A REVISED MONOD-TYPE RATE LAW PREDICTING VARIABLE SULFUR ISOTOPE FRACTIONATION FACTORS AS A FUNCTION OF MICROBIAL SULFATE REDUCTION RATE

BY

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THESIS

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ABSTRACT

Microbial sulfate reduction is associated with characteristic sulfur isotope partitioning, which can serve as a proxy for the rate of this reactivity in a wide variety of reducing environments. We demonstrate a new model for this functional relationship through the use of a modified Monod-type rate expression constrained by a novel set of experiments. A series of batch reactors containing an identical amount Desulfovibrio vulgaris, a strain of sulfate reducing bacteria (SRB), were subjected to differing continuous mass addition rates of formate to minimize growth and control the rate of sulfate reduction via electron donor limitation. The isotopic composition (δ³⁴S) of the unreacted sulfate pool was measured through time for each experiment. This approach resulted in five steady state reduction rates of 2.06, 1.22, 0.83, 0.52 and 0.28 µmol*hr⁻¹ that enriched the unreacted sulfate in ³⁴S, where each rate was associated with a characteristic enrichment factor (αobs) of 0.9976, 0.9962, 0.9938, 0.9924 and 0.9903, respectively. This relationship was used to calibrate a coupled set of isotope-specific Monod rate laws that were modified to incorporate (1) both minimum (α₁ = 0.998) and maximum (α₂ = 0.930) fractionation factors and (2) a rate-controlling electron donor factor (DF). These parameters constrain a model which produced realistic predicted shifts in the apparent fractionation factor (αobs) as a function of reduction rate in an electron donor limited system. Application of these parameters in the updated model accurately reproduced our data and thus offers a means to predict the relationship between αobs and sulfate reduction rate. We argue that this approach offers a reasonable approximation to more detailed microbial reactive network models, while still maintaining sufficient simplicity and versatility to allow incorporation into multi-component reactive transport simulations. Thus, the current study provides a foundation for accurate simulation of rate-dependent fractionation in open, transient, and through-flowing systems.
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# TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION ......................................................................................... 1
CHAPTER 2: METHODS .............................................................................................. 8
CHAPTER 3: EXPERIMENTAL RESULTS ................................................................. 11
CHAPTER 4: DISCUSSION ......................................................................................... 15
CHAPTER 5: CONCLUSIONS .................................................................................... 26
CHAPTER 6: FUTURE PROJECTS ............................................................................. 27
FIGURES ................................................................................................................... 28
TABLES .................................................................................................................... 40
REFERENCES ........................................................................................................... 44
CHAPTER 1: INTRODUCTION

Microbial sulfate reduction is a critical component of the global sulfur and carbon cycle as it supports more than 12% of global carbon oxidation and generation of hydrogen sulfide (Bowles et al., 2014). Sulfate reduction is ubiquitous in nearly all oxygen-limited environments and plays a vital role in the biogeochemistry of both terrestrial and marine ecosystems. Hydrogen sulfide readily reacts with toxic metal ions such as As, U, Cd, Pb, and Zn to form metal-sulfide minerals that have exceptionally low solubility under anoxic conditions (Stumm and Morgan, 1996; Kirk et al., 2004; Peltier et al., 2003). Generation of excess hydrogen sulfide, however, can have adverse effects on drinking water as it is toxic to humans in concentrations greater than 1 ppm (Guidotti, 2010). Furthermore, it is a costly problem in the context of oil field souring and the deterioration of petroleum related infrastructure such as pipelines, drilling equipment, and storage facilities (Postgate, 2013; Cord-Ruwisch et al., 1987). In total, the ubiquity of microbially mediated sulfate reduction necessitates an accurate and robust ability to detect and quantify its impact in a wide variety of open, heterogeneous and transient environments. A key means of testing quantitative models to ensure accurate prediction of the spatiotemporal distribution of sulfate reduction is through the characteristic partitioning of stable sulfur isotopes. This diagnostic tool can assist in more precise predictions of sulfate reduction rates, which aid in quantifying the total flux of sulfate and reduced organic carbon (and hydrogen) in a system.

The complete reductive pathway from sulfate to sulfide requires a transfer of eight electrons, the generation of multiple intermediate redox species, and consequentially a mass-discrimination between sulfur isotopes ($^{32}$S and $^{34}$S), where $^{32}$SO$_4^{2-}$ generally reacts faster than $^{34}$SO$_4^{2-}$. This mass-discrimination is a result of the relative difference in activation energy (i.e. breaking S-O bonds) between to the two isotopologues, which creates slight difference in the likelihood of each isotopologue to participate in the enzyme-catalyzed electron transfers. The observable result of this is a broad range of natural isotope ratios ($\delta^{34}$S) across sulfur-bearing compounds in both natural and engineered environments.

For the remainder of this work, we define the cumulative extent to which the isotope ratio is altered through each reaction or transport process across the complete sulfate reduction pathway as the observed fractionation factor ($\alpha_{obs}$) (Eq. 1.1).
\[
\alpha_{obs} = \frac{R_B}{R_A}
\]  
(Eq. 1.1)

Where \( R_A \) is the isotopic ratio in the residual reactant phase A (i.e. sulfate), and \( R_B \) is the isotopic ratio of the instantaneous product flux contributing to the product phase B (i.e. H\(_2\)S). Given the short half-life of intermediate species involved in this reductive pathway, the isotopic ratio of the instantaneous product flux is often assumed to be equivalent to the isotopic ratio of the instantaneous reactant flux. As a result, the isotopic ratio of the unreacted sulfate pool can be used to constrain \( \alpha_{obs} \).

Previous work has demonstrated that the overall observed fractionation factor for sulfate reduction is highly dependent on both the cell specific sulfate reduction rate (csSRR) and the form and availability of reduced organic carbon (Kaplan & Rittenberg, 1964; Sim et al., 2011). Additionally, the microbial species-specific physiology (Bruchert et al., 2001; Detmers et al., 2001), the concentration of sulfate (Bradley et al., 2016; Rees, 1973), temperature (Hoek, 2006; Johnston, 2007), and the availability of nutrients (e.g. Fe, P, N) (Sim et al. 2012), also influence the patterns of fractionation. Thus, the use of sulfur isotope signals as a proxy for modern and ancient biogeochemical processes is contingent upon the fidelity of models describing the relationship between species-specific (or consortia-specific) isotope fractionation and these controlling factors. In the present study, we seek to improve model predictions for the overall observed fractionation factor (\( \alpha_{obs} \)) between sulfate and sulfide by allowing for rate-dependent shifts between maximum and minimum fractionation factors (i.e. \( \alpha_1 \) and \( \alpha_2 \)), which represent the upper and lower fractionation limits reported for sulfate reducing bacteria.

1.1 Detailed Microbial Reactive Network Models

Models for stable sulfur isotope fractionation associated with sulfate reduction have been under development since the late 1950s (Harrison & Thode, 1958) and vary widely in parameterization and complexity. Such models predict variations in observable sulfur isotope ratios based on the premise that (1) microbial redox reactions rely on enzyme-catalyzed transfer of electrons through the respiratory chain and (2) there is a fixed fractionation factor for each enzymatic step involving a redox reaction in this chain. This framework was adopted by Rees (1973) to construct a kinetic reactive pathway incorporating three reversible steps followed by a final irreversible step. This approach provided one of the first predictions of isotopic partitioning due to the balance of forward and backward fluxes across a multi-step reaction network and
identified conditions under which potentially large fractionation may occur. The Rees model is still widely accepted under the condition that intercellular pools of S-bearing species remain constant for a given steady state reduction rate, but further work has explicitly described additional intracellular steps and coexisting pathways (i.e. the trithionate pathway), which contribute both additional parameterization and a more nuanced understanding of the overall reductive process (Cypionka, 1995 chapter; Brunner & Bernasconi, 2005).

For instance, a generic model was used by Antler et al., (2017) to determine the isotopic fractionation associated with distinct reactive steps along the dissimilatory sulfate reduction (DSR) pathway. This model, which was slightly modified for this demonstration, is composed of five steps (four reversible) and accommodates multiple reduced sulfur intermediates (i.e. trithionate and thiosulfate) (Fig. 1.1). Where each arrow represents a kinetic reaction with an associated rate constant \(k\) and fractionation factor \(\alpha\). The result is a model capable of predicting a variable overall observed fractionation factor \(\alpha_{\text{obs}}\) as a function of the net sulfur species flux across every step. This model features an initial uptake step (step 1) which is likely an active cellular process involving a sulfate permease enzyme rather than passive diffusion (Pilsyk and Paszewski, 2009; Madigan et al., 2003); it is thought the negative charge of sulfate would prohibit diffusive uptake since the cytoplasmic membrane is also negatively charged during normal cell function (i.e. H' pumping). Enrichment along this initial forward step, though small, should be apparent as the membrane-bound sulfate permease enzyme catalyzes a kinetic process, and thus, exhibits preferential transport of the lighter isotope. The next step (step 2) involves the conversion of intercellular sulfate into APS (adenosine-5'-phosphosulfate), which is requires an ATP and exhibits a small fractionation factor (Farquhar et al., 2003). A significant mass discrimination \(\alpha_3 = 25\%\) is thought to be associated with step 3 (Rees, 1973), involving the forward and reverse reaction of the reduction of APS to sulfite. Step 3 also facilitates the first reductive step as the oxidation state of sulfate transitions from (+6) to (+4). Step 4 involves the reduction of sulfite to sulfide (+4 to -2), ultimately representing a six-electron transfer via dissimilatory sulfite reductase (Dsr), which is exhibits a large fractionation factor \(\alpha_4 = 25\%\). In this model (Fig 1.1), *S²⁻ represents a multitude of reduced S intermediates (e.g. thiosulfate and trithionate). The final step (step 5) represents transport of the completely reduced sulfur species into the surrounding environment and is likely associated with a small enrichment factor.
(Brunner and Bernasconi, 2005). We show this step as irreversible because it is unlikely that bisulfide or sulfide would reenter the cell.

