

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE
ENGINEERING AND TECHNOLOGY

**APPLICATIONS OF BACTERIAL QUORUM QUENCHING WITH
RHODOCOCCUS SP. BH4 FOR EFFECTIVE BIOFOULING CONTROL IN
MBR**

Ph.D. THESIS

Börte KÖSE MUTLU

Environmental Engineering Department

Environmental Sciences and Engineering Doctorate Program

SEPTEMBER 2015

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

**MEMBRAN BİYOREAKTÖRLERDE MEMBRAN BİYOTIKANMASINI
ENGELLEMEK AMACIYLA *RHODOCOCCUS SP. BH4* İLE BAKTERİYEL
QUORUM QUENCHING UYGULAMALARI**

DOKTORA TEZİ

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To my parents, sister and husband,

FOREWORD

In today's world, the gradual reduction in the availability of clean water resources coupled with the need for potable and usable water has resulted in the development and increasing use of water treatment technologies. Membrane technologies represent just one means of obtaining high-quality treated water, and their application is very much in the process of development and improvement. One of the most common membrane processes involves the use of a membrane bioreactor (MBR) to treat domestic wastewater. While MBRs are typically more effective than conventional wastewater treatment systems, like many similar treatment processes, they have distinct disadvantages. In the case of MBRs, the Achilles heel is fouling.

This thesis describes the use of novel studies that utilized a quiet innovative mechanism to prevent membrane biofouling in the MBR. The results of the research indicated that the approach employed was successful in lab-scale MBR applications.

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ABBREVIATIONS

3D	: Three Dimension
AHL	: N-acyl Homoserine Lactone
AI	: Autoinducer
APHA	: American Public Health Association
BSA	: Bovin Serum Albumin
CEB	: Cell Entrapping Bead
CLSM	: Confocal Laser Scanning Microscopy
COD	: Chemical Oxygen Demand
DGGE	: Denaturing Gradient Gel Electrophoresis
DMF	: Dimethylformamide
DNA	: Deoxyribonucleic Acid
DPD	: 4,5-dihydroxy-2,3-pentanedione
EPS	: Extracellular Polymeric Substances
F:M	: Food/Mass
HRT	: Hydraulic Retention Time
HSL	: Homoserine Lactone
KOI	: Kimyasal Oksijen İhtiyacı
KORANET	: Korean Scientific Cooperation Network with the European Research Area
LB	: Luria Bertani
LMH	: Liter/square meter/hour
MBR	: Membrane Bioreactor
MEC	: Magnetic Enzyme Carrier
MEM-TEK	: Prof. Dr. Dincer Topacık National Research Center on Membrane Technologies
MLSS	: Mixed Liquor Suspended Solids
MLVSS	: Mixed Liquor Volatile Suspended Solids
MV	: Microbial Vessel
NF	: Nanofiltration
ORP	: Oxidation-Reduction Potential
PCR	: Polymerase Chain Reaction
PVA	: Polyvinyl Alcohol
PVDF	: Polyvinylidene Fluoride
QQ	: Quorum Quenching
QS	: Quorum Sensing
RMCF	: Rotating Microbial Carrier Frame
SMP	: Soluble Microbial Product
SRT	: Sludge Retention Time
TMB	: Transmembran Basıncı
TMP	: Transmembrane Pressure
TN	: Total Nitrogen (Toplam Azot)
TP	: Total Phosphorus

TUBITAK : Scientific and Technological Research Council of Turkey
USA : United State of America
USACE : United States Army Corps of Engineers
UV : Ultraviolet
WEMT : Water Environment-Membrane Technology

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BACTERIAL QUORUM QUENCHING APPLICATIONS WITH *RHODOCOCCUS SP. BH4* FOR EFFECTIVE BIOFOULING CONTROL IN MBR FOR DOMESTIC WASTEWATER TREATMENT

SUMMARY

The increasing world population and development of industry have resulted in environmental problems like climate change and water scarcity. The membrane bioreactor (MBR) can be used to treat wastewater and provide a source of irrigation water. As such, it represents an innovative and sustainable solution that can be employed to alleviate existing water scarcity problems. While MBRs have a lot of advantages, they also have a distinct disadvantage: the fouling that results from the attachment of biomass to the membrane surface.

Scientists have only recently discovered that microorganisms can communicate with one another and show group behaviors in their environments. The creation of biofilm represents one example of the primary group behavior of microorganisms, which use “Quorum Sensing” (QS) to increase the effectiveness with which they can protect themselves against external factors and to prolong their life cycles more easily. Species that can produce signal molecules and species that can use signal molecules as carbon sources live together in the same ecological environments. Species that use signal molecules as a substrate may interfere with intraspecies and between species communications because they use signal molecules for their own lifecycles. The use of these species to degrade signal molecules in order to interrupt communication between microorganisms is referred to as quorum quenching (QQ). In this study, a QQ mechanism was adapted to an MBR and an innovative approach to prevent and control biofilm formation during MBR operation was suggested and researched by experimenting with different QQ bacteria immobilization configurations.

The scope of this PhD thesis was as follows. First, the QS mechanism was proven through an experiment in an activated sludge system. The QQ microorganisms were then isolated from the activated sludge mixed liquor and their QQ activities were determined. These QQ bacteria were examined for their QQ applicability during MBR operation by using different immobilization media. Microbial vessel and cell entrapping beads (CEBs) were preferred as immobilization media because they are the only two unique QQ MBR application media examples described in the literature. In addition to these two media, a new medium was also trialed. The main aim of these studies was to identify transmembrane pressure (TMP) profile differences between the control MBR and the QQ MBR, which were a conventional MBR and an MBR that incorporated QQ media respectively. The expectation was that the use of QQ products in the QQ MBR would result in a reduction in TMP values. In this thesis, two different QQ bacteria were supplied from the WEMT laboratory: *Rhodococcus sp. BH4* and *Pseudomonas sp. 1A1*. Since the QQ activity of *Rhodococcus sp. BH4* was determined to be higher than the QQ activity of *Pseudomonas sp. 1A1*, *Rhodococcus sp. BH4* was preferred and used as the QQ bacteria of choice to immobilize the media in this study.

Microbial vessel studies were carried out using two different QQ bacteria amounts. Microbial vessels were manufactured from microfiltration hollow fiber membranes, and QQ bacteria were immobilized in the fibers. After the manufacture and QQ activity determination, the microbial vessel was located in the reactor in a stable position. Microbial vessel applications could, on average, result in 44% TMP reduction during MBR operations. The microbial vessel application had a medium level QQ effect. In addition to this, there was an approximately 32% difference in QQ effect between the batch QQ activity determination bioassay result and the reduction in TMP during MBR application. This difference resulted from the immobility of a microbial vessel in the reactor, which resulted in low food/mass (F:M) ratios in the microbial vessels and low lactonase (QQ enzyme) dispersion in the reactor. Following the microbial vessel studies, six QQ bead studies were conducted under different operation conditions. QQ beads were manufactured using alginate or polymer as the base immobilization material. QQ bacteria immobilization was carried out by adding QQ bacteria to the alginate or polymer dope solution. After manufacturing, the QQ beads were added to the reactor and moved dispersedly throughout the reactor during the operation. A reduction in TMP in the QQ MBR with the addition of QQ beads was observed, and the average reduction in TMP across the six studies was 90%. Also, the addition of the QQ beads to the QQ MBR resulted in a 70% and 38% reduction in the concentrations of the protein and carbohydrate content of extracellular polymeric substances (EPS_p and EPS_c) respectively. As such, the results indicated that the application of QQ beads to the QQ MBR successfully decreased TMP in the MBR.

Scientists have recently discovered that the center of QQ beads can be anoxic. In this regard, a one-step MBR study was carried out with polymeric beads in which immobilized nitrification/denitrification bacteria from the anoxic tank of a real MBR plant were utilized. The aim of this study was to eliminate the anoxic tank, mission of which is to remove nitrogen forms, from the flowchart and an adjustable nitrogen removal capacity by adding these special beads to the MBR tank without pausing the aeration. A very high QQ effect (94%) coupled with a 7% and 21% increase in COD and TN removal efficiencies respectively was observed. As such, the results indicated that a one-step MBR operation may represent an effective alternative to advanced wastewater treatment.

Long-term studies of the QQ beads revealed that they are effective as an immobilization medium because they have a high QQ effect, but are also ineffective as an immobilization medium because they have low mechanical durability. QQ beads can easily destroy themselves and mix into the activated sludge. These destroyed QQ bead particles and leaked QQ bacteria result in excess sludge and modify the structure of the activated sludge. However, it is the high F:M ratio and the additional physical cleaning effect on the membrane surface via mobility that allows the QQ bead to achieve such high levels of biofouling prevention. In the light of the advantages and disadvantages of the microbial vessel and QQ bead applications, an innovative, more effective, and practically feasible application media was designed and manufactured. This new media was referred to as the rotating microbial carrier frame (RMCF). The RMCF incorporates a carrier frame that is manufactured from an inert material and several cubbyholes covered with a microfiltration membrane. After QQ bacteria immobilization in these cubbyholes, RMCF is located under the membrane module and rotated during the operation of the MBR. While the inert material incorporated in the RMCF can eliminate the disadvantages of QQ beads in terms of mechanical durability, rotational motion can eliminate the low F:M ratio disadvantage of the

microbial vessel application. RMCF was examined comprehensively for its QQ and physical cleaning effects during short- and long-term MBR operations.

Short- and long-term QQ MBR operations with an RMCF were tested. The results indicated that the use of an RMCF in the QQ MBR could result in a 65% reduction in TMP and a reduction in biofilm formation. Moreover, the RMCF decreased concentrations of EPS_p and EPS_c by 54% and 29%, respectively. The RMCF could be used over the course of 30 days without any external addition or intervention. In addition, 20% of the biofilm prevention effect of the RMCF resulted from the physical cleaning effect. The results indicated that a small increase in the amount of immobilized QQ bacteria could easily increase the percentage by which TMP decreased within RMCF applications. According to the MBR plant scale and the target QQ effect level, the number of RMCFs and cubbyholes of the RMCF can be increased and adjusted. Furthermore, the design of the RMCF can allow immobilized bacteria to be emptied and refilled. In this regard, the growth kinetic of *Rhodococcus* sp. BH4 was determined under the MBR operation conditions. The sludge retention time of *Rhodococcus* sp. BH4 was calculated and it was found that the number of immobilized QQ bacteria can be kept at the same level, and immobilized QQ bacteria can be kept alive in their exponential growth phase during operation by removing about 8% of immobilized QQ bacteria daily. In real-scale plants, the daily removal of immobilized QQ bacteria can be carried out using special lines.

QQ bacteria can affect microbial communities by degrading signal molecules and decreasing the concentration of EPS; these two factors are indissociable. The microbial community in the activated sludge was monitored throughout all these QQ application studies to check the possible effect of the QQ mechanism on the bacterial species. While short-term QQ applications had no significant effect on the microbial community, there was a significant increase in the number of bacterial species following long-term QQ applications. This change can be evaluated as follows: the degradation of signal molecules by QQ bacteria prevented some common species from being dominant in population equilibrium, and provided other rare bacterial species with the opportunity to gather and grow without any social stress. Quorum quenching bio-product usage results in TMP decreasing and energy saving. QQ bio-product usage results in a reduction in TMP and enhanced energy saving during MBR operations. Within the scope of this PhD thesis, a cost analysis was conducted to identify the energy savings percentages and QQ product manufacturing costs of three different QQ media. If manufacturing costs were not taken into consideration, cost savings were 12%, 24% and 23% for the microbial vessel, QQ bead, and RMCF applications respectively. On the other hand, if manufacturing costs were taken into consideration, cost savings were 6%, 15% and 21% for the microbial vessel, QQ bead, and RMCF applications respectively. These differences can be attributed to the fact that the manufacturing cost of the RMCF was the lowest due to design factors like inert material and bacteria regeneration line. To summarize, the feasibility of a QQ MBR was examined from several different perspectives. The QQ MBR, which has very limited application examples and research results in the literature, was successfully used for advanced wastewater treatment at a lower unit cost than a conventional MBR.

Keywords: Membrane Processes, MBR, Biofouling, Quorum Quenching, Energy Saving

EVSEL ATIKSULARIN ARITILMASINDA KULLANILAN MEMBRAN BİYOREAKTÖRLERDE MEMBRAN BİYOTIKANMASINI ENGELLEMEK AMACIYLA *RHODOCOCCLUS SP. BH4* İLE BAKTERİYEL QUORUM QUENCHING UYGULAMALARI

ÖZET

Hızla artan dünya nüfusu ve gelişen sanayi doğal kaynakları orantısız tüketmekte, bu tüketim iklim değişikliği ve su kıtlığı gibi önemli çevresel sorunları doğurmaktadır. Gittikçe artan su kıtlığına sürdürülebilir çözüm arayışlarından biri olarak öne çıkan membran biyoreaktörler (MBR), atıksuların membran sistemlerle arıtılıp sulama amaçlı olarak geri kazanımına dayanmaktadır. MBR sistemleri umut verici bir sistem olmasının yanı sıra tıkanmadan kaynaklı sınırlamalara da sahiptir. Tıkanmaların çoğu biyokütleinin membran yüzeyinde tutunarak birikmesinden kaynaklanan biyokirlenmedir.

Mikroorganizmaların bulundukları ortamlar içerisinde birbirleri ile iletişime geçerek yeterli sayıya ulaştıklarında topluluk davranışlarına yöneldikleri tıp bilim dalının son yıllarda ortaya çıkardığı bilimsel bir gerçektir. Biyofilm oluşturmak da mikroorganizmaların en başta gelen topluluk psikolojisi davranışlarıdır. Bir araya gelerek dış etkilere karşı korunmalarını arttırmak ve yaşamsal döngülerini sağlayabilmek amacı ile “Yetersayı etkisi” (*Quorum sensing*) mekanizmasını kullanırlar.

Ekolojik ortamlar içerisinde iletişim sinyallerini üretebilen türler olduğu gibi iletişim sinyallerini besin kaynağı olarak kullanabilen türler de bulunmaktadır. Bu türler kendi döngüleri için sinyal moleküllerini besin olarak kullanırken aslında bir araya gelmeye çabalayan türlerin birbirleri ve türler arasındaki iletişimlerini sekteye uğratmaktadır. Bu bakterilerin mekanizmalarının amaç doğrultusunda kullanılması da yeni keşfedilen “Yetersayı etkisi azaltımı” (*Quorum quenching*) olarak isimlendirilir. Bu mekanizmanın membran biyoreaktör işletimine adapte edildiği bu çalışma kapsamında etkili bakteri immobilizasyon konfigürasyonları denenerek MBR sistemlerinde biyokirlenmenin engellenmesi ve kontrolü için yenilikçi bir yaklaşım ortaya konulmuş ve araştırılmıştır.

Bu amaçla gerçekleştirilen tez kapsamında, ilk olarak aktif çamur sistemlerinde quorum sensing mekanizmasının varlığı kanıtlanmıştır. Ardından, aktif çamurun bünyesindeki karışık kültürden quorum quenching etkisine sahip mikroorganizmalar izole edilmiş, bunların quorum quenching aktiviteleri saptanmış ve MBR sistemlerde uygulanabilirlikleri çeşitli immobilizasyon medyaları ile ortaya konulmuştur. Immobilizasyon medyası olarak literatürde uygulamaları mevcut olan mikrobiyal vesel ve QQ boncuğun yanı sıra yeni bir medya da denenmiştir. Bu çalışmalarda ana amaç, kontrol MBR (konvansiyonel olarak işletilen MBR) ve QQ MBR (ilgili çalışmada kullanılan hedef immobilizasyon ürününün reaktöre ilave edilmesi ile işletilen MBR) lerinde oluşan transmembran basıncı (TMB)’nin profilleri arasındaki

farqları elde ederek, QQ mekanizmasının sonuçlarını gözlemlemektir. Mikrobiyal vesel ve QQ boncuk uygulamaları değişik akı ve askıda katı madde işletme koşullarında gerçekleştirilmiş ve etkili quorum quenching mekanizması sonuçları elde edilmiştir.

Bu tez kapsamında, QQ mekanizmasını gerçekleştirmek adına iki adet QQ bakterisi elde edilmiştir: *Rhodococcus sp.* BH4 ve *Pseudomonas sp.* 1A1. *Rhodococcus sp.* BH4 türünün QQ aktivitesinin *Pseudomonas sp.* 1A1 türünün QQ aktivitesinden yüksek olduğu belirlenmiş ve tez kapsamındaki MBR işletimleri BH4 türü kullanılarak hazırlanan QQ ürünler ile gerçekleştirilmiştir.

İki farklı bakteri miktarı ile gerçekleştirilen mikrobiyal vesel çalışmalarında kullanılan mikrobiyal veseller mikrofiltrasyon boşluklu fiber membranları kullanılarak üretilmiş ve fiberlerin içerisine QQ bakterileri izole edilmiştir. Mikrobiyal vesellerin QQ aktivitelerinin belirlenmesinin ardından hareketsiz duracak şekilde mikrobiyal veseller reaktörlere yerleştirilmiştir. Mikrobiyal vesel ile QQ MBR işletiminde %44'lük bir QQ mekanizması başarıları gözlenerek TMB değerlerinin artması engellenmiştir. Kesikli olarak gerçekleştirilen QQ aktivitesi deneyindeki QQ aktivitesi ile MBR işletiminde elde edilen başarı arasındaki %30'luk fark mikrobiyal veselin reaktör içerisinde hareketsiz durması gösterilebilir. Hareketsizlik, düşük F:M oranlarına ve laktonaz enziminin reaktördeki yayılımının kısıtlı kalmasına sebep olur.

Mikrobiyal vesel çalışmalarının ardından QQ boncuklar ile işletimler gerçekleştirilmiştir. QQ boncuklar aljinat/polimer kullanılarak üretilmiş ve bünyelerine QQ bakteri immobilize edilmiştir. Üretimlerinin ardından reaktörlere ilave edilen boncuklar reaktörler içerisinde tam karışım halindeyken reaktörler işletilmiştir. Tüm boncuk çalışmaları sonucunda ortalama %90'lık bir TMB azalışı elde edilmiştir. Buna ek olarak, QQ boncuk kullanımı hücre dışı polimeric maddelerin protein ve karbonhidrat içeriklerinin (EPSp ve EPSk) konsantrasyonlarını sırası ile %70 ve %38 oranlarında azaltmıştır. QQ boncuk kullanımının başarılı bir QQ mekanizması sağlayarak yüksek verimlerde TMB azalmasına sebep olduğu söylenebilir.

Bunlara ek olarak, tez kapsamında gerçekleştirilen bir diğer çalışma ise tek-adımlı MBR uygulaması ile aerobik ortamda azot gideriminin artırılmasına yönelik bir işletim olmuştur. Çalışmanın çıkış noktası, üretilen boncukların çekirdek kısımlarının anoksik koşullara sahip olması fikridir. Bu bağlamda üretilen boncukların anoksik kısımları kullanılarak aerobik MBR işletimi süresince anoksik koşullarda gerçekleşebilen reaksiyonların da gerçekleşmesine olanak sağlanmıştır. Bu bağlamda gerçek evsel atıksu ile A2O prosesi doğrultusunda işletilen bir MBR tesisinin anoksik tankından anoksik aktif çamur alınarak polimer boncuklara immobilize edilmiştir. Bu çalışmanın yenilikçi tarafı ise, bu anoksik boncuklar ile QQ boncukların beraber MBR'ye ilavesi ile geliştirilmiş azot giderimi mekanizması ile QQ mekanizmasının birbirine etkisi olmadan pozitif sonuçlarını görmektir. Kontrol MBR boş boncuklar ile QQ MBR ise anoksik ve QQ boncuklar işe işletilmiştir. %94 gibi oldukça yüksek bir QQ etkisinin yanı sıra sırası ile %7 ve %21 seviyelerinde kimyasal oksijen ihtiyacı (KOİ) ve toplam azot (TN) giderim verimi artışı elde edilebilmiştir. Sonuç olarak, tek-adımlı MBR işletimi ile, tesisin akım şemasından anoksik tankların da kaldırılabilmesi ya da çıkış standartlarının sağlanmasının zorlandığı mevsimlerde ek giderim veriminin sağlanması mümkündür.

Mikrobiyal vesel ve QQ boncuk uygulamaları esnasında gözlenen uygulama avantajları ve dezavantajları doğrultusunda daha verimli ve pratik bir uygulama

medyası tasarlanmış ve üretilmiştir. Üretilen yeni medya, döner mikrobiyal çerçeve (rotating microbial carrier frame-RMCF) olarak isimlendirilmiştir. RMCF, taşıyıcı bir çerçevenin üzerinin mikrofiltrasyon membran ile kaplanması sonucunda elde edilen odacıklara quorum quenching bakterilerin immobilize edilmesinin ardından bir karıştırıcının ucunda döndürülmek suretiyle uygulanır. RMCF inert bir malzemeden üretilerek, QQ boncuğun uygulama sırasında parçalanması dezavantajını, dönme hareketi ile mikrobiyal veselin uygulama sırasında düşük F:M oranı dezavantajını ortadan kaldırmaktadır.

RMCF'nin üretimi ardından, tez kapsamında tasarlanmış olan bu yenilikçi ürün MBR işletimlerinde denenmiştir. Gerçekleştirilen kısa ve uzun süreli MBR işletimleri sonucunda RMCF kullanımı ile biyofilm oluşumunun %65'lik bir başarı ile engellenebildiği görülmüştür. Bunun yanı sıra, RMCF kullanımı ile EPS_p ve EPS_k konsantrasyonlarında sırası ile %54 ve %29'luk azalma olduğu sonucu da elde edilmiştir. RMCF'nin MBR uygulaması sırasında quorum quenching etkisi ve dönme hareketi ile elde edilen fiziksel membrane temizleme etkisi kısa ve uzun süreli işletmeler ile ortaya konulmuştur. Yapılan testler sonucunda RMCF'nin biyofilm engelleme başarısının %20'sinin fiziksel yıkama etkisi olduğu sonucuna varılmıştır. RMCF'nin hem quorum quenching hem de fiziksel yıkama etkisi mekanizmaları sayesinde başarılı bir şekilde biyofilm oluşumunu engellediği görülmüştür. Ayrıca, RMCF kullanımında immobilize bakterilerinin miktarının artırılması ile MBR işletimindeki QQ verimi kolaylıkla artırılabilmiştir. MBR tesisinin boyutu ve hedeflenen biyofilm azaltımı seviyesine göre RMCF'nin ve/veya RMCF bünyesindeki ceplerin sayısı artırılarak kullanımı sağlanabilir.

Quorum quenching bakteriler bulundukları ortamdaki sinyal moleküllerini parçalayarak grup davranışlarını ortadan kaldırır ve EPS salınımını azaltır. Sonuç olarak, mikrobiyal kültür bu durumdan etkilenebilir. Mikrobiyal çeşitliliğin tüm tez boyunca gerçekleştirilen QQ çalışmalarından etkilenip etkilendiğini gözleyebilmek adına aktif çamurdan alınan kültür örnekleri ile denature gradyen jel elektroforez (Denaturing Gradient Gel Electrophoresis-DGGE) analizleri gerçekleştirilmiştir. Kısa süreli QQ MBR işletimleri mikrobiyal çeşitlilik üzerinde herhangi bir etkide bulunmazken, uzun süreli QQ MBR işletimleri sonucunda reaktör içerisindeki tür çeşitliliğinin arttığı görülmüştür. Bu tür çeşidi artışı, sinyal moleculerinin parçalanması sonucu popülasyon dengesi içerisinde daha baskın olan türlerin iletişimlerini sürdürerek baskın olma durumlarını sürdürmelerinin engellenmesi ve popülasyon dengesi içerisinde çekinik olan türlerin üzerindeki baskının kalkması sebebi ile çoğalarak karışık kültürde daha görünür bir yüzdesel oran elde edebilmeleri olarak yorumlanabilir.

Quorum quenching immobilizasyon medyalarının uygulamalarının sağladığı TMB azalimleri sayesinde MBR işletimindeki enerji sarfiyatlarında azalma gerçekleşecektir. Tez kapsamında çalışmaları gerçekleştirilen üç değişik medyanın enerji sarfiyatı azaltımlarını ve üretim maliyetlerini ortaya koymak amacıyla maliyet analizi gerçekleştirilmiştir. Sadece enerji sarfiyatı azalımı göz önüne alındığında QQ boncuk kullanımı en faydalı ve mikrobiyal vesel kullanımı en az faydalı tercihler iken, biyo-ürünlerin üretim maliyetleri de yansıtıldığında RMCF kullanımı en faydalı ve mikrobiyal vesel kullanımı en az faydalı tercih olmaktadır. Üretim maliyetleri dikkate alınmadığında toplam maliyetteki azalım oranları mikrobiyal vesel, QQ boncuk ve RMCF için sırası ile %12, %24 ve %23 iken, üretim maliyetleri dikkate alındığında bu yüzdelere sırası ile %6, %15 ve %21 olmaktadır. Bunun sebebi, RMCF'nin tasarımında

göze alınan hususlar doğrultusunda üretim maliyetinin düşük olması ve uzun süreli işletimler sırasında immobilize bakterilerin rejenerasyonuna izin vermesidir.

Tez kapsamında, MBR sistemlerde yenilikçi bir yaklaşım olan quorum quenching mekanizmasının uygulanabilirliği değişik bakış açıları ile ortaya konulmaya çalışılmıştır. Daha önce Çevre Mühendisliği uygulama alanları içerisinde oldukça yeni yer bulan ve literatürdeki mevcut uygulamaların kısıtlı olduğu quorum quenching mekanizması atıksu arıtımında kullanılmış ve birim atıksu arıtma maliyetleri önemli ölçüde azaltılabilmektedir.

Anahtar kelimeler: Membran Prosesler, MBR, Biyojik Tıkanma, Bakterilerin Sinyalizasyonunun Engellenmesi, Enerji Tasarrufu

1. INTRODUCTION

1.1 Purpose of Thesis

The main purpose of this thesis was to develop a new and effective method of preventing biofouling as an output of the membrane process, which is currently considered to be one of the most effective wastewater treatment options. Quorum sensing and quorum quenching mechanisms are relatively new concepts and, as such, there is a distinct lack of detailed studies on these technologies. This thesis describes research in which innovative products were employed to apply biological concepts to MBRs with the intention of controlling biofilm formation on the surface of the membrane during the operation of the reactor.

Over the course of the last twenty years, membrane bioreactor technology has represented one of the most important and innovative technologies for advanced wastewater treatment. However, there are a number of inherent problems associated with its use, and a large amount of research has focused on developing solutions that can solve these problems. One of the hottest research areas has been in the domain of environmental engineering. Recent studies indicate that some of the main characteristics of the bio-cake, which gradually forms on the membrane surface over time, plays an important role in the membrane's loss of permeability.

According to the results obtained from the various research studies that have examined membrane biofouling, extracellular polymeric substances (EPSs) and soluble microbial products (SMPs) are directly related to the biological fouling of membranes. In order to decrease the effect of EPSs and SMPs on the membrane, operation parameters—such as hydraulic retention time, sludge age, organic loading rate, etc.—or the characteristics of the membrane, such as the hydrophilicity, surface roughness, surface charge, etc.—need to be modified. The membranes themselves can be modified by using various chemical substances like polyelectrolytes, nanoparticles, and antibiotics.

At present, there are many examples of anti-biofouling studies that incorporate different approaches to solutions including material, engineering and chemistry sciences; however, all of these methods have some limitations in terms of the extent to which they can prevent biofouling. One of the biggest challenges in this area concerns the fact that biofilm formation is a totally natural process; as such, unnatural engineering and/or chemistry solutions have not yet demonstrated the ability to adapt to the dynamic processes involved.

In the current study, the bacterial quorum quenching mechanism was applied to membrane bioreactors using various QQ products. The results indicated that some bacteria species produce small molecules, named auto-inducers, to create an intracellular communication. When the concentration of these auto-inducers reaches a certain level, which can depend on the species, auto-inducers combine with receptor proteins. These proteins activate the target genes responsible for group behaviors. Bioluminesans, the production of antibiotics, virulence, the creation of biofilm and the creation of spores can be given as examples of such group behavior.

Since the concept of quorum sensing was first introduced, numerous studies have been conducted that use plants to prevent biofilm formation on medical devices. In some cases, these studies have been successful. In light of the achievements in this area, it was theorized that interrupting bacterial communication in activated sludge could reduce membrane biofouling and the more efficient operation of the MBR. This thesis describes the use of an innovative QQ product in which QQ bacteria immobilized was designed, manufactured and used in the MBR to determine the relationship between the QQ mechanism and biofouling.

It was expected that this innovative QQ product could reduce membrane biofouling. The prevention of membrane biofouling is an essential prerequisite for the efficient operation of MBRs. Furthermore, this thesis aimed to present new literature on an idea that did not have any application example in our country. In addition to these, the design utilized in the study provided a means through which the advantages and disadvantages of present immobilization media would be better understood. Within this regard, through bringing a new perspective to the literature, it was anticipated that this study would make an important contribution to research in this domain.

To summarize, this study identified a means by which the flux decrease resulting from degradation of the auto-inducers secreted by microorganisms in the activated sludge during the operation of MBR could be prevented through the use of manufactured QQ products. This use of QQ products resulted in a stable and continuous permeate while also reducing energy consumption and a reduction in the unit wastewater treatment cost.

1.2 Scope of Thesis

This thesis is organized as follows. First, a comprehensive literature review is presented that provides a detailed overview of the key concepts and terms associated with the topic, explains the development process and reviews the disadvantages and shortages of the anti-biofouling applications that are currently available. The literature review presents an introduction to the membrane processes, membrane bioreactors, the advantages and disadvantages of membrane bioreactors, and the biofouling problem. It then progresses to describe quorum sensing, quorum sensing and biofilm creation, quorum quenching, and quorum quenching and the prevention of biofilm. Finally, the literature review concludes by discussing contemporary quorum quenching MBR applications and their advantages and disadvantages.

Following the literature review, the experimental studies that were conducted as part of this research are described. Within the scope of this thesis, these are described in four sections. Each experimental study section contains an introduction, a material and method sub-section for the manufacturing of products, details of the operation conditions and analytical methods employed, and the results and discussion of the experimental results and their evaluations.

The first section describes a new approach to bacterial quorum quenching applications that is associated with biofouling control: the rotary microbial carrier frame (RMCF). This section explains the idea of a new immobilization media design, the process by which this was manufactured and its application within MBRs. The biofouling prevention capacity of this innovative product was investigated during both short- and long-term operations.

The second section describes quorum quenching MBR operations for biofouling control under different operation conditions and by using different immobilization

media. In this section, a comprehensive study was conducted with the objective of observing and comparing the quorum quenching activity of three different immobilization media: cell entrapping beads, microbial vessels and RMCFs, and to examine the effects of various MBR operation conditions using cell entrapping beads.

The third section describes a one-step membrane bioreactor: a combined MBR system for enhanced nitrogen removal and biofouling control. In this section, the extent to which a new MBR operation approach could simultaneously prevent biofouling and enhance nitrogen removal was assessed.

The fourth and final experimental study examined the effect of the bacterial growth phase on the quorum quenching activity of *Rhodococcus sp.* BH4. Within the scope of this fourth study, the growth kinetic of BH4 was determined, and a sludge age concept was applied to regenerate BH4 for quorum quenching activity sustainability during long-term MBR applications.

Within the scope of this thesis, an innovative immobilization medium named RMCF was designed and manufactured, and its QQ mechanism efficiency in the MBR was examined and compared using cell entrapping beads and a microbial vessel. All three of these immobilization media have their own advantages and disadvantages. It was possible to reduce transmembrane pressure (TMP) values during MBR operation, and this reduced the costs associated with advanced wastewater treatment.

The thesis concludes by evaluating the experimental results and cost analysis and presents recommendations for the future direction of the RMCF for pilot- and real-scale applications.

2. LITERATURE REVIEW

2.1 Introduction

In the contemporary world, scarcity has become a hot issue. In particular, many areas of the globe currently suffer from a lack of access to clean water and the need for potable and usable water has resulted in the development and increased use of water treatment technologies. According to forecasts produced by the Met Office (2006), by 2025, 5.1 billion people will have a lack of access to clean water, and by 2085 this figure will reach 6.5 billion. The wastewater that is currently discharged into the environment is the byproduct of both domestic and industrial processes. At present, domestic wastewater is accountable for the highest volumetric ratio in terms of total discharge. As such, as the population increases with the development of humanity, so too does the unit water consumption per capita and the unit pollution loads per capita. Furthermore, discharge standards that are designated according to the total daily mass of pollutant required to protect the quality of receiving waterbody necessitates the treatment of wastewater with high removal efficiencies.

Several types of wastewater treatment techniques are currently in use. These can be broadly classified as physical, chemical and biological. While physical treatment is preferred to remove big solid materials from wastewater, like debris, hairs, etc., chemical treatment is required to remove specific pollutants like heavy metals or phosphorus. Physical treatment is generally employed as the primary treatment mechanism and is used to help and protect the biological treatment units in which the secondary treatment processes take place. Biological wastewater treatment utilizes both conventional approaches, such as activated sludge systems and rotating biological contactors, and innovative applications like activated sludge systems with modifications and membrane bioreactors (MBR).

The membrane bioreactor utilizes a treatment technology that combines the activated sludge and membrane filtration processes. Membrane can be basically defined as a selective wall that can separate two different phases (Judd & Judd, 2006). While

pollutants cannot cut across through the membrane, treated water can be collected from behind it. Biological treatment in the MBR tank can be realized by the activated sludge via organic matter removal during the life cycles of the microorganisms that exist within it. MBR systems that can combine these two processes and achieve advanced wastewater treatment have many advantages. Since mixed liquor suspended solid (MLSS) concentrations, which are in the range of 2000-4000 mg/L in conventional systems, can be kept in the range of 12000-15000 mg/L in the MBR, the hydraulic retention time in the MBR is lower. This lower HRT value results in a smaller tank volume and, thus, entails that the tank can be placed in a smaller area (Judd et al., 2001; Judd, 2004). In addition to this, high MLSS values help MBR systems to tolerate shock loadings (Yigit, 2007). Moreover, there is no need for the use of clarifiers in the treatment plant because solids are separated from the mixed liquor by means of filtration (Ortiz et al., 2007). Comparison of activated sludge treatment system and membrane bioreactor system was schematically given in Figure 2.1.

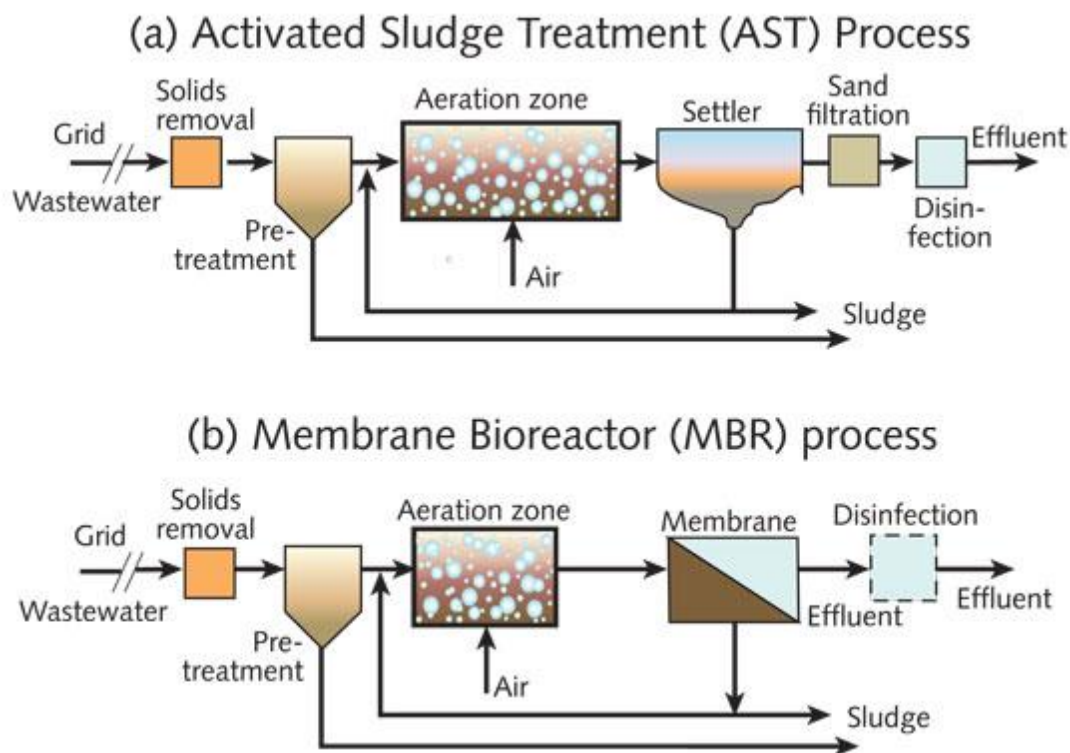


Figure 2.1 : Comparison of conventional activated sludge system and membrane bioreactor system, adapted from WaterWorld (2015).

