Effects of Microbial Quorum Sensing on Membrane Fouling and Characteristics of Extracellular Polymeric Substances in a Membrane Bioreactor Process

Dissertation

Effects of Microbial Quorum Sensing on Membrane Fouling and Characteristics of Extracellular Polymeric Substances in a Membrane Bioreactor Process

Graduate School of
Natural Science & Technology
Kanazawa University

Division of Environmental Design

1524052006

HONG PHUC NGUON

Chief supervisor: Ryo HONDA

January 2018

Dissertation

Effects of Microbial Quorum Sensing on Membrane Fouling and Characteristics of Extracellular Polymeric Substances in a Membrane Bioreactor Process

Graduate School of
Natural Science & Technology
Kanazawa University

Division of Environmental Design

1524052006

HONG PHUC NGUON

Chief supervisor: Ryo HONDA

January 2018

ACKNOWLEDGEMENTS

Firstly, I would like to appreciate examination committee: Prof. Ikemoto, Prof. Furuuchi, Prof. Kosui, and Prof. Hata, for their insightful and valuable comments which improved content of the dissertation.

Especially, I would like to express my sincere gratitude to my advisor Prof. Ryo Honda for his knowledge transfer to me, for continuous supports of my study and research, for his patience, motivation, and forgiven to me. His guidance helped me in all the time of research, writing of papers and this dissertation.

My sincere thanks also go to Prof. Ikemoto, Dr. Noguchi, Dr. Matsuura, and Dr. Tobino who provided valuable advice, suggestions about my research and who gave access to the laboratory and research facilities. Without their precious supports, it would not be possible to conduct this research.

I would like to thank my tutor, research-group members: Ms. Thanh, Ms. Tiang, Mr. Panchavinin, Mr. Ito, Mr. Yoshida, Mr. Aizawa and lab-mates: especially Ms. Sulfika, Mr. Yang for the assistances and discussions, for all parties and fun that we have had for three years. Also I thank my friends in the following Water Environmental Engineering Lab, especially Ms. Kanazawa and Ms. Guo, for supporting my laboratory works.

I would like to express my thanks to laboratory secretary Ms. Takeda for her help in paperwork and ordering; and to all teachers, staffs, friends and colleagues who willingly supported me for the years in Japan. I am really grateful to Mekong 1000 project for financial supports, to Kubota Company for search materials. I also thank to the community of Vietnamese students in Kanazawa “Vietkindai” supporting me for the years in Japan.

Last but not the least, I would like to thank my parents who have given me this wonderful life; my brothers, sisters and my lover for always supporting me spiritually throughout my study, work and life.

Contents

List of abbreviations

3D-EEM	Three-dimensional excitation-emission matrix
AHL	Acyl-homoserine lactones
AI	Auto-inducer
BSA	Bovine serum albumin
CAS	Conventional activated sludge process
CCA	Canonical correspondence analysis
DO	Dissolved oxygen
DOC	Dissolved organic carbon
DN	Dissolved nitrogen
ELSD	Evaporative light scattering detector
EPS	Extracellular polymeric substances
FTMS	Fourier transform mass spectrometry
HPI	Hydrophilic/ Hydrophilicity
HPO	Hydrophobic/ Hydrophobicity
HRT	Hydraulic retention time
HS	Humic substances
LB-EPS	Loosely-bound EPS
MBR	Membrane bioreactor
MLSS	Mixed liquor suspended solids
MW	Molecular weight
NGS	Next-generation sequencing
OTU	Operational taxonomic unit
OLR	Organic loading ratio
PN	Protein

PS	Polysaccharide
QS	Quorum sensing
QQ	Quorum quenching
SEC	Size-exclusion chromatography
SMP	Soluble microbial products
SRT	Sludge retention time
SS	Suspended solids
SVI	Sludge volume index
TB-EPS	Tightly-bound EPS
TMP	Transmembrane pressure
TN	Total nitrogen
TOC	Total organic carbon
TS	Total solids
VS	volatile solids

Chapter 1. Introduction

1.1. Background and Objective

Membrane bioreactor (MBR) is a combination of membrane modules with conventional activated sludge process (CAS) [1]. This technology is increasingly widely applied for treatments of wastewater, re-used and drinking water in microfiltration, ultrafiltration, nanofiltration processes [2]. Reverse osmosis and forward osmosis membranes have been also used for potable water reuse, for microalgae cultivation process as well [3, 4]. Number of "mega-scale" MBR plants (with capacity over one million cubic meter per day) have been continuously increasing, especially in China and the United States [5] since MBRs offer plentiful benefits including excellent quality of effluent, high concentration of mixed liquor suspended solids (MLSS), reduction in reactor volume and footprint [6]. However, widespread application of the MBR technology is seriously restrained by membrane fouling [1, 2, 6, 7, 8], which causes a reduction in productivity and an increase in operation and energy costs [8]. Membrane fouling can be classified into three categories based on foulant material, such as inorganic fouling, organic fouling, and biofouling [6, 9]. The organic fouling and biofouling caused via attachment of extracellular polymeric substances (EPS) and microbial growth (biofilm formation) attract more attentions since the fouling quickly recovers after being removed (after a few weeks even a few days) [2, 8, 9]. The term of "membrane fouling" in this study was used to mostly focus on organic fouling and biofouling via attachment of EPS and biofilm formation.

Extracellular polymeric substances are known as macromolecular biopolymers, naturally biosynthesized by prokaryotes and eukaryotes [8, 10]. EPS layers around microbial aggregates (e.g., biofilm and sludge floc, granules) function as protective barrier and

energy storage [11, 10]. Recently, EPS have been considered to play important roles in membrane fouling of MBR process. The possible reasons are: (i) EPS substances can condition surface and internal structure of membrane (membrane pores), encouraging attachment of others foulants on/ in the membrane [9, 12]; (ii) EPS cause pore clogging and deposit on membrane surface to from cake layer [8, 13]; (iii) EPS produced on membrane surface under microbial biofilm formation make the cake layer thicker and membrane fouling more serious [9]. In addition, EPS also directly determine surface properties of sludge flocs including charge, hydrophilicity/ hydrophobicity (HPI/ HPO), molecular weight (MW) distribution, which are important for stability of sludge flocs, attachment of foulants on membrane, biofouling development as well [8, 9]. For these reasons, EPS concentration and characteristics have been expected to determine the extent and severity of fouling condition [14]. However, fouling mechanism caused by EPS is not comprehensively understood. Moreover, possible correlations between EPS production and membrane fouling are still controversial among researches.

EPS production and decomposition are known as responses of microbes to external environmental conditions [10]. Microbes are found to produce more EPS under stressful conditions. EPS are also produced when nutrients are redundant and EPS are decomposed for energy demand when nutrients are insufficient. EPS production and decomposition from microbes in MBRs are believed to make fouling more severe. Therefore, bacterial community and EPS production are believed to be important factors of membrane fouling, which are reportedly affected by operational conditions [15, 16, 17]. Although a number of studies have attempted to investigate development and behavior of microbial community on membrane surface under different operation condition, information about microbial ecology of bio-cake layers are limited. For this reason, bacterial structure of

cake layer of MBRs is still one of the hottest topics in membrane fouling study.

To control membrane fouling, several approaches have been recommended and evaluated, including operational optimization, membrane cleanings, and biological controls [5, 17]. Recently, EPS production and biofilm formation causing membrane fouling have been found to have positive relation to microbial quorum sensing (QS) activity in MBRs [18, 19, 20, 21]. Therefore, quorum sensing inhibition is acknowledged to be a sustainable and promising approach to control membrane fouling. Quorum sensing is known as cell to cell communication among microorganisms to regulating their collective behaviors including biofilm formation [22]. As this reason, inhibition of microbial QS activity in a MBR is believed to reduce biofilm formation on membrane surface, resulting in a biofouling mitigation [23]. Bacteria, enzyme and natural compounds can be QS inhibitors and QS inhibition is also named quorum quenching (QQ) [21]. Some enzymes and bacteria are found to inhibit QS process and fouling control via these bacteria and enzymes has been increasingly investigated [24, 25]. Nonetheless, QQ bacteria need to be uncaptured in carriers while enzymes are too expensive [21, 24]. Natural compounds can be easier to be directly applied in practical MBRs than bacteria and enzymes. A few natural compounds (e.g., vanillin) are able to inhibit microbial QS in MBRs [21], which can be applied to reduce fouling severity. However, membrane fouling control via QS inhibitions requires more comprehensively experimental knowledge about possible effects of QS inhibition on EPS characteristics, bacterial community and membrane fouling.

Objectives of this study are: to understand characteristics of EPS in MBRs and to control membrane fouling via quorum sensing inhibition.

To achieve the mentioned objectives, extraction methods from literature were evaluated in EPS extraction efficiency and effects on EPS characteristics. Therefore, suitable methods were chosen to fractionate and extract EPS from MBR sludge. Besides, methods for EPS characterization was also developed in order to characterize crucial EPS components in MBRs, which contribute to development of membrane fouling.

Characteristics and production of EPS in a lab-scale MBR operated with two stages: constant flux and constant TMP were investigated. Bacterial community structure was also analyzed to investigate presence of quorum sensing bacteria in the MBR. Findings from the bacterial community and EPS characteristic analysis were necessary for investigation of membrane fouling control via QS inhibition.

To control membrane fouling, QS inhibitor (e.g., vanillin) was added into a lab-scale MBR; and effects of vanillin on regulation of QS, bacterial community and key EPS components contributing to fouling reduction were investigated.

1.2. Thesis Organization

This thesis was comprised of 8 chapters and main contents of each chapter were described as below. Organization of this thesis was shown in Figure 1.1.

Chapter 1 “Introduction” described current issues in MBR research and application, which are necessary to be investigated in details. They include EPS-related fouling, bacteria community structure and membrane fouling control via microbial QS inhibition. The objectives of this study were also stated in this chapter.

Chapter 2 “Literature Review” summarized updated-knowledge and findings from previous studies about EPS and membrane fouling in MBRs. Application of QS inhibition to control membrane fouling was also reviewed.

Chapter 3 “Materials and Methodology” summarized materials, analytical instruments and methods. This chapter also described property and operation of lab-scale MBRs of the study.

In chapter 4 “Optimum Methods for EPS Extraction”, efficient methods of EPS extraction reported in previous studies were comprehensively compared in extraction efficiency. Therefore, three suitable methods were chosen to extract and fractionate EPS from MBR sludge into soluble microbial products, loosely-bound EPS and tightly-bound EPS.

In chapter 5 “Characterization of EPS Causing Membrane Fouling”, EPS fractions of bulk sludge and cake layer were characterized in hydrophobic/ hydrophilic property and

molecular weight distribution. A two-dimensional matrix named polarity – molecular weight profile was developed to figure key EPS components importantly contributing to membrane fouling.

In chapter 6 “Bacterial Community Structure in a MBR under a Constant TMP and a Constant Flux”, bacterial community and EPS production in bulk sludge and cake layers were investigated to figure out fundamental microbes in MBR and membrane fouling. Furthermore, correlation of operation parameters, dominant bacteria in MBRs and EPS related foulants was evaluated.

In chapter 7 “Effect of Vanillin on Regulation of Quorum Sensing and Membrane Fouling”, EPS productions in bulk sludge and cake layer of MBR under vanillin addition were studied. Moreover, polarity – molecular weight profile of EPS under vanillin addition were evaluated. Besides, membrane performance and bacterial structure of cake layer were investigated. This chapter was conducted based on findings in Chapter 5 and 6.

In chapter 8 “Conclusions and Future Perspectives”, conclusions about suitable methods for EPS extraction from MBR sludge and fouling potentials of EPS fractions were stated. Potential impacts of QS inhibition on MBRs were described. Future research needs in biofouling control were also suggested.

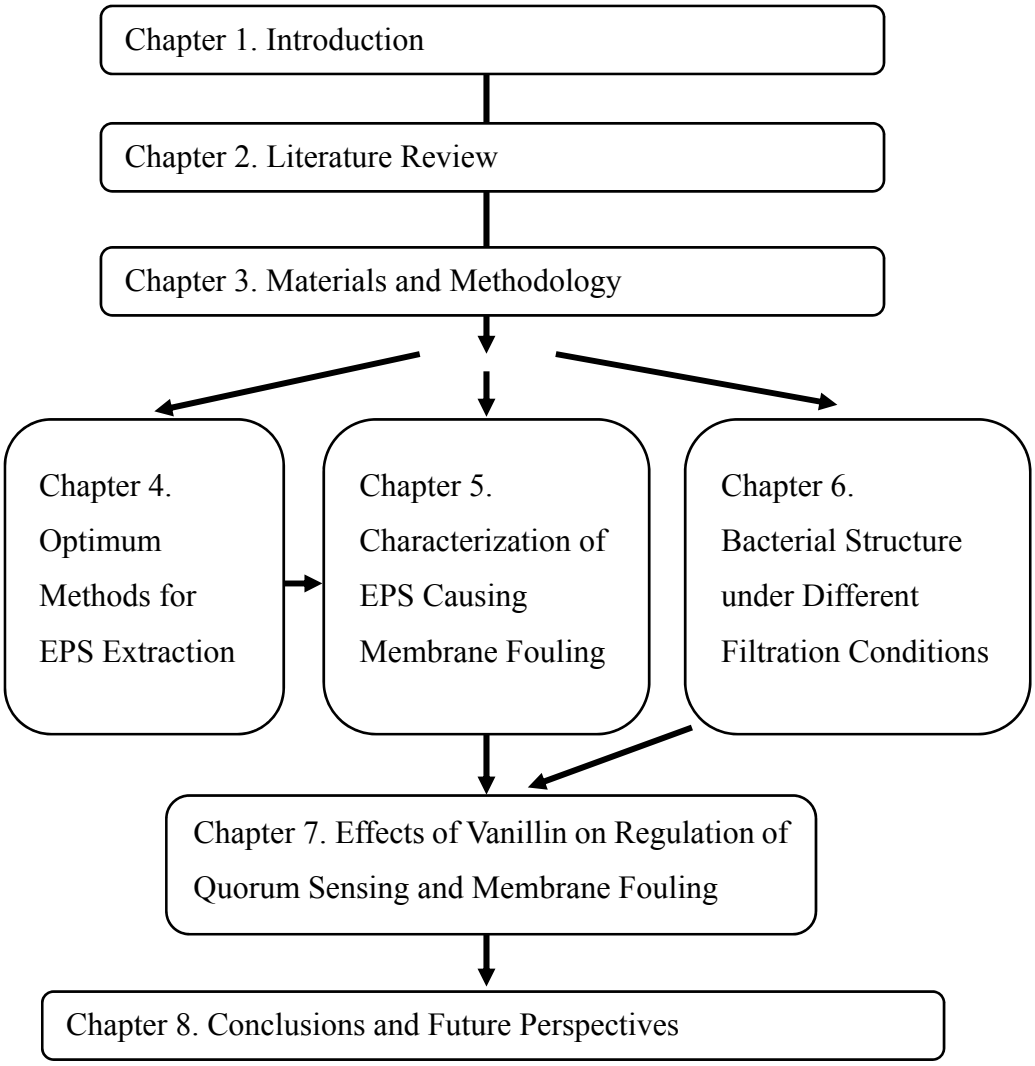


Figure 1.1. Diagram of thesis organization

Chapter 2. Literature Review

2.1. Membrane Fouling

2.2.1. Definition and mechanism of membrane fouling

Membrane fouling can be defined as deposition of suspended or dissolved substances on membrane surface or within membrane pores causing reduction of treatment performance [26]. Membrane fouling is also known as interactions between bulk sludge components (foulants) and membrane units [8], causing ether permeate flux decline under constant transmembrane pressure (TMP) operation or TMP increase under constant flux operation [1]. Membrane fouling is considered as a main barrier of widespread application of the MBR process [12] because it causes poorer membrane permeability, more serious flux decline, resulting in higher costs for more energy consumption, more frequent membrane cleaning and replacement [17]. Membrane foulings are usually classified into inorganic fouling, organic fouling, and biofouling according foulant material. Foulants in MBRs are also grouped as removable, irremovable and irreversible foulants [12]. The removable foulant is removed by physical cleaning and the irremovable foulant is eliminated by chemical cleaning, meanwhile the irreversible foulant cannot be removed by any approaches (Figure 2.1).

Constant flux operations are referred to be applied in practical MBRs, in which TMP profile of a membrane fouling process has been found to obtain three stages (Figure 2.2): sharp, prolonged, and rapid increases [8], equivalent to conditioning fouling, steady fouling and TMP jump, respectively [1]. The fouling process is usually summarized into three mechanisms (Figure 2.2): (i) pore narrowing/ clogging by substances having sizes less than or comparable to membrane pores; (ii) gel layer and cake layer formation via attachment of the greater-size substances, microbial cells on membranes; and (iii) biofilm

formation – growth of bacterial community on membrane surfaces [2, 8]. Potential foulants in MBRs can be small sludge flocs, individual cells, microbial products, non-degradable matters, natural organic matters, etc. [2]. These potential foulants form complex interactions together and with membrane units, making the fouling process really complicated [17]. Moreover, membrane fouling can be become more complicated due to effects of a number of operational parameters, being described as following.

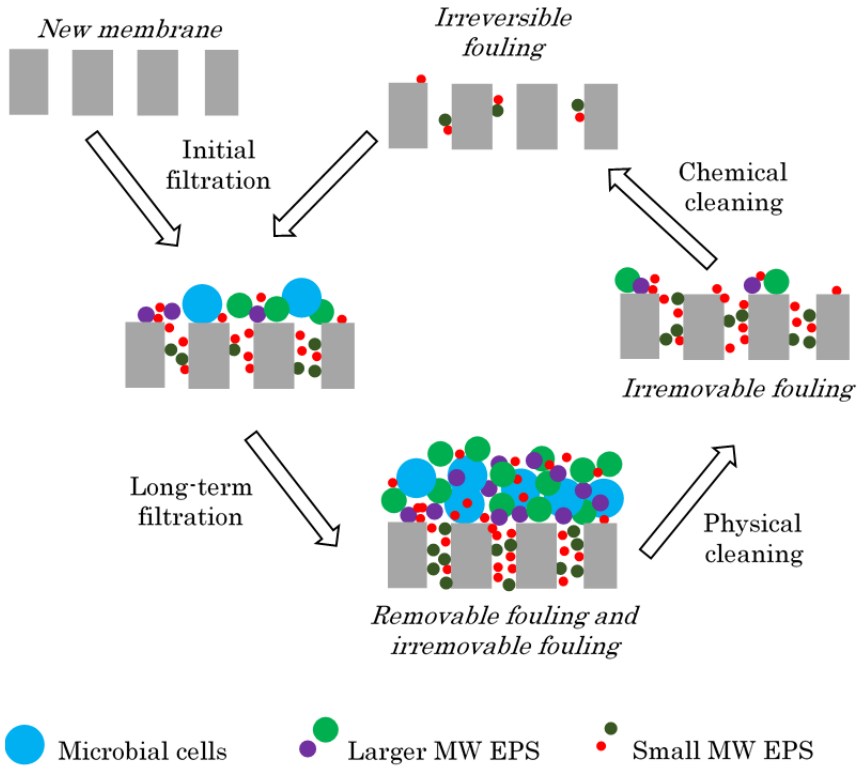


Figure 2.1. Illustration of removable, irremovable and irreversible foulings [12]

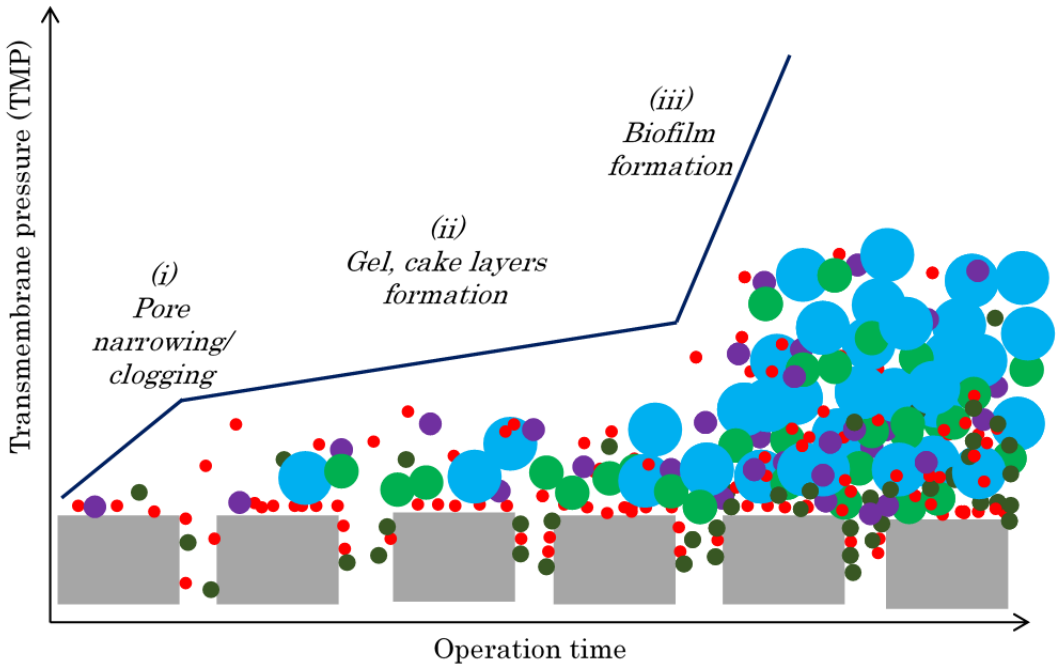


Figure 2.2. Illustration of TMP profile and membrane fouling mechanisms [2, 5, 8]

2.2.2. Affecting factors of membrane fouling

Effects on membrane fouling of operational parameters, mixed liquor properties, feed water characteristics and membrane properties have been briefly summarized in Figure 2.3 [17]. Membrane units are fouled faster under too long or short sludge retention time (SRT), affecting microbial community and further activated sludge characteristics [12, 14] whereas shorter hydraulic retention time (HRT) increases fouling rate because of increasing organic loading ratio (OLR) [27]. Different levels of air flow rate (or DO) and temperature strongly affect microbial community structure in bulk sludge, cake layers, hydrodynamic condition, and EPS characteristics, governing different rate in membrane fouling [17, 28, 29, 30, 31].

The OLR (or ratio of feeding to mass) and nutrient parameters (e.g., C/N, C/P ratios, cations, etc.) reportedly have influences in microbial structure, behavior, MLSS

concentration, and floc size, further influencing membrane biofouling [17]. In fact, higher OLRs increase EPS levels and facilitate cake layer formation on the membrane surface while low OLRs can encourage EPS degradation for microbial activities, being advantageous to fouling reduction. However, low OLRs for a long time prompt the release of low MW substances (e.g., humic substances) from EPS degradation [32], and the low MW substances can lead to serious fouling by membrane pore clogging [33]. Divalent cations (i.e. Mg^{2+}, Ca^{2+}) from feeding are important for formation and stabilization of microbial aggregation and sludge flocs, mitigating membrane biofouling [17, 34].

Sludge floc size was reported to have positive correlation with membrane permeability meanwhile higher MLSS levels and viscosity can return in higher TMP and greater membrane fouling resistance [17, 35]. In addition, investigations in effects of membrane characteristics reported that type and pore size of membranes have impacts on membrane fouling [36]. Besides, high hydrophilicity, asymmetric structure, interconnected pore morphology, and high surface porosity can lessen membrane fouling [37]. Membrane surface zeta potential and roughness are more vital in the interaction between EPS, sludge flocs and membranes than membrane hydrophilicity/ hydrophobicity [38].

Besides these factors mentioned above, EPS characteristics and components in MBR sludge should be considered as a central factor affecting membrane fouling because of their important roles in membrane fouling [8, 17], which is thoroughly discussed in the section 2.2.

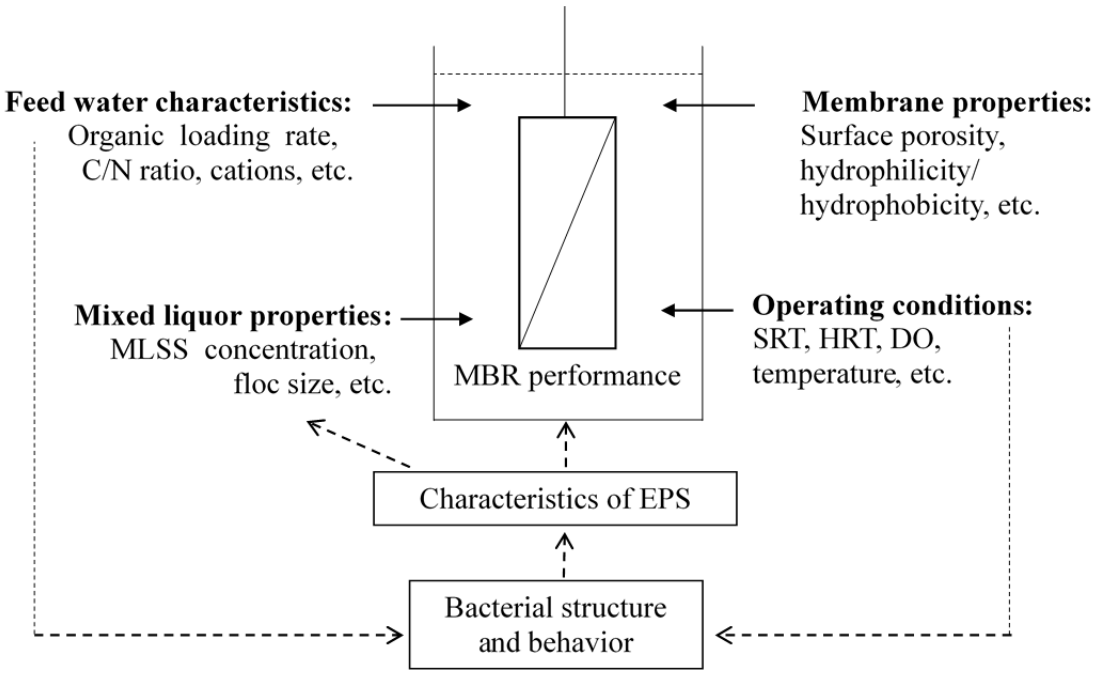


Figure 2.3. Factors affecting MBR performance and membrane fouling [1, 17].

2.2. Extracellular Polymeric Substances

2.2.1. EPS definition and studies

Extracellular polymeric substances are generally defined as macromolecular polymers secreted from microorganism. Actually, EPS in MBRs also contain the products of cellular lysis and macromolecular hydrolysis and organic matters from wastewater [11]. EPS can be soluble in water and bound in the matrix of microbial aggregates, named soluble and bound EPS, respectively [8]. Soluble EPS have been accepted to be identical concept of soluble microbial products (SMP) [8], being comprised of soluble compounds originating from cell lysis, extracellular diffusion and excretion, and feed substrates [39]. SMP can be released from EPS matrix and vice versa bound EPS also adsorb SMP into their matrix [40, 10] (Figure 2.4). The bound EPS form a complex matrix around microbial cells and function as a protective barrier around the bacteria, water retention and surface adhesion [39]. Recently, bound EPS have been fractionated into two kinds via based on their degree of binding with cells: loosely-bound EPS (LB-EPS) present near the cell and glue clusters of the outer layers of cell aggregates, and tightly-bound EPS (TB-EPS) present on the cell wall and bridging cells [41, 42].

Extracellular polymeric substances are comprised of biopolymers of polysaccharides, proteins, nucleic acids, lipids and other compounds [43]. These components contain many charged groups (e.g., carboxyl, phosphoric, sulfhydryl, phenolic, and hydroxyl groups) and apolar groups (e.g., aromatics and aliphatics in proteins, and hydrophobic regions in carbohydrates) [44]. These groups determine physical and chemical characteristics of EPS including surface charge, hydrophobicity/hydrophilicity, adhesion, etc. [8]. These functional groups also allow EPS to make hydrophobic and electrostatic

interactions, covalent bonds, and other non-covalent interactions to from a highly hydrated gel matrix surrounding microbial cells [10, 11]; therefore, EPS components determine mass transfer ability, adhesion and adsorption ability, surface charge, and stability of the microbial aggregates [11].

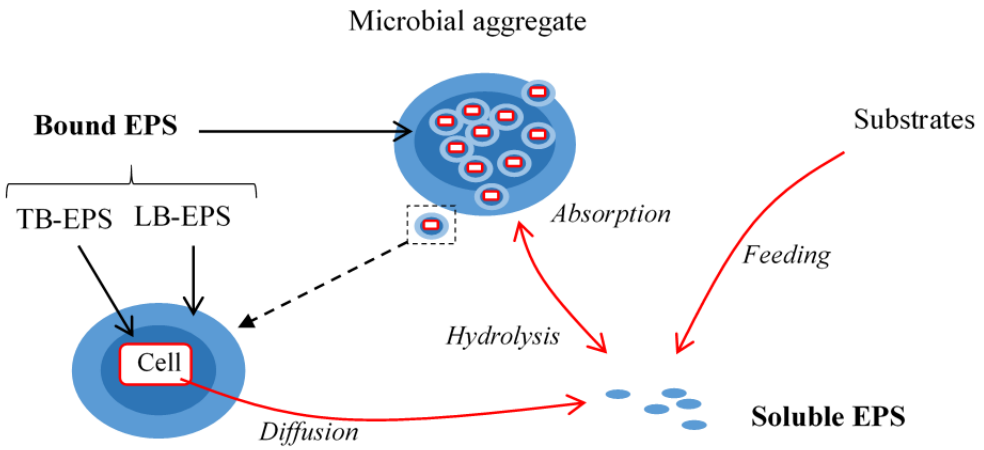


Figure 2.4. Schematic of EPS fractions and origin (modified form literatures **[1, 10]**)

Several methods have been applied for EPS extraction but no method has been standardized to distinguish each EPS fraction [42]. SMP are considered as soluble EPS, present separately from the cells and dissolve in the bulk liquid. They are usually collected via using centrifuge force [11]. In practice, different strengths in separation force of extraction methods have been used for LB- and TB-EPS fractionation. They include high speed centrifuge, ultra-sonication, cation exchange resin (CER), heat, alkaline treatment, acid treatment, enzymatic extraction, which have been summarized in a review paper of Sheng *et al*. [11]. These methods exhibit different mechanisms of extraction; hence, EPS extraction methods probably affect extraction efficiency and cell lysis level [11, 42, 45].

Extraction efficiency and cell lysis are considered as the primary evaluation for the optimum extraction method [9]. In general, more stringent methods have extraction efficiency, which is often indicated by volatile solids amount per one gram of sludge. However, stringent extraction may cause cell lysis, which results in leakage of intracellular compounds and over-extraction. Cell lysis can be indicated by DNA contents in the extract [46]. Therefore, a larger amount of total EPS with lower DNA content in the extract is a typical primary criterion in selection of EPS extraction methods. In other words, ratio of DNA content to EPS amount in the extract is considered as a criteria to evaluate for an optimum extraction method. Different extraction methods reportedly differ in characteristics of EPSs among the extracts under, including EPS components, zeta potential (surface charge), MW distribution, polarity (hydrophobicity/ hydrophilicity), etc. [45].

Conventional chemical colorimetric analyses can be used to quantify EPS components. Anthrone method and phenol–sulfuric acid method are usually employed to quantified polysaccharide content while the Lowry method and the Bradford method often are used for protein measurement [11]. Humic substance content can be measured by using a modified Lowry method by correcting the protein interference [47]. Nucleic acid content in EPS extract can be measured using the DAPI fluorescence method, or the diphenylamine method [43, 48]. Surface charge can be generally measured based on the electrophoretic mobility in an electric field using a zeta analyzer [8]. Size exclusion chromatography (SEC) equipped with UV-Vis, refractive index, evaporative light scattering detectors has been widely used to analyze molecular weight (size) distribution of substances in EPSs [45]. Hydrophilicity/hydrophobicity was assessed by analyzing free energy of interaction between two identical surfaces immersed in water [8] Relative

hydrophobicity (RH) in EPS is also expressed as the percentage of the reduction in aqueous phase concentration after extraction in n-hexane [34]. Fluorescence excitation-emission matrix spectroscopy and Fourier transform infrared spectrometry have also been used to characterize EPS basing on absorption wavelengths of EPS components and functional groups [45].

2.2.2. Roles of EPS in sludge flocculation and membrane fouling

Components of EPS play different roles in membrane fouling according to their own properties. Polysaccharides in EPS of MBRs are considered as primary membrane foulants while proteins and humic substances make the fouling more complex by participating in complix interaction with these primary foulants [5]. Moreover, polysaccharides are suggested to cause relatively reversible fouling [49] while protein and humic substances mainly contribute to irreversible fouling [50]. Polysaccharides are found to be a major composition of biopolymers in sludge supernatant and cake layer of MBRs [51]. Polysaccharides are highly rejected by MBR membrane because of large molecular weight, gelling property and low biodegradation rate [5, 52, 53]. Besides, polysaccharides were also found to participate in non-covalent interactions with proteins [50], which has high membrane-fouling potential [5]. Differently, proteins are found to and generally possess a higher rate of degradation and a higher affinity to sludge flocs than polysaccharides [54, 55]. Moreover, proteins are important for flocculation ability of sludge [56]. Therefore, ratio of protein to polysaccharide is more influential on sludge flocculation and fouling potential of EPS in MBR bulk sludge [8].

Humic substances with natural hydrophobicity and small molecular size are able to adsorb to membranes, subsequently condition membrane surface and narrow membrane

pores [5], which facilitate the deposition of other EPS components into conditioned membrane [33]. The humic substances on membrane surface and within membrane pores are able to make hydrophobic and electrostatic interactions with other EPS components such as polysaccharides and proteins [33, 57], which make fouling more complicated and serious. Low biodegradation property of humic substances make these substance highly accumulated in both sludge supernatant and bio-cake [52], increasing membrane filtration resistance.

EPS fractions are found to have different roles in sludge flocculation and membrane fouling. In fact, SMP content reportedly has a strong and direct relationship with permeate flux decline and membrane fouling rate [58]. During filtration, SMP components can be rejected by membrane, then accumulated in sludge supernatant at a high concentration, resulting in poor filterability [12]. They are also adsorbed onto internal structure and surface of the membrane, cause significant pore clogging and/or form gel layers on the membrane surface [6, 12]. Besides, SMP compositions (especially ratio of polysaccharides to proteins) also significantly influence membrane fouling [17]. Concentration and composition of SMP are really cared because of their notable contribution to membrane fouling [12].

Bound EPS are crucial in floc formation and stability, which is vital for membrane fouling reduction. For instance, higher content of bound EPS is reported to correspond to higher flocs stability. However, too low or high content of bound EPS content is found to have no effect on the specific resistance. Bound EPS content with 20 - 80 mg/g MLVSS play a significant role on MBR fouling [16]. Moreover, compositions and characteristics (hydrophobicity, surface charge, molecular weight) of bound EPS have effects on

flocculation of sludge flocs [8]. MBR sludge with small and unstable flocs results in increases of cake layer resistance and fouling rate [17]. Among EPS fractions, LB-EPS are reportedly more significantly correlated with membrane fouling in MBRs as compared to TB-EPS [59]. Higher content of LB-EPS results in a poorer flocculation ability and a lower settle ability, and looser sludge flocs, which increases fouling rate. However, higher TB-EPS contents are able to reduce occurrence of membrane fouling [60]. A high ratio of TB-EPS to LB-EPS offers a lower rate of total membrane resistance and membrane fouling [61]. From these findings, ratio of SMP to LB-EPS, and to TB-EPS is important in sludge flocculation and membrane fouling potential even though the fouling rate cannot always be correlated with the SMP concentration [62].

Characteristics of EPS such as MW distribution, degradation rate, polarity and surface charge have influences in fouling propensity. EPS components with MW larger than membrane pore size can be rejected [52]. Large molecules can be biodegraded as substrates for microbes and become smaller ones. The small molecules are expected to freely permeate through membrane pores; however, they can be absorbed into the membrane via several complex interactions, which include hydrophobicity, electrostatic double layer and van der Waals, etc. [8]. A number of studies have attempts to fractionate EPS in MBR sludge and foulants via their MW size, hydrophobicity/ hydrophilicity in order to understand the role and fate of EPS in MBRs [52, 63]. In general, composition, content and characteristic of EPS fractions have certain effects on sludge flocs and MBR membrane fouling; therefore, production EPS in MBRs is attracted more attention from researchers and engineers, which is discussed in details in next section.

2.2.3. EPS production in MBRs

EPS production can be affected by a number of factors, which are classified into feeding, MBR operations, and additives [8]. Characteristics of feeding such as carbon source [64, 65], C/N or C/P ratio [34, 66], nutrient loading rate [67], salinity and cations [68, 69] are reported to influence EPS production, which can be generally explained by differences in microbial metabolism [8]. For example, glucose feed has more EPSs production than acetate feed [65]. In addition, the higher rate of organic loading reportedly increases EPS content and facilitate cake layer formation [67]. Moreover, decreases of C/N and C/P ratios via increasing nitrogen and phosphorus concentration lead to change in bacterial structure and prompt the release of bound EPS components to form new SMP [66], giving higher rate of irreversible fouling resistance. Furthermore, salinity shock (e.g., NaCl) is reported to have a significant increase in polysaccharide concentration of EPS [70]. Additionally, a lower ratio of Mg^{2+} to Ca^{2+} cause increases in relative hydrophobicity of EPS and in SMP release from bound EPS, resulting in higher deposition of EPS components on membrane surface [69].

Investigations in effects of operational parameters on EPS production have been summarized in the review paper of Lin *et al.* [8]. For instance, increases in feeding/mass ratio and temperature lead to rises of EPS production and/ or SMP release from bound EPS [71, 72, 73]. Likewise, an increase of MLSS concentration also increases EPS production [74, 75] Besides, too long and/ or too short HRT, SRT can cause increases in EPS decomposition and production [76, 77]. Furthermore, dissolved oxygen level has impacts on EPS production in MBRs [29].

Several studies showed that additions of additives have effects on EPS production.

Suspended biofilm carriers (e.g., AnoxKaldnes, K1 carriers) are reported to obtain a reduction of EPS content in bulk sludge and cake layer [78]. Moreover, powdered activated carbon can absorb SMP and increase EPS content in sludge flocs [79]. Similar with powdered activated carbon, other absorbent agents (e.g., zeolite, diatomite, bentonite, etc.) and coagulants (or flocculants) have been reported to increase absorption of SMP into sludge flocs, resulting in fouling mitigation [8]. Recently, biological approaches based on EPS reduction strategy have been investigated to mitigate membrane fouling [5, 80]. In fact, grazing by protozoans or metazoans were found to reduce biomass as well as EPS in MBRs, reducing membrane fouling [80]. Besides, enzymes and bacteria degrading protein and polysaccharide are reported to affect EPS production, disrupt biofilm formation as well as mitigate membrane fouling [5]. Additionally, inhibition of QS system can control EPS production [10], which would be described in details in the section 2.3.2.

2.3. Quorum Sensing-Based Fouling Control

2.3.1. Quorum sensing and quorum quenching

Microbes are reported to have a special process to communicate among individual cells, named quorum sensing [23]. In this process, single cells produce chemical signals (called auto-inducers), represent for cell population density [81]. The signals are exchanged among cells in surrounding environment [82]. Until reaching a threshold concentration, the signals combine with protein receptors to trigger gene expression [83]. This process allow microbes regulate their collective behavior including virulence, antibiotic production, biofilm formation, etc. [81]. A number of bacterial auto-inducers have been described, which are often classified into three groups: acyl-homoserine lactones (AHLs), oligopeptides and autoinducer 2 (AI-2) [22]. AHL-based QS process (Figure 2.4), named LuxI/LuxR QS circuit, are common among Gram-negative bacteria [22]. Recently, several studies investigating QS process in biological wastewater treatment have reported that EPS production, granule formation and biofilm formation are dependent on the AHL-based QS circuit [19, 84].

Quorum quenching is a technical term referred to a disruption of quorum sensing [85]. QQ may lead to inhibition of collective behaviors but it is not interfered with microbial survival and growth [21]. The AHLs-based QS process can be interrupted by (i) inhibition of AHL synthesis, (ii) degradation of AHLs, and (iii) interference with signal receptors (Figure 2.5) [83].

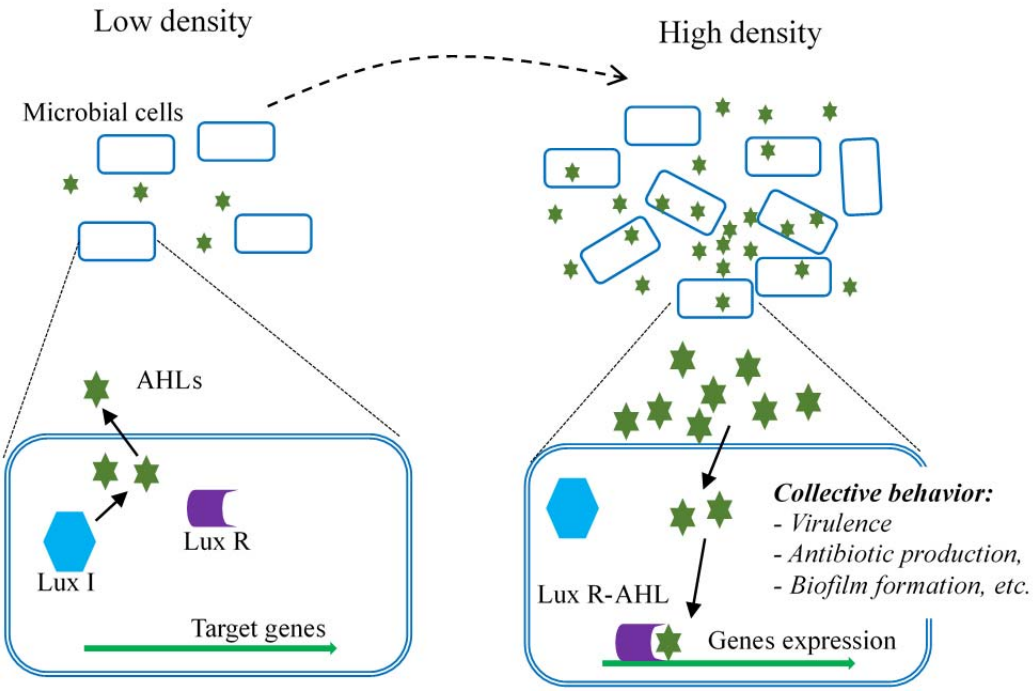


Figure 2.4. Schematic illustration of AHL-based QS process [83]

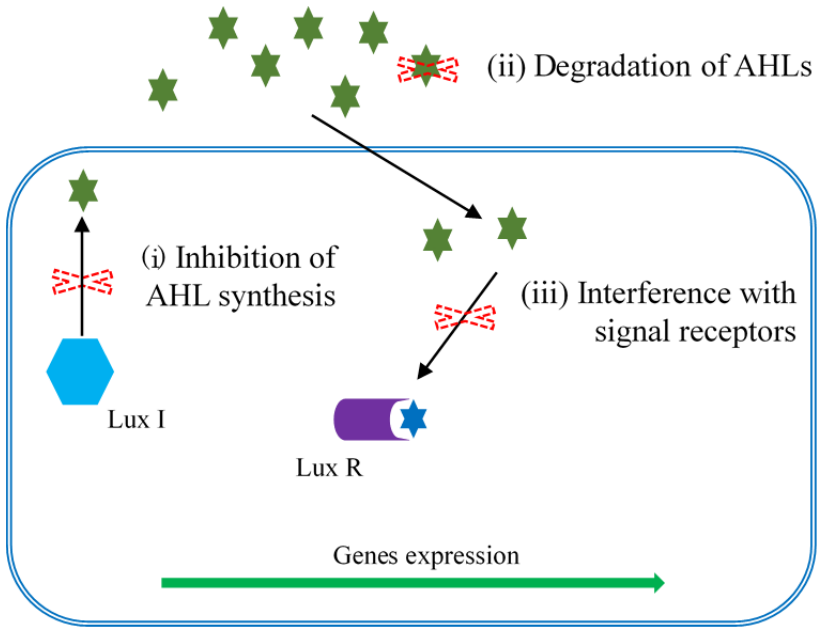


Figure 2.5. Schematic illustration of AHL-based QQ [83]

To inhibit AHL synthesis, utilization of necessary substrates for AHL synthesis have been used [82]. AHL synthesis also can be repressed via some compounds including purine nucleotides, and macrolide antibiotics [83]. In term of degrading AHL signals, bacteria (e.g., *Pseudomonas, Variovorax*) are found to metabolize AHL signals for their growth, while AHL-lactonases, AHL acylases and oxidoreductases are able break and/ or modified the structure of AHLs, causing a loss of signaling activity [83]. Lactone ring of AHL can be opened via alkaline pH and recovered under acidic pH. Finally, several compounds such as vanillin, furanones are found to compete or interfere with AHLs for binding to LuxR receptors [86, 87], which are major strategy to block QS process [23, 21].

