XJ106	98%	EF648154.1	*Flavobacterium ceti* strain : 454-2	93%	NR_042540.1
1	*Acinetobacter sp.* FY3	94%	JX393018.1	*Acinetobacter gyllenbergii* strain	94%	NR_042026.1
1	Bacterium enrichment culture clone phytdeg33	99%	JF834291.1	*Pseudacidovorax intermedius* strain CC-21	97%	NR_044241.1
1	*Cellvibrio fibrivorans* strain R-4079	99%	NR_025420.1	*Cellvibrio fibrivorans* strain R-4079	99%	NR_025420.1
1	*Comamonadaceae bacterium* MPsc	98%	AY651926.1	*Ottowia thiooxydans* strain K11	96%	NR_029001.1
1	*Flavobacteriaceae bacterium* HM0024	96%	FJ713810.1	*Chryseobacterium gregarium* strain : DSM 19109	92%	NR_042647.1
1	*Hydrogenophaga sp.* DD8b	98%	HQ113382.1	*Diaphorobacter oryzae* strain RF3	97%	NR_044472.1
1	*Thauera phenylacetica* strain B4P	99%	NR_027224.1	*Thauera phenylacetica* strain B4P	99%	NR_027224.1
1	Uncultured *Acinetobacter sp.* clone GI5-006-A01	97%	FJ192912.1	*Acinetobacter haemolyticus* strain DSM 6962	96%	NR_026207.1
1	Uncultured anaerobic bacterium clone A-2S	93%	AY953228.1	*Paludibacter propionicigenes* WB4 strain WB4	88%	NR_074577.1
1	Uncultured bacterium 16S rRNA gene, clone MRA1008	99%	FN428754.1	*Zoogloea ramigera* strain 106	97%	NR_026130.1
1	Uncultured bacterium clone 212dS0.50	99%	AY212659.1	*Aquabacterium fontiphilum* strain CS-6	97%	NR_044322.1
1	Uncultured bacterium clone 7E7	99%	JF826427.1	*Alicycliphilus denitrificans* BC strain BC	97%	NR_074585.1
1	Uncultured bacterium clone ACS7	99%	FJ375420.1	*Prolixibacter bellariivorans* strain F2	89%	NR_043273.1
1	Uncultured bacterium clone AFZEB_aaj72a12	96%	EU464857.1	*Pseudomonas vancouverensis* strain : DhA-51	90%	NR_041953.1
1	Uncultured bacterium clone AFZEB_aaj72c03	94%	EU464870.1	*Acinetobacter parvus* strain LUH4616	91%	NR_025425.1
1	Uncultured bacterium clone B24	98%	FJ660513.1	*Methyloversatilis universalis* FAM5 strain FAM5	98%	NR_043813.1
1	Uncultured bacterium clone B2-53	98%	JF922464.1	*Sphingobium amiense* strain Y	96%	NR_028622.1
1	Uncultured bacterium clone B3NR69D26	95%	AY957941.1	*Hydrogenophaga atypica* strain BSB 41.8	95%	NR_029023.1

1	Uncultured bacterium clone B-9	99%	HQ860538.1	*Dechloromonas agitata* strain CKB	97%	NR_024884.1
1	Uncultured bacterium clone BF23	99%	DQ327689.1	*Aquabacterium parvum* strain B6	99%	NR_024874.1
1	Uncultured bacterium clone BF-39	97%	HQ609643.1	*Flavobacterium psychrophilum* strain IFO 15942	94%	NR_040914.1
1	Uncultured bacterium clone BXHA59	99%	GQ480012.1	*Dechloromonas hortensis* strain MA-1	98%	NR_042819.1
1	Uncultured bacterium clone C2T	98%	DQ856536.1	*Propionivibrio dicarboxylicus* strain CreMal1	96%	NR_026477.1
1	Uncultured bacterium clone C43	99%	EU234269.1	*Rhodobacter changlensis* strain : JA139	97%	NR_042564.1
1	Uncultured bacterium clone C53	99%	EU234264.1	*Ferruginibacter lapsinanis* strain HU1-HG42	84%	NR_044589.1
1	Uncultured bacterium clone D169	99%	JX271927.1	*Terrimonas ferruginea* strain : DSM 30193	93%	NR_042494.1
1	Uncultured bacterium clone D-82	89%	HQ860677.1	*Dechloromonas agitata* strain CKB	88%	NR_024884.1
1	Uncultured bacterium clone EMIRGE	96%	JX222644.1	*Aquabacterium commune* strain B8	95%	NR_024875.1
1	Uncultured bacterium clone EV818CFSSAHH41	98%	DQ336991.1	*Pseudomonas alcaliphila* strain AL15-21	97%	NR_024734.1
1	Uncultured bacterium clone HglFeb4H8m	96%	JX016872.1	*Alkanindiges illinoisensis* strain MVAB Hex1	94%	NR_025254.1
1	Uncultured bacterium clone LJ4	99%	AY756593.1	*Dechloromonas agitata* strain CKB	97%	NR_024884.1
1	Uncultured bacterium clone MA-60-I98C	99%	HM141872.1	*Prolixibacter bellariivorans* strain F2	90%	NR_043273.1
1	Uncultured bacterium clone N1512_04	99%	EU104177.1	*Chitinophaga niastensis* strain JS0.1256-4	84%	NR_044560.1
1	Uncultured bacterium clone N86	96%	JQ655791.1	*Fluviicola taffensis* DSM 16823 strain DSM 16823	95%	NR_074547.1
1	Uncultured bacterium clone Q7689-ASSA	92%	JN391604.1	*Paludibacter propionicigenes* WB4 strain WB4	90%	NR_074577.1
1	Uncultured bacterium clone SBRFL34	98%	HQ158705.1	*Prolixibacter bellariivorans* strain F2	88%	NR_043273.1
1	Uncultured bacterium clone VHW_F_L10	99%	JQ085636.1	*Parabacteroides goldsteinii* 16S	93%	NR_043317.1
1	Uncultured *Bacteroidetes* bacterium clone	99%	JN981892.1	*Rheinheimera texasensis* strain A62-14B	99%	NR_043133.1
1	Uncultured *Bacteroidetes* bacterium MADSa49	92%	AB669250.1	*Parabacteroides goldsteinii*	88%	NR_043317.1
1	Uncultured *beta proteobacterium* clone Orbal D45	99%	AF450471.1	*Propionivibrio pelophilus* strain asp 66	99%	NR_024855.1
1	Uncultured *Betaproteobacteria bacterium* clone QEDN11CE04	99%	CU926352.1	*Propionivibrio pelophilus* strain asp 66	96%	NR_024855.1
1	Uncultured *Flavobacterium sp.* clone S0.12517	99%	JN217052.1	*Flavobacterium terrigena* strain DS-20	94%	NR_044006.1
1	Uncultured *Lactobacillales bacterium* clone 736	95%	JN173148.1	*Streptococcus sp.* strain SHV515	94%	NR_044912.1
1	Uncultured *Methylophilaceae bacterium* clone P36	99%	HE648207.1	*Methylotenera versatilis* 301 strain 301	98%	NR_074693.1
1	Uncultured *proteobacterium* clone R7C31	94%	DQ450182.1	*Fluviicola taffensis* DSM 16823 strain DSM 16823	92%	NR_074547.1

