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IMPACT OF ORGANIC LIGANDS ON THE BIOREDUCTIVE PRECIPITATION OF
HEXAVALENT URANIUM

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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DEDICATION

This dissertation is dedicated to

my parents

Mr. V. Rajagopalan and Mrs. Bagavathi Rajagopalan

brother

Mr. R. Lakshmi Narayanan

and uncle

Mr. C. R. Subramanian

whose love and sacrifice laid the foundation for my higher education.

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ABSTRACT

Microbial reduction of soluble hexavalent uranium (U^{6+}) to the less soluble tetravalent (U^{4+}) state has recently been proposed as a treatment option for selective uranium removal from complex waste streams. This research focuses on the impact of various organic ligands present in uranium containing waste streams on the microbial reduction and subsequent precipitation of dissolved uranium. A sulfate reducing bacteria (*Desulfovibrio desulfuricans*) and an iron reducing bacteria (*Shewanella alga* (BrY)) were used to study reductive precipitation of uranium.

Five organic ligands (acetate, malonate, oxalate, citrate and tiron) were chosen to evaluate their impact on bioreductive precipitation. These ligands varied significantly in their nature as well as affinity of association with hexavalent uranium. Batch anaerobic experiments, under non-growth conditions, were performed to evaluate U^{6+} reductive precipitation in the presence of each ligand. A bacterial concentration of $\sim 5 \times 10^8$ cells/mL was used. In all the experiments, an initial U^{6+} concentration of 100 mg/L was maintained. The test solutions contained H_2 gas as sole electron donor, U^{6+} as the primary electron acceptor and each ligand as the sole organic complexing agent. All experiments were conducted at constant ionic strength (0.17 M).

U^{6+} reduction experiments were performed using two concentrations of each target ligand. At the higher concentration of ligands used, $\sim 100\%$ of the U^{6+} introduced, initially complexed to the ligands. At the lower ligand concentration, $\sim 50\%$ of the U^{6+} complexed to the ligands. Microbial reduction of U^{6+} was evaluated by measuring the

decrease in U^{6+} concentration, over time, after cell incubation. In addition, the fate of uranium was determined by monitoring the concentration of bio-reduced uranium (U^{4+}) remaining in solution and the fraction removed via precipitation.

Experimental results indicated that, *D. desulfuricans* reduced U^{6+} rapidly from organic/inorganic monodentate complexes. In organic free and acetate containing test solutions, the bacteria reduced U^{6+} at an initial rate of $\sim 1.6 \times 10^{-3}$ mg/mg cell-hr. However, U^{6+} reduction in multidentate malonate, oxalate and citrate solutions was nearly twenty fold slower ($\sim 6 \times 10^{-5}$ mg/mg cell-hr). Results indicated that, (i) multidentate ligands non-competitively inhibited U^{6+} reducing enzymes, or (ii) hexavalent uranium chelated to multidentate ligands was not directly available for reduction for *D. desulfuricans*. However, > 90 % U^{6+} reduction was achieved after 96 hours of incubation in all organic solutions.

An exception to the above trend was observed in ternuclear tiron containing solutions. *D. desulfuricans* reduced U^{6+} at a rate ($\sim 3.2 \times 10^{-3}$ mg/mg cell-hr) much faster than that observed in binuclear citrate solutions. Differences in the structure (aromatic vs aliphatic) and functional group involved in complexation (hydroxyl vs carboxyl) appeared to have enhanced the U^{6+} reduction rate in tiron solutions.

Hexavalent uranium reduction in test solutions incubated with *Shewanella alga* (BrY) followed a different trend. BrY rapidly reduced hexavalent uranium in carbonate solutions. However insignificant U^{6+} reduction was observed in organic free ($NaNO_3$) solutions where U^{6+} formed uranyl hydroxide species. In acetate containing solutions,

U^{6+} reduction was slow ($\sim 4 \times 10^{-5}$ mg/mg cell-hr) as well as incomplete. In malonate, oxalate and citrate solutions, when $\sim 100\%$ of the U^{6+} was initially complexed to the ligands, reduction was very rapid ($\sim 2.4 \times 10^{-3}$ mg/mg cell-hr). However, in solutions containing $\sim 50\%$ uranyl hydroxide species and $\sim 50\%$ multidentate aliphatic complexes, reduction was slow ($\sim 4 \times 10^{-5}$ mg/mg cell-hr) and incomplete. It appeared that in solutions containing multidentate carboxyl U^{6+} complexes as well as uranyl hydroxyl species, BrY reduced U^{6+} essentially from the carboxyl complexes. Subsequent reduction appeared to continue through equilibrium redistribution of U^{6+} from hydroxyl to carboxyl species.

Once U^{6+} was bio-reduced to U^{4+} , subsequent precipitation appeared to be impacted by the type and concentration of individual ligand introduced into the test solution. In acetate and malonate solutions, almost all the bio-reduced uranium precipitated from the test solutions. However, in oxalate and citrate solutions, an increase in the ligand concentration decreased the amount of U^{4+} precipitated. In tiron containing solutions less than 10% of reduced uranium precipitated at the lowest concentration of ligand used.

In most cases the trends in uranium precipitation observed agreed with the prediction made using the thermodynamic constants for the association of tetravalent uranium with each ligand. This indicated that, while trends in bio-reduction of hexavalent uranium depended on the metabolism of microorganism used, subsequent precipitation of bio-reduced uranium was dictated by its thermodynamic constants for the association with test ligands.

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1. INTRODUCTION

Uranium is naturally radioactive and a known carcinogen and chemotoxin (Cothorn and Lappenbusch, 1983; Guglielmott, 1984). Contamination of ground and surface waters by uranium is a matter of great concern. Uranium containing waste streams are produced wherever uranium is processed or used (Straub, 1964). Operations such as crushing and grinding of ores, acid/alkali leaching, polishing and other end uses of uranium produce significant volumes of uranium contaminated wastewater. In addition to industrial wastewaters, uranium may be found in leachate from radioactive solid wastes burial trenches (Francis, 1980a,b; Schippers, 1995). Environmental restoration of uranium affected soils, using organic and inorganic extraction fluids, produces waste streams containing uranium and extractants (Francis, 1993). Moreover, some groundwaters naturally contain significant amounts of uranium (Bradford, 1990).

Industrial and environmental restoration waste streams generally contain several hundred mg/L of uranium (Straub, 1964, Francis, 1993). The uranium level in drinking water is currently unregulated, although a maximum contaminant level (MCL) of 20 $\mu\text{g/L}$ has been proposed (United States Environmental Protection Agency, 1991). The discharge limit of uranium in waste streams is site specific. It may vary from 1 $\mu\text{g/L}$ to several hundred $\mu\text{g/L}$ (Ally, 1995). Several techniques are currently used to remove uranium from contaminated waste streams. Chemical precipitation using coagulants and ion exchange are the two primary treatment operations used in many Department of

Energy (DOE) and other nuclear materials processing facilities (Straub, 1964; Burns, 1966). Although these techniques efficiently remove uranium from waste streams, certain inherent limitations exist in the treatment or disposal of secondary wastes produced. For example, chemical precipitation removes uranium from waste streams in a non-specific manner. In addition to uranium, this process removes several other Resource Conservancy and Recovery Act (RCRA) regulated heavy metals, producing large volumes of secondary mixed-wastes. Mixed-wastes are defined as wastes containing both a hazardous waste subjected to RCRA and a source, special nuclear, or by-product material subject to the Atomic Energy Act of 1954 (United States Code, 1994). To date, there is no disposal facility in the United States that will accept mixed-wastes (Code of Federal Regulation, 1994). Therefore, the mixed sludges produced at DOE facilities are indefinitely stored at treatment sites (Hale, 1988). Ion exchange techniques to remove uranium involve high operation and maintenance costs (McCready and Lakshmanan, 1986). In addition, the extent of uranium removal is affected by the limited number of binding sites available on the resins. Exchange resins, once saturated with uranium, are either regenerated using concentrated acids or disposed of as solid wastes (Straub, 1964). Regeneration of resins using acids produces highly corrosive uranium containing secondary waste streams. Disposal of resin as solid waste generates large volumes of radioactive solids (Lovely, 1992a).

Horikoshi (1979) and Tsezos (1982) proposed that sorption of uranium to live and dead microorganisms as a possible option to economically remove uranium from waste

streams. Uranium binds to biomass through several physico-chemical interactions during biosorption. However, microbial metabolism is not involved. This technique, though initially promising, did not effectively remove uranium from waste streams containing carbonate and heavy metals such as iron, nickel and zinc (Tsezos, 1984). In addition, limitations in available binding sites and the requirement of mechanically operated clarifiers in the treatment process may render this technique uneconomical (McCready and Lakshmanan, 1986).

High treatment costs and production of undesirable secondary mixed-wastes associated with various physico-chemical processes generated interest in developing alternate treatment approaches. A technique was sought to selectively remove uranium from waste streams to minimize mixed waste production. If uranium can be selectively removed from solution, the remaining waste stream can be economically treated as a RCRA waste using conventional treatment methods. In addition, after isolation into a smaller volume, uranium can either be disposed of at a lower cost or be recycled with minimal processing. A recent discovery that some microorganisms can enzymatically remove dissolved uranium provided such an option for selective uranium removal (Lovely, 1991, 1992a). Uranium in waste streams is generally found in the hexavalent oxidation (UO_2^{2+}) state (Truex, 1992) which is highly soluble (Allison, 1991). Selected microorganisms utilize hexavalent uranium as an electron sink during metabolic processes thereby altering its oxidation state to the tetravalent form which is sparingly soluble (Weast, 1974). Precipitation of tetravalent uranium as uraninite ($\text{UO}_2(\text{s})$) has been

reported (Lovely, 1991). Microbial enzymes generally act specifically on target compounds. Hence, this technique can selectively remove uranium from waste streams.

Although enzymatic precipitation appears to be a promising mechanism for selective removal of uranium from process waste streams, attempts to develop this technique are still in the preliminary stages. Uranium precipitation using iron and sulfate reducing bacteria has been demonstrated in synthetic bicarbonate solutions and in ground waters amended with bicarbonate (Gorby, 1992; Lovely, 1992a). Hexavalent uranium formed uranyl carbonate $((\text{UO}_2)_3(\text{CO}_3)_2)$ species in such solutions (Cochran, 1986). However, composition of process waste streams and leachate solutions can be much more complex than those previously evaluated. For example, various simple and complex organic compounds are used in ore extraction and process unit decontamination operations in uranium industries (Macaskie, 1991). In addition, uranium in subsurface environments readily associates with humic components (Slazy, 1958). Various organic chelators such as citrate, tiron, cyclohexanediaminetetraacetic acid (CDTA) and diethylenetriaminepentaacetic acid (DTPA) are widely used in soil decontamination operations (Francis, 1993). These organic compounds can complex with uranium and alter its speciation. Bioavailability of a compound during metabolism is often impacted by its speciation (Madsen and Alexander, 1986). For example, microorganisms develop transport systems to diffuse substrates to the proximity of target enzymes (Van Der Rest, 1991). Enzymes, then, accommodate substrates in active sites and catalyze metabolism. Transport systems as well as active sites are often specific to certain species of the target

compounds. It is not currently known, if hexavalent uranium (U^{6+}) complexed to various organic compounds can serve as an electron acceptor to enzymes of iron and sulfate reducing microorganisms. In addition to bioavailability, organic complexation can impact metabolic activity (toxicity) of microorganisms. Rothstein et al. (1948a) reported that uranyl ion (UO_2^{2+}), when not complexed to strong complexants, was toxic to yeast cells. Information related to possible toxic effects in uranium precipitation is not currently available.

In addition to their influence on microbial activity, organic compounds can also impact precipitation of bio-reduced uranium (U^{4+}). Tetravalent uranium has been reported to form soluble complexes with several multidentate organic compounds (Perrin, 1979; Macaskie, 1991). Waste stream components can chelate bio-reduced uranium and prevent its removal via precipitation.

Microbial reduction as well as subsequent precipitation of uranium is essential for removal of dissolved uranium using this mechanism. Therefore, it is imperative to evaluate the impact of various organic compounds on bio-reduction as well as precipitation, prior to developing this process to treat uranium containing waste streams and leachate solutions. The purpose of this research was to evaluate the influence of organic complexation on bioavailability of hexavalent uranium (U^{6+}) and precipitation of tetravalent uranium (U^{4+}) from organic containing waste streams. A sulfate reducing bacterium, *Desulfovibrio desulfuricans* and an iron reducing bacterium, *Shewanella alga* (BrY), were used to evaluate uranium bioavailability. The specific goals were to:

- (i) Evaluate the relationship between enzymatic reduction of hexavalent uranium and the nature of hexavalent uranium-organic binding
- (ii) Study the source of available hexavalent uranium (complexed or uncomplexed) for reduction in the presence of organic compounds and
- (iii) Evaluate the impact of organic ligands on the precipitation of bio-reduced uranium.

2. LITERATURE REVIEW

2.1 Chemical properties of uranium

2.1.1 Introduction

Uranium, a naturally occurring radioactive element was first isolated from pitchblende by Klaproth in the year 1789 (De Ment and Dake, 1945). It exists in three isotopic forms (U_{234} (0.006%), U_{235} (0.72%) and U_{238} (99.72%)) in the environment. The two principle ores of uranium are pitchblende and carnonite (Voegtlin, 1949). Pitchblende, a bluish-black material consists of a mixture of uranyl oxides that closely corresponds to the formula U_3O_8 . Carnonite is a canary yellow material containing potassium, uranium and vanadium corresponding to the formula $K_2O \cdot 2UO_3 \cdot V_2O_5 \cdot 3H_2O$. Although four valance forms of uranium have been identified (+3, +4, +5, +6), it is predominantly found in the tetra(4+) and hexavalent (6+) states. Process waste streams and contaminated soils generally contain uranium in the hexavalent form which is very soluble.

2.1.2 Inorganic chemistry of uranium

Uranium chemistry is very complex. Hexavalent as well as tetravalent uranium form a variety of complexes with organic and inorganic anions in the environment. Selected inorganic species of uranium and their free energy of formation are shown in Table I (Langmuir, 1986). The type of uranium species formed in the environment

Table I. Selected uranium species and their free energy of formation at 25° C and 1 atmosphere total pressure (Langmuir, 1978).

Mineral or Aqueous Species	ΔG_f° (Kcal/mol)
U^{4+}	-126.9
$UO_2(s)$ uraninite	-246.6
U_4O_9	-1022.4
U_3O_8	-805.4
UCl^{3+}	-160.1
$UHPO_4^{2+}$	-403.6
UO_2^{2+}	-227.7
UO_2OH^+	-276.5
$(UO_2)_2(OH)_2^{2+}$	-561.1
$(UO_2)_3(OH)_5^+$	-945.2
$UO_2CO_3^0$	-367.6
$UO_2(CO_3)_2^{-2}$	-503.2
$UO_2(CO_3)_3^{4-}$	-635.4
$UO_2CO_3(s)$ rutherfordine	-373.6

depends on its free energy of formation, concentration of various anions and cations present, pH and electron potential. Uranium speciation in simple surface water systems as well as in groundwaters containing significant amounts of carbonate are briefly discussed in this section. In surface waters and other aerobic environments, uranium predominantly remains in the hexavalent oxidation state forming various oxide and hydroxyl complexes (Truex, 1992). At a $\text{pH} < 3$, hexavalent uranium is associated with two oxygen molecules to form uranyl ion (UO_2^{2+}) (De Ment and Dake, 1945). At pH between 3 and 6 uranyl ion tends to hydrolyze and form several soluble polymeric species. Langmuir (1978) reported that UO_2OH^+ , $(\text{UO}_2)_2(\text{OH})_2^{2+}$ and $(\text{UO}_2)_3(\text{OH})_5^+$ are the three major uranyl hydroxide species in the environment. Above pH 6.5 uranium begins to precipitate from solution as smectide ($\text{UO}_2(\text{OH})_2$ (s)). The extent of smectide precipitation varies with the total uranium level and the solution pH (Allison, 1991). Under aerobic conditions, tetravalent uranium is generally unstable and tends to oxidize to the hexavalent state. However, when the electron potential is altered ($\text{Eh} < 0.2$ volts) inducing anaerobic conditions, uranyl ion reduces to the less soluble tetravalent state and precipitates as uraninite (UO_2 (s), $K_{\text{sp}} = 10^{-60}$) (Langmuir, 1978). In general, under acidic and neutral pH levels, uraninite precipitates at $\text{Eh} < 0.2$ volts. However, under alkaline conditions, a much lower Eh level is required to facilitate tetravalent uranium formation.

Unlike surface waters, groundwaters contain a significant concentration of carbonate (Cochran, 1986). Hexavalent uranium forms several soluble complexes with carbonate (Langmuir, 1978). In groundwaters, polymeric UO_2CO_3^0 , $\text{UO}_2(\text{CO}_3)_2^{2-}$ and

$(\text{UO}_2)_2(\text{CO}_3)^{4-}$ complexes predominate at pH above 4. A fraction of uranyl ion tends to precipitate as rutherfordine (UO_2CO_3 (s)) between pH 4 and 6. However, at pH > 6.5, uranium forms very soluble $(\text{UO}_2)_2(\text{CO}_3)_3^{4-}$ species and remains in solution. Although tetravalent uranium is reported to complex with carbonate under anaerobic conditions, information related to the strength of the complex is not currently available (Voegtlin, 1949). It has been reported that under anaerobic conditions, the preferred reaction of U^{4+} is to precipitate as uraninite rather than complex with carbonate (De Ment and Dake, 1945; Voegtlin, 1949).

Figure 1 shows the distribution of uranium species at various pH and Eh conditions (Langmuir, 1978). A total uranium concentration of 10^{-6} M and a CO_2 level of 10^{-2} atmosphere were used in determining speciation. At pH < 5, under aerobic conditions (Eh > 0.2 volts), uranium essentially remains as uranyl ions (UO_2^{2+}). When Eh was decreased to < 0.2 volts, hexavalent uranium reduced to the tetravalent form and precipitated as uraninite. Although minerals such as U_4O_9 (s) and amorphous UO_2 were formed, uraninite was the predominant solid under these conditions. At pH > 5, under aerobic conditions, uranium formed various uranyl carbonate species. Uranyl ion did not hydrolyze appreciably. In addition, alkaline conditions required stricter anaerobic conditions (lower Eh) for the formation of insoluble tetravalent uraninite. At pH > 11, uranyl ion did not reduce to tetravalent state at pE levels as low as -0.6 volts. It is to be noted that the speciation shown in Figure 1 is the distribution under equilibrium

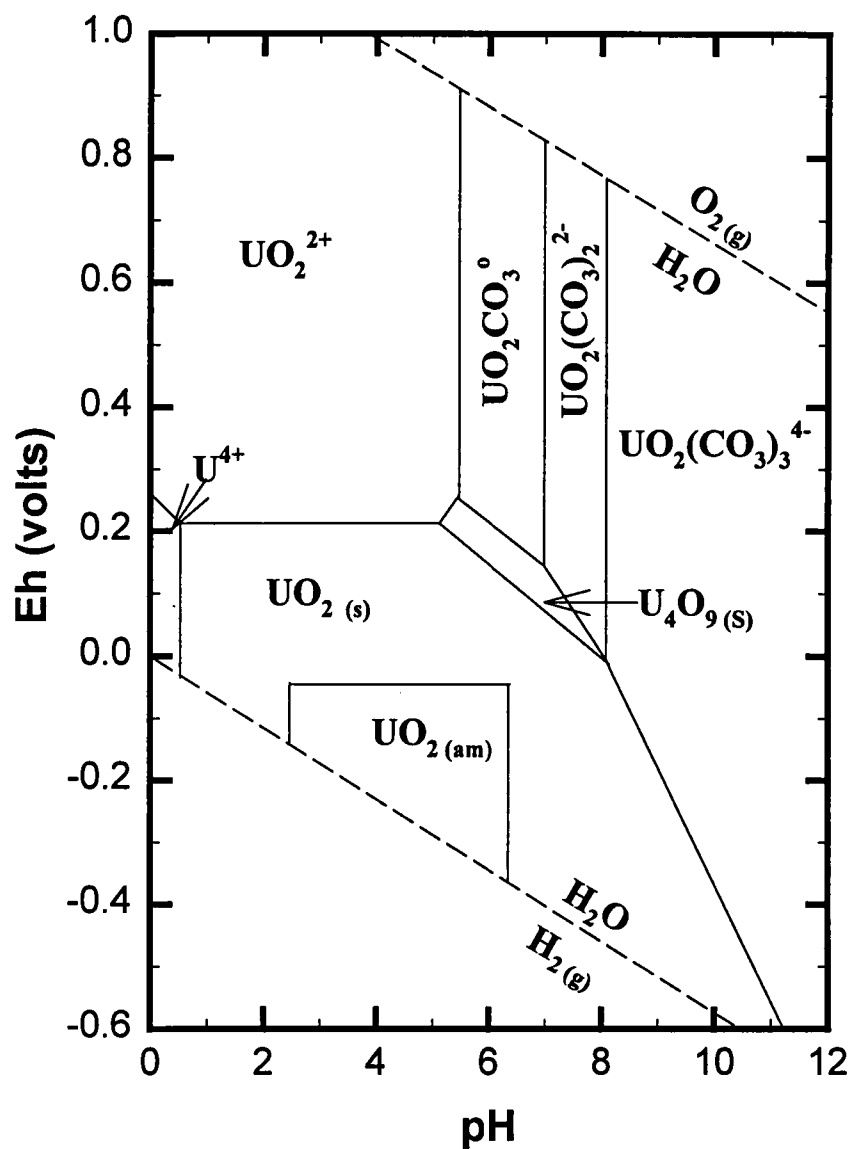


Figure 1. Eh - pH diagram in the U-O₂-CO₂-H₂O system at 25° for $P_{\text{CO}_2}=10^{-2}$ atm. Total uranium = 10^{-6} M. (Langmuir, 1978)

conditions. However, the rate at which the distribution is achieved depends on the chemical kinetics of species formation (Sposito, 1981).

In addition to carbonate, inorganic ligands such as phosphate, sulfate, nitrate, fluoride and chloride complex with uranyl ion in the environment. However, carbonate forms stronger complexes with uranyl ion than other inorganic ligands evaluated (Voegtlin, 1949).

2.1.3 Organic chemistry of uranium

Complexes formed between metals and organic ligands can be characterized by the type and affinity of binding. The type of binding formed is defined by the number of ligand functional groups and their orientation in the complex. For example, a complex involving the interaction of one ligand functional group with a metal is referred to as a monodentate complex. Complexes involving two and three functional group interactions are referred to as bi and tridentate complexes, respectively. In general, if more than one functional group is involved in complexation, the complexes are called chelates.

The affinity of a ligand for a metal can be represented by a stability (formation) constant. A 1:1 ligand-metal (M-L) complex can be represented by the equation



where,

m^+ = charge of the metal

l^- = charge of the ligand

The formation constant of the ML complex is expressed as

$$K = \frac{ML^{(m-l)}}{[M^{m+}] \times [L^{-l}]} \quad (2)$$

When more than one molecule of the ligand complexes with the metal the following expression holds



where,

q = moles of metal ion involved in the reaction

r = moles of ligand involved in the reaction

and the overall formation constant is expressed as

$$\beta = \frac{[M_qL_r^{(qm-rl)}]}{[M^{m+}]^q [L^{-l}]^r} \quad (4)$$

The constants K and β represent the strength of the complex formed (Evans, 1989).

The type of bonding and formation constants for uranyl ion/organic ligand complexes formed varied widely. Table II lists selected UO_2^{2+} -Organic complexes and their nature and affinity of complexation reported in literature. Ramamurthy and Santappa (1970) reported that cis dicarboxylic maleic acid formed a bidentate complex with uranyl ion while trans dicarboxylic fumaric acid formed a monodentate complex. It appears that orientation of electrophilic groups play a major role in the nature of the complex formed. Multidentate organics such as citrate, malate and tartarate formed binuclear complexes with uranyl ion (Nunes, 1982, 1986, 1987; Markovites, 1972; Rajan and Martell, 1965). In a binuclear complex, two metal cations form the nucleus of the complex surrounded by

Table II. Selected UO_2 -Organic complexes and their nature and affinity of complexation

Ligand	Complex	Nature of Complex	Log β	Reference
Acetate	$\text{UO}_2\text{CH}_3\text{COO}^+$	Monodentate	2.61	Ahrland, 1951 I = 0.1 M, T = 25° C
	$\text{UO}_2(\text{CH}_3\text{COO})_2$		4.9	
	$\text{UO}_2(\text{CH}_3\text{COO})_3^{-1}$		6.3	
Formate	$\text{UO}_2(\text{HCOO})^{+1}$	Monodentate	1.86	Magon, 1972 I = 1.0 M, T = 20° C
	$\text{UO}_2(\text{HCOO})_2$		3.05	
	$\text{UO}_2(\text{HCOO})_3^{-1}$		3.52	
Benzoate	$\text{UO}_2(\text{C}_6\text{H}_5\text{COO})^{+1}$	Monodentate	4.01	Ramamurthy and Santappa, 1968 I = 0.1 M, T = 30° C
Phenyl Acetate	$\text{UO}_2(\text{C}_7\text{H}_7\text{OCOO})^{+1}$	Monodentate	4.12	Ramamurthy and Santappa, 1968 I = 0.1 M, T = 30° C
Maleic Acid	$\text{UO}_2(\text{C}_4\text{H}_4\text{O}_4)$	Bidentate	1.95	Ramamurthy and Santappa, 1968 I = 0.1 M, T = 30° C
	$\text{UO}_2(\text{C}_4\text{H}_4\text{O}_4)_2$		6.16	
Oxalic Acid	$\text{UO}_2(\text{C}_2\text{H}_2\text{O}_4)$	Bidentate	5.99	Havel, 1969
	$\text{UO}_2(\text{C}_2\text{H}_2\text{O}_4)_2$	Bidentate	10.64	I = 1.0 M, T = 25° C
	$\text{UO}_2(\text{C}_2\text{H}_2\text{O}_4)_3$	Bidentate	11.0	
Citric Acid	$\text{UO}_2\text{C}_6\text{H}_7\text{O}_7$	Bidentate	7.4	Rajan and Martell, 1965 I = 0.1 M, T = 25° C
	$2(\text{UO}_2(\text{C}_6\text{H}_8\text{O}_7))$	Binuclear	18.87	
Humic Acid	N/A	N/A	6.7	Li, 1980, I = 0.01M, T = N/A

N/A - Not Available, I - Ionic Strength, T = Temperature.

the ligands involved in chelation. Uranium complexation with aromatic, phenolic ligands are generally bi/multinuclear in nature involving one or more external hydroxyl groups (Bartusek and Sommer, 1965). Uranium in sediments was found to associate with plant debris and humus containing substances (Slazy, 1958). Both hexavalent and tetravalent uranium complexed with humic acid components in the environment (Vine, 1958). Li et al. (1980) studied binding of UO_2^{2+} and U^{4+} to humic, fulvic and tannic acid components. They reported that both these ions readily complexed to the organic ligands at $\text{pH} > 3$. Affinity of hexavalent uranium to the ligands followed the order: fulvic acid ($K=2.7 \times 10^7$) $>$ humic acid ($K=5.3 \times 10^6$) $>$ tannic acid ($K=2.3 \times 10^6$). For U^{4+} , the order was: humic acid ($K=9.5 \times 10^6$) $>$ tannic acid ($K=8.5 \times 10^6$) $>$ fulvic acid ($K=4.4 \times 10^6$).

Note that hexavalent and tetravalent uranium readily form a variety of complexes with organic and inorganic ligands in the environment. Therefore, any attempts to isolate uranium from contaminated streams should consider possible influences of these ligands in separation procedures.

2.2 Sources of uranium contamination in wastewaters

Dissolved uranium is found in the environment wherever uranium is processed or used (Straub, 1964). Processes such as uranium mining, ore concentration, polishing and final use of refined uranium generate waste streams containing significant amounts of uranium (Straub, 1964). In addition, uranium is found in trench leachates from

radioactive material burial sites (Francis, 1980a,b). Remediation of uranium contaminated soils using organic and inorganic extraction liquids result in uranium containing waste streams (Francis, 1993). The composition of these waste streams depends on the source of their generation. The nature of treatment required to decontaminate these streams, in turn, is significantly influenced by the composition. In the following sections, various sources and composition of uranium waste streams are briefly discussed.

2.2.1 Composition of process wastewaters

Uranium ore extraction processes generally include crushing and grinding followed by leaching of ore using sulfuric acid or sodium carbonate (Straub, 1964). The leachate is separated using ion exchange resins or solvent extraction procedures. Uranium from the extract is then stripped using an aqueous solution and precipitated with alkaline reagents. These processes, termed as mining and milling of ores, generate uranium waste stream containing sulfate (acid leachate), carbonate (alkaline leachate), metals and some organics.

Uranium precipitated in milling processes is further purified by solvent extraction and a series of chemical processes, producing waste streams containing large quantities of nitrate, metals, hydrocarbon and fluorocarbon oils and organic acids. Table III shows the chemical composition of three process waste streams obtained from the Y-12 West End Treatment Facility, Oak Ridge, Tennessee (1992). This treatment facility receives waste

Table III. Chemical composition of three process waste streams obtained from the Y-12 West End Treatment Facility

Contaminant	Waste Stream #1	Waste Stream #2	Waste stream #3
Calcium	62 mg/L	7900 mg/L	126 mg/L
Copper	0.6 mg/L	9.1 mg/L	16.1 mg/L
Iron	1.8 mg/L	6.1 mg/L	1643 mg/L
Nickel	2.0 mg/L	0.1 mg/L	236 mg/L
Phosphorus	26 mg/L	20 mg/L	1074 mg/L
Silicon	6.8 mg/L	4.3 mg/L	25 mg/L
Sodium	3100 mg/L	4300 mg/L	1640 mg/L
Nitrate	645 mg/L	21000 mg/L	81400 mg/L
Sulfate	3350 mg/L	860 mg/L	9820 mg/L
Zinc	0.14 mg/L	0.01 mg/L	7.0 mg/L
TOC	3770 mg/L	5180 mg/L	2590 mg/L
U ⁶⁺	62 mg/L	121 mg/L	25 mg/L
pH	7.0	7.0	3.2

waters from various operations involved in uranium processing at the location. In general these waste streams contain substantial concentrations of anions such as sulfate and nitrate, metals such as chromium, nickel, and iron and organic acids such as citrate.

2.2.2 Composition of trench leachates

Francis (1980 a,b) collected leachates from four low level radioactive waste disposal sites containing various radionuclides and analyzed the wastes for various organic compounds. The major compounds detected were benzoic acid, 2-ethyl hexanoic acid, hexanoic acid, methyl butyric acid, pentanoic acid and naphthalene. These compounds were reported to be derived from synthetic and natural organic wastes such as contaminated cellulosic materials, scintillation liquids, solvent and decontamination fluids buried in trenches and their biological decomposition products. Trench leachates are often pumped to waste treatment facilities prior to final discharge.

2.2.3 Composition leachates from uranium contaminated soils

Various organic and inorganic compounds are being used to extract uranium from contaminated soils during environmental restoration treatment processes. Process waste streams generally contain a significant amount of the complexant used, a small fraction of soil organic matter, uranium and other metals initially sorbed to soil. Francis et al., (1992) conducted extensive studies on the use of various extractants to decontaminate soils containing uranium. They reported that carbonate, which forms stable complexes

with uranium, is very effective in soil decontamination. Nearly 60 to 80 % of the uranium was extracted from soils using carbonate leaching. The extract contained very low concentrations of secondary soil constituents such as iron, aluminum, calcium and silica. Organic complexants such as citrate, tiron, CDTA and DTPA removed 25 to 80% uranium from soil. The waste produced contained trace aluminum, calcium and silica in addition to complexants and uranium.

2.3 Removal of uranium from waste streams

Various techniques used to remove uranium from waste streams can be classified as (i) physicochemical processes of non-biological origin, (ii) physicochemical processes of biological origin (sorption) and (iii) enzyme mediated metal precipitation (active/metabolic immobilization).

2.3.1 Current treatment processes for uranium removal

Chemical precipitation and ion exchange techniques are the most widely used physicochemical treatments for uranium removal (Burns, 1966). Chemical precipitation involves flocculation of colloids (added as coagulants) which adsorb, entrap or otherwise remove suspended matter from solution (Straub, 1964). Alum, ferrous sulfate, phosphate and lime-soda are some of the commonly used coagulants in the removal of radionuclides from waste streams. Although precipitation removes 70 to > 95% of metals in solution, a

major shortcoming of this technique is its non-specific nature. Coagulation, in addition to removing the target metal (uranium), removes other RCRA regulated metals producing a large volume of secondary wastes. For example, approximately 10% of the total volume of waste was collected as sludge from radioactive waste using calcium-iron-phosphate coagulants at Harwell, UK (Burns, 1966). The large volume of mixed sludge produced results in high cost of treatment/disposal of the solids.

Various synthetic resins have been used to remove radionuclides from drinking water and waste waters using ion exchange techniques (McCready and Lakshmanan, 1986, Zang, 1994, Clifford, 1994). In general, resins such as IR-400 that contained hydroxyl functional groups, and IR-120, a mixed bed of lithium form resin were reported to remove uranium efficiently from waste streams. However, several limitations existed in the use of this technique in waste treatment. Installation and operation costs of ion exchange processes are generally very high. In addition, other cations such as ferric iron and nickel successfully compete with uranium for exchange sites (McCready and Lakshmanan, 1986). Cothorn (1990) reported that attempts to regenerate uranium exchanged resins were incomplete and unsuccessful. Approximately 5 to 85 % of the bound uranium was removed. In addition to the above concerns, production of a large volume of wastes, upon disposal of exchange resins, and generation of highly corrosive uranium containing regenerants limited the use of ion-exchange techniques to remove uranium from waste streams (Lovely, 1992a, McCready and Lakshmanan, 1986).

2.3.2 Biosorption of uranium to microbial cells

Cell walls of microorganisms are chemically very active due to the presence of compounds such as proteins, carbohydrates and polysaccharides (Geesy and Jang, 1990). The mechanism of sorption can be ion exchange, electrical attraction, Van Der Waals attraction, hydrolysis, precipitation, transport across membrane and/or complexation (Tsezos and Volsky, 1981, Gaad, 1990).

Various fungal and bacterial cells have been found to biosorb uranium. Tsezos (1982) reported that uranium sorbed to the cell wall of *Rhizopus arrhizus* through interactions with chitin functional groups. However, sorption mechanisms varied from species to species and with the nature of metal immobilized. Friis and Myers-Keith (1986) studied sorption of uranium to *S. longwoodensis* and found that sorption was predominantly due to complexation with surface and cytoplasmic phosphate groups of the cells. Biosorption does not involve microbial metabolic activity and it occurs with living as well as dead cells. Removal of aqueous metals using such non metabolic processes is called passive metal immobilization (Brierly, 1990).

Although biosorption effectively removed dissolved uranium, there are some limitations involved with this mechanism. Horikoshi (1979) studied sorption of uranium to *S. elongatus* and found that uranium uptake was strongly affected by the presence of carbonate. Uranium formed stable complexes with carbonate in solution which did not sorb to the cells. In addition, the presence of counter ions such as Fe^{2+} , CO_3^{2-} , Cu^{2+} , Ni^{2+} and Zn^{2+} greatly reduced biosorption of uranium to various microorganisms (Tsezos,

1984, McCready, 1986). Since, biosorption is a physicochemical phenomenon, the amount of uranium sorbed is severely limited by the number of binding sites available on the cell wall. Also, McCready (1986) reported that the requirement of mechanical clarifiers and centrifuges in the treatment process to remove uranium via sorption may prove this technique uneconomical.

2.3.3 Enzymatic precipitation of uranium

The limitations in various physicochemical processes generated interest in finding an alternate technology for dissolved uranium removal. Several researchers investigated the possibility of uranium immobilization using direct/indirect microbial metabolic activities. Kauffman et al. (1986) proposed that sulfide produced by the metabolism of various *desulfovibrio* species can precipitate uranium from mine waters. Unlike biosorption, precipitation using microbial metabolism is generally not limited by availability of binding sites. However, Lovely (1991) demonstrated that sulfide was a poor precipitant of uranium. In addition, several metals in waste streams formed insoluble salts with sulfide. Hence, use of this technique to treat process waste streams may produce a large volume of mixed-waste. Macaskie (1992) reported that a *citrobactor sp.* can accumulate uranyl phosphate through enzymatic action. *Citrobactor* liberated inorganic phosphate during metabolism which complexed with uranium and precipitated as uranyl phosphate. The amount of uranium precipitated was approximately 900 % of the cellular dry weight. Although this mechanism was effective in uranium

removal from synthetic waste streams, ligands such as carbonate, citrate and other multidentate organics generally found in process waste streams may preferentially complex with uranium and prevent its precipitation via phosphate interaction.

Francis et al., (1991, 1994) isolated a *Clostridium* species that enzymatically immobilized uranium. This bacterium transported soluble hexavalent uranium inside the cell wall and reduced it to tetravalent and trivalent forms. Reduced uranium precipitated intracellularly. The mechanism of uranium transport and the enzymes responsible for reductive precipitation have not been evaluated. Hence, the impact of chemical speciation on uranium bioreductive precipitation is not currently known. In addition, intracellular uranium precipitation may limit the amount of uranium bio-reduced by the cells.

Recently, researchers have found that some iron and sulfate reducing bacteria, under anaerobic conditions, can reduce soluble hexavalent uranium (U^{6+}) to insoluble tetravalent uranium (U^{4+}) via direct enzymatic action. Hexavalent uranium was utilized as an electron sink by these microorganisms during metabolism. Bio-reduced uranium precipitated from solution as uraninite (Lovely, 1991, Lovely and Phillips 1992a). Uranium immobilization using direct enzymatic action appeared to be advantageous over the other techniques previously discussed due to the following reasons: (i) microbial enzymes generally act on specific compounds, (hence, the sludge produced may be limited to the uranium solids and other compounds acted upon by the enzymes), (ii) unlike biosorption, enzymatic precipitation is not limited by binding sites, (Lovely and

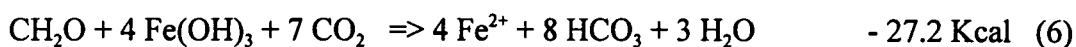
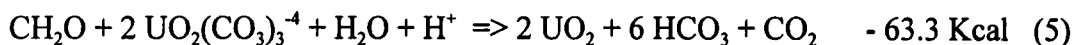
Phillips (1992) reported that the amount of uranium enzymatically precipitated was at least 20 fold larger than the uranium immobilized via other techniques), (iii) these microorganisms can precipitate uranium from carbonate complexes. Iron reducing microorganisms (*Geobacter metalireducens*, *Shewanella putrefaciens*, *Shewanella alga*) and sulfate reducing bacteria (*Desulfovibrio desulfuricans*, *Desulfovibrio vulgaris*) have been reported to enzymatically reduce uranium (Lovely, 1993a). Mechanisms of uranium reduction, metabolic diversity and issues related to removal of uranium from complex waste streams using these microorganisms are discussed in the following section.

2.4 Microorganisms involved in enzymatic uranium reduction

2.4.1 *Geobacter Metalireducens* (GS-15)

Lovely and coworkers (1987, 1993b) isolated this iron reducing bacteria from bottom sediments of the Potomac River, Va. GS-15 is a rod, non-motile, non-spore forming gram negative bacteria, 2 to 4 μm long and 0.5 μm thick. It is a strict anaerobe with little tolerance for oxygen. It was the first organism reported to grow by completely oxidizing an organic substrate (acetate) while utilizing ferric iron as the terminal electron acceptor. Upon evaluating the ability of this microorganisms to utilize various electron acceptors, Lovely (1991) reported that GS-15 can grow by reducing soluble hexavalent uranium to insoluble tetravalent uranium. It appeared that this mechanism, if properly developed, can be used as a potential treatment technique to remove dissolved uranium

from contaminated waste streams. Based on the following thermodynamic calculations of Cochran (1986),



Lovely suggested that microorganisms can obtain more energy from organic oxidation coupled to U^{6+} reduction than that obtained from ferric iron reduction.

GS-15 was reported to reduce uranium using a membrane bound c-type cytochrome (Lovely, 1993a). Uranium reduction appeared to be linked to energy yielding phosphorylation reactions. GS-15 was metabolically versatile in that, in addition to acetate, it utilized aliphatic organic compounds such as ethanol, propionate, butyrate, isobutyrate, valerate, pyruvate, propanol, butanol and aromatic compounds such as toluene, benzoate, benzaldehyde, benzyl alcohol, hydroxo benzene, phenol and p-cresol as electron donors. In addition, this bacteria utilized Mn^{4+} , Cr^{6+} , colloidal gold, silver and nitrate as electron acceptors during metabolism. However, GS-15 did not utilize glucose, malate, lactate, formate or hydrogen as a carbon/electron source. The bacterium did not utilize oxygen, sulfate, elemental sulfur, selenate or thiosulfate as electron acceptors.

The high metabolic diversity of GS-15 and its ability to grow using U^{6+} as an alternative electron acceptor suggested that this bacterium may be used to treat uranium from groundwaters and process waste streams. However, its extreme sensitivity to

oxygen and slow growth rate are major concerns in utilizing this bacteria for treating complex waste streams (Truex, 1992).

The potential of GS-15 to reduce U^{6+} in a synthetic bicarbonate solution has been demonstrated. In addition, the bacteria removed uranium from a groundwater sample amended with bicarbonate (Gorby, 1992). However, the ability of this microorganism to precipitate uranium from complex waste streams containing various organic chelators, metals and competing electron acceptors has yet to be demonstrated.

2.4.2 *Shewanella putrefaciens*

S. putrefaciens is a manganese reducing bacterial strain isolated from the anaerobic sediments of Oneida Lake, New York (Myers and Nealson, 1988). This bacterium grew by coupling U^{6+} reduction to oxidation of lactate, formate or H_2 . Lactate was incompletely oxidized to acetate during uranium reduction. It appeared to follow the following stoichiometry while using H_2 gas as electron donor,



The enzymes involved in uranium reduction have not been isolated. However, a c type cytochrome has been identified in the process (Lovely, 1989). In addition, *S. putrefaciens* reduced uranyl ion faster than *Geobacter metalireducens* (Lovely and Phillips, 1992a; Peyton, 1995). Unlike *Geobacter metalireducens*, this bacterium did not utilize a variety of aliphatic (acetate, succinate, oxalate) and aromatic (benzoate, phenol, p-cresol) hydrocarbons as the carbon/electron source. However, *S. putrefaciens* was

more versatile in utilizing electron acceptors in the environment. In particular, this bacterium grew facultatively by utilizing oxygen as a terminal electron acceptor. In addition, *S. putrefaciens* has utilized nitrate, nitrite, ferric iron, sulfite and thiosulfate and fumarate as electron acceptors. Sulfate, however, did not serve as an electron acceptor.

2.4.3 *Shewanella alga* (BrY)

Caccavo et al., (1992) isolated a dissimilatory iron reducing bacteria from the sediments of Great Bay Estuary, New Hampshire. This isolate was a facultative, gram negative rod type bacteria. Currently classified as *Shewanella alga* (formerly BrY), this bacteria was found to reduce U^{6+} when lactate or H_2 was utilized as the electron donor. In addition to Fe^{3+} and U^{6+} , this bacterium used O_2 , Mn^{4+} , thiosulfate and fumarate as electron acceptors. Nitrate was poorly reduced by BrY. In addition to lactate, BrY used citrate, fumarate and maleate as carbon sources. However, energy for growth was obtained only from H_2 and lactate oxidation. The mechanism of enzymatic reduction has not been established. Caccavo (1995) fractionated the cells into soluble and membrane portions and tested each fraction for uranyl ion reducing ability. A c type cytochrome found in both fractions of the bacteria was believed responsible for metal reduction (Caccavo, 1995). In addition, electron transport appeared to proceed through quinones. Unlike GS-15 and *S. putrefaciens*, growth of BrY using U^{6+} as the electron acceptor has not yet been established. Peyton et al., (1995) reported that, under non-growth conditions, BrY

reduced uranium from carbonate complexes more efficiently than other bacteria reported to reduce hexavalent uranium.

The fact that BrY can grow under anaerobic conditions, rapidly utilize U^{6+} and oxidize various complex organic compounds as carbon/ energy sources projected this bacterium as a strong candidate for the treatment of uranium in environmental waste streams. In addition, since BrY cannot reduce sulfate, additional metal precipitation as sulfide salts can be prevented by using this bacterium. However, the ability of BrY to reduce uranium has so far been demonstrate only in carbonate solutions. The possible influence of various organic/inorganic compounds on uranium reduction has not been evaluated.

2.4.4 *Desulfovibrio desulfuricans*

Until early 1992, only two iron reducing microorganisms (GS-15 and *S. putrefaciens*) were reported to reduce U^{6+} . However, deposits of uranium have been found in the environment where iron reducing microorganisms were not dominant. In particular, copious amounts of uraninite precipitates were found in sulfidogenic subsurface environments (Klinkhammer, 1991). This suggested that U^{6+} can be precipitated by mechanisms other than enzymatic precipitation mediated by iron reducers. Non-enzymatic precipitation mediated by sulfide was initially identified as the mechanism resulting in uranium deposits in these environments (Kauffman, 1981). However, studies performed by Lovely and coworkers (1991) to evaluate abiotic uranium

precipitation using sulfide salts indicated that sulfide was a poor reductant of uranium and abiotic processes may not account for the reduced uranium found. In addition, a hard acid does not strongly associate with soft base and vice versa. According to classification by Pearson (1966), uranium is a hard acid and sulfide a soft base. This suggested that uranium precipitation as a sulfide salt may not be the major mechanism of removal in sulfidogenic environments. Lovely and Phillips (1992a) observed that a sulfate reducing bacteria, *Desulfovibrio desulfuricans*, precipitated uranium in an anaerobic bicarbonate solution containing no sulfate or any other reductant. This bacterium used lactate as the sole electron donor and uranium as sole electron acceptor. These observations suggested that uranium deposits found in sulfidogenic zones were not solely due to non-enzymatic precipitation caused by sulfide but were also due to enzymatic precipitation caused by some sulfate reducing bacteria.

Lovely and Phillips (1992a,b) evaluated uranium reduction using *D. desulfuricans* in the presence of various electron donors, acceptors and trace metals. The bacterium was able to utilize lactate or H_2 gas as an electron donor during uranium reduction, under non-growth conditions. In addition, co-presence of sulfate did not alter the rate or extent of uranium reduction. Neither was sulfate reduction affected by the presence of uranium. It appeared that *D. desulfuricans* reduced sulfate and uranium using different enzyme mechanisms. In addition, the rate of U^{6+} reduction was faster than that of sulfate reduction. Table IV shows the electron transfer reactions for sulfate and UO_2^{2+} reduction using various electron donors. The free energy for the reactions for UO_2^{2+} reduction,

Table IV. Electron transfer reactions for UO_2^{2+} and SO_4^{-2} reduction

Reaction	ΔG° (KJ/mol)	E° volt
<u>1) $\text{H}_2/\text{UO}_2^{2+}$</u> $1/2 \text{H}_2 \rightleftharpoons \text{H}^+ + \text{e}^-$ $1/2 \text{UO}_2^{2+} + \text{e}^- \rightleftharpoons \text{UO}_{2(s)}$ $1/2 \text{H}_2 + 1/2 \text{UO}_2^{2+} \rightleftharpoons \text{UO}_{2(s)} + \text{H}^+$ 	-39.5	0.41
<u>2) $\text{H}_2/\text{SO}_4^{-2}$</u> $1/2 \text{H}_2 \rightleftharpoons \text{H}^+ + \text{e}^-$ $1/8 \text{SO}_4^{-2} + \text{H}^+ + \text{e}^- \rightleftharpoons 1/4 \text{S}^{-2} + \text{H}_2\text{O}$ $1/2 \text{H}_2 + 1/8 \text{SO}_4^{-2} \rightleftharpoons 1/8 \text{S}^{-2} + 1/2 \text{H}_2\text{O}$ 	-14.8	0.153
<u>3) $\text{CH}_3\text{COO}^-/\text{UO}_2^{2+}$</u> $1/8 \text{CH}_3\text{COO}^- + 1/2 \text{H}_2\text{O} \rightleftharpoons 1/4 \text{HCO}_3^- + 9/8 \text{H}^+ + \text{e}^-$ $1/2 \text{UO}_2^{2+} + \text{e}^- \rightleftharpoons \text{UO}_{2(s)}$ $1/8 \text{CH}_3\text{COO}^- + 1/2 \text{H}_2\text{O} + 1/2 \text{UO}_2^{2+} \rightleftharpoons 1/4 \text{HCO}_3^- + 9/8 \text{H}^+ + 1/2 \text{UO}_{2(s)}$ 	-21.4	0.22
<u>4) $\text{CH}_3\text{COO}^-/\text{SO}_4^{-2}$</u> $1/4 \text{CH}_3\text{COO}^- + 1/2 \text{H}_2\text{O} \rightleftharpoons 1/2 \text{HCO}_3^- + 5/4 \text{H}^+ + \text{e}^-$ $1/4 \text{SO}_4^{-2} + \text{H}^+ + \text{e}^- \rightleftharpoons 1/4 \text{S}^{-2} + 1/2 \text{H}_2\text{O}$ $1/4 \text{CH}_3\text{COO}^- + 1/4 \text{SO}_4^{-2} \rightleftharpoons 1/2 \text{HCO}_3^- + 1/4 \text{H}^+ + 1/2 \text{S}^{-2}$ 	6.55	-0.067
<u>5) $\text{CH}_2\text{O}/\text{UO}_2^{2+}$</u> $1/2 \text{CH}_2\text{O} + 1/2 \text{H}_2\text{O} \rightleftharpoons 1/2 \text{CO}_2 + \text{H}^+ + \text{e}^-$ $\text{UO}_2^{2+} + \text{e}^- \rightleftharpoons \text{UO}_{2(s)}$ $1/2 \text{CH}_2\text{O} + 1/2 \text{H}_2\text{O} + \text{UO}_2^{2+} \rightleftharpoons 1/2 \text{CO}_2 + \text{H}^+ + \text{UO}_{2(s)}$ 	-137	0.526
<u>6) $\text{CH}_2\text{O}/\text{SO}_4^{-2}$</u> $1/4 \text{CH}_2\text{O} + 1/4 \text{H}_2\text{O} \rightleftharpoons 1/4 \text{CO}_2 + \text{H}^+ + \text{e}^-$ $1/8 \text{SO}_4^{-2} + 9/8 \text{H}^+ + \text{e}^- \rightleftharpoons 1/8 \text{HS}^- + 1/2 \text{H}_2\text{O}$ $1/4 \text{CH}_2\text{O} + 1/8 \text{SO}_4^{-2} \rightleftharpoons 1/4 \text{CO}_2 + 1/8 \text{H}^+ + 1/4 \text{H}_2\text{O} + 1/8 \text{HS}^-$ 	-50.7	1.47

released on an unit electron transfer basis, was more than that released during sulfate reduction. This suggested that uranyl ion reduction was favored over sulfate reduction in the environment. Among several heavy metals tested, only copper was found to be toxic to *desulfuricans*. Metals such as Mo, Va, Ni, Co and Zn did not impact uranium reductive precipitation.

However, attempts to grow *D. desulfuricans* using uranium as the sole electron acceptor were not successful (Lovely and Phillips, 1992a,b). This suggested that the bacterium reduced uranyl ion through a pathway that did not yield energy sufficient to support growth. Enzymes responsible for uranium reduction by *D. desulfuricans* have not been isolated. However, cytochrome c_3 in the soluble fraction of the periplasmic region was found to be responsible for U^{6+} reduction for the sulfate reducing bacteria *D. vulgaris* (Lovely, 1993c).

Genus *Desulfovibrio*, a common sulfate reducer, is versatile in its ability to use various electron donors and acceptors and adapting to different environment. These bacteria can use H_2 , formate, lactate and pyruvate as electron acceptors during anaerobic respiration. In addition, they can metabolize aromatic hydrocarbons such as phenol, toluene and hydrocarbons from crude oil constituents (Mort, 1994, Hanzen, 1994 and Voorduow, 1995). Fermentative growth using cholin has also been reported (Voorduow, 1995).

