Biofouling of an Aerated Membrane Reactor: Four Distinct Microbial Communities

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Abstract

Membrane fouling is a significant operational challenge in the application of membranes in drinking and wastewater treatment. Microbial association with the surfaces of membranes and the extracellular polymeric substances (EPS) associated with biofilms contribute to fouling. Within the microbial community, there are polysaccharide producers and consumers. A better understanding of the microbial community at the interfaces of the membrane material will be important in supporting next-generation cleaning strategies. A laboratory-scale mesophilic-aerated membrane bioreactor using sheet polyvinylidene fluoride microfiltration membrane was used as a model system. The microbial community was characterized from four distinct compartments: mixed liquor suspended biomass (MLSB); the membrane cake (i.e., extramembrane loosely bound [EMLB]); the foulant layer (i.e., extramembrane tightly bound [EMTB]), and the intracore membrane biofilm (ICMB). Electron microscope images revealed an established biofilm in the ICMB. Microbial structures were observed in the macrovoids of the asymmetric membrane and the open architecture of the support web. This is the first study to acknowledge the presence of ICMB. The community structure of all four compartments (i.e., MLSB, EMLB, EMTB, and ICMB) was characterized at the genus level and results indicate four distinct microbial communities exist. Composition of the ICMB compartment most closely aligns with MLSB and is distinct from the community found in the contiguous EMTB compartment. Results identify the need to characterize the microbial community, and conditions for growth and EPS production, in both the EMTB and ICMB compartments to understand irreversible fouling. Biofilm characterization in the EMLB compartment is insufficient.

Keywords: biomass compartmentalization; extramembrane and intramembrane biofilms; flocs; loosely and tightly bound biofilms; membrane bioreactor; suspended and attached biomass

Introduction

MEMBRANES ARE USED extensively in wastewater treatment for biosolids/effluent separation in conjunction with suspended or hybrid growth systems. Membrane bioreactors (MBRs), which incorporate both suspended growth (SG) and a membrane-based biofilm (i.e., attached growth [AG]), outperform conventional activated sludge facilities in terms of effluent quality, reliability, reduced footprint, and lower sludge production (Trussell *et al.*, 2005; Judd, 2006). The biofilm functionally contributes to the removal of nutrients (Meng *et al.*, 2009; Kumar *et al.*, 2010). The Inter-

national Union of Pure and Applied Chemistry (IUPAC) working party on membrane nomenclatures defines “fouling” as “the process resulting in a loss of performance of a membrane due to deposition of suspended or dissolved substances on its external surfaces, at its pore opening or within its pores” (Koros *et al.*, 1996). Fouling is more than particle attachment as biofilm growth occurs wherever there is sufficient substrate.

Membrane fouling decreases the flux and increases in the transmembrane pressure are needed to maintain a constant effluent flux. Fouling results in increased costs and operational restrictions and is a major disadvantage for membrane technologies. Much effort has been spent on characterizing and managing fouling in MBRs (Piasecka *et al.*, 2012; Guo *et al.*, 2015). Fouling consists of a cake layer that can be removed mechanically and an irreversible foulant chemically bound to the membrane. Traditionally, the membrane foulant

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is considered a black box completely ignoring its complex biology (Malaeb *et al.*, 2013). Mitigation of fouling occurs using physical (e.g., backwashing, backpulsing, and air sparging) and chemical (e.g., acids, bases, oxidants, chelating agents, polymeric coagulants, and surfactants) cleaning methods that often fail to adequately control biofouling (Meng *et al.*, 2017). Next-generation strategies will be situational specific going beyond physical/chemical treatments and may include those altering the community structure [e.g., quorum quenching (Bouayed *et al.*, 2016); enzymatic disruption; energy uncoupling; cell wall hydrolysis; and the use of microbial predation and bacteriophages (Malaeb *et al.*, 2013)].

Extracellular polymeric substance (EPS) is composed of carbohydrates (polysaccharide [PS]), proteins (PN), nucleic acids, lipids, and humic substances. The primary components are carbohydrates and proteins (75–90%) (More *et al.*, 2014). EPS secretion is influenced by the physical, chemical, and biological conditions of the growth medium. Growth conditions influencing EPS production are: carbon to nitrogen (C/N ratio) (Han *et al.*, 2015), pH and temperature of the incubation media (Dogan *et al.*, 2015), and aeration intensity (Ma *et al.*, 2012). EPS composition is influenced by a complex and symbiotic relationship between EPS-producing, and non-EPS-producing cells. EPS can be degraded by bacteria as an energy source during substrate shortages (More *et al.*, 2014).

Meng *et al.* (2017), as a consequence of a literature review, holds that EPS PSs are the primary membrane foulant in MBR systems. In addition to the large size of PSs, their gelling properties strongly contribute to their high fouling propensity. Many investigations have focused on identifying bacteria-producing PSs (More *et al.*, 2012; Shahnavaz *et al.*, 2015) or characterizing the PSs involved in biofilms (Czaczky and Myszka, 2007).

Traditionally, fouling studies have characterized the cake and a portion of the irreversible fouling in support of cleaning strategy development. This study examines the EPS and the bacterial community at a higher spatial resolution. Four compartments (Fig. 1) are considered: mixed liquor suspended biomass (MLSB), extramembrane loosely bound (EMLB), extramembrane tightly bound (EMTB), and intracore membrane biofilm (ICMB). Inclusion of the ICMB

compartment is novel to this study. The bacterial community was characterized at the genus level thereby identifying if the communities in each compartment were distinct. In addition, in-depth community analysis can further our understanding of fouling mechanisms.

Materials and Methods

Sludge production as a function of COD:N:P ratio was investigated experimentally using an MBR (Hao and Liao, 2015; Hao *et al.*, 2016) and this study further characterizes the membranes and SG.