Assignment of clones obtained from the **R1** to their closest relative						
No of clones	Closest relative	Max identity	Accession number	Closest cultured relative	Max identity	Accession number
6	Uncultured bacterium clone C53	95-99 %	EU234264.1	*Ferruginibacter lapsinanis* strain HU1-HG42	83-85%	NR_044589.1
6	Uncultured *Flavobacterium sp.* clone SB97	90-99%	JQ723659.1	*Flavobacterium suncheonense* strain GH29-5	89-95	NR_043655.1
2	Uncultured bacterium clone B-22	99%	HQ860531.1	*Acidovorax temperans* strain PHL	99%	NR_028715.1
3	Uncultured *Flavobacterium sp.* clone S0.12517	93-98%	JN217052.1	*Flavobacterium saliperosum* strain AS 1.3801	90-94%	NR_043481.1
1	*Acinetobacter sp.* CGMCC 6052	99%	KC422446.1	*Acinetobacter gyllenbergii* strain : RUH 422 = NIPH 2150 = ACI 651	98%	NR_042026.1
1	*Acinetobacter sp.* CIP 102637	93%	JQ638581.1	*Acinetobacter parvus* strain LUH4616	93%	NR_025425.1
1	*Alicycliphilus sp.* R-24611	98%	AM084014.1	*Alicycliphilus denitrificans* BC strain	98%	NR_074585.1
1	*Arcobacter cryaerophilus* isolate CCUG 17802	97%	AY314755.1	*Arcobacter cryaerophilus* strain A 169/B	96%	NR_025905.1
1	Bacterium J10	99%	FJ418599.1	*Methylobacillus flagellatus* strain K	99%	NR_043691.1
1	*Candidatus Magnospira bakii* clone BM16	92%	AF087091.1	*Fluviicola taffensis* DSM 16823 strain	87%	NR_074547.1
1	*Cellvibrio fibrivorans* strain R-4079	98%	NR_025420.1	*Cellvibrio fibrivorans* strain R-4079	98%	NR_025420.1
1	*Chitinophagaceae bacterium* NYFB	85%	JQ033715.1	*Ferruginibacter lapsinanis* strain HU1-HG42	84%	NR_044589.1
1	*Clostridium sp.* 6-44 gene	95%	AB596885.1	*Alistipes shahii* WAL 8301 strain	87%	NR_043319.1
1	Enrichment culture bacterium LB-P clone LB-P	96%	AF538773.1	*Thermomonas brevis* strain R-13291	96%	NR_025578.1
1	*Flavobacterium columnare* strain cl41	97%	EU294416.1	*Flavobacterium filum* strain EMB34	92%	NR_043767.1
1	*Fluviicola taffensis* DSM 16823 strain DSM 16823	96%	NR_074547.1	*Fluviicola taffensis* DSM 16823 strain DSM 16823	96%	NR_074547.1
1	*Paludibacter propionicigenes* WB4 strain	91%	NR_074577.1	*Paludibacter propionicigenes* WB4 strain	91%	NR_074577.1
1	*Pedobacter kwangyangensis* strain CW39	86%	EF693742.1	*Nubsella zeaxanthinifaciens* strain TDMA-5	86%	NR_041478.1
1	*Pseudomonas sp*. TN12	92%	HQ860327.1	G*essardii* strain CIP 105469	90%	NR_024928.1
1	*Rhodobacter sp.* oral taxon C30 strain WC014	97%	HM099648.1	*Rhodobacter sphaeroides* strain 2.4.1	96%	NR_029215.1
1	*Shigella sp.* 4092	98%	FJ405321.1	*Shigella dysenteriae* Sd197 strain Sd197	98%	NR_074892.1
1	*Terrimonas sp.* JJ008	88%	JN679215.1	*Ferruginibacter lapsinanis* strain HU1-HG42	87%	NR_044589.1
1	*Thauera phenylacetica* strain B4P	96%	NR_027224.1	*Thauera phenylacetica* strain B4P	96%	NR_027224.1
1	*Thauera sp.* TS4	98%	EU073070.1	*Thauera sp*. MZ1T strain MZ1	98%	NR_074711.1
1	Uncultured bacterium clone 86	95%	GU225967.1	*Hydrogenophaga bisanensis* strain K102	92%	NR_044268.1
1	Uncultured bacterium clone AFZEB_aaj72a11	93%	EU464856.1	*Pseudomonas nitroreducens* strain IAM 1439	90%	NR_042435.1

1	Uncultured bacterium clone ASSO-61	98%	JN391659.1	Thauera aminoaromatica strain S0.5	98%	NR_027211.1
1	Uncultured bacterium clone BXHA7	99%	GQ479966.1	*Pseudoxanthomonas kaohsiungensis* strain J36	98%	NR_043070.1
1	Uncultured bacterium clone BXHB2	95%	GQ480053.1	*Chitinophaga sancti* strain IFO 15057	88%	NR_040917.1
1	Uncultured bacterium clone eff109	99%	JN245776.1	*Haliscomenobacter hydrossis* DSM 1100 = ATCC 27775	89%	NR_042316.1
1	Uncultured bacterium clone EV818SWSAP20	99%	DQ337065.1	*Aquabacterium commune* strain B8	98%	NR_024875.1
1	Uncultured bacterium clone FGL12_B54	96%	FJ437844.1	*Owenweeksia hongkongensis* strain UST20020801	88%	NR_040990.1
1	Uncultured bacterium clone FrsFiS0.59	91%	JF748025.1	*Helicobacter anseris* strain MIT 04-9362	87%	NR_043798.1
1	Uncultured bacterium clone Hyd4 1	98%	KC189671.1	*Pseudomonas aeruginosa* PAO1	96%	NR_074828.1
1	Uncultured bacterium clone Hyd82	99%	KC189708.1	*Cellvibrio fibrivorans* strain R-4079	98%	NR_025420.1
1	Uncultured bacterium clone inf98	99%	JN245836.1	*Parabacteroides goldsteinii*	92%	NR_043317.1
1	Uncultured bacterium clone JdFBDF2-26	98%	JQ678573.1	*Pseudomonas alcaligenes* strain IAM12411	97%	NR_043419.1
1	Uncultured bacterium clone LJ4	99%	AY756593.1	*Dechloromonas agitata* strain CKB	98%	NR_024884.1
1	Uncultured bacterium clone M0111_60	99%	EU104057.1	*Erysipelothrix inopinata* strain 143-02	91%	NR_025594.1
1	Uncultured bacterium clone M0509_05	99%	EU104095.1	*Haliscomenobacter hydrossis* DSM 1100	87%	NR_042316.1
1	Uncultured bacterium clone MS041	99%	FR691501.1	*Fusibacter paucivorans* strain SEBR 4211	94%	NR_024886.1
1	Uncultured bacterium clone N-1	99%	JX040322.1	*Desulfomicrobium baculatum* DSM 4028 strain	99%	NR_074900.1
1	Uncultured bacterium clone N-15	99%	JX040327.1	*Fluviicola taffensis* DSM 16823 strain DSM 16823	94%	NR_074547.1
1	Uncultured bacterium clone P092904_P1G01	99%	HQ385529.1	*Comamonas odontotermitis* strain Dant 3-8	95%	NR_043859.1
1	Uncultured bacterium clone Q7689-ASSA	99%	JN391604.1	*Paludibacter propionicigenes* WB4 strain	96%	NR_074577.1
1	Uncultured bacterium clone SHCB0574	94%	JN697815.1	*Desulfonatronum thiodismutans* strain MLF1	84%	NR_025163.1
1	Uncultured bacterium clone SL-169	96%	JF497840.1	*Methylocaldum szegediense* strain OR0.5	88%	NR_026064.1
1	Uncultured bacterium clone WW1_a49	92%	GQ264182.1	*Chitinophaga pinensis* DSM 2588	87%	NR_074566.1
1	Uncultured bacterium isolate 1112865261585	86%	HQ120546.1	*Rhizobium daejeonense* strain L61; KCTC 12121 1	86%	NR_042851.1
1	Uncultured bacterium, clone D2871	97%	AJ617874.1	*Dechloromonas agitata* strain CKB	95%	NR_024884.1
1	Uncultured *Rhodocyclaceae bacterium* gene isolate: PCP-BT-5	97%	AB723844.1	*Propionivibrio pelophilus* strain asp 66	96%	NR_024855.1
1	Unidentified *eubacterium* from anoxic bulk soil clone BSV73	90%	AJ229217.1	*Prolixibacter bellariivorans* strain F2	88%	NR_043273.1

