Effects of Quorum Quenching on the Microbial Community of Biofilm in an Anoxic/Oxic MBR for Wastewater Treatment

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Introduction

Membrane bioreactors (MBRs) are increasingly used for wastewater treatment. They provide efficient removal of organic pollutants, and effective water reclamation by allowing a high concentration of mixed liquor suspended solids (MLSS) and low production of excess sludge compared with conventional activated sludge systems [25]. However, biofouling remains one of the main problems in MBRs because it results in decreased plant productivity/permeate yield, membrane lifespan, and energy efficiency [8].

Yeon et al. [40] revealed that quorum sensing (QS) is strongly related to biofilm formation on the membrane surface in a laboratory-scale MBR. Furthermore, they demonstrated that biofilm formation (biofouling) could be substantially mitigated by quorum quenching (QQ) using an enzyme (i.e., acylase) [17, 41]. However, enzymatic QQ has cost and stability issues. These problems require more effective QQ methods. Oh et al. [29] proposed a new QQ strategy using the isolation of indigenous QQ-bacteria, which produce the QQ-enzyme, from activated sludge and developed a carrier to entrap QQ-bacteria. Bacterial QQ is an attractive approach to control biofouling in MBRs because it increases the treatment efficiency and consumes less additional energy. Recently, researchers isolated new QQ-bacteria and developed different types of carriers to increase QQ activity [5, 6, 18, 19] and this approach has been fully investigated in a larger pilot-scale MBR using...
actual wastewater [23]. Although information regarding the microbial community of biofilm is crucial for the development of QQ strategies, the available information is not sufficient. Only a few studies have reported that enzymatic QQ had an effect on the development of microbial composition in biofilm in a laboratory-scale MBR (a 2.5 L reactor with a hollow fiber membrane) [16]. However, this study did not consider the QQ mechanisms in terms of the microbial ecology. Although, microbial community diversity influences microbial system function [2], this study did not address the effect of QQ on the community diversity of the biofilm. In addition, the microbial community shift of biofilm from a conventional-MBR has already been observed in previous studies [11, 24, 26], implying a need for further investigation of the effect of QQ on the microbial community in biofilm.

In this study, we observed the microbial community based on transmembrane pressure (TMP) and time (days). Multivariate statistical techniques (e.g., principal component analysis and correspondence analysis) were used to reveal how TMP and time affect the microbial community composition. Such techniques are often too simplistic to explain the complex interactions in microbial systems [20]. Therefore, the main objectives of this study were to elucidate the change of the microbial community and to determine the effects of bacterial QQ on the microbial community in the biofilm using high-throughput sequencing (an Illumina MiSeq platform).

Materials and Methods

MBR Systems
The laboratory-scale submerged MBR consisted of interconnected anoxic (2.5 L) and aerobic (5 L) tanks. Synthetic wastewater (the composition of which is described in Table S1 in the supplementary file) was fed first into an anoxic tank equipped with an agitator. The feed COD and total nitrogen (TN) were 539.2 ± 19.3 and 39.9 ± 2.3 mg/l, respectively. The removal efficiency of COD was 98–100% and that of nitrogen was 73–83%. The mixed liquor overflowed from the anoxic tank into the aerobic tank in which two membrane modules were immersed. The aerobic tank was equipped with a coarse bubble diffuser connected to a blower (2.1 L/min aeration rate) that mixed the activated sludge and maintained the dissolved oxygen concentration at 2.5 mg/l and above. The rate of recirculation flow from the middle of the aerobic tank to the anoxic tank was maintained at two times the feed flow rate, Q. The screen (hole size: approximately 1 mm) was placed in the oxic tank so that vacant or QQ-beads were constantly retained in the oxic tank. The concentrations of the MLSS were maintained at 6,404 ± 797 mg/l in the anoxic tank and 8,199 ± 854 mg/l in the aerobic tank. The hydraulic retention time and solid retention time were maintained at 8 h and 30 days, respectively. Both tanks were maintained at a temperature of 25 ± 3°C and pH of 6.4 to 7.8. The two identical membrane modules were mounted vertically within the aerobic tank. One was used to maintain the MBR operation, and the other was used for biofilm sampling. The effective area of the hollow fiber membrane module was 370 cm² for water filtration and 155 cm² for biofilm sampling. New membrane modules for biofilm sampling were used in each run to collect the biofilm. Each membrane module for biofilm sampling has a value of resistance similar to that of the membrane (Table S2). Polyvinylidene fluoride hollow fiber microfiltration membranes (ZeeWeed, GE-Zenon, USA) with a membrane pore size of 0.04 μm were used. Two sets of MBR operations were run under the same conditions. Each reactor was operated at a filtration flux of 281/m²·h to maintain the MBR operation. The permeate of the filtration module was used to maintain the water level in the MBR through a level sensor and three-way valve. The membrane module for biofilm sampling was operated at a filtration flux of 201/m²·h. The permeate of the sampling module was recycled in the oxic tank. After a filtration period of 59 min, the membrane module for biofilm sampling was relaxed for 1 min. TMP is an important membrane performance parameter, and the extent of membrane biofouling is quantified by monitoring changes in TMP [12]. TMP variations were monitored for four runs. In the first and second runs, the TMP profiles of the conventional (without vacant beads) and QQ-MBR were compared and then vacant beads were inserted into the conventional MBR (vacant-MBR). The TMP profiles of the vacant and QQ-MBRs were also compared at the third and fourth runs. QQ-beads were injected in the oxic tank at an injection volume of 1.2% of the total volume of the oxic tank.