Flat sheet polyvinylidene fluoride microfiltration membranes (Shanghai SINAP Membrane Science & Technology Co., Ltd., China) were used in module construction. The membrane is characterized as having a pore size of 0.3 μm (Hao *et al.*, 2016). The operating cycle was 4-min permeation followed by 1 min of relaxation. As an operating strategy, an applied pressure was varied to maintain a constant flux of $10 \pm 0.5 \text{ L/min}$. During normal operation, transmembrane pressure (TMP) was 3.5 KPa with excursions as high as 12 KPa. At the conclusion of the study, the TMP was 9 KPa.

Sample preparation

Mixed liquor suspended biomass. A 100 mL aliquot of mixed liquor (ML) was taken from the daily wastage and used either immediately or stored at 4°C for up to 24 h. A subsample of 50 mL was centrifuged at 9,000 g for 10 min at 4°C. The pellet was washed twice with the final pellet becoming MLSB. The washing procedure entailed centrifuging 50 mL at 9,000 g for 10 min at 4°C, decanting the sample, and replacing the decant with an equal volume of 0.1 M phosphate saline buffer (pH 7.0).

Extramembrane loosely bound. The membrane was removed from the module and $\sim 15 \text{ cm}^2$ was cut from the center of the membrane module to collect the representative sample. The membrane sample was submerged in a 100 mL of 0.1 M phosphate saline buffer, in a 200 mL centrifuge tube, and vortexed for 2 min (Vortex-Genie 2; Fisher). The membrane was removed and the solution containing the biomass

FIG. 1. Composite membrane with compartments identified.

slurry was poured into a 100 mL centrifuge tube. The tube was centrifuged at 9,000 *g* for 10 min. The pellet was resuspended in 10 mL of 0.1 M phosphate saline and identified as EMLB.

Extramembrane tightly bound. After removal of EMLB, the membrane was scraped using a clean glass microscope slide. The membrane surface was viewed using scanning electron microscope (SEM) and confocal laser scanning microscope (CLSM) to confirm the entire biofilm profile had been removed. The scrapings were resuspended in 10 mL of phosphate saline buffer and centrifuged at 9,000 *g* for 10 min at 4°C. After decanting, the pellet was resuspended and centrifuged once more under the above conditions. The final pellet was identified as EMTB.

Intracore membrane biofilm. After completely removing EMLB and EMTB from the surface, the membrane was placed in a 100 mL of phosphate saline buffer in a 150 mL beaker. The beaker was placed in an ultrasonic cleaner (Branson Model 2510R-MT; Branson Ultrasonic) for 30 min to remove foulant, if present. After this treatment, the membrane was rinsed in sterile water and layered on a paper towel for 5 min to remove excess water.

The membrane was cut into small pieces using sterilized surgical scissors and stored at -80°C. Samples were frozen using liquid nitrogen and ground to a powder using a chilled mortar and pestle. The powder was used as a representative of IMCB.

Microbial ecology

The DNA was extracted from all four biomass samples with soil DNA extraction kit (Mo Bio Laboratories, Inc.) and was amplified using PCR for amplicon preparations. The V3–V4 region of the 16S rRNA gene was amplified using primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACH VGGGTATCTAATCC), with sample-specific barcodes. The community DNA was used for the deep sequencing-based microbial diversity analyses. Extracted community DNA samples (from MLSB, EMLB, EMTB, and IMCB), in 20 μ L aliquots, were sent to the Genome Quebec Research and Testing Laboratory (Montreal, Quebec, Canada) for 16S rRNA gene sequencing (MiSeq; Illumina) and analyses.

Bioinformatics analyses

16S rRNA gene sequences were used to generate an operational taxonomic unit (OTU) table and a corresponding FASTA file. These files were generated from the rRNA Tagger companion pipeline. Taxonomy assignment of OTUs was performed using the Ribosomal Database Project (RDP) classifier with a modified Greengenes training set built from a concatenation of the Greengenes database. Then, from these classified OTUs, diversity metrics were obtained by aligning OTU sequences on a Greengenes core reference alignment (DeSantis *et al.*, 2006) using the PyNAST aligner (Price *et al.*, 2010). Alignments were filtered to keep only the hypervariable region part of the alignment. A phylogenetic tree was then built from that alignment with FastTree (Price *et al.*, 2010). Alpha (observed species) and beta (weighted, unweighted UniFrac, and Bray–Curtis distances) diversity metrics, taxonomic classifications, UPGMA clustering, and

OTUs heatmap were computed using the QIIME software suite (DeSantis *et al.*, 2006). Sequence data were deposited at the European Nucleotide Archive (ENA) (accession name: PRJEB13415; www.ebi.ac.uk/ena/data/view/PRJEB13415).

Microscopy and imaging

Scanning electron microscopy. Virgin and fouled membranes (~10×10 mm) were generated by cutting the membrane with a sterile surgical scalpel. Top and lateral views allowed visualization of the skin and intramembrane, respectively. Three samples were taken and prepared for each view. Samples were fixed by submerging in 2% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.0) for 2 h. The fixed samples were washed thrice; washing consisted of immersing the fixed samples in 0.1 M phosphate buffer. Then samples were post-fixed in 1% OsO₄ for 30 min, washed with buffer twice, and dehydrated with a graduated series of ethanol solutions (50%, 70%, 80%, 90%, neat, neat, neat). Samples were critical point dried and then mounted on carbon tape and sputter coated in 20 nm gold with an Emitech K550 Sputter Coater. A Hitachi S-2300 SEM (Tokyo, Japan) was used to capture micrographs. All images were acquired digitally and analyzed using URSA 200 software (Mektech, Inc., Hillsdale, NJ).