It should be noted that many, if not most, of the individual steps in the reaction networks of most common SRB strains have been shown to exhibit reversibility. Each of these steps, both forward or reverse, which induce a fractionation, are hereafter referred to as ‘branching points’ (e.g., Farquhar et al., 2003). However, the reverse branching points are typically assumed to be negligible (Brunner and Bernasconi, 2005) Thus, a key inference from detailed models such as the one presented here is that the observable fractionation factor ($\alpha_{obs}$) reflects a balance between the net forward and net reverse fluxes through each branching point (e.g., Brunner et al. 2012), and that each of these branching points exhibits a unique and fixed fractionation factor.

1.2 Functional Relationship Models

While current developments characterizing the DSR reactive network often focus on the use of multi-isotope tracers to constrain the flux across individual intracellular pathways (Antler et al., 2017; Brunner and Bernasconi, 2005), application of sulfur isotope ratios as proxies for contemporary microbial activity in natural and engineered systems is accomplished with comparatively simplified models, which inherently restrict or eliminate the ability to treat variability in the overall observed fractionation factor (Druhan et al., 2012, 2014; Berna et al., 2009; Sherwood Lollar et al., 2011; Sherwood Lollar et al., 1999; Ahad et al., 2000; Bolliger et al., 1999; etc.). Thus, a clear disparity exists between the predictive models used to describe highly controlled experimental conditions, and the quantitative treatment of natural, heterogeneous and transient systems.

Considering the most simplified case, where the csSRR is operating at the maximum feasible rate, the microbial reactive pathway behaves like a unidirectional process and the corresponding fractionation factor is constant and dictated by the first branching point (i.e. uptake). Isotope partitioning in such idealized conditions, typical of some engineered bioremediation sites and eutrophic systems, are thus reliably quantified using a simple distillation or Rayleigh-type model with a single observed (i.e. ‘effective’) fractionation factor (Criss, 1999; Druhan et al., 2014; Bottcher et al, 1990; Richnow et al., 2003; Heraty et al., 1999). Though employed extensively, many examples exist in which this single fractionation factor is not suitable to describe observations in biologically-mediated redox environments for a variety
of stable isotope systems (Berna et al., 2009; Aravena et al., 1998; Barker & Fritz, 1981; Schroth et al., 2001; Brandes & Devol, 1997). The reason for this variability in the fractionation factor is thought to derive from the unique conditions of energy limited environments (i.e. subsurface continent and marine sediments), in which sulfate reducing bacteria operate at very slow respiration rates (Whitman et al., 1998; Kallmeyer et al, 2012), which we anticipate to be spatially and temporally variable.

In order to predict isotope fractionations in systems that exhibit a range of $\alpha_{\text{obs}}$, the unidirectional first-order model (i.e. one branching point) may be expanded to incorporate reversibility through a second reactive step, which leads to the general form of isotope-enabled Monod kinetics (Fig 1.2) – commonly referred to as a “two-step” model under the assumptions specified by Monod kinetics (Monod, 1949). Here [S] is the concentration of the substrate (e.g. sulfate), [Bio] is the concentration of biomass in the system that is available to metabolize the substrate, [Bio-S] represents the concentration of all intercellular substrate species in the system (i.e. enzyme complexed and/or reduced species), and [P] is the concentration of the resulting product species (e.g. HS'). In this framework each arrow represents a kinetic reaction that doubles as a branching point; all of which have a characteristic rate constant ($k$) and fractionation factor ($\alpha$). The first forward branching point ($k_1$, $\alpha_1$) corresponds to substrate uptake and the reverse branching point ($k_{-1}$, $\alpha_{-1}$) corresponds to the process by which the substrate, that may be enriched, can leave the cell. The second forward reaction ($k_2$, $\alpha_2$) serves as an approximation to all subsequent metabolic steps and is associated with the largest possible fractionation factor ($\alpha_2$) that can be produced by combining these branching points. The extent to which $\alpha_2$ is expressed in the external substrate is thus a function of the overall rate, whereby slower rates create a situation in which the intercellular substrate pool has time to (1) become enriched by $\alpha_2$ and (2) leave the cell at a rate which competes with the net forward rate. As the overall rate approaches a maximum, the reverse process no longer exhibits significant control on the rate and is effectively suppressed. This situation leads to the dampening fractionations associated with $\alpha_2$ because any enriched intercellular substrate that leaves the cell is overwhelmed by the net forward mass flux.

Using this isotope-specific version of the Monod general form (Fig.1 2.), a rate law can be derived for each isotopologue of the substrate (Eq. 1.2, 1.3) (Druhan et al., 2014). Additionally, these rate law(s) take into account the concentration of the electron donor on the overall rate (i.e. a dual-Monod formulation).
\[ r = k_2 [Bio_T] \left( \frac{[D]}{[D] + K_D'} \right) \left( \frac{[A]}{[A] + K_A \left( 1 + \frac{[A']}{K_A} \right)} \right) \]  

(Eq. 1.2)

\[ r' = k_2' [Bio_T] \left( \frac{[D]}{[D] + K_D} \right) \left( \frac{[A']}{[A'] + K_A' \left( 1 + \frac{[A]}{K_A'} \right)} \right) \]  

(Eq. 1.3)

Where \( r \) (\( r' \)) (mol\*L\(^{-1}\)*T\(^{-1}\)) is the rate of the common (rare) isotopologue and \( k_2 \) (\( k_2' \)) (mol\*cell\(^{-1}\)*T\(^{-1}\)) is the rate constant term associated with the forward reaction for the common (rare) isotopologue. \( Bio_T \) (cell\*L\(^{-1}\)) is the concentration of the total biomass in the system, \([A']\) is the concentration of the electron acceptor (i.e. the substrate) for the common (rare) isotopologue, \([D]\) is the concentration of the electron donor, \( K_A \) (\( K_A' \)) (mol\*L\(^{-1}\)) is the half-saturation term for the common (rare) isotopologue of the electron acceptor (Eq. 1.4 (Eq. 1.5)), and \( K_D \) (mol\*L\(^{-1}\)) is the half-saturation term corresponding to the electron donor.

\[ K_A = \frac{k_2 + k_{-1}}{k_1} \]  

(Eq. 1.4)

\[ K_A' = \frac{k_2' + k_{-1}'}{k_1'} \]  

(Eq. 1.5)

Where \( k_1 \) (\( k_1' \)) and \( k_{-1} \) (\( k_{-1}' \)) are the rate constants for uptake and substrate release from the cell, respectively, for the common (rare) isotopologue. It is important to mention that the half-saturation expression(s) (Eq. 1.4 and Eq. 1.5) represent a balance between the three kinetic reactions, and consequently determine the conditions under which fractionation will approach \( \alpha_1 \) or \( \alpha_2 \). Furthermore, the quotient of the concentration of electron acceptor \([A']\) by the sum of the electron acceptor and corresponding half-saturation term in Eq. 1.2 and 1.3 is a value ranging between 0 – 1 and is hereafter referred to as the “Monod term”.

\[ \frac{[A']}{[A'] + K_A' \left( 1 + \frac{[A]}{K_A'} \right)} \]
These coupled isotope-enabled dual-Monod rate laws are potentially capable of treating both fixed (Rayleigh-style) fractionation as well as variable $\alpha_{\text{obs}}$ as a function of the overall rate within a reactive transport framework. Thus far, applications of these expressions have employed the simplifying assumption that the second, irreversible step of the reactive pathway (Fig. 1.2) is the only branching point (Druhan et al 2012, 2014; Wanner et al., 2014; Jamieson-Hanes et al., 2012). This effectively reverts the Monod-type rate expression(s) to an isotopic model equivalent to a fixed fractionation factor, or a Rayleigh-style distillation process. Thus, at present, isotope-enabled reactive transport simulations of microbial sulfate reduction are limited because they do not consider the extensive experimental observations and theoretical predictions for variable $\alpha_{\text{obs}}$ as a function of SRR. In this study, we seek to reconcile this disconnect through further development of two-step models capable of treating both the reversible and irreversible components as branching points. We test the fidelity of this approach against a unique dataset in which csSRR is precisely controlled, by prohibiting biomass growth, such that the relationship between rate and the observed fractionation factor is accurately constrained.
CHAPTER 2: METHODS

