EVALUATION OF CHROMIUM REMEDIATION IN GROUNDWATER:

INVESTIGATIONS USING MICROBIAL FUEL CELLS AND ELECTROKINETIC SYSTEMS

by

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Dedication

This dissertation is dedicated to my wonderful parents, Chung Hsiung Hsu and Li Chiu-Tuan Hsu, and my brother, Andrew Hsu. Without their love and support, none of this would be possible.
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Abstract

Hexavalent chromium (CrVI) is a common metallic carcinogen heavily used in industrial applications such as electroplating and leather tanning. Improper handling and disposal, along with the high solubility of CrVI, have led to widespread contamination of soil and water systems. Several remediation methods have been proposed, including biologically based techniques.

Bioremediation of CrVI is a promising approach due, in part, to the ability of the technique to rapidly lower CrVI concentrations. The research presented here focuses on the development of a CrVI remediation approach based on microbial fuel cell (MFC) technology. MFC technology has been proposed as a source of renewable energy and as a remediation tool. While much work has been done on developing the technology as a renewable energy source, relatively little work has been performed to assess its capabilities as a remediation tool.

To address this shortcoming, the research presented here will showcase several aspects of using an MFC as a remediation tool. These include (i) a predictive modeling approach for biological CrVI remediation, (ii) an evaluation of Shewanella bacteria as biocatalysts, (iii) the selection and analysis of mixed communities in a CrVI-reducing MFC, and (iv) the integration of a CrVI-reducing MFC with an electrokinetic system.

These findings show that CrVI removal to low parts-per-billion concentration levels is possible with MFC systems and that integration with existing technologies is a valuable application to consider when designing remediation strategies.
Chapter 1: Introduction

The protection of freshwater supplies is of urgent importance in preventing serious water shortage problems in the United States and around the world. Drinking water sources previously considered polluted are requiring cleanup by remediation because current potable water reservoirs will be unable to meet future demands. In 2002, Vörösmarty et al. reported that most of the midwestern and western United States faced severe fresh water scarcity in 1985. Furthermore, the United States Department of the Interior has identified several major metropolitan areas that have a substantial likelihood of experiencing water shortages in the near future. From 1998 – 2002, low precipitation and high temperatures imposed drought-like conditions on much of the United States. Global warming models also forecast the loss of annual fresh water availability due to decreased snow pack and loss of uncaptured precipitation to the ocean. Remediation of contaminated reservoirs and water systems can provide additional water resources that can be utilized to meet future public and industrial water needs. This research intends to present an approach towards developing novel microbial fuel cell technology for application to chromium containment and consequent removal from subsurface environments. The approach detailed here can be extended to other contaminants in soil and groundwater environments, providing new and effective technology to complement state-of-the-art remediation technologies.

1.1 Nature of the Problem

Chromium is a metallic species widely used in industrial applications including metal plating, leather tanning, and dye manufacture. Historically, chromium has also been added to process waters used for cooling towers because it was considered an inexpensive corrosion inhibitor. Inadequate or inappropriate handling of waste often allows for the introduction of
chromium into the environment. The two major forms found in the environment are hexavalent, \( \text{Cr}^{VI} \), and trivalent, \( \text{Cr}^{III} \), species. Large doses of \( \text{Cr}^{VI} \) have been linked to cancer, skin ulcers, and other maladies.\(^7\) The high mobility and solubility of \( \text{Cr}^{VI} \) frequently results in contamination of soils, surface waters, and groundwater. In contrast, trivalent species are generally considered innocuous in the environment because of its lack of mobility as an insoluble species and its beneficial use as a trace nutrient in plant and animal nutrition.\(^8,9\) Recently, however, \( \text{Cr}^{III} \) has been linked to deleterious effects in both bacterial and animal cells.\(^10,11\)

The highest reported contamination of resources directly used for drinking water were 6 ppm (Midland, TX) and 0.5 ppm (Hinkley, CA).\(^12\) The United States Environmental Protection Agency (EPA) has identified more than 700 contaminated Superfund sites listing chromium as a pollutant of concern.\(^13\) \( \text{Cr}^{VI} \) has been classified as one of the most common metals found at US EPA Superfund sites.\(^14\) Exposure to public health and resulting impacts became highlighted as media outlets began to report on cases of widespread contamination. Popular concern regarding environmental \( \text{Cr}^{VI} \) contamination of drinking water sources was ignited following the release of the 2000 Hollywood film, *Erin Brokovich*, which highlighted the \( \text{Cr}^{VI} \) groundwater contamination by the Pacific Gas and Electric Company and the carcinogenic effects alleged by the local populace of Hinkley, CA.\(^15,16\)

Additionally, more than 100 sites requiring cleanup by the Department of Energy (DOE) were identified in preliminary studies as needing hazardous waste cleanup and legacy monitoring.\(^17\) Preliminary surveys of the major remediation sites list heavy metals, such as chromium, and/or radionuclides as contaminants of concern.\(^18\) For example, chromium
Figure 1.1: Superfund sites requiring remedial efforts and maintenance can be seen marked with 🚭. Contamination is widespread in almost all states with varying levels of soil and groundwater contamination.
concentrations at the Hanford Site in a recent survey were reported to be higher than 2 ppm, 40 times the California EPA maximum concentration value for groundwater. At all of these sites (both EPA and DOE monitored), contamination often threatens local groundwater and/or surface water supplies.

The research described here presents a new approach to chromium remediation using microbial fuel cell (MFC) technology and its integration with electrokinetic technology. The essence is the incorporation of CrVI reducing microbes with microbial fuel cells, resulting in a system that can both produce usable electrical energy as well as reduce and trap the resulting CrIII, leading to its facile removal from the environment. This approach eliminates the shortcomings of existing approaches, such as electrokinetic remediation, (discussed later) and introduces additional advantages. While the research was focused on the remediation of CrVI, this approach can be extendable to other metals and radionuclides including uranium and technetium.

1.2 Behavior in natural environments

Two main forms of chromium can be found in the natural environment, CrIII and CrVI, both of which have toxicity concerns. The trivalent state is considered insoluble in aqueous solution and has therefore not been the cause of much alarm. The hexavalent state, however, is of great concern because it is highly soluble and has long been listed as a carcinogenic chemical. The dominant form found in an aquifer depends to a large extent on the redox potential of the contaminated system (Figure 1.2). Under oxidizing conditions, chromium is readily oxidized to its hexavalent state which has significant mobility in subsurface aquifers as one of two chromate species. In contrast, under reducing conditions the dominant species of chromium involve the trivalent state, including Cr3+, Cr(OH)2+, Cr(OH)3, and Cr(OH)4−. Moreover,
the speciation of these oxidized or reduced forms is clearly determined by both redox potential and pH. Depending on the chemical composition of the groundwater, chromium may form complexes with hydroxyl, sulfate, ammonium, cyanide, fluoride, chloride, and natural organic matter. For these reasons, the amount of solid, precipitated Cr$^{III}$ will be highly dependent on site-specific groundwater aquifer characteristics.
Figure 1.3. (a) pH and concentration relationships for soluble Cr$^{VI}$ species. (b) Chromate-dichromate relationships shown for pH 4.

Possible species of Cr$^{VI}$ include HCrO$_4^-$, CrO$_4^{2-}$, H$_2$CrO$_4$, and Cr$_2$O$_7^{2-}$. Distributions between these species is dependant both on pH and Cr$^{VI}$ dissolved in solution (Figure 1.3).$^{26}$ From these speciation diagrams, the pH of common natural water systems will generally force the speciation of Cr$^{VI}$ species to be either HCrO$_4^-$ or CrO$_4^{2-}$. As stated previously, total Cr$^{VI}$ contamination of drinking water sources has not exceeded 6 ppm (0.12 mM). Thus, the dimeric Cr$^{VI}$ species (Cr$_2$O$_7^{2-}$) will not be encountered in most groundwater systems unless the concentration exceeds 1500 ppm (=30 mM).
1.3 Toxicity of Chromium

The carcinogenic effects of chromium exposure have been attributed to the accumulation of chromium within the cell. Primary accumulation in the cell generally manifests itself as Cr\textsuperscript{III} precipitation due to uptake of Cr\textsuperscript{VI} from the environment followed by intracellular reduction of the species. Cr\textsuperscript{VI} toxicity is a well documented fact and public exposure to the compound is well regulated.\textsuperscript{27}

The toxic effect of Cr\textsuperscript{III} was not well-understood until recently because it was believed that intracellular chromium did not significantly accumulate in its trivalent form.\textsuperscript{20} However, new studies have shown uptake of Cr\textsuperscript{III} from the environment is possible and may account for mutagenic effects if accumulated amounts are significant. Evidence of human health impacts have also been shown in several DNA interaction studies with exposures as low as 5 \textmu M Cr\textsuperscript{III}.\textsuperscript{10,11} In 2002, Plaper \textit{et al.} reported evidence that Cr\textsuperscript{III} was responsible for damage to DNA and inhibition of a topoisomerase essential in DNA replication and transcription. Accumulation of Cr\textsuperscript{III} was shown in both eukaryotic and prokaryotic cell lines to cause diminished viability in exposed cell lines and cultures.\textsuperscript{28} Cr\textsuperscript{III} has also been shown to have further effects on cell viability when accumulation occurred within the bacterial cells.\textsuperscript{29}

Impacts of chromium uptake and accumulation in cells have clearly been associated with mutations and carcinogenic effects. These effects have been linked to exposure to both forms of chromium. For these reasons, it is imperative that concentrations of total chromium in water supplies be controlled and its exposure to populations mitigated. Given widespread uncertainties concerning exposure and uptake, chromium remediation by reductive precipitation alone is not a foolproof strategy for mitigating exposure. In fact, the more prudent
strategy is likely to incorporate complete removal from contaminated sites, eliminating any risks of exposure and oxidative remobilization.

1.4 Regulations

Regulatory agencies have provided guidelines for acceptable chromium drinking water concentrations to reduce exposure. International bodies such as the Commission of European Communities and the World Health Organization recommend 50 ppb as the maximum allowable limit.\(^{27}\) On a national level, the United States EPA has declared 100 ppb to be the Maximum Contaminant Level (MCL) in drinking water.\(^{20}\) State-defined limits tend to be even more stringent. For example, the California EPA has set drinking water MCLs at 50 ppb and in the State of Washington chronic exposure limit for aquatic species is set at 10 ppb.\(^{30}\) Furthermore, the draft Public Health Goal (PHG) in California was proposed to be 2.5 ppb total chromium or 0.06 ppb for hexavalent chromium.\(^{28}\) The draft PHG recommendation for California was further reduced in 2010 to 0.02 ppb.\(^{31}\) MCL levels may be further reduced as more extensive toxicity studies are performed and public demand for lower pollutant levels escalates. In response to the challenge posed by these low water standards, scientists and engineers must begin developing efficient and cost-effective treatment technologies.

1.5 Current State of Technology – Physicochemical

To meet the current drinking water standards, a variety of physical and chemical removal methods have been investigated. Aggarwal \textit{et al.} have published research regarding adsorption on powdered activated carbon.\(^{32}\) Meanwhile, other researchers have used various forms of activated carbon to investigate removal of chromium at different pH values.\(^{33}\) Use of reductants such as ferrous iron to reduce hexavalent chromium has also been widely studied.\(^{34}\)
The spectrum of physical and chemical treatment methods is too broad to fully summarize in this work. The limitations of methods currently employed are discussed below.

Chemical reduction-precipitation reactors and adsorption columns used to remove chromium from aqueous solution are expensive and can be ineffective for highly contaminated water. Chemical reduction requires multiple unit operations and the storage of strong acids used in the reduction pose storage and handling challenges. Physical removal by adsorption maintains chromium in its highly carcinogenic form and the disposal of spent media and contaminated cleaning solutions require special safety measures and hazardous waste management protocols.

1.6 Current State of Technology – Electrokinetic

One emerging physical technology is the application of electrical potential to the subsurface, also known as electrokinetic remediation. First noticed in 1809 by Ruess, application of a DC electrical field will cause movement of pore fluid. Application of this electroosmotic phenomenon to soil stabilization was further explored in the mid 1960's by a wide variety of researchers. Recently, the technique has been applied in the remediation of contaminated soils by driving the movement of contaminants along with the pore fluid towards a charged electrode where it may be removed or treated. However, the process still requires that pore fluid be extracted for treatment, or that contaminated soils must still be excavated for cleaning.

1.7 Current State of Technology – Biological

An alternative to physical or chemical treatment is bacterial reduction of hexavalent chromium to the trivalent species, allowing the formation of a chromium precipitate. Several microbial species have been shown to be able to chemically reduce (and render insoluble) mobile pollutants. For example, kinetic data published in 1998 by Schmieman et al.
compares a wide range of mixed culture treatment of hexavalent chromium and suggests the feasibility of in situ remediation. A biological growth model by Vainshtein et al. suggests a consortium of sulfate reducers and denitrifiers may be used in CrVI reduction under anaerobic conditions. Tebo and Arias also report a mixed culture of bacteria able to reduce hexavalent chromium at concentrations up to about 70 ppm (1.35 mM). Bioreactors, functioning as an ex situ treatment technique, were found to be capable of achieving CrVI removal to levels of about 10 ppm.

Microbial CrVI removal by precipitation as CrIII is a promising concept, but current environmental regulations require removal to substantially lower levels than those reported from the previous studies mentioned in the preceding paragraph. This represents a gap in knowledge regarding both the extent, kinetics, and toxic effects of the microbial CrVI reduction. An important goal of this research is to explore the extent of microbial chromium removal at low ppb levels, establish kinetic parameters describing the reduction process and to determine ways to enhance microbial reduction of CrVI, either by increasing resistance to chromium toxicity or enhancing reduction rates. This knowledge will be essential for the design of a reactor, based on microbial fuel cell principles.

1.8 Current State of Technology – Microbial Fuel Cells

The present work involves (i) the development of a thorough understanding of the microbial physiology of CrVI reduction and (ii) the engineering and integration of these bacteria in microbial fuel cells (MFCs). This will result in a system that will reduce CrVI and trap the resulting CrIII, leading to its removal from the environment. The basics of MFC design and operation are discussed in the ensuing paragraphs.
While space prohibits a detailed history of the development of MFC systems, it may suffice to say that from the early 1900s it was clear that microbial systems could establish electron gradients and currents, and as of 1962 that such systems could be studied in the laboratory using organic electron donors.\textsuperscript{58} These studies paved the way for current concepts of electrical generation by fuel cells, from the oxidation of organic waste products to the markedly different uses that are being proposed.\textsuperscript{59,60}

Traditional MFCs rely on bacteria to catalyze the oxidation of a substrate in the anodic chamber of the MFC system and transport electrons from the electron donor to the cathode electrode where an appropriate electron acceptor is then reduced (Figure 1.4). In principle, any respirable organic compound could be used as an electron donor in the anode. Electron donors that have been shown to be effective in MFCs include lactate, glucose, and organics found in wastewater.\textsuperscript{61,62}

In principle, nearly any electron acceptor could be used on the cathode side of the MFC; however the majority of MFC studies reported in literature utilize ferricyanide or oxygen as primary electron acceptors on the cathode side of the MFC. The use of oxygen as the electron acceptor in the cathode requires an electrode plated with expensive catalysts such as platinum.\textsuperscript{63}

The use of MFCs for renewable power generation and wastewater treatment is an area of current research. Power output maximization studies focus on minimizing internal resistance by altering electrode structure, enhancing species diffusion or transport, and testing different ion exchange membrane material.\textsuperscript{64-68} Another area of research is in domestic wastewater treatment where MFCs could supplement the power requirements for sewage treatment with energy generated while reducing the biological oxygen demand (BOD) and nutrient levels of the
Figure 1.4. General principles of operation for (a) air breathing MFCs and (b) MFCs employing biological catalysts at both the anode and cathode.
MFC technology could also be applied to other waste streams containing high organic concentrations such as food processing plants. Other MFC applications currently being developed include their use as a power source for remote sensing operations.

Development of biologically active cathodes has allowed the use of MFCs to be extended for contaminant remediation. This application has been explored in poised half cells (cells containing only one electrode with an applied potential from an external power source). More recently, mixed community cathodes at high chromium concentrations, 3 – 80 ppm, were reported. Such reports are promising with respect to treating relatively high CrVI concentrations, however, the research presented here show the reduction of CrVI levels from low ppm levels to low ppb levels to meet drinking water standards.
Chapter 1 References


Chapter 2: Research Scope and Objectives

2.1 Research Objectives

A primary objective of this research was to develop and present a feasible method for hexavalent chromium remediation and reclamation in groundwater systems. The design of a treatment method revolves around the dissimilatory metal reduction performed by environmental bacteria, primarily from the *Shewanella* genus. These bacteria have been shown to couple oxidation with the reduction of metals in the environment. Of particular interest is the transformation of soluble hexavalent chromium to an insoluble trivalent form.

One of the first steps in this research was to obtain some physiological information describing the reduction of chromium and how environmental factors may influence the effectiveness of biological Cr<sup>VI</sup> reduction. These environmental factors include temperature, carbon source, and pH. Each of these factors can be well controlled in experimental batch systems which provide some operating criteria that can be used to optimize future systems.

An important part of this research was to determine an appropriate model describing biological Cr<sup>VI</sup> reduction. A series of models was evaluated and model was chosen that best fit experimental data. The predictive ability of this model for a different reactor system was then performed. Information gained by using this model will be used in future work to predict Cr<sup>VI</sup> removal in microbial fuel cell reactors.

This research also investigated the feasibility of utilizing fuel cell technology for metal remediation. The use of microbial fuel cells has largely been focused on oxidation of organic wastes. The experiments performed and described in this dissertation demonstrate the applicability of fuel cell technology in metal remediation. First, demonstration of biocatalytic
ability of *Shewanella* in batch reactors and at the microbial fuel cell cathode with various oxidants (electron acceptors) was performed, followed by detailed studies of Cr$^{VI}$ reduction. While the experiments described here focus mainly on hexavalent chromium, extension of this research should be applicable to other metal pollutants and more complex microbial communities.