Confocal laser scanning microscopy. Small flat pieces of each fouled membrane (~10×10 mm) were cut from the membrane module and directly stained. A cocktail of fluorescent stains concanavalin A (Con-A; Life Technologies), calcofluor white (Sigma-Aldrich, St. Louis, MO), and fluorescein isothiocyanate were used for distribution and localization of α -PS, β -PS, and protein, respectively (Aqeel *et al.*, 2016). Propidium iodide and syto-9 (Life Technologies) stains were used for visualizing dead and live cells, respectively, on the biofoulants. Working stain solutions were prepared in 0.1 M phosphate-buffered saline (pH 7.0). Probes were made from stock solutions by dissolving in filter-sterilized (pore size, 0.20 μ m) 0.1 M phosphate-buffered saline except DNA stains, which were prepared in DNase-free water. A working solution contained each of the five probes at a concentration of 5 mg/L. Approximately 1 mL of the solution was applied to the membrane samples. The samples were incubated in the dark at room temperature (22°C ± 2°C) for 30 min. The samples were carefully rinsed with 0.1 M phosphate buffer (pH 7.0) twice to remove unbound probes.

Immediately after the staining procedure, the biofilms were analyzed using CLSM (Zeiss LSM 710) system with three different visible light lasers, covering six excitation wavelengths. The excitation and emission spectra of fluorophores were as identified by Mahendran *et al.* (2012). Different objective lenses (i.e., 10× and 20× water immersion and 63× oil immersion lens) were used for imaging. The microscope had a spectrophotometer for each detector channel allowing the design of custom filters to maximize sensitivity and minimize crosstalk and record emission spectra. The microscope was set up by examining the sample for bright objects, and photomultiplier tube settings (gain and black level) were adjusted to provide full-range pixel values. The pinhole for each scanned channel was adjusted to scan all channels with an identical optical slice thickness during imaging. By setting a threshold, pixels with intensity below the threshold were assigned to the background. Z-stack images

were obtained beginning at the top of the biofilm with optical slices taken every $4\text{ }\mu\text{m}$ through the biofilm surface. Signals were recorded using an appropriate excitation and emission lengths as described previously (Aqeel *et al.*, 2016). The images were processed to measure the intensity of surface area covered by cells, and proteins and PSs using the confocal assistant software supplied by the manufacturer (ZEN 2012) (Mahendran *et al.*, 2012; Aqeel *et al.*, 2016).

Results and Discussion

Performance data

This study made use of ML and membranes generated by (Hao and Liao, 2015; Hao *et al.*, 2016) and detailed in associated publications. Operating conditions key to understanding the microbial community is the operating temperature (35°C) and glucose feed ($2,500\text{ mg COD/L}$). The ML concentration and sludge age was $8,450 \pm 325\text{ mg/L}$ and 15 days, respectively. The reactor was operated under a constant flux of $2.8 \times 10^{-6}\text{ m/s}$. The TMP baseline was $\sim 3\text{ kPa}$ with maximum values of 12 kPa (Hao and Liao, 2015). The system's resistance is variable because of the non-Newtonian behavior of the ML [i.e., the viscosity of the slurry changes with the applied shear (Zsirai *et al.*, 2012)]. The viscosity ranges from 0.16 to $0.43\text{ mPa}\cdot\text{s}$ as calculated from empirical correlations that consider both temperature and solids concentration (Psoch and Schiewer, 2008; Yang *et al.*, 2009). Based on baseline and maximum TMP measurements, fouling accounts for $\sim 75\%$ of the overall system resistance.

Microscopic analysis

Composite membranes, an asymmetric membrane bonded to an open-architecture support web, are used in this study. The asymmetric membrane and the support web are oriented

(Hao and Liao, 2015) toward the wastewater and permeate side, respectively. Images of the virgin membrane show the top as an irregular network of comparably sized pores (Fig. 2a). A cross-sectional image identifies a network of large intracconnected teardrop voids with their opening oriented toward the wastewater/membrane interface (Fig. 2e). The integrity of some pores was compromised by breakage (Fig. 2c).

In the EMTB compartment, a compact biofilm with individual cells visible is adjacent to the membrane skin (Fig. 2b). Bacterial cells are predominantly rods (length $0.5\text{--}1.0\text{ }\mu\text{m}$) and spheres (diameter $0.25\text{--}0.5\text{ }\mu\text{m}$). Within the IMCB compartment, a spatially varying biofilm exists. When viewed from the membrane's skin, growth is seen within the opening of the macrovoids (Fig. 2g). Close to the EMTB–IMCB interface, a compact biofilm exists with individual cells being visible. A cross-sectional view of the macrovoids shows that further into the void a diffuse biofilm dominated by EPS exists (Fig. 2f). Higher SEM resolution images of the support-web identify patchy biofilm growth (Fig. 2d). The morphology of the biofilm in the IMCB differs spatially suggesting local changes in substrate and/or hydraulic conditions.

Growth in the ICMB compartment could be a significant source of resistance as partial blockage of the macrovoids and the support web may cause preferential flow paths. In addition, episodic detachment of the ICMB biofilm could explain previously puzzling fluctuations in permeate quality. An operational change from relaxation to backwashing could impact IMCB character and its fouling properties.

The pore size at the wastewater–membrane interface is $\sim 0.3\text{ }\mu\text{m}$ and would be expected to exclude bacteria from entry into the membrane. Growth within the membrane is likely owing to ultramicrobacteria (i.e., bacterial cells with a diameter $<0.3\text{ }\mu\text{m}$) (Torrella and Morita, 1981) and

FIG. 2. Scanning electron microscope images of membrane used in aerobic submerged MBR (a) virgin membrane as seen from wastewater/biofilm interface; (b) compactly packed biofilm in the EMTB compartment; (c) virgin support web; (d) arrow indicates bacteria present on the permeate side of the support web; (e) cross-sectional view of macroporous voids located below the membrane skin and microcolonies of bacteria; (f) high-resolution image [highlighted area of image (e)] showing biofilms attached on macroporous voids. (g, h) Cross-section view of used membrane after cake layer removal from outer membrane skin showing macroporous voids exists below the membrane skin, and microcolonies of bacteria were present within voids. EMTB, extramembrane tightly bound; MBR, membrane bioreactor.