Initially *Desulfuricans* were considered to be strict anaerobes, having little tolerance to oxygen. However, later studies indicated that several species of this bacteria

contained enzymes *superoxide dismutase* and *catalase* which detoxify free oxygen radicals. Postgate (1979) and Dilly and Cypionka (1991) reported that under an environment containing low oxygen concentrations, *Desulfovibrio* can survive for 3 to 20 hours metabolizing lactate, pyruvate and malate. In addition, sulfate reducing bacteria can enzymatically reduce several metals such as Fe^{3+} , Cr^{6+} and arsenate (Lovely, 1993a).

Due to the adaptability of *Desulfuricans* to survive under aerobic and anaerobic conditions and their ability to oxidize various fatty acids and aromatic hydrocarbons, *D. desulfuricans* was considered as a candidate for treating uranium from complex waste streams. Although *Desulfovibrio* species can utilize carboxylic acids such as lactate, pyruvate and succinate (Hansen, 1994) as carbon sources, information related to the oxidation or possible toxicity of multidentate carboxylic acids such as citrate, nitrilotriacetic acid (NTA) and ethylenediaminetetraacetic acid (EDTA), which are used in current uranium decontamination processes, is not available. In addition, possible co-production of metal sulfide salts in waste streams containing metals and sulfate may increase the amount of solids produced during enzymatic uranium precipitation.

2.4.5 *Desulfovibrio vulgaris*

In addition to *D. desulfuricans*, the sulfate reducing bacterium *Desulfovibrio vulgaris* reduced uranium in anaerobic bicarbonate solutions. The rate and extent of reduction was similar to that observed with *D. desulfuricans*. Lovely et al. (1993) investigated mechanisms of U^{6+} reduction by *D. vulgaris* and reported that cytochrome c_3

located in the soluble fraction of the periplasmic region catalyzed reductive precipitation of uranium.

Adaptation to oxygen containing environments and metabolic diversity of *D. vulgaris* are similar to that of *D. desulfuricans*. The ability of this bacteria to reductively precipitate uranium from complex waste streams is not currently known.

2.5 Importance of chemical speciation on bioavailability

Microorganisms in the environment utilize various organic and inorganic compounds for cell synthesis, as electron donors and electron acceptors, during metabolism. However, bioavailability of a compound is often influenced by its chemical speciation (Madsen and Alexander, 1985). A substrate that is readily bioavailable under certain conditions may not be available upon alteration of its chemical state. To date, reductive precipitation of uranium has been demonstrated only in synthetic bicarbonate solutions and in groundwaters amended with bicarbonate. The chemical composition of process waste streams and leachate solutions containing uranyl ion can be more complex than those previously evaluated. Many compounds in these waters can complex with uranyl ion and alter its speciation. The influence of various compounds present in a waste stream on bioreductive uranium precipitation must be evaluated in order to provide optimum conditions for effective uranium removal. In this section, limitations in

bioavailability of certain metals and organics due to changes in their speciation are discussed.

2.5.1 Bioavailability of organic compounds when complexed to metals

Brynhidsen and Rosswall (1989) evaluated degradation of citrate complexed to various metals in solution using a *Klebsiella sp.* The bacteria readily utilized citrate from Mg^{2+} complexes, however, the ligand (citrate) was not degraded when complexed to Cd^{2+} , Cu^{2+} or Zn^{2+} . *B. subtilis* (Wellecke, 1973) was found to degrade citrate readily from bivalent Ca^{2+} , Mg^{2+} , Mn^{2+} and Ba^{2+} complexes. However, citrate from a monodentate potassium (K^+) complex was not degraded. Limited citrate bioavailability was not due to metal toxicity to the bacteria. Citrate degradation primarily occurs intracellularly. Transport of substrate across the cell membrane to the proximity of the target enzymes is an essential step for intracellular metabolism. Such systems may or may not be sensitive to substrate speciation. *B. subtilis* utilized citrate using a transport system that required the ligand to be complexed at adjacent carboxyl groups by a bivalent cation (Bergsura, 1983). This resulted in rapid degradation of citrate from Ca^{2+} , Mg^{2+} , Mn^{2+} and Ba^{2+} complexes but not from the potassium (K^+) complex. Recently, Francis (1992) conducted systematic studies to evaluate citrate biodegradation from several metal complexes using *P. fluorescens*. The bacterium readily degraded citrate from monodentate and bidentate metal complexes (H^+ , Ca^{2+} , Ni^{2+} , Fe^{3+}). The bacterial cells did not degrade citrate from binuclear (UO_2^{2+}) complexes. However, when the membranes of the cells were

fractionated, the membrane free fraction of the cells readily degraded citrate from the UO_2^{2+} complex. This indicated that citrate degradation using *P. fluorescens* may be affected by speciation of the ligand.

In addition to membrane transport, structural compatibility of the substrate to the enzymes also appeared to impact ligand degradation. For example, cell free extracts of *P. fluorescens* degraded citrate from UO_2^{2+} complex. However, the extracts did not degrade citrate from tridentate Cu or Cd complexes (Joshi-Tope and Francis, 1995). The latter two cations were not toxic to the bacteria. The authors suggested that the hydroxyl group of citrate which participated in Cd^{2+} and Cu^{2+} complexation sterically hindered the enzymes involved in citrate degradation.

2.5.2 Bioavailability of metals complexed to organic ligands

Several researchers have studied the bioavailability of metals when complexed to organic ligands (Cox, 1980, Huyer and Page, 1989). Although the chemical speciation of metals has been reported to alter bioavailability of several metals, no systematic study has been performed to evaluate the governing mechanisms involved. For example, Huyer and Page (1989) reported that cell free extracts from *A. vinelandii* contained *ferrireductase* enzymes which reduced ferric iron complexed to various organic compounds at different rates. The enzyme reduced ferric iron faster from citrate complexes than from azotochelin complexes. Reduction from an azotobactor complex was the slowest. The mechanism responsible for the differences has not been identified.

Similarly, an enzyme of *P. aeruginosa* (*Ferripyochelin iron reductase*) reduced iron from a pyochelin- Fe^{3+} complex but not from a citrate complex or from organic free solution (FeCl_3). The reasons for the differences have not been evaluated. A *Pseudomonas sp.* (K62) reduced inorganic mercury (Hg^{2+}) to volatile elemental mercury (Hg^0) using the enzyme *mercury reductase*. However, it required an additional enzyme (*organomercurial lyase*) to reduce the metal from organic complexes. The latter enzyme cleaved the carbon-mercury bond and liberated Hg^{2+} from the complex. Once liberated it was reduced by *mercury reductase*. A review of the literature indicated that little, if any, work has been performed to evaluate the factors influencing the bioavailability of metals from organic complexes.

In summary, chemical speciation affects bioavailability primarily in two ways: (i) limitations in transport of electron donors/acceptors across the cell membrane, and (ii) limitations in the structural compatibility of the substrate with the target enzymes. It is not clear if either one or both of these factors could affect uranyl ion bioreduction by iron or sulfate reducing bacteria. Uranyl reduction studies, to date, have been conducted in bicarbonate solutions. Uranium forms a $(\text{UO}_2)_3(\text{CO}_3)_2^{2-}$ polymeric complex in carbonate solution. Information related to uranium reduction from UO_2^{2+} , uranyl hydroxide or organic complexes is not currently available.

2.6 Summary

Uranium in the environment can associate with various organic and inorganic compounds. Current treatment practices to remove uranium from waste streams are generally non-specific and uneconomical. Enzymatic reduction of soluble hexavalent uranium to insoluble tetravalent uranium appears to be a promising technique to immobilize uranium. However, the impact of organic complexants on uranyl ion reduction has not been evaluated. Mechanisms of metal reduction by microorganisms indicate that organic complexation can either alter the rate of bioreduction or require additional enzymes to facilitate bioavailability. In addition, information related to the influence of organic ligands on precipitation of bio-reduced uranium (U^{4+}) is not currently available. Further research is required to optimize treatment procedures to facilitate effective uranium removal from complex waste streams using bio-reductive precipitation techniques.

3. RESEARCH OVERVIEW

3.1 Introduction

Microbially mediated reduction and subsequent precipitation of uranium has been demonstrated in synthetic bicarbonate solutions and bicarbonate amended groundwaters (Lovely, 1991, 1992a). However, the chemical composition of uranyl ion containing waste streams is often more complex than those previously tested. For example, waste streams generated from uranium ore extraction and environmental restoration processes contain several simple and complex organic compounds (Straub, 1964; Francis, 1980a,b; Francis, 1993). Waste stream constituents can chemically interact with uranium thereby limiting microbial reduction or precipitation reactions. The hypothesis that bioavailability of uranyl ion depended on the type and strength of the organic complex formed was tested in this research. In particular, the role of organic ligands in biological reduction and precipitation of uranium was evaluated. Microbial numbers, activity, organic ligand concentration and ligand toxicity to microorganisms during uranyl ion bioreduction were closely monitored to assist in data interpretation.

This section briefly describes the various experiments performed in this research and the rationale behind their selection. Figure 2 lists the steps behind the approach adopted. Each experiment is outlined in this chapter while a more detailed description of each method is given in chapter 4.0.

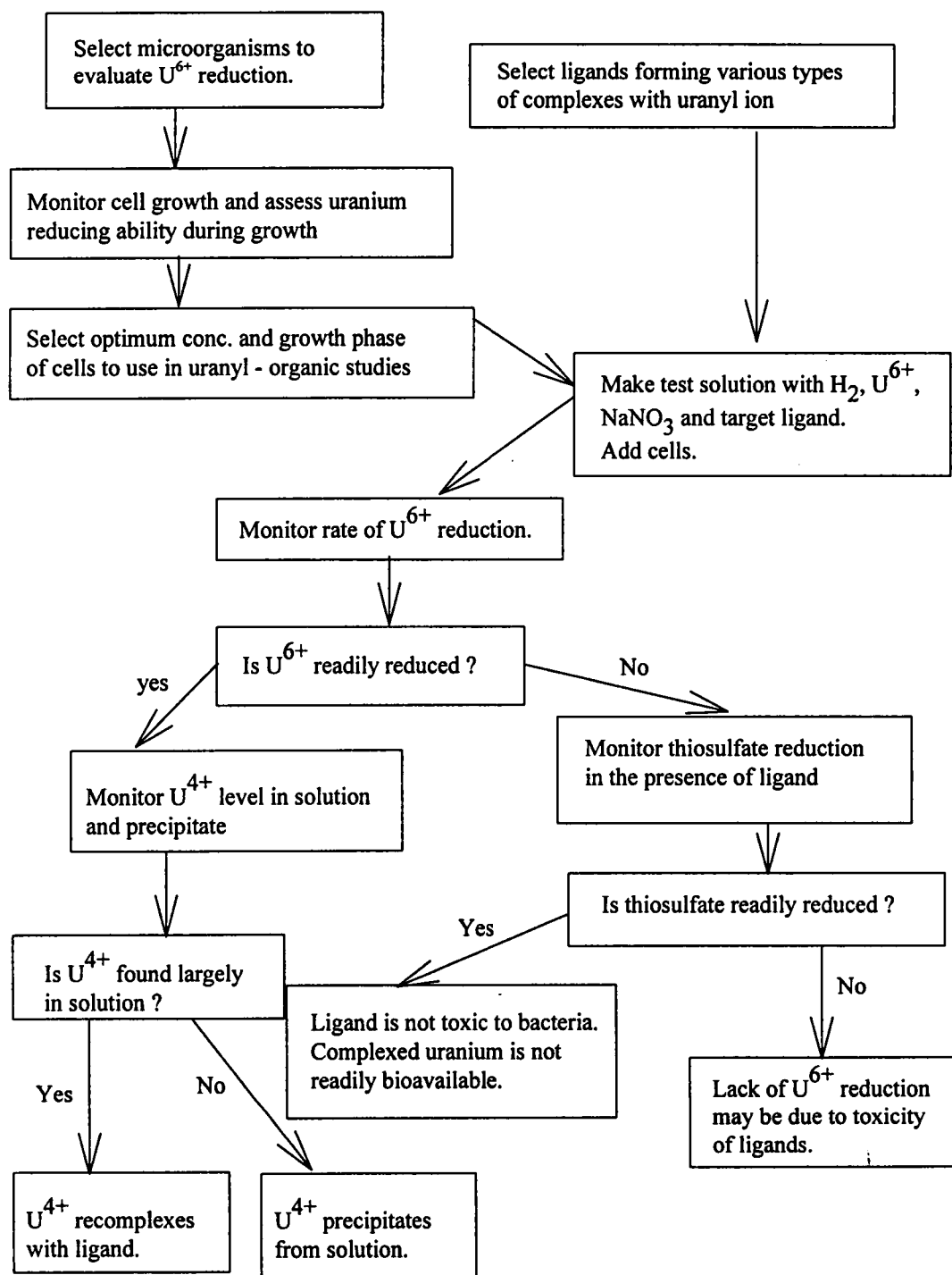


Figure 2. Schematic diagram showing the experimental approach used in the evaluation of complexed U^{6+} bio-reductive precipitation in this research

3.2 Microbial selection and characterization

Selection of microorganisms for this research was based on their demonstrated ability to utilize uranyl ion during metabolic processes. A sulfate reducing bacteria *Desulfovibrio desulfuricans* and an iron reducing bacterial strain *Shewanella alga* (BrY) were selected for this research. *Desulfovibrio desulfuricans* is primarily an anaerobic microorganism (Krieg and Holt, 1984) with limited tolerance to oxygen (Dilling, 1990). This microorganism reduces hexavalent uranium using cytochrome C₃ located in the soluble periplasmic fraction. However, it does not gain energy to sustain growth through uranyl ion reduction (Lovely, 1992b, 1993c). The iron reducing bacterial strain *Shewanella alga* (BrY) is facultative, that is, it can grow under aerobic as well as anaerobic conditions (Caccavo, 1992). Whether the bacterium can utilize hexavalent uranium as the sole electron acceptor for growth has yet to be established. However, other iron reducing microorganisms which reduce hexavalent uranium (*Geobacter metalireducens*, *Shewanella putrefaciens*), have been reported to grow employing U⁶⁺ as the sole electron acceptor (Lovely, 1993a). This indicated that sulfate and iron reducing microorganisms reduced hexavalent uranium using different mechanisms. The two metabolically diverse bacteria were chosen to evaluate reductive precipitation of uranium from organic complexes.

The bacterial growth pattern and the ability to reduce uranium at various phases of growth in organic ligand-free solutions were initially determined. These studies were performed in order to : (i) determine the growth phase in which these bacteria efficiently

reduce hexavalent uranium and (ii) maintain the same concentration of biomass and the same growth phase in all subsequent experiments. Bacterial growth was evaluated by monitoring the increase in cell numbers using the Acridine Orange Direct Count (AODC) technique. The efficiency of uranyl ion reduction at different growth phases was monitored using procedures described in chapter 4.0.

3.3 Ligand selection

The following criteria were considered in selection of ligands to facilitate hypothesis testing: (1) sufficient information was available in the literature regarding complexation of uranyl ion with each ligand selected, (2) selected ligands varied widely in their nature of binding as well as their binding affinity with uranyl ion, and (3) test ligands were representative of those generally found in uranium-containing wastewaters. Based on the above considerations, five ligands were chosen for study (Table V).

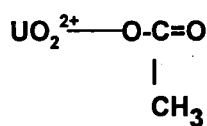
The nature of binding between uranyl ion (UO_2^{2+}) and the chosen ligands varied widely. For example, each molecule of acetate complexed with uranyl ion through the interactions of one carboxyl functional group (monodentate complexation) (Ahrland, 1951). In contrast, molecules of malonate and oxalate complexed with uranyl ions through the interactions of two carboxyl functional groups (bidentate complexes) (Rajan and Martell, 1967; Havel, 1969). In general, complexes involving multi-functional groups interactions of ligand are called chelates (Stumm and Morgan, 1981).

TABLE V. Ligands selected and their nature of association with hexavalent uranium

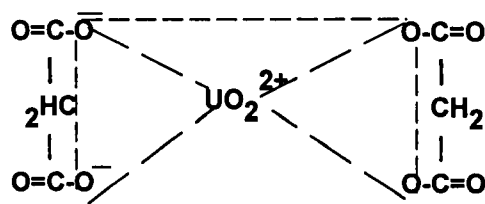
LIGAND	TYPE OF COMPLEX	STABILITY CONSTANT	
		LOG K	LOG β
Acetate	Monodentate	$10^{3.21}$	$10^{7.33}$
Malonate	Bidentate	$10^{5.6}$	$10^{10.1}$
Oxalate	Bidentate	$10^{6.5}$	$10^{11.3}$
Citrate	Binuclear	$10^{18.9}$	$10^{18.9}$
Tiron	Polynuclear	$10^{15.9}$	10^{55}

Citrate formed a binuclear complex with UO_2^{2+} , that is, the complex contained two uranyl ions surrounded by two molecules of citrate (Rajan and Martell, 1965; Nunes, and Gil, 1987). Two carboxyl and one hydroxyl group of each ligand participated in complexation. Similarly, a ternuclear complex was formed between tiron and uranyl ions (Gustafson, 1960; Bartusek and Sommer, 1965). Representative complexes formed between uranyl ions and the test ligands in this research are shown in Figure 3. Note that, with the exception of tiron, all ligands were aliphatic and associate with uranyl ion primarily through carboxyl ion interactions. Tiron, which is aromatic, complexes with uranyl ion through hydroxyl ion interactions.

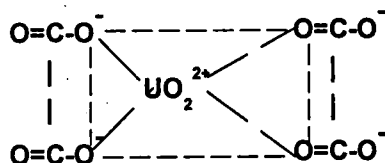
In addition to the nature of association, the affinity of the ligands for uranyl ions varied widely. The formation constants (K , β) of the complexes formed between the test ligands and uranyl ion differed by several orders of magnitude. For example, acetate formed the weakest complex with uranyl ion, with stability constants K , β of $10^{2.6}$ and $10^{7.3}$, respectively. Tiron formed very strong uranyl complexes with stability constants K , β of $10^{9.0}$ and 10^{55} , respectively. In addition to the diversity of types of complexes formed, the ligands were representative of those generally found in uranyl ion-contaminated waste streams. Acetate represents various straight chain aliphatic acids found at radioactive waste disposal sites (Francis, 1980a). Both the sulfate and iron reducing microorganisms are reported to survive in acetate containing solutions without



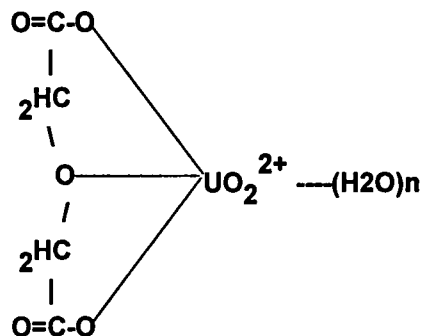
(i) Acetate (Ahrlund, 1951)



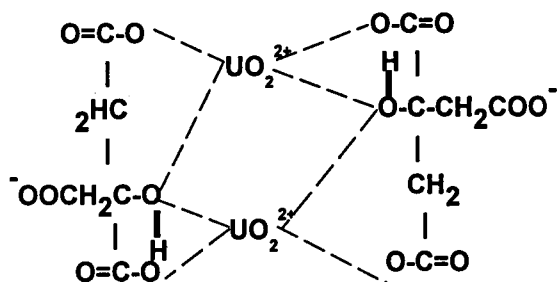
(ii) Malonate (Rajan and Martell, 1967)



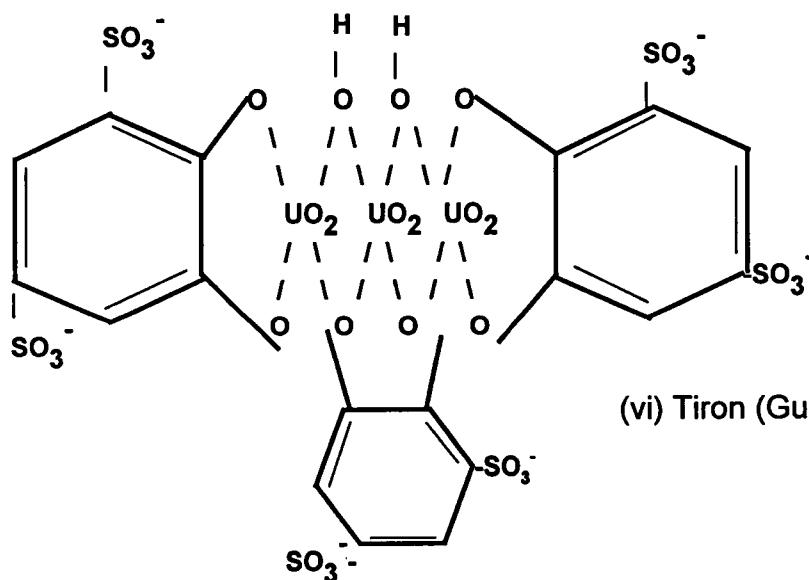
(iii) Oxalate (Rajan and Martell, 1967)



(iv) Oxydiacetate (Cassol, 1973)



(v) Citrate (Rajan and Martell, 1965)



(vi) Tiron (Gustafson, 1960)

Figure 3. Schematic of representative complexes formed between uranyl ion and various organic ligands used in this research.

any toxic effect of the ligand. Citrate and tiron are used to leach uranium from contaminated soils (Francis, 1993). In addition, citrate can be used as a carbon source by the strain BrY. Hence, these ligands were chosen to obtain a better insight regarding bioavailability of organically complexed uranyl ion.

3.4 Uranyl ion reduction and distribution

Bioavailability of a pollutant appears to be influenced by its physical state and its chemical speciation in the environment. The rate of microbial utilization of a substrate in its free or weakly complexed state may be quite different from that of its strongly complexed form. In this research, batch anaerobic experiments were performed to determine bioreduction of hexavalent uranium (UO_2^{2+}) complexed with each of the chosen ligand. Procedures used to maintain anaerobic conditions are explained in section 4.1. In all the experiments, an initial hexavalent uranium concentration of ~100 mg/L was used. This represented a typical uranium concentration found in process wastewater obtained from the Y-12 West End Treatment Facility, Oak Ridge, TN (Table III). Experiments were performed using two concentrations of each ligand to evaluate the extent of complexation on bioreduction. In test solutions containing the higher concentration of each ligand, almost all the uranyl ion (UO_2^{2+}) initially present was complexed to the ligand. At the lower ligand concentration evaluated, approximately 50% of the uranyl ions initially present in solution were complexed to each ligand. The

remaining uranyl ions formed various organic $((\text{UO}_2)(\text{OH})\text{ligand})$ and inorganic uranyl hydroxide $((\text{UO}_2)_2(\text{OH})_y)$ species. The concentration of each ligand used and the complexes formed are given in section 4.4.5. In order to selectively evaluate the influence of each test ligand on uranyl ion bioreduction, no other organic compound was introduced during testing. H_2 gas was provided as the electron source in all experiments. Experiments were conducted with microorganisms grown to optimum phase determined by initial characterization studies (Section 3.2). Microbial utilization of uranyl ion was determined by monitoring the decrease in concentration of hexavalent uranium (U^{6+}) over time. The trend in reduction observed in organic solutions was compared with that observed in organic-free systems to determine if organic complexation influenced U^{6+} bioavailability.

The success of bioreductive uranium precipitation as a viable treatment option depends not only on the bioavailability of U^{6+} but also the subsequent precipitation of tetravalent uranium (U^{4+}) upon reduction. The solubility of metal ions is often influenced by the presence of various ligands in solution (Stumm and Morgan, 1981). A metal which is insoluble in the absence of organic ligands may form highly soluble complexes in their presence. Hence, it is imperative to monitor the fate of microbially reduced uranium in the presence of organic ligands such that proper conditions which maximize uranium removal from solution can be determined. In addition to monitoring decreases in U^{6+} levels in solution, the distribution of uranium in various phases (such as the level of bioreduced uranium (U^{4+}) in solution, the fraction of precipitated uranium and the

hexavalent uranium sorbed to microbial mass) was also determined. The concentration of each organic ligand was also monitored over time to determine if reduction and/or precipitation of uranium was influenced by simultaneous degradation of the test ligand by the bacteria.

3.5 Toxicity of ligands to microorganisms

If bioavailability of uranyl ion (as an electron acceptor) was limited by the presence of organic ligands, the following two mechanisms may prevail: (i) change in uranyl ion speciation caused by complexation (chelate effect), and (ii) toxicity of ligands to the microorganisms. Therefore, experiments were conducted to assess toxicity of organic ligands to the microorganisms. To evaluate toxicity, the ability of bacteria to utilize thiosulfate ($\text{S}_2\text{O}_3^{2-}$) as an electron acceptor in the presence and absence of organic ligands was evaluated. Thiosulfate, being an anion, does not readily complex with the organic ligands tested (Sillen and Martell, 1964). Hence, any limitation in the utilization of thiosulfate in the presence of the test organic ligands was presumed to be due to toxic effects of ligands to the bacteria.

4.0 MATERIALS AND METHODS

4.1 Microorganisms and culture conditions

A sulfate reducing bacteria, *Desulfovibrio desulfuricans* obtained from the American Type Culture Collection (ATCC 29577), Rockville, MD was used in this research. Sulfate reducers, as a group, are anaerobic microorganisms. They are reported to grow either by anaerobic respiration of sulfate using lactate, formate and simple alcohols as the carbon/electron source or by fermentation of pyruvate or choline (Krieg and Holt, 1984). In this research, *D. desulfuricans* was grown in a nutrient rich medium (Table VI) containing lactate as the major electron and carbon source and sulfate as the terminal electron acceptor (Lovely, 1992b). The following procedure was used in the preparation of growth medium. Initially, a stock medium was prepared by adding bicarbonate, mineral salts, vitamins and nutrients to deionized distilled water (1 L). This bicarbonate buffered medium pH was approximately 6.8. Anaerobic conditions in the stock medium were ensured by following a protocol developed by Hungate (1969). The medium, upon heating to near boiling, was deoxygenated by purging with an anaerobic gas mixture (5% H₂ : 10% CO₂ : 85% N₂) for five minutes. Heating hastened the diffusion of dissolved oxygen out of the medium. Prior to purging, trace levels of oxygen present in the gas mixture were removed by passing the gas through a hot copper column (320° C) filled with copper filings in a reduced state. To ensure that the copper filings were in a reduced state, a H₂ rich gas mixture (80% H₂ : 20% CO₂) was passed through

Table VI. *Desulfovibrio desulfuricans* growth medium composition

Constituent	Concentration
NaHCO ₃	2.5 g/L
NH ₄ Cl	1.5 g/L
NaH ₂ PO ₄	0.6 g/L
KCl	0.1 g/L
MgSO ₄	1.5 g/L
Na ₂ SO ₄	2.0 g/L
Yeast Extract	2.0 g/L
Polypeptone	5.0 g/L
Lactate	20 ml/L ¹
Vitamins	Trace ²
Minerals	Trace ²

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

2 - composition in appendix I

the filings for 10 minutes prior to medium deoxygenation. Both the nitrogen rich and hydrogen rich gas mixtures were certified gases (99.5 % purity) obtained from National Welders Co., Knoxville, TN. The deoxygenated medium was then quantitatively transferred in 54 ml volumes, using a pipette into 100 ml anaerobic serum bottles. Prior to medium transfer, oxygen present in the empty serum bottles was removed by purging with a N₂ rich (5% H₂:10% CO₂: 85% N₂) gas mixture for 30 seconds. After the medium was transferred, trace oxygen in the head space of the bottles was removed by purging with the anaerobic (N₂ rich) gas mixture for one minute. During head space deoxygenation the bottles were closed with butyl rubber stoppers (Belco glass co., Vineland, NJ), while the tip of the deoxygenating needle was still in the head space.

Subsequently, the needle was gently pulled out while the stopper prevented air from entering into the tube. The bottles were then sealed with aluminum caps and autoclaved to ensure sterility. Prior to cell inoculation, 6 ml of L-cystiene HCl (2 g/L stock), which is a strong reducing agent, was added to each serum bottle. This treatment scavenged trace levels of oxygen trapped in the medium and expedited microbial growth. The cell culture obtained from ATCC was transferred using a syringe into two bottles containing growth medium. The bottles were incubated at 32° C for three days to generate a growing culture. Subsequently, three mls of growing bacterial culture were transferred to serum bottles containing 60 ml of growth medium twice a week and placed in an incubator (32° C) to maintain a growing culture. The pipettes, syringe and needles used in the transfer

of medium and cell culture were flushed with an oxygen free gas mixture (5% H₂: 10% CO₂: 85% N₂) prior to use.

An inoculum of the iron reducing bacteria *Shewanella alga* (BrY) was obtained from Dr. Frank Caccavo, University of Oklahoma, Norman, OK. This facultative bacteria was grown anaerobically in a medium containing lactate as the primary carbon and electron source and ferric iron as the terminal electron acceptor. The constituents of the medium used to grow BrY are shown in Table VII (Caccavo, 1992). Culture techniques similar to those used to grow *D. desulfuricans* were followed.

4.2 Cell characterization studies

A major goal of this research was to evaluate the impact of organic complexation on the bioavailability of hexavalent uranium. In order to selectively study the role of complexation, it was imperative that other factors which could affect uranyl ion bioavailability were controlled. For example, the rate and extent of uranyl ion reduction may be influenced by the phase of cell growth and the concentration of cells used in experiments. Therefore, studies were initially conducted to characterize the growth pattern of the two bacteria in order to ensure that the same number of cells in the same phase of growth were added in each experiment. This section describes the protocol followed in the experiments conducted to characterize bacterial growth.

Table VII. *S. alga* (BrY) growth medium composition

Constituent	Concentration
NaHCO ₃	2.50 g/L
KCl	0.10 g/L
NH ₄ Cl	1.50 g/L
NaH ₂ PO ₄ H ₂ O	0.60 g/L
Sodium Acetate	6.00 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	Trace ²
Minerals	Trace ²

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

2 - Composition in appendix I

Figure 4 shows the steps followed to characterize the growth of cells and their uranyl ion reducing ability in different phases of growth. Sample tubes were prepared by adding 9 ml of growth medium to each of 50 anaerobic pressure tubes (25 ml). Cells in the growing culture (Section 4.1) were enumerated using an epifluorescence microscopic technique (AODC procedure, Section 4.8), and appropriately diluted in anaerobic pressure vials (2.5 g/L NaHCO₃) to a final concentration of 10⁴ cells/mL. Subsequently, one ml of the diluted culture was added to each of fifty tubes containing 9 ml of growth medium which resulted in an initial concentration of 10³ cells/mL. Upon inoculation, the tubes were incubated at 32° C. Cell growth was determined by monitoring the number of cells over time (upto 120 hours) using the AODC procedure. During the first 48 hours of incubation, samples were analyzed at 4 hour intervals. Subsequently, they were analyzed at intervals of 12 hours. At each sampling period, subsamples in the tubes were analyzed in duplicate employing sacrificial sampling, that is, samples from two tubes generated data at one time period. Repeated sampling from the same tube for different time periods was not performed.

As part of microbial characterization, a study was conducted to observe the uranium removal efficiency at various phases of cell growth. Cells were collected at different phases of growth from four pressure tubes by centrifugation (7500g, 20 minutes, 4° C). After decanting the supernatant, cells were washed twice by resuspending in 2 ml of anaerobic bicarbonate solution (2.5 g/L) followed by centrifugation at 7500g for 10 minutes at 4° C. The cells were then suspended in 2 ml anaerobic bicarbonate solution

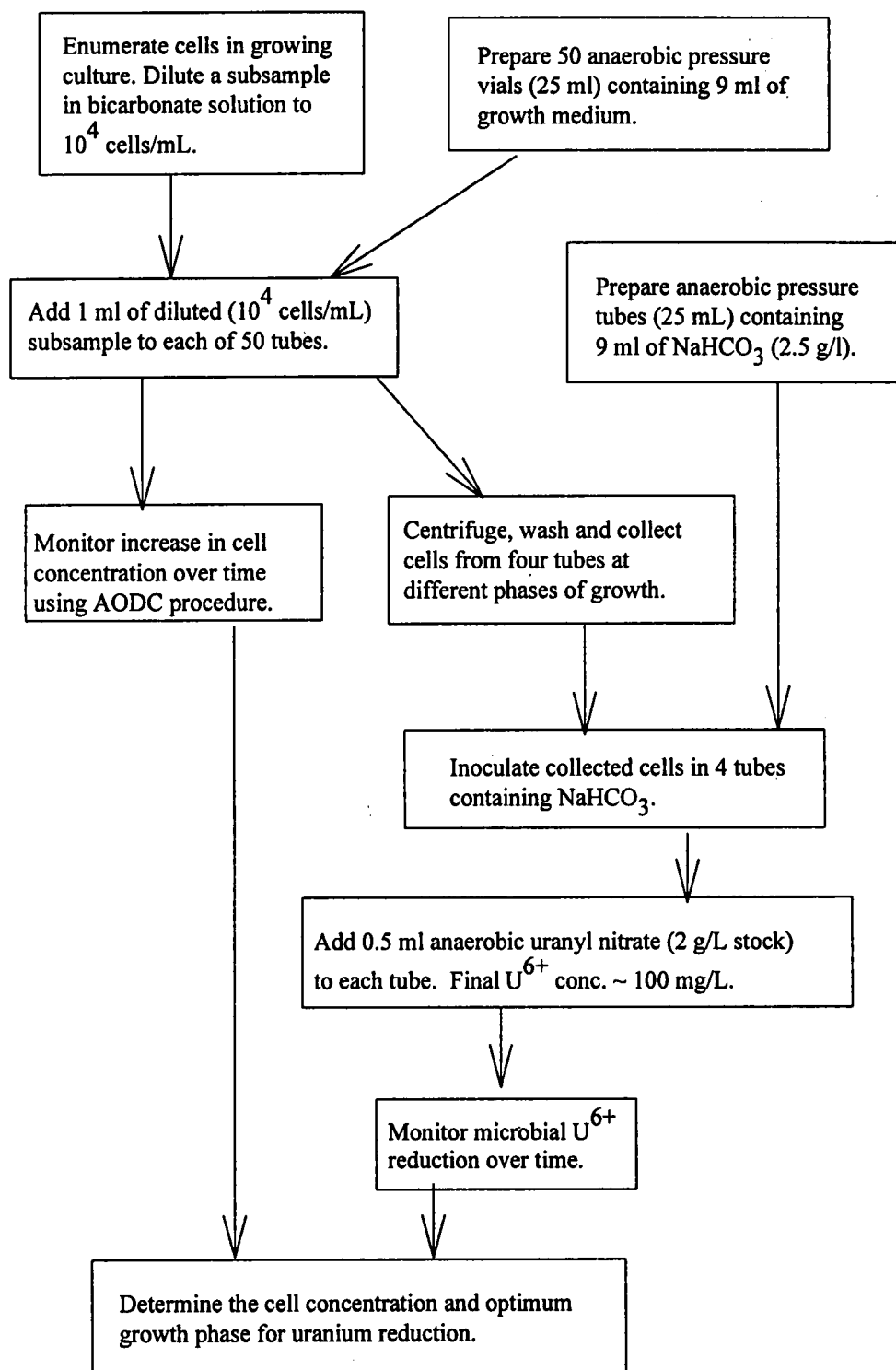


Figure 4. Schematic diagram showing various steps in cell growth characterization study.

(2.5 g/L) and transferred (using a sterile syringe) into four 25 ml anaerobic pressure tubes containing 9 mls buffer solution (2.5 g/L NaHCO_3 , 10 mM lactate). A 0.5 ml solution of uranyl acetate (2 g/L stock) was added to each tube, using a syringe, which resulted in an initial U^{6+} concentration of approximately 100 mg/L. The bicarbonate washing solution and the uranyl stock solutions were deoxygenated using Hungate's (1969) procedure prior to use. All transfers of cells and stock solutions were performed with pipettes, syringes and needles that were flushed with an oxygen free gas mixture (5% H_2 : 10% CO_2 : 85% N_2). Reduction of hexavalent uranium by the cells was monitored by measuring the decrease in U^{6+} concentration over time. Details of uranium analysis are given in section 4.6.

4.3 Uranyl ion properties and stock solution preparation

Reagent grade uranyl (U^{6+}) acetate and uranyl nitrate (Fluka Chemical Company, Ronkonkoma, NY) were used to prepare U^{6+} stock solutions (2 g/L) for biotransformation experiments. The salts were used as received, at their commercially available purity level of 99% for stock solution preparation. The stock was then deoxygenated using Hungate's (1969) procedure and used in uranium reduction experiments. A high purity (99.5%) uranyl nitrate solution (1000 mg/L), obtained from Ricca Chemical Company (Arlington, TX) was used to calibrate the uranium analyzer.

4.4 Selection of experimental conditions for uranyl ion biotransformation

4.4.1 Introduction

The bacterial characterization studies yielded information regarding the growth pattern and uranium reducing efficiency of the organisms. The results were used to insure that the same concentration of cells in the same phase of growth were added to test solutions in subsequent experiments with organic ligands. Microorganisms were maintained in a basal metabolic (non-growth) mode in all experiments using organic ligands, that is, various nutrients and vitamins required for growth were not provided. This arrangement eliminated the possible interactions of target ligand and/or uranyl ion with vitamins and minerals during UO_2^{2+} bioreductive precipitation. H_2 gas was provided as the electron donor rather than lactate to eliminate possible complexation of uranyl ion with lactate in the presence of target ligands. Uranyl ion was provided as the primary electron acceptor, that is, neither sulfate nor ferric iron were added in these experiments. This measure eliminated possible interactions such as sulfide mediated uranium precipitation, coprecipitation of uranium with iron and competitive inhibition caused by competing electron acceptors. In summary, the test solutions contained H_2 gas as the sole electron donor, uranyl ion as the sole electron acceptor and each target ligand as the sole organic complexing agent.

As previously discussed (section 3.3), six ligands which varied widely in the type and affinity of binding with uranyl ion were chosen for study. Each target ligand can

interact with uranyl ion to form a variety of species. The nature and extent of the various species formed depended on the pH, concentration of uranyl ion and target ligand, association and protonation constants of various species, and ionic strength. Biodegradation of a compound is often impacted by its chemical speciation (Madison and Alexander, 1985). The goal of this research was to evaluate the impact of selected 'uranyl ion-ligand' species on UO_2^{2+} bioavailability. Hence, the test solution composition was selected such that the target UO_2^{2+} -ligand species predominated. The following paragraphs describe the criteria used in the selection of test solution composition.

4.4.2 pH

pH influenced the speciation of hexavalent uranium and microbial viability. At neutral and alkaline pH levels, uranium predominantly formed hydrolyzed species and did not complex well with organic ligands (Allison, 1991). Lower pH levels ($\text{pH} < 5$), inhibited microbial growth. Hence, the initial pH was adjusted to 6.0 in all experiments.

4.4.3 Stability constants for uranium-ligand species

The stability constants for metal-ligand species indicate the relative affinity of the metal and ligand to form a particular species. For a given condition, stability constants of individual species determine the relative distribution of these species in solution. In addition, stability constants play an important role in the selection of solution composition to favor the formation of specific species. Stability constants for uranyl-

ligand species for this research were obtained from Sillen and Martell (1964), Smith and Martell (1986), Perrin (1979) and Brainard (1993). The constants for various species are shown in Table VIII. Note that the various species formed between the five aliphatic ligands and uranyl ion do not involve hydroxyl ion interactions. However, for the pH utilized in all experiments (6.0), uranium tends to hydrolyze and form complexes with ligands involving hydroxyl ion interactions (Feldman, 1954; Courtney, 1958). Information related to the stability constants of such species for the five aliphatic ligands are not currently available in the literature. Hence, an empirical equation was developed to predict the stability constants for the uranyl-hydroxide/ligand species which form. This equation was developed by relating the difference in the stability constants for 'Metal Hydroxide (MOH)' species and 'Metal Ligand (ML)' species to the stability constant for 'Metal Hydroxide Ligand (MOHL)' species available for metal (Fe^{3+} , Al^{3+} , Cu^{2+} , Cd^{2+} , Cr^{3+} , Ni^{2+} , Zn^{2+})- ligand (citrate, acetate, CDTA, EDTA) species. In developing this equation it was hypothesized that, the affinity of a metal to form metal hydroxide organic (MOHL) complexes is related to its tendency to hydrolyze as well as the affinity of the unhydrolyzed metal to complex with the target organic ligand. The stability constants obtained from literature for various complexes are shown in Table IX. Figure 5 shows the relationship between the constants. The empirical relationship obtained by regression analysis of the data was

$$\text{Log } K_{(\text{MOHL})} = 0.326 + 0.798 (\text{Log } K_{(\text{ML})} + \text{Log } K_{(\text{MOH})}) \quad (10)$$

Table VIII. Stability constants of various 'uranium - ligand' species

Ligand	UO ₂ ²⁺ - Ligand Species	Stability Constant (Log K)
Acetate	(UO ₂ CH ₃ COO) ⁺¹	3.21
	UO ₂ (CH ₃ COO) ₂	2.15
	UO ₂ (CH ₃ COO) ₃ ⁻¹	1.97
Malonate	UO ₂ (C ₃ H ₄ O ₄)	5.60
	UO ₂ (C ₃ H ₄ O ₄) ₂ ⁻²	4.50
Oxalate	UO ₂ (C ₂ H ₄ O ₄)	6.50
	UO ₂ (C ₂ H ₄ O ₄) ₂ ⁻²	4.20
	UO ₂ (C ₂ H ₄ O ₄) ₃ ⁻⁴	0.60
Citrate	UO ₂ (C ₆ H ₈ O ₇)	7.40
	UO ₂ (C ₆ H ₈ O ₇) ₂	18.87
Tiron	UO ₂ -L	15.90
	UO ₂ HL	6.40
	(UO ₂) ₂ (OH)L ₂	-8.90
	(UO ₂) ₃ (OH) ₂ L ₃	2.80
	(UO ₂) ₃ (OH) ₃ L ₃	9.00

Table IX. Stability constants for metal-ligand, metal-hydroxide and metal-hydroxide ligand species obtained from literature, used in the determination of empirical relationship.

Metal Ligand	- Log K_{ML}	Log K_{MOH}	Log $K_{ML} + \text{Log } K_{MOH}$	Log K_{MOHL}
Fe ³⁺ - Citrate	-2.2	13.5	11.0	10.12
Fe ³⁺ - NTA	-2.2	17.9	15.7	13.5
Fe ³⁺ - EDTA	-2.2	27.7	25.5	19.9
Fe ³⁺ - DTPA	-2.2	31.2	29.0	20.9
Al ³⁺ - Citrate	-5.0	9.9	4.9	6.4
Al ³⁺ - NTA	-5.0	13.7	8.7	8.4
Al ³⁺ - CEDTA	-5.0	19.1	14.1	12.8
Al ³⁺ - DTPA	-5.0	21.9	16.9	13.7
Cu ²⁺ - Citrate	-7.7	7.2	-0.5	2.5
Cu ²⁺ - NTA	-7.7	14.4	6.7	4.8
Cu ²⁺	-7.7	20.5	12.8	8.5
CEDTA	-7.7	11.4	3.7	2.8
Cu ²⁺ - IDA	-4.0	26.8	22.8	19
Cr ³⁺ - Acetate	-7.7	12.0	4.3	1.5
Zn ²⁺ - NTA	-7.7	18.2	10.5	6.0
Zn ²⁺ - EDTA	-9.9	12.8	2.9	1.5
Ni ²⁺ - NTA	-9.9	20.1	10.2	7.6
Ni ²⁺ - EDTA	-10.1	11.1	1.0	-0.6
Cd ²⁺ - NTA	-10.1	18.2	8.1	4.1
Cd ²⁺ - EDTA	-2.1	17.1	15.0	21.3
Th ⁴⁺ - NTA				

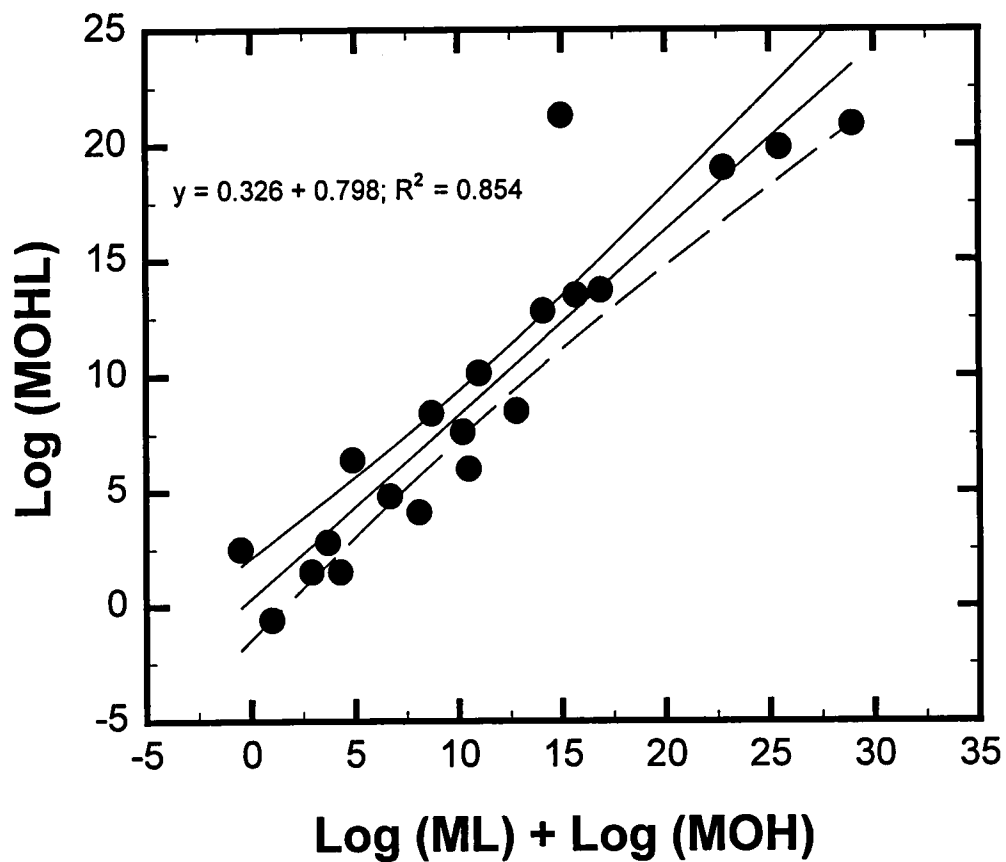


Figure 5. Relationship between Log (ML) , Log (MOH) and Log (MOHL) for various metal-ligand species obtained from literature. Variation in the predicted values at 90 % confidence interval also is shown.

where,

$K_{(\text{MOHL})}$ = Stability constant for (MOHL) species

$K_{(\text{ML})}$ = Stability constant for (ML) species

$K_{(\text{MOH})}$ = Stability constant for (MOH) species

The nature of chemical interactions represented by the empirical relationship is not currently known. However, the stability constants of 'metal hydroxide ligand (MOHL)' complexes for each ligand tested correlated well with the ionic potential (z/r) and the charge density (z/r^2) of the metal involved in complexation (Appendix II). Note that ionic potential and charge density of metals are related to their potential to outer sphere complexes, that is, complexation without damage to their hydration shell (Sposito, 1980). Since hexavalent uranium and the functional groups of the ligands used often tend to form outer sphere complexes (Pearson, 1968a), this equation was used to estimate the stability constants of various uranyl hydroxide ligand ($\text{UO}_2(\text{OH})\text{Ligand}$) species used in this research. The constants obtained are given in Table X. The values presented in Tables VIII & X were used to determine the uranium speciation (initial) in test solutions.

4.4.4 Ligand concentration

Uranyl reduction experiments were conducted with two different initial concentrations of each selected ligand. In one set of experiments, the ligand was added to sufficiently complex most of the uranyl ion (UO_2^{2+}) initially added to solution. In the

Table X. Stability constants of 'UO₂(OH)Ligand' species obtained from the empirical relationship

Ligand	Species	Stability Constant (Log K)*
Acetate	UO ₂ (OH)CH ₃ COO	-1.91
Malonate	UO ₂ (OH)(C ₃ H ₄ O ₄)	-0.31
Oxalate	UO ₂ (OH)(C ₂ H ₄ O ₄)	0.85
Citrate	UO ₂ (OH)(C ₆ H ₈ O ₇)	1.76

* Log stability constant values varied by $\sim \pm 1.5$ units at 90 % confidence interval of regression

second set of experiments, a lower ligand concentration was used to initially complex approximately 50% of the uranyl ion in solution. The computer speciation program MINTEQA2, using the stability constant values reported in Tables VIII & X was utilized to determine the concentration of ligand required for each experiment. The concentration of ligands and predominant ligand species (%) predicted in solution for each case is shown in Table XI.

As can be seen, at the higher concentration of acetate, malonate, oxalate and citrate, > 97 % of the hexavalent uranium formed exclusive 'uranyl ion-organic ligand' species with each ligand, that is, < 3 % UO_2^{2+} formed hydrolyzed species. At lower concentrations, nearly 50% of the uranyl ions formed 'UO₂²⁺-organic ligand' species. The remaining uranium (50%) either formed organic complexes involving hydrolyzed uranyl species or remained as inorganic uranyl hydroxide compounds. At both high and low tiron concentrations, hexavalent uranium complexation occurred only through uranyl hydroxide interactions. No exclusive 'UO₂²⁺-tiron' species formed. At the lower concentration of tiron used, 50% of uranyl ion formed inorganic uranyl hydroxide complexes.

Note that, the ligand concentrations were chosen based on the stability constants obtained from literature and that predicted using the empirical relationship developed (equation 10). Statistical analysis of the empirical relationship yielded a deviation of $\pm 10^{1.5}$ units on stability constants at 90 % confidence interval (Figure 5). Varying the stability constants of the $\text{UO}_2(\text{OH})$ Organic ligands by this range varied the % distribution

Table XI. Ligand concentration and distribution of various species in test solutions

LIGAND	CONC. (M)	UO ₂ - LIGAND SPECIES	UO ₂ (OH)LIGAND SPECIES	INORGANIC SPECIES
Acetate	0.17	UO ₂ (CH ₃ COO) ₃ ⁻¹ (91.2%) UO ₂ (CH ₃ COO) ₂ (5.8%)	UO ₂ (OH)CH ₃ COO (2.5 %)	
		UO ₂ (CH ₃ COO) ₃ ⁻¹ (23.1%) UO ₂ (CH ₃ COO) ₂ (8.8%) UO ₂ (CH ₃ COO) ₁ ⁺¹ (4.3%)	UO ₂ (OH)CH ₃ COO (23 %)	UO ₂ OH ⁺¹ (1.6 %) (UO ₂) ₃ (OH) ₅ ⁻¹ (38 %)
	0.03			
	0.01	UO ₂ (C ₃ H ₄ O ₄) ₂ (98%)	UO ₂ (OH)(C ₃ H ₄ O ₄) (1.2%)	
Malonate	0.00075	UO ₂ (C ₃ H ₄ O ₄) ₂ (33.9%) UO ₂ (C ₃ H ₄ O ₄) (5.5%)	UO ₂ (OH)(C ₃ H ₄ O ₄) (9.3%)	UO ₂ OH ⁺¹ (1.7 %) (UO ₂) ₂ (OH) ₂ (1.0%) (UO ₂) ₃ (OH) ₅ ⁻¹ (48.4 %)
	0.01	UO ₂ (C ₂ H ₄ O ₄) ₃ (22.8%) UO ₂ (C ₂ H ₄ O ₄) ₂ (75.2%)	UO ₂ (OH)(C ₂ H ₄ O ₄) (1.6%)	
Oxalate	0.00075	UO ₂ (C ₂ H ₄ O ₄) ₂ (30.7%) UO ₂ (C ₂ H ₄ O ₄) (11.9%)	UO ₂ (OH)(C ₂ H ₄ O ₄) (36.5%)	UO ₂ OH ⁺¹ (1.2%) (UO ₂) ₃ (OH) ₅ ⁻¹ (18.8 %)
	0.005	(UO ₂ (C ₆ H ₆ O ₇)) ₂ (100%)		
Citrate	0.0002	(UO ₂ (C ₆ H ₆ O ₇)) ₂ (49.7%)		(UO ₂) ₃ (OH) ₅ ⁻¹ (49.7%)
	0.0004		UO ₃ (OH) ₂ L ₃ (100%)	
Tiron	0.0002		UO ₃ (OH) ₂ L ₃ (50%)	(UO ₂) ₃ (OH) ₅ ⁻¹ (49.7%)
	0.0002			

of each species predicted (Table XII). A decrease in the stability constant by $10^{1.5}$ units did not appreciably change the speciation in most of the test solutions. However, an increase in the value decreased the % distribution of $(\text{UO}_2)_x\text{Organic}_y$ species by 30 to 40 % in acetate, malonate and oxalate solutions. However, solutions containing high concentrations of each ligand contained $\sim 50\%$ more $(\text{UO}_2)_x\text{Organic}_y$ complex than the solution containing lower level of the test ligand. The variation in the stability constants had no impact on the speciation in citrate solutions. In this research, various species of uranyl ion formed in test solutions was not directly determined. It was assumed that the speciation predicted (Table XI) using the empirical relationship (equation 10) prevailed in all test solutions. Data interpretation and elucidation of reductive precipitation mechanisms were made using this assumption.

