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Linking solids retention time to the composition, structure, and hydraulic resistance of biofilms developed on support materials in dynamic membrane bioreactors



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ABSTRACT

Biofilms have permeable structures, which can be used as membranes for solids separation, e.g., dynamic membrane bioreactors (DMBRs) that relied on the biofilm developed on the support material with large apertures for filtration. This study found that the biofilm formed on the support material in DMBRs under different solids retention time (SRTs) had diverse structures, which in turn determined the hydraulic resistance and filtration performance. At 5-day SRT, a thin but compact gel-like biofilm layer (porosity = 27.5%) was formed on the support materials, which had a higher hydraulic resistance of approximately 4.9×10^{11} m⁻¹. As a result, the transmembrane pressure (TMP) rose dramatically every 20-40 days' operation. A thick but porous biofilm layer (porosity > 60% and hydraulic resistance $< 2.5 \times 10^9$ m⁻¹) was formed at longer SRTs of 20 and 40 days and the TMP could keep consistently low (< 20 Pa) for more than 180 days. Therefore, a longer SRT could facilitate the formation of a porous biofilm layer on the support material, which was of critical importance for achieving long-term and low-pressure filtration. The biofilm porosity was negatively correlated with the protein/polysaccharide ratio of its extracellular polymeric substance (EPS). Extending SRT would reduce the Live/Dead cell ratio of biofilm, thus lowering EPS production while increasing the porosity. Moreover, extending SRT promoted the growth of protozoa (e.g., Euglypha) that inhabited the biofilm, which could maintain the biofilm porous and mitigate the support layer biofouling though feeding and movement.

1. Introduction

Biofilm, which is an association of microorganisms in which microbial cells stick to each other within a self-produced and slimy matrix of extracellular polymeric substance (EPS), has been widely used to degrade contaminants in water [1,2]. Since biofilms have dynamic and permeable structures, they can also be used as membranes to separate solids in water, e.g., dynamic membrane bioreactor (DMBR) [3-5]. DMBRs use cheaper filtration materials with large apertures (e.g., stainless steel grids and polyester meshes) as the support material. Biofilm is formed on the surface of support material and/or inside the large apertures during the long-term operation. The rejection of solids is accomplished by the combination of self-formed biofilm (also named dynamic membrane) and support material. DMBRs have similar

advantages as microfiltration (MF) or ultrafiltration (UF) MBRs. However, DMBRs can achieve low-pressure filtration and the fouled support materials can be easily cleaned.

For the MF/UF MBRs, any sludge deposition or biofilm growth on the membrane surface and any accumulation of biofoulants inside the membrane pores are not desired since they will significantly increase the membrane filtration resistance and decrease the permeability. For DMBRs, however, a self-formed biofilm is necessary for solid rejection and, as a result, the performance of DMBRs is highly impacted by the characteristics of biofilm developed on the support material. The bioactivity is usually concerned if using biofilm to degrade contaminants. When using biofilm as a membrane to separate solids in DMBRs, however, researchers become more concerned about its hydraulic resistance. The hydraulic resistance of biofilm is highly

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determined by its compositions and physical structures, e.g., pores/ channels, thickness, and porosity [6,7]. Biofilms are mainly composed of different organics, e.g., live cells, dead cells, and EPS [8–10]. Desmond et al. [11] reported that the physical structure and hydraulic resistance of biofilm were mainly determined by the EPS matrix. Some other studies, however, suggested that bacterial cells were more resistant to permeation than EPS [12]. In addition to the biofilm compositions, predation could alter the biofilm structures as well [13]. A porous and heterogeneous structure was developed with predation, whereas a flat, compact, and thick structure was developed in the absence of predation [13]. These previous studies suggested that both compositions and predation could influence biofilm physical structures and in turn hydraulic resistance.

Solids retention time (SRT) is one of the most important control parameters in the bioreactors, which could dominantly impact the substrate concentrations in the effluent and biomass properties, including mixed liquor suspended solids (MLSS), biomass activity, soluble microbial products (SMP) production, EPS content, microbial community, and population of bacterial predators [14–16]. Therefore, SRT can significantly impact the characteristics of biofilm developed on support materials in DMBRs. Unfortunately, the links of SRT to the biofilm compositions, physical structures, and hydraulic resistance in DMBRs have not been well determined.

Four submerged DMBRs were operated respectively with 5-, 10-, 20-, and 40-day SRTs for approximately 200 days in this study. The filtration performance and the compositions and structures of the bio-film developed on the support materials in the four reactors were investigated. Furthermore, this study also tries to reveal how SRT impacts the biofilm compositions (e.g., EPS, live and dead cells, and protozoa) with further effect on biofilm physical structures (e.g., porosity and thickness) and in turn filtration resistance.

