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GRADUATE COLLEGE

ANAEROBIC REDUCTION OF HEXAVALENT CHROMIUM BY SUBSURFACE MICROORGANISMS

DISSERTATION SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

TAMARA L. MARSH

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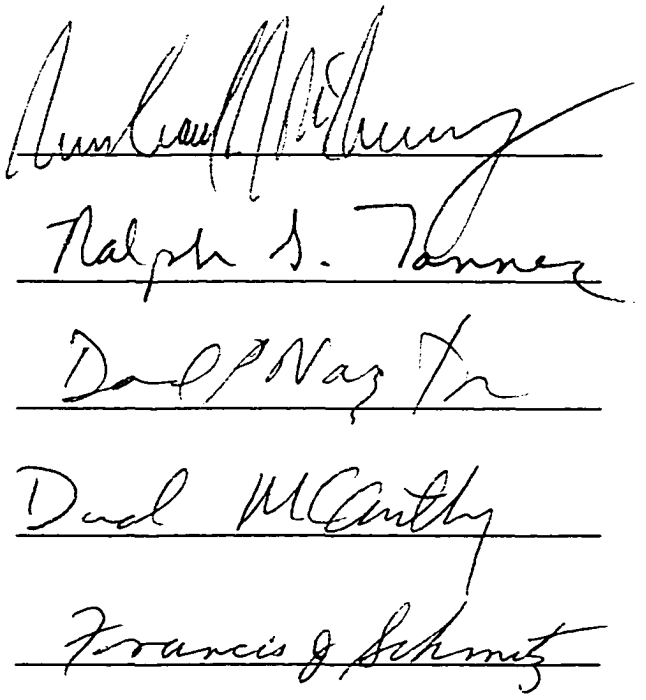
1999

ANAEROBIC REDUCTION OF HEXAVALENT CHROMIUM BY SUBSURFACE MICROORGANISMS

A DISSERTATION

APPROVED FOR THE DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY



DEDICATION

I dedicate this dissertation to my family, without whose loving support I could never have made it this far. To my parents, who gave me the world, and taught me to go after my dreams, no matter how crazy they may seem. To my mother, for encouraging her daughters to be everything and anything they wanted, and then telling us to do it better than anyone else. Only you know how many times I wanted to walk away from everything. I love you most for listening to me whine and then encouraging me to "stand up straight, kick yourself in the pants, and give the guy in front of you one, too." To my father, who despaired of me ever finishing school, fearing that I would forever remain a "college kid." Our political and enrivonmental debates brought me here and I love you for that. Unfortunately for you, I think I will always be a "damned environmentalist," but I know you love me in spite of that. Finally, to my sisters and brother, thanks for all the love and laughs we have had through the years. Without that I couldn't have made it.

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Abstract

Anaerobic sediments from an aquifer contaminated with landfill leachate were used to study the potential for microbial reduction of Cr(VI) to Cr(III). The lightly colored, sandy sediments slowly reduced Cr(VI) in viable, but not heat-killed, microcosms indicating a biological process. Microcosms containing sandy sediments and mineral medium were amended with various electron donors to determine those most important for biological Cr(VI) reduction. More than 500 μM Cr(VI) was reduced when formate, lactate, hydrogen, and glucose, but not benzoate or acetate, were added as electron donors. Chromate reduction in microcosms was not inhibited by the addition of sulfate, selenate, or Fe(III). However, the presence of nitrate partially inhibited Cr(VI) reduction. Nearly complete inhibition of Cr(VI) reduction was observed when microcosms were shaken in the presence of oxygen. The addition of molybdate to the microcosms did not affect Cr(VI) reduction in sandy sediments until very high concentrations (40 times the Cr(VI) concentration) were used. The addition of bromoethanesulfonic acid (BESA) in amounts less than, or slightly greater than, the Cr(VI) concentration partially inhibited Cr(VI) reduction in the microcosms. An anaerobic Cr(VI)-utilizing enrichment was obtained that was dependent upon hydrogen for both growth and Cr(VI) reduction. Growth and Cr(VI) reduction by the enrichment were completely inhibited by the addition of formaldehyde, partially inhibited by molybdate, and completely unaffected by

BESA. No methane was produced by the enrichment, which reduced about 750 μM Cr(VI) in less than six days. These studies showed that Cr(VI) reduction in sandy aquifer sediments is a biologically mediated, anaerobic process that is inhibited by oxygen and nitrate. In addition, electron donors which resulted in increased available hydrogen showed a greater extent of Cr(VI) reduction.

ANAEROBIC REDUCTION OF HEXAVALENT CHROMIUM BY SUBSURFACE MICROORGANISMS

CHAPTER I

LITERATURE REVIEW

Introduction

Chromium is a trace metal found naturally in U.S. soils and rocks with concentrations ranging from 1 to 2000 mg kg^{-1}, and averaging 54 mg kg^{-1} (Westbrook, 1991; Shacklette and Boerngen, 1984). Chromium-bearing domestic ores, usually chromite ($FeCr_2O_4$), crocoite ($PbCrO_4$), or chrome ochre (Cr_2O_3), can contain up to 270 g Cr kg^{-1} (Shupack, 1991; Dragun and Chiasson, 1991). Chromium is recovered directly from its ores by a process of roasting, leaching, filtration, acidification, and drying (Shupack, 1991). Chromium obtained from the mining of ores has many industrial applications in metal finishing and electroplating industries, petroleum refining, electric-power production, leather tanning, wood preservation, pulp and paper production, textile manufacture, ceramics, pyrotechnics and electronics (Darrin, 1956, Förstner and Wittman, 1981). Chromium compounds are also used in the production of pigments, fungicides, magnetic tapes, catalysts, and synthetic rubies for lasers (Shen and Wang, 1995; Patterson, 1985). In 1978, chromium

discharges from these industrial activities was estimated to be 4,000 pounds per day by the U.S. Environmental Protection Agency (EPA) (Towhill et al., 1978). Increased regulation has not brought about a decrease in the discharge volume (Palmer and Puls, 1994). In the U.S. alone, 0.5 million tons of chromium were consumed in 1996, constituting approximately 16% of world chromite ore production (Mineral, 1997). In fact, annual worldwide production of chromite ores is almost 9×10^9 kg (Palmer and Wittbrodt, 1991). Unfortunately, improper storage, treatment, and disposal practices have led to many areas of chromium contamination in both soils and groundwater. Currently in the United States, 635 Superfund sites exist which list chromium as one of the contaminants (U.S. EPA, 1999).

Chromium Chemistry and Toxicity

Chromium can exist in six valence states, from 0 to +6, but is generally encountered as the trivalent [Cr(III)] or hexavalent [Cr(VI)] form (Palmer and Puls, 1994). Trivalent chromium exists naturally in the environment while the presence of hexavalent chromium is almost always due to the industrial practices of man (Nieboer and Nriagu, 1988). Chromium is an intriguing element because the trivalent form is a required essential nutrient in mammalian systems, contributing to the regulation of carbohydrate metabolism, yet it can be toxic in very high doses (U.S. EPA, 1998). Hexavalent chromium can be toxic, mutagenic, teratogenic, and carcinogenic with even moderate exposure (U.S. EPA, 1984; Bartlett, 1991; Cervantes, 1991). Focusing on toxicity and exposure potential, the U.S. EPA recently designated chromium,

and its compounds, as one of seventeen chemicals posing the greatest threat to human health (Boutacoff, 1991).

Trivalent Chromium. Cr(III) is the most stable form of chromium. This stability can be attributed to the structure of the cations which exist in an octahedral form and therefore cannot readily cross biological membranes (Park et al., 1996). Cr(III) is 100-fold less toxic than Cr(VI) and forms minerals which are not very soluble or mobile in natural waters (WHO, 1988). Thus, trivalent chromium compounds are considered biologically unavailable and non-carcinogenic. While Cr(III) has been classified as a Class D chemical (not carcinogenic to humans) it has been shown to have toxic effects in humans and animals, affecting both the respiratory and reproductive systems (U.S. EPA, 1998).

In mammals, Cr(III) plays an essential role in normal glucose, protein, and fat metabolism and aids lowering of blood glucose levels by binding to glucose receptor sites, causing decreased uptake of glucose (Goto and Kida, 1995). The recommended daily intake of trivalent chromium is 50 to 200 μg/d for adults (EPA, 1998). However, regardless of dose or dietary status, only 1-3% of ingested inorganic trivalent chromium compounds are absorbed by the body, emphasizing the difficulty involved in transporting Cr(III) across membranes for cellular use (Donaldson and Barreras, 1966). Some synthetic Cr(III) complexes on the market, with lipid soluble organic anions as ligands, such as picolinate, cross membranes fairly readily and are touted to aid in weight loss (Petrilli and DeFlora, 1978). There are, however, conflicting reports

as to the safety of these chromium-containing aids, particularly in reference to self-dosing and excessive consumption, given the potential toxic effects described above (Standeven et al., 1989).

Hexavalent Chromium. Cr(VI) is generally considered to pose the greatest risk to human health because of its toxicity and mobility in the environment. Water-soluble Cr(VI) compounds, existing as oxides or oxyanions, penetrate the cell membrane easily in the form of the tetrahedral chromate anion (CrO_4^{2-}), using the general anion transport system (Dartsch et al., 1998). Cr(VI) is much more toxic than Cr(III), for both acute (short-term) and chronic (long-term) exposures (ATSDR, 1991; WHO, 1988). In fact, Cr(VI) has been listed as a Class A human carcinogen by inhalation, and its toxicity varies greatly among a wide variety of chromium compounds (IRIS, 1996). The Reference Dose (RfD) (maximum ingestable dose before risk for chronic non-cancer effects) for Cr(VI) is 0.005 $mg \cdot kg^{-1} \cdot d^{-1}$ while the RfD for Cr(III) is 200 times higher (1 $mg \cdot kg^{-1} \cdot d^{-1}$) (EPA, 1988). The $1/ED_{10}$ value, a measure of the carcinogenic potency of a chemical, for Cr(VI) is 390 mg/kg/d, placing this metal form in the high category under Superfund's ranking for carcinogenic hazards (WHO, 1988).

Effects of Chromium on Health

Hexavalent chromium compounds enter eucaryotic cells via sulfate anion channels (Ottenwaelder and Wiegand, 1988; Wiegand et al., 1988). Once Cr(VI) has crossed the cell membrane it is reduced intracellularly by redox-active enzymes and small molecules (e.g. cytochrome P-450s, glutathione,

cysteine, ascorbate), resulting in the formation of unstable intermediates (i.e. short-lived Cr(V) and Cr(IV) species) and radical species which attack DNA. The resulting DNA damage in the form of chromium-DNA adducts, radical adducts, DNA cross-links, and DNA strand breaks interferes with the normal function of the DNA template in replication and transcription (Dartsch et al., 1998; Standeven and Wetterhahn, 1989; Bridgewater et al., 1994; Xu et al., 1996). These alterations in the DNA cause chromosomal aberrations and sister-chromatid exchanges (Sarto et al., 1982, 1983; Hamamy et al., 1987). Within the cell, reduction of Cr(VI) to Cr(III) is a prerequisite for chromate toxicity, because only the trivalent chromium form is able to interact in vivo with critical target molecules inside a cell (Wolf et al., 1989). So while Cr(III) cannot enter cells itself, its toxicity is manifested once Cr(VI) is reduced to Cr(III) intracellularly. Cr(III) complexes formed in situ must gain entry to the nucleus and only Cr(III) is detected bound to DNA. Thus it may be that only the Cr(III) formed *in situ* is toxic. Petrilli and De Flora (1978) discovered that Cr(VI) compounds are only mutagenic once a metabolic reduction has transformed the molecules. In contrast, it has been speculated that the Cr(V) state is responsible for toxicity and that further reduction to the trivalent form renders this metal nearly harmless (Vonburg and Liu, 1993; Aiyar et al., 1990; Kortenkamp et al., 1990). Further studies are necessary to determine whether chromium toxicity is caused by the trivalent ion or the reduction intermediates Cr(V) and Cr(IV).

Within the body, chromium is not concentrated in one particular region or organ. However, for acute (short-term) and chronic (long-term) inhalation exposures the respiratory tract is the major target organ for both Cr(VI) and Cr(III) toxicity. The effects seem to be similar, although Cr(III) is less toxic. Dypsnea, coughing, and wheezing were reported from a case of acute exposure to Cr(VI), while perforations and ulcerations of the septum, bronchitis, decreased pulmonary function, pneumonia, asthma, and other respiratory effects have been noted from chronic exposure (U.S. EPA, 1998). Other effects noted from acute inhalation exposure to very high concentrations of Cr(VI) include gastrointestinal and neurological effects, while dermal exposure causes skin burns (ATSDR, 1993; WHO, 1988). Acute animal tests, such as the LC_{50} and LD_{50} tests in rats, have shown Cr(VI) to have extreme toxicity from inhalation and oral exposure, while Cr(III) has been shown to have only moderate toxicity from oral exposure (ATSDR, 1993; RTECS, 1993).

Impairment of gestation and complications during pregnancy and childbirth have been reported in female workers exposed to chromium at the workplace (Shmitova, 1978; 1980). Toxic effects on embryo and fetus in experimental animals after exposure to Cr(VI) have been reported (Trivedi et al., 1989; Junaid et al., 1995, 1996 a,b; Kanojia et al., 1996; Elbeteiha and Al-Hamood, 1997). Recent studies have shown that exposure of female mice to chromium results in fewer implantations, causing decreased fertility and litter size (Kanojia et al., 1998). Oral animal studies have reported severe developmental effects in mice, such as gross abnormalities and reproductive

effects including decreased litter size, reduced sperm count, and degeneration of the outer cellular layer of the seminiferous tubules (ATSDR, 1993). No reproductive effects from inhalation exposure to Cr(VI) have been reported.

It has been reported that upon ingestion the kidney is the main target organ of chromium toxicity since acute exposure to Cr(VI) produces acute necrosis of the renal tubules in humans (Franchini et al., 1978; Langard and Norseth, 1986). Recent work suggests that liver cells are also highly sensitive to Cr(VI), although to a lesser degree than kidney cells, resulting in 50% hepatocellular necrosis after exposure to 50 µmol/l Cr(VI) (Dartsch et al., 1998).

Chromium Contamination in the Environment

The general population is exposed to chromium, generally as Cr(III), by eating food, drinking water, and inhaling air that contains this element. The average daily intake from these sources is estimated to be approximately 0.01 to 0.03 µg, 2.0 ppb, and 60 µg, respectively (ATSDR, 1993). However, given the toxic effects of this metal in greater than trace amounts, the contamination of natural environments with chromium is a cause for public health concern (U.S. EPA, 1984).

Chromium in Water and Soil Systems. Chromium occurs naturally in water from mineral weathering, sediment load, and precipitation. Unpolluted lakes and rivers have a mean of more than 2 µg/l versus 0.3 µg/l Cr in ocean water (Cary, 1982). A frequently recommended limit for chromium in natural waters is 0.05 mg l^{-1} of Cr(VI), based in part on harmful effects to man (U.S. EPA, 1976; FWPCA, 1968). Sources of chromium pollution of surface waters include

domestic waste water, metal manufacturing, and sewage sludge. Amounts of Cr(VI) in excess of 1.7 ppm in effluent water are unacceptable to health authorities (U.S. EPA, 1975). Furthermore, the U.S. EPA has mandated that the Maximum Contaminant Level (MCL) for chromium in drinking water be 0.1 mg l^{-1} (U.S. EPA, 1998). Chromium from contaminated freshwater sources eventually moves into the ocean via river systems. The half-life of chromium in lake waters is 4.6 to 18 years, compared to a residence time in ocean waters of 25,000 to 40,000 years (Campbell and Yeats, 1981).

Hexavalent chromium is the dominant form in polluted soils, surface and groundwaters and is of particular concern because this form has been demonstrated to be toxic to animals at low concentrations as well as being highly mobile, often leading to detection in groundwater (Calder, 1988; Deutsch, 1972). Redox reactions are the basis for the cycling of chromium in soils, freshwater, and marine systems. In groundwater, Cr(VI) predominates under oxidizing conditions, such as in shallow aquifers within a few meters of the water table (Wade et al., 1993). However, Cr(III) can predominate under more reducing conditions, such as in deeper anoxic groundwaters (Calder, 1988). Cr(III) is usually lost from water sources as a result of the hydrolysis of Cr(III) complexes to insoluble hydroxides, particularly chromium hydroxide, and subsequent accumulation in sediments. This precipitation of chromium hydroxide is thought to be the predominant fate of chromium in freshwater (Callahan et al., 1979).

Soluble ionic forms of Cr(VI) in soil are attenuated by leaching, adsorption, precipitation, uptake by living cells, or reduction to Cr(III). Adsorption of Cr(VI) onto soil clay particles is enhanced by acidity and other factors that increase the positive charges on soil colloids (Bartlett and James, 1988). Leaching is often evidenced by the presence of hexavalent chromium in groundwater. In fact, the U.S. EPA has mandated that disposal of any contaminated soils or solids must have a leachate with no more than 5 mg/l of Cr (U.S. EPA, 1987). Organic matter in soil, such as humic substances, can reduce Cr(VI) to Cr(III) (Lovley et al., 1999). Like adsorption, chemical reduction proceeds more readily under acidic rather than neutral or basic conditions. There is no evidence that chromium undergoes biological alterations in the soil (Oak Ridge National Laboratory: Chromium, 1990).

Chromium in the Atmosphere. Most of the chromium-containing particles in ambient air come from anthropogenic sources such as coal-burning steel and iron manufacturing factories, chromium chemical and cement production facilities, and rust inhibitors containing chromium in cooling towers (IARC, 1990). Chromium concentrations in air ranging from 0.01 to 1.3 ng/m^3 can result from natural sources, such as wind-blown dust and volcanic eruptions (Pacyna and Nriagu, 1988; Fishbein, 1976; IARC, 1986). Particle-bound chromium in the atmosphere is often transported and later deposited. Particles less then 10 μm in diameter can remain airborne for long periods of time, whereas larger particles are quickly deposited. It is believed that atmospheric fallout is a major contributor to the lack of chromium tolerance in

many ecosystems, mainly due to the toxic effects on the organisms comprising the ecosystem (Wade et al., 1993).

Current Chromium Treatment Strategies

While trivalent chromium can be toxic at very high doses, in the environment these ions are nearly immobile. Thus, primary concern is for environmental contamination by hexavalent chromium. Industrial wastewater containing chromium must undergo treatment to remove chromium before release into the environment. The most commonly used current treatment technology involves chemical reduction of the Cr(VI) to Cr(III) with iron and sulfur containing solutions ($FeSO_4$, Na_2SO_3, $NaHSO_3$, and $Na_2S_2O_5$) followed by precipitation. Since chemical reduction generally occurs only in a narrow range of pH (pH 2-3), these methods require the use of chemical additives not only for chromate reduction, but also for pH adjustment (Fujii et al., 1990). Other chemical treatments use ion exchange and adsorption of the chromium onto coal, activated carbon, alum, kaolinate, and fly ash, or electrodepositing in which wastewater is electrically charged and the chromium is precipitated onto an immobile matrix (Zhao and Duncan, 1997; Srivastava et al., 1986). In extreme cases where chromium contamination of soil has occurred, remedial measures such as excavation or pumping of contaminated subsurfaces, are implemented. These methods, while effective, can be quite costly, requiring high energy input or large quantities of chemical reagents, and can create other forms of waste with unique environmental concerns. The process of site excavation, for example, can cost more than $100 per ton to remove chromium

(Higgins et al., 1998). In addition, this method may pose unacceptable health risks from airborn contaminants during the excavation. Therefore there is a need for the development of low-cost easily available materials that can remediate chromium economically.

