Evaluation of toluene transformation in an Extractive Membrane Bioreactor (EMBR)

Loh, Aron Shu Tien

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Evaluation of toluene transformation in an Extractive Membrane Bioreactor (EMBR)

ARON LOH SHU TIEN

SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING

2020

Evaluation of toluene transformation in an Extractive Membrane Bioreactor (EMBR)

ARON LOH SHU TIEN

School of Civil and Environmental Engineering

**A Thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Master of Engineering**

2020

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarized materials, and has not been submitted for a higher degree to any other University or Institution.

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Aron Loh Shu Tien

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I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

18 June 2020

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Date

Associate Professor, Zhou Yan

Authorship Attribution Statement

This thesis contains material from 0 number paper(s) published in the following peer reviewed journal(s) where I was the first and/or corresponding author.

The contributions of the co-authors are as follow:

- Associate Professor Zhou Yan provided guidance and project direction.
- All sample preparation was conducted by me in AEBC for chemical analysis and microscopy.
- I prepared the draft of the manuscript. The manuscript was revised by Associate Professor Zhou Yan and Professor Wang Rong
- I conducted the fabrication of synthetic membrane in SMTC
- Lim Huai Xun Josh assisted on the fabricating the support layer of the membrane
- Dr Jin Mengyi taught me on the fabrication of ZIF-8@PDMS/PVDF membrane

18 June 2020

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Date

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Aron Loh Shu Tien

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Summary

This thesis presents the findings of a lab-scale microaerobic Extractive Membrane Bioreactor (EMBR) on toluene treatment under oxygen limiting condition (no aeration). A comparison study was carried out between fabricated 4wt% ZIF-8@PDMS/PVDF membrane and commercial PDMS membrane, in terms of overall flux performance and biofilm formation.

This membrane bioreactor utilizes a non-porous membrane as a barrier between the wastewater and the biological growth medium. The membrane extracts the targeted organic compounds from the wastewater feed and transport into the opposite side of the aqueous bio medium, which allows biodegradation to take place without exposing to the hostile composition of the wastewater.

The lab-scale microaerobic EMBR's maximum toluene elimination capacity per effective membrane area was found to be 1.18 and 1.34 g/m² hour on the commercial and synthetic membrane, respectively. The synthetic membrane was found to outperform the commercial membrane throughout the experiment. The commercial PMDS membrane with 100 μm thickness shows a mass transfer coefficient, k_o of $2.6 \pm 0.6 \times 10^{-5}$ m/s. Similarly, a 4wt% ZIF-8@PDMS/PVDF membrane with an average thickness of 13.7 ± 0.7 μm shows a mass transfer coefficient, k_o of $3.4 \pm 0.3 \times 10^{-5}$ m/s. As the experiment progress, the growth of the biofilm on the membrane surface formed a layer of resistance which reduces the mass transfer of toluene. The formation of biofilm reduced the overall mass transfer by 60~70%, and signs of decreasing toluene removal efficiency were noted after operation for 17 days. Thus, cleaning was conducted to recover the mass transfer performance, and it was operated for another 17 days again. Based on the microbial community results, *Zoogloea* and *Acidovorax* were found to the main toluene degraders in the system. The feasibility of operating the EMBR in a low energy and operation cost is possible, by eliminating the need for aeration and using mixed culture.

CHAPTER 1: Introduction

1.1 Background

Over the years, the global oil demand has been increasing despite the effort to use renewable and sustainable resources for substitution and with the United States being the largest consumer in 2018 (Agency, 2019). As a result, the use of aromatic chemical compounds like Benzene, Toluene, Ethylbenzene and Xylene (BTEX) have been increasing drastically (Dobashi et al., 2019). The major source of benzene, toluene and xylenes are produced from petroleum naphtha, which contributes about 70% of the world's supply reported by Meyers (2004). However, these chemical compounds can be found in the environment due to improper disposal of industrial effluent, accident events like oil spills, leaks from pipelines and storage tanks (Mitra and Roy, 2011, Liu et al., 2005). Hence, there are cases reported that BTEX can be found in contaminated groundwater, surface water and drinking water sources (Fayemiwo et al., 2017, Mitra and Roy, 2011).

When gasoline was spilt in water, 90% of the gasoline components in the water-soluble fraction are found to be BTEX reported by Mitra and Roy (2011). Thus, water is a common reagent used in petroleum and petrochemical industries for distillation, extraction, cooling and washing (Walker et al., 2013). Apart from that, produced waters from oil and natural gas refineries are commonly used to maintain reservoir pressure. Moreover, it is added with chemical additives to enhance the oil/water separation process. As a result, the produced waters contain a high amount of monoaromatics compounds like BTEX and inorganic compounds. The concentration level of these compounds varies depending is an offshore or onshore platform (Veil et al., 2004). These oil extraction activities are water-intensive and were reported 3 to 20 times more amount of water is used to produce oil, the ratio varies from well to well and the life of the well (Stephenson, 1992, Larson, 2018, Dórea et al., 2007). Hence, if the produced water is left untreated and discharge into the environment, it can cause toxicity effects on living organisms (Veil et al., 2004).

Another concerning factor was that BTEX were classified as volatile organic compounds (VOCs), emission sources from gas stations, surface coating, electronic manufacturing, household products, petroleum products and exhaust from

automobiles which posed adverse harmful effects on the people and the environment (Barros et al., 2019, Kuranchie et al., 2019, Shuai et al., 2018, Lin et al., 2017, Lim et al., 2014, Shen et al., 2018, Lan and Minh, 2013). They are commonly found in household products like paints, cosmetics, glue, rubber and plastics (Bolden et al., 2015, Sack et al., 1992). Due to their volatility and solubility, there were studies reported about the environment contaminations of BTEX in surface water, groundwater, soil and air (CDW, 2014, Do Rego and Pereira Netto, 2007, WHO, 2005, Habeebullah and Hassanien, 2012).

Based on their toxicological profile, prolonged exposure to BTEX can cause neurological impairment, harmful effects on respiratory system and organs failure (ATSDR, 2017, ATSDR, 2010, ATSDR, 2007, Mitra and Roy, 2011). Aside from that, studies have shown that BTEX are genotoxic compounds which cause oxidative stress damage to the human body, chromosomal alterations and increase the potential risk of DNA damage due to long term exposure (Xiong et al., 2016, Makwela, 2013, Villalba-Campos et al., 2016).

It has been a considerable challenge for petroleum industries and oil refineries for their treated effluents or produced water to meet the discharge limits, in order to prevent the possible harmful impact done to the environment and people, especially BTEX (Fayemiwo et al., 2017, Radelyuk et al., 2019). The stringent discharge guidelines that were established by the environmental protection agencies highlight the toxicity of untreated effluent from petroleum industries. For example, the acceptable limits for BTEX in drinking water are Benzene 10 µg/L, Toluene 700 µg/L, Ethylbenzene 300 µg/L and Xylene 500 µg/L as established by WHO (2008). The maximum discharge limit for oil/grease compounds is 42 mg/L and 29 mg/L on average in produced water EPA (2017).

Despite the implementation of environmental regulations, oil refineries in developing and developed countries continue to discharge their untreated wastewater into the natural water bodies partly because there is no incentive for efficient wastewater treatment reported (Radelyuk et al., 2019). The conventional wastewater treatment process in most refineries consists of the primary, secondary and tertiary treatment process, including hydrocyclone, dissolved air flotation,

activated carbon filters, microfiltration and ultrafiltration, etc. (Larson, 2018, Arthur et al., 2005).

However, the disadvantages of these conventional treatment processes are energy-intensive and have a high maintenance cost (Li et al., 2013). Furthermore, Aljuboury et al. (2017) reported that the use of physical treatment technology is often accompanied by pretreatments, such as chemical coagulation to handle the complexity of petroleum wastewater. Many technologies were explored to obtain effective removal of COD and BOD in petroleum wastewater, such as membrane separation technology, biological and chemical processes. Aljuboury et al. (2017) further mentioned that membrane separation treatment process has found to be effective compared to conventional physical treatment processes, by using specific pore size filtering medium to intercept the large size particles and allows water to pass through. Aside from that, it removes the need of chemical dosing which leads to an economic approach (Salahi et al., 2015, Li et al., 2013, Nasiri et al., 2017). To further improve its performance, it was designed in a combination of the conventional biological wastewater treatment process, which also known as the membrane bioreactor (MBR). Hence, with the right operation optimisation on the MBR system, they can perform effectively in BOD, COD and nitrogen removal.

There are numerous studies conducted on the treatment of petroleum wastewater using MBR, and the results show high removal of COD, BOD, TOC, TSS and VSS (Razavi and Miri, 2015, Ayseasatekin and Mayes, 2009, Hua et al., 2007). A hollow fibre membrane bioreactor (HF-MBR) study conducted by Razavi and Miri (2015) on petroleum refinery wastewater, showing an efficiencies removal of COD 82%, BOD 89%, TSS 98% and VSS 99%. Another study conducted by Scholz and Fuchsm (2000) was able to obtain a 99.99 % hydrocarbon removal on oily wastewater with HRT of 13.3 hours. Mafirad et al. (2011) reported 95 % COD overall removal treating synthetic oily wastewater.

However, the major drawback of these pressure-driven processes is membrane fouling which leads to high cleaning cost and lowers the lifetime of the membrane (Aljuboury et al., 2017). The cause of membrane fouling was due to oil disposition which resulted in the blockage of membrane pores (Marco Capodici et al., 2017).

In addition, the petroleum refineries effluent's quality has become complex with a high concentration of heavy metals and salts, especially produced water which contains additives resulted in a TDS ranging from 2000 – 150000 mg/L (Arthur et al., 2005). Besides that, the pH of the produced water varies on the types of the reservoir and the geological fields, which can be acidic if it is from a natural gas platform, ranging from 3.5 – 5.5 reported by (Veil et al., 2004). Produced water from onshore refineries reported by Mueller et al. (1996) contains about 50-1000 mg/L of oil and grease, and 50-350 mg/L of TSS. As for produced water from oil fields, Chakrabarty et al. (2008) reported at a range of 100 – 100 mg/L or more of oil and grease. As a result, this complex feed poses an upset risk to the biological reactor, causing a potential risk of microbes inhibition or inactivation.

Fakhru'l-Razi et al. (2010) reported that TDS greater than 1000 mg/L in produced water can reduce pollutant removal efficiencies significantly. Another study conducted by Reid et al. (2006) stated when an immersed bioreactor was exposed to 5000 mg/L of salt, it causes an inhibitory effect of salt shocking to COD and BOD removal. Apart from that, Mannina et al. (2016) showed that when a MBR system which acclimatized to 10 g/L of NaCl exposed to 20 g/L of NaCl and 20 mg/L of total petroleum hydrocarbons (TPH), the COD removal dropped was due to the inhibitory effect caused by salinity and hydrocarbons. Hence, the complexity of the produced water and petroleum wastewater has been the main challenge for petroleum refineries, due to the high amount of TDS and recalcitrant compounds which affects the biokinetics on sludge growth, characteristics and removal performance (Zaffaroni et al., 2016).

In order to tackle this issue, a pre-treatment of the chemical or physical process is required to remove the inhibitory compounds before sending into the biological treatment process. As a result, it will incur additional operation cost in pretreatment and capital cost of having multiple treatment units (Zaffaroni et al., 2016). Hence, the selection of treatment processes for refineries to be sustainable and viable are based on considerations like, the complexity of the wastewater, a define effluent quality to meet, operation cost and capital cost (Zaffaroni et al., 2016, Aljuboury et al., 2017, Li et al., 2013).

An EMBR system was introduced by Livingston (1993a) to overcome this situation by being a point source biological treatment process. The performance of the EMBR system depends on the microbial activity, the partition coefficient of the targeted organic compound in between phases (feed solution, receive solution and membrane), the chemical and physical properties of the membrane and the hydrodynamic boundary layers on both sides (Cocchini et al., 1999). Its unique feature minimises the abrupt organic loading exposure to the microbes in a controlled environment, by implementing hydrophobic polydimethylsiloxane (PDMS) membrane between the feed side and biological unit (also known as the receive side), which allows permeation of targeted organic pollutant. This system is driven by the concentration difference of the targeted organic pollutant between the feed and receive side. Over time, a layer of biofilm is formed on the surface of the membrane facing the receive side, which aids in removing the organic pollutants. Hence, there is a steep concentration gradient across from the feed side to the receiving side, allowing the targeted organic pollutant compounds to be diffused across the membrane and biofilm in a controlled manner. Moreover, this system avoids direct contact between the biomedium and wastewater feed. Thus, the microbial activity will not be affected by the toxic compounds, like heavy metals, salts and even high or low pH condition, provided the membrane's chemical resistance is compatible with the feed. This unique feature allows the implementation of EMBR in "direct" treatment (Livingston, 1994).