Assignment of clones obtained from the **S0.125** to their closest relative						
No. of clones	Closest relative	Max identity	Accession number	Closest cultured relative	Max identity	Accession number
16	Uncultured bacterium clone SL-119	90-99%	JF497786.1	*Ralstonia solanacearum* GMI1000 strain GMI1000	93-95%	NR_074551.1
9	Uncultured bacterium clone SINH1066	98-99%	HM127904.1	*Arcobacter nitrofigilis* strain CI	94-95%	NR_025906.1
3	Uncultured bacterium clone BF2-6	96-97%	HM584330.1	*Geobacter thiogenes* strain K1	96%	NR_028775.1
2	*Simplicispira metamorpha*	98-99%	AB680538.1	*Simplicispira metamorpha* strain DSM 1837	98-99%	NR_044941.1
1	*Arcobacter cryaerophilus* strain W2-1	98%	JX392996.1	*Arcobacter cryaerophilus* strain A 169/B	98%	NR_025905.1
1	*Beta proteobacterium* INA17	96%	AB599792.1	*Propionivibrio limicola* strain GolChi1	93%	NR_025455.1
1	*Beta proteobacterium* SAK18	99%	AB607321.1	*Propionivibrio limicola* strain GolChi1	94%	NR_025455.1
1	*Candidatus Accumulibacter sp.* JJ007	99%	JN679214.1	*Propionivibrio limicola* strain GolChi1	94%	NR_025455.1
1	*Flavobacterium sp.* 3AR1-35	97%	GU295965.1	*Flavobacterium hercynium* strain : WB 4.2-33	96%	NR_042520.1
1	*Janthinobacterium sp.* HC7-17	97%	JF313035.1	*Janthinobacterium lividum* strain DSM 1522	97%	NR_026365.1
1	*Paludibacter propionicigenes* WB4 strain WB4	97%	NR_074577.1	*Paludibacter propionicigenes* WB4 strain WB4	97%	NR_074577.1
1	*Proteocatella sphenisci* strain PPP2	92%	NR_041885.1	*Proteocatella sphenisci* strain PPP2	92%	NR_041885.1
1	*Pseudomonas sp.* TH6(2011)	99%	JN695702.1	*Pseudomonas grimontii* strain CFML 97-514	99%	NR_025102.1
1	*Pseudomonas veronii*	99%	AB494444.1	*Pseudomonas veronii* strain CIP 104663	99%	NR_028706.1
1	Uncultured bacterium clone 13	99%	JN256094.1	*Arcobacter cryaerophilus* strain A 169/B	98%	NR_025905.1
1	Uncultured bacterium clone 3C003280	98%	EU801901.1	*Methylotenera versatilis* 301 strain 301	97%	NR_074693.1
1	Uncultured bacterium clone 6-12W42	91%	KC179073.1	*Aquaspirillum arcticum* strain IAM 14963	90%	NR_040898.1
1	Uncultured bacterium clone aaa96f04	87%	DQ817154.1	*Aquitalea denitrificans* strain 5YN1-3	85%	NR_044535.1
1	Uncultured bacterium clone B-22	99%	HQ860531.1	*Acidovorax temperans* strain PHL	99%	NR_028715.1

1	Uncultured bacterium clone DP3.1.10	97%	FJ612117.1	*Alistipes finegoldii* DSM 17242 strain CIP 107999	87%	NR_043064.1
1	Uncultured bacterium clone E10	99%	EU864468.1	*Dysgonomonas mossii* DSM 22836 strain CCUG 43457	83%	NR_025484.1
1	Uncultured bacterium clone F4	93%	FJ230935.1	*Mitsuaria chitosanitabida* strain 3001	92%	NR_040786.1
1	Uncultured bacterium clone FL261	92%	HM481387.1	*Arcobacter butzleri* RM4018 strain RM4018	95%	NR_074573.1
1	Uncultured bacterium clone M17-10-B18	99%	JQ088394.1	*Cloacibacterium* normanense strain :CCUG 46293	99%	NR_042187.1
1	Uncultured bacterium clone RS-C13	99%	KC541147.1	*Acidovorax defluvii* strain BSB411	99%	NR_026506.1
1	Uncultured bacterium clone SHCB0574	89%	JN697815.1	*Geobacter grbiciae* strain TACP-5	84%	NR_041826.1
1	Uncultured bacterium clone SINH1066	99%	HM127904.1	*Arcobacter nitrofigilis* strain Cl	94%	NR_025906.1
1	Uncultured bacterium partial clone AV5-38	97%	AM181910.1	*Geobacter thiogenes* strain K1	96%	NR_028775.1
1	Uncultured *Bacteroidetes bacterium* clone Cf2-37	99%	GQ502503.1	*Cloacibacterium normanense* strain :CCUG 46293	98%	NR_042187.1
1	Uncultured *Bacteroidetes bacterium* clone UMAB-cl-136	94%	FR749761.1	*Flavobacterium cucumis* strain R0.5A45-3	92%	NR_044107.1
1	Uncultured *Betaproteobacteria bacterium* clone QEDN3DA03	90%	CU925839.1	*Clostridium bartlettii* DSM 16795	90%	NR_027573.1
1	Uncultured *epsilon proteobacterium* 1014 clone 1014	98%	AB030587.1	*Sulfurospirillum multivorans* strain K	98%	NR_044868.1
1	Uncultured *epsilon proteobacterium* BioIuz K34	90%	AF324539.1	*Arcobacter cryaerophilus* strain A 169/B	88%	NR_025905.1
1	Uncultured *epsilon proteobacterium* clone ATB-LH-5962	99%	FJ535175.1	*Arcobacter butzleri* RM4018 strain RM4018	94%	NR_074573.1
1	Uncultured *Fusobacteria bacterium* clone u114	99%	GQ850557.1	*Clostridium rectum* strain PCL	92%	NR_029271.1
1	Uncultured *gamma proteobacterium* clone EXY4	99%	EU567150.1	*Acinetobacter junii* strain DSM 6964	97%	NR_026208.1
1	Uncultured *Hydrogenophaga sp.* clone DS062	99%	DQ234146.2	*Macromonas bipunctata* strain IAM 14880	98%	NR_040903.1
1	Uncultured *Pseudomonas sp.* clone JT58-31	95%	AB189350.1	*Pseudomonas veronii* strain CIP 104663	94%	NR_028706.1
1	Uncultured *Pusillimonas sp.* clone De3217	99%	HQ183860.1	*Pusillimonas noertemannii* strain BN9	96%	NR_043129.1

Assignment of clones obtained from the **S0.5** to their closest relative						
No. of clones	Closest relative	Max identity	Accession number	Closest cultivated relative	Max identity	Accession number
6	Uncultured bacterium clone SINH1066	99%	HM127904.1	*Arcobacter nitrofigilis* strain CI	94-97%	NR_025906.1
6	Uncultured bacterium clone SINH737	99-98%	HM128224.1	*Arcobacter nitrofigilis* strain CI	94-95%	NR_025906.1
6	Uncultured *gamma proteobacterium* clone ATB-LH-5976	97-99%	FJ535199.1	*Acinetobacter haemolyticus* strain DSM 6962	95-98%	NR_026207.1
5	*Giesbergeria anulus*	99%	AB680703.1	*Giesbergeria anulus* strain IAM 14948	99%	NR_040900.1
2	*Arcobacter sp.* HME6665	99%	HM590830.1	*Arcobacter cibarius* strain LMG 21996	95-96%	NR_042218.1
2	Uncultured *Acinetobacter sp.* clone JI44F070	98-99%	GU356096.1	*Acinetobacter junii* strain DSM 6964	92-97%	NR_026208.1
2	Uncultured *gamma proteobacterium* clone EXY4	99%	EU567150.1	*Acinetobacter haemolyticus* strain DSM 6962	97%	NR_026207.1
1	*Acinetobacter sp.* zol-02	99%	JQ782897.1	*Acinetobacter haemolyticus* strain DSM 6962	97%	NR_026207.1
1	*Aeromonas sobria* strain JCM 2139	99%	AB472942.1	*Aeromonas sobria* strain 208	99%	NR_037012.2
1	Arcobacter cloacae strain SW28-13T	98%	HE565360.1	*Arcobacter butzleri* RM4018 strain RM4018	94%	NR_074573.1
1	*Arcobacter suis* CECT 7833 strain F41	98%	FJ573216.1	*Arcobacter nitrofigilis* strain CI	94%	NR_025906.1
1	*Giesbergeria kuznetsovii* strain D-412	98%	NR_043187.1	*Giesbergeria kuznetsovii* strain D-412	98%	NR_043187.1
1	Iron-reducing bacterium enrichment culture clone HN19	99%	FJ269054.1	*Bacteroides nordii* strain WAL 11050	92%	NR_043017.1
1	Paludibacter propionicigenes WB4 strain WB4	96%	NR_074577.1	Paludibacter propionicigenes WB4 strain WB4	96%	NR_074577.1
1	Stenotrophomonas daejeonensis strain MJ03	98%	GQ241320.1	Stenotrophomonas acidaminiphila strain AMX 19	98%	NR_025104.1
1	Uncultured Acidovorax sp. clone BPS_CK2	99%	HQ857618.1	Acidovorax temperans strain PHL	99%	NR_028715.1
1	Uncultured Acinetobacter sp. clone 248	95%	JN082574.1	Acinetobacter haemolyticus strain DSM 6962	93%	NR_026207.1
1	Uncultured Acinetobacter sp. clone BER_e12	98%	JQ815599.1	Acinetobacter gyllenbergii strain RUH 422	97%	NR_042026.1
1	Uncultured bacterium BA2	97%	AF087043.1	Fluviicola taffensis DSM 16823 strain DSM 16823	93%	NR_074547.1
1	Uncultured bacterium clone ASSO-69	97%	JN391664.1	Zoogloea oryzae strain A-7	96%	NR_041286.1
1	Uncultured bacterium clone DP10.1.18	96%	FJ612320.1	Arcobacter butzleri RM4018 strain RM4018	96%	NR_074573.1
1	Uncultured bacterium clone DP10.1.2	95%	FJ612311.1	Arcobacter butzleri RM4018 strain RM4018	93%	NR_074573.1
1	Uncultured bacterium clone NBBSP0109_41	99%	JQ072702.1	Cloacibacterium normanense strain CCUG 46293	99%	NR_042187.1
1	Uncultured bacterium clone RS-C13	99%	KC541147.1	Acidovorax defluvii strain BSB411	99%	NR_026506.1
1	Uncultured bacterium clone RS-C16	98%	KC541150.1	*Aquabacterium commune* strain B8	97%	NR_024875.1

