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# Determination of Kinetic Coefficients for the Simultaneous Reduction of Sulfate and Uranium by *Desulfovibrio desulfuricans* Bacteria

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# Determination of Kinetic Coefficients for the Simultaneous Reduction of Sulfate and Uranium by *Desulfovibrio desulfuricans* Bacteria

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Abstract

Uranium contamination of groundwaters and surface waters near abandoned mill tailings piles is a serious concern in many areas of the western United States. Uranium usually exists in either the U(IV) or the U(VI) oxidation state. U(VI) is soluble in water and, as a result, is very mobile in the environment. U(IV), however, is generally insoluble in water and, therefore, is not subject to aqueous transport. In recent years, researchers have discovered that certain anaerobic microorganisms, such as the sulfate-reducing bacteria *Desulfovibrio desulfuricans*, can mediate the reduction of U(VI) to U(IV). Although the ability of this microorganism to reduce U(VI) has been studied in some detail by previous researchers, the kinetics of the reaction have not been characterized. The purpose of this research was to perform kinetic studies on *Desulfovibrio desulfuricans* bacteria during simultaneous reduction of sulfate and uranium and to determine the phase in which uranium exists after it has been reduced and precipitated from solution. The studies were conducted in a laboratory-scale chemostat under substrate-limited growth conditions with pyruvate as the substrate. Kinetic coefficients for substrate utilization and cell growth were calculated using the Monod equation. The maximum rate of substrate utilization ($k$) was determined to be 4.70 $days^{-1}$ while the half-velocity constant ($K_S$) was 140 mg/l COD. The yield coefficient (Y) was determined to be 0.17 mg cells/mg COD while the endogenous decay coefficient ($k_d$) was calculated as 0.072 $days^{-1}$. After reduction, U(IV) precipitated from solution in the uraninite ($UO_2$) phase. Uranium removal efficiency as high as 90% was achieved in the chemostat.

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# Table of Contents

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# Section 1
# Introduction

Uranium mining was an important economic activity in the western part of the United States for many years following World War II. However, since no new nuclear power plants are being built and since less expensive uranium is available from foreign sources, this activity has declined in recent years and most uranium mines have been abandoned. In most cases, the mill tailings piles associated with the mineral processing plants near those mines have been left in place. Most of the mill tailings piles contain low concentrations of uranium which was not economically recoverable during mining operations. Infiltration from precipitation can cause this uranium to be leached from the pile and transported to surface water or ground water supplies (Thomson and Heggen, 1983). As a result, there is great interest in determining methods to remediate uranium contaminated soils and groundwater. The reduction of highly soluble U(VI) species to insoluble U(IV) species may play an important role in controlling uranium contamination and is the subject of this research.

In the natural environment, uranium usually exists in either the U(VI) or U(IV) oxidation state. Under oxidizing conditions, uranium is stable in the soluble U(VI) oxidation state (Brookins, 1988). The low pH of acidic mine drainage water also enhances the solubility of uranium and can result in its transport into groundwater or surface water supplies. By reducing uranium to the insoluble U(IV) oxidation state, it may be precipitated from solution and bound in the soil matrix. This investigation focuses on the use of microorganisms to reduce U(VI) to insoluble U(IV) species.

The purpose of this study was to investigate the ability of *Desulfovibrio desulfuricans*, a sulfate-reducing bacteria, to mediate the uranium reduction reaction. This microorganism has been used by previous researchers to reduce U(VI) to U(IV), but the kinetics of the process have not been characterized (Lovley and Phillips,

1992b). In this research, kinetic studies were conducted in a laboratory-scale chemostat and the kinetic coefficients for substrate utilization and cell growth were calculated for the Monod equation. In addition, studies were also conducted to identify the U(IV) mineral phase.

The next section of this report contains a review of the available literature on this topic. The literature review is followed by two sections which discuss general chemical and microbiological aspects and the materials and methods used in this research. The experimental results and a discussion of those results are then presented. Finally, conclusions are made concerning the ability of *Desulfovibrio desulfuricans* bacteria to remove uranium from an aqueous waste stream.

# Section 2
# Literature Review

The ability of microorganisms to use compounds other than oxygen as the terminal electron acceptor in respiration under anaerobic conditions has been known for many years. However, the ability of anaerobic microorganisms to reduce certain metals, such as iron and manganese, has only recently been discovered. Much of the previous research in this area has been conducted by Derek R. Lovley and his collaborators at the U.S. Geological Survey in Reston, Virginia. In the late 1980s, Lovley and his co-workers isolated an Fe(III) and Mn(IV)-reducing microorganism from freshwater sediments of the Potomac River and designated it GS-15. In a subsequent series of studies, they grew GS-15 in an anaerobic medium with acetate, ethanol, butyrate, or propionate as the sole electron donor and Fe(III), Mn(IV), or nitrate as the sole electron acceptor (Lovley and Phillips, 1988). Fe(III) was optimally reduced to Fe(II) at pH 6.7 to 7 and at 30 to 35°C. Mn(IV) was reduced to Mn(II) and nitrate was reduced to ammonia. This study was the first demonstration that microorganisms can completely oxidize organic compounds with Fe(III) or Mn(IV) as the sole electron acceptor. In another study, Lovley and his co-workers grew GS-15 in an anaerobic medium with toluene as the sole electron donor and Fe(III) oxide as the electron acceptor and found that growth of the microorganism coincided with Fe(III) reduction (Lovley and Lonergan, 1990). Toluene was completely oxidized to $CO_2$ and magnetite ($Fe_3O_4$) was the iron end product. GS-15 was the first microorganism known to couple the oxidation of aromatic compounds to the reduction of Fe(III).

Subsequently, Lovley and his co-workers turned their attention to uranium and began to investigate the ability of GS-15 (now called *Geobacter metallireducens*) and other microorganisms to reduce U(VI) to U(IV). In a 1991 study, they found that microbial reduction of U(VI) by GS-15 was much faster than commonly cited

abiological mechanisms for U(VI) reduction (Lovley, Phillips, Gorby and Landa, 1991). In 1992, they used *Shewanella putrefaciens*, another iron-reducing microorganism, and *Desulfovibrio desulfuricans*, a sulfate-reducing bacteria, to reduce U(VI) (Gorby and Lovley, 1992; Lovley and Phillips, 1992a; Lovley and Phillips, 1992b). In the latter experiments, sulfate and U(VI) were reduced simultaneously and resulted in the extracellular precipitation of the U(IV) mineral uraninite ($UO_2$). Enzymatic reduction of U(VI) by *Desulfovibrio desulfuricans* was much faster than nonenzymatic reduction of U(VI) by sulfide, even when cells of the bacteria were added to provide a potential catalytic surface for the nonenzymatic reaction. In addition, *Desulfovibrio desulfuricans* reduced U(VI) faster than GS-15. During the experiments, the concentration of uranium in aqueous streams was decreased from initial concentrations as high as 2.4 mM to final concentrations below 0.1 mM.

Earlier work in this area was also performed by Jim W. Kauffman and others at Kerr-McGee Corporation in Oklahoma City, Oklahoma (Kauffman, Laughlin and Baldwin, 1986). They used bacteria belonging to the genus *Clostridium* and *Desulfovibrio* to reduce the concentration of uranium, selenium, molybdenum and sulfate in mine water. However, Kauffman and his co-workers concluded that uranium was not reduced from U(VI) to U(IV) by a biological process. Instead, they believed that hydrogen sulfide ($H_2S$) produced by the sulfate-reducing bacteria reduced U(VI) by a chemical reaction.

