BACTERIAL AND ENZYMATIC QUORUM QUENCHING AGENTS TO CONTROL BIOFOULING IN MEMBRANE BASED TREATMENT SYSTEM

by

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No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Institute of Environmental Sciences and Engineering (IESE) in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Field of Environmental Science, Institute of Environmental Sciences and Engineering (IESE), National University of Sciences and Technology, Islamabad, Pakistan.

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"In the Name of Allah, the most Beneficent, the most Merciful"

DEDICATION

This PhD work is dedicated to

My Parents

Without whom none of my success would be possible…..

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Abstract

Membrane bioreactor (MBR) has gained increasing popularity and extensive application in wastewater reclamation worldwide due to its exceptional effluent quality, high biodegradation efficiency and low sludge production. However, membrane biofouling caused by formation of biocake or deposition of microbial flocs, due to its membrane clogging phenomenon, results in a significant decline in the treatment efficiency of the MBR systems. Membrane scientists have been more focused towards investigating the physicochemical based fouling control strategies, while ignoring the microbiological mechanism and agents responsible for biofouling. Generally, it is very important to clearly investigate and establish a cause of the problem to be tackled with. Therefore, investigation of mediators responsible for cell to cell bacterial communication or quorum sensing (QS), an important cause leading to formation of mature biofilm, is imperative prior to selection of biofouling control strategy.

This study aimed to comprehensively investigate the factors which may contribute to membrane biofouling and examine their changes in the presence of antifouling mechanism under steady state and transient loads. Further, it was hypothesized that in tandem effect of chemical and biological based antifouling strategies could further enhance the overall efficacy of fouling control in submerged MBR treating pharmaceutical wastewater.

In this context, prevalence of quorum sensing bacteria and corresponding signal molecules was examined in MBR, treating synthetic wastewater, during Phase 1 of the study. Among acyl homoserine lactones (AHLs) producing strains, *P. kilonensis* and *Psychrobacter* sp. were scrutinized as the most dominant biofilm forming groups. These bacteria were considered as potential strains which need to be addressed while considering QS inhibition mechanism. Whereas, all bacterial strains showed inherently different biofilm forming tendencies, irrespective of their growth rate, verifying the role of QS agents responsible for biofilm development. The amount of EPS required to form the matured colonies varies depending on the type, genus or species.

Biofouling is also considered as a major operational challenge in MBR technology. Controlling the biofilm by the action of interspecies quorum quenching (QQ) has received a significant attention over the past few years. Phase 2 of the study was designed to investigate the antifouling bacterial consortium for improved biofouling control in the MBR operation. Potential quenching strains including *Enterobacter cloaca*, *Delftia* sp., and *Pseudomonas* sp. were utilized to control biofouling in the MBR operated in the continuous mode. The MBRs with QQ consortium experienced almost three times less biofouling as compared to Conventional MBR leading to significant decrease in AHLs concentration in the biocake without compromising the permeate quality. Comparatively lower concentration of bound extracellular polymeric substances (EPS), in MBRs with QQ consortium, has restricted the bacterial adhesion to membrane, resulting in enhanced membrane permeability. Polymeric beads with entrapped bacteria appeared to alter the sludge characteristics like decrease in sludge particle and floc size.

Efficacy of QQ mechanism under transient conditions, such as a continuous increase in organic loading rate (OLR), was examined in Phase 3. Modified QQ consortium comprised of bacteria that could release either acylase or lactonase (QQ enzymes) and degrade both AHLs and autoinducer-2 (AI-2) was used to investigate the QQ mechanism in lab-scale MBRs under steady state (fixed OLR) and transient conditions (varying OLR). During the steady state the QQ consortium, both in immobilized and suspensions, has retarded biofouling by a factor of 3.04 ± 0.5 and 2.96 ± 0.7, respectively, as compared to the conventional MBRs. However, a continuous increase in OLR (transient condition) greatly reduced the effectiveness of QQ bacteria: where the fouling rates were reduced by a factor of 1.8 ± 0.3 and 1.4 ± 0.1 for immobilized beads and suspensions, respectively. Despite the significant reduction in the concentration of signal molecules in the mixed liquor and sludge cakes, a significant increase in EPS, especially the loosely-bound EPS of the mixed liquor was found to be major contributor to membrane fouling, while polysaccharides being a dominant contributor to membrane fouling over proteins.

The QQ strategy was further strengthen by integrating it with powdered activated carbon (PAC) and backwash cycling for fouling control. Successful application of modified PAC-alginate

beads was verified in a lab scale MBR treating synthetic pharmaceutical wastewater. A 450% increase of cycle time was achieved in MBR with QQ strains entrapped PAC beads and backwash as compared to conventional MBR. Moreover, substantial reduction in signal molecules showed the effectiveness and prolonged survival of QQ strains within PAC-alginate (PACQQ) beads. Interestingly, continuous suspension of PACQQ beads increased sludge floc size which further enhanced sludge dewaterability. These QQ entrapped PAC beads provide a novel MBR with less biofouling and high removal efficiency of pharmaceutical compounds like trimethoprim, sulfamethoxazole, carbamazepine, diclofenac and triclosan up to 71±15, 80±7.34, 63±21, 82±8.26 and 67±22% respectively. Moreover, consistency in the biodegradation of all PhAC adsorbed on QQ beads shows the effectiveness of PAC-QQ beads.

Chapter 1

INTRODUCTION

1.1. BACKGROUND

Reclamation of wastewater to reduce freshwater burden is imperative due to increasing water shortage, which requires an economical and efficient treatment system. The wastewater treatment using Activated Sludge Process (ASP) for the transformation of organic and inorganic pollutants to CO_2 and water has contributed greatly to the improvement of aquatic environments worldwide (Moura et al., 2009). However, efficiency of ASP is greatly influenced by design and operation of treatment system as it generates large quantities of excess sludge and requires large aeration and sedimentation tanks. Due to compliance of stringent water quality regulations, membrane bioreactor (MBR) - combinations of bioreactors and membrane filtration units for biomass retention- has emerged as one of the innovative options in advanced wastewater treatment technology owing to its distinctive advantages over conventional ASP (Lin et al., 2014).

Compared to ASP, MBR technology features various distinct advantages, including excellent effluent quality, higher volumetric loading, less sludge production, smaller footprints, process flexibility towards influent changes, and improved nitrification (Mustafa et al., 2016). These advantages, together with more stringent

discharge standards have given remarkable impetus to the extensive research and applications of MBR for biological wastewater treatment systems.

However, membrane fouling, due to organic, inorganic or colloidal particles may cause severe flux decline (Amy, 2008), shorten membrane life span, increases energy and chemical consumption, still remains a bottleneck that limits the widespread application of MBR (S.-R. Kim et al., 2013). Among various types of fouling, membrane biofouling, caused by formation of biocake (or deposition of microbial flocs) on membrane surface, has attracted a great deal of attention due to its strong negative effects including membrane clogging and low treatment efficiency (Lee et al., 2014; Siddiqui et al., 2015).

Various measures such as changing hydro-dynamical parameters (aeration rate and air diffuser position, relaxation/filtration mode and backwashing), pre-treatment of feed by coagulation/flocculation, chemical (acid, base and oxidant) cleaning, and addition of polymer or powdered activated carbon (PAC), have been attempted to mitigate membrane biofouling (Kobayashi et al., 2003; Liu et al., 2010; Yang et al., 2013). These conventional fouling control methods have been proven to alleviate membrane fouling efficiently during early operational stage only, and they all have a limitation of drastic permeability loss as the operation progresses.

Recently, advanced molecular techniques have exposed the biofilm formation, intrinsically a natural biological process, as the main constituent that results into ultimate membrane fouling (S.-R. Kim et al., 2013; Shrout and Nerenberg, 2012). Likewise, it is well accepted that cake layer formation on membrane surface accounts

for over 80% of the total filtration resistance in most MBR studies (Miura et al., 2007; Wang et al., 2007). This suggests that retardation of biofilm formation could be a more direct solution to control biofouling than conventional approaches based on the physico-chemical principles.

One of the important phenomenon behind formation of matured biofilm is quorum sensing (QS) i.e. regulation of bacterial communication via cell to cell signaling (Chong et al., 2012). A large number of gram-negative bacteria produce acyl homoserine lactones (AHLs) as signal molecules or autoinducers (AI-2) (J W Costerton et al., 1995). Once the concentration of AHLs or AI-2 reaches upto certain threshold level, the phenomenon of QS turned "ON" and this cell-cell communication allows bacteria to coordinate gene expression and regulate the formation of biofilm (Christiaen et al., 2011).

Many researchers have focused towards fouling control strategies ignoring the importance of mechanism and agents responsible for biofouling. Although biofilm can be formed by a single bacterium, but MBR always consists of diverse bacterial communities belonging to a variety of different genera (Yun et al., 2006). Therefore, it is essential to know the dominant microbial community structure as a prerequisite for fundamental understanding of biofouling aspects influenced by QS in engineered reactors, and to assess how designs and operations might be improved by keeping the signaling mechanism into account.

For the past few years, a biological mechanism based antifouling approach – bacterial quorum quenching (QQ), which introduces QQ enzyme-producing bacteria to

MBRs, either in liquid cultures (Cheong et al., 2014; Jahangir et al., 2012) or immobilized in alginate beads (S.-R. Kim et al., 2013), has been adopted to reduce cell-to-cell communication among bacteria, thereby retarding biofilm development on the membrane. Successful applications of single QQ bacteria i.e. *Rhodococcus* sp. to control biofouling in lab (Kim et al., 2015; S.-R. Kim et al., 2013; Maqbool et al., 2015; Oh et al., 2012) as well as pilot scale MBRs (Lee et al., 2016) have been reported. As the biocake consists of diverse group of QS bacteria releasing a variety of signal molecules to communicate among themselves. These diverse groups of signal molecules could be very difficult to get mineralized by a single QQ strain. A possible strategy could be to utilize a consortium of bacterial strains with overlapping capacities to mineralize the signal molecules which could result in more efficient biofouling control in MBR.

Further, previous studies have proved the effectiveness of QQ mechanisms for biofouling control under steady conditions; however, efficacy of QQ under transient conditions has not been studied yet. An increase in organic loading rate (OLR), a typical transient condition, may trigger increased EPS production, causing EPS bulking and increased biofouling in MBRs (Abdollahzadeh and Bonakdarpour, 2013; Shariati et al., 2013). This poses a challenge to the application of QQ for biofouling control under transient conditions, as QQ bacteria are believed to disrupt QS molecules, thus reducing EPS production and biofouling (Kim et al., 2015; S.-R. Kim et al., 2013). Therefore, it is important to comprehensively investigate the factors which may contribute to membrane biofouling, and examine their changes in the presence of QQ bacteria under transient loads.

In spite of the fact that QQ strategy is a practically viable option, few studies have reported reduction in floc size as the major drawback of QQ beads due to continuous suspension of immobilized bacteria within the bioreactor (Jiang et al., 2013; Lee et al., 2016). Microbial floc size is one of the important factors of the floc characteristics and is related to membrane-biofouling; therefore it is important to maintain the floc size in the mixed liquor to maintain the efficiency of the MBR system. Further, fouling due to pore blockage could not be tackled through QQ mechanism since it is restricted to cake layer reduction only. Role of powdered activated carbon (PAC), in mitigating the membrane biofouling and maintaining the floc size, has been studied earlier (Lee et al., 2009; Pirbazari et al., 1992). This study aimed to supplement the QQ strategy by demonstrating the combined impact of PAC, QQ and backwash in fouling control.

1.2. SCOPE AND OBJECTIVES

In this work the well-established quorum sensing inhibiting strategy has been examined by applying consortium of QQ bacteria in MBR under transient and steady state conditions to gain a better understanding of the underlying relationship between QQ and biofouling. Further, combined effect of QQ strategy, backwash and PAC addition was monitored to overcome the drawback of QQ mechanism alone and improve the overall efficacy of fouling control.

The study was based on the following objectives:

i. Profiling of quorum sensing agents in lab scale MBR

ii. Biofouling control through consortium of quorum quenching bacteria in MBR

iii. Effect of transient conditions on the performance efficacy of quorum quenching mechanism

iv. Combined effect of PAC and QQ on simultaneous removal of pharmaceutical compounds and foulants behavior of MBR

Based on above mentioned objectives, research work was conducted in four phases in laboratories of the Institute of Environmental Sciences and Engineering (IESE), National University of Sciences and Technology (NUST), Islamabad, Pakistan and Advanced Environmental Biotechnology Centre (AEBC), Nanyang Technological University (NTU), Singapore.

Chapter 2

LITERATURE REVIEW

Water is the most important and fundamental ingredient of life on earth. However, increasing population growth coupled with unsustainable water practices in the field of agriculture and industry have stressed the water availability all over the world. The unhealthy relationship of industrial activity, urbanization, economic growth, unsustainable development, climate change and environmental pollution has increased fear of water scarcity, which is a biggest threat in recent times.

The population of Pakistan is now estimated to be more than 180 million. With the current growth rate of 1.8%, population of the country is expected to have doubled by the year 2025. Estimates exposed the fact that Pakistan's water resources had declined from 5000 m^3 per capita to less than 1,000 m^3 per capita to be declared as water stressed country. Around 200 MG domestic wastewater is being cleared to surface waters daily. While collection level is no more than 50 % nationally (<20 % in rural), however, only 10 % of collected wastewater is effectively being treated (World Bank, 2006). Importantly, absence of regular monitoring program and national environmental quality standards (NEQS) are further increasing complexity of the problem. Thus, efficient and cost effective water and waste treatment plants should be developed, and efforts be made for recycling of wastewater to make it reusable for agricultural, domestic and industrial purposes.

2.1. WASTEWATER TREATMENT OPTIONS

Industrial, agricultural and domestic wastewater treatment has an important role in solving the global problem of water pollution. Wastewater treatment plants uses physical and biochemical procedures to minimize organic matter, eliminate pathogenic organisms and enhance water quality, so that water can be reused or discharged into the environment with nominal concerns.

Various wastewaters treatment technologies are being applied such as aerated lagoons, activated sludge process, coagulation, flocculation etc. (Bolong et al., 2009). Where, biological processes may remove more than 90% of biological oxygen demand (BOD) in wastewater systems (Sheng et al., 2008). However, major global concern still prevails i.e. majority of compounds are persistent organic pollutants, owing to their resistance to conventional treatments such as coagulation, biological oxidation, adsorption, ion exchange and chemical oxidation (Moreira et al., 2017). As a result, they have been detected in rivers, lakes, oceans and even drinking waters all over the world. This constitutes a serious environmental health problem mainly due to their toxicity and potential hazardous health effects (carcinogenicity, mutagenicity and bactericidality) on living organisms, including human beings (Damalas and Eleftherohorinos, 2011; Kümmerer, 2009).

2.2. MEMBRANE BIOREACTOR (MBR)

2.2.1. Concept and Process Description

With increasing demand for clean and fresh water, many new technologies of water purification are being developed. Since last two decades, membrane technology has emerged as a worthwhile treatment to comply with existing and pending water quality regulations (Diagne et al., 2012; Meng et al., 2009). MBR, a combination of biological degradation and membrane filtration, has been recognized as one of the key technology for the separation of contaminants from polluted sources thus purifying contaminated waters besides others. Membranes are selective barriers that separate two different phases, allowing passage of certain components and retention of others (Figure 2.1). The driving force for transport in membrane processes is may be a gradient of pressure, chemical potential, electrical potential or temperature across the membrane (Li et al., 2008).

Wastewater is continuously supplied to reactor which contains activated sludge. Organic pollutants in wastewater stream are consumed by microorganisms in activated sludge as substrates for growth, maintenance and endogenous metabolism (Yeon, 2009). Whereas, membrane processes rely on a physical separation, usually with no addition of chemicals in the feed stream and no phase change, thus stand out as alternatives to conventional processes (i.e. distillation, precipitation, coagulation/flocculation, adsorption by active carbon, ion exchange, biological treatment) for chemical, pharmaceutical, biotechnological and food industries (Drioli and Giorno, 2009).

2.2.2. Submerged and Side Stream MBR

Two main MBR designs exist with the membrane module either located externally to the bioreactor (side stream MBR) or immersed directly into it (submerged MBR) (Figure 2.2). In side stream configuration, membrane unit is independent of bioreactor. Feed enters the bioreactor where it contacts biomass. A recirculation pump provides a cross flow velocity at the membrane surface, reducing deposition of suspended solids. The transmembrane pressure (TMP) and crossflow velocity, which define the operation of membrane, are both generated from a pump (Le-Clech et al., 2006; Yeon, 2009).

In submerged MBR system, biological degradation process and membrane separation are incorporated in the same tank. Under these circumstances, TMP is derived from hydraulic head of the water above the membrane. Further, coarse aeration is provided to the system not only to provide oxygen to the biomass, but also to limit particle deposition at membrane surface (Le-Clech et al., 2006).

Both submerged and side stream systems have advantages and disadvantages respectively. Especially, energy consumption during side stream MBR operation tends to be greater than during submerged MBR operation due to high rate liquid recirculation required to prevent membrane fouling and maintain high permeate flux rates. Whereas, aeration of bulk liquid provides necessary cross-flow velocity at membrane surface in submerged MBR process. More than half of operating MBRs are configured as submerged systems due to lower operating, pumping cost and comparatively less frequent cleaning than side stream systems (Yeon, 2009).

2.2.3. Global Market for MBR

The MBR has been widely used in water reuse systems in building, industry, sanitary treatment and municipal wastewater treatment. More than 2,500 MBR plants have been installed worldwide (Hanft, 2008). Valued at an estimated rate of $ 337 million in 2010 to $ 627 million by 2015. Global MBR market is rising at an average annual growth rate of 13.2%, faster than the larger market for advanced wastewater treatment systems (Figure 2.3). MBR systems have increased in competitiveness against traditional solutions due to reduction in cost and lower energy consumption levels. A final overarching driver behind global growth of MBR market is a steady decrease in price and energy consumption levels compared with earlier models sold in 1990s. This has been achieved by research and development progress. As per BCC research report, technological innovation, streamlined manufacturing, value chain control and optimization have allowed major competitors to reduce prices by between 5-30%, with a tangible reduction in energy consumption. These two changes to MBR units have helped drive them forward in a cost sensitive market where competition between various technological solutions is strong.

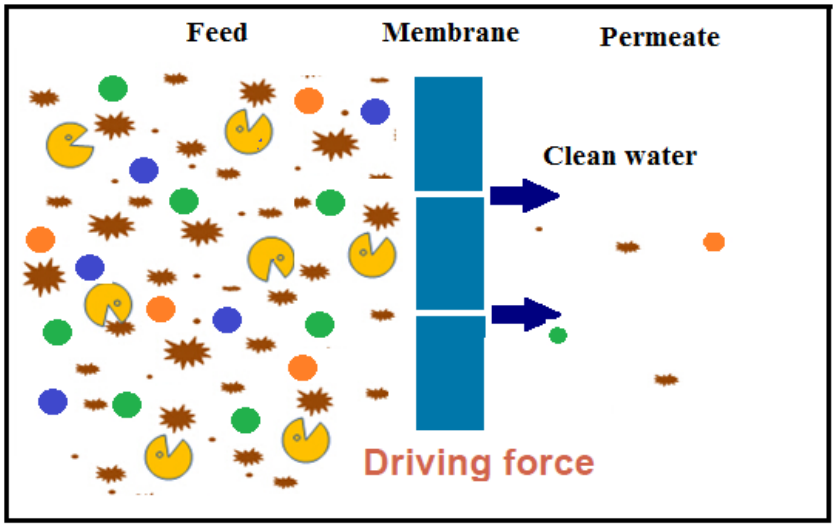


Figure 2.1. Process diagram of membrane bioreactor (MBR)

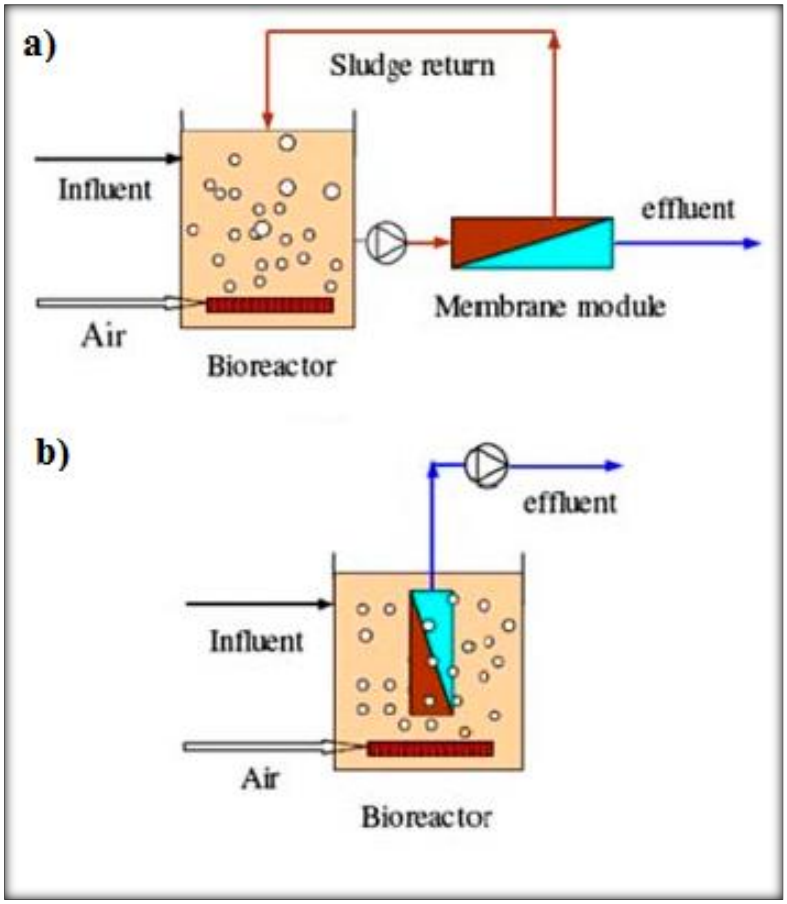


Figure 2.2. General configuration of (a) Side stream MBR, (b) Submerged MBR

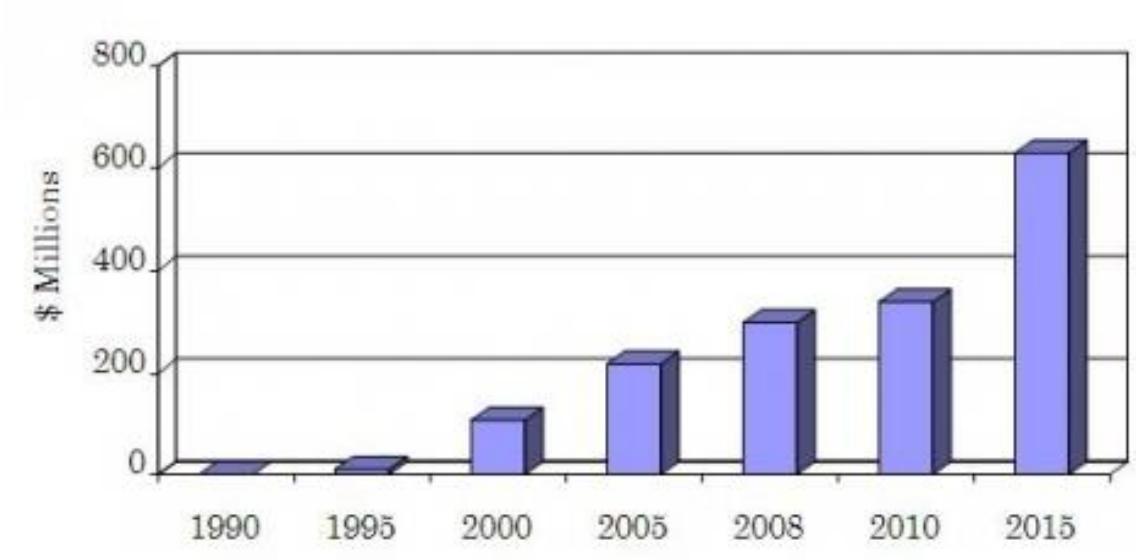


Figure 2.3. MBR market from 1990-2015 (BCC Research)

2.3. RESTRICTIONS IN MBR

To some extent, MBR systems are constrained primarily by:

i. Greater system complexity: membrane separation demands additional operational protocols relating to maintenance of membrane cleanliness thereby resulting in requirement of skilled staff (Chong et al., 2010).

ii. Higher capital and operating costs: membrane component of the MBR incurs a significant capital cost over and above that of an activated sludge process (Zhang et al., 2016).

Therefore, despite the growing acceptance of MBR technology, technical demand to overcome the perceived drawbacks of MBR such as its complex and small scale nature, high costs and operator skill requirements, has also been escalated. Among all, membrane fouling remains the primary hindrance for its universal and large scale applications (Ajmani et al., 2012; Kim et al., 2015). Hence, over 70% of MBR researches has been dedicated to identify, investigate, control, and model membrane fouling (Chang et al., 2002; Yeon, 2009).

2.4. MEMBRANE FOULING

Membrane fouling could decrease permeate flux and productivity, increase energy consumption of gas scouring and elevate cleaning frequency, which would shorten membrane lifespan and lead to higher replacement costs (Pidou et al., 2009). Hence, prosperous and effective applications of membrane technology has been critically limited and hampered by membrane fouling. Membrane fouling results from

interaction of membrane material and components present in the activated sludge. The latter includes substrate components, cells, cell debris and microbial metabolites such as extracellular polymeric substance (EPS) and soluble microbial products (SMP). Further, fouling of membranes also depends on many parameters such as membrane material, surface roughness (Elimelech et al., 1997), surface chemistry, porosity, operational conditions and of course feed water quality (Nyström et al., 1995).

2.4.1. Internal Fouling

Internal fouling is caused by adsorption and deposition of solutes and fine particles within the internal structure of membranes, e.g. adsorption of foulants to pore walls which is known as pore narrowing or pore blocking (Iritani, 2013; Le-Clech et al., 2006).

2.4.2. External Fouling

The deposition of particles, colloids and macromolecules on membrane surfaces leads to external fouling. It is also termed 'fouling layer' on membrane surfaces. Generally, external fouling may be divided into two kinds of fouling layers: cake layer due to accumulation of retained solids on membrane, and gel layer resulted from precipitation of soluble macromolecules, colloids, and inorganic solutes (van den Brink et al., 2013).

External fouling may be of various types including organic, inorganic or biological based on the biological and chemical characteristics of membrane foulants

(Wang et al., 2014), although the boundary between these classifications is not rigid and definitions of different fouling types may overlap (Malaeb et al., 2013).

2.4.2.1. Organic fouling

The initial cause of membrane fouling is organic fouling. It is related to molecular shape, size and chemical characteristics of organic matter. Chemical characteristics may include polar, steric, functional group and stability to form hydrogen bonds (Shon et al., 2009). Thus, organic fouling is primarily caused by deposition of proteins, amino sugars, polysaccharides, humic acids, polyhydroxyaromatics, and other organic substances (either soluble or colloidal) originated from feed water or microbial secretion. Fan et al. (2001) recognized potential foulants in order as hydrophilic neutrals > hydrophobic acids > transphilic acids. Moreover, organic fouling is generally considered as irreversible and requires careful chemical treatment.

In particular, a gel layer may be formed in MBRs with sub-critical flux operation due to continuous organic fouling (Metzger et al., 2007; Wang et al., 2012). SMP and EPS are regarded as key membrane organic foulants in MBRs.

2.4.2.2. Inorganic fouling

Chemical precipitation of inorganic crystals and/or biological precipitation of inorganic-organic complexes are the major causes of inorganic fouling or scaling in MBRs (Costa et al., 2006; Meng et al., 2009). Presence of inorganic ions may enhance membrane fouling. As in most cases, metal cations interact with some functional groups of organic matters (biopolymers) to form chelating polymers (organic-inorganic

complexes) (Myat et al., 2014). If the saturation concentrations are exceeded on the membrane surfaces, these metals cations such as calcium, magnesium, iron, phosphorus and aluminum and anions like sulphates, carbonates, phosphates and hydroxyl may react and cause chemical precipitation.

In addition, inorganic particulates existing in the systems may also attach on to membrane surfaces or block membrane pores to cause inorganic fouling (Zhang et al., 2012). Besides, some inorganic ions could interact with some groups of organic pollutants, enter cake layer and crosslink the structure, then make it denser and lead to higher fouling (Choo et al., 2008). For instance, trichloroethylene caused severe membrane fouling in presence of $CaCl_2$ and $NaHCO_3$.

2.4.2.3. Biofouling

Biofouling is considered as one of the most serious operational challenge in MBR application. Among all types of fouling, biofouling is a dynamic, complex and relatively slow process that involves interactions not yet thoroughly understood. It is formed due to deposition, accumulation, metabolism and growth of microorganisms on membrane surfaces. Colonization of membrane surfaces with microorganisms is one of the important factor in biofouling process (Ma et al., 2013). Release of organic matters e.g., SMP and EPS via microorganisms, is the major contributor to membrane biofouling. Severe biofouling might occur and sludge cake layer will be formed on membrane surfaces when MBRs operated under supra-critical flux operation mode (Giraldo and Lechevallier, 2006). The dominant foulants for biofouling (sludge cake) are biosolids but also include part of organic matters.

Biofouling represents the “Achilles heel” of membrane process because microorganisms may multiply over time; even if 99.9% of them are removed, there are still enough cells remaining which may continue to grow at the expense of biodegradable substances in the feed water (Flemming et al., 1997). Biofouling has been known as a contributing factor to more than 45% of all membrane fouling and has been reported as a major problem in nanofiltration (NF) and reverse osmosis (RO) membranes specifically (Komlenic, 2010).

Biofouling possess several adverse effects on membrane systems (Murphy et al., 2001; Nguyen et al., 2012) such as:

i. Formation of a low permeability biofilm on membrane surface thereby resulting into decline of membrane flux.
ii. Increased feed pressure and differential pressure may require to maintain the same production rate due to biofilm resistance.
iii. Acidic by products, concentrated at the membrane surface, causes membrane biodegradation, e.g. cellulose acetate membrane are found to be more susceptible to being biodegraded.
iv. Accumulation of dissolved ions in the biofilm at membrane surface reduce the quality of product water, thereby increasing chances of concentration polarization
v. Energy consumption increases due to higher pressure being required to overcome the flux decline and biofilm resistance.

2.5. FACTORS AFFECTING BIOFOULING IN MBR

As illustrated in Table 2.1, factors affecting membrane fouling may be classified into six groups (Chang et al., 2002; Le-Clech et al., 2006; Nguyen et al., 2012): membrane type, feedwater characteristics, operating conditions, hydrodynamic conditions, biomass characteristics and microbial diversity in sludge.

2.5.1. Feedwater Characteristics

Feedwater influence the process of membrane fouling indirectly, by altering the sludge characteristics. Feedwater characteristics may impact the membrane fouling in following ways:

- As reported by Al-Halbouni et al. (2008), increase in temperature leads to biomass deterioration, reduction in protein content in EPS, increase in SMP and turbidity. Whereas, low temperature correlated to the occurrence of filamentous bacteria in mixed liquor, which ultimately produce more SMP in the sludge.
- Moreover, higher salinity influence the biochemical properties and physical properties of activated sludge directly by increasing the EPS and SMP concentrations along with decrease in membrane permeability (Reid et al., 2006).
- Significant role of metal cations in formation of fouling layers cannot be ignored. These ions work as a bridge between the deposited cells and biopolymers, thereby help in formation of a dense cake layer. Presence of magnesium ion is beneficial in reducing membrane fouling due to bridging of

negatively charged biopolymers and enhancement of microbial aggregation. Whereas, calcium ion reduces the growth of filamentous bacteria as a result of Ca bridges and increases hydrophobicity of EPS. Therefore, the fouling rate is lower in the reactor with the higher Mg:Ca ratio (Arabi and Nakhla, 2009; Kim and Jang, 2006; Meng et al., 2009).

- High food to microorganism (F/M) ratio or organic loading rate (OLR) decreases the sludge filterability and lowers the filtration index by inducing the generation of SMP and more bound EPS (Meng et al., 2007; Rosenberger and Kraume, 2003).

2.5.2. Membrane Characteristics

Membrane materials and properties have long been considered important factors that may affect membrane fouling. Module design plays a significant role in determining the performance of MBRs by affecting the hydrodynamic conditions, which in turn, influences the fouling rate. The packing density of flat sheet and hollow fibers, porosity, hydrophobicity and roughness of membrane, specific location of aerator under the membranes and choice of aerator are critical design parameters for MBRs (Nguyen et al., 2012). A detailed comparison of various membrane configuration and their link to fouling tendency is elaborated in Table 2.2. Selection of membrane module is determined by type of application, economic consideration and functionality of the module.

2.5.3. Operating Conditions

Control aeration in MBR is essential. Low level of dissolved oxygen (DO) leads to accumulation of high molecular weight compounds in sludge suspension and a less porous biofilm with worse filterability, while a high aeration rate leads to breakage of sludge flocs and production of EPS and SMP (Fan and Zhou, 2007; Jin et al., 2006).

Lower hydraulic retention time (HRT) results in release of EPS from bacterial cells, which is responsible for rise in SMP and sludge deflocculation. Decreased HRT also affects MBR performance and causes over growth of filamentous bacteria and formation of large and irregular flocs. However, very high HRT may increase energy consumption and also leads to an accumulation of foulants (Fallah et al., 2010; Meng et al., 2007; Wang et al., 2009).

Short solid retention time (SRT) is one of the most important operating parameters affecting MBR performance, in particular membrane fouling. Short SRT has negative impact on membrane due to high concentrations of SMP and bound EPS, particularly polysaccharides in the supernatant. At shorter SRT, concentrations of deposited EPS on membrane are much higher than membrane at higher SRT. Although prolonged SRT is conducive to sludge concentration and minimize excess biomass production, nevertheless too long SRT accelerates fouling due to large amount of foulants and high sludge viscosity. Therefore, it is recommended that optimum SRT of MBRs should be controlled at 20–50 days depending on HRT and feedwater quality (Al-Halbouni et al., 2008; Keskes et al., 2012; Meng et al., 2009).

2.5.4. Hydrodynamic Conditions

Hydrodynamic conditions including shear stress, velocity, air scouring and flux have a crucial impact on MBR performance. In MBR, bubble size and bubble flow rate play significant roles in hydrodynamic conditions and energy demand. Air scouring may prolong the MBR operation (Sofia et al., 2004), whereas Psoch and Schiewer (2006) reported the improvement in membrane flux through air-sparging. Larger bubbles or bubble-induced shear reduces fouling significantly (Wicaksana et al., 2006).

Membrane permeating flux determines the mass flow of mixed liquor to membrane surfaces and, thus, affects sludge cake formation. Thus, enhancement of hydrodynamic conditions is one of the effective approaches to mitigate membrane fouling in MBRs, since hydrodynamic conditions has close relation with aeration intensity, bubble size, membrane module configuration, MLSS concentration and sludge viscosity, etc. Further, module characteristics and operating mode also control the hydrodynamic environment and provide the key to successful membrane operation.

2.5.5. Mixed Liquor Characteristics

As the mixed liquor suspended solids (MLSS) concentration increases, concentration of both protein and carbohydrate fractions of EPS and SMP increases leading to increase in fouling potential thereby higher cake resistance (Chang and Kim, 2005). Moreover, increase in MLSS affects the distribution of particle sizes to smaller particles, leading to decrease in mean particle size (Yigit et al., 2008).

Sludge viscosity is also considered as one of the crucial parameter that determines membrane permeability. As shown by Liao et al. (2004), higher viscosity is related to lower permeate flux and higher trans-membrane pressure (TMP). Further, other parameters like nutrients availability, floc texture/size and dissolved organic matter (DOM) in sludge directly impacts the membrane fouling. For instance, nitrogen deficiency increases protein level in EPS, associated with good settling (low SVI) and high negatively charged floc surfaces. Whereas, phosphorous deficiency increases SVI values, decreases protein content of EPS and surface charge of flocs in activated sludge (Sponza, 2002).

