The the Graduate Council:

I am submitting herewith a thesis written by Xiaoyan Zhang entitled "Metal Bioreduction by *Shewanella alga* in the Presence of Co-electron Acceptors under Non-growth and Growth Conditions". I have examined the final copy of this thesis for form and content and recommended that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Environmental Engineering.

Kevin G. Robinson, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor
and Dean of the Graduate School

METAL BIOREDUCTION BY *SHEWANELLA ALGA* IN THE PRESENCE OF CO-ELECTRON ACCEPTORS UNDER NON-GROWTH AND GROWTH CONDITIONS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Xiaoyan Zhang

August 1998

ACKNOWLEGEMENT

I would like to thank my major professor Dr. Kevin Robinson for his support and guidance throughout this research. I would also like to thank Dr. Chris Cox and Dr. Gregory Reed for being my committee members and their insightful comments and suggestions.

Special thanks to Dr. John Sanseverino, who spent a lot of time teaching me microbial culture technique and help me whenever I needed. Thanks also go to the other members of Center for Environmental Biotechnology Laboratory: Hae-jin Woo, Fu, Kristen Kelly, Zabin Raman, Julia, Bruce, Keith Lactore and Leon Wright, who made my laboratory life much easier and pleasant.

I owe thanks to Dr. Ganesh Rajagopalan, Dr. Michael Treux, Dr. Yuri Gorby and Ms. Nancy Valentine at Pacific Northwest Laboratory at Richland, WA, who helped me to obtain valuable information through personal communications.

ABSTRACT

Anaerobic microbial reduction of soluble hexavalent uranium (U (VI)) has been reported for iron and sulfur reducing bacteria. A facultative anaerobic iron reducer, *Shewanella alga* is considered one of the most promising strains for uranium biotreatment because it is easy to grow and can effectively reduce U (VI). The purpose of this research is to further evaluate the potential of this organism in uranium waste biotreatment. The research goals include investigating possible growth of *S. alga* using U (VI) as the sole electron acceptor and exploring the impact of iron on uranium reduction under both non-growth and growth conditions. Metal reduction and microbial growth kinetics were also studied and the kinetic coefficients determined to predict the performance of *S. alga* for metal reduction.

All batch tests were conducted in duplicate using 100 mL serum bottles. The seed *S. alga* culture was grown in an anaerobic medium containing ferric citrate as the electron acceptor. U (VI) and/or Fe (III) were introduced into test solutions as terminal electron acceptors. Each test solution contained 2.5 g/L of sodium bicarbonate (pH buffer) and 2mL/L of 60% w/w lactate syrup (equivalent to 20 mM lactate) as the electron donor. For growth coupled studies, a chemically defined medium which included nutrients essential for microbial growth was used. The initial Fe (III) and/or U (VI) concentrations used were ten times higher than those used in non-growth studies in order to support measurable cell mass. All experiments were conducted at constant pH (8.4) and temperature (35°C).

The Monod model was used to quantify the kinetic data for Fe (III) and U (VI) reduction under non-growth conditions. Results show that the maximum specific

reduction rate for Fe (III) was ~5.13 /hr, more than ten times higher than that for U (VI) (~0.39 /hr). The half-saturation constant for Fe (III) was ~36.8 mg/L, two times lower than that for U (VI) (~74.04 mg/L). This suggests that *S. alga* has a higher reduction capability and a higher enzymatic affinity for Fe (III) than U (VI) when lactate serves as the electron donor.

When both metals were present in test solutions under non-growth conditions, *S. alga* preferentially reduced Fe (III). This was most likely because Fe (III) reduction does not require as low a redox potential as U (VI) and was therefore more thermodynamically favorable. Subsequent U (VI) reduction after significant Fe (III) reduction appeared to be impeded by poising the electron potential caused by the [Fe (II)/Fe (III)] redox couple. At low initial Fe (III) concentrations, U (VI) reduction occurred slowly after Fe (III) reduction, suggesting a weak Eh buffering effect. At high initial Fe (III) concentrations, no U (VI) reduction was measured during the experimental duration. It's believed that, high initial Fe (III) levels produced high [Fe (II)/Fe (III)] ratios which result in strong Eh buffering. Such buffering may poison the system by keeping the Eh value higher than that required for U (VI) reduction. The presence of U (VI) had no impact on Fe (III) reduction kinetics. Tests also showed that Fe (II) initially added had a negative impact on U (VI) reduction.

Growth of *S. alga* using U (VI) as the sole terminal electron acceptor was demonstrated under batch conditions. Unlike *D. desulfuricans*, *S. alga* grew in a minimum salt medium with lactate as the electron donor/carbon source and U (VI) as the electron acceptor. The rate of microbial growth was four times higher than that previously

reported for another iron reducer, *Geobacter metalireducens*. The maximum specific growth rate was estimated to be 0.0215 /hr for U (VI) reduction, and 0.139 /hr for Fe (III) reduction.

Growth-coupled reduction was more complicated because active microbial activities, such as oxidative phosphorylation and cellular synthesis/division, were involved (Bailey and Ollis, 1986). The individual reduction rate of either metal was slower when both metals were present in the medium compared to the rate when one of them was absent. The impact of one metal on the initial reduction rate of the other was both concentration dependent. A combination of initial concentrations of 10 mM Fe (III) and 5 mM U (VI) impeded 90% of the reduction of both metals. Unlike non-growth results, reduction of U (VI) occurred during iron reduction when coupled with microbial growth. In addition, the presence of U (VI) inhibited iron reduction. The reasons are believed associated with growth mechanisms.

Similar to *G. metalireducens* and *Shewanella putrefaciens*, *S. alga* grew when U (VI) served as the sole electron acceptor when lactate was the electron donor. But unlike *D. desulfuricans*, which utilized two different enzyme systems for sulfate and uranium reduction, the reduction of U (VI) and Fe (III) by *S. alga* seemed to share the same active enzyme. Under non-growth conditions, the presence of Fe (III) delayed U (VI) reduction. The Fe (II)/Fe (III) redox couple subsequently inhibited U (VI) reduction by keeping the electron potential of the medium higher than that required for the reaction to occur. While under growth conditions, the reduction of U (VI) and Fe (III) occurred simultaneously.

However, reduction was slower and incomplete for each metal compared to that when one of them was absent.

The significance of growth using U (VI) as the sole electron acceptor by *S. alga* is important. Combined with other advantages of this bacterium, such as the high tolerance to oxygen, the high uranium reduction rate, and no sulfide co-precipitation problems, a continuous flow treatment process using this organism appears promising for selective reduction of U (VI) at a high rate.

Regarding results in the presence of both electron acceptors, predictions could be made on iron containing uranium wastewater treatment. A delay in uranium reduction is expected if *S. alga* cell suspensions are used. The duration of the delay is Fe (III) concentration dependent. Above certain iron concentrations, the iron redox couple could possibly poise the system such that U (VI) reduction could never occur. However, U (VI) reduction will occur slowly, although incompletely, when cells are placed in a growth medium. It's suggested that for a uranium waste containing high levels of sulfate and low levels of iron (less than 0.2 mM), *S. alga*, rather than *D. desulfuricans* should be utilized.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURE **PAGE**

LIST OF TABLES

TABLE **PAGE**

CHAPTER I

INTRODUCTION

With the rapid development of industry, environmental contamination due to the presence of heavy metals has become a serious problem. Metals present in soils and waters primarily come from the leakage of industrial waste material, which may disrupt the growth of plants, animals and even human beings (Groot, 1995). Pollution due to radioactive metals such as uranium is of great concern due to the carcinogenic nature of such materials.

Uranium is found typically in process waste streams and contaminated soil and groundwater at U.S. Department of Energy (DOE) facilities and uranium ore mining process (Francis, 1993). As in nature, uranium predominantly exists in the soluble hexavalent form (UO_2^{2+}) or insoluble tetravalent form (UO_2) (Katz & Rabinowitch, 1955). Both inorganic and organic chemistry of uranium is very complex. Temperature, pH, Eh, the nature and concentration of various ions and chelating reagents (especially organic acids) can impact uranium speciation and subsequent distribution (Langmuir, 1978). Currently, wastewater treatment technologies utilized for uranium removal are chemical precipitation, biosorption and biotransformation.

Metal biotransformation is an alternative waste treatment technique in which harmful metal ions are transformed to other less harmful valences with the meditation of microbial metabolism. A number of metal ions, such as Fe (III), U (VI), Cr (VI), Co (VI) and Mn (IV), can be reduced in the natural environment. While geologists are concerned

with the impact of biotransformation on the cycling of metals, environmental engineers have found that biotransformation of metals (especially uranium) can be a cost-effective treatment option for selective metal removal. For example, reduced tetravalent uranium is unstable and will precipitate from wastewater as uraninite (UO_2). Such biological transformation of uranium can result in a 75 fold reduction in the volume of final radioactive waste (Truex *et al*, 1995).

Two types of microorganisms, iron and sulfate reducers, have been found to date which utilize hexavalent uranium as an alternative terminal electron acceptor for anaerobic enzymatic respiration. Lovely and coworkers isolated iron reducers, *Geobacter metalireducens* (1987) and *Shewanella putrefaciens* (1989) and a sulfate reducer, *Desulfovibrio desulfuricans* (1992) from natural anaerobic sediments. Caccavo *et al.* (1992) isolated a dissimilatory iron reducing bacterium, *Shewanella alga* from the sediment of Great Bay Estuary, New Hampshire. Among all the microorganisms reported responsible for U (VI) reduction, *G. metalireducens* and *S. Putrefaciens* grew using U (VI) as the sole terminal electron acceptor (Lovley, 1991 & 1992). However, both microorganisms have little tolerance to oxygen, which limited their application in engineered treatment processes. More tolerant to oxygen, *D. desulfuricans* and *S. alga* have proved capable of growing to a high density and reducing uranium at a relatively high rate. However, since uranium-contaminated wastewater usually contains other materials such as sulfate, *D. desulfuricans* may not be the best treatment organism. Because sulfate is the primary electron acceptor utilized by *D. desulfuricans,* co-reduction of sulfate to sulfide during uranium reduction can result in metal sulfide co-precipitation

with other metals. The noxious odor and large volume of final sludge caused by metal sulfides may cause *D. desulfuricans* to be less desirable than *S. alga*. In addition, *D. desulfuricans* was reported not able to grow using uranium as the sole electron acceptor (Lovley and Phillips, 1992). Continuous cell supply during the non-growth reduction then becomes necessary. Alternatively, *S. alga* may be the better choice for microbial mediated metal biotransformation. However, since iron (the primary electron acceptor utilized by *S. alga*) is usually present in uranium-containing wastewater, the impact of iron on U (VI) reduction using *S. alga* should be established before this organism can be utilized in treatment reactors. Whether *S. alga* can couple uranium reduction with cellular growth is currently unknown.

S. alga, a dissimilatory Fe (III) and Mn (IV) reducing bacterium, was first isolated by Dr. Caccavo from the bottom of the Great Bay Estuary, New Hampshire in 1993. The isolate was determined to be a facultative anaerobic gram-negative rod. This bacterium was found to grow anaerobically in a defined medium with hydrogen or lactate as the electron donor and Fe (III) as the electron acceptor. U (VI) was utilized as an alternative electron acceptor and reduced to U (IV) by cell suspensions under non-growth conditions. In addition to Fe (III) and U (VI), *S. alga* also used oxygen, Mn (IV), thiosulfate and fumarate as electron acceptors. Sulfate can not be reduced by this organism. In addition to lactate, *S. alga* utilized citrate, fumarate and malete as carbon sources. However, energy for growth was obtained only from hydrogen or lactate oxidation. Peptone *et al.* (1995) reported that under non-growth conditions, *S. alga* reduced uranium from carbonate complexes more efficiently than any other bacterium studied.

In order to further evaluate the potential of *S. alga* in uranium-contaminated wastewater treatment, the following questions must be answered. First, can *S. alga* grow using uranium as the sole electron acceptor? Second, will the presence of iron impact uranium reduction? If yes, how? The overall goal of this research is to answer these questions. The specific objectives are to:

- Demonstrate batch growth of *S. alga* using U (VI) as the sole electron acceptor;
- Obtain kinetic coefficients for Fe (III) and U (VI) reduction and microbial growth; and
- Investigate the impact of iron on uranium reduction under non-growth and growth conditions.

CHAPTER II

LITERATURE REVIEW

2.1 Heavy metals in aquatic sediments and wastewater

2.1.1 Introduction

The history of metal pollution can trace back to the industrial revolution or even earlier (Forstner, 1991). The problems caused by metal contamination are more complicated because of the instability of species resulted from the impacts of environmental factors such as pH, alkalinity, salinity, redox potential, and the presence of soluble inorganic and organic ligands (Groot, 1995). Metal cycling in natural sediments has a great impact on the ecology and geochemistry of waterways. Mobilization of chemicals stored in sediments as a consequence of alteration of certain environmental conditions may result in a chain of events like delayed and sudden occurrence of harmful effects, which is termed as a chemical time bomb (CTB) (Stigliani *et al.*, 1991). Studies show that metal cycling is influenced by both physicochemical erosion/deposition and microbial involved metal metabolism (Walter & Winfried, 1991). Metals present in soil and water may disrupt the growth of plants, animals and even human beings. Metals in soil come from the leakage of industrial waste material and are subject to strict environmental laws and regulations (Peters & Shem, 1991). Current available *in-situ* metal remediation technologies include soil extraction, electrokinetics, phytoremediation and containment (EPA report, 1995). For *ex-situ* treatment, chemical extraction with subsequent biological treatment is a common design (Peters & Shem, 1991).

2.1.2 Biomeditated redox reactions in aquatic sediments

The main reactions occurring in the sediment are chemical redox transformations, precipitation and biological degradation of organic compounds. Microbially mediated reactions are dependent upon a set of limiting concentrations of successive external electron acceptors such as O_2, NO_3^-, Mn (IV), Fe (III) and SO_4^{2-} (Cappellen and Wang, 1995). Table 1 lists the range of limiting concentrations of terminal electron acceptors reported for aquatic environments.

Table 1: Limiting concentrations of terminal electron acceptors in respiration pathways (Adopted from Cappellen and Wang, 1995).

Pathway	Limiting Reactant	Limiting Concentration	Environment
Aerobic respiration	Dissolved O_2	1-10 μM	Marine Freshwater
Denitrification	Dissolved NO_3	50-80 μM 4-20 μM	Marine Freshwater
Mn reduction	Solid Mn (IV)	1-10 μmole/g	Marine Freshwater
Fe reduction	Solid Fe (III)	1-50 μmol/g	Marine Freshwater
Sulfate reduction	Dissolved SO_4	1600 μM 60-300 μM	Marine Freshwater

At the upper layer of the sediment, organic biodegradation is usually coupled with oxygen consumption. At the deep layer, where oxygen is depleted, organisms can couple organic substance degradation with the utilization of alternative electron acceptors, such as NO_3^- and SO_4^{2-} or, metals such as Fe (III) and Mn (VI). During organic decomposition, the most thermodynamically favorable oxidants are utilized first. The least thermodynamically favorable oxidants are then utilized until all the oxidants are

consumed (Froelich *et al*, 1979). Figure 1 (Stumm and Morgan, 1981) shows the sequence of microbially mediated redox processes occurring in order of decreasing thermodynamic feasibility. The reduction of ferric oxide occurs when Eh of the system lies below –50 mV. The quantity of biological growth resulting from inorganic and organic metabolism is related to the free energy released by the mediated reaction [$-\Delta G°$] and the efficiency of microorganisms in capturing this energy (McCarty, 1971). Table 2 presents the free energy distribution of a series of electron acceptors coupled with the degradation of the same complex carbohydrate organic molecule.

Table 2: Oxidation reactions for the decomposition of organic matter, Gibbs Free Energy is given per mole of glucose (adopted from Froelich, 1979).

Reaction	G (kJ/mole)
Oxygen $138O_2+C_{106}H_{263}O_{110}N_{16}P\rightarrow$ $106\ CO_2+16\ HNO_3+\ H_3PO_4+122\ H_2O$	-3190
Nitrate $94.4\ HNO_3+\ C_{106}H_{263}O_{110}N_{16}P\rightarrow$ $106\ CO_2+55.2\ N_2+\ H_3PO_4+177.2\ H_2O$	-3030
MnO_2 $236\ MnO_2 + C_{106}H_{263}O_{110}N_{16}P+ 472\ H^+\rightarrow$ $8\ N_2+ 106CO_2+H_3PO_4+236\ Mn^{2+}+ 366\ H_2O$	-3000
Fe_2O_3 $212\ Fe_2O_3 + C_{106}H_{263}O_{110}N_{16}P +848\ H^+\rightarrow$ $106\ CO_2+16\ NH_3+H_3PO_4+424Fe^{2+}+530H_2O$	-1410
SO_4^{2-} $53SO_4^{2-}+ C_{106}H_{263}O_{110}N_{16}P \rightarrow$ $106\ CO_2+16NH_3+H_3PO_4+53S^{2-}+106H_2O$	-380
Methanogensis $C_{106}H_{263}O_{110}N_{16}P \rightarrow$ $53CO_2+53CH_4+16NH_3+H_3PO_4$	-350

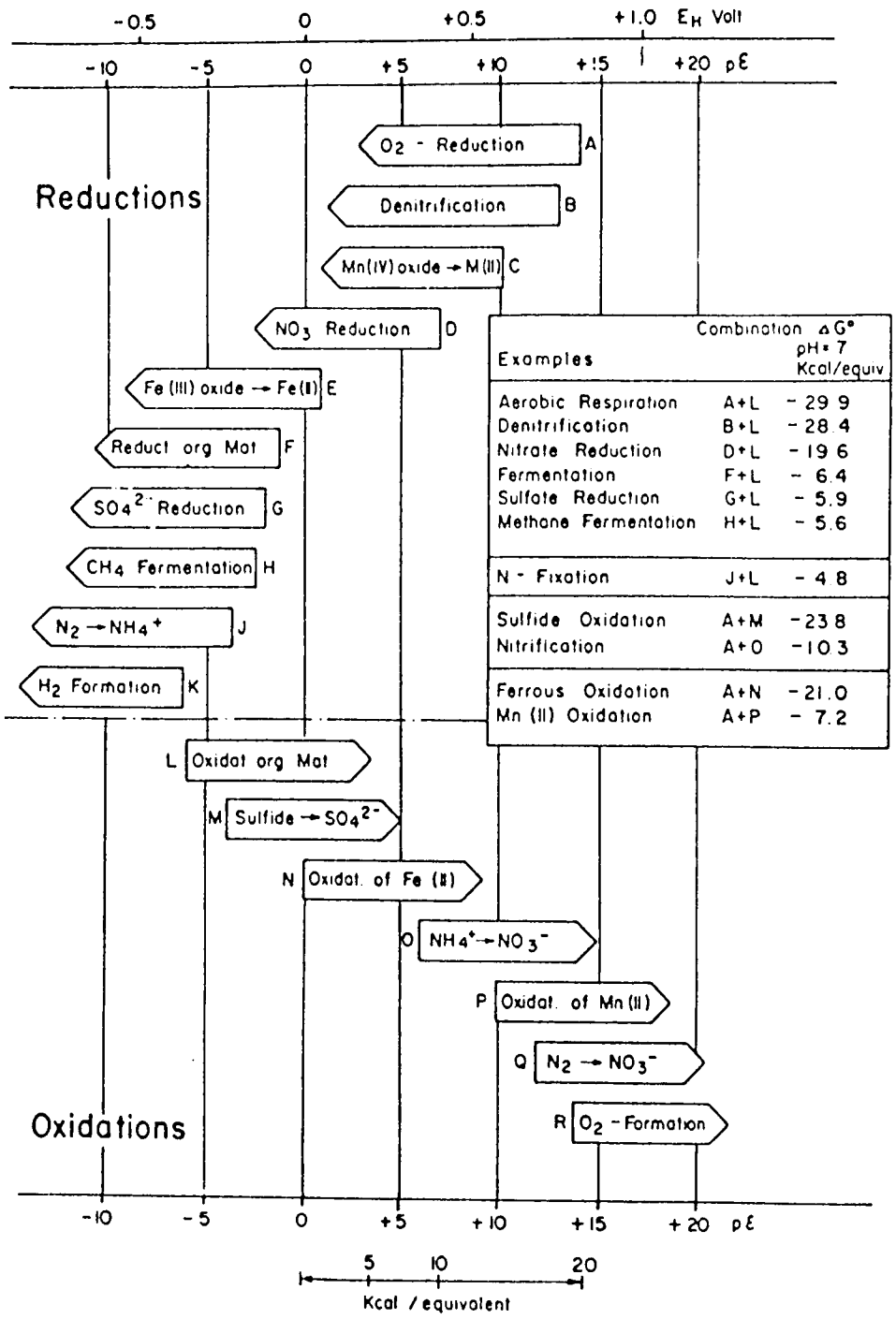


Figure 1: The sequence of microbially mediated redox processes (Adopted from Stumm and Morgan, 1981).

It can be seen that microorganisms obtain the highest energy from oxygen utilization, which suggests that microorganisms prefer to utilize oxygen more than any other electron acceptor listed. However, in most sediments, manganese and ferric oxide reductions are found more readily because oxygen and nitrate are not found in high concentrations relative to these metal oxide phases. The succession of oxidants for microbial decomposition of organic matter indicates that there are discrete zones in the sediment porewater, which show the buildup and depletion of Mn (II) followed by the buildup and depletion of Fe (II) in the deeper sediment. Most iron and manganese reactions are microbially catalyzed (Lovley, 1991). Recent studies by Lovely *et al.* (1992 & 1993) identified a group of bacteria responsible for organic degradation coupled with reduction of metals such as Fe (III) and U (VI).

2.1.3 Heavy metal removal from contaminated soil and water

Heavy metal contamination of subsurface and groundwater results from various activities including the following: application of industrial waste, application of fertilizers and pesticides, mining operations, smelting operations, automobile battery production, metal plating/metal finishing operations, vehicle emissions and fly ash from combustion/incineration processes (Peters and Shem, 1991).

Metal concentrations in the aquifer are greatly controlled by the interactions with surrounding materials. A variety of mechanisms influence the partition of metals between the solid phase and the solute phase and hence affect the leachability of metals from contaminated soil. Those processes could be dissolution/precipitation, sorption/exchange

and complexation/biological fixation (Elliott and Peters, 1991). Complexation dramatically increases metal solubility. Complexing agents such as ethylenediaminetetraacetic acid (EDTA) and nitrilotriacetic acid (NTA) appear to be potential metal extractants (Ku & Peters, 1991). Lovley (1988) and Caccavo (1992) reported that NTA or citrate chelated ferric ion was more readily utilized by a series of iron reducing bacteria. Recently some researchers combined organic acid extraction with *ex-situ* biotreatment for radioactive metal mobilization. Gorby *et al.* (1997) reported that cobalt EDTA complex can be utilized by an iron reducing bacterium *Shewanella alga* as a terminal electron acceptor. Lovely *et al* (1992, 1993 & 1995) found out that uranium carbonate extractant was highly bioaccessible to a wide range of sulfur and iron-reducing bacteria. As the most important and common reaction in the natural environment, iron reduction is discussed in the following section.

2.1.3.1 Iron chemistry and removal

Iron is the sixth abundant element and the most abundant metal in the universe. On the surface of earth, iron is found in a number of ores, primarily in the form of magnetite (Fe_3O_4), hematite (Fe_2O_3), siderite ($FeCO_3$) and pyrites (FeS_2) (Silver, 1993). Iron has two common oxidation states, Fe (III) and Fe (II). In the presence of air or warm dilute nitric acid, Fe (II) can be oxidized to Fe (III). Iron hydroxide is very insoluble (Silver, 1993). Fe (II) can form a wide range of complexes while Fe (III) forms strong complexes with some oxygen-containing ligands such as citrate. Ferric citrate is readily utilized by microorganisms in a reducing environment.

Dissimilatory Fe (III) reduction has a greater overall environmental impact than microbial reduction of any other metal. Microbial Fe (III) reduction has been directly shown or implied as an important process in the following phenomena: organic matter decomposition in a variety of fresh water, estuarine, and marine sediments; the decomposition of aromatic hydrocarbons in contaminated aquifers; and the generation of undesirably high concentration of dissolved iron in deep pristine aquifers (Lovley, 1993).

2.1.3.2 Uranium chemistry and removal

Uranium is now recognized as a ubiquitous element with a wide distribution in nature. Its radioactive property and disintegration products like radium make it easy to detect and estimate. The best available estimate of the mean uranium content in the surface of the earth's crust is $4x10^{-6}$ g per gram of rock, which is far greater than other metals like silver or mercury, which are not considered excessively rare (Katz & Rabinowitch, 1951). Uranium appears to be a normal component of soil, which is in concentrations varying from 10^{-4} to 10^{-9} percent by weight (Hoffman, 1941; 1942; 1943). Uranium forms a series of complex minerals, among which uraninite may be considered as the primary mineral form. Natural uraninite crystals are isomorphous with CeO_2 and ThO_2 and give the same x-ray patterns as the synthetic uranium dioxide. They are always more or less strongly oxidized so that their actual component lies between UO_2 and $UO_{2.67}$ (U_3O_8) with tetravalent uranium usually predominant (Katz & Rabinowitch, 1951).

Uranium chemistry is very complex. Hexavalent uranium as well as tetravalent uranium forms a variety of complexes with organic and inorganic anions in the environment. The type of uranium species formed in the environment depends on its free energy of formation, concentration of various anions and cations present, pH and electron potential (Ganesh, 1995). In surface waters and other aerobic environments, uranium predominantly remains in the hexavalent oxidative state forming various oxide and hydroxyl complexes (Truex, 1992). In groundwaters, hexavalent uranium forms several soluble complexes with carbonate (Langmuir, 1978). Uranium in carbonate complexes has been found to be highly bioreducible using cell suspensions of several bacteria under lower redox potentials (Lovley *et al.*, 1992, 1993 & 1995).

The early geochemical literature suggested that U (VI) reduction in an anaerobic environment resulted from the non-enzymatic reduction of U (VI) by sulfide or H_2. However, neither sulfide nor hydrogen is an effective U (VI) reductant at the temperatures and pHs typical of most aquatic sediments or ground waters, and the sterilization of anaerobic sediments inhibits U (VI) reduction (Lovley *et al.*, 1991). These findings suggest U (VI) reducing bacteria are responsible for U (VI) reduction in many environments. The reduction of U (VI) in marine sediments is the most globally significant sink for dissolved uranium (Anderson *et al*, 1989; Klinkhammer *et al*, 1991; Veeh *et al*, 1967). The reductive precipitation of uranium from groundwater is considered to be the major mechanism for the formation of some sandstone or roll-type uranium ores (Hostetler *et al.*, 1962; Jensen, 1958; Langmuir, 1978).

Microbial reduction of uranium may also be used to remove uranium from contaminated waters and soils. A uranium contaminated waste stream usually is a mixed waste, containing a wide range of other metals such as iron, nickel, zinc and calcium, and inorganics like sulfate (Ganesh, 1995). Such characteristic of wastewater requires a high selectivity for uranium regarding the treatment technology. Currently there are three major types of mechanisms for uranium treatment (Gadd, 1988). They are chemical precipitation, biosorption and biotransformation. Biotransformation is considered the most promising option because of its high selectivity and the relative small volume of the final sludge produced.

Biotransformation is a biotreatment technology in which organisms change the original chemical form of metals into other less toxic forms by enzymatic reduction, oxidation, methylation and demethylation (Wood and Wang, 1983). Microbial uranium reduction belongs to this category. At redox potential below –200 mV, several types of microorganisms can utilize hexavalent uranium (UO_2^{2+}) as a terminal electron acceptor for anaerobic respiration and/or cellular growth. The reduced uranium hence precipitates from the waste stream in an insoluble tetravalent form (uraninite UO_2). Microbial uranium reduction is a cost-effective treatment technique because it reduces radioactive waste volume by a factor of 75 (Truex, 1995). Microorganisms involved in enzymatic uranium reduction are discussed in the following section.

2.2 Microorganisms involved in uranium biotransformation

From 1991 when Lovely *et al.* reported the first uranium reducing bacterium *Geobacter Metalireducens* till today, several anaerobes have been reported capable of U

(VI) utilization. According to the primary electron acceptor utilized, those organisms can be classified into two categories, sulfur reducers and iron reducers. Information on these bacteria available to date is collected and summarized in Table 3.

2.2.1 The common and different characteristics of these microorganisms

These uranium-reducing microorganisms have some common characteristics. First, their activities were all found in natural anaerobic sediments, the low redox potential environment. Secondly, other than U (VI), they can utilize various other electron acceptors available in sediments, such as Fe (III), Mn (IV), SO_4^{2-} or NO_3^-. In addition, they can utilize hydrogen as the electron donor and organic substances (abundant in the sediment) as the carbon source/electron donor. Third, it seems that there is one specific enzyme responsible for uranium reduction, either located in the periplasmic region or cell membrane.

