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Quorum quenching, biological characteristics, and microbial community dynamics as key factors for combating fouling of membrane bioreactors

Syed Salman Ali Shah, Luigi De Simone, Giuseppe Bruno, Hyeona Park, Kibaek Lee, Massimiliano Fabbricino, Irini Angelidaki and Kwang-Ho Choo

Membrane fouling is a major challenge in membrane bioreactors (MBRs) for wastewater treatment. This study investigates the effects of disturbance and solid retention time (SRT) on quorum-quenching (QQ) MBRs relative to antifouling efficacy and microbial community change. The fouling rate increases with the applied disturbance at a short SRT, counteracting the antifouling effect of QQ; however, it decreases with QQ at a long SRT. The microbial community appears to be responsible for such MBR behaviors. Several bacterial species belonging to the biofilm-forming group are dominant after disturbance, resulting in substantive membrane fouling. However, the balance between the bacterial species plays a key role in MBR fouling propensity when stabilized. *Koflera Flava* becomes dominant with QQ, leading to reduced membrane fouling. QQ makes the MBR microbial community more diverse, while lowering its richness. QQ with long SRT would be a favorable operational strategy for effective MBR fouling control.

**INTRODUCTION**

Membrane fouling is the major technical challenge limiting the broader adoption of membrane bioreactors (MBRs) in wastewater treatment and reclamation\(^1\)--\(^3\). The key factors involved in membrane fouling include wastewater composition, membrane and membrane module characteristics, and operating conditions. The interaction between mixed liquor constituents and membranes in an MBR appears to be the factor most responsible for membrane fouling\(^4\). Solid retention time (SRT) is an important parameter influencing mixed liquor constituents (including activated sludge) in MBRs\(^5\). Researchers have found that SRT affects MBR fouling propensity, because biomass content increases with increasing SRT, simultaneously reducing the food-to-microorganism ratio\(^6\). The SRT had additional influences on MBR biopolymer characteristics, such as the excretion of extra-cellular polymeric substances (EPS) and soluble microbial products (SMPs), both of which are considered major membrane foulants\(^7\). Previous research reported that EPS and SMP concentrations decreased with increased SRT, thus alleviating fouling\(^8,9\). However, a contradictory report showed greater degrees of membrane fouling at high SRTs\(^10\). Therefore, further studies on SRT's effects on MBR membrane-fouling propensities are needed.

SRT changes the microbial ecology of activated sludge in an MBR, thus influencing biofouling behaviors\(^11\)--\(^13\). Biofouling occurs when biofilms form on membrane surfaces and is known to be closely linked to microbial cell-to-cell communication through small diffusible signal molecules, known as quorum sensing (QS)\(^14\). There are several approaches to inhibit the bacterial QS system, a process known as quorum quenching (QQ), to interfere with biofilm formation\(^15,16\). The first QQ approach used a QQ enzyme (porcine kidney acylase)\(^17\). This strategy targeted the signal molecule N-acetylhomoserine lactones, successfully demonstrating MBR-biofouling retardation. Microbial QQ strategies for MBR-biofouling control using indigenous QQ bacteria rather than QQ enzymes have also been developed. The isolate *Rhodococcus* sp. BH4 was the first QQ bacterium used for MBR-biofouling control\(^18\). Since then, several indigenous QQ bacteria, such as *Bacillus* sp. T5\(^19\), *Delftia* sp. T6\(^19\), *Bordetella hinzii* S3\(^20\), *Enterococcus* sp. HEMM-1\(^21\), *Acinetobacter bereziniae* strain (ATCC 17924) homolog\(^22\), and *Acinetobacter* sp. DKY-1\(^23\), have been isolated and tested for their QQ activities and membrane-biofouling control capabilities\(^16,24\).

Recently, novel indigenous facultative QQ strains (*Pseudomonas* sp. KS2 and KS10), which have ambidextrous biofouling control activities for both aerobic and anaerobic MBRs, were isolated and characterized\(^25\).

It has also been reported that QS and QQ bacteria coexist in activated sludge in MBRs, meaning that the antagonism between these types of bacteria could influence sludge properties, bioflocculation, and fouling\(^16,26\). Another study showed that SRT altered the abundance of bacterial groups due to QS and QQ activities in the MBR microbial community, which influenced the biofilm formation responsible for membrane biofouling\(^27\). However, SRT's effects on QQ media-assisted antifouling efficacies and MBR microbial community structures are not yet well elucidated.

