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TITLE PAGE

Title:

Membrane biofouling in a wastewater nitrification reactor: microbial succession from autotrophic colonization to heterotrophic domination

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Abstract

Membrane biofouling is a complex process that involves bacterial adhesion, extracellular polymeric substances (EPS) excretion and utilization, and species interactions. To obtain a better understanding of the microbial ecology of biofouling process, this study conducted rigorous, time-course analyses on the structure, EPS and microbial composition of the fouling layer developed on ultrafiltration membranes in a nitrification bioreactor. During a 14-day fouling event, three phases were determined according to the flux decline and microbial succession patterns. In Phase I (0-2 days), small sludge flocs in the bulk liquid were selectively attached on membrane surfaces, leading to the formation of similar EPS and microbial community composition as the early biofilms. Dominant populations in small flocs, e.g., *Nitrosomonas*, *Nitrobacter*, and *Acinetobacter* spp., were also the major initial colonizers on membranes. In Phase II (2-4 d), fouling layer structure, EPS composition, and bacterial community went through significant changes. Initial colonizers were replaced by fast-growing and metabolically versatile heterotrophs (e.g., unclassified *Sphingobacteria*). The declining EPS polysaccharide to protein (PS:PN) ratios could be correlated well with the increase in microbial community diversity. In Phase III (5-14 d), heterotrophs comprised over 90% of the community, whereas biofilm structure and EPS composition remained relatively stable. In all phases, AOB and NOB were constantly found within the top 40% of the fouling layer, with the maximum concentrations around 15% from the top. The overall microbial succession pattern from autotrophic colonization to heterotrophic domination implied that MBR biofouling could be alleviated by forming larger bacterial flocs in bioreactor suspension (reducing autotrophic colonization), and by designing more

45 specific cleaning procedures targeting dominant heterotrophs during typical filtration
46 cycles.

47 **Key words:** nitrification, biofouling, biofilm structure, bacterial succession, submerged
48 MBR

49

1. Introduction

The submerged membrane bioreactor (MBR) is a well-developed process that combines membrane filtration with biological treatment, providing better effluent quality and lower sludge production compared to the conventional activated sludge processes (Judd 2011). A significant portion of MBR operating cost is attributed to fouling control. Membrane fouling occurs as a result of the accumulation of solutes, colloids and bacterial flocs on membrane surfaces, and biofouling contributes to about 45% of the overall fouling (Komlenic 2010). Traditional methods developed for mitigating membrane biofouling are mainly based on physico-chemical principles, and factors modulating biofouling behaviors have been extensively studied, including feed characteristics, membrane properties, and reactor operation conditions (Ahmed et al. 2007, Park et al. 2006, Tsuyuhara et al. 2010). However, a comprehensive understanding of biofouling mechanisms from a microbial ecology perspective is still limited, hampering the applications of innovative, biological fouling control strategies, including enzymatic disruption of extracellular polymeric substances (EPS) excretion, quorum quenching, and biofilm disruption by adding bacteriophage (Xiong and Liu 2010). In order to transform these novel solutions into more effective, long-term fouling control strategies, it remains important to study biofilm structure, EPS and microbial community succession in mixed-culture SMBR systems.

Compared with natural environments or media-based biofilm reactors, MBR provides a unique habitat for biofilm growth. The transmembrane pressure generates a drag force that dominates the transport of particulates, colloids, cells, and nutrients from the mixed liquor to the membrane surfaces. One of the typical membrane fouling mechanisms is

standard blocking, where small particles pass the membrane pores and a finite number adhere to the walls causing pore blockage (Nguyen et al. 2010). Only a few studies have examined the relationship between small sludge flocs in a MBR and the initial microbial colonizers on membrane surfaces (Piasecka et al. 2012, Zhang et al. 2006a, Gao et al. 2011). These studies observed that the biofilm community succession could be influenced by the enhanced flux of nutrients and metabolites within the fouling layer, and bulk sludge and the fouling layer microbial communities could be different. However, how various physical and biological factors contribute to the fouling layer microbial community succession throughout the fouling process remains unknown.