The biological reduction of hexavalent chromium to the trivalent form may present a viable alternative treatment strategy, whereby microorganisms would reduce the Cr(VI) in solution to Cr(III). Biological reduction of chromate would have several advantages over current treatment methods. First, chromate reduction would occur around a neutral pH and at normal temperatures. Second, there would be no requirement for large quantities of chemical reagents. Finally, microbiological reduction would ensure a low energy requirement and small excess sludge production. However, in order to implement such a treatment regime a deeper understanding of the mechanisms involved in microbial chromium reduction is necessary. The following review of the literature summarizes current knowledge of the processes involved in microbial reduction of hexavalent chromium.

Microbial Reduction of Hexavalent Chromium

A diversity of microorganisms including, sulfate-reducers, nitrate-reducers, iron-reducers, and even cyanobacteria have been reported to have chromium-reducing capabilities (Guan et al., 1993; Garnham and Green, 1995; Fude et al., 1994). Most of the microorganisms shown to reduce chromate are members of the following genera: *Achromobacter*, *Aeromonas*, *Agrobacterium*, *Bacillus*, *Comamonas*, *Desulfovibrio*, *Enterobacter*, *Escherichia*, *Geobacter*,

Geovibrio, *Pseudomonas*, *Shewanella*, and *Streptomyces* spp (Bopp et al., 1983; Das and Chandra, 1990; Gvozdyak et al., 1987; Ishibashi et al., 1990; Kvasnikov et al., 1990; Lovley, 1993; Nealson and Little, 1997). One group of microorganisms notably absent from this list are members of the Archaea. To date no methanogenic, halophilic, or thermophilic archaebacteria have been reported to reduce Cr(VI) to the trivalent form, although genome sequences have shown that some of these organisms possess genes for heavy metal reductases and ATPases (Smith et al., 1997).

Chromium Reduction by *Pseudomonas* sp. The first group of bacteria shown to have chromate-reducing activity is the pseudomonads. Species capable of reducing chromium within this genus include: *Pseudomonas fluorescens*, *P. aeruginosa*, *P. mendocina*, *P. ambigua*, *and P. putida* (Bopp et al., 1983; Cervantes and Ohtake, 1988; Dhakephalkar et al., 1996; Horitsu et al., 1983; Ishibashi et al., 1990). While exhibiting many similarities in physiology, the mechanisms by which these organisms reduce chromate can be quite different. Several species have been shown to have plasmid-conferred resistance to chromate while others are able to reduce chromate via an enzymatic system independent of any plasmid effects.

Pseudomonas dechromaticans, isolated from industrial sewage in 1977, was the first organism reported to undergo chromate reduction in this group of organisms. *P. dechromaticans* could use chromate or dichromate as a terminal electron acceptor during anaerobic respiration, however, the mechanism of reduction was never elucidated (Romanenko and Koren'kov, 1977).

Subsequently, a number of organisms were identified that utilized Cr(VI), generally chromate or dichromate, as a terminal electron acceptor during anaerobic respiration (Lebedeva and Lyalikova, 1979; Kvasnikov et al., 1985; Gvozdyak et al., 1987). Again, the mechanism by which these organisms reduced chromium was unclear and very little evidence existed to show that chromate reduction was linked to growth.

In 1983, a chromate-resistant strain of *Pseudomonas fluorescens* (LB300) was isolated from chromium-contaminated river sediment (Bopp et al., 1983). Chromate resistance and reduction in *P. fluorescens* LB300 was plasmid-associated, since the loss of the plasmid (pLHB1) resulted in a simultaneous loss of chromate reduction. One percent of the cultures showed loss of the plasmid when cells were cultured over five consecutive transfers without chromate. Ninety percent of the cultures showed loss of the plasmid upon curing with mitomycin C. Attempts to transform a chromate-sensitive strain of *P. fluorescens* with pLHB1 were successful, however, no transformation occurred in *P. putida* or *Escherichia coli*. Attempts to impart chromate resistance via conjugation were successful in *E. coli* AC80, and the chromate-sensitive strain of *P. fluorescens* but not in five other *E. coli*, three *P. putida*, and two *Klebsiella pneumoniae* strains. It was determined *that P. fluorescens* LB300 was 200-fold more resistant to chromate and 2-fold more resistant to dichromate than the plasmid-free, chromate-sensitive counterpart (Bopp et al., 1983).

Further studies with *P. fluorescens* LB300 showed that chromate resistance was related to reduced uptake of chromate relative to the chromate-sensitive strain. Ohtake et al. (1987) showed that chromate was transported into the cell via the active transport system for sulfate. When the sulfate transport system was repressed during growth with cysteine, the cells were much more resistant to chromate than were cells grown with djenkolic acid, a depressor of the transport system. Some uptake of chromate occurred even when the transport system was repressed by cysteine, perhaps due to partial chromate transport by a second transport system without involvement of the sulfate carrier system. Kinetic studies showed that the V_{max} for chromate transport in LB300 was 2.2 fold less than the plasmid-free, chromate-sensitive strain, with a value of 0.076 nmol/min/mg protein, although the K_m for chromate transport was the same for both organisms at 19 μM chromate (Ohtake et al., 1987).

Initial studies with *P. fluorescens* LB300 did not address reduction of chromate, only resistance. Strain LB300 was indeed capable of reducing chromate during aerobic growth with glucose as the electron donor. Anaerobic reduction of chromate only occurred when acetate was added as the donor and the chromate concentration was less than 50 μg/ml. Under aerobic growth conditions with glucose, strain LB300 showed a lag in growth upon addition of 500 μg/ml chromate. This lag was more pronounced with 1000 μg/ml chromate. Chromate reduction occurred in resting cells, regardless of growth conditions, as long as a suitable electron donor was provided. Interestingly, resting cells of

the plasmid-free, chromate-sensitive strain reduced chromate as well as LB300. Furthermore, resting cells of chromate-sensitive *P. putida* strain AC10 also catalyzed chromate reduction. These results showed that plasmid-mediated chromate resistance and chromate reduction are independent processes and that chromate-sensitive and -resistant strains of *P. fluorescens* are both equally able to reduce chromate. Comparison of the rates of chromate reduction by chromate-grown cells and cells grown without chromate indicated that the chromate reductase activity is constitutive since an increased rate was not observed in chromate-grown cells (Bopp and Ehrlich, 1988).

Cell-free extracts of *P. fluorescens* reduced chromate when either glucose or NADH were added. It was concluded that the reduction of chromate by strain LB300 is enzymatic, with some or all of the enzymes necessary for transfer of electrons from NADH to chromate being membrane associated. Further, chromate reduction was inhibited by both cyanide and azide, suggesting that an electron transport system was involved (Bopp and Ehrlich, 1988). The studies performed with *P. fluorescens* LB300 to date suggest that the bacterial reduction of chromate is not the basis for chromate resistance in this organism (Cervantes and Silver, 1992).

Batch and continuous cultures of *P. fluorescens* LB300 also reduced hexavalent chromium aerobically at a neutral pH with citrate as a carbon and energy source (DeLeo and Ehrlich, 1994). Isolate LB300 reduced the majority of chromate in batch cultures, reducing as much as 99.7% of an initial 1125 mg/L Cr(VI). Shaking the cultures to increase aeration also increased the rate

of reduction. In continuous culture, strain LB300 lowered the influent Cr(VI) concentration by a minimum of 57%, with the amount of Cr(VI) reduced increasing with longer retention time in the chemostat. DeLeo and Ehrlich (1994) reported that the accumulation of Cr(III) in the medium decreased the rate of Cr(VI) reduction since Cr(III) did not precipitate out of solution, presumably due to chelation by citrate, which allowed the Cr(III) to remain in solution. Based on these studies *P. fluorescens* LB300 showed some potential for use in the treatment of industrial waste streams and bioremediation of contaminated soils, sediments, and groundwater. However, further research is still necessary to determine an ideal carbon and energy source, chromate concentration, and oxygen concentration for the application of this organism to chromium-contaminated environments.

In a collection of 326 clinical *P. aeruginosa* strains, only eight isolates showed resistance to chromate (Cervantes and Ohtake, 1988). Five of these isolates transferred the resistance through conjugation to *P. aeruginosa* strain PU21. Analysis of the plasmid DNA revealed that in all five cases only one plasmid was found, a 100 kb plasmid designated pUM505. Cells of PU21 containing this plasmid were not affected by 4 mM chromate. As was the case with *P. fluorescens* LB300, reduced uptake of chromate appeared to be the mechanism of resistance in *P. aeruginosa* PU21. The presence of the plasmid pUM505 caused a 5-fold decrease in the uptake of chromate. This result was also seen in *P. aeruginosa* PU21 containing the antibiotic multiresistance

plasmid pMG6 (Cervantes and Ohtake, 1988). Thus, a common mechanism of resistance appeared on at least three separate plasmids.

Whereas chromate resistance genes were found to be plasmid-associated and chromate reduction genes to be chromosomal in *P. fluorescens* LB300, *P. mendocina* MCM B-180 containing the plasmid pARI180 was the first reported case of both resistance and reduction genes encoded by a plasmid (Dhakephalkar et al., 1996). This plasmid appeared more stable than that in *P. fluorescens* LB300 since MCM B-180 could be transferred 20 times in chromate-free medium without loss of either resistance or reducing capability. Strain MCM B-180, an organism isolated from sewage, was resistant to up to 30 mM Cr(VI) and could reduce up to 2 mM Cr(VI) (Dhakephalkar et al., 1996; Salunkhe et al., 1998). A cured (plasmid-free) strain of MCM B-180, was unable to grow or reduce any chromate in the chromate-containing medium. These findings suggest that although some mechanism in addition to Cr(VI) reduction seems to be responsible for chromate resistance in this organism, chromate reduction plays an important role in chromate resistance of *P. mendocina* MCM B-180 (Dhakephalkar et al., 1996).

Bench-scale trials with this organism showed that complete reduction of chromate occurred in 44 h when *P. mendocina* and molasses were added to sterile soil. When *P. mendocina* and molasses were added to non-sterile soil complete reduction of chromate required ~36 h. Soil that had not been inoculated with *P. mendocina* showed no significant chromate reduction after 7 days of incubation. This suggested that *P. mendocina* MCM B-180 was

unaffected by indigenous microflora and that those organisms played little or no role in detoxifying the soil. Wheat seedlings were grown in chromium-containing soil, chromium-free soil, and soil treated with *P. mendocina* MCM B-180. Seedlings in the *P. mendocina* treated soil appeared the same as those grown in chromium-free soil, while seedlings grown in chromium-contaminated soil were small and misshapen. This successfully showed detoxification of soil as a result of chromate reduction by *P. mendocina* MCM B-180 (Salunkhe et al., 1998).

Horitsu et al. (1983) studied a chromate-tolerant *P. ambigua* strain (G-1) obtained from activated sludge and isolated chromate-sensitive derivatives to study the mechanism of chromate resistance. Strain G-1 was able to grow in medium containing up to 20 mM chromate. It was believed that decreased uptake was again the mechanism of resistance as the chromate-sensitive derivative accumulated six times more chromate than did the resistant parent strain G-1 (Horitsu et al., 1983). The reduced accumulation of Cr(VI) in isolate G-1 was attributed to a thick envelope which prevented permeation of Cr(VI) into the cells. Strain G-1, like many other pseudomonads, was also able to reduce chromium aerobically. Cell-free extracts of *P. ambigua* G-1 were able to reduce 80 μM Cr(VI) within 30 minutes (Horitsu et al., 1987). The chromate-reducing enzyme in G-1 required NADH as a hydrogen donor, but not NADPH. Chromate-sensitive mutants of isolate G-1 exhibited a specific activity of the reducing enzymes that was one fourth to one tenth that of the parent strain. The chromate reductase reported in *P. ambigua* G-1 appears similar to that of *P.*

fluorescens LB300 in that it can use NADH as an electron donor for the reduction of chromate. In addition to NADH, intact cells of *P. fluorescens* LB300 were able to utilize exogenous glucose as an electron donor (Ohtake et al., 1987). This study suggested that chromate reduction by *P. ambigua* G-1 may be part of the basis for resistance to chromate (Horitsu et al., 1987).

Efforts were made to further elucidate the mechanism of chromate reduction in *P. ambigua* G-1 by purifying the chromate reductase. Suzuki et al. (1992) purified the soluble chromate-reducing enzyme from this bacterium and found it to be 65 kDa in molecular weight by gel filtration. SDS-polyacrylamide gel electrophoresis suggested that the subunit weight was 25 kDa. It was concluded that this enzyme was di- or trimeric in structure, although this has not yet been confirmed. The optimal reducing conditions for this enzyme were 50°C and pH 8.6, although the enzyme was active over a range of temperatures and pHs. Kinetic analysis showed a K_m value of 13 μM for chromate reduction and a V_{max} of 27 nmol/(min·mg) of protein (Suzuki et al., 1992).

It was previously reported that the chromate reductase of *P. ambigua* G-1 was unable to use NADPH as an electron donor (Horitsu et al., 1987) . On the contrary, work carried out by Suzuki et al. (1992) showed that the enzyme was active in the presence of either NADH or NADPH, although NADPH gave only 63% of the activity seen with NADH. These cofactors were essential for enzyme activity. This work represented the first stoichiometric balance between donor depletion and chromate reduced, with 3 moles of NADH required to reduce 1 mol of Cr(VI) to Cr(III). Interestingly, a Cr(V) intermediate was found, requiring

one electron from an NADH molecule for formation. The Cr(V) intermediate was then proposed to accept two more electrons from NADH molecules for reduction to the trivalent form (Suzuki et al., 1992). At this time it is unknown if reduction of Cr(V) to Cr(III) occurs in a single step, or as two separate steps.

P. putida PRS2000, another organism capable of aerobic chromate reduction, has been shown to contain a similar NADH- or NADPH-dependent chromate reductase as that found in *P. ambigua* G-1 (Ishibashi et al., 1990). The enzyme was found in the soluble protein fraction, rather than the membrane fraction of the cells. The rate of chromate reduction increased linearly with additions of either NADH or NADPH. Kinetic analysis showed that the K_m of the enzyme was 40 μM for chromate and the V_{max} was 6 nmol/(min·mg) protein. Chromate-reductase activity was inhibited by the presence of mercury or silver anions (Ishibashi et al., 1990). The activity described for *P. putida* appears similar to chromate-reducing activity reported in *P. ambigua*; however, further studies would be required to validate this conclusion.

Chromium Reduction by *Escherichia coli*. Shen and Wang (1993) reported that *Escherichia coli* ATCC 33456 anaerobically reduced hexavalent chromium via an enzymatic process. *E. coli* ATCC 33456 reduced about 300 μM Cr(VI) in 12 hours when approximately 10^{10} cells were added to Cr(VI)-containing medium. Whole cell reduction of Cr(VI) was partially inhibited by the presence of oxygen. The Cr(III) measured in spent media accounted for 98% of the total chromium, with 2% found in the cell pellet. Shen and Wang concluded that chromium reduction occurred on the cell surface because Cr(VI)

reduced intracellularly to Cr(III) would remain in the cell until the cell membrane was disrupted. The formation of insoluble Cr(III) on the cell surface was thought to offer protection to the cells from the toxic effects of Cr(VI) (Shen and Wang, 1993).

Supernatant fluids of cell extract reduced chromium under both aerobic and anaerobic conditions and to nearly the same degree as whole cells, indicating that chromium reduction was predominantly carried out by soluble reductase activity. Cr(VI) reductase reduced Cr(VI) in the absence of any electron donor, leading to the conclusion that endogenous stores were utilized. However, Cr(VI) reduction was enhanced by the addition of the external donor, NADH. The chromium reductase of this organism was heat labile and not affected by respiratory inhibitors such as cyanide, azide, and rotenone. Cytochromes *b* and *d* were identified in the membrane fraction of cell extracts but the membrane fraction alone could not catalyze Cr(VI) reduction. Instead, cytochrome difference spectra analysis indicated that these respiratory chain cytochromes required the presence of the soluble Cr(VI) reductase to mediate electron transport to Cr(VI). The low dissipation rate of the proton motive force appeared to limit the respiratory-chain-linked electron transport to Cr(VI). This was concluded when the addition of 2,4-dinitrophenol, an uncoupler of electron transport and oxidative phosphorylation stimulated Cr(VI) reduction activity in whole cells (Shen and Wang, 1993).

Since many industrial wastes contain a mixture of toxic inorganic and organic compounds *E. coli* ATCC 33456 was used in a defined coculture with

the phenol degrader, *Pseudomonas putida* DMP-1 (Shen and Wang, 1995). The coculture reduced Cr(VI) and degraded phenol simultaneously under aerobic growth conditions, resulting in reduction of about 140 μM Cr(VI) and depletion of 7 mM phenol in 48 hours. Phenol was supplied as the sole carbon source during growth and *P. putida* DMP-1 quickly dominated the population in the coculture, with phenol degradation commencing without a lag period. Several degradation intermediates were detected in the culture but it is unknown which substrate produced by *P. putida* was utilized by *E. coli* for growth and Cr(VI) reduction. Both the rate and extent of Cr(VI) reduction and phenol degradation were affected by the cell numbers of each organism in the coculture. Although Cr(VI) reduction occurred as a result of *E. coli* metabolism, the rate of phenol degradation by *P. putida* may become a rate-limiting factor for Cr(VI) reduction at a low population ratio of *P. putida* to *E. coli*, since metabolism of *P. putida* supplies the electron donor for *E. coli* growth and Cr(VI) reduction. Finally, Cr(VI) reduction by *E. coli* was not inhibited by phenol until concentrations in excess of 9 mM were present, but phenol degradation by *P. putida* was very sensitive to relatively low concentrations of Cr(VI). Although this might be problematic in a bioremediation or treatment regime this work showed that a mixture of defined organisms could effectively treat more than one type of industrial waste (Shen and Wang, 1995).

Chromium Reduction by *Enterobacter* sp. Another extensively studied biological system for Cr(VI) reduction involves the use of *Enterobacter cloacae* strain HO1 (Wang et al., 1989). This organism was originally isolated

in 1989 from the activated sludge of a municipal wastewater treatment plant (Wang et al., 1989; Ohtake and Hardoyo, 1992). Strain HO1 was resistant to up to 10 mM chromium under both aerobic and anaerobic conditions, however, chromate reduction under growing conditions was a strictly anaerobic process, with 500 μM Cr(VI) being reduced in 60 hours. The rate of chromate reduction was dependent upon cell density. Wang et al. (1989) reported that chromate was used as a terminal electron acceptor for growth of *Enterobacter cloacae* strain HO1. Cells could be grown anaerobically without chromate but did not achieve high optical density. Upon addition of chromate the turbidity of the culture increased significantly, along with a decrease in the chromate concentration.

