FE(III) AND CR(VI) REDUCTION IN ALKALINE MEDIA USING SOAP LAKE ALKALIPHILES

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of MICHAEL ROBERT VANENGELEN find it satisfactory and recommend that it be accepted.

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Abstract

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Chromium, in its hexavalent state, is one of the most widely utilized industrial metals, and is therefore present in many industrial waste water streams. Given the carcinogenic and mutagenic consequences of Cr(VI) exposure, the release of Cr(VI) into the environment is a major concern. In its trivalent state, chromium is both non-toxic and, due to its lower environmental mobility, exhibits limited environmental impact. For this reason, the reduction of Cr(VI) to Cr(III) remains the principle method for the treatment of chromium-containing waste. However, traditional chemical and electrochemical methods are expensive and produce large volumes of sludge, fueling the search for alternative methods of Cr(VI) reduction. The ability of many species of microbes to reduce Fe(III) to Fe(II) has led a number of researchers to consider microbial Cr(VI) reduction as a possible alternative. Although many reports of microbial Cr(VI) reduction are currently in circulation, very few have utilized alkaliphilic bacteria in their studies. Alkaliphilic Cr(VI) reduction is advantageous because in elevated pH environments, Cr(III) produces insoluble hydroxides which can easily be recovered. Soda lakes represent the most stable alkaline environments on earth, and are therefore widely used as sources of alkaliphilic bacteria. As part of the Soap Lake Microbial Observatory, alkaliphilic communities taken from the sediment of the monimolimnion layer of Soap Lake have been tested for anaerobic Fe(III) and Cr(VI) reduction capability. The goal of

this research will be to present one of the few reports of microbial metal reduction under alkaline, anaerobic conditions.

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DEDICATION

I would first like to thank my family for their encouragement and support throughout my academic career. Specifically, I would like to thank my parents, John and Susan VanEngelen, for all the sacrifices they made, both of their time and finances, allowing me to be home-schooled, and to spend four years at Whitworth College. I also greatly appreciate their continued love and encouragement over the past three years spent at Washington State University, and for what I'm sure will be continued generosity and support while I attend Montana State University. This work is also dedicated to my two super-neat younger sisters, Jennifer and Kristen VanEngelen, and to my older sister Tamara Cullen, her husband Rich Cullen, and their daughter Mercedes Lorraine. The great hospitality that Jon and Jen Springstead, and Dick and Paula Cullen have showed me over the past few years have been greatly appreciated. Thank you for opening your homes to me time and again. I wouldn't be here if it weren't for the four best friends and influences one could hope for. To Tim Beal, Dave Hannah, Zach Liljenburg, and Jon Sprigstead this work is also dedicated. Finally, I would like to thank Starbucks Coffee Company for giving me employment and a place to study while at WSU.

CHAPTER 1

BACKGROUND

This background will first attempt to introduce the reader to the chromium industry and its potential impact on the environment and public health. Since such large quantities of chromium waste gets landfilled every year, the behavior of chromium in soil systems will be discussed briefly, followed by an overview of various techniques aimed at the remediation of chromium contaminated sites. Next, the role of metal reducing bacteria in the environment and their potential remedial capabilities will be introduced. Specific attention will then be given to Cr(VI) reducing bacteria and their potential use in the remediation of chromium contaminated sites. The remedial application of alkaliphilic bacteria will be then be introduced. Since environmental chromium contamination can be prevented by proper treatment of chromium containing wastewater, wastewater treatment methods will be discussed with the intention of elucidating the potential role of alkaliphilic bacteria in this process. Once the usefulness of alkaliphilic bacteria has been established, a physiological description of these bacteria will be provided. Since many alkaliphiles are isolated from soda lakes, a description of soda lakes will follow, with special attention given to Soap Lake in Washington State, the source of the alkaliphiles used in this study. A final section will draw appropriate conclusions and provide the impetus for the research described in this report.

1. 1 THE CHROMIUM INDUSTRY

In 2004, the United States consumed 300,000 metric tons (Mt) of chromium, representing only 10% of the worldwide total (Papp, 2005). Chromium has many applications for metals, chemicals, and refractories. Because of these important applications, chromium maintains a large and growing global market (Papp, 1997; 2003; 2005).

Chromium is most often mined as chromite, $FeOCr_2O_3$, of which 17.0 Mt were produced worldwide in 2004 (Papp, 2005). Over half of the world's supply of chromite ore comes from South Africa, where 8.0 Mt were mined in 2005 (Papp, 2005). The remaining four of the top five producers, listed in order of decreasing production, are as follows: Kazakhstan (3.2 Mt), India (2.3 Mt), Zimbabwe (0.73 Mt, 2003 total), and Finland (0.55 Mt, 2003 total). Since chromite ore prices are on the order of $100/Mt (Papp, 2005), chromite mining is a very important economic activity.

The vast majority of the world's chromite is utilized by the metallurgical industry, which in 2003 accounted for 91.2% of all chromate consumption (Papp, 2004). The remaining chromite that year went to the chemical industry (5.2%), the foundry industry (2.8%), and the refractory industry (0.8%).

Chromium's chief application within the metallurgical industry is in the production of stainless steel. The performance of the stainless steel industry is therefore the most important factor dictating worldwide demand for chromium (Papp, 2004). Ferrochromium is the intermediate between chromite ore and stainless steel, representing the most consumed chromium containing material (Papp, 2004). In 2003, 5.849 Mt of ferrochromium were produced worldwide (Papp, 2004).

Various chromium chemicals have been proven useful for a range of purposes, such as pigments and metal finishing. Between 80-90% of tanned leather is tanned with chromium chemicals (Papp, 2004). Chromium has also been used in wood preservatives. However this particular use is discouraged by the United States Environmental Protection Agency (USEPA) over health concerns associated with the arsenic also present in the preservatives (USEPA website).

Owing to its high melting point and chemical inertness, chromium has found use in the production of refractory bricks, mortars and metal castings. More recently, however, use of chromium in the refractory industry has been declining over environmental and technological issues (Papp, 2004).

Given its widespread application, it is to be expected that large amounts of chromium waste are annually released into the environment. According to the USEPA, 32,589.6 Mt of chromium and chromium compounds were released into the environment in 2003, about half of which was land-filled (USEPA, 2005). Owing to its potential toxicity, as will be discussed in the next section, chromium contamination resulting from land-filling is a major environmental and human health concern.

1.2 CHROMIUM AND HUMAN HEALTH

Chromium, predominantly in its trivalent form, can be found in different concentrations throughout the human body including the liver, brain, blood, and kidneys (National Academy of Sciences (NAS), 1974; Mertz, 1969). It is the fifteenth most abundant element in the human body (Love, 1983). However, the biological effects of chromium depend very heavily on its valence state (NAS, 1974). While Cr(III) has

proven biological benefits, Cr(VI) is considered toxic. Most of the toxic properties of Cr(VI) are attributed to its high oxidizing potential and the ease with which it can permeate biological membranes (NAS, 1974).

