BIODEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS
(PAHS) USING MICROBIAL FUEL CELLS (MFCS) IN THE
CONTAMINATED SEDIMENT AND WASTEWATER

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DEPARTMENT OF CIVIL AND ENVIRONMENTAL ENGINEERING
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2016
To my dad and mum,

for their love and support
Declaration

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

Mohammad Sherafatmand

05 January 2016
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Summary

Environmental pollution is one of the major problems taking place today. Our earth is increasingly contaminated and polluted and no one else to blame, but us. The effect of pollution on soil and water is quite disturbing and can result in huge disturbance in the ecological balance and health of living being on the earth. Among various contaminants, polycyclic aromatic hydrocarbons (PAHs) due to their persistency and effects on the environment have received significant attentions in order to be removed or to be kept under the threshold limit. So, different strategies have been utilized to remove PAHs from the soil and water; physical/chemical methods that are known and used for a long time and bioremediation methods. However, all these conventional methods, due to their non-environmental friendly nature (secondary contamination), high cost and low efficiency, could not be good solutions. Therefore, an environmental-friendly method is needed for the treatment of contaminated sediments and wastewater.

Microbial Fuel Cells (MFCs) have been receiving significant attentions in the last decade due to their ability to breakdown contaminants and generate electricity simultaneously. However, they could be a great solution to treat contaminated sediments and wastewater if the microorganisms would be able to breakdown PAHs in these systems.

In this study, three types of microbial fuel cells (MFCs) were developed to remove PAHs from contaminated sediment and wastewater. MFC is a device that
uses microorganisms to generate electricity through the oxidation of organic matters present in the sediment or wastewater.

In aerobic-Sediment Microbial Fuel Cells (SMFCs), four SMFC reactors in aerobic cathodic condition were implemented to investigate the feasibility of PAHs bioremediation in the contaminated sediment (Chapter 4). Air was constantly supplied to provide enough electron acceptor (oxygen) in the water column. The results revealed significant rates of removal in PAHs (i.e., naphthalene, acenaphthene and phenanthrene) from the sediment. The SMFCs achieved 41.7, 31.4 and 36.2% PAHs removal in aerobic environment for naphthalene, acenaphthene and phenanthrene, respectively. In addition, this study also showed that SMFCs can increase TOC removal in the sediment. The SMFCs showed 52% TOC removal from the sediment, while it was only 27% for the non-SMFC reactor. This stimulation could be attributed to altering physical and chemical properties of the sediment by applying a potential difference and more activated medium for microorganisms provided by electrochemical systems. This finding was verified with the results of pyrosequencing analysis that revealed the presence of different microbial communities in the systems with electrodes. These results have shown that aerobic-SMFCs can have major implications for in-situ bioremediation of PAH-contaminated sediment.

Therefore, it was also shown that the aerobic-SMFC reactors containing PAHs could generate power density as great as the first 10 days for the next 30 days, which confirmed the PAHs degradation in the systems. However, for the control reactor (without PAHs), the power density dropped significantly after 10 days.
This study has taken the first step of scaling up electrochemical systems (i.e., SMFCs) for both sediment cleaning and power generation.

Although the results of the aerobic-SMFCs demonstrated the feasibility of bioremediation process, it should be noted that in the real environment, there are many anaerobic zones that the concentration of oxygen is not adequate for the process. So, four anaerobic-SMFCs were constructed to investigate the rate of PAHs removal, TOC removal as well as electricity output and microbial analysis (Chapter 5). Nitrate and sulfate were added as electron acceptors to the water column (cathodic compartment). The results showed the significance effectiveness of electrochemical systems on bioremediation rates (i.e., PAHs and TOC) and also the power output. It was found that 76.9%, 52.5% and 36.8% of naphthalene, acenaphthene and phenanthrene were removed during the process. Similar to the aerobic-SMFCs, the higher removal rates of bioremediation was due to the stimulation provided by electrodes.

The notable different performances observed in the aerobic and anaerobic phases were largely due to two reasons: first, the different reduction potential of electron acceptors (oxygen in the aerobic and nitrate/sulfate in the anaerobic phases) and second, the different microbial communities involved in the systems. For instance, in the phylum level, *Proteobacteria* comprised between 70-76% of the total sequences in the aerobic phase, while it was between 93-96% in the anaerobic phase. And also in the class level, *β-proteobacteria* was 55.72% in the aerobic, while it was 76.46% in the anaerobic.
In the last part of the study, single-chambered air-cathodes MFCs were constructed to examine the effect of the electrochemical systems on the treatment of PAH-contaminated wastewater (Chapter 6). In the batch study, it was found that better electricity output, higher COD and TOC removals could be achieved in the presence of PAHs (i.e., naphthalene, acenaphthene and phenanthrene) in the wastewater. For instance, COD was removed by 91% in the duplicated reactors with PAHs while it was only 66% in the duplicated reactors without PAHs. In addition, all PAHs were degraded with higher efficiencies in the closed-circuit (1000 Ω) compared to the open-circuit reactor.

In the continuous study, a HRT of 12 h was found to be the optimum hydraulic retention time among 2, 4, 8, 12 and 24 h. Therefore, similar to the batch mode, better efficiencies were obtained in the duplicated reactors with PAHs at a HRT of 12 h compared to those without PAHs, except electricity output and TOC removal. Electricity (i.e., voltage and power density) generated from the MFC reactors without PAHs was higher.

However, this chapter showed that single-chambered air-cathode MFC reactors used in this study could be used not only for biodegradation of persistent compounds such as PAHs (i.e., naphthalene, acenaphthene and phenanthrene) but also for even higher electricity output and removal efficiencies in COD and TOC of wastewater.
The results of this study indicated the potential of electrochemical systems (i.e., SMFCs or single-chambered air-cathode MFCs) in in-situ biodegradation of sediment and wastewater contaminated with persistent compounds such as PAHs.
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<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>Acen</td>
<td>Acenaphthene</td>
</tr>
<tr>
<td>AS</td>
<td>Anaerobic sludge</td>
</tr>
<tr>
<td>BMFC</td>
<td>Benthic microbial fuel cell</td>
</tr>
<tr>
<td>CCV</td>
<td>Closed circuit voltage</td>
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<tr>
<td>CD</td>
<td>Current density</td>
</tr>
<tr>
<td>CE</td>
<td>Counter electrode</td>
</tr>
<tr>
<td>COD</td>
<td>Chemical oxygen demand</td>
</tr>
<tr>
<td>CV</td>
<td>Cyclic voltammetry</td>
</tr>
<tr>
<td>DGGE</td>
<td>Denaturing gradient gel electrophoresis</td>
</tr>
<tr>
<td>DOM</td>
<td>Dissolved organic matter</td>
</tr>
<tr>
<td>DWW</td>
<td>Domestic wastewater</td>
</tr>
<tr>
<td>EAM</td>
<td>Electrochemically-active microorganism</td>
</tr>
<tr>
<td>$E_{an}^0$</td>
<td>Maximum potential obtained at the anode</td>
</tr>
<tr>
<td>$E_{cat}^0$</td>
<td>Maximum potential obtained at the cathode</td>
</tr>
<tr>
<td>$E_{emf}$</td>
<td>Electromotive force</td>
</tr>
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<td>Efficiency</td>
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<td>Electrode</td>
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<tr>
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<td>Faraday’s constant</td>
</tr>
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<td>GFE</td>
<td>Graphite felt electrode</td>
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<tr>
<td>GPE</td>
<td>Graphite plate electrode</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>GS/MS</td>
<td>Gas chromatography/mass spectrometry</td>
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<td>Hydraulic retention time</td>
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<td>Current</td>
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<td>Ion chromatography</td>
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<td>Inorganic carbon</td>
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<td>$K_{ow}$</td>
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<td>Loss on ignition</td>
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<td>MFC</td>
<td>Microbial fuel cell</td>
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<td>Multi-walled carbon nanotubes</td>
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<td>Open circuit voltage</td>
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<td>Polycyclic aromatic hydrocarbons</td>
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<td>Persistent organic pollutant</td>
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<td>Reference electrode</td>
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<td>Symbol</td>
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</tr>
<tr>
<td>$R_{\text{ext}}$</td>
<td>External resistance</td>
</tr>
<tr>
<td>$R_{\text{int}}$</td>
<td>Internal resistance</td>
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<tr>
<td>SMFC</td>
<td>Sediment microbial fuel cell</td>
</tr>
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<td>Sediment organic matter</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>TC</td>
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</tr>
<tr>
<td>TDS</td>
<td>Total dissolved soil</td>
</tr>
<tr>
<td>TEA</td>
<td>Terminal electron acceptor</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TKN</td>
<td>Total Kjeldahl nitrogen</td>
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<tr>
<td>TN</td>
<td>Total nitrogen</td>
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<td>TOC</td>
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<tr>
<td>TPH</td>
<td>Total petroleum hydrocarbons</td>
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<td>TSS</td>
<td>Total suspended solid</td>
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<tr>
<td>$V_{\text{cell}}$</td>
<td>Cell voltage</td>
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<tr>
<td>VSS</td>
<td>Volatile suspended soil</td>
</tr>
<tr>
<td>WE</td>
<td>Working electrode</td>
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<tr>
<td>CE</td>
<td>Coulombic efficiency</td>
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<tr>
<td>$\eta_{\text{act}}$</td>
<td>Activation loss</td>
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<td>$\eta_{\text{con}}$</td>
<td>Concentration loss</td>
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<tr>
<td>$\eta_{\text{ohmic}}$</td>
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Journal Publications and Conference Presentations

1. Mohammad Sherafatmand, How Yong Ng, “Using sediment microbial fuel cells (SMFCs) for bioremediation of polycyclic aromatic hydrocarbons (PAHs)”, Bioresource Technology Journal.

2. Mohammad Sherafatmand, Tan Ying Hui, How Yong Ng, “PAH-Contaminated Soil Treatment Using a Sediment Microbial Fuel Cell (SMFC)”. The 5th International Meeting on Microbial Electrochemistry and Technologies, Arizona, US.


Chapter 1 Introduction

1.1 Background

Environmental pollution is one of the major problems taking place today. Our earth is increasingly contaminated and polluted and no one else to blame, but us. The pollution is the result of the presence of contaminants including toxic compounds, radioactive materials and other foreign harmful chemicals. The effect of pollution on soil and water is quite disturbing and can result in huge disturbance in the ecological balance and health of living being on the earth. So, strong regulatory programs to minimize soil and water contamination need to be introduced. Among various contaminants, polycyclic aromatic hydrocarbons (PAHs) due to their persistency and effects on the environment have received significant attentions in order to be removed or to be kept under the threshold limit. Therefore, different strategies have been utilized to remove PAHs from the soil and water; physical/chemical methods that are known and used for a long time and bioremediation methods. Among these strategies, physical/chemical methods are less desirable because they are generally expensive and also create secondary pollutions. Bioremediation that can be divided into aerobic and anaerobic methods is more favorable due to their environmental-friendly nature. These biological methods use microorganisms, mostly bacteria to break down PAHs. Among these biological methods, aerobic treatment has been well-studied compared to anaerobic but due to its limitations in the environments with absent or limited oxygen, it cannot be widely used. Hence, anaerobic treatment has been
identified as the main treatment remedy to these situations. However, so far, due to low efficiency and the time-consuming processes, anaerobic treatment is still a subject to debate that needs more comprehensive investigations.

1.2 Conventional PAHs Removal Methods

The methods to remove PAHs from the environment can be classified as physical/chemical methods and biodegradation methods.

1.2.1 Physical/Chemical Methods

These methods include extraction, chemical-oxidation, photo-oxidation, electro-kinetic and thermal processes. Among these methods extraction, oxidation and thermal processes are the most widely used. In different types of extraction (solvent extraction, supercritical fluid and subcritical fluid extractions) processes, even though with the differences, typically two steps are involved; desorption from binding site followed by elution from the solid into the extraction fluid (Ahn et al., 2008; Deshpande et al., 1999; Khodadoust et al., 2000; Rababah & Matsuzawa, 2002; Silva et al., 2005; Zhou & Zhu, 2007). The problems associated with these methods are slow legal acceptance, low efficiency, secondary contamination, high cost and the non-environmental-friendly nature (Hawthorne & Grabanski, 2000).

For the other common physical/chemical method - oxidation, oxidation agents such as ozone (O₃), potassium permanganate (KMnO₄) and activated persulfate (Na₂S₂O₈) convert PAHs into acid, alcohol and aldehyde derivatives that are more
soluble and degradable (Brown et al., 2003). The advantage of oxidation process is the high removal rate although the costs are expensive (Pizzigallo et al., 1998). In thermal methods, heat is employed to either destroy or volatilize PAHs (Acharya & Ives, 1994; Gan et al., 2009). Nevertheless, the oxidation and thermal methods are also not environmental-friendly and economic.

1.2.2 Biodegradation Methods

Biodegradation methods refer to utilizing or stimulating the activities of microorganisms to degrade pollutants from the environment. Biodegradation can be carried out in-situ either by adjusting nutrients (biostimulation) (Head, 1998; Lovley, 1995; Yu et al., 2005) or adding inoculum of microorganisms (bioaugmentation) (Odokuma & Dickson, 2003). Biodegradation is occurred by breaking down the PAHs using living organisms either in the presence of oxygen (aerobic) or without the oxygen (anaerobic). In aerobic bioremediation, microorganisms use PAHs as a carbon source and produce carbon dioxide (CO₂) – if it is complete – or a smaller (more degradable) compound – if it is not complete – and water (Gan et al., 2009) (more details in Chapter 2 Literature Review). One of the advantages of this method is that microorganisms involved in this process have been widely investigated (Alleman & Leeson, 1999). Another advantages of this method over physical/chemical methods could be its environmental-friendly nature, safety and efficiency (Mohan et al., 2006). However, in the environment that oxygen is absent or limited such as aquifer and marine sediment, aerobic bioremediation cannot be applicable or economic viable (Bakermans et al., 2002b). In these situations, anaerobic microorganisms will
degrade the pollutants with other electron acceptors such as nitrate (NO$_3^-$) or sulfate (SO$_4^{2-}$) (more details in Chapter 2 Literature Review). Although the anaerobic process is not as efficient as the aerobic process or the anaerobic process is not as well-studied as the aerobic process, the anaerobic process is more economic and applicable in many situations.

From the above review, it can be seen that removal of PAHs from the environment especially from the soil is a serious issue and so far has been addressed by existing methods of physical/chemical and biodegradation. Although physical/chemical methods have achieved high efficiency, they are generally not recommended due to their non-environmental-friendly natures and the high costs. Biodegradation methods are more desirable, but the aerobic and anaerobic processes are still cannot be widely adopted due to the abundance of polluted-anaerobic- environments and low efficiency, respectively. However, these challenges highlight and imply the importance of comprehensive studies to develop new ways of biological degradation of PAHs.

1.3 Biological Degradation Using Microbial Fuel Cells (MFCs)

A MFC is a device that generates electricity by bacterial oxidation of substrates that are either organic or inorganic (Logan, 2008; Logan, 2006; Potter, 1911; Rabaey & Verstraete, 2005). This can be achieved when bacteria switch from a natural electron acceptor such as oxygen or nitrate to an insoluble acceptor such as the MFC anode. Microorganisms break down the substrate (i.e. organic carbon) and produce electrons and protons. The electrons then flow through an external
resistor to a cathode, at which the electron acceptor is reduced (Rabaey & Verstraete, 2005). This concept has been widely used in different studies of wastewater treatment. Most of these studies have focused on simple and degradable pollutants (organic carbons) (Logan, 2008).

A sediment microbial fuel cell (SMFC) is a type of MFC that has recently attracted significant attentions (Huang et al., 2011b; Rezaei et al., 2007) due to its unique property of removing organic compounds from the soil/sediment. SMFCs typically consist of an anode buried in a reduced matrix (soil) and a cathode in the overlaying, oxidized water layer (Logan, 2008; Rezaei et al., 2007; Tender et al., 2002a). However, there is no detailed research into the ability of MFC/SMFC for bioremediation of complex compounds such as PAHs. However, it is worthwhile to extend the MFC principles to investigate the degradation of PAHs. Contaminated soil or wastewater with PAHs could be used as the substrate to the MFCs and since the condition in anodic chamber of MFCs is anoxic, anaerobic degradation will be the main treatment process.

1.4 Research Objectives and Significance

Studies reported the possibility of PAHs degradation (as one the most recalcitrant pollutants) by anaerobic microorganisms even though with low efficiencies. On the other hand, MFCs have shown themselves as promising devices for removal of contaminants. However, more comprehensive research is required to investigate the possibility of PAHs degradation with better efficiency in association with MFCs. The aim of this study was to examine the degradation of
PAHs in the soil and wastewater-contaminated sites. The result of this study may contribute to a better understanding of PAHs anaerobic degradation and may offer an inexpensive method for cleaning the soil and wastewater environments. The focus of this study was to study on the feasibility of the above process. More specifically, factors such as pH, design and electrode spacing of MFC for achieving better efficiencies were not evaluated in this study.

1.5 Thesis Organization

This dissertation is organized into a total of seven chapters including this introduction chapter followed by literature review (chapter 2), materials and methods (chapter 3), results and discussions (chapter 4, 5 and 6) and finally conclusion and recommendations for the future works (chapter 7). Figure 1.1 below gives an overview of all chapters.
Chapter 2 Literature Review

2.1 Polycyclic Aromatic Hydrocarbons (PAHs)

2.1.1 Basic Properties

2.1.1.1 General

Polycyclic aromatic hydrocarbons (PAHs), also known as polyarenes or polynuclear aromatic hydrocarbons, are products of incomplete combustion. Different types of PAHs are formed based on combustion temperature, where high temperatures (i.e. cooking process) create simple PAHs and low temperatures (i.e. smoldering) result in more complex PAHs (Harvey, 1998; Harvey, 1997). They
are formed of two or more rings of carbon and hydrogen atoms bonded in either a linear, angular or clustered way (Dabestani & Ivanov, 1999; Harvey, 1997; Sims & Overcash, 1983). Generally, PAHs are toxic and very persistent organic pollutants that are widely distributed in the environment (Björseth et al., 1979; Cerniglia, 1992; Gao & Zhu, 2004; Haeseler et al., 1999).

2.1.1.2 Molecular Weight

PAHs are either classified as low molecular weight (LMW) PAHs if they have two or three fused rings or high molecular weight (HMW) PAHs if they have four or more fused rings. LMW PAHs are degraded and volatilized more rapidly than HMW PAHs (Harvey, 1997). As molecular weight increases, hydrophobicity/lipophilicity increases, water solubility decreases, vapor pressure decreases and the compound will have a more recalcitrant (difficulty to degrade) structure. HMW PAHs persist in the environment because of low volatility, resistance to leaching and their recalcitrant nature (Jones et al., 1996; Wild & Jones, 1995). Molecular weights of 16 priority PAHs are provided in Table 2.1.

Table 2.1 US EPA’s 16 priority pollutant PAHs and selected properties. Adapted from Bojes and Pope (2007); Lundstedt (2003)

<table>
<thead>
<tr>
<th>PAH name</th>
<th>Number of rings</th>
<th>Molecular weight (g/mole)</th>
<th>Solubility in water (mg/L)</th>
<th>Vapor pressure (Pa)</th>
<th>Log (K_{ow})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>2</td>
<td>128.17</td>
<td>31</td>
<td>11.866</td>
<td>3.37</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>3</td>
<td>154.21</td>
<td>3.8</td>
<td>0.500</td>
<td>3.92</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>3</td>
<td>152.2</td>
<td>16.1</td>
<td>3.866</td>
<td>4.00</td>
</tr>
<tr>
<td>Anthracene</td>
<td>3</td>
<td>178.23</td>
<td>0.045</td>
<td>3.40 x 10^{-3}</td>
<td>4.54</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>3</td>
<td>178.23</td>
<td>1.1</td>
<td>9.07 x 10^{-2}</td>
<td>4.57</td>
</tr>
<tr>
<td>Fluorene</td>
<td>3</td>
<td>166.22</td>
<td>1.9</td>
<td>0.432</td>
<td>4.18</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>4</td>
<td>202.26</td>
<td>0.26</td>
<td>1.08 x 10^{-3}</td>
<td>5.22</td>
</tr>
<tr>
<td>Benz[a]anthracene*</td>
<td>4</td>
<td>228.29</td>
<td>0.011</td>
<td>2.05 x 10^{-3}</td>
<td>5.91</td>
</tr>
<tr>
<td>PAH</td>
<td>Ring</td>
<td>Molecular Weight</td>
<td>Log <em>P</em></td>
<td>Octanol-Water Partition Coefficient (Kow)</td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td>------</td>
<td>------------------</td>
<td>---------</td>
<td>------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Chrysene*</td>
<td>4</td>
<td>228.29</td>
<td>0.0015</td>
<td>1.04 x 10⁻⁶</td>
<td>5.91</td>
</tr>
<tr>
<td>Pyrene</td>
<td>4</td>
<td>202.26</td>
<td>0.132</td>
<td>5.67 x 10⁻⁴</td>
<td>5.18</td>
</tr>
<tr>
<td>Benzo[a]pyrene*</td>
<td>5</td>
<td>252.32</td>
<td>0.0038</td>
<td>6.52 x 10⁻⁷</td>
<td>5.91</td>
</tr>
<tr>
<td>Benzo[b]fluoranthe*</td>
<td>5</td>
<td>252.32</td>
<td>0.0015</td>
<td>5.67 x 10⁻⁴</td>
<td>5.80</td>
</tr>
<tr>
<td>Benzo[k]fluoranthe*</td>
<td>5</td>
<td>252.32</td>
<td>0.0008</td>
<td>1.28 x 10⁻⁸</td>
<td>6.00</td>
</tr>
<tr>
<td>Dibenz[a,h]anthracene*</td>
<td>6</td>
<td>278.35</td>
<td>0.0005</td>
<td>2.80 x 10⁻⁹</td>
<td>6.75</td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene*</td>
<td>6</td>
<td>276.34</td>
<td>0.00026</td>
<td>1.33 x 10⁻⁸</td>
<td>6.50</td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]pyrene*</td>
<td>6</td>
<td>276.34</td>
<td>0.062</td>
<td>1.87 x 10⁻⁸</td>
<td>6.50</td>
</tr>
</tbody>
</table>

*The U.S. EPA has classified PAH in italics as possible human carcinogens

2.1.1.1 Structure

PAHs are also classified into two groups based on ring structure: alternant and non-alternant (Li, 2010). Alternant PAHs such as anthracene, phenanthrene and chrysene are derived from benzene by fusion of additional six-membered benzoic rings, and contain fewer than eight benzoic rings (Harvey, 1998). Non-alternant PAHs may contain rings with fewer than six carbon atoms in addition to six-membered rings. This group is extremely broad in structure and greatly increases PAH diversity (Harvey, 1998). Examples of four-, five- and six-membered rings are fluorine and fluoranthene (Dabestani & Ivanov, 1999; Harvey, 1997). Figure 2.1 shows the structure of US EPA’s 16 priority pollutant PAH (Lundstedt, 2003).
2.1.1.2 Solubility and Vapor Pressure

Solubility of PAHs depends on temperature, pH and ionic strength (Pierzynski et al., 2000). Generally solubility is estimated by: (i) chemical structure: by increasing the number of benzene rings in a PAH compound, solubility decreases (Wilson & Jones, 1993) and (ii) octanol-water partition coefficients ($K_{ow}$): there is an inverse relationship between $K_{ow}$ and solubility, which is calculated by the following equation:

$$K_{ow} = \frac{\text{amount of organic chemical in octanol (mg/L)}}{\text{amount of organic chemical in water (mg/L)}}$$

Equation 2.1

Vapor pressure defines the point at which PAHs in the solid state either evaporate into a gaseous form or condense back to a solid state. The higher the vapor
pressure (at normal temperatures), the more volatile the compound is. Naphthalene (11.866 Pa) is more volatile and would readily evaporate more rapidly than dibenz[a,h]anthracene (2.80 \times 10^{-9} \text{ Pa}) at room temperature (Mackay & Callcott, 1998). PAHs vapor pressures are important for determining risk associated with dredge sediments, transfer between resources as well as field sampling and lab safety. Table 2.1 presents the vapor pressure of all 16 PAHs.