The final objective of this research was to explore the integration of an electrokinetic system with the microbial fuel cell system. Electrokinetic systems are reported to have been used in directing soluble ions and charged particulates in subsurface environments. In standard electrokinetic applications, the electrodes are often poisoned or covered by non-conductive precipitates as reducing conditions are encountered at the cathode. By directing pollutants towards an *in situ* treatment system such as a microbial fuel cell, the life of the electrodes may be extended and the transformed pollutants can be collected in the fuel cell chambers and recovered. The integration of biological remediation with physical electrokinetics is attractive since the two processes can complement each other and enhance pollutant recovery in the subsurface.

2.2 Research Scope

The scope of the research conducted is outlined below:

A. A series of physiological batch reactor studies was designed and operated with *Shewanella* bacteria for estimating reducing capabilities. These experiments were designed to systematically observe several different strains of bacteria and identify strains that most accurately represent the wide range of chromium reduction rates. Environmental conditions such as pH, temperature, and
electron donors were also varied in order to determine approximate ideal operating conditions.

B. A set of models describing Cr\textsuperscript{VI} reduction were evaluated for use as predictive models. Biokinetic parameters for these models were determined and the most suitable model describing experimental data was determined.

C. The feasibility of utilizing \textit{Shewanella} as a cathodic biocatalyst in a microbial fuel cell was determined. Previous studies have shown that \textit{Shewanella} are able to catalyze oxidation reduction at the anode of such devices. This series of experiments will demonstrate the ability to utilize \textit{Shewanella} to also catalyze the reduction reaction occurring at the cathode. Different strains were used at the cathode to determine differences in power production, efficiency, and overall effectiveness of chromium reduction.

D. Mixed consortia of bacteria were enriched from wastewater inoccula for use in MFC systems. The stability and effectiveness of these consortia were evaluated at both the anode and cathode, with respect to Cr\textsuperscript{VI} reduction. Strategies for enrichment and establishment of these communities was also evaluated.

E. A small, bench-scale electrokinetic system was designed and operated to mimic \textit{in situ} groundwater conditions. Fate and transport of chromium in this system was characterized and Cr\textsuperscript{VI} removal effectiveness was evaluated.

F. A MFC system was introduced to complement the electrokinetic system and investigate its ability to enhance chromium removal from the saturated zone of a soil system. The effectiveness of this system was evaluated and compared with the performance of the electrokinetic-only system.
Chapter 3: Bioreactor kinetic studies investigating CrVI reduction by *Shewanella* spp.

3.1 Introduction

Hexavalent chromium is a common environmental contaminant which is very mobile in the environment due to its high solubility.¹ Contamination of drinking water sources, including groundwater and surface water, is largely due to improper handling during industrial applications.² Its widespread occurrence has prompted the need to explore means of remediation and removal from the environment.

Bioremediation has been proposed as a promising technology of CrVI remediation that may be used for *in situ* treatment processes.³⁴ Examples of organisms used in bioremediation studies include sulfate reducers and metal reducers, notably members of the *Shewanella* genus.⁵⁻⁸ In order to implement these bacteria as engineering tools, models predicting rates and physiological mechanisms have been proposed.⁸⁻¹¹ In order to use the models effectively, biokinetic parameters must be determined for specific communities or microorganisms to be used.

Bioreactor kinetic experiments were performed with the intention of determining kinetic parameters governing biological chromate reduction utilizing *Shewanella* as model organisms. Based on previous physiological and genomic work, these organisms are well-described metal reducers and have been shown to be able to perform chromate reduction, in addition to other heavy metals.¹²⁻¹⁴ Additionally, metal reduction models employed in these studies may aid in the development of treatment systems by providing a predictive modeling tool.
One reactor system that shows a great deal of promise for energy generation and heavy metal reduction are microbial fuel cells (MFCs). These systems have been studied heavily in recent years as potential renewable energy sources.\(^{15,16}\) MFCs have also been proposed recently as remediation tools to remove heavy metals such as Cr\(^{VI}\).\(^ {17,18}\) In order to engineer this type of reactor system, a predictive model describing the biological phenomena occurring will be necessary to describe chromium reduction.

To this end, a series of batch reactor studies has been performed to evaluate potential models to describe the chromate reduction process. In these experiments, several kinetic models were investigated to determine the most appropriate model to describe experimental data. Furthermore, different experimental conditions (pH, temperature, and electron donor) were examined and their effect on chromate reduction was assessed.

### 3.2 Scope

This series of experiments consisted of several components. First, bio kinetic parameters were estimated from batch experiments performed with each of the *Shewanella* species tested (Table 3.1). From these batch experiments, data was fit to several models by nonlinear regression and the best model describing Cr\(^{VI}\) reduction was determined. Another set of experiments served to test the predictive capabilities of the model on a larger reactor system with different biomass concentrations. Thus, possible application of the model to other reactor systems was evaluated for different *Shewanella* species. Finally, the effects of experimental parameters (pH, temperature, and electron donor) affecting transformation of Cr\(^{VI}\) were evaluated.
3.3 Materials and Methods

Preliminary biokinetic studies

For this study, seven *Shewanella* species including *S. baltica* OS155, *S. frigidimarina* NCIMB 400, *S. putrefaciens* 200, *S. putrefaciens* CN-32, *S. loihica* PV-4, *S. oneidensis* MR-1, and *S. amazonensis* SB2B, were obtained from Pacific Northwest National Laboratory (PNNL) and stored at -80°C. These species were streaked on Luria-Broth, Miller agar plates and incubated aerobically overnight at 30°C. Individual colonies were transferred to a modified minimal media (Table 3.2) with a 30 mM lactate concentration and grown overnight at 30°C. These cultures

<table>
<thead>
<tr>
<th>Species</th>
<th>Location of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. baltica</em> OS155</td>
<td>Deep anoxic water basin (Baltic Sea)</td>
</tr>
<tr>
<td><em>S. frigidimarina</em> NCIMB 400</td>
<td>Coastal seawater (North Sea)</td>
</tr>
<tr>
<td><em>S. putrefaciens</em> 200</td>
<td>Crude-oil pipeline (Alberta, Canada)</td>
</tr>
<tr>
<td><em>S. putrefaciens</em> CN-32</td>
<td>Anaerobic sandstone (New Mexico, USA)</td>
</tr>
<tr>
<td><em>S. loihica</em> PV-4</td>
<td>Hydrothermal sea vent (Hawaii, USA)</td>
</tr>
<tr>
<td><em>S. oneidensis</em> MR-1</td>
<td>Freshwater lake sediment (Lake Oneida, New York, USA)</td>
</tr>
<tr>
<td><em>S. amazonensis</em> SB2B</td>
<td>Shallow marine sediment (Amazon River delta)</td>
</tr>
</tbody>
</table>
were subsequently diluted with 50 mM PIPES buffer to an optical density at 600 nm (OD$_{600}$) of 0.5, corresponding to approximately $10^9$ CFU mL$^{-1}$.

Table 3.2. Composition of minimal media used for growing cultures of *Shewanella* species

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Conc (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIPES buffer</td>
<td>5.00 x 10</td>
</tr>
<tr>
<td>Sodium Hydroxide</td>
<td>1.25 x 10$^2$</td>
</tr>
<tr>
<td>Ammonium Chloride</td>
<td>2.80 x 10</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>1.00</td>
</tr>
<tr>
<td>Sodium phosphate, monobasic</td>
<td>4.00</td>
</tr>
<tr>
<td>Sodium Lactate</td>
<td>variable on application</td>
</tr>
<tr>
<td>Nitritriacetic Acid</td>
<td>7.85 x 10$^{-2}$</td>
</tr>
<tr>
<td>Magnesium Sulfate Heptahydrate</td>
<td>1.22 x 10$^{-1}$</td>
</tr>
<tr>
<td>Manganese Sulfate Monohydrate</td>
<td>2.96 x 10$^{-2}$</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>1.71 x 10$^{-1}$</td>
</tr>
<tr>
<td>Ferrous Sulfate Heptahydrate</td>
<td>3.60 x 10$^{-3}$</td>
</tr>
<tr>
<td>Calcium Chloride Dihydrate</td>
<td>6.80 x 10$^{-3}$</td>
</tr>
<tr>
<td>Cobalt Chloride Hexahydrate</td>
<td>4.20 x 10$^{-3}$</td>
</tr>
<tr>
<td>Zinc Chloride</td>
<td>9.54 x 10$^{-3}$</td>
</tr>
<tr>
<td>Cupric Sulfate Pentahydrate</td>
<td>4.01 x 10$^{-4}$</td>
</tr>
<tr>
<td>Aluminum Potassium Disulfate Dodecahydrate</td>
<td>2.11 x 10$^{-4}$</td>
</tr>
<tr>
<td>Boric Acid</td>
<td>1.62 x 10$^{-3}$</td>
</tr>
<tr>
<td>Sodium Molybdate Dihydrate</td>
<td>1.03 x 10$^{-3}$</td>
</tr>
<tr>
<td>Nickel Chloride Hexahydrate</td>
<td>1.01 x 10$^{-3}$</td>
</tr>
<tr>
<td>Sodium Tungstate</td>
<td>7.58 x 10$^{-4}$</td>
</tr>
<tr>
<td>Biotin</td>
<td>8.19 x 10$^{-5}$</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>4.53 x 10$^{-5}$</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>4.86 x 10$^{-4}$</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>1.33 x 10$^{-4}$</td>
</tr>
<tr>
<td>Thiamine HCl monohydrate</td>
<td>1.41 x 10$^{-4}$</td>
</tr>
<tr>
<td>Nicotinic Acid</td>
<td>4.06 x 10$^{-4}$</td>
</tr>
<tr>
<td>d-Pantothenic Acid, Hemicalcium Salt</td>
<td>2.10 x 10$^{-4}$</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>7.38 x 10$^{-7}$</td>
</tr>
<tr>
<td>p-Aminobenzoic Acid</td>
<td>3.65 x 10$^{-4}$</td>
</tr>
<tr>
<td>Thiocetic Acid</td>
<td>2.42 x 10$^{-4}$</td>
</tr>
<tr>
<td>L-Glutamic Acid</td>
<td>1.36 x 10$^{-1}$</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>1.15 x 10$^{-1}$</td>
</tr>
<tr>
<td>DL-serine</td>
<td>1.90 x 10$^{-1}$</td>
</tr>
</tbody>
</table>
Experiments were performed in an anaerobic chamber (Coy Labs, Inc; Grass Lake, MI) with an atmosphere of 5% H₂ and 95% N₂. 250 mL of each diluted species culture was transferred to a 500 mL glass reactor inside the anaerobic chamber. Cr⁶⁺ (as K₂Cr₂O₇) was added to achieve an initial concentration of 0.1 mM (5.2 ppm). Mixing was performed by magnetic stir plate operating at approximately 100 rpm. Cultures were continuously mixed and samples taken at regular intervals over 3 hours.

**Biomass quantification**

Biomass was quantified primarily by measuring absorbance of suspended cultures at 600 nm using a UV-Visible spectrophotometer (Model DU530; Beckman Coulter, Inc.; Brea, CA). Relation of absorbance to both volatile suspended solids and colony forming units was determined by EPA method 1684 and the dilution-to-extinction method.¹⁹,²⁰

**Cr⁶⁺ measurement**

Samples from the anaerobic reactors were immediately placed on ice after collection to prevent further bioreduction of Cr⁶⁺ in the sample. After samples were collected from all reactors for a given time point, biomass was removed by centrifugation at 17,000g for 5 minutes. Supernatant was immediately collected and evaluated for Cr⁶⁺ by reaction with diphenylcarbazide (DPC) in acidic solution followed by absorbance readings at 540 nm by UV-Visible spectrophotometer. Absorbance measurements were then correlated with concentrations by using a standard calibration curve for samples above 0.01 mM.²¹

For samples below 0.01 mM, the limit of detection for the DPC method, an ion chromatography method was used.²²-²⁴ This method utilizes separation on an anion exchange column (Model AS7 250/4, Dionex Corporation; Sunnyvale, CA) followed by post-column
derivitization and measurement with a UV-Visible spectrophotometer (Model AD25, Dionex Corporation; Sunnyvale, CA) set at 530nm. The flow rate of the eluent [0.25 M (NH₄)₂SO₄, 0.1 M NH₄OH] was 1 mL min⁻¹, while the flow rate of the post-column derivitization reagent (0.002 M DPC, 10% methanol, 0.5M H₂SO₄) was 0.5 mL min⁻¹.

**Evaluation of preliminary biokinetic parameters**

Four *Shewanella* species, PV-4, MR-1, strain 200, and SB2B, were chosen for investigation using 1 liter reactors based on performance of the preliminary biokinetic experiments. These were chosen to represent the range in rates determined from the preliminary biokinetic experiments. The purpose of these experiments was to evaluate the application of the best biokinetic model determined from the preliminary studies. Parameters from the best fit model were used to predict the behavior of these systems.

Initial cultures were grown as before except the OD₆₀₀ of the reactor cultures was adjusted to 0.150, approximately 10⁸ CFU mL⁻¹, with 50 mM PIPES buffer. For each strain, triplicate batch reactor studies were performed as follows. 1 L of each diluted culture was added to separate 1.1 L batch reactors (Figure 3.1) equipped with N₂ gassing ports. Mixing was achieved by magnetic stir plates operating at 100 rpm. Temperature control was maintained by immersion of reactors in a temperature controlled water bath. Reactor vessels were stirred under N₂ purging for 2 hours before a Cr⁶⁺ stock solution was added to bring the Cr⁶⁺ concentration in each reactor to 0.1 mM. Samples were collected at regular time intervals over 10 hours.
Effects of temperature and pH

Cultures of *S. putrefaciens* 200 were grown in standard minimal media as described before. This species was chosen because its rate constant was near the median of the observed rates for mesophilic strains. Stock cultures were grown in minimal media from frozen stock preparations as before at 30°C. Once sufficient cell density had developed, these stock cultures were diluted to an OD$_{600}$ of 0.150 using sterile minimal media.

![Figure 3.1. Batch reactor setup for larger scale Cr$^{III}$ reduction experiments](image-url)
1 liter of diluted cultures was placed into a set of triplicate reactors that were prepared for each temperature to be tested. These reactors were then transferred to a temperature controlled water bath and allowed to equilibrate under anaerobic conditions (N₂ purge) for 2 hours. Continuous stirring was provided by magnetic stir plates operating at 100 rpm. The following temperatures were tested for this study: 20°C, 25°C, 30°C, 35°C, and 40°C.

The working range for PIPES buffer is 6.1 – 7.5. Therefore, it would not be useful in controlling pH at the values tested in the pH-variation experiments (6, 7, and 8). Similar methodology as described for the temperature studies was used to prepare the culture for pH studies except a phosphate buffer was substituted for PIPES buffer. Diluted cultures were distributed to reaction vessels and placed into a 20°C water bath and allowed to equilibrate under anaerobic conditions (N₂ purge) for 2 hours. Cr⁶⁺ was introduced as before to bring exposure concentrations in each reactor vessel to 0.1 mM. Samples were taken at regular time intervals over 4 hours and Cr⁶⁺ measurements were performed utilizing the DPC and IC methods.

**Effect of Carbon Source on Chromium Reduction**

*S. putrefaciens* strain 200 and *S. amazonensis* SB2B were utilized as primary candidates for this set of experiments. Each species was grown in minimal media with sodium lactate (30mM) as the carbon source following the previously described procedures. Upon completing overnight growth, cells were isolated by centrifugation of 50 mL aliquots at 6000g. Supernatant was decanted and the cell pellet washed three times using minimal media containing no carbon source. Cells were then resuspended in a minimal media containing one of eight carbon source mixtures (Error! Not a valid bookmark self-reference.). Sterile media was added to achieve an OD₆₀₀ of 0.150 after resuspension of the cell pellet.
Table 3.3. Organic molecules tested for coupling with Cr\textsuperscript{VI} reduction

<table>
<thead>
<tr>
<th>Carbon source mixtures to be tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
</tr>
<tr>
<td>Lactate &amp; Fumarate</td>
</tr>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>Citrate</td>
</tr>
<tr>
<td>n-Acetyl-d-Glucosamine</td>
</tr>
<tr>
<td>Adenosine</td>
</tr>
<tr>
<td>d-Fructose</td>
</tr>
<tr>
<td>Molasses (food grade)</td>
</tr>
</tbody>
</table>

20 mL of the resuspended culture was then transferred to 40 mL anaerobic bottles and allowed to become oxygen limited over 2 hours. The cultures were then dosed with Cr\textsuperscript{VI} from a 20 mM Cr\textsuperscript{VI} (as K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7}) stock solution to achieve a starting concentration of 0.1 mM Cr\textsuperscript{VI}. Samples were drawn from the bottles after a period of 2 hours. Biomass was immediately separated by centrifugation and supernatant analyzed by the DPC method.

3.4 Determining model parameters

*Modified Monod Model (MMM)*

To estimate chromium reduction kinetics, a modified version of the Monod kinetic model was considered.\textsuperscript{25} To describe substrate change with time, the following expression (Equation 1) of the Monod model was modified to account for experimental conditions.\textsuperscript{19}
\[
\frac{dS}{dt} = - \frac{1}{Y} \frac{\mu_{\text{max}} SX}{K_s + S}
\]

where \( S \) is substrate concentration, \( t \) is time, \( Y \) is the biomass yield coefficient, \( \mu_{\text{max}} \) is the maximum specific growth rate, \( X \) is the biomass concentration, and \( K_s \) is the half saturation constant.

The cultures were inoculated from cultures in the late log – stationary phase of batch growth. Therefore, it would not be unreasonable to assume that biomass would not change appreciably within the first hour of exposure to chromium and lactate. For this reason, it is assumed that the amount of biomass in the reactor is constant for the first 60 minutes after inoculation.