Preparation of the QQ-Beads
The quorum quenching bacteria used in this study were Rhodococcus sp. BH4 [29]. The strain was incubated in LB broth for 1 day (30°C, 200 rpm) for the preparation of the QQ-beads. The QQ-beads were prepared using the dripping method, and the material and solidification method were modified to reinforce the stability of the QQ-beads, which are composed of an alginate matrix that decomposes in real wastewater [23]. The concentration of dried Rhodococcus sp. BH4 in the beads was approximately 3.5–3.8 mg per gram of beads. The average size and density of the QQ-beads were approximately 4.3–4.6 mm and 1.0–1.1 g/ml, respectively.

Bioassay of N-Acyl-Homoserine Lactone (AHL)-Degrading Activity
To determine the AHL-degrading activity of the QQ-beads, 50 QQ-beads were incubated in 20 ml of C8-HSL (N-(decanoyl)-γ-homoserine lactone; Sigma-Aldrich, USA) in a Tris–HCl buffer (50 mM, pH 7.0) for 2 h (30°C, 200 rpm). The concentration of C8-HSL was 200 nM. Every 30 min, the supernatants were sampled to measure AHL-degrading activity. The concentrations of the C8-HSL molecules were measured via the luminescence
method [28] using the reporter strain of *Agrobacterium tumefaciens* A136 [10].

**Analytical Methods**

The concentration of the MLSS was measured in duplicate by standard methods [1]. To measure the soluble COD and TN concentrations, all of the samples (50 ml per sample) were centrifuged at 2,760 × g and the supernatants were filtered using a 0.45 µm hydrophilic polypropylene membrane filter (Pall Corp., USA). The filtered samples were measured for COD and TN in duplicate using Hach digestion vials and a DR 4000U spectrophotometer (Hach Co., USA). The dissolved oxygen concentration was measured by a DO meter (YSI, Model DO200, USA). The pH was determined using pH meter (iSTEK, Model pH-20N, Korea). To determine the hydrodynamic resistance of the membrane (Rw), the new membrane module was inserted in 5 L of distilled water and operated at a filtration flux of 20 L/m²·h. The Rw was calculated using the measurement of TMP and resistance of the membrane equation \( R_w = \Delta P / \eta J \) [27], and the water viscosity was assumed to be 0.00089 Pa·s (at 25°C) (Table S2).

**Sampling for Analysis of the Microbial Community in the Biofilm**

The biofilm was collected to analyze the microbial community. Samples of the biofilm were taken from the membrane module at TMPs of 15, 25, and 45 kPa in the first and third runs. Samples of the biofilm were taken from the membrane module at the time when the TMPs of the conventional- and vacant-MBRs reached 15, 25, and 45 kPa in the second and fourth runs. Four strings of hollow fiber membranes (47.7 cm² effective areas) with biofilm were cut from the membrane module for each sampling point. After cutting the membrane, the membrane module was sealed with the ethylene-vinyl acetate copolymer. The cut membranes with biofilm were then dipped in distilled water for 5 min to remove activated sludge. After soft washing, the pieces of the membrane with biofilm were transferred to a 50 ml conical tube.