### 2.1.1.3 Toxicity

It has long been known that PAHs can have serious deleterious carcinogenic and mutagenic effects to human health (Connell et al., 1997; Leahy & Colwell, 1990). PAHs present a risk to the environment, especially when they enter the food chain through contact, inhalation or ingestion (Schoeny & Poirier, 1993). Adverse respiratory effects, inducing the number of toxic effects from genotoxicity, mutagenic, tumornicity and carcinogenicity have been demonstrated experimentally (NAS, 1983; Schoeny & Poirier, 1993). Mutation of organisms could be a direct result of the genotoxicity of PAHs, which is due to their ability to form stable and depurinating DNA adducts (Chakravarti et al., 1995).

The evidence of carcinogenicity of PAH-containing complexes is strong and convincing. Their carcinogenicity is different and depends on their molecular weight. For instance, the HMW PAHs and their metabolites are more suspected to be mutagenic and carcinogenic (Cerniglia, 1992). Table 2.2 Shows the carcinogenicity of 16 priority PAHs (IARC, 1987; NAS, 1983; Schoeny & Poirier, 1993).
Table 2.2 The carcinogenicity of 16 PAHs

<table>
<thead>
<tr>
<th>PAHs</th>
<th>IARC</th>
<th>US EPA</th>
<th>NAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Anthracene</td>
<td>3</td>
<td>D</td>
<td>0</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>3</td>
<td>D</td>
<td>0</td>
</tr>
<tr>
<td>Fluorene</td>
<td>3</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>3</td>
<td>D</td>
<td>+</td>
</tr>
<tr>
<td>Benz[a]anthracene*</td>
<td>2A</td>
<td>B2</td>
<td>+</td>
</tr>
<tr>
<td>Chrysene*</td>
<td>3</td>
<td>B2</td>
<td>0/+</td>
</tr>
<tr>
<td>Pyrene</td>
<td>3</td>
<td>D</td>
<td>0</td>
</tr>
<tr>
<td>Benzo[a]pyrene*</td>
<td>2A</td>
<td>B2</td>
<td>++</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene*</td>
<td>2B</td>
<td>B2</td>
<td></td>
</tr>
<tr>
<td>Benzo[k]fluoranthene*</td>
<td>2B</td>
<td>B2</td>
<td></td>
</tr>
<tr>
<td>Dibenzo[a,h]anthracene*</td>
<td>2A</td>
<td>B2</td>
<td></td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene*</td>
<td></td>
<td>D</td>
<td>+</td>
</tr>
</tbody>
</table>

Indeno[1,2,3-cd]pyrene*

1. IARC (International Agency for Research on Cancer): 2A>2B>3
   2A – probably carcinogenic to humans;
   2B – possibly carcinogenic to humans;
   3 – not classifiable as to carcinogenicity to humans.

2. US EPA (US Environmental Protection Agency):
   B2 – probable human carcinogen (sufficient evidence in animas and inadequate or no
evidence in humans);
   D – unclassifiable as to human carcinogen

3. NAS (National Academy of Science):
   0 – no carcinogenic;
   0/+ - uncertain carcinogenic;
   + - probable carcinogenic
   ++ - carcinogenic

The relative toxicity of PAHs can be measured using LD_{50} values (the legal dose
in 50% of cases). These are expressed as milligrams of toxic materials per
kilograms of the subject’s body weight that will cause death in 50% of cases
(Bamforth & Singleton, 2005). Table 2.3 presents the LD_{50} values of some
representative PAHs.
Table 2.3 LD\textsubscript{50} of some representative PAHs

<table>
<thead>
<tr>
<th>Material</th>
<th>Number of carbon rings</th>
<th>LD\textsubscript{50} Value (mg/kg)</th>
<th>Test subject</th>
<th>Exposure route</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>2</td>
<td>533-710</td>
<td>Male/female mice respectively</td>
<td>Oral</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>3</td>
<td>750</td>
<td>Mice</td>
<td>Oral</td>
</tr>
<tr>
<td>Anthracene</td>
<td>3</td>
<td>&gt;430</td>
<td>Mice</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>4</td>
<td>100</td>
<td>Mice</td>
<td>Intravenous</td>
</tr>
<tr>
<td>Pyrene</td>
<td>4</td>
<td>514</td>
<td>Mice</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>5</td>
<td>232</td>
<td>Mice</td>
<td>Intraperitoneal</td>
</tr>
</tbody>
</table>

Data taken from the Risk Assessment Information System (RAIS)

2.1.2 PAHs in Environment

PAHs are widespread in the environment and can be found in the air, soil, water and biological systems (Björseth et al., 1979; Cerniglia, 1992; Gao & Zhu, 2004; Haeseler et al., 1999). Additionally, PAH compounds transfer between these resources, i.e., leaching of PAH from a soil resource into ground water or transporting of PAH particles in the atmosphere (Wick et al., 2011). Figure 2.2 shows a diagram of transfer of PAHs in the environment.
2.1.2.1 PAHs in Atmosphere

PAHs in atmosphere can be originated from other resources such as water, soil and biological resources. Atmospheric PAHs levels are usually higher in the winter time due to combustion products from heating and thermal decomposition (Greenberg et al., 1985; Harvey, 1997). For instance, in North America, the PAH concentration is in the range of 3.7 to 450 ng m\(^{-3}\). Nitrated PAHs which are the result of gas-phase reaction of PAHs with nitrous oxides in the atmosphere are in lower concentration than non-nitrated PAHs. But nitrated PAHs are of concern due to their persistency and their carcinogenic potential which is higher than non-nitrated PAHs (Bamford et al., 2003).

The fate of atmospheric PAHs is influenced by whether the PAHs are in the gaseous or particulate form (Wick et al., 2011). The residence time of PAH
particles can be varied from one to two weeks, which allows them to travel a long way (Atkinson & Arey, 1994). The presence of PAHs in the atmosphere is a big concern due to their volatilization nature and ease of transport, but since this study is focused on contaminated soil and water, the removal of atmospheric PAHs is outside the scope of this thesis.

2.1.2.2 PAHs in Water

The main sources of PAHs in water bodies are runoff of polluted ground sources, pollution of rivers and lakes wastewater discharge, oil spills and atmospheric particulate matters (Dabestani & Ivanov, 1999; Latimer & Zheng, 2003). As mentioned in Table 2.1, due to the low solubility of PAHs in the water, they can be found in very low concentrations and even for discharges into the environment, they tend to adsorb to the solid matters such as sediment and soil (to be discussed in the next section). For instance, low PAH concentrations have been reported in water: marine waters with the levels of non-detected to 11 µg L\(^{-1}\) and wastewater in North America and European municipalities with levels of less than 1 to 625 µg L\(^{-1}\) (Latimer & Zheng, 2003).

2.1.2.3 PAHs in Soil and Sediment

PAHs tend to accumulate in the sediments and soils due to their hydrophobicity and low solubility in the water (Juhasz & Naidu, 2000). PAH levels in the sediment and soil can range from µg kg\(^{-1}\) to g kg\(^{-1}\) depending on the sources and places. Apart from the main industrial discharge and contamination, one of the main cause of PAH accumulation in the soils is atmospheric deposition after long-
range transport (Greenberg et al., 1985). It even sometimes hit the levels of mg kg\(^{-1}\) i.e. in Welsh soils (0.1-55 mg kg\(^{-1}\)) (Jones et al., 1989).

Levels of PAHs in the sediment and soils have increased in the past 100-150 years because of growing industrial activities and discharges. PAH concentration in urban industrial soils can be 10-100 times higher those in remote areas (Wild & Jones, 1995). For instance, the concentrations of 821, 5863 and 18704 mg kg\(^{-1}\) have been reported in petrochemical site, creosote production site and wood preserving site, respectively. However, these numbers show the critical and fatal situation of contaminated soil sites that need to be treated.

As reported above, PAHs are persistent contaminants with carcinogenic properties that can be found in all environments and also can be transferred easily and accumulate in the soil and sediment. In the below section, existing methods for removal of PAHs from the environment in particular from soil are discussed.

### 2.1.3 PAHs Removal

The existing methods for removal of PAHs from contaminated soil and sediment are classified into physical/chemical methods and biological methods (Gan et al., 2009). The following subsections provide a summary of these methods with more focus on the biological.


### 2.1.3.1 Physical/Chemical Methods

These methods include extraction, oxidation, thermal and high-energy electron beam irradiation (Cerniglia, 1992; Hawthorne & Grabanski, 2000; Khodadoust et al., 2000). Among these methods, the first three have been more commonly used.

#### 2.1.3.1.1 Extraction

Oxidation can be used in different ways such as surfactant solubilization, solvent extraction, super- and sub-critical fluid extractions. The common base of all these extraction techniques is employing different solvents to extract and concentrate PAHs from the contaminated environment (Paterson et al., 1999; Smyth et al., 1999). In this method, there are two steps involved in the extraction of PAH from the soil matrix: desorption from binding site in the solid matrix followed by elution from the solid into the extraction fluid (Ahn et al., 2008; Deshpande et al., 1999; Khodadoust et al., 2000; Rababah & Matsuzawa, 2002; Silva et al., 2005; Zhou & Zhu, 2007). The problem associated with these methods are low efficiency, secondary pollution, high cost and slow legal acceptance (Hawthorne & Grabanski, 2000).

#### 2.1.3.1.2 Oxidation

In oxidation techniques, oxidizing agents such as Fenton’s reagent, ozone (O₃), potassium permanganate (KMnO₄) and activated persulfate (Na₂S₂O₈) convert PAHs into acid, alcohol and aldehyde derivatives, which are more soluble and degradable (Bogan & Sullivan, 2003; Brown et al., 2003; Ferrarese et al., 2008; Flotron et al., 2005). The efficiency of oxidation reaction can be improved by
adding catalysts such as metal oxides (Pizzigallo et al., 1998). Although the oxidation methods result in high removal rate of PAHs, they cannot be widely used due to the high cost and non-environmentally nature of the process (i.e. production of intermediates which can be more toxic than the parent compound) (Pizzigallo et al., 1998).

2.1.3.1.3 Thermal

In thermal methods, heat is employed to either destroy or volatilize PAHs (Acharya & Ives, 1994; Gan et al., 2009). Incineration of soil/sediment at high temperature (870⁰C to 1200⁰C) effectively breaks down PAHs. Therefore, thermal desorption is a physical treatment process that applies heat to volatilize organic compounds from the soil, sediment and sludge. Then a vacuum system sweeps the volatized compounds into a gas treatment system for the secondary treatment or disposal. As the nature of the method shown, these methods are also not environmental-friendly, economic and may increase the risk of the volatile secondary pollution.

2.1.3.2 Biological Methods

Microbial communities including bacteria, fungi and algae can biologically degrade PAH compounds during direct metabolism or cometabolism (Lundstedt, 2003). Bioremediation (bioreclamation or biorestoration) can be described as “the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state” (Mueller et al., 1997). The main principle of this process is to remove pollutants from the natural environment and/or convert them
to a less harmful product (Bamforth & Singleton, 2005). Bioremediation strategies are enhanced to speed up the microbial activities of indigenous microorganisms either by adjusting nutrients (biostimulation) or adding inoculum of microorganisms with known pollutant transformation abilities (bioaugmentation). During biostimulation process, electron acceptors (oxygen, sulfate, nitrate, iron and manganese) and electron donors (organic carbon and hydrogen) are often added to improve the activity of in-situ organisms (Head, 1998; Lovley, 1995; Vogel, 1996; Yu et al., 2005). Bioaugmentation involves the addition of external microorganisms (indigenous or exogenous), which have the ability of degradation (Odokuma & Dickson, 2003) and is employed in the areas where in-situ biodegradation potential is lacking (Li, 2010). These biodegradation processes can be divided into aerobic and anaerobic degradations that will be discussed in the following subsections.

2.1.3.2.1 Aerobic Biodegradation

Aerobic bacteria use oxygen as an electron acceptor to break down PAHs into smaller compounds, producing either carbon dioxide (CO$_2$) – complete mineralization – or metabolites – partial degradation - and water as the final products (Gan et al., 2009). A variety of aerobic bacteria, fungi and enzymes have been specified as the species that can use PAHs as carbon and energy sources (Haritash & Kaushik, 2009). Tables 2.4 and 2.5 show the aerobic bacterial species capable of PAHs degradation.
Table 2.4 Aerobic bacterial species reported to degrade polycyclic aromatic hydrocarbons (2 to 3 rings) in recent years. Data taken from Li (2010)

<table>
<thead>
<tr>
<th>Organisms</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td></td>
</tr>
<tr>
<td>Acinetobacter calcoaceticus</td>
<td></td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td></td>
</tr>
<tr>
<td>Burkholderia cepacia 2A-12</td>
<td>(Lal &amp; Khanna, 1996)</td>
</tr>
<tr>
<td>Burkholderia sp. BS3702, Burkholderia sp. BS3770, Pseudomonas fluorescens BS3760, Pseudomonas putida BS202-P1, Pseudomonas putida BS3701, Pseudomonas putida BS3750, Pseudomonas putida BS590-P</td>
<td>(Jacques et al., 2008) (Kim et al., 2003) (Balashova et al., 1999)</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>(Molina et al., 2009)</td>
</tr>
<tr>
<td>Enterobacter</td>
<td>(Jacques et al., 2008)</td>
</tr>
<tr>
<td>Gordonia polyisoprenivorans, Mycobacterium fortuitum, Microbacteriaceae, Microbacterium sp. Neptunomonas naphthovorans Pseudomonas sp. Pseudomonas fluorescens 5R Pseudomonas putida BS202, Pseudomonas putida BS238 Pseudomonas putida G7 Pseudomonas stutzeri Rhodococcus sp. NCIMB112038 Xanthobacter polyaromaticivorans</td>
<td>(Hedlund et al., 1999) (Molina et al., 2009) (Leblond et al., 2001) (Kozlova et al., 2004) (Lee et al., 2003b) (McNally et al., 1999) (Kulakov et al., 2000) (Hirano et al., 2004)</td>
</tr>
<tr>
<td>Fluorene</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas sp. Sphingomonas sp. LB126 V. fischeri (strain NRRL-B-11177)</td>
<td>(Selifonov et al., 1993) (van Herwijnen et al., 2003) (Hirman et al., 2007)</td>
</tr>
<tr>
<td>Anthracene</td>
<td></td>
</tr>
<tr>
<td>Mycobacterium frederiksbergense LB501T Pseudomonas sp. KBM-1, Pseudomonas sp. W-2 Rhodococcus sp. Sphingomonas sp. LB126 Stenotrophomonas sp. Xanthobacter polyaromaticivorans</td>
<td>(Buchholz et al., 2007) (McNally et al., 1998) (Dean-Ross et al., 2001) (van Herwijnen et al., 2003) (Molina et al., 2009) (Hirano et al., 2004)</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td></td>
</tr>
<tr>
<td>Organisms</td>
<td>References</td>
</tr>
<tr>
<td>-----------</td>
<td>------------</td>
</tr>
<tr>
<td><strong>Pyrene</strong></td>
<td></td>
</tr>
<tr>
<td>Acinetobacter sp.</td>
<td>(Ramirez et al., 2001)</td>
</tr>
<tr>
<td>Burkholderia cepacia 2A-12</td>
<td>(Gao et al., 2006)</td>
</tr>
<tr>
<td>Mycobacterium austroafricanum</td>
<td>(Kim et al., 2003)</td>
</tr>
<tr>
<td>Mycobacterium sp. PYR-1</td>
<td></td>
</tr>
<tr>
<td>Mycobacterium sp.6PY1</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas saccharophila P15</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas sp. KBM-1, Pseudomonas sp. SAG-R</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas sp. W-2</td>
<td></td>
</tr>
<tr>
<td>Rhodococcus sp.</td>
<td></td>
</tr>
<tr>
<td>Sphingomonas sp.</td>
<td></td>
</tr>
<tr>
<td>Sphingomonas paucimobilis EPA 505</td>
<td></td>
</tr>
<tr>
<td>Alcaligenes denitrificans</td>
<td></td>
</tr>
<tr>
<td>Mycobacterium austroafricanum</td>
<td></td>
</tr>
<tr>
<td>Mycobacterium sp. AP1</td>
<td></td>
</tr>
<tr>
<td>Mycobacterium sp. SNP11</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas paucimobilis</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas saccharophila P15</td>
<td></td>
</tr>
<tr>
<td>Sphingomonas paucimobilis EPA 505</td>
<td></td>
</tr>
<tr>
<td>Sphingomonas sp. LB126</td>
<td></td>
</tr>
</tbody>
</table>

**Benz[a]anthracene**

<table>
<thead>
<tr>
<th>Organisms</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas saccharophila P15</td>
<td></td>
</tr>
<tr>
<td>Sphingomonas paucimobilis EPA 505</td>
<td></td>
</tr>
</tbody>
</table>

**Chrysene**

<table>
<thead>
<tr>
<th>Organisms</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas saccharophila P15</td>
<td></td>
</tr>
<tr>
<td>Sphingomonas paucimobilis EPA 505</td>
<td></td>
</tr>
</tbody>
</table>

**Benzo[b]fluoranthene**

<table>
<thead>
<tr>
<th>Organisms</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphingomonas paucimobilis EPA 505</td>
<td></td>
</tr>
<tr>
<td>Compound</td>
<td>Organism</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Benzo[a]fluoranthene</td>
<td>Sphingomonas paucimobilis EPA 505</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>Burkholderia cepacia VUN 10,001</td>
</tr>
<tr>
<td></td>
<td>Mycobacterium sp.</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas saccharophila P15</td>
</tr>
<tr>
<td></td>
<td>Sphingomonas paucimobilis EPA 505</td>
</tr>
<tr>
<td></td>
<td>Sphingomonas yanoikuyae JAR02</td>
</tr>
<tr>
<td>Dibenz[a,h]anthracene</td>
<td>Burkholderia cepacia VUN 10,001</td>
</tr>
<tr>
<td></td>
<td>Sphingomonas paucimobilis EPA 505</td>
</tr>
<tr>
<td>Coronene</td>
<td>Burkholderia cepacia VUN 10,001</td>
</tr>
</tbody>
</table>

Beside bacteria, there are several fungi known to have the property of degradation of recalcitrant pollutants. *Lignolytic, Phanerochaete chrysosporium, Bjerkandera adusta* and *Pleurotus ostreatus* are the common PAH-degrading fungi (Andersson & Henrysson, 1996; Eggen & Majcherczyk, 1998; Field et al., 1992; Kennes & Lema, 1994).

Biosorption and metabolic activities are the main mechanisms of algae to metabolize PAHs through both monooxygenase and dioxygenase pathways (Li, 2010; Luan et al., 2006; Tam et al., 2001). *Prokaryotic* and *eukaryotic photoautotrophic marine algae*, *cyanobacterium Agmenellum quadruplicatum PR6* are some of algae capable of PAH degradation (Narro et al., 1992).

As can be seen from the above discussion, aerobic remediation is a well-studied and efficient technique for PAHs degradation but it cannot be applicable for all environments and situations such as aquifer or marine sediments due to lack of oxygen (Bakermans et al., 2002a). Therefore, anaerobic environments can be developed in aerobic environments such as contaminated soil or sediments (Anderson & Lovley, 1997). This is due to the depletion of oxygen during aerobic
aspiration by in-situ microbial communities. This oxygen is not replenished at the same rate of depletion, so anaerobic zone will be formed. Thus for such situations or environments with the limited amount of oxygen, aerobic degradation will not seem economic due to high cost of aeration. With the above discussion, anaerobic degradation can play an important role in such circumstances.

2.1.3.2.2 Anaerobic Biodegradation

As mentioned above, when oxygen is absent or limited, biodegradation can occur anaerobically. Contrary to aerobic respiration, in anaerobic biodegradation, microorganisms use other electron acceptors such as nitrate, sulfate, iron, manganese and carbon dioxide (Coates et al., 1996; Meckenstock et al., 2000; Zhang et al., 2000). On the other hand, PAHs are a common contaminant of anaerobic environments such as aquifers and marine sediments (Bakermans et al., 2002a; Bewley & Webb, 2001; Coates et al., 1996; Meckenstock et al., 2000; Sharak Genthner et al., 1997). However, despite the importance of the anaerobic biodegradation, little is known about the process.

Generally anaerobic bacteria can be classified by two criteria. First, anaerobic bacteria can be separated into obligate anaerobe and facultative anaerobe by their tolerance to oxygen. Second, anaerobic bacteria can be classified in terms of compounds used as electron acceptors (Kraig, 2000). As earlier mentioned, the common electron acceptors utilized by anaerobic bacteria are nitrate (NO$_3^-$), manganese (Mn$^{4+}$), iron (Fe$^{3+}$), sulfate (SO$_4^{2-}$) and CO$_2$. The bacteria involving with these compounds are called as nitrate reducer, manganese reducer, iron reducer, sulfate reducer and methanogen, respectively (Schlesinger & Bernhardt,
These compounds are ranked based on their redox potential (E\textsubscript{h}) that shows the tendency of the environment to receive the supply electrons (Zehnder, 1988). Table 2.6 illustrates the redox potential (E\textsubscript{h}) and released energy during different electron reactions (Levett, 1990; Zehnder, 1988).

### Table 2.6 The redox potential (E\textsubscript{h}) and released energy through different electron reactions (pH=7)

<table>
<thead>
<tr>
<th>Reduction Reaction</th>
<th>E\textsubscript{h} (mV)</th>
<th>Released energy (Ve)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O\textsubscript{2} to H\textsubscript{2}O</td>
<td>812</td>
<td>29.9</td>
</tr>
<tr>
<td>NO\textsubscript{3}\textsuperscript{-} to NO\textsubscript{2}\textsuperscript{-}</td>
<td>747</td>
<td>28.4</td>
</tr>
<tr>
<td>Mn\textsuperscript{4+} to Mn\textsuperscript{2+}</td>
<td>526</td>
<td>23.3</td>
</tr>
<tr>
<td>Fe\textsuperscript{3+} to Fe\textsuperscript{2+}</td>
<td>-47</td>
<td>10.1</td>
</tr>
<tr>
<td>SO\textsubscript{4}\textsuperscript{2-} to H\textsubscript{2}S</td>
<td>-221</td>
<td>5.9</td>
</tr>
<tr>
<td>CO\textsubscript{2} to CH\textsubscript{4}</td>
<td>-244</td>
<td>5.6</td>
</tr>
</tbody>
</table>

Below is the summary of the works that have been conducted on different types of anaerobic bioremediation including nitrate-reducing, manganese-reducing, iron-reducing, sulfate-reducing and methanogenic bioremediation.

#### 2.1.3.2.2.1 Nitrate-reducing Bioremediation

There are relatively more studies on nitrate-reducing bioremediation comparing with other anaerobic conditions. Generally, the amount of nitrate added depends on the concentration of PAHs (=10-20 mM) (Chang et al., 2002; Sharak Genthner et al., 1997). Calcium nitrate Ca(NO\textsubscript{3})\textsubscript{2}, potassium nitrate (KNO\textsubscript{3}) and sodium nitrate (NaNO\textsubscript{3}) are the common sorts of nitrate used. There are several investigations on biodegradation of naphthalene, 3-4 and 5-6 rings PAHs under nitrate-reducing condition (al-Bashir et al., 1990; Ambrosoli et al., 2005;
Grishchenkov et al., 2000; Li, 2010; Macrae & Hall, 1998; Mihelcic & Luthy, 
1991; Quantin et al., 2005; Rockne & Strand, 2001).

2.1.3.2.2.2 Manganese-reducing Bioremediation

There are only two studies on biodegradation of PAHs under manganese-reducing 
condition, which are summarized in Table 2.7 whereby manganese is usually 
added in the form of manganese oxide (MnO₂) (Li, 2010).

