Selection of quorum quenching (QQ) bacteria for membrane biofouling control: effect of different Gram-staining QQ bacteria, *Bacillus* sp. T5 and *Delftia* sp. T6, on microbial population in membrane bioreactors

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ABSTRACT

This study aimed to address the gap in understanding how the microbial community present within quorum quenching-membrane bioreactor (QQ-MBRs) changes during the operations by investigating the behavior of two different types of QQ bacteria, *Bacillus* sp. T5 and *Delftia* sp. T6. The anti-biofouling effects of T5 and T6 in the QQ-MBR were 85% and 76%, respectively. According to the Illumina HiSeq results, when the QQ-MBR was operated with Gram-positive bacteria, T5, in the mixed liquor a reduction was observed in Gram-positive bacteria and Gram-negative bacteria population increased. In contrast, when the QQ-MBR was operated with Gram-negative bacteria, T6, Gram-negative bacteria population reduced and an increase in Gram-positive bacteria observed. As such, the outputs of the Illumina analysis revealed that use of Gram-negative QQ bacteria in the reactor induced a Gram-positive microbial community and vice versa. This indicates that a close interaction occurs between indigenous Gram-negative and positive bacterial phyla, and *Bacillus* sp. T5/*Delftia* sp. T6 is fundamental to the performance of MBRs. This is the first study demonstrating such a relationship and assistance selecting QQ bacteria/strategy in an effective way.

Key words | *Bacillus* sp. T5, bacterial community, *Delftia* sp. T6, membrane bioreactors, molecular analysis, quorum quenching

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INTRODUCTION

Membrane bioreactors (MBRs) are commonly employed in advanced wastewater treatment processes, and previous research has concluded that their performance outperforms traditional activated sludge approaches (Drews 2010; Meng *et al.* 2017). However, during its operation, microorganisms accumulate on the membrane surface in MBRs and this results in biofouling on a long-term basis. Despite the significant developments that have emerged in this area, to date scientists are still looking for an effective method to prevent biofilm formation. Therefore, membrane biofouling remains a significant issue that undermines MBR sustainability.

Previous studies have found that cell-to-cell signals, also known as quorum sensing (QS) signals, play a regulatory role in controlling the membrane surface biofilm that develops during the operation of an MBR (Davies *et al.* 1998). Researchers have found that quorum quenching (QQ) methods that interfere with QS signals can effectively reduce biofouling. Significant effort has been invested in the identification of approaches that block QS signals (Yeon *et al.* 2009a; Yeon *et al.* 2009b). Bacterial QQ represents a promising approach to control biofilm formation in MBRs because it enhances the efficiency of the treatment and requires less energy than alternative approaches (Oh *et al.* 2013; Köse-Mutlu *et al.* 2015; Ergön-Can *et al.* 2017). However, to date QQ bacteria was investigated as a means of impeding QS signals based on the level of acyl-homoserine lactone (AHL) degradation and no effort has been made to assess the impact that the QQ bacteria has on the microbial community.

Although a solid understanding of the microbial community present within the biofilm is critical to the development of effective QQ strategies, knowledge in this

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area is insufficient. Therefore, the effect of different QQ bacteria having a variety of properties on the microbial community present in an MBR is a requirement.

In the current study, we assessed the relationship between the microbial community and two different QQ bacteria, *Bacillus* sp. T5 (Gram-positive) and *Delftia* sp. T6 (Gram-negative), which were exhibiting significant AHL degradation. The objective of this research was to use the Illumina sequencing method to identify how different QQ bacteria impact the relative abundance of the microbial community during QQ-MBR processes. It is anticipated that the findings of this research will lead to an enhanced knowledge of the relationships that exist between QQ bacteria and the bacterial community structures present in MBRs and, consequently, the development of effective methods for controlling biofouling and enhancing the performance of MBRs.