4.4.5 Ionic strength

To eliminate any possible influence caused by variation in ionic strength, all uranium reduction experiments were performed at the same ionic strength. An ionic strength of 0.17M was maintained in all experiments. Studies by Kucsmas (1994) found no negative impact on microbial uranium reduction at this ionic strength. In test solutions containing no organic ligands, sodium nitrate salt was added as the background electrolyte to maintain the appropriate ionic strength. In addition, sodium nitrate was added to test solutions where the organic ligand and uranyl ion introduced were not

Table XII. Variation in the distribution of UO_2^{2+} species in test solutions upon change in the predicted stability constant of $\text{UO}_2(\text{OH})$ Ligand species by $\pm 10^{1.5}$

LIGAND	CONC. (M)	UO_2 - LIGAND SPECIES	$\text{UO}_2(\text{OH})$ LIGAND SPECIES	INORGANIC SPECIES
Acetate	0.17	$\text{UO}_2(\text{CH}_3\text{COO})_3^{-1}$ (93 - 51.8 %) $\text{UO}_2(\text{CH}_3\text{COO})_2$ (5.9 - 3.3%)	$\text{UO}_2(\text{OH})\text{CH}_3\text{COO}$ (1 - 44.6 %)	
		$\text{UO}_2(\text{CH}_3\text{COO})_3^{-1}$ (26.3 - 1.2 %) $\text{UO}_2(\text{CH}_3\text{COO})_2$ (10 - 1.2 %) $\text{UO}_2(\text{CH}_3\text{COO})_1^{+1}$ (4.9 - 0 %)	$\text{UO}_2(\text{OH})\text{CH}_3\text{COO}$ (55 - 94.9 %)	$\text{UO}_2\text{OH}^{+1}$ (1.8 - 0 %) $(\text{UO}_2)_3(\text{OH})_5^{-1}$ (1.2 - 0 %)
Malonate	0.01	$\text{UO}_2(\text{C}_3\text{H}_4\text{O}_4)_2$ (99 - 72.5%)	$\text{UO}_2(\text{OH})(\text{C}_3\text{H}_4\text{O}_4)$ (0 - 27.5 %)	
		$\text{UO}_2(\text{C}_3\text{H}_4\text{O}_4)_2$ (37.2 - 8 %) $\text{UO}_2(\text{C}_3\text{H}_4\text{O}_4)$ (5.9 - 1.6 %)	$\text{UO}_2(\text{OH})(\text{C}_3\text{H}_4\text{O}_4)$ (0 - 87 %)	$\text{UO}_2\text{OH}^{+1}$ (1.7 - 0 %) $(\text{UO}_2)_2(\text{OH})_2$ (1.0%) $(\text{UO}_2)_3(\text{OH})_5^{-1}$ (54 - 2.6 %)
Oxalate	0.01	$\text{UO}_2(\text{C}_2\text{H}_4\text{O}_4)_3$ (37 - 16.4 %) $\text{UO}_2(\text{C}_2\text{H}_4\text{O}_4)_2$ (5.9 - 51 %)	$\text{UO}_2(\text{OH})(\text{C}_2\text{H}_4\text{O}_4)$ (0 - 32 %)	
		$\text{UO}_2(\text{C}_2\text{H}_4\text{O}_4)_2$ (45 - 3 %) $\text{UO}_2(\text{C}_2\text{H}_4\text{O}_4)$ (16 - 0 %)	$\text{UO}_2(\text{OH})(\text{C}_2\text{H}_4\text{O}_4)$ (35.6 - 96 %)	$(\text{UO}_2)_3(\text{OH})_5^{-1}$ (1.5 - 0 %)
Citrate	0.005	$(\text{UO}_2(\text{C}_6\text{H}_8\text{O}_7))_2$ (100 - 99%)		
		$(\text{UO}_2(\text{C}_6\text{H}_8\text{O}_7))_2$ (49.8 - 49 %)		$\text{UO}_2\text{OH}^{+1}$ (0 - 1.5 %) $(\text{UO}_2)_2(\text{OH})_2$ (0 - 1.2%) $(\text{UO}_2)_3(\text{OH})_5^{-1}$ (45 - 46.8%)
	0.0002			

sufficient to yield the reference ionic strength. The amount of sodium nitrate added in each case was determined using MINTEQ.

Note that nitrate ion, present as a background electrolyte, can serve as an electron acceptor for the bacteria used in this research. Kucsmas (1994) performed experiments to evaluate the impact of nitrate on hexavalent uranium bioreduction. Results from his experiments indicated that, in test solutions containing ~ 100 mg/L U^{6+} , a nitrate concentration of 1000 mg/L (~ 0.16 M) did not significantly affect U^{6+} bioreduction. Hence it was assumed that the amount of sodium nitrate used in this research, to yield the reference ionic strength (0.17 M), should not impact U^{6+} reduction by the microorganisms.

4.5 Sample preparation

The previous section described the various steps involved in determination of solution composition for biotransformation experiments. The protocol followed in the preparation of each test solution is described in this section.

Batch experiments, using 25 ml anaerobic pressure tubes, were conducted to evaluate biotransformation of organically complexed uranium. Initially, stock solutions (1 L) were prepared by adding each target ligand and sodium nitrate in distilled deionized water. The amount of ligand and sodium nitrate added was obtained from MINTEQ. The pH of the stock solutions was adjusted to 6.0 using NaOH (0.1 M) or HNO_3 (0.1 M)

before filter sterilization (0.2 μ m). Sterilized solutions were deoxygenated using procedures described previously, and dispensed in 9 ml volumes, using sterile pipettes, into the 25 ml pressure tubes. Upon transfer of test solutions, the tubes were closed with butyl rubber stoppers and sealed with aluminum caps. A 0.5 ml uranyl nitrate solution (2 g/L deoxygenated stock) was added by syringe, which resulted in an initial concentration of ~ 100 mg/L total hexavalent uranium in each tube. Solutions were then equilibrated for 48 hours using a platform shaker (Controlled Environmental Shaker, Brunswick, NJ) set at 100 rpm to allow for equilibrium complexation between uranyl ion and each test ligand. The time provided was sufficient for equilibrium complexation to occur (Bates, 1967). H₂ gas was then provided as electron donor by injecting 20 mls of an 80% H₂: 20% CO₂ gas mixture into the head space of each pressure tube. The tubes were gently shaken end to end by hand several times to allow for equilibration of H₂ gas with each sample solution. The amount of H₂ gas thus provided was sufficient to allow complete uranyl ion reduction by the bacteria in each sample (Appendix III). Cells grown to a preevaluated optimum phase in nutrient rich medium (60 hours for *D. desulfuricans*, 24 hours for *S. alga*) were centrifuged (7500g, 20 minutes, 4° C), washed twice with anaerobic test solution (containing no uranium) and injected into the 'uranyl ion-ligand' containing pressure tubes using a syringe. The tubes were then incubated at 32° C. They were maintained in a stationary condition and were not shaken during bioreduction. Heat killed cells were used as abiotic controls. In control experiments, cells collected by centrifugation were boiled for 30 minutes, flushed with nitrogen gas

and inoculated into uranyl ion containing pressure tubes. Hexavalent uranium reduction in these tubes was evaluated over a period of 96 hours. Test solutions were analyzed at 0, 0.33, 1.0, 1.5, 2.33, 4, 6, 12, 24, 48, and 96 hours after incubation. Subsamples in pressure tubes were analyzed for uranium in duplicate, employing a sacrificial sampling technique.

4.6 Uranium analysis

4.6.1 Analyzer and principle

A Kinetic Phosphorescence Analyzer (KPA-11, Chemchek Instruments, Richland, WA) was used to measure uranium in all samples. This instrument measures the level of hexavalent uranium (oxidized state of uranium - U^{6+}) in the sample. Oxidized and reduced forms of uranium are differentiated by differences in their phosphorescence property. The oxidized form of uranium, upon excitation, phosphoresces by emitting a characteristic green light. In the reduced state, uranium is essentially nonluminescent (Brina and Miller, 1992, 1993). KPA-11 uses a laser beam which is pulsed through a dye nitrogen source (2,2'-([1,1'-biphenyl]-4,4'-diyl)-2,1-ethenediyl)bis-benzenesulfonic acid, disodium salt (stelbene 420)) prior to exciting the sample. Pumping the laser through the nitrogen dye provides the wavelength (425 nm) required to excite hexavalent uranium. The instrument records the phosphorescence of the excited uranyl ion as luminance

intensity using a photon counting unit. The intensity was counted at fixed time intervals (time gates) during the life time of phosphorescence (~ 200 to $300 \mu\text{s}$). The summed intensity of the sample was compared with that of a standard uranium solution to determine the hexavalent uranium concentration in the sample. During analysis, the luminance intensities of a reference (patent protected, Chemchek Instruments) solution was also simultaneously measured by the photon counter to correct for instrumental changes such as laser fluctuation and temperature drifts. In addition, a quenching agent, URAPLEX (patent protected compound, Chemchek Instruments) was added to each sample to quench the luminescence caused by non-uranium constituents in the sample.

4.6.2 Instrument calibration

A uranyl nitrate stock solution (1000 mg/L) obtained from Ricca Chemicals, Arlington, TX (99.5% purity) was used as the primary analytical standard. Since the instrument response was reported by the manufacturer to be linear up to a hexavalent uranium concentration of 5 mg/L , internal standards (0.5 , 1 and 2 mg/L) were prepared by appropriately diluting the uranyl nitrate stock solution with distilled deionized water. Care was taken in preparing these standards, such that, standards contained the same amounts of ligand, sodium nitrate and other constituents of the test solutions analyzed. A blank standard containing no uranium but all the other constituents was used to account for background luminescence. Standards were analyzed by mixing 1 ml of standard solution with 1.5 ml of complexing reagent described earlier.

4.6.3 Uranium distribution during bioreduction

The uranyl ion added to the test solution was subjected to various physical, chemical and biological activities during microbial treatment. As a result, during incubation, uranium in solution was present in different forms such as (i) U^{6+} in solution, (ii) reduced uranium (U^{4+}) in solution, (iii) reduced uranium as a precipitate and (iv) sorbed uranium. It was essential to know the distribution of uranium in the various phases to better understand the fate of uranium during bioreduction. The procedure followed to determine the various forms of uranium is described below. In this research, precipitated uranium was defined as the fraction of uranium retained on a 0.2 μm filter. Soluble uranium was defined as the fraction passing through the filter (filtrate). Acrodiscs (Gelman Scientific, Ann Arbor, MI) were used to filter the samples prior to analysis. Preliminary experiments indicated that the amount of soluble uranium sorbed to the filter material was insignificant. Lovely (1993c) reported that, although the rate of U^{4+} precipitation was enhanced due to nucleation by intact cells, the extent of removal was not enhanced via sorption of reduced uranium (U^{4+}) to cells. Therefore, in this research, no distinction was made between reduced uranium that precipitated from solution and reduced uranium sorbed to microbial cells.

4.6.3.1 Oxidized Uranium (U^{6+}) in Test Sample

At each sampling time, a pressure tube was taken from the incubator and gently but thoroughly shaken by hand to produce a homogenous solution. A subsample of the

homogenous solution (~ 2 ml) was collected by syringe and dispensed into a 15 ml polystyrene tube (Corning Inc., Corning, NY). A 100 μl aliquot of this subsample was mixed with 4.9 ml or 9.9 ml of deoxygenated distilled water (1:50 or 1:100 dilution). One ml of the diluted sample was mixed with 1.5 ml of complexing reagent and analyzed for uranium. Since reduced uranium can be oxidized within 30 minutes upon aeration (Voegtlin, 1949), samples were analyzed as soon as they were collected to accurately determine the concentration of hexavalent uranium (U^{6+}).

4.6.3.2 Oxidized Uranium (U^{6+}) in Solution

To measure this fraction, a subsample (~ 2 ml) of the homogenous solution was collected using a syringe and filtered through a 0.2 μm acrodisc syringe filter (Tuffryn membrane, Gelman Science, Ann Arbor, MI). Preliminary experiments performed indicated that < 5 mg/L uranium was lost due to sorption to the filter (Lovely, 1992, Kucsmas, 1994). A 100 μl subsample of the filtrate was diluted with 4.9 (or 9.9) ml of deoxygenated water, mixed with 1.5 ml of complexing reagent and analyzed for U^{6+} immediately.

4.6.3.3 Sorbed U^{6+}

The difference between the hexavalent uranium (U^{6+}) levels in the unfiltered and filtered (filtrate) samples yielded the level of uranium (U^{6+}) associated with solids.

4.6.3.4 Total Uranium in Sample

The total uranium level in each sample ($U^{4+} + U^{6+}$) was determined by oxidizing the unfiltered sample with nitric acid prior to uranium measurement. This treatment converted U^{4+} in the sample to U^{6+} which was then measured. 100 μ l of unfiltered sample (well mixed) was added to 0.5 ml of 2N nitric acid and diluted with 4.4 ml of deionized water (1:50 dilution). The volume and strength of nitric acid used were based on preliminary experiments. One ml of this oxidized, diluted sample was mixed with 1.5 ml of URAPLEX and then analyzed. Since oxidation of uranium due to aeration had no impact on total uranium analysis, samples were not required to be analyzed immediately upon collection. These samples were stored at 4° C until analyzed.

4.6.3.5 Total Uranium in Solution

Total uranium in solution ($U^{6+} + U^{4+}$) was measured by oxidizing filtrate (0.2 μ m) samples using 2N nitric acid. The same procedure described in the previous section was followed in analyzing the concentration of uranium.

4.6.3.6 Concentration of reduced uranium (U^{4+}) in Solution

The difference between the concentration of total uranium in solution (filtrate) and that of oxidized uranium (U^{6+}) in solution yielded the concentration of reduced uranium (U^{4+}) which remained in solution.

4.6.3.7 Amount of Uranium Precipitated

The difference between the total uranium level in an unfiltered sample and the total uranium level in a filtered sample (filtrate) yielded the amount of reduced uranium precipitated during bioreduction.

4.7 Analysis of organic ligands

Microorganisms can reduce U^{6+} in the presence of complexing agents using various mechanisms. Some bacteria may utilize the uncomplexed as well as the complexed fraction of uranium with equal preference. Other bacterial strains may reduce only the uncomplexed portion of uranium in the solution. Microorganisms may oxidize organic ligands during metabolism and utilize the uranium thus liberated. To determine if *D. desulfuricans* or *Shewanella alga* (BrY) utilized uranium upon oxidation of ligands, ligand concentrations in solution were monitored during uranium bioreduction. Ligand concentrations were measured in experiments utilizing the higher concentration of each ligand.

Acetate and citrate levels were measured using 'High Performance Liquid Chromatography (HPLC)' (Model 410, Perkin Elmer, Norwak, CT). A spherisorb C8, 5 μ m, 250 mm X 4.6 mm column (Alltech Chromatography, Deerfield, IL) was used to separate these ligands in their acidic state. A 0.2 M phosphoric acid solution was used as the mobile phase at a flow rate of 0.8 ml/minute. The weaker inorganic phosphoric acid

eluent converted acetate and citrate to their protonated acidic state. The C8 column then selectively separated the organic acids due to their hydrophobic nature. A LC90 UV detector (Perkin Elmer, Norwak, CT) at 210 NM wavelength was used to detect the ligands. Samples were filtered using 0.2 μ m filters and diluted appropriately prior to analysis. A 20 μ l sample volume was injected for analysis. Test solutions were analyzed in duplicates. Reagent grade sodium salts of the ligands (Malincrodt Specialty Chemical Co., Paris, KY) were used as standards for instrument calibration.

Malonate and oxalate were analyzed using a Dionex, Ion Exchange chromatography. An IonPack, AS4A-SC anion exchange column (4mm X 250mm), coupled with a self regenerating suppresser unit (ASRS-1 (4mm) (Dionex Chromatography, Sunnyvale, CA) was used to separate the ligands. The mobile phase used was a 0.18mM carbonate /0.17mM bicarbonate solution at a flow rate of 1.5 ml/minute. The ligands remained as their bivalent anions in the mobile phase and were separated through anion exchange by the column. A conductivity detector (ED 40, Dionex) was used to measure the concentration of ligands. The test solutions (1 mL) were filtered and diluted prior to injection.

Tiron was analyzed using a Beckman DU-70 spectrophotometer (Beckman Co., Fullerton, CA) (Brainard, 1994). Initially, a scan analysis (200nm through 600 nm) was performed to identify a maximum absorption wavelength for tiron. It was observed that tiron strongly absorbed at 300 nm and that absorbance increased linearly with

concentration at this wavelength. Filtered (0.2 μm) test solutions (2 mL) were analyzed at 300 nm to measure tiron concentration during uranium bio-reduction.

4.8. Prediction of reduced uranium distribution

As mentioned in Section 4.6.3.6 the distribution bio-reduced uranium was experimentally determined in this research. In addition, an attempt was made to compare the results with the speciation predicted using thermodynamic constants for association of reduced uranium with organic ligands. However, complete information related to the association of U^{4+} with test ligands are not currently available. Tetravalent Thorium ion (Th^{4+}) whose atomic number (90) and ionic radius (1.02 $\text{\AA}$) are similar to that of U^{4+} (92, 0.97 $\text{\AA}$, respectively) is reported to associate with ligands in a manner similar to that of tetravalent uranium (Brainard, 1993). Thorium ion formed several multiligand complexes with acetate, malonate and oxalate (Table XIII, Martell and Smith, 1977). Complete information related to the association of Th^{4+} with citrate and tiron are not currently available. Hence, the distribution of U^{4+} under experimental conditions was predicted using the thermodynamic constants of Th^{4+} and compared with the experimentally observed results in acetate, malonate and oxalate solutions.

Table XIII. Stability constant for Th^{4+} -Organic complexes

Ligand	Complex	Log Stability Constant
Acetate	$\text{U}^{4+}(\text{CH}_3\text{COO}^-)$	3.89
	$\text{U}^{4+}(\text{CH}_3\text{COO}^-)_2$	6.94
	$\text{U}^{4+}(\text{CH}_3\text{COO}^-)_3$	9.01
	$\text{U}^{4+}(\text{CH}_3\text{COO}^-)_4$	10.3
	$\text{U}^{4+}(\text{CH}_3\text{COO}^-)_5$	11.0
Malonate	$\text{U}^{4+}(\text{C}_3\text{H}_4\text{O}_4)$	7.42
	$\text{U}^{4+}(\text{C}_3\text{H}_4\text{O}_4)_2$	5.28
Oxalate	$\text{U}^{4+}(\text{C}_2\text{H}_2\text{O}_4)$	8.23
	$\text{U}^{4+}(\text{C}_2\text{H}_2\text{O}_4)_2$	8.8
	$\text{U}^{4+}(\text{C}_2\text{H}_2\text{O}_4)_3$	6.0

4.9 Cell enumeration

4.9.1 Introduction

Cellular growth in culture medium was monitored using the Acridine Orange Direct Count (AODC) procedure. A modified protocol of the American Society of Testing and Materials (ASTM, Designation D4454-85) was followed as it enables the detection and enumeration of aquatic bacteria. The materials used and the procedures followed for AODC analysis are outlined below.

4.9.2 Apparatus and materials used for analysis

1. Fluorescence microscope (ZEISS, West Germany) with an immersion objective lens (1000 x magnification) and a high pressure mercury lamp (200 W) as UV light source.
2. 25 mm vacuum filtration funnel/support assembly (Baxter Scientific, McGaw Park, IL)
3. Sterile, 0.2 μm pore size, 25 mm diameter Polycarbonate membrane black filters (Costar company, Cambridge, MA).
4. 3"x1" micro cover slides and 18 mmx18mm cover glasses (Baxter scientific products, McGaw park, IL).
5. Immersion oil (Stephens Scientific, Riverdale, NJ), syringes and needles.
6. Acridine Orange dye (Baxter Scientific, McGaw Park, IL).

4.9.3 Reagents

Phosphate buffer containing 40.83 g KH_2PO_4 and 121.9 g K_2HPO_4 per liter D.I water. pH of the buffer was adjusted to 7.2 using 0.1 M NaOH.

Acridine Orange Dye Solution was prepared by adding 0.1 g acridine orange dye to 100 ml phosphate buffer and diluting it to 1 liter with D.I water.

4.9.4 Staining procedure and slide preparation

The polycarbonate membrane was placed on the vacuum filter unit and soaked with 2 ml of phosphate buffer for ~ 1 minute. The buffer was then vacuum filtered. One ml of growth media containing bacteria was dispensed into the filter funnel and vacuum filtered. Cells retained on the membrane were then soaked in 3 ml acridine dye solution for ~ 3 minutes. The excess dye was filtered, the membrane removed and air dried for 15 seconds then placed on a clean microslide containing ~ 2 drops of immersion oil. Another drop of the oil was added to the top of the membrane which was then covered with the cover slip.

4.9.5 Counting cells using microscope

The cover slide containing the membrane was placed on the mount of the microscope and stained cells were counted using incident fluorescent illumination set at a violet light wavelength (410 nm). The cells were counted randomly in 25 fields within

the stained portion of the membrane. The bacterial density of the sample was calculated using the formula

$$\text{Number of bacterial cells / ml} = 1.77 \times 10^4 \text{ n/d} \quad (11)$$

where,

n = average number of bacteria counted per micrometer field

d = dilution factor of the sample

1.77×10^4 = membrane conversion factor based on the wet area of 25 mm membrane, micrometer area and cover slip thickness.

4.10. Kinetic parameters for U^{6+} reduction

A Michaelis-Menten kinetic expression based on the hyperbolic rate model (Goring, 1972) was used in the prediction of kinetic parameters for hexavalent uranium reduction in this research. According to this model, the rate of U^{6+} reduction can be expressed as

$$\frac{d[\text{U}^{6+}]}{dt} = \frac{V_m [\text{U}^{6+}]}{(K_m + [\text{U}^{6+}])} \quad (12)$$

where

t = time of cell incubation (hour)

V_m = maximum substrate utilization rate (mg U^{6+} /mg biomass-hr)

K_m = half saturation coefficient (mg/L)

X = biomass dry weight (mg)

$[U^{6+}]$ = hexavalent uranium concentration (mg/L)

The above equation was then linearized upon integration to aid in parameter estimation. The linearized form obtained is (Sulfito, 1983; Barrio-Lage, 1986):

$$\frac{t}{[\ln(S_o/S_t)]} = \frac{1}{V_m} X \frac{[S_o - S_t]}{[\ln(S_o/S_t)]} + \frac{K_m}{V_m} \quad (13)$$

Where

S_o = Initial U^{6+} concentration (mg/L)

S_t = U^{6+} concentration after incubation time 't' (mg/L)

The U^{6+} level measured over time after cell inoculation in each experiment was fitted to the equation by treating the terms in the square brackets as independent variables and $1/V_m$ and K_m/V_m as the parameters to be estimated. A graphics program (Sigma Plot, Jandel Scientific) was used to obtain the kinetic parameters V_m and K_m by linear regression analysis. Subsequently, the initial rate of U^{6+} reduction

$$K_i = \frac{(V_m \times S_o)}{(K_m + S_o)} \text{ mM h}^{-1}$$

was calculated to aid in the comparison of U^{6+} reduction obtained in various experiments. A higher value of K_i indicated faster reduction of hexavalent uranium in the test solution. The cell numbers were converted to dry weight using a conversion of 10^{-7} mg/cell (Rothestein, 1948). An initial cell concentration of 5×10^8 cells/mL introduced into the test solutions was used in parameter estimation. Note that test solutions for this research

were maintained under non-growth conditions. A fraction of cells in the solutions may decay under such conditions. However, for comparison of U^{6+} reduction, kinetic parameters were estimated using the initial cell concentration provided in the test solutions.

4.11 Statistical methods

The trends in U^{6+} reduction obtained in various experiments were compared using a one way analysis of variance procedure (ANOVA) to determine statistical differences. In all analyses, a null hypothesis (H_0) that no differences in trend occurred was tested. A failure of null hypothesis indicated a significant difference in the trends. In statistical analysis, the probability of rejecting the null hypothesis when it is true is often called significance level (α) of the test. Unless mentioned otherwise, in all the analysis the probability was maintained at 5% ($\alpha = 0.05$) in this research. Level of probability Duplicate values of U^{6+} concentrations obtained at each time period in different experiments were used in data analysis. The Statistical Analysis Systems (SAS) program was used for analysis. SAS analysis yielded the level of significance (p) at which values were significantly different. A value of $p < 0.05$ indicated that the trends were different at a 5 % level of significance.

In addition, a non-parametric statistical analysis 'Cox and Struat test' was performed to test if the U^{6+} reduction trends observed in various experiments were

statistically different (Conover, 1980). In this analysis, the differences in % U^{6+} reduction at different time periods between the experiments were calculated. Subsequently, the differences were analyzed to determine if they increased significantly over time. An increase in the differences indicated a significant difference in the rate of U^{6+} reduction between the experiments. The trends were determined at 5 % level of significance ($\alpha = 0.05$). Data for statistical analysis were obtained from the binomial distribution table (Conover, 1980). A sample procedure and results from statistical analysis are shown in Appendix VII.

In addition, the U^{6+} levels measured in unfiltered and filtered test solutions in individual experiments were analyzed to see if significant levels of U^{6+} sorbed to cells in the test solution.

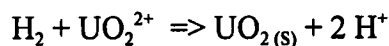
5. CHEMISTRY OF URANIUM IN ORGANIC TEST SOLUTIONS

5.1 Introduction

In this chapter, an attempt has been made to theoretically predict various chemical changes that can occur in organic containing test solutions during hexavalent uranium reduction mediated by H₂ gas oxidation. This exercise is done to aid in the interpretation of results obtained in various experiments performed using the sulfate and iron reducing bacteria. Note that the predictions made in this chapter does not consider the metabolism of microorganisms or the kinetics of various reactions. Only the equilibrium speciation based on the thermodynamic properties were used.

5.2 pH profile during U⁶⁺ reduction

In this research H₂ gas was provided as the electron donor for hexavalent uranium reduction. This reaction is reported to proceed the following stoicheometry (Lovely, 1991)



that is, reduction of one mole of U⁶⁺ would produce 2 moles of H⁺ ions which may impact the pH of the test solution. The magnitude of pH change is governed by the of the organic ligands present. The procedure used to predict the pH profile in acetate

containing test solutions is explained below. pH profile in other test solutions were predicted using the same procedure.

5.2.1 pH profile in acetate solution

The protonation constant (pK_a) of acetate is 4.76 (Stumm, 1980) and the initial pH of the test solution was 6.0. The solution contained an initial U^{6+} concentration of ~ 100 mg/L (0.00042 M) and an acetate concentration of 0.17 M. The distribution of acetate into its deprotonated (acidic, CH_3COOH) and protonated (CH_3COO^-) form at pH 6.0 can be determined by (Snoeyink and Jenkins, 1980)

$$\begin{aligned} \frac{[CH_3COOH]}{[CH_3COO^-]} &= \frac{[H^+]}{K_a} \\ &= \frac{10^{-6.0}}{10^{-4.76}} = 0.057 \end{aligned}$$

(i.e) $[CH_3COOH] = 0.0091 \text{ M}$

$[CH_3COO^-] = 0.1609 \text{ M}$

A reduction of 0.0001 M (~ 23.8 mg/L) U^{6+} would produce 0.0002 M of H^+ ions. Since the hydroxyl ion concentration (10^{-8} M) is negligible compared to acetate concentration, we can assume that the H^+ produced would react completely with acetate. This would result in an increase in the CH_3COOH concentration by 0.0002 M and a decrease in the

CH₃COO⁻ level by the same extent. Hence the pH resulting from the reduction of 0.0001 M of U⁶⁺ will be

$$\begin{aligned} \text{pH} &= \text{pK}_a + \text{Log} \frac{[\text{CH}_3\text{COO}^-]}{[\text{CH}_3\text{COOH}]} \\ &= 4.76 + \text{Log} \frac{0.1598}{0.0092} \\ &= 5.97 \end{aligned}$$

Similarly when 0.0002 M (~ 47.6 mg/L) of U⁶⁺ is reduced the resulting pH would be

$$\begin{aligned} &= 4.76 + \text{Log} \frac{0.1596}{0.0094} \\ &= 5.98 \end{aligned}$$

After 99% of U⁶⁺ reduction, solution pH would be

$$\begin{aligned} &= 4.76 + \frac{0.1592}{0.00978} \\ &= 5.97 \end{aligned}$$

Although ~0.0008 M of H⁺ ion is generated during U⁶⁺ reduction, pH does not change significantly due to high buffering capacity of acetate under test conditions. The protonation constants of the ligands tested are given in Table XIV. The pH profiles calculated using the above procedures for U⁶⁺ reduction are shown in Figures 6 & 7.

Table XIV. Protonation constants of organic ligands used

Ligand	pKa
Acetate	$pK_{a(1)} = 4.76$
Malonate	$pK_{a(1)} = 2.85$ $pK_{a(2)} = 6.10$
Oxalate	$pK_{a(1)} = 1.19$ $pK_{a(2)} = 4.21$
Citrate	$pK_{a(1)} = 3.06$ $pK_{a(2)} = 4.74$ $pK_{a(3)} = 5.40$
Tiron	$pK_{a(1)} = 7.6$ $pK_{a(2)} = 12.5$

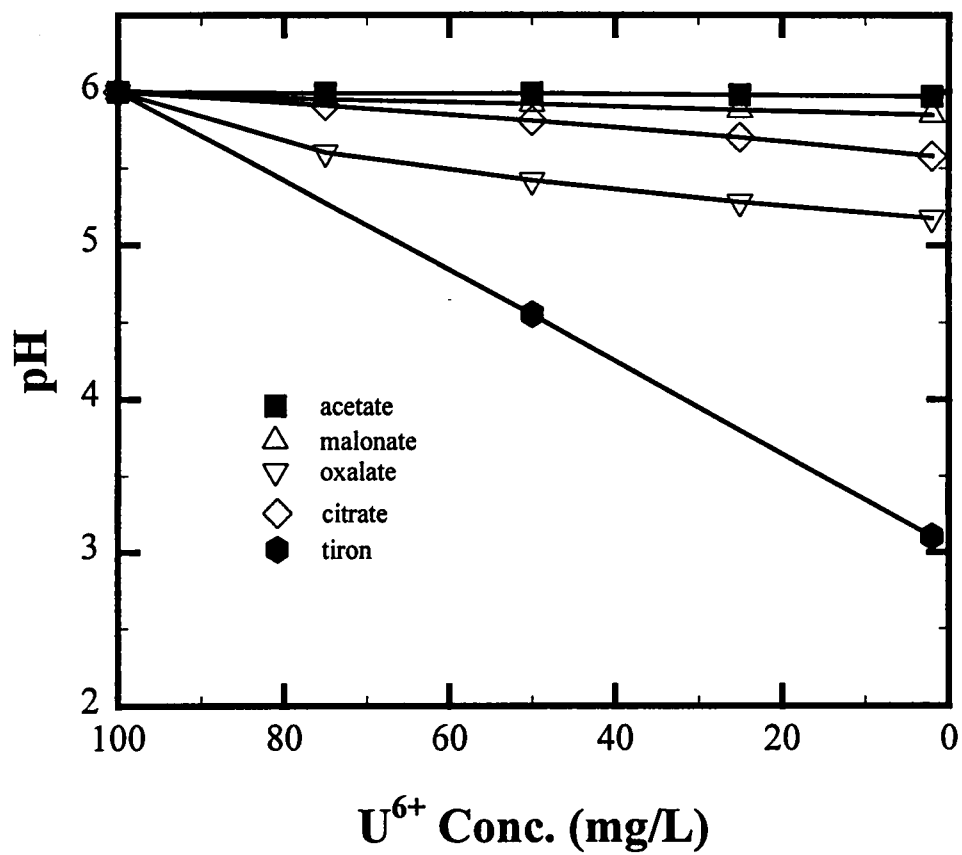


Figure 6. Calculated pH levels in organic test solutions when U^{6+} was reduced to U^{4+} upon oxidation of H_2 gas. Ligand concentrations used initially complexed $\sim 100\%$ U^{6+} in test solutions.

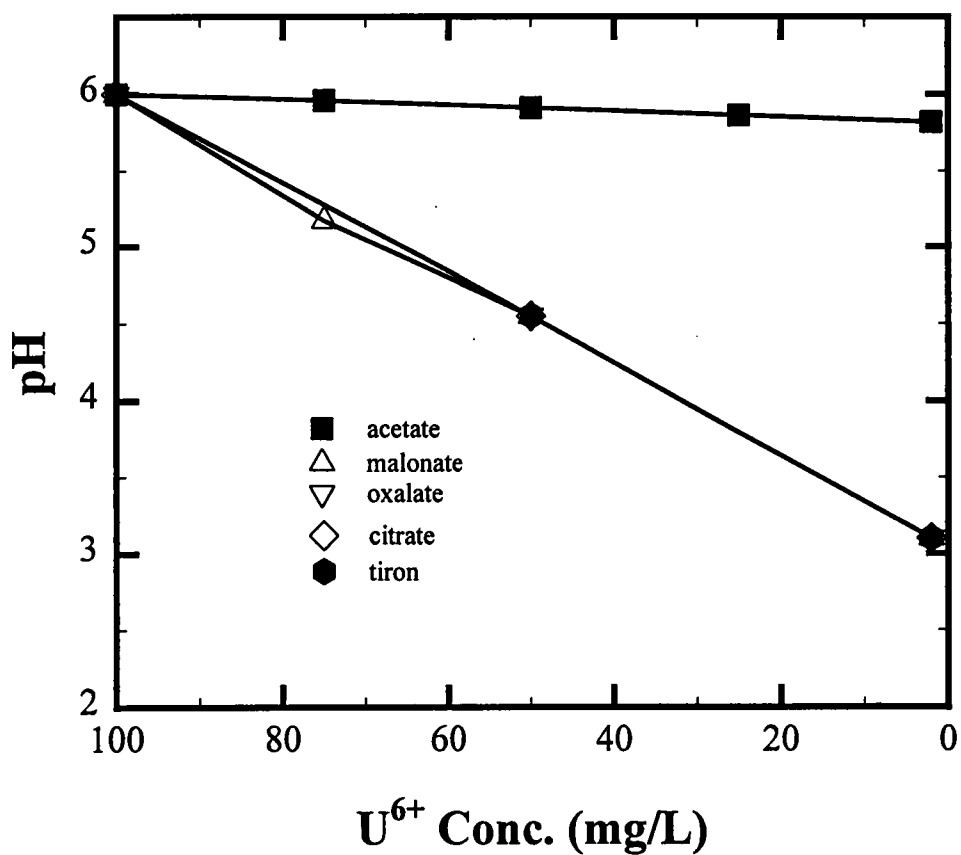


Figure 7. Calculated pH levels in organic test solutions when U^{6+} is reduced to U^{4+} upon oxidation of H_2 gas. Concentrations of ligands used initially complexed $\sim 50\%$ U^{6+} in test solutions.

Calculations indicated that in iron containing test solutions and solutions containing lower concentrations of malonate, oxalate and citrate the ligands used were not sufficient to buffer the H^+ ion produced, resulting in rapid decrease in pH upon U^{6+} reduction.

5.3. Distribution of UO_2^{2+} -organic species during U^{6+} reduction

A major goal of this research was to study the impact of UO_2^{2+} speciation on bioreduction. As discussed in Section 4.4, pH of the solution often impacts speciation. The % distribution of $(UO_2)_x(Organic\ ligand)_y$ species for various levels of U^{6+} reduction was calculated using the pH profile predicted for U^{6+} reduction. The calculations were made by MINTEQA2 using the stability constants obtained from literature and those estimated using the empirical relationship (Equation 10). The distribution obtained are shown in Figure 8. The predictions indicated that, at the highest concentrations of ligands used, the % distribution of $(UO_2)_x(Organic\ ligand)_y$ did not change significantly during U^{6+} reduction. However, at the lower concentrations of ligands used, the distribution varied during U^{6+} reduction. As shown in Figure 8 solutions initially contained ~ 50 % of the added U^{6+} as $(UO_2)_x(Organic\ ligand)_y$ species. The remaining U^{6+} formed various uranyl hydroxide species. Reduction of ~ 0.0002 M of U^{6+} decreased solution pH. This resulted in an increase in the % distribution of remaining U^{6+} into $(UO_2)_x(Organic\ ligand)_y$ species by ~ 90 %. A rapid decrease in pH decreased OH^- concentration resulting in an increase in $(UO_2)_x(Organic\ ligand)_y$. After ~ 99 % of U^{6+}

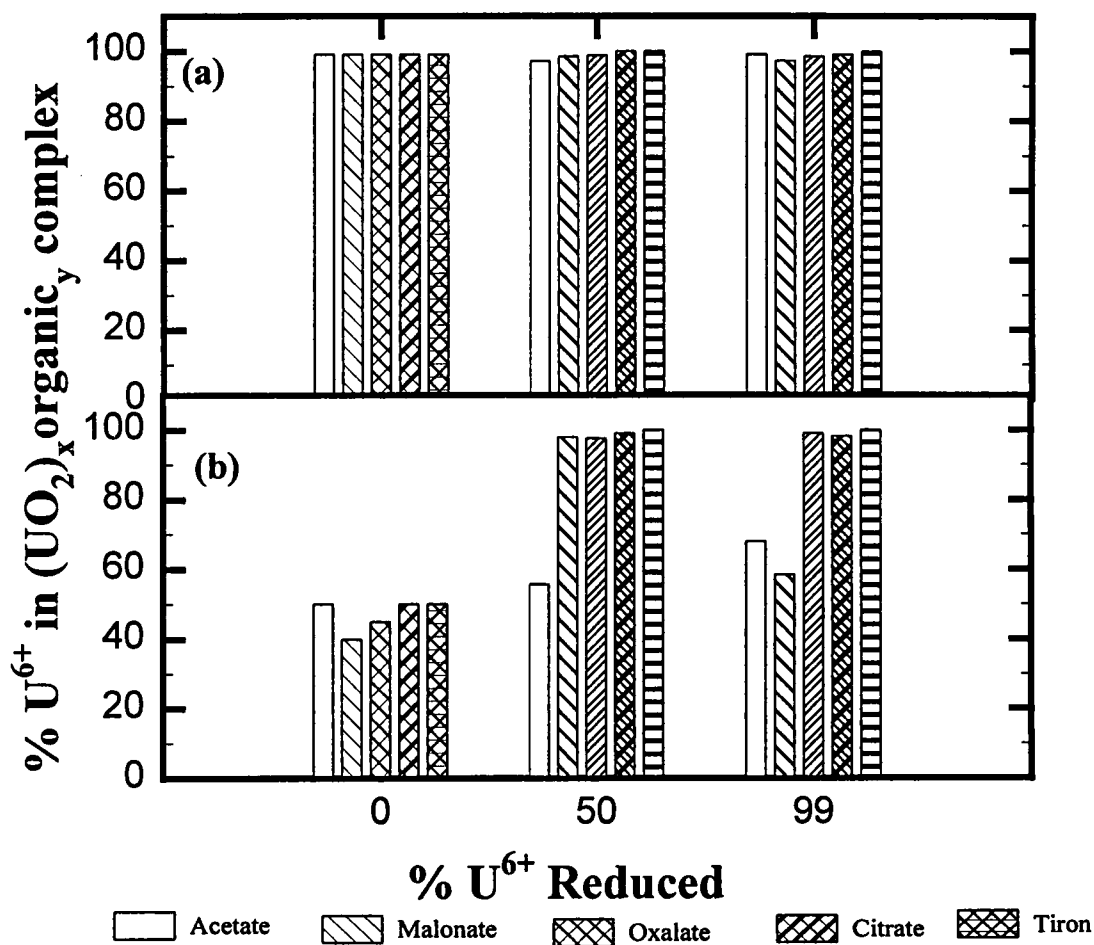


Figure 8. % Aqueous uranium predicted to form $UO_2)_x$ -Organic_y complexes at various stages of U^{6+} reduction. (a) Ligands initially complexed ~100% U^{6+} . (b) Ligands initially complexed ~50% U^{6+} .

reduction, solution pH was ~ 3.1 and a significant fraction of remaining U^{6+} formed $(UO_2)_x(\text{Organic ligand})_y$ species and a small fraction of U^{6+} formed uncomplexed, unhydrolyzed UO_2^{2+} species.

5.4 Distribution of reduced uranium (U^{4+}) in test solutions

As discussed in section 4.8, the association constants of Th^{4+} to complex with test ligands were used in the prediction of U^{4+} speciation. Predictions were made for acetate, malonate and oxalate containing solutions. The association constants for citrate and tiron were not available and hence no predictions were made. The final pH shown in Figures 6 & 7 were used in the calculations. Predictions made using MINTEQ indicated that, in acetate, malonate and oxalate solutions, at either concentrations of ligands used, all of the reduced uranium should precipitate from solution as uraninite (Figure 9).

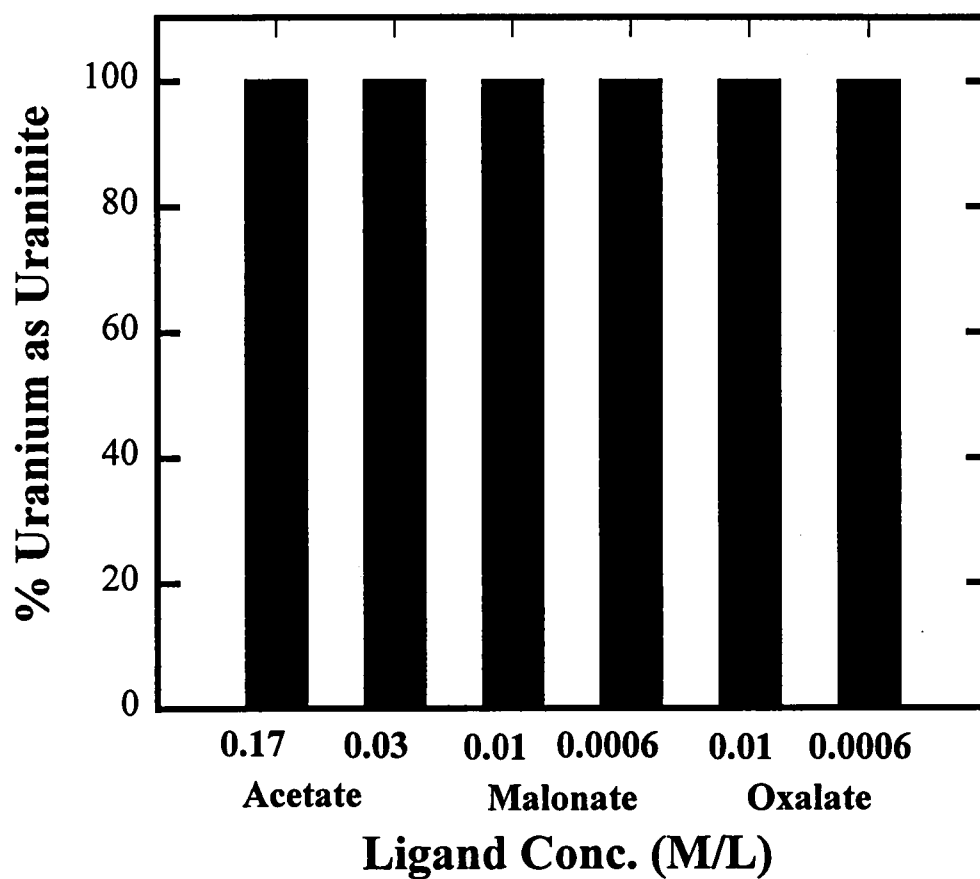


Figure 9. % of total uranium predicted to precipitate as uraninite in test solutions. Calculations were made using Th^{4+} - organic association constants and final pH predicted to prevail in test solutions.

6. CHARACTERIZATION OF MICROBIAL GROWTH

6.1 Introduction

Results of the studies conducted to characterize growth of the two bacteria are presented and discussed in this section. These studies were performed as batch experiments, where the bacteria were grown in closed pressure tubes. Both *D. desulfuricans* and BrY grew asexually by binary fission using the substrate in the growth medium. Growth of the bacteria was monitored by measuring cell numbers using the AODC technique. An estimate of growth doubling time has been made for the two bacteria. In addition, the efficacy of the bacteria to reduce uranium at different phases of growth was determined and the results are presented. Finally, based on these studies, a choice was made on the growth phase and the number of cells to be used in subsequent uranium reductive precipitation experiments.

6.2 Growth of *D. desulfuricans*

Figure 10 shows the pattern of *D. desulfuricans* growth over time in the nutrient rich medium. Lactate served as the electron and carbon source and sulfate was the electron sink. The medium contained an initial concentration approximately 0.8×10^3 cells/ml. During the first fourteen hours of incubation lag phase of growth was observed.

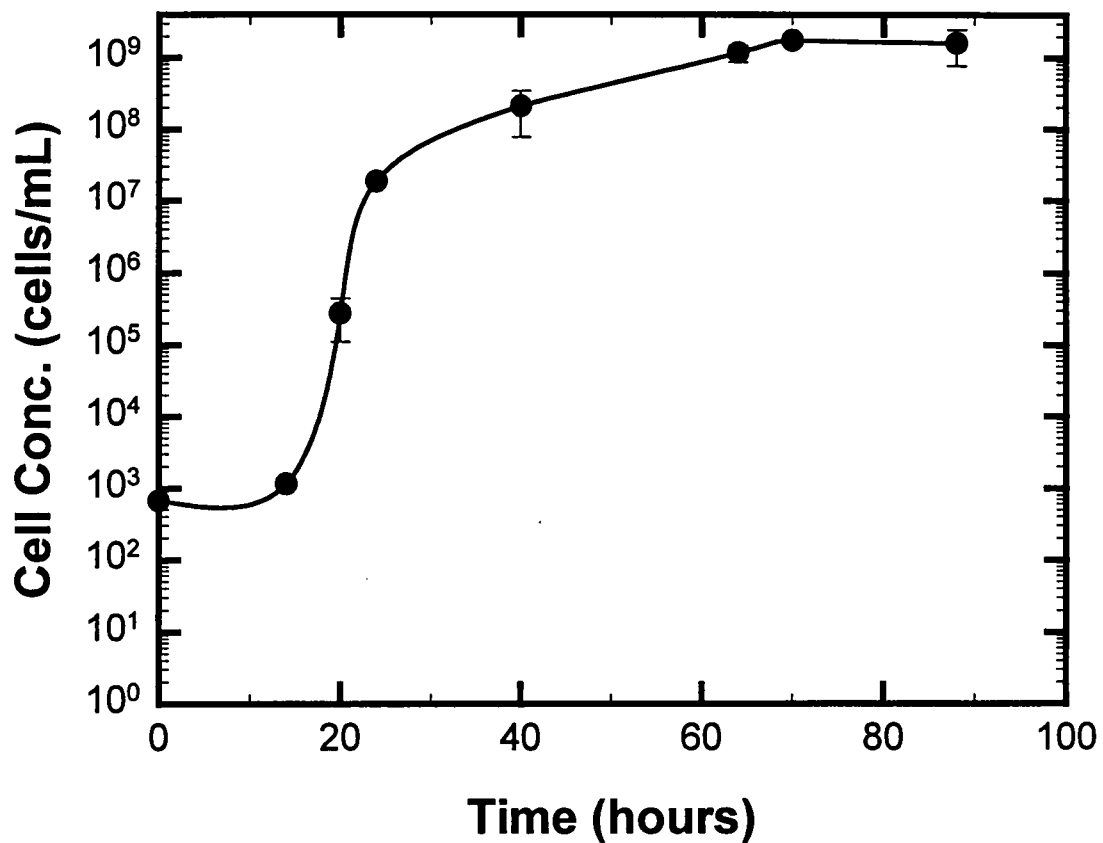


Figure 10. Growth pattern of *Desulfovibrio desulfuricans* in nutrient rich medium. Lactate was the carbon/energy source and sulfate was the terminal electron acceptor.

During lag phase, microorganisms acclimate to the new environment and develop enzyme systems to metabolize the available substrate. Therefore, lag phase is characterized by longer cell generation time and slower rate of growth.

Cell numbers increased exponentially from 1.2×10^3 at 14 hours to 1.85×10^7 cells/ml after 24 hours of incubation. During this phase, the acclimated bacteria grew at a maximum rate, utilizing the unlimited supply of nutrients in the medium. The magnitude of exponential growth rate is generally expressed in terms of cell doubling time. A low doubling time would indicate a high rate of cellular growth. A doubling time of 0.71 hour for *D. desulfuricans*, at log phase (between 14 and 24 hours of incubation) under the growth conditions used in this research, was calculated using the formula

$$T_d = \frac{T_l \times \text{Log } 2}{\text{Log } X_f - \text{Log } X_i} \quad (14)$$

Where,

T_d = Cellular doubling time (hours)

T_l = Duration of log phase (hours)

X_f = Cell concentration at the end of log phase (cells/ml)

X_i = Cell concentration at the beginning of log phase (cells/ml)

The doubling time for microorganisms vary from a few minutes to a few days (Metcalf and Eddy, 1980). It varies with the type of microorganism, substrate used, energy yielded from substrate utilization, initial substrate level, temperature, pH, reactor

conditions (batch or continues flow) and initial cell concentration. Doubling times for the sulfate reducing bacteria *Desulfuromonos acetoxidans* (Roden and Lovely, 1993), the iron reducing bacteria BrY (Caccavo *et. al.* 1992) and the iron reducing bacteria *Geobacter metalireducens* (Lovely *et. al.* 1991) under anaerobic conditions have been reported as 12.9, 24 and 3.2 hours respectively. All of these studies were conducted with an initial cell concentration of approximately 10^7 cells/ml which is about four orders of magnitude higher than the initial cell concentration used in this research. These doubling times are more than an order of magnitude longer than that observed with *D. desulfuricans* in this research. In addition to the difference in the type of bacteria and substrate used, a possible limitation in nutrient level caused by high initial cell concentrations could have resulted in the high doubling times in their research. In evaluating various cell growth characteristics, it is essential to note that the growth pattern of bacteria in a closed system is not only a basic property of the cells but also a result of their interaction with the environment in the closed system (Monod, 1949).

After 24 hours of incubation, a decrease in the rate of *desulfuricans* growth was observed. Cell numbers gradually increased to 1.5×10^9 cells/ml after 78 hours of incubation. This declining growth phase is generally characterized by a decrease in growth rate due to exhaustion of substrates and/or nutrients required for growth (Monod, 1949).

Between 70 and 88 hours of incubation, the cell concentration remained unchanged. This stationary phase is characterized by exhaustion of a limiting nutrient

and crowding of cells in the medium. The medium contained approximately 2×10^9 cells/ml of *desulfuricans* during this phase. Wilkinson (1975) reported that the constant cell concentration during this period can either result from a balance between the growth and decay of cells in the medium or as a result of cells remaining in a state of suspended animation.