The biggest advantage of MBRs is that effluent can find several beneficial usage areas, like irrigation. It is perhaps due to these advantages that the use of MBRs has increased in recent years. According to existing studies in this domain, the global use of MBRs

has grown at a rate of between 10 and 15%, and the total value of the global MBR market is expected to witness positive growth in the near future (BCC, 2008; Frost & Sullivan, 2013).

The current market research indicates that MBR technology is increasingly being viewed as a modern and reliable technology; however, uncertainty remains as to the relatively high risks and costs associated with the use of MBRs versus conventional technologies (Frost & Sullivan, 2003). Furthermore, specific concerns have been raised regarding the fouling problem that is associated with the operation of MBRs. One of the fundamental limitations of all membrane processes is that they result in pollution. Rejected pollutants and microorganisms in the mixed liquor tend to attach to the surface of the filtration membrane. Fouling can result in flux reduction under the constant transmembrane pressure (TMP) condition or TMP increase under the constant permeate flowrate condition because of the permeability=flux/TMP relationship, and these changes result in an increase in the unit cost of wastewater treatment (Judd & Judd, 2006). Strategies that are specifically designed to prevent fouling as an output of the MBR operation can be broadly grouped into five different approaches: 1) Physically treating the feed, 2) Physically and chemically cleaning the membrane, 3) Reducing the flux, 4) Increasing the aeration rate, and 5) Making chemical or biochemical modifications to the mixed liquor (Judd & Judd, 2006). Since all of these operation strategies increase the unit treatment cost, researchers have invested a significant amount of effort in identifying alternative methods of preventing fouling that minimize this cost. Studies on the fouling problem have gradually increased in recent years and generally involve the modification of membrane material or module configuration. However, many people view these studies as insufficient because the modifications proposed are costly and have largely failed to stand the test of time during long-term MBR operations. The reason for this insufficiency is that the creation of biofilm, and the gradual increase of this biofilm, is a totally natural process. Membrane biofouling in the MBR is generally associated with extracellular polymeric substances (EPS) and soluble microbial products (SMP) (Chang & Lee, 1998; Cho & Fane, 2002; Nagaoka et al., 1996; Rosenberger & Kraume, 2002). EPS is a structural material of microbial aggregations, such as biofilm, floc and activated sludge liquor, and a term used for macromolecules like carbohydrates, proteins, lipids and other polymeric substances on the surfaces of the cells or in the gap between the cells

(Flemming & Wingender, 2001; Jang et al., 2005). EPS, with its unstable and heterogeneous structure, can create a highly hydrated gel, and this gel structure forms a barrier against the permeate flow during the membrane filtration process (Nielson & Jahn, 1999). Besides, SMP is used for definition of cellular compounds created during cell decay, synthesis, etc. (Laspidou & Rittmann, 2002; Li et al., 2005).

The key aspect of biofilm creation that involves a totally natural process is quorum sensing (QS). Quorum sensing is the communication between species using signalization. Quorum sensing microorganisms show several types of group behaviors including EPS and SMP secretions and biofilm creation. Microorganisms in communication with each other start to accumulate in the biofilm and create a bio-cake that has less porosity. In order to prevent this situation, signalization mechanisms have to be interrupted, and this interruption mechanism is known as quorum quenching (QQ). Although the application of QQ in MBRs is a relatively new topic in the area of anti-biofouling MBR studies, there are some examples of the use of QQ within MBRs. For the purposes of this thesis, existing QQ MBR studies were compiled by evaluating their advantages and disadvantages, and informative explanations on the future potential of this new vision were formulated.

2.2 Quorum Sensing

Quorum sensing (QS) can be basically described as a mechanism by which bacteria are produced and chemical signal molecules, known as autoinducers (AIs), are released to allow cell-to-cell communication. When the concentration of AI molecules reaches a threshold level as a function of cell density, bacteria sense their density and a coordinated change in bacterial behavior is induced (Williams et al., 2007). Using these QS signal-response systems, bacteria regulate gene expression in response and synchronize particular behaviors such as bioluminescence, antibiotic production, virulence, biofilm formation, the production of soluble microbial products (SMP) and extracellular polymeric substances (EPS) (Jiang et al., 2013; Waters & Bassler, 2005). A schematic drawing of quorum quenching mechanism is given in Figure 2.2.

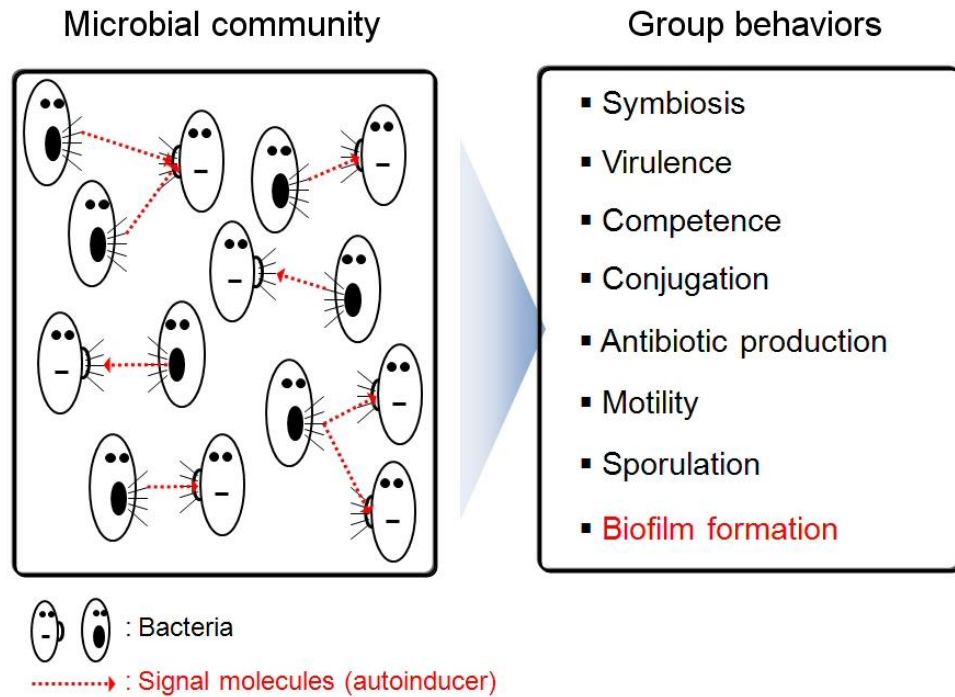


Figure 2.2 : Quorum sensing mechanism and group behaviours, adapted from WEMT (2015).

Although several QS systems are known, N-Acyl-homoserine lactones (AHLs) are the most common signaling molecules used by mainly Gram-negative bacteria and peptide-based signaling systems are used by mainly Gram-positive bacteria (Kim et al., 2014; Parsek & Greenberg, 2005). Additionally, both Gram-negative and Gram-positive bacteria produce autoinducer-2 (AI-2), which is a member of a family of signaling molecules used in quorum sensing that can facilitate interspecies cell-to-cell signaling.

The first observed QS system based on AHL autoinducer and its cognate regulatory circuit was that of the bioluminescent marine bacterium *Vibrio fischeri*, which was examined as a model for quorum sensing in most Gram-negative bacteria. At low cell densities in natural seawater, *V. fischeri* is non-luminescent. However, when it is proliferated to high cell densities in the laboratory, a *V. fischeri* culture bioluminesces with a blue-green light. Significantly, this bioluminescent bacterium is commonly found in symbiotic relationships with marine animals like the bobtail squid (Whitehead et al., 2001). While the squid host uses the bacterial-produced light for counter illumination to mask its shadow as an anti-predation tactic, the bacteria profits from the nutrient-rich squid and grows to high cell density that is unachievable in seawater. Two regulatory proteins, called LuxI and LuxR, are essential for QS control of

bioluminescence in *V. fischeri*. LuxI is the AI synthase enzyme in *V. fischeri*, and it catalyzes the formation of the AHL. Then, AHL molecules freely diffuse out of the cell membrane and accumulate. At high AHL concentrations (high cell density), LuxI is bound by LuxR and this complex activates transcription of the LuxICDABE operon. In the absence of an AHL signal, LuxR is inactive. At low cell densities, the AHL signals produced by LuxI diffuse passively out of the cell following a concentration gradient. Thus, the lux operon is not expressed. Different types of AHL-based quorum sensing molecules are produced by different bacteria. AHLs are composed of homoserine lactone (HSL) rings that carry acyl chains that differ from C4 to C18 in length (Ng & Bassler, 2009). A large number of other Gram-negative bacteria possess LuxIR-type proteins and communicate with AHL signals for intraspecies quorum sensing (Waters & Bassler, 2005).

While Gram-negative bacteria use AHLs as auto-inducers, Gram-positive bacteria mostly use secreted peptides as autoinducers for quorum sensing. Peptides are encoded in genes and are made as a larger precursor protein (pro-peptides) inside the cell. These are then further processed to small linear or cyclic peptides during secretion. According to Parsek & Greenberg (2005):

In contrast to AHL-based signaling, peptide signals are not detected inside the cell. In some cases, a membrane bound sensor protein belonging to the two-component signal transduction family interacts with the peptide. Peptide-bound sensor then activates an associated response regulator, which modulates expression of quorum-sensing-regulated genes. (p. 1)

The AI-2 quorum-sensing system was first described in *Vibrio harveyi* and has been implicated in interspecies communication (Surette & Bassler, 1998). In all AI-2-producing bacteria, the precursor for the AI-2 signal is 4,5-dihydroxy-2,3-pentanedione (DPD), the product of the reaction catalyzed by the LuxS enzyme. Homologs of luxS exist in hundreds of Gram-negative and Gram-positive bacteria, consistent with a role for AI-2 in interspecies communication that allows other luxS-encoding bacteria in a particular environment to contribute to the overall cell-density information (Federle & Bassler, 2003; Xavier & Bassler, 2005). Quorum sensing signals and QS-controlled behaviors in some bacteria are listed in Table 2.1.

Table 2. 1 : Quorum sensing signals and QS-controlled behaviors in some bacteria, adapted from Dobretsov et al. (2009).

QS signals	Bacteria	Signal synthase	Process controlled by QS	References
3-oxo-C6-HSL	<i>Vibrio fischeri</i>	LuxI	Bio-luminescence	Nealson et al., 1970; Eberhard et al., 1981
3-oxo-C10-HSL	<i>Vibrio anguillarum</i>	VanI	Virulence	Defoirdt et al., 2004
3OH-C4-HSL	<i>Vibrio harveyi</i>	LuxM	Bio-luminescence, biofilm production	Waters & Bassler, 2005
C4-HSL	<i>Pseudomonas aeruginosa</i>	RhII	Biofilm maturation, adhesion	Waters & Bassler, 2005
3-oxo-C12-HSL	<i>P. aeruginosa</i>	LasI	Virulence production	Pearson et al., 1994
3-oxo-C8-HSL	<i>Agrobacterium tumefaciens</i>	TraI	Conjugal transfer of the virulence plasmid	Cha et al., 1998; Fuqua et al., 2001
C4-HSL C6-HSL	<i>Aeromonas hydrophyla</i> , <i>A. salmonicida</i>	AhyI AsaI	Biofilm formation, enzyme production	Swift et al., 1999
C6-HSL	<i>Chromobacterium violaceum</i>	CvI	Violacein, antibiotics and enzyme production	Cha et al., 1998
C4-HSL	<i>Serratia marcescens</i>	SwrI	Swarming	Miller & Bassler, 2001
C6-HSL Oxo-C6-HSL C8-HSL	<i>Yersinia enterocolytica</i> , <i>Y. pseudotuberculosis</i>	YenI YpsI	Motility Aggregation	Miller & Bassler, 2001
Group I thiolactone	<i>Staphylococcus aureus</i>	AIP-I	Virulence	Lyon et al., 2000

Table 2. 1 (continued) : Quorum sensing signals and QS-controlled behaviors in some bacteria, adapted from Dobretsov et al. (2009).

QS signals	Bacteria	Signal synthase	Process controlled by QS	Reference
Cyclic thiolactone	<i>S. aureus</i>	AIP-II	Virulence	Zhang & Dong, 2004
Group III thiolactone	<i>S. aureus</i>	AIP-III	Virulence	Lyon et al., 2000
Group IV thiolactone	<i>S. aureus</i>	AIP-IV	Virulence	Lyon et al., 2000
ADPITRQWGD	<i>Bacillus subtilis</i>	ComX	Sporulation	Waters & Bassler, 2005
ERGMT	<i>Bacillus subtilis</i>	CSF	Competence, Sporulation	Waters & Bassler, 2005
EMRLSKFFRD FILQRKK	<i>S. pneumoniae</i>	CSP	Competence	Waters & Bassler, 2005
g-butyrolactones g-butyrolactone	<i>Streptomyces griseus</i>	A-factor	Induce biosynthesis of antibiotics	Waters & Bassler, 2005
Diketopiperazines (DKP)	<i>Pseudomonas aeruginosa</i>	*	Cross-species communication	Holden et al., 1999
Cyclo(Ala-I-Val)	<i>E. coli</i>			
and cyclo(1-Pro-I-Tyr)	<i>Proteus mirabilis</i>			
	<i>Citrobacter freundii</i>			
	<i>Enterobacter sp.</i>			
Furanosyl borate diester	<i>Vibrio harveyi</i>	AI-2	Luminescence	Chen et al., 2002
(2R,4S) -2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran	<i>Salmonella enterica</i> <i>sv Typhimurum</i>	AI-2	Virulence gene expression	Miller et al., 2004

* So far no evidence of enzymatic synthesis of DKP by these bacteria

2.3 The Role of Quorum Sensing on Biofilm

Surfaces are important microbial habitats that generally provide enormous access to nutrients, protection from predation and environmental stresses, and a means for cells to remain in a favorable habitat without being washed away. Biofilm formation occurs when bacterial cells grow on surfaces. Bacterial cells that attach to a surface combine

through an adhesive matrix secreted by the cells. The matrix is composed of a variety of polysaccharides and proteins. Biofilms utilize nutrients more easily for microbial growth and help prevent the detachment of cells on dynamic surfaces, such as in flowing systems. Biofilms may contain only one or two species or, more commonly, many species of bacteria. As the organisms adhere to a surface, they keep signaling to one another, and ultimately an expression of genes-related biofilm is initiated (Figure 2.3). For example, the major intracellular signaling molecules are acylated homoserine lactones in *Pseudomonas aeruginosa*. As these lactones accumulate according to population density, they are released and subsequently recognized by adjacent cells. The signaling lactones then control the expression of genes that contribute to biofilm formation:

At least four reasons have been proposed for the formation of biofilms. First, biofilms are a means of microbial self-defense that increase survival. Second, biofilm formation allows cells to remain in a favorable niche. Third, biofilms form because they allow bacterial cells to live in close association with each other. Finally, biofilms seem to be the typical way bacterial cells grow in nature. The biofilm may be the “default” mode of growth for prokaryotes in natural environments, the latter of which differ dramatically in nutrient levels from the rich liquid culture media used in the laboratory. (Madigan et al., 2010, p. 14-15).

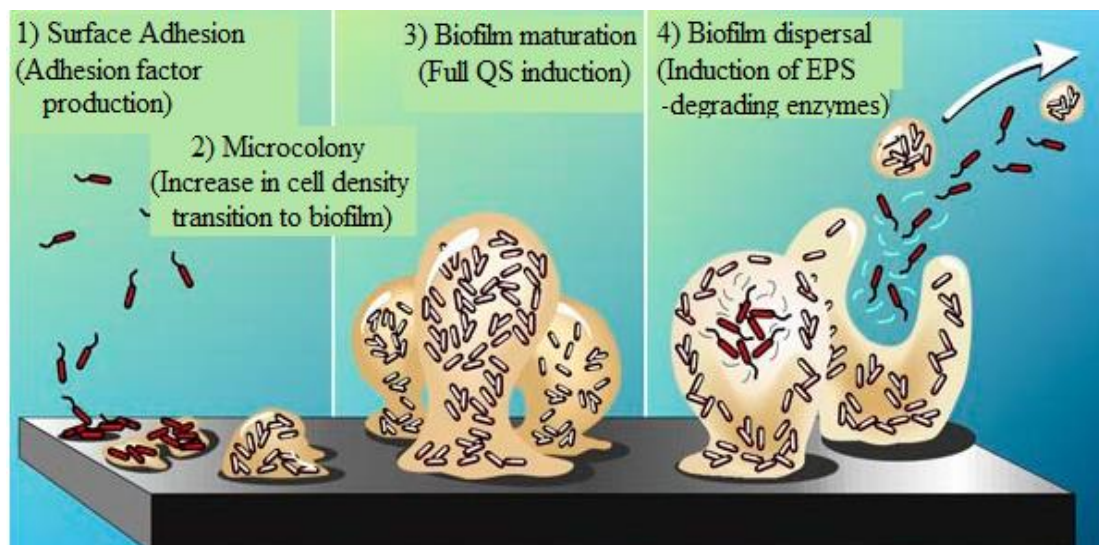


Figure 2.3 : Quorum sensing mechanism and group behaviours, adapted from Montana (2015).

2.4 Determination of Quorum Sensing

Since the signal molecules are produced at relatively low concentrations, conventional methods are unable to accurately detect them. However, bioassays can be precise, and they can be quickly and effectively performed to detect the autoinducers that diffuse out of the cell. Most of the biosensors in use are genetically modified microorganisms that are used for the detection of exogenous AHL signal molecules. They cannot produce their own AHL autoinducers because regulatory components of the quorum sensing system have been genetically removed. Biosensors have generally been dependent upon the use of lacZ reporter fusions in an *E. coli* or *A. Tumefaciens* genetic background or on the inhibition of the purple pigment violacein in *Chromobacterium violaceum* (Brelles-Mariño & Bedmar, 2001).

2.5 Enzymatic Quorum Quenching

The most common approaches to control bacterial growth in a given environment involve the use of antibiotics; however, it is a well-known fact that bacteria species can develop resistance to these anti-bacterial agents (Wopperer et al., 2006; García-Aljaro et al., 2008). In addition, the main aim of biofouling prevention during membrane bioreactor operation is not to kill or deactivate all bacteria because a high level of death and deactivation may reduce the efficiency with which pollutants are removed. It is widely recognized that N-acyl homoserine lactone (AHL)-mediated quorum sensing plays the main role in biofilm creation. As such, some researchers have suggested the use of a new paradigm “quorum quenching” to control the group behaviors of bacteria without affecting their lifecycles (Uroz et al., 2009). Because the number of Gram-negative bacteria is higher than the number of Gram-positive bacteria in the activated sludge, AHL-mediated quorum quenching has been investigated. There are three main AHL-mediated quorum quenching mechanisms (schematically shown in Figure 2.4): 1) Blocking AHL synthesis in the sender cell, 2) Deactivating AHL via enzymatic destruction, and 3) interfering with signal receptor in the receiving cell (Lade et al., 2014).

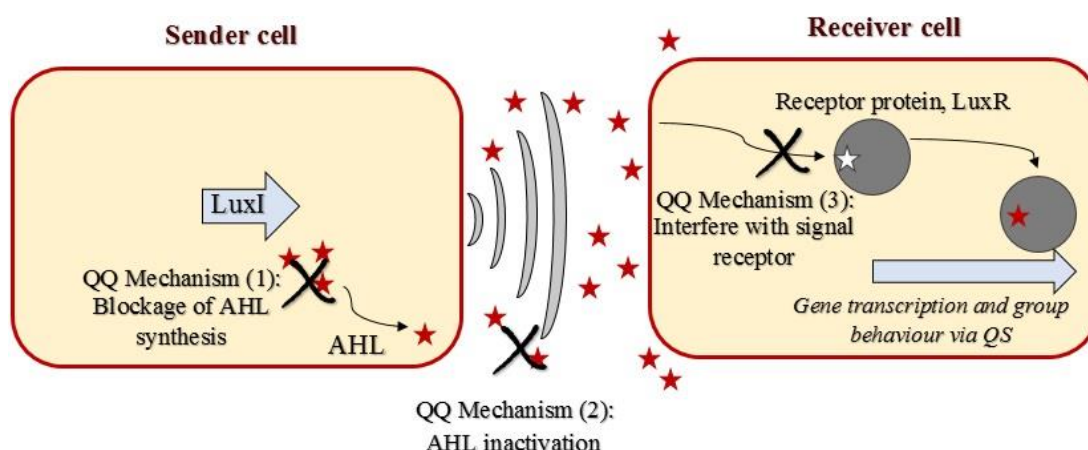


Figure 2.4 : Main AHL-mediated quorum quenching mechanisms.

As mentioned above, the first mechanism involves blocking AHL synthesis. The inhibition of AHL synthesis by blocking LuxI-type synthase proteins is possible and, since AHL is synthesized from S-adenosylmethionin, the analogues of this amino acid can be used to block AHL synthesis (Geske et al. 2008; Parveen & Cornell, 2011). Additional research has found that erythromycin can block AHL synthesis at the ribosomal level through the use of an unknown mechanism (Rasmussen & Givskov, 2006).

The second mechanism, AHL degradation, is the most well known mechanism. Enzymatic destruction of AHL molecules can be realized by different types of enzymes, and this prevents AHL accumulating in the environment. Seven different AHL degradation enzymes have been described in the literature. While some of them prefer the degradation pathway, others use the modification pathway (Figure 2.2). The name of quorum quenching enzymes and the final product/s that result from AHL degradation are presented in Figure 2.5.

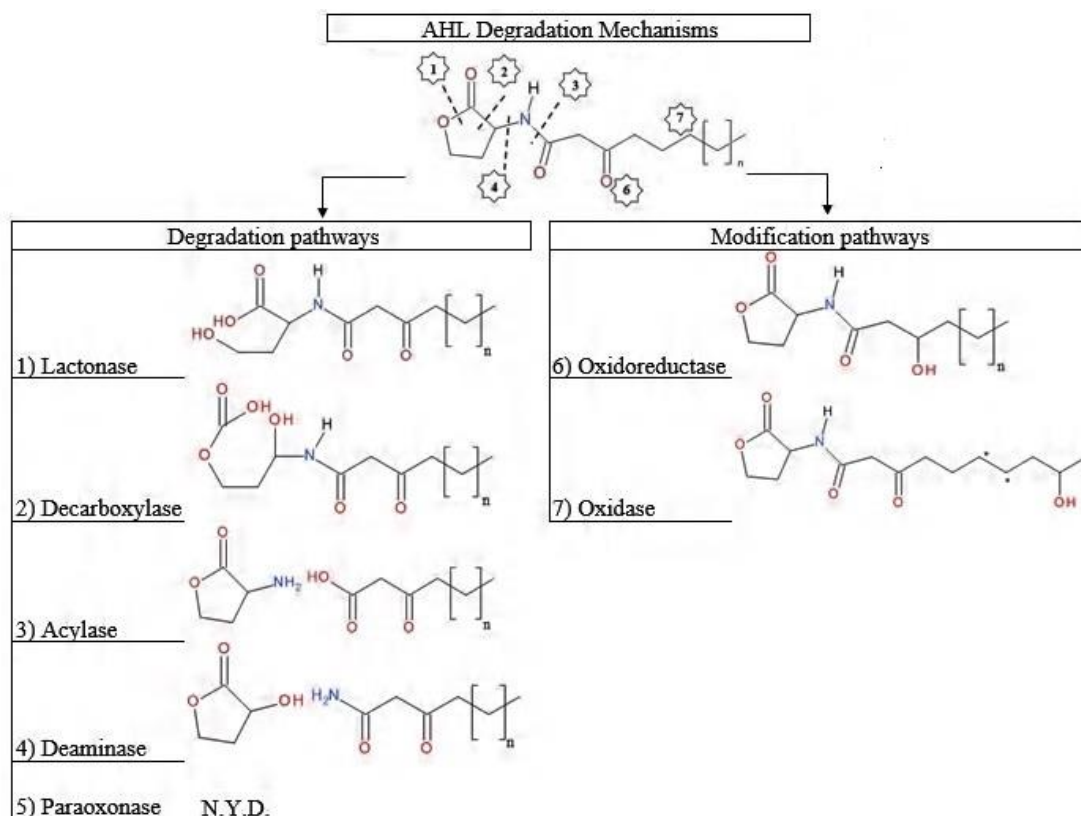


Figure 2.5 : Enzymatic AHL degradation mechanism and final products (N.Y.D.: Not yet determined).

While lactonases open the homoserine (HSL) ring by disruption of the bond at the left of the double-bonded oxygen (Dong et al.; 2000; Dong et. al., 2001; Zhang et al., 2002) and create acyl-homoserine lactone, decarboxylases open this ring by disrupting the bond at the right of the double bonded oxygen (Dong & Zhang, 2005). At present, no studies have investigated the mechanism of decarboxylase with a certain bacteria specie. In addition, acylases, which has synonyms in the literature like aminase and amidohydrolysis, cleave the acyl side chain from the HSL ring (Perveen & Cornell, 2011; Leadbetter & Greenberg, 2000) and create fatty acid and homoserine lactone. Even though there are no examples of studies that have been carried out with a certain bacteria specie, deaminase can also cleave the acyl side chain from the HSL ring, but only with a different point. Deaminases create final products as a HSL chain with OH⁻ and a side chain with NH₂ (Dong & Zhang, 2005). The last example of the QQ enzymes that carry degradation pathway is paraoxonase. Paraoxonase is an enzyme that is sourced from mammalian cells, and information about its mechanism has not yet been exactly determined; however, research has proven that it can both disrupt the chain and cleave the side chain of AHL (Billecke et al., 2000; Draganov et al., 2000; Teiber et al., 2003; Chun et al., 2004). On the other hand, two enzymes can carry the

modification pathway: oxidoreductase and oxidase. While oxidoreductase can convert the oxygen that is double bonded to the side chain of AHL to OH⁻ (Chowdhary et al., 2007; Uroz et al., 2005), oxidase modifies the last bond of the side chain (Uroz et al., 2005). Species that use these QQ enzymes are listed in Table 2.2.

Table 2. 2 : Quorum quenching species according to the enzyme types.

QQ Enzymes	Source/Bacterial strains	Gene/ Protein	AHL type	References
Lactonase	Mammalian cell	Lactonase	C6-HSL, 3-oxo-C12-HSL	Chun et al., 2004 Yang et al., 2005
	Fungi	n.d.	C6-HSL	Götz et al., 2007
	As. Niger IAN 2094	Glocono lactonase	Lactone ring hydrolysis	Ogawa et al., 2002
	<i>A. Tumafaciens</i> c58	AttM	3-oxo-C8-HSL	Zhang et al., 2002
	<i>Bacillus</i> sp. 240B1	AiiA	C8-HSL	Dong et al., 2000 Wopperer et al., 2006
	<i>B. anthracis</i>	AiiA	C6-HSL, C8-HSL, C10-HSL	Ulrich, 2004
	<i>B. cereus</i>	AiiA	C6-HSL, C8-HSL, C10-HSL	Dong et al., 2002
	<i>B. mycoides</i>	AiiA	C6-HSL, C8-HSL, C10-HSL	Dong et al., 2002
	<i>B. thuringiensis</i>	AiiA	3-oxo-C6-HSL, C6-HSL, C8-HSL	Lee et al., 2012
	<i>A. Tumafaciens</i> C58	AiiB	3-oxo-C6-HSL, C6-HSL, C8-HSL, C7-HSL, 3-oxo-C8-HSL	Carlier et al., 2003
	<i>A. Tumafaciens</i>	AiiB	C4-HSL, 3-oxo-C6-HSL, C6-HSL, C6-HSL, 3-oxo-C8-HSL, C8-HSL, C10-HSL	Liu et al., 2007
	<i>Ge. Kaustophilus</i> HTA426	GKL	C6-HSL, 3-oxo-C8-HSL, C8-HSL, C10-HSL, 3-oxo-C12-HSL	Chow et al., 2010
	<i>M. testaceum</i> StLB037	AiiM	3-oxo-C6-HSL, C6-HSL, 3-oxo-C8-HSL, C8-HSL, 3-oxo-C10-HSL, C10-HSL	Wang et al., 2010
	<i>My. Paratuberculosis</i> K-10	MCP	C7-HSL, 3-oxo-C8-HSL, C8-HSL, C10-HSL, C12-HSL	Chow et al., 2009

Table 2. 2 (continued) : Quorum quenching species according to the enzyme types.

QQ Enzymes	Source/Bacterial strains	Gene/ Protein	AHL type	References
Lactonase	<i>My. Tuberculosis</i>	PPH	C4-HSL, 3-oxo-C8-HSL, C10-HSL	Afriat et al., 2006
	<i>Ochrobactrum sp. T63</i>	AidH	C4-HSL, C6-HSL, 3-oxo-C6-HSL, 3-oxo-C8-HSL, C10-HSL	Mei et al., 2010
	<i>So. Silverstris StLB046</i>	AhlS	C10-HSL	Morohoshi et al, 2012
	<i>Sul. Solfataricus P2</i>	SspPox	3-oxo-C8-HSL, C8-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL	Merone et al., 2005 Elias et al., 2008
	<i>Rho. Erythropolis W2</i>	QsdA	3-oxo-C6-HSL, C6-HSL, 3-oxo-C8-HSL, C8-HSL, 3-oxo-C10-HSL, C10-HSL, 3-oxo-C12-HSL, C12-HSL, 3-oxo-C14-HSL, C14-HSL	Uroz et al., 2008
	<i>Acidobacteria sp.</i>	QIcA	3-oxo-C6-HSL, C6-HSL, C7-HSL, 3-oxo-C8-HSL, C8-HSL, 3-oxo-C10-HSL, C10-HSL	Riaz et al., 2008
	<i>Arthrobacter sp. IBN110</i>	AhlD	C4-HSL, 3-oxo-C6-HSL, C6-HSL, 3-oxo-C8-HSL, C8-HSL, 3-oxo-C10-HSL, C10-HSL	Park et al., 2003
	<i>Agrobacterium radiobacter K84</i>	AiiS	Broad spectrum	Uroz et al., 2009
	<i>Klebsiella pneumonia KCTC2241</i>	AhlK	C6-HSL, 3-oxo-C6-HSL	Park et al., 2003
	<i>Rhodococcus sp. BH4</i>	n.d.	Broad spectrum	Park et al., 2006 Uroz et al., 2008
Paraoxonase	Mammalian cells	Pon 1,2,3	Long chain AHL	Yang et al., 2005

Table 2. 2 (continued) : Quorum quenching species according to the enzyme types.

QQ Enzymes	Source/Bacterial strains	Gene/ Protein	AHL type	References
Acylase	Porcine	Acylase I	C4-HSL, C6-HSL, 3-oxo-C8-HSL, C8-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL	Lynch et al., 2002 Paul et al., 2009 Sauer et al., 2001 Xu et al., 2003 Jiang et al., 2013 Kim et al., 2011 Yeon et al., 2009b
	<i>Ralstonia</i> sp. XJ12B	AiiD	3-oxo-C8-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL	Lin et al., 2003
	<i>Anabena</i> sp. PCC7120	AiiC	C4-HSL, C14-HSL	Romeo et al., 2008
	<i>Pseudomonas</i> sp. PAI-A	PvdQ	3-oxo-C10-HSL, C10-HSL, 3-oxo-C12-HSL, C12-HSL, C14-HSL, C16-HSL	Huang et al., 2003 Sio et al., 2006
	<i>P. syringae</i> B728a	HacA	C8-HSL, C10-HSL, C12-HSL	Shepherd & Lindow, 2009
	<i>P. syringae</i> B728a	HacB	C6-HSL, C8-HSL, C10-HSL, C12-HSL	Shepherd & Lindow, 2009
	<i>R. solanacearum</i> GMI1000	Aac	C7-HSL, C8-HSL, 3-oxo-C8-HSL, C10-HSL	Chen et al., 2009
	<i>Shewanella</i> sp. MIB015	Aac	C8-HSL, C10-HSL, C12-HSL	Morohoshi et al., 2008
	<i>Streptomyces</i> sp. M664	AhlM	C8-HSL, C10-HSL, 3-oxo-C12-HSL	Park et al., 2005
	<i>P. aeruginosa</i>	QuiP	C6-HSL, C8-HSL, C10-HSL, C12-HSL	Chan et al., 2011
	<i>Pseudomonas</i> sp. 1A1	n.d.	C6-HSL, C8-HSL, 3-oxo-C8-HSL, C10-HSL, 3-oxo-C10-HSL, C12-HSL, 3-oxo-C12-HSL	Cheong et al., 2013
	<i>V. pradoxus</i>	n.d.	n.d.	Zhang et al., 2002 Carlier et al., 2003
	<i>R. eutropha</i>	AiiD		Lin et al., 2003
	<i>Comamonas</i>	n.d.		Uroz et al., 2006
	<i>Rhodococcus</i>	n.d.	Broad spectrum	Lithgow et al., 2000

Table 2. 2 (continued) : Quorum quenching species according to the enzyme types.

QQ Enzymes	Source/Bacterial strains	Gene/ Protein	AHL type	References
Oxidoreductase	<i>B. megaterium</i> CYP102A1	P450BM3	Oxidizes; C12-HSL, 3-oxo-C12-HSL, C14-HSL, 3-oxo-C14-HSL, C16-HSL, C18-HSL, C20-HSL	Chowdhary et al., 2007
	<i>Burkholderia</i> sp. GG4	n.d.	Reduces; 3-oxo-AHL	Chan et al., 2011
	<i>Rho. Erythropolis</i> W2	n.d.	Converts; C8-HSL to C14-HSL	Uroz et al., 2005
	Rhodococcus	n.d.	Long 3-oxo-AHL	Lithgow et al., 2000
Oxidase	Bacillus megaterium CYP102A1	P450BM-3	Long chain AHL	Bassler & Losick, 2006

The last AHL-mediated quorum quenching mechanism involves interfering with the signal receptor (Koch et al., 2005; Chen et al., 2011). AHL analogues that compete with receptors have been tested in order to prevent microorganism from receiving QS signals. The analogues can be produced by using side chains of AHL. These compounds interact with LuxR, the receptor protein responsible for AHL, and result in the elimination of AHL (Hentzer et al., 2002).

2.6 Biofouling Prevention via Quorum Quenching in MBR

Within the scope of this review, examples of quorum quenching MBR applications that have been developed for the purposes of preventing the biofouling of the filtration membranes located these MBRs were examined and compiled. A list of the studies examined is presented in Table 2.3. These studies are listed according to application methods, such as immobilization media type. Grouping the research in this way also resulted in them being generally being listed chronologically because application methods have changed over time as existing methods have been examined, evaluated and improved. The application method, quorum quenching bacteria specie (if applicable), QQ enzyme type, advantages and disadvantages are presented in this table. The quorum quenching mechanism efficiencies, which are expressed in percentages, were extracted from the sources or calculated using the TMP profiles given in the sources.

Table 2. 3 : Quorum quenching MBR applications.

Immobilization medium	QQ bacteria	QQ enzyme	Advantages	Disadvantages	References
-	-	Acylase	QQ activity (57%)	Cost and instability of enzyme	Yeon et al., 2009a
Magnetic enzyme carrier	-	Acylase	QQ activity (66%)	Cost and instability of enzyme	Yeon et al., 2009b
Magnetic enzyme carrier	-	Acylase	QQ activity (58%)	Cost and instability of enzyme	Kim et al., 2012
Polymeric microbial vessel	<i>Rhodococcus sp.</i> BH4	Lactonase	QQ activity (50%), inert immobilization media material	Low F:M ratio, low QQ enzyme distribution in MBR	Oh et al., 2012
Polymeric microbial vessel	<i>Rhodococcus sp.</i> BH4	Lactonase	QQ activity (60%), inert immobilization media material, higher QQ enzyme distribution in MBR via recirculation	Low F:M ratio	Jahangir et al., 2012
Polymeric microbial vessel	<i>Pseudomonas sp.</i> 1A1	Acylase	QQ activity (58%), inert immobilization media material	Low F:M ratio	Cheong et al., 2013
Ceramic microbial vessel	<i>Pseudomonas sp.</i> 1A1	Acylase	QQ activity (80%), inert immobilization media material, higher F:M ratio via inner feed	Low QQ enzyme distribution in MBR	Cheong et al., 2014

Table 2. 3 (continued) : Quorum quenching MBR applications.

