Characteristics of different fractions of microbial flocs and their role in membrane fouling
H. J. Lin, W. J. Gao, K. T. Leung and B. Q. Liao

ABSTRACT
Characteristics of different fractions (small flocs vs. large flocs) of sludge flocs from a submerged anaerobic membrane bioreactor treating thermomechanical pulping (TMP) whitewater were determined using various analytic techniques, including extraction and chemical analysis of extracellular polymeric substances (EPS), particle size analyzer, and polymer chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE). The results showed that the fraction of smaller flocs contained a higher level of bound EPS and had a higher fractal dimension as compared to the fraction of larger flocs. PCR-DGGE analysis indicated that there were significant differences in microbial community between the fraction of smaller flocs and large flocs. The microbial community of the smaller flocs was similar to that of the sludge cake layers, indicating the pioneering role of the microbial community in smaller flocs in membrane fouling. These findings provide a new insight in the difference of membrane fouling potential between smaller flocs and larger flocs fraction.

Key words | extracellular polymeric substances, fractional dimension, membrane fouling, microbial community, small flocs, submerged anaerobic membrane bioreactor

INTRODUCTION
Due to the stringent discharge criteria, increasing space constraints and desired flexibility for future expansion and upgrade, submerged anaerobic membrane bioreactors (SAfMBRs), which combine the advantages of the submerged membrane bioreactors (SMBRs) and anaerobic processes, have received much attention in recent years (Hu & Stuckey 2006; Jeison & van Lier 2006; Liao et al. 2006; Huang et al. 2008; Lin et al. 2009). However, membrane fouling and its consequences in terms of plant maintenance and operating costs remain the critical limiting factors affecting the widespread application of SMBRs to wastewater treatment (Jeison & van Lier 2006; Lin et al. 2009).

Previous studies had focused on various factors affecting the membrane fouling in SMBRs, including hydrodynamic conditions, membrane materials and module design as well as feed-biomass properties (nature and concentration of feed, particle size, sludge concentration, extracellular polymeric substances (EPS), hydrophobicity, surface charge, etc.) (Meng et al. 2009). It has been proposed that the small flocs in bulk sludge play a critical role on membrane fouling. The small flocs are actually aggregates formed by microorganisms, colloids, bound EPS, and cell debris. It has been reported that flocs had a size that is close to or smaller than the size of membrane pores, would penetrate into the membrane pores easily and then block pores (Bae & Tak 2005), which causes serious membrane fouling problems. Many experimental studies and practical applications have indicated that sludge deposition on the membrane surface is the main cause of membrane fouling in SMBRs. Previous studies have indicated that small flocs had a strong tendency to deposit on the membrane surface (Bae & Tak 2005; Lin et al. 2009), corresponding to a high membrane fouling rate. It is also widely accepted that small flocs would form a denser and less porous cake layer or gel layer which has a higher specific resistance.

To date, the explanations for these phenomena are mainly based on the particle size of flocs. For example, small flocs have a strong tendency to deposit on a membrane surface and this is generally explained by the fact that the back transport...
velocity of particles decreased with their size (Belfort et al. 1994). Both small flocs and large flocs are the source of cake sludge. However, recent studies showed that the cake layer deposited on the membrane surface in the SMBR system has a filtration resistance of around 10^{14} m/kg (Chu & Li 2005), which is much higher than the value of 10^{11} m/kg determined for activated sludge during dewatering (Buyukkamaci 2004). In SMBR systems, small flocs only account for a small fraction of the total sludge suspension in terms of volume, while they seem to take major responsibility for the membrane fouling in SMBR systems (Wisniewski & Grasmick 1998; Ng & Hermanowicz 2005). Therefore, the particle size of the flocs alone could not satisfactorily explain the observation that small flocs play a critical role on membrane fouling. By reviewing the literature, we can extend the hypothesis that besides their size, small flocs have different characteristics, such as EPS, fractional dimension and bacterial community, from large flocs and bulk sludge. However, to date, few attempts have been made to characterize small flocs. A detailed characterization of different fractions of microbial flocs will be conducive to a better understanding of membrane fouling, and it will also facilitate to develop effective fouling control strategies and to optimize operational parameters in SMBRs.

