LONG-RANGE EXTRACELLULAR ELECTRON TRANSPORT BY DISSIMILATORY METAL-REDUCING BACTERIA ACROSS A PHYSICAL SEPARATION

BY

KYLE MICHELSON

DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Environmental Engineering in Civil Engineering in the Graduate College of the University of Illinois at Urbana-Champaign, 2018

Urbana, Illinois

Doctoral Committee:

Professor Albert Valocchi, Chair
Professor Charles Werth, Director of Research
Professor Robert Sanford
Assistant Professor Jeremy S. Guest
ABSTRACT

Nanopores in anaerobic sediments are stores of secondary minerals like Fe and Mn oxides that are often the most abundant electron acceptors for microbial respiration. The reduction of these minerals is catalyzed almost exclusively by dissimilatory metal-reducing bacteria (DMRB) that are defined by their ability to gain energy by coupling the oxidation of organic compounds and hydrogen to the reduction of metals. While DMRB are known to use a variety of electron transport mechanisms to reduce Fe and Mn minerals in contact with their outer membrane, little is known about their capacity to reduce sequestered minerals deep within pore spaces that are too small for cell passage.

To study long-range extracellular electron transfer (LR-EET) to sequestered minerals, microfluidic reactors with the novel ability to separate DMRB from the insoluble Mn(IV) mineral birnessite were etched into silicon using photolithography or electron beam lithography. The central feature of the microfluidic reactors was a 1.5 μm thick wall containing an array of <200 nm deep pores that allowed the diffusion of solutes but not the passage of cells. The nanoporous wall bifurcated two parallel flow channels; one containing cells, and the other containing a mineral. Bacteria were completely prevented from crossing the wall as demonstrated using brightfield microscopy, fluorescent staining, and scanning electron microscopy (SEM). At the completion of an experiment, a novel method was used to debond the anodically bonded reactors for high-resolution imaging using SEM. Microfluidic reactors were reused by cleaning between experiments using a newly developed protocol.

The first mechanism studied was reduction of birnessite by microbial nanowires using *Geobacter sulfurreducens* KN400. The nanoporous wall in these experiments was composed of an array of pillars separated by <200 gaps (i.e. nanopores) to provide nanowires access to the entire
depth of deposited birnessite. Using optical microscopy and Raman spectroscopy, it was demonstrated that birnessite can be reduced up to 15 μm away from cell bodies, similar to the reported length of *Geobacter* nanowires. Inhibition of nanowire production showed that nanowires were essential for reducing birnessite across the nanoporous wall by LR-EET, but not for reducing birnessite by direct contact. In the latter case, birnessite reduction was likely the result of electron transfer from outer membrane c-Cyts. Reduction across the wall required reducing conditions, provided by *Escherichia coli*, and an exogenous supply of riboflavin. Riboflavin was found to act not as a diffusible electron shuttle, but as a bound redox cofactor. The high binding affinity of riboflavin reported for outer membrane c-type cytochromes (c-Cyts) suggests that riboflavin was bound to OmcS, a c-Cyt that decorates the nanowire surface. Upon addition of a soluble electron shuttle (i.e., AQDS), it was also demonstrated that reduction extends up to 40 μm into a layer of birnessite, well beyond the reported nanowire length of 15 μm.

The second mechanism studied was the reduction of birnessite by electron shuttling using *Shewanella oneidensis* MR-1. The nanoporous wall in these experiments was composed of a series of highly uniform <200 nm slits along the top of a continuous wall in response to the increased ability of these bacteria to penetrate narrow pore spaces. It was demonstrated that birnessite reduction was driven by the endogenous production of riboflavin (RF) and flavin mononucleotide (FMN), which mediate redox reactions as diffusion-based shuttles or c-Cyt bound cofactors. Experiments with mutants that lacked flavin exporters showed that birnessite reduction is controlled by the concentration of flavin in the system. Addition of exogenous flavin to cells lacking flavin exporters restored birnessite reduction to wild-type rates in the microfluidic reactors. Experiments with mutants lacking conductive nanowires showed that nanowires were not responsible for birnessite reduction by LR-EET, and that the metal-reducing (Mtr) pathway,
currently believed to be critical for efficient reduction of insoluble metal oxides, is not required for high rates of reduction by LR-EET. These results suggest the existence of alternative electron pathways for metal reduction, which may be investigated in future work. It was also demonstrated that S. oneidensis can decouple growth from metabolism, potentially expanding the conditions under which metal reduction can be possible in the natural environment.

The results presented in this dissertation may lead to more accurate estimations of mineral redox cycling in anaerobic sediments, improved models of contaminant transport, and broadened understanding of carbon exchange between atmospheric and terrestrial ecosystems. Shewanella and Geobacter spp. are widely used in other applications such as energy production and wastewater treatment, and the results in my dissertation may aid in the design of more efficient bioelectrical systems.
ACKNOWLEDGEMENTS

I would like to express my gratitude towards my adviser, Professor Charles Werth, for his guidance, support, and enormous patience throughout my years of graduate research. I am especially grateful for the many opportunities I’ve been given to explore my own research ideas. I would like to give my thanks to Professors Rob Sanford, Al Valocchi, and Jeremy Guest for their help in organizing and fine-tuning my research proposal during the preliminary exam, and for giving important feedback that influenced my work in the past several years. Special thanks must be given to Professor Rob Sanford and Dr. Masaru Nobu for their aid in helping me culture microorganisms early in my PhD. I thank Professor Glennys Mensing for her help in designing and fabricating microfluidic reactors at the Micro and Nanotechnology Laboratory (MNTL), and to Dr. Xin Xu for introducing me to photolithography.

I appreciate the support of my wife, Xiangchen Huo, who was never shy in providing advice about organization, time management, and prioritization of research. I appreciate the support of my parents, Eric and Dorene Michelson, who have supported me emotionally and financially during my time as a graduate student. I am also happy that my younger brother, Brett Michelson, has managed to advance in career and in life while I’ve been away from home.

I am grateful to Dr. Shaoying Qi for maintaining the laboratories, fixing broken equipment in a timely manner, and offering career and research advice. I thank the Werth Group for their help and support, and hope that I have been able to advance their own research projects during my time at the University of Illinois and the University of Texas at Austin. A special thanks to Reinaldo Alcalde and Lang Zhou, who worked with me on my microfluidics experiments and now have the burden of passing my knowledge down to the next generation. I also thank Dr. Shengkun Dong for his support, encouragement, and friendship.
# TABLE OF CONTENTS

**CHAPTER 1: INTRODUCTION**
- 1.1 BACKGROUND .......................................................................................................................... 1
- 1.2 RESEARCH OBJECTIVES .......................................................................................................... 8
- 1.3 EXPERIMENTAL APPROACH................................................................................................... 9
- 1.4 FIGURES .................................................................................................................................. 12
- 1.5 REFERENCES ............................................................................................................................ 15

**CHAPTER 2: FABRICATION OF A MICROFLUIDIC REACTOR WITH A NANOPOROUS WALL TO STUDY LR-EET BY NANOWIRES AND ELECTRON SHUTTLES**
- 2.1 INTRODUCTION ...................................................................................................................... 20
- 2.2 EXPERIMENTAL APPROACH ................................................................................................. 21
- 2.3 FIGURES .................................................................................................................................. 28
- 2.4 REFERENCES ............................................................................................................................ 33

**CHAPTER 3: NANOWIRES OF GEOBACTER SULFURREDUCENS REQUIRE REDOX COFACTORS TO REDUCE METALS IN PORE SPACES TOO SMALL FOR CELL PASSAGE**
- 3.1 ABSTRACT .............................................................................................................................. 34
- 3.2 INTRODUCTION ....................................................................................................................... 35
- 3.3 MATERIALS AND METHODS ................................................................................................. 38
- 3.4 RESULTS AND DISCUSSION .................................................................................................. 47
- 3.5 FIGURES AND TABLES ........................................................................................................... 59
- 3.6 REFERENCES ............................................................................................................................ 73

**CHAPTER 4: FLAVIN DIFFUSION AND RECYCLING BY SHEWANELLA ONEIDENSIS MR-1 CAN DRIVE METAL REDUCTION ACROSS A PHYSICAL SEPARATION**
- 4.1 ABSTRACT .............................................................................................................................. 78
- 4.2 INTRODUCTION ....................................................................................................................... 79
- 4.3 MATERIALS AND METHODS ................................................................................................. 81
- 4.4 RESULTS AND DISCUSSION .................................................................................................. 87
- 4.5 FIGURES AND TABLES ........................................................................................................... 97
- 4.6 REFERENCES ............................................................................................................................ 112

**CHAPTER 5: CONCLUSIONS**
- 5.1 CONCLUSIONS ....................................................................................................................... 117
- 5.2 CONTRIBUTIONS ..................................................................................................................... 119
- 5.3 FUTURE WORK ....................................................................................................................... 121
- 5.4 REFERENCES ............................................................................................................................ 123
1.1 Background

Microbially mediated redox transformations of iron and manganese are important biogeochemical reactions that affect carbon turnover\(^1\), contaminant transport\(^2\), trace metal speciation\(^3\), and macronutrient bioavailability\(^4,5\). In anaerobic nonsulfidogenic soils and sediments, Mn(IV) and Fe(III) reduction is driven by dissimilatory metal-reducing bacteria like members of the *Geobacter* and *Shewanella* genus\(^6\). The majority of Mn(IV) and Fe(III) in anaerobic sediment systems consists of insoluble metal oxides. Since bacterial cell envelopes are impermeable to metal oxides, enzymatic reduction requires an extension of the respiratory machinery to the outer membrane\(^7\). Bacteria have evolved several extracellular mechanisms to overcome this physical limitation, including localization of c-type multiheme cytochromes (c-Cyts) to the outer membrane (OM), reduction of soluble electron shuttles, and production of electrically conductive nanowires\(^8-11\). Electron shuttles and nanowires allow for long-range extracellular electron transport (LR-EET) beyond the outer membrane of the cell, but little is known about the extent and mechanisms of LR-EET to Mn(IV) and Fe(III) oxides trapped in pore spaces that are too small for cell passage.

**Biogeochemistry of Fe and Mn oxides**

Microbially-mediated redox transformations of iron and manganese in marine and freshwater sediments play important roles in the biogeochemical cycling of other elements and the exchange of energy at the aerobic-anaerobic interface\(^4,12\). The availability of essential macronutrients like nitrogen, phosphorus, and sulfur is affected by the redox state of iron and manganese as a result of microbial activity\(^13,14\). Ammonia oxidizing (anammox) bacteria, for
example, couple iron oxidation to nitrate reduction\textsuperscript{15}. In anoxic sediments, sulfate reducing bacteria produce elemental sulfur and sulfide species that can react abiotically with iron and manganese oxides\textsuperscript{16}. Oxidation of organic carbon coupled to the reduction of iron and manganese produces carbon dioxide and drives the exchange of carbon between terrestrial and atmospheric ecosystems\textsuperscript{17}. Syntrophic interactions between dissimilatory metal-reducing bacteria (DMRB) and denitrifying anaerobic methane oxidizers (DAMO) reduce iron and oxidize methane, with important implications for global carbon and iron cycling\textsuperscript{18}. An illustration of redox reactions coupled to iron reduction and oxidation is presented in Figure 1.1.

Insoluble oxides of manganese and iron, such as ferrihydrite (FeOOH•xH\textsubscript{2}O) and birnessite [(Na, K)\textsubscript{0.6}(Mn\textsuperscript{4+}, Mn\textsuperscript{3+})\textsubscript{2}O\textsubscript{4} • 1.5H\textsubscript{2}O], play important roles in the mobility and redox cycling of a wide range of metals (e.g. Cu, Ni, Zn), metalloids (e.g. As, Se), and radionuclides (e.g. U, Tc) due to their high sorptive capacities\textsuperscript{2,14}. For example, the concentration of dissolved uranyl species at concentrations similar to those at highly contaminated sites was found to decrease by several orders of magnitude in the presence of birnessite\textsuperscript{19}. The adsorption of positively charged species to iron and manganese oxides is partly due to their high cation exchange capacities\textsuperscript{2,20}. In addition, poorly crystalline iron and manganese oxides that account for a significant fraction of insoluble Fe(III) and Mn(IV) in anaerobic sediments tend to possess high surface areas that further increase their sorptive capacity\textsuperscript{21,22}. While the reductive dissolution of iron and manganese oxides often results in the solubilization of adsorbed species, the microbial reduction of structural iron in clays actually leads to an increase in the cation exchange capacity of the clay mineral, and therefore its sorptive capacity\textsuperscript{23}. Changes in the oxidation state of solid-phase iron and manganese as a result of microbial activity can therefore affect the mobility of contaminants in complex ways.
**Extracellular electron transport mechanisms**

Structural Fe(III) and pure mineral phases of Fe(III) and Mn(IV) are often the most abundant electron acceptors for microbial respiration in anaerobic nonsulfidogenic soils and sediments\(^6\). However, their reduction by bacteria is complicated due to the structure of cell walls that are impermeable to solid-phase minerals\(^7\). In response to this physical limitation, bacteria have evolved several mechanisms to transport electrons to their outer membrane in a process referred to as extracellular electron transport (EET).

EET between a low redox potential donor (e.g. acetate) to a high redox potential acceptor (e.g. birnessite) is achieved through a sequence of redox reactions that are catalyzed by an electron transport chain spanning the inner and outer membrane\(^24\). The process begins when electrons from a cytoplasmic donor are released into the quinone pool of the inner membrane. Electrons in the quinone pool are then transferred through a sequence of multiheme c-type cytochromes with different redox potentials resulting in the generation of a proton gradient and the generation of ATP\(^24\). While the EET process is similar across different species of DMRB, the cytochromes involved vary depending upon the species. In this thesis, we look at EET by *Geobacter sulfurreducens* and *Shewanella oneidensis*, two model DMRB that are studied for their application in bioremediation\(^25\), wastewater treatment\(^26\), and energy generation\(^27\). In *S. oneidensis* MR-1, electrons are transported from the quinone pool to the outer membrane via the Mtr pathway, which consists of inner membrane c-Cyt CymA, periplasmic c-Cyt MtrA, outer membrane scaffolding protein MtrB, and outer membrane c-Cyts OmcA and MtrC (Fig. 1.2)\(^28\). The decaheme c-Cyts OmcA and MtrC, which can serve as terminal reductases, are translocated across the outer membrane through MtrB via a type II secretion pathway where they can interact with Fe(III) and Mn(IV) oxides\(^29\). In *G. sulfurreducens*, electrons are transported from the quinone pool to the
periplasm via inner membrane c-Cyt MacA\textsuperscript{24}. Electrons are then transported from the periplasmic c-Cyt PpcA to a variety of outer membrane c-Cyts (e.g. OmcS, OmcZ) that act as terminal reductases for Fe(III) and Mn(IV) oxides (Fig. 1.2\textsuperscript{30,31}). Localization of c-Cyts to the outer membrane is a strategy used by both \textit{G. sulfurreducens} and \textit{S. oneidensis} for efficient reduction of Fe(III) and Mn(IV) oxides that are in direct contact with the outer membrane\textsuperscript{9,32}. However, cells that are physically separated from an electron accepting surface have evolved additional strategies to transport electrons over longer distances in a process referred to as long-range extracellular electron transport (LR-EET). This can occur via electron shuttling of cytochrome-bound or freely soluble electron shuttles, and the production of conductive ‘nanowires’ that differ in structure and composition between bacterial species.

Electron shuttling is mediated by organic molecules that serve as electron carriers between bacteria and an electron-accepting surface. The most common electron shuttles endogenously produced by DMRB are flavins, which include riboflavin (RF), flavin mononucleotide (FMN), and to a lesser extent flavin adenine dinucleotide (FAD)\textsuperscript{33}. These redox active molecules can abiotically reduce iron oxide minerals like ferrihydrite and lepidocrocite when treated with a reducing agent\textsuperscript{34}. The addition of RF or FMN to cultures of \textit{G. sulfurreducens} and \textit{S. oneidensis} is observed to dramatically increase the rate of Fe(III) and Mn(IV) oxide reduction\textsuperscript{35}, although the mechanism of electron shuttling is a subject of ongoing debate. There is no experimental evidence to support the role of flavins as diffusion-based shuttles in \textit{G. sulfurreducens}. Rather, the high binding affinity of flavins to \textit{Geobacter} cytochromes suggests that flavins may act as cytochrome-bound redox cofactors\textsuperscript{36,37}. This may explain why current production in anode grown biofilms of \textit{G. sulfurreducens} was unaffected when culture supernatant was replaced with fresh media\textsuperscript{38}. In \textit{S. oneidensis}, the opposite results were observed. Current production in membrane bioreactors
sharply declined when supernatant containing flavins was replaced with fresh media, suggesting that electron shuttling in *S. oneidensis* is diffusion-based\textsuperscript{39}. However, shifts in redox potential upon addition of flavins to *Shewanella* biofilms were consistent with a one-electron transfer reaction, which only occurs when flavins are bound to c-Cyts. These contradictory results suggest that flavin shuttling may be more complicated in *S. oneidensis*. Exogenous electron shuttles like the quinone moieties of humic substances can also be used by DMRB to shuttle electrons to Fe(III) and Mn(IV) oxides. Experiments using anthraquinone-2,6-disulfonate (AQDS), a humic acid analogue, showed similar rate enhancements of ferrihydrite and birnessite reduction in cultures of *S. oneidensis* and *G. sulfurreducens*\textsuperscript{40,41}. Electron shuttling by AQDS is expected to contribute to metal reduction in organic rich sediments. Recently, the possibility of electron shuttling by thiols has been proposed. The addition of cysteine to cultures of *G. sulfurreducens* and *S. oneidensis* accelerates the reduction of Fe(III) oxides and iron-containing smectite clays\textsuperscript{42,43}. Microbial sulfate reduction in anoxic marine sediments is associated with increases in thiol concentrations, particularly those of 3-mercaptopropionic acid and glutathione that accumulate in the porewater at nM to μM concentrations\textsuperscript{44}. Sulfur-containing amino acids have also been detected in anoxic coastal sediments at concentrations between 100-100 μM\textsuperscript{45}. Flavins, humic acid, and thiols are the only known electron shuttles that are capable of Fe(III) and Mn(IV) oxide reduction. DMRB are capable of synthesizing and secreting flavins and thiols, although thiols can also be present in the environment. DMRB are capable of quinone production as well, but their use as extracellular shuttles has fallen out of favor over the past decade with no evidence to support their role in Fe(III) and Mn(IV) reduction. Rather, exogenous quinones acquired from soil organic matter degradation are now thought to be the only quinone shuttles used by DMRB in natural environments. Solubilization of Fe(III) and Mn(IV) may also be possible with the aid of endogenously produced...
chelators (i.e. siderophores), but experimental evidence suggests that this is not a major pathway of reduction\textsuperscript{46}.