In addition, factors exposing stress to the microbiomes, such as shear forces, starvation conditions, dissolved oxygen (DO) content, and the presence of antibiotics, also affect MBR membrane fouling by altering mixed liquor characteristics\(^28--30\). The recommended velocity gradient range for biological treatment systems is 40–80 s\(^{-1}\), but higher levels of shear can be applied to MBRs, to minimize membrane fouling. Aeration intensity affected the hydrodynamics and DO levels of mixed liquor in the MBR, but the effects diminished at high biomass levels (>10 g/L) due to increased sludge viscosity\(^32\). Starvation or low food-to-microorganism ratio

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1Department of Environmental Engineering, Kyungpook National University, Daegu, Republic of Korea. 2Department of Civil, Architectural, and Environmental Engineering, University of Naples Federico II, Naples, Italy. 3Advanced Institute of Water Industry, Kyungpook National University, Daegu, Republic of Korea. 4Department of Biotechnology and Bioengineering, Chonnam National University, Gwangju, Republic of Korea. 5Department of Chemical Engineering, Technical University of Denmark, Lyngby, Denmark. 6email: chookh@knu.ac.kr

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increased mixed liquor biopolymer content, leading to increased membrane fouling. Low DO levels (<0.1 mg/L) led to a greater fouling tendency (7.5 times) than high DO concentrations (>3.0 mg/L) because of denser membrane-biofouling layer formation at low DO levels. The effects of environmental stress on QQ in MBR are thus of great interest, because real MBR facilities often encounter such situations.

Therefore, in this study we investigated the impact of SRT and operational disturbance on antifouling efficacy and microbial community structure in MBRs with and without QQ. A stressful environment (starvation with high shear) was implemented in the middle of MBR operation while monitoring membrane fouling pattern and microbial community structure changes. We also evaluated key MBR characteristics, such as biopolymer production, mixed liquor properties, and treatment efficiencies, and their correlations with MBR fouling rates.

RESULTS AND DISCUSSION

Effects of imposed disturbance and SRT on QQ-based antifouling efficacy

Figure 1 shows the fouling rate profiles of MBRs over time under different operating conditions, summarizing the average fouling rates of each phase. Two representative transmembrane pressure (TMP) profiles, the average fouling time, and the number of MBR runs at each phase are also provided in Supplementary Fig. 1 and Supplementary Table 1. In Phase 1, the average fouling rates for Reactors 1 and 2 were nearly the same, i.e., there was no statistically significant difference between the reactors. This confirms that the MBRs were in identical states, in terms of fouling behavior when operated in the conventional mode. In Phase 2, the average fouling rates of Reactors 1 and 2 were also almost the same, showing that the QQ effect on fouling mitigation appears to be insignificant. Unlike previous reports on the QQ effect on antifouling efficacy, it was unclear whether QQ played a role in fouling control under Phase 2 conditions. One possible reason for the difference is SRT, which will be further investigated and discussed later. After disturbance (2 d starvation with a high shear rate of 103 s⁻¹) was applied to both MBRs at the beginning of Phase 3, both MBRs experienced severe fouling phenomena, with sharp increases in the average fouling rate at >40 kPa/d. No QQ effect was observed in this phase either. The applied disturbance may have caused a drastic change in mixed liquor characteristics, probably including the microbial community structure, while aggravating fouling propensities, which will be discussed further in later sections.

To examine how SRT affects membrane fouling in the MBRs, we increased SRT from 50 to 75 d in Phase 4. The average fouling rate of Reactor 1 slightly decreased with vacant beads (which contain no QQ bacteria), whereas that of Reactor 2 decreased more significantly with QQ beads (corresponding to 47% of that of Reactor 1). With the longer SRT, it appears that QQ affected biofouling mitigation. When Reactor 1 was switched to conventional mode in Phase 5, its membrane fouling rate was slightly reduced compared to Phase 4. This implies that the vacant beads had no effect on fouling mitigation and in fact may have caused membrane fouling. A previous study also reported that membrane fouling increased when the media added were trapped inside the membrane fibers. However, Reactor 2 exhibited a notably slower fouling rate, which corresponds to 55% of that of Reactor 1. Thus, the biofouling control due to QQ was evident at long SRT (75 d). The effect of SRT on QQ will be discussed further in later sections, along with the time-series data of MBR operational performance and microbial community.