2.3.2. Status and trends of quorum sensing-based fouling control

The first investigations in QS-based fouling control are introduced via a group of researchers at Seoul National University, who mainly and widely focus on application of QQ enzymes and bacteria. Quorum quenching enzymes including AHL-acylase and AHL-lactonase have shown their potentials to be used as an enzymatic quorum quenching to control membrane fouling [21]. Acylase I (Porcine kidney) reportedly reduces biofilm formation by *Aeromonas hydrophila* and *Pseudomonas putida* on three different surfaces [88]. To from biofilm, microbes produce more EPS to facilitate attachment and matrix formation; hence, biofilm formation is usually quantified as EPS content present in an area unit. Acylase was found to inhibit quorum sensing process of bacteria to from biofilm on the membrane, reducing membrane fouling [89]. QQ enzymes (e.g., acylase) are immobilized onto membrane surface or special carriers to prevent being permeated with MBR effluent [89]. Special carriers include magnetic enzyme carrier [90], magnetically-separable mesoporous silica [91], beads [92]. The immobilized

enzymes have effects on sludge characteristics and biofouling, but not on pollutant degradation [92]. QQ enzymes are demonstrated to have no impact on microbial communities in bulk sludge of MBRs but cause a decrease in the proportion of quorum sensing bacteria, and a delay in TMP rise-up [93]. The application of pure enzymes to inhibit QS process in MBRs are effective to mitigate membrane fouling but it is costly and limited by a low stability [5].

Due to the limitation of QQ enzyme, quorum quenching bacteria who themselves can synthesize QQ enzymes are introduced to disrupt QS process of other microbes. This strategy is considered as an interspecies quorum quenching, which is also promising to alleviate biofilm formation and membrane fouling [24]. Since then, QQ bacteria have been being isolated, investigated and applied for MBR fouling control [94]. QQ isolates (e.g., *Rhodococcus* sp., *Pseudomonas* sp.) encapsulated in "microbial-vessels" show successful control of membrane fouling in lab-scale MBRs [24, 95]. Since bacterial QQ isolates are vulnerable when being added directly into MBR sludge, carriers for them have been developed to optimize bacterial cell viability and fouling mitigation efficiency [96]. Shape of carriers including beads [97], cylinders [98], and sheets [99] also have impact on fouling control by having physical contact with membrane surface. QQ bacteria entrapping beads applied in pilot-scale MBRs show significant mitigation of membrane fouling [25]. QQ activity of the beads in pilot MBRs can retain for over four months. Several studies reported that QS and QQ bacteria coexist in biological wastewater treatments including MBRs [83]. Therefore, main targets of QQ-based fouling control should be to enhance QQ enzyme expression of native QQ bacteria in MBRs as well as to apply bacterial QQ beads in full scale MBRs [5].

Several natural compounds have been found to inhibit microbial QQ activity while not affecting microbial growth. Natural compounds as quorum sensing inhibitors include the extracts of furanone, ajoene, iberin, allin, vanillin, etc., which have been summarized in the review paper of Lade *et al.* [21]. However, only a few investigations of these compounds for membrane fouling control have been reported. For example, addition of Piper betle extract in ultrafiltration MBR treating textile effluent can make a reduction of EPS content in cake layer and a mitigation of membrane biofouling [100]. Moreover, a vanillin dose of 250 mg/L reduces 31 percentage of EPS content in cake layer of MBRs for wastewater treatment [18]. Recently, vanillin also has been investigated to prevent 52 % biofilm formation via bacterial multi-species in wastewater culture [20]. Vanillin (1200 mg/L) is reported to reduce 79 % biofilm formation via marine bacteria, over 40 % polysaccharide content of EPS, and over 20 % dead cell on membrane surface [101]. These findings show potential application of these natural compounds (especially vanillin) as a QS inhibitor to control membrane fouling. However, before being widely applied in larger scale MBRs, the compounds should be comprehensively investigated in possible effects on treatment performance, microbial community and behavior.

Chapter 3. Materials and Methodology

3.1. Analytical methods

All methods employed in the study are described in this section. They include analyses for sludge property, EPS characteristics and components, as well as for bacterial communities.

3.1.1. Analysis for sludge property

3.1.1.1. Total solids and volatile solids

Concentrations of total solids (TS) and volatile solids (VS) were analyzed according to APHA standard methods [102]. Clean evaporating dishes were ignited at 550 °C for 2 h in a muffle furnace, then cooled and stored in a desiccator until needed. They were weighed just before being used. A sample of 10-25 mL was poured into the prepared dish, which was being dried in a water bath at 100 °C before placed in an oven at 103-105 °C for at least 20 h. After cooled in a desiccator, the dried dish was weighed. The weighed dish with dried sample was ignited at 550 °C for 20 min a muffle furnace, then weighed again after cooled in a desiccator. Concentration of TS and VS was calculated based on the following equations:

$$TS\ [g/L] = (W_{105} - W_0) \times 10^3 / V \qquad \text{(Equation 3.1-1)}$$

$$VS\ [g/L] = (W_{550} - W_{105}) \times 10^3 / V \qquad \text{(Equation 3.1-2)}$$

, where:

W_{105} = weight of a dish with sample dried at 105 °C [g]

W_{550} = weight of a dish with sample ignited at 550 °C [g]

W_0 = weight of a vacant dish [g]

V = volume of a sample [mL]

3.1.1.2. Suspended solids

Concentrations of suspended solids (SS) were analyzed according to APHA standard methods [102]. Clean evaporating dishes were dried at 105 °C for 2 h in an oven, then cooled and stored in a desiccator until needed. They were weighed just before being used. A twenty-five mL sample was centrifuged at 6000 ×G for 10 min. Supernatant was discarded and remaining pellet was re-suspended with the same volume of pure water prior to being centrifuged again. The pellet of the second centrifuge was transferred into the prepared dish, which was dried in a water bath at 100 °C before placed in an oven at 103-105 °C for at least 20 h. After cooled in a desiccator, the dried dish was weighed again. A concentration was calculated based on the following equation:

$$SS\ [g/L] = (W_{ss} - W_0) \times 10^3 / V \qquad \text{(Equation 3.1-3)}$$

, where:

Wss = weight of a dish with sample [g]

W_0 = weight of a vacant dish [g]

V = volume of a sample [mL]

3.1.1.3. Sludge volume index

Sludge volume index (SVI) was analyzed according to APHA standard methods [102]. SVI is the volume in milliliters occupied by 1 g of a suspension after 30 min settling. One liter of sludge was placed in a one-liter volumetric cylinder. The volume occupied by suspension was determined after 30 min. Concentration of suspended solids was also measured as the previous mention. SVI was calculated based on the following equation:

$$SVI\ [mL/g] = V_{30}/SS \qquad \text{(Equation 3.1-4)}$$

, where:

V_{30} = volume occupied by suspension after 30 min [mL/L]

SS = concentration of suspended solids [g/L]

3.1.1.4. pH and dissolved oxygen

Values of dissolved oxygen (DO) and pH were measured by a DO/pH meter (DM-32P, TOA-DKK, Japan) equipped with a DO electrode (OE-270AA, TOA-DKK, Japan) and a pH combination electrode (GST-2739C, TOA-DKK, Japan).

3.1.2. Analysis for EPS extract

3.1.2.1. Total organic carbon

Concentrations of total organic carbon (TOC) and dissolved organic carbon (DOC) were measured by a TOC analyzer (TOC-V_{CPN}, Shimadzu, Japan). TOC concentrations of suspension samples were analyzed after being homogenized by using ultra-sonication (Sonifier VC-505, Sonics & Materials, USA) at 8 W/mL for 10 min. The sample for DOC was filtrated with a cellulose-acetate filter membrane (0.45 μm, Advantec-Toyo, Japan).

3.1.2.2. Total nitrogen

Concentrations of total nitrogen (TN) and dissolved nitrogen (DN) were measured by a TN analyzer (TNM-1, Shimadzu, Japan) equipped with a TOC analyzer (TOC-V_{CPN}, Shimadzu, Japan). TN concentrations of suspension samples were analyzed after being homogenized by using ultra-sonication (Sonifier VC-505, Sonics & Materials, USA) at 8 W/mL for 10 min. The sample for DN was filtrated with a cellulose-acetate filter membrane (0.45 μm, Advantec-Toyo, Japan).

3.1.2.3. Polysaccharide

Polysaccharide (PS) concentration was measured by phenol-sulfuric acid method [103]. One mL of phenol solution (5 % w/w) was added to 1 mL of sample. Subsequently, 5 mL of concentrated sulfuric acid (96-98 %) was added into sample solution. The mixture was briefly vortexed and left to stand for 10 min. The sample was wortexed again, then placed in a water bath at 25–30 °C for 20 min. Absorbance at 490 nm of sample was measured by a spectrophotometer (DR3900, Hach, Germany). Glucose was used to make a standard curve and the unit of polysaccharide concentration was mg glucose/L.

3.1.2.4. Proteins and humic acid

Protein and humic acid concentration was analyzed by using modified Lowry method [47]. One mL of sample was added with 1.4 mL of Lowry solution, which was comprised of the mixture 2 % sodium carbonate, 1.5 % copper sulfate pentahydrate, and 1 % sodium tartrate (100:1:1). After a brief wortex, sample mixture was placed in dark for 10 min before added with 0.2 mL Folin's reagent (1 mol/L). Once again, the sample mixture was mixed and placed in dark for 30 min. Absorbance at 750 nm was measured by a spectrophotometer (DR3900, Hach, Germany). The protein concentrations was determined from a standard curve of bovine serum albumin (BSA). The unit of protein concentration was mg BSA/L. Protein measurement is interfered by humic compounds. Without copper sulfate pentahydrate in Lowry solution, the colour developed by BSA decreased to 20% but no decrease was observed for humic acids [47]. The final concentrations of protein and humic acid was corrected according to the following equations

$$Abs_{protein} = 1.25\ (Abs_{total} - Abs_{blind}) \qquad \text{(Equation 3.2-1)}$$

$$Abs_{humic} = Abs_{blind} - 0.2\ Abs_{protein} \qquad \text{(Equation 3.2-2)}$$

, where:

Abs_{total} = total absorbance with $CuSO_4$

Abs_{blind} = total absorbance without $CuSO_4$

$Abs_{protein}$ = absorbance due to proteins

Abs_{humic} = absorbance due to humic compounds

3.1.2.5. Nucleic acid

Nucleic acid concentration was quantified by diphenylamine method [104]. One mL of sample was mixed with 1 mL diphenylamine reagent (1.5 g diphenylamine in 100 mL glacial acetic acid, 1.5 mL concentrated sulfuric acid, 1 mL acetaldehyde). The sample mixture was incubated at 25-30 °C for 20-24 h. Absorbance at 660 nm was measured by a spectrophotometer (DR3900, Hach, Germany). Salmon DNA was used to make a standard curve.

3.1.2.6. Relative hydrophobicity

Relative hydrophobicity (RH) of protein and polysaccharide in SMP and EPS were conducted based on the study of Arabi and Nakhla [34]. The procedure was as follows: a 20-mL sample was agitated with 20 mL n-hexane in a shaker of 200 rpm for 30 min. the mixture was left for separation for 30 min, then aqueous phase was collected. Protein and carbohydrate concentrations in the original sample and the collected aqueous phase were analyzed. The RH was expressed as percentage reduction of concentration after extraction with n-hexane, compared to original concentration. The relative hydrophobicity was calculated as the following equation:

$$RH\ [\%] = (C_b - C_a) \times 10^2 / C_b \qquad \text{(Equation 3.2-3)}$$

, where:

C_a = concentration of proteins (or polysaccharides) after extraction,

C_b = original concentration of proteins (or polysaccharides).

3.1.2.7. Three-dimensional excitation-emission matrix fluorescence spectra

An EPS sample was filtered with a filter (0.45 μm, Advantec-Toyo, Japan) prior to analysis. Three-dimensional excitation-emission matrix (3D-EEM) spectrum of the filtered sample was measured using a fluorescent spectrophotometer (FP-8200, Jasco,

Japan). The 3D-EEM spectrum was obtained with subsequent scanning emission spectra from 200 to 550 nm at 5 nm increments by varying the excitation wavelength from 200 to 550 nm at 5 nm increments. Detected peaks were classified according to Chen *et al.* [105].

3.1.2.8. Size-exclusion chromatography

Molecular weight size was analyzed via using a preparative high-performance liquid chromatography system (Agilent 1260) equipped with an evaporative light scattering detector (ELSD). A size-exclusion column (OHpak SB–806 M HQ, Shodex, Japan) was used with pure water as the mobile phase at 0.5 mL/min. A hundred µL sample was injected after filtration by 0.45 µm filters. The detection by ELSD was conducted under evaporating temperature of 60 °C and nebulizer temperature of 30 °C. Dextran with molecular weights at 1 kDa, 12 kDa, 50 kDa, 500 kDa, 670 kDa, and 1100 kDa (Sigma-Aldrich, Denmark) were used as standards. The SEC chromatograms of these standard were shown in Figure 3.1.

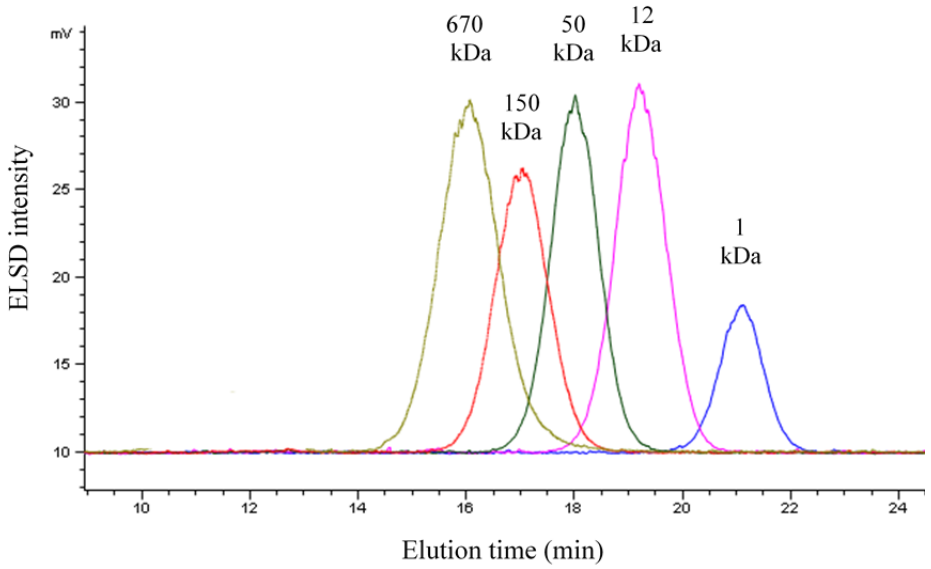


Figure 3.1. Size elution chromatogram of dextran as molecular weight standards

3.1.2.9. Reversed-phase chromatography

To analyze polarity (hydrophobicity/hydrophilicity) of substances in EPS extracts, reversed-phase chromatography was conducted via using a preparative HPLC (Agilent 1260, Germany) equipped with a reversed-phase column (C18M 4D, Shodex, Japan) and an ELSD. Solvent of methanol: water (70:30) was used as mobile phase for the separation. One-hundred μL of sample was injected after filtration by a 0.45 μm filter. Uracil, propanol, aniline, chloroform, and humic acid (Cat. no. 082-04625, Wako, Japan) extracted in NaOH 0.1 mol/L were used as reference materials. Chromatograms of these materials were shown in Figure 3.2. The HPLC was also equipped with a fraction collector (Agilent 1260, Germany), which allow to fractionate and collect fractions based on retention time.

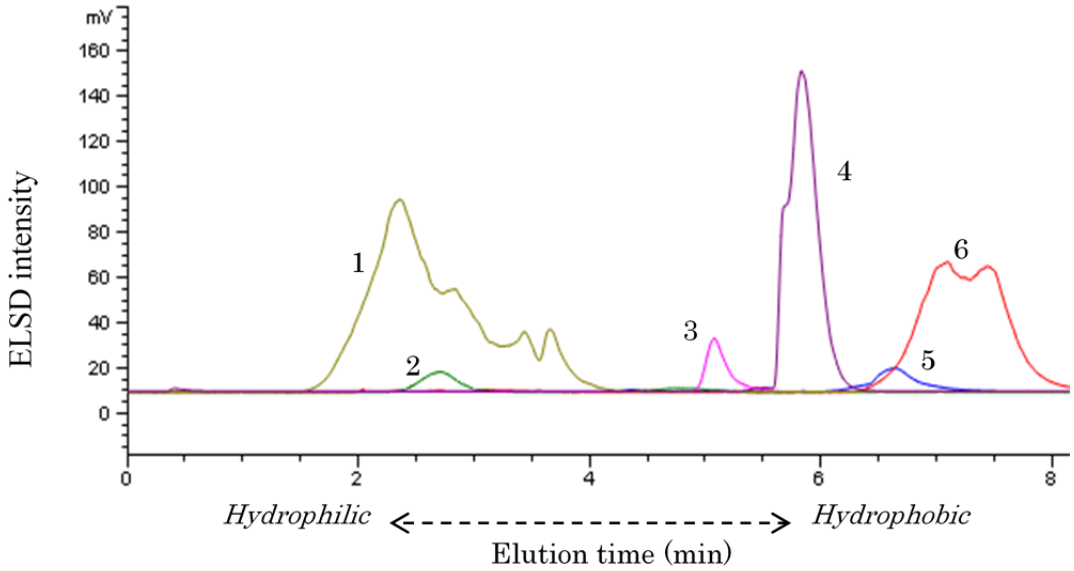


Figure 3.2. Reversed-phase chromatograms of reference materials

#1. EPS extract, #2. Uracil, #3. Humic acid extract, #4. Propanol, #5. Aniline, and #6.

Chloroform.

3.1.3. Analysis for bacterial community

This section shows general procedures of bacterial community analysis in Chapter 6 and 7. Detailed parameters and conditions of analysis were described in section of materials and methods of corresponding chapters.

V3-V4 regions of 16S rRNA genes were sequenced under next-generation sequencing (NGS) method via Illumina Miseq platform for bacterial community analysis. Workflow for sequence library preparation was conducted as Figure 3.3, according to "16S Metagenomic Sequencing Library Preparation" [106]. DNA extract from environmental samples (e.g., bulk sludge, cake layer) was used as templates in the 1^{st}-PCR to amplify specific target sequences, in which a primer pair for the PCR was appended with overhang adaptors (linkers). The 2^{nd}-PCR was conducted to amplify 1^{st}-PCR products and add sequencing adaptors. To sequence many samples at once, barcodes or indices (unique sequences of 10-12 bp) were also appended into the overhang adaptors and sequencing adaptors. These barcodes (or indices) were used for sample sorting and identification in downstream analyses. The 2^{nd}-PCR amplicons were quantified and pooled prior to be sequenced via Illumina Miseq platform based on paired-end reading method.

After sequencing, raw data were trimmed to remove fragments with low-quality, short sequences. Then, trimmed reads were merged to make paired-end library. The paired-end library was checked and remove chimeric sequences prior to be used for bacterial community analysis via Quantitative Insights into Microbial Ecology -QIIME (e.g., de novo OTU picking and diversity analyses).

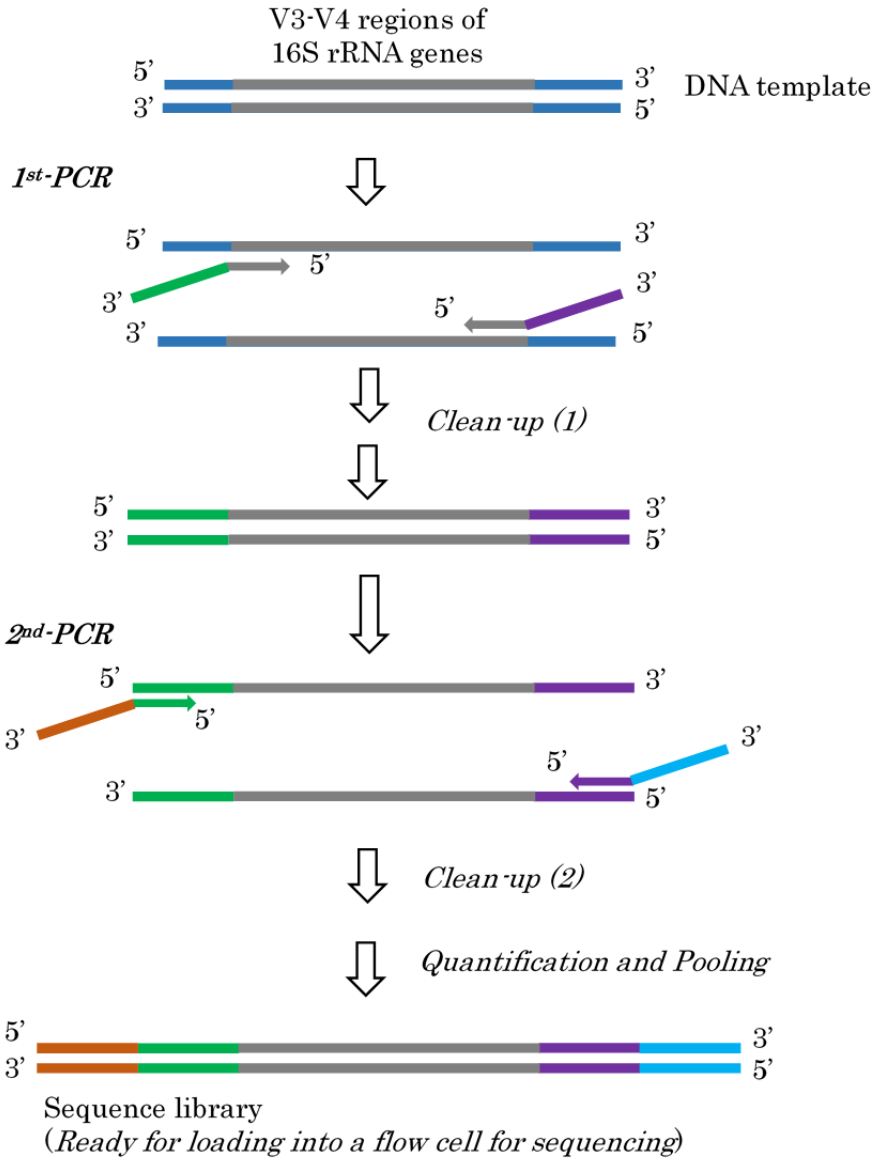


Figure 3.1. Workflow for sequence library preparation [106]

3.1.4. AHL detection and QS bioassay

3.1.4.1. AHL extraction

AHLs in MBR sludge were extracted in ethyl acetate. A 250 mL-sludge sample was destructed by sonication for 10 min before mixed with equal volume of ethyl acetate. The mixture was shaken in a shaker 250 rpm for 30 min then separated via a fractionation funnel. After the first extraction, the residue water phase was extracted again with equal volume of ethyl acetate. The solvent phase from the both extraction were mixed and concentrated by a rotary evaporator. The concentrated extract was dried up under nitrogen stream, then dissolved in 250 µL of acetonitrile for storage.

3.1.4.2. AHL detection via Fourier transform mass spectrometry

AHLs in extract was detected and quantified according to Cataldi *et al*. [107]. Chromatographic separation of the compounds was made using a TSKgel ODS-C18 column. Separation was conducted at a 0.2 mL/min flow rate of mobile phase comprised of 0.1 % formic acid in MilliQ water and 0.1 % formic acid in acetonitrile (1:1). N-acyl homoserine lactones (ranging from C4-C12, and for 3-oxo substituents and 3-hydroxy substituents) as in Table 3.1 were detected based on positive ion ESI-MS at operational conditions: source voltage 4.5 kV, heated capillary temperature 250 °C, capillary voltage -250 °C and tube lens -100 V. Full-scan accurate mass spectra was ranged from m/z 100 to 400, which coupled with precursor ion scan mode. By this way, relevant information for identification and confirmation, e.g., retention time, molecular weight and fragmentation, was obtained.

The limit of detection for each AHL is 0.1-1 µg/L. Sample was filtered through a 0.45

μm filter so as to analyze FTMS. Data acquisition and analysis were accomplished using the Xcalibur software (v2.3, Thermo Electron). Identification and quantification of target compounds was performed using the accurate mass of the protonated molecule within a mass window of 5 ppm. The chromatographic raw data were imported and elaborated. The concentration of AHLs were calculated based on the accurate mass values together with chromatographic retention times from the reference standards in order to identify unknown and known AHLs in activated sludge.

Table 3.1. List of AHL standard in FTMS analysis

AHL type		m/z range
N-butanoyl-L-homoserine lactone	C4-HSL	172.0960-172.0977
N-hexanoyl-L-homoserine lactone	C6-HSL	200.1271-200.1291
N-heptanoyl-L-homoserine lactone	C7-HSL	214.1427-214.1448
N-octanoyl-L-homoserine lactone	C8-HSL	228.1583-228.1606
N-decanoyl-L-homoserine lactone	C10-HSL	256.1894-256.1920
N-dodecanoyl-L-homoserine lactone	C12-HSL	284.2206-284.2234
N-3-oxo-hexanoyl-L-homoserine lactone	3O-C6-HSL	214.1063-214.1085
N-3-oxo-octanoyl-L-Homoserine lactone	3O-C8-HSL	242.1375-242.1399
N-3-oxo-decanoyl-L-homoserine lactone	3O-C10-HSL	270.1686-270.1713
N-3-oxo-dodecanoyl-L-homoserine lactone	3O-C12-HSL	298.1998-298.2028
N-3-hydroxy-octanoyl-L-homoserine lactone	3OH-C8-HSL	244.1531-244.1556
N-3-hydroxy-decanoyl-L-homoserine lactone	3OH-C10-HSL	272.1843-272.1870

3.1.4.3. Quorum sensing bioassay

Agrobacterium tumefaciens NTL4 (pZLR4) and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) were employed as a microbial biosensor and an indicator of bioassay test for quorum sensing process and quorum sensing inhibition. Transcription for beta-galactosidase of the *A. tumefaciens* NTL4 was modified to be regulated via AHL-based QS process. Under the presence of AHL, the *A. tumefaciens*

produces beta-galactosidase, which can oxidize 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (colorless) to form 5,5'-dibromo-4,49-dichloro-indigo (colored blue) [108]

To conduct QS bioassay test, quorum sensing signal (e.g., AHL standards, extracts) was added into a mixture of 5 mL LB-medium with 100 μL inoculant of *A. tumefaciens* NTL4 (optical density at 600 nm – $OD_{600} \approx 2$), 5 μg/mL gentamicin, 60 μg/mL X-gal. Subsequently, the mixture was incubated in a glass-tube shaker (250 rpm) in 30 °C for 12 h. After inoculation, color of the mixture changes from yellow (color of LB-medium) to blue (or green), indicating an occurrence of QS process.

To test quorum sensing inhibition, quorum sensing inhibitor (e.g., vanillin) and 0.5 nmol C6-HSL (quorum sensing signal) were added into a mixture of 5 mL LB-medium with 100 μL-inoculant of *A. tumefaciens* NTL4 ($OD_{600} \approx 2$), 5 μg/mL gentamicin, 60 μg/mL X-gal. Subsequently, the mixture was incubated in a glass-tube shaker (250 rpm) in 30 °C for 12 h. After inoculation, colored blue of the mixture with vanillin and C6-HSL is lower in colored density than that of the mixture with only C6-HSL.

To test QS signals production by microbes in bulk sludge and cake layer, 100 μL sample of bulk sludge (20 mg sample of cake layer) was incubated in 5 mL LB-medium at 30 °C for 12 h. The culture was added 100 μL *A. tumefaciens* NTL4 ($OD_{600} \approx 2$), 15 μg/mL gentamicin, 60 μg/mL X-gal, 0.5 mL LB-medium, and incubated again at 30 °C for 12 h. Cultivation mixture turns from yellow to blue, indicating that microbes in sludge were able to produce AHLs during their growth.

3.2. Lab-scale MBR operation

Lab-scale membrane bioreactors were operated for investigations about EPS characteristics, bacterial community structure and fouling control via QS inhibition in Chapter 5, 6 and 7, respectively. This section describes general information of MBR setups and operation

Membrane bioreactors were operated under aerobic condition to treat artificial sewage. Photograph and diagram of the MBR operation were showed in Figure 3.4. Membrane units of microfiltration flat sheet with the average pore size at 0.2 μm (Kubota, Japan) were immersed in aeration tank to separate biomass of activated sludge and treated water. For MBR sludge seed, activated sludge (MLSS = 1.5 g/L) was collected from an aeration tank of a conventional activated sludge process in Kanazawa city of Japan. Ingredients of the sewage are showed in Table 3.2, which was simulated for real influent wastewater in a wastewater treatment plant in Kanazawa city. Oxygen was supplied from a compressor with a high rate of air flow (10 L/min), which was also employed to stir bulk sludge in MBR reactor.

Concentrated sewage (2X) was made for 4 day usage and kept in a fridge. A peristaltic pump connected with a floating switch was used to mix the concentrated sewage with RO water (1:1) and automatically feed the MBRs. Organic loading rate was controlled at 40–60 mg TOC/g per day. Another peristaltic pump was used to withdraw effluent. It was operated under an intermittent on/off circuit, programmed with 4 min of filtration and 1 min of relaxation. HRT was kept at 11-13 h, while SRT was operated to maintain MLSS concentration ranging from 5 to 7 g/L after acclimation period, in which sludge

was not withdrawn. Temperature was controlled in a range of 18-22 °C. Values of DO, pH, temperature, and TMP were captured via a recorder. Treatment performance of the MBR was evaluated based on TOC removal efficiency; therefore, TOC concentrations in influent and effluent were weekly analyzed. Permeate flux was calculated based on accumulated effluent volume of whole day.

When membrane was fouled (criterial TMP at 60 kPa or 50 % flux reduction), the fouled membrane was taken out of aeration tank and cleaned by brushing as Figure 3.5. The cleaning employed a “glass tube cleaning brusher” to remove biomass on the membrane surface. The foulant removed by brushing was considered as a cake layer sample. The cleaned membrane was re-operated for next filtration cycle. After 4 times being cleaned via physical cleaning, fouled membrane was cleaned via chemical cleaning. Sodium hypochlorite solution (0.1 % of active chlorine) was added into membrane module and soaked for 30 min. Subsequently, a new filtration was continued.

Table 3.2. Ingredients of artificial sewage (mg/L)

Glucose	250
Peptone	40
Yeast extract	40
$NaHCO_3$	190
KH_2PO_4	10
$CaCl_2$	99
$FeCl_3 \cdot 6H_2O$	2
NH_4Cl	80
$MgSO_4 \cdot 7H_2O$	80
$Mg(NO_3)_2 \cdot 6H_2O$	3

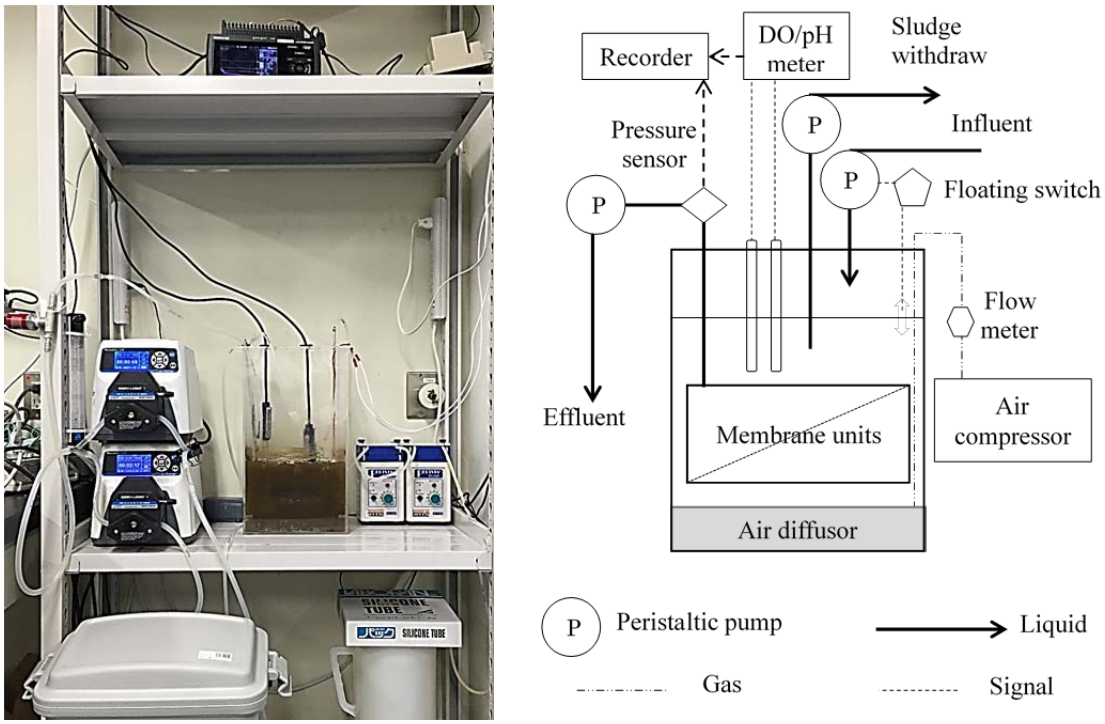


Figure 3.4. Photograph and diagram of MBR operation in Chapter 5 and 6

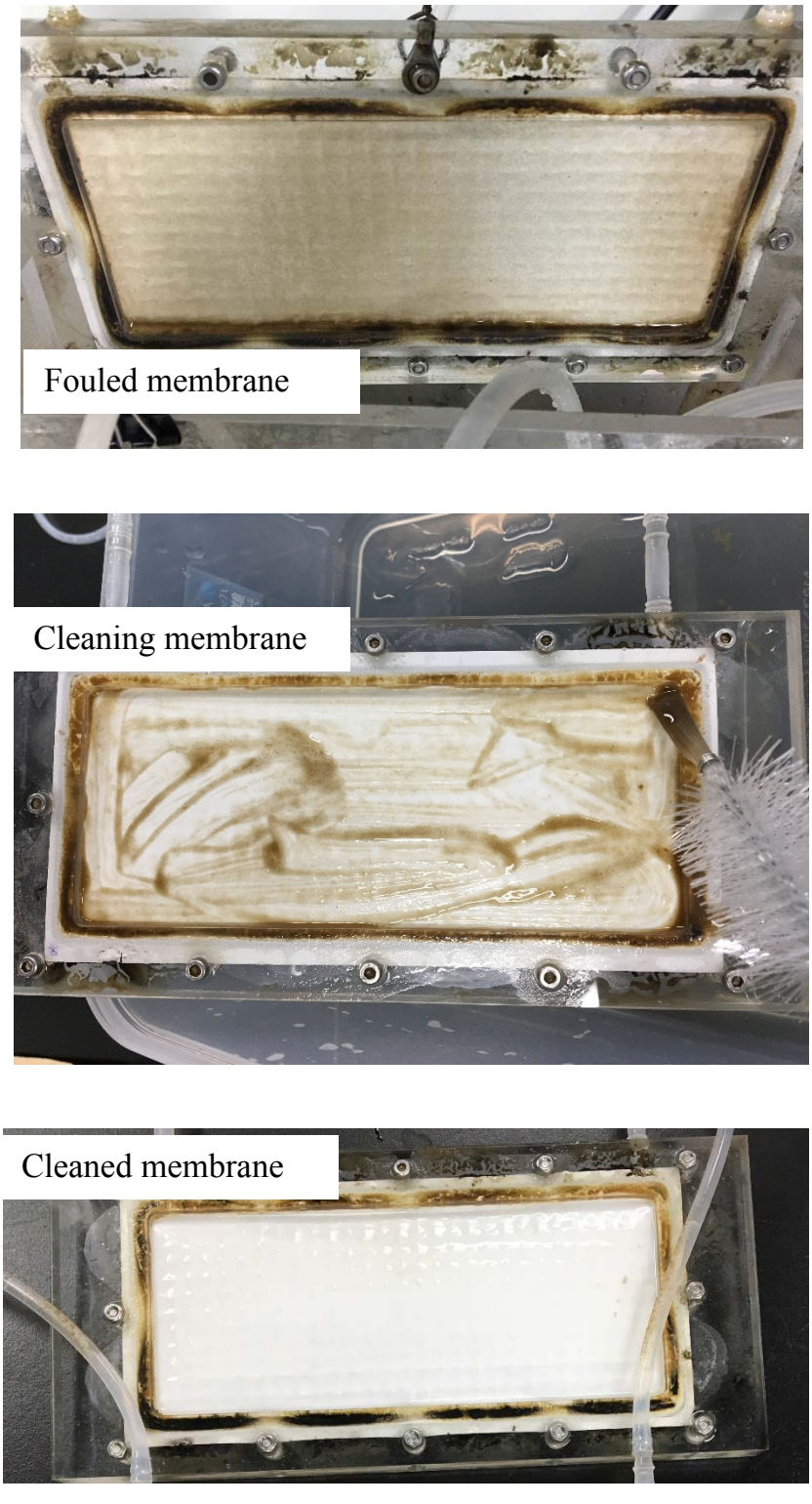


Figure 3.5. Physical cleaning and cake layer collection from fouled membrane

Chapter 4. Optimum Methods for Extraction of Extracellular Polymeric Substances

4.1. Introduction

In 1995, Frolund *et al*. [47] extracted EPS from activated sludge using cation exchange resin (CER) and reported EPS components for first time. Nielsen and Jahn [41] later described a typical procedure for EPS extraction that consisted of pretreatment, extraction and purification. Various EPS extraction methods have since been developed and investigated. Extracellular polymeric substances are often classified from their degree of binding with cells. EPS fractions such as SMP, LB-EPS, and TB-EPS were classified by strength of separation force from the cell in extraction methods. SMP are often collected as supernatant of centrifuged sludge, while LB-EPS are often collected after detachment from the cell by sonication. For TB-EPS extraction, various materials and methods have been used including CER, sodium hydroxide, and heating [43, 48, 46].

During extraction process, cell lysis can happened and intracellular compounds (e.g., nucleic acid) can leak out and cause over-extraction. For this reason, the ratio of EPS content to DNA content in the extract can be considered as an indicator for cell lysis. EPS extraction methods have been compared in many studies in terms of extraction efficiency and possible cell lysis. However, their results are often inconsistent with each other, probably because of differences in samples and extraction conditions. Moreover, extraction efficiency for EPS components may depend on the extraction methods, which is not well understood. Therefore, extraction methods should be carefully selected based on the target components or analyses in the downstream characterization of EPS.

This chapter investigated effects of extraction methods on EPS characteristics by comparing total extraction efficiency, cell lysis and EPS compositions (e.g., polysaccharide and protein contents, 3D-EEM spectra and MW distribution). The most

appropriate methods which obtained high extraction efficiency, less cell lysis and less effect on EPS characteristics, were chosen to extract and fractionate EPS from samples of MBR bulk sludge and cake layer in this thesis.

4.2. Materials and Methods

Activated sludge samples were taken in March, April, and May of 2016 from the aeration tank of a municipal wastewater treatment plant in Kanazawa, Japan. Total solids (TS), volatile solids (VS), suspended solids (SS), and sludge volume index (SVI) of each sample were analyzed within 4 hours of collection according to the Standard Methods, which were described in section 3.1.1. Characteristics of the sludge samples are shown in Table 4.1.

Procedure of EPS extraction and fractionation are summarized in Figure 4.1. Each extraction was triplicated for each sludge sample. Fifty-mL of activated sludge was used for each extraction. A fraction from the filtration of sludge supernatant with a disposable membrane filter (0.45 μm, Advantec-Toyo, Japan) after centrifugation at 6,000 × g for 10 min was collected as SMP. The remaining sediment after SMP extraction was re-suspended with pure water to the initial volume to extract LB-EPS. The re-suspended sample was treated with ultra-sonication at 3.5 W/mL at a depth of 5 cm (half the solution level) for 2 min. The sonication conditions were selected from the optimum conditions according to Han *et al*. [109]. The sonicated liquor was centrifuged at 13,000 × g for 15 min, after which the supernatant was collected as LB-EPS.

The residual solids of LB-EPS extraction were re-suspended with pure water to extract TB-EPS. The extraction of TB-EPS was conducted using three different methods. The three methods were chosen according to previous studies [47, 48, 110] in which they are evaluated as efficient methods for EPS extraction. In chapter 4, these methods were comprehensively compared in the efficiency to extract TB-EPS. The extractions was conducted as the following:

(i) Cation exchange resin (CER): CER (Na-form, 20-50 mesh, Dowex, Sigma-Aldrich) with a dose of 70 g/g VS-sludge was mixed with LB-EPS extraction residue and stirred (approximately 600 rpm) at 4°C for 1 h.

(ii) Combination of formaldehyde and sodium hydroxide (HCHO/NaOH): 0.3 mL of 36–38 % HCHO solution was added into 30 mL of LB-EPS and stirred at 4°C for 1 h, after which 20 mL of 1 mol/L NaOH solution was added and the samples were stirred at 4 °C for 1 h.

(iii) Combination of sodium hydroxide and heating (NaOH/Heat): 20 mL of 1 mol/L NaOH was added into 30 mL of LB-EPS extraction residue and stirred at 4 °C for 1 h, then heated at 80 °C for 10 min in a water bath.

The TB-EPS extraction samples were centrifuged at 13,000 × g for 15 min, after which the supernatant was collected as TB-EPS.

Residues of HCHO and NaOH in the extracts were removed with dialysis tubing membrane (Biotech CE Tubing, MWCO: 100-500 Da) submerged in pure water for 24 h. A 50 mL sample of TB-EPS extract was treated with the dialysis tubing membrane via submerged in pure water for 24 h. Pure water in dialysis membrane filtration was changed 3 times at 2-4 h, 6-8 h and 10-14 h.

Extraction efficiency was defined as volatile solids in extracted EPS per total VS in the sludge. Concentrations of VS, TOC, TN, protein, polysaccharide, humic acids, and DNA were analyzed as described in 3.1. These measurements were conducted in triplicate. 3D-EEM and MW distribution of each EPS fractions were also measured was description in 3.1.2.

Table 4.1. Characteristics of activated sludge samples for EPS extraction (n=4).

