MECHANISTIC ASPECT OF ELECTROCHEMICAL SIGNAL
IN A MICROBIAL FUEL CELL - BASED BIOSENSOR

by

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ABSTRACT

Due to contamination of effluent from industrial plants by toxic chemicals, the development of rapid and continuous water toxicity monitoring is an important issue. Complex influent flows into wastewater treatment plants (WWTPs) alter the routine operational condition and reduce the efficiency of the treatment process. The objective of this research is to develop a real-time device for monitoring WWTPs. A microbial fuel cell (MFC)-based biosensor is designed to detect the electrochemical signals in response of disruption by contaminants. A MFC-based biosensor can serve as a water quality emergency alarm for preventing harsh water impact. The activated sludge utilized in the experiment has DO concentration 2 ~ 6 mg/L; MLSS ranged from 2500 ~ 3000 mg/L; C: N: P ratio of 100: 5: 1 in nearly 99% COD removal efficiency. In the experiment, a current is generated from substrates in the synthetic wastewater instead of from the microbial metabolism. Of the metals tested Cu(II) contributes the largest current changes relative to metal ion concentration. The redox reaction of substrates is eliminated as a cause. A possible cause with divalent metals is a concentration gradient in which pH provides the most current variation. There is no significant influence of current change corresponding to pH in trivalent metals. Some secondary factors, such as ionic strength and species distribution, are also discussed. Furthermore, theoretical proton concentration variation is calculated and compared with experimental
data to understand the difference between an idea model and realistic situation. The experimental data show two orders of magnitude differences from theoretical values. It is assumed that other impacts, such as sludge interaction and membrane transportation, may influence signal variation. In addition, the current variation curve equation fitted mathematically is not only an index to differentiate metal species, but also can be used to calculate concentration of metal ions. However, the accuracy of detection needs to be enhanced.
Chapter 1
INTRODUCTION

Contamination of natural ecosystem has become more and more serious. The pollutants, including heavy metals, and organic/inorganic contaminants, flow into water sources and cause deleterious harm not only for human health but also for other organisms and the whole environment. With the rapid increase population, the water scarcity issue has affected almost one-fifth of the world’s population. Approximately 500 million people live in areas without sufficient water sources while another 1.6 billion people settle in water shortage regions where lack the water treatment infrastructure to utilize rivers and lakes as water sources.[1,2]

The water scarcity issue results not only from natural but also from human-made effects. The freshwater on the planet is quite sufficient for usage by 7 billion people. The water resources, however, are distributed unevenly and numbers of them are overused, wasted, and unsustainably managed.[3] In order to solve troublesome water shortage affair, people investigate novel water sanitizing technologies. On the other hand, they attempt to improve water treatment efficiency by continuously monitoring water quality in wastewater treatment plants (WWTPs).

There are various components contained in influent of WWTPs. The industrial and municipal wastewater quality fluctuates all the time and influences the operational efficiency of WWTPs. The irregular concentration of organic or inorganic contaminants, such as carbonic substrates and nitrate compounds, would alter the routine operational condition of WWTPs. In addition, other toxins like heavy metal
complexes flowing from industrial areas cause serious damage and irrevocable impact of WWTPs. At present, the majority of wastewater quality monitoring methods, including electrochemical and optical methods, are operated off-site and take a long period of time in analysis.[4–6]

Development of an in-situ and real-time device to monitor WWTPs influent water quality and installment of an instantaneous detection warning alarm are the major goals. For the past decades, biosensors have been studied and applied in some of the practical usage. Many kinds of biosensor based on the biological interaction between living organisms (e.g. algae, daphnia, fish, and microorganisms) and organic/inorganic compounds are designed to detect various parameters in water quality.[7] However, most of the biosensors require an external power supply and specialized equipment that restrict the development of the technique. Furthermore, there is a long response time, which reduces its value as a real-time monitoring application.

In recent years, microorganisms have become popular in toxicity testing. They take advantage of short generation time, cheap cost, and good sensitivity to toxins to be utilized as biosensors.[8,9] Bacteria can be used for toxicity detection by measuring microbial growth rate, bioluminescence intensity, metabolic byproducts concentrations, and other electrochemical signals.[4,10,11]

Microbial fuel cell (MFC) has been widely discussed for a period of time since the global energy crisis. Different from the traditional fuel cells producing electrical power electrochemically through catalytic oxidation of chemical at the anode, MFC uses bacteria instead of metallic catalysts to convert the energy in chemical bonds in organic matter to electrical energy through catalytic reactions of microorganisms.[12,13] The motivation of finding alternative energy sources
reinvigorates interests in MFC as a method to generate electricity or hydrogen gas from biomass without net carbon emission into ecosystem. Unfortunately, the renewable energy applications of MFC are restricted by the low power density level production.

Most of the applications of MFC studies focused on BOD measurement, which had an excellent correlation between BOD concentration in wastewater samples and MFC electrical power output.[14,15] In addition, it has been discovered that the concentrations of biodegradable organic matters in wastewater are proportional to power output of MFC generated by anaerobic electrogenic bacteria.[16] Investigators tested MFC as a biosensor for wastewater quality monitoring since the electrical power output of MFC depends on the microbial activity.[17–19] The toxins in the influent of WWTPs could disturb microbial metabolism and shift the microbial activity. They expected to determine the concentrations of toxins in wastewater by monitoring electrochemical signals generated from microbial metabolisms.

The objective of the research is to develop a real-time sensitive device for monitoring WWTPs by using a MFC-based biosensor to detect current changes with response of metabolic disruption by contaminants. The altered electrochemical signals are employed to determine the inhibition by toxins of microbial activity. The MFC biosensor could be exploited as an emergency alarm for preventing harsh water impact in WWTPs and respond in a short time. In this work, the electrochemical signals produced by MFC biosensor would be further investigated to determine the mechanisms of power generation, including redox reaction, concentration gradient, and pH effect.
Chapter 2

LITERATURE REVIEW

2.1 Biofuel Cell

2.1.1 Introduction

With the increasing demand of energy throughout the world, the global energy crisis has become an urgent issue. It is difficult for the current supply of fossil fuel to fulfill the rising needs for the future. Moreover, the global warming and air pollutions problems urge the development of alternative and renewable energy techniques. Biofuel cells provide one of the solutions to the energy depletion situation.

Biofuel cells are devices that convert biochemical energy to electricity, hydrogen, and other fuel. The growing development of biofuel cells arose from United State spacecraft propulsion power program in the late 1950s. The research designed and fabricated an economical space power supply system which made it possible to use waste disposal to generate power. Between the early 1960s and the late 1970s, the advance of spacecraft power supply performance was impressive.[20] Furthermore, some of the electrical utility companies dedicated themselves to generate electricity by inorganic fuel cells. In Sweden, an electrical generating station used biomass to create hydrogen by an inorganic fuel cell reacting with oxygen.[21] The improved technique was also applied in medical treatment. In the late 1970s, the biofuel cell began to be utilize as an in vivo power supply for artificial heart or cardiac pacemakers.[22]
For many years, several limitations, such as high cost and extreme temperature conditions, hindered the usage of enzymes and microorganisms as catalysts in biofuel cells. Widespread research on biocatalysts solved problems of high operating cost and improved the performance under mild conditions. A number of reviews on bio-electrochemistry have been summarized.[23–27] Diverse applications of biofuel cells have appeared in wastewater remediation process, BOD sensor, micro-scale devices, and other utilizations.[15,26,28–30]

2.1.2 Classification of Biofuel Cell

There are diverse types of biofuel cell design with different metabolisms for energy generation. The major types of biofuel cells are defined by the different types of biocatalysts and distinct cell formats. The three main categories of biofuels are showed below:

1. Biofuel cells convert organic fuel to electricity by enzymes.
2. Biofuel cells convert organic fuel to electricity by microorganisms.
3. Biofuel cells generate electricity from sunlight by combination with the photochemical system.

There are some other types of fuel cells that utilize organic wastes, such as agricultural byproducts and animal sewage, as primary fuel to produce substrates like hydrogen or ethanol. These secondary fuels are then used to work with conventional biofuel cells.[31–33] In the absence of direct generation of power by bio-electrochemical technique, these types of fuel cells hardly fall within the scope of discussion.
2.1.2.1 Enzyme-based Biofuel Cell

The concept of enzymatic biofuel cells have been known for a half century since the first enzymatic biofuel cell was demonstrated in 1964 with glucose as the substrate fuel and glucose oxidase and D-amino acid oxidase as the biocatalysts.[34] This inspired a wide range of researches for enzymatic fuel cells. [23,35]

As the name implies, enzymatic biofuel cells employ enzymes to catalyze the oxidation of the fuel. The benefit of using enzymes as catalysts is that enzymes are natural catalysts which are cheaper and more environmentally friendly than conventional metallic catalysts. There is no need for contaminants treatment. However, the development of enzymatic biofuel cells are still restricted by short lifetimes, stability, poor power densities, and low efficiency of oxidation of fuels. Most of the enzymes in human bodies live only a few days. The lifetime should be enhanced for more than months or years in practical applications. In addition, enzymes that flow around the cells cannot easily transfer electrons to electrode surface. For using enzymes as catalysts in a fuel cell, they must be immobilized to improve the cell efficiency. Furthermore, adding mediators boosts the performance of fuel cell by helping electrons transportation but how to hold mediators in a continuous feeding biofuel cell is a big challenge.[34,36,37]

During the last decade, much research and momentous improvements have been achieved in solving the problems of short lifetime and low power density. One strategy is to immobilize and stabilize enzymes on the electrode surface. With the recent progresses in nanotechnology development, large surface areas providing enzyme attachment from nanostructure materials can increase enzyme loading and facilitate reaction kinetics while improving power generation.[36,38] Nanoparticles, nanotubes, nanofibers, and other nanomaterials have been broadly examined as carriers
for enzymatic catalysts immobilization. [39–42] Furthermore, some of the researches stabilize and immobilize enzyme by utilizing micellar polymers. With micellar polymers, the lifetime of enzymes increase from a few hours to a few weeks, even to one year long. [43,44]

Although many efforts have been made in addressing the problems of poor power density and short lifetime, the enzymatic biofuel cells still have difficulties in application as a power generation device. Most of the researches of enzymatic biofuel cells focus on special applications, such as sensors, portable power supplies, and in vivo implantable devices. [26,45,46] Despite the fact that several drawbacks have still restricted enzymatic biofuel cells for practical applications, they are a promising technology with renewable power and will play an essential role in energy market for the future by better understanding and improvement.

2.1.2.2 Microorganism-based Biofuel Cell

Because of the limitation in enzymatic biofuel cells application, many studies in microorganism-based biofuel cells have been made for several decades. There are three considerable advantages of using microorganisms as catalysts. The first benefit is that microorganism can remain much longer than enzyme does under normal operating condition and thereby solve the problem of short lifetime in enzyme biofuel cells. Another advantage of using bacteria as catalysts is that they could accelerate the chemical reaction more completely than enzyme does. More kinds of biofuels can be oxidized easily by cells than by enzymes and this improves the power generation. Moreover, microorganisms are less sensitive to contaminants than enzymes which makes microbial biofuel cells much popular in practical applications. [30,47]
The primary shortcoming of using microorganisms as catalysts for power generation is that microorganisms could hardly transport electrons produced by metabolism or fermentation from the inner cell to the outer electrode surface. Numerous reviews have been published for improving electrons transfer across the cell membranes.[17,25,47,48] The electron-transfer mediator addition is one of the feasible methods. The electrochemical mediator molecules carry electrons between microbial cells and electrode system.[8,49,50] Nonetheless, the chosen mediators using in microbial biofuel cells must be able to travel through microbial cell membranes and be harmless to the microorganisms. Many specific kinds of microorganisms, such as iron reducing bacteria, have been isolated which could directly transfer electrons to electrode.[51,52] These studies show that some bacterial strains can transfer electron without the aid of mediators.

It is believed that mediator-less microbial fuel cell could be improved by modifying other operating parameters like electrode materials, biofuels, and microbial species. For example, microbial cells have been immobilized on the electrode surface to enhance cell efficiency.[53] Instead of bacterial immobilization, other researches focus on mediator immobilization on the anode.[54] There are more recent reports of using mixed microbial communities as catalysts to improve power output.[55] A further study combines microorganisms with enzymes together in the catalysis system.[56] Diverse alternatives to the earlier system have advanced microbial fuel cells technology to a higher level and still improve the performance of the cell efficiency.
2.1.2.3 Photoelectrochemical Biofuel Cell

Photosynthesis cells transfer light energy to electrochemical energy and finally to chemical potential energy stored in chemical bonds. The traditional photosynthesis process utilizes photovoltaic materials, such as dye-sensitized or semiconductor-based solar cells. Some alternatives to conventional solar cells have been developed to use enzyme, or even microorganisms to catalyze the oxidation reaction. These kinds of cells combined photosynthetic approaches and biofuel cell mechanism are called photoelectrochemical biofuel cells.

![Diagram of enzyme-based photoelectrochemical biofuel cell](image)

**Figure 2.1** Schematic diagram of enzyme-based photoelectrochemical biofuel cell adapted from [57].

Many of the applications have been published with the link between photovoltaic and biofuel cells. The cyanobacteria were immobilized on an electrode surface and catalyze the reaction of water oxidation. The generated electrons could be transferred from the anode through an external circuit to the cathode and made available for the
reduction reaction of oxygen. The light conversion efficiency reached up to 2-2.5% upon the illumination of 30-40 μW/cm².[58] Instead of microorganisms, photosynthetic cells working in combination with enzyme-catalyzed biofuel cells have also been reported in the recent year. Photoreaction occurred at the photoanode and converted NADPH to NADP⁺. NADP⁺ then be reduced and gains electrons by oxidizing organic compounds. The process yields oxidized fuel and hydrogen ions which could pass through the ion-permeable membrane between two-compartment cells and yield electrical power. [57]

2.2 Microbial Fuel Cell

The requirement of alternative energy sources is urgent. Due to unsustainable fossil fuels and increasingly severe global warming issues, the interest in renewable energy technologies has been extensively raised. The development of microbial fuel cell (MFC) has been investigated for decades and being selected as one of the top 50 best inventions for 2009 by Time Magazine.[59] MFC technology provides energy in the form of electricity by microbial metabolism which breaks the limitation of using only purified fuel to generate electrical power. The following sections based on review articles show the fundamentals of MFC, MFC reactor design, factors that influence the efficiency of MFC, currently use and limitations and potential future applications.

2.2.1 Principle of MFC

The original concept of the biofuel cell traces back to 1780s. Galvani’s experiments discovered the dead frog’s leg muscles twitched when contacting with a static electricity generator and inspired the studies in bioelectricity and nervous system in organisms.[60] A long period time after Galvani’s work, Grove made a fuel cell
which successfully recombined oxygen and hydrogen to generate water and electrical current in 1839.[61] Until 1910, the use of microorganisms in a biofuel cell was established. Potter, a professor in University of Durham, revealed that the microorganisms could produce a little amount of electricity.[62] Twenty years after Potter’s studies, Cohen developed a microbial fuel cell that could generate an electrical voltage higher than 35 volts based on the principles demonstrated by Potter.[63] The dramatic development of MFC technology was prompted by space program in late 1950s and applied in spacecraft for power generation by waste disposal in MFC system. The currently researches of MFC are focusing on improvement of power density output and further applications than producing electrical energy.

Unlike traditional fuel cells that produce electricity electrochemically through catalytic oxidation of chemicals at the anode, MFCs use microorganisms as a catalyst instead of a metallic catalyst to generate electricity. MFCs could convert chemical energy in chemical bonds of organic compounds to electrical power through catalytic reactions of bacteria. Microorganisms play a role as catalyst for release of electrons from organic matters and move them to different electron carriers. [11,25,64] There are several mechanisms of electron transfer, such as direct electro transfer (DET) in the tricarboxylic acid (TCA) cycle. The oxidation of organic fuel produces electrons that are transported through metabolic pathway in the microbial cells. Electrons obtained from organic fuel oxidation move across the inner membrane, periplasm and outer membrane. At the end of the respiratory chain, a final enzyme in the microbial cell interacts with the electrode to connect the internal biological circuit to the electric circuit and convert potential energy into electrons that flow in the external circuit for power generation.[11,65]
2.2.2 Structure of MFC

The MFC consists of an anode that accepts electrons from organic matter oxidation by microorganisms, and a cathode that converts electrons to electron acceptors. In a conventional MFC device, it consists of two chambers that are separated by a cation exchange system, typically proton exchange membrane for the most general practical application. Due to complex designs of two-compartment reactor, a one-compartment MFC omits the cathodic chamber by exposing the cathode electrode straight to the air [11,13,25,66].

Two-chamber MFC design has a widely used reactor built in an H shape. A traditional two-compartment MFC contains an anodic chamber and a cathodic chamber, divided by a proton permeable membrane. The membrane allows protons to pass through and restricts organic biomass diffusion. The anodic chamber is filled with
microorganisms and biofuel and yields electrons after oxidation. Electrons flow from anode to cathode through an external circuit. The reactors can be designed in diverse practical shapes to optimize the MFC system. Up-flow style with cylindrical shape reactor has been invented to scale up easily in practical application in wastewater treatment plant.[67] The miniature size of MFC is originated to enhance power density within 2cm-diameter reactor.[68]

![Diagram](image)

**Figure 2.3** Schematics of three types of single-compartment MFC adapted from [17,69,70].

There are some difficulties to expand two-compartment MFCs in real applications. Therefore, a simple design that eliminates the need for the cathodic chamber has been fabricated. A porous air-cathode is designed for proton transfer from electrolyte in anodic chamber (figure 2.3 (a)).[69] Another innovation combines proton exchange membrane with carbon cloth cathode. A multi-functional cathode is designed to simplify MFC structure (figure 2.3 (b)).[17,71] Furthermore, one type of single-chamber MFC associates both anode and cathode in a single chamber. A proton exchange membrane is merged with a plastic tube to build air-porous cathode that is surrounded by numerous anodes (figure 2.3 (c)).[70]
2.2.3 Factors

In order overcome electrochemical barriers and optimize the power generation efficiency, the design of MFC reactors has been altered with many distinct materials and the systems are operated under various configurations. The difference of operating conditions includes pH, temperature, microbial communities, biofuel source, electrode material, membrane type, and whether mediator addition or not. The effect of main factors would be discussed respectively in the following sections.

2.2.3.1 Microorganism

It has been know over one hundred years that microorganisms could produce electrical power since Potter discovered *Escherichia coli* and *Saccharomyces* yields electricity with platinum electrode.[62] The interest in MFC power generation was boosted enormously since the discovery in the 1960s that addition of exogenous mediators enhances the current density and power output extremely.[19] Electron mediators solve the problem of inhibition in the transfer of electrons from cell membranes to electrode since the microbial outer membrane constitutes by peptidoglycans and lipopolysaccharides.