Immobilization medium	QQ bacteria	QQ enzyme	Advantages	Disadvantages	Reference
Sodium alginate beads	-	Acylase	QQ activity (88%) , high F:M ratio, extra physical cleaning effect	Cost and instability of enzyme, low mechanical strength of bead, inseparable from AS	Jiang et al., 2013
Sodium alginate beads	<i>Rhodococcus sp.</i> BH4	Lactonase	QQ activity (90%), high F:M ratio, extra physical cleaning effect	Low mechanical strength of bead, inseparable from AS	Kim et al., 2013
Magnetically-separable mesoporous silica	-	Acylase	QQ activity (94%), highly stable and separable material, high F:M ratio, extra physical cleaning effect	Cost and instability of enzyme	Lee et al., 2014
Sodium alginate bead covered with polymeric membrane layer	<i>Rhodococcus sp.</i> BH4	Lactonase	QQ activity (87%), high F:M ratio, extra physical cleaning effect, Higher mechanical strength of bead	Inseparable from AS	Kim et al., 2015

Yeon and his colleagues conducted the first QQ MBR study in 2009. They applied a QQ mechanism by the direct addition of an enzyme (Yeon et al., 2009). In this study, a quorum quenching mechanism efficiency of 57% was achieved, and a huge contribution to the literature was realized. Their research indicated that quorum quenching can be applied to MBRs and that more than half of the associated biofouling can be prevented. However, the findings also revealed that acylase had been rapidly consumed alongside the increasing concentration of AHL, which is secreted by the microorganisms in the activated sludge, and this consumption resulted in the need for the addition of continuous acylase to the reactor. Yeon et al. (2009b) then carried out a second study on QQ MBR in which they manufactured magnetic enzyme carriers (MECs) to immobilize free acylase enzymes. The main advantage of this study was that it presented a potential method of separating the immobilization media from activated sludge. By using MECs, higher quorum quenching activities were obtained than those secured in the first study. Another study that involved the use of free acylase was that conducted by Kim et al. (2012). While this study was quite similar to Yeon et al.'s first study, the main aim of the research was to verify the effect that the quorum quenching mechanism had on the microbial community in activated sludge. A similar quorum quenching mechanism efficiency was achieved in this study and, again, the researchers concluded that biofouling could be prevented. Acylase enzyme can be commercially supplied by the manufacturer; however, the cost of this chemical is high and direct use of this enzyme causes an increase in the cost of research studies. As such, the researchers concluded that the direct enzyme application may not represent a feasible option for quorum quenching applications because there is a need to achieve a balance between reducing costs and preventing biofouling.

Following the study with MECs, a number of bacterial quorum quenching studies have been conducted. In these studies, quorum quenching bacteria were immobilized into the various immobilization media, and these immobilization media were added to the MBRs in order to create quorum quenching. Five different immobilization media techniques are typically preferred in the quorum quenching MBR studies described in existing literature. The type of immobilization media is an important parameter that affects the efficiency of the quorum quenching mechanism during the bacterial QQ applications. Firstly, Oh et al. (2012) carried out a bacterial quorum quenching study by using a polymeric microbial vessel in which *Rhodococcus* sp. BH4 was

immobilized (Oh et al., 2012). The polymeric microbial vessel was stimulated as a small tube containing 10-12 hollow fiber membranes. In this study, biofouling was prevented with a success rate of 50%. The reason as to why a lower quorum quenching mechanism activity than expected was attributed to the low F:M ratio for *Rhodococcus sp.* BH4 and low dispersion of QQ enzyme in the reactor. In light of this result, Jahangir et al. (2012) conducted a study with a polymeric microbial vessel in which *Rhodococcus sp.* BH4 was immobilized, and the vessel was located near the membrane module. The researchers found that the location of the vessel in the reactor had a direct effect on the efficiency of the quorum quenching mechanism because the vessel was not mobile, and QQ enzyme dispersion was limited. The last study that utilized a polymeric microbial vessel was conducted through the immobilization of a different QQ bacteria, *Pseudomonas sp.* 1A1 (Cheong et al., 2013). The researchers achieved quorum quenching with a similar efficiency to that demonstrated in earlier polymeric microbial vessel studies. After that, Cheong et al. (2013) realized a study with the same bacteria specie, but employed a different immobilization medium, the ceramic microbial vessel. The ceramic microbial vessel can be described as a ceramic tube that contains lumens. In this study, they tried to increase the F:M ratio in the vessel with an inner mode feeding. Thus, they achieved a higher quorum quenching mechanism efficiency.

Since microbial vessels have no mobility, cell entrapping beads are generally preferred as an immobilization medium for quorum quenching MBR studies. In this regard, two different studies have employed the use of sodium alginate beads (Jiang et al., 2013; Kim et al., 2013). While Jiang and his colleagues immobilized acylase enzyme into the beads, Kim and his colleagues immobilized *Rhodococcus sp.* BH4 into the beads. Jiang and his colleagues achieved biofouling prevention and energy saving; however, the high cost of the enzyme remained an issue with this application. However, Kim et al. realized an important contribution to existing understanding of QQ MBRs, and presented a QQ mechanism application that offered high efficiency and long-term operational capabilities.

In addition to the high F:M ratio and effective enzyme distribution properties of the beads, it can be said that another advantage of cell entrapping beads is the extra physical cleaning effect that is achieved as a result of their mobility. The only

disadvantage of the sodium alginate usage is that they cannot be separated from activated sludge because they do not have a supporting property, like magnetism. Moreover, they can be destructed in the activated sludge, and this results in excess sludge because their material is not inert like microbial vessels.

Another study conducted by Lee et al. (2014) can be given as an example of a successful quorum quenching MBR operation. In this study, the researchers immobilized acylase into magnetic beads, and presented an approach that combined all the advantages of previous studies. However, it may be said that the cost of the enzyme can be a disadvantage of this study. The quorum quenching mechanism efficiency was quite high, and biofouling prevention was achieved in this study. Lastly, Kim et al. (2015) conducted a study with sodium alginate beads covered with a polymer to increase the stability and mechanical strength of the beads. They found that the increase in TMP could be postponed with a very high quorum quenching efficiency of 87%. When the objective and the results of all present studies are examined and evaluated, it becomes apparent that each of the existing approaches has their own advantages and disadvantages and offers some elements of superiority to others. In this regard, it appears that the development of QQ MBR studies could be achieved by designing innovative immobilization media.

2.7 Determination of Quorum Quenching

The methods by which the quorum quenching activities of QQ products manufactured in the QQ MBR studies mentioned above were determined were also examined within the scope of this review. As previously mentioned, the first QQ MBR study was carried by Yeon et al. (2009a). This study employed a detailed bio-assay method to determine the AHL degradation capacity of a structure in which QQ bacteria was immobilized. Yeon and his colleagues opted to use *Agrobacterium tumefaciens* A136 as a biosensor for AHL detection, and they based this decision on existing research (Fuqua et al., 1996). In fact, the main base of this method was based on the work of Kawaguchi et al. (2008). Kawaguchi and his colleagues supplied the bacteria from Fuqua's research team and their method was published in 2008.

One year later, Yeon et al. employed this bio-assay assay with some minor modifications. In this bio-assay, QQ products were placed in a tube containing 200 nM

C8-HSL and samples were periodically taken to determine the AHL concentrations of the samples. A136 was incubated overnight with spectinomycin and tetracycline. This incubated A136 solution was diluted using LB agar and antibiotics at a 1:9 ratio. After that, X-gal was added to the mixture and agar plates were prepared. The idea of X-gal usage was mentioned by Kawaguchi et al. (2008). Samples were loaded into the wells, prepared on the agar plates, and kept overnight for incubation. During the incubation, the AHL in the samples was linked to TraR protein in A136 and activated the target genes and, as a result, β -galactosidase was synthesized. This β -galactosidase degraded the X-gal, and a blue color developed. The blue colors spread around the wells, and the diameters of these zones were in direct proportion to the AHL concentrations. By using the equation between the diameters and known AHL concentrations, the concentration of AHL in the sample could be calculated.

The literature review indicated that the determination of QQ activity in the QQ MBR studies conducted after 2013 typically employed alternative methods that were, again, based on the work of Yeon et al. (2009), but were modified. While the mechanism between AHL and A136 was the same, luminometer was preferred over indicating agar plate. At this point, beta-glo started to play a role in the method. In fact, as with the indicating agar method, this method was also mentioned in the work of Kawaguchi et al. (2008); however, the first trial was seen after five years. In this method, as a result of A136 and AHL relationship, β -galactosidase was created. At the end of a 30 min incubation of β -galactosidase and beta-glo, xyluciferin was synthesized, and this chemical resulted in luminescence. The intensity of this luminescence was in direct proportion to the AHL concentration, and could be determined using a luminometer. As a result of using a luminometer as opposed to indicating agar to determine the AHL, it was possible to reduce the duration of the experiment. The QQ activity could be calculated on the same day, and this created the possibility of repeatability across multiple studies. The schematic drawing of these two methods of AHL determination is presented in Figure 2.6.

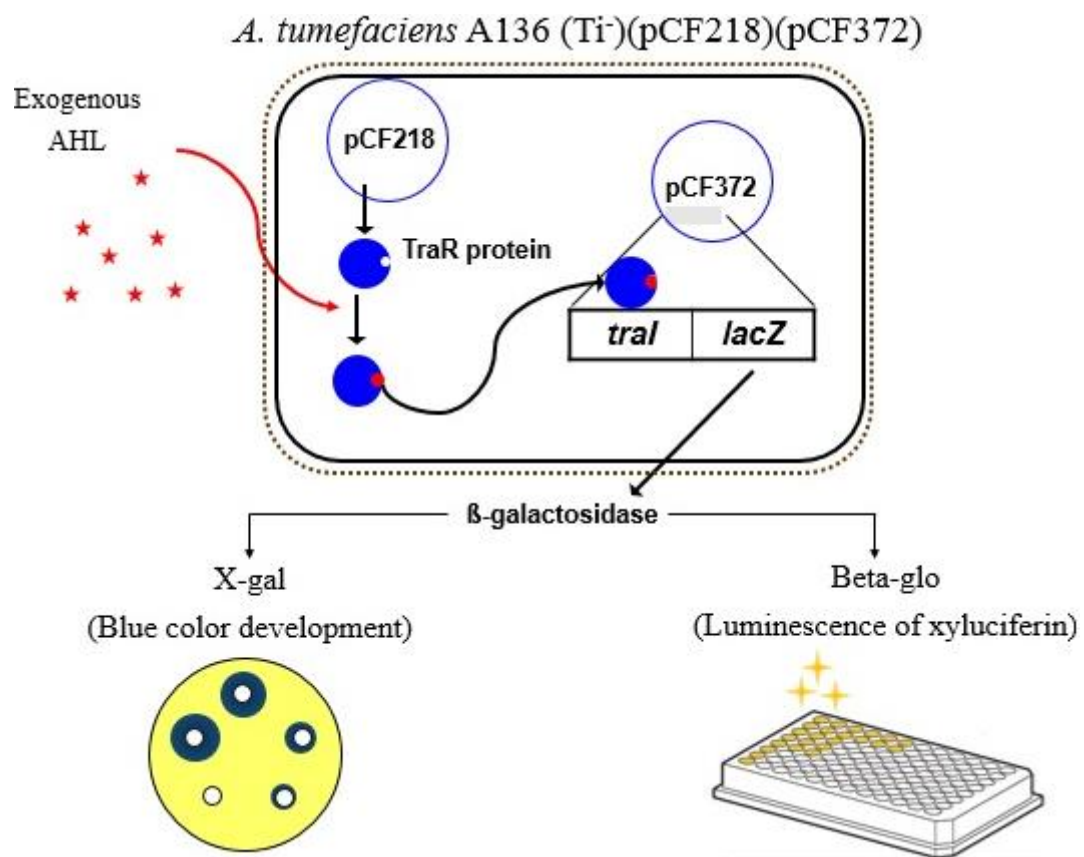


Figure 2.6 : Bio-assay methods for AHL determination in QQ MBR studies.

3. A NEW APPROACH TO BACTERIAL QUORUM QUENCHING APPLICATIONS FOR BIOFOULING CONTROL: ROTARY MICROBIAL CARRIER FRAME

3.1 Purpose and Scope

Bacterial quorum quenching (QQ) is a new approach for controlling of membrane fouling in membrane bioreactors (MBRs). In this study, a novel bacterial immobilization media named rotary microbial carrier frame (RMCF) was designed and applied to inhibit biofilm formation in a MBR to overcome the durability problem of cell entrapping bead (CEB) and low F:M ratio inside a microbial vessel. RMCF was prepared by entrapping QQ bacteria (*Rhodococcus sp.* BH4) onto polycarbonate frame covered with microfiltration membrane. QQ effect was examined with short- and long-term operations by using two different bacteria amounts. In MBRs operated with RMCF, transmembrane pressure (TMP) increasing rate was decreased with 65% efficiency. The prevention of biofouling was resulted from both physical (shear force) and biological (quorum quenching) effects of RMCF, and it was seen that RMCF could sustain its QQ effect over long-term operations. Biofilm formation inhibition of RMCF was confirmed visually using Confocal Laser Scanning Microscopy (CLSM). In addition, microbial population dynamics in MBRs were examined with Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis and it was found that RMCF had an effect on microbial diversity. RMCF usage could be applied via its renewable, refillable and durable property at the plant scale of MBR.

3.2 Introduction

Membrane bioreactor (MBR) technology has recently received considerable attention due to its high quality effluent for municipal and industrial wastewater treatment (Judd & Judd, 2006; Drews, 2010). Although it has numerous advantages over conventional wastewater treatment processes, membrane fouling is one of the major drawbacks and process limitations of MBRs (Lade et al., 2014). Membrane fouling in MBR, primarily

caused by microbial deposition/growth and microbial product accumulation on membranes, is known as biofouling (Wu & Fane, 2012). The control of membrane biofouling in MBR has been recognized as a key factor for reducing the operating and maintenance costs through energy saving biofilm formation on the membrane surface lead to several adverse effects on MBR performance, such as significant increase of transmembrane pressure (TMP), reduction in flux and membrane lifespan (Hai et al., 2013).

In recent years, a novel approach has been demonstrated that the quorum sensing mechanism is directly correlated with biofouling in membrane bioreactors (Yeon et al., 2009a). Quorum sensing is a biological communication mechanism between microorganisms which assessing their population densities based on small diffusible signalling molecules called autoinducer like N-acyl homoserine lactones (AHL). When it reaches to an adequate level, QS induces some bacterial behaviors such as bioluminescence, antibiotic production, virulence, biofilm formation and some bacterial activities such as production of soluble microbial products (SMP) and extracellular polymeric substances (EPS), exocellular enzyme secretion via activating the transcription of specific genes. Thus, quorum quenching, based on interruption of cell-to-cell communication between microorganisms, has found an application field in MBR as an innovative approach.

Quorum quenching is an efficient mechanism for inhibition of biofilm in the MBR for advanced wastewater treatment. The quorum quenching causes to prevent of biofouling, by which autoinducer-mediated quorum sensing, is interrupted. Three basic QS-based membrane biofouling control strategies have been reported (Zhang et al. 2002): (i) blockage of AHL production, (ii) interference with the signal receptor, and (iii) inactivation of AHL signal molecules.

Since the concept of quorum quenching was used to control of biofouling in MBRs by Yeon et al. (2009a), various strategies have been developed. Firstly, they investigated the effects of acylase added in a batch type of MBR on the transmembrane pressure. And then, a magnetic enzyme carrier (MEC) which immobilized acylase onto magnetic particles was produced (Yeon et al. 2009b). Furthermore, acylase and chitosan were deposited and cross-linked on the NF membrane surface (Kim et al. 2011). Bacteria, having quorum quenching activity were also immobilized in a hollow fiber membrane (Oh et al., 2012; Jahangir et al., 2010), because the enzymes have

disadvantages such as instability and purification cost. Subsequently, quorum quenching bacteria were encapsulated into the alginate beads and biofouling was inhibited successfully (Kim et al., 2013), Cheong and his colleagues designed ceramic microbial vessel to enhance the QQ bacterial viability, as relatively higher F:M ratio inside the vessel rather than inside the hollow fiber membrane (2014). All of the studies mentioned above were successful as progressive applications.

Previous studies showed that QQ beads are non-durable and QQ vessels have small food-to-microorganism ratio. In this study, a module named “rotary microbial carrier frame” (RMCF) has been designed as an alternative to present bacterial QQ studies such as QQ beads and QQ vessels in MBR. The module consists of cubbyholes filled with QQ bacterium was coated with the membrane to provide bacterial entrapment. In addition, RMCF can be reused and is effective on creation of nutrition area for bacteria and minimization of bacterial and polymeric residue.

3.3 Material and Methods

3.3.1 Reagents

Spectinomycin was supplied as a powder (Sigma Aldrich, USA), and the stock solution was stored at 4°C, in the dark. Tetracycline was also supplied as powder form (Sigma Aldrich, USA) and stock solution was stored at -20°C. Commercial C8-HSL from Cayman (USA) was preferred as representative chemical for signal molecules and the stock solution was stored at -20°C. X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) was supplied from Sigma Aldrich with its powder form and X-Gal solution was stored at -20°C and in dark after prepared by using dimethylformamide (DMF) which is bought from Merck (Germany).

3.3.2 Bacterial strains and growth conditions

Agrobacterium tumefaciens A136 (Ti⁻)(pCF218)(pCF372) was used for detection of N-acyl homoserine lactone (AHL) signal molecules as a biosensor strain (Yeon et al., 2009a; Kawaguchi et al., 2008; Oh et al., 2013). *A. tumefaciens* A136 was cultured on Luria Bertani (LB) medium containing spectinomycin (50 mg/L) and tetracycline (4.5 mg/L) to maintain the two plasmids that provide the AHL response system. *Rhodococcus sp.* BH4, isolated from a real MBR plant by Oh et al. (2012), was used

as a QQ bacterium and cultured on LB broth and incubated in a 30°C rotary shaker (160 rpm) for 24 hours.

3.3.3 Quorum quenching activity test

QQ activities of both free and immobilized cells in RMCF were measured respectively. AHLs were detected using the indicating agar plate method adopted from Park et al. (2001). Indicating agar plate was prepared by mixing (ratio of 9:1) LB-agar and an overnight culture of *A. tumefaciens* A136 including spectinomycin (50 mg/L), tetracycline (4.5 mg/L), and X-gal (0.2 g/L). AHL degradation by *Rhodococcus sp.* BH4 was occurred in the reaction tube with the initial concentration of C8-HSL with 200 nM. The reaction mixtures were loaded into the well of plates overlaid with *A. tumefaciens* A136. The residual amounts of AHL were calculated using the relationship equations based on the color zone size and known amounts of AHLs.

3.3.4 Manufacturing of RMCF

The mobile module composed of a polycarbonate frame including four cubbyholes was covered with a polyvinylidene fluoride (PVDF) membrane (Microdyn Nadir GmbH, Germany). PVDF membrane has a nominal pore size of 0.20 µm and thickness of 210–250 µm. *Rhodococcus sp.* BH4 culture was filled into each cubbyholes with an amount of 1 ml (~25 mg dry weight of biomass per ml) using a sterilized syringe. Polycarbonate frame was completely sealed with glue avoiding cell leaking. Cubbyholes have inlet and outlet channels to refill with cell suspension. Three of the mobile modules were mounted to impeller before submerging to MBR. A photography and illustrations of RMCF were given in Figure 3.1. Quorum quenching mechanism of RMCF in MBR was shown in Figure 3.2.

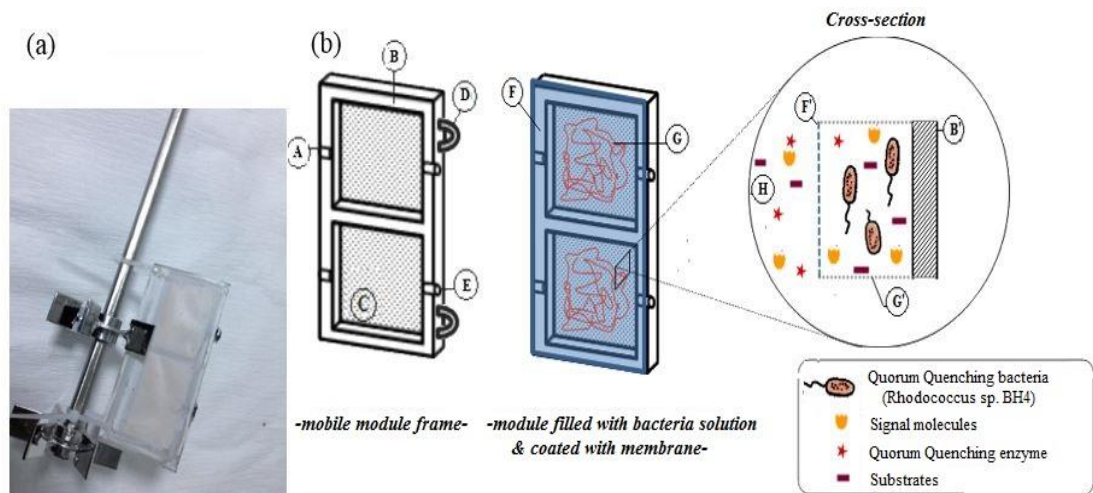


Figure 3.1 : Pictures of the mobile module: (a) photography, (b) illustration of the empty and coated frames (A: bacteria discharge line, B: frame, C: empty cubbyhole, D: fastener, E: bacteria filling line, F: coating membrane, G: immobilized bacteria, H: mixed liquor, B': cross section of frame, F': cross section of coating membrane).

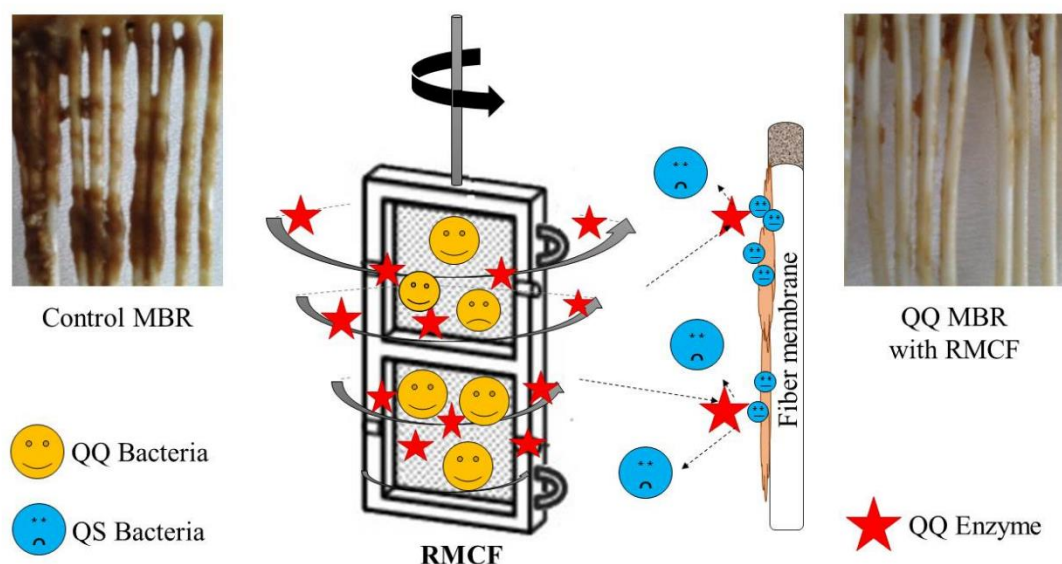


Figure 3.2 : Quorum quenching mechanism of RMCF in MBR.

3.3.5 Membrane bioreactors

Short term and long term quorum quenching activities of RMCF were studied in MBRs. Two parallel MBRs were operated under the same operating conditions as the MBR-A and MBR-B. While MBR-A was a conventional MBR, MBR-B was a parallel operated MBR with Quorum Quenching product. Scheme of the MBR system was depicted in Figure 3.3. Activated sludge was taken from an advanced biological wastewater treatment plant in Istanbul, Turkey, and prior to the MBR operations, it was acclimated to synthetic wastewater. The composition of the synthetic wastewater

was as follows (mg/L): glucose, 400; yeast extract, 14; bactopeptone, 115; $(\text{NH}_4)_2\text{SO}_4$, 104.8; KH_2PO_4 , 21.75; MgSO_4 , 15.63; FeCl_3 , 0.075; CaCl_2 , 2.45; MnSO_4 , 1.8; and NaHCO_3 , 255.5. MBRs were connected to a computer and controlled by an automation program, according to the target ranges of operating parameters like water levels, pH, temperature, oxidation-reduction potential (ORP) values and dissolved oxygen concentrations (Table 3.1). MBR-A and MBR-B were operated under the constant flux condition by using microfiltration system consisting of hollow fiber membrane module. The effective area of the hollow fiber membrane module was around 100 cm^2 . The hydraulic retention time (HRT) and sludge retention time (SRT) were 13 h and 30 d, respectively. The mixed liquor suspended solid (MLSS) concentrations in MBRs were maintained within the range of 11000-12000 mg/L. Five different experimental studies were carried out to see the effects of QQ bacteria as well as RMCF on membrane fouling in short- and long-term operations. Operating strategies in the MBR experiments were given in Table 3.2.

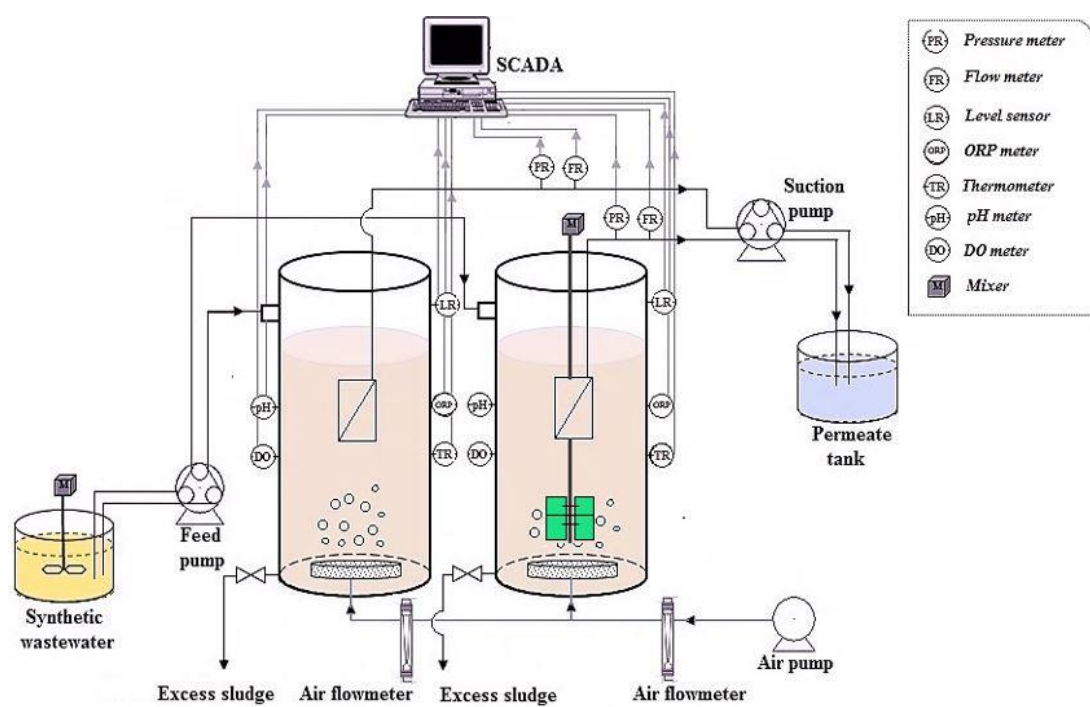


Figure 3. 3 : Schematic description of the MBR system.

Table 3. 1 : Operation parameters and conditions of the MBR.

Parameter	Value/Information
Working volume (L)	5
pH	6.8~7
Dissolved oxygen (mg/L)	4.2-6.5
ORP (mV)	170-200
Temperature (°C)	20±2
SRT (d)	30
HRT (h)	13
Membrane type:	Philos MegaFluxI
Membrane area (m ²)	0.01
Flux (LMH)	60
COD removal efficiency (%)	~ 95
MLSS (mg/L)	12,000
Feed COD (mg/L)	440
Seeding sludge	Paşaköy WWTP

Table 3. 2 : Experimental procedure for MBR operation.

Operation Study	MBR-A	MBR-B	Aim of the operation
Study I	Conventional MBR	RMCF with 8 mg BH4/cm ³ cubbyhole	Short term QQ effect of RMCF
Study II	Conventional MBR	RMCF with 12.5 mg BH4/cm ³ cubbyhole	Short term QQ effect of RMCF (different QQ bacteria amount)
Study III	Conventional MBR	Vacant RMCF	Short term anti-fouling effect of rotation
Study IV	Conventional MBR	Vacant RMCF	Long term anti-fouling effect of rotation
Study V	Conventional MBR	RMCF with 8 mg BH4/cm ³ cubbyhole	Long term QQ effect of RMCF
Study VI	Conventional MBR	RMCF with 12.5 mg BH4/cm ³ cubbyhole	Long term QQ effect of RMCF (different QQ bacteria amount)

3.3.6 Analytical methods

During MBR operation some important parameters like MLSS, COD, TKN, total phosphorus (TP), EPS and SMP were analyzed. MLSS, EPS, and SMP concentrations of the mixed liquor were determined to check and control the parameters that are directly related with biofouling. On the other side, COD, TKN and TP concentrations in feed and permeate flows were analyzed to check whether proper and efficient microfiltration processes in MBR-A and MBR-B were achieved or not. MLSS, COD,

TKN and TP were determined according to Standard Methods (APHA, 2005). After separation of EPS and SMP, protein and carbohydrate analysis were carried with Lowry (Lowry et al., 1951) and Phenol sulfuric acid (Dubois et al., 1956) methods, respectively. Samples were measured by a UV spectrophotometer (Hach Lange, DR 500; 660 nm; 490 nm) by using standard solution prepared with Bovine Serum Albumin (BSA) and glucose solutions, respectively.

Molecular analysis of the samples taken from activated sludge were realized to check microbial community changes in MBR-A and MBR-B. DNA extraction was carried according to manufacturer's instructions (ZymoResearch, Irvine, CA, USA) after mechanical disruption through bead beating. DGGE-PCR of extracted DNAs was performed with U968-GC (5'-AAC GCG AAG AAC CTT AC-3) forward and L1401 (5'-GCG TGT GTA CAA GAC CC-3) reverse primers (Nübel et al., 1996) specific for the variable region between V6 and V8 of the 16S rRNA gene. U968-GC had a 40-base GC-clamp (CGC CGG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G) linked to the 5' end of the primer to improve the separation of the DNA fragments. The cycling conditions for DGGE-PCR was as follows; initial denaturation at 95°C for 5 min then, 35 cycles of denaturation at 95°C for 30 s, primer annealing at 56 for 40 s and extension at 72°C for 1 min. After the last cycle, a final elongation of 72°C for 5 min and immediate cooling to 4°C were applied. The GC clamp PCR-products were separated in 8% (w/v) polyacrylamide gels (37.5:1 acrylamide:bisacrylamide) containing a linear 35-58% denaturant gradient (100% denaturant corresponds to 7 M urea and 40% deionized formamide) in a 1 X (TAE) buffer at a constant voltage of 100 V and a temperature of 60°C for 16 h.21 Gels were stained using silver nitrate and scanned at GS-800TM Calibrated Densitometer (Bio-Rad, Hercules, CA, USA). The processing of the DGGE gels was performed using BioNumerics software package Gel Compar II Software version 6.5 (Applied Maths, Kortrijk, Belgium). The DGGE banding patterns reflecting microbial consortia were examined by clustering analysis. The gel was normalized and the dendrogram of DGGE patterns were constructed using an Unweighted Pair Group Method with an Arithmetic Mean method. The Dice correlation (binary) coefficient matrix was applied. Since the clustering analysis of the PCR-DGGE patterns could be affected by various factors, such as position bias in gels, band assignment, and different settings in the BioNumerics software, the optimal position tolerance and optimization setting

were calculated using the tolerance and the optimization analysis program supplied with the BioNumerics software package to ensure that there were better matches for the band patterns.

The biofilm on a membrane specimen was stained with 100 μL SYTO9 (Molecular Probes, USA) for 20 min in the dark to visualize bacterial cells. Biofilm, after staining was observed using CLSM (C1 plus, Nikon, Japan).

3.4 Results and Discussion

3.4.1 Effect of RMCF with *Rhodococcus* sp. BH4 on AHL degradation

Quorum quenching activity of *Rhodococcus* sp. BH4 was tested by degradation of C8-HSL in the aqueous solution. And the degradation efficiency of C8-HSL with the free cells was measured to be 95% in the reaction time of about 20 minutes (Figure 3.4).

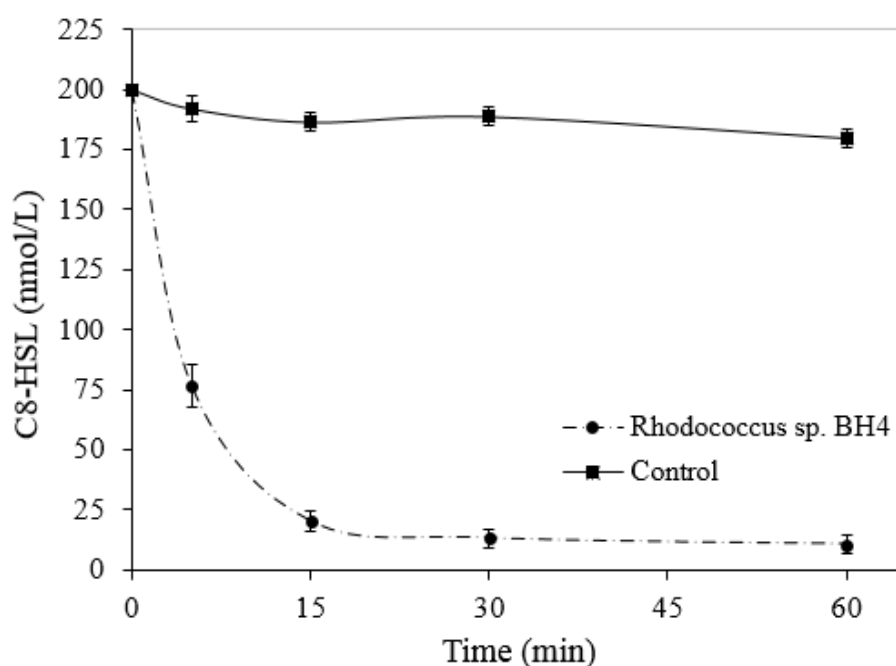


Figure 3. 4 : Quorum quenching mechanism of RMCF in MBR.

The AHL degradation potential of RMCF containing 12.5 mg BH4/cm³ cubbyhole was determined also by using C8-HSL as a signal molecule. As shown in Figure 3.5, the degradation of C8-HSL with the RMCF was measured to be 65% in the reaction time of 120 min. While the adsorption of C8-HSL on the vacant RMCF was negligible, significant decrease in the concentration of C8-HSL was achieved by the RMCF inside 12.5 mg BH4/ cm³.

