Journal of Membrane Science Letters 2 (2022) 100023

Contents lists available at ScienceDirect

Journal of Membrane Science Letters

journal homepage: www.elsevier.com/locate/memlet

Linking endogenous decay and sludge bulking in the microbial community to membrane fouling at sub-critical flux

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ARTICLE INFO

Keywords:
Membrane fouling
Microbial community
Membrane bioreactors
Biofouling layer
Sludge bulking

ABSTRACT

This study examined membrane fouling and associated microbial taxa in a membrane bioreactor operating at a sub-critical flux condition using next-generation amplicon sequencing. The membrane was operated at a sub-critical flux, thus, fouling was not observed until endogenous decay. The observed fouling could be attributed to endogenous decay which was driven by nutrient deficiency at high sludge age and low food-to-microorganisms ratio (decreasing from 0.15 to 0.09 gBOD/gMLVSS.d). Endogenous decay resulted in a sharp decrease of the number of species and evenness between different species (49.7 and 58.9% compared to the inoculum, respectively). The release of dissolved organic matters and cell debris from endogenous decay as well as the excessive growth of filamentous bacteria, e.g. *Thiotrichales* were the main contributors to membrane fouling. The relative abundance of *Thiotrichales* significantly correlated with TMP (Pearson R = 0.996, *p*-value <0.001), indicating this order's contribution to membrane fouling. Other dominant orders in the mixed liquor after endogenous decay such as *Rhizobiales, Burkholderiales, Rhodospirillales* and *Myxococcales, Flavobacteriales* can produce extracellular polymeric substances and aggravating membrane fouling. Fouling layers possess highly similar microbial composition with the mixed liquor, with some filamentous microbial orders, e.g. *Corynebacteriales* and *Oligoflexales* showing increased relative abundance by 6.83 and 5.64 folds, respectively.

1. Introduction

Membrane bioreactor (MBR) offers numerous advantages over the conventional activated sludge process, including better effluent quality and smaller footprint. A recent life cycle assessment conducted by Banti et al. (2020) also showed that MBR has significantly lower environmental impacts e.g. eutrophication potential and global warming potential compared with conventional activated sludge (CAS) treatment plant. Since 2008, more than 2500 MBR plants have been constructed worldwide (Krzeminski et al., 2017). The estimated global MBR treatment capacity was 20 GLD (gigalitres per day) in 2019 (Judd, 2019). Nevertheless, membrane fouling remains the most challenging issue in MBR operation and limits MBR widespread application. High energy consumption and operational costs associated with membrane cleaning and replacement made retrofitting from CAS to MBR a controversial topic when considering both economical and management aspects (Hao et al., 2018; Gao et al., 2021). Thus, characterization of membrane fouling, optimization of operating conditions, and development of novel methods for fouling mitigation have attracted great interest (Meng et al., 2017).

Operating conditions such as permeate flux, solids retention time (SRT), food-to-microorganisms (F/M) ratio, as well as sludge characteristics can have considerable impacts on fouling rate (Van den Broeck et al., 2012; Wu et al., 2013; Pan et al., 2010). Researchers have reach a consensus that operation below the critical flux can minimize membrane fouling (Meng et al., 2017; Bacchin et al., 2006; Field et al., 1995), due to the restrain of foulants deposition on the membrane surface (Li et al., 2014; Kimura et al., 2008). Nguyen et al. (2016) reported that applying a low flux of 2 LMH (liters per square meter per hour) can delayed fouling onset for more than 30 days when treating hospital wastewater treatment. Thanh et al. (2013) also observed lower fouling rate at low operating fluxes (1.2 and 2.4 LMH) in MBR treatment of high strength leachate from a solid waste transfer station. In addition, operating at sub-critical flux (20 LMH) can result in lower formation of polysaccharides, protein, and high molecular weight organics (~ 48 kDa) than operating above critical flux condition (40 LMH) (Johir et al., 2012). SRT is another major influencer on membrane fouling, however, the impact of SRT reported in the literature was controversial (Ouyang and Liu, 2009; Han et al., 2005; Deb et al., 2022; Fu et al., 2017). Lower membrane fouling rate was achieved at elevated SRT of 40+ days or even complete sludge retention (Van den Broeck et al., 2012; Ouyang and Liu, 2009). In contrast, Han et al. (2005) found that membrane fouling increased with SRT since sludge particles were more severely deposited on the membrane surface at longer SRT (100 days vs. 30–70 days).