For this reason, we will also assume the \( K_s \) is much greater than \( S \) because of the low concentrations of \( \text{Cr}^{\text{VI}} \) used in this study. Taking the biomass and substrate concentration assumptions into account gives a pseudo-first order rate equation (Equation 2). Using these considerations, this modified Monod model was tested for fit to experimental data.

\[\frac{dS}{dt} \approx - \left( \frac{1}{Y} \frac{\mu_{\text{max}} X}{K_s} \right) S = -k' S \quad \text{(2)}\]

**Non-growth models**

Heavy metal decay models under non-growth conditions have been proposed for mixed consortia of bacteria. \( \text{Cr}^{\text{VI}} \) reduction by *Shewanella* is considered to be a non-growth process, where growth with \( \text{Cr}^{\text{VI}} \) as the sole electron acceptor has been reported to be non-existant or severely impaired. Thus, these non-growth models should be applicable to \( \text{Cr}^{\text{VI}} \) reduction by
Shewanellae. Adoption of these models for Cr\textsuperscript{VI} reduction is presented below and represents three scenarios which may be occurring during this reduction.

The first non-growth model proposed by Boonchayaanant et al. assumes a constant biomass amount.\textsuperscript{9} This model, similar to the situation presented with the MMM, results in a pseudo-first order reaction rate. The governing differential equations (Equation 3 and Equation 4) and exact solution (Equation 5) are presented.

\[
\frac{dS}{dt} = -kXS \tag{3}
\]

\[
\frac{dX}{dt} = 0 \tag{4}
\]

\[
S = (S_{t=0})e^{-k(S_{t=0})t} \tag{5}
\]

The second non-growth model introduces the concept of a “transformation capacity,” \(T_c\), that suggests the presence of a finite limit to heavy metal reduction per unit biomass.\textsuperscript{9,11} That is to say, that each bacterium can only reduce a certain amount of Cr\textsuperscript{VI} before being inactivated. The equations describing this “Transformation Capacity Model” (TCM) are shown in Equations 6–9.

\[
\frac{dS}{dt} = -kXS \tag{6}
\]

\[
\frac{dX}{dt} = X|_{t=0} - \frac{S|_{t=0}}{T_c} \tag{7}
\]

\[
\frac{dS}{dt} = -k \left( X|_{t=0} - \frac{S|_{t=0}}{T_c} \right) S \tag{8}
\]

\[
S = (S_{t=0}) \frac{X|_{t=0} - \frac{S|_{t=0}}{T_c}}{X|_{t=0} - (S_{t=0})e^{-k(X|_{t=0} - \frac{S|_{t=0}}{T_c})t}} \tag{9}
\]
The third non-growth model suggests that active biomass will decrease according to a first order decay rate with a decay constant, \( b \). The form of this “Biomass Decay Model” (BDM) is presented as follows.

\[
\frac{dS}{dt} = -kXS \tag{10}
\]

\[
\frac{dX}{dt} = -bt \tag{11}
\]

\[ X = X|_{t=0}e^{-bt} \tag{12} \]

\[
\frac{dS}{dt} = -k(X|_{t=0}e^{-bt})S \tag{13}
\]

\[
S = (S|_{t=0})e^{\frac{kX|_{t=0}(e^{-bt}-1)}{b}} \tag{14}
\]

### 3.5 Computational Approach

Data was fit to these models by nonlinear regression methods to estimate each of the model parameters in Matlab (version 2010b). The built-in function, \textit{nlinfit}, was utilized, incorporating the robust variation of the Levenberg-Marquardt algorithm for least squares fits. This variation uses a iterative reweighting of least squares values in a bisquare weight function to minimize the effects of outliers on model fits.

The differential equations were then solved using the built-in Matlab function, \textit{ode45}, using the model parameters estimated by the nonlinear regression. This function uses an adaptive implementation of Runge-Kutta Method where the fourth and fifth order approximations are compared to determine allowable step sizes between solver steps.

The sum of squares approach (SSA) was used to evaluate goodness of fit. This approach is summarized as follows and produces coefficient of determination, \( r^2 \), relating how well the
proposed model describes the experimental data with value closer to 1 indicating better model fits.

\[
\text{Sum of Squares}_{\text{total}} = \sum (y_{\text{model}} - \bar{y})^2 
\]  \hspace{1cm} (15)

\[
\text{Sum of Squares}_{\text{error}} = \sum (y_{\text{model}} - y_{\text{experimental}})^2 
\]  \hspace{1cm} (16)

\[
 r^2 = 1 - \frac{\sum (y_{\text{model}} - y_{\text{experimental}})^2}{\sum (y_{\text{model}} - \bar{y})^2} 
\]  \hspace{1cm} (17)

### 3.6 Results and Discussion

**Initial Cr\textsuperscript{VI} reduction tests**

The results of the initial batch reactor experiments with 7 *Shewanella* species are shown in Figure 3.2. The error bars represent a 95% confidence interval of the triplicate average. The results show different reduction rates for the species tested.

Fitting the first 60 minutes of data (corresponding to a 50% reduction in Cr\textsuperscript{VI}) to the MMM gives rate constants presented in Table 3.4. Figure 3.3 shows the fit of the model to experimental data. This data was used primarily because large deviations from this model begin to occur after 60 minutes.

Because the non-linear fitting approach is an iterative process that requires an initial prediction of parameters, the rate constants generated by this analysis were used as initial guesses for non-linear fitting of the data to each of the proposed models. Correlation coefficients for each model were determined by the SSA. Best fits to experimental data, as evaluated by the SSA, were observed with the TCM (Table 3.5). Thus, the TCM was used as the predicting model in both sensitivity analysis and in the rest of the experimental analyses.
Evaluation of the first 60 minutes of data under the MMM does appear to predict, to within an order of magnitude, the rate constant. This prediction is also able to give a reasonable comparison of rates between each of the species tested. However, at times greater than 60 minutes, this approximation does not appear to model the data. This inaccuracy at longer time scales necessitates the use of a more descriptive model, such as the others presented in this paper.

The TCM provided the best fits to experimental data for the *Shewanella* tested. The transformation capacity parameter for these strains appears to be very similar, implying that the method of inactivation for all these strains is similar. One explanation of this would be one that involves precipitation of CrVI around the periphery of the cell, blocking access to the active chromate reducing enzymes. Since the difference in sizes among the *Shewanella* are negligible,

<table>
<thead>
<tr>
<th>Strain</th>
<th>$k'$ (min$^{-1}$)</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. loihica</em> PV-4</td>
<td>0.0463</td>
<td>0.969</td>
</tr>
<tr>
<td><em>S. amazonensis</em> SB2B</td>
<td>0.0172</td>
<td>0.951</td>
</tr>
<tr>
<td><em>S. oneidensis</em> MR-1</td>
<td>0.0102</td>
<td>0.971</td>
</tr>
<tr>
<td><em>S. baltica</em> OS155</td>
<td>0.0566</td>
<td>0.799</td>
</tr>
<tr>
<td><em>S. putrefaciens</em> CN-32</td>
<td>0.0172</td>
<td>0.977</td>
</tr>
<tr>
<td><em>S. frigidimarinana</em> NCIMB 400</td>
<td>0.0329</td>
<td>0.948</td>
</tr>
<tr>
<td><em>S. putrefaciens</em> 200</td>
<td>0.0245</td>
<td>0.996</td>
</tr>
</tbody>
</table>
Figure 3.2. Bioreactor studies for Cr\textsuperscript{VI} reduction by different Shewanella species
Figure 3.3. Linearized data and model fit for pseudo-first order reaction rate for Cr$^{VI}$ reduction
Figure 3.4. Transformation capacity model predictions using kinetic constants from fitting experimental data to governing equations.
Table 3.5. Model parameter estimates for the transformation capacity model

<table>
<thead>
<tr>
<th>Strain</th>
<th>Tc x 10^19 (mol CrVI CFU⁻¹)</th>
<th>k x 10¹¹ (mL CFU⁻¹ min⁻¹)</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. loihica</em> PV-4</td>
<td>1.318</td>
<td>5.63</td>
<td>0.9756</td>
</tr>
<tr>
<td><em>S. amazonensis</em> SB2B</td>
<td>1.169</td>
<td>2.08</td>
<td>0.9773</td>
</tr>
<tr>
<td><em>S. oneidensis</em> MR-1</td>
<td>1.169</td>
<td>1.31</td>
<td>0.9854</td>
</tr>
<tr>
<td><em>S. baltica</em> OS155</td>
<td>1.212</td>
<td>28.3</td>
<td>0.9529</td>
</tr>
<tr>
<td><em>S. putrefaciens</em> CN-32</td>
<td>1.158</td>
<td>1.95</td>
<td>0.9944</td>
</tr>
<tr>
<td><em>S. frigidimarina</em> NCIMB 400</td>
<td>1.208</td>
<td>3.28</td>
<td>0.9821</td>
</tr>
<tr>
<td><em>S. putrefaciens</em> 200</td>
<td>1.199</td>
<td>3.11</td>
<td>0.9931</td>
</tr>
</tbody>
</table>
this would result in the observed transformation capacities. Evidence of this type precipitation with heavy metals, including chromium, has been shown in previous studies.\textsuperscript{8,30,31}

A sensitivity analysis of this model was then performed by simulating model responses resulting from changes in model parameters. Such an analysis help determined which parameters would affect reduction rates most significantly. The effects of changing the initial concentration ($S_0$), transformation capacity ($T_c$), and initial biomass ($X_0$) were all evaluated individually by using a deviation of 50\% from the values used in the best model fit to the experimental data. These results are shown in Figure 3.5 – Figure 3.7.

Sensitivity tests performed on all the parameters of the TCM seem to indicate that the most important of the biokinetic parameters was the initial biomass concentration. Changes in initial $\text{Cr}^{VI}$ concentrations (Figure 3.5) did not appear to have a pronounced since all scenarios predicted almost complete removal within 180 minutes. Deviation in transformation capacity by 50\% also did not appear to change the model predictions (Figure 3.6). This would be expected if the amount of chromium reduced per bacteria under these experimental conditions was not near the transformation capacity, implying that negligible amounts of biomass were inactivated under these conditions.

On the other hand, changes in biomass concentration appeared to have a large effect. Moreover, the TCM seemed to be more greatly affected by a biomass decrease as opposed to an increase (Figure 3.7). A decrease of initial biomass by 50\% appeared to almost double the amount of time required for complete removal of $\text{Cr}^{VI}$, where an increase by 50\% seemed to have relatively little effect. This result suggests that there is an optimal amount of biomass required to achieve high reduction rates, but that increases above this level would have little
Figure 3.5. Sensitivity of the TCM to variation of the initial Cr$^{VI}$ concentration by different *Shewanella* species
Figure 3.6. Sensitivity of the TCM to variation of the transformation capacity by different *Shewanella* species
Figure 3.7. Sensitivity of the TCM to variation in initial biomass concentration by different Shewanella species
benefit, a useful conclusion for optimization of larger reactor systems. Moreover, this shows that biomass is a critical variable that should be closely monitored in any future systems.

From the data presented, all reduction rates are on the same order of magnitude with the exception of S. baltica OS155, which, according to the all the fitted models, exhibits a rate constant predicted to be about an order of magnitude larger than the other strain (Table 3.5). It should be noted that while the reduction rates do not differ by orders of magnitude, these differences may be important to consider when designing reactor systems.

S. baltica OS155, along with S. frigidimarina NCIMB 400, was isolated from a cold-temperature marine environment and may not perform well under relatively high temperature or low salinity conditions. Utilizing these species would add additional variables that would need to be closely monitored, contributing to increase cost and effort. For this reason, these strains were not considered in parameter evaluation experiments.

**Preliminary model parameter evaluation**

An evaluation on the predictive capabilities of the TCM was performed by solving the model equations using conditions imposed during the 1 liter reactor experiments. For the model, only the first 200 minutes were evaluated, corresponding to more than 99% CrVI removal for the PV-4, the fastest species (Figure 3.8). The results and $r^2$ values (Table 3.6) show relatively good agreement of predicted values with experimental values for PV-4 and MR-1, and poorer agreement for SB2B and Strain 200 (Figure 3.9).

1 liter reactors were employed to determine if model parameters generated from small reactor systems would be useful at predicting behavior of systems operating at larger volumes under different starting conditions. The experimental data was compared with model predictions using the initial reactor conditions for these systems (Figure 3.9). Based on $r^2$ values,
Figure 3.8. Cr(VI) reduction in 1 L reactors by selected Shewanella species
Figure 3.9. Data for 1 liter reactor experiments and model prediction using constants generated from the initial Cr$^{VI}$ reduction tests.
the model still predicts the observations for MR-1 and PV-4. For SB2B and Strain 200, the model predictions appear to be high, indicating the values for the rate constant or the transformation capacities were too low (Table 3.6).

<table>
<thead>
<tr>
<th>Strain</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. loihica</em> PV-4</td>
<td>0.8945</td>
</tr>
<tr>
<td><em>S. amazonensis</em> SB2B</td>
<td>0.7343</td>
</tr>
<tr>
<td><em>S. oneidensis</em> MR-1</td>
<td>0.9504</td>
</tr>
<tr>
<td><em>S. putrefaciens</em> 200</td>
<td>0.5376</td>
</tr>
</tbody>
</table>

Discrepancies between model predictions and experimental data suggests that in order to model future reactor systems, the model may need to be adjusted or tuned for specific architecture and conditions. Furthermore, in some cases, such as Strain 200, very poor fits may result from using parameters in other systems. Thus, the application of the TCM should be evaluated for specific cases.

**Effect of pH and Temperature on Chromium Reduction**

By testing a range of pH values between 6 and 8, it was determined that optimal pH for Cr⁶⁺ reduction should occur at neutral pH values (Figure 3.10). By varying the temperature, the thermal stability of the reduction mechanism was evaluated (Figure 3.11). For the temperatures evaluated, the highest average reduction rates were found around room temperature (25°C). High reduction rates were also observed with temperatures ranging from 35 – 40°C, but these
Figure 3.10. pH effects on the rate constant, $k$, evaluated through fitting data to the “transformation capacity” model.

Figure 3.11. Temperature effects on the rate constant, $k$, evaluated through fitting data to the “transformation capacity” model.
rates were about 30% lower than those observed at 25°C. These results are in accord with the findings by Shi et al. regarding temperature and pH effects on purified cytochromes from *Shewanella* believed to be utilized in metal reduction.\(^\text{32}\)

Tests on the effects of pH and temperature show the sensitivity of chromate reduction to different reactor conditions. The reduction of chromate by *Shewanella* species appears to be highly sensitive to pH. In contrast, rate constants for the temperature range tested appear to be roughly on the same order of magnitude. While an optimum pH was obtained, it does not appear that temperature control of treatment systems will be as critical as pH and the availability of a suitable electron donor. These tests indicate that tight control of pH is necessary and may be critical in engineered reactor systems. Furthermore, the studies show that temperature control may not be as critical, and that a range of temperatures can be tolerated in these systems.

*Effect of Carbon Source on Chromium Reduction*

*S. putrefactions* 200 and *S. amazonensis* SB2B were evaluated in batch reactors for their ability to reduce Cr\(^{VI}\) with different electron donors. These were chosen to represent average reduction rates by the *Shewanella* species tested and because of their different isolation locations (Strain 200 was isolated from an oil pipeline; SB2B was isolated from a marine sediment.). The results (Figure 3.12) show that lactate and molasses had the greatest observed reduction, followed by adenosine. The other electron donors tested did not appear to result in significant Cr\(^{VI}\) reduction over the time period evaluated. Furthermore, the addition of an alternative electron acceptor (fumarate) did not appear to influence reduction with lactate as the electron donor.
Figure 3.12. Effect of electron donor on Cr(VI) reduction
The specificity of electron donor suggests that not all organic compounds may be used by *Shewanella* species in Cr\(^{VI}\) reduction. However, a complex organic mixture, such as molasses, was able to be utilized in Cr\(^{VI}\) reduction. In fact, lactate and molasses based Cr\(^{VI}\) reduction were nearly identical. Lesser reduction was seen with adenosine, and essentially no reduction was coupled to the remaining electron donors. Molasses is often considered a waste by-product of sugar refining, and its use as an inexpensive substrate in Cr\(^{VI}\) reduction can be explored in future work.

### 3.7 Conclusion

These studies show a direct comparison among *Shewanella* strains reducing chromate under various reactor conditions. The experimental data were fit to various models to predict model parameters and to determine model that best describes the Cr\(^{VI}\) reduction process. From the model analyses, it was possible to determine that a “transformation capacity” model described the experimental best. Sensitivity tests on model parameters reveal that biomass concentration and rate constant were the most important parameters and that an optimal biomass concentration would exist for a given system.

Model parameters obtained from preliminary studies agreed reasonably with the reactors operating under different conditions for a few of the species. While adjustment of these parameters will be needed to predict larger scale systems, this approach shows the usefulness of using the TCM parameters in predicting chromate reducing systems.

Sensitivity of the rate to changes in pH and temperature were also determined. The reduction process appeared to be very sensitive to pH, where decrease of one unit from neutral decreased the pseudo-first order reduction rates more than an order of magnitude (0.06 min\(^{-1}\))
to 0.002 min\(^{-1}\)). On the other hand, differences in temperature from 20 – 40 °C were not as pronounced with pseudo-first order rate constants in the range of 0.03 to 0.06 min\(^{-1}\).

The results presented here show that the TCM is capable of describing Cr\(^{VI}\) reduction by several different *Shewanella* species and that certain reactor conditions would greatly affect the amount and rate of reduction. Moreover, they present the most important parameters to optimize when considering the design and operation of other reactor systems, such as microbial fuel cells.
Chapter 3 References


Chapter 4: Evaluation of microbial fuel cell *Shewanella* biocathodes for treatment of chromate contamination

4.1 Introduction

Chromium is a metallic species widely used in industrial applications including metal plating, leather tanning, and dye manufacturing. Inadequate or inappropriate handling of waste often allows for the introduction of chromium into the environment. The two major forms found in the environment are the hexavalent, Cr\textsuperscript{VI}, and trivalent, Cr\textsuperscript{III}, species. Exposure to Cr\textsuperscript{VI} has been linked to cancer, skin ulcers, and other diseases\textsuperscript{1} The high mobility and solubility of Cr\textsuperscript{VI} frequently results in contamination of soils, surface waters, and ground water. In contrast, the trivalent species is generally considered innocuous in the environment due to its lack of mobility as an insoluble species\textsuperscript{2,3} Thus, reduction of Cr\textsuperscript{VI} to Cr\textsuperscript{III} has been proposed as an effective mechanism for limiting exposure and movement in the natural environment.