Effects of QQ, disturbance, and SRT on biopolymer production

Figure 2a–d show EPS and SMP variations during MBR operations with and without QQ at different SRT values. The EPS and SMP data normalized to mixed liquor suspended solids (MLSS) are also provided in Supplementary Fig. 2. During Phase 1, when the MBRs were operated in the conventional mode, the EPS-carbohydrate (EPS-C) and EPS-protein (EPS-P) levels were similar in both MBRs (~20 and 80 mg/L, respectively). Notably, however, there was only ~3% probability that the EPS-P level between Reactors 1 and 2 occurs by chance. One possible explanation is that the microbial communities in both the reactors should change as a result of the provision of synthetic wastewater, leading to alterations in metabolic products. A previous study also reported that fluctuations in EPS-P level at the beginning of MBR operation were observed due to bacterial acclimation to new environments. In Phase 2, the EPS-C concentration decreased by ~13.5% in Reactor 1 compared with that of Phase 1, but decreased by 33.1% in Reactor 2. However, the EPS-P concentrations in both reactors remained virtually unchanged. It seemed that the lower EPS levels with QQ did not virtually contribute to fouling mitigation, possibly because its levels were still too high to make a perceptible
Fig. 2  Biopolymer concentrations and mixed liquor characteristics according to phases in the two MBRs. a EPS-carbohydrates (EPS-C); b EPS-proteins (EPS-P); c SMP-carbohydrates (SMP-C); d SMP-proteins (SMP-P); e mixed liquor suspended solids (MLSS); and f floc size. The box is determined by the 25th and 75th percentiles, whereas the whiskers are determined by the 5th and 95th percentiles. The white square symbol inside each bar represents the average value of each parameter.
reduction in membrane fouling. In Phase 3, the EPS-C concentration in Reactor 1 increased by ~6%, whereas it increased by ~39.3% in Reactor 2. This substantial EPS-C increase may have resulted in severe membrane fouling, even in the presence of QQ beads. A previous study reported that increased EPS production was strongly correlated with environmental stresses such as shear and starvation. It was thought that the disturbance at the beginning of Phase 3 may have caused a similar phenomenon. When the SRT was increased to 75 d in Phase 4, the EPS levels in the two MBRs decreased. In Reactor 1, EPS-C and EPS-P concentrations declined by ~15.6% and 11%, respectively, whereas their concentrations decreased by ~74.4% and 21.65%, respectively, in Reactor 2. Similarly, previous studies also reported that EPS production was reduced with long SRT values. In Phase 5, EPS-C and EPS-P contents continued to decrease in Reactor 2; however, no further decrease was observed in Reactor 1. The higher EPS content caused preferential attachment of biomass onto the membrane surface so as to form cake layers.

![Fig. 3 Variations in the microbial community structures with phases in MBRs. a Species-level bacterial community composition. b Species-level principal component analysis (PCA) result using the dominant species given in Supplementary Table 2.](image)

S.S.A. Shah et al. npj Clean Water (2021) 19 Published in partnership with King Fahd University of Petroleum & Minerals
The reduced EPS production associated with the QQ strategy correlates with previous findings. It is thus believed that membrane fouling could be mitigated by the presence of QQ media.

The SMP-carbohydrate (SMP-C) and SMP-protein (SMP-P) levels were also similar in both reactors in Phase 1 (~5 and 4.5 mg/L, respectively). In Phase 2, there were slight changes in both. When disturbance was applied in Phase 3, the SMP levels in both reactors significantly increased. The SMP-C and SMP-P concentrations in Reactor 1 increased by ~45.3% and ~36.1%, respectively, and their respective increases in Reactor 2 were more significant at ~62.4% and ~110%. These results agree well with previous studies, which reported that the disturbance imposed on microorganisms induced the release of microbial polymeric substances, in addition to substrate limitations. When the SRT was increased to 75 d in Phases 4 and 5, the SMP-C and SMP-P concentrations started decreasing, and the decline was more significant with QQ. For instance, in Phase 5, Reactor 2 had the lowest SMP-C and SMP-P levels, at ~54.2% and ~62.4% lower than these respective values in Phase 4. In addition, the longer SRT contributed to decreased SMP levels when comparing Reactor 2 between Phases 2 and 5. This result is consistent with previous studies, which reported that the presence of QQ media reduced soluble biopolymer contents in MBRs. Another previous study also reported that increasing the SRT in MBRs alleviated the effect of QQ. Caused a slight decrease in biomass concentration compared to that of Phase 2. At the longer SRT (75 d) in Phases 4 and 5, the MLSS concentration increased to 2650 mg/L. It is natural to expect that increased microbial yield, converting the resource (food) to more biologically active biomass (biomass concentration). This increase in biomass concentration increased to 2650 mg/L. It is natural to expect that increased microbial yield, converting the resource (food) to more biologically active biomass (biomass concentration).