2. Materials and methods

2.1. Bioreactor setup and operation

Four lab-scale DMBRs with an effective volume of 12 L (Fig. S1) were operated in parallel. A flat-sheet membrane module with a filtration area of 0.068 m^2 was submerged into each reactor. The 3-D printed frame was wrapped with nylon mesh having an averaged pore size of approximately 25 μ m. The mesh was used as the support layer for biofilm growth. A fine bubble diffuser was installed below the membrane module in each reactor. The air supply rate for each reactor was similar (approximately 1.2 L-air/min), which could provide sufficient dissolved oxygen (> 2 mg/L) and mix.

To evaluate the effect of SRT on the filtration performance, the four reactors were operated at 5-, 10-, 20-, and 40-day SRTs, respectively. The SRT was controlled by daily sludge discharge and the discharge volume for each reactor was equal to the actual water volume in that reactor divided by the controlled SRT. Synthetic wastewater, which contained chemical oxygen demand (COD) of 180 mg/L and ammonia and 48 mg-N/L, was used to feed the reactors at a constant flow rate of approximately 16.7 mL/min. At the original water level, the HRT was approximately 12 h. The COD and ammonia in the influent were provided with glucose and ammonium bicarbonate, respectively. In addition, trace elements were added into the influent, which was described previously [17]. The seed sludge was taken from another well-operated bioreactor in our laboratory [18]. During the operation, the treated water passed the membrane at a nearly constant flux of $14.7 \,\mathrm{Lm}^{-2} \,\mathrm{h}^{-1}$, which was driven by the water head loss between the reactor water level and the outlet (Δ H) (Fig. S1). Each reactor had a maximum operation water head loss of 25 cm (ΔH_{max}). Along with the increasing filtration resistance, the water level in the reactor would increase to provide a greater transmembrane pressure (TMP) and then maintain the flux constantly. Once the operation TMP reached 2500 Pa, the membrane module was taken out and flushed with tap water. The equations used for calculating the flux, TMP, and resistance were given in S1.

The four reactors ran at an ambient temperature of 15-26 °C for about 200 days. The dissolved oxygen (DO) concentration was maintained above 2 mg/L and the pH ranged from 7.0 to 8.0. During the long-term operation, the outflow rate, effluent turbidity, operation water head loss (i.e., TMP), DO, pH, and temperature for each reactor were monitored regularly. The concentrations of effluent COD and ammonia and mixed liquor suspended solids (MLSS) in each reactor were monitored as well. Analytical methods for DO, turbidity, MLSS, COD, and ammonia were described previously [18].

2.2. Activated sludge characterization

2.2.1. Particle size, apparent viscosity, FTIR, and bioactivity

A Malvern Mastersizer 2000 (Worcestershire, UK) with a measuring range from 0.02 to 2000 µm was used to characterize the size distribution of sludge floc in the four reactors. Each sample was measured in triplicate. The activated sludge was also observed by an inverted fluorescence microscope (Olympus-IX53, Tokyo, Japan). The apparent viscosity of activated sludge and permeate at different SRTs was measured using a rotational viscometer (NDJ-5S, Bonsai Instrument Technology Co., Ltd, Shanghai, China) under room temperature. A Bruker EQUINOX 55 fourier-transform infrared spectrometer (FTIR) was used to analyze the major functional groups of activated sludge at different SRTs. Before analysis, the activated sludge was dried at 70 °C. The maximum specific oxygen uptake rate (SOUR_{max}) of activated sludge for COD degradation, which was used to represent the biomass activity, was measured by a pulse-flow PF-8000 aerobic/anaerobic respirometer (RSA, USA). In this measurement, sodium acetate was used as the COD. The detailed experimental protocols for SOUR measurements were described previously [19]. To further characterize the bioactivity under different SRTs, the live and dead cells in activated sludge were characterized based on confocal laser scanning microscopy (CLSM) analysis. The same analysis protocols used for the biofilm, which are described in the following Sections 2.3.3 and 2.3.4, were employed to analyze the sludge.