An emerging technology for exploiting bacterial reduction-oxidation systems is that of microbial fuel cells (MFCs)\textsuperscript{4,5} This technology has recently been proposed for pollution remediation, capitalizing on the reducing power supplied by the anode compartment and the catalytic abilities of biocathodes\textsuperscript{6,7} In particular, several studies utilizing enrichment communities as biocathode communities have been reported for Cr\textsuperscript{VI} contamination\textsuperscript{8-10} While these studies show reduction of chromium at ppm concentration levels, drinking water regulations require removal to low ppb concentrations, orders of magnitude below what was reported in these studies\textsuperscript{11} To demonstrate the feasibility of MFC systems to reduce chromium to levels acceptable for drinking water regulations, data is presented here examining the
utilization of single strains of bacteria as the sole biocatalyst at the cathode of a two-chamber MFC.

Using a mixed community does not allow us to directly understand both what organisms are active at the electrode surface and the mechanisms for electron transfer, essentially a “black box” approach. Knowledge of *Shewanellae*, on the other hand, allows us to use them as model organisms since their physiology and genomes have been studied extensively.\textsuperscript{12-15} By comparing the behavior between these *Shewanellae*, systems biology approach can be used to understanding the mechanisms behind electron transfer at the cathode. Understanding these mechanisms is critical if these systems are to be optimized and mixed microbial consortia are to be understood. For these reasons, several *Shewanella* strains were examined as single-strain cathode bicatalysts (Table 4.1).

**Table 4.1. *Shewanella* strains used as biocathode catalysts for these experiments.\textsuperscript{15}**

<table>
<thead>
<tr>
<th>Species Name</th>
<th>Discovery location</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Shewanella oneidensis</em> MR-1</td>
<td>Lake Oneida, New York, USA (freshwater sediment)</td>
</tr>
<tr>
<td><em>Shewanella putrefaciens</em> W3-18-1</td>
<td>Pacific Ocean sediment, Washington, USA (coastal sediment)</td>
</tr>
<tr>
<td><em>Shewanella amazonensis</em> SB2B</td>
<td>Amazon River delta, Brazil (coastal sediment)</td>
</tr>
<tr>
<td><em>Shewanella</em> sp. ANA-3</td>
<td>Woods Hole, Massachusetts, USA (brackish estuary)</td>
</tr>
<tr>
<td><em>Shewanella loihica</em> PV-4</td>
<td>Loihi seamount, Hawaii, USA (hydrothermal sea vent)</td>
</tr>
<tr>
<td><em>Shewanella</em> sp. MR-4</td>
<td>Black Sea, 5 m depth (oxic salt water)</td>
</tr>
</tbody>
</table>
The selected *Shewanellae* represent a variety of physiologies and genetic differences. All strains contain the metal-reducing gene locus (mtrABC) known to be important for anode biocatalysis along with a variety of other multi-heme cytochromes, some unique to individual strains.\(^{16}\) Comparison of these strains will help us to identify those strains with the most favorable transfer mechanisms for organisms operating at the cathode. This allows future research to focus on determining the gene products and/or physiological responses responsible for high performance biocatalysis.

The *Shewanella* strains used here have not been reported in any biocathode studies. First, biocatalytic abilities of these strains at the cathode utilizing fumarate reduction was tested. Fumarate was chosen as the initial electron acceptor based on earlier studies with MR-1 which show no interference by fumarate and its metabolic by-product (succinate) with Cr\(^{VI}\) reduction.\(^ {17}\) Following an initial period of fumarate reduction, each strain was examined under chromate reducing conditions. The data shown here detail the first comparisons using *Shewanella* strains as biocathodes, under both non-toxic, fumarate-reducing, conditions and toxic, chromate-reducing, conditions.

### 4.2 Materials and Methods

*Fuel Cell System Construction*

The fuel cell systems utilized in this research were similar to those described by Rhoads et al. with two 500 mL compartments which serve as anode and cathode chambers (Figure 4.1).\(^ {18}\) In these fuel cells, the electrodes were prepared from reticulated vitreous carbon (80 ppi; ERG Aerospace; Oakland, CA) cut to a size of 5 cm x 10 cm x 0.5 cm. The electrode material was pretreated by soaking with an ethanol/water (95%/5%) mixture followed by an acid wash with
Figure 4.1. Side-view schematic of fuel cell reactor used in fumarate and chromate biocathode studies
1 M HCl. Electrodes were then rinsed with ultrapure H₂O and dried at 105°C. Graphite rod leads were attached to pretreated electrodes with conductive epoxy. Stranded stainless steel wire was attached at the free end of the rods with vinyl tape to serve as connections from the electrode to the exterior of the fuel cell. Care was taken to ensure that this connection was above the working liquid level in the fuel cell chamber to avoid interaction of the stainless steel with bacterial cultures and electrolyte.

A cation exchange membrane (CMI-7000; Membranes International, Inc.; Glen Rock, NJ) was utilized to separate the anode and cathode chambers. The membrane was prepared by hydration in 50 mM PIPES for 24 hours prior to use.

**Preparation of bacterial cultures**

Cultures of *Shewanella* species (Table 4.1) were prepared by inoculating 10 mL of LB Broth, Miller (BD; Franklin Lakes, MD) from -80°C glycerol stocks obtained from a collection of *Shewanella* maintained by the Nealson Lab at the University of Southern California. This culture was allowed to incubate for 16 hours at 30°C at 100 rpm. This overnight culture was diluted 1:1000 to inoculate a bacterial media containing 50 mM PIPES buffer, 90 mM NaOH, 28 mM NH₄Cl, 1.3 mM KCl, 4.3 mM NaHPO₄, trace minerals, trace vitamins, and 15 mM sodium lactate at pH 7. This was allowed to incubate for 18-24 hours at 30°C at 100 rpm and diluted with 50 mM PIPES buffer to reach a cell density of approximately 10⁷ CFU/mL (OD₆₀₀ = 0.2).

250 mL of MR-1 was added to the anode compartment of the fuel cell to serve as the anodic biocatalyst. To evaluate various *Shewanella* as the sole cathodic biocatalyst, 250 mL of a single *Shewanella* strain to be tested was added to the cathode compartment. The fuel cell apparatus was then connected to a prepurified nitrogen gas line. Nitrogen gas was fed through sterile gas filters (0.2 µm) to each chamber of the fuel cell at a rate of 40 mL per minute. Fine frit
glass gas dispersers (Chemglass, Inc.; Vineland, NJ) were utilized for gas sparging to ensure adequate mass transfer.

**Operation and Characterization of Fuel Cell Systems**

Anode and cathode electrodes were connected to a 10 ohm resistor and the system was allowed to equilibrate for 2 – 4 hours. After achieving a baseline voltage, aliquots of lactate and fumarate were injected into the anode and cathode compartments, respectively, to reach final concentrations of 4 mM each (a stoichiometric excess of 2 mM lactate).

The electrodes were disconnected from the resistor at this point and the system was allowed to equilibrate for several hours until a stable open circuit potential was reached. Calomel reference electrodes were used to determine electrode potentials and a linear polarization resistance curve was generated by employing a potentiostat (Reference 600; Gamry Instruments; Warminster, PA) using the anode as the working electrode and cathode as the reference and counter electrodes.

Following the completion of the polarization tests, the electrodes were connected to a 10 ohm resistor and generated voltage was monitored using a digital multimeter and data acquisition system (Model 2700; Keithley Instruments, Inc.; Cleveland, OH). Once voltage decreased to initial levels (indicating exhaustion of fumarate), a 1040 ppm (20 mM) Cr(VI) solution was injected into the cathode compartment to increase the Cr(VI) concentration level to 2.5 ppm. This injection was repeated every three days for a total of three semi-batch operation cycles. Samples were taken for chromium and organic acid analysis during both fumarate and chromium reduction.
**HPLC analysis**

In order to monitor the levels of organics, anolyte and catholyte samples were taken during three time periods: (1) immediately after feeding, (2) at peak voltage, (3) after reaching baseline. The samples were centrifuged for 5 minutes at 17,900×g and the supernatants were collected and stored at −20°C. Samples were then acidified by addition of sulfuric acid to bring the final concentration of sulfuric acid to 12.5 mM. After overnight incubation at 4°C to facilitate precipitation of PIPES buffer, samples were filtered through a 0.22 μm syringe filter. An Agilent 1100 Series HPLC system (1100 series; Agilent, Inc.; Santa Clara, CA), using a reverse phase C18 column (Synergi-Hydro 4 μm 250 mm/4.6 mm; Phenomenex; Torrance, CA, USA) for separation, equipped with diode array detector (G1315B; Agilent, Inc.; Santa Clara, CA) set at 210 nm. Chromatograms were generated using a mobile phase of 2.5 mM sulfuric acid with a flow rate of 0.5 mL min⁻¹ at ambient temperature.

**Ion chromatography analysis**

The presence of Cr⁶⁺ was measured by taking samples before, during, and after each injection period. These samples were adjusted to pH 10 by addition of a 0.25 M (NH₄)₂SO₄/1M NH₄OH buffer solution and incubated at 4°C until analysis. Samples were then filtered through a 0.22 μm syringe filter prior to injection to the ion chromatography (IC) system. An IC system (850 Professional; Metrohm AG; Switzerland) with a post-column derivitization coil and UV-Vis absorbance detector (Lambda 1010; Metohm AG; Switzerland) set at 530 nm was used for Cr⁶⁺ analysis. For separation, a 1000 μL sample injection loop was used with an anion exchange column (Metrosep A Supp 10 250/4.0; Metrohm AG; Switzerland). Chromatograms were generated with an eluent (10 mM LiOH, 78 mM Li₂SO₄) flow rate of 0.8 mL min⁻¹ and a post-
column derivitization reagent (0.5 g L⁻¹ diphenylcarbazide, 0.5 M H₂SO₄, 10% methanol) flow rate of 0.4 mL min⁻¹.

Electron microscopy

Electron microscopy was employed to observe attached biomass and to determine the probable locations of reduced chromium species on the electrode surface. Following each experiment, sections of electrodes were extracted and fixed in 2.5% gluteraldehyde. Following fixation, samples were prepared by gradual ethanol dehydration and hexamethyldisilizane substitution.²⁰,²¹ The samples were then mounted on aluminum stubs and carbon coated. Electron microscopy and elemental analysis were performed using a scanning electron microscope (JSM-6610LV; JEOL USA, Inc.; Peabody, MA) operated with a 10 keV accelerating voltage and equipped with an energy dispersive spectroscopy (EDS) detector (JSM 6490; EDAX Inc.; Mahwah, NJ).

Theoretical electrode potential and electron transfer comparisons

Reactions and standard potentials for lactate oxidation and fumarate reduction from literature were compared with observed open circuit cell potentials.²² The chromate reduction standard potential was calculated from a published redox equilibria constant and adjusted for reactor conditions.² Redox potentials for conditions found in the fuel cell reactor were also calculated from standard potentials.⁴ This relationship between reactor conditions and standard potentials is given by the Nernst equation:

\[
E = E^\circ - \frac{RT}{nF} \ln \frac{\prod_{p} a_p}{\prod_{r} a_r}
\]

where E is the potential at reactor conditions, E^\circ is the standard potential, R is the universal gas constant, T is the absolute temperature, n is the number of electrons transferred per reaction, F
is Faraday’s constant, \(a_p\) is the activity of the reaction products, and \(a_r\) is the activity of the reactants.

The potential at the cathode under open circuit conditions is given by the following equation:

\[
E_{\text{cathode}} = E_{\text{reduction}} - (\eta_{\text{activation}} + \eta_{\text{concentration}}) \quad (2)
\]

where \(E_{\text{cathode}}\) is the cathode potential and \(E_{\text{reduction}}\) is the theoretical potential of the reduction half reaction. The overpotentials \((\eta_{\text{activation}} + \eta_{\text{concentration}})\) refer to losses due to activation potential and concentration polarization. Ohmic losses are not expected to be significant during open circuit measurements since no current is being transferred. Mass transfer losses due to concentration polarization are also expected to be minimal since measurements are being done at peak fumarate concentrations. Therefore, the differences in cathode potentials that were observed between \textit{Shewanella} strains are associated with activation overpotentials and governed by how these strains perform electron transfer within the electrode-biofilm-oxidant system.

As a measure of efficiency, comparisons were made between the measured electron transfer from the anode compartment \((e_{\text{charge}}^-)\) and the electron transfer required to explain the observed reduction/oxidation change \((e_{\text{chem}}^-)\). When the ratio of \(e_{\text{chem}}^-\) to \(e_{\text{charge}}^-\) is 1, then the charge transfer likely proceeds through a 3 electron reduction reaction. Values less than one indicate more charge was accepted by the cathode system than was necessary for the reduction reaction to occur. This condition implies that additional electron sinks were present in the reactor system in the cathode chamber. On the other hand, values greater than one indicate that an insufficient amount of electrons were transferred to yield the observed chemistry, suggesting that 1) an additional source of electrons was present to facilitate the reduction at the
cathode, 2) $\text{Cr}^{VI}$ was removed by other means, such as physical adsorption, and/or 3) unstable $\text{Cr}^{V}$ and $\text{Cr}^{IV}$ species were formed from a 1 or 2 electron transfer reaction and subsequently further reduced by other species in the cathode compartment.

### 4.3 Results and Discussion

**Fumarate reduction**

While all the selected strains of *Shewanella* are known to be capable of electron transfer to the anodic electrode,\textsuperscript{16,23} previous reports have not determined whether these strains of *Shewanella* are capable of catalyzing the cathodic reduction reaction in a bioelectrochemical system. Thus, the first set of experiments was aimed at verifying activity and evaluating performance at the cathode. For this purpose, fumarate reduction at the cathode was evaluated based on both the known physiology of *Shewanella* species\textsuperscript{13,15,24} and previously reported work with *Geobacter* species.\textsuperscript{25-27}

Anodic and cathodic potentials were measured versus a standard calomel electrode (Table 4.2). These potentials can be compared with calculated theoretical potentials under conditions experienced in the fuel cell reactor. The anode potentials were all similar in magnitude, an expected result since the conditions and species were the same at the anode for all experiments. The cathode potentials show wide and unexplained variability, probably due to the different species, specifically their interaction with the cathode electrode and differences in physiology. As an estimate of losses, these potentials can be compared to the calculated potential ($E$) of the fumarate half reaction under the given cathode conditions of $+0.137 \text{ V vs Standard Hydrogen Electrode (SHE)}$. Because more negative values indicate losses due to the species and bioelectrochemical system,\textsuperscript{4} those species with more positive values should be
Table 4.2. Open circuit potentials of fumarate reducing microbial fuel cells

<table>
<thead>
<tr>
<th>Cathode Species</th>
<th>Anode Potential vs SHE mV</th>
<th>Cathode Potential vs. SHE mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR-1</td>
<td>-778 ± 2.61</td>
<td>-713 ± 299</td>
</tr>
<tr>
<td>W3-18-1</td>
<td>-661 ± 70.3</td>
<td>-452 ± 132</td>
</tr>
<tr>
<td>SB2B</td>
<td>-656 ± 68.4</td>
<td>-343 ± 263</td>
</tr>
<tr>
<td>ANA-3</td>
<td>-746 ± 71.2</td>
<td>-518 ± 138</td>
</tr>
<tr>
<td>PV-4</td>
<td>-709 ± 56.3</td>
<td>-536 ± 93.4</td>
</tr>
<tr>
<td>MR-4</td>
<td>-701 ± 118</td>
<td>-456 ± 117</td>
</tr>
</tbody>
</table>

capable of more effective electron transfer (lower activation losses), and consequently more power generation. For example, the cathode potential of an MR-1 biocathode appears much lower than that of the SB2B biocathode. Based on this, it would appear that the MR-1 biocathode would suffer greater overpotential losses, and thus perform less efficiently than an SB2B biocathode.

As shown in Figure 4.2, linear polarization results show maximum power densities ranging from 10.2 to 59.4 nW cm\(^{-2}\) (26.5 to 154 nW cm\(^{-3}\)) for MR-1 and W3-18-1, respectively. Current measurements during this determination show maximum current densities between 0.5 A m\(^{-3}\) for MR-1 and 2.4 A m\(^{-3}\) for W3-18-1. These values are lower, but on the same order of
magnitude as current densities reported for fumarate-reducing systems utilizing *Geobacter* species and poised electrodes.\textsuperscript{26,27}

Reduction of fumarate to succinate requires a 2 electron transfer reaction. Previous studies with *Geobacter* have shown that incomplete oxidation of fumarate may occur, leading to a lower amount of charge transfer from the electrode to fumarate than would be predicted for full reduction to succinate. For example Gregory et al. showed a 1:1 electron consumption to succinate production ratio, implying that only half of the necessary electrons required for fumarate reduction were transferred from the electrode.\textsuperscript{25} On the other hand, Strycharz et al. reported an exact stoichiometric match (2:1) of electrons transferred to succinate production.\textsuperscript{27} Comparisons between the results reported in this paper and those of previous studies may be difficult to make due to the differences in reactor architecture and its essential components.
The ratio of $e_{\text{chem}}$ to $e_{\text{charge}}$ is shown in Figure 4.3. The values here also show an imbalance, where electrons for succinate production are not completely accounted for by electrons transferred from the anode compartment. The ratios reported here represent a deficiency greater than the 1:1 succinate to electron ratio (corresponding to an $e_{\text{chem}}/e_{\text{charge}}$ of 2) reported by Gregory et al., which they attributed to incomplete fumarate oxidation. In the results for this study, analysis shows that between 10.6 (MR-4) and 57.4 (SB2B) times the observed electron transfer would be required to account for the observed succinate production.