2.1 Culture Incubation

One liter of growth media for live cultures was prepared using a 2.0 L Erlenmeyer flask sparged with a constant N\textsubscript{2} stream to maintain anaerobic conditions. First, 1.00 L of 18.2 M\text{Ω} water was added to the 2.0 L Erlenmeyer flask and allowed to degas while stirred and gently heated for ~30 minutes. The following were made as concentrated stock solutions then added via pipette for a target concentration: KH\textsubscript{2}PO\textsubscript{4} (2 mM), CaCl\textsubscript{2} (1.67 mM), MgCl\textsubscript{2} (1.9 mM), FeCl\textsubscript{2} (1 mM), NH\textsubscript{4}Cl (2 mM), MnCl\textsubscript{2} (0.63 mM), H\textsubscript{3}BO\textsubscript{3} (0.03 mM), ZnCl\textsubscript{2} (0.07 mM), CoCl\textsubscript{2} (0.07 mM), NiSO\textsubscript{4} (0.089 mM), CuCl\textsubscript{2} (0.05 mM), NaMoO\textsubscript{4} (0.02 mM), Na\textsubscript{2}SeO\textsubscript{4} (0.57 μM), and Na\textsubscript{2}WO\textsubscript{4} (1.65 µM). The media was brought to a boil and then cooled in an ice bath to room temperature before 0.840 g (10.0mM) NaHCO\textsubscript{3} and 0.030 g (0.25 mM) L-cysteine – as a reductant – were added. Finally, CO\textsubscript{2(g)} was introduced with the N\textsubscript{2} stream until the pH of the media reached 7.2 and remained constant.

Ten 160 mL glass serum bottles were degassed simultaneously, and 100 mL of media was added to each serum bottle using a degassed 60 mL syringe and flexible silicone tubing; these transfers were made on a balance to ensure volume precision. Once the media had been transferred to the serum bottles, they were allowed to degas for another 10 minutes before butyl rubber stoppers were quickly inserted into the opening of the bottles and sealed with an aluminum crimp. A long (4") 22-gauge needle was inserted into the liquid and the same gas (CO\textsubscript{2} + N\textsubscript{2}) was vented through a second short needle to avoid loss of any liquid for ~30 minutes to remove any O\textsubscript{2}. The bottles were then autoclaved on a 20-minute liquid cycle. Once at room temperature, anaerobically prepared stock solutions of sterile Na-sulfate, Na-Pyruvate, and Na-acetate were added to the serum botte to reach target concentrations of approximately 2.0, 16.0, and 3.0 mM, respectively. Additionally, 1.0 mL of yeast (1.00 g*L\textsuperscript{-1}) extract and 1.0 mL of Wolfe’s vitamins (ATCC®; Medium 2672) were added to all of the growth media bottles.

A pure culture of *Desulfovibrio vulgaris* str. Hildenborough (DZMZ 644, ATCC 29579) was used to inoculate one serum bottle (2.0 mL of inoculum). The cultures grew and respired for 20 days, at which time the pyruvate concentrations fell below 0.10 mM. Anions were measured on a Metrohm 761 Comact Ion Chromatograph (IC) coupled to a Metrosep A SUPP 5-150 column. second transfer was made using 4.0 mL of inoculum into the same sterile growth media, which respired and grew for 33 days and pyruvate concentrations fell below 0.10 mM. A
third transfer into the same sterile media with 4.0 mL of inoculum grew and respired for 16 days and pyruvate concentrations fell below 0.10 mM. This third generation of *D. vulgaris* was used for reactor experiments.

### 2.2 Reactor Operation

The method described in section 2.1 was implemented to prepare the ten 160 mL serum bottles (reactors) with 100.0 mL of growth media. However, there was no pyruvate in these serum bottles. Instead, sulfate (4.0 mM target), formate (1.0 mM target for pump-controlled rate reactors and ~16.0 mM target for the maximum rate reactor), and acetate (2.0 mM target for pump-controlled rate reactors and 5.0 mM target for the maximum rate reactors) were added to each vessel before inoculation. The third generation of *D. vulgaris* was diluted into each reactor (10.0 mL inoculum into 100 mL medium) and metabolism proceeded for 48 hours. After this initial respiration interval, formate mass addition began through the use of a syringe pump for the controlled rate reactors, while the maximum rate reactor and associated replicate proceeded with the initial formate and sulfate amendments.

A method adopted form He and Sanford (2004) was implement for controlled rate reactors (Fig 2.1). Four 2.5 mL syringes (SGE™ Gas Tight PTFE Luer Lock), plus replicates, were filled with four distinct formate and bromide solutions. The syringes held a formate(bromide) stock with a concentration of 50.0(50.0), 100.0(200.0), 200.0(100.0), and 400.0(200.0) mM for Rate(s) 1 through 4, respectively. These syringes were loaded onto a ten-channel syringe pump (KD Scientific™; model no. KDS-230), which was programmed to dispense a volumetric flux (q) of 0.02 mL/hr. Peek tubing (1/16” OD x 0.01” ID) was used to connect the syringes to stainless steel needles (22-gauge x 4.0”) that were inserted into the bioreactors and further submerged into the reactor liquid; this avoided the formation of droplets on the needle tip which would have resulted in large pulses of formate as opposed to continuous injection. A shut off valve (KD Scientific™; model no. P-732) was fixed along the injection line in order seal the reactors during injectate refilling. All reactors and replicates, including the maximum rate reactors, were placed onto an orbital shaker table (~80 r.p.m.) and covered with a cardboard box lined with aluminum foil to minimize exposure to light. The experiment was carried out in open air in a laboratory that was 21 ± 2.0°C.
The reactors were vented weekly with a sterile needle (i.e. ~2 sec gas release) to avoid excessive pressure build up in the headspace. A sterile and degassed syringe and needle was used to sample the reactors; the volume of the sample was determined by predicting the sulfate concentration, such that there was enough sulfate for isotopic analysis. The volume of each reactor was tracked and used for mass calculations. Once the sample was pulled from the reactors, it was filtered through a 0.20 µm nylon membrane. A small portion of the filtrate was used for anion analysis (e.g. sulfate, formate, acetate, chloride, bromide) and the remaining filtrate received a barium chloride solution (10 eq. according to predicted sulfate concentrations) to precipitate barium sulfate solid for isotopic analysis. The BaSO₄(s) was chilled at 2.0°C overnight to facilitate more precipitation for improved yields. The BaSO₄(s) slurry was then drawn into a syringe and filtered through a reusable syringe filter (Millipore™ Swinnex Filter Holder, 13mm) and the solid was collected onto a 0.22 µm membrane. The original slurry vessel was rinsed twice with 18.2 MΩ and filtered through the same membrane and filter holder to improve yield and further remove any impurities. The membrane was removed from the holder and the solid was rinsed into a 4.0 mL glass vial and placed on a hot plate (~80°C) to dry.

Dried BaSO₄(s) samples were analyzed in duplicate at the Indiana University Stable Isotope Research Facility (SIRF) in Bloomington, IN using a Thermo delta V coupled to an Elemental Analyzer inlet. Isotopic values (δ³⁴S) of samples were compared to the International Atomic Energy Agency (IAEA) standards for sulfur (S1, S2, S3) and reported on the Vienna Canyon Diablo Troilite (VCDT) scale. Uncertainty was ± 0.3‰ for this method.
CHAPTER 3: EXPERIMENTAL RESULTS

Four controlled steady state sulfate reduction rates were achieved by mass delivery of formate, and a fifth, maximum steady state rate was achieved by one initial amendment of formate in stoichiometric proportion with sulfate (Table 3.1). The rate of sulfate reduction was calculated based on the change of dilution corrected sulfate mass ($M_{SO_4}$) with time. The criteria for steady-state reduction was established based on the linear regression of the sulfate mass through time, such that an $R^2 \geq 0.950$ was required for the linear regression. When deviation from this steady state criteria was observed, the corresponding isotopic data was not included in subsequent interpretation.

The extent to which the population grew in these systems was not directly constrained, but it is assumed to be minimal due to (1) the choice of electron donor (2) the relatively high starting biomass concentration and (3) very little acetate mass loss which would be used as a carbon source for growth. A linear regression through the dilution corrected sulfate mass through time was used to calculate the rate. Based on these methods, we suggest that there was no significant cell growth during this experiment.

Each rate exhibited a characteristic observed fractionation factor, which increased with decreasing rate. The bromide delivered along with the formate served as a conservative tracer to monitor injection consistency and aided in identifying a leak in one replicate. Since chloride is a conservative tracer that can only be removed from the system through sampling, it was used to normalize other species concentrations, primarily acetate, to further quantify uncertainty from the anion analysis.