METHODS AND MATERIALS

MBR systems

Two laboratory-scale submerged MBRs comprising two identical aerobic tanks, the control reactor with vacant beads without QQ bacteria, and the QQ reactor with QQ beads were designed (Figure 1). Two sets of MBR operations for T5 and T6 were run under the same conditions. Each reactor was operated at a filtration flux of 23 L/m^2 h to maintain the MBR operation. Activated sludge was obtained from a wastewater treatment plant (İstanbul, Turkey) and acclimated with the synthetic wastewater. The composition of the synthetic wastewater (ww) was as follows (mg/L): glucose, 500; urea, 100; $(NH_4)_2SO_4$, 50; KH_2PO_4, 50; $MgSO_4$ $7H_2O$, 50; NaCl, 50; $CaCl_2$ $2H_2O$, 10; and $NaCO_3$, 100. Dissolved oxygen concentration was maintained between 5.5–6 mg/L. Actual volume of reactor and mixed liquor suspended solids (MLSS) concentration of each reactor were maintained at 4.5 L and 11,000–11,200 mg/L for operation with T5, and 11,200–11,700 mg/L for operation with T6, respectively. The hydraulic retention time (HRT) and sludge retention time (SRT) were maintained at 19 h and 30 d, respectively. The two identical, submerged polyvinylidene fluoride (PVDF) hollow fiber membrane modules with an effective area of 88 cm^2 were used in both reactors for both MBR operations. In order to quantify the degree of membrane biofouling, changes in transmembrane pressure (TMP), one of the critical membrane performance parameters was observed. Changes in TMP were monitored via the fully automated system, Supervisory Control and Data Acquisition (SCADA) for both operations. The efficiency of mitigating biofouling formations was estimated by comparing the areas under the TMP curves of the control and QQ reactors for each run.

QQ bacteria: *Bacillus* sp. T5 and *Delftia* sp. T6 and preparation of the immobilization media

QQ bacteria *Bacillus* sp. T5 (Accession no: KR705939) and *Delftia* sp. T6 (Accession no: KR705940) were used

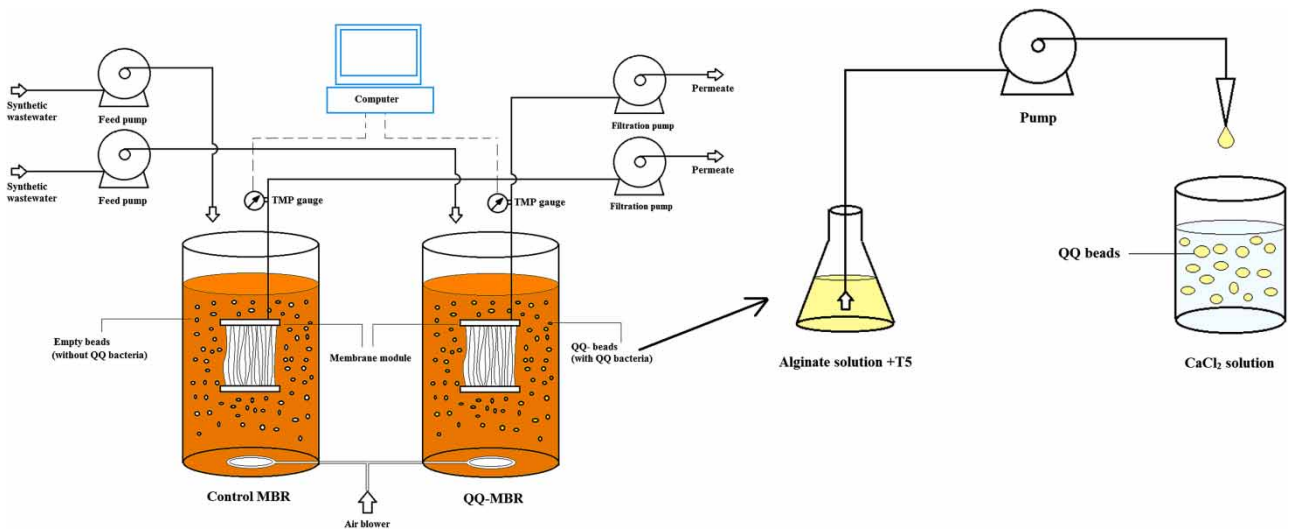

Figure 1 | Schematic diagram of laboratory-scale MBR systems and preparation of the beads.

(Yavuztürk Gül & Koyuncu 2017). Gram staining was done according to the procedure described in the literature (Beveridge 2001). The growth rate of T6 was evaluated in both Luria-Bertani (LB) medium and synthetic ww by measuring the optical density at a wavelength of 660 nm (OD_{660}). The specific growth rate of QQ bacteria was calculated from the logarithmic scale of the growth curve.

Sodium alginate (Sigma-Aldrich) beads were used as a cell immobilization media. *Bacillus* sp. T5/*Delftia* sp. T6 were immobilized to the beads as described in the literature (Kim *et al.* 2013b). Vacant beads were prepared without the addition of T5/T6 for the control reactors (Figure 1). The concentration of *Bacillus* sp. T5/*Delftia* sp. T6 was approximately 9 mg/mL in the beads. The average diameter of the beads was approximately 3 mm.