2.2.2. EPS extraction and measurement

During the operation, the contents of tightly bound extracellular polymeric substance (TB-EPS) in activated sludge from the four reactors were measured. Each sample was measured in triplicate. The heat treatment method, which was one of the most effective methods with less disruption to cells, was used to extract TB-EPS [14]. The samples were firstly centrifuged at 4000 rpm for 15 min and the supernatant was removed. The remaining biomass was re-suspended with saline water (0.9% NaCl solution) and then centrifuged again at 4000 rpm for 15 min. After discharging the supernatant, the remaining biomass was re-suspended again with 0.9% NaCl solution and heated in a water bath at 80 °C for 30 min. Finally, the mixed liquor was centrifuged at 4000 rpm for 15 min and then the supernatant was used for TB-EPS analysis after filtration by a $0.45\,\mu m$ membrane. The contents of EPS were normalized as the sum of carbohydrate and protein since they are the main components of EPS. The carbohydrate in EPS was measured using the H₂SO₄/Anthranone oxidation method with glucose as standard. The protein was determined by a modified Lowry method with BSA (bovine serum albumin, Sigma fraction V, 96%) as the standard.

2.3. Characterizing the biofilm on/inside the support materials

2.3.1. Scanning electron microscopy (SEM)

The support material was cut from the module for SEM analysis at the end of the operation. To examine the biofilm inside the mesh pores, a sample with surface biofilm scraped was prepared as well. The detailed steps for the dehydration process referred to a previous study [20]. After dehydration, the samples were coated with aurum–platinum alloy. A Zeiss Ultra 55 FESEM was used to observe the samples. For the

Table 1

Summary for the reactor performance under different SRTs.

Parameters	SRT(days)			
	5	10	20	40
Effluent COD (mg/L)	18.7 ± 8.3	11.3 ± 8.5	6.4 ± 5.8	5.4 ± 4.9
COD removal (%)	89.6	93.7	95.7	97
Effluent NH ₃ -N (mg/L)	4.2 ± 7.9	0.05 ± 0.04	0.04 ± 0.06	0.04 ± 0.03
NH ₃ -N removal (%)	91.5	99.9	99.9	99.9
MLSS (g/L)	0.33 ± 0.08	0.82 ± 0.12	1.34 ± 0.20	2.16 ± 0.27
Effluent turbidity (NTU)	0.64 ± 0.21	0.72 ± 0.22	0.52 ± 0.08	0.45 ± 0.14
Sludge viscosity (mPa·s)	1.19	1.06	1.42	1.87
F/M ratio (kg-COD/kg-MLSS)	0.55	0.22	0.13	0.08

Mean value \pm standard deviation.n > 20 for effluent COD, NH₃-N, turbidity, and MLSS.

40-day SRT samples, a SEM of LEO153VP (Carl Zeiss, Germany) was used.

2.3.2. EPS extraction and measurement

The TB-EPS in the biofilm on the surface and inside the mesh pores were also measured using the same method as described above. At the end of the operation, the tightly-bound biofilm on the surface of the support layer was scraped off for EPS analysis. Then the residual TB-EPS in the remaining support material was measured. Each sample was measured in triplicate. FTIR analysis was also conducted for the scrapped off biomass.

2.3.3. CLSM image acquisition

Four pieces of mesh samples with a size of approximately 0.5×0.5 cm were cut randomly from each module. Two of the four samples with surface biofilm scrapped off were used to investigate the biofilm or biofoulants inside the mesh pores. Of the two pairs of samples, one was used to investigate the distribution of protein and polysaccharides and the other was used for the live and dead cells analysis. Fluoresceins of isothiocyanate (FITC), Concanavalin A (ConA), Calcofluor white (CW), SYTO9, and Propidium iodide (PI) were used to stain and probe the proteins (EPS), α -D-glucopyranose polysaccharides (EPS), live cells, and dead cells, respectively [21,22].

The collected samples were immediately stained under dark conditions. Specifically, to investigate the distribution of protein and polysaccharides, the sample was first stained with 20 μ L FITC solution (10 g/L) for 30 min, before which a bit of sodium bicarbonate (1 M) buffer was added to maintain pH of 9. In addition, sodium bicarbonate buffer can keep the amine group in a non-protonated form. Next, Con A solution (0.25 g/L) was added and then the sample was incubated for 30 min. Finally, Calcofluor white (0.3 g/L) was added to incubate for another 30 min. The second sample was immersed in SYTO9 (10 μ M) and Propidium iodide (60 μ M) solution for 30 min. For investigating the live and dead cells, a fresh sample was immersed in the solution containing both SYTO9 (10 μ M) and Propidium iodide (60 μ M) for 30 min. After each staining step, PBS solution was utilized to wash the labeled sample several times to remove the extra probes.