Table 2.7 Studies on biodegradation of PAHs under Mn(IV) condition

<table>
<thead>
<tr>
<th>PAH</th>
<th>Experimental condition</th>
<th>Experimental results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>Bicarbonate, sulfate, iron, manganese, or nitrate was added to five sediment columns with Nap contamination, 25-200 μM.</td>
<td>Nap was partly removed with Mn(IV) addition. No effect was observed when adding an easier degradable substrate.</td>
<td>(Langenhoff et al., 1996)</td>
</tr>
<tr>
<td>Pyrene</td>
<td>Creosote-contaminated soil was amended with manganese oxide (MnO₂), incubated at low-oxygen tensions (0-6% O₂). Adding manganese oxide in concentrations of one or two times the stoichiometric requirement for mineralization of the total creosote.</td>
<td>MnO₂ amendments had no statistically significant effect on the reduction of PAHs when compared to the un-amended control.</td>
<td>(Nieman et al., 2001)</td>
</tr>
</tbody>
</table>

2.1.3.2.2.3 Iron-reducing Bioremediation

Iron (III) is another electron acceptor that can be used for anaerobic degradation 
of PAH-contaminated soils (Coates et al., 1996; Kraig, 2000; Langenhoff et al., 
1996; Ramsay et al., 2005). The important factor in biodegradation in this method 
is the form of Fe (III). For instance, iron (III) phosphate (FePO₄) inhibits the 
biodegradation, but not for iron (III) citrate (C₆H₆FeO₇) (Kraig, 2000).
2.1.3.2.2.4 Sulfate-reducing Bioremediation

The abundance of sulfate in marine environments causes sulfate-reducing condition as an effective way of PAH biodegradation (Chang et al., 2002; Coates et al., 1996; Kraig, 2000; Langenhoff et al., 1996). Based on literature, sulfate-reducing bioremediation is the most effective anaerobic way to degrade PAH-contaminated soils (Kraig & Sambhunath, 1998). Calcium sulfate (CaSO_{4}) is the best sulfate source due to its low solubility in water and consequently, not inhibiting the bacterial activities (Li, 2010).

2.1.3.2.2.5 Methanogenic Bioremediation

Methanogenic condition is very common to be expected in the most reducing environments. A few studies claimed that methanogenic biodegradation of PAHs is even better than nitrate-reducing degradation, while it is been also reported that there is no significant PAH degradation specially for HMW compounds (Chang et al., 2002; Sharak Genthner et al., 1997; Trably et al., 2005).

Adding carbon dioxide/bicarbonate, dextrene or sodium hydrogen carbonate can enhance the efficiency of methanogenic biodegradation (Kraig, 2000; Kraig & Sambhunath, 1998; Sharak Genthner et al., 1997). However, there are only few studies on methanogenic bioremediation of PAHs, thus more comprehensive works are needed to reveal information about the mechanisms of the process.

As discussed above, it is preferred to remove PAHs from the environment (i.e. soil and water) with biological methods rather than physical/chemical methods due to their environmental-friendly natures of the process and also the economical
consideration. Among the biological methods, anaerobic biodegradation needs more attention since there are many anaerobic environments that cannot be treated by aerobic microorganisms due to the lack of oxygen molecules. However, anaerobic PAHs-contaminated soils need to be treated in a better way since the efficiency of anaerobic bioremediation is low. In the following section, the fundament of microbial fuel cells (MFCs) are presented and it is discussed how these biological processes (i.e., anaerobic biodegradation) can be improved by MFCs.

2.2 Microbial Fuel Cells (MFCs)

2.2.1 Principles of MFCs

A microbial fuel cell (MFC) is a device that generates electricity by bacterial oxidation of substrates that are either organic or inorganic (Logan, 2008; Logan et al., 2006; Potter, 1911; Rabaey, 2005). This can be achieved when bacteria switch from a natural electron acceptor such as oxygen or nitrate, to an insoluble acceptor such as the MFC anode. This transfer can occur either via membrane-associated compounds or soluble electron shuttles. The electrons then flow through an external resistor to a cathode, at which the electron acceptor is reduced (Rabaey, 2005). In this process, which involves a wide range of microorganisms (Logan, 2009; Lovley, 2008), organic hydrocarbons would be degraded at the anode of the MFC.
An MFC system typically consists of an anode and a cathode chambers separated either with or without a proton exchange membrane (i.e. SMFC cases that will be discussed later) (Figure 2.3).

![Figure 2.3 Schematic illustration of a MFC. Substrate (●), proton (○), electron (●), oxygen (●) and water (●).]

At the anode, substrate (organic matter) is oxidized, producing electrons, protons and usually carbon dioxide. For example, when acetate is the fuel, its oxidation reaction is:

\[
\text{CH}_3\text{COO}^- + 2\text{H}_2\text{O} \rightarrow 2\text{CO}_2 + 8\text{H}^+ + 8\text{e}^- \quad \text{Equation 2.2}
\]

As shown in Figure 2.2, the generated protons migrate from the anode chamber to the cathode through the membrane, which at the same time restricts the oxygen
diffusion from the cathode chamber into the anode chamber. The produced electrons are transferred to the anode and then flow through the external circuit. At the cathode chamber, electrons, protons and the final electron acceptor (i.e., oxygen, nitrate, sulfate, etc.) meet each other to form water as below:

\[ 4H^+ + O_2 + 4e^- \rightarrow 2H_2O \]  

Equation 2.3

However, in MFCs, electricity is generated as a combination of electrochemistry and biofilm kinetics. The key processes taking place in the biofilm of the MFC has been summarized by Rittmann et al. (2008): (i) mass transport: substrates transport within the biofilm and reach the bacteria; (ii) microbial processes (cell growth and respiration): the electrochemically-active microorganisms (EAMs) oxidize the substrates (electron donors) and electrons and protons are produced; (iii) the electrical potential gradient: the electrons produced transferred between the cell and from the cell to the electrode; and (iv) proton transport: the protons produced during the oxidation transported out of the biofilm.

### 2.2.2 Voltage Generation by MFCs

The useful energy, \( V_{\text{cell}} \) (V) that can be harvested from different voltages of anode and cathode is less than its predicted thermodynamic value (\( E_{\text{emf}} \)) due to three different losses called activation losses, ohmic losses and mass transport losses (Logan et al., 2006; Rittmann et al., 2008). The real output voltage (\( V_{\text{cell}} \)) can be calculated as follows by subtracting the voltage losses:

\[ V_{\text{cell}} = E_{\text{emf}} - (\eta_{\text{act}} + \eta_{\text{ohmic}} + \eta_{\text{conc}}) \]  

Equation 2.4
where \( \eta_{\text{act}} \) is the activation loss due to reaction kinetics, \( \eta_{\text{ohmic}} \) is the ohmic loss from ionic and electronic resistances and \( \eta_{\text{conc}} \) is the concentration loss due to mass transport limitations (Logan et al., 2006; Rittmann et al., 2008).

In MFC, the measured cell voltage \( (V_{\text{cell}}) \) (V) is usually a linear function of the current \( (I) \) (A), and can be described simply as follows:

\[
V_{\text{cell}} = E_{\text{emf}} - IR_{\text{int}}
\]

Equation 2.5

where \( IR_{\text{int}} \) or internal resistance is the sum of all the voltage losses mentioned above due to reaction kinetic, ohmic resistance and mass transport limitations.

### 2.2.2.1 Maximum Voltage

The theoretical overall cell electromotive force \( (E_{\text{emf}}) \) of a MFC can be calculated from the Gibbs free energy, which is defined as the potential difference between the cathode and anode (Logan et al., 2006):

\[
E_{\text{emf}} = E_{\text{cat}} - E_{\text{an}}
\]

Equation 2.6

where \( E_{\text{cat}} \) and \( E_{\text{an}} \) are the maximum potentials of anode and cathode, respectively. The potential of each electrode can be determined by the Nernst equation (Rittmann et al., 2008):

\[
E_{\text{electrode}} = E_{\text{electrode}}^0 - \frac{RT}{nF} \ln \left( \frac{[\text{red}]}{[\text{ox}]} \right)
\]

Equation 2.7

where \( E_{\text{electrode}}^0 \) (V) is the standard free energy at pH = 7, R is the universal gas constant (8.314 J/mol K), T is the operation temperature (K), \( n \) is the number of electrons transferred, F is the Faraday constant (96,485 Coulombs/mol), \([\text{ox}]\) and \([\text{red}]\).
[red] are the concentrations of the oxidized and reduced compounds, respectively, and \( \gamma \) and \( \beta \) are their corresponding stoichiometric coefficients.

The open circuit voltage (OCV) is the cell voltage that can be measured when circuit is kept open for some time. Theoretically, it should be equal to \( E_{\text{emf}} \) but due to various losses it is lower than \( E_{\text{emf}} \) (Logan et al., 2006; Wen et al., 2009).

### 2.2.2.2 Voltage Losses

2.2.2.2.1 Electron-quenching Reactions

A portion of electrons is consumed by microorganisms for their growth in the process of fermentation, methanogenesis or respiration (Pham et al., 2009).

2.2.2.2.2 Activation Losses

The loss that comes from overcoming the barrier for the electron transfer from microorganisms toward the electrode is called activation overpotential or activation losses (Clauwaert et al., 2008).

2.2.2.2.3 Ohmic Losses

Ohmic losses is the result of the resistance in electron and ions transfers through the electrodes/external circuit and the membrane/electrolytes, respectively (Logan et al., 2006).

2.2.2.2.4 Mass Transfer Losses

The transport of substrate to the anodic biofilm and the transfer of products outside of the biofilm will cause mass transfer losses, which is more significant
when the concentration of substrate is low. Another portion of electrons can be lost in this process that cause current production (Clauwaert et al., 2008).

### 2.2.3 Microorganisms in MFCs

There are a variety of microorganisms in anodic biofilm of MFCs. However, only some of those bacteria that are able to transfer electrons to a chemical or material are important in MFC. They are called exoelectrogens: "exo-" for exocellular and "electrogens" based on the ability to transfer electrons to a chemical or material that is not the immediate electron acceptor. These bacteria are so far known to transfer the electrons via two mechanisms: electron shuttling and nanowires (Logan, 2008). Table 2.8 presents a list of main exoelectrogens involved in MFCs. Although the recent techniques have suggested a greater diversity of exoelectrogens, still information on electron transfer mechanisms is insufficient and more comprehensive studies is needed. Generally marine sediments, soil and activated sludge are rich sources of exoelectrogens that makes this study easier (Niessen et al., 2004a; Zhang et al., 2006). Therefore, recently a few studies have investigated the microbiology of the SMFCs in particular that will be presented in the following section under SMFC.

**Table 2.8 Exoelectrons present in anodic biofilm of MFCs**

<table>
<thead>
<tr>
<th>Microbes</th>
<th>Substrate</th>
<th>Application</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Actinobacillus succinogenes</em></td>
<td>Glucose</td>
<td>Neutral red as electron mediator</td>
<td>(Park &amp; Zeikus, 2000)</td>
</tr>
<tr>
<td><em>Aeromonas hydrophila</em></td>
<td>Acetate</td>
<td>Mediator-less MFC</td>
<td>(Pham et al., 2003)</td>
</tr>
<tr>
<td><em>Alcaligenes faecalis</em>,</td>
<td></td>
<td>Mediator-less MFC</td>
<td></td>
</tr>
<tr>
<td><em>Enterococcus gallinarum</em>,</td>
<td></td>
<td>Selfmediate consortia</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Glucose</td>
<td>isolated from MFC with a maximal level</td>
<td>(Rabaey et al., 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>of 4.31 Wm$^{-2}$</td>
<td></td>
</tr>
</tbody>
</table>
As can be seen in the above sections, MFCs are interesting devices that can be used for biological treatment of a large variety of substrates while producing power simultaneously. Therefore, it has been tried in different studies both in the lab-scale or pilot-scale designs to make them economically and technically viable, but there is no significant successful case (i.e. in MFCs for wastewater treatment) mainly due to the low power density or slow rate of substrate degradation. However, the only type of MFCs that has been scaled up so far is sediment microbial fuel cells (SMFCs) (Tender et al., 2008). In the following section, SMFCs will be introduced with details information regards their basic concept, operating condition and their applications, including how they can be used for the treatment of PAHs-contaminated soils and sediments.
2.3 Soil/Sediment Microbial Fuel Cells (SMFCs)

Many studies have investigated MFCs from different aspects: design, substrate (Pant et al., 2010), operating conditions and as well as types. However, from the treatment points of view, MFCs have been proven themselves to be significantly effective, but due to their low power density or slow rate of substrate degradation, they have not been successfully scaled up. The only type of MFCs that has been scaled up so far is sediment microbial fuel cell (SMFC) (Tender et al., 2008).

SMFCs are simple to construct and typically consist of an anode buried in the sediment/soil and a cathode in the overlaying water (Logan, 2008; Tender et al., 2002b). However, the knowledge obtained from other types of MFC especially wastewater-fueled MFC which has been investigated the most, cannot be directly applied for SMFCs due to substantially different influential factors and different operating conditions (Li & Yu, 2015). Considering SMFC’s abilities in scaling up, they deserve more comprehensive investigations. Following sections will examine SMFC’s currently in use, effects of operating conditions, microbiology and as well as advances in power generation and bioremediation.

2.3.1 Operational Conditions in SMFCs

There are many factors affecting the performance of a SMFC (controllable or uncontrollable) such as pH, aeration, temperature, surface area ratio, external resistance, electrode spacing and electrical conductivity. In the below sub-
sections, these factors will be reviewed and will be shown how these factors can affect the performance of SMFCs in terms of electricity or degradation.

2.3.1.1 pH

The pH of most media that are used for bioremediation or waste fermentation is significantly low due to acidogenic fermentation, which is not favorable for metabolisms of local bacteria. However, power generation/bioremediation could be improved by addition of buffer such as sodium carbonate (Venkata Mohan et al., 2008). Below is the list of items that can be affected by variation of pH.

2.3.1.1.1 Internal Resistance

The pH difference between anodic and cathodic chambers has been observed to change the internal resistance of SMFCs. Internal resistance decreases with increasing pH difference between anode and cathode solution. For the pH differences of 2 and zero units, the internal resistances of 523 and 547 Ω were reported respectively. (Jadhav & Ghangrekar, 2009; Sajana et al., 2013).

2.3.1.1.2 Power Generation

In SMFC, power generation has been observed to follow the same increasing or decreasing trends. By decreasing pH, power generation would be decreased because lower pH might have reduced the enrichment of electrogenic bacteria during biofilm formation. Sajana et al. (2014a) reported a power production of 3.96 mW/m² at pH of 6.5 and 4.52 mW/m² at pH of 8.5. Gil et al. (2003) and He et al. (2008) have also reported that lower pH resulted lower power generation.
and the optimal pH has been suggested to be between 7 and 8 or 8 and 10, depending on the configuration.

2.3.1.1.3 COD Removal
The effect of pH on chemical oxygen demand (COD) removal has been observed to be in inverse relationship (Zhang et al., 2011). COD removal rate increases with decrease in pH. For instance, Sajana et al. (2014a) reported decrease in COD removal rate (from 3.81 g/m²d to 1.77 g/m²d) when pH was increased from 6.5 to 8.5.

2.3.1.1.4 TKN Removal
Different feed pH will give different total kjeldahl nitrogen (TKN) removal rate. Sajana et al. (2014a) reported that by increasing feed pH from 6.5 to 8.5, TKN removal rate increased from 0.017±.001 g/m²d to 0.024±0.05 g/m²d.

2.3.1.2 Aeration
One of the limiting factors with all MFCs including SMFCs is cathodic reaction, which is commonly the reduction of oxygen. The performance of the SMFC is reduced when oxygen as the final electron acceptor has been depleted. Thus for compensation, aeration near cathode could be provided. Hong et al. (2009) examined the effect of dissolved oxygen (DO) level at the cathode on current production by adjusting the DO level to 0, 3, 5 and 7 mgO₂/l. They observed that no current was produced from SMFC with DO concentration of 0 mg/l. Furthermore, the DO level must be kept above 3 mg/l to have the optimum performance. Therefore, He et al. (2007) illustrated improvement in performance
of SMFC by using a rotating cathode to supply oxygen. The maximum power density was improved from 29 to 49 mW/m² (69% improvement). Sajana et al. (2013) also showed the significant differences between the performance of SMFC with and without aeration as shown Table 2.9.

Table 2.9 Performance of SMFC operated with and without aeration.

<table>
<thead>
<tr>
<th></th>
<th>With Aeration</th>
<th>Without Aeration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average COD removal eff. (%)</td>
<td>79.4±1.4 (35.26% improvement)</td>
<td>58.7±2</td>
</tr>
<tr>
<td>Average TN removal eff. (%)</td>
<td>44±5.4 (62.96% improvement)</td>
<td>27±6</td>
</tr>
<tr>
<td>Average OCV (mV)</td>
<td>499±0.8 (39% improvement)</td>
<td>356±7.4</td>
</tr>
<tr>
<td>Average sustainable PD (µW/m²)</td>
<td>18.8±1.3 (8.67% improvement)</td>
<td>17.3±1.3</td>
</tr>
<tr>
<td>Average maximum PD (µW/m²)</td>
<td>106.7±1.1 (106% improvement)</td>
<td>51.8±0.8</td>
</tr>
</tbody>
</table>

2.3.1.3 Temperature

Sediment temperature has been reported to varied from 15 to 33°C (excluding winter) by seasonal variation (Lei et al., 2005). Hong et al. (2009) showed that maximum power density increased with temperature. They observed 3.4 and 1.5 folds higher power density generation at 35°C compared to 10 and 20°C, respectively. Liu et al. (2005) also demonstrated 10% loss of power output by decreasing temperature from 32 to 20°C. However, the optimum operating temperature was found to be between 20-25°C even though anaerobic activities are still possible at lower temperature (e.g. 10°C) (Grady et al., 1999). Therefore, for treatment wise, Sajana et al. (2013) showed lower performance efficiency in TKN removal rate while higher efficiency in COD removal was observed by increasing the temperature.
2.3.1.4 Surface Area Ratio

Hong et al. (2009) examined the effect of the ratio of apparent geometric surface area of the anode and cathode by varying from 1:1, 1:1/2, 1:1/5 to 1:1/10 (anode:cathode) to investigate the catalytic activity of the cathode on current production. They found out that the surface ratio must be at least 1:1/5 to capture the current peak. The reason that they used smaller cathode compared to anode while it is common to operate larger anodes is due to substantially lower anodic reaction (i.e., oxidation of organic matter in sediment).

2.3.1.5 External Resistance

External load affects the performance of SMFC by controlling the flow of electrons from the anode to the cathode (Sajana et al., 2014a). Jang et al. (2004), Venkata Mohan et al. (2008) and Jadhav and Ghangrekar (2009) demonstrated that by lowering the external resistance, COD removal rates are increased. Sajana et al. (2013) also reported higher COD and TKN removal efficiencies in open-circuited SMFC than the SMFC with 100-Ω external load. According to the findings of Hong et al. (2009), by increasing the external load, current density could be decreased while power density was increased. This unexpected result for the power output is due to the fact that power (P) is equal to the square of the current (I) times the value of the external resistance (R); \( P = I^2 R \). It means that more power can be achieved either by an increase in current or the external load.
2.3.1.6 Electrode Spacing

COD and TN removal efficiencies would be slightly better in SMFCs with less electrode spacing according to Sajana et al. (2014a). With a longer distance between the electrodes, the diffusion of oxygen into the anode chamber is minimal and this might improve COD and TN removal efficiencies. From electricity point of view, it is common to generate less power density when the electrode spacing is increased. Power density increased by 10.92% when the electrode spacing was reduced from 100 cm to 50 cm (Sajana et al., 2014a). Liu et al. (2005) also reported 60% improvement in power generation by reducing the distance from 4 to 2 cm. Hong et al. (2009) also examined the effect of distance between electrodes by varying from 12, 20, 40, 80 and 100 cm. They found that current generated from SMFC decreased as the electrode spacing was increased. Power density increased from 0.37 to 1.01 mW/m² by increasing the distance from 12 to 100 cm. From the polarization curve diagrams, it can be interpreted that ohmic losses was much greater at a spacing of 100 cm compared to 12 cm. However, power generation can be attributed to the ohmic losses, which depends on electrode spacing, meaning that by decreasing the electrode spacing, protons would have less distance to travel (Logan et al., 2006).

2.3.1.7 Electrical Conductivity

In MFCs, ohmic losses can be reduced by increasing solution conductivity to the maximum tolerated by the local bacteria (Logan et al., 2006). Schamphe laire et al. (2008) reported that power generation was more difficult to obtain from
freshwater than salt-water system due to lower electrical conductivity. Hong et al. (2009) also demonstrated that SMFCs in marine environments could produce power density of four to five times greater than in freshwater environments.

2.3.2 Microorganisms in SMFCs

Microbial communities in SMFCs play two different roles. First, extracting electrons from microbial metabolisms and delivering it to the anode by EAMs (Li et al., 2014). Secondly, many bacteria can break down complex organic matters to utilize substrate for EAMs (Borole et al., 2011). In general, some major phylogenetic groups of EAMs have been identified on anodic electrode such as *delta-, gamma-, beta-proteobacteria* (Bond et al., 2002; Logan et al., 2005; Lovley, 2006; Nielsen et al., 2007), *nitrospira* and *chloroflexi* (Yan et al., 2012).