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https://doi.org/10.1016/j.memlet.2022.100023
Received 25 December 2021; Received in revised form 16 March 2022; Accepted 26 March 2022

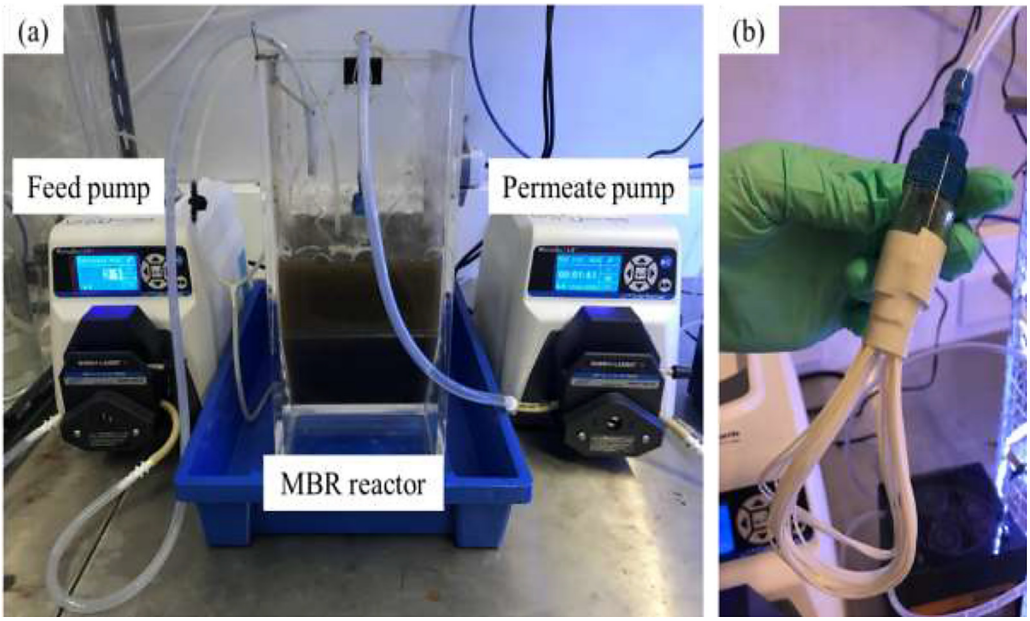

Fig. 1. (A) Laboratory-scale membrane bioreactor setup and (B) hollow-fiber membrane module.

The substrate deficient state (low F/M ratio) created by long SRT can also reduce specific bioactivity and trigger endogenous respiration in microbial cells (Ouyang and Liu, 2009; Han et al., 2005). These conditions are prone to excessive growth of filamentous bacteria (sludge bulking) in the reactor. Filamentous bacteria have greater capacity of energy storage and easier access to nutrient compare to other bacteria (Pan et al., 2010), making them more resilient under limited substrate conditions. Most previous studies found that membrane fouling behaviour induced by bulking sludge was significantly more severe in comparison with normal sludge (Tian et al., 2011; Li et al., 2008; Meng and Yang, 2007). However, these studies mainly focused on examining sludge characteristics using visual/physiochemical analyses such as floc morphology, relative hydrophobicity, three-dimensional excitation-emission matrix fluorescence spectroscopy (Pan et al., 2010; Meng and Yang, 2007; Meng et al., 2006a, 2006b; Wang et al., 2010). Investigation of the microbial community presented in bulking sludge will provide more insights into sludge bulking impact on membrane fouling.

This study aims to investigate the fouling mechanism and effect of filamentous bacteria on fouling under long-term operation at sub-critical flux and infinite SRT (complete sludge retention). Next-generation sequencing on Illumina Miseq platform was performed to characterize the microbial community profile in the mixed liquor and fouling layer over time. Results from this study can provide the fundamental understanding for fouling mitigation under sub-critical flux condition through optimizing operating conditions.

2. Materials and methods

2.1. Laboratory-scale membrane bioreactor system setup

A laboratory-scale aerobic MBR system was used in this study (Fig. 1). The MBR was equipped with a 5 L reactor, two identical hollow fibre membrane modules (Evoqua, Australia), two peristaltic pumps, a digital pressure gauge, and an air pump connected to a diffuser for aeration. The two peristaltic pumps (Masterflex L/S, USA) were used for feeding and permeate extraction. Each of the two membrane modules consisted of 20 polyvinylidene difluoride fibers with a nominal pore size of 0.04 μm and a length of 30 cm (effective surface area of 0.02 m^2). One membrane module was submerged in the reactor but was not connected with the pump to examine the biofouling layer under static condition (denoted ST-BF or biofouling layer on static membrane), the other module was connected with the permeate pump to examine the biofouling layer with permeation (denoted as PM-BF or biofouling layer on membrane with permeation). The digital pressure gauge was a high-resolution pressure sensor (± 0.1 kPa, John Morris Group, Australia), which was installed between the membrane module and the permeate pump for continuous monitoring of the transmembrane pressure (TMP). The reactor's working volume were maintained at 3 L. The air pump (AquaOne, Australia) aerated the reactor at an air flowrate of 0.4 L/min via a diffuser at the bottom of the reactor.