2.5.6. Bacterial Communication

Biofouling caused by formation of biocake, or deposition of microbial flocs, on membrane surface, has attracted a great deal of attention due to its strong negative effects including, but not limited to, membrane clogging resulting in low treatment efficiency (Lee et al., 2014; Siddiqui et al., 2015). Biofouling occurs as a result of two mechanisms: (i) colonization of membrane surfaces with microorganisms and (ii) production of membrane foulants by microorganisms in mixed-liquor. Both the microbes and their products contribute to membrane fouling. These biofilms, comprising of polymeric substances, are important for bacterial survival in their social system. Where, these bacteria communicate each other, by a phenomenon known as quorum sensing (QS), using chemical signals or autoinducers (Shrout and Nerenberg, 2012; Xiong and Liu, 2010). Yeon (2009) demonstrated the evidence of quorum sensing activity in MBR and could correlate quorum sensing with membrane fouling.

Table 2.1. Factors influencing microbial adhesion to membrane surfaces (Chang et al., 2002; Nguyen et al., 2012)

Microorganisms	Membrane Surface	Feed water	Operating conditions	Mixed liquor	Hydrodynamic conditions
Type of species	Chemical composition	Temperature	Configuration	MLSS	Shear stress
Composition of mixed population	Surface tension	pH	HRT/SRT	Floc texture	Velocity
Growth phase	Surface charge	Dissolved inorganics	Aeration	Floc size	Air scouring
Microbial density	Hydrophobicity	Dissolved organic matter	TMP	Viscosity	Flux
Availability of nutrients	Porosity	Viscosity	Cross flow velocity	Dissolved matter	-----
Hydrophobicity	Conditioning film	Suspended matter	-----	-----	-----
Charge on flocs	Roughness	F/M	-----	-----	-----

Table 2.2. Qualitative comparison of various membrane configuration (Shon et al., 2009)

Characteristics	Tubular	Flat sheet	Spiral wound	Capillary	Hollow fiber
Packing density	Lowest	Lower	Low	High	Higher
Fouling tendency	Lowest	Lower	Low	High	Higher
Investment cost & installed area	Highest	Higher	High	Low	Lower
Ease of cleaning	Best	Better	Good	Poor	Poorer
Operating cost	Highest	Higher	High	Low	Lower
Membrane replacement	Yes/No	Yes	No	No	No

2.5.6.1. Quorum sensing and biofilm development

Quorum sensing (QS) is a means of bacterial communication by synthesizing, secreting and responding to small signal molecules or autoinducers. These autoinducers mediated cell-to-cell signaling allows bacteria to coordinate gene expression (Dobretsov et al., 2009). Figure 2.4 explains the QS mechanism and types of signal molecules involved. These autoinducers produced by bacteria accumulate in the extracellular environment. Once the concentration of autoinducers reaches a particular "threshold concentration", QS genes and phenotypes are switched "on" (Lade et al., 2014a). Phenotypes may include biofilm formation, virulence, bioluminescence and toxin production. Moreover, autoinducer-mediated quorum sensing system is associated with almost all stages of biofilm formation such as initial surface attachment, bacterial growth, maturation, and detachment of aged cells.

Generally, autoinducers have been divided into two major groups: acyl homoserine lactones (AHLs), used by gram negative bacteria and oligopeptides, used by gram-positive bacteria (Figure 2.4b) (Waters and Bassler, 2005). However, it has been shown that bacterial QS is not limited to these two classes of molecules. There are other molecules such as the universal autoinducer (AI-2), quinolones, hydroxyl ketones, and bradyoxetin (Lade et al., 2014a; Ochiai et al., 2013).

Fluid dynamic forces are the major mechanism for transporting microorganisms to membrane surface. The attachment of microbial cells to membrane surface is the first step of membrane biofouling leading to formation of biofilm layer. The biofilm may comprise populations of different types of microorganisms (e.g., bacteria, algae,

protozoa and fungi). As demonstrated in Figure 2.5, initial microbial attachment is mediated by electrokinetic and hydrophobic interactions, and is generally followed by cell growth and multiplication at the expense of soluble nutrients in the feed water or adsorbed organics on the membrane surface (Roy et al., 2011). The sequence of biofilm formation includes (a) adsorption of organic species and suspended particles on the wetted membrane surface to form a conditioning film; (b) transport of microbial cells to the conditioning film; (c) attachment of microbial cells to the membrane surface; (d) growth and metabolism of the attached microorganisms and biofilm development; (e) limitation of biofilm growth by fluid shear forces (detachment process) to achieve a steady state fouling resistance The extracellular polymeric substances (EPS) excreted by microorganisms anchor the cells to substratum and stimulate additional microbial colonization on the membrane surface (Lade et al., 2014a; Nguyen et al., 2012).

As microorganisms within a biofilm live in a matrix of hydrated extracellular polymeric substances that form their immediate environment, microorganisms account for less than 10% of dry mass whereas matrix of extracellular materials, which is mostly produced by the organisms themselves, may account for over 90% (Flemming and Wingender, 2010). Under most environmental conditions, organic carbon compounds provide nutrients for growth and energy supply to the biomass. The attachment of microorganisms to membrane surface is affected by factors such as membrane material (substratum nature), roughness of membrane surface, hydrophobicity and membrane surface charge.

2.5.6.2. Quorum sensing signaling

It has been verified that formation of biofilm induced by QS demonstrates negative impacts on filtration process in MBRs (Khor et al., 2007). Many gram-positive and gram-negative bacteria use QS signal circuits to coordinate a diverse array of physiological behaviors. In general, gram-positive bacteria use processed oligopeptides and gram-negative bacteria use acyl homoserine lactones (AHLs) as signal molecule to coordinate their behaviors. List of signal molecules and their physic-chemical properties is described in Table 2.3.

2.5.6.2.1. Mechanism of bacterial signaling in gram-negative bacteria

The AHL-mediated quorum sensing system requires three major components to function: (i) AHL signal molecule, (ii) AHL synthase protein to produce AHL signal, and (iii) a regulatory protein which responds to the surrounding concentration of AHLs (Lade et al., 2014a).

As represented in Figure 2.6a, signal molecules are produced by an AHL synthase gene Lux I at low concentration and are distributed in and around the cell. At lower cell densities, Lux I is constitutively expressed at a low, basal level and thus AHLs get accumulated in the surrounding (Rasmussen and Givskov, 2006) At high concentration of AHLs, the signal-receptor protein complex forms and gets activated. The activated signal-receptor complex in turn forms dimers with other activated AHL-lux R complexes and functions as transcriptional regulators controlling the expression of quorum sensing regulated target genes. At a certain cell density, also known as

"quorum size," the transcription of quorum sensing genes gets triggered and results in the expression of various phenotypes (Shrout and Nerenberg, 2012).

2.5.6.2.2. Mechanism of bacterial signaling in gram-positive bacteria

In gram positive bacteria, QS systems generally consist of three components (as indicated in Figure 2.6b), a signaling peptide known as autoinducing peptide (AIP) and a two-component signal transduction system that specifically detects and responds to an AIP. In further contrast to AHL signals, cell membrane is not permeable to AIP but rather a dedicated oligopeptide transporter, largely an ABC transporter, is required to secrete AIP into the extracellular environment (Claverys et al., 2006).

Most of signaling peptides in Gram-positive bacteria typically consist of 5–25 amino acids and some contain unusual side chains. Detection of signaling peptides in Gram-positive bacteria mediated by a two-component signal transduction system, consists of a membrane-associated, histidine kinase protein sensing the AIP, and a cytoplasmic response regulator protein enabling the cell to respond to the peptide via regulation of gene expression (Li and Tian, 2012).

2.5.6.3. Role of extra cellular polymeric substances (EPS) in quorum sensing

EPS are mainly high molecular weight secretions of microorganisms and consist of various organic substances such as polysaccharides, proteins, humic acids, uronic acids, nucleic acids and lipids. EPS bind the microbes together in a three-dimensional gel like highly hydrated matrix and so affect the physico-chemical characteristics of the microbial aggregates such as mass transfer, surface characteristics, adsorption ability

and stability (Sheng et al., 2010). Spatial and component distribution of EPS in sludge floc is quite heterogeneous, and are mainly divided into two categories 1) Soluble EPS, 2) Bound EPS (Figure 2.7). Soluble EPS, weakly bound to cells or dissolved in the surrounding solution, consist of soluble macromolecules, colloids, and slimes. Whereas bound EPS contain heaths, capsular polymers, condensed gels, loosely bound polymer and attached organic materials (Conrad et al., 2003). EPSs surrounding bacteria possibly possess a dynamic double-layered structure where tightly bound EPSs (TB-EPSs) forms inner layer and loosely bound EPSs (LB-EPSs) diffuses in outer layer. LB-EPSs are of highly hydrated matrix, and tend to form a dispersible and loose slime layer without an obvious edge (dispersible part) (Figure 2.7a). It was reported that LB-EPSs more significantly correlated with membrane fouling in MBRs as compared to TB-EPSs (Ramesh et al., 2007).

Soluble EPS are sometimes referred to as soluble microbial products (SMP) (Rosenberger and Kraume, 2003). Soluble EPS have greater binding capacity for organic matter than bound EPS (Pan et al., 2010).

EPS could contribute to biofilm formation or membrane biofouling in following ways (Nguyen et al., 2012):

- Establish the structural and functional integrity of microbial biofilms, and significantly contribute to organization of biofilm community.
- Contribute to the mechanical stability of biofilms, enabling them to withstand considerable shear forces.

- Create scaffolds with suitable physical characteristics and interconnected pore structures that promote cell attachment.

Moreover, it was also found that EPS content and floc size directly contribute to membrane biofouling. Lin et al. (2011) demonstrated that small flocs in sludge suspension contained more specific bacterial populations which produced higher level of EPSs, and therefore were prone to adhere to membrane surface. It has also been reported that deflocculation of sludge flocs would produce a great amount of colloids and solutes in sludge suspension, which resulted in severe pore blocking of membrane and formation of second membrane cake layer (Meng and Yang, 2007). Cell adhesion to solid surfaces is inhibited by electrostatic interaction at low EPS concentration but enhanced by polymeric interaction at high EPS concentration (Tsuneda et al., 2003).

2.6. BIOFOULING CONTROL STRATEGIES IN MBR

As previously described, fouling is the most critical factor for determining performance of MBR system. Therefore, various techniques have been explored to overcome membrane fouling generally and biofouling specifically through engineering, material and chemical approach.

2.6.1. Status of Commonly Applied Biofouling Control Strategies

Many studies focused on biofouling control via optimizing hydraulic conditions. Membrane fouling was also controlled by changing the configuration of system. Kim et al. (2008) evaluated effect of vertical position of the submerged membrane on fouling

and reported that fouling could be alleviated only in the upper zone due to reduction in biomass concentration and then applied this configuration to both lab-scale reactor and pilot plant.

Various conventional physical (such as back-pulsing, back-flushing, air sparging) and chemical (using acids, bases, chelating agents, oxidants, polymeric coagulants, flocculants, surfactants) cleaning methods often fail to adequately control biofouling. It is well reported fact that frequent chemical cleaning may impair the permeate quality (Tao et al., 2005), increases the chances of irrecoverable membrane fouling thereby reduces membrane lifespan (Judd, 2008), and may produce undesirable waste streams that need to be disposed of properly (Brepols et al., 2008).

In addition, Calderón et al. (2011) demonstrated that backwashing and chemical cleaning using sodium chlorite were found to be ineffective in complete removal of biofouled layers from the surface of MBR membranes. And remaining populations supported rapid re-growth of biofilm, leading to regeneration of a similar microbial community structure due to microbial adaptability to the conditions. In fact, it is easier to control the biofilm formed under natural conditions in the long-term than one formed where biocides have been applied. Bacteria surviving after a biocide application may develop adaptability and defense mechanisms, which render them even more resistant to the biocide when reapplied (Baker and Dudley, 1998).

The basic idea of chemical approach is to remove the major foulants such as small colloid, biopolymers using chemicals such as zeolite, coagulant, charged polymer and activated carbon etc. Lee et al. (2001) reported that with the addition of natural

zeolite, membrane permeability was greatly enhanced by formation of rigid floc that had lower specific resistance than control activated sludge without zeolite.

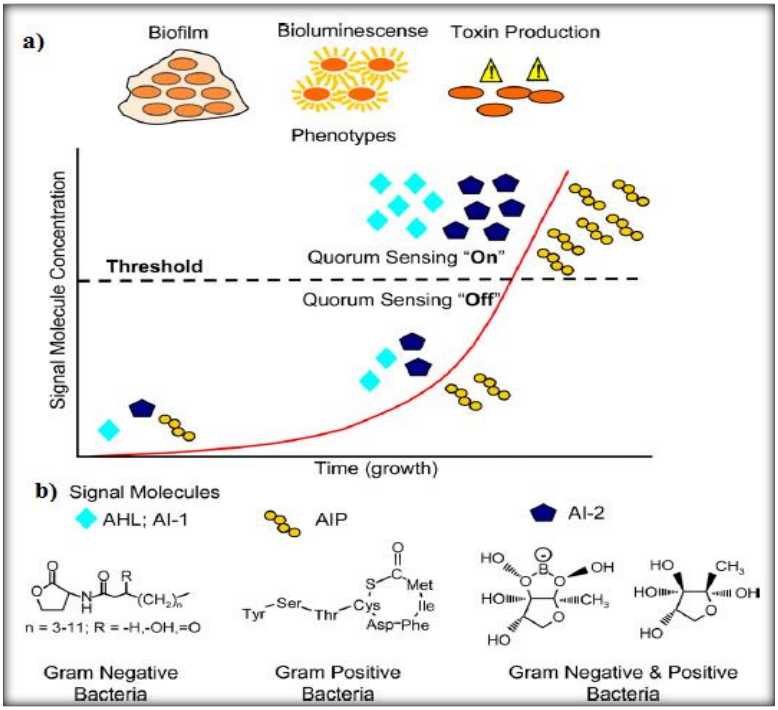


Figure 2.4. Schematic of a) Quorum sensing switch and types of signal molecules. (b) Three main families of auto-inducers in various bacteria (Roy et al., 2011)

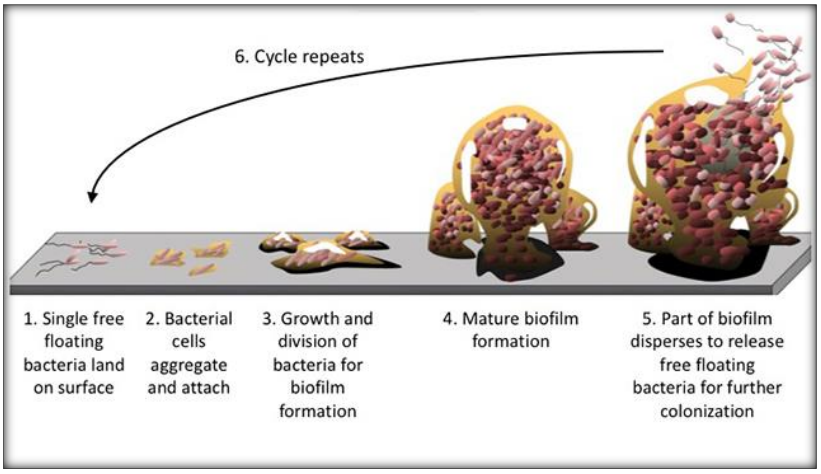


Figure 2.5. Stages of biofilm development

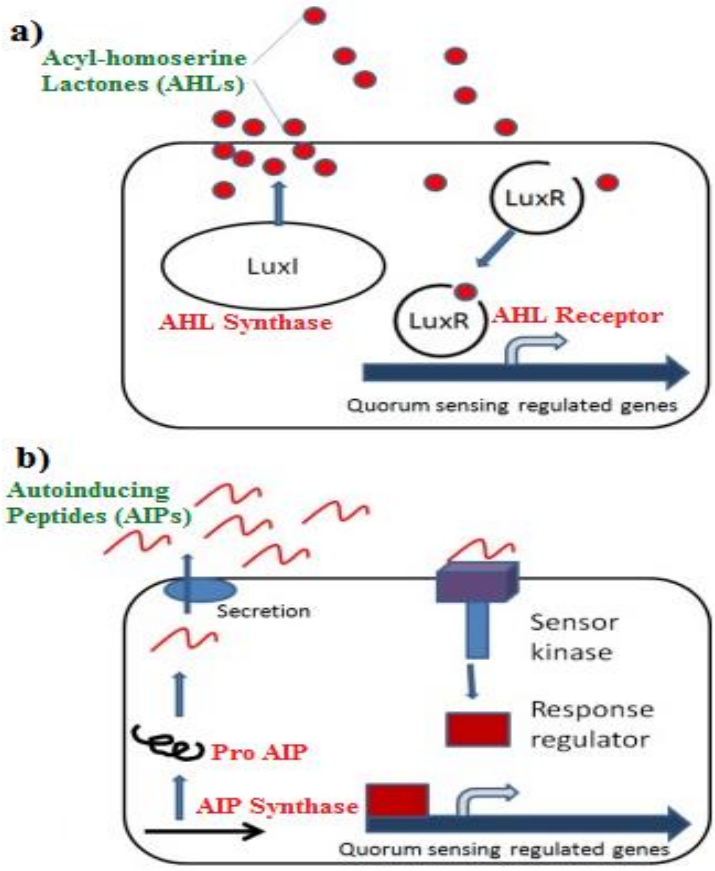


Figure 2.6. Three points of quorum sensing interception, signal generating, signal sequestration & signal receptor mechanism in a) Gram-negative bacteria b) Gram-positive bacteria

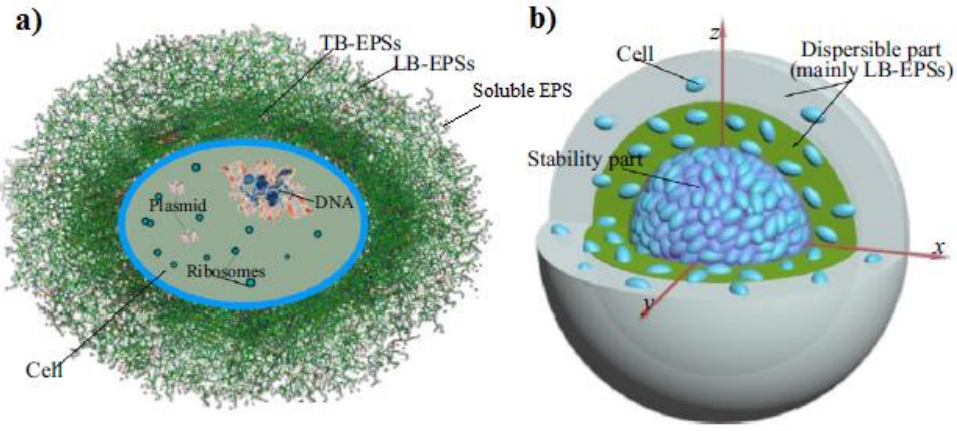


Figure 2.7. Schematic of EPS distribution in a) Cell structure b) floc size (Lin et al., 2014)

Further, adding different kinds of flocculants in MBRs could alter the properties of activated sludge and cake layer and may lead to enhanced membrane performance (Deng et al., 2016). However, all these approaches may delay but could not halt the process of biofilm formation, the actual basis of biofouling.

Membrane modification has been revealed as a successful control strategy to reduce fouling and improve flux consistency. But its applicability is limited as it is challenging to modify membrane structures without impacting the separation performance or occluding membrane pores (Mansouri et al., 2010). Moreover, many coatings do not show long term chemical and mechanical stability. Pasmore et al. (2001) elaborated that membrane characteristics such as roughness, surface charge, hydrophobicity and material only prolong the rate of initial bacterial adhesion tested under specific or controlled conditions. Even membrane surfaces, those were most resistant to formation of biofilm, have been reported to show a 40% surface coverage within two days.

However, many reports have shown experimentally the importance of the biological mechanism on the permeability loss in the MBR i.e. membrane biofouling. As a result, recent MBR researches placed their research focus on the revealing of fouling phenomena under biological frame. Conventionally, biological based antifouling strategies might prove to be valuable and may potentially enhance the effectiveness of currently applied cleaning protocols. These strategies refer to use of certain chemicals that target a specific molecule or mechanism that is responsible for attachment, communication, motility or growth of microbial cells.

2.6.2. Biological Approach – Quorum Quenching

Several studies have been reported to identify and eradicate the factors causing membrane biofouling including variation in hydrodynamic conditions i.e. aeration rate and position, relaxation and filtration mode and backwashing (Siddiqui et al., 2015). Chemical addition methods such as acid/base cleaning, oxidants, coagulant and polymers, however all these approaches have limitations in terms of mitigation of biofilm formation as it is a natural process. Therefore, it seems that a biological problem in the form of quorum sensing may only be dealt with a biological solution using quorum quenching (QQ) which is the disruption and hydrolyzation of signal molecules (Maqbool et al., 2015).

A revolutionary anti-fouling strategy was proposed by Lee's group at Seoul National University (SNU), South Korea in 2009. A QS inhibition strategy by Yeon (2009) demonstrated that AHL type QS system of gram-negative bacteria generally offers three points of attack: The signal generator (LuxI homologue), the signal molecule (AHL) and the signal receptor (LuxR homologue). Therefore, AHL QS inhibition strategy may be divided into blockage of AHL synthesis, interference with signal receptor and inactivation of AHL molecules (Figure 2.8). This discovery opens a new avenue to manage the behaviors of bacteria and control biofouling in membrane systems.

To date, least investigated strategy to interfere with quorum sensing is blockage of AHL production. A second QS control approach is to prevent the signal from being perceived by the bacteria using chemicals (QS inhibitor). These QS inhibitors can be

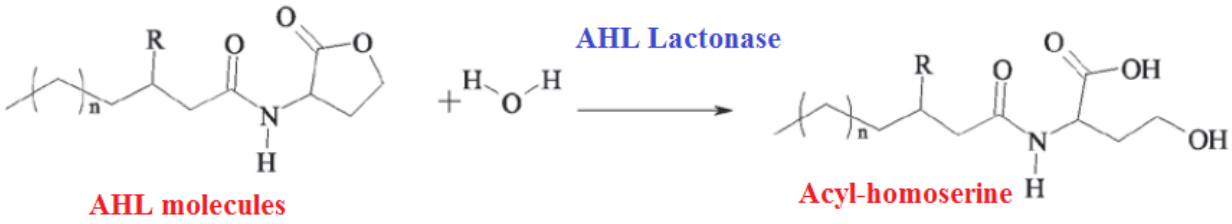
obtained from both natural source like penicilic acid or patulin and chemical synthesis. The third QS control strategy is inactivation or complete degradation of AHL molecules. This may be achieved by both chemical degradation and enzymatic destruction.

2.6.2.1. Enzyme based quorum quenching mechanism

AHL inactivation scheme based on QQ could be defined as the enzymatic degradation of signal molecules. Some bacteria produce lactonase, oxidoreductase or acylase, which disrupts cell to cell communication by hydrolyzing the amide linkage or cyclic ester of AHL molecules respectively (Figure 2.9).

2.6.2.1.1. AHL lactonase

Lactonolysis of AHLs may be accomplished by enzymatic activity. Members of the genus Bacillus including *Bacillus cereus*, *Bacillus mycoides*, and *Bacillus thuringiensis* produce an enzyme, AiiA, which is specific for AHLs degradation (Lee et al., 2002). Enzymes released via *Bacillus* sp. reduces the amount of bioactive AHL molecules through catalyzing the ring opening reaction. It has been reported that up to 20 mM 3-oxo-C6 HSL may be completely inactivated within 2 hours by a suspension culture producing the enzyme (Yeon, 2009). This shows that enzymatic degradation of AHLs would be useful as a means of biocontrol.

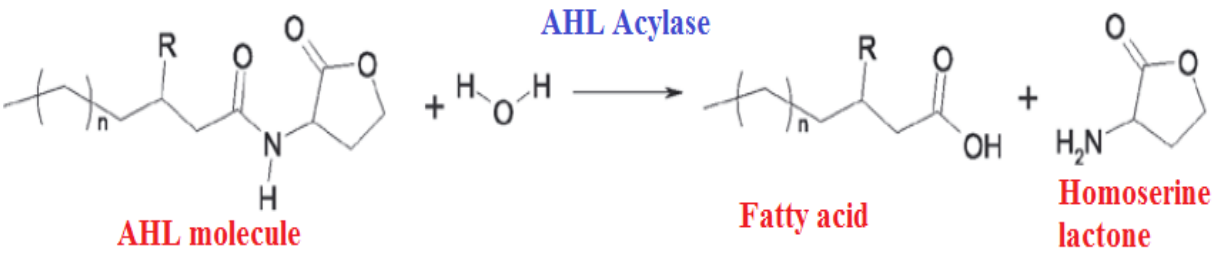


Moreover, release of AHL lactonases is not limited to *Bacillus* species. Various bacteria including *Pseudomonas aeruginosa* (Uroz et al., 2003), *Arthrobacter* sp., *Klebsiella pneumonia* (Park et al., 2003), *Agrobacterium tumefaciens* and *Rhodococcus* sp. (Park et al., 2006) have been found to produce AiiA homologues. A drawback of this lactonolysis reaction is that it is reversible at acidic pH. If the environment is not alkaline, a ring-opened AHL molecule spontaneously undergoes ring formation, regardless of the method by which it was opened either chemical or enzymatic (Cámara et al., 2002).

Sitanggang et al. (2014) also developed an enzymatic membrane bioreactor system supported by a suitable control design to enhance lactonase synthesis. They suggested that optimisation of reaction conditions, such as HRTs, agitation speeds, ionic strength of the media, etc. may possibly further enhance continuous synthesis of lactonase, thereby retard membrane fouling.

2.6.2.1.2. AHL acylase

AHL autoinducers may also be degraded by acyl-amide cleavage which is catalyzed by acylase enzyme. Xu et al. (2003) have shown the degradation of AHLs through porcine kidney acylase and reduction of the biofilm growth in aquarium water sample.



Yeon et al. (2009) demonstrated the evidence of quorum sensing activity in MBR and correlated quorum sensing with membrane fouling. They observed that AHL activity was low during the early stages of filtration, intensified around the point at which TMP showed the first signs of its typical exponential rise and was fully developed when fouling was severe. They proved that addition of acylase may inactivate AHL by amide bond cleavage as a novel fouling control strategy. After having proven the feasibility of quorum quenching by free enzymes in a lab-scale MBR equipped with Zenon modules, the group have overcome the technological limitations of using free enzymes by applying magnetic enzyme carriers. These may be readily retained by the membrane and recovered by magnetic capture, and show a high stability which led to a significant delay in fouling (Lee et al., 2014).

2.6.2.2. Bacterial quorum quenching

Enzymatic quorum quenching has proven its potential as an effective approach for biofouling control in MBRs for advanced wastewater treatment. Several groups of bacteria known to produce quorum quenching enzymes have been reported and their role could be further elaborated as economically feasible antibiofouling tool in MBR.

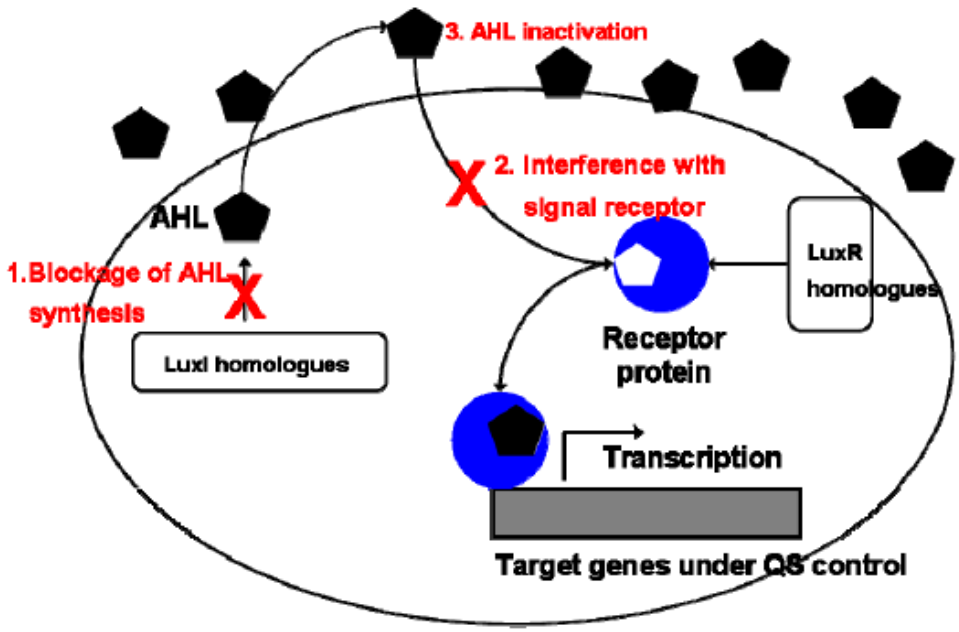


Figure 2.8. Three approaches to control acyl homoserine lactones (AHLs) (Yeon, 2009)

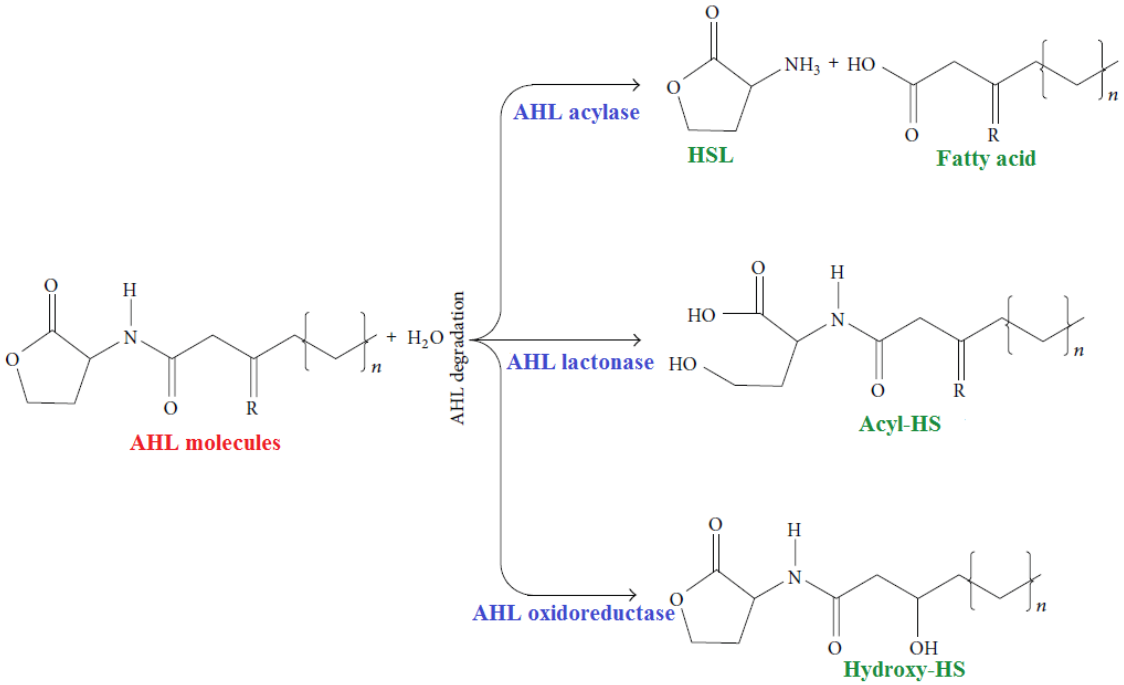


Figure 2.9. Quorum quenching enzymes and their AHLs degradation mechanism

This interspecies quorum quenching mechanism present in bacterial cells thus help to resolve practical issues concerned with extraction and purification cost of free enzyme as well as its stability. In view of this, Oh et al. (2012) investigated inhibition of quorum sensing in MBR by two quorum quenching bacteria, a recombinant *E. coli* which produces AHL-lactonase and a real MBR isolate *Rhodococcus* sp. BH4.

Moreover, an internal submerged MBR equipped with quorum quenching microbial vessel showed much lower biofouling than conventional MBR. Since then, biofouling control technique based on quorum quenching has attracted a lot of attention, and it is now viewed as a promising alternative for mitigating membrane biofouling.

The discovery of quorum quenching mechanisms in several bacterial species represents a new milestone in quorum sensing and quorum quenching research. Further, a microbial vessel encapsulated with indigenous isolated *Pseudomonas* sp. 1A1 has been found effective in the inhibition of AHLs mediated membrane biofouling in a lab-scale MBR (Cheong et al., 2013).

However, various factors such as vessel material, pore structure, inner volume of vessel, and amount of quorum quenching bacteria have been found to affect the microbial vessel performance and should be considered into account while designing further microbial vessel containing anti biofouling strategies.

To overcome the limitations of quorum quenching microbial vessel, Kim et al. (2013) demonstrated cell entrapping beads as an alternative method of bacterial quorum quenching. The quorum quenching activity of cell entrapping beads has also inhibited generation of EPS in biofilm cells and thus formed loosely bound biofilms. Successful

application of quorum quenching bacteria to control biofouling in MBR systems in lab (Kim et al., 2015; S.-R. Kim et al., 2013; Maqbool et al., 2015; Oh et al., 2012) as well as pilot scale (Lee et al., 2016) has been reported. These studies have demonstrated the quorum quenching strategy as a practically viable option.

Considering the essential roles of AHL-mediated quorum sensing in biofilm formation by gram-negative bacteria, degradation or disruption of AHLs signals with quorum quenching enzymes produced by other bacteria appears to be a promising alterative for controlling membrane biofouling. Therefore, strategies of disrupting the AHL-mediated quorum sensing with special emphasis on control of membrane biofouling by quorum quenching bacteria are of special concern in this study.

2.7. COUPLING MBR TECHNOLOGY WITH ADSORPTION AND QUORUM QUENCHING

As quorum sensing regulates a broad spectrum of microbial phenotypes, the characteristics of sludge in MBR and reactor performance should be examined comprehensively to exclude any potential side effects of quorum quenching on MBR operation (Choudhary and Schmidt-Dannert, 2010). Further, application of only quorum quenching technique in MBR may not be sufficient to control all types of fouling keeping MBR performance and sludge characteristics intact. Thus, focused have been emphasized to integrate various fouling control strategies simultaneously.

Recently, QQ strategy coupled to chlorine injection was applied in MBR to monitor the activity of QQ bacteria and degree of biofouling and compared with

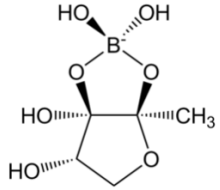
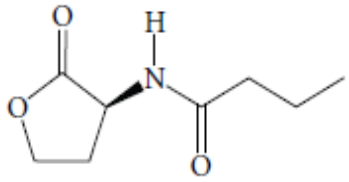
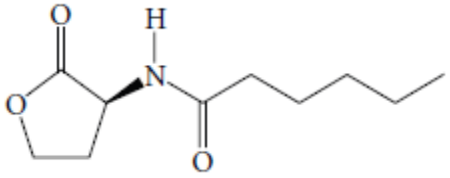
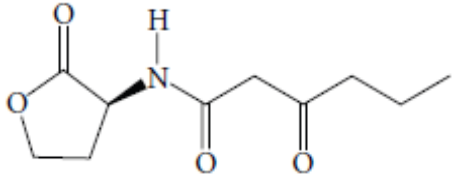
physical/chemical cleaning strategies. Integrated effect of QQ and chlorination saved the filtration energy up to 74 % in membrane bioreactors. Moreover, chlorine injection contributed to a reduction in the chemically reversible resistance, whereas quorum quenching reduced the physically reversible filtration resistance markedly while mitigating biomass attachment (Weerasekara et al., 2016).