A CLSM of Zeiss LSM880 (Germany) was used to scan the samples. The scanned regimes were $859 \times 859 \,\mu\text{m}^2$, respectively. Fluorescence emission was determined in a series of XY images. Each image corresponded with each of the Z position (depth). The optical sections were scanned from the surface to its base in 3.3 μ m and 1 μ m sections respectively for EPS and Live/Dead cells, to obtain a good resolution in the YZ plane for 3D images [21]. Movie files generated from the image stack were saved as uncompressed AVI files. The specific operating conditions and parameters are shown in Table S1.

2.3.4. CLSM image analysis and quantification

After obtaining the CLSM images, the thickness, biovolume, coverage, and distribution in depths for the stained components could be determined using digital image analysis of the CLSM optical thin sections in each of the channels [21,23,24]. The quantification in the series of XY images was determined using Image proplus 6.0 and ZEISS confocal software (ZEN 2.0). The images of each fluorochrome were defined as "channels" in this analysis. For each image, the area occupied (μm^2) by the pixels in each channel was measured, which determined the averaged area occupied by the component in each section. The averaged thickness for the surface biofilm is equal to the sum of all individual section. The biovolume of each component, including the live cell, dead cell, carbohydrate, and protein, in the scanned regime, was equal to the sum of the product of average coverage and thickness in each section. The ratios of Live/Dead cells and protein/polysaccharide (PN/PS) were determined based upon their biovolumes. The porosity for the surface biofilm is given as the percentage of void volume (the difference between the total volume and the biovolumes for all of the analyzed components) in the total volume. The total volume is equal to the product of CLSM image area and averaged biofilm thickness.

3. Results

3.1. Reactor performance

Table 1 summarizes the treatment performance of DMBR with different SRTs under the steady state conditions. Though all of the reactors had good removal efficiency for COD and ammonia, their removal efficiency still increased as SRT was prolonged. This was in agreement with a previous study which found that both COD and ammonia removal efficiency increased with increasing SRT in the complete mixed activated sludge systems [17]. After the biofilm was formed, the effluent turbidity for the four SRTs was always less than 2 NTU with averaged values of less than 1 NTU (Fig. 1(a)), which again evidenced that the coarse-pore support material with self-formed biofilm could achieve excellent solid separation. During the 200 days' operation, the reactors were operated with a constant flux and thus the TMP change was directly associated with the filtration resistance.

As shown in Fig. 1(b), the 5-day SRT reactor was operated with a low TMP of less than 20 Pa for 40 days and then the TMP increased rapidly to approximately 2000 Pa within 5 days. After that, the mesh filter was taken out and flushed with tap water. From 45th day to 180th day at 5-day SRT, the mesh filter was cleaned for five times and each operation cycle lasted for 20–40 days. At 10-day SRT, the first and second operation cycles lasted for 64 and 75 days, respectively. The TMP at 20-day SRT was always below 20 Pa throughout the whole experiment. Though the filter was fouled at 40-day SRT during the start-up period, it was operated with a low TMP of less than 20 Pa for more than 180 days after the first cleaning. The results shown in Fig. 1(b) demonstrated that the rapid rise in TMP for the membrane (biofilm + support material) occurred more frequently with decreasing SRT, which was consistent with the results observed by Duan et al. (2011) [25]. However, the mechanisms behind this phenomenon in



Fig. 1. Change in (a) the effluent turbidity and (b) the operational transmembrane pressure (TMP) under different SRTs.

DMBR had not been well studied previously.

3.2. MLSS concentration, apparent viscosity, particle size distribution, and biomass activity

The MLSS concentration, sludge floc size distribution, and apparent viscosity of mixed liquor could impact the filtration process. A few studies indicated that the membrane fouling and resistance could increase with increasing sludge concentration and apparent viscosity [26,27]. In this study, both the MLSS concentration and apparent viscosity for activated sludge increased with increasing SRT (Fig. 2 and Table 1). Conversely, the rapid rise in TMP occurred less frequently



Fig. 2. MLSS concentration, specific oxygen uptake rate (SOUR) for COD biodegradation, food/microorganism (F/M) ratio, and live/dead (L/D) cell ratio under different SRTs.

with increasing SRT (Fig. 1(b)). This indicated that the rapid rise in TMP or higher hydraulic resistance at low SRT did not result from the MLSS concentration and apparent viscosity. Fig. S2 shows that the sludge at different SRTs had similar size distribution. The similarity in the sludge floc size suggested that the differential filtration performance among SRTs was not contributed by particle size. The increase in the MLSS concentration at a greater SRT will reduce the food/microorganism (F/M) ratio, which further impacts the biomass activity, i.e., SOUR. Fig. 2 shows that both the SOUR and F/M ratio decreased with increasing SRT, which were negatively correlated with the frequency of rapid rise in TMP. The decrease in the SOUR was due to the decrease in the ratio of Live/Dead cells (Fig. 2 and Fig. S3). As SRT increased, more active cells would die due to endogenous decay [28].