In short, BTEX contamination in the environment water bodies and drinking water is a known severe issue. It poses potentially adverse health effects when people are exposed over the acceptable limit through inhalation and/or oral consumption. Furthermore, people are unknowingly exposed to these compounds daily. Emission pathways from cosmetics, paints, rubber, plastic, vehicle exhaust and gas stations are the sources. On the other hand, it has been a challenge for petrochemical and refineries to find a sustainable and effective treatment on their oily wastewater and produced water to meet the stringent guidelines established by the environmental agencies. However, the current treatment technologies used in refineries pose a high operating and maintenance cost, which is not a sustainable approach in handling the complex influent. Besides that, the membrane separation process like MBR has its

limitations like membrane fouling, inhibitory shock on microbial activity, and pretreatment is required. As a result, it leads to high chemical cleaning cost, high capital cost due to the additional pretreatment processes and shortens the lifetime of the membrane. Although the MBR has its limitations, it is still commonly used as a treatment process in refineries. The EMBR is introduced to overcome this current issue. The EMBR's unique feature has a potential for treating oily wastewater or produced water as a point source biological treatment process without the need of any pretreatment unit.

Thus, this thesis will present the findings and data to determine whether the EMBR can be a feasible and sustainable wastewater treatment option for petroleum industries and refineries. The aims of this study are to (1) evaluate the toluene removal performance in a microaerobic (no aeration) EMBR system using commercial and a novel synthetic membrane, (2) determine the mass transfer coefficient k_o and the recovery of k_o using chemical cleaning, (3) understand the microbial profile and dynamics, and (4) identify degradation by-products and degradation pathways.

Organization of thesis

This thesis is written in a total of 7 chapters. The highlights and scope of each chapter are mentioned below:

Chapter 1:

This chapter emphasises the serious problem of BTEX contaminations, harmful health effects and its sources. The major challenges faced by petrochemical industries and refineries on treating oily wastewater and produced water with the current treatment technologies. In addition, a brief introduction of EMBR, and the objectives and highlights of this study are presented.

Chapter 2:

This chapter will focus on the toxicology of toluene on humans and animals, biodegradability of toluene, introduction of EMBR and introduction of ZIF-8 PDMS@PVDF membrane.

Chapter 3:

The details of the experimental setup, system operating conditions and procedures and analytical methods will be mentioned in this chapter.

Chapter 4:

The microbial inhibition effects of toluene at different toluene concentration levels. The identification of toluene intermediate byproducts and the proposed biodegradation pathways are presented in this chapter.

Chapter 5:

A comparison study between commercial and synthetic PDMS membranes, in terms of toluene removal, overall mass transfer coefficient and the recovery of overall mass transfer coefficient after chemical cleaning, was carried and reported.

Chapter 6:

The biofilm profile on both membranes, in terms of thickness, viable biomass percentage, morphology, EPS composition, roughness, adhesive energy and the organic composition of the biofilm, was discussed.

Chapter 7:

The last chapter summarises the findings and concludes the outcome of this study. More importantly, the recommendations for future study and the feasibility of using EMBR as an industrial application are proposed.

CHAPTER 2: Literature review

Outline:

- 2.1. Toluene
- 2.2. EMBR
- 2.3. ZIF-8 PDMS@PVDF membrane
- 2.4. Summary of literature review
- 2.5. Scope and objectives

2.1. Toluene

2.1.1 Environmental fate

Toluene is a human-made monoaromatic hydrocarbon compound which can be found mainly in petroleum products (EPA, 1994). In the environment, it can be found in coal and petroleum. Toluene is widely used in many products like gasoline blending, paints, cosmetics, adhesives, production of rubber and plastics. It is also a common raw feedstock chemical used in the synthesis of chemicals like trinitrotoluene, benzoic acid, benzyl chloride, phenol and benzene (WHO, 2004, EPA, 1994). It was reported as the top 10 chemicals found in consumer products by EPA (2014). Furthermore, it is a colourless flammable liquid and contains a distinct benzene-like smell (Canada, 1992, ATSDR, 2017). Aside from that, it is classified as a volatile organic compound which volatilizes quickly into the atmosphere, due to emitting sources from automobile exhaust and cigarettes. Hence, it is common to detect toluene in urban areas where there is heavy traffic. There have been numerous reports stating that toluene can be found in air, surface water, groundwater and soil (ATSDR, 2017, WHO, 2004).

It was reported by EPA (1994) that 86 % of the produced toluene would end up in the biosphere and lifetime expected to be from 4 days to several months. In Canada, the atmospheric release of toluene from solvent use and emission from automobiles account for 51% and 38% respectively (Canada, 1992). A groundwater toluene contamination study was conducted in Portugal, and results reported that 93.5 % of the water samples exceed the guidelines for drinkable water 700 ug/L, it was due to

illegal discharge from industries (Barberes et al., 2018). In addition, there are cases of toluene in groundwater from industrially polluted sites found to be as high as 3500 ug/L (Leusch and Bartkow, 2010). Once toluene is released into the environment waters, it can be biodegraded aerobically or anaerobically depending on the presence of the toluene degrading microorganisms (Parales et al., 2008).

Table 1: Physical and chemical properties of toluene

Properties	Value
Molecular weight (g/mol)	92.14
Melting point (°C)	95 -94.5
Boiling point at 760 mm Hg (°C)	110.6
Density (g/cm ³)	
At 25°C	0.8623
At 20°C	0.8667
Colour	Clear
Odour	Benzene-like
Solubility (mg/L)	
In water at 20°C	500
Vapour pressure (mm Hg)	
At 20°C	21.9
At 30°C	36.7
At 40°C	59.3
Flashpoint (°C)	4.4
Odor threshold (mg/L)	0.024
Taste threshold (mg/L)	0.14

Modified from (EPA, 1994, Alexander et al., 1982)

There are many ways that toluene can enter the human body from the contaminated air, water and soil, via inhalation, ingestion and skin adsorption (ATSDR, 2017). Even though a major amount of the toluene will be removed from the body via metabolizing into less harmful compounds such as hippuric acid, but a small amount will be accumulated in the body fat issue depending on the exposure frequency (ATSDR, 2017).

2.1.2 Toxicity of toluene

The harmful effects of toluene have been well studied on both animals and humans. Toluene is classified as a neurotoxin, irritants and genotoxic compound (Timothy

and Lily, 2012, Kuranchie et al., 2019). The oral LD50 for rats is between 2.6 to 7.5 g/kg body weight, repeatedly exposing to toluene can cause central nervous system depression and potential adverse effects on liver, kidney, lungs and reproductive effects (Canada, 1992, ATSDR, 2017). Even though toluene is not considered carcinogenic, but it can inhibit cells growth and pose several detrimental health effects on animals and humans (Ekpenyong and Asuquo, 2017, Kuranchie et al., 2019).

The symptoms of nervous systems effects can be temporary and/or permeant. The signs of temporary health effects, such as headaches, dizziness, tiredness, nausea and loss of appetite, which affects day to day work productivity. A study conducted by Echeverria et al. (1989) reported that there is an increasing pattern of incidence of sleep and headache in the test subjects when exposed to toluene levels of 75 mg/L (283 mg/m³) and 150 mg/L (566 mg/m³). Moreover, another study reported by Ekpenyong and Asuquo (2017) that occupation exposure between 100 to 150 mg/L of toluene can lead to short-term memory loss, reduced dexterity and reduced vigilance. Furthermore, signs of permeant health effects, such as impaired the ability to think, hearing loss, colour vision loss, decreased mental ability and even death at high exposure level. Besides that, inhaling a significant amount of toluene during the pregnancy period can lead to mental and growth retardation in the children (WHO, 2000, ATSDR, 2017).

Studies had reported on the health effects of petrol pump attendants at the gas station. Petrol pump attendants had higher oxidative stress damage and signs of genotoxicity which leads to DNA damage, due to a higher exposure risk to toluene from the gasoline (Xiong et al., 2016, Ekpenyong and Asuquo, 2017). Makwela (2013) reported that there is a distinct relation between job exposure and DNA damage.

There are numerous studies had reported on the toxicity effects of toluene in the environment, which affects several species at different trophic levels, such as algae and fish and amphibians in the aquatic environment. For freshwater phytoplankton species such as *Skeletonema costatum*, *Amphidinium carterae*, *Cricosphaera*

carterae, and *Dunaliella tertiolecta* show signs of slower growth with increasing toluene concentration levels greater than 10 mg/L, reported by Dunstan et al. (1975).

Fish species such as the rainbow trout, *Oncorhynchus mykiss*, and coho salmon, *Oncorhynchus kisutch*, have a 96-hour LC₅₀ of 5.8 mg/L and 5.5 mg/L, respectively (Galassi et al., 1988, Moles et al., 1981). Moles et al. (1981) reported that the weight and length of the coho salmon decreased with increasing concentrations of toluene, which indicates the signs of growth inhibition; the lowest-observable-effect-concentration (LOEC) and no-observed-effect-concentration (NOEC) reported 2.8 mg/L and 1.4 mg/L, respectively. A long term continuous toluene exposure study conducted by Black et al. (1982), found that the LC_{50s} for continuous exposure were 0.02 mg/L for trout, 0.39 mg/L for a frog, and 0.85 mg/L for a salamander.

The effects of toluene on microorganisms are well studied. Due to the partition coefficient of toluene (hydrophobic nature), it can be easily adsorbed into the cell membrane (Sikkemat et al., 1994). Thus, it leads to an accumulation of toluene which disrupts the cell membrane functions, like increasing the membrane fluidity, swelling of membrane walls, increasing the permeability of the membrane for ions and alter the phospholipid composition of the membrane (Sikkemat et al., 1994, Calderón-Guzmán et al., 2005). Only certain toluene tolerant strains can adapt and utilized toluene as a carbon source for energy. Weber (1995) reported that *Pseudomonas putida* strains in response to the toxicity of toluene, these strains change the fatty acid composition of the cell membrane into high trans/cis ratio of the unsaturated fatty acids, which alters the partition coefficient of toluene in the membrane.

2.1.3 Removal of toluene in wastewater technologies

There are several wastewater treatment technologies used in petrochemical and refineries. However, each different treatment applications has its pros and cons. This literature review will focus on the current wastewater treatment processes and challenges faced on toluene removal.

The composition of produced water consists of aromatic compounds (BTEX), dissolved organics, suspended solids, microorganisms, dissolved gases and high

amount of salt (Nasiri et al., 2017). The complexity of the produced water makes it challenging to meet the environmental regulations or for reusing purpose. Hence, the selection of treatment technologies depends on, the characteristics of feed, a define water quality to meet, cost and sustainability for plant operation (Zaffaroni et al., 2016).

The common treatment method is to remove the TDS from the feed first, by using pretreatment processes such as thermal, ion-exchange or membrane technologies. However, these treatment technologies usually incur high operation cost, high energy consumption and disposal of brine solution (Nasiri et al., 2017).

A study conducted by Yu et al. (2018), using a MBR on synthetic oil refinery wastewater consisting 20 mg/L of toluene shows high toluene removal efficiency of more than 99% with a SRT greater than 10 days. However, the study also did mention that the drawback of using MBR was the membrane fouling due to proliferation of bulking and foaming bacteria.

Sahar Saghafi (2010) reported the toluene removal was 65% and 80% with a HRT of 24 hours and 72 hours, respectively, by using an up-flow anaerobic packed bed reactor (UAPB) bioreactor with a pure culture of *Pseudomonas putida*. In that study, it did mention that the drop in pH was due to the generation of intermediate by-products.

Trusek-Holownia and Noworyta (2015) reported using a MBR system with 2 separation modules on BTEX removal, using *Pseudomonas fluorescens*. A MF/UF module as a first step filtration and NF/RO module as a second step separation. It reported a batch process of 120 mg/L toluene removal after a residence time of 200 hours.

Another study was conducted by Ishak and Malakahmad (2013) using Fenton (Advance Oxidation) treatment process as a pretreatment process on refinery wastewater which consists of 39.83 mg/L of toluene and other organic compounds. It was reported that the biodegradability improves BOD₅/COD ratio from 0.27 to 0.43. However, it did not mention the limitations of the study and how much toluene was oxidized.

Mustapha (2018) conducted a study on the treatment of petroleum refinery wastewater with constructed wetlands as an environmentally friendly and economical approach. It reported that the removal of BTEX was ranging 50 to 100 %, but the toluene concentration in the influent was relatively low between 0.03 to 0.07 mg/L. The advantages of using constructed wetlands are low energy consumption, maintenance cost and operation cost (Gorgoglione and Torretta, 2018). Moreover, Rani et al. (2011) mentioned that wetlands have a simplicity in design, low sludge production cost, and creates potential biodiversity. However, the major disadvantage of using constructed wetland for industrial wastewater treatment is the low organic loading. Therefore, pretreatment is always required before discharging into the constructed wetlands (ITRC, 2003)

Another way of removing toluene from wastewater was using anaerobic treatment process, which poses an attractive treatment solution due to the production of biogas. Enright et al. (2007) reported toluene removal between 55 to 99% in a granular sludge bed-anaerobic filter bioreactor treating an effluent containing 5 to 104 mg/L of toluene, during 630 days of operation. The results mentioned about the inhibition effects caused by the toxicity of toluene which hinders its removal performance.