Microbial nanowires are conductive appendages with nanometer-scale widths and micrometer-scale lengths that are produced by a variety of bacteria, most notably \textit{S. oneidensis} and \textit{G. sulfurreducens}\textsuperscript{10,47,48}. It is currently believed that nanowires allow cells to respire metal oxides and aqueous phase toxic species from a distance\textsuperscript{49}. Thicker biofilms and greater current production is observed when nanowire production is expressed in \textit{G. sulfurreducens}, suggesting that nanowires also form an electrical conduit between biofilm cells and allow those not in direct contact with an electron donor or acceptor to participate in energy generating redox reactions\textsuperscript{50,51}. Their role in \textit{S. oneidensis} is less clear, however, since electron shuttling dominates in metal oxide reduction, and electron transport in \textit{Shewanella} biofilms appears to be mediated by endogenously produced flavins\textsuperscript{35}. The nanowires of \textit{S. oneidensis} were recently discovered to be extensions of their outer membrane\textsuperscript{47}. Electron hopping between outer membrane c-Cyts OmcA and MtrC, which are the terminal reductases of the Mtr pathway, is responsible for their conductivity\textsuperscript{11,47}. Deletion of these two cytochromes results in the production of nonconductive nanowires\textsuperscript{11}. In vivo observation of nanowire formation, which occurs under electron acceptor limited conditions, revealed a maximum length of 9 μm\textsuperscript{47}. The diameter of \textit{Shewanella} nanowires is 50-150 nm as shown by SEM and STM\textsuperscript{48}. The nanowires of \textit{G. sulfurreducens} have a similar length, but much smaller diameter of around 3-5 nm as measured by AFM\textsuperscript{10,52,53}. In contrast to the nanowires of \textit{Shewanella}, those of \textit{Geobacter} are intrinsically conductive and composed of repeating units of PilA protein\textsuperscript{54}. \textit{G. sulfurreducens} requires nanowires for efficient metal oxide reduction as demonstrated using nanowire-deficient mutants\textsuperscript{10,55}. Their nanowires may also protect the cell from periplasmic accumulation of toxic metals and radionuclides such as uranium, which would
otherwise pass through the cell membrane in their oxidized and soluble forms and precipitate upon reduction\textsuperscript{49}. Outer membrane c-Cyt OmcS decorates the surface of \textit{Geobacter} nanowires but is not responsible for their conductivity since the spacing of individual cytochromes is too far for electron tunneling\textsuperscript{56,57}. Rather, it appears that OmcS may be an intermediate electron carrier involved in the transfer of electrons between the nanowire and an electron acceptor. The mechanism of electron transfer through \textit{Geobacter} nanowires is a subject of intense debate that is more fully described in Chapter 3. Similar nanowires have been reported in the cyanobacterium \textit{Synechocystis} strain PCC 6803, and the thermophilic bacterium \textit{Pelotomaculum thermopropionicum}, suggesting that proteinaceous nanowires may be a common feature of a diverse range of bacteria\textsuperscript{48}.

**The role of nanowires and electron shuttles in reducing sequestered metal oxides**

Electron transport via nanowires and electron shuttles has primarily been studied in biofilms and individual cells, with a focus on mechanisms of conduction and applications in bioelectrochemical systems. However, metal reduction by LR-EET in sediment nanopores across physical separations has largely been overlooked. Little research has been done to investigate the extent to which microbes have access to sequestered metal oxides, or the conditions under which this may occur. The importance of nanopores is reflected in their enormous contribution to porosity and surface area in sediments, particularly in B-horizon soils where nanopores with diameters <100 nm account for 10-40\% of the porosity and more than 90\% of the surface area\textsuperscript{58}. During the weathering of primary minerals, poorly crystalline iron and manganese oxides such as ferrihydrite precipitate in nanopores that are smaller than the diameter of a bacterial cell\textsuperscript{59}. However, the contribution of DMRB to Fe(III) and Mn(IV) reduction in nanopores is often neglected because these minerals are physically inaccessible to bacteria. It is important to understand the contribution
of LR-EET to the reduction of sequestered Fe(III) and Mn(IV) oxides to improve our understanding of iron and manganese redox cycling in anaerobic sediments. This would also affect models of contaminant transport, the mobility of macronutrients and trace elements, and the flux of carbon between the subsurface and atmosphere. Several studies have investigated metal reduction in nanopores using glass or alginate beads filled with iron oxides, but little could be ascertained about the mechanism of electron transport during Fe(III) reduction. These experiments were limited in their ability to distinguish between different LR-EET mechanisms, measure in-situ redox reactions, and visualize pore-scale phenomena in real-time. New experimental platforms and analytical techniques are needed to study metal reduction through nanopores across physical separations and quantify electron flux via different LR-EET mechanisms.

1.2 Research objectives

The overall objective of this work is to investigate LR-EET into nanopores across a physical separation. Specifically, this involves testing the relative contribution of different LR-EET mechanisms on the reduction of sequestered metals, measuring reduction rates under different flow conditions, and quantifying electron flux between a biofilm and an environmentally relevant electron acceptor (e.g. birnessite). This will allow us to predict the extent to which different LR-EET mechanisms enhance reaction rates within the physical mixing zone in anaerobic sediments. The primary objectives of this work are the following:

1) To develop a novel experimental platform that can physically separate bacteria from an electron acceptor: Decoupling EET from LR-EET requires the creation of a physical barrier between cells and an electron acceptor. The physical barrier must be customized to account for differences in bacteria phenotype and LR-EET strategy (i.e. nanowires, electron shuttles). Quantifying the relative importance of different LR-EET strategies requires the
creation of an experimental platform that is compatible with multiple analytical tools for real-time, in-situ measurements of electron flux.

2) To measure the rate, extent, and mechanism of LR-EET via nanowires: *Geobacter sulfurreducens* is a model nanowire-producing DMRB. While electron transport through nanowires has been measured in previous work, it is not known if these results translate to metal reduction across physical separations, or whether redox mediators (e.g. flavins) play a role in mediating electron transfer between the nanowire and an electron acceptor.

3) To measure the rate, extent, and mechanism of LR-EET via electron shuttles: *Shewanella oneidensis* is a model electron-shuttling DMRB. There is ongoing debate over whether flavins act as diffusion-based shuttles or bound cofactors, and new evidence to support shuttling by thiols. Most experiments to date have tested electron shuttling when cells are in direct physical contact with an electron acceptor, but little is known about the capacity of electron shuttles to reduce sequestered metal oxides.

1.3 Experimental approach

LR-EET was investigated in silicon-etched microfluidic reactors using *G. sulfurreducens* KN400 and *S. oneidensis* MR-1 as model DMRB, and the Mn(III/IV) mineral birnessite as a representative metal oxide. Microfluidic reactors were fabricated in a cleanroom using photolithography or electron beam lithography. The fabrication process is discussed in detail in Chapter 2 of this thesis. Microfluidic reactors were inoculated under sterile conditions and stored in a temperature-controlled environment. DMRB were grown in bicarbonate-buffered anaerobic minimal media. A vitamin-free casein hydrolysate was also added to cultures of *S. oneidensis* MR-1 to aid in growth. Solutions were infused by syringe pump, stored in gastight syringes, and delivered to reactors through gas impermeable PEEK tubing. A picture of the experimental set-up...
is shown below in Figure 1.3. Birnessite was synthesized by oxidizing manganese chloride in a basic solution of potassium permanganate. Anaerobic conditions were maintained by the addition of a reducing agent at a concentration that did not result in the abiotic reduction of birnessite. *Escherichia coli* K-12 was added to cultures of *G. sulfurreducens* KN400 to provide additional oxygen scavenging ability.

While the media composition and growth conditions slightly varied between *G. sulfurreducens* KN400 and *S. oneidensis* MR-1, the same sequence was followed for birnessite fixation, inoculation, and growth. Birnessite was fixed to the nanoporous wall by manual injection of a colloidal solution of birnessite through one channel of the reactor. Once a layer of birnessite was deposited at the wall, cells were inoculated at the other inlet. Fumarate, or a combination of fumarate and nitrate as soluble electron acceptors were infused through the channel containing birnessite to aid in biofilm formation at the nanoporous wall. Once a biofilm was established, fumarate and nitrate were removed and cells were forced to rely on birnessite as the sole electron acceptor.

Multiple analytical tools were used to differentiate between different LR-EET mechanisms, measure birnessite reduction, and quantify electron flux. Optical microscopy was used to measure the extent of birnessite reduction across the wall. Fluorescent microscopy was used to monitor the redox state of biofilm cells and detect contamination across the nanoporous wall. Raman spectroscopy was used to identify minerals in-situ and differentiate between birnessite and the solid-phase reduction product identified as rhodochrosite. High performance liquid chromatography (HPLC) was used to quantify the concentration of flavin and thiol electron shuttles in the effluent. Inductively coupled plasma – optical emission spectroscopy (ICP-OES) was used to quantify dissolved manganese in the effluent. Scanning electron microscopy (SEM)
was used to image biofilm and extracellular polymeric substances (EPS) after completing an experiment.
1.4 Figures

Figure 1.1. Summary of notable redox reactions between macronutrients and iron oxides adapted from Melton et al. Note that many redox reactions involving iron oxides also occur with manganese oxides.
Figure 1.2. An illustration showing the electron transport pathways of *G. sulfurreducens* (left) and *S. oneidensis* (right), adapted from Kracke et al\textsuperscript{24}. OmcZ and OmcS (not shown) are important for metal reduction in *G. sulfurreducens* (left). The Mtr pathway, which includes MtrABC and OmcA are important for metal reduction in *S. oneidensis* (right).
Figure 1.3. Experimental set-up with microscope, computer, and incubators (top) and a close-up of a microfluidic reactor and syringe pump inside an incubator (bottom).
1.5 References


(28) Szeinbaum, N.; Burns, J. L.; DiChristina, T. J. Electron transport and protein secretion


(54) Malvankar, N. S.; Vargas, M.; Nevin, K. P.; Franks, A. E.; Leang, C.; Kim, B.-C.; Inoue,


CHAPTER 2: FABRICATION OF A MICROFLUIDIC REACTOR WITH A
NANOPOROUS WALL TO STUDY LR-EET BY NANOWIRES AND ELECTRON
SHUTTLES

2.1 Introduction

Quantifying electron flux across a physical barrier and differentiating between mechanisms of LR-EET requires a precisely machined experimental platform that is compatible with multiple analytical techniques during its operation. A silicon-etched microfluidic reactor with a glass coverslip was chosen as the best experimental platform for several reasons: 1) Its 2D architecture allows a clear view of the biofilm and deposited birnessite 2) The glass coverslip that seals the reactor is compatible with brightfield and fluorescent microscopy, as well as Raman spectroscopy 3) Sterile conditions can be easily maintained 4) Nanoscale features can be etched in silicon with high precision and reproducibility 5) There is minimal lag time when changing solutions or flow rates. Microfluidic reactors were simple in design and consisted of two parallel flow channels that were bisected by a physical barrier. One channel contained bacteria, and the other an insoluble electron acceptor (i.e. birnessite). Physical separation between bacteria and birnessite was achieved using a thin, nanoporous wall that allowed the diffusion of solutes but not the physical passage of cells. For the study of LR-EET via nanowires, the wall was fabricated as series of pillars with nanopores that spanned the entire depth of the reactor. For the study of LR-EET via electron shuttling, the wall was fabricated as a continuous structure with a series of slits. The fabrication process and rationale behind the various wall designs is described in the following sections of this chapter along with a cleaning method for reactor reuse, and a novel method for removing the glass coverslip.
2.2 Experimental approach

Fabrication of a microfluidic reactor for the study of LR-EET via nanowires

The study of LR-EET via nanowires of *G. sulfurreducens* KN400 required a wall with nanopores that spanned the entire depth of the reactor. This is because nanowires are anchored to the cell membrane and possess a length that is similar to the minimal depth of the reactor needed to allow passage of birnessite particles without blocking the inlets. Nanopores etched only at the top of the reactor, for example, may not allow nanowires to reach to the bottom of the wall on the adjacent side. As a result, the extent of birnessite reduction would be difficult to quantify.

An illustration of the fabrication process is shown in Figure 2.1. Silicon wafers (P/boron, 100 mm dia., 10-20 Ω-cm) were degreased by rinsing in acetone, isopropyl alcohol (IPA), and water. Remaining organics were removed in SC-1 solution consisting of a 1:1:5 v/v ratio of 28% ammonium hydroxide (NH$_4$OH), 30% hydrogen peroxide (H$_2$O$_2$), and water set at 80 °C. A 135 nm layer of silicon dioxide was grown on the silicon wafers by dry oxidation in a tube furnace at 1100 °C. Next, wafers were coated in a monolayer of the adhesion promoter AP8000. ZEP520A electron beam resist was then spun on the front side of the wafer at 3000 rpm to a thickness of 400 nm followed by a prebake for 3 minutes at 180 °C. A digitized image of the nanoporous wall was created in AutoCAD and etched into the resist layer using electron beam lithography (EBL) at a dose of 90 μC/cm$^2$, a current of 1 nA, and voltage of 50 kV. The exposed pattern was developed in o-xylene to expose the underlying silicon dioxide then rinsed in a 3:1 v/v solution of methyl isobutyl ketone (MIBK) to IPA to improve resolution. The exposed silicon dioxide was partially etched by reactive ion etching (RIE) in a CF$_3$/O$_2$ plasma for 4 minutes at 90W. The remaining silicon dioxide was removed in a 6:1 v/v ratio of 40% ammonium fluoride (NH$_4$F) to 49%
hydrofluoric acid (HF), also referred to as a buffered oxide etch (BOE). The remaining ZEP520 resist was removed in an O2/Ar plasma at 300W.

The reactor channels were created using photolithography as shown in Figure 2.1. SPR220-3.0 photoresist was spun on the wafers at 3000 rpm to a thickness of 3.0 µm and prebaked for 2 minutes at 115 °C. A digitized image of the reactor channels was created in AutoCAD and etched in chrome on a soda lime glass mask for photolithography. The pattern was exposed with UV light on a H-line mask aligner at 20 mW/cm² for 20 seconds then developed in a 4:1 v/v solution of H2O:AZ400K developer until clear. This was followed by a post-exposure bake for 5 minutes at 115 °C. Wafers were then descummed in an O2/Ar plasma at 300W for 30 seconds. The exposed silicon dioxide was removed by BOE.

This process was repeated for the etching of ports, except that SPR220-7.0 photoresist was spun on the wafers at 3000 rpm to a thickness of 7.5 µm. A soda lime glass mask containing the pattern of the ports was exposed with UV light on a H-line mask aligner at 20 mW/cm² for 30 seconds. A thicker layer of photoresist as required for through-etching of the ports. This layer of photoresist also protected the patterns for the nanoporous wall and channels that were previously etched into the silicon dioxide layer. After removing the exposed silicon dioxide by BOE, the wafers were attached to a carrier wafer using vacuum grease and etched through using inductively coupled plasma - deep reactive etching (ICP-DRIE). The etch recipe was a standard Bosch process¹ for anisotropic etching with alternating plasmas of SF₆ and C₄F₈. Photoresist was removed using an O₂/Ar plasma at 300W to expose the etched silicon dioxide containing the pattern for the nanoporous wall and channels.

The final etching step involved transferring the pattern of the nanoporous wall and channels from silicon dioxide to silicon. Due to the high selectivity of the Bosch process for silicon
over glass, the 135 nm thick silicon dioxide layer was used as a protective mask for the nanoporous wall and channels, which were etched to a depth of approximately 10 μm. The silicon wafers were then separated from the carrier wafer, and the vacuum grease was removed in a piranha bath consisting of concentrated sulfuric acid and 30% hydrogen peroxide in a 2:1 v/v ratio. The remaining silicon dioxide was removed by BOE. After silicon etching, the pillars that formed the nanoporous wall measured 1.0 x 1.0 μm. However, the nanopores between each pillar were 0.6 μm wide, which are not small enough to prevent bacteria from passing through. Nanopores were closed by depositing polysilicon on the wafer by low pressure chemical vapor deposition (LPCVD). This increased pillar area to 1.4 x 1.4 μm while decreasing nanopore width to 0.2 μm as measured by SEM and shown in Figure 2.2. To seal the reactors, Pyrex 7740 glass wafers were anodically bonded to the etched silicon wafers at 900 V and 400 °C. This results in a bond strength between 10-20 MPa, which is greater than the fracture strength of glass. Once bonded, PEEK NanoPort assemblies were glued over the etched ports to allow them to be connected to tubing.