Effects of QQ, disturbance, and SRT on mixed liquor characteristics and biological treatment efficiencies

Mixed liquor characteristics, such as MLSS and floc size, were monitored over time (Fig. 2e, f). The MLSS concentration in both MBRs from Phases 1–3 varied in the range of 2100–2250 mg/L. Disturbance (starvation with shear) at the beginning of Phase 3 caused a slight decrease in biomass concentration compared to that of Phase 2. At the longer SRT (75 d) in Phases 4 and 5, the MLSS concentration increased to 2650–2900 mg/L. It is natural to expect that increased microbial yield, converting the resource (food) to more biologically active biomass. Operational parameters such as QQ, SRT, and disturbance did not yield significant changes in floc size during the entire study, although fluctuations were possible. In this study, there was a slight increase in floc size with QQ in Phase 5. The microbial floc size is a function of several factors, such as QS, QQ, nutrients, and operational conditions. A recent study reported that there was a negative correlation between floc size and EPS level, because the excessive EPS played a role in reducing the hydrophobicity of flocs and, thereby, weakening the cells’ attachment. It is thus seen that the reduced EPS content may help enhance the floc aggregation, possibly resulting in greater floc sizes.

The biological treatment efficiencies of the two MBRs were evaluated in terms of removals of chemical oxygen demand (COD), total organic carbon (TOC), total nitrogen (TN), and total phosphorus (TP) (Supplementary Fig. 3a–d). The effects of SRT and QQ on these removal efficiencies were almost negligible, although disturbance caused a slight decrease in organics removal. The result coincided with the increased SMP level with shear in Phase 2. At the longer SRT (75 d) in Phases 4 and 5, the SMP-C and SMP-P concentrations started decreasing, and the decline was more significant with QQ. For instance, in Phase 5, Reactor 2 had the lowest SMP-C and SMP-P levels, at ~54.2% and ~62.4% lower than these respective values in Phase 4. In addition, the longer SRT contributed to decreased SMP levels when comparing Reactor 2 between Phases 2 and 5. This result is consistent with previous studies, which reported that the presence of QQ media reduced soluble biopolymer contents in MBRs. Another previous study also reported that increasing the SRT in MBRs alleviated the effect of QQ. Caused a slight decrease in biomass concentration compared to that of Phase 2. At the longer SRT (75 d) in Phases 4 and 5, the MLSS concentration increased to 2650 mg/L. It is natural to expect that increased microbial yield, converting the resource (food) to more biologically active biomass (biomass concentration).

Microbial community structure change

Figure 3a and Supplementary Table 2 show microbial community variations relative to phases, with clear microbial community structure shifts between phases. Two species were dominant in the seed sludge: Dokdonella immobilis (11.31%) and Sphaerotilus natans (14.91%). After inoculation in the laboratory MBRs, the dominance of these species diminished and other species, being adapted to the synthetic feed, flourished instead (Phase 1). In Phase 2, Thiobrix eikelboomii (15%–18%) and Panacibacter ginsenosidivorans (11%–14%), which were negligible in the seed sludge, became dominant in both reactors. In addition, the relative abundances of Kofleria flava and Flavibacteria antarctica increased to 7.57% and 6.95%, respectively. These two species were more abundant in Reactor 1 than Reactor 2. Lastly, the major species of the seed sludge, such as D. immobilis, S. natans, and Terrimonas lutea, became minority species (<0.5%) in Phase 2. The effects of QQ on the species-level microbial community appeared to be minimal, although some of the major species (T. eikelboomii and P. ginsenosidivorans) were less abundant in Reactor 2. This demonstrates that the adaptation of the sludge to the laboratory conditions led to dramatic changes in the microbial community composition; however, at short SRT, the community was not significantly impacted by QQ.

A drastic change in the microbial community occurred with the disturbance applied at the beginning of Phase 3. Spingomonas piscinae, Pedobacter glucosidilyticus, and F. antarctica, all of which belong to the previously reported biofilm-forming bacterial classes of Sphingobacteria and Alphaproteobacteria, boomed after the disturbance. This result supported the occurrence of severe membrane fouling in Phase 3 as already shown above (see Fig. 1); however, there was a marginal difference in fouling between the reactors with vacant and QQ beads. It seemed that the disturbance counteracted the QQ effect.

When the biological treatment performances were stabilized from the disturbance of Phase 3, along with the longer SRT (see Supplementary Fig. 3), T. eikelboomii, P. ginsenosidivorans, and K. flava returned to the community in Phase 4, but to different degrees, possibly owing to the QQ application. T. eikelboomii (32.93%), F. antarctica (10.25%), S. piscinae (5.93%), P. glucosidilyticus (5.58%), and P. ginsenosidivorans (4.43%) were the dominant species without QQ, whereas T. eikelboomii (21.08%), K. flava (12.79%), and P. ginsenosidivorans (5.47%) were dominant with QQ. Two species, F. antarctica and K. flava, were distinct between the two reactors. The microbial communities of Phase 4 differed slightly from those of Phase 2, where D. immobilis and K. flava were major species in Reactor 1. The result indicates that the microbial community recovers from the disturbance when returned to the community in Phase 4, but to different degrees, possibly owing to the QQ application.