Other workers have also investigated the microbial transformations of various metals. Dvorak, Hedin, Edenborn and McIntire (1992) used anaerobic reactors to treat metal-contaminated water. Concentrations of aluminum, cadmium, manganese, iron, nickel and zinc were decreased by over 95% in the reactors. Silver (1987) found that iron-oxidizing bacteria in mill tailings piles are responsible for the oxidation of Fe(II) in the mineral pyrite ($FeS_2$) to Fe(III) which results in the generation of sulfuric acid ($H_2SO_4$). Sulfuric acid reacts with minerals such as uranium, thorium and radium in

the mill tailings piles to cause the solubilization of the heavy metals and radioactive nuclides. The migration of those substances into groundwater and surface water can cause contamination of those water systems.

Additional information concerning sulfate-reducing bacteria was provided by other workers. Middleton and Lawrence (1977) calculated kinetic coefficients for microbial sulfate reduction by a mixed culture of sulfate-reducing bacteria which utilized acetic acid as the substrate. They found that the Monod equation can be used to model the growth of bacteria in the system and that the effect of temperature on the system can be modeled using an Arrhenius type relationship. Thomson (1987) used strongly reducing conditions produced by sulfate-reducing bacteria to precipitate various metals and metalloids (copper, chromium and selenium) from an aqueous stream. Thomson also conducted studies on the growth kinetics of a mixed culture of sulfate-reducing bacteria and determined that only chromium inhibited bacterial growth in the chemostat. For every case that was studied, the system was able to achieve greater than 90% removal of the metals. Magee, Ensley and Barton (1978) conducted kinetic studies on *Desulfovibrio vulgaris* and *Desulfovibrio gigas* bacteria. They found that efficiency of growth varied with electron donor-acceptor combinations and that differences in energy coupling occurred with the various bacterial strains.

Also included in the literature review were several books and papers that cover the general topic of uranium solubility. Brookins (1988) calculated Eh-pH diagrams for numerous elements, including uranium. The Eh-pH diagram for the U-C-O-H system was used during the course of this research. Anderson (1987) found that uranium was not reduced chemically by hydrogen sulfide in the deep seawater Cariaco Trench despite the anoxic conditions that exist in the basin. This suggests that U(VI) is not reduced to insoluble U(IV) in marine systems, even in the presence of hydrogen sulfide. Thomson and Heggen (1983) provide an overview of uranium mining and milling activities in the western U.S. along with a discussion on uranium geochemistry.

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# Section 3

# Chemical and Microbiological Aspects

## A. Uranium Chemistry

The reduction of U(VI) to U(IV) plays an important role in the formation of uranium deposits. Likewise, forcing a change in the oxidation state of uranium could serve as a mechanism for the bioremediation of uranium-contaminated waters. The chemistry of uranium in water can be conveniently summarized in an Eh-pH diagram. Figure 3-1 shows the Eh-pH diagram for the U-C-O-H system.

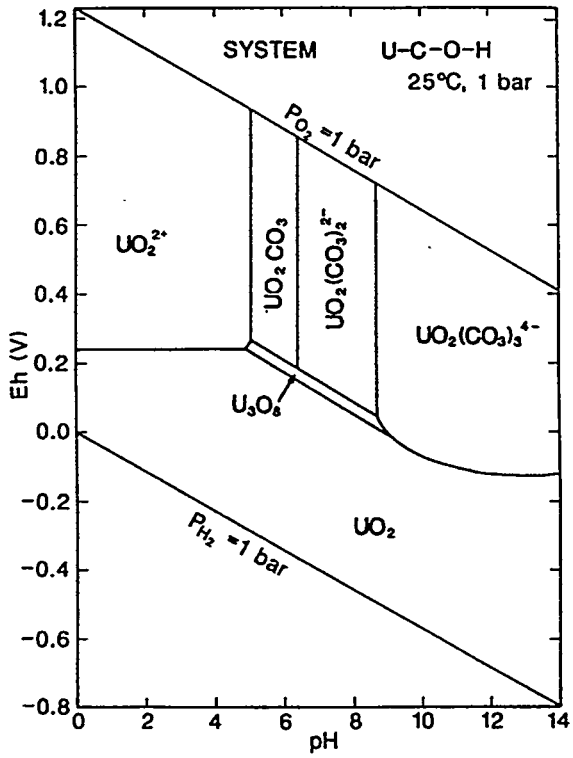


Figure 3-1: Eh-pH Diagram for the U-C-O-H System
Total Concentration of U = $10^{-6}$ M, C = $10^{-3}$ M
(Brookins, 1988)

Under oxidizing conditions and moderate to high pH, U(VI) forms highly soluble uranium-carbonate complexes. Under oxidizing conditions and low pH, U(VI) forms $UO_2^{2+}$, which is also highly soluble in water. However, under reducing conditions over the entire pH range, uranium is stable as U(IV) and forms the insoluble compound uraninite ($UO_2$). Microbial reduction requires a strong anaerobic environment represented by a low Eh. When uranium is present, the microorganisms can substitute U(VI) as the terminal electron acceptor in place of Fe(III) or sulfate. The general chemical reaction for the sulfate reduction process is shown in Equation 3-1.

$$\text{Organics} + SO_4^{2-} + \text{Nutrients} \longrightarrow \text{Biomass} + HS^- + CO_2 + H_2O + \text{Products} \qquad (3\text{-}1)$$

In this reaction, the organic compound serves as the substrate (electron donor and energy source) and sulfate serves as the terminal electron acceptor. The reaction is mediated by sulfate-reducing bacteria such as *Desulfovibrio desulfuricans*. Most sulfate-reducing bacteria require a low molecular weight organic acid, such as lactate, acetate or pyruvate as the substrate. A summary of the sulfur cycle is shown in Figure 3-2. The reaction shown in Equation 3-1 is represented on the right-hand side of Figure 3-2 where sulfate ($SO_4^{2-}$) is reduced to sulfide ($S^{2-}$) by anaerobic bacteria.

When oxidized uranium, U(VI), or certain other oxidized metals are also present, the following reaction may also occur (Lovley, Phillips, Gorby and Landa, 1991).

$$\text{Organics} + \text{U(VI)} + SO_4^{2-} + \text{Nutrients} \longrightarrow \text{Biomass} + \text{U(IV)} + HS^- + CO_2 + H_2O + \text{Products} \qquad (3\text{-}2)$$

Again, sulfate-reducing bacteria mediate this reaction where low molecular weight organic compounds serve as the substrate. In this reaction, U(VI) serves as the terminal electron acceptor in addition to the sulfate. As shown in Figure 3-1, the reduction of U(VI) to U(IV) causes uranium to be precipitated from solution possibly in the form of uraninite ($UO_2$).