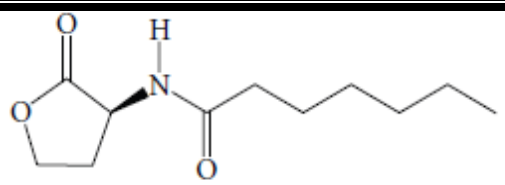
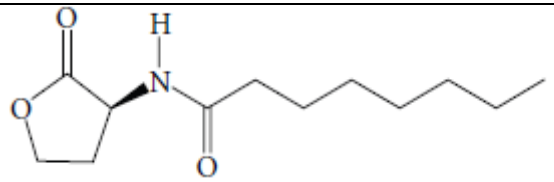
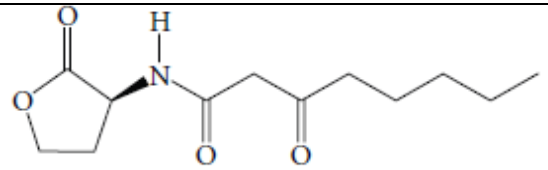
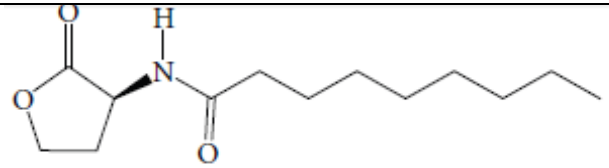
Proper additives adsorb SMP and colloids, increase EPSs content in flocs, enlarge flocs size, and thus mitigate membrane fouling in MBRs (Lin et al., 2013). The effect of addition of powdered activated carbon (PAC) on trans-membrane pressure (TMP) or flux in membrane filtration has also been reported (Wang et al., 2016). Some researchers have found that addition of PAC neither attenuates nor exacerbates membrane fouling because PAC selectively adsorbs non foulant natural organic matter (NOM) molecules irrespective of NOM characteristics and inorganic compounds present in water (Hu and Stuckey, 2007; Yoo et al., 2014). In contrast, some reports have indicated that PAC could worsen membrane fouling due to strong attachment of NOM-bound PAC particles to the membrane surface and then formed a close-packing PAC cake layer and blocked the membrane pores, causing an increase in filtration resistance (Ren et al., 2014). Moreover, Lin et al. (2013) also verified increase in bound EPS level and protein to polysaccharide ratio due to PAC addition. Other researchers have reported that PAC may control membrane fouling and even retard irreversible fouling. This effect is attributed to the adsorption of membrane foulants by PAC; subsequent biodegradation of organic substances (especially polysaccharides and proteins) by the biologically active PAC; enhanced scouring of membrane surface from

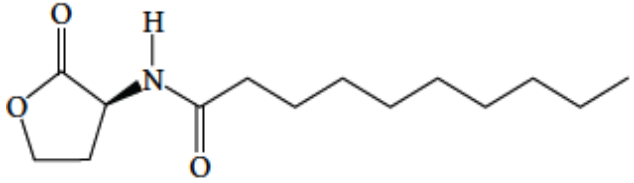
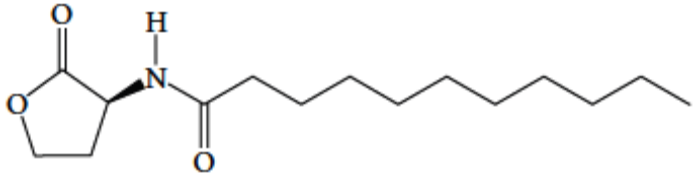
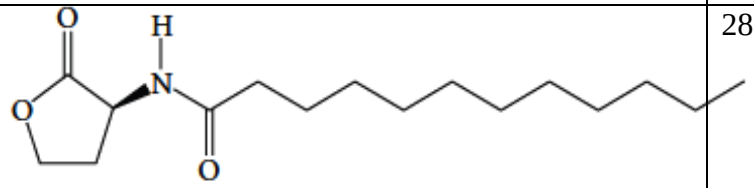
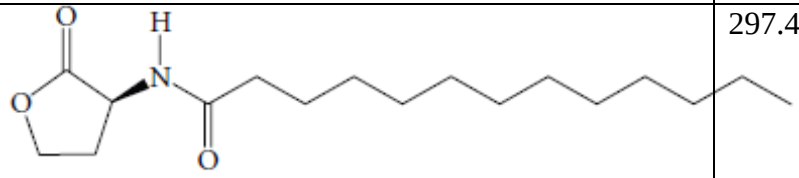
collision effects; and positive effect of PAC on the strength of sludge flocs (Gao et al., 2014; Shin et al., 2014). PAC is a potential alternative that could also be combined with QQ to enhance the adsorption of contaminants and prevent membrane fouling.

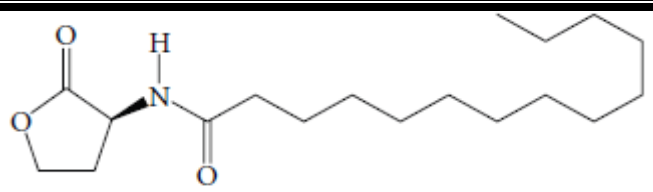
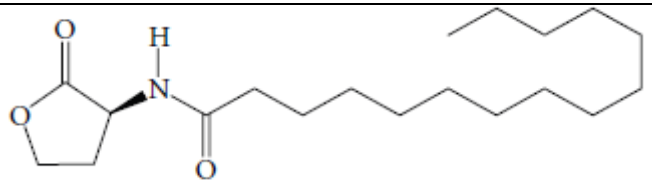
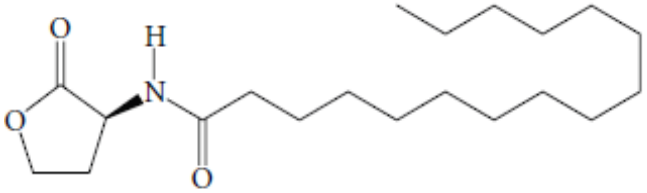
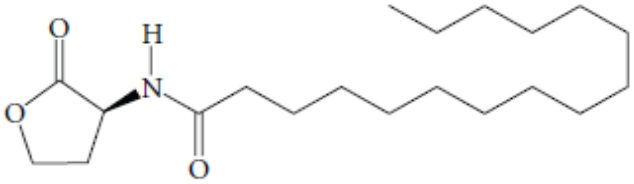
Presence of immobilized QQ beads may alter the sludge characteristics specifically reduction in floc size which may greatly impact membrane fouling. Therefore combination of PAC and QQ could also be a viable solution to control membrane fouling in MBR treating micro pollutant or pharmaceutical wastewater.

Table 2.3. List of signal molecules and their physicochemical properties (Lade et al., 2014b)

Autoinducers	Synonym	Structure	Molecular weight	Producing microorganisms
Furanosyl borate diester	C5H10BO7		192.94	*Pasteurella, Photorhabdus, Haemophilus, Bacillus*
N-Butanoyl-L-homoserine lactone	C4-HSL	 (N-[(3*S*)-Tetrahydro-2-oxo-3-furanyl]-butanamid)	171.2	*A. hydrophila, A. salmonicida, P.aeruginosa, S. liquefaciens*
N-Hexanoyl-Lhomoserine lactone	C6-HSL	 (N-[(3*S*)-Tetrahydro-2-oxo-3-furanyl]-hexanamide)	199.25	*Chromobacterium violaceum, Edwardsiella tarda, Bur. cepacia, S. marcescens*
N-(3-Oxohexanoyl)-Lhomoserine lactone	3-oxo-C6-HSL	 (3-Oxo-N-[(3*S*)-tetrahydro-2-oxo-3-furanyl]-hexanamide)	213.20	*V. fischeri, Enterobacter agglomerans, Erwinia carotovora, Pectobacterium chrysanthemi*

N-Heptanoyl-Lhomoserine lactone	C7-HSL	 (N-[(3*S*)-Tetrahydro-2-oxo-3-furanyl]-hexanamide)	213.30	*Ed. tarda, S. marcescens* SS-1
N-Octanoyl-Lhomoserine lactone	C8-HSL	 (N-[(3*S*)-Tetrahydro-2-oxo-3-furanyl]-octanamide)	227.30	*Bur. cepacia*, *Rho. Rubrum*
N-(3-Oxooctanoyl)-Lhomoserine lactone	3-oxo-C8-HSL	 (3-Oxo-N-[(3*S*)-tetrahydro-2-oxo-3-furanyl]-octanamide)	241.30	*Rho. rubrum*, *Agrobacterium tumefaciens*, *Rhizobium* sp.strain NGR234, *Rhizobium leguminosarum* bv. *Viciae*
N-Nonanoyl-Lhomoserine lactone	C9-HSL	 (N-[(3*S*)-Tetrahydro-2-oxo-3-furanyl]-nonanamide)	241.30	*Er. carotovora* strain SCC 3193

N-Decanoyl-Lhomoserine lactone	C10-HSL	 (N-[(3*S*)-Tetrahydro-2-oxo-3-furanyl]-decanamid)	255.35	*Rho. rubrum, Er. carotovora* strain SCC 3193, and *Bur. Vietnamiensis*
N-Undecanoyl-Lhomoserine lactone	C11-HSL	 (N-[(3*S*)-Tetrahydro-2-oxo-3-furanyl]-undecanamide)	269.40	*P. aeruginosa* strain PAO1
N-Dodecanoyl-Lhomoserine lactone	C12-HSL	 (N-[(3*S*)-Tetrahydro-2-oxo-3-furanyl]-dodecanamide)	283.41	*Klebsiella pneumonia, P. aeruginosa*, and *Sinorhizobium meliloti* Rm1021
N-Tridecanoyl-Lhomoserine lactone	C13-HSL	 (N-[(3*S*)-Tetrahydro-2-oxo-3-furanyl]-tridecanamide)	297.4	*Yersinia pseudotuberculosis*

N-Tetradecanoyl-Lhomoserine lactone	C14-HSL	 (N-[(3*S*)-Tetrahydro-2-oxo-3-furanyl]-tetradecanamide)	311.46	*Y. pseudotuberculosis, Proteus Mirabilis*
N-Pentadecanoyl-Lhomoserine lactone	C15-HSL	 (N-[(3*S*)-Tetrahydro-2-oxo-3-furanyl]-pentadecanamide)	325.50	*Y. pseudotuberculosis*
N-Hexadecanoyl-Lhomoserine lactone	C16-HSL	 (N-[(3*S*)-Tetrahydro-2-oxo-3-furanyl]-hexadecanamide)	339.50	*Rh. Capsulatus*
N-Octadecanoyl-Lhomoserine lactone	C18-HSL	 (N-[(3*S*)-Tetrahydro-2-oxo-3-furanyl]-octadecanamide)	367.70	*Si. Meliloti*

Chapter 3

MATERIALS AND METHODS

3.1. PHASE 1

Microbial population dynamics and profiling of quorum sensing agents in MBR

3.1.1. Extraction and Detection of AHLs

AHLs were extracted from activated sludge of MBR installed at IESE as per method described by S.-R. Kim et al. (2013). Briefly, activated sludge sample (20 mL) was centrifuged at 4000 rpm for 15 min to remove large flocs. The supernatant and ethyl acetate was mixed in a ratio of 1:1. The mixture was vortexed at 120 rpm for 2 h, and organic layer was collected using a separatory funnel. Cell debris were removed by centrifugation at 4000 rpm for 10 min. The supernatant was then dried in a rotary evaporator at 30 ^{o}C and residue was dissolved in 300 µl of methanol. *N*-octanoyl-*DL*-homoserine (C_8-HSL), *N*-hexanoyl-*DL*-homoserine (C_6-HSL) and *N*-butanoyl-*DL*-homoserine (C_4-HSL), purchased from Sigma-Aldrich, were used as standards. These standards were dissolved in methanol to obtain 1 mg/mL stock solution. Working solutions were prepared by mixing 20 µl of stock solution with 980 µl of methanol. Analysis were performed using a water/methanol (35:65) as mobile phase, UV detector along with column C18 (Gemini) was used for HPLC (Perkin Elmer) analysis. AHL standards and samples were injected into HPLC system at a flow rate of 0.25 mL/min.

3.1.2. Isolation of Dominant Bacterial Diversity

Activated sludge samples were collected from semi pilot scale MBR set up treating synthetic wastewater. The dominant microbial consortia were isolated by serial dilution of samples up to 10^{-9}. Each dilution (0.1 mL) was plated on different media including nutrient, Luria Bertani (LB) and tryptone soy agar and incubated at 37 ^{o}C for 24-48 h. Overall, thirty morphologically distinct colonies were isolated as per features described in Table 3.1 and confirmed through 16S rRNA analysis using polymerase chain reaction (PCR, thermocycler 9600).

3.1.2.1. Selection of quorum sensing bacteria

Among all isolated strains, screening of QS bacteria responsible for biofouling behavior was carried out using genetically modified organisms, *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* A136 (received from Water Environment - Membrane Technology lab, Seoul National University, South Korea), as biosensors.

3.1.2.2. Bioassay for quorum sensing bacteria

Bioassay, consisting of an indicating agar plate and bacteria to be tested, was carried out as per method described by Lade et al. (2014b). A fresh culture, of CV026 or A136 and LB agar were mixed in a ratio of 1:9 to prepare indicating agar plate. The indicating agar plates for CV026 was supplemented with kanamycin (20 µg/mL), and spectinomycin (50 µg/mL) and tetracycline (4.5 µg/mL) were added along with X-gal for A136 bioassay, respectively. All strains in the presence of

CV026 and A136 produced purple or blue pigmentation and were considered as potential QS or biofouling bacteria.

3.1.2.3. 16S rRNA sequencing analysis

DNA extraction of selected strains was performed using direct DNA extraction kit (Norgen, Canada) (Figure 3.1). All strains, including general bacterial population as well as QS bacteria, were amplified using universal primers, as listed in Table 3.2, through PCR and gel electrophoresis. After gel purification, sequencing analysis were conducted using services of Macrogen (Seoul, South Korea). Results obtained after sequencing were verified online using NCBI gene bank, http://www.ncbi.nlm.nih.gov/. After obtaining accession numbers, Eztaxon and MEGA 6 softwares were used to generate a phylogenetic tree.

3.1.2.4. Analysis for the biofilm formation

Biofilm formation ability of selected strains was observed using Microtitre plate assay (Figure 3.2). Briefly, wells of micro plate were filled with LB broth for 1h to perform conditioning at room temperature. Wells were then emptied and bacterial suspension along with LB agar was added to each well with a ratio of 1:100 µL. Plates were sealed with para film and incubated at 28 ^{o}C for 24, 48 and 72 h under static condition. A classical crystal violet assay was used to quantify biofilm (Lade et al., 2014b). Amount of crystal violet absorbed by biofilm was extracted with 200 µl ethanol (95%), per well for one h, and OD_{595nm} was measured with a UV-Vis spectrophotometer. A well containing sterile LB broth served as control.

3.1.2.5. Measurement of the bacterial cell hydrophobicity

Bacterial adhesion to the hydrocarbon (BATH) test was used to describe bacterial cell surface hydrophobicity as reported by Chao et al. (2014). Briefly, bacteria were grown in nutrient broth until the mid-logarithmic phase and centrifuged to separate cell pellete and then washed with phosphate urea magnesium (PUM) buffer containing (per litre): 17 g K_2HPO_4, 7.26 g of KH_2PO_4, 1.8 g urea and 0.2 g $MgSO_4.7H_2O$. Cells were again re-suspended in PUM buffer to an optical density of 1.0 measured at 400 nm. An aliquot (1.2 mL) of this suspension was transferred to a series of test tubes, to which increasing volume of hexadecane (0 to 0.2 mL) was added subsequently. The test tubes were vortexed for 15 min and allowed to stand for 2 min. After a complete phase separation, OD_{400} of aqueous phase was measured. A bacteria free buffer served as a reference blank.

Table 3.1. Attributes to study colony morphology

S. No	Morphological attributes	Measuring techniques
1.	Form	Circular, irregular, rhizoid
2.	Elevation	Flat, raised, convex, umbonate
3.	Size	Pinpoint, small, moderate or large
4.	Margin	Entire, lobate, undulate, filamentous
5.	Surface	Dull, shiny
6.	Pigmentation	Intracellular, extracelluar, non chromogenic
7.	Opacity	Opaque, translucent, transparent
8.	Gram reaction	Gram negative or positive

Table 3.2. Primers used in the study

Primers	Sequence (5`⟶3`)	Target genes (bp)	References
H^+	GAGTTTGATCCTGGCTCAG	16S rRNA (19)	(Cheong et al., 2013)
E^-	AGAAAGGAGGTGATCCAGCC	16S rRNA (20)	
518F	CCAGCAGCCGCGGTAATACG	16S rRNA (20)	(Lade et al., 2014)
800R	TACCAGGGTATCTAATCC	16S rRNA (18)	

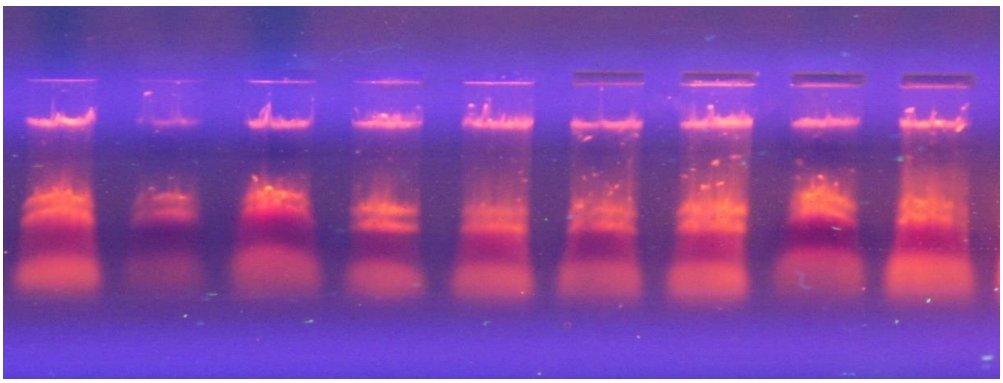


Figure 3.1. Gel picture for DNA extraction

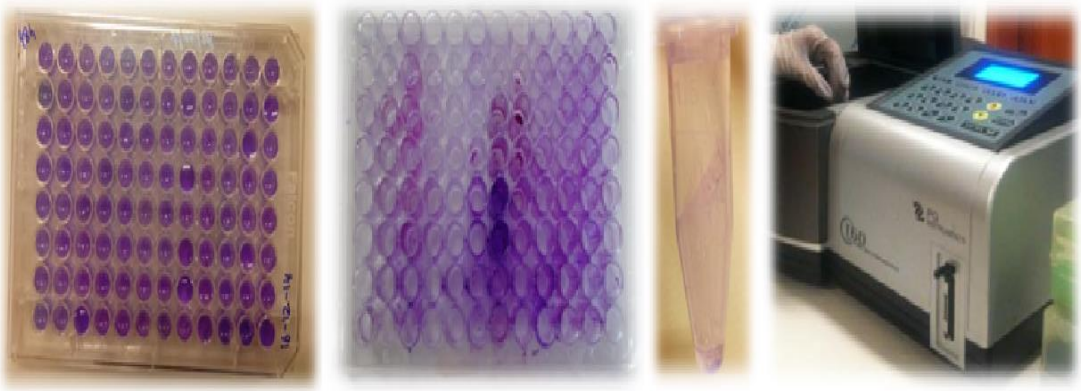


Figure 3.2. Crystal violet assay for the quantification of biofilm

3.2. PHASE 2

High performing antifouling bacterial consortium for submerged MBR

3.2.1. Isolation and Identification of Quorum Quenching Bacteria

For isolation of AHLs degrading or QQ bacteria, 250 µl of activated sludge was sampled from a lab scale MBR fed with synthetic wastewater. Each sample was separately seeded in a 200 µl of minimal medium containing 50 µl AHLs mixture, C6-HSL (2mM) and C8-HSL (1 mM), as sole carbon source and incubated for 3 days (Cycle 1). As per previously described recipe (Christiaen et al., 2011), minimal medium contained (per litre) 1 g of NaCl, 0.5 g of KCl, 0.4 g of $MgCl_2$, 0.1 g of $CaCl_2$, 0.15 g of Na_2SO_4, 2 g of KH_2PO_4, 2.25 g of Na_2HPO_4. The medium was acidified to pH 5.5 using 1 M HCl, and trace elements were added to final concentrations of 1 mg $FeCl_3$, 0.1 g $MnCl_2$ and 46 mg $ZnCl_2$ per litre. Medium was filter sterilized through a 0.22 µm filter and 100 µl of culture medium was transferred to 1 mL of fresh minimal medium containing AHLs and incubated again at 30 ^oC. After three enrichment cycles, 100 µl of aliquot was collected and spread over TSA. After incubation for 24 to 48 h, morphologically distinct colonies were separated and stored at -80 ^oC.

DNA extraction and identification procedure was same as described in section 3.1.2.3. Moreover, identified strains were further assessed in terms of their QQ enzyme location via bio agar assay as per method described by Cheong et al. (2013).

3.2.2. Detection of QQ Activity in Bacterial Isolates

QS inhibition selector strain (QSIS2) i.e. *P. aeruginosa* lasI rhlI double mutant harboring pLasB-SacB1 (received from Ghent University, Belgium), encoding an AHL-induced killing system, was cultured aerobically at 37 ºC in minimal medium supplemented with 0.5% glucose, 0.5% casamino acid and 80 µg/mL gentamicin (Christiaen et al., 2011; Rasmussen et al., 2005). To detect AHL degradation rate of isolates, 900 µl of 24 h culture in tryptone soy broth (TSB) was added in a mixture of AHLs containing C4-HSL and N-3-oxo-dodecanoyl-L-homoserine lactone (3OC12-HSL) (final concentration of 800 nM for each AHL in the reaction mixture). After incubation at 30 ºC for 24 h, reaction mixtures were sterilized upon filtration and added to 24-well micro titre plate containing QSIS2 biosensor. Non inoculated TSB with sterile MilliQ water and AHLs served as a negative (0% activation of QS) and positive control (100% activation of QS) respectively. Inverse relation of QSIS2 growth and remaining AHL level (%) was calculated as follows:

$$\text{Remaining AHL level (\%)} = \frac{(Final\ OD - Initial\ OD)}{Initial\ OD} \times 100$$

3.2.3. Preparation of Polymeric Beads

Polymeric beads, entrapped with single QQ strain, were prepared according to the method described by Kim et al. (2015) with minor modifications (Figure 3.3). Briefly, fresh culture of antifouling or QQ bacteria, grown in 10 mL LB broth, was centrifuged at 4000 rpm for 30 min and re-suspended in autoclaved water. 5 mL of bacterial suspension was mixed with 45 mL of sterile sodium alginate (2% w/v) and final suspension was dropped into 200 mL $CaCl_2$ solution (4% w/v) through a nozzle

at a rate of 1 mL/min using a peristaltic pump. For polymeric coating, pellets of polysulfone were dissolved in N-methyl-2-pyrrolidone (8% w/v) at 60 ^{o}C. Finally, alginate beads were dipped in polymeric solution for 15 s and stored in deionized water at 4 ^{o}C. Average size of beads with diameter and density of 4 mm and 1.8 mg/g alginate respectively was maintained throughout the study. Since separate polymeric beads were prepared for all QQ strains to avoid survival competence and settling issue, their equal proportion was added in MBR keeping the overall ratio of beads to original MBR volume as 2 %.

3.2.4. Bench Scale MBR Setup

Three bench-scale MBRs, each with a working volume of 5 L, were operated continuously (Table 3.3). These include (i) conventional MBR (C-MBR) (ii) MBR with antifouling consortium comprising of 3 indigenous isolates (AC_1-MBR) and (iii) MBR with antifouling consortium comprising of well reported *Rhodococcus* sp. BH4 (Kim et al., 2015) and 2 indigenous isolates (AC_2-MBR). Each reactor was immersed with hollow fiber membrane (Mitsubishi, Rayon) having pore size of 0.05 µm with surface area of 0.07 m^2 (Figure 3.4). Sludge (collected from full scale sewage treatment plant located at sector I-9, Islamabad-Pakistan) was acclimatized for one month with synthetic wastewater. Detailed composition of synthetic wastewater was similar to that mentioned in Table 3.4. MBRs were operated at an optimized filtration (8 min) and relaxation mode (2 min) using peristaltic pumps (Master flex, USA). During the study period, solids retention time (SRT) and hydraulic retention time (HRT) were maintained at 20 d and 4 h respectively, at an operational flux of 20 L/m^2/h.

3.2.4.1. Extraction and quantification of EPS through cation exchange resin

Cation exchange resin method (Frølund et al., 1996) was used for extraction of extra cellular polymeric substances (EPS). Briefly, 50 mL sludge sample, collected from MBR, was centrifuged (4000 rpm for 30 min at 4 ^{o}C) and supernatant was stored as soluble EPS. Sludge pellet was resuspended in phosphoric buffer and stirred for 1 h. After complete mixing, sample was centrifuged (4000 rpm for 15 min at 4 ^{o}C) and stored as loosely bound (LB) EPS. Whereas, for extraction of tightly bound (TB) EPS, sludge pellet was re-dissolved in buffer solution along with resin and centrifuged. Folin–ciocalteu phenolic reagent was used for protein (PN) analysis, and absorption was noted at 750 nm using a spectrophotometer (T60UV, PG Instrument, Britain). For quantification of total polysaccharides (PS), phenol–sulfuric acid (Dubois et al., 1956) method was employed and absorption was measured at 490 nm while concentrations were determined via standard curve using glucose.

3.2.4.2. Membrane resistance analysis

Membranes were evaluated in terms of their fouling potential using a simple resistance in series model (Choo and Lee, 1996):

$$R = \frac{\Delta P}{J\mu}$$

Where, R is hydraulic resistance (1/m), ΔP is the TMP rise (Pa), J is the operational flux ($m^3/m^2/s$), and μ is permeate dynamic viscosity (Pa.s). In MBR, total hydraulic resistance, Rt is the sum of cake layer resistance (Rc), pore blockage resistance (Rp) and intrinsic membrane resistance (Rm).

Rm was measured by filtering clean water through new or chemically cleaned membrane, while Rt was determined at end of each experiment by measuring flux and TMP cross the membrane when Milli-Q H_2O was filtered through. The cake layer was then removed gently with a sponge brush and membrane was submerged in Milli-Q H_2O to measure Rm + Rp. Rc was calculated by subtracting Rm + Rp from Rt.

3.2.4.3. Sludge characterization and permeate quality analysis

AHLs were extracted and detected from biocake and activated sludge of MBR as per method described in section 3.1.1 and concentrated 50 times prior to detection. Details of various sludge characteristics monitored during the entire study are listed in Table 3.5. Chemical Oxygen Demand (COD), Phosphate ($P\text{-}PO_4^-$) and ammonium nitrogen ($NH_4\text{-}N$) were measured regularly to monitor permeate quality, using Standard Methods (APHA et al., 2012). Spectrophotometric method was used to measure concentrations of nutrients.

Table 3.3. Description of QQ bacterial composition in MBRs

MBRs	QQ bacterial consortium	No. of beads in MBR (1ml = 15 beads)	Biomass concentration (g/L)	Operational flux ($L/m^2/h$)
C-MBR	Nil	Nil	6-8	20
AC_1-MBR	*Enterobacter cloaca* *Delftia sp.* *Pseudomonas sp.*	400 400 400	6-8	20
AC_2-MBR	*Rhodococcus* RBH4 *Delftia sp.* *Pseudomonas sp.*	400 400 400	6-8	20

Table 3.4. Composition of synthetic wastewater used in Phase 2

Chemicals	Chemical formula	Quantity (mg/L)
Glucose	$C_6H_{12}O_6$	500
Ammonium chloride	NH_4Cl	190
Potassium dihydrogen phosphate	KH_2PO_4	55.6
Calcium chloride	$CaCl_2$	5.5
Magnesium sulphate hepta hydrate	$MgSO_4.7H_2O$	5.7
Ferric chloride	$FeCl_3$	1.5
Mangnese chloride	$MnCl_2$	1
Sodium bicarbonate	$NaHCO_3$	pH adjustment

Table 3.5. Details of sludge characteristics measured during the study

Parameters	Units	Purpose	Instrument/Protocol
Dissolved oxygen (DO)	mg/L	Sludge characterization	Multimeter Fisher Scientific, XL600
Soluble oxygen uptake rate (SOUR)	mg/hr/g		
Zeta potential	mV		Malvern MRK, 570-01
Electrical conductivity (EC)	mS/cm		
Mixed liquor suspended solids (MLSS)	mg/L		APHA et al., 2012
Mixed liquor volatile suspended solids (MLVSS)	mg/L		
Sludge volume index (SVI)	mL/g	Sludge settleability	Imhoff cone
Capillary Suction Time (CST)	Sec	Sludge dewaterability	CST apparatus (Triton Canada) Ofite, US 294-50
Particle size	µm	Size distribution	Particle size analyzer (PSA) SALD-3101, Shimadzu
Floc size distribution	µm		Fluroscent microscope, Nikon-ECLIPSC

Figure 3.3. Alginate beads coated with polysulfone

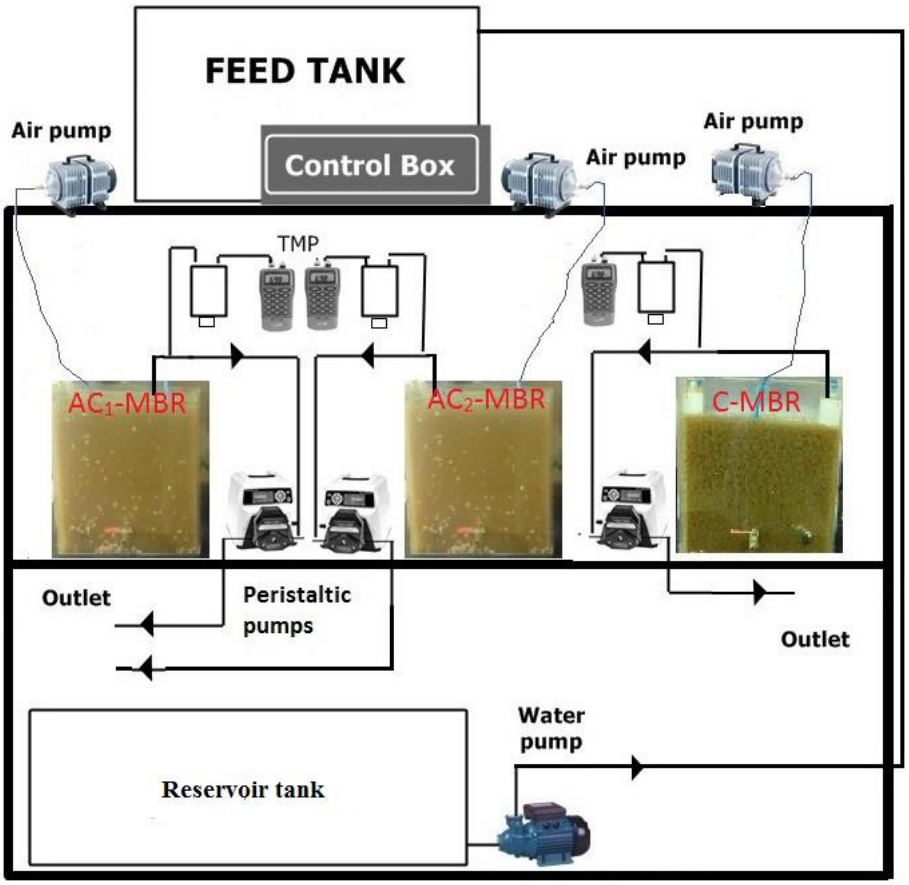


Figure 3.4. Schematic of lab scale MBRs setup used for Phase 2

3.3. PHASE 3

Insights into quorum quenching mechanisms to control membrane biofouling under transient conditions

3.3.1. Experimental Setup

Experiment was conducted under varying (Phase I) and fixed OLR (Phase II) in duplicate (Table 3.6). In Phase I four MBRs, each with a working volume of 450 mL; (i) control MBR (C-MBR), (ii) control MBR with empty beads (Cb-MBR), (iii) MBR with addition of QQ bacteria entrapped in polymer-coated alginate beads (QQb-MBR), and (iv) MBR with addition of QQ bacteria in broth (QQs-MBR) were set up and operated simultaneously (Figure 3.5). Under fixed OLR (Phase II), two more QQb-MBR were added in addition to four MBRs; when C-MBR, Cb-MBR and QQs-MBR fouled, one of the QQb-MBR was stopped, and levels of EPS and other fouling parameters in both MBRs were analyzed to gain better insight of QQ mechanism along with operation cycle. In Phase I, OLR was gradually increased from 4 to ~ 7 mg COD/ g VSS.hr via wasting sludge from MBRs; while in Phase II, OLR was maintained constantly via supplementing seed sludge.

Hollow fiber membranes with a pore size of 0.1 μm and surface area of 0.0045 m^2 were used in present study. Sludge (collected from Ulu Pandan Water Reclamation Plant, Singapore) was acclimatized to a synthetic wastewater for one month before being used as seed sludge. During study, SRT and HRT were maintained at 30 d and 12 h, respectively, at an operational flux of 8.5 $L/m^2/h$ (LMH) unless otherwise mentioned. A constant flux of 8.5 LMH was maintained to keep fouling at a reasonable rate.

3.3.2. Bacterial Immobilization

A QQ consortium comprising of four AHLs degrading strains *Enterobacter cloaca* (QQ13), *Microbacterium* sp. (QQ1), *Pseudomonas* sp. (QQ3), isolated from activated sludge (the 16S rRNA gene accession number: KR058854, KR058848 and KR058846, respectively), *Rhodococcus* sp. RBH4 (S.-R. Kim et al., 2013) and an AI-2 degrading *E. coli* strain ΔlsrRΔluxS (Thompson et al., 2015) were selected for the study. Polymeric beads, entrapped with a single QQ strain, were prepared according to the method described by Kim et al. (2015) with some modifications (section 3.2.3). For QQs-MBR, strain suspensions were directly added into the sludge. The QQ strains were entrapped separately in polymeric beads to avoid non-homogenous mixing, and added in equal proportions to MBRs keeping the overall ratio of beads to original MBR working volume at 2%.

3.3.3. Quantification of Signal Molecules

AHLs were extracted from the biocake and activated sludge (50 mL) of MBR as per method described in section 3.1.1. Samples were concentrated 100 times and analyzed using an Agilent 6460 (USA) QQQ triple quadrupole mass spectrometer (MS) equipped with an AJS electro-spray interface. The instrument settings were optimized as follows: interface voltage, 3.5 kV; drying gas temperature, 325 °C; drying gas (N_2) flow rate, 8 L/min; sheath gas temperature, 300°C; sheath gas (N_2) flow rate, 11 L/min. An Agilent 1290 Infinity II HPLC was used for chromatographic separation, which was achieved on a 100x2.1 mm Kinetex 2.6 µm Bi-phenyl column (Phenomenex, USA) using 0.1% formic acid (A) and methanol with 0.1% formic acid (B) as mobile phase at a gradient of 5 to 85% B within 12 min.

One milliliter of sample was used to analyze concentration of AI-2 in sludge supernatant via bioluminescence method using reporter strain *Vibrio harveyi* BB170 (Taga and Xavier, 2011). Concisely, fresh culture of BB170 (OD600 = ~0.3 to 0.5) was diluted 2000 times in a freshly-prepared AB medium. Duplicate mixture containing 10 % (v/v) sample along with 90 % (v/v) diluted BB170 culture was added to a microtitre plate and incubated at 30 ^{o}C with rotary mixing at 150 rpm. The same amount of Milli-Q water was used as for negative controls. The luminescence produced in each sample was measured using a microtitre plate reader (Infinite M200 Pro, Tecan, Switzerland) at a wavelength of 490 nm. Measurements were recorded every 30 min from 5 to 8 h, until negative control samples showed lowest amount of luminescence. A precursor of AI-2, 4,5-dihydroxy-2,3-pentanedione (DPD) (Omm Scientific, Dallas, TX, USA), was used as calibration standard (Annex IIIa).

3.3.4. Quantification of EPS through Heat Extraction Method

The heat extraction method (Li and Yang, 2007) was used for extraction of extracellular polymeric substances (EPS). Briefly, 15 mL of sludge was collected from MBRs, centrifuged at 4000 rpm and 4 ^{o}C for 30 min (this condition was also used for the following centrifugation steps unless otherwise stated), and supernatant stored as soluble EPS. The sludge pellet was re-suspended in 0.05 % (w/v) NaCl and homogenized; the sample was centrifuged (4000 rpm for 15 min at 4 ^{o}C) and supernatant stored as LB EPS. The sludge pellet was re-suspended in same NaCl solution and incubated at 60 ^{o}C for an hour; after centrifugation the supernatant was

stored as TB EPS. Detection protocol for protein and polysaccharides is same as illustrated in section 3.2.3.1.