However, some drawbacks in using mediators to improve MFC power generation efficiency. Most exogenous mediators are chemical compounds synthesized in the laboratory such as methylene blue, natural red, thionin, and EDTA.[53,69] The toxicity and instability of typical mediators hinders their use in MFC systems.
<table>
<thead>
<tr>
<th>Year</th>
<th>Microorganism</th>
<th>Application</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1985</td>
<td><em>Proteus mirabilis</em></td>
<td>Thionin as mediator</td>
<td>[72]</td>
</tr>
<tr>
<td>1987</td>
<td><em>Streptococcus lactis</em></td>
<td>Ferric chelate complex as mediators</td>
<td>[73]</td>
</tr>
<tr>
<td>1987</td>
<td><em>Lactobacillus plantarum</em></td>
<td>Ferric chelate complex as mediators</td>
<td>[73]</td>
</tr>
<tr>
<td>1987</td>
<td><em>Erwinia dissolven</em></td>
<td>Ferric chelate complex as mediators</td>
<td>[73]</td>
</tr>
<tr>
<td>1999</td>
<td><em>Shewanella putrefaciens</em></td>
<td>Mediator-less MFC</td>
<td>[74]</td>
</tr>
<tr>
<td>1999</td>
<td><em>Actinobacillus succinogenes</em></td>
<td>Neutral red or thionin as electron mediator</td>
<td>[75]</td>
</tr>
<tr>
<td>2001</td>
<td><em>Clostridium butyricum</em></td>
<td>First Gram-positive fermentative bacterium</td>
<td>[76]</td>
</tr>
<tr>
<td>2002</td>
<td><em>Desulfuromonas acetoxidans</em></td>
<td>Identified in a sediment MFC community</td>
<td>[77]</td>
</tr>
<tr>
<td>2003</td>
<td><em>Aeromonas hydrophila</em></td>
<td>Mediator-less MFC</td>
<td>[78]</td>
</tr>
<tr>
<td>2003</td>
<td><em>Geobacter sulfurreducens</em></td>
<td>Generate electricity without poised electrode</td>
<td>[79]</td>
</tr>
<tr>
<td>2003</td>
<td><em>Rhodoferax ferrireducens</em></td>
<td>Glucose and xylose as substrate, mediator-less MFC</td>
<td>[80]</td>
</tr>
<tr>
<td>2004</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Pyocyanin and phenazine-1-carboxamide as mediator</td>
<td>[81]</td>
</tr>
<tr>
<td>2005</td>
<td><em>Geothrix fermentans</em></td>
<td>Produced an unidentified mediator</td>
<td>[82]</td>
</tr>
<tr>
<td>2005</td>
<td><em>Geobacter metallireducens</em></td>
<td>Generate electricity in a poised potential system</td>
<td>[83]</td>
</tr>
<tr>
<td>2005</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>2-hydroxy-1,4-naphthoquinone (HQD) as mediator</td>
<td>[84]</td>
</tr>
<tr>
<td>2006</td>
<td><em>Shewanella oneidensis</em></td>
<td>Anthraquinone-2, 6-disulfonate (AQDS) as mediator</td>
<td>[68]</td>
</tr>
<tr>
<td>2006</td>
<td><em>Escherichia coli</em></td>
<td>Methylene blue as mediator</td>
<td>[85]</td>
</tr>
<tr>
<td>2008</td>
<td><em>Desulfovibrio desulfuricans</em></td>
<td>Reduced sulphate/sulphide in lactate substrate</td>
<td>[86]</td>
</tr>
<tr>
<td>2011</td>
<td><em>Comamonas testosteroni</em></td>
<td>Mediator-less MFC</td>
<td>[87]</td>
</tr>
<tr>
<td>2011</td>
<td><em>Clostridium ljungdahillii</em></td>
<td>Reduced carbon dioxide to multicarbon</td>
<td>[88]</td>
</tr>
<tr>
<td>2012</td>
<td><em>Eubacterium innosum</em></td>
<td>Sediment-type MFC</td>
<td>[89]</td>
</tr>
<tr>
<td>2014</td>
<td><em>β,γ-Proteobacteria</em></td>
<td>Air-cathode chamber, potato wastewater as substrate</td>
<td>[90]</td>
</tr>
</tbody>
</table>

An important advance was made at the end of 20th century when dissimilatory metal reduction bacteria were explored for electron transfer from cell membrane to electrode directly in the absence of exogenous mediators.[16,74] Other studies showed many pure cultures in anodic biofilms could generate high power efficiency. Although *Shewanella putrefaciens* was found by Kim et al. in 1999, various *Geobacter* strains are used in MFC, such as *Geobacteraceae sulfurreducens* and *Geobacter*
metallireducens.[74,79,83] Chaudhuri et al. and Pham et al. demonstrated Rhodoferax ferrireducens and Aeromonas hydrophila strains could also form biofilms on anodic surface and bioelectrochemically transfer electrons straight from membrane to electrode.[78,80]

In the past two decades, a huge number of studies published the description of microorganisms which have ability to produce electrical current by consuming organic biofuel. A list of microbes used in MFC shown in table 2.1 Some of these microbes that have ability to self-mediate transfer electrons extracellularly are called exoelectrogens or electricigen.[19] Traditional cellular respiration uses oxygen as the final electron acceptor in the electron transport chain (ETC). However, the final electron acceptor of an exoelectrogen can be solid mineral oxides or strong oxidizing agents in the aqueous solution, which means the anode can act as the final electron acceptor just like the solid mineral oxides and transfer electrons with various intracellular components and outer-membrane cytochromes from cell to anode in MFC.[52,91,92]

Most of the mediator-less MFCs are conducted with dissimilatory metal reducing microorganisms which are mainly associated with the classification of Geobacter, Shewanella, and Rhodoferax.[74,79,80,83] An exception of mediator-less MFC was shown with Clostridium butyricum that ferment glucose to acetate, CO₂, or other products.[76] The bulk of MFCs are operated with extracellular mediators which shuttle between microorganisms and electrode. Electron mediator addition would also enhance performance of MFC in dissimilatory metal reducing bacteria.[69] Some of the microbes produce intercellular mediators by themselves. For instance, Pseudomonas aeruginosa yields pyocyanin and phenazine-1-carboxamide for assisting electron transportation.[81,93]
Understanding how microbial ecology works in electrical power generation is a key to optimize the performance of MFC system. From pure culture to complex bacterial community, an in depth study of the electron transfer mechanisms between microorganisms and electrode would help us better recognize MFC issue. The small microbes might open a great door to a sustainable energy generation.

2.2.3.2 Substrate

Substrate is one of the significant biological factors that influence the electricity generation performance of MFC since organic biofuel provides not only nutrient source but also energy supply. To optimize MFC efficiency, various organic substrates have been tested. Substrate determines not only the dominant microbial community in anodic chamber, but also the power generation performance of MFC.[94] The energy productivity depends on the substrate source, concentration of organics, and degree of substrate oxidation.[95] To obtain more energy from organic substrate, the substrate should be oxidized to carbon dioxide completely with valuable electron transfer to electrode surface.

It’s hard to compare electricity efficiency with a variety of substrates since there are many operating conditions in different studies. The performance of MFC depends on the amount of microbial communities, the mass transfer between microorganisms and substrates, the bacterial kinetics (including the maximum specific growth rate, \( \mu_{\text{max}} \) and the bacterial affinity constant for the substrate, \( K_s \)), and the biomass loading rate.[55] The following paragraphs discuss some substrates commonly used in MFC.
2.2.3.2.1 Acetate

Acetate is the most common substrate utilized in MFC. It plays a role as carbon source to encourage electro-active microorganisms. The reason why acetate is generally used in MFC is that it is the end product of many microbial metabolic pathways for high molecular weight compounds.[77] Moreover, its inertness towards fermentations and conversions make it widely used.[65] In the MFC power generation performance, acetate is predominant than other simple substrates, like butyrate, propionate, and glucose. Acetate also has better power generation efficiency than complex substrates. However, the complex substrates provide more diverse microbial communities which improve the completion of oxidizing substrates.[65,96]

2.2.3.2.2 Glucose

Glucose is another popular substrate applied in MFCs system. In recent years, several studies reported that MFC had limited power output with glucose feeding as a substrate. The low coulombic efficiency results from the fact that glucose is a fermentable substrate which being consumed by diverse competitive metabolic pathways such as fermentation and biomethanation that hardly to produce electricity.[72,97] Nevertheless, glucose degradation will generate diverse byproducts, such as acetate and lactate, which microorganisms could exploit as nutrient and energy sources.

2.2.3.2.3 Lignocellulosic Biomass

Even though lingo-cellulosic biomass to be viewed as a promising feedstock, it cannot be utilized directly. Lignocellulosic biomass should be transferred to some low molecule weight compounds first such as monosaccharide that is a good source for power generation.[98] Some of the researches indicated that electricity power output
from consumption of lignocellulosic biomass is much less than that with glucose.[99] It means that lignocellulosic biomass is more difficult to utilize for power generation than glucose.[65,98]

2.2.3.2.4 Synthetic wastewater

Using wastewater as substrates could not only generate electrical power, but also accomplish wastewater treatment simultaneously. Several reviews used synthetic wastewater as substrates because they can change loading strength, conductivity and pH easily.[100,101] Some wastewater contains numerous electron donors such as reduced sulfur compounds that can enhance the performance of MFCs.[86] Some studies show that feeding biodegradable substrates slowly was more efficient probably due to the intermediates can increase the electricity generation dramatically.[96,97,101]

In the early stage, simple substrates such as acetate and glucose were widely used in MFC; recently, however, more and more researchers try other unconventional substrates with an aim of utilizing waste biomass or treating wastewater on one hand and generate more electrical power by MFC in the other.[102] Further studies utilized phenol and sulfide as single substrates with anodic oxidation and also decreased the toxicity of the target substrate.[103] Diverse substrates have been used in MFC for power generation as well as wastewater and toxicity treatment. It is promising that a greater variety of substrates will be utilized with the development of the technology in the coming years and substitute the current bioenergy that produced from food productions. The enhanced MFC system will generate a sustainable energy with wastes and renewable biomass.
2.2.3.3 Electrode

The structure and material of electrode could largely influence in electricity performance. To improve the efficiency of MFC, the electrode selection must be considered seriously. Different electrodes determine electrical conductivity, microbial compatibility, chemical stability and substrate oxidation. Both anode and cathode will be discussed respectively in the following parts.

2.2.3.3.1 Anode

There are many kinds of material for making anode. The most popular electrode material is carbon due to its high electrical conductivity, considerable surface area, and microbial culture friendly. The structure of carbon consists of graphite plate, graphite felt, graphite rod, granule, carbon fiber, carbon cloth, and carbon foam.[54,69,71,86] The most prevalent material for anodic electrode is plain graphite plate and graphite rod which is relatively cheaper, stable and has large surface area.

To improve the electrical generation efficiency, various approaches have been evolved. In some studies, anode was incorporated graphite, metal, and electron mediator to increase the performance of anode. Park et al. used composited graphite that associated with metal (trivalent iron) and mediator compounds (neutral red) which could enhance current production obviously.[104] Despite metal and mediator modification, conductive polymers have been exploited in anode construction. Among the polymers used in anode modification, polyaniline (PANI) is one of the most studied due to high electrical conductivity. Pt-Ru/PANI composites[105], TiO₂/NAPI composites[106], fluorinated PANI [107]and carbon nanotube/NAPI composites[108] are reported to produce greater current density. Another review demonstrated that gold electrode assembled with alkanethiol self-assembled monolayer would enhance
electron transfer which related to the length and functional groups of monolayer molecules.[109] Moreover, carbon cloth anode and phosphate buffer treatment with ammonia gas was reported to improve the current production in MFC.[110] In some cases, the growth of power density is corresponded to the water flow direction in continuous flow system. Changing flow direction eliminates the oxygen diffusion and helps proton transportation in some cases.[111]

### 2.2.3.3.2 Cathode

In the cathodic compartment, oxygen serves as the most dominant electron acceptor in MFC system since oxygen is sustainable, free cost, high oxidation potential (figure 2.4) and nontoxic with end product. However, oxygen reduction reaction is the main rate limiting-step of MFC power generation.[112] To improve the catalytic activity, the application of coating catalysts in cathode is necessary for accelerating the rate of oxygen reduction reaction. Platinum catalysts are commonly used in dissolved oxygen process and air-cathode system.[70,113] Platinum, however, cannot practically be operated to real applications due to expensive cost of noble metal. Alternative electron acceptors were examined to lower the cost, such as manganese dioxide[84], lead dioxide[114], and cobalt tetramethoxyphenylporphyrin (CoTMPP)[115]. These noble-metal free catalysts have high catalytic activity which minimize the amount of platinum used and the researches showed that the difference of performance is negligible.[115,116] Nonetheless, the cobalt modification should be considered carefully since cobalt metal is not environmentally friendly.
**Figure 2.4** The redox potential of electron donors and electron acceptors at pH 7 adapted from [117].

Exclusive of oxygen as an electron acceptor, several chemical compounds have been explored in improvement of MFC. According to its low overpotential in cathode and faster rate kinetics than oxygen reduction reaction, ferricyanide is one of the most popular electron acceptors in experiments [69] Some studies reported that ferricyanide generated higher power output than a dissolved oxygen cathode as a result of low energy loss [68,118,119] Unfortunately, the disadvantages of using ferricyanides as electron acceptors make it difficult to scale-up in practical application. The reoxidation by oxygen is insufficient, which costs more for regeneration of
ferricyanide. In addition, the leakage of ferricyanide through cation exchange membrane to anodic chamber decreases the power output.[120]

In addition to abiotic cathodes, microorganisms can also serve as catalysts in cathode. The advantages of biocathodes cause widely used in MFC. First, biocathodes could lower the operation expense because other metal and artificial catalysts are not required. In addition, the products produced by microbial metabolisms in biocathodes can be utilized. For example, algae yielded oxygen by photosynthesis and reduced the cost of oxygen supply.[121] Biocathodes could also remove the contaminants at the mean time of cathode reaction. The application was used in wastewater treatment with denitrification. The biocathode used denitrifying bacteria to reduce nitrate to nitrogen gas that gains the additional value of MFC system.[122]

2.2.3.4 Membrane

Most of the MFCs require a proton exchange membrane to separate the anode and the cathode chamber. Generally, the amounts of protons produced in the anodic chamber are totally the same as generated electrons. However, the transfer rate is not the case. Electrons transported from anode to cathode based on the potential gradient. On the other hand, protons migrate to the cathode merely by diffusion that is much slower than electron transfer.[17,28] The different transfer rate leads to a proton transfer rate through the membrane that is lower than the proton consumption rate in the cathodic chamber. Therefore, proton migration significantly affects the internal resistance of MFC.[123]

The most popular proton exchange membrane used in MFC is Nafion (Dupont™, Wilmington, DE) due to its favorable selectivity of proton permeability. Despite several researchers attempts to utilize more cost-effective membranes, such as Ultrex
CMI-7000 (Membranes International Inc., Ringwood, NJ) and pressed carbon paper, Nafion is still the first preference for current generation.[17,81] However, the competition of other cations influencing the performance of proton exchange membrane is inescapable. Typically, the concentration of these cations (mainly Na$^+$, K$^+$, NH$_4^+$, Ca$^{2+}$, Mg$^{2+}$) is five orders of magnitude higher than proton concentration in electrolytes at neutral pH condition, which leads to the obstacle of proton transportation.[112,124] Rozendal et al. explored the effect of cation transfer in Nafion$^\circledR$117 and discovered that roughly 74% of sulfonate residues were occupied by Na$^+$ and K$^+$. They also reported that the total numbers of positive charges of cations transferred was equal to the number of electrons transferred through external circuit, which demonstrated that the cations were transferred mainly by electroneutrality rather than concentration gradient force.[124] Because of the barrier of proton migration to cathode, the anodic chamber becomes acid with the accumulation of proton, whereas the cathodic chamber turns alkaline. This phenomenon results in anode potential enhancement and cathode potential decline, which lower the current output from MFC.[17,28] To avoid the pH difference between two compartments, using a low cation concentration electrolyte or adding phosphate buffer to restrain pH variation is a feasible scheme.[28,113]

In addition to the type of proton exchange membrane and the cation concentration of electrolyte, the ratio of membrane surface area to chamber volume and the distance between two electrodes would also influence the proton transfer efficiency. Oh and Logan established that larger surface area caused lower internal resistance in MFC.[125] Reviews showed that the performance became better when two electrodes are placed as close as possible.[113,126]
Other alternatives, such as using salt bridge substitutes proton exchange membrane, have been revealed. The salt bridge MFC compared with proton exchange membrane, however, produces lower power output because of high internal resistance observed.[64,83] Grzebyk and Pozniak synthesized interpolymer cation exchange membranes from the system polyethylene/poly(styrene-co-divinylbenzene) [PE/poly(St-co-DVB)] by sulfonation with a solution of chlorosulfonic acid in 1,2-dichloroethane.[127] Furthermore, Park and Zeikus prepared porcelain septum by kaolin.[69] Membrane-less MFC is also an advantageous choice to solve the membrane block problem.[113,126]

2.2.3.5 Mediator

MFC can be categorized into two general groups, mediator MFC and mediator-less MFC. Most of the microorganisms are electrochemically inactive, which means they cannot transfer electrons form the bacteria to the outer-membrane. The first MFC developed in the early 20th century was mediator MFC.[62] The mediator-less MFC did not been originate until the 1970s when dissimilatory metal reduction bacteria were discovered.[52]

Electron mediators play an important role in electron transportation for those microorganisms that are incapable migrate electron to the anode. Most of the mediators are chemical compounds which carry electrons generated from substrate oxidation to electrodes.[75,77] Electron mediators could easily be reduced and capture electrons in an oxidized state by penetrating bacterial membrane. Then the reduced mediators, which are also membrane permeable, diffuse out of the bacterial membrane and are re-oxidized to oxidized state by electron discharge to the electrode surface.
These sequential procedures increase the electron transfer rate and also enhance the power generation efficiency.

The selection of sufficient electron redox mediators is significant for MFC performance. Redox mediators must possess a redox potential that is positive enough compared to the intracellular electron carriers (such as NADH and reduced cytochromes) to grab electrons from them; on the other hand, the mediators potential should be negative enough compared to the anode to be re-oxidized at the electrode surface. In addition, mediators should be membrane permeable, soluble in electrolyte, and biodegradable to microorganisms.[128] Traditional synthetic mediators used in MFC systems contain neutral red, methylene blue, thionine, 2-hydroxy-1, 4-naphthoquinone (HNQ) and anthraquinone-2, 6- disulfonate (AQDS). However, these synthetic mediators are not stable and are regard as toxicants for microorganisms that hinder their utilization in MFC.[53,54,73,128] Instead of using synthetic mediators for electron transfer, several natural compounds (such as humic acid and sulphur) are demonstrated to have ability of electron transfer from cell membrane to electrode surface.[129]

The concept of medaitor-less MFC has been announced since Lovley determined dissimilatory metal reduction bacteria have the ability to transfer electrons extracellularly to the electrode.[52,92,129] Not until Kim et al. used Shewanella putrefaciens as catalysts for electricity generation, mediator-less MFC have been widely studied.[74] There are three main mechanisms of electron transfer in mediator-less MFC: (1) direct connection by outer membrane associated cytochromes; (2) building specialized appendages described as a conductive pili to link with electrode; (3) using endogenous secreted or excreted mediators from the microorganisms themselves.[92,130] Figure 2.5 shows three types of microorganisms transfer electrons
at an anode surface. At the beginning, dissimilatory metal reducing bacteria were
determine to transfer electrons by direct contact and exerted mediator
(shuttles).[51,93] With the advanced studies, a third mechanism has been
demonstrated with discovering of nanowire produced by *Shewanella oneidensis*.[131]
To improve the efficiency of MFC, better understanding the mechanisms of electron
transfer is necessary.