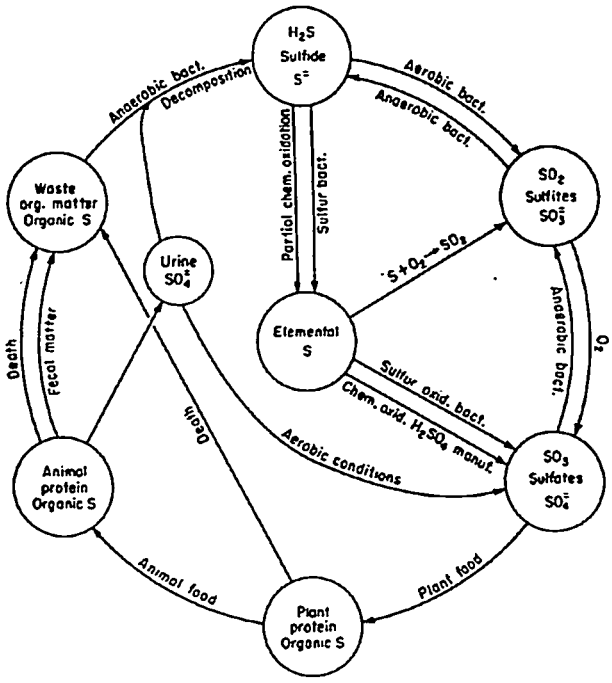


Figure 3-2: The Sulfur Cycle in Nature
(Sawyer and McCarty, 1978)

## B. Microbial Kinetics

Middleton and Lawrence (1977) conducted kinetic studies of microbial sulfate reduction. They used acetate as the organic carbon source and electron donor and fitted their results to the Monod equation for substrate-limited growth. Monod kinetics for substrate-limited growth were also assumed in this research and the kinetics of sulfate reduction were investigated in a continuous-flow, complete-mix chemostat. A schematic of the laboratory chemostat without recycle is shown in Figure 3-3 (Metcalf & Eddy, 1991).

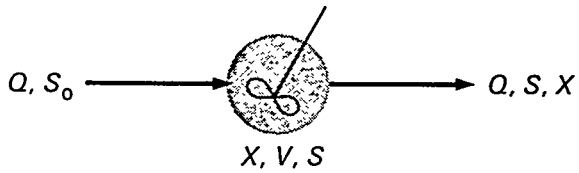


Figure 3-3: Schematic of a Chemostat without Recycle
(Metcalf & Eddy, 1991)

Where:

$V$ = chemostat volume

$Q$ = flow rate

$S_o$ = influent substrate concentration

$S$ = effluent substrate concentration

$X$ = biomass concentration in the chemostat

The mass balance equation for the substrate in the chemostat is shown in Equation 3-3 (Metcalf & Eddy, 1991).

$$V \frac{dS}{dt} = QS_o - QS + r_s V \tag{3-3}$$

Where:

$r_s$ = rate of substrate appearance due to microbial reaction

At steady state, dS/dt=0. Therefore, the mass balance reduces to the following equation.

$$0 = QS_o - QS + r_s V \tag{3-4}$$

Rearranging:

$$r_s = -\frac{1}{\theta} (S_o - S) \tag{3-5}$$

Where:

$\theta$ = hydraulic residence time (V/Q)

Assuming that Monod kinetics apply, Equation 3-6 can be written.

$$r_S = -\frac{kXS}{K_S + S} = \frac{1}{\theta}(S_O - S) \qquad (3\text{-}6)$$

Where:

$k$ = maximum rate of substrate utilization

$K_S$ = half-velocity constant

Also, the relationship between bacterial growth and substrate utilization can be written. This relationship is shown in Equation 3-7 (Metcalf & Eddy, 1991).

$$r_g = -Yr_S - k_dX \qquad (3\text{-}7)$$

Where:

$r_g$ = rate of bacterial growth

$Y$ = yield coefficient

$k_d$ = endogenous decay coefficient

Performing a mass balance on the chemostat shown in Figure 3-3 for biomass results in Equation 3-8.

$$V\frac{dX}{dt} = QX_O - QX + r_gV \qquad (3\text{-}8)$$

Where:

$X_O$ = biomass concentration in influent

At steady state and assuming that the biomass concentration in the influent is equal to zero, Equation 3-8 can be reduced to Equation 3-9.

$$0 = QX - (-Yr_S - k_d X)V \tag{3-9}$$

After rearranging and substituting for $r_S$, Equation 3-10 can be written.

$$\frac{1}{\theta} = Y\frac{S_O - S}{X\theta} - k_d \tag{3-10}$$

As a part of this research, values for the kinetic coefficients that are shown in Equations 3-6 and 3-7 were calculated.

## C. Uranium Removal

The purpose of this research was to remove uranium from the aqueous stream by using *Desulfovibrio desulfuricans* bacteria to reduce soluble U(VI) to insoluble U(IV). To determine the effectiveness of this process, the uranium removal efficiency can be written. This relationship is shown in Equation 3-11.

$$\eta = [1 - ([U_{eff}]/[U_{in}])] \cdot 100 \tag{3-11}$$

Where:

$[U_{in}]$ = concentration of uranium in influent

$[U_{eff}]$ = concentration of uranium in effluent

$\eta$ = uranium removal efficiency

In Equation 3-11, both $[U_{in}]$ and $[U_{eff}]$ are measured as U(VI).

## D. Sulfate-Reducing Bacteria

*Desulfovibrio desulfuricans* bacteria are strict anaerobes and are usually mesophilic (Postgate, 1979). Most members of the *Desulfovibrio* genus of bacteria have a curved shape and are fairly easy to isolate and purify. Mesophilic *Desulfovibrios* have an upper temperature limit of 45 to 48°C and their best growth occurs around 30°C. Optimum pH for growth is between 5.5 and 8.5. These bacteria were used in this research because they are abundant in nature and take part in the sulfur cycle (Figure 3-2). In addition, the ability of this microorganism to reduce uranium was demonstrated in previous research (Lovley and Phillips, 1992b). However, there has been some controversy among researchers over the exact mechanism of uranium reduction. Some early researchers concluded that sulfate was first microbially reduced to sulfide and then U(VI) was chemically reduced to U(IV) by the sulfide. However, Lovley showed that U(VI) is indeed microbially reduced simultaneously with the sulfate (Lovley and Phillips, 1992b). In Lovley's research, the addition of U(VI) to a culture which contained sulfate and bacteria did not decrease the rate of sulfate reduction. However, attempts to force U(VI) to act as the sole electron acceptor were not successful. When sulfate was not present, U(VI) was not reduced to U(IV) and no cell growth occurred.

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# Section 4
# Materials and Methods

## A. Introduction

The goal of this research program was to investigate and describe the kinetics of the sulfate-reducing bacteria *Desulfovibrio desulfuricans* during simultaneous reduction of sulfate and uranium. The research was conducted in a laboratory-scale, anaerobic chemostat using pyruvate ($CH_3COCOONa$) as the substrate. The experimental setup is shown in Figure 4-1.