![Figure 4.3. Efficiency ratio of hypothetical charge transfer requirements to observed transfers for a 2 electron reduction of fumarate to succinate for different Shewanella species](image)

The extra electron pool could be explained through a number of possibilities. For example, substances in the extracellular biofilm matrix could be metabolized or inactive biomass.
could be degraded by extant bacteria. Freguia et al. demonstrated the use of a storage molecule as a source of electrons for the anodic catalysis.\textsuperscript{28} Thus, the possibility of a similar molecule to provide electrons at the cathode cannot be discounted.

Possible mechanisms for the formation of such a substance have been presented in previous batch studies and genomic analysis. Tang et al. reported that during aerobic and anaerobic growth on lactate, MR-1 will allocate a portion of carbon to be sent through the glyoxolate shunt, presumably for more complex carbon molecules used in biosynthesis and growth.\textsuperscript{29} Heidelberg et al. also present the possibility of glycogen synthesis and utilization as a possible energy storage strategy.\textsuperscript{12} Regardless of the source of these electrons, the use of this extra pool decreases the amount of current transferred from the anode side, adversely affecting overall fuel cell performance with fumarate as the electron acceptor.

\textit{Chromate reduction}

Once fumarate reduction (succinate production) was completed, as indicated by a drop in current production (and later verified by HPLC analysis) the cathode compartments were injected with 20 mM Cr\textsuperscript{VI} stock solution to achieve a concentration of 2,500 ppb (48 μM). Cr\textsuperscript{VI} was measured periodically during this semi-batch operation over a three day period, at which point another spike of Cr\textsuperscript{VI} was injected into the system. The residual Cr\textsuperscript{VI} concentrations after 3 successive exposures are shown in Figure 4.4.

For the first exposure, Cr\textsuperscript{VI} levels were reduced to below 10 ppb for most species tested, and well below levels achieved with abiotic reduction. The exception was MR-1, where the remaining Cr\textsuperscript{VI} was almost 40 times this amount (365 ppb). The second injections showed more variability between the species. Both MR-4 and the sterile cathode were only able to bring Cr\textsuperscript{VI} concentrations to around 560 ppb. PV-4 and SB2B showed higher degrees of removal, with
concentrations between 100 – 200 ppb. MR-1, W3-18-1, and ANA-3 were all able to bring concentration to below 10 ppb. It is important to note that all species were able to reduce as well or better than the abiotic cathode for this Cr⁶⁺ exposure.

Figure 4.4. Residual chromium for each of the semi-batch chromium injection cycles for different Shewanella species

Final injections showed drastic differences from the first two Cr⁶⁺ exposures. For this injection, all species except for W3-18-1 showed an average residual chromium level between 700 and 2000 ppb, above that of the abiotic cathode (640 ppb). W3-18-1 catalyzed systems resulted in average residual chromium levels of 167 ppb. The relatively high concentrations of
residual chromium indicate a finite tolerance limit to exposure of Cr\textsuperscript{VI}, consistent with previously reported batch studies.\textsuperscript{30} For W3-18-1, this tolerance appears to be much higher than the rest of the species tested.

Additionally, the inability to remove Cr\textsuperscript{VI} to concentrations levels comparable to the abiotic cathode indicates the possibility of fouling of system components. Non-reducing biological material (such as cell debris, biofilm matrices, inactive/dead cells) may reduce active surface area at the electrode surface. Reduced chromium precipitates may also persist at the electrode surface after reduction, also resulting in a decrease in available surface area for reduction. The compounded effect of both biological fouling and chromium fouling serves to lower overall mass transport at the electrode surface, resulting in losses due to concentration polarization.

Figure 4.5 shows that the $e^{-\text{chem}}/e^{-\text{charge}}$ ratios for the initial cycle of chromium exposure are relatively similar for all biocathodes. These values range from 0.87 (MR-1) to 1.64 (SB2B), indicating that most of the chromium underwent a 3 electron reduction to Cr\textsuperscript{III} and that these electrons came from the cathode electrode. The slight increase of values greater than 1 indicates that either some removal of Cr\textsuperscript{VI} has occurred that may not be associated with electron transfer from the cathodic electrode.

The next exposure of chromium resulted in increased $e^{-\text{chem}}/e^{-\text{charge}}$ values suggesting that Cr\textsuperscript{VI} was removed by one or more of the following mechanisms: i) reduction by other unknown electron donors, ii) removal by adsorption to fuel cell surfaces, or iii) incomplete reduction to Cr\textsuperscript{V} or Cr\textsuperscript{IV}. While MR-1 still exhibited ratios comparable to those observed in the first Cr\textsuperscript{VI} exposure, other strains showed values ranging from 1.97 (W3-18-1) to 2.69 (SB2B). These values show that only around half of the electrons necessary for a 3 electron reduction
were passed from the anode side of the fuel cell. Studies have reported Cr\textsuperscript{VI} reduction by simple amino acids and natural organic matter which may explain the electrons unaccounted for by charge transfer from the anode side.\textsuperscript{2} Use of succinate as an electron source may also be possible, since MR-1 is reported to be able to oxidize succinate through TCA cycle enzymes.\textsuperscript{29,31}

![Figure 4.5. Ratio of hypothetical charge transfer requirements to observed transfers for a 3 electron reduction of Cr\textsuperscript{VI} to Cr\textsuperscript{III} for different Shewanella species](image)

Although the amount of Cr\textsuperscript{VI} reduced during the third exposure suggest little to no activity for most species, larger $\frac{e_{\text{chem}}}{e_{\text{charge}}}$ values were observed. This may indicate a higher dependence on the alternate electron sources. These sources likely include organic material, such as bacterial cell lysates, resulting from cell inactivation or death due to chromium toxicity.
High residual levels resulted from several of the *Shewanella* strains indicate that biological catalysis of chromate may have ceased and abiotic removal of chromate may be dominant during this period.

The sterile cathode results indicate that the anode biocatalysts clearly provide enough reducing power to achieve chromate reduction, but not to levels seen with the *Shewanella* biocathodes. The $e_{\text{chem}}^\text{charge}$ values for the sterile cathode also showed differences from the biological cathodes. The initial exposure of the sterile cathode resulted in an $e_{\text{chem}}^\text{charge}$ value of 0.80. For the second exposure, the $e_{\text{chem}}^\text{charge}$ value rose to 0.88. Both of these values are very close to the expected value for 3 electron reduction of Cr$^{\text{VI}}$. The third injection shows a relatively large $e_{\text{chem}}^\text{charge}$. This may be explained removal of Cr$^{\text{VI}}$ by other means such as adsorption to fuel cell surfaces or interactions with Cr$^{\text{III}}$ species. The abiotic cathode also maintained a lower average Cr$^{\text{VI}}$ residual for the third exposure in comparison to the *Shewanella* biocathodes. This would suggest that biological material (either biofilm components or cellular debris) may prevent any significant adsorption/precipitation interactions with fuel cell components from taking place during this third exposure of Cr$^{\text{VI}}$.

To investigate the different possibilities of fouling (chemical species, biomass, etc.) further, electron microscopy and elemental analysis were employed to observe the electrode surface and determine the location of chromium at the electrode-biomass environment and to examine the extent of biofilm coverage. The results are shown in Figure 4.6 and summarized in Table 4.3. The results show a variety of biofilm coverage, with W3-18-1 showing the greatest amount of bacteria attached to the electrode surface and PV-4 exhibiting the least. Furthermore, nodules of precipitated chromium were observed at the surface of several of the electrodes and associated with the biomass of certain species (MR-1, W3-18-1, SB2B, and
Figure 4.6. Scanning electron micrographs and elemental dispersive spectroscopy peaks for the determination of chromium in biocathode samples for each *Shewanella* species.
Table 4.3. Summary of electron microscopy and elemental analysis observations

<table>
<thead>
<tr>
<th>Species</th>
<th>Biomass attachment</th>
<th>Chromium seen as separate precipitates</th>
<th>Chromium associated with biomass</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR-1</td>
<td>Moderate</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>W3-18-1</td>
<td>High</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>SB2B</td>
<td>Moderate</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>ANA-3</td>
<td>Moderate</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>PV-4</td>
<td>Low</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>MR-4</td>
<td>Moderate</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>
It can be speculated from these observations that the mechanism of chromium reduction may be different for each *Shewanella* species and that factors such as biofilm attachment can influence Cr$^{VI}$ reduction as well.

Similar results were reported by Huang et al. indicating chromium reduction occurring at the cell surface and in the biofilm matrix.$^{10}$ Their results also indicate that there was no significant abiotic effect at the concentrations used. While biocathodes were able to reduce chromium to much lower levels than sterile cathodes in this study, reduction at the sterile cathode did seem to play a significant role at the concentrations of chromium used in the experiments presented here. This is in contrast to previous studies.$^{9,10}$ However, those studies used levels of chromium at least one order of magnitude above the concentrations used here, and as a result the abiotic effects of reduction may have been insignificant when compared to the overall reduction reported. Although oxidation states of chromium were not determined in our study, previous batch reactor studies with MR-1 have shown that biomass associated precipitates are predominantly Cr$^{III}$ with minor amounts of Cr$^{VI}$.32

**Mechanisms for electron transfer to electron acceptors**

In order to understand the abilities of *Shewanellae* performing reduction at the cathode, it is important to understand the fundamental electron transfer strategies starting from the cathode, flowing through the bacteria, and finally, leading to the terminal electron acceptor. There are several proposed mechanisms for electron transfer within the electrode-bacteria-oxidant system. These include direct electron transfer, use of an external mediator, or a combination of these.$^{6,7}$ The results presented here suggest that the electron transfer mechanisms for one species will show different behaviors based on the electron acceptor. Such
results should not be surprising when considering the electron transfer pathways of the different species and different electron acceptors.

Similar electron transfer pathways might be expected for fumarate reduction at the cathode when considering that only a single fumarate reductase has been reported to exist. The terminal fumarate reductase in MR-1 is a soluble periplasmic flavoprotein (FccA). Homologous genes to fccA appear in all strains tested, suggesting the key fumarate reduction enzyme is the same among all the selected Shewanella strains.

On the other hand, no common Cr\textsuperscript{VI} reducing enzyme or pathway has thus far been reported. Batch studies have shown Cr\textsuperscript{III} precipitates may be deposited on exterior cell surfaces and within the periplasm of cells. These observations suggest that reduction is occurring at both the cell surface and in the periplasm. Previous studies also indicate that multiple mechanisms of chromium removal may be significant for MR-1. Enzymes similar to the variety of reductases and multi-heme cytochromes found in MR-1 can also be found in all of the species tested, and imply that several different physiological mechanisms may be involved in both chromium reduction and interaction with the electrode surface. The built-in redundancy of this system may lead to the observed inefficiencies in electron transfer, but may also enable a greater chance for bacteria survival when toxic chemicals such as Cr\textsuperscript{VI} are encountered.

Variability exists not only in the electron transfer to the terminal electron acceptor, but also in the transfer of electrons between the bacteria and electrode surface. Important genes implicated at anode studies of MR-1 show that proteins required for metal reduction (MtrABC) appear to be necessary for transfer of electrons to a solid electrode surface. The numerous cytochromes harbored by Shewanella suggest that the electron transfer pathways between the cell surface and the interior of the cell are very complicated and not yet fully understood.
example, several paralogs of the mtrABC genes are seen in MR-1 and orthologs for these genes are present in all species tested. These genes are believed to form complex electron transfer pathways for transfer of electrons to the exterior of the cell and may be used for transfer of electrons from an electrode to the interior of the cell. The complexity and variability of these electron transfer pathways between species may explain the differences in power generation and coulombic efficiencies observed in the fuel cell reactor.

4.4 Conclusions

The studies here give evidence of a fumarate reducing biocathode by *Shewanella* strains and provide a comparison between these species using the same MFC architecture. The results show differences in efficiency and power output that are related to the strain catalyzing reduction at the cathode.

The present studies, in particular, demonstrate the ability of these species to reduce chromium concentrations to low levels (ppb) at the cathode. Furthermore, chromium precipitates are shown to be associated with the electrode surface and the biomass of certain *Shewanella* strains. Most studies have primarily utilized enriched consortia as biological catalysts to provide reduction of CrVI to parts per million levels. While those studies demonstrate reduction of CrVI to levels between 40 and 80 ppm, the studies reported herein demonstrate the feasibility of these systems to effectively lower CrVI concentrations from 5 ppm to less than 5 ppb levels. Considering the United States’s chromium drinking water standard of 100 ppb and the World Health Organization’s guideline of 50 ppb, removal of CrVI to these levels will be necessary if such systems will be used as remediation tools for contaminated water sources.

Evidence is also presented here that a variety of electron transfer pathways may be available to *Shewanella*, and that interspecies variability of these pathways likely accounts for
the discrepancies in efficiency and reduction capacity. If only a single pathway was utilized for electron transfer, then one would expect these values to be similar. The data presented here show that this is likely not the case, and that understanding these phenomena will be important in understanding mechanisms of electron transfer in biocathodes.

Future work will focus on determining the physiological mechanisms for electron transfer at the cathode (and for Cr$^{VI}$ reduction in particular). Understanding the fundamentals of electron transfer at the cathode will enable the design of more effective electrode materials and selection of more efficient biological catalysts. Enrichment of microorganisms for both enhanced resistance to Cr$^{VI}$ toxicity as well as enhanced electron transfer between electrodes will also be pursued. Additional efforts will focus on implementing larger scale reactor architecture as a prelude to large scale remediation efforts while simultaneously decreasing losses within the system to increase electron transfer efficiency.
Chapter 4 References


Chapter 5: Assessment of enriched bacterial communities and application to Cr\textsuperscript{VI} reduction in a microbial fuel cell

5.1 Introduction

Microbial fuel cells (MFCs) are electrochemical systems which have recently been considered for electrical energy generation, as well as metal remediation.\textsuperscript{1-5} Much of the work can be divided into two distinct categories: 1) those studies utilizing an enriched microbial community and 2) those utilizing model organisms (such as \textit{Geobacter} or \textit{Shewanella}).

One of the major advantages to using model organisms in MFCs is that they can be used to probe the processes of electron transfer within the MFC. While \textit{Geobacter} and \textit{Shewanella} have both been found in enrichment MFC cultures, an abundance of other bacterial groups were also observed and may play ecological roles in these communities.\textsuperscript{6,7} Thus, the use of single cultures in MFCs does not feasible in real-world systems, and the need to study how these different mixed microbial groups interact also necessary to understand how to optimize performance and output from these systems.\textsuperscript{8}

Several studies on mixed communities exist for enriched communities at the MFC anode. However, relatively few studies have reported on the MFC cathode.\textsuperscript{9-11} Of these, a few explore the possibilities of Cr\textsuperscript{VI} reduction in MFC communities,\textsuperscript{12,13} but examine the community structure of Cr\textsuperscript{VI}-reducing enrichments. Understanding the stability of these communities and their response to chromate stress is important when attempting to use these systems to remediate polluted environments. Moreover, this understanding of major bacterial groups in
these communities will provide a basis for possible biological models describing Cr\textsuperscript{VI} reduction and therefore the development of remediation strategies utilizing MFC technology.

To this end, this series of experiments was aimed at evaluating different strategies of establishing an electrode community, and how an enrichment community would respond when forced to perform Cr\textsuperscript{VI} reduction. Evaluation of electrode communities would involve the following considerations: (i) the power production between a pure culture of \textit{Shewanella oneidensis} MR-1 and enriched bacterial consortia from wastewater sludge under identical conditions, (ii) the power production and microbial population structures of MFCs enriched with different carbons sources, and (iii) MFC performance of bacterial communities when exposed to carbon compounds other than the one initially used for enrichment.

Bacterial communities were enriched (and electricity generation monitored) over a six-month period with six different electron donors: formate, acetate, lactate, succinate, n-acetyl-d-glucosamine (NAG), and uridine. The anode-attached and planktonic microbial communities were analyzed by polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE). In addition, based on the results from the molecular analysis of the bacterial population structures, cross-feeding experiments were conducted to test the stability of enriched bacterial communities and the ability of these enriched communities to utilize various electron donors.

Following this, establishment of a Cr\textsuperscript{VI} reducing MFC cathode was evaluated in two ways: (1) a direct enrichment (DE) method at a MFC cathode and (2) use of an established community from the above studies at an MFC cathode to catalyze Cr\textsuperscript{VI} reduction, or a subsequent enrichment (SE) method. The first method would be simultaneously enrich for microbes capable of electron transfer from the electrode and for those capable of Cr\textsuperscript{VI} reduction. This method has been used in previous studies to build electrically active biofilms on
electrode surfaces before employing them in MFC cathodes. The second method would involve an initial enrichment for electron transfer capabilities followed by selection for Cr\textsuperscript{VI} reduction capabilities.

5.2 Materials and Methods

MFC design and operation

Seven dual-chamber MFCs (Figure 5.1) were assembled as previously described,\textsuperscript{16} except that the anode chamber contained two graphite felt electrodes (GF-6-6, Electrolytica Inc., Amherst, NY, USA) with the following dimensions: 2.0 cm × 1.0 cm × 0.6 cm. Anode chambers were supplied with a continuous stream of sterile, ultrapure N\textsubscript{2} gas (20 mL/min) while cathode chambers were supplied with filtered compressed air (40 mL/min). Voltage across a 10 Ω external resistor connected to both the anode and cathode electrodes was recorded every 5 minutes with a high-impedance digital multimeter (Model 2700, Keithley Instruments Inc., Cleveland, OH, USA).