The differences among these microorganisms determined their application for uranium waste treatment. First of all, the ideal candidate should have a high reduction rate as well as reduction extent for uranium. It's been reported that *Shewanella alga* and *Desulfovibrio desulfuricans* reduced uranium at the highest rate under non-growth conditions (Lovley, 1992; Caccavo, 1995). High percent uranium removal has been demonstrated in uranium carbonate solutions and some organic solutions using both microorganisms. The ease of microbial growth and the density levels achieved are important factors in treatment scenarios. Strict anaerobes require extremely low redox potentials for enzyme activities, which are very difficult and costly to maintain in

Table 3: Microorganisms involved in uranium reduction

Category	**Iron reducing**			**Sulfur reducing**	
Name of Bacterium	*Geobacter Metalireducens*	*Shewanella putrefaciens*	*Shewanella alga* (BrY)	*Desulfovibrio desulfuricans*	*Desulfovibrio vulgaris*
First Isolation	1987, bottom sediment of Potomac River,VA	1988, sediment of Oneida Lake, New York	1992, Sediment of Great Bay Estuary, New Hampshire	1992	N/A
Tolerance to Oxygen	Strict anaerobe	Facultative	Facultative	Little tolerance	Little tolerance
Electron Donors/ Substrate	Acetate, Aliphatic/ Aromatic compounds	Lactate Hydrogen Formate	Lactate Citrate H_2	Lactate H_2 Formate Pyruvate	Lactate H_2 Formate Pyruvate
Electron Donors Cannot Be Utilized	Glucose Lactate Formate Hydrogen	Aliphatic/ Aromatic hydrocarbons	N/A	N/A	N/A
Electron Acceptors	Fe (III)/U (VI) Mn (IV)/Cr(VI) NO_3^-	Mn (IV)/O_2 U (VI)/Fe(III) SO_4^{2-}/ NO_3^-	O_2/Fe(III) Mn(IV) U(VI)	SO_4^{2-} Fe(III)/U(VI) Mn(IV) Cr(VI)	SO_4^{2-}/ Fe(III) U(VI) Mn(IV) Cr(VI)
Electron Acceptors Cannot Be Utilized	Oxygen Sulfate Elemental sulfur	N/A	Sulfate Nitrate (poor)	N/A	N/A
Uranium Reducing Enzyme	Membrane bound c-type cytochrome has been isolated (Lovely,1993)	C type cytochrome identified but not isolated	C type cytochrome found both in soluble cell fraction and membrane portion	C_3 type cytochrome in soluble fraction of the periplasmic region found responsible for U(VI) reduction	C_3 type cytochrome in soluble fraction of the periplasmic region found responsible for U(VI) reduction
Growth Using Uranium as the Sole TEA	Yes (Lovely, 1991)	Yes	Unknown	No (Lovely & Phillips, 1992)	Unknown

engineered systems. *D. desulfuricans* could only survive 3 to 20 hours in an environment containing a low concentration of oxygen (Postgate, 1979; Dilly, 1991). However, *S. alga* can grow at a high rate using oxygen as a terminal electron acceptor. Since most of the uranium bioreduction studies were demonstrated using cell suspensions under non-growth conditions, a sufficient amount of biomass then was required to ensure a high reduction rate. *Geobacter Metalireducens* and *Shewanella putrefaciens* grow at a relatively low rate compared to *S. alga* and *D. desulfuricans.* Therefore, their application has been largely restricted. Whether the organism can selectively reduce uranium from mixed contaminants is also an important concern. Uranium wastewater usually contains mixed components such as metals (including iron) and SO_4^{2-}. Sulfur reducing bacteria can reduce sulfate to sulfide (S^{2-}), which then precipitates with other metal ions as metal sulfide from the wastewater. This co-precipitation becomes a serious problem since most of the metals can form highly insoluble metal sulfide precipitates. Final sludge volumes can be significantly increased due to co-precipitation. *S. alga* displays an advantage compared to *D. desulfuricans* because this organism cannot utilize sulfate as an electron acceptor. However, whether the presence of iron in uranium-contaminated water may impact uranium reduction using *S. alga* is still an unknown.

In summery, among all the uranium reducing bacteria, *Desulfovibrio desulfuricans* and *Shewanella alga* are currently considered as the most promising strains. Both bacteria are found to be highly efficient in U (VI) reduction under non-growth conditions. In addition to U (VI), *D. desulfuricans* can utilize sulfate as the sole electron acceptor and obtain energy for growth, while *S. alga* can grow with poorly crystallized

ferric as the sole electron acceptor. Lovely (1992) reported that *D. desulfuricans* could not grow with U (VI) as the sole electron acceptor. However, this bacterium can obtain energy for growth from sulfate reduction while simultaneously reducing U (VI) using two different mechanisms. Lovely (1992) concluded that *D. desulfuricans* might be more practical in some applications compared to *S. alga* because soluble ferric may reoxidize U (IV) to U (VI). This assertion was based on the hypothesis that microbial reduction of U (VI) and Fe (III) by *S. alga* occur simultaneously. To confirm this assertion, studies on the impact of Fe (III) on U (VI) reduction using *S. alga* need to be conducted. Whether or not potential chemical reactions occurring during enzymatic U (VI) and Fe (III) reduction will affect uranium removal needs to be investigated.

2.2.2 *Shewanella alga*

Shewanella alga, a dissimilatory Fe (III) and Mn (IV) reducing bacterium, was first isolated by Dr. Caccavo from the bottom of Great Bay Estuary, New Hampshire in 1993 (Caccavo, 1993). The isolate was determined to be a facultative anaerobic gram-negative rod. Well-isolated colonies were pink, round, and glossy. Cells were straight rods 0.75 to 1.5 μm in length by 0.5 μm in width. They were motile and possessed one or two polar or bipolar flagella. This bacterium was found to grow anaerobically in a defined medium with hydrogen or lactate as the electron donor and Fe (III) as the electron acceptor. *S. alga* can also utilize U (VI) as the terminal electron acceptor during anaerobic respiration. In addition to Fe (III) and U (VI), *S. alga* also utilized oxygen, Mn (IV), thiosulfate and fumarate as electron acceptors. Nitrate was poorly reduced. Cells didn't

utilize sulfate, sulfide or elemental sulfur as electron acceptors. In addition to lactate, *S. alga* used citrate, fumarate and malete as carbon sources. However, energy for growth was obtained only from H_2 and lactate oxidation. Growth of *S. alga* using U (VI) as the sole electron acceptor has not yet been established. Peptone *et al.* (1995) reported that under non-growth conditions, *S. alga* reduced uranium from carbonate complexes more efficiently than other bacteria. However, further evaluation of this organism for uranium treatment is required.

- **The reduction kinetics**

Kinetic coefficients will help to evaluate the capability of *S. alga* for uranium reduction, to predict the reduction rate and extent, and assist process design. Previous research was focused on the capability of this organism for uranium reduction. Limited kinetic information has been reported.

- **Growth of *S. alga* using U (VI) as the sole terminal electron acceptor**

Uranium reduction has been demonstrated under non-growth conditions where cells maintain a minimum metabolism due to the absence of nutrients. Microbial growth using uranium as the sole electron acceptor has been demonstrated in *G. metalireducens* and *S. putrefaciens*. However, no growth was observed during the incubation of *D. desulfuricans*. As a strong candidate for uranium waste treatment, whether *S. alga* can grow using U (VI) as the sole electron acceptor is valuable information regarding the selection of microorganisms. Though in natural anaerobic sediments the level of hexavalent uranium is low and cannot support significant cell growth, microbial growth

using uranium as an alternative electron acceptor may have great significance for continuous uranium wastewater treatment.

- **Impact of Fe (III) to U (VI) reduction**

There are a variety of electron acceptors in natural anaerobic sediments. Hexavalent uranium is just one of them. Some researchers have done studies on the competition for electron donors between sulfur reducers and iron reducers in anaerobic sediments. The result indicated that iron reducers could outcompete sulfur reducers for electron donors like H_2 (Lovley *et al.*, 1992). Studies also have been conducted to observe the reduction pattern of *D. desulfuricans* in the presence of co-electron acceptors (SO_4^{2-} and U (VI)). At certain concentrations, uranium reduction and sulfate reduction take place simultaneously without any impact from each other. It was suggested that different enzyme systems might be involved in the utilization of these two electron acceptors. A study was performed to determine the kinetic coefficients for uranium reduction using a laboratory chemostat. Sulfate was added as a growth supporting electron acceptor while uranium as a limiting electron acceptor. Steady-state data were analyzed and 90% co-removal of U (VI) was found (Tucker, 1995). However, whether *S. alga* can utilize U (VI) and Fe (III) simultaneously has yet to be established. Such information is required to determine if the presence of Fe (III) in wastewater would have a negative impact on U (VI) removal using *S. alga* cells.

2.3 Models for enzymatic metal transformation

2.3.1 Mechanisms for microbial Fe (III) transformation

Microbial iron transformation has been well studied compared to U (VI) utilization. Iron uptake can be classified into three major mechanisms: iron assimilation by plants and animals as an essential component of protein and tissue; iron deposition by microorganisms; and microbial iron reduction and oxidation (Moore and Wilson, 1993). Microorganisms capable of Fe and Mn reduction are found in many different groups, ranging from highly aerobic bacteria and fungi to strictly anaerobic bacteria. The genetics of iron transformation during iron metabolism has been well studied. It involves a speciation specific transport system, a specific enzyme, as well as an ATP generating electron flow system.

Iron transport

Microbial Fe (III) reduction involves two iron forms, ferric oxide and water-soluble Fe (III) complex. Microbial utilization of Fe (III) requires the effective cellular binding, the transport of ferric ion across the outer membrane and the final release of Fe (II) inside the cell membrane. It's been long established that microbial metal utilization is greatly impacted by metal speciation. Cox (1980) reported that the bacterium *P. aeruginosa* reduced Fe (III) rapidly from a strong pyochelin complex. Fe (III) from a weak chloride complex was not reduced by this bacterium. A review of the literature also showed that the growth of *S. alga* as well as other iron reducing bacteria was also impacted by the speciation of Fe (III) (Lovley, 1988; Caccavo, 1992). *S. alga* grew rapidly when Fe (III) was chelated by organic ligands such as citrate or NTA. However,

the growth was significantly slowed when amorphous ferric hydroxide was provided as the electron acceptor. The reason why *S. alga* favors strong organic complexes for ferric transformation has not been elucidated. However, strategies for microbial iron assimilation have been postulated. Weinberg (1989) proposed at least four different strategies for iron assimilation by bacterial and fungal cells. The most common mechanism for iron acquisition involves the specific chelation of ferric iron by siderophores, either synthesized by the organism itself or by cells of other species or genera (Hider, 1984; Byers, 1987). The siderophores must have a high affinity for ferric ion and hence their evolutionary design has been dictated by chemical properties of the metal. Ferric-siderophore was then recognized by the outer membrane of microbial cells, which utilize siderophore-mediated iron accumulation (Hider, 1984; Clarke *et al.*, 1987). Once its transport has been assured across the outer membrane through the periplasm to the cytoplasmic membrane, iron is reduced by a ferric-siderophore reductase, located either on the cytoplasmic membrane, or within the cell. The reduced iron is then released from the ferric-siderophore. The iron free siderophore must either be modified, re-excreted or stored to ensure that it will not interfere with the intracellular iron utilization (Hartmann and Braun, 1980; Byers, 1987). In a well-studied citrate-dependant Fe (III) transport system, the uptake of ferric citrate requires the outer membrane protein Fec A as a receptor. Although citrate is required for induction, it cannot serve as a carbon or electron source (Frost and Rosenberg, 1973). This example is very similar to the situation where *S. alga* requires ferric citrate for efficient ferric reduction while citrate cannot serve

as an electron donor nor carbon source. Whether this strategy is also utilized for ferric biotransformation by *S. alga* is currently unknown.

Specific enzymes

Microbial metal reduction could be the direct reduction by enzyme or indirect reduction by metabolic end products. Enzymatic iron reduction is more important than the reduction by metabolic end products. Lovley *et al.* (1992) conducted several experiments to isolate the reductase from selected iron reducing and sulfur reducing bacteria. An active metal reductase (C_3 cytochrome), responsible for the reduction of crystalline ferric oxide, was isolated from *D. desulfuricans*. For iron reducer *G. metalireducens*, a membrane bound Fe (III) reductase as well as several soluble c-type cytochromes, which were distinct from nitrate reductase, appeared to be involved in the electron transport to Fe (III) and other metals. For another iron reducer *S. putrefaciens*, anaerobic growth was found to stimulate the production of c-type cytochromes in the outer membrane, though whether these cytochromes can reduce Fe (III) was not identified. In summary, it appeared that there was a specific metal reductase located either on cell membrane or inside the cell, which was responsible for Fe (III) reduction.

Energy and electron flow

Microbial Fe (III) reduction could occur through ferric assimilation (non-growth associated) and growth coupled ferric reduction (dissimulation). As early as 1992, no microorganisms had been found capable of coupling organic oxidation and ferric reduction with ATP synthesis. An Fe (III) reducing enzyme system was studied in cell-free extracts of *Vibrio sp.* isolated from English Lake. The iron reducing activity was

inhibited by NOQNQ, azide, and rotenone, suggesting that a respiration chain was involved. However, there was no increase in growth yield with ferric oxide provided as the sole terminal electron acceptor. It was assumed that the organism used its Fe (III) terminated electron transport system to dissipate excess reductant without synthesizing ATP (Zehnder, 1988). Yet another iron reducing enzyme system has been investigated in *Pseudomonas sp.* strain 200 isolated from cruel oil. Arnold *et al.* (1991) conducted spectrophotometric studies using non-growing whole cells and found that cells grown on high concentration of O_2 reduced chelated iron through an abbreviated electron transport chain. Cells grown on low concentration of O_2 increased their iron reducing activity six to eight fold, but the increase in the activity apparently was uncoupled with oxidative phosphorylation.

Iron reducer *G. metalireducens* was the first organism reported to couple Fe (III) reduction and organic/hydrogen oxidation with cellular growth. A series of iron reducing microorganisms have since been reported capable of iron utilization for ATP synthesizing phosphorylation. For example, Roden *et al.* (1996) reported that *S. alga* grew in a medium with synthetic goethite ($2.5x10^6$ cells produced/mmole of goethite) as the sole electron acceptor.

Uranium reduction mechanism

The mechanism for enzymatic U (VI) reduction by *G. metalireducens* and *S. putrefaciens* is ill defined. The fact that these microorganisms conserve energy to support growth by oxidizing non-fermentable substrates with U (VI) as the electron acceptor suggests that the electron transport-linked phosphorylation must be involved. Electron

transport to or through c-type cytochrome is likely based on the observation that U (VI) oxidizes the c-type cytochrome in whole-cell suspensions of *G. metalireducens* (Lovley, 1991). A U (VI) reductase has been isolated from *D. vulgaris*. The soluble fraction of *D. vulgaris* rapidly reduced U (VI) with H_2 as the electron donor. All the capacity for U (VI) reduction was lost when c-type cytochrome was removed from the soluble fraction. The reducing capacity was restored after the c-type cytochrome was added back (Lovley and Phillips, 1992). Caccavo *et al* (1995) attempted to isolate the enzyme responsible for U (VI) reduction from *S. alga*. Although the pure enzyme was not isolated, they observed that >90% of the uranium reduction capacity was associated with the membrane fraction of the cell. A "c" type cytochrome was found involved in U (VI) reduction. In addition, U (IV) precipitation using *S. alga* was extracellular. The result suggests that *S. alga* may not need to transport U (VI) across the cell membrane for reduction (Ganesh, 1995).

The mechanism of uranium reduction by various microorganisms is still limited within the information of overall reaction end products. Better understanding of detailed transport system and electron flow system will help engineers further apply those microorganisms into industrial waste treatment. The result will also be valuable to ecologists who are interested in microbial activity and heavy metal cycling in anaerobic sediments.

2.3.2 Non-growth and growth coupled reduction

Most of the previous studies on enzymatic uranium reduction were conducted under non-growth conditions. Non-growth conditions are defined as the environment in

which cells maintain a non-growing but active metabolism. Non-growth phase is ensured by not providing cells with essential nutrients, minerals and vitamins. The cell density is assumed to be conservative and cell growth yield assumed to be zero. However, the electron donor and acceptor are usually provided to complete the electron flow chain in order to supply energy required for cellular maintenance activities. Non-growth conditions do not last indefinitely. After remaining in a nutrient-free environment for a long time, cells will eventually shift to the decay phase. During laboratory uranium reduction studies, non-growth conditions were selected to observe microbial reduction capabilities for the following reasons. First, the non-growth medium contained the least number of components, which avoids any possible interference during uranium or iron analysis. Additionally, non-growth conditions provide relatively stable cell conditions, which are under easy control for initial microbial studies.

Therefore, non-growth studies appear to be a simplified and useful research tool. The constant cell density assumption breaks down when cells actually shift to a decay phase due to various reasons. For studies on growth kinetics and continuous culture, non-growth conditions can no longer meet the criteria required and the medium has to be eventually defined to support microbial growth.

Non-growth and growth conditions for bioreduction studies are different in many aspects. First, cells encounter a different medium environment. As previously discussed, media used for non-growth and growth studies were dramatically different in composition. Each was prepared to meet a specific purpose. For non-growth studies, the medium (also referred as the test solution) only contained lactate as the electron donor

and Fe (III) or U (VI) as the electron acceptor. Nutrients essential for bacterial growth, such as N, P, minerals and vitamins, were not provided. The electron donor and acceptor in test solutions help enzymes to complete the electron flow chain by transferring electrons from lactate to Fe (III) or U (VI). Cells in turn, obtain energy for maintenance activities, such as ion transport across the membrane, and repair of broken or damaged cellular material. Cells in test solutions were assumed to maintain a non-growth phase due to the shortage of essential nutrients required for protein, RNA and DNA synthesis.

For growth coupled bioreduction studies, the media contain sufficient substances such as the electron exchange couple, carbon source, nutrients, minerals and vitamins. Cells obtain relatively higher energy not only for maintenance but also for protein synthesis and cell divisions. Cells will also eventually shift to the decay phase if any essential substance becomes limited or depleted or, due to the presence of toxic products.

The difference in medium components determines the different behavior of microorganisms. The inoculum for non-growth studies was cells in the stationary growth phase. At this phase, cells are still metabolically active and produce secondary metabolites though the net growth rate is zero. Primary metabolites are growth-related products and secondary metabolites are non-growth related. During the stationary phase, one or more of the following phenomena may take place:

- Total cell mass concentration may stay constant, but the number of viable cells may decrease.
- Cells may not be growing but may have an active metabolism to produce secondary metabolites. Cellular regulation changes when concentrations of certain metabolites

(carbon, nitrogen, and phosphate) are low. Secondary metabolites are produced as a result of metabolite deregulation.

The above phenomena describe how enzymes respond during the stationary phase of batch growth. However, for non-growth reduction, what would happen after the stationary cells were inoculated into a new nutrient-free environment (test solutions)? According to the above information, cellular regulation will change when concentrations of certain metabolites such as N and P change to zero from the nutrient rich growth medium to test solutions. As a result, secondary metabolites (different from the growth related primary metabolites) are produced. The number of viable cells may drop, which suggests the total reduction capacity may decrease. Fortunately most of the uranium reduction studies were conducted within 24 hours, where the constant viable cell density may not be such a bad assumption.

Some researchers (Gorby *et al*, 1995) concluded that non-growth reduction was similar to the enzyme-catalyzed reaction. Recall the role of enzymes in chemical reactions: enzymes lower the activation energy of the reaction catalyzed by binding the substrate and forming an enzyme-substrate complex. Enzymes do not affect the free-energy change or the equilibrium constant. Chemical reactions catalyzed by enzymes could greatly improve reaction rates by a factor about 10^8. In addition to the high efficiency, enzymes are highly specific to the substrate they catalyzed. Some enzymes require certain cofactors, usually metal ions or coenzymes for proper function.

The growth-coupled batch reduction appears more complicated because both the cell density and the substrate concentration are changing continuously. It can provide valuable information on growth parameters for a limiting substrate or nutrient. Batch growth kinetic data will help in a continuous flow system design. Cells in the growth medium, upon inoculation, will selectively take up the dissolved nutrients and convert them into biomass. Then cells will go through a typical batch growth cycle, which includes the following five phases, lag phase, exponential phase, deceleration phase, stationary phase and death phase. It is the exponential phase where most of the modeling occurs. During the exponential phase (also known as the logarithmic growth phase), cells multiply rapidly, and the cell mass and the cell density increase exponentially over time. Cells, upon growing, produce primary growth-associated metabolites and create new enzymes.

For growth, the energy requirement is much higher because oxidative phosphorylation is involved. The living cells are able to couple energy-yielding reactions with chemical reactions and other functions that do not occur appreciably unless the energy is supplied. The energy obtained through electron chains is typically stored and shuttled in a convenient high-energy intermediate such as ATP. The cell uses this energy to perform three types of work: chemical synthesis of large and complex molecules (growth), transport of ionic and neutral substance into or out of cell or its internal organelles, and mechanical work required for cell division and motion. The energy for biosynthesis is usually utilized at a high efficiency, usually greater than 20 percent.

The differentials existing between non-growth and growth reactions can be better understood by the presence of a series of iron reducing microorganisms. Although iron can be utilized as a terminal electron acceptor with organic carbon as an electron donor, no growth has been observed during the whole period of incubation. It suggests that the energy obtained through this electron flow chain is not sufficient for biosynthesis. Some authors concluded that Fe (III) apparently was reduced by side reactions of NO^{3-} or O^{2-} terminated respiratory chains (Zimmermann, 1984). Others think that this organism used its Fe (III) terminated electron transport system to dissipate excess reductant without synthesizing ATP (Zehnder, 1988). In 1992, Lovely *et al* isolated an iron-reducing microorganism (*G. metalireducens)* capable of coupling Fe (III) reduction and acetate oxidation with cellular growth. Later, additional iron reducing bacteria, including *S. alga,* have been reported to be able to grow using chelated Fe (III) as the sole electron acceptor. In summary, different from such dissimilatory iron reducers, there exist a group of microorganisms which cannot couple iron reduction with growth even when all the necessary nutrients are provided. It indicates that those microorganisms probably utilize iron in the way different from those growth-coupled iron reducers.

In addition to Fe (III), U (VI) can also play two different roles in the same microorganism. The sulfur reducing bacterium, *Desulvibrio desulfuricans,* was found to reduce uranium under non-growth conditions although growth using uranium as the sole electron acceptor was not successful (Lovley and Phillips, 1992). The authors didn't give any explanation for the result observed. According to previous studies on iron reducers, the possible reasons might be that either the organism used the uranium terminated

transport system to dissipate excess reductant without synthesizing ATP or, the energy obtained through uranium reduction was too low to conduct biosynthesis.

In summary, non-growth microbial reactions and growth coupled reactions have significant differences in microbial growth status, cellular product formation and energy requirements. It's been demonstrated that *S. alga* can reduce U (VI) under non-growth conditions. However, whether *S. alga* can also couple U (VI) reduction with growth is to be established. It's also remained unknown whether the impact of iron on uranium reduction would be the same for *S. alga* under non-growth and growth conditions.

2.3.3 Multiple substrates/enzymes system

2.3.3.1 Multiple substrates reacting on a single enzyme or a single microorganism

Some enzymes can utilize more than a single substrate. In such cases, various reactants compete with each other for the limited active enzyme sites. The most obvious examples of such enzymes are hydrolytic deploymerase, which act on many identical bonds, regardless of the length of the polymer segment (Shular, 1991). When the substrate takes the form of a dimer, trimer, or oligommer, the kinetic parameters characterizing the reaction may change from the values applicable to large polymeric units. Some hydrolytic enzymes exhibit selectivity for different regions of the bio-polymer chain (Bailey and Ollis, 1986). The kinetics describing the reaction of mixture substrates catalyzed by one enzyme can be illustrated by the following example:

$E + S_1 = ES_1$ $\quad\quad$ K_1 = dissociation constant for ES_1 complex

$E + S_2 = ES_2$ K_2 = dissociation constant for ES_2 complex

Slow steps:

$ES_1 \rightarrow E + P_1$ k_1 = formation constant for product P_1

$ES_2 \rightarrow E + P_2$ k_2 = formation constant for product P_2

Here, each substrate (S_1, S_2) binds a certain fraction of the enzyme (E). P_1 and P_2 are products for substrate S_1 and S_1 respectively. Then the overall rate expressions are:

$$-\frac{dS_1}{dt} = V_1 = \frac{\frac{k_1 ES_1}{K_1}}{1 + \frac{S_1}{K_1} + \frac{S_2}{K_2}}$$

and

$$-\frac{dS_2}{dt} = V_2 = \frac{\frac{k_2 ES_2}{K_2}}{1 + \frac{S_1}{K_1} + \frac{S_2}{K_2}}$$

where V_1 = degradation rate of substrate 1

V_2 = degradation rate of substrate 2

E = concentration of enzyme.

The rate expression for substrate 2 catalyzed by one enzyme without the presence of substrate 1 is:

$$-\frac{dS_2}{dt} = V_2 = \frac{\frac{k_2 ES_2}{K_2}}{1 + \frac{S_2}{K_2}} = \frac{k_2 ES_2}{K_2 + S_2}$$

The equations above indicate that if two reactions are catalyzed by the same enzyme, the individual velocities are slower in the presence of both substrates than in the absence of one of the substrates. This fact has been used advantageously to determine whether the same enzyme in an undefined sample acts upon both substrates or each substrate's reaction is catalyzed by a separate enzyme (Bailey and Ollis, 1986).

The section above discussed the single enzyme reaction in the presence of multiple organic substrates, where they serve as carbon sources and/or electron donors. The following section will discuss a single enzyme reacting with multiple electron acceptors, in most cases they are oxidized metals or nitrogen/sulfur oxides. Lovely *et al* (1991) conducted a study on the co-reduction of two electron acceptors by non-growing whole cells of *D. desulfuricans*. The result indicated that sulfate and uranium were reduced simultaneously. The presence of sulfate (the primary terminal electron acceptor from which the microorganism obtains growth energy), did not have any effect on uranium reduction. The presence of uranium did not have any impact on sulfate reduction either. The authors concluded that there might be two different enzyme systems involved. In this case, not the single enzyme but the single microorganism with two different enzyme systems was involved.

2.3.3.2 Multiple enzymes or organisms reacting on a single substrate

There are many practical situations where more than one organism or enzyme competes for one single substrate. The best example could be the sediment environment,

where various facultative and anaerobic microorganisms compete for the same organic compounds. Lovley *et al.* (1987) studied sulfate reduction and methane production in the zone of Fe (III) reducing sediments. The result showed that addition of amorphic iron oxyhydroxide to sediments, where sulfate was the predominant terminal electron acceptor, inhibited sulfate reduction by 86-100%. In a similar manner, the addition of iron also inhibited methane production in sulfate-depleted sediments. The inhibition was believed due to the limitation of low concentration of organic carbon such as acetate, or hydrogen (electron donor) in sediments where iron reduction was a predominant process. The author further concluded that when Fe (III) was in the form which iron reducers can readily use, iron reducing microorganisms can inhibit sulfate reduction and methane production by outcompeting sulfur reducers and methanogen reducers for electron donors.

2.3.3.3 Inhibition and poising

Certain compounds can bind to enzymes and reduce their activities. These compounds are known to be enzyme inhibitors. Enzyme inhibitions may be irreversible or reversible. Irreversible inhibitors, such as heavy metals (lead, mercury and others), reduce enzyme activities by forming stable complexes with enzymes. Such inhibitions may be reversed by using chelating agents like EDTA and citrate. Reversible inhibitors may dissociate more readily from the enzyme after binding. There are three types of reversible inhibitions, competitive, non-competitive, and uncompetitive inhibitions. The substrate may act as an inhibitor in some cases.

Competitive inhibition

Competitive inhibitors are usually substrate analogs and compete with the substrate for active sites of the enzyme. The net effect of competitive inhibition is a decrease in enzyme affinity (high Ks) with a constant maximum reduction rate. Competitive inhibition can be overcome by using high concentration of substrate.

Non-competitive inhibition

Non-competitive inhibitors are not substrate analogs. Inhibitors bind to the enzyme at a site other than the target substrate-binding site and behaves as though it were removing the enzyme from the solution. The net effect of non-competitive inhibition is a decrease in the maximum reduction rate with a constant half-saturation constant (Devlin, 1986). High substrate concentrations would not overcome non-competitive inhibition. Other agents need to be added to block binding of the inhibitor to the enzyme.

Uncompetitive inhibition

Uncompetitive inhibitors bind to the enzyme-substrate complex only and have no affinity for the enzyme itself. The net effect of uncompetitive inhibition is a reduction in both the maximum reduction rate and the half-saturation constant.

Poising

Changes in the physicochemical properties of the medium would also cause a negative impact on enzymatic metal reduction by poisoning the system without direct enzyme attack. The possible causes for poising could be various environmental factors such as unfavorable pH, ion strength, electron potential, osmotic pressure, or the presence

of toxic compounds (Blanch, 1996). Kucsmas (1994) found that the presence of sulfate could delay uranium bioreduction when sulfate concentration was higher than 10,000 mg/L. He proposed that the delay might be caused by ironic strength poisoning or sodium poisoning. Lovley and Phillips (1992) found that copper lowered the uranium reduction rate when copper concentration was higher than 40 μM. Heavy metals such as copper poisoned the cells by biding to proteins and resulted in a deactivation. Most of the anaerobic processes produce acid, which cause a decrease in pH. If a proper amount of base is not added in time to neutralize the system, microorganisms will be poisoned by the products produced by themselves.

CHAPTER III

MATERIALS AND METHODS

3.1 Microorganism culture conditions

An iron reducing bacterium, *Shewanella alga* (formerly BrY) was obtained from Dr. Frank Caccavo, University of New Hampshire. This facultative bacterium was reported to grow either aerobically in rich tryptic soy broth or in an anaerobic medium containing ferric ion as the terminal electron acceptor and lactate or hydrogen as the electron donor. In this study, *S. alga* was grown under both aerobic and anaerobic conditions to compare differences in growth environment on the efficiency of subsequent metal reduction.

For aerobic growth, 30 grams of dehydrolized tryptic soy broth (DIFCO, product No. 0370-05-7) were added to 1 liter of deionized-distilled water. The medium was stirred until the broth completely dissolved then the pH was adjusted to 6.8 using 0.1 N NaOH. Aliquots of medium (50 mLs) were transferred to several 200 mL flasks. The medium flasks were tightly affixed with cotton caps and immediately autoclaved at 120°C (27 PSI) for 30 minutes. After the medium had cooled to room temperature, the solution was inoculated with frozen cells. Frozen screw-capped culture tubes were taken from a low temperature freezer (– 80°C) and placed at room temperature for 30 seconds. A small amount of culture from the frozen cell tube was removed using a previously sterilized inoculating loop, and inserted immediately into the cooled growth medium. A

sterilized cotton cap was then inserted immediately into the neck of the flask before incubation at 35°C with shaking at 150 rpm.

For anaerobic growth of *S. alga*, a stock medium was initially prepared according to Table 4. Ferric citrate (12.5 g) was added to 0.8 L deionized-distilled water and heated (with stirring) until dissolved. The solution was cooled to room temperature and the pH adjusted to 6.8 using 5N/0.1N NaOH (adjusted to 6 using 5N then to 6.8 using 0.1N). Bicarbonate, minerals, nutrients and vitamins were then added sequentially. The final volume was adjusted to 1 L using deionized-distilled water. The following protocol, developed by Hungate (1969), was used to ensure anaerobic conditions in the stock medium. The medium, upon heating to boiling, was deoxygenated by purging with an anaerobic gas mixture (5% H_2 : 10% CO_2: 85% N_2) for five minutes. Heating hastened the diffusion of dissolved oxygen out of the medium. Prior to purging, the gas was passed through a hot column (320°C) filled with copper fillings in a reduced state to remove any trace oxygen in the gas mixture. An H_2 rich gas mixture (80% H_2: 20% CO_2) was passed through the column for 10 minutes prior to deoxygenating to ensure the copper fillings were in a reduced state. Both the nitrogen and hydrogen gases were certified (purity 99.5%) from National Welders Co., Knoxville, TN. The deoxygenated medium was then quantitatively dispensed, using a 25 mL pipette, into 100 mL serum bottles. Prior to transfer, oxygen in the empty serum bottles was removed by purging with an N_2 rich gas mix for 30 seconds. After transferring, trace oxygen in the headspace of the serum bottles

Table 4: Composition of anaerobic growth medium of *S. alga*

Constituent	Final Concentration
NaHCO3	2.5 g/L
KCl	0.10 g/L
NH_4Cl	1.50 g/L
$NaH_2PO_4.H_2O$	0.60 g/L
Lactate	20 ml/L[1]
Ferric Citrate	12.25 g/L
Peptone	5.00 g/L
Yeast Extract	2.00 g/L
Vitamins	10ml/L[2]
Minerals	10ml/L

1. -60% w/w syrup (Sigma Chemical Co., St. Louis, MO)

2. - Composition in appendix I.

was removed by purging with the N_2 rich gas for 1 minute. Serum bottles were closed with butyl rubber stoppers (Belco glass Co., Vineland, NJ) while purging the headspace. Purging needles were pulled out gently while the stopper was pressed into place. After all the medium was dispensed, aluminum caps were then used to seal each bottle. The medium was autoclaved within 2 hours at liquid cycle (120°C, 27 PSI). Cell cultures grown 16 hours in the aerobic growth medium were transferred into each serum bottle using a sterilized syringe. The pipettes, syringes and needles used in the transfer of medium were flushed with an oxygen free gas mixture prior to use.