E. cloacae strain HO1 was able to reduce as much as 2 mM Cr(VI) under anaerobic conditions (Komori et al., 1989). Incomplete reduction of Cr(VI) was observed between 3 and 5 mM and concentrations greater than this proved lethal to the cells. The yellow color of the culture gradually turned white and turbid as the chromate concentration decreased, presumably due to formation of insoluble trivalent chromium. The rate of reduction was estimated to be about 1.0×10^{-8} umol·h^{-1} per cell, although this rate decreased with increasing concentrations of dissolved oxygen. When the oxygen concentration exceeded 4.5 ppm chromate reduction was inhibited, at least until cell respiration occurred and depleted the oxygen (Komori et al., 1990a). Redox potential was measured and found to decrease from -60 mV to -220 mV during chromate reduction (Komori et al., 1989). It was at first believed that oxygen was not the limiting

factor in chromate reduction but rather redox potential. However, redox potentials as low as -200 mV were measured and chromate reduction still did not proceed if dissolved oxygen was present (Komori et al., 1990).

Chromate reduction by strain HO1 occurred between pH values of 6.0 and 8.5, with an optimum of 7.0-7.8 (Komori et al., 1989). Growth and chromate reduction of strain HO1 occurred at temperatures ranging from 20-40°C, with an optimum from 30-37°C. Interestingly, some chromate reduction occurred at temperatures at and above 50°C, although growth was completely inhibited (Komori et al., 1989). Chromate reduction was partially inhibited by molybdate, vanadate, tungstate, chlorate, sulfate, or sulfite. Growth and chromate reduction were inhibited by penicillin, cycloserine and chloramphenicol (Wang et al., 1990).

Electron donors which successfully supported chromate reduction in strain HO1 included acetate, aspartate, ethanol, glutamate, malate, succinate, and glycerol (Ohtake et al., 1990; Komori et al., 1989). Glucose, citrate, lactate, and pyruvate supported anaerobic growth, but only limited reduction of Cr(VI) was observed with these organic compounds. Growth of strain HO1 was stimulated upon addition of casamino acids, casitone, tryptone, and yeast extract. Aspartate, isocitrate, malate, oxalate, and tartrate stimulated chromate reduction by growing cultures when they were added to the basal medium along with casamino acids. Several amino acids were also shown to stimulate the rate of chromate reduction by washed cells, including alanine, glutamine, glycine, methionine, and threonine. Chromate reduction was also observed

upon addition of isoleucine, leucine, proline, and valine, but at a slower rate (Ohtake et al., 1990).

Attempts were made to identify the portion of the HO1 cell responsible for chromate reduction. Wang et al. (1991) demonstrated that the chromate reductase activity was preferentially associated with the membrane fraction of *E. cloacae* HO1 cells. Right-side-out membrane vesicles prepared from *E. cloacae* HO1 showed high rates of anaerobic chromate reduction. Both cell-free filtrate and supernatant were unable to reduce chromate, even under anaerobic conditions. However, once cells were resuspended in fresh chromium-containing medium chromate reduction rapidly resumed. In fact, a cell density of 2 X 10^7 cells/ml completely reduced 500 μM Cr(VI) within 5 hours. Reductase activity was lost upon exposure of the membrane vesicles to oxygen or heat treatment (100° C for 1 minute) (Wang et al., 1991).

The conclusion reached from these studies was that Cr(VI) reduction took place on the cell surface, forming insoluble chromium hydroxide in the external medium. Chromate reduction on the cell surface and formation of insoluble chromium hydroxide would favor protection of the cells from the toxicity of Cr(VI) (Wang et al., 1990). As an industrial benefit, formation of external chromium hydroxide would facilitate collection of the reduced chromium from solution. These studies showed that Cr(VI) was reduced to Cr(III) under anaerobic conditions and its reduction was caused by the respiratory chain system of the cell membrane. However, the Cr(VI) reductase has not yet been purified, and the details of the reaction mechanism are still unclear. A study of the chromate

reductase activity of *Enterobacter aerogenes* determined that chromate reductase activity was induced markedly by nitrite under anaerobic conditions (Clark, 1994). Thus, it seemed likely that chromate reduction was catalyzed by the nitrite reductase. There is currently no evidence to suggest that chromate reduction by *E. cloaecae* and *E. aerogenes* are related, although this may be a possibility.

Studies were undertaken to test the suitability of *E. cloacae* strain HO1 for Cr(VI) reduction and removal from wastewater. Komori et al. (1990b) demonstrated a method for removing Cr(VI) from solution by placing grown HO1 cells into a dialysis tubing pouch and then placing this pouch into solutions containing Cr(VI). Strain HO1 successfully removed about 90% of the total concentration when initial concentrations were less than 4 mM (Komori et al., 1990b; Ohtake and Hardoyo, 1992). This system has the advantage that precipitated chromium can be collected inside the bag. Cr(VI) diffuses into the bag and is subsequently reduced. Trivalent chromium is insoluble and remains within the bag. Unfortunately, this system may not lead to a cost-effective treatment strategy on a large scale (Komori et al., 1990b). In addition, a bench scale trial of this method showed that chromate reduction was limited by chromium diffusion into the bag. On an industrial level this method would be very problematic.

Ohtake et al. (1990) performed studies on the detoxification and removal of toxic chromate in an industrial effluent using strain HO1 as a "bioreductant." However, most chromate-bearing wastewaters from industrial processes

generally contain heavy metal cations such as Cu^{2+} Cd^{2+}, Zn^{2+}, and Ni^{2+} (Germain and Patterson, 1974). The cations Ag^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} were tested to determine their effects on the survivability of isolate HO1, as well as chromate-reducing activity. Effluent from a manufacturing process of copper-chromium catalysts was obtained and amended with suitable carbon and energy sources. Unfortunately, all cations tested strongly inhibited chromate reduction by *E. cloacae* strain HO1 (Ohtake and Hardoyo, 1992; Hardoyo et al., 1991; Ohtake et al., 1990). If chromate was removed from wastewater, and thus separated from the other metals, using an ion-exchange membrane, then strain HO1 could reduce it. Alternatively, if the wastewater was significantly diluted these cations would be present at less than toxic levels. These methods may not present a cost-effective treatment option for removing Cr(VI) from industrial wastes.

Chromium Reduction by Iron- and Sulfate-Reducing Bacteria. The most recently described metal-reducing microorganisms have been members of the gamma, delta, or epsilon subgroups of the proteobacteria. Organisms such as *Shewanella alga* and *S. putrefaciens* represent the gamma subgroup of the proteobacteria, while the delta and epsilon subgroups are represented mainly by sulfate- or iron-reducing organisms (Nealson, 1997). It is in the delta subgroup that most sulfate-reducing bacteria are placed. Many organisms with the ability to reduce sulfate to sulfide are capable of utilizing other electron acceptors such as chromate.

Two mechanisms have been reported to explain the reduction of alternate electron acceptors by sulfate-reducing bacteria. First, microorganisms may directly reduce the electron acceptor via an enzymatic mechanism. A second possibilitiy is that the bacteria produce hydrogen sulfide which acts as the reducing agent for Cr(VI), as has been observed in marine environments (Smillie et al., 1981). Smillie et al. (1981) showed that microbially-produced sulfide was responsible for reduction of Cr(VI) to Cr(III) in coastal sediments which had been impacted with tannery effluent. Very little Cr(VI) was measured in these sediments, however, the Cr(III) was precipitated together with organic matter into the sediment. Indeed, microbially-produced sulfide has also been shown to mediate manganese reduction in marine environments (Burdige and Nealson, 1986). The rate-limiting step in this mode of manganese reduction, as well as indirect Cr(VI) reduction, would be bacterial sulfide production. In 1994, a consortium of sulfate-reducing bacteria was reportedly tolerant to large concentrations (~50 mM) of Cr(VI), with rapid reduction and a first-order rate constant of 0.1518 h^{-1} (Fude et al., 1994). Fude et al. (1994) concluded that sulfide production by the consortium was responsible for this reduction. Similarly, a pure culture of *Desulfobacterium*, grown with sulfate and Cr(VI) completely reduced the chromium indirectly when high levels of sulfide had been produced (Karnachuk, 1995).

Direct enzymatic reduction of Cr(VI), as well as other metals, has been reported in pure cultures of sulfate-reducing bacteria. For example, *Desulfovibrio vulgaris* and *Geobacter metallireducens*, both members of the

delta proteobacteria, have been shown to reduce hexavalent chromium as well as other electron acceptors. Indeed, *D. vulgaris*, a sulfate-reducing bacterium, and *G. metallireducens*, an iron-reducing bacterium, have both been shown to reduce Cr(VI) via the c-type cytochromes. Chromate, as well as gold, silver, and mercury were shown by Lovley et al. (1993a) to be reduced by the c-type cytochromes of *G. metallireducens*. Cell suspensions of *D. vulgaris* rapidly reduced Cr(VI) when H_2 was added as the donor. To identify the enzyme responsible for Cr(VI) reduction in *D. vulgaris*, cells were broken in a French pressure cell and fractionated into soluble and membrane-bound protein fractions by centrifugation. Hydrogenase was purified from the soluble protein fraction and, when combined with cytochrome c_3, Cr(VI) reduction occurred. Neither hydrogenase alone nor cytochrome c_3 alone could reduce Cr(VI) (Lovley and Phillips, 1994). Lovley and Phillips (1994) determined that chromate-reducing activity was due to cytochrome c_3 activity, the same source of uranium(VI) reduction in the same organism (Lovley et al., 1993b). However, these studies were not performed under growing conditions and no coupling of growth and metal-reduction was shown.

In addition to Cr(VI)-reduction capability, *D. desulfuricans* has also been reported to reduce uranium, although other metals were not examined (Lovley and Phillips, 1992). A recent report by Tucker et al. (1998) showed reduction of Cr(VI), Mo(VI), Se(VI), and U(VI) by immobilized cells of this sulfate-reducer. Either H_2 or lactate could serve as the electron donor for these reactions leading to removal of these metals from solution. Heat-killed cells and cells with no

electron donor did not reduce the metals in solution. Metal reduction was observed at temperatures from 10°C to 57°C, but the highest metal removal occurred at 37°C for all the metals tested, indicating an enzymatic reduction (Tucker et al., 1998). This study demonstrated that dissimilatory reduction of chromate, molybdate, selenate, and uranate by immobilized cells of *D. desulfuricans* may be a practical method for waste treatment in a continuous flow process. However, more work is needed to determine nutrient and substrate requirements, as well as scale-up techniques.

Recently, Tebo and Obraztsova (1998) isolated *Desulfotomaculum reducens*, a sulfate-reducing bacteria which could grow with sulfate, Cr(VI), U(VI), Mn(IV), or Fe(III) as the terminal electron acceptor. Growth and metal reduction of this organism occurred when lactate was supplied as the electron donor at 37°C. The mechanism by which this organism reduces Cr(VI) has not yet been elucidated. This report represents the first clear demonstration that sulfate-reducing bacteria can grow by coupling the oxidation of organic compounds with the reduction of Cr(VI) to Cr(III), Mn(IV) to Mn(II), Fe(III) to Fe(II) or U(VI) to U(IV), and the first to demonstrate that Cr(VI) reduction supports anaerobic growth in any organism (Tebo and Obraztsova, 1998).

Summary of Chromium Reduction and Research Objectives

As described in this literature review, many organisms have the ability to reduce hexavalent chromium to the less toxic trivalent form, employing a variety of mechanisms. This ability has led to the suggestion that Cr(VI)-reducing microorganisms may be useful "bioreductants" for removing hexavalent

chromium from industrial wastes, and chromium-contaminated soils and groundwaters. However, the practical application of such technology requires knowledge of the physiology and biochemistry of the chromate-reducing reactions. To some degree this information is still lacking. For instance, there has been only one report with conclusive evidence to show that Cr(VI) reduction yields energy to support growth, and it has been suggested that Cr(VI) reduction is catalyzed by enymes that have other functions, and thus substrates other than Cr(VI) (Lovley, 1993). Furthermore, no studies have been conducted to investigate factors affecting the rate and extent of chromate reduction in natural systems. Thus, this study was begun to determine the potential of microbial Cr(VI) reduction in aquifers.

Microbial chromium reduction offers the potential to prevent the migration and to reduce the toxicity of Cr(VI) in aquifers. However, it is not known under what redox conditions bacterial reduction occurs, the types of organisms involved in this reaction, or the mechanisms used by aquifer microorganisms to carry out chromium reduction. For example, it is not known if Cr(VI) reduction in an aquifer would occur under highly reducing conditions, such as sulfate-reducing or methanogenic, or if more oxidized conditions, such as nitrate-reducing or aerobic, are required. Shen et al. (1996) showed that the more oxidizing conditions (nitrate-reducing and aerobic) stimulated the rate and extent of Cr(VI) reduction during anaerobic degradation of benzoate. In this report oxygen or nitrate were added initially to stimulate anaerobic degradation and reduction, which occurred once oxygen or nitrate had been depleted (Shen

et al., 1996). Guan et al. (1993) also reported Cr(VI) reduction by a consortium of denitrifying bacteria. However, the existence of strictly anaerobic bacteria such as *Desulfotomaculum reducens*, which can gain energy for growth by reducing Cr(VI), indicates that more highly reduced conditions may be required. Work with soils and sediments has also indicated that highly reduced conditions may be required for heavy metal reduction (Turick et al., 1996; Fude et al., 1994; Francis et al., 1991; Masscheleyn et al., 1990). A greater understanding of the microbiology involved in chromium reduction and of the interaction between biotic and abiotic factors is needed to predict the fate of chromium in the environment and to accurately assess the chromium contamination risks. Specifically, the research objectives of this dissertation were:

1.) To investigate the important environmental parameters that govern the rate and extent of microbial reduction of chromium in aquifer sediments such as the effects of different electron donors, competing electron acceptors, source of nutrients, chemical inhibitors, pH, and temperature.

2.) To understand the microbiological processes involved in microbial chromium reduction in aquifers.

3.) To determine the redox conditions under which the microbial reduction of Cr(VI) occurs.

Once the optimum conditions for chromate reduction are defined, the use of Cr(VI)-reducing organisms will become a more defined, and less problematic, approach to treating environmental contamination.

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CHAPTER II

PHYSIOCHEMICAL FACTORS AFFECTING CHROMATE REDUCTION BY AQUIFER MATERIALS

Abstract

Aquifer sediments from Norman, OK were used to study the potential for microbial reduction of Cr(VI) to Cr(III). Black, clay-like sediments rapidly reduced Cr(VI) in both autoclaved and viable microcosms, indicating an abiotic mechanism. Lightly colored, sandy sediments slowly reduced Cr(VI) in viable microcosms, indicating a biological process. Cr(VI) reduction in sandy sediments typically consisted of two phases; first, a rapid reduction of Cr(VI) in the first 7-10 days of incubation, followed by a period of slower Cr(VI) reduction. Cr(VI) reduction in these sediments had a pH optimum of 6.8 and temperature optima of 22°C and 50°C. Nearly complete inhibition of Cr(VI) reduction was observed when sandy sediments were shaken in the presence of oxygen. The addition of nitrate to sandy sediments but not sulfate, selenate, or ferrous iron inhibited Cr(VI) reduction. When electron acceptors were supplied in combination with Cr(VI), reduction of Cr(VI) occurred only in the absence of nitrate. No loss of sulfate, or production of Fe(II), occurred in the presence of Cr(VI). The addition of molybdate to the microcosms did not affect Cr(VI) reduction in sandy sediments until very high concentrations (40 times the Cr(VI) concentration) were used. Interestingly, the addition of bromoethanesulfonic acid (BESA) in amounts less than, or slightly greater than, the Cr(VI)

concentration partially inhibited Cr(VI) reduction in sandy sediments. These sandy sediments produced methane in the absence of BESA. A methanogenic enrichment was obtained from sandy sediments that was capable of reducing Cr(VI) during growth. However, the enrichment produced methane only when Cr(VI) was absent. This indicates that a shift in electron flow from methane production to Cr(VI) reduction may have occurred. These studies showed that Cr(VI) reduction in sandy aquifer sediments is a biologically-mediated, anaerobic process that is inhibited by oxygen and nitrate. The lack of sulfate reduction and sulfide production, as well as a lack of inhibition of Cr(VI) reduction by molybdate, argues against an indirect mechanism for Cr(VI) reduction, whereby the sulfide produced during sulfate reduction chemically reduces Cr(VI). Rather, Cr(VI) reduction may be mediated by a community of microorganisms which normally uses methane production as the terminal electron accepting process.

Introduction

Industrial practices including electroplating, leather tanning, pigment manufacture, corrosion inhibition, and fungicide production generate large quantities of chromium-laden wastewater which must be treated before discharge (Wang and Shen, 1995; Förstner and Wittman, 1981). On a global scale, about 170,000 tons of chromium waste are released into the environment annually (Gadd and White, 1993). The widespread use of chromium as well as the improper disposal of chromium-laden wastes has led to areas of serious environmental contamination. Currently in the United States, 635 Superfund sites exist which list chromium as one of the contaminants (U.S. EPA, 1999). The prevalence of chromium contamination has resulted in the classification of

chromium as one of the seventeen most important environmental toxicants by the U.S. Environmental Protection Agency (Boutacoff, 1991).

Chromium exists in oxidation states ranging from 0 to +6, although, only two states, Cr(III) and Cr(VI), are commonly observed in environmental samples (Palmer and Puls, 1994). The valence state and relative solubility of chromium is dependent on environmental conditions (pH, temperature, redox potential) and the presence of other organic and inorganic molecules (Moore and Ramamoorthy, 1983). Trivalent chromium, or Cr(III), an essential trace nutrient in the human diet, has relatively low toxicity and is nearly insoluble at neutral pH, and is thus nearly immobile in the environment (Anderson and Kozlovsky, 1985). Conversely, hexavalent chromium, or Cr(VI), is acutely toxic, mutagenic, teratogenic, and carcinogenic. In addition, Cr(VI) is highly mobile in the environment, mainly due to its soluble nature (Bartlett, 1991; Cervantes, 1991).

Common treatment technologies for removal of chromium from industrial waste include ion exchange, electrodepositing, and chemical reduction with iron and sulfur containing solutions ($FeSO_4$, Na_2SO_3, $NaHSO_3$, and $Na_2S_2O_5$) followed by precipitation (Zhao and Duncan, 1997). These methods, while effective, can be quite costly, requiring high energy input or large quantitites of chemical reagents, and can create other forms of waste with unique environmental concerns. The biological reduction of Cr(VI) to Cr(III) may provide a less costly approach to soil and aquifer remediation.

Although many bacterial strains have been shown to mediate reduction of Cr(VI) to Cr(III), few studies have examined the *in situ* potential of microbial Cr(VI) reduction in aquifers (Lovley, 1993). The stimulation of existing microbial populations may result in microbial chromium reduction, potentially decreasing the migration of Cr(VI) in aquifers. However, it is not clear whether Cr(VI) reduction will occur under oxidizing conditions (aerobic or nitrate-reducing) or

highly reducing conditions (e.g. sulfate-reducing, iron-reducing or methanogenic). Thermodynamic predictions suggest that Cr(VI) reduction will occur after oxygen and nitrate depletion but prior to ferric iron- or sulfate-reduction (Ahmann, 1997). Many bacteria are known to reduce chromium under aerobic conditions (Bopp and Ehrlich, 1988; Horitsu et al., 1983) However, other facultative organisms, such as *Enterobacter cloacae,* reduce Cr(VI) only under anaerobic conditions. Strictly anaerobic iron-reducing and sulfate-reducing bacteria require, by nature, highly reducing conditions (Tebo and Obraztsove, 1998; Wang et al., 1989; Ohtake and Hardoyo, 1992). In addition to a direct enzymatic mechanism for Cr(VI) reduction, Cr(VI) can also be reduced indirectly whereby microbially reduced iron or sulfur species chemically reduce the Cr(VI) (Fude et al., 1994; Smillie et al., 1981). Thus, determining where chromate reduction occurs in relation to other terminal electron accepting processes is an essential step for the development of a remediation strategy for chromium-contaminated aquifers. The potential for microbially mediated Cr(VI) reduction in aquifers, the physical and chemical parameters that control this process, and the redox conditions under which Cr(VI) reduction occurs were assessed in this study.