MFC anode inoculation and enrichment

To serve as a comparison to the enrichment cultures, \textit{S. oneidensis} MR-1 was inoculated to MFC No. 1 as described by Bretschger \textit{et al.},\textsuperscript{16} except that the lactate concentration was 7 mM instead of 18 mM. Sludge was obtained from the Los Angeles County Joint Water Pollution Control Plant. Equal volumes of activated sludge and anaerobic digest were mixed and filtered through 11 μm paper filters (Grade 1, Whatman, Piscataway, NJ, USA). Filtrate was then diluted five fold with sterile 50 mM PIPES buffer at pH 7.0. This mixture was used as the initial inoculum for the enrichment of MFCs No. 2 – 7.
Figure 5.1. (a) Side view and (b) front view of the small MFC used in these anode enrichment studies.
Twenty-five mL of each inoculum was injected into the anode chamber of a pre-sterilized MFC. The chambers were supplied with N₂ (anode) or air (cathode) until a stable baseline voltage was achieved. Lactate was used as the electron donor for the MR-1 MFC while lactate, succinate, NAG, acetate, formate and uridine were used in MFCs No. 2 – 7. Each electron donor concentration was set to 2 mM to begin each experiment. Since voltage fluctuations directly correspond with electron donor consumption with saturation occurring at maximum peak values,¹⁶ voltage was allowed to plateau and decrease to initial baseline values after each injection.

**Evaluation and characterization of MFC performance**

Total transferred charge was calculated based on the area under the current versus time curve. Current (I) was calculated based on Ohm’s Law, I = V/R. Maximum current densities were calculated using the average peak current values over 3 hours for each feeding divided by the projected surface area of 7.6 cm². In order to evaluate the maximum power output for each fuel cell, linear sweep voltammetry was used to generate polarization curves for each fuel cell system. MFCs were allowed to reach a maximum voltage after injection of appropriate electron donors and equilibrate at open circuit conditions for approximately 2 - 4 hours until the open circuit potential (OCP) was stable over 10 seconds. A potentiostat (Reference 6000, Gamry Instruments Inc., Warminster, PA, USA) was then utilized to conduct a polarization curve starting at OCP and ending at 0 V. Rate of voltage change was set to 0.1 mV/s. Generated I-V data were then utilized to calculate maximum power output.
Sample collection and DNA extraction

Initial fuel cell inoculum (0.5 ml) was centrifuged (17,900g for 5 minutes) and the pellet was collected for DNA extraction. For each MFC, pellets from 0.3 mL planktonic suspension (17,900×g for 5 minutes) were collected and frozen until DNA extraction. A slice of the anode electrode (=0.3 g) from each MFC was rinsed briefly by sterile 50 mM PIPES buffer and used for analysis of attached microbial communities. Genomic DNA was extracted by UltraClean™ Soil DNA kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer’s protocol. DNA concentrations were estimated based on 260 nm absorbance using a spectrophotometer (ND-1000, NanoDrop Products, Wilmington, DE, USA).

PCR-DGGE

PCR-DGGE was performed as described by Kan et al.,17 except 1) DGGE primers were 1070f and 1392r(GC)18 and 2) the linear gradient of the denaturants was from 40% - 70% instead of 40% - 65%. The DGGE gel was stained with SYBR Gold and photographed with a CCD camera mounted on a UV transluminater (UVP, Upland, CA, USA). As previously described, representative bands were excised and sequenced, and phylogenetic trees were constructed using MacVector 10.0 software package (MacVector Inc., Cary, NC, USA).17 These sequence data have been submitted to the GenBank databases under accession No. GU056316-GU056345.

Assessment of community stability

Similar population structures were identified based on the presence or absence of DGGE bands. In order to test the stability and robustness of the enriched fuel cell community, alternative electron donors were introduced to similar bacterial communities and voltage/current production was monitored. Once a response was observed, the original
enriching electron donor was re-supplied to determine any changes to the characteristic voltage response after the initial enrichment.

On reestablishment of the characteristic voltage, unique communities at the lactate and acetate oxidizing anode were selected for the creation of frozen stock cultures for Cr\textsuperscript{VI} experiments. These cultures were prepared by placing sections of the electrode in sterile 30% glycerol, followed by storage at -80°C. Lactate and acetate were selected based on their representation of two major communities discovered through PCR-DGGE analysis.

Cr\textsuperscript{VI} enrichment using anode communities

Frozen stock cultures from acetate and lactate oxidizing communities were recovered by grinding the section of frozen electrode in 5 mL of Luria-Bertani, Miller media under anaerobic conditions. This suspension was then injected into the anode compartment sterile fuel cells of the same design used in the anode enrichment, except that graphite felt disks (39 mm diameter) were used as the anode (Figure 5.2a). 1 mL of filtered wastewater was also injected to the anode to augment the frozen inoculum.

These communities were allowed to recover by introducing either lactate or acetate to the anode compartment until a characteristic voltage response was established. Community stability was tested by subsequent injections of electron donor (lactate, succinate, NAG, acetate, formate and uridine) followed by recovery of characteristic voltage responses with original electron donors.

At this point, a DNA sample from both the planktonic and attached communities was taken for PCR-DGGE analysis. The polarity of the fuel cell was then switched by replacing the platinum cathode with a lactate oxidizing MR-1 culture. Cr\textsuperscript{VI} was introduced to the mixed community to serve as an electron acceptor. In this manner, the MR-1 culture would help to fix
Figure 5.2. MFC setup used in (a) recovery of enrichment communities from frozen stock and (b) Cr\textsuperscript{VI} enrichment studies.
the other electrode at a negative potential, forcing the enrichment to change from an oxidizing to a reducing metabolism.

Cr\textsuperscript{VI} was introduced weekly to the cathode at a concentration of 0.1 mM. Samples were taken immediately after Cr\textsuperscript{VI} was added to the cathode and after three days determine Cr\textsuperscript{VI} by ion chromatography (Chapter 4). Three days was chosen as a benchmark for reduction since it was shown previously that *Shewanella* are able to complete removal within this time frame (Chapter 4). After 30 days of operating the enrichment, DNA from the electrodes and the catholyte were extracted and analyzed by PCR-DGGE.

**Direct Cr\textsuperscript{VI} enrichment**

For direct enrichment of Cr\textsuperscript{VI} reducing communities, the same filtered wastewater enrichment used in the anode enrichments was utilized. The same fuel cell setup as before was used with the exception that round (39 mm diameter) graphite felt electrodes were used as the anode and cathode (Figure 5.2b). For these experiments, an MR-1 anodic culture oxidizing lactate was used to bias the cathode potential. A reactor enriching for a Cr\textsuperscript{VI}-reducing community was also setup to examine the differences due to enrichment in the fuel cell.

Cr\textsuperscript{VI} (as $\text{K}_2\text{Cr}_2\text{O}_7$) was introduced to the cathode at a concentration of 0.1 mM. Each cathode was sampled weekly to determine the extent of chromate reduction by ion chromatography as in Chapter 4. Once Cr\textsuperscript{VI} was determined to be removed by more than 90%, the Cr\textsuperscript{VI} concentration was increased by additional introduction of $\text{K}_2\text{Cr}_2\text{O}_7$ to a concentration of 0.1 mM. Upon achieving a removal efficiency greater than 99%, the microbial community from the catholyte and on the electrode surface was analyzed by PCR-DGGE.
**High pressure liquid chromatography (HPLC)**

In order to monitor the consumption of electron donors, HPLC samples were taken from anode planktonic suspension at four time points: (1) immediately after feeding, (2) at peak voltage, (3) during voltage decline, and (4) after reaching baseline. The samples were centrifuged for 5 minutes at 17,900g and the supernatants were collected and stored at –20°C. Samples were acidified by addition of sulfuric acid to bring final concentration of sulfuric acid to 12.5 mM. After being held overnight at 4°C, acidified samples were filtered through a 0.22 µm syringe filter. An Agilent 1100 Series HPLC system with a 20 µL sample loop, G1311A quaternary pump, and a G1315B diode array detector at 210 nm was utilized to analyze the filtrate. Separation occurred using a 4 µm, 250 mm x 4.6 mm, reverse phase C18 column (Synergi-Hydro, Phenomenex, Torrance, CA, USA). Chromatograms were generated using a mobile phase of 2.5 mM sulfuric acid with a flow rate of 0.5 mL/min at ambient temperature.

**Scanning electron microscopy (SEM)**

After reproducible voltage responses to electron donor injection were experienced from MFCs, one of the graphite felt electrodes in the anode compartment (Figure 5.1) was retrieved for electron microscopy and bacterial community analysis. A section of the electrode for each MFC was fixed in 2.5% glutaraldehyde for at least 24 hours until drying by gradual ethanol dehydration followed by critical point drying. The desiccated samples were coated with evaporated carbon and viewed by a field emission scanning electron microscope (1550 VP, Zeiss-LEO, Oberkochen, Germany) using in-lens and below-lens secondary electron detectors operating with a 10.0 keV accelerating voltage.
5.3 Results and Discussion

Current production and electron donor consumption by MR-1 and enriched bacterial consortia

Table 5.1 summarizes the results of the anode enrichments. Current density, power density, and energy efficiency are presented for comparison among enrichments. MR-1 is also presented as a comparison to single-strain fuel cell performance.

The monoculture of MR-1 and microbial community enrichments with different carbon sources were evaluated by comparing current density responses over time upon electron donor injection (Figure 5.3). Decreases in current density were indicative of consumption of corresponding carbon compounds. HPLC analysis indicated that lactate, NAG, acetate, and uridine decreased quickly after injection and were not detected when current density had reached a minimum baseline (data not shown). For three of these organic acids (lactate, NAG, and uridine), the presence of acetate as an intermediate product was detected by HPLC shortly after the injection but was completely consumed as current production progressed. Formate decreased after feeding but remained at a constant concentration of 0.7 mM, suggesting that formate was only partially consumed. Data for succinate was irresolvable by HPLC analysis due to high background signals in the samples (data not shown).

MR-1 inoculated fuel cells showed a relatively strong response to the initial lactate injection but current production slowly diminished with subsequent injections (Figure 5.3a). In contrast, enrichment fuel cells did not produce a significant response until about 100 hours, indicating that a suitable bacterial population had developed over that period of time.
<table>
<thead>
<tr>
<th>MFC No.</th>
<th>Original Inoculums</th>
<th>Enrichment electron donor</th>
<th>Max. current density resulting from consumption of 2 mM electron donor ($\mu$A cm$^{-2}$)</th>
<th>Max. power density based on linear polarization sweep (mW m$^{-2}$)</th>
<th>Estimated heat of formation, $\Delta H^\circ_f$ (kcal mol$^{-1}$)</th>
<th>Max. electrical energy from 2 mM electron donor (kcal mol$^{-1}$)</th>
<th>Efficiency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MR1</td>
<td>lactate</td>
<td>8.9</td>
<td>13.0</td>
<td>-136.3</td>
<td>0.072</td>
<td>0.053</td>
</tr>
<tr>
<td>2</td>
<td>sludge</td>
<td>lactate</td>
<td>68.9</td>
<td>107.3</td>
<td>-136.3</td>
<td>1.049</td>
<td>0.770</td>
</tr>
<tr>
<td>3</td>
<td>sludge</td>
<td>succinate</td>
<td>100.5</td>
<td>67.2</td>
<td>-180.0</td>
<td>1.387</td>
<td>0.771</td>
</tr>
<tr>
<td>4</td>
<td>sludge</td>
<td>NAG</td>
<td>127.9</td>
<td>149.7</td>
<td>-232.3</td>
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</tr>
<tr>
<td>5</td>
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<td>193.5</td>
<td>221.1</td>
<td>-95.4</td>
<td>2.494</td>
<td>2.614</td>
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<td>6</td>
<td>sludge</td>
<td>formate</td>
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<tr>
<td>7</td>
<td>sludge</td>
<td>uridine</td>
<td>62.0</td>
<td>233.5</td>
<td>-92.4</td>
<td>2.302</td>
<td>2.492</td>
</tr>
</tbody>
</table>

Table 5.1. Description of inoculums and characteristics for each of the MFCs enriched from different carbon sources.
Subsequent injections in these cells resulted in both increased peak current densities and more total charge transferred per injection. Current production by bacterial communities enriched with lactate, acetate, and formate stabilized after 300 hours (Figure 5.3b,e,f). Fuel cells with succinate, NAG, and uridine did not yield reproducible responses to carbon source injection until after 700 hours (Figure 5.3c,d,g). In addition, power density yields among the enriched MFCs varied significantly for each given electron donor. The average peak current densities generated from bacterial consortia after stabilizing (39.9-193.5 μA/cm²) were 4.5 to 22 times higher than those generated from systems operating with only MR-1 at the anode (8.9 μA/cm²). Compared to MR-1, mixed bacterial communities also showed a maximum power density that varied from 67.2 mW/m² to 233.5 mW/m², higher than the 13.0 mW/m² resulting from MR-1 inoculated fuel cells (Table 5.1).

Enrichment of bacterial communities from original inoculums was a process of dynamic change, as shown in the current density plots (Figure 5.3). For example, the fuel cell enriched with succinate initially generated a two-peak current density profile, but the latter peak started to diminish from 600 hours and disappeared after 900 hours, suggesting the possibility of community shift during the enrichment. The dynamic aspect of the enrichment community is a finding in line with a recent report where MFCs exhibited dynamic population structures strongly correlated to the enriching environmental conditions during operation. Moreover, this aspect of enrichment cultures underscores the necessity for further quantitative investigations of microbial ecology and microbe-microbe interactions within MFCs. However, limited sampling opportunities (typically a single time point at the end of the experiment) of the anode for molecular analysis are allowed in most of the current fuel cell designs. Nonetheless,
Figure 5.3. Current response for MFCs containing (a) MR-1 biofilms, (b) lactate enrichments, (c) succinate enrichments, (d) acetate enrichments, (e) NAG enrichments, (f) formate enrichments, and (g) uridine enrichments
more frequent and non-invasive sampling for molecular techniques is necessary to investigate population dynamics during the enrichment processes.

*Energy conversion efficiency of MFCs*

An evaluation of MFC energy efficiency was approximated based on estimated enthalpies of formation and maximum MFC electrical energy produced from a single carbon source injection. Such a value gives an estimate on the relative efficiency of transformation from chemical to electrical energy in each of the MFCs. While coulombic efficiency has been previously utilized to supply mass balanced efficiency comparisons, gaps in knowledge regarding metabolic pathways by mixed microbial communities preclude its usage in the current study. Furthermore, lack of published experimental thermodynamic data prevents the usage of Gibbs free energy in this comparison. As an estimate, standard enthalpies of formation, \( \Delta H^\circ_f(g) \), were estimated from standard tabulated bond energies.\(^{21}\) Peak electrical energy output was calculated from integration of the power vs time peak for the injection producing the highest power (Table 5.1).

Energy efficiency ratings varied among the different MFCs with no trends related to heat of formation or peak current density. Conversion of acetate had the highest percentage (2.614\%) and complex molecules (NAG and uridine) also exhibited relatively high degrees of conversion (2.492\% and 1.017\%, respectively). Other common organic compounds such as lactate, succinate, and formate appeared to be converted at a lower efficiency (0.151 – 0.771\%). The fuel cell inoculated with only MR-1 yielded the lowest efficiency of 0.053\%. 
**SEM observations of biofilms on anode electrodes**

SEM micrographs showed that microbial biofilms formed on the anode surfaces of all the MFCs (Figure 5.4) MR-1 formed a thin biofilm with most of the cells directly attached on the graphite fibers. In contrast, the enriched cells showed different sizes and shapes of bacterial cells, films, and aggregates that appeared scattered on the graphite fibers. Enriched microbial cells from lactate, acetate, and formate distributed on the electrode surface in a more uniform manner while thick and irregular biofilms were found in succinate and uridine-fed MFCs. In addition to individually attached microbial cells, “clumps” with accumulated cells occurred in most of the enriched bacterial communities. Closer observation showed that the “clumps” contained clusters of diverse microbial cells.

**Planktonic and anode-attached bacterial communities**

DGGE analysis showed that the bacterial communities attached on the anode surface were notably different from those in the original sludge inoculums and distinct from the planktonic populations as well (Figure 5.5). In general, planktonic populations numerically showed more dominant bands than anode-attached bacteria. Jaquard-based clustering analysis on DGGE band patterns (band absence or presence) indicated that anode-attached bacterial assemblages enriched by lactate, NAG, and uridine were similar while bacterial communities enriched by succinate, acetate, and formate were similar (Figure 5.6).

Phylogenetic analysis (Figure 5.7) showed that the original sludge inoculums contained mainly β-Proteobacteria (bands s1 and s5), γ-Proteobacteria (bands s3, s6, and s7), Firmicutes (band s4), and Bacteroidetes (band s2). In enriched planktonic bacterial communities, β-Proteobacteria (bands p5, p6, p13, and p14), Firmicutes (bands p2, p3 and p12), and
Figure 5.4. SEM micrographs of anode electrodes for (a) MR-1 biofilms, (b) lactate enrichments, (c) succinate enrichments, (d) acetate enrichments, (e) NAG enrichments, (f) formate enrichments, and (g) uridine enrichments.
Bacteroidetes (bands p7, p8, and p9) were dominant groups (Fig. 4a and 4b). In contrast, δ-Proteobacteria, β-Proteobacteria, and α-Proteobacteria dominated in bacterial communities attached to the MFC anodes. *Geobacter* sp. 1 (bands a5 and a9) and *Ideonella* sp. (band a6) were major groups enriched by lactate, NAG, and uridine while *Geobacter* sp. 2 (band a2), *Azonexus* sp. (bands a3, a7, and a8), and *Aquaspirillum* sp. (band a4) were found in fuel cells enriched by succinate, acetate, and formate. Band a10 failed sequencing and was excluded in the phylogenetic analysis.

Sludge inoculums were dominated by Proteobacteria, Firmicutes, and Bacteroidetes, consistent with previous studies. However, the enriched bacteria populations (attached and planktonic) were significantly different from the original innoculum and were different from previous reports, likely resulting from different carbon sources and starting inoculums used in previous studies.