In this study, a laboratory-scale SAnMBR treating thermomechanical pulping (TMP) whitewater was operated for over six months in order to investigate characteristics of different fractions of microbial flocs and their role in membrane fouling. The bulk sludge was fractionated into small flocs fraction and large flocs fraction using centrifugation procedure. The bound EPS, fractal dimensions, and bacterial communities of small flocs and large flocs, as well as some property parameters of bulk sludge and cake sludge were examined and compared. The results obtained in this study could provide new insights into the role of different flocs fractions in membrane fouling.

**MATERIAL AND METHODS**

**Experimental setup and operation**

A laboratory-scale SAnMBR system was constructed and used in this study. The schematic diagram of this set-up was described in Lin et al. (2009). A flat sheet microfiltration membrane module with a total membrane area of 0.03 m² was mounted vertically within the reactor (10 L). All membranes used in this study were made of polyvinylidene fluoride (PVDF) materials using the phase inversion method. The molecular weight cut off (MWCO) was characterized as 70,000 Da. At the base of the membrane module, a stainless steel tube diffuser was located, and headspace biogas was recirculated by a gas recycle pump (Masterflex Console Drive, Model 7520-40, Thermo Fisher Scientific, USA) (1.5 L/min) to provide mixing and to control solids deposition over the membrane surface. A magnetic stirrer (Thermolyne Cimarec, model no: S47030) was located at the bottom of reactor to provide necessary mixing to the sludge liquid. The temperature was maintained constant at 37 ± 1°C by circulating warm water through the water jacket of the reactor. The pH was automatically adjusted to 7.0 ± 0.2 by a pH regulation pump and a pH electrode (Thermo Scientific, Beverly, MA) using 0.1 N NaOH solution.

The seeded sludge was obtained from an upflow anaerobic sludge blanket (UASB) reactor treating pulping wastewater at Tembec Industries Inc. (Temiscaming, Quebec). Thermomechanical pulping (TMP) whitewater from a local pulp and paper mill, which was enriched with NH₄Cl and KH₂PO₄ providing a COD:N:P ratio of 100: 2.6: 0.4, was pumped into the bottom of the bioreactor automatically by a feeding pump (Masterflex C/L 200 RPM, model 77120-70, Barnant Co., USA) which is controlled by a level sensor (Madison Co., USA) and controller (Flowline, USA). To prevent trace metal limitations of the methanogens, a trace element solution (Welander et al. 1999) was supplemented to the influent. The filtrate was intermittently obtained by using a peristaltic pump (Masterflex, C/L, Model 77120-70, Barnant, Co., USA) operating in the mode of 4-minutes-on and 1-minutes-off. Membrane flux was controlled by adjusting the pump speed and two calibrations were made each day. When the trans-membrane pressure reached 40 kPa, the reactor was shut down and a physical cleaning procedure was conducted. During the operation of the reactors, no sludge was discharged except for sludge sampling and sludge cake characterization. This corresponds to a sludge retention time of approximately 280 days.

**Analytical methods**

**Sludge liquor fractionation and sample pretreatment**

By using centrifugation, the bulk sludge can be fractionated into small flocs fraction and large flocs fraction liquor. A typical fractionation procedure in this study was centrifuging the bulk sludge at 1890 × g for 10 min. The sludge pellets deposited at the bottom of centrifuge tubes were re-suspended by gentle shaking to their original volume using a buffer consisting of 2 mM Na₃PO₄, 4 mM NaH₂PO₄, 9 mM NaCl and 1 mM KCl at pH 7. The fractionated sludge liquors could be then further treated according to the requirements of
specific analysis items. Serial sludge fractionations were conducted by centrifuging the bulk sludge at different centrifugation speeds for 10 min to assess the relationship of fractional dimensions with floc size.