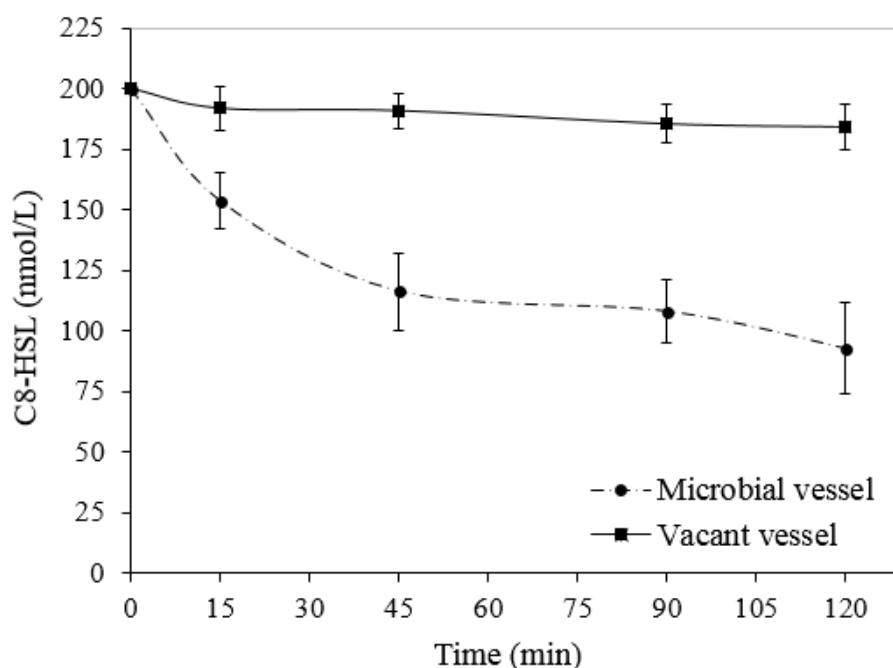


Figure 3. 5 : Quorum quenching mechanism of RMCF in MBR.

It can be said that RMCF has higher QQ activity potential than the studies based on polymeric (Oh et al., 2013) and ceramic microbial vessels (Cheong et al., 2014) have a QQ activity with approximately 48% and 38% (26.5 mg BH₄/cm³-lumen), respectively.

3.4.2 Application of the RMCF with *Rhodococcus sp.* BH4 to the continuous MBR

RMCF was applied to submerged MBRs to test its potential to inhibit biofouling in MBR. Two lab scale MBRs in continuous mode were operated under the same operating conditions except for the submersion of RMCF fixed to the end of the stirrer to one MBR (MBR-B) at the beginning of the operation. The rise of the transmembrane pressure (TMP) profiles of the MBR-A and MBR-B were compared to see and evaluate the inhibition of biofouling by RMCF. In addition to this, the effect of immobilized *Rhodococcus sp.* BH4 amount was also examined. With aim of this two different operation studies were carried: Study I and Study II. While at Study I immobilized *Rhodococcus sp.* BH4 amount was around 8 mg BH₄/cm³ cubbyhole, it was 12.5 mg BH₄/cm³ cubbyhole at Study II. Both operation studies were carried for 8 days. Operation strategies were pursued on continuous operation with cycles by using the same RMCF when TMP values of MBR-A reached at a certain value. As shown in Figure 3.6-a, it took around 3.6 d for the TMP to reach 350 mbar in the first

cycle of the MBR-A and TMP values of MBR-B were as much as half of the TMP values of MBR-A at the end of 3.6 d. This shows that RMCF can inhibit biofilm formation and decrease the TMP rise-up. The second cycles of operations for two MBRs were used to confirm the reproducibility of the discrepancy in the rate of the TMP rise-up. *Rhodococcus sp.* BH4 amount in the RMCF was increased at Study II. While the TMP reached 350 mbar in the first cycle of the MBR-A within 3.4 d, TMP values of MBR-B were around 120 mbar, as shown in Figure 3.6-b. With a small increase in quorum quenching bacteria resulted in an additional 15% decreasing on TMP values. RMCF could easily decrease the biofilm formation by the increasing amount of immobilized quorum quenching bacteria. Note that, the immobilized bacteria population in RMCF can be easily increased by increasing the cubbyholes numbers in the frame.

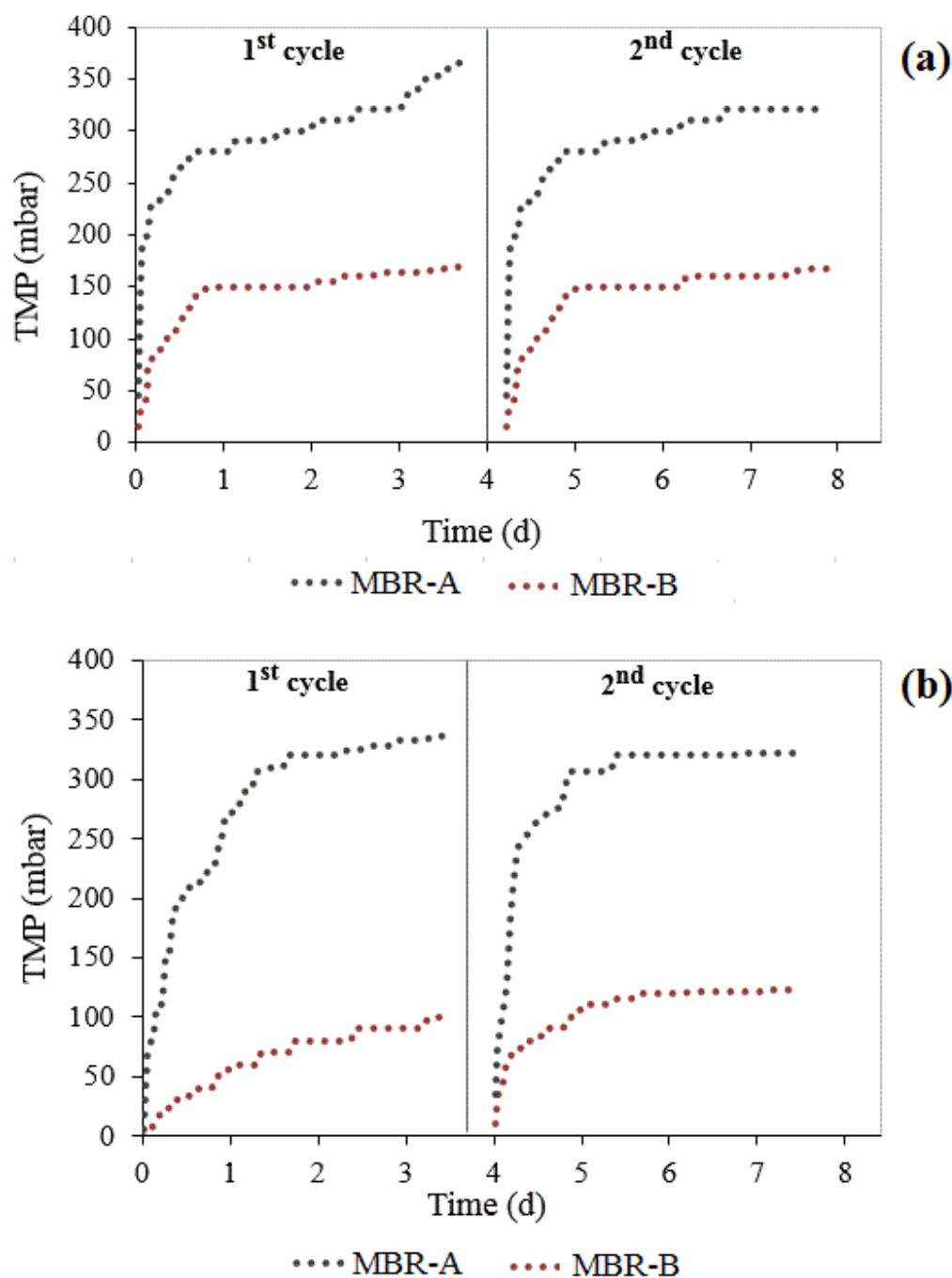


Figure 3. 6 : TMP profiles of MBR-A and MBR-B under the same operation conditions. (a) Study I; application of the RMCF with 8 mg BH₄/cm³ cubbyhole; (b) Study II; application of the RMCF with 12.5 mg BH₄/cm³ cubbyhole.

Oh et al. (2012) and Kim et al. (2013) have studied on different immobilization media as microbial vessel and cell entrapping bead (CEB) respectively by encapsulation the same quorum quenching bacteria, *Rhodococcus sp.* BH4. Oh, and his colleagues applied microbial vessel to a submerged MBR and observed that the microbial vessel resulted in lower biofouling compared with a conventional MBR. After that, Kim and his colleagues have tried to manufacture CEBs as an effective alternative to microbial

vessel (2013). Although a direct comparison between results obtained from RMCF and other quorum quenching MBR studies is quite difficult, it can be said that the significant effect of RMCF to reduce TMP increasing rate is better than reported by Oh et al. (2012) and can be described as lower than mentioned by Kim et al. (2013). The major disadvantage of microbial vessel usage was non-mobile property of immobilization carrier. On the other hand, the major disadvantage of CEB was thought that it cannot be removed from the mixed liquor and cannot be renewed. RMCF could provide a novel alternative approach to mitigate membrane fouling resulted from the inhibition of biofilm formation by quorum quenching and the prevention of biomass attachment on membrane surface due to shear forces created by rotation of RMCF located under the filtration module. Also, it has non-dispersible material and refillable structure.

In order to realize its preventing potential of biomass attachment caused by shear forces to the total anti-biofouling effect, which can be called as a physical washing effect, two parallel MBRs were operated for short-term (Study III) and long-term (Study IV). In these physical washing studies, while a conventional MBR was used as MBR-A, a vacant RMCF was submerged to MBR-B and rotated. While Study III was carried to see short term physical cleaning effect of vacant RMCF, the aim of Study IV was to see whether this possible physical cleaning effect can continue after long operation days or not. The results of physical washing studies can be seen in Figure 3.7-a and Figure 3.7-b.

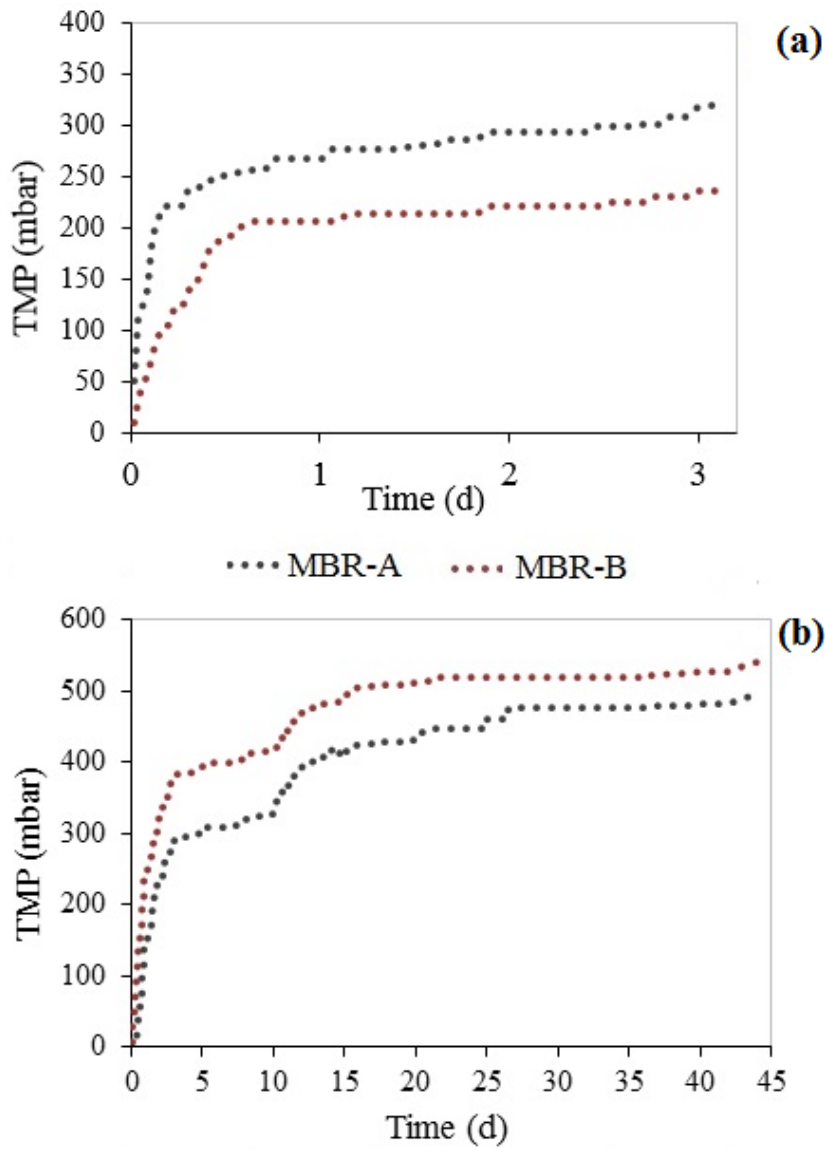


Figure 3. 7 : TMP profiles of MBR-A and MBR-B for physical washing effect studies. (a) Study III; short term physical washing effect of rotation; (b) Study IV; long term physical washing effect of rotation.

After a comprehensive evaluation of data from Study I, II, III and IV, contribution of physical washing effect caused by shear forces to total anti-biofouling effect was found out as approximately 16%. However, this effect decreased with time (Figure 3.7-a and 3.7-b) and it could be due to the effect of shear forces on biofilm is negligible when the bio-cake was formed on the membrane surface by rejected active particulate materials and growing microorganisms (Hwang et al., 2008).

3.4.3 Stability of quorum quenching activity of RMCF

It is important to maintain quorum quenching activity during MBR operations. The continuity of the quorum quenching activity in RMCF was determined by using TMP profiles obtained from long term operations of two parallel MBRs (Figure 3.8).

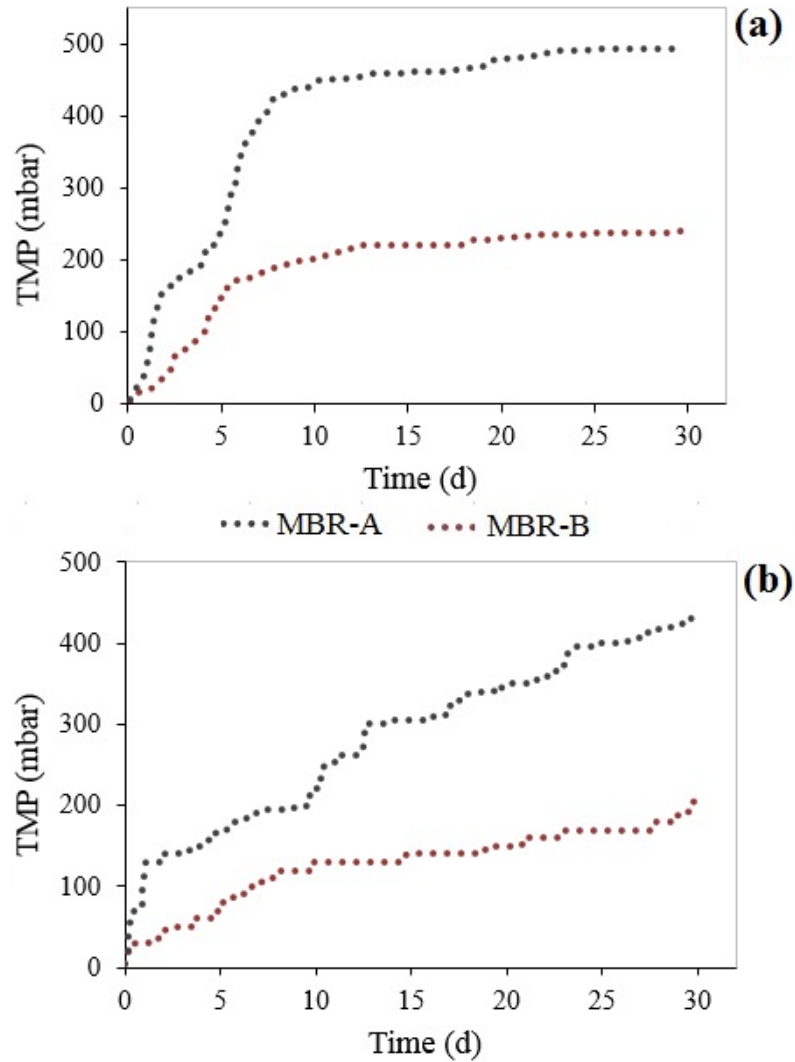


Figure 3. 8 : TMP profiles of MBR-A and MBR-B after long term operations. (a) Study V; long term application of the RMCF with 8 mg BH₄/cm³ cubbyhole; (b) Study VI; long term application of the RMCF with 12.5 mg BH₄/cm³ cubbyhole.

According to the TMP profiles in Figure 3.8, it can be said that quorum quenching bacteria can adapt very well to their new environment in the MBR as well as to their growth room inside the RMCF. After 30 days of operation, there were still QQ effect (~50%). Two different immobilized quorum quenching bacteria amounts were employed for long term operations and it was found out that continuity of quorum quenching activity is independent of cell concentration. To obtain another qualitative

evaluation of living *Rhodococcus sp.* BH4 bacteria in cubbyholes after long term operations, a separate experiment was designed. The used RMCF was taken off from MBR-B and under sterile conditions the layer at the rear of the cover membrane were slightly cut off and laid onto LB agar. In addition, samples taken from the bacteria solution in the cubbyholes of RMCF were spread onto LB agar. According to the morphology of BH4 on agar plate (figure not shown), it can be mentioned that *Rhodococcus sp.* BH4 was still alive in the cubbyholes of RMCF after 30 days operation. RMCF has a good potential of successful biofouling control in MBR at least one month without any further inputs or modifications. In addition, if quorum quenching bacteria lose their influence after a longer operation than 30 days, this design will allow to immobilize new QQ bacteria with its refill ability.

3.4.4 Effect of the RMCF on SMP and EPS production in the MBR

EPS and SMP in the mixed liquor can be analyzed and reported in terms of their polysaccharide and protein contents. In the literature (Jiang et al., 2013), it was mentioned that quorum quenching in the MBR can reduce EPS and SMP concentrations. In order to see the effect of the RMCF on EPS and SMP production in the mixed liquor, EPS and SMP concentrations were measured at the end of the Study VI (Figure 3.9).

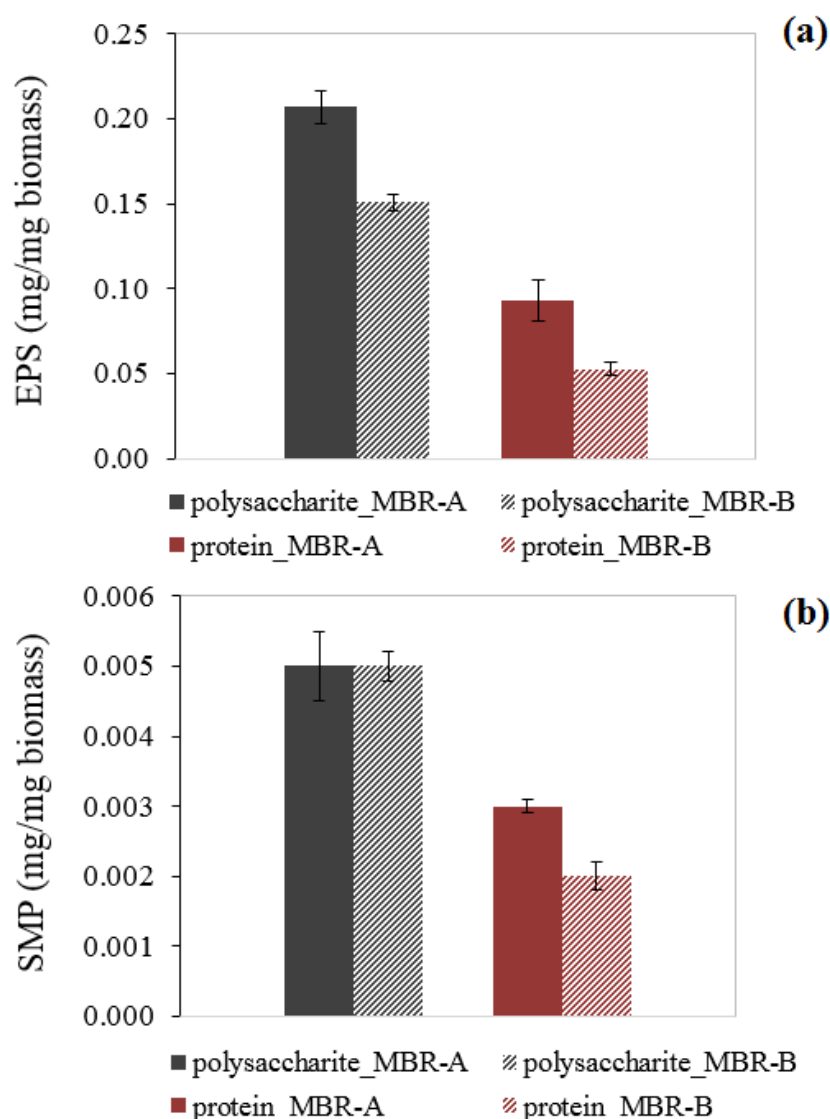


Figure 3.9 : (a) EPS and (b) SMP and their content concentrations in the mixed liquor of MBR-A and MBR-B (Error bar: standard deviation ($n=3$)).

As shown in Figure 3.9, the RMCF reduced polysaccharide and protein amount of EPS with efficiencies of 25% and 50%, respectively. On the other hand, there was only a slight difference between MBR-A and MBR-B from the point of the protein content of SMP. *Rhodococcus sp.* BH4 in the RMCF is presumed to have an effect on EPS production in the mixed liquor by quorum quenching by taking into account that concentrations at the beginning was similar. The usage of the RMCF can inhibit quorum sensing between cells by reducing the concentration of the AHL in the mixed liquor and decrease EPS production by microorganisms. Since these results correlate with the changes in microbial community in previous studies (Yeon et al., 2009a; Kim et al., 2013; Fuqua et al., 1994).

3.4.5 Effect of quorum quenching bacteria on the biofilm formation

The quorum quenching effect of RMCF was supported by CLSM results. In Figure 3.10, biofilm appearances were given in 3D form with plan and cross-section views in two groups: (i) the results of the quorum quenching effect (Study I) and (ii) the results of physical cleaning effect determination study (Study III).

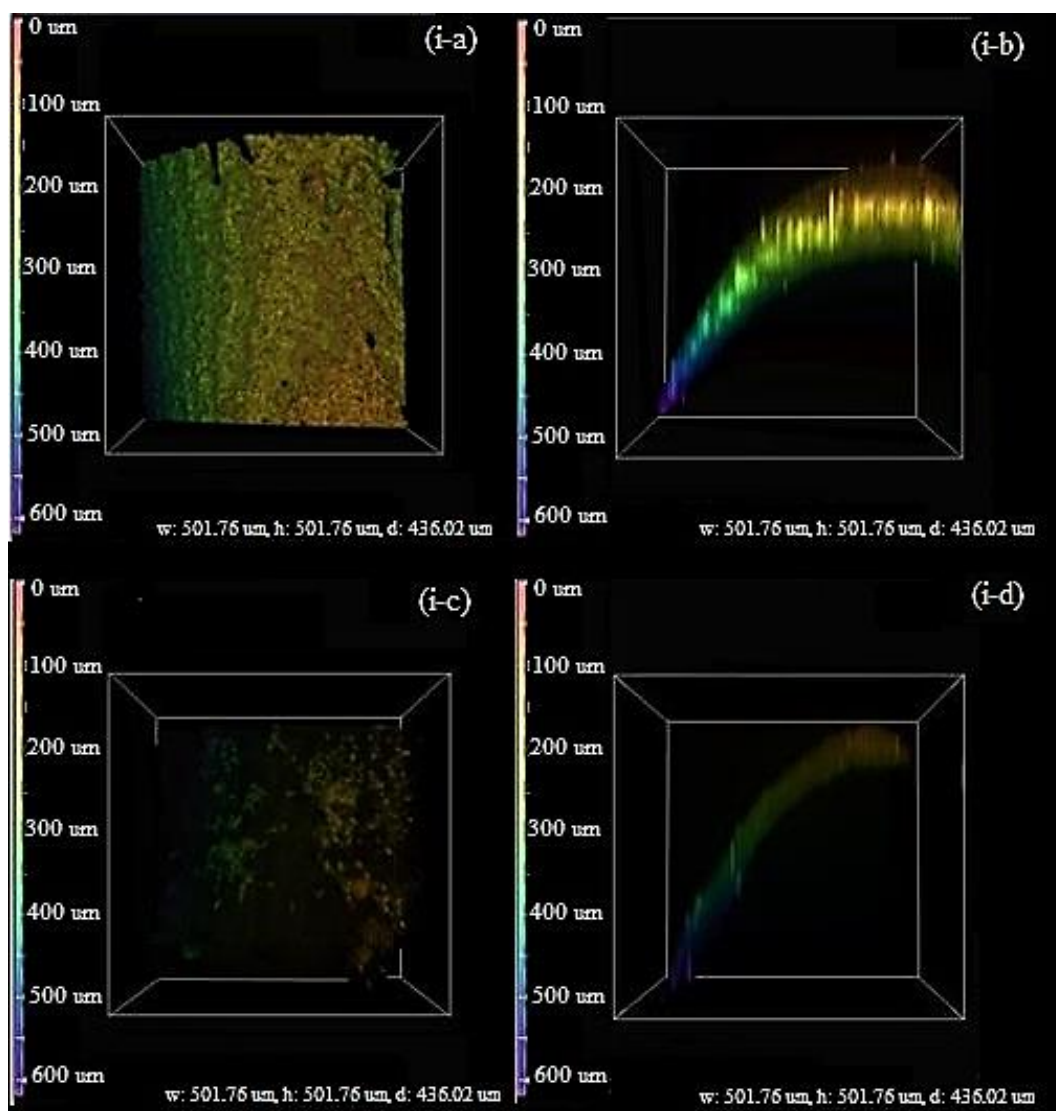


Figure 3. 10 : Reconstructed CLSM images of biofilm formed on the surface. (i) Quorum Quenching study; (ii) physical washing study. (a) MBR-A, plan view, (b) MBR-A, cross-section view, (c) MBR-B, plan view and (d) MBR-B, cross-section view at the end of the operation, stained with SYTO9. Magnification: X100. Image size: 501.78 μm X -501.78 μm .

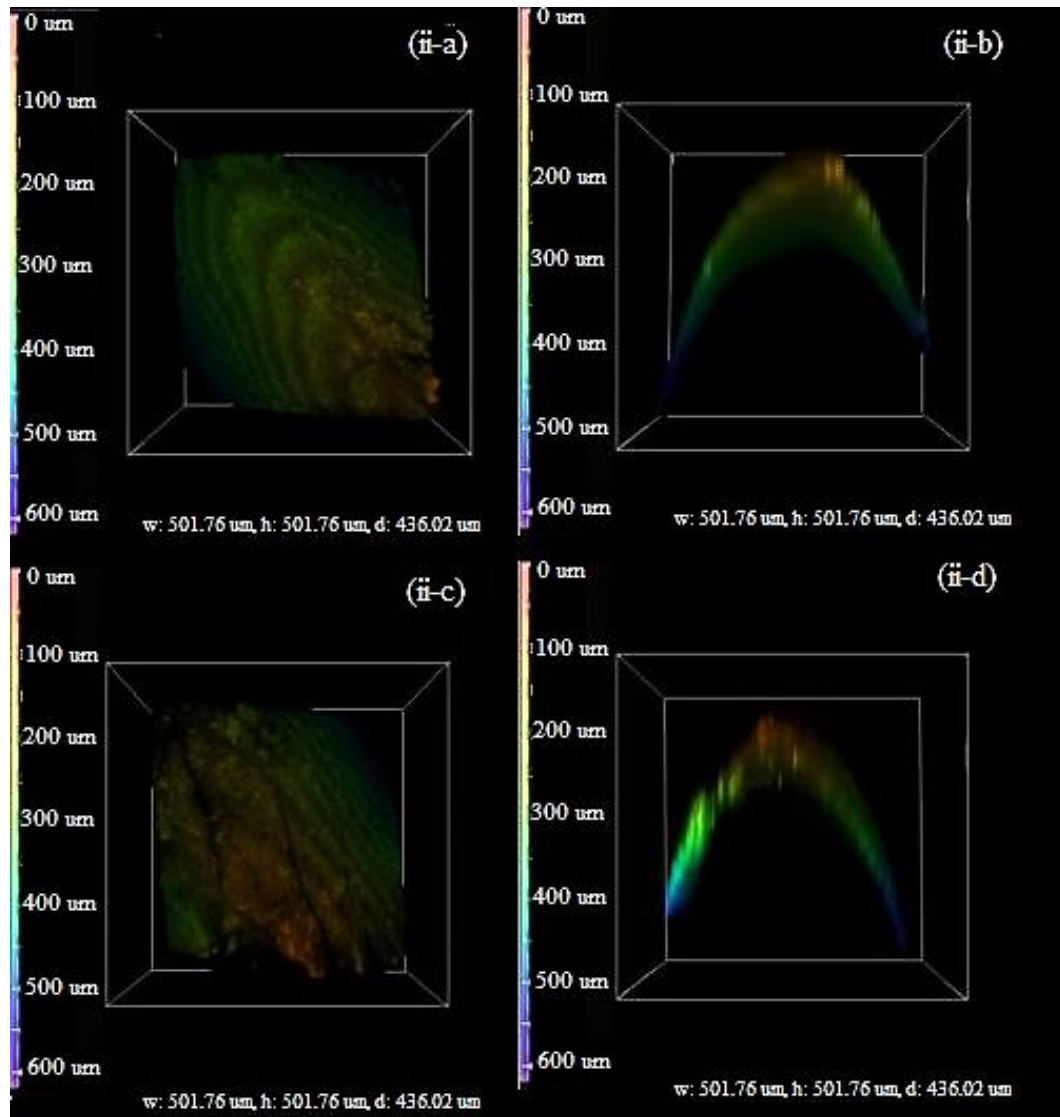


Figure 3.10 (continued) : Reconstructed CLSM images of biofilm formed on the surface. (i) Quorum Quenching study; (ii) physical washing study. (a) MBR-A, plan view, (b) MBR-A, cross-section view, (c) MBR-B, plan view and (d) MBR-B, cross-section view at the end of the operation, stained with SYTO9. Magnification: X100. Image size: 501.78 μm X -501.78 μm .

According to the CLSM images from the quorum quenching study, the amount of biofilm formed in the MBR-B was very few compared to the amount of biofilm formed in the MBR-A. These results agree with TMP profile results, and confirm the QQ effect of RMCF and biofilm formation prevention. Prevention of quorum sensing resulted in weakness about cell adhesion. CLSM images acquired after the physical cleaning study show that physical cleaning has a relatively small effect on the decreasing of biofilm amount.

3.4.6 Effect of quorum quenching on the biodegradation of organics

In addition to energy consumption balance during operation, providing of proper treatment performance is also important for wastewater treatment with MBR. COD, TKN, and TP removal efficiencies were monitored in MBR-A and MBR-B on the basis of their feed and permeate concentrations. COD, TKN and TP removal efficiencies were approximately 95%, 44% and 17%, respectively. There were no considerable difference of removal efficiencies between two parallel MBRs throughout the entire experimental period. RMCF application has not any effect on biological treatment processes.

3.4.7 Effect of quorum quenching on the microbial community

Quorum quenching bacteria can affect microbial community by degradation of signal molecules, and decrease EPS concentration and these two factors are indissociable. As mentioned above, EPS concentration in MBR-B was determined as lower than in MBR-A. It is a fact that this concentration difference results in decreasing about TMP increasing rate and biofilm thickness in the MBR-B. A further technique was used for determining the differences in microbial community in the mixed liquor of MBR-A and MBR-B by the time during RMCF application. The bacterial communities in the activated sludge after acclimation and at the end of operation were compared by PCR-DGGE analysis. PCR-DGGE profiles obtained from samples, which are depicted as MBR-A activated sludge (AS) after acclimation, MBR-A AS at the end of the operation, MBR-B AS after acclimation and MBR-B AS at the end of the operation, were given in Figure 3.11.

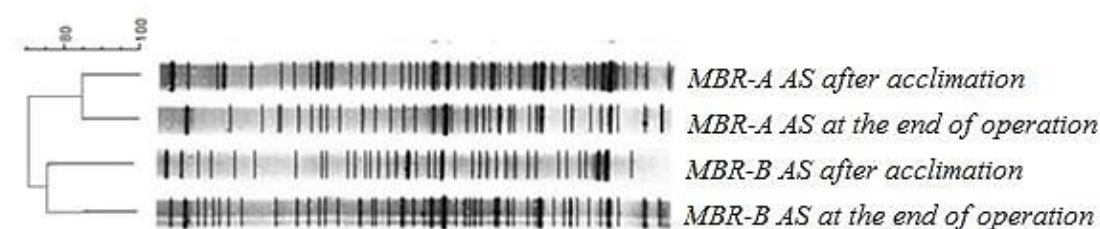


Figure 3. 11 : UPGMA dendrogram analysis for the assessment of similarity between DGGE profiles. The indices for the clustering analysis are optimization 0%; position tolerance 0.6%, minimum height and minimum surface 0% used for the comparison. 0.0–100%, indicating the entire length of each lane.

PCR-DGGE analyses show that a significant increasing in the number of bacteria species was observed in MBR-B after the RMCF application. It could be due to the

degradation of signal molecules by quorum quenching bacteria prevent some common species from being dominant in population equilibrium and provide other rare bacteria species with the opportunity to gather and grow without any social stress. In order to make a more detailed evaluation, the effect of signal molecule vanishing from the environment on bacterial community by time should be monitored during long operations.

3.5 Conclusion

In this study, *Rhodococcus sp.* BH4 was used as quorum quenching bacteria and QQ activity of BH4 was measured as 95%. Polymeric matrices used in CEB manufacturing have some disadvantages due to their unstable forms, leakage of cells and bacterial loss occurred while withdrawal of excess sludge from the MBR. On the other hand, microbial vessel has lower F:M ratio and stagnant property. RMCF was developed as an effective alternative to quorum quenching immobilization media. This design can provide mobility, material stability, and renewability to immobilization module and free living rooms for immobilized quorum quenching bacteria to grow without any releasing into the environment. The quorum quenching activity of RMCF was determined as 65%.

The usage of RMCF in MBR considerably decreased TMP increasing rate and membrane biofouling with an efficiency of 62% via both physical washing and quorum quenching effect without any effect on wastewater treatment performance. Since quorum quenching effect mitigated bacterial cluster trend, this paradigm has decreased EPS production. In addition to this, observation on increasing in the bacterial community diversity after the RMCF application was evaluated as recessive species were not dominated in the population because the bacteria which show the cluster behavior cannot find the proper conditions to come together. Finally, the RMCF showed AHL degradation continuity during 30 days of MBR operation and this result creates expectations for its potential in effective biofouling control in long term MBR operations. The application of quorum quenching bacteria immobilized in the RMCF could be expanded to the full scale of MBRs with its high feasibility, which is related with easy disassembling from MBR, renewable and QQ bacteria refillable properties of module structure and design, proper and enough room for quorum quenching bacteria growth, and the additive anti-biofouling effect of rotational motion.

4. QUORUM QUENCHING MBR OPERATIONS FOR BIOFOULING CONTROL UNDER DIFFERENT OPERATION CONDITIONS AND BY USING DIFFERENT IMMOBILIZATION MEDIA

4.1 Purpose and Scope

Biofouling is one of the major problems in reaching the goal of being the only preferable technology for advanced wastewater treatment for membrane bioreactors (MBR). In this study, the quorum quenching mechanism (QQ) was applied to MBR technology with prevention of group behavior of microorganisms via signaling. This study aims to prepare three different QQ immobilization media, which are 1) cell entrapping bead (CEB), 2) microbial vessel (MV) and 3) rotating microbial carrier frame (RMCF). Two different *Rhodococcus sp.* BH4 (QQ bacteria) amounts and three different fluxes were used to see their effects on QQ mechanism. The flux was more effective than QQ bacteria amount. TMP profiles showed that all QQ products were successful in anti-biofouling during MBR operation. While CEB was the most effective one, MV had the minimum influence on the transmembrane pressure (TMP) reduction. On the other hand, it was found out that RMCF is the most feasible one according to the cost analysis results. This study offers an idea about the potential of QQ applications in pilot- and real-scale MBR plants after examinations on different operation conditions and different QQ products.

4.2 Introduction

In today's world, advanced wastewater treatment to get available water has become crucial because of gradual decrease in fresh water resources. As known by the researchers who work on advanced wastewater treatment, membrane bioreactor combining membrane technology and biological treatment process is one of the most preferred alternatives (Melin et al., 2006). Furthermore, it is becoming more popular every day via high effluent quality and operation conditions, which decrease area requirement (Ven der Roest et al., 2002; Gao et al., 2009; Visvanathan et al., 2015).