The most significant health concern surrounding Cr(VI) exposure is due to the carcinogenic properties of Cr(VI). Chromium(VI) is considered a carcinogen based predominantly on reports concerning respiratory cancer in occupationally exposed groups (Langård, 1983). Reports of this kind have been generated in the US as early as 1948 (Malche, 1948), in Europe as early as 1890 (Malche, 1948; Burrows p. 14), and have continued ever since (Leonard, 1980; Norseth, 1981; Mancuso, 1997a; 1997b). In relation to the issue of its carcinogenicity, Cr(VI) is also considered a mutagen, in light of its ability to interfere with DNA synthesis (Leonard, 1980), although certain metabolic pathways have been shown capable of deactivating Cr(VI) mutagenicity (Petrilli, 1978).

In addition to an increased risk of respitory cancer, Cr(VI) is also known to be a skin irritant, causing allergic contact dermatitis (Nethercott, 1994; Seishima, 2003), and causes skin ulcerations sometimes referred to as “chrome holes” (Bloomfield, 1928; Williams, 1997). Other respiratory problems caused by Cr(VI) exposure include nasal septum lesions (Bloomfield, 1928; Kou, 1997).

While Cr(VI) is considered extremely toxic, Cr(III) is much less toxic and is an essential element in the human diet (Mertz, 1979; NAS, 1974). Due to its function within the glucose tolerance factor complex, Cr(III) is an integral part of glucose metabolism, so much so that Cr(III) deficiencies can lead to symptoms normally associated with diabetes (Mertz, 1969).

The potentially toxics effects of chromium exposure has led the USEPA to limit the amount of total chromium in drinking water to 100 parts per billion (ppb) (USEPA, 2003). A recent report has demonstrated that this limit is sufficient in preventing Cr(VI) in tap water from becoming a threat to human health (Paustenbach, 2003).

1.3 CHROMIUM REMOVAL FROM INDUSTRIAL WASTE WATERS

In general, heavy metals present in waste waters are concentrated in order to minimize the volume of substance in need of disposal. This usually occurs through precipitation of the metallic species (Eckenfelder, 2000) or the adsorption and accumulation of the metallic species onto a surface (Aksu, 2002; Eckenfelder, 2000). Although there are many variations, current technologies of chromium removal from waste water revolve around these two basic strategies (Eckenfelder, 2000). This section will discuss some of the various methods of chromium removal, both currently in use and in development, with separate attention given to precipitation based methods and adsorption based methods. In addition, the shortcomings of these methods will be addressed, with the hope of elucidating the potential contribution of alkaliphilic microorganisms to the process of chromium containing waste water treatment.

1.3.1 Precipitation Methods

Chromium is usually discharged into waste water as Cr(III) or Cr(VI) (Rana, 2004). In aqueous solutions, Cr(VI) exists as highly soluble oxyanions such as chromate (CrO_4^{-2}), dichromate ($Cr_2O_7^{-2}$), and their protonated counterparts, the relative abundance of each ion being determined by the pH of the solution and the Cr(VI) concentration

(Garrels, 1965; Richard, 1991; Latimer, 1952; Bartlett, 1976). Chromium(III) on the other hand forms insoluble hydroxides in aqueous solutions over a wide range of pH values (Rai, 1987; Barrera-Diaz, 2003). Therefore, complete precipitation can only be expected to take place upon prior reduction of all hexavalent chromium to trivalent chromium (Eckenfelder, 2000).

The general equations governing Cr(VI) reduction in aqueous media can be written as follows:

$$Cr_2O_7^{-2} + 6e^- + 14H^+ \Leftrightarrow 2Cr^{+3} + 7H_2O$$
$$CrO_4^{-2} + 3e^- + 8H^+ \Leftrightarrow Cr^{+3} + 4H_2O$$

Electrons can be supplied chemically by a reducing agent, one of the most common being ferrous sulfate ($FeSO_4$) (Eckenfelder, 2000; Philipot, 1984; Zotter, 1992). The reduction of Cr(VI) by $FeSO_4$ is favored under acidic conditions (pH = 2.5-3), as indicated by the reaction stoichiometry, thus necessitating the addition of acid (Eckenfelder, p. 143; Philipot, 1984). Complete reduction requires the addition of a large excess of ferrous sulfate, ranging from 16g- $FeSO_4$/ to 22g- $FeSO_4$/ g-Cr(VI) (Philipot, 1984; Eckenfelder, 2000).

Once reduced, the chromium can be precipitated as a hydroxide according to the following reaction, traditionally facilitated by the addition of lime ($Ca(OH)_2$) (Brady, 1995; Eckenfelder, 2000):

$$Cr^{+3} + 3OH^- \Leftrightarrow Cr(OH)_3$$

Thermodynamic and experimental evidence suggest that precipitation of $Cr(OH)_3$ is favored most between pH 8.5-10 (Barrera-Diaz, 2003; Rai 1987), although precipitation has been carried out under near neutral pH values (Philipot, 1984; Zotter, 1992).

The process outlined above has the ability to produce effluent chromium concentrations as low as 50 ppm, which is within the range of allowable chromium effluent discharge by federal standards (USEPA website). However, the large excess of reagents required produces large amounts of sludge, leading to expensive and difficult disposal issues, issues which have largely become the prime motivation for seeking alternative methods of chromium removal from waste water (Martinez, 2000; Tiravanti, 1997; Perez-Candela, 1995; Aksu, 2002; Agarwal, 1984; Rana, 2004; Brady, 1995; Petruzzelli, 1994; Paulson, 1952). In general, sludges can account for up to 25% of the total volume of treated industrial waste water (Colin, 1995).

Electrochemical methods have been developed as a means of providing an alternative to the purely chemical addition of reducing agents (Goeringer, 2000; Martinez, 2000; Barrera-Diaz, 2003). In these studies, reductants, in the form of Fe(II) (Martinez, 2000; Barrera-Diaz, 2003) or Cu(II) (Goeringer, 2000), are supplied through the dissolution of a sacrificial anode into solution. Although sludge production is reduced, these methods suffer from (1) mass transport limitations, and (2) passivation of the electrodes due to salt build-up on the electrodes.

The shortcomings of precipitation-based methods have made adsorption an attractive means of chromium concentration and removal from waste water streams. Indeed, adsorption techniques have received attention for the last half-centrury (Paulson, 1952; Eckenfelder, 2000) and will be discussed below.

1.3.2 Adsorption Methods

While there are few avenues for exploration within precipitation-based methods of chromium removal, adsorption provides a wide range of options, many of which have been studied extensively. Some of the more common adsorption methods will considered here.