![Diagram of electron transfer](image)

**Figure 2.5** Schematic of diverse redox mediators transfer electrons between
bioelectrochemical active microorganism and anode adapted from [13].
2.2.4 Application of MFC

With the better understanding of how microorganisms transfer electrons and improvement of MFC operational system, more and more profitable applications are performed in practice. MFCs have been a promising technology for sustainable energy generation. The most practical application is producing electricity in conjunction with treating wastewater. Other useful applications of MFC have appeared with specialized modifications.

2.2.4.1 Electricity Generation

Due to the huge electricity requirement in water and wastewater infrastructure, many efforts have been made to reduce the cost in energy supply. In U.S., roughly 4% of whole electricity produced is utilized for the treatment process of water and
wastewater, and it costs approximately $25 billion annually in water and wastewater treatment. [132] Therefore, the replacement of using MFC in wastewater treatment makes the operation more economical. The most frequent and favorable applications of MFC power generation occur in wastewater treatment. The energy recovery at wastewater treatment plant contributes not only to reduction of process cost but also to energy sustainability. However, how to scale-up the system into conventional plant treatment unit in a cost-effective way is a critical concern.

The advantage of power generation by MFC system is that chemical energy from microbial metabolism can directly be converted to electricity without heat loss. [80] Unfortunately, the power density produced by MFC is excessively low to apply in general power output. Therefore, some small devices with little power demand are achievable. The MFC could be used to provide electricity for automatic robot’s energy supply [29], and apply in remote-area sensing device, such as sediment MFC harvests power from organic matters in sediments. [133]

2.2.4.2 Wastewater Treatment

Substrate is cost-free from wastewater and it advances the development of MFC technology. It is estimated that a domestic wastewater treatment plant contains 9.3 times higher energy than it used in general aeration process treatment. [134] The application of alternatives, such as air-cathode utilizing and anaerobic method, could not only eliminate the air demand but also decrease the amount of disposal sludge. [135]

The MFC system has been used in wastewater treatment process since 1991. [136] In MFC wastewater treatment system, most of the organic can be consumed. Organic
molecules, such as acetate and butyrate, can be degraded to harmless carbon dioxide and water. Some specific chemical compounds could be removed through the MFC treatment process. For example, sulfide compounds are widespread in organic wastewater system. MFC could convert dissolved sulfide to elemental sulfur with particular microorganisms.[137] In addition to easily metabolized organic contaminants, several xenobiotics, such as nitrilotriacetic acid (NTA), are degraded under MFC conditions.[138] Furthermore, MFC treatment could produce 50 to 90% less sludge disposal.[139]

### 2.2.4.3 Hydrogen Gas Generation

Instead of producing electrical power, it is possible to generate hydrogen gas directly at the cathode of modified MFC with organic matter oxidation.[18,140] Typically protons generated on anode compartment would bind with oxygen and form water. In hydrogen production process, protons and electrons released at the anode are recombined together to create hydrogen gas at cathode with additional potential of 0.11V supply.[18] Even though it requires excess voltage, hydrogen gas generation by MFC is still more efficient than direct water electrolysis and traditional bacterial fermentation. As the studies mentioned, approximately 8-9 moles of hydrogen gas could be generated with glucose oxidation, which first gains 2-3 moles acetate per mole glucose at fermentation stage and converts to 2.9 moles of hydrogen gas per acetate with bio-electrochemically assisted microbial reactor (BEAMR) process.[140] The benefit of producing hydrogen gas rather than electrical power is that hydrogen gas can be concentrated and collected for future use.[139]
2.2.4.4 Biosensor

MFC is a beneficial tool to understand the environmental conditions by natural response monitoring and data analysis since it can generate power to maintain continuous monitoring in-situ, especially in sediment and deep oceanic area where have difficulties to replace batteries. The microbial biosensors are commonly used in evaluation of Biochemical Oxygen Demand (BOD) in water sample.[15,141] The first BOD sensor used by MFC demonstrated in 1977. Karube et al. immobilized Clostridium butyricum on a platinum anode and measured the hydrogen and formate generated from microorganisms to estimate the BOD.[142] After that, numerous of works reported that MFC is a valuable biosensor with good proportionality between BOD value and current produced.[14,15,143] With the advance of MFC technology, the MFC biosensor can detect BOD with in-situ, on-line, and real-time monitoring and replace the conventional method that takes five days for measurement.

MFC biosensors are also applied as on-site pollutant detectors to monitor contaminants in environmental water samples.[12,144] To avoid the uncertainty caused by toxicants, there is a need for real-time detecting tools to monitor the concentration of those contaminated compounds in environment. Based on the metabolisms of microorganisms, a MFC biosensor was exploited for phenol detection.[145] In addition, many of the researches focus on evaluation of heavy metal toxicity by MFC biosensors in nature and in a wastewater treatment plant.[146,147] Various bacteria have been utilized as sensors to detect different pollutants. For example, Pseudomonas putida F1 was used for detection of BTEX (benzene, toluene, ethylbenzene, and xylenes)[10] and Escherichia coli was chosen for monitoring DCP (3, 5-dichlorophenol)[148]. MFC biosensor provides a highly sensitive and accurate detecting technique that analyzes the impact of toxic chemicals on microorganisms.
2.3 Biosensor

2.3.1 Introduction

A biosensor is equipment that using biological identification compartments, such as enzymes, cofactors, proteins, antibodies, and microorganisms as indicators to produce quantitative signals correlated with the concentration of analytes.[149,150] At the beginning, an enzyme is commonly used in biosensor application due to its high selectivity and sensitivity.[9] Microorganisms gradually replaced enzymes as the most popular biological elements since microbes are cheaper, easier to be prepared extensively, and better stability in-situ environment.[150]

2.3.2 Sensing Techniques

There are various methods to detect biological response from indicators. The most widely utilized techniques in biosensor monitoring are electrochemical and optical process.

2.3.2.1 Electrochemical Biosensor

Based on the principle of detection, electrochemical methods can be classified into several categories, such as amperometry, potentiometry, conductometry, voltammetry, and MFC.

Amperometric techniques have been widely used in environmental application.[9] The current is produced by the electroactive metabolism of redox reaction and being recorded. In amperomeric process, an extra potential between working and reference electrode is supplied, and electrodes are connected with a sensitive amperemeter to continuously monitor current variations.[151]
In potentiometry method, the difference of potential between working and reference electrodes is detected which correlates with the concentration of the analytes.[6] However, the requirements of extremely sensitive reference electrode and selective working electrode hinder the application of potentiometry in microbial biosensor.[152]

Voltammetry combines amperometry and potentiometry that measures both the current and potential changes. Voltammetry is adaptable in electrochemical analysis. By detecting current density, the concentration of specific chemical compounds can be recognized with different current peak position and peak area. Cyclic voltammetry (CV) technique is the most widely exploited in analytical process.[153]

Unlike amperometric, potentiometric, and voltammetric procedures that measure with electrodes, the conductometric process is operated by analytic solution conductivity measurement. The changes in solution conductivity result from the depletion or expansion of ionic matter by microbial metabolisms. Even though the fast conductometric method could simplify the detecting system, the difficulty of identifying the conductivity changes limits the development of conductometry.[152]

MFC is another electrochemical method applied in biosensor. MFC used microorganisms as catalysts to oxidize biomass and convert chemical energy to electricity. Generated electricity varies with substrates consumption and toxicity inhibition of metabolic activity; it indicates the chemical concentration in water samples. MFC are commonly applied in water quality detection for in-situ analysis.[25,154]
2.3.2.2 Optical Biosensor

Optical microbial biosensor detectors can be categorized in three major types, which are fluorescent, bioluminescent, and colorimetry. The observed optical signal is generated from the interaction between microorganisms and chemical compounds. The optical signal intensity is used to estimate the correlated concentration of target compounds.

Fluorescent detecting method is the most popular optical sensing technique due to its simple structure and elementary biological technique. The fluorescent signal can be produced directly from some specific microorganisms, such as *Pseudomonas fluorescens* producing green fluorescent protein.\(^{[155]}\) Another mechanism of fluorescent microbial biosensor is detecting the changes of diffused light intensity from exogenous fluorescent components by microbial metabolism.\(^{[156]}\)

In contrast to the fluorescent biosensor using protein calculation, bioluminescent biosensor exploits enzyme activity measurement. Some of the microorganisms have particular reporter genes that accelerate the oxidation of reactants (FMNH\(_2\), oxygen, and RCHO) and emit luminescence.\(^{[157]}\) A luminescent biosensor can be utilized without any exogenous substrate addition; and it is more sensitive and cost-effective in monitor than fluorescent biosensor.\(^{[4]}\)

By using a spectrophotometer, the colored compounds generated from chromogen element conversion by microbial activity can be detected easily.\(^{[158]}\) Colorimetry is a simple and cheap method for optical microbial biosensor and has been widely applied in environmental and food analysis.
2.3.3 Applications and Future Trends

Many microbial biosensor devices have been invested for toxicity measurement and monitoring. Microtox® technique based on bioluminescence intensity detection has been widely used for many years.[12] However, there are some disadvantages that restrict the toxicity monitoring. The optical toxicity bioassays are limited by cell populations and not applicable for high turbidity sample. In addition, the luminous bacteria must live in saline solution to control the osmotic pressure of microorganisms, which is not suitable for normal environmental water sample.[12,148]

Other analysis techniques are developed to improve the toxicity assessment. Lincoln Technology invested a rapid biosensor-based device called MICREDOX, which was initially used for biochemical oxygen demand (BOD) measurement.[148] MICREDOX method was applied to determine 50% effective concentration (EC\textsubscript{50}) for 3,5-dichlorophenol. Liu et al. used several different bacteria and fungus to compare IC\textsubscript{50} of phenol and nitrophenols.[148] Oh et al. developed a rapid toxicity detection based on sulfur-oxidizing bacteria (SOB) that oxidize sulfur compounds to sulfuric acid and produce sulfate ions.[159] The pH and electric current changes can reflect toxicity. Other researches originated an amperometric biosensor call ToxTell to detect toxicity of heavy metal ions. This biosensor could be organized with different kinds of microbial species to deliver complete data for evaluating toxicity.[144] Li et al. demonstrated a bioassay that can monitor water quality in real-time and determine the biotoxicity.[160]

In contrast to the requirement of redox mediators for enhancing electrochemical signal collection in many biosensors, the novel biosensors facilitate the toxicity assay process and eliminate the consumption of mediators.[161] Many toxicity biosensors use an electrochemical signal as indicators to determine the toxicants in water.
However, the electrochemical signal relies on electron transfer in redox reaction. The pH and chemical compounds would generally affect the electrochemical redox reaction. Most literature did not mention the substrate effect, concentration gradient, and pH variation that could influence the electrochemical signal in biotoxicity assay. To explain what mechanisms establish the electrochemical current with activated sludge in biosensor system is an important issue in the future.

2.4 Mechanism of Electron Transfer in MFC

It is well demonstrated that MFC is capable of electricity generation. Many efforts have been made for improving power density with device design and operation condition. However, a deeper understanding of how microorganisms transfer electron extracellularly to electrode surface might be an important thing for MFC development. The mechanisms of electron transfer in MFC can be divided into two primary types: direct electron transfer and Mediated electron transfer.

2.4.1 Direct Electron Transfer (DET)

DET was the first introduced method that noted microorganisms have the ability of electron transport.[74] DET is a mechanism of electron transfer with physical connection between microbial cell membrane and MFC electrode surface. It has been established that some electrochemical and electron transfer processes limit the power production in DET pathway.[28] The limiting steps are (1) oxidation of substrate, (2) electron transfer form cell membrane to electrode surface, (3) inner resistance in electrode and external circuit, (4) proton permeability through cation exchange membrane, and (5) electron acceptors reduction at cathode.
Some specific bacteria have been discovered that are capable of diverting the generated electrons from intracellular electron acceptors out of the cells to the anode. The most widely studied species are metal reducing bacteria, such as *Shewanella*,[74] *Geobacter*,[52] and *Rhodoferax*. It has been demonstrated in further studies that these metal reducing microorganisms possess membrane-bound cytochromes, outer membrane redox proteins, and extracellular electron acceptors.[162,163] Cytochromes contain heme groups with porphyrin rings that enclose the redox active center, metal ion. The non-conductive protein component, amino acids, would restrict the DET between active center and anode.[16,74] Therefore, many efforts have been made to enhance the DET pathway by reducing the barriers between redox active center of protein and electrode surface, such as nanostructure electrode and surfactant addition.[106,164]

With genetic analysis, some specific proteins that involved in DET process can be identified, but the mechanism of electron transfer is still unknown.[74] It has been demonstrated that several outer membrane cytochromes are important in DET but not necessary for electrical power generation.[163] Further evidence is needed to determine the DET mechanism from metal reducing bacteria to anode. Instead of physical bacterial contact by the first monolayer at electrode surface for DET, some particular species, such as *Geobacter* and *Shewanella*, can produce conductive structures that assist connection with anode. The conductive nanowires, pili, allow microorganisms exploit remote electron acceptors without direct contact.[11,130] The conductive compositions enhance the available distance of electron transfer and improve the electricity production performance. It has been addressed that *Geobacter reducens* with nanowires produced ten-times more electricity than without nanowire formation.[165] Even though some microorganisms can generate electrical power via
DET, they are capable of oxidizing few substrates with low molecular chemicals only. This obstacle inhibits the efficiency of power generation by DET and decreases the value in practical application.[11]

2.4.2 Mediated Electron Transfer (MET)

Due to the disadvantages of DET process, other electron transfer mechanisms have been established for power generation improvement. It has been confirmed that DET performance in electricity production is much lower than mediated electron transfer (MET). MET mechanism can be classified into different approaches: artificial mediators addition, self-mediator production, and indirect electron transfer via interaction of reduced metabolic products (fermentation and anaerobic respiration).

2.4.2.1 Exogenous Mediators

An exogenous mediator severs as a carrier to move electrons produced by substrate oxidation out of non-conductive cell membrane and approach to the anode surface. Cohen is the pioneer who utilized mediators in MFC system for the first time.[63] He tested organic and organic compounds to assist the progress of electron transfer and determined that both benzoquinone and potassium ferricyanide have ability to enhance current production effectively. After that, Bennetto and other researchers examined numerous compounds as MFC mediators consisted of phenazines, phenothiazines, phenoxazines, and quinones.[27,46,72,75,93,97] These redox mediators play an important role of electron shuttle which support some microorganisms, such as Escherichia coli, Bacillus, and Psedomonas, that are incapable of transferring electron extracellularly.[166]
However, the precise interactive mechanism between cells and exogenous mediators is still unrecognized. The only thing has been proved is that some mediators, such as neutral red, can catch electrons from reduced components in cell (NADH) and be reduced by enzymes.[69,75,167] Besides the benefits brought by exogenous mediators, there are some drawbacks that limit the application of artificial redox mediators. The requirement of mediator addition adds extra cost in MFC operation; especially in a continuous flow system. In addition, most of the exogenous mediators are not environmentally friendly and also cause harm to humans. Additional treatment is required for toxic effluents.

2.4.2.2 Self-produced Mediator

Some microorganisms can produce low-molecular mediators by themselves to carry electrons extracellularly. *Shewanella oneidensis* is the first confirmed species that transfer electron out of the cell via self-produced electron shuttles.[168] *Shewanella* strains secrete a quinone-like compound as a mediator to reduce iron (III) oxide. It has been determined that *Shewanella* can transport electrons to electron acceptor with long distance, which indirectly proved the mechanism of self-produced electron shuttles.[169,170] Moreover, other microorganisms, such as *Pseudomonas aerugionsa* and *Geothrix fermentans*, can also yield electron shuttles to enhance efficiency of electricity generation.[93,171] The detailed pathway of how mediators work, however, has not been identified.

Upon deeper investigation of self-mediated electron transfer, it is shown that electron shuttles produced in biosynthesis process cost an abundance of energy. The shuttles must be reused to produce a net energy output.[172] This inhibits the application in a continuous input system that releases the self-produced mediator
rapidly. Another restraint of self-produced electron shuttles is low efficiency conversion of substrates to electrical power. Although, the redox potential of electrochemically active compounds is more negative than outer membrane cytochromes that are involved in DET mechanism and gain higher energy, the incompletely oxidizing process of substrates decreases the effectiveness of energy transformation.[81,93] Furthermore, some microbes can only oxidize a limited number of organic compounds under anaerobic condition, which is not feasible in realistic use.[173]

2.4.2.3 Metabolic Products Interaction

Instead of using electrochemically active electron shuttles, electricity can be produced by oxidizing metabolic production in MFC system. Before discussing the interaction between reduced metabolites and anode, the mechanism of how bacteria generate energy for living should be considered deliberately. Different microorganisms utilize various organic or inorganic compounds as substrates, and degrade substrates through biological oxidation for energy production. The biological metabolism can be distinguished by three main approaches of distinct terminal electron acceptors: aerobic respiration, anaerobic respiration, and fermentation.

In aerobic respiratory reaction, microorganisms exploit organic or inorganic compounds as substrates to transfer electrons via glycolysis with sequential electron transfer chain and gradually transfer to oxygen as terminal electron acceptor for energy receiving. Under anoxic condition, particular bacteria employ nitrate, sulfate, carbon oxide, or metal ions as terminal electron acceptors. Based on the Gibbs free energy formula and redox potential of conjugate electron pairs, the energy gain in anaerobic condition is greatly lower than in aerobic environment. Fermentation occurs
at the anoxic condition without exogenous oxidants. The incomplete substrate oxidation is utilized as substrate and form energy. However, the energy conversion from fermentation process is considerably low due to majority of energy content in metabolic intermediates.

\[ \Delta G^\circ = -nFE^\circ \]  \hspace{1cm} (2-1)

Since the MFC system usually operates without oxygen, the aerobic respiration reaction would not be considered. In anaerobic oxidation reaction, the low reduction potential of terminal electron acceptors results in little energy generation. Only few anoxic microbes utilized for MFC system have been addressed. For example, sulfate serves as an electron acceptor for electron transfer.\[166\] *Desulfovibrio desulfuricans* is generally used in MFC system that catalyzes the reduction of sulfate (SO\(_4^{2-}\)) to sulfide (S\(^2-\)).\[136\] The reduced production, sulfide, is re-oxidized and releases eight electrons to anode for electricity production. However, sulfide is more likely to be oxidized to sulfur (S\(^0\)) than sulfate, which gives only two electrons to anode and decrease the electron transfer efficiency.\[174\] The solid sulfur accumulates on the anode surface would also lead to effectiveness of electricity generation. Moreover, the incompletely oxidation of substrate by *Desulfovibrio* species restricts the energy conversion.