	Total solids [g/L]	Volatile solids [g/L]	Suspended solids [g/L]	Total organic carbon [mg/L]	Total nitrogen [mg/L]	C/N ratio [-]	Sludge volume index [ml/g]
Average	1.6	1.2	1.4	174	144	1.3	116
SD	0.3	0.2	0.3	45	43	0.5	27

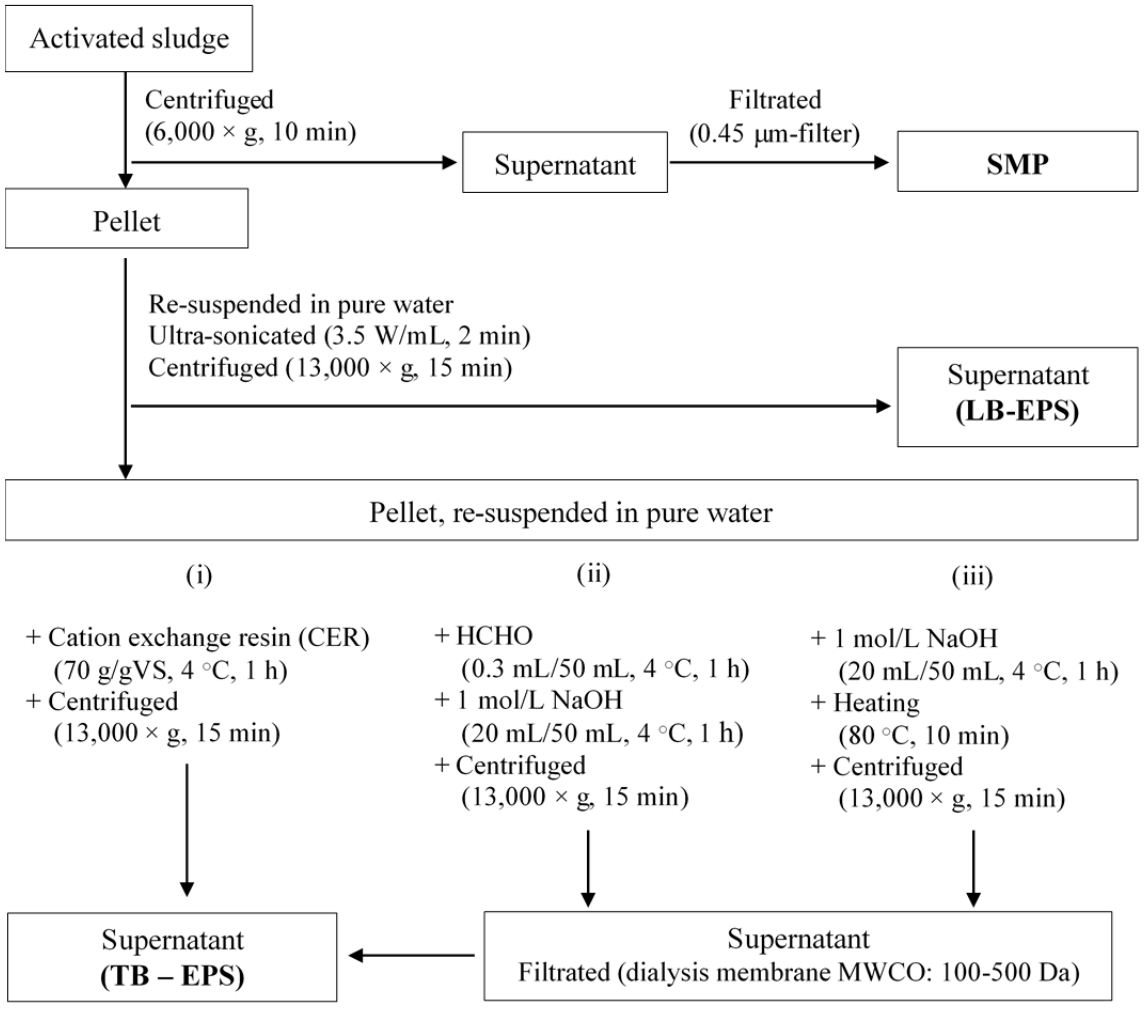
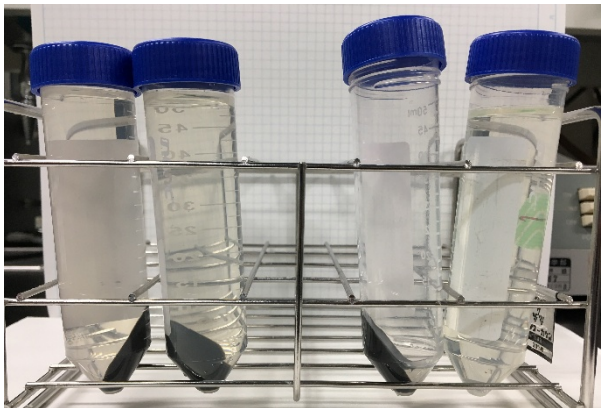
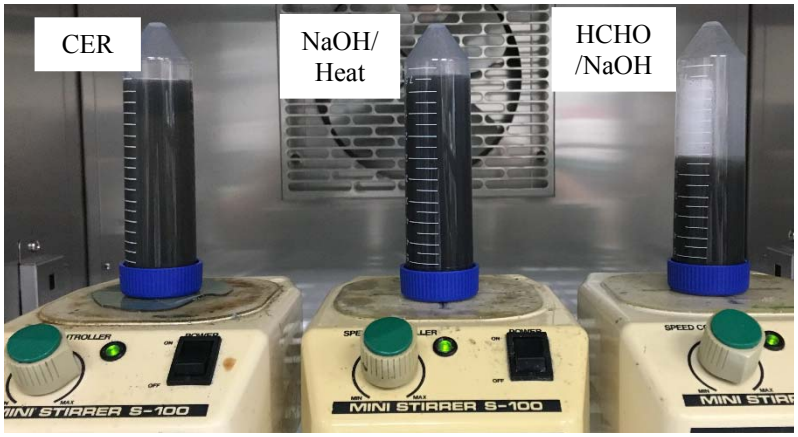


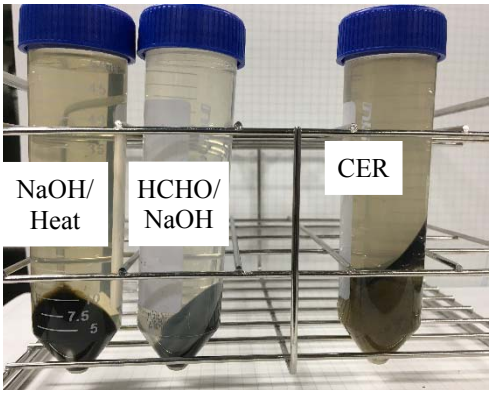
Figure 4.1. Procedure of EPS fractions with different extraction methods.



SMP extraction via centrifuge

LB-EPS extraction via ultra-sonication





TB-EPS extraction via different methods

Figure 4.2. Photographs of EPS extraction

4.3. Results and Discussion

4.3.1. EPS extraction efficiency and cell lysis

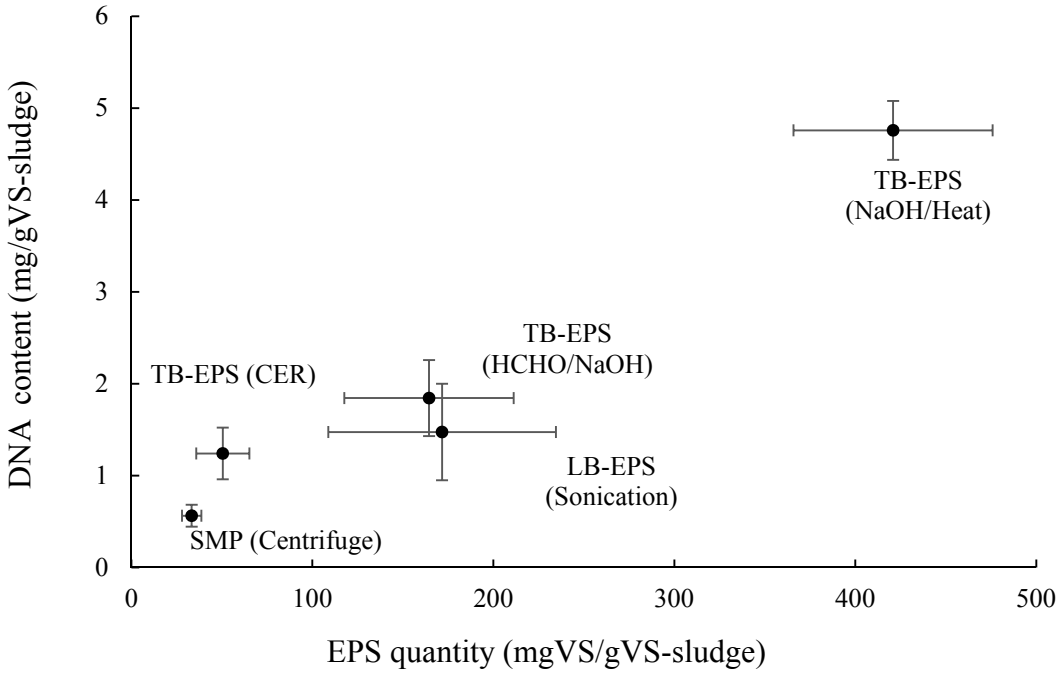
Concentrations of EPS in extracts are shown in Table 4.3. NaOH/Heat method showed the highest efficiency for TB-EPS extraction, followed by the HCHO/NaOH and CER methods. This tendency was similar for all surveyed samples, which indicated that NaOH/Heat method possibly is the best method to extract TB-EPS from activated sludge of MBRs.

Table 4.2. Concentrations of EPS in extracts [mg DOC/g VS]

EPS fraction	Methods	Sample #1	Sample #2	Sample #3	Sample #4	Mean	SD
SMP	Centrifuge	5.0	6.4	5.7	6.0	5.8	0.6
LB-EPS	Sonication	26.4	42.0	24.5	15.2	27.0	11.1
	CER	8.6	4.5	7.1	6.0	6.6	1.8
TB-EPS	HCHO/NaOH	52.3	28.9	30.4	44.9	39.1	11.4
	NaOH/Heat	138.1	115.1	141.8	168.2	140.8	21.8

Profile of EPS content – DNA content of each method was shown in Figure 4.3-A. The level of cell lysis was also largest in the NaOH/Heat method, followed by the HCHO/NaOH and CER methods. The results showed that NaOH/Heat caused more cell lysis than CER. Based on ratio of DNA content to EPS amount in the extract - a criteria to evaluate for the optimum extraction method, HCHO/NaOH and NaOH/Heat methods were better options than CER method since they owned a lower ratio of DNA to EPS than CER method did (Figure 4.3-B). In general, HCHO/NaOH method was found to be more appropriate for TB-EPS extraction than CER method.

A. DNA content and EPS content



B. Ratio of DNA to EPS

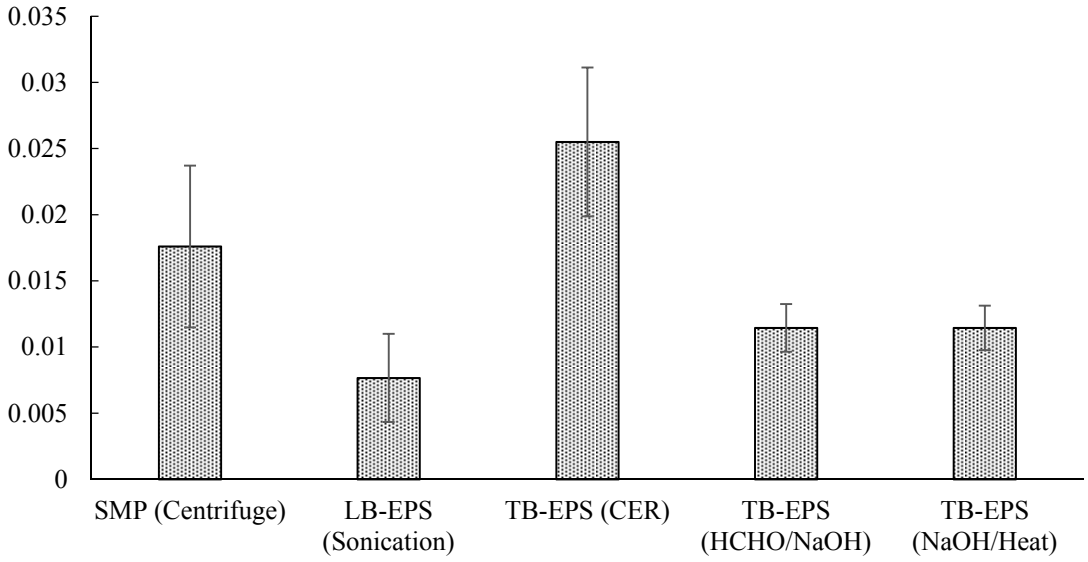


Figure 4.3. (A) EPS content and DNA concentration (B) ratio of DNA content to EPS under different extraction methods. Error bars were standard deviation (n=4).

4.3.2. EPS components

Protein was primary component in EPS extract and this component was slightly affect via extraction method (Figure 4.4). The protein contents in TB-EPS extracted by CER and HCHO/NaOH methods ranged from 510 to 670 mg BSA/g VS of TB-EPS and did not differ significantly. Moreover, TB-EPS extracted by the NaOH/Heat method showed a lower protein content of only 438 mg/gVS of TB-EPS. The low protein contents in TB-EPS under the NaOH/Heat method was probably because (i) a portion of the proteins was denatured and precipitated under heating to 80°C or (ii) hydrolyzed into amino acids. Therefore, the NaOH/Heat method may underestimate the protein content in TB-EPS. While, the sonication conditions in this chapter were considered to be sufficient to extract as much protein from the LB-EPS as possible with minimal cell lysis.

The contents of extracted polysaccharides in the TB-EPS fraction were similar among the methods, being approximately 140 mg/gVS of TB-EPS (Figure 4.4). The NaOH/Heat method extracted a slightly lower amount of polysaccharides than the other two methods. These results were consistent with those of other investigations of EPS in activated sludge that yielded 71–382 mg/gVS [46, 111, 112, 48]. Polysaccharides in the LB-EPS extracted by the sonication method were slightly higher than those in the TB-EPS. This was probably because more polysaccharides were present in the outer edge of the aerobic sludge flocs or granules than inside of the aggregates [110].

Additionally, Figure 4.4 also showed that humic substances in LB- and TB-EPS did not differ significantly among applied extraction methods. In TB-EPS, the humic substances content under the HCHO/NaOH and NaOH/Heat methods were slightly lower than

those under the CER method.

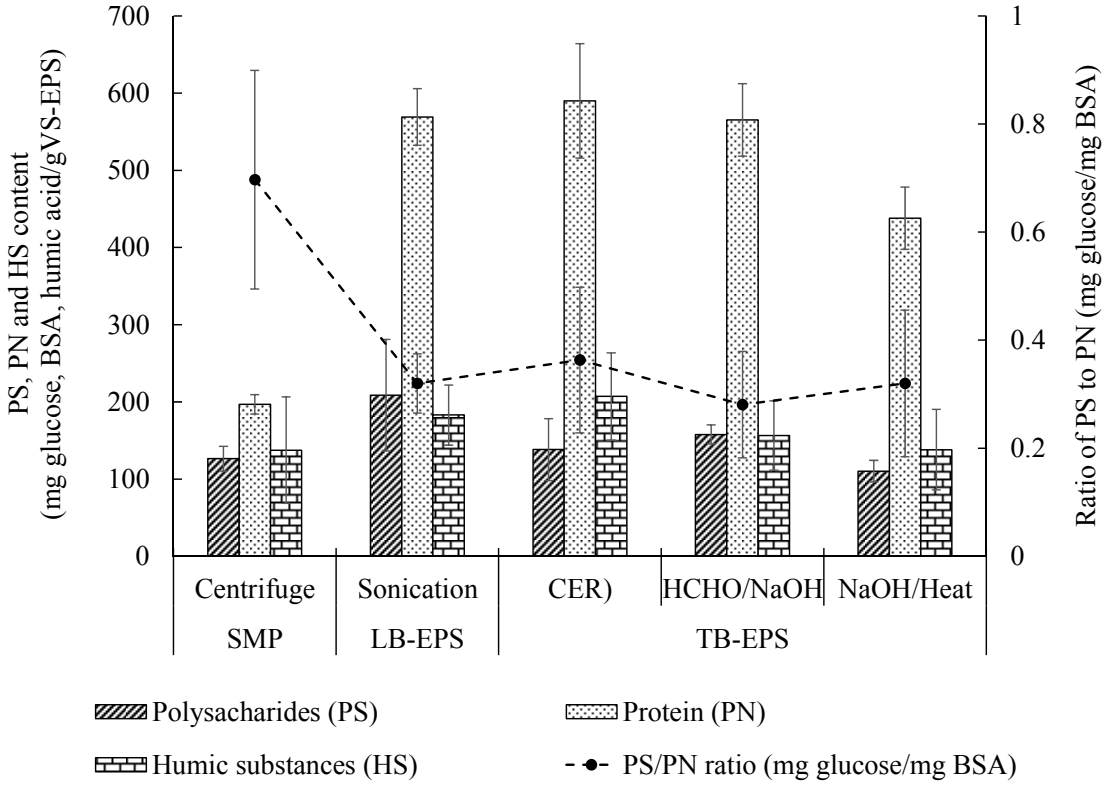


Figure 4.4. The polysaccharides (PS), proteins (PN) and humic substances (HS) contents in each EPS fraction extracted by different methods. The values are mean of the 4 sludge samples. Error bars stand for standard deviation.

A polysaccharide/ protein ratio (PS/PN) is also often reported to be an important indicator relating to membrane fouling. For instance, a lower PS/PN ratio increased floc hydrophobicity [113]. Thus, sludge flocs with a lower PS/PN ratio could aggravate membrane fouling by their deposition onto the membrane surface [17]. Furthermore, PS/PN ratio was found to decrease and then reach a stable level during biocake development [114]. However, different methods probably do not result in significant differences in the average of PS/PN ratios, as reported in past studies [46, 111, 112, 48].

Table 4.3. Extraction efficiency and EPS components under different methods from previous studies.

Method	Sample	VS	DNA	PN	PS	HS	Reference
		mg/g VS-sludge (mg/g SS-sludge)					
HCHO/ NaOH	*Activated sludge*	*164 (142)*	*1.7 (1.5)*	*91 (78)*	*26 (22)*	*26 (22)*	*This chapter*
	Activated sludge	165	0.4	55	41	50	[48]
	Activated sludge	198	7.9	29	14	24	[46]
	Activated sludge		0.34	36	44	52	[115]*
	Activated sludge	120	-	76	31	-	[112]
	Granular sludge	711	0.1	281	67	325	[111]
CER	*Activated sludge*	*51 (44)*	*1.1 (0.9)*	*30 (26)*	*7 (6)*	*10 (9)*	*This chapter*
	Activated sludge	58	0.14	18	13	16	[48]
	Activated sludge	198	7.9	29	14	24	[46]*
	Activated sludge	120	-	76	31	-	[112]
	Activated sludge	-	-	243	48	126	[43]
	Granular sludge	283	0.2	140	108	160	[111]
	Granular sludge	-	-	46	7	-	[110]
NaOH/ Heat	*Activated sludge*	*421 (361)*	*4.8 (4.1)*	*186 (159)*	*46 (39)*	*56 (48)*	*This chapter*
	Granular sludge	-	-	190	25	-	
	Activated sludge	-	-	96	22	-	[43] *(without heating)*
Heat	Activated sludge	57	1.2	21.2	11	5.7	[46]*
	Activated sludge	39	-	28	10	-	[112]
	Activated sludge	-	-	105	76	21	[116]
	Activated sludge			121	8	-	[43]
	Granular sludge	170	0.1	180	92	355	[111]
Sonication	*Activated sludge*	*172 (147)*	*1.5 (1.3)*	*98 (84)*	*31 (26)*	*32 (26)*	*This chapter*
	Activated sludge	-	(4.8)	(80)	(15)	(40)	[109]
	Activated sludge	26	1.3	10.7	3.9	2.8	[46]*
	Granular sludge	148	0.1	126	104	281	[111]

VS: Volatile solids; PN: Proteins; PS: Polysaccharides; HS: Humic substances

Data inside brackets are in mg/g SS-sludge

* Composition corrected assuming a ratio of volatile solids to total solids of 0.676 [42]

The amount of LB- and TB-EPS in this chapter exhibited typical values when compared with those of previous studies (Table 4.4). This order of stringency in extraction methods (NaOH/Heat > HCHO/NaOH > CER) was consistent with past studies [46, 111, 48]. Most of these studies have shown that the HCHO/NaOH method obtains higher extraction efficiency than the CER method, while the level of cell lysis does not differ significantly between methods.

Findings that proteins were present at much larger proportions than polysaccharides and humic substances, are consistent with previous studies (Table 4.4). The similar extraction efficiency of proteins between the CER and HCHO/NaOH method were also found in these studies [111, 112, 48]. The protein content in LB-EPS extracted using sonication at 570 mg/gVS was in the range of previous studies (Table 4.3). The sonication conditions in this chapter were considered to be sufficient to extract as much protein from the LB-EPS as possible with minimal cell lysis.

Polysaccharides in the LB-EPS extracted by the sonication method were slightly higher than those in the TB-EPS. This was probably because more polysaccharides were present in the outer edge of the aerobic sludge flocs or granules than inside of the aggregates [111]. Polysaccharides are often an important target component in studies of membrane fouling in MBRs. Many studies have reported that polysaccharides closely correlate with biofouling since polysaccharide content is much higher than protein content in biocake on the membrane surface [17, 52]. When polysaccharides are the main target component, selection of extraction methods does not cause significant differences.

4.3.3. 3D-EEM spectra

Five common peaks of 3D-EEM were detected in EPS fractions (Figure 4.5): peak A at Ex/Em of 225/290–320 nm (aromatic protein), peak F at 225/420 nm (fulvic acid-like substances), peak S at 275/290–320 nm (soluble microbial by-product-like substances), peak H at 275/430 nm (humic acid-like substances), and peak P at 350/430 nm (humic acid-like substances related hydrophobic acids). These peaks are also often found in the 3D-EEM spectra of EPS in activated sludge [112, 115, 117, 118]. Besides the five common peaks, two uncommon peaks were found: peak H1 at 490/515 nm belonging to the humic acid-like region in SMP, and peak F1 at 200/450 nm located at the fulvic acid-like region in all of the LB- and TB-EPS fractions. The balance of the peak intensity in the TB-EPS was diverse among CER, HCHO/NaOH and NaOH/Heat methods (Figure 4.5).

The Ex/Em location of the peak was also slightly different. In the CER method, the intensities of five common peaks were similar. Therefore, the CER method is appropriate for extraction of a wide range of diverse florescent substances in EPS from activated sludge. A variety of peaks were detected in past studies. Aromatic protein (peak A), soluble microbial by-product-like substances (peak S), humic acids (peak H), and fulvic acids (peak F) were detected in the EPS of MBR sludge [112, 119], anaerobic sludge [117], and activated sludge of a sequencing batch reactor [118]. In contrast, fulvic acid-like (peak F) and humic acid-like (peak H) substances were present at very low intensity or not detected when the HCHO/NaOH method was applied. In the NaOH/Heat method, aromatic protein (peak A) and fulvic acid-like (peak F) substances disappeared completely, while humic acid-like substances (peak H) appeared in higher proportion than in the HCHO/NaOH method. These results indicate that the CER

method can extract a broad range of fluorescent substances from activated sludge, while the HCHO/NaOH and NaOH/Heat methods cannot extract or have low efficiency to extract humic acid-like (peak H) and fulvic acid-like (peak F) substances. The low extraction efficiency of these substances during alkaline extraction is possibly because cell lysis by NaOH caused leakage of endoenzymes, which can degrade fulvic acids and aromatic proteins. Pan *et al*. [36] found a high intensity proportion of aromatic proteins and fulvic acids under HCHO/NaOH and HCHO methods in which a higher concentration of formaldehyde was applied than this chapter. These findings suggest that increased doses of formaldehyde could improve the detection of aromatic proteins and fulvic acids by alkaline extraction methods. However, HCHO may cause a reduction of humic substances. HCHO can not only generate cross-linking amino, hydroxyl, and carboxyl groups of proteins to fix the cell membrane structure, but can also protect acidic groups present in the structure of amino acids and humic substances via deprotonation under NaOH [42]. In alkaline extraction methods, residues of NaOH and HCHO were removed by filtration via dialysis membrane because the chemical residue interferes with analysis of VS to determine the extracted amount of EPS. The selected dialysis membrane may interfere with 3D-EEM analysis. 3D-EEM spectra were not interfered with when dialysis membrane with a molecular weight cut off (MWCO) of 100–500 Da was applied in this chapter (data not shown). However, filtration via dialysis membrane with a MWCO of 3,500 Da reduced humic acid-like substances [120], probably because smaller molecules of humic substances were also removed during filtration. Therefore, membrane with MWCO less than 500 Da should be applied to detect humic substances in 3D-EEM.

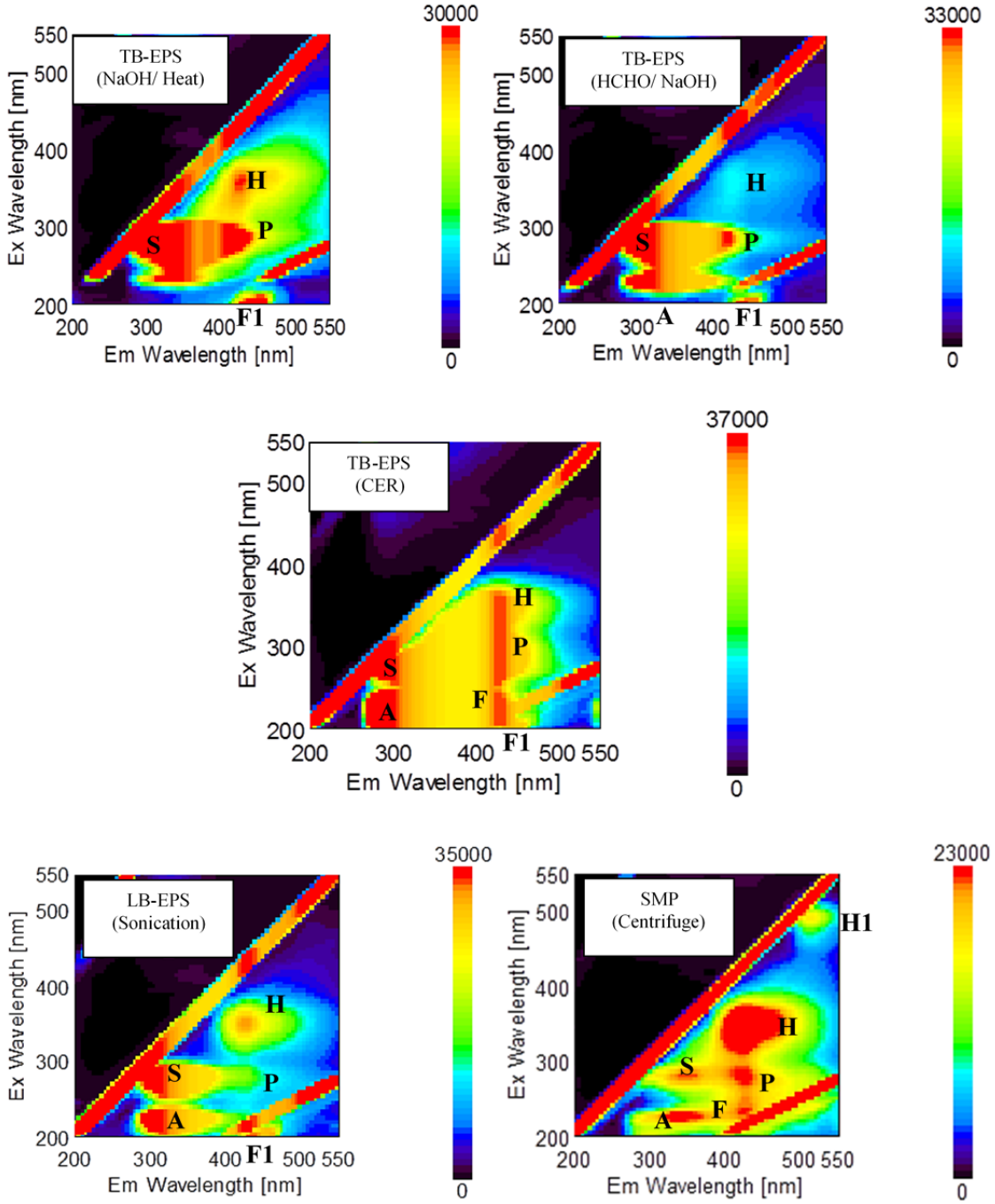


Figure 4.5. 3D-EEM fluorescence spectra of EPS fractions extracted by different methods.

Peak A: equivalent to aromatic protein, peak F: fulvic acid-like, peak S: soluble microbial by-product-like, peak H: humic acid-like, and peak P: related hydrophobic acids.

In LB-EPS extracted by sonication, hydrophobic acid-like (peak P) and fulvic acid-like (Peak F) substances were at very low intensity or not detected (Figure 4.5). These findings were very different when compared with those in SMP, in which hydrophobic acid-like substances (peak P) was predominant. The results shown in Figure 4.5 are somewhat similar to those of previous studies. Domíguez *et al*. [112, 119] found that more aromatic proteins (peak A) and soluble microbial by-product-like substances (peak S) were in LB-EPS, but more fulvic acids (peak F) and humic acids (peak H) were present in the SMP. These findings imply that sonication has low efficiency for the extraction of fulvic acids and humic acids from microbial aggregates.

4.3.4. Molecular weight profile

The apparent MW in EPS extract increased from SMP to LB-, and TB-EPS (Figure 4.6). Both LB- and TB-EPS extracts contained substances with a large MW. The main peak of LB-EPS exhibited a broader MW peak, ranging from 200 to 3,250 kDa (Figure 4.6-B), while the MW of TB-EPS ranged from 160 to >1,000 kDa (Figure 4.6-A). These very large components in the TB-EPS may have been lipoproteins and glycoproteins, which are complex polymers of proteins associated with lipids and polysaccharides. EPS proteins are reportedly more complex, with a larger MW than polysaccharides [121, 122]. Alkaline treatment by HCHO/NaOH and NaOH heat methods showed more peaks with a large MW, while TB-EPS obtained by the CER methods and LB-EPS by sonication showed only one peak with a MW of about 260 kDa. These findings imply that alkaline treatment is more efficient for separation of larger EPS molecules that are closely bound on the cell because HCHO/NaOH and NaOH/Heat methods not only dissociate a portion of the covalent bonds between EPS molecules and the cell by hydrolysis, but also increase the solubility of EPS by dissociation of acidic groups and

repulsion between negatively charged substances. Conversely, the CER method could only extract smaller EPS molecules with lower quantity because CER methods can only dissociate ionic bonds in EPS matrix through exchange of divalent cations, primarily Ca^{2+} and Mg^{2+}, which provide bridging bonds between two ionic substances, into monovalent Na+. A chromatogram of the MW of extracts obtained using the NaOH/Heat methods revealed that MWs shifted slightly lower than those acquired by the HCHO/NaOH method. This could be explained by heating of alkaline stimulating hydrolysis of macromolecules into smaller molecules [123]. The CER method is often employed to explore the MW profile of EPS [119, 124]. Moreover, it has been reported that CER extraction is preferable for MW analysis of EPS because it causes less change in MW than alkaline treatment [123, 119]. However, these studies targeted EPS with MWs < 700 kDa. The results of the present study revealed that CER cannot extract EPS larger than 500 kDa. Therefore, CER methods may not be appropriate for extraction of EPS molecules. The HCHO/NaOH method may be more appropriate when large molecule biopolymers are targeted.

The 3D-EEM spectra of MW fractions (MW-EEM profile) revealed more detailed differences among extraction methods. Although the CER method was not efficient at extracting EPS larger than 500 kDa, it was particularly inefficient at extracting soluble microbial by-product-like substances (peak S) larger than 450 kDa (Figure 4.7-A). No significant difference in aromatic proteins was observed in any of the MW fractions among the three extraction methods of TB-EPS. MW-EEM profiles indicated that proteins in EPS have a variety of molecular weights, independent of applied extraction methods. Bhatia *et al*. [124] also reported that the protein-like fraction of EPS was found in a wide range of MW from < 10 to > 600 kDa. Humic substances (peak H) were

not clearly detected in TB-EPS, probably because of dilution with the mobile phase in preparative HPLC. However, a slight peak was observed in all MW fractions of NaOH/Heat extract, while the smallest MW fraction was obtained with the HCHO/NaOH method. Moreover, humic substances were detected in all MW fractions larger than 1 kDa in SMP and LB-EPS (Figure 4.7-B and 4.7-C). Humic substances are reportedly one of the main components of EPS smaller than 6 kDa [124, 125]. The similarity of the MW-EEM profile between LB-EPS and SMP implies that unstable and degradable LB-EPS can be easily hydrolyzed from the matrix into SMP, while a portion of SMP can be adsorbed by the sludge flocs to LB-EPS [126]. Conversely, TB-EPS, which has a complex structure of macromolecular components, contributes to the structural stability of aggregates.

Consequently, selection of extraction methods greatly influences the MW profile of EPS extract. Stringent methods of HCHO/NaOH and NaOH/Heat had higher extraction efficiency, and were able to extract larger molecules from the inner layer of microbial aggregates (TB-EPS). However, the obtained MW profile should be carefully interpreted because alkaline extractants may cause changes in the MW distribution, as reported in previous studies [123, 119]. Heating caused a particularly large shift in the MW profile to lower size via hydrolysis of a portion of the EPS. Conversely, CER and sonication methods might cause less changes in the MW profile than alkaline extraction, although they are not very efficient for extraction of EPS >500 kDa.

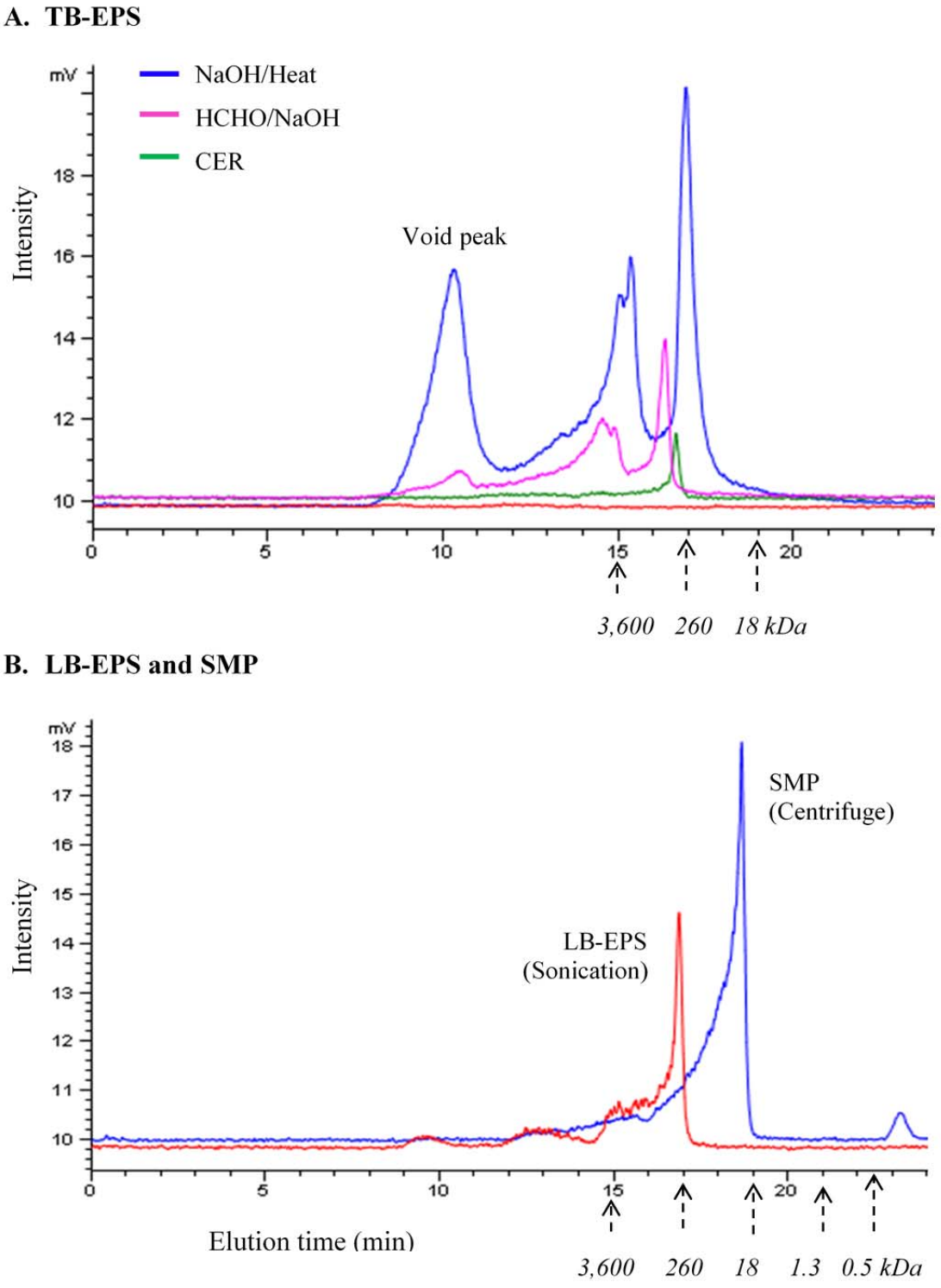


Figure 4.6. The molecular weight profile of TB-EPS (A), LB-EPS and SMP (B) extracted by different methods.

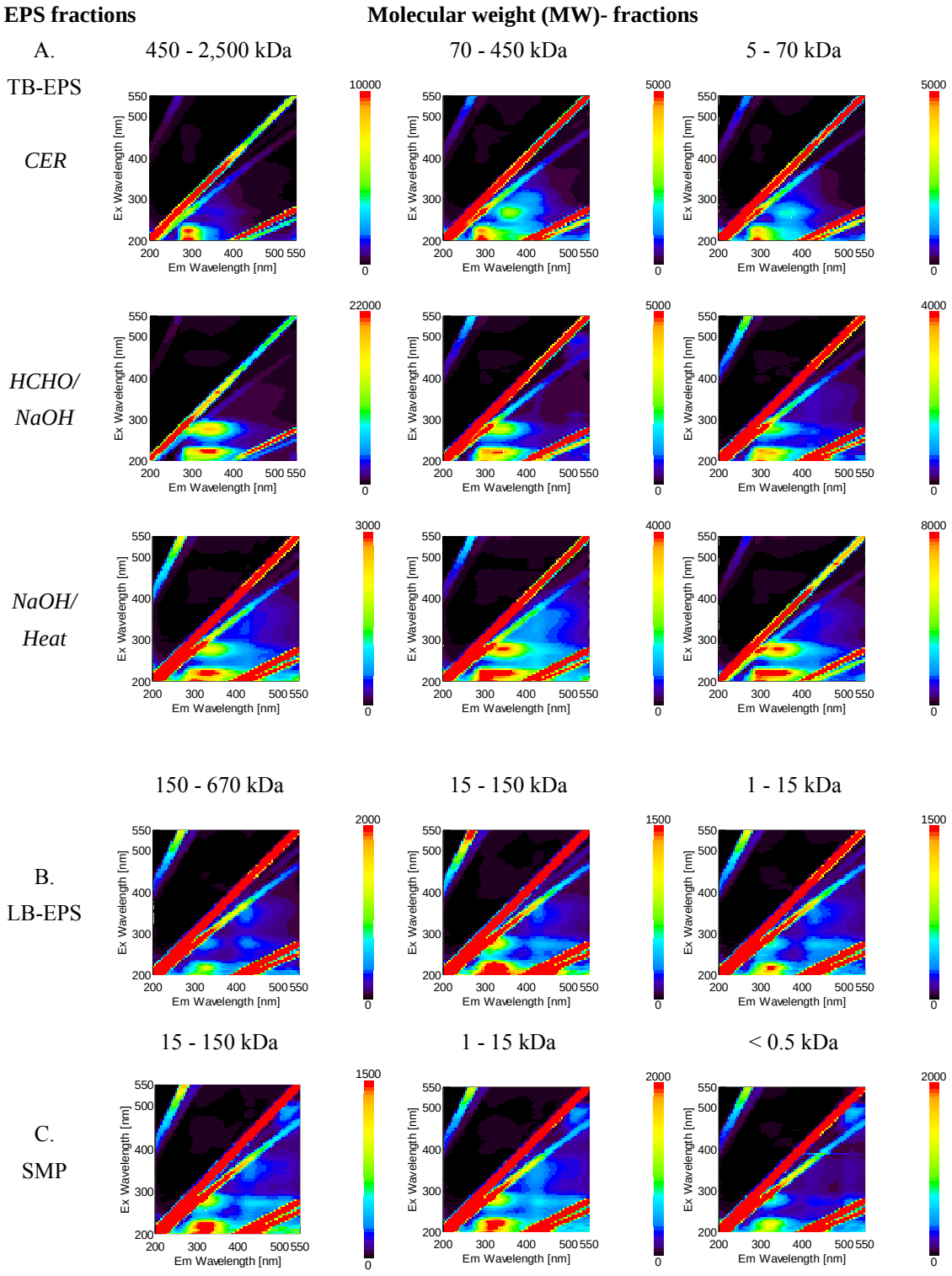


Figure 4.7. 3D-EEM spectra of molecular weight fractions collected via high pressure size exclusion chromatography from (A) TB-EPS, (B) LB-EPS, and (C) SMP.

4.4. Conclusions

Extraction methods affects not only extraction efficiency but also composition of proteins and polysaccharides, 3D-EEM spectra and MW distribution. Alkaline extraction has high efficiency but can cause cell lysis, which results in overestimation of EPS quantity. A combination of formaldehyde and alkaline is preferable for acquisition of high extraction efficiency with less cell lysis. There were no remarkable differences in protein and polysaccharide contents among extraction methods.

Upon 3D-EEM analysis, CER method is appropriate to detect a wide range of fluorescent organic substances, while alkaline extraction was less efficient for detection of humic acid-like substances. Additionally, MW profile analysis revealed that CER method could not extract EPS larger than 500 kDa and that it has an especially low extraction efficiency for soluble microbial by-product-like substances on 3D-EEM spectra. Alkaline extraction is more appropriate for extraction of large EPS molecules with a wide MW distribution. However, it should be noted that changes in MW distribution may occur in alkaline extraction, as the NaOH/Heat method of EPS showed a shift in the MW profile to the smaller side.

Finally, HCHO/NaOH was chosen for TB-EPS extraction for further experiments of this chapter because this method obtained high extraction efficiency, low cell lysis and less influence in 3D-EEM and MW distribution of EPS extract. Besides, parameters of centrifuge, sonication employed in this chapter (e.g., 6000 ×g for 10 min and 3.5 W/mL for 2 min) would be used for SMP, LB-EPS extraction, respectively. It was because the sonication conditions in this chapter were considered to be sufficient to extract as much LB-EPS as possible with minimal cell lysis.

Chapter 5. Characterization of EPS Causing Membrane Fouling

5.1. Introduction

Extracellular polymeric substances are reported to play pivotal roles in sludge flocculation and membrane fouling. For instance, SMP can permeate microfiltration and/or ultrafiltration membrane pores or adsorb in the pores then cause membrane plugging while membrane rejected microbial flocs and their EPS matrix accumulate on membrane surface forming a biofouling layer [26]. Poorer flocculation ability and lower settle ability of bulk sludge as the result of a higher level of LB-EPS reportedly cause an increase in cake layer resistance, as well as total membrane resistance [127]. SMP are reported to encourage a several-time increase in filtration resistance of cake layers [128]. Recently, it has reported that concentrations and characteristics of EPS are two important factors determining the extent and severity of fouling condition [14]. Among of EPS characteristics, polarity (HPO/ HPI) and MW size of EPS from cake layers and bulk sludge are the most vital indicator of MBR fouling since they are directly related to the interaction of EPS with the applied membrane.

Relative contribution of hydrophobic groups (e.g., alkyl, hydrophobic amine, and benzyl) and hydrophilic groups (e.g., hydroxyl, carboxyl, etc.) take responsibility for polarity of EPS [8]. Hydrophilic and hydrophobic substances in EPS can be fractionated by using adsorbent resins (e.g., Amberlite DAX-8, XAD-4 resins) [63]. The hydrophobicity of bulk sludge reportedly has different effluences in the dense of cake layer formation and the rate of TMP increase in MBRs with hydrophilic/ hydrophobic membranes [129]. Hydrophilicity/ hydrophobicity of membrane surface and foulant are essential factors for interactions between them when the two surfaces initially contact.

MW distribution of SMP and EPS have been indicated to impact on membrane fouling. Substances (100-300 kDa) SMP reportedly cause severe flux decline and pore plugging in microfiltration [130], while MW of EPS affect floc stability, and then play a role in membrane fouling [8]. Substances larger than 100 kDa (e.g., polysaccharides), which are mostly rejected by the membranes, accumulate and form cake layers [52]. Substances smaller than 100 kDa in SMP such as humic acid-like substances which are much smaller than the average size of microfiltration membrane pores (Figure 5.1), can be rejected by these membranes [131, 132]. It indicated that understanding in MW distribution of EPS-fraction components are necessary to control biofouling caused by EPS in bulk sludge.

Polarity and MW size of EPS are expected to have direct relation to attachment of EPS-related folants on membrane surface; therefore, this chapter determined characteristics of EPS in bulk sludge and cake layer via polarity-molecular profiling in order to figure out the contributions of key EPS fractions to membrane fouling in microfiltration process. To achieve this aim, EPS components were also characterized via 3D-EEM spectroscopy.