Table 3.6. Experimental setup and operating conditions

	Phase I	Phase II
Experimental conditions	Varying organic loading rate	Fixed organic loading rate
MBRs setup	Control MBR (C-MBR) Control MBR with vacant beads (Cb-MBR) MBR with QQ beads (QQb-MBR) MBR with QQ sludge (QQs-MBR)	
Reactor volume (mL)	450	450
Membrane type	Hollow fiber, PVDF	Hollow fiber, PVDF
Flux (LMH)	8.5	8.5
HRT (h)	12	12
SRT (d)	30	69

Figure 3.5. Batch scale MBRs set up for Phase 3

3.4. PHASE 4

In tandem effect of PAC and QQ on simultaneous removal of pharmaceutical compounds and membrane fouling control in submerged MBR

3.4.1. QQ Bacteria

A QQ consortium, mentioned in section 3.3.2 was selected for Phase 4 as well. The AHL-degrading strains were grown in LB broth at 30 °C for ~ 16 hours prior to immobilization in beads, while AI-2-degrading strain was grown in LB broth containing 100 mg/L of streptomycin at 37 °C for ~ 16 hours.

3.4.2. Beads Preparation

3.4.2.1. Vacant PAC beads for adsorption test

Batch adsorption experiments were performed to optimize PAC content in integrated PAC beads and bead dose. Alginate beads were prepared by mixing PAC (SAE2, Norit, the Netherlands) with Na-alginate solution (2% w/v) at different ratios (1% or 2%, Table 3.7) and dripping into $CaCl_2$ solution (4 % w/v) through a nozzle at a rate of 1 mL/min using a peristaltic pump; after soaking in the $CaCl_2$ solution for 3 hours, half of the beads were dipped in polysulfone solution (8 % w/v in N-methyl-2-pyrrolidone) for 10 seconds before transferring to Milli-Q H_2O and stored at 4°C until use, while remaining beads were transferred to Milli-Q H_2O directly. Beads without PAC were also prepared to evaluate adsorption to alginate beads alone.

3.4.2.2. Vacant and QQ-PAC beads for MBR

The optimal dose of PAC in the alginate beads obtained from the adsorption test was used in MBR test. The beads were prepared in similar manner as mentioned above, except that QQ bacterial suspension, which was obtained via harvesting the fresh-grown QQ culture at 4000 rpm for 30 min and resuspending in a phosphate buffer solution (PBS), was added (10% v/v) to PAC-Na-alginate mixture for QQ-entrapped PAC beads. The QQ strains were entrapped separately in the beads to avoid non-homogenous mixing, and added in equal proportions to MBRs.

3.4.3. Adsorption Tests

The traditional bottle-point method was used for adsorption test. Five pharmaceutical compounds (PhAC), namely trimethoprim (TrMP), sulfamethoxazole (SMX), carbamazepine (CBZ), diclofenac (DCF) and triclosan (TCS), were collectively used as target adsorbates. For each type of beads, five specific amounts of beads were added to 150-mL serum bottles containing 50 mL of PhAC solution to gain five different volume loads (1, 2, 5, 10 and 20%). The bottles were kept on a shaker at 150 rpm and 25 ^{o}C for 7 days (Table 3.7). Samples were collected from each bottle at predetermined intervals to estimate the time required for reaching equilibrium.

3.4.3.1. Extraction & detection of PhAC from water samples and beads/sludge

Solid-phase extraction (SPE) was used to extract PhACs from aqueous samples. 30 µm Oasis® HLB cartridge (Waters, Singapore), having 6 mL capacity,

was selected to concentrate analyte for further analysis (Figure 3.6). Briefly, water sample or reactor supernatant (50 mL) was centrifuged at 12,000 rpm for 5 min; 1 µg/L PhAC internal standards from a 1 mg/L stock in methanol was added before filtration with 0.45 µm Supor® membrane syringe filters (Pall, Singapore). Conditioning of SPE cartridge was acheived using acetone (5 mL) followed by Milli-Q H_2O (5 mL). Sample was loaded under gravity flow and dried under vacuum for 45 min. The cartridge was then sequentially eluted with 5 mL of methyl tert-butyl ether (MTBE)/acetone (9:1) and 5 mL acetone. The eluted volumes were combined, dried under vacuum to make final volume less than 1 mL, reconstituted to 1 mL using acetonitrile, and stored at -20 °C before analyzing through LC/MS/MS.

The amount of PhACs adsorbed onto sludge or beads were quantified. PhACs were extracted from sludge using 1 mL of mixed liquor sample from MBR. Sample was centrifuged at 6,000 rpm for 5 min and pellet was washed with Milli-Q H_2O once and re-suspended in 1 mL of MTBE/acetone (1:1) to which 1 µL of a 1 mg/L PhAC internal standard was added. Mixture was then vortexed at 3000 rpm for 2 min followed by centrifugation. The supernatant transferred to a glass test tube and dried under N_2. Resulting material was finally re-suspended in 150 µL of acetonitrile and filtered through a 0.22 µm PTFE syringe filter. Whereas, similar procedure was used for extraction of PhACs from beads, ten beads were randomly selected from each serum bottle and crushed into small fractions.

The PhACs were analyzed with a triple quadrupole mass spectrometer equipped with a Z-spray electro-spray interface (LCMS-8030, Shimadzu, Japan). The instrument settings were optimized as follows: interface voltage, 3.5 kV; desolvation line temperature, 250°C; heat block temperature, 400°C; desolvation gas

(N_2) flow rate, 3 L/min; drying gas (N_2) flow rate, 15 L/min; collision gas (argon) pressure, 230 kPa. The chromatographic separation was achieved on a 100x2.1 mm Kinetex 2.6 µm Bi-phenyl column (Phenomenex, USA) using 0.1% formic acid (A) and methanol with 0.1% formic acid (B) as the mobile phase at a gradient of 10% to 85% B within 12 min (Annex IV).

3.4.4. Lab Scale MBR Setup

A cylindrical acrylic reactor (working volume of 3.2L) equipped with a hollow fiber ultrafiltration PVDF membrane module (ZeeWeed, GE, US) with a pore size of 0.04 µm and surface area of 0.047 m^2 was operated as a conventional MBR (MBR-C), MBR with vacant PAC beads (MBR-PAC_V), and MBR with QQ-PAC beads (MBR-PAC_{QQ}) sequentially (Figure 3.7). For the latter two modes with beads inside, membrane was backwashed with effluent at the same flux as of filtration for 5 min every 2 hours. Each cycle was terminated when the TMP values reached 30 kPa; then, membrane was removed from the reactor, soaked in sodium hypochlorite solution (2%) for an hour and rinsed thoroughly with tap water prior to next run except for MBR-C where virgin membrane was used. A synthetic wastewater (Table 3.8) was used as the feed for MBR-C, while 5 PhAC were supplemented to feed at concentrations of ~ 2 µg/L for the others.

The sludge (collected from Ulu Pandan Water Reclamation Plant, Singapore) was acclimatized to the synthetic wastewater for one month before being used as seed sludge. Mixed liquor suspended solids (MLSS) concentration was maintained in typical range of MBR (5 ~ 6.5 g/L) throughout the study. The solids retention time (SRT) and hydraulic retention time (HRT) were maintained at 30 d and 6 h,

respectively, at an operational flux of 15 L/m^2/h (LMH). The beads were added to MBR at a volume ratio of 5%.

3.4.4.1. Characterization of soluble microbial products (SMP)

Size-exclusion chromatography, in combination with organic carbon and nitrogen detection (LC-OCD-OND) (DOC-LABOR, Germany) was used to characterize and quantify SMP. High-performance liquid chromatography (HPLC) (Agilent, USA) was used to deliver on-line purified mobile phase to an auto sampler and chromatographic columns. The refractive index (RI) detector was used for universal visualization of materials and ultraviolet (UV) light at 254 nm was used as detectors for specific visualizations of aromatic compounds such as proteins and humic acids (Yang et al., 2013). The molecular weight (MW) was calibrated using polyethylene glycol and polyethylene oxide standards with MW of 500, 70, 4, 0.6 and 0.1 kDa. Prior to analysis, samples from mixed liquor and permeate were filtered through 0.45 µm hydrophilic filters. The software from the manufacturer was used for data acquisition and processing (Huber et al., 2011).

3.4.4.2. Detection of PhAC, AHLs and AI-2 molecules through LC/MS/MS

An Agilent 6460 (USA) QQQ triple quadrupole mass spectrometer (MS) equipped with an AJS electro-spray interface was used for analysis of PhAC, AHLs and AI-2. One milliliter of sample was used to analyze concentration of AI-2 in sludge supernatant. A precursor of AI-2, 4,5-dihydroxy-2,3-pentanedione (DPD) (Omm Scientific, Dallas, TX, USA), was used as calibration standard. The DPD-M1CQ was analyzed with a triple quadrupole mass spectrometer equipped with a Z-spray electro-spray interface (LCMS-8030, Shimadzu, Japan) following separation

with a HPLC on a 2.1x100 mm Poroshell 120 2.7 µm SB-Aq column (Agilent, US). Chromatographic separation curves for AHLs, AI-2 and PhACs are shown in Annex I, III and IV respectively.

The instrument settings were optimized as follows for all measurements: interface voltage, 3.5 kV; drying gas temperature, 325 °C; drying gas (N_2) flow rate, 8 L/min; sheath gas temperature, 300°C; sheath gas (N_2) flow rate, 11 L/min. An Agilent 1290 Infinity II HPLC was used for chromatographic separation. For both PhAC and AHLs, a 100x2.1 mm Kinetex 2.6 µm Bi-phenyl column (Phenomenex, USA) was used with 0.1% formic acid (A) and methanol with 0.1% formic acid (B) as the mobile phase at a gradient of 10% (5% for AHLs) to 85% B within 10 min. For AI-2, a 2.1x100 mm Poroshell 120 2.7 µm SB-Aq column (Agilent, US) was used with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) as the mobile phase at a gradient of 35 to 85% B within 5.5 min.

Table 3.7. Batch scale experimental set up for optimization of PAC concentration

Sets	Beads composition			Beads to volume ratio (%)	Concentration of micro pollutants in solution
	Sodium alginate % (w/v)	PAC % (w/v)	Polysulfone coating % (w/v)		
Set 1	2	1	-	1, 2, 5, 10, 20	10mM
Set 2	2	2	-	1, 2, 5, 10, 20	10mM
Set 3	2	-	8	1, 2, 5, 10, 20	10mM
Set 4	2	1	8	1, 2, 5, 10, 20	10mM
Set 5	2	2	8	1, 2, 5, 10, 20	10mM

Table 3.8. Synthetic wastewater composition for lab scale MBR

Chemicals	Quantity (mg/L)
Glucose ($C_6H_{12}O_6$)	1000
NH_4Cl	190
$KH_2\ PO_4$	55.6
$CaCl_2$	5.5
$MgSO_4\ .7H_2O$	5.7
$FeCl_3$	1.5
$MnCl_2$	1
$NaHCO_3$	pH adjustment
Pharmaceutical compounds (PhACs)	**Quantity (µg/L)**
Triclosan (TCS)	2
Sulfamethoxazole (SMX)	2
Trimethoprim (TrMP)	2
Carbamazepine (CBZ)	2
Diclofenac (DCF)	2

Figure 3.6. Solid Phase Extraction (SPE)

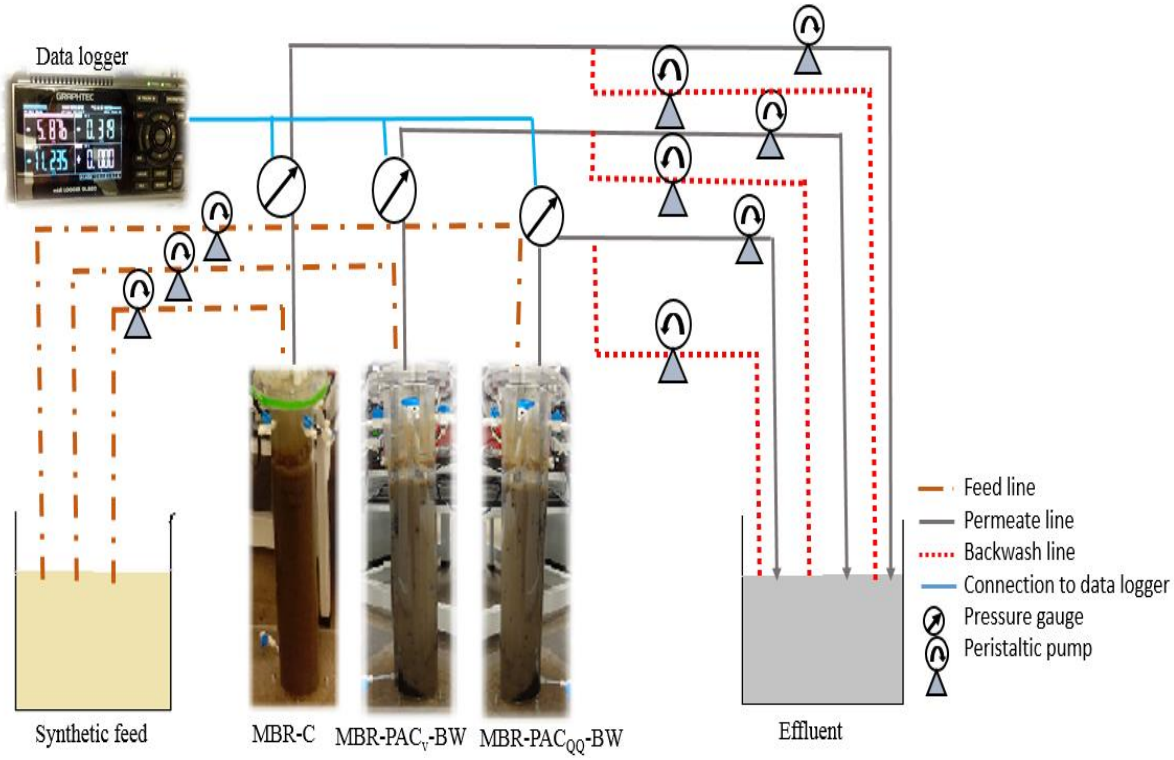


Figure 3.7. Schematic of lab-scale MBRs setup for Phase 4

Chapter 4

RESULTS AND DISCUSSION

4.1. PHASE 1

Microbial population dynamics and profiling of quorum sensing agents in MBR

Phase 1 was specifically designed to investigate microbial diversity and their characteristics in a newly fabricated semi pilot scale MBR system with effective volume of 35L. This is a well established fact that bacterial species of a certain MBR cannot be compared and generalized for other MBR systems. Hence, profiling of signal molecules and bacterial communities, present in activated sludge of MBR via 16S rRNA analysis, was carried out to understand the detailed picture of agents responsible for biofouling mechanism in MBR, being operated in environmental conditions of Pakistan. Furthermore, all strains were evaluated in terms of EPS production, types of AHL released and hydrophobicity to authenticate nature of biofouling bacteria, need to be targeted before selecting QS inhibition strategy.

4.1.1. Evidence of AHLs in Activated Sludge of MBR

The presence of signal molecules, produced by gram negative bacteria, was confirmed using two different reporter strains. During early phase of reactor operation, no small chain AHLs were detected via CV026, which might be attributed to lesser concentration of AHLs in activated sludge of MBR than the threshold amount required by reporter strains for their detection. However, all tested sludge

samples, upon reaching stable degradation phase, showed a blue and purple coloration, authenticating abundance of medium and long chain AHL molecules in various forms of sludge (Li et al., 2014). Moreover, HPLC analysis was also conducted to detect types of AHLs existing within the activated sludge. The presence of AHLs, i.e. C4-HSL, C6-HSL and C8-HSL was witnessed at the retention times of 5.6, 6.5 and 10.7 min respectively (Figure 4.1).

4.1.2. Isolation and Identification of Dominant Bacterial Diversity

Sludge characterization is an important indicator to evaluate dominant bacterial diversity and their role in overall treatment performance (Li et al., 2014). Therefore, a total of 30 dominant strains were isolated from activated sludge in a lab-scale MBR using several media. Out of them, 13 morphologically distinct strains were identified as dominant bacterial consortia, indicated in Figure 4.2. Phylogenetic analysis revealed the dominance of *Serratia* sp. (33%), *Pseudomonas* sp. (25%) and *Enterobacter* sp. (17%) in mixed liquor of MBR, while other strains belonged to *Raoultella*, *Bacillus*, and *Klebsiella* sp. Among all, *Serratia* sp. have been considered as efficient bioflocculant producing bacterium (Labbate et al., 2004), while, *Pseudomonas* sp. has been well reported for their diversity and adaptability in all kinds of environment. Dominance of *Pseudomonas* family in activated sludge may confirm evidence of denitrification in MBR (Khan et al., 2013). *Enterobacter* sp. has also been well reported for the development of mature biofilms within pilot-scale MBR (Lim et al., 2012; Xia et al., 2008). Furthermore, *Klebsiella* sp., has been studied for its ability to fix nitrogen in wastewater treatment. Hence, presence of these species may verify better nitrification (Waheed et al., 2013). All reported

species and their role in overall treatment performance have also been discussed by Chong et al. (2010) and Lade et al. (2014b).

4.1.3. Screening of AHLs Producing Bacteria

Among various signal molecules or autoinducers, such as oligopeptides of gram-positive bacteria, AHLs of gram-negative bacteria and AI-2 for interspecies bacterial communication (Yeon et al., 2009), research was focused on AHLs based QS as most of the strains were found to be gram negative (Figure 4.2 & 4.3). Studies have reported that AHLs have a variety of acyl chains and each bacterium use its own AHL to initiate QS. Therefore, two biosensor systems, A136 and CV026, were employed for screening of short and medium/long chain AHLs producing strains (Table 4.1). As a result, a total of 9 strains, as shown in Figure 4.3, were considered as potential QS bacteria, based on the appearance of blue and purple coloration using reporter strains. Almost all AHL producing strains were belonged to proteobacteria and most of the LuxI/R QS genes have also been identified within this phylum. Therefore, presence of QS regulation have already been confirmed by various studies in all isolates including *Staphylococcus* (Zhang et al., 2004), *Serratia* (Wei et al., 2006), *Pseudomonas* sp. (Juhas et al., 2005), *Klebsiella* (Balestrino et al., 2005), *Psychrobacter* (Jay et al., 2003) and *Delftia* sp. (Maisuria and Nerurkar, 2015). Hence, evidence of these species in production of AHLs in AS of MBR confirm their role in development of matured biofilms via regulating QS interspecies communication.

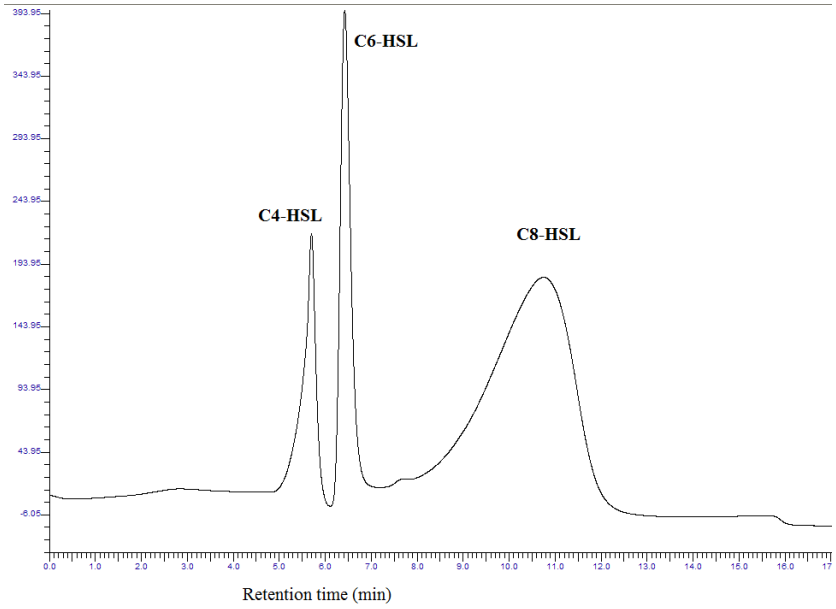


Figure 4.1. Presence of AHLs in activated sludge using HPLC

Table 4.1. AHLs production by QS strains using violacein and β-galactosidase assay

Accession No.	Strains	Biosensors	
		CV026	A136
KR058824	*S. aureus*	+++	+++
KR058825	*Serratia* sp.	+++	+++
KR058828	*P. flourescens*	---	+++
KR058831	*P. kilonensis*	---	+++
KR058836	*Pseudomonas* sp.	---	+++
KR058838	*Delftia* sp.	+++	---
KR058841	*P. aeruginosa*	---	+++
KR058842	*Klebsiella* sp.	---	+++
KR058844	*Psychrobacter* sp.	---	+++

* +++ Showed Purple (CV026) /Blue coloration (A136), ---- Showed no coloration

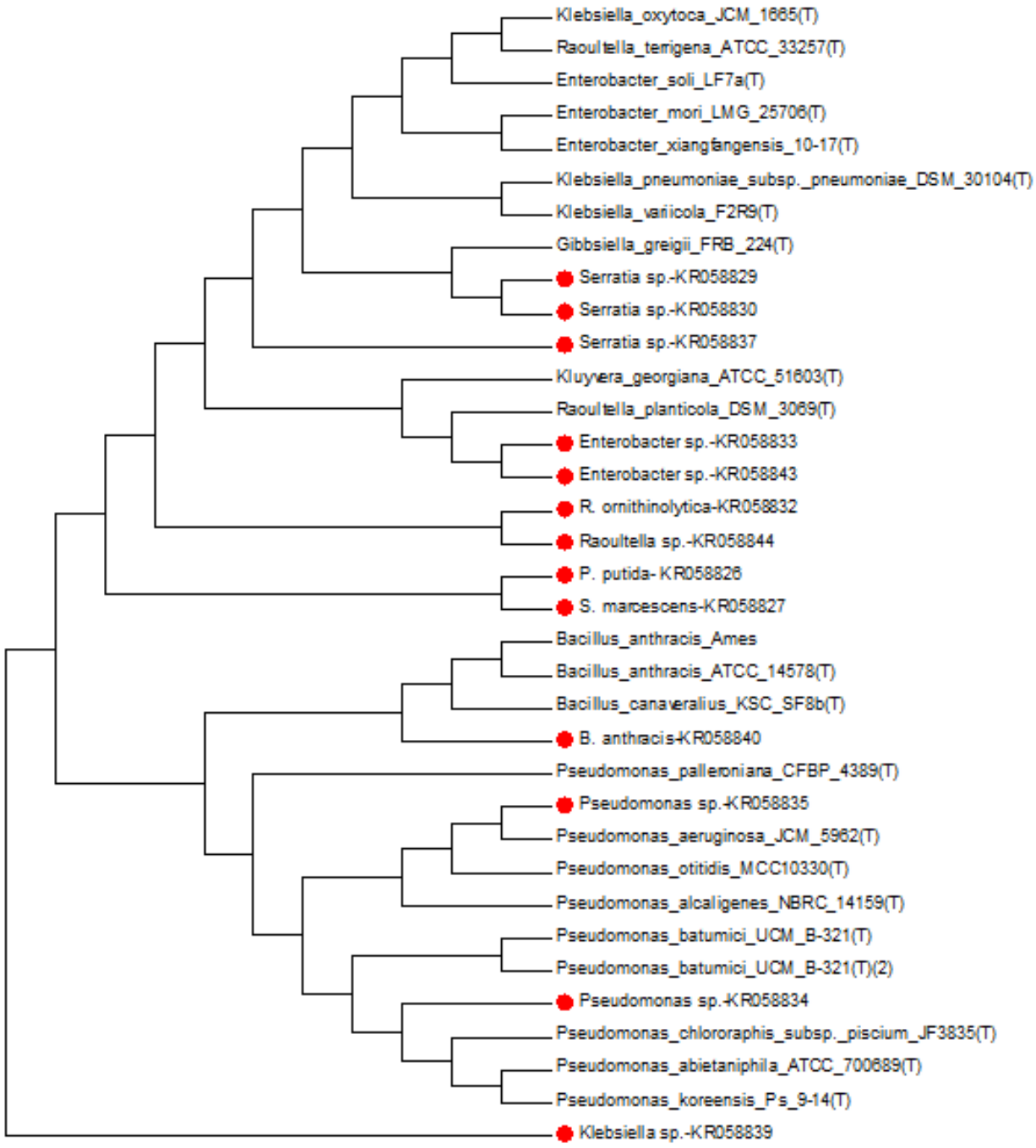


Figure 4.2. Phylogenetic tree of dominant bacterial diversity present in activated sludge of MBR (Species isolated in this study are highlighted with red dots)

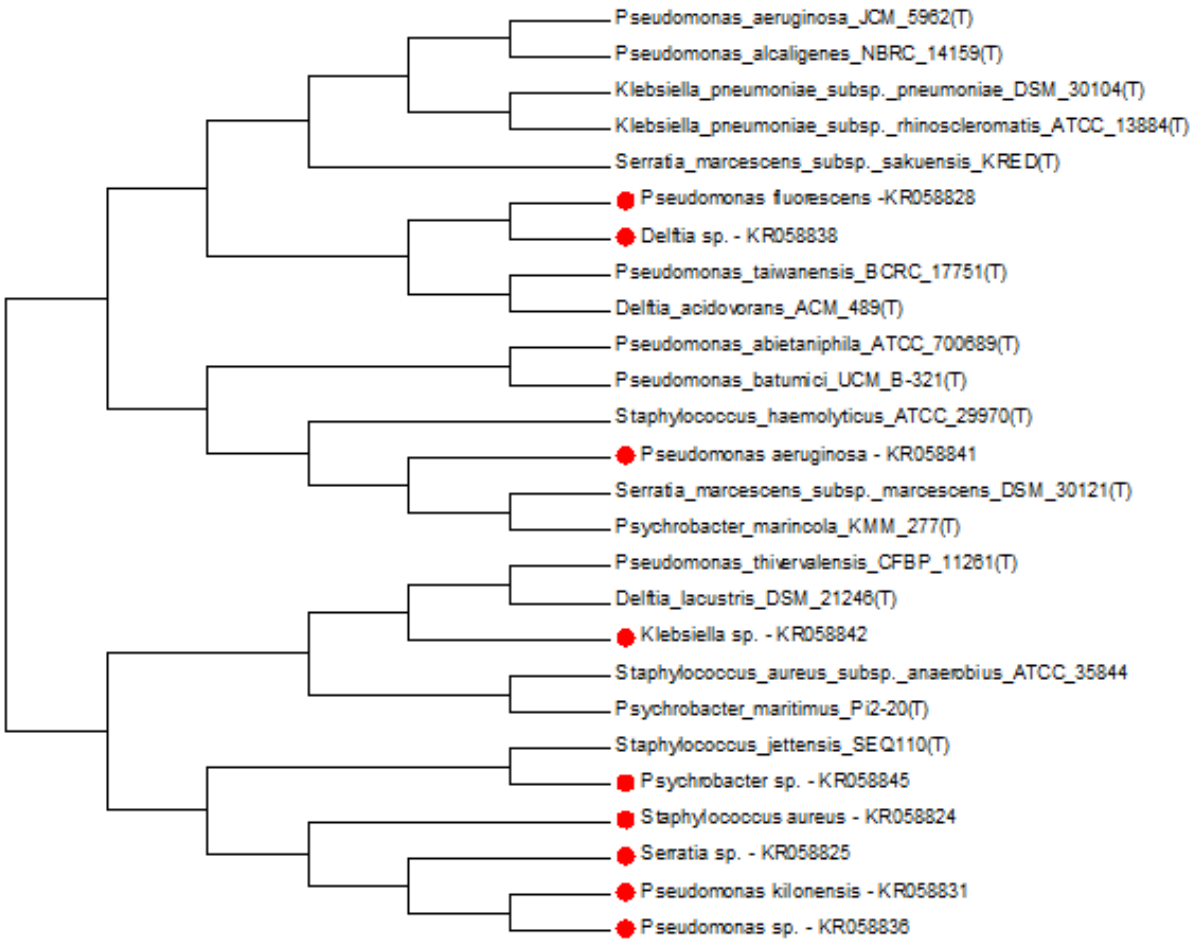


Figure 4.3. Phylogenetic tree of quorum sensing bacteria based on 16S rRNA gene sequences (Species isolated in this study are highlighted with red dots)

4.1.4. Correlation between Bacterial Growth Rate and Biofilm Formation Ability

Depending on growth rate and environmental conditions, most of the bacteria establish highly organized and complex communities in the form of biofilm. Development of matured biofilms highly depends on bacterial innate ability for survival and colonization on membrane surfaces. In the current study, an attempt was made to evaluate correlation between bacterial growth rate (Figure 4.4) and their biofilm formation ability (Figure 4.5) using crystal violet assay. Among dominant bacterial diversity, maximum growth rate of 0.47 ± 0.004 and 0.45 ± 0.004, after 72h of incubation, was indicated by *Pseudomonas* sp. and *Klebsiella* sp. respectively (Figure 4.4a); however, maximum biofilm formation ability of 0.38 ± 0.005 and 0.23 ± 0.02 was observed by *Raoultella ornithinolytica* and *Bacillus anthracis* respectively (Figure 4.5a). Biofilm formation of non-AHL producing species in the microtitre plate may explain role of other mechanisms like AI-2 etc. in QS (Shrout and Nerenberg, 2012).

Furthermore, to evaluate role of QS strains in development of biofilm, maximum growth rate of 0.61 ± 0.01, 0.41 ± 0.015 and 0.38 ± 0.001 in terms of OD at 655 nm was reflected by *Psychrobacter* sp., *P. aeruginosa* and *Klebsiella* sp. respectively (Figure 4.4b); however, maximum biofilm formation ability of 0.37 ± 0.015 and 0.23 ± 0.03 in terms of AHL production was observed by *P. kilonensis* and *Psychrobacter* sp. respectively (Figure 4.5b). Quantification of biofilm, at different time intervals, confirms that all strains seem to have different biofilm forming tendencies, irrespective of their growth rates (Lade et al., 2014b). Hence, it may be concluded that rate of biofilm formation does not always depend on bacterial

growth, rather there are some other agents (or chemical signals) responsible for gauging population density.

A significant increase in biofilm development may also be seen with prolonged incubation up to 72 h. After 24 h of incubation, i.e. first substrate degradation phase, amount of nutrients in broth may provide enough energy for biomass in sludge, inducing enhancement activity of chemical signal-based QS in biomass. As investigated by Jiang and Liu (2012), AHL-based QS is ATP dependent, which is subject to energy of metabolism. Moreover, an increase in incubation time also resulted into depletion of substrate, as a result, activity of AHL-based QS found to be reduced after 48 h to a low level, due to lack of energy that could be provided to microorganisms in sludge. However, with prolonged starvation, signal molecules-based QS might be recovered. As described by Ward et al. (2003), bacteria release more AHLs or other signal molecules to adapt to the hostile environment by enhancing cell to cell communication during formation of biofilm. Therefore, many species including *Salmonella*, *Serratia*, *Raoultella*, *Enterobacter*, *Klebsiella*, *Bacillus anthracis* (Figure 4.5a), *S. aureus*, *Pseudomonas* and *Psychrobacter* (Figure 4.5b) witnessed the same mechanism even after 72 h of incubation. Thus, it may be concluded that AHL-based QS is related to energy based metabolism in feast condition, however, in prolonged starvation, microorganisms would secrete more AHL-like molecules thereby protecting themselves to resist starvation (Li et al., 2014). Furthermore, it has also been reported earlier that some bacteria may modify their behavior in a coordinated fashion, via chemical signals (Shrout and Nerenberg, 2012). Current results of biofilm formation are in agreement with the previous

studies that may establish the correlation between QS signal molecules and biofilm formation.

4.1.5. Correlation among AHL-Based QS, EPS & Hydrophobicity

A significant factor in the development of biofilm growth is self-production of matrix material containing a mixture of EPS. However, very little is known about QS-regulated control of EPS regulation. Figure 4.6 shows release of EPS by short/long chain AHL-producing QS strains after 24 h of incubation. Results revealed a positive correlation between AHL-based QS and EPS production in case of *P. aeruginosa* and *Delftia* sp. where the concentration in terms of protein (PN) and polysaccharides (PS) content was found to be 397 and 379 mg/L, respectively, and their biofilm forming tendencies were found to be more or less same. Although *Psychrobacter* sp. produced less EPS as compared to other species, still showed maximum biofilm formation after 24 hrs (Figure 4.5b), so it may be anticipated that amount of EPS, required to form matured colonies, varies depending on type of genus or species. Some strains are required to release more EPS in order to form a primary scaffolding, that may aid in attachment to a surface as shown by *Pseudomonas* sp. (Whitchurch et al., 2002).

The ability of microorganisms to utilize any substrate depends on its growth prevalence and adherence to that particular substrate. Generally, it is observed that membrane surface is hydrophobic in nature unless treated to increase its hydrophilicity. It has been reported that more hydrophobic the bacterial cell surface, higher would be the interaction with membrane. In this study, bacterial relative hydrophobicity (RH) as percentage of bacterial cells adhering to hydrocarbon was

measured using BATH test (Chao et al., 2014). The highest hydrophobicity was observed for *Pseudomonas aeruginosa* that showed 31% decrease in OD of aqueous phase depicting migration of bacteria towards hydrocarbon phase of culture solution (Figure 4.6). *Serratia* and *S. aureus* also showed significant adhesion towards hydrocarbons, showing their ability to adhere to hydrophobic surface. While, all other strains seem to be more hydrophilic, hence may have more tendency to cause membrane biofouling. A positive correlation between EPS production and RH was witnessed in most of the strains while role of bacterial cell wall composition in hydrophobicity of cell surface has also been documented (Palmer et al., 2007). Another test, i.e. capability to aggregate salt, was performed where it was assumed that more the strain is hydrophobic, less salt would be required for aggregation that is in agreement with results of salt aggregation test (SAT) (Table 4.2).

4.1.6. Summary

The study highlighted prevalence of dominant as well as biofouling bacteria and their subsequent molecular diversity in an MBR system treating synthetic wastewater. Biofouling bacteria showed inherently different biofilm formation tendencies, which are not associated to their growth rates, depicts role of different factors (EPS release, cell hydrophobicity, etc.) in QS. *P. kilonensis* followed by *Delftia* sp. showed highest EPS production with correspondingly high biofilm formation making them most prominent QS strains. The study also demonstrated that amount of EPS required to form matured colonies varies depending on type of genus or species. Higher AHL concentration increases the biofilm formation however, QS is not solely regulated by one group of chemicals (e.g. AHLs) rather, non-AHLs

molecules also have a key role in QS mechanism, which needs to be addressed. This provides a guideline for developing QS inhibition strategy targeting these strains to control biofouling in MBR system.