Measurement of AHL degrading activity for immobilized QQ bacteria via bioassay

The N-octanoyl-homoserine lactone (C8-HSL) degrading activity of T5/T6 was identified according to the bioassay method described in previous studies (McLean *et al.* 2004; Lee *et al.* 2013). To determine the C8-HSL degrading activity of the beads, a 30 mL Tris-HCl (50 mM, pH = 7) buffer was prepared and C8-HSL was added to make the 200 nM final concentration. T5/T6 immobilized beads were incubated for an hour (31 °C, 180 rpm) and samples were taken at certain intervals. The concentrations of the residual C8-HSL molecules were measured on the basis of the calibration curve gained by color zone sizes, corresponding to each standard concentration of the C8-HSL.

Molecular analysis/DNA extraction, polymerase chain reaction amplification and HiSeq platform sequencing

DNA was extracted from 1,000 mg of samples via a PowerSoil DNA isolation kit (Mo Bio Laboratories, USA) according to the manufacturer's instructions. The extracted DNA was quantified using NanoDrop UV–Vis spectrophotometer (Thermo Scientifics, USA). The V4 regions of the 16S rRNA genes were amplified with the primers (length, ca. 250 bp) 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACVSGGGTATCTAAT-3′), which are specific to the V4 region. Illumina adapters and barcode sequences were added to the primers. Extracted DNA was amplified using polymerase chain reaction (PCR). The PCR cycling conditions were as follows: initial denaturation for 3 min at 94 °C, followed by 20 cycles of 45 s at 94 °C, 30 s at 53 °C, 90 s at 65 °C, and a final elongation step of 10 min at 65 °C (Shahi *et al.* 2016). Using the Wizard DNA Clean-Up System (Promega), all DNA samples were purified. The samples were measured using Qubit 2.0 Fluorometer (Invitrogen, USA). 16S rRNA genes were sequenced following the Illumina method (Illumina, Inc., USA) with paired-end read cycles. The methods suggested by Giongo *et al.* (Giongo *et al.* 2010) and Fagen *et al.* (Fagen 2012) were used for sequence raw data analysis and the identification of operational taxonomic units (OTUs). Sequence similarity of at least 80% was sustained as the domain and phylum. For each taxonomic rank OTUs abundance matrices were created via the total number of reads, which showed 16S rRNA sequences matching with the database. Matrices of each sample were divided by the total number of pairs for normalizing varying sequencing depths.

Analytical methods

The concentration of the MLSS and chemical oxygen demand (COD) were measured according to Standard Methods at 3 day intervals (*Standard Methods for the Examination of Water and Wastewater* 1998). Biofilm layers of the membrane surface were observed using confocal laser scanning microscopy after every operation (CLSM, C4, Nikon, Japan).

Statistical analysis

Statistical analysis was carried out using R 3.1.1 (www.r-project.org). To examine data normality, histogram, q-q plots and the Shapiro–Wilk's test were performed. Variance homogeneity was also determined by the Levene's test. One-way analysis of variance (ANOVA) or independent-samples *t*-test was used to check against the variations in QQ bacterium *Bacillus sp.* T5 and *Delftia sp.* T6 and bacterial community structures. The Tukey's test was used to provide multiple comparisons. Values of tests included mean and standard deviation. Important difference was detected at the $p < 0.05$ level of importance.

RESULTS AND DISCUSSION

QQ bacteria, activity of the beads

The growth of *Bacillus* sp. T5 was calculated in our previous study (Yavuztürk Gül *et al.* 2018). The growth rate of *Delftia* sp. T6 was calculated in both LB medium and synthetic ww and is shown in Figure 2. Each QQ bacteria grows faster in

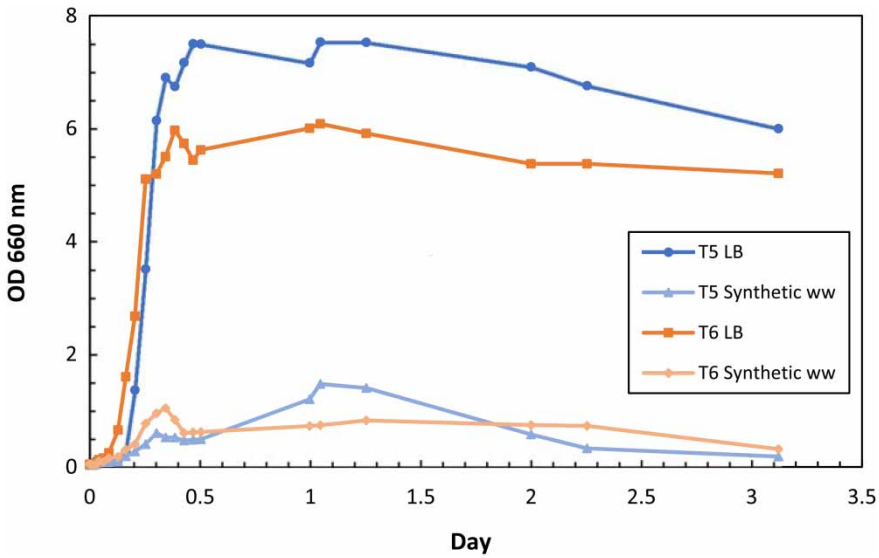

Figure 2 | Growth rate of *Delftia* sp. T6 and *Bacillus* sp. T5 in the LB medium and in the synthetic wastewater. The growth curves of *Bacillus* sp. T5 were illustrated using scientific data given by Yavuztürk Gül *et al.* (2018).