**Evaluation of filtration resistance**

Membrane resistance was analyzed using Darcy’s law as indicated in Equation (1):

\[
R_t = R_m + R_c + R_p = \frac{\Delta p_T}{\eta \cdot J}
\]

where, \(R_t\) is the total hydraulic resistance, \(R_m\) is the membrane resistance, \(R_p\) is the pore blocking resistance, \(R_c\) is the cake layer resistance, \(\Delta p_T\) is the trans-membrane pressure, \(\eta\) is the dynamic viscosity and \(J\) is the membrane flux.

Cake resistance is related significantly to cake specific resistance and cake mass as shown in Equation (2):

\[
R_c = \alpha \cdot m_c
\]

where \(m_c\) is the dry cake mass and \(\alpha\) is the specific cake resistance. The cake sludge was carefully scraped off from membrane surface using a plastic sheet, and then placed in a dryer at 105 °C for 24 h to obtain dry cake mass.

Specific cake resistance test was conducted in a 180 mL stirred filtration cell (Amicon, USA) using a PVDF flat-sheet membrane and operated at a constant pressure of 15 kPa by pressurized nitrogen from a gas cylinder following the method by Wisniewski & Grasmick (1998). The production of filtrate under pressure was continuously recorded by an electric balance. The specific resistance to filtration (\(\alpha\), m/kg) can be calculated by Equation (3):

\[
\alpha = \frac{2000 A^2 \Delta p_b}{\mu C}
\]

where \(\Delta p (15 \text{ kPa})\) is the pressure applied, \(A\) (0.00287 m²) the filtration area, \(C\) the total suspended solids (kg/m³), and \(b\) (s/m²) is the time-to-filtration ratio, which is the slope of the curve that is obtained by plotting the time of filtration to the volume of filtrate ratio (t/V) versus the filtrate volume (V).

**EPS extraction and measurement**

Extraction of bound EPS was based on a cation exchange resin (CER) (Dowex Marathon C, Na⁺ form, Sigma-Aldrich, Bellefonte, PA) method (Frolund et al. 1996): 100 mL sludge suspension was taken and centrifuged (IEC MultiRF, Thermo IEC, Needham Heights, MA, USA) at 18700 \(\times g\) for 10 min at 4°C. The sludge pellets were resuspended to their original volume using a buffer consisting of 2 mM Na₃PO₄, 4 mM NaH₂PO₄, 9 mM NaCl and 1 mM KCl at pH 7. Then, the sludge was transferred to an extraction beaker with buffer and the CER (80 g CER/BMLSS) added. The suspension was stirred (Corning 171 Scholar Stirrer, Corning, USA) for extraction of EPS for 2 hours at 4°C. The extracted EPS was recovered by centrifugation of the CER/sludge suspension for 20 min at 18700 \(\times g\) at 4°C in order to remove the CER and MLSS. The EPS was normalized as the sum of polysaccharide and protein, which were measured colorimetrically by the methods of Dubois et al. (1956) and Lowery et al. (1951), respectively. Bovine serum albumin (BSA) was used as a protein standard, and glucose was used as a polysaccharide standard.

**Floc size distribution and its structure**

The floc size distribution was determined by a Malvern Mastersizer 2000 instrument with a detection range of 0.02–2000 μm. The scattered light is detected by means of a detector that converts the signal to a size distribution based on volume. In this study, each sample was measured three times with a standard deviation of 0.1–4.5%.

The structure of the flocs was quantified in terms of fractal dimensions (\(D_f\)), which is calculated from the raw light scattering data from the Malvern Mastersizer 2000 according to the method by Spicer et al. (1998). The values of \(D_f\) are classically between 1.7 and 2.5 for biological aggregates; the high value of the \(D_f\) is related to compact and dense flocs.