Materials and Methods

Sample Collection. The potential of microbial processes to reduce Cr(VI) and the extent and rate that these processes occur were investigated using subsurface sediments from an aquifer underlying the Norman, Oklahoma municipal landfill (Beeman and Suflita, 1987). Subsurface sediments were collected by digging to the top of the water table (5-6 feet below the surface) with a post-hole digger and collecting aquifer material in sterile glass jars

(Beeman and Suflita, 1987). The jars were filled to capacity, sealed, and transported to the laboratory where they were stored in an anaerobic chamber at room temperature until use. Both black clay-like and light colored sandy sediments were collected from the landfill. No measurable Cr(VI) was detected in either type of sediment.

Microcosm Construction. Standard anaerobic techniques were used throughout the study (Balch and Wolfe, 1976). All manipulations with the aquifer material were done inside of an anaerobic chamber containing an atmosphere of approximately 5% H_2 in N_2 (Balch and Wolfe, 1976). The composition of the mineral medium has been previously described by Tanner (1989) and was prepared according to the procedure described by Balch and Wolfe (1976). Acetate (500 μM) was added to the medium to serve as the electron donor.

Microcosms were prepared in an anaerobic chamber by slurrying 5 g (wet weight) of sediment with 50 ml of sterile, anaerobic mineral medium in sterile serum bottles (160 ml capacity). No chemical reductant, such as cysteine sulfide, was added to the microcosms since reductants will chemically react with Cr(VI), making it impossible to discern biological Cr(VI) reduction. All serum bottles were sealed with butyl rubber stoppers, the head-spaces were evacuated and replaced with 80% N_2-20% CO_2 (125 kPa). The final pH of the microcosms was 7.2. Each bottle typically had an initial Cr(VI) concentration of approximately 500 μM, added from an anoxic sterile stock of K_2CrO_4. In some cases microcosms were prepared with groundwater instead of mineral medium.

Groundwater was collected from the well at the methanogenic site of the Norman landfill, at a depth of 4.7 meters. Triplicate microcosms were used for each treatment and heat-killed controls were included for each experiment. The heat-killed controls were prepared by autoclaving inoculated bottles twice at 121°C for 20 minutes, followed by the addition of 20 mg/L of $HgCl_2$ (final concentration) (Shen et al., 1996). Additions of other chemicals were made using sterile anoxic, stock solutions. Samples for Cr(VI) analysis were periodically taken using sterile, N_2-flushed syringes and needles. Unless otherwise stated, all incubations were carried out at room temperature in the dark.

Electron Acceptors. To determine the effect of exogenous electron acceptors on Cr(VI) reduction, the following compounds were added, singly or in combination, to Cr(VI)-containing sediment slurries at a concentration of 5 mM each: $NaNO_3$, Na_2SO_4, or Na_2SeO_4. Approximately 5 mM amorphous ferric oxyhydroxide (final concentration) was added as a source of Fe(III) which had been prepared according to Lovley and Phillips (1986). The fate of these electron acceptors was followed by measuring the reduction of nitrate, and the production of sulfide and ferrous iron, respectively. Selenate reduction was indicated by the formation of a red precipitate, presumably Se^0. To test the effect of oxygen on chromium reduction, the bottles were prepared as above with the exception that a sterile syringe plugged with cotton wool was placed in the butyl rubber stopper to allow oxygen exchange. Chromium reduction in

these microcosms was compared to reduction of Cr(VI) in anaerobic microcosms.

Chemical Inhibitors. The effect of increasing concentrations of inhibitors on Cr(VI) reduction was determined. Slurries were amended with bromoethanesulfonic acid (BESA) (0, 0.2, 2.0 and 20.0 mM), an inhibitor of methanogenic bacteria, or sodium molybdate (0, 0.2, 2.0 and 20.0 mM), an inhibitor of sulfate-reducing bacteria, to determine the relative contributions of these two groups of bacteria to Cr(VI) reduction.

Environmental Parameters. The effects of pH and temperature on the rate and extent of microbial Cr(VI) reduction were investigated. Microcosms (pH 7.2) were incubated at a range of temperatures and reduction of Cr(VI) was monitored. The effect of pH was tested with slurries incubated at room temperature (ca. 24°C). To study the effect of pH on Cr(VI) reduction, the mineral medium was modified to include 20 mM each of the following buffers: 2-(N-morpholine)-ethanesulfonic acid (MES) (pK_a of 6.15), Piperazine-N,N'-bis-(2-ethanesulfonic acid) (PIPES) (pK_a of 6.8), and N-2-hyroxyethylpiperazine-N'-3-propanesulfonic acid (HEPPS) (pK_a of 8.0). The pH of the medium was then adjusted by the addition of HCl or NaOH. Triplicate heat-killed slurries were constructed for each temperature and pH value to monitor abiotic reduction of chromium.

Enrichments Obtained from Aquifer Sediment. A Cr(VI)-reducing enrichment, which produced methane in the absence of Cr(VI), was obtained from aquifer sediment and serially transferred in a mineral salts medium with an

80% H_2; 20% CO_2 (125 kPa) head space and an initial Cr(VI) concentration of approximately 500 μM. The mineral medium contained the following components (per liter of deionized water): Tanner's metal solution (1989), 5 ml; Tanner's vitamin solution (1989), 10 ml; Pfennig's mineral solution (McInerney et al., 1978), 10 ml; yeast extract, 0.1 g; $NaHCO_3$, 10 g. The enrichment was maintained by transferring a 1 to 5% inoculum to fresh medium bi-monthly.

Similarly, a sulfate-reducing enrichment which was also able to reduce Cr(VI) was obtained and transferred into the mineral salts medium used in the original sediment-containing microcosms, with the addition of 2 mM lactate and either 5 mM sulfate or 500 μM Cr(VI). The enrichment was maintained by transferring a 1 to 5% inoculum to fresh medium bi-monthly.

Analytical Techniques. Cr(VI) was determined colorimetrically by reaction with diphenylcarbazide in acid solution (Urone, 1955; Clesceri, 1989). This procedure measures only hexavalent chromium, i.e., Cr(VI). Prior to total chromium determination, samples were digested with a combination of heat, and nitric and hydrochloric acids (Hach, Co., Loveland, CO). Total chromium was determined by the alkaline hypobromite oxidation method (Hach Co., Loveland, CO). The amount of Cr(III) was calculated from the difference between total chromium and Cr(VI). Nitrate and nitrite were measured colorimetrically using a commercially available assay (Hach Co., Loveland, CO). Sulfate concentrations were determined with a Dionex ion chromatograph. Sulfide analysis was performed colorimetrically as previously described (Tanner, 1989). Fe(II) was assayed by the colorimetric method

described by Lovley and Phillips (1986). Acetate was analyzed using an HPLC equipped with a BioRad 300- × 7.8-mm Aminex HPX-87H column and an isocratic mobile phase of 0.016 N H_2SO_4 at a flow rate of 0.9 ml/min (Amos and McInerney,1990). Methane was measured by gas chromatography (Beeman and Suflita, 1987).

Results

Biotic versus abiotic Cr(VI) reduction. Black sediments that were clay-like in appearance and texture reduced 500 µM Cr(VI) so rapidly that in many cases substantial losses of Cr(VI) occurred before initial sampling could take place. Re-amendment of these microcosms with an additional 500 µM Cr(VI) resulted again in a rapid reduction of Cr(VI) (Figure 1). With clay-like sediments, Cr(VI) reduction occurred in both heat-treated and unheated microcosms (Table 1), indicating that Cr(VI) reduction occurred in these sediments via an abiotic mechanism. Cr(VI) reduction in microcosms containing sandy sediments occurred more slowly than in clay-like sediments, with little or no loss of Cr(VI) in heat-killed controls (Table 1). Cr(VI) reduction in sandy sediments typically consisted of two phases; first, a phase of rapid reduction in the first 7-10 days of incubation, followed by a second period of slower Cr(VI) reduction (Figure 1). The slow period of Cr(VI) reduction is likely due to a kinetic limitation rather than loss of activity because re-amendment with Cr(VI) again resulted in a rapid phase of reduction followed by a slower period of Cr(VI) loss at concentrations of ~50 µM. Because it was possible to measure biological Cr(VI) reduction in sandy sediments, these were used for the

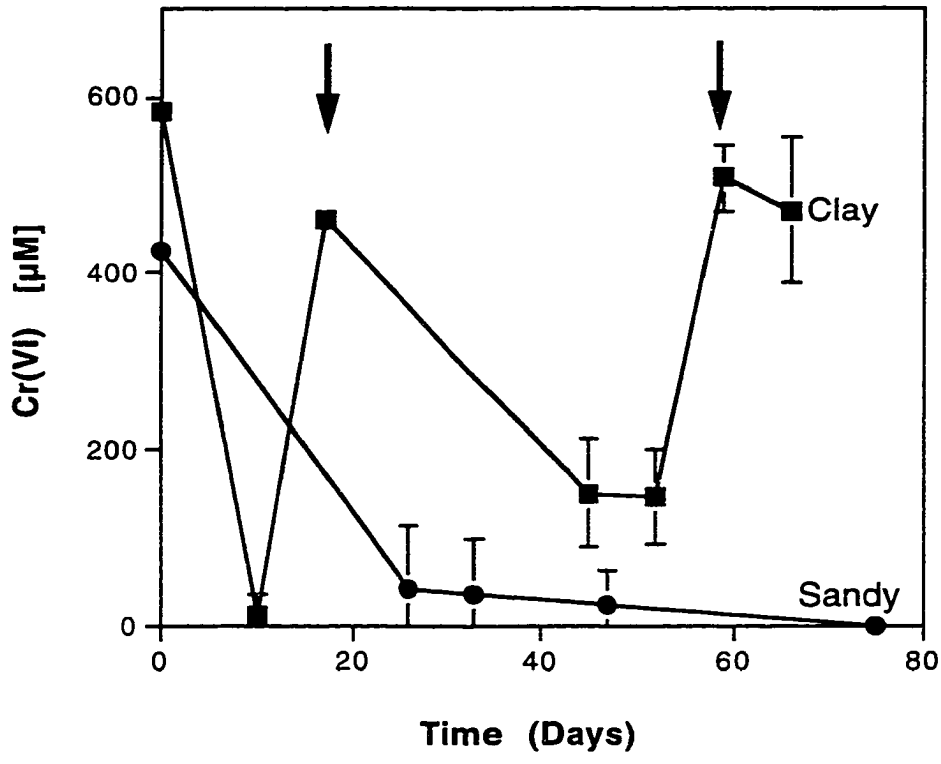

Figure 1. Comparison of sediment type on Cr(VI) reduction in viable microcosms. Arrows indicate re-amendment of Cr(VI).

Table 1. Comparison of Cr(VI) reduction by sandy and clay-like sediments from Norman aquifer.

	Sandy Sediment		Clay-like Sediment	
Treatment	**[Final Cr(VI)] (μM)[a]**	**Total μM Reduced**	**[Final Cr(VI)] (μM)[b]**	**Total μM Reduced**
None	23.5 ± 33[c]	402	BDL[d]	856
Autoclaved	402.0 ± 21	BDL	8.9 ± 13	606

[a]Concentration of Cr(VI) was measured after 47 days of incubation with no re-amendment of Cr(VI). The initial concentration was ~500 μM.

[b]Concentration of Cr(VI) was measured after 45 days of incubation with re-amendment of Cr(VI) after 17 days of incubation.

[c]Values stated are mean ± standard deviation of triplicate determinations.

[d]Below detection limits.

subsequent experiments on the factors that affect microbially mediated Cr(VI) reduction.

Chromium Reduction in the Presence of Other Electron Acceptors. Alternate electron acceptors were added to Cr(VI)-amended microcosms to determine their effect on Cr(VI) reduction. Oxygen was a strong inhibitor of Cr(VI) reduction. Microorganisms in aerobic microcosms which were incubated with shaking reduced only 12% of the initial Cr(VI) added (Table 2). In microcosms incubated in a stationary position, only 41% of the initial Cr(VI) added was reduced. In contrast, stationary and shaken microcosms prepared anaerobically reduced 75% and 84% of the Cr(VI) added, respectively, indicating that Cr(VI) reduction in these sediments was inhibited by oxygen.

The addition of either 5 mM sulfate, ferrous iron, or selenate, had no effect on Cr(VI) reduction, when compared to microcosms which had been amended with Cr(VI) as the only electron acceptor (Figure 2A). However, the addition of 5 mM nitrate affected the rate and extent of Cr(VI) reduction, resulting in reduction of only 69% of the Cr(VI) added, compared to 83% Cr(VI) reduction in microcosms without nitrate. The fate of the alternate electron acceptor was determined by analyzing the concentrations of either sulfate, ferrous iron, or nitrate. During a 65 day incubation period, sulfate concentrations did not change (Figure 2B) and sulfide was not detected. Similarly, no Fe(II) was ever detected, indicating an absence of Fe(III) reduction. In contrast, nitrate was completely reduced within the first 9 days of incubation (Figure 2B). Interestingly, when both nitrate and Cr(VI) were present, the reduction of both electron acceptors occurred simultaneously with similar rates but to a different extent. Selenate reduction was assumed when the microcosms turned red with elemental selenium precipitation by day 44 of incubation. No reduction of any

Table 2. Effect of Oxygen on Cr(VI) Reduction by Norman Aquifer Sandy Sediments

Treatment	[Initial Cr(VI)] (μM)	[Final Cr(VI)][a] (μM)	%Cr(VI) Reduced
Anaerobic Incubations			
Stationary	408 ± 14[b]	101 ± 73	75
Shaken	362 ± 39	58.3 ± 82	84
Aerobic Incubations			
Stationary	426 ± 4.7	251 ± 61	41
Shaken	408 ± 12	359 ± 20	12
Heat Killed Controls			
Anaerobic	325 ± 8.4	270 ± 2.1	17
Aerobic	492 ± 5.7	394 ± 1.5	20

[a]Concentration of Cr(VI) was measured after 51 days of incubation.

[b]Values stated are mean ± standard deviation of triplicate determinations.

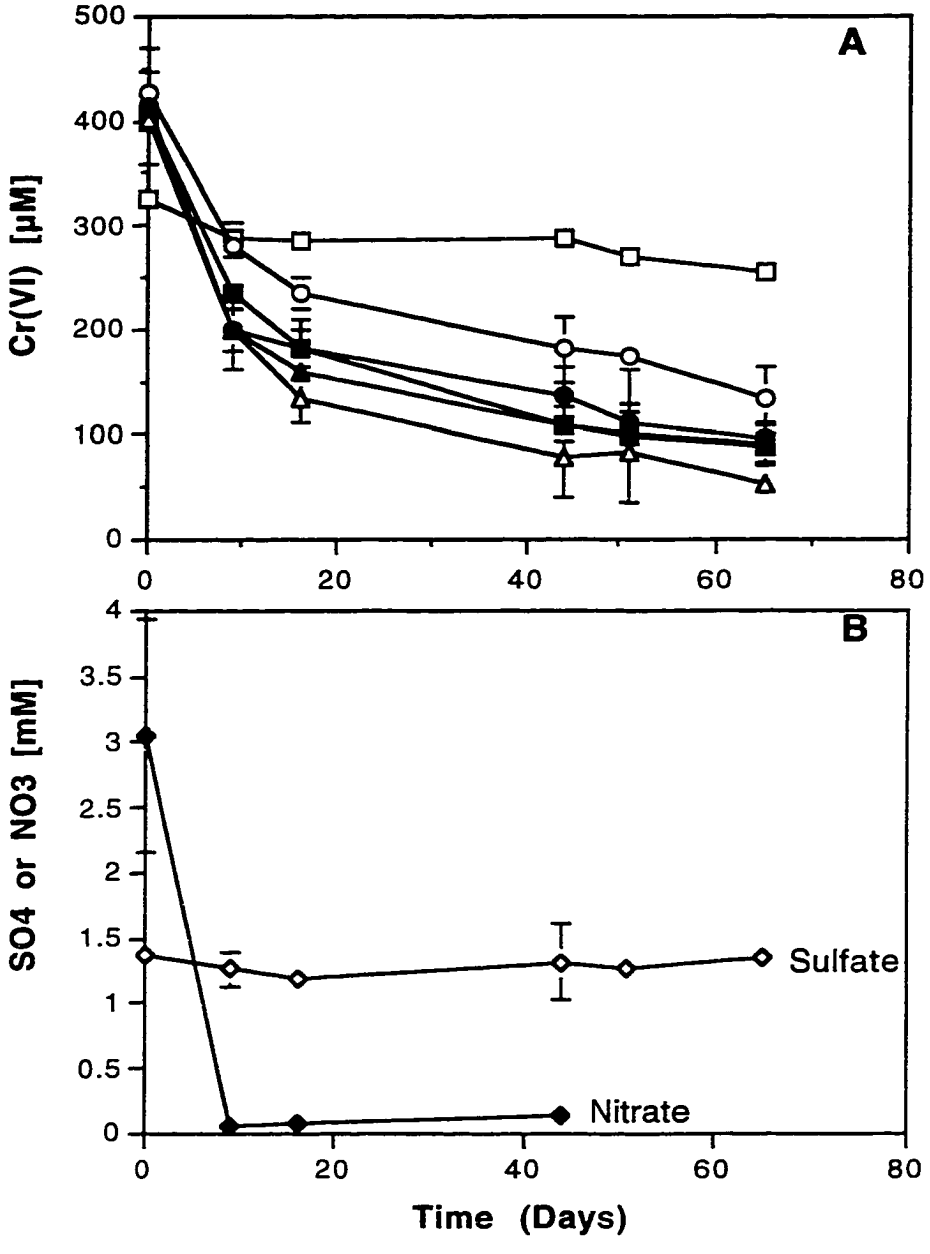

Figure 2A. Effect of alternate electron acceptors on Cr(VI) reduction by sandy aquifer sediments. Heat-killed controls were included for each condition. The data shown are typical for all heat-killed controls, regardless of the treatment.. (❑, Heat killed control; ■, Cr(VI) alone; ○, Cr(VI) with NO_3^- present; ●, Cr(VI) with Fe(III) present; ▲, Cr(VI) with SO_4^{2-} present; Δ, Cr(VI) with SeO_4^{2-} present)
Figure 2B. Fate of nitrate and sulfate in microcosms containing Cr(VI).

electron acceptors was observed in heat killed controls. Methane production was observed in slurries with final Cr(VI) concentrations less than 50 μM.