LB medium than in synthetic ww. The specific growth rates of T5 and T6 during the exponential growth phase were 0.45 h^{-1} and 0.44 h^{-1} in LB medium and 0.23 h^{-1} and 0.28 h^{-1} in synthetic ww, respectively. 9 mg/cm^3 cubbyhole QQ bacteria were immobilized to the sodium alginate beads, which were approximately 3 mm in diameter and spherical with a smooth surface. AHL degradation potential was measured for the QQ beads and vacant beads. As shown in Figure 3, the degradation efficiency of C8-HSL beads with T5 and T6 was measured as 77% and 71% in the reaction time of 120 min, respectively. The vacant beads without the QQ bacteria did not exhibit a considerable decrease in the C8-HSL concentration.

Anti-biofouling effect of QQ bacteria on MBR

Two laboratory-scale aerobic reactors (control and QQ) were operated in parallel under the same operating conditions. QQ beads and vacant beads were used in QQ-MBR and control MBR to observe their biofouling

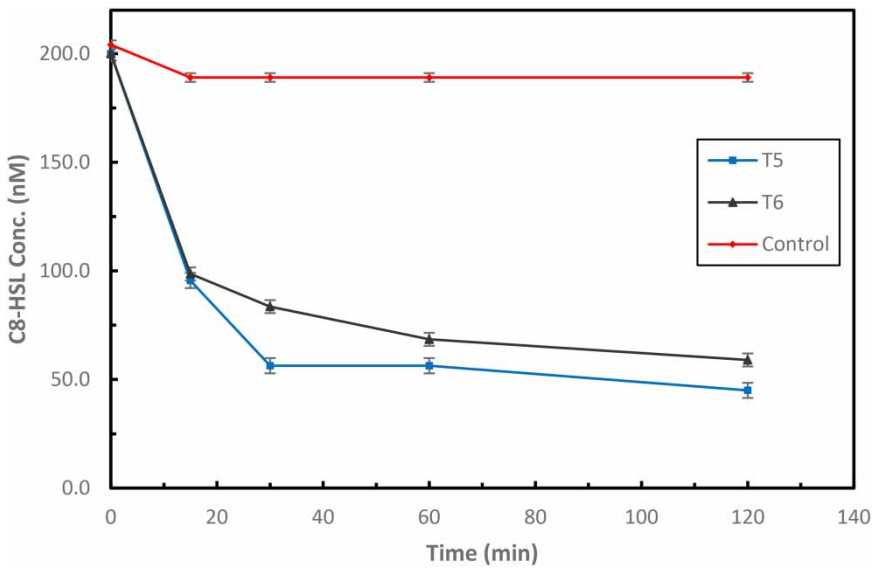

Figure 3 | Degradation of C8-HSL via QQ beads containing *Bacillus* sp. T5 and *Delftia* sp. T6. The C8-HSL degradation of QQ beads was determined after incubation in 2 mM AHL with QQ bacteria in Tris-HCl buffer. Control (vacant beads) was also tested to check the adsorption of C8-HSL. Error bar: standard deviation ($n = 3$).

inhibition potential. Inhibition of biofouling was detected by monitoring the rise of the TMP profiles of the control and QQ-MBR during the operations and comparing areas under the TMP curves to evaluate the anti-biofouling efficiency of T5 and T6 (Figure 4).

TMP rise was successfully controlled with tested QQ bacteria T5 and T6. In Figure 4, the control reactor reached a TMP value of 300 mbar in 20 d, whereas the QQ reactor with QQ beads reached a TMP value of 100 mbar within the same operation time with T5. In addition during the operation of T6, after 11 days of operation, TMP rose to 420 mbar and 150 mbar for control MBR and QQ-MBR, respectively. The QQ process controlled biofilm formation and extended the time required to reach the same TMP values. TMP values of QQ reactors were as much as one-third that of the TMP values of the control at the end of the operation period. According to the calculated area under the TMP curves, T5/T6 immobilized on QQ beads and mitigated biofilm formation by 85–76%, compared to control MBR. The results were consistent with our previous studies reporting the QQ effect of the same bacteria with different immobilization media (Yavuztürk Gül and Koyuncu 2017, Köse-Mutlu *et al.* 2015).