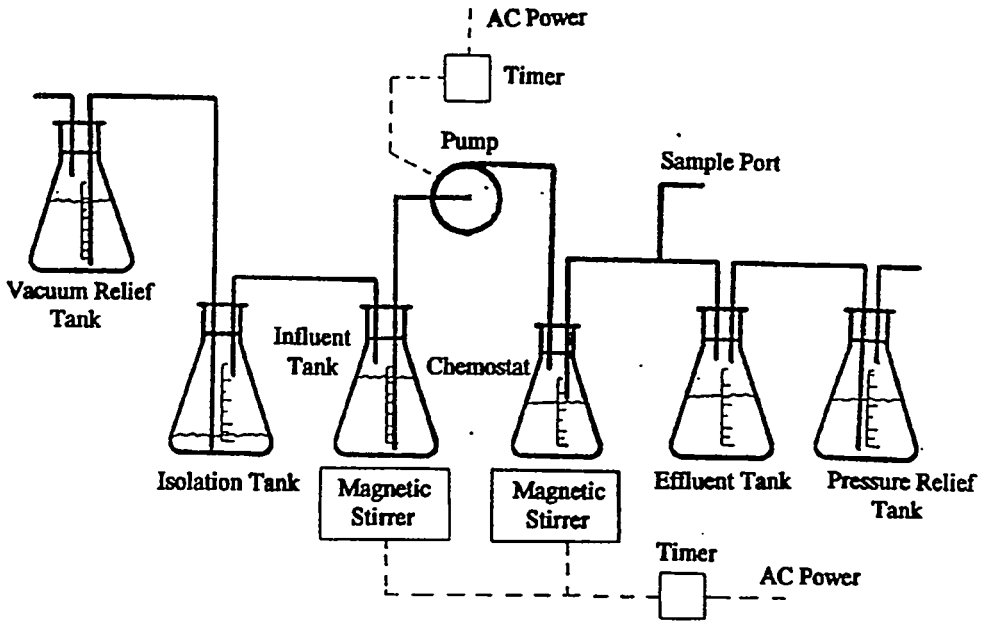


Figure 4-1: Experimental Setup

During the experiments, certain procedures were followed to prevent contamination of the chemostat with other strains of bacteria. All glassware, tubing and other laboratory apparatus were washed with standard dish washing detergent and rinsed with deionized water. Then, all solutions were added to the flasks and the entire assembly was autoclaved at a temperature of 120°C for 30 minutes. In addition, care

was taken to assure that an anaerobic environment was maintained in the influent tank, chemostat, and effluent tank. The atmosphere above the solution in each of these containers was purged with nitrogen gas for at least ten minutes before the start of the experiment and the flasks were sealed to prevent air leakage. All connections between the flasks were made with autoclavable tubing.

The vacuum relief tank, isolation tank, influent tank, effluent tank, and pressure relief tank each consisted of a 1 liter Erlenmeyer flask. The influent tank was mixed by a magnetic stirrer to assure that a well-mixed solution was always input to the chemostat. The magnetic stirrer was cycled on and off by an automatic timer since this type of equipment is not designed to operate continuously and may be a fire hazard if it is not allowed to cool. The purpose of the vacuum relief and isolation tanks was to prevent the creation of a vacuum in the influent tank as the media was withdrawn by the pump. The purpose of the pressure relief tank was to prevent an increase of pressure in the effluent tank as it received discharge from the chemostat.

The chemostat consisted of a 125 ml Erlenmeyer flask. A volume of 100 ml was maintained in the chemostat throughout the experiments. The chemostat was mixed by a magnetic stirrer. This magnetic stirrer was also operated by an automatic timer to prevent overheating. Insulation was also placed between the stirrer and the flask to prevent heating of the chemostat. The chemostat was maintained at room temperature (25°C) throughout the experiments.

The bacteria were grown in Legall's medium (Postgate, 1979). The contents of the medium are shown in Table 4-1. The pH of the medium was adjusted to 7.4 with a solution of 20% KOH. The same medium (without the bacteria) was also used as influent to the chemostat. Resazurin is a redox indicator that is colorless under reducing conditions and pink or blue (depending on the pH) under oxidizing conditions (Thomson, 1987). The transition from color to clear occurs at an Eh of approximately -81 mV. In the chemostat, sulfate ($SO_4^{2-}$) is first reduced to sulfite ($SO_3^{2-}$) which is

then reduced to sulfide ($S^{2-}$). The $SO_3^{2-}$-$HS^-$ redox transition occurs at an Eh of approximately -170 mV at a pH of 7. Use of the resazurin indicator made it easy to determine if reducing conditions were being maintained in the influent tank and chemostat.

| Constituent | Concentration (mg/l) | Concentration (mM) |
|---|---|---|
| Pyruvate | 11000 | 100.0 |
| $NH_4Cl$ | 2000 | 37.8 |
| $Na_2SO_4$ | 400 | 2.81 |
| $MgSO_4$ | 200 | 1.67 |
| $FeSO_4$ | 20 | 0.13 |
| $K_2HPO_4$ | 500 | 2.87 |
| Yeast Extract | 1000 | - |
| Resazurin | 1.0 | 0.004 |

Table 4-1: Contents of Legall's Medium

Seven experimental runs were conducted during this research. In the first five experimental runs, 1.0 mM (393.0 mg/l) of uranyl nitrate ($UO_2(NO_3)_2$) was added to the influent solution. Kinetic coefficients for the reduction of U(VI) to U(IV) were calculated using data from these first five experimental runs. Except for the flow rate, conditions in the system were identical for each run. The system was operated at

residence times ranging from 21 to 89 hours during these five experiments. Since the chemostat volume was small, a very low flow rate (between 1.1-4.8 ml/hr) was required. Most tubing pumps cannot be adjusted to maintain such a low flow rate so a timer was used to turn on the pump for a short period of time (2 minutes) during each hour. During that time, the pump output a higher flow rate (between 33-144 ml/hr) making the average flow rate equal to the required value. Therefore, the chemostat was only an approximation of a continuous-flow, complete-mix reactor.

Legall's medium was also used as the influent for the last two experimental runs. However, uranyl nitrate was not added to the influent during those experimental runs. The substrate concentration and biomass concentration from the chemostat effluent during the last two experimental runs were measured and the results were compared to the data from the first five experimental runs to determine if the presence of uranium in the chemostat inhibited the growth of *Desulfovibrio desulfuricans* bacteria.

## B. Analytical Methods

This research required the measurement of four parameters: 1) uranium concentration, 2) pyruvate ($CH_3COCOONa$) concentration, 3) volatile suspended solids (VSS) or biomass concentration, and 4) sulfate concentration. A summary of the analytical methods are shown in Table 4-2.

In order to perform the required analytical procedures, a 40 ml sample was taken from the sample port of the chemostat after each experimental run was completed. As soon as the sample was collected, 8 ml were drawn off and divided into four replicates of 2 ml each. These replicates were used to measure the concentration of uranium in solution. Another 4 ml of the sample were also drawn off and divided into four 1 ml replicates. Each of these 1 ml replicates were centrifuged for 10 minutes at 2500 rpm. The solid portion of each replicate was used to determine the biomass concentration. Another 4 ml of the original sample were also divided into four 1 ml replicates and

used to determine the substrate (pyruvate) concentration. The remainder of the sample (24 ml) was immediately frozen so that it could be used in case any test had to be repeated. Each of the analytical methods that are shown in Table 4-2 are described in the following sections.

| Parameter | Procedure | Method Description | Reference |
|---|---|---|---|
| Uranium Concentration | Colorimetric | hexanol extraction | Meloan et al. (1960) |
| Pyruvate Concentration | Colorimetric | toluene extraction | Friedman and Haugen (1943) |
| Biomass Concentration | Colorimetric | protein assay | Lowry et al. (1951) |
| Sulfate Concentration | Ion Chromatograph | Chromatography | Am. Public Health Assoc. (1992) |

Table 4-2: Summary of Analytical Methods

## C. Uranium Measurement

Uranium concentration in the influent and effluent was measured by a hexanol extraction technique using a Beckman Model 25 spectrophotometer (Meloan, Holkeboer and Brandt, 1960). In addition to the sample solution, six standard solutions with known concentrations of uranium were also prepared using the following procedure.