However, the predominant bacterial species depends on the environment. For example, *delta-proteobacteria* or *desulfuromonas* are ubiquitous in marine sediments while *geobacter* is widely existed in the freshwater sediments (Holmes et al., 2004). Although most of the studies have focused on the anodic microbiology, few studies have shown aerobic microbes predominant in the cathode such as *pseudomonas* and *novosphigobium* (Clauwaert et al., 2007; Erable et al., 2010).
2.3.3 Applications of SMFCs

2.3.3.1 Power Generation

One of the first applications of any type of MFCs including SMFCs is power generation. Many studies have been conducted on SMFC in order to increase the power generation to make it more viable and easy to scale up. In Table 2.10, it has been tried to highlight the main valuable works done for increasing the power output. Typically, open circuit voltage (OCV), current and power density (CD and PD), internal resistance (IR) and the longevity of the SMFC reactors have been investigated as the important indicators of generated power density.
<table>
<thead>
<tr>
<th>Design</th>
<th>Substrate Addition</th>
<th>Max. OCV (mV) (Improvement %)</th>
<th>Max. CD (mA m⁻²) (Improvement %)</th>
<th>Max. PD (mW m⁻²) (Improvement %)</th>
<th>IR (Reduction %)</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cylindrical, GPE</td>
<td>2% cellulose</td>
<td>976±2 (16%)</td>
<td>30.89 (77%) @open-circuited</td>
<td>8.47 (108%) @PC</td>
<td></td>
<td></td>
<td>(Sajana et al., 2014b)</td>
</tr>
<tr>
<td>Scaled-up (100L) GPE</td>
<td></td>
<td>830</td>
<td></td>
<td>18.6</td>
<td>@PC</td>
<td></td>
<td>(Yang et al., 2015)</td>
</tr>
<tr>
<td>Rotating cathode, Carbon cloth anode</td>
<td></td>
<td>50 (163%) @100Ω</td>
<td>49 (69%) @PC</td>
<td></td>
<td></td>
<td></td>
<td>(He et al., 2007)</td>
</tr>
<tr>
<td>GPE</td>
<td>20% graphite flake</td>
<td>578</td>
<td></td>
<td>33.25</td>
<td>@PC</td>
<td></td>
<td>(Lenin Babu &amp; Venkata Mohan, 2012)</td>
</tr>
<tr>
<td>Cylindrical, GPE</td>
<td>726 (46%)</td>
<td>1.989 (74%) @open-circuited</td>
<td>0.25 (125%) @PC</td>
<td>616</td>
<td>(27%)</td>
<td></td>
<td>(Sajana et al., 2013)</td>
</tr>
<tr>
<td>Graphite rods electrodes</td>
<td>Sodium acetate</td>
<td>72.27±6.37 (943%) @4600Ω</td>
<td>19.59±0.35 @4600Ω</td>
<td></td>
<td></td>
<td></td>
<td>(Sacco et al., 2012)</td>
</tr>
<tr>
<td>Self-stacked submersible</td>
<td></td>
<td>1120</td>
<td></td>
<td>294</td>
<td></td>
<td></td>
<td>(Zhang &amp; Angelidaki, 2012)</td>
</tr>
<tr>
<td>GPE at different depth (2-10 cm)</td>
<td></td>
<td>840</td>
<td></td>
<td>14.5 (45%) @PC</td>
<td>224 (-56%) @PC</td>
<td></td>
<td>(An et al., 2013)</td>
</tr>
<tr>
<td>Cylindrical, GFE, Heated at 150°C 3hrs</td>
<td></td>
<td>188 (40%)</td>
<td></td>
<td>20.1 (253%) @PC</td>
<td></td>
<td>Heating pretreatment has the most effect on increasing PD, LOI removal and DOM release</td>
<td>(Song &amp; Jiang, 2011)</td>
</tr>
<tr>
<td>Cylindrical, GFE served by MWNT</td>
<td>Algae (Chlorella vulgaris) to the cathode</td>
<td>246 (81%)</td>
<td></td>
<td>38 (138%)</td>
<td>489 (73%)</td>
<td>Algae could be used as oxygen supplier</td>
<td>(Wang et al., 2014)</td>
</tr>
</tbody>
</table>
2.3.3.2 Bioremediation

In natural water bodies, oxygen reduction reaction (ORR) is the dominant process in water column and the sediment surface layers (Li & Yu, 2015). In deeper sediments when anoxic environment is formed, other electron acceptors such as nitrate, sulfate and iron oxides can oxidize the organic matters. Organics can also be degraded via fermentation and methanogenesis (Kruger et al., 2008). This is the natural process of organic matter oxidation, but when it comes to bioremediation especially for persistent organic pollutants (POPs), it becomes difficult to break down due to usually insufficient availability of oxidation or reduction power (Eek et al., 2008; Kao et al., 2003; Kurt et al., 2012). This limitation can be alleviated by many microorganisms that are already in the nature through extracellular electron transport (Newman & Kolter, 2000). Electrons are naturally transferred to the abundant oxygen in overlaying water through various natural electron shuttles such as iron oxides minerals (Klupfel et al., 2014) or even via filamentous bacteria (Pfeffer et al., 2012). This process accelerates sediment bioremediation as can been seen in Figure 2.4 (Li & Yu, 2015). However, this natural route is typically weak especially for longer distances and some recalcitrant contaminants. Here, the SMFC may offer an interesting solution.
In SMFCs cathode is usually submerged in the overlaying water and is connected to the anode in the sediment. The anode acts as a powerful electron acceptor for the microorganisms nearby to enhance the oxidation of contaminants while cathode serves as a powerful final electron acceptor (Bond et al., 2002). Hong et al. (2010) investigated the effect of SMFC on alteration of physio-chemical properties of sediment organic matter (SOM). They found that SOM around the electrochemically-active electrodes becomes more humified, aromatic and polydispersed along with its partial degradation. Zhang et al. (2010) investigated the possibility of the electrodes that might serve as electron acceptors to stimulate the degradation of aromatic hydrocarbons in the sediment. Toluene, benzene and
naphthalene loss were stimulated in the process. Their results suggested that graphite electrodes can serve as electron acceptor to degrade aromatic hydrocarbon contaminated-soil. Morris and Jin (2012) tested a SMFC to determine if it facilitates degradation of total petroleum hydrocarbons (TPH). Results showed a degradation rate of 24% for TPH. Therefore, they found that natural biodegradation can be enhanced by nearly 12 folds. In addition, SMFCs have been effective even for more complex compounds such as polycyclic aromatic hydrocarbons (PAHs). Yan et al. (2012) investigated the effect of a SMFC on the degradation of phenanthrene and pyrene in freshwater sediment and found after 240 days, there is a significant difference (29.3%) between the degradation rate of natural attenuation and treatment with SMFC. Sajana et al. (2013) also evaluated the performance of SMFC in terms of COD removal and TKN removal of an aquaculture pond water. SMFCs demonstrated effective in-situ remediation of aquaculture water by removing 84.4% and 95.3% of COD and TKN within 27 days. Another potential application of SMFCs is for detoxification of heavy metals. For instances, Abourached et al. (2014) showed significant removal efficiency of heavy metals such as Cd (90%) and Zn (97%) simultaneously with high power generation (3.6 Wm⁻²). SMFCs can also be used for direct supply of electrons to the sediment. In this case cathode was placed in the sediment too and an external voltage was loaded for contaminants removal (Aulenta et al., 2007; Chun et al., 2013). Ueno and Kitajima (2014) demonstrated that by setting a certain oxidative potential (i.e. +300 mV vs. Ag/AgCl), they could raise oxidation reduction potential (ORP) in the sediment to the level of
inhibition for methanogenesis. In addition, many POPs degradation could be accelerated by polarized graphite electrodes as direct or indirect electron acceptor (Aulenta et al., 2011; Aulenta et al., 2013).

However, as it can be seen from the above, SMFCs have been promising devices for the purpose of bioremediation. This study has focused on the application of the SMFCs in biodegradation of persistent compounds (i.e., PAHs) from the contaminated soil.
Chapter 3 Materials and Methods

3.1 Introduction

This study was divided into three phases. Phase 1 focused on the design of SMFC reactors to investigate PAH bioremediation with aerobic cathodic chambers. Phase 2 followed the same objectives as phase 1 with the difference of anaerobic cathodic chambers. Phase 3 focused on the performance of wastewater-MFCs on removal of PAHs in two sub-phases of batch (Phase 3-1) and continuous (Phase 3-2). In all phases, characteristic analysis such as electrical, physio-chemical and microbiological was done. However, in this chapter, the Materials and Methods adopted for the three sub-sections of the three phases shall be discussed. Sub-section 3.1 gives information about the setup construction, materials and methods used in the first two phases (Phases 1 and 2) followed by sub-section 3.2, which is for wastewater-MFCs (Phase 3). In the sub-section 3.3, all details about the measurements and analysis will be provided.

3.2 Sediment MFCs

3.2.1 Sediment

Sediment (0-20 cm depth) and the lake water were collected from the MacRitchie Reservoir (Singapore). They were placed into clean polycarbonate jars and transported to the laboratory. All sediment were sieved through a 2-mm sieve to remove plant debris and other terrestrial leaves and then homogenized by mixing with a stainless steel spatula prior to use.
3.2.2 Reagents

PAHs (naphthalene, acenaphthene and phenanthrene), tetrahydrofuran (THF), methanol, 2-propanol, potassium nitrate and potassium sulfate were purchased from Sigma Aldrich. All standards and working solutions were stored at 4°C. Deionized water was obtained from a Mili-Q water purification system (Merck Milipore, Temecula, California, USA). Bakerbond SPE columns C18 (2.5g) for solid phase extraction (SPE) were purchased from Agilent Technologies.

3.2.3 Sample pretreatment (SPE Method) - PAHs Extraction

Since the samples were sediment, pretreatment of the samples according to the method developed by Kootstra et al. (1995) was carried out prior to analysis. The procedure of the pretreatment was as follows: a 10-g amount of soil was placed into a 50-ml tube with 20 ml of acetone and the mixture was shaken for 30 min. After centrifugation at 1,000 g for 5 min, exactly 10 ml of the mixture was then pipetted into a 100-ml volumetric flask together with 5 ml of 2-propanol. The sample was brought to 100 ml with HPLC-grade water.

C18 were conditioned with 1×3 ml of methanol, followed by two times of 3 ml of water-2-propanol (9:1, v/v). The 100-ml sample solution was loaded onto the SPE column under vacuum. Then the column was washed with 3 ml of methanol-water (50:50, v/v). The PAHs were eluted with two times of 1.5 ml of THF. The first 1.5 ml has to soak the cartridge for two minutes before eluting. After elution, the final THF extract passed through a filter and then was injected into the GC/MS. All flows through the cartridge were about 2 ml/min.
3.2.4 Sediment Characteristics

Sediment samples were analyzed for the amount of reducing compounds present (i.e., sulfate, nitrate, phosphate, iron and manganese). Sulfate, phosphate, iron, manganese and other ions were measured by extracting from the sediment and analyzing with an Ion Chromatograph (IC) (DIONEX-500, Thermo Scientific, Sunnyvale, California, USA). Nitrate was measured using the TNT NitroVer Test Kit and DR5000 UV-Vis spectrophotometer (Hach, Loveland, Colorado, USA). All extracts were filtered through a 0.45-µm GA-8 membrane filter before being analyzed by the IC or Spectrophotometer.

3.2.5 Substrate

3.2.5.1 PAHs
A solution of three selected PAHs (i.e., naphthalene, acenaphthene and phenanthrene) was added to the sediment to create contaminated sediment with PAHs for the study (50 ppm of each). First, PAHs were dissolved into THF (solvent) and then added to the sediment and mixed by a stainless steel spatula to get a homogenized mixture of sediment and PAHs.

3.2.5.2 Terminal Electron Acceptors (TEAs)
For aerobic SMFC reactors, no external TEA was added since oxygen was introduced in the cathodic chamber. However, for anaerobic SMFC reactors, nitrate and sulfate were added to provide potential TEAs for the reduction reaction. Nitrate and sulfate would be utilized for stimulating nitrate-reducing and sulfate-reducing bacteria, respectively, that have been known for the
biodegradation of PAHs in the soil in an anaerobic environment (Coates et al., 1996; Coates et al., 1997; Meckenstock et al., 2000; Zhang et al., 2000).

3.2.5.3 Final Substrate

The final substrates for aerobic and anaerobic SMFCs consist of PAHs and the TEAs (i.e., nitrate and sulfate only for anaerobic SMFC) were made by mixing PAH solution and TEA solution according to Table 3.1.

<table>
<thead>
<tr>
<th>Cathode environment</th>
<th>PAHs</th>
<th>TEA</th>
<th>Concentration</th>
<th>Reactor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic</td>
<td>No</td>
<td>Oxygen</td>
<td>—</td>
<td>AR2</td>
</tr>
<tr>
<td>Aerobic</td>
<td>Yes</td>
<td>Oxygen</td>
<td>—</td>
<td>AR1, AR3 &amp; AR4</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>No</td>
<td>Nitrate and sulfate</td>
<td>10 mM for each TEA</td>
<td>AnR2</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>Yes</td>
<td>Nitrate and sulfate</td>
<td>10 mM for each TEA</td>
<td>AnR1, AnR3 &amp; AnR4</td>
</tr>
</tbody>
</table>

3.2.6 Electrodes

Isomolded graphite plates were used as the electrodes in this experiment (Graphite Store Pte Ltd, Buffalo Grove, Illinois, USA). One graphite plate (10.16 cm × 10.16 cm × 0.318 cm) as the anode and one with the same dimensions as the cathode (Figs. 1a and c) were used for each SMFC reactor.

3.2.7 Construction & Operation

Eight SMFC reactors (AR1 to AR4 and AnR1 to AnR4 under aerobic and anaerobic environments, respectively), made of Plexiglas, were operated simultaneously. The bottom of AR1, AR3, AR4, AnR1, AnR3 and AnR4 were
filled with the prepared substrate as mentioned earlier and is summarized in Table 3.2. AR1 and AnR1 were reactors comprised of only lake water on top of the sediment without any electrodes, and they were used to determine the background bioremediation done by the indigenous microorganisms in a non-SMFC environment (denoted as non-SMFC reactors). AR3, AR4, AnR3 and AnR4 were duplicated SMFC reactors in aerobic and anaerobic environments, respectively, and were constructed by placing the electrodes in horizontal position at a height of 4 cm (anode) and 15 cm (cathode) from the bottom and then the lake water was added as the water column. AR2 and AnR2 were constructed similar as the duplicated SMFC reactors (i.e., AR3, AR4, AnR3 and AnR4), and the difference was that the substrate in them was the clean sediment (i.e., without PAHs) to monitor the background electricity generation. Figure 3.1 shows the schematic of non-SMFC & SMFC reactors in both aerobic and anaerobic conditions and Table 3.2 summarizes the details of the constructed reactors. The circuit was completed using a 1500-Ω resistor for each cell. Voltages for all reactors were monitored across the resistance every 30 min using a data acquisition system. For aerobic SMFC reactors (i.e., AR1 to AR4), air was introduced by a fine bubble diffuser suspended in the overlaying water near the cathodes in order to maintain oxic condition (i.e., dissolved oxygen concentration of 3-4 mg/L) in the cathode chamber (Hong et al., 2010). For anaerobic mode, all the SMFC reactors (i.e., AnR1 to AnR4) were completely closed using rubbers and caps to maintain anaerobic condition inside the reactors. All the SMFC reactors were operated at
ambient temperature (~27°C) and the water loss due to evaporation was compensated every two days by topping up with tap water.

![Figure 3.1 Schematic diagram of the experimental setup. (a) Aerobic SMFC (AR2, AR3 and AR4), (b) Aerobic non-SMFC (AR1), (c) Anaerobic SMFC (AnR2, AnR3 and AnR4) and (d) Anaerobic non-SMFC (AnR1).](image)

<table>
<thead>
<tr>
<th>Type</th>
<th>Reactor</th>
<th>SMFC</th>
<th>Contaminated sediment</th>
<th>Initial PAHs conc.</th>
<th>External TEA (nitrate &amp; sulfate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic</td>
<td>AR1</td>
<td>No</td>
<td>Yes</td>
<td>50 ppm (each)</td>
<td>No</td>
</tr>
<tr>
<td>Aerobic</td>
<td>AR2</td>
<td>Yes</td>
<td>No</td>
<td>—</td>
<td>No</td>
</tr>
<tr>
<td>Aerobic</td>
<td>AR3</td>
<td>Yes</td>
<td>Yes</td>
<td>50 ppm (each)</td>
<td>No</td>
</tr>
<tr>
<td>Aerobic</td>
<td>AR4</td>
<td>Yes</td>
<td>Yes</td>
<td>50 ppm (each)</td>
<td>No</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>AnR1</td>
<td>No</td>
<td>Yes</td>
<td>50 ppm (each)</td>
<td>Yes</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>AnR2</td>
<td>Yes</td>
<td>No</td>
<td>—</td>
<td>Yes</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>AnR3</td>
<td>Yes</td>
<td>Yes</td>
<td>50 ppm (each)</td>
<td>Yes</td>
</tr>
</tbody>
</table>
3.3 Wastewater MFCs

3.3.1 Electrolyte

Domestic wastewater (DWW) (COD of 300-400 ppm) was used as the inoculum and the fuel for the reactors. DWW was collected from the effluent of a primary clarifier of the Ulu Pandan Water Reclamation Plant in Singapore. Prior to feeding into the continuously stirred feed tank, the effluent was filtered with a screen of 150-µm pore size to remove the particles. Thereafter, the electrolyte was enriched by adding 1 ml anaerobic sludge (AS) per 1 liter of DWW. Other chemical parameters characterizing the wastewater are listed in Table 3.3.

Table 3.3 Characterization of influent domestic wastewater (DWW)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Compound</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD (ppm)</td>
<td>351±45</td>
<td>NO$_3^-$ (ppm)</td>
<td>1.26±0.87</td>
</tr>
<tr>
<td>TSS (ppm)</td>
<td>211±14</td>
<td>PO$_4^{3-}$ (ppm)</td>
<td>5.63±3.2</td>
</tr>
<tr>
<td>VSS (ppm)</td>
<td>204±74</td>
<td>SO$_4^{2-}$ (ppm)</td>
<td>54.23±14.3</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>27</td>
<td>Na$^+$ (ppm)</td>
<td>50.27±16.9</td>
</tr>
<tr>
<td>Conductivity (mS cm$^{-1}$)</td>
<td>0.915±0.15</td>
<td>NH$_4^+$ (ppm)</td>
<td>30.21±11.7</td>
</tr>
<tr>
<td>pH</td>
<td>7.84±0.7</td>
<td>K$^+$ (ppm)</td>
<td>6.35±2.6</td>
</tr>
<tr>
<td>F$^-$ (ppm)</td>
<td>0.2±0.1</td>
<td>Mg$^{2+}$ (ppm)</td>
<td>3.21±2.5</td>
</tr>
<tr>
<td>Cl$^-$ (ppm)</td>
<td>110.7±62</td>
<td>Ca$^{2+}$ (ppm)</td>
<td>20.36±8.3</td>
</tr>
</tbody>
</table>

3.3.2 Reagents

PAHs (naphthalene, acenaphthene and phenanthrene), tetrahydrofuran (THF), methanol, 2-propanol, potassium nitrate and potassium sulfate were purchased
from Sigma Aldrich. All standards and working solutions were stored at 4°C. Deionized water was obtained from a Mili-Q water purification system (Merck Milipore, Temecula, California, USA). Bakerbond SPE columns C18 (2.5g) for solid phase extraction were purchased from Agilent Technologies.

3.3.3 Sample pretreatment (SPE Method) - PAHs Extraction

Since the samples were wastewater, pretreatment of samples was carried out prior to analysis as follows: 5 ml of sample was placed into a 25-ml tube with 10 ml of acetone and the mixture was shaken for 30 min. After centrifugation at 1,000 g for 5 min, exactly 10 ml of the mixture was then pipetted into a 100-ml volumetric flask together with 5 ml of 2-propanol. The sample was brought to 100 ml with HPLC-grade water.

C18 were conditioned with 1×3 ml of methanol, followed by two times of 3 ml of water-2-propanol (9:1, v/v). The 100-ml sample solution was loaded onto the SPE column under vacuum. Then the column was washed with 3 ml of methanol-water (50:50, v/v). The PAHs were eluted with two times of 1.5 ml of THF. The first 1.5 ml has to soak the cartridge for two minutes before eluting. After elution, the final THF extract passed through a filter and then was injected into the GC/MS. All flows through the cartridge were about 2 ml/min.

3.3.4 Substrate

3.3.4.1 PAHs

A solution of three selected PAHs (i.e., naphthalene, acenaphthene and phenanthrene) was added to the wastewater to create contaminated solution with
PAHs for the study (50 ppm of each). First, PAHs were dissolved into THF and then added to the wastewater.

3.3.4.2 Terminal Electron Acceptors (TEAs)
Since all wastewater-MFCs were operated under air-cathode condition, it was tried to provide anoxic environment in anode chamber. Hence, all the feeds before being fed to the system were sparged by nitrogen gas for 5 minutes to remove the oxygen. After sparging, the feed tank was capped and ready for usage as the influent. However, in wastewater-MFCs, the TEA was oxygen from the air since two sides of the reactor (cathodes) were exposed to the air (Figure 3.2).

3.3.4.3 Final Substrate
The final substrates for the reactors in both the batch and continuous phases were prepared according to Table 3.4.

<table>
<thead>
<tr>
<th>Type</th>
<th>Reactor</th>
<th>Electrolyte &amp; Inoculum</th>
<th>PAHs Conc.</th>
<th>Circuit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch</td>
<td>BR1</td>
<td>DWW &amp; AS</td>
<td>No</td>
<td>Open</td>
</tr>
<tr>
<td>Batch</td>
<td>BR2 &amp; BR3</td>
<td>DWW &amp; AS</td>
<td>No</td>
<td>Closed (1000 Ω)</td>
</tr>
<tr>
<td>Batch</td>
<td>BR4</td>
<td>DWW &amp; AS</td>
<td>50 ppm (each)</td>
<td>Open</td>
</tr>
<tr>
<td>Batch</td>
<td>BR5 &amp; BR6</td>
<td>DWW &amp; AS</td>
<td>50 ppm (each)</td>
<td>Closed (1000 Ω)</td>
</tr>
<tr>
<td>Continuous</td>
<td>CR1</td>
<td>DWW &amp; AS</td>
<td>No</td>
<td>Open</td>
</tr>
<tr>
<td>Continuous</td>
<td>CR2 &amp; CR3</td>
<td>DWW &amp; AS</td>
<td>No</td>
<td>Closed (1000 Ω)</td>
</tr>
<tr>
<td>Continuous</td>
<td>CR4</td>
<td>DWW &amp; AS</td>
<td>50 ppm (each)</td>
<td>Open</td>
</tr>
<tr>
<td>Continuous</td>
<td>CR5 &amp; CR6</td>
<td>DWW &amp; AS</td>
<td>50 ppm (each)</td>
<td>Closed (1000 Ω)</td>
</tr>
</tbody>
</table>
3.3.5 Electrodes

Carbon fiber brushes and carbon clothes were used as anodes and cathodes, respectively (Fuel Cell Earth LLC, Woburn, Massachusetts, USA). Two carbon fiber brushes in the dimensions of 20 cm in length and 2 cm in radius as anodes and two carbon clothes in the dimensions of 28 cm × 12 cm × 0.2 cm as cathodes were used for each reactor.

3.3.6 Construction and Operation

In this study, 12 single-chambered MFCs (SCMFC) (BR1 to BR6 and CR1 to CR6 in batch and continuous modes, respectively) with air exposed cathodes were constructed in order to make MFCs more compact and simple with reduced cost of operation. An air-cathode MFC, where oxygen present in the air comes in contact with the cathode and works as electron acceptor, provides potential advantages over the two-chambered system. Each reactor consisted of a rectangular chamber with the dimensions of 28 cm × 12 cm × 0.2 cm and an effective anodic volume of 1.5 L. As shown in Figure 3.2, each reactor was fabricated by adding two carbon brushes inside the chamber (anodes), two silicon rubbers (for well-sealing), two Ti plates, two cathodes and two glass lids on the sides of the chamber. BR2, BR3, BR5 and BR6 in the batch mode and CR2, CR3, CR5 and CR6 in the continuous mode were duplicated reactors in the absence and the presence of PAHs, respectively, and were connected to an external load of 1000 Ω. BR1, BR4, CR1 and CR4 were the reactors served as control whereby their circuits were not completed by any external resistance and were directly
connected to the multimeter. Voltages of reactors were monitored across the resistance every 5 min in the batch and every minute in the continuous mode using a data acquisition system. Table 3.4 shows a summary of the details of the constructed reactors in each of the sub-phases. All the reactors were operated in ambient temperature (~27°C) and the water loss due to evaporation was compensated every two days by topping up with tap water.

![Schematic of SCMFC used in this study](image)

**Figure 3.2 Schematic of SCMFC used in this study**

### 3.3.7 HRT

In continuous phase (Phase 3.2), five different flow rates were used to investigate the effect of HRT on the performance of SCMFCs in terms of biodegradation rate and electricity generation by varying the HRT from 2 to 24 h (Table 3.5).
Table 3.5 Flow rates tested for the batch and continuous phases

<table>
<thead>
<tr>
<th>HRT (h)</th>
<th>Flow rate (ml min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>12.5</td>
</tr>
<tr>
<td>4</td>
<td>6.25</td>
</tr>
<tr>
<td>8</td>
<td>3.13</td>
</tr>
<tr>
<td>12</td>
<td>2.08</td>
</tr>
<tr>
<td>24</td>
<td>1.04</td>
</tr>
</tbody>
</table>

3.4 Measurement

3.4.1 Cell Voltages

Cell voltages (V) were measured using a data acquisition system (ARRAY M3500A, Array Electronic Co., Ltd., Nanjing, Jiangsu, China) connected to a computer.

3.4.2 Electrode Reference

Anode and cathode potentials were measured by an Ag/AgCl reference electrode (Metrohm Pte Ltd, Singapore).

3.4.3 Current/Power

Current (I) was calculated using the ohm’s law (R=V/I), where R is the external resistance (Ω) and V is the measured cell Voltage (V). Power (P) was calculated as P=VI and normalized by the anode surface area for phases 1 and 2 (SMFC) and normalized by the anode chamber volume for the phase 3 (wastewater MFCs). The maximum power density was measured by varying the external resistance from 50 kΩ to 25 Ω with 15 and 2 min of interval times for the SMFCs (Phases 1
and 2) and wastewater-MFCs (Phase 3), respectively, in order to allow the voltage to be stabilized. The internal resistances of the reactors were calculated using the slope of polarization curve as reported by Logan (2008).

### 3.4.4 Coulombic Efficiency

Although power (current) is generated in MFCs, the amount of power harvested is only a proportion of total amount of stored energy within the metabolized substrate (Logan, 2008). To determine the Coulombic efficiency of the system, the ratio of the number of electrons moving through the external circuit to the theoretical number of electrons produced from the substrate metabolism was calculated. Coulombic efficiency of MFCs in batch mode can be calculated as:

\[
CE = \frac{M \int_0^{t_b} I dt}{FbV_{An} \Delta COD}
\]

Equation 3.1

where \( M \) is the molecular weight of oxygen (32 g mol\(^{-1}\)), \( F \) is the Faraday’s constant, \( b \) is the number of electrons needed per mole of oxygen, \( V_{An} \) is the liquid volume of the anode chamber and \( \Delta COD \) is the change in COD over time.