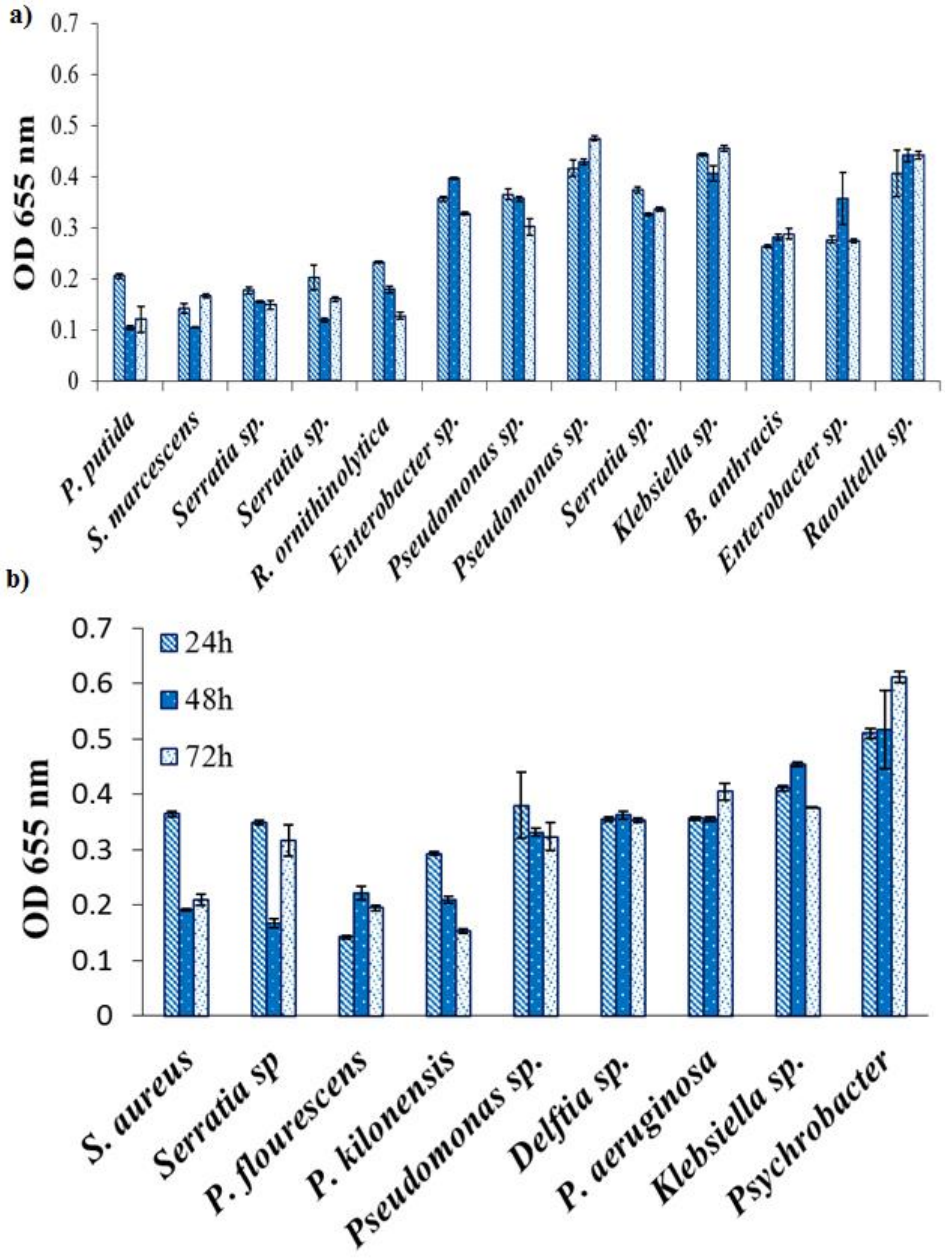


Figure 4.4. Bacterial growth rate of a) Dominant bacterial diversity present in activated sludge of MBR, b) AHL producing strains

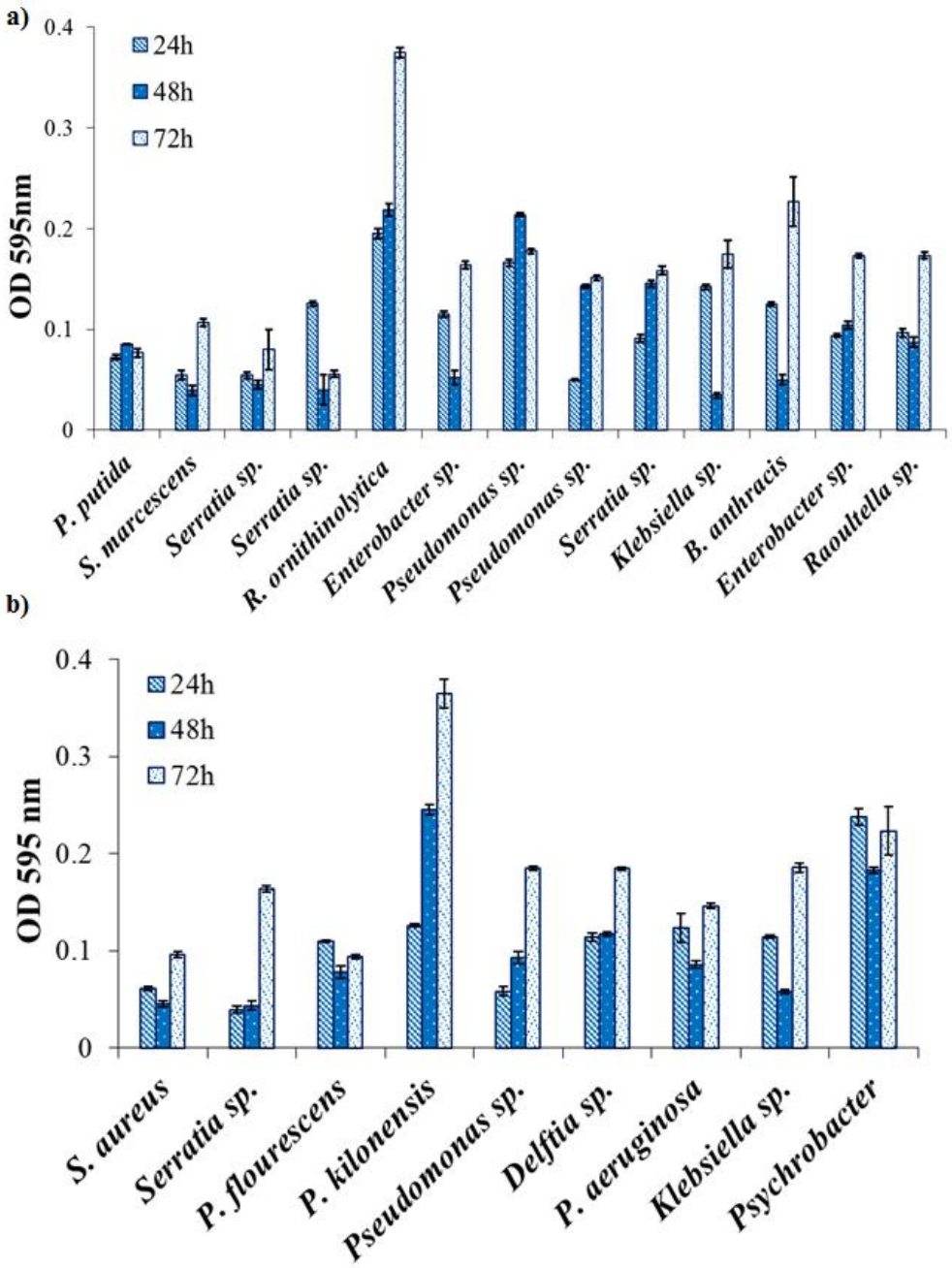


Figure 4.5. Biofilm formation assay of a) Dominant bacterial diversity present in activated sludge of MBR, b) AHL producing strain

Table 4.2. Aggregation of bacterial suspension under increasing influence of ammonium salt

Strains	Ammonium Salt Solution Concentration (M)*				
	0	**1**	**2**	**3**	**4**
S. aureus	0	2	2	2	2
Serratia sp.	0	1	1	2	2
P. flourescens	0	2	3	4	4
P. kilonensis	0	1	2	3	3
Pseudomonas sp.	0	1	2	2	3
Delftia sp.	0	2	3	4	4
P. aeruginosa	0	1	2	3	3
Klebsiella sp.	0	2	2	3	4
Psychrobacter sp.	0	2	2	3	4

* Arbitrary units 0-4, 0 for least and 4 for highest aggregation

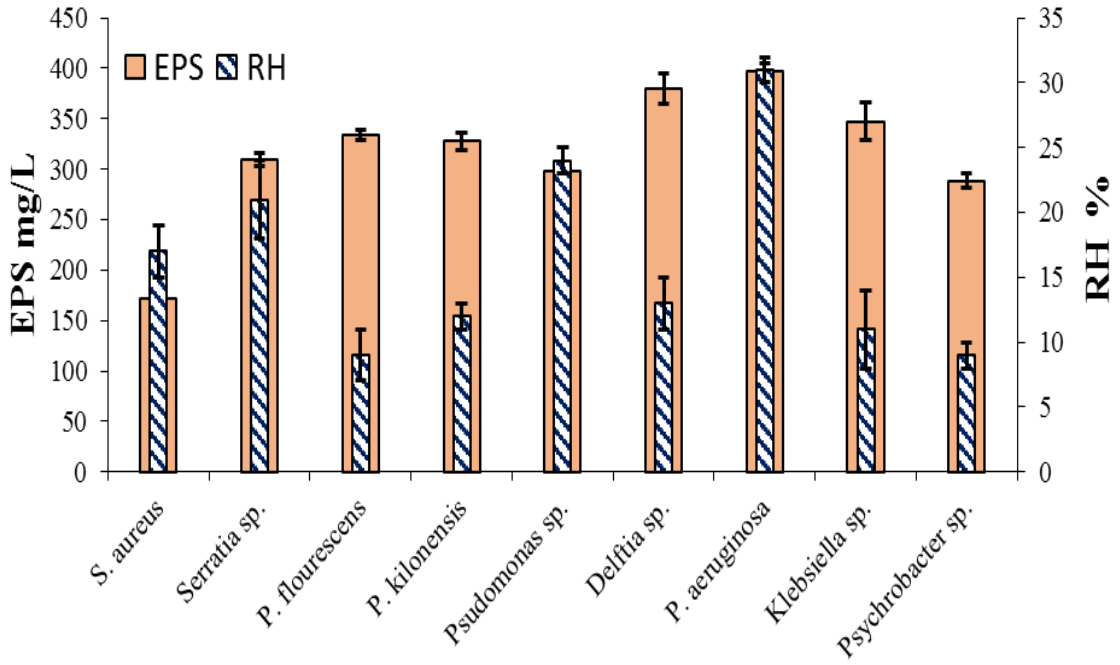


Figure 4.6. Extra cellular polymeric substances (EPS) and relative hydrophobicity (RH) content of AHLs producing strains

4.2. PHASE 2

High performing antifouling bacterial consortium for submerged MBR

Since biocake consists of diverse group of QS bacteria, releasing a variety of signal molecules to communicate among themselves. These diverse groups of signal molecules could be very difficult to get mineralized by a single QQ strain. A possible strategy could be to utilize a consortium of bacterial strains with overlapping capacities to mineralize signal molecules which could result in more efficient biofouling control in MBR. In Phase 2, QQ bacterial strains were screened and identified via 16S rRNA analysis. Consortium of potential QQ bacteria, entrapped within polymeric beads separately, was applied in submerged MBR to mineralize various signal molecules whereby reducing biofilm formation. Effect of QQ consortium on biocake formation, in terms of AHLs degradation and reduction in EPS release, was investigated and direct correlation was established to fouling determinants. Sludge characterization in terms of dewaterability, sludge settleability, microbial activity, and particle/floc size was also determined. Organic and nutrients removal rate of MBR with antifouling consortium (AC) vs. conventional MBR, was also carried out to assess performance efficiency of both MBRs.

4.2.1. Indigenously Isolated Antifouling Bacteria and their AHLs Degradation Ability

Gram negative bacteria being the dominant group in MBR system play a vital role in production of signals molecules like AHLs which is a major concern in

controlling membrane biofouling. These AHLs could be degraded by direct induction of enzymes (acylase, lactonase or oxidoreductase) to MBR system or using bacteria that release such enzymes. The latter approach has some advantages to tolerate a wide range of environmental conditions, whereas direct introduction of enzymes to MBR system requires relatively controlled environmental condition which may add additional operating costs. In the present study, AHL degrading QQ bacteria were indigenously isolated and identified though 16S rRNA approach (Figure 4.7). Evolutionary analysis classified all QQ isolates in 5 different families including *Enterobacteriaceae, Pseudomonadaceae, Microbacteriaceae*, *Alcaligenaceae* and *Aeromonadaceae*. Isolates belonging to genera *Microbacterium* sp., *Pseudomonas* sp., *Achromobacter* sp., *Delftia* sp. and *Aeromonas* sp. have already been reported as AHLs hydrolyzing bacteria (Christiaen et al., 2011; Huang et al., 2003; Kim et al., 2014; Wahjudi et al., 2011; Wang et al., 2010). However, QQ enzyme responsible for AHL degradation from other bacteria like *Enterobacter* sp., *Escherichia hermannii*, *Weissella cibaria*, *Salmonella* sp., *Yersinia* sp. has yet to be identified. Some species like *Pseudomonas* sp., *Enterobacter* sp., *Salmonella* sp. and *E. hermannii*, isolated as QQ bacteria, may also have a significant role in QS mechanism as their role in release of AHLs has been noticed in earlier studies (Taga and Bassler, 2003) which is not surprising as some bacteria may have the ability to produce and quench AHLs simultaneously.

Screening of antifouling or QQ bacteria in terms of their AHLs degradation capability was carried out using QSIS2 reporter strain. The QQ activity of already reported *Rhodococcus* sp. BH4 was considered as bench mark and compared with the isolates (Table 4.3). Among 13 isolates, *Enterobacter cloacae*, *Delftia* sp.,

Pseudomonas sp. and *Microbacterium* sp. have indicated higher QQ activity than *Rhodococcus* sp. BH4. Based on screening experiments, 2 different consortia, each comprising of 3 strains, were selected and compared for their AHLs quenching ability in MBR. Further, to target broader range of AHLs, selected consortium (indicated in Table 3.3) comprises of those strains that release either endoenzymes (lactonase) or exoenzymes (acylase).

4.2.2. Trans Membrane Pressure (TMP) Profile of MBRs

Three MBRs, C-MBR, AC_1-MBR and AC_2-MBR, were run simultaneously under identical operating conditions unless mentioned otherwise. Detailed description and bacterial composition of MBRs are mentioned in Table 3.3. All QQ strains were separately entrapped in polymeric beads to avoid settling problems related to beads density. MBRs were fed with same activated sludge having biomass concentration ~ 8 g/L.

Trans membrane pressure (TMP) is an important indicator to estimate membrane permeability as the extent of membrane fouling is directly linked with TMP build up. Dominant stages in overall TMP profile like initial rise due to pore blockage (stage I), slow rise because of accumulation of EPS on membrane surface (stage II) and a sharp build up (stage III) were therefore, critically monitored for all MBR systems (Zhang et al., 2006).

Figure 4.8 represents TMP profile for MBRs operated at constant flux (20 $L/m^2/h$) and experimental run was terminated at fixed TMP (~30kPa) for all reactors. Significant variations were observed in TMP build up pattern of all MBRs. The TMP rise of AC_1-MBR, AC_2-MBR and C-MBR was reached at 30 kPa in 39, 36 and 13

days respectively. Moreover, biofouling rate of MBRs were also compared to the MBR having single QQ bacteria.

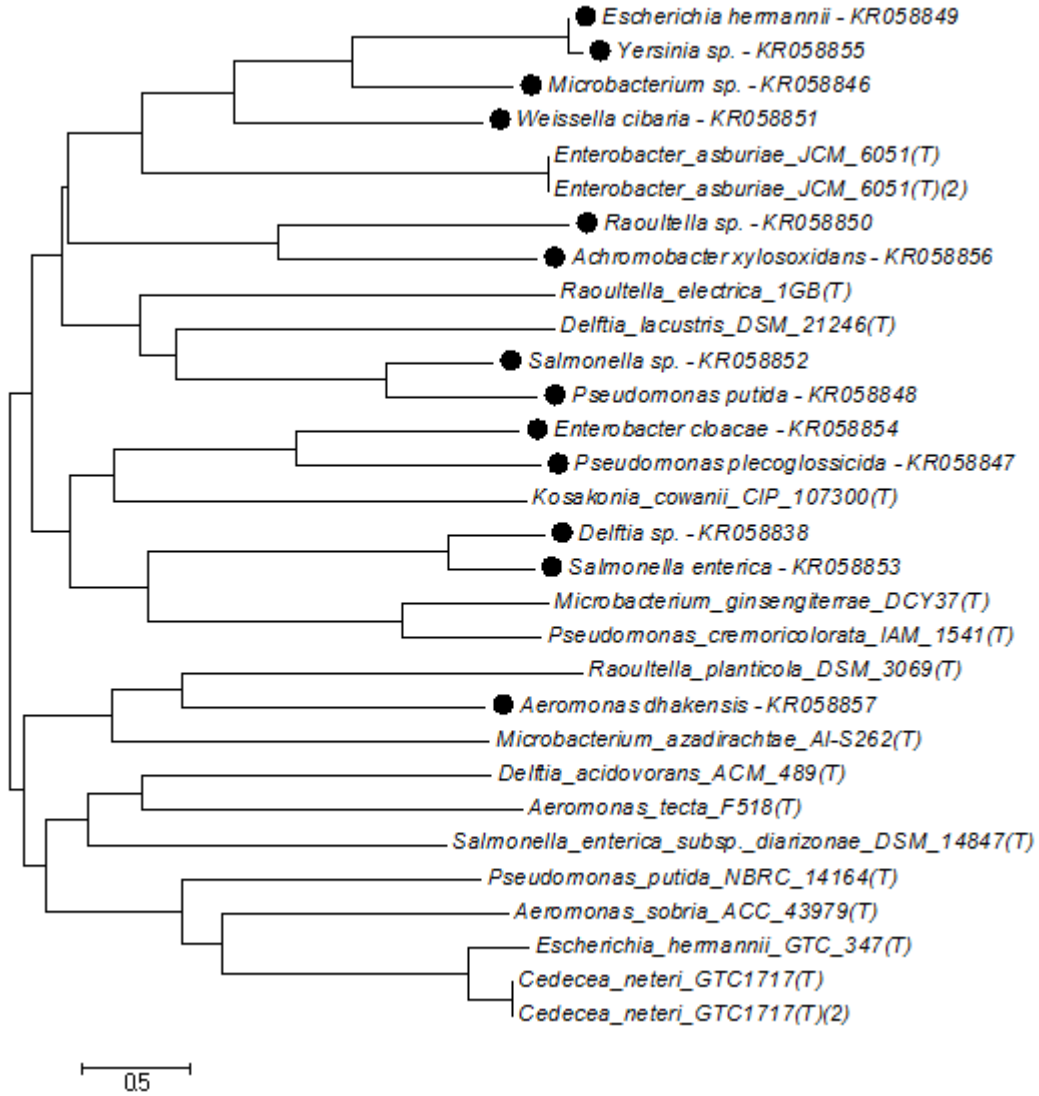


Figure 4.7. Neighbor-joining evolutionary linkage among indigenous quorum quenching strains using MEGA 6 software (Species isolated in this study are highlighted with dots)

Table 4.3. Indigenously isolated quorum quenching strains, their AHLs degradation ability and location of QQ enzyme

Isolates code	QQ strains	Accession no.	Remaining AHL levels %	Probable location of QQ enzyme
QQ0	*Delftia* sp.	KR058838	2	Endoenzyme
QQ1	*Microbacterium* sp.	KR058846	6.7	Endoenzyme
QQ2	*Pseudomonas plecoglossicida*	KR058847	9.6	Exoenzyme
QQ3	*Pseudomonas putida*	KR058848	5.12	Exoenzyme
QQ4	*Escherichia hermannii*	KR058849	13.95	-----
QQ7	*Enterobacter asburiae*	KR058850	22.8	------
QQ8	*Weissella cibaria*	KR058851	25	Exoenzyme
QQ10	*Salmonella* sp.	KR058852	32	Exoenzyme
QQ12	*Salmonella enterica*	KR058853	13	-----
QQ13	*Enterobacter cloacae*	KR058854	0.93	Exoenzyme
QQ14	*Yersinia* sp.	KR058855	47.4	Endoenzyme
QQ15	*Achromobacter*	KR058856	30.93	-----
QQ18	*Aeromonas dhakensis*	KR058857	14	Exoenzyme
BH4	*Rhodococcus* sp. BH4	------	9.4	Endoenzyme

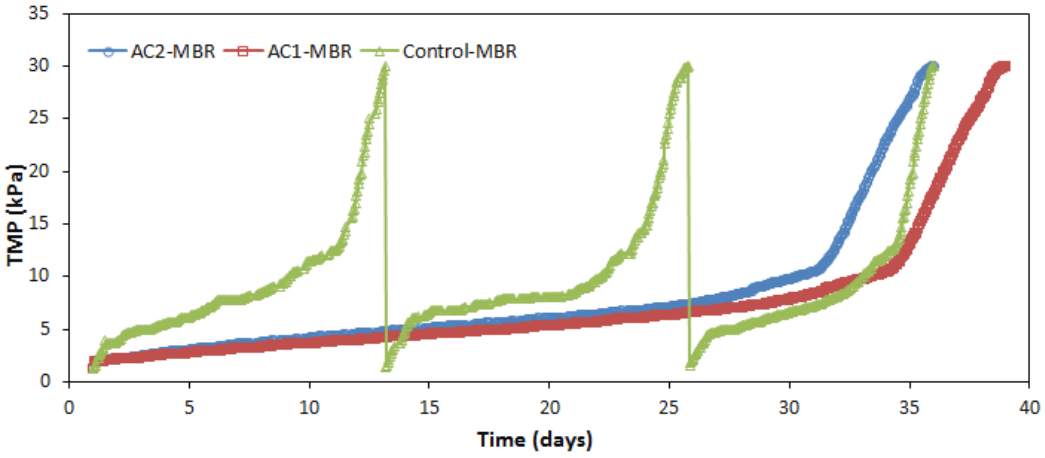


Figure 4.8. Transmembrane pressure (TMP) profile of conventional, AC_1 (Antifouling consortium-1) and AC_2 (Antifouling consortium-2)-MBR

The MBR system with single QQ bacteria (*Rhodococcus* sp. BH4*)* was fouled after 24 days compared to C-MBR. Whereas, fouling rate of MBR with single QQ bacteria was 62 % higher than AC_1-MBR. Faster fouling of C-MBR could be due to EPS or other biological products which were either deposited from bulk liquor or produced in biofilm thereby producing biocake and resulting in reduced membrane permeability (Hwang et al., 2012). It was important to note that the slope of TMP rise of C-MBR during stage III of TMP profile was thrice faster than AC_1-MBR and AC_2-MBR. A substantial difference in total attached biomass (TAB) was observed and it was noted to be 3.8 g for AC_1-MBR, 4.2 g for AC_2-MBR and 12.5 g for C-MBR. This might be the one probable role of quenching enzymes (lactonase or acylase), released by QQ strains, to reduce TAB and EPS (Lee et al., 2014).

During stage II, prolonged TMP build up time required by AC_1-MBR (30 d) and AC_2-MBR (28 d) than C-MBR (9 d) may further verify the significance of quenching mechanism in biofouling retardation. Average membrane fouling rate ($\Delta p/\Delta t$) for AC_1-MBR and AC_2-MBR was found to be 0.77 and 0.85 kPa/d respectively as compared to C-MBR (2.5 kPa/d). Overall, C-MBR experienced 3.5 and 3 times more biofouling than AC_1-MBR and AC_2-MBR respectively. Earlier study (Maqbool et al., 2015) reported 7 times less biofouling as compared to C-MBR using alginate beads entrapped with *Rhodococcus* sp. BH4. As alginate beads were modified with a polysulfone layer to reduce their deterioration rate, it may be anticipated that polymeric coating enhanced the effective life of bead but may reduce release of QQ enzymes into the MBR system. To monitor growth of bacteria inside the polymeric bead, SEM images were captured at regular intervals. A cross section of beads was also obtained to visualize the alginate content and polymer coating

(Figure 4.9). Further, few beads were randomly selected from both AC_1-MBR and AC_2-MBR to monitor growth of immobilized bacteria after 30 d of operation.

4.2.3. Effect of QQ Mechanism on Membrane Resistance

Comparatively, few patches of biocake layer were observed even after 35 d of study in case of both AC-MBRs than C-MBR. Membranes of all three MBRs were compared to further authenticate the role of quenching mechanism on biocake reduction. Under controlled conditions, a significant contribution of R_c was observed in case of C-MBR, where membrane undergoes 13 days to reach at TMP of 30 kPa (Figure 4.10). As enzymes released by QQ bacteria might have played some role in delaying the process of biofilm development over membrane surface via mineralization of signal molecules, this may further be linked with less Rc as compared to C-MBR. Thus, R_p contributed a key portion of R_t in fouling of both AC-MBRs than R_c. Overall, contribution of R_c in case of C-MBR, QQ- AC_1-MBR and AC_2-MBR was found to be 48, 24 and 31 % respectively.

4.2.4. Targeted AHLs in Biocake of MBR

Presence of medium chain AHLs (C_4HSL, C_6HSL and C_8HSL) in activated sludge of MBR has already been reported in our previous studies (Maqbool et al., 2015; Waheed et al., 2016). In the current study, biocake samples of C-MBR, AC_1-MBR and AC_2-MBR were analyzed using HPLC (Agilent 1290 Infinity II). Varying concentration of AHL standards including, C4-HSL, 3OC6-HSL, C6-HSL, 3OC8-HSL, C7-HSL, C8-HSL, C10-HSL, 3OC12-HSL, C12-HSL, 3OC14-HSL and C14-HSL, were run and their corresponding retention time was recorded (Figure 4.11). Among all, presence of C6-HSL, C8-HSL, C10-HSL, 3OC12-HSL and C14-HSL

was confirmed in biocake samples extracted from C-MBR. While, only 3OC6-HSL was detected in biocake of AC_1-MBR which was observed at retention time of 2.8 min, whereas 3OC6-HSL and C8-HSL, were detected at 2.8 and 3.45 min respectively in the biocake of AC_2-MBR. The possibility of AHLs conversion to other products via enzymes released by QQ consortium could be anticipated from the results of present study.

4.2.5. Influence of Quenching Mechanism on Sludge Characteristics and MBR Performance

The mixed liquor was sampled regularly to monitor effect of QQ on different sludge characteristics enlisted in Table 4.4. The DO level was maintained within the range of 4-6 mg/L in all three reactors. Maximum microbial activity in terms of SOUR, indicated by C-MBR, could be attributed to large and firmed flocs found in the activated sludge of C-MBR. As polymeric beads remained in suspension in MBRs with antifouling consortium (AC-MBRs), their role in reduction of floc and particle size is not very surprising. Since, floc size in both AC-MBRs was found to be relatively smaller and less compact than C-MBR, both reactors experienced loss of biomass with time and similar correlation between floc size, sludge settleability and biomass loss has also been reported by Basuvaraj et al. (2015). The dewatering and settling properties of sludge was measured in terms of CST and SVI respectively. Relatively high SVI (> 90 to < 140 mL/g) indicated by AC-MBRs than C-MBR (<120 mL/g), reveals effect of polymeric beads on deterioration of sludge size and hence reduced settleability.

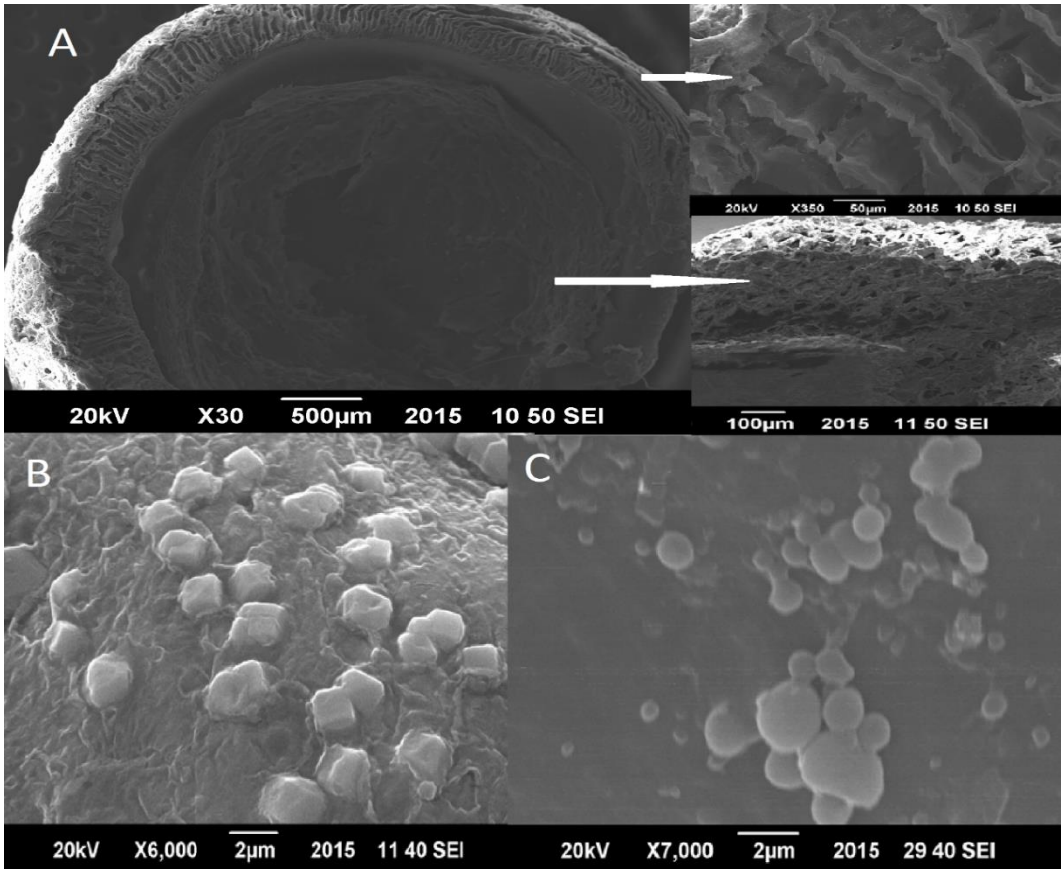


Figure 4.9. SEM images of QQ-bacteria entrapped polymeric bead (A) Cross-sectioning image of whole bead and polysulfone membrane matrix in the bead, (B & C) Inner surface of the polymeric bead

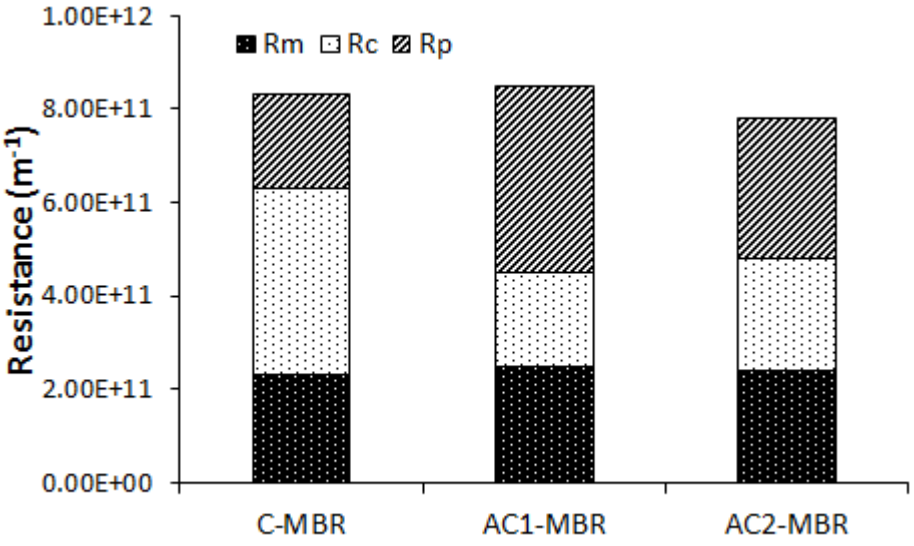


Figure 4.10. Membrane fouling resistances of three MBRs using resistance in-series model

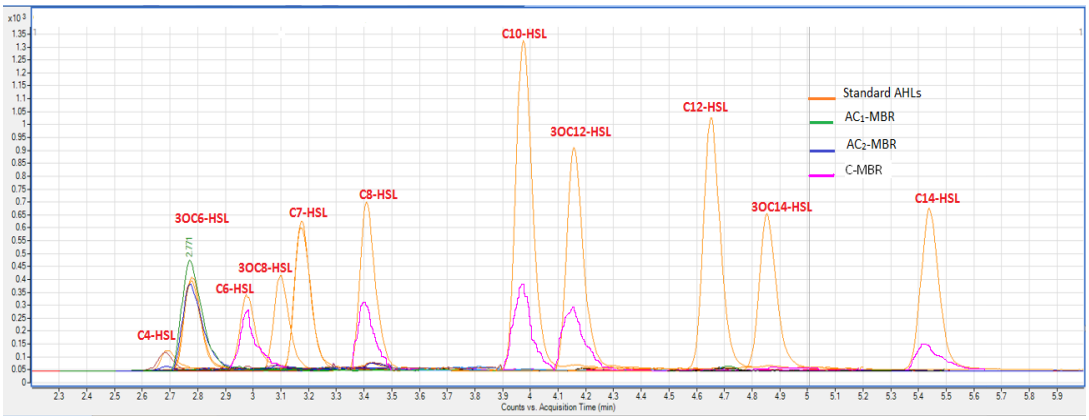


Figure 4.11. Chromatograph of AHLs and their corresponding retention times

Whereas, no significant effect of QQ beads in terms of CST was observed. The EPS components of mixed liquor and biocake were measured in terms of protein and polysaccharides as they accounted for 75 to 90% of EPS content (Tsuneda et al., 2003). In mixed liquor, a continuous increase in S-EPS concentration of AC-MBRs could be linked with the reduction in floc size (Figure 4.12A). Structure and size of biological flocs in activated sludge are directly correlated to chemical constituents of EPS (Wilén et al., 2003). As reported by Metzger et al. (2007), dissociation of sludge flocs results into a high S-EPS and eventually contributes to membrane pore blockage. Thus it could be anticipated that reduction in floc size may enhance EPS release up to some extent.

Slightly lower concentration of bound EPS was observed at the time of fouling for AC-MBRs, whereas, C-MBR showed a continuous rise for LB and TB EPS. For biocake, overall composition was found to be same i.e. TB-EPS concentration was significantly higher in all three reactors than LB-EPS and S-EPS

(Figure 4.12B). Lowest protein and polysaccharide concentration was observed in biocake of AC_1-MBR. Protein and polysaccharide concentrations were 2.24 and 2 times higher in C-MBR than AC_1-MBR. While AC_2-MBR indicated 1.2 times more concentration of protein and polysaccharide than AC_1-MBR. Hence, a positive correlation could be developed between EPS component and fouling rate (Gao et al., 2013) as the influence of quenching strategy in terms of EPS reduction is consistent with previous study (Maqbool et al., 2015). Removal efficiency of COD was found to be more than 90 % in all MBRs. Consistency in COD and nutrients removal in all three reactors illustrates that the presence of antifouling consortium did not affect performance efficiency of MBR.

4.2.6. Summary

Three indigenously isolated bacterial strains (*Enterobacter cloacae, Delftia* sp., and *Pseudomonas* sp.) showed higher QQ activity when applied in consortium as compared to other consortium containing *Rhodococcus* sp. BH4. Antifouling bacterial consortium entrapped in polymeric beads is much more effective (3 times) in controlling biofouling as compared to single or without QQ bacteria. The effluent quality was not significantly affected by QQ consortium with added advantage of 3X longer time required to foul the membranes. Contribution of cake layer resistance (R_c) in TMP rise for C-MBR was twice as compared to AC_1-MBR. Distinct effect of bacterial entrapped beads on sludge characteristics like sludge particle and floc size was observed as only drawback of polymeric beads.