Another metabolic pathway of MET in anaerobic environment is fermentation. Fermentative process applied in MFC system is more popular than anaerobic respiratory process since the products of fermentation are energy-rich. These products include hydrogen, formate, lactate, and ammonia.\[103,173,175\] Hydrogen is the most applicable electron carrier that many reports exhibited the use for electricity generation. In order to enhance the hydrogen yields from fermentation, some approaches focused on combination of dark fermentation and photo fermentation,
which improves the substrate conversion efficiency and increases the energy
generation.[176] In dark fermentative process, bacteria degrade substrates to hydrogen
and organic acid byproducts. Afterward the photoheterotrophic microbes, such as
Rhodobacter spaeroides, are utilized in photo-fermentative part for further oxidize the
remaining organic acids produced in dark fermentation.[177] Unfortunately, the
requirement of sufficient light source and equipment demand in photo fermentative
process burdens the application of fermentation in MFC system.
Chapter 3

MATERIALS AND METHODS

3.1 Experimental Framework

The experiment can be separated into three parts: (1) Activated sludge cultivation; (2) MFC-based biosensor design and system set up; (3) Toxicity test in the synthetic wastewater sample with different heavy metals compounds. In addition, the methods of metal speciation analysis by Visual MINTEQ software are demonstrated.

For the beginning of the experiment, sequencing batch reactor (SBR) wastewater treatment system has been utilized for activated sludge culture. Various parameters were detected to control the stability of microbial growth rates which contribute to the following toxicity test.

The MFC-like biosensor reactor was designed and the cultivated activated sludge was added to the biosensor reactor for heavy metal toxicity monitoring. To design a proper device, diverse components have been concerned. The main objective of this research was to develop a sensitive biosensor as an emergency alarm for continuously monitoring water quality in a wastewater treatment plant by measuring instantaneous signal variation. Accordingly, to provide a steady background signal from activated sludge was essential. After biosensor preparation, activated sludge was added and an electrometer device recorded the electrochemical signal data generated from microorganisms.

The toxicity test of a heavy metal was conducted after optimizing the biosensor monitoring system. An electrometer, as an analytical device, was connected with
electrodes to record the electrochemical signal variation. In order to get conspicuous current change, bio-electrochemical process was studied under various conditions with different heavy metals concentration.

3.2 Activated Sludge Cultivation

![Diagram of activated sludge cultivation procedure]

**Figure 3.1** Schematic of activated sludge cultivation procedure

The activated sludge sample was obtained from a wastewater treatment plant in Baltimore MD. The sludge solids were cultivated in p acrylic cylinder tanks (D: 14.1 cm; H: 51.2 cm) by SBR technology and feed on synthetic wastewater. The components of synthetic wastewater influent consisted of three major organic nutrients (C, N, and P), and some inorganic trace elements (such as Fe, Mn, and Cu). The C: N: P ratio was 100: 5: 1 in this aerobic cultivation system. Furthermore, alkalinity
addition should be considered during the treatment period. Alkalinity was the capacity of wastewater to neutralize acids. If no additional chemical compounds were added to compensate for alkalinity drop, pH in SBR system declined and ruined whole process. Sodium bicarbonate (NaHCO₃) was the most common chemical for alkalinity addition since it had pH of 8.3. It was profitable to supplement with bicarbonate species at a pH near neutrality.

**Table 3.1** Components of synthetic wastewater

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Chemical formula</th>
<th>Concentration</th>
<th>Molar Weight</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose Anhydrous</td>
<td>C₆H₁₂O₆</td>
<td>750 mg/L</td>
<td>180</td>
<td>Fisher, &gt;99.8%</td>
</tr>
<tr>
<td>Ammonium Sulfate</td>
<td>(NH₄)₂SO₄</td>
<td>377 mg/L</td>
<td>132.1</td>
<td>Aldrich, 99%</td>
</tr>
<tr>
<td>Potassium Phosphate</td>
<td>K₃PO₄</td>
<td>54 mg/L</td>
<td>212.3</td>
<td>Sigma, &gt;98%</td>
</tr>
<tr>
<td>Sodium Bicarbonate</td>
<td>NaHCO₃</td>
<td>300 mg/L</td>
<td>84.01</td>
<td>Acros, 99.7%</td>
</tr>
</tbody>
</table>

**Table 3.2** Activated sludge cultivation conditions

<table>
<thead>
<tr>
<th>Operation Method</th>
<th>Sequencing Batch Reactor (SBR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultivation Basin</td>
<td>Three cylinder-shaped reactors</td>
</tr>
<tr>
<td>Reactor Volume</td>
<td>8 L (D: 14.1 cm, H: 51.2 cm)/each</td>
</tr>
<tr>
<td>Wastewater Flow</td>
<td>5 L/day /each</td>
</tr>
<tr>
<td>MLSS Concentration</td>
<td>2500 ~ 3000 mg/L</td>
</tr>
<tr>
<td>Sludge Retention Time (SRT)</td>
<td>15 day</td>
</tr>
<tr>
<td>Dissolved Oxygen (DO)</td>
<td>2 ~ 4 mg/L</td>
</tr>
<tr>
<td>C: N: P Ratio</td>
<td>100: 5: 1</td>
</tr>
</tbody>
</table>
SBR process combined all the wastewater treatment steps into one individual tank while conventional facilities depend on multiple basins. Each basin contained 5 liter of activated sludge sample and treatment cycle took place in a single basin which scaled down the operation space. There are five procedures in the SBR treatment technology, including fill, react, settle, decant, and idle.

![Diagram of SBR process](image)

**Figure 3.2** Major process of SBR operation cycles

Under the fill procedure, the synthetic wastewater was pumped into sludge tanks. The influent brought nutrients and reacted with the microorganisms in the activated sludge. During the fill phase, both an aeration pump (Hydrofarm®, Active Aqua™ air pump) and a mechanical-mixing device (Thermolyne® Cimarec®, top stirring hotplate)
were used for biochemical reactions. Dissolved oxygen (DO) was monitored the while to prevent excess oxygen in activated sludge cultivation system.

In the react step, no synthetic wastewater entered the basin and the aerator and mixer were still on. The removal rate of organic compound greatly increased due to no additional organics loading. Microorganisms assimilated organic compounds in wastewater and transferred organics to biomass. Biochemical oxygen demand (BOD) removal and mixed liquor suspended solids (MLSS) were monitored to control the biomass enhancement and treatment efficiency.

After reacting completely for 24 hours, the activated sludge was allowed to settle without aeration and stirring. The sludge solids coagulated together and precipitated to the bottom of basin, separating a recognizable interface from transparent supernatant.

Once the settle process was complete, the supernatant was removed by a peristaltic pump (Cole Parmer®, Masterflex® peristaltic pump with speed control). The volume of effluent was 2.5 liter in a single tank, which equaled half of activated sludge volume. The pipet connected to the effluent pump should be away from the bottom of the basin to avoid disturbing the settled sludge. The pH was monitored for alkalinity control.

The idle phase happened between decant and the next fill procedure. In this period, some of the excess activated sludge would be pumped out for maintaining high concentration of MLSS. The wasted sludge could be collected to a digester or holding tank for future processing and disposal.
3.3 MFC-like Biosensor Design and Detecting System Set-up

In recent years, design of MFC devices has been extensively reviewed with enhanced performance. The MFC-like biosensor reactor in this experiment was designed based on the researches of MFC. A typical two-chambers MFC reactor consisted of an anodic chamber and a cathodic chamber separated by a proton exchange membrane (PEM).

The bioreactor chambers were made of acrylic plastic material with cubic shape. The volume of each chamber was 400 mL (L: 7.6 cm, W: 5.0 cm, H: 10.0 cm), and the thickness of the reactor wall was 0.6 cm. One side plane of the bioreactor chamber had a rectangular space (L: 6.0 cm, W: 5.0 cm) for PEM connection.

![Diagram of MFC-like biosensor reactor](image)

**Figure 3.3** Schematic of biosensor reactor operation
A 178 µm – thick PEM (Dupont™ Nafion® 117 perfluorinated membrane, L: 6.5 cm; W: 5.5 cm) was located between anodic and cathodic chamber during reactor operation. The PEM was attached to chambers with surroundings spread with waterproof silicone (GE®, Silicone II Kitchen & Bath Caulk), air-dried for 24-hour and secured with 6 stainless steel screws.

Two electrodes used in the experiment were made of graphite plate (Graphtek™ L.L.C, coarse extruded graphite plate) and cut down to the volume of 9 cm³ (L: 10 cm; W: 3 cm; H: 0.3 cm) for each. Both anode and cathode were drilled with a small hole at the top of the electrode for electric wire binding. Two copper wires were wrapped around the electrodes and fixed with silver paste (SPI Supplies®, Silver Paste Plus™) and covered with waterproof silicone (GE®, Silicone II Kitchen & Bath Caulk). The copper wires were linked with an electrometer device (Keithley, 614 Electrometer) for detecting electrical current.

For aeration operation, two plastic tubes were set up in the biosensor. The ring-shaped tubes with numerous holes were positioned under the bottom of chambers. The ends of tubes were sealed by parafilm (Parafilm M® film) while another ends were connected with air pump (Hydrofarm®, Active Aqua™ air pump) for aeration. The biosensor detecting system was operated in a batch mode. The anodic compartment was fed with 312 mL of cultivated activated sludge and synthetic wastewater for system set-up (half activated sludge and half synthetic wastewater). The cathodic chamber was filled with deionized water (DI water) in the same volume as the mixed sludge. Both compartments had aeration with 100 mL/min flow rate. A small stir bar was located in anodic compartment to prevent sludge precipitation. Two electrodes were established steadily in both chambers, and were connected to electrometer for
current monitoring. The digital data was recorded by software (DATAQ® Instruments, \textit{WinD}A\textsubscript{Q}® data acquisition software) in computer.

<table>
<thead>
<tr>
<th>Table 3.3</th>
<th>Biosensor detecting system operation condition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Operation Mode</strong></td>
<td><strong>Component</strong></td>
</tr>
<tr>
<td>Anodic</td>
<td>Activated sludge &amp; synthetic wastewater</td>
</tr>
<tr>
<td></td>
<td>Volume</td>
</tr>
<tr>
<td>Cathodic</td>
<td>Activated sludge &amp; synthetic wastewater</td>
</tr>
<tr>
<td></td>
<td>Component</td>
</tr>
<tr>
<td></td>
<td>Volume</td>
</tr>
<tr>
<td>Aeration flow rate</td>
<td>100 mL/min</td>
</tr>
<tr>
<td>Recording software</td>
<td>DATAQ® Instruments, \textit{WinD}A\textsubscript{Q}® data acquisition software</td>
</tr>
</tbody>
</table>

3.4 **Toxicity Test with Different Heavy Metals Compounds**

After adding activated sludge and synthetic wastewater into the anodic compartment for more than three hours to stabilize the background values, the prepared metal perchlorate solutions were injected into anodic chamber to determine the response of electrical signals and pH variation. Eleven different metal perchlorate solutions were prepared in the same concentration of 1 M, which were separately added into chamber with gradually increasing volumes (10, 40, 50, 100, 200, 400 μL) in the first day and another 200 μL (twice 100 μL addition) in the following day. Electrometer connected with two electrodes would record electrical current changes continuously. In addition, pH value was measured in both chambers before and after metal solution adding to determine the deviations.
### Table 3.4

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Chemical formula</th>
<th>Molar Mass</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper Perchlorate Hexahydrate</td>
<td>Cu(ClO₄)₂ · 6H₂O</td>
<td>370.53</td>
<td>Aldrich, 98%</td>
</tr>
<tr>
<td>Nickel Perchlorate Hexahydrate</td>
<td>Ni(ClO₄)₂ · 6H₂O</td>
<td>365.69</td>
<td>Alfa, 99%</td>
</tr>
<tr>
<td>Zinc Perchlorate Hexahydrate</td>
<td>Zn(ClO₄)₂ · 6H₂O</td>
<td>372.38</td>
<td>Alfa, 99%</td>
</tr>
<tr>
<td>Cadmium Perchlorate Hexahydrate</td>
<td>Cd(ClO₄)₂ · 6H₂O</td>
<td>419.41</td>
<td>Alfa, 99.9%</td>
</tr>
<tr>
<td>Cobalt Perchlorate Hexahydrate</td>
<td>Co(ClO₄)₂ · 6H₂O</td>
<td>365.93</td>
<td>Alfa, 99.9%</td>
</tr>
<tr>
<td>Lead Perchlorate Trihydrate</td>
<td>Pb(ClO₄)₂ · 3H₂O</td>
<td>460.09</td>
<td>Alfa, 95%</td>
</tr>
<tr>
<td>Manganese Perchlorate Hexahydrate</td>
<td>Mn(ClO₄)₂ · 6H₂O</td>
<td>361.93</td>
<td>Acros, 99%</td>
</tr>
<tr>
<td>Iron(II) Perchlorate Hydrate</td>
<td>Fe(ClO₄)₂ · xH₂O</td>
<td>254.75</td>
<td>Aldrich, 98%</td>
</tr>
<tr>
<td>Chromium Perchlorate Hexahydrate</td>
<td>Cr(ClO₄)₃ · 6H₂O</td>
<td>458.44</td>
<td>Alfa, 99.9%</td>
</tr>
<tr>
<td>Iron(III) Perchlorate Hydrate</td>
<td>Fe(ClO₄)₃ · xH₂O</td>
<td>354.19</td>
<td>Alfa, 94.8%</td>
</tr>
<tr>
<td>Aluminum Perchlorate Nonahydrate</td>
<td>Al(ClO₄)₃ · 9H₂O</td>
<td>487.47</td>
<td>Aldrich, 98%</td>
</tr>
</tbody>
</table>

Besides different metal perchlorate solutions and volumes, diverse synthetic wastewater components and concentrations were considered as factors which could influence the current variation. Blank samples with synthetic wastewater only were also determined in eleven different metal perchlorate solutions. The process in blank sample was the same as metal toxicity test.

#### 3.5 Analysis Equipment and Methods

In this research, various detecting devices monitored different items. The analytical approaches and equipment would be introduced respectively.
3.5.1 pH

The samples were extracted and detected by pH meter (Corning® digital pH/ion analyzer 350) combined with pH electrode (Corning® combination BNC electrode probe). A small vial (Thermo Scientific™ Reacti-Vial™) was used to collect samples in order to shrink the sampling volume to 3 mL. The pH analyzer was calibrated by 3 buffer solutions before sample analysis. The pH electrode was rinsed by DI water during the detecting approach for decreasing experimental errors.

3.5.2 Dissolved Oxygen (DO)

DO in activated sludge was measured by a DO meter (YSI 55 dissolved oxygen meter). There was a small chamber containing a porous silver anode filled with potassium chloride (KCl) electrolyte solution. The permeable membrane, covered the sensor, separated the electrodes from environment and allowed only gas to pass thorough. When a voltage was applied to sensor, oxygen passed through the membrane and reacted with the cathode causing a current flow.

3.5.3 Mixed Liquor Suspended Solids (MLSS)

MLSS measuring was a test to detect the total concentration of mixed liquor non-soluble solids in the aeration basin of an activated sludge system. The MLSS data was critical in determining the operational behavior. It was applied for deciding when to recycle or waste excess activated sludge.

The first step of MLSS test was to weigh a glass filter paper (Hach® 934-AH glass filters, 47 mm) to a constant weight (W1) after drying at a temperature between 103°C and 105°C in an oven. The filter holder (Pall® Life Sciences, Magnetic filter
funnels, 47mm, 300 mL) was placed on a vacuum flask (Pyrex® Glass heavy wall filtering flask, 500 mL) and the filter paper put on the top of the holder by tweezers. Mixed liquor sample was stirred evenly to ensure complete mixture and poured into filter holder with proper volume (V). DI water was run through the filter to rinse residual particles stuck to the filter paper and the wall of holder. After running the vacuum pump (Emerson vacuum air pump SA55NXGTE 4870) for three minutes, the filter was removed from the filter holder and placed into a dry oven for heating at a temperature between 103°C and 105°C for one hour. The filter was weighed for a second time \(W_2\) and the concentration of suspended solids in sample was calculated by the following formula.

\[
MLSS\left(\frac{mg}{L}\right) = \frac{W_2(mg) - W_1(mg)}{V(mL)} \times 1000
\]  \hfill (3-1)

3.5.4 Chemical Oxygen Demand (COD)

Oxygen demand is a significant parameter for measuring the amount of organic pollutants in water samples. The oxygen demand measurement was widely applied to evaluate the efficiency of wastewater treatment process. There were three ways to detect oxygen demand: Biochemical Oxygen Demand (BOD), COD, and Total Organic Carbon (TOC). The principle of the COD test was to oxidize organic compounds to CO\(_2\) and H\(_2\)O by adding strong chemical oxidant in acid solution. The advantages of COD test were rapid and frequent monitoring of wastewater treatment plant efficiency and water quality. It could also be utilized to estimate the BOD results.
The micro digestion method was used in this COD test to minimize reagent consumption and reduce required space for equipment. The samples were settled and extracted 2 mL supernatant to a COD vial (Hach® Company, COD digestion vials, 3-150 mg/L range). The first step of digestion was to turn on the COD reactor (Hach® Company, COD reactor) and preheat the temperature to 150°C. The sample vial and one blank vial was placed into pre-heated COD reactor for 2 hours. The vials were removed from the reactor and cooled down to room temperature. The outside wall of the vials was cleaned with a towel after setting program of colorimetric measurement (Hach® Company, DR-2000 UV-VIS spectrophotometer) for low range COD test. Placed the vials into adapter and closed the light shield and read the results.

![Diagram of COD test procedure](image)

**Figure 3.4** Diagram of COD test procedure

### 3.6 Metal Speciation Analysis

In results data analysis, the software, Visual MINTEQ, is utilized to calculate and predict the metal complexes species distribution in the system. Visual MINTEQ can measure the strength of the interaction between metal and ligands that produce metal complexes, which is called stability constant or equilibrium formation constant.

The experimental system contains various components which provides several ligands that form complexes with metal ions. The concentrations of components and
the aquatic system condition would influence metal species distribution significantly. The example of calculated equilibrium speciation of Cu(II) by Visual MINTEQ is displayed below.