1	Uncultured bacterium clone SJTU_F_11_78	99%	EF399556.1	*Bacteroides graminisolvens* strain XDT-1	99%	NR_041642.1
1	Uncultured bacterium clone US0.535M	97%	HM640996.1	*Giesbergeria anulus* strain IAM 14948	97%	NR_040900.1
1	Uncultured bacterium clone D242_27F_BAC2_013	97%	AB447719.1	*Paludibacter propionicigenes* WB4 strain WB4	96%	NR_074577.1
1	Uncultured bacterium clone SS_03	93%	AB473881.1	*Giesbergeria anulus* strain IAM 14948	93%	NR_040900.1
1	Uncultured bacterium isolate 1112842460703	97%	HQ119903.1	*Fluviicola taffensis* DSM 16823 strain DSM 16823	94%	NR_074547.1
1	Uncultured bacterium clone 784	99%	FR853657.1	*Shewanella putrefaciens* strain LMG 26268	98%	NR_044863.1
1	Uncultured *Bacteroidales bacterium* TCP-1	99%	AB721396.1	*Parabacteroides goldsteinii*	93%	NR_043317.1
1	Uncultured *Bacteroidetes bacterium* clone QEDN7AE12	99%	CU927836.1	*Paludibacter propionicigenes* WB4 strain WB4	90%	NR_074577.1
1	Uncultured *beta proteobacterium* clone IRD18H06	95%	AY947980.1	*Comamonas terrigena* strain IMI 359870	94%	NR_028719.1
1	Uncultured *Betaproteobacteria* bacterium clone QEDN11AF08	95%	CU926539.1	*Propionivibrio limicola* strain GolChi1	92%	NR_025455.1
1	Uncultured *epsilon proteobacterium* 1006	99%	AB030592.1	*Arcobacter butzleri* RM4018 strain RM4018	95%	NR_074573.1
1	Uncultured epsilon proteobacterium 1053	99%	AB030593.1	*Arcobacter nitrofigilis* strain CI	95%	NR_025906.1
1	Uncultured *Pseudomonas sp.* clone 4.6m32	99%	JN679142.1	Pseudomonas umsongensis strain Ps 3-10	98%	NR_025227.1

Assignment of clones obtained from the **S1** to their closest relative						
No. of clones	Closest Relative	Max identity	Accession number	Closest cultivated relative	Max identity	Accession number
7	Uncultured bacterium clone SINH1066	99%	HM127904.1	*Arcobacter nitrofigilis* strain CI	94-97%	NR_025906.1
4	Uncultured bacterium clone SINH737	99%	HM128224.1	*Arcobacter butzleri* strain ATCC 49616	94-95%	NR_043035.1
2	*Giesbergeria anulus* NBRC 14917	97-99%	AB680703.1	*Giesbergeria kuznetsovii* strain D-412	97-99%	NR_043187.1
2	Uncultured bacterium clone eff109	99%	JN245776.1	*Haliscomenobacter hydrossis* DSM 1100 strain DSM 1100 = ATCC 27775	89%	NR_042316.1
2	Uncultured *epsilon proteobacterium* 1006 gene	98-99%	AB030592.1	*Arcobacter cibarius* strain LMG 21996	92-94%	NR_042218.1
1	*Acetobacterium wieringae* strain DP9	99%	HQ384240.1	*Acetobacterium wieringae* strain DSM 1911	99%	NR_026324.1
1	*Acidovorax sp.* isolate G8B1	99%	AJ012071.1	*Acidovorax defluvii* strain BSB411	99%	NR_026506.1
1	*Acinetobacter sp.* CIP 102529	92%	JQ638580.1	*Acinetobacter parvus* strain LUH4616	91%	NR_025425.1
1	*Acinetobacter tjernbergiae* strain DSM 14971	98%	HE651928.1	*Acinetobacter haemolyticus* strain DSM 696	98%	NR_026207.1

1	*Aeromonas sp.* RA11	99%	FJ898302.1	*Aeromonas bestiarum* strain CIP 7430	99%	NR_026089.2
1	*Beta proteobacterium* pACH94	96%	AY297809.1	*Rhodocyclus tenuis* strain 2761	95%	NR_025839.1
1	*Giesbergeria giesbergeri* gene	99%	AB680537.1	*Giesbergeria giesbergeri* strain IAM 14949	99%	NR_040897.1
1	*Giesbergeria kuznetsovii* strain D-412	98%	NR_043187.1	*Giesbergeria kuznetsovii* strain D-412	98%	NR_043187.1
1	*Proteocatella sphenisci* strain PPP2	94%	NR_041885.1	*Proteocatella sphenisci* strain PPP2	94%	NR_041885.1
1	*Runella limosa* strain EMB111	99%	NR_043771.1	*Runella limosa* strain EMB111	99%	NR_043771.1
1	Uncultured *Arcobacter sp.* clone ATB-KS-13838	80%	JQ845776.1	*Arcobacter cibarius* strain LMG 21996	81%	NR_042218.1
1	Uncultured bacterium clone 053H11_B_DI_P58	92%	CR933245.1	*Giesbergeria giesbergeri* strain IAM 14949	92%	NR_040897.1
1	Uncultured bacterium clone 49	99%	JN648193.1	*Chitinophaga sancti* strain IFO 15057	89%	NR_040917.1
1	Uncultured bacterium clone a10	99%	HM467987.1	*Proteocatella sphenisci* strain PPP2	99%	NR_041885.1
1	Uncultured bacterium clone ACS7	99%	FJ375420.1	*Prolixibacter bellariivorans* strain F2	90%	NR_043273.1
1	Uncultured bacterium clone C22	99%	JX262578.1	*Shewanella putrefaciens* strain LMG 26268	99%	NR_044863.1
1	Uncultured bacterium clone C2T	97%	DQ856536.1	*Propionivibrio limicola* strain GolChi1	96%	NR_025455.1
1	Uncultured bacterium clone DSBR-B082	98%	AY302128.1	*Ferruginibacter lapsinanis* strain HU1-HG42	93%	NR_044589.1
1	Uncultured bacterium clone EMIRGE_OTU_S1t2d_183	87%	JX222631.1	*Simplicispira metamorpha* strain DSM 1837	86%	NR_044941.1
1	Uncultured bacterium clone EV821FW101601SAC11	99%	DQ226081.1	*Acinetobacter johnsonii* strain ATCC 17909	99%	NR_044975.1
1	Uncultured bacterium clone F-51	98%	HQ132435.1	*Leucobacter chromiireducens subsp. chromiireducens* strain	97%	NR_042287.1
1	Uncultured bacterium clone MBR0.583-102	89%	AY913837.1	*Paludibacter propionicigenes* WB4 strain	88%	NR_074577.1
1	Uncultured bacterium clone MC1_16S_72	89%	EU662641.1	*Ehrlichia muris* strain AS0.12545	100%	NR_025962. 1
1	Uncultured bacterium clone ncd221e04c	96%	HM266866.1	*Proteocatella sphenisci* strain PPP2	95%	NR_041885.1
1	Uncultured bacterium clone ncd673e07c1	99%	HM290092.1	*Acinetobacter parvus* strain LUH4616	99%	NR_025425.1
1	Uncultured bacterium gene clone: 0131	91%	AB286360.1	*Opitutus terrae* PB90-1 strain PB90-1	90%	NR_074978.1
1	Uncultured beta *proteobacterium* clone DFAW-050	99%	AY823962.1	*Dechloromonas agitata* strain CKB	97%	NR_024884.1
1	Uncultured beta *proteobacterium* clone F-49	99%	HQ132426.1	*Aquabacterium commune* strain B8	98%	NR_024875.1
1	Uncultured *Comamonadaceae* bacterium clone DS0.12571	91%	DQ234253.2	*Brachymonas denitrificans* strain AS-P1	94%	NR_025834.1
1	Uncultured *Delftia sp.* clone GI5-13-D06	94%	FJ192433.1	*Giesbergeria kuznetsovii* strain D-412	95%	NR_043187.1
1	Uncultured *Flavobacterium sp.* clone SB97	94%	JQ723659.1	*Flavobacterium terrae* strain R0.5A1-13	91%	NR_044096.1
1	Uncultured *Flavobacterium sp.* clone SB97	99%	JQ723659.1	*Flavobacterium terrae* strain R0.5A1-13	94%	NR_044096.1