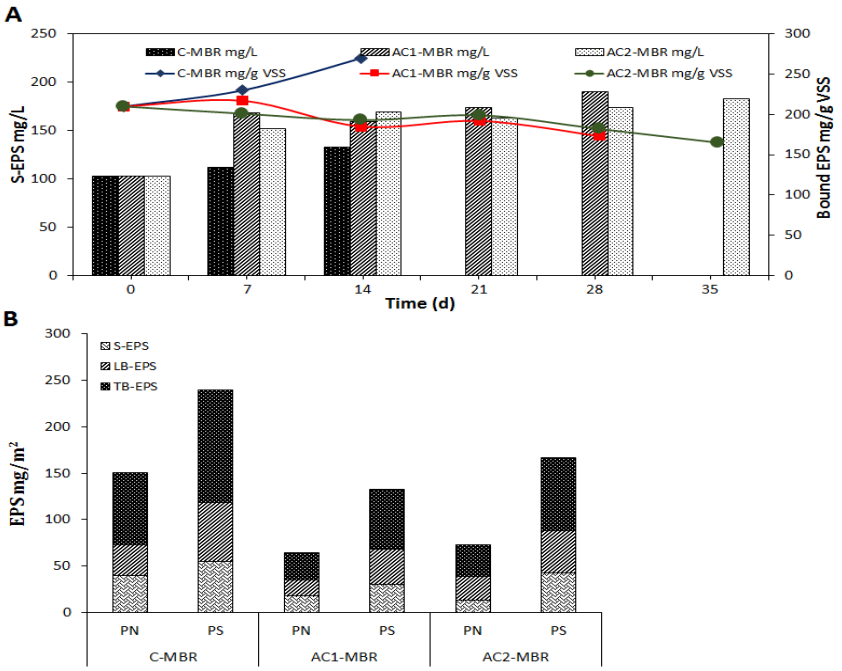


Figure 4.12. Extracellular polymeric substances (EPS) components in (A) mixed liquor and (B) biocake of MBRs (n=3)

Table 4.4. Sludge characterization and performance efficiency of MBRs

Parameters	Units	C-MBR	AC_1-MBR	AC_2-MBR
Dissolved Oxygen (DO)	mg/L	6.4 ± 0.4	6.23 ± 0.75	5.76 ± 1.2
Soluble Oxygen Uptake Rate (SOUR)	mg/g/hr	140 ± 5.0	105 ± 3.6	79 ± 7.0
Particle size	µm	80 – 130	55 – 75	64 – 80
Specific Cake Resistance (SCR)	m/kg	9.0×10^{13}	6.0×10^{13}	4.2×10^{13}
Capillary Suction Time (CST)	S	16 ± 4.0	18 ± 1	15 ± 1.0
Mixed Liquor Suspended Solids (MLSS)	g/L	8 ± 1.0	7 ± 0.5	8.5 ± 1.3
Chemical Oxygen Demand (COD) removal	%	93.5 ±1.4	95 ± 0.8	93 ± 1.3
NH_4-N removal	%	52 ± 1.5	47 ± 3.0	45 ± 2.4
PO_4^{3-}-P removal	%	48 ± 2.5	49 ± 2.8	50 ± 3.0

4.3. PHASE 3

Insights into Quorum Quenching Mechanisms to Control Membrane Biofouling under Transient Conditions

This phase aimed to investigate impact of OLR on QQ-based biofouling control. To achieve this objective, a consortium of QQ strains comprised of AHLs- and AI-2-degrading bacteria, both in immobilized and suspension forms, was applied to MBRs. The influence of QQ bacteria on EPS production, sludge floc size, and sludge filterability in terms of capillary suction time (CST) under both steady and transient organic loads was examined to gain a better understanding of the underlying relationship between QQ and biofouling. The concentrations of AHLs and AI-2 were also monitored to confirm QQ effects.

4.3.1. MBR Operation and Trans-membrane Pressure (TMP) Trends

The experiment was conducted in two separate phases, as described in Table 3.6. The DO levels in all reactors throughout the phases were maintained within 5.5 to 6.5 mg/L. The COD removal efficiency was more than 90 % in all MBRs (Table 4.5), and similar COD removal in QQ MBRs indicates that QQ beads or sludge did not affect the performance of MBRs. A gradual decrease in MLVSS concentration was carried out purposely in Phase I via wasting sludge and controlling a SRT of 30 d, thus creating a continuous increase in OLR. While in Phase II, all MBRs were supplemented with seed sludge at regular intervals to maintain a constant MLVSS and OLR (Figure 4.13).

To evaluate MBR performance, TMP was monitored for both experiments as the extent of membrane fouling is directly linked to TMP increase. Figure 4.14 shows TMP profiles of MBRs of both phases which were terminated when the TMP reached ~30 kPa. In Phase I (Figure 4.14a), no significant difference was observed between both control MBRs, as the overall fouling rates ($\Delta TMP/\Delta t$) for C-MBR and Cb-MBR were found to be 5.81 and 5.12 kPa/d, respectively. However, addition of QQ sludge or QQ beads in MBRs reduced the fouling rate by 29 and 49%, respectively, as compared to C-MBR under transient conditions. While in steady state (Phase II in Figure 4.14b), addition of QQ consortium, either in culture broth or in immobilized beads, significantly reduced the overall fouling rate by more than 66 % as compared to C-MBR, with QQ beads slightly better than QQ sludge. The vacant beads in Cb-MBR also reduced the fouling rate by 35%.

The similar TMP profiles of QQs-MBR and QQb-MBR in Phase II suggest that the QQ consortium was effective in fouling control when the MBR was in steady state, irrespective of whether it is in broth or in immobilized beads. However, under transient conditions, robust fouling control was achieved only when the QQ consortium was in immobilized beads. QQ strains may grow well in immobilized form since beads provide protective environment under harsh conditions Whereas, activity/growth of QQ strains was suppressed when they were added directly to sludge, which could be due to the presence of predators or QQ enzyme suppressors released by other competitors. Previous studies (Gao et al., 2013; Zhang et al., 2006) demonstrated three dominant stages in overall TMP profiles, including an initial rise due to pore blockage (stage 1), a gradual rise due to accumulation of EPS on membrane surface (stage 2), and a sharp jump resulted from biofilm formation (stage

3). In this study, a slower rise in TMP during stage 2 in QQ MBRs compared to control MBRs was observed for both Phases, indicating that QQ may have played a prominent role in retarding biofouling by decreasing EPS production and accumulation, thereby delaying cake layer formation.

4.3.2. Membrane Resistance and Foulant Characterization

Membrane resistances from all six reactors of Phase II were compared to further elucidate the mechanism of QQ (Figure 4.15). As evident, a clear decrease in the fraction of pore blockage resistance (Rp/Rt) with time was observed in QQb-MBR, from >70% on day 4, to ~ 45% on day 6, and further to ~ 40% on day 12 when it was fouled. The fraction of cake layer resistance (Rc/Rt) remained very low (<7%) on day 4 and 6, but increased to ~30% on day 10. This clearly indicates the strong inhibition of cake layer formation by QQ in early stage. Compared to other MBRs, fraction of pore blockage resistance (Rp/Rt) of both QQ MBRs (>40%) were greatest, when they were fouled. This suggests that more molecules or small particles had blocked membrane pores in QQ MBRs, as QQ had postponed the cake layer formation. Hence in QQ MBRs, pore blockage may be the leading factor that had led to final fouling.

The significantly higher portion of cake layer resistance (Rc/Rt) in C-MBR as compared to Cb-MBR indicates that beads souring had greatly reduced cake layer formation. This was also observed when QQs-MBR was compared to QQb-MBR.

Fouled membranes from all reactors were analyzed via Fourier transform infrared (FTIR) spectroscopy (AIM-8800, Shimadzu) detect any foulant composition changes posed by quenching mechanism. QQ strains either immobilized or added as

a suspension did not modify the membrane foulants, as the organic or functional groups in all membranes were found to be same, as shown in Figure 4.16. It can be inferred that quenching activity delayed biofilm formation, but kept the foulant composition intact. Further, foulant composition has also been discussed in section 3.5.2.

4.3.3. Effect of QQ on QS Signal Molecule

Biofilm formation (biofouling) could be mitigated substantially by disrupting signal molecules such as AHLs, oligopeptides or AI-2 using enzymes released by QQ bacteria (Shrout and Nerenberg, 2012). In the present study, role of QQ bacteria was confirmed by monitoring the concentrations of two signal molecules, AHLs and AI-2 using LC-MS and *V. harveyi* bioluminescence assays, respectively.

4.3.3.1. Quenching of AHLs

AHLs in seed sludge were analyzed to screen major types of QS signal molecules; C4-HSL, 3OC6-HSL, 3OC8-HSL and 3OC12-HSL were detected as the dominant AHLs present. Table 4.6 presents detailed quantitative results of AHLs in biocake, mixed liquor and effluents from each reactor. Various AHLs including C4-, 3OC6-, C6- and 3OC14-HSL were detected in mixed liquor of C-MBR and Cb-MBR, whereas they were not detected or lower than the quantification limit in QQs-MBR or QQb-MBR. This indicates that QQ consortium had quenched these QS molecules, thus reducing biofilm formation. High concentrations of C4-HSL and 3OC6-HSL in biocake sample of C-MBR and Cb-MBR as compared to that in QQ MBRs may signify the role of small chain signal molecules in the formation of

matured biofims. Moreover, degradation rate of various AHLs further justifies the potential of selected consortium (Annex II).

4.3.3.2. AI-2

The AI-2 concentration was determined by bioluminescence assay with 4,5-dihydroxy-2,3-pentanedione (DPD), a precursor of AI-2, as the calibration standard. The effectiveness of QQ bacteria in reducing AI-2 signal molecules is shown in Figure 4.17. The AI-2 concentrations in QQb-MBR were lowest when all MBRs were fouled in both Phases. However, AI-2 in QQs-MBR of Phase II was higher than that of C-MBR, though it was still lower than both C-MBR and Cb-MBR in Phase I. Higher concentrations of AI-2 in QQs-MBR could be due to the less survival rate of the *E. coli* strain *ΔlsrRΔluxS,* since the cells were not protected in the beads. A slow but gradual increase in AI-2 concentrations with time in QQb-MBR was also noticed. These may suggest that QQ consortium were effective in quenching AI-2, but only at early stage; with time the quenching effect vanished. Their quenching effect was more robust when they were immobilized in beads.

4.3.4. Effect of QQ on Sludge Characteristics

The mixed liquor was sampled regularly to monitor the effect of QQ on different sludge characteristics as listed in Table 4.5. The SOUR was measured as a representative of sludge respirometric activity, and Phase II showed higher activity compared to the Phase I. The SOUR of QQb-MBR was almost twice as higher that of control MBRs in both Phases. This shows that QQ phenomenon or disruption in bacterial signaling had a direct impact on metabolic activity of microbial cells; which could be related to starving conditions developed during interruption in bacterial

communications, or dominance of strains those require very high OUR (Garcia-Ochoa et al., 2010; Gomez et al., 2006).

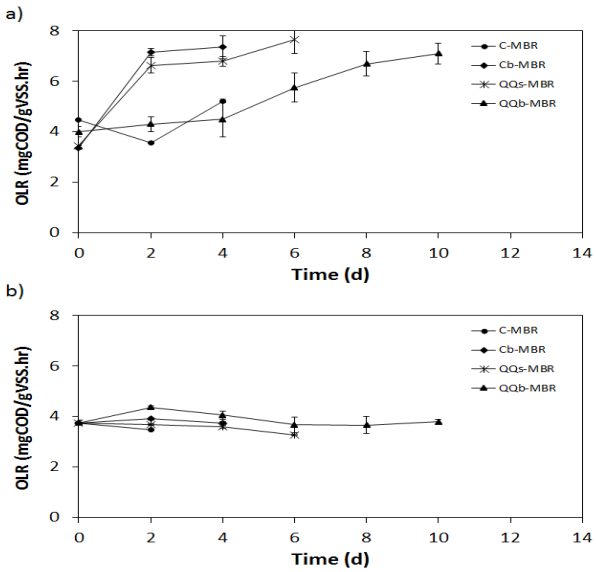


Figure 4.13. Organic loading rate (OLR) of (a) Phase I and (b) Phase II, Where: control MBR (C-MBR), (ii) control MBR with empty beads (Cb-MBR), (iii) MBR with addition of QQ bacteria entrapped in polymer-coated alginate beads (QQb-MBR), and (iv) MBR with addition of QQ bacteria in broth (QQs-MBR)

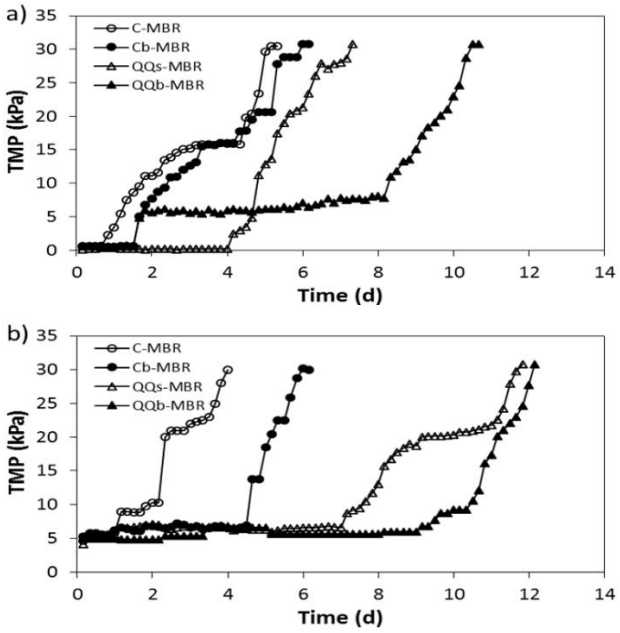


Figure 4.14. TMP profiles of (a) Phase I and (b) Phase II

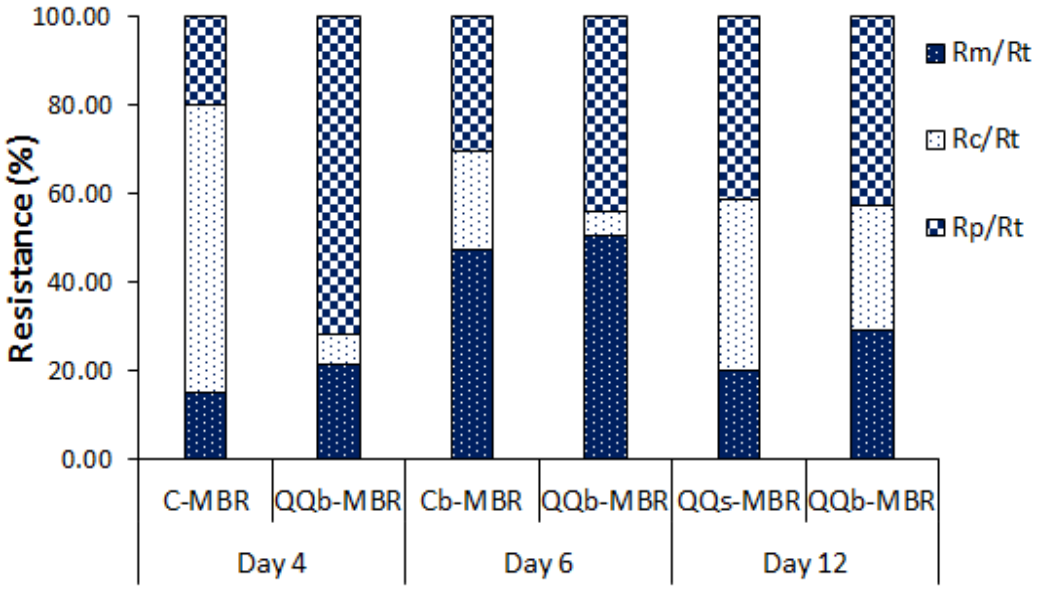


Figure 4.15. Membrane resistance analysis under steady state conditions

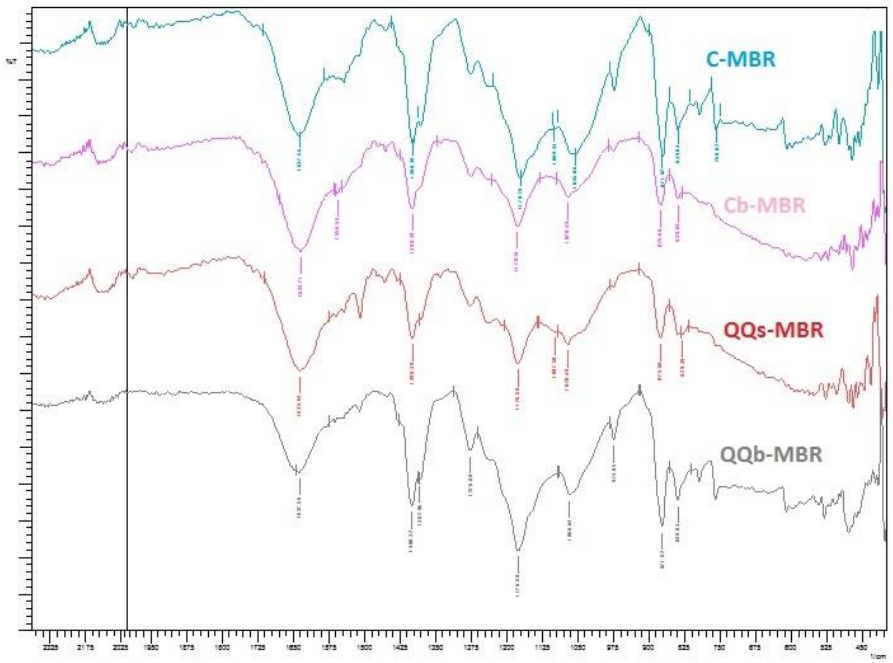


Figure 4.16. Membrane surface analysis using FTIR

Mean floc size for QQb-MBR was found to be 49 and 35 µm for Phases I and II respectively. Overall, sludge floc sizes in Cb-MBR and QQb-MBR were smaller than C-MBR and QQs-MBR in both phases, respectively; presence of beads in the former two MBRs contributed to this reduction in floc size. The dewaterability of sludge determined by CST is also an indication of its permeability in MBR (Table 4.5), and a high EPS content will increase sludge viscosity thus reducing its permeability. However, no clear impacts of QQ consortium on CST could be observed. Reduction in floc size still resulted in better sludge dewaterability, however, its effects on sludge settling property cannot be ignored.

4.3.5. QQ Effects on EPS Production

EPS, either in bound or soluble form, is considered to be the major component in cell flocs and biofilms which ultimately leads to membrane fouling in MBRs. In this context, soluble EPS (S-EPS), loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) extracted from the mixed liquor and sludge cake were analyzed for all phases.

4.3.5.1. EPS concentrations in the biocake

The protein and carbohydrate distributions in different EPS fractions extracted from sludge cake is shown in Figure 4.18. EPS fractions retained on membranes in the form of a fouling layer led to a high resistance on membrane surface, and a reduction in permeate flux (Lin et al., 2014). In the biocake sample, bound EPS was higher than soluble EPS in all phases. Bound EPS facilitates more in floc formation, and an increase in floc size tends to enhance floc deposition on the membrane surface, which ultimately leads to biocake formation (Chu and Li, 2005).

The total biocake EPS in Phase I was more than 3 times higher than Phase II, which indicates that the change in OLR had a dominant effect on total biocake EPS content. However, TB-EPS were in the same range (~0.95-2.2 g/m^2) amongst all MBRs in both phases except a little lower amount was observed in Cb-MBR of Phase II (Figure 4.18). The high S-EPS and LB-EPS contents in Phases I could have resulted from new or loose sludge flocs being deposited on the surface of compacted biocake. It also suggests that scouring effects of the beads were minimal for fouling control, which corresponded well to TMP profiles in Figure 4.14.

The total and especially TB-EPS in QQb-MBR were much lower on day 4 and 6 as compared to control reactors of the same day (Figure 4.18b), but they increased to levels higher than the control reactors when it was fouled. This corresponded well with the membrane resistance analysis.

4.3.5.2. EPS concentrations in the mixed liquor

Figure 4.19 shows the protein-carbohydrate ratio (EPS_P/EPS_C), as well as soluble and bound EPS concentrations at different time intervals in the mixed liquor. When MBRs were started, bound-EPS (more dominantly for LB-EPS) contents of all MBRs in Phase II were lower than those of Phase I, which could be the reason of higher SOUR. The prominent increases (from day 0 to day 2) in LB- and TB-EPS in Cb-MBR and QQs-MBR of Phase I corresponded well to the significant decreases in MLSS in the MBRs (Figure 4.20). But after that, both LB-EPS and TB-EPS in the two MBRs gradually decreased. The LB- and TB-EPS in QQb-MBR also increased since the start of MBR, but started to decrease gradually from day 8 and 4, respectively. The initial increase could be partially due to the decrease in MLSS

concentrations, which led to an increase in OLR and severe biofouling. Similar results were also reported in sludge granulation (K. S. Kim et al., 2013) and MBR operation (Trussell et al., 2006).

In Phase II, decreases in LB- and TB-EPS in all MBRs were clearly noticed from the beginning of operation. However, no significant differences between QQ MBRs and control MBRs were observed. Instead, S-EPS gradually increased in all MBRs in both Phases except of a slight decrease in QQb-MBR in Phase I before day 4. Several studies have directly correlated the S-EPS with biomass concentrations (Drews et al., 2008; Geng and Hall, 2007; Jeong et al., 2007). In our study, MLSS levels dropped substantially in Phase I; however, continuous rise in S-EPS was still observed. These results suggest that intensive aeration and fluidization of polymeric beads significantly reduced sludge floc size (Table 4.5), and dissociation of sludge flocs had resulted in decrease in bound EPS and increase in S-EPS and eventually contributed to membrane pore blockage (Meng et al., 2009; Metzger et al., 2007).

The ratio of EPS_P (EPS-protein) to EPS_C (EPS-carbohydrate) was also investigated to gain better understanding of QQ mechanism. In Phase I, all MBRs showed a decrease in EPSp/EPSc ratio of S-EPS with an increase in OLR, except for that of QQb-MBR, which slightly increased (Figure 4.19:a1). In contrast, a clear increase in EPS_P/EPS_C in sludge supernatant was observed in Phase II (Figure 4.19:b1). The gradual increase in EPS_P/EPS_C of LB-EPS was also observed in all MBRs in Phase II, while it remained unchanged in Phase I. However, for TB-EPS, a slight decrease in EPS_P/EPS_C was observed in both Phases. These results suggest that increase in OLR had more impacts on LB-EPS and S-EPS; it had maintained high fraction of polysaccharides in LB-EPS, which contributed to fouling more than

protein-like substances (Rosenberger et al., 2006; Yigit et al., 2008). The EPSp/EPSc ratio in LB-EPS corresponded well to higher concentrations of polysaccharides than protein in the biocake in Phase I, and higher protein contents in Phase II. This suggests that LB-EPS were more significantly correlated with membrane fouling compared with TB- and S-EPS, as they contributed to flocculation and sedimentation processes, which was also reported in literatures (Lin et al., 2014; Ramesh et al., 2007; Wang et al., 2009).

The biofouling was deferred by factors of 3.04 ± 0.5 and 2.96 ± 0.7 (mean ± standard deviation, n=2) in QQb-MBR and QQs-MBR, respectively, compared to C-MBR for Phase II. However, these factors decreased to 1.8 ± 0.3 and 1.4 ± 0.1 in Phase I when OLR kept increasing. A continuous increase in OLR, which resulted in significant EPS production and higher content of polysaccharide in LB-EPS, was the major contributing factor resulting in the impaired efficiency of immobilized QQ strains. The reduction in S-EPS concentration in the mixed liquor in MBRs containing QQ bacteria were reported previously (Lee et al., 2016; Maqbool et al., 2015). However, it was not observed in our study; instead, QQ consortium retarded EPS accumulation on the membranes.

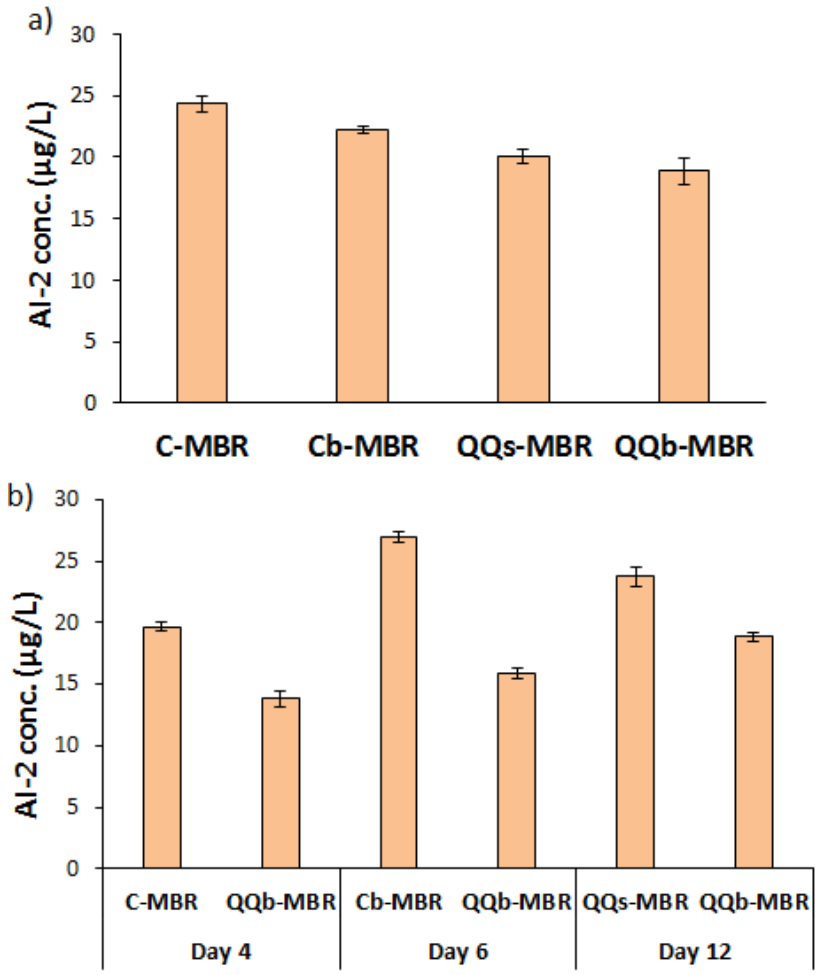


Figure 4.17. Level of autoinducer (AI-2) in mixed liquor of MBRs, (a) Phase I and (b) Phase II

Table 4.5. Sludge characterization and performance efficiency of MBRs (n=3)

Parameters	Phase I				Phase II			
	C-MBR	Cb-MBR	QQs-MBR	QQb-MBR	C-MBR	Cb-MBR	QQs-MBR	QQb-MBR
SOUR (mg/hr/g)	32.4	40.7	35.8	79.9	70.2	77.5	122.3	160.3
CST (s)	15.0	8.9	18.5	12.0	19.8	16.0	12.5	13.5
PSD mean (µm)	50.6	87.5	125.3	48.6	69.0	38.5	60.6	35.0
COD removal (%)	97.5	96.9	98.0	85.2	90.5	90.6	91.5	92.0

Table 4.6. AHLs concentrations under steady state conditions (µg/L)

Sample ID		C4-HSL	3OC6-HSL	C6-HSL	3OC8-HSL	C7-HSL	C8-HSL	C10-HSL	3OC12-HSL	C12-HSL	3OC14-HSL	C14-HSL
Sludge	Mixed liquor	25	<LOQ	ND	<LOQ	ND	ND	ND	0.66	ND	ND	ND
C-MBR	Mixed liquor	3.86	49	0.7	<LOQ	ND	ND	ND	ND	ND	18	ND
	Biocake	25	21	ND	<LOQ	ND	ND	ND	ND	ND	ND	ND
	Effluent	<LOQ	<LOQ	0.026	ND	ND	ND	ND	0.38	ND	ND	ND
Cb-MBR	Mixed liquor	5.26	68	ND	<LOQ	ND	ND	1.74	ND	0.06	70	ND
	Biocake	0.84	15	ND	<LOQ	ND	ND	0	ND	ND	ND	ND
	Effluent	ND	<LOQ	ND	ND	ND	ND	0	ND	ND	ND	ND
QQs-MBR	Mixed liquor	ND	<LOQ	ND	ND	ND	ND	0	ND	ND	ND	ND
	Biocake	ND	ND	ND	ND	ND	ND	0	ND	ND	ND	ND
	Effluent	ND	<LOQ	ND	ND	ND	ND	0	ND	ND	ND	ND
QQb-MBR	Mixed liquor		<LOQ	ND	ND	ND	ND	0	ND	ND	ND	ND
	Biocake	ND	<LOQ	<LOQ	<LOQ	ND	ND	0.1	ND	ND	ND	ND
	Effluent	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

*ND: Not detected, <LOQ: Less than of quantification limit

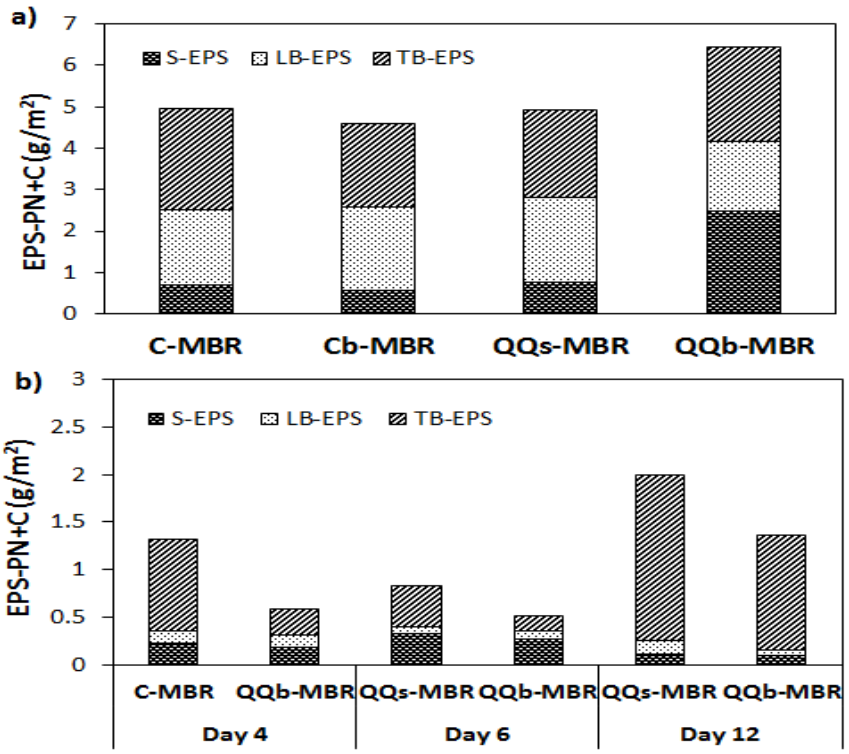


Figure 4.18. EPS concentration in the biocake samples (a) Phase I and (b) Phase II

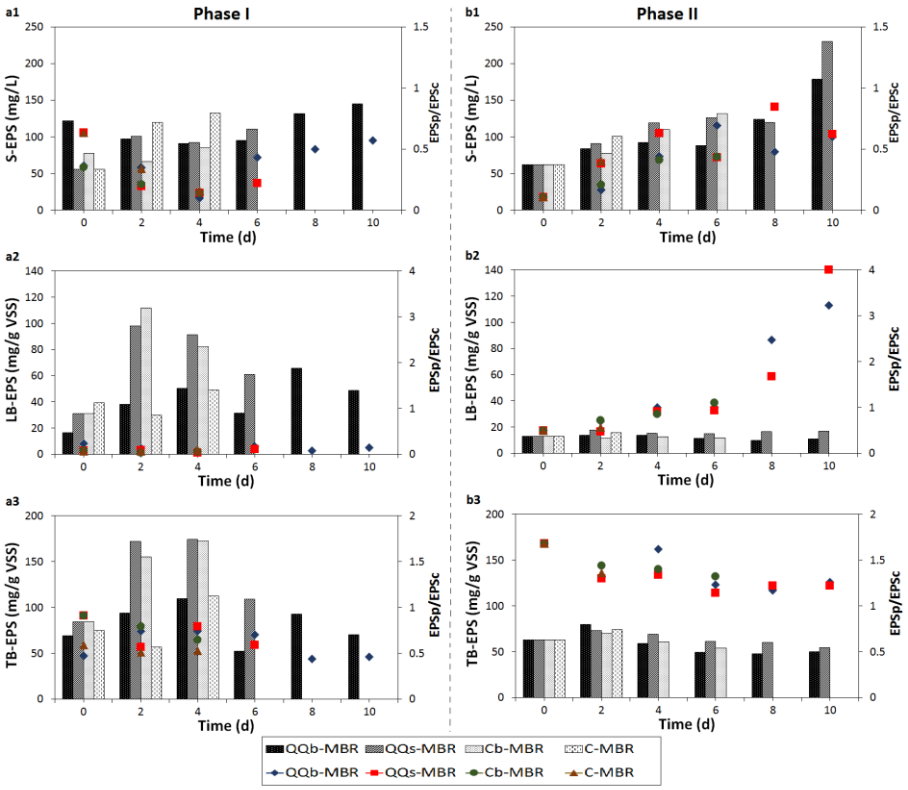


Figure 4.19. Extracellular polymeric substances (EPS) concentration in terms of soluble EPS (S-EPS), loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) in the mixed liquor of (a) Phase I and (b) Phase II

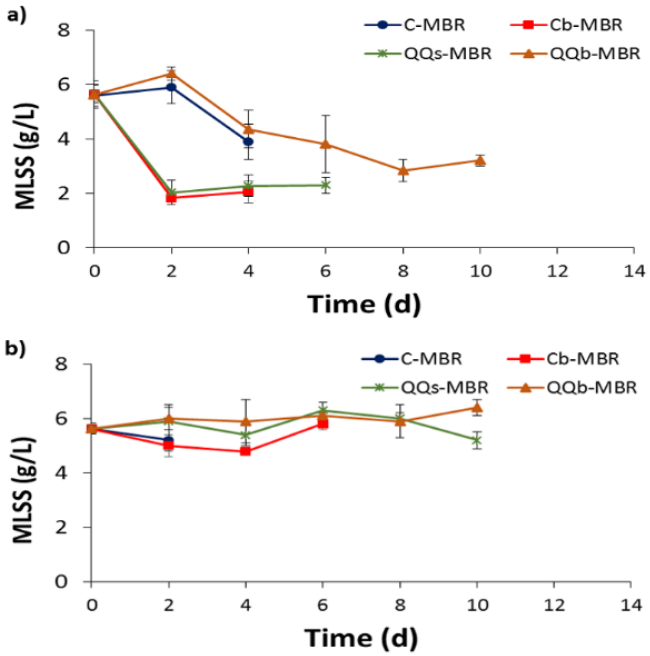


Figure 4.20. Mixed liquor suspended solids (MLSS) concentration of (a) Phase I and (b) Phase II

4.3.6. Summary

The QQ consortium in immobilized beads showed robust fouling control capacity under both steady state and transient condition. They remained effective only under steady state. The QQ consortium significantly reduced AHL levels in mixed liquor inside MBR and in sludge cake on membrane surface. However, they did not result in obvious decreases in EPS in mixed liquor.

LB-EPS of mixed liquor was the major contributor to membrane fouling, and within the EPS, polysaccharides contributed more to membrane fouling than proteins. The EPS in the cake layer had a composition like that of LB-EPS of the mixed liquor. A continuous increase in OLR resulted in higher EPS levels and higher polysaccharide ratios in LB-EPS, which in turn lead to faster fouling in the MBRs.

The QQ consortium retarded the biofilm formation on membranes; meanwhile, more S-EPS deposited on membranes and resulted in highest pore blockage resistance in QQ MBRs when they were fouled. The pore blockage could be a governing factor leading to final sharp increase in TMP in QQ MBRs.

4.4. PHASE 4

Tandem effects of PAC and QQ on fouling control and simultaneous removal of pharmaceutical compounds

This phase aimed to integrate QQ strategy with PAC addition as well as backwashing to improve overall efficacy of fouling control. Batch adsorption isotherm tests were conducted to determine optimal PAC dosage in integrated beads. In order to gain deep understanding of integrated fouling control strategy, production of soluble microbial products (SMP) and EPS, change of sludge floc size and sludge filterability, together with quorum quenching activity were continuously monitored. In addition, simultaneous removal of OMP owing to adsorption of PAC was also evaluated. The five pharmaceutical compounds (PhAC), namely trimethoprim (TrMP), sulfamethoxazole (SMX), carbamazepine (CBZ), diclofenac (DCF) and triclosan (TCS), were selected as target trace organic compounds based on their broad range of hydrophobicity (Table 4.7).