This study aimed at examining the microbial community changes and the associated fouling behaviors of ultrafiltration membrane (UF) in a lab-scale wastewater nitrification reactor. It is hypothesized that 1) initial membrane filtration favors the attachment of small flocs, and initial colonizers are closely related to species present in small flocs; 2) the biofilm community succession is dominated by the pressure-driven deposition of bulk microbes at an early stage and species interactions among autotrophs and heterotrophs at later stages. Specific objectives were to 1) investigate the bacterial populations in size-fractionated bulk sludge and their correlations with the early colonizers on membranes; 2) monitor the changes of biofilm structural parameters and EPS composition during biofouling; and 3) evaluate the biofilm community succession and the spatial distribution of major bacterial groups within the fouling layer. By depicting a more comprehensive and detailed picture of biofouling processes, this study can potentially inspire novel anti-fouling strategies for membrane bioreactors in general, and for aerobic submerged MBRs in particular.

2. Materials and Methods

2.1. Nitrifying bioreactor

A 20-L nitrification bioreactor was operated in continuous flow mode at ambient temperature with a hydraulic retention time (HRT) of 4 d and solids retention time (SRT) of 15 d (Figure 1). The seed sludge was obtained from a lab-scale nitrifying reactor operated under similar conditions (Ahn et al. 2011). The feed medium contained 500 mg-N/L ammonium and was devoid of organic carbon as described previously (Hockenbury and Grady 1977, Ahn et al. 2008). Reactor pH was automatically controlled at 7.5 ± 0.1 by adding 1 mol/L NaHCO_3 solution. Aeration was continuously provided at a rate of 1 L/min through flexible rubber tubing placed at the bottom edges, resulting in a constant level of dissolved oxygen in the bioreactor (2.2 ± 0.5 mg O_2 /L, specific aeration demand was about $0.6 \text{ m}^3/\text{m}^2/\text{h}$ during the operation of submerged membrane filtration). The bioreactor was monitored for its performance (ammonia, nitrite, nitrate, total and effluent chemical oxygen demand concentrations) using standard methods (Rice 2012).

Submerged membrane filtration was operated for different time periods (3h, 6h, 12h, 18h, 1d, 2d, 3d, 5d, 7d, and 14d) in the nitrification bioreactor using flat-sheet polysulfone membranes (avg. pore diameter = 86 nm) placed in plexiglass membrane cartridges. Membrane fabrication procedures and more details about UF operation are described in Supplementary Information. Triplicate membranes were operated simultaneously in the reactor for each time period, and completely sacrificed for imaging and community analysis. Air scouring on membrane surfaces was eliminated by positioning the membrane cartridge away from air diffusers. Permeate was driven across the membrane by a vacuum pump at a constant transmembrane pressure (TMP, 25 kPa),

weighed by a digital scale, and recorded by the LabView software (National Instruments, TX). The constant TMP instead of the constant flux mode was selected mainly to simplify the analysis of biofilm community variations caused by pressure-driven attachment. In addition, it has been suggested that UF performance could potentially get improved by allowing flux to decline naturally, i.e., a constant-TMP condition (Bourgeois et al. 2001). Freshly harvested membranes were cut into equal-width pieces (1.54×2.54 cm) and rinsed twice by gently dipping sequentially in 40 ml 1X PBS buffer. Two buffer washes were previously proven to be an optimal procedure for examining membrane fouling (Xue et al. 2014), i.e., 0-1 buffer wash was insufficient to remove planktonic cells, but >2 washes will remove loosely attached flocs. Rinsed membranes were analyzed freshly for biofilm structure, fixed for fluorescent *in situ* hybridization (FISH), or stored at -80 °C for less than a week prior to EPS and DNA extraction as detailed in the following sections.

2.2. Floc separation, size measurement, and EPS extraction

Activated sludge flocs collected from the nitrification reactor (prior to membrane operation) were size-fractionated by gravity settling. Briefly, 500 mL activated sludge was transferred into a 1 L separatory funnel. 20 mL from the bottom layer was collected after 30 min settling time to represent the large floc fraction, and another 60 mL was collected from the top after another 30 min settling to represent the small floc fraction. The floc size distribution in each fraction and the original bulk sludge was obtained by microscopic analysis. The micrographs were obtained with a Zeiss Axio Observer Z1 microscope (40X objective, phase contrast, Carl Zeiss AG, Germany) and analyzed for

particle size using ImageJ software. For each sample, over 50 images were taken and 5000-30000 particles were examined to set up a representative particle size distribution.