To determine when Cr(VI) reduction occurred in relation to other terminal electron accepting processes, slurries were prepared with combinations of chromate, sulfate, Fe(III), and nitrate. Very little loss of any electron acceptor occurred in these slurries and the experiment was repeated (data not shown). Fresh sediment was used in the repeat experiment but the rate of electron acceptor reduction was still very slow (Figure 3A), possibly suggesting that these combinations of electron acceptors can act as non-specific inhibitors. Some Cr(VI) was reduced in all slurries but the greatest extent of Cr(VI) reduction was observed in the absence of nitrate. Approximately half of the 1 mM nitrate added was reduced in the first 352 days of incubation when Cr(VI), sulfate, and Fe(III) were also present (Figure 4A). No sulfate reduction or production of Fe(II) was observed in Cr(VI)-amended microcosms. In contrast, when microcosms were not amended with Cr(VI), nitrate was completely reduced after 43 days, Fe(II) production began after approximately 30 days, and sulfate was slowly reduced between 50 and 352 days (Figure 4B).

In order to determine whether the microbial reduction of these electron acceptors could be stimulated microcosms were amended with a H_2-utilizing, Cr(VI)-reducing enrichment after 352 days of incubation and supplied with an 80% H_2 headspace to serve as the donor. Again, these microcosms showed reduction of Cr(VI) only in the absence of nitrate, which was previously shown to be inhibitory to chromate reduction (Figure 3B). After addition of the enrichment

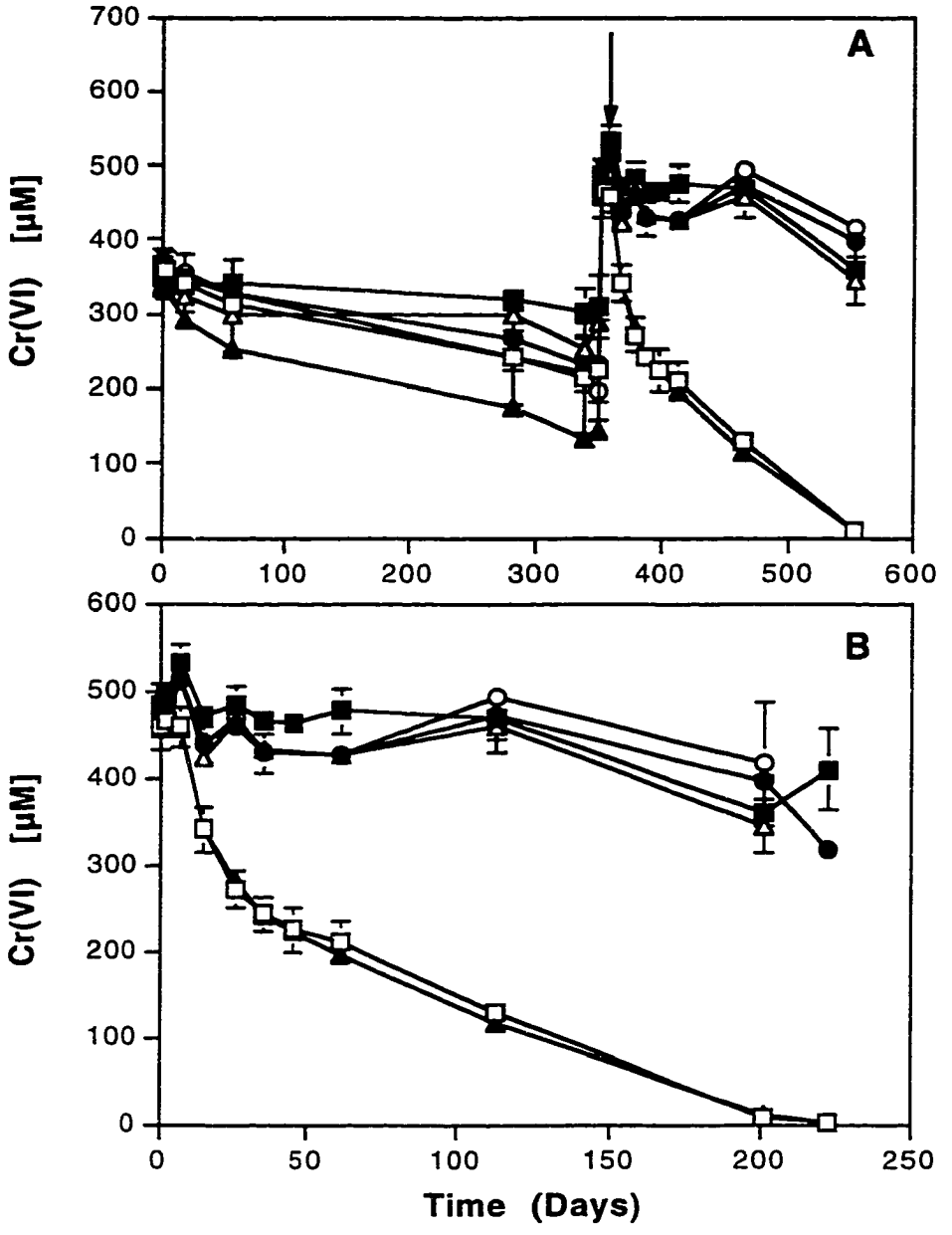

Figure 3A. Cr(VI) reduction in sandy aquifer sediment microcosms when combined with alternate electron acceptors. (Arrow indicates re-amendment with Cr(VI) *and* addition of Cr(VI)-reducing, H_2-utilizing enrichment. Heat killed controls were used for each condition. The line shown represents a typical set of heat killed controls.) Figure 3B. Enlargement of 3A after addition of Cr(VI)-reducing, H_2-utilizing enrichment. (■, Heat killed control; ❑, Cr(VI) alone; ●, Cr(VI), SO_4^{2-}, Fe(III), and NO_3^-; ○, Cr(VI), Fe(III), and NO_3^-; ▲, Cr(VI), SO_4^{2-}, and Fe(III); Δ, Cr(VI), SO_4^{2-}, and NO_3^-)

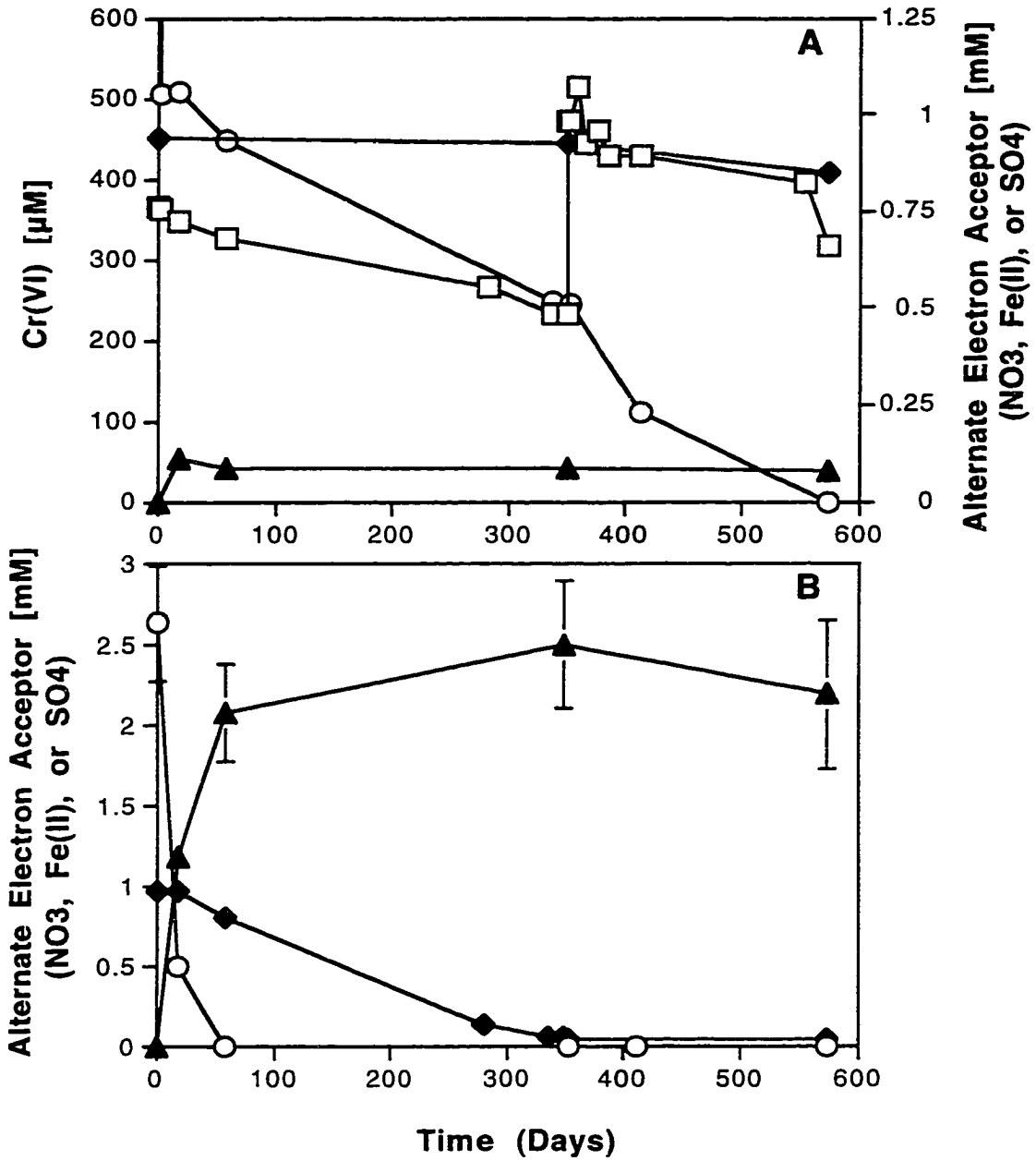

Figure 4A. Utilization of Cr(VI), nitrate, Fe(III), and sulfate when incubated in combination. Cr(VI) was reamended after 350 days of incubation. Figure 4B. Utilization of nitrate, Fe(III), and sulfate in the absence of Cr(VI). (❑, Cr(VI); ○, NO_3^-; ▲, Fe(II); ◆, SO_4^{2-})

all remaining nitrate was reduced in the next 222 days (Figure 4A). However, no Fe(III)- or sulfate-reduction was observed in the presence of Cr(VI).

The fate of the different electron acceptors is shown in Figure 5. Little reduction of any electron acceptors occurred in heat-killed controls. Maximum reduction of nitrate, Fe(III) and sulfate occurred in the absence of Cr(VI), indicating that the presence of Cr(VI) inhibits utilization of these electron acceptors. The maximum reduction of Cr(VI) occurred in microcosms amended with Fe(III) and sulfate, but not with the more thermodynamically favorable electron acceptor nitrate. This follows thermodynamic predictions whereby reduction of Cr(VI) would follow nitrate reduction, but precede reduction of Fe(III) and sulfate.

Chemical Inhibitors. Since several sulfate-reducing bacteria have been shown to reduce Cr(VI), either directly via an enzymatic mechanism (Tebo and Obraztsova, 1998; Lovley and Phillips, 1994), or indirectly due to the production of sulfide (Fude et al., 1994; Smillie et al., 1981), experiments were conducted to determine the effect of molybdate, an inhibitor of sulfate-reducing bacteria, on Cr(VI) reduction. Noticeable inhibition of Cr(VI) reduction occurred only in microcosms containing very high molybdate concentrations (20 mM) (Table 3). At 0.2 and 2.0 mM molybdate, Cr(VI) reduction occurred to the same extent as without molybdate (Table 3). However, a high concentration of molybdate (20 mM) also completely inhibited methanogenesis, suggesting that molybdate acted as a non-specific inhibitor of several microbial processes. Conversely, low levels of molybdate (0.2 or 2.0 mM) seemed to stimulate

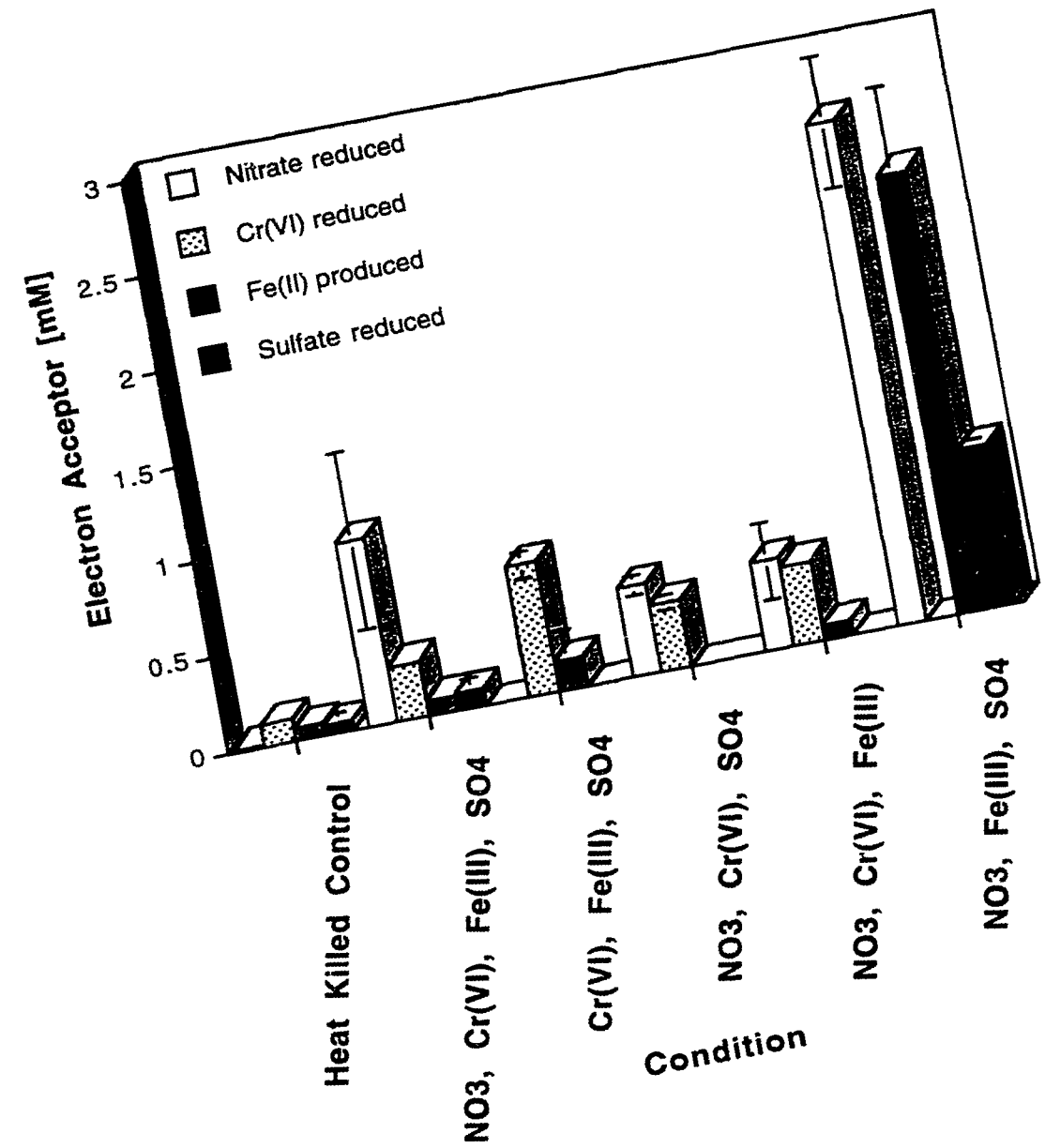

Figure 5. Fate of alternate electron acceptors when incubated in combination with or without Cr(VI). Values shown represent the change in electron acceptor concentration (see key) after 574 days of incubation.

Table 3. Effect of Inhibitor Concentration on Cr(VI) Reduction by Sandy Norman Aquifer Sediments.

Treatment	[Initial Cr(VI)] (μM)	[Final Cr(VI)][a] (μM)	%Cr(VI) Reduced
Molybdate			
0 mM	425.3 ± 11.3	23.48 ± 33.2	95
0.2 mM	427.7 ± 8.7	28.87 ± 40.8	93
2.0 mM	416.7 ± 12.9	44.15 ± 36.3	94
20.0 mM	364.4 ± 4.7	174.7 ± 38.9	52
BESA			
0 mM	425.3 ± 11.3[b]	23.48 ± 33.2	95
0.2 mM	402.7 ± 5.15	45.88 ± 64.9	89
2.0 mM	442.0 ± 5.94	80.30 ± 58.1	82
20.0 mM	444.9 ± 9.64	140.2 ± 34.0	68
Heat Killed Control	400.8 ± 12.2	402.4 ± 20.5	0

[a]Concentration of Cr(VI) after 47 day incubation period

[b]Values stated are Mean ± Std. Dev. of triplicate values

methanogenesis since microcosms amended with only 0.2 or 2.0 mM molybdate produced 2.98 µmol and 17.10 µmol of methane, respectively. No methane was detected in the heat-killed controls. The occurrence of Cr(VI) reduction at lower molybdate concentrations suggests that sulfate-reducing bacteria may not be important members of the microbial community in regard to Cr(VI) reduction.

The addition of bromoethanesulfonic acid (BESA), an inhibitor of methanogenic bacteria, also inhibited Cr(VI) reduction (Table 3). Inhibition of Cr(VI) reduction was observed with as little as 0.2 mM BESA, a concentration lower than that of the Cr(VI). Approximately 1 µmol of methane was produced in microcosms without BESA, while microcosms with 0.2 or 2.0 mM BESA produced only trace amounts of methane. In microcosms amended with 20.0 mM BESA no methane was detected. The inhibition of Cr(VI) reduction by BESA suggests that methanogens may be involved either directly or indirectly in Cr(VI) reduction. The addition of BESA disrupts the electron flow to methanogenesis and it is possible that several biological processes can be affected in the pathway, including electron flow to Cr(VI) reduction.

Sediment-free Enrichments. To confirm the biological potential for Cr(VI) reduction several enrichments were obtained from the sediment-containing microcosms. These enrichments allowed further testing of the role of sulfate-reducing and methanogenic bacteria in Cr(VI) reduction and the determination of whether biological Cr(VI)-reducing conditions could be sustained. Serially transferring sandy sediments into methanogenic mineral medium led to the development of the Cr(VI)-reducing activity away from the sediment. The enrichment obtained from these transfers was able to reduce 220 µM Cr(VI) within 28 days of incubation when H_2 was supplied as an electron donor. Methane was not detected when cultures were incubated with

Cr(VI). Incubation of the enrichment in the above medium with a cysteine-sulfide reducing solution (Balch and Wolfe, 1976) and no Cr(VI) led to production of methane (~114 µmol). The existence of a methanogenic, Cr(VI)-reducing enrichment as well as the inhibitory effects of BESA on Cr(VI) reduction indicate that most available electrons go to methane in the absence of Cr(VI) in these aquifer sediments.

In addition to the methanogenic enrichment, a sulfate-reducing enrichment was obtained that could reduce either 5 mM sulfate or 500 µM Cr(VI) in about 10 days. Continually transferring this enrichment in Cr(VI) containing medium led to loss of sulfate-reducing activity as well as decreased Cr(VI) reducing activity.