4.4.1. Optimization of PAC Beads Composition and Bead to Volume Ratio

In this study, five independent sets of batch-scale experiments with different bead composition and increasing bead doses (volume ratio of bead to reactor) from 1 to 20% for each set (Table 3.7) were conducted to optimize adsorption of PhACs. The aqueous concentrations of PhACs were monitored at regular intervals for 7 days to determine time to reach adsorption equilibrium. The adsorbed amounts of five PhACs increased with time, but major adsorption (>95%) finished within 1 day, which indicated high driving forces of adsorption (Larous and Meniai, 2016). A rapid

decrease in aqueous concentration of SMX was observed after 2 days, which was attributed to abiotic degradation. Hence, an equilibrium time of 1 d was selected for adsorption isotherm modelling.

Among adsorption isotherm models, Freundlich model is widely applied in heterogeneous systems especially for organic compounds or highly interactive species on activated carbon and molecular sieves (Foo and Hameed, 2010). Table 4.8 shows results of fitting the equilibrium data to Freundlich model and regressed constant values, where *k* is a constant indicative of the relative adsorption capacity of adsorbent and *n* is a constant indicative of intensity of adsorption (Hamdaoui and Naffrechoux, 2007). The R^2 (> 0.9) values for all PhAC in both sets 1 and 2 suggest the good fits of Freundlich model. It is noted that PhACs showed inherently various degrees of adsorption intensity (*n* values in Table 4.8) towards PAC, which corresponded well to their hydrophobicity. For further test, set 1 is more preferable in comparison to set 2, because of the almost same removal efficiency of micro pollutants with a lower amount of PAC. An increase in the bead dose from 1 to 5% led to an increase in adsorption, whereas further increase did not result in any significant change in adsorption; therefore, bead dose of 5% was considered as optimum and selected for further studies (Figure 4.21).

Table 4.7. Properties of selected pharmaceuticals

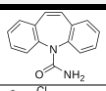
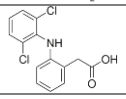
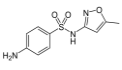
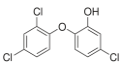
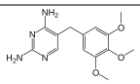
Compounds	Applications	log Kow	Solubility in H_2O (mg/L)	Structures	References
Carbamazepine (CBZ)	Antiepileptic	2.3-2.5	17.7		(Suárez et al., 2008)
Diclofenac (DCF)	Anti-inflammatory	4.5-4.8	2.4		(Suárez et al., 2008)
Sulfamethoxazole (SMX)	Antibiotic	0.5-0.9	610		(Suárez et al., 2008)
Triclosan (TCS)	Antibacterial and antifungal	4.76	10		(USFDA, 2008)
Trimethoprim (TrMP)	Antibiotic	0.9-1.4	400		(Suárez et al., 2008)

Table 4.8. Parameters derived from freundlich isotherm for adsorption of micro pollutants

Micro pollutants	Set 1			Set 2			Set 3			Set 4			Set 5		
	N	**K**	**R^2**	**N**	**K**	**R^2**	**N**	**K**	**R^2**	**N**	**K**	**R^2**	**n**	**K**	**R^2**
TrMP	3.2	0.2	0.9	2.9	0.1	0.9	0.2	0.0	0.8	1.9	0.0	0.8	2.3	0.01	0.9
SMX	2.1	0.0	0.8	2.5	0.0	0.9	0.4	0.0	0.6	0.7	0.0	0.7	0.9	0.00	0.8
DCF	4.8	0.4	0.9	6.0	0.4	0.9	0.7	0.0	0.7	1.9	0.0	0.8	2.5	0.01	0.9
CBZ	3.0	0.2	0.9	3.2	0.0	0.9	0.2	0.0	0.7	1.8	0.0	0.8	2.2	0.01	0.9
TCS	2.2	0.1	0.9	2.9	0.3	0.9	2.2	0.0	0.9	0.7	0.0	0.8	0.8	0.00	0.9

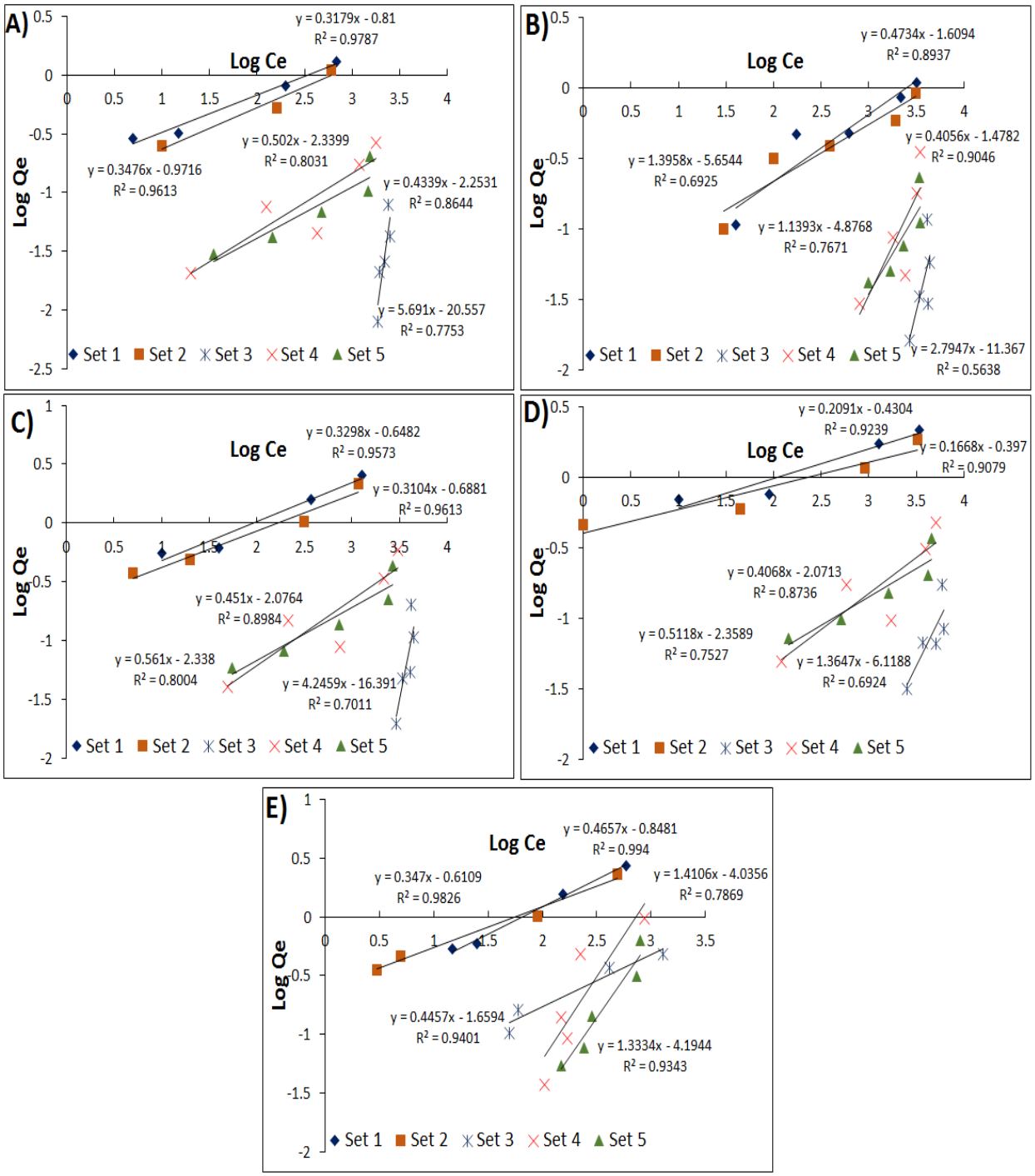


Figure 4.21. Adsorption isotherm at various bead to volume ratio for A) Trimethoprim B) Sulfamethoxazole, C) Carbamazepine, D) Diclofenac, E) Triclosan

4.4.2. Effect of PAC and QQ on Biofouling

Figure 4.22 shows the time-course of TMP in MBR-C, MBR-PAC_V and MBR-PAC_{QQ} at a fixed flux of 15 LMH. A continuous rise in intrinsic membrane resistance (R_m) was observed after every chemical cleaning (Table 4.9). A virgin membrane was used for MBR-C, which was fouled after 4 days of operation; the overall TMP rise was 3 and 6.5 times faster than that of MBR-PAC_V and MBR-PAC_{QQ}, respectively. In MBR-PAC_{QQ}, the TMP increased slowly until it reached 8 kPa, which corresponded to a delay in TMP jump by 450% as compared to MBR-C. Further, sharp rise in TMP was 2/3 slower than that in MBR-PAC_V. Overall, average fouling rate ($\Delta TMP/\Delta t$) for MBR-C, MBR-PAC_V and MBR-PAC_{QQ} was 0.3, 0.16 and 0.07 $kPa.h^{-1}$, respectively.

In an earlier study, addition of PAC resulted in an increase in filtration time by 25% (Villamil et al., 2016), whereas an increase in filtration time by 100% was achieved in this study through the combination of PAC and backwash. With the addition of QQ, filtration time further increased by 450% in MBR-PAC_{QQ}, this clearly depicts that integration of QQ strategy along with PAC impeded the membrane fouling to a maximum extent.

4.4.3. Effect of PAC and QQ on Membrane Resistances

To elucidate impacts of PAC and QQ mechanism on membrane fouling control, membrane resistance analysis was performed after each MBR run (Table 4.9). For MBR-C, cake resistance (R_c) was dominant contributing to 86% of total resistance (R_t), indicating strong cake layer formation on membrane surface within 4 days of operation. The contribution of R_c to R_t decreased to 50% in MBR-PAC_V,

which shows that surface scouring by vacant PAC beads and backwash reduced bacterial attachment to membrane surface. The reduction in cake layer resistance in the presence of PAC was also observed by Wu et al. (2017). Addition of QQ bacteria to PAC beads further reduced relative contribution of R_c by 50% as compared to MBR-PAC_V.

Since controlling pore blocking is essential for minimizing biofouling associated with MBR, backwash was introduced to reduce the pore block resistance (Rp) in MBR-PAC_V. A significant decrease in Rp in MBR-PAC_V by 67% as compared to that of MBR-C was achieved. However, with longer filtration time, irreversible pore blockage became more and more dominant; its contribution to R_t was maximum (43%) in MBR-PAC_{QQ}. These results indicate that integrating the QQ mechanism with PAC and backwash effectively reduced the biocake formation and physically reversible pore blockage, thus greatly extending the membrane cycle time.

4.4.4. Quenching QS Signal Molecules

Biofilm formation could be mitigated substantially by disrupting signal molecules such as AHLs, oligopeptides or AI-2 using enzymes released by QQ bacteria (Shrout and Nerenberg, 2012). In the present study, role of QQ bacteria was confirmed by monitoring concentrations of two types of signal molecules, AHLs and AI-2 using LC-MS. Figure 4.23A represents AHLs concentration in all three MBRs; substantial degradation of AHL signal molecules by QQ bacteria was obvious, while total concentration of AHLs in MBR-C and MBR-PAC_V was almost same. These results correspond to the previous findings (Kim et al., 2015; Lee et al., 2016; Maqbool et al., 2015). However, it should be noted that composition of AHLs were

completely different among three reactors; C8-HSLs, the dominant AHLs in both MBR-C and MBR-PAC_{QQ}, was not detected in MBR-PAC_V; $3OC_{12}$-HSL, an important one in both MBR-PAC_V and MBR-PAC_{QQ}, was also not detected in MBR-C; most of AHLs in MBR-PAC_V including C4-HSL, C10-HSL and C14-HSL were not detected in other 2 MBRs. These indicate that PAC in alginate beads might have altered microbial community within MBR, thus resulting in different AHL-mediated quorum sensing molecules. According to LaBarge et al. (2017), addition of activated carbon was also found to be effective for microbial enrichment. Moreover, influence of PAC on biomass, metabolic activity and floc stability in activated sludge process has been reported by Hu et al. (2015). This could further be linked to microbial composition. The on-going microbial community analysis will shed light on this.

The effectiveness of QQ bacteria in reducing AI-2 signal molecules is shown in Figure 4.23B, as MBR-PAC_{QQ} had lowest AI-2 concentration at day 4. However, gradual increase in AI-2 level afterwards suggest that AI-2-reducing strain in QQ consortia lost its quenching activity in long-term operation.

4.4.5. Effects of PAC and QQ on SMP Production and its Composition

Generation of SMP in MBR process is a serious concern, as they contribute directly to biofouling and effluent COD (Ghernaout et al., 2011), and bacterial re-growth and biofilm formation if permeate of MBR is recycled for various applications (Cai et al., 2016). In the present study, LC-OCD-OND was used to characterize SMP. The change in dissolved organic carbon (DOC) content with respect to initial level was compared for each MBR (Figure 4.24A), where

biopolymers, building blocks, low molecular weight (LMW) acids and neutrals were quantified as a part of hydrophilic (HI) DOC content.

By the time when the membrane was completely fouled, the total DOC concentration in MBR-C, MBR-PAC_V and MBR-PAC_{QQ} all increased as compared to their initial values. PAC may have adsorbed certain DOC initially but up to saturation in MBR-PAC_V and MBR-PAC_{QQ}; hence, longer operation time resulted in higher DOC concentrations at final stage. Interestingly, the DOC composition in MBR-PAC_{QQ} was significantly different from the other two. In MBR-PAC_{QQ}, DOC was primarily composed of LMW neutrals and hydrophobic (HB) DOC, which contributed to 76% and 20% of the total DOC content, respectively; whereas in the others, the sum of LMW neutrals and HB DOC were less than 50%. Furthermore, the building blocks, biopolymers and LMW acids, all decreased significantly in MBR-$PAC_{QQ,}$ while they all increased in the other two MBRs. Decrease in high MW organic compounds like biopolymers and building blocks in MBR-PAC_{QQ} could be due to the decomposition of these molecules to low MW organics (i.e. change in molecular size/weight etc.) as a result of prolonged suspension of PAC beads. These results suggest that the microbial variations in MBR-PAC_{QQ} may have biodegraded these building blocks, biopolymers and LMW acids in addition to QS molecules.

Earlier study by Pramanik et al. (2014), also verified the reduction of biopolymers (protein and polysaccharide) and building blocks (humic substances) through biodegradation and activated carbon adsorption respectively. Whereas, ultimate fouling in MBR-PAC_{QQ} could be due to reduction in adsorption capacity of the PAC with time because of partial blockage of pores by the microorganisms or macromolecules. Aromaticity of SMP in MBR-PAC_V and MBR-PAC_{QQ}

significantly reduced by 74 and 36% (Annex VI), respectively, which could be attributed to the strong affinity to aromatic compounds of PAC.

At the end of operation, the concentration of total protein and polysaccharides in MBR-C, MBR-PAC$_V$ and MBR-PAC$_{QQ}$ was found to be 27, 51 and 5 mg/L, respectively (Figure 4.24B). Significantly lower concentrations of these substances in MBR-PAC$_{QQ}$ authenticate the role of QQ mechanism in membrane fouling as also demonstrated by others (Kim et al., 2015; Lee et al., 2016; Maqbool et al., 2015). The concentration of polysaccharide was higher than LMW and HMW proteins in the mixed liquor of all MBRs except for MBR-PACv. These results together with the DOC compositions suggest that polysaccharides (biopolymer = polysaccharide + protein (HMW+LMW) more significantly contributed to cake layer formation, while the low MW organics would have entered the membrane pores partially or completely, resulting in hydraulically irreversible membrane fouling (Pramanik et al., 2015).

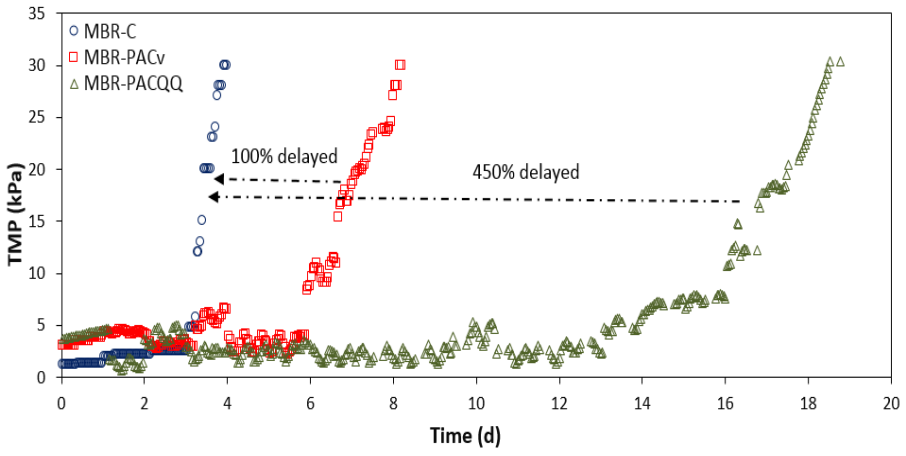


Figure 4.22. Trans-membrane pressure (TMP) trends of MBRs, Where: control MBR (C-MBR), (ii) control MBR with empty PAC beads (PAC$_V$-MBR) and (iii) MBR with addition of QQ bacteria entrapped in PAC beads (PAC$_{QQ}$-MBR)

Table 4.9. Relative distribution of filtration resistance using resistance-in-series model

Parameters	MBR-C (x 10^{12})	MBR-PAC$_V$ (x 10^{12})	MBR-PAC$_{QQ}$ (x 10^{12})
R_t (m^{-1})	21.2	8.3	7.2
$R_m + R_p$ (m^{-1})	3.05	4.1	5.43
R_m (m^{-1})	2.63	2.7	3.14
Rc (m^{-1})	18.1	4.2	1.8
R_p (m^{-1})	4.26	1.42	2.29
R_m / R_t (%)	12	32	43
Rc / R_t (%)	86	50	25
R_p / R_t (%)	2	17	32

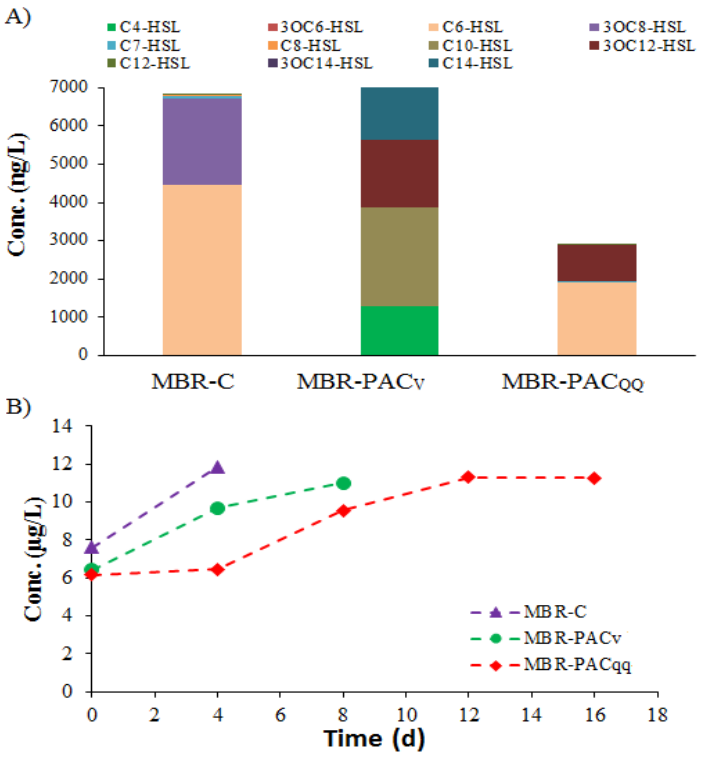


Figure 4.23. Remaining level of signal molecules in all MBRs detected through LC-MS A) AHLs concentration, B) AI-2 concentration

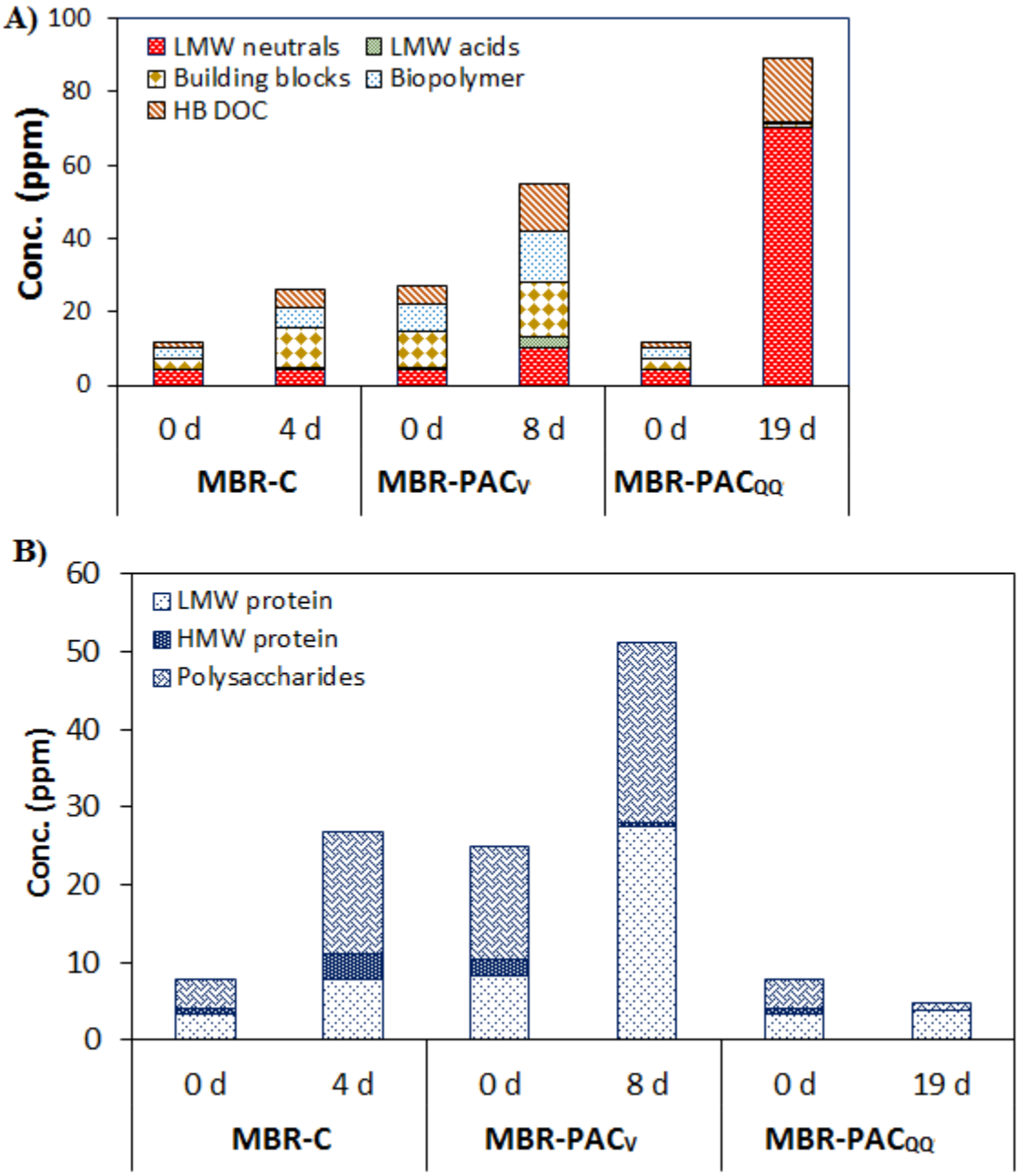


Figure 4.24. DOM in mixed liquor A) Concentration of hydrophobic and hydrophilic dissolved organic carbon B) Protein and polysaccharide distribution in MBRs

4.4.6. Effect of PAC and QQ on Sludge Properties

The MLSS and DO concentrations were maintained in the range of 5-6 g/L and 4-5 mg/L, respectively (Table 4.10). Oxygen uptake rate (OUR) and SOUR are frequently used to characterize respiratory activity, and to infer physiological state of cells (Garcia-Ochoa et al., 2010). The SOUR was same in MBR-C and MBR-PAC_V as shown in Table 4.10, whereas it doubled at the end of experiment in MBR-PAC_{QQ}. This shows that QQ phenomenon or disruption in bacterial signaling had a direct impact on metabolic activity of microbial cells; which could be related to (i) starving conditions developed during interruption in bacterial communications, (ii) dominance of strains required very high OUR (Garcia-Ochoa et al., 2010; Gomez et al., 2006), or (iii) amount of oxygen provided to the cell is less than amount consumed by the cell (Kirk and Szita, 2013). Since, DO level was maintained throughout the study, therefore either condition (i) or (ii) prevails.

The floc size distribution was monitored throughout the study. As shown in Table 4.10 and Figure 4.25, floc size in MBR-C did not change significantly, but it increased by 59 and 130% in MBR-PAC_V and MBR-PAC_{QQ}, respectively, by the end of operation. The microscopic images (Figure 4.25) also clearly shows the trend of increasing sludge floc sizes from MBR-C, to MBR-PAC_V, and further to MBR-PAC_{QQ}. The increase in sludge floc size has never been reported in QQ-supplemented MBRs, hence increase observed in this study was attributed to PAC in the QQ beads. Previous studies also showed that addition of PAC had changed sludge floc size (Massé et al., 2006). The smaller sludge flocs in MBR-PAC_V than those in MBR-PAC_{QQ} could be due to shorter contact time of PAC with the sludge. The larger flocs in MBR-PAC_{QQ} may contribute to better dewaterability, as

evidenced by the lowest CST values among all MBRs (Table 4.10), which was also reported by Maqbool et al. (2015). Change in floc size at regular intervals is shown in Annex V.

Figure 4.26 shows the protein-carbohydrate ratio (PN/C) of bound EPS, and concentration of bound EPS at different time intervals in all MBRs. The bound EPS increased in both MBR-C and MBR-PAC_V, and remained around 70 mg/g VSS when membrane was fouled; it also increased in MBR-PAC_{QQ} within the first 4 days, however, it decreased gradually after that and remained around 50 mg/g VSS when the membrane was fouled. This is in accordance with QQ effect (Lee et al., 2016). The initial sharp rises could be due to rejection of these compounds by membranes, thus resulting in accumulation over time inside the reactors (Meng et al., 2009). The relative high soluble EPS in MBR-PAC_{QQ}-BW could also be associated with large floc size (Wilén et al., 2003); where it may be anticipated that larger flocs in MBR-PAC_{QQ}-BW might have released higher amounts of soluble EPS. Maintenance of soluble EPS level afterwards could be attributed to entrapment of these molecules within large flocs or bacteria may reuse excess EPS as carbon source. It was reported that a higher EPS content corresponded to higher floc stability (Abdollahzadeh and Bonakdarpour, 2013) which further resulted in better settleability.

The zeta potentials of sludges from MBR-C and MBR-PAC_V remained relatively stable around -8 and -14 mV (Figure 4.26), respectively. The increase in absolute value of zeta potential might be because of the increase in PN/C ratio, as individual EPSs components like proteins, humic acids, uronic acids and nucleic acids in EPSs generally carry more abundant ionizable functional groups, which are the source of surface charges, than the polysaccharides (Liao et al., 2001).

Typically, lower zeta potential carries more negative charge which increases repulsive electrostatic interaction, thus affecting the agglomeration process. As indicated above, addition of PAC increased the sludge floc size, thereby, a sudden drop to below -18 mV in MBR-PACQQ after initial stabilization around -12 mV, still resulted in better dewaterability

4.4.7. Micro Pollutants Removal and MBR Performance

Figure 4.27 shows the removal efficiencies of selected pharmaceutical compounds in MBRs. The removal efficiencies of TrMP, SMX, CBZ, DCF and TCS were 71±15, 80±7.34, 63±21, 82±8.26 and 67±22%, respectively, in MBR-PAC_V; whereas the efficiencies increased slightly for all PhACs in MBR-PAC_{QQ}. CBZ showed the least removal efficiency among all the PhACs, which corresponded to previous studies (Zhang et al., 2016).

The PhACs adsorbed to sludge was also analyzed for both MBRs (Figure 4.27). Comparatively, less concentration of SMX, DCF and TCS was found in sludge; thus they could be considered as highly degradable. The average TrMP concentrations ranged from 0.18-0.91 µg/gVSS, while values for CBZ were 0.88 to 1.28 µg/gVSS in MBR-PAC_{QQ}. Therefore, removal of TrMP and CBZ could be due to membrane rejection than biodegradation.

Fate of all PhACs was calculated to compare biodegradation potential of vacant and QQ beads (Table 4.11). Consistency in biodegradation of all PhAC adsorbed on QQ beads shows the effectiveness of PAC-QQ beads. Furthermore, amount of pollutants adsorbed to PAC beads were quantified and shown in Table 4.12. Irrespective of better removal efficiencies achieved in the permeate of MBR-

PAC_{QQ} and same concentrations of adsorbate in sludge of both MBRs, concentrations of micro pollutants adsorbed to vacant PAC beads (after 8 d) was much higher than QQ bacteria-entrapped beads, measured after 18 d. It is well known that uptake or adsorption of micro pollutants generally depends upon availability of adsorption sites over the adsorbent (Hamdaoui and Naffrechoux, 2007). It can be inferred that the pollutants adsorbed to the PAC might be further utilized by the bacterial strains as their carbon source, hence regenerated some adsorption sites and enhanced removal efficiency. These findings suggest that simultaneous adsorption and biodegradation could be possible through PAC-QQ beads.

Similar COD removal efficiencies (93.0 ± 2.5, 95 ± 4 and 92.1 ± 1.5%) were achieved in MBR-C, MBR-PAC_V and MBR-PAC_{QQ} respectively. The addition of PACs or QQ did not result in any significant improvement in COD removal.

Table 4.10. Sludge characterization

Parameters (Units)	**MBR-C**	**MBR-PAC_V**	**MBR-PAC_{QQ}**
DO (mg/L)	4-5	4-5	4-5
MLSS conc. (mg/L)	5000-6000	5000-5700	5000-6500
SOUR (mg/g/hr)	59.0 ± 4.5	60.6 ± 2.4	96.73 ± 3.7
SVI (mL/g)	20 ± 3	42 ± 3.5	76 ± 2.5
CST (sec)	21.8 ± 0.5	18.0 ± 1.5	8.0 ± 0.3
PSA (μm)	50 ± 3 (0 d)	32 ± 6 (0 d)	72 ± 4 (0 d)
	44 ± 6.7 (4 d)	51 ± 3.5 (8 d)	164 ± 11 (19 d)

Table 4.11. Fate of PhACs in terms of biodegradability

Time (d)	Biodegradation = Q.Cin-Q.Ceff-Qs.Cs-ADS										
	MBR-PAC$_V$					Time (d)	MBR-PAC$_{QQ}$				
	TrMP	SMX	CBZ	DCF	TCS		TrMP	SMX	CBZ	DCF	TCS
2	4.1	2.7	3.9	5.4	9.9	4	4.6	1.1	3.4	4.6	5.3
4	4.5	2.9	4.6	5.4	2.8	8	3.5	2.5	3.4	5.8	6.2
6	0.7	-0.9	-1.3	-0.2	-0.2	12	6.4	0.9	5.3	6.0	3.6
8	12.4	6.8	13.2	1.5	-15.2	16	4.7	2.1	4.7	5.6	2.0
						18	5.4	2.1	4.2	4.9	2.5

Table 4.12. Contents of micro pollutants adsorbed to beads

Conc. (µg/mg)	MBR-PAC$_V$ (after 8 d)	MBR-PAC$_{QQ}$ (after 18 d)
TrMP	2.85 ± 0.21	9.09 ± 0.05
SMX	2.28 ± 0.03	0.72 ± 0.01
CBZ	3.27 ± 0.36	20.93 ± 0.16
DCF	1.38 ± 0.13	2.55 ± 0.01
TCS	1.46 ± 0.30	3.41 ± 0.01

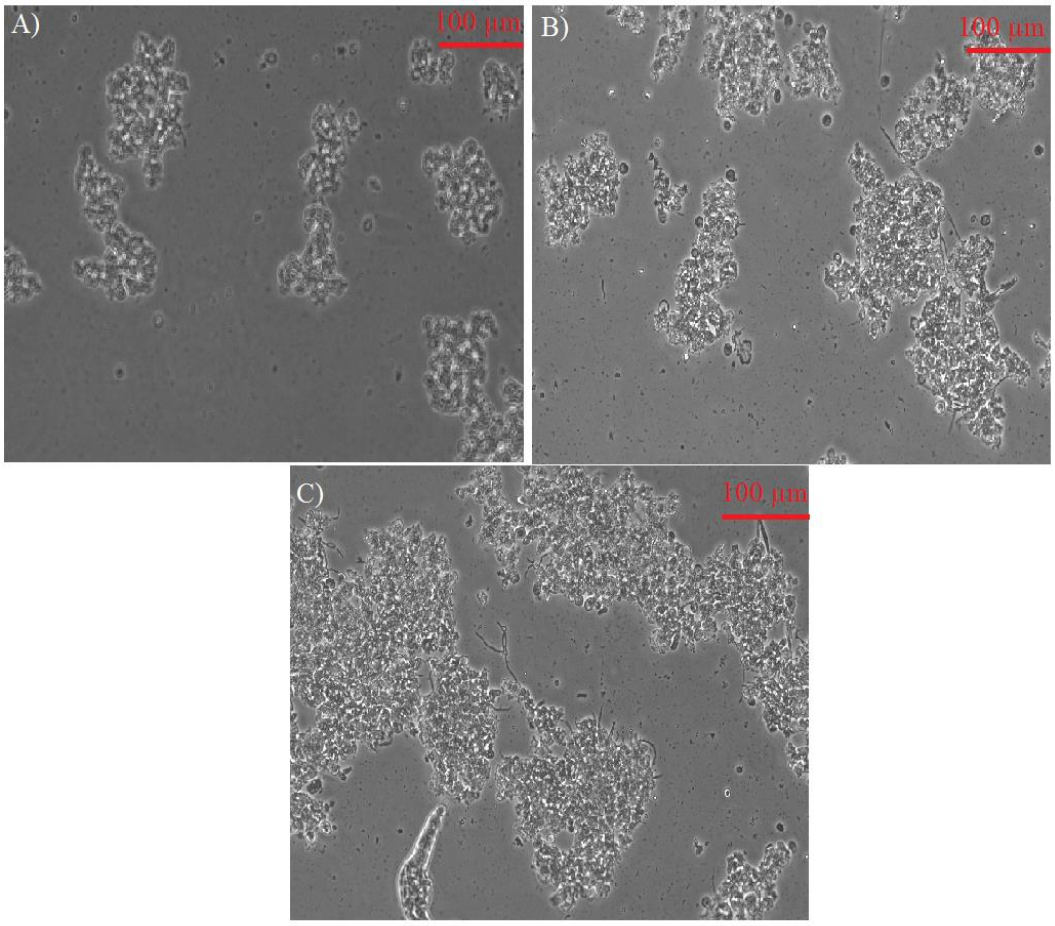


Figure 4.25. Microscopic observation of sludge floc size in (A) MBR-C (B) MBR-PAC_V and (C) MBR-PAC_{QQ}

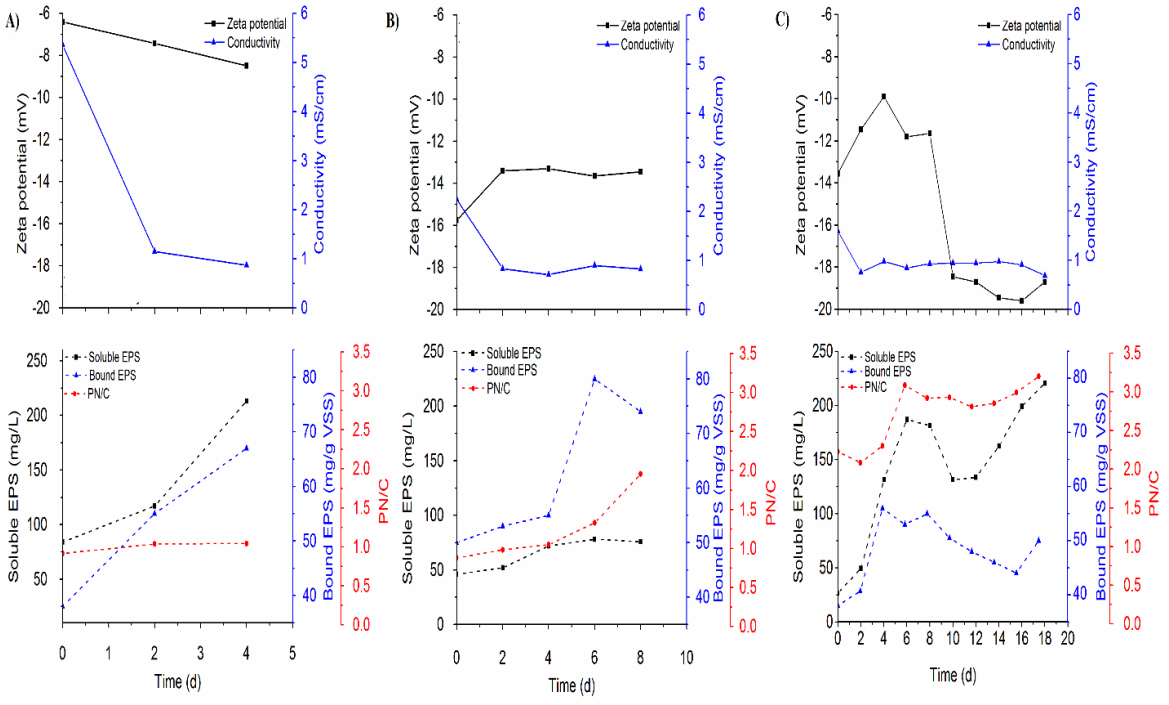


Figure 4.26. Zeta potential and extra cellular polymeric substances in (A) MBR-C (B) MBR-PAC_V and (C) MBR-PAC_{QQ}

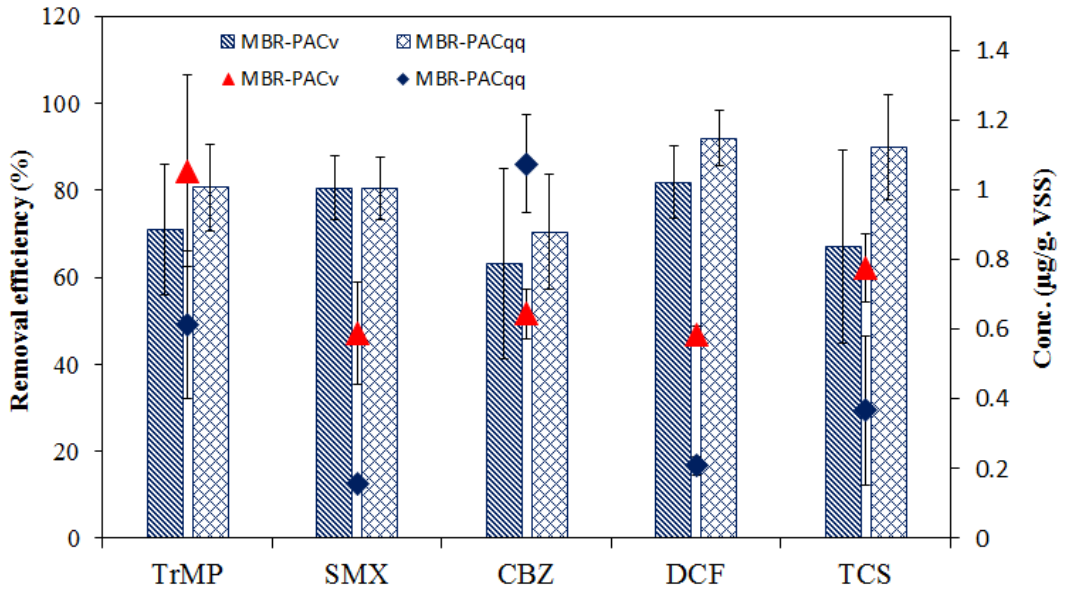


Figure 4.27. Average removal efficiencies of micro pollutants in permeate of MBRs and concentration of micro pollutants adsorbed to sludge The error bars represent standard deviation of multiple measurements (n = 5 for MBR-PAC_V, n = 6 for MBR-PAC_{QQ})

4.4.8. Effect of PAC on microbial diversity

The Illumina MiSeq high-throughput sequencing was used herein to analyze the microbial communities for better understanding of the effects of QQ or PAC in the MBRs. The PCoA plot (Figure 4.28) shows that there were significant differences in microbial communities between the biocake of the three MBRs, and between the sludge of MBR-PAC_V and MBR-PAC_{QQ}. Members of the phylum Proteobacteria was the most dominant group in all communities (Figure 4.29), with the OUT counts ranging from 34 to 55%; it was followed by Actinobacteria (19-36%) in all sludge or biockae communities. Members of the candidate phylum TM7 was a dominant group (26-30%) in the biocake of MBR-C, but it was barely present in other communities, and was largely replaced by the members of Cyanobacteria (11-22%) in MBR-PAC_V and by Bacteroidetes (24-25%) in the bioacke of MBR-PAC_{QQ}. The hydrodynamic forces generated by the beads could be the major factor leading to the difference in microbial communities between MBR-C and MBR-PAC_V, as similar influences on microbial communities resulted from shear forces were also observed in reverse-osmosis membranes (Ashhab et al., 2015). However, PAC could also have contributed to the shift in microbial community, although it had no impacts on microbial community structure in activated sludge in another study (Hu et al., 2015). The difference in microbial communities between MBR-PAC_V and MBR-PAC_{QQ} could be attributed to the QQ activity. These differences in microbial communities between MBRs have resulted in distinct AHL profiles (Figure 4.23) in MBRs.