2.1.4 Biodegradation pathways of toluene

Figure 1: Biodegradation pathways of toluene, retrieved from Parales et al. (2008)

There were numerous studies reported about the toluene-degrading strains, and toluene can be biodegraded under aerobic and anaerobic environment (as shown in Figure 1). Despite the stability of the benzene ring structure, microorganisms like bacteria and fungi have evolved and developed an enzyme to destabilize and metabolize toluene (Parales et al., 2008). The initial step of toluene degradation is the most difficult, due to the stability of the benzene ring. Hence, under the aerobic condition, the first initial degradation step of toluene undergoes hydroxylation which transforms it into 3-methyl catechol or having an OH group attached at the ortho, meta or para position of toluene, which makes the structure less stable (Parales et al., 2008). Halldorson et al. (2004) had reported that toxicity of toluene is reduced from 88 % to 33 % when toluene is bio-transformed into its intermediate byproducts which indicates that these compounds are much less harmful. The common toluene degrading bacteria usually belong to a genus-group of

Pseudomonas, which is known to carry a gene for degrading many toxic chemical compounds (Nahar et al., 2000).

On the other hand, for the anaerobic pathway, it requires fumarate to carry out the toluene degradation reaction which forms benzyl succinate (also known as benzyl succinic acid). It will then undergo a series of oxidation reactions to transform into benzyl-CoA. Benzyl-CoA will be further metabolised in the tricarboxylic acid cycle (TCA) cycle, also known as the Krebs Cycle (Parales et al., 2008). The known intermediate byproducts are benzyl succinic acid, benzyl fumaric acid and benzoate (Beller et al., 1995). Moreover, the notable strains that degrade toluene under anaerobic condition are, *Thauera Aromatica K172* and *Thauera Aromatica T1* (Parales et al., 2008). Besides that, there are also sulfate-reducing bacteria which able to degrade toluene under anaerobic condition (Beller et al., 1992b, Beller et al., 1992a, Edwards et al., 1992).

However, it is important to note that using pure toluene degrading strains are not practical in industrial applications, due to the complications like the risk of contaminations, high production of toxic byproducts, non-biodegradable secondary byproducts, high substrates and product purification cost (Jiang et al., 2017, Rajamanickam et al., 2017). There are several advantages of using mixed consortia over pure culture like, the production of other enzymes which are more resistant to the inhibition effects of toxic compounds and promote the exchange of genetic materials between the bacterial community of different species (Trusek-Holownia and Noworyta, 2015). Apart from that, mixed cultures can utilize mixed substrates, having the combination of different metabolic pathways which result in efficient substrate utilization and remove the need of sterile operating environment (Sabra and Zeng, 2014). Ultimately, it provides a cheap and readily available solution to handle complex substrates for biotechnology processes (Jiang et al., 2017, Sabra and Zeng, 2014).

There are studies reported the use of mixed consortium on toluene removal. Rajamanickam et al. (2017) reported that the inhibition concentration of toluene for mixed consortia is above 100 mg/L and the dominant species are *Pseudomonas*, *Bacillus* and *Escherichia coli*. While other study reported by Ince et al. (2009) that

the inhibition effects of toluene on anaerobic sludge, lowered the potential methane production with increasing toluene concentration levels and also mentioned that there is a negative impact on the methanogenic genera, *Methanosaeta spp.* The study conducted by Edwards and Grbic-Gali (1994) shows that anaerobes with different concentration of inhibitors, electron acceptors and donors like nitrite, sulfate, glucose and acetate can hinder the toluene degradation performance, the reported toluene inhibition concentration was found to be close to 165.8 mg/L.

Another study conducted by Sun et al. (2014) on methanogenic consortium was able to identify that the *Desulfosporosinus* phylotype was the key consumer of toluene, while other phylotypes, *Peptostreptococcaceae* and *Pseudonocardia* were assumed to consume the daughter compounds and produce methane precursors which further break down into biogas by the methanogens. Muccee and Ejaz (2018) were able to isolate twenty toluene-degrading strains from tannery effluent and suggested the exploitation of using these bacteria's toluene degrading ability for bioremediation. Feisther et al. (2015) was studying the degradation kinetics of BTX at approximately 50 mg/L using acclimatized activated sludge under aerobic condition but did not identify the strains. A similar study was conducted by Reardon et al. (2002) to develop a degradation kinetics model for the degradation of aromatics compounds such as toluene, benzene, ethylbenzene, phenol and other aromatic compounds by using pure cultures (*Pseudomonas putida* Fl and *Burkholderia* sp. strain JS150) and mixed cultures. The results showed that the interactions between the different species in the mixed culture have a significant effect on the biodegradation kinetics compared to pure strains. Reardon et al. (2002) explained that to develop a predictive modelling system for mixed cultures, the chemical interactions, species interactions, possible inhibitors, metabolic intermediates must be known first. Alvarez and Vogel (1991) reported the degradation of toluene by pure culture (*Pseudomonas* sp. CFS-215) and mixed cultures from aquifer slurry showed 48 mg/L day and 23 mg/L day, respectively.

Based on the previous studies conducted, the use of mixed cultures seems to be much practical and cost-effective in industrial applications. Even though it is difficult to predict the biodegradation kinetics of a mixed culture due to the unknown species interactions, chemical interactions and the dynamic changes in the

system, but it still provides a feasible solution in removing aromatic organic compounds, such as toluene, benzene and phenol. The challenge is to acclimatize the mixed consortium to toluene and promote the proliferation of toluene-degrading strains.

2.2. EMBR

2.2.1 Working principles of EMBR

Figure 2: Mass transfer process and working principles of EMBR

Andrew Guy Livingston (1993) conducted the first study on Extractive Membrane Bioreactor. The degradation of 3-chloronitrobenzene and phenol via EMBR were his initial studies (Livingston, 1993b, Livingston, 1993a). The results of his previous studies have shown that EMBR can be a promising solution to removing toxic organic compounds in wastewater with a silicone rubber membrane, also known as polydimethylsiloxane (PDMS). It acts as a barrier between the feed side and bio medium, rejecting inorganic species and water, but allows the targeted organic compounds to permeate through (as shown in Figure 2). Thus, the biodegradation can occur on the bio medium side smoothly without being exposed to the harsh feed conditions (e.g. extreme pH and salinity). Over the past decades, extensive studies had been conducted to determine the feasibility of using EMBR

for treating different kinds of hazardous organic waste. Examples of organic chemicals tested, 3 chloro-4-methylaniline (Antonietta Splendiani, 2000), toluene, dichloromethane (Jorge and Livingston, 2000), 1,2-dichloroethane (Santos and Livingston, 1994) and 3,4-dichloroaniline (Brookes and Livingston, 1994) and other VOCs.

The EMBR process poses several advantages compared to the conventional activated sludge process as mentioned by Jin et al. (2017), Livingston et al. (1998), Yeo et al. (2015), Biundo (1999). (1) EMBR can be operated under room temperature and atmospheric pressure condition. (2) It is a point source treatment process without the need for pretreatment. (3) The concentration gradient of targeted organic compound is always maintained steep across the membrane due to the ongoing biodegradation reaction. (4) The optimization of microbial activity at the biological side under a controlled environment, given that it is isolated away from the feed. (5) The process generates a small amount of waste stream, less than 1% of the supplied wastewater.

In short, the performance of the EMBR system depends on the acclimatized microbial activity, the partition coefficient of the targeted organic compound in between phases (feed solution, receive solution and membrane), the chemical and physical properties of the membrane and the hydrodynamic boundary layers on both sides (Cocchini et al., 1999). The working principle of the non-porous PDMS membrane follows the solution-diffusion model.

The overall mass transfer coefficient can be determined by using the following mass balance equation (1)

Given that:

$$V_r \frac{dC_r}{dt} = k_o A (C_f - C_r) \quad (1)$$

Where V_r (m^3) is the volume of the reactor; $\frac{dC_r}{dt}$ ($g/m^3 s$) is the rate of targeted solute concentration changes in the receiving side; k_o (m/s) is the overall mass transfer coefficient; A (m^2) is the effective area of the membrane; C_f and C_r (g/m^3) are the solute concentrations at the feed side and receiving side, respectively, at time t (s). Rearrange equation (1), to get equation (2)

$$k_o = \left(V_r \frac{dC_r}{dt} \right) / \{A(C_f - C_r)\} \quad (2)$$

The equation (2) above is followed closely to Liao et al. (2018b).

The mass transfer k_o can also be calculated based on the rate of targeted solute concentration changes in the feed side. Where $\frac{dC_f}{dt}$ (g/m³ s) is the rate of targeted solute concentration changes in the feed side; V_f (m³) is the volume of the feed.

$$k_o = \left(V_f \frac{dC_f}{dt} \right) / \{A(C_f - C_r)\} \quad (3)$$

Hence, assuming it is a resistances-in-series model for the targeted solute, the overall resistance can be calculated as the following below:

$$R_o = \frac{1}{k_o} = \frac{1}{k_f} + \frac{1}{k_{rs}} + \frac{1}{k_m} \quad (4)$$

Where R_o (s/m) is the overall resistance; k_f is the feed side mass transfer coefficient; k_{rs} is the receive side mass transfer coefficient; k_m is the mass transfer coefficient of the membrane.

However, as biofilm starts to grow on the surface of the membrane. The overall resistance is written like this.

$$R_o = \frac{1}{k_o} = \frac{1}{k_f} + \frac{1}{k_{rs}} + \frac{1}{k_m} + R_{biofilm} \quad (5)$$

Where $R_{biofilm}$ is the biofilm resistance on the membrane. It is followed closely to Yeo et al. (2016).

If the targeted solute is not detectable or below the detection limit on the receive side, thus $C_r \approx 0$. Equation (2) can be determined as the following below:

$$k_o = \left(V_r \frac{dCr}{dt} \right) / (AC_f) \quad (6)$$

The percentage reduction of k_o due to biofilm growth and cake layer attachment can be determined by calculating the normalized k_o

$$\text{Normalised } k_o = \frac{\text{Final } k_o}{\text{Initial } k_o} \times 100\% \quad (7)$$

Where the *Final* k_o is the k_o observed on that specific day of operation until the end of the experiment. Where the *Initial* k_o is the k_o observed on the first day of the experiment, according to Yeo et al. (2016).

By calculating the mass balance of toluene in the EMBR. The amount of toluene removed daily can be determined by the following equation below:

$$\text{Toluene removed daily } \left(\frac{\text{mg}}{\text{day}} \right) = (V_f C_{f \ t=0\text{hr}}) - (V_f C_{f \ t=24\text{hr}}) - (V_r C_{r \ t=24\text{hr}}) \quad (8)$$

Where $C_{f \ t=0}$ (mg/L) is the initial toluene concentration in the feed solution, $C_{f \ t=24\text{hr}}$ (mg/L) is the final toluene concentration in the feed solution after 24 hours and $C_{r \ t=24\text{hr}}$ (mg/L) is the final toluene concentration in the receive solution after 24 hours. $C_{r \ t}$ is assumed to be 0, thus $(V_r C_{r \ t=24\text{hr}})$ is equal to 0.

Thus, the toluene elimination capacity per membrane area can be determined by the following equation below:

$$\text{Toluene elimination capacity } \left(\frac{\text{g}}{\text{m}^2 \text{ hour}} \right) = \frac{\frac{\text{toluene removed daily}}{24000}}{\text{effective membrane area (m}^2\text{)}} \quad (9)$$

The overall toluene removal can be determined by the following equation below:

$$\text{Toluene removal } (\%) = \left(1 - \left(\frac{V_f C_{f \ t=24\text{hr}}}{V_f C_{f \ t=0\text{hr}}} \right) \right) \times 100\% \quad (10)$$

2.2.2 Configuration and challenges of EMBR

Recent studies have been exploring the methods to enhance the EMBR performance. For examples, the flow direction of the setup, the type of membrane, the orientation of the membrane have been investigated. The challenges of EMBR faced were the lack of efficient extractive membrane for enhancing organic mass transportation and the chemical stability, the uncontrollable biofilm growth on the membrane and the lack of biofilm control strategies (Liao et al., 2018a, Yeo et al., 2015, Loh et al., 2016).

Special extractive membranes were designed to improve the overall mass transfer of the targeted organic compound. The first PDMS coated on PVDF nanofibrous composite membrane reported by Jin et al. (2017) exhibits 4 times higher phenol overall mass transfer than commercial membrane. Similarly, Liao et al. (2018a) were able to design a novel nanofibrous composite membranes coating with superhydrophobic and superhydrophilicity surface, and both were able to achieve 10 times higher phenol extraction efficiency. Unlike the commercial PDMS membrane which reported having a thickness greater than $>0.2\text{mm}$ in past studies, these membranes were much thinner about $60\mu\text{m}$ thick which lowers the mass transfer resistance (Emanuelsson et al., 2003, Livingston, 1993a). Besides that, a comparison study conducted by Yeo et al. (2016) was done between crossflow setup and submerged setup, and it reported that submerged setup was better biofilm control due to the air scouring on the membrane surface, they further support that air scouring in submerged setup aids the reduce biofilm formation. Another study by Loh et al. (2016) reported thin film PDMS on PVDF exhibited 7.5 times higher overall mass transfer than commercial membrane.