3.2 Cell characterization studies

Since the growth phase of cell suspensions and cell density may affect the rate and extent of Fe (III)/U (VI) reduction, these factors were closely controlled. Studies were first conducted to characterize the growth pattern of *S. alga* in order to ensure that the same number of cells in the same growth phase were used for each study. Since *S. alga* can grow both aerobically and anaerobically, the impact of different electron acceptors supporting early bacterial growth on non-growth metal reduction was also examined. This section describes the protocol applied in the experiments for bacterial growth characterization.

For anaerobic growth characterization, sample tubes were prepared by adding 9 mLs of growth medium to 25 mL anaerobic pressure tubes. Cells in the growing culture were quantified using direct cell counting (AODC procedure) and protein analysis (protein assay kit, Pierce). One mL of diluted culture was added to each pressure tube to a

final concentration of 10^3 cells/mL. Upon inoculation, tubes were incubated at 35°C over time. Aliquots of the growing culture (0.1 mL) were withdrawn for direct cell counting and protein assay over time. The experiment lasted up to 60 hours to ensure that exponential growth was included in the characterization.

Studies were also conducted to evaluate metal reduction efficiency at various phases of cellular growth. Cells grown in ferric citrate medium were collected at 24 and 48 hours and centrifuged (4°C) at 7500 g for 20 minutes. After decanting the supernatant, cells were washed twice by resuspending in 100 mLs of anaerobic bicarbonate solution (2.5g/L) followed by centrifugation (4°C) at 7500 g for 10 minutes. The cells were then resuspended in the bicarbonate solution (10 mLs) and 1 mL of this cell suspension was transferred into each anaerobic pressure tube containing 9 mLs of test solution (2.5 g/L $NaHCO_3$, 20 mM lactate). Proper amounts of anaerobic stock uranyl nitrate or ferric citrate were added to each tube, resulting in the appropriate initial U (VI) or Fe (III) concentrations. The bicarbonate washing solutions and the metal stock solutions were deoxygenated using Hungate's methods prior to use. All transfer pipettes, syringes and needles were flushed with an oxygen free gas mixture (5% H_2: 10%CO_2: 85% N_2). Reduction of U (VI) by cell suspensions was monitored by measuring the decrease in soluble U (VI) concentration over time. However, reduction of soluble Fe (III) was monitored indirectly by difference. Soluble Fe (II) concentrations were directly measured over time and subtracted from the total iron levels initially added to obtain Fe (III) concentrations.

For aerobic cell characterization, cells were grown in 50 mLs of tryptic soy broth medium for up to 60 hours. Aliquots of the culture (0.1 mL) were taken for direct cell counting and protein assay over time. Metal reduction efficiency studies were also performed for 12 and 16 hour grown cells. The procedure was the same as that in the anaerobic studies except that the harvested cells were degassed following the Hungate (1969) procedure to minimize oxygen remaining in the culture.

3.3 Metal stock solutions preparation

Reagent grade uranyl nitrate (Fluka Chemical Company, Ronkonkoma, NY) and ferric citrate (Sigma Chemical Company) were used for metal stock solution preparation. The chemicals were used as received with a purity of 99%. Twenty grams per liter of uranyl nitrate (as U (VI)) and 5600 mg/L of ferric citrate (100 mmole/L as Fe (III)) were prepared by dissolving dry chemical powder into deionized-distilled water. The water and the stock container were autoclaved prior to metal addition to avoid possible metal precipitation caused by heating. Different uranyl substock concentrations were obtained by diluting the 20 g/L stock (84 mmole/L as U (VI)) with deionized-distilled water. Each substock solution was then degassed using Hungate's procedure. To prevent possible oxidation of ferrous iron by air, fresh stock was prepared by dissolving 1.3 g/L of ferrous chloride then degassed immediately before each experimental run.

Chemicals used for metal analysis calibration were a high purity (99.5%) uranyl nitrate solution (1000 mg/L) from Ricca Chemical Company (Arlinton, TX) and ferrous

ethylenediammonium sulfate ($C_2H_{10}N_2O_4S.4H_2O$, FW = 382.15 g/mole) from Sigma Chemical Company.

3.4 Selection of experimental conditions

- **Metal speciation**

The bacterial characterization studies provided information on the growth pattern of *S. alga* using Fe (III) or oxygen as the terminal electron acceptor. Additionally, information concerning Fe (III) and U (VI) biotransformation efficiency by *S. alga* was obtained. Results were used to ensure a constant bacterial growth phase and cell density in the non-growth metal transformation studies. For the growth coupled metal biotransformation studies, a different medium was used to provide the necessary nutrients and minerals for microbial growth while Fe (III) or U (VI) was provided as the sole terminal electron acceptor. For non-growth metal reduction studies, various nutrients and vitamins essential for microbial growth were not provided. Microorganisms were maintained in a bicarbonate (2.5g/L) test solution containing 20 mM lactate as the electron donor.

As discussed in the previous chapter, biotransformation of metals is largely impacted by the chemical speciation of target metals, which is dependent on pH and the organic/inorganic ligands present in solution. In order to focus on the enzymatic transformation of metals and to simplify data analysis, pH was held constant in all experiments and no additional metal-complexing ligands were added to the test solutions. pH was buffered in non-growth test solutions and growth medium through the addition of

sodium carbonate (2.5 g/L). Solution pH was approximately 8.5 and the buffer capacity was sufficient to ensure no pH drop in the final medium after metal bioreduction. Organic chelating agents have a great impact on the bioavailability of U (VI) and Fe (III) to *S. alga.* For Fe (III), *S. alga* was reported to prefer organic Fe (III) complexes like citrate and NTA (Lovley, 1988) while for U (VI), inorganic carbonate complexes were found bioavailable to *S. alga* (Ganesh, 1995). Therefore, ferric citrate and uranyl nitrate were provided as the source of the desired metal species. The bicarbonate concentration of 2.5 g/L was sufficient to chelate UO_2^{2+} as uranyl carbonate. Based on the high solubility of ferric citrate, this species remained in solution as the primary metal form in the experiment.

- **Medium Composition**

Medium for growth of cell suspensions:

Growth of seed culture to a high final cell density was required for non-growth metal transformation studies. Therefore extra nutrients (peptone and yeast extract) were included in the ferric citrate medium for seed culture growth as indicated in Appendix I.

Medium for non-growth metal reduction studies:

The medium for non-growth metal reduction contained primarily an electron donor and an electron acceptor. Microorganisms were maintained in the stationary phase where nutrients and vitamins essential for cell growth were not supplied. The medium for non-growth metal reduction studies (also referred as the test solution in this report) contained 2.5 g/L of bicarbonate to buffer pH and chelate hexavalent uranium.

Additionally, lactate (2 mL/L of 60% w/w sodium lactate, equivalent to 20 mM of lactate) was added as the electron donor. Desired levels of ferric citrate or uranyl nitrate were supplied as the electron acceptor for anaerobic respiration.

Medium for growth coupled metal reduction studies

The growth coupled metal transformation experiments were expected to provide kinetic information for cellular growth and metal reduction. Therefore a chemically defined medium was required especially for growth kinetics studies. Peptone and yeast extract were removed from the growth medium (Table 4) since the exact chemical composition of these materials is unknown. In addition, the yellow color resulting from addition of these two materials caused interference with uranium analysis. Since Fe (III) and U (VI) are both chemically active elements, minimum salts medium was preferred to eliminate any possible interactions of medium components with the metals. Phosphate dosage was reduced to 0.06 g/L to prevent uranyl phosphate precipitation. The amount of lactate added was calculated according to the amount of Fe (III) or U (VI) used.

3.5 Uranium analysis

Hexavalent uranium U (VI) was measured using a Kinetic Phosphorescence Analyzer (KPA-11, Chemchek Instruments, Richard, WA). A laser beam was pumped through a dye nitrogen source (2,2'- ([1,1'-biphenyl] –4,4'-dyldi-2,1-ethendiyl) bis-benzenesulfonic acid disodium salt (stelbene 420)) to provide a wavelength of 425 nm. This specific wavelength light then excited oxidized uranium to phosphoresce by emitting a characteristic green light. Hexavalent uranium and tetravalent uranium can be

differentiated by their phosphorescence (tetravalent uranium does not phosphoresce.). A proton counting unit was used to record phosphorescence of the excited uranyl ion as luminance intensity. The intensity was counted at fixed time intervals (time gate) during the lifetime of phosphorescence (~200 to 300 μs). The sum of the intensity at each time gate then was compared to a standard uranium solution to determine the hexavalent uranium concentration in the sample. To correct for laser fluctuations and temperature drifts, a reference solution was excited and measured simultaneously. A patent protected quenching agent (URAPLEX) was mixed with each sample to quench interfering luminescence by non-uranium components in the sample. High concentrations of ferrous chloride were found to interfere with uranium analysis by quenching the luminescence. Dilution was used to minimize such interference. All samples were passed through 0.2 μm filter Acrodiscs (Gelman Scientific, Ann Arbor, MI), therefore hexavalent uranium was reported as mg/L of soluble U (VI) in the sample.

Uranyl nitrate ($UO_2(NO_3)_2 \bullet H_2O$, 1000 mg/L, Ricca Chemicals) was used for KPA calibration. According to manufacture specifications, the KPA response for U (VI) is linear up to 5 mg/L using the high range of the instrument and linear up to 0.2 mg/L using the low calibration range. Therefore, standard concentrations of 0.5, 1, and 2 mg/L uranium nitrate were prepared and calibrated for the high range. Concentrations of 100 μg/L and 200 μg/L were prepared for low range calibration. To correct for interference from the non-growth and growth media, solutions were diluted appropriately and added to each standard to ensure that the same background matrix as that in the uranium sample was analyzed. A blank standard, containing no uranium but all the other constituents, was

measured to determine background luminescence. Solutions were analyzed by mixing 1 mL of standard or sample with 1.5 mL of URAPLEX.

3.6 Iron analysis

Iron analysis was conducted through direct measure of soluble Fe (II) in the sample using the ferrozine assay (Lawrence, 1970). The Fe (III) concentration was calculated as the difference between the total initial Fe (III) concentration (based on stock preparation) and the measured Fe (II) concentration at any time. Ferrozine, disodium salt of 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (Hach Chemical Co., Catalog No. 2304), was found to react with divalent iron to form a very stable and intensely colored species, which was highly soluble in water. The complex was purple colored and exhibited a single sharp peak with maximum absorbance at 562 nm (Lawrence, 1970). The magenta Fe $(ligand)_3^{3+}$ species will form completely in aqueous solution between pH values of 4 and 9. Once formed, the complex is stable in 1 N perchloride acid. Some heavy metals will react with ferrozine in competition with iron, but excess reagent can minimize such interference.

Ferrousethylenediammonium sulfate ($C_2H_{10}N_2O_4S.FeSO_4.4H_2O$, Sigma Chemical) was used for ferrous standard preparation. Standards (0.125, 0.250, 0.500 and 1.00 mM) were prepared in 1% HCl (1 mL of concentrated HCl/99 mLs of dH_2O). Ferrozine agent was prepared by dissolving 1 g/L ferrozine in 50 mM HEPES then adjusting pH to 7.0 using 0.1 N NaOH. Each standard (100 µL) was mixed with 5 mLs of ferrozine solution then measure at a wavelength of 540 nm. Samples were diluted in 1%

HCl and filtered through 0.2 μm Acrodiscs then measured within 15 minutes. The volume of sample taken and the amount of HCl used depended on the concentrations of Fe (II) in the sample. Higher concentrations of Fe (II) required more HCl. The interference from U^{4+} was corrected by using 10 or 20 mLs of ferrozine reagent instead of 5 mLs.

3.7 Protein assay

Protein was used as an indirect indicator of cellular growth. A BCA protein assay reagent kit (Pierce, product No. 23225) was utilized for total protein analysis. The kit contains BCA reagent A (sodium carbonate and bicarbonate, bicinchoninic acid and sodium tartrate in sodium hydroxide solution), reagent B (4% cupric sulfate) and an albumin standard. Sample analysis was conducted by simply mixing freshly prepared reagent A with B in a 1: 50 volume ratio and adding this solution to the sample before incubating at 37°C for 30 minutes. Absorbance was then measured at 562 nm. The following reactions occur:

1. Protein (peptide bonds) + $Cu^{2+} \rightarrow$ tetradentate-Cu^{1+} complex
2. Cu^{+1} + 2 Bicinchoninic Acid (BCA) $\rightarrow$ BCA-Cu^{1+}ternary complexes (purple colored)

The BCA Protein assay kit combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium (the birut reaction) with the highly sensitive and selective detection of cuprous cation (Cu^{1+}) using a unique reagent containing bicinchoninic acid. The purple color reaction product is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm

which is linear with increasing protein concentration over a broad working range between 20 μg/mL and 2000 μg/mL. The macromolecular structure of protein, the number of peptide bonds and the presence of four amino acids (cysteine, cystine, trytophan and tyrosine) are reported to be responsible for color formation with BCA.

Protein standards were prepared by diluting the BCA stock to 25, 125, 250, 500 and 750 mg/L. Reagent A was mixed with reagent B (50:1 volume ratio) for a working reagent. Two mLs of working reagent were added to each standard (0.1 mL) and mixed well. Deionized water (0.1 mL) was used as a blank. All tubes were then incubated at 37°C for 30 minutes. After incubation, the tubes were cooled to room temperature and the absorbance measured at 562 nm. Reducing reagents like Fe (II) and tetravalent uranium may interfere with protein analysis. It was observed that keeping the sample in the refrigerator (4°C) for over 2 days could oxidize most of the metals to a non-interfering state. Another separation method was used to eliminate metal interference. Protein samples (1 mL) were centrifuged in 1.5 mL mini centrifuge tubes (Fisher Scientific) at 50,000 rpm for 1-2 minutes. The supernatant was decanted and replaced by the same amount of bicarbonate buffer (2.5 g/L). The sample then was mixed using a sonic-vibrator and re-centrifuged until the color of solution, due to the presence of metals, disappeared. After this pretreatment, samples were then analyzed following the procedure discussed above.

3.8 Direct cell counting

Direct cell counting, also called viable counting, was performed to estimate the number of living microorganisms in a given sample. In general, organisms are first diluted in a liquid medium and then placed in an agar medium to produce some type of visual effect. The actual count is of bacterial colonies and expressed in colony-forming units (CFU). The facultative characteristic of *S. alga* allows aerobic culture in tryptic soy broth plates. The following procedure was used in this research.

One mL of well-mixed culture sample of *S. alga* was obtained during microorganism culture studies. The suspension was diluted in 9 mLs of sterilized phosphate buffer. One mL of well-mixed sample was removed and introduced into separate sterile dilution tubes. The agar plates were prepared ahead of time. A 2 liter solution of tryptic soy broth (30 g/L) was prepared and well mixed. 15 g/L of nutrient agar then were added. The medium flask was autoclaved immediately for 30 minutes. The sterilized medium was removed while hot and placed in a 60°C water bath for 30 minutes. The hot medium was poured into sterilized plates and allowed to solidify. The finished plates were then stored at room temperature until use. 100 μLs of culture sample (after 1-10 serial dilutions) were introduced into rotated agar plates to ensure proper mixing, then incubated at 35°C. After 16 hours, colonies in each plate were counted and viable cell number in the original culture was calculated using the following equation:

CFU = colony counts/ (initial sample volume x dilution rate)

The probable number of bacteria per milliliter of the original sample was calculated as the bacterial colony count in the dilutant multiplied by the dilution and initial volume used.

The key operation during this procedure is dilution. The dilution ratio was determined by the cell density in the sample. The best dilution ratio produced a final colony count in the range of 30-300. Insufficient dilution may cause the final cell colonies to be too numerous to count while over dilution will likely produce too few colonies which could be easily affected by the mixing of the dilutants. To minimize the errors during dilution, 1 to 10 serial dilutions were used.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Overview of microbial growth characterization

Results of aerobic and anaerobic growth studies using *Shewanella alga* are presented and discussed in this section. Batch experiments were conducted at 35°C in which *S. alga* was grown aerobically in a 250 mL flask (shaking at 150 rpm) or anaerobically in a closed 100 mL serum bottle (incubated motionless). Bacterial growth was monitored using both the direct counting method and the BCA protein assay kit (Pierce, product number # 23225). The relationship between cell number and total protein was calibrated and total protein then used as the indicator of cell growth in subsequent experiments. The efficacy of iron and uranium reduction by cell suspensions grown using molecular oxygen or Fe (III) as the terminal electron acceptor was investigated and results were compared. Metal reduction using *S. alga* cell suspensions in different growth phases was also evaluated. Finally choices were made on the terminal electron acceptor used for the growth of seed culture as well as the growth phase of the inoculum to be used in subsequent non-growth metal bioreduction experiments.

4.1.1 Growth of *S. alga*

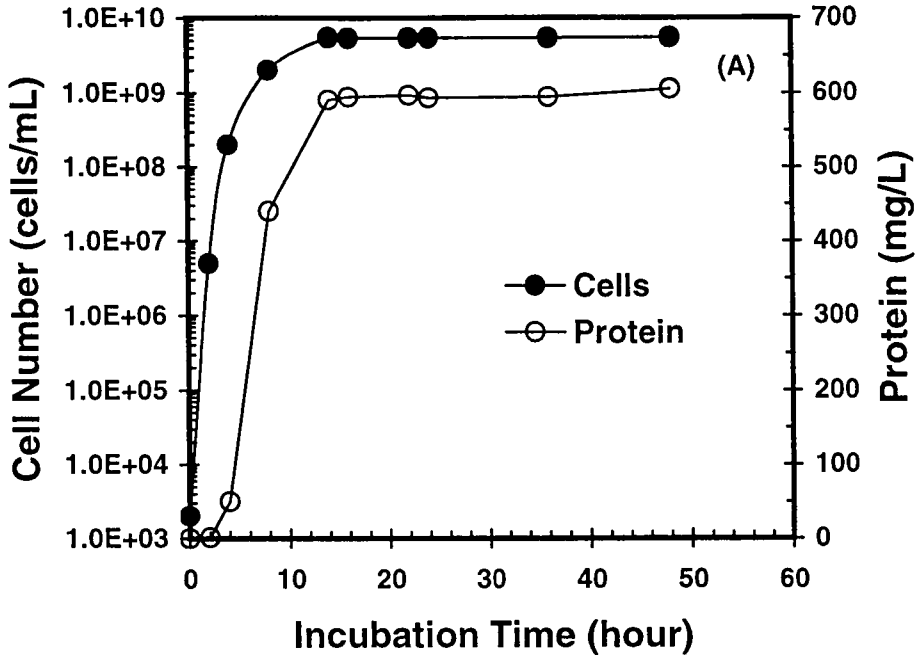
4.1.1.1 Growth of *S. alga* using oxygen as the terminal electron acceptor

The ability of the facultative bacterium *S. alga* to grow using oxygen as the terminal electron acceptor differentiates this organism from other U (VI) reducing

bacteria, which are primarily strict anaerobes. Aerobic growth conditions generally result in higher cell densities while avoiding the stringent requirement necessary to maintain anaerobic conditions. Therefore, utilization of *S. alga* for uranium treatment is highly desirable from an engineering perspective. Figure 2A presents the growth profile of *S. alga* in rich tryptic soy broth (TSB) medium. The medium contained an initial cell concentration of approximately $1.5x10^3$ cells/mL. The culture was shaken at 150 rpm to enhance oxygen transfer from the headspace to the aqueous phase, thereby minimizing any oxygen uptake limitation. During the incubation period, no obvious lag time was observed. Lag time is characterized as a period of adaptation of cells to a new environment, where microorganisms reorganize their molecular constituents and internal machinery (Shuler & Kargi, 1992). Lovely (1995) reported that some bacteria, whose enzyme systems were well developed, did not require a lag time before growth. Since the seed culture used in this experiment was transferred from the TSB medium 12 hours after the frozen cells were inoculated, it's apparent that pre-exposure of *S. alga* to the TSB medium results in enzyme production, which eliminates any lag period before growth.

During the first 12 hours of incubation, cell numbers multiplied rapidly from ~$1.5x10^3$ to ~$5x10^9$ cells/mL and cell protein levels increased from less than 1 to 590 mg/L. This period constitutes exponential growth in which the specific growth rate reaches a maximum and is zero order with respect to the nutrient or substrate concentration. Mathematically, this relationship can be expressed as:

$$\frac{dX}{dt} = \mu_{max} X$$



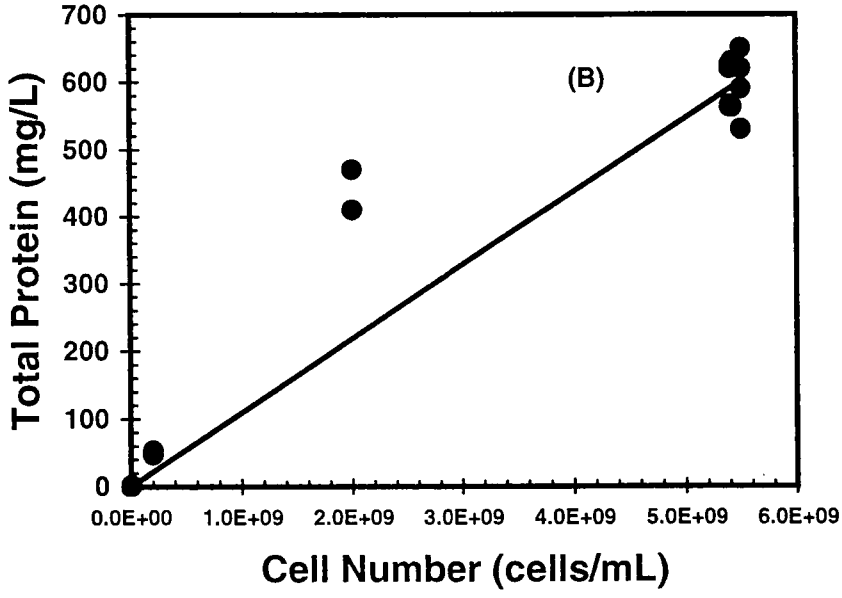


Figure 2.A) Cell number and protein concentration of *S. alga* in tryptic soy broth medium after inoculation. B) Total protein as a function of cell number of *S. alga* in TSB medium after inoculation.Oxygen was the electron acceptor.

where μ_{max} is the specific maximum growth rate (hr^{-1}), t is the incubation time after cell inoculation and X is the cell density at time t. Integration of the above equation yields:

$$\ln(\frac{X}{X_o}) = \mu_{max}\, t \quad or \; X = X_o\, e^{\mu_{max} t}$$

where X_o is the cell density or total protein concentration at t = 0. From a plot of $\ln(X/X_0)$ vs. t, the value of μ_{max} (slope of the line) can be determined. Using linear regression, μ_{max} was calculated to be 1.68 hr^{-1}. The maximum specific growth rate for aerobic microorganisms lies in the range of 0.50 to 3.0 hr^{-1} (Mynhier and Grady, 1975). The time required to double the microbial mass is given by:

$$\tau_d = \ln 2/\mu_{max} = 0.693/\mu_{max}$$

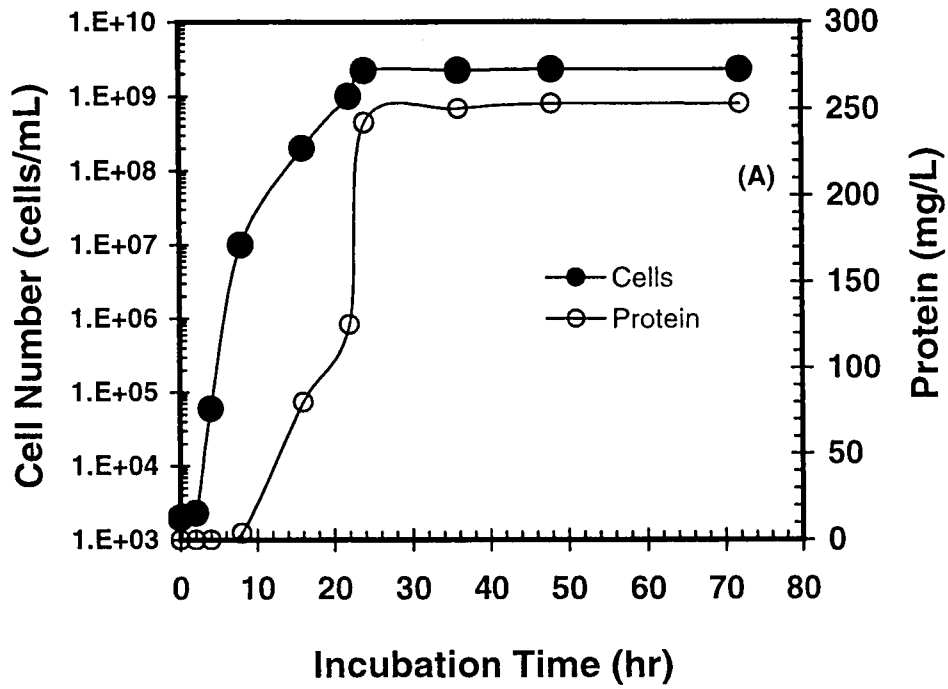
In this study the doubling time (τ_d) for aerobic growth of *S. alga* was calculated to be 0.413 hr.

After ~12 hours of incubation, there was no further increase in cell number or protein concentration. The cell number was maintained at ~5.5×10^9 cells/mL and protein concentration at ~600 mg/L. Although net growth is essentially zero during this stationary phase, cells remain metabolically active and produce secondary non-growth related metabolites (Shuler & Kargie, 1992). During this phase, cells must expend energy to maintain an energized membrane, transport of nutrients, and for essential metabolic functions such as mobility and repair of damage to cellular structures. Past reported uranium reduction studies were all performed using cells in this stationary phase (Lovley, 1992 & 1993; Caccavo, 1992). Therefore, non-growth conditions were selected in this study such that comparisons with previous results could be made.

As mentioned previously, the total protein concentration was used as an indicator of cellular growth in subsequent experiments. During a batch growth cycle, the concentrations of cellular components including total protein in a system change with time due to the increase in total biomass. The concentration of RNA (RNA/cell weight) varies significantly during a batch cycle. However, the protein concentration per cell remains fairly constant (Shuler & Kargie, 1992). Figure 2B shows the relationship between the total protein concentration and the cell number for *S. alga* during batch growth when oxygen served as the electron acceptor. It can be seen that as the cell number increased, the total protein concentration increased proportionally. The regression indicated a linear relationship with the slope of the line calculated to be approximately 10^{-10} mg protein/cell for *S. alga*. Due to scatter in the protein data, the slope obtained may have uncertainty associated with it. However, this result is comparable to the value of 1.1×10^{-10} mg protein/cell obtained by Gorby (1997) for *S. alga*.

4.1.1.2 Growth of *S. alga* using Fe (III) as the terminal electron acceptor

Growth of *S. alga* in a nutrient rich medium using Fe (III) as the terminal electron acceptor and lactate as the carbon source/electron donor is demonstrated in Figure 3A. The initial cell density remained relatively constant at ~1.5×10^3 cells/mL during the first 2 hours of incubation. This lag period can be explained by the fact that the seed culture was previously grown in the TSB medium, where aerobic conditions were maintained. It appears that the bacterium requires time to develop the appropriate enzymes for the new conditions. After the 2 hour lag period, cell numbers increased rapidly from ~2.0×10^3 to



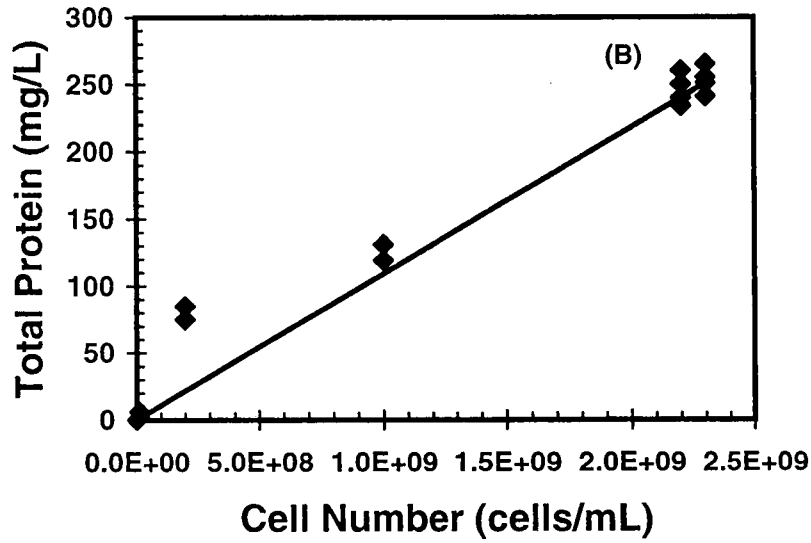


Figure 3.A) Cell number and protein concentration of *S. alga* in anaerobic ferric citrate medium after inoculation. B) Total protein as a function of cell number in ferric citrate medium after inoculation. Fe (III) was the electron acceptor.

~$1.5x10^9$ cells/mL during the next 18 hours of incubation and the total protein concentration increased from less than 1 to ~250 mg/L. The cell number and protein level then stabilized. It was observed that the color of the medium changed during cellular growth. Initially, the medium was dark brown due to the presence of ferric ions and peptone/yeast extract. After 20 hours of growth, the solution color turned to greenish yellow then became colorless after 22 hours. A black precipitate appeared after 26 hours of incubation. The color change was believed to be associated with Fe (III) reduction to Fe (II) and subsequent precipitation of ferrous compounds. Analysis showed medium pH values before and after Fe (III) reduction were 8.35 and 8.4 respectively, which indicated that precipitation was not due to changes in pH.