1.3.2.1 Ion-exchange resins:

Ion-exchange resins have been used in the electroplating industry since the 1950's (Paulson, 1952) and are still being studied and utilized today (Eckenfelder, 2000; Gode, 2003). Ion-exchange resins carry the benefit of high chromium removal efficiency, reduced sludge production and, upon regeneration, allow for the recycling of chrome species (Eckenfelder, p.458; Tiravanti, 1997, 1997; Petruzzelli, 1994). However, ion exchange resins are limited in that they are either Cr(III) or Cr(VI) specific (Gode, 2003, Tiravanti, 1997, 1997; Petruzzelli, 1994). Therefore, for complete chromium removal in streams where both Cr(III) and Cr(VI) are present, a reduction or oxidation process would have to precede adsorption onto the resin, or multiple resins would be necessary (Eckenfelder, 2000; Paulson, 1952). In addition, processes involving ion-exchange resins often require the use of large amounts of acid and base for resin regeneration (Paulson, 1952; Eckenfelder, 2000; Petruzzelli, 1994; Tiravanti, 1997), adding to the cost and equipment necessary for operation.

1.3.2.2 Activated carbon:

Chromium can also be adsorbed onto activated carbon, which has seen wide use due to its ability to remove both organic and inorganic aqueous contaminants (Aksu, 2002; Han, 2000; Lee, 1989). Activated carbon has specific surface areas on the order of 1000 m^2/g (Han, 2000), and can be produced using a wide range of cheap raw materials including agricultural byproducts, and even almond shells (Perez-Candela, 1995). In addition, adsorption data can easily be fitted using Langmuir and Freundlich isotherms and their corresponding parameter values (Ramos, 1994; Han, 2000). However, adsorption of Cr(III) and Cr(VI) are favored under different conditions. Cationic Cr(III) species are more likely to adsorb under higher pH conditions, while the adsorption of anionic Cr(VI) is favored under low pH conditions (Han, 2000). Thus, in mixed streams, total removal of chromium would require either two separate adsorptions, or the complete reduction or oxidation of all chromium species.

1.3.2.3 Carbon electrodes:

Adsorption can be facilitated electrochemically using carbon electrodes, particularly when chromium concentrations on the order of 1-10 mg/l (Golub, 1988; Rana, 2004). However, compared to other electrochemical methods, carbon electrodes aren't as energy efficient (Golub, 1988; Rana, 2004; Barrera-Diaz, 2003), and, depending on the design of the electrode, the process would once again require the use of either two separate electrochemical columns or single column in which a pH gradient would have to be maintained (Golub, 1988, Agarwal, 1984).

1.3.2.4 Biological methods of adsorption:

Metal adsorption using biomass, both viable and non-viable, has been investigated (Brady, 1995; Dias, 2000), and the application of this technique to chromium removal has also been studied (Aksu, 2002; Quintelas, 2001; Rivera-Utrilla, 2003). The efficiency of metal removal by biomass can be enhanced if a suitable support, such as a resin or activated carbon, is utilized (Aksu, 2002; Brady, 1995; Dias, 2000; Quintelas, 2001; Rivera-Utrilla, 2003). However, comparisons between biosorption techniques and traditional adsorption techniques have shown biosorption to be less efficient (Aksu, 2002).

1.3.3 SUMMARY

It is apparent that the methods described above are limited in that separate or sequential treatments are necessary to completely remove and dispose of chromium from waste water. Precipitation requires an oxidation step before precipitation can take place, and although regeneration steps aren't necessary with this method, the large amount of sludge produced makes it undesirable. Adsorption requires that Cr(III) and Cr(VI) be removed under different conditions, not to mention the additional regeneration steps often required.

Precipitation methods could be improved if more efficient means of electron donation were available, as this would make Cr(VI) reduction more efficient and reduce overall sludge production (Barrera-Diaz, 2003). In addition, if Cr(VI) reduction could take place in the pH range most conducive to Cr(III) precipitation, i.e. at pH ~ 9, the process of precipitation could take place in one step rather than two. To be discussed in

section 1.8, bacterial reduction of Cr(VI) has been well documented, but not under alkaline conditions which favor Cr(III) precipitation. It is at this point that the potential contribution of Cr(VI) reducing alkaliphilic bacteria should be recognized. Although alkaliphilic bacteria have seen application in the detergent industry and in the hide-dehairing process (Horikoshi, 1996; Ito, 1998), their potential application for wastewater treatment has yet to be investigated. The subject of alkaliphilic bacteria will be discussed in section 1.9.

1.4 CHROMIUM IN THE ENVIRONMENT

Chromium ranks 21st in crustal abundance, and can therefore be found naturally throughout the environment (Adriono, p.317). On average, soils contain between 10 and 150 parts per million (ppm) of chromium, although soils derived from ultramafic rocks can reach concentrations as high as 3400 ppm (Adriono, p.318). Natural causes such as these have accounted for ground-water concentrations of 200 ppb (Robertson, 1975). However, high chromium concentrations cannot always be attributed to natural causes.

The land-filling of chromium waste has lead to soil and ground-water contamination in the United States and across the globe (James, 2001; Morgado, 2001; Stepniewska, 2001; Khan, 2003). Concentrations as high as 7000 ppb were found in the ground-water near the chrome plating facility at the U.S. Coast Guard Support Center outside of Elizabeth City, North Carolina (Khan, 2003). An electroplating facility in north-central Portugal is responsible for topsoil concentrations ranging from 10-27,132 ppm. In the vicinity of a tannery waste lagoon in eastern Poland, chromium concentrations have reached 3,268 ppm in the soil and 120 ppb in the groundwater

(Stepniewska, 2001). Chromium is second only to lead among metals most commonly present in all matrices at Superfund sites in the United States (USEPA, 2000).

Of particular interest to residents of Washington State is the widespread contamination of the Department of Energy (DOE) site at Hanford, where chromate is a significant groundwater contaminant (Zachara, 2004). Soil samples from underneath waste tank SX-108 in the Vadose zone plume located in the west-central part of the Hanford site have measured chromium concentrations exceeding 500 ppm and range in pH from 7.2 up to 9.8 (Fredrickson, 2004).

Since such large amounts of chromium are deposited in the soil, and since soil contamination can lead to groundwater contamination, understanding the behavior of chromium in soil is very important if environmental impact is to be minimized or corrected.

1.5 CHROMIUM IN THE SOIL

Within pH-Eh ranges typically found in soils, chromium will exist in either the trivalent state as Cr^{+3} or CrO_2^-, or in the hexavalent state as $Cr_2O_7^{-2}$, or CrO_4^{-2} (Bartlett, 1976a). In addition to exhibiting drastically different behavior in the human body, as discussed earlier, Cr(III) species and Cr(VI) species behave differently in soil systems (Pettina, 1998; Fendorf, 2000; Bartlett, 1979).