After MBR operations, inhibition of biofilm formation on membrane surface was confirmed via confocal laser scanning microscopy (CLSM). From the CLSM image (Figure 5), it can be observed that the biofilm formation on the surface of membranes operated in the QQ reactor was thinner compared to the control MBRs. These data support the evidence of QQ effect on the inhibition of biofouling.

During the microfiltration process, COD removal efficiencies of control and QQ-MBR were evaluated at 96–98% and the differences in COD profiles of QQ and control reactors were negligible.

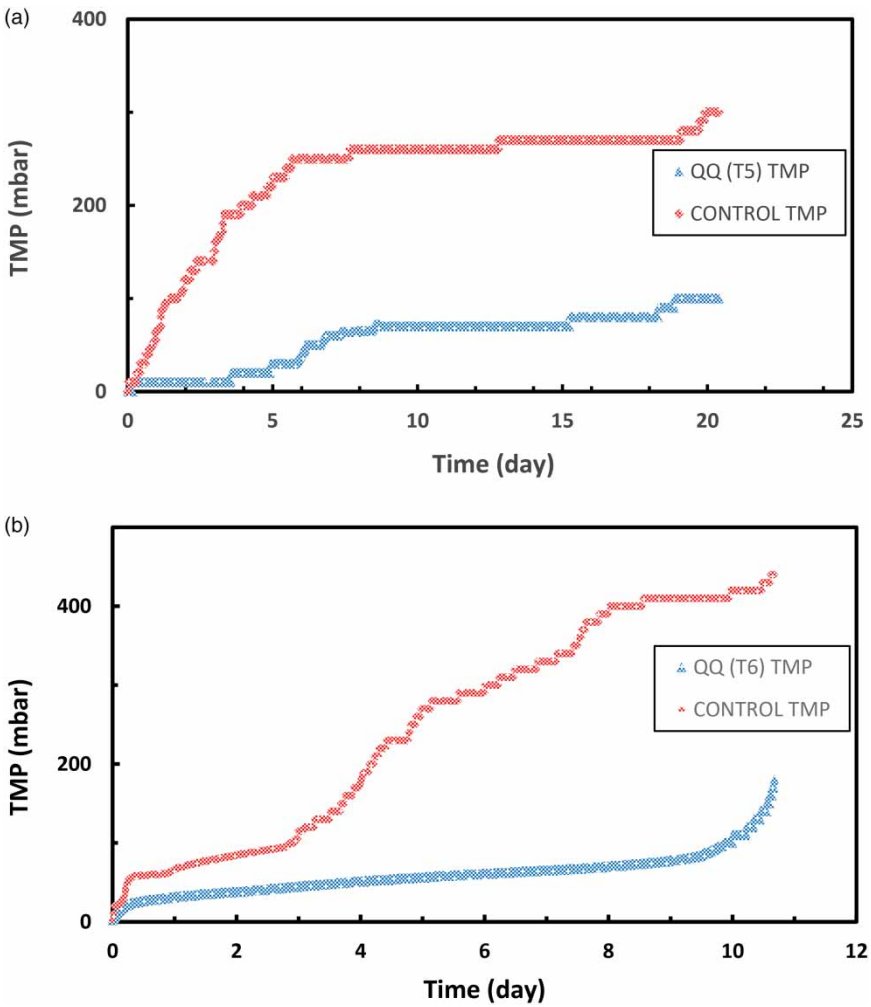

Figure 4 | Transmembrane pressure (TMP) profiles of the control and the QQ reactors during the MBR operation with vacant beads and QQ beads with *Bacillus* sp. T5 (a) and *Delftia* sp. T6 (b).