We designated samples as R1C1, R1C2….R4Q3. R indicates an experimental run, and C, V, and Q indicate conventional-, vacant-, and QQ-MBRs, respectively. The number following letter “R” indicates the order of experimental run and the number following letters “C”, “V”, and “Q” indicate the sampling order of biofilm. To compare biofilm and activated sludge, 50 ml activated sludge samples were collected at the first biofilm sampling time (R1C1, R1Q1, R2C1, R2Q1, R3C1, R3Q1, R4C1, and R4Q1) of each run from the oxic tank. As soon as the sampling was completed, DNA was extracted from all samples. The remaining samples were stored in a deep-freezer at −80°C. After removing the membrane from the membrane module, the filtration rate was decreased to maintain the filtration flux at 20 L/m²·h.

**DNA Extraction, PCR Amplification and MiSeq Platform Sequencing**

Sample preparation for DNA extraction was as follows: Two strings of hollow fiber membrane (4–5 cm) with biofilm were cut into small pieces and the pieces of the membrane with biofilm were inserted into a bead tube for DNA extraction. A total of 1.5 ml of activated sludge was harvested by centrifugation (10,000 × g, 1 min, 25°C), the pellet was resuspended in 0.5 ml of DI water, and the resuspended solution was injected into the bead tube for DNA extraction. DNA was extracted from the precipitates using a NucleoSpin Soil kit (Macherey-Nagel GmbH, Germany). The extracted DNA was eluted in 100 µl of elution buffer and was quantified using an ND-1000 spectrophotometer (Nanodrop Inc., USA).

Each sequenced sample was prepared according to the Illumina 16S Metagenomic Sequencing Library protocols. Quantification of DNA and the DNA quality were measured by PicoGreen and Nanodrop. The V3-V4 regions of the 16S rRNA genes were amplified with the primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′) [21]. Input gDNA (12.5 ng) was amplified with 16S V3-V4 primers and a subsequent limited-cycle amplification step was performed to add multiplexing indices and Illumina sequencing adapters. The final products were normalized and pooled using PicoGreen, and the sizes of the libraries were verified using a LabChip GX HT DNA High Sensitivity Kit (PerkinElmer, USA). Next, a sample was sequenced using the MiSeq platform (Illumina, USA) by Macrogen Incorporation (Seoul, Korea).

**Data Analysis for the Bacterial Community**

The sequencing data were divided into four runs. Each run consisted of two activated sludge samples and six biofilm samples. Each sequencing datum was trimmed and analyzed using the Mothur program ver. 1.35.1 [33]. The sequences were filtered by the standard operating procedure for MiSeq [22]. The primer sequences and low-quality sequences (length, <300 bp and 500 bp <; and with an ambiguity) were removed using the Mothur program. The average nucleotide length was 412 bp for the bacterial libraries. Chimeric sequences were removed using the UCHIME function with the abundant sequences as reference. All of the sequences were classified using the SILVA reference library. The sequencing reads obtained in this study were deposited into the DNA Data Bank of Japan (DDBJ) Sequence Red Archive (http://trace.ddbj.nig.ac.jp/dra) under Accession No. DRA004065.

For community analysis, operational taxonomic unit (OTU)-based approaches were conducted using Mothur software. The OTUs were determined at 3% dissimilarity. Each read was taxonomically assigned at the genus level with bootstrap values of more than 80%, resulting in a taxonomic classification from the phylum to genus levels. A total of 1,246,792 (Run1, 335,983; Run2, 361,310; Run3, 282,444; Run4, 267,055) effective sequences were obtained after the raw reads were cleaned. The sequences were randomly normalized to 1,000 sequences per sample from the original sequence libraries (27,490–99,569 sequences per sample after trimming) with 1,000 iterations to analyze the community diversity (i.e., diversity, evenness, and richness).
Statistical Analysis

Statistical analysis was carried out in the R environment (http://www.r-project.org). The values of community diversity (i.e., Chao 1 richness, Shannon diversity, and Shannon evenness) were divided into control- (conventional- and vacant-) and QQ-MBRs. The differences of the community diversity between the control- and QQ-MBRs were analyzed using the t-test.

OTU-based correspondence analysis (CA), a multivariate statistical technique, was applied to determine the relationships among the communities, using Canoco ver. 4.5 software (Microcomputer Power, USA). The sequences were randomly normalized to 27,490 sequences per sample for the correspondence analysis.

Results

QQ Activity of the QQ-Beads and Effect of QQ on MBR Biofouling

As shown in Fig. S1, the vacant-bead without the QQ-bacteria did not show a substantial decrease in the C8-HSL concentration, suggesting that its adsorption of C8-HSL was negligible. The AHL degradation rate of the QQ-beads was 0.062 ± 0.003 nmol C8-HSL/min for a 60-min reaction time.

QQ-beads were continuously applied to the MBRs to test their inhibition activity of biofouling in the MBRs (Fig. 1).