Another study conducted by Liao et al. (2018b) shown that thin-film composite PDMS layer on PVDF facing the receive solution (containing ultrapure water only) has a higher overall mass transfer coefficient than the PDMS layer facing the feed solution (containing a high amount of salt), due to internal concentration polarization. Furthermore, they also mentioned that having the PDMS layer facing the receive side promote lesser biofilm attachment on the smooth membrane surface.

In the previous studies mentioned by S.-F. Zhang (1998), Loh et al. (2016), Brookes and Livingston (1995) that liquid film resistance is negligible if the operating flow rate is high, whereas the biofilm and the membrane resistance affect the mass transfer significantly.

In most cases, biofilm was deemed to have a negative impact on the EMBR performance by increasing the overall mass transfer resistance, which lowers the mass transportation of the targeted organic compound. However, there were a few studies stated otherwise if the biofilm is at its optimal thickness can prevent the targeted organic compound from being air stripped, especially VOCs (Pavasant et al., 1996, Strachan et al., 1996, Santos and Livingston, 1995). It was reported by Yeo et al. (2016) that the active biofilm helps to maintain the pollutant concentration close to zero, thus maximizes the concentration gradient across the membrane. For most cases, the microbial community and biofilm formation profiles were not mentioned.

2.2.3 Removal of toluene in EMBR

An EMBR study was conducted by Jorge and Livingston (2000) to investigate the degradation performance of microbes when exposed to an alternating source of organic compounds, toluene and dichloromethane. A tubular PDMS membrane with a thickness of 0.35 mm was used and the overall mass transfer coefficient, k_o was 1.5×10^{-6} m/s. There were signs of toluene degradation, but the biofilm formation profile was not mentioned, and the operating conditions were not optimized.

Another EMBR study was conducted by Emanuelsson et al. (2003) to determine the toluene degradation under anoxic condition by manipulating nitrate conditions. The overall mass transfer coefficient, k_o was 9.5×10^{-6} m/s with a membrane thickness of 0.35 mm. Emanuelsson also mentioned that under nitrate limiting condition, the biofilm formed rapidly, and more soluble microbial products (SMPs) were generated. However, effective toluene removal was not observed in the system.

The most recent EMBR study on toluene removal was conducted by Mehdizadeh et al. (2011). using a pure aerobic strain *Alcaligenese faecalis*, and the substrate inhibition threshold was 180 mg/L. A tubular PDMS membrane with a thickness of

0.2 mm was used in that study and mass transfer coefficient, k_o was found to be 1.33×10^{-6} m/. The study claimed that using this strain will not cause any formation biofilm on the membrane surface after 9 days of operation. Besides that, it has a high degradation capability of toluene at 1 g/L. Mehdizadeh et al. (2011) suggested that operating the EMBR without any biofilm on the membrane offers lower mass transfer resistance, therefore improves the removal efficiency of EMBR.

The findings in previous studies show that toluene was being degraded during the experiment period, but toluene's metabolites were not mentioned in the stated studies. Besides that, toluene has a high vapour pressure. Hence, it is uncertain whether it was totally biotransformed or partially air stripped at the receive side under aerobic condition. Operating the system under oxygen limiting condition is much preferred compared to an aerated aerobic system. First, it lowers the operating cost without having aeration. Next, it eliminates the possibility of toluene being air stripped. Thus, an oxygen limiting condition was selected for this EMBR study.

2.3. ZIF-8@PDMS/PVDF membrane

2.3.1 Transportation model of ZIF-8@PDMS/PVDF membrane

In recent studies, researchers have been trying to fabricate high-efficient PDMS membrane to improve the flux of the targeted organic compounds in the EMBR. One of the notable studies was the fabrication of PDMS/PVDF composite membrane which was shown to have 4 times higher phenol extraction than commercial silicone rubber membrane, at mass transfer coefficient, k_o $4.1 \pm 0.3 \times 10^{-7}$ m/s after 2 weeks of operation reported by Jin et al. (2017). On the other hand, Liao et al. (2018a) was able to achieve 10 times higher phenol extraction than commercial silicone rubber membrane and mass transfer coefficient, k_o was $6.5. \pm \times 10^{-7}$ m/s after 31 days operation. A study was conducted by Jin et al. (2018) to improve the flux by adding nanofillers into the PDMS membrane using a high efficient ZIF-8@PDMS/PVDF membrane. It exhibits an astonishing improvement in phenol flux, at the mass transfer coefficient, k_o $35.7 \pm 1.1 \times 10^{-7}$ m/s. The addition of ZIF-8 (zeolitic imidazolate framework-8) nanofillers enhance the affinity between phenol and the membrane matrix, by improving molecular attraction forces.

Furthermore, this newly developed membrane has a “bi-mode” transport mechanism, a solution-diffusion transport mode and a pore-flow transport mode, unlike the commercial PDMS membrane has only a solution-diffusion transport mode mentioned by Jin et al. (2018). It suggested that 4wt% ZIF-8 was the optimum amount to enhance the k_o . Further adding ZIF-8 will decrease the k_o and increase the salt flux. The use of this high-efficient ZIF-8@PDMS/PVDF membranes is not well explored. Till now, only phenol was tested. Hence, the application on other types of organic compounds remains unknown. In this study, a flat sheet 4wt% ZIF-8@PDMS/PVDF nanofibrous composite membrane was fabricated to compare with a commercial flat sheet PDMS membrane on toluene extraction.

2.4. Summary of literature review

The conclusions from the literature reviews are all summarized below:

The global demand for toluene and petrochemical compounds will only keep increasing over the years. As a result, the environmental pollution of these compounds becomes inevitable. Furthermore, the toxicology of toluene conducted by past studies reported that it pose harmful health effects on human and the environment. Knowing that toluene can also upset conventional activated sludge treatment processes by inhibiting the biological processes. Besides that, the feed or produced water contains a high amount of salt, heavy metals and with extreme pH condition. Thus, pretreatments are often required, which resulted in additional operation and capital cost, making petrochemical and refineries challenging to meet the environmental discharge standards.

There were only a few EMBR studies conducted on toluene removal, in aerobic or anoxic condition. However, operating in aerobic condition could air strip toluene into the atmosphere which leads to an environmental problem and also could lead to an inaccurate reported mass transfer coefficient, k_o value. Thus, operating the EMBR under microaerobic condition was proposed. Apart from that, using pure toluene degrading strain is not feasible in industrial applications, due to the risk of contamination and the operation complexity on growing it. None of these EMBR

studies on toluene removal mention about the microbial community of toluene degrading strains, biofilm profile and the biotransformation of toluene.

The use of high efficient ZIF-8@PDMS/PVDF membranes on toluene extraction and how effective between ZIF-8@PDMS/PVDF membranes and commercial membrane on toluene removal are not explored.

2.5. Research objectives

1. The preliminary batch study is to determine the inhibition concentration level of toluene and toluene degradation kinetics of the acclimatized mixed culture.
2. To identify the toluene byproducts and its degradation pathways. Furthermore, to investigate the microbial profile and dynamics in the EMBR under microaerobic conditions.
3. A comparison study on toluene extraction performance between ZIF-8@PDMS/PVDF membranes and commercial PDMS membrane in a microaerobic EMBR setup.
4. To evaluate the biofilm profile, in terms of thickness, morphology, microbial community, adhesive strength and chemical composition.
5. To evaluate the performance of the microaerobic EMBR based on toluene removal, mass transfer coefficient k_o , toluene elimination capacity and the recovery of k_o using chemical cleaning.

CHAPTER 3: Materials and methods

Outline:

- 3.1 Synthesis of ZIF-8 nanofillers and membrane
- 3.2 Parent reactor
- 3.3 Toluene degradation batch experiment
- 3.4 Extractive membrane bioreactor (EMBR)
- 3.5 Membrane autopsy and foulant characterization
- 3.6 Analytical methods

3.1 Synthesis of ZIF-8 nanofillers and membrane

The synthesis of ZIF-8 nanofillers was followed closely to Jin et al. (2018). The ZIF-8 nanofillers were synthesized by pouring a solution of $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (reagent grade, 98 %, Sigma Aldrich) (0.11 g) in 25 mL ethanol (EMSURE® ACS, ISO, Reag. Ph Eur., Sigma Aldrich) into a solution of 2-methylimidazole (green alternative, 99 %, Sigma Aldrich) (2.27g) in 50 mL ethanol under stirring condition. The solution was stirred for 5 hours to allow the homogeneous formation of ZIF-8 nanofillers. The solution was then washed 3 times with fresh ethanol by centrifuging at 3000 rpm for 5 mins at 4 °C. Next, the solution was rewashed 3 times with fresh n-hexane (Merck chemicals) by centrifuging at 3000 rpm for 12 mins at 4 °C. The washing steps help to remove the unreacted reagents and by-products in the solution, to obtain high purity of ZIF-8 nanofillers. The washed ZIF-8 nanofillers were resuspended in n-hexane solution, with a pre-determined concentration (ZIF-8/n-hexane). The ZIF-8/n-hexane solution was used to mod the PDMS composite membrane later.

Similarly, the fabrication of flat sheet ZIF-8@PDMS composite membrane method was modified from Jin et al. (2018). The PVDF substrate was first immersed in 100 % ethanol for 10 mins, and it was rinsed with DI water later. The washed PVDF substrate was immersed in 50 wt% glycerol (Sigma Aldrich) as a final pre-wetting solution. The pre-wetted PVDF substrate was taped onto a clean glass plate and was dab with filter paper to remove any residual pre-wetting solution. It was placed onto

the automatic film applicator (Elcometer 4340, Elcometer Asia Pte. Ltd.) and ready to be coated. A PDMS/n-hexane solution was prepared by adding 5 wt% hydroxyl-terminated polydimethylsiloxane (PDMS supplied from Sigma Aldrich) into n-hexane solution and stirred for 1 hour at room temperature. Next, a pre-determined amount of synthesised ZIF-8/n-hexane solution was added into an empty glass bottle. The PDMS/n-hexane solution was added dropwise into the bottle containing ZIF-8/n-hexane solution under stirring via the “prime” technique. The mixed solution was stirred vigorously for 1 hour. Next, cross-linker 2.5 wt% tetraethyl orthosilicate (TEOS supplied from Sigma Aldrich) and 0.5 wt% dibutyltin dilaurate (DBTDL supplied from Sigma Aldrich) were added and stirred vigorously for several minutes until the solution becomes viscous. The solution was poured onto the PVDF substrate and was coat using the automatic film applicator (Elcometer 4340, Elcometer Asia Pte. Ltd.) with a knife height of 100 μm . The coated membrane was left drying for 30 mins at room temperature to allow the solvent to evaporate and solidify. Lastly, it was placed in the 80 $^{\circ}\text{C}$ oven for 24 hours to heat cure. Once the heat cure was done, it was immersed in 80 wt% ethanol solution for 10 mins, followed by ultrapure water for later usage. The commercial PDMS membrane with a thickness of 0.1 mm (Silex Ltd) was used in this study for comparison.

3.2 Parent reactor

Figure 3: Schematic diagram of the parent reactor setup

The bioreactor with a volume capacity of 2.2 L was set-up to enrich toluene degradation culture. It was inoculated with anaerobic sludge and had mixed liquor volatile suspended solids (MLVSS) of 5 - 10 g/L and a working volume of 1 ± 0.1 L. An easySense™ DO, pH and ORP probes were supplied from Mettler Toledo were connected to Mettler Toledo M200 transmitter to monitor the temperature, DO, pH and ORP of the bioreactor, shown in Figure 3. The dissolved oxygen (DO) in the reactor was recorded at 0 mg/L during the operation period, and pH was maintained at 7 ± 0.5 . The mixing speed was set at 180 rpm, and the reactor was operated at room temperature (24 ± 1 °C). The total dissolved solids (TDS) and conductivity of the bioreactor were kept below 5 g/L and 10 mS/cm, respectively. The $\text{NH}_4\text{-N}$ concentration was kept below 400 mg/L. It was fed with 25 to 30 mg/L toluene daily as a sole carbon source for more than 3 months before the EMBR was seeded. Before starting up the system, it was purged with nitrogen gas to remove any presence of oxygen and operated in a sealed condition. A 3 L Tedlar bag was partially filled with nitrogen gas and was attached to the bioreactor to provide positive overhead pressure to prevent any possibility of air entering into the system. A magnetic stirrer plate was placed beneath the bioreactor to provide adequate mixing to the bio medium. An automatic syringe pump (Cole Parmer) was used to supply the desired amount of concentrated feed solution to the bio medium at

adjustable time intervals and volume dosage. The concentrated feed was purged with nitrogen before loading onto the syringe pump.