In Phase 5, it appears that the microbial communities in both reactors smoothly succeeded from those of Phase 4. In Reactor 1 (conventional MBR), T. eikelboomii (36.75%) became dominant, T. lutea (7.49%) grew more, and K. flava (7.70%) returned to the community in Reactor 1. However, several species, such as S. piscinae (0.5%), P. glucosidilyticus (0.00%), and P. ginsenosidivorans (0.03%), virtually disappeared in Reactor 2. With QQ, the two species, T. eikelboomii and K. flava, dominated the microbial community, with K. flava occupying ~30% of the whole community. Flavobacterium cheonhonense and Tabrizicola aquatica were detected in relatively large quantities in Reactor 2, although their abundances were smaller than those of K. flava. The result indicates that K. flava needs more time to grow; however, it may contribute to antifouling efficiency. The correlation between K.
Microbial community balance between selection pressure on microbial community composition. Thus, the preventing their washout with sludge wastage. QQ can exert a help the growth of these two species in the MBRs while than 0.994, which indicates the detection of most bacterial species between 23,902 and 51,345, and the normalized number of reads The total effective readings of the 11 biomass samples were Microbial diversity change

Microbial community structure can be further understood with characteristics. K. flava belongs to the order myxobacteria, Gram-negative, rod-shaped aerobic bacteria, which are a subgroup of the phylum Proteobacteria. These bacteria are excellent producers of secondary metabolites with antibacterial and antifungal properties, and degrade complex biomolecules such as cellulose. In contrast, T. eikelboomii has been reported as a filamentous microbe that secretes extracellular polymers and causes sludge bulking in MBRs, resulting in biofilm formation and serious membrane fouling. The longer SRT (75 d) may help the growth of these two species in the MBRs while preventing their washout with sludge wastage. QQ can exert a selection pressure on microbial community composition. Thus, the microbial community balance between K. flava and T. eikelboomii may have eventually dictated membrane-biofouling outcome. QQ with longer SRT helps endogenous bacteria (e.g., K. flava) antagonistic to biofilm-forming bacteria (e.g., T. eikelboomii) grow, further leading to reduced membrane fouling.

Principal component analysis (PCA) results show the changes in the microbial community structures between the operating phases (Fig. 3b). With the acclimation of seed sludge in the laboratory MBRs fed with synthetic wastewater, the microbial community structure moved to the center (Phase 1), corresponding to the left and downward from the seed sludge position. The community structure further moved downward in Phase 2. The effect of QQ on the microbial community appeared to be insignificant at short SRT. However, the disturbance introduced in Phase 3 significantly changed the microbial community structure, moving the direction upward and to the left along the vertical axis. With stabilization in the presence of QQ (Phases 4 and 5), the microbial community structure returned to the position seen before the disturbance. Without QQ, however, the microbial community was clustered left of the center. The effect of QQ on PCA of microbial community structures was distinct from the one without QQ as the abundances of specific bacterial species changed with SRT, as discussed above. Thus, it is believed that QQ had an effect on the collective microbial community structure at long SRT and, consequently, the membrane-fouling propensity that can be induced by certain species of the microbial community was compromised.

Microbial diversity change

The total effective readings of the 11 biomass samples were between 23,902 and 51,345, and the normalized number of reads was 23,902. The coverage indices of all the samples were more than 0.994, which indicates the detection of most bacterial species with high data reliabilities. Table 1 provides the operational taxonomic units (OTUs) and α-diversity (Chao1, Shannon, and Inverse Simpson) indices at the normalized sequencing depth. Microbial community richness values of Reactors 1 and 2 in Phase 2 were significantly lower (28.6–30.5% and 21.6–26.7%) than those of the seed sludge and Phase 1 mixed liquors. After applying disturbance at the beginning of Phase 3, these values increased by 55.8% and 31.0%, respectively. The richness then decreased with stabilization in Phases 4 and 5. There were no significant differences between the reactors during each phase. These findings are similar to those from a previous study. The reduced richness in Phases 2 and 4 can be ascribed to the feed type changing from real to synthetic wastewater and to relief from the imposed disturbance, respectively. In contrast, the higher richness observed in Phase 3 can be attributed to disturbance. The Shannon and Inverse Simpson diversity indices showed similar trends to the Chao1 index, indicating the predominance of selective species with the adaptation of seed sludge in the laboratory MBRs, in addition to mixed liquor stabilization after disturbance. Interestingly, the microbial diversity was always greater with QQ than in the control. This indicates that QQ may disturb the existing microbial community, leading to a more diverse structure. In addition, diversity decreased with the longer SRT, possibly because of the survival and dominance of species more fit to environmental conditions with higher sludge ages.