In attached bacterial communities, the dominant sequences were affiliated with δ-Proteobacteria (*Geobacter* spp.), which shared high similarity with sequences retrieved from other MFC systems. Detected β-Proteobacteria in the attached community (Figure 5.7), were similar to denitrifying bacteria detected in wastewater treatment plants. The bacteria related to *A. caeni* may be involved in electricity generation because bacteria that were capable of nitrate reduction, such as MR-1 and *Geobacter* sp., have been demonstrated to be electrochemically active in MFCs. Another DGGE band (a4) associated with an α-Proteobacterium (*Aquaspirillum* sp.) occurred only in the attached and planktonic fuel cell communities enriched with lactate, succinate, acetate, or formate. Neither the physiology nor functional role(s) of this α-Proteobacterium, a group commonly found in soil and sludge, is apparent.
Figure 5.5. DGGE gel image of PCR product from anodic (a) and planktonic (p) cultures for each of the MFC enrichments. MR-1 and the original wastewater sludge (S) are also shown for comparison.
Figure 5.6. Clustering analysis of PCR-DGGE results based on a Jacquet distance matrix calculated from band presence or absence in the DGGE gel.
Figure 5.7. Phylogenetic tree analysis from PCR-DGGE analysis of communities from enrichment at the anode enrichment related to Proteobacteria with different electron acceptors and closest related sequences from Genbank. Selected bootstrap values were generated from 1000 resampling datasets.
Figure 5.8. Phylogenetic tree analysis from PCR-DGGE analysis of communities from enrichment at the anode enrichment related to Firmicutes and Bacteroidetes with different electron acceptors and closest related sequences from Genbank. Selected bootstrap values were generated from 1000 resampling datasets.

In comparison to attached bacterial communities, planktonic communities contained more dominant DGGE bands associated with β-Proteobacteria, Firmicutes, and Bacteroidetes (Figure 5.7, Figure 5.8), a finding in contrast to published findings suggesting a higher diversity in the attached community. The results shown here suggested that different carbon compounds enriched planktonic populations distinct from the attached communities. For instance, β-Proteobacteria and Bacteroidetes were only found in the NAG-enriched fuel cell while a band associated with α-Proteobacteria was obtained only from MFCs enriched with lactate, succinate,
acetate, and formate. The differences between the attached and planktonic populations suggest that MFC offers distinctly different niches serving various functions for electricity generation and community maintenance. Furthermore, possible electron transfer at relatively large distances from the electrode have been proposed.\textsuperscript{7,19,30-32} Therefore, it is reasonable to hypothesize that the planktonic community may be involved in both substrate oxidation and electricity generation.

\textit{Community stability in MFCs}

Similar bacterial populations occurred in MFCs enriched with lactate, NAG, and uridine ("Group A", MFCs 2, 4, and 7) and with succinate, acetate, and formate ("Group B", MFCs 3, 5, and 6). In order to test if the similar bacterial communities were able to utilize different electron donors, Group A fuel cells were fed with lactate, NAG, and uridine and Group B fuel cells were fed with succinate, acetate, and formate. All the MFCs utilized the provided alternative electron donors and generated electricity after feeding but showed different current density profiles when compared to the original fuel. In Group A, lactate and NAG generated higher current densities than uridine. In Group B, acetate and formate yielded higher current densities but succinate resulted in a more sustained and lengthy response. Finally, the current production for each cell returned to its normal pattern when switched back to its original fuel, suggesting that the bacterial communities were stable and that introduction of alternate electron donors did not significantly change their population structures.

\textit{Pure cultures vs. enriched bacterial community}

Single species cultures provide good models for mechanistic studies, but it is generally agreed that 1) mixed bacterial communities hold greater promise for large-scale implementation and that 2) power generated by pure cultures MFCs are lower than that produced by mixed
bacterial communities, although few direct comparisons of pure and mixed cultures have been conducted in the same MFC architecture and under identical conditions. As shown in this study, enriched bacterial communities generated significantly higher current densities and power yields than MR-1, although stabilization times for enriched communities were significantly greater. Furthermore, although alternative electron donors changed the current density response, mixed bacterial communities showed high stability by returning to initial responses after feeding of the initial carbon compounds. This observation suggested that established mixed cultures were able to derive energy from a variety of organic molecules, limiting their susceptibility to environmental variations such as alterations of substrate type, concentration, and availability.

Enriched fuel cell communities also showed higher energy efficiency with acetate and uridine based fuel cell communities performing the best. The MR-1 fuel cell had the lowest energy efficiency when oxidizing lactate compared with its enriched community counterpart. Interestingly, the fuel cells oxidizing complex molecules, NAG and uridine, yielded some of the highest efficiency numbers. These results suggested that mixed communities were adaptable and more efficient than single culture fuel cells when introduced to more complex reductants. However, the general low energy efficiency for all MFCs in the current study indicated that substantial electron and energy losses to processes other than power generation. For instance, the occurrence of fermentation in MFCs could not be excluded because fermentations were commonly found within sludge microbial communities, such as succinate and lactate fermentation by Clostridium species and methanogenic fermentation of formate and acetate. The low energy efficiency shows that an opportunity exists to improve MFC
performance by optimizing MFC design, hardware and operation, and microbial activity, in addition to meeting practical applications needs.

**Comparison of Cr\textsuperscript{VI} enrichment strategies**

Comparisons of enrichment strategies for Cr\textsuperscript{VI} removal at an MFC cathode were performed over one month. The removal efficiency over the 30 days of operation for each strategy is shown in Table 5.2. Development of enrichment efficiency after 10 days for direct enrichments (10.4\%) was less than the efficiency observed with using pre-established communities, 61.9\% and 48.8\% for acetate and lactate enriched communities, respectively. However, the directly enriched community rapidly became adept at Cr\textsuperscript{VI} removal, establishing the ability to complete removal after 3 days within 30 days of operation. This is in contrast to the acetate and lactate enriched communities which did not appear to be able to increase their removal efficiency.

**Table 5.2.** Removal efficiencies for Cr\textsuperscript{VI} enrichment from directly enriched, acetate enriched, and lactate enriched communities

<table>
<thead>
<tr>
<th>Enrichment Time</th>
<th>Removal Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Direct Enrichment</td>
</tr>
<tr>
<td>10 days</td>
<td>10.4</td>
</tr>
<tr>
<td>20 days</td>
<td>98.3</td>
</tr>
<tr>
<td>30 days</td>
<td>100</td>
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</table>
PCR-DGGE analysis shows very different communities were established depending on which enrichment strategy was used. The DE cultures in the fuel cell showed differences with Cr\textsuperscript{VI}-reducing community enriched in a batch reactor (Figure 5.9). The Cr\textsuperscript{VI} batch enrichment showed members of β-Proteobacteria and Spirochetes (Figure 5.10). On the other hand, the fuel cell enrichment showed dominant members from Actinobacteria and Firmicutes in addition to β-Proteobacteria (Figure 5.11). β-Proteobacteria members appeared to be common between these two enrichment cultures though the specific bands do not seem to be similar.

Examination of the SE Cr\textsuperscript{VI} enrichment cultures showed several changes from the initial inoculum. The δ-Proteobacteria and γ-Proteobacteria members seen in the inoculum did not appear to have persisted through the course of the enrichment. In fact both communities appeared to become dominated by α-Proteobacteria and β-Proteobacteria.

It should be noted that while DGGE was able to identify major groups within these different enrichment cultures, examination of the gel images show that each of these communities appears to be significantly different from one another. This indicates that the method of enrichment, whether DE or SE, will influence the final community composition when selecting for members capable of Cr(VI) reduction in a MFC cathode. Furthermore, the DE method appears to have selected for a more adaptable community since its ability to remove Cr\textsuperscript{VI} appeared to improve over time. The SE cultures did not appear to improve, suggesting that use of this technique may not be suitable for Cr\textsuperscript{VI} removal to low concentration levels, and therefore not suitable for developing effective Cr\textsuperscript{VI} reducing reactor systems with the MFC architecture.
Figure 5.9. DGGE gel image of PCR products from direct enrichment at the MFC cathode and of the batch Cr(VI) enrichment.
Figure 5.10. Phylogenetic tree analysis from PCR-DGGE analysis of the Cr\(^{VI}\) batch reactor enrichment and closest related sequences from Genbank. Selected bootstrap values were generated from 1000 resampling datasets.
Figure 5.11. Phylogenetic tree analysis from PCR-DGGE analysis of the direct MFC enrichment and closest related sequences from Genbank. Selected bootstrap values were generated from 1000 resampling datasets.
Figure 5.12. DGGE gel image of PCR products from using lactate and acetate enriched communities for Cr\textsuperscript{VI} enrichment.
Figure 5.13. Phylogenetic tree analysis from PCR-DGGE analysis of the acetate enriched community after Cr⁶⁺ enrichment and closest related sequences from Genbank. Selected bootstrap values were generated from 1000 resampling datasets.
Figure 5.14. Phylogenetic tree analysis from PCR-DGGE analysis of the acetate enriched community after Cr\textsuperscript{VI} enrichment and closest related sequences from Genbank. Selected bootstrap values were generated from 1000 resampling datasets.
5.4 Conclusions

MFCs with mixed communities provide a new platform for studying microbial physiology and biofilm ecology. For example, a well-defined MFC with varied electrical potentials will be a promising approach to enrich as yet unculturable microbes. Physiological features of these microbes will facilitate our understanding of critical microbiological processes such as extracellular electron transport, carbon compound utilization, etc. Furthermore, many unknowns remain with regard to the microbial ecology of MFCs.

Future work on this would involve further investigation at the microscopic level of these communities. Molecular characterization of MFC microbes in a real-time manner by FISH (fluorescence in situ hybridization) will locate the specific groups on the anode surface, and perhaps identify the most important active members of communities. In addition, unique genomic signatures or functional keystones genes should be possible to identify and characterize by metagenomic analysis of the enriched microbial communities, leading to the maturation of MFC microbiology.
Chapter 5 References


Chapter 6: Integrating electrokinetic technology with a microbial fuel cell system to enhance Cr\textsuperscript{VI} remediation

6.1 Introduction

Electrokinetic remediation has been proposed as a way of directing and removing heavy metals from subsurface environments.\textsuperscript{1,2} Commercial processes have also been developed and applied in field tests to determine its effectiveness as an in situ remediation technique.\textsuperscript{3,4} The ability of this technique to be applied over heterogeneous subsurface components and as a non-destructive in situ method are some of the most promising aspects of this technology.\textsuperscript{1-3}

Difficulties in applying the technique successfully are attributed, in part, to lack of understanding the geochemistry that governs the subsurface when an electric potential is applied.\textsuperscript{5} To overcome some of these limitations, enhancements such as addition of chelating agents or controlling the chemistry around the electrodes have been proposed.\textsuperscript{3,5} Specific challenges exist where reduction of contaminants, such as Cr\textsuperscript{VI}, may produce insoluble species that would potentially precipitate on the electrodes and interfere with the creation of the electric field.\textsuperscript{3}

To circumvent this shortcoming when applying the electrokinetic remediation technique for metals such as Cr\textsuperscript{VI}, the use of a microbial fuel cell (MFC) is proposed. Microbial fuel cells have been proposed for remediation purposes\textsuperscript{6,7} and could serve to protect electrokinetic electrodes by providing a reservoir for removal and collection of metal contaminants away from the electrode surface. Proper integration into an electrokinetic remediation system should
provide the following benefits: (1) prevention of metal precipitate formation and fouling at the electrokinetic electrode surface and (2) provide additional removal capacities by microbial reduction of heavy metals (that may not be achievable through application of electrokinetic remediation alone).

To demonstrate this integration in an electrokinetic system, this series of experiments presents the incorporation of an MFC system into the treatment of a bench-scale Cr$^{VI}$ contaminated clay system simulating a saturated zone in the subsurface. Experiments monitoring pH and Cr$^{VI}$ removal efficiency were performed to determine any potential advantages an integrated MFC-electrokinetic system would have over standard electrokinetic remediation application.

6.2 Materials and Methods

Electrokinetic Column

Vertical electrokinetic columns (Figure 6.1a) were constructed from section of acrylic pipe (1.5” I.D. x 2” O.D. x 12”). The column was split into three sections with a 10 cm center partition used to contain a clay-water mixture. The remaining sections on each end were separated from the center section by cellulose-fiberglass partitions and utilized as electrode reservoirs.

Sampling ports were fabricated at 1 cm intervals along the clay-water section along with an additional one for each electrode reservoir. The cellulose-fiberglass partitions were assembled such that they would both allow water to pass freely and remain impermeable to suspended clay particles. Electrokinetic electrodes were prepared by machining ¼” graphite plates (McMaster-Carr; Santa Fe Springs, CA, USA) into 1” disks. A 0.8 mm diameter titanium
Figure 6.1. Illustrations describing (a) the parts of the vertical electrokinetic column and (b) schematic of the microbial fuel cell used for integration.
wire (Alfa-Aesar; Ward Hill, MA, USA) was attached the graphite electrode inside the electrode reservoir and used to connect the electrode with an external power supply.

A buffered 3 ppm Cr\textsuperscript{VI} solution was prepared by mixing K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} with a 0.01M HCO\textsubscript{3}\textsuperscript{−} buffer (pH 7). The clay-water suspension was prepared by mixing pure kaolinite (Sigma-Aldrich; St Louis, MO; USA) with this buffered solution to achieve a 40% (wt/wt) clay suspension. The pH was maintained by addition of concentrated NaOH or HCl during mixing.

This suspension was used to fill the center compartment of the electrokinetic column. The same Cr\textsuperscript{VI} solution used to prepare the clay mixture was added to each of the electrode reservoirs. The ends of the columns were then sealed and the column was allowed to equilibrate for 24 hours. After equilibration, a 10V potential was applied across the electrodes to maintain a voltage gradient of 1 V cm\textsuperscript{−1} using an external power supply (HQ Power PS3003U; Fry's Electronics, Inc.; Manhattan Beach, CA, USA). A small pressure relief port at the top of the column was unsealed to allow the release of any gases formed during the experiment. Two different polarity configurations (Figure 6.2) were applied in separate experiments to determine

![Graphical representations of (a) Cr\textsuperscript{VI} migration when cathode is at the top of the column, and (b) when the anode is at the top.](image)

**Figure 6.2.** Graphical representations of (a) Cr\textsuperscript{VI} migration when cathode is at the top of the column, and (b) when the anode is at the top.
the effects on transport within the vertical column. Each of these experiments was performed in duplicate.

During operation each electrode reservoir and the center of the clay compartment were sampled every 24 hours for 5 days. Preliminary experiments (not shown) suggested that any changes in Cr$^{VI}$ and pH would become apparent within this timeframe. Each sample was centrifuged at 17,000g for 5 minutes to remove any clay particles. The supernatant from the centrifuged sample was analyzed for pH and Cr$^{VI}$ concentration.

**Microbial fuel cell preparation**

A microbial fuel cell (Figure 6.1b) was integrated into each electrokinetic experiment to assess its use in aiding Cr$^{VI}$ transport and removal. The acrylic microbial fuel cell (MFC) system used in the *Shewanella* species experiments (Chapter 4, Figure 1) was used for these experiments. For these experiments, *S. species* W3-18-1 was used as the cathode biocatalyst and *S. oneidensis* MR-1 was used as the anode biocatalyst.

For the anode compartment, MR-1 was first inoculated to Luria-Bertani, Miller broth and incubated overnight at 30°C. 250 μL of this overnight culture was used to inoculate 250 mL of minimal media (specified in the species experiments) and incubated overnight at 30°C while shaking at 100 rpm. These cultures were diluted to an OD$_{600}$ of 0.2 and transferred to the anode compartment of the fuel cell.

For the cathode compartment, W3-18-1 was utilized due to its performance in the species experiments (Chapter 4). Cultures were prepared in the same way as MR-1, except that cells from overnight minimal media culture were separated by centrifuge at 6000g for 15 min at 4°C. These cells were resuspended in 0.01 M HCO$_3^-$ buffer to give an OD$_{600}$ of 0.2 and then
transferred to the cathode compartment of the fuel cell. This transfer was performed to prevent components from minimal media from entering the electrokinetic column during operation.

The MFC was placed in operation as shown in Figure 6.3 where the anode compartment would be used in batch mode while the cathode compartment would be operated in a continuous mode. Fluid from the clay compartment was continuously pumped from a sampling port directly under the fiberglass partition to the bottom of the MFC cathode to minimize transfer of clay to the MFC. The MFC catholyte was then pumped from the top of the MFC into the top electrode reservoir. This pumping configuration would remain the same regardless of whether the electrokinetic anode or electrokinetic cathode was placed in the top reservoir.

![Diagram showing the integration of the MFC with the electrokinetic column](image)

**Figure 6.3.** Diagram showing the integration of the MFC with the electrokinetic column

Connections between the MFC and the electrokinetic column were made by flexible tubing (PharMed BPT; Cole-Parmer Instrument Company; Vernon Hills, IL, USA). Peristaltic pumps (Masterflex L/S Model EW-07524-40; Cole-Parmer Instrument Company; Vernon Hills, IL, USA) equipped with dual channel pump heads (Masterflex L/S EasyLoad II; Cole-Parmer Instrument Company; Vernon Hills, IL, USA) were used to transfer fluids between the column.
and the MFC cathode. The position for the MFC cathode outlet was chosen to maintain volume of catholyte volume (250 mL) to achieve a residence time of 7 hours.

Performance of the MFC was assessed by collection and analysis of anolyte and catholyte samples for pH and Cr\textsuperscript{VI} analysis in addition to monitoring MFC current production.

**Sample Analysis**

The pH of the supernatant was measured by using a pH electrode (Model 14231-178; VWR International LLC; Radnor, PA, USA). The sample was then prepared for ion chromatography analysis by increasing the pH to 10 by adding 0.25 M (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}/1M NH\textsubscript{4}OH. The samples were then passed through a 0.45 μm syringe filter before injection to the ion chromatography instrument.