A concentrated phosphorous and nitrogen nutrient solution was prepared with 4.5 g/L K_2HPO_4 (Merck) and 15 g/L NH_4Cl (VWR chemicals), to provide enough C: N: P ratio for biomedium growth. A concentrated pH buffer solution was prepared with 80 g/L $NaHCO_3$ (Sigma Aldrich) to provide buffering capacity for the bioreactor. Trace element solution which contains 1.25 g/L EDTA (Sigma Aldrich), 0.55 g/L $ZnSO_4 \cdot 7H_2O$ (Merck), 0.4 g/L $CoCl_2 \cdot 6H_2O$ (Sigma Aldrich), 1.275 g/L $MnCl_2 \cdot 4H_2O$ (Sigma Aldrich), 0.4 g/L $CuSO_4 \cdot 5H_2O$ (Alfa Aesar), 0.05 g/L $Na_2MoO_4 \cdot 2H_2O$ (Sigma Aldrich), 1.375 g/L $CaCl_2 \cdot 2H_2O$ (VWR chemicals), 1.25 g/L $FeCl_3 \cdot 6H_2O$ (Sigma Aldrich) and 44.4 g/L $MgSO_4 \cdot 7H_2O$ (VWR chemicals) was added as inorganic nutrients for the bio medium at 1.2 mL/L. At the beginning phase, to acclimatize the biomass to toluene, the bioreactor was fed with glucose and toluene at a low concentration. The concentration of toluene gradually increased until it became a sole carbon source. Occasionally, mixed liquor samples were drawn from the parent reactor to measure concentrations of toluene, metabolites, NH_4-N and MLVSS.

3.3 Toluene degradation batch experiment

The degradation test was conducted to determine the degradation capacity and toluene inhibition threshold. Six 100 mL glass bottles were prepared and were inoculated with toluene acclimatized biomass from the parent reactor at a concentration of 0.5 g/L MLVSS, shown in Figure 4A. N_2 purged tap water was used as diluent. The working volume was 100 mL which left a small headspace. The six bottles were labelled and dosed with different toluene concentration, i.e. 0, 100, 200, 300, 400 and 500 mg/L. The bottles were placed on the rotary shaker machine, operating at 180 rpm. Toluene, pH, NH_4-N , TDS and conductivity were measured daily. 2 mL of sample was drawn daily from each bottle to test for toluene and NH_4-N . The highest toluene degradation concentration was selected for metabolite test.

For the metabolite test, six 100 mL glass bottles were prepared and inoculated with toluene acclimatized biomass from the parent reactor at a concentration of 0.5 g/L

MLVSS. The six bottles were labelled based on the number days, 0, 1, 2, 3, 4 and 5, respectively. All bottles were dosed with the same toluene concentration which selected from toluene degradation batch result. Similarly, the bottles were placed on the rotary shaker. Every day, one bottle was taken out for sampling according to the stated number. 50 mL of the sample was drawn from the bottle and proceeded to solid phase extraction (SPE) prior to GC-MS analysis to determine the metabolites (Figure 4 C).

Figure 4: (A) Day 0, toluene degradation test; (B) Day 3 toluene degradation test; (C) Day 0, toluene metabolite test

3.4 Extractive membrane bioreactor (EMBR)

3.4.1 Setup

Figure 5: Schematic diagram of the setup

A crossflow and closed EMBR system was designed for the experiment, shown in Figure 5. In order to minimize toluene volatilization and adsorption, sealed glass bottles were used, and the connection tubing used was made of PTFE (Teflon) material. No aeration was provided to the system; thus, air stripping was minimised. A leak test was conducted on the commercial and synthetic membrane before seeding. The commercial thin silicone film membrane has a thickness of 100 μm and was purchased from Silex (clear, Shore hardness 30°). The membranes were mounted into the flow cell, and the effective area was 126 cm^2 (18 cm $\times$ 7 cm). The feed solution contained 1 L of 5 g/L NaCl, and the receiving solution contained 1 L of ultrapure water. Both sides were recirculated at a flow rate of 100 mL/min and TDS were measured periodically. The TDS value at feed side and receive side was around 4.5 ± 0.2 g/L and $1 \text{ mg/L} \pm 0.5 \text{ mg/L}$, respectively, indicating no leakage was detected, and salt flux was negligible.

3.4.2 Operating conditions

The microaerobic EMBR was operated in a semi-continuous process for 34 days (2 cycles). After the 1st cycle ended, chemical cleaning with 0.002 ~ 0.0025 %wt NaClO was conducted to remove the cake layer and biofilm to observe the recovery of k_o . The experiment was repeated twice on the commercial and synthetic membrane, respectively. Feed solution contained 100 mg/L of toluene and 5 g/L NaCl. It was recirculated at a flow rate of 100 mL/min at the feed side. The 0.4 L bioreactor was seeded with toluene acclimatised sludge from parent reactor at a biomass concentration of 0.5 g/L MLVSS. Bio medium on the receive side was recirculated at a flow rate of 400 mL/min. $\text{NH}_4\text{-N}$ and $\text{PO}_4\text{-P}$ in the bio medium were maintained at 8 ~ 10 mg/L and 2 mg/l, respectively, by dosing concentrated NH_4Cl and K_2HPO_4 stock to provide enough nutrients. A concentrated pH buffer solution was prepared with 80 g/L NaHCO_3 to provide buffering capacity in biomedium.

The EMBR system was operated in a closed system but not strict anaerobic conditions. Throughout the experiment, TDS was monitored on both sides, to ensure there was no leakage. The feed solution was always topped up to approximately 100 mg/L of toluene daily throughout the experiment. After topping up with toluene, the system was left to stabilize for 1 hour before sampling was conducted. 1 mL of sample was drawn at both feed and receive side at time intervals of 0.5 ~ 1 hour to measure toluene concentration measured via the High-Performance Liquid Chromatography (HPLC, Agilent 1260 Infinity). Volume losses due to sampling were topped with tap water purged with nitrogen gas to its original working volume. The fabricated membrane's active layer was facing the bio medium because it was reported by Liao et al. (2018b) that in this membrane configuration has a better mass transfer.

At the end of the operation (34 days), the fouled membrane was dismounted from the flow cell, and membrane autopsy was conducted to obtain cake layer and biofilm samples. The biofilm morphology and thickness were investigated using Field Emission Scanning Electron Microscopy (FESEM) and confocal microscopy. Extracellular polymeric substances (EPS) extraction was conducted on the cake

layer and biofilm to determine the protein and polysaccharide content. Liquid Chromatography – Organic Carbon Detection (LC-OCD) analysis was conducted on the bio medium, cake layer and biofilm to determine the dissolved organic matters in each individual biological component. A 50 mL of bio medium was drawn for solid-phase extraction (SPE) before Gas Chromatography Mass Spectrometry (GC-MS) for identification of metabolites. DNA extraction was conducted on the 2nd cycle of EMBR experiment with the commercial membrane at day 0, day 6, day 17 of the bio medium and the cake layer. Samples were sent for DNA analysis to determine the microbial dynamics and presence of toluene degrading microorganisms.

3.5 Membrane autopsy and foulant characterization

3.5.1 Membrane roughness

The roughness of the membrane was determined by atomic force microscopy (AFM). The instrument was operated non-contacted mode to determine the roughness of the membrane. The samples were cut into 1 cm × 1 cm pieces and were pasted on the sample disk firmly before analyzing. The results were reported in Rq (nm), Rz (nm) and Ra (nm). Rq represents the root mean square roughness. Rz is the ten-point average roughness based on the five highest peaks and five lowest valleys. Ra represents the roughness average. The cantilever used was Non-Contact Cantilever (NCSTER 10M, Parksytms), force constant 7.4 N/m. Several 5 µm × 5 µm areas were swept across the sample at different locations.

3.5.2 Membrane autopsy

The membrane autopsy method was according to Ho et al. (2016). The membrane with foulants was first rinsed gently to remove the loose cake layer. An exact 200 mL volume of ultrapure water was used to remove the cake layer, leaving a thin layer of biofilm on the membrane. Next, the membrane was sliced into three pieces of 3 cm × 4 cm at different locations of the membrane. The membrane samples were placed into a 25 mL 0.85 % NaCl solution and underwent ultrasonication for 2 mins to detach the biofilm from the membrane. The samples were filtered through a 30 mm Nylon syringe filter with pore size 0.45 µm prior TOC, LC-OCD, total

protein and total polysaccharide analysis. Besides, six pieces of 1 cm × 3 cm were sliced for confocal microscopy and field electron microscopy imaging.

3.5.3 Biofilm and membrane morphology

The morphology of the biofilm and membrane were determined by Field Emission Scanning Electron Microscopy (FESEM). The membrane samples were sliced into 1 cm × 3 cm pieces and were left to air dry at room temperature before analysis. As for the biofilm samples, they were prepared by fixation and ethanol dehydration. First, the samples were fixed with 2.5% glutaraldehyde for 4 hours. Next, the samples were washed with 0.1 M phosphate buffer 3 times for 10 mins each. After that, it underwent a series of ethanol dehydration 30 %, 50 %, 70% and 95% at 10 mins each. Finally, it was placed in 100 % ethanol for 15 mins for twice before it was left to air-dry overnight at room temperature. The dried samples were pasted on the sample disks, and a conductive coating was applied at 20 mA for 40 seconds. Images were taken at several locations across the sample to obtain a representative result.

3.5.4 Membrane and biofilm adhesion strength

The adhesion strength of the biofilm on the membrane and membrane were determined by Atomic Force Microscopy (AFM). The samples were sliced into 1 cm X 1cm pieces. For biofilm samples were prepared by fixation. The biofilm samples were fixed with 2.5% glutaraldehyde for overnight. Next, the samples were left to dry at room temperature overnight. The samples were pasted firmly onto the sample disk. The instrument was operated in contacted mode (pinpoint mode) to determine the adhesion strength. The cantilever used was Non-Contact Cantilever (NCSTER 10M, Parksytms), force constant 7.4 N/m. Multiple locations on the sample were tested to provide a representative result. The results were reported in force nN.

3.5.5 Biofilm and membrane thickness

The biofilm samples were also stained using LIVE/DEAD® BacLight™ Bacterial Viability Kit, L7012. The steps were followed exactly to the manufacturer protocol. The steps were followed exactly to the manufacturer protocol. 1.5 uL of Propidium

iodide and SYTO 9 dye each was added into 1 mL of 0.85% NaCl solution containing the biofilm sample. The samples were incubated at room temperature in the dark for 30 minutes. Next, the biofilm samples were rinsed with phosphate buffer solution for 3 times. Stained samples were pasted on the glass slide and imaged under the confocal laser scanning microscopy (CLSM). Images were taken from several positions to obtain a representative result. The biofilm volume and thickness were calculated using IMARIS software (Bitplane, version 7.1.3)

The membrane thickness was measured using Field Emission Scanning Electron Microscopy (FESEM). Prior to the measurement, the samples were sliced from the membrane and dried at room temperature to remove any moisture. Images were taken at several locations across the sample to obtain a representative result.

3.5.6 Extracellular polymeric substances (EPS) extraction

The EPS extraction was conducted on biofilm and sludge cake to determine the protein and polysaccharide composition. The extraction method was according to Sun et al. (2018) using heat extraction method. Briefly, the supernatant of the samples after centrifugation at 4000g was collected as soluble bound (SB) EPS. The solid pellet was resuspended to the original volume with pre-heated 70 °C 0.05% NaCl solution. After vortex for 1 min, the samples were centrifuged again, and the supernatant was collected as loose bound (LB) EPS. The remaining pellets were resuspended to their original volume again with 0.05% NaCl solution and mix well. Next, the samples were incubated at 60 °C for 30 mins. Lastly, the samples were centrifuged, and the supernatant was collected as tight bound (TB) EPS. All collected EPS samples were filtered through a 30 mm Nylon syringe filter with pore size 0.45 µm prior to protein and polysaccharide analysis. The total EPS of extracted biofilm was measured in the ultrasonicated samples mentioned in section 3.5.2. The EPS content per unit area of biofilm and cake layer can be determined by the following equation below:

$$\text{EPS content (mg/cm}^2\text{)} = \frac{\text{EPS} \left(\frac{\text{mg}}{\text{L}} \right) \times \text{extraction volume (L)}}{\text{effective membrane area (cm}^2\text{)}} \quad (10)$$

3.6 Analytical methods

3.6.1 Total dissolved solids (TDS)

The total dissolved solids (TDS) and conductivity were measured using Mettler Toledo, SevenGo Duo™ SG23 Meter, by inserting the measuring probe into the sample.

3.6.2 pH

The pH measurement was conducted using Thermo Scientific, Orion Star A111 pH meter, inserting the measuring probe into the sample.