6.3 Efficacy of uranyl ion reduction by *D. desulfuricans*

As a part of the cell characterization study, the ability of the bacteria to reduce hexavalent uranium at different stages of growth was evaluated. U^{6+} reduction using cells grown for 40, 64 and 88 hours was tested. At the former two time periods, cells were growing at a steady but slow (declining growth) rate. In the later time period, cells were in stationary phase. At each selected time, cells from six pressure tubes were centrifuged, washed and resuspended in six additional pressure tubes containing 100 mg/L uranium and 10 mM lactate in a bicarbonate (2.5 g/L) test solution. The initial cell concentrations used in the three time periods were 2.0×10^8 , 8×10^8 and 1.7×10^9 cells/ml, respectively. Prior to discussing the results of these experiments, it is to be noted that, at this early stage of this research, a deoxygenation unit located in the Applied Microbiology Laboratory at the Center for Environmental Microbiology, University of Tennessee, Knoxville, was used for preparing growth medium and test solutions. Lactate and bicarbonate solutions were deoxygenated, however, the uranium stock (2 g/L) used to

prepare test solutions for this experiment was not deoxygenated due to possible contamination of the equipment. As a result, the final test solutions contained trace amounts of dissolved oxygen. Since trace levels of dissolved oxygen may impact uranyl ion reduction, exact performance could not be determined. However, the *relative* impact of various cell growth phases on U^{6+} reduction could be evaluated. Figure 11 shows the decrease in aqueous U^{6+} concentration in test solutions inoculated with cells grown to various stages. Uranyl ion formed monodentate, polymeric uranyl carbonate complexes in these solutions. In the experiment conducted with cells grown for 40 hours, the hexavalent uranium level decreased from 88 mg/L to 27 mg/L in one day. Uranyl ion concentration did not decrease any further even after four days of incubation in the test solution. The 64 hour grown cells decreased aqueous U^{6+} from an initial concentration of 104 mg/L to approximately 17 mg/L in one day. In the test solutions inoculated with 88 hour grown cells, hexavalent uranium concentration decreased from 100 mg/L to 83 mg/L in one day. The aqueous U^{6+} concentration gradually decreased to 35 mg/L after four days and removal appeared to be linear over time. Cells grown for 64 hours reduced more hexavalent uranium (~ 87 mg/L) on the first day then cells grown for 40 (~ 60 mg/L) and 88 (~ 17 mg/L) hours during the same incubation time period. Based on these results, cells grown for 60 hours, at a concentration of $\sim 5.0 \times 10^8$ cells/ml were utilized in subsequent experiments. It is to be noted that the extent of U^{6+} reduction observed in these characterization experiments was poorer than that reported by other researchers. For example, Lovely et al., (1992b) reported that *D. desulfuricans* reduced 100 - 200

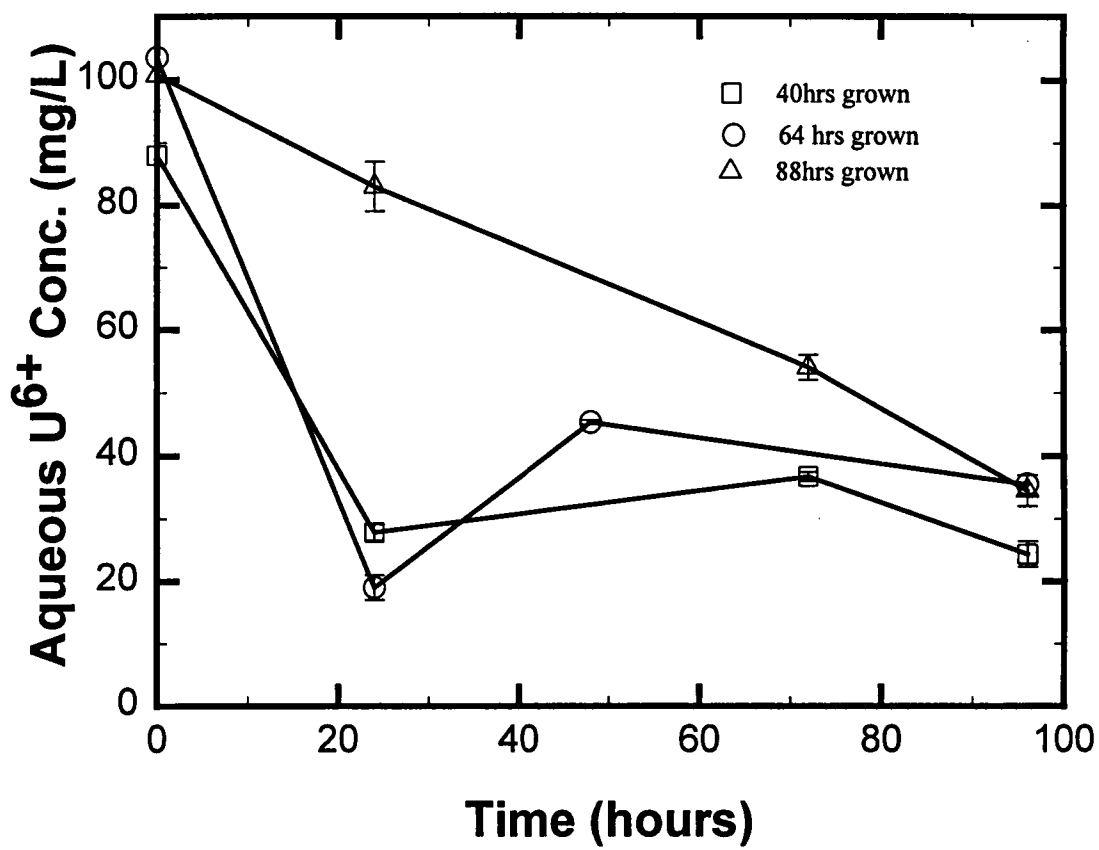


Figure 11. Aqueous U^{6+} levels in test solutions inoculated with *D. desulfuricans* at different growth phases.

mg/L U^{6+} in test solutions within one day. Trace oxygen present in the uranyl ion stock could have resulted in the lower U^{6+} reduction efficiencies observed in the characterization studies.

6.4 Growth of *Shewanella alga* (BrY)

Growth of BrY was characterized using the same procedures as those followed for *D. desulfuricans*. While lactate served as the electron donor, ferric iron was the electron acceptor for BrY growth. Figure 12 shows the pattern of BrY growth. The medium contained an initial cell concentration of approximately 1.5×10^3 cells/ml. The cell numbers rapidly increased to 1×10^8 cells/ml within the first 8 hours, with no apparent lag. After 8 hours, the rate of growth decreased and the medium contained nearly 7×10^8 cell/ml after 21 hours. No appreciable change in cell numbers was observed between 21 and 72 hours of incubation. In addition, the color of the medium also changed as the cells grew. Initially, the medium was dark brown in color due to the presence of excessive ferric iron. After 22 hours of growth, the medium turned greenish yellow, then became colorless after 24 hours. The color change was believed due to reduction of ferric iron over time. The cells produced a black precipitate after growing for about 30 hours in the medium. The composition of the precipitate is not currently known.

As can be seen from Figures 10 & 12, the growth of BrY differed from that of *D. desulfuricans* in some aspects. Unlike *D. desulfuricans*, BrY did not require an initial

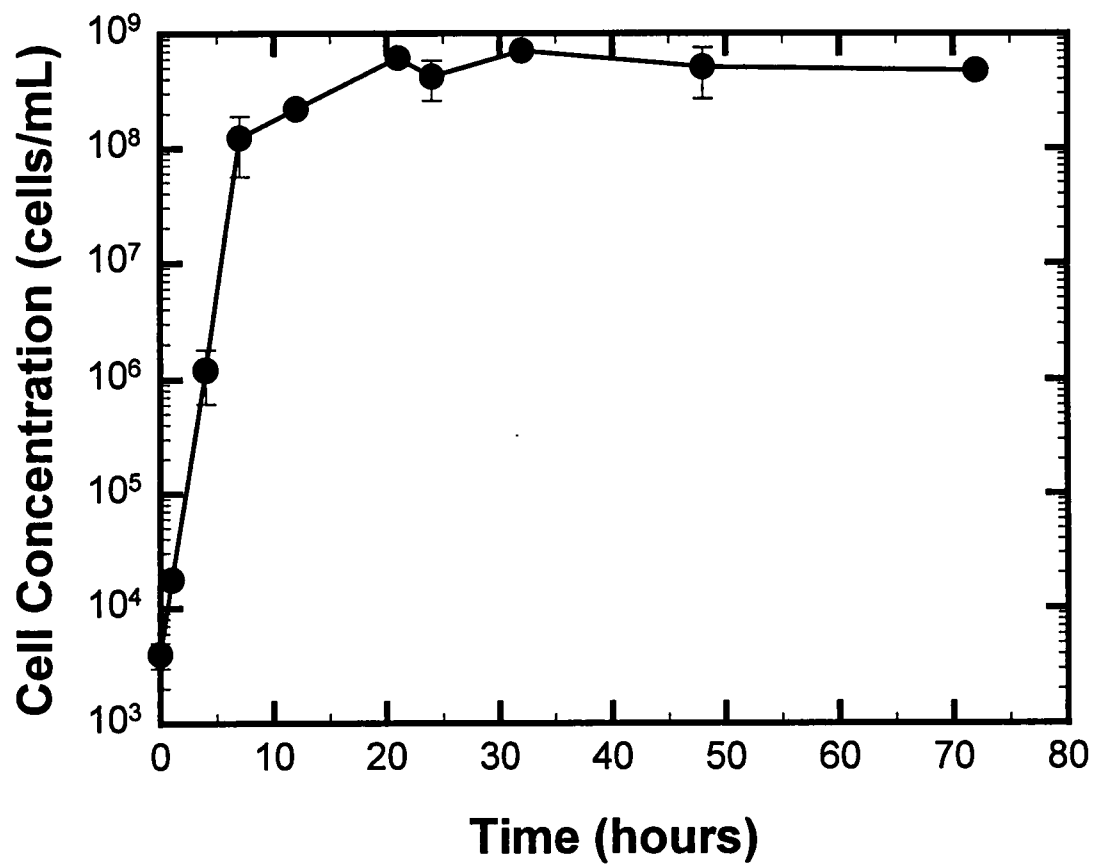


Figure 12. Growth of BrY in nutrient rich medium. Lactate was carbon/energy source and ferric iron was the electron acceptor.

acclimation period (lag phase). Monod (1949) reported that certain bacteria whose enzyme systems are well developed do not require an acclimation period to grow. In addition, BrY did not require a lag period to grow with poorly crystalline ferric oxide as the electron acceptor (Caccavo, 1992). During exponential growth, BrY grew faster than *D. desulfuricans*. The BrY doubling time was 31 minutes while *desulfuricans* doubled every 43 minutes. BrY yielded a free energy of 158 kJ for oxidation of one mole of lactate using ferric iron as the electron sink, while *desulfuricans* yielded 104 kJ for lactate oxidation (Stumm and Morgan, 1981). Metcalf and Eddy (1980) reported that, under similar environments, microbial growth would be directly proportional to the energy obtained from substrate utilization. The difference in growth rate observed between the two bacteria could have been caused by the difference in energy yielded during lactate oxidation.

6.5 Uranium reduction by BrY

The ability of BrY to reduce hexavalent uranium after 24 hours (declining growth phase) and 48 hours (stationary phase) of growth was evaluated. As can be seen in Figure 13, hexavalent uranium levels decreased from ~ 85 mg/L to ~ 25 mg/L in four hours of incubation in the test solutions. Most of the U^{6+} initially present was reduced within 24 hours. In this and subsequent U^{6+} reduction experiments, test solutions were prepared using a new deoxygenation unit established at the Center for Environmental

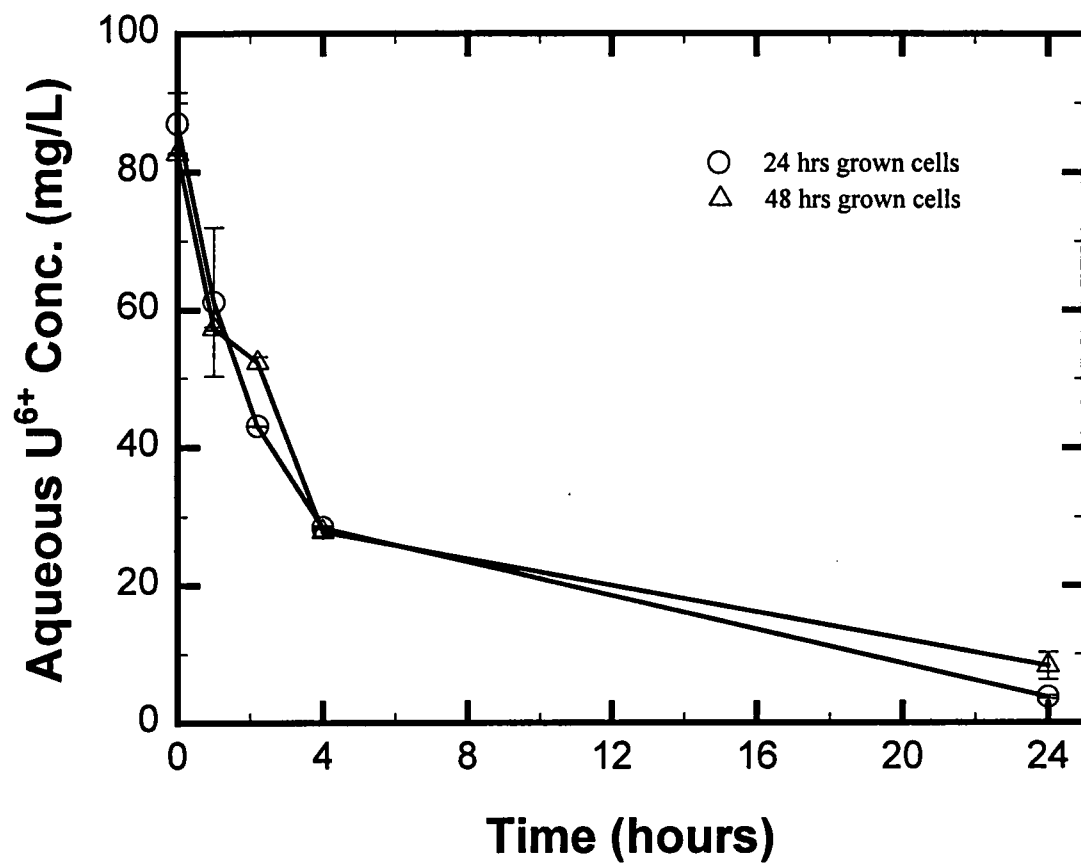


Figure 13. Aqueous U^{6+} level in test solutions inoculated with BrY at different growth phases.

Biotechnology laboratory located at the University of Tennessee. This unit was used to deoxygenate all solutions including the uranium stock. The rate of uranium reduction observed was similar to that reported by Caccavo et al. (1992). In addition, no appreciable difference in the rate of uranium reduction was observed between the cells grown to two different phases. However, as mentioned in section 5.4, after 30 hours of cell growth, a black precipitate formed in the growth medium. Attempts to isolate cells from the precipitate prior to incubation in U^{6+} containing test solutions were not successful. Note that such a precipitate did not form at 24 hours of cell growth. In order to eliminate any possible influence of this precipitate, it was decided to use cells grown for 24 hours in experiments with organically complexed uranium. The growth medium contained a cell concentration approximately 5×10^8 cells/ml after 24 hours of incubation which was used to inoculate subsequent U^{6+} -containing test solutions.

In summary, cells in a declining growth phase (60 hours for *D. desulfuricans* and 24 hours for BrY) at a concentration of $\sim 5 \times 10^8$ cells/ml were used in all subsequent uranium reduction experiments.

7. REDUCTIVE PRECIPITATION OF ORGANICALLY COMPLEXED URANYL ION BY *D. desulfuricans*

7.1 Introduction

In this chapter, results of various experiments conducted to evaluate uranium bioreductive precipitation using the sulfate reducing bacteria *D. desulfuricans* are discussed. In addition, results of experiments performed to identify possible mechanisms of U^{6+} reduction are presented and discussed. All U^{6+} bioreduction tests were conducted as batch experiments in 25 ml anaerobic pressure tubes, using 10 ml volume test solutions at 32° C. Hexavalent uranium (~ 100 mg/L) was used as the primary electron acceptor and H_2 gas was provided as the electron donor for the bacteria. Ligand concentrations were adjusted such that, a) essentially all of the hexavalent uranium (U^{6+}) initially added was complexed by the ligand, or b) approximately 50% of the U^{6+} was initially complexed. Sodium nitrate was added as the background electrolyte to maintain a constant ionic strength (0.17 M) in all experiments, unless stated otherwise. Number (~ 5×10^8 cells/ml) and growth phase (late log) of cells added to each test solution were similar. Test solutions contained no other nutrients, vitamins or minerals to aid in microbial activity. In each of the experiments, U^{6+} concentrations in unfiltered and filtered (0.2 μm) samples as well as total uranium ($U^{6+} + U^{4+}$) concentrations in unfiltered and filtered samples were determined. Ligand concentrations were monitored during microbial utilization of uranyl ion. An experiment was also conducted in which the test

solution contained only the background electrolyte, H₂ gas and hexavalent uranium but no organic ligand.

7.2 Uranium biotransformation in organic free (NaNO₃) solution by *D. desulfuricans*

In this section, results from U⁶⁺ bioreductive precipitation experiments performed using organic free (NaNO₃) test solutions are presented. Procedures used to determine the distribution of U⁶⁺ and bioreduced uranium (U⁴⁺) are explained. A detailed interpretation of the trends observed during biotransformation also are presented. Note that the same procedures used in data interpretation in this experiment were used in all experiments performed using *D. desulfuricans* and BrY in this research. Hence these procedures will not be reiterated during the discussion of the trends observed in subsequent experiments. The organic free test solution initially contained uranyl nitrate (~ 100 mg/L U⁶⁺) and the background electrolyte (NaNO₃) at an ionic strength of 0.17 M. MINTEQ predicted that, upon introduction, uranyl ion formed monodentate, polymeric hydroxyl complexes ((UO₂)_x(OH)_y) in the test solution. After cell inoculation, the amount of U⁶⁺ reduced by microorganisms was determined by monitoring the decrease in U⁶⁺ level over time. Figure 14 depicts the biotransformation of U⁶⁺ in organic free test solution upon inoculation with *D. desulfuricans*. The unfiltered test solution initially contained ~ 90 mg/L hexavalent uranium. After four hours of incubation, the test solution contained only ~ 5 mg/L U⁶⁺ indicating that >90 % of the hexavalent uranium

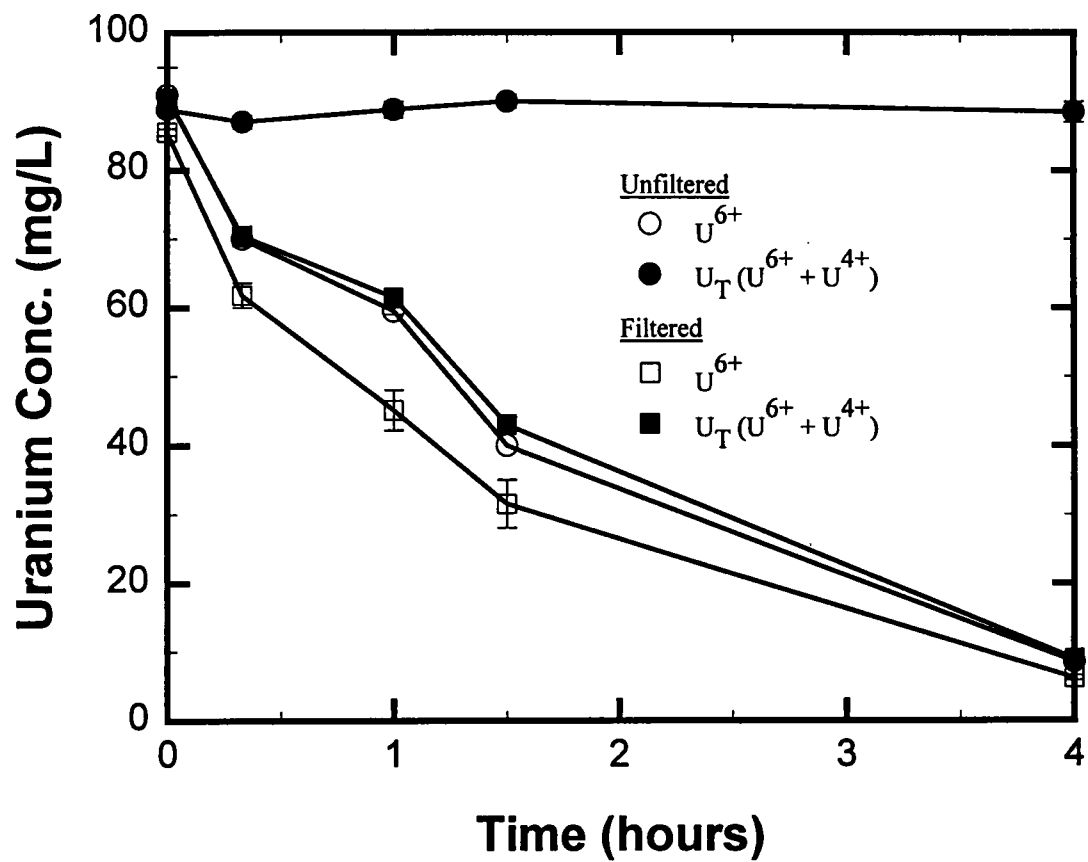


Figure 14. Uranium distribution in organic free ($NaNO_3$) solution after inoculation with *D. desulfuricans*.

initially present was reduced by the sulfate reducing bacteria. Results indicated that hexavalent uranium from monodentate, polymeric uranyl hydroxide species was readily bioavailable for reduction by *D. desulfuricans*. This trend was similar to the U^{6+} reduction observed in bicarbonate solution, where uranyl ion formed polymeric monodentate complexes with carbonate (Lovely, 1992a). In addition, near identical U^{6+} reduction trends observed in carbonate solutions (Lovely, 1992a) and nitrate solutions (this research) indicated that presence of nitrate had no negative impact on uranyl ion bioreduction by *D. desulfuricans*.

The fraction of U^{6+} dissolved in test solution was determined by measuring the U^{6+} level in the filtrate (0.2 μ m) during cell incubation. As can be seen from the figure, U^{6+} level in the filtrate decreased from 88 to 5 mg/L within four hours. The difference between U^{6+} level in unfiltered and filtered test solutions yielded the amount of U^{6+} sorbed to various solids (biomass, uraninite, filter material) during bioreduction. During the initial stages of incubation (~ 2 hours) the U^{6+} level in the filtrate was slightly lower than that in the unfiltered solution. However, after four hours, the U^{6+} levels were essentially the same. Statistical analysis of the data indicated no significant difference in U^{6+} levels between unfiltered and filtered test solutions after four hours of incubation. Results indicated that little, if any, U^{6+} sorbed to solids after four hours. *D. desulfuricans* did not appreciably sorb U^{6+} from solutions in any of the experiments conducted in this research (Appendix IV).

The precipitation of bio-reduced uranium was determined by measuring the total uranium level ($U^{6+} + U^{4+}$) in each test solution upon oxidation by 0.2N nitric acid. Note that the kinetic phosphorescence analyzer selectively measures only hexavalent uranium in solution. Treatment with nitric acid oxidized tetravalent uranium in the test solution to the hexavalent state. Total uranium ($U^{6+}+U^{4+}$) in solution was then measured as hexavalent uranium using the analyzer. The unfiltered test solution contained approximately 90 mg/L total uranium throughout the test period, which was consistent with the concentration of uranium initially introduced into the test solution. This indicated that acid oxidation effectively converted all forms of uranium to the U^{6+} state and that insignificant amount of uranium was lost due to volatilization. Total uranium in the filtrate decreased from ~ 90 mg/L to 5 mg/ after four hours. The difference in the total uranium level between the unfiltered and filtrate solutions yielded the fraction of uranium sorbed to solids and that precipitated as uraninite and retained on the filter. As mentioned earlier in this section little, if any, U^{6+} sorbed to the solids during bio-reduction, suggesting that the difference in total uranium level yielded the amount of uranium reductively precipitated by *desulfuricans*. Results from organic free experiments indicated that more than 90 % of the uranium reductively precipitated from solution and was retained on the filter. In addition, the total uranium ($U^{6+}+U^{4+}$) concentration in the filtrate was slightly higher than the hexavalent uranium (U^{6+}) concentration for the first ~1.5 hours of this experiment. This difference yielded the amount of tetravalent uranium (U^{4+}) which was dissolved in the test solution. The presence of U^{4+} in the filtrate during

the initial phase of the experiment indicated a small lag period between U^{6+} reduction and initiation of precipitation. However, at the end of four hours, the U^{6+} level and the total uranium level in the filtrate were essentially the same indicating that all of the bio-reduced uranium precipitated from solution. Note that in this experiment, the amount of uranium precipitated was not directly measured but was calculated as the difference in total uranium level between the unfiltered and filtered solutions. However, in a separate experiment performed in the presence of acetate, the amount of uranium retained on the filter upon precipitation was collected, measured and compared with the level calculated by difference. Results (Appendix V) indicated that the levels determined by difference yielded near accurate measure of the uranium precipitated from test solution. Hence, to simplify analyses, precipitated uranium was not directly measured but was calculated by difference in all the experiments in this research. In organic free solution, during the first two hours of incubation, a precipitate was not clearly visible to the naked eye and no appreciable deposition was observed at the bottom of the tubes. However, after approximately three hours of incubation, formation of precipitate was observed and after four hours of incubation, a large quantity of black precipitate (apparently uraninite) was noted at the bottom of the pressure tubes. Similar precipitate deposition was reported by Lovely et al.(1992).

For a better understanding of the procedures used, the distribution of uranium in the test solution, determined by direct measurement and that determined indirectly are

provided in Table XV for three time periods (0, 20 minutes and four hours) during this experiment.

Kinetic parameters for bioreduction of hexavalent uranium for all experiments were obtained using the Michaelis-Menten hyperbolic rate model (Goring, 1972). Figure 15 shows the linearized data for U^{6+} reduction in organic free ($NaNO_3$) solution by *D. desulfuricans*. Regression analysis of the data yielded a maximum substrate utilization rate (V_m) of 3.6×10^{-3} mg U^{6+} /(mg biomass-hour). Note that a conversion value of 10^{-7} mg/cell was used in the rate calculation. The half saturation constant and the initial rate of U^{6+} reduction obtained were 112 mg/L and 1.6×10^{-3} mg/(mg biomass-hr), respectively.

7.3 Bioavailability of U^{6+} to *D. desulfuricans* in organic solutions

In this section, results pertinent to the bioavailability of U^{6+} to *desulfuricans* in the presence of various organic ligands are presented and discussed. The next two sections discuss the mechanisms of hexavalent uranium reduction by *desulfuricans* and subsequent precipitation of bio-reduced uranium in organic solutions. Figure 16 shows the U^{6+} levels in unfiltered test solutions containing various organic ligands after inoculation with *D. desulfuricans*. The ligands were predicted to initially complex ~ 100 % of the U^{6+} introduced. U^{6+} levels observed in organic free ($NaNO_3$) solutions also are presented for comparison. Figure 17 depicts U^{6+} concentration in test solutions where the ligands were predicted to initially complex ~ 50 % of the U^{6+} . In both figures, > 90 % of

Table XV. Distribution of uranium in organic free (NaNO₃) solution after inoculation with *D. desulfuricans*

Time	Measured Uranium Concentration (mg/L)				Calculated Uranium Concentration (mg/L)		
	Total uranium in test solution (i)	U ⁶⁺ in test solution (ii)	U ⁶⁺ in filtrate (iii)	U ⁶⁺ +U ⁴⁺ in filtrate (iv)	U ⁶⁺ sorbed (v) = (ii) - (iii)	U ⁴⁺ in solution (vi) = (iv) - (iii)	U ⁴⁺ precipitated (vii) = (i) - (iv) - (v)
0	91	91	88	90	3	2	0
20minutes	88	68	61	68	7	7	13
4 hours	91	5	4	5	1	1	85

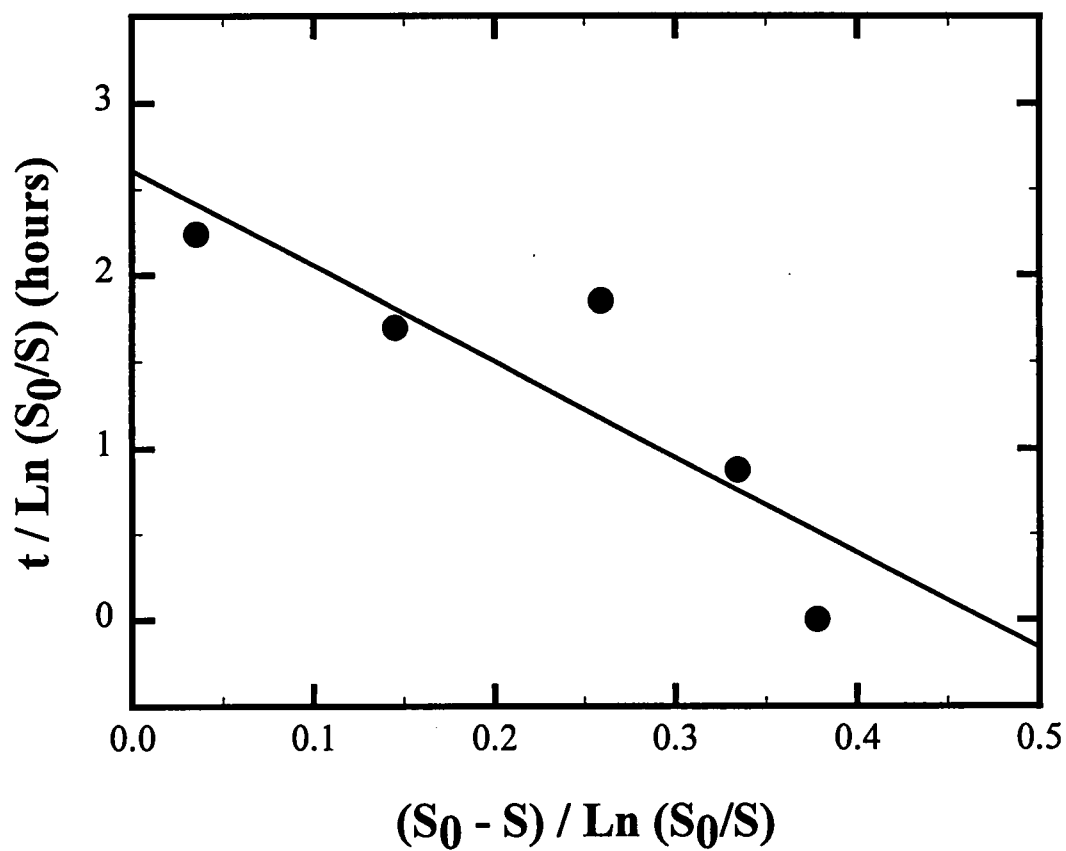


Figure 15. Michealis-Menten fit for U^{6+} reduction in organic free ($NaNO_3$) test solution after incubation with *D. desulfuricans*.

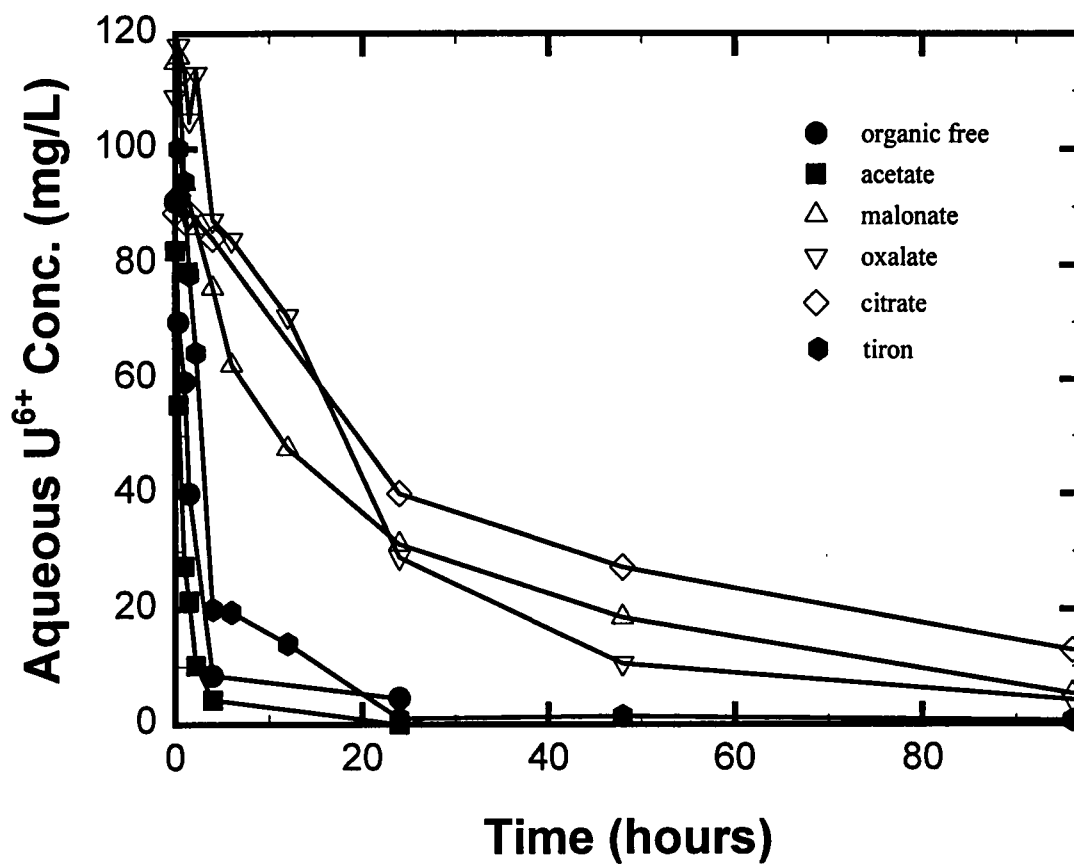


Figure 16. U^{6+} levels in solutions containing various ligands after inoculation with *D. desulfuricans*. Ligands initially complexed $\sim 100\%$ U^{6+} .

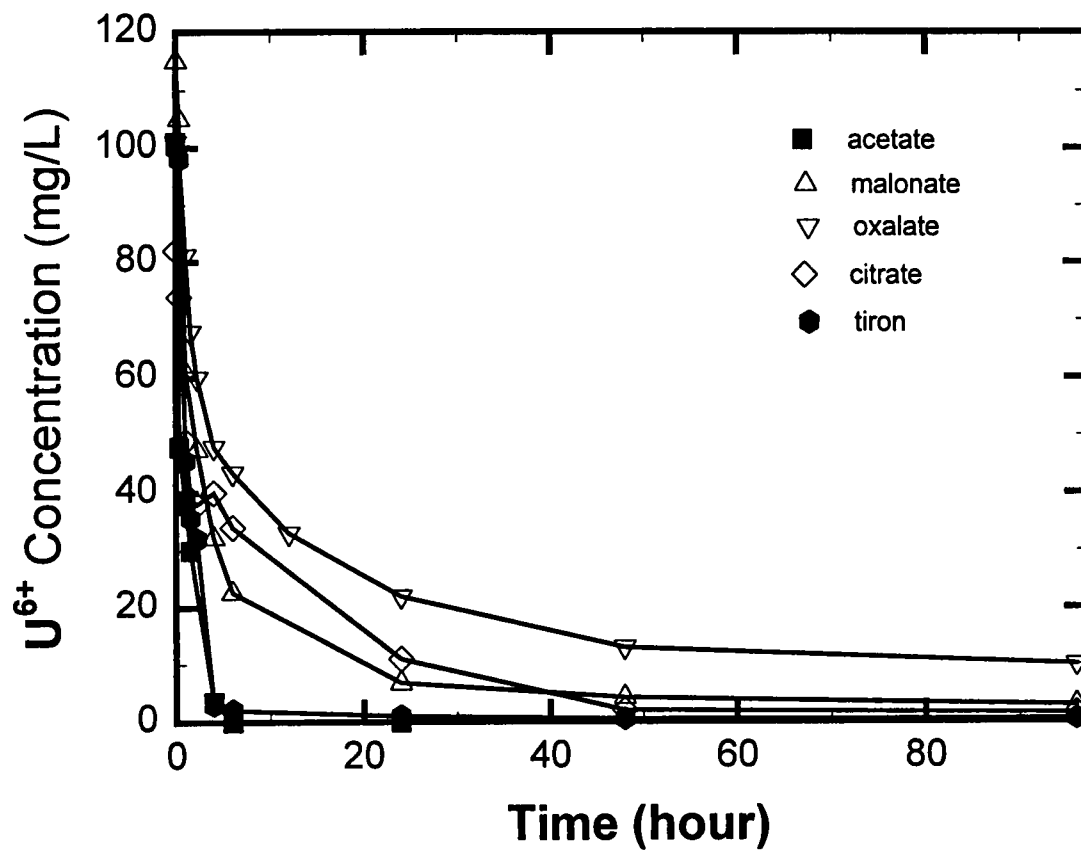


Figure 17. U⁶⁺ concentration in solutions containing various ligands after inoculation with *D. desulfuricans*. Ligands initially complexed ~50% U⁶⁺.

the hexavalent uranium was reduced after 96 hours of cell incubation. Results indicated that U^{6+} was bioavailable to *D. desulfuricans* as electron acceptor in the presence of each ligand tested. The extent of U^{6+} bio-reduced was not affected by the nature of complex formed or the levels of such complexes present. At the highest concentrations of organic ligands used (Figure 16), the bacteria reduced uranyl ion from monodentate acetate, bidentate malonate and oxalate, and bi/polynuclear citrate and tiron complexes. In addition, *desulfuricans* reduced U^{6+} from solutions containing a significant level of (~ 50 %) U^{6+} -organic species and uranyl hydroxide species. A review of literature yielded no information regarding the factors that can impact the bioavailability of metals for reduction (electron acceptors) from metal-organic complexes. However, studies on the bioavailability of organic ligands (electron donors) from such complexes indicated that (i) transport requirements of ligands across cell membrane (Brynhidsen and Rosswall, 1989) and (ii) structural incompatibility of the complexes formed to the active sites of the target enzymes (Joshi-Tope and Francis, 1995) can limit the extent of their bioavailability. Significant level of U^{6+} reduction observed in this research indicated that neither of the two factors prevented *desulfuricans* from using U^{6+} as electron acceptor from organic complexes. Information related to the structural specificity of U^{6+} species to uranium reducing enzyme of *desulfuricans* is not currently available. However, experiments performed by Lovely et al. (1993c) yielded some information related to the transport requirement of hexavalent uranium. The authors reported that *desulfuricans* reduced U^{6+} using the enzyme *cytochrome c3* which is located between the cell membrane and the cell

wall (periplasmic region), and uraninite precipitated extracellularly. This suggested that diffusion across a membrane was not required for uranyl ion bioreduction. While transport across a cell membrane may limit citrate degradation from certain complexes, the lack of such a requirement for transport may have facilitated uranyl ion reduction from all complexes studied.

7.4 Mechanism of U^{6+} reduction in organic solutions

Although *D. desulfuricans* reduced U^{6+} from each of the complex studied, the rate of reduction varied with the type of complex formed. In organic free ($NaNO_3$) solutions and in solutions containing low and high concentrations of acetate > 95% of the U^{6+} was reduced within four hours of cell inoculation. However, in solutions containing high concentrations of malonate, oxalate and citrate, the bacteria required ~ 96 hours to reduce > 90 % of U^{6+} . Based on the stability constants used in this research, MINTEQ predicted U^{6+} to form monodentate complexes in $NaNO_3$ and acetate solutions. At the high concentrations of malonate, oxalate and citrate used a significant level of (> 98 %) of U^{6+} was predicted to form multidentate complexes. Tables XVI & XVII show the kinetic parameters obtained for U^{6+} reduction by *desulfuricans* in various test solutions. The initial rate of U^{6+} reduction (K_i) in solutions containing monodentate U^{6+} species was nearly 20 fold higher than that in solutions predicted to contain multidentate complexes. Statistical analysis indicated that the rates were significantly ($\alpha = 0.05$) different. This

Table XVI. Kinetic parameters for U^{6+} reduction in various test solutions by *D. desulfuricans*. Ligands initially complexed ~ 100 % of the U^{6+} .

LIGAND	Maximum Substrate Utilization Rate. V_M (mg U^{6+} /mg biomass-hr).	Half Saturation Coefficient K_M (mg/L)	Initial U^{6+} K_i (mg U^{6+} /mg biomass-hr)	Reduction Rate
$NaNO_3$	3.6×10^{-3}	112	1.6×10^{-3}	
Acetate	3.2×10^{-3}	57	1.9×10^{-3}	
Malonate	1.7×10^{-4}	104	9×10^{-3}	
Oxalate	1.8×10^{-4}	104	8.9×10^{-3}	
Citrate	1.4×10^{-4}	116	6.0×10^{-3}	
Tiron	1.3×10^{-3}	121	5.7×10^{-4}	

Table XVII. Kinetic parameters for U^{6+} reduction in test solutions by *D. desulfuricans*. Ligands initially complexed ~ 50 % of hexavalent uranium.

LIGAND	Maximum Substrate Utilization Rate. V_M (mg U^{6+} /mg biomass-hr).	Half Saturation Coefficient K_M (mg/L)	Initial U^{6+} Reduction Rate K_i (mg U^{6+} /mg biomass-hr)
Acetate	4.0×10^{-3}	100	1.9×10^{-3}
Malonate	6.4×10^{-4}	105	3.4×10^{-4}
Oxalate	3.8×10^{-4}	117	1.8×10^{-4}
Citrate	2.6×10^{-4}	74	1.4×10^{-4}
Tiron	1.0×10^{-3}	119	4.6×10^{-4}

indicated that, although the extent of hexavalent uranium bioavailability was not affected, certain factors altered the rate of bioreduction by *D. desulfuricans* in multidentate organic solutions. The slower reduction of U^{6+} from multidentate/ binuclear complexes may be due to one or several of the following factors: (i) preferential reduction of nitrate in the background electrolyte over U^{6+} in selected test solutions by the bacterium, (ii) the lack of direct availability of chelated U^{6+} as an electron acceptor (chelate effect), (iii) due to the toxicity of ligands to the microorganisms. A detailed discussion pertinent to the impact of each mechanism is presented in the following paragraphs. Some additional experiments were performed in this research to validate or negate the impact of each mechanism. Results of these experiments also are presented and discussed.

7.4.1 Preferential reduction of nitrate in selected test solutions

Among the test solutions used in this research, organic free ($NaNO_3$) and tiron test solutions contained the highest concentration of nitrate. If indeed the microorganisms preferred nitrate as an electron acceptor over hexavalent uranium (first factor), the impact should be more severe in these two solutions than in malonate or citrate containing solutions. However, results indicated that U^{6+} reduction was significantly faster in $NaNO_3$ and tiron solutions than in malonate, oxalate and citrate solutions. This suggested that the differences in the rate of U^{6+} reduction was probably not due to the presence of nitrate in test solutions.

7.4.2 Toxicity of ligands to microorganisms

Evidence in the literature indicated that either chelation effect (second factor) or toxicity (third factor) can influence the microbial utilization electron donor/acceptor from metal-organic complexes. For example, Firestone and Tiedje, (1975) reported NTA degradation from Cu^{2+} , Cd^{2+} and Zn^{2+} complexes using a *pseudomonas sp.* was not affected by chelation effect but was affected by metal toxicity to the bacterium. However, Joshi-Tope and Francis, (1995) observed that cell free extracts of *P. fluorescens* degraded citrate more rapidly from weaker complexes than from stronger complexes. Their results suggested that chelation retarded the rate of citrate degradation. More importantly, evidence in literature provide conflicting information related to the impact of multidentate aliphatic ligands to sulfate reducing bacteria. Colleran (1995) reported that certain sulfate reducing bacteria can grow rapidly by degrading multidentate aliphatic ligands such as succinate, fumarate, malate, oxalate, citrate using citric acid cycle of metabolism. Their work suggested that these ligands were not toxic to the bacteria. Recently, Roden and Lovely (1993) reported that *D. desulfuricans* grew slowly in nutrient rich media inoculated with 50 mM of citrate. However, the authors did not elucidate if the slow growth was due to ligand toxicity to the bacteria or due to possible chelation of certain metals essential for growth.

In this research, to identify the factor (chelation or toxicity) responsible for slower U^{6+} reduction from malonate, oxalate and citrate complexes additional experiments were performed. The following hypothesis was tested in these experiments. If chelation

limited the reduction of U^{6+} , then *desulfuricans* should readily metabolize an electron acceptor that is not complexed, in the presence of these ligands. On the contrary, if the ligands were toxic, then the reduction of an electron acceptor that is not complexed should also be affected in the presence of these ligands. Thiosulfate ($S_2O_3^{2-}$), an anion which does not complex readily with the organic ligands tested (Sillen and Martell, 1964) but can be utilized by *desulfuricans* as an electron acceptor (Krieg and Holt, 1984), was used to test this hypothesis. Test solutions for thiosulfate experiments were prepared as described in section 4.5 for U^{6+} reduction experiments, with the exception that, instead of U^{6+} , a 0.5 ml of sodium thiosulfate (4 g/L deoxygenated stock) solution was added to each pressure tube resulting in an initial $S_2O_3^{2-}$ concentration of 200 mg/L. Concentrations of ligands that initially complexed $\sim 100\%$ uranyl ion were used. Upon inoculation with *desulfuricans* (5×10^8 cells/mL), subsamples were collected over a period of 24 hours, filtered (0.2 μm) and the filtrate analyzed for thiosulfate concentration using an anion exchange column (Dionex chromatography, Sunnyvale, CA). Figure 18 shows thiosulfate concentration in test solutions after cell inoculation. Bacteria reduced thiosulfate in organic free as well as organic containing solutions. In organic free solution, the thiosulfate concentration decreased from 242 to 145 mg/L in 24 hours (a reduction of ~ 77 mg/L). *Desulfuricans* reduced 73, 118 and 140 mg/L thiosulfate from malonate, oxalate and citrate solutions, respectively, after 24 hours. Although concentrations varied widely in duplicate samples, results indicated that *desulfuricans* can reduce thiosulfate from organic ligand containing solutions as well as organic ligand free

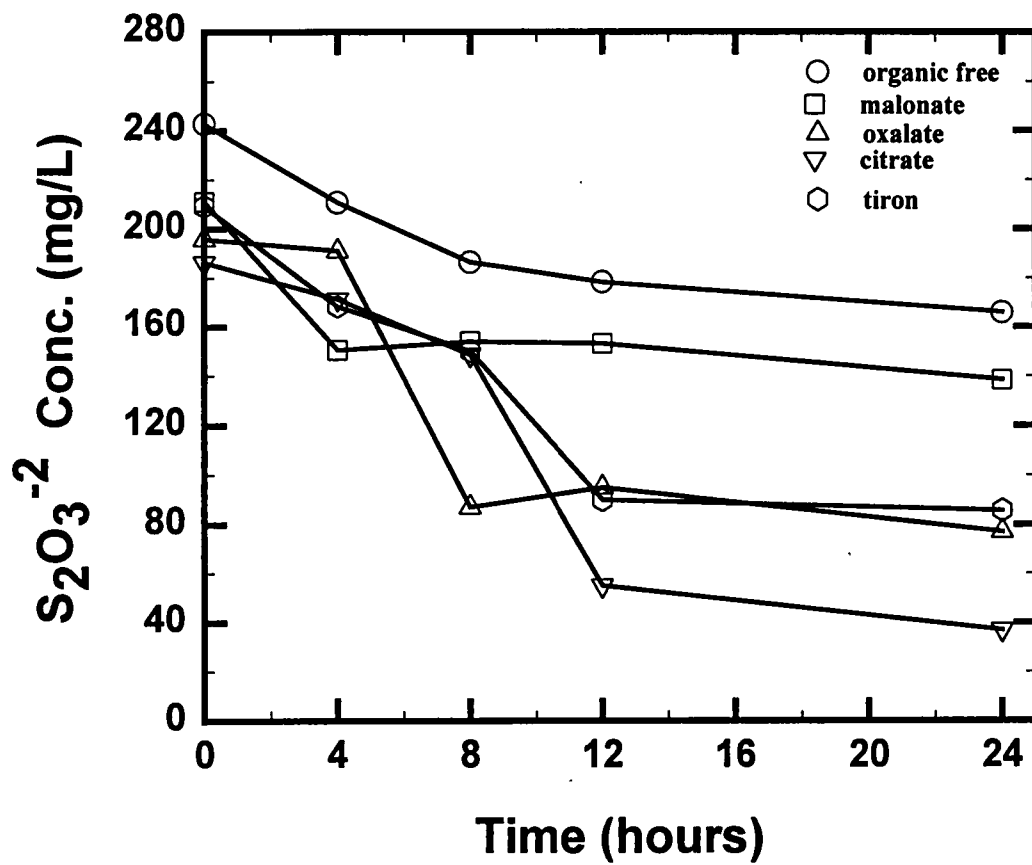


Figure 18. Thiosulfate concentration in solutions containing various ligands after inoculation with *D. desulfuricans*.

solutions. Results suggested that at the concentrations used, the multidentate ligands were not toxic to the viability of *D. desulfuricans*.

Although experiments using thiosulfate indicated no inhibitory effect to the viability of the bacteria, it is possible that these ligands may selectively inhibit the U^{6+} reducing enzyme of the bacteria. The possibility of such an inhibition was evaluated using the kinetic parameters obtained in this research (Tables XVI & XVII). Note that the maximum U^{6+} utilization rate (V_m) varied by more than an order of magnitude among the test solutions used. In general, the V_m values in monodentate U^{6+} containing solutions were ~ 20 fold higher than the those observed in multidentate U^{6+} complexes. In addition, a decrease in the multidentate ligand concentration increased the maximum U^{6+} utilization rate (V_m) by ~ 2 fold. Results indicated that the presence of malonate, oxalate and citrate decreased the maximum U^{6+} reduction rate of *desulfuricans* and the impact was proportional to the level of these ligands present. Kinetic parameters indicated that, although the V_m values varied widely, the half saturation coefficient (K_m) did not vary significantly in the presence of multidentate organic ligands. The K_m value was ~ 100 mg U^{6+} /L in organic free ($NaNO_3$) solution and it varied $< 20\%$ in the presence of malonate, oxalate or citrate. A decrease in the ligand concentrations did not appreciably change the K_m values (Table XVII). Devlin (1986) reported that a variation in V_m value with constant K_m results from non-competitive inhibition caused to the enzymes by certain inhibitory compounds in the solution. A non-competitive inhibitor binds to the enzyme at a site other than the target substrate binding site and behaves as though it were

removing the active enzyme from the solution, resulting in a decrease in the V_m value (Devlin, 1986). However, at such a condition, the K_m of the remaining active enzymes does not vary. A schematic diagram explaining the mechanism of non-competitive inhibition is shown in Figure 19. In the context of this research, a non-competitive inhibition would indicate a possible binding of the ligands at a site other than U^{6+} reducing site at cytochrome C_3 , resulting in enzyme inactivation. Decrease in the V_m values would correspond to a decrease in the fraction of active enzymes. Multidentate ligands, at the higher concentration used, complexed to a substantial concentration of cytochrome C_3 in test solution thereby decreasing the fraction of active enzymes available for reduction. A decrease in active enzyme concentration could have decreased the maximum U^{6+} reduction rate at the cell concentration (5×10^8 cells/ml) used. A decrease in the concentration of multidentate ligands ($\sim 50\%$ U^{6+} complexed) probably increased the fraction of active enzymes resulting in a two fold increase in the maximum U^{6+} reduction rate in test solutions (Table XVII). A near constant K_m value would indicate that the fraction of enzyme which was not inactivated by the ligands reduced U^{6+} with an efficiency at which they reduced U^{6+} in the absence of the ligands. Although trends in kinetic parameters (V_m , K_m) obtained indicated a possible non-competitive inhibition to U^{6+} reduction, information related to the binding of multidentate organic ligands to the sites at cytochrome C_3 of *desulfuricans* are not currently available. Elucidation of such a mechanism inhibiting U^{6+} reduction requires further investigation.