The $\delta^{34}$S versus IAEA sulfur standards (plotted on the VCDT scale) of the precipitated BaSO$_4$(s) were compared with the associated dilution corrected sulfate mass fraction ($M/M_0$) in each reactor for each sampling interval. The fractionation factor for each reactor was calculated using a Rayleigh distillation model, which is appropriate due to the fact that the reactors are (1) closed systems and (2) exhibit one fractionation factor during for steady state sulfate reduction rate. The details of each bioreactor experiment are discussed further below.
3.1 Rate 1

Rate 1 replicate reactors exhibited the slowest reduction rate of all four experiments at a steady state value of 0.28 µmol sulfate/hr (R² = 0.986). Between replicates, the average sulfate concentration decreased from 3.67 ± 0.09 mM to 0.58 ± 0.01 mM during the total 1359.25 hours of injection (Fig 3.1). Steady-state was achieved for 1,004 hours beginning at the time of inoculation, and formate was held constant (0.12 ± 0.03 mM) throughout the duration. As sulfate concentrations approached 0.82 ± 0.01 mM at 1194 hours, the formate began to accumulate. There was no change in the chloride normalized acetate mass indicating no loss of the initial acetate mass due to cell activity. The calculated αobs based on reaction progress (i.e. M/M₀) and measured δ³⁴S values was 0.9903 ± 0.0007 for both replicate reactors over the 1,004 hours determined to exhibit steady state reduction (Fig 3.2).

3.2 Rate 2

At steady state, Rate 2 replicate reactors achieved a sulfate reduction rate of 0.52 ± 0.02 µmol/hr (R² = 0.994 ± 0.004). However, for one replicate, steady state reduction began after a 48-hour delay due to a leak at the syringe-feed tube connection, which prevented injectate delivery for 48 hours after pumping began. Between the replicate reactors, the average sulfate concentration decreased from 3.68 ± 0.02 mM to 0.65 ± 0.06 mM during the total 667.50 hours of injection (Fig. 3.3). A steady state reduction rate was achieved for 621 hours beginning at inoculation for one reactor, and 573 hours beginning at 48 hours after injection began for the replicate that was delayed due to the leak. During steady state, formate was held constant at 0.16 ± 0.02 mM until sulfate concentrations approached 0.88 ± 0.11 mM after 621 hours, at which point formate began to accumulate in both reactors. The chloride normalized acetate mass in each replicate exhibited an 8 ± 4 µmol (3.35 ± 1.17%) decrease in initial acetate mass due to cell activity. The calculated αobs for replicate reactors for the 621 and 573-hour period of steady state reduction was 0.9924 ± 0.0008 (Fig. 3.4).
3.3 Rate 3

Rate 3 replicate reactors achieved a steady state sulfate reduction rate of 0.83 ± 0.01 \( \mu \text{mol/hour} \) (\( R^2 = 0.987 \pm 0.002 \)). The average sulfate concentration between replicate reactors decreased from 3.58 ± 0.01 mM to 0.55 ± 0.02 mM during the total 478.50 hours of injection (Fig 3.5). Steady state reduction was achieved for 430 hours beginning at the time of inoculation. In one reactor (hollow markers), a steady state formate concentration of 0.19 ± 0.07 mM was observed. However, in the replicate reactor (filled markers), formate concentrations were less stable and exhibited an increase of formate to 0.69 mM at 120 hours, but this did not affect the reduction rate and is thought to be caused by an analytical error and/or a sample that did not reflect the true concentration at that time. Neglecting this isolated spike in concentration, formate in both reactors was held at 0.22 ± 0.10 mM for the period of steady state reduction. After 430 hours, formate began to accumulate as sulfate dropped below 0.58 ± 0.01 mM. Bromide measurements were not performed beyond 287 hours and thus, bromide concentration is unknown for the final 143 hours of steady state reduction. However, the sulfate reduction rate did not deviate from steady-state during this period, and further, formate began to accumulate after the period of steady state, indicating that normal injectate delivery persisted throughout this 143-hour period. In both reactors the chloride normalized acetate concentration for the entirety of the experiment exhibited 5 ± 4 \( \mu \text{mol} \) (2.28 ± 1.82%) loss of the initial acetate mass due to cell activity. The \( \alpha_{\text{obs}} \) calculated from the unreacted sulfate pool was 0.9938 ± 0.0010 for the 430-hour period of steady state sulfate reduction (Fig. 3.6.).

3.4 Rate 4

Rate 4 replicate reactors achieved a steady state sulfate reduction rate of 1.22 ± 0.01 \( \mu \text{mol/hour} \) (\( R^2 = 0.966 \pm 0.006 \)). For both reactors, the average sulfate concentration decreased from 3.34 ± 0.01 mM to 0.23 ± 0.04 mM over a total 287 hours of reduction (Fig 3.7). The standard deviation for formate concentrations were 80%-100% of the average concentrations and thus formate was considered to be relatively unstable in Rate Experiment 4. This instability is likely associated with the delivery of a high concentration injectate (400 mM), which is then subsequently mixed into the incubation vessel through the action of the shaker table. Despite the unstable nature of formate, a linear trend in the sulfate reduction rate was observed and did not
change significantly throughout the 287 hours of reaction. Thus, we maintain that a steady state reduction was achieved across the complete 287 hours beginning at inoculation. In one duplicate (hollow markers), a leak occurred between 0 and ~12 hours, which slowed formate addition. This leak was fixed immediately, and the targeted reduction rate was re-established before the next sampling point at 48 hours. Bromide concentrations demonstrate stable injection aside from the leak. The chloride normalized acetate concentrations show that the loss of initial acetate mass due to cell activity was 10 ± 4 µmols (4.24 ± 1.06%) over the 287-hour period and the $\alpha_{\text{obs}}$ calculated using a Rayleigh model was 0.9962 ± 0.0006 during steady state sulfate reduction (Fig. 3.8).

3.5 Maximum Rate

The Maximum Rate Experiment did not implement controlled delivery of electron donor. Instead, replicate reactors containing 4.52 ± 0.04 mM sulfate and 17.26 ± 0.04 mM formate at the time of inoculation were allowed to respire freely beginning at the same time as inoculation of all other reactors (Fig. 3.9). After 262 hours of metabolism sulfate and formate concentrations decreased to 0.56 ± 0.05 mM and 1.63 ± 0.10 mM, respectively (Fig 3.8). We note that the reaction proceeded in stoichiometric fashion throughout the duration of the experiment.

Though formate did not limit the reaction, a steady state sulfate reduction rate of 1.65 ± 0.03 µmole*hr\(^{-1}\) ($r^2 = 0.974 ± 0.009$) was achieved for a 262-hour period. These two reactors received 80.0% of the biomass compared to Rate(s) 1 – 4. Therefore, we correct the overall reduction rate (i.e. increase by 20.0%) to 2.06 ± 0.04 µmole*hr\(^{-1}\), such that the csSRR is normalized to the biomass within the pump-controlled reactors. The chloride normalized acetate concentrations in this reactor demonstrated a 0.5% decrease in the initial acetate mass over the 262-hour period and the $\alpha_{\text{obs}}$ was calculated as 0.9976 ± 0.0004 (Fig 3.10).

A relationship between the rate of sulfate reduction for each reactor and the corresponding observed fractionation factor was clearly exhibited, such that as the rate of reduction decreased, $\alpha_{\text{obs}}$ increased (Fig. 3.11). This relationship is discussed in further detail below.
CHAPTER 4: DISCUSSION

4.1 Model development

The results of these growth-regulated experiments clearly indicate a functional relationship between the observed fractionation factor and the sulfate reduction rate (Fig. 3.11). This is consistent with the results of many prior studies (Harrison and Thode, 1958; Rees, 1973; Bolliger et al., 2001; Rudnicki et al., 2001; Brunner and Bernasconi, 2005; Kaplan and Rittenberg, 1964). The reactors that received a constant injectate via pumping were designed to support simplified parameterization that is both accurate and unambiguous. This is accomplished through three principle means. First, we negate cell growth so that every cell within a reactor respires at the same rate, thus fixing the csSRR in each experiment and the biomass across all experiments. Second, precise control of the formate mass delivery rate to the cell population enables quantitative constraint of the electron donor availability as a fixed parameter value for each rate. Third, all other nutrients for cell respiration exist in excess, such that microbial activity is limited by the electron donor. Additionally, the use of one bacterial strain eliminates any disparity between intracellular reactive pathways, such that a single $\alpha_{obs}$ can be treated as a result of a single observable SRR. We note that the maximum rate experiment, as a result of negligible cell growth, proceeded with steady state reduction, and therefore supports accurate and unambiguous parameterization similar to the pump-fed reactors.

The reported datasets are thus appropriate to validate a novel modeling approach designed to predict the rates and associated isotope fractionations in electron-donor limited systems. We develop this model using a new modified Monod half-saturation term, which operates as a variable rather than a fixed value, and a new electron donor expression. This expanded modeling capability is first accomplished by revision of the 'dual-Monod' rate expression (Rittmann and McCarty, 2001; Jin and Bethke, 2003), where instead of treating the electron donor in the same manner as the electron acceptor (i.e. Monod term), we introduce a nondimensional scaling factor, hereafter referred to as the “donor factor” $(D_F)$. The pump-fed reactors facilitated the instantaneous and near-complete oxidation of formate as it entered the reactor. As a result, use of a typical 'dual-Monod' rate expression for the electron donor was inappropriate for our purposes. Further, the concentration of sulfate was above the threshold concentration for a zero-order rate regime (Rees, 1973; Wing and Halevy, 2014; etc.) during steady state reduction, and therefore did not exhibit significant control on the rate for the
majority of the experiment. The new expression for the overall rate of sulfate reduction (Eq. 4.1) incorporating the $D_F$ is given as:

$$r = k_2 D_F [Bio_T] \frac{[SO_4^{2-}]}{[SO_4^{2-}] + K_{1/2}^*}$$

(Eq. 4.1)

Where $k_2$ (mol*cell$^{-1}$*time$^{-1}$) is the maximum specific growth rate, $D_F$ is the donor factor which ranges from 0 - 1, $[Bio_T]$ (cell*L$^{-1}$) is the concentration of biomass in the system, $[SO_4^{2-}]$ is the concentration of the substrate, and $K_{1/2}^*$ (mol*L$^{-1}$) is our modified substrate-specific half-saturation term (Eq. 4.2), which includes the donor factor as a scaling parameter representing microbial adaptation to environmental stressors (discussed in detail below).

$$K_{1/2}^* = \frac{D_F k_2 + k_{-1}}{k_1}$$

(Eq. 4.2)

In the modified half-saturation term $K_{1/2}^*$, $D_F$ and $k_2$ are consistent with the definitions given for Eq. 4.1, and $k_{-1}$ and $k_1$ are the rate constants associated with the reversible reaction as described by the general functional form of the Monod rate law (Fig. 1.2).