3.6.3 Dissolved organic matters (DOM)

The dissolved organic matters in the biological system were detected using Liquid Chromatography – Organic Carbon Detection (LC-OCD), according to Stewart et al. (2013). The samples were first tested for Total Organic Compounds (TOC) to ensure that the TOC value is < 5 mg/L. Injection volume was 4000 µL and the run time was 130 mins. The 5 L mobile phase buffer solution contains 12.5 g KH₂PO₄ (Merck 1.04873) and 7.5 g Na₂HPO₄·2H₂O (Merck 1.06580). The 5 L acidification solution contains 2.5 g potassium peroxydisulfate (e.g. Fluka 60489) and 20 mL of phosphoric acid (H₃PO₄ 85% p.A. e.g. Fluka 79620). The organic compounds in the sample were separated in the column and were identified based on their retention time. The results were reported in biopolymers, humic substances, building blocks, low molecular weight (LMW) acids and low molecular weight neutral compounds in percentage values for each individual component in the biological system.

3.6.4 Toluene

The concentration of toluene in feed solution and bio-medium was measured via the High-Performance Liquid Chromatography (HPLC, Agilent 1260 Infinity) equipped with a Gemini-NX 3µ, 110A, 100*2.0 mm, Phenomenex 18C column. The collected samples were measured immediately after sampling and filtered through a 30 mm Nylon syringe filter with pore size 0.45 µm. The filtered sample was placed in a 2 mL screw cap vial with blue caps, septum PTFE red / silicone white / PTFE

red and was measured at wavelength 254 nm, operating temperature 25 °C, operating pressure 181 bars and flowrate at 0.5 mL/min. The mobile phase used was ultrapure water (Millipore Co.) and acetonitrile (Merck) at 50:50. The metabolites were also measured at wavelength 210 nm, operating temperature 25 °C, operating pressure 210 bars and flowrate at 0.5 mL/min. The mobile phase used was ultrapure water with 0.1% orthophosphoric acid (v/v) and acetonitrile at 70:30.

3.6.5 Degradation metabolites

The metabolites assay was conducted to identify the possible toluene degradation pathway. A solid-phase extraction (SPE) was done for sample preparation before GC-MS analysis to detect metabolites. The SPE method was modified from Anumol et al. (2013). An Oasis HLB Extraction Cartridge, 20 cc 1g LP was used. The cartridge was conditioned by stepwise addition of 10 mL methanol and 10 mL ultrapure water by using a vacuum pump without letting the cartridge running dry. Next, load 50 mL volume of sample onto the cartridge at the rate of 10 mL/min. The cartridge was rinsed with 10 mL of ultrapure water and dry under vacuum for 10 mins. The eluant was discarded. The dried cartridge was further eluting by stepwise addition of 5 mL of methanol (VWR chemical) and 5 mL of 10/90 % methanol/MTBE (v/v) solution. The cartridge was dried under vacuum for 10 mins again. The eluent was collected and underwent nitrogen purge drying till 500 µL. Next, methanol was added to a final volume of 1 mL. The eluent was filtered through a 13 mm, 0.2 µm pore size PTFE syringe filter media and kept in a 2 mL screw cap vial for GC-MS analysis.

Gas Chromatography (Agilent Technologies 7890B GC) equipped with a Mass Spectrometry (Agilent Technologies 5977A MSD) was employed. The operating parameters were followed according to Muccee et al. (2019). The column and the operating parameters were as follows: the Agilent Technologies column with 30 m × 250 µm thickness × 0.25 µm I.D; the injection port temperature was 250 °C; the oven temperature program was held at 110 °C for 2 min, followed by a temperature increase of 10 °C /min to 200 °C. Next, the temperature was further increased to 280

°C at a rate of 5 °C /min. The injection volume was 1 µL. The identification of these compounds was based on their mass spectrum similarities with the database library. Metabolites assay was further measured by using LC-MS/MS (Agilent Technologies 1290 Infinity II (LC), Agilent Technologies 6460 Triple Quad LC/MS) equipped with Gemini-NX 3µ, 110A, 100*2.0 mm, Phenomenex 18C column. The operating parameters were followed closely to Abu Laban et al. (2009). The mobile phase consists of (A) water with 0.1 % acetic acid (v/v) and (B) acetonitrile. Eluent of A: B was kept at 80:20 for 3 mins. Next, the eluent (B) was increased from 20 % to 90 % within 10 mins and A: B was kept 10:90 for 3 mins. The flow rate was kept at 0.3 mL/min and injection volume at 50 µL. The column temperature was set at 35 °C.

3.6.6 Ammonium (NH₄-N)

NH₄-N was measured according to standard methods (APHA) . The method for protein assay was Lowry's method which carried out using the Pierce BCA *Protein Assay Kit* (Thermo Fisher Scientific). In brief, 0.5 mL of sample, was added with 12 mL of ultrapure water. Next, 3 drops of mineral stabiliser were added, followed by another 3 drops of polyvinyl alcohol. Finally, 1 mL of Nessler Reagent was added, and the sample was measured immediately at wavelength 420 nm using a UV-Visible Spectrophotometer (Shimadzu, UV-1800). The NH₄-N calibration curve was prepared using NH₄Cl as standards at a range of 0 to 100 mg/L. Mineral stabilizer, polyvinyl alcohol and Nessler reagent were supplied from Hach.

3.6.7 Polysaccharide

The method used for polysaccharide assay was the phenol-sulfuric acid method from Dubois et al. (1956). The samples were filtered through a 30 mm Nylon syringe filter with pore size 0.45 µm prior to polysaccharide assay. 1 mL of sample was drawn into a test tube, followed by adding 1 mL of 5 % phenol. Next, 5 mL of 96 % sulfuric acid was added and waited for 30 mins. It was analyzed at wavelength 490 nm using UV-Visible Spectrophotometer (Shimadzu, UV-1800). The

polysaccharide calibration curve was prepared using glucose as standards at a range of 0 to 200 mg/L.

3.6.8 Protein

The method for protein assay was Lowry's method which carried out using the Pierce BCA Protein Assay Kit by Thermo Fisher Scientific. The samples were filtered through a 30 mm Nylon syringe filter with pore size 0.45 µm before protein assay. The procedures were followed closely to the instruction manual of the kit. It was analyzed at wavelength 562 nm using UV-Visible Spectrophotometer (Shimadzu, UV-1800). The protein calibration curve was prepared using bovine serum albumin as standards at a range of 0 to 250 mg/L.

3.6.9 Total Organic Compounds (TOC)

The TOC assay was measured by using a TOC analyser (Shimadzu, TOC-LCSH, TNM-L, ASI-L). Samples were diluted accordingly into a 24 mL vial and were placed onto the autosampler for TOC measurement.

3.6.10 Microbial community

The DNA of the samples was extracted by using the FastDNA® SPIN Kit for Soil. The DNA of the pellet was extracted by using the FastDNA® SPIN Kit for Soil. In brief, the cake and bio medium samples were drawn into a clear 2 mL microcentrifuge tube and underwent centrifuge for 5 mins, 4 °C at 14000 rpm. The supernatant was collected for TOC and LC-OCD analysis, and the pellet was stored in the -80 °C for DNA extraction. The extracted DNA samples were freeze-dried before submitting to Shanghai Meiji Biomedical Technology Co., Ltd for DNA sequencing analysis. The DNA samples were undergone metagenomics analysis. The microbial community in the samples were categorized based on their classification levels (domain, genus, species, OTU) and the relative abundance. The DNA sequencing method was based on their protocol to analysis the sample. Multiple software and instruments were used to categorize the samples into

different classification levels and display them in evolutionary tree analysis, venn diagrams, circos diagrams, diversity analysis or OTU statistics depending on the user preference.

In this case, the circos diagrams were selected to determine the relationship between samples and the raw data were extracted out to identify the major species which degrades toluene.

3.6.12 Mixed liquor suspended solids and volatile suspended solids (MLSS & MLVSS)

The procedures were followed closely to APHA (1999). 1 mL of sample was filtered through a pre-weighed glass fibre filter (GC-50 was supplied by Adventec). The test was conducted in triplicates. The samples were dried at 105 °C in the oven for more than 2 hours and followed by cooling in the desiccator. The mass of the cooled samples was recorded as MLSS. Next, the samples were further ignited at 550 °C for 1 hour in the furnace and followed by cooling in the desiccator. Finally, the reduction of samples mass was recorded as MLVSS.

CHAPTER 4 Toluene degradation metabolites

Outline:

4.1 Introduction

4.2 Material and methods

4.3 Results and discussion

4.4 Conclusion

4.1 Introduction

The objective of this experiment was (1) to determine the presence of toluene degrading microbes under a microaerobic condition, (2) to investigate the inhibition threshold and identify the toluene degradation pathway. In previous studies, the degradation of toluene pathway is well studied under aerobic or anaerobic conditions (Parales et al., 2008, Muccee et al., 2019, Shinoda et al., 2004). In brief, the common metabolites found from toluene aerobic degradation were benzyl alcohol, o-cresol, p-cresol, m-cresol and catechol. On the other hand, the common metabolite found in toluene anaerobic degradation is benzyl succinate (Beller et al., 1992b, Beller., 2000). It will be interesting to find out how acclimatized mixed cultures adapt to the changing environment.

In previous studies, there are microbes able to degrade toluene under aerobic and anaerobic conditions. *Thauera* sp. Strain DNT-1 discovered by Shinoda et al. (2004) mentioned that this strain contains the key enzymes for aerobic and anaerobic toluene degradation. *Acidovorax caeni* and *Flavobacterium* were discovered in the sewage treatment system and found to degrade toluene under aerobic and anaerobic conditions (Yamaguchi et al., 2018). Aside from that, microorganisms discovered from petroleum contaminated sites and aquifer were found to survive under microaerobic and aerobic conditions (Táncsics et al., 2018, Táncsics et al., 2020). *Zoogloea* was reported to degrade toluene under microaerobic conditions in aquifer using monooxygenation mechanism (Táncsics et al., 2020).

4.2 Material and methods

The batch experiment was conducted in serum bottles and the sampling method were described in section 3.3.1. The analytical measurements were TDS, pH, NH₄-N, toluene and metabolites were mentioned in section 3.6. The inhibition concentration threshold obtained from the toluene degradation test was selected for the metabolite test to determine the pathway of toluene degradation under microaerobic condition.

4.3 Results and discussion

4.3.1 Toluene inhibition threshold

The batch experiment had a limited amount of headspace in each bottle, where minor amount of oxygen was present. In this experiment, there was an obvious sign of decreasing trend for 100, 200 and 300 mg/L of toluene based on pH, NH₄-N and TDS, shown in Figure 6. On the other hand, 0, 400 and 500 mg/L showed no signs of any changes. The decrease in pH indicates the formation of acids, which were due to toluene undergoing TCA cycle. At a concentration level of 300 mg/L, toluene seemed to be the inhibition threshold for the biomass. At a concentration level of 400 mg/L and above, it led to the deactivation of the microbial activity.

Figure 6: (A) pH VS Time; (B) NH₄-N VS Time; (C) TDS VS Time

4.3.2 Identification of metabolites

300 mg/L of toluene was selected for microaerobic metabolite test in a batch experiment. Samples were taken daily from the serum bottles to till 5 days consecutively. SPE sample preparation was conducted prior to GC/MS analysis. Based on the GC MS spectrum results, the toluene's metabolites were found to be benzyl alcohol, o-cresol, p-cresol and m-cresol (shown in Figure 7A, B). The identification of these compounds was based on their mass spectrum similarities with the library database. There was also small traces of 3 methyl catechol and 4 methyl catechol detected. The results suggested that toluene underwent aerobic pathway given that all metabolites detected were attracted with an OH bond. Furthermore, these metabolites were being mineralized over time. The initial step of toluene degradation was the most difficult, due to the stability of the benzene ring structure. Hence, under the aerobic condition, the first initial degradation step of toluene undergoes dehydrogenation or hydroxylation which transform it into 3-methyl catechol or having an OH group attached at the ortho, meta or para position of toluene, which makes the structure less stable (Parales et al., 2008). These compounds further underwent a tricarboxylic acid cycle (TCA) cycle, which

generates low molecular weight acids. The retention time for these compounds were, benzyl alcohol (3.11 mins), o-cresol (3.3 mins), p-cresol (3.85 mins), m-cresol (3.85 mins), 3 methyl catechol (6.22 mins) and 4 methyl catechol (6.25 mins). It seems that it is possible to operate the EMBR under microaerobic conditions with adequate headspace for oxygen supply. Furthermore, supplying 300 mg/L or less toluene concentration in feed solution will be ideal for the EMBR, to prevent any inhibition effects towards the biological activity.

Based on the GC-MS mass spectrum, as shown in Figure 8, the identification of the metabolites was based on their m/z ratio similarities with the library database. Given that benzyl alcohol, o-cresol, p-cresol and m-cresol shares the same molecular weight with different -OH placement, the mass spectrum fragments were the same. The notable mass spectrum fragments are 108, 90 and 79 m/z . They were identified based on their retention time (based on polarity) and relative abundance ratio. The previous study conducted by Ramalho et al. (2020) reported that o-cresol could be differentiated from m-cresol and p-cresol based on retention time. Similarly, the identification of 3 methyl catechol and 4 methyl catechol were identified based 124, 107 and 78 m/z . Another study by Li et al. (2018) reported 3 methyl catechol and 4 methyl catechol were identified based on their retention time. The identification of these compounds was further support from National Institute of Standards and Technology Chemistry WebBook (Linstrom and W.G. Mallard).