The Achilles heel of this technology with growing popularity is biofouling that is resulted from biofilm creation on filtration membrane during treatment process by the mixed liquor suspended solids in reactor (Judd, 2004).

Bio-cake is formed as a result of exponential increase of biofilm on the membrane surface and cellular polysaccharide and protein components, which could be designated as extracellular polymeric substances (EPS) and soluble microbial products (SMP) (Kimura et al., 2005; Chang et al., 2002; Ng et al., 2006; Ramesh et al., 2007; Drews et al., 2008). Flux of the filtration membrane begins to fall rapidly because of bio-cake with decreasing porosity (Gkotsis et al. 2014). Increasing operation pressures in order to ensure the continuity of permeate flux results in an increase in unit cost of wastewater treatment. In the literature, it can be seen that there are a variety of research studies based on chemistry, engineering, etc. to create a solution for biofouling in MBR (Koseoglu et al., 2008; Koseoglu-Imer et al., 2013; Rahimi et al., 2015; Xiang et al., 2001; Kimura et al., 2005; Ahmed et al., 2007).

Some of these above mentioned solutions could be inadequate from time to time, since the biofilm blocking feature of some of these could be defeated by time or have high costs. Indeed, the main point of this inadequacy is that biofouling is a totally natural and biological process. Quorum sensing mechanism, which is a quite new concept, can be explained as the communication via signalization and group behavior exhibition by microorganism (Chen et al., 2005; Waters & Bassler, 2005; Miller & Bassler, 2003). The most common one of these group behaviors is biofilm creation (Hammer & Bassler, 2003; Barrios et al., 2006; Li et al., 2002). Communication mechanism can be interrupted via degradation of N-acyl homoserine lactone (AHL), which is secreted for signalization, by bacteria and this can be called bacterial quorum quenching (Lin et al., 2003; Dong & Zhang, 2005; Sio et al., 2006; Yang et al., 2005; Wang et al., 2004).

Bacterial quorum quenching mechanism can adapt to MBR operation by using various immobilization media in which quorum quenching bacteria are immobilized. There are two main bacterial quorum quenching applications that take place in literature: cell entrapping bead and microbial vessel (polymeric/ceramic) (Kim et al., 2013; Oh et al., 2012, Cheong et al., 2014). The main aim of bacterial quorum quenching applications is to decrease the transmembrane pressure (TMP). TMP profile related with biofouling could be affected by operation conditions including the flux. The higher the flux causes the higher the TMP values (Suworno et al., 2014). With this reason; first of all, the

effects of operation conditions on bacterial quorum quenching were studied by using only one immobilization media type, which is preferred as cell entrapping bead because of its common usage. Furthermore, it can be said that several successful quorum quenching MBR operation studies were carried with CEB and microbial vessels. However, CEB and microbial vessel have disadvantages like low mechanical durability and low F:M ratio for immobilized bacteria, respectively. Within this scope, after the effect of MBR operation conditions on bacterial quorum quenching was examined by using CEB, potentials on prevention of biofouling and decreasing on unit wastewater treatment cost by using different immobilization media such as; cell entrapping bead, microbial vessel and rotating microbial carrier frame (RMCF) were also studied and tried to be determined under the same operation conditions.

4.3 Material and Method

4.3.1 Regents

Commercial C8-HSL (Cayman, USA) was preferred as representative chemical for signal molecules and the stock solution of C8-HSL was stored under -20°C temperature condition. X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) was supplied from Sigma Aldrich (USA) with its powder form and X-Gal solution was stored under -20°C temperature and dark conditions after prepared with dimethylformamide (DMF) bought from Merck (Germany). Spectinomycin was supplied as a powder from Sigma Aldrich (USA) and the stock solution of spectinomycin was stored under -4°C temperature and dark conditions. Tetracycline was also supplied with its powder form from Sigma Aldrich (USA) and stock solution was kept at -20°C temperature. Luria Bertani (LB) medium was prepared from its granular form according to the supplier (Merck, Germany) instructions. Lastly, sodium alginate was supplied from Sigma Aldrich (Germany).

4.3.2 Preparation of QQ products

4.3.2.1 Bacterial strains and growth conditions

Agrobacterium tumefaciens A136 (Ti)(pCF218)(pCF372) (Kawaguchi et al., 2008; Yeon et al., 2009; Oh et al., 2013) was preferred to use for detection of N-acyl homoserine lactone (AHL) signal molecules by using it as a biosensor strain. A.

tumefaciens A136 was cultured on LB medium containing spectinomycin (50 mg/L) and tetracycline (4.5 mg/L) in order to get two plasmids which can provide the AHL response system. This incubated A136 was used for Quorum Quenching activity bioassay. *Rhodococcus sp.* BH4, which was used as QQ bacteria for this study, cultured on LB broth and incubated in a 30°C rotary shaker (160 rpm, Nüve, Turkey) for 24 hours.

4.3.2.2 Cell entrapping beads

CEBs were prepared according to the method obtained from (Kim et al., 2013). 100 ml sodium alginate (4% w/v) and 300 ml CaCl₂ (3% w/v) solutions were prepared and autoclaved by using steam sterilizer (Nüve OT032, Turkey). After *Rhodococcus sp.* BH4 bacteria solution was centrifuged for 10 min (6000 rpm, Hettich 320R, Germany), it was added to the sodium alginate solution. This mixture was distilled into CaCl₂ solution which is placed on a magnetic stirrer (WiseStir MSH-A, Germany). Created beads were stored at 4°C for 8 hours to increase their strength. At the end of 8 hours, beads were stored in physiological saline for the future time.

4.3.2.3 Microbial vessels

Microbial vessel was prepared by using hydrophilic polyvinylidene fluoride (PVDF) hollow fiber microfiltration membranes (Philos Co. Ltd, Korea) that have 0.2 µm nominal pore size and this method was taken from (Oh et al., 2012). A small 10 cm length hollow fiber module, which has a closed and an open end, was manufactured by using 0.2 cm² membrane. After *Rhodococcus sp.* BH4 bacteria solution was centrifuged for 10 min (6000 rpm, Hettich 320R, Germany), it was pumped into the fibers by using a peristaltic pump (Watson Marlow 323D, USA).

4.3.2.4 Rotating microbial carrier frame (RMCF)

RMCF is a module composed of a polycarbonate frame and four cubbyholes. The cubbyholes of RMCF were covered with PVDF microfiltration membrane (Microdyn Nadir GmbH, Germany) that has a nominal pore size of 0.20 µm and thickness of 210–250 µm. After *Rhodococcus sp.* BH4 bacteria solution was centrifuged for 10 min (6000 rpm, Hettich 320R, Germany), it was filled into each cubbyholes by using a syringe. The polycarbonate frame of RMCF was sealed with special glue in order to

avoid cell escape and each cubbyhole of RMCF has a small channel with 0.75 mm diameter for bacteria solution renewal.

4.3.3 Quorum quenching activity test

QQ activities of free cells and immobilized cells in beads, microbial vessels and RMCF were measured by using a bioassay. In this bioassay, AHL concentrations were determined with indicating agar plate method adopted from Park et al. (2001). Indicating agar plate was prepared by mixing (ratio of 9:1) LB-agar and an overnight culture of A136 that includes spectinomycin (50 mg/L), tetracycline (4.5 mg/L) and X-gal (0.2 g/L). Samples taken regularly from the reaction tube were loaded into the wells of this indicating agar plate. As a result of chain reaction of C8-HSL, A136 and X-Gal, the blue color was developed. C8-HSL concentration decreases with time by the degradation of AHL by free or immobilized QQ bacteria could be determined by using the relationship equations related with blue color zone areas and standard AHL concentrations.

After QQ activity test, QQ media were added into the MBRs and used during operations. In Figure 4.1, schematic drawings of immobilization media and their locations in the MBR can be seen. While CEBs are in a dispersed mode, microbial vessel is in a stable mode in the MBR. On the other hand, RMCF is submerged into the MBR by using an impeller top and rotated during operation via a rotor.

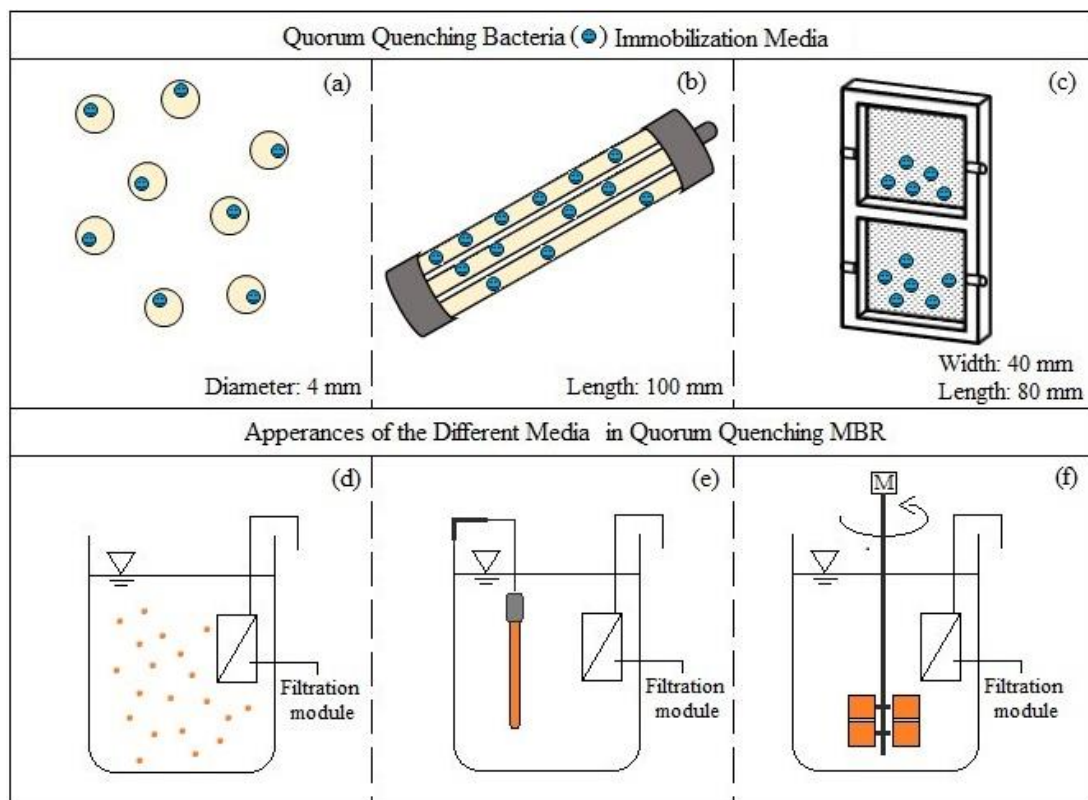


Figure 4.1 : Different QQ immobilization media (a: CEB, b: microbial vessel, c: RMCF, d: locations of moving CEBs in a MBR, e: location of a microbial vessel in a MBR and e: location and rotation of a RMCF in a MBR).

4.3.4 MBR system

Within the content of quorum quenching MBR studies, two parallel MBRs were simultaneously operated under the same operation conditions. While one MBR named as control MBR was operated as a conventional MBR, the other one named as QQ MBR was operated with QQ product additions. In this study, MBRs were operated under the constant flux condition. Scheme of the MBR system was given in Figure 4.2.

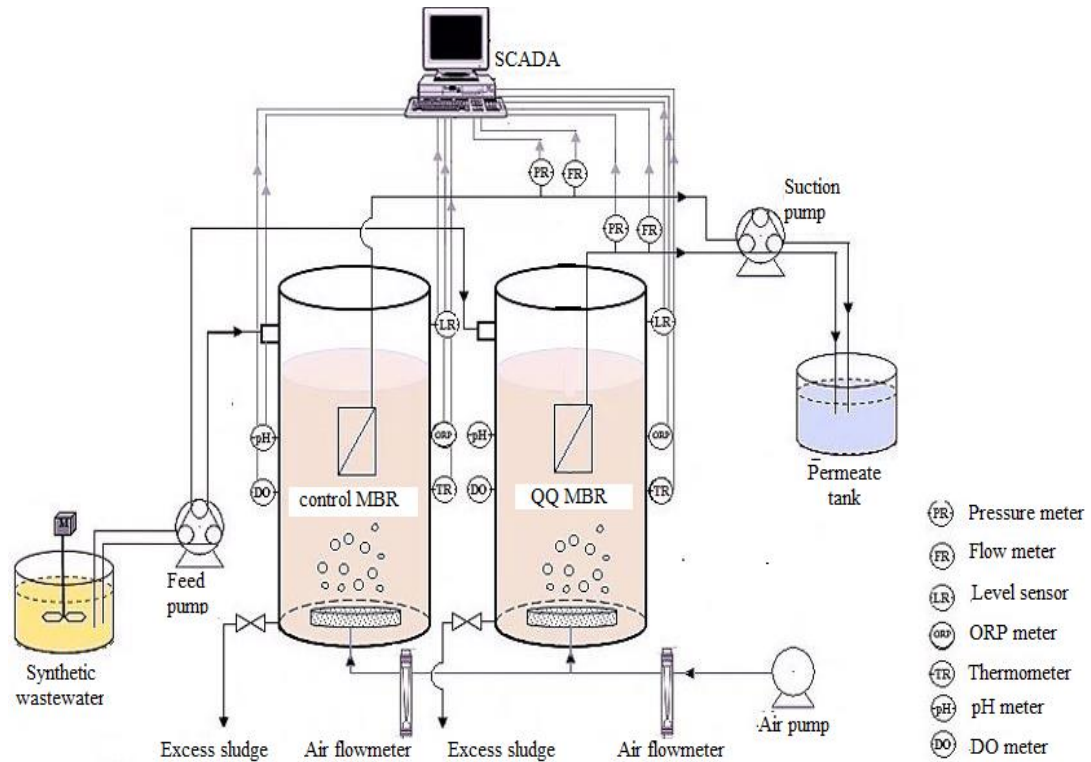


Figure 4.2 : MBR system.

Activated sludge was supplied from Paşaköy Advanced Biological Wastewater Treatment Plant in Istanbul (Turkey) and it was acclimated to synthetic wastewater prior to the MBR operations. The composition of the synthetic wastewater can be listed as follows (mg/L): glucose, 400; yeast extract, 14; bactopectone, 115; $(\text{NH}_4)_2\text{SO}_4$, 104.8; KH_2PO_4 , 21.75; MgSO_4 , 15.63; FeCl_3 , 0.075; CaCl_2 , 2.45; MnSO_4 , 1.8; and NaHCO_3 , 255.5. Control MBR and QQ MBR were connected to a computer and controlled by using an automation program that can adjust the values of operating parameters like water levels, pH, temperature, oxidation-reduction potential (ORP) and dissolved oxygen concentration according to the desired levels listed in Table 4.1. Membrane module was prepared by using hollow fiber microfiltration membrane supplied from Philos Co. Ltd. (MegaFlux I, Korea).

Table 4. 1 : Operation parameters and conditions of the MBR.

Parameter	Unit	Value
Working volume	L	5
pH	-	6.8~7
Dissolved oxygen	mg/L	4.2-6.5
ORP	mV	170-200
Temperature	°C	20±2
SRT	d	30
HRT	h	13
Membrane area	m ²	0.01
COD removal efficiency	%	~ 95
Feed COD	mg/L	440

4.3.5 Experimental systematic

With the aim of this study, a two stage experimental systematic was carried. While the first stage of the experimental systematic intended to determine the relationship of QQ effect with the immobilized QQ bacteria amount and flux, the aim of the second stage was the comparison of three different immobilization media about their quorum quenching effects. This first stage can be described as an optimization study. The reason why CEB was selected as the immobilization media for this part of the study was that this immobilization media is one of the main and the most common immobilization media in the literature. Operating strategies for the optimization experiments were given in the Table 4.2. The experimental systematic of the second stage can be seen in Table 4.3. During all these studies, mixed liquor suspended solid (MLSS) concentrations in MBRs were maintained within the range of 12000-13000 mg/L.

Table 4. 2 : Experimental systematic used for the comparison of QQ effects obtained under the different operation conditions.

Study	QQ bacteria amount in immobilization media	Flux (LMH)	MLSS (g/L)	Aim of the operation
CEB I	1.5 mg BH4/cm ³	20	12-13	QQ effect determination
CEB II	7.5 mg BH4/cm ³	20	12-13	Effect of immobilized QQ bacteria amount on QQ effect
CEB III	7.5 mg BH4/cm ³	30	12-13	Effect of an increase of 50% in flux on QQ effect
CEB IV	7.5 mg BH4/cm ³	50	12-13	Effect of an increase of 50% more in flux on QQ effect

Table 4. 3 : Experimental systematic used for the comparison of QQ effects obtained via different quorum quenching media.

Study	QQ bacteria amount in immobilization media	Flux (LMH)	MLSS (g/L)	Aim of the operation
CEB IV	7.5 mg BH4/cm ³	50	12-13	QQ effect of CEB in high-flux MBR operation
MV	7.5 mg BH4/cm ³	50	12-13	QQ effect of MV in high-flux MBR operation
RMCF	7.5 mg BH4/cm ³	50	12-13	QQ effect of RMCF in high-flux MBR operation

4.3.6 Analytical methods

During MBR operation some important parameters like MLSS, COD, TKN and TP were analyzed. While MLSS concentrations of the mixed liquor were determined to check and control the parameters that are directly related with biofouling, COD, TKN and TP concentrations in feed and permeate flows were analyzed to check whether proper and efficient microfiltration processes in control MBR and QQ MBR were achieved or not. MLSS, COD, TKN and TP were determined according to Standard Methods (APHA, 2005).

4.4 Results and Discussion

4.4.1 QQ MBR operations under different operation conditions

4.4.1.1 Quorum quenching activities of CEBs in a standard AHL solution

Optimization studies on effect of operation conditions were carried as four QQ MBR operations by using CEBs according to the experimental systematic given in Table 4.2. The aim of this study was to see the effects of immobilized QQ bacteria amount and flux on the QQ mechanism efficiency. It is important to determine the QQ effect (AHL degradation rate) of manufactured CEBs in a standard AHL solution by using bioassay before adding to the reactor for healthy evaluations of different QQ mechanism efficiencies resulted from the operation conditions. This bioassay is based on AHL, signal molecule, degradation rate with time in a standard 200 nM C8-HSL solution. The rate of degradation in the standard AHL solution is directly related with the immobilized QQ bacteria amount. AHL degradation rates of four different CEBs,

which have two different immobilized QQ bacteria amounts as 1.5 and 7.5 mg BH4/cm³, are given in Figure 3.

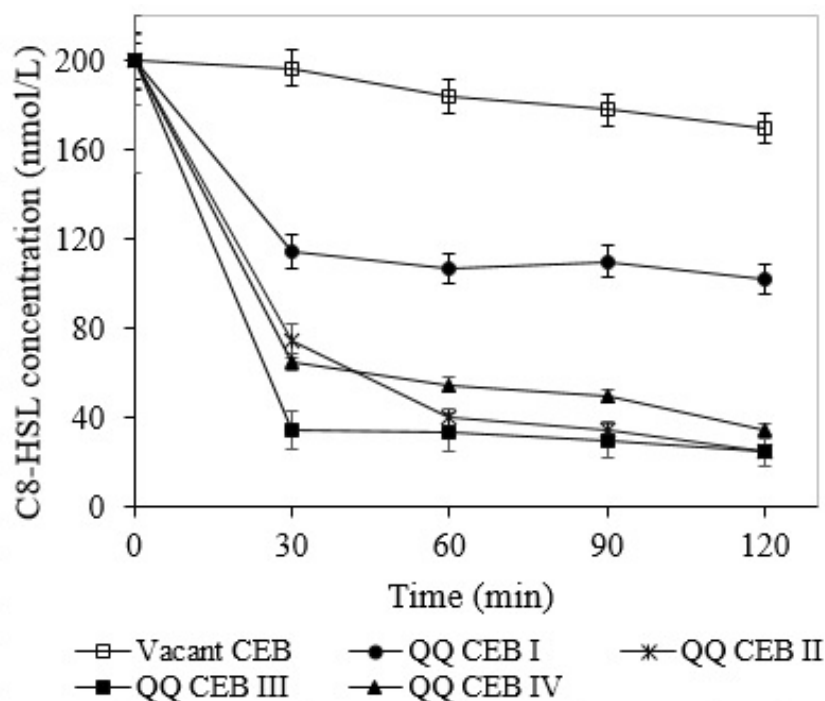


Figure 4.3 : QQ activities of CEBs (vacant beads did not include QQ bacteria).

As seen in Figure 4.3, while CEB that includes 1.5 mg BH4/cm³ could degrade 49% of 200 nM C8-HSL in 120 min, CEBs that include 7.5 mg BH4/cm³ could show QQ mechanism efficiencies, which can be defined as the degradation efficiencies of AHL in a certain time, as the average of 85.8%±2.3 in the same duration. It can be said that QQ mechanism efficiency could increase with the increase of the immobilized QQ bacteria amount and three CEBs including 7.5 mg BH4/cm³ had quite close QQ mechanism efficiencies. Vacant CEB that was used as a control sample for this study did not include bacteria, and it was seen that this CEB showed also a small amount decrease on AHL concentration in the environment (~9.5%). This decrease could be evaluated as the surface adsorption of the CEB and this is negligible.

4.4.1.2 Quorum quenching activities of CEBs in the MBR

After QQ mechanism efficiency determination, CEBs were used in MBRs. In these four studies, while control MBR was operated with vacant CEBs, QQ MBR was operated with QQ CEBs having the same amount. Control MBR and QQ MBR were operated parallel and under the same and constant flux conditions. At the beginning of each study, activated sludge of the control MBR and QQ MBR was totally mixed in a

tank and added to the control MBR and QQ MBR after it was divided into two. New membrane modules were used for each operation. The rate of TMP increase in control and QQ MBR were determined by using the TMP profiles obtained from both reactors. It is a known fact that TMP values increase with the biofouling formation in time. Areas under the TMP graphs drawn after operations were calculated via taken the integrals of the formulas of TMP profiles, and fouling reductions were parameterized via percentage statement of the differences between these areas. TMP profiles for four studies can be seen in Figure 4.4.

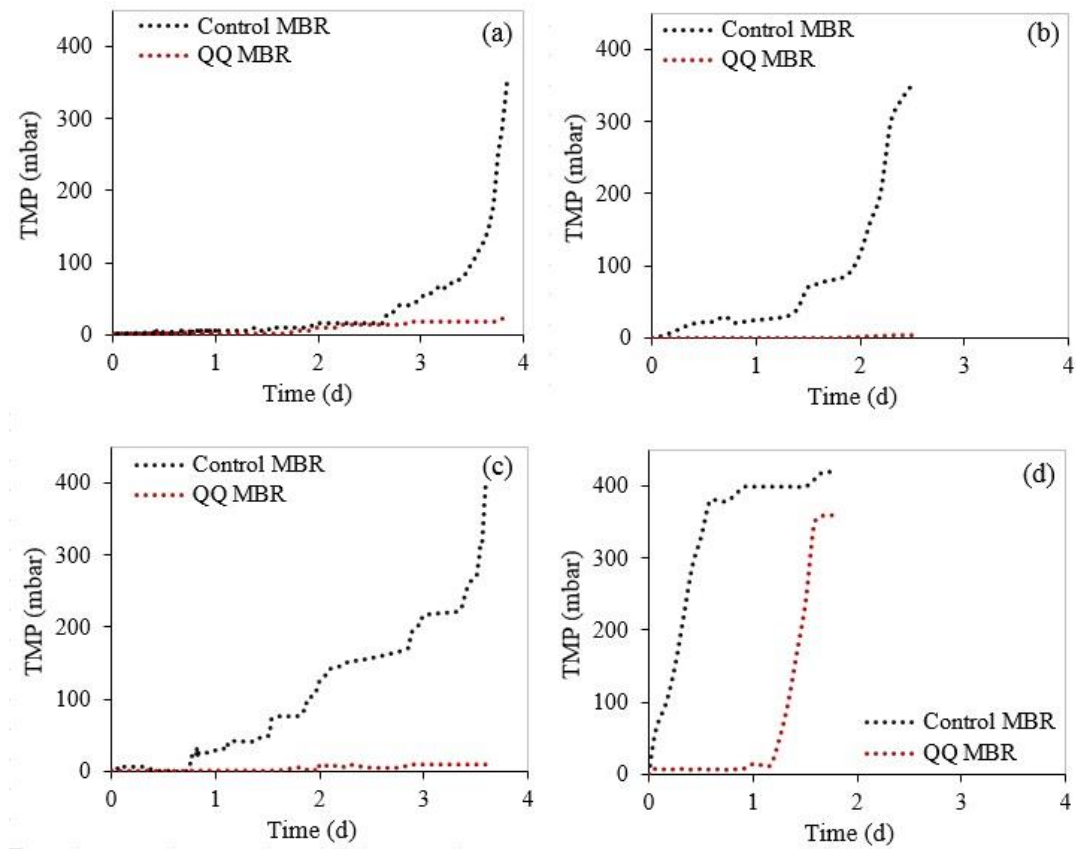


Figure 4.4 : TMP profiles obtained from CEB studies for different operation conditions (a: study CEB I, b: study CEB II, c: study CEB III and d: study CEB IV).

As seen in Figure 4, while TMP values increased via fouling in the control MBRs, TMP values in QQ MBR were kept lower than TMP values of control MBRs. This means that in all studies, quorum quenching was achieved. The first CEB study was carried under low flux and low immobilized QQ bacteria amount conditions, and biofouling could be prevented with 78.7% efficiency. In the second study, while flux value was kept the same with the first study, the amount of the immobilized QQ bacteria was increased four times and 99.3% biofouling prevention efficiency was

achieved with a 21% efficiency increase. After the second study, the flux was increased to see the efficiency of the same amount immobilized QQ bacteria under the harder conditions. In this study, study CEB III, the efficiency about biofouling prevention was found as 96.1%. It can be said that efficiency decreased with flux increase; however, high quorum quenching efficiency could be achieved for this flux. It is a fact that very high flux values could result in rapid and irreversible fouling in MBR treatment. In the fourth study, control MBR and QQ MBR were operated as high-flux MBRs and it was desired to see the potential quorum quenching effect of immobilized QQ bacteria on rapid fouling. Finally, quorum quenching efficiency was 77.7% for this study. This efficiency result is close to the result of the first study. In the light of these results, it can be mentioned that four times increase in the immobilized QQ bacteria amount could hardly meet the one and a half times increase in flux. As a result, the effect of flux on TMP increase was higher than the effect of the immobilized QQ bacteria amount on TMP decrease.

4.4.1.3 Removal of organics

It is necessary to check whether microfiltration process had properly been carried during the MBR operation or not to be able to mention about the quorum quenching mechanism as a result of TMP decrease. In this regard, COD and TKN removal efficiencies of control MBR and QQ MBR were determined. It can be said that efficiencies were similar for control MBR and QQ MBR and quorum quenching did not affect the removal of organics during operation (Figure 4.5).

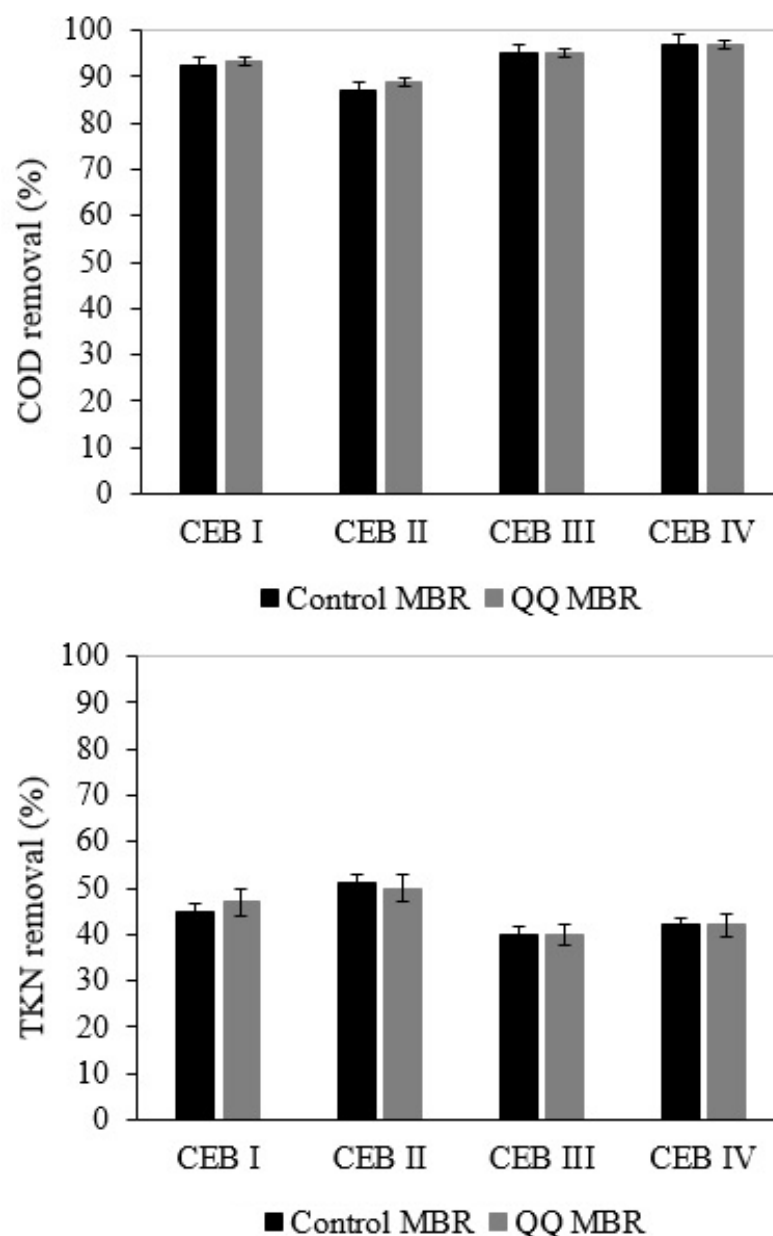


Figure 4.5 : COD and TKN removal efficiencies obtained from CEB studies for different operation conditions.

4.4.2 QQ MBR operations with different immobilization media

4.4.2.1 Quorum quenching activities of immobilization media in a standard AHL solution

Because it was found out that flux has higher an effect on the quorum quenching efficiency than immobilized QQ bacteria amount, three different immobilization media were aimed to be compared via their efficiencies under the high flux conditions. With this aim, two different immobilization media (MV and RMCF) were also

manufactured with the same amount of QQ bacteria in the study CEB IV for MBR operations. AHL degradation rates of the vacant and QQ bacteria immobilized vessel and RMCF in a standard AHL solution were determined before operations and given in Figure 4.6 with study CEB IV results.

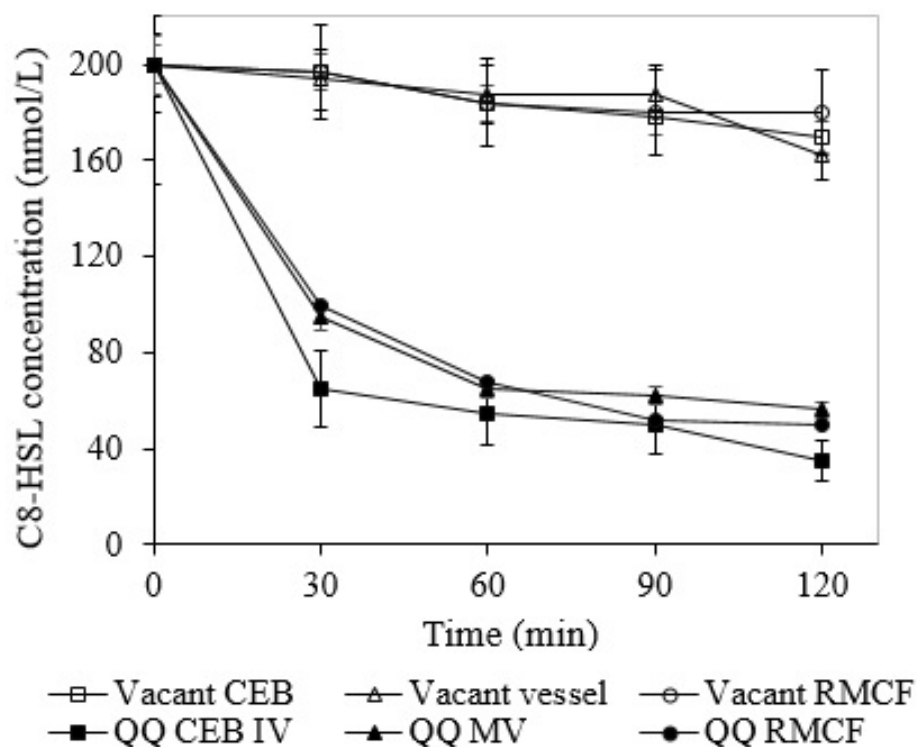


Figure 4.6 : QQ activities of CEB, MV and RMCF.

It can be seen in the figure that the immobilization media material or design has no effect on QQ activity potential. The only parameter was the amount of immobilized QQ bacteria for a batch study in a standard solution. While decreasing of the AHL amount in the solution by vacant immobilization media was $13.6\% \pm 3.6$, it was averagely $76.5\% \pm 4.5$ for CEB, MV and RMCF.

4.4.2.2 Quorum quenching activities of immobilization media in the MBR

After the bioassays, QQ mechanism efficiency studies of MV and RMCF were carried with MBR operations. Firstly, MV study was realized with a parallel MBR operation by using a control MBR operated with a vacant vessel and a QQ MBR operated with the microbial vessel in which BH4 is immobilized. After MV study, RMCF study was realized with the same conditions. For MV and RMCF studies, control MBR and QQ MBR were operated under the same and constant flux conditions. At the beginning of these each two studies, activated sludge of the control MBR and QQ MBR was totally

mixed and added to the control MBR and QQ MBR after it was divided into two again. New membrane modules were used for these operations like CEB studies. The TMP profiles obtained from MV and RMCF studies are given in Figure 4.7.

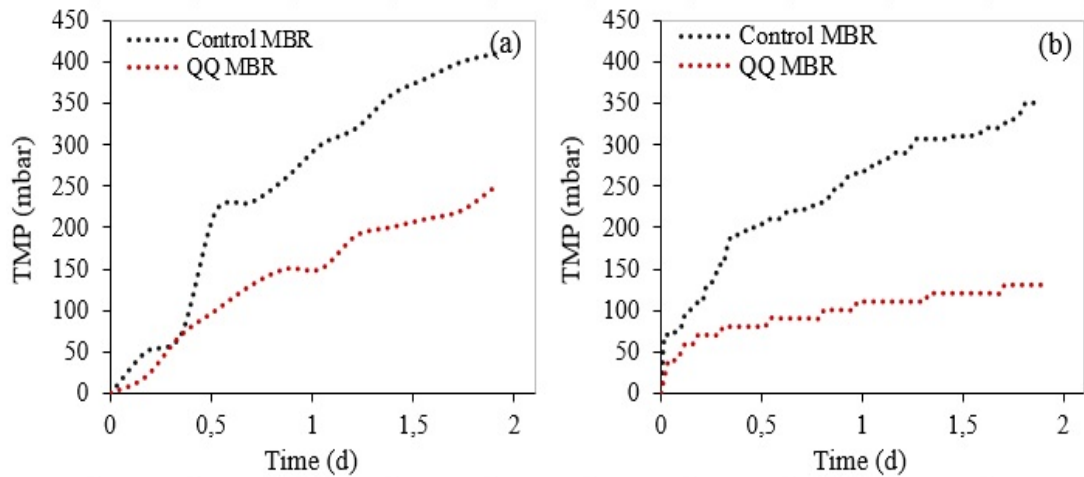


Figure 4.7 : TMP profiles obtained from MV and RMCF studies (a: study MV, b: study RMCF).