3.3. SEM images for the biofilm on the surface and inside the mesh pores

Fig. 3 shows that the biomass concentration per unit area of the surface biofilm increased from $2.3 \pm 0.3 \text{ g-SS/m}^2$ at 5-day SRT to 8.9 ± 1.9 and $11.3 \pm 3.6 \text{ g-SS/m}^2$ at 10- and 20-day SRTs, respectively. At 40-day SRT, it decreased to approximately 4.8 g-SS/m^2 . Interestingly, the filtration resistance did not positively correlate to the biomass concentration on the mesh surface. This indicated that the thickness or the biomass concentration per unit area of the biofilm was not the crucial factor of determining the overall hydraulic resistance.

The SEM images show that the structures of biofilm formed under different SRTs were significantly different (Fig. 4). A thin but dense gellike layer was formed on the mesh surface at 5-day SRT (Fig. 4 (a)). The SEM image with greater amplification (Fig. 4(b)) shows that the biofilm formed at 5-day SRT was compact and nonporous. After scraping the surface layer, it was clearly seen that a mass of biopolymers tightly adhered to the inner structure of the support layer and thus most of the pores were blocked. As a result, the operation TMP increased significantly at 5-day SRT (Fig. 1(b)). At 10- and 20-day SRTs, however, abundant filaments intertwined together and formed a thick but porous biofilm layer. Probably due to over dehydration, the biofilm for 10- and 20-day SRTs shown in the SEM images were cracked. As shown in



Fig. 3. Biomass concentration per unit area of biofilm on mesh surface under different SRTs.

Fig. 4(f) and (i), although a significant amount of biomass had adhered to the pore walls at 10- and 20-day SRTs, they were not completely blocked. As a result, the combination of porous biofilm layer and unblocked support layer had provided excellent particle rejection with low TMP < 20 Pa. At 40-day SRT, the mesh surface was covered by some scattered microbial clusters, bacterial filaments, and biopolymers, as shown in Fig. 4(j) and (k). Though some biomass adhered on the pore walls, the mesh pores were not significantly blocked after 180 days' operation. The size for most pores, however, were still reduced significantly and, as a result, low effluent turbidity was achieved at 40-day SRT (Fig. 1(a)).

Fig. 4 indicated the diversity of biofilm structures under various SRTs. A previous study found that without the formation of a dynamic membrane, the effluent turbidity could be greater than 20 NTU for a mesh filter with the same pore size [18]. The low effluent turbidity (Fig. 1(a)) suggested that the combinations (biofilm + support material) under all of the tested SRTs were effective to reject particles. However, the difference in the biofilm structures had resulted in the inconsistent TMP changes under various SRTs. The formation of a gellike and nonporous biofilm had increased the filtration resistance dramatically at 5-day SRT. The mesh filter with porous biofilm, however, could reject particles effectively with a low TMP. Moreover, the porous biofilm could prevent the particles from depositing in the mesh pores



Fig. 4. Scanning electron microscope (SEM) images for the biofilm on the surface and inside the mesh pores. (a), (b), and (c) for 5-d SRT; (d), (e) and (f) for 10-d SRT; (g), (h) and (i) for 20-d SRT; and (j), (k) and (l) for 40-d SRT. Some *Euglypha* observed in the biofilm were circled in red. The SEM image for the fresh mesh is shown in Fig. S5. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



Fig. 5. (a) Concentrations of tightly bound extracellular polymeric substance (EB-EPS) per unit biomass in the surface biofilm (SB) (i.e., biofilm on the mesh surface) and activated sludge (AS); (b) TB-EPS concentration per unit area of the surface biofilm and biofilm inside the mesh pores (MP); and (c) ratio of protein/poly-saccharides (PN/PS) for the EPS in AS, SB, and MP.

and kept the support layer unblocked [29,30]. Therefore, forming a porous biofilm layer is critically important for the success of DMBR.

It is worth to notice that lots of protozoa (e.g., *Euglypha*) were observed on/in the biofilms and even inside the mesh pores at 10-, 20-, and 40-day SRTs. *Euglypha* is commonly found in activated sludge and biofilms [31]. They are predators of bacteria like nitrifiers [32]. The protozoa would feed on the biofilm and also shuttle back and forth in the biofilm and even in the mesh pores, which could help to maintain the biofilm porous and support layer unblocked [33].