Procedure to Measure Uranium Concentration

1. Start with 5 ml of sample. Centrifuge for 10 minutes at 2500 rpm. Take 2 ml of the liquid and place in a test tube.
2. Add 1 ml of 0.1 M Bis-Tris buffer solution to test tube.

3. Prepare Benzohydroxamic acid reagent (0.137 g of Benzohydroxamic acid in 10 ml of $H_2O$). Add 1 ml to test tube.

4. Add 2 ml of hexanol to test tube. Mix on vortex shaker. Extract 1 ml of upper layer.

5. Place the solution in the spectrophotometer and record absorbance at 380 nm.

After measuring the absorbance of the six standard solutions, a calibration curve was prepared. The absorbance of the sample solutions from the influent and the effluent were then measured and the concentration of uranium in each sample was determined from the calibration curve.

**D. Measurement of Pyruvate Concentration**

Pyruvate was used as both the carbon source and the electron donor for the bacteria. In the chemostat, pyruvate was oxidized to acetate by the following reaction.

$$4CH_3COCOO^- + HSO_4^- \longrightarrow 4CH_3COO^- + 4CO_2 + HS^- \qquad (4\text{-}1)$$

Previous studies, including Middleton and Lawrence (1977) and Thomson (1987) used lactic acid or acetic acid as the substrate. However, Middleton and Lawrence (1977) found that the value for the yield coefficient (Y) predicted by thermodynamics for cell growth with pyruvic acid is higher (0.24 mg cells/mg pyruvic acid) than for lactic acid (0.12 mg cells/mg lactic acid) or for acetic acid (0.062 mg cells/mg acetic acid). In addition, Magée, Ensley and Barton (1978) found that the efficiency of the conversion of free energy to adenosine triphosphate (ATP) during the electron donor-acceptor reaction for cellular biosynthesis is higher for pyruvate (34.8%) than for lactate (22.6%). Therefore, in order to optimize the growth conditions in the chemostat, pyruvate was used as the substrate for this research.

In order to determine the rate of substrate utilization, the concentration of pyruvate in the influent and effluent had to be measured. Pyruvate was measured by a colorimetric method (Friedman and Haugen, 1943). Six standard solutions containing 0 to 1 mM of pyruvate were prepared using the following procedure. Solutions containing samples from the chemostat influent and effluent were also prepared by the same procedure. Test tubes containing each of the standard solutions and the sample solutions were placed in a Beckman Model 25 spectrophotometer and the absorbance was recorded at 520 nm. The absorbance values from the standard solutions were used to calculate a calibration curve. After establishing the calibration curve, the concentration of pyruvate in the influent and effluent samples were determined.

Procedure to Measure Pyruvate Concentration

1. Start with 1 ml of the effluent solution.
2. Deprotinate the solution with 2 ml of 20% trichloroacetic acid (TCA).
3. Remove the precipitate by centrifugation.
4. Take 2 ml of the centrifugate and add it to a large test tube containing 0.8 ml of 0.1% 2,4 dinitrophenylhydrazine in 2 N HCl.
5. After 10 minutes, add 3 ml of toluene to the test tube.
6. Shake the tube vigorously on a vortex mixer and allow it to set for ten minutes. Resolve (if necessary) the two solvent fractions by centrifugation.
7. Remove 2 ml of the toluene layer and place it in a large test tube.
8. Add 3 ml of 10% $Na_2CO_3$ to the test tube.
9. Mix the suspension, remove 2 ml of the carbonate layer and place it in a test tube containing 2.5 ml of 2.4 N NaOH.
10. Develop the color for ten minutes.
11. Record the absorbance at 520 nm.

## E. Determination of VSS Concentration

Since Monod kinetics were assumed for substrate utilization, knowledge of the concentration of biomass in the chemostat was required. Typically, a gravimetric method is used to measure biomass concentration. However, the gravimetric procedure will not work in an anaerobic system due to the high concentration of solids in the chemostat. Therefore, the Lowry Method for protein measurement was used to determine the biomass concentration (Lowry, Rosebrough, Farr and Randall, 1951). This method measures the protein in a fixed volume of effluent from the chemostat using a colorimetric technique. Since protein constitutes a known percentage of the cells of *Desulfovibrio desulfuricans* bacteria, it was possible to calculate the biomass concentration in the chemostat once the protein concentration had been determined.

Lowry Method for Protein Measurement

1. Prepare Reagent A (100 g of $Na_2CO_3$ in 1 L of 0.5 N NaOH).
2. Prepare Reagent B (1 g of $CuSO_4$ in 100 ml of $H_2O$).
3. Prepare Reagent C (2 g of Potassium Tartate in 100 ml of $H_2O$).
4. Prepare Reagent D (15 ml of Reagent A; 0.75 ml of Reagent B; 0.75 ml of Reagent C). Reagent D must be prepared immediately before the test.
5. Prepare Reagent E (5 ml of 2.0 N Folin Ciocalteu Phenol Reagent in 50 ml of $H_2O$). Reagent E must be prepared immediately before the test.
6. Take 5 ml of sample. Centrifuge for 10 minutes at 2500 rpm. Pour off liquid.
7. Dissolve pellet in 1 ml of 3.0 N NaOH. Place in 60°F water bath for 30 minutes until pellet is dissolved.
8. Add 1 ml of Reagent D. Wait for 10 minutes.
9. Add 3 ml of Reagent E.
10. Record absorbance on spectrophotometer at 500 nm.

In addition to the sample, six standard solutions with known protein concentrations were also prepared and a calibration curve was calculated. The concentration of protein in the effluent was determined from the calibration curve. The biomass concentration was then determined from Equation 4-2.

$$X = \frac{P}{f} \tag{4-2}$$

Where:

X = biomass concentration in the chemostat

P = protein concentration in the chemostat

$f$ = fraction of protein in cells

For *Desulfovibrio desulfuricans* bacteria, the average percentage of protein in a typical cell has been determined to be equal to 60% (Lichstein and Oginsky, 1965). Therefore, parameter $f$ in Equation 4-2 is equal to 0.60.

## F. Determination of Sulfate Concentration

Sulfate concentrations in the chemostat were measured with a Dionex 2010i ion chromatograph following Procedure 4500-$SO_4^{2-}$ B in *Standard Methods for the Examination of Water and Wastewater* (American Public Health Association, 1992). Before analysis in the ion chromatograph, three standard solutions were prepared and the samples were filtered using a 0.45 μm filter.