TABLE 1. EXTRACELLULAR POLYMERIC SUBSTANCES COMPOSITION AND ENUMERATION OF LIVE AND DEAD CELLS DETERMINED USING FLUORESCENT/CONFOCAL LASER SCANNING MICROSCOPE

Compartment	PN	PS	PN:PS	Live:Dead
MLSB	15.16	1.11	14.11	3.56
EMLB	28.48	4.28	6.65	1.13
EMTB	35.91	34.78	1.03	1.56

EMLB, extramembrane loosely bound; EMTB, extramembrane tightly bound; FL, fluorescent; MLSB, mixed liquor suspended biomass; PN, protein; PS, polysaccharide.

ultramicrocells [bacteria possessing a volume of $<0.1 \mu\text{m}^3$ and a small size of genome (from 3.2 to 0.58 Mb) (Duda *et al.*, 2012)]. They are formed during the starvation–survival process. Ultramicrocells were isolated from drinking water systems and identified as belonging to cultured and uncultured members of *Proteobacteria*, *Actinobacter*, and *Firmicutes* (Silbaq, 2009). Ultramicrocells were detected after filtration of a municipal secondary effluent through track-etched membranes with pore diameters of 0.2, 0.1, and $0.05 \mu\text{m}$ (Sadar Ghayenia *et al.*, 1999). The cells were unculturable on standard basal microbiological agar. Gram-negative model bacteria, SW8, was filtered in parallel with the secondary effluent. The viability of the bacteria was evaluated based on electron chain activity. Viability of SW8 was $\sim 50\%$ after filtration. Field emission SEM provided evidence for entrapment of bacteria within the membrane matrix.

EPS composition was characterized using Fluorescent/CLSM (Table 1). EPS composition is affected by sludge age with higher PN:PS ratios corresponding with lower sludge age (Silva *et al.*, 2016). The ranking from highest to lowest PN:PS ratio is: EMTB, EMLB, and MLSB compartments. The trend is consistent with values reported for both SG and AG systems (Mahendran *et al.*, 2012; Silva *et al.*, 2016). MLSB is expected to have a lower sludge age than biofilm compartments because of the substrate and oxygen availability. Substrate diffusion decreases across the biofilm and thus EMLB is expected to have a lower sludge age than EMTB. Experimentally derived PN:PS are consistent with this hypothesis.

Communities analysis

Alpha diversity, within sample species richness, was determined using a rarefaction curve (Fig. 3). Alpha diversity values, in ranked order of most to less diverse, are as follows: IMCB, EMLB, MLSB, and EMTB. The beta diversity between compartment was characterized using the unweighted UniFrac (i.e., based on species richness) and weighted UniFrac (i.e., based on species richness and abundance), respectively. The unweighted UniFrac finds the diversity of IMCB comparable with EMLB (Supplementary Fig. S1a). The weighted UniFrac denotes the beta diversity of the ICMB/MLSB, and EMTB/EMLB compartments as comparable (Supplementary Fig. S1b). Both alpha and beta diversity values indicate IMCB is most comparable with MLSB rather than the contiguous EMTB compartment.

FIG. 3. Alpha diversity as shown by rarefaction curve.

The contribution of four phyla to the total richness (Supplementary Fig. S2) was examined and in all cases, ~250 OTU were common to all four compartments. Analysis of MLSB and EMLB compartments identified almost 60% of the OTU as common. A comparison between EMLB and EMTB compartments found 50% and 74% of the compartment total, respectively, as common. In the final comparison, common OTU accounts for 85% and 50% of the total for EMTB and ICMB, respectively.

Bacteria from seven phyla comprise 73–95% of the microbial community of the four compartments: *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Tenericutes*, *Proteobacteria*, and TM7 (now Candidate phylum *Saccharibacteria*) (Fig. 4). The composition of the MLSB compartment is distinct from both EMTB and EMLB (Fig. 4). Many researchers have made this same observation previously (Pia-secka *et al.*, 2012; Gao *et al.*, 2013; Silva *et al.*, 2016). *Proteobacteria* (mostly α -, β -, and γ -*Proteobacteria*) are one of the most abundant bacterial groups in wastewater systems (Kwon *et al.*, 2010). In the MLSB compartment, this phylum is predominant, whereas in EMLB and EMTB compartments, it accounts for only 35% of the population and the *Bacteroidetes* phylum is predominant (42–51% of population). The phylum *Chloroflexi* is only present at appreciable levels (i.e., >1%) in the MLSB compartment. In the MLSB compartment, the distribution of *Proteobacteria* is as follows: α (30%), β (25%), and γ (3%). The distribution for EMTB/EMLB and ICMB is as follows: α (15%), β (13–15%), and γ (7–10%) and α (30%), β (13–15%), and γ (10%), respectively.

Environmental conditions (i.e., temperature and dissolved oxygen) of this study are comparable with those of the mill treatment pond inoculum. The microbial community of an MABR treating synthetic paper mill wastewater was identified as belonging to α -*Proteobacteria*, β -*Proteobacteria*, and γ -*Proteobacteria*, the *Cytophaga-Flavobacterium-Bacteroides* clade, and an uncultured bacterium of division TM7 were identified (Zhang *et al.*, 2006). Several waterborne bacteria can cause diseases in human beings (e.g., *Legionella* and *Campylobacter*). However, the growth of these bacteria is suppressed in engineered and natural aquatic systems because of environmental and operational conditions (McKay, 1992). In this study, the dominant bacterial genera are non-pathogenic.