The true novelty of this approach lies in the modified half-saturation term, which is no longer a constant for a given microorganism as it has been treated previously (Eppley et al., 2009; Jin and Bethke, 2003; Smith and Klug, 1981). Instead, the value of this parameter is a variable that decreases with decreasing available organic carbon (i.e. smaller $D_F$). In standard Monod kinetics, the half-saturation term denotes the substrate concentration at which half of the maximum rate is achieved and as such, it quantifies the balance between effective forward and reverse reactive pathways by dictating the conditions under which reversibility (i.e. substrate leaving the cell) exhibits significant control on the overall rate. Further, it is an approximation to the ensemble of intercellular steps (i.e. the weighted expression of multiple Michaelis-Menton parameter values) that govern the observable behavior of a cell. Thus, if the supply of enzymes for a particular process is adjusted as the cell responds to its environment, the apparent value of the half-saturation ‘constant’ would also be anticipated to change.

This behavior is consistent with prior work showing clear evidence for an apparent decrease in the half-saturation value in association with a higher substrate affinity (Li et al.,

16
Jin et al. (2013) reported variation in the sulfate-associated half-saturation value by at least two orders of magnitude as a function of the concentration of sulfate (Fig. 4.1). In each study, a half-saturation value was determined for a unique consortia of sulfate reducers in either marine, brackish, or freshwater environments. In marine and brackish consortia, where sulfate concentrations are high, relatively large $K_{1/2}$ values are observed, whereas freshwater consortia exhibit relatively small $K_{1/2}$ values associated with comparatively low sulfate concentrations.

Though this observational relationship does not explain the mechanism(s) by which (or the timescale over which) a microorganism can alter its half-saturation constant, it does indicate that half-saturation values can be considerably different for the same overall process. Therefore, we justify the current modeling approach in which relatively small changes (less than one order of magnitude) in the half-saturation value within the modified Monod rate expression are implemented as a function of electron donor availability. The outcome is that our adjusted model honors the observed behavior, wherein high sulfate conditions promote large half-saturation values, whereas low sulfate conditions promote small half-saturation values, thus incorporating the ability of a microbial population to optimize their substrate affinity in order to survive in a range of environmental conditions.

Critically, modifying the half-saturation term does not alter predictions for the total rate of sulfate reduction in sulfate-rich environments from that of a classic Monod model (e.g. eq. 1.2) because such a small change in the half-saturation value (within the range created by eq. 4.2) imposes a negligible shift in the overall rate. Instead, the need for this modification arises from the treatment of sulfur isotopes, and therefore the model is designed to support variable observed fractionation factors while imparting minimal impact on the total rate of reduction.

Through this modified functional form, we introduce the unique capacity to assign two distinct fractionation factors, $\alpha_1$ (Eq. 4.3) and $\alpha_2$ (Eq. 4.4), as the smallest observable fractionation factor demonstrated by these experiments ($\sim 0.998$), and the approximate maximum fractionation limit exhibited by sulfate reducers (0.930) (Sim et al, 2011; Wing and Halevy, 2014; Bradley et al, 2016; Leavitt et at, 2013), respectively.
\[
\alpha_1 = \frac{34k_1}{32k_1} = 0.998
\]
(Eq. 4.3)

\[
\alpha_2 = \frac{34k_2}{32k_2} = 0.930
\]
(Eq. 4.4)

Where \(\alpha_1\) is the fractionation factor assigned to the first forward step corresponding to the general Monod expression (Fig. 1.2), and \(\alpha_2\) is the fractionation factor assigned to the second irreversible step. As a result, the calculated overall fractionation factor is bounded within the range designated by \(\alpha_1\) and \(\alpha_2\). Notably, the \(\alpha\) assigned to the reverse reaction \((k_1)\) is non-fractionating \((\alpha_1 = 1.000)\) because the reversibility serves as a pathway by which \(\alpha_2\) is expressed, and therefore any fractionation for this step would be redundant.

This approach thus serves as a generalized, isotope-enabled approximation to the complexity of fractionating processes (i.e. forward and reverse branching points) known to occur during the complete reduction of sulfate to sulfide through a modified Monod-type expression with two branching points. The coupled isotope-enabled rate laws for sulfate reduction, originally developed by Druhan et al, (2014) (Eq. 1.2, 1.3) are modified to incorporate \(D_F\) and \(K_{1/2}^*\), and implementation of the corresponding \(^{32}\)S and \(^{34}\)S specific parameters are, for the first time, treated in both the maximum specific growth rate and half saturation terms (Eq. 4.5, Eq. 4.6).

\[
^{32}r = D_F^{32}k_2[Bio_T] \frac{[^{32}SO_4^{2-}]}{[^{32}SO_4^{2-}] + ^{32}K_{1/2}^* \left(1 + \frac{[^{34}SO_4^{2-}]}{^{34}K_{1/2}^*}\right)}
\]
(Eq. 4.5)

\[
^{34}r = D_F^{34}k_2[Bio_T] \frac{[^{34}SO_4^{2-}]}{[^{34}SO_4^{2-}] + ^{34}K_{1/2}^* \left(1 + \frac{[^{32}SO_4^{2-}]}{^{32}K_{1/2}^*}\right)}
\]
(Eq. 4.6)

Here, \(D_F\) and \([Bio_T]\) are common to both isotopes and the modified half-saturation expressions (Eq. 4.7, Eq. 4.8) become:
\[ \frac{32K_{1/2}^*}{32k_1} = \frac{D_F^{32}k_2 + 32k_{-1}}{32k_1} \]  
(Eq. 4.7)

\[ \frac{34K_{1/2}^*}{34k_1} = \frac{D_F^{34}k_2 + 34k_{-1}}{34k_1} \]  
(Eq. 4.8).

In equations 4.5-4.8, The functional treatment of \( k_2 \) as a parameter scaled by \( D_F \) is consistently implemented both in the overall rate expression and within the modified half-saturation terms. This product adjusts the overall value of the rate and the half-saturation term to reflect the current environmental conditions of the system. Further, the appearance of \( D_F \) as a multiple of both the maximum rate constant \( (k_2) \), and within the denominator of the Monod term\( (K_{1/2}^*) \), creates a competition between these factors. This opposition results in overall isotope-specific rate expressions that do not scale linearly with \( D_F \), as discussed in further detail below.

4.2 Model Behavior

A set of parameter values (Table 4.1) that correlate with a reasonable natural system were chosen to illustrate the behavior of the model across a broad range of reduction rates during electron donor limited conditions. A total of 500 simulations were performed across the complete range of \( D_F \) between 0 – 1 at a uniform interval \( (\Delta D_F = 0.002) \), using an initial sulfate concentration of 3.5mM, each for 1000 hours \( (\Delta t = 0.5hrs) \). It is worthwhile to note that the full range in \( D_F \) results in a range of \( 32K_{1/2}^* \) \( (34K_{1/2}^*) \) values for the chosen parameter set between 0.150 (0.141) and 1.018 (0.950). We again emphasize that this relatively minor change in the two half-saturation terms has a small influence on the predicted overall rate, however, it exerts a significant influence on the corresponding \( \alpha_{obs} \).

These calculations yield a predictive relationship between rate and \( \alpha_{obs} \) (Fig 4.2), where each simulated rate has been normalized to the maximum rate \( (D_F = 1.0) \). As described above, the resulting \( \alpha_{obs} \) is bounded between \( \alpha_1 \) and \( \alpha_2 \) across the complete range of normalized rates and produces the anticipated increase in fractionation as the overall rate of the reaction decreases.
A key behavior of this model is that a unique and constant $\alpha_{\text{obs}}$ is predicted for every steady state reduction rate (Fig 4.3), regardless of the progress of the reaction, or the depletion of sulfate. This is an important benchmark for the model because we wish to emphasize the ability to accommodate systems where the rate of the reaction is zero-order with respect to sulfate and limited by the availability of the electron donor, as is typical of many natural environments (Whitman et al., 1998; Wenk et al., 2018). The extent of the reaction for each simulation, illustrated as the change in concentration of sulfate relative to the initial value ($C/C_0$), is thus associated with a unique and constant $\alpha_{\text{obs}}$. Though $\alpha_{\text{obs}}$ remains constant for a given rate, the change in alpha does not scale linearly with changing rate (Fig. 4.3). Therefore, a key prediction of the proposed model is that $\alpha_{\text{obs}}$ is more sensitive, and thus offers better constraint, on csSRR as the overall rate of the reaction decreases, or as environmental conditions become more oligotrophic.