Between the two oxidation states, Cr(III) tends to get held much more tightly by soils, especially clay soils (Bartlett, 1976a). It has a strong tendency to adsorb onto soil surfaces (Pettina, 1998), coordinate with oxygen and nitrogen containing ligands (Bartlett, 1976a), and, depending on the pH, can precipitate as insoluble hydroxides (Rai,

1987). Chromium(III) therefore exhibits limited environmental mobility (Fendorf, 2000; Jardine, 1999). Oxidation of Cr(III) to Cr(VI) typically occurs at high redox potentials, normally associated with aerated soils (Bartlett, 1976a; Tokunaga, 2003). At high pHs, Mn is a strong oxidizer of Cr(III) (Bartlett, 1979).

Unlike Cr(III), Cr(VI) doesn't form any insoluble compounds, and isn't likely to coordinate with or adsorb to soil constituents (Tokunaga, 2003; Fendorf, 2000; Weng, 2001). It therefore exhibits much greater environmental mobility, thus enhancing its overall environmental threat (Bartlett, 1976b; Fendorf, 2000). This is especially true in alkaline soils, where Cr(VI) is more easily leached compared to neutral soils (Weng, 2001). Chromium(VI) can be reduced by natural organic matter present in soils (Fendorf, 2000; Jardine, 1999). Inorganic reduction can be carried out by H_2S or Fe(II), the former being favored at pH < 5.5 and the later favored at pH > 5.5 (Fendorf, 2000). Biological processes also contribute to the reduction of Cr(VI) in soils (Fendorf, 2000; Tokunaga, 2003; Tseng, 2002).

1.6 CHROMIUM REMEDIATION

The physical removal of chromium from contaminated soils is very costly and difficult (Lovley, 2000). For this reason, chromium clean-up is almost exclusively associated with immobilization and detoxification rather than the complete removal of chromium species. In other words, reduction of Cr(VI) species to Cr(III) is the fundamental goal of most chromium remediation strategies (Lovley, 1997b, 2000a; 2001b; Khan, 2003; Alowitz, 2002; Melitas, 2001; 2002). In the previous section, natural mechanisms of Cr(VI) reduction in soils was discussed, including biotic reduction by

bacteria and abiotic reduction by organic and inorganic materials. Several strategies are being developed to enhance one or more of these naturally occurring processes.

Jardine et al. suggested amending contaminated soils with natural organic matter (NOM) to reduce and subsequently precipitate or adsorb hexavalent chromium (Jardine, 1999). However, the reaction kinetics were only favorable in acidic soils ($pH \leq 4$), thus limiting its applicability.

Amendment using inorganic reducers as an in situ remediation technique has also received attention. In particular, permeable reactive barriers (PRBs) have been developed and even successfully implemented (Lovley, 2001; Melitas, 2001). The USEPA regards PRBs as a proven technology in this regard (USEPA, 2000). These barriers take advantage of iron's ability to reduce Cr(VI) by introducing zerovalent iron metal into groundwater plumes (Alowitz, 2002; Melitas, 2001; 2002). The kinetic description of this process is 0.5 order in $[H^+]$ (Melitas, 2001), and subsequently alkaline conditions lead to inefficient Cr(VI) reduction (Alowitz, 2002). PRBs are also limited in that they aren't very useful when it comes to source area remediation (Khan, 2003).

Sodium dithionate ($Na_2S_2O_4$), a reducing agent often used by chromium consuming industries, was used by Khan et al. for in situ treatment of a source Cr(VI) contamination site (Khan, 2003). Over the course of 48 weeks, ground-water Cr(VI) concentrations were reduced below the detection limit of 10 ppm. The reaction stoichiometry proposed by Khan suggests high pH values are favored by this reaction.

Recent studies have dealt with the role of bacterial Cr(VI) reduction in soils, and the remedial potential this role carries (Fendorf, 2000; Tokanaga, 2003; Tsang, 2002). Recognition of the importance of metal reducing bacteria, particularly in anaerobic

subsurfaces, was only given attention in the last decade (Lovley, 1997a). Since then, metal reducing bacteria have been incorporated into many bioremediation schemes (Fennell, 2001; Lee, 1998; Lloyd, 2001; Lovley, 2000a; 2000c; 2001). Given the growing importance of this topic, it will be appropriately elaborated in the next section.

1.7 BACTERIAL METAL REDUCTION

Metal reducing bacteria are responsible for most subsurface Fe(III) reduction, and are therefore very important to the ecology of subsurface environments (Lovley, 1991a; 1991b; 1997a). This is particularly important since reduction of metallic soil components, primarily iron and manganese, strongly affects the distribution of toxic metals in subsurface environments (Lovley, 1991a; 2000a). In addition to secondary effects, many metal reducing bacteria are capable of directly reducing and immobilizing a variety of metal toxins (Lovley, 1997b; 2000b).

In most anaerobic sedimentary environments, Fe(III) is the most abundant electron acceptor, and therefore is the species most likely to get reduced by local metal reducing bacteria (Lovley, 2000; Schröder, 2003). Iron reducing bacteria (IRB) couple the oxidation of organic molecules to carbon dioxide with Fe(III) reduction (Lovley, 1991a; 1991b; 1997a). Organic contaminants are among the molecules IRB have been shown capable of oxidizing, thus making IRB applicable in bioremediation technology (Lovley, 2000a; 2000b; 2000c; Shen, 1995). These microbes have found use as a less expensive alternative for the remediation of petroleum products such as benzene (Lovley, 2000d), and chlorinated solvents including perchloroehtene (PCE) and trichloroethene (TCE) (Fennell, 2001; Lee, 1998; Lovley, 2000a).

Although many IRB are capable of reducing and immobilizing toxic metals such as U(VI) (Lovley, 2001), Tc(VII) (Lloyd, 2000), and Cr(VI) (Lovely, 1997a), progress in developing methods of microbial metal detoxification has lagged behind bacterial detoxification of organic contaminants (Lovley, 2000a). In addition, among the methods of detoxification discussed so far, most have involved the use of aerobic bacteria, which presents a problem since oxygen, a scarce commodity in most contaminated sub-surfaces, is required by aerobes for growth (Lovley, 2001). For this reason, bacteria equipped to grow and reduce metal toxins in anaerobic environments are gaining interest.

1.8 BACTERIAL Cr(VI) REDUCTION

Many bacterial isolates known to reduce Fe(III) have been used in Cr(VI) reduction experiments, particularly among the *Cellulomonas* (Sani, 2002), *Shewanella* (Guha, 2001; Liu, 2002; Viamajala, 2003; 2004), and *Geobacter* (Liu, 2002) species. Sulfate reducing bacteria have demonstrated Cr(VI) reducing capability, including *Desulfovibrio* (Lovley, 1994; Wang, 1997; Wielinga, 2001; Viera, 2003), and *Desulfotomaculum* (Tebo, 1998) species. Denritrifying bacteria of the genus *Pseudomonas* have also been used in many Cr(VI) reduction experiments (Shen, 1995; Wang, 1997; Konovalova, 2003). Consortia and novel species of Chromium(VI) reducing bacteria have been isolated from a variety of Cr(VI) contaminated sites including soils (Badar, 2000; Camargo, 2003; Jeyasingh, 2005), sludges (Ohtake, 1992; Nozawa, 1998), and waste effluents (Pattanapipitpaisal, 2001a; Faisal, 2004).