Proteobacteria (mostly α -, β -, and γ -*Proteobacteria*) are one of the most abundant bacterial groups in wastewater system (Kwon *et al.*, 2010). Community composition is affected by dissolved oxygen (DO) (Ma *et al.*, 2012). Experimental evidence was generated by two MABR that differed only in the operating DO. The first reactor with high aeration (RH) was operated under a relatively high DO of 3–5 mg/L, whereas the second reactor with low aeration (RL) was operated under a lower DO (1–2 mg/L). The main subgroups of *Proteobacteria* present in both reactors, in order of decreasing abundance are as follows: β -*Proteobacteria*, α -*Proteobacteria*, δ -*Proteobacteria*, γ -*Proteobacteria*, and ϵ -*Proteobacteria*. In reactor RH, β -*Proteobacteria* and γ -*Proteobacteria* decreased in abundance by 41.5% and 66.6%, respectively compared with RL. The investigators

FIG. 4. Community analysis of each compartment.

TABLE 2. GENUS-LEVEL CHARACTERIZATION RESULTS IN MIXED LIQUOR SUSPENDED BIOMASS, EXTRAMEMBRANE LOOSELY BOUND, EXTRAMEMBRANE TIGHTLY BOUND, AND INTRACORE MEMBRANE BIOFILM COMPARTMENTS

<i>Phylum</i>	<i>Class</i>	<i>Order</i>	<i>Family</i>	<i>Genus</i>	<i>Compartment</i>			
					<i>MLSB</i> (%)	<i>EMLB</i> (%)	<i>EMTB</i> (%)	<i>ICMB</i> (%)
Actinobacter	Actinobacter	Actinomycetales	Bogoriellaceae		1	2	0	3
Actinobacter	Actinobacter	Actinomycetales	Microbacteriaceae	<i>Subtercola</i> spp.	6	1	0	0
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	<i>Dysgonomonas</i> spp.	6	40	14	12
Actinobacter	Actinobacteria	Actinomycetales	Flavobacteriaceae		1	5	41	9
Chloroflexi	Anaerolineae				11	1	0	0
Firmicutes	Bacilli	Bacillales	Listeriaceae		0	0	2	0
Firmicutes	Clostridia	Clostridiales	Symbiobacteriaceae		1	0	0	2
Proteobacteria	α -Proteobacteria		Bradyrhizobiaceae	<i>Blastobacter</i> spp.	0	0	0	4
Proteobacteria	α -Proteobacteria	Caulobacterales	Caulobacteraceae	<i>Caulobacter</i> spp.	1	1	1	1
Proteobacteria	α -Proteobacteria	Rhizobiales	Cohaesibacteraceae		7	2	1	1
Proteobacteria	α -Proteobacteria	Rhizobiales	Methylocystaceae	<i>Pleomorphomonas</i> spp.	3	0	1	13
Proteobacteria	α -Proteobacteria	Rhodospirillales	Rhodospirillaceae	<i>Rhodocista</i> spp.	18	3	4	1
Proteobacteria	α -Proteobacteria	Rhizobiales	Xanthobacteraceae	<i>Xanthobacter</i> spp.	7	4	1	2
Proteobacteria	α -Proteobacteria				0	3	1	4
Proteobacteria	β -Proteobacteria	Burkholderiales	Comamonadaceae	<i>Caldimonas</i> spp.	0	0	4	0
Proteobacteria	γ -Proteobacteria	Oceanospirillales	Litnicolaceae		2	2	5	3
Proteobacteria	γ -Proteobacteria	Enterobacteriales			1	5	5	7
TM7	TM7-Group 1				7	3	1	4
TM7	TM7-Group 3				7	12	8	16
Unclassified bacteria					13	3	2	2
Identified minor contributors (<1%)					2	6	3	4

Procabacteriaceae belonging to the β -Proteobacteria class has been excluded, and revised abundance numbers net of its contribution have been calculated.

infer, based on the identification of members of two subgroups as pioneers of surface colonization (Jinhua *et al.*, 2006; Miura *et al.*, 2007; Meng *et al.*, 2009; Lim *et al.*, 2012), that reductions in these groups result in the reduced fouling experienced in reactor RH.

Gao *et al.* (2011) used an anoxic/aerobic MBR to investigate the impact of DO on fouling. Aeration was constantly adjusted to maintain the DO at the setpoint value (4.0, 2.0, and 0.5 mg/L). The authors concluded EPS generated on the surface of the membrane is mostly responsibility for fouling, especially under low DO conditions. The AG had a higher PN:PS ratio than did the SG, thereby suggesting protein as a major cause of fouling. The community composition of the AG, as identified through 16s rDNA gene community fingerprint (denaturing gradient gel electrophoresis [DGGE]), was more sensitive to DO changes than the SG. *β-Proteobacteria* was identified as a major contributor to irreversible fouling. At higher aeration rates, *β-Proteobacteria* became the most dominant fouling bacteria (Miura *et al.*, 2007).

More in-depth analysis was completed with characterization being achieved at the genus level and higher (Table 2). *Procabacteriaceae* spp. accounted for 88% of *β-Proteobacteria* in the MLSB compartment. In MAC, *Procabacteriaceae* spp. accounted for 9–13% of the *β-Proteobacteria*. *Procabacteriaceae* spp. belongs to the *β-Proteobacteria* class and lives synergistically within its amoebae host (e.g., *Acanthamoeba*), which can be metabolically active or exist as a stress-induced cyst (Barker and Brown, 1994). To provide an equitable basis for comparison between compartments, comparisons are based on revised abundance values (RAV) which are net of the *Procabacteriaceae* spp. contribution.