## G. Determination of Kinetic Coefficients

The purpose of this research was to determine the following kinetic coefficients; 1) $k$, the maximum rate of substrate utilization, 2) $K_S$, the half-velocity constant, 3) Y, the

specific yield, and 4) $k_d$, the endogenous decay coefficient. The first two coefficients were determined by rearranging Equation 3-6 to obtain Equation 4-3.

$$\frac{X\theta}{S_o - S} = \frac{K_s}{k}\frac{1}{S} + \frac{1}{k} \qquad (4\text{-}3)$$

This equation was then used to plot the experimental data on a linear scale in the form of $y = mx + b$ where 1/S was plotted on the x-axis and $X\theta/(S_o-S)$ was plotted on the y-axis. Using linear regression, a straight line was fit to the data points. The values of the first two kinetic coefficients were determined from the plot since the slope of the line is equal to $K_s/k$ and the y-intercept of the line is equal to $1/k$.

Likewise, the values for the kinetic coefficients Y and $k_d$ were also determined by plotting the experimental data on a linear scale using Equation 4-4 with $(S_o-S)/X\theta$ plotted on the x-axis and $1/\theta$ plotted on the y-axis.

$$\frac{1}{\theta} = Y\frac{S_o - S}{X\theta} - k_d \qquad (4\text{-}4)$$

A line was also fit to these data points and the values of the kinetic coefficients were determined. On this plot, the slope of the line is equal to Y and the y-intercept of the line is equal to $-k_d$.

# Section 5
# Experimental Results

Data was collected and analyzed from seven experimental runs. Data from the first five experimental runs were used to calculate the kinetic coefficients. Data from the last two experimental runs were used to determine if the presence of uranium in the chemostat inhibited bacterial growth. The resazurin indicator was only added to the medium during the first two experimental runs since it showed that it was not difficult to maintain reducing conditions in the chemostat. Legall's medium, shown in Table 4-1, was used as the influent to the chemostat during each experimental run. Uranium was added to the medium for the first five experimental runs.

The concentration of pyruvate in the influent to the chemostat was only measured for the last four experimental runs. The concentration of uranium in the influent was only measured for the fourth and fifth experimental run. Measurement of the influent concentrations of uranium and pyruvate were not made during the first three experimental runs because it was not recognized, until after the first three runs were complete, that these values needed to be measured to account for small weighing errors during the preparation of the medium and for fluid losses during autoclaving. Therefore, in order to correct this problem, the measured influent concentrations for uranium and pyruvate were averaged and those values were used for the influent concentrations for the first three runs.

Data collected from the seven experimental runs are shown in Table 5-1. The concentrations for uranium, pyruvate and volatile suspended solids were measured using the methods described in the Section 4.

The kinetic coefficients were calculated using the data from the first five experimental runs. Figure 5-1 shows the Lineweaver-Burke plot that was used to calculate the maximum rate of substrate utilization ($k$) and the half-velocity constant

($K_S$). Figure 5-2 shows the plot that was used to calculate the specific yield (Y) and the endogenous decay coefficient ($k_d$).

| Run No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Chemostat Volume (V) (ml) | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Total Flow Volume (ml) | 641 | 658 | 524 | 527 | 510 | 550 | 513 |
| Test Duration (hr) | 135 | 243 | 266 | 334 | 455 | 162 | 407 |
| Average Flow Rate (Q) (ml/hr) | 4.75 | 2.71 | 1.97 | 1.58 | 1.12 | 3.40 | 1.26 |
| Residence Time ($\theta$) (hr) | 21.1 | 36.9 | 50.8 | 63.3 | 89.3 | 29.5 | 79.3 |
| Influent Pyruvate Concentration ($S_O$) (mg/l) | 10831 | 10831 | 10831 | 10878 | 10784 | 10722 | 10901 |
| Effluent Pyruvate Concentration (S) (mg/l) | 4933 | 3134 | 1744 | 482 | 105 | 4274 | 66 |
| Influent Uranium Concentration ($U_O$) (mg/l) | 232.0 | 232.0 | 232.0 | 233.0 | 231.0 | 0.0 | 0.0 |
| Effluent Uranium Concentration (U) (mg/l) | 107.1 | 92.8 | 65.1 | 31.4 | 26.2 | 0.0 | 0.0 |
| VSS Concentration (X) (mg/l) | 896 | 1046 | 1333 | 1215 | 1467 | 1176 | 1866 |
| Influent Sulfate Concentration (mg/l) | 445.0 | NA | NA | NA | 439.0 | NA | NA |
| Effluent Sulfate Concentration (mg/l) | 80.0 | NA | NA | NA | 25.0 | NA | NA |
| Uranium Removal Efficiency ($\eta$) (%) | 53.8 | 60.0 | 71.9 | 86.5 | 88.7 | NA | NA |

Table 5-1: Summary of Experimental Results

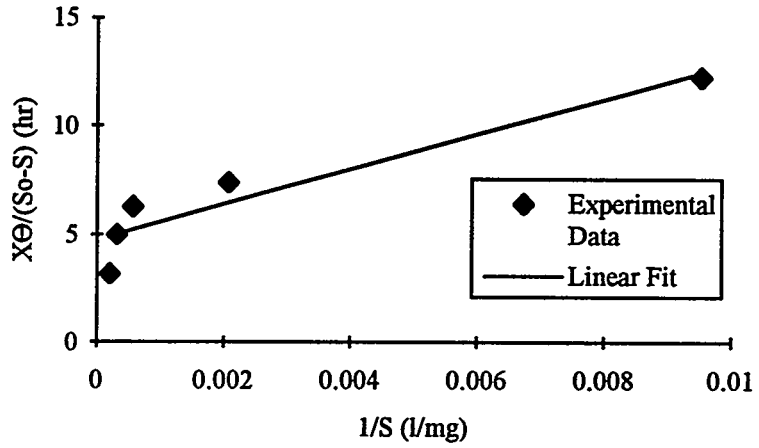


Figure 5-1: Determination of the Maximum Rate of Substrate Utilization ($k$) and the Half-velocity Constant ($K_S$)

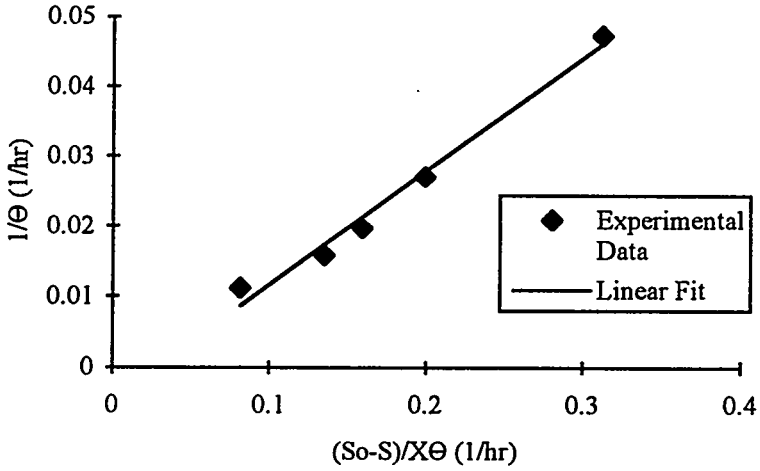


Figure 5-2: Determination of the Specific Yield (Y) and the Endogenous Decay Coefficient ($k_d$)

The kinetic coefficients were calculated using a least-squares fit to the points on the plots shown in Figures 5-1 and 5-2. The $r^2$ value for the linear fit to the data in Figure 5-1 was 0.89 and for the data in Figure 5-2, $r^2$ was equal to 0.98. Table 5-2 summarizes the calculated values for the kinetic coefficients for this system. Table 5-2 also includes results from Middleton and Lawrence (1977) and Thomson (1987). Also

included in Table 5-2 are the upper and lower 95% confidence levels for the kinetic coefficient values that were calculated in this study.