To quantify the bound EPS, 15 mL sludge liquor from each fraction was filtered through a 0.22- μ m membrane filter and the collected sludge was processed for EPS extraction using the formaldehyde and NaOH method (Liu and Fang 2002). Protein and polysaccharide concentrations in the extracted EPS were measured by the standard Lowry (Total Protein Kit, Sigma-Aldrich, MO) and the phenol-sulfuric acid methods, respectively (Eaton et al. 2005). The bound EPS content in each fraction was calculated as protein or polysaccharide concentrations per unit TSS of sludge, and the TSS was measured according to the standard method (Rice 2012).

2.3. Imaging analysis for membrane biofilm structure

The rinsed membrane biofilm samples were mounted on glass slides with double-sided tape. An extra layer of the double-sided tape was placed outside each end of the membrane to create a space and to prevent the cover slide from disturbing the biofilm structure. The biofilm samples were examined by confocal laser scanning microscopy (CLSM) and subject to imaging and quantitative structural analysis. The total biofilm cells were fluorescently tagged by nucleic acid stain SYTO 9 (2.5 μ mol/L, Sigma-Aldrich, MO). Three EPS markers were applied to target the polysaccharide (Alexa 633 conjugated Concanavalin A and Wheat Germ Agglutinin, 10 μ g/ml each, Life Technologies, MA) and protein (Sypro Orange, 5 μ g/ml) components (Yang et al. 2006, Strathmann et al. 2002). A detailed description of the fluorescent dyes and an example of CLSM EPS staining image can be found in Supplementary Information (Table S1 and Figure S1). The sampled biofilms were incubated with SYTO 9 for 30 min at room

temperature in the dark followed by another 30 min incubation with a mixture of the EPS markers and mounted on glass slides with a SlowFade antifade reagent (Life Technologies, MA). The fluorescently labeled biofilms were analyzed with a CLSM (Leica TCS SP2, Germany) equipped with a 63x oil-immersion objective. Images were acquired at 1 μm step size of the z-stack, and six fields per sample were randomly selected for statistical evaluation. The acquired confocal images were processed by the COMSTAT software for biofilm structural analysis, including total biofilm volume, average thickness, roughness coefficient, diffusion distance, and surface to biovolume ratio (Heydorn et al. 2000).

2.4. Fluorescent *in-situ* hybridization (FISH)

The bulk sludge and membrane biofilms were fixed with 4% paraformaldehyde for 2h at 4 °C, followed by rinsing with PBS/ethanol (1:1 v/v) solution and storing at -20 °C prior to the hybridization procedures as described previously (Benakova and Wanner 2013). The prefixed biofilms were dehydrated in a series of ethanol solutions (50%, 80%, and 96%) for 3 min each. The dried samples were incubated with a hybridization mix containing 1 ng/ μL of each oligonucleotide probe at 46 °C for 4 hr and then washed in a preheated washing buffer for 15 min in a 48 °C water bath. Afterwards, the samples were dipped in cold DI water, air dried, and mounted with SlowFade (Life Technologies, MA). A total of 20 membrane biofilm samples were examined and 160 hybridization were performed. Planktonic activated sludge in the mixed liquor was fixed and spotted on gelatin-coated slides for FISH analysis.

The oligonucleotide probes used included EUB338 for total bacteria, Nso1225 for β -ammonia oxidizing bacteria (AOB, including *Nitrosomonas* and *Nitrosospira*), and NIT3

together with Ntspa712 for the predominant nitrite oxidizing bacteria (NOB, *Nitrobacter* and *Nitrospira*) (Benakova and Wanner 2013). The nonsense probe nonEUB338 was applied as a control for nonspecific staining (Christensen et al. 1999). The relative abundance of nitrifying bacteria in total bacteria was estimated as a ratio of AOB or NOB hybridized cell volume to the volume of total bacteria by image analysis using COMSTAT.