(A)

Day 0 (full view)

Day 1 (full view)

Day 2 (full view)

Day 4 (full view)

Day 5 (full view)

(B)

Day 1 (closed up view)

Figure 7:(A) Full spectrum view of metabolites identified over time; (B) Closed up spectrum view of metabolites identified on day 1.

Figure 8: GC-MS mass spectrum comparison between sample (top) and library database (bottom): (A) Benzyl alcohol, (B) P- cresol and M-cresol ; (C) O-cresol; (D) 4 methyl catechol

4.3.3 The identification of metabolites in parent reactor

Based on the LC/MS results, it was found that benzyl succinate was found in the parent reactor. This suggested that toluene was undergoing anaerobic degradation in the parent reactor. The identification of this metabolite was based on the mass spectrum fragment of 207 m/z, 163 m/z and 117 m/z which reported by Abu Laban et al. (2009), as shown in Figure 9B. Benzyl succinate will further undergo Beta oxidation reactions to form Benzyl-CoA. Next, it will further be broken down into low molecular acids in the TCA cycle reported by Parales et al. (2008), shown in Figure 9C. The mass spectrum fragment results are agreeable with previous studies (Beller, 2002, Abu Laban et al., 2009).

Figure 9: (A) LC/MS spectrum, blank; (B) LC/MS spectrum, parent reactor; (C) Proposed anaerobic toluene degradation pathway, retrieved from Paraless et al. (2008)

4.4. Conclusion

The results suggested that the presence of facultative microbes was able to degrade toluene under anaerobic conditions and microaerobic conditions. The microbial community was able to adapt to both operating conditions. However, operating under anaerobic condition the toluene degradation capacity will be much lower, as mentioned before the parent reactor was fed with 25 to 30 mg/L daily. As a result, it will not be ideal for operating the EMBR in anaerobic condition, because concentration difference is the driving force in EMBR operation. Besides that, it

was difficult to maintain an anaerobic environment in that EMBR configuration because of the possible air leakage through the tube and fittings. Hence, the EMBR will be operating in microaerobic condition without any aeration provided. The identification of these species in the EMBR setup will be mentioned in the subsequent experiment results.

CHAPTER 5 Toluene removal and biotransformation in EMBR

Outline:

5.1 Introduction

5.2 Material and methods

5.3 Results and discussion

5.4 Conclusion

5.1 Introduction

In this experiment, the microaerobic EMBR was carried on the commercial and synthetic membrane for 34 days (2 cycles of 17 days). The comparison of both membranes was based on k_o , normalized k_o , recovery of k_o , toluene removal and toluene elimination capacity. Besides that, the membrane's morphology and thickness were investigated, to determine how membrane properties can affect the biofilm growth and the toluene extraction. The metabolites and the dissolved organic matters of the EMBR were measured at the end of the experiment. At the end of the experiment, the fouled membrane was taken out for membrane autopsy and foulant characterization.

5.2 Material and methods

The EMBR experiment configuration, as described in section 3.4. The analytical measurements were TDS, pH, $\text{NH}_4\text{-N}$, toluene, metabolites and natural organic compounds were mentioned in section 3.6. The experiment was being conducted twice on both commercial and synthetic membrane. The experiment was conducted for 17 days, followed by a chemical cleaning to observe the recovery of k_o and was operate for another 17 days. Similarly, the morphology and thickness measurement of both membranes were described in section 3.5

5.3 Results and discussion

5.3.1 Toluene removal performance

The mass transfer coefficient performance on both membranes are shown in Figure 10A, B. Throughout the experiment, TDS was monitored on both sides to ensure there was no leaking. Based on the results, the synthetic ZIF8 membrane was found to outperform the commercial membrane. The initial k_o was found to be $3.4 \pm 0.3 \times 10^{-5}$ m/s and $2.6 \pm 0.6 \times 10^{-5}$ m/s in the synthetic membrane and commercial membrane, respectively. Similarly, the maximum toluene capacity was found to be 1.18 and 1.34 g/m² hour on the commercial and synthetic membrane, respectively. In the first few days of operation, the k_o and toluene diffusion capacity on both membranes dropped significantly. After 1 day of operation, the reduction of normalised k_o in synthetic and commercial membranes were found to be 40.2 % and 31.6 %, respectively. Such reduction was due to the sludge cake attachment on the membrane surface, which contributes an additional resistance layer for mass transfer. After 4 days of operation, the k_o values were stabilized in both membranes, only a gentle decreasing trend was observed due to the biofilm growth over time. At the end of 17 days, the toluene elimination capacity was found to be 0.33 and 0.58 g/m² hour on the commercial and synthetic membrane, respectively (Figure 10D).

After chemical cleaning, it was observed that the commercial membrane was able to recover back to its initial k_o , unlike the synthetic membrane, it does not fully recovered backs to its original k_o . The reason was due to the irreversible biofilm attachments on the rough surface of the synthetic membrane. The findings were supported by the AFM results, showing that both synthetic membrane and the commercial membrane was a Ra value of 52.689 nm and 27.460 nm, respectively, Figure 11A, B. The synthetic membrane was about twice as rough as the commercial membrane. Rz value was also twice higher, which means that there are deeper valleys or depressions across the membrane surface. The reason was due to the particle aggregation of ZIF-8 nanofillers embedded in the PDMS matrix. Jin et al. (2018) reported 1-2 μ m size of ZIF-8 nanofillers were observed on a 8 wt% ZIF-8@PDMS membrane surface. As a result, it promoted biofilm adhesion and reduced

the possibility of the biofilm from being washed away during chemical cleaning. Thus, it was difficult to remove the biofilm attachments totally on the synthetic membrane. Therefore, it explained the sharper drop in the initial normalised k_o value and the lower recovery of normalised k_o value in comparison with the commercial membrane. Despite that, the synthetic membrane still outperforms the commercial membrane in terms of toluene removal efficiency and elimination capacity, shown in Figure 10C, D. The use of highly efficient membrane allows the better mass transfer of toluene which allows the microbes to degrade more.

Throughout the experiment, the receive side was always found to have low or no trace of toluene. Only the exception of the start of the experiment, where biofilm was not formed and matured. Once the biofilm is formed, the microorganisms residence inside the biofilm and cake layer were able to degrade toluene quickly. As a result, the synergy between the extracellular polymeric matrix and the high-density of active microorganisms led to the efficient removal of toluene. However, as the biofilm and cake layer becomes thicker over time, the toluene removal performance of the EMBR system decreases due to high mass transfer resistance in the biofilm and cake layer from the receiving solution (Figure 10C) (Kumar et al., 2012).

Figure 10: (A) overall mass transfer coefficient throughout the experiment; (B) Normalized overall mass transfer coefficient throughout the experiment; (C) Toluene removal performance; (D) Toluene elimination capacity.

Figure 11: (A) Surface roughness of the commercial membrane; (B) Surface roughness of the synthetic membrane.

5.3.2 Toluene degradation metabolites

At the end of EMBR experiment on day 34, metabolites in biomedium were analysed to determine the toluene degradation pathway. The major degradation metabolites were found to be benzyl alcohol, o-cresol, p-cresol and m-cresol. There was also a small trace of 3-methyl catechol and 4-methyl catechol detected. The detection of these metabolites indicates the presence of aerobic or microaerobic microbes in the EMBR. Although the system was sealed and operated without aeration, there could be air leakage from the fittings, oxygen diffusion from headspace and oxygen permeation from the feed solution side. It was believed that most of the toluene underwent toluene monooxygenase and toluene oxidase, instead of dioxygenase pathway due to the limited amount of oxygen supply. The proposed toluene degradation pathways are shown in Figure 12.

The dissolved organic compounds in each different components of the biological system were different (Figure 13). LMW neutrals consist of alcohols, aldehydes, ketones, and amino acids (Stewart et al., 2013). The biofilm layer has a high amount

of LMW neutrals ($75 \pm 1\%$) which could present the by-products of toluene, given that o-cresol, p-cresol and m-cresol falls under the category of alcohols. The biofilm on the membrane surface was exposed directly to the diffused toluene and biotransformation occurred within the biofilm; this explains the high percentage of LMW neutrals content. On the other hand, the dissolved organic compounds in bio medium were different. The LMW neutrals was $20.3 \pm 0.2\%$, LMW acids was $5.3 \pm 0.7\%$, biopolymers were $39.6 \pm 2.6\%$ and building blocks was $34.8 \pm 2.1\%$, degraded compounds profile in the biomedium. Besides, the TOC concentration was 20 ± 10 mg/L and no trace of toluene was detected in the bio medium during 17 days. This indicates that there was no accumulation of by-products in the biomedium.

The relatively stable toluene removal throughout the experiment suggests that there were no signs of toluene's metabolites inhibition. The previous study reported that the toxicity of toluene is reduced from 88% to 33% when toluene is biotransformed into its intermediate by-products (Halldorson et al., 2004). However, at the end of the experiment, traces of metabolites, e.g. o-cresol, p-cresol and m-cresol, were detected in the feed solution side. This indicates that a minor portion of metabolites produced within the biofilm and cake layer was fluxed back into the feed solution, due to the concentration difference at both sides (Figure 14).

Figure 12: Proposed aerobic toluene degradation pathways, modified from Parales et al. (2008)

Figure 13: Dissolved organic compounds, commercial membrane

Figure 14: Detection of metabolites in feed solution at the end of the experiment

5.3.3 Morphology and thickness of both membranes

The morphology and thickness of both membranes were investigated to understand how it affects the biofilm attachment growth and extraction performance. The

thickness of both membranes was analysed by SEM (Figure 15). The synthetic membrane was found to have an overall thickness of $81.4 \pm 4.3 \mu\text{m}$ and the active PDMS layer thickness of $13.7 \pm 0.7 \mu\text{m}$. The commercial membrane has a thickness of $100 \mu\text{m}$. The thin PDMS active layer on the synthetic membrane lowers the mass transfer resistance, allowing more toluene to flux through. This explains the higher k_o performance of the synthetic membrane in comparison with the commercial membrane. The spaces between the fibres offer no resistance and aid in diffusion. Furthermore, the pocket of spaces within the support layers suggest that it is not ideal for the synthetic membrane to operate the active layer facing towards the feed side and exposing the support layer to the receive side. It will be difficult to remove the biofilm which may be embedded inside the support layers. The recovery k_o will be thus much lower compared to the current result, if the orientation is the other way around reported by Liao et al. (2018b).

Figure 15: (A) Thickness of PDMS layer of the synthetic membrane; (B) Overall thickness of the synthetic membrane; (C) Thickness of commercial membrane

5.4 Conclusions

The findings show that the ZIF-8@PDMS/PVDF membrane outperformed the commercial membrane in the microaerobic EMBR based on the k_o and toluene removal results shown in (Figure 10). The initial k_o value of the synthetic membrane and commercial was $3.4 \pm 0.3 \times 10^{-5}$ m/s and $2.6 \pm 0.6 \times 10^{-5}$ m/s, respectively. The synthetic membrane has a slightly higher k_o value than the commercial membrane throughout the experiment. The thin PDMS layer and the beneficial enhancement from the ZIF-8 nanofillers improves the k_o of the synthetic membrane. Furthermore, the toluene elimination capacity of the synthetic membrane at the start until the end of the experiment was still higher than the commercial membrane. It was evident that cake attachment and biofilm growth contribute to an additional mass transfer resistance which resulted in a similar reduction in normalized k_o in both membranes. Thus, the overall toluene removal and elimination capacity were lowered. The surface roughness of the membrane does play a significant role in cake attachment and biofilm growth, and the recovery of k_o after chemical cleaning. The low recovery of k_o after chemical cleaning in the synthetic membrane, indicates that there was irreversible biofouling on the membrane surface. The metabolites were identified in the biomedium and TOC results show no signs of metabolites accumulation. However, the detection of metabolites in the feed solution shows the limitation of this EMBR configuration.

CHAPTER 6 Biofilm profile and microbial dynamics in EMBR

Outline:

6.1 Introduction

6.2 Material and methods

6.3 Results and discussion

6.4 Conclusion

6.1 Introduction

The findings from this experiment will provide a more in-depth insight into microbial dynamics in the microaerobic EMBR at the genus and/or species level. In previous EMBR studies on toluene removal, most were either using pure strains or did not mention the microbial community in the EMBR (Jorge and Livingston, 2000, Emanuelsson et al., 2003, Mehdizadeh et al., 2011). On the other hand, microbial community studies on toluene removal in other industrial application did mention. One of the studies reported by Yu et al. (2018) that *Actinobacteria* was found in high abundance and it played a role in degrading aromatic hydrocarbons in submerged MBR treating synthetic oil refinery wastewater containing toluene and anthracene. Another study on BTEX removal reported by Akmirza et al. (2017), shows that *proteobacteria* was the main predominant phylum found in an anoxic bio trickling filter. Similarly, a study conducted by Yamaguchi et al. (2018) toluene gas treatment using downflow hanging sponge reactor reported that phylum *proteobacteria* was the most abundant and *Acidovorax* spp. was detected throughout the experiment phase. On the other hand, a shake flask study on petroleum hydrocarbon removal under microaerobic conditions found that *Acinetobacter* (66.3%), *Pseudomonas* (11%) and *Acidovorax* (11%) reported by Revesz et al. (2020). Another shake flask study conducted by Tánácsics et al. (2018)

on BTEX contaminated aquifer, reported mostly were *Quatrionicoccus* spp., *Zoogloea* spp., and uncultured *Rhodocyclaceae* were found based on the 16S rRNA gene. The findings from these studies could only give a rough guideline and what to expect from in the microaerobic EMBR. More importantly, it will be interesting to find out the different microbial community between the attached growth (extracellular polymeric layer) and suspended growth over time. Furthermore, it is crucial to understand how the community change affects the toluene removal performance.