Several experimental factors affect observed Cr(VI) reduction rates, although reported reduction rates are typically on the order of 10^{-4} M/h (Fendorf, 2000). One of the

most crucial is the presence or absence of oxygen, since the toxics effects of Cr(VI) can vary between these two conditions. Using fumarate as the terminal electron acceptor, Viamajala et al. (2004) found that *Shewanella oneidensis* MR-1 cultures were more sensitive to Cr(VI) toxicity under anaerobic conditions, experiencing growth inhibition at Cr(VI) concentrations of 0.015 mM. Similar inhibition under aerobic conditions was seen at Cr(VI) concentrations of 0.035 mM. Aerobic and anaerobic experiments with *Shewenella alga* (BrY-MT) ATCC 55627 showed little difference in Cr(VI) reduction rates, except at high Cr(VI) concentrations (5.0 mM Cr(VI)) at which point more reduction was observed under aerobic conditions (Guha, 2001). Under aerobic conditions, *Citrobacter* sp. N14 exhibited greater growth, but after 7.5 days only removed about 60% of the initial 0.1 mM of Cr(VI) present, possibly due to the reoxidation of the Cr(III) speices. Although anaerobic conditions resulted in less than half as much growth, about 90% Cr(VI) removal was achieved over 7.5 days (Pattanpipitpaisal, 2002).

Since Cr(VI) is toxic, bacterial Cr(VI) tolerance is another important factor. Some of the more resistant bacterial strains have come from Cr(VI) contaminated sites. Camargo et al. found bacteria living in alkaline (pH = 9) soil contaminated with dichromate able to withstand Cr(VI) concentrations as high as 0.048 M (Camargo, 2003). Soil samples taken from a contaminated site near a chromate plant in India yielded bacteria able to withstand and reduce 1 mM of Cr(VI) (Jeyasingh, 2005).

Although Cr(VI) concentration can directly affect whether or not reduction takes place, recent studies suggest that Cr(VI) concentration exerts limited affect on actual reduction rates (Shen, 1995; Wang, 1997; Pattanapipitpaisal, 2001a; Viamajala, 2003). Reduction rates of 7.1×10^{-4} mM-Cr(VI) $\cdot$ day^{-1} $\cdot$ mg-protein^{-1} were observed over initial

Cr(VI) concentrations ranging from 0.29 mM to 1.8 mM using *Bacillus* isolates (Wang, 1997). Pattanapipitpaisal et al. (2001a) reported a reduction rate of 21.4 mM-Cr(VI) · day^{-1} · mg-$protein^{-1}$ over Cr(VI) concentrations ranging from 0.1 mM to 0.5 mM using *Microbacterium* sp. MP30. *Shewanella oneidensis* MR-1 exhibited similar reduction rates over the range of .04 mM to .12 mM Cr(VI) (Viamajala, 2003). In these cases, an initial, fast reduction was followed by a slow reduction, both of which appeared to be independent of initial Cr(VI) concentration. Viamajala et al. (2003) used a dual-enzyme kinetic model to explain this behavior. According to this model, Cr(VI) reduction is carried out independently by two mechanisms: one fast and susceptible to deactivation, and one slow but stable. Experiments with *S. oneidensis* MR-1 yielded maximum specific reduction rates for each enzyme. Depending on the growth conditions, the stable enzyme exhibited calculated rates on the order of 10^{2} mM-Cr(VI) · day^{-1} · mg-$protein^{-1}$, while rates of the deactivating enzyme were on the order of 10^{1} mM-Cr(VI) · day^{-1} · mg-$protein^{-1}$.

Pattanapipitpaisal et al. (2001a) reported the effect of initial concentrations on Cr(VI) reduction rates. Cell concentrations of *Microbacterium* sp. MP30 ranging from $3.4 - 3.0\times10^{3}$ mg-protein · L^{-1} gave reduction rates ranging from $5.3\times10^{-2} - 9.4\times10^{-2}$ mM-Cr(VI) · day^{-1}, respectively. Although overall rates increased, specific rates decreased from 0.3 to 5.8×10^{-4} mM-Cr(VI) · day^{-1} · mg-$protein^{-1}$. This inverse relationship has been reported for a variety of species.

Choice of substrate, or electron donor, is another important factor affecting not only Cr(VI) reduction rates, but also the economics of a proposed remediation strategy. While substrates such as tryptic soy broth (TSB) and lactate are among the more

commonly used electron donors in Cr(VI) reduction studies, acetate has also received attention, as it is inexpensive and therefore a more economical alternative (Pattanapipitpaisal, 2002). Acetate, like lactate, also carries the benefit of being chemically defined. However, minimal media, such as media containing acetate, result in reduced reduction rates. In a study of Cr(VI) reduction by *Shewanella algy* BrY under different growth conditions, Guha et al. (2001) found a direct relationship between reduction rates and substrate richness. Bacteria grown in brain heart infusion broth, the richest substrate among three used in this experiment, were the fastest to grow and subsequently exhibited faster Cr(VI) reduction rates. TSB and glucose were also tested, with TSB, second in richness, causing higher growth and reduction rates that glucose. Biotic Cr(VI) reduction can also be carried out in the absence of substrate, as demonstrated by Sani et al. (Sani, 2002). However, this study showed that reduction rates were greater in the presence of substrate, namely lactate.

Bacteria can reduce Cr(VI) indirectly when a suitable electron shuttle is present in the media. Chromium(VI) containing media can be amended with Fe(III), which, upon bacterial reduction to Fe(II), can couple reoxidation to Fe(III) with Cr(VI) reduction to Cr(III) (Wielinga, 2001; Lee, 2003). The presence of Fe(III) or Fe(II) can therefore enhance Cr(VI) reduction capability. Anthroquinone-2,6-disulfonate (AQDS), a compound commonly used as a humic substance analogue, can also act as an electron shuttle and enhance Cr(VI) reduction rates (Fredrickson, 2000).

Bacteria capable of Cr(VI) reduction have recently been used in the construction of bioreactors. In these systems, bacteria are immobilized to prevent cell washout (Konovalova, 2003). High surface area supports (Battaglia-Brunet, 2004), including

polyvinyl alcohol beads (Pattanapipitpaisal, 2001b), and agar-agar films formed on cellulose acetate membranes (Konovalova, 2003) have been used for this purpose. These reactors can be operated in batch or continuous flow settings, adding to their versatility (Battaglia-Brunet, 2004; Pattanapipitpaisal, 2001b). However, immobilization leads to a loss of efficiency since it slows reduction rates (Pattanapipitpaisal, 2001b). Cell concentrations on the order of 10% w/v were therefore needed by Pattanapipitpaisal et al. and Konovalova et al. to achieve 100% Cr(VI) removal in four days. Experiments conducted by Battaglia-Brunet et al. employed protein concentrations of $0.25 - 0.375$ mg-protein/ml (corresponding to about 0.034% w/v (Peyton, 1992)), and typical experiment times were 160 days.