Fig. 1. TMP profile of each run.
A circle indicates a sampling point of the biofilm from the membrane module.

A. Run 1
- Conventional-MBR
- QQ-MBR

B. Run 2
- Conventional-MBR
- QQ-MBR

C. Run 3
- Vacant-MBR
- QQ-MBR

D. Run 4
- Vacant-MBR
- QQ-MBR
Two laboratory-scale MBRs in continuous mode were operated in parallel under identical operating conditions except for the addition of QQ-beads to one MBR. The rise of the TMP profile of the conventional- and QQ-MBRs was compared to evaluate the inhibition of biofouling by QQ activity (Figs. 1A and 1B).

As shown in Fig. 1A, the conventional-MBR took 14.3 days for the TMP to reach 47 kPa in the first run, whereas the QQ-MBR took 28.3 days for the first run. QQ mitigated the formation of biofilm and extended the time required to reach a TMP of 45 kPa by 97%, compared with the conventional-MBR. The effect of QQ on the mitigation of biofouling was reconfirmed in the next run. In the second run, the rise of the TMP profiles of the conventional-MBR took 9.8 days for the TMP to reach 45 kPa, while the TMP of the QQ-MBR was maintained at 10–12 kPa. In the third run, a vacant-bead without QQ-bacteria was injected into the conventional-MBR (vacant-MBR). As shown in Fig. 1C, the vacant-MBR took 17.4 days for the TMP to reach 45 kPa in the third run, whereas the QQ-MBR took 35.8 days for the first run. QQ mitigated the formation of biofilm and extended the time required to reach a TMP of 45 kPa by 106%, compared with the vacant-MBR. The effect of QQ on the mitigation of biofouling was reconfirmed in the next run. The fourth runs showed that the rise of the TMP profiles of the vacant-MBR took 12.9 days for the TMP to reach 44 kPa. The TMP profile of the QQ-MBR was still maintained at a low level.

**Microbial Community Diversity of the Biofilm**

Ribosomal tag MiSeq platform sequencing was applied to analyze the community diversity of the biofilm. The levels of the Chao1 richness, Shannon diversity index, and Shannon evenness were estimated at a sequence divergence of 3% (Table 1). Statistical analysis (t-test) showed that the level of community diversity (richness, diversity, and evenness) did not significantly differ between the control-MBR (conventional- and vacant-MBRs) and QQ-MBR in the first, second, and third runs (p-value > 0.05). The diversity (p-value: 0.01) and evenness (p-value: 0.02) of the QQ-MBR were significantly greater than those of the vacant-MBR in the fourth run (p-value < 0.05). The richness did not differ between the vacant- and QQ-MBRs.

**Correspondence Analysis**

Correspondence analysis was applied to distinguish between communities on the basis of their OTU composition (Fig. 2). The first and second axes of the CA plot explained 34.3% and 24.1% of the community composition variations, respectively (Fig. 2). The CA plot shows the difference between the inoculum community (activated sludge) and biofilm communities. The microbial communities of the biofilm were categorized into two distinct groups: conventional- and vacant-MBRs vs. QQ-MBR.