1	Uncultured organism clone ELU0157-T387-S-NIPCRAMgANb_000062	99%	HQ806210.1	*Cloacibacterium normanense* strain CCUG 46293	99%	NR_042187.1
1	Uncultured *proteobacterium* clone AEP-eGFP-peri_2	83%	FJ517734.1	*Candidatus Midichloria mitochondrii IricVA* strain	77%	NR_074492.1
1	Uncultured *Shewanella sp.* clone T313G8	79%	HM438583.1	*Shewanella oneidensis* strain MR-1	78%	NR_036917.1

Summary

Wastewater is commonly treated using the activated sludge process, during which the wastewater pollutants are utilized by heterotrophic microorganism and converted to CO_2, water and biomass. The activated sludge process is robust and discharge demands for treated water are met, however, the potential energy of the wastewater, deriving from the organic pollutants, is destroyed upon microbial degradation. Only a minor amount of energy can be recovered, when a part of the biomass which is produced during the activated sludge process is digested anaerobically, and converted into the energy carrier methane. Therefore it would be more sustainable to convert the organic matter present in the wastewater into a suitable energy carrier or valuable chemicals e.g. volatile fatty acids (VFAs) using anaerobic treatment technologies. Anaerobic treatment is common in practice; however, it is limited to high strength or warm industrial wastewater. Yet, with an appropriate up-concentration step for organic matter, anaerobic treatment could also become feasible for the relatively cold municipal wastewater.

In this thesis the up-concentration of wastewater organic matter using a bioflocculation process is investigated in high loaded membrane bioreactors (HL-MBR), characterized by extremely short solid (typically < 1d) and hydraulic (typically < 1 h) retention times. During the bioflocculation process, wastewater organic matter is flocculated with the help of biopolymers, produced by microorganisms. Only a minor amount of the wastewater organic matter is utilized by bacteria to grow and to produce the biopolymers. Thus, most of the potential energy can be recovered from the wastewater when the concentrated stream produced in the HL-MBRs is treated anaerobically to produce methane or VFAs, which would increase the sustainability of wastewater treatment processes substantially.

Following a general introduction **Chapter 2** describes the effect of applying different extremely short retention times (0.125-5 d) on the bioflocculation process in HL-MBRs and the role of extracellular polymeric substances (EPS). The aim was to find a suitable SRT to operate HL-MBRs, at which bioflocculation is maximized and mineralization of organic matter is low. Bioflocculation efficiency increased from 59% at SRT of 0.125 d to 98% at SRT of 0.5 d which was accompanied by an increase in the concentration of (sludge) bound EPS. A redistribution was observed between supernatant and bound EPS when the SRT was prolonged, meaning that free supernatant EPS became (sludge) bound

during the bioflocculation process. Mineralization of organic matter was limited at SRTs ranging up to 1 d, but increased to 32% when SRT was prolonged to 5 d. Thus, it was suggested to operate a HL-MBR at SRT of 0.5 d because at this SRT an optimum between maximum flocculation efficiency and low mineralization of organic matter could be achieved. Additionally, applying shorter SRTs resulted in higher membrane fouling potentials, mainly because of low bioflocculation efficiency. Furthermore, (sludge) bound EPS concentrations found in the HL-MBRs were compared with those extracted from several full and pilot scale wastewater treatment systems, including a full scale AB treatment plant, operated at similar short SRTs as the HL-MBRs and a full scale MBR operated at much longer SRT of 50 d. Similar EPS concentrations were found in the sludge from the full scale AB-treatment plant and the HL-MBR sludge. Additionally it was observed that the EPS concentration firstly increased (until SRT of 1 d) and decreased when the SRT was prolonged further to 50 d.

Changes in operational parameters such as the dissolved oxygen (DO) concentration may affect the bioflocculation process in HL-MRs. Therefore, **Chapter 3** describes the effect of different DO concentrations on the bioflocculation process in HL-MBRs. Two HL-MBRs were operated in parallel with DO concentrations of 1 mg/L (LDO) and 4 mg/L (HDO). A DO concentration of 1 mg/L was chosen because it was assumed that this concentration would not yet be limiting for the microbiology in the reactor. It was found that the HL-MBR operated at high DO concentration (4 mg/L) showed a better bioflocculation process, characterized by higher flocculation efficiency of 92% compared to 69% in the LDO reactor. The increased flocculation efficiency could be attributed to higher EPS concentrations in the HDO reactor. Also a higher concentration of multivalent cations (calcium, aluminum, iron) was distributed to the sludge in the HDO reactor. Furthermore the sludge of the HDO reactor was characterized by bigger flocs, better settleability and lower membrane fouling potential, meaning that elevated DO concentrations were beneficial to the bioflocculation process and overall sludge properties.

In **chapter 4** the bacterial population present in the (solid) sludge fraction, the supernatant and the inflow wastewater of three HL-MBRs operated at different SRTs (0.125 d, 0.5 d and 1 d) is studied to identify the role of bacterial species in the bioflocculation process of wastewater organic matter. Using a PCR-DGGE approach it was shown, that the bacterial communities in the (solid) sludge fraction and in the supernatant were significantly different from each other. Furthermore, the DGGE profile of the HL-MBR operated at SRT of 0.125 d was different from that of the inflow wastewater, demonstrating that already at this short SRT a distinct bacterial community developed. Bioflocculation efficiency, however, was low at this short SRT. As the SRT was prolonged,

bioflocculation efficiency increased which was accompanied by an increased in the abundance of *Bacteroidetes* from 27.5% at SRT of 0.125 d to 46.4% at SRT of 1 d in the (solid) sludge fraction, suggesting that those bacteria were involved in the bioflocculation process. At the same time the abundance of *Proteobacteria* in the (solid) sludge fraction decreased, meaning that the *Proteobacteria* were outcompeted at longer SRTs by slower growing *Bacteroidetes* with better substrate affinity. In the supernatant higher abundances of *Proteobacteria* were found, especially caused by the high abundance *Epsilonproteobacteria*. Clone library analysis revealed high occurance of *Arcobater*, which was exclusively detected in the supernatant. Since *Arcobacter* was recently recognized as water born pathogen, wastewater treatment in MBRs could avoid the distribution of *Arcobacter*. Besides *Arcobacter*, other species known for their EPS production such as *Acinetobacter* were found in the supernatant, suggestion that also supernatant bacteria are involved in the bioflocculation process.