For continuous flow systems, the Coulombic efficiency can be determined based on the current in the steady state as follows:

\[
CE = \frac{MI}{FbQ\Delta COD}
\]

Equation 3.2

Where \( Q \) is the volumetric influent flow rate and \( \Delta COD \) is the difference between influent and effluent COD.
3.4.5 Cyclic Voltammetry

Bio-electrochemical behavior of mixed communities in the reactors was studied by cyclic voltammetry (CV) using a potentiostat system (Metrohm Autolab B.V., Utrecht, Netherlands). All the assays were performed by considering anode as the working electrode (WE) and cathode as the counter electrodes (CE) against Ag/AgCl reference electrode (RE).

3.4.6 Ion chromatography (IC)

Samples were first filtered through a 0.45-μm pore-sized membrane (GN-6 grid 47-mm, Gelman Science, Pall Corporation, Ann Arbor, Mich.) before the measurement. Cations of Na⁺, NH₄⁺, K⁺, Mg²⁺, Ca²⁺ and anions of F⁻, Cl⁻, NO₂⁻, NO₃⁻, Br⁻, PO₄³⁻, and SO₄²⁻ were measured using an Ion Chromatogram (DIONEX-500 fitted with GP50 Gradient pump and CD20 conductivity detector) equipped with IonPac CS12A cation and IonPac AS9-HC anion columns.

3.4.7 Chemical Oxygen Demand (COD)

The chemical oxygen demand (COD) of the feed and effluent was measured using the DR5000 UV-Vis spectrophotometer (Hach). For preparation of the COD tubes, 1.5 ml of digestion solution and 3.5 ml of acidic reagent were added to the empty tubes. Then 2.5 ml of sample was poured and heated at 150°C for two hours. The samples were filtered through a glass microfiber filter (0.45-μm pore-sized, Whatman, Maidstone, UK) prior to analysis. The percentage of COD removal was calculated as:
where $\text{COD}_{\text{in}}$ is the influent COD (mg L$^{-1}$) and $\text{COD}_{\text{out}}$ is the effluent COD (mg L$^{-1}$). COD test preparation protocol can be found in Appendix I.

### 3.4.8 Total Organic Carbon (TOC)

Total organic carbon (TOC) of water and sediment samples were analyzed using a Total Carbon Analyzer (Shimadzu TOC-L, Shimadzu Corporation, Kyoto, Japan) and a Solid Sample Module (SSM) associated with TOC-V, respectively.

### 3.4.9 Liquid Chromatography – Organic Carbon Detection (LC-OCD)

Characterization of dissolved organic matters (DOMs) was determined using a liquid chromatography - organic carbon detection (LC-OCD) (DOC-Labor, Karlsruhe, Germany), which was equipped with a size exclusion column, Toyopearl HW-50S (Tosoh, Tokyo, Japan).

### 3.4.10 Total Suspended Soil (TSS) and Volatile Suspended Solid (VSS)

The TSS and VSS were determined according to the Standard Methods (APHA, 2005). The glass microfiber filters (GF/F, Whatman, Maidstone, UK) were rinsed with 25 mL of DIW and heated in a furnace at 550°C for 20 min prior to analysis. Then samples were filtered through the filter to collect the TSS and then dried at 105°C for 1 h. After that, samples were cooled to the room temperature in a desiccator before being weighed. To determine the VSS, the filter with the
collected TSS was further heated at 550°C for 20 min and weighed after being cooled in the desiccator.

3.4.11 pH

The pH values of the feed and effluent were measured using a pH meter (F-54BW, HORIBA LTD, Kyoto, Japan) to determine conditions for the bacterial growth and the behavior of the MFCs. The pH was maintained in the range of 6 to 8 to obtain the best performance out of SMFCs and wastewater-MFCs.

3.4.12 UV–Vis Spectrophotometer

A DR 5000 UV–vis spectrophotometer (Hach, Loveland, Colorado, USA) was used for measuring the nitrate concentration and COD. The kit used for the nitrate was the TNT NitraVer 50 tests, high range (0–30 mg/l NO₃⁻N) and the kit for measuring COD was prepared based on the protocol in Appendix I.

3.4.13 Gas Chromatography/Mass Spectrometry (GC/MS)

A Shimadzu 2010 GC system with a mass spectrometer detector (MS) were used for the determination of PAH concentration. The following analytical conditions were used: capillary column used was the DB-5 MS (30 m, 0.25 mm, 0.25 µm film thickness); the pressure was 44.2 kPa; the injector temperature was maintained at 300°C, the initial temperature used was 70°C and the sample was held for 2 min; the temperature was then increased at a rate of 15°C/min until it reached the first isotherm of 200°C and then the sample was further held for 4 min; the temperature was further increased a rate of 5°C/min to reach the second
isotherm at 300°C and the sample was further held for 5 min. Ion source and interface temperatures were maintained at 250°C and the peaks captured by the SIM method. PAHs were identified by the retention times and characteristic ions of the identified compounds.

3.4.14 Biological Analysis

3.4.14.1 Scanning Electron Microscopy (SEM)
Anodic biofilm and the membrane surfaces were observed under a scanning electron microscope (SEM). The anode graphite plates, carbon clothes and membrane sections were first soaked in a 2.5% Glutaraldehyde solution for 30 min at room temperature (25°C) for fixation. They were then rinsed with ethanol, which was used in an ascending concentration order of 25, 50, 75, 90, and 100%, in order to dehydrate the samples. Prior to observation, the samples were critical-point dried and coated with gold in a sputtering device. An SEM (XL-30-FEG, Philips, Germany) was used to take images of the anodic biofilm and fouled PEM surface.

3.4.14.2 DNA Extraction
Samples from different points of the surface of electrodes and the matrixes (i.e. sediment, wastewater) were taken and their DNAs were extracted by a PowerMax Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California, USA) according to the manufacturer’s instructions (Appendix II).

3.4.14.3 Denaturing Gradient Gel Electrophoresis (DGGE)
Cloned 16S rRNA gene fragments were analyzed by denaturing gradient gel electrophoresis (DGGE) to screen clones prior to sequencing. DGGE analysis was
conducted using the D-Gene DGGE system (Bio-Rad, Hercules, California, USA). Polyacrylamide gels (10% polyacrylamide, acrylamide:N,N'-methylenebisacrylamide, 37.5:1; 0.75 mm thick; 16×16 cm) were run in a 1 X TAE buffer (40mM Tris-acetate, 1mM EDTA at pH 8.3). A gradient ranging from 30 to 60% denaturant (100% denaturant is 7M urea plus 40% vol/vol formamide in 1 X TAE) was used. Gels were run at 60°C for 4 h at a constant 200 V and stained for 30 min in SYBR green I (Sigma, Poole, UK; diluted 1/10,000 in 1 X TAE). Stained gels were viewed and documented using a Fluor-S Multilmager (Bio-Rad, Hercules, CA, USA). Clones with different migration characteristics in the DGGE analysis were selected for sequence determination.

**3.4.14.4 Microbial Community Diversity Analyses by Pyrosequencing**

The extracted DNA from replicates of each sample set was pooled with equal amount (DNA concentration was determined by a NanoDrop system). Subsequently, the 16S rRNA gene fragments from the pooled DNA of each sample set were amplified by polymerase chain reaction (PCR) using DreamTaq Green PCR Master Mix (Thermo Scientific, Sunnyvale, California, USA), with two commonly applied primer sets: 343F (5'-TACGGRAGGCAGCAG-3') (Nossa et al., 2010) /926R (5'- CCGTCAATTYYTTTRAGTTT-3') (Liu et al., 1997) for bacterial populations and 341F (5'-CCT ACG GGR SGC AGC AG-3') (Baker et al., 2003) / 958R (5'-YCC GGC GTT GAM TCC AAT T-3') (DeLong, 1992) for archaeal populations. To conduct pyrosequencing, barcodes were incorporated between the 454 adaptor and forward primers. The PCR amplification program consisted of an initial denaturing at 95°C for 5 min,
followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 45 s, and finally an extension at 72°C for 10 min. The amplicons with different barcodes were then mixed in equal concentration and sequenced by a Roche 454 GS-FLX Titanium sequencer (Roche, USA). Raw sequences from pyrosequencing were screened, the adaptors, barcodes and primers were trimmed, and those sequences less than 200 bp or containing ambiguous bases were excluded. The taxonomic identities of the sequences were then assigned using the Classifier program of the RDP-II, with a minimum confidence level of 80%. The rarefaction curves were generated, and the Chao 1 and abundance-based coverage estimator (ACE) indices were calculated to compare the microbial diversity and richness between the inocula and adapted biomasses according to the literature (Zheng et al., 2013). The relative abundance values of bacterial or archaeal groups were expressed as percentage of the whole bacterial or archaeal community DNA sequences, respectively.
Chapter 4 Biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs) in the Contaminated Soil Using Sediment Microbial Fuel Cells (SMFC) – Aerobic Cathodic Chamber

4.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic compound that consists of two or more fused benzene rings and/or pentacyclic molecules that are arranged in various structural configurations. They are highly recalcitrant molecules that can persist in the environment due to their hydrophobicity and low water solubility (Bamforth & Singleton, 2005; Cerniglia, 1992). PAHs are ubiquitous in the environment, and originate from either natural or anthropogenic sources (Bamforth & Singleton, 2005).

PAHs are one of the most prevalent contaminants found in soils (Bamforth & Singleton, 2005; Liebeg & Cutright, 1999). The origin of PAH contaminated soils include anthropogenic sources such as abandoned manufactured gas sites, leaking underground storage tanks, wood treatment sites and industrial processes (Liebeg & Cutright, 1999). Natural processes can also provide a source of PAHs such as volcanic eruptions and forest fires (Blumer, 1976). For instance, they have been found in marine sediments such as San Diego Bay, California and the Central Pacific Ocean (Coates et al., 1997; Ohkouchi et al., 1999).

Biostimulation & Bioaugmentations are widely known technologies to remediate hydrocarbons-polluted sites (Amezgua-Allier et al., 2012; Amezgua-Allier et al.,
Biodegradation is occurred by breaking down of PAHs using microorganisms, either in the presence of oxygen (i.e., aerobic condition) or without oxygen (i.e., anaerobic condition). A variety of aerobic bacteria, fungi and enzymes have been specified as the species that can use PAHs as carbon and energy sources (Haritash & Kaushik, 2009). The limiting factors with aerobic biodegradation such as a very thin layer of oxic zone in the soil, high aeration cost and the tendency of PAHs to be accumulated in the soil rather than dissolving in water or suspending in air, show the significance and potential of anaerobic biodegradation (Liang et al., 2007). PAHs are a common contaminant in anaerobic environments such as soil and sediment (Coates et al., 1996; Coates et al., 1997; Sharak Genthner et al., 1997). Even anaerobic zones could be developed in aerobic environments due to the depletion of oxygen during aerobic respiration and oxygen not being replenished at the same rate (Bedessem et al., 1997). This suggests that anaerobic zones could be easily established and thus anaerobic biodegradation would occur.

Anaerobic biodegradation of organic pollutants is an important pathway in nature due to the ability of microorganisms in removal of organic compounds under anoxic conditions (Huang et al., 2011a). Basically, anaerobic biodegradation needs an electron acceptor such as Fe (III) oxides, nitrate or sulfate. Due to the abundance of sulfate in the soil, especially marine environments, bioremediation of PAHs in many instances would be most effective under sulfate-reducing conditions (Chang et al., 2002; Kraig, 2000). The possibility of oxidation of PAHs under sulfate-reducing conditions has been investigated by Coates et al. (1996).
A microbial fuel cell (MFC) is a device that generates electricity by bacterial oxidation of substrates that are either organic or inorganic in nature (Logan, 2008; Rabaey et al., 2006; Rezaei et al., 2007). This can be achieved when bacteria switch from a natural electron acceptor such as oxygen or nitrate, to an insoluble acceptor such as the MFC anode. This transfer can occur either via membrane-associated compounds or soluble electron shuttles. The electrons then flow through an external resistor to a cathode, at which the electron acceptor is reduced (Rabaey et al., 2005). In this process, which involves a wide range of microorganisms (Logan, 2009; Lovley, 2008), organic hydrocarbons would be degraded at the anode of the MFC.

A sediment microbial fuel cell (SMFC) is a type of MFC that has recently attracted significant attentions (Huang et al., 2011a; Rezaei et al., 2007) due to its unique property of removing organic compounds from the soil/sediment. SMFCs typically consist of an anode buried in a reduced matrix (soil) and a cathode in the overlaying, oxidized water layer (Logan, 2008; Rezaei et al., 2007; Tender et al., 2002b). However, there is no detailed research into the ability of MFC/SMFC for bioremediation of complex compounds such as PAHs. All the former studies have been done on non-complex compounds present in the soil/sediment except the studies on Chitin 20 and Chitin 80 done by Rezaei et al. (2007) and anaerobic biodegradation of diesel by Morris and Jin (2012). This chapter investigated the ability of SMFC for bioremediation of PAHs in contaminated soil under aerobic condition provided in the cathodic chamber by introducing air bubbles.
This phase of the research was aimed to investigate the feasibility of PAHs removal using SMFCs in aerobic cathodic compartment. The SMFC reactors were constructed based on the procedures mentioned in Chapter 3. Their performance in PAHs removal and electricity generation is discussed below.

4.2 Results and Discussions

4.2.1 Electricity

Figure 4.1 shows the polarization curve and power density of aerobic SMFC reactors at day 9. The maximum open-circuit voltage (OCV) of the duplicated aerobic SMFC reactors (i.e., AR3 and AR4) and the aerobic control (i.e., AR2) were found to be 0.72 ± 0.02 and 0.82 V, respectively. The maximum power density of the duplicated aerobic SMFC reactors (i.e., AR3 and AR4) was 5.77 ± 1.14 mW/m², while that of the AR2 was 8.67 mW/m². Since the surface area of the anodes and cathodes were similar in this experiment, it did not matter which one was used for the normalization. The calculated internal resistances (IR) based on the slope of the polarization curves (Figure 4.1) were 437 ± 70 and 337 Ω for the duplicated SMFC reactors (i.e., AR3 and AR4) and AR2, respectively. The reason behind this difference between the AR2 and the duplicated SMFC reactors (i.e., AR3 and AR4) could be due to the complex compounds (PAHs) that were present in the AR3 and AR4. In other words, with all experimental conditions being similar except the absence or presence of PAHs in the SMFC reactors, the differences in electricity generation by the control and SMFC reactors with PAHs could probably be attributed to the different dominant microbial communities that
had been developed in the control and the SMFCs reactors with PAHs. With different microbial communities, the rate of electron production, due to different proton pumping rate, would be different. Therefore, higher electrical performance and lower internal resistance were resulted in the AR2. Although there was a difference between the duplicated SMFC reactors (i.e., AR3 and AR4) and the control (i.e., AR2), all their internal resistances were much lower than those in the literature reported by Rezaei et al. (2007) (1297 Ω for Chitin 20 and 1762 Ω for chitin), Logan et al. (2006) and Cheng et al. (2006). Therefore, although the internal resistances in this study were significantly lower than others reported in the literature, unlike the expectation, maximum power generation (5.8 mW/m²) was not remarkable. This could be due to the fact that no catalyst was applied in the cathodes of the SMFC reactors used in this study, as it has been shown by many studies that catalyst would increase the power generation significantly (Cheng et al., 2006; Zhao et al., 2005).

Figure 4.1 Electricity performance - polarization curve (PC) and power density (PD) - of the aerobic SMFC reactors.
Figures 4.2a and b present the current and power densities of the SMFC reactors over 45 d. As can be seen from all figures, the duplicated SMFC reactors (i.e., AR3 and AR4) had a different trends than the control reactor (i.e., AR2) or previous SMFC performances reported by Hong et al. (2010). In either the control reactor (AR2) or the literature, current density (or power density) decreased significantly after 10, 12 or 27 d, respectively, while in the duplicated SMFC reactors (AR3 and AR4), it remained between 16.33 and 20.77 mA/m2 over the whole 45 d. However, by comparing the difference between the control reactor (AR2) and the duplicated SMFC reactors (i.e., AR3 and AR4), the impact of PAHs on the SMFC reactors were significant - PAHs were been degraded and been consumed as a substrate. The minimum fluctuation in the current (or power) density observed in the AR3 and AR4 (Figure 4.2) suggested that PAHs had been removed at a constant rate. This observation was confirmed with the removal rate of PAHs measured using GC/MS as discussed in the next section (4.2.2).
4.2.2 PAHs Removal

The analysis of PAHs biodegradation in the control SMFC reactor (AR1) and the duplicated SMFC reactors (i.e., AR3 and AR4) showed the effect of SMFCs on
biodegradation rate. By sampling three times over 45 d from different parts of anode compartments at day 10, 25 and 45 and comparison with the initial PAH concentration, a significant removal of PAHs was observed in the sediment. The results are summarized in Table 4.1 and Figure 4.3.

Table 4.1 PAHs removal efficiency (%) in aerobic SMFC reactors.

<table>
<thead>
<tr>
<th>PAHs Compounds</th>
<th>Duplicated SMFCs (AR3 &amp; AR4)</th>
<th>Non-SMFC Control (AR1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D10</td>
<td>D25</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>13.5 ± 4.7%</td>
<td>25.8 ± 1.6%</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>12.1 ± 1.7%</td>
<td>23.3 ± 1.1%</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>8.2 ± 1.3%</td>
<td>19.9 ± 1.8%</td>
</tr>
</tbody>
</table>

D10: Sample collected on day 10; D25: Sample collected on day 25; D45: Sample collected on day 45

Figure 4.3 PAHs removal efficiency (%) in the SMFC and non-SMFC reactors. (a) Naphthalene (b) Acenaphthene and (c) Phenanthrene.

As it can be seen in Table 4.1 and Figure 4.3, there was a significant difference in the rate of PAH removal between the control SMFC reactor (i.e., AR1) and the duplicated SMFCs (i.e., AR3 and AR4). It showed the effectiveness of
electrochemical systems for biodegradation of PAHs, as demonstrated by the AR3 and AR4. Duplicated SMFC reactors (i.e., AR3 and AR4) were able to remove 41.7±1.7, 34±0.3 and 36.2±0.9% of the initial naphthalene, acenaphthene and phenanthrene, respectively, while those of the AR1 were 12.6, 9.8 and 11.3%, respectively. In other words, it can be inferred that the microbial communities that were formed on the surface of electrodes in the anode chamber could stimulate the biodegradation of PAHs. Since anaerobic condition was maintained in the anode chamber (sediment), only anaerobic microorganisms were responsible for the biodegradation of PAHs. Figure 4.4 shows the voltammetric cycle (CV) of the duplicated reactors (R3 & R4) that showed good redox activities in the SMFC reactors.

Figure 4.4 Voltammogram profile of the duplicated reactor (AR3 and AR4).

Ion chromatograph (IC) analysis of the initial sediment and lake water showed that there were negligible amounts of other electron acceptors such as nitrate, sulfate and Mn (IV) present. So biodegradation by sulfate-reducing or nitrate-
reducing bacteria contributed insignificantly to PAHs biodegradation. It was postulated that PAHs could diffuse out of the sediment into the overlaying water, where biodegradation then occurred. However, GC analysis for PAHs in the overlaying water in all the SMFC reactors showed that all PAHs concentrations were negligible (Figure 4.3).

The difference of biodegradation rates of PAHs compounds individually in aerobic or anaerobic cathodic conditions showed the different PAH biodegradation capability of microorganisms and also, different properties of PAHs in terms of bioavailability. Simpler compounds are typically being degraded much easier and more rapidly (Lefebvre et al., 2009). For instance, in this study, the removal efficiencies of naphthalene (41.7%) were much higher compared to those of the acenaphthene (34.1%) or phenanthrene (36.2%) because naphthalene is a much simpler organic compound. On the other hand, this claim could not be generalized as acenaphthene is a simpler compound compared to phenanthrene, but has a lower removal rate in duplicated SMFC reactors.

4.2.3 Total Organic Carbon (TOC)

Total organic carbons (TOC) were monitored in the water and sediment samples during the process to determine the biodegradation of PAHs by the SMFC reactors. Figure 4.5 shows the differences in the TOC/TOC\(^0\) ratio in the SMFC reactors during the operation in the aerobic condition. It was found that these ratios for the duplicated SMFC reactors (i.e., AR3 and AR4) was higher than those of the AR1 (control 1, which was a non-SMFC reactor with PAHs
contaminated sediment) and AR2 (control 2, which was a SMFC with non-polluted sediment). Comparison of the AR1 with the duplicated SMFC reactors (i.e., AR3 and AR4) showed that on day 45, 52% of the initial TOC were consumed by the microorganisms in the duplicated SMFCs (AR3 and AR4), while only 37% of the initial TOC were removed in the AR1. It means that microorganisms had been more active in the SMFC reactors and consequently, more PAHs had been degraded in SMFC reactors compared to the non-SMFC reactor. When the results of the AR2 were compared to those of the duplicated reactors (i.e. AR3 and AR4), the presence of PAHs in the sediment matrix served as the organic substrate for the SMFC reactors, achieving TOC removal of 52% on day 45, while only 27% of the initial TOC were consumed in the AR2. It could be due to different microbial communities that were formed, which will be discussed in detail in the section 4.2.4.

Figure 4.5 TOC/TOC⁰ ratio of the sediment in aerobic SMFC reactors.
4.2.4 Analysis of Pyrosequencing Results

In order to understand the fundamentals and also maintain the high performance of the SMFC reactors, it is necessary to study the microbial communities involved in the process. Generally, in the literature, only anodic biofilm has been studied but in this work, the microbial communities of all reactors in sediment, anodic and cathodic biofilm were investigated.

In this section, samples which were taken from these three parts of the reactors (sediment, anode and cathode surfaces) were labeled as shown in Table 4.2.

Table 4.2 Labels used for the samples taken from sediment, anodic and cathodic biofilms.

<table>
<thead>
<tr>
<th>Label</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR1-S</td>
<td>Aerobic phase, Sediment sample, Reactor 1 (control, non-SMFC), with PAHs</td>
</tr>
<tr>
<td>AR2-S</td>
<td>Aerobic phase, Sediment Sample, Reactor 2 (control, SMFC), without PAHs</td>
</tr>
<tr>
<td>AR3-S</td>
<td>Aerobic phase, Sediment Sample, Reactor 3 (SMFC), with PAHs</td>
</tr>
<tr>
<td>AR2-A</td>
<td>Aerobic phase, Anode surface, Reactor 2 (control, SMFC), without PAHs</td>
</tr>
<tr>
<td>AR3-A</td>
<td>Aerobic phase, Anode surface, Reactor 3 (SMFC), with PAHs</td>
</tr>
<tr>
<td>AR2-C</td>
<td>Aerobic phase, Cathode surface, Reactor 2 (control, SMFC), without PAHs</td>
</tr>
<tr>
<td>AR3-C</td>
<td>Aerobic phase, Cathode surface, Reactor 3 (SMFC), with PAHs</td>
</tr>
</tbody>
</table>

To study the underlying mechanisms in the reactors, the bacterial communities from different parts of reactors including sediment matrix, anode surface (anodic biofilm) and cathode surface (cathodic biofilm) were investigated and compared using 454-pyrosequencing technology. As shown in Table 4.3, a total of 33,444 effective sequences were obtained after filtering from raw sequences with average sequence length of 423 bp.
Table 4.3 Summary of pyrosequencing data for sediment, anodic and cathodic biofilms samples.

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Sequences</td>
<td>4381</td>
<td>2706</td>
<td>4544</td>
<td>5586</td>
<td>5022</td>
<td>2761</td>
<td>4783</td>
<td>3661</td>
</tr>
<tr>
<td>OTUs</td>
<td>661</td>
<td>537</td>
<td>610</td>
<td>632</td>
<td>522</td>
<td>269</td>
<td>464</td>
<td>440</td>
</tr>
<tr>
<td>ACE</td>
<td>1744</td>
<td>1416</td>
<td>2434</td>
<td>3369</td>
<td>1675</td>
<td>1323</td>
<td>1796</td>
<td>1441</td>
</tr>
<tr>
<td>Chao</td>
<td>1379</td>
<td>1006</td>
<td>1686</td>
<td>1982</td>
<td>1224</td>
<td>720</td>
<td>1256</td>
<td>983</td>
</tr>
</tbody>
</table>

Generated at 97% similarity threshold.