2.5. DNA extraction and 16S rRNA gene amplicon sequencing

DNA was extracted from the bulk sludge and membrane-attached biofilms with the FastDNA Spin Kit for Soil (MP Biomedicals, CA) according to the manufacturer's instructions. DNA concentrations were measured by the Qubit 2.0 Fluorometer (Life Technologies, MA). The hypervariable V4 region of 16S rRNA gene widely used for taxonomic classification was then amplified with dual-indexed primers 515F and 806R as described elsewhere (Kozich et al. 2013). The PCR amplicons were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, MI) and quantified by Qubit. Equimolar aliquots of each PCR product were pooled to give ~1 µg of total DNA in 100 µL. The size, quantity and quality of amplicons were measured using an Agilent DNA 1000 chip and a 2100 BioAnalyzer (Agilent, CA). The pooled amplicon library was sequenced on Illumina MiSeq Personal Sequencer with a v2 paired-end read.

Sequence data were processed using the Mothur program (Schloss et al. 2009), yielding an average of ~100,000 sequences per sample (average read length: 250 bp) after quality control and chimera screening. The unique sequence reads were clustered into 1709 operational taxonomic units (OTUs) at 97% similarity, which were then blasted

against the Greengenes database for 16S rRNA genes. Bacterial diversity of the bulk biomass and biofilm at different ages was evaluated with the Chao1 calculator in Mothur.

2.6. Multivariate analysis

In order to discriminate the community composition among bulk sludge and biofilm samples, sequence counts of total OTUs were transformed into relative abundance and non-metric multidimensional scaling (NMDS) was performed based on Bray-Curtis similarities. Unsupervised clustering was performed using the “Heatplus” package in the R program to correlate biofilm community composition with filtration performance (Ploner 2014). Input data are the Pearson’s correlation coefficients (r values) measuring the linear correlations between the abundance of top 40 most abundant OTUs and the three most important environmental variables (flux, PS:PN ratio, and biofilm thickness, determined based on the redundancy analysis described below). Initially, sequencing data were log-transformed, standardized by sample norm and centered by species, and 8 environmental variables were selected for redundancy analysis (RDA, Table S1). The importance of the 8 explanatory variables in explaining population dynamics of the top 40 most abundant OTUs at different biofilm ages, or their “marginal effects” were evaluated by using the RDA package in CANOCO 4.5 (Table S2). Marginal effect stands for the amount of variability in species data that would be explained by a constrained ordination model using that variable as the only explanatory variable (Lepš and Šmilauer 2003). Eventually, permeate flux, PS:PN ratio, and biofilm thickness were the most influential variables to microbial species data, and were used for Pearson’s correlation tests above.

3. Results

3.1. Reactor performance and flux variations

The nitrification reactor was operated at steady state for 120 days prior to this study (ammonia removal: 99.2 ± 0.5 %, nitrite accumulation: < 0.1 mg N/L and mixed liquor volatile suspended solids, or MLVSS: 635.2 ± 21.3 mg/L). When membrane filtration was conducted, changes in the ammonia, nitrite and MLVSS concentrations were not statistically significant. Nitrate in the effluent decreased from 462.1 ± 13.4 mg N/L (without membrane filtration) to 444.0 ± 3.8 mg N/L (with filtration, $p < 0.05$).

Without backwashing or membrane cleaning, permeate flux rapidly declined within the first 1-2 days, stayed relatively constant in the following 20 days, and completely ceased on day 34 (Figure S2). The six operating periods (3h, 6h, 12h, 18h, 1d, 2d, 3d, 5d, 7d, and 14d) were determined in order to collect sufficient samples from both the rapid- and slow-flux decline phases. The membrane fouling process, despite of different operating periods, displayed identical flux decline patterns, assuring the consistency and reproducibility of EPS and community analyses for biofilm samples.