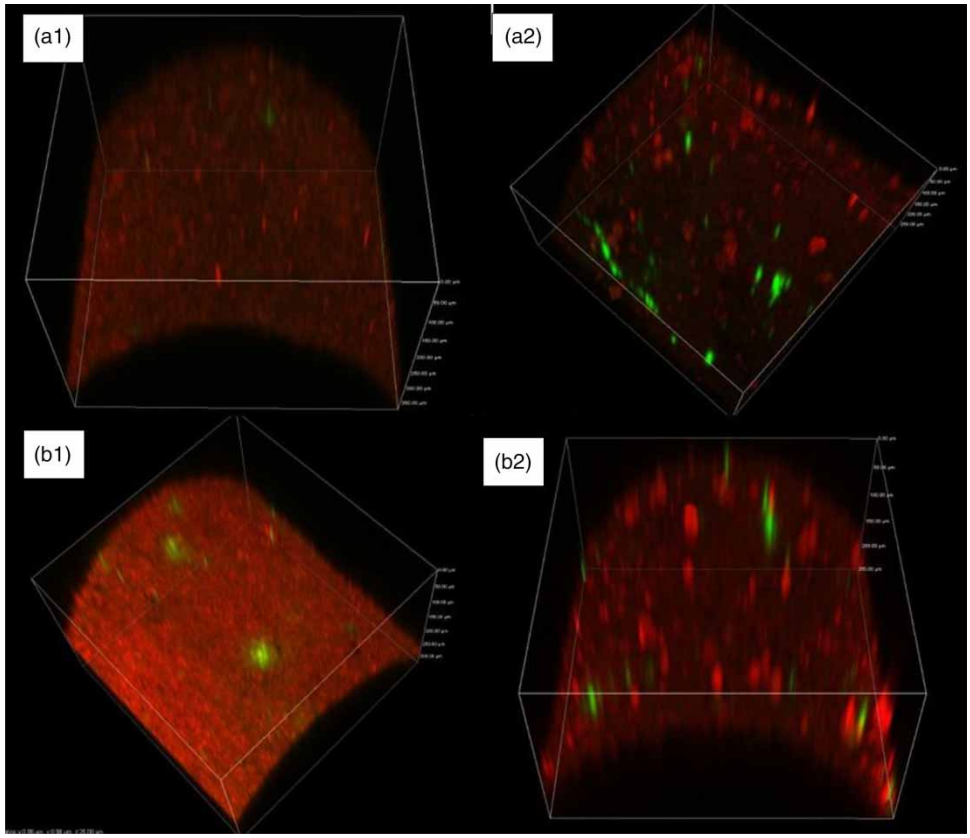

Figure 5 | The CLSM images of biofilm formed on the membrane surfaces. (a1) Control MBR of T5 operation, (a2) QQ-MBR of T5 operation, (b1) Control MBR of T6 operation, (b2) QQ-MBR for T6 operation. Magnification: ×40. Image size: 501.76 μm × 501.76 μm.

Differentiation of bacterial composition of the mixed liquor

Illumina analysis was performed to assess differentiation in the bacterial community. Bacterial composition of the mixed liquor in the control and the QQ reactor was determined (Figure 6). The phyla *Proteobacteria, Actinobacteria,* and *Bacteroidetes* were found to be the most dominant in the control and QQ reactors for each run, which was consistent with previous studies (Teplitski *et al.* 2004; Miura *et al.* 2006; Lim *et al.* 2012; Kim *et al.* 2013). It was found that the average relative abundance of *Proteobacteria* was 31% in the control MBR, whereas it was 40% in the QQ-MBR in the first run (T5 operation), 34% in the control, and 28% in the QQ reactors in the second run (T6 operations). It is observed that the QQ effect of T5 caused a decrease in the abundance of the *Actinobacteria*, whereas an increase in the abundance of *Proteobacteria* and *Bacteroidetes* was observed. The QQ effect of T6 caused an increase in the amount of *Actinobacteria* while *Proteobacteria* and *Bacteroidetes* abundance was decreased. When phylum results were examined, it was observed that the QQ mechanisms of T5 and T6 have the opposite effect on microbial community composition. The results of the class abundance have been compared in order to examine and understand this opposite effect further. The average relative abundances of the dominant bacteria classes in T5 and T6 operations are shown in Figure 7. In the first operation with the T5, abundances of bacilli, bacteroidea, and *Chloroflexia* increased over time in the QQ-MBR, whereas *Acidimicrobiia* and *Acidobacteria* decreased over time in the QQ-MBR. There were no significant differences in the relative compositions of the same classes in the control reactor ($P < 0.05$). In the second run with T6, bacilli, bacteroidea, and *Chloroflexia* decreased over time in the QQ-MBR, whereas *Acidimicrobiia* and *Acidobacteria* increased over time in the QQ-MBR. The abundances of the same classes were not significantly changed in the control reactor ($P < 0.05$). In this study, it was demonstrated that the QQ mechanism of different QQ bacteria (a Gram-positive and a Gram-negative bacteria) had an opposite effect on microbial taxa in the QQ reactor compared with the control. Furthermore, it was observed that Gram-negative QQ bacteria (T6) increased the relative abundances of