Fabrication of a microfluidic reactor for the study of LR-EET via electron shuttling

The fabrication process is shown in Figure 2.3. Slight variations in the pillars that resulted in some nanopores being slightly wider than 200 nm did not lead to contamination of the adjacent channel by *G. sulfurreducens* KN400. However, *S. oneidensis* MR-1 was able to pass through. Investigation of electron shuttling by *S. oneidensis* MR-1 therefore required the fabrication of another type of wall with narrower pores that were consistently <200 nm. Since *S. oneidensis* was expected to use soluble electron shuttles for LR-EET, a continuous wall with slits (i.e. nanopores) etched only on the top of the wall would not be expected into interfere with measurements of electron flux.
Silicon wafers were degreased as before and treated in a piranha bath consisting of concentrated sulfuric acid and 30% hydrogen peroxide in a 2:1 v/v ratio to remove all organics. Next, wafers were coated in a monolayer of the adhesion promoter hexamethyldisilazane (HMDS). Wafers were then spin coated with AZ1518 photoresist at 3000 rpm for a thickness of 1.8 μm and prebaked at 110 °C for 1 minute. A digitized image of 3 x 3 μm slits patterned on a soda lime glass mask was transferred to the photoresist with UV light using an i-line mask aligner. Exposure was carried out at 10 mW/cm² for 7 seconds, then wafers were developed in MF-26A (2.3% tetramethylammonium hydroxide) developing solution until clear. Slits were etched by reactive ion etching (RIE) to a depth of 180-200 nm using a combination of HBr (25 sccm) and Cl₂ (4 sccm) plasmas at a power of 180 W. The depth of the slits was measured by optical profilometry. After stripping the photoresist in acetone and IPA, AZ1505 photoresist was spun on the wafers at 1500 rpm to achieve a thickness of 1.0 μm. A prebake at 110 °C for 1 minute was followed by UV exposure for 3 seconds at 10 mW/cm² using a soda lime glass mask that contained the pattern of the 1.5 μm wall and channels. The wafers were developed in MF-26A until clear. The slits that were etched in the previous step were protected by the photoresist. Next, the wafers were etched to a depth of 10 μm using ICP-DRIE to form the wall and channels as shown in Figure 2.4. After stripping the photoresist and degreasing the wafers, AZ1529 photoresist was pooled on the etched surface and spun at 1000 rpm, yielding a thick and nonuniform protective layer. Resist was prebaked for 15 minutes at 110 °C. Ports were then ultrasonically drilled through the silicon wafers at 20 kHz and 100 W. After degreasing in acetone and IPA and cleaning in a piranha bath, a 135 nm thick layer of silicon dioxide was grown on the surface at 1100 °C. Silicon wafers were anodically bonded to Borofloat 33 glass wafers to seal the reactor, and PEEK NanoPort assemblies were glued over the etched ports.
Cleaning microfluidic reactors for reuse

Formation of biofilm and precipitation of byproducts from birnessite reduction made the reuse of microfluidic reactors difficult. EPS is particularly resistant to chemical treatment and mechanical shearing due to its chemically complex and sticky nature. The composition of EPS varies between different microorganisms, but is generally composed of proteins, lipids, polysaccharides, and extracellular DNA. The stickiness of biofilms is affected by different EPS components at different stages in biofilm growth. In Pseudomonas aeruginosa, whose EPS has been well-studied, DNA was found to be critical for early biofilm attachment to surfaces while polysaccharides played a greater role in the attachment of mature biofilms. A variety of methods have been developed to remove EPS from surfaces such as pipes, reverse-osmosis (RO) membranes, and food processing plants. These include hydrolysis of EPS with acid or base, solubilization of biofilm with surfactants (e.g. sodium dodecyl sulfate), destabilization with chelating agents (e.g. ethylene diamine tetraacetate) mechanical shearing by ultrasonication, reaction of specific biofilm components with enzymes (e.g. lipase, protease, deoxyribonuclease), and dissociation of biofilm with quorum sensing compounds that induce detachment of cells among other technologies. Since EPS is complex, a combination of these methods may be used to achieve higher removal efficiency.

The cleaning protocol used to remove manganese solids and EPS involved treatment with acid, protease, and surfactant as shown in Table 2.1. Manganese solids were dissolved by infusing 3,000 pore volumes of 5% H$_2$SO$_4$ and 10 mM oxalic acid. The protein fraction of EPS was digested by infusing 12,000 pore volumes of 10% v/v serine protease (Savinase, 16 U/g) at pH 9.5. Remaining cells and EPS were removed by infusing 3,000 pore volumes of 1% w/v sodium dodecyl sulfate at pH 11. After cleaning, reactors were inspected using brightfield and fluorescence
microscopy. Cells and EPS did not appear to be present. Cleaning was followed by autoclaving all materials to ensure sterile conditions for latter experiments.

**Debonding microfluidic reactors**

The bond strength between silicon and glass after anodic bonding is typically in the range of 10-20 MPa, which is similar to the fracture strength of glass. It is therefore difficult to open a bonded reactor. However, post-experimental analysis with high-resolution imaging (e.g. SEM) requires the removal of the anodically bonded glass cover. Several methods exist to debond silicon and glass, but they require high heat or voltage, intermediate layers, or additional steps in the fabrication process. Cathodic debonding of silicon and glass requires high voltage and temperatures of 400 °C. These conditions would damage biological material such as nanowires and are therefore not recommended for post-experimental analysis of biofilms. Intermediate layers such as thin adhesives between silicon and glass result in weaker bond strength, and dissolution of the intermediate layer may affect microbial activity. The use of low temperature eutectic metals (e.g. Au-In) as intermediate layers results in a more complicated fabrication process, and temperatures required to separate the silicon and glass, which are in excess of 150 °C, may still damage delicate biological structures. Surface modification of the silicon wafer to decrease the overall bonding strength is another method used to debond anodically bonded reactors. This typically involves the etching of silicon to create surface defects that reduce the area of silicon in conformal contact with the glass. While the local bond strength is typical of anodic bonding, the bond strength averaged over the entire wafer is lower. This facilitates the separation of silicon and glass but requires extra steps in the fabrication process.
A novel method to separate anodically bonded glass and silicon wafers without damaging biological material was developed for high-resolution post-experimental analysis of microbial nanowires and EPS. This method does not involve intermediate layers or extra steps in the fabrication process, making it ideally suited for biological experiments that cannot be performed using traditional soft materials such as polydimethylsiloxane (PDMS). The method first involves roughing the silicon and glass surfaces. In this method, 150 grit silicon carbide sandpaper was used. Two aluminum blocks with flat surfaces were also roughened by sandpaper to increase the surface area for bonding and remove the native oxide. The surface area of the aluminum blocks should be slightly larger than the surface area of the microfluidic reactor. Next, chromic acid was synthesized by dissolving 1.8 g potassium dichromate in 35 mL of water, then adding 15 mL concentrated sulfuric acid. The aluminum blocks were submerged in chromic acid for 15 minutes at 70 °C, then washed thoroughly with DI water. Treatment of aluminum in chromic acid increases surface area significantly improves adhesive bonding strength. The aluminum blocks were dried using compressed air, then placed in an oven at 60 °C for 15 minutes. Loctite E-60HP Epoxy Structural Adhesive was applied to the bonding surface of both aluminum blocks. This adhesive was chosen for its high peel and shear strength, particularly on glass and acid-etched aluminum. The anodically bonded wafer was placed between the two aluminum blocks and allowed to cure at 25 °C for 24 hours to reach full strength. The blocks were separated by applying a sudden bending moment on either side of the wafer. Two wrenches were used to apply torque. An SEM image of a debonded reactor is shown in Figure 2.5.
2.3 Figures

Figure 2.1. Side view process diagram for reactor fabrication. Cleaning wafer (A) growth of thermal oxide (B) spincoating of ZEP520A e-beam resist (C) e-beam patterning of wall and channels (D) CF$_4$ plasma etch of exposed oxide (E) stripping of ZEP520A (F) spincoating of SPR220 (G) pattern transfer by exposure to UV light (H) developing exposed resist (I), BOE of exposed silicon dioxide (J) stripping of SPR220 (K) silicon deep etch to 10 microns (L) stripping of remaining oxide by BOE (M) LPCVD of polysilicon to close the gap between pillars to <200 nm (N) DRIE of ports (O) and anodic bonding of silicon wafer to glass (P).
Figure 2.2. SEM image of pillars after closing the gaps (i.e. nanopores) to < 200 nm.
Figure 2.3. Plane view process diagram for reactor fabrication. Spinning AZ1518 photoresist (A), UV exposure (B), RIE plasma etch of slits to 180-200 nm (C) stripping of AZ1518 (D), spinning of AZ1505 photoresist (E), UV exposure (F), DRIE of channels (G), stripping of AZ1505 photoresist (H), anodic bonding (I).
Figure 2.4. SEM image of nanoporous wall with 180-200 nm deep slits.
Figure 2.5. SEM image of a debonded reactor showing the glass coverslip removed from the wall and channels.
2.4 References

(1) Laermer, F.; Schilp, A. Method of anisotropically etching silicon, 1996.


CHAPTER 3: NANOWIRES OF GEOBACTER SULFURREDUCTENS REQUIRE REDOX COFACTORs TO REDUCE METALS IN PORE SPACES TOO SMALL FOR CELL PASSAGE

Accepted by *Environmental Science & Technology*, 2017


3.1 Abstract

Members of the *Geobacteraceae* family are ubiquitous metal reducers that utilize conductive ‘nanowires’ to reduce Mn(IV) and Fe(III) oxides in anaerobic sediments. However, it is not currently known if and to what extent the Mn(IV) and Fe(III) oxides in soil grains and low permeability sediments that are sequestered in pore spaces too small for cell passage can be reduced by long-range extracellular electron transport via *Geobacter* nanowires, and what mechanisms control this reduction. We developed a microfluidic reactor that physically separates *Geobacter sulfurreducens* from the Mn(IV) mineral birnessite by a 1.4 μm thick wall containing <200 nm pores. Using optical microscopy and Raman spectroscopy, we show that birnessite can be reduced up to 15 μm away from cell bodies, similar to the reported length of *Geobacter* nanowires. Reduction across the nanoporous wall required reducing conditions, provided by *Escherichia coli*, and an exogenous supply of riboflavin. Our results discount electron shuttling by dissolved flavins, and instead support their role as bound redox cofactors in electron transport from nanowires to metal oxides. We also show that upon addition of a soluble electron shuttle (i.e., AQDS), reduction extends beyond the reported nanowire length up to 40 μm into a layer of birnessite.
3.2 Introduction

Microbially mediated redox transformations of iron and manganese are important biogeochemical reactions that affect carbon turnover\(^1\), contaminant transport\(^2\), trace metal speciation\(^3\), and macronutrient bioavailability\(^4,5\). In anaerobic nonsulfidogenic soils and sediments, Mn(IV) and Fe(III) reduction is driven by dissimilatory metal-reducing bacteria like *Geobacter sulfurreducens*\(^6\). The majority of Mn(IV) and Fe(III) in anaerobic sediment systems consists of insoluble metal oxides. Because bacterial cell envelopes are impermeable to these insoluble oxides, enzymatic reduction requires an extension of the respiratory machinery to the outer membrane\(^7\). Bacteria have evolved several extracellular mechanisms to overcome this physical limitation, including localization of c-type multiheme cytochromes (c-Cyts) to the outer membrane (OM), reduction of soluble electron shuttles, and production of electrically conductive nanowires\(^8-11\). Electron shuttles and nanowires allow for long-range extracellular electron transport (LR-EET) beyond the outer membrane of the cell, but little is known about the extent and mechanisms of LR-EET to Mn(IV) and Fe(III) oxides trapped in pore spaces too small for cell passage.

Soluble electron shuttles are naturally present in groundwater as redox-active quinone moieties of humic substances, contributing to mineral dissolution in organic rich sediments\(^12\). In environments with low concentrations of organic matter, some bacteria may synthesize and export riboflavin (RF) or flavin mononucleotide (FMN) to use as aqueous phase, endogenous electron shuttles that can greatly enhance rates of respiration on insoluble metal oxides\(^13\). However, the efficiency of electron shuttling may be decreased by advection and diffusion of shuttles away from shuttle producing biofilms, or by nonspecific adsorption onto sediments\(^14\).

Nanowires, in contrast, facilitate electron transfer to extracellular electron acceptors by direct contact beyond the outer membrane of the cell\(^15,16\). The nanowires of *G. sulfurreducens*,
which measure 3-5 nm in diameter and 10-20 μm in length\textsuperscript{10,17}, are retractable\textsuperscript{18} like other Type IV pili but also conductive\textsuperscript{10}. Their ability to retract may be particularly advantageous in flow through environments and is speculated to play a role in the removal of reduction products that adhere to their nanowires\textsuperscript{18}. The mechanism of electron transport through \textit{G. sulfurreducens} nanowires has generated controversy\textsuperscript{19}. One hypothesis is that nanowires possess metallic-like conductivity due to π-orbital overlap of closely spaced aromatic amino acids\textsuperscript{17}. This is supported by measurements of charge propagation along pili proteins using electrostatic force microscopy\textsuperscript{20}, nanowire topography and conductivity using scanning tunneling microscopy\textsuperscript{21}, and spacing between pilin-subunits using X-ray diffraction\textsuperscript{22}. An alternative is that conductivity is imparted by multi-step electron hopping between aromatic residues or closely spaced c-Cyts\textsuperscript{23,24}, and this is supported by quantum mechanical calculations\textsuperscript{25}, molecular dynamics simulations\textsuperscript{26}, and charge transport measurements through purified pili\textsuperscript{27}. \textit{G. sulfurreducens} biofilms also exhibit conductivity\textsuperscript{17,22}, which is imparted by cell-associated nanowires and c-Cyts\textsuperscript{28} dispersed within extracellular anchoring polysaccharide (\textit{xap})\textsuperscript{29}. Regardless of the mechanism of electron transport, conductivity in nanowires and/or biofilms is needed for LR-EET to metal oxides\textsuperscript{10,26}.  

The long and narrow geometry of nanowires is thought to facilitate the respiration of metal oxides trapped in or behind pore spaces too small for cell passage (e.g., <500 nm), but this and the spatial extent of respiration could be hindered by the production of insoluble reduction products that block access of nanowires to metal oxides. The significance of such pores, often referred to as nanopores because their size is <1000 nm\textsuperscript{30}, is reflected in their enormous contribution to pore volume and reactive surface area in sediments\textsuperscript{30,31}. For example, 10-40\% of the pore volume and >90\% of the surface area has been measured in pore spaces smaller than 100 nm in B-horizon soils that have high mineral content\textsuperscript{31}. These pore spaces are often filled with or formed from
Mn(IV) and Fe(III) oxides that act as electron sinks for microbial respiration and sorbents of toxic metals and radionuclides\textsuperscript{3,32}. The reductive dissolution of Fe and Mn bearing minerals can mobilize groundwater contaminants like arsenic\textsuperscript{33}.

Despite the intrinsic conductivity of nanowires, there is evidence to suggest that the terminal electron transfer step from \textit{Geobacter} nanowires to metal oxides requires OmcS\textsuperscript{34,35}, an OM c-Cyt that was found in one study to decorate the outer surface of its nanowires\textsuperscript{36}. Furthermore, recent evidence from differential pulse voltammetry implicates c-Cyt bound flavin cofactors as the terminal electron donors\textsuperscript{37,38}. If correct, then riboflavin or FMN bound to c-Cyts on \textit{Geobacter} nanowires, not c-Cyts themselves, would be responsible for electron transfer to Fe(III) and Mn(IV) oxides. However, the importance of flavins in LR-EET through nanowires to metal oxides has not been experimentally tested, and the contribution of pili-associated OmcS to the reduction of iron oxides is not known.

The objectives of this work are to determine if and to what extent \textit{Geobacter} nanowires can reduce metal oxides trapped in pore spaces too small for cell passage, and if a redox cofactor is required for this mode of respiration. We hypothesized that nanowires will be able to penetrate common mineral pore spaces, given their relatively small diameter of 3-5 nm\textsuperscript{17}, and that the buildup of solid phase reduction products can block their access to occluded metal oxides. We also hypothesize that bound metal co-factors are required for metal oxide reduction based on the work of Okamoto et al.\textsuperscript{38}, but it is not clear if these will be endogenously produced in sufficient quantities. To test our hypotheses, we developed a silicon-etched microfluidic reactor that physically separates \textit{G. sulfurreducens} strain KN400 from the Mn(VI) mineral birnessite with a 1.4 µm thick wall containing <200 nm pores. The microfluidic reactor is coated with a 100 nm thick layer of silicon dioxide, an analog of quartz with respect to composition and surface charge\textsuperscript{39}. \textit{G.}
*Geobacter sulfurreducens* was chosen for its reported production of conductive nanowires\(^{10,40}\), and the well-documented association of these nanowires with metal oxide respiration\(^{10,34,40}\). The hyperpiliated wild-type strain KN400 was chosen because of its enhanced ability to attach to smooth surfaces under flow-through conditions as compared to strain PCA from which it was isolated\(^{41}\).

The extent of birnessite reduction across the nanoporous wall without and with an exogenous supply of the redox cofactor riboflavin was monitored at 24 °C by optical microscopy and Raman spectroscopy. Birnessite reduction across the wall would support LR-EET, and reduction extending up to the reported nanowire length (i.e., 15 μm) or greater would support LR-EET by nanowires or soluble electron shuttles, respectively. Birnessite reduction only in the presence of an exogenous supply of riboflavin would support the requirement of this co-factor for LR-EET, and the spatial extent of birnessite reduction would indicate wither the riboflavin is bound to nanowires or freely diffusing in solution. Control experiments were also performed to evaluate alternative LR-EET transfer mechanisms.