The discussion on the pyrosequencing results shall be divided into three sections of sediment, anodic and cathodic biofilms.

### 4.2.4.1 Bacterial Community Composition

#### 4.2.4.1.1 Sediment

Various groups of bacteria are involved in the substrate decomposition in the process. Figure 4.6 shows the phylum level distributions of bacterial populations among different sediment samples. It can be observed that *Proteobacteria* was the dominant phylum, comprising between 70.58 and 76.21% of that total bacterial sequences, which is in agreement with literature regards the dominant phyla in the sediment (Lee et al., 2003a). Among the reactors, the AR2-S showed a bit less abundance of *Proteobacteria*, which reveals the effect of PAHs on the growth of these bacteria. It was followed by *Acidobacteria* comprising between 5.74 and 17.18%. The significant difference in abundance of *Acidobacteria* in the AR2-S (17.18%) compared to the AR1-S (9.29%) and AR3-S (5.74%) showed incompatibility of these bacteria in the presence of PAHs. Therefore, significantly higher *Firmicutes* in AR3-S (10.59%) compared to the AR1-S (1.07%) and AR2-
S (0.37%) showed the dominance and proliferation of these bacteria in the presence of PAHs in an electrochemical system.

![Bacterial community compositions (phylum level) of sediment in reactors AR1, AR2 and AR3.](image)

**Figure 4.6 Bacterial community compositions (phylum level) of sediment in reactors AR1, AR2 and AR3.**

For investigation of more specific taxa within the phylum, the class level distribution within *Proteobacteria* is illustrated in Figure 4.7a. It can be observed that in all sediment samples, *β*-proteobacteria was the dominant class of bacteria comprising between 35.75 and 55.52% of total bacterial sequences. It was followed by *α*-proteobacteria accounting for 13.01 to 29.31% of the whole bacterial population. Notably, higher abundance of *β*-proteobacteria in the AR3-S (55.52%) and AR2-S (44.35%) compared to the AR1-S (35.75%) reveals the effect of electrochemical systems on the bacterial communities. In addition, *β*-proteobacteria has been identified as a class of bacteria in the process of electricity generation (Lee et al., 2003a; Phung et al., 2004). Even between the reactors with electrochemical systems, the AR3-S with 55.52% compared to the
AR2-S with 44.35% suggested better condition for the growth of this class of bacteria (β-proteobacteria). In contrast to β-proteobacteria, Figure 4.7a showed that α-proteobacteria was found more in the AR1-S (29.31%) than reactors with electrochemical systems (AR2-S (14.38%) and AR3-S (13.01%)), which suggested less possible contribution of this class to the electricity production and bioremediation. The higher abundance of δ-proteobacteria in the AR2-S (5.43%) compared to the AR1-S (3.10%) and AR3-S (2.38%) can be attributed to the possibility that this class of bacteria is not compatible with PAHs.

Moreover, as mentioned before, Acidobacteria was the second most important phylum in the sediment samples. The class level distribution of this phylum is illustrated in Figure 4.7b. Acidobacteria_Gp1 was the dominant class in the phylum comprising between 3.68 and 17.11% of total bacterial sequences and followed by Holophagae by accounting up to 4.34% within the phylum in the AR1-S. Significantly higher abundance of Acidobacteria_Gp1 found in the AR2-S (17.11%) compared to the AR1-S (4.95%) and AR3-S (3.68%) revealed the incompatibility of these taxa with PAHs even though they played a role in electricity generation.

Another two important taxa of bacteria which were accounted for up to 10.08 and 2.67% of the total bacterial sequences were Clostridia and Sphingobacteria from Firmicutes and Bacteroidetes phyla, respectively. Notably, the abundance of Clostridia in the AR3-S was 10.08%, while in the AR1-S and AR2-S were 0.94 and 0.15%, respectively. These significant differences might suggest the synergic effect of PAHs and the electrochemical system on the growth of the taxa. On the
other hand, the population of *Sphingobacteria* in the AR1-S (2.67%) compared to the AR2-S (1.37%) and AR3-S (0.26%) showed the effect of electrochemical systems on this bacteria.

Figure 4.7 Bacterial community compositions (class-level) of sediment in reactors AR1, AR2 and AR3. (a) Proteobacteria (b) Acidobacteria
Figure 4.7 Bacterial community compositions (class level) of sediment in reactors AR1, AR2 and AR3. (a) Proteobacteria (b) Acidobacteria. Considering the great potential for biodegradation of PAHs in the contaminated sediment using electrochemical systems (SMFC), the bacterial genus-level distribution was further assessed and the populations of dominant genera are been listed in Table 4.4. It was found that a large number of sulfate-reducing bacteria populations (*Desulfotherosporinus* and *Desulfitobacterium*) were detected in the AR3-S, while it was under the detection limit for the AR1-S and AR2-S. These bacteria are capable of breaking down several groups of hardly biodegradable compounds such as PAHs (Abed et al., 2011; Sherry et al., 2013). In addition these bacteria have also been identified as exoelectrogens in the literature (Park et al., 1997).

Another interesting result found from the genus-level distribution was the significant abundance of *Massilia* and *unclassified_Burkholderiales_incertae_sedis* in all the reactors especially in the reactors with electrodes, which showed that these bacteria were favorable for growing and degradation in the presence of electrodes. The contribution of genus *Novispirillum*, *Rubellimicrobium*, *Magnetospirillum* and *Geothrix* in the AR1-S were significantly higher than the AR2-S and AR3-S, which revealed the incompatibility of these bacteria in the presence of electrodes and consequently, it can be concluded that these bacteria were not responsible for the higher PAHs biodegradation in the AR3-S.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Relative Abundance (%)</th>
<th>Phylum</th>
<th>AR1-S</th>
<th>AR2-S</th>
<th>AR3-S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Massilia</td>
<td>Proteobacteria</td>
<td>8.17</td>
<td>14.78</td>
<td>15.29</td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td>----------------</td>
<td>------</td>
<td>-------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>Desulfosporosinus</td>
<td>Firmicutes</td>
<td>0</td>
<td>0</td>
<td>6.36</td>
<td></td>
</tr>
<tr>
<td>Desulfitobacterium</td>
<td>Firmicutes</td>
<td>0</td>
<td>0</td>
<td>2.22</td>
<td></td>
</tr>
<tr>
<td>Novispirillum</td>
<td>Proteobacteria</td>
<td>7.46</td>
<td>0.11</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>Rubellimicrobium</td>
<td>Proteobacteria</td>
<td>3.24</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Magnetospirillum</td>
<td>Proteobacteria</td>
<td>2.81</td>
<td>0</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>Geothrix</td>
<td>Acidobacteria</td>
<td>4.20</td>
<td>0.04</td>
<td>2.02</td>
<td></td>
</tr>
<tr>
<td>Unclassified Bukholderiales incertae sedis</td>
<td>Proteobacteria</td>
<td>17.92</td>
<td>24.61</td>
<td>33.82</td>
<td></td>
</tr>
</tbody>
</table>

4.2.4.1.2 Anodic Biofilm

In all anodic biofilm samples that were taken from the surface of electrodes, *Proteobacteria* was the dominant phylum comprising between 89.33 and 92.68% of total bacterial sequences (Figure 4.8a). Then it was followed by *Bacteroidetes*, *Actinobacteria* and *Acidobacteria*. The dominancy of *Proteobacteria* was in agreement with the findings of Phung et al. (2004) and Lee et al. (2003a). However, comparison of bacterial communities of anodic biofilms and sediment samples showed more abundance of *Proteobacteria* in the anodic biofilms than the sediment samples, which revealed their greater growth on the surface of electrodes. Similar to the sediment samples, *Acidobacteria* phylum was found to be notably in the AR2-A, compared to under-the-limit in the AR3-A. Comparison of the bacterial abundance between the anodic biofilms and the sediment samples showed that this phylum of bacteria were not growing well on the surface of electrodes especially in the duplicated reactor (AR3-A).

For further investigation on the bacteria in the anodic biofilms, the class-level distribution of samples is illustrated in Figure 4.8b. It can be observed that *β-proteobacteria* was the dominant class within *Proteobacteria* phylum comprising
from 53.33% in the AR2-A to 65.66% in the AR3-A of the total bacterial sequences. It was followed by the $\alpha$-proteobacteria, $\delta$-proteobacteria and $\gamma$-proteobacteria. In contrast to the sediment samples, the abundance of $\alpha$-proteobacteria in the AR3-A (17.31%) was higher than the AR2-A (14.75%), which showed the ability of this class of bacteria to grow on the surface of electrodes in an electrochemical system. The same trend was observed for the $\gamma$-proteobacteria. In addition, there is a significant difference between the numbers of $\delta$-proteobacteria in the AR2-A (16.22%) and the AR3-A (under the detection limit), which showed the incompatibility of the bacteria for growing on the surface of electrodes in a competition with other classes of Proteobacteria (i.e., $\beta$-proteobacteria, $\alpha$-proteobacteria and $\gamma$-proteobacteria) in the presence of PAHs.
Further assessment on the genus-level distribution of samples can be found in Table 4.5. As it can be seen, there were many genera (i.e., Massilia,
Ochrobactrum, Pseudomonas, Rhodococcus, Halomonas, Desulfosporosinus, Desulfitobacterium, Vibrio, Arthrobacter, Staphylococcus and Unclassified Bukholderiales incertae sedis) present in the duplicated reactor’s biofilm (AR3-A), which their abundances were significantly higher than the control reactor (AR2-A). Their higher abundance in the AR3-A suggested that the biofilm formed in the AR3 was due to a better environment for the growth of these genera. On the other hand, there were some genera which their abundances were significant in the AR2-A, while it was under the detection limit in the AR3-A. This demonstrated that not all the bacteria were capable of growing on the surface of electrodes in the presence of PAHs. This environment may not be favorable for some genera such as Cystobacteraceae, Ralstonia and Brevundimonas. In addition, there were a few genera that were found almost with the same abundance in both biofilms (i.e., AR2-A and AR3-A) such as Methylobacterium and Sphingomonas, showing their independencies from the presence of PAHs in the system.

Table 4.5 Dominant genera involved in the anodic biofilms of the reactors AR2 and AR3.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Phylum</th>
<th>Relative Abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Massilia</td>
<td>Proteobacteria</td>
<td>13.59 19.63</td>
</tr>
<tr>
<td>Ochrobactrum</td>
<td>Proteobacteria</td>
<td>1.81 4.89</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>Proteobacteria</td>
<td>0.88 2.79</td>
</tr>
<tr>
<td>Rhodococcus</td>
<td>Actinobacteria</td>
<td>0.95 1.77</td>
</tr>
<tr>
<td>Halomonas</td>
<td>Proteobacteria</td>
<td>0.23 1.48</td>
</tr>
<tr>
<td>Desulfosporosinus</td>
<td>Firmicutes</td>
<td>0 1.15</td>
</tr>
<tr>
<td>Desulfitobacterium</td>
<td>Firmicutes</td>
<td>0 1.08</td>
</tr>
<tr>
<td>Vibrio</td>
<td>Proteobacteria</td>
<td>0.04 0.98</td>
</tr>
<tr>
<td>Arthrobacter</td>
<td>Actinobacteria</td>
<td>0 0.51</td>
</tr>
<tr>
<td>Staphylococcus</td>
<td>Firmicutes</td>
<td>0.05 0.47</td>
</tr>
<tr>
<td>Cystobacteraceae</td>
<td>Proteobacteria</td>
<td>15.70 0</td>
</tr>
<tr>
<td>Ralstonia</td>
<td>Proteobacteria</td>
<td>2.83 0</td>
</tr>
</tbody>
</table>
4.2.4.1.3 Cathodic Biofilm

Cathodic biofilm was not expected to play a key role in biodegradation process since the majority of microorganisms and organic carbons (PAHs or others) were in the sediment. Despite this fact, it was valuable to investigate microorganisms involved in the cathodic biofilms. However, in the phylum-level distribution, *Proteobacteria* was the dominant phyla comprising 89.99% in the AR2-C and 86.89% in the AR3-C of total bacterial sequences. It was followed by *Bacteroidetes* comprising 4.52 and 5.49% in the AR2-C and AR3-C, respectively. Therefore, in the class-level distribution, similar as the anodic biofilm and the sediment samples, *β-proteobacteria* was the dominant class of bacteria comprising 57.62% in the AR2-C and 45.23% in the AR3-C, followed by *α-proteobacteria* and *γ-proteobacteria*.

Table 4.6 provides the list of genera that were dominant and significant to take into account in the cathodic biofilm. As it can be observed, there were many genera that were found significantly in higher abundance in the AR3-C than the AR2-C such as *Bradyrhizobium, Methylophilus, Acidovorax, Sphingomonas, Sphingobium, Peredibacter, Nitrospira, Bosea* and *Geothrix*. Therefore, there are some genera that are not favorable for the growth in the environment containing PAHs such as *Massilia, Brevundimonas* and *Rhodococcus*. However, as it can be
seen, there were not many differences between the control (AR2-C) and the duplicated ones (AR3-C), which was reasonable due to the tendency of PAHs to be adsorbed onto the sediment and not dissolving into the water.

Table 4.6 Dominant genera involved in the cathodic biofilms of the reactors AR2 and AR3.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Phylum</th>
<th>AR2-C</th>
<th>AR3-C</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bradyrhizobium</em></td>
<td><em>Proteobacteria</em></td>
<td>2.07</td>
<td>5.44</td>
</tr>
<tr>
<td><em>Methylophilus</em></td>
<td><em>Proteobacteria</em></td>
<td>1.05</td>
<td>4.48</td>
</tr>
<tr>
<td><em>Acidovorax</em></td>
<td><em>Proteobacteria</em></td>
<td>0.02</td>
<td>3.66</td>
</tr>
<tr>
<td><em>Sphingomonas</em></td>
<td><em>Proteobacteria</em></td>
<td>2.63</td>
<td>4.21</td>
</tr>
<tr>
<td><em>Sphingobium</em></td>
<td><em>Proteobacteria</em></td>
<td>0</td>
<td>1.28</td>
</tr>
<tr>
<td><em>Peredibacter</em></td>
<td><em>Proteobacteria</em></td>
<td>0</td>
<td>1.28</td>
</tr>
<tr>
<td><em>Nitrospira</em></td>
<td><em>Nitrospirae</em></td>
<td>0.31</td>
<td>1.28</td>
</tr>
<tr>
<td><em>Bosea</em></td>
<td><em>Proteobacteria</em></td>
<td>0.44</td>
<td>1.31</td>
</tr>
<tr>
<td><em>Geothrix</em></td>
<td><em>Acidobacteria</em></td>
<td>0</td>
<td>0.46</td>
</tr>
<tr>
<td><em>Massilia</em></td>
<td><em>Proteobacteria</em></td>
<td>16.83</td>
<td>10.98</td>
</tr>
<tr>
<td><em>Brevundimonas</em></td>
<td><em>Proteobacteria</em></td>
<td>3.49</td>
<td>1.48</td>
</tr>
<tr>
<td><em>Rhodococcus</em></td>
<td><em>Actinobacteria</em></td>
<td>1.38</td>
<td>0.41</td>
</tr>
<tr>
<td><em>Methyllobacterium</em></td>
<td><em>Proteobacteria</em></td>
<td>4.01</td>
<td>4.29</td>
</tr>
<tr>
<td><em>Unclassified Bukholderiales incertae sedis</em></td>
<td><em>Proteobacteria</em></td>
<td>35.58</td>
<td>19.61</td>
</tr>
</tbody>
</table>

4.3 Conclusions

To date, bioaugmentation and biostimulation are widely known technologies to remediate hydrocarbon-polluted sites (Amezcua-Allieri et al., 2012), whereby the addition of nutrients or microorganisms is required. Recently, in-situ remediation of contaminated soils has received considerable attention due to its many advantages such as low cost and the avoidance of secondary pollution (Huang et al., 2011a; Rulkens, 2005). However, this phase of the study had demonstrated a new way of increasing the rate of PAHs remediation by harvesting the electrons...
generated via an external circuit and stimulation of microorganism by means of a SMFC in an aerobic cathodic environment. This stimulation could be due to the alteration of the physical and chemical of soil properties by applying a potential difference and providing a more activated medium for microorganisms by the SMFCs (Hong et al., 2010). In other words, SMFCs might stimulate indigenous microorganisms and make them more active for electron generation and transferring.

However, SMFC was found capable of biodegrading complex PAHs in sediment. The SMFCs achieved 41.7, 31.4 and 36.2% PAHs removal in aerobic environment for naphthalene, acenaphthene and phenanthrene, respectively. In addition, this study also showed that SMFCs could stimulate TOC removal in the sediment. The SMFCs showed 52% TOC removal from the sediment, while it was only 27% for the non-SMFC reactor. Analysis of pyrosequencing results revealed significant differences in microbial community between the duplicated reactors and the control reactors (i.e., AR1 and AR2).

The results of the anaerobic phase of this study will be presented and a comparison between aerobic and anaerobic cathodic environment shall be discussed in the chapter 5.
Chapter 5 Biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs) in the Contaminated Soil Using Sediment Microbial Fuel Cells (SMFC) – Anaerobic Cathodic Chamber

5.1 Introduction

Although most of hydrocarbons including PAHs are known to be degraded more in oxic environments, most contaminated sediments are anoxic. In fact, the pure oxic zone of polluted sediments typically forms only at the surficial levels of the sediment and the majority of compounds are in anoxic zones. Consequently, treatment of these zones requires significant oxygen introduction which is not economic. On the other hand, a typical MFC is a half biological system because only the anode side contains electrochemically-active microorganisms while the cathodic compartment is abiotic. However, it can be fully biological process if other compounds such as nitrate or sulfate accept the electrons through a biological process on the surface of the cathode (biocathode).

Biocathode may have advantages over abiotic cathodes for several reasons. First, they lower the cost of construction and operation. Second, they increase the operational sustainability of the cathode (He & Angenent, 2006; Rabaey, 2008). Third, the microbial metabolism may be utilized to remove unwanted compounds (Lovley, 1991).

In this chapter, the performances of SMFCs with anaerobic cathodic condition (biocathode) in removal of PAHs from contaminated sediment were investigated.
As mentioned earlier in chapter 3, nitrate and sulfate were added as the final electron acceptors (TEAs).

5.2 Results and Discussions

5.2.1 Electricity

The polarization curve (PC) and power density (PD) of anaerobic SMFC reactors are shown in Figure 5.1. As can be seen, the maximum PD of the duplicated reactors (i.e., AnR3 and AnR4) was found to be 1.98±0.5 mW/m² while that of the AnR2 was 3.30 mW/m². In addition, based on the slope of the linear part of the PC, the internal resistances (IR) were 522±2 and 900 Ω for the duplicated reactors (i.e., AnR3 and AnR4) and the control SMFC (AnR2), respectively. The reason behind this difference could be due to the presence of PAHs that developed different microbial communities on the anode electrode. Consequently, with different microbial communities, the rates of the electron production were different. However, it is important to note that the internal resistance (R\text{int}) should not be interchangeably used with ohmic losses (R\text{Ω}). The data reported in this study (all phases) is truly internal resistance (R\text{int}) and not the ohmic losses (R\text{Ω}) because as can be seen in Figure 5.1, due to non-linear and rapid loss of voltage at low current, the below equation will be used:

\[
E_{\text{emf}} = OCV^* - IR_{\text{int}}
\]

Equation 5.1
Where OCV* is determined by extrapolation and is not the true OCV. In other words, \( R_{\text{int}} \) includes anode and cathode overpotentials. Thus those anodic and cathodic microbial kinetics (only anodic here) can affect \( R_{\text{int}} \) (Logan, 2008).

![Figure 5.1 Electricity performances - polarization curve (PC) and power density (PD) - of the anaerobic SMFC reactors.](image)

By comparing the results of PC, maximum PD and IR obtained from this phase (anaerobic) with the aerobic phase (Chapter 4), significant differences could be found. For instance, the maximum OCV of the duplicated reactors (i.e., AnR3 and AnR4) was 0.61 V while that of aerobic duplicated SMFC (i.e., AR3 and AR4) was 0.72 V. Another example of differences could be in the maximum PD where the maximum PD of anaerobic phase was 1.98 mW/m² while that of aerobic was 5.77 mW/m². The same differences could be observed in favor of aerobic SMFC reactors in IR, average current density (Ave. CD) and average PD. Table 5.1
summarizes the differences between the duplicated aerobic and anaerobic SMFC reactors in terms of electricity performances.

**Table 5.1 Comparison of electrical performances of the duplicated aerobic and anaerobic SMFCs.**

<table>
<thead>
<tr>
<th></th>
<th>Aerobic</th>
<th>Anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max. OCV (V)</td>
<td>0.72</td>
<td>0.61</td>
</tr>
<tr>
<td>Max. PD (mW/m²)</td>
<td>5.77</td>
<td>1.98</td>
</tr>
<tr>
<td>Ave. CD (mA/m²) @ 1500Ω</td>
<td>20.27</td>
<td>15.46</td>
</tr>
<tr>
<td>Ave. PD (mA/m²) @ 1500Ω</td>
<td>6.08</td>
<td>3.59</td>
</tr>
<tr>
<td>IR (Ω)</td>
<td>437</td>
<td>522</td>
</tr>
</tbody>
</table>

However, these significant differences could be due to two main reasons. Firstly, fewer electrons were transferred through the external circuit since a portion of electrons released from the oxidation process were consumed by the nitrate-reducing and sulfate-reducing bacteria under anaerobic cathodic environment. Secondly, less energy was available with nitrate than oxygen as an electron acceptor as indicated by a lower redox potential for nitrate (NO₃⁻/0.5N₂, E₀=0.74V) than oxygen (0.5O₂/H₂O, E₀=0.82V) (Logan, 2008).

### 5.2.2 PAHs Removal

By sampling two times over 45 d from the anode compartments of the reactors at day 1 and 45, a significant removal of PAHs was observed in the sediment. The results are summarized in Table 5.2 and Figure 5.2.

**Table 5.2 PAHs removal efficiency (%) in anaerobic SMFC reactors.**

<table>
<thead>
<tr>
<th>PAHs Compounds</th>
<th>Duplicated SMFCs (AnR3 &amp; AR4)</th>
<th>Non-SMFC Control (AnR1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D45</td>
<td>D45</td>
</tr>
</tbody>
</table>

93
<table>
<thead>
<tr>
<th>PAH</th>
<th>SMFC Efficiency</th>
<th>AnR1 Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>76.9±0.12</td>
<td>29.3±0.05</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>52.5±0.04</td>
<td>29.0±0.03</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>36.8±0.04</td>
<td>12.3±0.03</td>
</tr>
</tbody>
</table>

D45: Sample collected on day 45

Figure 5.2 PAHs removal efficiency (%) in the SMFC and non-SMFC reactors. (a) Naphthalene (b) Acenaphthene and (c) Phenanthrene.

As mentioned earlier, the AnR1 as the control reactor measured the background natural bioremediation of PAHs in the sediment. Thus by comparing it with the duplicated SMFC reactors (i.e., AnR3 and AnR4), the effectiveness of the electrochemical systems could be shown. Duplicated SMFC reactors were able to remove 76.9±0.12, 52.5±0.04 and 36.8±0.04% of the initial naphthalene,acenaphthene and phenanthrene, respectively, while those of the AnR1 (control)
were 29.3, 29.0 and 12.3%, respectively. However, these significant differences between the control and the duplicated reactors could be attributed to the different microbial communities that were formed on the surface of electrodes, which will be discussed later.

The nitrate and sulfate that had been added to the reactors as the potential TEAs were measured during the operation. It was found that nitrate was the dominant TEA as it was consumed by 38.7%, while that of sulfate was only 13.2% in the sediment (Figure 5.3). The lower rate of sulfate reduction compared to nitrate revealed that sulfate-reducing bacteria would take a longer time to adopt than nitrate-reducing bacteria.

In addition, GC analysis for the overlaying water samples showed that amount of PAHs diffused was negligible which is reasonable due to their high hydrophobicity.