<p>| Table 1. Diversity indexes and evenness of the biofilm samples retrieved from the conventional-, vacant-, and QQ-MBRs. |</p>
<table>
<thead>
<tr>
<th>TMP (kPa)</th>
<th>MBR</th>
<th>Sample</th>
<th>Chao 1 richness</th>
<th>Shannon diversity</th>
<th>Shannon evenness</th>
<th>Time (days)</th>
<th>MBR</th>
<th>Sample</th>
<th>Chao 1 richness</th>
<th>Shannon diversity</th>
<th>Shannon evenness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Conventional-MBR</td>
<td>C1</td>
<td>320</td>
<td>3.57</td>
<td>0.70</td>
<td>3</td>
<td>Conventional-MBR</td>
<td>C1</td>
<td>340</td>
<td>3.68</td>
<td>0.71</td>
</tr>
<tr>
<td>28</td>
<td>C2</td>
<td>335</td>
<td>4.01</td>
<td>0.77</td>
<td>6</td>
<td>C2</td>
<td>322</td>
<td>3.77</td>
<td>0.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>C3</td>
<td>324</td>
<td>3.59</td>
<td>0.70</td>
<td>10</td>
<td>C3</td>
<td>367</td>
<td>4.13</td>
<td>0.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>QQ-MBR</td>
<td>Q1</td>
<td>318</td>
<td>3.91</td>
<td>0.76</td>
<td>3</td>
<td>QQ-MBR</td>
<td>Q1</td>
<td>325</td>
<td>3.71</td>
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<td>25</td>
<td>Q2</td>
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<td>6</td>
<td>Q2</td>
<td>341</td>
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<tr>
<td>52</td>
<td>Q3</td>
<td>344</td>
<td>3.91</td>
<td>0.75</td>
<td>10</td>
<td>Q3</td>
<td>342</td>
<td>4.22</td>
<td>0.80</td>
<td></td>
<td></td>
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<tr>
<td>16</td>
<td>Vacant-MBR</td>
<td>V1</td>
<td>313</td>
<td>3.87</td>
<td>0.75</td>
<td>5</td>
<td>Vacant-MBR</td>
<td>V1</td>
<td>355</td>
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<td>0.75</td>
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<tr>
<td>30</td>
<td>V2</td>
<td>390</td>
<td>4.27</td>
<td>0.80</td>
<td>11</td>
<td>V2</td>
<td>408</td>
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<tr>
<td>50</td>
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<td>0.84</td>
<td>13</td>
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<td>331</td>
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<td>0.85</td>
<td>5</td>
<td>QQ-MBR</td>
<td>Q1</td>
<td>355</td>
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<tr>
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<td>0.84</td>
<td>11</td>
<td>Q2</td>
<td>407</td>
<td>4.29</td>
<td>0.81</td>
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</tr>
<tr>
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<td>Q3</td>
<td>366</td>
<td>4.61</td>
<td>0.86</td>
<td>13</td>
<td>Q3</td>
<td>462</td>
<td>4.64</td>
<td>0.84</td>
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</table>

The asterisk indicates the significant differences between the vacant- and QQ-MBRs (p < 0.05).
The microbial communities in Fig. 2 showed patterns of variation by TMP and time. The relationships between the bacterial composition and variations (TMP and time (days)) were investigated using the value of the slope from the linear regression equation. Figs. 3 and 4 show the relationship between the variations (TMP and time) and scores on the first ordination axis of the CA plots in Fig. 2.

A change in bacterial composition was observed in Fig. 3A by TMP. The absolute value of the slope (the change rate of bacterial composition) was $3.13 \times 10^{-2}$ and $1.48 \times 10^{-2}$ in the conventional- and QQ-MBR, respectively. The change rate of microbial composition in the conventional-MBR was 2.1-fold higher than in the QQ-MBR. The change of the microbial composition is observed in Fig. 3B by sampling time (days). The absolute value of the slope was $2.68 \times 10^{-2}$ and $2.32 \times 10^{-2}$ in the conventional- and QQ-MBR, respectively. The change rate of the microbial composition in the conventional-MBR was 1.1-fold higher than in the QQ-MBR. The TMP of the conventional-MBR samples increased from 16 to 46 kPa, whereas the TMP of the QQ-MBR was maintained at 10–12 kPa.

Fig. 4 shows the difference in the change of the bacterial composition between the vacant- and QQ-MBRs. A change in the microbial composition is observed in Fig. 4A by biofouling (TMP). The absolute value of the slope was $3.90 \times 10^{-2}$ and $2.31 \times 10^{-2}$ in the vacant- and QQ-MBR,
respectively. The change rate of the microbial composition in the vacant-MBR was 1.7-fold higher than that in the QQ-MBR. The change of the microbial composition can be observed in Fig. 4B by sampling time (days). The TMP of the vacant-MBR increased from 15 to 44 kPa, whereas the TMP of the QQ-MBR was maintained at 11–13 kPa. The absolute value of the slope was $16.2 \times 10^{-2}$ and $3.26 \times 10^{-2}$ in the vacant- and QQ-MBR, respectively. The change rate of the microbial composition in the vacant-MBR was 5.0-fold higher than in the QQ-MBR. The change rate of the microbial community in non-QQ-MBRs (conventional- and vacant-MBRs) was higher than in the QQ-MBR.

Microbial Composition in Biofilm

Phylyotype analysis was performed (Fig. 5). The phyla Proteobacteria and Bacteroidetes were most dominant in the biofilm. The average relative abundances of the dominant bacteria species are listed in Table 2 and more specific relative abundances of bacteria species are listed in Table S3. These bacteria existed abundantly in the biofilm. In the first run, OTU 1 (*Thiothrix*), OTU 2 (*Rhodobacter*), OTU 3 (unclassified), and OTU 9 (Gp4) were more abundant in the conventional-MBR than in the QQ-MBR, whereas OTU 5 (*Dokdonella*), OTU 7 (Sphingobacteriales), and OTU 10 (Nannocystineae) were less abundant in the

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**Fig. 3.** Linear relationship between variations (TMP and time) and scores on the first ordination axis 1 of correspondence analysis plots in Run 1 and Run 2.