Note that in this nutrient rich medium, peptone and yeast extract served as a readily available source of N and P for rapid microbial growth (DIFCO Catalog, 1997-1998). Because those materials are synthesized from animal tissues, the poor purity violates the requirement of a chemically defined medium during kinetic studies. In addition, the yellow color caused by peptone/yeast extract solution severely interfered with uranium analysis. Therefore, for later growth-coupled reduction experiments, peptone and yeast extract were omitted to avoid analytical interference from constitutes and to ensure that the growth medium was chemically defined. However, they were added during seed culture growth to insure generation of a sufficient amount of cell mass for subsequent experiments.

Growth of *S. alga* using Fe (III) as the terminal electron acceptor was evaluated using two defined constants. The maximum specific growth rate (μ_{max}) was calculated to be 0.4 hr^{-1} and the doubling time (τ_d) was 1.73 hr using the same numerical methods as described in the aerobic growth study. When comparing growth using oxygen as the terminal electron acceptor (μ_{max} =1.68 hr^{-1}, τ_d = 0.413 hr) vs. Fe (III), *S. alga* grew faster and to a higher cell density when oxygen was present. This result is compatible with the theory that bacteria normally obtain more energy for growth when utilizing oxygen as the terminal electron acceptor. Froelich (1979) showed that for the decomposition of a complex carbohydrate, microorganisms obtain 3190 KJ of energy per mole of glucose when using oxygen as the electron acceptor, more than twice that obtained through Fe (III) reduction (1410 KJ/mol).

The relationship between the total protein concentration and the cell number during anaerobic growth of *S. alga* is shown in Figure 3B. The same linear regression method discussed in the aerobic growth section (Figure 2B) was used and a value of ~$1x10^{-10}$ mg protein/cell was obtained. Again there was uncertainty associated with this value due to the scattered data, however, this result was the same as that obtained for aerobic growth ($1x10^{-10}$ mg protein/cell). Therefore, the electron acceptor used to support growth appears to have no impact on the average protein content per cell of *S. alga*.

4.1.2 Efficacy of *S. alga* for metal reduction

Since *S. alga* can utilize both molecular oxygen and Fe (III) as terminal electron acceptors for cellular growth, the efficacy of metal reduction by *S. alga* cell suspensions grown using these two different electron acceptors was evaluated and compared. A determination of whether uranium reduction using stationary cells requires the presence of iron or oxygen as the electron acceptor during the growth of seed culture was then made.

4.1.2.1 Fe (III) reduction by aerobically grown *S. alga*

Cells grown for 12 hours (late exponential phase) and 16 hours (early stationary phase) in tryptic soy broth medium were harvested by centrifugation, washed twice in an anaerobic bicarbonate buffer, diluted to an initial cell density of about $3x10^7$ cells/mL and then transferred into deoxygenated test solutions. Test solutions contained 2.5 g/L of sodium bicarbonate and 2 mL/L of sodium lactate (60% w/w syrup, Sigma Chemical). Anaerobically prepared ferric citrate stock was then added as the sole electron acceptor. Fe (II) concentration and cell density were monitored over time. Figure 4 shows the Fe (III) reduction pattern using *S. alga* in the two different growth phases. From Figure 4A, Fe (III) levels decreased rapidly from 0.5 mM to 0.08 mM during the first 7 hours of incubation. The increase in Fe (II) levels is shown in Figure 4B. More than 80% of the Fe (III) initially present was reduced within 7 hours. No lag time was observed. Gorby *et al.* (1995) reported that *S. alga* developed a special Fe (III)-reducing enzyme during the late exponential phase in TSB medium. Therefore, *S. alga* does not require a lag period for

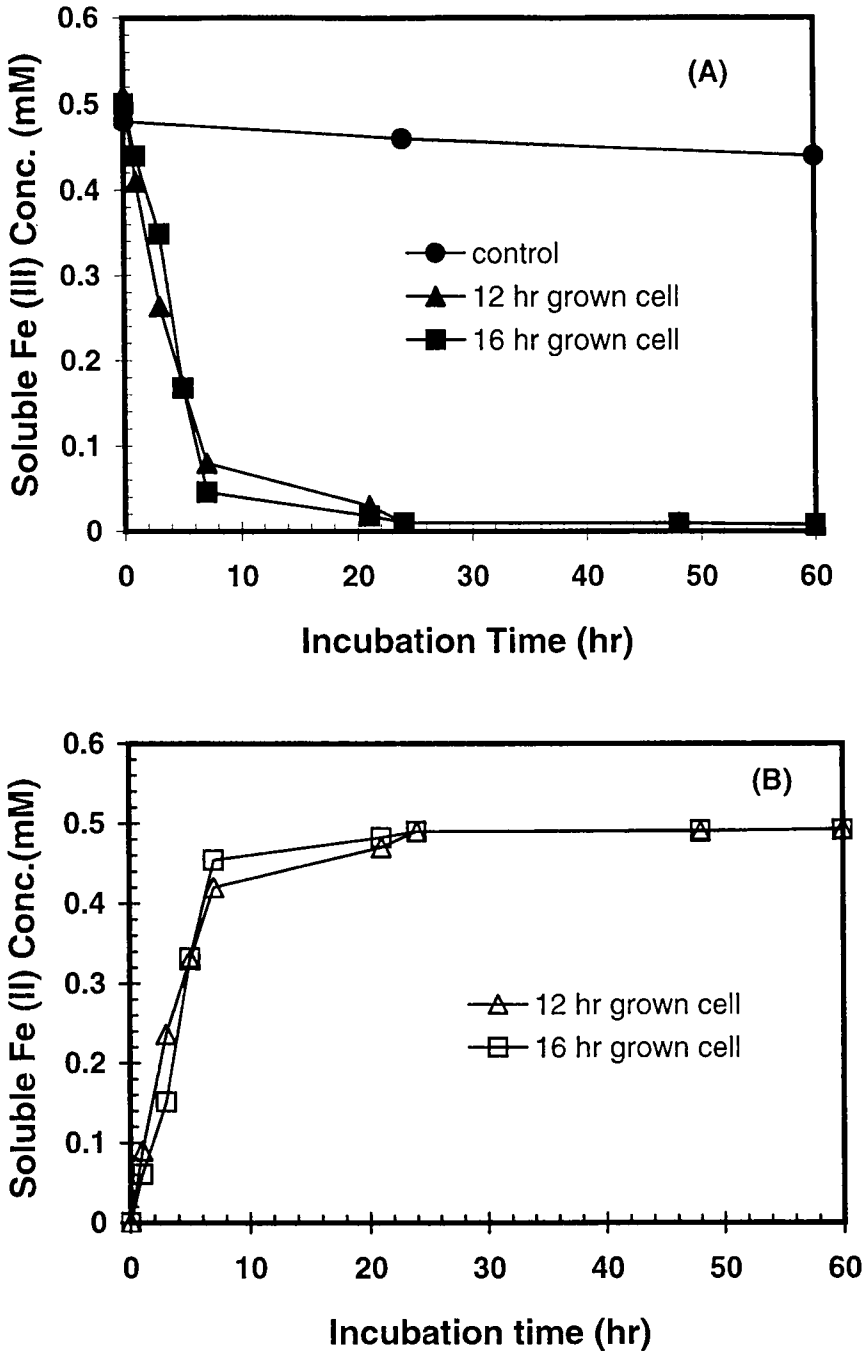


Figure 4. Fe (III)(A)/Fe (II)(B) levels as a function of time after inoculation with 12 & 16 hr aerobically grown *S. alga* cells. Fe (III) level in sample without inoculum was used as a control.

Fe (III) reduction because the Fe (III)-reducing enzyme is already in place during late exponential growth in TSB media. This specially designed enzyme can be the result of adaptation to a sediment environment where oxygen is limited while ferric oxides are abundant. The reduction rate for the two different growth phases was determined using the slope of the linear part of the curve. The reduction rate for Fe (III) using 12 hour grown cells was calculated as 0.0593 mM/hr and 0.0658 mM/hr using 16 hour grown cells. The difference in rates was less than 10%. There appeared to be little difference in rates or extent of Fe (III) reduction between these two growth phases.

4.1.2.2 U (VI) reduction by aerobically grown *S. alga*

U (VI) reduction using *S. alga* cell suspensions harvested at 12 and 16 hours in TSB medium was also evaluated (Figure 5). A lag period (up to ~8 hr) was observed after inoculation. U (VI) was reduced from 0.45 mM to 0.02 mM over the entire 48 hours of incubation for each growth phase (>95.6% reduction). U (VI) levels decreased linearly from 0.45 mM to 0.05 mM during the 6 to 24 hour incubation period. The reduction rate (slope of the linear part of curve) was estimated to be 0.0228 mM/hr (0.0114 mM-electrons transferred/hr) for 12 hour grown cells and 0.0232 mM/hr (0.0166 mM-electrons transferred/hr) for 16 hour grown cells. There were 2 moles of electrons transferred for every mole of U (VI) reduced to U (IV). Again, no significant difference in rates or extent of reduction was observed between these two growth phases during the time course of this experiment. Compared to the average Fe (III) reduction rate

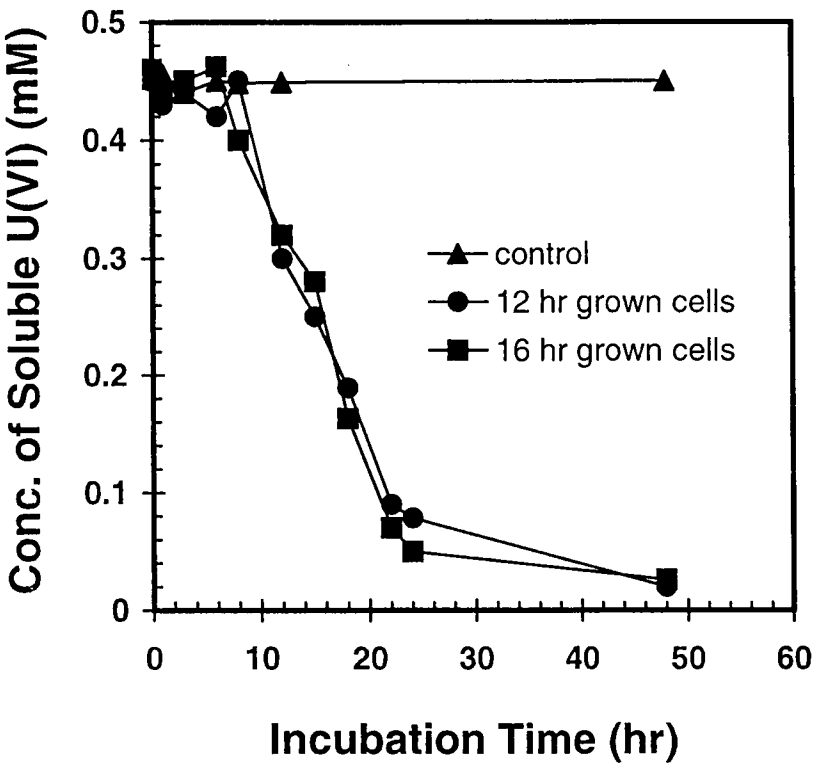


Figure 5. U(VI) levels as a function of time after inoculation with 12 & 16 hr aerobically grown *S. alga* cells. U(VI) level in the medium without cell inoculation was used as a control.

(0.06255 mM-electrons transferred/hr), aerobically grown *S. alga* cells had a 3 fold higher reduction rate for Fe (III) than U (VI).

The lag time observed during U (VI) reduction but not Fe (III) reduction using aerobically grown *S. alga* suspensions may be due to a number of factors. As cited previously, *S. alga* develops a special Fe (III)-reducing enzyme during growth in TSB media. However, there is no information on whether *S. alga* can develop a special U (VI)-reducing enzyme, or, whether Fe (III) and U (VI) share the same reducing enzyme. Since U (VI) is less abundant than Fe (III) in the natural environment, it is likely that *S. alga* has developed an enzyme system for the more abundant Fe (III) compound.

Additionally, the difference in the redox potential required for complete reduction of each metal might account for the lag difference. The energy and the corresponding redox potentials associated with various metabolic reactions can vary significantly. The redox potential required for Fe (III) reduction is in the range of –100 to -50 mV (Stumm and Morgan, 1981), well above the required redox potential for U (VI) reductive precipitation, which is less than – 200 mV (Lovley, 1992). The redox reaction cannot occur unless the system reaches the required electron potential. The presence of trace oxygen will dramatically increase the redox potential of a solution. As mentioned in the previous chapter, the degassing method used in this experiment only approximated an anaerobic environment. Measurements were not made to determine if trace amounts of oxygen remained in the test solution. For solutions inoculated with cell suspension grown from an oxygen rich environment, it may be more difficult to completely eliminate the presence of dissolved oxygen. Although no actual redox potentials were measured during

this study, oxygen carryover in the test solution was most probably responsible for the lag time observed during studies using aerobically grown cells. Lag time will terminate when the remaining oxygen has been consumed and subsequently U (VI) becomes the sole electron acceptor in the medium.

4.1.2.3 Fe (III) reduction using anaerobically grown cells

As a facultative microorganism, *S. alga* was also reported capable of growth using Fe (III) as the terminal electron acceptor in addition to oxygen (Caccavo, 1992). Fe (III) reduction using *S. alga* cell suspensions initially grown on Fe (III) is demonstrated in Figure 6. The seed culture was grown in ferric citrate medium for 24 hours (late exponential phase) and 48 hours (early stationary phase) and then harvested and transferred into the respective test solutions. The initial inoculum was diluted to ~$3x10^7$ cells/mL. As Figure 6 shows, Fe (III) levels decreased rapidly from ~ 0.5 mM to less than 0.02 mM during incubation. Approximately 96% of Fe (III) initially present was reduced within 60 hours. Reduced ferric formed a black precipitate which remained at the bottom of the serum bottle. The soluble Fe (III) level in the control remained relatively constant. Fe (III) reduction occurred after inoculation without any apparent lag. The lack of lag time can be explained by the enhanced enzyme adaptability to the same nutrient environment before and after cell transfer. During the first 8 hours of incubation, Fe (III) concentrations dropped linearly from ~0.5 mM to ~0.08 mM. The slope of the linear part of the line was calculated as 0.0594 mM/hr for 24 hour grown cells and 0.0558 mM/hr for

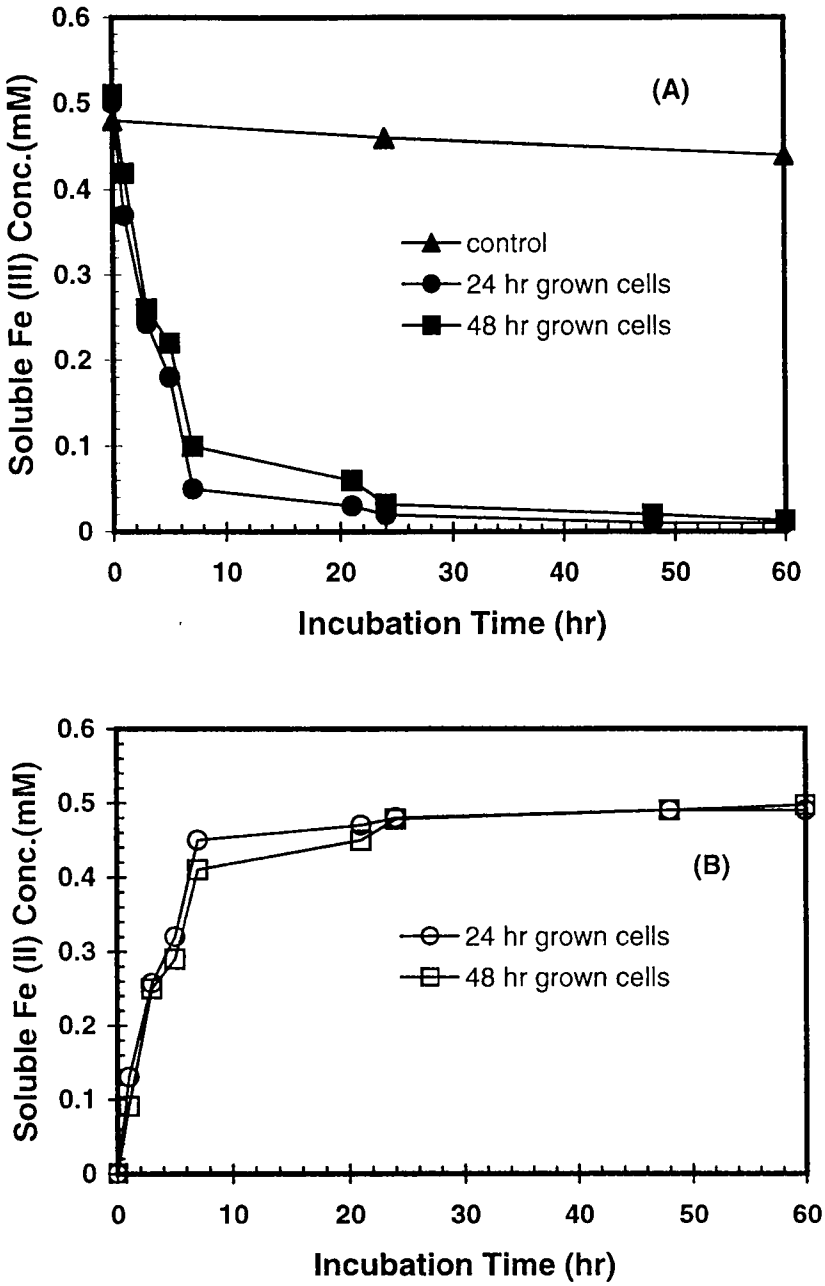


Figure 6. Fe (III) (A)/Fe (II) (B) levels as a function of time after inoculation with 24 & 48 hr anaerobically grown *S. alga* cell suspensions. Fe (III) in the medium without inoculum was used as a control.

48 hour grown cells using linear regression. There was no appreciable difference in rates or overall extent of reduction between the 24 and 48 hour grown cells. The initial reduction rate (average 0.0576 mM/hr) and the overall reduction extent (96%) were within 10% of the values obtained for cells grown using oxygen as the terminal electron acceptor (Figure 2, average 0.06255 mM/hr and 95.6%). It appears that aerobically and anaerobically grown *S. alga* cells are equally effective in reducing Fe (III). Since the aerobic environment supports a higher cell density and is much easier to maintain compared to the anaerobic environment, growing cells under aerobic conditions would be more favorable with respect to Fe (III) biotreatment.

4.1.2.4 U (VI) reduction using anaerobically grown *S. alga*

Figure 7 shows the U (VI) reduction pattern by *S. alga* cell suspensions initially grown on Fe (III). The initial cell inoculum was approximately $3x10^8$ cells/mL. U (VI) levels dropped from ~0.45 mM to ~0.05 mM during 50 hours of incubation without an apparent lag. Approximately 88.9% of the hexavalent uranium initially present was reduced within 48 hours. U (VI) levels in the control stayed relatively constant. Hexavalent uranium was reduced linearly from 0.45 mM to 0.2 mM within the first 6 hours. Reduction rates (the slopes of the linear parts of the lines) were estimated to be 0.019 mM electron transferred /hr using 48 hours grown cells and 0.02 mM/hr using 24 hours grown cells. No significant difference was observed between 24 and 48 hour grown cells. Using anaerobically grown *S. alga* cell suspensions, the U (VI) reduction rate was 67% slower than the average Fe (III) reduction rate.

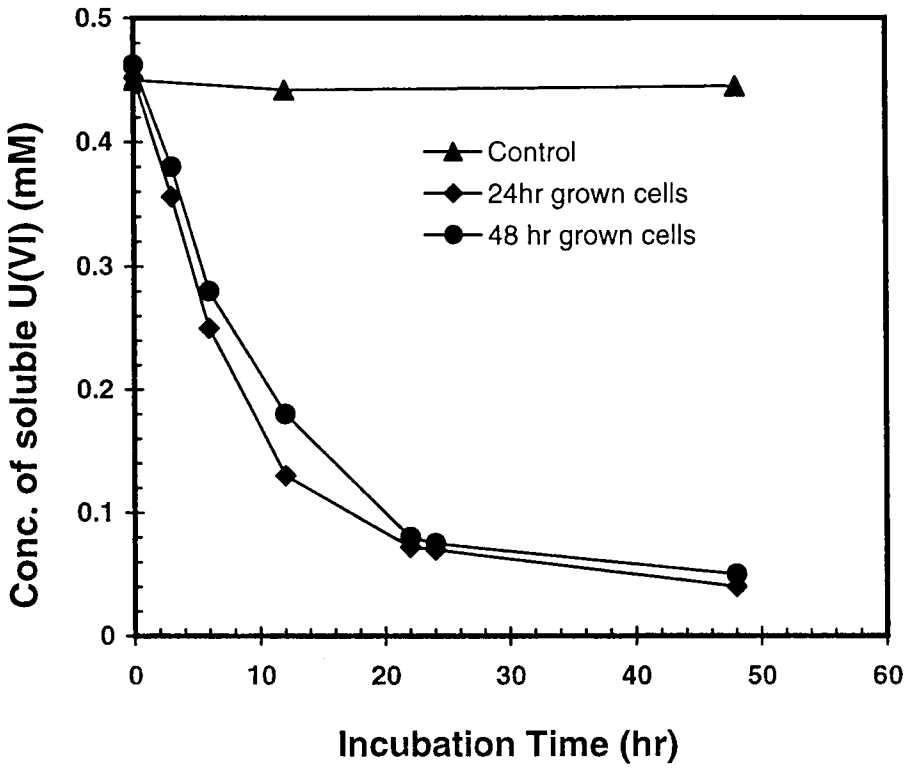


Figure 7. U(VI) levels as a function of time after inculation with 24 & 48 hr anaerobically grown *S. alga* cell suspensions. U (VI) level in the medium without cell inoculation was used as a control.

Compared to uranium reduction using aerobically grown cells (Figure 5), there was a significant difference in the lag time though the reduction rate (0.014 mM/hr for aerobically grown cells and 0.02 mM for anaerobically grown cells) and extent (90% for aerobic cells and 88.9% for anaerobic cells) were relatively close to each other. Since there was no apparent lag time in uranium reduction using *S. alga* cells grown on Fe (III), it appeared that the enzyme required for U (VI) reduction was present. It may be that the enzyme system required for iron reduction is the same one needed for uranium reduction. If this is the case, then the lag time observed during uranium reduction using aerobically grown cells was not due to the difference in enzyme adaptability. It was likely that carryover of trace oxygen to the anaerobic medium resulted in the lag period observed for uranium reduction using aerobically grown cell suspensions.

4.1.3 Summary

In summary, *S. alga* grew both to a high cell density (~10^9 cells/mL) when oxygen or Fe (III) served as the electron acceptor and lactate as the electron donor/carbon source. However, the fact that the cell doubling time for anaerobic growth (1.73 hr) was approximately four fold of that for aerobic growth (0.413 hr) suggests that *S. alga* had a preference for oxygen as the terminal electron acceptor.

Cell suspensions initially grown using oxygen or Fe (III) as the terminal electron acceptor were equally effective in subsequent Fe (III) reduction studies. For uranium reduction, an 8 hours lag time was observed using cells initially grown on oxygen. The

lag period was believed due to trace oxygen carryover. The rate and extent of subsequent U (VI) reduction were comparable with values obtained using cells initially grown on Fe (III). This result suggests that for uranium reduction using stationary phase *S. alga* cells, the presence of Fe (III) is not required during the initial cell growth phases. Late exponential cells and early stationary cells were tested separately and no appreciable difference was observed between these two growth phases either for Fe (III) or U (VI) reduction.

Growth of *S. alga* using Fe (III) as the terminal electron acceptor resulted in a 4 fold lower doubling time and 3 fold lower cell density (stationary phase) compared to values obtained using oxygen. Based on these preliminary results, *S. alga* grows faster and to a higher density using oxygen as a terminal electron acceptor. However, the lag time observed during uranium reduction studies using aerobically grown cells complicated data analysis. Therefore, Fe (III) was selected as the terminal electron acceptor for growth of seed culture rather than oxygen to prevent oxygen carryover. Additionally, cells were grown for 24 hours before inoculation in subsequent non-growth experiments.

4.2 Metal reduction by *S. alga* under non-growth conditions

In this section, results of various experiments performed to collect kinetic data on U (VI) and Fe (III) reduction using *S. alga* suspensions are presented. Additionally, the impact of competing electron acceptors on metal reduction is also evaluated under non-

growth conditions. Non-growth conditions exist when essential nutrients for cellular growth are not provided. Bacteria are only supplied with an electron donor (lactate) and an electron acceptor (U (VI) or Fe (III)). Cells in such test solutions can utilize the electron pair for minimum respiration and obtain energy for cellular maintenance.

Kinetics coefficients for metal reduction and microbial growth are estimated using the Monod model. The initial levels of ferric ion and hexavalent uranium were chosen based on the equivalence of electrons transferred. As the following reactions show, one mole of electrons are transferred for every mole of Fe (III) reduced, while for U (VI) there are two.

$$U\ (VI) + 2e^{-} \rightarrow U\ (IV)$$

$$Fe\ (III) + e^{-} \rightarrow Fe\ (II)$$

Therefore, the initial Fe (III) concentrations used were approximately twice that of the initial U (VI) concentrations utilized. A protein assay was used to monitor microbial growth. For all experiments lactate served as the electron donor.

S. alga cell suspensions were grown in the nutrient rich medium with Fe (III) as the terminal electron acceptor for 24 hours, harvested by centrifugation, washed and diluted to $3x10^7$ cells/mL and finally inoculated into 100 mL serum bottles. The test solutions contained 2.5 mg/L of sodium bicarbonate to buffer the pH at ~8.4 and 2 mL/L of sodium lactate (60% w/w syrup) (final lactate concentration ~20 mM). U (VI) and Fe (III) from anaerobic stock solutions were added before inoculation. The levels of soluble

U (VI) and Fe (II) were continuously monitored using the KPA and ferrozine methods, respectively. Cell density was assumed to be approximately constant during the course of the non-growth studies (Ganesh, 1995), however, no cell density measurements were made to confirm this assumption.

4.2.1 Fe (III) reduction by *S. alga* cell suspensions

Microbial reduction of Fe (III) to Fe (II) has been well studied and documented. However, kinetic data for Fe (III) reduction by *S. alga* cell suspensions are not available. Such information should provide valuable comparisons with uranium kinetic data and lead to general metal reduction mechanisms.

Figure 8A depicts Fe (III) reduction by *S. alga* cell suspensions under different initial iron concentrations (0.5 mM, 0.2 mM, 0.1 mM and 0.05 mM). At the highest initial concentration, Fe (III) decreased rapidly from 0.5 mM to 0.05 mM during first 7 hours of incubation. The reduction was linear during this time frame. After 7 hours, little additional Fe (III) reduction was observed. For the 0.2 mM concentration, 90% of the Fe (III) reduction occurred within 5 hours while at concentrations of 0.1 mM and 0.05 mM, it required ~3 hours for 90% of the Fe (III) to be reduced. The slopes of the linear part of the Fe (III) concentration curves were used to determine reduction rates. The initial cell density of $3x10^7$ cells/mL (3 mg/L as protein) was assumed to remain constant during these experiments since no nutrients were added.

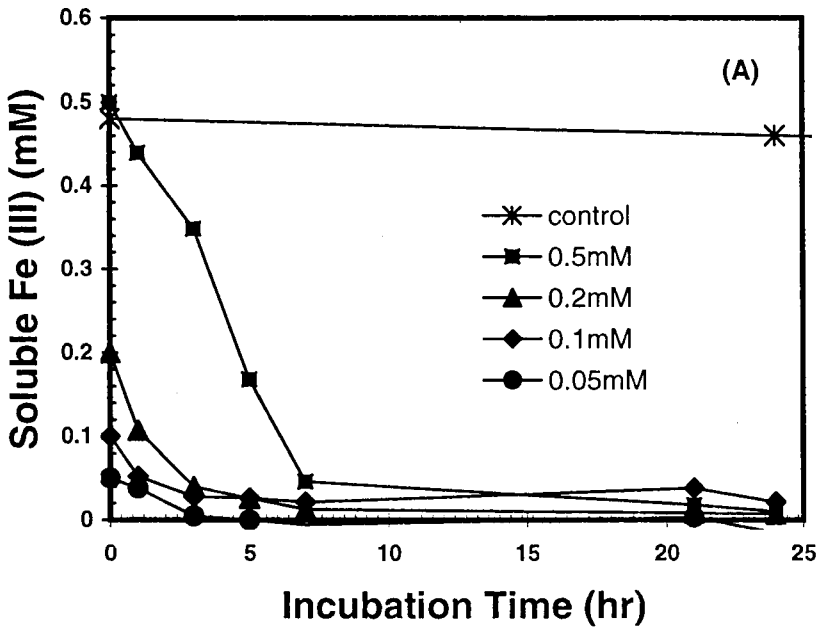


Figure 8. A) Fe (III) reduction under different initial levels using *S. alga* cell suspensions.

The method used to assess the kinetic parameters is obtained from Goring (1972). This method was derived from the Monod model,

$$-\frac{dS}{dt} = \frac{V_{max}[S]}{K_s + [S]} = \frac{kX[S]}{K_S + [S]}$$

where S is the limiting substrate concentration (i.e. iron concentration in this experiment). The half-saturation constant (K_s) is defined as the initial iron concentration at which the corresponding specific Fe (III) reduction rate is half the maximum rateV_m is the maximum reduction rate and k is the maximum specific reduction rate (V_m= kX). X is the biomass concentration and t is the incubation time. Integration of above equation with V_m expression yields:

$$-V_{max}\, t = [S_o] - [S] + K_s \ln\left(\frac{[S_o]}{[S]}\right)$$

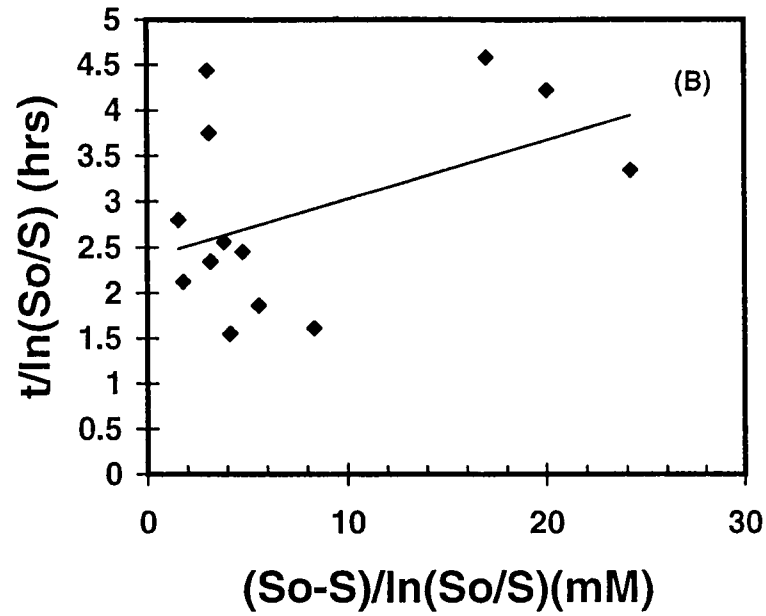
Rearrange:

$$\frac{t}{\ln\left(\frac{[S_o]}{[S]}\right)} = \left(-\frac{1}{V_{max}}\right)\left[\frac{[S_o]-[S]}{\ln\left(\frac{[S_o]}{[S]}\right)}\right] + \frac{K_s}{V_{max}}$$

For the Fe (III) reduction studies, V_{max} is the maximum Fe (III) reduction rate (mg Fe (III)/L•hr), K_s is the half saturation constant (mM as Fe (III)), S_o is the initial Fe (III) concentration (mM), and S_t is the Fe (III) concentration at time t (mM).