**Confocal laser scanning microscopy (CLSM) analysis**

Samples were stained before examined by an upright CLSM system (Leica DM RE microscope connected to a Leica TCS SP2, Leica, Germany) with 3 different visible light lasers, covering 6 excitation wavelengths. To observe EPS on the cake layer, two different probes were collectively applied: Concanavalin A, Alexa Flour 633 conjugate (5 mg/L, Invitrogen) to target the polysaccharides with (α-Man, α-Glu (Polysaccharide) and SYPRO orange (Invitrogen) to target all the proteins. The membrane samples were placed and stained in 5 cm dia. petri-plates and incubated in the dark at room temperature for 30 min. After staining, all the samples were washed gently three times with a phosphate buffer to remove unbound probes. After washing, the treated samples were immediately observed in CLSM. Signals were recorded in the green channel (excitation 488 nm, emission 570 nm) for proteins and red channel (excitation 633 nm, emission 647 nm) for polysaccharide. The confocal assistant software
supplied by the manufacturer (Leica Confocal Software (LCS), version 2.61) was used to conduct the image analysis.

**Microbial Community Study**

A volume of 1 mL mixed liquor was collected from each sludge sample and centrifuged for 3 min at 15000 rpm. The supernatant was discarded and the cell pellet was resuspended in 50 μl sterile deionized water. Bacterial genomic DNA was extracted from the pellet using Fecal DNA isolation kit (MoBio Laboratories, Solana Beach, CA, USA) according to the protocol described by the manufacturer.

To amplify the V3 region of bacterial 16S ribosomal RNA gene (rDNA) from the DNA extracted, the primer set, 514 f-GC (5'-GC-clamp-CCTAGGGAGGCAGCAG-3') and 534r (5'-ATTACCGCGGCTGCTGG-3') was used. A PCR program was performed on a 50-μl PCR mixture containing 1.0 U of Taq DNA polymerase (Fermentas Life Sciences, Canada), 30 pmol of each primer, 1 × PCR buffer, 1.5 mM MgCl₂, 200 μM dNTP (Fermentas Life Sciences, Canada) and 2 μl of the 10-fold diluted purified sludge DNA extract. PCR cycling was carried out in a Hybrid Thermocycler (Thermo Electron Corp., USA) under the following conditions: an initial denaturation at 94 °C for 5 min followed by 35 cycles consisting of denaturation at 94 °C for 1 min, primer annealing at 56 °C for 1 min, and primer extension at 72 °C for 1 min. A final extension step was conducted at 72 °C for 5 min prior to cooling at 4 °C.

Denaturing gradient gel electrophoresis (DGGE) of the PCR products was performed with a 16 × 16-cm 10% polyacrylamide gel maintained at 60 °C in a 7 L of Tris-acetate-EDTA (TAE) buffer (20 mM Tris-acetate, 0.5 M EDTA, pH 8.0) using a D-code DGGE system (BIORAD Laboratories, USA). Gradient gels were prepared with 25 and 65% denaturant (100% denaturant defined as 7 M urea plus 40% v/v formamide). Between 0.5 and 1 μg of the amplified DNA was loaded per well and run at 35 V for 16 h. Gels were stained in 150 mL TAE buffer containing 15 μL 10 000 × concentrated SYBR Green I stain (Fermentas Life Sciences, Canada) for 1 h and destained in 350 mL TAE for 1 h, and then photographed with a digital camera (SynGene a division of Synoptics Ltd, UK).

**RESULTS AND DISCUSSION**

**Filtration performance**

The SAnMBR for the treatment of TMP whitewater was continuously operated for over six month. During stable operation period, for the applied filtration flux of approximately 8.2 L/m² h and biogas sparging rate of 1.5 L/min, the membrane becoming severely fouled required 14 days. When TMP reached about 40 kPa, sludge cake layer over the membrane surface could be clearly observed, demonstrating cake formation was the main cause of the eventually severe membrane fouling. This corresponded to cake resistance of 1.5 × 10¹³ m⁻¹. The dry mass of cake sludge collected from the membrane surface was measured as 0.11 kg/m², according to Equation (2), the filtration resistance of the cake sludge was then estimated to be around 1.34 × 10¹⁴ m/kg.