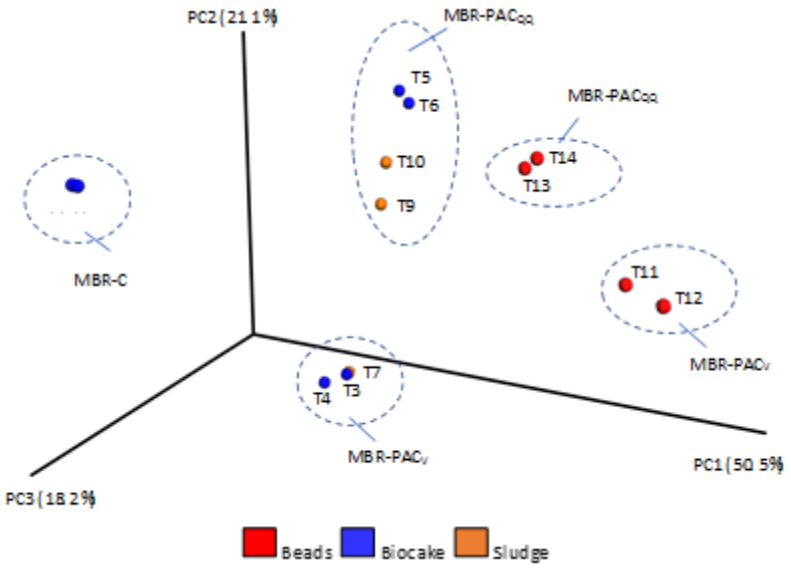


Figure 4.28. Principle coordinate analysis (PCoA) plot of weighted UniFrac distance comparing variation in MBR communities. T1-T12 are the sample names

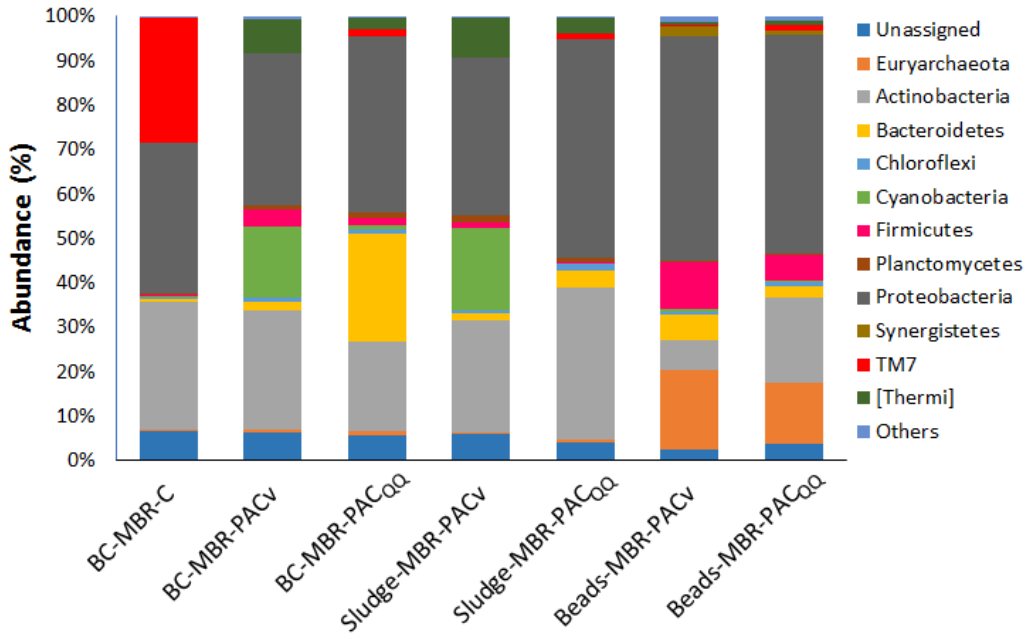


Figure 4.29. Relative abundance of major bacterial and archaeal phyla (>1%) in the communities of biocake (BC), sludge and beads in the MBRs. Each bar shows the average of duplicate samples except for 'Sludge-MBR-PACv', which is the result of only one sample

It is worth noticing that the microbial community of the biocake of MBR-PAC$_{QQ}$ was distinct from that of the sludge in the same reactor; the abundance of

Bacteroidetes was greatly reduced to 3-5% in the sludge, while the composition of Proteobacteria and Actinobacteria was increased to 48-50% and 32-36%, respectively, in the sludge, from 39-41% and 19-21% in the biocake. This difference in microbial communities between biocake and bulk sludge in MBRs was widely observed in full-scale MBRs, where the fluctuations in HRT, F/M or MLSS created selective pressures on biocake formation (Jo et al., 2016). However, in laboratory studies (internal communications), the microbial communities in biocake were very similar to those in bulk sludge. *Spirosoma* and an unidentified genus of the family Comamondaceae were the two dominant genera in biocake, which accounted for ~22% and ~14% of the total OUT counts, respectively; while two unidentified genera of the family Methylocystaceae and Streptomycetaceae were the dominant genera in the bulk sludge, with the abundance of ~17% and ~10%, respectively. Members of *Spirosoma* were observed to grow only as biofilms in a fresh river under defined successional stages (Lyautey et al., 2005). Bacteroidetes was also an important portion in biocake of an anaerobic MBR under ultrasonic treatment, which increased the production of proteinaceous EPS (Yu et al., 2012). Hence, with major inhibition of polysaccharide production in EPS, the QQ activity might have disturbed the balance of microbial community in MBR-PAC_{QQ} biocake and favored the growth of *Spirosoma*.

Figures 4.28 and 4.29 also show that very distinct microbial communities formed in PAC_V and PAC_{QQ} beads; Proteobacteria was still the most dominant phylum (46-55%) in beads, but methanogens of Euryarchaeota replaced a large portion of Actionbacteria and formed the second (15-21%) and third (13-15%) most dominant phylum in PAC_V and PAC_{QQ} beads, respectively. The genus *Methanosaeta*

was almost the sole (>99.5%) member of Euryarchaeota in both beads. In addition, members of Firmicutes increased to 8-13% and 5-7% in PAC_V and PAC_{QQ} beads, respectively. Both *Methanosaeta* and *Firmicutes* were widely present in methanogenic reactors (Rotaru et al., 2014). These results suggest that oxygen cannot diffuse into the alginate beads and reducing conditions form inside the beads; hence, methanogenesis could be an active metabolism inside the PAC_V and PAC_{QQ} beads. The methane produced by methanogens in MBRs might have been utilized by members of Methylocystaceae, a family comprises of many methane-oxidizing bacteria (Strong et al., 2015) in the MBRs. The presence of both oxidizing and reducing conditions may have contributed to the high biodegradation of PhACs inside both reactors.

Members of the same genera of the QQ strains except for the AI-2 degrading *E.coli* strain were all present in PAC_{QQ} beads, however, their abundance is very low (< 0.5% of total OUT counts). The presence of these microbes does not guarantee the survival and activity of QQ strains, since these genera except for *Microbacterium* were also present (< 0.5% of total OUT counts) in PAC_V beads. Hence, the microbial communities in beads were primarily influenced by the sludge in reactors. The absence of *Escherichia* in microbial community of PAC_{QQ} beads explains the increasing AI-2 level in MBR-PAC_{QQ} reactor (Figure 4.23B).

4.4.9. Summary

This study investigated the combined impact of PAC, QQ and backwash in membrane fouling control. Successful application of modified PAC-alginate-QQ beads was also investigated in a laboratory-scale MBR treating synthetic pharmaceutical wastewater. A 450% increase of cycle time was achieved in MBR with QQ strains entrapped PAC beads and backwash (MBR-PAC-QQ) as compared to control MBR (MBR-C). Substantial reduction in signal molecules showed the effectiveness and survival of QQ strains within PAC-alginate beads. Addition of PAC increased the floc size which further enhanced the sludge dewaterability. These QQ entrapped PAC beads provide a novel approach to mitigate biofouling and improve organic micropollutants (OMP) removal efficiency.

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

The research work, aimed at addressing the control of membrane biofouling through quorum quenching mechanism, conclude as follows.

5.1. CONCLUSIONS

Prevalence of quorum sensing bacteria and corresponding signal molecules was examined in MBR treating synthetic wastewater. Major findings of Phase 1 were as follows:

- Irrespective of growth rate, biofouling bacteria have inherently different biofilm formation tendencies which authenticates the role of various factors (EPS release, bacterial hydrophobicity, etc.) in QS.
- *Pseudomonas* sp. followed by *Delftia* sp. were scrutinized as the most dominant groups which need to be addressed while considering QS inhibition mechanism.
- The study also demonstrated that amount of EPS required to form the mature colonies varies depending on type of genus or species.
- Higher AHL concentration increases the biofilm formation however, QS is not solely regulated by one group of chemicals (e.g. AHLs) rather, non-AHLs molecules also have a key role in QS mechanism, which needs to be targeted.

A consortium of indigenously isolated QQ bacteria, with enhanced capacities to mineralize various signal molecules, was selected to control membrane biofouling in Phase 2. Main highlights of Phase 2 are:

- Antifouling bacterial consortium, comprising of *Enterobacter cloacae*, *Delftia* sp., and *Pseudomonas* sp., entrapped in polymeric beads were much more effective in controlling biofouling as compared to the single or without QQ bacteria.
- The effluent quality was not affected by QQ consortium with added advantage of 3X longer time required to foul the membranes.
- Distinct effect of bacterial entrapped beads on sludge characteristics like sludge particle and floc size was observed as only drawback of polymeric beads.

Phase 3 was designed specifically to examine role of quenching mechanism under steady state (fixed OLR) and transient conditions (varying OLR). Consortium of QQ strains was further modified to mineralize not only AHL's but another major class of signal molecules i.e. AI-2. Main findings are as follows:

- QQ consortium in immobilized beads showed robust fouling control capacity under both steady state and transient condition.
- Quantitative analysis revealed reduction in AI-2 and AHL levels in mixed liquor as well as sludge cake of MBR with QQ consortium.
- LB-EPS of mixed liquor was found to be major contributor to membrane fouling, and within the EPS, polysaccharides contributed more to membrane fouling than proteins.

- The pore blockage could be a governing factor leading to the final sharp increase of TMP in QQ MBRs.

Further, following the findings of Phase 2 and 3, Phase 4 was designed to overcome pore blockage and reduction of floc size via backwash and PAC addition. Therefore, combined impact of PAC, QQ and backwash in membrane fouling control was investigated. Where,

- Successful application of modified PAC-alginate beads was verified in a lab scale MBR treating synthetic pharmaceutical wastewater. Moreover, substantial reduction in signal molecules showed effectiveness and survival of QQ strains within PAC-alginate beads.
- A 450% increase of cycle time was achieved in MBR with QQ strains entrapped PAC beads and backwash as compared to control MBR.
- Addition of PAC increased floc size which further enhanced sludge settleability.
- QQ entrapped PAC beads provide a novel MBR with less biofouling and high removal efficiency of organic micro pollutants (OMP).

5.2. RECOMMENDATIONS

In order to further improve efficacy of QQ mechanism and address existing obstacles, future researches should be focused on the following aspects:

- Immobilization not only provides nutrients for target bacteria without competing with other bacteria but also protects them from environmental stress. Hence, continuous searching for more effective QQ bacteria as well

as more stable immobilization materials and relatively simple immobilization methods is still highly desirable for biofouling control in MBRs.

- Meanwhile, experiments should also be conducted to correlate the amount of QQ enzymes released by certain QQ bacteria, required to degrade signal molecules per unit time. This information would be very effective to optimize addition of QQ bacteria within MBR and to scale up results to pilot scale treatment systems and verify their effectiveness using real wastewater.
- Online monitoring of biofilm architecture and formation should be observed to elucidate role of fouling control techniques in retarding membrane fouling. Certainly, improved understanding and potential applications of AHL-based QS and QQ will help to engineer biological wastewater treatment systems successfully.

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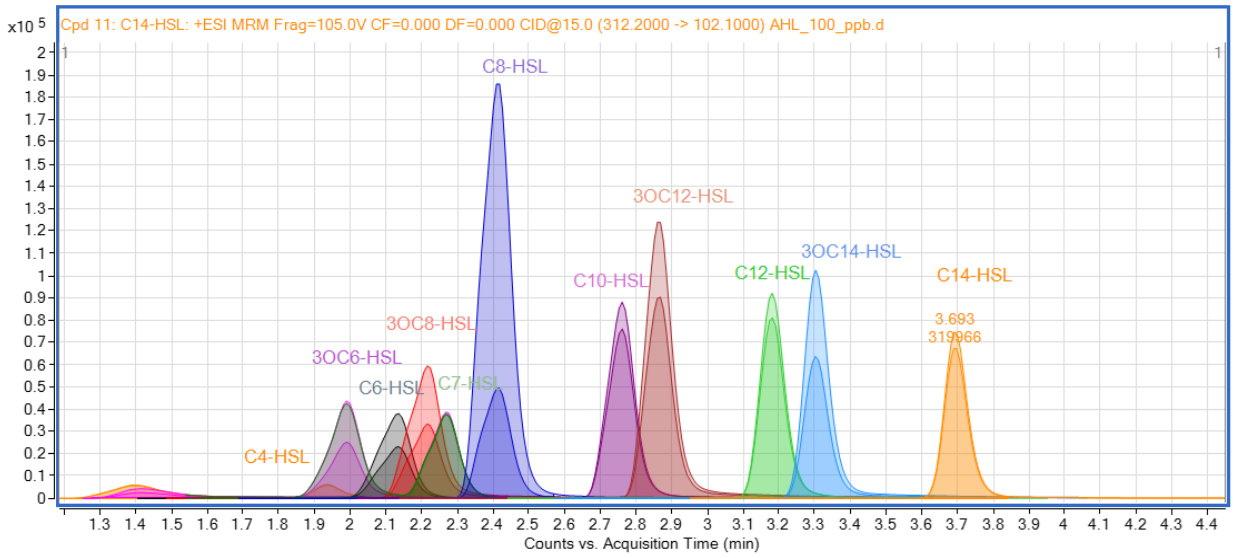

Figure Ia. Chromatogram of AHLs standards

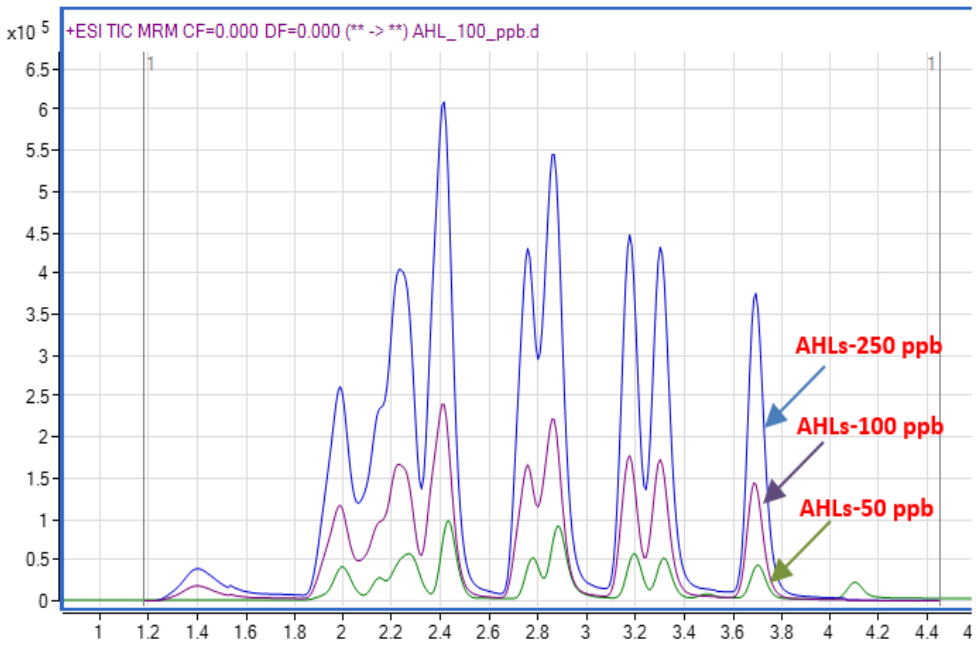

Figure Ib. Varying concentration of AHLs standards for quantitative analysis via LC-MS/MS

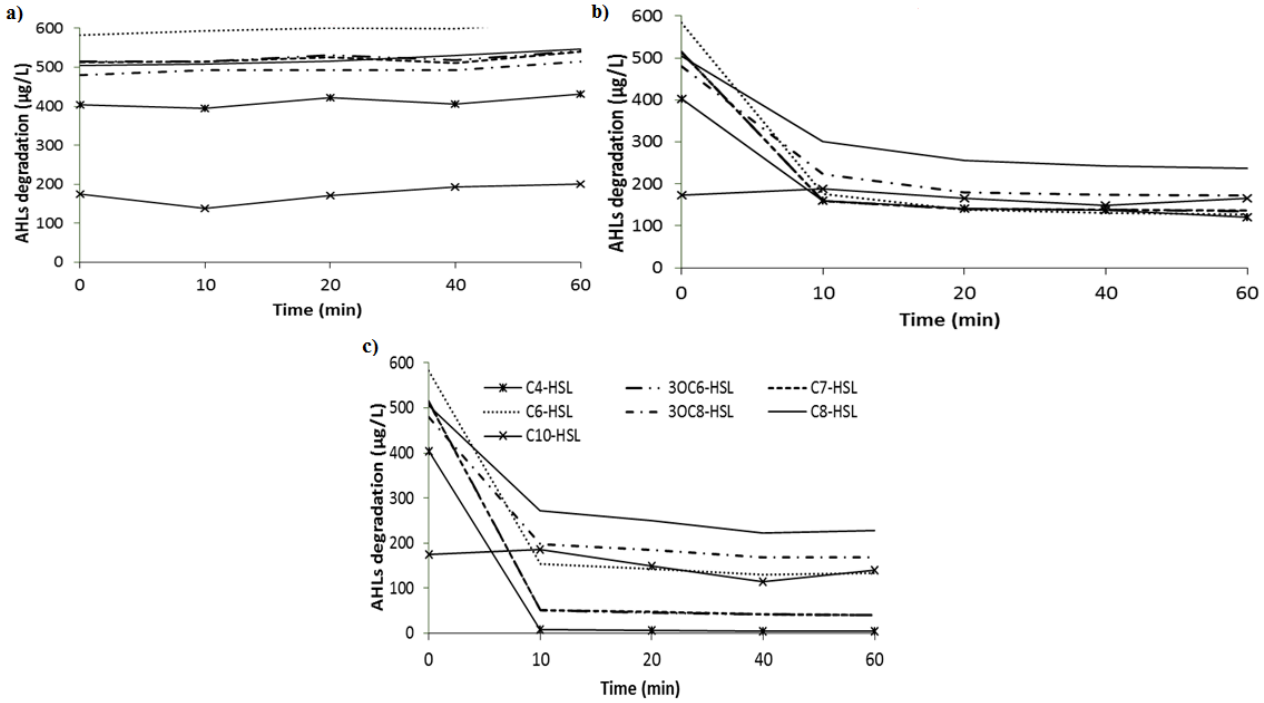

Figure II. Level of AHLs a) under controlled conditions b) in the presence of *Rhodococcus* BH4 c) QQ consortium

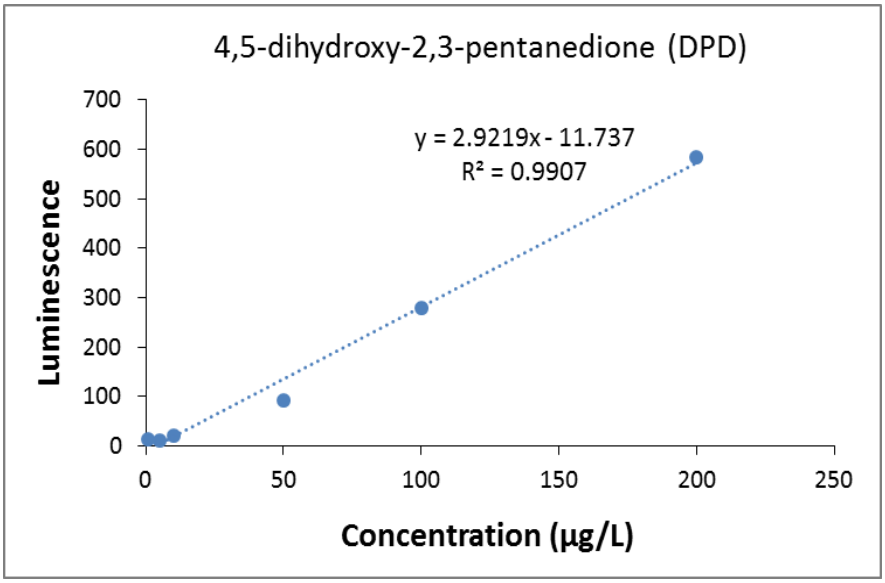

Figure IIIa. Calibration curve of DPD standard used for AI-2 quantification via BB170 assay

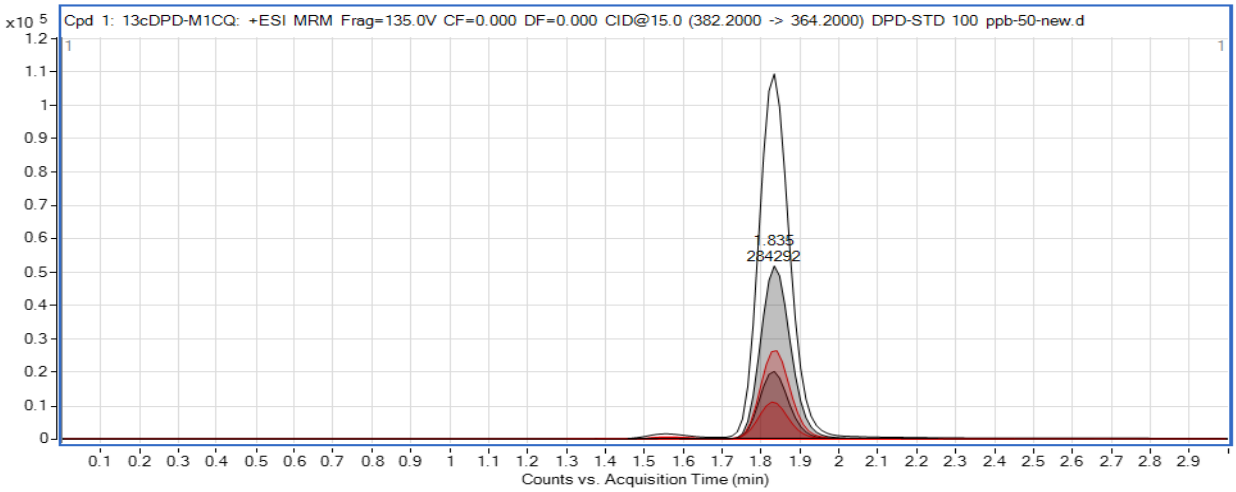

Figure IIIb. Calibration peak of DPD standard used for AI-2 quantification via LC-MS/MS

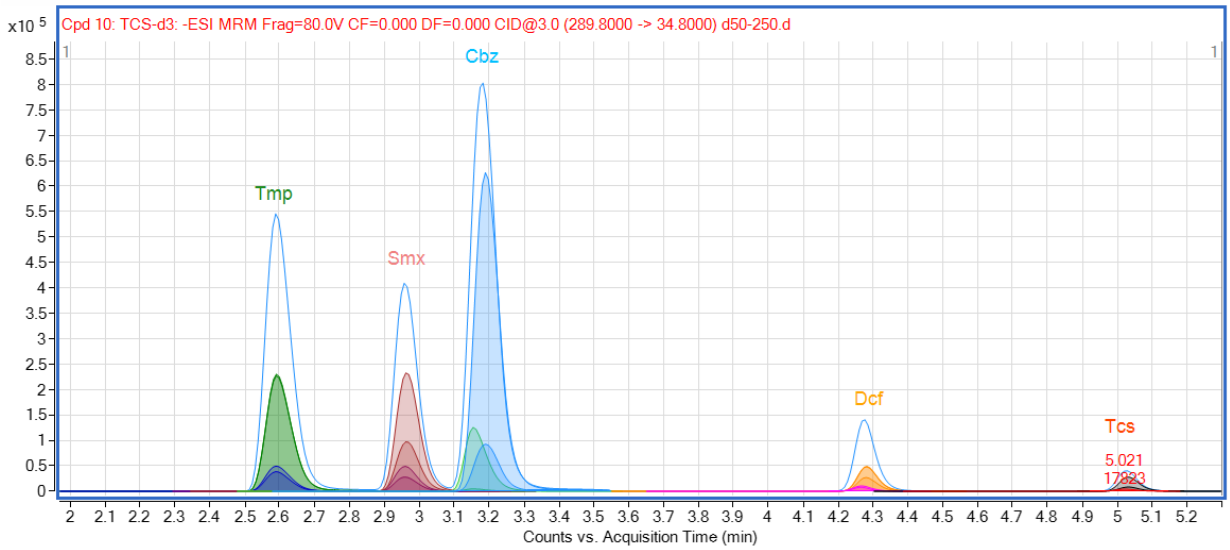

Figure IV. Chromatographic separation of PhACs at varying concentration via LC-MS/MS

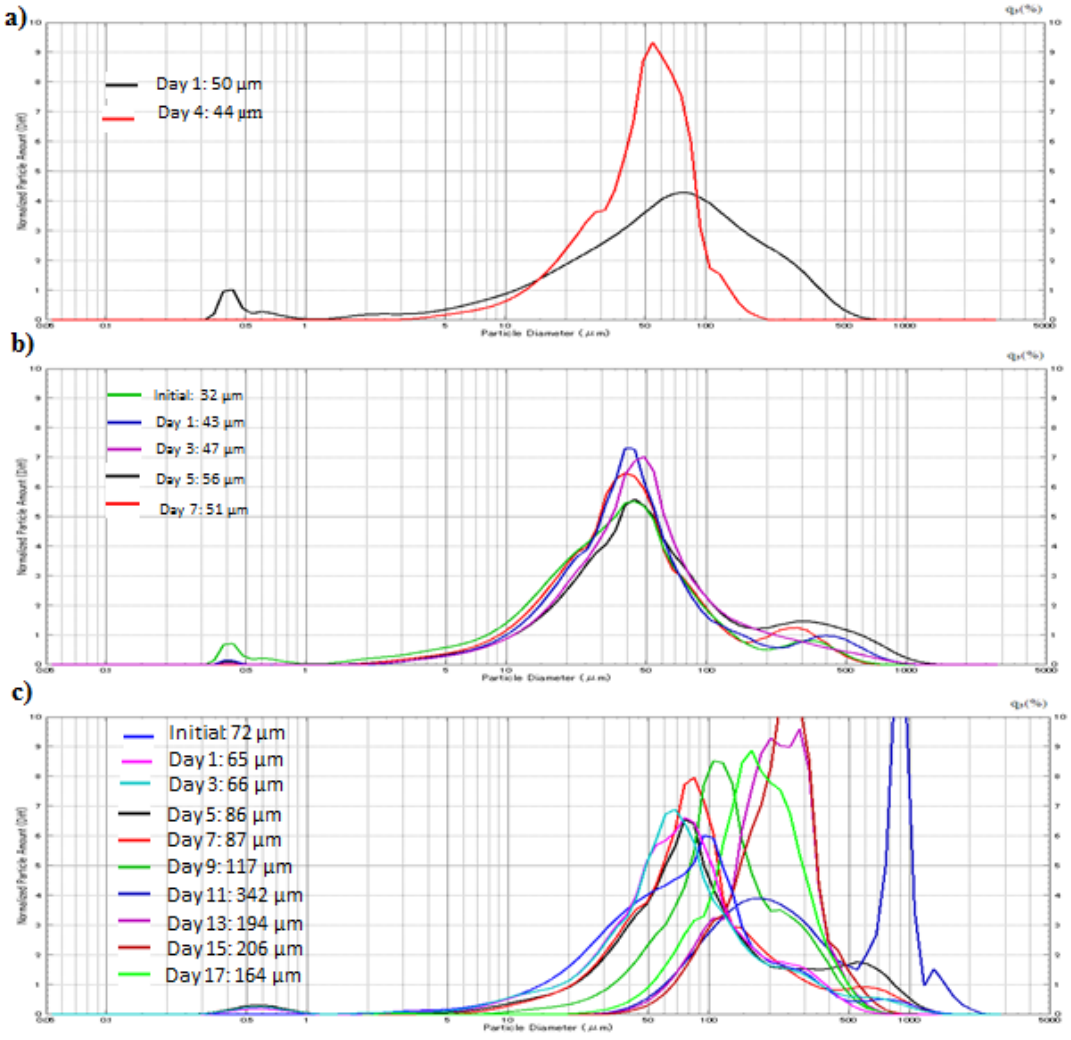

Figure V: Floc size distribution of a) MBR-C, b) MBR-PAC$_V$ and c) MBR-PAC$_{QQ}$ at regular intervals in Phase 4

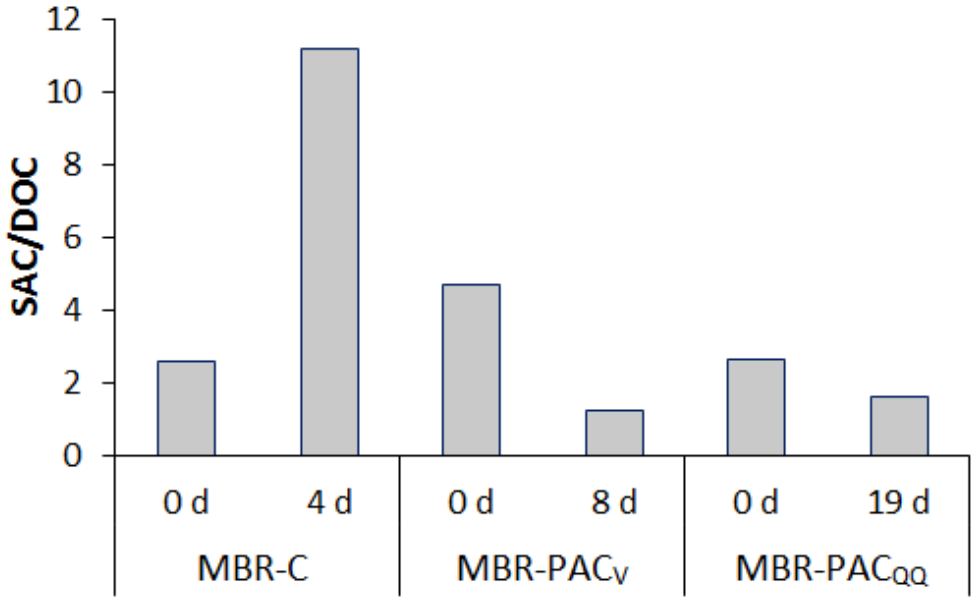

Figure VI: Aromaticity (SAC/DOC) in the mixed liquor of all MBRs

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