Table 1. Microbial diversity indices for the experimental samples in different phases.

| Phase | Reactor | Reads | OTUs | Chao1 | Shannon | Inverse Simpson | Good’s coverage |
|-------|---------|-------|------|-------|---------|-----------------|----------------|
| Seeding | Seed sludge | 24,717 | 651 | 547 | 4.42 | 0.96 | 0.997 |
| 1 | R1 | 33,996 | 634 | 532 | 4.49 | 0.98 | 0.997 |
| 2 | R1 | 23,902 | 386 | 380 | 3.57 | 0.93 | 0.994 |
| 3 | R1 | 44,800 | 645 | 592 | 4.60 | 0.98 | 0.998 |
| 4 | R1 | 41,879 | 640 | 562 | 4.64 | 0.98 | 0.997 |
| 5 | R1 | 49,216 | 594 | 502 | 4.08 | 0.94 | 0.998 |
| 6 | R1 | 47,136 | 594 | 502 | 4.08 | 0.94 | 0.998 |
| 7 | R1 | 48,539 | 416 | 329 | 3.04 | 0.83 | 0.998 |
| 8 | R1 | 51,345 | 422 | 350 | 3.21 | 0.89 | 0.998 |

Correlation analysis for MBR fouling and biological characteristics

Figure 4 summarizes the Spearman’s correlation coefficients between MBR membrane fouling, mixed liquor characteristics, treatment efficiencies, and microbial communities using the entire experimental data obtained during this study. The fouling rate had strong, positive correlations (r > 0.7) with SMP-C, SMP-P, and the relative abundance of four individual microbial species (i.e., F. antarctica, P. glucosidilyticus, S. piscinae, and T. carbonis). SMP had a lot stronger correlations with fouling rates than EPS, although the actual amounts of the former were a lot smaller than those of the latter. The result indicates that the soluble biopolymers present in the bulk liquid play a more important role in membrane fouling, possibly due to their direct deposition onto the membrane surface. The strong, negative correlations of fouling rates with COD and TOC removal efficiencies support the above explanation. In particular, P. glucosidilyticus and S. piscinae, which had the highly strong correlations with membrane fouling (r > 0.87), accordingly exhibited strong correlations with SMP. Notably, T. eikelboomii, which was the most abundant in Reactor 1 of Phases 4 and 5, had a relatively weak negative correlations (r = −0.32) with membrane fouling and so not as strong as did K. flava and membrane fouling will be further discussed in later sections.

Microbial diversity change

The total effective readings of the 11 biomass samples were between 23,902 and 51,345, and the normalized number of reads was 23,902. The coverage indices of all the samples were more than 0.994, which indicates the detection of most bacterial species...
The decrease in the relative abundance of *T. eikelboomii* in Reactor 2 of Phase 5 should be associated with "QQ, but the species might still have been contributing to membrane fouling, as discussed above. As expected, the microbial diversity indices between OTUs and Chao1, as well as Shannon and inverse Simpson, were found to be strongly correlated. However, the fouling rate did not have strong correlations with any of the microbial diversity indices (−0.05 ≤ r ≤ 0.55), although the microbial diversity was always higher in the presence of QQ (see Table 1).

On the other hand, the content of EPS-C and EPS-P had strong, negative correlations with MLSS levels. The biomass increase was accompanied with longer SRT, so the aged sludge produced less EPS amounts leading to the floc size decline (corresponding to a negative correlation, i.e., r = −0.52). Notably, the floc size had a strong, positive correlation (r = 0.84) with TN removal, suggesting that simultaneous nitration and denitration possibly occurred with larger biological flocs. In addition, *D. immobilis* showed a strong positive correlation (r = 0.84) with TP removal, proposing its role as a potential phosphate uptake strain.

Overall, the relationships between MBR parameters (e.g., fouling rates, mixed liquor characteristics, biological treatment efficiencies, and microbial species dominance) helped better understand the fouling patterns and biological performances in the MBRs with and without QQ.

In summary, the QQ effect on MBR antifouling efficacy was clearer when the SRT was extended from 50 to 75 d, although the disturbance (starvation with shear) aggravated membrane fouling, which counteracted the positive QQ effect. QQ yielded a significant biopolymer production decrease with the longer SRT. Accordingly, organic substance removal showed relatively strong, negative correlations with MBR-fouling propensity. MBR microbial communities showed dynamic responses to the feed change, QQ, disturbance, and SRT. With disturbance, *F. antarctica*, *S. piscinae*, and *P. glucosidilyticus* dominated the microbial community leading to substantive membrane fouling. However, the microbial community balance between *T. eikelboomii* and *K. flavo* played a key role in fouling propensity under stabilized conditions. The correlation analysis showed strong positive relationships between membrane fouling rate and the abundance of several microbial species (*F. antarctica*, *P. glucosidilyticus*, *S. piscinae*, and *T. carbonis*). However, there was no strong correlation between *T. eikelboomii* and membrane fouling propensity, possibly due to the antagonism by *K. flavo*, and vice versa.