The use of fixed $\alpha_1$ and $\alpha_2$ parameters suggests that this model may not be appropriate for sulfate-limited systems where sulfate concentrations approach or fall below the characteristic half-saturation concentration. However, previous work has shown that sulfate-limited systems exhibit a relatively minor variability in $\alpha_{\text{obs}}$ as a result of microbial activity (Rees, 1973; Bradley et al., 2016). The current formulation predicts a fixed $\alpha_{\text{obs}}$ for a given value of $D_F$ across a broad range of $C/Co$ far below the value of the characteristic half-saturation term. Thus, we proceed in implementing the model framework for our current experimental conditions, emphasizing systems operating at sulfate concentrations near or above the concentration associated with the modified half-saturation term, but suggest that this model may be more generally applicable. Testing of this extension to electron acceptor limitation is beyond the scope of the current study.

As a final check of model behavior before turning to current datasets, we note that the isotope-enabled rate expressions (Eq. 4.5, 4.6) are derived such that the abundance of $^{34}$S and $^{32}$S are represented by their corresponding concentrations. As a result, there may be a potential for the initial and subsequently fractionated concentrations of $^{34}$SO$_4^{2-}$ and $^{32}$SO$_4^{2-}$ to impact the corresponding calculated $\alpha_{\text{obs}}$. We verify the absence of any such model behavior via comparison of two separate sets of simulations across three distinct reduction rates ($D_F = 0.25, 0.50, 0.75$) over a 1000-hour timespan (Fig. 4.4). In the first set of simulations, the isotopic composition of the initial sulfate pool (3.84 mM) is approximately equivalent to the natural abundance of sulfur ($^{34}$S/$^{32}$S = 0.042), whereas in the second set of simulations, the ratio has been (extremely)
artificially enriched ($^{34}\text{S}/^{32}\text{S} = 1$). This comparison indicates that substantial differences in the isotopic abundance of the sulfate pool have a negligible impact on the observed fractionation factor predicted by this model.

4.3 Model Validation

Having verified appropriate model behavior, we now turn to the experimental datasets generated in this study to validate the fidelity of the simulations. All parameters are either taken from the literature or constrained by the experimental conditions and reported datasets (Table 4.1, 4.2). Specifically, we implement known values for the initial sulfate concentration and isotope ratios, the electron donor addition rate, and relative biomass concentration based on the experimental design. The resulting range of observed sulfate reduction rates are used to constrain the maximum specific rate constant ($k_2$), and the corresponding observed fractionations are used to constrain the minimum fractionation factor $\alpha_1$, while $\alpha_2$ is taken from previous studies (Sim et al, 2011; Wing and Halevy, 2014; Bradley et al, 2017; Leavitt et al, 2013). It is important to note that the fixed values for all parameters described above, including both $k_2$ and $\alpha_1$, are common to all simulations of the complete set of bioreactor experiments. The only parameter which changes between simulations of the experiments is the value of $D_F$.

The donor factor in our model, for each rate, is established relative to the maximum rate experiment which was conducted under eutrophic conditions and exhibited minimal signs of growth. As a result, we assign this $D_F = 1.0$, such that any $D. vulgaris$ of this population could not achieve a faster csSRR during formate oxidation at $21^\circ\text{C}$. All rate simulations are then assigned a $D_F$ which scales linearly with the rate of sulfate reduction relative to this maximum value, resulting in a range spanning $D_F = 1.0, .575, 0.392, 0.245, 0.132$.

All five rate simulations agree with the experimental datasets for a common parameter set (Fig 4.5). Over the majority of the time series, both the measured and modeled sulfate concentrations exhibit a linear rate, but this trend eventually breaks down towards the completion of the experiment. We interpret this behavior as a result of diminishing substrate concentration causing beginning to affect the rate of sulfate reduction. The model predicts this behavior as the concentration approaching the value of the half-saturation term for each simulation. As noted earlier, these experiments were designed to observe isotopic shifts that are associate with steady state reduction, thus we compare the simulated isotopic trends with the data corresponding to the
periods of steady state reduction only (Fig 4.5). This comparison demonstrates that the model can accurately predict the characteristic $\alpha_{\text{obs}}$ as a function of steady state rate, incorporating two fractionation factors and a range of appropriately scaled $D_F$ values.

We further demonstrate the fidelity of this model via comparison of the experimental and predicted relationship between $\alpha_{\text{obs}}$ and rate (Fig. 4.6). It is clear that the behavior exhibited by experiments here are consistent with the model predictions. We emphasize that the competition between two threshold fractionation factors as a function of rate is consistent with many prior studies suggesting that all observed fractionation factors are bounded between theoretical maximum and minimum limits (Rees, 1973; Brunner and Bernasconi, 2005; Farquhar et al., 2003; Bradley et al, 2011; Detmers et al., 2001; Wing and Halevy, 2014; Antler, 2017; Johnston et al., 2007).

The principle advancement of this new model is that it serves as simplified expression that describes the patterns of sulfur isotope fractionating during sulfate reduction. Due to its simplicity and unambiguous parameterization, the model can serve as an effective update within a reactive transport framework. This stands to significantly improve our interpretations of microbial reactivity in a vast range of anoxic and suboxic environments; from aquifer systems, to marine and lacustrine sediments and water columns, and into the deep subsurface.

### 4.4 Isotope Biogeochemistry in Open Systems

The ability to monitor and predict microbial reactions in both terrestrial and marine environments is heavily contingent upon interpretation of the isotopic signature imparted by such activity. Thus far, the use of stable isotopes to detect the presence of microbial respiration in open through-flowing systems has been successful in a multitude of both natural and engineered environments, but in order to quantify the extent of microbial activity, a fractionation factor for the microbial reaction of interest must be defined. A number of previous studies have demonstrated the ability to quantify the extent of microbial reactivity in an open system by assigning one ‘effective’ fractionation factor within a reactive transport framework (e.g. Berna et al., 2010; Massmann et al., 2003; Druhan et al., 2012), but this technique is predominantly suited for systems, or small domains of a system, that exhibit eutrophic conditions, such as a stimulated bioremediation site. For instance, during a U remediation experiment, Druhan et al., (2012) demonstrated that one fractionation factor ($\alpha = 0.9873$) assigned to microbial sulfate reduction in
a reactive transport model was sufficient in order to accurately simulate the SRB population during three acetate injections (5 mM target concentration) across a ~2.5 m long flow path in a shallow alluvial aquifer. Since acetate, unlike formate, is a carbon source for sulfate reducers, this study required model treatment of SRB population growth, which implies that any catabolic metabolism was approaching a maximum rate. In a similar study, Druhan et al., (2014) used sediments from the same site (i.e. comparable microbial consortia) in a 1-meter-long flow-through column and again accurately predicted the isotopic enrichment of the unreacted sulfate using one fractionation factor ($\alpha = 0.985$). In this experiment, acetate and sulfate were pumped in at 9.7 and 8.8 mM, respectively, which constitutes eutrophic conditions. In both of these studies, an isotope-enabled Monod rate law, capable of predicting one constant $\alpha_{\text{obs}}$, reproduced the sulfur isotopic values associated with sulfate reduction while being implemented within a reactive transport framework.

In both cases presented, the SRB population was experiencing negligible energy limitation across the distances measured (~2.5 and 1.0 m). Therefore, csSRRs were approaching a maximum across these domains, which should produce a constant and small $\alpha_{\text{obs}}$. However, the conditions simulated in these studies did not reflect the conditions in domains further from the injection site where we would anticipate electron donor concentrations to decrease as a function of distance and/or depth (e.g. Massmann et al., 2003), primarily due to prior reactions and dilution (Fig. 4.7). We expect that within these increasingly donor-limited domains, csSRRs will deviate from maximum and $\alpha_{\text{obs}}$ will increase as a function of distance due to subsequent oxidation or dilution of the electron donor. It is in these evolving regimes that we suggest the application of our model, which offers the capability of predicting sulfur isotope fractionations in domains extending beyond an initial eutrophic regime. The resulting predicted fractionation factor as a function of distance may be embedded within a reactive transport framework, and thus support the expanded capability to treat transient behavior. We highlight that our modifications on this approach do not incorporate additional variables or unexpected behavior (i.e. $\alpha_2 \geq \alpha_{\text{obs}} \geq \alpha_1$) and provides the capacity to predict variable fractionation factors for systems that will inevitably evolve as a function of distance and time (i.e. subsequent reactions and transport). A key result of the current model is the potential for enhanced continuum-scale forecasting of microbial reactivity informed by variable fractionation factors. This will improve
our ability to monitor and predict the extent of sulfate reduction in a vast range of natural and engineered systems.