Environmental Parameters. Cr(VI) reduction occurred at all tested temperatures, with the exception of 4°C. Little loss of Cr(VI) occurred in heat killed controls until 65°C, when some abiotic reduction (about 88 µM Cr(VI)) could be seen (Figure 6). From this data a first-order rate constant for Cr(VI) reduction was calculated for each temperature. It was determined that chromium reduction in these sediments appeared to have two optimal temperatures, 22°C and 50°C (Figure 7). As this was an uncommon result the work was repeated with identical results. The Cr(VI) reduction rate constant for 22°C was 0.11 $days^{-1}$ and 0.16 $days^{-1}$ for 50°C. The existence of two temperature optima in these sediments indicates that potentially two separate groups of microorganisms are present in the aquifer which are capable of Cr(VI) reduction. However, given the ambient temperature of the aquifer, it is likely that the group with the lower temperature optimum would be the major participant in these sediments.

Similarly, Cr(VI) reduction occurred at all the pH values tested with little or no loss of Cr(VI) in heat killed controls. Significant loss of Cr(VI) occurred at

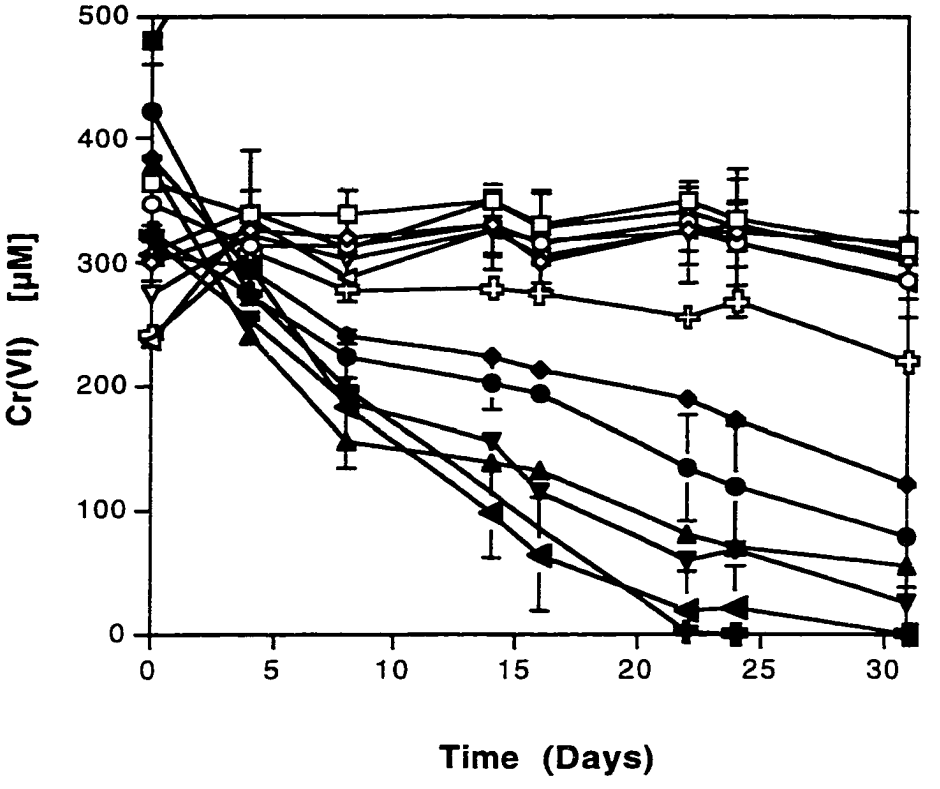

Figure 6. Effect of temperature on Cr(VI) reduction by sandy aquifer sediments. (Open symbols represent heat-killed controls and closed symbols represent viable microcosms. ■, 4°C; ◆, 22°C; ●, 30°C; ▲, 37°C; ▼, 45°C; ◀ 50°C; ✚, 65°C).

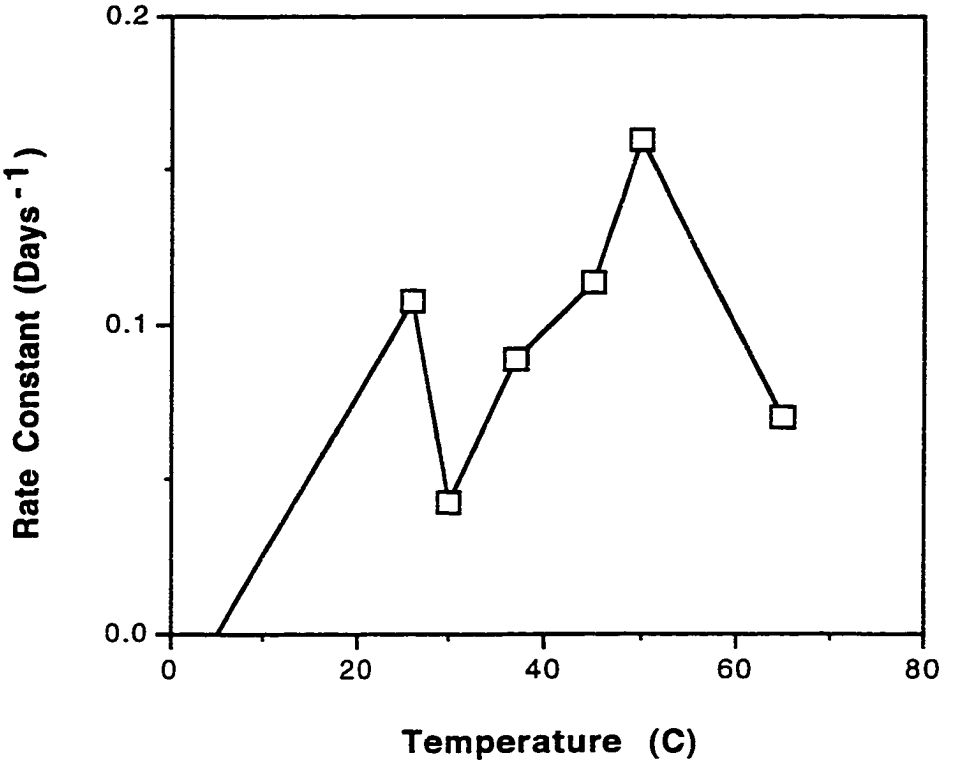

Figure 7. Cr(VI) reduction rate constant vs. temperature. (Values have been corrected for abiotic loss of Cr(VI).)

pHs ranging from 5.7 to 9.3. Again, first-order rate constants were calculated and revealed that the pH optimum for Cr(VI) reduction in these sediments was 6.8 (Figure 8).

Effect of Groundwater. In order to determine how closely the microcosms simulated the aquifer system, microcosms were constructed which contained groundwater instead of the mineral medium. Cr(VI) reduction in these microcosms was compared to Cr(VI) reduction in microcosms containing basal medium. Cr(VI) reduction proceeded very rapidly in groundwater microcosms (Figure 9). In fact, after 10 days Cr(VI) was completely reduced in groundwater microcosms while only half of the Cr(VI) had been reduced with medium. Microcosms containing groundwater were re-spiked twice more with Cr(VI) and rapidly reduced the Cr(VI) to less than detection levels while approximately 100 μM still remained in microcosms containing medium.

It was unknown what component(s) of the groundwater were stimulating Cr(VI) reduction in these sediments. It was possible that the groundwater was supplying electron donors, nutrients, or even microorganisms. Therefore, microcosms were constructed with sediment and either untreated groundwater or groundwater which had been filter-sterilized. In a 65 day incubation period, total reduction of 440 μM Cr(VI) occurred in microcosms containing untreated groundwater (Figure 10), while microcosms containing filter-sterilized groundwater reduced only 244 μM Cr(VI). The addition of groundwater stimulated the rate and extent of Cr(VI) reduction in these sediments, most likely because higher cell numbers were necessary for complete Cr(VI) reduction. It is probable that the increase in Cr(VI) reduction by groundwater is due to live particles rather than any inert particles present in the groundwater since no Cr(VI) reduction occurred in heat-killed controls of groundwater microcosms (Figure 10).

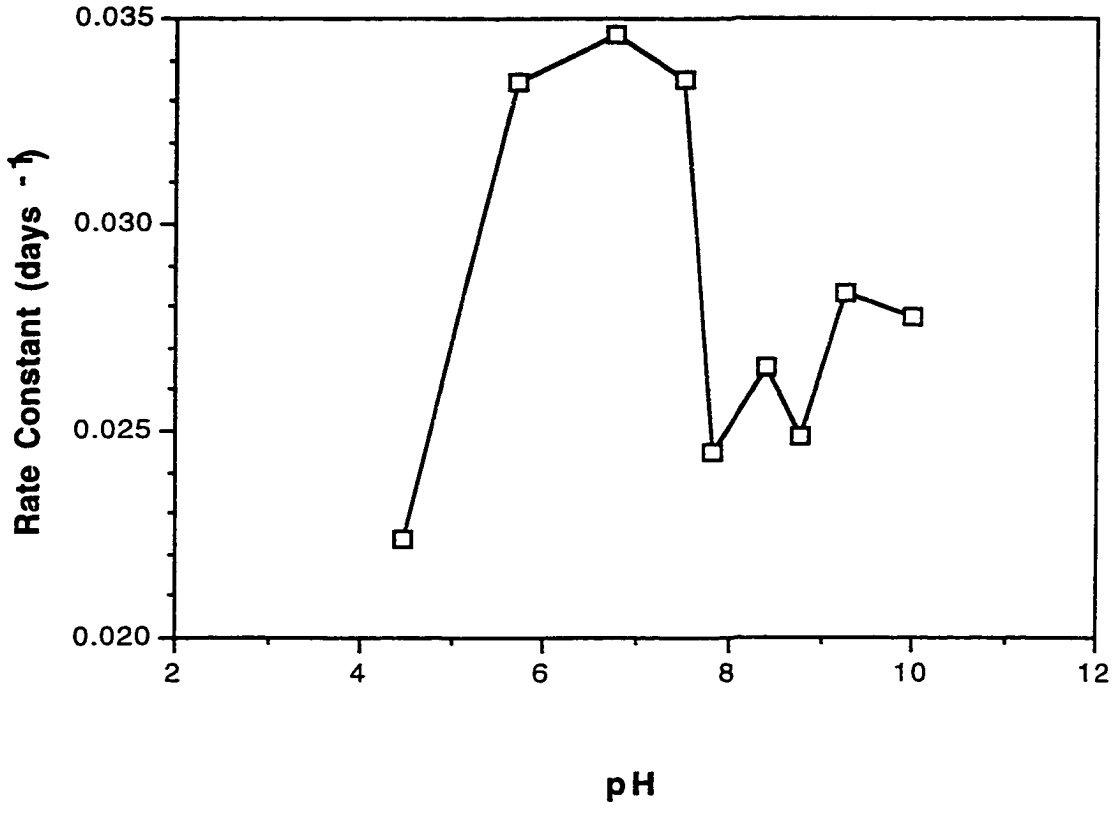

Figure 8. Cr(VI) reduction rate constant vs. pH. (Values have been corrected for abiotic loss of Cr(VI).)

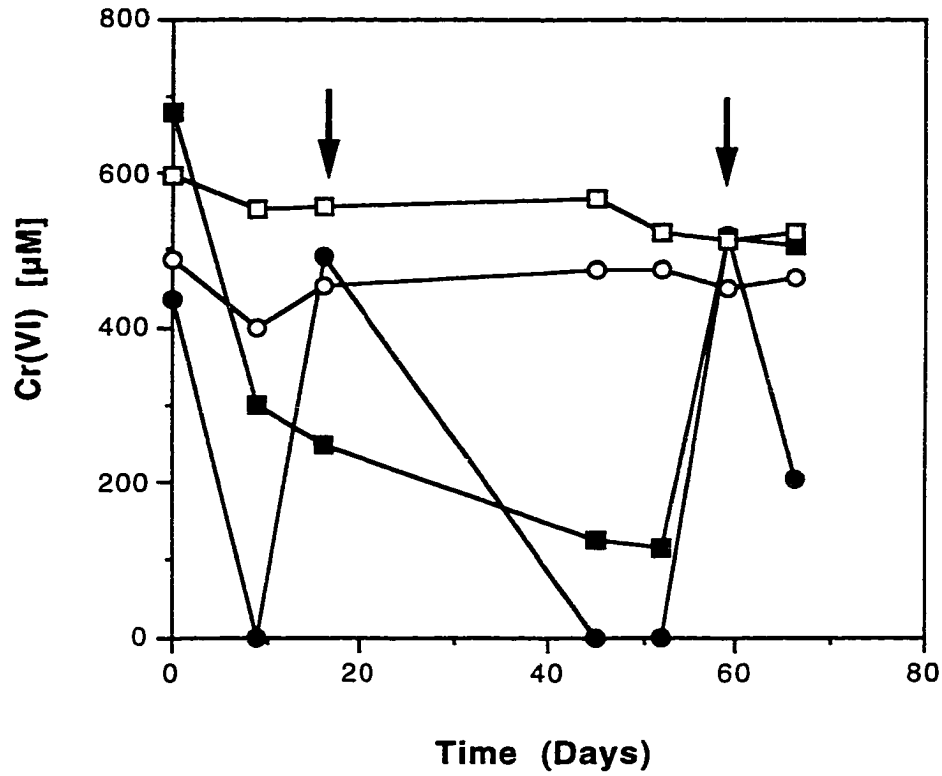

Figure 9. Effect of groundwater on Cr(VI) reduction by sandy aquifer sediments. (Arrows indicate re-amendment with Cr(VI). ❑, Basal medium- Heat killed control; ■, Basal medium; ○, Groundwater- Heat killed control; ●, Groundwater)

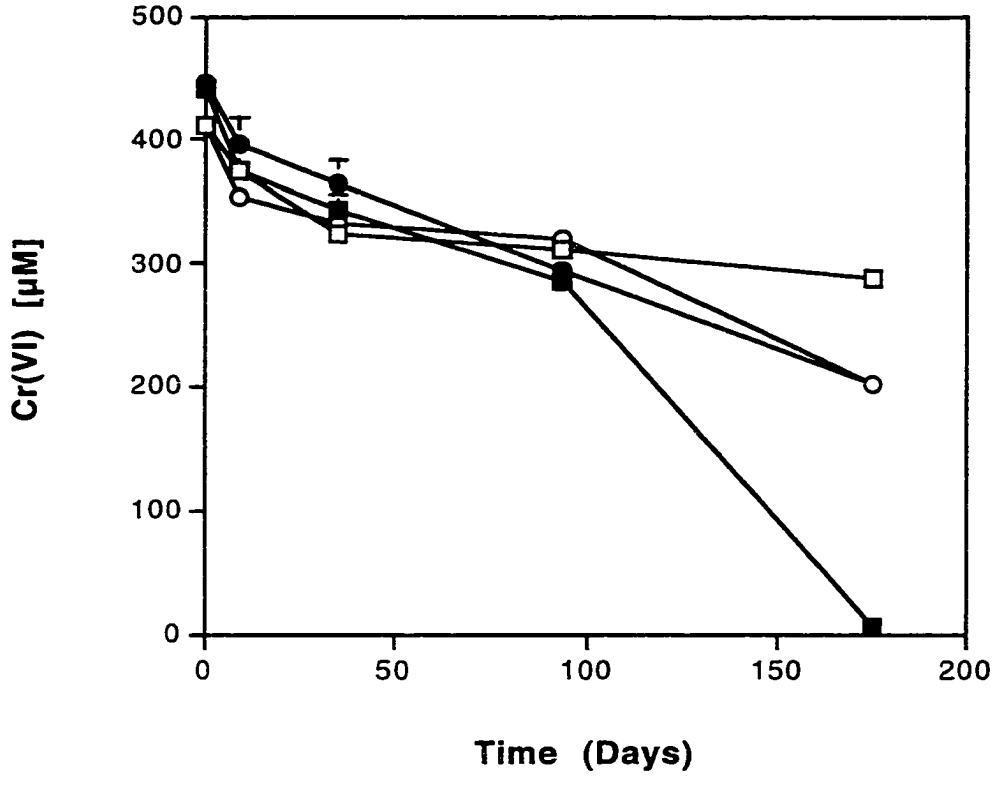

Figure 10. Comparison of Cr(VI) reduction by microcosms with viable groundwater or filter-sterilized groundwater. (❑, Groundwater- Heat killed control; ■, Groundwater; ○, Filter sterilized groundwater- Heat killed control; ●, Filter sterilized groundwater)

Discussion

Cr(VI) is a strong oxidant and is reduced abiotically in the presence of electron donors commonly found in soils such as aqueous Fe(II), ferrous iron minerals, reduced sulfur, and soil organic matter (Palmer and Puls, 1994; Eary and Rai, 1988). In addition to abiotic reduction, many microorganisms are able to mediate reduction of Cr(VI) to the trivalent form (Wang and Shen, 1995). These studies show that the sandy aquifer sediments from the Norman landfill have the potential for Cr(VI) reduction, with a pH optimum of 6.8 and temperature optima of 22°C and 50°C. The existence of pH and temperature optima confirms that Cr(VI) reduction in these sediments is a biotic process. By altering physiochemical factors such as pH, temperature, and inhibitor cocentration it was possible to alter the rate and extent of Cr(VI) reduction, with little loss of Cr(VI) in heat killed controls, clearly showing that Cr(VI) reduction is a biological process in these sediments. Thus, the stimulation of naturally occurring microorganisms may be an effective method for treating chromium contamination in aquifers. On the other hand, in certain sediments, such as clay-like sediments, stimulation of microbial populations is probably not needed for Cr(VI) reduction. We observed a rapid chemical reduction of Cr(VI) in clay-like sediments. The black appearance of these sediments suggests the presence of ferrous iron minerals and/or reduced sulfur compounds (collectively forming iron sulfides), both of which are known to chemically reduce Cr(VI) (Eary and Rai, 1988; Wiberg, 1965).

When electron acceptors were supplied in combination with Cr(VI), nitrate utilization occurred first. Chromate reduction then proceeded, presumably followed at a later time by Fe(III) and sulfate-reduction, although these two reactions were never observed in the presence of Cr(VI). In the absence of Cr(VI), reduction of Fe(III) and sulfate occurred once nitrate was

depleted. These results could be predicted based on the redox potentials reported for these reactions (Table 4) (Ahmann, 1997). The use of oxygen as a terminal electron acceptor would be the most thermodynamically favorable reaction while nitrate is slightly less favorable, based on the change in free energy per electron at low hydrogen concentrations. Reduction of nitrate would be followed by chromate reduction, and at some point following reduction of Cr(VI), Fe(III) and sulfate reduction would occur.

The inhibition of biological Cr(VI) reduction by oxygen and nitrate indicates that this process in sandy sediments is predominantly an anaerobic one, requiring redox conditions lower than that reported for oxygen. This is further supported by the fact that sulfate-reducing and methanogenic enrichments, which by their very nature require highly reduced conditions, also reduced Cr(VI). Although some Cr(VI) reduction occurred in aerobic microcosms, this was most notable in stationary microcosms where the development of anaerobic zones would be favored. Our results suggest that the depletion of oxygen in sandy aquifers may be required for effective Cr(VI) removal.