In these two studies, while TMP values of control MBR increased in time rapidly, TMP values of QQ MBR increased more slowly. This prevention of the rapid TMP increase in QQ MBR is a result of QQ mechanism. MV and RMCF could result in 44% and 59.7% TMP decrease during operation, respectively. If the results of the CEB IV (Figure 4.6-d), MV (Figure 4.7-a) and RMCF (Figure 4.7-b) were evaluated together, it can be said that CEB is the most successful one for biofouling control under the high flux condition. As seen, RMCF had also an important achievement for biofouling control with prevention around 60%. The minimum efficiency was obtained from the microbial vessel study and these results are similar to previous studies in the literature (Kim et al., 2013; Oh et al., 2012). First of all, it can be mentioned that the reason why the highest efficiency obtained from CEB study is the high F:M ratio for immobilized QQ bacteria in the CEB and physical cleaning of the membrane surface via the mobility of CEB in the reactor. In addition to this, RMCF has motion property and provide higher F:M ratio when it is compared to microbial vessel. Furthermore, the rotation motion of the RMCF under the membrane module also helps to create shear forces and physical cleaning even if it is not a totally spread media through the reactor like CEB. The biggest disadvantage of microbial vessel is immobility as an immobilization medium.

4.4.2.3 Removal of organics

It was also checked whether microfiltration process had properly been carried during the MV and RMCF studies or not to be able to mention about the quorum quenching mechanism as a result of TMP decrease. COD and TKN removal efficiencies of control MBR and QQ MBR were also determined. The COD and TKN removal efficiencies for study CEB IV, study MV and study RMCF were given in Figure 4.8 together. It may be said that efficiencies were similar for control MBR and QQ MBR and quorum quenching did not affect the removal of organics during operations.

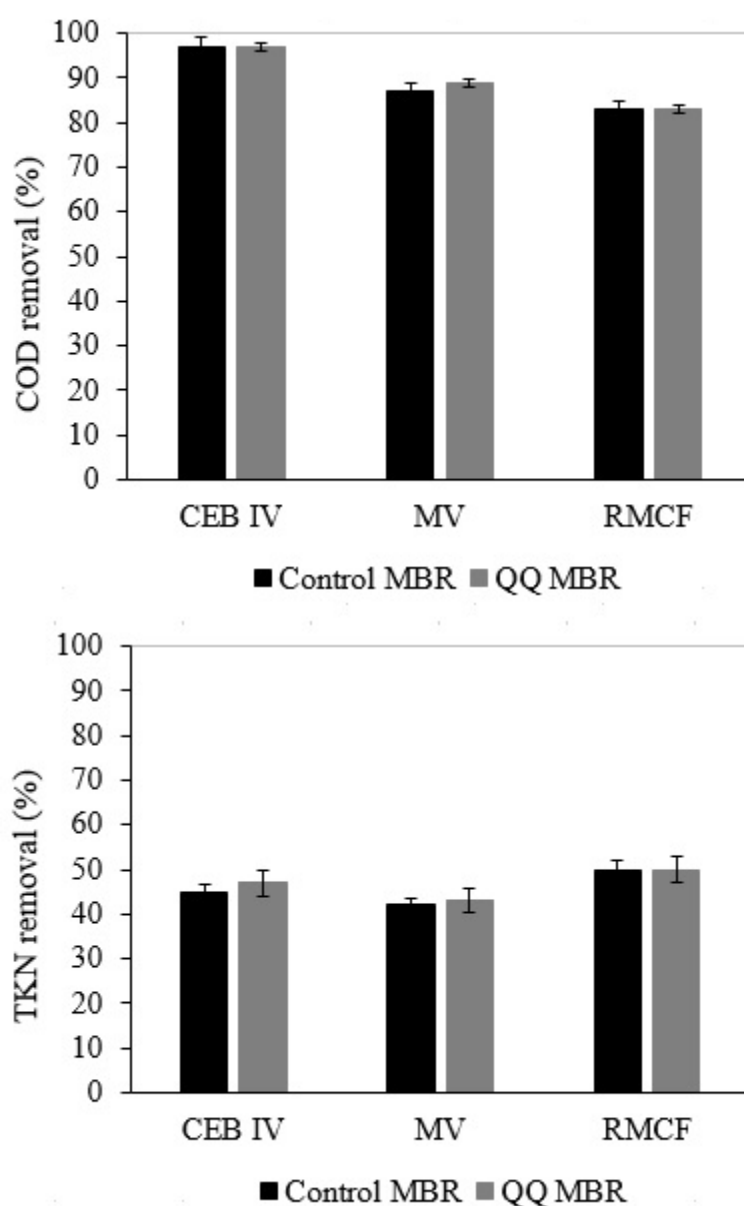


Figure 4.8 : COD and TKN removal efficiencies obtained from CEB IV, MV and RMCF studies.

4.4.3 Energy saving and cost analysis

It is a well-known fact that the successful prevention of biofouling results in reduction of TMP values and this reduction minimizes the spending item for suction pump power, which is one of the most important spending items. Although immobilization media have remarkable high success on TMP decrease, the main point that should be taken into consideration is their manufacturing costs and lifetimes. For instance, the material of CEB shows generally low durability and CEBs should be renewed periodically. Because MV and RMCF are manufactured from inert and durable material, they do not need to be renewed during long-term operations. In this regard, cost analysis with the manufacturing costs and without the manufacturing costs were done to see the rational reduction on unit wastewater treatment cost via quorum quenching.

The unit energy cost of domestic wastewater treatment with MBR technology is approximately 0.8 kWh/m³ (Engelhardt, 2010). MBR operation cost items with their contributions to the total operation cost can be listed as: feed pumps (10%), activated sludge aeration (20%), membrane aeration (36%), suction pumps (21%), online control (7%) and chemical cleaning (6%) (Bentem et al., 2010; Decarolis & Adham, 2007). The using of CEB, MV and RMCF can reduce the costs of two items like suction pump and chemical cleaning via their biofouling prevention in direct proportion to their own QQ mechanism efficiencies in MBR. In addition to this, CEB and RMCF have a reduction on the membrane aeration cost of 10% and 20%, respectively. Total operation costs for MBR operation with/out QQ product were determined and the gained rational reductions on the total operation cost via QQ product usage are given in Figure 4.9-a. The results if manufacturing costs were also involved in total operation costs are given in Figure 4.9-b.

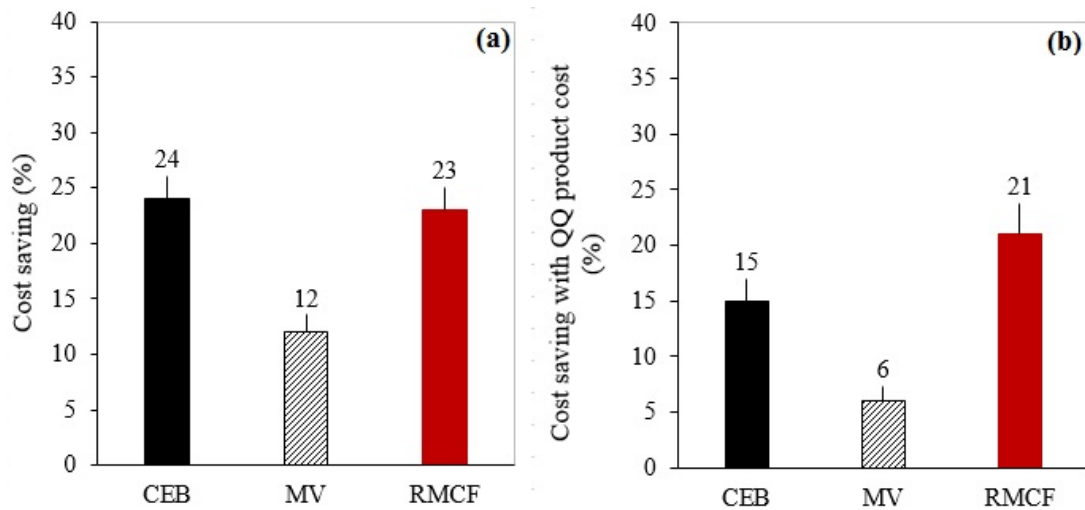


Figure 4.9 : Cost savings via QQ MBR operations.

By looking at Figure 4.9-a, it can be said that while CEB and RMCF can create cost savings of 24% and 23% via their higher biofouling prevention efficiencies respectively, the usage of MV could only reduce the cost 12%. Because of its immobile property MV fell behind about cost saving. Besides, high manufacturing cost and renewal need of CEB may cause to reduce the saving from 24% to 15%. There is no need for material renewal for MV and RMCF. However, the manufacturing cost of MV is higher than the manufacturing cost of RMCF. Because of this reason while RMCF may provide a cost saving of 23%, MV can only save 6%. To summarize, it can be said that the most feasible immobilization media is RMCF and the unit cost of domestic wastewater treatment with MBR technology would be 0.61 kWh/m³ with RMCF preference.

4.5 Conclusion

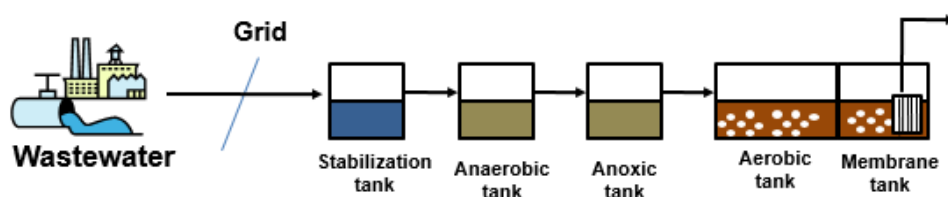
In this study, the effects of QQ bacteria amount and flux on application of QQ mechanism to MBR technology were examined. With the results of the studies, it can be said that the flux increase can affect dramatically the QQ mechanism in the MBR. After that, the biofouling prevention efficiencies of three different QQ immobilization media under the same conditions, in which QQ mechanism had difficulty in working properly, were studied. It was seen that CEB is very successful in biofouling prevention; however, RMCF is more feasible for pilot- and real-scale MBR plants when the manufacturing costs of QQ products are taken into consideration.

5. ONE-STEP QQ MEMBRANE BIOREACTOR: A COMBINED MBR SYSTEM FOR ENHANCED NITROGEN REMOVAL AND BIOFOULING CONTROL

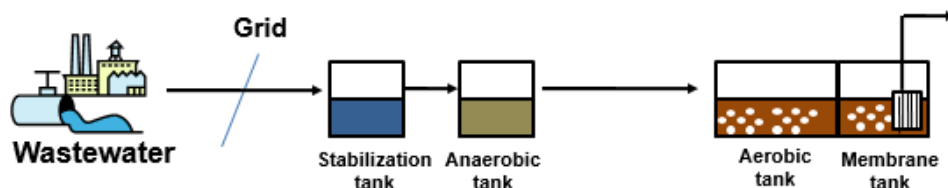
5.1 Purpose and Scope

An innovative treatment process combination, one-step membrane bioreactor (MBR) was developed to enhance biological nitrogen removal and prevent biofouling, and its performance was investigated. With these two aims of the domestic wastewater treatment, two parallel submerged membrane bioreactors were operated under the 30 l/m²/h flux and continuous aeration conditions. While one reactor, Control MBR, contained vacant beads, the other one, QQ MBR, was operated with two different bead additions: 1) anoxic sludge immobilized PVA beads and 2) quorum quenching (QQ) immobilized PVA beads. According to the batch test and bio-assay results, the denitrification rate of anoxic bead in the synthetic wastewater was 0.067 mg nitrate/mg MLVSS.h, and the AHL degradation efficiency of QQ beads was found out as 90%. Parallel operation of the submerged MBR showed that QQ beads could prevent the biofouling with 94% efficiency and anoxic bead resulted in 7% and 21% increase on COD and TN removal efficiencies, respectively. Within this regard, it can be said that this study represented results that will may be beneficial for operators and researchers related with pilot- and real-scale MBRs. The concept of one-step QQ MBR was schematically shown in Figure 5.1.

Conventional MBR :



One-step QQ MBR :



- Advantages

1. Decrease plant area
2. Stable TN removal adjusting the number of bead
3. Strong physical washing effect (vs. QQ-MBR)

Figure 5.1 : One-step QQ MBR concept.

5.2 Introduction

In today's World, increasing population and industrialization have gradually increased environmental pollution and decreased the amount and the quality of usable water. Socioeconomic development results in harder domestic wastewater characterization and higher quality targets for receiving water bodies. Nowadays, most of the developed and developing countries have started to apply discharge limits according to the total maximum daily loads of organic pollutants for the sustainability of receiving body quality (Stow et al., 2003; USACE, 2006; Chen et al., 2009; Hsu et al., 2009; Havens & Schelske, 2001). This means that the higher flowrates result in the lower discharge concentrations, and these lower discharge limits may cause problem for wastewater treatment plants located in cities having high populations.

As it is known, MBR technology has been preferred in wastewater treatment plants with high flowrate ($5000 \text{ m}^3/\text{d}$ - $100000 \text{ m}^3/\text{d}$) via its several advantages (Lesjean et al., 2009; Zheng et al., 2010). MBR, which lets control of mixed liquor suspended solids (MLSS) concentrations in the tank during the operation (Fan et al., 1994), can help reduce the area need of aeration tanks. Because MBR is a combined technology that

get biological treatment and filtration processes together (Melin et al., 2006), precipitation tanks can be removed from the flowchart of the wastewater treatment plant. Within this regard, it can be said that MBR preference can reduce wastewater treatment plant area. However, this preference has no effect on the area of anaerobic and anoxic tanks that are related with phosphorus and nitrogen removals (Kraume et al., 2004). In order to fit the strict nitrogen discharge limits, the volume of anoxic tank (V_{anoxic}) should be increased, and sometimes this increase could not be sufficient. Kraume et al. (2003) mentioned that a volume ratio $V_{\text{anoxic}}/V_{\text{aerobic}}$ of 1:1 for MBR plants with pre-denitrification while this ratio is 1:3 for conventional wastewater treatment plants. Because of this reason, researches have carried several studies on enhanced nitrogen removal for MBRs (Ahn et al., 2003; Shin et al., 2005; Wang et al., 2008; Lee et al., 2009).

The usage of bacteria with specific and different aims after immobilization into the beads is a common application in biotechnology (Bashan, 1986; Bashan et al., 2002; Idris & Suzana, 2006; Gonzalez & Bashan, 2000; Ha et al., 2009). Besides, it was discovered that the center area of beads having a diameter larger than 1.8 mm could be anoxic (Annesini et al., 2000). Within this regard, it is possible to increase the nitrogen removal efficiencies with a continuous aerated MBR operation with the addition of beads in which a mixed culture of denitrifiers and nitrifiers is immobilized. Small anoxic areas in these beads could be used for reactions carried under anoxic conditions. Thus, anoxic tanks may be removed from the flowchart of wastewater treatment plant, and nitrogen removal efficiency can be adjusted according to changing wastewater characteristic by adding of these beads with the desired amount. In this way, both area need reduction and fitting the discharge limits can be achieved.

One of the most important disadvantages of MBR technology is biofouling as indicated before in various studies (Koseoglu-Imer et al., 2013; Sweity et al., 2011; Trouve et al., 1994). Moreover, there are studies on quorum quenching (QQ) MBR applications which were successful in biofouling prevention (Oh et al., 2012; Yeon et al., 2009; Cheong et al., 2013; Kim et al., 2013). In this study, a method of immobilization of bacteria into the beads was used to combine biofouling prevention via QQ mechanism with QQ bacteria and organic matter removal enhancement via denitrification/nitrification mechanism with anoxic activated sludge in a continuous aerated MBR operation for synthetic domestic wastewater treatment.

5.3 Material and Method

5.3.1 Reagents

A commercial chemical C8-HSL from Cayman (USA) was used to represent signal molecules and the stock solution of this chemical was stored at -20°C temperature. Two antibiotics were used in this study. The first one, spectinomycin, was supplied as a powder from Sigma Aldrich (USA) and the stock solution of this was kept under +4°C temperature and dark conditions. The other one, tetracycline was also supplied from Sigma Aldrich (USA) with its powder form and the stock solution was stored at -20°C temperature like C8-HSL. X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) chemical from Sigma Aldrich (USA) was used for bio-assay and supplied with its powder form and X-Gal solution was stored under -20°C temperature and dark conditions after prepared with dimethylformamide (DMF, Merck, Germany). Luria Bertani (LB) medium for bacterial growth was supplied from Miller (US). Lastly, polyvinyl alcohol (PVA), calcium chloride (CaCl_2) and boric acid for bead production were supplied from Sigma Aldrich (Korea).

5.3.2 Preparation of anoxic beads

Anoxic beads were prepared according to the method obtained and modified from Kim et al. (2013), and Hashimoto and Furukawa (1987). 100 ml PVA (2% w/v) and 1000 ml boric acid- CaCl_2 (7% w/v boric acid and 4% w/v CaCl_2) solutions were prepared and autoclaved by using steam sterilizer. Activated sludge to be immobilized into the beads were taken from the anoxic tank of a pilot-scale MBR plant (Seoul, Korea) which is used for domestic wastewater treatment. After activated sludge was centrifuged for 15 min (12000 rpm), it was added to the PVA solution after it was re-suspended by using 10 ml of sterilized distilled water. This PVA-bacteria mixture was dropped by using a syringe needle into boric acid- CaCl_2 solution that is placed on a magnetic stirrer. These beads were stored at 4°C temperature for 8 hours. At the end of 8 h, beads were stored in distilled water at room temperature until MBR operation. The bacterial content of PVA beads 20 mg anoxic bacteria bacteria/ml PVA solution.

5.3.3 Preparation of quorum quenching beads

QQ beads were also prepared according to the same method used for anoxic bead production. 100 ml PVA (2% w/v) and 1000 ml boric acid- CaCl_2 (7% w/v boric acid

and 4% w/v CaCl_2) solutions were prepared and sterilized. *Rhodococcus sp.* BH4 (KCTC 33122), QQ bacteria for this study, cultured on LB broth and incubated at 30°C temperature for 24 hours (Kim et al., 2013; Oh et al., 2012). After cultured *Rhodococcus sp.* BH4 bacteria solution was centrifuged for 15 min (12000 rpm), it was mixed with PVA solution after re-suspension with 10 ml of sterilized distilled water. This PVA-bacteria mixture was dripped via a syringe needle into boric acid- CaCl_2 solution and beads in the solution were stirred. After these beads were stored at 4°C temperature for 8 hours, they were stored in distilled water at room temperature until MBR operation. The anoxic bacterial content of PVA beads 20 mg anoxic bacteria/ml PVA solution. A symbolic drawing of anoxic and quorum quenching beads was given in Figure 5.2.

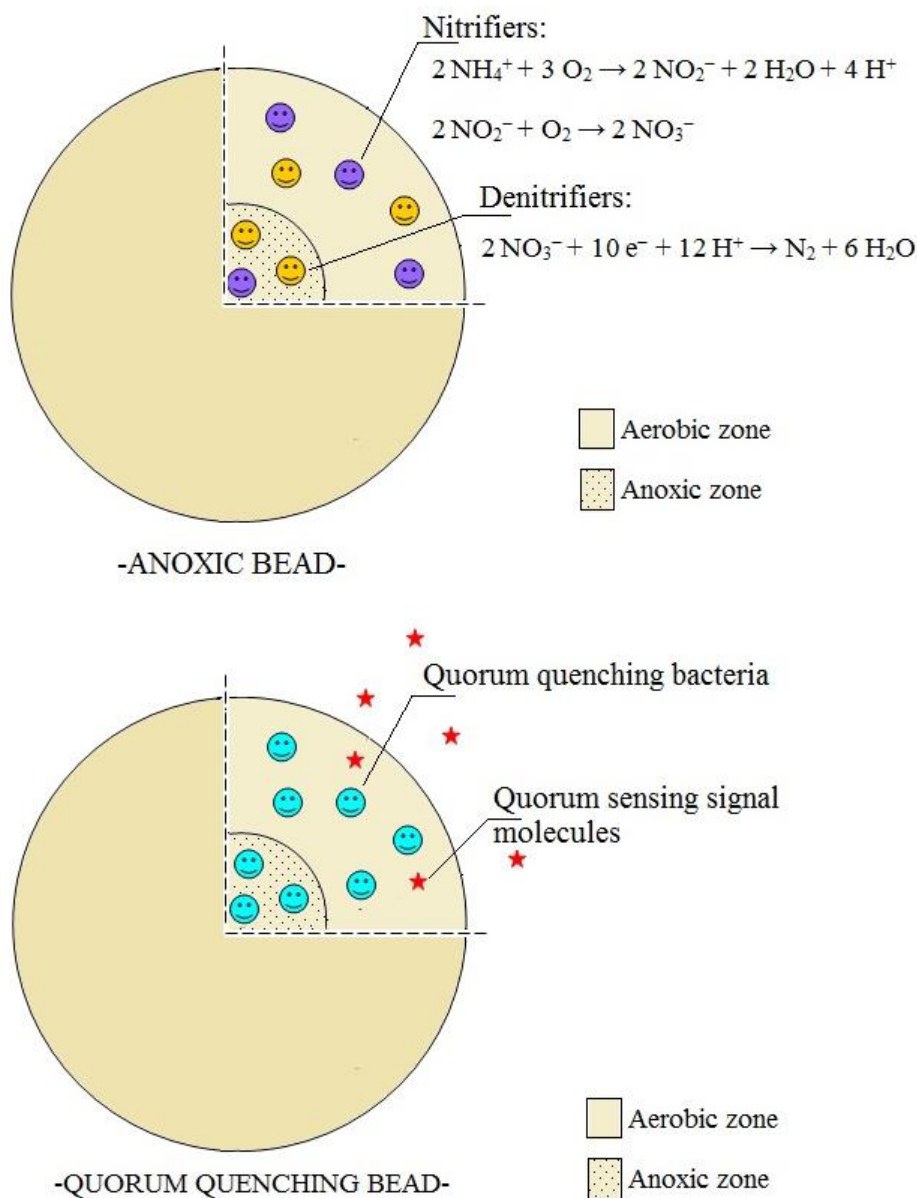


Figure 5.2 : Symbolic cross-section of anoxic and QQ beads.

5.3.4 Quorum quenching activity test

N-acyl homoserine lactone (AHL) signal molecules were detected by using *Agrobacterium tumefaciens* A136 (Ti⁻)(pCF218)(pCF372) (Kawaguchi et al., 2008; Yeon et al., 2009; Oh et al., 2013) as a biosensor strain. *A. tumefaciens* A136 was cultured on LB medium containing spectinomycin (50 mg/L) and tetracycline (4.5 mg/L) to get two plasmids, which can provide the AHL response system. This incubated A136 was used for bioassay on quorum quenching activity determination. QQ activities of free cells (anoxic sludge and BH4), and immobilized cells in anoxic and QQ beads were measured. The AHL levels were quantitatively determined using

a bioassay with the luminescence substrate Beto-Glo (Promega, USA) (Kawaguchi et al., 2008). 5µl of AHL samples were loaded into a 96-well-plate with 95µl of an overnight incubated A136. After incubation for 90 min at 30°C temperature, 30µl of Beta-Glo were added into each well. As a result of chain reaction of C8-HSL, A136 and Beta-glo during a second incubation for 30 min at 30°C, the luminescence of xyluciferin was developed. The intensity of xyluciferin is directly proportional to the amount of beta-galactosidase released from the *A. tumefaciens* A136 biosensor. These intensities were recorded using a luminometer (Synergy 2, Bio-Tek, USA). A calibration curve was created by using intensities from the standard AHL solutions of C8-HSL. Each bioassay was conducted in triplicate to assess repeatability.

5.3.5 Batch study on denitrification rates

A batch study on determination of denitrification rates of anoxic bead in a NaNO_3 solution including 50 ppm NO_3^-) and in a synthetic domestic wastewater. The composition of the synthetic wastewater can be listed as follows (mg/L): glucose, 400; yeast extract, 14; bactopectone, 115; $(\text{NH}_4)_2\text{SO}_4$, 104.8; KH_2PO_4 , 21.75; MgSO_4 , 15.63; FeCl_3 , 0.075; CaCl_2 , 2.45; MnSO_4 , 1.8; and NaHCO_3 , 255.5. In the batch study, there two parallel reactors with 400 ml volume. They were operated under the anoxic and batch conditions with magnetic stirrers. While one reactor was operated with 100 vacant beads, the other one was operated with 100 anoxic beads. NO_3^- concentrations in NaNO_3 solution, and NO_3^- and COD concentrations in synthetic wastewater were routinely determined to calculate denitrification rates for both conditions. Batch reactors can be seen in Figure 5.3.

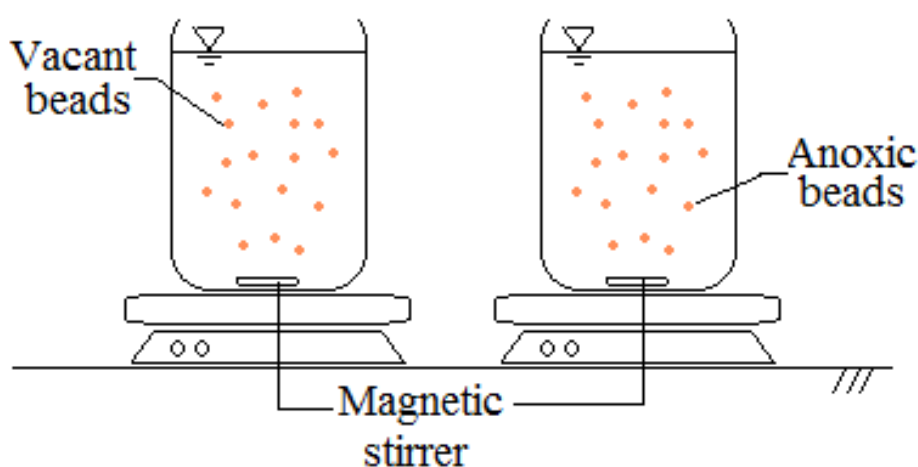


Figure 5. 3 : Symbolic cross-section of anoxic and QQ beads.

5.3.6 MBR operation

After QQ activity test, beads were added into the lab-scale MBRs, and used during operations. Within the content of quorum quenching MBR studies, two parallel MBRs were simultaneously operated under the same operation conditions. While one MBR named as control MBR was operated with vacant beads containing no QQ bacteria, the other one named as QQ MBR was operated with anoxic beads and QQ beads. In this study, MBRs were operated under the constant flux ($30 \text{ l/m}^2/\text{h}$ condition and the transmembrane pressure (TMP) was monitored to see biofouling level. Scheme of the MBR system was given in Figure 5.4. Activated sludge was supplied from Tancheon Wastewater Treatment Plant in Korea and it was acclimated to synthetic wastewater prior to the MBR operations. The Membrane module was prepared by using poly (vinylidene) fluoride hollow fiber microfiltration membrane supplied from GE-Zenon (ZeeWeed500, USA). Operating parameters and conditions of the lab-scale MBR were given in Table 5.1.

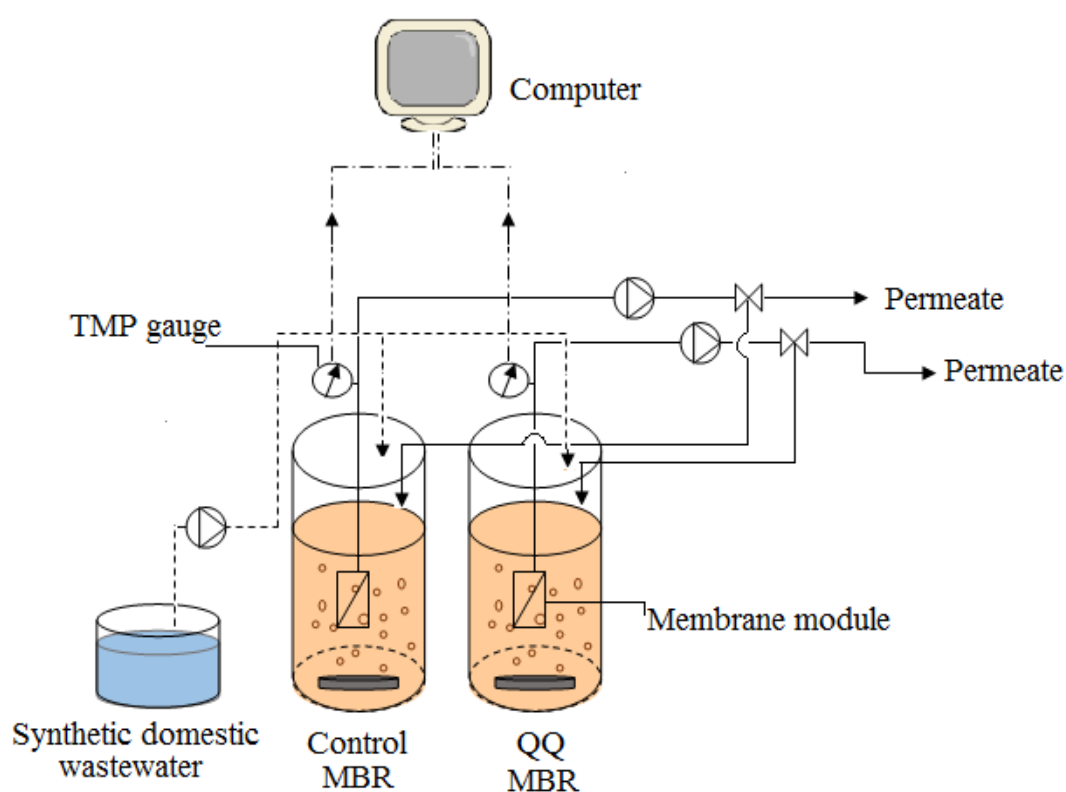


Figure 5.4 : Schematic diagram of the lab-scale MBR.

Table 5. 1 : Operation parameters and conditions of the lab-scale MBR.

Parameter	Unit	Value
Working volume	L	2.6
pH	-	6.8~7
Temperature	°C	25±1
SRT	d	30
HRT	h	5.4
COD removal efficiency	%	~ 95
Feed COD	mg/L	440
MLSS	mg/L	~10.000

5.3.7 Analytical methods

NO₃-N concentrations in the solution during batch study were determined by using ion chromatography (DIONEX, ASI4 column, Korea). MLSS, COD and TN were analyzed during continuous MBR operation. While MLSS concentrations of the mixed liquor were determined to check and control the parameter that are directly related with biofouling, COD and TN concentrations in feed and permeate flows were analyzed to check whether proper and efficient treatment processes in control MBR and QQ MBR were achieved or not. MLSS, COD and TN were determined according to Standard Methods (APHA, 2005). EPS contents in the bio-cakes, which are extracted using ultra-sonification, were determined at the end of the operation. Protein and carbohydrate analysis were carried with Lowry (Lowry et al., 1951) and Phenol sulfuric acid (Dubois et al., 1956) methods, respectively. Samples were measured with a UV spectrophotometer (Hach Lange, DR 500; 660 nm; 490 nm) by using standard solution prepared with BSA and glucose solutions. At the end of the operation, modules were taken out from the reactors and the 3D structure of biofilms was visually monitored with confocal laser scanning microscope (CLSM, C1 plus, Nikon, Japan). Biofilms were fluorescently stained with SYTO9 according to the instructions from the suppliers (Molecular Probes, Eugene, OR). Z-section image stacks (5µm) of each sample were reconstructed using IMARIS software (Bitplane AG, Switzerland).

5.4 Results and Discussion

5.4.1 QQ activities of bacteria and beads

QQ activities of pellets from incubated *Rhodococcus sp.* BH4 and anoxic activated sludge taken from treatment plant were determined before bead productions. *Escherichia coli* was preferred as a control sample for this bio-assay because it is not

a QQ bacteria specie. AHL degradation rate results of the three samples were given in Figure 5.5. While anoxic activated sludge did not show any QQ activity, BH4 could degrade more than 94% of C8-HSL in 60 min. After QQ activity determination with free cells, PVA beads were manufactured using prepared pellets. PVA beads can be seen in Figure 5.6.

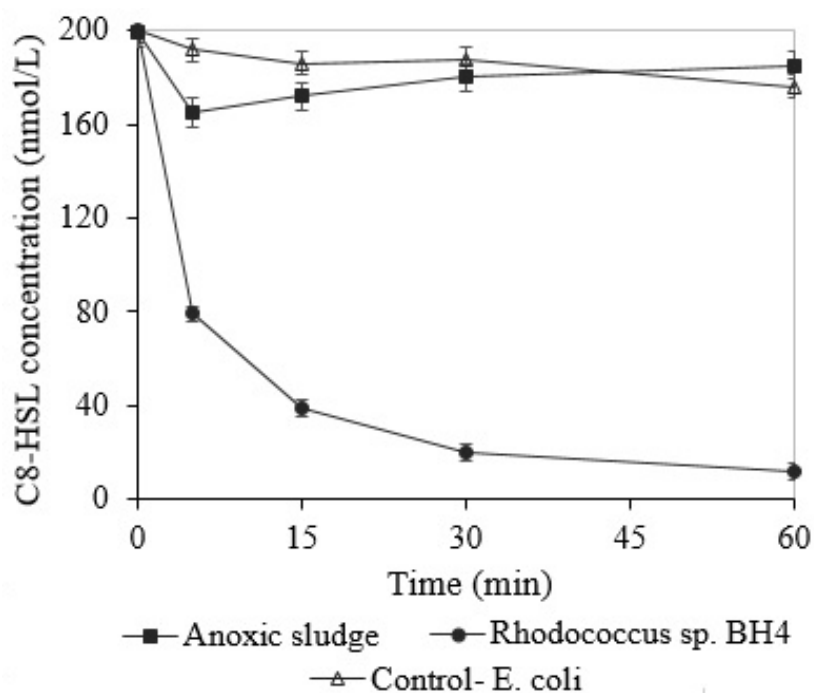


Figure 5.5 : QQ activities of free microorganisms from activated sludge and *Rhodococcus sp.* BH4.

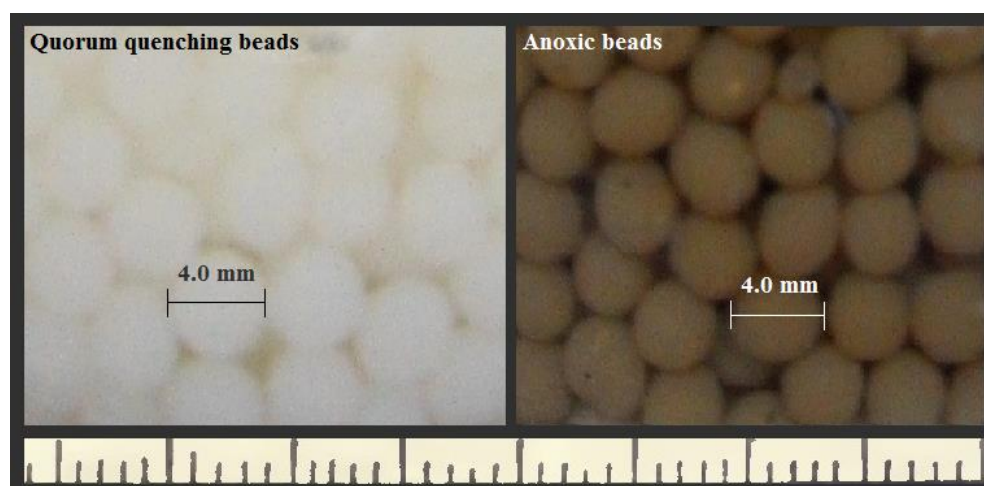


Figure 5.6 : Photographs of QQ and anoxic bead with a scale (left: QQ beads, right: anoxic beads).

As seen in the figure, both beads could be manufactured with the same diameter. While anoxic beads which was named as anoxic beads because of its including were brown,

QQ beads were opaque and white-salmon. Vacant beads that have no images in this manuscript were more transparent and white. QQ activities of three different beads (vacant, anoxic and QQ beads) were determined using bio-assay with a standard AHL solution. The aim of this determination was to prove that a possible TMP increase prevention was not resulted by anoxic beads. Figure 5.7 shows the results of AHL degradation rates of the three beads. According to the results in Figure 5.7, it can be said that control and anoxic beads had a small amount decrease of AHL concentrations via adsorption and QQ beads could degrade AHL with 90% efficiency.