2.2. Operating protocol

Activated sludge was collected from a wastewater treatment plant (New South Wales, Australia) and used as the inoculum. The MBR was fed with synthetic wastewater to ensure a consistent composition of carbon, nitrogen, and phosphorus for microbial growth. The synthetic feed has COD: TN: TP = 150: 6.5: 1, which is similar to the municipal sewage. In details, the synthetic feed solution (influent) contains the following ingredients in mg/L: glucose (600), peptone (100), urea (35), KH_2PO_4 (17.5), $MgSO_4$ (17.5), $FeSO_4$ (10), and sodium acetate (225) as described in previous studies (Nguyen et al., 2022; Nguyen et al., 2013). The average total organic carbon (TOC) of the synthetic feed was 238.8 ± 17.0 mg/L. The MBR was operated at a flux of 6.25 LMH corresponding to a hydraulic retention time of 24 h. Prior to the main experimental period and biomass collection for analysis, the MBR was acclimatised under the same operating condition for one month.

The performance of the MBR was evaluated by monitoring effluent, influent, and mixed liquor twice per week. Samples were analysed for pH, dissolved oxygen (DO) concentration, TOC, nitrate concentration, mixed liquor suspended solids (MLSS), and mixed liquor volatile suspended solids (MLVSS). MLSS and MLVSS were measured according to the standard method 2540D. TOC was analysed using a TOC-V_{CSH} analyser (Shimadzu, Japan). Nitrate concentration was measured using ion chromatography (Thermo Scientific, Australia). DO concentration of the MBR was maintained at 6.5 ± 1.3 mg/L.

2.3. DNA extraction and quality monitoring

Duplicate samples of the inoculum were collected at the beginning of the experiment. Duplicate samples of the mixed liquor were collected at the end of the acclimatisation period (day 39), and at severe fouling

stage (day 102 and day 142). Biofouling layer on the surface of static membrane (denoted as ST-BF) and membrane with permeation (denoted as PM-BF) were collected at severe fouling stage (day 142) by sonication (72 W, 43 ± 2 kHz) for 2 min in Milli Q water, following by centrifuge (3500 rpm, 10 min).

Each sample was mixed with 100% v/v ethanol (1:1 v/v) and stored at -20 °C prior to DNA extraction. Genomic DNA extraction was carried out using DNAeasy PowerSoil Pro Kit (50) (Qiagen) following the manual's instructions. The integrity, purity and concentration of the extracted DNA were evaluated by NanoDrop® spectrophotometer. DNA concentration of all samples was normalized to 10 ng/μl using DNase/Pyrogen-Free Water before sending to the sequencing facility.

2.4. Amplicon sequencing and bioinformatics analysis

The universal primer set Pro341F (5'-CCTACGGGNBGCASCAG-3') and Pro805R (5'- GACTACNVGGGTATCTAATCC-3') was used to amplify 16S rRNA V3 – V4 regions of the microbial community. Paired-end amplicon sequencing (2 × 300 bp) was carried out on the Illumina MiSeq platform (Australian Genome Research Facility, Melbourne, Australia).

Raw reads were imported into Quantitative Insights into Microbial Ecology (QIIME) 2 (version 2019.10) for computational analysis (Bolyen et al., 2019). Quality filtering, denoising (primer and read trimming), paired-end reads merging, dereplication, chimera filtering and feature clustering (≥ 97% similarity) were performed using the q2-dada2 denoise-paired plugin (Callahan et al., 2016). Forward reads were truncated at position 280 and reverse reads were truncated at position 250 in the 3' end due to decrease in quality. The parameter *min-fold-parent-over-abundance* was set to 8 in the denoising step. Reads were mapped back to amplicon sequence variants (ASV) with a minimum identity of 97% to obtain the number of reads in each feature.