Figure 5.3 C/C₀ ratio of the nitrate and sulfate in the sediment in anaerobic SMFC reactors. (a) Nitrate (b) Sulfate.
5.2.2.1 Anaerobic vs. Aerobic (PAHs Removal)

Another interesting result observed from comparing the two phases was the significant difference between the performance of aerobic and anaerobic SMFC reactors in the biodegradation of PAHs. For instance, the duplicated aerobic SMFC reactors (i.e., AR3 and AR4) were able to remove 41.7±1.7, 34±0.3 and 36.2±0.9% of the initial naphthalene, acenaphthene and phenanthrene present, respectively, while those of the duplicated anaerobic SMFC reactors (i.e., AnR3 and AnR4) were 76.9±0.12, 52.5±0.04 and 36.8±0.04%, respectively. This significant difference could be due to the fact that PAHs could be degraded under anaerobic conditions, in the presence of TEAs such as nitrate and sulfate (Coates, 1997; Coates, 1996; Meckenstock et al., 2000; Zhang X, 2000). The PAH removal rates by the control SMFC reactors also confirmed this claim since the AR1 was able to remove 12.6, 9.8 and 11.3% of the initial naphthalene, acenaphthene and phenanthrene present, respectively, while those of the AnR1 was 29.3, 29.0 and 12.3%, respectively. However, it was observed that PAHs are more susceptible to biodegradation in anaerobic SMFCs with TEAs such as nitrate and sulfate, rather than in aerobic SMFCs. This is because nitrate and sulfate not only served as the electron acceptors of PAHs degradation in the cathodic chamber, but also in the anodic chambers as the local electron acceptors of the SMFCs. This observation has also been reported in literature, whereby nitrate- and sulfate-reducing bacteria enhanced PAHs biodegradation (Coates, 1997; Coates, 1996; Meckenstock et al., 2000; Zhang X, 2000).
5.2.3 Total Organic Carbon (TOC)

The TOC analysis of sediment and water samples showed the effectiveness of electrochemical systems in the removal of TOC. Figure 5.4 shows the TOC/TOC\textsuperscript{0} ratio of sediment samples after 45 days of operation. As it can be seen, the duplicated reactors (i.e., AnR3 and AnR4) were able to remove 67% of the initial TOC, while that of the AnR1 (background natural biodegradation) and the AnR2 (SMFC without PAHs) were 31 and 41%, respectively. This suggests that microorganisms were more active in the duplicated reactors followed by the AnR1 and AnR2. Based on these results, it can be implied that reactors with electrodes (i.e., AnR2, AnR3 and AnR4) generally had a better performance in TOC removal due to different microbial communities.

These findings were similar to the findings of the aerobic phase that aerobic duplicated reactors achieved 52% TOC removal while it was only 37 and 27% for the AR1 and AR2. In addition, the better performance of SMFC reactors in anaerobic cathodic condition compared to aerobic cathodic condition could be due to impossibility of oxygen diffusion from the overlaying water (cathodic chamber) into the sediment (anodic chamber).
5.2.4 Analysis of Pyrosequencing Results

Similar to the aerobic phase, microbial communities of the anodic and cathodic biofilms as well as sediment were studied to elucidate the differences observed in the previous sections such as electricity and bioremediation performances between the duplicated SMFC and the control reactors. Different types of samples studied are summarized in Table 5.3.

Table 5.3 Different types of samples taken from the sediment, anodic and cathodic biofilms for analysis.

<table>
<thead>
<tr>
<th>Label</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AnR1-S</td>
<td>Anaerobic phase, Sediment sample, Reactor 1 (control, non-SMFC), with PAHs</td>
</tr>
<tr>
<td>AnR2-S</td>
<td>Anaerobic phase, Sediment sample, Reactor 2 (control, SMFC), without PAHs</td>
</tr>
<tr>
<td>AnR4-S</td>
<td>Anaerobic phase, Sediment Sample, Reactor 4 (SMFC), with PAHs</td>
</tr>
<tr>
<td>AnR2-A</td>
<td>Anaerobic phase, Anode surface, Reactor 2 (control, SMFC), without PAHs</td>
</tr>
</tbody>
</table>

Figure 5.4 TOC/TOC\textsuperscript{0} ratio of the sediment in anaerobic SMFC reactors.
Table 5.4 shows the summary of pyrosequencing data obtained from the microbial communities of sediment, anodic and cathodic biofilms. It can be seen that a total of 20,240 effective sequences were obtained after filtering the raw sequences with an average sequence length of 424 bp.

<table>
<thead>
<tr>
<th>Bacterial Community</th>
<th>AnR1-S</th>
<th>AnR2-S</th>
<th>AnR4-S</th>
<th>AnR2-A</th>
<th>AnR4-A</th>
<th>AnR4-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Sequences</td>
<td>2825</td>
<td>3084</td>
<td>5120</td>
<td>4318</td>
<td>2974</td>
<td>1919</td>
</tr>
<tr>
<td>OTUs</td>
<td>339</td>
<td>389</td>
<td>525</td>
<td>887</td>
<td>299</td>
<td>277</td>
</tr>
<tr>
<td>ACE</td>
<td>1757</td>
<td>3228</td>
<td>3448</td>
<td>2388</td>
<td>1109</td>
<td>1203</td>
</tr>
<tr>
<td>Chao</td>
<td>874</td>
<td>1492</td>
<td>1692</td>
<td>1827</td>
<td>810</td>
<td>659</td>
</tr>
</tbody>
</table>

Generated at 97% similarity threshold.

5.2.4.1 Bacterial Community Composition

5.2.4.1.1 Sediment

The phylum-level distribution of sediment samples is shown in Figure 5.5. It can be seen that *Proteobacteria* was the most dominant phylum comprising between 93.95 and 96.48% of the total bacterial sequences, which is in agreement with the literature findings (Lee et al., 2003a). Higher abundance of the *Proteobacteria* in the reactors with electrodes (i.e., AnR2-S and AnR4-S) revealed the effectiveness of electrochemical systems in the growth of these bacteria. It was also shown that the second-dominant phylum after the *Proteobacteria* was * Acidobacteria*. In contrast
to the *Proteobacteria*, the *Acidobacteria* phylum was not compatible with electrochemical systems since it comprised only 1.39% in both the AnR2-S and AnR4-S, while it was 3.82% in the AnR1-S (i.e., the reactor without electrodes).

![Bacterial community compositions (phylum level) of sediment in reactors AnR1, AnR2 and AnR4.](image)

**Figure 5.5** Bacterial community compositions (phylum level) of sediment in reactors AnR1, AnR2 and AnR4.

**Anaerobic vs. Aerobic (Phylum-level)**

By comparing anaerobic results with aerobic ones in the phylum-level, it can be observed that the proportions of *Proteobacteria* in the anaerobic phase (i.e., 96.48% in the AnR4-S) were significantly higher than those in the aerobic phase (i.e., 76.21% in the AR3-S), which suggested that anaerobic reactors provided a more favorable environment for the growth of these bacteria. Another significant difference between these two phases was in the abundance of *Acidobacteria*. This phylum was found in the aerobic phase comprising between 5.74 and 17.18% of
the total sequences, while it was almost negligible in anaerobic phase. Similar trend was observed for _Bacteroides_, whereby it was nearly two to three times higher in the aerobic than the anaerobic cathodic environment.

For more detailed investigation, the class-level distribution within the _Proteobacteria_ was analyzed, which is illustrated in Figure 5.6. Similar to the aerobic phase, _β-proteobacteria_ was the dominant taxa comprising between 74.42 and 76.46% of the total sequences in the sediment. It was followed by _α- and γ-proteobacteria_.

**Anaerobic vs. Aerobic (Class-level)**

By comparing the class-level distributions of the _Proteobacteria_ in both the aerobic and anaerobic cathodic environment, it can be seen that in the anaerobic reactors, more _β-proteobacteria_ was found compared to the aerobic reactors. _β-proteobacteria_ was responsible for up to 55.72% of the AR3-S, while it was 76.46% of the AnR4-S. Another difference was in the abundance of the _α-proteobacteria_ in the control reactors (i.e., AR1-S and AnR1-S). This class of bacteria was responsible for 29.31% of the total bacterial sequences in the AR1-S, while it was only 13.56% in the AnR1-S. In addition, the number of _δ-proteobacteria_ was nearly negligible (below 0.32% in anaerobic), while it was up to 5.43% in the aerobic phase. This revealed that firstly, anaerobic condition might not be suitable for _δ-proteobacteria_ and secondly, it might have different impacts on the some classes (i.e., _β-proteobacteria_).
Figure 5.6 Bacterial community compositions. Class-level distribution of *Proteobacteria* in sediment of reactors AnR1, AnR2 and AnR4.

For a better microbial assessment, genus-level distributions of samples were studied. The list of these genera can be found in Table 5.5. It can be seen, unlike the aerobic phase, that the abundance of the dominant genera in all reactors did not change significantly. However, in the anaerobic phase, differences probably will be more observable in the anodic biofilm (discussed in the next section) because the main biofilm that was responsible for electrochemical systems was formed on the surface of anodes.

Table 5.5 Dominant genera involved in the sediment samples of the reactors AnR1, AnR2 and AnR4.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Phylum</th>
<th>AnR1-S</th>
<th>AnR2-S</th>
<th>AnR3-S</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Brevundimonas</em></td>
<td>Proteobacteria</td>
<td>3.04</td>
<td>3.31</td>
<td>4.61</td>
</tr>
<tr>
<td><em>Vibrio</em></td>
<td>Proteobacteria</td>
<td>0.00</td>
<td>0.19</td>
<td>0.27</td>
</tr>
<tr>
<td><em>Aquamicrobium</em></td>
<td>Proteobacteria</td>
<td>0.14</td>
<td>0.58</td>
<td>0.18</td>
</tr>
<tr>
<td>Microorganism</td>
<td>Phylum</td>
<td>AnR4</td>
<td>AnR2</td>
<td>Unclassified Proteobacteria</td>
</tr>
<tr>
<td>---------------------------</td>
<td>----------------</td>
<td>------</td>
<td>------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>Ochrobactrum</td>
<td>Proteobacteria</td>
<td>1.27</td>
<td>1.78</td>
<td>1.11</td>
</tr>
<tr>
<td>Massilia</td>
<td>Proteobacteria</td>
<td>16.00</td>
<td>15.63</td>
<td>16.54</td>
</tr>
<tr>
<td>Corynebacterium</td>
<td>Actinobacteria</td>
<td>1.42</td>
<td>0.03</td>
<td>0.14</td>
</tr>
<tr>
<td>Methylobacterium</td>
<td>Proteobacteria</td>
<td>5.38</td>
<td>4.8</td>
<td>4.77</td>
</tr>
<tr>
<td>Novosphingobium</td>
<td>Proteobacteria</td>
<td>0.67</td>
<td>0.49</td>
<td>0.29</td>
</tr>
<tr>
<td>Marinospirillum</td>
<td>Proteobacteria</td>
<td>1.42</td>
<td>1.39</td>
<td>1.27</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>Proteobacteria</td>
<td>1.06</td>
<td>0.71</td>
<td>0.76</td>
</tr>
<tr>
<td>Unclassified Burkholderiales incertae sedis</td>
<td>Proteobacteria</td>
<td>55.15</td>
<td>53.18</td>
<td>55.41</td>
</tr>
</tbody>
</table>

5.2.4.1.2 Anodic Biofilm

The microbial communities of biofilms formed on the surface of the anode electrodes were assessed. As it can be observed in Figure 5.7a, similar to the sediment samples, the *Proteobacteria* was the dominant phylum accounting for 61.65% in the AnR2-A and 96.20% in the AnR4-A of the total bacterial sequences that was in agreement with the literature findings (Lee et al., 2003a; Phung et al., 2004). However, there was a significant difference in the abundance of *Proteobacteria* between the control reactor (AnR2-A) and the duplicated reactor (AnR4-A). It was found that *Proteobacteria* formed 96.20% of the total bacterial in the AnR4-A, while surprisingly it was only 61.65% for the AnR2-A, which showed the effect of presence of PAHs in AnR4-A. On the other hand, higher *Bacteroidetes* was found in the AnR2-A (24.90%) than AnR4-A (1.41%).

Therefore, there was another difference in the abundance of *Nitrospirae* phylum. Although Yan et al. (2012) reported *Nitrospirae* as the most dominant phylum by 30.3% in their system contaminated with phenanthrene and pyrene, in this study, no *Nitrospirae* was detected in the AnR4-A and it only made up 4.52% of the total bacterial in the AnR2-A. In addition, similar as the sediment samples, the
abundance of Acidobacteria was under the detection limit in the AnR4-A, while it was 2.01% in the AnR2-A.

**Anaerobic vs. Aerobic (Phylum-level)**

By comparing the results of the anodic biofilm community in the anaerobic with aerobic phase, some significant differences were found. There was a significant difference between the abundance of Proteobacteria, Bacteroidetes and Actinobacteria in the aerobic and anaerobic phases. For instance, the abundances of these phyla, respectively, were 89.33 and 3.49 in the AR2-A, while those were 61.65 and 24.90% in the AnR2-A. Moreover, the abundance of Actinobacteria in the AR3-A was 4.13%, while only 1.61% in the AnR4-A. However, these differences showed how small changes in the condition of a reactor could affect the microbial communities and consequently, the performances in removal and electricity efficiencies.

For more investigation of involved bacteria in the anodic biofilms, the class-level distribution of Proteobacteria is illustrated in Figure 5.9b. It can be observed that β-proteobacteria was the dominant class within Proteobacteria phylum comprising from 34.39% in AnR2-A to 72.46% in AnR4-A of total bacterial sequences. It was followed by α-proteobacteria and γ-proteobacteria. In contrast to sediment samples, the abundance of α-proteobacteria in AnR4-A (18.63%) was higher than AnR2-A (13.90%) which showed the interest of this class of bacteria for growing on the surface of electrodes in an electrochemical system in the presence of PAHs. In addition, there was a significant difference between the
numbers of $\delta$-proteobacteria in AnR2-A (3.87%) and AnR4-A (under the detection limit) which showed the incompatibility of this class of Proteobacteria for growing on the surface of electrodes in a competition with other classes of Proteobacteria (i.e. $\beta$-proteobacteria and $\alpha$-proteobacteria) in the presence of PAHs.

Figure 5.7 Bacterial community compositions of anodic biofilms in reactors AnR2 and AnR4. (a) Phylum level. (b) Class-level within Proteobacteria Phylum.
The abundances of the dominant genera in the AnR4-A were substantially different than the AnR2-A (control). For instance, *Massilia* comprised 16.64% of the total bacterial sequences in the AnR4-A, while it was only 5.33% for the AnR2-A. Another example could be *Methylobacterium* which was found to be 5.72% and 1.30% in the AnR4-A and the AnR2-A, respectively. On the other hand, there were also some genera that were found more in the AnR2-A compared to the AnR4-A such as *Nitrospira, Thiobacillus, Geothrix and Clostridium XI*. The list of differences between the AnR2-A and the AnR4-A are shown in Table 5.6. However, these notable differences between the duplicated SMFC and the control reactors showed that dominant genera could be changed by the different substances (i.e., PAHs in the duplicated reactors).

**Table 5.6 Dominant genera involved in the anodic biofilms of the reactors AR2 and AR4.**

<table>
<thead>
<tr>
<th>Genus</th>
<th>Phylum</th>
<th>Relative Abundance (%)</th>
<th>AnR2-A</th>
<th>AnR4-A</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Massilia</em></td>
<td>Proteobacteria</td>
<td>5.33</td>
<td>16.61</td>
<td></td>
</tr>
<tr>
<td><em>Methylobacterium</em></td>
<td>Proteobacteria</td>
<td>1.30</td>
<td>5.72</td>
<td></td>
</tr>
<tr>
<td><em>Sphingomonas</em></td>
<td>Proteobacteria</td>
<td>1.16</td>
<td>2.62</td>
<td></td>
</tr>
<tr>
<td><em>Ochrobactrum</em></td>
<td>Proteobacteria</td>
<td>1.11</td>
<td>1.88</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>Proteobacteria</td>
<td>0.25</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td><em>Thauera</em></td>
<td>Proteobacteria</td>
<td>0.19</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td><em>Aquamicrobium</em></td>
<td>Proteobacteria</td>
<td>0.09</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td><em>Rhodococcus</em></td>
<td>Actinobacteria</td>
<td>0.25</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td><em>Pontibaca</em></td>
<td>Proteobacteria</td>
<td>0.00</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td><em>Bradyrhizobium</em></td>
<td>Proteobacteria</td>
<td>0.05</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td><em>Corynebacterium</em></td>
<td>Actinobacteria</td>
<td>0.05</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td><em>Nitrospira</em></td>
<td>Nitrospirae</td>
<td>4.52</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td><em>Thiobacillus</em></td>
<td>Proteobacteria</td>
<td>3.61</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td><em>Geothrix</em></td>
<td>Acidobacteria</td>
<td>0.86</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td><em>Clostridium XI</em></td>
<td>Firmicutes</td>
<td>0.44</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td><em>Unclassified Bukholderiales incertae sedis</em></td>
<td>Proteobacteria</td>
<td>14.47</td>
<td>51.75</td>
<td></td>
</tr>
</tbody>
</table>
5.2.4.1.3 Cathodic Biofilm

The Phylum-level distribution of the cathodic biofilms showed that similar to the anodic biofilms, *Proteobacteria* was the dominant phylum accounting for 94.89% in the AnR4-C. Therefore, in the class-level distribution, β- and α-proteobacteria comprised 57.01 and 35.54% of the total bacteria in the cathodic biofilm of the AnR4-C.

Since the big difference between the phases (i.e., aerobic and anaerobic) was in the condition provided in the cathodic chamber, it was important to compare the microbial communities of the cathodic biofilms. Table 5.7 provides a list of genera which were different between the AR3-C and AnR4-C. It can be observed that many bacteria with higher populations were found more in the aerobic cathodic biofilm (AR3-C) than in the anaerobic cathodic biofilm (AnR4-C). For instance, the abundance of genera such as *Bradyrhizobium*, *Methylophilus*, *Sphingomonas*, *Acidovorax* and etc were significantly higher in the aerobic cathodic biofilm. On the other hand, there were a few genera that were growing much better in the anaerobic condition provided in AnR4 such as *Massilia*, *Altererythrobacter*, *Brevundimonas* and etc.

Table 5.7 Comparison of dominant genera in cathodic biofilms of reactors AR3 (aerobic) and AnR4 (anaerobic).

<table>
<thead>
<tr>
<th>Genus</th>
<th>Phylum</th>
<th>Relative Abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bradyrhizobium</em></td>
<td><em>Proteobacteria</em></td>
<td>AR3-C: 5.44, AnR4-C: 2.61</td>
</tr>
<tr>
<td><em>Methylophilus</em></td>
<td><em>Proteobacteria</em></td>
<td>AR3-C: 4.48, AnR4-C: 0.05</td>
</tr>
<tr>
<td><em>Sphingomonas</em></td>
<td><em>Proteobacteria</em></td>
<td>AR3-C: 4.21, AnR4-C: 2.24</td>
</tr>
<tr>
<td><em>Acidovorax</em></td>
<td><em>Acidobacteria</em></td>
<td>AR3-C: 3.66, AnR4-C: 0</td>
</tr>
<tr>
<td><em>Bosea</em></td>
<td><em>Proteobacteria</em></td>
<td>AR3-C: 1.31, AnR4-C: 0.05</td>
</tr>
<tr>
<td><em>Sphingobium</em></td>
<td><em>Proteobacteria</em></td>
<td>AR3-C: 1.28, AnR4-C: 0</td>
</tr>
</tbody>
</table>
SMFCs were found capable of biodegrading complex PAHs in the contaminated sediment. The SMFCs achieved 76.9, 52.5 and 36.8% PAHs removal in an anaerobic environment for naphthalene, acenaphthene and phenanthrene, respectively, which were significantly higher compared to an aerobic phase. This significant difference could be due to the fact that PAHs could be degraded under anaerobic conditions, in the presence of TEAs such as nitrate and sulfate (Coates et al., 1996; Coates et al., 1997; Meckenstock et al., 2000; Zhang et al., 2000). The PAH removal rates by the control SMFC reactors also confirmed this claim since the AR1 was able to remove 12.6%, 9.8% and 11.3% of the initial naphthalene, acenaphthene and phenanthrene present, respectively, while those of the AnR1 was 29.3%, 29.0% and 12.3%, respectively. However, it was observed that PAHs are more susceptible to biodegradation in anaerobic SMFCs with TEAs such as nitrate and sulfate, rather than in aerobic SMFCs. This is because nitrate and sulfate not only served as the electron acceptors of PAHs degradation in the cathodic chamber, but also in the anodic chambers of the SMFCs.
In addition, this study also showed that SMFCs can stimulate TOC removal in the sediment. The SMFCs showed 67% TOC removal from the sediment, while it was only 31% for the non-SMFC reactor. Analysis of pyrosequencing results revealed significant differences of microbial community in duplicated reactors from the control reactors (AnR1 and AnR2) in terms of population and types of bacteria. In addition, comparison of these results with the aerobic phase revealed substantial differences in microbial communities of the sediment, anodic and cathodic biofilms. However, this phase (anaerobic) demonstrated the great ability of SMFCs in bioremediation of soil sites contaminated by persistent compounds such as PAHs.
Chapter 6 Investigation of Single-chambered Air-cathode Microbial Fuel Cells for the Treatment of PAH-contaminated Wastewater

6.1 Introduction

At present, only 3-4% of the wastewater in the world is recycled and that leaves a huge potential for water reuse to solve water scarcity problem. MFCs were introduced for the wastewater treatment by (Habermann & Pommer, 1991), and domestic wastewater contains a multitude of organic compounds that can be used as the fuel in MFCs. MFCs can potentially halve the electricity needed for a treatment process. They have been promising in the lab-scale for the treatment of wastewater. For instance, MFCs yield 50-90% less sludge production and also many organic compounds such as acetate (Biffinger et al., 2008), glucose (Lee et al., 2008), lactate (Manohar & Mansfeld, 2009) and propionate (Oh & Logan, 2005) can be thoroughly broken down to carbon dioxide and water.

In this chapter, the performance of single-chambered air-cathode MFCs for the treatment of PAH-contaminated wastewater was investigated. This chapter is being divided into two sections - batch and continuous studies. In each section, the performances of the reactors in electricity output, COD removal, TOC removal and PAHs were monitored.

As mentioned in chapter 3, three single-chambered air-cathode MFCs were operated simultaneously (Figure 6.1).
6.2 Batch Study - Results and Discussions

6.2.1 Electricity (Batch Study)

Figure 6.2 shows the open-circuit voltage (OCV) and closed-circuit voltage (CCV) – external resistance of 1000 Ω - of all reactors during the operation in the batch phase. As can be seen in Figure 6.2, the performances of the reactors in the presence of PAHs were significantly better than those reactors without PAHs. The maximum OCV of the control reactor with PAHs (i.e., BR1) and the control reactor without PAHs (i.e., BR4) were found to be 0.63 and 0.58 V, respectively. In addition, the maximum CCV (an external resistance of 1000 Ω) in the duplicated reactors with PAHs was 0.36 V, while it was only 0.21 V without
PAHs. As it is observed, voltages in the BR5 and BR6 (with PAHs) lasted significantly longer than those in the BR2 and BR3 (without PAHs). For instance, after 15 days, the voltage of BR2/3 was almost less than 0.01 V. This could be due to the biodegradation of PAHs in the BR5 and BR6, which was confirmed by the GC/MS results.

Figure 6.2 Output voltage harnessed from the single-chambered air-cathode MFC reactors.

Figure 6.3 shows the polarization curves (PCs) and power densities (PDs) of the reactors. As it can be seen, the maximum power density of the duplicated MFC reactors with PAHs (i.e., BR5 and BR6) was 304 mW/m$^3$, while that of duplicated MFC reactors without PAHs was 190 mW/m$^3$. Since it was practically impossible to calculate the surface area of the anode brushes, all the densities such as power density (PD) and current density (CD) were reported based on the anodic volume
However, a possible explanation for better electrical performance in the reactors with PAHs compared to those without PAHs could be due to the more carbon source provided in the reactors containing PAHs. In addition, as it was found in the previous two chapters in the SMFCs’ studies, PAHs can affect the community and the population of microorganisms. Consequently, with different microbial communities, the rate of electron production, due to different proton pumping rate, would be different resulting dissimilar electrical performances.