3.4. FTIR analysis

As shown in Fig. S4, the eight FTIR spectra curves shown similar profiles, suggesting that the biomass in both activated sludge and biofilm under different SRTs had similar functional groups. Peaks in the vicinity of 3,430, 2,929, and $1074 \,\mathrm{cm^{-1}}$ were attributed to the stretching of O–H bond in the hydroxyl functional groups, C–H bond, and C–O or C–O–C bonds from polysaccharides, respectively [34,35]. Three characteristic bonds for protein at about 1,656, 1,548, and 1247 cm⁻¹ were also observed in the spectra, which were unique to the secondary structure of proteins, namely amides I, II, and III, respectively [36,37]. These results indicated the presence of polysaccharides and proteins in the bulk sludge and biofilm. Jarusutthirak et al. (2002) reported that the asymmetrical stretching peak could be observed at 1715 cm⁻¹ when humic acids were present. This peak, however, was not detected, indicating a low concentration of humic acids in our samples.

3.5. EPS in activated sludge and biofilms on the surface and inside the mesh pores

The TB-EPS concentration per unit biomass in activated sludge and surface biofilm under different SRTs are shown in Fig. 5(a). Obviously, the protein was the major quantified TB-EPS component in the surface biofilm. Its concentration in the surface biofilm at 5-day SRT (56.2 mg/ g-VSS) was significantly greater than that at 10- and 20-day SRTs (25.9 and 29.1 mg/g-VSS, respectively). However, the polysaccharide concentrations were similar from 5- to 20-day SRTs. Interestingly, both protein and polysaccharide concentrations in the surface biofilm at 40day SRT were greater than those at other SRTs, probably due to greater endogenous metabolism at a long SRT that produced more EPS from cell lysis [38]. For the EPS in activated sludge, the protein was still the major component under all of the tested SRTs. From 5-day to 20-day SRTs, the activated sludge contained much greater concentrations of both protein and polysaccharide than the dynamic layer did. Unlike EPS in the dynamic layer, both concentrations in activated sludge were similar among different SRTs. This result likely suggests that a distinct correlation between the activated sludge and surface biofilm in terms of EPS concentration did not exist.

Fig. 5(b) shows the EPS concentration per unit area of surface biofilm and support layer (i.e., biofilms inside the mesh pores). Protein was still the major EPS in the support layer. Only at 5-day SRT, the support layer had a greater EPS concentration than the surface biofilm did. Among different SRTs, the protein concentrations in the support layer were similar. A consistent correlation between EPS concentrations and hydraulic resistance or rapid rise in TMP under various SRTs could not



Fig. 6. Integrated confocal laser scanning microscopy (CLSM) images of EPS in the surface biofilm layer at (a) 5-day, (b) 10-day, (c) 20-day, and (d) 40-day SRTs; Integrated CLSM images of EPS in the mesh pores at (e) 5-day, (f) 10-day, (g) 20-day, and (h) 40-day SRTs; (i) fresh mesh. The colors of light green, red, and blue stand for proteins (FITC), α -D-glucopyranose polysaccharides (ConA), and β -D-glucopyranose polysaccharides (CW), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

be found. Instead of EPS quantity, the PN/PS ratio is a parameter that is more related to membrane biofouling [39]. As shown in Fig. 5(c), the PN/PS ratio in the surface biofilm decreased from 5.5 at 5-day SRT to 3.3 and 2.1 at 10 and 20-day SRTs, respectively, suggesting a positive correlation between the PN/PS ratio and the rapid rise in TMP or hydraulic resistance. Under the same SRT, the PN/PS ratios for the biofilm on the surface and inside the mesh pores were very close, indicating that the biomass in both layers had similar EPS compositions. Previous studies indicated that polysaccharide was generally considered as a hydrophilic substance, while the protein was more hydrophobic [40]. A greater PN/PS ratio could enhance the hydrophobicity and stickiness of the biofilm [41]. As a result, the biofilm at the 5-day SRT was compact and a gel layer was developed with high hydraulic resistance. At 10-day SRT or more, however, the biofilm was loose and porous and a gel layer was not formed.