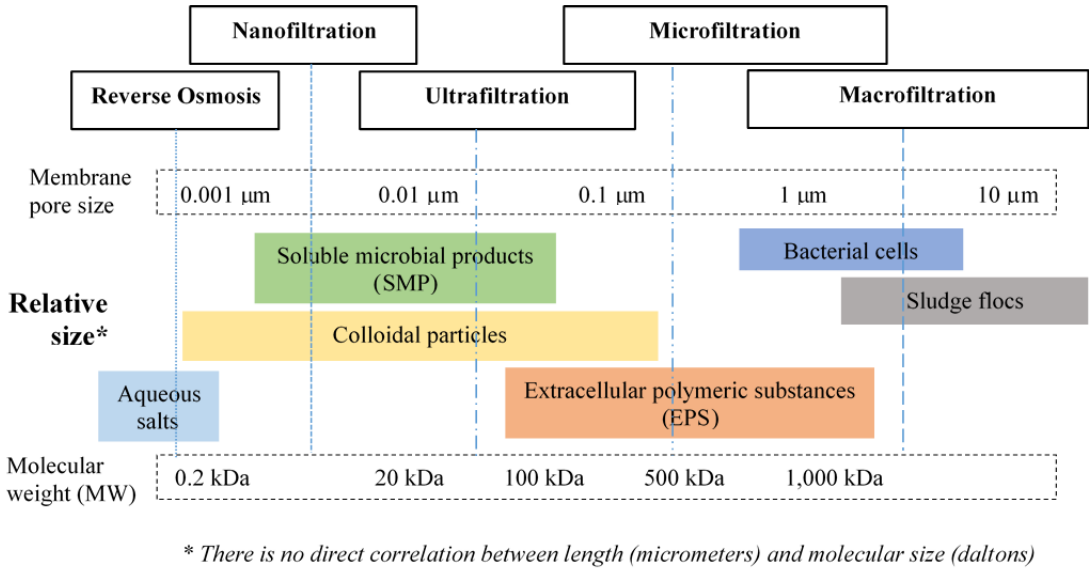


Figure 5.1. Membrane pore size of filtration processes and relative size of foulants [8].

5.2. Materials and Methods

Bulk sludge and cake layer were sampled from a lab-scale MBR, which was operated as descriptions in 3.2. Membrane units were immersed in aeration tank to separate biomass of activated sludge and treated water. This study employed microfiltration flat sheet made of chlorinated polyethylene with the average pore size at 0.2 μm (maximum 0.4 μm, Kubota, Japan). Transmembrane pressure was operated at the constant level of 80 kPa. Fouling development was monitored by permeate flux, which ranged 5.6-18.2 LMH. Operational conditions were summarized in table 5.1.

Table 5.1. MBR operation condition

Operational parameters	Value
Working volume	2 L
Membrane area	152 cm^2
MLSS	5 - 7 g/L
Filtration/relaxation	4 min/ 1 min
HRT	12 - 14 h
SRT	28 days
Physical cleaning	brushing (weekly)
Temperature	18 - 22 ^oC
Organic loading rate	40 - 60 mg TOC/g·day
TOC influent	120 - 160 mg/L
TOC effluent	4 - 8 mg/L
TOC removal	93 - 98 %

Membrane was physically cleaned every 7 days. The foulant removed by brushing was considered as a cake layer sample. The physically cleaned membrane was rinsed several times with pure water, then was backwashed with 50 mL of pure water at the rate of 5

mL/min. Chemical cleaning was conducted once to observe change in components of membrane permeate in the initial stage of filtration. In chemical cleaning, NaClO (0.1 % of active chlorine) was added into membrane module and soaked for 2 hours.

EPS in MBR bulk sludge extraction were fractionated into SMP, LB- and TB-EPS. The three fractions were extracted via centrifuge, ultra-sonication and HCHO/NaOH, respectively based on findings in chapter 4. Cake layers were collected from membrane surface every week, re-suspended with 150 mL pure water. LB- and TB-EPS of cake layers were extracted under ultra-sonication and alkaline-formaldehyde methods, respectively, in the same manner as extraction from bulk sludge. EPS fractions from bulk sludge and cake layer were characterized via relative hydrophobicity, HPI/ HPO, MW distribution, and 3D-EEM. These measurements were conducted as described in 3.1.2.

The detected EPS components were plotted in a two-dimensional matrix of polarity and molecular weight (hereinafter called polarity-molecular weight profile). To make the profile, firstly, one-hundred μL sample of EPS was injected after filtration by 0.45 μm filters into reversed phase preparative HPLC for polarity chromatography, which was described in 3.1.2. From the polarity chromatogram, detected peaks were designed to be fractionated and collected by a fraction collector. Second, the collected fractions were injected into a SEC to investigate its molecular weight distribution, which was also described in 3.1.2. Since there is a limitation in injection volume (100 μL) the injection was conducted 8 - 10 times in order to get sufficient volume of polarity fractions for further analyses such as SEC and 3D-EEM.

5.3. Results and Discussion

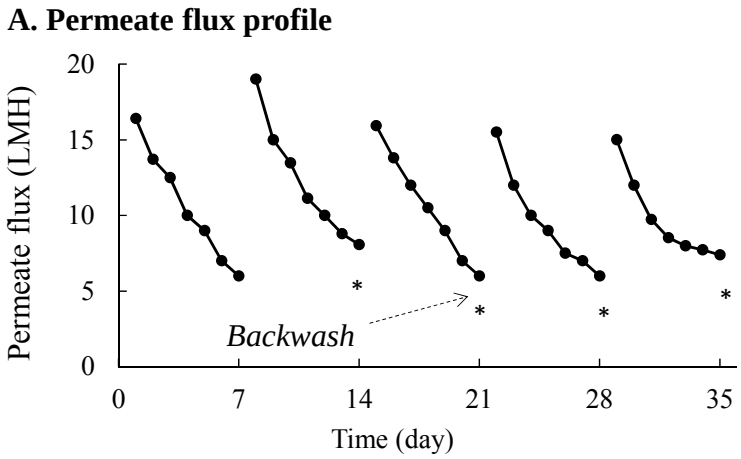
5.3.1. Relatively hydrophobic proteins and polysaccharides

The average contribution of relatively hydrophobic substances (e.g., proteins and polysaccharides) in EPS of bulk sludge were comparably higher than that in cake layers (Figure 5.2). It showed that bulk sludge possibly contained a higher level in hydrophobic adhesion than cake layers. The hydrophobic adhesion could enhance adsorption ability of bulk sludge at membrane surface. The result was inconsistent with a previous study of Arabi and Nakhla [34], who found that contributions of hydrophobic substances in bulk sludge and of cake layers are similar and affected by wastewater property.

RH proteins in SMP, LB-, and TB-EPS accounted for 50, 24, and 37 % in total, respectively (Figure 5.2). They were much higher than the contribution of RH polysaccharide (7, 20 and 6 %, respectively). On the contrary, in LB-, and TB-EPS of cake layers, RH polysaccharides were present at higher level than corresponding proteins (26 and 17 % compared with 13 and 5 %, respectively). The result showed that RH polysaccharides tended to adsorb membrane and became a main component of cake layers while RH proteins were highly present in SMP and EPS of bulk sludge. This finding was consistent with finding of Massé [54] in which proteins are reported to possess higher affinity to sludge flocs than hydrophobic polysaccharides.

Hydrophobic substances of SMP (mainly proteins, less polysaccharides) could contribute to pore clogging in this case since a high concentration of hydrophobic substances in SMP reportedly contributes to higher fouling rate due to its higher potential for blocking membrane pores [34]. In addition because Liao [133] found that LB-EPS with more

hydrophobicity and less negative charge could own low bio-flocculation, causing more release of SMP from EPS matrix. In this case, a high level of hydrophobic proteins in LB-EPS of bulk sludge might cause de-flocculation of sludge flocs, resulting in the high ratio of hydrophobic proteins in the SMP. It implied that hydrophobicity of SMP and LB-EPS of bulk sludge directly relates to fouling development in MBR.



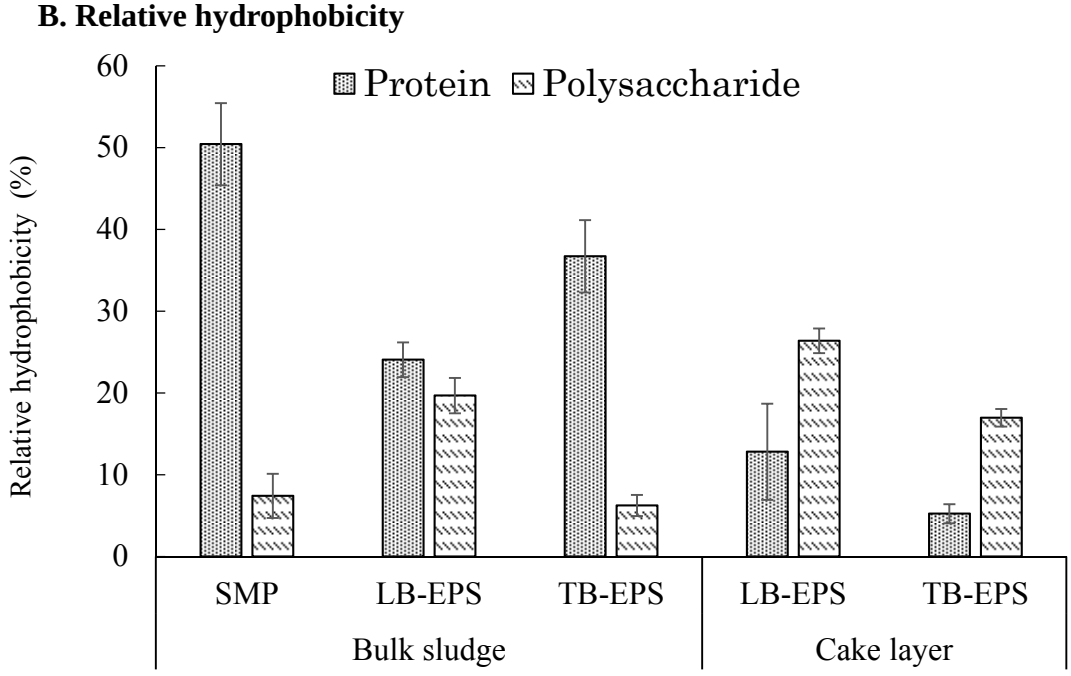


Figure 5.2. (A) Permeate flux profile and (B) Relative hydrophobicity of EPS fractions from bulk sludge and cake layers. Error bar represents standard deviation (n=4).

5.3.2. Hydrophobic/hydrophilic substances of EPS fractions

Polarities of SMP, LB-, and TB-EPS of bulk sludge differed on reversed phase chromatograms. SMP and MBR permeate possessed a large peak at 5-6 min of retention time, relevant to a hydrophobic peak while TB-EPS owned a hydrophilic peak at 2-4 min of retention time. LB-EPS of bulk sludge exhibited a bimodal chromatogram of hydrophilic and hydrophobic substances (Figure 5.3). Substances of SMP showed a great hydrophobic property; therefore, the SMP owned a large amount of free hydrophobic groups to form hydrophobic interactions. In bulk sludge, the TB-EPS were highly hydrophilic while LB-EPS showed presence of both hydrophobic and hydrophilic substances. The presence of hydrophobic and hydrophilic substances of LB-EPS can indicate two tendencies of detachment away or involvement in the EPS matrix of bulk sludge. In contrast, both LB- and TB-EPS of cake layers showed a similar chromatogram with a hydrophilic peak, as well as TB-EPS of bulk sludge (Figure 5.3). In general, the SMP largely comprised of hydrophobic substances which mainly contain proteins, while the EPS in bulk sludge and cake layers largely contributed by hydrophilic substances. Both results from reverse phase chromatography and relatively hydrophobic analysis indicated that hydrophilic substances of bulk sludge were mainly protein whereas those of cake layers were largely polysaccharides. In this chapter, the hydrophobic substances in SMP could be adsorbed by the membrane [134], conditioning pores and surface of membrane. The absorbed hydrophobic substances facilitated the accumulation of hydrophilic LB-EPS of bulk sludge (e.g., polysaccharides) on the conditioned membrane to form a cake layer [33]. Hydrophobicity/ hydrophilicity of SMP and EPS is expected to play an important role in membrane fouling [8], which is governed by complicated physical–chemical interactions between the membrane and initial foulants , followed by foulant-foulant interactions [5].

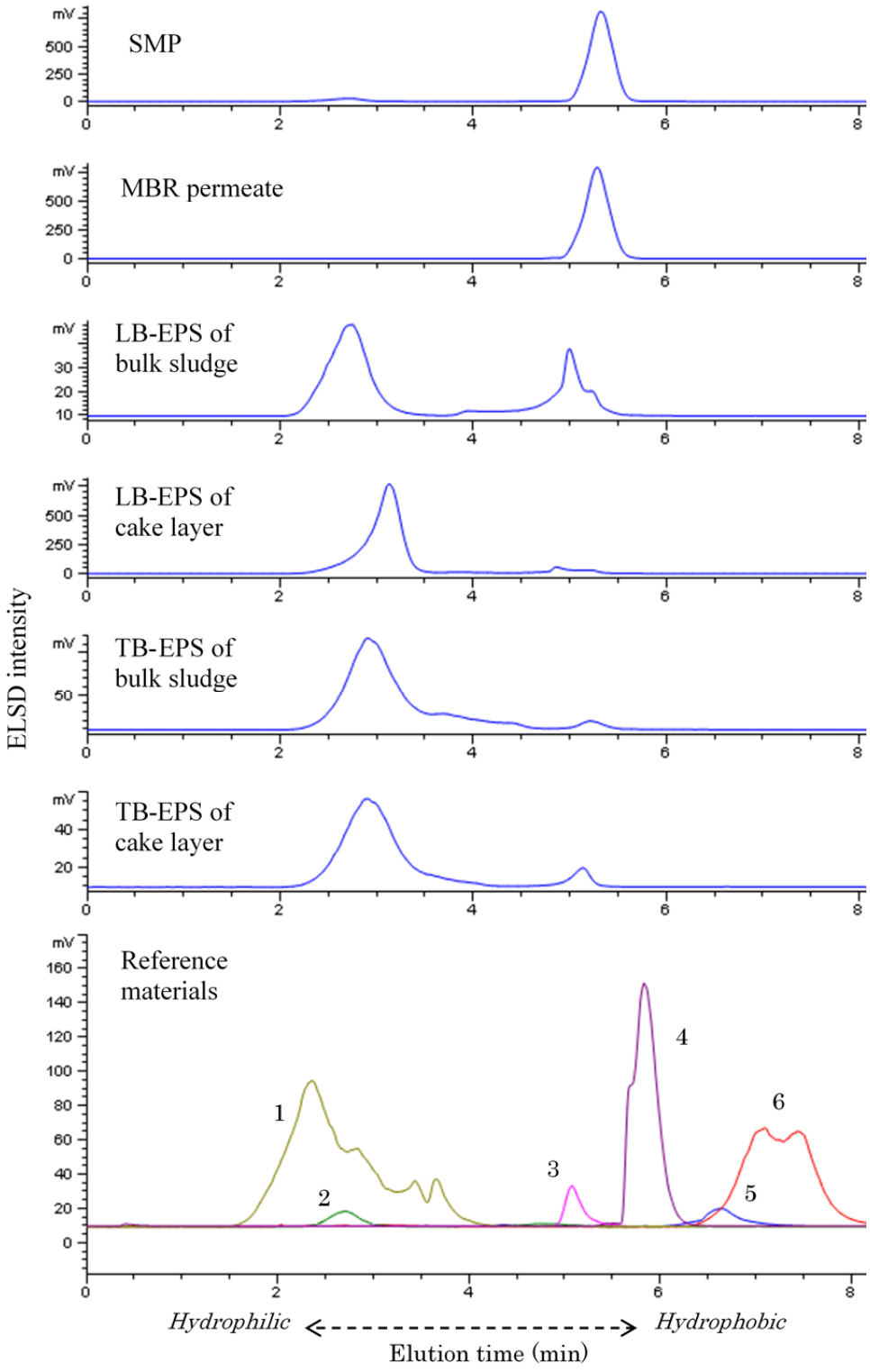


Figure 5.3. Polarity profile of EPS fractions of bulk sludge cake layers

Reference materials: #1. EPS extract, #2. Uracil, #3. Humic acid extract, #4. Propanol, #5. Aniline, and #6. Chloroform.

5.3.3. Molecular weight distribution of EPS fractions

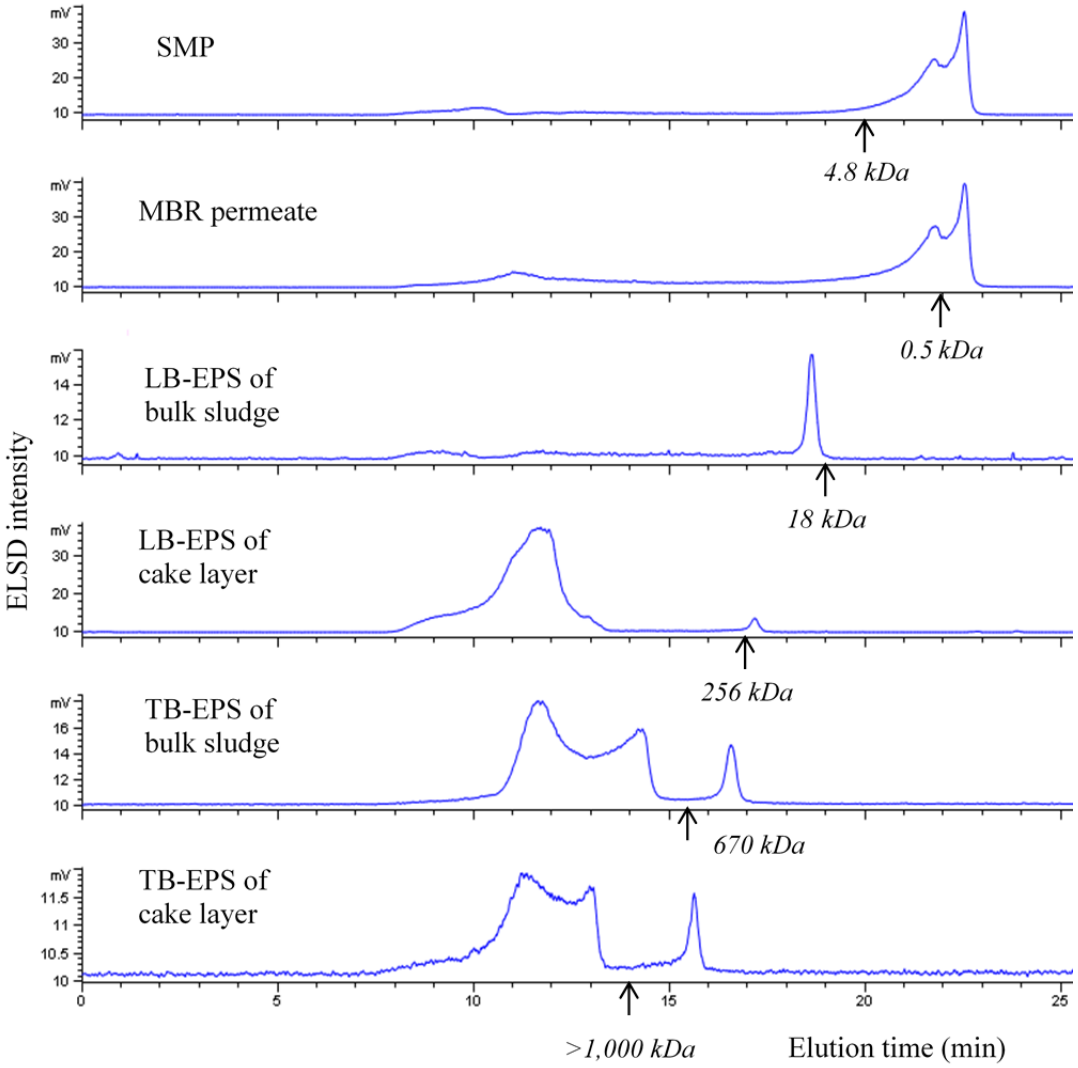


Figure 5.4. Molecular weight distribution of EPS fractions of bulk sludge and cake layers under high-pressure size exclusion chromatography.

Molecular weight of SMP was smaller than those of EPS of bulk sludge (Figure 5.4). SMP and MBR permeate shared a similar MW distribution, largely containing < 20 kDa substances whereas LB-EPS of bulk sludge mainly comprised of biopolymers of 100-670 kDa. TB-EPS contained substances ranging from 670 kDa to over 1,100 kDa. The MW of LB-EPS from bulk sludge was lower than that of cake layers (Figure 5.4). The finding of

SMP containing lower 100 kDa in our study was consistent with past studies [125, 135], in which by using HPSEC-UV detector and RID, they have reported that these substances can be proteins, polysaccharides, humics. The EPS of cake layers contained large MW than the SMP and their MW distribution ranged from to 450 kDa to over 1,100 kDa which was consistent with previous studies, ranging 488 – 3,397 kDa [135]. Especially, Xiong *et al*. [135] found that the peak of 488 kDa substances in cake layers gradually shift to 838 kDa in a longer-filtration operation. This finding is consistent with the phenomena of the larger MW of cake layers than bulk sludge in our study. Transition of slowly or non-biodegradable substances (proteins, polysaccharides, even glycoprotein) can be a reasonable explanation for the increase in the MW distribution of cake layers. The transition of these substances possibly formed larger molecule compounds, which were not found in LB-EPS of bulk sludge.

5.3.4. Polarity-molecular weight profile

The detected EPS components were plotted in a two-dimensional matrix of polarity and molecular weight (hereinafter called polarity-molecular weight profile). Figure 5.5 showed polarity-MW profile of SMP, EPS components of bulk sludge and cake layers. Hydrophobic substances smaller than 20 kDa were mainly found in SMP of bulk sludge (Figure 5.5). 3D-EEM spectra analysis revealed that the hydrophobic fraction in SMP contained a large ratio of humics (humic acid-like and fulvic acid-like substances) to proteins (e.g., tryptophan protein-like, aromatic protein-like substances). MBR permeate also contained hydrophobic substances smaller than 20 kDa (Figures 5.3 and 5.4). 3D-EEM spectra of this fractions in MBR permeate was also similar to those in SMP (data not shown). This indicates that hydrophobic fraction in SMP is transported through micropores and appeared in permeate. Hydrophobic substances smaller than 20 kDa were

also found in LB-EPS of bulk sludge. This implies the close relations of SMP and LB-EPS of bulk sludge. Hydrophobic fractions of 20-450 kDa in LB-EPS of bulk sludge contained humic acid-like and fulvic acid-like substances as well as aromatic protein and tryptophan protein-like substances on 3D-EEM spectra (Figure 5.6-B). These peaks were also found in 3D-EEM spectra of SMP. Therefore, hydrophobic fraction of 20-450 kDa in LB-EPS of bulk sludge is possibly one of the potential origin of hydrophobic substances in SMP [8] when the fraction in LB-EPS of bulk sludge is decomposed into smaller molecules.

Hydrophilic substances as large as 100-670 kDa were found only in LB-EPS of bulk sludge (Figure 5.5). 3D-EEM spectra showed this fraction contained aromatic and tryptophan protein-like substances (Figure 5.6). Interestingly, this fraction was not found in cake layer nor in permeate. Therefore, this fraction is possibly trapped inside the membrane or degraded on the membrane surface, as discussed in next section.

Hydrophilic substances larger than 450 kDa were found in TB-EPS of bulk sludge as well as LB- and TB-EPS of cake layers. These fractions had similar 3D-EEM spectra, having a high level of aromatic protein and tryptophan protein-like but no humics (Figure 5.5 and 5.6-C). Moreover, hydrophobic substances larger than 450 kDa were also found in the three samples and showed similar 3D-EEM spectra. These results indicated that LB- and TB-EPS of cake layers were highly similar with TB-EPS of bulk sludge (Figure 5.5). Therefore, TB-EPS of bulk sludge is probably deposited to be a main component of EPS in cake layer.

Several studies have investigated relations of polarity and molecular weight of membrane

foulants by using adsorbent resins (e.g., XAD resin) [63, 136, 57, 137, 138]. In these studies, a sample was first fractioned with resin into two fractions: hydrophilic and hydrophobic ones, then MW of each fraction was analyzed with size-exclusion HPLC. In this chapter, preparative reverse-phase HPLC was employed to fraction a sample by polarity. Its chromatogram could offer more detailed profile of a sample, and compounds separated by their polarity. Subsequently, we can decide the number of fractions to be collected from the obtained chromatogram. Therefore, we can get precisely targeted fractions by using this method. Moreover, polarity-MW profile from the method can show MW distribution of hydrophobic and hydrophilic fractions as in previous studies [63, 57]. With a fraction collector, this method also allow collect polarity-MW fractions for further analysis such as 3D-EEM in the study (Figure 5.6).

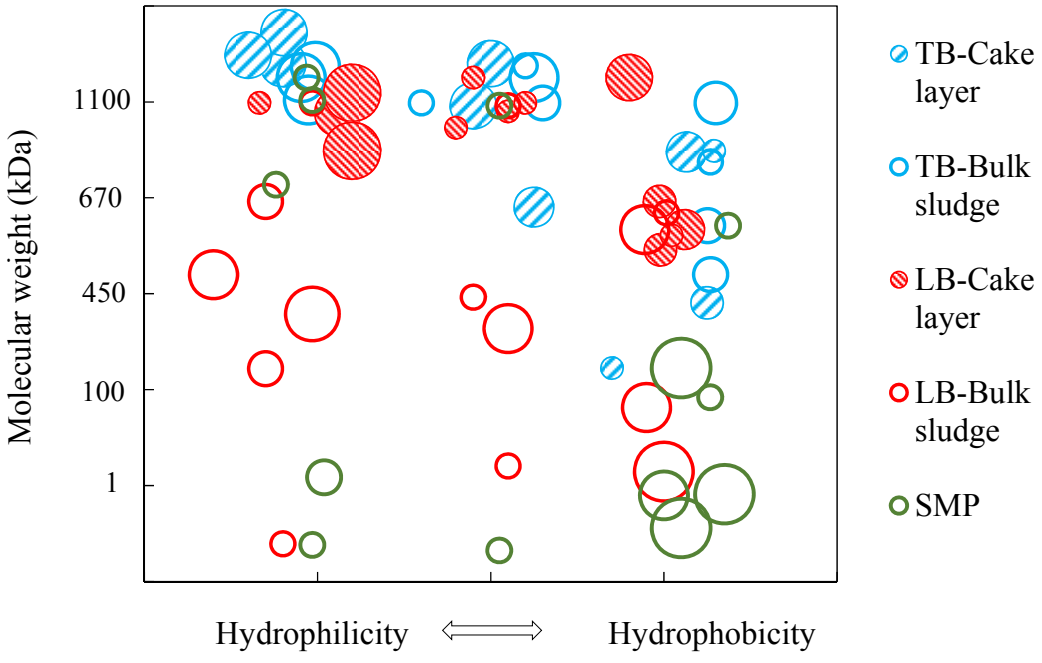
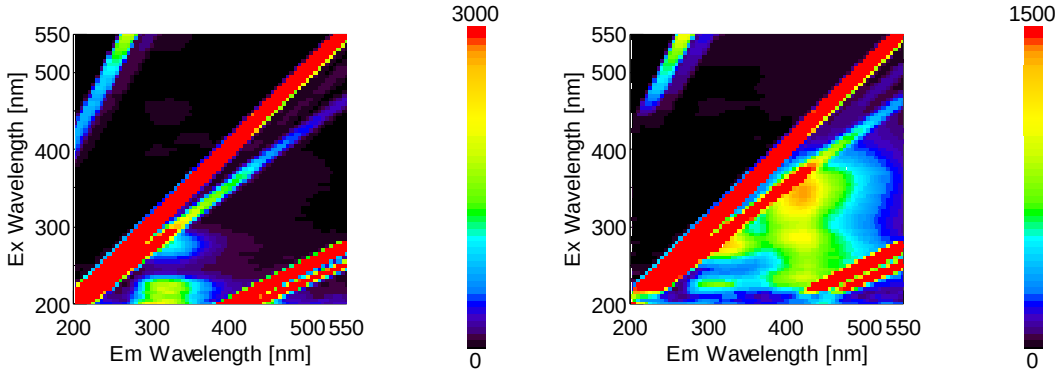


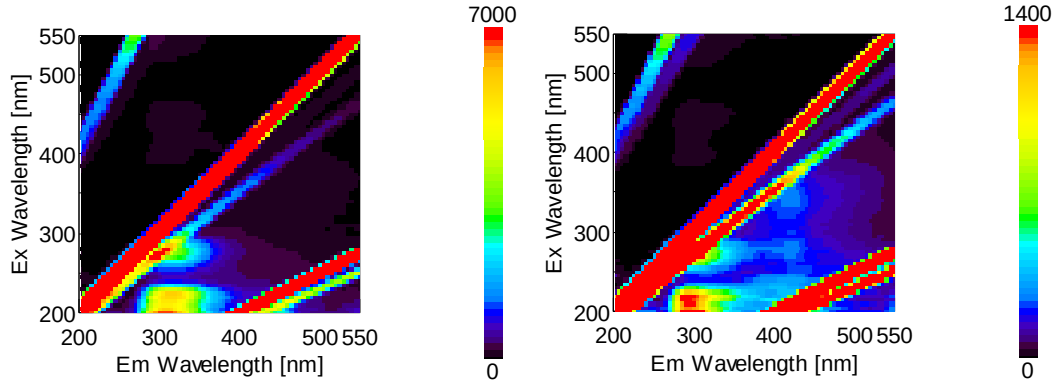
Figure 5.5. Polarity – molecular weight profiling of EPS fractions in bulk sludge and cake layers (n=4). Dot size represents relative presence of relevant substances in EPS fractions.

A. SMP*



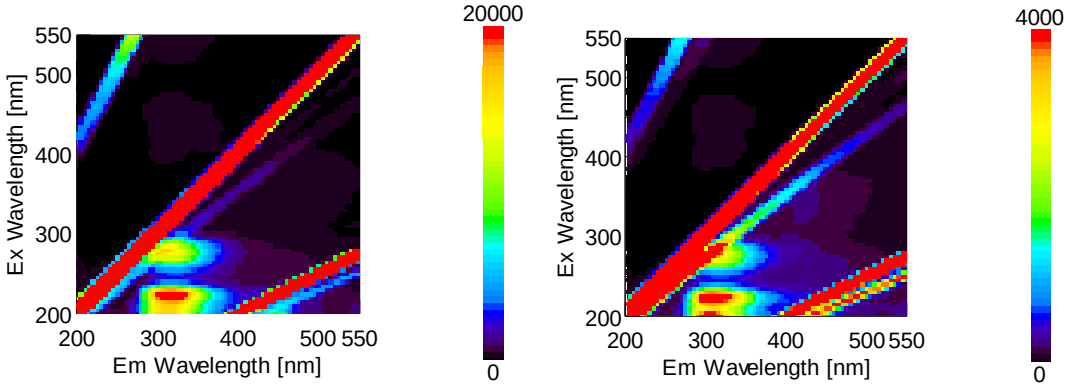
< 20 kDa hydrophilic fraction < 20 kDa hydrophobic fraction

B. LB-EPS of bulk sludge



20-450 kDa hydrophilic fraction 20-450 kDa hydrophobic fraction

C. TB-EPS of bulk sludge**



>450 kDa hydrophilic fraction >450 kDa hydrophobic fraction

Figure 5.6. 3D-EEM matrix spectra of polarity-MW fractions of EPS fraction in bulk sludge and cake layers. (*) 3D-EEM of polarity-MW fractions in SMP was similar with that in permeate; (**) 3D-EEM of polarity-MW fractions in TB-EPS of bulk sludge.

5.3.5. Role and fate of EPS components in MBR fouling

Soluble microbial products in supernatant of bulk sludge could contain hydrophobic substances smaller than 100 kDa (Figure 5.5), which are expected to freely permeate ultrafiltration and microfiltration membrane. However, hydrophobic humics smaller than 20 kDa are found to adsorb on membrane surface and onto membrane pore [55]. Inside of membrane pores, these hydrophobic humic substances are adsorbed on the membrane (Figure 5.9-A). There are two evidences for membrane conditioning by SMP. The first evidence was that MW distribution of SMP was much larger than that of permeate for the initial 5 days after chemical cleaning (Figure 5.7). This suggests that a part of SMP was adsorbed or rejected by the membrane. The second evidence was that 3D-EEM spectra and MW distribution of MBR permeate changed day by day at the initial stage of filtration, while those of SMP did not change (Figure 5.7). This is probably because membrane surface condition had changed every day, suggesting that some substances attached on the membrane pores.

The adsorbed humic substances can incorporate with other hydrophilic compounds to narrow the pore of membrane and cause internal membrane clogging (Figure 5.9-B). LB-EPS in bulk sludge contained a 100-670 kDa hydrophilic fraction. This fraction probably plays an important role in membrane fouling, because it was not found in MBR permeate nor on cake layer. This size of fraction can enter membrane pores or be deposited on the blocked pore. When it enter the membrane pore, it possibly attached on the internal membrane structure which has been conditioned by hydrophobic humic substances (Figure 5.9-B). Because backwash effluent did not contain this fraction, it can be a major irreversible foulant. When this fraction is deposited on the blocked pore or membrane surface, it is probably degraded into smaller molecules. This degraded fraction

was possibly trapped on the internal structure of membrane or pass through the membrane. Analyses of the backwash effluent showed presence of hydrophobic and hydrophilic substances of 35 kDa (Figure 5.8). This 35 kDa fraction in backwash effluent is probably a degraded fraction of LB-EPS, because its size is larger than SMP. Consequently, the deposited fraction of LB-EPS in bulk sludge was decomposed into smaller molecules, pulled into conditioned micropores, then remain inside the pores.

Hydrophilic substances larger than 670 kDa, which were main components of the TB-EPS in bulk sludge, were also found in LB- and TB-EPS of cake layers (Figure 5.5). This result indicated that TB-EPS in bulk sludge was deposited on the membrane surface and formed cake layers (Figure 5.9-C). After the initial deposition of cake layer, EPS production from live microbe cells as well as intercellular compounds from dead cells can make biocake layers thicker, causing serious fouling (Figure 5.9-C). EPS matrix of cake layers were mainly comprised of large hydrophilic substances, which contained a high proportion of polysaccharides and were larger than 450 kDa or even over 1,100 kDa. These large hydrophilic substances can originate from (i) substances in SMP rejected by membrane [52], (ii) aggregation of non-degradable molecules of bulk sludge [55], (iii) metabolites of microbe aggregates [8], and (iv) inner-cellular compounds or cell body of dead cells in the layers [12].

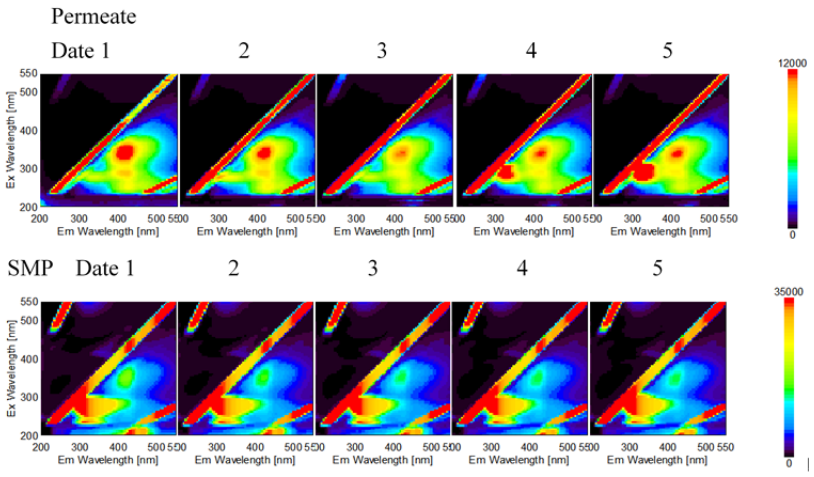
Physical cleaning can only remove cake layers on fouled membrane but not foulants in membrane pores, which are considered as irreversible foulants, when physically cleaned membrane is applied for a new filtration cycle (Figure 5.9-D). The irreversible fouling quickly becomes severe, caused by abundant biopolymers and humic substances in SMP readily plugging the narrowed pores. Hydrophobic and electrostatic interactions of humic

and hydrophilic polysaccharides [5] are considered to keep the foulants in the membrane pores [33], [this case]. Consequently, non-covalent network formed by hydrophilic proteins and polysaccharides can make irreversible foulants more complicated [5, 139].

The foulants inside the membrane were collected by backwashing with pure water to determine their characteristics of protein and polysaccharide components, 3D-EEM, polarity and MW distribution. The foulants contained 20-100 kDa hydrophobic and hydrophilic substances of protein and polysaccharide (Figure 5.8). Under 3D-EEM spectra, tryptophan protein-like substances and aromatic proteins were detected but humic acid-like substances were not. This implied that humic acid-foulants cannot be removed by only physical cleaning nor backwash. Kimura *et al*. [140] reported that NaClO obtains a higher removal efficiency for irreversible foulants of humic substances than NaOH and HCl. These facts in our study indicate low MW hydrophobic and hydrophilic substances contribute to pore clogging and irreversible fouling. Firstly, small hydrophobic humics are adsorbed by membrane, which conditions membrane surface and narrows membrane pores. Subsequently, larger hydrophilic compounds (protein and polysaccharides) not only plug the narrowed pores but also accumulate on the conditioned membrane surface. However, complicated interactions between membrane and foulants, as well as those among foulants, still need to be understood. Especially, biotransformation of the biopolymers in EPS of cake layers requires more researches in the future. The finding in polarity-MW profile of this chapter also implies that any method can encourage the absorption SMP into EPS matrix of bulk sludge to reduce SMP quantity in supernatant can be a good strategy to control biofouling. In addition, inhibiting formation of gel layer by reducing or breaking hydrophobic and electrostatic interaction of humics and 100 - 450 kDa hydrophilic substances on MBR membrane

surface would be also effective to reduce irreversible fouling. Minimizing hydrophobic content of SMP and EPS of bulk sludge, for instance by quorum quenching [92] may reduce attachment of hydrophilic biopolymers into/ onto membrane, lessening membrane fouling rate in MBR.

A. Excitation emission matrix spectra of SMP and permeate



B. Molecular weight distribution of SMP and permeate

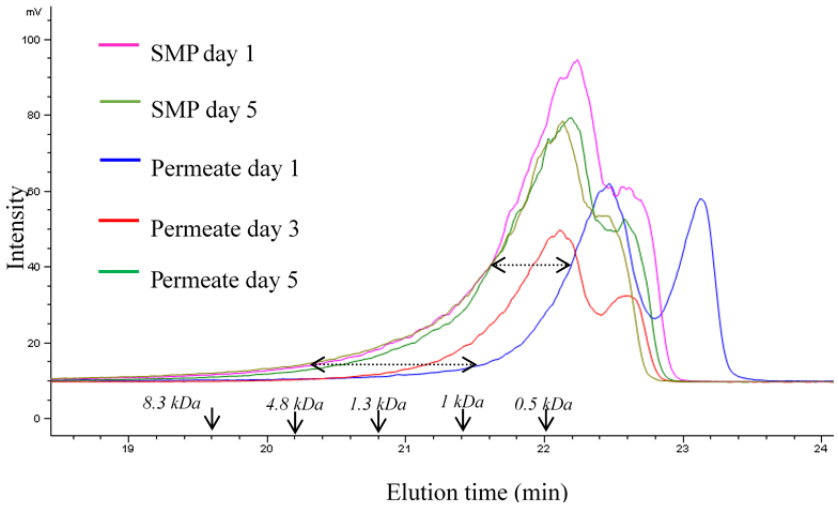
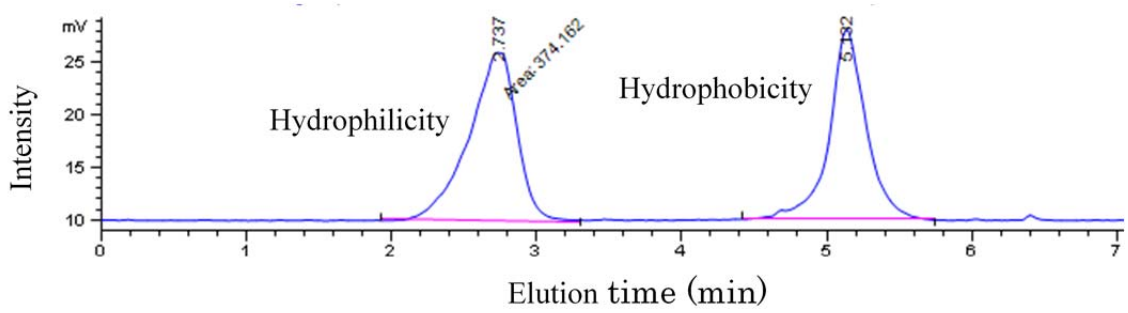


Figure 5.7. EEM spectra and MW of SMP and permeate in 5 days after chemical cleaning.

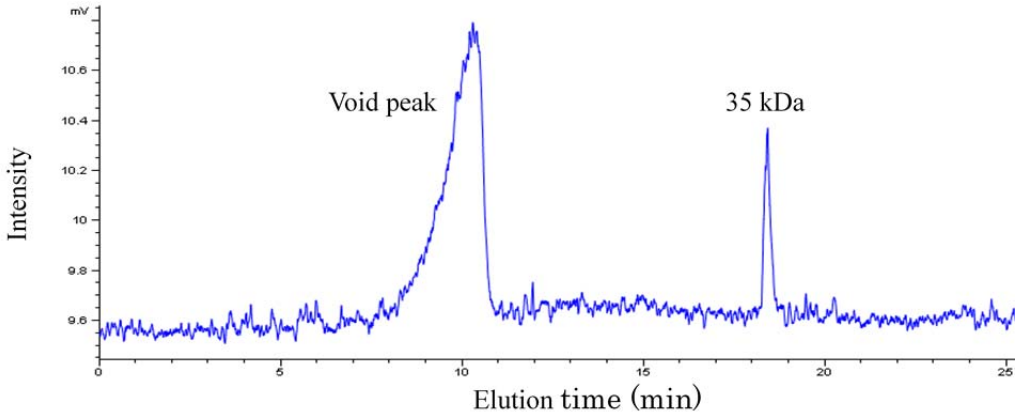
MW distribution of SMP was much larger than that of permeate for the initial 5 days after chemical cleaning. This suggests that a part of SMP was adsorbed or rejected by the membrane. In addition 3D-EEM spectra and MW distribution of MBR permeate changed day by day at the initial stage of filtration, while those of SMP did not change. This is probably because membrane surface condition had changed every day,

suggesting that some substances attached on the membrane pores.

A. Polarity chromatogram of foulant



B. Molecular weight chromatogram of foulant



C. Excitation emission matrix spectra of foulant

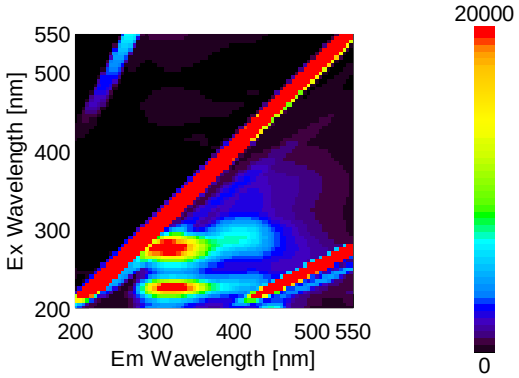


Figure 5.8. Polarity, MW and EEM spectra of pore-foulant collected by backwashing.

Foulant collected by back-washing of physically cleaned membrane was comprised of hydrophobic and hydrophilic substance of 35 kDa. The foulants contained large amount of tryptophan protein-like substances and aromatic proteins but less humic acid-like substances (Ex/EM: 340/405). The humic substances were hard to be removed by physical cleanings (e.g., backwashing), possibly causing

irreversible fouling.