### 3.3 Materials and methods

**Materials**

All reagents were of ACS reagent grade or cell culture grade and used without further purification. Solutions were prepared with distilled, deionized water (DDW; 18.2MΩ cm, Millipore Co.) and delivered to the microfluidic reactor in 2.5 mL Hamilton gastight syringes.

**Bacteria growth conditions**

*Geobacter sulfurreducens* KN400 and *Escherichia coli* K-12 were cultured in anoxic,
bicarbonate-buffered freshwater medium at pH 6.9 containing the following per liter of distilled water: 0.5 g NH₄Cl, 0.14 g KH₂PO₄, 0.2 g MgCl₂·6H₂O, 0.15 g CaCl₂·2H₂O, and 2.5 g NaHCO₃. This was supplemented with a non-chelated SL-10 trace elements solution and a selenite plus tungstate solution. Vitamins, chelating agents, and chemical reductants were omitted to minimize the risk of abiotic reduction and dissolution. All cultures in the microfluidic reactor were maintained at the nanowire inducing temperature of 24 °C, except for an experiment set at 31 °C to investigate LR-EET at temperatures reported to inhibit nanowire expression. Batch cultures were grown under similar conditions.

**Preparation for microfluidic reactor inoculation**

Growth medium was dispensed into 125 mL serum bottles, sparged with N₂:CO₂ (80:20) and sealed with butyl rubber stoppers before autoclaving. Sodium sulfide was added to bottles at a concentration of 30 μM to reduce media prior to inoculation. *G. sulfurreducens* was revived from a frozen stock of the same generation and cultured at 24 °C with 8 mM acetate and 3 mM birnessite to verify metal-reducing activity. Cells were then transferred into fresh media with 11 mM acetate and 20 mM fumarate. Once planktonic, cells were infused by syringe into the microfluidic reactor, which was placed inside an environmental chamber set to 24 °C. In one set of experiments, *G. sulfurreducens* was maintained at 31 °C to prevent the formation of nanowires. *E. coli* was revived from frozen stock and cultured at 30 °C in freshwater media supplemented with 5 mM glucose as the sole carbon and energy source. Cells were infused through the microfluidic reactor after being diluted in freshwater medium to 5% of the stock concentration.

All solutions infused through the microfluidic reactor were prepared in the same media with the following modification: The sulfide concentration was decreased from 30 μM to 5 μM.
Sulfide was added to scavenge oxygen remaining in the gastight syringes after filling. Syringes containing the redox indicator resorufin showed that the reducing potential of 5 μM sulfide was maintained for less than 2 hours in the absence of *E. coli*. Since syringes were stored overnight to remove gas bubbles, we did not consider sulfide as an active reductant during infusion. Long-term abiotic controls in the microfluidic reactor showed no reduction of birnessite by sulfide, and experiments with *E. coli* and 5 μM sulfide in the absence of *G. sulfurreducens* were also negative for birnessite reduction. The combination of 5 μM sulfide and 0.5 mM glucose allowed for fast reduction and sustained redox buffering between -130 mV ≤ E_p ≤ -370 mV as evidenced by color changes in syringes containing the redox indicators indigo carmine (E_m^7 = -130 mV) and benzyl viologen (E_m^7 = -370 mV). Reduced conditions in the reactor were confirmed with resorufin (E_m^7 = -110 mV) by exploiting the fluorescent properties of the dye in its oxidized state. Redox indicators were only used in preliminary experiments to investigate the effect of redox potential on birnessite reduction.

**Batch culture experiments**

*G. sulfurreducens* and *E. coli* were grown on birnessite in separate pure cultures and also in co-culture to explore the effect of *E. coli* on birnessite reduction. Pure cultures of *G. sulfurreducens* were grown in 125 mL serum bottles with 3 mM birnessite and 11 mM acetate, while pure cultures of *E. coli* and co-cultures also contained either 0.5 mM or 5 mM glucose. Six replicates were performed for each condition. Additional batch cultures of *G. sulfurreducens* and *E. coli* were used to explore reduction of birnessite at elevated temperature and collect reduced material for analysis by X-ray powder diffraction (XRD). In these experiments, pure cultures and co-cultures were maintained at 31 °C to prevent nanowire formation and explore whether *G.
*sulfurreducens* is capable of reducing birnessite by direct contact at elevated temperature. Reduced solids from six replicates were poured into 50 mL centrifuge tubes, washed and centrifuged 3 times in DI water, dried at room temperature, and ground into a powder by mortar and pestle for XRD analysis. Batch experimental details are listed in Table 3.1.

**Batch adsorption experiments**

Sorption of riboflavin to *Geobacter* was evaluated in batch experiments. Briefly, *Geobacter* was grown in replicate 20 mL bottles of media on 5 mM fumarate and 10 mM acetate until biofilms began to form (5-7 days). We then added 5 mM birnessite to the bottles, and upon initial reduction (within 2 days, as indicated by the presence of white rhodochrosite precipitates), aqueous samples were taken and analyzed for background riboflavin. Immediately thereafter, the bottles were spiked with the equivalent of 300 nM riboflavin, mixed for 20 minutes, and then aqueous samples were again taken and analyzed. Control bottles with only media and birnessite were also prepared, spiked with 300 nM riboflavin, shook for 20 minutes, and then analyzed. The background riboflavin concentration in the bottles with *Geobacter* was 93.5 ± 1.2 nM, but in the controls, it was not detected. After spiking with 300 nM riboflavin, the riboflavin concentration was 341 ± 0.6 nM in bottles with *Geobacter*, and 287 ± 0.6 nM in the controls. The decrease in the bottles with *Geobacter* from 380.5 nM (i.e., 287+93.5) to 341 ± 0.6 nm indicates sorption occurred, and the larger decrease for the bottles with *Geobacter* compared to the bottles with only birnessite indicate sorption was to cell material. To further probe the sorption mechanism, the bottles with *Geobacter* were incubated an additional day, and then samples were taken for riboflavin analysis. Immediately thereafter, the bottles were spiked with 1 U/g of a serine protease (i.e., Savinase) and sampled for analysis. Serine protease digests outer membrane cytochromes...
without killing or lysing the cells; this is expected to release bound riboflavin due to conformational changes induced in the cytochromes. We measured 226 ± 2 nM in the bottles before Savinase addition, and 355 ± 4 nM after Savinase addition, indicating that riboflavin was sorbed to *Geobacter* cytochromes. The Savinase had no effect on riboflavin concentration in the control bottles with only media and birnessite.

**Birnessite synthesis**

The Mn(IV) mineral birnessite \[((\text{Na, K})_{0.6}(\text{Mn}^{4+}, \text{Mn}^{3+})_2\text{O}_4 \cdot 1.5\text{H}_2\text{O})\] was synthesized by oxidizing manganese chloride in a basic solution of potassium permanganate according to the protocol of Villalobos et al. The product was stored as a suspension in 0.01 mol/L NaCl at pH 8 and is reported to be structurally stable under these conditions. Fresh batches of birnessite were prepared every 3 months and analyzed by Raman spectroscopy before each experiment to verify the purity and stability of the mineral. TEM of freshly precipitated birnessite showed that individual crystals were <100 nm in length (Fig. 3.1), similar to the range reported by Villalobos et al. While this is smaller than the size of the gaps in our wall, high pressure fixation of birnessite on the nanoporous wall by aggregation and straining of solids at the pillars results in a stable layer of material that does not migrate once deposited.

**Microfluidic reactor fabrication**

Microfluidic reactors were etched in silicon using a combination of photolithography and electron beam lithography (EBL). A schematic illustrating the major steps of fabrication is shown in Figure 3.2. ZEP520A electron beam resist was spin coated on a silicon wafer, and a digitized image of a nanoporous wall was transferred into the resist using a JEOL-6000 FSE EBL system.
(JEOL Ltd., Japan). Microfluidic channels were etched to a depth of 10 µm by inductively coupled plasma - deep reactive ion etching (ICP-DRIE) on a Plasma-Therm Versaline DSE (Plasma-Therm LLC, USA) with SF$_6$ and C$_4$F$_8$. The nanoporous wall was 1 cm long and consisted of an alternating series of pillars and gaps. After etching, the pillars measured 1.0 x 1.0 µm in area and gaps were 0.6 µm wide. Nanopores were closed by depositing a uniform layer of polysilicon on the wafer by low pressure chemical vapor deposition (LPCVD), resulting in pillars that were 1.4 x 1.4 µm in area while the gaps (i.e. nanopores) were ≤ 0.2 µm wide. Two inflow and two outflow ports approximately 1 mm in diameter were etched by ICP-OES. The reactors were encapsulated by anodically bonding the silicon wafers to Pyrex 7740 glass wafers at 900 V and 400 °C. Once bonded, PEEK NanoPort assemblies (IDEX Corporation, Illinois, USA) were glued over the drilled ports and connected to 0.01” ID PEEK tubing. PEEK and ETFE fittings connected the tubing to gastight syringes that were driven by a NE-4002X microfluidic syringe pump (New Era Pump Systems Inc., New York, USA).

**Microfluidic reactor experiments**

All solutions were continuously delivered to the two reactor influent channels from gastight syringes using a microfluidic syringe pump. Microfluidic reactors, tubing, and syringe pumps were stored together in an environmental chamber maintained at 24 °C ± 1 °C, except for the experiment that was maintained at 31 °C ± 1 °C. Reactors were disinfected for 12 hours with 1 M acetic acid and flushed with 10 pore volumes of sterile DI water. Next, birnessite was infused through one of the flow channels. The effluent port in this channel was closed to induce cross flow so that solid particles would be pushed towards the wall, strained at the pillars, and concentrated into a packed and immobile layer. The resulting layer of birnessite had a thickness of 40-60 µm. After fixation,
both outlet ports were opened, *G. sulfurreducens* KN400 was infused with acetate in media through the top channel, and 1 mM sodium fumarate in media was infused through the bottom channel containing birnessite. In this initial stage, acetate served as the electron donor, and fumarate as the electron acceptor (which diffused across the nanoporous wall and mixed with acetate); birnessite did not react in the presence of added fumarate. The addition of fumarate was temporary and was used because it promoted cell attachment at the nanoporous wall. Once cells were observed at the wall, both channels were infused with fresh media amended in different ways to start an experiment. The different amendments tested in the microfluidic reactor are 1) 10 mM acetate only, 2) 10 mM acetate, 0.5 mM glucose and a 5% transfer of *E. coli* K-12, 3) Amendment 2 + 100 nM riboflavin, and 4) Amendment 2 + 100 nM anthraquinone-2,6-disulfonate (AQDS). Triplicate experiments were performed with each condition, with the exception of Amendment 4 that was performed in duplicate. Microfluidic control experiments with Amendments 1-4 were performed without *G. sulfurreducens* to test for abiotic reduction of birnessite by media constituents, and for *E. coli* mediated reduction by electron shuttling. Abiotic reduction by hydrogen (expected during glucose fermentation) and reduced cellular lysate was tested with Amendments 1 and 2, respectively.

**Cleaning and sterilization**

Biofilm, birnessite, and rhodochrosite in microfluidic reactors left over from previous experiments were removed by successive infusions of 0.3 M HNO3, 20% (v/v) serine protease ≥ 16 U/g, 1% (w/v) sodium dodecyl sulfate, and 75% isopropyl alcohol. NanoPorts were detached and reactors were briefly soaked in boiling Piranha solution containing 2:1 (v/v) concentrated sulfuric acid and 30% H2O2 to remove glue from the surface. Reactors were then sterilized by
autoclaving and reassembled in a biological safety cabinet. These steps were also performed on newly fabricated reactors to ensure similar surface chemistry between experiments. Solutions prepared for infusion were kept sterile by autoclaving gastight syringes before filling, autoclaving chemical stocks with the exception of riboflavin and AQDS, and performing all critical work in a biological safety cabinet. Riboflavin and AQDS were sterilized by filtration through sterile 0.22 μm syringe filters.

**Raman spectroscopy**

Mineralogical analyses were performed in-situ on a Horiba LabRAM HR Evolution confocal Raman system (HORIBA Scientific, Kyoto, Japan). Raman spectra were taken between 0 and 1200 cm⁻¹ using a 532 nm DPSS laser in a backscattering configuration. The laser was calibrated daily using silicon as a reference material, and reference spectra from the RUFF mineral database were used to identify minerals inside the reactor. Birnessite spectra were obtained at 5 mW and averaged over 3 successive scans with an acquisition time of 60 seconds. Rhodochrosite spectra were obtained at 2.5 mW and averaged over 15 successive scans with an acquisition time of 10 seconds.

**Light microscopy**

Brightfield and fluorescence images were taken on a Nikon Eclipse TI-E (Nikon, Kobe, Japan) inverted microscope integrated with the Raman system. Autofluorescence of cytochrome C was detected by exciting *G. sulfurreducens* cells within a range of 340-380 nm and filtering emission between 435-485 nm through a 400 nm dichroic mirror. The dead cell stain propidium iodide and the redox dye resorufin were excited between 540-580 nm, and fluorescence was
detected through a 595 nm dichroic mirror and 600-660 nm emission filter. The live cell stain Syto-9 was excited between 457-487 nm, and fluorescence was detected through a 495 nm dichroic mirror and 502-538 nm emission filter. Fluorescent images were taken with an Andor Zyla 5.5 camera and color images with a Lumenera Infinity 3-1UR camera.

HPLC analyses

Effluent was analyzed every 24 hours by reversed-phase high performance liquid chromatography (HPLC) for riboflavin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) using a Shimadzu LC-2040C HPLC, and Shimadzu C18 reversed-phase column (50 mm x 2.1 mm with 1.9 μm particle size). Separation was achieved at 45 °C with an isocratic concentration of 30% methanol versus an aqueous solution of acetic acid at pH 4.7, for 3.5 minutes, at a flow rate of 0.2 mL/min. A Shimadzu RF-20 fluorescence detector was used with an excitation wavelength of 450 nm and an excitation wavelength of 520 nm, and peak area was calculated using LabSolutions Lite LC/GC software, Ver. 5.82.

XRD analyses

XRD patterns were collected at room temperature in transmission mode on a Rigaku R-Axis Spider diffractometer (Rigaku, Tokyo, Japan) with an image plate detector using a graphite monochromator with CuKα radiation (λ = 1.5418Å). The R-Axis Spider was operated using Rigaku’s RINT Rapid Version 2.3.8 diffractometer control program. The powder sample was mounted on a Hampton Research CryoLoop. The 2D image plate data was converted to a conventional 1D powder pattern using Rigaku’s 2DP Version 1.0 data conversion program. The data were analyzed using Bruker Analytical’s DiffracPLUS, EVA (V. 2009) software.
TEM analyses

Bright field transmission electron microscope (TEM) images were taken on a JEOL 2010f operated at 200kV with a Gatan OneView camera.

3.4 Results and discussion

Microfluidic reactor platform with nanoporous wall

A photograph of the microfluidic flow cell is shown in Figure 3.3a. The two 250 μm wide channels are apparent from the bifurcated inlet and outlet. The channels come together in the center and are separated by a nanoporous wall as shown in Figure 3.3b. A close-up SEM image of the wall before anodic bonding is shown in Figure 3.3c. The nanoporous wall contains thousands of uniform rectangular columns spaced <200 nm apart. A conceptual illustration of the experimental setup with the nanoporous wall is shown in Figure 3.3d. The G. sulfurreducens cells are confined to one channel; at ~500 nm wide they are too large to pass through the nanopores\textsuperscript{10}. Birnessite is the electron acceptor, and is located on the opposite side, and also within the nanopores, while acetate serves as the electron donor and is continuously delivered through both channels. In order for respiration to occur, the cells are forced to access and reduce birnessite through the nanopores once the limited quantity of birnessite in the nanopores is reduced. Porous beads filled with iron oxides have previously been used to investigate electron shuttling\textsuperscript{47,48}, but this method is not practical for the study of nanowires because the extent of reduction inside pores due to nanowire penetration cannot be measured in real-time, and in-situ analyses are limited to measurements of aqueous metal concentration. Our microfluidic platform, with its 2D geometry, allows us to
observe reduction in real-time, analyze mineral redox state, and measure the spatial extent of reduction beyond the cell barrier.

**Birnessite reduction by direct contact required the removal of trace oxygen**

We initially grew *G. sulfurreducens* in direct contact with birnessite inside one channel of our microfluidic reactor as a positive control. As previously noted, acetate was the electron donor, and birnessite was fixed at the wall and served as the electron acceptor. *G. sulfurreducens* was infused through the same channel as the birnessite layer. Surprisingly, we did not observe reduction of birnessite after 4 weeks of continuous infusion of acetate, even though the free energy of reaction for acetate oxidation coupled to birnessite reduction under reactor conditions is energetically favorable (i.e., -607 kJ/mol)\(^{49}\), and we observed complete birnessite reduction by *G. sulfurreducens* under the same conditions (i.e., same media with *G. sulfurreducens* and birnessite) in static batch experiments within 8-10 days (Table 3.1).