3.6. CLSM analysis for the biofilm on the surface

The reconstructed 3D CLSM images for the EPS distributed in the biofilm are shown in Fig. 6(a–d) and the 2D images scanned at 1–10 μ m above the support layer were exhibited in Figs. S6(a–d). The biofilm had non-uniform structures with uneven protrusions. Obviously, protein (green) coverage occupied the majority of CLSM image and only small areas were α -D-glucopyranose polysaccharides (red) and β -D-glucopyranose polysaccharides (red) and β -D-glucopyranose polysaccharides (blue). Proteins were aggregated into clusters and compact, while polysaccharides were well dispersed. The ratios of PN/PS based on CLSM analysis were 2.54, 2.50, 1.22, and 1.34 at 5-, 10-, 20-, and 40-day SRTs (Fig. 8), respectively, again

suggesting that proteins were the major EPS in the surface biofilm. Although the values of PN/PS ratio determined by CLSM analysis was different from those measured by extraction method (Fig. 5(c)), its change trend with SRT was consistent, both decreasing with the increase in SRT. The 3D reconstruction CLSM side-view images suggested that the surface biofilm at SRTs of 5-, 10-, 20- and 40-day had a mean thickness of 95, 195, 270, and 161 μ m, respectively (Fig. 8). This is agreement with the result shown in Fig. 3 and again suggested that the filtration resistance was not mainly determined by the thickness of biofilm. Wang et al. also reported that the hydraulic resistance of biofilm was not only affected by the layer thickness and EPS concentration, but also by the biofilm structure and morphology [30].

Fig. 7(a–d) shows the reconstructed 3D CLSM images for the live and dead cells in the biofilm, with the 2D images exhibited in Fig. S7 (ad). Live cells were aggregated into clusters, while dead cells were dispersed relatively well. The Live/Dead cell ratios in the surface biofilm at 5-, 10-, 20-, and 40-day SRTs were 1.05, 0.90, 0.59, and 0.30 (Fig. 8), respectively. This indicated that SRT also had a great impact on the cells in the surface biofilm and more active cells would die as a result of endogenous decay or predation at a greater SRT, which could lead to different outputs of EPS [42]. Therefore, the lower ratio of Live/Dead cell might also contribute to the loose and porous biofilm at the extended SRTs.

3.7. CLSM analysis for the biofilm inside the mesh pores

The biofilm inside the mesh pores could also work as biofoulants. Fig. 6(e-h) shows that a significant amount of proteins and



Fig. 7. Integrated confocal laser scanning microscopy (CLSM) images of cells on the surface biofilm layer at (a) 5-day, (b) 10-day, (c) 20-day, and (d) 40-day SRTs; Integrated CLSM images of cells in the mesh pores at (a) 5-day, (b) 10-day, (c) 20-day, and (d) 40-day SRTs; (i) fresh mesh. The colors of light green and red stand for live cells (SYTO9) and dead cells (PI), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



Fig. 8. Correlations of filtration resistance with the biofilm structural parameters under different SRTs determined based on confocal laser scanning microscopy (CLSM) analysis. The hydraulic resistance was calculated using the flux and TMP data when the membranes were sampled. (R_t = resistance; PN/ PS = protein/polysaccharide; L/D = Live/Dead).

polysaccharides accumulated in the mesh pores under various SRTs. Proteins were still the major EPS, as shown in Figure Fig. 6. The CLSM images, however, could not exhibit the detailed structures and morphology of biofilm inside the pores. The SEM images clearly showed that, though biofilm or biofoulants had accumulated in the pores with SRTs \geq 10 days, enough water flow pathways still existed. The support material used in this study had a pore size of approximately 25 µm. As a

result, the live cells could easily grow on the pore walls (Fig. 7(e–h)), which would foul the support layer. On the other hand, the live cells would release hydrolase which could hydrolyze the EPS compounds [43] and then mitigated the biofouling, especially under long SRTs. This might be a possible mechanism for lower biofouling in the support layer that occurred during the long-term operation of DMBR with an extended SRT. The pore size of MF/UF membranes was smaller than most of the bacteria and, as a result, EPS were the major biofoulants inside the pores and the biofouling became irreversible.

4. Discussion

The combination of the self-formed biofilm and the support material provides filtration in DMBR and their structure and hydraulic resistance determine the filtration performance. The biofilm formed on the support materials under different SRTs had very diverse structures (Fig. 4). The low effluent turbidity (Fig. 1(a)) suggested that all of the biofilms were effective to reject solids. However, the occurrence of a rapid rise in TMP decreased significantly with increasing SRT. The TMP rising occurred every 20–40 days at 5-day SRT, while the reactors with 20-and 40-day SRTs could be operated with a low TMP < 20 Pa (hydraulic resistance < $2.5 \times 10^9 \text{ m}^{-1}$) for more than 180 days. Further study indicated that the difference in the filtration performance under various SRTs did not result from the physical properties of activated sludge. However, it positively correlated to the F/M ratio and biomass activity (Fig. 2).