Many examples of bacterial Cr(VI) reduction have been reported, although virtually all of them were conducted at near neutral pH values. Few reports of bacterial Cr(VI) reduction under alkaline conditions have been published (Yi, 2004). Understanding alkaline Cr(VI) reduction is important because Cr(VI) is more mobile and therefore a greater environmental threat in alkaline soils (Weng, 2001). In addition, Cr(III), the product of Cr(VI) reduction, is least soluble in alkaline environments, particularly near pH = 9 (Rai, 1987). Iron(II) mediated Cr(VI) reduction rates are also favored at pH values near 9 (Pettine, 1998). Bacterial reduction of Cr(VI) under alkaline conditions therefore has obvious benefits for the remediation of contaminated high pH environments. Bacteria capable of Cr(VI) reduction under alkaline conditions also have potential use in the treatment of chromium containing wastewater, as mentioned previously in section 1.3. A discussion of bacteria capable of growth in alkaline media will be considered here.

1.9 ALKALIPHILES

Bacteria are categorized as alkaliphilic when optimal growth takes place at pH values of 9 or greater. Alkaliphiles can be further categorized as obligate if pH values of 8.5-9.0 are absolutely required for growth, or as facultative if growth can occur near neutral pH (Yumoto, 2002). Growth in high pH environments forces alkaliphiles to face many, often interrelated physiological challenges particularly concerning pH homeostasis (Booth, 1999; Krulwich, 1997; 1998), cell wall components (Aono, 1995; Kaieda, 1998; Krulwich, 1983; Tsujii, 2002), and bioenergetics (Hicks, 1995; Skulachev, 1999; Yumoto, 2002).

The chemiosmotic mechanism of hydrogen transfer states that bacteria spend energy expelling H^+ ions against a concentration gradient, subsequently developing a chemical potential, $\Delta\mu_{H^+}$, across the cytoplasmic membrane. This potential, known as the proton motive force (PMF), represens the sum of the proton concentration gradient (ΔpH) and trans-membrane electrical potential ($\Delta\Psi$, negative inside) and is utilized for ATP synthesis (Mitchell, 1961; Skulachev, 1999; Yumoto, 2002). This process implies that cytoplasmic pH is higher, or more alkaline, than the surrounding media. Since alkaliphiles grow in environments where the ambient pH can be over 2 units above the internal pH (Hicks, 1995; Krulwich, 1997), a drastic reduction of ATP synthesis, resulting from the reduced $\Delta\mu_{H^+}$, might be the expected result. However, ATP sythesis is "manifestly robust" in alkaliphiles (Hicks, 1995).

One way to maintain a sufficient $\Delta\mu_{H^+}$ in an alkaline environment would be to generate a trans-membrane electric potential large enough to compensate for the adverse

ΔpH (Skulachev, 1999). While $\Delta\Psi$ in alkaliphiles do tend to be greater than for bacteria grown at neutral pH (Horikoshi, 1982), measured $\Delta\Psi$ values have not been shown to be sufficient to completely overcome the adverse ΔpH (Krulwich, 1998). Indeed, the necessary $\Delta\Psi$ would be too large and would likely lead to the breakdown of the bacterial membrane (Skulachev, 1999). Simply switching the direction of $\Delta\Psi$ from inside negative to inside positive would orient ΔpH and $\Delta\Psi$ in the same direction, thus resolving the conflict. However, no such $\Delta\Psi$ orientation is known to exist (Skulachev, 1999). Rather, alkaliphiles are believed to have various alternative mechanisms, both passive and active, which are used to maintain cytoplasmic pH near neutrality, in addition to generating the energy required for growth (Krulwich, 1997).

The active mechanisms are primarily concerned with the successful establishment of a Na^+ electrochemical gradient to replace the unfavorable H^+ electrochemical gradient (Kaieda, 1998; Krulwich, 1983; 1997; 2001; Yumoto, 2002). This gradient of Na^+ ions is established by Na^+ / H^+ antiporters, which expel Na^+ ions out of the cell while simultaneously importing protons, and other Na^+ pumping mechanisms (Kaieda, 1998; Krulwich, 1983; 2001). Sodium ions can then reenter the cell through one of many Na^+/solute symports and, among motile bacteria, during the process of flagellar rotation (Krulwich, 1997; Yumoto, 2002). Not only does this action acidify the cytoplasm, the stoichiometry of the Na^+ / H^+ antiportation contributes to the establishment of an adequate $\Delta\Psi$ (Krulwich, 2001). It is possibly for this reason that all alkaliphiles studied to date exhibit a sodium requirement, with the concentration of sodium required varying among different species (Horikoshi, 1982; Krulwich, 2001).

Passive mechanisms include the presence of highly negatively charged phospholipids, particularly phosphatidyglycerol, within the plasma membrane serving to attract the protons needed to maintain the relatively low cytoplasmic pH (Hicks, 1995; Yumoto, 2002). Many Gram-positive alkaliphiles also posses anionic cell wall macromolecules which can serve as proton traps (Aono, 1995; Tsujii, 2002). High concentrations of acidic cytochromes are also associated with alkaliphiles (Hicks, 1995; Yumoto, 1997; 2002). These cytochromes assist in pH homeostasis, in addition to contributing to a more energetically efficient electron transport mechanism across the cytoplasmic membrane (Yumoto, 1997; 2002).

Despite all the research conducted, many questions relating to the physiology of alkaliphiles have yet to be answered (Yumoto, 2002; Krulwich, 1997). Since most of the alkaliphiles studied so far have been aerobic *Bacillus* species (Horikoshi, 1996; Yumoto, 2002), there exists the possibility that entirely new mechanisms will be discovered as investigations expand to include a greater variety of bacterial species (Krulwich, 1997). Therefore, studying alkaliphilic bacteria from various sources, including soda lakes such as Soap Lake in Washington State, could easily add to our overall understanding of alkaliphilic microorganisms.

1.10 SOAP LAKE: A MEROMICTIC SODA LAKE

Soda lakes represent the most stable alkaline environments on earth and are therefore excellent sources of alkaliphilic bacteria (Jones, 1998). Soda lakes tend to develop in closed basins in arid regions where high evaporation rates contribute to high salt and carbonate concentrations (Hutchinson, 1957). Due to high carbonate concentrations, soda lakes can maintain a pH as high as 11.5 (Jones, 1998). Soda lakes are further characterized by large concentrations of alkali metals, including Na^+ and K^+, and low concentrations of alkaline earth metals, such as Ca^{2+} and Mg^{2+}, which would otherwise precipitate the carbonates and reduce the pH of the lake (Duckworth, 1996; Jones, 1998; Sorokin, 2005). While soda lakes might not appear conducive to life, they are in fact very biologically productive (Jones, 1998; Duckworth, 1996), predominantly due to massive microbial blooms (Jones, 1998). The microbial diversity of soda lakes have been the subject of many reviews currently in circulation (Duckworth, 1996; Jones, 1998; Sorokin, 2005).