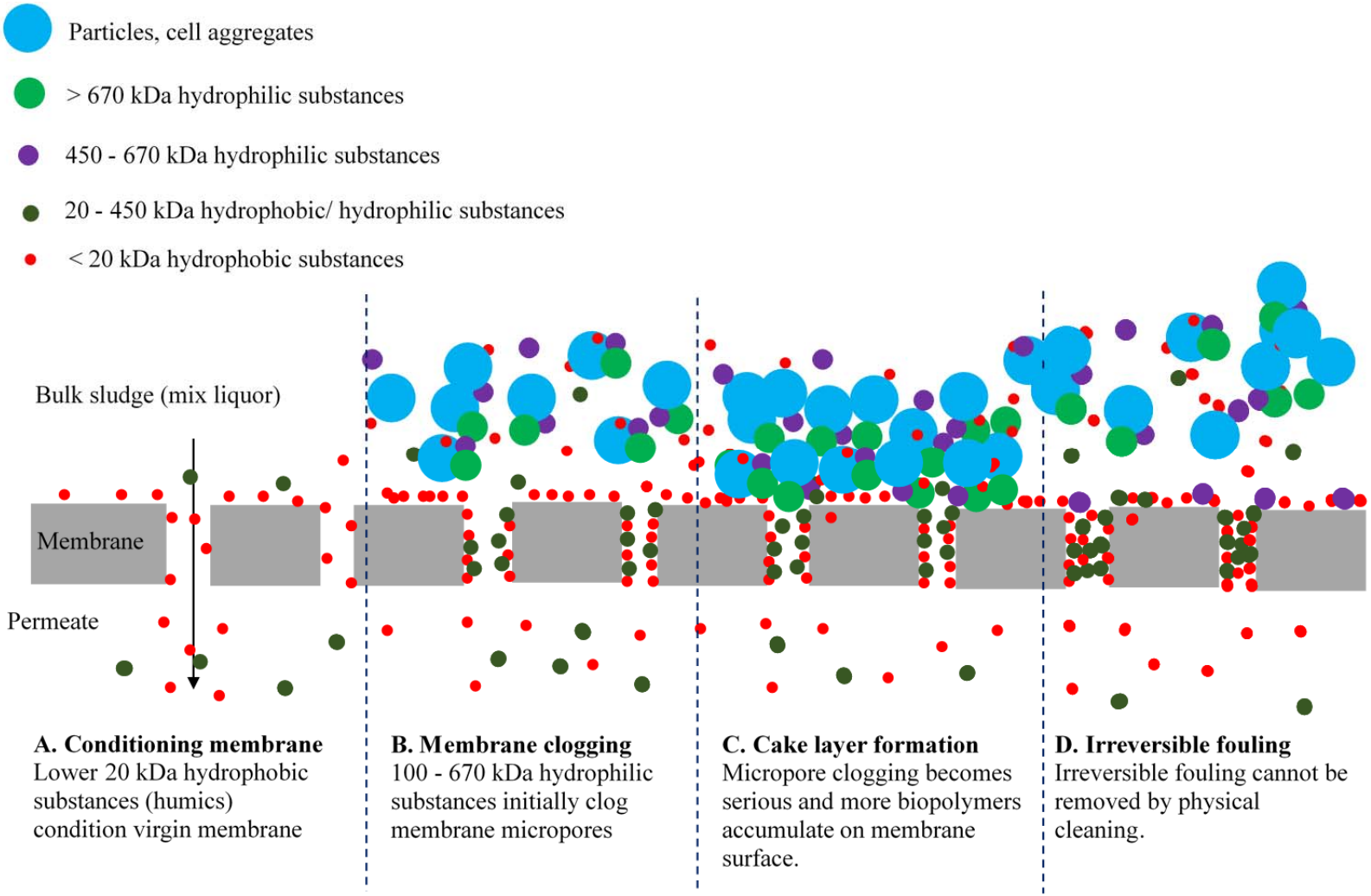


Figure 5.9. Schematic illustration of suggested fouling process in a MBR.

5.4. Conclusions

Polarity-MW profiling conducted as the procedure in this chapter was appropriate to determine characteristics of EPS extracted from sludge and cake layer of MBRs.

Based on the polarity-molecular weight profile of EPS fractions, key EPS components causing membrane foulants were be tracked. The results showed that hydrophilic substances as large as 100-670 kDa was found only in LB-EPS of bulk sludge but not in that of cake layers nor in MBR permeate. Hydrophobic substances smaller than 20 kDa were mainly found in SMP of bulk sludge. Hydrophilic substances larger than 670 kDa was mainly found in TB-EPS of bulk sludge and in LB- and TB-EPS of cake layer.

These findings suggest a possible mechanism of fouling in a microfiltration process (e.g., application of membrane pore 0.2 μm and chlorinated polyethylene material):

- Surface and micropores of virgin membrane are conditioned by hydrophobic substances smaller than 20 kDa in SMP and LB-EPS. Besides, hydrophobic substances from LB-EPS can be released into SMP, contributing to pore narrowing.
- Hydrophilic biopolymers as large as 100-670 kDa in bulk sludge plug into narrowed micropores of the conditioned membrane, causing irreversible fouling.
- Hydrophilic substances larger than 670 kDa of cake layer were possibly from TB-EPS fraction of bulk sludge and produced from microbes on membrane surface to form biofilm.

Chapter 6. Bacterial Community Structure under Different Filtration Conditions

6.1. Introduction

In membrane fouling process, SMP components initially accumulated on membrane surface form gel layers [17], offering a favorable condition for attachment of other foulants and pioneer microbes. The pioneer microbes including individual bacterial cells and in small aggregates may attach to the surface during a short period of filtration [141]. These pioneer bacteria reproduce and grow to make a new community on membrane surface. Microbial cells of the new community and EPS and their EPS and development form cake layers [17], increasing total membrane resistance by causing cake layer fouling. Some bacteria (e.g., *Xanthomonadaceae, Pseudomonadaceae, Enterobacteriaceae,* etc.) are found to have higher potential causing fouling than others [142]. Structure and behavior bacterial community of bulk sludge are believed to be important factors of membrane fouling. Recently, pioneer bacteria on MBR membrane surface responsible for cake layer formation have been listed [141, 143]. Investigation in pure culture of pioneer bacteria has revealed that fouling potential of each microbe depends on its own surface properties [144], determined by EPS content of hydrophilic organic matter and polysaccharides, surface charge, water content [142]. In other words, pioneer bacteria obtain different tendency to attach on membranes and different potentials causing cake layer fouling.

EPS production and decomposition are known as microbial behavior to response to environmental condition [10]; Similar with EPS production and membrane fouling, bacterial structure in bulk sludge have been reported to be also affected by operational factors such as aeration, stress condition (e.g., salinity shock), MLSS concentration [31, 15, 145]. Moreover, Guo *et al.* [15] found that some bacterial genera had very high positive correlation with EPS components and fouling rate. Hence, effects of

operational condition (e.g., filtration condition) on bacterial structure and EPS production should be investigated.

Membrane bioreactor process can be operated under either constant permeate flux or constant TMP and fouling caused ether an increase of TMP or a decline of permeate flux, respectively [6]. TMP and flux are consider as indicator parameters of fouling development [146]. At a constant flux operation, TMP is observed to increase gradually in initial stage, followed by a TMP jump stage [6]. The gradual rise of TMP is expected to be contributed by attachment of SMP and EPS from bulk sludge whereas the TMP jump is reportedly caused by EPS produced by the deposited microbial cells [147]. It could say that TMP jump is a result of cake layer formation on membrane surface contributed by the growth and EPS secretion of predominant microbial pioneers. At a constant flux, Gao *et al*. [148] found when MBR become more fouled, indicated by the TMP increase, bacterial consortia in cake layers become more diverse. Moreover, different flux operation was found to affect bacterial diversity and EPS content in cake layers [149, 150]. In laboratory-based membrane fouling studies, constant TMP operation is usually applied [146]; however, effects of the fixed TMP on the development of cake layer have not investigated yet. In this chapter, a lab-scale MBR was operated under different filtration conditions: a constant flux operation, followed by a constant TMP operation to investigate effect of filtration condition on microbial community in bulk sludge and cake layer.

6.2. Materials and Methods

A lab-scale The MBR was operated under two stages: Stage 1 was under a constant permeate flux of 9-11 L/m^2/h for three months, followed by Stage 2 of a constant TMP operation of 80 kPa for next three months. Operational parameters were summarized in Table 6.1. Sludge properties including VS, SS, DOC, etc. was analyzed via methods described in 3.1.1. EPS from bulk sludge and cake layer was fractionated as those in Chapter 5. EPS content and protein, polysaccharide concentrations were determined via methods described in 3.1.2.

Bacterial communities in bulk sludge and cake layer were analyzed as the workflow in section 3.1.3, with details as below. DNA from bulk sludge and cake layer was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA), which was used for DNA template for the 1st PCR to amplify V3-V4 regions of 16S rRNA genes. The 2nd-PCR and NGS sequencing via Illumina Miseq platform based on paired-end reading were conducted at Hokkaido System Science Co. Ltd. Sequences of adaptors-primers and PCR conditions for two PCRs were showed in Table 6.2. Raw sequencing data were trimmed by using Trimmomatic (v0.3.3) to remove sequences with low quality reads. FLASH (v1.2.11) was used to make paired-end library. Chimeric sequences in the paired-end library was removed via USEARCH 6.1 function in QIIME pipeline (v1.9.1) prior to conduct “de novo OTU picking and diversity analyses” (http://qiime.org/tutorials/tutorial.html). The analysis in QIIME pipeline was conducted based on reference database of Greengenes library (97 % identity). The number of effective reads per sample used for OTU picking was shown in Table 6.3. Phylogenetic trees of abundant OTUs and reference QS, QQ bacteria was conducted under MEGA6

(version #6140226) with neighbor-joining method. Statistical analysis including clustering analysis, canonical correspondence analysis (CCA) were conducted via Rstudio (v3.4.1) employed Vegan-package (v2.4-0).

Table 6.1. Operational condition under two stages

Operational condition	**Stage 1**	**Stage 2**
Flux	9-11 LMH	-
TMP	-	80 kPa
Working volume	2 L	2 L
Membrane area	152 cm^2	152 cm^2
MLSS	5 - 7 g/L	5 - 7 g/L
Filtration/relaxation	4 min/ 1 min	4 min/ 1 min
HRT	11-13 h	-
SRT	28 days	28 days
Physical cleaning	weekly	weekly
Temperature	18-22 ^oC	18-22 ^oC
Organic loading rate	40-60 mg TOC/g·day	40-60 mg TOC/g·day
TOC influent	120-160 mg/L	120-160 mg/L

Table 6.2. Primer sequences and PCR conditions

Overhang and primer sequences for 1st-PCR [151]:
Forward overhang adaptor: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3'
Reverse overhang adaptor: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3'
Forward primer (341F): 5'-CCTACGGGNGGCWGCAG-3'
Reverse primer (785R): 5'-GACTACHVGGGTATCTAATCC-3'
Conditions for 1st-PCR:
95°C for 3 min
25 cycles of:
98°C for 20 seconds
60°C for 15 seconds
72°C for 45 seconds
72 °C for 5 min
Hold at 4 °C
Primer sequences for 2nd-PCR:
Forward primer: 5'-CTGTCTCTTATACACATCTGACGCTGCCGACGA-3'
Reverse primer: 5'-CTGTCTCTTATACACATCTCCGAGCCCACGAGAC-3'
Conditions for 2nd-PCR:
95°C for 3 minutes
8 cycles of:
95°C for 30 seconds
55°C for 30 seconds
72°C for 30 seconds
72°C for 5 minutes
Hold at 4°C

Table 6.3. Number of effective sequence reads per each sample after trimming.

	Stage 1				**Stage 2**			
Sampling date	07	35	56	77	104	125	153	183
Sample name	# 1.1	# 1.2	# 1.3	# 1.4	# 2.1	# 2.2	# 2.3	# 2.4
Bulk sludge (B)	4,099	12,507	7,780	3,436	12,532	8,259	6,318	2,898
Cake layer (C)	4,637	2,881	2,608	1,920	6,182	6,682	9,554	8,862

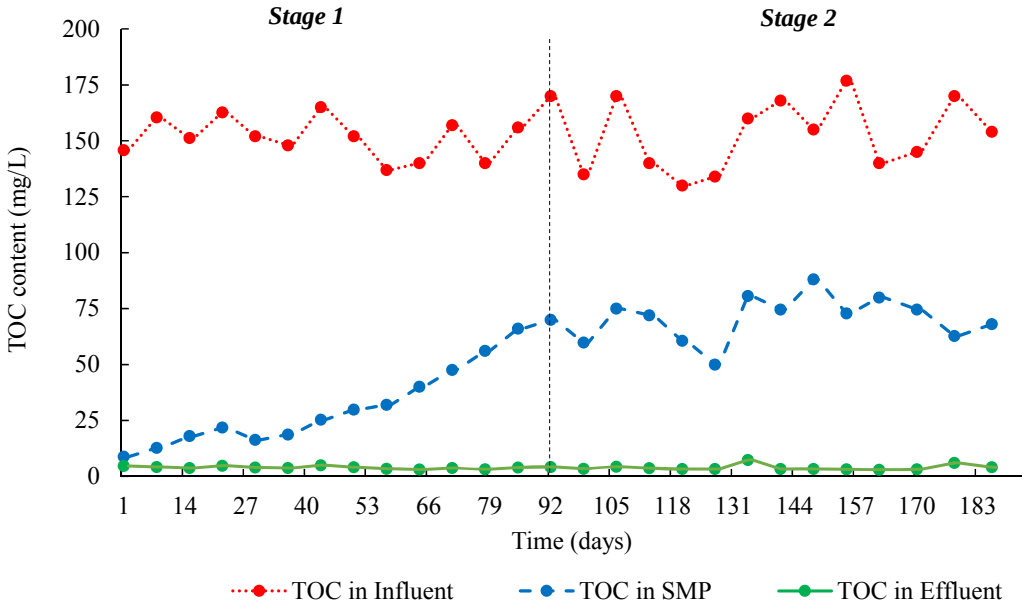
6.3. Results and Discussion

6.3.1. MBR performances

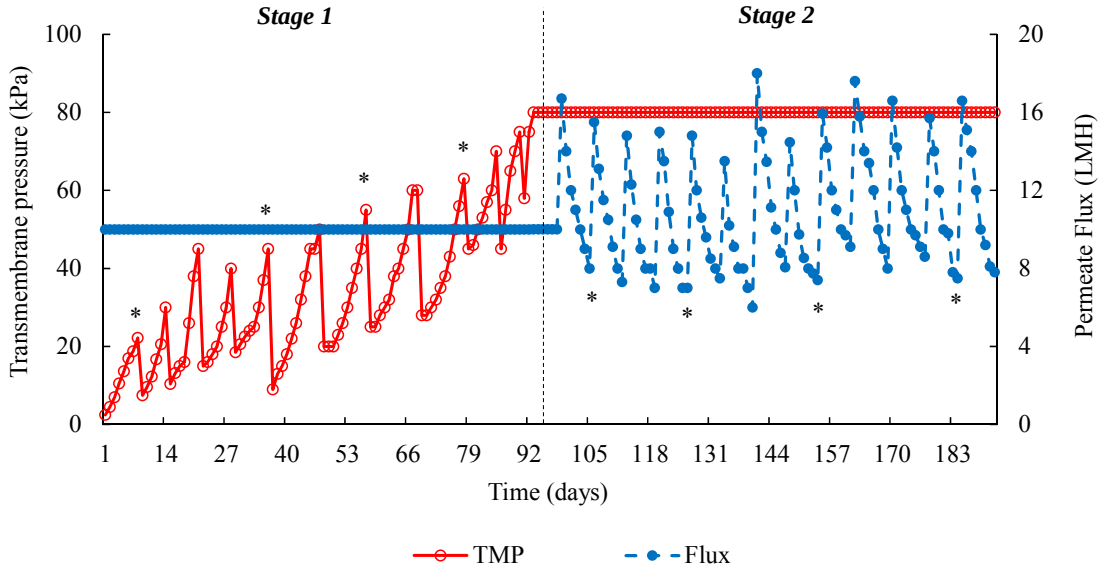
Operated MBR achieved a high and stable efficiency to remove organic matters, eliminating 92-98 % TOC of influent. Average TOC content in permeate effluent of Stage 1 – constant flux was 4.0 ± 0.9 (SD) mg/L, which was comparable with that of Stage 2 – constant TMP (Figure 6.1-A).

During operation period, Stage 1 obtained a permeability of 0.27 ± 0.08 LMH per kPa under an averaged TMP of 40 ± 17 (SD) kPa (Figure 6.1-B) while Stage 2 exhibited a permeability of 0.18 ± 0.02 LMH per kPa under an average flux of 10.8 ± 2.9 LMH. This finding revealed that permeability of Stage 1 was statistically higher than that of Stage 2; therefore, membrane fouling in Stage 1 was less severe than in Stage 2. Within a filtration circle, TMP of Stage 1 obtained a rise of 30 ± 6 kPa after 7 days of filtration, while flux of Stage 2 dropped from 14.6 to 7.2 LMH (Figure 6.1-B). In addition, TMP of Stage 1 increased slowly in the first days then increased sharply in the next days, whereas permeate flux of Stage 2 dropped rapidly then slightly. A similar profile of TMP was found in the study of Arabi and Nakhla [34], in which MBRs are also operated under constant fluxes.

A. TOC removal profile



B. Transmembrane pressure and Flux profile



(*) sampling points for bacterial community analysis

Figure 6.1. TOC removal profile (A), transmembrane pressure and flux profiles (B) of Stage 1 – constant flux of 9-11 LMH and Stage 2 – constant TMP of 80 kPa. The data in every plot represent the average value of a whole day.

A gradual increase of SMP from 5 to 70 mg/L (Figure 6.1-A) possibly caused the TMP development during the operation of Stage 1 since SMP concentration reportedly have a close correlation to membrane fouling rate [58]. Fouling in Stage 2 occurred earlier than that in Stage 1 (Figure 6.1-B) because of its high level of initial flux [146] and a high content of SMP at 60–80 mg/L (Figure 6.1-A). In Stage 2, physical cleaning recovered almost total permeability; however, permeate flux quickly declined by the attachment of soluble foulants (e.g., SMP) on membrane surface under very high fluxes [152] caused by the constant TMP mode. The findings indicated that MBR fouling in a constant TMP operation possibly happened earlier and more seriously than in a constant flux one. Besides, the two stages exhibited different tendencies of SMP quantity (Figure 6.1-A), which may result from a variation in the structure and behavior of bacterial community under the two operational modes

6.3.2. Bacterial structure in bulk sludge

After acclimation period, bacterial structure in bulk sludge at Stage 1 significantly shifted from that of seeding sludge (Figure 6.2-A and 6.2-B). Relative abundances of class *Gammaproteobacteria* increased, meanwhile those of *Planctomycetia*, and *Alphaproteobacteria* decreased. *Gammaproteobacteria* reportedly have an increase in their abundance after an acclimation [153]. They are also able to produce more EPS than others bacteria [154]. In addition, Stage 1 witnessed a growth in the relative abundance of *Comamonadaceae*, *Xanthomonadaceae* and *Chitinophagaceae,* who became the most predominant families in bulk sludge (Figure 6.3-A). Some genus of *Comamonadaceae* and *Chitinophagaceae* can be vectors for distinguishing between aerobic and anaerobic MBRs [135], while *Xanthomonadaceae* are one of core phenotypes in biological nutrient removal process [155].

Stage 2 exhibited higher contributions of classes of *Proteobacteria* in bacterial community of bulk sludge than Stage 1 (Figure 6.2-B and 6.2-C). In fact, relative abundance of *Betaproteobacteria* increased from 24.5 to 35% while Grammaproteobacteria slightly increase. At family level, *Comamonadaceae*, *Xanthomonadaceae* were two of the most abundant families in bulk sludge as Stage 1. However, contribution in the community from 22 to 32% for *Comamonadaceae* and from 17 to 22% for *Xanthomonadaceae* (Figure 6.3-A). These findings revealed that the constant TMP operation (Stage 2) had certain influences on structure of bacterial community. The constant TMP operation was found to cause too high and too low fluxes, resulting in a fluctuation in nutrient loading ratio. The nutrient loading ratio reportedly cause changes in structures of microbial community and sludge floc [17].

Bacterial community in bulk sludge was comprised of *Proteobateria*, *Bacteroidetes*, and others; 63, 18, and 19 %, respectively. That structure was fairly consistent with those in previous studies [153, 31], but inconsistent with the investigation of Choi *et al*. [156], in which *Bacteroidetes* are the most abundant. The structure of bacterial community is reportedly affected by several factors, including stress condition [15], MLSS concentration [145], influent composition [157] and filtration condition (this chapter). Top three abundant families in bulk sludge were *Comamonadaceae, Xanthomonadaceae* and *Chitinophagaceae* (Figure 6.3-A), which are frequently found in bulk sludge of MBRs treating domestic wastewater [158, 15, 159].

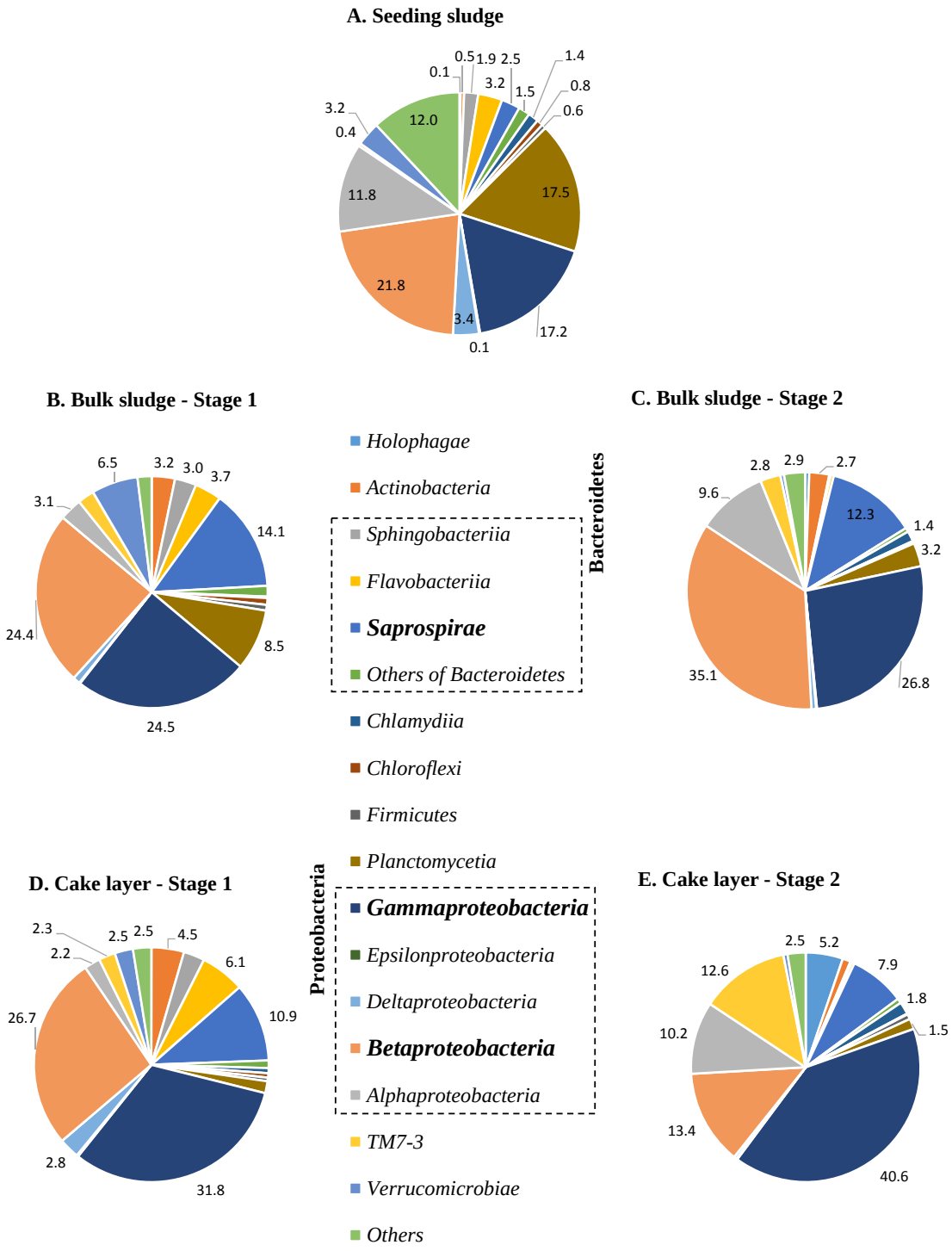


Figure 6.2. Community structure at class level of bulk sludge and cake layer at Stage 1 – constant flux of 9-11 LMH and Stage 2 – constant TMP of 80 kPa. Data of bulk sludge and cake layer represent average values of n=4.

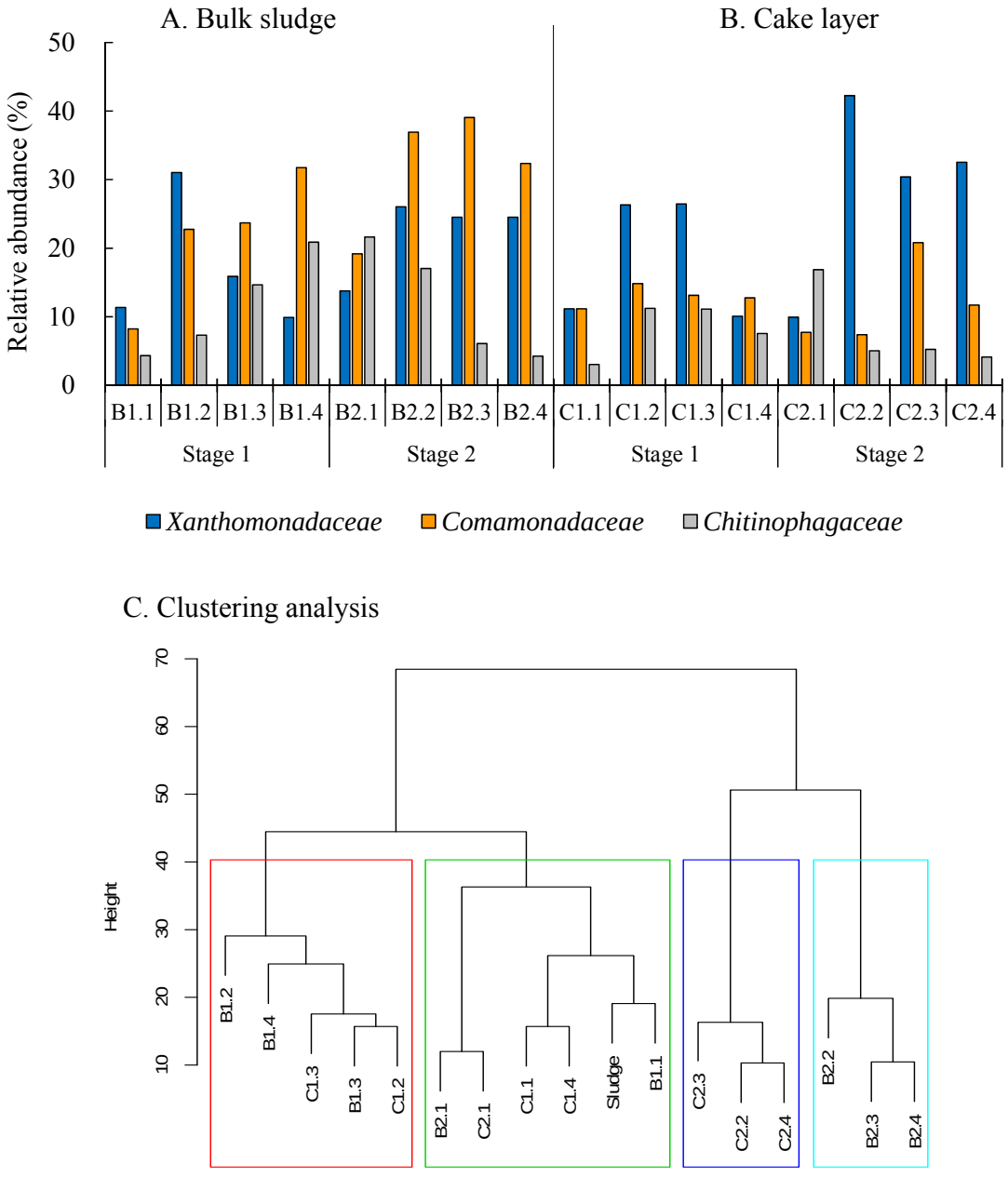


Figure 6.3. Profile of the top three abundant families in (A) bulk sludge and (B) cake layer. (C) Hierarchical cluster analysis conducted by based on bacterial structure at genus level under Ward's method. B1.1-4 and C1.1-4 stand for bulk sludge and cake layer in Stage 1 sampled in date 7, 35, 56, and 77. B2.1-4 and C2.1-4 stand for bulk sludge and cake layer in Stage 2 sampled in date 104, 125, 153, and 183.

6.3.3. Bacterial structure in cake layer

Bacterial structure in cake layer of Stage 1 was fairly comparable to that of bulk sludge, largely contributed by *Betaproteobacteria* and *Gammaproteobacteria.* However, major differences were that cake layer contained more *Gammaproteobacteria*, and less *Planctomycetia*, *Verrucomicrobiae* than bulk sludge (Figure 6.2-B and 6.2-D). This finding was consistent with previous studies [149, 143, 153, 31]. *Gammaproteobacteria* reportedly prefer to adhere to membrane surface [153, 30], and produce more EPS than others bacteria [154]. In addition, isolates of *Gamaproteobacteria* (e.g., *Xanthomonadaceae*) can secrete EPS with high level of filtration resistance [142]. Moreover, at genus level, some genera of *Xanthomonadaceae* (e.g., *Rhodanobacter*) were highly abundant in cake layer but less in bulk sludge (Figure 6.4); therefore, these bacteria exhibited a higher potential of membrane adhesion than other bacteria. These findings revealed that *Xanthomonadaceae* bacteria possibly had a major contribution to cake layer formation and membrane fouling at Stage 1.

Under Stage 2, cake layer contained more *Gammaproteobacteria*, but less *Betaproteobacteria, Saprospirae* than bulk sludge did (Figure 6.2-C and 6.2-E). Moreover, in cake layer *Gammaproteobacteria* were more predominant than *Betaproteobacteria*, over 40 % compared to 13.4 % in total abundance (Figure 6.2-C). Furthermore, *Xanthomonadaceae* and *Comamonadaceae* were still the most abundant families in cake layers, with average contribution at 29 % and 12 % in total respectively (Figure 6.3-C). However, *Xanthomonadaceae* were more abundant in cake layer than in bulk sludge, meanwhile *Comamonadaceae* were only abundant in bulk sludge (Figure 6.3-B). These findings revealed that some genera of *Xanthomonadaceae* (e.g.,

Rhodanobacter) possibly tend to make their biofilm on membrane surface (Figure 6.4) while *Comamonadaceae* may prefer to live individually or in aggregates of bulk sludge.

Distinction in bacterial structure between cake layer and bulk sludge under constant TMP operation became more significant than under the constant flux (Figure 6.3-C). Under the constant flux operation, bacterial structures of bulk sludge and cake layer were similar (Figure 6.2-B and 6.2-D). However, under the constant TMP operation, bacterial structure of cake layer was significantly different from that of bulk sludge (Figure 6.2-C and 6.2-E). These findings indicated that operation have effects on evolution of bacterial community in bulk sludge and initial development of bacterial community on membrane surface.

Interestingly, under both operation modes, *Xanthomonadaceae* were the most abundant in cake layer, meanwhile *Comamonadaceae* were the most abundant in bulk sludge (Figure 6.3-A and 6.3-B). The phenomenon of *Xanthomonadaceae* attachment on membrane surface may related to surface property of bacterial cells [142]. This phenomenon possibly related to collective behaviors of these bacteria, including biofilm formation on membrane surface. Biofilm formation causing membrane fouling in MBR is reported to a positive relation with AHL signal of microbial QS process [19, 21]. Some genera of *Xanthomonadaceae* (e.g., *Stenotrophomonas, Lyzobacter*) and of *Comamonadaceae* (e.g., *Acidovorax, Variovorax, Delftia*) have been known to produce AHL as quorum sensing bacteria [160, 161]. However, in some study these genera are also considered as quorum quenching bacteria due to its AHL degradation [162]. Two AHL signals were found to be present in the MBR under Stage 1. The signals included C4-HSL, C6-HSL, C8-HSL, etc. (Table 6.4). Detected type and concentration of AHLs were various and different among two stages. It may be because of difference of bacterial structures. Moreover, C6-HSL

and C8-HSL were not detected in Stage 2. A possible reason is degradation via AHL degrading bacteria. This finding was not mean that C6-HSL and C8-HSL-based QS process in MBR under Stage 2 did not occurred or lower than that under Stage 1. However, it could say that QS activity occurred in the MBR under two stages and collective behaviors regulated by the QS activity possibly impacted on membrane fouling as previous studies [19].

Family [Order]	Genera	Bulk sludge								Cake layers							
		Stage 1				Stage 2				Stage 1				Stage 2			
		date 07	*date 35*	*date 56*	*date 77*	*date 104*	*date 125*	*date 153*	*date 183*	*date 07*	*date 35*	*date 56*	*date 77*	*date 104*	*date 125*	*date 153*	*date 183*
Holophagaceae	Unclassified	0.1	0.0	0.0	0.0	0.0	0.2	1.1	1.0	0.1	0.0	0.0	0.0	4.6	6.9	4.6	4.8
Intrasporangiaceae	Unclassified	1.0	1.2	3.5	1.0	0.0	0.1	0.3	1.4	1.1	4.8	9.3	0.7	0.1	0.1	0.4	0.2
[Saprospirales]	Unclassified	0.8	1.2	6.1	1.2	0.0	0.0	0.0	0.0	0.3	2.7	7.2	0.3	0.0	0.0	0.1	0.0
Chitinophagaceae	Unclassified	3.5	5.6	14.0	20.7	21.6	17.0	6.1	4.2	2.7	9.9	10.9	7.6	16.9	5.0	5.2	4.1
Cryomorphaceae	Unclassified	0.0	2.0	6.0	3.4	0.0	0.0	0.0	0.4	0.0	5.0	4.3	0.5	0.0	0.0	0.0	0.0
Flavobacteriaceae	*Flavobacterium*	0.2	0.2	0.1	0.1	0.0	0.0	0.1	0.0	4.1	0.4	0.2	0.5	0.1	0.0	0.0	0.4
Gemmataceae	*Gemmata*	13.5	3.4	0.6	0.2	0.2	0.0	0.0	0.0	0.6	0.9	0.2	0.0	0.1	0.0	0.0	0.0
Isosphaeraceae	Unclassified	1.2	0.9	2.7	4.0	5.2	0.7	3.3	2.3	0.1	1.0	1.0	0.2	0.4	0.2	1.3	2.0
Planctomycetaceae	*Planctomyces*	2.3	1.1	0.8	0.2	0.1	0.0	0.7	0.1	0.2	1.2	0.1	0.1	0.1	0.1	1.2	0.4
Caulobacteraceae	*Phenylobacterium*	0.0	0.0	0.0	0.0	0.4	1.3	1.5	5.3	0.0	0.0	0.0	0.0	0.6	1.3	1.5	0.9
[Ellin329]	Unclassified	0.2	0.2	0.3	0.2	1.0	1.0	2.9	2.5	0.8	0.3	0.1	0.2	1.6	3.8	5.1	3.9
Rhodobacteraceae	Unclassified	0.2	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.3	0.1	0.0	0.0	0.0	0.0	0.0	0.0
[Rickettsiales]	Unclassified	0.5	0.0	0.0	0.0	1.0	3.3	2.2	0.7	1.1	0.3	0.1	0.1	2.0	4.9	2.2	4.3
Sphingomonadaceae	*Novosphingobium*	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Comamonadaceae	Unclassified	4.7	16.6	9.8	15.1	0.4	0.4	0.3	0.1	2.5	11.2	8.3	1.9	0.3	0.0	0.0	0.7
Comamonadaceae	*Comamonas*	1.0	0.4	0.5	2.2	1.8	3.8	1.1	0.6	7.9	0.4	0.6	3.5	1.0	2.0	1.9	2.8
Comamonadaceae	*Hydrogenophaga*	0.1	0.1	0.2	0.2	9.2	7.2	6.8	3.4	0.0	0.1	0.2	0.1	4.2	1.5	3.1	1.3
Comamonadaceae	*Hylemonella*	0.1	4.3	11.9	13.3	0.6	0.1	0.0	0.1	0.1	2.7	3.5	1.0	0.6	0.0	0.0	0.2
Comamonadaceae	*Roseateles*	0.4	0.1	0.3	0.2	6.3	25.3	30.6	28.2	0.1	0.1	0.1	0.1	1.6	3.8	15.7	6.7
Oxalobacteraceae	Unclassified	0.3	0.0	0.0	0.0	0.1	0.1	0.1	0.1	2.6	0.1	0.0	0.0	0.0	0.0	0.0	0.0
Procabacteriaceae	Unclassified	0.1	0.0	0.6	0.7	0.1	0.0	0.1	0.2	6.3	0.1	1.6	10.5	0.0	0.0	0.1	0.4
Aeromonadaceae	Unclassified	1.8	2.6	3.1	0.8	0.9	0.1	0.2	0.1	5.8	0.9	4.3	1.8	0.6	0.3	0.7	0.9
Legionellaceae	*Tatlockia*	0.0	0.0	0.1	0.0	3.1	1.9	1.7	0.3	0.1	0.0	0.1	0.2	0.5	10.3	4.2	5.9
Moraxellaceae	*Acinetobacter*	0.8	0.3	0.4	0.5	0.2	0.0	0.1	0.1	1.6	0.4	0.4	10.1	0.0	0.0	0.1	0.0
Pseudomonadaceae	*Pseudomonas*	0.1	4.6	7.2	0.6	2.2	0.5	2.0	1.2	0.2	0.6	5.6	5.2	0.8	6.9	6.0	6.7
Thiotrichaceae	*Thiothrix*	2.7	0.1	0.0	0.0	0.0	0.0	0.0	0.1	3.9	0.1	0.0	0.1	0.1	0.1	0.1	0.0
Xanthomonadaceae	Unclassified	10.0	29.4	12.1	8.3	10.7	23.8	18.6	12.8	4.7	16.5	8.0	2.6	6.7	11.8	11.5	10.0
Xanthomonadaceae	*Rhodanobacter*	0.7	1.5	3.6	1.2	2.5	2.1	5.7	11.4	1.1	9.5	17.4	2.9	3.2	30.2	18.7	22.3
[TM7-3]	Unclassified	7.3	1.1	0.4	0.4	10.3	0.2	0.3	0.2	5.7	1.5	0.5	0.7	12.0	3.1	1.0	1.5
Verrucomicrobiaceae	Unclassified	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Verrucomicrobiaceae	*Prosthecobacter*	8.5	6.2	0.3	10.9	1.1	0.0	0.8	0.1	2.9	4.9	0.5	1.7	0.4	0.0	1.9	0.0

Figure 6.4. Relative abundance of dominant genera in bulk sludge, cake layer of 2 stages.

Table 6.4. Concentration of AHL signals in bulk sludge of two stages [ng/L].

Type of AHLs	C4-HSL	C6-HSL	C8-HSL	3O-C10-HSL	3OH-C10-HSL
Stage 1	250	2.5	54	Not detected	12.3
Stage 2	40	Not detected	Not detected	13.8	22.4

6.3.4. EPS of bulk sludge and cake layer

Cake layer contained more LB-EPS than TB-EPS. In contrast, bulk sludge contained less LB-EPS than TB-EPS (Figure 6.5-A). These findings were consistent with previous studies [163, 14]. The content of LB-EPS in cake layer was much higher than that in bulk sludge, meanwhile the content of TB-EPS in cake layer was comparable with that in bulk sludge. EPS in cake layer possibly was not only contributed from bulk sludge from but also directly secreted from pioneer bacteria on membrane surface. Furthermore, a high content of SMP was found in the MBR supernatant (Figure 6.5-A). SMP can cause pore clogging and gel layer formation [17], increasing membrane filtration resistance and TMP (Figure 6.1). SMP can be contributed from EPS hydrolysis and/ or adsorbed into EPS matrix of bulk sludge, closely related to flocculation ability and stability of bulk sludge [8].

Polysaccharides were overwhelming in LB-EPS of cake layer while proteins were predominant in LB- and TB-EPS of bulk sludge (Figure 6.5-B and 6.5-C). These findings indicated that polysaccharides were major EPS-related foulants. PS/PN ratios in LB-EPS of cake layer was greater than that of bulk sludge, while PS/PN ratios of TB-EPS in cake layer and bulk sludge were fairly similar (Figure 6.5-D). Finding revealed a difference in characteristics of EPS between cake layer and bulk sludge. In addition, a high concentration of PS and a PS/PN ratio over than 1 were found in SMP and LB-EPS of cake layer (Figure 6.5-B and 6.5-D). It revealed that polysaccharides were highly rejected by MBR membrane, subsequently accumulated in sludge supernatant and greatly adsorbed into membrane surface [5] Controlling EPS components is expected to mitigate membrane fouling because polysaccharides are the primary foulants in MBRs [5] while proteins are important for flocculation ability of sludge [56].

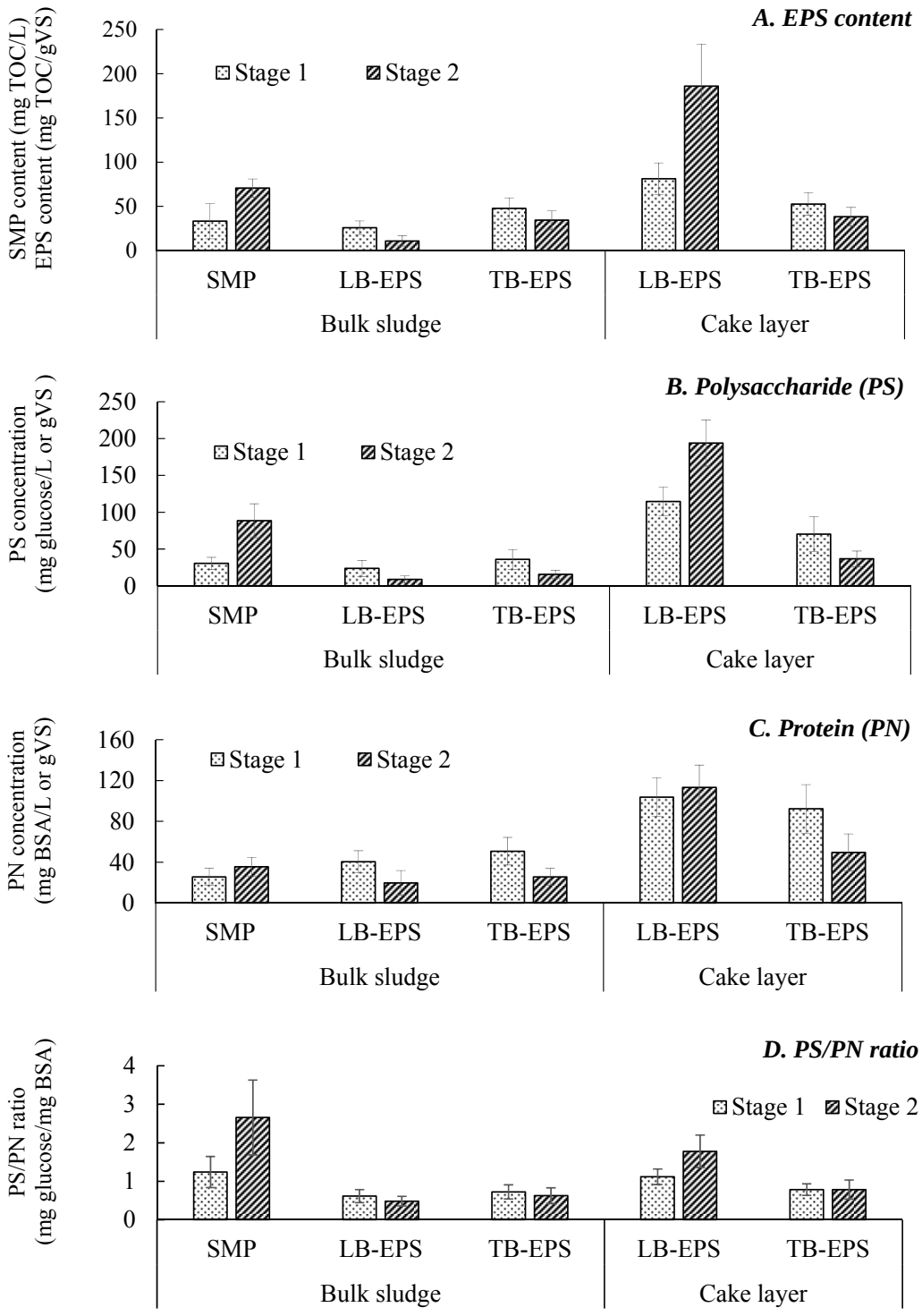


Figure 6.5. (A) EPS content, (B) polysaccharide concentration, (C) protein concentration and (D) ratio of polysaccharides to proteins in bulk sludge and cake layer at Stage 1 – constant flux of 9-11 L/m^2/h and Stage 2 – constant TMP of 80 kPa (n=13).