6.2 Material and methods

The membrane autopsy was conducted prior to any biofilm analysis. The measurement of the biofilm morphology, biofilm thickness and volume are described in section 3.5. In short, after images were taken from confocal microscopy, the IMARIS software was used to calculate the biofilm thickness and volume. The EPS extraction is the heat extraction method followed closely to Sun et al. (2018). Protein and polysaccharide were conducted on the extracted EPS samples, described in section 3.5.6. The DNA extraction was followed correctly to FastDNA® SPIN Kit for Soil's protocol. The extracted samples were sent to Shanghai Meiji Biomedical Technology Co., Ltd for DNA sequencing analysis

6.3 Results and discussion

6.3.1 Biofilm morphology and EPS

Based on SEM images, there was a wide diversity of microorganisms on the membrane surface, mainly rod and cocci shaped microorganisms (Figure 16B, C). These microorganisms were probably the main players of degrading toluene, given that they were in direct contact with the diffused toluene from the membrane surface. Besides that, colonies on the membrane surface and some portion of the bared membrane can be seen. This suggests that the membrane roughness could also play an important role in biofilm formation. The previous study conducted by

Liao et al. (2018b) mentioned that roughness of the membrane surface is critical for biofilm accumulation in the EMBR process.

The EPS extraction and analysis of the cake and biofilm layer were mentioned in section 3.5.6. The EPS of biofilm and cake layer The biofilm and cake layer EPS content in both membranes were about the same (Figure 17A, B). It is worth noting that the high amount of protein presented in the cake layer, which can promote the adhesion on the hydrophobic PDMS membrane. Besides from that, the high protein content also indicates the inhibitory effects of toluene and the enzymatic activity of toluene mineralization for nutrient acquisition (Flemming and Wingender, 2010). The inhibitory effects of toluene can cause microorganisms to increase membrane wall fluidity and decrease bilayer stability (Weber, 1995). In order to adapt the toxicity of toluene, microorganisms may have to change the lipid composition of the cell wall membrane to adjust the toluene partition, and this can be done by increasing the protein content (Weber, 1995). In comparison between both membranes, the biofilm thickness was relatively the same (commercial vs synthetic membrane) $10.8 \pm 2.2 \mu\text{m}$ and $11.8 \pm 2.8 \mu\text{m}$, respectively. Although the synthetic membrane has a higher flux capability, the influence on biofilm thickness and growth was negligible. There is a notable thickness difference between the biofilm layer and the cake layer based on visual observation. Hence, the sludge cake layer has a more significant influence on the reduction of k_o than the biofilm.

Based on the adhesive strength results, it seems that both membranes shared about the same adhesive strength values (Table 2). Interestingly, both clean membranes have higher adhesive energy than their respective membranes with biofilm. The reason could be due to the rubbery texture and hydrophobic properties of the PDMS layer which encourages the attachment of cake layer and biofilm growth. A more sensitive cantilever would be required to measure the biofilm adhesive strength energy accurately with a lower spring constant. Nonetheless, the current results were enough to prove that biofilm's adhesive energy was lower than the clean membranes.

Figure 16: (A) Clean membrane; (B) Biofilm: rod shaped microorganisms; (C) Biofilm: cocci shaped microorganisms; (D) Biofilm under confocal microscopy, top view

Figure 17: (A) Synthetic membrane, EPS content; (B) Commercial membrane, EPS content

Table 2: Biofilm thickness and adhesive strength between commercial and synthetic membrane

	<i>Commercial membrane</i>	<i>ZIF-8 membrane</i>
<i>Biofilm thickness (μm)</i>	10.8 ± 2.2	11.8 ± 2.8
<i>Adhesive strength, w/o biofilm (J)</i>	$4.7 \pm 0.96 \times 10^{-15}$	$5.0 \pm 1.2 \times 10^{-15}$
<i>Adhesive strength, with biofilm (J)</i>	$3.5 \pm 0.74 \times 10^{-16}$	$2.3 \pm 0.67 \times 10^{-16}$

6.3.2 Microbial dynamics and community

At the end of the experiment, samples from the bio medium and sludge cake layer were sent for metagenomic sequencing. The biofilm layer samples were too little to form sludge pellet for extraction. Hence, an assumption was made that it shares a similar microbial community as the sludge cake layer. Table 3 represents the OTU, Sequence, Shannon, Simpson, Chao and Coverage of the samples in the EMBR. The Shannon index for all samples was about the same ranging from 4.6 to 4.9, and the Simpson index was low, ranging between 0.026 to 0.056, this indicates that low biodiversity in the EMBR. The OTU in the EMBR did not change much from Day 0 (4473) to Day 17 (4492), this suggested that the microbial community was able to adapt well and there were no signs of cell inhibition. At the phylum level, samples were consisting about the same microorganism division, except for the Day 0 (parent reactor) shown in Figure 18. The notable divisions were *Proteobacteria*, *Firmicutes* and *Bacteroidetes*. The highest relative abundance was *Proteobacteria* in all samples. The phylum *Proteobacteria* was found abundant in aerobic conditions and played a role in organic matters degradation (Nascimento et al., 2018, Hu et al., 2012).

The results shown in Figure 19A, B, shows a wide diversity of microorganisms present in the system, the change of microbial community over time and the microbial community in a different component of the biological system. The notable genus/species were *Zoogloea* and *Acidovorax* were found in the bio medium and cake layer. This suggested that they were the main degraders of toluene and metabolites in the EMBR. Interestingly, at the end of the experiment, the cake layer contains a high percentage of *Zoogloea*, presented 20.8% of the

microbial population and a relatively low presence of *Acidovorax* (0.5%). *Zoogloea* and *Acidovorax* were 4% and 11% in the biomedium, respectively. *Zoogloea* was reported to degrade toluene in microaerobic condition, using it as a sole carbon source and energy to thrive (Táncsics et al., 2020, Táncsics et al., 2018). Due to the oxygen limiting condition, toluene undergoes ring monooxygenation instead of dioxygenation, which explains why benzyl alcohol, o-cresol, p-cresol and m-cresol were detected, but not catechol. The study by Táncsics et al. (2020) also mentioned that *Zoogloea* was involved in the initial reaction step of toluene mineralization. *Acidovorax* was found to degrade toluene under aerobic environment and microaerobic environment (Medina and Peimbert, 2011, Yoshikawa et al., 2017). Secondly, *Acinetobacter baumannii* and *Acinetobacter nosocomialis* are also abundant shown in Figure 19B. They were reported to be capable of degrading toluene and other aromatic hydrocarbon compounds such as phenol, ethylbenzene and benzene (Huang et al., 2014, Rochman, 2016).

Table 3: OTU's alpha diversity of DNA samples in the EMBR

<i>Samples</i>	<i>OTU</i>	<i>Sequence</i>	<i>Shannon</i>	<i>Simpson</i>	<i>Chao</i>	<i>Coverage (%)</i>
<i>Day 0</i>	4473	131,171	4.9	0.026	1452	99.8
<i>Day 6</i>	4224	127,010	4.8	0.036	1491	99.8
<i>Day 17</i>	4492	130,490	4.9	0.029	1437	99.8
<i>Cake</i>	4326	114,441	4.6	0.056	1445	99.8

Figure 18: Phylum level, (A) Day 0; (B) Day 6; (C) day 17; (D) Cake layer

6.4 Conclusions

In summary, the microbial community results indicate the presence of toluene degrading microorganisms under a microaerobic EMBR system. *Zoogloea* and *Acidovorax* are the crucial groups of microorganisms involving toluene, and its metabolites degradation. It was certain that toluene underwent ring monooxygenation by *Zoogloea*. The microbial dynamic was continually changing according to the operating conditions throughout the experiment. At the beginning of the experiment, the microbial community needed time for adjustment and proliferate. The EPS composition of the biofilm suggested that the microorganisms were able to adapt well on the membrane surface by producing a higher amount of protein in the biofilm matrix to maintain its cell wall membrane stability and resist against toluene's toxicity. At the same time, the development of biofilm and cake layer can host the active microorganism to degrade the flux toluene. However, the downside would be poor mass flux performance due to the thickness of the biofilm and cake layer were beyond an acceptable level. The suspended growth and attached growth of the biological side have their significant roles to play a part in toluene removal.

CHAPTER 7 Conclusion and recommendations

Outline:

7.1 Introduction

7.2 Conclusion

7.3 Recommendations

7.1 Introduction

This chapter will conclude the findings and the recommendations for future work for the EMBR study. It will also emphasize on limitations and challenges faced, and suggest what appropriate methodology and recommendations to improve this study for future research.

7.2 Conclusion

The synthetic membrane outperforms the commercial membrane in terms of toluene elimination capacity and k_o . The reduction of k_o in both membranes was about the same due to biofilm and cake layer. After operating for 17days, the toluene capacity dropped from 1.18 to 0.33 g/m² hour for commercial membrane and 1.34 to 0.58 g/m² hour for synthetic membrane. The membrane roughness does have an impact on biofilm growth, cake attachment and the recovery of k_o . The development of biofilm and cake layer lowers the mass transfer of toluene from the feed side. Even though the synthetic membrane performs better than the commercial membrane throughout the experiment, but in both cases, the biofilm and cake layer was the drawback on the EMBR's performance. Thus, a biofilm control strategy is necessary. In order to achieve that, frequent cleaning can be carried out to reduce the overall mass transfer resistance which mentioned by previous studies (Yeo et al., 2016, Splendiani et al., 2006). It is a challenge to implement a biofilm control strategy without affecting the biological performance and extraction rate at the same time. A series of reactors or membrane models can be lined up with alternative operation and maintenance.

Operating the EMBR under a microaerobic condition with mixed cultures offers several advantages. First, it eliminates the need for aeration, which reduces the operating cost and air stripping of volatile compounds. Second, the use of low-cost acclimatised sludge. Third, the use of mixed cultures offers several advantages, like the exchange of genetic materials within the microbial community, more resistant to toxic compounds and having combination of different metabolic pathways (Trusek-Holownia and Noworyta, 2015, Sabra and Zeng, 2014). In this study, *Zoogloea* and *Acidovorax* were the dominant vital players of toluene removal in this micro aerobic EMBR. The microbial dynamics in the EMBR, provide a more in-depth insight into how a mixed community of microbes adapt to the environment and mineralized toluene. The findings from this study highlight the EMBR's potential of treating aromatic organic compounds under microaerobic conditions with the right acclimatised strains microbial community.

According to the results obtained in this study, the following conclusions can be drawn:

- (1) The 4 wt% ZIF-8@PDMS/PVDF synthetic membrane outperforms the commercial membrane in terms of toluene elimination capacity and k_o . The reduction of k_o in both membranes was about the same due to the formation of biofilm and cake layer.
- (2) The membrane roughness indeed had an impact on biofilm attachment and the recovery of k_o . The high protein content in EPS of biofilm and cake layer promotes better bio adhesion with the hydrophobic surface of the membrane. Besides that, the microorganisms adapt to the toxicity of toluene by increasing protein and/or EPS content.
- (3) *Zoogloea* and *Acidovorax* were the major degraders in toluene removal in this microaerobic EMBR. The microbial dynamics findings provide an

insight into how a mixed community of microbes adapt to the environment and mineralized toluene.

- (4) The identified degradation by-products are o-cresol, p-cresol and m-cresol, indicating that toluene ring monooxidation (at para, meta and ortho position) and toluene ring oxidation are the major toluene degradation pathways in the microaerobic EMBR.

7.3 Recommendation

Based on the challenges faced in this study, the following recommendations for future work are suggested:

- (1) A possible solution to prevent biofilm and cake formation would be changing the configuration of the EMBR system. For example, it can be configured into an External EMBR to prevent the biological system in contact with the membrane.
- (2) Investigating the feasibility of removing other VOC compounds in the microaerobic EMBR, e.g. Benzene, Ethylbenzene and Xylene
- (3) Apart from using air scouring or chemical cleaning as a biofilm control strategy, investigating other possible biofilm control strategies to provide a long term and feasible approach to keep the process in operation.
- (4) An online system to measure the thickness of foulant (biofilm and cake layer) would be ideal to understand the formation of foulant over time and to determine the appropriate time to carry out cleaning.

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