3.2. Sludge fractioning, EPS and bacterial community composition

Triplicate bulk sludge aliquots were harvested from the bioreactor on days 1, 5 and 14 during a 14-day membrane filtration period. The small and large flocs had a median diameter of 7.2 and 27.6 μm , respectively (Figure S3). The total EPS concentration (in mg EPS/g sludge TSS), and the polysaccharide-EPS to protein-EPS ratio (PS:PN) were significantly different for the two fractions (Figure 2). Specifically, small flocs had 40.6% higher total EPS than large flocs, and the PS:PN ratios were also significantly higher in the small flocs (1.33 ± 0.19 v.s. 0.32 ± 0.07 , $p < 0.05$). The bulk sludge demonstrated

medium-level EPS concentrations and PS:PN ratios compared with the two fractions (Figure 2).

Nitrosomonas, *Nitrobacter* and *Nitrospira* spp. were the predominant nitrifiers in all three types of samples. It is further revealed that the above typical AOB and NOB populations comprised 31.7 ± 0.05 % and 11.7 ± 1.4 % of the small floc community, and $20.3\% \pm 0.12$ % and 4.4 ± 0.01 % of the big floc community, respectively (Figure 2). Amplicon sequencing data indicated that dominant heterotrophic bacteria from the sludge samples were in the families of Sphingomonadaceae and Comamonadaceae (Figure 7). Both families belong to the phylum Proteobacteria and their relative abundances in the bulk sludge communities were 25.13 ± 0.22 % and 11.01 ± 0.13 %, respectively. The overall community composition of small flocs, large flocs and the bulk sludge were further presented in an NMDS plot in Figure 3a.

3.3. Biofilm structure and EPS composition

Figure 4 indicated the changes in total biovolume, average thickness, and PS:PN ratio with respect to biofilm age. Biofilm thickness increased rapidly during the first 2 days of filtration, and stabilized around 80-100 μm after day 5. The increase in biovolume followed an almost identical trend as thickness. The PS:PN ratio of biofilm samples, as quantified by CLSM imaging, decreased from approximately 3.2 ± 1.2 on day 1 to 0.2 ± 0.07 on day 5. The ratios were comparable with those obtained by standard Lowry and phenol-sulfuric acid methods. Despite of different quantification approaches, the PS:PN ratio of the early biofilm samples (≤ 2 days) were closer to that of small flocs. Other biofilm structural parameters (e.g. roughness coefficient, diffusion distance, and

surface to biovolume ratio) demonstrated fluctuating patterns during the first 2 days, and stabilized as the fouling layer matured (Figure S5).

3.4. Biofilm microbial community

In Figure 3a, the NMDS plot displayed the temporal variations of biofilm community structure, and samples more similar to each other were ordinated closer. Early biofilm communities (within the first hours) were closer to small flocs than the bulk sludge or large flocs. Initial colonizers were related to *Nitrosomonas*, *Nitrobacter* and *Nitrospira* species. A dramatic community structure shift was in accordance with the rapid changes in flux and biofilm structure at the early stage. The shift in biofilm community structure appeared to slow down with an increase in biofilm age after day 2. The microbial community diversity of biofilm samples, as indicated by the Chao1 index, was consistently higher than the bulk sludge and increased as biofilm aged (Figure 3b). Heterotrophic populations eventually comprised over 90% of the total bacterial community, and exhibited a high phylogenetic diversity with *Bacteroidetes* and *Proteobacteria* as the dominant bacterial phyla ($47.1 \pm 8.3\%$ and $24.4 \pm 2.6\%$, averaged from day 5-14). In addition, a Pearson's correlation test revealed a strong negative relationship between PS:PN ratios and biofilm diversity ($r = -0.81$, $n=8$).

FISH images in Figure 5a-d displayed the fouling layer development process from small cell aggregates to a thick, fully established biofilm. Figure 5e further indicated the spatial distribution of major bacterial groups within the fouling layer. Most AOB and NOB clusters were situated within the top 40% of the fouling layer, with the highest abundance appearing at approximately 15% from the surface, regardless of the biofilm age (Figure S6). Nitrifiers were rarely found in the deeper layer and their abundances

decreased over time at all depth. In comparison, heterotrophs were increasingly found in the deeper layers and also across time. In Figure 6, population dynamics based on family-level taxonomy revealed substantial microbial community succession during the 14-day filtration period. Nitrifying populations continuously decreased, and the fouling layer was primarily dominated by heterotrophs with higher diversity over time. Nevertheless, members of the emerging phyla such as *Firmicutes* and *Chloroflexi* were of low abundance in the community (<2%) even by the end of the filtration period. Interestingly, Proteobacteria, Bacteroidetes, and nitrifiers as classified at the taxonomic level of family exhibited remarkable similarities to other studies on biofouling in aerated membrane reactors (Ma et al. 2013).