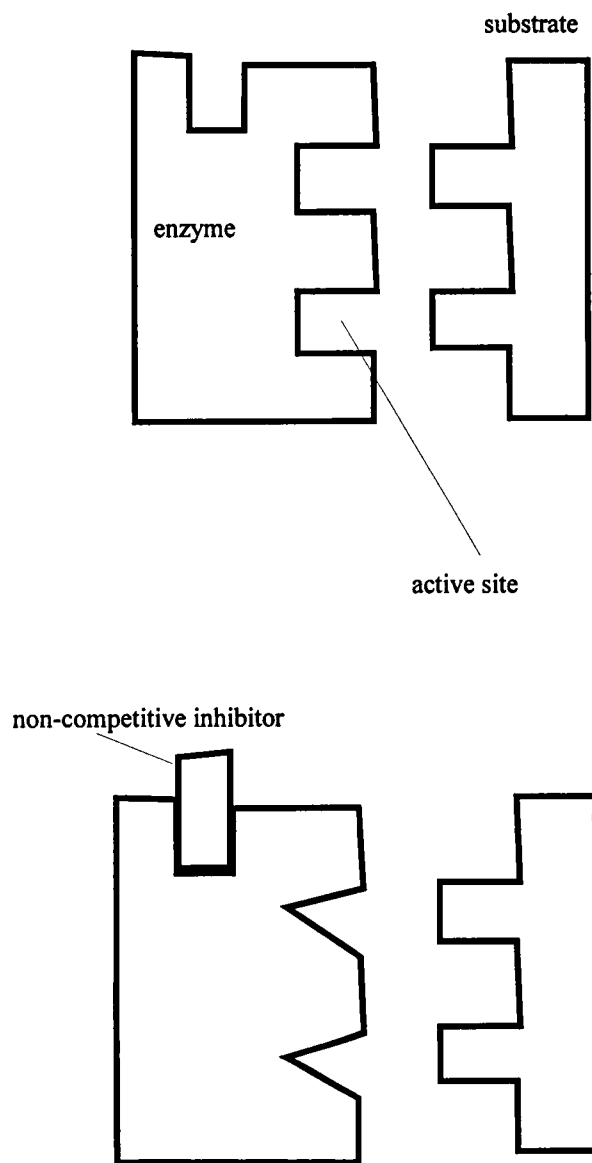


Figure 19. Schematic representation of non-competitive inhibition (Atlas, 1984)

7.4.3 Effect of chelation on U^{6+} bioavailability

If *cytochrome C₃* was not non-competitively inhibited by multidentate organic ligands, then, the differences observed in the rate of U^{6+} reduction may be due to chelation effect (third factor). Evidences in the literature indicated that microorganisms utilize electron donor/acceptors from metal organic complexes using a variety of mechanisms (Cox, 1980, Francis, 1992, Firestone and Tiedje, 1975). However, to date, no systematic study has been performed to determine the relationship between metabolic characteristics of the microorganisms and the mechanism of complexed compound utilization. In general, microorganisms appear to utilize compounds from metal-organic complexes using three mechanisms. In one case, during metal uptake from organic complexes, the microorganisms first hydrolyzed the complexing ligand. The released metal was then rapidly utilized. *E.coli* is reported to use this mechanism during iron assimilation (Porra, 1972). Enterochelin, an organic siderophore complexing ferric iron, was first hydrolyzed to N,2,3-dihydroxybenzoylserine by the enzyme *enterochelin esterase* of *E. coli*. This released ferric iron for heme synthesis of *E.coli*. In the second case, microorganisms initially utilized substrate only from the uncomplexed or weakly complexed species. Subsequent utilization then proceeded through the equilibrium redistribution of remaining substrate between weak and strongly complexed species. For example, *P. fluorescens* is reported to biodegrade citrate only from uncomplexed or weak bidentate metal species but not from strong tridentate or binuclear complexes (Francis, 1992). In the third case, microorganisms attacked substrates from all the complexes with

equal preference. During heme synthesis, *B.subtilis* obtained iron from ferrisiderophore complexes using this mechanism. Ferric iron complexed to multidentate siderophore '2,3-dihydroxy benzoic acid' was directly reduced using the enzyme *ferrisiderophore reductase*. Firestone and Tiedje (1975) reported that under nontoxic conditions, *pseudomonas sp.* attacked free NTA as well as metal complexed NTA simultaneously for metabolism.

Figure 20 shows the ligand concentrations in various experiments during U^{6+} (100 % complexed) reduction by *D. desulfuricans*. No appreciable degradation of the ligands was observed after 96 hours of cell incubation. This indicated that *desulfuricans* did not reduce hexavalent uranium using the first mechanism, that is, through degradation of complexing ligand. To evaluate if *desulfuricans* reduced U^{6+} only from monodentate complexes (second mechanism) or from mono and multidentate complexes simultaneously (third mechanism), the following discussion is presented. According to Michealis-Menton enzyme kinetics, microbial enzymes utilized electron donors/acceptors at varying rates, depending on the concentration of readily available fraction (Lineweaver, 1935). When the concentration of target compounds was not high enough to saturate the active sites of the enzymes, the enzymes utilized compounds at rates proportional to the amount of compounds available. Under these conditions, an increase in the level of target compound caused more active sites to engage in metabolism, resulting in an increase in the rate of utilization. The relationship between target compound availability and rate of utilization has been reported to be linear (first order)

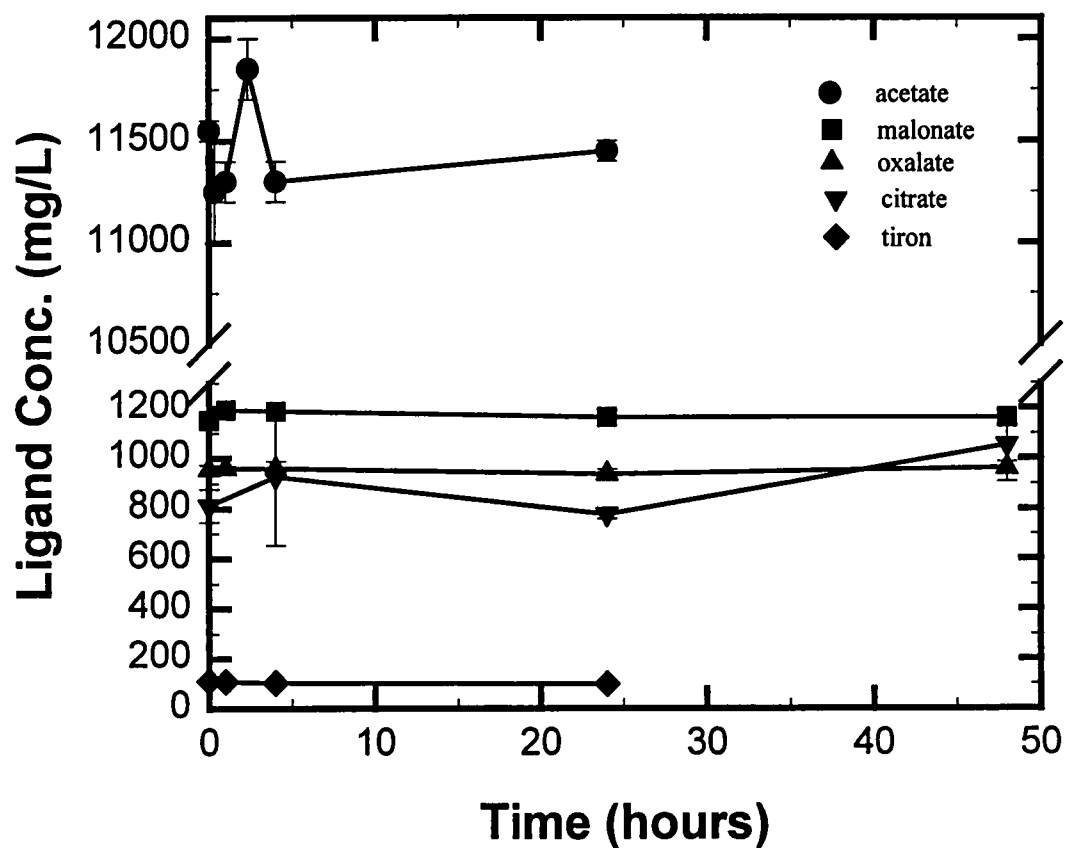


Figure 20. Concentrations of organic ligands during U^{6+} bioreduction by *D. desulfuricans*. Ligands initially complexed $\sim 100\%$ U^{6+} .

under such target compound limiting conditions. Once the active sites were saturated, increase in compound concentrations had no impact on rate of utilization. A zero order kinetics (same rate for varying concentrations) prevailed under such conditions. The ability of microorganisms to utilize compounds (electron donors/acceptors) at varying rates based on available initial concentrations, under limiting conditions, was used to evaluate the source of available U^{6+} to *desulfuricans*, in this research.

The experimental approach adopted to determine the source of available U^{6+} consisted of two parts. In the first part, (i) the relationship between available U^{6+} and rate of reduction and, (ii) the limiting conditions for U^{6+} reduction were evaluated. A series of tests were first conducted in organic free solutions to evaluate the ability of *desulfuricans* to reduce U^{6+} at varying initial concentrations. Test solutions were prepared according to the procedure outlined in section 4.5, with the exception that the initial U^{6+} concentrations were varied from 1 mg/L to ~ 400 mg/L and the ionic strength of the test solutions was maintained at 0.08 M by the addition sodium bicarbonate. All the hexavalent uranium added formed monovalent carbonate species in the test solutions. The initial rate of reduction (K_i) was measured as described in Section 4.10. Unfiltered test solutions were analyzed for U^{6+} concentration. Figure 21 depicts the microbial reduction of U^{6+} from solutions containing varying initial U^{6+} concentrations. *Desulfuricans* upon inoculation reduced U^{6+} more rapidly in test solutions containing higher initial U^{6+} levels. A linear increase in the rate of initial U^{6+} reduction (K_i) upto the highest initial concentration utilized (~ 400 mg/L) indicated that, substrate limiting conditions prevailed in the test

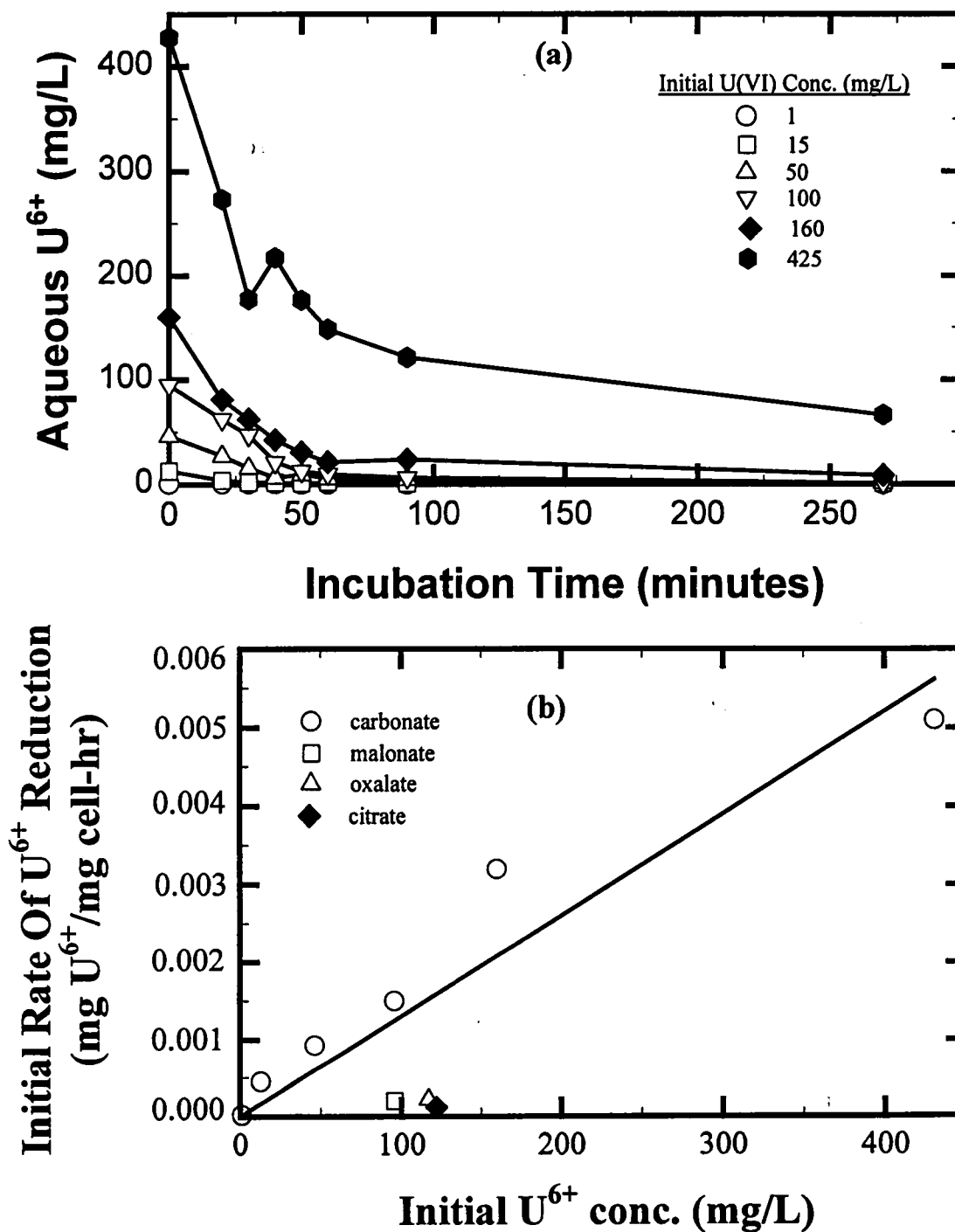


Figure 21. (a) U^{6+} level in carbonate solutions containing varying initial U^{6+} concentration after inoculation with *D. desulfuricans*. (b) Relationship between initial U^{6+} concentration and initial rate of reduction.

solution even at this U^{6+} concentration. Based on this observation, experiments were performed to evaluate the ability of *desulfuricans* to reduce U^{6+} in the presence of complexing agents. Test solutions ($I = 0.08$ M) containing ~ 100 mg/L U^{6+} , target ligand and H_2 gas were prepared. In malonate and oxalate solutions nearly 98 % of the uranium was initially complexed and remaining 2 % formed monodentate complexes. In citrate solution more than 99% uranium was complexed and less than 1 % uranium formed monodentate complexes. Figure 21(b) shows the initial rate of U^{6+} reduction in carbonate, malonate, oxalate and citrate solutions. The bacterium reduced U^{6+} at an initial rate of $\sim 2 \times 10^{-4}$ (mg/mg cell-hr) from all the organic solutions. Note that this rate was significantly slower than that observed in carbonate solution ($\sim 10^{-3}$ mg/mg cell-hr) containing identical initial U^{6+} concentration. According to Michaelis-Menten Kinetics, this trend suggested that introduction of complexing agents decreased the pool of available U^{6+} to *desulfuricans*. Comparison of results indicated that U^{6+} reduction observed in organic solutions containing ~ 100 mg/L of U^{6+} was identical to that observed in monodentate carbonate species containing an initial U^{6+} level of < 10 mg/L. Note that this (~ 2 mg/L) was the level of monodentate form of U^{6+} initially present in the multidentate organic solutions. From these results, it appeared that, in multidentate organic solutions *desulfuricans* indeed reduced U^{6+} only from monodentate complexes. Subsequent reduction proceeded through replenishment of monodentate U^{6+} resulting from equilibrium redistribution of multidentate complexed U^{6+} .

Figure 22 shows U^{6+} reduction in high and low concentrations of aliphatic ligand containing solutions. Note that in solutions containing lower concentrations of test ligands ($\sim 50\%$ U^{6+} complexed) the rate of U^{6+} reduction was faster than that observed at the highest concentrations of multidentate ligand containing solutions. The initial reduction rate obtained using the kinetic parameters (Tables XVI & XVII) indicated a two to five fold faster reduction in lower organic ligand containing solutions. The differences in the rates were statistically significant ($\alpha = 0.05$). If chelate effects rather than non-competitive inhibition affected U^{6+} reduction, the trends shown in Figure 22 can be explained as below. In acetate containing solutions, all the U^{6+} initially formed monodentate uranyl acetate and uranyl hydroxide complexes. No multiligand chelation occurred in these solutions. The rate of reduction observed was identical to that observed in organic free ($NaNO_3$) and high acetate containing solutions, where all the added U^{6+} formed monodentate complexes. These results indicated that regardless of the species formed, $((UO_2)_x(OH)_y)$, or $UO_2(\text{acetate})_2$, *desulfuricans* rapidly reduced U^{6+} from monodentate complexes. In low malonate, oxalate and citrate containing solutions, $\sim 50\%$ of the U^{6+} initially formed bidentate/binuclear complexes. The remaining uranyl ion formed uranyl hydroxide species. Analysis of results indicated that the reduction rates were statistically faster than that observed in high concentration of multiligand containing solutions. An increase in the rate of reduction upon a decrease in the multidentate ligand concentration probably resulted from an increase in the ($\sim 50\%$) monodentate U^{6+} concentration. Note that, in these solutions the initial level of monodentate U^{6+} species

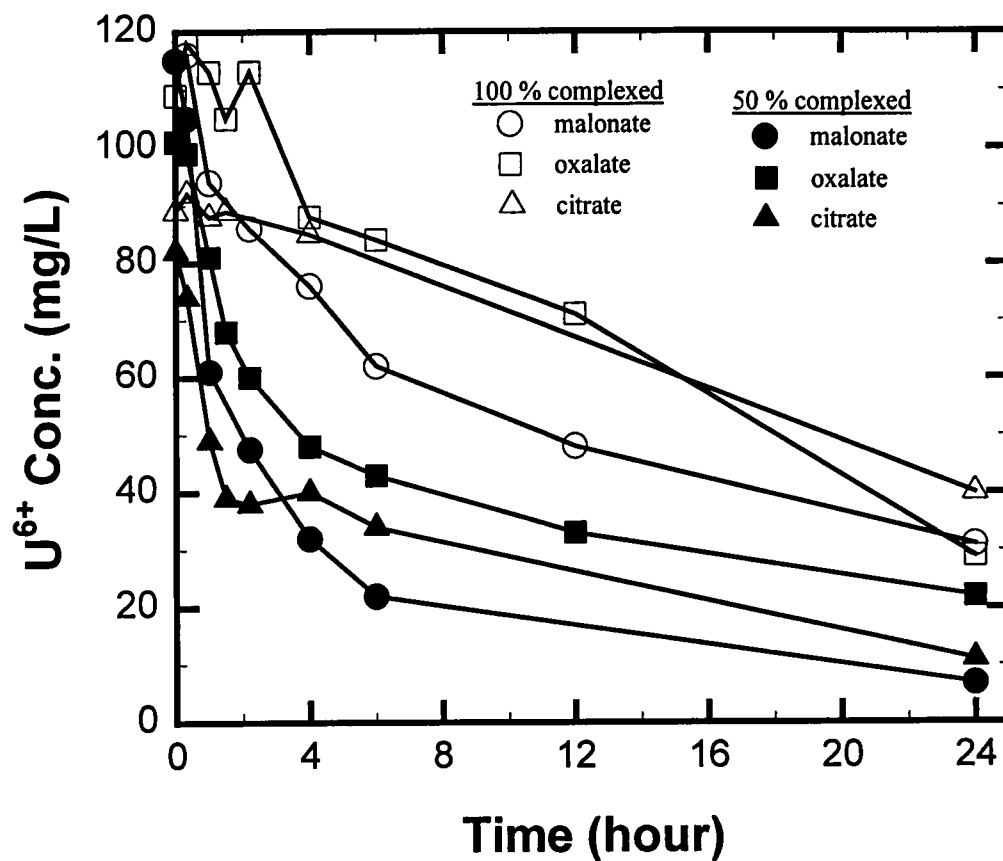


Figure 22. U^{6+} reduction in test solutions containing low and high concentrations of malonate, oxalate and citrate after inoculation with *D. desulfuricans*.

(50 %) was lower than that in acetate solutions (100 %). The rates of U^{6+} reduction also were slower than that observed in acetate solutions. Statistical analysis using the ANOVA procedure indicated that U^{6+} reduction in lower acetate solution was significantly faster than that in lower malonate, oxalate and citrate solutions. However, Cox and Stuart procedure (Conover, 1980) did not yield a significant difference in the rate between lower acetate and malonate solutions. Results suggested that, at the lower concentration of malonate, oxalate and citrate used, *D. desulfuricans* probably reduced U^{6+} using two mechanisms. Initially, the bacteria rapidly reduced the fraction of U^{6+} from monodentate species as indicated by the rapid reduction in the first 4 to 24 hours of reduction. Once the monodentate U^{6+} was reduced, further reduction continued through continues replenishment of monodentate U^{6+} through equilibrium redistribution of remaining multidentate complexed U^{6+} . Although a qualitative estimate has been made to explain the differences in the trend in U^{6+} reduction, an accurate quantitative estimation was limited by the following factors : (i) the activity of the bacteria decreases with time in test solution under non-growth conditions. Peyton et al (1994) reported that the activity of the iron reducing bacteria 'BrY' decreases in test solutions by ~ 40% after nearly eight hours of incubation. However, no information regarding the activity of desulfuricans in organic test solutions is currently available. (ii) Oxidation of H_2 gas to H^+ by the bacteria during U^{6+} bioreduction may decrease the solution pH (Figure 7, Section 5.2). As shown in Figure 8 change in pH level may alter U^{6+} speciation in test solution, rendering data interpretation difficult.

An exception to the chelation effect on U^{6+} bioreduction was observed for the ternuclear tiron- U^{6+} complexes. *D. desulfuricans* reduced U^{6+} from tiron solutions (~ 100% & 50 % complexed) much faster than in malonate, oxalate and citrate solutions (Figures 16 & 17, Tables XVI & XVII). The initial rate of U^{6+} reduction (K_i) in tiron solutions (~ 5×10^{-4} mg U^{6+} /mg cell-hr) was nearly five fold higher than that in other solutions (~ 9×10^{-5} mg U^{6+} /mg cell-hr). This difference could have resulted from the differences in the nature of tiron- U^{6+} complex. Note that, unlike other ligands studied, tiron complexed with uranium exclusively through hydroxyl group interactions ($(UO_2)_3(OH)_2(Tiron)_3$) rather than carboxyl group interactions. This difference in functional group association may have enhanced the rate of U^{6+} reduction from tiron complexes. Functional groups involved in complexation have been reported to impact degradation of citrate (Joshi-Tope and Francis, 1995). Cell free extract of *P. fluorescens* degraded citrate from binuclear UO_2^{2+} complexes but not from tridentate Cd^{2+} and Cu^{2+} complexes that involved hydroxyl group of citrate in complex formation. The authors suggested that hydroxyl ion interactions altered the structural compatibility of the complex and limited bioavailability of citrate. In this research, the rate of U^{6+} reduction was enhanced in tiron complex involving hydroxyl group interactions. It may also be possible that the three aromatic tiron molecules did not structurally protect U^{6+} from microbial enzymes, as did the branched aliphatic malonate, oxalate and citrate molecules. The above factors together could have enabled *desulfuricans* to attack uranyl ion rapidly

from tiron complexes. However, a better understanding of these interactions requires further investigation.

7.5 Precipitation of bio-reduced uranium

Although *desulfuricans* reduced hexavalent uranium from each of the organic complexes evaluated, the success of bio-reduced uranium precipitation as a treatment option depends on subsequent precipitation of reduced uranium. In this research the fate of uranium was evaluated by measuring the total uranium ($U^{6+}+U^{4+}$) in the filtrate after inoculation with the bacteria. Results indicated that precipitation of bio-reduced uranium appeared to be impacted by the type as well as the concentration of ligand present in solution. Figure 23 shows the total uranium level in the filtrate (dissolved uranium) when ~ 100% of the uranyl ion was initially complexed to various ligands. In acetate solutions, the dissolved uranium level rapidly decreased, indicating that uranium, reduced by *desulfuricans*, precipitated from solution. This indicated that, unlike hexavalent uranium (U^{6+}), bio-reduced (U^{4+}) uranium did not complex well with acetate in solution. Predictions made using estimated solution pH and association constants of Th^{4+} (Section) indicated precipitation of U^{4+} as uraninite in acetate solutions. Significant (< 90 %) removal of bio-reduced uranium from acetate solution observed in this research agreed with the speciation predicted using MINTEQA. Results indicated that while reduction of

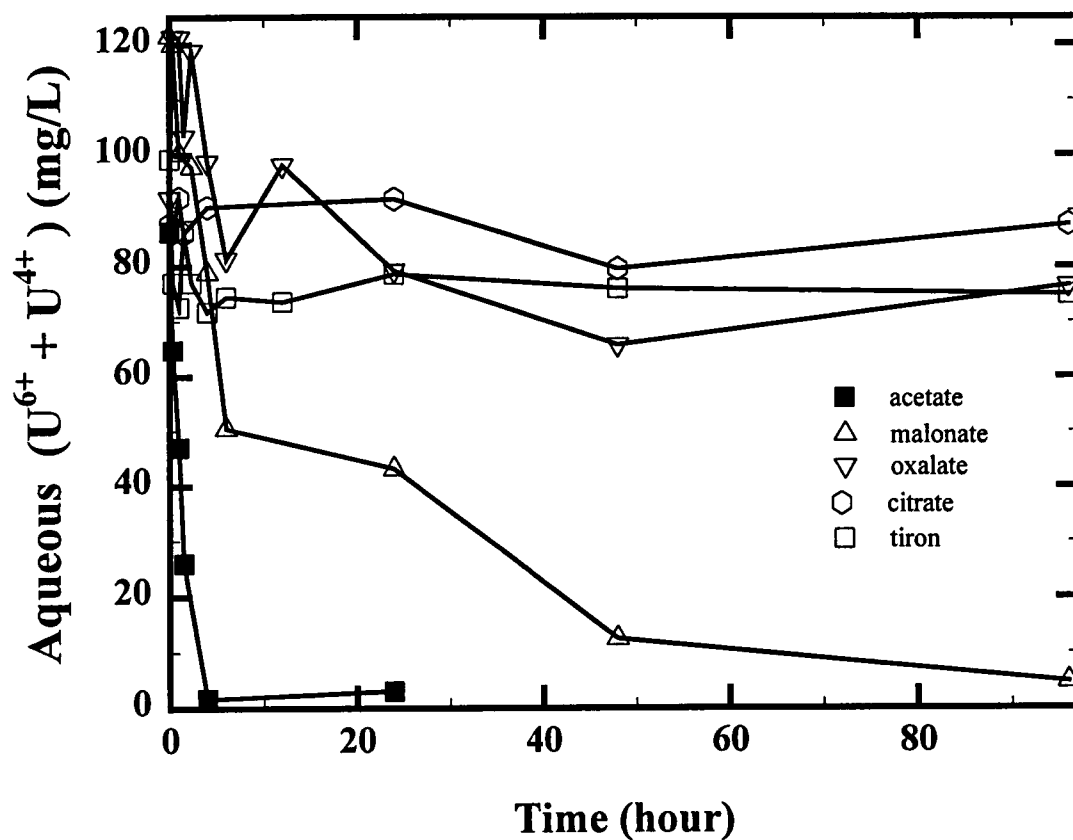


Figure 23. Total uranium ($U^{6+} + U^{4+}$) concentration in filtrate during U^{6+} bioreduction by *D. desulfuricans*. Ligands initially complexed $\sim 100\%$ U^{6+} .

U^{6+} in solution was governed by the metabolism of *desulfuricans*, precipitation of U^{4+} depended only on the thermodynamic constant for the association of U^{4+} with the ligand.

The decrease in dissolved uranium level in malonate solution over time was slower than that in acetate solution. Approximately 96 hours was required for near total uranium removal. Total uranium level in the filtrate remained essentially identical to U^{6+} level suggesting that precipitation of U^{4+} occurred with very little lag period. Results indicated that bioreduction of U^{6+} rather than subsequent U^{4+} precipitation limited the rate of uranium removal in malonate solution. The fate of U^{4+} in malonate solution predicted using thermodynamic constants of Th^{4+} using MINTEQ indicated precipitation as uraninite from test solution. As found in the acetate experiment, observed results agreed well with the predicted speciation in the malonate solution.

Unlike acetate and malonate solutions, the dissolved uranium level did not decrease in oxalate solutions over time. The filtrate contained ~ 80 % total uranium initially added even after 96 hours of incubation. However, almost all the hexavalent uranium was bioreduced by *desulfuricans* during this time, indicating that uranium upon bioreduction complexed with oxalate and remained in solution. These results differed from the predictions made using thermodynamic constants of Th^{4+} . MINTEQ predicted precipitation of U^{4+} as uraninite under experimental conditions. This suggested that Th^{4+} may not be a good surrogate to predict the fate of reduced uranium in oxalate solutions. Results indicated that oxalate not only impacted the rate of U^{6+} reduction by *desulfuricans* but also prevented the precipitation of tetravalent uranium through complexation.

Figure 23 also shows that reduced uranium did not precipitate from the citrate containing solutions. More than 80 % of the bio-reduced uranium was in solution after 96 hours of incubation. As observed with oxalate, the presence of citrate affected both the rate of uranium reduction as well as subsequent precipitation of bio-reduced uranium. Thermodynamic constants for the association of citrate with U^{4+} or Th^{4+} are not currently available. Hence no attempt was made to predict possible speciation of U^{4+} in citrate solution.

Although a substantial amount of U^{6+} was reduced within four hours in tiron solutions, no appreciable decrease in the total uranium level was observed in the filtrate. Bio-reduced uranium apparently complexes with tiron in solution thereby preventing UO_2 precipitation. Complete information related the association of U^{4+} or Th^{4+} with tiron is not currently available. Hence, no attempt was made to theoretically predict the speciation of bio-reduced uranium in the test solution.

Figure 24 depicts total dissolved uranium in test solutions over time, when the ligands initially complexed ~ 50% of the U^{6+} introduced. The bio-precipitation trend in low acetate concentration solution was similar to that observed in high acetate solution. This indicated that, in the range of concentrations tested in this research, acetate did not affect precipitation of bio-reduced uranium. In the low malonate concentration solutions, the decrease in dissolved uranium concentration was slower than that in acetate solution, but was faster than that in high malonate solution. The precipitation trend followed the trend in the reduction of hexavalent uranium, indicating that U^{6+} reduction dictated the

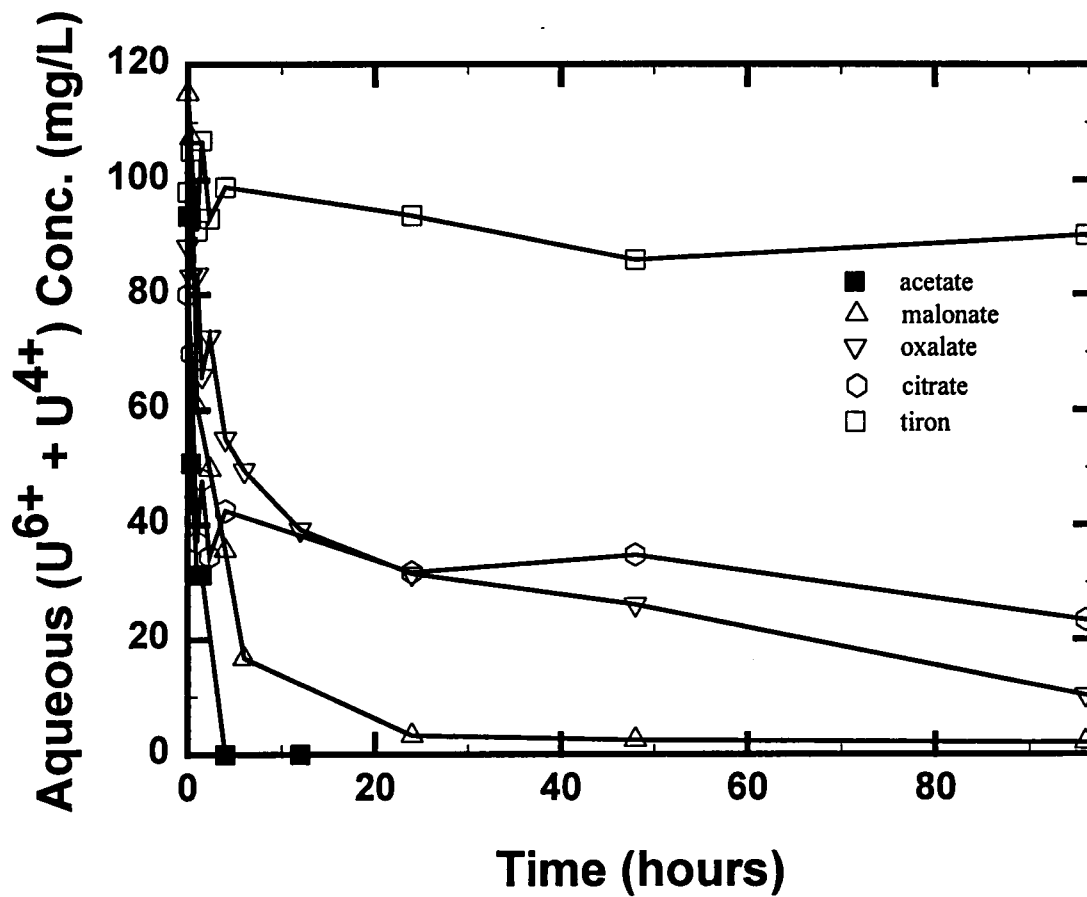


Figure 24. Total uranium ($U^{6+} + U^{4+}$) in filtrate in the presence of various ligands after inoculation with *D. desulfuricans*. Ligands initially complexed ~50% U^{6+} .

rate of uranium removal in malonate solution. As observed in low malonate solution, the uranium level in the filtrate of the low oxalate solution gradually decreased. After 96 hours of incubation, >90 % of the uranium initially present precipitated from solution. The speciation of U^{4+} predicted using 'MINTEQ' using the lower concentration of oxalate (0.0007 M/L), indicated near total precipitation of U^{4+} as uraninite. Relatively faster U^{6+} reduction, followed by U^{4+} precipitation in low oxalate solution, compared to slower U^{6+} reduction with no appreciable precipitation in high oxalate solution indicated that the rate of U^{6+} reduction as well as subsequent precipitation of U^{4+} were affected by the concentration of oxalate in solution.

Dissolved uranium in citrate solutions decreased in a manner similar to that observed in oxalate solution. When 50 % of the hexavalent uranium was initially complexed, ~ 75 % of the uranium was removed from solution via precipitation. Results indicated that the concentration of citrate in test solutions may affect the rate of uranium reduction as well as the precipitation of bio-reduced uranium. Tiron appeared to have a very high affinity for complexation with reduced uranium. Although near total U^{6+} reduction occurred within four hours, ~ 20 % uranium precipitated even after 96 hours of incubation. Note that the amount of tiron used (0.0002 M) complexed only ~ 50% of the U^{6+} in solution. However, U^{6+} once reduced to U^{4+} was totally complexed by tiron indicating that tiron complexed more readily with tetravalent uranium than it did with hexavalent uranium. Precipitation of uranium was much lower the presence of the aromatic tiron ligand solution than in the presence of the aliphatic ligands tested. Results

indicated that, low concentrations of ligands such as tiron in waste streams may affect uranium reductive precipitation due to recomplexation.

In general, among the ligands tested, tetravalent uranium (U^{4+}) precipitated in the presence of ligands that complexed weakly with hexavalent uranium (acetate; $\text{Log } \beta = 7.2$; malonate; $\text{Log } \beta = 10.1$). Bio-reduced uranium complexed with ligands that formed strong complexes with U^{6+} , (oxalate, citrate and tiron; $\text{Log } \beta = 11.2, 18.87$ and ~ 55 , respectively) and remained in solution. In addition, speciation predicted using thermodynamic constants agreed well with the observed results in most cases. This indicated that precipitation of bio-reduced uranium essentially depended on its thermodynamic constants for association of U^{4+} with the ligands.

7.6 Summary

Results indicated that organic ligands affect the rate of U^{6+} bio-reduction as well as precipitation of bio-reduced uranium. In general, solutions in which U^{6+} was predicted to form monodentate complexes *desulfuricans* rapidly reduced hexavalent uranium. In solutions where U^{6+} was predicted to form predominantly (< 98 %) multidentate aliphatic complexes the rate of reduction was significantly slow. Investigations of bio-reduction mechanisms indicated that, either non-competitive inhibition to U^{6+} reducing enzyme or the inability of *desulfuricans* to directly reduce chelated uranium resulted in the differences in the rate of U^{6+} reduction. However, aromatic structure of tiron and/or the

hydroxyl ion interactions of tiron- U^{6+} complexation facilitated faster U^{6+} reduction form multidentate complexes. Once hexavalent uranium was bio-reduced, U^{4+} precipitation appeared to be governed by thermodynamic constants for association with the ligands tested.

Figures 25, 26 and Table XVIII summarize the impact of each ligand on uranium removal via bio-reductive precipitation. In acetate and malonate solutions (weaker U^{6+} complexes) bio-reduction dictated the removal of dissolved uranium. Precipitation of uranium as soon as hexavalent uranium was reduced to tetravalent state by *desulfuricans*. Oxalate and citrate affected the rate of U^{6+} reduction as well as the extent of U^{4+} precipitation, depending on the concentrations used. At the highest concentrations of these ligands tested, U^{6+} reduction was very slow. In addition, insignificant level of U^{4+} precipitated. However, a decrease in the concentration of these ligands significantly increased the rate of U^{6+} reduction and the extent of U^{4+} precipitation. The highest concentration of tiron used had no impact on the rate of U^{6+} bio-reduction. However, tiron prevented reduced uranium precipitation at the lowest concentration used.

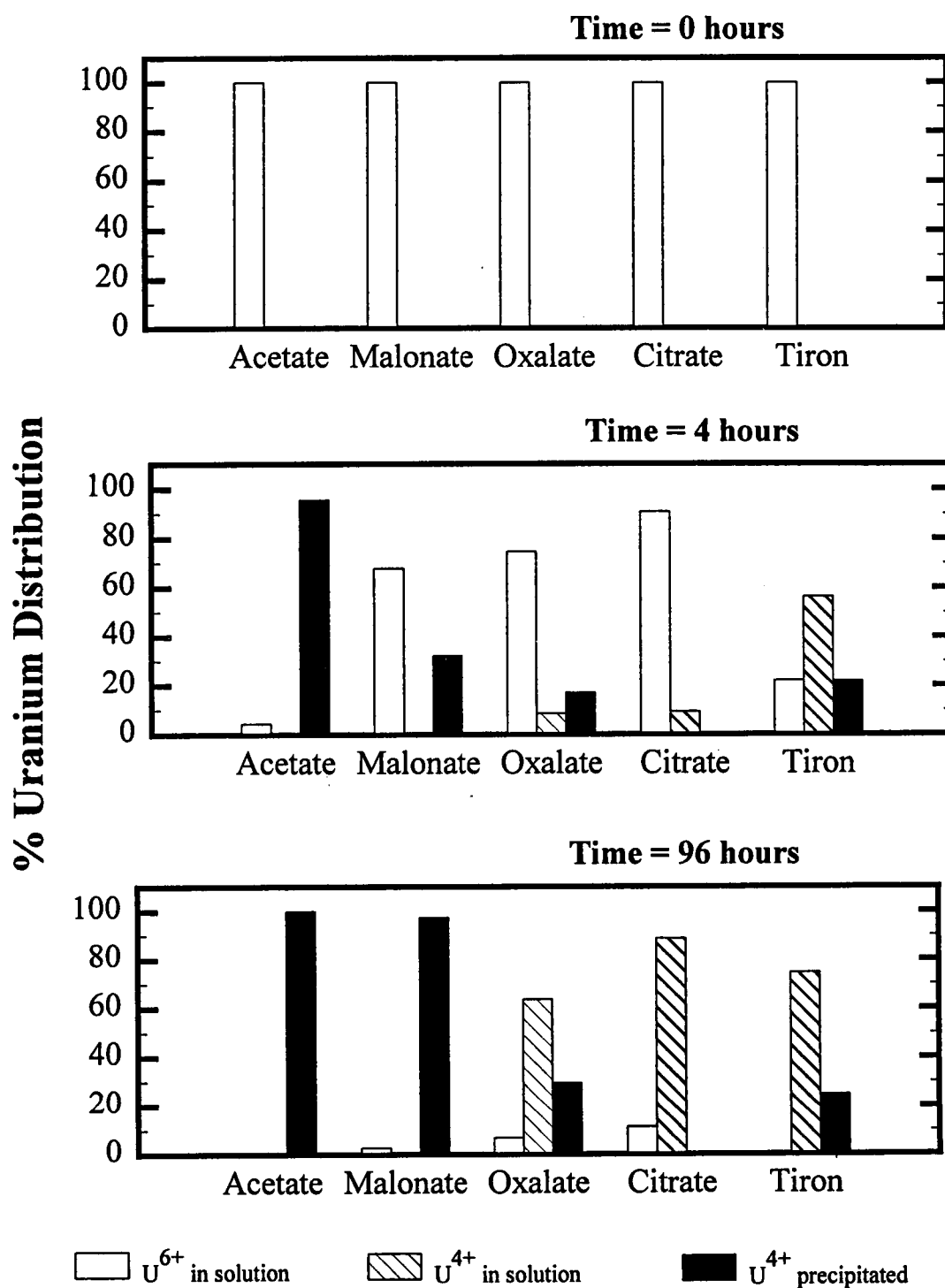


Figure 25. Distribution of uranium complexed to ligands during bioreduction by *D. desulfuricans*. Ligands initially complexed ~100 % U^{6+} .

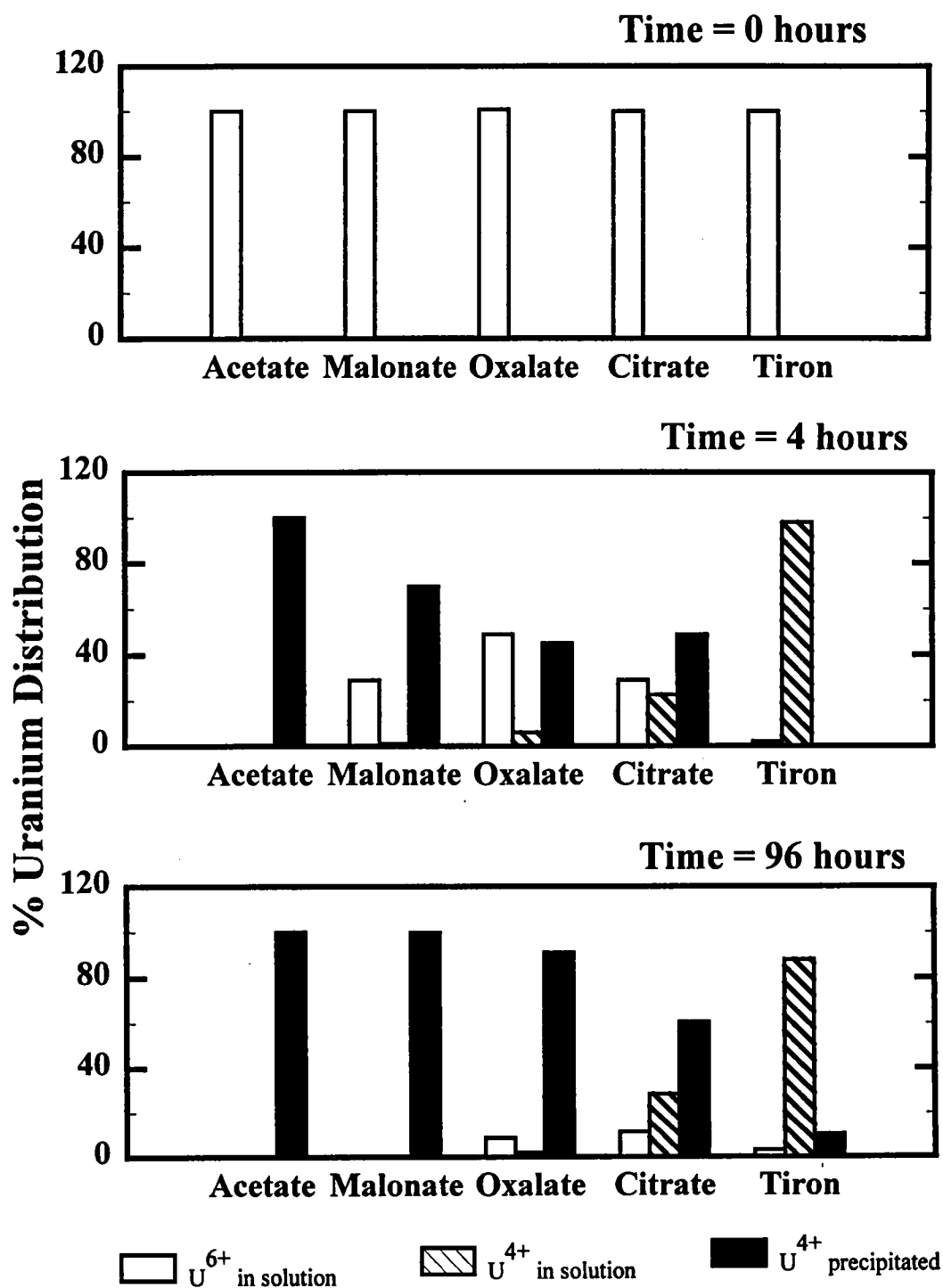


Figure 26. Distribution of uranium complexed to ligands during bioreduction by *D. desulfuricans*. Ligands initially complexed $\sim 50\% \text{U}^{6+}$.

Table XVIII. Impact of various ligands on removal of uranium via reductive precipitation by *D. desulfuricans*

LIGAND	U ⁶⁺ REDUCTION	U ⁴⁺ PRECIPITATION	CRITICAL FACTORS IN URANIUM REMOVAL
Acetate	No impact.	Precipitation occurred immediately upon U ⁶⁺ reduction.	Rate of reduction dictated uranium removal.
Malonate	Impacted rate of U ⁶⁺ reduction. Rate depended on the conc. of ligand.	No impact. Precipitation occurred immediately after reduction.	Rate of reduction dictated uranium removal.
Oxalate	Impacted rate of U ⁶⁺ reduction. Rate depended on the conc. of ligand.	Precipitation depended on ligand concentration. U ⁴⁺ precipitated at low (0.0006M) ligand conc. but not at high (0.01M) ligand conc.	Conc. of oxalate dictated rate of U ⁶⁺ reduction and U ⁴⁺ precipitation, which affected uranium removal.
Citrate	Impacted rate of U ⁶⁺ reduction. Rate depended on the conc. of ligand.	Precipitation depended on conc. of ligand. Increase in citrate conc. decreased U ⁴⁺ precipitation.	Conc. of ligand affected both U ⁶⁺ reduction and precipitation.
Tiron	No impact. U ⁶⁺ was readily reduced at high/low tiron concentrations.	No precipitation occurred. Even the lower conc. of tiron complexed almost all the reduced uranium.	Precipitation affected uranium removal.

8. REDUCTIVE PRECIPITATION OF ORGANICALLY COMPLEXED URANYL ION BY *Shewanella alga* (BrY)

8.1 Introduction

In this section, results from uranium biotransformation experiments using the iron reducing bacterial strain BrY are presented. Interpretation and a detailed discussion of results also are presented. As with *D. desulfuricans*, experiments using BrY were conducted under non-growth conditions. After cell inoculation, subsamples were collected from pressure tubes over time and analyzed for U^{6+} and total uranium levels. The same procedures followed in experiments using *D. desulfuricans* to determine uranium distribution and data interpretation were followed. In addition, experiments performed to predict possible mechanisms of U^{6+} utilization by BrY are presented and discussed.

8.2 Bioavailability of hexavalent uranium to BrY

Figures 27 & 28 show the hexavalent uranium levels in unfiltered test solutions containing various organic ligands, after inoculation with BrY. In addition, U^{6+} levels in organic free ($NaNO_3$ and carbonate) solutions are also presented for comparison. Results ($\alpha = 0.05$) indicated that the trend in U^{6+} reduction varied with the type of uranyl ion species formed in different test solutions. Unlike *desulfuricans* which reduced

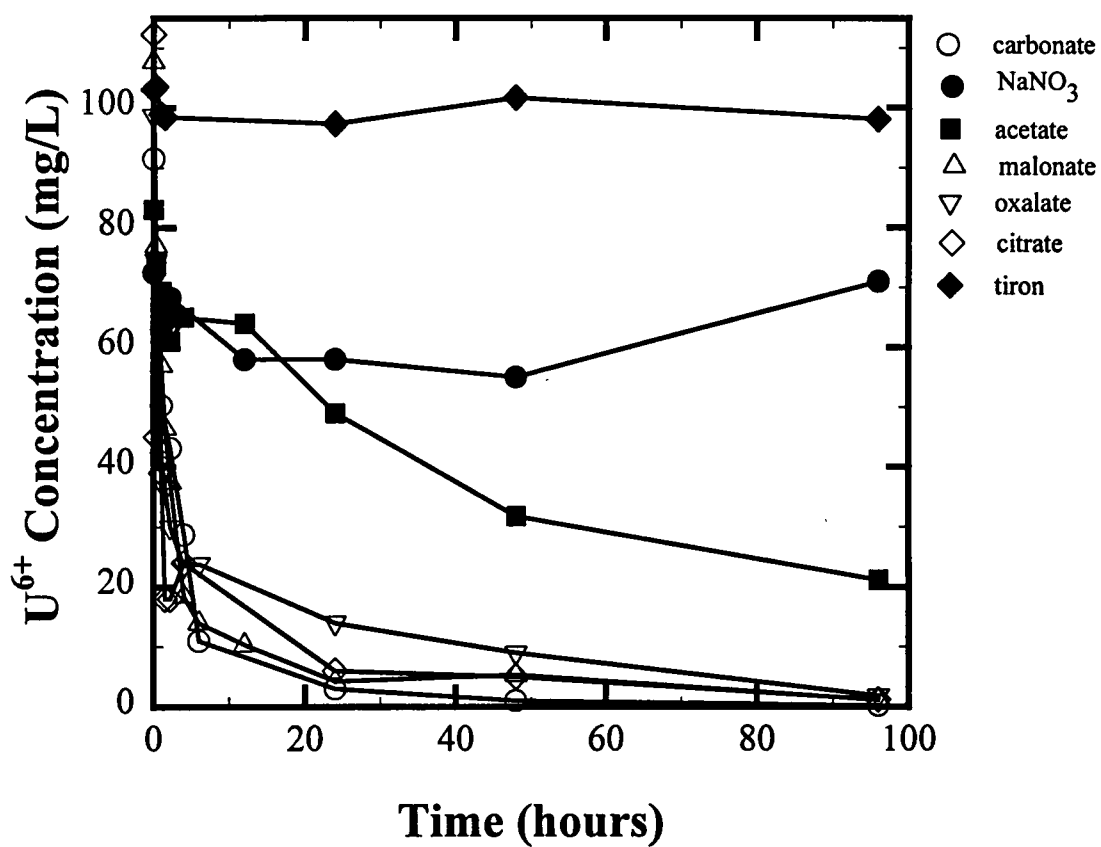


Figure 27. U^{6+} level in solutions containing various ligands after inoculation with BrY. Ligands initially complexed $\sim 100\%$ U^{6+} .

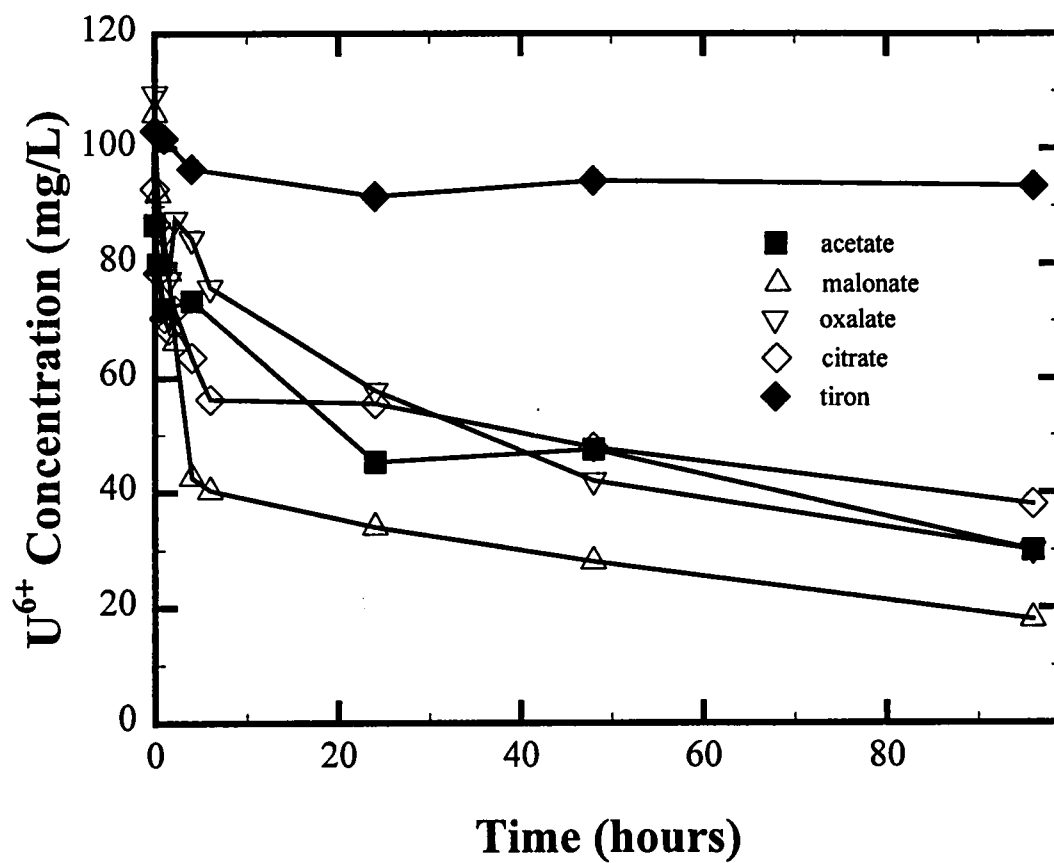


Figure 28. U^{6+} level in solutions containing various ligands after inoculation with BrY. Ligands initially complexed ~50% U^{6+} .

hexavalent uranium from all the solutions tested, BrY did not reduce U^{6+} in organic free ($NaNO_3$) and tiron solutions. Note that all of the uranyl ion initially added was predicted to hydrolyze and form uranyl hydroxide and/or ternuclear $(UO_2)_3(OH)_2(Tiron)_3$ species in these solutions. Almost all the uranium introduced to the ligand free and tiron solutions was in the hexavalent state after 96 hours of incubation. In addition, bioreduction was incomplete in ligand solutions where $\sim 50\%$ of the U^{6+} predicted to initially form uranyl hydroxide species. U^{6+} reduction was incomplete also in acetate containing ($\sim 98\%$ complexed) solutions. The test solutions where BrY incompletely reduced U^{6+} visually appeared different from that incubated with *D. desulfuricans*. After one or two hours of cell incubation, BrY cells in solutions aggregated and deposited at the bottom of the test vials, whereas *D. desulfuricans* remained suspended in all test solutions. Deposition of cells may have affected bacterial (BrY) activity and resulted in incomplete U^{6+} reduction after 96 hours. In carbonate, malonate, oxalate and citrate solutions ($\sim 100\%$ complexed), U^{6+} reduction was rapid and essentially complete after 96 hours.