The mechanisms involved in the bioflocculation process are until now not very well understood. Several mechanisms such as double layer interactions (described by the DVLO theory), divalent cation bridging (DCB) theory and the alginate theory as a sub-set of the DCB theory, are proposed. In **Chapter 5** the effect of cations and the impact of the characteristics of EPS on the bioflocculation process are investigated. A HL-MBR was operated with a synthetic bactopeptone wastewater feed, which was characterized by very low ion concentration compared to real municipal wastewater. As a result, poorly flocculated biomass developed in the HL-MBR. Subsequently, jar test were performed to investigate the effect of cation addition on the floc formation of the poorly flocculated peptone biomass. It was found that flocs were formed when 10 mEq/L cations (Na^+, Ca^{2+}, Al^{3+}) were added to the biomass. The flocs formed with Ca^{2+} and in particular those formed with Al^{3+} were more compact and bigger than the flocs formed with Na^+. Also the flocculation efficiency, measured as a decrease in turbidity, with increasing valence of the cation. This observation could fully be explained by the DVLO theory. In a second set of jar tests, the influence of the characteristics of EPS on bioflocculation of kaolin clay particles was studied in the presence of cations (Na^+, Ca^{2+}, Al^{3+}). Different types of EPS were extracted from sludge obtained from various wastewater treatment systems, including municipal and industrial wastewater treatment systems. Flocculation activities with Ca^{2+} and Al^{3+} were similar, however, the extent of flocculation depended greatly on the type of EPS and the treatment system they were obtained from. Furthermore, EPS originating from municipal wastewater treatment systems did not show flocculation activity with Na^+, whereas the EPS extracted from the treatment systems treating industrial and synthetic wastewater did flocculated with Na^+. The flocculation of kaolin clay particles in the presence of cations with EPS was most likely caused by polymer-ion interaction as described by the DCB theory. Interestingly, EPS extracted from

sludge from the industrial wastewater treatment plant, treating high salinity wastewater, flocculated with Na^+, suggesting that wastewater characteristics and probably also operational parameters determine the characteristics of EPS and therewith their flocculation activity with different types of cations.

A general discussion and outlook is provided in **Chapter 6**. It was shown that HL-MBRs should be operated at SRT of 0.5 d. Additionally, elevated dissolved oxygen concentrations were beneficial for the bioflocculation process and reduced membrane fouling. Bacteria from both the supernatant as well as the solid sludge fraction are likely to be involved in the bioflocculation process. Two major mechanisms namely double layer interaction and cation bridging with EPS could be identified to be involved in the bioflocculation process in HL-MBRs. Further research should be conducted to (1) optimize up-concentration of organic matter to make e.g. VFA production from the concentrated stream feasible and (2) to investigate the role of EPS characteristics on the bioflocculation process.

Samenvatting

Afvalwater wordt gewoonlijk behandeld met het actief slib proces, tijdens dit proces worden de afvalwaterverontreinigingen gebruikt door heterotrofie micro-organismen en omgezet naar CO_2, water en biomassa. Het actief slib proces is robuust en de lozingseisen voor het effluent worden behaald. Echter, de potentiele energie van de organische verbindingen in het afvalwater wordt vernietigd door de microbiële afbraak. Slechts een miniem deel van de energie kan worden teruggewonnen wanneer de biomassa, geproduceerd tijdens het actief slib proces, anaeroob wordt vergist en omgezet naar de energiedrager methaan. Daarom zou het duurzamer zijn wanneer de organische stof in afvalwater wordt omgezet d.m.v. anaerobe technologie naar een geschikte energiedrager of waardevolle chemicaliën, b.v. vluchtige vetzuren. Anaerobe behandeling wordt veelvuldig toegepast voor de behandeling van warm afvalwater of van afvalwater met een hoge concentratie organische verbindingen, maar is niet geschikt voor het relatief koude en verdunde huishoudelijk afvalwater. Door de toepassing van een geschikte methode om het organische materiaal te concentreren zou anaerobe behandeling echter wel geschikt worden voor de behandeling van huishoudelijk afvalwater.

In dit proefschrift wordt het concentreren van de organische verbindingen in afvalwater d.m.v. een bioflocculatie proces onderzocht in zwaarbelaste membraanbioreactors (ZB-MBR's) met extreem korte slib (typisch < 1 dag) en hydraulische (typisch < 1 uur) verblijftijden. Tijdens het bioflocculatie proces worden de organische verbindingen in het afvalwater geflocculeerd d.m.v. biopolymeren geproduceerd door micro-organismen. Slechts een miniem deel van de organische verbindingen in het afvalwater worden door de bacteriën aangewend voor groei en de productie van biopolymeren. Daarom kan de bulk van de potentiele energie uit het afvalwater worden teruggewonnen wanneer het concentraat van de ZB-MBR's anaeroob wordt behandeld, resulterend in de productie van methaan of vluchtige verzuren, welk de duurzaamheid van het afvalwaterbehandelingsproces aanzienlijk doet stijgen.

Na een algemene introductie beschrijft **Hoofdstuk 2** het effect van de toepassing van extreem korte retentietijden (0,125 - 5 dagen) op het bioflocculatie proces in ZB-MRB's en de rol van extracellulaire polymerische verbindingen. Het doel was het vinden van een slibverblijftijd, voor het bedrijven van

ZB-MBR's, waarbij de bioflocculatie maximaal en de mineralisatie van de organische verbindingen minimaal is. De bioflocculatie efficiëntie steeg van 59% bij een slibverblijftijd van 0,125 dag naar 98% bij een slibverblijftijd van 0,5 dag, welk werd vergezeld door een toename van de concentratie van aan-slib-gebonden extracellulaire polymerische verbindingen. Wanneer de slibverblijftijd werd verlengd trad er een herverdeling op tussen de extracellulaire polymerische verbindingen in het supernatant en die gebonden aan het slib. Dit betekend dat de vrije extracellulaire polymerische verbindingen in het supernatant tijdens het bioflocculatie proces werden gebonden aan het slib. Mineralisatie van de organische verbindingen was beperkt bij slibverblijftijden van 1 dag of korter, maar nam toe tot 32% wanneer de slibverblijftijd werd verlengd naar 5 dagen. Vanwege de hoge flocculatie efficiëntie en lage mineralisatie is voorgesteld om de ZB-MBR's te bedrijven bij een slibverblijftijd van 0,5 dag. Bovendien resulteerde kortere slibverblijftijden tot een hoger membraanvervuilingspotentiaal, met name vanwege de lagere bioflocculatie efficiëntie. Verder zijn de aan-slib-gebonden extracellulaire polymerische verbindingen concentraties in ZB-BMR's vergeleken met die van verschillende full- en pilotschaal afvalwaterbehandelingsinstallaties, inclusief één full-scale AB behandelingsinstallatie bedreven bij een vergelijkbare korte slibverblijftijd als de ZB-MBR's en één full-sale MBR bedreven bij een veel hogere slibverblijftijd van 50 dagen. Vergelijkbare concentraties van extracellulaire polymerische verbindingen werden gevonden in het slib van de full-scale AB behandelingsinstallatie en het ZB-MBR slib. Daarnaast is er gevonden dat de concentratie van extracellulaire polymerische verbindingen eerst toenam (tot een slibverblijftijd van 1 dag) en afnam wanneer de slibverblijftijd werd verlengd tot 50 dagen.

Veranderingen in operationele parameters zoals de opgeloste zuurstof concentratie beïnvloeden mogelijk het bioflocculatie proces in ZB-MBR's, dit is daarom onderzocht in **hoofdstuk 3**. Twee ZB-MBR's zijn in parallel bedreven, één bij een opgeloste zuurstof concentratie van 1 mg/L en één bij 4 mg/L. De verwachting is dat een zuurstof concentratie van 1 mg/l niet limiterend is voor de microbiologie. De bioflocculatie efficiency was hoger bij 4 mg/L (92%) dan bij 1 mg/L (69%). De hogere flocculatie efficiëntie kon worden gerelateerd aan een hogere concentratie extracellulaire polymerische verbindingen. Verder was de concentratie multivalente kationen (calcium, aluminium, ijzer) in het slib hoger bij een bij een zuurstof concentratie van 4 mg/L vergeleken bij een zuurstof concentratie van 1 mg/L. Het slib in de ZB-MBR met de hogere zuurstof concentratie werd gekarakteriseerd door grotere vlokken, een betere bezinkbaarheid en een lager membraanvervuilingspotentieel. Dit betekend dat een verhoogde zuurstof concentratie gunstig is voor het bioflocculatie proces en de eigenschappen van het slib.