The simplest complex is hydroxo-complex which is the formation of metal ions and hydroxide ion. Equation 3.2 shows the hydrolysis reaction of Cu(II). However, water is not the sole component in the system. Metal ion ($M^{n+}$) would combines with other ligand ($L$) and form $ML_n$ complex. The formation constant ($K$) can be expressed by the equilibrium concentration of species (equation 3.3). There are some other components that complex with Cu(II) ions and form diverse complexes in solution. Equation 3.4 presents all possible Cu(II) species that chelate with component existing in the system.

\[
Cu^{2+} + H_2O = CuOH^{+} + H^+ \quad \log^* K_1 = -8.0 (I = 0) \tag{3.2}
\]

\[
M + nL = ML_n \quad K = \frac{[ML_n]}{[M][L]^n} \tag{3.3}
\]

\[
[Cu(II)]_{f} = [Cu^{2+}] + [CuOH^{+}] + [Cu(OH)_2] + [Cu(OH)_3] + [Cu(OH)_4]^{2-} + [Cu_2OH_2(OH)_2] + [Cu_2(OH)_3] + [Cu_3(OH)_4]^+ + [Cu_{2}HCO_3] + [CuCO_3^{2-}] + [Cu_{2}(CO_3)_2] + [Cu_{2}(CO_3)(OH)] + [Cu_{2}(CO_3)_{2}] + [Cu_{2}(CO_3)(OH)] + [Cu_{2}(CO_3)_{2}]
\tag{3.4}
\]

Each metal forms different metal complexes even in the same conditions. The concentrations of components in the system are listed in table 3.5. The formation constants of metal speciation are listed in Appendix. All the equilibrium constants are at the condition of 25°C and 1 atm. The injection of different concentration of metals
would influence the pH value in the whole system. Therefore, one concentration of metal injection would correspond to one specific pH value. The speciation of different metal solution is calculated by Visual MINTEQ software under different pH condition. Visual MINTEQ software also considers the ionic strength effect, which activity coefficient is related to ionic strength of chemical species. Ionic strength is a function of all the concentration of ions in solution multiples square of ionic charge numbers (equation 3.5). Through Davies equation (equation 3.6), activity coefficients are modified. Equation 3.7 shows the experimental ionic strength with activated sludge in this system. The composition matrix for the equilibrium speciation of ligands and metals are displayed in Appendix.

\[
I = \frac{1}{2} \sum_{i=1}^{n} C_i Z_i^2 \tag{3.5}
\]

\[
-\log f_z = 0.5 z_z i \left[ \frac{\sqrt{I}}{1+\sqrt{I}} - 0.15 I \right] \tag{3.6}
\]

\[
I = \frac{1}{2} \begin{Bmatrix}
Na^+ (+1)^2 + CO_3^{2-} (-2)^2 + NH_4^+ (+1)^2 + SO_4^{2-} (-2)^2 \\
+ K^+ (+1)^2 + PO_4^{3-} (-3)^2
\end{Bmatrix} \tag{3.7}
\]

<table>
<thead>
<tr>
<th>Table 3.5</th>
<th>Concentrations of components in the system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Component</td>
<td>(CO_3^{2-})</td>
</tr>
<tr>
<td>Concentration (mM)</td>
<td>1.79</td>
</tr>
</tbody>
</table>
Chapter 4

RESULTS AND DISCUSSION

4.1 Activated Sludge Cultivation Monitoring

Before using an MFC-like biosensor for heavy metal toxicity detection, the activated sludge utilized in this system should be controlled and the microbial growth rate should be stabilized. Several parameters are monitored to adjust activated sludge in a continuously stable condition. Testing would include the determination of pH and dissolved oxygen (DO) in SBR tank; the measurement of mixed liquor suspended solids (MLSS); and the COD of aeration tank effluent.

4.1.1 pH

The pH values in the cultivated tank were detected for monitoring the alkalinity in wastewater system. The pH was satisfactory near 7.0. The low pH usually results from nitrification and lack of alkalinity in the wastewater. DO could be decreased enough to limit nitrification in activated sludge. However, ammonia in the system would be accumulated without nitrification and reduce the quality of effluent. The provision of alkalinity by sodium bicarbonate (NaHCO₃) addition was a proper way to raise the pH and remain pH stable in neutrality.

At the beginning of activated sludge cultivation, pH altered intensely from neutral to pH 2. The extreme acid environment harmed microbial metabolism and influenced the system dramatically. To control pH near neutrality, NaHCO₃ alkalinity
addition was considered in the cultivation process. Figure 4.1 shows the pH values of SBR cultivated system detected in three cylinder tanks after NaHCO₃ addition. There was no significant difference among three columns. Although column 3 had greater pH fluctuation than others, all of them had pH between 6.4 and 8.0. The results establish that NaHCO₃ was a profitable chemical for pH adjustment in this system.

![Figure 4.1](image)

**Figure 4.1** The pH values of activated sludge cultivated in three columns. The feeding substrates consisted of 750 mg/L C₆H₁₂O₆, 377 mg/L (NH₄)₂SO₄, 54 mg/L K₃PO₄, and 300 mg/L NaHCO₃ with SBR operational method.

4.1.2 Dissolved Oxygen (DO)

The requirement of oxygen was necessary for microorganisms in activated sludge to oxidize biofuels and obtain energy for living. Deficient oxygen would make microorganisms work inefficiently and slow down the aerobic bacteria. Furthermore, some of the anaerobic by-products would increase with unpleasant smell and affect the whole process operation. Enough oxygen must be supported in the aeration basin to
stabilize the efficiency of microbial oxidation reaction, especially in the influent with high BOD concentrations.

Figure 4.2   DO measurement of activated sludge cultivation in three different columns: (a) column 1 (b) column 2 (c) column 3.
Figure 4.2  Continued.

As figure 4.2 presents above, DO in all of the three columns dropped down to approximate 1 mg/L after refilling synthesis wastewater into tanks, and were gradually elevated to 6 mg/L within 100 minutes. The reason why DO diminished to low oxygen concentration was the oxygen demand of microorganisms for oxidizing substrates in influent. The oxygen dissolved in water was consumed immediately to ensure the complete bio-degradation. In column 3 (Figure 4.2 (c)), the level of oxygen raised to a balanced point slowly during the first three days. The situation was changed as same as the trends exhibited in column 1 and column 2 after replacing a new aeration stone.

In addition to understanding oxygen consumption with time after refilling influent, the stability of DO in aeration tanks is important. Figure 4.3 displays the DO values after adding new substrates for 2 hours. The amounts of DO in column 1 and column 2 showed comparably stable than in column 3. Maintaining the level of oxygen in activated sludge process is an important issue. The filamentous microorganisms would become dominant species with DO less than 1 mg/L and
hindered the operation efficiency. On the other hand, it causes problem when DO reaches to high. The floc particles floated to the surface and reduced the contact between bacteria and substrates. The routine monitoring of DO is necessary for system operation by DO meter.

![Graph showing DO measurements over time in three columns](image)

**Figure 4.3** DO measurements of activated sludge in three columns after influent filling for 2 hours.

### 4.1.3 Mixed Liquor Suspended Solids (MLSS)

Microorganisms in activated sludge mixed with wastewater and grow on the suspended solids. The mixture of microorganisms and wastewater is described as mixed liquor. With the process of bio-floculation, microbial mixtures stick together and form floc particles. Microorganisms capture and consume substrates in the wastewater and produce biomass. The mass of microorganisms should sustain in a steady condition. The balance between substrates (Food) and microorganisms (Mass) is known as F:M ratio.
The adjustment of the F:M ratio is another critical issue for maintaining system with an appropriate condition. Microorganisms grabbed most of the organic matters in synthetic wastewater immediately. However, it took much time for consuming substrates in wastewater and transferring into new biomass. The mass concentration of microorganisms that controls in the system is a function of MLSS in aeration tanks. The excess MLSS must be discharged for effective treatment. Conversely, increasing the amounts of microorganisms or reducing organic loading helps to lower the F:M ratio. MLSS is a significant factor in activated sludge system maintenance.

![Graph](image)

**Figure 4.4** MLSS measurements of activated sludge in three columns after new substrates addition for 24 hours. Substrate components: 750 mg/L \(\text{C}_6\text{H}_{12}\text{O}_6\); 377 mg/L \((\text{NH}_4)_2\text{SO}_4\); 54 mg/L \(\text{K}_3\text{PO}_4\); 300 mg/L \(\text{NaHCO}_3\).

As figure 4.4 presents, the ranges of MLSS in three columns established between 2000 mg/L and 3500 mg/L. The concentration of microbial biomass indicates the growth of microorganisms in aeration basin. Columns 2 had higher MLSS than other columns, which lived in a favorable condition for metabolism. Moreover, the level of
MLSS in column 2 had more steady balance than others that lay in the range between 3250 mg/L and 3500 mg/L.

4.1.4 Chemical Oxygen Demand (COD)

BOD is the amount of oxygen required by microorganism to decompose organic matters. It depends on the dissolved organic matters in the water samples. In general, BOD values are around 30 to 70% of COD. Since all the substances, such as glucose, ammonium sulfate, and potassium phosphate, contained in synthetic influent are biodegradable, the value of BOD is totally equal to COD. Therefore, COD test was performed to measure the amount of organics instead of BOD measurement due to faster and more convenient in water quality analysis.

\[ C_6H_{12}O_6 + 6O_2 \xrightarrow{\text{Microbes}} 6CO_2 + 6H_2O \quad \Delta G^\circ = -2843 \text{kJ/mol} \tag{4.1} \]

The COD value exhibits the amount of substances in the water. The COD was measured after feeding 800 mg/L of organic matters for 24 hours. The residual organic matters remained in water sample would be identified via COD analysis, and the metabolic activity of microorganisms in activated sludge can be predicted. The carbon sources provided in this system came from glucose (C\textsubscript{6}H\textsubscript{12}O\textsubscript{6}) that was oxidized completely to CO\textsubscript{2} and H\textsubscript{2}O with microbial catalysis. (Equation 4.1) In addition to major carbon substrate, other nutrients are required for microbial growth. Nitrogen and phosphorous are the predominant sources in activated sludge cultivation.

The optimum ratio of C: N: P in aerobic treatment is commonly thought to be 100: 5: 1. Ammonium sulfate ((NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}) and potassium phosphate (K\textsubscript{3}PO\textsubscript{4}) were
added into system following the general proportion in aeration tank. Trace elements, including Fe, Na, K, Ca, Mg, were also needed and were supplied from tap water for activated sludge reproduction.

![Figure 4.5](image)

**Figure 4.5**  COD removal percentage of activated sludge cultivation in three columns after 800 mg/L of COD substrates addition for 24 hours. Substrate components: 750 mg/L C₆H₁₂O₆; 377 mg/L (NH₄)₂SO₄; 54 mg/L K₃PO₄; 300 mg/L NaHCO₃.

Figure 4.5 reveals the percentage of COD removal with initial COD 800 mg/L in three activated sludge basins. After one-day metabolism by microorganisms in activated sludge, the efficiency of organic matters consumption was favorable. The COD removal capability was higher than 97 % for all aeration tanks. Comparing the results of three different columns, column 1 had better and more stable removal efficiency that exceeded 99 %.

According to all the monitoring data of activated sludge cultivation for a period of time, microorganisms in column 1 was the best choice for toxicity test in MFC-like
biosensor. In conclusion, the activated sludge utilized in biosensor device was selected from column 1 with DO concentration roughly 2 ~ 6 mg/L, MLSS ranged from 2500 to 3000 mg/L, and C: N: P ratio of 100: 5:1 in nearly 99% COD removal efficiency. Based on the results of DO variation verses time, the toxicity test would be operated 3 hours after influent refilling.

4.2 Blank Experiment

To design a highly sensitive biosensor for heavy metal toxicity detection, it is important to understand what leads to current generation from MFC-like biosensor consideredly. The background values of current generated from substrates themselves should be withdrew in advance. In control test, the electric current was detected by electrometer without activated sludge addition. The components of influent and the concentration of the nutrients were inspected respectively.

4.2.1 Different Components of Substrates Influence Current Change

The main components in cultivating activated sludge contained glucose, ammonium sulfate, potassium phosphate, and sodium bicarbonate. The current generated from these four chemicals individually are displayed in figure 4.6. Cu(II) was used as a target pollutant in blank experiment. Different volume of 1 M Cu(II) solutions were added into anodic chamber.
Figure 4.6  Current changes with different Cu(II) concentration addition measured under four components contained in synthetic wastewater: (a) 2.08 mM C₆H₁₂O₆; (b) 1.43 mM (NH₄)₂SO₄; (c) 0.13 mM K₃PO₄; (d) 1.79 mM NaHCO₃. Experimental condition: pH = not controlled; Ionic strength = not controlled; Temperature = 25°C; Electrodes = graphite plate; Δtₜₐₚ = 1 min.

In figure 4.6 (a) and (b), the current variation with each concentrations of copper perchlorate addition altered slightly in the range between -0.08 μA and +0.07 μA. The accumulative current fluctuation varied between -0.1 μA and +0.05 μA. It showed that the current contribution from 2.08 mM of glucose and 1.43 mM of ammonium sulfate were negligible. The current change provides from the rest of substrates consisted in
synthetic wastewater is presented in figure 4.6 (c) and (d). Accumulative current difference increased rapidly in both situation when Cu(II) concentration was less than 2.5 mM and reached to a steady point when Cu(II) concentration approached to 3 mM. The ultimate accumulative current changes in 0.13 mM of potassium phosphate and 1.79 mM of sodium bicarbonate reached to approximately 0.7 μA and 0.9 μA respectively.

![Graph](image)

**Figure 4.7** The pH change with accumulative Cu(II) concentration in anode under four different nutrient solutions. Concentration: 2.08 mM C₆H₁₂O₆; 1.43 mM (NH₄)₂SO₄; 0.13 mM K₃PO₄; 1.79 mM NaHCO₃.

The structure of biosensor operated in toxicity test was a two-chamber device that separated two compartments by a proton exchange membrane (Dupont™ Nafion® 117 perfluorinated membrane). In general, proton is the only ion capable to pass through Nafion® 117 PEM and recombined with electrons transported via external circuit at the cathode to produce electric current. The major factor that influences the current change should be proton concentration, which means pH value in solution. However,
the pH measured by pH meter seems no significant differences between four nutrients. All the pH values ranged from initial neutral pH to around 5.5.

**Figure 4.8** Comparison of different substrate components combinations with accumulative current changes. Experimental condition: pH = not controlled; Ionic strength = not controlled; Temperature = 25°C; Electrodes = graphite plate; Δtexp = 1 min. Concentration: 2.08 mM C₆H₁₂O₆; 1.43 mM (NH₄)₂SO₄; 0.13 mM K₃PO₄; 1.79 mM NaHCO₃. Substrate contains C₆H₁₂O₆, (NH₄)₂SO₄, K₃PO₄, and NaHCO₃.

Some reviews determined that several cations with small ionic radius have ability to move through the Nafion™117 membrane as well as proton. The order of ionic radius is H⁺ (< 0.5 Å) < Na⁺ (0.95 Å) < K⁺ (1.33 Å) < NH₄⁺ (1.43 Å). The demonstration can explain why potassium phosphate and sodium bicarbonate provided current changes but glucose and ammonium sulfate did not.

To confirm the assumption, more permutation combinations of four major nutrients had been examined to analyze the current differences by electrometer. Figure 4.8 shows various combinations of substrates with current changes. The data in figure
4.8 revealed the same results from figure 4.6. Glucose and ammonium sulfate were not capable of current changes. Although mixing with potassium phosphate or sodium bicarbonate, the accumulative current variations remained, even decreased somewhat.

On the other hand, the combinations of potassium phosphate and sodium bicarbonate enhanced the current changes. Potassium phosphate and sodium bicarbonate provided roughly 0.7 and 0.9 μA individually, and contributed over 1.2 μA when incorporation. Current change was invariable with additional ammonium sulfate supplement. Furthermore, the current variation in the synthetic wastewater containing four substrates is also showed in figure 4.8. It had the greatest current change signals yet no significant difference.

### 4.2.2 Different Concentrations of Substrates Influence Current Change

The experiment of substrates proportion added into anodic compartment had been examined. In this test, lead perchlorate trihydrate (Pb(ClO₄)₂ · 3H₂O) performed as a target heavy metal pollutant and was added into system with different concentrations successively. Three different substrate ratios tested in the experiment consisted of substrate only, half of substrate with tap water, and quarter of substrate with tap water.
Figure 4.9  Comparison of different substrate proportions added into anodic compartment verses accumulative current changes. 100% of substrate: 4.16 mM C₆H₁₂O₆; 2.86 mM (NH₄)₂SO₄; 0.26 mM K₃PO₄; 3.58 mM NaHCO₃.

As the results exposed, the current difference increased with the augmentation of substrate percentage in system. The accumulative current changes in substrate only rose up to 5.35 μA, which was twice higher than half of substrate and 15-times higher than quarter of substrate. It was apparent that concentrations of substrate contained in biosensor reactor determined the current changes significantly.

In addition, the current change in substrate with activated sludge was analyzed to make comparison with the current change in substrate without sludge. The average current fluctuation in samples of half substrate half sludge approached to 0.9 μA, which was much lower than the sample composed of half substrate with tap water (2.3 μA). The results established that activated sludge would decrease the current changes instead of increase.
In conclusion of the control experiment, the major electric current was not originated microbial metabolism in activated sludge, instead, the current was generated from substrates themselves. It is predicted that some cations with smaller ionic radius had ability of passing through Nafion®117 membrane used in the biosensor device. Moreover, The current variation with sludge addition also depended on metal species. The metal effect would be discussed in next section.

4.3 Heavy Metal Toxicity Test

The toxicity test of heavy metal was experienced after optimizing biosensor system and analyzing the background value without activated sludge addition. The bio-electrochemical signals were detected by electrometer which connected with anode and cathode in the biosensor device. According to the results shows figure 4.2, the toxicity test would be operated 3 hours after activated sludge being stabilized in the biosensor. Different concentrations were analyzed to better understand the electric current changes with heavy metal contamination.

4.3.1 Divalent Metals

Eight divalent metals, including Mn, Fe(II), Co, Ni, Cu, Zn, Cd, and Pb, were tested in toxicity experiments. All the heavy metal toxins were prepared in the form of 1M metal perchlorate solutions, and were injected into anodic chamber with increasing dosages. The next toxicant addition took place when current change stabilized after prior toxicant injection. Every toxicity experiment was operated thrice. The current fluctuations with all distinct metals were compared with control groups.
4.3.1.1 Copper

Figure 4.10 Comparison of current change with (a) discrete (b) accumulative Cu(II) concentration injection between sludge and control group. Experimental Condition: pH = not controlled; Ionic strength = not controlled; Temperature = 25°C; Electrodes = graphite plates; t_{exp} = 1 min. Substrate contains 2.08 mM C_{6}H_{12}O_{6}, 1.43 mM (NH_{4})_{2}SO_{4}, 0.13 mM K_{3}PO_{4}, and 1.79 mM NaHCO_{3}.

The result of respective heavy metal toxicity test was established in two distinct figures. Figure 4.10 (a) shows the current variation with metal concentration in each
injection. It is obvious that current change rose to greatest value then dropped down with the increasing concentration. With the Cu(II) concentration under 0.3 mM, activated sludge had greater current change than the sample without sludge. The control group reached to the highest point, 3.44 μA, at the concentration of 0.64 mM.

Another result expression displays in figure 4.10 (b) comparing the accumulative current change with accumulative metal concentrations between activated sludge sample and control sample. The figure implies the sludge in anodic chamber was barely affected by current variation. The accumulative current experimented a noticeable increase, which contributed large current change to 12 μA in both treatment and control group. This clearly indicates that current change was independent from activated sludge in Cu(II) toxicity test.

4.3.1.2 Nickel

The result shown in different heavy metal was completely dissimilar. Figure 4.11 (a) presents Ni(II) concentration verse current change, which hardly to find a relative top point as same as the result in Cu(II). In addition, the current fluctuation, ranged from 0.14 to 0.46 μA, was quite smaller than Cu(II) did. There was no significant trend in current change with increasing Ni(II) concentration, especially in sludge sample that ranged from 0.15 μA at the initial point to 0.24 μA at the end point.