**Fig. 4.** Linear relationship between variations (TMP and time) and scores on the first ordination axis 1 of correspondence analysis plots in Run 3 and Run 4.
conventional-MBR than in the QQ-MBR. The relative compositions of OTU 4 (unclassified), OTU 6 (Chloroflexi), and OTU 9 (Cytophagaceae) were similar between the conventional- and QQ-MBRs in the biofilm.

In the second run, OTU 1 (*Thiothrix*), OTU 2 (Chloroflexi), OTU 3 (Bacteroidetes), OTU 7 (unclassified), and OTU 9 (Chlamydiales) were more abundant in the conventional-MBR than in the QQ-MBR, whereas OTU 4 (*Haliscomenobacter*) and OTU 8 (*Lactococcus*) were less abundant in the conventional-MBR than in the QQ-MBR. The abundances of OTU 5 (*Rhodobacter*), OTU 6 (Cytophagaceae), and OTU 10 (Chloroflexi) were similar between the conventional- and QQ-MBRs.

In the 3rd run, OTU 1 (*Thiotrix*), OTU 2 (*Tolumonas*) and OTU 3 (Chloroflexi) were more abundant in the vacant-MBR than in the QQ-MBR, whereas OTU 4 (*Haliscomenobacter*), OTU 6 (*Rhodobacter*), OTU 7 (*Pseudomonas*), OTU 8 (*Dokdonella*), OTU 9 (Gp4), and OTU 10 (*Nitrospira*) were less abundant in the vacant-MBR than in the QQ-MBR. The relative abundance of OTU 5 (Cytophagaceae) was similar in the biofilms of the vacant- and QQ-MBRs.

In the fourth run, OTU 1 (*Thiothrix*), OTU 2 (Sphingomonadaceae), OTU 6 (Chloroflexi), and OTU 10 (Parachlamydiaceae) were more abundant in the vacant-MBR than in the QQ-MBR, whereas OTU 3 (*Nitrospira*), OTU 4 (*Rhodobacter*), OTU 5 (Chloroflexi), OTU 7 (Rhodocyclaceae), and OTU 9 (Ferruginibacter) were less abundant in the vacant-MBR than in the QQ-MBR. The relative abundance of OTU 8 (*Geminicoccus*) in the biofilm was similar between the vacant- and QQ-MBRs.

**Discussion**

**QQ Activity of the QQ-Beads and Effect of QQ on MBR Biofouling**

The TMP rise-up was stably delayed for 87 days of membrane filtration. Thus, it was speculated that the QQ activity of the QQ-beads was maintained during membrane
filtration (87 days). Previous studies reported that the QQ activity of the QQ bacteria maintained its stability for at least 100 days in a laboratory-scale MBR [15] and that the stability of the QQ-beads in terms of the QQ activity, viability, and mechanical strength was maintained well for at least 100 days in actual wastewater [23].

Fig. 2 shows the TMP profiles in the conventional-, vacant-, and QQ-MBRs. The TMP rise-up was delayed by approximately 100–110% in the QQ-MBR by the addition of QQ-beads to the oxic tank. The effect of QQ on membrane biofouling is similar to a previous observation in a three-stage (anaerobic/aerobic/membrane tanks) MBR system [23]. The previous studies reported that the signal molecules (AHLs) were reduced in a QQ-MBR using *Rhodococcus* BH4 (QQ-bacteria) [19, 29, 37]. Thus, it was speculated that the TMP rise-up was delayed by the reduction of the signal molecules.

**Microbial Community Diversity of Biofilm**

Biofilm is believed to have a complex and dynamic microbial community. Hence, alterations of the community diversity of these microbial resources are thought to influence the development of biofouling in MBRs. In this study, the diversity of microbial communities was measured in a biofilm. The community diversity did not differ across TMP and time in each run. The evenness differed by QQ but not the richness and diversity in the fourth run. These results indicated that the variations of the TMP and time had no effect on the bacterial community diversity but the effect of QQ gradually favored the development of an even community, because only the fourth run had a significantly different evenness with the QQ. The diversity and evenness of the biofilm were increased in the QQ-MBR because the AHL quorum-sensing bacteria in the biofilm were decreased by the effect of QQ, which is similar to previous observation from a study that the relative