In **hoofdstuk 4** wordt de bacteriële populatie in de slibfractie, het supernatant en het afvalwater, waarmee de ZB-MBR's worden gevoed onderzocht bij verschillende slibverblijftijden (0,125 dag, 0,5 dag en 1 dag). Het doel is het identificeren van de rol van individuele bacterie soorten tijdens de bioflocculatie van organische verbindingen in afvalwater. M.b.v. PCR-DGGE werd aangetoond dat de microbiële samenstelling in de slibfractie significant verschild van die in het supernatant. Verder werd aangetoond dat de microbiële samenstelling in ZB-MBR's bedreven bij een slibverblijftijd van 0,125 dag anders is dan de microbiële samenstelling in het afvalwater waarmee de ZB-MBR's werden gevoed, dit toont aan dat zelfs bij een korte slibverblijftijd er een onderscheidende bacteriële gemeenschap wordt gevormd. De bioflocculatie efficiëntie was laag bij een dergelijke korte slibverblijftijd. Wanneer de slibverblijftijd werd verlengd nam de bioflocculatie efficiëntie toe, dit werd vergezeld door een toename van *Bacteroidetes* in de slibfractie (van 27,5% bij een slibverblijftijd van 0,125 dag naar 46.4% bij een slibverblijftijd van 1 dag), dit suggereert dat *Bacteroidetes* zijn betrokken bij het bioflocculatie proces. Tegelijkertijd nam de talrijkheid van *Proteobacteria* in de slibfractie af. Dit betekend dat de *Proteobacteria* werden weggeconcurreerd bij langere slibverblijftijden door de *Bacteroidetes. Bacteroidetes* groeien langzamer maar hebben een hogere substraataffiniteit. In het supernatant werd een hogere *Proteobacteria* concentratie gevonden, voornamelijk *Epsilonproteobacteria.* Een clone library analyse liet verder een hoge *Arcobacter* concentratie zien, maar alleen in het supernatant. Recentelijk is *Arcobacter* geïdentificeerd als een pathogeen die via het water wordt verspreid. Daarom moet de verspreiding van *Arcobacter* tijdens afvalwaterbehandeling worden voorkomen. Behalve *Arcobacter* zijn er ook andere soorten in het supernatant gevonden die extracellulaire polymerische verbindingen produceren, wat suggereert dat ook de bacteriën in het supernatant betrokken zijn bij het bioflocculatie proces.

Er is nog veel onbekend over de mechanismen die een rol spelen in het bioflocculatie proces. Verschillende mechanismes zijn genoemd, zoals dubbellaag interacties (beschreven door de DVLO theorie), de divalent kation overbrugging theorie en een afgeleide daarvan (de alginaat theorie). In **hoofdstuk 5** worden het effect van kationen en de eigenschappen van de extracellulaire polymerische verbindingen op het bioflocculatie proces onderzocht. Een ZB-MBR werd gevoed met synthetisch afvalwater met bactopeptone. Dit synthetische afvalwater bevatte een lage ijzerconcentratie vergeleken met het echte huishoudelijke afvalwater. Dit resulteerde in een slechte flocculatie van de biomassa in de ZB-MBR. Vervolgens zijn er batch tests gedaan om te onderzoeken wat het effect is van de additie van kantionen op de vlokformatie van de op peptone gekweekte biomassa. Er werden vlokken gevormd wanneer 10 MEq/L kationen (Na^{+}, Ca^{2+}, Al^{3+}) weren toegevoegd aan de biomassa. De vlokken gevormd met Ca^{2+} en met name de vlokken gevormd met

Al^{3+} waren compacter en groter dan de vlokken gevormd met Na^{+}. Ook de flocculatie efficiëntie, bepaald m.b.v. de afname in troebelheid, was hoger naarmate de kationvalentie hoger was. Deze resultaten worden volledig verklaard door de DVLO theorie. Tijdens een tweede serie batch experimenten is de invloed van de eigenschappen van extracellulaire polymerische verbindingen op de bioflocculatie van kaolin-kleideeltjes in aanwezigheid van kantionen (Na^{+}, Ca^{2+}, Al^{3+}) onderzocht. Verschillende typen extracellulaire polymerische verbindingen zijn onttrokken uit het slib van verschillende afvalwaterbehandelingsinstallaties, inclusief huishoudelijke en industriële afvalwaterbehandelingsinstallaties. Flocculatie activiteiten met Ca^{2+} en Al^{3+} waren vergelijkbaar, hoewel de mate van flocculatie afhankelijk was van het type extracellulaire polymerische verbindingen en de behandelingsinstallatie waaruit ze waren verkregen. Bovendien was er geen flocculatie activiteit met de extracellulaire polymerische verbindingen van een huishoudelijk afvalwaterbehandelingsinstallatie en Na^{+}, terwijl er wel flocculatie was met Na^{+} en extracellulaire polymerische verbindingen verkregen uit installaties die industrieel of synthetisch afvalwater behandelden. De flocculatie van kaolin-kleideeltjes in aanwezigheid van kantionen en extracellulaire polymerische verbindingen werd hoogstwaarschijnlijk veroorzaakt door polymeer-ion interactie zoals beschreven door de divalent kation overbrugging theorie. Er was flocculatie met Na^{+} en extracellulaire polymerische verbindingen onttrokken aan slib van een installatie die industrieel afvalwater met een hoog zoutgehalte behandelde. Dit doet vermoeden dat dat de eigenschappen van het afvalwater zelf, en waarschijnlijk ook de bedrijvingscondities, de eigenschappen van extracellulaire polymerische verbindingen bepalen en dus ook de flocculatieactiviteit met verschillend kationen beïnvloeden.

Hoofdstuk 6 voorziet in een algemene discussie en voortuitzicht. Het is aangetoond dat ZB-MBR's dienen te worden bedreven bij een slibverblijftijd van 0,5 dag. Daarnaast is aangetoond dat een verhoogde opgeloste zuurstof concentratie heilzaam werkt op het bioflocculatie proces en leid tot verminderde membraanvervuiling. Bacteriën in zowel het supernatant als in de vaste slibfractie spelen naar alle waarschijnlijkheid een rol in het bioflocculatie proces. Twee voorname mechanismen, namelijk dubbellaag interactie en kation overbrugging met extracellulaire polymerische verbindingen, spelen een rol in het bioflocculatie proces in ZB-MBR's. Verder onderzoek is nodig om (1) de concentratie van organische verbindingen te optimaliseren, om het bijvoorbeeld mogelijk te maken vluchtige vetzuren te maken van het MBR concentraat, en om (2) de rol van de eigenschappen van de extracellulaire polymerische verbindingen op het bioflocculatie proces te onderzoeken.

Acknoledgement

So, here we are: at the end of a story that was often thought not to be finished at all. I am looking back at 5 years of fun and frustration, hard work and happy holidays, old habits and new experiences and uncountable liters of wastewater but countable thesis pages. Yet, I would not have made it this far without the help of various people.

First of all I want to thank Gert-Jan Euverink for hiring me as a master-thesis student at Wetsus in 2009. This gave the start to my succeeding career at Wetsus. Also thanks for closing the cycle by recently hiring me once more!

I would like to thank Cees and Johannes for giving me the opportunity to conduct my work at such an inspiring and multidisciplinary water research institute as Wetsus.

Special thanks goes to Hardy. Thanks for your never ending ideas about experiments, and your limitless trust that one day we will get it right. Although I had to grow a thick skin, your constructive criticism played a major part in the success of this thesis. It also contributed a lot to my personal growth.

I would like to thank Huub for taking over the task to be my promotor. You were always available for questions and suggestions for experimental improvement and intervened before situations would become complicated. Thank you very much!

I also want to thank Arie for the fruitful discussions about membrane, flats sheets or tubular, backwaschable or handwaschable… and the felt millions of SEM pictures we took together in this dark, hot and mysterious SEM room. Also thanks for all the good talks during long drives to theme meetings and PhD defenses and the one or other appel and egg ☺.