The first 5 hours of data for each iron concentration in Figure 8A were fitted to the equation by calculating the terms [t/ln (S_o/S)] and [(S_o-S)/ln (S_o/S)] then evaluating the terms (-1/V_m) and (K_s/V_m). Excel graphic was used to obtain the kinetic parameters V_m and K_s by linear regression analysis. The maximum specific reduction rate (k) was then calculated using the maximum reduction rate V_{max} obtained divided by the concentration of protein used.

A plot of [t /ln ([S_o]/[S])] vs. [([S_o] - [S])/ Ln ([S_o]/[S])] is shown in Figure 8B. The mean slope (-1/V_m) equals 0.065 (standard error 0.038), and the mean intersection (K_s/V_m) equals 2.392 (standard error 0.400). Statistic results are shown in Appendix III. The half saturation constant (K_s) was estimated to be in a range between 0.26 and 1.05 mM as Fe (III). The maximum reduction rate (V_m) was determined to be between 6.48 and 24.3 mg/L•hr. Both ranges are for a 95% confidence interval. The specific reduction rate (k) was then calculated to be between 2.16 and 8.1 mg Fe (III)/ mg protein•hr. As can be seen from Figure 8B, iron experimental data had a large error associated with. However, the average kinetic coefficients obtained using the Goring method effectively predicted iron concentrations over time (Figure 8C). The experimental iron levels after 5 hours of incubation were higher than those predicted by the model. Such deviation was probably due to product inhibition, which lowers the reduction rate over time. Truex *et al.* (1995) reported a half-saturation constant of 3.4 mg/L and a maximum specific reduction rate of ~2 /hr at 30°C (pH = 6.8) for iron reduction using the same bacterium in a continuous flow system.



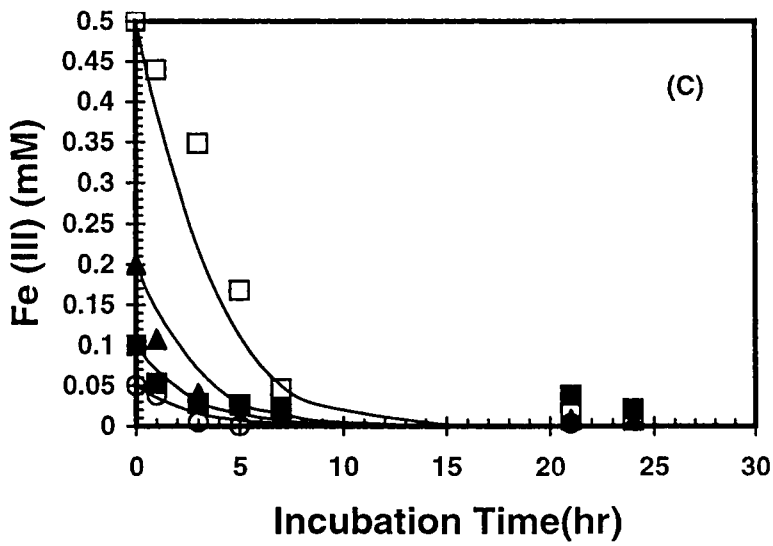


Figure 8. B) Goring (1972) method to estimate the kinetics coefficients for iron reduction; C) Model predicted iron concentrations (smooth curves) and experimental iron levels (isolated points) as a function of time.

4.2.2 U (VI) reduction by *S. alga* cell suspensions

Microbial reduction of soluble U (VI) to insoluble U (IV) is an important process in the global uranium cycle and also may be a useful technique for removing uranium from contaminated environments (Gorby *et al.*, 1992; Lovley *et al.*, 1992). *Geobacter metalireducers* (GS-15) was the first microorganism found to use U (VI) as the electron acceptor when acetate was provided as the electron donor. *S. putrefaciens* also grew using U (VI) as the sole electron acceptor when H_2 was provided as the electron donor. Several *Desulfovibrio* species can enzymatically reduce U (VI) using either lactate or H_2 as the electron donor. However, attempts to grow *D. desulfuricans* with U (VI) as the sole terminal electron acceptor have not been successful (Lovley *et al.*, 1992). Among all the microorganisms found to be responsible for U (VI) transformation, *S. alga* has drawn considerable attention because of its tolerance to oxygen and high uranium removal efficiency. Although a number of studies have been conducted to demonstrate the efficacy of microbial reduction, few of them have investigated reduction kinetics.

U (VI) reduction experiments using *S. alga* cell suspensions were conducted under the same laboratory conditions as the Fe (III) reduction studies. As figure 9A indicates, *S. alga* cell suspensions significantly reduced up to 1.7 mM hexavalent uranium within 60 hours. Residual U (VI) concentrations ranged from 0.008 to 0.63 mM (2 to 150 mg/L) for initial uranium levels of 0.105 to 1.89 mM (25 to 450 mg/L). The overall percent removal was ~65% at initial U (VI) levels of 0.84 mM (200 mg/L) and above, but increased to ~90% at initial U (VI) levels of 0.42 mM (100 mg/L) and below.

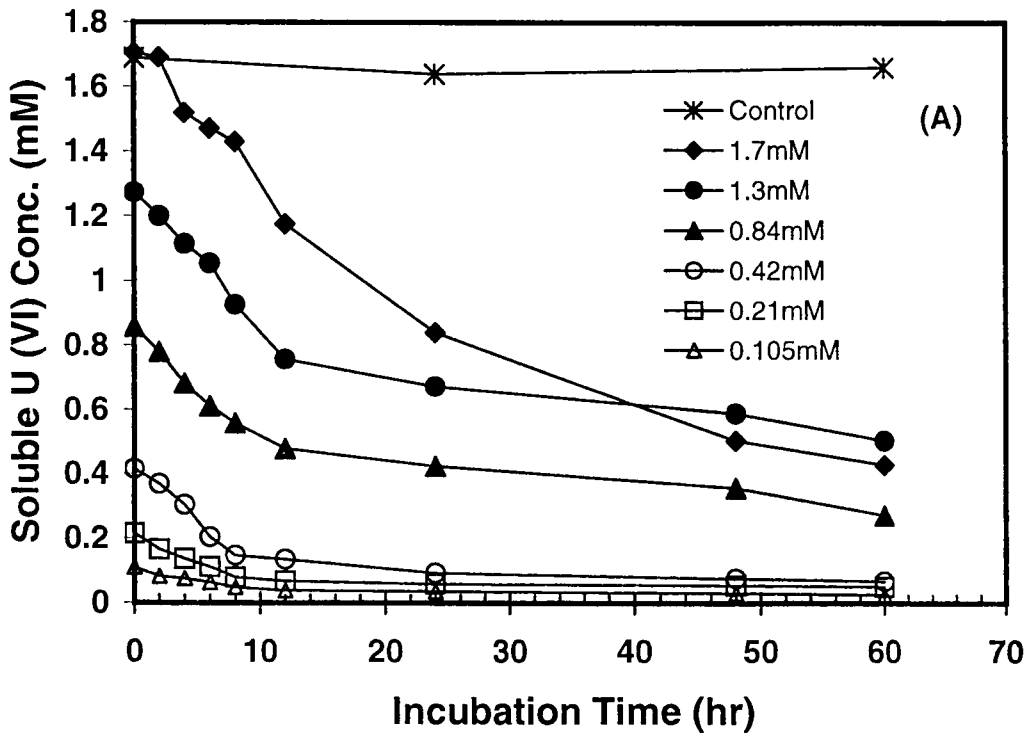
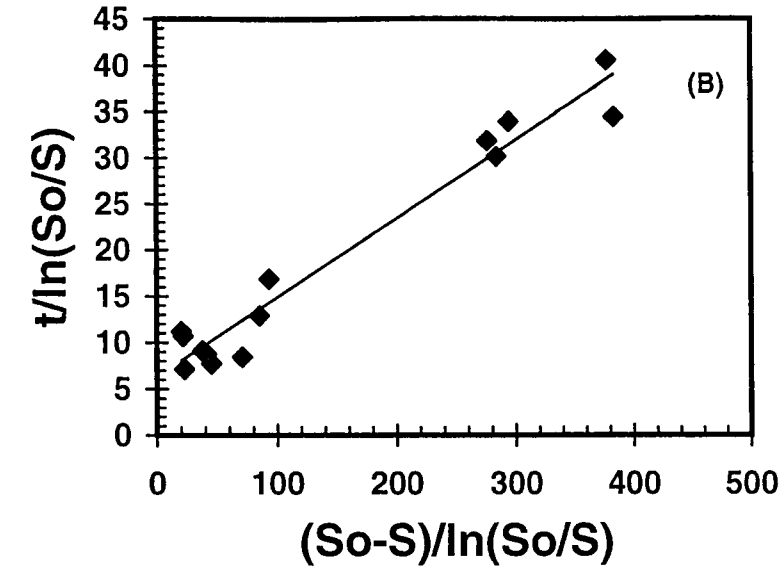


Figure 9. A) U(VI) reduction under different initial levels using *S.alga* suspensions.

Initial reduction rates were constant for the first 8 hours at initial U (VI) concentrations below 0.42 mM; for 12 hours at initial U (VI) concentrations of 0.84 and 1.26 mM; and for ~26 hours at 1.89 mM.

The same data analysis method as that used in the iron experiment was utilized to calculated the kinetic coefficients for uranium reduction. The first 7 hours of data for each uranium concentration in Figure 9A were fitted to the equation by calculating the terms $[t/\ln (S_o/S)]$ and $[(S_o-S)/\ln (S_o/S)]$ then evaluating $(-1/V_m)$ and (K_s/V_m). Excel graphic was used to obtain the kinetic parameters V_m and K_s by linear regression analysis. The maximum specific reduction rate (k) was then calculated using the maximum reduction rate V_{max} obtained divided by the concentration of protein used.

Figure 9B shows a plot of $[t /\ln ([S_o]/[S])]$ vs $[([S_o] - [S])/ \text{Ln} ([S_o]/[S])]$. The mean slope $(-1/V_m)$ equals 0.085 (standard error 0.0051), and the mean intersection (K_s/V_m) equals 6.32 (standard error 1.03). Statistic results are presented in Appendix III. The half saturation constant (K_s) was estimated to be in a range between 0.256 and 0.364 mM as U (VI). The maximum reduction rate (V_m) was determined to be between 10.74 and 12.66 mg/L•hr. Both ranges are in a 95% confidence interval. The specific reduction rate (k) was then calculated to be between 0.358 and 0.422 mg U (VI)/ mg protein•hr. Figure 9B indicates a very good linear relationship with a relative small error compared to iron studies. Model predicted uranium levels over time, using the average kinetic coefficients obtained, are indicated by the solid lines in Figure 9C. Experimental uranium data appear as isolated data points. The model predicts uranium data quite well during the



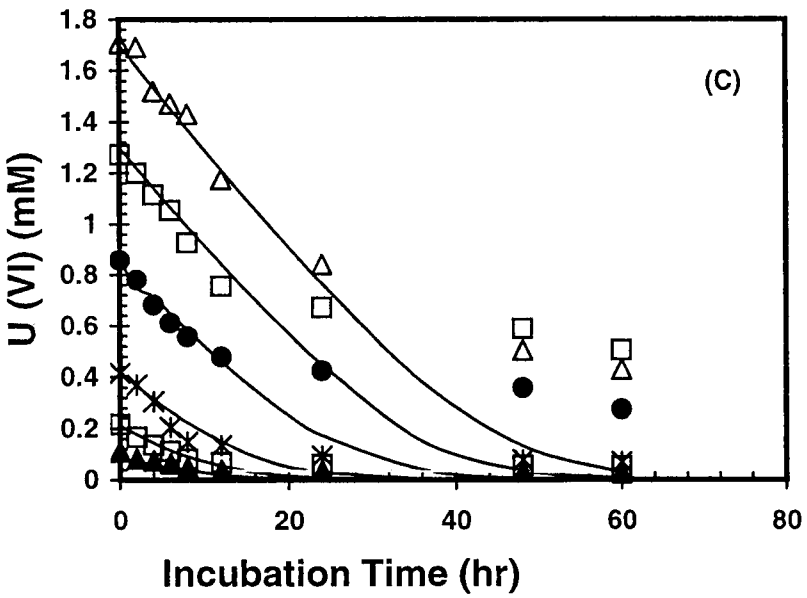


Figure 9. B) Goring (1972) method to estimate the kinetic coefficients for uranium reduction; C) Model predicted U (VI) levels (smooth curves) and experimental U (VI) levels as a function of time.

first 12 hours. After that, the experimental U (VI) levels were usually higher than those predicted by the model. Again, product inhibition may be responsible for the difference. Ganesh (1995) reported kinetic parameters for uranium reduction by *S. alga* in carbonate containing solutions. The maximum specific reduction rate was 2.7×10^{-3} mg U (VI)/mg cell dry weight-hr while the half-saturation constant was 102.3 mg/L. The maximum rate determined by Ganesh (1995) was much smaller than that obtained in this study because the author assumed a cellular mass of 1 mg dry weight per 10^7 cells during conversion. A value of 1 mg protein per 10^{10} cells was experimentally determined in this study. When the same cell protein conversion ratio (10^{-10} mg protein/cell) is used for Dr. Ganesh's data, an equivalent maximum rate of 2.7 mg U (VI)/mg protein-hr is obtained, which is an order of magnitude different than the value of .39 mg U (VI)/mg protein-hr obtained in this study.

4.2.3 Comparison of reduction of Fe (III) and U (VI) by *S. alga* cell suspensions

Based on the kinetic coefficients estimated, the half-saturation constant (K_s) for U (VI) reduction was ~50 mg/L, almost 10 times higher than that of Fe (III) (6 mg/L). K_s is a coefficient indicating the affinity of microorganisms to a limiting substrate or nutrient. Since the electron donor (lactate) was added in excess, the electron acceptor was the primary limiting factor in these experiments. Therefore, K_s indicates the affinity of *S. alga* to the available metal. The higher the K_s, the lower the affinity would be. The data suggest that *S. alga* had a much higher affinity for Fe (III) than for U (VI). Additionally, the maximum specific Fe (III) reduction rate was 0.9 /hr, 3 times higher than the U (VI)

reduction rate (0.3 /hr). The result indicates that *S. alga* reduced Fe (III) faster than U (VI).

The fact that *S. alga* can utilize Fe (III) at a higher rate and has a higher affinity for Fe (III) over U (VI) will likely impact bioreductive uranium treatment when Fe (III) is present in uranium-contaminated water. The presence of Fe (III) could possibly delay or even inhibit U (VI) reduction due to enzymatic competition. However, if *S. alga* utilizes U (VI) and Fe (III) using two different mechanisms in the way that *D. desulfuricans* utilizes sulfate and U (VI), there will be little problem. To date there is no evidence to show that *S. alga* shares the same reducing enzyme for both U (VI) and Fe (III). The following study on metal reduction by *S. alga* in the presence of both U (VI) and Fe (III) ions was conducted to address the issue of preferential metal utilization by this bacterium.

4.2.4 Metal reduction by *S. alga* cell suspensions in the presence of co-electron acceptors

In this section, metal reduction data in the presence of co-electron acceptors by *S. alga* cell suspensions are presented. Ferric citrate and uranium nitrate were added to provide Fe (III) and U (VI) as co-electron acceptors for anaerobic microbial respiration. Lactate was provided as the electron donor. Bicarbonate was added to buffer the test solution (pH = 8.4). Since Ganesh (1995) reported that citrate had no negative impact on uranium reduction by *S. alga*, extraneous citrate reactions were not considered. Lactate may chelate Fe (III) or U (VI) to some extent, however, no stability constants are currently available. Since bicarbonate and citrate are strong chelating agents of U (VI)

and Fe (III), respectively and the lactate concentration used in this experiment was far below that of bicarbonate and citrate, the possible reactions between lactate/Fe (III) and lactate/U (VI) were assumed negligible.

Figure 10 presents the reduction profile of *S. alga* in the presence of both electron acceptors. Ferric citrate and uranium nitrate were added from anaerobic stock to provide the initial Fe (III) and U (VI) concentrations 0.45 mM and 0.9 mM, respectively. Based on the equivalent electron transferred, the molar concentration of Fe (III) used was twice that of U (VI). Test solutions with the addition of one of the two metals were used as controls. As figure 10A shows, 0.9 mM Fe (III) was significantly (>90%) reduced to Fe (II) during 48 hours of incubation in test solutions with both metals present. The reduction rate is almost linear within the first 6 hours. Fe (III) levels in the control sample (no U (VI)) followed almost the same pattern as that with both metals added. While it seems that the presence of U (VI) (0.45 mM) has no impact on the rate or extent of Fe (III) reduction, the presence of Fe (III) does have a significant effect on U (VI) reduction. Figure 10B presents U (VI) levels in control samples (without iron) and in test solutions containing both metals. Over the 48 hour incubation period, the U (VI) concentration (0.45 mM) remained essentially unchanged in test solutions containing Fe (III). However, U (VI) levels in control samples without Fe (III) decreased to less than 0.02 mM. It is clear that when both metals were present (U (VI) = 0.45 mM, Fe (III) = 0.9 mM), *S. alga* preferentially utilized Fe (III). No U (VI) reduction was measured during or after Fe (III)

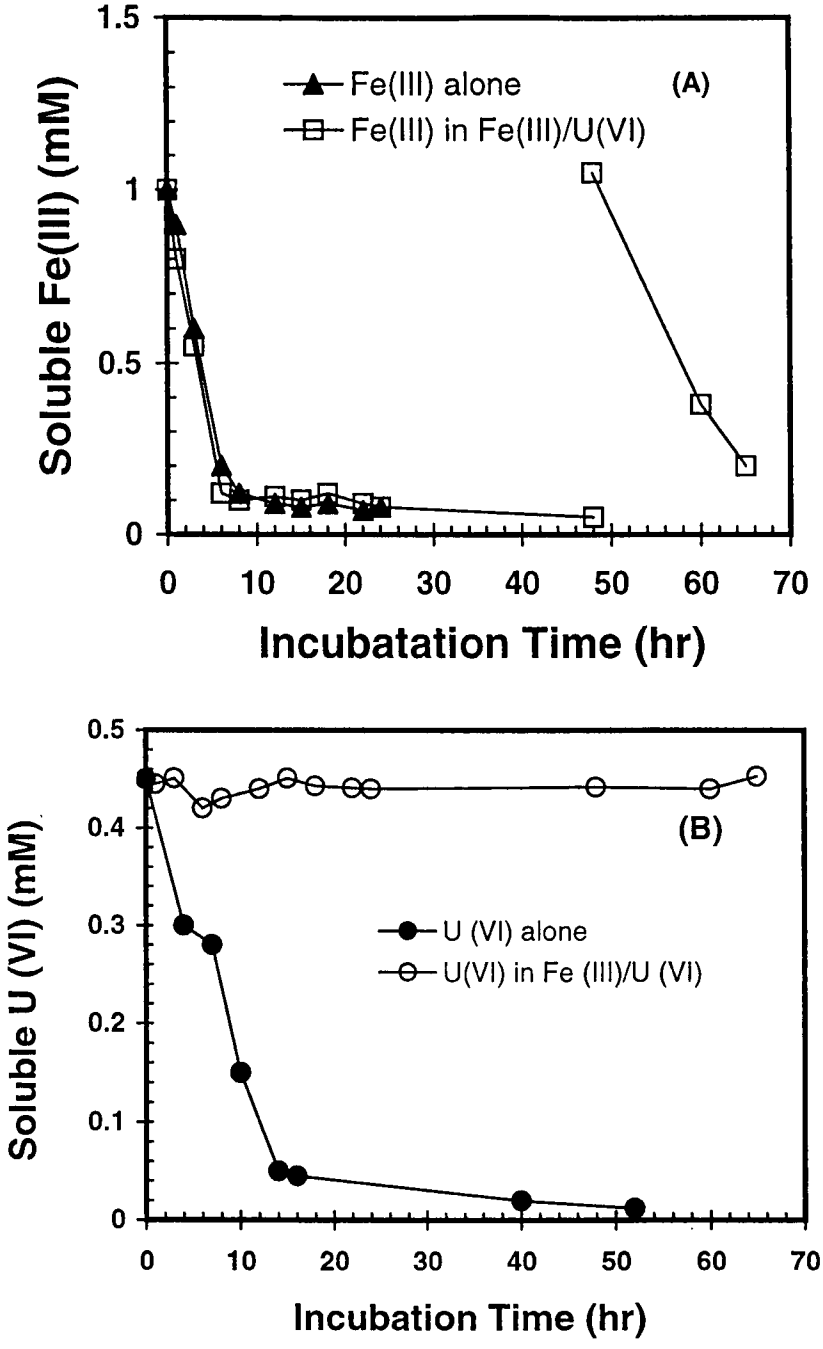


Figure 10. Ferric ion A) and hexavalent uranium B) levels as a function of time after inoculation of *S. alga* cell suspenstions. At t= 48 hr, additional 1.0 mM Fe (III) was added in A).

reduction. After 48 hours, additional Fe (III) was added into the same test solutions and further Fe (II) product was observed, which suggests that the lack of U (VI) reduction was neither due to the limitation of the electron donor (lactate) nor enzyme inactivity. It's believed that the presence of Fe (III) or Fe (II) caused the lack of U (VI) reduction.

To investigate if U (VI) reduction can occur at lower Fe (III) concentrations, initial iron levels were varied and metal concentrations measured over time. Figure 11A shows U (VI) reduction (initial concentration = 0.5 mM) in the presence of 0.05 mM, 0.1 mM, 0.2 mM and 0.5 mM of Fe (III). At initial Fe (III) concentrations greater than or equal to 0.2 mM, no uranium reduction was observed over the 240 hour experimental duration. At initial Fe (III) concentrations of 0.05 mM and 0.1 mM, U (VI) reduction occurred only after an initial lag period (20 hours). Fifty to sixty percent of U (VI) initially present was reduced within 250 hours of incubation. There was no appreciable difference in U (VI) reduction rates between the two (0.0018 mM/hr for 0.1 mM, 0.022 mM/hr for 0.05 mM). As a control, U (VI) reduction without Fe (III) occurred rapidly immediately after inoculation. More than 90% of the initially present U (VI) was reduced within 50 hours. The U (VI) reduction rate was higher in the control (0.0129 mM/hr) than that in the presence of Fe (III) (at 0.05 and 0.1 mM). All these results indicate that initial Fe (III) concentration does impact U (VI) reduction. As the initial Fe (III) concentrations decreased to levels below 0.2 mM, U (VI) reduction occurred though only after a lag period and at a lower rate/extent. At initial Fe (III) concentrations greater than 0.2 mM, no U (VI) reduction was observed even after >90% of the Fe (III) had been utilized. The

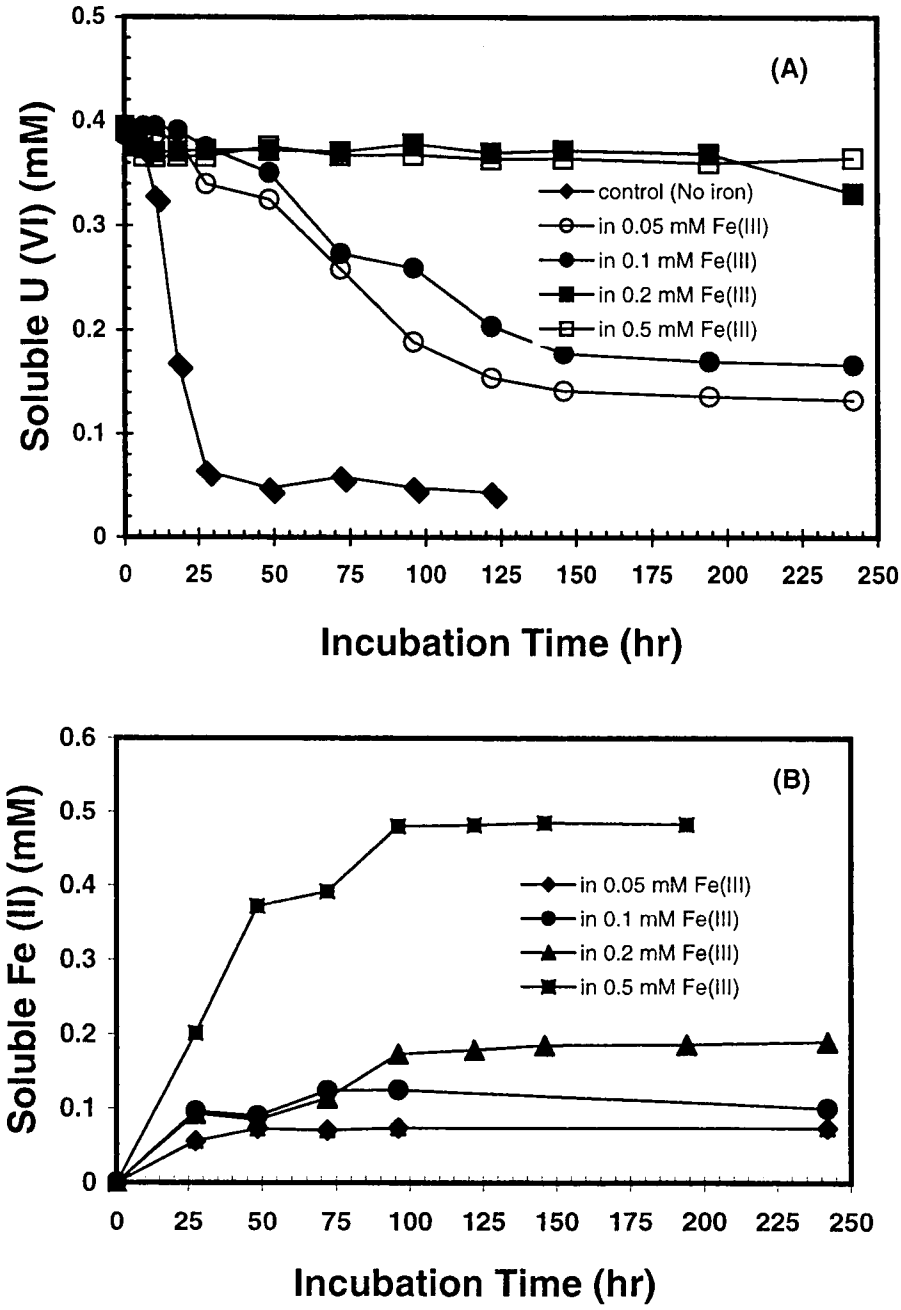


Figure 11. A) U(VI) profile in the presence of different levels of Fe(III) using *S. alga* cell suspensions. U (VI) level in sample without iron was used as a control. B) Fe(II) profile in the corresponding Fe(III)/U(VI) solutions.

reason no uranium reduction was measured in high initial Fe (III) solutions may be due to differences in the electron potential values required for these two metals. An Eh value of -200 mV is needed for U (VI) reduction, while only a value between -100 and –50 mV is required for Fe (III) reduction. Thus the electron potential buffering effect caused by the Fe (II)/Fe (III) redox couple, which keeps the Eh of the system above that required for U (VI) reduction, may explain the impact of high Fe (III) levels on U (VI) reduction. Redox reactions involve an equilibrium between the oxidant and the reductant. pε (pε = -log{ε}) gives the electron activity at equilibrium and measures the relative tendency of a solution to accept or transfer electrons. For the reaction $\Sigma n_i A_i + ne = 0$, where A_i designates the participating species and n_i is the numerical coefficient of each species, the following relationship exists:

$$p\varepsilon = p\varepsilon^\circ + (1/n) \log(\Pi\{Ai\}^{ni})$$

and

$$p\varepsilon^\circ = (1/n) \log K;$$

where the quantity of pε is the electron activity when all species other than electrons are at unit activity and K is the equilibrium constant. The Nernst equation gives the relations in terms of electron potential values (E_H):

$$E_H = E_H^\circ + \frac{2.3RT}{nF} \log \frac{\pi_i\{ox\}^{ni}}{\pi\{red\}^{nj}}$$

where E_H° is the standard redox potential related to the free energy change (ΔG°) or the equilibrium constant (K) of the reaction:

$$E_H^\circ = -\frac{\Delta G^\circ}{nF} = \frac{RT}{nF} \ln K$$

E_H is the redox potential at temperature T and n_j is the numerical value of the reductant. Similar to a pH buffering couple, an oxidant and its conjugate reductant, such as [Fe (II)]/[Fe (III)], can also form a couple that poises electrical potential (Stumm and Morgan, 1981). High initial iron concentrations resulted in stronger buffering capacities, which resulted in a greater negative impact on uranium reduction. Above all, the fact that U (VI) reduction was Fe (III) concentration dependent suggests the possibility of Eh poising and/or product inhibition.

The Fe (II) profile (figure 11B) may help to explain the delay of U (VI) reduction with < 0.2 mM Fe (III). At initial Fe (III) concentrations of 0.05 and 0.1 mM, it took about 25 hours for approximately 90% of the Fe (III) initially present to be reduced. It seems that at low initial Fe (III) concentrations, U (VI) reduction occurred only after significant Fe (III) reduction. This, once again, demonstrates the preference of *S. alga* for Fe (III) over U (VI) under non-growth conditions even at low Fe (III) concentrations. Stumm and Morgan (1981) showed that, in a closed O_2 limiting aqueous system, bioreduction of alternative electron acceptors will proceed in an order from high to low redox potential of each electron acceptor from an energetic standpoint. Since the reduction of U (VI) requires a lower Eh (-200 mV) than Fe (III) (-50 ~ -100 mV), it's clear that *S. alga* will preferentially reduced Fe (III). The reduced Fe (II) product and the

residual Fe (III) ions form an electron potential buffering pair which keeps the Eh value of test solutions stable at a relatively high value. However, since the current degassing device has no quantitative control on the redox potential, this parameter was not directly studied in this work.