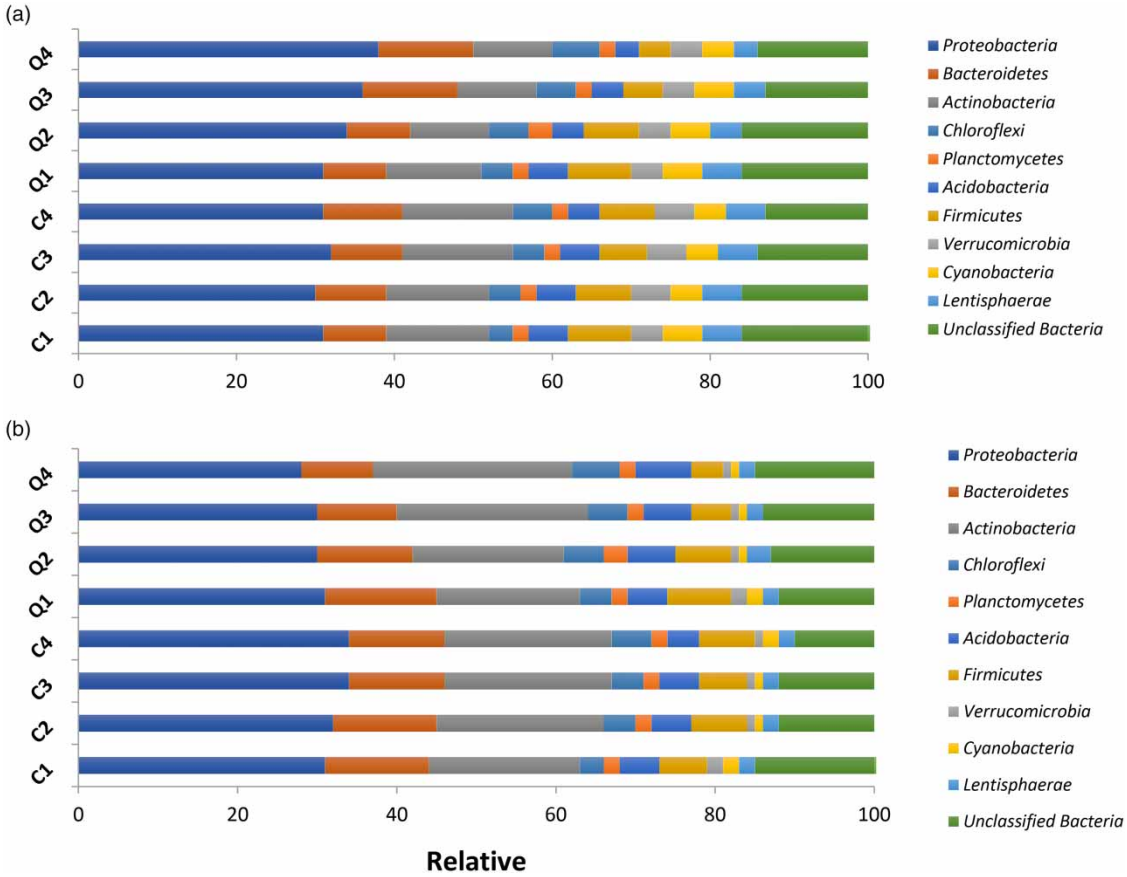

Figure 6 | Dominant bacterial phyla in the MBRs (mixed liquor) and changes in the relative abundances of the dominant phyla during QQ process with T5 (a) and T6 (b) (C: Control, Q: QQ reactor, samples were taken at 2 day intervals for T6 operation and 5 day intervals for T5 operation).

Gram-positive classes and decreased Gram-negatives. On the contrary, Gram-positive QQ bacterium (T5) increased relative abundances of Gram-negative classes and decreased Gram-positive bacteria. The microbial groups affected by the T5 and T6 were different and the shifting effects of the QQ bacteria on the bacterial population dynamics were in the exact opposite direction. It can be inferred that the opposite effect mechanism of the QQ bacteria may have caused two different consequences: (1) the amount of certain microbial groups increased in mixed liquor because they may be more likely to form biofilm or (2) depending on the QQ mechanism, they may be prevented from binding biofilm and survive on mixed liquor, thus their population increased for this reason. This mechanism may also occur because of different enzyme activity of T5 and T6. Although *Bacillus* sp. T5 produce AHL lactonase, *Delftia* sp. T6 produce AHL acylase enzyme for degrading AHL molecules (Maisuria & Nerurkar 2015; Yavuztürk Gül & Koyuncu 2017). Most *Delftia* species are able to degrade or transform a wide range of organic and inorganic compounds. Lately, it was found that an AHL-inactivating enzyme from *Delftia* sp. VM4, identified as AHL acylase, showed distinctive similarity with α/β-hydrolase fold protein (Maisuria & Nerurkar 2015).