| Study | $k$ (days$^{-1}$) | $K_S$ (mg COD/L) | Y (mg cells/mg COD) | $k_d$ (days$^{-1}$) |
|---|---|---|---|---|
| Tucker (1994) | 4.7 | 140.0 | 0.170 | 0.072 |
| Lower 95% Confidence Level | 4.0 | 94.1 | 0.130 | 0.064 |
| Upper 95% Confidence Level | 6.9 | 243.4 | 0.210 | 0.080 |
| Middleton and Lawrence (1977) | 7.1 | 99.0 | 0.060 | 0.0 |
| Thomson (1987) | 16.9 | 2.8 | 0.065 | - |

Table 5-2: Calculated Kinetic Coefficients for Three Experimental Studies

Middleton and Lawrence (1977) used a mixed culture of bacteria in the chemostat and acetic acid as the substrate. Thomson (1987) also used a mixed culture of bacteria and utilized lactic acid as the substrate. All three studies were conducted at a temperature of 25°C.

A plot of uranium removal efficiency versus residence time for the first five experimental runs is shown in Figure 5-3. This plot shows a direct relationship between uranium removal efficiency and the residence time.

The last two experimental runs were conducted to determine if the presence of uranium in the chemostat inhibited the growth of bacteria. Table 5-3 compares the concentration of substrate and biomass in the effluent for experimental runs of nearly equal residence time with and without uranium in the influent solution.

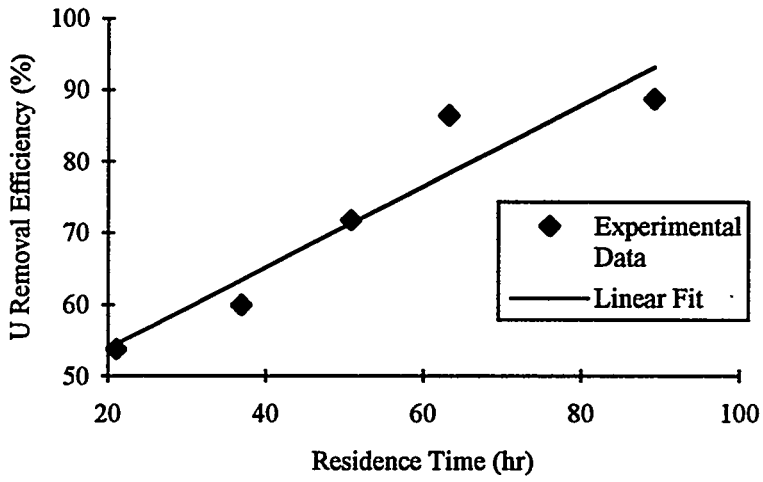


Figure 5-3: Uranium Removal Efficiency vs. Residence Time in the Chemostat

| Run No. | Residence Time (hr) | Influent Uranium Concentration (mg/l) | Effluent Substrate Concentration (mg/l) | Effluent Biomass Concentration (mg/l) |
|---|---|---|---|---|
| 1 | 21.1 | 232.0 | 4933 | 896 |
| 6 | 29.5 | 0.0 | 4274 | 1176 |
| 4 | 89.3 | 233.0 | 105 | 1476 |
| 7 | 79.3 | 0.0 | 66 | 1866 |

Table 5-3: Comparison of Biological Growth in the Chemostat for Experimental Runs With and Without Uranium

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# Section 6
# Discussion

## A. Introduction

The results of this study provide a mathematical description of the growth kinetics of *Desulfovibrio desulfuricans* sulfate-reducing bacteria under the specified conditions. The kinetic coefficients that were determined as a part of this research may be used in mathematical models to develop expressions for the rate of substrate utilization and cell growth. However, there are several aspects of this research that merit further discussion. These include: 1) uranium removal mechanism, 2) justification of the use of the substrate-limited growth model, and 3) uranium inhibition of reaction kinetics.

## B. Uranium Removal Mechanism

It was shown that *Desulfovibrio desulfuricans* bacteria were able to remove soluble uranium from an aqueous stream during simultaneous reduction of sulfate and uranium. The efficiency of uranium removal was shown to be a function of the residence time in the chemostat (see Figure 5-3). It was assumed that the process that occurred in the chemostat was the microbial reduction of soluble U(VI) to insoluble U(IV). The mass of uranium removed from the aqueous stream under steady-state conditions (and assumed to be reduced) can be calculated by the mass balance equation shown below.

$$M_U = ([U_{in}] - [U_{eff}]) \cdot Q \cdot t \qquad (6\text{-}1)$$

Where:

$M_U$ = mass of uranium removed in chemostat

$[U_{in}]$ = concentration of uranium in influent

$[U_{eff}]$ = concentration of uranium in effluent

$t$ = time

In this equation, both $U_{in}$ and $U_{eff}$ are soluble species in the U(VI) oxidation state and were directly measured by the method described in Section 4. $M_U$ represents the mass of uranium removed from solution in the chemostat during system operation and is assumed to be equal to the mass of uranium reduced from U(VI) to U(IV). However, since U(IV) was not directly measured as a part of this research, it was not possible to determine the exact mechanism for uranium removal. U(VI) could have been reduced to the insoluble U(IV) oxidation state by an extracellular process such as chemical reduction by the sulfide ($S^{2-}$) ion. Alternatively, U(VI) could have been reduced by the bacteria by an intracellular biological process. In order to investigate the mechanism for uranium removal that actually occurred in the chemostat, a sample of effluent from the fifth experimental run ($\theta$ = 89.3 hours) was viewed under a Transmission Electron Microscope (TEM). A photograph from the TEM is shown in Figure 6-1.

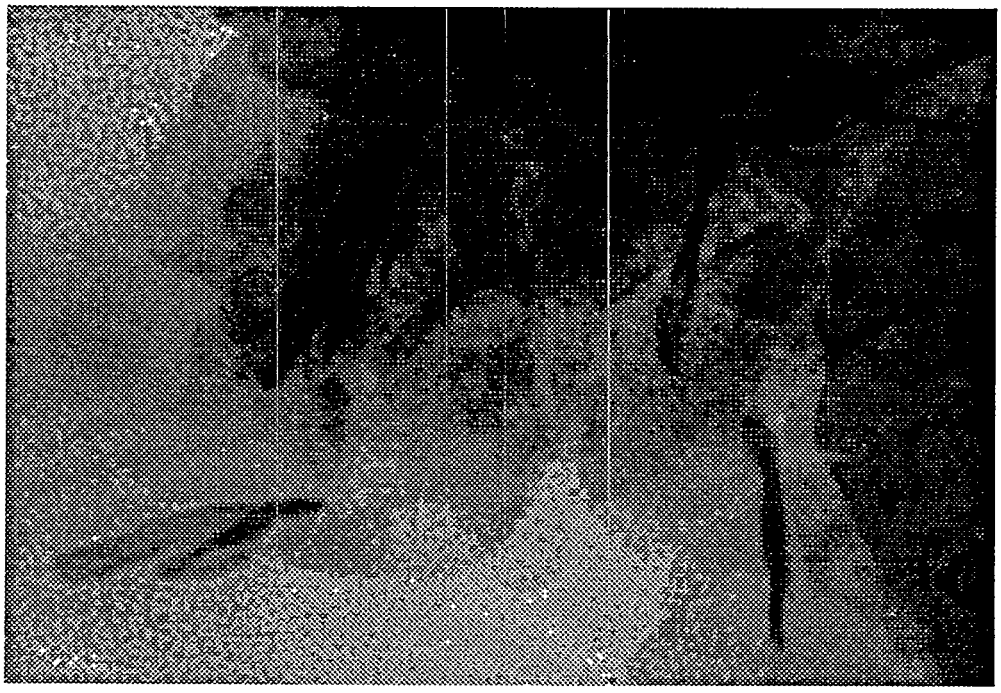

Figure 6-1: TEM Photograph of Uranium Accumulation Outside of Bacterial Cells in the Chemostat