Taxonomy was assigned to features using the q2-feature-classifier (Bokulich et al., 2018) classify-sklearn Naïve Bayes taxonomy classifier against the SILVA database (release 132) (Glöckner et al., 2017; Yilmaz et al., 2013; Quast et al., 2012) with a confidence of 0.7. All features were aligned with mafft (Wu et al., 2013) and used to construct phylogenetics tree with FastTree2 (Price et al., 2010) via the q2-phylogeny align-to-tree-mafft-fasttree pipeline. Diversity metrics (Bray-Curtis dissimilarity) were estimated using q2-diversity core-metrics-phylogenetic pipeline after samples were rarefied (subsampled without replacement) to 66,500 sequences per sample.

3. Results and discussion

3.1. Membrane bioreactor performance and fouling development under stress condition

Fig. 2 shows the basic performance of MBR in three phases: acclimatisation period (day 1–39), stable operation (day 40 – 86), endogenous decay and severe fouling (day 87 onwards). During the acclimatisation period, as expected, the biomass growth as well as TOC and nitrate concentration in the mixed liquor and permeate showed considerable fluctuation. Nevertheless, the TOC removal efficiency was high, in the range of 97.4–98.7%. After the acclimatisation period, the MBR system showed stable biological treatment performance until day 86. TOC removal efficiency increased to 99.1–100%, biomass concentration increased steadily from around 4.3 g/L on day 39 to 7.5 g/L on day 86. MLVSS/MLSS ratio increased from 0.90 ± 0.05 to 0.94 ± 0.03, in other words, active sludge accounts for most of the solids content in the reactor.

In the last phase from day 87, endogenous decay resulted in loss in cell mass due to oxidation of internal storage products (Fig. 2). Along with the decreased sludge concentration, this process released a large amount of dissolved organic matter (indicated by rapid rise in TOC concentration in the mixed liquor – Fig. 2D), soluble nitrogen (Fig. 2C) probably in the form of ammonia as presented in Eq. (1).

$$C_5H_7O_2N + 5O_2 \rightarrow 5CO_2 + NH_3 + 2H_2O \quad (1)$$

Stress conditions including nutritional stress, extreme temperatures and oxidative stress are common factors to trigger microbial endogenous decay (Rice and Bayles, 2008; Hazan et al., 2004). Since sludge withdrawal was not conducted except for performance monitoring, the MBR operated in a high SRT of 265 days (high sludge age) and with a continuously decreasing F/M ratio (from 0.15 on day 4 to 0.09 gBOD/gMLVSS.d on day 86), which can result in decreased sludge production as identified in a previous study (Van Loosdrecht and Henze, 1999). Thus, nutritional stress is likely to be the cause of endogenous decay in this MBR. At the same time with endogenous decay, TMP increased exponentially from 18.6 to 40.8 kPa in 10 days and reached 42 kPa on day 100 (Fig. 2B), suggesting that membrane fouling was related to endogenous decay and its successive events e.g. rise in TOC and changes in microbial community (Section 3.2).

3.2. Microbial succession in the mixed liquor after endogenous decay

Bioinformatics analysis of MBR mixed liquor revealed significant shift in both microbial diversity and composition, especially after endogenous decay. The decrease in diversity of the mixed liquor compared to the inoculum (25% of number of species observed and 7.2% of evenness) during the acclimatisation phase (day 0 to 39) could be attributed to the adaptation of microbes to the new environmental conditions (Table 1). Endogenous decay resulted in another sharp decrease of diversity (day 102), with species richness and evenness 33 and 55.7% lower than day 39. The change in species evenness (Shannon index) caused by endogenous decay was statistically significant (Student t-test, *p*-value 0.01), indicating the predominance of a few microbial species in the mixed liquor. Both biofouling layers on the surface of static and permeate membrane showed reduced number of species and evenness compared to mixed liquor (Table 1). The number of observed species and evenness in the permeate module fouling layer were slightly lower than those of the static module fouling layer, indicating the impact of permeation drag.

The most dominant order in the mixed liquor after endogenous decay were *Thiotrichales* (Fig. 3), accounting for 66.1 ± 3.6 % of the mixed liquor community. *Thiotrichales* is filamentous bacteria involved in activated sludge bulking (Guo and Zhang, 2012). Besides *Thiotrichales*, other filamentous bacteria including *Saccharibacteria* and *Caldilineales* were also detected in the community (Kindaichi et al., 2016). It has been reported that long SRT and low F/M induces the growth of filamentous microorganisms (Dunkel et al., 2018; Kowalska et al., 2016). Sludge that has been retained too long can become septic, lose its activity, and consequently can deplete the necessary DO in the reactor, which favours the growth of filamentous bacteria (Zaidi et al., 2020). In addition, the nutrient compounds in the simulated feed are readily biodegradable and accessible to filamentous bacteria, due to their morphology.