Soap Lake ($47^o 23' N.$, $119^o 30' W.$) is a soda lake located in the Grand Coulee Basin, a semi-arid region in central Washington State (Walker, 1974). The Grand Coulee Basin is a closed basin home to a series of lakes of increasing salinity, starting with Deep Lake and ending with Soap Lake (Edmonson, 1965). Soap Lake has no effluent, and only loses moisture by evaporation (Edmonson, 1965; Walker, 1974). It is high in carbonates (Hutchinson, 1957 p.568; Oremland, 1993; Rice, 1988) and sodium (Rice, 1988), has an average pH of 9.7 (Oremland, 1993), and a productivity of 391 $g \cdot C \cdot m^{-2} \cdot yr^{-1}$ (Oremland, 1993).

Another unique feature of Soap Lake is its meromixis, or chemical stratification. This term applies to lakes which are divided horizontally into two parts, or layers, of differing chemical composition. The top layer, where circulation and mixture can periodically occur is called the mixolimnion layer. The lower layer, perennially stagnant and unable to undergo periodic mixing, is called the monimolimnion layer. The two layers meet at a boundary known as the chemocline (Hutchinson, 1958; 1937). This stratification results in differing salinities between the two layers. The salinity of the monimolimnion layer of Soap Lake is 141 g/L (Oremland, 1993), compared to 18 g/L in the mixolimnion layer (Oremland, 1993; Walker, 1974). Since meromictic stability is directly related to the difference in density between the monimo- and mixolimnion layers (Walker, 1974), the relative densities between the layers of Soap Lake lead many to believe that Soap Lake has maintained its meromictic state for at least the last 2,000 years (Oremland, 1993; Rice, 1988).

The age and alkalinity of Soap Lake makes it an ideal source of alkaliphilic bacteria (Pinkart, 2001). Indeed, novel species and even novel genera of alkaliphiles have been recently isolated from Soap Lake (Dimitriu, in press), although their potential for industrial applications has yet to be determined.

1.11 CONCLUSIONS

Studying the metal reducing capability of alkaliphilic bacteria taken from Soap Lake therefore has the potential to benefit many areas of interest, including bioremediation of high pH soils contaminated with chromium, including the DOE site at Hanford, and the treatment of chromium containing wastewater. In addition, such

research will add to our overall understanding of alkaliphilic bacteria, since reports of alkaliphilic metal reduction are so scarce.

CHAPTER 2

INTRODUCTION

In 2004, the United States consumed 300,000 metric tons of chromium, representing only 10% of the worldwide total (Papp, 2005). The vast majority of the world's chromium is utilized by the metallurgical industry, which in 2003 accounted for 91.2% of all chromate consumption (Papp, 2003). Various chromium chemicals have proven useful for a range of purposes, such as pigments and metal finishing for example. Around 80-90% of tanned leather is tanned with chromium chemicals, and chromium has also been used in wood preservatives (Papp, 2003). Given its widespread application, it is no surprise that large amounts of chromium waste are annually released into the environment. According to the Environmental Protection Agency (EPA), 32,589.6 Mt of chromium and chromium compounds were released into the environment in 2003, about half of which was land-filled (EPA Toxics Release Report, 2005).

The biological effects of chromium depend heavily on its valence state (National Academy of Sciences, 1974). While Cr(III) has proven biological benefits (Shapcott and Hubert, 1979; National Academy of Sciences, 1974; Mertz, 1969), Cr(VI) is considered carcinogenic (Malche, 1948; Mancuso, 1997a; 1997b), and mutagenic (Leonard, 1980), Chromium(VI) can also cause skin disorders including dermatitis (Nethercott, 1994; Seishima, 2003), and ulcerations (Bloomfield, 1928; Kou, 1997). Owing to its potential toxicity, chromium contamination is a major environmental and human health concern.

Since such large amounts of Cr are deposited in the soil, understanding the behavior of Cr in soil is important if environmental impact is to be minimized or corrected. Within pH-Eh ranges typically found in soils, Cr will exist in either the

trivalent state as Cr^{+3} or chromite (CrO_2^-), or in the hexavalent state as chromate (CrO_4^{-2}) or dichromate ($Cr_2O_7^{-2}$) (Bartlett, 1976a). Between the two oxidation states, Cr(III) is often bound tightly by soils (Bartlett, 1976a), has a strong tendency to adsorb onto soil surfaces (Pettina, 1998), coordinate with oxygen and nitrogen containing ligands (Bartlett, 1976a), and, depending on the pH, can precipitate as insoluble hydroxides (Rai, 1987). Chromium(III) therefore exhibits limited environmental mobility (Fendorf, 2000; Jardine, 1999). Unlike Cr(III), Cr(VI) does not form any insoluble compounds, and is not likely to coordinate with or adsorb to soil constituents (Tokunaga, 2003; Fendorf, 2000; Weng, 2001). Chromuim(VI) therefore exhibits much greater environmental mobility, thus enhancing its overall environmental threat (Bartlett, 1976b; Fendorf, 2000). This is especially true in alkaline soils, where Cr(VI) is more easily leached compared to neutral soils (Weng, 2001).

The reduction of Cr(VI) species to Cr(III) is the fundamental goal of most chromium remediation strategies (Lovley, 1997b, 2000a; 2001b; Khan, 2003; Alowitz, 2002; Melitas, 2001; 2002). Abiotic organic (Jardine, 1999) and inorganic (Alowitz, 2002; Khan, 2003; Melitas, 2001; 2002) Cr(VI) reductants have been investigated. Many recent studies have addressed the role of bacterial metal reduction in soils, and its remedial potential (Fendorf, 2000; Tokanaga, 2003; Tsang, 2002). This is particularly important since reduction of metallic soil components, primarily iron and manganese, strongly affects the distribution of toxic metals in subsurface environments (Lovley, 1991a; 2000a). In addition, many metal reducing bacteria are capable of directly reducing and immobilizing a variety of toxic metal, including Cr(VI) (Lovley, 1997b; 2000b). Enriching contaminated soils with carbon sources can accelerate bacterial growth and

subsequently enhance bacteria-mediated Cr(VI) reduction (Lloyd, 2001). Although many species of metal reducing bacteria are capable of reducing and immobilizing Cr(VI), progress in developing methods of microbial metal detoxification has lagged behind bacterial detoxification of organic contaminants (Lovley, 2000a).