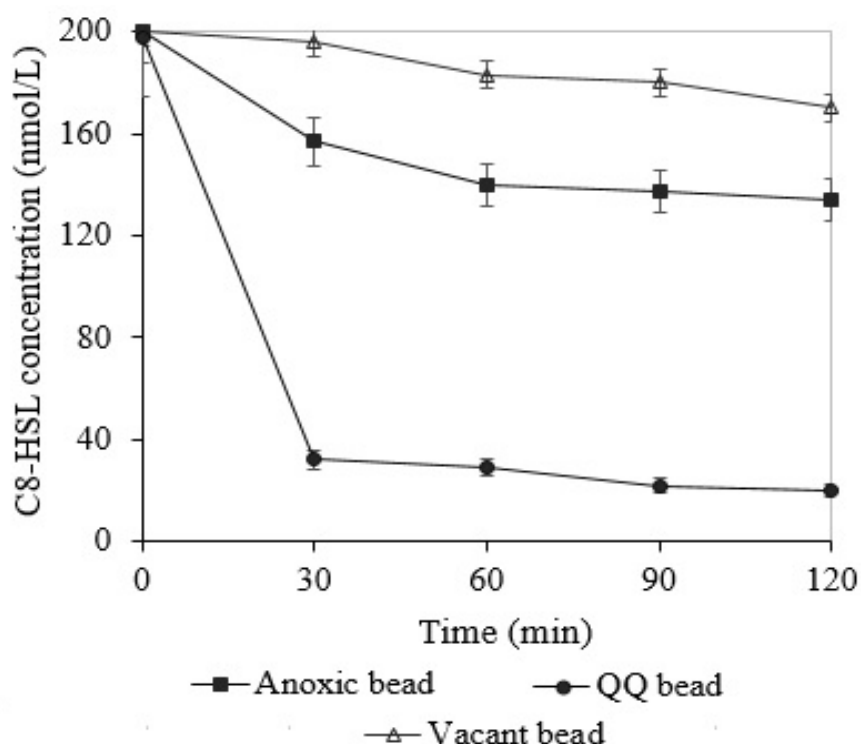


Figure 5. 7 : QQ activities of immobilized anoxic sludge and *Rhodococcus sp.* BH4.

5.4.2 Denitrification rates

After the anoxic bead production, it was aimed to determine denitrification rates of anoxic beads with batch tests during 3 hours. Within this regard, $\text{NO}_3\text{-N}$ and COD removals in synthetic wastewater and $\text{NO}_3\text{-N}$ removals in standard NaNO_3 were determined. The results of these studies were presented in Figure 5.8.

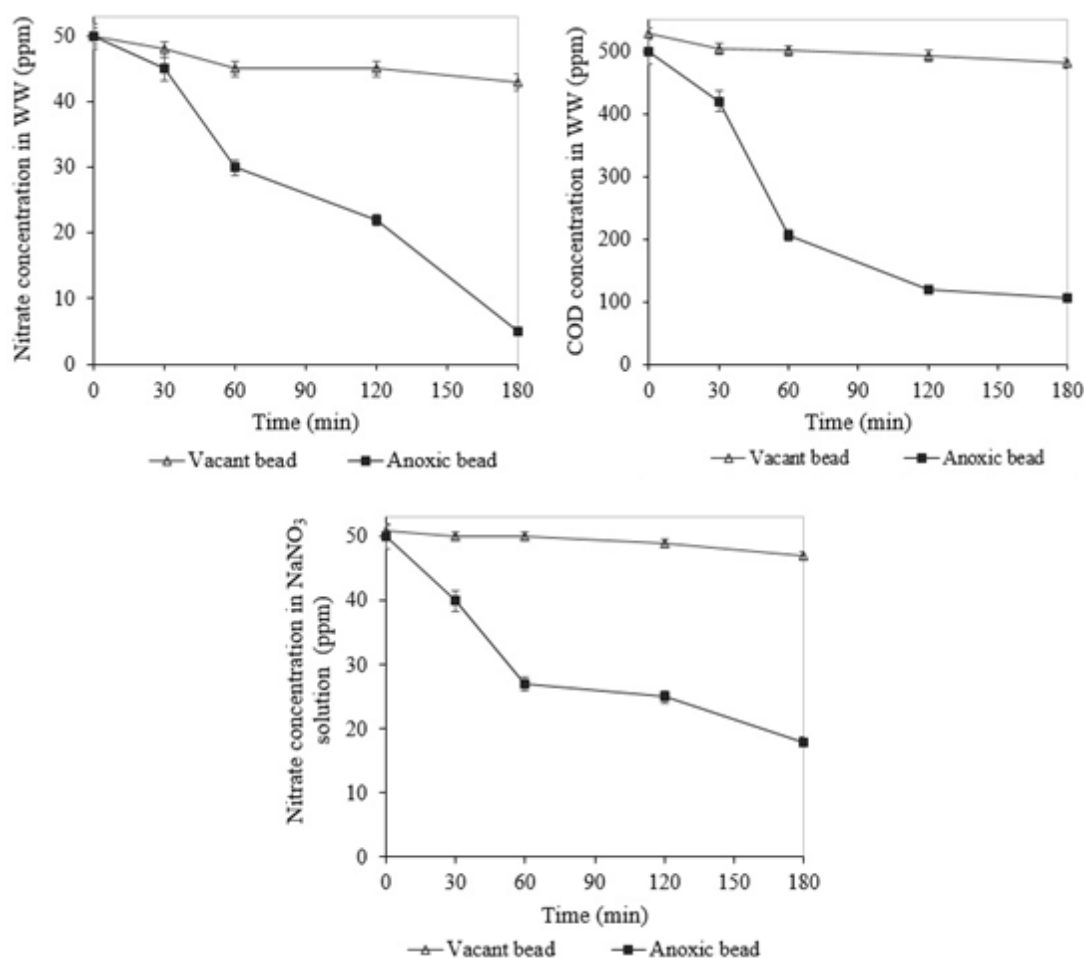


Figure 5.8 : Batch study results on nitrate and COD removals.

According to the results of the study carried with synthetic wastewater; it can be seen that while vacant beads did not remove any organic matter in the environment, anoxic beads could consume most of the nitrate and carbonaceous organic matters in 180 min. It may be said that denitrification bacteria in anoxic beads could maintain their life cycles and denitrification process. The denitrification rate in synthetic wastewater was found out as 0.067 mg nitrate/mg MLVSS.h when the ratio between the anoxic volume to the total volume of each bead was taken into the consideration. This denitrification rate fits to the optimum range about denitrification rates for domestic wastewater treatment (Liwarska-Bizukojc, 2012). The denitrification rate in standard was calculated as 0.048 mg nitrate/mg MLVSS.h and this is lower than the denitrification rate in the synthetic wastewater. The difference between the rates may be explained with the faster reactions by means of the other including of the synthetic wastewater.

5.4.3 Biofouling inhibition by QQ beads in a continuous MBR

After bio-assay and batch study, it was aimed to see the high AHL degradation capacity of QQ beads and the possible extra addition to nitrogen removal efficiencies of anoxic beads in a continuous MBR operation. While Control MBR was operated with 200 ml vacant beads, QQ MBR was operated with 100 ml anoxic beads and 100 ml QQ beads. Two parallel MBRs were operated under the same flux conditions during 5 days. TMP profiles obtained from Control MBR and QQ MBR are given in Figure 5.9. Moreover, the appearances of the membranes of Control MBR and QQ MBR can be seen in Figure 5.10.

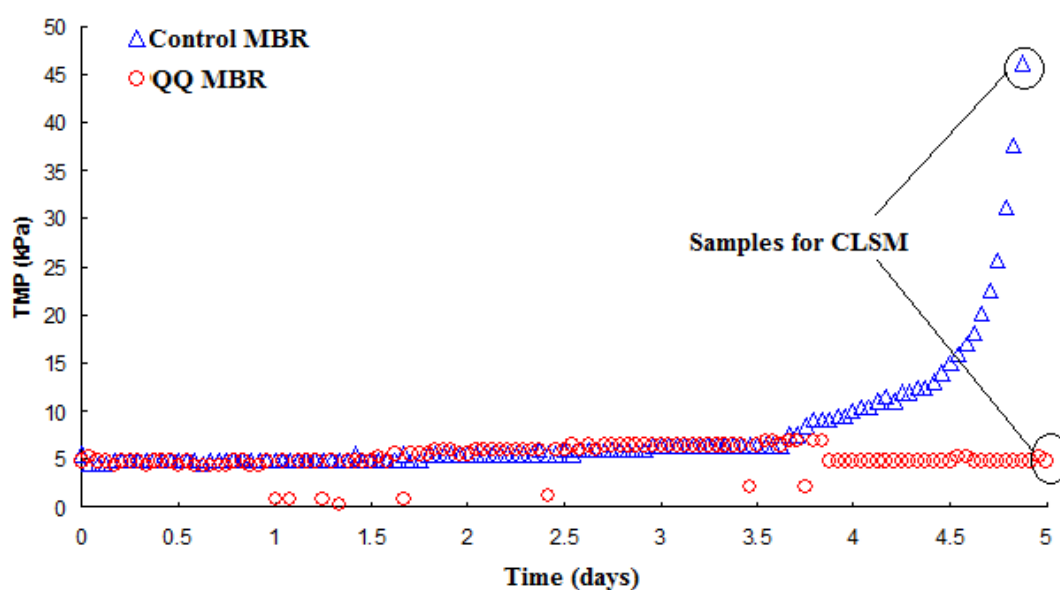


Figure 5.9 : TMP profiles obtained from Control MBR and QQ MBR operations.



Figure 5. 10 : The appearances of the membranes of Control MBR and QQ MBR.

As seen in Figure 5.9, TMP values had started to increase rapidly at the end of the 3.5th operation day and membrane was totally fouled on 5th operation day because of biofilm creation. After membrane in the Control MBR was fouled, the operation was ended. During these 5 days, the TMP increase in QQ MBR was achieved to be prevented via quorum quenching beads. QQ mechanism efficiency, biofouling prevention efficiency, was found out as 94% by calculating the difference between the areas under the TMP graphs. Sampling time for confocal analysis was shown in Figure 5.9. These samples were examined with confocal microscopy and images were given in Figure 5.11. At the end of the operation, bio-cake layers on the membrane surfaces were extracted and EPS contents were determined (Figure 5.12).

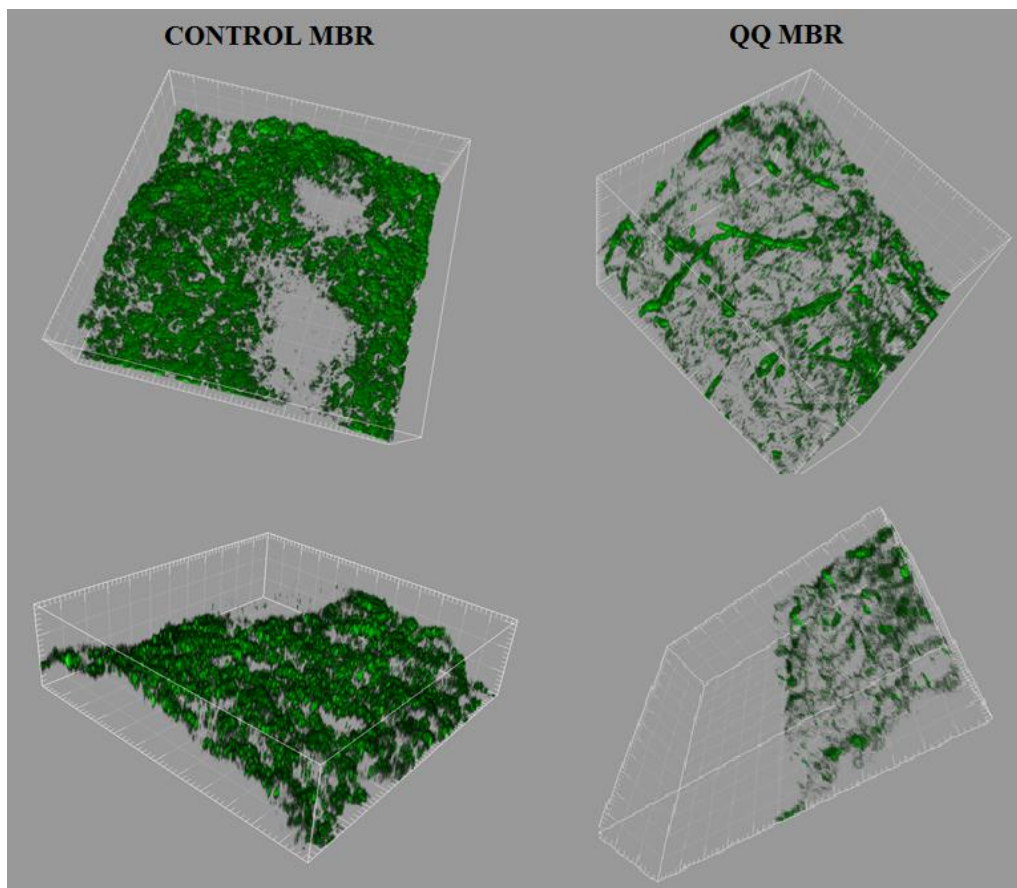


Figure 5.11 : CLSM image of the biofilm layers on the membrane surfaces (x: 1213 μm , y: 1213 μm). Note that no colors appear in membrane layer because the fluorescence dyes were adsorbed onto the membrane layer during the staining step.

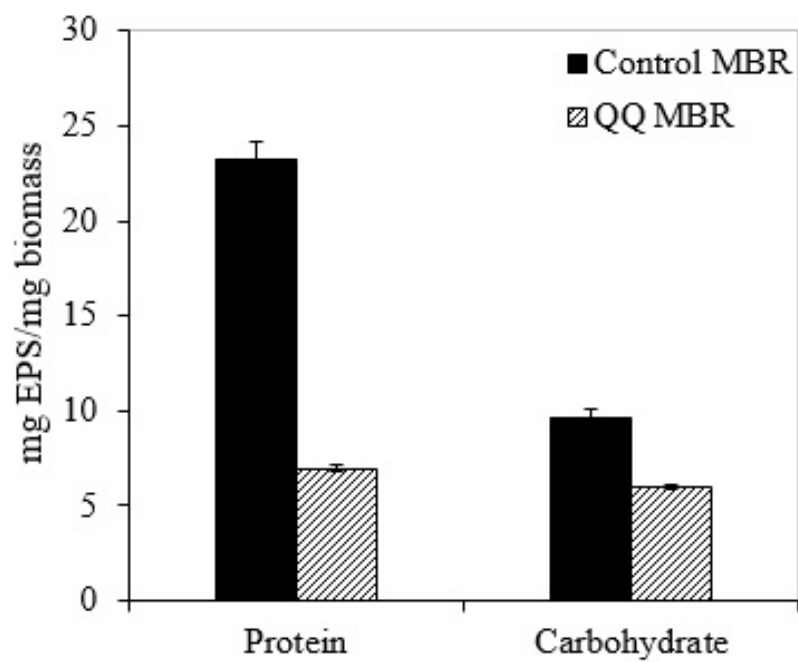


Figure 5.12 : EPS contents in the bio-cakes on the membrane surfaces.

Confocal images went along with TMP results, and while there is a thick biofilm layer on the fiber membrane of Control MBR, the biofilm layer on the fiber membrane from QQ MBR was rare and thinner. Biofouling could be successfully prevented by using QQ beads during operation. These results coincide with the results of previous studies (Kim et al., 2013; Kim et al., 2014). Moreover, during the operation 70% and 38% decrease could be achieved in the protein and carbohydrate content of the attached biomass, respectively. EPS secretion by microorganisms that were attached to the membrane surfaces was prevented by quorum quenching activity, and especially protein secretion, which is mentioned in the literature as the most difficult one to be prevented, could be suppressed in this study. The different point of this study was to use QQ beads and anoxic bead together, and it was seen that anoxic beads had no any effect on the quorum quenching mechanism during a continuous MBR operation.

5.4.4 Effect of anoxic beads on the MBR performance about removal of organics

The other one of two main aims of one-step MBR study was to enhance the organic matter removal, especially nitrogen removal, via anoxic beads while aeration was continuously realized during the whole operation. In order to see the differences, COD and TN concentrations in feed and permeate of Control MBR and QQ MBR were determined daily. The removal efficiencies obtained from Control MBR and QQ MBR were given in Figure 5.13. As known from previous studies (Kim et al., 2013), QQ beads had no any effect on the removal of organics. Because of this reason, it can be said that in this study anoxic beads resulted in 7% and 21% increase on COD and TN removal efficiencies, respectively.

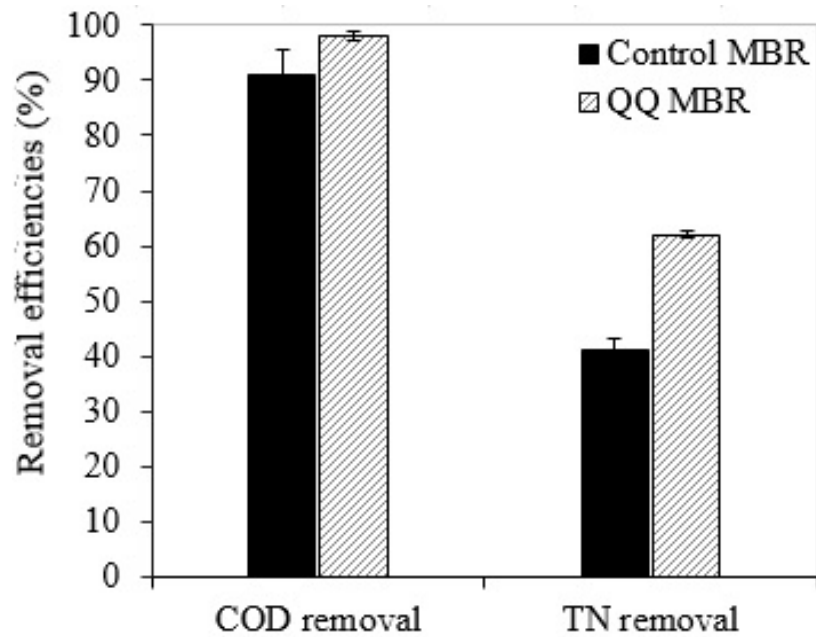


Figure 5.13 : COD and TN removal efficiencies obtained from Control MBR and QQ MBR during the operation.

5.5 Conclusion

To summarize, an unprecedented combination of modification of the MBR operation was studied. The aim was to see the results of a study, which combined QQ mechanism and enhanced nitrogen removal in a joint biological reactor by using PVA beads with target immobilized microorganisms. Biofouling could be prevented successfully via QQ bead usage during MBR operation. In addition to this, organic matter removal could also be enhanced via special beads performed as a mixed culture of denitrification and nitrification bacteria. Within this regard, it could be claimed that this study suggested a new idea that will be beneficial for pilot- and real-scale MBR applications for residential areas for which area need and discharge limits are important, and put that idea into effect.

6. EFFECT OF BACTERIAL GROWTH PHASE ON QUORUM QUENCHING ACTIVITY OF *RHODOCOCCUS SP. BH4*

6.1 Purpose & Scope

In this study, it was tried to give a new point of view to the quorum quenching MBR applications. There are two dead heads of quorum quenching MBR applications: 1) a suitable and comfortable immobilization medium for bacteria, but then easily dispersible medium; or 2) Durable and inert immobilization medium, but then unsuitable medium for bacterial growth and low F:M conditions. The decrease of quorum quenching activity because of the aging and/or death of immobilized quorum quenching bacteria can be a problem especially during long-term operations. It is possible to maintain the quorum quenching activity with bacteria removal and regeneration. Within this regard, the growth kinetic of *Rhodococcus sp. BH4* was revealed and the net growth rate was determined in order to decide the bacteria removal percentage. The regeneration time was calculated as 28.3 days. Moreover, it was found out that bacteria regeneration had a positive effect on the quorum quenching activity. While un-regenerated group lost 10% of their quorum quenching activities in four days, regenerated group could maintain their quorum quenching activities.

6.2 Introduction

The biggest problem of membrane bioreactors that are an important option for advanced wastewater treatment is biofouling. Because of biofouling, the unit flowrate of treated water decreases and the unit cost of wastewater treatment increases. Within this regard, various scientific studies have been carried in order to prevent the biofouling. The idea of biological solution for this biological problem was created after seeing the high costs and unstability of the studies based on chemistry. It was mentioned and proven that bacteria can communicate and they are more complex creatures than thought. The quorum quenching mechanism for inhibition of biofilm creation, which is caused by quorum sensing, was adapted to environmental

engineering, and quorum quenching MBR studies have been carried. As seen in the literature review, biofouling prevention has been achieved in many studies carried since 2009 until today.

It is a well-known fact that the growth of the bacteria occurs in some continuous phases. There are four growth phases: 1) Lag phase, 2) Log (exponential growth) phase, 3) stationary phase and 4) decay (death) phase. Bacteria acclimate in the lag phase. While the number of bacteria does not change in the lag phase, the size of them change. They start to reproduce rapidly in the log phase. After the log phase, bacteria come to the stationary phase in which these considerable reproduced bacteria encounter with the limited food. They maintain their lives in an equilibrium for a while, and the number of bacteria does not change in this phase because the rate of growth and death are equal. After that, the final phase, death phase, comes. In this phase, food is not enough for bacteria and the number of bacteria starts to decrease because of the decay (Amrita University, 2015). These growth phases can be determined using the growth graphic created with routinely measured optical densities of incubated culture. While the time needed for the passing from acclimation term to growth phase can be stated using minutes, this time can be expressed with hours or even with days. These periodic intervals in bacterial lifetime are important for the variety and rate of their actions. They can wait to come some special phases for the activation of some actions or the rate of the actions can depend on the phases even if this action occurs during the whole lifetime of bacteria. Biofilm formation, resistance to the antibiotics, quorum sensing, quorum quenching and virulence can be given as examples of these bacterial actions (Mercier et al., 2002; Gad et al., 2004; Lee & Falkow, 1990; Szostkova et al., 1999; Cherchi & Gu, 2011; Maalej et al., 2004). Bacteria can realize the quorum sensing and quorum quenching in high levels during their exponential growth phases in which bacterial vital activities are at the highest point (Wagner et al., 2002). Within this regard, it was thought that the high and sustained quorum quenching activity of a culture can be achieved by keeping this culture always in its log phase.

Within the scope of the thesis, the achievement of the RMCF about biofilm prevention was revealed with experimental study results. The main point of the RMCF design was an inert and undispersable material/structure, and the opportunity of immobilized bacteria renewal by using possible discharge/filling lines during pilot- or full-scale MBR plant operation. The growth rate (μ) and the decay rate (k_d) of *Rhodococcus sp.*

BH4 in the MBR conditions can be used to calculate net growth rate and the sludge age. The excess bacteria can be removed according to the sludge age, and bacteria can be kept in exponential growth phase. Within this regard, the sludge age concept was adapted to the immobilized bacteria solution into the RMCF, and a study was planned with the idea of daily bacteria removal from the cubbyholes of RMCF. Batch and continuous studies were carried for growth kinetic and QQ activity determination. After that, MBR operation was realized in order to see the effect of immobilized bacteria removal from the immobilization media.

6.3 Material and Method

6.3.1 Reagents

Spectinomycin was supplied as a powder (Sigma Aldrich, USA), and the stock solution was stored at 4°C, in the dark. Tetracycline was also supplied as powder form (Sigma Aldrich, USA) and stock solution was stored at -20°C. Commercial C8-HSL from Cayman (USA) was preferred as representative chemical for signal molecules and the stock solution was stored at -20°C. X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) was supplied from Sigma Aldrich with its powder form and X-Gal solution was stored at -20°C and in dark after prepared by using dimethylformamide (DMF) which is bought from Merck (Germany). Microfiltration membrane was a polyvinylidene fluoride (PVDF) membrane (Microdyn Nadir GmbH, Germany), and had a nominal pore size of 0.20 μ m and thickness of 210–250 μ m.

6.3.2 Bacterial strains and growth conditions

Agrobacterium tumefaciens A136 (Ti⁻)(pCF218)(pCF372) was used for detection of N-acyl homoserine lactone (AHL) signal molecules as a biosensor strain (Yeon et al., 2009a; Kawaguchi et al., 2008; Oh et al., 2013). *A. tumefaciens* A136 was cultured on Luria Bertani (LB) medium containing spectinomycin (50 mg/L) and tetracycline (4.5 mg/L) to maintain the two plasmids that provide the AHL response system. *Rhodococcus sp.* BH4, isolated from a real MBR plant by Oh et al. (2012), was used as a QQ bacterium and cultured on LB broth and incubated in a 30°C rotary shaker (160 rpm) for 24 hours.

6.3.3 Batch study on mass flux

The mass flux of microfiltration membrane that is used for covering the RMCF surface was determined. Within this regard, an experimental setup was prepared. This experimental setup included a beaker, in which synthetic wastewater having 150 mg/L COD was, and a pocket from a MF membrane in which distilled water was (Figure 6.1). The composition of the synthetic wastewater was as follows (mg/L): glucose, 133; yeast extract, 4.67; bactopectone, 38.3; $(\text{NH}_4)_2\text{SO}_4$, 34.9; KH_2PO_4 , 7.25; MgSO_4 , 5.21; FeCl_3 , 0.025; CaCl_2 , 0.817; MnSO_4 , 0.6; and NaHCO_3 , 85.17. Osmotic pressure and total organic matter (TOC) concentration in the synthetic wastewater (outside environment) and in the distilled water (inside environment) were routinely determined. Sampling was continued until the osmotic pressures on the outside and on the inside were the same.

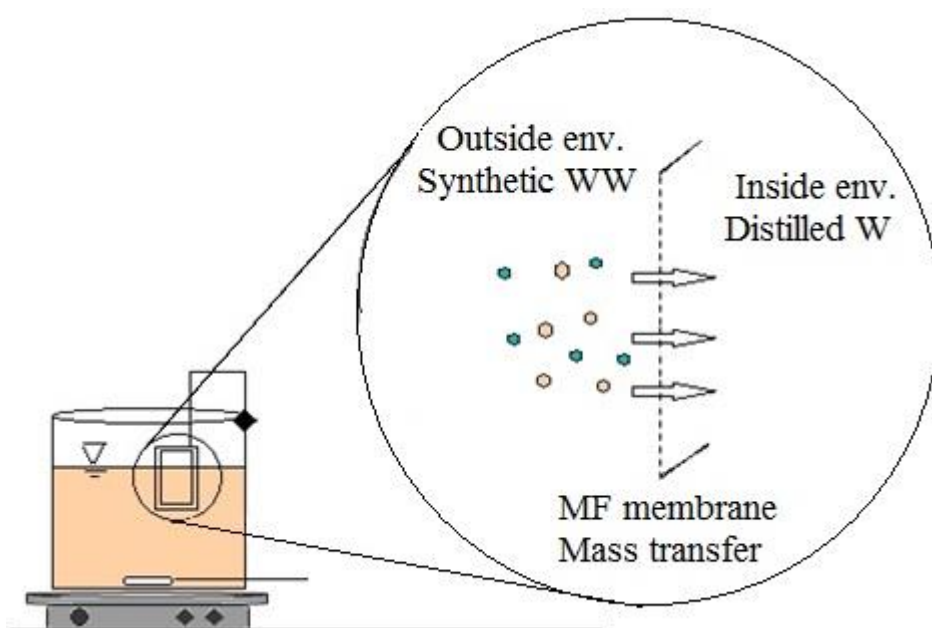


Figure 6.1 : Experimental setup for mass flux determination (WW: wastewater, W: water, env.: environment).

6.3.4 Batch study on growth kinetic

After the mass flux was determined, growth kinetic of *Rhodococcus* sp. BH4 in the reactor conditions was tried to be revealed by using a batch study. With this aim, a batch experimental setup was prepared. Inoculated BH4 bacteria were centrifuged and BH4 with the same amount as RMCF studies added into the synthetic wastewater. The composition of the synthetic wastewater was as follows (mg/L): glucose, 133; yeast

extract, 4.67; bactopectone, 38.3; (NH₄)₂SO₄, 34.9; KH₂PO₄, 7.25; MgSO₄, 5.21; FeCl₃, 0.025; CaCl₂, 0.817; MnSO₄, 0.6; and NaHCO₃, 85.17. During the incubation of BH4 in this synthetic wastewater, the optical density of the bacterial solution was routinely measured by using a UV spectrophotometer. Moreover, TOC concentrations were also measured in order to see the rate of organic matter removal by the microorganisms. According to the results of these measurements, μ and k_d coefficients were found out (Equation 6.1 and Equation 6.2) and sludge retention rate (Equation 6.3) was calculated. μ and k_d coefficients were found out by using the slope of the exponential growth and exponential decay phases, respectively (Orhon & Artan, 1997).

$$\mu = [\ln(OD_t) - \ln(OD_0)] / \Delta t \quad (6.1)$$

$$k_d = -[\ln(OD_t) - \ln(OD_0)] / \Delta t \quad (6.2)$$

$$1/\theta_x = \mu - k_d \quad (6.3)$$

6.3.5 Continuous study on quorum quenching activities

In the light of idea about the positive effect of bacteria regeneration on the quorum quenching activity, an experimental study was realized with continuous feed. Sterilized synthetic wastewater, which is the same as the used one in the growth kinetic study, was used as inoculation media, and COD concentration was kept constant and at 150 mg/L according to the previously determined organic matter removal rates. 10 mg BH4 were inoculated during 4 days. This study was carried with two groups: 1) control group and 2) regenerated group. While control group was inoculated without any interception, a determined volume of regenerated group was removed from the flask according the sludge retention time calculations. This experiment was carried under the aseptic conditions. Experimental setup can be seen in Figure 6.2. QQ activities of these two groups were daily determined by adding 200 nM C8-HSL and taking samples at 0., 10. and 30. minutes. AHLs were detected using the indicating agar plate method adopted from Park et al. (2001). Indicating agar plate was prepared by mixing (ratio of 9:1) LB-agar and an overnight culture of *A. tumefaciens* A136 including spectinomycin (50 mg/L), tetracyclin (4.5 mg/L), and X-gal (0.2 g/L). AHL degradation by *Rhodococcus* sp. BH4 was occurred in the reaction tube with the initial concentration of C8-HSL with 200 nM. The reaction mixtures were loaded into the

well of plates overlaid with *A. tumefaciens* A136. The residual amounts of AHL were calculated using the relationship equations based on the color zone size and known amounts of AHLs.

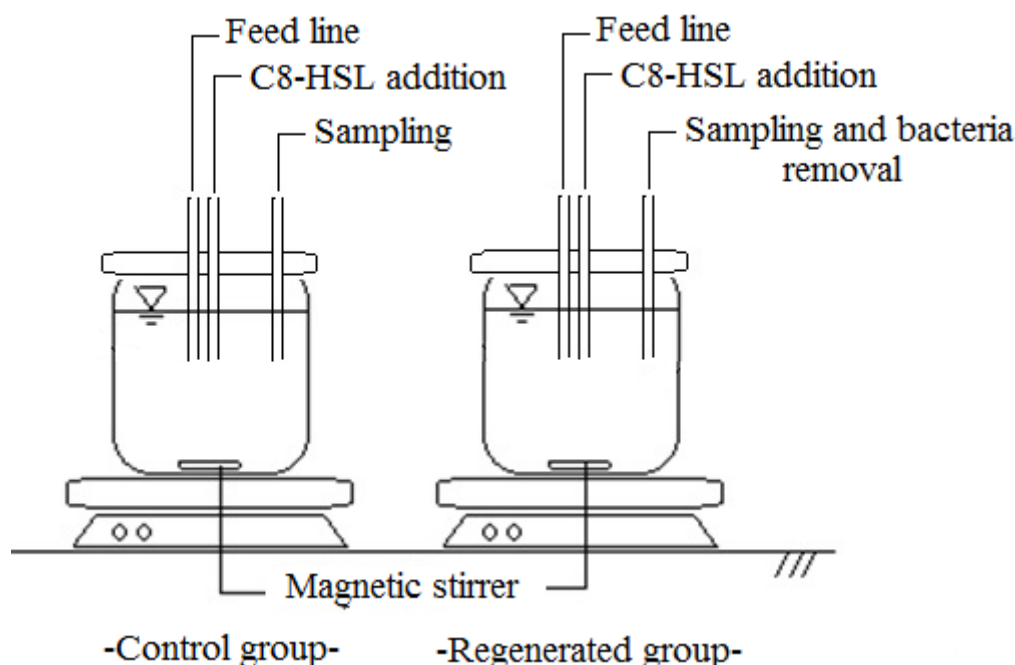


Figure 6.2 : Experimental setup.

6.3.6 MBR operation

Two parallel MBRs were operated under the same operating conditions as MBR-A and MBR-B. While MBR-A was operated with RMCF, MBR-B was a parallel operated MBR with RMCF having regeneration lines. Scheme of the MBR system was depicted in Figure 6.3. Activated sludge was taken from an advanced biological wastewater treatment plant in Istanbul, Turkey, and prior to the MBR operations, it was acclimated to synthetic wastewater. The composition of the synthetic wastewater was as follows (mg/L): glucose, 400; yeast extract, 14; bactopectone, 115; $(\text{NH}_4)_2\text{SO}_4$, 104.8; KH_2PO_4 , 21.75; MgSO_4 , 15.63; FeCl_3 , 0.075; CaCl_2 , 2.45; MnSO_4 , 1.8; and NaHCO_3 , 255.5. MBRs were connected to computer and controlled by an automation program according to target ranges of operating parameters like water levels, pH, temperature, oxidation-reduction potential (ORP) values and dissolved oxygen concentrations (Table 6.1). MBR-A and MBR-B were operated under the constant flux condition by using microfiltration system consisting of hollow fiber membrane module. The effective area of the hollow fiber membrane module was around 100 cm^2 . The hydraulic retention time (HRT) and sludge retention time (SRT) were 13 h and 30

d, respectively. The mixed liquor suspended solid (MLSS) concentrations in MBRs were maintained within the range of 11000-12000 mg/L.

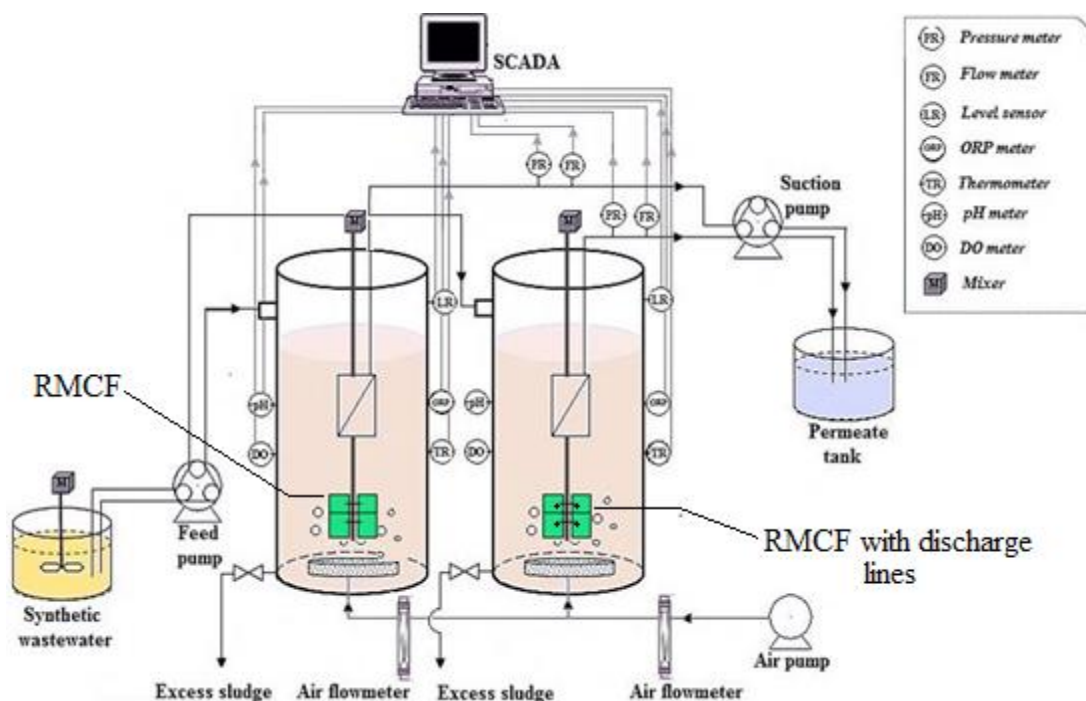


Figure 6.3 : Schematic description of the MBR system.

Table 6. 1 : Operation parameters and conditions of the MBR.

Parameter	Value/Information
Working volume (L)	5
pH	6.8~7
Dissolved oxygen (mg/L)	4.2-6.5
ORP (mV)	170-200
Temperature (°C)	20±2
SRT (d)	30
HRT (h)	13
Membrane type:	Philos MegaFluxI
Membrane area (m ²)	0.01
Flux (LMH)	60
COD removal efficiency (%)	~ 95
MLSS (mg/L)	12,000
Feed COD (mg/L)	440
Seeding sludge	Paşaköy WWTP

6.3.7 Analytical methods

MLSS and COD were determined according to Standard Methods (APHA, 2005). TOC concentrations were measured using TOC instrument (Shimadzu, Japan).