<table>
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<th>OTU No.</th>
<th>Classification</th>
<th>Conventional-MBR</th>
<th>QQ-MBR</th>
<th>Classification</th>
<th>Conventional-MBR</th>
<th>QQ-MBR</th>
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<td>1</td>
<td>Thiothrix</td>
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<td>19.7 ± 5.9</td>
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<td>4.5 ± 0.7</td>
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<td>2.3 ± 0.6</td>
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<tr>
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<td>4.2 ± 0.5</td>
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<td>3.3 ± 0.8</td>
<td>4.1 ± 0.7</td>
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<td>Dokdonella</td>
<td>2.9 ± 1.3</td>
<td>4.0 ± 0.4</td>
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<td>2.1 ± 0.6</td>
<td>2.2 ± 0.4</td>
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<tr>
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<td>Chloroflexi*</td>
<td>1.5 ± 1.0</td>
<td>1.6 ± 0.7</td>
<td>Cytophagaceae*</td>
<td>2.3 ± 0.6</td>
<td>2.7 ± 1.9</td>
</tr>
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<td>7</td>
<td>Sphingobacteriales*</td>
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<td>2.7 ± 0.5</td>
<td>Unclassified*</td>
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<td>2.1 ± 0.9</td>
</tr>
<tr>
<td>8</td>
<td>Gp4*</td>
<td>1.9 ± 0.4</td>
<td>1.6 ± 0.4</td>
<td>Lactococcus</td>
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<td>3.7 ± 1.9</td>
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<td>9</td>
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<td>Chlamydiales*</td>
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<td>1.5 ± 0.1</td>
<td>1.3 ± 0.1</td>
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</table>

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<th>QQ-MBR</th>
<th>Classification</th>
<th>Vacant-MBR</th>
<th>QQ-MBR</th>
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<td>19.9 ± 13.0</td>
<td>6.5 ± 2.1</td>
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<tr>
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<td>Chloroflexi*</td>
<td>2.6 ± 0.6</td>
<td>1.5 ± 0.3</td>
<td>Nitrospira</td>
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<td>2.4 ± 0.3</td>
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<tr>
<td>4</td>
<td>Halosorbenovibacter</td>
<td>1.6 ± 1.6</td>
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<td>Rhodobacter</td>
<td>1.7 ± 0.9</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>5</td>
<td>Cytophagaceae*</td>
<td>2.5 ± 1.0</td>
<td>2.3 ± 1.6</td>
<td>Chloroflexi*</td>
<td>1.8 ± 0.6</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>6</td>
<td>Rhodobacter</td>
<td>1.2 ± 0.6</td>
<td>2.3 ± 0.4</td>
<td>Chloroflexi*</td>
<td>2.4 ± 1.9</td>
<td>0.9 ± 0.4</td>
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<tr>
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<td>2.2 ± 0.9</td>
</tr>
<tr>
<td>8</td>
<td>Dokdonella</td>
<td>1.8 ± 1.5</td>
<td>2.4 ± 0.8</td>
<td>Geminicoccus</td>
<td>1.6 ± 0.6</td>
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<tr>
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<td>Parachlamydiaceae*</td>
<td>2.7 ± 3.7</td>
<td>1.5 ± 1.4</td>
</tr>
</tbody>
</table>

The OTU was classified at the genus level, and the asterisk indicates that the OTU was assigned at the taxonomic classification level.
Correspondence Analysis

The CA plot shows the difference between the inoculum community (activated sludge) and biofilm communities. This result is similar to the results of many previous studies. Previous studies have reported differences between activated sludge and biofilm communities in laboratory-scale MBRs [24, 31].

The microbial communities of the biofilm were categorized into two distinct groups: conventional- and vacant-MBRs vs. QQ-MBR. A previous study reported that QQ had an effect on the proportion of the quorum-sensing bacteria with AHL-like autoinducers (such as Enterobacter, Pseudomonas, and Acinetobacter) using the enzymatic QQ method at the genus level [16]. Thus, it was speculated that QQ had an effect on the microbial composition of the biofilm.

A considerable temporal change of the microbial composition was observed in both the control- (conventional- and vacant-MBRs) and QQ-MBRs as the TMP increased (Figs. 3A and 4A). However, the variation in the microbial composition was smaller in the QQ-MBR than in the control-MBR. The variation in microbial composition in the QQ-MBR was less than that of the control-MBR at the same sampling time, because QQ delayed the TMP rise (Figs. 3B and 4B). These results indicated that the change of the microbial composition coincided with the development of the biofilm and that QQ reduced the variation in the microbial composition in the biofilm. Gao et al. [11] reported that the succession of microbial communities might be the cause of membrane fouling in laboratory-scale A/O MBRs. Thus, the reduction of variation in the microbial composition in the QQ-MBR might affect the development of the biofilm.