Furthermore, this model may be readily extended to other microbially fractionated stable isotope systems – such as reduction of heavy metal contaminants including Cr, U, and Se. The microbially catalyzed reductive pathways for these metals are in many ways comparable to that of sulfate reduction and therefore exhibit similar isotopic fractionation patterns (i.e. rate-dependence for a particular strain or consortia) (Beard et al., 2004; Sikora et al., 2008; Kitchen et al., 2012; Ellis et al., 2003; Basu et al., 2014). Thus, the conceptual models associated with microbial reduction of Cr, U, and Se apply the same principles as the conceptual models for sulfate reduction, whereby the fractionation factor associated with the rate-limiting intercellular branching point is most heavily expressed in the bulk fluid during steady state.

At present, the use of stable isotopes as a proxy for the extent of microbial reactivity in open systems is either (1) appropriate for eutrophic systems where cell-specific reduction rates are approaching a maximum (i.e. one ‘effective’ fractionation factor) (Druhan et al., 2012, 2014; Berna et al., 2010), or (2) semiquantitative, such that the maximum and minimum biological fractionation factors, typically determined by batch experiments, are used to estimate the total variability in the extent of microbial reactivity (e.g. Raddatz et al., 2011) (Fig. 4.8). The latter results in a significant amount of uncertainty in quantifying the extent of microbial reactivity, which implies that the impact of additional factors (e.g. dilution) on the species concentration will also be inaccurate (Fig. 4.8).

For instance, a reactive transport model for an open system that has been assigned one $\alpha_{\text{obs}}$ that is too small for the microbial reaction will overpredict the extent of microbial reactivity (Fig. 4.8). Such interpretations have implications for the fate and transport of aqueous contaminants (e.g. Cr, Se, U, Zn, Cd, Pb, As), where accurate simulations for microbial reactions are necessary in order to determine the validity of contaminant attenuation predictions. Biostimulation is by no means the only strategy for remediation, as several others have proven effective for a multitude of contaminated sites. For example, the pump and treat method or installation of a permeable reactive barrier are both effective strategies for contaminant remediation, but they are extremely costly when compared to biostimulation (e.g. an electron donor injection) or natural attenuation (Blowes, 2002). We suggest that the capabilities presented
by our model can improve predictions for contaminant attenuation as a result of microbial reactivity, thus supporting more cost-effective remediation strategies in field-scale systems.
CHAPTER 5: CONCLUSIONS

The characteristic fractionation factor exhibited by microorganisms during sulfate reduction serves as a tracer for predicting the extent of this reaction in an open system. There exists a functional relationship between this fractionation factor and the rate of sulfate reduction. This relationship was calibrated for *D. vulgaris* through a series of batch reactors (Fig. 2.1) whereby the csSRR was controlled by continuous injection of formate and we observed a fractionation factor associated with each steady state sulfate reduction rate (Fig. 4.5). This relationship was used to parameterize and modify an isotope-enabled Monod rate expression, that generates a variable $\alpha_{\text{obs}}$ as a function of rate and is bounded between a maximum and minimum fractionation factor (i.e. $\alpha_2 \geq \alpha_{\text{obs}} \geq \alpha_1$). This model behavior was accomplished through two principal means: (1) implementation of a new electron donor factor ($D_F$) that normalizes the observed reduction rate to the maximum reduction rate for a constant biomass – as determined in this experiment – and (2) functional treatment of the isotope specific half-saturation term which shift in value according to $D_F$. Further, the changes in the half-saturation terms for both isotopes are small and fall within the range of observed values for sulfate reduction (Fig 4.1).

We emphasize that this study serves as a link between the typical approach to characterizing microbial reactivity in a reactive transport framework (i.e. one ‘effective’ $\alpha$) and the empirical fractionating behavior of sulfate reducing bacteria and other redox catalyzing microorganisms. This can further improve modeling capabilities in open and transient systems where labile organic carbon is the rate limiting substrate.
CHAPTER 6: FUTURE PROJECTS

To further parameterize and test the fidelity of this model for its intended use within a reactive transport framework, a 50 cm flow-through column (Fig. 5.1) has been constructed in order to directly demonstrate variable $\alpha_{\text{obs}}$ as a function of distance from the inlet. The experiment is to be run under conditions similar to that of the batch reactors in this study, such that it will be inoculated with *D. vulgaris* and formate (4 mM target concentration) will be pumped through the inlet with a high background sulfate (4 mM target concentration). The design of the column incorporates five 10 cm segments that are separated by a high porosity zone (i.e. significantly higher than the sediment) that can be sampled. This high porosity zone and that occurs at the end of each 10 cm segment supports a well-mixed flux weighted average fluid that can be sampled every 10 cm. Each segment will be inoculated with the same biomass of *D. vulgaris*, which is a motile organism. In order to guarantee that a constant biomass is held within each section in of the column, a 0.65 $\mu$m filter membrane will be placed between each segment. As such, the biomass for each segment will remain constant, and therefore, the measured rate of sulfate reduction in each domain can be normalized to a known biomass so that the csSRR in each domain can be estimated.

Using this technique, we expect that the steady state concentration of formate will decrease with each subsequent domain, and therefore the csSRR will decrease with each subsequent domain. This should create a situation in which each domain exhibits a characteristic fractionation factor, that increases as a function of distance from the inlet. If successful, this will be the first time, to our knowledge, that variable fractionation as a function of distance in a through-flowing system has been demonstrated. Additionally, this experiment may improve parameterization of a reactive transport model that we intend on applying to field-scale systems.
**FIGURES**

**Fig 1.1.** Five-step dissimilatory sulfate reduction model (modified from Rees, 1973; Brunner and Bernasconi, 2005; Antler et al, 2017). Every enzyme-catalyzed reaction has an associated rate constant (\(k\)) and fractionation factor (\(\alpha\)). The reduced sulfur species \(*S^2*\) represents intermediates that may occur in the cell such as trithionate and thiosulfate.

**Fig 1.2.** General reaction from for isotope-enabled Monod kinetics adopted from Druhan et al., (2014). Rate constants (\(k\)) and fractionation factors (\(\alpha\)) for each step are fixed parameters. Step 1 represents the same substrate uptake and release processes shown in Fig. 1.1, however, Step 2 is an approximation to all steps downstream from Step 1 (i.e. Step 2–5 from Fig. 1.1).

**Fig 2.1.** Diagram for experimental method (replicates not shown). A syringe pump maintained a constant volumetric flux (q) of 0.02 mL*hr\(^{-1}\) for a set of eight 2.5 mL gas-tight syringes throughout the duration of the experiment. The valves were used to seal the reactors when the injectate needed to be refilled.
**Figure 3.1.** Concentration data as a function of time for Rate 1 reactor (filled markers) and a replicate (hollow markers) reactor exhibiting a steady state sulfate reduction rate of $0.28 \pm 0.00 \, \mu\text{mol}\cdot\text{hr}^{-1}$: (A) sulfate (triangles) and formate (circles); (B) bromide, (C) chloride (diamonds) and acetate (squares); (C) a linear regression was fit to acetate ($R^2 = 0.950$) and chloride ($R^2 = 0.936$) to account for any loss of the initial acetate mass, which was found to be $0 \pm 4 \, \mu\text{mol}$ ($0.00 \pm 7\%$).

**Figure 3.2.** (A) Residual sulfate mass ($M_{\text{SO}_4}$) through time for Rate 1 reactor (filled markers) and a replicate (hollow markers) reactor. Linear regression for Rate 1 reactor is shown as a dashed line ($R^2 = 0.986$) and a dotted line ($R^2 = 0.985$) for the replicate. (B) Measured $\delta^{34}\text{S}$ values versus IAEA sulfur isotope standards (VCDT scale) as a function of the mass fraction of sulfate remaining ($M/M_0$). The dotted line shows a Rayleigh model with a single fractionation factor ($\alpha = 0.9903$), and solid lines represent $\pm 0.7\%$ on this value.
Figure 3.3. Concentration data as a function of time for Rate 2 reactor (filled markers) and a replicate (hollow markers) exhibiting a steady state sulfate reduction rate of $0.52 \pm 0.02 \mu$mol*hr$^{-1}$. (A) sulfate (triangles) and formate (formate); (B) bromide, (C) chloride (diamonds) and acetate (squares); (C) a linear regression was fit to acetate ($R^2 = 0.904$) and chloride ($R^2 = 0.886$) to account for any loss of the initial acetate mass, which was found to be $8 \pm 4 \mu$mol ($3.35 \pm 1.17\%$). Rate 1 reactor achieved steady state reduction 48 hours after the replicate reactor due to a leak that disrupted formate mass addition.

Figure 3.4. (A) Residual sulfate mass ($M_{SO_4}$) through time for Rate 2 reactor (filled markers) and a replicate (hollow markers) reactor. Linear regression for Rate 2 reactor is shown as a dashed line ($R^2 = 0.990$) and a dotted line ($R^2 = 0.998$) for the replicate. (B) Measured $\delta^{34}S$ values versus IAEA sulfur isotope standards (VCDT scale) as a function of the mass fraction of sulfate remaining ($M/M_0$). The dotted line is a Rayleigh model with a single fractionation factor ($\alpha = 0.9924$), and the two solid lines represent $\pm 0.8\%$ on this value.
Figure 3.5. Concentration data as a function of time for Rate 3 reactor (filled markers) and a replicate reactor (hollow markers) exhibiting a steady state sulfate reduction rate of 0.83 ± 0.01 µmol*hr⁻¹: (A) sulfate (triangles) and formate (circles); (B) bromide, (C) chloride (diamonds) and acetate (circles); (C) a linear regression was fit to acetate (R² = 0.961) and chloride (R² = 0.955) to account for any loss of the initial acetate mass, which was found to be 5 ± 4 µmols (2.28 ± 1.82%).