The accumulative current variation exhibits in figure 4.11 (b). An obvious increasing slope are shown in both experiment and control sample when Ni(II) accumulative concentration was below 2.5 mM. However, the total accumulative current change was still minor after 3.2 mM Ni(II) injection. The total accumulative current change in control sample was 2.5, which was twice larger than sludge sample.
Figure 4.11 Comparison of current change with (a) discrete (b) accumulative Ni(II) concentration injection between sludge and control group. Experimental Condition: pH = not controlled; Ionic strength = not controlled; Temperature = 25°C; Electrodes = graphite plates; Δt_{exp} = 1 min. Substrate contains 2.08 mM C_8H_{12}O_6, 1.43 mM (NH_4)_2SO_4, 0.13 mM K_3PO_4, and 1.79 mM NaHCO_3.
4.3.1.3 Zinc

Figure 4.12 Comparison of current change with (a) discrete (b) accumulative Zn(II) concentration injection between sludge and control group. Experimental Condition: pH = not controlled; Ionic strength = not controlled; Temperature = 25°C; Electrodes = graphite plates; Δt_irp = 1 min. Substrate contains 2.08 mM C₆H₁₂O₆, 1.43 mM (NH₄)₂SO₄, 0.13 mM K₃PO₄, and 1.79 mM NaHCO₃.
Zinc toxicity effect of current fluctuation is presented in figure 4.12 (a). The current change increased gradually when Zn(II) concentration beneath 0.64 mM. The control group without activated sludge had larger current change than experiment group, and approached to 0.93 μA at Zn(II) concentration of 0.16 mM.

Figure 4.12 (b) shows the accumulative current change in Zn(II) toxicity test. The difference between sample with and without activates sludge is conspicuous. The accumulative current variation achieved to 2.3 in control sample, which was nearly 80% greater than sample with activated sludge. This indicates that sludge addition declined the current variation instead enhancement.

4.3.1.4 Lead

Different from the results shown in the previous heavy metals that current change reached to a top point and dropped down, the current variation increased with the Pb(II) concentration augmentation (figure 4.13 (a)). The greatest current change experimented at the Pb(II) concentration of 1.28 mM; however, the standard deviation was big. In addition, control group provided 60% larger current fluctuation than experiment sample with activated sludge.

The accumulative current variation with Pb(II) concentration addition is presented in figure 4.13 (b). The equality of current fluctuation between sludge and blank appeared at low accumulative Pb(II) concentration. The blank sample contributed greater current variation after Pb(II) concentration was above 0.64 mM. Besides, the reproducibility of current change data was not as good as other metals with sludge.
Figure 4.13  Comparison of current change with (a) discrete (b) accumulative Pb(II) concentration injection between sludge and control group. Experimental Condition: pH = not controlled; Ionic strength = not controlled; Temperature = 25°C; Electrodes = graphite plates; $\Delta t_{\text{exp}} = 1$ min. Substrate contains 2.08 mM $\text{C}_6\text{H}_{12}\text{O}_6$, 1.43 mM $(\text{NH}_4)_2\text{SO}_4$, 0.13 mM $\text{K}_3\text{PO}_4$, and 1.79 mM $\text{NaHCO}_3$. 
4.3.1.5 Cadmium

Figure 4.14 Comparison of current change with (a) discrete (b) accumulative Cd(II) concentration injection between sludge and control group. Experimental Condition: pH = not controlled; Ionic strength = not controlled; Temperature = 25°C; Electrodes = graphite plates; Δt_{exp} = 1 min. Substrate contains 2.08 mM C₆H₁₂O₆, 1.43 mM (NH₄)₂SO₄, 0.13 mM K₃PO₄, and 1.79 mM NaHCO₃.
Figure 4.14 (a) shows the current variation with various concentration of Cd(II) injection. The current change was limited with comparison of other metal solutions. The range of current change with Cd(II) concentration was from 0.01 µA to 0.07 µA while control group provided even less.

The big distinctness of accumulative current fluctuation between Cd(II) and the metal mentioned above was that sample with activated sludge contributed greater current fluctuation than control sample. (figure 4.14 (b)) However, the total accumulative current change after 3.2 mM Cd(II) injection was lower than 0.4 µA. This implies that Cd(II) addition had poor influence in current change.

4.3.1.6 Cobalt

For all the heavy metal examined in the toxicity test, Co(II) contributed the most negligible current variation. In figure 4.15 (a), the current variation with activated sludge was near zero even Co(II) concentration increased to 1.28 mM. Typically, the current reduced when metal solution injected into system. The current, however, in Co(II) blank experiment rose when Co(II) concentration over 1.28 mM (A negative current change shown in figure 4.15 (a) with high Co(II) concentration injection represented an increase in current).

The enhanced current resulted in negative current change with accumulative Co(II) concentration in control group. The accumulative current fluctuation in sample with activated sludge was approximately to 0.07 µA, which was the smallest current change of all tested metal.
Figure 4.15 Comparison of current change with (a) discrete (b) accumulative Co(II) concentration injection between sludge and control group. Experimental Condition: pH = not controlled; Ionic strength = not controlled; Temperature = 25°C; Electrodes = graphite plates; $\Delta t_{exp} = 1$ min. Substrate contains 2.08 mM C$_6$H$_{12}$O$_6$, 1.43 mM $(\text{NH}_4)_2\text{SO}_4$, 0.13 mM K$_3$PO$_4$, and 1.79 mM NaHCO$_3$. 
4.3.1.7 Iron

Figure 4.16 Comparison of current change with (a) discrete (b) accumulative Fe(II) concentration injection between sludge and control group. Experimental Condition: pH = not controlled; Ionic strength = not controlled; Temperature = 25°C; Electrodes = graphite plates; Δ$t_{exp}$ = 1 min. Substrate contains 2.08 mM C$_6$H$_{12}$O$_6$, 1.43 mM (NH$_4$)$_2$SO$_4$, 0.13 mM K$_3$PO$_4$, and 1.79 mM NaHCO$_3$. 
In Fe(II) toxicity test, the results are totally contrasting to other heavy metals. The current increased instead of decrease with Fe(II) addition whether sludge existed or not. The current change rose from 0.1 µA at low Fe(II) concentration to 1.35 µA at high Fe(II) concentration with activated sludge. In control sample, the current change was twice larger than experimental sample, which approached to 3.16 µA at end point.

Comparing the accumulative current change between sludge and control group, the blank sample had higher value. The accumulative current variation gradually exaggerated with the accumulative Fe(II) concentration in sludge sample while it reached to a steady level of 8 µA in blank sample. It is assumed that Fe(II) would oxidized to a relatively stable species, Fe(III), and generate electrons to increase the current output. Reaction 4.2 indicates Fe(II) oxidation reaction occurred at anode, and reaction 4.3 shows oxygen reduction reaction at cathode. The overall reaction (reaction 4.4) has a positive \( E_{\text{cell}} \) value of 0.458 V, which could self-produce electricity in the system.

**Anode:** \( \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + e^- \quad E_{\text{ca}} = -0.771 V \) (4.2)

**Cathode:** \( \text{O}_2(g) + 4\text{H}^+ + 4e^- \rightarrow 2\text{H}_2\text{O}(l) \quad E_{\text{ref}}^o = 1.229 V \) (4.3)

**Overall:** \( 4\text{Fe}^{2+} + \text{O}_2(g) + 4\text{H}^+ \rightarrow 4\text{Fe}^{3+} + 2\text{H}_2\text{O}(l) \quad E_{\text{cell}}^o = 0.458 V \) (4.4)

### 4.3.1.8 Manganese

Even though current slightly rose at low Mn(II) concentration, the sludge sample exhibited an obvious slope of current variation with Mn(II) increasing injection and the value reached to 0.033 µA at Mn(II) concentration of 1.28 mM. The
control test had the same trend as experiment sample, which current increased at the beginning and dropped to 0.07 μA at the end.

![Graph](image)

**Figure 4.17** Comparison of current change with (a) discrete (b) accumulative Mn(II) concentration injection between sludge and control group. Experimental Condition: pH = not controlled; Ionic strength = not controlled; Temperature = 25°C; Electrodes = graphite plates; Δt exp = 1 min. Substrate contains 2.08 mM C₆H₁₂O₆, 1.43 mM (NH₄)₂SO₄, 0.13 mM K₃PO₄, and 1.79 mM NaHCO₃.
The accumulative current change displays in figure 4.17 (b). Both experimental and control group had a light growth of current change with the accumulative Mn(II) concentration from 0.032 mM to 0.64 mM. When the accumulative concentration of Mn(II) was above 0.64 mM, the current started declined. Compared with the active sludge sample that had an accumulative current change of 0.08 μA, the blank sample revealed a wider current variation, which approximately to 0.18 μA.

The current variations of eight different divalent metals are discussed respectively through discrete and accumulative concentration of metal ions. The calculated current difference is between the current of prior injection and the current of after injection. However, the value of prior injection current has considerable relevance to the concentration of metal ions that have been already injected into system. Besides, it is difficult to apply accumulative current variation data for realistic monitoring situation. Therefore, the value of x-axis and y-axis are adjusted to make the diagrams more useful in practical detection.

In figure 4.18 (a), the x-axis represents the difference of metal concentration between two injections, and y-axis is current variation between injection before and after. On the other hand, x-axis is adjusted to accumulative concentration of metal ions in figure4.18 (b). The comparison of figure 4.18 (a) and (b) demonstrates that the current variation is more related to the increased dosage of metal ions than the total concentration of metal ions. The current declines with the difference of metal concentration increases except Fe(II) ions. There is no apparent changes of current in Cd(II), Co(II), and Mn(II) ions.
Figure 4.18  The current variation of (a) metal concentration difference (b) accumulative metal concentration in different divalent metal ions. Experimental Condition: pH = not controlled; Ionic strength = not controlled; Temperature = 25°C Electrodes = graphite plates; $\Delta t_{\text{exp}} = 1$ min. Substrate contains 2.08 mM C₆H₁₂O₆, 1.43 mM (NH₄)₂SO₄, 0.13 mM K₃PO₄, and 1.79 mM NaHCO₃.
4.3.2 Trivalent metals

Unlike using a bunch of divalent metals, three trivalent metals, including chromium, iron, and aluminum, were experimented for toxicity tests. All of the metals were prepared as 1M metal perchlorate solutions, and injected into system with different dosages. The dilute effect could be neglected due to the injection volume cost 0.32% of working volume.

4.3.2.1 Chromium

The current change with different concentration of Cr(III) addition in sludge and control sample are shown in figure 4.19 (a). There was no clear current variation when Cr(III) concentration was less than 0.32 mM. The current declined noticeably after Cr(III) concentration surpassed 0.64 mM in experimental and control sample.

In figure 4.19 (b), it exhibited the accumulative current fluctuation with accumulative Cr(III) concentration in the anodic chamber. The accumulative current fluctuation gradually increased with the accumulating Cr(III) concentration. The total current change approached to 1.58 μA and 2.39μA in sludge and blank sample respectively.
Figure 4.19  Comparison of current change with (a) discrete (b) accumulative Cr(III) concentration injection between sludge and control group. Experimental Condition: pH = not controlled; Ionic strength = not controlled; Temperature = 25°C; Electrodes = graphite plates; $\Delta t_{exp} = 1$ min. Substrate contains 2.08 mM C$_6$H$_{12}$O$_6$, 1.43 mM (NH$_4$)$_2$SO$_4$, 0.13 mM K$_3$PO$_4$, and 1.79 mM NaHCO$_3$. 
Iron is the only heavy metal that obtained divalent and trivalent ions examined in toxicity test. Contrary to the results shown in figure 4.20 (a), Fe(III) injection resulted
in a large current decrease. In experiment group, the current dropped from 0.003 μA at Fe(III) concentration of 0.032 mM to 3.06 μA at Fe(III) concentration of 1.28 mM. In addition, the control group without any sludge addition had greater current variation, which approached to 12.76 μA when 1.28 mM of Fe(III) injected into system.

Figure 4.20 (b) displays the accumulative current change with accumulative Fe(III) concentration addition. The blank sample had 4-times larger current change than sludge sample did. However, the accumulative current variation in sludge sample, 7.38 μA, was extremely larger than other metals except Cu(II). For iron metal, Fe(III) is the most stable metal ions in environment. If Fe(II) compounds is injected into system, it would first be oxidized to Fe(III) by releasing electrons to others and form a more stable Fe(III) type. This could explains why current increased when Fe(II) injection and began declined when Fe(III) addition.

4.3.2.3 Aluminum

The last metal experimented in toxicity test is Al(III). The current fluctuation in Al(III) injection is shown in figure 4.21 (a) was insignificant compared with other two trivalent metals. Different from the results of Cr(III) and Fe(III) that current change had direct proportion with metal concentration injection, the current change in both experimental and control group gradually increased when Al(III) concentration reached to 0.64 mM and descended at the 1.28 mM Al(III).
Figure 4.21 Comparison of current change with (a) discrete (b) accumulative Al(III) concentration injection between sludge and control group. Experimental Condition: pH = not controlled; Ionic strength = not controlled; Temperature = 25°C; Electrodes = graphite plates; Δt_{exp} = 1 min. Substrate contains 2.08 mM C_6H_{12}O_6, 1.43 mM (NH_4)_2SO_4, 0.13 mM K_3PO_4, and 1.79 mM NaHCO_3.

The accumulative current change result is exhibited in figure 4.21 (b). The trend of blank sample and sludge sample were familiar with the tendency in Cr(III) and
Fe(III), which control sample had a larger accumulative current variation than activated sludge contained sample. In addition, the accumulative current change in Al(III) increased rapidly with increasing accumulative Al(III) concentration and the variation rate slowed down after accumulative Al(III) concentration was above 1.28 mM. The final accumulative current variation met 0.52 µA and 0.86 µA in sludge and blank sample individually.

Figures are modified for practical application in three trivalent metal ions. By comparing figure 4.22 (a) and (b), the current variation shows more relevant to difference of metal concentration than accumulative concentration. Fe(III) contributes 10-times larger than Cr(III) and Al(III) ions.
Figure 4.22  The current variation of (a) metal concentration difference (b) accumulative metal concentration in different trivalent metal solutions. Experimental Condition: pH = not controlled; Ionic strength = not controlled; Temperature = 25°C; Electrodes = graphite plates; $\Delta t_{\text{exp}} = 1$ min. Substrate contains 2.08 mM C$_6$H$_{12}$O$_6$, 1.43 mM (NH$_4$)$_2$SO$_4$, 0.13 mM K$_3$PO$_4$, and 1.79 mM NaHCO$_3$. 
4.4 Comparisons and Discussion

To comprehensively understand the difference of current variation between various metal ions, three main influences, substrate, metal ions, and pH effect, would be discussed respectively.

4.4.1 Substrate Effect

Four fundamental substrates, including glucose, ammonium sulfate, potassium phosphate, and sodium bicarbonate, were experienced separately without activated sludge addition as it shown in figure 4.6. The result of current change demonstrates that glucose and ammonium sulfate contributed little current generation in the system; on the other hand, potassium phosphate and sodium bicarbonate provided most of the current production. Although activated sludge was injected to biosensor reactor, the current change altered insignificantly. It is assumed that residual substrates that consumed by microorganisms incompletely were added to biosensor reactor with activated sludge, and increased the concentration of substrate. This hypothesis indicates that the current measured in this system produced mainly from electrochemical reaction between chemicals instead of biological oxidation mechanisms.

In electrochemistry field, Nernst equation gives a formula that calculates the electromotive force of the full cell at different temperature and concentration of chemicals at equilibrium. Full cell and half-cell of Nernst equations are as follows:

\[
\text{Full Cell: } E_{\text{cell}} = E_{\text{cell}}^{\circ} - \frac{RT}{nF} \ln Q
\]  

(4.5)
Anode: \[ E_{\text{ex}} = E_{\text{ox}}^\circ - \frac{RT}{nF} \ln \frac{[O]}{[R]} \] 

Cathode: \[ E_{\text{red}} = E_{\text{red}}^\circ - \frac{RT}{nF} \ln \frac{[R]}{[O]} \] \[ \text{(4.7)} \]

In equation 4.5 to 4.7, E is the potential (electromotive force) at certain temperature; \( E^\circ \) represents the standard potential at standard state, which is at the condition of 25°C, 1 atm pressure, and with solute concentration of 1M; R is universal gas constant (R=8.314 J/K mol); T is the absolute temperature in unit K; n represents the number of transferred electrons; F is Faraday constant, 96485 C/mol; Q is the reaction quotient; [O] and [R] represents the concentration of oxidant and reductant.

The electrochemical cells can be divided into two categories: electrolytic cells and galvanic cells. Electrolytic cells need external power supply while galvanic cells generate power by themselves. It is not necessary for extra power input in this biosensor, which yields electric power spontaneously. Therefore, the MFC-based biosensor is regarded as a galvanic cell.

There is a spontaneous redox reaction proceeded and provides cell an electric potential in galvanic cell system. According to the Gibbs free energy equation (equation 4.8), \( \Delta G \) should be negative if the reaction is spontaneous. Besides, the standard cell potential (\( E_{\text{cell}}^\circ \)) is the sum of two half-cell standard potentials (equation 4.9). Therefore, the total of \( E_{\text{ex}}^\circ \) and \( E_{\text{red}}^\circ \) must be positive.

\[ \Delta G_{\text{cell}}^\circ = -nFE_{\text{cell}}^\circ \] \[ \text{(4.8)} \]

\[ E_{\text{cell}}^\circ = E_{\text{ex}}^\circ + E_{\text{red}}^\circ \] \[ \text{(4.9)} \]
Table 4.1 lists some possible half reactions and standard reduction potentials in this biosensor system. Oxygen is a good oxidant due to its high oxidation state. In this system, cathodic chamber merely consists of DI water with air aeration. Oxygen is the most possible electron acceptor to consumed electrons generated from anode. Oxygen plays a role of oxidant and reduces itself to form water in the cathode. In order to keep $E^0_{cell}$ in positive values, the oxidative potential must be higher than -1.23 V. However,
some chemical anions, such as perchlorate, sulfate, and phosphate, are in the highest oxidation state. They have no abilities to donate electrons. In addition, cations including sodium and potassium could contribute electrons and oxidized to solid phase while oxidation potential is lower than -1.23 V. The achievable electron donors that drive cell a spontaneous power output are glucose and ammonium.

Glucose could be oxidized completely to carbon dioxide, and generate protons and electrons. These produced protons and electrons were recombined with oxygen and reduced to water. Two half reactions and full cell reaction are shown as follow:

**Anode:** \( C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^- \quad E_{an}^{\circ}(W) = 0.43V \) (4.10)

**Cathode:** \( O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \quad E_{red}^{\circ}(W) = 0.82V \) (4.11)

**Overall:** \( C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \quad E_{cell}^{\circ}(W) = 1.25V \) (4.12)

Theoretical cell potential can be calculate by Nernst equation. The standard cell potential should be adjusted by pH. Due to the standard glucose oxidation potential is measured at pH 7, it is assumed that the system in the condition of pH 7. However, the concentration of glucose and the partial pressure of gases at equilibrium are unknown. It is hard to predict the overall potential produced by glucose oxidation in the system when reaction is balanced.