QQ affected both the diversity of the microbial community present in the reactor and the relative abundances of these microbes in the community. Furthermore, these effects varied according to the type of QQ bacteria. These findings are significant and can navigate ongoing research efforts to identify and develop a new QQ strategy. The development of a comprehensive molecular analysis of the microbial community present in MBRs could play a critical role in the identification of membrane biofouling control methods and the selection of the most appropriate QQ bacteria to achieve control objectives. It can be concluded that these new findings between microbial community structure with QQ and biofouling characteristics may lead to choosing suitable bacteria/strategy for different biotechnological applications which may be used in studies that aim to control different bacterial groups via QQ. Developments of additional methods for selecting QQ bacteria other than screening

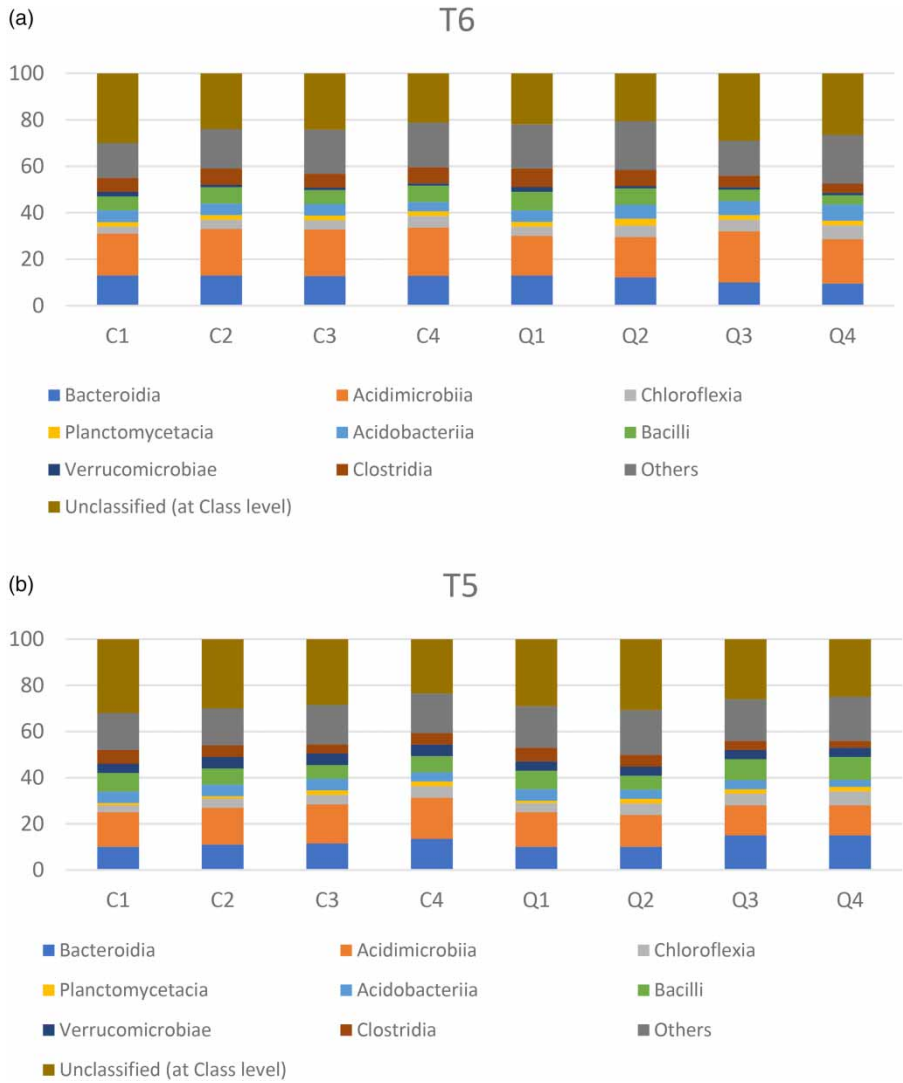

Figure 7 | Relative abundance of dominant bacterial classes in control and QQ reactor for operation with T6 (a) and T5 (b). Samples were taken from the mixed liquor 2 day interval for T6 operation and 5 day interval for T5 operation).

AHL activity is needed. These findings also provide significant information for an insight into the QQ approach, and future research may be conducted on the effect of different QQ bacteria on biofilm composition in the QQ-MBR.

CONCLUSION

The findings of the MBR operations and Illumina HiSeq sequences indicated that *Bacillus* sp. T5 and *Delftia* sp. T6 had a strong impact on the performance of MBRs. Anti-biofouling effect of T5 and T6 was evaluated as 85% and 76%. The results of the molecular tests revealed that Gram-positive QQ bacterium, T5, has a positive impact on Gram-negative species, whereas gram positives species was negatively affected. For Gram-negative T6, this situation was completely reversed. This was ultimately reflected in the microbial population dynamics. Consequently, assessment of different Gram-staining QQ bacteria may highlight the effects of QQ on the population dynamics. It also increases selection methods for QQ bacteria/strategy because past selection methods only considered QQ activity for MBR operations and different biotechnological applications.

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