The calculated internal resistances (IR) based on the slope of the polarization curves (Figure 6.3) were 15 and 13 Ω for the reactors with PAHs (i.e., BR5 and BR6) and without PAHs (i.e., BR2 and BR3), respectively, which were significantly low compared to the literature. However, as mentioned in the previous chapters, it is very important to note that the internal resistance ($R_{\text{int}}$) should not be interchangeably used with ohmic losses ($R_\Omega$).

![Figure 6.3 Electrical performance - polarization curve (PC) and power density (PD) - of the duplicated reactors with and without PAHs.](image)

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Figures 6.4a and b show the current and power densities of the MFC reactors over 26 d. As can be seen from all figures, the duplicated reactors with PAHs (i.e., BR5 and BR6) had better performances compared to reactors without PAHs (i.e., BR2 and BR3) in terms of highest density and longevity. The BR5 and BR6 were able to generate a maximum power density of 86 mW/m$^3$, which was almost three times more than the maximum power density generated by the BR2 and BR3 (30 mW/m$^3$). Therefore, both graphs (CD and PD) demonstrated that reactors with PAHs could generate power for a longer time even though it was low compared to the first 6 days. However, as discussed in the previous two chapters, this longevity of reactors in the presence of PAHs was attributed to the PAHs degradation although the differences between the reactors with and without PAHs were not in the same order as those in the sediment.
Figure 6.4 Current density (a) and power density (b) of duplicated MFC reactors.
6.2.2 COD Removal (Batch Study)

Figure 6.5 shows the COD/COD\textsuperscript{0} ratio of the reactors during the time. As it can be seen, the highest COD removal was observed in the duplicated reactors with PAHs (i.e., BR5 and BR6) with a total COD removal of about 91%. It was followed by the BR4 (83%), the BR2 and BR3 (66%) and finally, the BR1 (63%). Although it was unexpected, the results suggested that reactors with PAHs (i.e., BR4, BR5 and BR6) were able to achieve higher COD removal, and also duplicated reactors with external resistance (i.e., BR5/BR6 or BR2/BR3) could remove significantly more than reactors with short circuit (i.e., BR4 and BR1). A possible explanation is that as it was observed in the SMFCs, PAHs can affect the microbial community on biofilms and consequently, the performances of the reactors.

Figure 6.5 COD removal of the reactors during the time in the batch study.
6.2.3  TOC Removal (Batch Study)

Total organic carbon (TOC) of wastewater in all reactors is shown in Figure 6.6. The results appeared to confirm the same trend observed in COD removal. Duplicated reactors with PAHs (i.e., BR4 and BR5) achieved the highest TOC removal by 98%, followed by the BR4 (90%), BR2/BR3 (81%) and BR1 (74%) at day 28.

Figure 6.6 TOC/TOC⁰ ratios of MFC reactors during 28 days of operation.

6.2.4  PAHs Removal (Batch Study)

The analysis of PAHs biodegradation in the control MFC reactor (i.e, BR4) and the duplicated MFC reactors (i.e., BR5 and BR6) showed the effectiveness of electrochemical systems on biodegradation rate. By sampling eight times over 28
days from the effluent of anode compartments at day 0, 4, 8, 12, 16, 20, 24 and 28 and comparison with the initial PAH concentration, a significant removal of PAHs was observed in the wastewater. The results are summarized in Table 6.1 and Figure 6.7.

Table 6.1 PAHs removal efficiency (%) in the single-chambered air-cathode MFC reactors.

<table>
<thead>
<tr>
<th>PAHs Compounds</th>
<th>Duplicated reactors (BR5 &amp; BR6)</th>
<th>Control reactor (BR4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D12</td>
<td>D28</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>14</td>
<td>52</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>30</td>
<td>42</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>26</td>
<td>47</td>
</tr>
</tbody>
</table>

D12: Sample collected on day 12; D28: Sample collected on day 28

Figure 6.7 $C/C^0$ ratio of PAHs in the single-chambered air-cathode MFCs. (a) Naphthalene (b) Acenaphthene and (c) Phenanthrene.
The effectiveness of air-cathode MFCs in biodegradation can be observed in the different performances between the duplicated reactors (i.e., BR5 and BR6) and the control MFC (BR4). Naphthalene, acenaphthene and phenanthrene were removed by the rate of 52, 42 and 47%, respectively, in the duplicated reactors, while their removal were 34, 27 and 35% in the open-circuit control reactor (BR4), respectively.

6.3 Continuous Study – Results and Discussions

In the continuous phase, six reactors labeled CR1 (control – W/O PAHs - short circuit), CR2 and CR3 (duplicated reactors – W/O PAHs - 1000Ω), CR4 (control – with PAHs – short circuit) and CR5 and CR6 (duplicated reactors – with PAHs - 1000Ω) were operated in different HRTs ranging from 2 h to 24 h. First, the reactors without PAHs (i.e., CR1, CR2 and CR3) were operated for all HRTs mentioned above and then based on the performance in COD and TOC removal, the optimum HRT was chosen to be used for the rest of the experiment.

6.3.1 COD Removal (Continuous Study)

The performance of MFC reactors (i.e., CR1, CR2 and CR3) in different HRTs is shown in Figure 6.8a. These graphs indicated that the optimum performance could be obtained at a HRT of 12 h. COD of the influent wastewater was removed by the efficiencies of 28, 30, 37, 46 and 43% at HRTs of 2, 4, 8, 12 and 24 h, respectively, in the duplicated reactors (i.e., CR2 and CR3), while 12, 16, 26, 36 and 39% were removed, respectively, in the CR1. In addition, similar to the batch
mode, the duplicated reactors (i.e., CR2 and CR3) showed better performance compared to the open-circuit reactor (i.e., CR1).

As mentioned above, the optimum HRT of 12 h was chosen to achieve good removal efficiency. Thus the rest of the experiments with PAHs were done at a HRT of 12 h. Figure 6.8b shows the effectiveness of MFC reactors with PAHs for COD removal rate. The duplicated reactors with PAHs (i.e., CR5 and CR6) were able to achieve 52% COD removal, while 46% COD removal was achieved for the duplicated reactors without PAHs (i.e., CR2 and CR3).

![Figure 6.8 Performances of single-chambered air-cathode MFC reactors in COD removal. (a) COD/COD\(0\) ratio in the absence of PAHs in different HRTs. (b) Comparison of COD removal efficiencies in the presence and absence of PAHs in HRT12.](image)

### 6.3.2 TOC Removal (Continuous Study)

Total organic carbon (TOC) of the reactors in the absence of PAHs was measured in different HRTs ranging from 2 h to 24 h. Figure 6.9 shows TOC (a), TC (b), IC (b) and TN (d) of the samples compared to their initial amount in the influent wastewater. Similar as the COD removal, it can be seen that a HRT of 12 h obtained the optimum performance among all the HRTs tested. The duplicated reactors (i.e., CR2 and CR3) were able to remove 7, 25, 32, 53 and 55% of the
initial TOC at HRTs of 2, 4, 8, 12 and 24 h, respectively, while in the control reactor (i.e. CR1), 2, 35, 26, 32 and 36% of the TOC was removed, respectively. The TOC results confirmed that a HRT of 12 h was the optimum retention time considering the removal rate and the size of the reactor.

Figure 6.9 Analyses operated by TOC-L analyzer in different HRTs. (a) Total Organic Carbon (TOC) (b) Total Carbon (TC) (c) Inorganic Carbon (IC) and (d) Total Nitrogen (TN).

Figure 6.10 shows the differences of MFC reactors in TOC, TC, IC and TN removals in the absence and the presence of PAHs at a HRT of 12 h. As can be seen, unlike COD removal, the duplicated MFC reactors without PAHs (i.e., CR2 and CR3) were able to remove slightly more TOC compared to the CR5 and CR6.
6.3.3 PAH Removal (Continuous Study)

After selecting the HRT of 12 h as the optimum retention time of the continuous study, PAHs removal was studied only at this HRT. Figure 6.11 shows the performance of the MFC reactors in biodegradation of naphthalene (a), acenaphthene (b) and phenanthrene (c). As can be observed, closed-circuit MFCs (i.e., CR5 and CR6) provided a better condition for the removal of all the three PAHs compared to the open-circuit MFC (i.e., CR4). For instance, the duplicated reactors (i.e., CR5 and CR6) were able to remove 12.5±1.6, 14.3±2.2 and 12.0±2.1% of the initial naphthalene, acenaphthene and phenanthrene, respectively, while those of the control reactor (i.e., CR4) were 8.8, 6.8 and 8.0%,
respectively. The results suggested that closed-circuit MFCs could achieve higher PAHs removal. Although it was expected to obtain higher removal rate for acenaphthene than naphthalene due to its more complex structure, less removal was observed.

![Figure 6.11 C/C₀ ratios of PAHs in HRT12. (a) Naphthalene (b) Acenaphthene and (c) Phenanthrene.](image)

6.3.4 Electricity (Continuous Study)

The maximum open circuit voltage (OCV) generated from the MFC reactors were found to be 0.69 V in the CR1 and 0.68 V in the CR4, and the maximum closed-circuit voltage (CCV) were 0.51±0.02 V in the CR2 and CR3 and 0.43±0.01 V in the CR5 and CR6. As can be seen in Figure 6.12, unlike the batch mode, the duplicated reactors in the absence of PAHs produced higher voltage and
consequently, higher current and power densities. This dissimilarity with the batch study can be explained by the operation time of the reactors in each mode. In other words, for the continuous mode (Figure 6.12), the PAHs loading was much higher with a HRT of 12 h compared to that of the batch mode, which had a retention time of 28 days. Therefore, more PAHs could be degraded in the continuous mode and caused a higher electrical performance.

![Electrical performances of the duplicated of single-chambered air-cathode MFC reactors at a HRT of 12 h.](image)

**Figure 6.12** Electrical performances of the duplicated of single-chambered air-cathode MFC reactors at a HRT of 12 h. (a) Voltage (b) Current Density and (c) Power Density.

Figure 6.13 shows the polarization curves (PCs) and the power densities (PDs) of the duplicated reactors in the absence and the presence of PAHs. As it can be seen, unlike the batch mode, reactors without PAHs reached a higher maximum power density (327 mW/m³ in the CR2/3 and 253 mW/m³ in the CR5/6). Internal
resistances (IRs) were calculated based on the slope of linear part of PCs, and were found to be \(42 \, \Omega\) in the CR2 and CR3 and \(38 \, \Omega\) in the CR5 and CR6. Similar to the batch mode, it was observed that there was insignificant difference between the reactors with PAHs and without PAHs. This revealed that in wastewater, the presence of PAHs did not impact much on the power generation by MFCs, unlike the significant difference observed in the bioremediation of PAHs in sediment by SMFCs (Chapters 4 and 5). Another difference found between the batch and continuous phases was the orders of difference in the IRs. Results showed that IRs in the range of \(15 \, \Omega\) in the batch study while it was \(40 \, \Omega\) on average in the continuous study. Thus this suggested that in the batch mode, either protons could be transferred easier or faster anodic kinetic due to more enriched biofilm could be happened.

![Polarization curves (PCs) and power densities (PDs) of duplicated reactors in HRT12.](image)

Figure 6.13 Polarization curves (PCs) and power densities (PDs) of duplicated reactors in HRT12.
6.4 Conclusion

This chapter examined the performance of air-cathode wastewater-MFC reactors in terms of PAHs biodegradation, COD and TOC removals, electricity outputs in the batch and continuous phases. In the batch study, it was found that better electricity output, higher COD and TOC removals could be achieved in the presence of PAHs (i.e., naphthalene, acenaphthene and phenanthrene) in the wastewater. For instance, COD was removed by 91% in the duplicated reactors with PAHs, while it was only 66% in the duplicated reactors without PAHs. In addition, all PAHs were degraded with higher efficiencies in the closed-circuit (1000 Ω) compared to the open-circuit reactor.

In the continuous study, a HRT of 12 h was found to be the optimum hydraulic retention time among 2, 4, 8, 12 and 24 h. Similar to the batch mode, better efficiencies were obtained in the duplicated reactors with PAHs at a HRT of 12 h compared to those without PAHs except electricity output and TOC removal. Electricity (i.e., voltage, power density) generated from the MFC reactors without PAHs was higher.

However, this chapter showed that single-chambered air-cathode MFC reactors used in this study could be used not only for biodegradation of persistent compounds such as PAHs (i.e., naphthalene, acenaphthene and phenanthrene) but also for higher electricity output and removal efficiencies in COD and TOC of wastewater.
Chapter 7 Conclusions, Limitations and Recommendations

The purpose of this study was to investigate the application of electrochemical systems for the treatment of PAH-contaminated sediments and wastewater. The results are divided into three chapters: aerobic-SMFC, anaerobic-SMFC and single-chambered air cathode MFC.

In aerobic-SMFCs, four SMFC reactors in aerobic cathodic condition were implemented to investigate the feasibility of PAHs bioremediation in the contaminated sediment (Chapter 4). Air was constantly supplied to provide enough electron acceptor (oxygen) in the water column. The results revealed significant rates of removal in PAHs (i.e., naphthalene, acenaphthene and phenanthrene) from the sediment. The SMFCs achieved 41.7, 31.4 and 36.2% PAHs removal in an aerobic environment for naphthalene, acenaphthene and phenanthrene, respectively. In addition, this study also showed that SMFCs can increase TOC removal in the sediment. The SMFCs showed 52% TOC removal from the sediment, while it was only 27% for the non-SMFC reactor. This stimulation can be attributed to altering physical and chemical properties of the sediment by applying a potential difference and more activated medium for microorganisms provided by electrochemical systems (Hong et al., 2010). This finding was verified with the result of pyrosequencing analysis that revealed the presence of different microbial communities in the systems with electrodes. These results have shown that aerobic-SMFCs can have major implications for in-situ bioremediation of PAH-contaminated sediment.
It was also shown that the aerobic-SMFC reactors containing PAHs could generate power density as great as the first 10 days for the next 30 days, which confirmed the PAHs degradation in the systems. However, for the control reactor (without PAHs), power density dropped significantly after 10 days. This study has taken the first step of scaling up electrochemical systems (i.e., SMFCs) for both sediment cleaning and power generation. However, it should be noted that although good efficiencies in PAHs removal were achieved by the SMFCs compared to the control reactors, the rate of these removals were still lower than the conventional physical/chemical methods such as advanced oxidation processes (AOPs). Thus further research work is still required to improve PAHs removal by SMFCs such as addition of nutrients to the sediment/soil in order to provide better condition for microorganisms.

Although the results of the aerobic-SMFCs demonstrated the feasibility of bioremediation process, it should be noted that in the real environment, there are many anaerobic zones because the concentration of oxygen is inadequate for the process. Therefore, four anaerobic-SMFCs were constructed to investigate the rate of PAHs removal, TOC removal as well as electricity output and microbial analysis (Chapter 5). Nitrate and sulfate were added as electron acceptors to the water column (cathodic compartment). The results showed the significance effectiveness of electrochemical systems on bioremediation rates (i.e., PAHs and TOC) and also the power output. It was found that 76.9%, 52.5% and 36.8% of naphthalene, acenaphthene and phenanthrene were removed during the process.
Similar to the aerobic-SMFCs, the higher removal rates of bioremediation was due to the stimulation provided by electrodes.

The notable different performances observed in the aerobic and anaerobic phases were largely due to two reasons: first, the different reduction potential of electron acceptors (oxygen in the aerobic and nitrate/sulfate in the anaerobic phases) and second, the different microbial communities involved in the systems. For instance, in the phylum level, *Proteobacteria* comprised between 70-76% of the total sequences in the aerobic phase, while it was between 93-96% in the anaerobic phase. And also in the class level, *β-proteobacteria* was 55.72% in the aerobic phase while it was 76.46% in the anaerobic phase.

In the last part of the study, single-chambered air-cathodes MFCs were constructed to examine the effect of the electrochemical systems on the treatment of PAH-contaminated wastewater (Chapter 6). In the batch study, it was found that better electricity output, higher COD and TOC removals could be achieved in the presence of PAHs (i.e., naphthalene, acenaphthene and phenanthrene) in the wastewater. For instance, COD was removed by 91% in the duplicated reactors with PAHs, while it was only 66% in the duplicated reactors without PAHs. In addition, all PAHs were degraded with higher efficiencies in the closed-circuit (1000 Ω) compared to the open-circuit reactor.

In the continuous study, a HRT of 12 h was found to be the optimum hydraulic retention time among 2, 4, 8, 12 and 24 h. Therefore, similar to the batch mode, better efficiencies were obtained in the duplicated reactors with PAHs at a HRT
of 12 h compared to those without PAHs except electricity output and TOC removal. Electricity (i.e. voltage, power density) generated from the MFC reactors without PAHs was higher.

The results showed that single-chambered air-cathode MFC reactors used in this study could be used not only for biodegradation of persistent compounds such as PAHs (i.e. naphthalene, acenaphthene and phenanthrene) but also for even higher electricity output and removal efficiencies in COD and TOC of wastewater.

In summary, based on all chapters, it can be found that inexpensive electrochemical systems could have significant impacts on removal of persistent compounds such as PAHs. They could be environmental-friendly substitutes to the conventional methods for PAHs removal. However, as mentioned in the scope of this study, optimization and improvement of removal were not central to this study. Thus, further researches are needed to improve the performance of the system. Moreover, electricity generation which is one of primary goals of MFCs was not the priority in this study and all the efforts were concentrated on treatment performances. Therefore, future studies could focus on improving electricity generation simultaneously with bioremediation. For instance, efficiencies (both electrical and biodegradation) could be improved by using different electrode and reactor configurations or even bioaugmentation.
References


Manganese as a Cathodic Reactant. Environmental Science & Technology. 39(12), 4666-4671.


Appendix I

**COD Tube test preparation protocol** inspired from “centre d'expertise d'analyse environnementale du Québec”

**General precaution:** all reagents employed are toxics and extremely corrosives.
Use appropriate PPE (gloves, lab coat, goggles, and covered shoes)

**Preparation of Solutions**

**Solution of digestion**

- High range (100 – 1,000 mg O₂ L⁻¹): in a volumetric flakes of 1 L, add 500 mL of ultrapure water and then dissolve 10.2 g of K₂Cr₂O₇. Then, slowly add 167 mL of H₂SO₄ (concentrated) and 33.3 g of HgSO₄. Shake until complete dissolution, let it cool down and fill it up with ultrapure water.

- Low range (0 – 100 mg O₂ L⁻¹): dilute per 10 the solution from high range by pouring 100 mL of this solution into a volumetric flask of 1 L. Fill it up with ultrapure water.

**Acidic reagent**

- Pour 23.4 g of Ag₂SO₄ into a bottle of 2.5 L (4.25 kg) of concentrated H₂SO₄. Let it mix during 1 - 2 days until Ag₂SO₄ is completely dissolved (don’t forget to shake it just before each use).
N.B.: The solution can be prepared with less sulfuric acid, but still the amount of Ag$_2$SO$_4$ should be 5.5 g per kg of sulfuric acid. The solution needs to be kept in the dark.

**Standard solution**

- H$_2$SO$_4$ (9 N) solution: in a volumetric flask of 1 L, dilute 250 mL of H$_2$SO$_4$ in about 600 mL of ultrapure water. Let it cool down and fill it up with ultrapure water.

- Standard solution of 10,000 mg O$_2$ L$^{-1}$: in a volumetric flask of 250 mL, dissolve 2.125 g Potassium hydrogen phthalate (C$_8$H$_4$KO$_4$) in about 200 mL of ultrapure water and fill it up with ultrapure water.

N.B.: Potassium hydrogen phthalate should be dried in oven before. This solution can be kept 2 years in the fridge at 4°C.

- Standard solution of 1,000 mg O$_2$ L$^{-1}$: in a volumetric flask of 100 mL, pour 10 mL of standard solution at 10,000 mg O$_2$ L$^{-1}$ into about 80 mL of ultrapure water. Add 0.5 mL of H$_2$SO$_4$ (9 N) and fill it up with ultrapure water.

- Standard solution to establish calibration curve of high range (100, 300, 500, 800 and 1,000 mg O$_2$ L$^{-1}$): in volumetric flasks of 100 mL, pour 1, 3, 5, 8 and 10 mL (respectively) of standard solution at 10,000 mg O$_2$ L$^{-1}$ into about 80 mL of ultrapure water. Add 0.5 mL of H$_2$SO$_4$ (9 N) and fill it up with ultrapure water.
Standard solution to establish calibration curve of low range (10, 30, 50, 80 and 100 mg O₂ L⁻¹): in volumetric flasks of 100 mL, pour 1, 3, 5, 8 and 10 mL (respectively) of standard solution at 1 000 mg O₂ L⁻¹ into about 80 mL of ultrapure water. Add 0.5 mL of H₂SO₄ (9 N) and fill it up with ultrapure water.

### Preparation of Tubes

- Add slowly 1.5 mL of digestion solution in each clean tube.

N.B.: Use the appropriate digestion solution to the range that is wanted (high or low).

- Then add slowly 3.5 mL of acidic reagent.
- Then cap the tubes and shake them from left to right (not up to down). Be careful the reaction is extremely exothermic.

N.B.: The tubes can be kept in the dark during 1 year.

### Dosage of Standard Solutions and Samples

- Pour 2.5 mL of standard (or sample) into COD tube. Then cap the tubes and shake them from left to right. Be careful the reaction is extremely exothermic.
- Heat the tubes at 148°C during 2 h.
- Then let cool down the tubes at room temperature.
• Insert the tubes into the DR5000 UV-Vis spectrophotometer (Hach) in order to measure the absorbance.

N.B.: Don’t forget to clean the external tubes before absorbance measurement. Select the appropriate measurement method:
- High range: measurement at 605 nm
- Low range: measurement at 410 nm (programme 344 / “N nitrate HR TNT 30.0 mg/L”)

• Draw the calibration curve (Absorbance as a function of standard concentrations) of low range (linear decrease) and high range (linear increase).

• Calculate the COD values of samples with the calibration curve.
Appendix II

DNA Extraction Protocol instructed by MO BIO Laboratories, Inc.

PowerSoil® DNA Isolation Kit

Prepare Sample
- Add soil sample to PowerSoil® Bead Tube
- Add Solution C1
- Attach to Vortex Adapter
- Vortex

Cell Lysis
- Add Solution C2
- Incubate at 4°C

Centrifuge

Inhibitor Removal Technology®
- Add Solution C3
- Incubate at 4°C

Centrifuge

Bind DNA
- Add Solution C4
- Load into Spin Filter

Centrifuge

Wash
- Wash with Solution C5

Centrifuge

Elute
- Elute with Solution C6

Elute
- Elute with Solution C6

Alternate Protocol for PowerSoil® Midi Spin Filter Adapter

Vacuum

Centrifuge
1. To the PowerBead Tubes provided, add 0.25 grams of soil sample.

2. Gently vortex to mix.

3. Check Solution C1. If Solution C1 is precipitated, heat solution to 60°C until dissolved before use.

4. Add 60 µl of Solution C1 and invert several times or vortex briefly.

5. Secure PowerBead Tubes horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1-24) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.

Note: If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.

6. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x g for 30 seconds at room temperature. CAUTION: Be sure not to exceed 10,000 x g or tubes may break.

7. Transfer the supernatant to a clean 2 ml Collection Tube (provided).

Note: Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.

8. Add 250 µl of Solution C2 and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.

10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean 2 ml Collection Tube (provided).

11. Add 200 µl of Solution C3 and vortex briefly. Incubate at 4°C for 5 minutes.

12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.

13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean 2 ml Collection Tube (provided).

14. Shake to mix Solution C4 before use. Add 1200 µl of Solution C4 to the supernatant and vortex for 5 seconds.

15. Load approximately 675 µl onto a Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Load the remaining supernatant onto the Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature.

**Note:** A total of three loads for each sample processed are required.

16. Add 500 µl of Solution C5 and centrifuge at room temperature for 30 seconds at 10,000 x g.

17. Discard the flow through.
18. Centrifuge again at room temperature for 1 minute at 10,000 x g.

19. Carefully place spin filter in a clean 2 ml Collection Tube (provided). Avoid splashing any Solution C5 onto the Spin Filter.

20. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).

21. Centrifuge at room temperature for 30 seconds at 10,000 x g.

22. Discard the Spin Filter. The DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20°C to -80°C). Solution C6 contains no EDTA.