The fractionated small flocs fraction and large flocs fraction liquor had different particle size distributions (mean size of 8.6 μm for small flocs fraction vs. 30.7 μm for large flocs fraction). Calculation of the data obtained from batch filtration tests showed that the small flocs fraction liquor had a specific resistance of 1.26 × 10¹⁴ m/kg, which was over 6 times and almost 3 times more than that of large flocs fraction liquor and bulk sludge liquor, respectively, and comparable to the estimated specific filtration resistance of cake layer shown in above. These results indicate that the small flocs fraction in the sludge liquor has a very different fouling behavior from the large flocs fraction, and it might be largely responsible for the high cake layer filtration resistance. Considering all the three sludge liquors have the same supernatant, the different fouling behavior should be attributed to the characteristics of flocs.

**CLSM analysis**

Upon fluorescent staining, both proteins (green in Figure 1) and polysaccharides (red in Figure 1) became visible. Figure 1 gives the direct evidence of the presence of proteins and polysaccharides in fouling layers. Moreover, analysis of porosity showed that the fouling layers formed with small flocs fraction sludge had a lower porosity (0.49 ± 0.05 vs. 0.53 ± 0.03 vs. 0.63 ± 0.01 for small, intermediate, and large flocs fraction liquor, respectively).
0.64 ± 0.01 for that formed with large flocs fraction sludge). This illustrates that the small flocs fraction sludge could result in the formation of a denser cake layer. It should be noted that the fouling layers were formed after short term filtration (24 min), a more significant differences in porosity between them could be expected if long term filtration applied. The images also indicate that EPS may be involved in cake formation. Besides flocs size, EPS may have some effects on the different filtration performance between the small and large flocs fraction sludge.

**Bound EPS**

Figure 2 presents the comparison of bound EPS values measured for the small, large flocs fraction sludge and bulk sludge. Statistical analysis using analysis of variance (ANOVA) verified that the differences in protein, polysaccharide and total EPS among the three sludge liquors were all statistically significant (p < 0.01). Some studies (Bae & Tak 2005; Lin et al. 2009) found that small flocs had a strong tendency to deposit on the membrane surface. It is generally explained with their lower back transport velocity compared to the large flocs (Belfort et al. 1994). Previous studies didn’t measure other characteristics of small flocs. To our best knowledge, it is the first time revealed from our study that the small flocs have higher bound EPS. It was reported that filterability of sludge decreased with an increase in bound EPS (Nagaoka et al. 1996). Tsuneda et al. (2003) reported that sludge adhesion is enhanced by polymeric interactions when the EPS content is increased. It seems that EPS act as “glue”, facilitating sludge attachment on membrane surface. Therefore, the higher bound EPS in small flocs could be at least partially responsible for their stronger tendency to deposit on the membrane surface.

**Fractal dimension analysis**

Figure 3 shows the variations of fractal dimension of sludge flocs with the mean flocs size. Fractal dimension decreased as the flocs size increased. Statistical analysis shows that mean flocs size has a strong negative correlation (r² = -0.914, p = 0.00) with fractal dimensions. That means the reduction in mean flocs size is associated to a more compact flocs structure. This is probably due to the fact that the small flocs, i.e. dispersed bacteria and small colonies have a higher density than the large flocs with more bridging between biopolymers. According to the Carman-Kozeny equation for cake layer filtration, for a given porosity and biomass amount, specific resistance of cake layer is controlled by flocs size. This study revealed another important contributor of the specific resistance of cake layer. Compact flocs have been reported to yield a denser cake layer (Lencki & Riedl 1999). According to this, the higher fractal dimension of small flocs would certainly provide additional resistance to the cake layer.