Since the product of Fe (III) reduction is Fe (II), the impact of Fe (II) on U (VI) reduction was then studied to evaluate possible product toxicity. Ferrous ion was added from an anaerobic ferrous chloride stock. The stock was prepared by dissolving ferrous chloride powder into sterilized deionized water and then degassed. Aliquots of Fe (II) stock were added to serum bottles using a gas-flushed syringe to obtain final Fe (II) concentrations of 0.05, 0.1, 0.2 and 0.5 mM. The same amount of U (VI) (0.52 mM) was then added. Figure 12 presents U (VI) concentration profiles in the presence of different initial Fe (II) concentrations. Uranium levels decreased over time in all of the experimental sets. However, both the rate and extent of U (VI) reduction were retarded in solutions containing Fe (II) compared to the control (without iron). At higher Fe (II) concentrations (≥0.2 mM), 0.38 mM (90 mg/L) of U (VI) initially present was reduced to 0.084-0.105 mM (20-25 mg/L) within 120 hours with a transformation efficiency of ~75%. At lower Fe (II) concentration (0.05 mM), the residual U (VI) level was ~0.05 mM (12 mg/L) with a relatively higher transformation percent (~90%), which was close to that in the control. The rates of U (VI) reduction also decreased as the initial Fe (II) levels

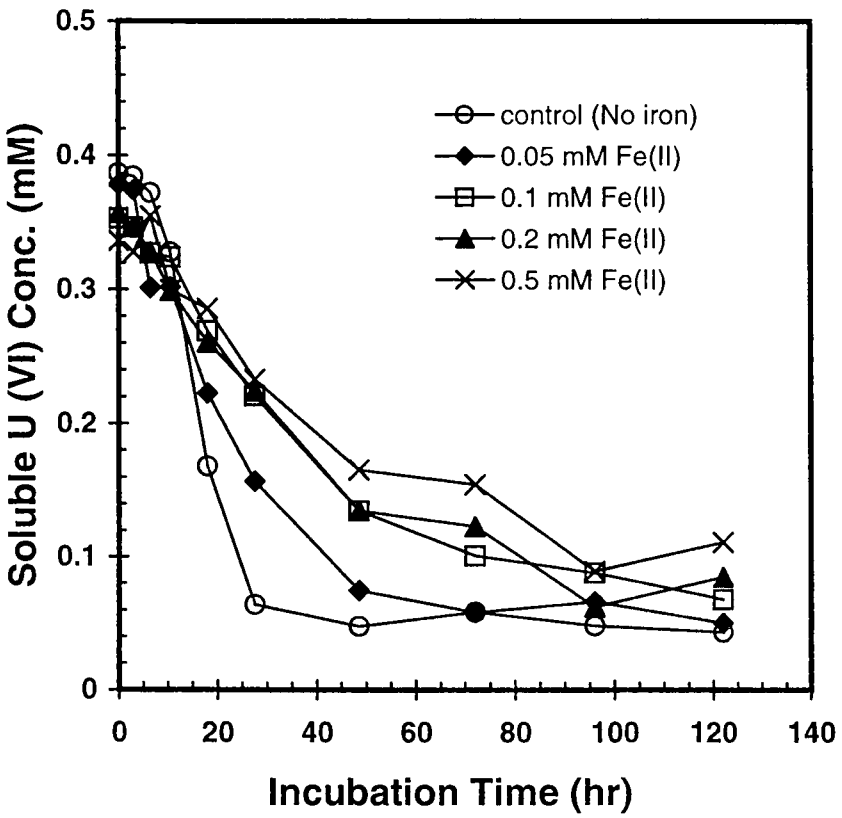


Figure 12. U (VI) levels as a function of time in the presence of different initial Fe (II) concentrations using *S. alg* cell suspensions.

increased. The slope of the linear portion of each concentration curve was calculated to determine the U (VI) reduction rate. The rate of U (VI) reduction without Fe (II) was predicted using the Monod model and kinetics coefficients previously determined (the maximum specific rate (V_{max}) used in the calculations was 0.3 hr^{-1} and the half-saturation constant (K_s) was 50 mg/L.). The U (VI) reduction rate (V) at an initial concentration of 90 mg/L was calculated as 5.8 mg/L•hr using the following relationship (protein concentration = 30 mg/L):

$$V = \frac{V_{max} X S}{K_s + S} = \frac{(0.3 hr^{-1})(30 mg/L)(90 mg/L)}{50 mg/L + 90 mg/L} = 5.8 mg/L \bullet hr$$

The impact of Fe (II) on the rate of U (VI) reduction was further investigated in Figure 13. The percent of impact on the U (VI) reduction rate was calculated as the difference between the calculated initial rate of U (VI) reduction in the presence of Fe (II) and the U (VI) reduction rate predicted in the absence of Fe (II) (5.8 mg/L hr) divided by the latter. As Figure 13 shows, an initial Fe (II) concentration as low as 0.1 mM had a ~60% impact on the U (VI) reduction rate. The impact was linear at concentrations below 0.1 mM. At concentrations above 0.1 mM, the impact remains constant at ~65%. The reason why Fe (II) ion had a negative impact on U (VI) reduction is probably an example of product inhibition. Enzymatic reactions can be simplified as follows:

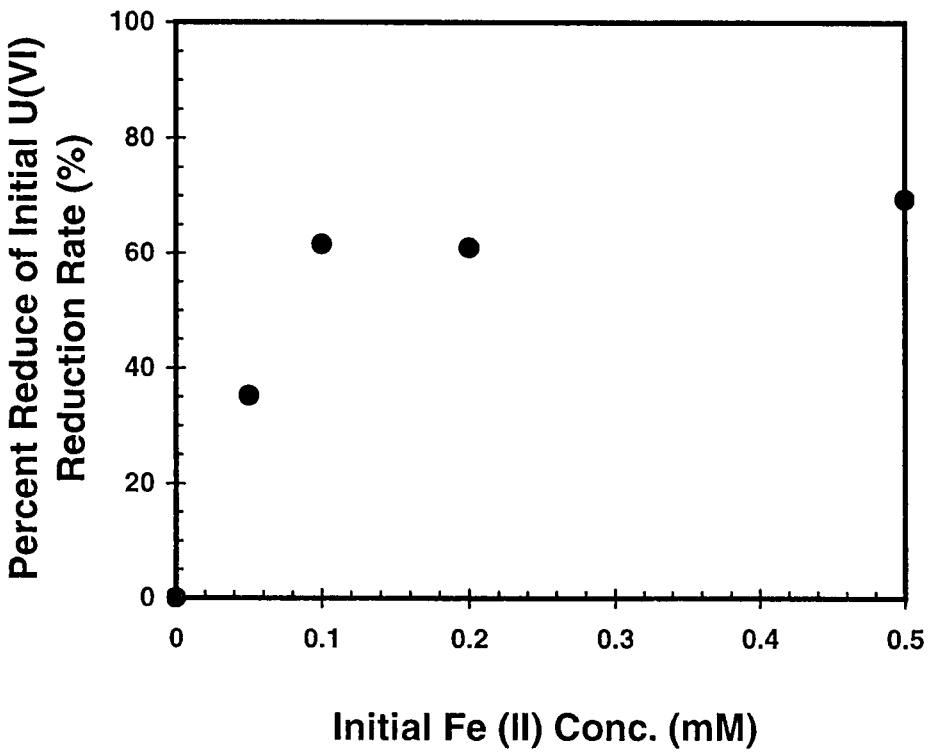


Figure 13. Percent reduce in the initial U(VI) reduction rate as a function of initial Fe(II) concentration using *S. alga* cell suspensions.

$$E + S \Leftrightarrow ES;$$

$$ES + P \Rightarrow EP \Leftrightarrow E + P$$

where S is the substrate, E is the enzyme, ES and EP are the intermediate enzyme-substrate and enzyme-product complexes. In some cases products can bond to the active enzyme sites and form an enzyme-product complex, which can result in a lower rate of substrate utilization. In this system, Fe (II) could inhibit U (VI) reduction by forming the enzyme-Fe (II) complex. The reason why Fe (II) inhibition was not observed during Fe (III) reduction was likely due to the relative affinity of Fe (II) to enzyme over U (VI) and Fe (III).

Metal impacts on biologically meditated uranium reduction have been studied for *D. desulfuricans*. Results showed that for the various metals tested (iron, copper, nickel, vanadium and zinc), only copper above 40 μm inhibited uranium reduction by *D. desulfuricans* (Lovley & Phillips, 1992). Copper above 0.1 mM totally inhibited uranium reduction. Vanadium inhibited U (VI) reduction in a short-term period (1.5 hours) but not long term (20 hours).

The results clearly indicate that, unlike *D. desulfuricans*, which reduces sulfate and uranium simultaneously, *S. alga* utilizes Fe (III) more readily than U (VI) as the electron acceptor under non-growth conditions. Microbial reduction in the presence of multiple electron acceptors has been studied for several strains. Lovley (1992) has done an extensive study on the co-reduction of U (VI)/SO_4^{2-} using *D. desulfuricans* cell

suspensions. Sulfate was added to a solution containing 0.3-0.4 mM uranium to obtain 1.5-2.0 mM sulfate. They found that sulfate has no significant effect on U (VI) bioreduction, and the addition of U (VI) did not influence the rate of sulfate reduction; the two electron acceptors were reduced simultaneously. The author concluded that *D. desulfuricans* may utilize two different mechanisms for U (VI) and SO_4^{2-} reduction. In contrast, Kucsmas (1994) found that the presence of sulfate could affect the uranium reduction rate for sulfate concentrations higher than 10,000 mg/L (U (VI) = 100 mg/L) or 5000 mg/L (U (VI) = 10 mg/L). The author proposed the delay might be caused by ionic strength inhibition or sodium inhibition.

4.3 Metal reduction by *S. alga* coupled with growth

Non-growth conditions provide stationary cells with minimum salts (only electron donor/acceptor, no nutrients) to maintain cellular respiration. However, continuous fresh cell supply becomes a problem when applying this technique to a treatment scenario. For engineered applications, it would be highly desirable if cells could grow and reduce metals in the same medium. However, metal treatment results may differ under growth conditions because important and complex microbial activities such as oxidative phosphorylation and cellular synthesis/division occur. Such activities are limited under non-growth conditions. Subsequently, the impact on reduction rates and/or the reduction sequence of the metals available may differ under growth conditions.

The difference between growth and non-growth conditions on organism performance can be illustrated by other iron reducing microorganisms. While certain organisms can utilize Fe (III) as a terminal electron acceptor, no growth has been observed during incubation suggesting that the energy obtained through this electron flow chain is not sufficient for biosynthesis. Some authors concluded that Fe (III) apparently was reduced by side reactions of the NO_3^- or O_2 terminated repirationary chains of these bacteria (Zimmermann, 1984). Others believe organisms use Fe (III) terminated electron transport systems to dissipate excess reductant without synthesizing ATP (Zehnder, 1988). In 1992, Lovely *et al.* isolated an iron-reducing microorganism (*Geobacter metalireducens*), the first organism which can couple Fe (III) reduction and organic oxidation with growth. After that, additional iron reducing microorganisms (including *S. alga*) have been reported to grow using chelated Fe (III) as the sole electron acceptor.

In addition to Fe (III), U (VI) also plays two different roles in the same microorganism – *D. desulfuricans.* This sulfur reducing bacterium was found to utilize U (VI) as a terminal electron acceptor under non-growth conditions. However, growth using U (VI) as the sole electron acceptor was not successful. The author didn't provide an explanation for the difference observed. According to studies on iron reducers, the possible reasons might be either that the organism uses a uranium-terminated system to dissipate excess reductant without synthesizing ATP, or the energy obtained through this reaction is too low to conduct biosynthesis.

In summary, non-growth microbial reactions and growth coupled reactions have significant differences in microbial growth status, cellular product formation and energy consumption. These differences have been demonstrated by microorganisms that can conduct certain biotransformation reactions for respiration but not for growth. However, it's unclear as to how those differences will impact metal degradation rates and microbial preference when multiple metals are available.

4.3.1 Fe (III) reduction by *S. alga* coupled with growth

It's been established in this work that *S. alga* can not only utilize Fe (III) for anaerobic respiration, but can also obtain energy from reduction for cellular growth. In this experiment, kinetics of microbial growth and metal reduction were quantified. The growth medium was different from that used for seed culture during microbial characterization. Peptone and yeast extracts were not added to ensure the medium was chemically defined and also to prevent interference with U (VI) analysis. Growth medium in this study was also different from test solutions used in non-growth studies in that nutrients, such as N and P, were added to support cellular growth. A higher concentration of Fe (III) (10 mM-ten times higher than that used in non-growth experiments) was provided as the sole electron acceptor to support measurable cell mass. Lactate was used as the sole electron donor/carbon source. Protein was used as the indicator for cell growth. The growth coupled reduction pattern is show in Figure 14. Fe (II) product accumulated from 0 to 7.5 mM during ~120 hours of incubation, while the total protein concentration increased from ~30 mg/L to 150 mg/L. Fe (III) levels were calculated and

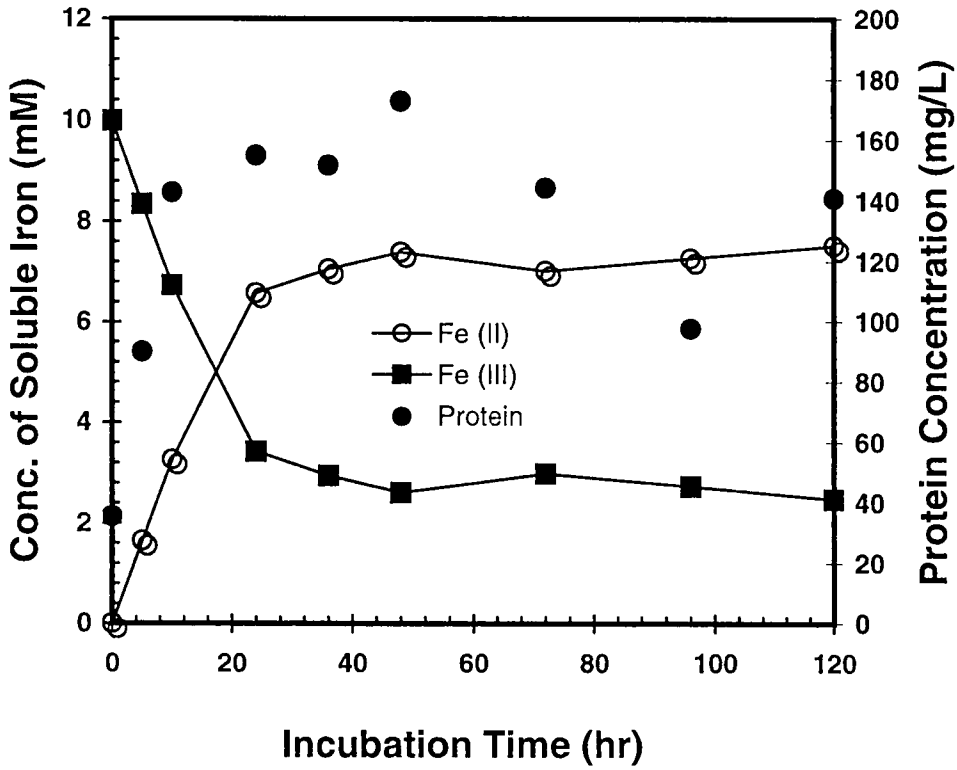


Figure 14. Iron and protein levels in the growth medium as a function of time after inoculation with *S. alga* cells.

presented in this plot. During the first 24 hours of incubation, the decrease in Fe (III) level was linear and characterized as a first order reaction. After 24 hours, the Fe (III) level remained constant at 2.5 mM. The protein level was linear throughout the first 10 hours of incubation, which corresponded to the exponential growth phase. After that, it remained relatively constant at ~150 mg/L. The maximum specific growth rate (μ_{max}) was calculated as 0.139 mg protein/mg Fe (III)•hr. The doubling time (τ_d) was 4.9 hr. Compared to results obtained from growth characterization, the maximum specific reduction rate was 0.4 /hr and doubling time was 1.73 hr for Fe (III) reduction in a growth medium with the addition of pepton and yeast extract. It seems that due to the presence of pepton/yeast extract, the growth rate increases 2-3 times. A possible explanation could be due to the presence of unidentified electron acceptors in these synthesized materials. This result also confirms the necessity to use a chemically defined medium in a kinetic study. Compared to the kinetic coefficients reported by Truex *et al.* (1995) for a CSTR system at pH = 6.8 and temperature = 35°C (maximum specific growth rate = 0.142 /hr; growth yield = 0.071 mg biomass/mg Fe (III)), the specific growth rate is very close to what was determined in a batch system.

Growth of *S. alga* on different amounts of Fe (III) (0.1 mM, 1.0 mM and 10 mM) is presented in Figure 15. Fe (II) accumulated and the protein concentration increased over time. After 10 hours of incubation, both the Fe (II) and protein levels reached saturation and remained constant. Initial Fe (III) concentrations of 0.1 mM, 1 mM and 10

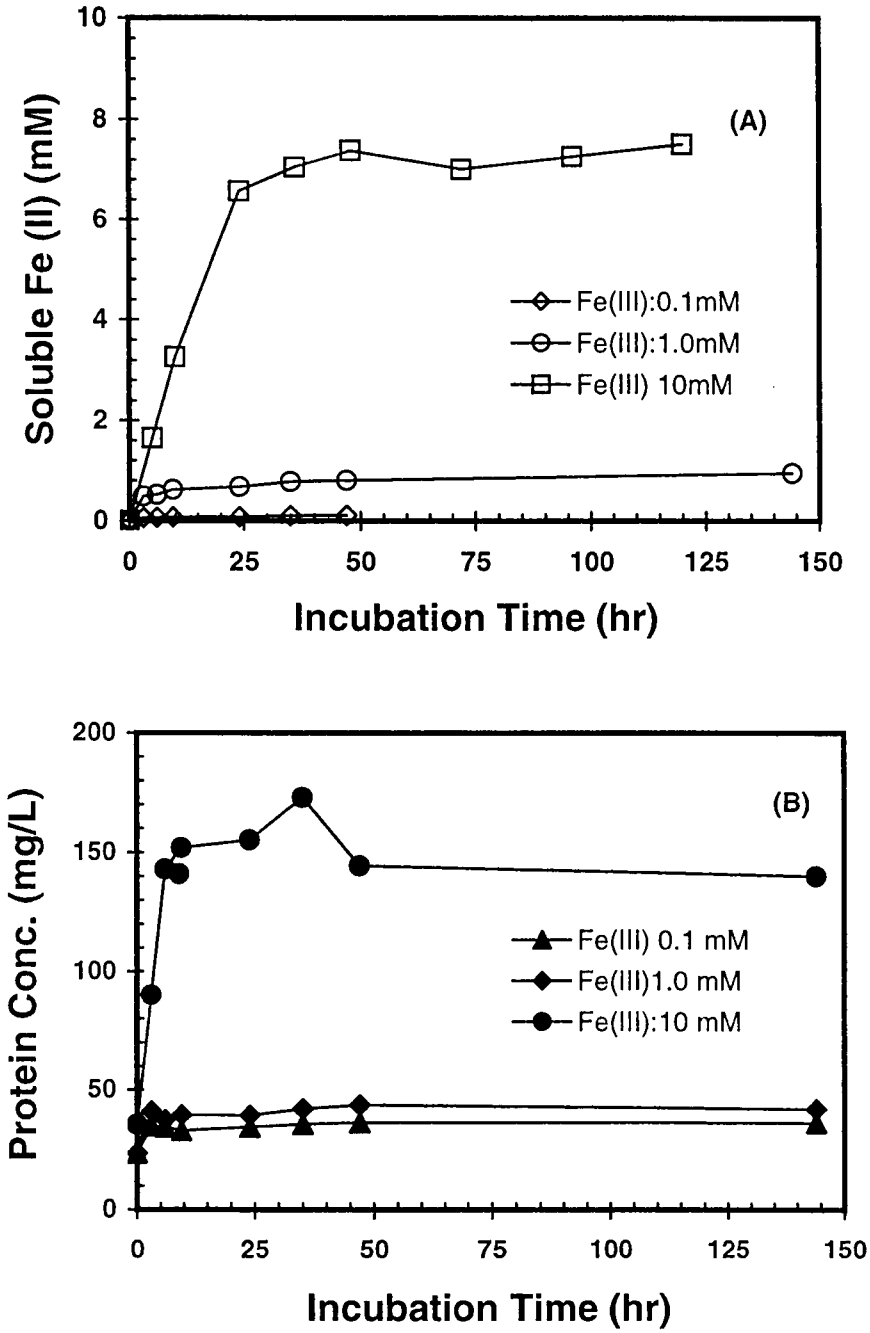


Figure 15. Fe (II) (A) and protein (B) profiles of *S. alga* in growth medium for different initial Fe (III) levels.

mM supported ~3, 20 and 140 mg/L of protein respectively. There was no limit to the maximum growth when up to 10 mM Fe (III) was provided as the terminal electron acceptor, which indicates no substrate inhibition.

There was a significant color change during cellular growth. Initially the color of the medium was dark red due to excessive ferric ions. After one day's incubation, the medium turned colorless after most of the ferric was reduced to ferrous ion. Unlike growth in the medium containing peptone and yeast extract, there was only a small volume of black precipitate generated at the bottom of the serum bottle rather than the total black homogeneous solution observed when these constituents were present. The unknown black precipitate in the seed culture was rather difficult to separate from the cells during centrifugation, which was then carried over to subsequent reduction experiments. This suggests that separation problems caused by the unknown black precipitate during seed culture preparation could be minimized by cutting the dosage of peptone and yeast extract.

4.3.2 U (VI) reduction by *S. alga* coupled with growth

Uranium reduction by cell suspensions under non-growth conditions has been reported for iron reducing bacteria (*S. alga* and *G. metaliputrificans*) and a sulfur-reducing bacterium (*D. desulfuricans)* (Lovley & Phillips, 1992). However, microbial growth using U (VI) as the sole terminal electron acceptor has only been demonstrated for the iron reducing strains *G. metaliputrificans* and *S. putrefaciens*. Due to the strict

anaerobic environment required for reductive precipitation of U (IV) by *G. metaliputrificans*, the application of this organism for uranium contaminated groundwater remediation has been greatly limited. *S. alga*, a facultative anaerobe, not only is able to reduce uranium at a high rate but also is able to grow using oxygen as the terminal electron acceptor. Though the presence of oxygen inhibits U (VI) reduction, it is not toxic to cells. If cellular growth using U (VI) was demonstrated for *S. alga*, this organism could be a good alternative to *G. metaliputrificans* for uranium biotreatment. Continuous viable cell supply under non-growth conditions would not be necessary. Experiments were performed to demonstrate growth of *S. alga* using U (VI) as the sole electron acceptor in a batch system. The medium used was the same as that used in the Fe (III) growth studies. U (VI) was added from uranium nitrate stock. Seed culture was diluted and then inoculated into the serum bottle. Protein concentrations and soluble U (VI) levels were measured over time. The protein level in samples containing cells but no U (VI) was monitored as a control. Figure 16 profiles the growth of *S. alga* using 4.2 mM U (VI) as the sole electron acceptor. Initially present ~4.2 mM (1000 mg/L) U (VI) was reduced to ~1.26 mM (300 mg/L) during 120 hours of incubation. Cell mass correspondingly increased from ~ 30 mg/L to ~120 mg/L. Cell mass in control samples remained relatively unchanged (~ 30 mg/L). The results clearly demonstrate that *S. alga* can utilize hexavalent uranium for cellular growth in a batch system. As compared to growth of the previous reported iron reducing bacterium *G. metaliputrificans* (Lovley, 1992), 8 mM (1900 mg/L) U (VI) was reduced to 2.2 mM (~523 mg/L) within 24 hours with subsequent cell growth from $3x10^7$ to $13x10^7$ cells/mL. For *S. alga*, within the first 24

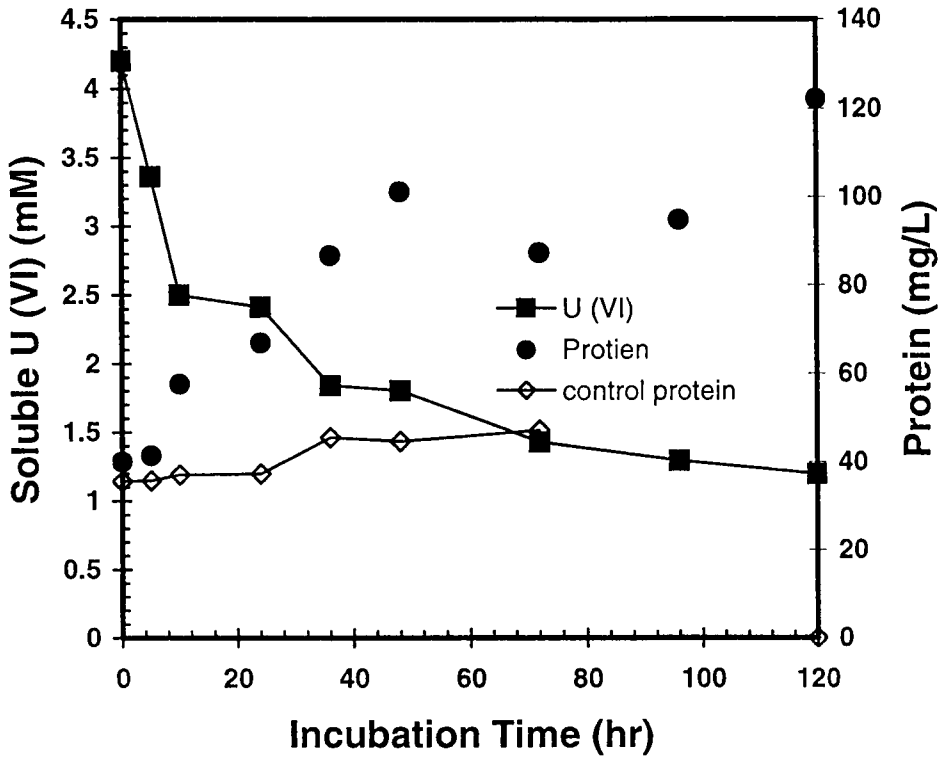


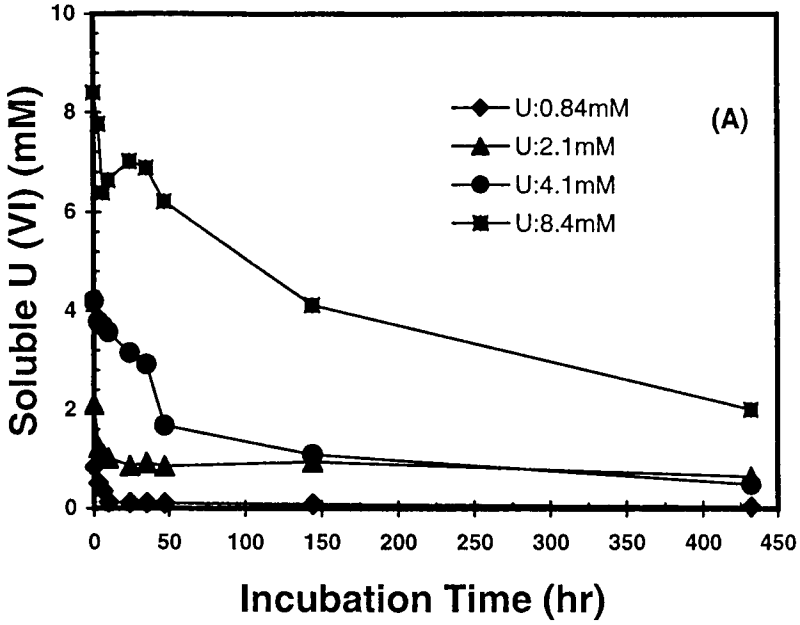
Figure 16. U (VI) and protein profiles in the growth medium after incubation with *S. alga* cells. Protein level in the medium without addition of U(VI) was used as a control.

hours of incubation, the U (VI) level decreased from 1000 mg/L (4.2 mM) to ~580 mg/L (2.44 mM) while the protein concentration increased from 30 mg/L to ~70 mg/L. The growth rate of *S. alga* (1.7 mg/L protein•hr) is 3 times higher than that of *G. metaliputrificans* (0.42 mg/L protein•hr) (assuming 10^{-10} mg protein/cell).

Using the same numerical methods as those described in the Fe (III) growth kinetic studies, the maximum specific growth rate (μ_{max}) was calculated to be 0.0215 mg protein/ mg U (VI) •hr and the doubling time was 32.2 hr. Compared to that of Fe (III) reduction, *S. alga* grew six times faster when utilizing Fe (III) rather than U (VI) as the electron acceptor.

In order to collect uranium inhibition information, the growth and reduction pattern under different concentrations of uranium was evaluated and presented in Figure 17. Up to 8.4 mM U (VI) was reduced, and the corresponding protein concentrations increased as the initial U (VI) levels increased. U (VI) levels at 0.84, 2.1, 4.2 and 8.4 mM support protein levels of 40, 65, 110 and 200 mg/L, respectively. Similar to Fe (III), no metal inhibition was observed within the U (VI) concentrations used.