In this photograph, uranium is clearly visible outside of the cells of *Desulfovibrio desulfuricans* bacteria. Uranium appears as long, slender, needle-like structures which are darker than the nearby bacteria cells. Although the mechanism for uranium removal cannot be determined from this photograph, it can clearly be seen that uranium accumulates outside of the bacteria cells.

An electron diffraction test was conducted to determine the structure of the reduced uranium compound. The electron diffraction pattern is shown in Figure 6-2. Table 6-1 compares the measured electron diffraction pattern to the standard electron diffraction pattern for $UO_2$. Since the measured pattern closely matches the standard pattern, the conclusion can be made that the reduced uranium compound in the chemostat is uraninite ($UO_2$) which is the phase predicted under reducing conditions in the U-C-O-H system shown in Figure 3-1 (Brookins, 1988). However, the confirmation of the presence of the uraninite phase still does not determine the mechanism of uranium reduction since this compound could have been formed by an extracellular chemical reduction process or by an intracellular biological reduction process.

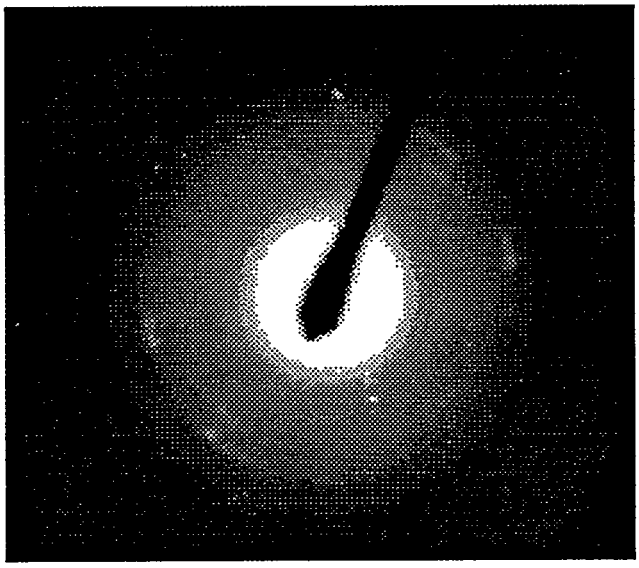

Figure 6-2: Electron Diffraction Pattern for $UO_2$

| Ring | Measured Diameter (Angstroms) | $UO_2$ Diameter (Angstroms) | Error (%) |
|---|---|---|---|
| 111 | 3.25 | 3.15 | 3.2 |
| 002 | 2.86 | 2.73 | 4.7 |
| 022 | 2.01 | 1.93 | 4.1 |
| 113 | 1.64 | 1.65 | 0.6 |

Table 6-1: Comparison of Measured Electron Diffraction Pattern to Standard $UO_2$ Electron Diffraction Pattern

## C. Substrate-limited Growth Model

The mathematical model that was developed in this research for the kinetics of *Desulfovibrio desulfuricans* bacteria assumed that substrate-limited growth occurred in the chemostat. However, there are conditions where other substances, such as the electron acceptor ($SO_4^{2-}$), can also begin to limit growth. Therefore, it is important to determine the minimum concentration of the electron acceptor in the chemostat below which the substrate-limited growth assumption is no longer valid. Middleton and Lawrence (1977) conducted batch studies on sulfate-reducing bacteria to determine this minimum concentration. They found that the concentration of sulfate in the chemostat that is maintained at 25°C should be at least 10 mg/l. Another study has reported that sulfate concentrations above 17 mg/l are required in the chemostat to maintain substrate-limited growth conditions (Pomeroy and Bowlus, 1946).

Sulfate concentrations in the chemostat for the first and fifth experimental run were measured. Sulfate concentration in the influent was 445 and 439 mg/l, respectively.

Sulfate concentrations in the chemostat under steady-state conditions varied between 25 mg/l for the experimental run with the longest residence time ($\theta$ = 89.3 hours) to 80 mg/l for the run with the shortest residence time ($\theta$ = 21.1 hours). Therefore, it is concluded that substrate-limited growth conditions existed in the chemostat during each experimental run.

### D. Uranium Inhibition of Reaction Kinetics

Based on the data shown in Table 5-3, it can be concluded that uranium inhibits growth of biomass in the chemostat. This conclusion is only valid for a uranium concentration of 1 mM (238.0 mg/l) which was the concentration in the influent during the first five experimental runs. This conclusion is validated by analyzing the results from the other experimental studies that are summarized in Table 5-2. Since the value of $k$ is smaller and the value of $K_S$ is larger for this research when compared to the values obtained by Middleton and Lawrence (1977) and Thomson (1987) it appears that the introduction of uranium into the system as an alternative electron acceptor decreases the rate of substrate utilization by the bacteria especially since pyruvate is more efficient at converting free energy to ATP than lactate or acetate (Magee, Ensley, and Barton, 1978). The value for the specific yield (Y) was higher for pyruvate than for lactate or acetate as predicted by thermodynamics but not as high as the theoretical value calculated by Middleton and Lawrence (1977). This would also support the conclusion that uranium inhibits growth in the chemostat.

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# Section 7
# Summary and Conclusions

Monod kinetics were used to describe the growth of the sulfate-reducing bacteria *Desulfovibrio desulfuricans* that utilized both sulfate and U(VI) as the terminal electron acceptor. Values of the kinetic coefficients were calculated for this model using a continuous-flow, complete-mix chemostat at a temperature of 25°C under anaerobic conditions. These kinetic coefficients included: 1) $k$, the maximum rate of substrate utilization, 2) $K_S$, the half-velocity constant, 3) Y, the specific yield, and 4) $k_d$, the endogenous decay coefficient. Based on the results of this study, the following conclusions can be made:

1. Monod kinetics can be used to model the growth of *Desulfovibrio desulfuricans* which utilize pyruvate as the substrate and both sulfate and U(VI) as the terminal electron acceptor.

2. Efficiency of the removal of U(VI) from the aqueous stream is dependent on the residence time in the chemostat. As residence time increases, uranium removal efficiency also increases.

3. The exact mechanism of uranium reduction (intracellular or extracellular) cannot be determined from the results of this research. After uranium is reduced and precipitated from solution, it accumulates outside of the bacteria cells in the uraninite ($UO_2$) phase as predicted by thermodynamics.

4. Bacterial growth is inhibited by the presence of uranium in the chemostat.

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# Section 8
# Acknowledgments

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# Section 9

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