Sulfate reduction, giving rise to sulfide production, can provide an indirect mechanism for Cr(VI) reduction, whereby sulfide reacts with Cr(VI) to cause immediate abiotic reduction. By a similar mechanism, Fe(II) can also indirectly reduce Cr(VI) to Cr(III). However, Cr(VI) reduction was not stimulated if either sulfate or Fe(III) were added and Cr(VI) was reduced by enrichments in medium which contained very little sulfate. Furthermore, the lack of sulfide production or sulfate reduction, and the lack of inhibition of Cr(VI) reduction by equimolar concentrations of molybdate argue against an indirect mechanism for Cr(VI) reduction. Instead, Cr(VI) reduction may be mediated by a microbial community which, in the absence of Cr(VI), uses methane production as the

Table 4. Gibbs' free energies for terminal electron accepting processes coupled to H_2 oxidation.

Reaction	$\Delta G'$ (kJ/mol e^-)[a, b]
$1/2\ MnO_2\ (s) + H^+ + 1/2\ H_2 \rightarrow 1/2\ Mn^{2+} + H_2O$	-94.06
$1/5\ NO_3^- + 1/5\ H^+ + 1/2\ H_2 \rightarrow 1/10\ N_2\ (g) + 3/5\ H_2O$	-86.44
$1/3\ CrO_4^{2-} + 5/3\ H^+ + 1/2\ H_2 \rightarrow 1/3\ Cr^{3+} + 4/3\ H_2O$	-45.02
$Fe(OH)_{3(am)} + 2\ H^+ + 1/2\ H_2 \rightarrow Fe^{2+} + 3\ H_2O$	-43.89
$1/8\ SO_4^{2-} + 1/8\ H^+ + 1/2\ H_2 \rightarrow 1/8\ HS^- + 1/2\ H_2O$	- 0.42
$1/8\ HCO_3^- + 1/8\ H^+ + 1/2\ H_2 \rightarrow 1/8\ CH_4 + 3/8\ H_2O$	+ 0.67

[a] All values are taken from Ahmann (1997) except for methane production which was calculated from data in Thauer et al., 1977.

[b] $P_{H2} = 10^{-6.6}$ atm; $P_{N2} = 0.78$ atm; $P_{O2} = 0.21$ atm; $P_{CH4} = 2.45 \times 10^{-4}$ atm; pH = 7.0;

$[Mn^{2+}] = [NO_3^-] = [CrO_4^{2-}] = [Cr^{3+}] = [Fe^{2+}] = [SO_4^{2-}] = [HS^-] = [HCO_3^-] = 10^{-6}$ M

terminal electron-accepting process. This is supported by the fact that Cr(VI) reduction in sandy sediments is inhibited by BESA and a methanogenic enrichment has been obtained that can also reduce Cr(VI).

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CHAPTER III

HYDROGEN BIOAVAILABILITY AS A MEASURE OF POTENTIAL MICROBIAL CHROMATE REDUCTION

Abstract

Biological Cr(VI) reduction was studied in anaerobic sediments from an aquifer in Norman, OK. Microcosms containing sediment and mineral medium were amended with various electron donors to determine those most important for biological Cr(VI) reduction. More than 800 μM Cr(VI) was reduced when formate, lactate, hydrogen, and glucose, but not benzoate or acetate, were added as electron donors. From these sediments, an anaerobic Cr(VI)-utilizing enrichment was obtained that was dependent upon hydrogen for both growth and Cr(VI) reduction. Growth and Cr(VI) reduction by the enrichment were completely inhibited by the addition of formaldehyde, partially inhibited by molybdate, and completely unaffected by bromoethane sulfonic acid (BESA). No methane was produced by the enrichment, which reduced about 750 μM Cr(VI) in less than six days. Dissolved hydrogen concentration was used as an indicator of the terminal electron accepting process occurring in the sediments. Sediments with chromate metabolized hydrogen to a concentration below the

detection limits of the mercury vapor gas chromatograph. Similar findings have been reported for nitrate- and manganese-reducing conditions. In sediments without chromate, the hydrogen concentration was about 8 nM, a concentration comparable to that for methanogenic conditions. When the methanogenic microcosms were amended with 500 μM Cr(VI), the dissolved hydrogen concentration quickly fell below the detection levels. These results showed that hydrogen concentration under chromate-reducing conditions became very low, as low as reported for nitrate- and manganese-reducing conditions, a logical result considering that the free energy changes for these reactions are similar. The utilization of formate, lactate, hydrogen, and glucose as electron donors for Cr(VI) reduction indicates that increasing the availability of hydrogen results in a greater capacity for Cr(VI) reduction. This conclusion is supported by the existence of an enrichment dependent upon hydrogen for growth and Cr(VI) reduction.

Introduction

Chromium-containing compounds have many industrial applications including electroplating, leather tanning, pigment manufacture, corrosion inhibition, and fungicide production. These industries generate large quantities of waste which must be treated before discharge. The amount of chromium wastes released annually is estimated to be 170,000 tons (Gadd and White, 1993). The widespread use of chromium as well as the improper disposal of by-products and wastes has led to areas of serious environmental

contamination. Currently in the United States, 635 Superfund sites exist which list chromium as one of the contaminants (U.S. EPA 1999).

Chromium can exist in six valence states from 0 to +6, but is generally encountered as the trivalent [Cr(III)] or hexavalent [Cr(VI)] form (Palmer and Puls, 1994). Trivalent chromium, an essential trace element in the human diet, has relatively low toxicity and is nearly insoluble at neutral pH, and thus, nearly immobile in the environment (Anderson and Kozlovsky, 1985). Conversely, hexavalent chromium is acutely toxic, mutagenic, teratogenic, and carcinogenic. In addition, Cr(VI) is highly mobile in the environment, mainly due to its soluble nature (Bartlett, 1991; Cervantes, 1991). Focusing on its toxicity and exposure potential, the U.S. Environmental Protection Agency recently designated chromium, and its compounds, as one of seventeen chemicals posing the greatest threat to human health (Boutacoff, 1991).

The valence state and relative solubility of chromium is dependent on a variety of environmental conditions (redox potential, pH, and temperature) and the presence of other organic and inorganic molecules (Moore and Ramamoorthy, 1983). Oxidation-reduction (redox) reactions can greatly influence the fate and mobility of these organic and inorganic compounds in both pristine and contaminated aquifers. Most of the significant redox reactions taking place in aquifers, such as nitrate reduction, Fe(III) reduction, sulfate reduction, and methane production, are microbially catalyzed. Lovley and Goodwin (1988) have proposed that H_2 concentrations in groundwater may indicate which terminal electron accepting process (TEAP) is dominant in a

given system, with each TEAP having a specific range for its hydrogen concentration.

Many bacterial strains have been shown to mediate reduction of Cr(VI) to Cr(III), however, few studies have examined the in situ potential of microbial reduction in aquifers (Bopp and Ehrlich, 1988; Gvozdyak et al., 1987; Horitsu et al., 1987; Kvasnikov et al., 1985; Lebedeva and Lyalikova, 1979; Lovley, 1993; Romanenko and Koren'Kov, 1977; Wang et al., 1989). The stimulation of existing microbial populations with bioavailable electron donors may result in microbial chromium reduction, potentially preventing the migration, and reducing the toxicity, of Cr(VI) in aquifers. However, it is not clear whether highly reducing conditions (e.g. sulfate-reducing, iron-reducing, or methanogenic) are needed, or whether microbial Cr(VI) reduction will occur under more oxidized conditions (aerobic or nitrate-reducing). Examination of electron donors as stimulants for microbially mediated Cr(VI) reduction in aquifers, as well as the H_2 concentrations during this process were assessed in this study.

Materials and Methods

Sample collection and microcosm construction. Subsurface sediments from an aquifer underlying the municipal landfill in Norman, Oklahoma were collected by digging to the top of the water table (5-6 feet below the surface) with a post-hole digger and collecting aquifer material in sterile glass jars. The jars were filled to capacity, sealed, and transported to the

laboratory where they were stored in an anaerobic chamber at room temperature until use.

Microcosms were prepared in an anaerobic chamber by placing 5 g (wet weight) of sediment into sterile serum bottles (160 ml capacity) and adding 50 ml of sterile, anaerobic mineral medium. The composition of the mineral medium has been previously described by Tanner (1989) and was prepared according to the procedure described by Balch and Wolfe (1976). No reductant was added to the microcosms. All serum bottles were sealed with butyl rubber stoppers, the gas head space was exchanged with 80% N_2; 20% CO_2 (125 kPa), and the final pH of the microcosms was 7.2. Each microcosm typically had an initial Cr(VI) concentration of approximately 500 µM, added from a sterile stock of K_2CrO_4, which was replenished after complete reduction. Triplicate microcosms were used for each treatment and heat-killed controls were included for each experiment. The heat-killed controls were autoclaved twice at 121°C for 20 minutes and then amended with 20 mg/L $HgCl_2$. All incubations were carried out at room temperature in the dark. Samples were collected regularly from each microcosm.

Effect of Electron Donor. To determine the effect of electron donor, microcosms were incubated without donor until the rate of Cr(VI) reduction slowed, to exhaust any endogenous electron donor present in the sediment. After 161 days the microcosms were amended with donor and re-amended with Cr(VI) before an additional 360 day incubation period began. During this

second incubation period microcosms were re-amended with Cr(VI) on one occasion, 217 days after donor additions.

Determination of H_2 concentration under Cr(VI)-reducing conditions. Microcosms were constructed as above with 5 g (wet weight) sediment, 50 ml of groundwater and 500 µM Cr(VI). Hydrogen was added with a Hamilton syringe to give an initial soluble concentration of approximately 300 nM (~0.8%). Microcosms were stored in a stationary position at room temperature in the dark.

Analytical Methods. Cr(VI) concentration was determined colorimetrically by reaction with diphenylcarbazide in acid solution, having a detection limit of approximately 5 µM Cr(VI) (Clesceri, 1989; Urone, 1955). The coefficient of variation was 4.7%. Hydrogen was quantitated with a mercury vapor reduction gas analyzer (Seiler et al., 1980). Fatty and aromatic acids were analyzed using an HPLC equipped with a BioRad 300- × 7.8-mm Aminex HPX-87H column and an isocratic mobile phase of 0.016 N H_2SO_4 at a flow rate of 0.9 ml/min (Amos and McInerney, 1990). Benzoate was analyzed by an HPLC equipped with an Alltech Econosphere C18 column (250- × 4.6-mm, reverse-phase) and a UV detector set at 254 nm. The HPLC was operated at a flow rate of 1.0 ml/min using a mobile phase of 80% (v/v) sodium acetate (50 mM, pH 4.5) and 20% (v/v) acetonitrile. Protein concentrations were determined using bicinchoninic acid (Smith et al., 1985). At the conclusion of each experiment, methane production was measured by gas chromatography (Beeman and Suflita, 1987).

Enrichment for Cr(VI)-reducing, H_2-utilizing consortium. A Cr(VI)-reducing, H_2-utilizing enrichment was obtained from aquifer sediment and serially transferred in a mineral salts medium with a with 80% H_2; 20% CO_2 (125 kPa) head space and an initial Cr(VI) concentration of approximately 750 μM. The mineral medium contained the following components (per liter of deionized water): Tanner's metal solution (1989), 5 ml; Tanner's vitamin solution (1989), 10 ml; Pfennig's mineral solution (McInerney et al., 1978), 10 ml; yeast extract, 0.1 g; $NaHCO_3$, 10 g. The enrichment was maintained by transferring a 1-5% inoculum to fresh media bi-monthly.

Results

Effect of Electron Donor. More Cr(VI) reduction was observed with aquifer sediments when formate, lactate, hydrogen, glucose, and benzoate, were supplied as electron donors (Table 1). Microcosms amended with acetate reduced less Cr(VI) than microcosms with no exogenous donor. Little loss of Cr(VI) occurred in heat killed controls, indicating that reduction of Cr(VI) in viable microcosms was microbiologically mediated.

Measurement of the donors revealed that formate (2.1 mM) was completely used and the 1.6 mM lactate added was converted to about 1.2 mM acetate. Hydrogen, initially 80% of the gas headspace, was below detection limits by mercury vapor gas chromatography at the conclusion of the experiment. Glucose (approximately 58 mM) was converted to about 80 mM acetate, 4 mM propionate, 9 mM isobutyrate, and 3 mM butyrate. Taking into

Table 1. Effect of Electron Donor on Chromate Reduction by Aquifer Material

	Initial [Cr(VI)] (μM)	Final [Cr(VI)][a] (μM)	Total Cr(VI) Added (μM)	Total Cr(VI) Reduced[b,c] (μM)	% of Electrons Available From Donor [d]
Heat Killed Control	509 ± 42[e]	404 ± 38	510 ± 42	106 ± 11	
No Donor	458 ± 9	115 ± 159	820 ± 128	344 ± 166	
Formate	699 ± 190	83 ± 134	1174± 179	1050± 73	17
Lactate	615 ± 143	5 ± 1	932 ± 322	899 ± 370	16
Hydrogen	561 ± 37	5 ± 1	1018± 259	895 ± 250	2.3
Glucose	568 ± 63	245 ± 48	1068± 165	823 ± 166	0.25
Benzoate	740 ± 246	230 ± 218	892 ± 180	510 ± 63	80
Acetate	549 ± 67	255 ± 31	549 ± 67	294 ± 48	0

[a]Concentration of Cr(VI) was measured after 360 day incubation period.

[b]Value shown is additive based on Cr(VI) amendments.

[c]Data has been corrected for Cr(VI) loss prior to donor addition.

[d]Value shown was corrected for Cr(VI) loss when no exogenous donor was supplied.

[e]Values shown are mean ± standard deviation.

account methane production (~ 1.8 mmol) and an assumed production of CO_2 (one CO_2 produced for every 2 carbon compound produced) resulted in a carbon recovery of about 91 percent. Approximately 62 μM of the 639 μM benzoate added was used and no depletion of acetate (2.1 mM) occurred during the 360 day incubation period. At the conclusion of the experiment methane was measured. Microcosms supplied with H_2 produced 0.84 mmol of methane, and glucose amended microcosms produced 1.8 mmol methane. Lactate and benzoate amended microcosms produced 0.04 mmol of methane, 0.05 mmol of methane was formed when formate was the donor. Little methane (0.001 mmol) was detected in acetate amended and unamended microcosms. Cr(VI) reduction accounted for about 80% of the electrons available from benzoate and about 16-17% of the electrons available from lactate and formate. Glucose and hydrogen amended microcosms were primarily fermentative and methanogenic, respectively, with these processes accounting for the majority of the electrons available.

Cr(VI)-reducing, H_2-utilizing consortium. From these sediments a Cr(VI)-reducing, H_2-utilizing enrichment was obtained, which was maintained sediment-free for over two years. This enrichment was able to reduce approximately 750 μM Cr(VI) in six days under growing conditions when hydrogen was supplied as the donor (Figure 1). Some growth, but no chromium reduction, occurred in the absence of hydrogen.

Growth and Cr(VI) reduction by the enrichment were completely inhibited in the presence of 10 mM formaldehyde (Figure 2). The enrichment

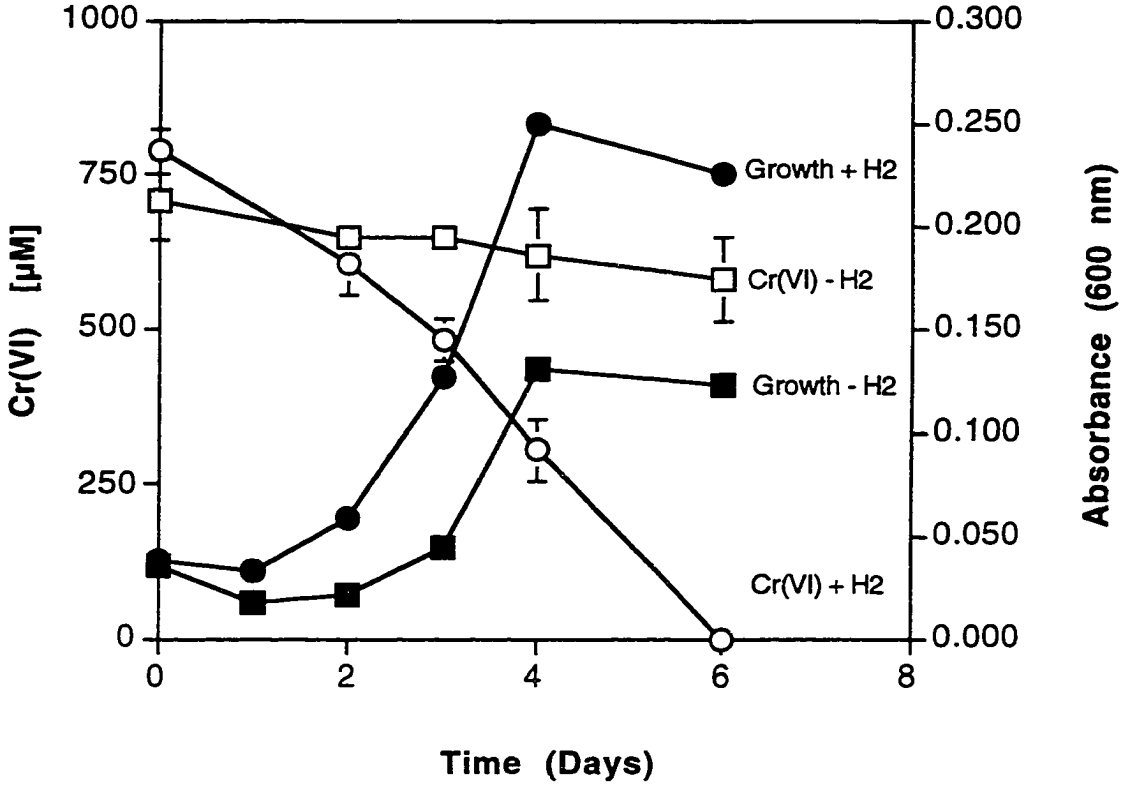

Figure 1. Growth and chromate reduction by H_2-utilizing, Cr(VI)-reducing enrichment. (Error bars represent the standard deviation of triplicate samples, if no error bars then standard deviation is less than width of the point.)