In general, in inorganic ($NaNO_3$) as well as organic (Tiron) solutions where all the uranyl ion was predicted to hydrolyze and form various species prior to cell inoculation, U^{6+} was not bioavailable to BrY. Incomplete but significant ($\sim 80\%$) reduction of U^{6+} occurred in test solutions containing same amount of uranyl carboxyl species uranyl hydroxide species. These results suggested that BrY probably utilized U^{6+} from carboxyl species but not from hydroxyl species. In addition, U^{6+} in a monodentate complex with a low stability constant (acetate, $\log K = 2.6$) was incompletely reduced.

BrY completely utilized hexavalent uranium from multidentate aliphatic complexes (malonate, oxalate, citrate) and from the monodentate complex (carbonate) with a high stability constant ($\text{Log } K \simeq 7.3$).

8.3 Mechanism of U^{6+} utilization by BrY

As discussed earlier, several researchers have studied the bioavailability of organics and metals from metal-organic complexes (Wellecke, 1973; Cox 1980). Results in general indicated that, once the complex has diffused to the proximity of the enzymes, microorganisms utilized the target molecule more readily from weaker complexes than from stronger complexes. For example, the efficiency of *P. fluorescens* to metabolize citrate decreased with an increase in the stability constant of metal-citrate complexes tested (Joshi-Tope and Francis, 1995). The bacterium degraded citrate faster from a weaker Fe^{3+} complex ($\text{Log } K = 2.6$) than from stronger Zn^{2+} complex ($\text{Log } K = 4.9$). In addition, citrate degradation from stronger uranyl ion complex ($\text{Log } K \sim 18$) was the least efficient. Similarly, the iron assimilating bacterium *A. vinelandii*, reduced ferric iron faster from weaker citrate complex than from stronger azotochelin complex (Huyer and Page, 1989). However, very little information related to the preferential utilization of metal electron acceptors from stronger complexes over weaker complexes has been reported. For example, the bacterium *P. aeruginosa* reduced ferric iron rapidly from stronger pyochelin complex (Cox, 1980). Fe^{3+} from weaker chloride complex was not

reduced by this bacteria. However, the mechanisms favoring ferric iron utilization from organic complexes or the factors preventing reduction from chloride species have not been elucidated.

In this research, BrY reduced U^{6+} rapidly from stronger oxalate and citrate complexes. Reduction from weaker acetate and hydroxyl complexes was less efficient. In addition, hexavalent uranium, upon hydrolysis, was not available from a stronger organic (tiron) complex. A review of literature indicated that the growth of BrY as well as the iron reducing bacteria was impacted by the speciation of ferric iron provided as terminal electron acceptor (Lovely, 1988; Caccavo, 1992). In general, these microorganisms grew rapidly ferric iron complexed to organic ligands such as citrate or NTA was provided as the terminal electron acceptor. However, the bacterial growth was significantly slow using amorphous ferric hydroxide. The factors that negatively impacted the growth using the hydrolyzed species of ferric iron have not been elucidated. The trends in U^{6+} reduction from organic complexes and hydrolyzed species using BrY in this research were similar to the growth using iron from such complexes. The dependence of U^{6+} bioavailability to BrY on uranyl ion speciation may be due to one or several of the following factors: (i) requirement of species specific transport systems to diffuse U^{6+} across cell membrane; (ii) preferential reduction of nitrate over U^{6+} in selected test solutions; (iii) deposition of cells at the bottom of pressure tube, which resulted in limited accessibility of dissolved uranyl ion to the cells; (iv) specificity of U^{6+} reducing enzymes to the speciation of uranium and/or (v) toxicity of certain U^{6+} species to BrY.

The contribution of each mechanism to the differences observed in U^{6+} reduction by BrY is discussed in the following paragraphs. Additional experiments performed to aid in the interpretation of mechanisms are also presented and discussed.

8.3.1 Requirement of speciation specific transport system

Caccavo, et al. (1995) attempted to isolate BrY enzymes responsible for U^{6+} reduction. 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 and a 'c' type cytochrome was involved in U^{6+} reduction. In addition, U^{4+} precipitation using BrY was found to be extracellular. These observations suggested that BrY did not require transport of U^{6+} across the cell membrane for reduction. Hence, it appeared that the lack (or slow rate) of uranyl ion reduction observed in organic free ($NaNO_3$), acetate and tiron solutions was not due to U^{6+} transport limitation across the cell membrane.

8.3.2 Preferential reduction of nitrate in selected test solutions

Lack of U^{6+} reduction in organic free ($NaNO_3$) solutions which contained a very high concentration of nitrate (0.17 M) suggested that BrY may utilize nitrate more readily than U^{6+} as electron acceptor. However, U^{6+} reduction was incomplete in high acetate solution which contained no nitrate ions, whereas a rapid U^{6+} reduction occurred in high citrate solution which contained a very high concentration of nitrate (> 0.15 M). In addition, the trend in U^{6+} reduction in high citrate containing solution was essentially

similar to that observed in carbonate solution which contained no nitrate ions. This suggested that the lack of (or incomplete) reduction of U^{6+} observed in selected test solutions was probably not due to the presence of nitrate ion as the background electrolyte.

8.3.3 Mass transfer limitations due to cell deposition

BrY, within 2 to 3 hours of incubation, settled to the bottom of pressure tubes containing organic free ($NaNO_3$) and tiron solutions. The reasons for cell aggregation and settling are not currently known. It may be possible that, transport limitations of dissolved uranyl ion resulting from deposition of cells limited rate of U^{6+} reduction. Robinson et al. (1994) evaluated the ability of *D. desulfuricans*, contained inside dialysis bags, to reduce hexavalent uranium. Diffusion of dissolved uranium across the membrane rendered U^{6+} bioavailability to *D. desulfuricans* difficult. However, approximately 40 to 90 % U^{6+} was found to be available for reduction after 96 hours of incubation. Hence, if diffusion limited U^{6+} bioavailability upon BrY deposition, reduction comparable to that observed with *desulfuricans* contained in dialysis bags should have been observed after 96 hours. Total lack of U^{6+} reduction observed in $NaNO_3$ and tiron solutions indicated that limitation due to diffusion alone did not limit uranium bioavailability in these solutions.

8.3.4 Structural specificity of complexes for U^{6+} reducing enzymes

It appeared that the limited bioavailability of certain U^{6+} species to BrY may be due to enzyme specificity and/or toxic effects of selected species to the bacteria. The importance of U^{6+} speciation on bioavailability was also observed in this research using the sulfate reducing bacteria *D. desulfuricans*. The bacterium was unable to readily access U^{6+} from multidentate aliphatic organic complexes. Similarly, cell free extracts of the citrate degrading bacteria *P. fluorescens*, did not degrade citrate from tridentate Cu^{2+} and Cd^{2+} complexes (Joshi-Tope and Francis, 1995). Hindrance caused by the structure of complexes was reported to inhibit citrate degradation. Enzymes responsible for U^{6+} reduction by BrY have not been isolated or sufficiently characterized. Hence, information related to the structural specificity for hexavalent uranium utilization is not currently known.

8.3.5 Toxicity of selected U^{6+} species to BrY

Finally, toxic effects of different uranyl ion species on microorganisms have been evaluated by various researchers (Rothstein, 1948; Tuovinen and Kelly, 1974a,b; Sugio et al. 1982). Tuovinan and Kelly (1974a,b) evaluated toxicity of various U^{6+} species to iron oxidizing acidophilic bacteria *Thiobacillus ferrooxidans*. They observed that, when U^{6+} formed uncomplexed UO_2^{2+} species, activity of bacteria was negatively impacted. Addition of 5 to 100 mM of U^{6+} inhibited ferrous iron oxidation, CO_2 fixation and cell viability. The authors reported that the cells appeared plasmolyzed when

observed under electron microscope. Toxic effects to *T. ferrooxidans* were alleviated upon complexation of uranyl ion with EDTA. No study has so far been performed regarding toxicity of uranyl hydroxide, UO_2 -acetate or $(\text{UO}_2)_3(\text{OH})_2(\text{tiron})_3$ species on bacterial or yeast cells. In order to investigate if indeed exposure to certain species of U^{6+} inhibited BrY in this research, additional experiments were performed in which cells inoculated in various test solutions were observed using an electron microscope. For microscopic analysis, test solutions containing various ligands, prepared using procedure described in section 4.5, were inoculated with 5×10^8 cells/mL. After incubating for twelve hours cells were fixed with a 3 % solution of glutaraldehyde in cacodylate buffer (pH=7.2) for two hours at 60 ° C. Subsequently they were dehydrated with a series of ethyl alcohols and embedded in epon resin. Sections, approximately 900 - 1100 Å thickness, were cut with a diamond knife using a Reichert ultramicrotome and mounted on copper grids. Pictures were made using an H600 - Hitachi electron microscope at an accelerating voltage of 75 kV. For comparison, electron micrographs of cells grown in nutrient rich medium and cells inoculated in organic free (NaNO_3) solution containing no uranium were also made.

Figures 29 through 33 show electron micrographs of BrY in growth and test solutions. Pictures of cellular sections in nutrient rich growth medium showed the presence of a large number of healthy cells (Figure 29). Most of the cells retained their shape and their interior was replete with cellular material, as indicated by the dark structures. A small number of cells were in a state of decay, with outer membranes



Figure 29. Electron micrograph of thin sections of BrY after growth in nutrient rich media for 12 hours.

peeling away from cells in addition to depletion of cellular materials, as indicated by patches of white region. Sections made from cells in the citrate solution contained a substantial fraction of healthy cells (Figure 30). U^{6+} was rapidly reduced in the presence of citrate. However, the fraction of healthy cells was less than that found when growth medium was used. The non-growth conditions in test (citrate) solution could have reduced the number of healthy cells. Micrographs of $NaNO_3-UO_2^{+2}$ and tiron- UO_2^{2+} solutions (Figures 21, 32) showed a substantial fraction of unhealthy cells. Although a very small fraction of healthy cells was observed, most of the cells lacked intracellular material in addition to a swollen nucleotide. This indicated that incubation of cells in these solutions affected the activity of cells. Although the interior composition was affected, cells in the $NaNO_3-UO_2^{2+}$ and tiron- UO_2^{2+} solutions retained their shapes. This indicated that the BrY membrane was not lyzed as reported for *T. ferrooxidans* upon exposure to UO_2^{2+} . It appeared that the nature of inhibition to BrY in $NaNO_3$ /tiron solutions was different than that experienced by *T. ferrooxidans* in uranium solutions. In addition, the electron micrograph of BrY cells in organic free test solution containing no uranium (Figure 33) contained a large number of unhealthy cells. This suggested that the decrease in the number of healthy cells in U^{6+} containing organic free and tiron solutions was not due to any direct toxic effect of uranium. Non-availability of U^{6+} as an electron acceptor rather than toxicity appeared to have produced the unhealthy cells in these solutions. However, elucidation of the mechanism which resulted in non-utilization of U^{6+} from organic free and tiron solutions needs further investigation.

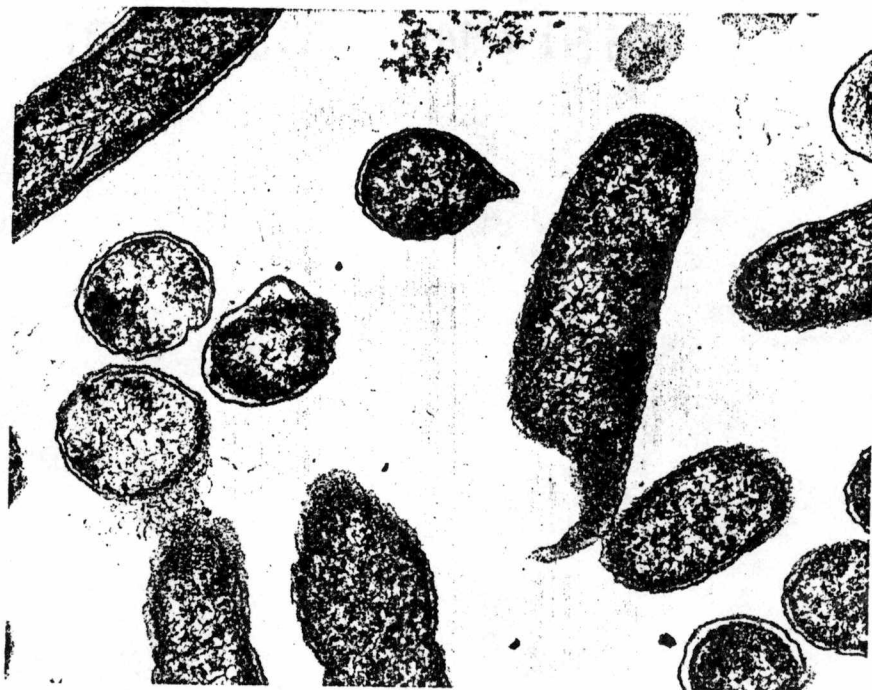


Figure 30. Electron micrograph of thin sections of BrY after incubation in citrate - U^{6+} containing test solution for 12 hours.

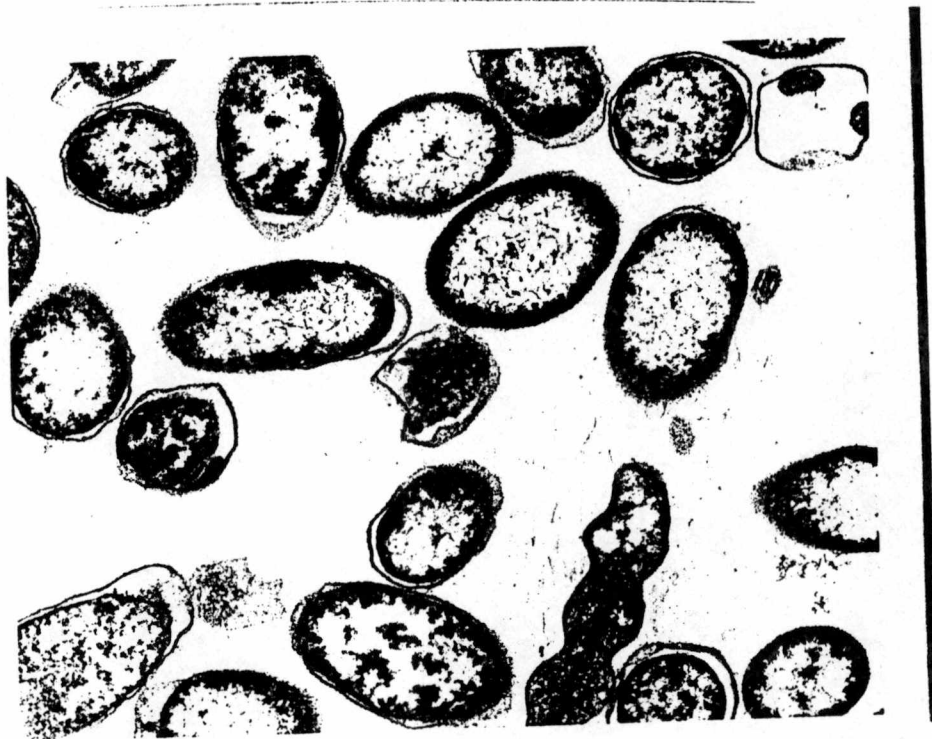


Figure 31. Electron micrograph of thin sections of BrY after incubation in organic free (NaNO_3) - U^{6+} containing test solution for 12 hours.

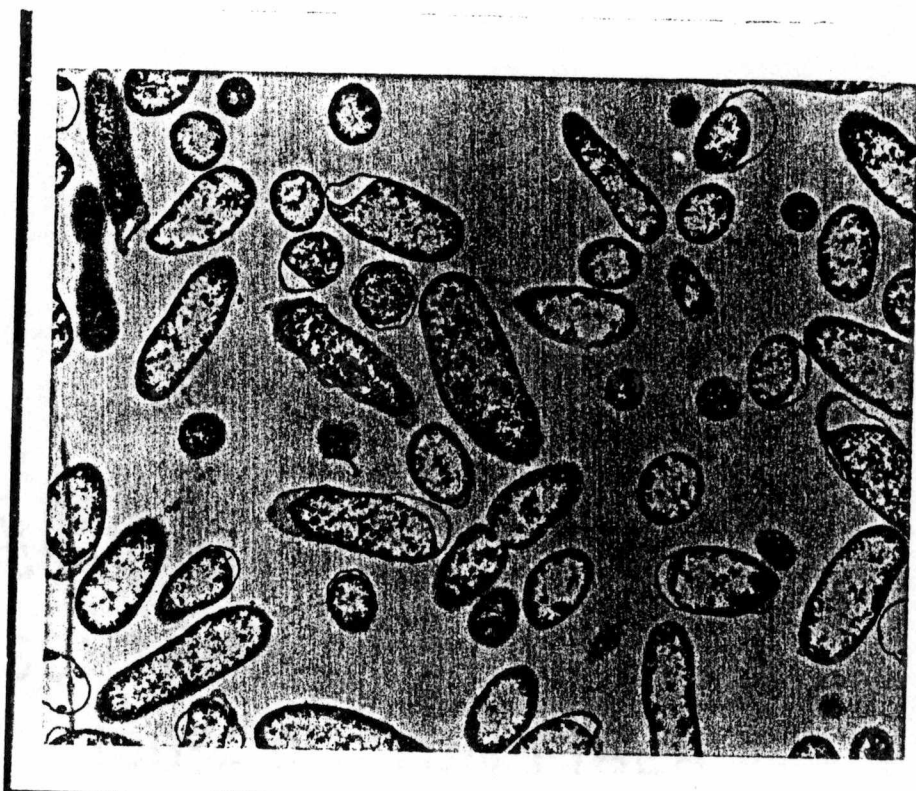


Figure 32. Electron micrograph of thin sections of BrY, after incubation in tiron - U^{6+} containing test solution for 12 hours.

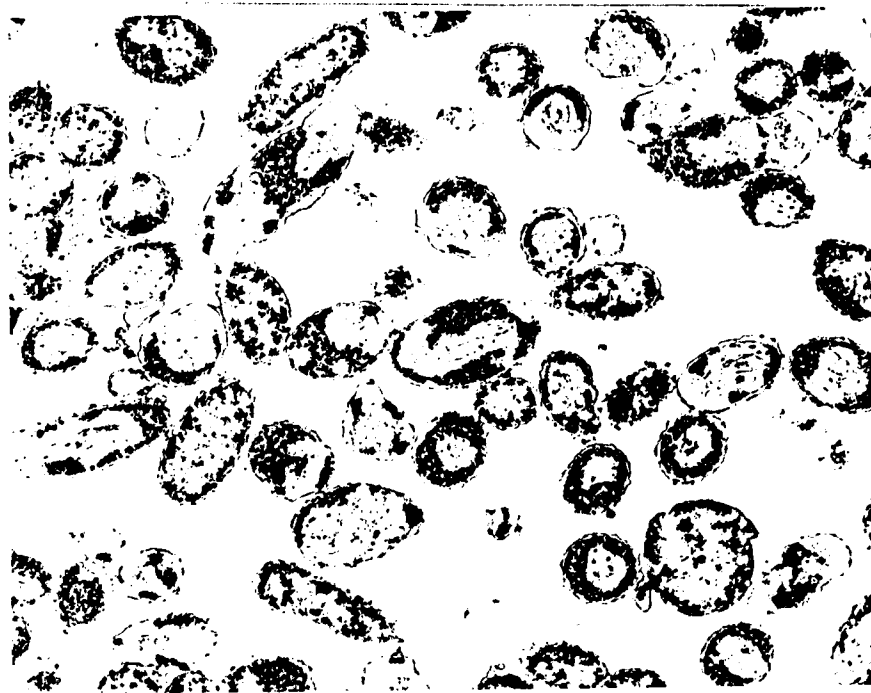


Figure 33. Electron micrograph of thin sections of BrY, after incubation in organic free (NaNO_3) test solution for 12 hours. The solution did not contain U^{6+} .

In addition to toxicity via plasmolysis, uranyl ion is reported to inhibit cellular activity using other mechanisms. For example, Rothstein, et al. (1948,1949, 1950) conducted extensive studies to investigate inhibitory effects of different uranium species to yeast cells. Their studies indicated that, when U^{6+} formed uncomplexed UO_2^{2+} species in test solution, it negatively impacted sugar metabolism of yeast cells. It was also observed that uncomplexed uranyl ion did not kill the cells and that inhibition was reversible. When the researchers introduced complexing agents such as carbonate and citrate, sugar metabolism was restored, suggesting that inhibitory effects were speciation depended. Their studies indicated that sugar metabolism required bivalent cations such as Mg^{2+} , Mn^{2+} and Ca^{2+} to be associated with selected polyphosphate groups on the cell surface. Polyphosphates, upon complexation with these cations, participated in sugar metabolism. However, UO_2^{2+} successfully competed with these cations for binding with polyphosphates and inactivated sugar metabolism of the yeast cells. Nearly 6.7×10^7 uranium molecules sorbed to each cell to result in 100% inhibition. In a separate study Cochrane and Tull (1958) observed that uranyl ion inhibited spore respiration of *Neurospora sitophila*. Anaerobic respiration was found to be 20 fold more sensitive to uranyl ion toxicity than aerobic respiration. Maxwell et al., (1971) reported that in addition to sugar metabolism, uranyl ions inhibited the uptake of amino acid (glycine), vitamin (biotin) and disaccharide (maltose) by yeast cells.

Comparison of results obtained using BrY in this research with that of Rothstein and other researchers on uranyl ion toxicity yielded several similarities as well

as differences. The similarities include the sorption of uranium to cells and the effects of chelating ligands (carbonate and citrate) on cellular activity. For example, in organic free (NaNO_3) and tiron containing test solutions $\sim 5 \text{ mg/L}$ of hexavalent uranium sorbed to cells (Appendix VI). Assuming a cellular mass of $1 \text{ mg per } 10^7 \text{ cells}$ (Rotheistein, 1948), approximately $2.35 \times 10^{12} \text{ U}^{6+}$ molecules sorbed to each BrY cell. This sorption is orders of magnitude higher than the amount of uranium sorbed to yeast cells during 100 % inhibition in Rotheistein's (1948) studies. As found with yeast cells, uranyl ions sorbed to BrY could have affected the activity of BrY cells.

Secondly, complexation of uranyl ion with carbonate and citrate enabled BrY to rapidly reduce hexavalent uranium. This observation was comparable to the alleviation of uranium toxicity to yeast cells after the introduction of these ligands. In addition to the similarities mentioned, it should be noted that U^{6+} reduction using BrY was evaluated under anaerobic conditions. Microorganisms have been reported to be more sensitive (~ 20 fold) to uranium toxicity under anaerobic conditions than under aerobic conditions (Cochrane and Tull, 1958).

These observations regarding uranyl ion sorption, role of citrate and carbonate and U^{6+} reduction under anaerobic conditions suggested possible inhibition of uranyl ion to BrY cells through complexation with polyphosphate or other related cellular functional groups. However, experimental conditions using BrY differed from that used for yeast cells in some aspects. For example, toxicity studies using yeast cells were conducted under growth conditions. Sugar and other organic compounds were provided as

carbon/electron source for the cellular growth. Bivalent cations such as Ca^{2+} and Mn^{2+} were provided to facilitate phosphorylation. In contrast, U^{6+} reduction using BrY in this research were evaluated under non-growth conditions. No carbon source, other than the test ligand was provided during bioreduction. H_2 gas was the electron donor for U^{6+} reduction. It is not currently known if uranyl ion can inhibit microbial transformation of H_2 gas. In addition, no bivalent cations such as Ca^{2+} , Mn^{2+} or Mg^{2+} were provided during U^{6+} reduction. It is not known if uranyl ion from hydroxyl complexes competed with intracellular/periplasmic cations of BrY during H_2 / UO_2^{2+} biotransformation. However, comparison of kinetic parameters for U^{6+} reduction in various test solutions (Tables XIX & XX) indicated that maximum U^{6+} utilization rate (V_m) observed in multidentate organic solutions was nearly forty fold higher than that observed in acetate solutions. The half saturation coefficient (K_m) for U^{6+} reduction was essentially the same ($100 \pm 20 \text{ mg U}^{6+}/\text{L}$) in most of the test solutions. This trend indicated the possibility of a non-competitive inhibition occurring to microbial enzymes in test solutions (Devlin, 1986). A non-competitive inhibition is caused by the binding of an inhibitory species to a site in the enzyme other than the active site of target substrate binding. The trend in kinetic parameters obtained in this research suggested the possibility of uranyl hydroxide or uranyl acetate molecules binding to selected sites in the U^{6+} reducing enzyme complex thereby inhibiting U^{6+} reduction.

In summary, experiments using BrY indicated that hexavalent uranium from multidentate aliphatic complexes was readily available for bioreduction. However,

Table XIX. Kinetic parameters for U^{6+} reduction in various test solutions by BrY. Ligands initially complexed ~ 100 % of hexavalent uranium.

Ligand	Maximum Substrate Utilization Rate. V_m (mg U^{6+} /mg biomass-hr)	Half Saturation Constant K_m (mg/L)	Initial U^{6+} Reduction Rate. K_i (mg U^{6+} /mg biomass-hr)
Carbonate	2.7×10^{-3}	102.3	1.24×10^{-3}
Acetate	4×10^{-5}	78.5	2×10^{-3}
Malonate	2.4×10^{-3}	116.2	1.1×10^{-3}
Oxalate	2.44×10^{-3}	114	1.25×10^{-3}
Citrate	2.46×10^{-3}	93	1.3×10^{-3}

Table XX. Kinetic parameters for U^{6+} reduction in various test solutions by BrY. Ligands initially complexed ~ 50 % of hexavalent uranium.

Ligand	Maximum Substrate Utilization Rate V_m (mg U^{6+} /mg biomass-hr)	Half Saturation Constant K_m (mg/L)	Initial U^{6+} Reduction Rate K_i (mg U^{6+} / mg biomass- hr)
Acetate	3×10^{-3}	86	1.5×10^{-3}
Malonate	1.1×10^{-4}	95	5.7×10^{-3}
Oxalate	5.4×10^{-4}	1023	5.2×10^{-3}
Citrate	2.0×10^{-3}	83.3	1.1×10^{-3}

evidences indicated that of uranyl hydroxide or acetate species complexed with polyphosphate or other cellular functional groups and non-competitively inhibited U^{6+} reduction. However, complete information related to such a toxicity is not currently available.

8.4 Rate of U^{6+} reduction in organic solutions by BrY

Although, UO_2^{2+} reduction using BrY was limited in $NaNO_3$, tiron and other solutions involving hydrolysis of uranyl ion, the bacterium rapidly reduced hexavalent uranium from carbonate, malonate, oxalate and citrate (> 98% complexed) solutions. In addition, the rate of reduction from monodentate carbonate solution as well as that predicted to from multidentate malonate, and citrate were essentially identical (Tables XIX & XX). Analysis of results yielded no statistical ($\alpha = 0.05$) difference in U^{6+} reduction in these solutions. The monodentate complexed U^{6+} levels (< 2 mg/L) in malonate, oxalate and citrate solutions represented substrate limiting conditions for BrY cells (Peyton, 1995). Identical U^{6+} reduction trends observed in monodentate (carbonate) and multidentate (malonate, oxalate and citrate, 100 % complexed) solutions indicated that BrY directly attacked U^{6+} from all the complexes evaluated. Note that the sulfate reducing bacterium *D. desulfuricans* used in this research selectively reduced hexavalent uranium from monodentate complexes.

In the test solutions where the ligands initially complexed ~ 50% of the U^{6+} , the rate of U^{6+} reduction was lower than that observed in solutions containing higher

concentration of each multidentate ligand (Figure 34). However, reduction appeared to be more efficient than that observed in organic free (NaNO_3) solution, where U^{6+} was not reduced after ninety six hours of incubation. Note that in organic free (NaNO_3) solutions, all of the uranyl ion added was formed uranyl hydroxide species. In solutions containing low concentrations of organic ligands, MINTEQ predicted $\sim 50\%$ U^{6+} to complex with the ligand through carboxyl group interactions. Results indicated that formation of such uranyl-organic complexes enhanced the bioavailability of hexavalent uranium to BrY. It appeared that BrY reduced U^{6+} in two stages in these solutions. Initially the bacterium readily utilized hexavalent uranium complexed to the organic ligands, as indicated by the rapid U^{6+} reduction observed in the first four hours of incubation (~ 40 to 50%). Once the organic complexed U^{6+} was reduced, further reduction likely continued through equilibrium redistribution of uranyl hydroxide among uranyl-organic and uranyl hydroxide species. Uranyl ion reduction resulting from the combination of the two mechanisms appeared to be slower. However, a quantitative estimate on the rate and extent of each stage was rendered difficult due to the following reasons, (i) the influence of uranyl hydroxide species (non-availability vs partial inhibition) is not currently known, (ii) the decrease in the activity of the cells over time, under the non-growth conditions provided, may affect the efficiency of U^{6+} reduction.

Similar speciation related bioavailability was observed also by Francis et al. (1992). The authors reported that, *P. fluorescens* readily degraded citrate when the ligand was not complexed to metals. However, no degradation occurred when citrate was

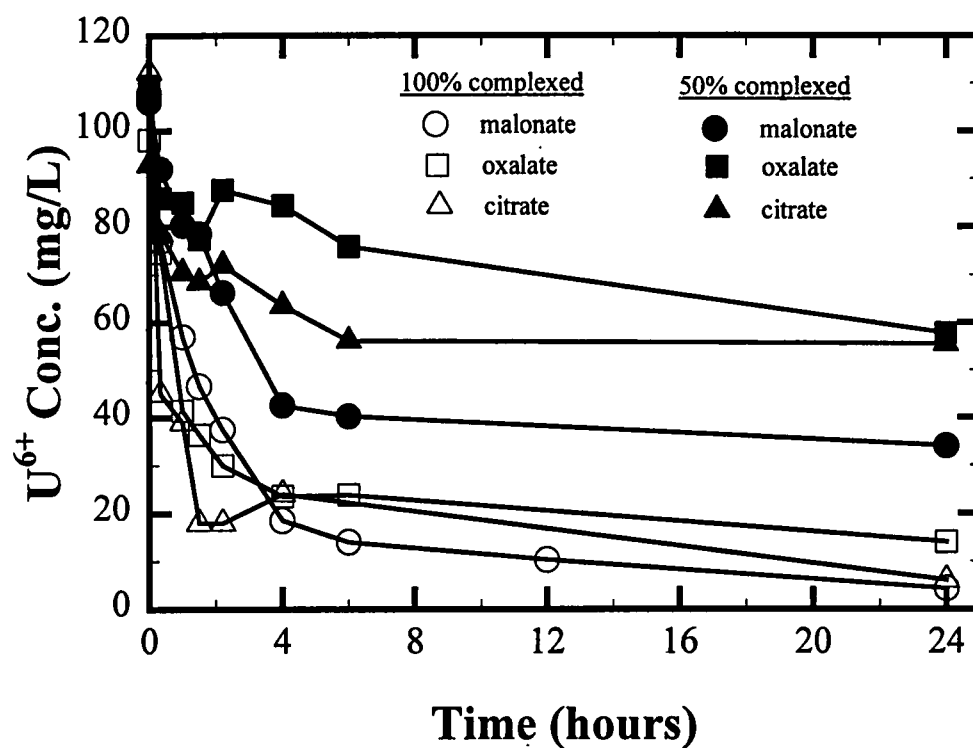


Figure 34. U^{6+} reduction in test solutions containing high and low concentrations of malonate, oxalate and citrate after inoculation with BrY

complexed to uranyl ion. In test solutions which contained 50 % uncomplexed citrate and 50 % UO_2^{2+} complexed citrate, the ligand degradation was slow.

Statistical analysis ($\alpha = 0.05$) indicated that, with the exception of tiron, no appreciable difference in U^{6+} reduction was observed among individual test ligands. This suggested that the source of available U^{6+} was essentially the same (~50% U^{6+} from multidentate complex) in each solution.

8.5 Precipitation of bio-reduced uranium

Although the type of bacterium (*D. desulfuricans* or BrY) impacted the trends in U^{6+} reduction, bacterial strains appeared to have no impact on the extent of bio-reduced uranium precipitation. Precipitation appeared to be impacted by the type and concentration of individual ligands introduced into the test solution. Figure 35 shows the total uranium ($\text{U}^{6+} + \text{U}^{4+}$) measured in filtrate samples after inoculation with BrY when ~100% U^{6+} was initially complexed to the ligands. In acetate solution, all the bio-reduced uranium precipitated from solution. U^{4+} had very little affinity for acetate. The preferred reaction, under the experimental conditions, was precipitation from solution. In addition, the total uranium concentration in the filtrate was essentially the same as the U^{6+} level (Appendix VI), indicating uranium, after reduction, precipitated with no appreciable lag period. Incomplete U^{6+} reduction (~80%) resulted in incomplete uranium removal in acetate solutions incubated with BrY.

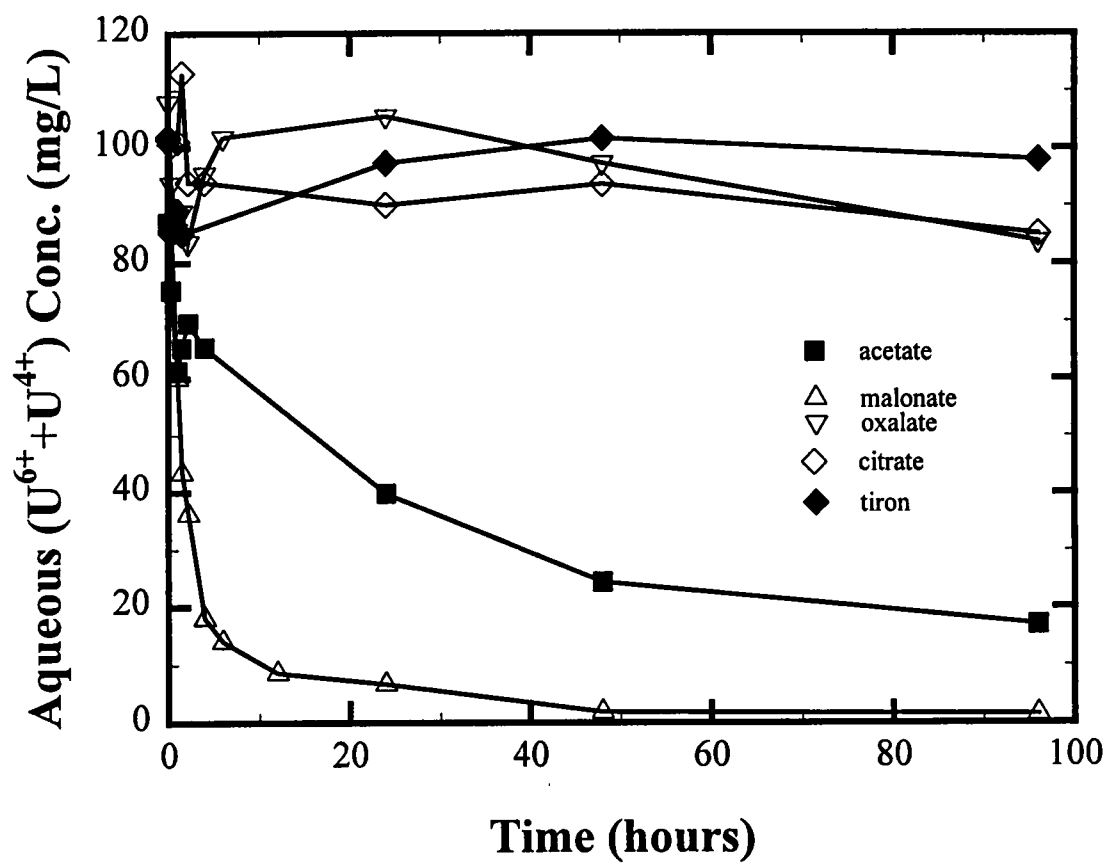


Figure 35. Total uranium ($U^{6+} + U^{4+}$) concentration in filtrate during U^{6+} bioreduction by BrY. Ligands initially complexed $\sim 100\%$ U^{6+} .

Total uranium level in malonate solutions decreased more rapidly and the extent of uranyl ion reduction was greater than in acetate solution. Filtrate samples contained less than 5 mg/L after 24 hours of incubation. Rapid reduction of uranium led to the rapid rate of removal via precipitation. U^{4+} precipitated from solution rather than recomplexing with malonate. Tetravalent uranium precipitation in acetate and malonate solutions was similar to that observed in the presence of *desulfuricans* and that predicted using thermodynamic constants.

Unlike acetate and malonate solutions, total dissolved uranium concentrations did not appreciably decrease in oxalate solutions. However, BrY reduced most of the hexavalent uranium during this period. This indicated that U^{4+} readily complexed with oxalate and remained in solution. Results differed from the predictions made using Th^{4+} stability constants were that observed in experiments conducted with *desulfuricans*.

Reduced uranium did not precipitate in citrate solutions. Although > 90% U^{6+} was reduced within four hours of incubation, more than 80% of the reduced uranium remained in solution even after 96 hours. Similar to oxalate, the presence of citrate did not impact U^{6+} reduction by BrY, however, it impacted uranium removal due to recomplexation with U^{4+} .

Since hexavalent uranium was not bio-reduced in tiron solution, it was not removed via reductive precipitation. Note that in the experiments using *desulfuricans*, U^{4+} precipitation was affected due to recomplexation with tiron. These results suggested

that U^{6+} reduction and possible U^{4+} precipitation may be affected in tiron containing solutions incubated with BrY.

Figure 36 shows total uranium concentration in filtrate samples after inoculation with BrY when ~50% U^{6+} was predicted initially complex to ligands. In all but tiron containing test solutions, filtrate samples contained ~ 20 to 30 % of the initial uranium added after 96 hours of incubation. The decrease in total uranium concentration closely followed the trend in U^{6+} reduction in these solutions. This indicated that bioreduction of U^{6+} by BrY dictated removal of uranium from these solutions. In addition U^{4+} precipitation appeared to occur with no appreciable lag period. However, since all these solutions contained ~50% uranyl hydroxide complexes, bioreduction was generally slower than that observed in solutions containing high concentration of ligands.

In summary, the extent of U^{4+} precipitation varied widely in test solutions incubated with BrY. In organic free ($NaNO_3$) and tiron containing solutions, little, if any uranium was removed via precipitation. Non-availability of U^{6+} for bioreduction resulted in the lack of uranium removal in these solutions. In solutions where BrY reduced U^{6+} to U^{4+} the trends in precipitation were similar to that observed in experiments using *D. desulfuricans*. Note that, under the experimental conditions used, bioreduced uranium precipitated as uraninite, essentially due to abiotic processes. Metabolism of microorganisms was not involved in precipitation. Once U^{6+} was reduced to U^{4+} , differences in bacterial metabolism (*desulfuricans* vs BrY) had little impact on the subsequent precipitation process. Fate of the reduced uranium was rather governed by its

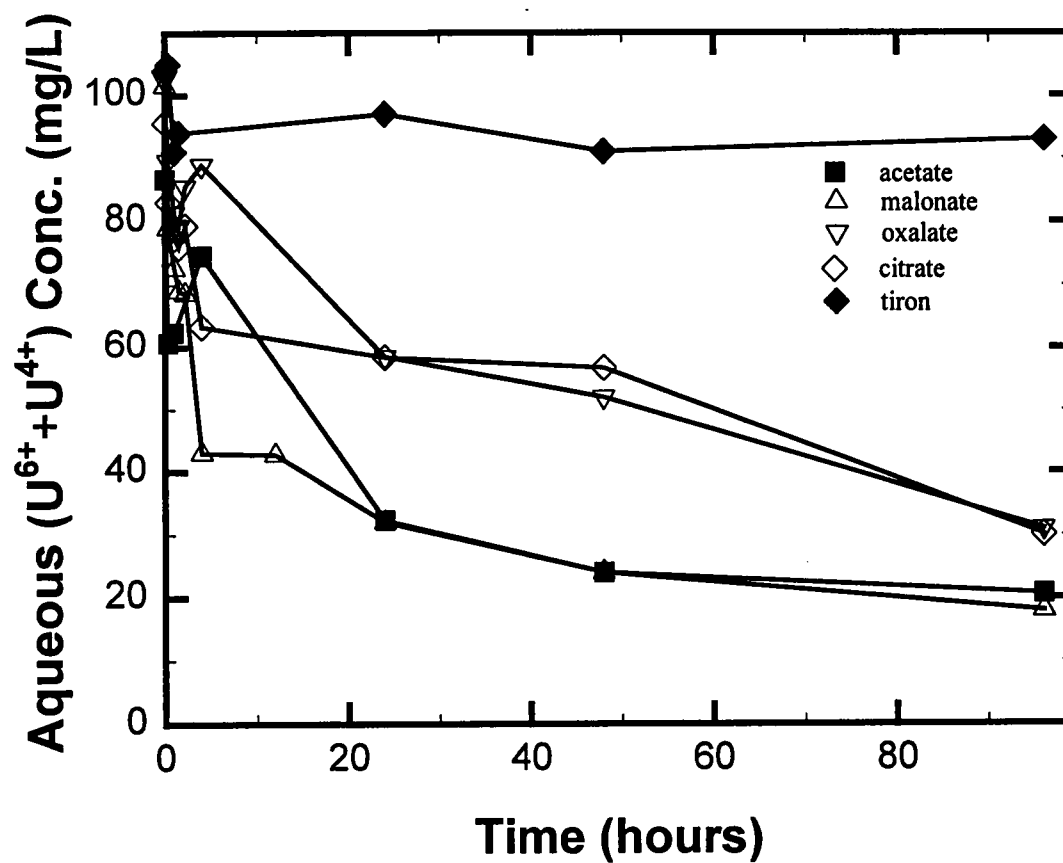


Figure 36. Total uranium (U⁶⁺+U⁴⁺) concentration in filtrate during U⁶⁺ bioreduction by BrY. Ligands initially complexed ~ 50% U⁶⁺.

thermodynamic (stability) constants for complexation with various ligands used. As discussed earlier, thermodynamic constants of Th^{4+} were used to predict the fate of bio-reduced uranium in acetate, malonate and oxalate solutions. MINTEQA2 predicted precipitation of U^{4+} from acetate, malonate and oxalate solutions. The prediction indicated recomplexation of U^{4+} with oxalate when the ligand concentration was increased. Results similar to that predicted were observed in test solutions incubated with both BrY and *D. desulfuricans*. Since association constants for binding of citrate with U^{4+} or Th^{4+} are not available, no prediction was made. In general, it appeared that the fate of bio-reduced uranium was essentially dictated by its thermodynamic constants for association with the ligands.

In addition, for test solutions where U^{4+} precipitated, the rate of removal varied. Unlike the extent of precipitation, the rate of precipitation appeared to be influenced by the bacterial activity. In test solutions where uranium precipitated, the rate at which BrY reduced U^{6+} appeared to impact the rate of uranium removal.

8.6 Summary

Figures 37, 38 and Table XXI summarize the impact of individual organic ligands on uranium removal via BrY bio-reductive precipitation. Experimental results indicated that organic ligands influenced bioavailability of hexavalent uranium to BrY as well as subsequent precipitation of bio-reduced tetravalent uranium. It appeared that BrY

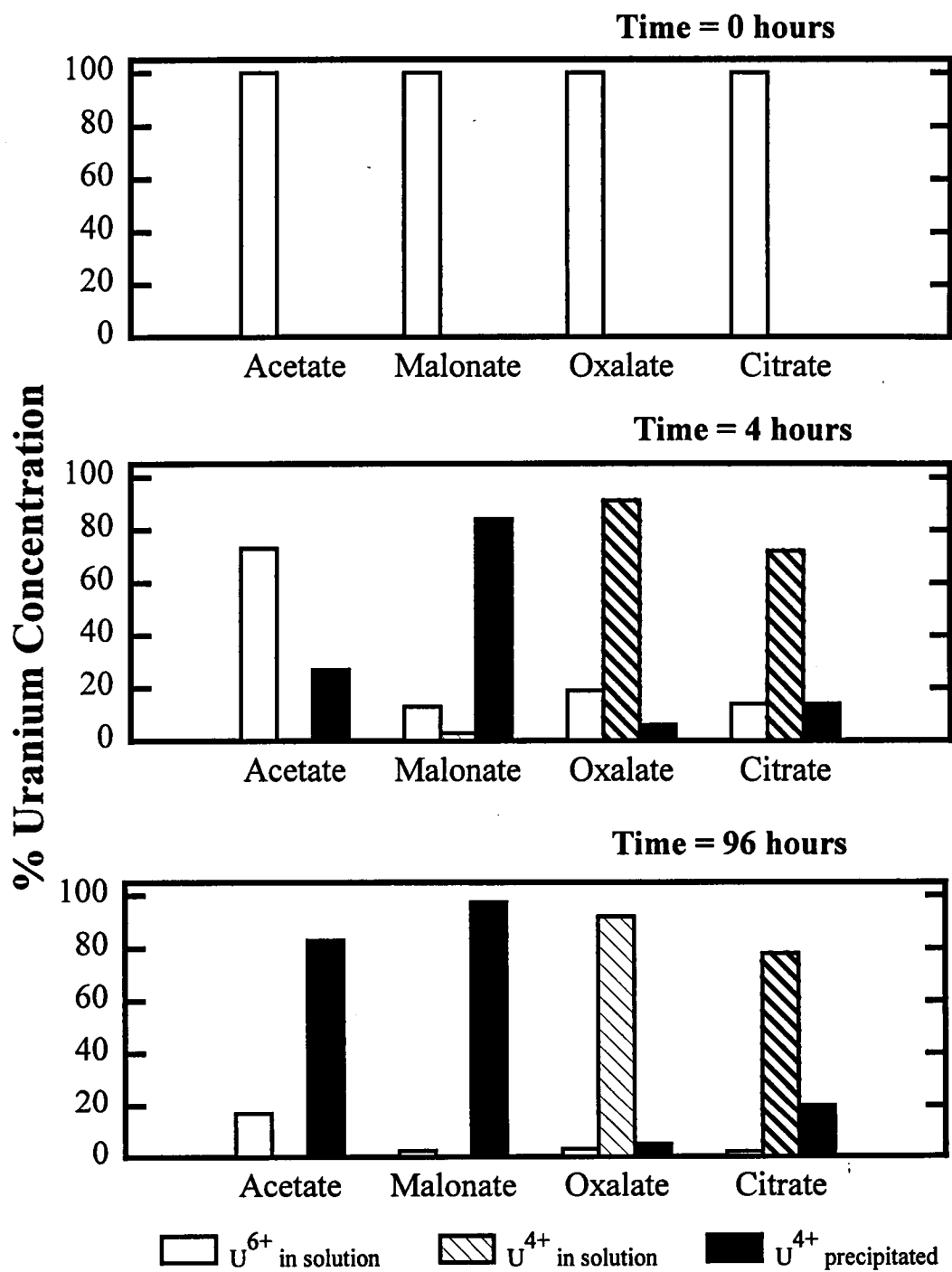


Figure 37. Summary of uranium distribution in solutions containing various organic ligands after inoculation with BrY. Ligands initially complexed ~100% U⁶⁺.

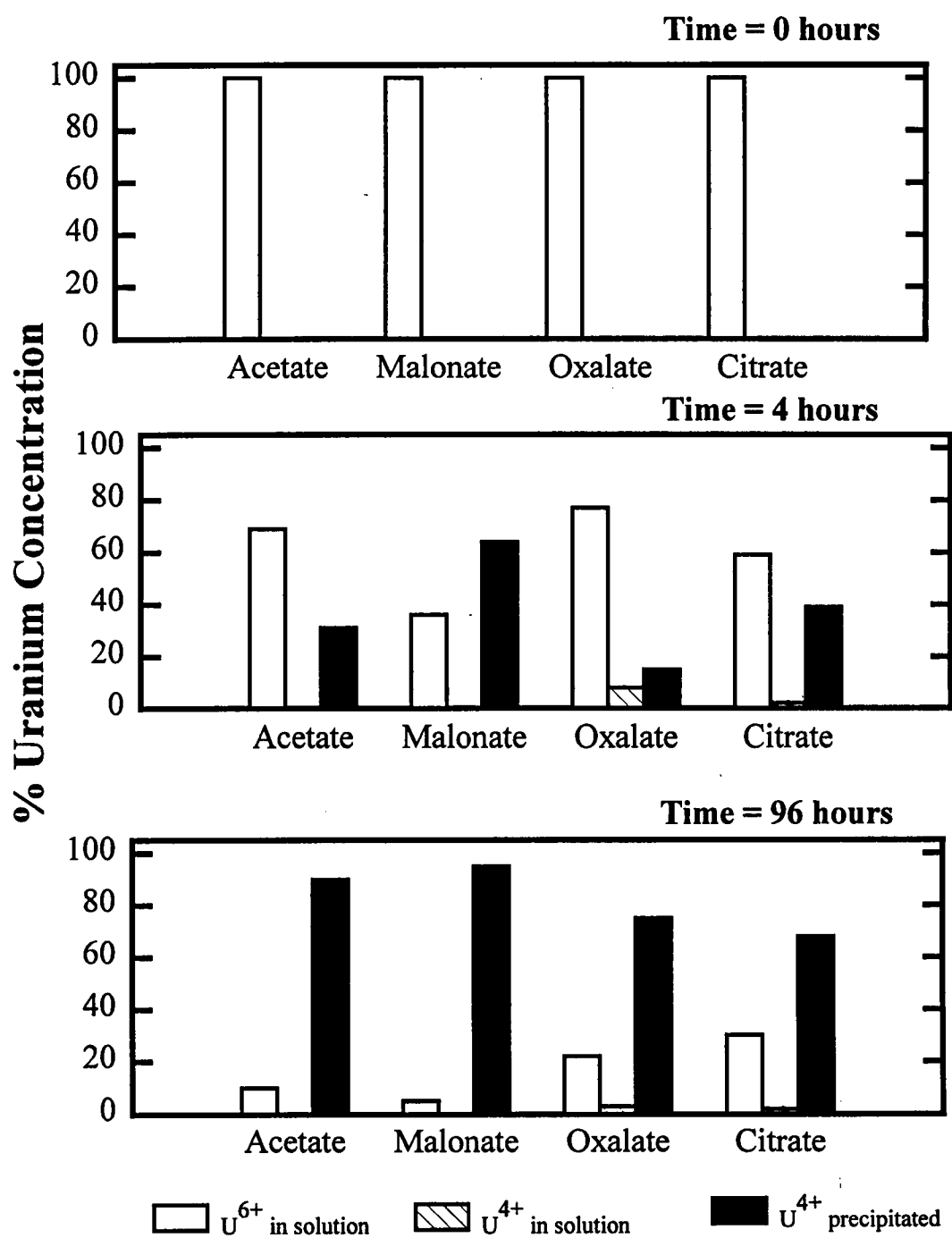


Figure 38. Summary of uranium distribution in solutions containing various organic ligands, after inoculation with BrY. Ligands initially complexed ~50% U⁶⁺.