The MLSB compartment is composed of aerobic bacteria (e.g., *Rhodocista* spp., members of *Cohaesibacteraceae*, *Xanthobacter* spp., members of *Flavobacterium*, *Subtercola* spp.), facultative anaerobes (e.g., members of *Cohaesibacteraceae*, members of *Flavobacterium*, *Dysgonomonas* spp.), chemolithotrophic (e.g., *Xanthobacter* spp.), and obligate anaerobes (e.g., *Chloroflexi Anaerolineae*). *Anaerolineae*, a class in the phylum *Chloroflexi* (Yamada and Sekiguchi, 2009), was present in the MLSB compartment in moderate amounts (14%) and nearly absent in all other compartments ($\leq 1\%$). The presence of obligate anaerobes in moderate proportions suggests regional oxygen deficits possibly caused by the high oxygen demand of the rapidly biodegradable glucose substrate. Flocs range in size between 1 and 300 μm (Hao and Liao, 2015).

TM7 when present in complex microbial communities may exhibit pleomorphic characteristics (i.e., the ability of some microorganisms to alter their shape or size in response to environmental conditions). TM7 is divided into three subdivisions: subdivision 1—filamentous morphology (Group 1); or subdivisions 2–3—nonfilamentous morphology (e.g., rods or cocci) (Groups 2–3) (Hugenholtz *et al.*, 2001). TM7 has been isolated from wastewater treatment plants in Denmark (Group 1) and Japan (Group 3) with relative abundances of $\sim 3\%$ (Kindaichi *et al.*, 2016). Phylogenetic analysis of previously isolated activated sludge clones identifies them as subphyla I and III (Yamada and Sekiguchi, 2009). Surveys of full-scale treatment plants have identified both subphyla as present with the dominant subphylum being site specific (Bjornsson *et al.*, 2002).

In the MLSB compartment, subphylum 1 and subphylum 3 members equally contribute to the overall value of 7% based

on RAV. The ratio of subphylum 1:subphylum 3 in other compartments are as follows: EMLB (1:4), EMTB (1:8), and ICMB (1:3.8). Total abundance values for the EMLB, EMTB, and ICMB compartments are 15%, 9%, and 19%, respectively. Literature values of 3% (Kindaichi *et al.*, 2016) are lower than this study's values of 9–19% based on RAV and the 8–18% based on total values. TM7 may play an important role in biofilm structure and results of this study suggest it may be underreported.

Dysgonomonas spp. includes five species including strain WJDL-1 that exhibits ligninolytic activity under facultative anaerobic conditions (Duan *et al.*, 2016). It was isolated in the laboratory under environmental conditions comparable with those of this study (pH 6.8, COD:N ratio of 6, 33°C). The genus is capable of degrading wheat straw, alginate (i.e., a PS), starch, and acetate. In the MLSB compartment, *Dysgonomonas* spp. accounts make a marginal contribution, 7–8% of the RAV, whereas in the EMLB compartment, it dominates the biofilm, accounting for 40% of the RAV. In the other biofilm compartments, EMTB and ICMB, it accounts for 14% and 12% the RAV, respectively.

Possible linkages between bacteria–substrate–EPS production/degradation are shown in a conceptual model (Weissbrodt *et al.*, 2014). It identifies *Sphingomonadaceae*, *Xanthomonadaceae*, and *Rhodocyclaceae* families, along with *Competibacter* and *Rhizobiales* as EPS producers. *Rhizobiales* are present in the MLSB, EMLB, EMTB, and ICMB at the following levels based on RAV: 17%, 4%, 3%, and 16%, respectively. Levels in the MLSB and ICMB compartments are comparable and higher than other compartments. *Flavobacterium*, a clade of *Flavobacteriaceae*, has been characterized as floc-forming bacteria producing EPS to bind cells together (Gerardi, 2002). *Flavobacteriaceae* members dominate the EMTB compartment (41%), whereas in other compartments (MLSB, EMLB, and ICMB) the family is present in low proportions (1–9% of RAV). Numerous members of the family *Flavobacteriaceae* are adept at the digestion of macromolecules such as proteins and polymers.

Weissbrodt *et al.* (2014) developed a conceptual model to describe a granular sludge ecosystem originating from reactors operated for full biological nutrient removal under alternating aerobic and anaerobic conditions. It is a useful framework for examining the current system. Protein hydrolysis was carried out by *Chloroflexi* and TM7 under all conditions, and *Sphingobacteriales* and *Cytophaga* under aerobic conditions. Polysaccharide hydrolysis is carried out by the same consortium of bacteria with the exclusion of TM7 and addition of *Tetrasphaera*. Based on the microbial consortium of this experimental system, it is proposed that *Dysgonomonas* spp. be considered as capable of PS degradation.

Conclusions

This study illustrates why cleaning strategies should not be based on the characterization of membrane cake (EMLB). The one size fits all approach while easier is invalid. Novel to this study is the report of biofilm formation within the membrane (ICMB). The morphology of the biofilm differs spatially thereby suggesting further resolution of the communities. The community analysis performed suggests it is distinct from growth in all other compartments but closest to MLSB.

EPS, and PSs in particular, is considered to be the primary contributor to overall fouling in MBRs (Meng *et al.*, 2017). Community analysis at the genus level can provide information on the presence and absence of PS producers and degraders. In this study, there are two noteworthy observations. In the EMLB compartment, *Dysgonomonas* dominates 40% of the RAV. Contributions in all other compartments are 7–14%. *Flavobacterium*, a clade of *Flavobacteriaceae*, has been characterized as floc-forming bacteria producing EPS to bind cells together (Gerardi, 2002). *Flavobacteriaceae* members dominate the EMTB compartment (41%), whereas in other compartments (MLSB, EMLB, and ICMB) the family is present in low proportions (1–9% of RAV). Numerous members of the family *Flavobacteriaceae* are capable of degrading polymeric substrates, including proteins and polymers.