6.3.5. EPS characteristic under different filtration conditions

EPS content in cake layer at Stage 2 was significantly greater than that at Stage 1 meanwhile EPS content in bulk sludge at Stage 2 was lower than that at Stage 1 (Figure 6.5-A). LB-EPS in cake layer increased from 81 to 186 mg/g VS, whereas LB-EPS in bulk sludge decreased from 26 to 11 mg/g VS. Both TB-EPS in cake layer and TB-EPS in bulk sludge declined from 53 to 38 and 48 to 34 mg/g VS, respectively. Differently, SMP content in sludge supernatant increased from 33 to 71 mg/L. The decrease of TB-EPS in bulk sludge revealed that de-flocculation might occur under Stage 2 since TB-EPS content reportedly encourage the generation of larger flocs [61]. The de-flocculation could make more SMP be released from EPS matrix of bulk sludge, resulting in the greater content of EPS in cake layer.

The constant TMP operation possibly affected the flocculation and stability of sludge flocs. At the first days of a filtration circle, initial flux was much higher than critical flux but very low at the end of filtration cycle (Figure 6.1). Flux changes resulted in a wide range of HRT change from 6 h to 16 h. Short HRT has been reported to cause an increase of SMP and EPS in bulk sludge, and result in an increase of fouling rate [76]. However, too long HRT leads to a low organic loading rate and lack of substrates for microbes in sludge. When substrates are deficient, bacteria in bulk sludge need to decompose their EPS matrix into SMP for their growth. The decomposition of EPS matrix make sludge flocs unstable and de-flocculated. Deflocculated flocs and degraded EPS with low molecular weight also increase fouling rate and irreversible fouling (Chapter 5).

Stage 2 showed significant increases in PS content of SMP and LB-EPS in cake layer (Figure 6.5-B). In contrast, it caused drops in both PS and PN contents of TB-EPS in cake

layer, LB- and TB-EPS in bulk sludge (Figure 6.5-B and 6.5-C). This phenomena made PS/PN ratios of SMP and LB-EPS in cake layer reach higher while those of TB-EPS in cake layer, LB- and TB-EPS in bulk sludge be remained (Figure 6.5-D). The constant TMP operation obtained an effect on SMP components. The constant TMP operation caused de-flocculation, resulting in more polysaccharides and proteins released from EPS matrix. Proteins are highly permeated by MBR membranes while polysaccharides are greatly rejected by the membranes and accumulated in MBR supernatant [5].

SMP fraction contained tryptophan protein-like and humic acid-like substances. LB-, TB-EPS of bulk sludge and cake layer contained one more substance: aromatic proteins (Figure 6.6). In a previous mention, constant TMP (Stage 2) was expected to cause de-flocculation, making more SMP be released from LB-EPS of bulk sludge. This hypothesis was possibly evidenced by an increase of the humic acid-like substances in SMP and a decrease of that in LB-EPS in bulk sludge under Stage 2 (Figure 6.6-A and 6.6-B). As a result of the higher content in SMP, more humic acid-like substances are found in LB-EPS of cake layer (Figure 6.6-C). Higher level of humic acid substances in SMP and LB-EPS of cake layer could related to severe fouling under the constant TMP operation since these substances reportedly are potentially irreversible foulants (Chapter 5), and encourage membrane attachment of other foulants [33]. Stage 2 made peak location of humic acid-like substances in TB-EPS of cake layer and bulk sludge shifted from Ex/Em of 340/420 nm into 320/405 nm and 395/485 nm (Figure 6.6-D and 6.6-E). This location shift might be caused by changes in functional groups of humic acid-like substances [164]. The increases of function groups of the humic acid-like substances in TB-EPS of cake layer and bulk sludge was expectedly resulted from bio-decomposition of EPS matrix for microbial growth under too long HRT as previous mention.

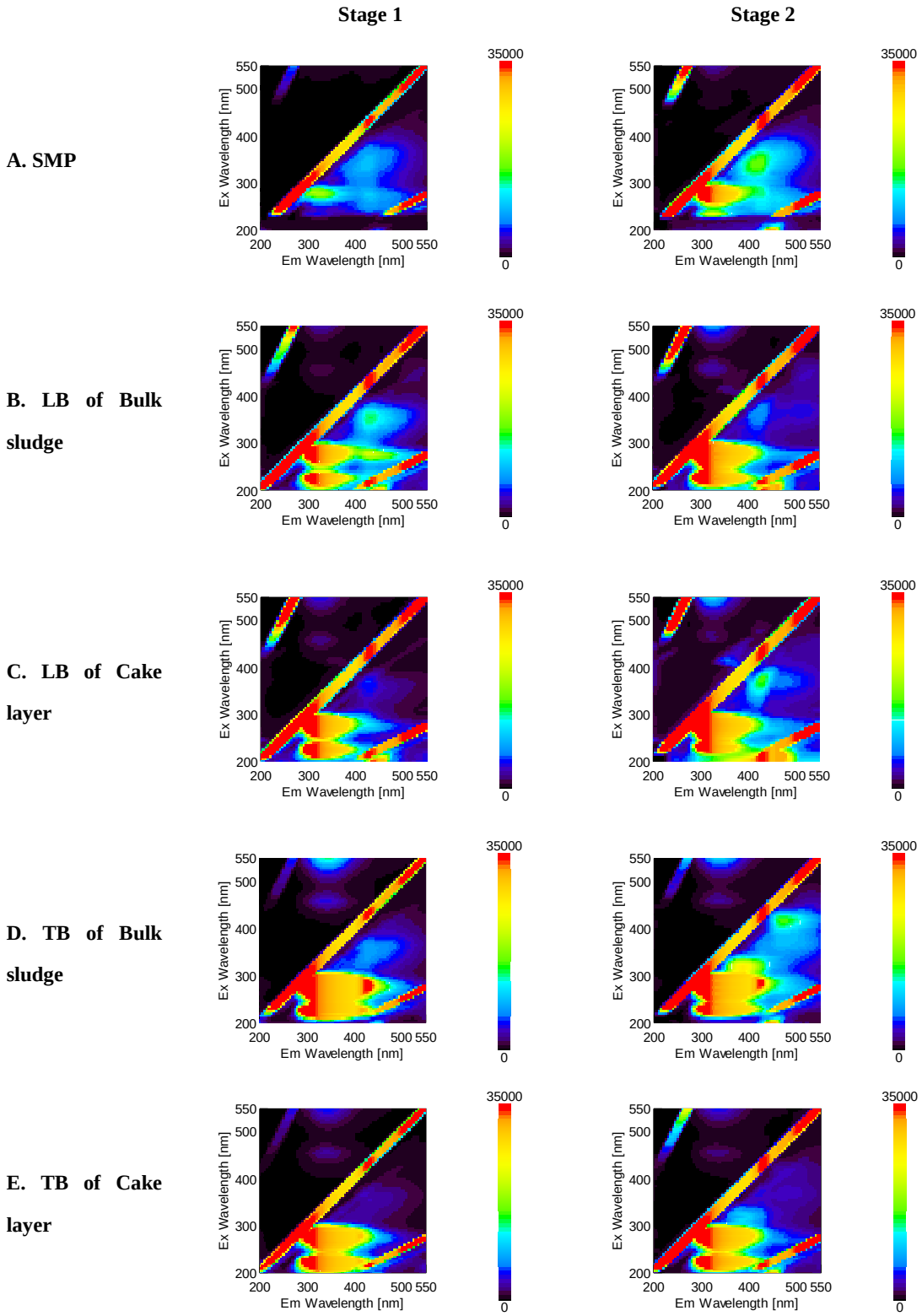


Figure 6.6. Three-dimensional excitation-emission matrix fluorescence spectra of SMP and EPS fractions from bulk sludge and cake layers

6.3.6. Correlation between bacterial community and EPS

Under the constant flux operation (Stage 1), most of abundant OTUs in bulk sludge (B1.1-4) including OTU_2, 11, 15 and 602 were also found to be abundant in cake layer (C1.1-4) (Figure 6.7). Differently, at the constant TMP operation (Stage 2), OTUs_6, 26, 29, 37, and 353 were abundant in only cake layer (C2.1-4) while OTUs_3, 4, 5, 8, and 30 were predominant in bulk sludge (B2.1-4). The finding suggested that operation mode may have an impact on the evolution of bacterial community in MBR sludge, and the structure of new bacterial consortia on MBR membrane surface.

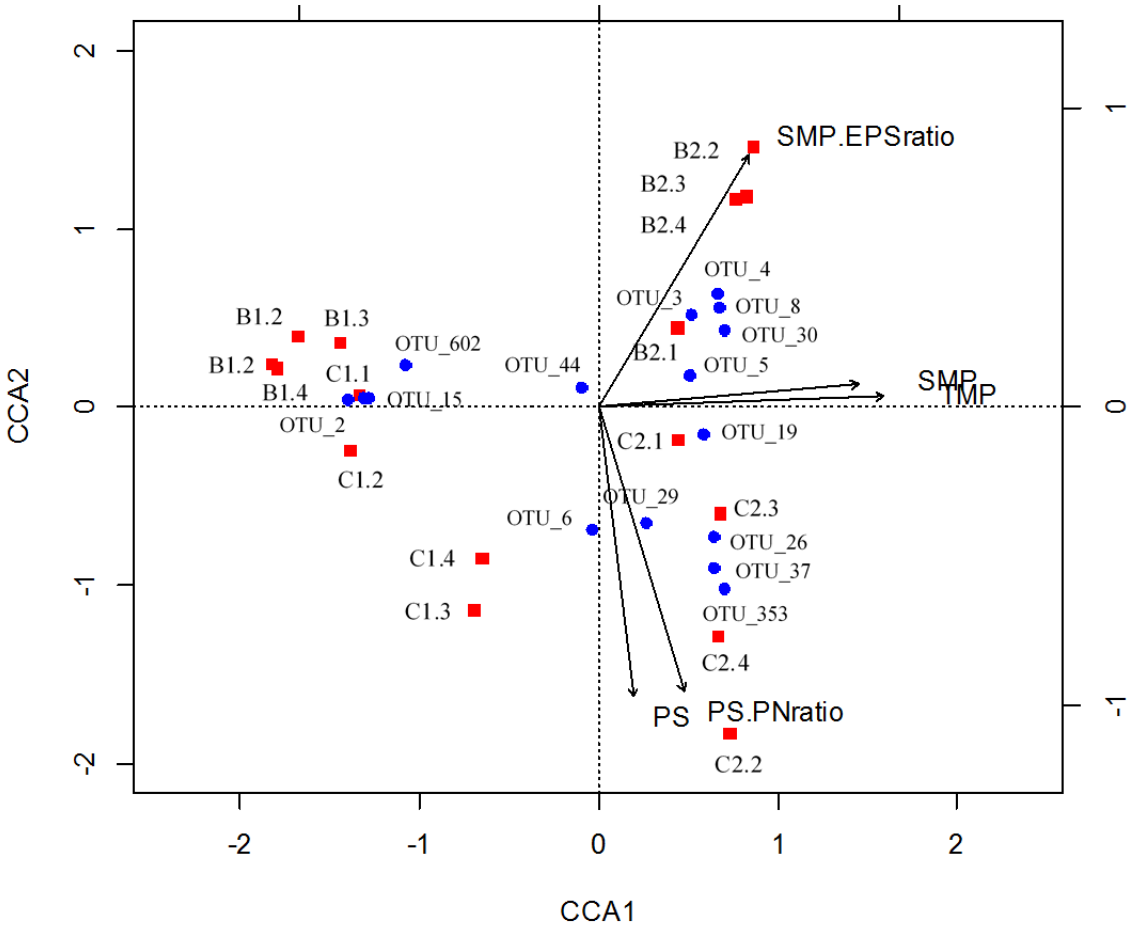


Figure 6.7. (A) Canonical Correspondence analysis (CCA) of the most OTUs in relation to EPS production and operation parameters.

(CCA1: 45.7 %; CCA2: 18.8 %; $p < 0.05$; B1.1-4 and C1.1-4 stand for bulk sludge and cake layer in Stage 1 sampled in date 7, 35, 56, and 77. B2.1-4 and C2.1-4 stand for bulk sludge and cake layer in Stage 2 sampled in date 104, 125, 153, and 183).

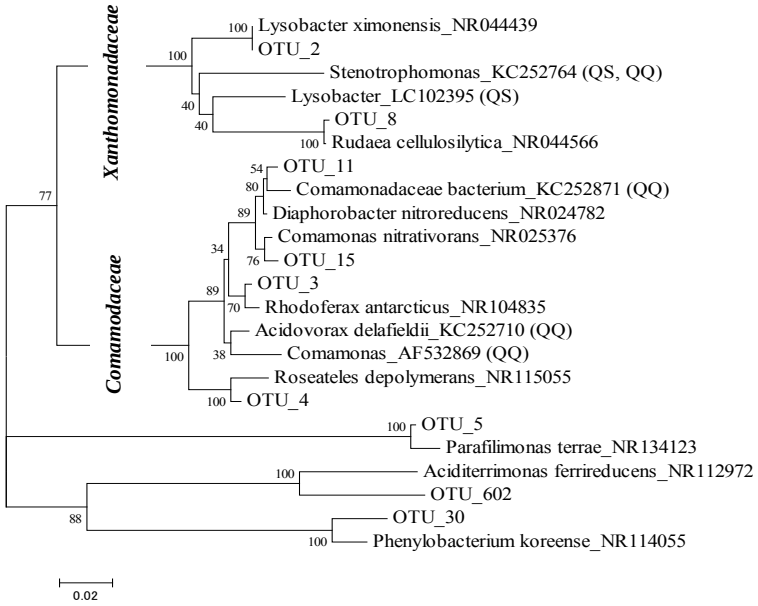
Vector of SMP and EPS content was close to the abundant OTUs in bulk sludge while vector of EPS composition (PS and PN) closely related to the abundant OTUs in cake layer (Figure 6.7). This phenomena revealed that the content and composition of SMP and EPS in MBRs have close relation with specific bacterial genera [15, 165]. However, membrane fouling represented by vectors of TMP increase and flux decrease, equivalent to Stage1 and Stage 2 was found to be not clearly correlated to neither abundant OTUs in bulk sludge nor cake layer (Figure 6.7).

The abundant OTUs in bulk sludge owning positive correlation with SMP and EPS content (Figure 6.7) were phylogenetically close to genera of *Comamonadaceae* and *Xanthomonadaceae* (Figure 6.8-A). OTUs_4, 11 and 15, for example, were close to depolymerizing, quorum quenching and denitrifying bacteria of *Comamonadaceae*, respectively. OTUs_2 and 8 were close to genera *Lysobacter* and *Rudaea*, whose are known to have ability to carbohydrate utilization. In the MBR of this chapter, these predominant bacteria might play an important role in organic matter removal.

Interestingly, the abundant OTUs in cake layer with clear correlation to EPS composition mainly belonged to *Xanthomonadaceae* (Figure 6.8-B). Especially, OTUs_6, 44 and 353 were closest to *Rhodanobacter*, being known as denitrification bacteria in activated sludge and biofilm of an autotrophic denitrification process. It seems that *Rhodanobacter* in the MBR exhibited higher potential to be adsorbed onto membrane surface than others such as *Comamonadaceae* bacteria. *Rhodanobacter* are also reportedly found to have positive correlation to EPS production and membrane fouling in saline-stress and Ca(II)-stress MBRs [15, 165]. It was clear that the content and composition of EPS obtained positive correlation with some specific bacteria in bulk sludge and cake layer under

constant TMP operation. However, bacterial community in MBR is diverse and affected by several operation parameters. For this reason, future investigation should focus more on the sludge or bio-cake microbiota and pure-cultured strains from MBR sludge in order to achieve deeper information on fouling mechanisms [5].

A. Phylogenetic tree of OTUs in bulk sludge



B. Phylogenetic tree of OTUs in cake layer

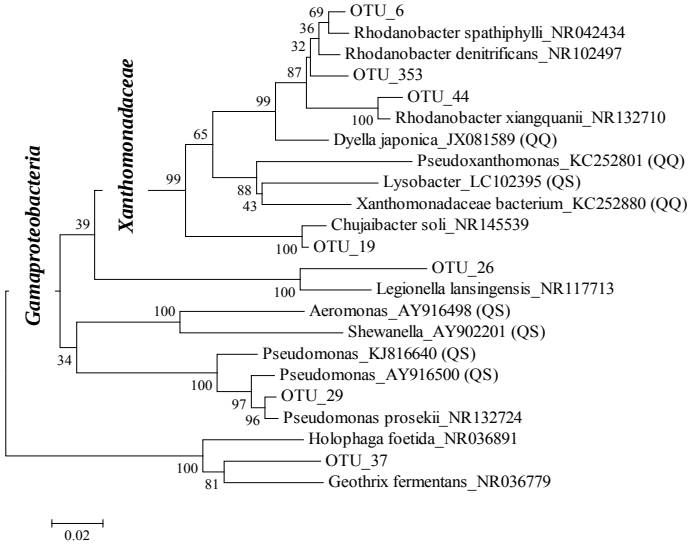


Figure 6.8. Phylogenetic tree of the most abundant OTUs in bulk sludge and cake layer (Reference quorum sensing, quorum quenching bacteria from previous studies [161,

94].

6.4. Conclusions

Bacterial structures in bulk sludge and cake layer (or biofilm) in MBR significantly change under changing from constant flux operation to constant TMP operation. A constant operation of high TMP enhances discrepancy in bacterial structure between cake layer and bulk sludge.

Changing from constant flux to constant TMP causes reduction of polysaccharide and protein components in LB- and TB-EPS of bulk sludge and TB-EPS of cake layer However, it causes an significant increase of polysaccharide content in SMP and LB-EPS of cake layer.

Abundant OTUs phylogenetically close to bacteria which involve biofilm, quorum sensing and quorum quenching bacteria. They have correlation to polysaccharide content and EPS production. The changing filtration condition affects bacterial community structure and further possibly influences bacterial behaviors (e.g., EPS production, biofilm formation), causing membrane fouling.

Comamonadaceae, Xanthomonadaceae, and *Chitinophagaceae* are dominant in MBR bulk sludge but only *Xanthomonadaceae* are highly abundant in cake layer on membrane surface. Besides, quorum sensing signals (e.g., C4-HSL and C6-HSL) and QS bacteria were detected in bulk sludge of the MBR, which can be a primary condition to investigate fouling control via QS inhibition.

Chapter 7. Effect of Vanillin on Regulation of Quorum Sensing and Membrane Fouling

7.1. Introduction

The findings in Chapter 6 showed that some bacteria were greatly abundant in cake layer while other were abundant in bulk sludge of MBR. These bacteria included *Comamonadaceae* and *Xanthomonadaceae*, which were known as AHL related-QQ, QS bacteria, respectively. Their behaviors regulated via QS process (e.g., EPS production, biofilm formation) were possibly affected membrane fouling in a MBR. Especially, *Xanthomonadaceae* are reported as EPS producers [166]. Besides, cake layer formation on membrane surface causes a sharp increase of TMP in MBRs, which has been known as a result of biofilm formation by microbial growth.

Recently, presence of AHL signals in MBRs has been reported to have a link with biofilm formation and membrane biofouling [19, 93, 21]. The finding encourage several attempts to control membrane fouling by disruption AHL-based QS process in MBRs. QS inhibition (or quorum quenching) via the application of enzymes [89], quorum quenching bacteria [94] and natural compounds (e.g., vanillin) [18] revealed that this strategy is promising for membrane fouling control.

Among natural compounds, vanillin known as food flavoring agent is more referred to apply as QS inhibitor than furanone because of its cheapness and safety. In 2006, extract from vanilla beans was found to act as a QS inhibitor [167]. Since then, inhibition of quorum sensing mechanism by vanillin was investigated [86], in which vanillin was found to inhibit C4-HSL, C6-HSL, C8-and 3-oxo-C8-HSL. Vanillin addition is reported to significantly lessen membrane fouling in RO MBRs [101, 168]. Effect of vanillin on biofilm formation via single culture as *Aeromonas hydrophila* and via multi-species

such as species of *Comamonas, Enterobacteriaceae, Pseudomonas, Stenotrophomonas*, etc. have been investigated [86, 20].

Effect of different doses of vanillin on biofilm formation have been investigated. For instance, Ponnusamy *et al*. [86] found that increasing vanillin doses from 62.5, 125 to 250 mg/L caused a gradual increase in deduction of biofilm formation via *Aeromonas hydrophila*. However, Nam *et al.* [18] found that a dose of 375 mg/L vanillin showed a lower reduction in protein component of EPS than doses of 125 and 250 mg/L. They also found that feeding 250 mg/L vanillin made a reduction of MBR membrane fouling, which took 21 days to reach 40 kPa compared with 3 days in the control one. The dose of 250 mg/L vanillin reduce 16 % EPS in bulk sludge and 30 % EPS in cake layer. These findings revealed that vanillin is able to inhibition QS process and reduce EPS production in MBRs, further to reduce membrane fouling development. However, effects of vanillin on key EPS components causing membrane fouling (e.g., hydrophobic fraction of SMP and hydrophilic fraction in LB-EPS, according to findings in Chapter 5) have been not investigated yet. Moreover, effects of QS inhibition on bacterial community structure of bulk sludge and cake layer (or biofilm) on membrane surface have not been reported. Hence, this chapter aimed to investigate positive effects of vanillin on EPS production, characteristic and bacterial community, and further mitigating membrane fouling.

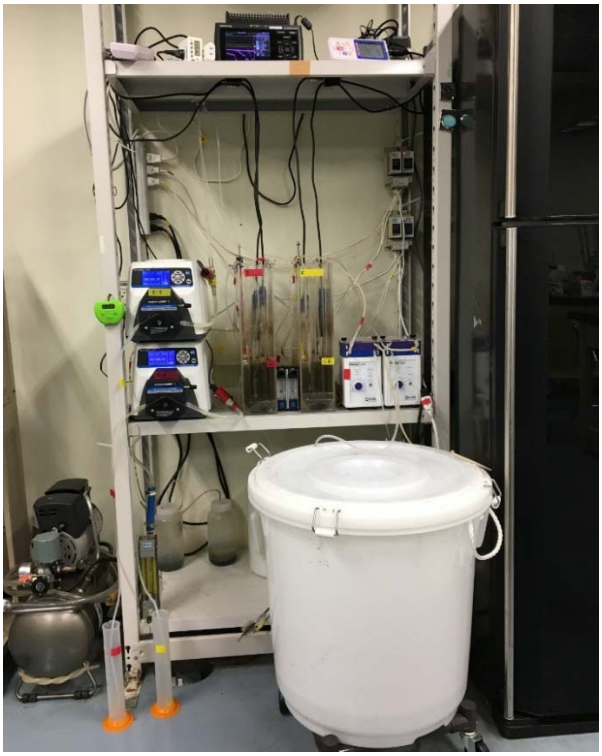
7.2. Materials and Methods

Two lab-scale membrane bioreactors were operated to treat artificial sewage as described in 3.2. Bulk sludge from the MBR of Chapter 6 was used as sludge seed for these two new MBRs. Photograph and diagram of two MBRs was showed in Figure 7.1. One MBR was continuously feed with artificial sewage with 250 m/L vanillin solution (MBR-vanillin) and another MBR was feed only with artificial sewage as a control MBR (MBR-control). The dose of vanillin was according to the study of Nam *et al*. [18]. The constant flux of 14 LMH was manually operated via control accumulated volume of effluent. TMP observation was recorded via a data logger. The effluent volume was manually measured twice per day. Working volume, HRT, and SRT were at 1.6 L, 7-8 h, and 25 days, respectively. Operational parameters of SRT, original MLSS, and temperature were set at 25 days, 6 g/L, 18-22 °C, respectively.

MBR influent and effluent were sample twice a week for TOC measurement. When TMP reached a criteria (over 60 kPa), fouled membrane was physically cleaned and cleaned membrane was applied for next filtration cycle. Foulants collected from physical cleaning was considered as cake layer samples and centrifuged supernatant of cake layer was considered as SMP of cake layer. EPS of cake layer were also fractionated into LB-, and TB-EPS as in Chapter 6. These EPS fractions of bulk sludge and cake layer were characterized in concentration, components and polarity-MW profile as in Chapter 5.

Quorum sensing inhibition via vanillin was tested on biosensor *Agrobacterium tumefaciens* NTL4 (pZLR4). Microbes in bulk sludge and cake layer were determined in

their ability to produce AHL signal. AHL signals in the MBRs were also extracted and identified by FTMS. These analyses were conducted as description in section 3.1.4.



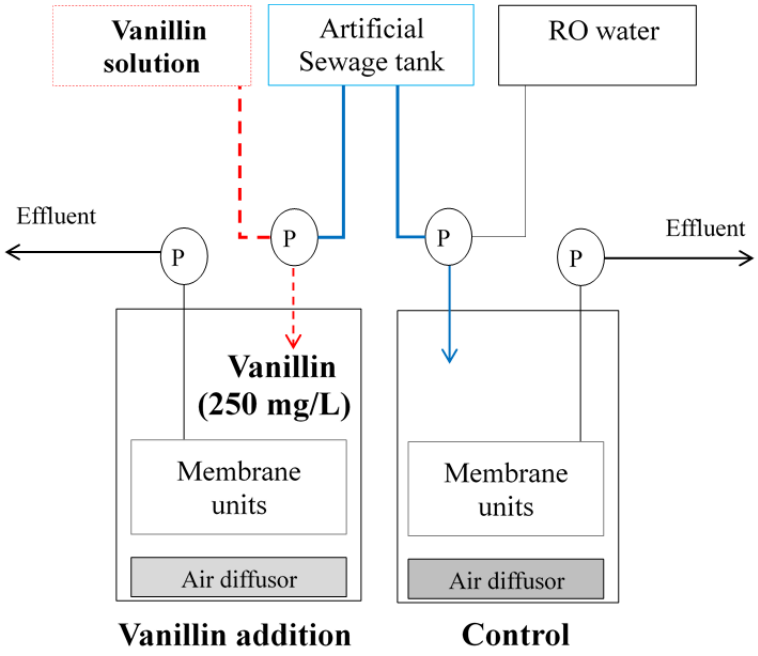


Figure 7.1. Photograph and diagram of MBR operation for QS inhibition via vanillin

Bacterial communities in bulk sludge and cake layer were analyzed as below. DNA from bulk sludge and cake layer was extracted using the PowerSoil DNA Isolation Kit as in Chapter 6. DNA library preparation, NGS sequencing and taxonomic analysis were conducted via the service of Seibutsu-Giken. Sequences of adaptors-primers and PCR conditions for DNA library preparation were showed in Table 7.1. Amplicon library was sequenced via Illumina Miseq platform based on paired-end reading. Low quality reads (< Q20, < 150 bp) were removed prior to be merged via FLASH software under criteria (e.g., fragment length ≥ 280 bp, overlapped sequence ≥ 10 bp, merged sequence ≥ 420). Chimeric sequences were removed via UCHIME function of USEARCH program (97% sequence similarity threshold of Greengenes database). Taxonomic analysis was conducted via QIIME (v1.9.1) with reference database of Greengenes library (default parameters). Number of effective reads was 3,720 and 4,408 for bulk sludge and cake layer of MBR-control; 4,086 and 3,730 for bulk sludge and cake layer of MBR-vanillin.

Table 7.1. Primer sequences and PCR conditions

Overhang and primer sequences for 1st-PCR:
Forward overhang adaptor: 5'- ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'
Reverse overhang adaptor: 5'- GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'
Forward primer (341F): 5'- CCTACGGGNGGCWGCAG-3'
Reverse primer (805R): 5'- GACTACHVGGGTATCTAATCC-3'
Conditions for 1st-PCR:
94°C for min
(22-35) cycles: 94°C for 30 seconds-55°C for 30 seconds-72°C for 30 seconds
72 °C for 5 min
Hold at 4 °C
Primer sequences for 2nd-PCR:
Forward primer: 5'- ACACTCTTTCCCTACACGACGC-3'
Reverse primer: 5'- GTGACTGGAGTTCAGACGTGTG-3'
Conditions for 2nd-PCR:
94°C for 2 minutes

12 cycles: 94°C for 30 seconds-60°C for 30 seconds-72°C for 30 seconds
72°C for 5 minutes
Hold at 4°C

7.3. Results and Discussion

7.3.1. MBR performance and bulk sludge property

Vanillin addition did not significantly affect TOC removal efficiency of MBR process. Both MBRs were ability to remove up to over 96 % TOC of influent (Table 7.3). Theoretically, a dose of 250 mg vanillin per liter contains 152 mg carbon, which made an increase of TOC influent, 326 mg/L compared with 161 mg/L in the control. Moreover, TOC in effluent of MBR-vanillin was slightly higher than that in MBR-control possibly due to vanillin residues (Table 7.3). Two MBRs was operated at the same SRT of 25 days and initial MLSS of 6 g/L; however, the MLSS of MBR-vanillin gradually increased and doubled after one month. This finding revealed that vanillin was possibly utilized as source of carbon. It is because a few species: *Sphingomonas paucimobilis, Rhodococcus jostii* are able to degrade vanillin for their growth [169, 170]. Beside, microbial growth is reportedly not inhibited via vanillin except for *Photobacterium phosphoreum* [171]. Vanillin addition did not negatively affect TOC removal and bacterial growth.

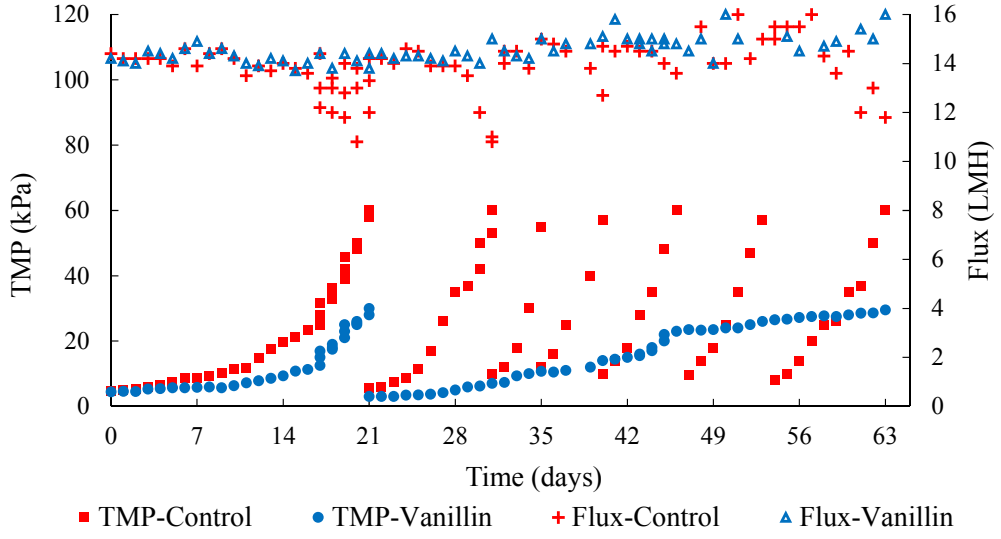
Vanillin addition were able to mitigate membrane fouling. Figure 7.2-A showed that TMP in MBR-control sharply increased after 17 days, then jumped up and reached to over 60 kPa after 21 days. Meanwhile, TMP of MBR-vanillin gradually increased and reached 30 kPa after 21 days. In the second filtration cycle, TMP of MBR-control reached 60 kPa after 10 days while that of MBR-vanillin reached 30 kPa after 42 days. In general, fouling increases when MLSS increases [172]. However, vanillin addition

made MBR maintain at a low level of TMP even though MLSS nearly got a double (Table 7.3). Similar as this study, Nam *et al*. [18] also found that vanillin addition can delay membrane fouling for 27 days and slightly increase MLSS. The reduction of membrane fouling possibly was as result from positive effects of vanillin on sludge floc, EPS production and characteristic as well as biofilm formation on membrane surface.

Table 7.3. Treatment performance of two MBRs (n=20)

	Influent [mg TOC/L]		Effluent [mg TOC/L]		TOC removal [%]		MLSS [g/L]	
	Control	Vanillin	Control	Vanillin	Control	Vanillin	Control	Vanillin
Mean	152	312	6.2	7.3	96.0	97.7	6.7	11.8
SD	43	73	2.4	1.5	2.2	0.5	1.2	2.5

A. Development of TMP



B. Sludge flocculation

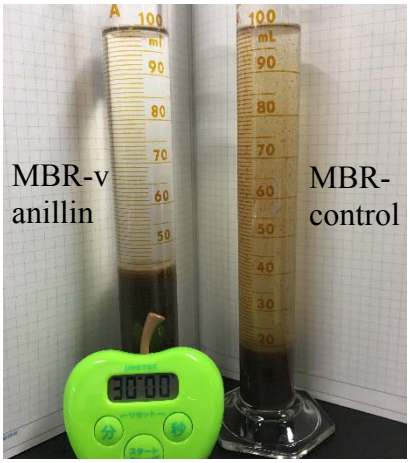


Figure 7.2. (A) TMP development and (B) sludge flocculation in two MBRs

7.3.2. EPS in Bulk sludge

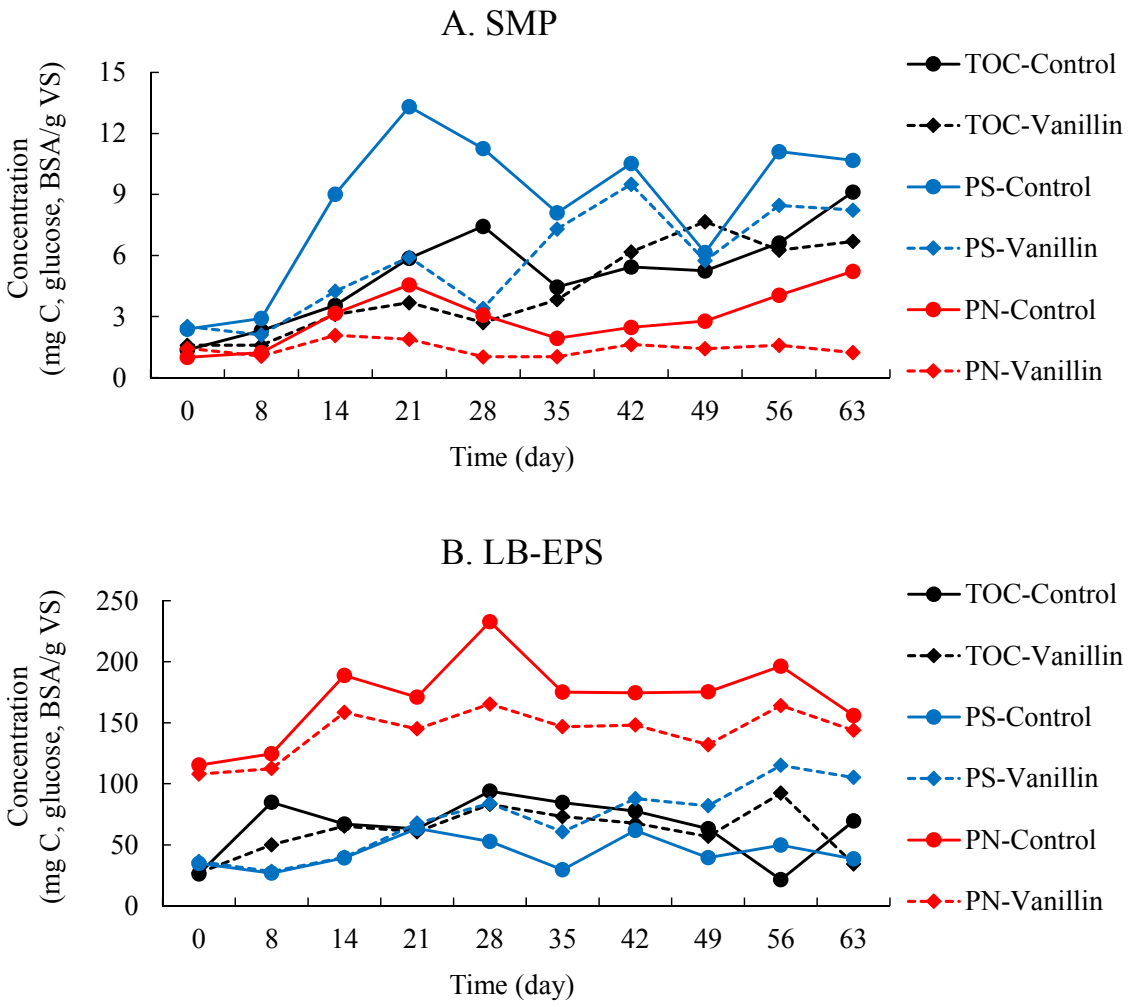
a. EPS content and components of EPS in bulk sludge

Vanillin addition slightly reduced EPS production in bulk sludge. Vanillin caused a reduction of SMP concentration (Figure 7.3-A). SMP concentration (mgC/g VS-sludge) in MBR-vanillin was 15% lower than that of MBR-control. Moreover, Figure 7.3-A showed that vanillin reduced both polysaccharide and protein components of SMP, in which polysaccharides were the primary component. Reduction of SMP possibly resulted in the delay of TMP development (Figure 7.2) because SMP were found to be highly adsorbed into membrane at initial stage of fouling (Chapter 5).

Vanillin addition reduced LB-EPS production in bulk sludge, in which vanillin highly reduced protein content – primary component of LB-EPS (Figure 7.3-B), LB-EPS in bulk sludge has been considered to highly cause irreversible fouling (Chapter 5) and a source SMP when being detached from the sludge flocs. Vanillin also caused an increase of polysaccharide component in LB-EPS (Figure 7.3-B). LB-EPS possibly adsorbed polysaccharide component in SMP to increase flocculation. It was because

settling ability of bulk sludge in MBR-vanillin was better than that in MBR-control (Figure 7.2-B).

Similar with LB-EPS, TB-EPS was greatly reduced via vanillin addition, in which vanillin highly reduced protein content – primary component of TB-EPS (Figure 7.3-B). Since TB-EPS in bulk sludge was found to deposit on membrane surface forming cake layer (Chapter 5), reduction of TB-EPS in bulk sludge via vanillin resulted in the reduction of membrane fouling in MBR-vanillin.



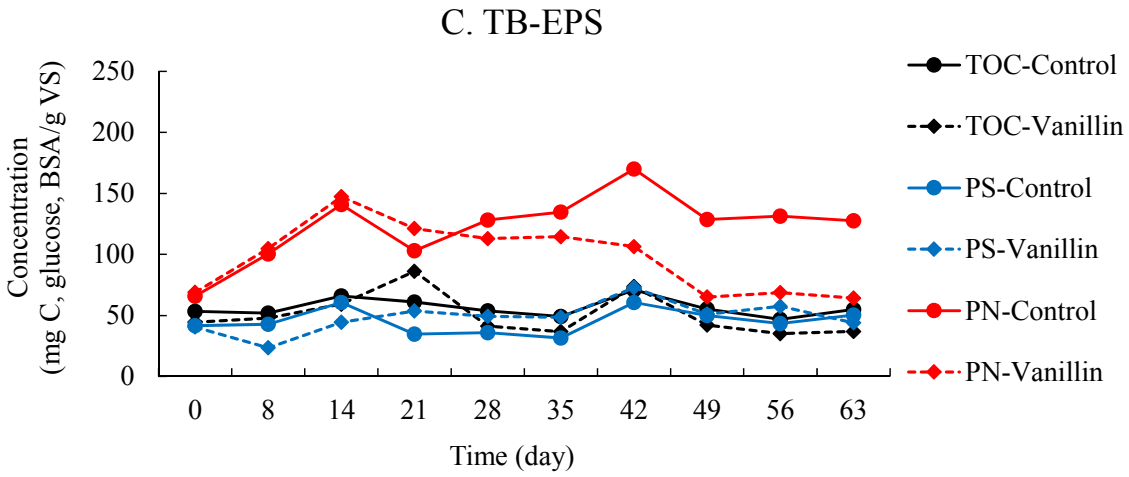


Figure 7.3. EPS content (TOC) and EPS components (PS, PN) in bulk sludge

In general, vanillin addition reduced EPS production in bulk sludge, as a previous study [18], reducing 16 % in SMP, 6 % in LB-EPS and 10.5 % of TB-EPS (calculation based on TOC concentration). Vanillin addition obtained effects on ratio of polysaccharide to protein in SMP and EPS of bulk sludge. In comparison with MBR-control, averaged ratio of PS to PN in SMP and EPS increased from 3.1 to 4.6 ($p \leq 0.05$) and from 0.3 to 0.5 ($p \leq 0.01$), respectively.

b. Polarity-MW profile of EPS in Bulk sludge

Vanillin addition reduced hydrophobic intensity of SMP but did not affect MW distribution of SMP. SMP in MBR-vanillin were comprised of slightly-hydrophobic substances, peaking at 3.3-3.5 min (Figure 7.4-A), which was similar with that in MBR-control. However, intensity of hydrophobic substances was much lower than that in MBR-control even though analyzed samples of the two MBRs were adjusted at a similar TOC concentration. Figure 7.4-B showed that MW distribution of SMP was not affected via vanillin addition. In general, polarity-MW profile of SMP (Figure 7.4-C) revealed that SMP largely contained hydrophobic substances lower than 100 kDa (as a finding in Chapter 5).

Vanillin reduced hydrophobicity ratio and increased MW of LB-EPS in bulk sludge. LB-EPS in bulk sludge contained slightly-hydrophobic and highly-hydrophobic substances, with elution time of 3.3-3.5 min and 3.8-4.0 min, respectively (Figure 7.5-A). Intensity of slightly-hydrophobic substances and highly-hydrophobic substances in MBR-control were similar, while highly-hydrophobic substances in MBR- vanillin was much lower than slightly-hydrophobic substances (Figure 7.5-A). Therefore, the most effect of vanillin addition on polarity of LB-EPS decreased ratio of

highly-hydrophobic substances to slightly hydrophobic substances. Besides, vanillin addition tend to make substances in LB-EPS larger than 1100 kDa and smaller 100 kDa (Figure 7.5-B), compared with 132 kDa in MBR-control. In general, LB-EPS under vanillin addition were largely comprised of > 1100 kDa and < 100 kDa-hydrophobic substances while LB-EPS of MBR-control highly contained hydrophobic substances of 100-670 kDa (Figure 7.5-C). 100-670 kDa hydrophobic substances were potential to be released into SMP causing pore membrane fouling (Chapter 5).

Vanillin reduced ratio of hydrophobicity to hydrophilicity but did not affect MW distribution of TB-EPS in bulk sludge. TB-EPS in two MBRs obtained hydrophilic, slightly-hydrophobic and highly-hydrophobic substances, with elution time at 1.9-2.1 min, 3.3-3.5 min and 3.8-4.0 min, respectively (Figure 7.6-A). Hydrophobic substances were predominant in TB-EPS of MBR-control while hydrophilic substances were predominant in MBR-vanillin (Figure 7.7-A). Moreover, Figure 7.6-B showed that MW distribution of TB-EPS was not significantly affected via vanillin addition. In general, TB-EPS were largely comprised of hydrophilic and hydrophobic substances with larger than 1100 kDa (Figure 7.6-C).