Table XXI. Impact of various ligands on uranium removal via reductive precipitation using BrY

LIGAND	U ⁶⁺ REDUCTION	U ⁴⁺ PRECIPITATION	CRITICAL FACTOR
Acetate	Partially inhibited. Slow reduction.	No impact. Precipitation occurred immediately after U ⁶⁺ reduction.	Rate of U ⁶⁺ reduction dictated uranium removal.
Malonate	No impact at high malonate conc. Decrease in ligand conc. inhibited rate of reduction.	Precipitation occurred upon U ⁶⁺ reduction.	Conc. of ligand inversely affected rate of U ⁶⁺ reduction. Uranium removal depended on U ⁶⁺ reduction.
Oxalate	No impact at high ligand conc. Low concentration affected rate of U ⁶⁺ reduction.	Precipitation depended on ligand conc. U ⁴⁺ precipitated at low (0.0006M) conc., and not at high (0.01M) conc.	Low ligand conc. affected U ⁶⁺ reduction. High ligand conc. affected U ⁴⁺ precipitation.
Citrate	Rate of reduction varied inversely with the conc. of citrate used.	Increase in ligand conc. decreased the amount of U ⁴⁺ precipitation.	Low citrate conc. affected U ⁶⁺ reduction. High ligand conc. affected precipitation.
Tiron	Near total inhibition for U ⁶⁺ reduction.	N/A	Unavailability of U ⁶⁺ for reduction affected impacted uranium removal.

N/A - Not applicable

readily bio-reduced uranyl ion from monodentate carbonate complexes and multidentate aliphatic complexes involving hydroxyl ion interactions. In solutions containing a mixture of uranyl-carboxyl and uranyl-hydroxyl species, BrY reduced U^{6+} essentially from carboxyl complexes. Subsequent reduction proceeded due to the replenishment of carboxyl-complexed uranyl ion from the hydroxyl species through equilibrium redistribution. Such a reduction, mediated by redistribution, enabled BrY to utilize a significant fraction of uranyl ion which was initially predicted to form hydroxyl species. However, after bio-reduction, precipitation of U^{4+} appeared to be governed by its association constants for complexation with test ligands. Tetravalent uranium precipitated from acetate, malonate and low concentration oxalate containing solutions. The extent of U^{4+} precipitation observed in BrY experiments was identical to that observed in experiments using *desulfuricans*.

9. COMPARISON OF U^{6+} REDUCTIVE PRECIPITATION BY *D. desulfuricans* AND *S. alga* (BrY)

Results presented in chapters 6.0 and 7.0 indicated that the two bacteria used in this research reduced hexavalent uranium using different mechanisms. The sulfate reducing bacterium *D. desulfuricans*, appeared to reduce U^{6+} readily from monodentate organic/inorganic complexes, as indicated by the rapid reduction in organic free ($NaNO_3$, carbonate) and acetate solutions. *D. desulfuricans*, however, did not reduce hexavalent uranium readily from multidentate organic, aliphatic, complexes involving carboxyl group interactions. The initial U^{6+} reduction rate (K_1) for monodentate complexes was more than an order of magnitude higher than that for multidentate organic complexes. In addition, results indicated that *D. desulfuricans* readily accessed hexavalent uranium from ternuclear aromatic tiron complexes. Tiron complexes involved interactions of four hydroxyl ions. Such a complexation, probably did not protect U^{6+} from the enzymes reducing it.

The iron reducing bacterium *S. alga* (BrY) appeared to reduce hexavalent uranium rapidly from multidentate aliphatic complexes and stronger monodentate carbonate complexes. However, reduction from weaker monodentate acetate complexes was slow and incomplete. In addition, uranyl ion from species involving hydroxyl ion interactions appeared to be unavailable as electron acceptor to BrY.

Tables XXII & XXIII summarize the differences in kinetic parameters for U^{6+} reduction in test solutions after inoculation with the two bacteria. In addition, % U^{6+} reduced by the two bacteria after 4 hours of incubation in various solutions are shown in Figure 39, to understand the differences better. The organic ligands initially complexed ~98% of U^{6+} in all these cases. As can be seen, with the exception of carbonate, in each of the test solution, the two bacteria reduced U^{6+} at significantly different rates. In monodentate test solutions the initial rate of U^{6+} reduction (K_i) using *desulfuricans* was more than an order magnitude higher than that using BrY. In multidentate aliphatic solutions initial rate of U^{6+} reduction using BrY was more than an order magnitude faster than that in the presence of *desulfuricans*. Such differences in U^{6+} reduction resulted from differences in the nature of enzymes involved in reduction. For example, *D. desulfuricans* reduced uranyl ion using enzyme cytochrome c_3 located at the soluble (periplasmic) fraction of the cell (Lovely, 1993c). In addition, the enzyme did not appear to be a component of electron transport chain. *Desulfuricans* did not grow using U^{6+} as terminal electron acceptor (Lovely, 1992b). In contrast, BrY reduced hexavalent uranium using a multi-component enzyme system which included a 'c' type cytochrome (Caccavo, 1995). The enzyme was primarily associated with the membrane fraction of the cell. In addition, the enzyme appeared to be involved in electron transport processes. Such differences in the U^{6+} reducing enzyme composition, location and metabolic functions probably lead to the differences in reduction trends observed using the two bacteria. However, in solutions where U^{6+} was readily available for reduction (monodentate for

Table XXII. Comparison of kinetic parameters for U^{6+} reduction in various test solutions incubated with *D. desulfuricans* and BrY. The organic ligands initially complexed $\sim 100\%$ U^{6+} .

Ligand	Max. U^{6+} Reduction Rate V_m (mg U^{6+} / mg cell - hr)		Half Saturation Constant K_m (mg U^{6+} / L)		Initial U^{6+} Reduction Rate K_i (mg U^{6+} / mg cell - hr)	
	<i>desulfuricans</i>	BrY	<i>desulfuricans</i>	BrY	<i>desulfuricans</i>	BrY
NaNO ₃	3.6×10^{-3}	-	112	-	1.6×10^{-4}	-
Carbonate	2.9×10^{-3}	2.7×10^{-3}	94.5	102	1.5×10^{-3}	1.2×10^{-3}
Acetate	3.2×10^{-3}	4.0×10^{-3}	57	78.5	1.9×10^{-3}	2.0×10^{-3}
Malonate	1.7×10^{-3}	2.4×10^{-3}	104	116	9.0×10^{-5}	1.1×10^{-3}
Oxalate	1.8×10^{-3}	2.4×10^{-3}	104	114	8.9×10^{-5}	1.3×10^{-3}
Citrate	1.4×10^{-3}	2.5×10^{-3}	117	93	6.0×10^{-5}	1.3×10^{-3}
Tiron	1.3×10^{-3}	-	121	-	5.7×10^{-4}	-

Table XXIII. Comparison of kinetic parameters for U^{6+} reduction in various test solutions incubated with *D. desulfuricans* and BrY. The organic ligands initially complexed $\sim 50\%$ U^{6+} .

Ligand	Max. U^{6+} Reduction Rate V_m (mg U^{6+} / mg cell - hr)		Half Saturation Constant K_m (mg U^{6+} / L)		Initial U^{6+} Reduction Rate K_i (mg U^{6+} / mg cell - hr)	
	<i>desulfuricans</i>	BrY	<i>desulfuricans</i>	BrY	<i>desulfuricans</i>	BrY
Acetate	4.0×10^{-3}	3.0×10^{-5}	100	86	1.9×10^{-3}	1.5×10^{-5}
Malonate	6.4×10^{-4}	1.1×10^{-4}	105	95	3.4×10^{-4}	5.7×10^{-5}
Oxalate	3.8×10^{-4}	5.4×10^{-4}	117	102	1.8×10^{-4}	5.2×10^{-5}
Citrate	2.6×10^{-4}	2.0×10^{-5}	78	83	1.4×10^{-4}	1.1×10^{-5}
Tiron	1.0×10^{-3}	-	119	-	4.6×10^{-4}	-

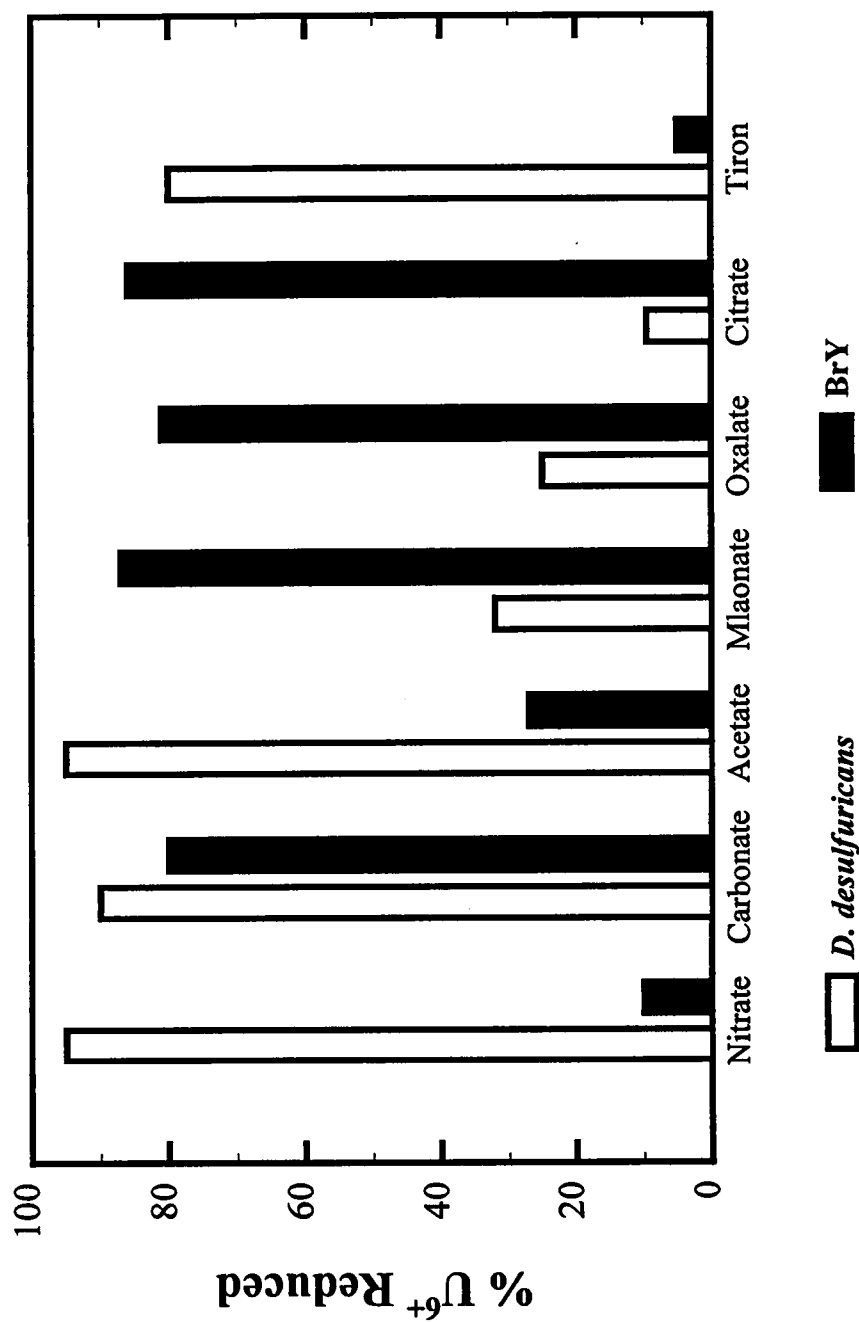


Figure 39. Amount of U^{6+} reduced by the *D. desulfuricans* and BrY in various test solutions after incubation for four hours.

desulfuricans and multidentate for BrY) the two bacteria reduced U^{6+} essentially at identical rates ($\sim 1.5 \times 10^{-3}$ mg U^{6+} /mg cell-hr). Multidentate organic ligands inhibited U^{6+} reduction by *desulfuricans* by non-competitive inhibition or through chelate effect. Uranyl hydroxide and uranyl acetate species non-competitively inhibit U^{6+} reduction by BrY. The exact nature of inhibition to either bacteria requires further investigation. However, the impact of inhibition on U^{6+} reduction appear to be the same. For example, an inhibition to U^{6+} reduction was often associated with a significant decrease in maximum U^{6+} reduction rate (V_m). However, the half saturation coefficient (K_m) for U^{6+} reduction remained essentially same (~ 100 mg/L) in most of the cases.

Although trends in U^{6+} reduction varied with the type of bacterium used, subsequent precipitation of bio-reduced uranium appeared to be unaffected by the metabolism of the two bacteria (Figure 40). Trends in precipitation generally followed the speciation predicted at equilibrium, using thermodynamic constants, for reduced uranium (U^{4+}) binding with various ligands. Any differences in the rate or extent of precipitation observed in the experiments were essentially due to the differences in the rate and extent of U^{6+} reduction by the two bacteria.

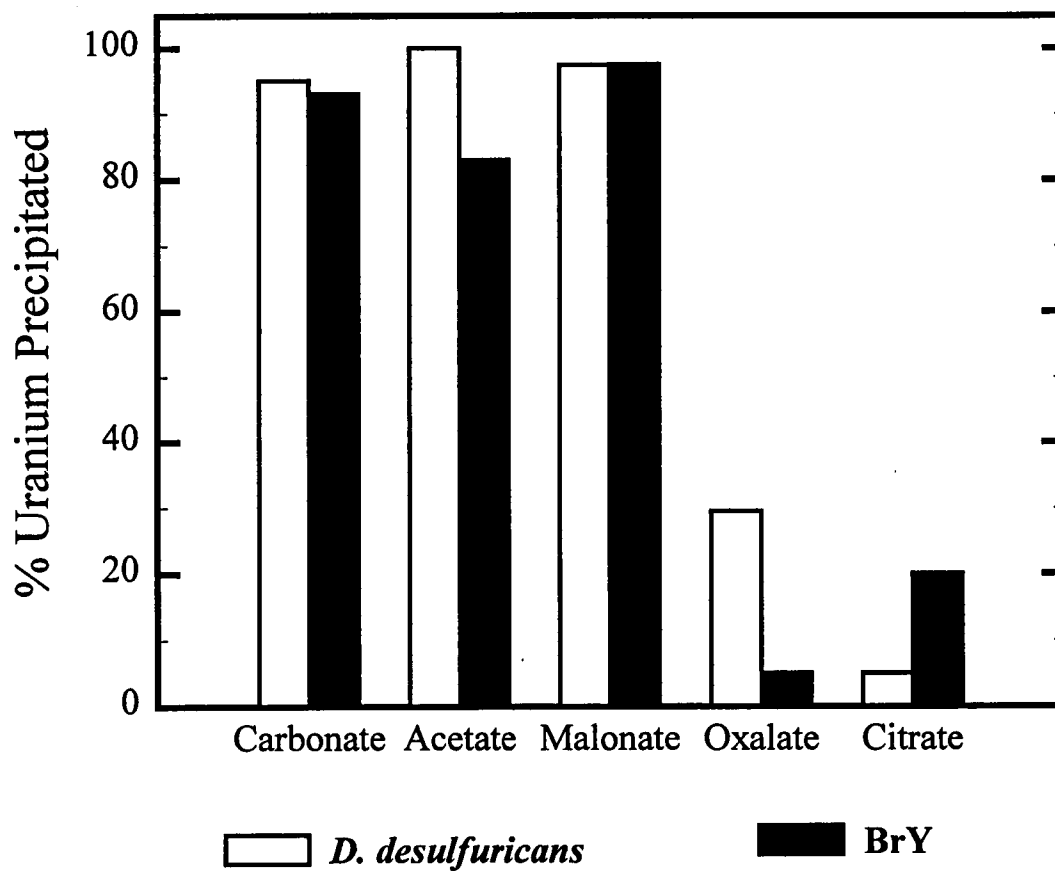


Figure 40. Amount of uranium precipitated after 96 hours in various test solutions incubated with *D. desulfuricans* and BrY.

10. IMPLICATIONS

Results obtained in this research indicated that uranium containing waste streams need to be pretreated prior to uranium removal using bioreductive precipitation processes. The type of pretreatment required will depend on the composition of the waste stream. Results from various experiments suggested that, from a waste treatment perspective, uranium containing streams can be classified into the following four groups: (i) surface and ground waters containing uranium but little or no organic matter; (ii) waste streams produced in environmental restoration processes through carbonate leaching of contaminated soils; (iii) mixed process waste streams containing a variety of simple and complex organic compounds used in uranium ore extraction and purification processes; and (iv) waste streams produced by soil decontamination processes using organic chelators such as citrate, EDTA, tiron.

When the composition of ground and surface waters containing uranium is similar to the organic free (NaNO_3 , carbonate) or acetate containing test solutions used in this research, rapid U^{6+} removal should occur. That is, bioreductive precipitation using *desulfuricans* can be effectively utilized. Use of BrY to treat such streams may require addition of carbonate to eliminate possible formation of uranyl hydroxide species in solution. Uranyl ion in carbonate containing leachate solutions can likely be removed using *D. desulfuricans* as well as BrY. These waste solutions generally contain very little

organic matter (Francis, 1993). The concentration and composition of organic matter present will govern the need for pretreatment of these leachate solutions.

Various organic compounds present in mixed process waste streams may impact the rate of U^{6+} reduction as well as subsequent precipitation of reduced uranium, when inoculated with either of the two bacteria studied. Selected organic compounds may need to be removed from process waste streams prior to reductive precipitation of uranium. Phillips et al.(1992) reported that oxidation of organic matter using hydrogen peroxide can facilitate subsequent uranium precipitation. Use of biological treatment processes such as activated sludge process may also aid in degradation of organic matter from the waste streams. Warren (1974) and Francis (1992) reported that biodegradation of organic ligands from metal complexes may be negatively impacted due to restrictions in the transport of complexed substrate across cell membrane. Biological degradation of organic compounds from UO_2^{2+} containing process waste streams may encounter similar limitations. Such transport limitations involved in biological treatment may be eliminated by the addition of carbonate salts to the waste streams. Carbonate which strongly complexes with uranyl ion, can liberate organic compounds from U^{6+} complexes resulting in their degradation. Once organic compounds are degraded from waste stream, uranyl ion can be precipitated using *desulfuricans* as well as BrY.

Finally, waste streams resulting from environmental restoration of uranium affected soils using organic chelators generally contain very high concentrations of organics. Results from this research indicated that certain organic chelators (citrate,

tiron) have a strong tendency to associate with reduced uranium (U^{4+}) and prevent its precipitation. Degradation of these chelators may be required to facilitate hexavalent uranium removal. Dodge and Francis (1994) demonstrated that citrate from a UO_2^{2+} complex can be degraded by photooxidation. In addition, EDTA when complexed to Fe^{3+} was reported to degrade via photooxidation (Means, 1980). However, degradation of other chelators associated with uranium, using photooxidation, has not been currently demonstrated. Organics from these waste streams may be degraded using pure culture bacteria capable of utilizing the target ligand as a carbon/energy source. Magne et al., (1974) reported that some heterotrophic microorganisms degraded organic compounds from uranium containing waters. Degradation of organics resulted in yellow and black uranium deposits in their research. The nature of organic compounds present and the mechanisms of uranium precipitation were not elucidated in their research. However, results strongly suggested that microorganisms can degrade organic compounds and facilitate uranium precipitation from solution. Bacteria such as *B. subtilis* and *P. fluorescens* can metabolize citrate from waste streams (Willecke, 1973; Francis, 1992). The ability of a catechol degrading bacteria such as *Azotobacter sp.* and *P. putida* (Rochkind, 1986) to degrade structurally similar tiron needs to be evaluated. Several soil and sewage microorganisms have been reported to degrade EDTA from wastewaters (Tiedje, 1975). Possible limitations in transport of chelators across cell membranes can be eliminated either by using cell free extracts of these bacteria or by altering uranyl ion

speciation by the addition of carbonate salts. Once the complexants are degraded, *D. desulfuricans* as well as BrY can be used to remove uranium from these waste streams.

11. CONCLUSIONS AND RECOMMENDATIONS

11.1 CONCLUSIONS

1. Presence of organic ligands impacted the bioreduction of hexavalent uranium by *D. desulfuricans*.
2. *D. desulfuricans* rapidly decreased U^{6+} in solutions predicted to contain monovalent U^{6+} species.
3. In solutions predicted to contain multidentate aliphatic ligands the initial rate of U^{6+} reduction by *D. desulfuricans* decreased by more than an order of magnitude.
4. Multidentate aliphatic ligands inhibited U^{6+} reduction by *desulfuricans* either by non-competitively binding to a site in uranium reducing enzyme or by restricting the direct availability of chelated uranium to the bacteria.
5. *Shewanella alga* (BrY) reduced hexavalent uranium rapidly from multidentate aliphatic complexes.
6. Hydrolysis of hexavalent uranium and uranium complexed to monodentate acetate complex decreased the initial rate of U^{6+} reduction by BrY by more than an order of magnitude.
7. Presence of organic ligands negatively impacted the precipitation of bio-reduced uranium.

8. Metabolic differences in U^{6+} reduction by the two bacteria did not alter the fate of bio-reduced uranium in organic solutions. Essentially same amount of U^{4+} recomplexed with organic ligands or precipitated from solution in the presence of the two bacteria used.

11.2 RECOMMENDATIONS

This section briefly describes a few uncertainties prevailed in the selection of experimental conditions and interpretation of results obtained in this research. Elimination of these uncertainties through further research may aid in a better evaluation of the impact of organic ligands on U^{6+} bio-reductive precipitation.

1) Under some environmental conditions hexavalent uranium tends to hydrolyze prior to complexing with organic ligands. The stability constants for the association of hydrolyzed uranium with several organic ligands are not currently available. In this research, an empirical relationship (Equation 10, Section 4.4) was developed to estimate the stability constants of such complexes formed and data were interpreted assuming that these values prevailed. Additional research needs to be performed in to validate the accuracy of this empirical estimations.

2) Experimental results indicated that precipitation of bio-reduced uranium was dictated by the affinity of U^{4+} to complex with the ligands present. However, stability constants for several U^{4+} -organic complexes are not currently available. These values need to be experimentally obtained.

3) Multidentate aliphatic ligands inhibited the rate of U^{6+} reduction in the presence of *desulfuricans* by non-competitive enzyme inhibition or by restricting the direct availability of chelated uranium. Uranyl hydroxide species and weaker acetate complexation appeared to affect U^{6+} reduction by *Shewanella alga* by non-competitive inhibition. However, the exact mechanisms by which these bacteria were inhibited are not currently known. These mechanisms can be elucidated by characterization of U^{6+} reducing enzymes and evaluation of structural compatibility of various U^{6+} species to the enzyme active sites. Elucidation of the relationship between the bacterial metabolism and the nature of inhibition will help in the selection of bacteria and optimum conditions for dissolved uranium removal.

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APPENDICES

APPENDIX I

CONCENTRATION OF NUTRIENTS AND MINERALS USED IN BACTERIAL GROWTH MEDIUM

COMPOUND	CONCENTRATION (mg/L)
Biotin	2×10^{-3}
Folic acid	2×10^{-3}
Pyridoxine HCl	10×10^{-3}
Riboflavin	5×10^{-3}
Thiamin	5×10^{-3}
Nicotinic acid	5×10^{-3}
Pantothenic acid	5×10^{-3}
B 12	0.1×10^{-3}
p- Aminobenzoic acid	5×10^{-3}
Thioctic acid	5×10^{-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	0.13
CuSO ₄	0.01
AlK(SO ₄) ₂	0.01
H ₃ BO ₂	0.01
Na ₂ MoO ₄	0.025
NiCl ₂ 6H ₂ O	0.024
Na ₂ WO ₄	0.025

APPENDIX II. Relationship between formation constants and ionic properties of metal-organic complexes

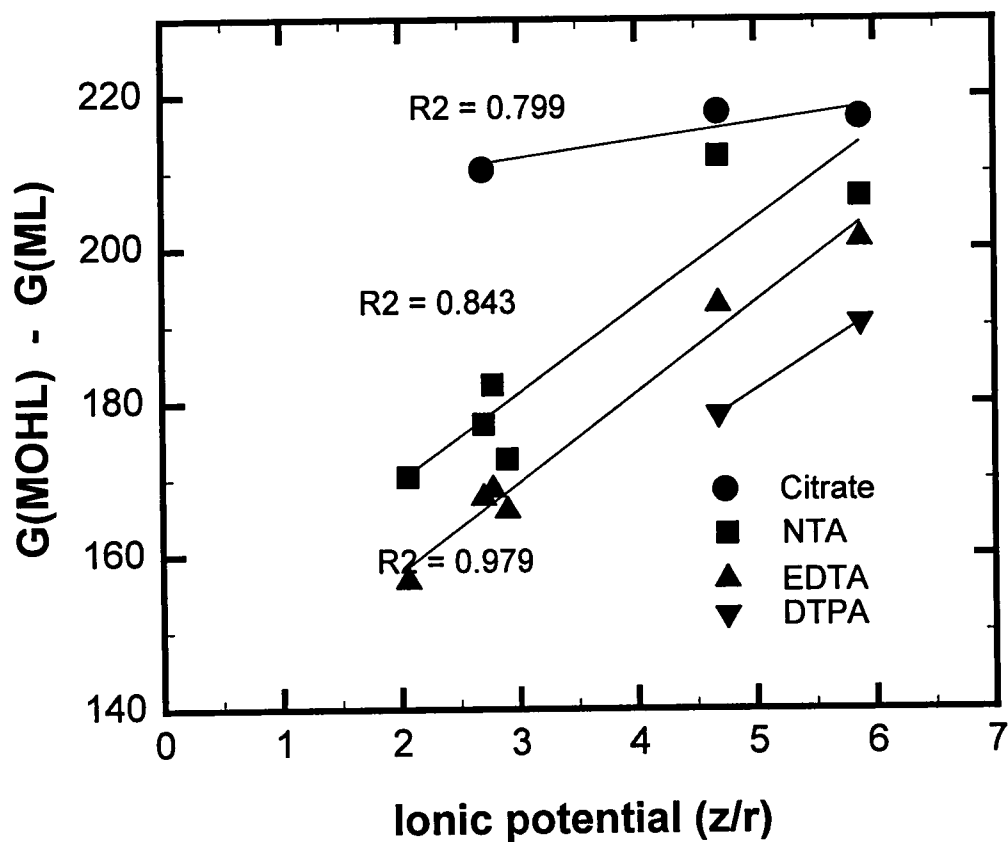


Figure II.1. Relationship between Ionic potential and formation constants

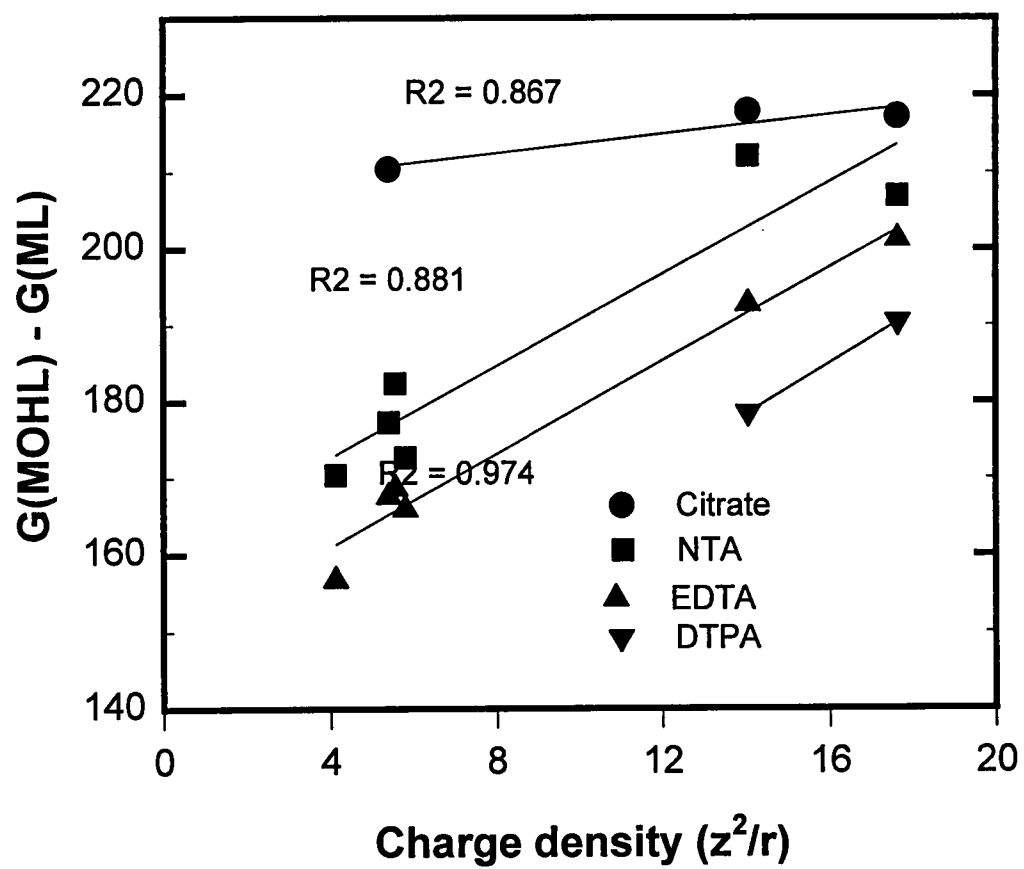
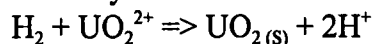


Figure II.2. Relationship between charge density and formation constants

APPENDIX III

Determination of H₂ gas concentration in test vials during U⁶⁺ reduction

i) Stoichiometry of U⁶⁺ reduction



ii) Moles of U⁶⁺ used in test solution $\simeq 100/238 \simeq 0.42 \times 10^{-3} \text{ M}$

Therefore, moles of H₂ required $\simeq \underline{0.42 \times 10^{-3} \text{ M}}$

iii) Gas mixture introduced into test solution

$\simeq 20 \text{ ml}$ of 80% H₂:20% CO₂ (by weight) in 15 ml head space.

iv) Mole fraction of H₂ in head space

$$= [0.8/\text{MW of H}_2] / \{[0.8/\text{MW of H}_2] + [0.2/\text{MW of CO}_2]\}$$

$$= [0.8/2] / \{[0.8/2] + [0.2/44]\}$$

$$= 0.9887$$

v) Mole fraction of CO₂ in head space $= 1 - 0.9887 = 0.0113$

vi) Total pressure of gas in head space $= [20\text{ml gas mixture} / 15 \text{ ml head space volume}] \times$

$$1 = 1.33 \text{ atm}$$

vii) Partial pressure of H₂ in head space $= 1.33 \times 0.9887 = \underline{1.31 \text{ atm}}$

$$= 1.31 \times 1.01325 \times 10^2 \text{ kPa} = \underline{132.7 \text{ kPa}}$$

viii) Mole fraction 'x' of H₂ gas in test solution

$$= \text{partial pressure of H}_2 \text{ in head space} / \text{Henry's constant at } 1.31 \text{ atm (Perry, 1984)}$$

$$= 1.31 / 7.75 \times 10^4$$

$$= 1.75 \times 10^{-5}$$

ix) Concentration of H₂ gas in test solution

$$= 1.75 \times 10^{-5} \times \text{molar concentration of H}_2\text{O}$$

$$= 1.75 \times 10^{-5} \times 55.45 \text{ mol/L} = \underline{0.9 \times 10^{-3} \text{ M}}$$

Therefore the conc. of H₂ gas dissolved in test solution was $\sim$ two fold higher than that required for U⁶⁺ reduction. In addition, the total H₂ gas provided in head space was orders of magnitude higher than that required for the reduction of 100 mg/L U⁶⁺.

APENDIX IV. Uranium distribution in test solutions containing various organic ligands after inoculation with *D. desulfuricans*.

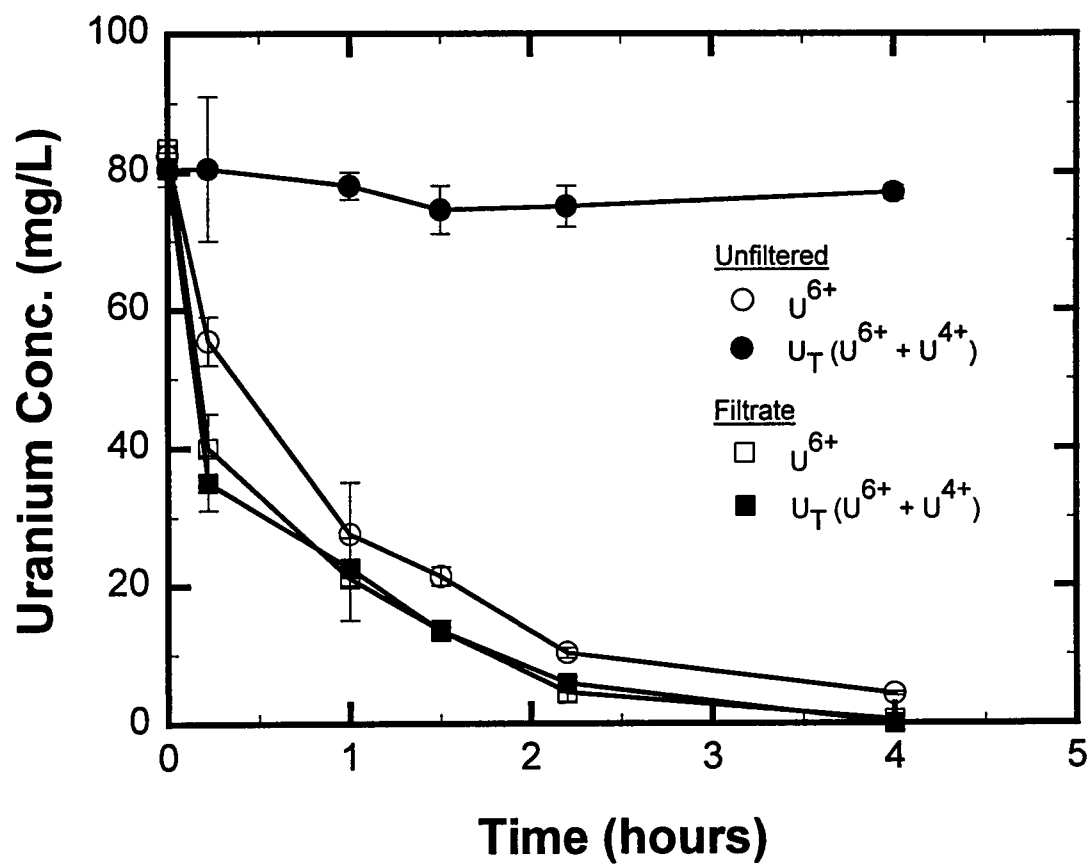


Figure IV.1. Uranium levels in acetate solutions after inoculation with *D. desulfuricans*. Acetate initially complexed ~98% U^{6+} .

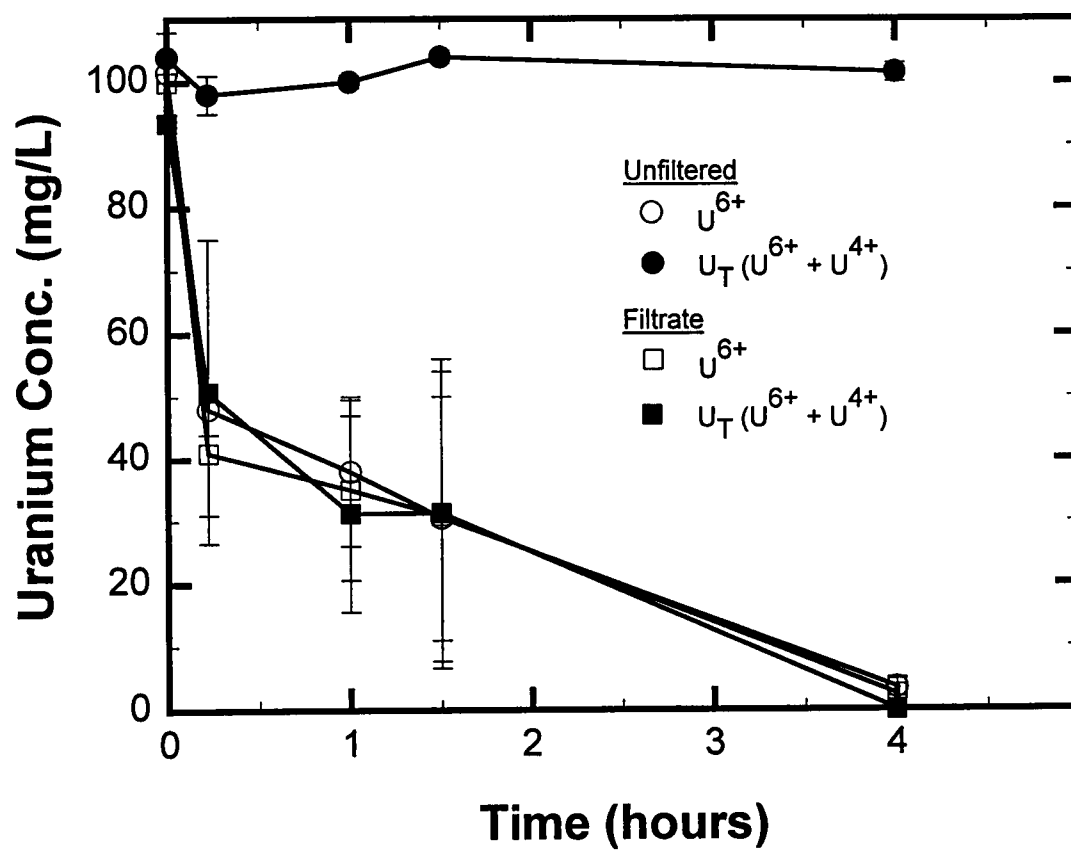


Figure IV.2. Uranium levels in acetate solutions after inoculation with *D. desulfuricans*. Acetate initially complexed ~50% U^{6+} .

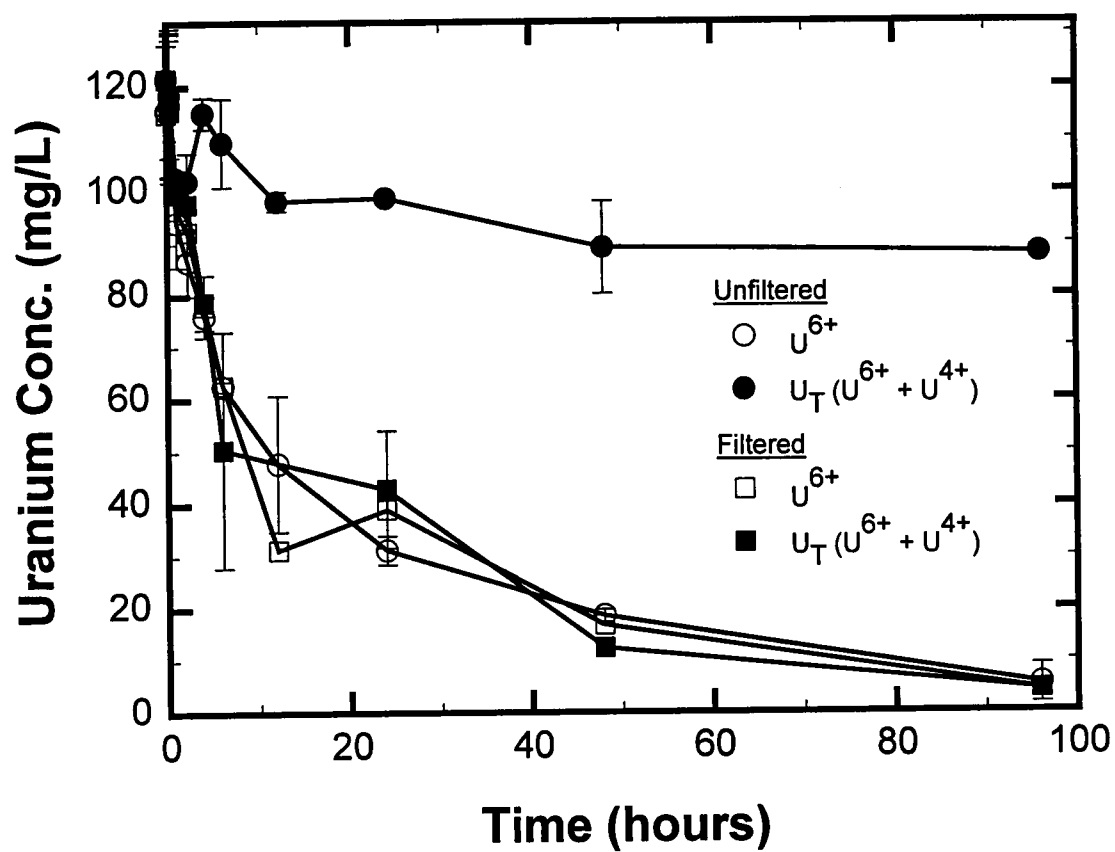


Figure IV.3. Uranium distribution in malonate solutions after inoculation with *D. desulfuricans*. Malonate initially complexed ~98% U^{6+} .

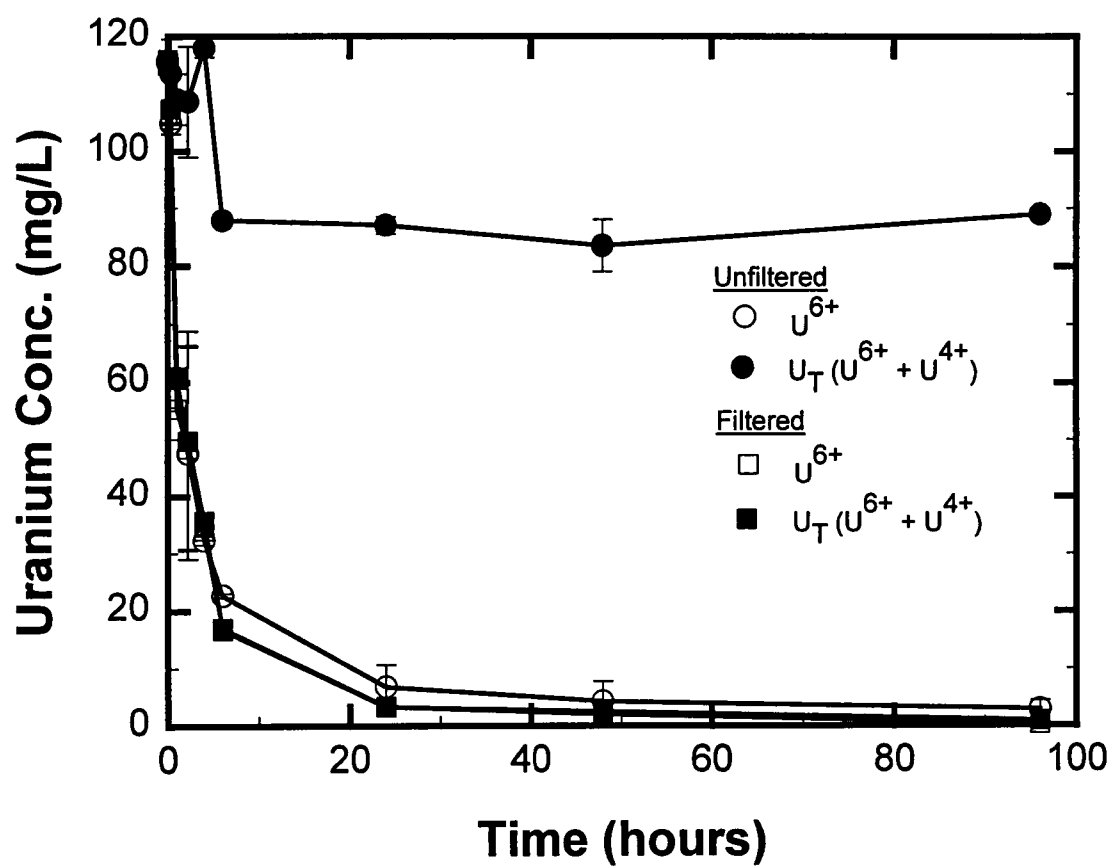


Figure IV.4. Uranium distribution in malonate solutions after inoculation with *D. desulfuricans*. Malonate initially complexed $\sim 50\%$ U^{6+} .

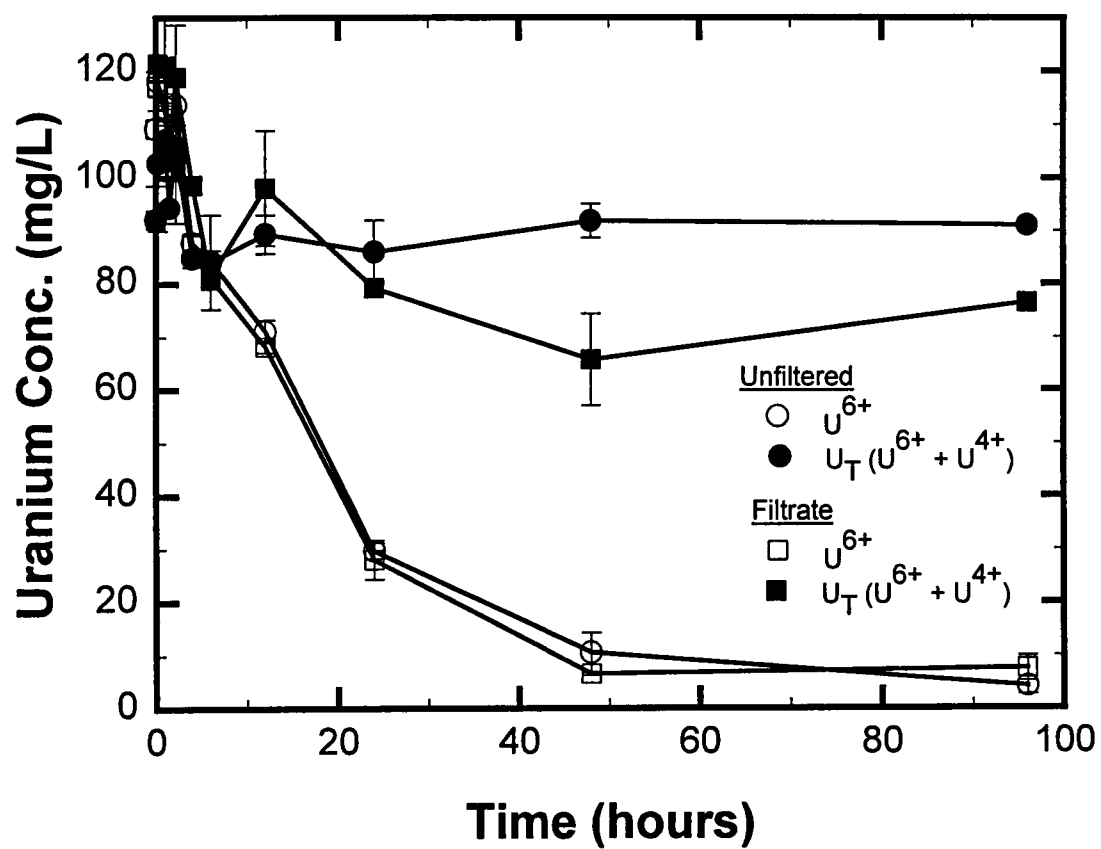


Figure IV.5. Uranium distribution in oxalate solution after inoculation with *D. desulfuricans*. Oxalate initially complexed ~98% U^{6+} .

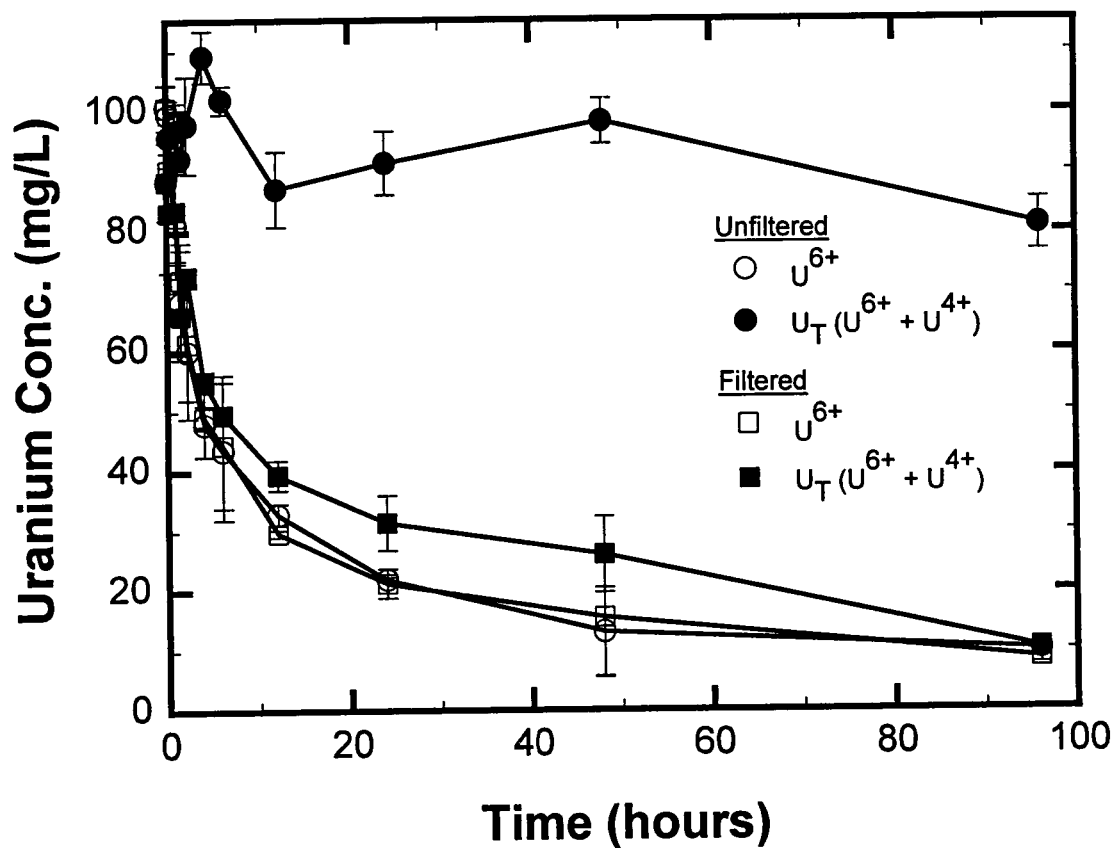


Figure IV.6. Uranium distribution in oxalate solution after inoculation with *D. desulfuricans*. Oxalate initially complexed ~50% U^{6+} .