**Microbial community**

Figure 4 compiles the DGGE profiles of the small, large flocs fraction sludge and cake layer sludge sampled at stable stage of operation. The fractions of small flocs and large flocs showed significant differences in microbial community structure. For example, some prominent bands (bands 6, 7, 8, 9 and 11) were detected in the fraction of small flocs while those were not detectable in the fraction of large flocs. In contrast, some populations represented by bands 1, 2, 13, 16, 17, 18, 19 and 20 almost disappeared in the fraction of small flocs compared to the fraction of large flocs. This result indicated that floc size significantly affects the microbial diversity. This may be explained by the different
micro-environment due to floc size which results in the development of different microbial populations. It is clear from Figure 4 that bands 6, 7, 8, 9, 11 were prominent in both small flocs and cake layer sludge lanes. Cluster analysis of 16S rDNA profiles of bacterial communities showed that the similarity of communities between small flocs and cake layer (91%) was larger than that between large flocs and cake layer (73%). Considering small flocs sludge only accounted for a small fraction (3% for the flocs with size <5 μm) of total amount of bulk sludge, the results indicate selectively attachment of small flocs on membrane surfaces occurred.

Previous studies (Verhoef et al. 2002; Zhang et al. 2006) have suggested some bacterial strains would produce large amount of EPS materials. It was also reported that some bacteria, like Acinetobacter, may play an important role in bridging different bacterial populations in suspended growth activated sludge systems (Malik et al. 2003). This study revealed that small flocs had higher level of bound EPS, denser structure, and a very different microbial community. In above context, there seems to exist specific bacterial populations being responsible for producing more EPS and presenting more bridging in small flocs. These bacteria may play a pioneering role in sludge attachment on membrane surfaces as they have more bound EPS. This result implies that membrane fouling control in SAnMBR systems should focus on these bacterial populations rather than the total microbial community in the bulk sludge. From the comparison of bacterial communities between small flocs and large flocs, it seems that the bacterial populations indicated by bands 6, 7, 8, 9 and 11 took these roles. In order to make a better understand of membrane fouling and develop effective fouling control strategies, further studies regarding identify of these bacterial populations and their ecology and physiology are needed.

Role in membrane fouling

Based on the filtration resistance estimation and examination of the characteristics of different floc fractions, the following membrane fouling mechanism underlying the role of the different floc fraction in SMBR systems can be summarized.

Cake layer formation was the main cause of membrane fouling. There are two opposite actions that control the rate of cake formation: permeation drag and back transport (Belfort et al. 1994). All sludge constituents are attached to the membrane surface by permeation drag, but are also detached by back transport. A decrease in particle size itself would decrease back transport velocity of the particles and thus increase cake formation. Besides their size, the small flocs had higher level of bound EPS, denser structure, and a very different microbial community. The richer bound EPS and denser structure would be the results of the existence of specific bacterial populations in small flocs. These bacteria appeared to be responsible for producing more EPS and presenting more bridging in small flocs, which result in the selectively attachment of small flocs on membrane surface, and present a denser cake layer. The findings in this study expand the insights into the role of different microbial fractions in membrane fouling, and imply that membrane fouling control in SAnMBR systems should focus on these specific bacteria.

CONCLUSIONS

This study investigated characteristics of different fractions of microbial flocs and their role in membrane fouling. Based on
the results presented in this study, the following conclusions can be drawn:

- Specific filtration resistance of small flocs fraction was found to be higher than that of large flocs fraction and bulk sludge liquor, and comparable to that of cake layer.
- The small flocs had higher bound EPS and fractal dimension compared to large flocs and bulk sludge.
- The small flocs and large flocs had different microbial communities, indicating that floc size significantly affects the microbial diversity. Some specific bacterial populations appeared to be responsible for producing more EPS and presenting more bridging in small flocs. It, therefore, would be desirable to identify these bacterial populations and understanding their ecology and physiology for establishing a general understanding of membrane fouling and develop effective fouling control strategies.

The findings from this study suggest that the difference in sludge characteristics (bound EPS, fractal dimension, and microbial community) provides new insight in difference of membrane fouling potential between smaller flocs and larger flocs fraction.

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