Figure 7 demonstrated the results from a hierarchical clustering analysis based on the Pearson's correlation coefficients between the abundances of the top 40 OTUs and three environmental variables, i.e., permeate flux, EPS PS:PN, and biofilm thickness. OTUs within each cluster exhibited high homology in successional patterns. Relative abundances of the representative OTUs in each cluster were plotted against the biofilm age to reveal temporal variations. In Cluster A, the decreases of populations such as *Nitrosomonas*, *Nitrospira*, *Nitrobacter* were positively correlated with the decline in permeate flux and PS:PN ratios. Microbial populations that stayed at relatively stable abundance (Cluster B) or increased over time (Cluster C) were mainly related to heterotrophs. Some of the heterotrophs were capable of N-fixation or denitrification, e.g., *Bradyrhizobium*, *Hypomicrobium*, and *Mesorhizobium*. Most OTUs in the early biofilm samples had similar concentrations as in the small flocs, indicating a potential link between small flocs and initial colonizers as reflected earlier in Figure 3a.

4. Discussion

4.1. Biofouling stages defined by filtration performance, biofilm structure and composition

Three to four stages were normally defined to depict membrane fouling development in MBRs, based on either flux or TMP changes (Zhang et al. 2006b, Marshall and Daufin 1995). In this study, a typical 3-stage flux decline was observed during the 14-day filtration. Phase I (approximately 0-2 days) was primarily characterized by the rapid flux decline and dramatic changes in PS:PN ratio, biofilm structure and microbial community (Figure S2, 3a and 4). Fouling in this phase was likely governed by physical factors, but contributions from biotic processes such as microbial EPS excretion and consumption could not be ruled out. Permeate drag and back transport facilitated the deposition of suspended particles onto the membrane surface, causing the rapid pore blockage and flux decline. A few studies have highlighted the potential roles that small particles play during initial fouling (Bae and Tak 2005, Lin et al. 2011). Their postulations were indirectly proven by correlating permeate flux drop and the filtration resistance of different sludge constituents. In this study, we observed similar PS:PN ratios, overall community structure, and percentages of dominant populations (Figure 2, 3a and 6) between the small sludge flocs and early biofilms. These direct evidences suggested that the preferential deposition of small flocs was indeed occurring when filtration just initiated, potentially due to their higher levels of bound EPS, smaller back transport velocity and denser structure compared with large flocs (Liu et al. 2012). Dominant populations in the small flocs, such as *Nitrosomonas* and *Nitrobacter* also served as the initial colonizers on the membrane. The membrane surface was soon fully conditioned by small flocs, favoring

further deposition of large flocs via non-selective attachment. The robustness and resistance of nitrifying colonizers to detachment is critical to biofilm development (Tijhuis et al. 1995). Giving the differences between small and large flocs in their PS:PN ratio and microbial composition (Figure 2 and 3a), the shift from selective- to unselective-attachment might contribute to the rapid changes in the PS:PN and microbial composition of biofilms in Phase I.

Phase II (approximately 2-4 days) exhibited alleviated rates of flux decline (Figure S2), decreases in PS:PN ratio (Figure 4), and active microbial successions (Figure 3a and 6). Variations in biofilm EPS composition were observed and this could result from the dynamic production and consumption of polysaccharide and protein by different microorganisms in the fouling layer. More specifically, protein EPS, rather than polysaccharide might be preferentially secreted by the nitrifying colonizers, e.g., *Nitrosomonas* (Stehr et al. 1995). Furthermore, the hydrolysis and biodegradation rates of protein EPS are usually much slower than polysaccharide, causing a manifest loss of polysaccharide but not protein in the EPS matrix (Malamis and Andreadakis 2009, Zhang and Bishop 2003). Some initial colonizers (cluster A) have been recognized as the aggregate-forming bacteria or “bridging bacteria”, such as *Nitrosomonas*, *Nitrobacter*, *Pseudomonas*, and *Acinetobacter* species (Malik et al. 2003, de Lima et al. 2013, Nogueira and Melo 2006). These populations potentially mediated the rapid establishment of biofilm consortium in Phase II, but were later on out-competed by rapidly growing heterotrophs (Figure 6). Utilization of EPS by these heterotrophs, primarily polysaccharide EPS, was evidenced by the decreased PS:PN ratios, as well as the more abundant and diverse heterotrophic community. Although the concentrations of