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Author Disclosure Statement

The authors declare that the research is in compliance with ethical standards, and they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2

References

- Aqeel, H., Basuvaraj, M., Hall, M., Neufeld, J.D., and Liss, S.N. (2016). Microbial dynamics and properties of aerobic granules developed in a laboratory-scale sequencing batch reactor with an intermediate filamentous bulking stage. *Appl. Microbiol. Biotechnol.* 100, 447.
- Barker, J., and Brown, M.R.W. (1994). Trojan Horses of the microbial world: Protozoa and the survival of bacterial pathogens in the environment. *Microbiology* 140, 1253.
- Bjornsson, L., Hugenholtz, P., Tyson, G.W., and Blackall, L.L. (2002). Filamentous Chloroflexi (green non-sulfur bacteria) are abundant in wastewater treatment processes with biological nutrient removal. *Microbiology* 148, 2309.
- Bouayed, N., Dietrich, N., Lafforgue, C., Lee, C., and Guigui, C. (2016). Process-oriented review of bacterial quorum quenching for membrane biofouling mitigation in membrane bioreactors (MBRs). *Membranes* 6, 1.
- Czaczyk, K., and Myszk, K. (2007). Biosynthesis of extracellular polymeric substances (EPS) and its role in microbial biofilm formation. *Polish J. Environ. Stud.* 16, 799.
- DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72, 5069.
- Dogan, N., Doganli, G., Dogan, G., and Bozkaya, O. (2015). Characterization of extracellular polysaccharides (EPS) produced by thermal bacillus and determination of environmental conditions affecting exopolysaccharide production. *Int. J. Environ. Res.* 9, 1107.
- Duan, J., Liang, J., Wang, Y., Du, W., and Wang, D. (2016). Kraft Lignin biodegradation by *Dysgonomonas* sp. WJDL-Y1, a new anaerobic bacterial strain isolated from sludge of a pulp and paper mill. *J. Microbiol. Biotechnol.* 26, 1765.
- Duda, V.I., Suzinar, N.E., Polivtseva, N., and Boronin, A.M. (2012). Ultramicrobacteria: Formation of the concept and contribution of ultramicrobacteria to biology. *Microbiol.* 81, 379–390.
- Gao, D., Fu, Y., and Ren, N. (2013). Tracing biofouling to the structure of the microbial community and its metabolic products: A study of the three-stage MBR process. *Water Res.* 47, 6680–6690.
- Gao, D., Fu, Y., Tao, Y., Li, X., Xing, M., Gao, X., and Ren, N. (2011). Linking microbial community structure to membrane biofouling associated with varying dissolved oxygen concentrations. *Bioresour. Technol.* 102, 5626.
- Gerardi, H.H. (2002). Floc formation. In *Settability Problems and Lost Solids in the Activated Sludge Process*. John Wiley & Sons, Inc., Hoboken, New Jersey, pp. 15–21.
- Guo, X., Miao, Y., Wu, B., Ye, L., Yu, H., Liu, S., and Zhang, X. (2015). Correlation between microbial community structure and biofouling as determined by analysis of microbial community dynamics. *Bioresour. Technol.* 197, 99.
- Han, X., Wang, Z., Ma, J., Zhu, C., Li, Y., and Wu, Z. (2015). Membrane bioreactors fed with different COD/N ratio wastewater: Impacts on microbial community, microbial products, and membrane fouling. *Environ. Sci. Pollut. Res.* 22, 11436.
- Hao, L., and Liao, B.Q. (2015). Effect of organic matter to nitrogen ratio on membrane bioreactor performance. *Environ. Technol.* 36, 2674.
- Hao, L., Liss, S.N., and Liao, B. (2016). Influence of COD:N ratio on sludge properties and their role in membrane fouling of a submerged membrane bioreactor. *Water Res.* 89, 132.
- Hugenholtz, P., Tyson, G.W., Webb, R.I., Wagner, A.M., and Blackall, L.L. (2001). Investigation of candidate division TM7, a recently recognized major lineage of the domain bacteria with no known pure-culture representatives. *Appl. Environ. Microbiol.* 67, 411.
- Jinhua, P., Fukushi, K., and Yamamoto, K. (2006). Bacterial community structure on membrane surface and characteristics of strains isolated from membrane surface in submerged membrane reactor. *Sep. Sci. Technol.* 41, 1527.
- Judd, S. (2006). *Principles and Applications of Membrane Bioreactors in Water and Wastewater Treatment. The MBR Book*. Oxford: Elsevier Ltd.
- Kindaichi, T., Yamaoka, S., Uehara, R., Ozaki, N., Ozaki, A., Albertsen, M., Nielsen, P.H., Nielsen, J. (2016). Phylogenetic diversity and ecophysiology of candidate phylum saccharibacteria in activated sludge. *FEMS Microbiol. Ecol.* 92, 1.