Suspecting that the diffusion of trace oxygen through our influent tubing was inhibiting respiration, we attempted to detect its presence using the fluorescent redox indicating dye resorufin. The reversible reduction of resorufin to the colorless dihydroresorufin requires the elimination of oxygen in addition to a negative redox potential. At pH 7, this occurs at approximately -110 mV (vs the standard hydrogen electrode) at a typical resorufin concentration of 4 μM\(^{50}\). While the infusing syringes remained colorless, media inside the reactor was fluorescent, indicating the presence of oxygen. We suspect that the presence of oxygen was responsible for the lack of birnessite reduction, since the leaking of trace oxygen inside *Geobacter* fuel cells has been reported to decrease current production\(^{51}\). The addition of *E. coli* to these fuel cells resulted in increased current. Similarly, Straub et al. amended batch experiments set up for ferrihydrite reduction by *G.
sulfurreducens with E. coli K-12 to lower the redox potential\textsuperscript{52}. They observed ferrihydrite reduction after 8-10 weeks of incubation in anoxic, non-reduced media with only G. sulfurreducens, but this was shortened to 1-2 weeks with amended E. coli.

We repeated the microfluidic experiment but added E. coli K-12 after a biofilm of G. sulfurreducens was established. A timeline of solutions added to the reactor is shown in Table 3.2. Initial tests with resorufin showed no fluorescence, which indicated that E. coli was able to create an anoxic and reduced environment within the reactor. The difference in fluorescence between reduced and non-reduced conditions is clearly visible as shown in Figure 3.4. Under these new conditions, we observed reduction after a lag period of 4 days post E. coli addition (Fig. 3.5), and after 9 days (post E. coli addition) the 100 µm layer of birnessite was fully reduced. In a control experiment set up the same way but without G. sulfurreducens, no reduction of birnessite by E. coli was observed in the microfluidic reactor. These results were also reflected in batch culture experiments, where birnessite was reduced by G. sulfurreducens after 8-10 days in pure culture and 5-6 days in co-culture with E. coli (Table 3.1). The lag time was shorter in the latter case, which may be a consequence of the faster onset of reducing conditions. No visible reduction of birnessite was observed by E. coli in the absence of G. sulfurreducens (Table 3.1).

**LR-EET across the nanoporous wall by nanowire expressing G. sulfurreducens required riboflavin**

After demonstrating birnessite reduction by direct contact, we investigated LR-EET to birnessite across the nanoporous wall. As previously noted, birnessite was fixed against the wall in one channel, and G. sulfurreducens was infused through the opposite channel and allowed to form a biofilm on the opposite side of the same wall (i.e., Fig. 3.3d). Once a biofilm was established,
both channels were infused with Amendment 3 consisting of 10 mM acetate, 0.5 mM glucose, and *E. coli* (Table 3.2). The infusion of identical solutions through both channels eliminated gradients in chemical concentration and redox potential. A small amount of birnessite (<10%) passed through the nanopores during fixation and redeposited as a thin (3-5 µm) layer on the wall in direct contact with *G. sulfurreducens* (Fig. 3.6), where the percentage is based on projected areas of fixed birnessite from imaging. This 3-5 µm layer was reduced 2 days after *E. coli* infusion, and only birnessite across the wall remained to sustain growth. However, no further reduction was observed after 3 weeks of continuous operation. The reduction of birnessite within the nanopores but not beyond the wall indicates that the extent of birnessite reduction is not connected to the production of aqueous Mn(II) (which could serve as a reducing agent) or conductivity of the birnessite. That the latter mechanism is not active is expected, given that the conductivity of birnessite (10^-5-10^-6 S/m) is many orders of magnitude lower than that of silicon.

A recently proposed bound cofactor model of electron transport in *Geobacter* and *Shewanella* spp. suggests that self-secreted or exogenously supplied flavins bound to OM c-Cyts, including riboflavin and FMN, may enable rapid and efficient metal reduction under environmental conditions. Therefore, we repeated the experiment above under the same conditions, except that we also added 100 nM riboflavin to the infusing syringes with acetate, glucose, and *E. coli*. The binding of flavins to OM c-Cyts has been indicated by current production of anode respiring *Geobacter* cells during media replacement experiments, and by differential pulse voltammetry. Also, we measured direct sorption of riboflavin to *G. sulfurreducens* cells in batch experiments, and riboflavin desorption from these cells using a serine protease; the latter suggests membrane cytochromes were involved in binding. Our microfluidic experiments, however, are the first to explore the role of flavins associated with OM c-Cyts on the long-range
reduction of metal oxides, and this is possible because of the physical separation created between *G. sulfurreducens* and birnessite.

With the addition of 100 nM riboflavin, we observed birnessite reduction through the nanopores within 5 days (Fig. 3.6a,b), and reduction of birnessite to rhodochrosite (MnCO$_3$) was confirmed in-situ using backscattering Raman spectroscopy (Fig. 3.6c,d). We note that production of rhodochrosite in bicarbonate buffered media was reported previously$^{55}$ and identified in our own batch cultures using X-ray powder diffraction (XRD) (Fig. 3.7). Birnessite reduction occurred up to 15 µm across the wall, but no further, indicating a spatial limit to LR-EET. This observation was consistent throughout the reactor and replicated three times. The spatial extent of reduction matches reports of nanowire length$^{17}$, and is evidence that nanowires fully extend into nanopores to maximize access to metal oxides. While the length and conductivity of nanowires has been measured by TEM and conductive AFM$^{10,17}$, this is the first evidence to our knowledge that nanowires can reduce metals up to their length and into spaces too small for cell passage. Previous work has attributed metal reduction to the presence of nanowires, but there are no quantitative measurements of the length scale over which metal oxides can be reduced by nanowires. We note that no reduction of birnessite was observed in microfluidic or batch culture controls with *E. coli* and 100 nM riboflavin in the absence of *G. sulfurreducens*.

The requirement of an exogenous supply of flavins to support LR-EET was surprising because *G. sulfurreducens* contains the nrdR-ribDE2BAH-nusB flavin synthesis region and the SO_0702 gene that encodes for the flavin export protein$^{37}$. In batch cultures incubated for several days with *Geobacter*, fumarate, and birnessite, we detected riboflavin (93.5 ± 1.2 nM), but no FMN, by HPLC. In the effluent of our microfluidic reactor, riboflavin was below the limit of detection (c.a. 800 pM) in the absence of *E. coli* or exogenous riboflavin, suggesting that either
the rate of riboflavin production by *Geobacter* is slow relative to the rate that these solutes leave the microfluidic reactor by advection, or that separation of *Geobacter* cells from the birnessite by the nanoporous wall suppresses riboflavin production. In microfluidic experiments with *E. coli* in the absence of exogenous riboflavin, riboflavin and FMN were detected at concentrations below 10 nM (typically 1-5 nM). Since *E. coli* K-12 lacks flavin transporters\(^5^6\), these were likely released by cell lysis. Concentrations of endogenously produced riboflavin in the nanoporous wall were estimated from the measured effluent concentration (1-5 nM) and are similar to this measured value. Hence, it appears that flavins are not secreted at sufficient rates by *G. sulfurreducens* or *E. coli* to promote birnessite reduction across the nanoporous wall under our flow conditions.

We explored metabolic activity of *G. sulfurreducens* in the microfluidic reactor using the fluorescent dye RedoxSensor Green, which is an indicator of bacterial reductase activity\(^5^7\) and a proxy for respiratory activity in *G. sulfurreducens*\(^4^0\). Metabolic activity during LR-EET was uniform throughout the 10-20 μm thick biofilm (Fig. 3.8), suggesting that cells far away from the nanoporous wall contributed to current production. This is consistent with reports of conductivity in thick *Geobacter* biofilms\(^5^8\).

**Birnessite reduction patterns and control experiments support reduction via nanowires**

Time-lapse images taken after the onset of birnessite respiration across the nanoporous wall show reduction until 7 days, followed by an abrupt stop once a uniform reduction zone distance of 15 μm was reached along the entire length of the 1 cm long wall (Fig. 3.9). This pattern is inconsistent with mineral dissolution or cell passage through nanopores. Dissolution would affect both sides of the mineral layer due to infusion of identical solutions through both channels, but the outer edge of birnessite in the channel without *G. sulfurreducens* remained unchanged. The
spatial distribution of *G. sulfurreducens* was periodically monitored by fluorescence microscopy to determine if cells had penetrated the nanopores. Individual cells could be resolved through birnessite and rhodochrosite by exploiting the autofluorescence of reduced OM c-Cyts\(^59\), but none were observed across the wall. Post-experimental Live/Dead staining was also performed after fixing cells in 2.5% glutaraldehyde\(^60\) and dissolving manganese solids with a mixture of 4 mM ethylenediaminetetraacetic acid (EDTA) and oxalic acid at pH 4. Fluorescence microscopy confirmed that cells were only present in one channel of the reactor (Fig. 3.10). In preliminary reactor designs with wider nanopores, cells frequently crossed the nanoporous wall. In such cases, the reduction front was equidistant at the point of cell penetration, cells were visible by fluorescence microscopy, and the entire birnessite layer was quickly reduced.

Additional control experiments were performed to rule out birnessite reduction by either hydrogen or reduced cellular lysate. *E. coli* can produce H\(_2\) during glucose fermentation, which has the potential to abiotically reduce fixed birnessite. Lysed cells may also release cytochromes into solution, which could drive birnessite reduction. In both microfluidic reactor control experiments, no birnessite reduction was observed after 3 weeks. In batch culture experiments, however, sterile lysate reduced birnessite over the course of several weeks (Table 3.1).

**Greater spatial limit to LR-EET observed when soluble electron shuttles are provided**

Media replacement experiments have revealed differences in the way that flavins are utilized by different microbial species. For example, low current densities in microbial fuel cells (MFCs) were produced by *Shewanella oneidensis* when the MFC was flushed with minimal salts buffer, indicating that flavins were used as soluble mediators\(^61\). In contrast, MFCs inoculated with *G. sulfurreducens* showed no change in current density upon medium replacement, indicating that
flavins were being utilized as bound redox mediators. To determine the role of soluble electron shuttles on the reduction of birnessite, we repeated the microfluidic experiment in duplicate with 100 nM of the humic acid analog anthraquinone-2,6-disulfonate (AQDS) in place of 100 nM riboflavin. AQDS mediates electron transfer through a well-known shuttling mechanism and was shown to significantly enhance rates of Fe(III) and Mn(IV) reduction by *G. sulfurreducens* and a variety of other metal reducing bacteria in batch experiments. AQDS was added to the microfluidic reactor with acetate, glucose, and *E. coli*, after a biofilm of *G. sulfurreducens* was established. Within 8 days, birnessite reduction across the nanoporous wall was observed. In contrast to the experiment with riboflavin, the birnessite reduction front with AQDS present did not stop at 15 μm but proceeded across the entire 40 μm thickness of the fixed birnessite layer (Fig. 3.9). This result is consistent with soluble electron shuttling rather than electron transfer via nanowires and suggests that birnessite reduction in the presence of riboflavin instead of AQDS is not due to electron shuttling. Batch culture experiments with *E. coli* and AQDS in the absence of *G. sulfurreducens* showed no birnessite reduction, discounting electron shuttling by *E. coli*; this is consistent with previous work showing that *E. coli* is unable to reduce or respire AQDS. We calculated that the time scale for diffusion of AQDS and riboflavin in our 250 μm wide channels is on the order of 60 seconds, suggesting that mass transfer is not limiting the observed birnessite reduction that occurs on the order of days.

**LR-EET across the nanoporous wall did not occur at higher temperatures**

We explored the effect of elevated temperature on LR-EET by repeating the microfluidic experiment with Amendment 3 at 31 °C. Pili expression is thermoregulated in many bacteria, and there is evidence using planktonic cells that *G. sulfurreducens* do not produce nanowires above
Our results are shown in Figure 3.11; at 31 °C, the thin layer of birnessite that deposited on the *Geobacter* side of the wall after fixation was rapidly reduced, indicating that cells were active inside the reactor, but birnessite across the nanoporous wall remained unchanged. In batch experiments, reduction of birnessite by pure cultures of *G. sulfurreducens* was accelerated at the higher temperature, and more so with the addition of riboflavin or AQDS (Table 3.1), indicating that these redox cofactors are still used at higher temperatures. These results support the absence of nanowire production in biofilms of *G. sulfurreducens* at 31 °C in our microfluidic reactor, and are consistent with nanowire mediated reduction of birnessite across the nanoporous wall at 24 °C.

We note, however, that recent experiments with wild type (WT) and a pilB mutant of *G. sulfurreducens* show greater electroactivity in the former, when each is grown as biofilm on a graphite electrode at 30 °C. The mutant lacks the pilB pilus assembly motor, and the authors reasonably assert that WT *G. sulfurreducens* produces nanowires at 30 °C which promote greater electroactivity. These results appear at odds with ours, and possible reasons (e.g., physical separation of cells from the metal oxide electron acceptor versus direct contact with an anode) require further study.

**Estimation of riboflavin concentration in the nanoporous wall**

In the absence of added riboflavin (i.e., only endogenously produced riboflavin), the concentration of riboflavin in the effluent of the microfluidic reactor could be lower than in the nanopores because of dilution from the influent flow, assuming that riboflavin production is occurring mainly in the *Geobacter* biofilm and not by planktonic *E. coli* cells. However, since the time scale of diffusion across a channel \( t = L^2/D = (0.0250 \text{ cm})^2/(4 \times 10^{-6} \text{ cm}^2/\text{sec}) = 156 \text{ sec} \) is on the order of the fluid retention time in the reactor \( t = 60 \text{ sec} \), we assumed these concentrations
would be quite close. To more rigorously evaluate this assumption, we identified a steady state two-dimensional analytical solution provided by Seagren et al. (1994), which assumes advection of clean fluid over a surface which is at a constant concentration, and the following governing equation and boundary conditions:

\[
\frac{\partial C}{\partial t} = \frac{\nu_x}{\partial x} = D_z \frac{\partial}{\partial z} \left( \frac{\partial C}{\partial z} \right) \tag{1}
\]

\[
C(x,z = \infty) = 0 \quad (semi - infinite\ domain) \tag{2}
\]

\[
C(x,z = 0) = C_s \quad for \ 0 \leq x \leq L_x \tag{3}
\]

\[
C(x = 0,z) = 0 \tag{4}
\]

where \( \nu_x \) (0.017 cm/sec) is the velocity of water flowing parallel to the biofilm surface, \( C \) is the riboflavin concentration at any location at or above the biofilm surface along the entire length of the nanoporous wall, \( x \) is the longitudinal distance along the wall, \( L_x \) (1 cm) is the length of the wall, \( z \) is the distance away from the biofilm surface into the flow channel, and \( D_z \) (4x10^{-6} cm^2/sec) is the riboflavin diffusion coefficient. The analytical solution to the above governing equation and boundary conditions is:

\[
F_{avg} \left\lceil \frac{M}{t} \right\rceil = 2 \nu_x C_s A \frac{D_z}{\nu_x L_x \pi} \tag{5}
\]

where \( F_{avg} \) represents the mass averaged flux from biofilm at the biofilm-water interface, which is equal to the flux of riboflavin in the effluent from one channel of the reactor, i.e., \( F_{avg} = F_{effluent} \), and \( A \) (0.001 cm x 1 cm) is the biofilm area at the biofilm-water interface. The value of \( F_{effluent} \) can be calculated from the water flow rate in a channel, \( Q \), and the measured riboflavin effluent concentration, \( C_{effluent} \), as follows:
\[ F_{\text{avg}} = F_{\text{effluent}} = QC_{\text{effluent}} = 1.25 \times 10^{-7} \text{ nmol/min} \] (6)

Solving for \( C_s \) in Equation 5, we obtain 7 nM. This is only slightly greater than the measured value of 5 nmol/L measured in the reactor effluent. Therefore, the concentration of riboflavin in the nanoporous wall is likely only slightly more than 5 nM, and much less than the 100 nM added to the reactor to stimulate birnessite reduction across the wall.

**LR-EET implications**

Our experiments are the first to probe nanowire mediated metal oxide reduction through nanopores and quantify the distance over which reduction can occur (i.e. 15 \( \mu \)m) when cells are physically separated from an electron acceptor. A summary of results is presented in Table 3.3, illustrating the conditions required to reduce birnessite at different distances from the cell. We were concerned about solid phase reduction products (i.e. rhodochrosite) redepositing on the birnessite surface and preventing LR-EET through the nanopores, but this was not observed in our reactors. However, at higher bicarbonate concentrations or high pH values, this could potentially limit reduction.

Birnessite reduction limited to 15 \( \mu \)m across the nanoporous wall in our microfluidic reactor required an exogenous supply of riboflavin, consistent with the requirement of nanowire associated redox cofactors (i.e. riboflavin) for LR-EET in *G. sulfurreducens*. In natural systems, riboflavin and FMN may be supplied in sufficient quantities by other microorganisms, particularly in multispecies biofilms. We note that we were unable to directly observe nanowires in our reactor. Also, we also did not observe biofilm across the nanoporous wall, but this could have gone undetected and an *xap* matrix rich in redox active proteins (e.g., c-Cyts) could have promoted birnessite reduction. These data gaps motivate the need for further study.
Our experiments suggest that *G. sulfurreducens* can reduce metal oxides through nanopores using soluble electron shuttles (i.e. AQDS), and that reduction by this mechanism extends beyond the reported length of their nanowires. In fact, we observed reduction of the entire 40 μm thick layer of deposited birnessite. Mathematical models of electron flux through biofilms of anode-respiring bacteria have suggested that electron shuttling is effective at distances >10 μm only if shuttle concentrations are in the mM range, several orders of magnitude higher than our influent concentration of AQDS (i.e., 100 nM). Hence, these models need to be revisited.