Microbial Composition in Biofilm

The phyla Proteobacteria and Bacteroidetes were the most dominant in the biofilm, which is consistent with previous observations that showed Proteobacteria and Bacteroidetes to be the dominant phyla in a biofilm [24, 31]. Other studies reported that Proteobacteria and Bacteroidetes were dominant phyla in activated sludge [14, 36, 42]. Activated sludge is the sole inoculum for biofilm formation on the membrane in the MBRs. Thus, it was speculated that Proteobacteria and Bacteroidetes were dominant phyla in the biofilm in this study. In particular, the average relative abundance of Proteobacteria was 53% in the conventional- and vacant-MBRs, whereas it was 49% in the QQ-MBR. The QQ-MBR had a smaller proportion of Proteobacteria than the conventional- and vacant-MBRs. Case et al. [4] suggested that eavesdropping on QS bacteria may be prevalent among the Proteobacteria, because LuxI and LuxR homologs were the only found proteobacterial genomes using the 512 completed bacterial genomes as a data set. This hypothesis may be one of the main reasons for the different composition of Proteobacteria between the conventional- and vacant-MBRs and the QQ-MBR. Bacteroidetes was reported to have been detected in various environments, including freshwater, soil, and compost, and to play an important role in the anaerobic decomposition of complex organic materials in marine sediments [30].

The average relative abundance of Thiothrix sp. in the conventional- and vacant-MBRs was higher than that in the QQ-MBR in the biofilm. Thiothrix species are filamentous, colorless, and sulfur-oxidizing bacteria that may form rosettes and gonidia and deposit sulfur when grown in the presence of sulfide or thiosulfate [38]. A previous study reported that they were found to be the principal bacterial components of aquatic biofilms causing biofouling in selected municipal water storage tanks, private wells, and drip irrigation systems [3], and Thiothrix were observed as the dominant population in biocake in the laboratory-scale A/O MBRs [26]. They reported that Thiothrix could secrete extracellular polymers in the infancy of membrane fouling, because it is a filamentous microbe. It is not clear whether Thiothrix sp. produce signal molecules for quorum sensing or not. It was reported that a member of Thiothrix has the LuxR homolog for the detection of AHLs without LuxI (BioProject: PRJNA51139) for the synthesis of the signal molecules. Diggle et al. [7] reported that AHLs produced by other bacteria could have an effect on the bacteria that detect AHLs but do not produce them using a Pseudomonas aeruginosa mutant (PA01 lecA::luxIAlasI). They showed that the addition of AHLs increased the population of the P. aeruginosa mutant. Thus, we speculate that Thiothrix populations could be affected by QQ because their members might possess the LuxR homolog gene.

Rhodobacter has the ability to remove nitrogen [13] and has been shown to control cellular aggregation based on QS [32]. They reported that the floc size was decreased by QS. However, the average relative composition of Rhodobacter sp. in the QQ-MBR decreased in the first and second runs, while it increased in the third and fourth runs. The role of the QS system in Rhodobacter sp. is not yet fully elucidated in biofilms, and it is not clear that the QS system can control the biofilm growth.

Previous studies reported that Pseudomonas spp. produce
AHL signal molecules [9, 35]. However, Pseudomonas was more abundant in the QQ-MBR than in the vacant-MBR in the third run. Many signal molecules (i.e., AHL or autoinducer-1, autoinducer-2, autoinducer-3, cholerae autoinducer-1, Pseudomonas quinolone signal (PQS), and autoinducer peptides) are involved in bacterial quorum-sensing systems [43] and Pseudomonas spp. produce AHLs as well as PQS. It has been reported that PQS is involved in biofilm formation [34]. Thus, the abundance of Pseudomonas sp. in the QQ-MBR suggested that there was another signal molecule (e.g., PQS) involved in the biofilm formation of Pseudomonas.

Some bacteria (e.g., Haliscomenobacter sp., Dokdonella sp., Nitrospira sp., and Lactococcus sp.) remained or increased in abundance in the QQ-MBR. Many types of signal molecules are involved in bacterial quorum-sensing systems, and microbes without quorum-sensing systems may exist in biofilms.

In this study, we observed that QQ had an effect on the evenness of the microbial community structure, bacterial composition, and change rate of the bacterial composition in biofilms. These findings provide insight for the development of a QQ strategy.

Acknowledgments

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References