**Anode:** \( E_{an} = (0.43) - \frac{0.05916}{24} \log \frac{P_{CO_2}^5}{[C_6H_{12}O_6]} \) (4.13)

**Cathode:** \( E_{red} = (0.82) - \frac{0.05916}{4} \log \frac{1}{P_{O_2}} \) (4.14)

**Overall:** \( E_{cell} = E_{an} + E_{red} = (1.25) - \frac{0.05916}{4} \log \frac{P_{CO_2}}{[C_6H_{12}O_6]^6 P_{O_2}} \) (4.15)
Similarly, the value of generated potential from ammonium oxidation is unpredictable since the equilibrium concentration of nitrate and nitrite in biosensor reactor are uncertain. The overall standard cell potential of ammonium oxidation is positive at pH 6 (equation 4.21). According to figure 4.8, the accumulative current remained unchanged with metal toxicants injection when electrolyte contained glucose or ammonium sulfate individually. It is considered that the primary current measured by electrometer is not associated with glucose or ammonium oxidation at anode, even though positive standard potentials contribute spontaneous redox reaction hypothetically. There should be other causes that predominate the current changes.

\[ \text{Anode: } NH_4^+ + 2H_2O \rightarrow NO_2^- + 8H^+ + 6e^- \quad E_{\text{ox}}^0 = -0.90V \quad (4.16) \]

\[ \text{Cathode: } O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \quad E_{\text{red}}^0 = 1.23V \quad (4.17) \]

\[ \text{Overall: } 2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 2H_2O + 4H^+ \quad (4.18) \]

\[ \text{Anode: } E_{\text{ox}} = \{ -0.90 - \frac{8 \times 0.05916}{6} \log[H^+] - \frac{0.05916}{6} \log[NH_4^+] \} \quad (4.19) \]

\[ \text{Cathode: } E_{\text{red}} = \{ 1.23 + \frac{4 \times 0.05916}{4} \log[H^+] - \frac{0.05916}{4} \log[O_2] \} \quad (4.20) \]

\[ \text{Overall: } E_{\text{cell}} = E_{\text{ox}} + E_{\text{red}} = \{ -0.43 + 0.88 \} - \frac{0.05916}{12} \log[NH_4^+] - \frac{0.05916}{12} \log[NO_2^-] \quad (4.21) \]

\[ = \{ 0.45 \} - \frac{0.05916}{12} \log[NH_4^+] - \frac{0.05916}{12} \log[NO_2^-] \]

4.4.2 pH Effect

In this toxicity analysis experiment, the biosensor device consisted of two compartments separated by a proton exchange membrane, which allowed only proton
pass through. In anodic chamber, activated sludge and synthetic wastewater were added as well as metal perchlorate pollutants. On the other hand, cathodic only comprised DI water. It is hypothesized that the produced current may derive from the concentration gradient of proton, which indicates that the biosensor device is a concentration cell.

Concentration cell is a kind of galvanic cell which can generate electric power spontaneously. Two half reactions at anode and cathode are equivalent except the concentration of chemicals. The standard oxidation potential at anode is the inverse number of standard reduction potential at cathode, and results in zero standard cells potential (reaction 4.22 to 4.24). The generated potential results from different concentration of reactants between two compartments. The subscript (a) and (c) in reaction 4.22 to 4.24 distinct reactants concentration in anodic and cathodic chamber.

**Anode:** \( \text{2H}_2\text{O} \rightarrow \text{O}_{2(c)} + 4\text{H}^+_{(c)} + 4\text{e}^- \quad E^{\circ}_{\text{ox}} = -1.23\text{V} \) \hspace{1cm} (4.22)

**Cathode:** \( \text{O}_{2(c)} + 4\text{H}^+_{(c)} + 4\text{e}^- \rightarrow 2\text{H}_2\text{O} \quad E^{\circ}_{\text{red}} = 1.23\text{V} \) \hspace{1cm} (4.23)

**Overall:** \( \text{O}_{2(c)} + 4\text{H}^+_{(c)} \rightarrow \text{O}_{2(c)} + 4\text{H}^+_{(c)} \quad E^{\circ}_{\text{cell}} = 0\text{V} \) \hspace{1cm} (4.24)

\[
\begin{align*}
\text{Anode:} & \quad E^{\circ}_{\text{ox}} = -1.23 - \frac{0.05916}{4} \log \frac{P_{\text{O}_{2(c)}}}{P_{\text{H}^+_{(c)}}} \\
\text{Cathode:} & \quad E^{\circ}_{\text{red}} = 1.23 - \frac{0.05916}{4} \log \frac{1}{P_{\text{O}_{2(c)}}} \\
\end{align*}
\]

(4.25) (4.26)

In addition, the developed potential can be calculated by Nernst equation. Two half-cell reactions are shown above. The full cell potential is the combination of oxidation potential and reduction potential, and standard cell potential is balanced off.
To simplify the equation, it is assumed that the oxygen partial pressure at equilibrium in both anode and cathode are the same. The final equation of full concentration cell can be abbreviated to equation 4.31. If pH in anode larger than pH in cathode, the reaction occurs spontaneously. On the contrary, the reaction won’t happen when the electrolyte in cathode has higher pH.

\[
E_{\text{cell}} = E_{\text{an}} + E_{\text{cat}} = E_{\text{cell}}^\circ - \frac{RT}{nF} \ln Q
\]  

(4.27)

\[
E_{\text{cell}} = (-1.23 + 1.23) - \frac{0.05916}{n} \log \frac{P_{O_2(a)}}{P_{O_2(c)}} - \frac{P_{H^+(a)}}{P_{H^+(c)}}
\]  

(4.28)

\[
E_{\text{cell}} = -0.05916 \left\{ \log P_{O_2(a)} + \log P_{H^+(a)} - \log P_{O_2(c)} - \log P_{H^+(c)} \right\}
\]  

(4.29)

\[
E_{\text{cell}} = -0.05916 \left( \log P_{O_2(a)} - \log P_{O_2(c)} \right)
\]  

(4.30)

\[
E_{\text{cell}} = 0.05916 \left( pH_{(a)} - pH_{(c)} \right)
\]  

(4.31)

To prove the hypothesis of pH effect, the theoretical pH of synthetic wastewater with different components has been calculated and displayed in figure 4.23. The pH in figure 4.23 presents the theoretical pH value in anodic chamber with metal perchlorate injection. It reveals that pH of glucose and ammonium sulfate were below 6 after copper perchlorate solution addition, and slightly decreased to pH 5 at the end point. Every other synthetic wastewater sample had various initial pH. The pH declined with increasing concentration of copper perchlorate solution, and the final pH set at the range between 5.8 and 6.2. Compared with figure 4.8 which exhibited the accumulative current change in different substrate components, the final pH is
associated with accumulative current variation. The higher the final pH in anodic compartment is, the larger the accumulative current fluctuation has.

![Figure 4.23](image)

**Figure 4.23** Theoretic pH variation in anodic chamber with Cu(II) addition in different substrate components. Concentration: 2.08 mM C₆H₁₂O₆; 1.43 mM (NH₄)₂SO₄; 0.13 mM K₃PO₄; 1.79 mM NaHCO₃. Substrate contains C₆H₁₂O₆, (NH₄)₂SO₄, K₃PO₄, and NaHCO₃.

Figure 4.24 correlates the pH in anode with accumulative current change in different substrate combination condition. An obvious negative correlation between theoretical pH in anode and accumulative current variation has been demonstrated except glucose and ammonium sulfate. The result corresponded to the assumption that pH would affect the current change. As equation 4.31 shows, the decreasing pH in anode side leads to fewer potential yield, and raises the current variation as well.
Figure 4.24  The relationship between theoretical pH in anodic chamber and accumulative current change contributed by substrate compositions in different synthetic wastewater. Experimental Condition: pH = not controlled; Ionic strength = not controlled; Temperature = 25°C; Electrodes = graphite plates; $\Delta t_{\text{exp}} = 1$ min. Concentration: 2.08 mM $C_6H_{12}O_6$; 1.43 mM $(NH_4)_2SO_4$; 0.13 mM $K_3PO_4$; 1.79 mM $NaHCO_3$. Substrate contains 2.08 mM $C_6H_{12}O_6$, 1.43 mM $(NH_4)_2SO_4$, 0.13 mM $K_3PO_4$, and 1.79 mM $NaHCO_3$.

However, accumulative current variation is irrelevant to pH change in anode with glucose and ammonium sulfate individually. It is supposed that the initial pH of glucose and ammonium sulfate fewer than 7 causes no influence in current change. Comparing with the pH of DI water in cathodic chamber, the electrolyte in anode was more acid than cathode, which means that the proton concentration is greater. The electricity would not be produced in the condition based on equation 4.31.
Figure 4.25  Theoretical pH in anode with Pb(II) perchlorate addition in different substrate proportion. 100% of substrate contains 4.16 mM C₆H₁₂O₆, 2.86 mM (NH₄)₂SO₄, 0.26 mM K₃PO₄, and 3.58 mM NaHCO₃.

The same consequence displays in figure 4.25. The theoretical pH reduces with the increasing concentration of Pb(II) perchlorate. As figure 4.9 presents, high proportion of substrate causes greater current change. However, high substrate percentage also leads to better pH buffer capacity, and reduce the pH variation. The sample containing one quarter of substrate with rest of tap water in figure 4.25 has the largest pH fluctuation but the smallest current change in figure 4.9. It is presumed that some factors other than pH influence current changes.
Figure 4.26  Theoretical ionic strength in anodic chamber with Pb(II) perchlorate addition in different substrate proportion. 100% of substrate contains 4.16 mM C$_6$H$_{12}$O$_6$, 2.86 mM (NH$_4$)$_2$SO$_4$, 0.26 mM K$_3$PO$_4$, and 3.58 mM NaHCO$_3$.

Ionic Strength measuring the concentration of ions in electrolyte is one of the main characteristics in electrolyte solution. The amount of electrolyte concentration badly influences in solubility, dissociation, and activity coefficient. Equation 4.6 and 4.7 establishes the Nernst equation at anode and cathode, respectively. The symbols of concentration of oxidant and reductant shown in the formula, however, are not conscientious. Due to the identity of activity coefficients at low species concentration, activities in the Nernst equation are usually simplified by concentrations. As equation 4.32 shows, activity is the product of activity coefficient and species concentration. Therefore, it is more thorough to use chemical activity of species instead of species concentration, and modified the Nernst equation of half-cell to equation 4.33 and 4.34.

\[ \alpha_x = \gamma_x C_x \quad (4.32) \]
Anode:  \[ E_{\text{ox}} = E_{\text{ox}}^{\circ} - \frac{RT}{nF} \ln \frac{a_{\text{ox}}}{a_{\text{red}}} \]  \( (4.33) \)

Cathode:  \[ E_{\text{red}} = E_{\text{red}}^{\circ} - \frac{RT}{nF} \ln \frac{a_{\text{red}}}{a_{\text{ox}}} \]  \( (4.34) \)

Before calculating chemical activity, the activity coefficient must be computed. The activity coefficients are related to ionic strength of chemical species. Ionic strength is a function of all the concentration of ions in solution multiples square of ionic charge number (equation 4.35). Typically, multivalent ions provide predominantly to the ionic strength. Through Debye–Hückel equation (equation 4.36) and ionic strength of solution, activity coefficient can be calculated. The extended Debye–Hückel equation (equation 4.37) are used when ionic strength is higher than 0.01 M. (For aqueous solutions at 25 °C; \( A = 0.51 \text{ mol}^{1/2}\text{dm}^{3/2}; B = 3.29 \text{ nm}^{-3} \text{mol}^{-1/2}\text{dm}^{3/2}; a \) is adjustable parameter which corresponds to the ionic size.)

\[ I = \frac{1}{2} \sum_{i=1}^{n} C_i Z_i^2 \]  \( (4.35) \)

\[ \log \gamma = -Az^2 \sqrt{I} \]  \( (4.36) \)

\[ \log \gamma = -Az^2 \frac{\sqrt{I}}{1 + Ba \sqrt{I}} \]  \( (4.37) \)

Figure 4.27 establishes the theoretical pH modified by ionic strength at anode. It shows that ionic strength rarely affects pH when considering activity coefficients. The trend of three different proportions of substrate is almost the same as figure 4.25. The result demonstrates that ionic strength of electrolyte solution would not influence the current changes.
Figure 4.27  Theoretical pH adjusted by ionic strength in anodic chamber with Pb(II) perchlorate addition at different substrate proportion. 100% of substrate contains 4.16 mM C\textsubscript{6}H\textsubscript{12}O\textsubscript{6}, 2.86 mM (NH\textsubscript{4})\textsubscript{3}SO\textsubscript{4}, 0.26 mM K\textsubscript{3}PO\textsubscript{4}, and 3.58 mM NaHCO\textsubscript{3}.

4.4.3 Metal Ions Effect

In toxicity test, eight divalent metal ions and three trivalent metal ions were experienced. All of them have been discussed in the previous sections individually. Here comes the integration of divalent and trivalent metal ions to compare the difference between each of them.

According to the data shown in figure 4.28 (a), all of the divalent metals lead to accumulative current decline except iron metal. It is assumed that Fe(II) is unstable, which easily supplies electron to others and form a relatively stable state as Fe(III). Therefore, divalent iron in the system played a role of electron donor, and caused accumulative current enhancement (∑ΔI at Y axis in figure 4.28 (a) represents accumulative current decrease after metal ions injection; Positive values mean current decline, and negative values mean current ascent.)
Figure 4.28  (a) The accumulative current change and (b) the accumulative current change per unit concentration of metal ions in different divalent metal concentration. Experimental Condition: pH = not controlled; Ionic strength = not controlled; Temperature = 25°C; Electrodes = graphite plates; Δt_{exp} = 1 min. Substrate contains 2.08 mM C₆H₁₂O₆, 1.43 mM (NH₄)₂SO₄, 0.13 mM K₃PO₄, and 1.79 mM NaHCO₃.

As figure 4.28 (a) describes, Cu(II) contributed the largest current fluctuation, which approximate 12 μA. The accumulative current changes in divalent metal ions
are listed below in sequence: zinc, lead, nickel, cadmium, manganese, cobalt, and iron. The range of current variation from zinc to cobalt was between 0.08 to 2.57 \( \mu \text{A} \). The increasing divalent metal concentration contributed little current change other than Cu(II) and Fe(II).

Figure 4.28 (b) depicts accumulative current change per unit metal concentration in different divalent metal ions. Most of divalent metals had higher current variation per metal concentration at low metal concentration. Fe(II) was the only exception that raised current variation per metal concentration with increasing metal concentration injection. On the basis of figures shown above, the sequence of current variation per metal concentration is the same as accumulative current change. Apart from Cu(II) and Fe(II), divalent metal ions make little contribution to current variation. One molar concentration of metal ion changes solely provides less than 5 mA changes.

The pH and ionic strength effect are also considered in heavy metal toxicity test. Figure 4.29 (a) and (b) displays the theoretical pH and ionic strength changes in anodic chamber with different concentration of divalent metal solution injection, respectively.

In figure 4.29 (a), copper solution addition causes the greatest pH changes. The value declines from pH 8 to 6.2. According to reaction 4.31, the produced electrical potential reduces when pH in anode declines. The accumulative current change proves the hypothesis that pH in anodic compartment would influence the amount of generated current. However, Pb(II) perchlorate injection, which leads to 1.5 units of pH variation, provides limited current changes. On the contrary, Zn(II) supplies higher current changes even though pH varies 0.66 units. Other metals contributing poor current changes have minor pH drop, which are under 0.5 pH units.
Figure 4.29  Theoretical (a) pH and (b) ionic strength variation in different divalent metal ions with accumulative metal ions concentration. Substrate contains 2.08 mM C₆H₁₂O₆, 1.43 mM (NH₄)₂SO₄, 0.13 mM K₃PO₄, and 1.79 mM NaHCO₃.

Comparing Ni(II) and Mg(II) metal ions in figure 4.29 (a), the pH variations are identical. However, similar pH variation does not bring about comparable results in
accumulative current changes. This indicates that not only pH but also other factors influence current variation. Ionic strength of anodic electrolytes is regard as an affect factor.

As figure 4.29 (b) exhibits, the metals with higher pH have larger ionic strength except Pb(II) perchlorate, which has the smallest ionic strength. Based on Debye–Hückel equation (reaction 4.36), activity coefficient is inverse relationship with ionic strength. Thus, activity of Pb(II) is larger than Cu(II) and other metals yet few differences. In conclusion, the cause of difference between accumulative current variation and pH change remains questionable after considering ionic strength effect. Other effect factors, such as activated sludge chelation, ion transport number, and concentration polarization, might influence the electrochemical reaction in system.

In addition to divalent metal ions comparison, the produced current difference between three trivalent metals is discussed as above. In trivalent metal toxicity test, iron ions contributed the greatest current variation but the standard deviation was large, too. Comparing with Cr(III), Fe(III) provided almost 5-times higher of current change, and 15-times larger than Al(III). Even though contrasting with all divalent metals shown in figure 4.28 (a), the accumulative current change supplied by trivalent iron ions exceeded most of them other than Cu(II).
Figure 4.30 (a) The accumulative current change and (b) the accumulative current change per unit concentration of metal ions in different trivalent metal concentration. Experimental Condition: pH = not controlled; Ionic strength = not controlled; Temperature = 25°C; Electrodes = graphite plates; $\Delta t_{exp} = 1$ min. Substrate contains 2.08 mM $\text{C}_6\text{H}_{12}\text{O}_6$, 1.43 mM ($\text{NH}_4$)$_2\text{SO}_4$, 0.13 mM $\text{K}_3\text{PO}_4$, and 1.79 mM $\text{NaHCO}_3$.

The only thing looks unusual is the curve line depicted in figure 4.30 (a). The accumulative current variation commonly increases immediately at low metal solution.
concentration, and retards to a smooth level at high concentration of metal solution. However, the current remained stable when Fe(III) concentration was under 1.5 mM, and had a sharp rise when Fe(III) surpassed 2.5 mM. It is determined that the current change increases with increasing Fe(III) concentration. The reason bring about this particular results may be membrane blocking by Fe(III) ions in anodic chamber. It was noticed that proton exchange membrane turned obscure after Fe(III) toxicity test. In addition, the reproducibility of Fe(III) toxicity test was obviously lower than normal. It is believed that Fe(III) having larger ionic radius inhibits proton transportation between two chambers and leads to extreme decrease of current output.

Three trivalent metals are also analyzed with accumulative current change per unit concentration of metal ions, which is presented in figure 4.30 (b). It displays similar trend as divalent metals in chromium ions while no significant varies in aluminum. Fe(III) had a opposite tendency with others as it shows in figure 4.30 (a), which provided 2.3 mA per unit molar To further understand what account for the results, pH and ionic impact are illustrated as follow.

The theoretical pH in trivalent metals decreases more severely than divalent metals with same accumulative concentration of metals, especially Fe(III) and Al(III).

It declines to pH 3.2 and pH 4.6 by adding 3.2mM concentration of Fe(III) and Al(III) injection, respectively. Extreme pH drop, however, does not lead to significant current change. It seems that theoretical pH variation with three trivalent metals has no direct relation with accumulative current change. The ionic strength of trivalent metals presented in figure 4.31 (b) has no obvious difference between three metal ions.
Figure 4.31 Theoretical (a) pH and (b) ionic strength variation in different trivalent metal ions with accumulative metal ions concentration. Substrate contains 2.08 mM C₆H₁₂O₆, 1.43 mM (NH₄)₂SO₄, 0.13 mM K₃PO₄, and 1.79 mM NaHCO₃.
Figure 4.32 The species fraction distribution with different concentration of trivalent metals in addition of (a) Cd(III) (b) Fe(III) (c) Al(III) perchlorate solution. Experimental Condition: Ionic strength = not controlled; Temperature = 25°C; Substrate contains 2.08 mM C₆H₁₂O₆, 1.43 mM (NH₄)₂SO₄, 0.13 mM K₃PO₄, and 1.79 mM NaHCO₃.