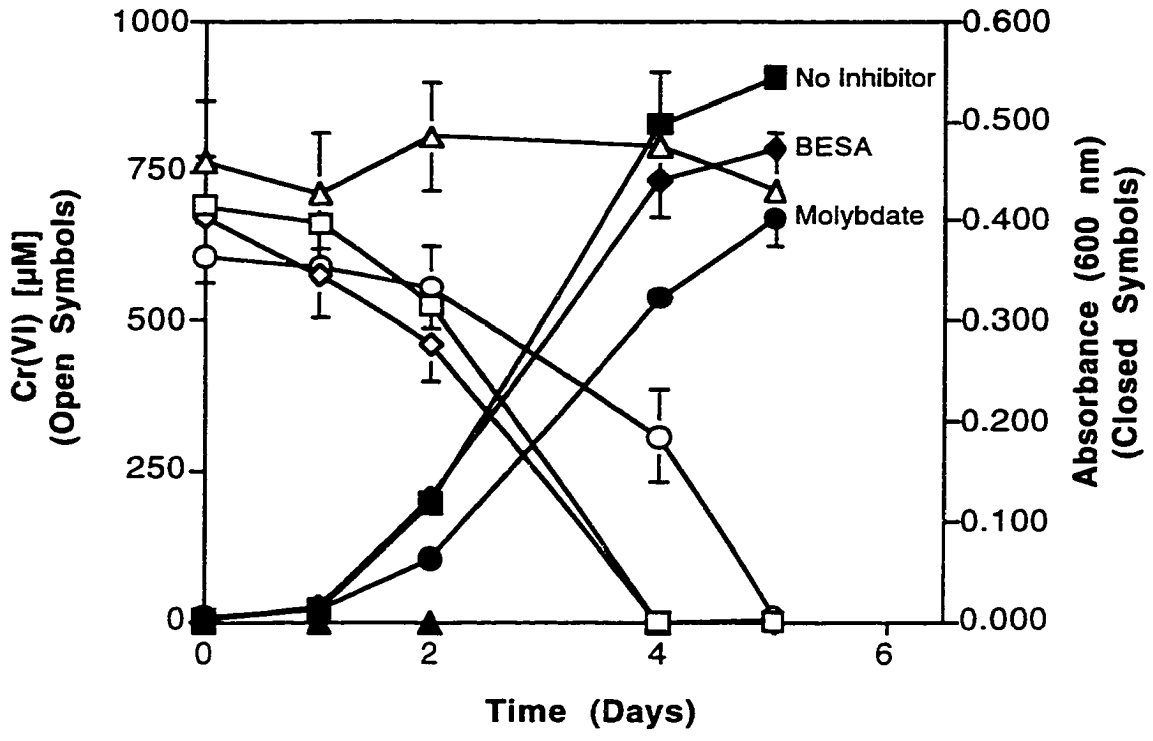

Figure 2. Effect of inhibitors on growth and chromate reduction by H_2-utilizing, Cr(VI) reducing enrichment. (■,❑ No inhibitor; ●,○ Molybdate; ◆,◊ BESA; ▲,△ Formaldehyde)

was able to grow and reduce Cr(VI) when either 10 mM bromoethane sulfonic acid (BESA), an inhibitor of methanogenic bacteria, or 10 mM molybdate, an inhibitor of sulfate-reducing bacteria, were present. However, molybdate appeared to partially inhibit growth of the enrichment since the optical densities were not equivalent to cultures with no inhibitor present. In addition, cultures grown with molybdate required an additional day to completely reduce the Cr(VI). Furthermore, analysis of protein concentration revealed a lower biomass yield when the enrichment was incubated with molybdate (Figure 3). There was no significant difference between final protein concentrations of cultures grown without inhibitor and cultures grown with BESA. No methane was produced by the enrichment at any time. Based on the lack of methane production and tolerance to BESA it was concluded that methanogenic bacteria were not important members of this consortium. However, inhibition of growth and Cr(VI) reduction by molybdate may indicate that a sulfate-reducing organism is present in the enrichment. Neither growth nor Cr(VI) reduction were affected by the addition of 40 mM NaCl, included as an ionic strength control (data not shown).

It was determined that 37°C was optimal for both rate of Cr(VI) reduction and growth by the enrichment, with a maximum O.D. of nearly 0.4 and reduction of 750 μM Cr(VI) in 5 days (Figure 4). The final protein concentration was greatest at 37°C (25 μg/ml), with 24°C and 30°C each yielding about 18 μg/ml protein. Room temperature (24°C) and 30°C also supported growth and Cr(VI) reduction. At these temperatures, Cr(VI) reduction proceeded slightly slower

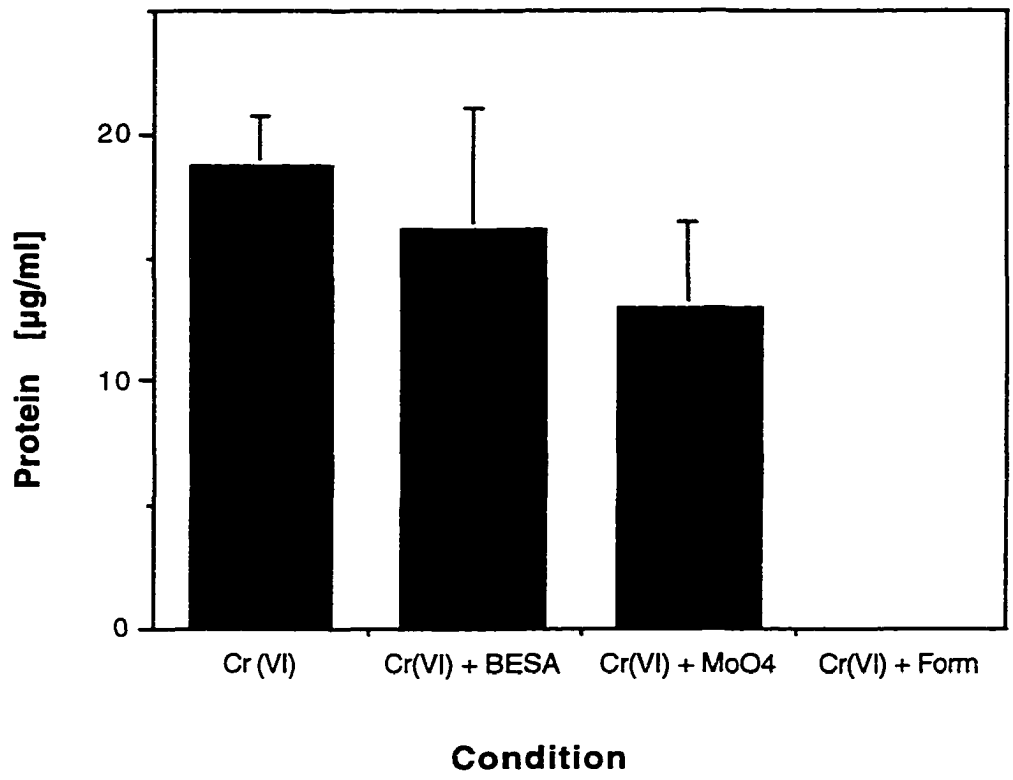

Figure 3. Protein concentration of H_2-utilizing, Cr(VI)-reducing enrichment after growth with inhibitors. (BESA, Bromoethane sulfonic acid: MoO4, Molybdate: Form, Formaldehyde)

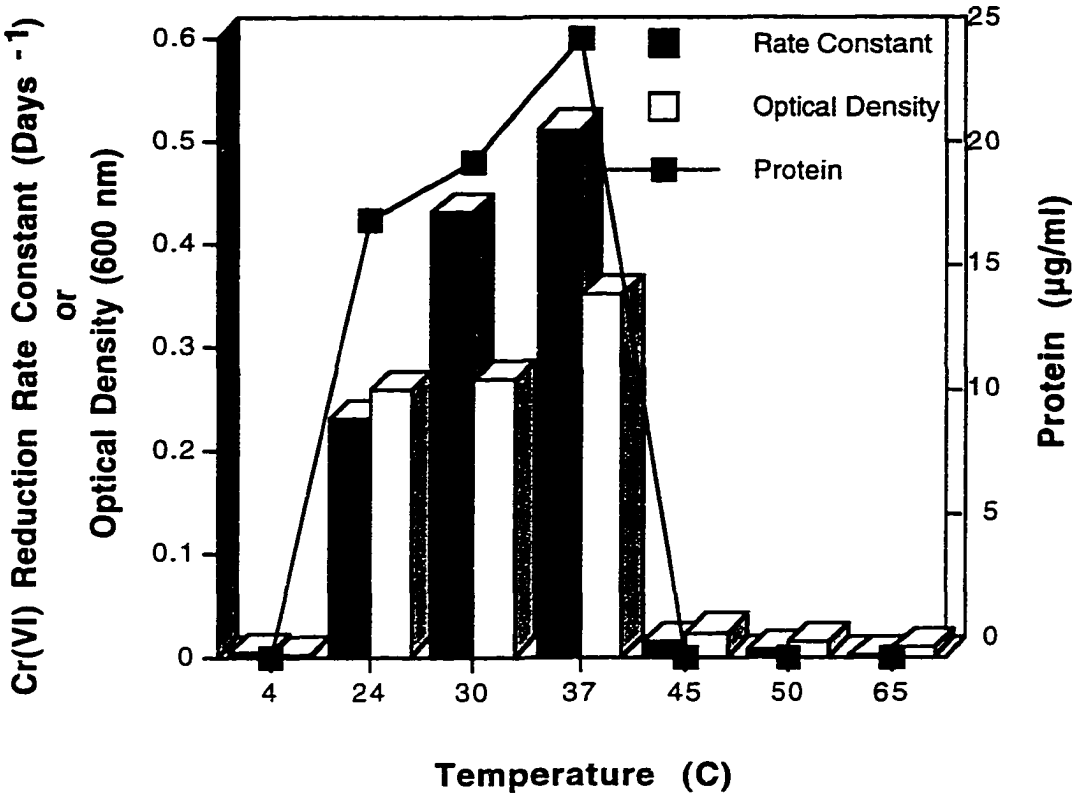

Figure 4. Effect of temperature on H_2-utilizing, Cr(VI)-reducing enrichment.

than at 37°C, however, significantly less growth occurred, even after extending the incubation period to 8 days. No growth or Cr(VI) reduction occurred at 4°C, or above 45°C.

H_2 concentration under Cr(VI)-reducing conditions. The hydrogen concentration under Cr(VI) reducing conditions was determined to be below detection limits, a value previously reported when nitrate- or manganese-reduction was the terminal electron accepting process (Figure 5). After 34 days, bottles were re-amended with hydrogen and the concentrations again fell to below detection limits. No loss of Cr(VI) could be measured due to the low amount of added hydrogen and no methane was detected when Cr(VI) was present. Microcosms which did not have chromate added reached a dissolved hydrogen concentration of 5.6 nM, which falls in the range reported for methanogenesis as the terminal electron accepting process (5-10 nM) (Lovley and Goodwin, 1988). Upon re-amendment with hydrogen, the concentration of dissolved hydrogen fell to 9.7 nM and was stable for over 50 days. The addition of 500 μM Cr(VI) caused the hydrogen concentration to fall below detection levels, as was found for the Cr(VI)-reducing microcosms (Figure 5).

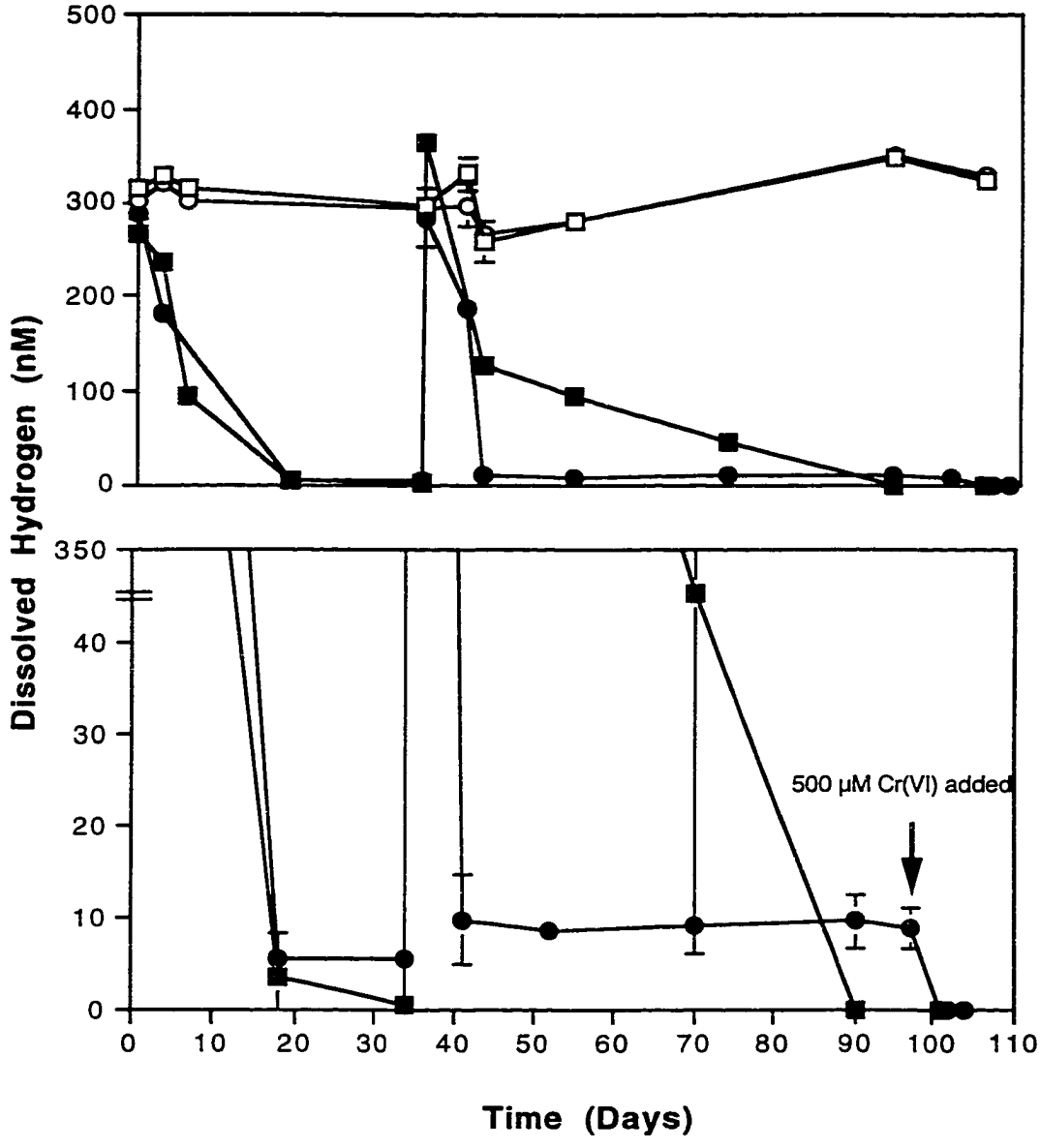

Figure 5. Hydrogen concentration determination for Cr(VI)-reducing conditions with aquifer sediments. (Bottom graph has expanded axis of top graph. ❑, Cr(VI)-reducing heat killed control; ■, Cr(VI)-reducing; ○, Methanogenic heat killed control; ●, Methanogenic.)

Discussion

Cr(VI) is a strong oxidant that can be reduced abiotically in the presence of electron donors commonly found in soils such as aqueous Fe(II), ferrous iron minerals, reduced sulfur, and soil organic matter (Eary and Rai, 1988; Palmer and Puls, 1994). In addition to abiotic reduction, many microorganisms have been shown to mediate reduction of Cr(VI) to the trivalent form (Wang and Shen, 1995, Lovley, 1993). Our studies show the potential for stimulating Cr(VI) reduction by naturally occurring microorganisms. Addition of electron donors which increase the bioavailable hydrogen for microbial use, such as formate, lactate, hydrogen, and glucose resulted in a greater extent of Cr(VI) reduction. Benzoate, which is more difficult to degrade thermodynamically with hydrogen production, and acetate, that may be degraded without any hydrogen production, did not stimulate Cr(VI) reduction to the extent of the other electron donors. Thus, the addition of suitable electron donors may be an effective method for treating chromium contamination in aquifers due to stimulation of organisms endogenous to the aquifer.

The existence of a Cr(VI)-reducing enrichment which is dependent upon H_2 for both growth and Cr(VI) reduction supports the conclusion that increasing the availability of hydrogen promotes greater reduction of Cr(VI). The rate of Cr(VI) reduction by the enrichment was slightly decreased in the presence of molybdate, possibly indicating that a sulfate-reducing organism may be part of this consortia. Indeed, several known sulfate-reducing bacteria have been reported to reduce Cr(VI), as well as other group VI elements such as Se(VI),

Mo(VI), and U(VI) (Tebo and Obraztsova, 1998; Tucker et al., 1998; Lovley and Phillips, 1994; and Lovley et al., 1993)

This study represents the first report of the hydrogen concentration during Cr(VI)-reducing conditions. Our results indicate that the hydrogen concentration during Cr(VI) reducing conditions is the same as that reported for nitrate- and manganese-reducing conditions (Lovley and Goodwin, 1988). Furthermore, methanogenic microcosms became Cr(VI)-reducing with Cr(VI) addition, and subsequently reached an identical H_2 concentration as those initially amended with Cr(VI). The fact that the hydrogen concentration is identical to that reported for nitrate- and manganese-reducing conditions is logical, given the Gibbs' free energy changes for these reduction reactions when coupled to hydrogen oxidation (Table 2) (Ahmann, 1997, Thauer et al., 1977). The Gibbs' free energy change for Cr(VI) reduction is less favorable than nitrate or manganese reduction but much more favorable than sulfate reduction or methanogenesis. This is also consistent with previous experiments in which Cr(VI) was added in combination with NO_3^-, Fe(III), and SO_4^{2-}. Cr(VI) reduction was found to occur simultaneously with nitrate reduction but prior to iron or sulfate reduction. When Cr(VI) was present no Fe(II) or $S^=$ production was observed. However, in the absence of Cr(VI), nitrate utilization was followed by Fe(II) production, and later, sulfate reduction (See Chapter 2, Figure 4).

The utility of our observations are two-fold. First, understanding the processes that stimulate naturally occurring microorganisms to reduce Cr(VI) is

Table 2. Gibbs' free energies for terminal electron accepting processes coupled to H_2 oxidation.

Reaction	$\Delta G'$ (kJ/mol e^-)[a, b]
$1/2\ MnO_2\ (s) + H^+ + 1/2\ H_2 \rightarrow 1/2\ Mn^{2+} + H_2O$	-94.06
$1/5\ NO_3^- + 1/5\ H^+ + 1/2\ H_2 \rightarrow 1/10\ N_2\ (g) + 3/5\ H_2O$	-86.44
$1/3\ CrO_4^{2-} + 5/3\ H^+ + 1/2\ H_2 \rightarrow 1/3\ Cr^{3+} + 4/3\ H_2O$	-45.02
$Fe(OH)_{3(am)} + 2\ H^+ + 1/2\ H_2 \rightarrow Fe^{2+} + 3\ H_2O$	-43.89
$1/8\ SO_4^{2-} + 1/8\ H^+ + 1/2\ H_2 \rightarrow 1/8\ HS^- + 1/2\ H_2O$	- 0.42
$1/8\ HCO_3^- + 1/8\ H^+ + 1/2\ H_2 \rightarrow 1/8\ CH_4 + 3/8\ H_2O$	+ 0.67

[a] All values are taken from Ahmann (1997) except for methane production which was calculated from data in Thauer et al., 1977.

[b] $P_{H2} = 10^{-6.6}$ atm; $P_{N2} = 0.78$ atm; $P_{O2} = 0.21$ atm; $P_{CH4} = 2.45 \times 10^{-4}$ atm; pH = 7.0;

$[Mn^{2+}] = [NO_3^-] = [CrO_4^{2-}] = [Cr^{3+}] = [Fe^{2+}] = [SO_4^{2-}] = [HS^-] = [HCO_3^-] = 10^{-6}$ M

essential to improving current remediation strategies of contaminated sites by optimizing Cr(VI)-reducing conditions. For example, addition of electron donors which make more hydrogen available for microbial use, thereby resulting in a greater extent of Cr(VI) reduction, offers a straight forward approach to the treatment of Cr(VI)-contaminated aquifers. Second, knowing the H_2 concentration during Cr(VI)-reducing conditions allows monitoring of contaminated aquifers to determine when Cr(VI) reduction, in relation to utilization of other terminal electron acceptors, commences and ceases.

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