Filamentous bacteria are the backbones within sludge flocs and play pivotal roles in floc formation and floc stability by assisting the aggregation of sludge and colloids (Li et al., 2020; Banti et al., 2021). Previous works have reported that the presence of a small quantity of filamentous microorganisms can increase porosity of the sludge and lower sludge adhesion on the membrane surface (Banti et al., 2017, 2020). For example, *Thiotrichales* was presented in the mixed liquor after acclimatisation (day 39) at 13.1%, but did not lead to any membrane fouling effect. Nevertheless, the predominance of *Thiotrichales* after endogenous decay (relative abundance > 60%) indicates excessive growth of filamentous bacteria, which can have detrimental effects on membrane permeation. Bulking sludge showed two to three times higher cake layer resistance than normal sludge (Meng et al., 2006). In addition, the bulking flocs with irregular shape can easily accumulate on the membrane surface and intertwisted on the membrane fibers due to their irregular morphology (Meng et al., 2006a, 2006b). The fixing action of filamentous bacteria results in more foulants adhering to membrane and enhance their

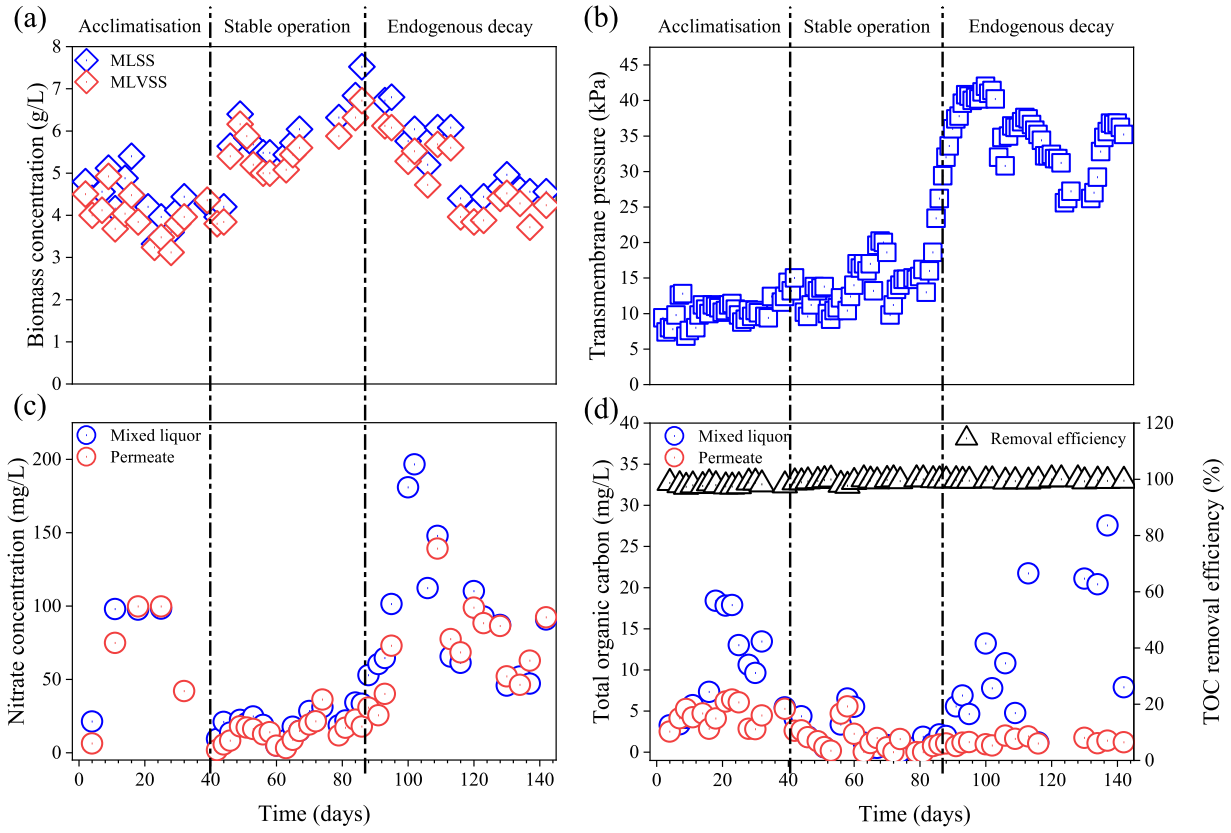

Fig. 2. Biomass growth, total organic carbon concentration and removal, nitrate concentration and transmembrane pressure in the MBR during the experimental period.