While our experiments used birnessite as a representative metal oxide, electron transfer via nanowires is likely relevant to a variety of compounds sequestered in nanopores, including iron oxides, soil organic matter, organic contaminants, and radionuclides. The release of metal contaminants upon reductive dissolution of manganese and iron oxides is particularly important for groundwater remediation, and future experiments are needed to explore how this may affect bioremediation efforts. The mechanism of electron transport through nanowires, the integration of intracellular and extracellular electron transport components, and the regulatory networks associated with metal reduction also remain areas of controversy and future research.
3.5 Figures and tables

Figure 3.1. TEM image of dried birnessite particles with individual crystals < 100 nm.
Figure 3.2. Process diagram for reactor fabrication. (A) spincoating of ZEP520A e-beam resist (B) E-beam patterning of wall and channels (C) CF$_4$ plasma etch of exposed oxide (D) silicon deep etch to 10 microns (E) stripping of remaining oxide by BOE (F) LPCVD of polysilicon to close the gap between pillars to <200 nm (G) ultrasonic drilling of ports and (H) anodic bonding of silicon wafer to glass.
Figure 3.3. Photograph of microfluidic flow cell (A), SEM image of wall separating the channels (B), SEM image of 1.4 x 1.4 μm wide pillars and 200 nm gaps (C) and illustration of cells separated from birnessite by the wall with extended nanowires and bound riboflavin (D).
Figure 3.4. Fluorescent image of reactor taken during infusion of resorufin with or without E. coli, with arrows indicating flow direction and dashed lines showing the boundary of the channel opposite the nanoporous wall.
Figure 3.5. Influence of *E. coli* on birnessite reduction by direct cell contact. No reduction is observed with *G. sulfurreducens* in pure culture (A). Birnessite is fully reduced after 9 days with the addition of *E. coli* in co-culture (B).
Figure 3.6. Brightfield image of birnessite (MnO$_2$) before reduction (A) and rhodochrosite (MnCO$_3$) after 5 days of biological reduction (B). Raman spectra of (C) birnessite and (D) rhodochrosite match those in the RUFF mineral database for each mineral. Real-time acquisition of Raman spectra confirms the formation of rhodochrosite from birnessite inside the reactor.
Figure 3.7. X-ray powder diffraction pattern of precipitates collected from several batch experiments following birnessite reduction. Reference data for rhodochrosite are shown in red.
Figure 3.8. Metabolic staining of biofilm with RedoxSensor Green during LR-EET of birnessite.
Figure 3.9. Time lapse images of birnessite reduction observed upstream (a), center (b), and (c) downstream in the reactor.
Figure 3.10. Dead cell staining with propidium iodide after inactivation by glutaraldehyde. Note the absence of cells below the nanoporous wall at the centerline.
Figure 3.11. Progression of birnessite reduction by *G. sulfurreducens* KN400 grown at 31 °C ± 1 °C, showing reduction of birnessite by direct contact, but not by LR-EET.
Table 3.1. Summary of conditions and results for batch culture experiments.

<table>
<thead>
<tr>
<th>Observation</th>
<th>G. sulfurreducens</th>
<th>E. coli</th>
<th>Temp. (°C)</th>
<th>RF / AQDS†</th>
<th>Days required to reduce Mn</th>
</tr>
</thead>
<tbody>
<tr>
<td>No reduction by media components*</td>
<td>×</td>
<td>×</td>
<td>24, 31</td>
<td>X,✓</td>
<td>×</td>
</tr>
<tr>
<td>No reduction by E. coli*</td>
<td>×</td>
<td>✓</td>
<td>24, 31</td>
<td>X,✓</td>
<td>✓</td>
</tr>
<tr>
<td>Reduction by G. sulfurreducens</td>
<td>✓</td>
<td>×</td>
<td>24</td>
<td>X,✓</td>
<td>8-10</td>
</tr>
<tr>
<td>Reduction stimulated by E. coli</td>
<td>✓</td>
<td>✓</td>
<td>24</td>
<td>X,✓</td>
<td>5-6</td>
</tr>
<tr>
<td>Activity increased at 31 °C</td>
<td>✓</td>
<td>×</td>
<td>31</td>
<td>X,✓</td>
<td>6-8</td>
</tr>
<tr>
<td>Reduction by concentrated lysate</td>
<td>×</td>
<td>✓</td>
<td>24</td>
<td>X</td>
<td>21-30</td>
</tr>
</tbody>
</table>

* Control experiments, × not present or observed, ✓ present or observed
† Experiments performed with riboflavin or AQDS, or neither
Table 3.2. Experimental set-up and flow conditions.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Inlet</th>
<th>Component</th>
<th>Flow Rate (nL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Top + Bottom</td>
<td>Sterilize flow channels</td>
<td>250</td>
</tr>
<tr>
<td>2</td>
<td>Top + Bottom</td>
<td>Purge flow channels with media</td>
<td>250</td>
</tr>
<tr>
<td>3</td>
<td>Bottom</td>
<td>Immobilize birnessite in bottom flow channel</td>
<td>Manual</td>
</tr>
<tr>
<td>4</td>
<td>Top</td>
<td>Inoculate G. sulfurreducens in top flow channel</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>Bottom</td>
<td>Infuse 1 mM fumarate in bottom flow channel</td>
<td>125</td>
</tr>
<tr>
<td>5</td>
<td>Top + Bottom</td>
<td>Infuse <em>E. coli</em> + 0.5 mM glucose + 10 mM acetate +</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>riboflavin / AQDS</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.3. Summary of conditions and results for microfluidic experiments

<table>
<thead>
<tr>
<th>Observation</th>
<th>G. sulfurreducens</th>
<th>E. coli</th>
<th>Temp. (°C)</th>
<th>RF</th>
<th>AQDS</th>
<th>EET (≤ 15 μm)</th>
<th>LR-EET (&gt; 15 μm)</th>
<th>LR-EET &gt; 31 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>No reduction by media</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>X</td>
<td>24, 31</td>
<td>X, ✓</td>
<td>X, ✓</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>No reduction by E. coli</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>✓</td>
<td>24, 31</td>
<td>X, ✓</td>
<td>X, ✓</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>No reduction by H₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>X</td>
<td>24</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>No reduction by lysate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>✓</td>
<td>24</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>EET requires low potential</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>✓</td>
<td>X</td>
<td>24</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Only EET w/o flavins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>✓</td>
<td>✓</td>
<td>24</td>
<td>X</td>
<td>✓</td>
<td>✓</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Only EET at 31 °C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>✓</td>
<td>✓</td>
<td>31</td>
<td>✓</td>
<td>X</td>
<td>✓</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>LR-EET w/ added flavins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>✓</td>
<td>✓</td>
<td>24</td>
<td>✓</td>
<td>X</td>
<td>✓</td>
<td>✓</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>AQDS extends reduction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>✓</td>
<td>✓</td>
<td>24</td>
<td>X</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
</tbody>
</table>

* Control experiments, X not present or observed, ✓ present or observed
3.6 References


CHAPTER 4: FLAVIN DIFFUSION AND RECYCLING BY SHEWANELLA

ONEIDENSIS MR-1 CAN DRIVE METAL REDUCTION ACROSS A PHYSICAL SEPARATION

Will Submit to Proceedings of the National Academy of Sciences, 2018

Kyle Michelson, Reinaldo Alcalde, Robert Sanford, Albert Valocchi, Charles Werth, Flavin diffusion and recycling by *Shewanella oneidensis* MR-1 can drive metal reduction across a physical separation

4.1 Abstract

Electron shuttling of soluble redox mediators is an adaptation evolved by some species of dissimilatory metal-reducing bacteria (DMRB) to obtain energy from the reduction of insoluble metal oxides that predominate in anaerobic groundwater environments. The diffusion of electron shuttles into pore spaces that are smaller than the diameter of a bacterial cell is believed to give DMRB access to sequestered metal oxides, particularly those of iron and manganese, that were previously assumed to be biologically unavailable. However, the role of electron shuttling in the reduction of sequestered metals remains unclear. We fabricated a microfluidic reactor with a physical barrier between the microbe-mineral interface to explore mechanisms of extracellular electron transfer in *Shewanella oneidensis* MR-1 to the manganese oxide mineral birnessite. Real-time measurements of mineral redox state coupled to aqueous phase analysis of putative shuttles and soluble manganese allowed us to quantify the number of electrons transferred to manganese oxide via different electron transport pathways. Thiols are the dominant electron shuttles produced under continuous flow on the basis of concentration, but metal reduction across a physical separation is controlled by redox cycling of endogenous flavins. The expression of cytochromes
MtrC and OmcA had no effect on the rate of flavin-mediated metal reduction, which suggests the existence of alternative electron transfer pathways.

4.2 Introduction

Most bacteria obtain energy in the form of ATP by mediating intracellular redox reactions between electron donors (e.g. acetate) and electron acceptors (e.g. nitrate). In anaerobic sediments and engineered systems, however, bacteria are frequently exposed to conditions in which extracellular electron sinks exist in abundance but soluble electron acceptors are unavailable for intracellular respiration. Microbial fuel cells, for example, use solid phase anodes made of carbon and steel as electron acceptors\(^1\). Anaerobic sediments are enriched in Fe(III) and Mn(IV) minerals that are unable to pass through the bacterial cell wall\(^2\). While some species of bacteria respond to electron acceptor limitations by entering a dormant state of decreased metabolic activity, others have evolved mechanisms that generate energy through the transport of respiratory electrons to the outer membrane in a process referred to as extracellular electron transport (EET)\(^3\). Dissimilatory metal-reducing bacteria (DMRB), most notably of the *Shewanella* and *Geobacter* genus, have evolved several EET strategies to generate ATP from the reduction of extracellular electron acceptors. These include the localization of c-type cytochromes (c-Cyts) to the outer membrane\(^4-7\), production of electrically conductive nanowires\(^8,9\), secretion of extracellular polymeric substances (EPS) with embedded c-Cyts\(^10,11\), and shuttling of redox mediators (e.g. flavins)\(^12-14\).

The remarkable metabolic versatility of DMRB, which are able to reduce a variety of organic and inorganic substrates in addition to metals\(^15,16\), has led to their application in bioremediation\(^17\), wastewater treatment\(^18\), and energy generation\(^19\). However, most experiments designed to elucidate EET mechanisms and optimize performance in bioreactors have been
conducted while DMRB were in direct contact with an electron accepting surface. In anaerobic sediments where DMRB thrive, physical separations between the cell and an electron acceptor are ubiquitous. Here, structural iron and metal oxides like Fe(III) and Mn(IV) are concentrated in narrow pore spaces that are smaller than the diameter of a bacterial cell\textsuperscript{20}. The reduction of structural iron in clays leads to significant changes in soil structure as a result of decreases in surface area and cation exchange capacity\textsuperscript{21}. Microbial reduction of Fe(III) and Mn(IV) oxides plays an important role in the transport and redox cycling of other compounds including macronutrients\textsuperscript{22}, trace elements\textsuperscript{33}, and environmental contaminants\textsuperscript{24,25}. In B-horizon soils, which have high iron and manganese content, pores with diameters <100 nm account for 10-40\% of the porosity and more than 90\% of the surface area\textsuperscript{26}. There is sufficient evidence to assert that nanowires can mediate EET across physical separations <10-15 $\mu$m based on in vivo measurements of nanowires length\textsuperscript{9}, and experiments in a microfluidic cell where a DMRB (i.e., \textit{G. sulfurreducens}) reduced Mn(IV) oxide this same distance across a barrier that prevented cell passage but allowed nanowire extension\textsuperscript{27}. At longer physical separations, there is evidence that EET can be mediated by diffusion-based electron shuttling\textsuperscript{27}, but the mechanisms of this process remain obscure. Specifically, what electron shuttles mediate this process, and can these shuttles be recycled over micrometer diffusion distances.

We explored long-range EET over physical separation distances up to 60 microns through narrow pore spaces in a silicon-etched microfluidic reactor that physically separated \textit{Shewanella oneidensis} MR-1 from the naturally occurring Mn(IV) mineral birnessite. We chose \textit{S. oneidensis} MR-1 because of its engineering and environmental relevance\textsuperscript{28}, and because it can endogenously produce at least two types of electron shuttles, flavins and thiols; their reduction is affected by the Mtr pathway\textsuperscript{29-31}, which forms an electrical conduit between the inner membrane and outer
membrane c-Cyts OmcA and MtrC\textsuperscript{32,33}. We measured electron production from \textit{S. oneidensis} MR-1 via soluble electron shuttles in the reactor effluent, and quantified electron consumption for birnessite reduction from real-time images of birnessite conversion to rhodochrosite under varying flow conditions. Our results show for the first time that diffusion-based electron shuttling of flavins, and not thiols, controls EET at all flow regimes, and that each flavin is reused between 22-60 times by redox cycling between MR-1 and birnessite depending on flow conditions. Mutants lacking outer membrane c-Cyts MtrC and OmcA reduced birnessite at wild-type rates, challenging the role of these cytochromes in long-range EET and suggesting possible redundancy in reduction pathways that should motivate future research. The results presented here broaden our understanding of electron flux across physical separations and may aid in the design of more efficient bioelectrical systems or bioremediation strategies. For example, keeping electron shuttling microorganisms physically separate from an electrode may reduce fouling in microbial fuel cells, while the addition of shuttles to groundwater amendments may enable the reduction of sequestered contaminants.

4.3 Materials and methods

Materials

Reagents were ACS reagent grade or cell culture grade and used without further purification. For manganese analysis by ICP-OES, all reagents were trace metals grade. All media solutions were prepared with distilled, deionized water (DDW; 18.2MΩ cm).
**Birnessite synthesis**

Birnessite [(Na, K)0.6(Mn$^{4+}$, Mn$^{3+}$)$_2$O$_4$ · 1.5H$_2$O] was synthesized by oxidizing manganese chloride in a basic solution of potassium permanganate$^{34}$, and as described in our recent work$^{27}$. Fresh batches of birnessite were prepared every 3 months and analyzed by Raman spectroscopy before each experiment to verify the purity of the birnessite. TEM images from our previous work showed that birnessite particles were < 100 nm in length$^{27}$.

**Batch culture conditions**

*Shewanella oneidensis* MR-1 was cultured in 100 mL of anoxic, bicarbonate-buffered freshwater medium at pH 7.0 containing the following per liter of distilled water: 0.5 g NH$_4$Cl, 0.14 g KH$_2$PO$_4$, 0.2 g MgCl$_2$·6H$_2$O, 0.15 g CaCl$_2$·2H$_2$O, and 2.5 g NaHCO$_3$. This was supplemented with a 2% w/v solution of cysteine-free casamino acid digest, a non-chelated SL-10 trace elements solution, and a selenite plus tungstate solution$^{35}$. Vitamins and chelating agents were omitted to minimize the risk of abiotic reduction and dissolution. Sulfur was provided by sulfide or methionine at a concentration of 12.5 μM. Lactate was supplied as the electron donor at a concentration of 20 mM. Batch cultures were grown in an incubator at 25 or 30 °C ± 1 °C.

**Microfluidic reactor fabrication**

Microfluidic reactors were etched in silicon using photolithography. A schematic illustrating the major steps of fabrication is shown in Figure 4.1. AZ1518 photoresist was spun on silicon wafers. A digitized image of the slits (i.e. nanopores) in a chrome plated soda lime glass mask was transferred into the photoresist by UV light. Exposed silicon was etched to a depth of 180–200 nm with HBr and Cl$_2$ using a Plasma-Therm RIE 790 (Plasma-Therm LLC, USA). The
depth of the etched slits was measured using optical profilometry. Next, AZ1505 photoresist was spun on silicon wafers. A digitized image of the wall and channels in a chrome plated soda lime glass mask was transferred into the photoresist by UV light. Exposed silicon was anisotropically etched by ICP-DRIE to a depth of 10 μm with SF₆ and C₄F₈ using a Plasma-Therm Versaline DSE (Plasma-Therm LLC, USA). Two inflow and two outflow ports approximately 1 mm in diameter were ultrasonically drilled into the wafers to create ports. The reactors were encapsulated by anodically bonding the silicon wafers to Borofloat 33 glass wafers at 900 V and 400 °C. Once bonded, PEEK NanoPort assemblies (IDEX Corporation, Illinois, USA) were glued over the drilled ports and connected to 0.01” ID PEEK tubing. PEEK and ETFE fittings connected the tubing to gastight syringes that were driven by a NE-4002X microfluidic syringe pump (New Era Pump Systems Inc., New York, USA).

**Microfluidic reactor setup and operation**

Flow was delivered to the microfluidic flow channels at a rate of 2.2 μL/h, corresponding to a linear velocity of 0.017 cm/s and a residence time of 90 seconds. Reactors were stored in the dark to prevent photodegradation of flavins and maintained at a temperature of 25 °C ± 1 °C. Solutions were stored in 2.5 mL gastight syringes and delivered by syringe pump through 0.005” ID PEEK tubing. All solutions including media and stocks were sterilized by autoclaving or filtration. All materials including syringes, fittings, and tubing were autoclaved. Reactors were disinfected for 12 hours with 1 M acetic acid and flushed with 3,000 pore volumes of sterile DI water before starting an experiment.

Experiments consisted of four stages. In Stage 1, birnessite was infused through one channel as a colloidal solution and immobilized at the barrier wall by inducing cross flow. This is
achieved by closing the effluent port on the birnessite side and opening the effluent port on the bacteria side. After fixing the birnessite, the reactor was inoculated in Stage 2 by infusing fumarate grown *S. oneidensis* MR-1 through the adjacent channel until several thousand cells deposit on the surface. In Stage 3, flow is closed off to the bacteria side while the top channel is infused with 20 mM lactate as the electron donor and a combination of 0.5 mM nitrate and 5 mM fumarate as soluble electron acceptors. Chemotaxis towards the wall is observed within hours, and flow through the birnessite side was maintained for 12-24 hours until a biofilm was established at the wall. In Stage 4, both channels were infused with media containing only lactate and essential media components so that birnessite remains as the sole electron acceptor. These stages are summarized in Table 4.1 In some experiments, we infused Syto-9 and propidium iodide as live and dead cell stains, respectively, to determine cell viability during reduction and probe for cell passage across the wall after the completion of an experiment. The metabolic stain RedoxSensor Green was infused in one experiment to determine if cells were metabolically active during birnessite reduction.