Osmotic pressures were determined with a 3250 model instrument of Advanced Instruments Inc. (USA).

6.4 Results and Discussion

6.4.1 Mass flux

Firstly, the mass flux of the microfiltration membrane that is used for coverage of the RMCF and in which *Rhodococcus sp.* BH4 is immobilized was tried to be determined. The change of the osmotic pressures of the inside and outside environments in time can be seen in Figure 6.4. In addition to this, organic matter concentrations in these environments were determined and mass flux was calculated.

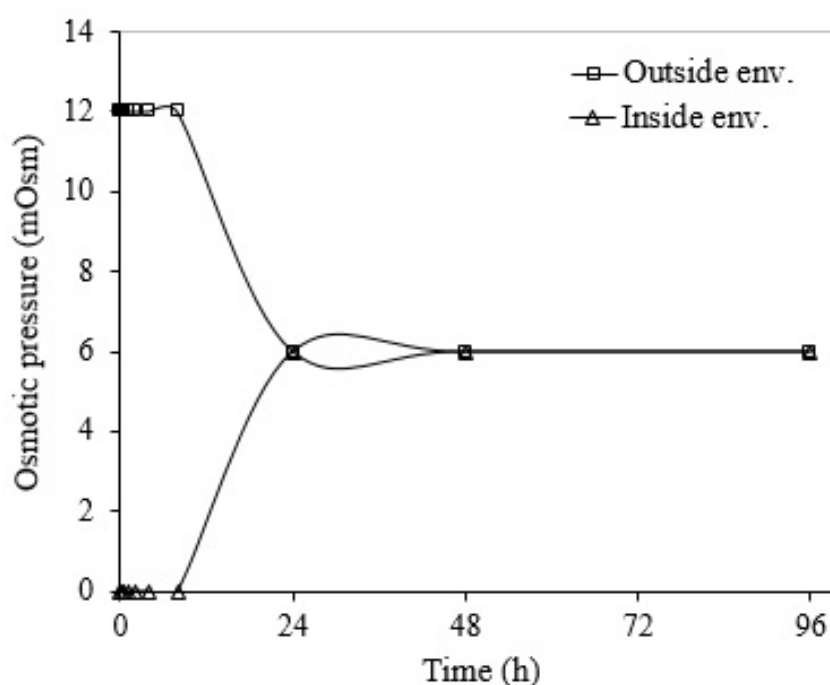


Figure 6.4 : Osmotic pressure profile (env.: environment).

At the end of 8th hour, microfiltration membrane totally soaked, and osmotic pressures had started to change. The osmotic pressures in two environments were equal at the end of 12th hour. Moreover, mass flux was calculated as $2.26 \text{ mg.m}^{-2}\text{h}^{-1}$.

6.4.2 Growth kinetics

After the mass flux was determined, a growth kinetic study was carried. The growth kinetic study was realized under the initiated reactor conditions, and the result of the

study was given in Figure 6.5. Growth phases of *Rhodococcus* sp. BH4 given in Figure 6.6. were determined by using data obtained from Figure 6.5.

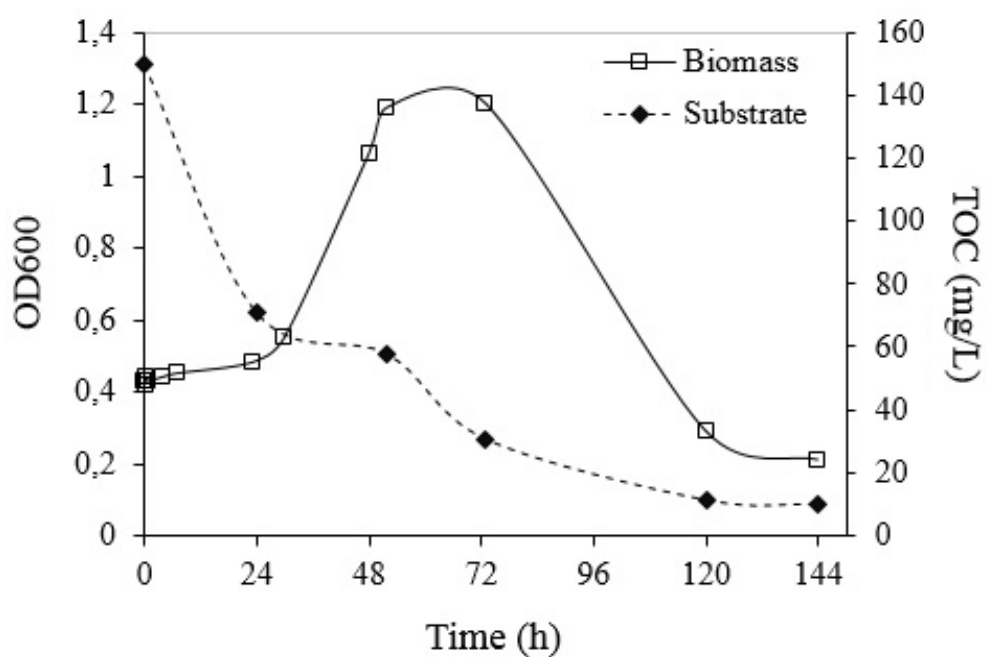


Figure 6.5 : Growth kinetic.

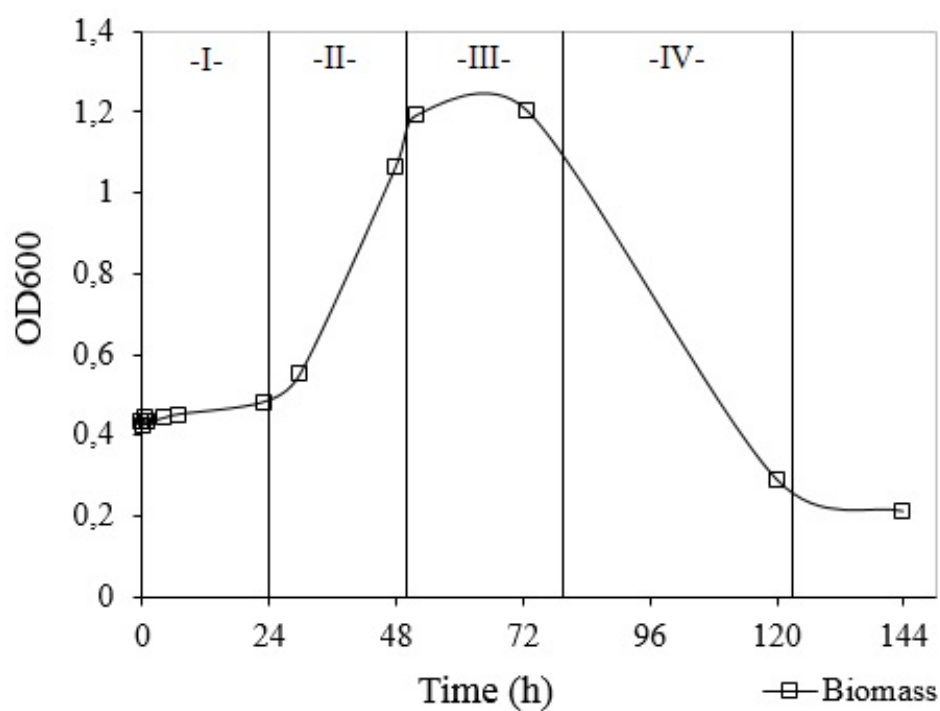


Figure 6.6 : Growth phases (I: lag phase, II: exponential growth phase, III: stationary phase, IV: exponential growth phase).

Equations 6.1, 6.2 and 6.3, and data from Figure 6.6 were used and sludge age of *Rhodococcus sp.* BH4 was determined as 28.3 days. This means that removal of 7% of the total bacteria amount may help the bacteria regeneration.

6.4.3 Effect of regeneration on quorum quenching activities

An experimental study with a control group was carried in order to check the possible effect of the bacterial regeneration on the quorum quenching activity. QQ activities of control and regenerated groups during the 4 days were daily determined. AHL degradations were given in Figure 6.7. Collective daily results were given in Figure 6.8.

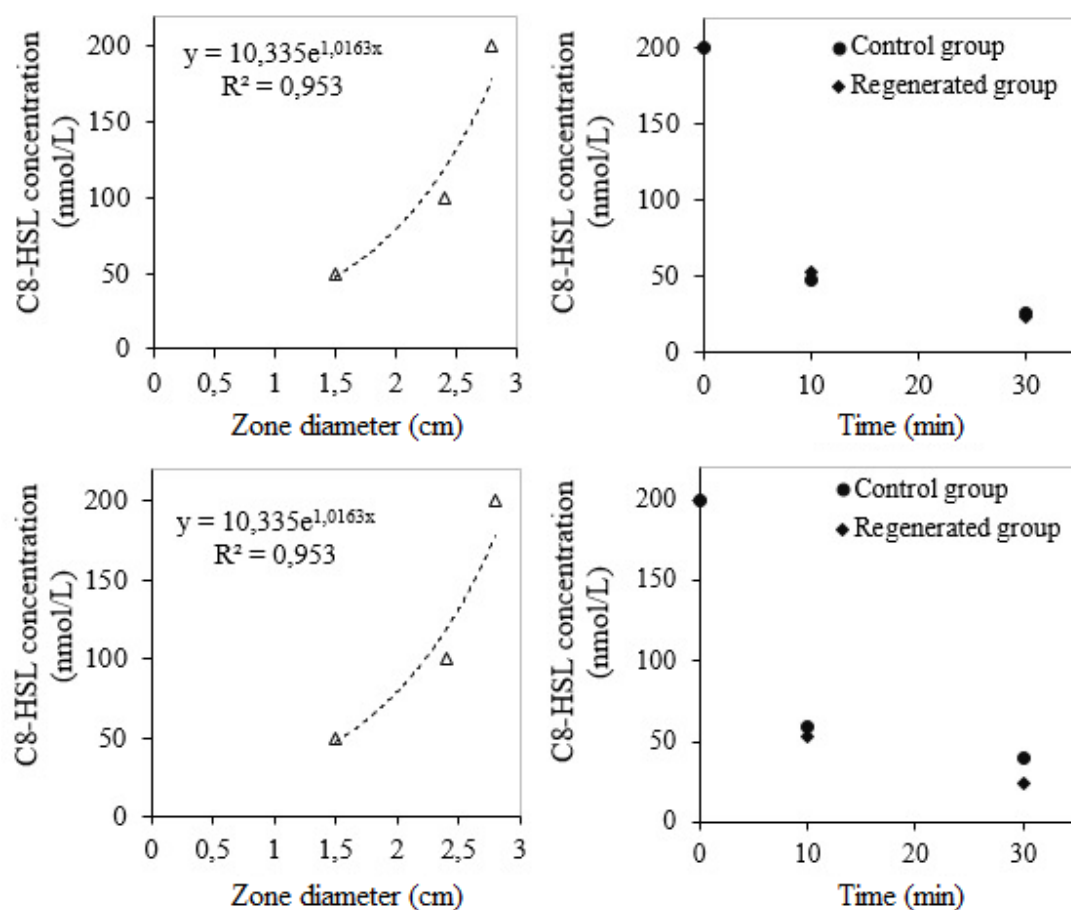


Figure 6.7 : QQ activities obtained from the regeneration study.

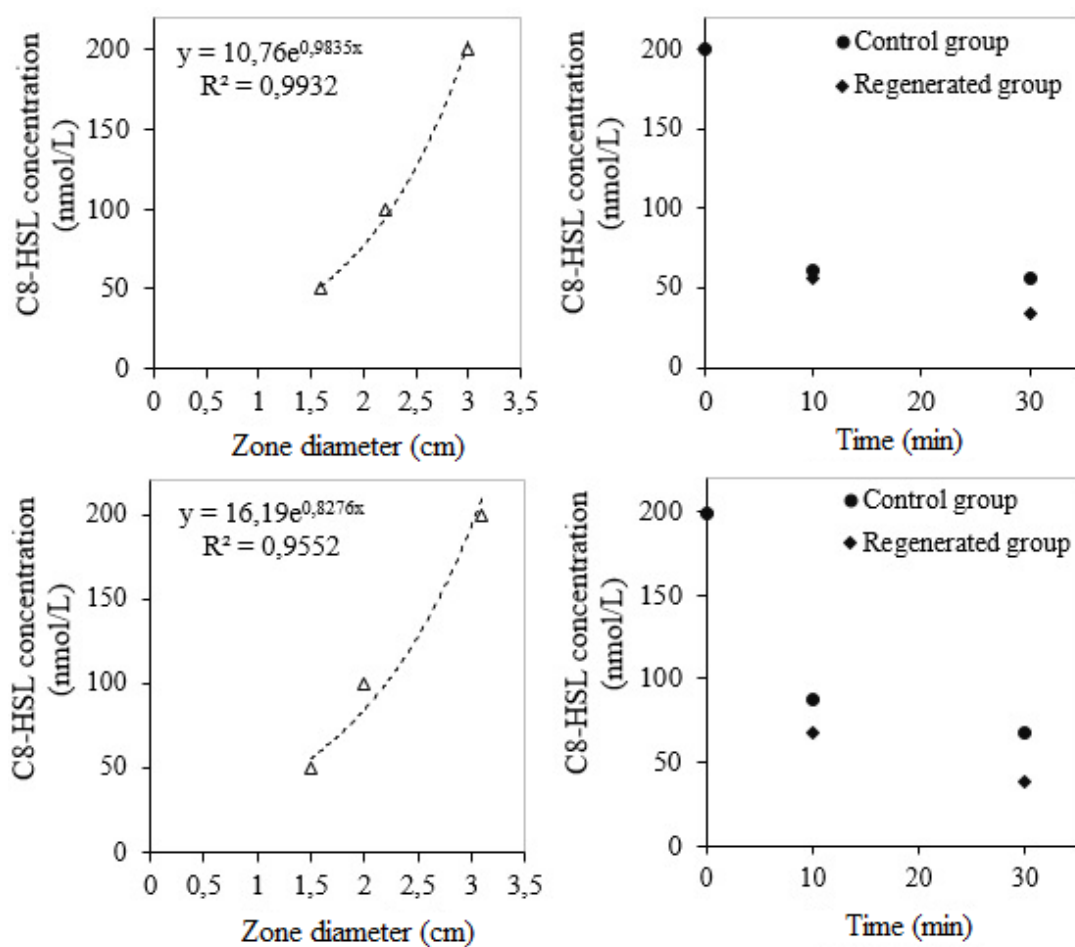


Figure 6.7 (continued) : QQ activities obtained from the regeneration study.

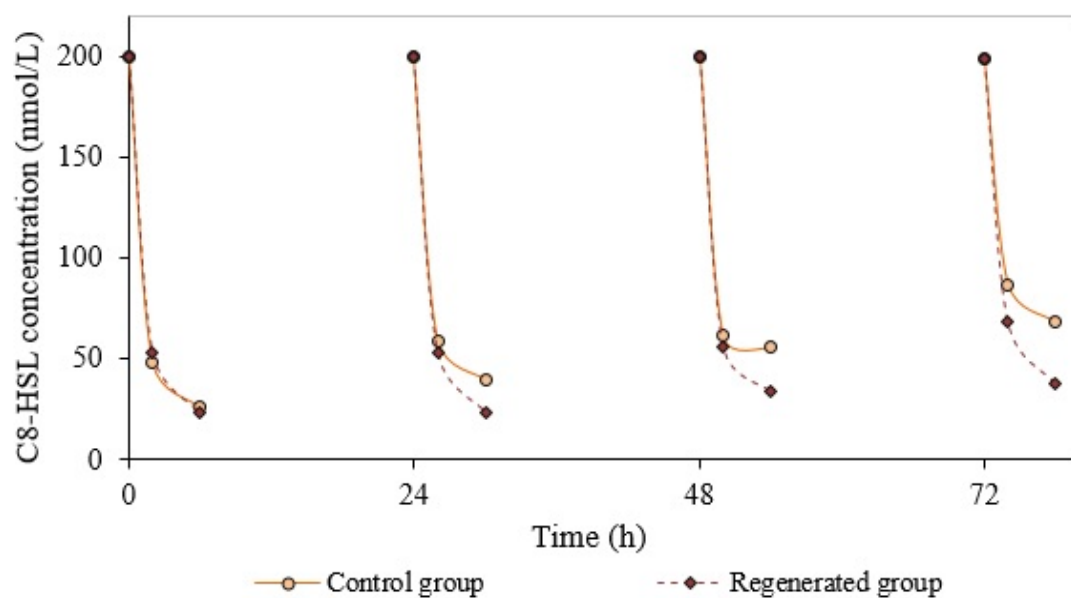


Figure 6.8 : The effect of regeneration of QQ activity.

If Figure 6.8 is examined, it can be said that while regenerated group could save its quorum quenching activity, control group started to lose its quorum quenching activity after 48 hours. In the light of this study, it may be mentioned that bacteria removal from the cubbyholes of the RMCF can result in higher quorum quenching activities.

6.4.4 Effect of regeneration on quorum quenching activities during the MBR operation

In order to examine the effect of bacteria regeneration in QQ MBR operation, two parallel MBRs were operated during two weeks. TMP profiles obtained from operations were given in Figure 6.9.

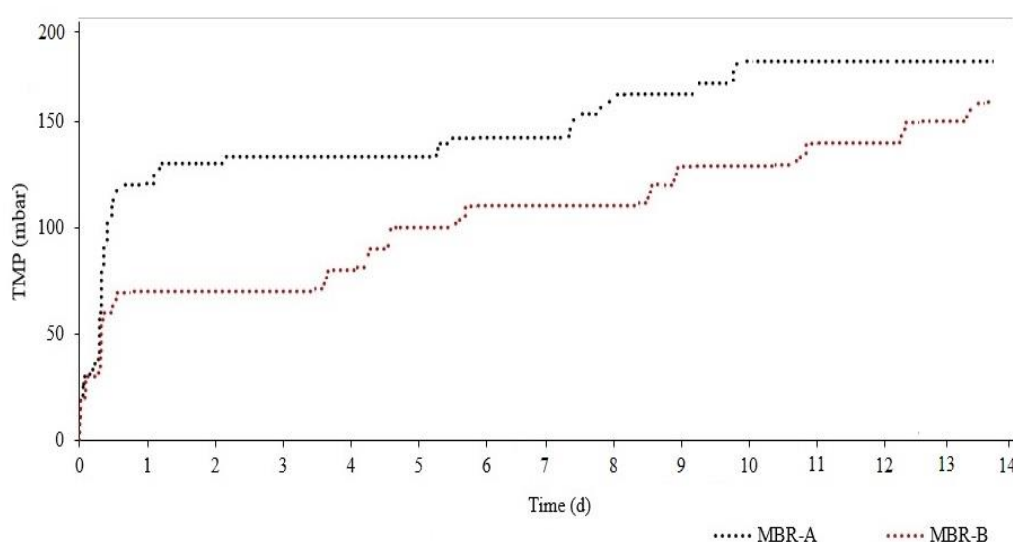


Figure 6.9 : TMP profiles obtained from the regeneration study.

According to these TMP profiles, it can be said that regeneration of bacteria resulted in an additive effect on the biofouling prevention via quorum quenching. There is a slight difference between the TMP profile of MBR-A and the TMP profile of MBR-B. While TMP values reached to 135 mbar in MBR-A, it was around 65 mbar in MBR-B. Because these both MBRs were operated as QQ MBRs, TMP values could not reach at high values during these 6 days of operation.

6.5 Conclusion

Within the scope of this study, the possible positive effect of bacterial regeneration on the quorum quenching activity was examined. The faster living of bacteria during their

log phases increases the efficiencies of many bacterial activities. Because of this reason, it was thought that a definite percentage, which was decided according to the net growth rate, of the *Rhodococcus sp.* BH4 solution in the RMCF can be removed, and this can be adapted to the MBR operation. Finally, it can be said that the application of this idea was succesful during MBR operation, and it will be possible, especially for pilot- and full-scale MBR plants with bacteria discharge lines.

7. CONCLUSIONS

The main aim of the studies carried out within the scope of this thesis was to create a solution for biofouling, which currently represents one of the biggest problems associated with the use of MBR technology for effective advanced wastewater treatment. The method chosen for this solution was biological and based on the quorum quenching mechanism, which prevents the formation of biofilm on the membrane surface by interrupting bacterial signaling. This thesis combined consideration of the materialized QQ mechanism, which is very new and innovative, with experimental results to increased awareness of the importance of the topic. Studies can be listed according to the following characteristics:

- Proof of existence of quorum sensing mechanism in the MBRs,
- Quorum quenching bacteria isolation from the MBR in the prospect of relevance between quorum sensing and quorum quenching in an environment,
- Determination of quorum quenching activities of isolates,
- MBR operation with quorum quenching bacteria addition by using different immobilization media,
- QQ MBR operations with cell entrapping beads and microbial vessel, and increasing the originality of the thesis with the idea of RMCF in the light of the results obtained from CEB and MV studies,
- The design and manufacture of RMCF,
- Determination of the differences between the TMP profiles obtained from different immobilization media and immobilized quorum quenching bacteria amount matches,

- Contribution of the experimental results obtained from the treatability studies and microscopic examinations of the membrane fouling studies,
- Comparison of all immobilization media in terms of energy saving, and
- Cost analysis based on energy savings with the preference of quorum quenching applications for real scale MBR plants.

7.1 Quorum sensing and isolation of quorum quenching bacteria

It is important and necessary to prove the existence of the quorum sensing mechanism in the environment in which the quorum quenching mechanism is applied in order to prevent biofilm creation. In this regard, the existence of quorum sensing in two parallel MBRs used for QQ MBR studies was determined. After quorum sensing bio-assays, it was seen that quorum sensing existed in the activated sludges used in the MBRs operated in both WEMT and MEM-TEK laboratories. Since the results indicated that the quantity of the quorum sensing can depend on bacterial community, activated sludges were mixed together and added to the parallel reactors after being separated into two.

Quorum quenching bacteria isolation was realized in WEMT by using the samples taken from the MBRs used for domestic wastewater treatment according to the enriched medium method. Two different isolates were found: *Rhodococcus sp.* BH4 and *Pseudomonas sp.* 1A1. Pure cultures were obtained using the single colony isolation technique and glycerol stocks were prepared for the studies. After the quorum quenching activities of the free cells had been determined, both species (BH4 and 1A1) degraded 90% of signal molecules in the environment in only 15 min. While, as expected, the study proved that bacteria have high quorum quenching activities, the research also found that BH4 exhibits higher quorum quenching activity than 1A1. In this regard, during this thesis study, *Rhodococcus sp.* BH4 was preferred as QQ bacteria for QQ MBR studies.

7.2 Design and development of a new QQ immobilization medium: RMCF

Within the scope of this thesis, QQ cell entrapping beads and microbial vessel applications that have rare examples in the literature were studied. Five CEB and two MV studies were conducted. It can be said that the sodium alginate matrices used in CEB manufacturing have some disadvantages due to their unstable forms, and the leakage of cells and bacterial loss that occurred as the excess sludge was removed from the MBR. On the other hand, the microbial vessel had a lower F:M ratio and stagnant property. RMCF was developed as an effective alternative to quorum quenching immobilization media. This design can provide mobility, material stability, and renewability to immobilization modules and free living rooms for immobilized quorum quenching bacteria to grow without being released into the environment. RMCF was applied to submerged MBRs to test its potential to inhibit MBR biofouling. Eight QQ MBR operations with RMCF were conducted.

The biofouling inhibition potential of the RMCF was studied in RMCF studies. In addition, the effect of the amount of the immobilized *Rhodococcus sp.* BH4 was also examined. While, in Study I, the amount of immobilized *Rhodococcus sp.* BH4 was around 8 mg BH4/cm³ cubbyhole, it was 12.5 mg BH4/cm³ cubbyhole in Study II. The TMP profiles indicated that RMCF can inhibit biofilm formation and decrease TMP, and a small increase in quorum quenching bacteria resulted in an additional 15% decrease in the TMP values.

Although a direct comparison between the results obtained in RMCF and other quorum quenching MBR studies is quite difficult, the extent to which RMCF can reduce the increase in TMP was, perhaps, most effectively reported by Oh et al. (2012) and Kim et al. (2013). These studies indicated that RMCF could provide a novel alternative approach to mitigate the membrane fouling that results from the inhibition of biofilm formation by quorum quenching and the prevention of biomass attachment on membrane surface due to the shear forces created by the rotation of the RMCF located under the filtration module. The contribution that the physical washing effect caused by the shear forces made to the total anti-biofouling effect was approximately a 16%. Moreover, it has a non-dispersible material and refillable structure.

The study recognized the importance of maintaining quorum quenching activity throughout long-term MBR operations, and a QQ effect (~50%) was observed after 30

days of operation. Besides, RMCF reduced polysaccharide and the protein amount of EPS with an efficiency of 25% and 50% respectively. According to the CLSM images produced during the quorum quenching study, the amount of biofilm formed on the membrane surface was very thin for QQ MBR. These results agree with TMP profile results, and confirm the QQ effect of RMCF and biofilm formation prevention. The CLSM images acquired after the physical cleaning study showed that physical cleaning also decreases biofilm. No considerable difference in terms of the removal efficiencies were observed between the two parallel MBRs throughout the entire experimental period. The application of RMCF did not have any effect on the biological treatment processes. The bacterial communities in the activated sludge after acclimation and at the end of operation were compared using PCR-DGGE analysis. PCR-DGGE analyses showed that, after the RMCF application, a significant increase in the number of bacteria species was observed in the MBR that used the QQ mechanism. This may have been due to the degradation of signal molecules by quorum quenching bacteria, which prevent some common species from being dominant in population equilibrium and provide other rare bacteria species with the opportunity to gather and grow without any social stress.

7.3 The effects of the flux and the immobilized QQ bacteria amount on the QQ activity, and cost analysis

The second main study aimed to prepare three different QQ immobilization media: 1) cell entrapping beads (CEB), 2) microbial vessel (MV) and 3) a rotating microbial carrier frame (RMCF). In addition, two different *Rhodococcus sp.* BH4 amounts and three different fluxes were used to test their effects on the QQ mechanism. The first part of this study involved an optimization study, which concluded two different bacteria amounts (1.5 and 7.5 mg BH4/cm³). While CEB that included 1.5 mg BH4/cm³ could degrade 49% of 200 nM C8-HSL in 120 min, CEBs that included 7.5 mg BH4/cm³ could degrade 85.8%±2.3 of C8-HSL over the same duration. In light of this, it could be argued that the efficiency of the QQ mechanism may increase as the amount of the immobilized QQ bacteria increases. Furthermore, three CEBs, including 7.5 mg BH4/cm³, exhibited similar QQ mechanism efficiencies.

After the efficiency of the QQ mechanism was determined, these four CEBs were used in the MBRs over a short period of time. While the control MBR was operated with

vacant CEBs, the QQ MBR was operated with the same amount of QQ CEBs. TMP values increased via fouling in the control MBRs, while the TMP values in the QQ MBR were kept lower than those in the control MBRs. Quorum quenching was achieved in all studies.

The first CEB study was conducted under low flux and low immobilized QQ bacteria amount conditions, and biofouling could be prevented with 78.7% efficiency. In the second CEB study, while the flux value was kept the same as the first CEB study, the amount of immobilized QQ bacteria was increased to 7.5 mg BH4/cm³, and 99.3% biofouling prevention efficiency was achieved with a 21% efficiency increase. After the second CEB study, the flux was increased to test the efficiency of the same amount of immobilized QQ bacteria under the hardest conditions. In the third CEB study, the achievement was 96.1%. It is a fact that very high flux values result in rapid and irreversible fouling in MBR treatment. In the fourth CEB study, the control MBR and QQ MBR were operated as high-flux MBRs with the objective of examining the potential quorum quenching effect of immobilized QQ bacteria on rapid fouling. The achievement was 77.7% in this study. This efficiency result was close to the results of the first study. To summarize, it can be said that the results indicated that increasing the amount of immobilized QQ bacteria by four did not counteract the one and a half times increase on the flux. Finally, the effect of the flux on TMP increase was higher than the effect of the immobilized QQ bacteria amount on TMP decrease.

The second part of the study tested three different immobilization media. Since earlier tests had indicated that the flux has a higher effect on the quorum quenching efficiencies than the amount of immobilized QQ bacteria, three different immobilization media were compared in terms of their efficiencies under high flux conditions. In this regard, two different immobilization media, MV and RMCF, were also prepared with the same amount of QQ bacteria employed in the study on CEB IV for short-term MBR operations. In these two studies, the rates by which the TMP was increased were decreased in QQ MBRs. MV and RMCF could result in a 44% and 59.7% TMP decrease during the operations, respectively. To sum up, the results of this study indicated that CEB is the most successful immobilization media alternative for biofouling control under high flux conditions. Furthermore, RMCF also played an important role in biofouling control with a prevention rate of around 60%. The minimum efficiency was obtained from the microbial vessel study, and these results

are similar to previous studies described in the literature. The reason why the highest efficiency was obtained from the CEB study could be attributed to the high F:M ratio for immobilized QQ bacteria in the CEB and the physical cleaning of the membrane surface via the mobility of CEB in the reactor. In addition, RMCF also has motion property and provides higher F:M ratio when it is compared to the microbial vessel. Even if it is not a totally spread media through the reactor, like CEB, the rotation motion of the RMCF under the membrane module created a physical cleaning effect. The biggest disadvantage of the microbial vessel related to immobility. Quorum quenching did not affect the removal of organics during these operations.

Following the second part of the study, which was carried out by using three different immobilization media, a comprehensive cost analysis was produced. A real-scale MBR plant that had a 1000 m³/d flowrate was taken as an example, and the costs of operating this plant over six months duration were calculated for two different situations: 1) conventional MBR plant operation and 2) QQ MBR plant operation with *Rhodococcus sp.* BH4 immobilized into different media. According to the results of this cost analysis, while CEB and RMCF can create cost savings of 24% and 23% respectively via their higher biofouling prevention efficiencies, the usage of MV could only reduce the cost by 12%. Because of its immobile property, MV was not as cost effective. On the other hand, high manufacturing costs and renewal need of the CEB reduced the saving from 24% to 15% in view of the manufacturing costs of the CEB. There is no need for material renewal for MV and RMCF; however, the manufacturing cost of MV is higher than the manufacturing cost of RMCF. While RMCF may provide a cost saving of 23%, MV can only save 6% in view of the manufacturing costs associated with the use of this immobilization media. To conclude, the most feasible immobilization media is RMCF, and the unit cost of domestic wastewater treatment associated with using this media with MBR technology would be 0.61 kWh/m³. As such, while CEB very successfully prevented biofouling, RMCF was more feasible for pilot- and real-scale MBR plants when the manufacturing costs of QQ products were taken into consideration.

7.4 One-step MBR

Thirdly, a one-step MBR study was conducted. One-step MBR is an innovative treatment process combination that was developed to enhance biological nitrogen removal and prevent biofouling at the same time. In this study, while control MBR contained vacant beads, QQ MBR was operated with two different bead additions: 1) anoxic sludge immobilized PVA beads and 2) quorum quenching (QQ) immobilized PVA beads. Anoxic activated sludge did not show any QQ activity in the standard AHL solution (QQ activity bio-assay). Besides, BH4 could degrade more than 94% of C8-HSL in 60 min.

Following the anoxic bead production, the denitrification rates of these beads were determined by using batch tests. While vacant beads did not remove any organic matter in synthetic wastewater, anoxic beads consumed most of the nitrate and carbonaceous organic matters. Denitrification bacteria in anoxic beads could maintain their lifecycles and denitrification process. While the denitrification rate of anoxic beads in synthetic wastewater was calculated to be 0.067 mg nitrate/mg MLVSS.h, when the ratio between the anoxic volume and the total volume of each bead was taken into the consideration, the denitrification rate in the standard solution was found to be 0.048 mg nitrate/mg MLVSS.h. This rate was lower than the denitrification rate in the synthetic wastewater because of the faster reactions by means of the other matters in the synthetic wastewater.

After the batch studies had been completed, a one-step QQ MBR was operated. In the control MBR, TMP values increased rapidly at the end of the 3.5th operation day, and the membrane was totally fouled on the 5th day of operation. On the other hand, the TMP increase in QQ MBR was prevented by using quorum quenching beads. The QQ mechanism efficiency was calculated to be 94%. Confocal images went along with the TMP results. The biofilm layer on the fiber membrane from the QQ MBR was rare and thinner. Furthermore, a 70% and 38% decrease in the protein and carbohydrate content respectively of the attached biomass was achieved during the operation. In this study, anoxic beads resulted in a 7% and 21% increase in the COD and TN removal efficiencies respectively. To summarize, this study suggested that one-step QQ MBR can be useful for pilot- and real-scale MBR plants located in residential areas in which discharge limits are important and strict.

7.5 The effect of bacterial regeneration on the QQ activity

The last part of the thesis involved a study on the regeneration of *Rhodococcus sp.* BH4. The rate of the performances of bacteria can depend on the phases of growth kinetic, even if this performance is shown during the whole lifetime of the bacteria. Biofilm formation, resistance to antibiotics, quorum sensing, quorum quenching and virulence are some of these bacterial performances. As a result of this, it was anticipated that higher quorum quenching activity can be achieved in their log phases. The growth kinetic of BHS and the QQ activities of this specie at different times were determined using batch and continuous studies. The regeneration time was determined to be 28.3 days. In addition to this, the quorum quenching activity had a positive effect on the bacterial regeneration. MBR operation was conducted to verify the effect of immobilized bacteria removal on the TMP decrease. The TMP results indicated that the regeneration of the bacteria had a positive effect on the biofouling prevention via quorum quenching. To summarize, the application of the regeneration concept to the QQ MBRs was successful during MBR operation; as such, the development of pilot- and full-scale MBR plants with bacteria discharge lines is feasible.

7.6 Storage conditions and reactivation of prepared QQ CEBs

Finally, an additional study was conducted on the storage conditions and reactivation of manufactured CEBs. According to the results of this study, CEBs should be stored at +4°C temperature in physiological water. While they can lose their quorum quenching activities in time when they are stored at room temperature, they can freeze at temperatures under +4°C. Within the scope of the scientific research project, which this thesis was based on, some hydrogel CEBs, including *Rhodococcus sp.* BH4, were prepared in the WEMT Laboratory and then transferred to the MEM-TEK Laboratory under the optimum storage conditions. These beads were reactivated after transfer with an overnight incubation in LB, and their QQ activities were the same as those identified at the beginning of the activity. After reactivation, they were tried in the MBR operation, and a TMP prevention of 99.6% was achieved. The results of this study open a new window into the anti-biofouling MBR operation market through presenting the idea that manufactured CEBs can be transferred from the manufacturer to the purchaser.

7.7 Final remarks and recommendations

To conclude, the results obtained from the studies carried out within the scope of this PhD thesis correspond to the results obtained from the studies that were conducted on the innovative quorum quenching mechanism in existing literature. The results indicate that membrane biofouling can be successfully controlled and prevented by means of quorum quenching mechanism applications during MBR operations. Thus, the increase of TMP values can be both decreased and considerably postponed. The application of a QQ mechanism in pilot- and real-scale MBR plants can significantly reduce the costs of operation. Besides, the manufacturing costs of quorum quenching bio-products are relatively lower than the costs of other anti-biofouling chemical and engineering solutions. The main and important point is that the choice as to which optimum immobilization medium to employ should take into consideration the manufacturing costs and the efficiency of the quorum quenching mechanism employed. The application of quorum quenching bacteria immobilized in the RMCF could be expanded to full-scale MBRs with its high feasibility, which is related to easy disassembling from MBR, renewable and QQ bacteria refillable properties of module structure and design, proper and sufficient room for quorum quenching bacteria growth, and the additive anti-biofouling effect of rotation motion.

In the view of the findings of this study, the following recommendations can be made for future studies:

- New quorum quenching bacteria species from different environments may be isolated and examined more detailed in terms of their structures and QQ activities.
- The usage of QQ CEBs can be developed with the new materials and reactivation studies.
- Various designs of innovative immobilization media should be studied in the future to get the highest feasibility for pilot- and real scale MBR plant operations.
- The further studies on the positive effect of bacterial regeneration on the quorum quenching activity may reflect the positive effect more healthier in terms of microbial and molecular structure.

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