The color change associated with uranium reduction and microbial growth was noticeable. Initially, the color of the medium was yellow due to the large amount of hexavalent uranium present. After one day's incubation, the medium turned dark and a



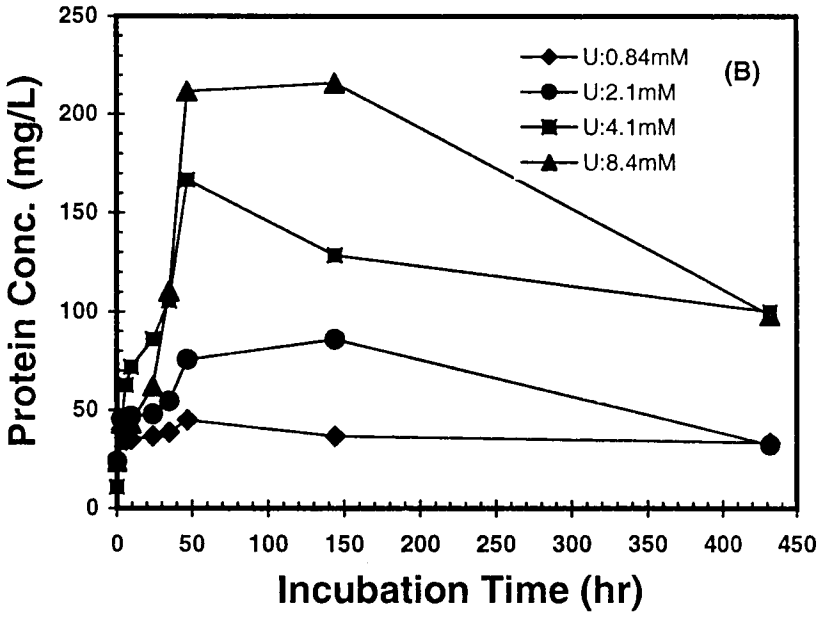


Figure 17. U(VI) (A) and protein (B) profiles of *S. alga* in growth medium for different initial U (VI) levels.

black precipitate started to accumulate at the bottom of the serum bottle. The black precipitation was believed to be uraninite, the reduction product of hexavalent uranium under low redox potential. Additionally, a large volume of dark purple floc formed during incubation of the 5 mM uranium sample. The floc which formed in 2.5 mM and 1 mM solutions appeared to be dark and black whereas floc in 10 mM samples appeared to be a dark yellow color. In general, living cell suspensions of *S. alga* showed a purple color and these cells were homogeneously distributed throughout the solution while dead cells (after autoclaving) formed a purple colony ball sticking together floating in the solution. Therefore, the dark purple color in the 5 mM uranium solutions may be the color reactions of the purple cells themselves and the black uraninite precipitate. Various concentrations of uranium supported different amounts of cell mass, which in turn, produced a given amount of uraninite precipitate. The combination of cells and uraninite precipitate contributes to the final color of the floc.

4.3.3 Metal reduction by growth coupled *S. alga* in the presence of both electron acceptors

Figure 18 presents the result of metal reduction and microbial growth in the presence of both electron acceptors. For an incubation time of 70 hours, the Fe (III) concentration decreased from 10 mM to 8 mM (20% biotransformation) while the U (VI) concentration decreased from 4.2 mM to 3.8 mM (9.5% biotransformation). When these metals were added to test solutions separately, Fe (III) levels decreased rapidly from 10 mM to 2.2 mM (78% biotransformation) and U (VI) levels dropped from 4.2 mM to 1.5

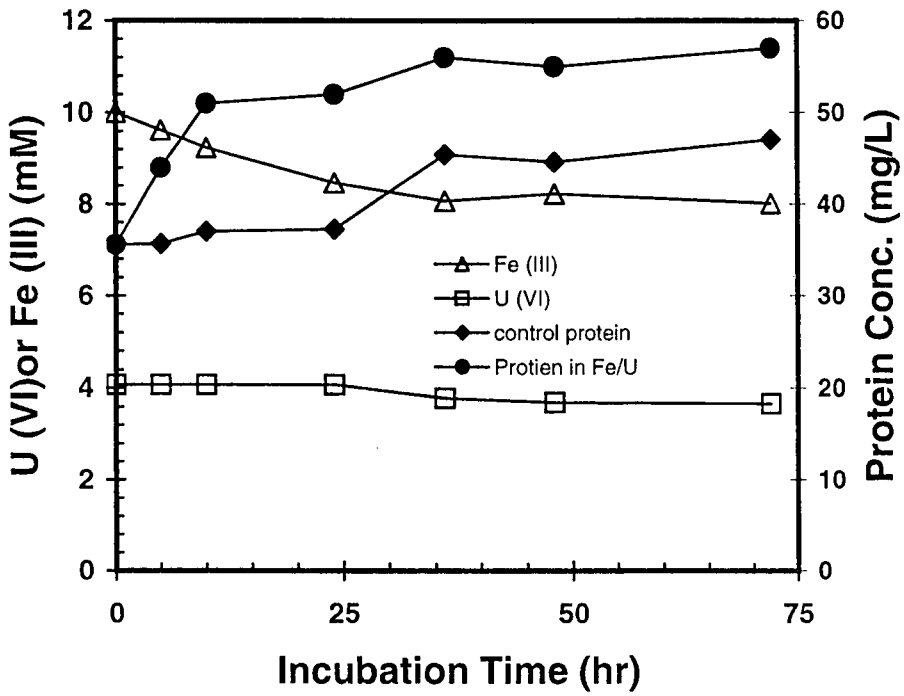


Figure 18. Metal and protein profiles of *S. alga* in growth medium using U (VI) and Fe (III) as co-electron acceptors. Protein in medium without addition of metals was used as a control.

mM (64% biotransformation) within 70 hours. The result indicates that one metal inhibits microbial utilization of the other. The fact that reduction of each metal was slower when co-electron acceptors were present indicates that *S. alga* probably utilizes U (VI) and Fe (III) through the same enzyme system. Consequently the competition for the limited enzyme sites between the two metals results in the slowing of both metal reduction rates. To further investigate the relationship between these two terminal electron acceptors, the impact of one metal on the other in addition to microbial growth was studied.

4.3.3.1 U (VI) impact on Fe (III) growth coupled reduction

Different uranium levels seemed to have different impacts on growth coupled reduction of 10 mM ferric citrate. As Figure 19A shows, 0.084 mM U (VI) had little impact on Fe (III) reduction whereas uranium levels of 0.42 mM and 0.84 mM slowed the rate of Fe (III) reduction as well as lowered the overall extent of reduction. No uranium reduction occurred during the 120 hour incubation period. Figure 19B indicated higher uranium levels lowered the overall production of cell mass. The impact of initial uranium concentration on the Fe (III) reduction rate is shown in Figure 20. The percent impact increased from 0 to 78% up to a U (VI) concentration of 0.42 mM (100 mg/L). At U (VI) levels higher than 100 mg/L, the percent impact on biotransformation rate remained relatively constant at ~80%.

The presence of 10 mM Fe (III) totally inhibited uranium reduction by *S. alga.* This result is the same as that found under non-growth conditions. However, the fact that U (VI) inhibited Fe (III) reduction when coupled with microbial growth was different

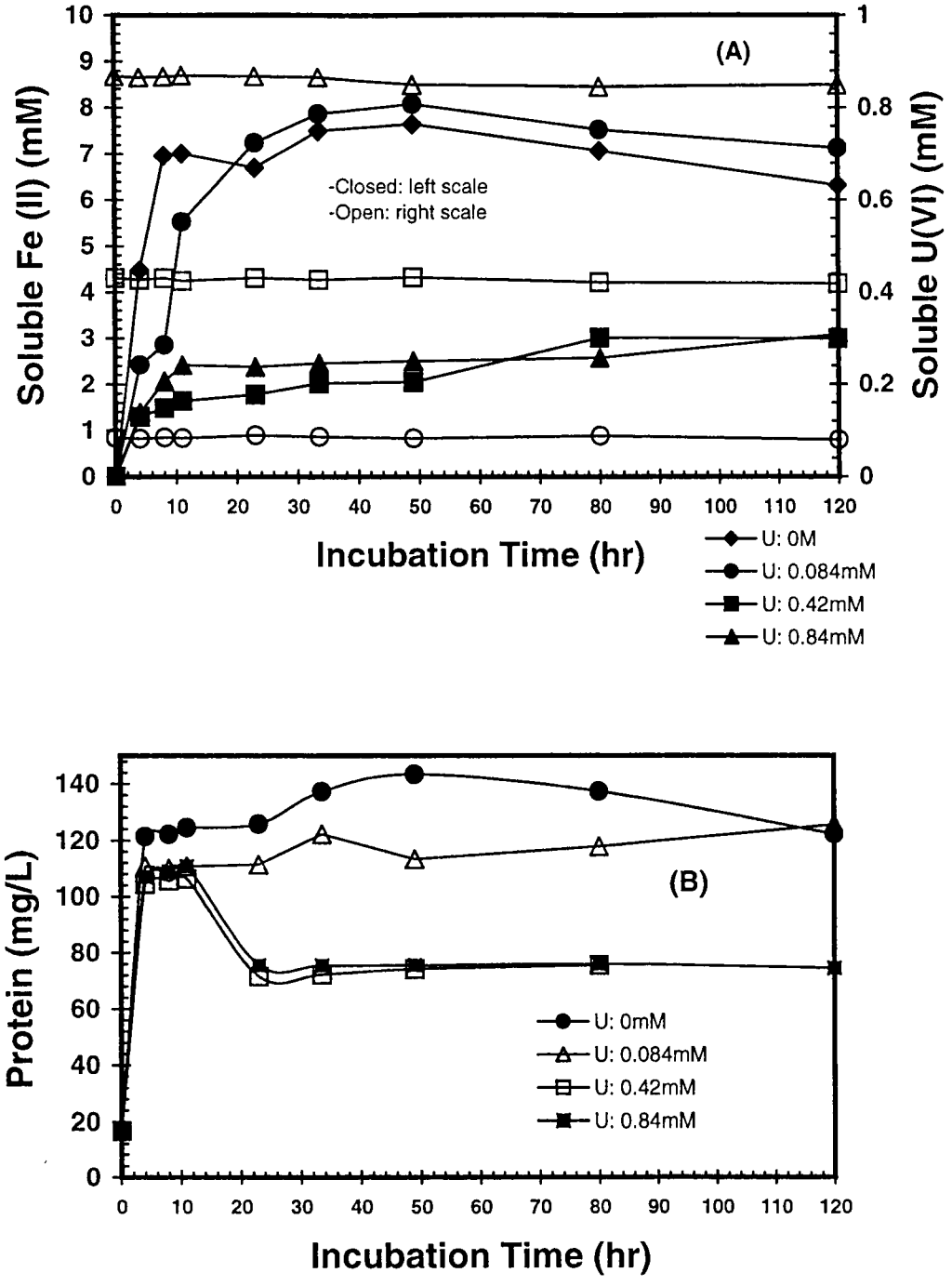


Figure 19. Metal (A) and protein (B) profiles of *S. alga* in growth medium for different levels of U(VI).

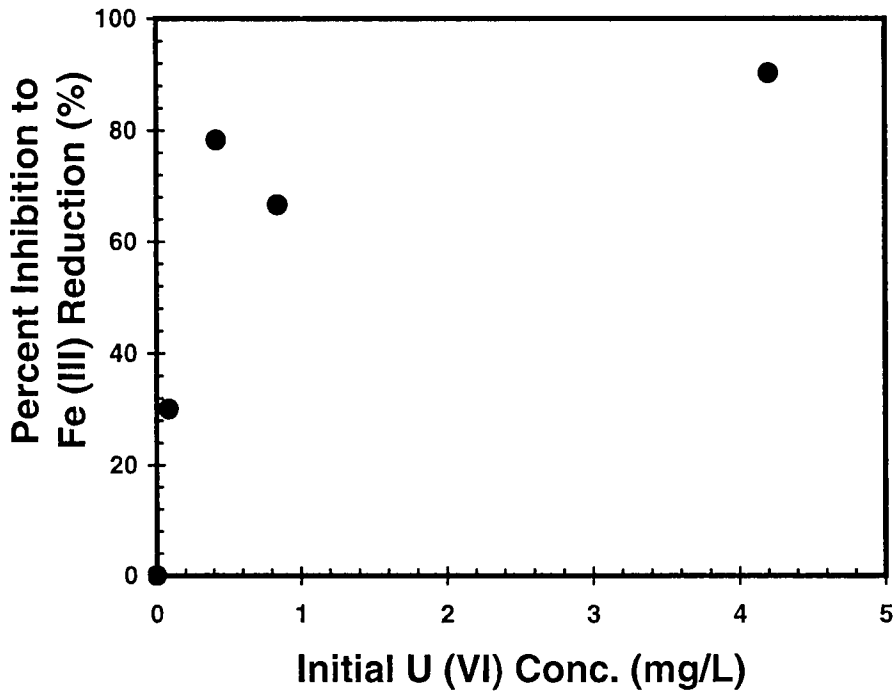


Figure 20. Percent inhibition to Fe (III) reduction as a function of initial U (VI) concentration by growth coupled *S. alga.*

from the non-growth results. Under non-growth conditions (Figure 21A), Fe (III) reduction patterns without U (VI) are similar to those with U (VI) (0.42 mM). That is, the presence of U (VI) did not alter the kinetics of Fe (III) reduction under non-growth conditions (Figure 21B). *S. alga* preferentially reduces Fe (III) as if U (VI) was not present. The reasons why U (VI) inhibited iron reduction when coupled with growth is believed to be growth associated.

4.3.3.2 Impact of Fe (III) to U (VI) reduction by growth coupled *S. alga*

Figure 22 shows the U (VI) (4.2 mM) reduction and microbial growth pattern under different initial Fe (III) concentrations (0.1, 0.2 and 1.0 mM). Serum bottles without inoculation were used as controls. For 4.2 mM U (VI) initially added, 20% soluble U (VI) was lost due to interactions with the medium. A yellowish precipitate was observed at the bottom of the control serum bottle after one day. The soluble hexavalent uranium remained constant in the control after one day. Approximately 4.2 mM U (VI) was reduced to ~1.47 mM (350 mg/L) within 150 hours of incubation (65% conversion). Residual U (VI) levels in 0.1, 0.2 and 1.0 mM of Fe (III) were 2.1, 2.94 and 3.15 mM, with 50%, 30% and 25% conversion, respectively. Initial rates of U (VI) reduction decreased as Fe (III) concentrations increased from 0 to 1 mM. Similar to non-growth studies, reduction of U (VI) occurred when the initial Fe (III) concentration was below 1 mM although both rates and extent of U (VI) reduction slowed as Fe (III) levels increased.

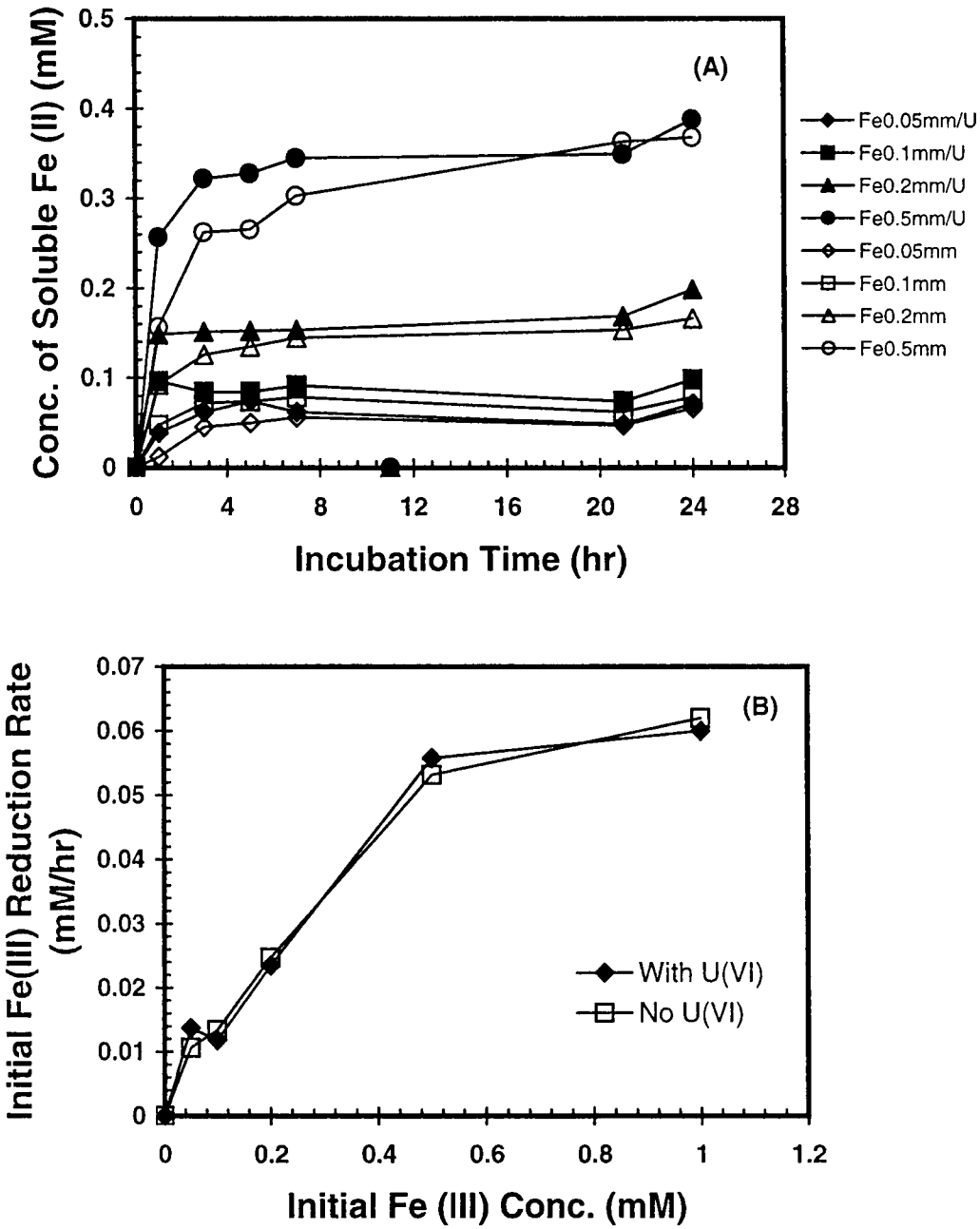


Figure 21. A) Fe (II) profile with U(VI) (0.42 mM) after inoculation with *S. alga* cell suspensions. B) Initial Fe(III) reduciton rates as a function of initial Fe (III) level with and without U(VI).

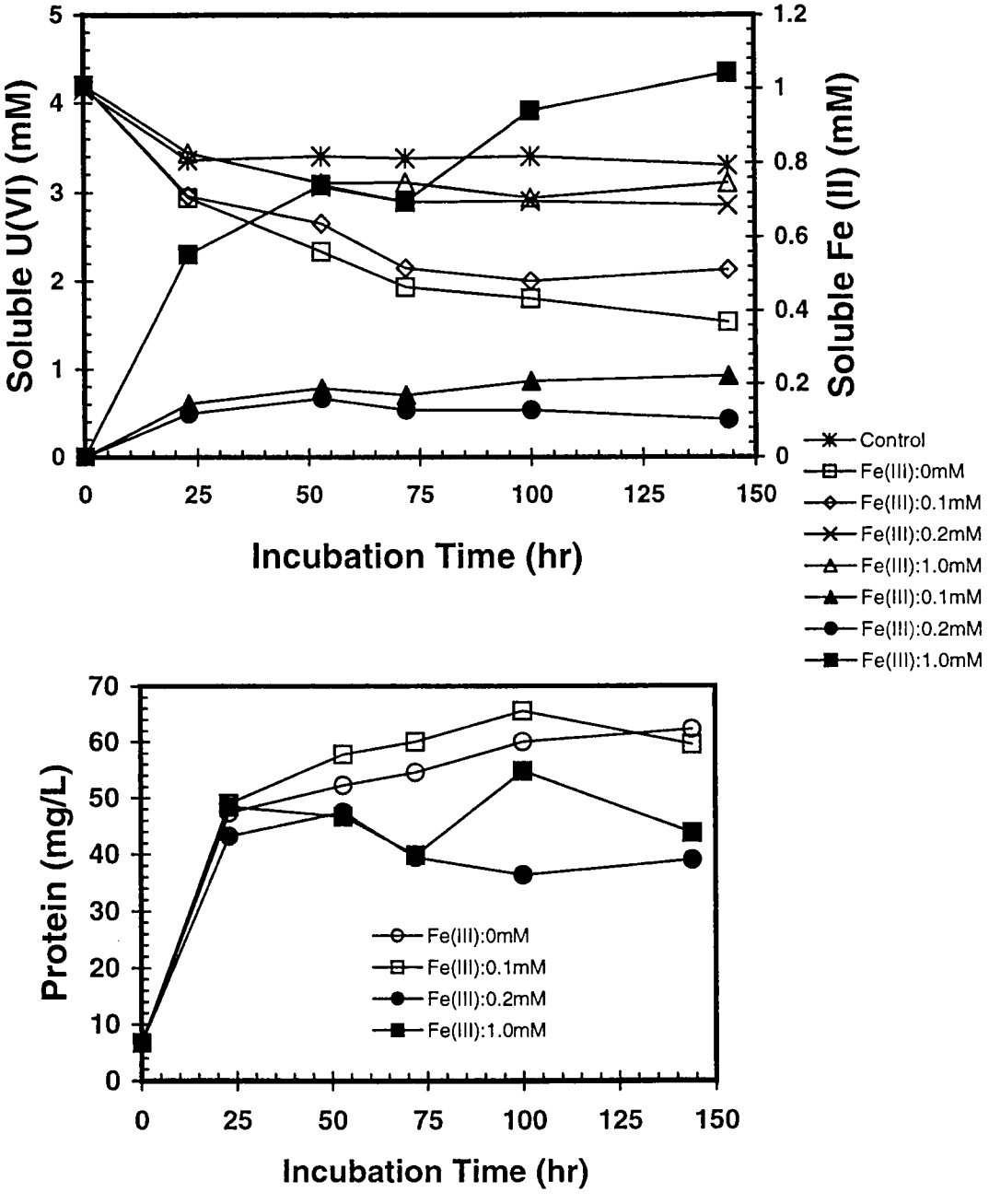
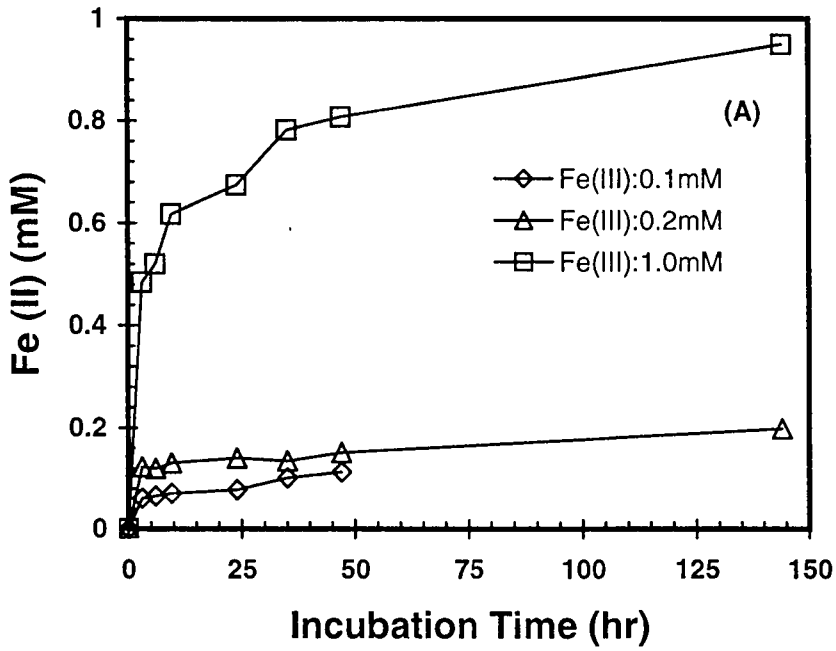


Figure 22. Fe (II)/U (VI) (A) and protein (B) profiles of *S. alga* in growth medium for different iron levels. U (VI) level = 4.2 mM.

Regarding microbial growth using 4.2 mM U (VI) as the terminal electron acceptor in the presence of Fe (III), Figure 22B indicated that Fe (III) levels had an impact on the biomass concentrations supported. Note that U (VI) (4.2 mM) was the major growth supporting electron acceptor compared to Fe (III) (≤ 1 mM). As the Fe (III) concentrations increased, U (VI) reduction rates decreased and subsequently the biomass produced also decreased. Thus, the more uranium reduced, the more biomass was generated. According to the graph, maximum uranium reduction (>50%) occurred with 0 and 0.1 mM Fe (III), therefore, more protein (70 mg/L) was produced. For iron concentrations of 0.2 and 0.5 mM, 30% U (VI) reduction occurred, therefore, less protein (~50 mg/L) was generated.

Iron reduction without uranium is shown in Figure 23. It only required 5 hours for 90% of 0.1 and 0.2 mM Fe (III) to be reduced, and ~50 hours for 90% of 1.0 mM Fe (III) to be reduced. The result confirms the conclusion reached in the previous section that the presence of U (VI) inhibits Fe (III) reduction when coupled with microbial growth (Figure19 & 20). Combining the data for Fe (III) and U (VI), it seems that reduction of U (VI) and Fe (III) occur simultaneously although both at a lower rate. Unlike the non-growth studies (figure 11: Fe (III): 0.05/0.1 mM), there was an obvious lag time when the U (VI) level remained unchanged while Fe (III) was continuously reduced. As discussed previously in the section on the impact of U (VI) on growth coupled Fe (III) reduction, the two electron acceptors compete for the limited enzyme sites, which results in the lower rates of both reductions.



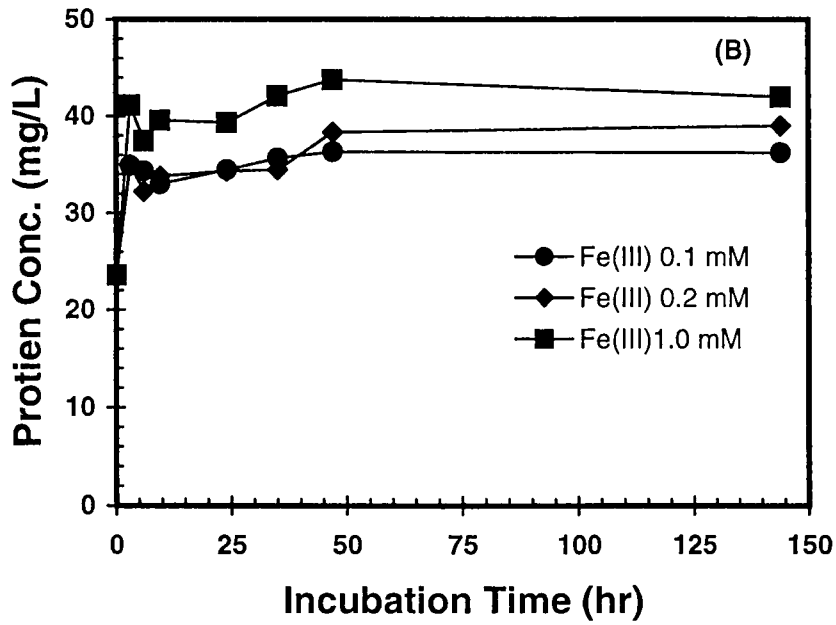


Figure 23. Fe (II) (A) and protein (B) profiles of *S. alga* in growth medium for low initial Fe (III) concentrations.

4.4 Summary

S. alga cell suspensions grown on oxygen and Fe (III) were equally effective for U (VI) and Fe (III) reduction. The lag time observed during U (VI) reduction using cells grown on oxygen was believed due to oxygen carryover. 12 hr and 16 hr cells grown on oxygen, as well as 24 hr and 48 hr cells grown on Fe (III), were effective in reducing both metals.

S. alga has a higher reduction capacity as well as a higher affinity for Fe (III) than U (VI). Batch growth of *S. alga* using U (VI) as the sole electron acceptor was successful. The growth rate was a factor of 3 higher than the value determined for another reported iron reducer, *D. metaliputrificans*. Metal reduction and microbial growth kinetic coefficients are summarized in Table 5.

Under non-growth conditions, Fe (III) was preferentially reduced by *S. alga* in the presence of U (VI). Subsequent U (VI) reduction was dependent on the initial Fe (III) levels. At high initial Fe (III) concentrations, no U (VI) reduction was measured. This may be due to the fact that *S. alga* preferentially utilizes Fe (III) over U (VI) because uranium reduction requires a much lower redox potential compared to iron reduction. While Fe (III) was reduced to Fe (II), the redox couple Fe (II)/Fe (III) poised the system by keeping the Eh level above that required for U (VI) reduction. Higher initial Fe (III) levels produce a higher poising effect, which results in a higher Eh value and therefore less uranium reduction.

Table 5: Kinetic coefficients of *S. alga* for metal bioreduction (pH = 8.4, T= 35°C)

Parameters	Max. Specific Reduction Rate (k)(hr^{-1})	Half-saturation Constant (K_s) (mM)	Max. Specific Growth Rate (μ_m) (hr^{-1})
Fe (III)	5.13 ± 2.97 (95%)	0.66 ± 0.4 (95%)	0.139
U (VI)	0.39 ± 0.023 (95%)	0.31 ± 0.054 (95%)	0.0215

Under growth conditions, reduction of U (VI) and Fe (III) was measured simultaneously. The individual rate of metal reduction when both were present together was slower than that when one was absent. That is, the presence of one metal impeded the reduction of the other. It is mostly likely that U (VI) and Fe (III) reduction shared the same active enzyme system, for which both metals were competing. Unlike non-growth conditions where *S. alga* selectively reduced Fe (III) over U (VI), U (VI) and Fe (III) were utilized simultaneously by growing *S. al*ga cells.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

- *S. alga* cell suspensions grown on oxygen and Fe (III) had the same reduction efficiency for U (VI) and Fe (III). The lag time observed during U (VI) reduction using cells grown on oxygen was believed due to oxygen carryover. Cells grown at 12 hr and 16 hr on oxygen, as well as cells grown at 24 hr and 48 hr on Fe (III), were equally effective for both U (VI) and Fe (III) reduction.

- *S. alga* grew in a chemically defined medium containing U (VI) as the sole electron acceptor with lactate as the electron donor. The maximum specific growth rate was estimated to be 0.0215 /hr, six times less than that for Fe (III) (0.139 /hr).

- *S. alga* has a higher affinity and a high reduction capacity for Fe (III) than U (VI). The maximum specific reduction rate for U (VI) was ~0.39 /hr, more than ten times less than that for Fe (III) (~5.13 /hr); The half-saturation constant for U (VI) was ~74.04 mg/L, two times greater than that for Fe (III) (~36.8 mg/L).

- Under non-growth conditions, *S. alga* preferentially reduced Fe (III). Subsequent U (VI) reduction was dependent on the initial Fe (III) concentration. The lack of

U (VI) reduction at high initial Fe (III) levels was probably due to Eh poising caused by the Fe (II)/Fe (III) redox couple.

- Under growth conditions, reduction of U (VI) and Fe (III) occurred simultaneously. Individual metal reduction rates, in the presence of both metals, were slower than when one of them was absent. It appears that *S. alga* utilizes the same enzyme for U (VI) and Fe (III) reduction.