- Koros, W., Ma, Y., and Shimidzu, T. (1996). Terminology for membranes and membrane-processes (IUPAC Recommendations 1996). *Pure Appl. Chem.* 68, 1479.
- Kumar, A., Chilongo, T., Dewulf, J., Ergas, S.J., and Van Langenhove, H. (2010). Gaseous dimethyl sulphide removal in a membrane biofilm reactor: Effect of methanol on reactor performance. *Bioresour. Technol.* 101, 8955.
- Kwon, S., Kim, T., Yu, G., Jung, J., and Park, K. (2010). Bacterial community composition and diversity of a full-scale integrated fixed-film integrated fixed-film activated sludge system as investigated by pyrosequencing. *J. Microbiol. Biotechnol.* 20, 1717.
- Lim, S., Kim, S., Yeon, K., Sang, B. I., Chung, J., and Lee, C. (2012). Correlation between microbial community structure and biofouling in a laboratory scale membrane bioreactor with synthetic wastewater. *Desalination* 287, 209.
- Ma, J., Wang, Z., Yang, Y., Mei, X., and Wu, Z. (2012). Correlating microbial community structure and composition with aeration intensity in submerged membrane bioreactors by 454 high-throughput pyrosequencing. *Water Res.* 47, 859.
- Mahendran, B., Lishman, L., and Liss, S.N. (2012). Structural, physicochemical and microbial properties of flocs and biofilms in integrated fixed-film activated sludge (IFFAS) systems. *Water Res.* 46, 5085.
- Malaeb, L., Le-Clech, P., Vrouwenvelder, J.S., Ayoub, G.M., and Saikaly, P.E. (2013). Do biological-based strategies hold promise to biofouling control in MBRs? *Water Res.* 47, 5447.
- McKay, A.M. (1992). Viable but non-culturable forms of potentially pathogenic bacteria in water. *Lett. Appl. Microbiol.* 14, 129.
- Meng, F., Chae, S., Drews, A., Kraume, M., Shin, H., and Yang, F. (2009). Recent advances in membrane bioreactors (MBRs): Membrane fouling and membrane material. *Water Res.* 43, 1489.
- Meng, F., Zhang, S., Oh, Y., Zhou, Z., Shin, H., and Chae, S. (2017). Fouling in membrane bioreactors: An updated review. *Water Res.* 114, 151.
- Miura, Y., Watanabe, Y., and Okabe, S. (2007). Membrane biofouling in pilot-scale membrane bioreactors (MBRs) treating municipal wastewater: Impact of biofilm formation. *Environ. Sci. Technol.* 41, 632.
- More, T.T., Yadav, J.S.S., Yan, S., Tyagi, R.D., and Surampalli, R.Y. (2014). Extracellular polymeric substances of bacteria and their potential environmental applications. *J. Environ. Manage.* 144, 1.
- More, T.T., Yan, S., John, R.P., Tyagi, R.D., and Surampalli, R.Y. (2012). Biochemical diversity of the bacterial strains and their biopolymer producing capabilities in wastewater sludge. *Bioresour. Technol.* 121, 304.
- Piasecka, A., Souffreau, C., Vandepitte, K., Vanysacker, L., Bilad, R.M., De Bie, T., Hellemans, B., Meester, L.D., Yan, X., Declerck, P., Vankelecom, I.F.J. (2012). Analysis of the microbial community structure in a membrane bioreactor during initial stages of filtration. *Biofouling* 28, 225.
- Price, M.N., Dehal, P.S., and Arkin, A.P. (2010). FastTree 2—Approximately maximum-likelihood trees for large alignments. *PLoS ONE* 5, e9490.
- Psoch, C., and Schiewer, S. (2008). Long-term flux improvements by air sparging and backflushing for a membrane bioreactor, and modelling permeability decline. *Desalination* 230, 193.
- Sadar Ghayenia, S.B., Beatson, P.J., Fanea, A.J., and Schneider, R. (1999). Bacterial passage in microfiltration membranes. *J. Membr. Sci.* 153, 71.
- Shahnavaz, B., Maroof, S., Karrabi, M., and Mashreghi, M. (2015). Characterization and molecular identification of extracellular polymeric substance (EPS) producing bacteria from activated sludge. *J. Cell. Mol. Res.* 7, 86.
- Silbaq, F.S. (2009). Viable ultramicrocells in drinking water. *J. Appl. Microbiol.* 106, 106.
- Silva, A.F., Antunes, S., Saunders, A., Freitas, F., Vieira, A., Galinha, C.F., Nielsen, P.H., Barreto Crespo, M.T., Carvalho, G. (2016). Impact of sludge retention time on the fine composition of the microbial community and extracellular polymeric substances in a membrane bioreactor. *Appl. Microbiol. Biotechnol.* 100, 8507.
- Torrella, F., and Morita, R.Y. (1981). Microcultural study of bacterial size changes and microcolony and ultramicrocolony formation by heterotrophic bacteria in seawater. *Appl. Environ. Microbiol.* 41, 518.
- Trussell, R., Adham, S., and Trussell, R. (2005). Process limits of municipal wastewater treatment with the submerged membrane bioreactor. *J. Environ. Eng.* 131, 410.
- Weissbrodt, D.G., Shani, N., and Holliger, C. (2014). Linking bacterial population dynamics and nutrient removal in the granular sludge biofilm ecosystem engineered for wastewater treatment. *FEMS Microbiol. Ecol.* 88, 579.
- Yamada, T., and Sekiguchi, Y. (2009). Cultivation of uncultured Chloroflexi Subphyla: Significance and ecophysiology of formerly uncultured Chloroflexi 'Subphylum I' with natural and biotechnological relevance. *Microbes Environ.* 24, 205.
- Yang, F., Bick, A., Shandalov, S., Brenner, A., and Oron, G. (2009). Yield stress and rheological characteristics of activated sludge in an airlift membrane bioreactor. *J. Membr. Sci.* 334, 83.
- Zhang, K., Choi, H., Dionysiou, D. D., Sorial, G. A., and Oerther, D. B. (2006). Identifying pioneer bacterial species responsible for biofouling membrane bioreactors. *Environ. Microbiol.* 8, 433.
- Zsirai, T., Buzatu, P., Maffettone, R., and Judd, S. (2012). Sludge viscosity—The thick of it. The MBR (Membrane Bioreactors) Site: Process Biology, 4 April 2012 <https://www.thembrsite.com/features/sludge-viscosity-in-membrane-bioreactors-the-thick-of-it/>