Nitrosomonas, *Nitrobacter* and *Nitrospira* decreased in a constant proportion in Phase II, their spatial distribution within the fouling layer was relatively consistent throughout the fouling process. AOB and NOB were present in the top 30-40% (Figure S6) and their highest percentages in the communities were found at the 15% distance from the surface, which may represent an optimal point with balanced substrates, metabolic intermediates and products for autotrophic growth. However, a dynamic modeling of diffusion, reaction, and species interaction is required to test this hypothesis. Abiotic factors (e.g., selective attachment or detachment of specific groups) played a less significant role in explaining the overall population variations in Phase II, as the microbial composition of the surface layer in this phase was very similar to the bulk sludge (Figure S6).

Phase III (5-14 days) exhibited a highly structured fouling layer with a localized stabilization in physical structure, EPS, and bacterial composition (Figure 3a and 4). It is observed that nitrifiers could release metabolic products to support heterotrophic growth (Ni et al. 2011). The continuous removal of these metabolic products could be achieved by heterotrophs in deeper layers. Most of the top OTUs presented in Figure 7 were microbial populations identified in globally distributed full-scale activated sludge systems, including the co-occurring nitrifiers (e.g., *Nitrosomonas* and *Nitrospira*), denitrifiers (e.g., *Hyphomicrobium*, *Comamonas*, *Acidovorax* and *Xanthobacter*) and N-fixing bacteria (e.g., *Mesorhizobium* and *Bradyrhizobium*) (Ju et al. 2013). Among them, the relative abundance of an unclassified *Sphingobacteria* increased by approximately 3 times during Phase II and III, and became the most dominant OTU in the matured fouling layer (Cluster C in Figure 7). This could be attributed to the fact that *Sphingobacteria* are strong competitors for uncommon carbon compounds (Krieg et al. 2011), and have been

frequently detected at full-scale wastewater treatment processes (Albertsen et al. 2012). In comparison, heterotrophic populations remaining at relatively constant percentages during Phase II and III (Cluster B) were likely less competitive for energy and carbon sources, including soluble microbial product (SMP) (Merkey et al. 2009). These observations could be exemplified by the two nitrogen-fixing heterotrophs *Mesorhizobium* (Cluster C) and *Bradyrhizobium* (Cluster B) found in the biofouling layer, which have indeed been identified as the fast-growing and slow-growing populations, respectively (Monza et al. 2006).

4.2. Practical implications for membrane fouling control

Compared with traditional activated sludge process, the new aerobic granular sludge technology is capable of retaining compact and dense microbial granules (Buitrón et al. 2014). Our results suggested that aerobic granular reactors coupled with submerged membranes could be more effective in biofouling control than conventional AS-based SMBR, as the membrane conditioning process leading to initial flux drop can be mitigated by forming larger bacterial flocs in granular reactors. The second implication of this study is associated with membrane cleaning procedures (e.g., backwash and chemical cleaning). Certain bacterial populations exhibited strong correlations with performance parameters (e.g., unclassified *Sphingobacteria*, Figure 6) and could rapidly regrow after backwashing. Monitoring of these populations could serve as an effective biological indicator and complement traditional indicators such as TMP and flux to better understand membrane fouling mechanisms. Accordingly, cleaning procedures could be optimized based on the bacterial succession patterns during typical filtration cycles. Thirdly, emerging biofouling control strategies, such as polymer or nanoparticle based

membrane surface modification, quorum quenching, inhibition on EPS excretion, or biofilm disruption by bacteriophage have been proposed (Xiong and Liu 2010). These strategies could be used to specifically target initial colonizers and rapid-growing species. For example, all bacteria use species-specific autoinducers for activating quorum sensing (Lade et al. 2014), and the information about fouling layer community structure is useful for screening quorum quenching molecules.