**METHODS**

**Feed wastewater**

We prepared synthetic wastewater (composition listed in Supplementary Table 3) every day in the laboratory. Before being fed into the MBRs, the synthetic wastewater was put in a fridge (4 °C). The levels of COD, TOC, TN, and TP in the synthetic wastewater were 200 ± 10, 49.0 ± 5.0, 35 ± 2.5, and 7.0 ± 1.5 mg/L, respectively.
Preparation of QQ beads

We prepared QQ beads containing *Rhodococcus* sp. BH4 (8 mg dry mass per mL) and two polymers, such as sodium alginate (Junsei Chemical Co, Ltd, Japan) and polyvinyl alcohol (Wako Chemicals, Japan), with slight modification. Briefly, we cultured colonies of QQ bacteria (*Rhodococcus* sp. BH4) on a Luria–Bertani (LB) agar plate and used a single colony to obtain the liquid seed culture in an LB broth (50 mL). The BH4 cells were further cultivated by adding 500 μL of the seed culture to 100 mL of LB broth, after which the culture was separated using a centrifuge (13,700 × g, 10 min, 4 °C). The BH4 cell pallets were collected and resuspended in 5 mL of deionized water. We then prepared a polymer solution containing polyvinyl alcohol and sodium alginate in deionized water with a respective mass ratio of 10 : 1 : 100. To prepare QQ beads, the polymer solution (20 mL) and BH4 resuspension (5 mL) were mixed, and the resulting mixture was added dropwise into a cross-linking solution containing 1.13 M boric acid and 0.27 M CaCl2 using a syringe pump (1 mL/min). The mixture was soaked in the solution for 4 h and then stabilized in 0.5 M Na2SO4 solution for an additional 8 h. Vacant beads (i.e., beads without QQ bacteria) were prepared following the same techniques described above, but using an equivalent volume of deionized water instead of the BH4 resuspension.

MBR operations

Two identical laboratory-scale submerged MBRs, each comprising a 2 L rectangular reactor, Kolon polyvinyl difluoride (PVDF) hollow fiber membrane, and an air diffuser were designed, built, and operated under prearranged conditions (Fig. 5 and Table 2). The reactor volume was kept constant at 2 L using a conductivity level sensor and the reactor temperature (mixed liquor) was kept at 25 °C by circulating water from a temperature-controlled water bath through the jacket that surrounded the reactor. The PVDF membrane had a pore size of 0.1 μm, but the effective surface area (94.2 or 113.4 cm²) changed according to the membrane flux (25 or 30 L/m² h). The aeration rate was set to 1.0 L/min, equivalent to 72 s⁻¹ as a velocity gradient value. Membrane operation was cyclical, with 19 min suction under a vacuum followed by 1 min relaxation (i.e., no permeate flow). The operation cycle was executed based precisely on the given inputs in an LG K7M-DR30S programmable logic controller (Korea). Each reactor’s TMP was continuously collected in a data acquisition system consisting of an SMC ZSE40F pressure sensor (Japan), a Metex M-3850D digital multitester (Korea), and a notebook computer. The MBR operation schemes were divided into five phases, which are detailed in Table 2. In Phase 1, the two reactors were inoculated using seed sludge collected from the Shincheon Municipal Wastewater Treatment Plant, Daegu, Korea, after which the reactors were operated in

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**Table 2. Summary of MBR operating conditions.**

| Phase | Operation time (d) | SRT (d) | Operation mode | BH4 concentration (mg/L of reactor volume) |
|-------|--------------------|---------|----------------|------------------------------------------|
|       | Reactor 1          | Reactor 2 | Reactor 1       | Reactor 2                                 |
| 1     | 1–30               | 50      | Conventional    | 0                                         |
| 2     | 31–60              | 50      | Vacant beads    | QQ beads                                 |
| 3     | 61–75              | 75      | Vacant beads    | QQ beads                                 |
| 4     | 76–135             | 75      | Vacant beads    | QQ beads                                 |
| 5     | 136–165            | 75      | Conventional    | QQ beads                                 |