Ion chromatography was performed to determine the Cr\textsuperscript{VI} concentrations in each of the 3 sections of the electrokinetic column. An IC system (850 Professional; Metrohm AG; Switzerland) with a post-column derivitization coil and UV-Vis absorbance detector (Lambda 1010; Metohm AG; Switzerland) set at 530 nm was used for Cr\textsuperscript{VI} analysis. Details regarding flow rates and eluent compositions can be found in Chapter 4.

**Electron microscopy**

Electron microscopy was employed to observe the MFC electrode surface. Following each experiment, sections of electrodes were extracted and fixed in 2.5% gluteraldehyde. Following fixation, samples were prepared by gradual ethanol dehydration and hexamethyldisilizane substitution.\textsuperscript{8,9} The samples were then mounted on aluminum stubs and carbon coated. Electron microscopy and elemental analysis were performed using a scanning electron microscope (JSM-6610LV; JEOL USA, Inc.; Peabody, MA) operated with a 10kV
accelerating voltage and equipped with an energy dispersive spectroscopy (EDS) detector (JSM 6490; EDAX Inc.; Mahwah, NJ).

6.3 Results and Discussion

Electrokinetic application (no MFC)

These experiments were anticipated to show the extent of remediation that would be accomplished by applying the electrokinetic technique alone. Additionally, these results show the fluctuations in pH caused by electrolysis in the electrode reservoirs and the direction of CrVI migration. Thus, experimental design can be verified by comparing these results with previously reported studies and a basis for comparison to the integrated systems can be established.

Initial CrVI concentrations for the columns after the 24 hour equilibration period indicate that approximately a third of the chromium was bound by adsorption to column and clay particles. This is in agreement with preliminary adsorption experiments (Figure 6.4).

The results from the column operating with the electrokinetic anode in the top reservoir (Figure 6.5) show only slight removal of CrVI from the bottom reservoir (cathode reservoir). The clay compartment shows almost no change from the initial concentration of 1930 ppb. The top reservoir showed some accumulation at first to 2130 ppb, but then CrVI concentrations began to slowly decrease over the 5 day period to a final concentration of 1237 ppb.

The pH for the anode reservoir dropped very sharply from its initial value of 7 to 2.38 within 24 hours. A slight decrease over the 5 day period to a final pH of 1.99 was observed. The clay compartment was able to maintain near-neutral pH levels through the third day. The pH of this compartment then began to decrease over the next two days to a final pH of 6.1. The pH of
Figure 6.4. Adsorption isotherm of Cr$^{VI}$ on kaolinite from preliminary experiments
Figure 6.5. pH and Cr\(^{VI}\) concentrations for the (a) top reservoir, (b) the clay compartment, and (c) the bottom reservoir when operated with the electrokinetic anode in the top reservoir.
the cathode reservoir was maintained around 7 for the first 24 hours, and then began to slowly rise to a final pH of 10.7 after 5 days of operation.

The column operating with the electrokinetic cathode in the top reservoir (Figure 6.6) gave slightly different results. The Cr\textsuperscript{VI} concentrations in the anode compartment began to decrease at a faster rate than when the anode was at the top. More removal was also observed in the cathode reservoir and clay compartment with final Cr\textsuperscript{VI} concentrations of 87 ppb and 97 ppb, respectively.

The pH changes with the cathode in the top reservoir appeared to follow a trend similar to that of having the anode at the top. The cathode reservoir pH level increased to 11.80 after 24 hours and stayed at this level for the remaining 4 days. The clay compartment remained near neutral for 24 hours and then began to increase to a final pH of 11.74. The pH of the anode reservoir decreased over 2 days to a value 3.04 and remained at this low pH for the remaining 3 days.

The application of electric potential to the column appears to have significant effects on both the accumulation of Cr\textsuperscript{VI} and the pH of the aqueous solution. The direction of Cr\textsuperscript{VI} migration should be towards the anode because of the negative charge of the HCrO\textsubscript{4}\textsuperscript{−}, and CrO\textsubscript{4}\textsuperscript{2−} ionic species. During operation with the anode in the top reservoir, upwards Cr\textsuperscript{VI} migration from the cathode compartment accounts for the observed increase in Cr\textsuperscript{VI} concentration in the clay compartment and anode reservoir. Additionally, low pH values have been shown to inhibit anionic species transport\textsuperscript{5}, and this may explain the lack of migration from the bottom (cathode) compartment once the pH of the clay compartment begins to decrease. The concentration of in the top reservoir does not appear to increase after migration had stopped and eventually begins to decrease. This is likely due to the reducing power at the electrode itself\textsuperscript{1,2} and reduction of
Figure 6.6. pH and Cr\textsuperscript{VI} concentrations for the (a) top reservoir, (b) the clay compartment, and (c) the bottom reservoir when operated with the electrokinetic cathode in the top reservoir.
Cr\textsuperscript{VI} to Cr\textsuperscript{III} coupled to mineral (iron or manganese) oxidation that may be found in trace amounts within the clay mixture.\textsuperscript{10}

When the cathode is in the top reservoir, an accumulation effect does not appear to be seen. The Cr\textsuperscript{VI} concentration at the top (cathode) reservoir, the clay compartment, and the anode reservoir decrease continuously. This indicates migration to the anode was occurring as a result of Cr\textsuperscript{VI} transport from the cathode reservoir and clay compartments. The lack of accumulation in the anode suggests that reduction by the aforementioned mechanisms would account for the observed decrease in concentration.

The pH of the aqueous solution in the proximity of the electrodes is dominated by the reduction of H\textsuperscript{+} at the cathode and the oxidation of OH\textsuperscript{−} ions at the cathode.\textsuperscript{1,2} Reduction of H\textsuperscript{+} to H\textsubscript{2} would decrease the proton concentration resulting in the observed pH increase. Oxidation of OH\textsuperscript{−} to O\textsubscript{2} would result in the decrease in pH seen at the anode. The effects of these oxidation and reduction reactions are clearly noticeable within 24 hours for these systems.

Integrated electrokinetic system with a microbial fuel cell

The integration of the MFC into the electrokinetic system gave very different Cr\textsuperscript{VI} concentration removal profiles and pH changes. The setup with the electrokinetic anode in the top reservoir (Figure 6.7) showed very rapid removal for the top (anode) reservoir and the clay compartment. The Cr\textsuperscript{VI} concentrations in the anode reservoir decreased to 18 ppb within 24 hours and to 1 ppb after 48 hours, where it remained for the remainder of the experiment. The Cr\textsuperscript{VI} concentration in the clay compartment decreased to 224 ppb within 48 hours and continued to decrease to a final concentration of 25 ppb. The concentration in the cathode reservoir showed less rapid decrease, but the final concentration (347 ppb) was still much lower than concentrations experienced during electrokinetic operation without the MFC (1930 ppb). The
Figure 6.7. pH and Cr\textsuperscript{VI} concentrations concentrations for the (a) top reservoir, (b) the clay compartment, (c) the bottom reservoir, and (d) in the MFC cathode when operated with the electrokinetic anode in the top reservoir.
concentration of CrVI in the MFC spiked briefly in the first 24 hours to 961 ppb, but then decreased over the next 4 days to a final concentration of 40 ppb.

The pH of the system for the top (anode) reservoir and clay compartment did not vary much at all for the first 4 days, after which the anode reservoir pH decreased to 3.48 and the pH in the clay compartment increased to 11.38. The pH of the cathode reservoir slowly increased over the 5 days to a final pH of 10.88. The pH of the MFC cathode remained near neutral for the entire 5 day operation.

When the cathode was in the top reservoir (Figure 6.8), similar decreases in CrVI for the clay compartment were observed, where the final concentration after 5 days was 22 ppb. The concentration in the cathode compartment decreased to 228 ppb after 24 hours and to 21 ppb after 5 days. The anode reservoir showed a slow decrease over the 5 day test period to a final concentration of 1269 ppb. The CrVI concentration in the MFC cathode increased after 24 hours to 206 ppb and then steadily fell to a final concentration of 18 ppb.

The pH of the cathode showed a slow increase to 9.89 after 5 days. The pH of the clay compartment remained near 7 for the first 24 hours and the rose over the next 4 days to a pH of 9.86. The pH of the anode reservoir slowly decreased from the initial pH of 7 to a final pH of 5.97 after 5 days. The pH of the MFC cathode in this setup increased over the 5 day period to a pH of 9.5.

Electron microscopy was employed to observe the graphite electrode of the MFC (Figure 6.9). Much of the surface of the electrode appeared to be a clay-biomass mixture. Elemental analysis (not shown) was able to pick up the aluminosilicate signature of the clay. In fact, this signal dominated the spectrum collected by the elemental detector, resulting in little to no detection of chromium.
Figure 6.8. pH and Cr⁶⁺ concentrations for a) top reservoir, b) the clay compartment, c) the bottom reservoir, and d) in the MFC cathode when operated with the electrokinetic cathode in the top reservoir.
Figure 6.9. Electron micrographs of the MFC cathode surface
Integration of the MFC system resulted in a marked change in the rates of pH and Cr\textsuperscript{VI} reduction. For both scenarios (electrokinetic anode or cathode in the top reservoir), the top reservoir and clay compartments both experienced rapid Cr\textsuperscript{VI} reduction and removal to levels below those achieved with electrokinetics alone. When the anode was on top, low (≈1 ppb) Cr\textsuperscript{VI} concentrations were achieved very rapidly. Based on these observations as well as the lack of Cr\textsuperscript{VI} accumulation in the MFC cathode, reduction at the MFC cathode likely played a significant role in Cr\textsuperscript{VI} removal. Similarly, the importance of the MFC cathode also plays an important role in reducing Cr\textsuperscript{VI} concentrations in the clay compartment. Low concentrations in the clay compartment may also provide additional driving force for Cr\textsuperscript{VI} migration from the cathode reservoir in this scenario.

When the cathode was at the top, similar rapid decreases in Cr\textsuperscript{VI} were observed for the top (cathode) reservoir and the clay compartment. These would be expected since Cr\textsuperscript{VI} migration is directed towards the bottom (anode) reservoir and removal from the column would be accelerated by cycling Cr\textsuperscript{VI} containing solution from the column into the MFC cathode where it would be subsequently removed. The anode reservoir did not show a rapid removal of Cr\textsuperscript{VI} or its removal to low concentration levels. This may be due in part to the pH level of the bottom reservoir; the pH was only moderately acidic (pH=6) when compared to electrokinetic operation without the fuel cell (pH=3). Thus, low pH appears to accelerate reduction of Cr\textsuperscript{VI} at the electrode in these systems.

pH changes with MFC operation also appeared to be more moderate than operation without the MFC. This may be due in part to the additional buffering capacity within the MFC cathode. This would prove to be an added benefit of MFC integration, as pH control in
electrokinetic systems is essential in maintaining a sustained remediation effort and is the subject of several electrokinetic studies.11-13

6.4 Conclusions

Establishment of an applied electrokinetic potential across vertical clay column showed varying degrees of success with respect to Cr\textsuperscript{VI} removal. The pH in the electrode compartments was not very stable and reached extreme pH values within 24 hours of operation. In these systems, Cr\textsuperscript{VI} migration towards the anode was observed, followed by reduction to Cr\textsuperscript{III}. This reduction likely proceeds through electrolysis reactions at the anode electrode or through coupling with metal oxidation from the clay matrix.

Integration of an integrated electrokinetic-MFC system appeared to moderate the pH change in the electrokinetic column when compared to the systems without an integrated MFC. Furthermore, operation of the MFC allowed removal of chromium to concentration levels that were an order of magnitude lower than those experienced by operating a system without a MFC. This series of studies serves as a proof-of-concept supporting the idea that the MFC serves to augment the capabilities of electrokinetic remediation. Moreover, the integration of the MFC allows for Cr\textsuperscript{VI} removal to around 1 ppb, within current drinking water standards established by the United States\textsuperscript{14} and the World Health Organization.\textsuperscript{15}

Future work will consist, in part, of utilizing a natural soil or clay to create a more heterogeneous matrix within the clay compartment that would mimic a more natural system. Additional work will focus on optimizing operational parameters such as the applied electrokinetic voltage, pH control, and residence time of fluid in the MFC cathode. Determining the effects of these parameters will lead to the development of larger dynamic systems and eventually to establishment of robust predictive modeling for field application design.
Chapter 6 References


Chapter 7: Summary, Conclusions, and Recommendations

7.1 Summary and Conclusions

*Bioreactor kinetic studies*

The use of models as prediction tools for reactor design was evaluated with the ultimate goal of finding a suitable model to predict \( \text{Cr}^{VI} \) in a microbial fuel cell system. By fitting \( \text{Cr}^{VI} \) reduction data, the speed of \( \text{Cr}^{VI} \) reduction for eight *Shewanella* strains were determined. Several models describing \( \text{Cr}^{VI} \) reduction were proposed and fit to experimental data. The best fit of these models was a “Transformation Capacity Model.”

A sensitivity analysis of biokinetic parameters of the “Transformation Capacity Model” appeared to indicate that the initial biomass concentration was a major influencing parameter. Threshold concentration and initial \( \text{Cr}^{VI} \) also appeared to affect the rate and extent of reduction, but not to the same degree as the initial biomass amount.

Sensitivity of reduction rates to pH and temperature were also considered. These studies demonstrated that pH was another important parameter to consider when designing future reactor systems. The effects of electron donor on \( \text{Cr}^{VI} \) reduction were also determined to be very important.

The “Transformation Capacity Model” was used to predict reactor behaviors for selected *Shewanella* species. It was determined that the model was able to predict \( \text{Cr}^{VI} \) reduction for these reactor systems. Nonetheless, some fine tuning of model parameters to specific applications would be necessary to increase accuracy. Overall, this model was successful in predicting experimental behavior within an order of magnitude.
Microbial fuel cell evaluations with Shewanella species

An evaluation of microbial fuel cell (MFC) technology utilizing *Shewanella* as cathodic biocatalysts was performed for fumarate reduction and Cr\(^{VI}\) reduction. Catalyzing fumarate reduction, *Shewanella* strains show a maximum power generation of between 10.2 and 59.4 nW cm\(^{-2}\). Comparisons between product formation and current transfer indicate either incomplete oxidation of fumarate or utilization of a separate source of electrons.

Similar comparisons under chromate reducing conditions indicate initial utilization of the electrode as the sole electron source followed by an imbalance between electrons transferred from the anode and the observed chromium reduction. Furthermore, it was shown that all *Shewanella* were initially capable of reducing Cr\(^{VI}\) to very low concentration levels. However, only *S. species* W3-18-1 was capable of Cr\(^{VI}\) removal to low concentrations after repeated exposures.

These studies also show that fuel cell systems, with *Shewanella* species acting as the sole biocatalysts at the cathode, are capable of achieving reduction of chromium concentrations to less than 5 ppb, well within acceptable guidelines established by regulatory agencies. This validates their use as a tool in removing Cr\(^{VI}\) contamination in drinking water sources.

Enrichment studies with microbial fuel cells

A comparison of enrichment strategies for establishing a Cr\(^{VI}\) reducing community in a MFC was completed and community development and structure was investigated. Two distinct strategies were examined: (i) a direct enrichment (DE) at the cathode of an MFC, and (ii) the subsequent enrichment (SE) of a Cr\(^{VI}\) reducing community after its establishment by enrichment at the anode of an MFC.
To evaluate enrichment of anodic population, several different electron donors were used at the MFC anode. Electricity production by bacterial communities enriched from wastewater sludge with lactate, succinate, N-acetyl-D-glucosamine (NAG), acetate, formate, and uridine were monitored in dual-chamber microbial fuel cells (MFCs). Stable electricity production was observed after 300 hours for communities enriched from lactate, acetate, and formate, while communities enriched with succinate, NAG, and uridine stabilized only after 700 h. The average peak current densities and maximum power densities generated from bacterial consortia were significantly higher than those generated from pure cultures of *Shewanella oneidensis* MR-1.

Two distinct communities emerged from these studies, which were then used for SE selecting for Cr VI reduction. Comparison of these communities with the one generated by DE show that while SE communities are able to immediately remove a significant amount of Cr VI initially, DE cultures appear to quickly adapt for Cr VI reduction and eventually outperform SE cultures. Differences between all of these communities were also highlighted by DGGE, implying that materials and techniques used in for enrichments are important and will have a large impact on the resultant community structure.

*Integration with electrokinetic systems*

To demonstrate the effectiveness of MFCs, integration with an electrokinetic system was performed. While an electrokinetic system alone was able to remove some Cr VI from a model system, MFC integration allowed for removal to low parts per billion concentration levels. Moreover, the integration of the MFC appeared to enhance Cr VI transport through a simulated saturated aquifer and prolong the useful life of the electrokinetic electrodes.
These studies also show that pH is an important variable to monitor during treatment with electokinetic technology. The extremes in pH caused by the electrolysis reactions at the electrodes inhibits migration of Cr\textsuperscript{VI} and control of pH will be of great importance when considering large scale electrokinetic treatment strategies.

Altogether, this set of experiments provides proof that an integrated MFC system is capable of achieving almost complete removal of Cr\textsuperscript{VI}. Moreover, the integration of the MFC system improved the performance and efficiency of the electrokinetic system.

7.2 Future recommendations

The following recommendations are proposed to guide future work:

- Extended operation of a larger Cr\textsuperscript{VI} reducing microbial fuel cell operating under continuous flow should be evaluated to identify possible challenges that may exist with scale-up or implementation into treatment systems.

- Investigation into the mechanisms of electron transfer within Cr\textsuperscript{VI} reducing fuel cell should be evaluated.

- Enhancement of MFC architecture design and materials needs to be done to increase efficiency and power production while maintaining the extent of Cr\textsuperscript{VI} removal.

- Additional work needs to be done to evaluate the microbial community and ecology with in microbial fuel cells. In particular, the active members of mixed communities should be accurately identified and efficient methods of selecting for these members must be developed.
• Incorporation of the threshold concentration model into existing electrokinetic transport models should be performed to provide a way to predict future, large-scale treatment systems.

• Investigation of model heterogeneous soil systems with an integrated MFC-electrokinetics system should be done to understand Cr\(^{VI}\) transport behavior in natural systems.
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