Table 1
Changes in number of observed species and evenness (Shannon index). ST-BF: biofouling layer on static membrane, PM-BF: biofouling layer on membrane with permeation.

Sample	Number of observed species	Evenness (Shannon index)
Inoculum	1588.5 ± 67.5	8.25 ± 0.04
Day 39 (after acclimatisation)	1191	7.65
Day 102 (after endogenous decay)	798.5 ± 20.5	3.39 ± 0.07
Day 142	802.5 ± 26.5	4.15 ± 0.13
ST-BF	778	3.64
PM-BF	654	3.21

clinging intensity, which worsen the membrane permeability seriously (Meng et al., 2006a). Sludge bulking also contribute to higher extracellular polymeric substances (EPS) and soluble microbial products (SMP) concentration (Pan et al., 2010; Meng et al., 2006b), and variation in the EPS (higher protein/polysaccharides ratio), leading to an increase in sludge hydrophobicity and surface negative charge (Shen et al., 2020) and aggravating membrane fouling.

The relative abundance of *Thiotrichales* in the mixed liquor significantly correlated with TMP (Pearson R = 0.996, p-value <0.001), indicating their possible contribution to membrane fouling. Results from this study is in consistent with previous report that exponential increase in TMP due to uncontrolled growth of filamentous bacteria in MBR (Banti et al., 2021; Deng et al., 2016) and observation by Meng et al. (Meng et al. (2006a; 2006b) that membrane fouling was most serious under the sludge bulking condition. *Rhizobiales* was the second most dominant order in the community (relative abundance of 9.4 ± 1.9%). The high abundance of *Rhizobiales* could be attributed to its involvement in nitrification process (Cydzik-Kwiatkowska et al., 2016; Zhou et al., 2020) of the ammonia release from endogenous decay. Members of *Rhizobiales* have also been reported to produce polar adhesive holdfasts and fimbrae (Brigmon et al., 2003; Quintero et al., 1998; Marcondes de Souza et al., 2014). Other dominant orders (relative abundance > 1%) such as *Burkholderiales, Rhodospirillales* and *Myxococcales, Flavobacteriales* can produce EPS, colloids, and biosurfactant which increase their ability to attach to the membrane surfaces (Vuko et al., 2020; Vu et al., 2009; Takimoto et al., 2018; Jo et al., 2016; Dang and Lovell, 2015).

3.3. Effects of endogenous decay and sludge bulking on biofouling layer

As noted in Section 3.2, diversity indices of the permeation module fouling layer (PM-BF) were slightly lower than those of the static module fouling layer (ST-BF). Dominant microbial orders of the biofouling layers were similar with the mixed liquor, with the static module fouling layer (ST-BF) possessing higher similarity to mixed liquor than the permeation module fouling layer (PM-BF) (Fig. 4). In the absence of permeation drag (ST-BF), the biofouling layer is formed mostly by adhesion and gravity deposition. Thus, it is expected that composition of the static module biofouling layer shows more similarly to the mixed liquor than the permeation module fouling layer. The most dominant order was *Thiotrichales*, accounted for 69.8 and 75.3% in the static and permeate module fouling layer, respectively. Several microbial taxa show increased relative abundance in the PM-BF compared to the mixed