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**Fig. 5 Reactor configurations.** Schematic of the laboratory-scale MBRs operated under different conditions, which are detailed in Table 2.
conventional mode (i.e., with no media). In Phases 2–4, Reactor 1 was operated with vacuum beads added (control MBR) and Reactor 2 was operated with QW beads added (QQ MBR). In Phase 5, Reactor 1 was switched back to conventional mode, but Reactor 2 was maintained as a QQ MBR. The media content of both vacuum and QW beads in the MBRs was 1.25% (v/v) and the corresponding BH4 concentration in the QQ MBR was 100 mg/L of reactor volume. The MBRs were all operated at a constant flux of 30 L/h in Phases 1 and 2, and at 25 L/m² h in Phases 3–5. The hydraulic retention time was set to 7.43 h, but the SRT was adjusted to 50 d for Phases 1–3 and to 75 d for Phases 4 and 5. MBR operations during the experimental runs were stopped when the TMP reached ~50 kPa, after which the fouled membrane was replaced. The average membrane fouling rate (kPa/d), which was defined as the increase in TMP from 4–6 kPa (initial TMP value) to ~50 kPa per elapsed time, was estimated. Notably, we never mixed the MBR-activated sludge between the two reactors during the entire experimental period (165 d) after seeding. Biomass samples for microbial community analysis were collected at the end of each phase. At the beginning of Phase 3 (during the first 2 d), disturbance (i.e., starvation with high shear) was imposed on both reactors to disturb their respective microbial communities. A high shear rate (corresponding to a velocity gradient of 103 s⁻¹) was imparted on the MBR mixed liquor by reducing the mixed liquor volume to 1 L at an air flow rate of 1 L/min. No feed was supplied during this period.

**Analytical methods**

SMF and EPS amounts were determined following methods described below. A 50 mL mixed liquor sample taken from the MBR was centrifuged at 4000 r.p.m. (2951 × g) for 20 min and the resulting supernatant was passed through a 0.45 μm filter (Millipore, USA). The filtrate was then utilized to analyze SMF components (i.e., carbohydrates and proteins) and the sediment (pellet) was suspended again in a 0.9% NaCl solution (50 mL). The suspension was placed in an 80 °C oven for 30 min for EPS extraction and then removed from it to cool down to room temperature. The sample proceeded through a centrifuge (4000 r.p.m., 20 min) and the supernatant was filtered through a 0.45 μm membrane filter to collect the EPS fraction. The concentrations of SMF and EPS-proteins (i.e., SMP-P and EPS-P) were determined according to a modified Lowry method, whereas the concentrations of SMP and EPS-carbohydrates (i.e., SMP-C and EPS-C) were determined by the phenol–sulfuric acid method. The dichromate method was adopted to determine COD using a Metrohm 809 Titrandos automatic titrator (Switzerland), TOC, TN, and TP were measured using a TCC analyzer (Sievers 800 TOC Analyzer, USA), Hach Persulfate Digestion Test ‘N Tube method, and Humas HT-TP-H Molybdio Vanadate method, respectively. The levels of MLSS were determined based on dried weight at 103 °C and the microbial floc size was measured using a Beckman Coulter LS 13320 laser diffraction particle size analyzer.

**Gene sequencing and assays**

The microbial community analysis of the seed sludge and MBR mixed liquor samples was conducted. Briefly, DNA was extracted using a DNeasyPowerSoil Kit (Qiagen, Hilden, Germany) and then the extracted DNA was quantified using Quant-IT Picogreen (Invitrogen). The sequencing library preparation was performed following the Illumina 16 Metagenomic Sequencing Library protocols to amplify the V3–V4 region (341F–805R). The universal polymerase chain reaction primer pair used for the amplifications was: V3-F: 5′-TCGTCAGCAGCGTCAGATGTATAAGAGACAGTCCTACGGGNGGCWGCAG-3′, V4-R: 5′-TCGTCGGCAGCGTCAGATGTATAAGAGACACAGCCTACGGGNGGCWGCAG-3′. The MiSeq™ platform (Illumina, San Diego, USA) was employed to carry out the paired-end (2 × 300 bp) gene sequencing by the Macrogen (Korea). All sequence reads were compared to data from the Silva rRNA database using the basic local alignment search tool and taxonomic assignments of the sequence reads were performed using the National Center for Biotechnology Information Taxonomy Database (USA). The reads were normalized with the single_rarefaction.py script from the QIME software with parameter -d 23902 (the lowest depth number of sequences to subsample per sample) and α-diversity indices (Chao1, Shannon, and Inverse Simpson) were calculated with the normalized sequencing depth to avoid any bias using the diversity function in the R package “vegan”. We performed PCA of microbial community structures using Origin 2019 software. The Chao1, Shannon, and Inverse Simpson values, which respectively represent the richness, diversity, and evenness of the microbial community, were estimated using microbial community data.

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**AUTHOR CONTRIBUTIONS**

S.S.A.S. conducted the experiments, analyzed the data, and wrote the original draft. L.D.S. and G.B. conducted the experiments. H.P. and K.L. supported experimental methodology. M.F. and I.A. revised the manuscript. K.H.C. supervised the research and revised the manuscript.

**COMPETING INTERESTS**

The authors declare no competing interests.
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Correspondence and requests for materials should be addressed to K.-H.C.

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