The biofilm formed at 5-day SRT contained a large amount of gellike substances. These biofilms were tightly adhered to the support material to form a thin but compact gel layer and most of the mesh pores were completely blocked. As SRT extended to 10 days or more, some filamentous bacteria worked as skeletons, which were surrounded by live cells, dead cells, and EPS, to form thicker but porous biofilms. Although a significant amount of biofilm accumulated inside the mesh pores, enough water flow pathways still existed. It was reported that the dynamic membrane formed on the support material consisted of a cake layer and an underlying gel layer [44]. In this study, however, a gel-like layer was found only at 5-day SRT. Therefore, SRT had played a significant role in the biofilm structures. Forming a porous biofilm layer is the key to achieving long-term low-pressure filtration in DMBR. The porous biofilm can serve as a barrier for rejecting and preventing fine particles from depositing in the mesh pores.

The gel-like biofilm at 5-day SRT had the greatest values of both PN/PS ratio and Live/Dead cell ratio. The values for both ratios decreased significantly as SRT increased (Figs. 5 and 8). The porosity for the biofilm, however, generally increased with increasing SRT (Fig. 8), suggesting that the porosity of biofilm was negatively correlated with the ratios of PN/PS or Live/Dead cell. Polysaccharides are solely hydrophilic substances, while proteins are generally more hydrophobic [40,45]. Thus, increasing PN/PS ratio could promote the formation of a compact gel layer and then decrease the biofilm porosity and permeability. As shown in Fig. 7, the live cells were aggregated into clusters, while dead cells were dispersed relatively well. In addition, the cells in the biofilm would impact EPS production. Consequently, the porosity of biofilm was negatively correlated with the Live/Dead cell ratio.

In addition to the PN/PS ratio, we assume that the species and biochemical properties for the proteins in the EPS could also change with SRT, which had a further impact on the biofilm structures. The EPS were from either substrate degradation during cell growth or cell lysis as a result of endogenous decay [40]. The 5-day SRT reactor had a higher F/M ratio (Fig. 2) and so the substrate concentrations of COD and ammonia in the reactor were greater than those under the extended SRTs (Table 1). As a result, its biofilm had a greater Live/Dead cell ratio (Fig. 8) and more proteins could be produced from cell growth. An extended SRT had decreased F/M ratio and then lowered the substrate concentrations in the reactor. Consequently, the biofilm had a smaller Live/Dead cell ratio and more proteins could be yielded from cell lysis. Possibly, the species and biochemical properties for the proteins from both sources were different [40], which might also impact the compactness and stickiness of biofilm. Unfortunately, the analysis used in this study and most references could not distinguish between them.

In addition to EPS compositions, the protozoa which inhabited the biofilm could also have significantly impacted its structures and support layer biofouling. Many *Euglypha* were observed (Fig. 4) in the biofilm with SRT \geq 10 days. These small protozoa would prey on the bacteria and dig into the biofilm [46] and even the mesh pores, which could help to maintain the biofilm porous and mitigate biofouling of the support layer. More protozoa were observed in the biofilm with a longer SRT, which could be one of the reasons for the thinner dynamic layer formed at 40-day SRT. In MF/UF MBRs, the protozoa could not pass the membrane pores to mitigate the biofouling. However, more studies are needed to determine the roles of protozoa in the formation of porous biofilm and unblocked support layer.

5. Conclusion

The biofilm formed on the support materials under different SRTs had very diverse structures and all of them were effective to reject solids. However, the hydraulic resistance and the occurrence of a rapid rise in TMP for the combination of biofilm and support material increased significantly with decreasing SRT. At 5-day SRT, a thin but compact gel-like layer was formed and the mesh pores were blocked significantly. As a result, the TMP rose significantly every 20–40 days. The TMP kept consistently low for more than 180 days' operation at 20-and 40-day SRTs since a longer SRT promoted the formation of a thick

but porous biofilm layer. Therefore, forming a porous biofilm layer was critically important for the long-term and stable operation of DMBRs. The porosity of biofilm was negatively correlated with the protein/polysaccharide (PN/PS) in the biofilm. Extending SRT lowered the Live/Dead cell ratio (decreasing EPS production) and increased the population of protozoa (e.g., *Euglypha*, feeding, and movement) in the biofilm, both of which increase the biofilm porosity and mitigate the support layer biofouling.

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Appendix A. Supplementary data

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