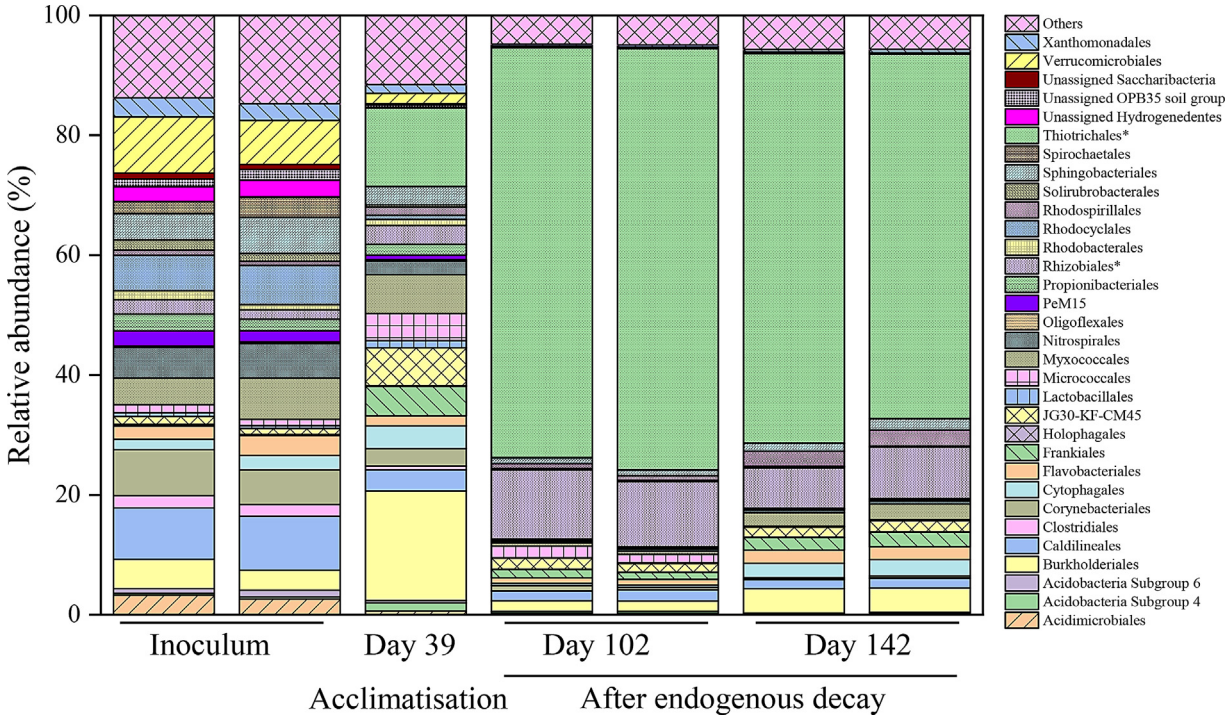

Fig. 3. Changes in microbial composition in the mixed liquor during different phases of MBR operation.

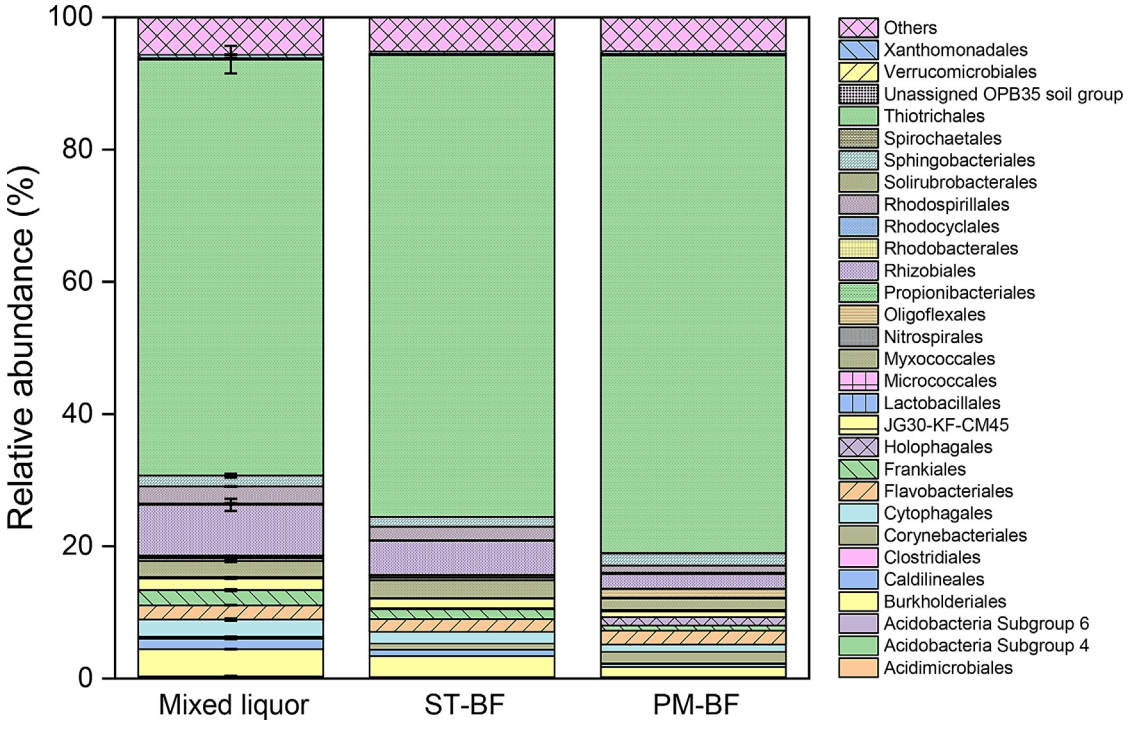

Fig. 4. Microbial composition of mixed liquor, biofouling layer on static membrane (ST-BF) and biofouling layer on membrane with permeation (PM-BF).