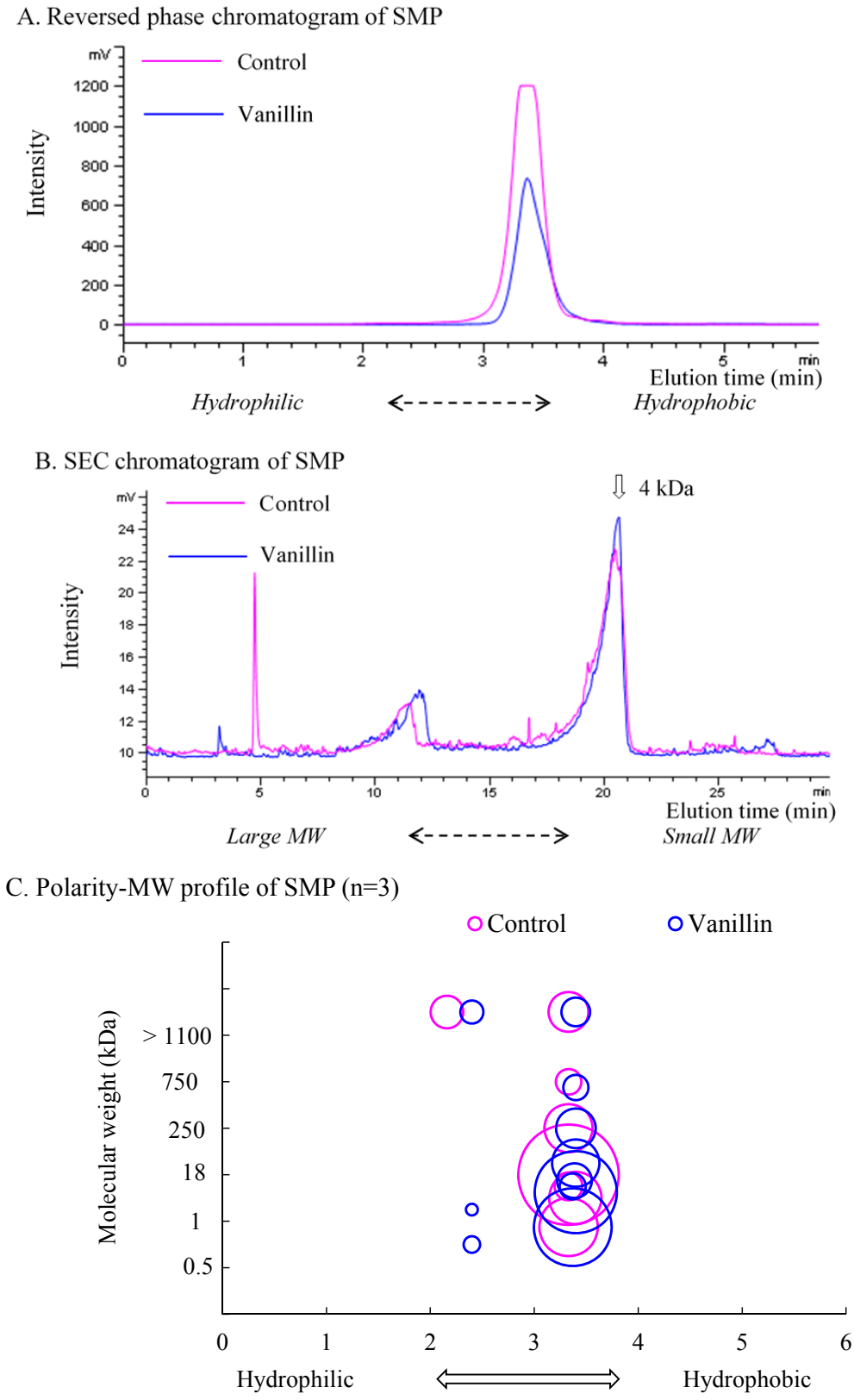


Figure 7.4. Reversed-phase, SEC chromatograms, and polarity-MW profile of SMP. (TOC concentration of analyzed sample in MBR-vanillin and MBR-control were similar).

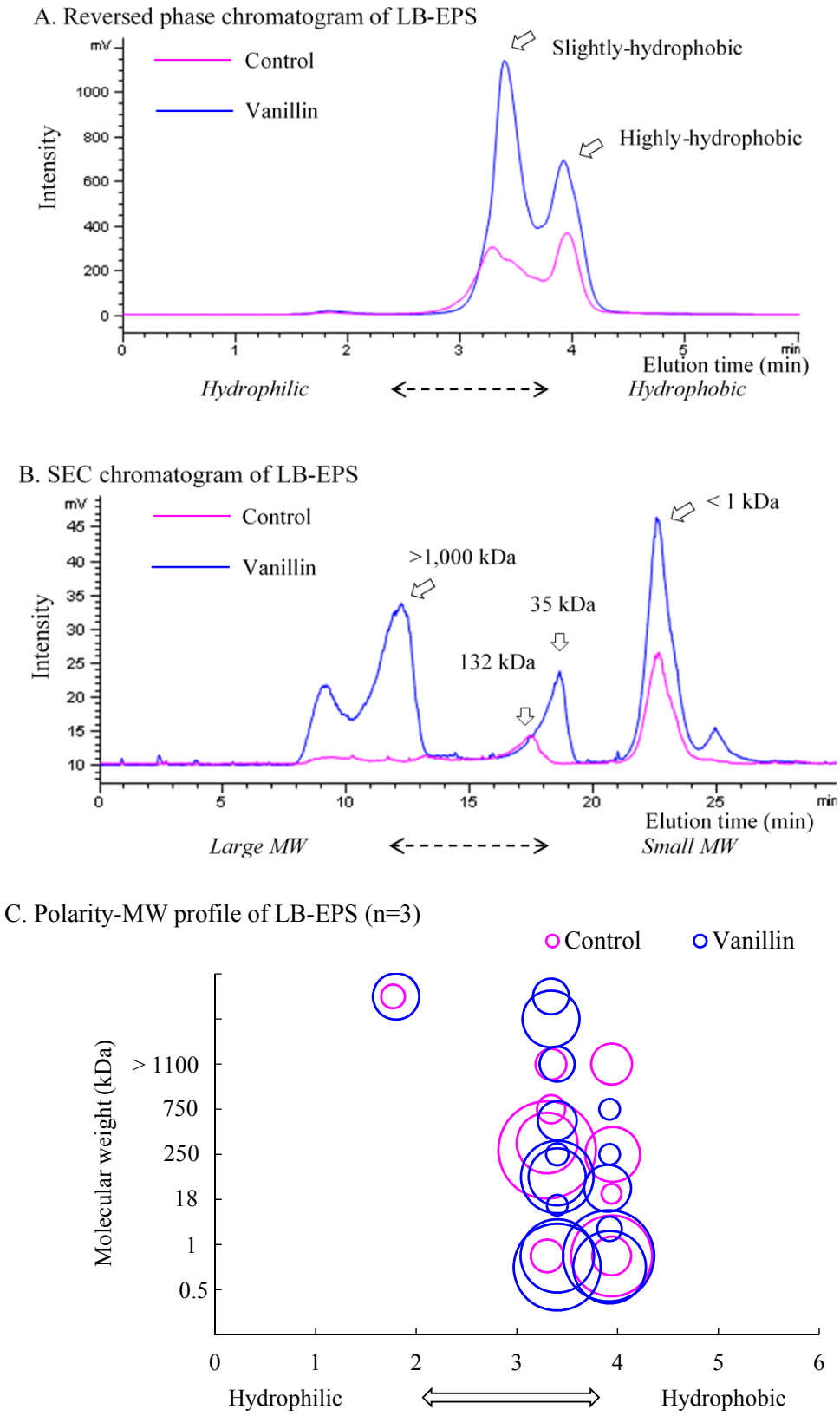


Figure 7.5. Reversed-phase, SEC chromatogram, and polarity-MW profile of LB-EPS. (TOC of the analyzed sample in MBR-vanillin was 1.7 time higher than that of MBR-control).

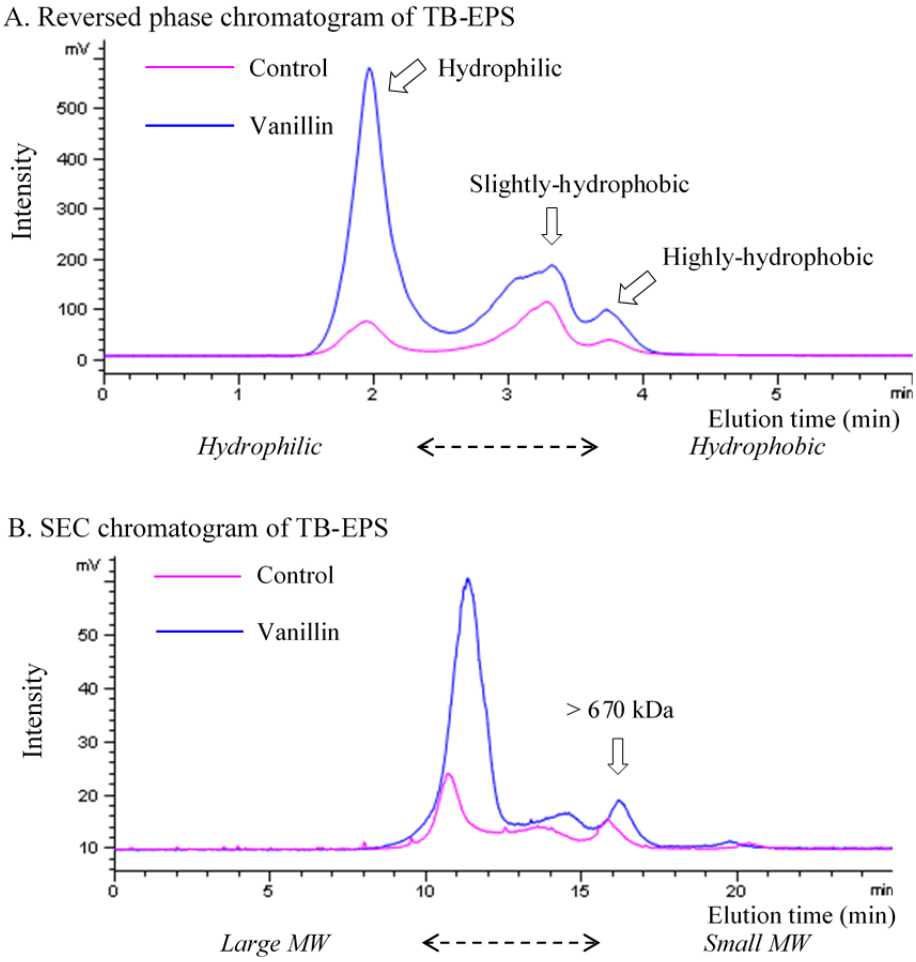


Figure 7.6. Reversed-phase, SEC chromatogram, and polarity-MW profile of TB-EPS. (TOC of the analyzed sample in MBR-vanillin was 2.5 time higher than that of MBR-control).

7.3.3. EPS in Cake layer

Vanillin addition reduced 36 % cake layer formation on membrane surface. Dried weight of cake layer in MBR-vanillin was much lower than that in MBR-control, 0.5 compared to 0.7 (mg VS/cm^2 of membrane). Moreover, vanillin reduced 44 % EPS content in cake layer, containing 290 mgC/g VS-cake layer, compared with 520 mgC/g in MBR-control (Figure 7.7-A). Nam *et al*. [18] found that vanillin causes 31 % reduction of EPS in cake layer.

Vanillin also caused effect on EPS components of cake layer, in which it caused high reduction of polysaccharide component of SMP and protein component of LB-EPS (Figure 7.7-B and C). This finding was inconsistent with the study of Nam *et al.* [18], in which only polysaccharides significantly decrease. Besides, vanillin showed different effects on components of EPS fractions. Vanillin made PS/PN ratios decrease in SMP but increase in LB- and TB-EPS (Figure 7.7-D).

Vanillin addition caused reductions in hydrophobic substances but did not affect MW of SMP, LB- and TB-EPS in cake layer. Figure 7.8-A and B showed that cake layer were comprised of hydrophobic and hydrophilic substances of larger than 250 kDa. Vanillin addition reduced hydrophobicity and increased hydrophilicity of SMP and LB-EPS. Besides, vanillin greatly reduced hydrophobicity of TB-EPS in cake layers.

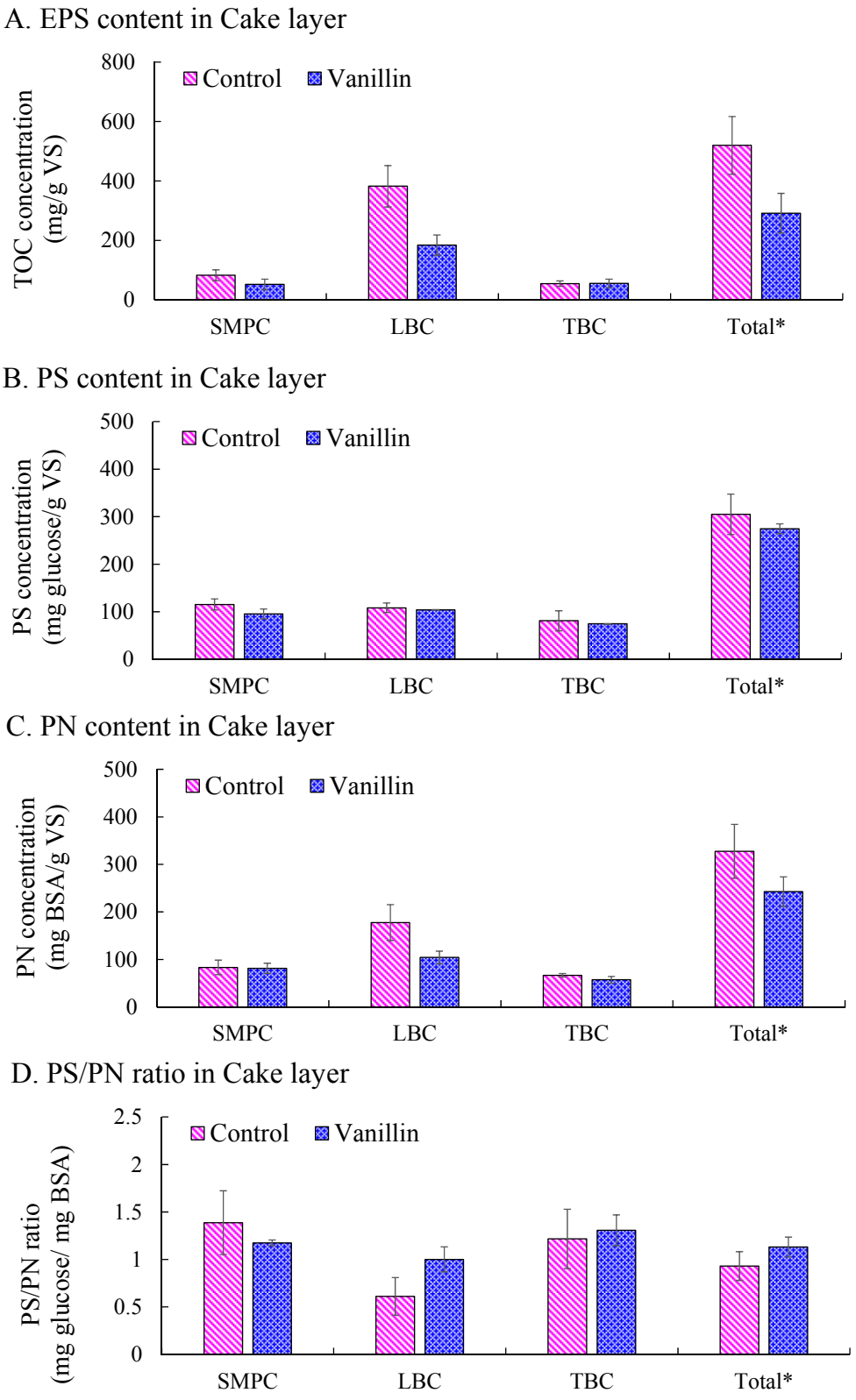
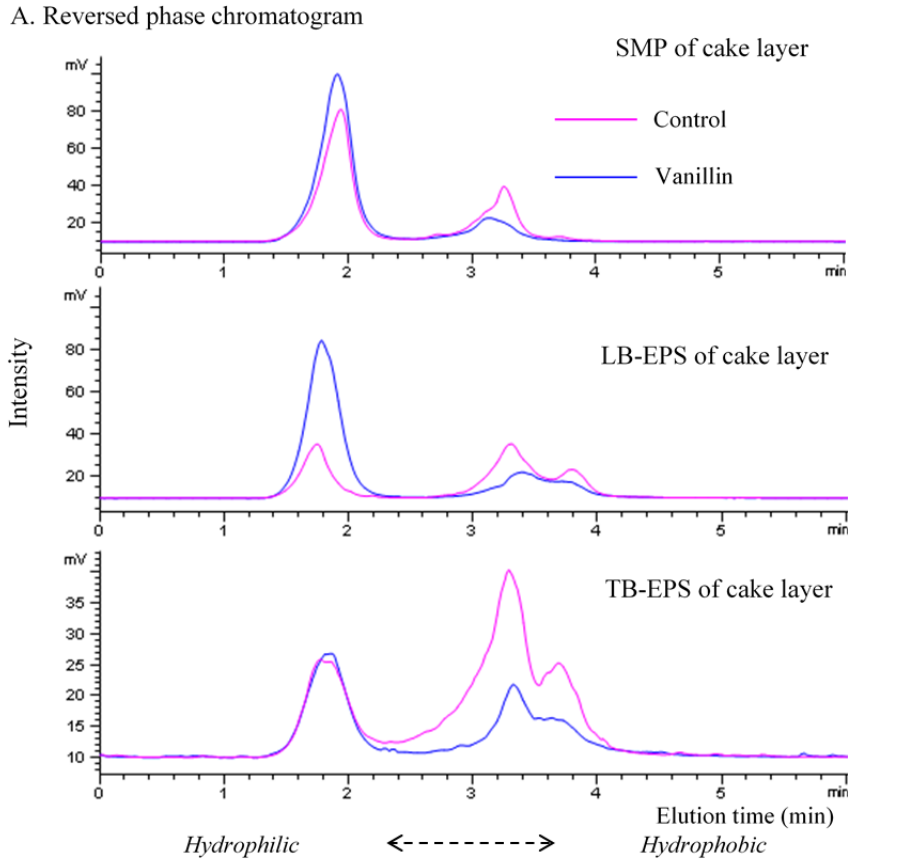


Figure 7.7. Content and components of EPS in Cake layer.

(Total is sum of SMP, LB- and TB-EPS; SMPC, LBC, TBC were stood for SMP, LB- and TB-EPS of cake layer, respectively; n=2 for MBR-vanillin, 3 for MBR-control).



B. Polarity-MW profile of Cake layer (n=2)

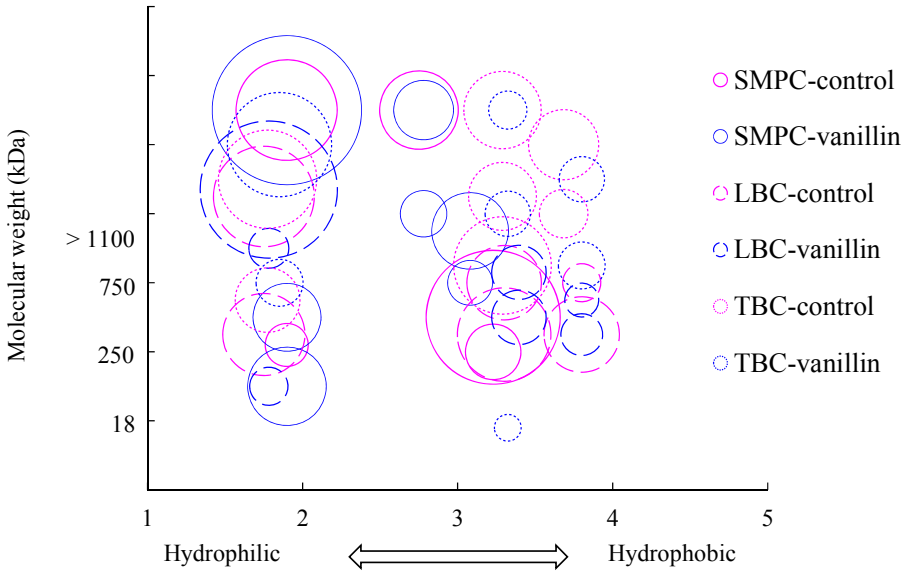


Figure 7.8. Reversed-phase chromatogram and polarity-MW profile of EPS in Cake layer.

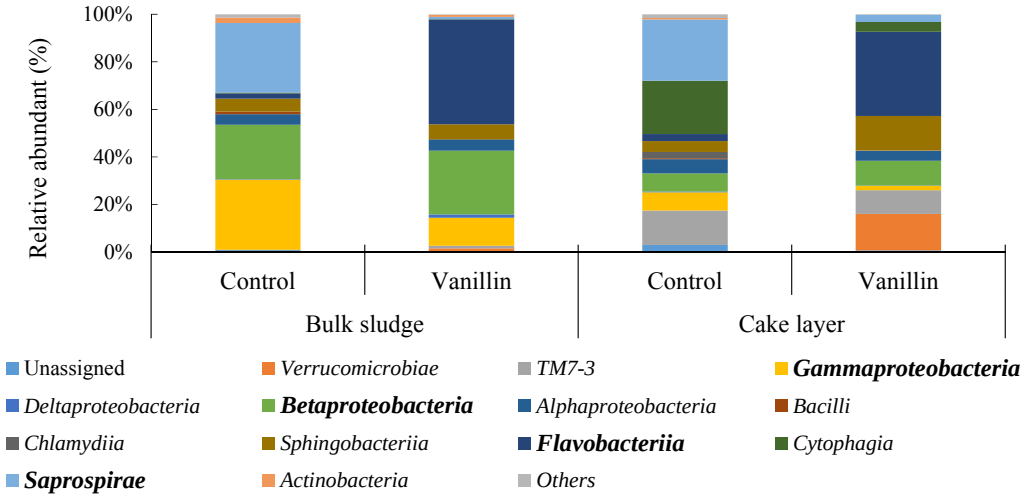
(TOC concentrations of analyzed samples in MBR-vanillin and MBR-control were similar).

7.3.4. Bacterial community

Similar with bacterial community in chapter 6, *Gammaproteobacteria* (30 %), *Saprospira*e (29 %), *Betaproteobacteria* (22 %), and were the most abundant in bulk sludge of MBR-control (Figure 7.9-A). However, *Flavobacteriia* (44 %) were the most abundant in MBR-vanillin, followed by *Betaproteobacteria* (27 %), and *Gammaproteobacteria* (12 %). At family level, *Chitinophagaceae, Xanthomonadaceae* and *Comamonadaceae* were dominant in MBR-control, while *Flavobacteriaceae* were the most dominant in MBR-vanillin, followed by *Comamonadaceae*, and *Xanthomonadaceae* (Figure 7.9-B). These results showed that vanillin addition affected bacterial structure in MBR bulk sludge, causing predominance of *Flavobacteriaceae*, which were reported to positively related to sludge flocculation [173].

Under presence of vanillin, bacterial community in cake layer was significantly different from that in bulk sludge, as well as from that of cake layer in MBR-control (Figure 7.9-C). It was largely contributed of genus *Fluviicola, Prosthecobacter, Pedobacter* (Figure 7.9-B), which were found to preferentially attach to membrane causing fouling [174, 175, 176]. Genera of *Chitinophagaceae* and *Cytophagaceae* were the most dominant in cake layer of MBR-control, being reported predominant in cake layer [177]. These bacteria have not been reported as QS or QQ bacteria in MBRs. QS inhibition via QQ bacteria was reported to shift bacterial structure of cake layer from bulk sludge [178].

A. Bacterial structure at class level



B. Most abundant genera

Class_Family_Genus	Bulk sludge		Cake layer	
	Control	Vanillin	Control	Vanillin
*Saprospirae_Chitinophagaceae*_Unclassified genus	29.2	0.8	25.4	2.4
*Gammaproteobacteria_Xanthomonadaceae*_Unclassified genus	22.1	11.7	3.9	1.7
*Betaproteobacteria_Comamonadaceae*_ Unclassified genus	15.0	7.8	3.5	1.6
Gammaproteobacteria_Xanthomonadaceae_Lysobacter	5.8	0.0	2.4	0.0
*Betaproteobacteria_Comamonadaceae*_ Unclassified genus	1.8	13.5	0.5	6.9
Flavobacteriia_Cryomorphaceae_Fluviicola	0.3	0.8	2.5	27.4
*Cytophagia_Cytophagaceae*_Unclassified genus	0.2	0.0	20.4	0.3
Flavobacteriia_Flavobacteriaceae_Flavobacterium	0.2	43.0	0.0	7.9
TM7-3_Unclassified family_Unclassified genus	0.1	1.1	2.6	9.9
TM7-3_Rs-045_Unclassified genus	0.1	0.0	11.9	0.0
Verrucomicrobiae_Verrucomicrobiaceae_Prosthecobacter	0.0	1.1	0.0	15.4
Sphingobacteriia_Sphingobacteriaceae_Pedobacter	0.0	0.0	0.0	13.4

C. Clustering analysis

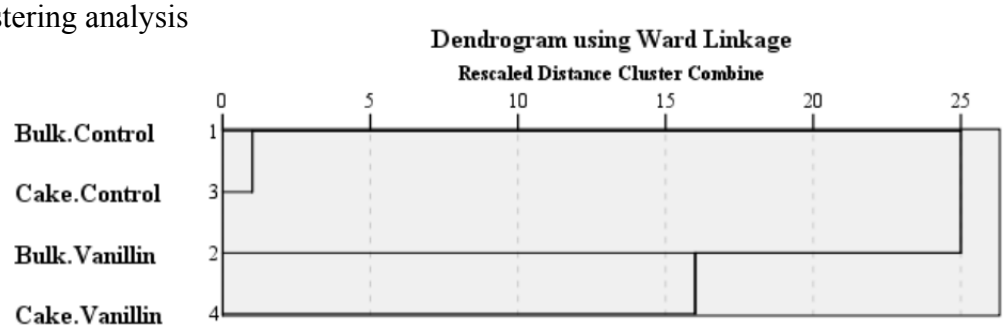


Figure 7.9. Bacterial community analysis of bulk sludge, cake layer sample of date 21; (A) community structure at class level, (B) the most abundant genera and (C) clustering analysis based on data of OTUs table.

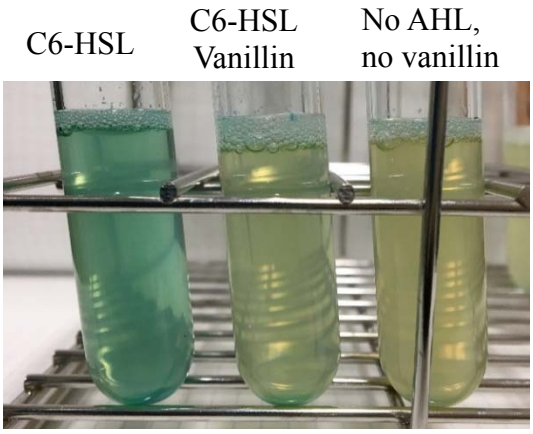
7.3.5. Inhibitory activity of vanillin against bacterial quorum sensing

Quorum sensing inhibition via vanillin was tested biosensor bacteria *Agrobacterium tumefaciens* NTL4 (pZLR4). The biosensor bacteria are genetically modified to produce β-galactosidase regulated by AHL-based QS process. After inoculation, vanillin did not affect growth of biosensor bacteria, which was evaluated via optical density at 600 nm. Moreover, blue coloration of the mixture with vanillin and C6-HSL is lower than that of the mixture with only C6-HSL (Figure 7.10-A). It revealed that vanillin possibly inhibit QS process of *A. tumefaciens*, which resulted that less β-galactosidase was produced to oxidize X-gal. So far, interference with AHLs in interaction with receptors [86] is a possible inhibitory of vanillin that has been accepted among researchers [21, 23].

Analysis of FTMS showed that some signals were present in MBR bulk sludge and AHL production was not affected via vanillin addition. C4-HSL was detected in AHL extract from MBR-control and MBR-vanillin in date 48 at fairly similar concentration (6.5 ng/L and 4.7 ng/L, respectively). Others signals (e.g., C6-HSL) were detected in extracts from bulk sludge before adding vanillin they were not detected in both MBR-vanillin and MBR-control. Possible reasons was that their concentrations in sample too low and lower than detection level of the FTMS method. Moreover, in QS bioassay test of bulk sludge and cake layer), cultivation mixtures turned from yellow to blue (Figure 7.10-B). It indicated that microbes in bulk sludge and cake layer in both MBR-control and MBR-vanillin were able to produce AHLs signal. In addition, vanillin did not inhibit microbial growth in bulk sludge, cake layer of MBR-vanillin and microbial synthesis of AHL production. It possibly was because AHL producers (e.g., *Flavobacterium*, *Xanthomonadaceace* bacterium, *Sphingomonas*) [145] were found dominant in bulk sludge and cake layer of MBR-vanillin (Figure 7.9-B). Blue coloration

in cultivation of cake layer from MBR-control was lowest among samples (Figure 7.10-B), which was because of low relative abundance of the AHL producers (Figure 7.9-B).

A. QS inhibition of *Agrobacterium tumefaciens* NTL4 (pZLR4) via vanillin



B. Bioassay test for AHL production of microbes in bulk sludge and cake layer

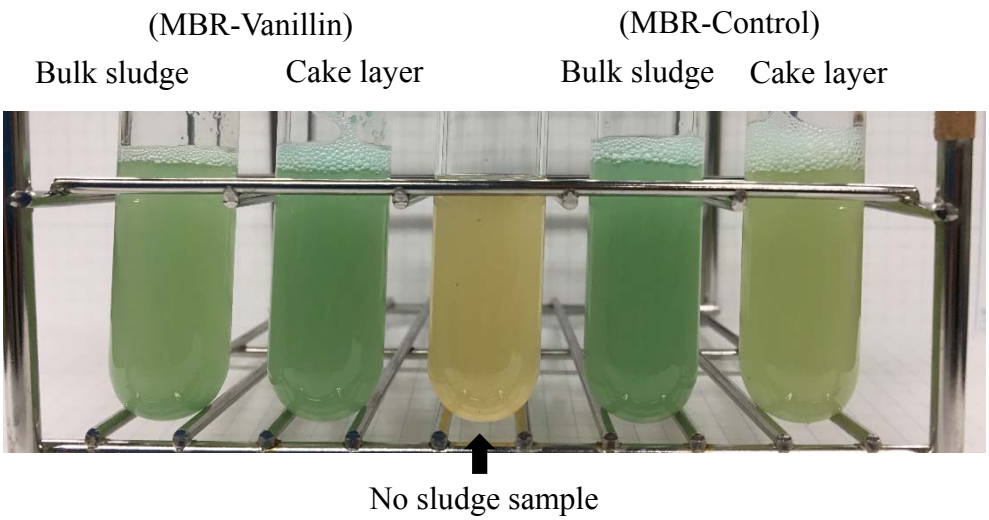


Figure 7.10. (A) QS inhibition of *Agrobacterium tumefaciens* NTL4 (pZLR4) via vanillin (Reduction of green-blue color indicated vanillin partly inhibit QS process). (B) Bioassay test for AHL production of microbes in bulk sludge and cake layer in

MBR-control and MBR-vanillin (Appearance of green-blue color indicated AHL production ability).

Quorum sensing inhibitions via disruption of QS signal synthesis and via degradation of QS signal directly cause reduction of QS signals. However, the inhibition via interference with QS signal receptors (e.g., vanillin) was only evaluated by based on microbial downstream activities regulated by QS process (e.g., biofilm formation). Reduce biofilm formation of MBR sludge using vanillin was reported [18], in which reduction of biofilm formation was determined by amount of EPS extracted from membrane surface incubated with MBR sludge in a shaker at 150 rpm for 24 h.

7.3.6. Membrane fouling control by vanillin addition

Similar with membrane fouling control via QQ bacteria [24], vanillin addition into MBR was expected to inhibit microbial quorum sensing process for biofilm formation on membrane surface, reducing membrane fouling [18]. In this study, vanillin was found to have some effects on bacterial quorum sensing, community structure, EPS production and characteristics in bulk sludge and cake layer.

In bulk sludge, presence of vanillin led to an increase of relative abundance of QS bacteria – *Flavobacterium* [179] and decreases of EPS producer – *Xanthomonadaceae* and EPS hydrolyzer – *Chitinophagaceae* [166] (Figure 7.9-B). *Xanthomonadaceae* bacterium were also found to have a relation to SMP and EPS production (Chapter 6). Therefore, vanillin reduced abundance of EPS producers, possibly resulting in the reduction of EPS production in SMP, LB-EPS and TB-EPS in bulk sludge (Figure 7.3). Besides, the abundance of *Flavobacterium* was important for sludge flocculation,

especially in hydrophobic fraction [180]. Low concentration of hydrophobic substances in SMP and LB-EPS and higher flocculation possibly resulted in reduction of attachment of EPS-foulants on membrane surface, which was important for initial fouling stage.

On membrane surface of MBR, vanillin possibly inhibited microbial QS process on membrane surface, in which QS bacteria including *Pedobacter, Flavobacterium* [180], and EPS producers (*Xanthomonadaceae* bacterium) [166] were highly abundant (Figure 7.9-B). Hence, vanillin might cause a reduction in EPS production from microbes on membrane surface, contributing the reduction of cake layer formation in MBR-vanillin (Figure 7.7). In addition, vanillin addition decreased concentrations of hydrophobic fraction in SMP, LB- and TB-EPS of cake layer (Figure 7.8), which possibly offered advantages for reductions of TMP development and membrane fouling.

7.4. Conclusions

Vanillin is able to inhibit microbial quorum sensing in bulk sludge and cake layer on membrane surface, resulting in reduction of membrane fouling via offering some positive effects:

- Vanillin causes shifts in bacterial structure: increasing abundance of *Flavobacterium* (AHL producers) in MBR bulk sludge, but reducing *Xanthomonadaceae* and *Chitinophagaceae* (which are known as EPS producers and hydrolyzers). Under presence of vanillin, *Fluviicola, Prosthecobacter, Pedobacter* are abundant only in cake layer (biofilm).

- In Bulk sludge, vanillin reduces productions of polysaccharides in SMP and proteins in LB-EPS, hydrophobic substances in bulk sludge, which lessens attachment of these foulants on membrane surface (lessening gel layer formation).

- On membrane surface, vanillin addition mostly decreases productions of proteins in SMP and LB-EPS of cake layer, and hydrophobic substances in cake layer (especially in TB-EPS), but increases MW distribution of TB-EPS, which can be as results of reduction in biofilm and cake layer formation.

Chapter 8. Conclusions and Future Perspectives

8.1. Conclusion

Characterization of EPS in MBRs is vital to understand membrane fouling because EPS are considered as primary foulants and play important role in membrane fouling development. These below conclusions were drawn from results of the conducted experiments:

Choosing EPS extraction method is important to study membrane fouling because characteristics of EPS (e.g., concentration of protein component, molecular distribution, and 3D-EEM spectra) are effected via extraction methods. A combination of sodium hydroxide and heat exhibits a great extraction efficiency but causes a high level of cell lysis. Application of cation exchange resin can extract various substances but obtains a low efficiency of EPS amount. A combination of formaldehyde and sodium hydroxide can not only extract a great amount of EPS but also cause less cell lysis. These methods do not significantly affect ratio of polysaccharide to protein but cause some impacts on molecular weight distribution and 3D-EEM spectra of EPS extracts.

EPS are potential source of membrane foulants in MBR. Each EPS fraction, polarity fraction and molecular weight size obtained its own different fate to cause membrane fouling. Hydrophobic substances smaller than 20 kDa in SMP (highly released from LB-EPS of bulk sludge) quickly condition micropores of membrane. Subsequently, hydrophilic biopolymers as large as 100-670 kDa in LB-EPS of bulk sludge plug narrowed micropores of the conditioned membrane, causing irreversible fouling. While, hydrophilic substances larger than 670 kDa in TB-EPS of bulk sludge deposit on membrane surface to form cake layers.

EPS content and compositions in bulk sludge and cake layer are affected via MBR operational condition (e.g., constant flux, constant TMP). These conditions also influence bacterial structure and behavior. In a comparison with a constant flux, a constant TMP operation can increase contents of polysaccharides and humic substances in MBR supernatant. Furthermore, this operation possibly enhance distinctions in bacterial structures between bulk sludge and cake layer. Moreover, under the constant TMP operation, *Comamonadaceae* bacteria were highly abundant in bulk sludge and positively correlate to SMP and EPS concentration while *Xanthomonadaceae* bacteria greatly adsorb into membrane surface and closely correlate to polysaccharide concentration in EPS.

Vanillin addition causes a shift of bacterial structure in cake layer (biofilm) from the structure of bulk sludge, reducing EPS producers and hydrolyzers (e.g., *Xanthomonadaceae* and *Chitinophagaceae*).

Hydrophobicity/hydrophicity and content of EPS in bulk sludge and cake layer of MBRs was impacted by microbial quorum sensing process. Inhibition of QS process via vanillin addition can reduce attachment of EPS-related foulant on membrane surface, and lessen cake layer, biofilm formation. These reductions altogether contribute to a mitigation of membrane fouling.

8.2. Future Perspectives

Successful application of quorum sensing inhibition to control membrane fouling in this study shows realistic prospects in membrane fouling management, which encourages making researches in larger scale MBRs.

In investigation of bacterial community in MBRs, some families are specifically predominant in bulk sludge and cake layer; however, the question “what are their functions” has not been asked yet. Developments of sequencing technology (e.g., NGS) and RNA database allow researchers infer microbial functions and activities of a whole community without isolation steps. Through RNA sequencing, doubts about QS process in MBRs will be clearer. For instance, mRNA genes expression for Lux I and Lux R proteins are available at a high level, which is a clearer evidence of QS activity than the presence of AHL signal.

Vanillin addition shows positive effects on mitigation of membrane fouling however, the concentration of vanillin is too high; therefore, it is difficult to be applied in larger scale MBRs. A combination of vanillin (at lower dose) and other QS inhibitors (e.g., QQ bacteria) should be investigated to enhance efficiency of membrane fouling control.

Quorum quenching and quorum sensing bacteria have been found to co-exist in MBRs; therefore, utility of native QQ bacteria in MBRs is possible to disrupt QS activity and control membrane fouling. To do this, optimum operational condition for growth of native QQ bacteria in MBRs is needed.

References

[1] P. Le-Clech, V. Chen and T. A. G. Fane, "Fouling in membrane bioreactors used in wastewater treatment," *J. Memb. Sci.,* vol. 284, no. 1-2, pp. 17-53, 2006.

[2] B. Wu and A. G. Fane, "Microbial relevant fouling in membrane bioreactors: Influencing factors, characterization, and fouling control," *Membranes,* vol. 2, no. 3, pp. 565-584, 2012.

[3] W. Luo, H. V. Phan, M. Xie, F. I. Hai, W. E. Price, M. Elimelech and L. D. Nghiem, "Osmotic versus conventional membrane bioreactors integrated with reverse osmosis for water reuse: Biological stability, membrane fouling, and contaminant removal," *Water Res.,* vol. 109, pp. 122-134, 2017.

[4] R. Honda, W. Rukapan, H. Komura, Y. Teraoka, M. Noguchi and E. M. V. Hoek, "Effects of membrane orientation on fouling characteristics of forward osmosis membrane in concentration of microalgae culture," *Bioresour. Technol.,* vol. 197, pp. 429-433, 2015.

[5] F. Meng, S. Zhang, Y. Oh, Z. Zhou, H. S. Shin and S. R. Chae, "Fouling in membrane bioreactors: An updated review," *Water Res.,* vol. 114, p. 151–180, 2017.

[6] P. Gkotsis, D. Banti, E. Peleka, A. Zouboulis and P. Samaras, "Fouling Issues in Membrane Bioreactors (MBRs) for Wastewater Treatment: Major Mechanisms, Prevention and Control Strategies," *Processes,* vol. 2, no. 4, p. 795–866, 2014.

[7] A. Drews, "Membrane fouling in membrane bioreactors-Characterisation, contradictions, cause and cures," *J. Memb. Sci.,* vol. 363, no. 1-2, pp. 1-28, 2010.

[8] H. Lin, M. Zhang, F. Wang, F. Meng, B. Q. Liao, H. Hong, J. Chen and W. Gao, "A critical review of extracellular polymeric substances (EPSs) in membrane bioreactors: Characteristics, roles in membrane fouling and control strategies," *J. Memb. Sci.,* vol. 460, pp. 110-125, 2014.

[9] T. Nguyen, F. A. Roddick and L. Fan, "Biofouling of water treatment membranes: A review of the underlying causes, monitoring techniques and control measures," *Membranes,* vol. 2, no. 4, pp. 804-840, 2012.

[10] Y. Shi, J. Huang, G. Zeng, Y. Gu, Y. Chen, y. Hu, B. Tang, J. Zhou, Y. Yang and S. L., "Exploiting extracellular polymeric substances (EPS) controlling strategies for performance enhancement of biological wastewater treatments: An overview," *Chemosphere,* vol. 180, pp. 396-411, 2017.

[11] G. P. Sheng, H. Q. Yu and X. Y. Li, "Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review," *Biotechnol. Adv.,* vol.

28, no. 6, pp. 882-894, 2010.

[12] F. Meng, S. R. Chae, A. Drews, M. Kraume, H. S. Shin and F. Yang, "Recent advances in membrane bioreactors (MBRs): Membrane fouling and membrane material," *Water Res.,* vol. 43, no. 6, pp. 1489-1512, 2009.

[13] L. Vanysacker, B. Boerjan, P. Declerck and I. F. J. Vankelecom, "Biofouling ecology as a means to better understand membrane biofouling," *Appl. Microbiol. Biotechnol.,* vol. 98, no. 19, pp. 8047-8072, 2014.

[14] A. F. Silva, S. Antunes, A. Saunders, F. Freitas, A. Vieira, C. F. Galinha, P. H. Nielsen, M. T. B. Crespo and G. Carvalho, "Impact of sludge retention time on the fine composition of the microbial community and extracellular polymeric substances in a membrane bioreactor," *Appl. Microbiol. Biotechnol.,* vol. 100, no. 19, pp. 8507-8521, 2016.

[15] X. Guo, Y. Miao, B. Wu, L. Ye, H. Yu, S. Liu and X. Zhang, "Correlation between microbial community structure and biofouling as determined by analysis of microbial community dynamics," *Bioresour. Technol.,* vol. 197, pp. 99-105, 2015.

[16] J. Cho, K. G. Song, S. H. Lee and K. H. Ahn, "Sequencing anoxic/anaerobic membrane bioreactor (SAM) pilot plant for advanced wastewater treatment," *Desalination,* vol. 178, no. 1-3, pp. 219-225, 2005.

[17] L. Deng, W. Guo, H. H. Ngo, H. Zhang, J. Wang, J. Li, S. Xia and Y. Wu, "Biofouling and control approaches in membrane bioreactors," *Bioresour. Technol.,* vol. 221, p. 656–665, 2016.

[18] A. N. Nam, J. H. Kweon, J. H. Ryu, H. Lade and C. H. Lee, "Reduction of biofouling using vanillin as a quorum sensing inhibitory agent in membrane bioreactors for wastewater treatment," *Membr. Water Treat.,* vol. 6, no. 3, pp. 189-203, 2015.

[19] K. M. Yeon, W. S. Cheong, H. S. Oh, W. N. Lee, B. K. Hwang, C. H. Lee, H. B. and Z. Lewandowski, "Quorum sensing: A new biofouling control paradigm in a membrane bioreactor for advanced wastewater treatment," *Environ. Sci. Technol.,* vol. 43, no. 2, pp. 380-385, 2009.

[20] X. Si and X. Quan, "Prevention of multi-species wastewater biofilm formation using vanillin and EPS disruptors through non-microbicidal mechanisms," *Int. Biodeterior. Biodegrad.,* vol. 116, pp. 211-218, 2017.

[21] H. Lade, D. Paul and J. H. y. Kweon, "Quorum quenching mediated approaches for control of membrane biofouling," *Int. J. Biol. Sci.,* vol. 10, no. 5, pp. 550-565, 2014.

[22] Y. H. Li and X. L. Tian, "Quorum Sensing and Bacterial Social Interactions in Biofilms,"

Sensors, vol. 12, pp. 2519-2538, 2012.

[23] M. F. Siddiqui, M. Rzechowicz, W. Harvey, A. W. Zularisam and G. F. Anthony, "Quorum sensing based membrane biofouling control for water treatment: A review," *J. Water Process Eng.,* vol. 7, pp. 112-122, 2015.

[24] H. S. Oh, K. M. Yeon, C. S. Yang, S. R. Kim, C. H. Lee, S. Y. Park, J. Y. Han and J. K. Lee, "Control of membrane biofouling in MBR for wastewater treatment by quorum quenching bacteria encapsulated in microporous membrane," *Environ. Sci. Technol.,* vol. 46, no. 9, pp. 4877-4884, 2012.

[25] S. Lee, S. K. Park, H. Kwon, S. H. Lee, K. Lee, C. H. Nahm, S. J. Jo, H. S. Oh, P. K. Park, K. H. Choo, C. H. Lee and T. Yi, "Crossing the Border between Laboratory and Field: Bacterial Quorum Quenching for Anti-Biofouling Strategy in an MBR," *Environmental Science and Technology,* vol. 50, no. 4, pp. 1788-1795, 2016.

[26] W. J. Koros, Y. H. Ma and T. Shimidzu, "Terminology for membranes and membrane processes," *J. Memb. Sci.,* vol. 120, p. 149–159, 1996.

[27] P. Babatsouli, I. Palogos, E. Michalodimitraki, C. Costa and N. Kalogerakis, "Evaluation of a MBR pilot treating industrial wastewater with a high COD/N ratio," *J. Chem. Technol. Biotechnol.,* vol. 90, no. 1, pp. 26-33, 2014.