5. Conclusions

In this study, the UF membrane biofouling process in a nitrification SMBR was characterized with regard to biofilm structure, EPS composition and microbial community succession. Three phases were determined mainly based on the patterns of flux changes. Various physical and biological factors governing the fouling behaviors and microbial community composition of the fouling layer were identified in each phase.

- Under constant transmembrane pressure, permeate drag force selectively attached small bacterial flocs onto membrane surfaces;
- Initial colonizers on membrane surfaces originated from the small flocs and profoundly influenced the fouling behavior and bacterial succession later on;
- During the autotroph- to heterotroph-dominated microbial succession, a constant nitrifier-active region can be defined within the fouling layer regardless of the biofilm age;
- Certain bacterial populations were well correlated with filtration performance, and therefore could serve as promising indicators for membrane fouling.

Although this study investigated the membrane biofouling mechanisms in an engineered system, the approach taken and the microbial ecology findings could be

437 useful for general biofilm research. For example, examining biofouling based on biofilm
438 thickness, chemical composition of EPS, and microbial community succession patterns
439 with intensive and short-interval sampling scheme could provide insights into broader
440 biofilm formation processes.

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Figure 1. A schematic diagram of the nitrification reactor and submerged membrane filtration setup in this study.

Figure 2. Microbial composition (based on FISH analysis), protein- and polysaccharide-EPS (based standard Lowry and the phenol-sulfuric acid methods) of size fractionated sludge flocs. Error bars represent one standard deviation from biological triplicates.

Figure 3. a) Non-metric multidimensional scaling (NMDS) ordination diagram derived from the Bray-Curtis similarities in the microbial community composition of triplicate bulk sludge and biofilm samples (based on sequencing). b) Microbial community diversity of bulk sludge and biofilm at different ages (based on sequencing). Error bars represent one standard deviation from biological triplicates.

Figure 4. Changes of EPS polysaccharide to protein (PS:PN) ratio, biovolume and biofilm thickness during the biofouling process. All parameters were obtained by confocal laser scanning microscopy. Error bars represent one standard deviation from biological triplicates.

Figure 5. CLSM images of membrane biofilms hybridized with FISH probes. Green: AOB (Nso1225), Red: NOB (Ntspa712 and NIT3), Blue: total bacteria (EUB338). (a-d) composite CSLM projection images of biofilms sampled at $t=6h$, $12h$, $5d$, $14d$, respectively; (e) a vertical cross-section view of 5-day biofilm with surface reconstruction by Imaris. Images were selected from locations with average thicknesses and biomasses closest to the average of all points analyzed. Microcolonies appeared as mixtures of multiple probes were not due to unspecific binding, but a result of the image projection. AOB appeared to be teal due to the bindings of Nso1225-EUB338 probes, and NOB appeared to be pink due to the bindings of Ntspa712-NIT3-EUB338 probes to the cells.

Figure 6. Relative abundance of microbial families in samples of small sludge, big sludge, bulk sludge, and biofilm samples. Data were generated by taxonomic annotation of the amplicon sequences of 16S rRNA genes.

Figure 7. Unsupervised clustering of the top 40 most abundant OTUs based on their correlations with permeate flux, biofilm thickness and EPS PS:PN ratio, and the microbial succession of representative OTUs in each cluster. Population dynamics were obtained from 16S rRNA gene sequencing analysis, and all OTUs were annotated to the best possible taxonomy levels. Open symbols to the left of all curves: concentrations in the sludge samples; filled symbols: concentrations in the biofilm samples. Error bars represent one standard deviation from triplicate sequencing reads.

HIGHLIGHTS

- Permeate drag force selectively attached small bacterial flocs to the membrane.
- Initial colonizers were dominated by nitrifiers and outcompeted by heterotrophs later.
- A constant nitrifier-active region was identified at the top of the fouling layer.
- Bacterial species abundances can serve as biomarkers for membrane fouling.