Figure 3.6. (A) Residual sulfate mass (Mₘₐₜ) through time for Rate 3 reactor (filled markers) and a replicate (hollow markers) reactor. Linear regression for Rate 1 (R² = 0.965) and the replicate (R² = 0.983) are shown as a dot-dash line as they are indistinguishable. (B) Measured δ³⁴S values versus IAEA sulfur isotope standards (VCDT scale) as a function of the fraction of sulfate remaining (M/M₀). The dotted line is a Rayleigh model with a single fractionation factor (α = 0.9938), where gray shading represents ±1.0‰ on this value.
Figure 3.7. Concentration data as a function of time for Rate 4 reactor (filled markers) and a replicate reactor (hollow markers) exhibiting a steady state sulfate reduction rate of 1.22 ± 0.01 µmol*hr⁻¹: (A) sulfate (triangles) and formate (circles); (B) bromide, (C) chloride (diamonds) and acetate (squares); (C) a linear regression was fit to acetate ($R^2 = 0.845$) and chloride ($R^2 = 0.904$) to account for any loss of the initial acetate mass, which was found to be 10 ± 4 µmols (4.24 ± 1.06%).

Figure 3.8. (A) Residual sulfate mass ($M_{SO4}$) through time for Rate 4 reactor (filled markers) and a replicate (hollow markers) reactor. Linear regressions for Rate 4 reactor is shown as a dashed line ($R^2 = 0.961$) and a dotted line ($R^2 = 0.973$) for the replicate. (B) Measured $\delta^{34}$S values versus IAEA sulfur isotope standards (VCDT scale) as a function of the mass fraction of sulfate remaining ($M/M_0$). The dotted line is a Rayleigh model with a single fractionation factor ($\alpha_{obs} = 0.9962$), where the two solid lines represent ±0.6‰ uncertainty on this value.
**Figure 3.9.** Formate (circles) and sulfate (triangles) concentration data as a function of time for the Maximum Rate reactor (filled markers) and a replicate reactor (hollow markers) showing a steady state sulfate reduction rate of $1.65 \pm 0.03 \mu\text{mol*hr}^{-1}$ – no biomass correction. A secondary axis for sulfate (right) demonstrates the reaction proceeding in a stoichiometric fashion.

**Figure 3.10.** (A) Residual sulfate mass ($M_{SO_4}$) through time for Maximum Rate reactor (filled markers) and a replicate reactor (hollow markers). Linear regression for Maximum Rate ($R^2 = 0.983$) reactor and the replicate ($R^2 = 0.965$) shown as a dot-dash line as the two are indistinguishable. (B) Measured $\delta^{34}$S values versus IAEA sulfur isotope standards (VCDT scale) as a function of the mass fraction of sulfate remaining ($M/M_0$). The dotted line is a Rayleigh model with a single fractionation factor ($\alpha = 0.9976$), where solid lines represent $\pm 0.4\%$ uncertainty on this value.

**Figure 3.11.** Comparison of the bulk reactor sulfate reduction rate and the observed fractionation factor. Each marker represents a reactor and replicate. Relationship demonstrates that an increasing rate exhibits a decreasing fractionation factor.
Figure 4.1. Half-saturation ($K_{1/2}$) values for sulfate reducing consortia measured in a variety of natural environments showing variation of over two orders of magnitude (modified from Jin et al., 2011). Half-saturation values increase with increasing available sulfate.

Figure 4.2. Rate versus observed fractionation. The overall fractionation asymptotically approaches $\alpha_2 (0.930)$ at slow rates, and similarly approaches $\alpha_1 (0.998)$ at fast rates.
Figure 4.3. Observed enrichment factor versus reaction progress for 500 discrete and uniformly distributed rate simulations taking place for 1000 hours. Non-uniform vertical spacing between individual simulations demonstrate the sensitivity of $\alpha_{\text{obs}}$ with respect to rate, such that $\alpha_{\text{obs}}$ becomes less sensitive as rate increases. A secondary y-axis (right) was added to illustrate the approximate value of $D_F$ as it varied for each rate simulation.

![Graph showing observed enrichment factor versus reaction progress for 500 discrete rate simulations](image)

Figure 4.4. Results of two simulations (colors and dashes). The green ($D_F = 0.25$), magenta ($D_F = 0.5$), and blue ($D_F = 0.75$) illustrate the model results associated with an initial natural S-isotope abundance ($^{34}\text{SO}_4^{2-}/^{32}\text{SO}_4^{2-} = 0.042$), while corresponding dashed lines incorporate the same donor factors, but the initial $\text{SO}_4^{2-}$ is heavily enriched ($^{34}\text{SO}_4^{2-}/^{32}\text{SO}_4^{2-} = 1$). (A) Total sulfate versus time showing negligible effect on the reduction rate as a function of initial isotopic abundance. (B) Concentration of sulfate in natural log space versus corresponding $\delta^{34}\text{S}$ demonstrating no effect on predicted isotope fractionation.

![Graph showing results of two simulations](image)
Figure 4.5. Results of four rate simulations (solid lines) plotted with observations from experimental Rate(s) 1 – 4 (markers) where filled and hollow markers represent replicate reactors. Green, red, magenta, and blue represent measured values and associated model predictions Rate(s) 1, 2, 3, and 4, respectively. Only $D_f$ varies between each simulation, aside from initial sulfate concentration for blue, where $D_f$ is 0.132, 0.245, 0.392, and 0.575 for green, red, magenta, and blue model predictions, respectively. (A) Concentration of sulfate versus time for four distinct steady state sulfate reduction rates. (B) $\delta^{34}S$ (vs. VCDT) values versus natural log of sulfate concentrations demonstrate a characteristic $\alpha_{obs}$ for every steady state rate.
**Figure 4.6.** Results of one rate simulation (solid line) plotted with observations from the Maximum Rate reactor (filled marker) and a replicate (hollow markers) – $D_F$ for this simulation is 1.00. (A) Concentration as a function of time for the Maximum Rate reactor(s) exhibiting steady state reduction. (B) $\delta^{34}S$ (vs. VCDT) values versus natural log of sulfate concentrations showing a characteristic $\alpha_{obs}$ for the steady state reduction rate.

**Figure 4.7.** The observational relationship between $\alpha_{obs}$ and rate compared to the same relationship as predicted by this model – i.e. the experimental data overlaid onto figure 5.2. Every marker represents a result from a single rate replicate experiment.
Conceptual illustration of predicted $\alpha_{\text{obs}}$ as a function of distance from an organic carbon source (e.g. formate) in an open, through-flowing system. The light grey shaded region represents a ‘fast’ rate regime where labile organic carbon is in abundance and fractionation is expected to be near-constant and small (i.e. one “effective” fractionation factor). The dark grey shaded region represents an ‘intermediate’ and ‘slow’ rate regime where the electron donor availability exhibits increasing control on the rate of sulfate reduction and the fractionation factor is both variable and increasing with increasing distance. The ‘intermediate’ rate region correlates with the conditions exhibited by the experiments in this study, but the model presented is capable treating systems that are significantly more oligotrophic.

Figure. 4.8.
Figure 4.9. Conceptual diagram demonstrating significant potential uncertainty associated with interpretation of microbial reactivity when using a maximum and a minimum fractionation factor observed from batch experiments. This diagram considers the uncertainty based on the magnitude of variable $\alpha_{\text{obs}}$ from this study (i.e. 8.0‰). The model presented in this study aims to close this uncertainty gap (red) with continuum-scale predictions for $\alpha_{\text{obs}}$ as a function of electron donor availability, which is treated as a proxy for reduction rates.

Figure 5.1. Photograph of the complete flow-through column (A) and a close-up photograph of one connecting section (B). Sampling takes place at 10 cm intervals from the well-mixed zone (i.e. high porosity). This effective created five representative elementary volumes within the single column. The filter membrane prohibits microorganism from of their respective domains. The column is packed with 1.0 mm diameter well-rounded pure quartz sand and the dark coloration is a combination of microorganisms and precipitated iron sulfide minerals. Each $\alpha$ labeled on the column is indicative of the increasing $\alpha_{\text{obs}}$ we expect to observe, such that $\alpha_5 > \alpha_4 > \alpha_3 > \alpha_2 > \alpha_1$. 
# TABLES

Table 3.1 Experimental results from Rate(s) 1 – 4 and Maximum Rate reactors and replicates (a and b)

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### Table 4.1 Rate constants and biomass for *D. Vulgaris*

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### Table 4.2 Initial isotope specific SO$_4^{2-}$ concentrations determined by natural abundance ($^{34}$S = 0.042%) and $D_F$ and $K^*_{1/2}$ value for each simulation as scaled by the SRR with respect to the maximum rate experiment. $D_F$ and $K^*_{1/2}$ are fixed parameters for each simulation and $^{34}$SO$_4^{2-}$ and $^{34}$SO$_4^{2-}$ are initial concentrations.

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REFERENCES