During each experiment, reactor effluent was collected every 24 hours for analysis. The effluent was diluted in 50 μL sterile DI water, resulting in a total sample volume of 100 μL. Samples were weighed to account for variations in effluent volume, then passed through a 13 mm 0.22 μL PES syringe filter. The 80-90 μL remaining after filtration was divided for analysis of flavins, thiols, and aqueous manganese.

**Image analysis**

The microfluidic reactor was imaged using a Nikon Eclipse TI-E inverted microscope integrated with a Horiba LabRAM HR Evolution confocal Raman system. High resolution black
and white images for the determination of birnessite, rhodochrosite, and biofilm area by optical thresholding were taken with an Andor Zyla 5.5 camera as previously described\textsuperscript{27}. Optical thresholding was performed using Nikon NIS software. Fluorescent images were also taken with an Andor Zyla 5.5 camera. Autofluorescence of c-Cyts produced by \textit{S. oneidensis} MR-1 was imaged through a 400 nm dichroic mirror at excitation and emission wavelengths of 340-380 nm and 435-485 nm, respectively. The dead cell stain propidium iodide was excited between 540-580 nm. Fluorescence was detected through a 595 nm dichroic mirror at an emission wavelength of 600-660 nm. The live cell stain Syto-9 and the metabolic stain RedoxSensor Green were excited between 457-487 nm. Fluorescence was detected through a 495 nm dichroic mirror at an emission wavelength of 502-538 nm. Color images were taken with a Lumenera Infinity 3-1UR camera.

**Derivatization of thiols**

Thiols were reduced with tris(2-carboxyethyl)phosphine (TCEP) and derivatized with 4-Chloro-3,5-dinitrobenzotrifluoride (CNBF) according to the protocol of Zhang et al.\textsuperscript{37} to prepare them for analysis using ion-pairing reverse-phase high performance liquid chromatography (HPLC). Briefly, 80 \( \mu \)L of reactor effluent filtered through a 0.2 \( \mu \)m PES syringe filter was reacted at room temperature for 10 minutes with 10 \( \mu \)L of a 50 mM aqueous solution of TCEP in borate buffer (200 mM, pH 7) to reduce disulfide bonds\textsuperscript{38}. Next, 5 \( \mu \)L of EDTA (50 mM, pH 7) in aqueous solution was added to attenuate thiol oxidation\textsuperscript{38}. This was followed by the addition of 50 \( \mu \)L borate buffer (200 mM, pH 7) and 30 \( \mu \)L methanol. Finally, 10 \( \mu \)L of 10 mM CNBF in methanol was added to derivatize the thiols. After 30 minutes at room temperature, the derivatization was quenched by the addition of 10 \( \mu \)L of 2 M HCl.
Quantification of manganese

The manganese content of birnessite was determined by digesting a known mass of birnessite powder in 10 mM oxalic acid and 2% HCl, then measuring manganese concentration by ICP-OES. The average oxidation state (AOS) of birnessite was determined by permanganate back titration using the protocol of Cui et al\textsuperscript{39}. The concentration of manganese in a deposited layer of birnessite within a microfluidic reactor was determined by crushing the reactor into a fine powder using high purity yttria zirconia beads, digesting the birnessite in 10 mM oxalic acid and 2% HCl, then analyzing by ICP-OES.

Manganese solids inside the reactor were identified in real-time on a Horiba LabRAM HR Evolution confocal Raman system. Raman spectra were taken between 0 and 1200 cm\textsuperscript{-1} using a 532 nm DPSS laser in a backscattering configuration. The laser was calibrated daily using silicon as a reference material, and reference spectra from the RUFF mineral database were used to identify minerals inside the reactor. Birnessite spectra were obtained at 5 mW and averaged over 3 successive scans with an acquisition time of 60 seconds. Rhodochrosite spectra were obtained at 2.5 mW and averaged over 15 successive scans with an acquisition time of 10 seconds. Aqueous Mn(II) was analyzed on a Varian 710-ES ICP-OES after acidification of samples in 10 mM oxalic acid and 2% HCl.

Quantification of redox mediators

Reactor effluent was collected every 24 hours and analyzed for flavins and thiols by HPLC using a Shimadzu LC-2040C HPLC, and Shimadzu C18 reversed-phase column (50 mm x 2.1 mm with 1.9 μm particle size). Separation of flavins was achieved at 45 °C with an isocratic concentration of 30% methanol versus an aqueous solution of acetic acid at pH 4.7 and a flow rate
of 0.2 mL/min. A Shimadzu RF-20 fluorescence detector was used with an excitation wavelength of 450 nm and an emission wavelength of 520 nm. Thiols were analyzed after reduction with TCEP and derivatization with CNBF. The mobile phase consisted of acetonitrile (eluent A) and water with 0.1% (v/v) TFA (eluent B). Separation was achieved at 0.12 mL/min and 40 °C using a gradient as follows (expressed as the proportion of A): 0-3.0 min, 30-75%; 3.0-6.0 min, 75%; 6.0-9.0 min, 75-30%. A photodiode array (PDA) set to an absorbance maximum of 230 nm was used to detect derivatized thiols.

4.4 Results and discussion

The nanoporous wall physically separates *S. oneidensis* MR-1 from birnessite

The main feature of our microfluidic reactor is a thin, nanoporous wall measuring 1.5 μm in width and 2 cm in length. The wall bisects two parallel flow channels that are 250 μm wide and 10 μm deep. An array of slits (i.e. pores) measuring 3 μm in length and 180 nm in depth are etched into the top of the wall at 3 μm intervals to allow the diffusion of solutes between each channel but not the passage of cells. An SEM image of the nanoporous wall with 180 nm deep slits is shown in Figure 4.2a. An illustration of the experimental set-up, with partially reduced birnessite separated from a biofilm of *S. oneidensis* MR-1, is shown in Figure 4.2b.

Autofluorescence of c-Cyts in *S. oneidensis* MR-1 was used to monitor the location of cells within the reactor and check for passage across the wall (Fig. 4.3). In earlier reactor designs with deeper slits, cell passage was visible within the thin layer of rhodochrosite, and cell motility within rhodochrosite could be observed using brightfield microscopy. With our present design, we did not observe any cells across the wall. In preliminary experiments, the BacLight Live/Dead
stain was introduced into both flow channels of a reactor to enhance cell visualization, and no cell passage across the nanoporous wall was observed. In another set of preliminary experiments, biofilms of *S. oneidensis* MR-1 were grown in the absence of birnessite to provide a clear view of the cells by brightfield and fluorescence microscopy. As shown in Figure 4.4, cells were retained in one channel of the reactor. Also, no microbial growth was observed when effluent from the flow channel with birnessite was used to inoculate bottles containing media, 20 mM lactate, and 15 mM fumarate. Together, these results demonstrate the effectiveness of the nanoporous wall in preventing the passage of *S. oneidensis* MR-1.

**Birnessite reduction across a physical separation is biologically driven**

Birnessite reduction across the nanoporous wall by *S. oneidensis* MR-1 in a representative experiment is shown in Figure 4.5a, where reduction of the dark brown birnessite to tan colored rhodochrosite is indicated from bright field images. In all experiments, birnessite reduction began after a lag period of 1-3 days, which we attribute to the acclimation of *S. oneidensis* MR-1 to the sudden absence of fumarate and nitrate in Stage 4. Within 10 days from the start of reduction, the 40-60 μm layer of birnessite was completely reduced to soluble Mn(II) and a solid product that was identified as rhodochrosite (MnCO₃) by Raman spectroscopy (Fig. 4.5b).

The cumulative production of Mn(II) from birnessite reduction was determined based on the average oxidation state (AOS), manganese content, and density of birnessite in the microfluidic reactor. The AOS, determined by permanganate back titration, is 3.96 ± 0.03, similar to reported values. The manganese content (i.e., Mn/birnessite), determined by ICP-OES on a mass fraction basis, is 0.44 ± 0.02, also similar to expected values. The density of birnessite in the reactor, determined by pulverizing a reactor containing a known area of birnessite and analyzing via ICP-
OES, is 0.298 ± 0.017 g/cm$^3$, corresponding to about 8 μg of birnessite in each reactor. Based on these values and the unit decrease in birnessite area of time, the Mn(II) production rate is 437 pmol/day, with 4009 ± 405 pmol Mn(II) produced after 10 days as shown in Figure 4.6.

In two experiments, the sulfide we provided as a sulfur source to *S. oneidensis* was replaced with an equal concentration of methionine as a control, to test for indirect reduction of birnessite via sulfur-mediated electron shuttling$^{41}$. A conceptual figure showing this pathway of reduction is shown in Figure 4.7. The Mn(II) production in the microfluidic reactor was unaffected by the replacement of sulfide with methionine (Fig. 4.8), which *S. oneidensis* can use as the sole sulfur source$^{42}$. This indicated that biological production of sulfide and abiotic reaction of sulfide with birnessite was not a significant pathway of reduction. Another microfluidic reactor experiment was performed with no *S. oneidensis* MR-1 as a control and showed no reduction or dissolution of birnessite after one month of continuous flow; this demonstrates that the loss of birnessite over time is not due to abiotic reduction or dissolution with media. Particle tracking using surface defects as reference points showed no internal movement of birnessite within the immobilized layer. This discounts the possibility that the migration of particles accounts for the disappearance of birnessite.

**Nanowires are not required for long-range EET in the microfluidic experiments**

We tested the role of nanowires and of the Mtr pathway in general by repeating our microfluidic experiments with ΔomcA ΔmtrC double mutant cells. Outer membrane c-Cyts OmcA and MtrC, which serve as terminal reductases of the Mtr pathway$^{43}$, are localized on nanowire surfaces and responsible for their conductivity. Deletion of OmcA and MtrC results in the production of nonconductive nanowires$^{29,44}$. 
In batch culture, we observed significantly slower and incomplete reduction of birnessite with the ΔomcA ΔmtrC double mutant compared to wild-type cells and expected to observe similar results in our microfluidic reactor. Surprisingly, rate of Mn(II) production was not significantly different between wild-type and double mutant cells (Fig. 4.9). The reduction of birnessite across the wall cannot be explained by nanowires since their conductivity is dependent on the presence of OmcA and MtrC. We also note that the maximum reported length of nanowires from in vivo measurements is 9 μm, while the extent of birnessite reduction exceeded 60 μm. Based on these results, we suspect that alternative c-Cyts and redox proteins were responsible for the observed reduction, possibly with the aid of soluble electron shuttles (e.g. flavins, thiols). It is also worth noting that elimination of MtrC and OmcA does not cause the reduction rate of Mn(IV) and Fe(III) oxides to drop to zero, suggesting redundancy in EET pathways.

**Flavins control reduction despite higher thiol concentrations**

After eliminating the role of nanowires, effluent collected from the original experiment containing wild type *S. oneidensis* MR-1 opposite birnessite in the microfluidic reactor was analyzed for electron shuttles. Riboflavin (RF) and flavin mononucleotide (FMN) were consistently detected by HPLC at masses of 5.12 ± 0.28 and 12.79 ± 0.63 pmol, respectively, as the combined mass from both channels measured over a 24-hour collection period (Fig. 4.10). This corresponds to approximately 100 nM RF and 250 nM FMN, respectively. Also, flavins collected from the effluent of the birnessite channel accounted for 12-15% of the flavin mass, indicating that diffusion of flavins across the wall was occurring. Flavin adenine dinucleotide (FAD) is not secreted by *S. oneidensis* MR-1 in anaerobic culture but was detected in trace amounts most likely as a result of cell lysis.
To test the role of flavins more thoroughly, we repeated our experiments with a mutant lacking *bfe*, the bacterial FAD transporter\(^{14}\). In batch culture, we observed an order of magnitude decrease in the concentration of both RF and FMN despite wild-type rates of birnessite reduction. We observed similar results in fumarate grown cultures as reported in the literature. In the microfluidic reactor, however, we observed a dramatic decrease in the reduction rate by approximately 5 times, corresponding to a decrease in RF and FMN concentrations by 6 and 8 times, respectively (Fig. 4.11). Mn(II) production was restored to wild-type rates by providing *bfe* cells with an exogenous supply of RF and FMN at concentrations similar to those produced by wild-type cells (Fig. 4.11). This result discounts the possibility that pleiotropic effects resulting from the mutation may have influenced the rate of birnessite reduction. Wild-type reduction rates in batch culture may be due to more efficient recycling of flavins under static conditions despite their lower concentration. It is also possible that flavins play a less important role in mediating electron transfer when the cell is in direct contact with birnessite as in batch culture.

A portion of the effluent was reduced using tris(2-carboxyethyl)phosphine (TCEP) and derivatized with 4-Chloro-3,5-dinitrobenzotrifluoride (CNBF) to detect thiols\(^{46}\). We detected the presence of three thiols including cysteine, homocysteine, and glutathione. Cysteine was periodically present at concentrations between 200-800 nM, while homocysteine and glutathione were detected in trace amounts near the limit of detection. Cysteine production began several days after the start of Stage 4, and its concentration in the effluent was not correlated to the rate of birnessite reduction. Therefore, it does not appear that thiols control birnessite reduction by LR-EET.

No other mechanisms of reduction or chelation are reported in *S. oneidensis* MR-1 that would account for the observed reduction of birnessite by LR-EET. Siderophores, which
microorganisms excrete to solubilize iron in iron-limited environments, were not expected to play a role in birnessite reduction since soluble iron was present in the media. Previous work has also shown that siderophores produced by *S. oneidensis* MR-1 are not involved in Fe(III) solubilization under anaerobic conditions\(^47\). A decrease in MnO\(_2\) reduction was observed when the SO3030 gene for siderophore synthesis was deleted, but mutants were also deficient in outer membrane c-Cyts that may have affected reduction rates\(^48\).

We also explored the possibility of conductive EPS being secreted across the wall. To date, 42 types of c-Cyts have been detected on the outer membrane of *S. oneidensis* MR-1\(^49\), and 20 redox proteins have been extracted from EPS\(^50\). Outer membrane c-Cyts OmcA and MtrC are associated with the EPS of *S. oneidensis* MR-1 during hematite reduction\(^7,51\), and may be responsible for its reported conductivity\(^11\). We ran two additional experiments to investigate the potential of LR-EET via the secretion of EPS. After birnessite was reduced, remaining manganese solids were dissolved in 10 mM oxalic acid at pH 4. The reactors were dried at 60 °C and opened, then analyzed by SEM. We observed no presence of EPS across the wall in either reactor (Fig. 4.12), conclusively showing that EPS was not involved in birnessite reduction.

**Diffusion-based shuttling and recycling of flavins accounts for the electron transfer imbalance**

We ran an additional pair of experiments with variations in flow rate to test the role of diffusion-based electron shuttling. The ports to the birnessite channel were initially closed to limit the loss of soluble species, then opened once half of the birnessite layer was reduced. After several days, the birnessite ports were closed again. If diffusion-based electron shuttling and flavin recycling were driving birnessite reduction, we would expect the opening of these ports to coincide
with a decrease in the Mn(II) production rate due to the fast removal of flavins from the purged flow channel, and the near zero concentration boundary created at the outer edge of the unreduced birnessite. In line with this prediction, we observed a significant decrease in the Mn(II) production rate immediately following the onset of flow in the birnessite channel (Fig. 4.13). During stopped flow, Mn(II) production was 1002 ± 152 pmol/day. During continuous flow, this decreased to 396 ± 14 pmol/day. During the final stopped flow phase, the reduction rate increased to 1271 ± 67 pmol/day. A decrease Mn(II) production at the onset of continuous flow is also consistent with a recycling mechanism. If a shuttle produced within the biofilm were to react only once with birnessite across the wall, then flow in the birnessite channel and the loss of the shuttle in the effluent would not be expected to affect the reduction rate.

**LR-EET implications**

Cumulative electron equivalents transferred for birnessite reduction (Fig. 4.6) can be compared to cumulative electron equivalents available from flavins (Fig. 4.10) to determine if the latter are present in sufficient quantities to reduce the former. We assume that each flavin molecule is capable of a 2-electron transfer to 1.02 Mn atoms with an AOS of 3.96. Under continuous flow and stop flow conditions, the numbers of electron equivalents generated per unit time from flavins are 18 and 22 pmol/day, respectively. In contrast, the number of electron equivalents consumed for birnessite reduction are 396 and 1271 pmol/day respectively. This means for flavins to be responsible for birnessite reduction, they must be reused at least 22 times, and up to 60 times under stop flow conditions. Given the evidence of diffusion-based electron shuttling, we assert that this discrepancy is due to the recycling of flavins across the wall between their reduced and oxidized states. Such a recycling mechanism is consistent with lower rates of reduction during continuous
flow, where flavins that diffuse through the birnessite layer are immediately removed from the reactor.