There are other characteristics besides pH variation that predominate current change in trivalent metals. According to figure 4.32 illustrating species fractions with pH in three trivalent metals, three trivalent metals prefer to form metal compounds than stay in free metal ions. There is no free Cr(III) ion existing in the solution of pH
higher than 4. Moreover, Fe(III) free ion species merely comprise less than 10% of total Fe(III) species. Al(III) species consist of more than 30% of Al(III) free ions, which is the highest percentage free ions in three trivalent metals.

Comparing species distribution with divalent metal ions displays in figure 4.33, free metal ions are the predominant species for all of eight divalent metals. The percentage of free metal ion mostly ranges between 60 and 80 whether at low or high metal concentration. In addition to free metal ions, other metallic species contribute less than 15 percentages. There are two exceptions, Cu(II) and Pb(II), in divalent metals, which account for low percentage of free metal ions at low metal concentration. Soluble metal carbonate is the predominant species that comprises almost 80 percent of total species distribution at low metal concentration.

The fluctuation of species fraction may influence the current variation. The metal that shift species distribution apparently would contribute noticeable current change. On the other hand, there is no significant current change with stable metal species distribution.
Figure 4.33 The species fraction distribution with concentration of divalent metals in addition of (a) Cu(II) (b) Ni(II) (c) Zn(II) (d) Pb(II) (e) Cd(II) (f) Co(II) (g) Fe(II) (h) Mn(II) perchlorate solution. Experimental Condition: Ionic strength = not controlled; Temperature = 25°C; Substrate contains 2.08 mM C₆H₁₂O₆, 1.43 mM (NH₄)₂SO₄, 0.13 mM K₃PO₄, and 1.79 mM NaHCO₃.
The metal speciation is calculated by Visual MINTEQ software. The distribution of metal complexes in different metal ion solutions can be measured. The results of metal speciation can be used in data modification. The concentration of total metal ions can be replaced by the concentration of free metal ions.

Theoretically, the metal complexes formed by metal ions and ligands contribute no current changes. The current is generated from free ions transporting in the solution. Therefore, it is more reasonable to plot current variation diagrams with concentration of free metal ions instead of concentration of total metal concentration. Figure 4.34 (a) and (b) depict the current variation with different free metal concentration in eight divalent metals.

With different distribution fraction of free metal ions, it provides distinct x values in different metal ion solutions. In diagrammatic modification of divalent metals, only Cu(II) and Pb(II) alter obviously. Most of the divalent metals are composed from 60% to 80% of free metal ions. However, Cu(II) consists of 2.5% to 60% Cu$^{2+}$, and Pb(II) contains 4.5% to 55% Pb$^{2+}$. There is no modification of free trivalent metals due to low percentages of free metal concentrations.
Figure 4.34 The current variation of (a) free metal concentration difference and (b) accumulative free metal concentration in different divalent metal solutions. Experimental Condition: pH = not controlled; Ionic strength = not controlled; Temperature = 25°C; Electrodes = graphite plates; Δt_{exp} = 1 min. Substrate contains 2.08 mM C₆H₁₂O₆, 1.43 mM (NH₄)₂SO₄, 0.13 mM K₃PO₄, and 1.79 mM NaHCO₃.
Figure 4.35  The relationship between the slope of current variation divided by free metal concentration difference and stability constant of divalent metal hydroxide.

The changes of x values in Cu(II) and Pb(II) cause an increasing in slope of curves displayed in figure 4.24. The slope is in a unit of μA/ mM, which represents how many current changes per one mM of free metal ion concentration. The larger the slope is, the greater the current variation occurs. It is assumed that the current variation has connection with the concentration gradient between two compartments, especially pH value. The relevance of current changes and pH fluctuation should be proved. Therefore, figure 4.35 has been developed to figure out their connection.

In figure 4.35, the current variation per unit free metal ion concentration difference is irrelevant to the equilibrium constant of metal hydroxide. All the slopes of current variation divided by free metal concentration difference in figure 4.34 are smaller than 0.5. In addition, the $r^2$ values of these linear regression equations are low. Table 4.2 presents the values of divalent metal regression equations in figure 4.34.
<table>
<thead>
<tr>
<th></th>
<th>Cu</th>
<th>Ni</th>
<th>Zn</th>
<th>Pb</th>
<th>Cd</th>
<th>Co</th>
<th>Fe</th>
<th>Mn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope</td>
<td>0.236</td>
<td>0.104</td>
<td>0.108</td>
<td>0.458</td>
<td>0.018</td>
<td>-0.052</td>
<td>-1.286</td>
<td>0.046</td>
</tr>
<tr>
<td>Intercept</td>
<td>1.828</td>
<td>0.149</td>
<td>0.367</td>
<td>0.057</td>
<td>0.039</td>
<td>0.027</td>
<td>-0.428</td>
<td>-0.006</td>
</tr>
<tr>
<td>$r^2$</td>
<td>0.013</td>
<td>0.86</td>
<td>0.055</td>
<td>0.925</td>
<td>0.06</td>
<td>0.782</td>
<td>0.628</td>
<td>0.886</td>
</tr>
</tbody>
</table>

**Table 4.2** The linear regression equation values of current variation curves in different divalent metals

**Figure 4.36** The relationship between the slope of current variation divided by metal concentration difference and stability constant of divalent metal hydroxide adapted from Wang’s group. [144]

The result also compares with other research to see the difference. In the research from Wang’s group, they used a p-benzoquinone-mediated amperometric biosensor to detect the toxicity of heavy metals, including Cu(II), Cd(II), Zn(II), Hg(II), Cr(VI) and Pb(II). [144] The results of current variation in five divalent metals injection are modified in the same form as figure 4.35. Even though the tested metal ions are the same, the metallic reagents used in Wang’s group are totally different. In addition, the experimental conditions, such as detecting device and the microbial species, are
distinct from the type we used in this experiment. The expression of metal concentration is another divergence that influences the data comparison. However, it shows no obvious distinction between figure 4.35 and figure 4.36. There is no direct correlation between the slope and stability constant of metal hydroxide.

In order to confirm the relationship between current changes and proton concentration variation, theoretical value of proton changed from current is calculated by conversion equation (equation 4.38). In equation 4.38, I is the current changed in a certain time period (coul/sec); T represents the detecting time period (second); F is the Faraday constant (96485 coul/mole); M is the theoretical proton concentration changed that contribute current variation (mole).

\[
\frac{I(\text{coul/sec}) \cdot T(\text{sec})}{F(\text{coul/mole})} = M(\text{mole})
\]  

(4.38)

**Figure 4.37** The theoretical and experimental proton concentration variation with different Cu(II) perchlorate concentration addition.
In this experiment, the current change detected by electrometer was in microampere level \((10^{-6} \text{ A})\), and the detecting duration took within 1 minute. The proton concentration variation is calculated by experiment data of current change with injection of metal perchlorate, and compares with the theoretical proton concentration. Here shows the example of divalent copper perchlorate solution. As figure 4.37 depicts, the theoretical proton changes differ by 2 orders of magnitude from experimental value. It seems that there is no variation of proton concentration with calculation from current change monitored by equipment.

Cu(II) contributed the largest current changes in this experiment. But the experimental value in Cu(II) injection was much lower than idea quantity, not to mention than other metals. It is supposed that the reactor in the system is a concentration cell which generates electricity spontaneously mainly by proton concentration gradient. However, some secondary factors influence the current production. To further understand the mechanism of current variation, other impacts, such as sludge interaction and membrane transport rate, should be considered.

The ultimate goal of this experiment is the application of biosensor in WWTPs for continuous detection. In order to distinguish different metal ion species and the concentration of heavy metal ions, some mathematical equations have been developed to predict the presence of distinct metal ion species in real water samples. These equations shown in table 4.3 represent the trends of current variation with accumulative concentration of metal ions. Each metal, including divalent and trivalent metals, acquires their own fitting equation which establishes an index to tell the difference of metal species.
Table 4.3 The fitting curve equations and $r^2$ values in different metal ions

<table>
<thead>
<tr>
<th>Metal species</th>
<th>Equations</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu(II)</td>
<td>$y = 2.6191 \ln(x) + 8.7274$</td>
<td>0.9824</td>
</tr>
<tr>
<td>Ni(II)</td>
<td>$y = 0.2475 \ln(x) + 0.8414$</td>
<td>0.9343</td>
</tr>
<tr>
<td>Zn(II)</td>
<td>$y = 0.5702 \ln(x) + 1.8185$</td>
<td>0.9615</td>
</tr>
<tr>
<td>Pb(II)</td>
<td>$y = 0.2987 \ln(x) + 0.768$</td>
<td>0.8518</td>
</tr>
<tr>
<td>Cd(II)</td>
<td>$y = 0.0768 \ln(x) + 0.2197$</td>
<td>0.8981</td>
</tr>
<tr>
<td>Co(II)</td>
<td>$y = 0.0125 \ln(x) + 0.067$</td>
<td>0.6574</td>
</tr>
<tr>
<td>Fe(II)</td>
<td>$y = -1.3811 \ln(x) - 3.5875$</td>
<td>0.8672</td>
</tr>
<tr>
<td>Mn(II)</td>
<td>$y = 0.0181 \ln(x) + 0.0265$</td>
<td>0.6245</td>
</tr>
<tr>
<td>Cr(III)</td>
<td>$y = 0.3535 \ln(x) + 0.9503$</td>
<td>0.8461</td>
</tr>
<tr>
<td>Fe(III)</td>
<td>$y = 0.039 e^{1.7685x}$</td>
<td>0.8044</td>
</tr>
<tr>
<td>Al(III)</td>
<td>$y = 0.1326 \ln(x) + 0.2953$</td>
<td>0.8095</td>
</tr>
</tbody>
</table>

All the equations displayed in table 4.3 perform the current change tendency with different heavy metal ions injection. Most of the equations are fitted by logarithmic type except Fe(III), which is exponential type. $r^2$ values are shown to demonstrate the correspondence of trend equations and experimental data points. Excluding Co(II) and Mn(II), $r^2$ values of fitting equation exceed 0.8 in all metal ions. However, Cu(II), Ni(II), and Zn(II) are the only three metal ions which obtain $r^2$ value above 0.9.

Figure 4.38 depicts the partial enlargement of accumulative current change in different divalent metal ions. It ignores extreme data points, Cu(II) and Fe(II), and extends the difference between other divalent metals. As the figure 4.38 shows above, Zn(II) has relatively larger current change. Next to Zn(II), Ni(II) and Pb(II) contribute the largest current variation. Unfortunately, the error bar of Zn(II) and Pb(II) are
expansive which have low quality of reproducibility. In addition, $r^2$ values presented in table 4.3 illustrate that the fitting curve equations are not suitable for experimental results. Further experimental parameters need to be modified and considered for the sake of characterizing different metal ions in the samples.

![Graph](image)

**Figure 4.38** Partial enlargement of accumulative current change in different divalent metal concentration. Experimental Condition: pH = not controlled; Ionic strength = not controlled; Temperature = 25°C; Electrodes = graphite plates; $\Delta t_{\text{exp}} = 1$ min. Substrate contains 2.08 mM C₆H₁₂O₆, 1.43 mM (NH₄)₂SO₄, 0.13 mM K₃PO₄, and 1.79 mM NaHCO₃.

The fitting equation not only can differentiate various metal ion species but also can predict the concentration of metal ions. The slope of straight line that is tangent to the curve equations can estimate the total metal ionic concentration. Most of the current change curve equations shown in table 4.3 are logarithmic curve, which the slope of tangent line decreases with the increasing metal ionic concentration (x value). Each tangential point of the curve corresponds to a specific metallic concentration. However, this estimating method can only apply to the metals that have obvious and
only one bend. Linear line doesn’t have slope variations; polynomial curve obtaining order above 3 doesn’t have one-to-one correspondence values.

Therefore, the metal species and metal ionic concentration can be predicted by fitting curve equations and the slope of tangent lines in this system. However, the accuracy of calculation is not good enough due to large error bar and low \( r^2 \) values. To improve the precision, more experimental parameters should be considered and adjusted to increase the reproducibility.
Chapter 5

CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

In activated sludge cultivation stage, four main parameters including pH, dissolved oxygen (DO), mixed liquor suspended solid (MLSS) and chemical oxygen (COD) were detected. According to all of monitoring data of activated sludge cultivation for more than one month, microorganisms in column 1 is the best choice for toxicity test in MFC-like biosensor. Therefore, the activated sludge utilized in biosensor toxicity monitoring experiment is selected from column 1 with DO concentration roughly 2 ~ 6 mg/L, MLSS ranged from 2500 to 3000 mg/L, and C: N: P ratio of 100: 5:1 in nearly 99% COD removal efficiency. In addition, the toxicity detection experiment is operated 3 hours after influent refilling based on the results of DO variation verses time.

Based on the data of control experiment without activated sludge addition, the major electric current is generated from substrates in synthetic wastewater themselves instead of being originated by microbial metabolism of activated sludge. It is predicted that some cations with smaller ionic radius had ability of passing through Nafion® 117 membrane used in the biosensor device.

Moreover, the proof to explain why substrates with activated sludge increased 20% of current change in Cu(II) perchlorate solution addition are considered. The hypothesis of current change enhancement is that residual substrates consumed
incompletely entered biosensor reactor with activated sludge mixed liquor and increased the concentration of substrates. The current variation with sludge addition also depends on metal species.

According to metal toxicity test in eight divalent metals and three trivalent metals, Cu(II) contributes the largest current changes with addition of different metal ion concentration. Excluding the possibility of oxidation of glucose and ammonia sulfate, the most probability that generates electricity spontaneously from biosensor is concentration gradient between anodic and cathodic electrolytes. To confirm the biosensor as a concentration cell, the theoretical pH variation is calculated and compares with current changes of metal perchlorate injection. Based on equation 4.31, the produced electrical potential declines with the pH in anode decreases. It is supposed that the initial pH of glucose and ammonium sulfate at pH 7 causes no influence in current change. In addition, the current drop derives from the same reason as pH of electrolyte in anode decline with increasing metal perchlorate concentration.

Furthermore, according to divalent metal results, the current raises instead of declines with addition of Fe(II) perchlorate solution. It is assumed that Fe(II) is unstable, which easily supplies electron to others and form a relatively stable state as Fe(III). Therefore, Fe(II) in the system plays a role of electron donor, and causes accumulative current enhancement.

Even though theoretical pH variation has correlation with accumulative current changes in divalent metals, there is no significant influence of current changes that corresponds with pH variation in trivalent metals. Some other factors, such as ionic strength and metal ionic species distribution, are considered. With the calculation of Debye–Hückel equation (equation 4.36), ionic strength affects activity coefficients barely. On the other hand, metal free ions are the predominant species in most of the
divalent metals and the species distributions are steady with increasing metal ion concentration, except Cu(II) and Pb(II). It is hypothesized that the fluctuation of species fraction may influence the current variation. The metal that shift species distribution apparently would contribute noticeable current change. On the other hand, there is no significant current change with stable metal species distribution.

Theoretical proton concentration variation is calculated by equation 4.38, and compares with experimental results to comprehend the difference between ideal model and realistic situation. The experimental data contribute two order of magnitude differences with theoretical values. It is assumed that some secondary factors, such as activated sludge interaction and ionic membrane transportation would influence electrochemical signal fluctuation.

The accumulative current variation curves of eleven metal ions have been fitted as mathematical equations to differentiate the presence of different heavy metal species. Most of the curves are fitted by logarithmic type except Fe(III) fitted by exponential type. Only three of all metals have $r^2$ values higher than 0.9, which means that the fitting equations are not equitable to the experimental data points. In addition, the fitting equations and the slope of tangent lines can predict the metallic concentration. Each tangential point of the curve corresponds to a specific metallic concentration. However, the accuracy of prediction is not good enough due to wide error bar of accumulative current variation. To improve the precision of metal species and metal ionic concentration, more experimental parameters, such as microbial species, structure of biosensor, and membrane selectivity, should be modified and considered deliberately.
5.2 Future Work

The ultimate goal of this research is to apply this MFC-based biosensor to realistic WWTPs and operates continuous detection of wastewater quality. However, the results of the experiment can only tell the presence of different metal ions and the concentration imprecisely. Some of the experimental parameters can be modified and make the biosensor detection more accurate.

In this experiment, activated sludge is used as a microbial catalyst. However, the microbial metabolism in activated sludge is complex. There are numerous of microbial species in activated sludge that influence the electron transfer directly to electrode surface. As the experimental results mentioned previously, microbes contribute little electrical power in this system. Pure microbial culture utilizing may increase the electricity production and improve the sensitivity of biosensors.

During the experimental process, it is discovered that current signals varies seriously when only external circuit wire changes. Since the electrochemical signals detected by electrometer are small in microampere unit, a minor adjustment that alters the inner resistance would affect the signal display. The consistency of experimental conditions in is an important issue in the research. Any replacement of materials used in system including electrode, wire, and membrane, should beware of.

Furthermore, proton exchange membrane is another factor that influence the electrochemical signals significantly. The PEM used in this experiment is Nafion® 117, which theoretically only allows proton pass through. However, it is considered that some of small cations could transfer across the membrane to cathode side and varies the redox reaction in biosensor system. Some researches have proved that several cations, such as Na⁺ and K⁺, would pass through Nafion® 117, and other bigger cations could also pass across the membrane with longer time. It is suggested to detect the
concentration of all cation species with IC in both chambers. To simplify the reactor into one-chamber system is an alternative. In one-chamber system, air cathode could replace the PEM and discharge the current impact from PEM to make bioreactor simpler.

In toxicity test, eleven different metal perchlorate solutions are injected into reactor with increasing dosages (10, 40, 50, 100, 200, and 400 μL of 1 M solutions). The experiment of using other injection dosages (addition of 100 μL of toxicant at one time replaces three separate injection with 10, 40, and 50 μL) should be conducted to prove injection volumes of contaminants would affect current variation or not. To consider different injection frequency could further understand the concentration impact in this system and improve the precision of metal concentration detection.

In addition, one heavy metal target pollutant is added into system at one time in toxicity test. However, real wastewater contains complex pollutants that have strong interaction between each other. A complicated toxicity test involving more than two toxicants must be experimented for better understanding the between contaminants interactions and impacts on current variation. This experiment would make the biosensor more suitable for analyzing realistic wastewater sample and enhance the ability in practical application.
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Appendix A

COMPOSITION MATRIX OF EQUILIBRIUM SPECIATION

Table A.1 Composition matrix for the calculated equilibrium speciation of ligands

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Table A.11 Composition matrix for the calculated equilibrium speciation of Fe(III)

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<th>$H^+$</th>
<th>$CO_3^{2-}$</th>
<th>$SO_4^{2-}$</th>
<th>$PO_4^{3-}$</th>
<th>$ClO_4^{-}$</th>
<th>log $K$</th>
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