The growth of the facultative bacterium *S. alga* using uranium as the sole electron acceptor has significant implications regarding continuous uranium waste treatment. *S. alga* is the third microorganism reported to couple U (VI) reduction with cellular growth. Since the other two microorganisms are strict anaerobes, their application has been largely restricted. Comparatively, *S. alga* grows in an oxygen rich environment and can reduce uranium at a high rate and efficiency. Therefore, *S. alga* is the strongest candidate regarding continuous uranium waste treatment. In addition, since there is no difference in U (VI) removal efficiency using cells grown on oxygen and Fe (III), *S. alga* could grow in an oxygen rich environment to a late exponential phase and harvested followed by an exhaustive de-oxygenation before inoculation into the bioreactor.

Unlike *D. desulfuricans*, *S. alga* seems to utilize U (VI) and Fe (III) using the same enzyme system. The presence of Fe (III) outcompeted U (VI) under non-growth conditions. High initial Fe (III) levels could totally impede U (VI) reduction probably by

Fe (II)/Fe (III) Eh buffering of the solution. Co-reduction of U (VI) and Fe (III) was not observed in non-growth studies. However, under growth conditions, U (VI) and Fe (III) reduction occurred simultaneously but at slower rates. In summery, though *S. alga* does not have the sulfide co-precipitation problems that *D. desulfuricans* has, the presence of iron in typical uranium wastewater would possibly delay uranium reduction due to thermodynamic competition. Subsequent uranium reduction may never occur due to strong redox buffering. If growth conditions are used, then reduction of U (VI) and Fe (III) will be incomplete. However, for a high sulfate and low iron (less than 0.2 mM) containing uranium waste, *S. alga* is recommended over *D. desulfuricans*. The uranium removal efficiency can be expected as high as 80%.

REFERENCE

REFERENCE

Bailey, J.E. & Ollis, D.F., 1986, in *Biochemical Engineering Fundamentals*, McGraw-Hill Book Company, p119

Benefield L. D. & Randall C. W., 1980, in *Biological Process Design for Wastewater Treatment*, Prentice-Hall, Inc., Englewood Cliffs, NJ, p210

Blanch, H.W. & Clark, D.S., 1996, in *Biochemical Engineering*, Marcel Dekker, Inc., p196

Caccavo, F. Jr., Blackmore, R. P., Lovley, D. R., 1992, "A Hydrogen Oxidizing Fe (III) Reduction Microorganism From the Great Bay Estuary, New Hampshire", *Applied and Environmental Microbiology*, 58(10): 3211-3216

Caccavo, F., Jr., Gorby, Y. A., Mickinerney, M. J., 1995, "Characterization of a Metal Reductase Complex in the Dissimilatory Metal-reducing Bacterium *Shewanella alga* strain BrY", Poster presented at the 95th ASM General Meeting, Washington, D.C., (May)

Chu, L. L., 1995, "Co-bioprecipitation of Uranium and Selected Heavy Metals in Sulfate Containing Solution", M.S. Thesis, The University of Tennessee, Knoxville

Crichton, R. R., 1991, In *Inorganic Biochemistry of Iron Metabolism*, Ellis Horwood, p59-76

Elliott, H. A. and Peters, R.W., 1991, "Decontamination of Metal-polluted Soils Using Chelating Agents", *Removal of Heavy Metals from Groundwaters*, CRC press, Inc.

Forstner U., 1993, "Land Contamination by Metals: Global Scope and Magnitude of Problem", *Metal Speciation and Contamination of Soil*, Lewis Publisher

Gaad, G. M., 1990, In *Microbial Mineral Recovery*, Ehrlich, H. L., Brierly, C. L., Eds., McGraw Hill Publishing Co., New York, Chapter 11

Ganesh, P., 1995, "Impact of Organic Ligands on the Bioreductive Precipitation of Hexavalent Uranium", Ph.D. Dissertation, The University of Tennessee, Knoxville

Gessa, C., 1983, In *Inorganic Chemistry Acet.* 80, L53

Gorby, Y. A., Lovley, D. R., 1992, "Enzymatic Uranium Precipitation", *Environmental Science and Technology*, 26:205-207

Gorby, Y.A., Nancy V., 1997, Personal Communications

Groot, A.J.D., 1995, "Metals and Sediments: A Global Perspective", *Metal Contaminated Aquatic Sediments*, Ann Aebor Press, Inc., p1-13

Harrison, P.M., 1990, "Ferritin", In *Iron Transport and Storage*, CRC Press, p81-101

Hungate, R. E., 1969, "A Roll Tube Method for Cultivation of Strict Anaerobes", In *Methods in Microbiology*, 3B: 117-132

Kalman Burger and Laszle Nagy, 1990, "Metal Complexes of Carbonates and Sugar-type Ligands", In *Biocoordination Chemistry: Coordination Equilibrium in Biologically Active Systems,* Ellis Horwood, p 267

Katz, J. J. and Rabinowitch E., 1951, *The Chemistry of Uranium*, Dover Publications, Inc., New York, p68-100

Ku, Y. and R.W. Peters, 1986, "The Effect of Weak Chelating Agents on the Removal of Heavy Metals by Precipitation Processes", *Environmental Progress*, 5:147-153

Lawrence, A. W., 1971, "Application of Process Kinetics to Design if Anaerobic Processes", in *Anaerobic Biological Treatment Processes*, F. G. Pohland, Symposium Chairman, American Chemical Society, Cleveland, Ohio

Leigh, J. G., Moore, G. R. and Wilson, M. T., 1993, "Biological Iron", In *Chemistry of Iron*, Blackie Academic & Professional, p181-189

Lovley, D. R., Philips, E.J.P., 1986, "Organic Matter Mineralization With Reduction of Ferric Iron in Anaerobic Sediments", *Applied Environmental Microbiology*, April, 683-689

Lovley, D. R., Philips, E.J.P., 1986, "Availability of Ferric Ion for Microbial Reduction in Bottom Sediments of the Freshwater Tidal Potomac River", *Applied Environmental Microbiology*, October, 751-757

Lovley, D. R., 1987, "Anaerobic Production of Magnetite by a Dissmilatory Iron Reducing Microorganism", *Nature,* 330:252-254

Lovley, D. R., Philips, E.J.P., 1987, "Rapid Assay for Microbially Reducible Ferric Iron in Aquatic Sediments", *Applied Environmental Microbiology*, April, 1536-1540

Lovley, D. R., Philips, E.J.P., 1988, "Novel Mode of Microbial Energy Metabolism: Organic Carbon Oxidation Coupled to Dissimilatory Reduction of Iron and Manganese", *Applied and Environmental Microbiology*, 54(6): 1472-1480

Lovley, D. R., Phillips, E.J.P., Longergan, D. J., 1989, "Hydrogen and Formated Oxidation Coupled to Dissimilatory Reduction of Iron and Mangnese", *Applied and Environmental Microbiology*, 55: 770-776

Lovley, D. R., and Lonergan, D. J., 1991, "Anaerobic Oxidation of Toluene, Phenol, and p-Cresol by the Dissimilatory Iron-Reducing Organism, GS-15", *Applied Environmental Microbiology*, June, 1858-1864

Lovley, D. R., Phillips, E.J.P., Gorby, Y. A., Landa, E. R., 1991, "Biological Reduction of Uranium", *Nature*, 350(4): 413-416

Lovley, D. R., Phillips, E.J.P., 1992a, " Bioremediation of Uranium Contamination with Enzymatic Uranium Reduction", *Environmental Science and Technology*, 26(11): 2228-2234

Lovley, D. R., Phillips, E.J.P., 1992b, "Reduction of Uranium by *Desulfovibrio desulfuricans*", *Applied and Environmental Microbiology*, 58(3): 850-856

Lovley, D. R., 1993a,"Dissiilatory Metal Reduction", *Annual Reviews in Microbiology*, 47:263-290

Lovley, D. R., Widerman, P. K., Woodward, J. C., Phillips, E.J.P., 1993c, "Reduction of Uranium by Cytochrome C3 of *Desulfovibrio vulgaris", Applied and Environmental Microbiology,* 59(11): 3572-3576

Luther, G.W., 1995, "Trace Metal Chemistry in Porewaters", *Metal Contaminated Aquatic Sediments,* Ann Arbor Press, Inc., P66-73

Mandel, L., 1966, *Complex Compounds of Uranium*, Academy of Science of the U.S.S.R., Moskva, p12-30, p331

McCarty, P.L., 1971, in *Organic Compounds in Aquatic Environments*, S.D. Faust and J.V.Hunter, Eds., Dekker, New York, p495

McCready, R.G.C., Lakshmanan, V. I., 1986, In *Immobilization of Ions by Biosorption,* Eccles, H., Hunt, S., Eds, Ellis Harwood Limited, U.K., Chapter 5

Monod, J., 1949, "The Growth of Bacterial Cultures", *Annual Review of Microbiology*, 3: 371-394

Peters, R. W., Shem L., 1993, "Treatment of Soils Contamination with Heavy Metals", *Metal Speciation and Contamination of Soil*, Lewis Publisher

Silver, J., 1959, "Introduction to Iron Chemistry", *Chemistry of Iron*, Blackie Academic & Professional, p2-13

Shular, M.L. & Kargi Fikret, 1992, in *Bioprocess Engineering Basic Concepts*, Prentice Hall, New Jersey, p10-97, p148-209

Stucki, J.W., Bailey, G.W., Gan H., 1995, "Redox Reactions in Phyllosilicates and Their Effects on Metal Transport", *Metal Speciation and Contamination of Soil*, 121-171

Stumm W. and Morgan, J. J., 1981, In *Aquatic Chemistry*, John Wiley & Sons Publication, p418-490

Tsezos, M. and Volesky. B, 1982, "The mechanism of Uranium Biosorption by *Rhizopus arrihizus*", *Biotechnology and Bioengineering*, 24: 385-401

Tsezos, M., 1984, "Recovery of Uranium from Biological Adsorbents-Desorption Equilibrium", *Biotechnology and Bioengineering*, 26: 973-981

Treux, M. E., Gorbey, Y. A., 1992, "Uranium Biotransformation", Proceedings of Soil Decontamination 93: *Technology Targeting – Radionuclides and heavy metals, Conference-9306198*, Department of Energy, Gatingburg, TN

Tucker, M.D., 1995, "*Determination of Kinetic Coefficients for the simultaneous Reduction of Sulfate and Uranium by Desulfovibrio desulfuricans Bacteria*", DOE Primary Report Number: SAND—94-0686

Wenzel, W.W. and Winfried, E. H. Blum, 1993, "Assessment of Metal Mobility in Soil-Methodological Problems", *Metal Speciation and Contamination of Soils*, Lewis Publisher

Weast, R.C., 1969, In *Handbook of Chemistry and Physics*, 50th ed., West Palm Beach, FL, Pb252

Zehnder, A.J., 1988, In *Biology of Anaerobic Microorganisms*, p493-496

APPENDICES

APPENDIX I

Aerobic TSB medium for seed culture:

Tryptic Soy Broth 30g/L

pH adjusted to 6.8 using 1N NaOH
Shaking at 150 rpm at 35°C

Anaerobic ferric citrate medium for seed culture:

Constituents	Final Concentration
$NaHCO_3$	2.5 g/L
KCl	0.10 g/L
NH_4Cl	1.5 g/L
$NaH_2PO_4H_2O$	0.6 g/L
Lactate	20 ml/L[1]
Ferric Citrate	12.25 g/L
Peptone	5.00 g/L
Yeast Extract	2.00 g/L
Vitamins	5 ml/L[2]
Minerals	5 ml/L[2]

Anaerobic test solution for non-growth metal reduction:

Constituents	Concentration
$NaHCO_3$	2.5 g/L
Lactate	2 ml/L[1]

Anaerobic medium for growth-coupled metal reduction:

Constituents	Concentration
$NaHCO_3$	2.5 g/L
KCl	0.10 g/L
NH_4Cl	1.5 g/L
$NaH_2PO_4H_2O$	0.6 g/L
Lactate	ml/L[1]
Ferric Citrate or Uranium Nitrate	g/L g/L
Vitamins	5 ml/L[2]
Minerals	5 ml/L[2]

1- 60% w/w syrup, sigma chemical
2- Components see appendix II.

APPENDIX II

Components for vitamins and minerals mixture:

Compound	Concentration (mg/L)
Biotin	$2x10^{-3}$
Folic acid	$2x10^{-3}$
Pyridoxine HCl	$10 x10^{-3}$
Ribnflavin	$5 x10^{-3}$
Thiamin	$5 x10^{-3}$
Nicotinic acid	$5 x10^{-3}$
Pantothenic acid	$5 x10^{-3}$
B12	$0.1 x10^{-3}$
p-Aminobenzoic acid	$5 x10^{-3}$
Thioctic acid	$5 x10^{-3}$
NTA	1.5
$MgSO_4$	3.0
$MnSO_4$	0.5
NaCl	1.0
$FeSO_4$	0.1
$CaCl_2$	0.1
$CoCl_2$	0.1
$ZnCl_2$	0.13
$CuSO_4$	0.01
$AlK(SO4)_2$	0.01
H_3BO_2	0.01
Na_2MoO_4	0.025
$NiCl_26H_2O$	0.024
Na_2WO_4	0.025

APPENDIX III

ANALYTICAL RESULTS

From Figure 4

Results from Fe (III) reduction using 12 & 16 hr aerobically grown cells

	Fe (II) (mM)		Fe (III) (mM)	
time (hr)	12 hr grown	16 hr grown	12 hr grow	16 hr grown
0	0	0	0.51	0.5
1	0.09	0.060528	0.41	0.439472
3	0.236	0.15132	0.264	0.34868
5	0.33	0.332	0.17	0.168
7	0.42	0.454	0.08	0.046
21	0.47	0.482	0.03	0.018
24	0.49	0.49	0.01	0.01
48	0.49	0.491	0.01	0.009
60	0.493	0.492	0.007	0.008

From Figure 5

Uranium reduction using 12 & 16 hr grown cells

	U (VI) conc. (mM)		
time (hr)	12 hr cells	16 hr cells	Control
0	0.45	0.46	0.452
1	0.43	0.44	0.456
3	0.44	0.45	0.44
6	0.42	0.462	0.45
8	0.45	0.4	0.448
12	0.3	0.32	0.449
15	0.25	0.28	107.1
18	0.189	0.163	
22	0.09	0.07	
24	0.0785	0.05	
48	0.02	0.026	0.45

From Figure 6

Fe (III) reduction using 24 & 48 hr anaerobically grown cells

	Fe(II) (mM)		Fe (III) (mM)		
Time (hr)	24 hr grown	48 hr grown	24 hr grown	48 hr grown	Control
0	0	0	0.5	0.51	0.5
1	0.130135	0.091	0.369865	0.419	
3	0.257244	0.25	0.242756	0.26	
5	0.32	0.29	0.18	0.22	
7	0.45	0.41	0.05	0.1	
21	0.47	0.45	0.03	0.06	
24	0.48	0.478	0.02	0.032	0.48
48	0.49	0.49	0.01	0.02	
60	0.49	0.497	0.01	0.013	0.43

From Figure 7

U (VI) reduction using 24 & 48 hr anaerobically grown cells

	U (VI) conc. (mM)		
time (hr)	24hr grown	48 hr grown	Control
0	0.452	0.462	0.45
3	0.356	0.38	
6	0.25	0.28	
12	0.13	0.18	0.442
22	0.072	0.08	
24	0.07	0.075	
48	0.04	0.05	0.445

From Figure 8 A

Fe (III) reduction kinetics

Time (hr)	Fe(III) (0.05mM)	Fe(III) (0.1mM)	Fe(III) (0.2mM)	Fe(III) (0.5mM)	Control for 0.5mM
0	0.05	0.1	0.2	0.5	0.5
1	0.03789	0.05259	0.10769	0.37087	
3	0.0046	0.02787	0.0744	0.24578	
5	6.4E-05	0.02636	0.06533	0.168	
7	-0.00599	0.02131	0.05524	0.046	
21	0.00309	0.03796	0.04666	0.018	
24	-0.01608	0.02131	0.03405	0.01	0.46

From Figure 8 B

Goring method

(so-s)/ln(so/s)	t/ln(so/s)
4.13	1.55
3.16	2.34
3.09	3.75
3.008324	4.437
1.799	2.12
1.566	2.8
8.35	1.61
5.567	1.864
4.776	2.45
3.83	2.56
24.2	3.347
20.0464	4.22
17	4.58

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.461385
R Square	0.212876
Adjusted R Square	0.141319
Standard Error	0.988635
Observations	13

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	2.907688	2.907688	2.974925	0.112511
Residual	11	10.75139	0.977399		
Total	12	13.65908			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	2.392037	0.400047	5.979392	9.19E-05	1.511539	3.272534	1.511539	3.272534
X Variable	0.064976	0.037671	1.724797	0.112511	-0.01794	0.14789	-0.01794	0.14789

From Figure 9

Uranium reduction kinetics

time (hr)	1.7mM	1.3mM	0.84Mm	0.42mM	0.21mM	0.105mM	Control for 1.7mM
0	1.7058824	1.2731092	0.8576891	0.4159664	0.2155462	0.1092437	1.6890756
2	1.6911765	1.2002101	0.7798319	0.3693277	0.1663866	0.082563	
4	1.5189076	1.114916	0.6819328	0.305042	0.1365546	0.075042	
6	1.4714286	1.0544538	0.6105042	0.2042017	0.1114706	0.0638655	
8	1.4298319	0.9267227	0.5579832	0.1470588	0.0796639	0.0487395	
12	1.1764706	0.7563025	0.4789916	0.1344538	0.0672269	0.0386555	
24	0.8403361	0.6722689	0.4243697	0.092437	0.0588235	0.0357143	1.6386555
48	0.5042017	0.5882353	0.3571429	0.0756303	0.0546218	0.0306723	
60	0.4285714	0.5042017	0.2731092	0.0672269	0.0504202	0.0252101	1.6596639

From Figure 9B

Goring method

(so-s)/ln(So/S)	t/Ln(so/s)
93.34	16.82
85.12	12.8967
70.836	8.433
22.677	7.1423
21.676	10.652
20.12	11.178
45.197	7.726
41.187	8.763
37.563	9.0989
294.239	33.92
283.76	30.147
276.163	31.84
383.32	34.456
377.412	40.582

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.978904
R Square	0.958254
Adjusted R Square	0.954775
Standard Error	2.618602
Observations	14

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1888.794	1888.794	275.4517	1.22E-09
Residual	12	82.28492	6.857077		
Total	13	1971.078			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	6.319445	1.028701	6.14313	5E-05	4.078097	8.560792	4.078097	8.560792
X Variable	0.085346	0.005142	16.59674	1.22E-09	0.074142	0.096551	0.074142	0.096551

From Figure 10

Co-reduction of Fe (III) and U (VI) using cell suspensions

time (hr)	U(VI) in Fe (III)/U (VI)	Fe(III) in Fe(III)/U(VI)	Fe(III) alone	Time (hr)	U (VI) alone
0	0.45	1	1	0	0.45
1	0.445	0.8	0.9	4	0.3
3	0.451	0.55	0.6	7	0.28
6	0.42	0.12	0.2	10	0.15
8	0.43	0.1	0.12	14	0.05
12	0.44	0.11	0.09	16	0.045
15	0.451	0.1	0.08	40	0.02
18	0.443	0.12	0.09	52	0.012
22	0.441	0.09	0.07		
24	0.44	0.08	0.08		
48	0.442	0.05			
60	0.44	0.38			
65	0.453	0.2			

From Figure 11

Impact of Fe (III) on U (VI) reduction using non-growing cells

	U (VI) conc. (mM)				
T (HR)	in 0.05 mM Fe(III)	in 0.1 mM Fe(III)	in 0.2 mM Fe(III)	in 0.5 mM Fe(III)	control (No iron)
0	0.3865546	0.394958	0.394958	0.390756	0.386555
3	0.387395	0.391597	0.37563	0.386134	0.38416
6.5	0.387563	0.394571	0.373782	0.366029	0.371807
10.5	0.3863992	0.394958	0.370168	0.365546	0.327731
18	0.3823529	0.390756	0.370588	0.366387	0.168067
27.5	0.3396639	0.37479	0.372269	0.365966	0.063739
48.5	0.3249706	0.350714	0.371987	0.375777	0.047529
72	0.2581513	0.273067	0.370504	0.366807	0.058235
96	0.1885462	0.259139	0.377899	0.367697	0.048067
122	0.1542017	0.203782	0.369328	0.363542	0.043277
146	0.1415966	0.177731	0.371849	0.364286	
194	0.1360798	0.169752	0.368908	0.360134	
242	0.1328571	0.166517	0.331092	0.364706	

Fe (II) profiles

Time (hr)	0.05mM Fe(III)	0.1mM Fe(III)	0.2mM Fe(III)	0.5mM Fe(III)
27.5	0.056	0.096	0.093	0.201
48.5	0.073	0.09	0.085	0.372
72	0.071	0.124	0.114	0.392
96	0.074	0.125	0.173	0.48
122			0.179	0.482
146			0.185	0.485
194			0.186	0.483
242	0.074	0.1	0.19	

From Figure 12

Impact of Fe (II) on uranium reduction

T (HR)	0.05 mM Fe(II)	0.1 mM Fe(II)	0.2 mM Fe(II)	0.5 mM Fe(II)	Control (No iron)
0	0.3781513	0.352941	0.357143	0.336134	0.386555
3	0.3739496	0.346218	0.346639	0.327899	0.38416
6.5	0.3012605	0.326891	0.327731	0.354622	0.371807
10.5	0.3016807	0.323992	0.299046	0.299437	0.327731
18	0.2226891	0.268908	0.260504	0.285714	0.168067
27.5	0.1567227	0.220588	0.22437	0.232773	0.063739
48.5	0.0744958	0.134622	0.134622	0.165282	0.047529
72	0.0579874	0.10063	0.122815	0.154328	0.058235
96	0.066063	0.087941	0.062181	0.08916	0.048067
122	0.0499076	0.067815	0.084714	0.110924	0.043277
146		0.067227	0.1	0.117437	
194		0.063025	0.068592	0.067227	
242					

From Figure 13

Fe (II) impact on U (VI) reduction rate

Initial Fe(II) Conc(mM)	Reduction rate(mg/lhr)	Percent Inhibition (%)
0	3.0692	0
0.05	1.99	35.16226
0.1	1.1793	61.57631
0.2	1.197	60.99961
0.5	0.9364	69.49042

From Figure 14

Growth of *S. alga* on Ferric citrate

time (hr)	Fe (II) (Mm)	Fe (III) (mM)	Protein (mg/L)
0	0	9.99	35.55556
5	1.65	8.34	90
10	3.26	6.73	142.8889
24	6.56981	3.4201861	155.1111
36	7.0471	2.9428996	151.8889
48	7.38369	2.6063057	172.8889
72	7.01148	2.978518	144.4444
96	7.26615	2.7238464	97.66667
120	7.5137	2.4762985	141

From Figure 15

Growth of *S. alga* on different amount of iron

	Fe(III) 0.1 mM		Fe(III) 1.0 mM		Fe(III) 10 mM		
t(hr)	Fe(II) (mM)	protein (mg/L)	Fe(II) (mM)	protein (mg/L)	T (hr)	Fe(II) (mM)	protein (mg/L)
0	0	23.58667	0	23.58667	0	0	35.55556
3	0.061032	34.88889	0.484224	41.11111	5	1.65	90
6	0.065572	34.33333	0.519532	37.44444	10	3.26	142.8889
9.5	0.070616	33	0.616881	39.55556	24	6.569814	151.8889
24	0.077678	34.44444	0.673374	39.33333	36	7.0471	155.1111
35	0.10088	35.66667	0.78182	42.11111	48	7.383694	172.8889
47	0.112986	36.33333	0.80704	43.77778	72	7.011482	144.4444
144		36.23	0.95	42	96	7.266154	140
					120	7.513701	141

From Figure 16

Growth of *S. alga* on U (VI)

Time (hr)	U (VI) (mM)	Protein (mg/L)	control protein (mg/L)
0	4.201680672	40	35.55555556
5	3.361344538	41.380952	35.67724868
10	2.500159664	57.560847	37.01587302
24	2.412911765	66.873016	37.24867725
36	1.841176471	86.719577	45.45502646
48	1.803445378	101.15344	44.64021164
72	1.430168067	87.301587	47.08465608
96	1.294117647	94.777778	
120	1.197478992	122.22222	

Form Figure 17

Growth of *S. alga* on different amounts of uranium

	U:0.84mM		U:2.1mM		U:4.2mM		U:8.4mM	
t(hr)	U (VI) (Mm)	Protein (mg/L)	U (VI) (Mm)	Protein (mg/L)	U (VI) (Mm)	Protein (mg/L)	U (VI) (Mm)	Protein (mg/L)
0	0.840336	23.58667	2.10084	23.58667	4.201681	10.80667	8.403361	23.58667
3	0.508403	40.11111	1.235294	45.44444	3.781513	45.55556	7.773109	43.22222
6	0.352941	34.33333	1.088235	45.22222	3.697479	62.66667	6.386555	44
9.5	0.12605	34.77778	1.016807	46.66667	3.571429	71.88889	6.638655	43.22222
24	0.113445	36.66667	0.865546	47.77778	3.151261	86	7.016807	62.11111
35	0.109244	38.55556	0.92437	54.44444	2.915966	105.5556	6.890756	110.4444
47	0.105042	44.88889	0.857143	75.55556	1.680672	166.6667	6.218487	211.7778
144	0.084034	36.66667	0.945378	86	1.092437	128.5556	4.117647	216
432	0.042017	33.33333	0.647059	32.22222	0.491597	100	2.012605	98.22222

From Figure 18

Co-reduction of U (VI) and Fe (III) using growth coupled *S. alga*

Time (hr)	U (VI)	Fe (III)	Protein in Fe/U	control protein
0	4.075630252	10	35.55556	35.555556
5	4.071428571	9.62	53	35.677249
10	4.073109244	9.24	60	37.015873
24	4.07194958	8.4684088	75	37.248677
36	3.781512605	8.06592088	86.77778	45.455026
48	3.687789916	8.22442276	91.44444	44.640212
72	3.659079832	8.01071236	92.22222	47.084656

From Figure 19

Impact of uranium on growth coupled Fe (III) reduction

	U (VI) Conc. (mM)				Protein Conc. (mg/L)			
time (hr)	U: 0mM	U: 0.084mM	U: 0.42mM	U: 0.84mM	U: 0mM	U: 0.084mM	U: 0.42mM	U: 0.84mM
0	0	0	0	0	16.72222	16.772	16.772	16.772
4	4.4681304	2.4200724	1.293146	1.3871388	121.3333	110.77777	104.44444	107
8	6.9624078	2.854419	1.4841	2.0619096	122.1111	110	105.55555	108.88888
11	7.0059414	5.5297566	1.639436	2.4151254	124.4444	110.7777	106.22222	110.77777
23	6.6986338	7.237461	1.775973	2.37456	125.7778	111.33333	71.555555	75.555555
33.5	7.4978711	7.8647406	2.019365	2.453712	137.2222	122.22222	72.222222	75.444444
49	7.6391574	8.0754828	2.043111	2.4992244	143.3333	113.33333	74.111111	75.555555
80	7.0684715	7.5233976	3.006787	2.5734294	137.3333	117.77777	75.555555	76
120	6.3163296	7.12368	2.987988	3.077034	122.2222	125.55555		74.444444

From Figure 20

Impact of uranium on growth coupled iron reduction

Initial U(VI) Conc.(mM)	Initial Iron Reduction Rate (mM Fe(III) hr)	Percent Inhibition (%)
0	0.6491	0
0.084034	0.4541	30.03
0.420168	0.1408	78.31
0.840336	0.2166	66.63
4.201681	0.063	90.29

From Figure 21

Impact of uranium on iron reduction using cell suspensions

	Fe (II) Conc. (mM)							
time (hr)	Fe0.05 MM/U	Fe0.1 mM/U	Fe0.2 mM/U	Fe0.5 MM/U	Fe0.05 mM	Fe0.1 mM	Fe0.2 mM	Fe0.5 mM
0	0	0	0	0	0	0	0	0
1	0.03884	0.09634	0.14829	0.25674	0.01211	0.04741	0.09231	0.15636
3	0.06154	0.08423	0.15132	0.32231	0.0454	0.07213	0.1256	0.26229
5	0.07415	0.08423	0.15233	0.32786	0.04994	0.07364	0.13467	0.26531
7	0.06204	0.0913	0.15334	0.34501	0.05599	0.07869	0.14476	0.30264
21	0.04842	0.07364	0.16847	0.34955	0.04691	0.06204	0.15334	0.36317
24	0.07112	0.09785	0.19873	0.38788	0.06608	0.07869	0.16595	0.36821

From Figure 22

Impact of iron on growth coupled uranium reduction

	U(VI) Conc.(mM)				Protein Conc.(mg/L)			
time (hr)	Fe(III): 0mM	Fe(III): 0.1mM	Fe(III): 0.2mM	Fe(III): 1.0mM	Fe(III): 0mM	Fe(III): 0.1mM	Fe(III): 0.2mM	Fe(III): 1.0mM
0	4.142857	4.201681	4.201681	4.201681	6.7	6.7	6.7	6.7
23	3.361345	2.941176		3.441176	47.33333	49	43.22222	48.44444
53	3.403361	2.336134	3.067227	3.105042	52.22222	57.77778	47.44444	46.66667
72	3.382353	1.932773	2.894958	3.113445	54.55556	60	39.44444	39.88889
100	3.403361	1.802521	2.903361	2.941176	60	65.55556	36.33333	54.77778
144	3.302521	1.537815	2.857143	3.109244	62.22222	59.55556	39	43.77778

From Figure 23

Growth of *S. alga* on low iron concentrations

	Fe(III) 0.1 mM		Fe(III) 0.2 mM		Fe(III)1.0 mM	
t(hr)	Fe(II) (mM)	protein (mg/L)	Fe(II) (mM)	Protein (mg/L)	Fe(II) (mM)	protein (mg/L)
0	0	23.58667	0	23.58667	0	23.58667
3	0.061032	34.88889	0.123074	35	0.484224	41.11111
6	0.065572	34.33333	0.12	32.22222	0.519532	37.44444
9.5	0.070616	33	0.13064	33.77778	0.616881	39.55556
24	0.077678	34.44444	0.140223	34.33333	0.673374	39.33333
35	0.10088	35.66667	0.13417	34.44444	0.78182	42.11111
47	0.112986	36.33333	0.15132	38.33333	0.80704	43.77778
144		36.23	0.198	39	0.95	42

VITA

Xiaoyan Zhang was born in NanTong, China in 1973. She obtained her Bachelors of Science degree in Environmental Engineering from East China University of Science and Technology in 1995. After graduation, she worked as Environmental, Health and Safety Coordinator in Carrier Corporation (China). In August 1996 she attended the University of Tennessee, Knoxville pursuing her Master of Science degree in Environmental Engineering. She received her degree August 1998.