Extracellular electron transport remains a controversial topic, and new evidence continues to challenge existing models. Flavin electron shuttling by *S. oneidensis* MR-1, for example, was originally proposed as a diffusion-based process mediated through a single two-electron transfer mechanism\(^\text{13}\). In support of this model, an immediate drop in current density was observed in membrane bioreactors when supernatant containing RF and FMN was flushed with minimal salts buffer\(^\text{52}\). However, recent work with *S. oneidensis* MR-1 supports a bound-cofactor model where flavins form transient complexes with outer membrane c-Cyts and transport electrons via two successive one-electron transfers\(^\text{53}\). Electrochemical measurements of anode biofilms are consistent with a one-electron transfer to birnessite via semiquinone flavin derivatives\(^\text{54}\). The binding of RF and FMN to OmcA and MtrC, respectively, is also associated with semiquinone formation and rate enhancements in electron transfer\(^\text{31}\). In another study, a mutant was isolated that reduced Mn(IV) to Mn(III) at wild-type rates but was deficient in Mn(III) reduction to Mn(II)\(^\text{32}\). This result suggests that *S. oneidensis* MR-1 possesses an electron transport pathway capable of one-electron transfer. Despite mounting evidence that flavins transport electrons as bound-cofactors when the cell is in direct contact with an electron accepting surface, diffusion-based shuttling may still play a role across physical separations. In microbial fuel cells, for example, greater current production is observed when cells are grown on nanopatterned electrodes with narrow pore as compared to flat electrodes\(^\text{55}\). Fe(III) oxide in nanoporous beads was also reduced by *S. oneidensis* MR-1, though a redox mediator was not identified in these experiments\(^\text{56}\).

Electron transport across a physical separation is relevant to both natural and engineered systems, but experimental difficulties in creating a separation and distinguishing between different
EET mechanisms has been a major limitation. We successfully built an experimental platform that meets these requirements, and systematically tested the contribution of known EET mechanisms using birnessite as an electron acceptor. The results from our microfluidic experiments show that diffusion-based shuttling of endogenously produced flavins drives electron transport across a physical separation. The reduction of birnessite under continuous flow at linear velocities greater than those encountered in sandy aquifers suggests that this mechanism of electron transport is possible at all flow regimes in groundwater environments. We were surprised that reduction proceeded across the entire layer of immobilized birnessite with a thickness of 40-60 μm, since diffusion-based shuttling of flavins at physiological concentrations is predicted to be effective only at distances < 1 μm\(^{57}\). In our previous work with *Geobacter sulfurreducens* KN400, reduction was limited to 15 μm and attributed to nanowires\(^{27}\). However, the observation that wild-type rates of birnessite reduction were observed in \(\Delta omcA \Delta mtrC\) double mutants is inconsistent with nanowire reduction and electron transfer via bound flavins. These results suggest that electron acceptors sequestered deep within narrow pores spaces, including iron and manganese oxides, metalloids (e.g. selenium), and radionuclides (e.g. uranium) may be accessible to *Shewanella* spp. and other microorganisms capable of diffusion-based electron shuttling. We were also surprised that cysteine, which was produced at order of magnitude higher concentrations than flavins, was not correlated with birnessite reduction. Disulfide bond reduction is observed in *Shewanella* spp., and Fe(III) oxide reduction is stimulated when cultures are amended with either cystine or cysteine\(^{58}\). However, thiol redox cycling requires dimer formation, which may be inefficient at low thiol concentrations.

The results presented here suggest that conventional models of electron transport in anaerobic sediments should be revised to account for diffusion-based electron shuttling within
narrow pore spaces and across physical separations. Incorporation of physical separations in bioelectrical systems and membrane bioreactors may also be used to reduce problems such as membrane fouling. Injecting electron shuttles at contaminated sites or stimulating the growth of shuttle-producing DMRB may also improve the efficiency of bioremediation.
4.5 Figures and tables

![Diagram](image)

Figure 4.1. Plane view process diagram for reactor fabrication. Spinning AZ1518 photoresist (A), UV exposure (B), RIE plasma etch of slits to 180-200 nm (C) stripping of AZ1518 (D), spinning of AZ1505 photoresist (E), UV exposure (F), DRIE of channels (G), stripping of AZ1505 photoresist (H), anodic bonding (I).
Figure 4.2. SEM image of the silicon-etched nanoporous wall at a 45-degree incline (A), and illustration of birnessite reduction to rhodochrosite by flavin electron shuttling and a recycling mechanism (B).
Figure 4.3. Autofluorescence of c-Cyts of *S. oneidensis* MR-1 inside the microfluidic reactor during birnessite reduction.
Figure 4.4. Brightfield microscopy image showing biofilm of *S. oneidensis* MR-1 in the upper channel being retained by the nanoporous wall.
Figure 4.5. Time progression of birnessite reduction across the nanoporous wall over 10 days by *S. oneidensis* MR-1 (A) and corresponding Raman spectra of birnessite and rhodochrosite taken in-situ (B). Images were taken after the lag phase at the onset of reduction.
Figure 4.6. Cumulative Mn(II) production by *S. oneidensis* MR-1 in the microfluidic reactor from birnessite reduction as measured by area reduced.
Figure 4.7. Illustration of biological sulfate reduction coupled to abiotic reduction of Fe(III) by sulfide\textsuperscript{41}. 
Figure 4.8. Mn(II) production in the microfluidic reactor by wild-type *S. oneidensis* MR-1 growing with sulfide or methionine as a sulfur source.
Figure 4.9. Mn(II) production in the microfluidic reactor by wild-type (WT), and OmcA/MtrC mutant cells of *S. oneidensis* MR-1.
Figure 4.10. Cumulative flavin production (FMN, RF, cumulative) by *S. oneidensis* MR-1 during birnessite reduction in the microfluidic reactor.
Figure 4.11. Mn(II) production in the microfluidic reactor by wild-type (WT), bfe export mutant (bfe), and bfe export mutant cells of *S. oneidensis* MR-1 supplemented with FMN and RF (bfe + flavin). Flavin supplementation consisted of 5 pmol RF and 15 pmol FMN per day.
Figure 4.12. SEM image of the birnessite side of the nanoporous wall after debonding a microfluidic reactor.
Figure 4.13. Mn(II) production under different flow conditions. Flow through the bacteria channel was continuous. Flow through the birnessite channel was either stopped (white) or continuous (blue).
Table 4.1. Description of stages in microfluidic experiments where the top channel refers to that containing *S. oneidensis* MR-1, and the bottom channel refers to that containing birnessite. All solutions were made in minimal media with 20 mM lactate.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Channel</th>
<th>Component</th>
<th>Flow Rate (nL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bottom</td>
<td>Immobilize birnessite on nanoporous wall</td>
<td>Manual</td>
</tr>
<tr>
<td>2</td>
<td>Top</td>
<td>Inoculate <em>S. oneidensis</em> MR-1</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>Bottom</td>
<td>Infuse 0.5 mM nitrate and 5 mM fumarate</td>
<td>125</td>
</tr>
<tr>
<td>4</td>
<td>Top, Bottom</td>
<td>Infuse media without electron acceptor</td>
<td>40</td>
</tr>
</tbody>
</table>
Table 4.2. Summary of conditions and results from microfluidic experiments.

<table>
<thead>
<tr>
<th>Observation</th>
<th>S. oneidensis</th>
<th>Methionine</th>
<th>Δbfe</th>
<th>ΔOmcA/MtrC</th>
<th>Stop / Continuous</th>
<th>Mn(II) production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mr-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No reduction by media’</td>
<td>X</td>
<td>X, ✓</td>
<td>X</td>
<td>X</td>
<td>C</td>
<td>None</td>
</tr>
<tr>
<td>No reduction by sulfide’</td>
<td>✓</td>
<td>✓</td>
<td>X</td>
<td>X</td>
<td>C</td>
<td>Normal</td>
</tr>
<tr>
<td>LR-EET not by nanowires</td>
<td>✓</td>
<td>X</td>
<td>X</td>
<td>✓</td>
<td>C</td>
<td>Normal</td>
</tr>
<tr>
<td>Flavins drive LR-EET</td>
<td>✓</td>
<td>X</td>
<td>✓</td>
<td>X</td>
<td>C</td>
<td>Low</td>
</tr>
<tr>
<td>LR-EET by recycling</td>
<td>✓</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>S, C</td>
<td>Normal, Low</td>
</tr>
</tbody>
</table>

* Control experiments, X not present or observed, ✓ present or observed
4.6 References


(13) Marsili, E.; Baron, D. B.; Shikhare, I. D.; Coursolle, D.; Gralnick, J. A.; Bond, D. R.


CHAPTER 5: CONCLUSIONS

5.1 Conclusions

Two types of microfluidic reactors were successfully fabricated to test different mechanisms of LR-EET. These reactors were similar except in the design of a nanoporous barrier that physically separated bacteria from an electron acceptor (i.e. birnessite). The first type of barrier, designed as a series of pillars with <200 nm gaps, was fabricated by electron beam lithography and gave Geobacter nanowires access to birnessite throughout its depth. The second type of barrier, designed as a series of <200 nm slits etched along the top of a continuous wall, was fabricated by photolithography and was suited to retaining highly motile Shewanella cells. The microfluidic reactors described in Chapter 2 of this dissertation provided a clear view of the biofilm and deposited birnessite, and were compatible with optical microscopy and Raman spectroscopy for real-time, in-situ measurements of biofilm growth, birnessite reduction, and mineral redox state. A cleaning protocol was successfully developed to remove EPS containing a variety of macromolecules, and significantly reduced the time and cost of fabricating new microfluidic reactors for every experiment. A novel method to debond the anodically bonded reactors was also developed for post-experimental analysis by high resolution microscopic techniques (e.g. SEM, AFM). This method is unique in that it doesn’t require high temperature, high voltage, or harsh chemicals that would otherwise damage biological structures.

Microfluidic experiments with G. sulfurreducens KN400, described in Chapter 3 of this thesis, showed that their nanowires had the capability of reducing birnessite up to 15 μm from cell bodies. Previous work has shown that nanowires are conductive¹, and that current density on produced by anode biofilms increases when nanowires are expressed². Other experiments have
shown that the absence of nanowires leads to a decrease in the reduction rate of Fe(III) oxides.

The work described in Chapter 3 of this dissertation is the first to test the ability of nanowires to reduce metals across a physical barrier and measure the length over which this occurs. Other mechanisms of LR-EET were systematically excluded by changing experimental conditions (e.g. at temperatures that result in nanowire-deficient wild-type cells) and performing the appropriate controls. This is also the first work to experimentally test the “flavin cofactor” model of electron transport along nanowires. When nanowires were first discovered, it was believed that electrons were transferred directly from the nanowire to an extracellular electron acceptor. More recently, it was shown that nanowires do not mediate redox reactions directly despite their intrinsic conductivity. Instead, it was argued, the terminal electron transfer step was carried out by c-Cyts that decorate the nanowire surface. Immunogold labeling with anti-OmcS antibodies showed that OmcS was localized on Geobacter nanowires and was also required for Fe(III) oxide reduction.

The work described in Chapter 3 of this dissertation builds upon this model and shows that an additional redox mediator is required to reduce an extracellular electron acceptor (e.g. birnessite). Our results suggest that the terminal electron transfer step in LR-EET is mediated not by c-Cyts on the nanowire surface, but by flavins that bind to these c-Cyts.

Microfluidic experiments with S. oneidensis MR-1, described in Chapter 4 of this thesis, showed that electron shuttling can reduce birnessite over distances exceeding 60 μm. A major controversy in the field is whether flavin electron shuttles transfer electrons as bound or diffusion-based electron shuttles. Recent evidence suggests that flavins transport electrons in two successive one-electron transfer steps as c-Cyt bound cofactors when cells are in direct contact with an extracellular electron acceptor. However, few experiments have tested electron transport by S. oneidensis MR-1 across a physical separation. We show that flavins, particularly RF and FMN,
drive LR-EET as diffusion-based electron shuttles as indicated by stop flow and continuous flow experiments and results using the \textit{bfe} flavin export mutant. Most importantly, our results suggest that flavins are recycled multiple times between their oxidized and reduced states as evidenced by the discrepancy between the electron equivalents consumed by birnessite and produced by flavins. In addition, the thiols cysteine, homocysteine, and glutathione did not appear to drive birnessite reduction despite being produced at much higher concentrations, albeit inconsistently. We also show that nanowires play little, if any role in LR-EET across a physical separation using the OmcA/MtrC double knockout mutant that produces nonconductive nanowires. We expected nanowires to drive reduction at distances <10 \textmu m since \textit{Shewanella} nanowires extend up to 9 \textmu m from cell bodies\textsuperscript{8}. However, reduction rates were not significantly different between the OmcA/MtrC mutant and wild-type cells within this region. This result also suggests that the Mtr pathway, considered to be the dominant pathway for metal reduction in \textit{S. oneidensis} MR-1\textsuperscript{9}, is not essential for LR-EET.

\textbf{5.2 Contributions}

This research led to the development of novel microfluidic reactors with the ability to physically retain bacteria on one side of a thin, nanoporous wall. While each microfluidic platform was designed to test a specific strategy of LR-EET to the mineral birnessite, we imagine the use of these reactors across a broad range of disciplines whenever small liquid volumes, thin physical barriers, and a nonreactive surface are desired. The method developed to debond anodically bonded reactors at ambient conditions may also be useful for post-experimental analysis by high-resolution analytical tools.

\textit{G. sulfurreducens} is frequently the dominant microorganism present in anaerobic
sediments that are undergoing Fe(III) reduction. Our finding in Chapter 3 of this dissertation that the nanowires of *G. sulfurreducens* can penetrate nanopores and reduce sequestered birnessite, a representative metal oxide, has implications for metal reduction in nanopores that are present in the natural environment. This is significant because Fe(III) and Mn(IV) oxides are concentrated in nanopores that are too small for cell passage, and their reductive dissolution affects carbon flow, contaminant transport, and the biogeochemical cycling and mobility of macronutrients and trace metals. An increasing number of bacterial species are being discovered that possess conductive nanowires based on Type IV pili, which are similar in structure to those of *G. sulfurreducens*. While our experiments studied LR-EET via nanowires in a single species of DMRB, our results may be extended to other bacterial species that thrive in diverse range of environments. For example, conductive nanowires are produced by oxygenic phototrophic cyanobacteria as well as thermophilic, fermentative bacteria. Thus, we can imagine that metal reduction and oxidation in nanopores may occur in anaerobic and aerobic environments over a wide range of temperature.

Diffusion-based electron shuttling of flavins has fallen out of favor recently with experimental evidence supporting their role as bound cofactors, but the results presented in Chapter 4 of this dissertation show that diffusion-based electron shuttling can drive metal reduction in nanoporous sediment systems. We show that reduction is not limited to 15 μm as in the case with LR-EET via nanowires, but can extend >60 μm through nanopores. Our stop flow and continuous flow experiments show that diffusion-based electron shuttling can occur at any flow regime in groundwater environments since the linear flow rate during continuous flow experiments is similar to that in coarse media. In anerobic sediments, flavins produced by *S. oneidensis* MR-1 and other microorganisms may also facilitate LR-EET by nanowire-producing DMRB like *G. sulfurreducens* by acting as bound factors on c-Cyts that decorate the nanowire surface. Our results
also suggest that adding an exogenous supply of flavins to contaminated groundwater sites may remove persistent sources of contamination. For example, U(VI) is primarily sorbed to clay minerals, particularly nanoporous chlorite clays, and desorption of U(VI) is observed shortly after pump and treat. Through electron shuttling, it may be possible to directly reduce this persistent source of contamination by reducing U(VI) to insoluble U(IV).

5.3 Future work

This dissertation explored two important mechanisms of LR-EET across a physical separation and advances our understanding of electron flux in porous media. The results presented here also raise many questions related to contaminant transport, redox cycling in aerobic sediments, electron transport in multispecies biofilms, and fundamental mechanisms of electron transport via nanowires and electron shuttles.

The release of metal contaminants upon reductive dissolution of sequestered manganese and iron oxides was not explored in this thesis, but we believe this is an important area to study in future work. Biofilm thickness, pH, flow rate, and other factors may determine whether released contaminants will be reduced within the biofilm or released into the porewater. This will have implications for the fate and transport of groundwater contaminants.

All experiments described in this dissertation were performed under anaerobic conditions. However, we believe that LR-EET may also occur in aerobic environments where Fe(III) and Mn(IV) are abundant. Anaerobic microenvironments exist deep within soil grains, and are reported to contain both Mn(IV) and Fe(III) oxides as well as adsorbed contaminants such as arsenic. Similar microenvironments may be present in the interior of thick biofilms if oxygen is depleted by aerobic bacteria or facultative anaerobes on the surface. The lack of oxygen as a terminal
electron acceptor may drive metal reduction deep in the biofilm. This could be tested with \textit{S. oneidensis} MR-1, which is a facultative anaerobic, or in multispecies biofilms containing both aerobic and anaerobic bacteria in syntrophic relationships.

Our findings in Chapter 3 and Chapter 4 of this dissertation also raise questions about LR-EET from a mechanistic perspective that could be explored in future work. For example, experiments with OmcS mutants could be conducted with \textit{G. sulfurreducens} to directly test our hypothesis that flavins bound to c-Cyts that decorate the nanowire surface mediate the terminal electron transfer step to an extracellular electron acceptor. The binding affinity of flavins to c-Cyts of \textit{Geobacter} could also be tested with surfactants or proteases that denature c-Cyts, therefore releasing them into solution. Our finding that MtrC and OmcA, the terminal reductases of the Mtr pathway, are not required for LR-EET in \textit{S. oneidensis} MR-1 challenges their perceived role in the reduction of Fe(III) and Mn(IV) oxides. Interestingly, the absence of these c-Cyts inhibited birnessite reduction significantly in batch culture, but not in the microfluidic reactor. This implies redundancy in LR-EET pathways that should be explored in future work. Our results also suggest that different reduction pathways may be responsible for metal reduction by direct contact and across a physical separation.
5.4 References