[28] L. De Temmerman, T. Maere, H. Temmink, A. Zwijnenburg and I. Nopens, "The effect of fine bubble aeration intensity onmembrane bioreactor sludge characteristics and fouling," *Water Res.,* vol. 76, pp. 99-109, 2015.

[29] D. Gao, Y. Fu, Y. Tao, X. Li, M. Xing, X. Gao and N. Ren, "Linking microbial community structure to membrane biofouling associated with varying dissolved oxygen concentrations," *Bioresour. Technol.,* vol. 102, no. 10, pp. 5626-5633, 2011.

[30] D. Gao, Z. Wen, B. Li and H. Liang, "Membrane fouling related to microbial community and extracellular polymeric substances at different temperatures," *Bioresour. Technol.,* vol. 143, pp. 172-177, 2013.

[31] Z. Ma, X. Wen, F. Zhao, Y. Xia, X. Huang, D. Waite and J. Guan, "Effect of temperature variation on membrane fouling and microbial community structure in membrane bioreactor," *Bioresour. Technol.,* vol. 133, p. 462–468, 2013.

[32] S. Wu and C. Lee, "Correlation between fouling propensity of soluble extracellular polymeric substances and sludge metabolic activity altered by different starvation conditions," *Bioresour. Technol.,* vol. 102, no. 9, pp. 5375-5380, 2011.

[33] K. Kimura, R. Ogyu, T. Miyoshi and Y. Watanabe, "Transition of major components in

irreversible fouling of MBRs treating municipal wastewater," *Sep. Purif. Technol.,* vol. 142, pp. 326-331, 2015.

[34] S. Arabi and G. Nakhla, "Impact of protein/carbohydrate ratio in the feed wastewater on the membrane fouling in membrane bioreactors," *J. Memb. Sci.,* vol. 324, no. 1-2, p. 142–150, 2008.

[35] T. Kornboonraksa and S. H. Lee, "Factors affecting the performance of membrane bioreactor for piggery wastewater treatment," *Bioresour. Technol.,* vol. 100, no. 12, pp. 2926-2932, 2009.

[36] N. Dizge, G. Soydemir, A. Karagunduz and B. Keskinler, "Influence of type and pore size of membranes on cross flow microfiltration of biological suspension," *J. Memb. Sci.,* vol. 366, no. 1-2, pp. 278-285, 2011.

[37] P. van der Marel, A. Zwijnenburg, A. Kemperman, M. Wessling, H. Temmink and W. van der Meer, "Influence of membrane properties on fouling in submerged membrane bioreactors," *J. Memb. Sci.,* vol. 348, no. 1-2, pp. 66-74, 2010.

[38] M. Zhang, B. Liao, X. Zhou, Y. He, H. Hong, H. Lin and J. Chen, "Effects of hydrophilicity/hydrophobicity of membrane on membrane fouling in a submerged membrane bioreactor," *Bioresour. Technol.,* vol. 175, pp. 59-67, 2015.

[39] C. Laspidou and B. Rittmann, "A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass," *Water Res.,* vol. 36, pp. 2711-2720, 2002.

[40] B. J. Ni, B. E. Rittmann and H. Q. Yu, "Soluble microbial products and their implications in mixed culture biotechnology," *Trends in Biotechnol.,* vol. 29, no. 9, pp. 454-463, 2011.

[41] P. Nielsen and A. Jahn, "Chapter 3. Extraction of EPS," in *Microbial extracellular polymeric substances: characterization, structure and function*, Berlin Heidelberg, Springer-Verlag, 1999, pp. 49-72.

[42] C. Pellicer-Nàcher, C. Domingo-Félez, a. G. Mutlu and B. F. Smets, "Critical assessment of extracellular polymeric substances extraction methods from mixed culture biomass," *Water Res.,* vol. 47, no. 15, pp. 5564-5574, 2013.

[43] B. Frolund, R. Palmgren, K. Keiding and ". P. H. Nielsen, "Extraction of extracellular polymers from activated sludge using a cation exchange resin," *Water Res.,* vol. 30, no. 8, pp. 1749-1758, 1996.

[44] H. Flemming and A. Leis, "Sorption properties of biofilms," in *Encyclopedia of Environmental*, New York, NY, USA, John Wiley & Sons, Inc., 2002, pp. 2958-2967.

[45] C. Kunacheva and D. C. Stuckey, "Analytical methods for soluble microbial products (SMP) and extracellular polymers (ECP) in wastewater treatment systems: A review," *Water Res.,* vol. 61, pp. 1-18, 2014.

[46] S. Comte, G. Guibaud and M. Baudu, "Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and EPS complexation properties: Part I. Comparison of the efficiency of eight EPS extraction methods," *Enzyme Microb. Technol.,* vol. 38, no. 1-2, pp. 237-245, 2006.

[47] B. Frolund, T. Griebe and P. H. Nielsen, "Enzymatic activity in the activated-sludge floc matrix," *Appl. Microbiol. Biotechnol.,* vol. 43, no. 4, pp. 755-761, 1995.

[48] H. Liu and H. H. P. Fang, "Extraction of extracellular polymeric substances (EPS) of sludges," *J. Biotechnol.,* vol. 95, no. 3, pp. 249-256, 2002.

[49] A. Resosudarmo, Y. Ye, P. Le-Clech and V. Chen, "Analysis of UF membrane fouling mechanisms caused by organic interactions in seawater," *Water Res.,* vol. 47, no. 2, pp. 911-921, 2013.

[50] F. Neemann, S. Rosenberger, B. Jefferson and E. J. McAdam, "Non-covalent protein-polysaccharide interactions and their influence on membrane fouling," *J. Memb. Sci.,* vol. 446, pp. 310-317, 2013.

[51] S. A. Huber, A. Balz, M. Abert and W. Pronk, "Characterisation of aquatic humic and non-humic matter with size-exclusion chromatography - organic carbon detection - organic nitrogen detection (LC-OCD-OND)," *Water Res.,* vol. 45, no. 2, pp. 879-885, 2011.

[52] F. Meng, Z. Zhou, B. J. Ni, X. Zheng, G. Huang, X. Jia, S. Li, Y. Xiong and M. Kraume, "Characterization of the size-fractionated biomacromolecules: Tracking their role and fate in a membrane bioreactor," *Water Res.,* vol. 45, no. 15, p. 4661–4671, 2011.

[53] T. Seviour, M. Pijuan, T. Nicholson, J. Keller and Z. Yuan, "Gel-forming exopolysaccharides explain basic differences between structures of aerobic sludge granules and floccular sludges," *Water Res.,* vol. 43, no. 18, pp. 4469-4478, 2009.

[54] A. Massé, M. Spérandio and C. Cabassud, "Comparison of sludge characteristics and performance of a submerged membrane bioreactor and an activated sludge process at high solids retention time," *Water Res.,* vol. 40, no. 12, p. 2405–2415, 2006.

[55] Z. Zhou, F. Meng, S. R. Chae, G. Huang, W. Fu, S. L. X. Jia and G. H. Chen, "Microbial transformation of biomacromolecules in a membrane bioreactor: Implications for membrane fouling investigation," *PLoS ONE,* vol. 7, no. 8, p. e42270, 2012.

[56] B. M. Wilén, B. Jin and P. Lant, "The influence of key chemical constituents in activated

sludge on surface and flocculating properties," *Water Res.,* vol. 37, no. 9, pp. 2127-2139, 2003.

[57] X. Zheng, M. T. Khan and J. P. Croué, "Contribution of effluent organic matter (EfOM) to ultrafiltration (UF) membrane fouling: Isolation, characterization, and fouling effect of EfOM fractions," *Water Res.,* vol. 65, pp. 414-424, 2014.

[58] X. Zhang, X. Yue, Z. Liu, Q. Li and X. Hua, "Impacts of sludge retention time on sludge characteristics and membrane fouling in a submerged anaerobic???oxic membrane bioreactor," *Appl. Microbiol. Biotechnol.,* vol. 99, no. 11, pp. 4893-4903, 2015.

[59] Z. Wang, Z. Wu and S. Tang, "Extracellular polymeric substances (EPS) properties and their effects on membrane fouling in a submerged membrane bioreactor," *Water Res.,* vol. 43, no. 9, pp. 2504-2512, 2009.

[60] Y. Liu, Z. Liu, A. Zhang, Y. Chen and X. Wang, "The role of EPS concentration on membrane fouling control: Comparison analysis of hybrid membrane bioreactor and conventional membrane bioreactor," *Desalination,* vol. 305, pp. 38-43, 2012.

[61] H. Azami, M. H. Sarrafzadeh and M. R. Mehrnia, "Fouling in membrane bioreactors with various concentrations of dead cells," *Desalination,* vol. 278, no. 1-3, pp. 373-380, 2011.

[62] A. Drews, M. Vocks, U. Bracklow, V. Iversen and M. Kraume, "Does fouling in MBRs depend on SMP?," *Desalination,* vol. 231, no. 1-3, pp. 141-149, 2008.

[63] H. Yamamura, K. Okimoto, K. Kimura and Y. Watanabe, "Hydrophilic fraction of natural organic matter causing irreversible fouling of microfiltration and ultrafiltration membranes," *Water Res.,* vol. 54, pp. 123-136, 2014.

[64] F. Ye, G. Peng and Y. Li, "Chemosphere Influences of influent carbon source on extracellular polymeric substances (EPS) and physicochemical properties of activated sludge," *Chemosphere,* vol. 84, no. 9, p. 1250–1255, 2011.

[65] X. Y. Li and S. F. Yang, "Influence of loosely bound extracellular polymeric substances (EPS) on the flocculation, sedimentation and dewaterability of activated sludge," *Water Res.,* vol. 41, no. 5, pp. 1022-1030, 2007.

[66] B. Wu, S. Yi and A. G. Fane, "Effect of Substrate Composition (C/N/P ratio) on Microbial Community and Membrane Fouling Tendency of Biomass in Membrane Bioreactors," *Sep. Sci. Technol.,* vol. 47, p. 440–445, 2012.

[67] B. Wu, S. Yi and A. G. Fane, "Microbial community developments and biomass characteristics in membrane bioreactors under different organic loadings," *Bioresour. Technol.,* vol. 102, no. 13, p. 6808–6814, 2011.

[68] L. De Temmerman, T. Maere, H. Temmink, A. Zwijnenburg and I. Nopens, "Salt stress in a membrane bioreactor: Dynamics of sludge properties, membrane fouling and remediation through powdered activated carbon dosing," *Water Res.,* vol. 63, pp. 112-124, 2014.

[69] S. Arabi and G. Nakhla, "Impact of magnesium on membrane fouling in membrane bioreactors," *Sep. Purif. Technol.,* vol. 67, no. 3, pp. 319-325, 2009.

[70] D. Jang, Y. Hwang, H. Shin and W. Lee, "Effects of salinity on the characteristics of biomass and membrane fouling in membrane bioreactors," *Bioresour. Technol.,* vol. 141, pp. 50-56, 2013.

[71] L. Dvořák, M. Gómez, M. Dvořáková, I. Růžičková and J. Wanner, "The impact of different operating conditions on membrane fouling and EPS production," *Bioresour. Technol.,* vol. 102, no. 13, pp. 6870-6875, 2011.

[72] E. Abdollahzadeh Sharghi and B. B., "The study of organic removal efficiency and halophilic bacterial mixed liquor characteristics in a membrane bioreactor treating hypersaline produced water at varying organic loading rates," *Bioresour. Technol.,* vol. 149, pp. 486-495, 2013.

[73] W. J. Gao, X. Qu, K. T. Leung and L. B. Q., "Influence of temperature and temperature shock on sludge properties, cake layer structure, and membrane fouling in a submerged anaerobic membrane bioreactor," *J. Memb. Sci.,* Vols. 421-422, pp. 131-144, 2012.

[74] S. Lee and M. H. Kim, "Fouling characteristics in pure oxygen MBR process according to MLSS concentrations and COD loadings," *J. Memb. Sci.,* vol. 428, pp. 323-330, 2013.

[75] L. D. Chabalina, J. B. Ruiz, M. R. Pastor and D. P. Rico, "Influence of EPS and MLSS concentrations on mixed liquor physical parameters of two membrane bioreactors,," *Desalination Water Treat.,* vol. 46, pp. 46-59, 2012.

[76] N. Fallah, B. Bonakdarpour, B. Nasernejad and M. R. Alavi Moghadam, "Long-term operation of submerged membrane bioreactor (MBR) for the treatment of synthetic wastewater containing styrene as volatile organic compound (VOC): Effect of hydraulic retention time (HRT)," *J. Hazard. Mater.,* vol. 178, pp. 718-724, 2010.

[77] Z. Ahmed, J. Cho, B. R. Lim, K. G. Song and K. H. Ahn, "Effects of sludge retention time on membrane fouling and microbial community structure in a membrane bioreactor," *J. Memb. Sci.,* vol. 287, no. 2, pp. 311-218, 2007.

[78] L. Jin, S. L. Ong and H. Y. Ng, "Fouling control mechanism by suspended biofilm carriers addition in submerged ceramic membrane bioreactors," *J. Memb. Sci.,* vol. 427, pp. 250-258, 2013.

[79] S. Jamal Khan, C. Visvanathan and V. Jegatheesan, "Effect of powdered activated carbon (PAC) and cationic polymer on biofouling mitigation in hybrid MBRs," *Bioresour. Technol.,* vol. 113, pp. 165-168, 2012.

[80] Y. Tian, Z. Li and L. Y., "Changes in characteristics of soluble microbial products and extracellular polymeric substances in membrane bioreactor coupled with worm reactor: Relation to membrane fouling," *Bioresour. Technol.,* vol. 122, pp. 62-69, 2012.

[81] C. M. Waters and B. L. Bassler, "QUORUM SENSING: Cell-to-Cell Communication in Bacteria,"," *Annu. Rev. Cell Dev. Biol.,* vol. 21, no. 1, pp. 319-346, 2005.

[82] W. R. Galloway, J. T. Hodgkinson, S. D. Bowden, M. Welch and D. R. Spring, "Quorum Sensing in Gram-Negative Bacteria : Small-Molecule Modulation of AHL and AI-2 Quorum Sensing Pathways," *Chem. Rev.,* vol. 44, pp. 28-67, 2011.

[83] J. Huang, Y. Shi, G. Zeng, Y. Gu and C. G., "Chemosphere Acyl-homoserine lactone-based quorum sensing and quorum quenching hold promise to determine the performance of biological wastewater treatments : An overview," *Chemosphere,* vol. 157, pp. 137-151, 2016.

[84] C. H. Tan, K. S. Koh, C. Xie, M. Tay, Y. Zhou, R. Williams, W. J. Ng, S. A. Rice and S. Kjelleberg, "The role of quorum sensing signalling in EPS production and the assembly of a sludge community into aerobic granules," *ISME J.,* vol. 8, no. 6, pp. 1186-1197, 2014.

[85] N. B. Turan, S. D. Chormey, C. Büyükpınar, G. O. Engin and S. Bakirdere, "Quorum sensing: Little talks for an effective bacterial coordination," *Trends Anal. Chem.,* vol. 91, pp. 1-11, 2017.

[86] K. Ponnusamy, D. Paul and J. H. Kweon, "Inhibition of Quorum Sensing Mechanism and Aeromonas hydrophila Biofilm Formation by Vanillin," *Environ. Eng. Sci.,* vol. 26, no. 8, pp. 1359-1363, 2009.

[87] K. Ponnusamy, D. Paul, Y. S. Kim and J. H. Kweon, "2(5H)-Furanone: A prospective strategy for biofouling-control in membrane biofilm bacteria by quorum sensing inhibition," *Brazilian J. Microbiol.,* vol. 41, no. 1, pp. 227-234, 2010.

[88] D. Paul, Y. S. Kim, K. Ponnusamy and K. J. H., "Application of Quorum Quenching to Inhibit Biofilm Formation," *Environ. Eng. Sci.,* vol. 26, no. 8, pp. 1319-1324, 2009.

[89] J. H. Kim, D. C. Choi, K. M. Yeon, S. R. Kim and C. H. Lee, "Enzyme-immobilized nanofiltration membrane to mitigate biofouling based on quorum quenching," *Environ. Sci. Technol.,* vol. 45, no. 4, pp. 1601-1607, 2011.

[90] K. M. Yeon, C. H. Lee and J. Kim, "Magnetic enzyme carrier for effective biofouling

control in the membrane bioreactor based on enzymatic quorum quenching," *Environ. Sci. Technol.,* vol. 43, no. 19, pp. 7403-7409, 2009.

[91] B. Lee, K. Yeon, J. Shim, S. Kim, C. Lee, J. . Lee and J. Kim, "Effective Antifouling Using Quorum-Quenching Acylase Stabilized in Magnetically-Separable Mesoporous Silica," *Biomacromolecules,* vol. 15, no. 4, pp. 1153-1159, 2014.

[92] W. Jiang, S. Xia, J. Liang, Z. Zhang and W. W. Hermanowicz, "Effect of quorum quenching on the reactor performance, biofouling and biomass characteristics in membrane bioreactors," *Water Res.,* vol. 47, no. 1, pp. 187-196, 2013.

[93] H. Kim, H. Oh, S. Kim, K. Lee, K. Yeon, C. Lee, S. Kim and J. Lee, "Microbial population dynamics and proteomics in membrane bioreactors with enzymatic quorum quenching," *Appl. Microbiol. Biotechnol.,* vol. 97, no. 10, pp. 4665-4675, 2013.

[94] A. L. Kim, S. Y. Park, C. H. Lee, C. H. Lee and J. K. Lee, "Quorum quenching bacteria isolated from the sludge of a wastewater treatment plant and their application for controlling biofilm formation," *J. Microbiol. Biotechnol.,* vol. 24, no. 11, pp. 1574-1582, 2014.

[95] W. S. Cheong, C. H. Lee, Y. H. Moon, H. S. Oh, S. R. Kim, S. H. Lee, C. H. Lee and J. K. Lee, "Isolation and identification of indigenous quorum quenching bacteria, Pseudomonas sp. 1A1, for biofouling control in MBR," *Ind. Eng. Chem. Res.,* vol. 52, no. 31, pp. 10554-10560, 2013.

[96] W. S. Cheong, A. R. Kim, S. R. Oh, S. H. Lee, K. M. Yeon, C. H. Lee and J. K. Lee, "Design of quorum quenching microbial vessel to enhance cell viability for biofouling control in membrane bioreactor," *J. Microbiol. Biotechnol.,* vol. 24, no. 1, pp. 97-105, 2014.

[97] S. R. Kim, H. S. Oh, S. J. Jo, K. M. Yeon, C. H. Lee, D. J. Lim, C. H. Lee and J. K. Lee, "Biofouling control with bead-entrapped quorum quenching bacteria in membrane bioreactors: Physical and biological effects," *Environ. Sci. Technol.,* vol. 47, no. 2, pp. 836-842, 2013.

[98] S. Lee, S. H. Lee, K. Lee, H. Kwon, C. H. Nahm, C. Lee, P. K. Park, K. H. Choo, J. K. Lee and H. S. Oh, "Effect of the shape and size of quorum quenching media on biofouling control in membrane bioreactors for wastewater treatment," *J. Microbiol. Biotechnol.,* vol. 26, no. 10, pp. 1746-1754, 2016.

[99] C. Nahm, D. Choi, H. Kwon, S. Lee, S. Hyun, K. Lee, K. Choo and J. L. C. P. P. Lee, "Application of quorum quenching bacteria entrapping sheets to enhance biofouling control in a membrane bioreactor with a hollow fi ber module," *J. Memb. Sci.,* vol. 526, pp.

264-271, 2017.

[100] S. Lee, S. K. Park, H. Kwon, S. H. Lee, K. Lee, C. H. Nahm, S. J. Jo, H. S. Oh, P. K. Park, K. H. Choo, C. H. Lee and T. Yi, "Crossing the Border between Laboratory and Field: Bacterial Quorum Quenching for Anti-Biofouling Strategy in an MBR," *Environ. Sci. Technol.,* vol. 50, no. 4, pp. 1788-1795, 2016.

[101] M. F. Siddiqui, L. Singh, A. W. Zularisam and M. Sakinah, "Biofouling mitigation using Piper betle extract in ultrafiltration MBR," *Desalin. Water Treat.,* vol. 51, no. 37-39, pp. 6940-6951, 2013.

[102] L. Katebian, E. Gomez, L. L. D. Skillman, G. Ho and S. C. Jiang, "Inhibiting quorum sensing pathways to mitigate seawater desalination RO membrane biofouling," *Desalination,* vol. 393, p. 135–143, 2016.

[103] A. D. Eaton, L. S. R. E. W. Clesceri and A. E. Greenberg, Standard Methods for the Examination of Water and Wastewater,, 21st edition, Washington DC: American Public Health Association, 2005.

[104] M. DuBois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and P. A. Smith, "Colorimetric method for determination of sugars and related substances," *Anal. Chem.,* vol. 28, p. 350–356, 1956.

[105] K. Burton, "A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid," *Biochem. J.,* vol. 62, no. 2, pp. 315-324, 1956.

[106] W. Chen, P. Westerhoff, J. A. Leenheer and K. Booksh, "Fluorescence Excitation-Emission Matrix Regional Integration to Quantify Spectra for Dissolved Organic Matter," *Environ. Sci. Technol.,* vol. 37, no. 24, p. 5701–5710, 2003.

[107] T. R. Cataldi, G. Bianco and S. Abate, "Profiling of N-acyl-homoserine lactones by liquid chromatography coupled with electrospray ionization and a hybrid quadrupole linear ion-trap and Fourier-transform ion-cyclotron-resonance mass spectrometry (LC-ESI-LTQ-FTICR-MS).," *J Mass Spectrom.,* vol. 43, no. 1, pp. 82-96, 2008.

[108] K. Tang, Y. Zhang, M. Yu, X. Shi, T. Coenye, P. Bossier and X. H. Zhang, "Evaluation of a new high-throughput method for identifying quorum quenching bacteria," *Sci. Rep.,* vol. 3, no. 1, p. 2935, 2013.

[109] X. Han, Z. Wang, C. Zhu and Z. Wu, "Effect of ultrasonic power density on extracting loosely bound and tightly bound extracellular polymeric substances," *Desalination,* vol. 329, pp. 35-40, 2013.

[110] B. S. Mcswain, R. L. Irvine, M. Hausner and P. a. Wilderer, "Composition and Distribution of Extracellular Polymeric Substances in Aerobic Flocs and Granular Sludge Composition and Distribution of Extracellular Polymeric Substances in Aerobic Flocs and Granular Sludge," *Appl. Environ. Microbiol.,* vol. 71, no. 2, pp. 1051-1057, 2005.

[111] P. D'Abzac, F. Bordas, E. Van Hullebusch, P. N. L. Lens and G. Guibaud, "Extraction of extracellular polymeric substances (EPS) from anaerobic granular sludges: Comparison of chemical and physical extraction protocols," *Appl. Microbiol. Biotechnol.,* vol. 85, no. 5, p. 1589–1599, 2010.

[112] L. Domínguez, M. Rodríguez and D. Prats, "Effect of different extraction methods on bound EPS from MBR sludges. Part I: Influence of extraction methods over three-dimensional EEM fluorescence spectroscopy fingerprint," *Desalination,* vol. 261, no. 1-2, pp. 19-26, 2010.

[113] Y. Tian, L. Chen, S. Zhang, C. Cao and S. Zhang, "Correlating membrane fouling with sludge characteristics in membrane bioreactors: An especial interest in EPS and sludge morphology analysis," *Bioresour. Technol.,* vol. 102, no. 19, pp. 8820-8827, 2011.

[114] H. Lu, Z. Xue, P. Saikaly, S. P. Nunes, T. R. Bluver and W. T. Liu, "Membrane biofouling in a wastewater nitrification reactor: Microbial succession from autotrophic colonization to heterotrophic domination," *Water Res.,* vol. 88, pp. 337-345, 2016.

[115] S. S. Adav and D. J. Lee, "Extraction of extracellular polymeric substances from aerobic granule with compact interior structure," *J. Hazard. Mater.,* vol. 154, no. 1-3, pp. 1120-1126, 2008.

[116] X. Guo, J. Liu and B. Xiao, "Evaluation of the damage of cell wall and cell membrane for various extracellular polymeric substance extractions of activated sludge," *J. Biotechnol.,* vol. 188, pp. 130-135, 2014.

[117] G. P. Sheng and H. Q. Yu, "Characterization of extracellular polymeric substances of aerobic and anaerobic sludge using three-dimensional excitation and emission matrix fluorescence spectroscopy," *Water Res.,* vol. 40, no. 6, pp. 1233-1239, 2006.

[118] B. J. Ni, F. Fang, W. M. Xie, M. Sun, G. P. Sheng, W. H. Li and H. Q. Yu, "Characterization of extracellular polymeric substances produced by mixed microorganisms in activated sludge with gel-permeating chromatography, excitation-emission matrix fluorescence spectroscopy measurement and kinetic modeling," *Water Res.,* vol. 43, no. 5, pp. 1350-1358, 2009.

[119] L. Domínguez Chabaliná, M. Rodríguez Pastor and D. P. Rico, "Characterization of soluble

and bound EPS obtained from 2 submerged membrane bioreactors by 3D-EEM and HPSEC," *Talanta,* vol. 115, p. 706–712, 2013.

[120] X. Pan, J. Liu, D. Zhang, X. I. Chen, L. Li, W. Song and J. Yang, "A comparison of five extraction methods for extracellular polymeric substances (EPS) from biofilm by using three-dimensional excitation-emission matrix (3DEEM) fluorescence spectroscopy," *Water SA,* vol. 36, no. 1, pp. 111-116, 2010.

[121] T. Görner, P. D. Donato, M. H. Ameil, E. Montarges-Pelletier and B. S. Lartiges, "Activated sludge exopolymers: Separation and identification using size exclusion chromatography and infrared micro-spectroscopy," *Water Res.,* vol. 37, no. 10, pp. 2388-2393, 2003.

[122] C. Garnier, T. Görner, B. S. Lartiges, S. Abdelouhab and P. De Donato, "Characterization of activated sludge exopolymers from various origins: A combined size-exclusion chromatography and infrared microscopy study," *Water Res.,* vol. 39, no. 13, p. 3044–3054, 2005.

[123] S. Comte, G. Guibaud and M. Baudu, "Effect of extraction method on EPS from activated sludge: An HPSEC investigation," *J. Hazard. Mater.,* vol. 140, no. 1-2, pp. 129-137, 2007.

[124] D. Bhatia, I. Bourven, S. S. F. Bordas, E. D. v. Hullebusch, S. Rossano, P. N. L. Lens and G. Guibaud, "Fluorescence detection to determine proteins and humic-like substances fingerprints of exopolymeric substances (EPS) from biological sludges performed by size exclusion chromatography (SEC)," *Bioresour. Technol.,* vol. 131, p. 159–165, 2013.

[125] S. Lyko, T. Wintgens, D. Al-Halbouni, S. Baumgarten, D. Tacke, K. Drensla, A. Janot, W. Dott, J. Pinnekamp and T. Melin, "Long-term monitoring of a full-scale municipal membrane bioreactor-Characterisation of foulants and operational performance," *J. Memb. Sci.,* vol. 317, no. 1-2, pp. 78-87, 2008.

[126] J. L. Urrea, S. Collado, P. Oulego and M. Díaz, "Effect of wet oxidation on the fingerprints of polymeric substances from an activated sludge," *Water Res.,* vol. 105, pp. 282-290, 2016.

[127] Y. Liu, Z. Liu, A. Zhang, Y. Chen and X. Wang, "The role of EPS concentration on membrane fouling control: comparison analysis of hybrid membrane bioreactor and conventional membrane bioreactor," *Desalination,* vol. 305, pp. 38-43, 2012.

[128] F. Wang, M. Zhang, W. Peng, H. Y. H. Lin, J. Chen, H. Hong, A. Wang and H. Yu, "Effects of ionic strength on membrane fouling in a membrane bioreactor," *Bioresour. Technol.,* vol. 156, pp. 35-41, 2014.

[129] T. Nittami, H. Tokunaga, A. Satoh, M. Takeda and K. Matsumoto, "Influence of surface hydrophilicity on polytetrafluoroethylene flat sheet membrane fouling in a submerged

membrane bioreactor using two activated sludges with different characteristics.," *J. Memb. Sci.,* vol. 463, p. 183–189, 2014.

[130] Y. X. Shen, K. Xiao, P. Liang, J. Y. Sun, S. J. Sai and X. Huang, "Characterization of soluble microbial products in 10 large-scale membrane bioreactors for municipal wastewater treatment in China," *J. Memb. Sci.,* vol. 415–416, p. 336–345, 2012.

[131] C. Jacquin, G. Lesage, J. Traber, W. Pronk and M. Heran, "Three-dimensional excitation and emission matrix fluorescence (3DEEM) for quick and pseudo-quantitative determination of protein and humic-like substances in full-scale membrane bioreactor (MBR).," *Water Res.,* vol. 118, pp. 82-92, 2017.

[132] R. K. Henderson, N. Subhi, A. Antony, S. J. Khan, K. R. Murphy, G. L. Leslie, V. Chen, R. Stuetz and P. Le-Clech, "Evaluation of effluent organic matter fouling in ultrafiltration treatment using advanced organic characterisation techniques," *J. Memb. Sci.,* vol. 382, pp. 50-59, 2011.

[133] B. Q. Liao, D. G. Allen, I. G. Droppo, G. G. Leppard and S. N. Liss, "Surface properties of sludge and their role in bioflocculation and settleability," *Water Res.,* vol. 35, no. 2, pp. 339-350, 2001.

[134] Z. Wang, J. Cao and F. Meng, "Interactions between protein-like and humic-like components in dissolved organic matter revealed by fluorescence quenching," *Water Res.,* vol. 68, pp. 404-413, 2015.

[135] Y. Xiong, M. Harb and P. Y. Hong, "Characterization of biofoulants illustrates different membrane fouling mechanisms for aerobic and anaerobic membrane bioreactors," *Sep. Purif. Technol.,* vol. 157, pp. 192-202, 2015.

[136] G. R. Aiken, I. Mcknight, I. Thorn and E. M. Thurman, "Isolation of hydrophilic organic acids from water using nonionic macroporous resins," *Org. Geochem.,* vol. 18, pp. 567-573, 1992.

[137] L. L. Wei, K. Wang, Q. L. Zhao, J. Q. Jiang, X. J. Kong and D. J. Lee, "Fractional, biodegradable and spectral characteristics of extracted and fractionated sludge extracellular polymeric substances," *Water Res.,* vol. 46, p. 4387–4396, 2012.

[138] D. Shapiro and E. I. Karavanova, "Fractionation of dissolved organic matter on the XAD resin," *Moscow Univ. Soil Sci. Bull.,* vol. 69, pp. 139-145, 2014.

[139] F. Neemann, S. Rosenberger, B. Jefferson and E. J. McAdam, "Non-covalent protein-polysaccharide interactions and their influence on membrane fouling," *J. Memb. Sci.,* vol. 446, pp. 310-317, 2013.

[140] K. Kimura, K. Tanaka and Y. Watanabe, "Microfiltration of different surface waters with/without coagulation: Clear correlations between membrane fouling and hydrophilic biopolymers," *Water Res.,* vol. 49, pp. 434-443, 2014.

[141] K. Zhang, H. Choi, D. D. Dionysiou, G. A. Sorial and D. B. Oerther, "Identifying pioneer bacterial species responsible for biofouling membrane bioreactors," *Environ. Microbiol.,* vol. 8, no. 3, p. 433–440, 2006.

[142] S. Ishizaki, T. Fukushima, S. Ishii and S. Okabe, "Membrane fouling potentials and cellular properties of bacteria isolated from fouled membranes in a MBR treating municipal wastewater," *Water Res.,* vol. 100, p. 448–457, 2016.

[143] A. Piasecka, C. Souffreau, K. Vandepitte, L. Vanysacker, R. Bilad, T. de Bie, B. Hellemans, L. de Meester, X. Yan, P. Declerck and I. Vankelecom, "Analysis of the microbial community structure in a membrane bioreactor during initial stages of filtration," *Biofouling,* vol. 28, no. 2, pp. 225-238, 2012.

[144] S. Bala Subramanian, S. Yan, R. D. Tyagi and R. Y. Surampalli, "Extracellular polymeric substances (EPS) producing bacterial strains of municipal wastewater sludge: Isolation, molecular identification, EPS characterization and performance for sludge settling and dewatering," *Water Res.,* vol. 44, no. 7, p. 2253–2266, 2010.

[145] S. J. Jo, H. Kwon, S. Y. Jeong, C. H. Lee and T. G. Kim, "Comparison of microbial communities of activated sludge and membrane biofilm in 10 full-scale membrane bioreactors," *Water Res.,* vol. 101, pp. 214-225, 2016.

[146] D. J. Miller, S. Kasemset, D. R. Paul and B. D. Freeman, "Comparison of membrane fouling at constant flux and constant transmembrane pressure conditions," *J. Memb. Sci.,* vol. 454, p. 505–515, 2014.

[147] Z. Zhou, F. Meng, X. He, S. R. Chae, Y. An and X. Jia, "Metaproteomic analysis of biocake proteins to understand membrane fouling in a submerged membrane bioreactor," *Environ. Sci. Technol.,* vol. 49, no. 2, p. 1068–1077, 2015.

[148] D. W. Gao, Z. D. Wen, B. Li and H. Liang, "Microbial community structure characteristics associated membrane fouling in A/O-MBR system," *Bioresour. Technol.,* vol. 154, p. 87–93, 2014.

[149] L. N. Huang, H. De Wever and L. Diels, "Diverse and distinct bacterial communities induced biofilm fouling in membrane bioreactors operated under different conditions," *Environ. Sci. Technol.,* vol. 42, no. 22, p. 8360–8366, 2008.

[150] B. K. Hwang, W. N. Lee, P. K. Park, C. H. Lee and I. S. Chang, "Effect of membrane

fouling reducer on cake structure and membrane permeability in membrane bioreactor," *J. Memb. Sci.,* vol. 288, no. 1-2, p. 149–156, 2007.

[151] A. Klindworth, E. Pruesse, T. Schweer, J. Peplies, C. Quast, M. Horn and F. O. Glöckner, "Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies," *Nucleic Acids Res.,* vol. 41, no. 1, pp. 1-11, 2013.

[152] T. N. P. Nguyen, Y. C. Su, J. R. Pan and C. Huang, "Comparison of membrane foulants occurred under different sub-critical flux conditions in a membrane bioreactor (MBR)," *Bioresour. Technol.,* vol. 166, p. 389–394, 2014.

[153] S. Lim, S. Kim, K. M. Yeon, B. I. Sang, J. Chun and C. H. Lee, "Correlation between microbial community structure and biofouling in a laboratory scale membrane bioreactor with synthetic wastewater," *Desalination,* vol. 287, pp. 209-215, 2012.

[154] D. Hu, Z. Zhou, X. Shen, H. Wei, L. M. Jiang and Y. Lv, "Effects of alkalinity on membrane bioreactors for reject water treatment: Performance improvement, fouling mitigation and microbial structures," *Bioresour. Technol.,* vol. 197, pp. 217-226, 2015.

[155] D. G. Weissbrodt, N. Shani and C. Holliger, "Linking bacterial population dynamics and nutrient removal in the granular sludge biofilm ecosystem engineered for wastewater treatment," *FEMS Microbiol. Ecol.,* vol. 88, no. 3, p. 579–595, 2014.

[156] J. Choi, E. S. Kim and Y. Ahn, "Microbial community analysis of bulk sludge/cake layers and biofouling-causing microbial consortia in a full-scale aerobic membrane bioreactor," *Bioresour. Technol.,* vol. 227, pp. 133-141, 2017.

[157] N. M. Shchegolkova, G. S. Krasnov, A. A. D. A. A. Belova, S. L. Kharitonov, K. M. Klimina, N. V. Melnikova and A. V. Kudryavtseva, "Microbial community structure of activated sludge in treatment plants with different wastewater compositions," *Front. Microbiol.,* vol. 7, pp. 1-15, 2016.

[158] S. Xia, J. Li, S. He, K. Xie, X. Wang, Y. Zhang, L. Duan and Z. Zhang, "The effect of organic loading on bacterial community composition of membrane biofilms in a submerged polyvinyl chloride membrane bioreactor," *Bioresour. Technol.,* vol. 101, no. 7, pp. 6601-6609, 2010.

[159] H. V. Phan, F. I. Hai, I. Zhang, J. Kang, W. E. Price and L. D. Nghiem, "Bacterial community dynamics in an anoxic-aerobic membrane bioreactor - Impact on nutrient and trace organic contaminant removal," *Int. Biodeterior. Biodegrad.,* vol. 109, pp. 61-72, 2016.

[160] A. Valle, M. J. Bailey, A. S. Whiteley and M. Manefield, "N-acyl-L-homoserine lactones

(AHLs) affect microbial community composition and function in activated sludge," *Environ. Microbiol.,* vol. 6, no. 4, pp. 424-433, 2004.

[161] C. H. Tan, K. S. Koh, C. Xie, J. Zhang, X. H. Tan, G. P. Lee, Y. Zhou, W. J. Ng, S. Rice and S. Kjelleberg, "Community quorum sensing signalling and quenching: microbial granular biofilm assembly," *npj Biofilms and Microbiomes,* vol. 1, pp. 1-9, 2015.

[162] H. Yu, G. Xu, F. Qu, G. Li and H. Liang, "Effect of solid retention time on membrane fouling in membrane bioreactor: from the perspective of quorum sensing and quorum quenching," *Appl. Microbiol. Biotechnol.,* vol. 100, no. 18, p. 7887–7897, 2016.

[163] Y. Ding, Y. Tian, Z. Li, W. Zuo and J. Zhang, "A comprehensive study into fouling properties of extracellular polymeric substance (EPS) extracted from bulk sludge and cake sludge in a mesophilic anaerobic membrane bioreactor," *Bioresour. Technol.,* vol. 192, pp. 105-114, 2015.

[164] J. Chen, B. Gu, E. J. LeBoeuf, H. Pan and S. Dai, "Spectroscopic characterization of the structural and functional properties of natural organic matter fractions," *Chemosphere,* vol. 48, no. 1, pp. 59-68, 2002.

[165] Y. Miao, X. Guo, W. Jiang, X. X. Zhang and B. Wu, "Mechanisms of microbial community structure and biofouling shifts under multivalent cations stress in membrane bioreactors," *J. Hazard. Mater.,* vol. 327, pp. 89-96, 2017.

[166] E. Szabó, R. Liébana, M. Hermansson, O. Modin, F. Persson and B. M. Wilén, "Microbial Population Dynamics and Ecosystem Functions of Anoxic/Aerobic Granular Sludge in Sequencing Batch Reactors Operated at Different Organic Loading Rates," *Front. Microbiol.,* vol. 18, pp. 1-14, 2017.

[167] J. H. Choo, Y. Rukayadi and J. K. Hwang, "Inhibition of bacterial quorum sensing by vanilla extract," *Lett. Appl. Microbiol.,* vol. 42, no. 6, pp. 637-641, 2006.

[168] S. Kappachery, D. Paul, J. Yoon and J. H. Kweon, "Vanillin, a potential agent to prevent biofouling of reverse osmosis membrane," *Biofouling,* vol. 26, no. 6, pp. 667-672, 2010.

[169] E. Masai, Y. Yamamoto, T. Inoue, K. Takamura, H. Hara, D. Kasai, Y. Katayama and M. Fukuda, "Characterization of ligV Essential for Catabolism of Vanillin by Sphingomonas paucimobilis SYK-6," *Biosci. Biotechnol. Biochem.,* vol. 71, no. 10, pp. 2487-2492, 2007.

[170] H. P. Chen, M. Chow, C. C. Liu, A. Lau, J. Liu and L. D. Eltis, "Vanillin catabolism in Rhodococcus jostii RHA1," *Appl. Environ. Microbiol.,* vol. 78, no. 2, pp. 586-588, 2012.

[171] OECD SIDS, "Vanilin," UNEP, Geneva, 1996.

[172] N. O. Yigit, I. Harman, G. Civelekoglu, H. Koseoglu, N. Cicek and M. Kitis, "Membrane

fouling in a pilot-scale submerged membrane bioreactor operated under various conditions," *Desalination,* vol. 231, no. 1-3, pp. 124-132, 2008.

[173] F. Guo, S. H. Zhang, X. Yu and B. Wei, "Variations of both bacterial community and extracellular polymers: The inducements of increase of cell hydrophobicity from biofloc to aerobic granule sludge," *Bioresour. Technol.,* vol. 102, no. 11, pp. 6421-6428, 2011.

[174] Q. Zhang, Y. Jie, W. Loong, J. Zhang, A. G. Fane, S. Kjelleberg, S. A. Rice and D. McDougald, "Characterization of biofouling in a lab-scale forward osmosis membrane bioreactor (FOMBR)," *Water Res.,* vol. 58, pp. 141-151, 2014.

[175] V. Nagaraj, L. Skillman, G. Ho, D. Li and A. Gofton, "Characterisation and comparison of bacterial communities on reverse osmosis membranes of a full-scale desalination plant by," *npj Biofilms and Microbiomes,* vol. 3, no. 1, p. 13, 2017.

[176] L. Bereschenko, G. Heilig, M. Nederlof, M. van Loosdrecht, A. Stams and G. Euverink, "Molecular Characterization of the Bacterial Communities in the Different Compartments of a Full-Scale Reverse-Osmosis Water Purification Plant," *Appl. Environ. Microbiol.,* vol. 74, no. 17, p. 5297–5304, 2008.

[177] J. Luo, P. Lv, J. Zhang, A. G. Fane, D. McDougald and S. A. Rice, "Succession of biofilm communities responsible for biofouling of membrane bioreactors (MBRs)," *PLoS ONE,* vol. 12, no. 7, pp. 1-23, 2017.

[178] S. J. Jo, H. Kwon, S. Y. Jeong, S. H. Lee, H. S. Oh, T. Yi and C. H. Lee, "Effects of Quorum Quenching on the Microbial Community of Biofilm in an Anoxic/Oxic MBR for Wastewater Treatment," *J. Microbiol. Biotechnol,* vol. 26, no. 9, p. 1593–1604, 2016.

[179] I. Wagner-Döbler, V. Thiel, L. Eberl, M. Allgaier, A. Bodor, S. Meyer, A. Ebner, A. Hennig, R. Pukall and S. Schulz, "Discovery of complex mixtures of novel long-chain quorum sensing signals in free-living and host-associated marine alphaproteobacteria," *ChemBioChem,* vol. 6, no. 12, pp. 2195-2206, 2005.

[180] J. Lv, Y. Wang, C. Zhong, Y. Li, W. Hao and J. Zhu, "The microbial attachment potential and quorum sensing measurement of aerobic granular activated sludge and flocculent activated sludge," *Bioresour. Technol.,* vol. 151, pp. 291-296, 2014.

[181] X. Su, Y. Tian, H. Li and C. Wang, "New insights into membrane fouling based on characterization of cake sludge and bulk sludge: an especial attention to sludge aggregation," *Bioresour. Technol.,* vol. 128, pp. 586-592, 2013.

[182] T. P. Nguyen, N. P. Hankins and N. Hilal, "A comparative study of the flocculation behaviour and final properties of synthetic and activated sludge in wastewater treatment,"

Desalination, vol. 204, no. 1-3, pp. 277-295, 2007.

[183] S. S. Branda, Å. Vik, L. Friedman and R. Kolter, "Biofilms: The matrix revisited," *Trends in Microbiol.,* vol. 13, no. 1, pp. 20-26, 2005.

[184] A. C. Fonseca, R. S. Summers, A. R. Greenberg and M. T. Hernandez, "Extra-cellular polysaccharides, soluble microbial products, and natural organic matter impact on nanofiltration membranes flux decline," *Environ. Sci. Technol.,* vol. 41, no. 7, pp. 2491-2497, 2007.

[185] Z. Geng and E. Hall, "A comparative study of fouling-related properties of sludge from conventional and membrane enhanced biological phosphorus removal processes," *Water Res.,* vol. 41, no. 19, pp. 4329-4338, 2007.

[186] J. Ma, Z. Wang, Y. Yang, X. Mei and Z. Wu, "Correlating microbial community structure and composition with aeration intensity in submerged membrane bioreactors by 454 high-throughput pyrosequencing," *Water Res.,* vol. 47, no. 2, pp. 859-869, 2013.

[187] J. R. Pan, Y. C. Su, C. Huang and H. C. Lee, "Effect of sludge characteristics on membrane fouling in membrane bioreactors," *J. Memb. Sci.,* vol. 349, no. 1-2, p. 287–294, 2010.