Very warm thanks goes to the other members of the MBR theme in particular: Antoine, Judita, Lieven and Christina. Antoine, thanks for always having suggestions to improve my graphs. Judita and Lieven, thanks for the many, many, many Thursday morning MBR meetings (especially those without supervisors!) and all the laughter, wailing and discussions about MBRs. In this regard I would also like to thank the *Membranes for water treatment and re-use* theme at Wetsus for all the fruitful discussions during theme meetings.

Christina, I am running out of words when thinking about how to express my gratitude to you. You were not only my most awesome MBR theme colleague and my dirty-labcoated-shit-reactor-buddy, we also became inseparable friends. You were there when the shit (not only the one in the reactors) was about to take over and frustrations needed to be overcome; but also when happy times needed

to be celebrated. We made it!!!!!! Throughout bad and happy days, with wine, bratwurst and hundreds of kilometers of running.

I also want to say thanks to my permanent office mates Martijn, Taina, Pom, Vitautas. Thanks Martijn for your never ending patience to listen to whatever I had to say and for keeping on trying to teach me dutch; I would probably not be able to speak it that well, if I would have not started practicing with you. And I forgive you for laughing about my initial pronunciation. You are welcome! Pom, thank you for the discussions about VFA's (uum, I mean carboxylates) and for keeping my reactors alive! I really highly appreciate it.

Speaking about reactors, those guys only exist(ed) because of the help of the technical team at Wetsus. Thank you Jan and Harm for fixing leaks, building aerator plates and spending countless hours to repair the inflow wastewater units. I appreciate your fearless fight through the smell, corn grains and all the other nasty stuff we encountered when the wastewater did not flow as it should.

Continuing with the lab, I have to say that all the experimental work would have not been possible without the help of a couple of excellent students: Lingjun, Karolina, Daniel, Billy, Maik and Virginia. Thanks for all the good work and for even working in the lab the one or other Saturday. Maik, I wish you lots of fun, luck and happy moments during your very own PhD experience. You will do just fine!

I would like to mention a few people that may not explicitly contributed to the work described in this precious book, but can be held liable for contributing to a happy live in Leeuwarden: Charu, thanks for helping me to keep the good spirit, for the yoga evenings, cups of coffee at Douwe Egberts and subs at Subway. My lovely (dinner) friends: Petra, Jos, Bob and Irene! Thanks for all the good food, great talks, fun and BABIES! We will draw level soon ;) I hope we will continue our dates, in Leeuwarden or Austria. You are always welcome!

Alex and Claudia, thanks a lot for all the, Grill-, Wein-, und Spiel Abende, Damenspitze, and for letting me use your horses/ponies to improve my horse back riding skills. Alex, thanks for the good times during the 2 years of house sharing. Without you my start in the Netherlands would have been much more lonesome! I am still looking back with pleasure to the time when "renovating" the house in Tainaburg!

Meine Maedels daheim: Anika und Alexa! Thanks for sharing the passion for water. Das hat uns zusammen gebracht! Ob Erkrath oder Solingen, Erkelenz oder Österreich, ich bin sicher wir werden immer ein Plaetzchen zum Fruehstuecken und zum vor-weihnachtlichen-dinieren finden ☺.

Sandra, meine Liebe! Danke dass du es immer bedenkenlos akzeptiert hast, wenn ich mal in der grossen und mal nicht so grossen Welt umher gezogen bin. Unsere Freundschaft hatte stehst Bestand und du weisst, falls es dich doch noch woanders hin verschlaegt bin ich die Erste die auf deiner Matte steht!

Sarah Jane, many thanks to you for all the good times in Canada, for sharing your flat with me whenever I decided that it would be time to come back to Halifax, for all the great movies you showed me, for all the dinner, lunches and tea. I will always remember your teapot warmer (is that the right word or is it as wrong as water boiler?). Very special thanks for coming all the way from Canada to do me the honour and be my paranymph. I know I can ALWAYS count on you!
Herr Bergmair and husband in spe: Thanks for always being my tower of strength!! Thanks for all your patience and for teaching me serenity also during the stormiest times. Nua net hudln ☺ Auch deiner Famile und der Kommune am Theklaweg gilt ein grosser Dankeschoen fuer die herrlichen Zeiten/Urlaube in Österreich und die herzliche Freundschaft.

Meiner eigenen Familie gebuert das wohl groesste Dankeschoen. Danke an Mama und Papa dass ihr mir IMMER das ermoeglicht habt was ich wollte und stehts voll und ganz hinter mir standet, auch wenn Lena in Canada und Lena in der Arktis das ein oder andere mal sicherlich ein mulmiges Gefuehl ausgeloest hat. Nicht zuletzt jedoch hat diese Unterstuetzung mich dahin gebracht wo wir heute stehen.

About the author

Lena Faust was born on March 24th 1984 in Duisburg, Germany. In 2004 she started to study water science at the University of Duisburg-Essen. Lena finished her Master Degree in 2009 by completing a thesis on Gel-Layer formation and particle fouling in membrane bioreactors at Wetsus, centre of excellence for suitanable water technology in the Netherlands. In December 2009 Lena started her PhD project and investigated the bioflocculation process of wastewater organic matter in membrane bioreactors for wastewater treatment. This work was performed under the supervison of Wageniningen University and the Sub-department of Environmental Technology. The practical work for her PhD thesis was performed at Wetsus in Leeuwarden.

Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment

D I P L O M A

For specialised PhD training

The Netherlands Research School for the Socio-Economic and Natural Sciences of the Environment (SENSE) declares that

Lena Faust

born on 24 March 1984 in Duisburg, Germany

has successfully fulfilled all requirements of the Educational Programme of SENSE.

Wageningen, 3 December 2014

the Chairman of the SENSE board	the SENSE Director of Education
Prof. dr. Huub Rijnaarts	Dr. Ad van Dommelen

The SENSE Research School has been accredited by the Royal Netherlands Academy of Arts and Sciences (KNAW)

KONINKLIJKE NEDERLANDSE
AKADEMIE VAN WETENSCHAPPEN

The SENSE Research School declares that **Ms Lena Faust** has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 84.2 EC, including the following activities:

SENSE PhD Courses

- SENSE PhD Introductory course: Environmental Research in Context (2010)
- Research Context Activity: Co-organiser of the Wetsus Water Challenge Science & Technology, Leeuwarden (2010)
- Biological Processes for Resource Recovery in Environmental Technology (2010)
- Basic Statistics (2011)

Other PhD and Advanced MSc Courses

- Microbial Ecology in Wastewater treatment, Aalborg University Denmark (2010)
- Principles of Anaerobic Wastewater Treatment, Lettinga Associates Foundation (2011)
- Techniques for Writing and Presenting Scientific Papers, Wageningen University (2011)
- Publishing using Word, Wageningen University (2011)
- Working Safely in Laboratories, Wageningen University (2011)
- Advanced Course Environmental Biotechnology, Delft University (2013)
- New Frontiers in Microbial Ecology, Wageningen University (2013)

Management and Didactic Skills Training

- Supervision of seven MSc thesis students and one internship student (2010-2013)
- Internal Business Challenge (Wetsus): funding and patenting innovative ideas (2011)
- Organisation of Bioflocculation workshop, University of Leuven, Belgium (2012)

Oral Presentations

- *High loaded MBR for improved Energy recovery from sewage.* Wetsus-Edingburgh University Workshop, 4 February 2010, Edinburgh, Scotland
- *Bioflocculation in high loaded MBRs.* Bioflocculation workshop KU Leuven, 12 December 2012, Leuven, Belgium
- *High loaded MBRs for improved energy recovery from sewage*. Dutch membrane society meeting, 27 November 2012, Leeuwarden, The Netherlands

SENSE Coordinator PhD Education

Dr. ing. Monique Gulickx

This work was performed in the cooperation framework of Wetsus, centre of excellence for sustainable water technology (www.wetsus.nl). Wetsus is co-funded by the Dutch Ministry of Economic Affairs and Ministry of Infrastructure and Environment, the European Union Regional Development Fund, the Province of Fryslân and the Northern Netherlands Provinces. The authors like to thank the participants of the research theme “Membrane processes and operation for wastewater treatment and reuse” for the fruitful discussions and their financial support.

Cover design by Rando Wiltschek.
Printed by GVO drukkers & vormgevers B.V., Ede – The Netherlands