liquor, including *Corynebacteriales* (6.83 folds), *Oligoflexales* (5.64 folds) and *Holophagales* (27.3 folds). Both *Corynebacteriales* and *Oligoflexales* order consist of filamentous bacteria, e.g. *Mycobacterium* and *Gordonia*, explaining for their higher abundance. Meanwhile, *Holophagales* is an anaerobic taxa that may develop in the inner side of the thick biofouling layer where oxygen is depleted. It has been established that the overgrowth of filamentous bacteria in sludge suspension could result in the formation of a thick (~200 μm) and loose cake layer, compared to a compact and thin (~20 μm) fouling layer at small quantity of filamentous bacteria (Meng et al., 2006a, Sun et al., 2007).

The spike in amount of foulants released by endogenous decay together with the overgrowth of filamentous bacteria inevitably led to severe membrane fouling, despite the sub-critical flux condition. The low drag force provided by sub-critical flux could not avoid the attachment of filamentous bacteria on the membrane surface. Thus, it is necessary to prevent endogenous decay and excessive growth of filamentous bac-

teria in order to mitigate MBR fouling while operating at sub-critical flux condition, through adjustments of operating parameters such as SRT, F/M and DO concentration. SRT could be adjusted by manually change the sludge wasting rate based on the F/M ratio or MLSS concentration. In this study, endogenous decay occurred at low F/M ratio (below 0.1 gBOD/gMLVSS.d). These results suggest the need for regular removal of activated sludge to maintain F/M higher than this threshold of 0.1 gBOD/gMLVSS.d. The results also highlight the role of filamentous bacteria overgrowth as the underlying cause of membrane fouling under sub-critical flux condition. Several strategies to control the proliferation of filamentous bacteria have also been suggested. Banti et al. (2021) successfully control filamentous bacteria at medium level using a step-aerating MBR, leading to low TMP values ≤ 2 kPa for more than 90 days of operation. Implementing successive anaerobic and aerobic reactors can also limit/suppress *Thiothrix*-caused bulking in dairy wastewater treatment plants (Donkin, 1997). In addition to optimizing the operating parameters, filamentous bacteria can be controlled by chlorination or biocide addition (Guo et al., 2012). Henriet et al. (2017) investigated several strategies to control filamentous bulking caused by *Thiothrix* species in full-scale wastewater treatment plants over 1.5 year. They suggested that polyaluminium chloride addition and volatile fatty acids reduction could not permanently solve the fouling problem, while periodic starvation to avoid endogenous decay could be used to reduce fouling at high sludge age.

4. Conclusions

Under the sub-critical flux condition, nutrient deficiency due to high sludge age and low F/M ratio (decreased from 0.15 to 0.09 gBOD/gMLVSS.d) led to the occurrence of endogenous decay. Once endogenous decay occured, severe membrane fouling can occur even at a sub-critical flux. Membrane fouling during endogenous decay was triggered by the release of dissolved organic matter and cell debris as well as the excessive growth of filamentous bacteria, e.g. *Thiotrichales*. The relative abundance of *Thiotrichales* was positively correlated with TMP increase (Pearson R = 0.996, p-value <0.001). Other dominant orders in the mixed liquor after endogenous decay such as *Rhizobiales, Burkholderiales, Rhodospirillales* and *Myxococcales, Flavobacteriales* can produce EPS and aggravating membrane fouling. Fouling layers possess highly similar microbial composition with the mixed liquor, with static module showing higher similarity with mixed liquor than permeate module. A few filamentous microbial orders, e.g. *Corynebacteriales* and *Oligoflexales* showing increased relative abundance by 6.83 and 5.64 folds in the permeate module compared to the mixed liquor, respectively.

Funding

Funding was received for this work.

All of the sources of funding for the work described in this publication are acknowledged below:

- Research Training Scholarship from the University of Technology Sydney to Quynh A Nguyen.
- Research funding from the Australia Research Council, Australia through the ARC Research Hub for Energy efficient Separation (IH170100009).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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