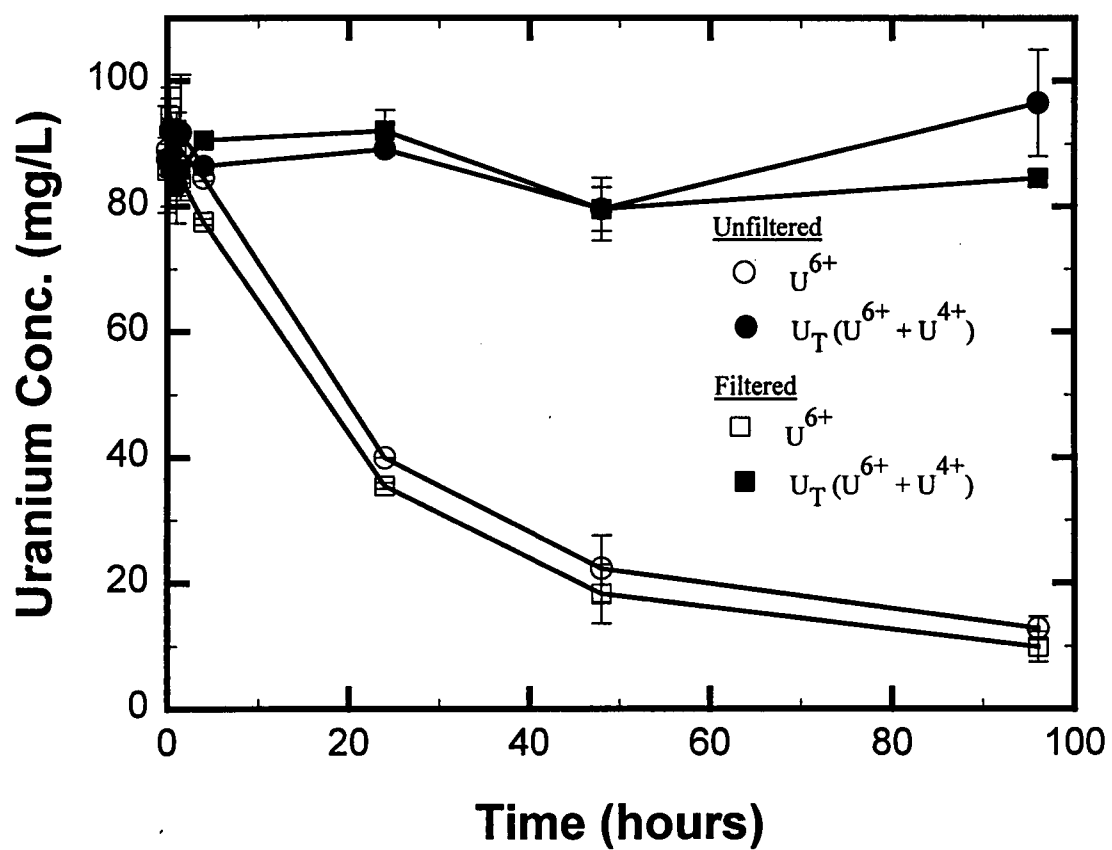


Figure IV.7. Uranium distribution in citrate solution after inoculation with *D. desulfuricans*. Citrate initially complexed ~100% U^{6+} .

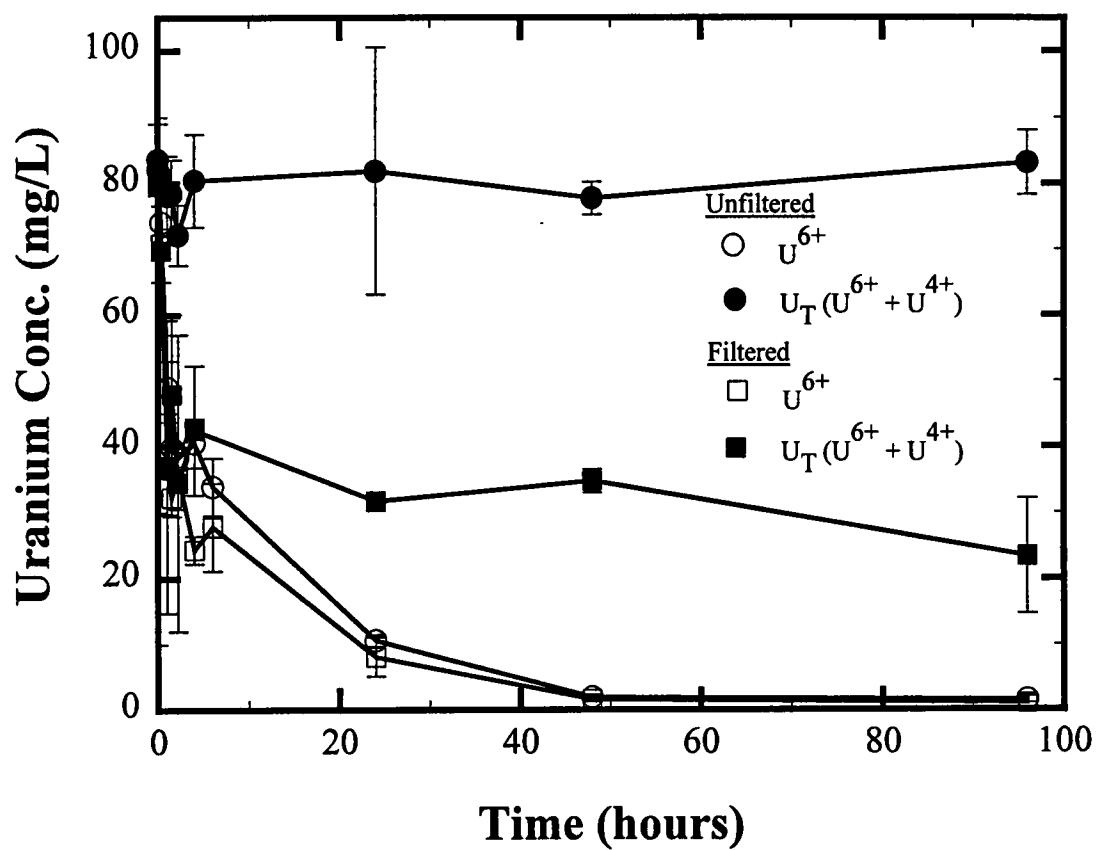


Figure IV.8. Uranium distribution in citrate solution after inoculation with *D. desulfuricans*. Citrate initially complexed ~50% U^{6+} .

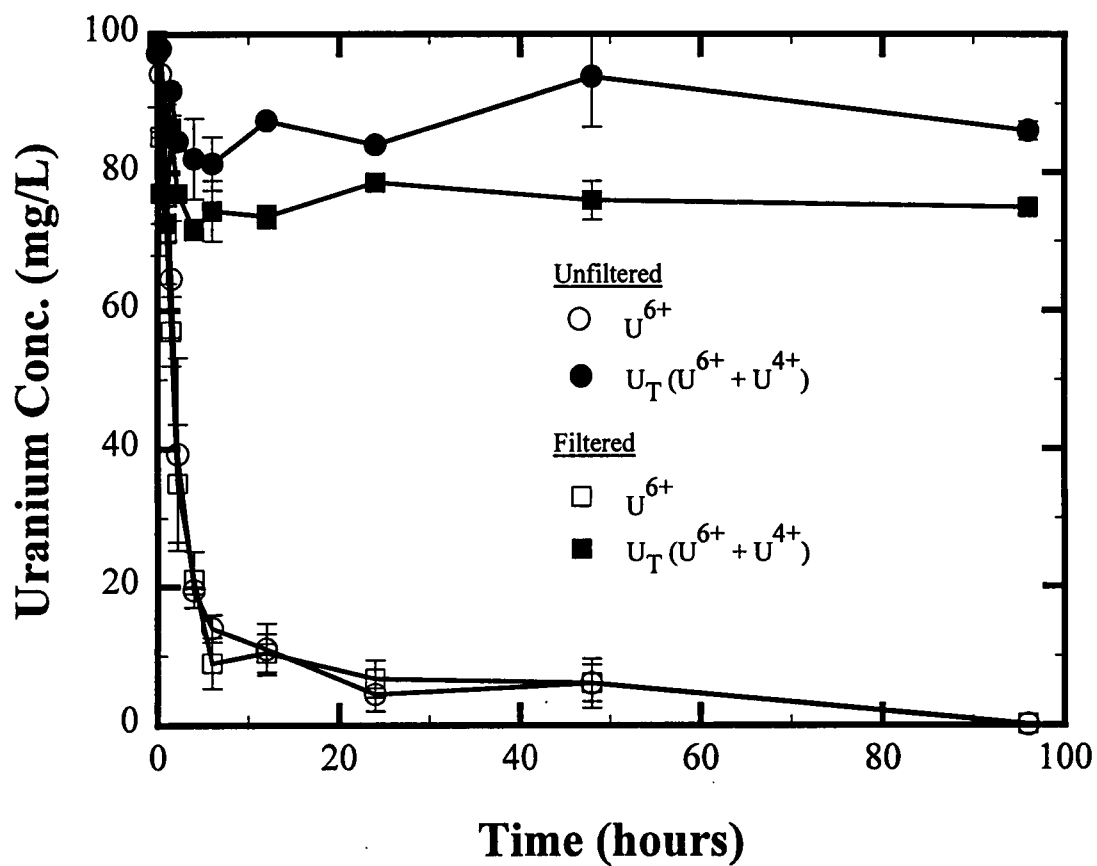


Figure IV.9. Uranium distribution in tiron solution after inoculation with *D. desulfuricans*. Tiron initially complexed ~100% U^{6+} .

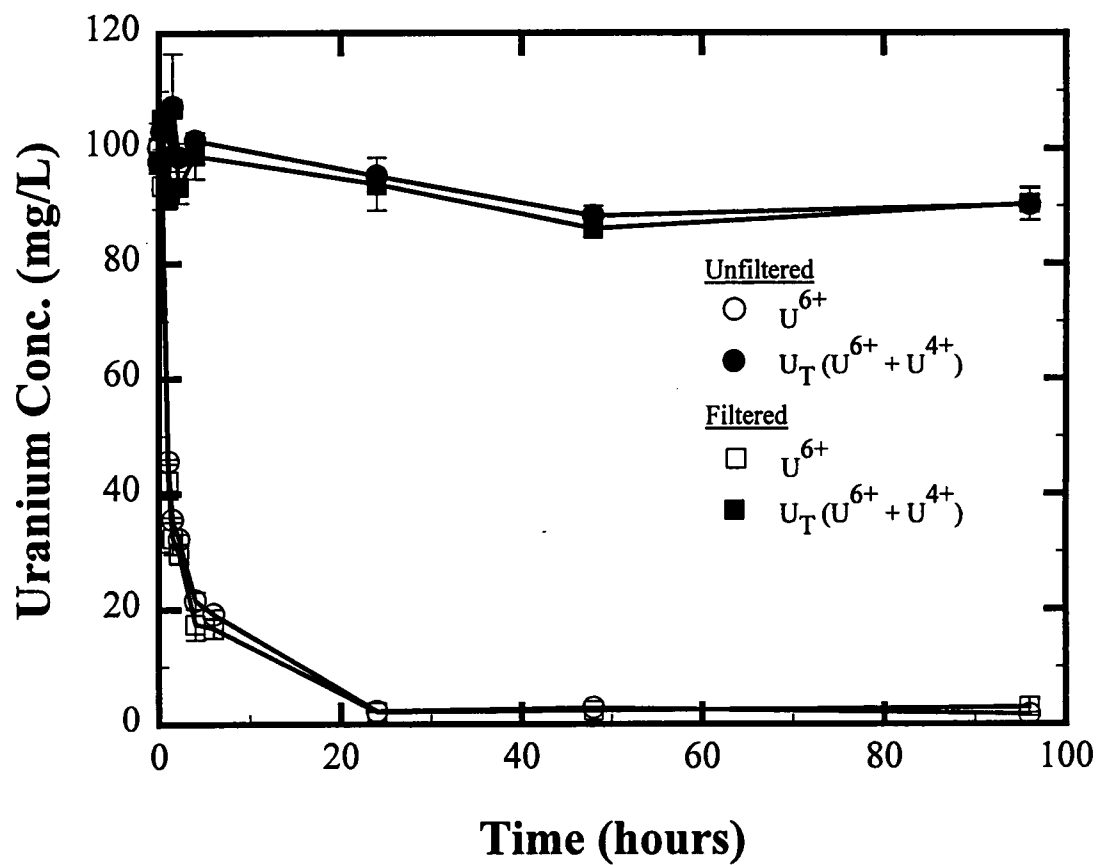


Figure IV.10. Uranium distribution in tiron solution after inoculation with *D. desulfuricans*. Tiron initially complexed ~50% U^{6+} .

APPENDIX V. Amount of uranium precipitated in acetate solutions measured directly and calculated by difference.

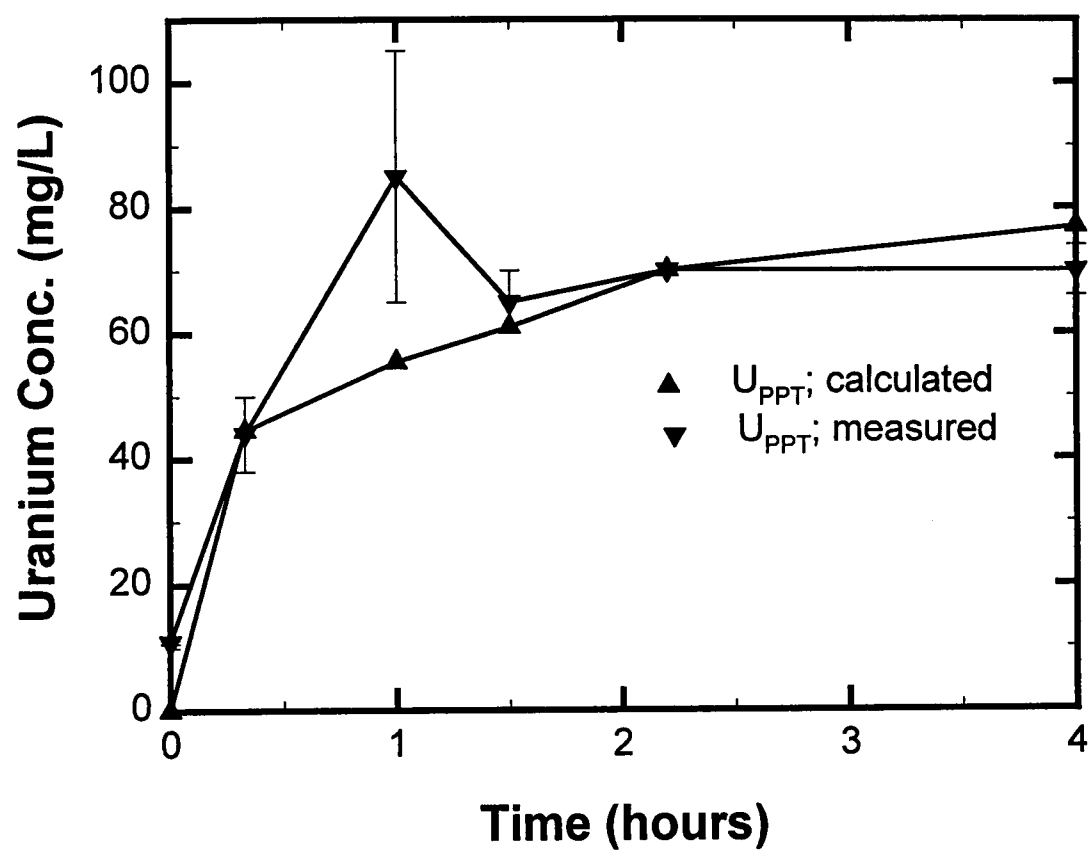


Figure V. 1. Amount of uranium precipitated in acetate solutions.

APPENDIX VI. Uranium distribution in test solutions containing various organic ligands after inoculation with BrY

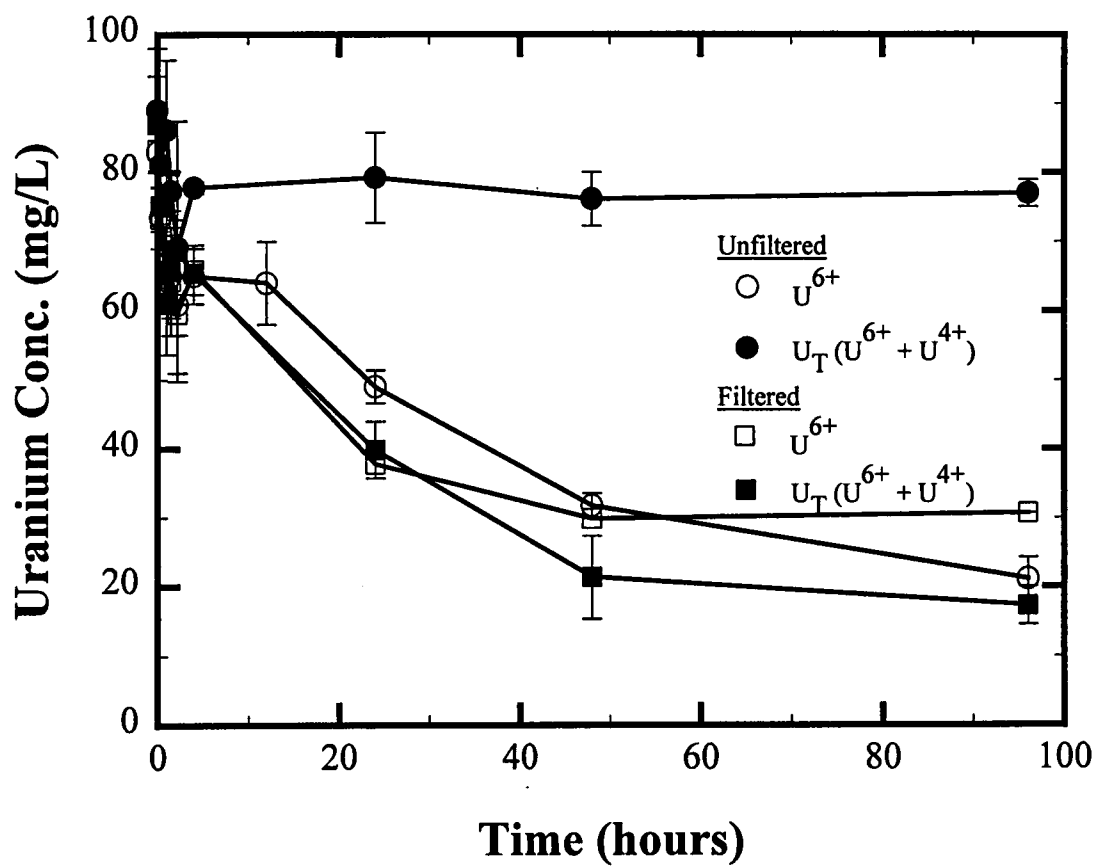


Figure VI.1. Uranium distribution in acetate solution after inoculation with BrY. Acetate initially complexed $\sim 98\%$ U^{6+} .

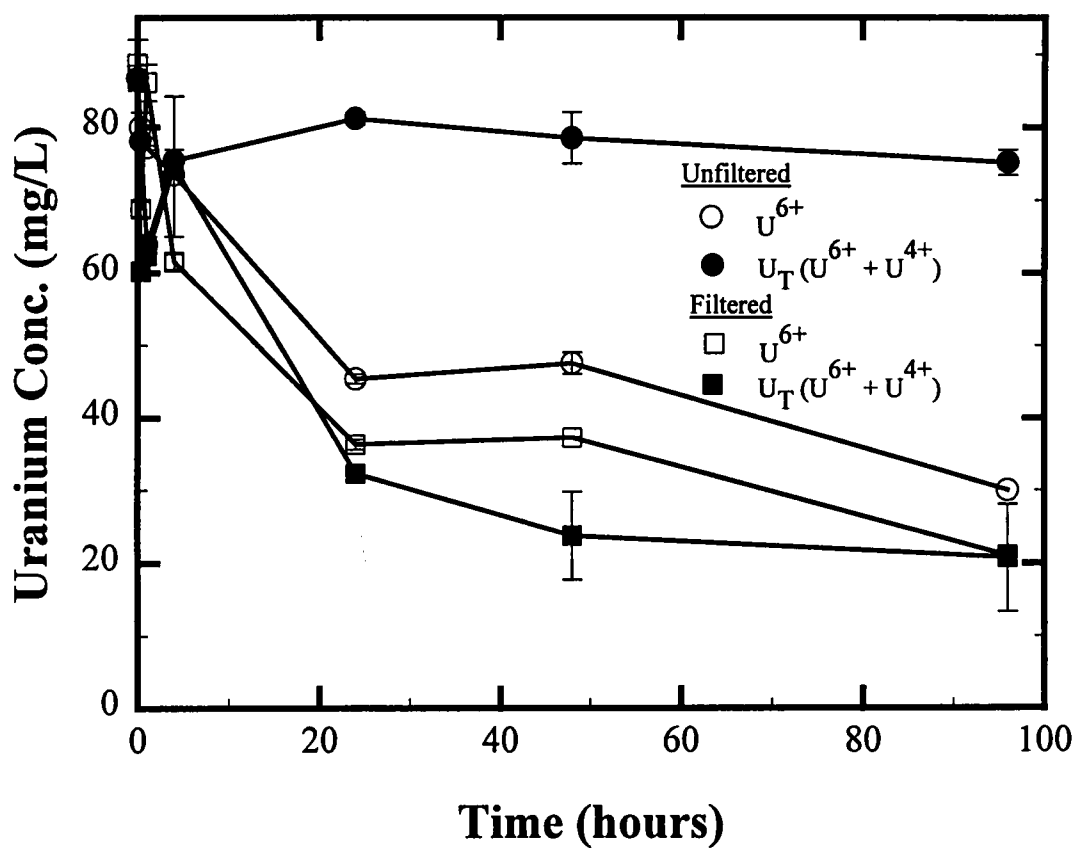


Figure VI.2. Uranium distribution in acetate solution after inoculation with BrY. Acetate initially complexed $\sim 50\% U^{6+}$.

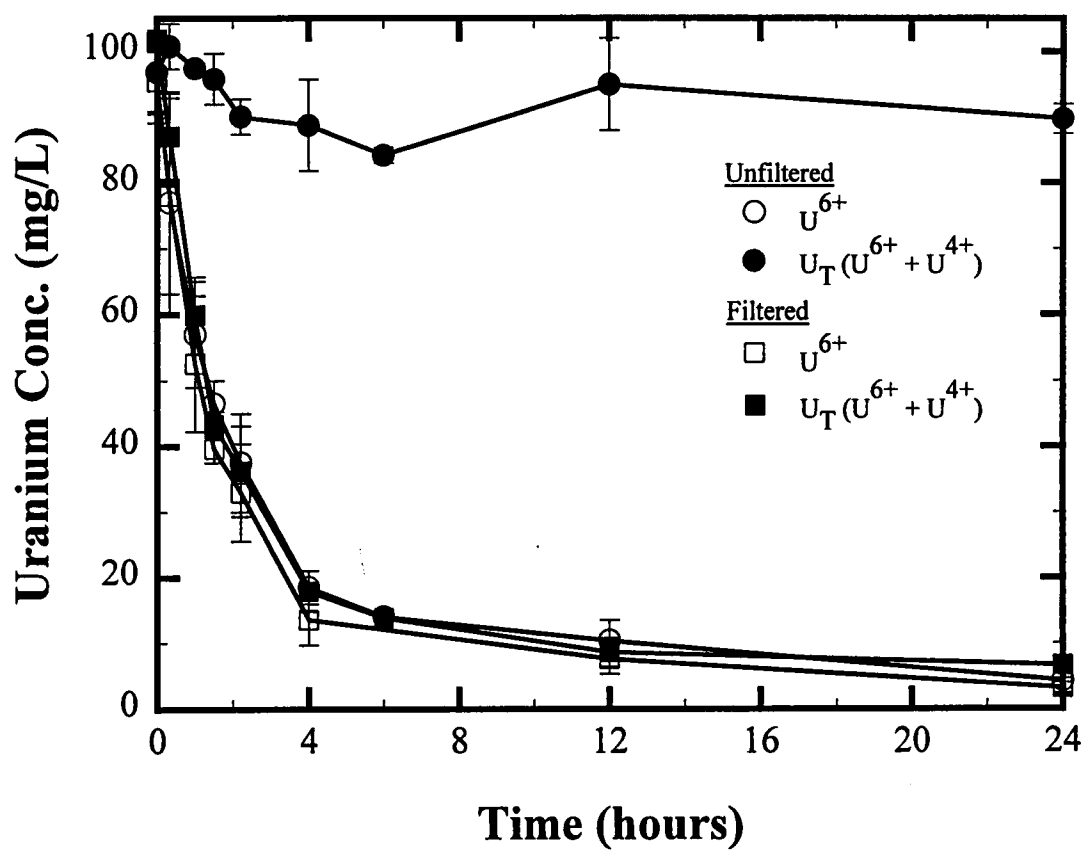


Figure VI.3. Uranium distribution in malonate solution after inoculation with BrY. Malonate initially complexed $\sim 100\%$ U^{6+} .

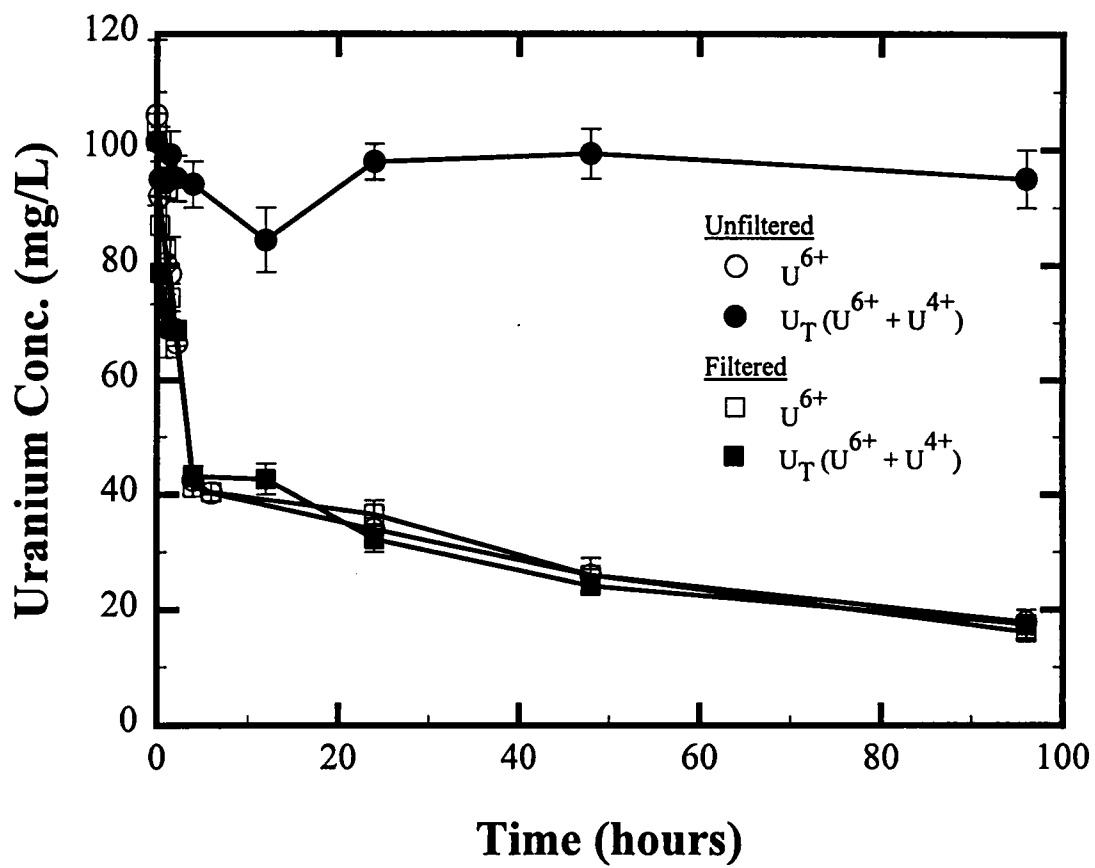


Figure VI.4. Uranium distribution in malonate solution after inoculation with BrY. Malonate initially complexed $\sim 50\% U^{6+}$.

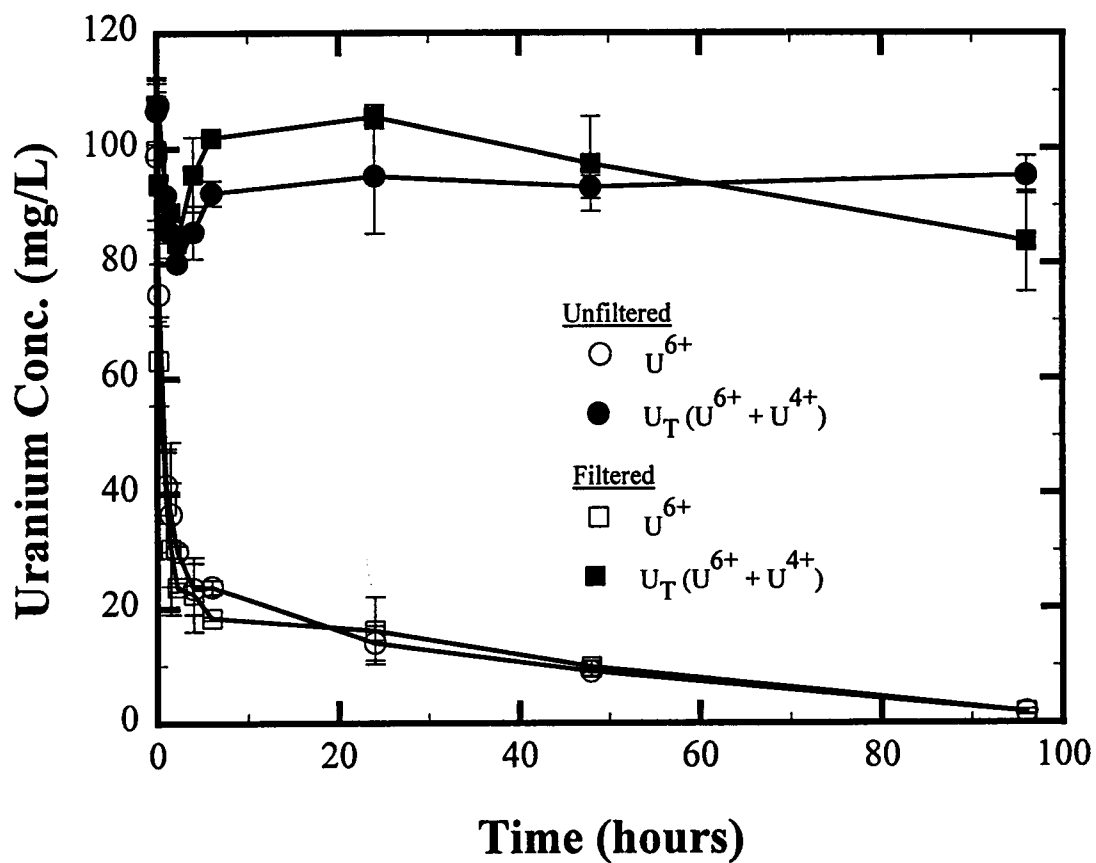


Figure VI.5. Uranium distribution in oxalate solution after inoculation with BrY. Oxalate initially complexed $\sim 100\%$ U^{6+} .

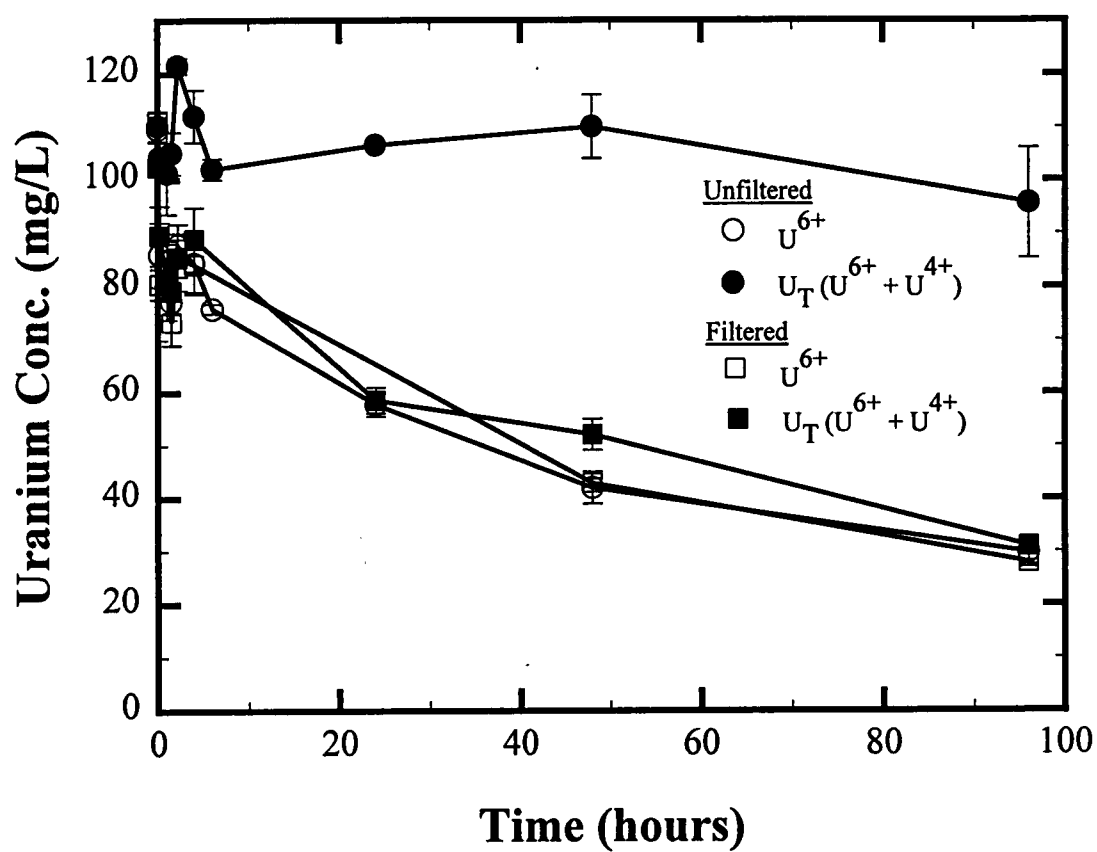


Figure VI.6. Uranium distribution in oxalate solution after inoculation with BrY. Oxalate initially complexed $\sim 50\% U^{6+}$.

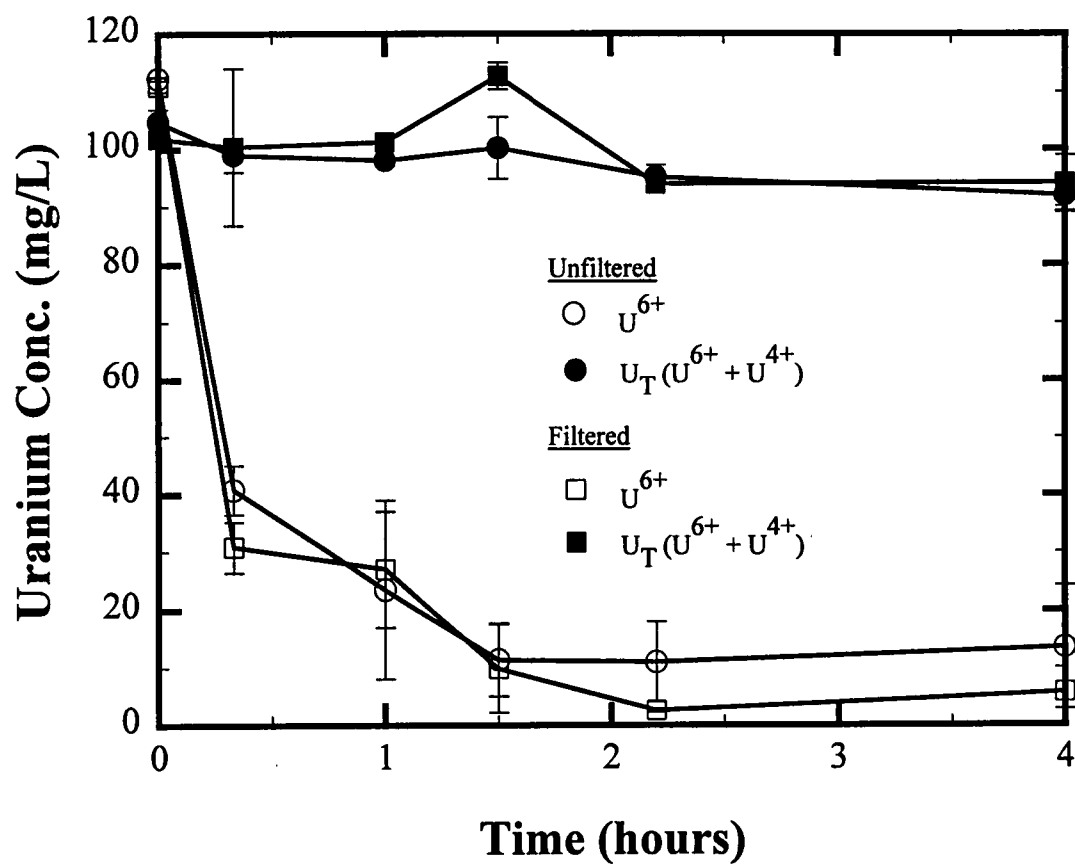


Figure VI.7. Uranium distribution in citrate solution after inoculation with BrY. Citrate initially complexed $\sim 99\% U^{6+}$.

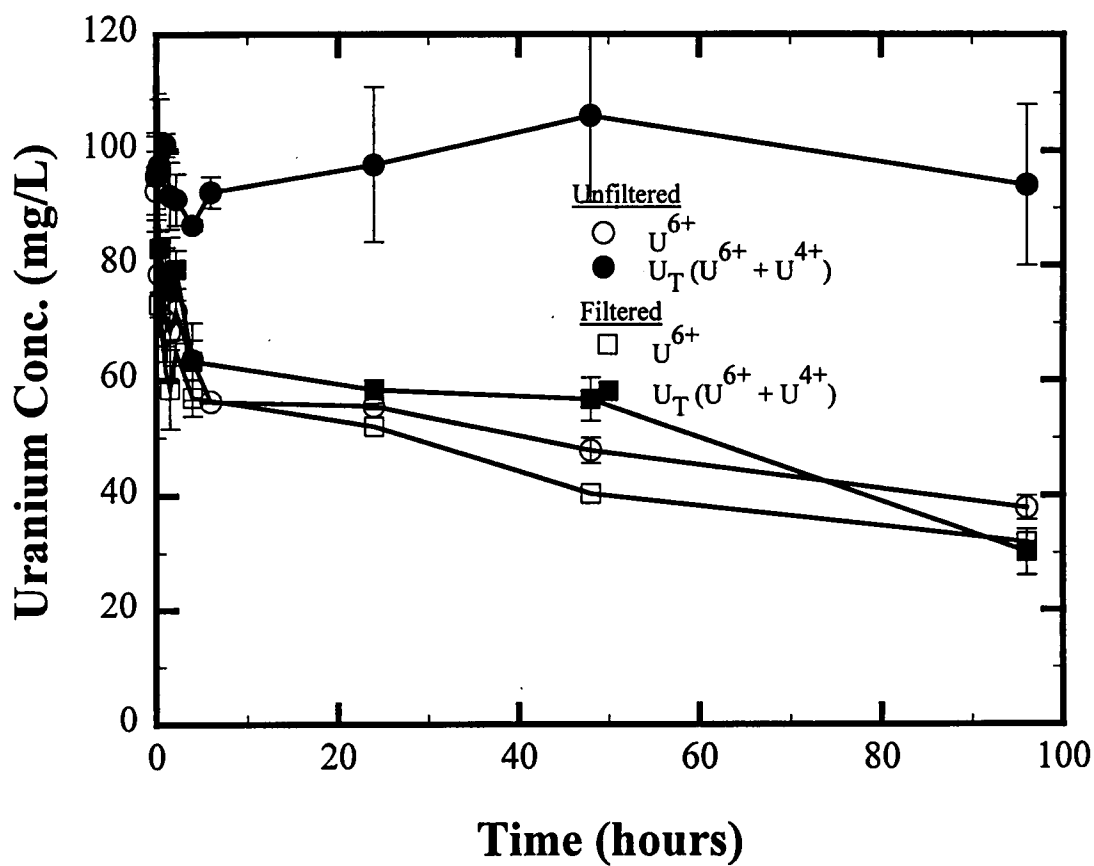


Figure VI.8. Uranium distribution in citrate solution after inoculation with BrY. Citrate initially complexed $\sim 50\% U^{6+}$.

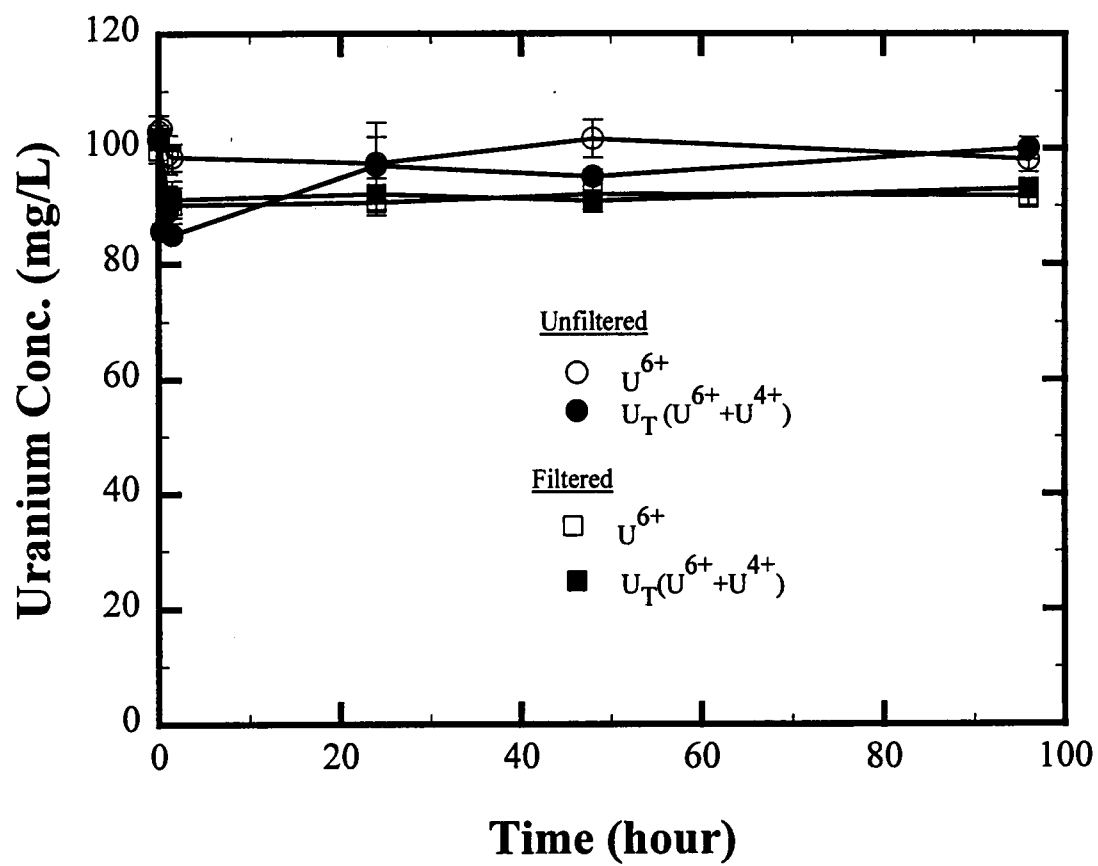


Figure VI.9. Uranium distribution in tiron solution after inoculation with BrY. Tiron initially complexed $\sim 99\%$ U^{6+} .

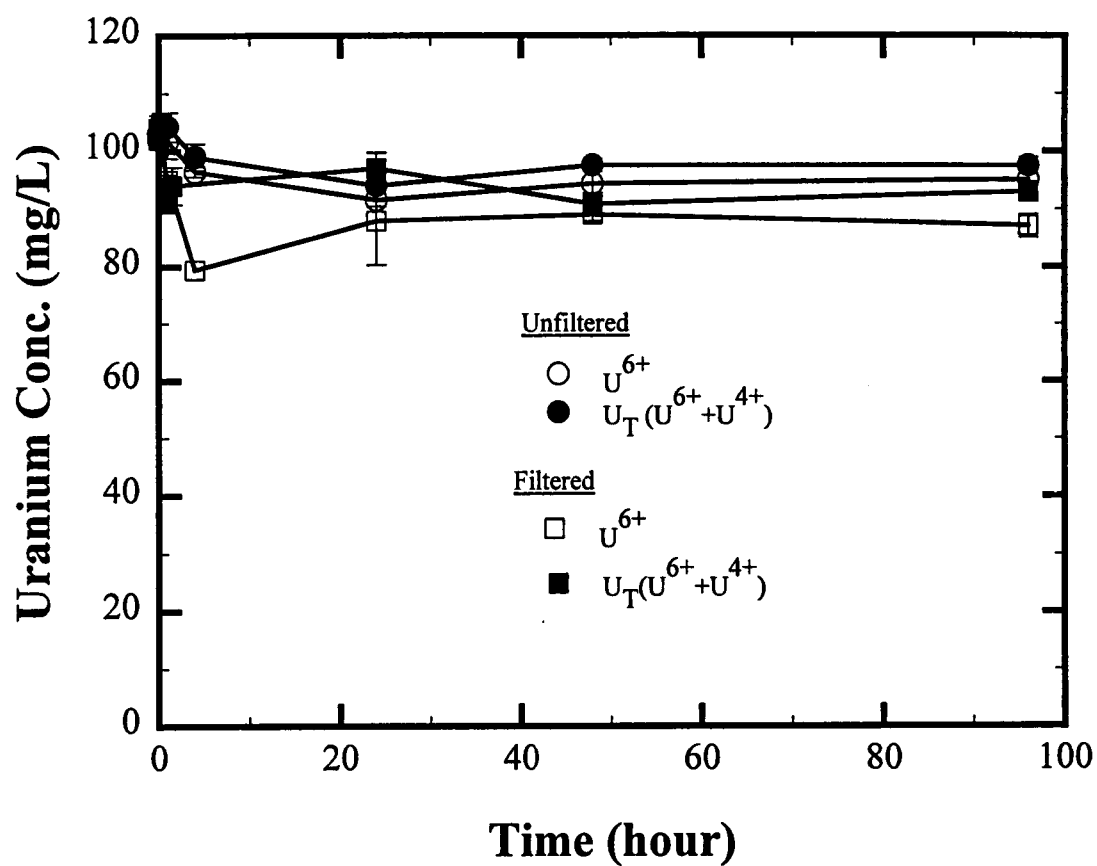


Figure VI.10. Uranium distribution in tiron solution after inoculation with BrY. Tiron initially complexed $\sim 50\% U^{6+}$.

APPENDIX VII. Cox and Stuart Test for Analysis of Trends

Cox and Stuart test (Conover, 1980) is a non-parametric, binomial test which is used to test if a trend exists between two events. In this research, this test was used to compare the trends in U^{6+} reduction between various experiments performed. Procedure used in one case (U^{6+} reduction by *D. desulfuricans* in acetate and malonate solutions) is explained below in detail. Same procedure was used in all the comparisons, the results of which are also included in this section.

1. U^{6+} reduction trends in high (100 % U^{6+} complexed) malonate and acetate solutions by *D. desulfuricans*:

Hypothesis tested : H_0 : No difference in U^{6+} reduction between malonate and acetate solutions.

H_1 : Reduction in malonate solution is slower than that in acetate solution.

The following Table shows the % U^{6+} remaining in solutions over time.

		% Of initial U^{6+} in solution					
		Malonate		Acetate			
No	Time (hr)	Sample #1 (a)	Sample #2 (b)	Sample #1 (c)	Sample #2 (d)	e = (a) - (c)	f = (b) - (d)
1	0	100	100	100	100	0	0
2	0.3	115	89.8	84.4	93.0	30.6	-3.2
3	1.0	87.7	74.9	63.3	59.0	24.4	15.9
4	1.3	80.0	70.0	3.1	24.3	76.9	45.7
5	4.0	61.7	70.0	0.5	0.2	61.2	69.8
6	24.0	46.0	25.0	0.1	0.1	45.9	24.9

Column 'e' depicts the difference in the % U^{6+} between sample #1 of malonate and acetate solutions and column 'f' shows the difference in sample # 2 between the two solutions.

A positive value in column e & f would indicate the presence of a higher of U^{6+} in malonate solution than in acetate solution, thereby indicating a slower reduction in the presence of malonate. A consistent increase in the difference over time would indicate a trend, that, U^{6+} reduction in malonate is consistently slower than that in acetate solution.

Let N be the number of values in each column.

Now, pair the entries in columns e & f as (f_i, f_{c+i}) where

$c = N/s$, if N is even and

$c = (N-1)/2$, if N is odd.

The pairs obtained are (0, 76.9)

(30.6, 61.2)

(24.4, 45.9)

Similarly, pair the values in column f as

(0, 45.7)

(-3.2, 69.8)

(15.9, 24.9)

Let, n = total number of united pairs = 6

If reduction in malonate solution is consistently slower than that in acetate solution, e_{c+i} will be consistently greater than e_i and f_{c+i} will be consistently greater than f_i .

Count the total number of pairs where $e_{c+i} > e_i$ and $f_{c+i} > f_i$.

In this case e_{c+i} is greater than e_i and f_{c+i} is greater than f_i in all the cases.

Hence $T = 3 + 3 = 6$

From the binomial distribution table (Appendix 3, Conover, 1980) calculate the value y corresponding to total number of pairs (n), and α close to 0.05. The value thus obtained is termed as 't'.

In the current problem, for $n = 6$, at $p=0.5$ (binomial distribution), α closer to 0.05 is 0.1094. The corresponding t value is 1.

The null hypothesis that no difference in trend exists is valid if

$$T < n - t.$$

In this case, $T = 6$

$$n - t = 6 - 1 = 5.$$

Note that T is not less than $n - t$. Therefore the null hypothesis is rejected. Results indicate that the rate of U^{6+} reduction in malonate solution is statistically slower than that in acetate solution.

Results from other experiments using *desulfuricans*

i) Oxalate vs Acetate (100% complexed)

$n = 6, t = 1$ and $T = 6$. Hence, reduction is significantly slower in oxalate solution.

ii) Citrate vs Acetate (100% complexed)

$n = 6, t = 1$ and $T = 6$. Hence, reduction is significantly slower in citrate solution.

iii) Tiron vs Acetate (100% complexed)

$n = 4, t = 0$ and $T = 4$. Hence, reduction is significantly slower in Tiron solution.

iv) Malonate vs Acetate (50% complexed)

$n = 6, t = 1$ and $T = 3$. Hence, no difference in the reduction rates.

v) Oxalate vs Acetate (100% complexed)

$n = 6, t = 1$ and $T = 5$. Hence, reduction is significantly slower in oxalate solution.

vi) Citrate vs Acetate (100% complexed)

$n = 6, t = 1$ and $T = 5$. Hence, reduction is significantly slower in citrate solution.

vii) Tiron vs Acetate (50% complexed)

$n = 6, t = 1$ and $T = 3$. Hence, no difference in the reduction rates.

viii) Acetate 100% U^{6+} complexed vs Acetate 50% complexed

$n = 6, t = 1$ and $T = 2$. Hence, no difference in reduction rates.

ix) Malonate 100% U^{6+} complexed vs Malonate 50% complexed

$n = 6, t = 1$ and $T = 5$. Hence, reduction rates are significantly different.

x) Oxalate 100% U^{6+} complexed vs Oxalate 50% complexed

$n = 8, t = 1$ and $T = 8$. Hence, reduction is significantly slower in high oxalate solution.

xi) Citrate 100% U^{6+} complexed vs Citrate 50% complexed

$n = 6, t = 1$ and $T = 5$. Hence, reduction is significantly slower in high citrate solution.

xii) Tiron 100% U^{6+} complexed vs Tiron 50% complexed

$n = 8, t = 1$ and $T = 4$. Hence, no difference in the reduction rates.

U^{6+} reduction using BrY

i) Acetate vs Malonate (100% complexed)

$n = 6$, $t = 1$ and $T = 6$. Hence, reduction is significantly slower in acetate solution.

ii) Acetate vs Malonate (50% complexed)

$n = 6$, $t = 1$ and $T = 4$. Hence, no difference in the reduction rates.

iii) Acetate 50 % U^{6+} complexed vs Acetate 100% complexed

$n = 6$, $t = 1$ and $T = 4$. Hence, no difference in the reduction rates.

iv) Malonate 50 % U^{6+} complexed vs Malonate 100% complexed

$n = 8$, $t = 1$ and $T = 8$. Hence, reduction is significantly slower in low malonate solution

v) Oxalate 50 % U^{6+} complexed vs oxalate 100% complexed

$n = 8$, $t = 1$ and $T = 8$. Hence, reduction is significantly slower in low oxalate solution.

vi) Citrate 50 % U^{6+} complexed vs citrate 100% complexed

$n = 6$, $t = 1$ and $T = 5$. Hence, reduction is significantly slower in low citrate solution.

vii) Malonate vs oxalate (50% complexed)

$n = 8$, $t = 1$ and $T = 8$. Hence, reduction is significantly slower in low oxalate solution.

VITA

Rajagopalan Ganesh was born in India in 1964. He graduated with a Bachelor of Engineering degree in Civil Engineering from the University of Allahabad, India in 1986. He worked for four years as a design engineer with M/S Consulting Engineering Services (India) Limited, New Delhi, India. Ganesh pursued his M.S. degree in Environmental Engineering at Virginia Tech from Fall semester, 1990. After graduation from Virginia Tech, Ganesh entered the University of Tennessee, Knoxville in Fall semester, 1992 for his Ph.D. degree in Civil Engineering.