Reports of metal reduction in alkaline conditions using alkaliphilic bacteria are rare (Khijniak, 2003; Ye, 2004). Bacteria are labeled alkaliphilic if optimum growth occurs at pH $\geq$ 9 (Yumoto, 2002). Alkaliphilic Cr(VI) reduction is important for bioremediation efforts because Cr(VI) contamination has been reported in high pH soils (Camargo, 2003), and since Cr(VI) is more mobile in high pH soils than in neutral soils (Weng, 2001). Since Cr(VI) reduction products are least soluble at pH= 9 (Rai, 1987), alkaliphilic Cr(VI) reducing bacteria could potentially be useful in the precipitation and removal of Cr(VI) from industrial wastewater.

Soda lakes represent the most stable alkaline environments on earth and are therefore excellent sources of alkaliphilic bacteria (Jones, 1998). The microbial diversity of soda lakes have been the subject of many reviews currently in circulation (Duckworth, 1996; Jones, 1998; Sorokin, 2005). Soap Lake ($47^{o}23'N.$, $119^{o}30'W.$), a meromictic soda lake located in the Grand Coulee Basin in central Washington State, has an average pH of 9.7 (Oremland, 1993). The age and alkalinity of Soap Lake makes it an ideal source of alkaliphilic bacteria (Pinkart, 2001). Novel species and even novel genera (Dimitriu, in press) of alkaliphiles have been recently isolated from Soap Lake, although their potential for industrial and remedial applications has yet to be determined.

As part of the Soap Lake Microbial Observatory, alkaliphilic bacteria were collected from Soap Lake, Washington, to quantify their ability to anaerobically reduce

Fe(III) and Cr(VI) in alkaline (pH = 9) media. Monod kinetics were used to quantify metal reduction rates for both growth and non-growth experiments using acetate and lactate as substrate.

CHAPTER 3

MATERIALS AND METHODS

Two types of anaerobic experiments were performed, one using a consortium enriched from the sediment of the monomolimnion layer of Soap Lake and one using an isolate taken from the surface of the mixolimnion layer of Soap Lake. Each set of experiments focused on measurement of dissimilatory Fe(III) and Cr(VI) reduction by the bacterial samples. Chromium(VI) reduction was also performed in the presence of Fe(III) to test the electron shuttling potential of Fe(III). Initial Cr(VI) concentrations of 100 μM were used in all experiments involving Cr(VI). Initial Fe(III) concentrations of 2.0 mM and 0.5 mM were used for Fe(III) reduction and experiments utilizing Fe(III) and Cr(VI), respectively. Aqueous ferric citrate ($C_6H_5FeO_7$) (Sigma) was used as the Fe(III) source to maximize reduction rates (Di Christina, 2000; Liu, 2002), and aqueous potassium dichromate ($K_2Cr_4O_7$) (Sigma) was used as the Cr(VI) source. Abiotic, metal free, and heat-killed cell controls were performed parallel to each experiment. All experiments were carried out in 150 mL serum bottles sealed and crimped with thick butyl stoppers and aluminum caps. Bottles containing ferric citrate were wrapped in aluminum foil to prevent exposure to light. Metal reduction experiments were conducted in duplicate. Reagents were of the highest quality commercially available.

3.1 Media Composition:

Growth media contained the following, listed on a per liter basis: 17.5 g NaCl (Fisher), 0.25 g KH_2PO_4 (Fisher), 0.5 g NH_4Cl (Fisher), 0.1 g yeast extract (Bacto, BD & Co.), 4.0 g $Na_2B_4O_7$ (Fisher), and, depending on the experiment, either 170 mM acetate

(Sigma) or 20 mM lactate (Fisher) was added as chemically defined substrate. Non-growth media contained, also on a per liter basis: 17.5g NaCl, 4.0g $Na_2B_4O_7$, and either 170 mM acetate or 20 mM lactate, depending on the experiment. Media pH was raised to 9.0 using 10 M NaOH. Prior to use, all media was autoclaved for 15 min. at 121°C. Media was made anaerobic by bubbling with 0.2 μm filter sterilized 99.99% nitrogen gas (Air Liquide) under 15 psig for 30 min. Bottles were then pressurized to 15 psig using the same gas stream.

3.2 Sample Collection and Enrichment:

Samples were collected from the anaerobic sediment of the monomolimnion layer of Soap Lake 23 m below the surface, and from the aerobic mixolimnion layer just below the lake surface. Samples were stored in airtight plastic bottles and placed on ice in a cooler. Sediment samples were bubbled with nitrogen gas for ~30 sec. prior to being stored. Samples were taken back to Washington State University and used to inoculate anaerobic growth media containing 5mM ferric citrate and 170 mM acetate the same day. Cells were transferred three times into fresh media to ensure medium to facilitate enrichment of Fe(III) reducing bacteria.

3.3 Non-Growth experiments using the mixolimnion samples:

After the third transfer, a sample was taken from the mixolimnion enrichment and plated on tryptic soy agar (TSA) (BD & Co.). Two days later, individual colonies appeared, one of which was streaked onto a fresh TSA plate. After two days, individual colonies appeared on the second plate. One of these colonies was picked off the plate,

and suspended in 5 mL of acetate containing growth medium. One mL of the liquid culture was used to inoculate 50 mL of aerobic, acetate containing growth medium, and another 1 mL was used to inoculate aerobic, lactate containing medium. These liquid cultures were allowed to reach late-log growth phase before being separated into 1.8 mL stock cultures in sterile 20% glycerol (Fisher) solution, and stored at -84°C.

Stock cultures were used to inoculate 500 mL serum bottles containing 100 mL of either acetate or lactate containing aerobic growth media. The excess volume in the serum bottle helped to ensure excess oxygen. After 2 days, biomass absorbance measured at 620 nm had leveled out, indicating the cells had reached the stationary phase. The cells were then pelleted by centrifugation for 5 min at 6,000 rpm, and washed with non-growth media. This was repeated three times to ensure thorough washing. The washed cells were then suspended in 300 mL of corresponding non-growth media. The culture was separated into 150 mL serum bottles, amended with the proper metal solution to bring the final liquid volume in each bottle to 50 mL, and then made anaerobic. Measurements were taken at $t = 0$ days, and the bottles were then placed on shakers rotating at 130 rpm in a 35°C temperature controlled environment.

3.4 Growth experiments using monomolimnion samples:

After the third transfer, two 1 mL samples were taken from the monomolimnion enrichment. One was used to inoculate anaerobic acetate containing growth medium, and the other was used to inoculate anaerobic lactate containing medium. Each inoculum had 2 mM Fe(III) citrate. Cultures were allowed to reach late-log growth phase before being

separated into 1.8 mL stock cultures in an anaerobic glove box in a sterile 20% glycerol solution and stored at -84°C.

In an anaerobic glove box, stock cultures were used to inoculate 150 mL serum bottles containing fresh anaerobic growth medium amended with aqueous metal. A final liquid volume of 100 mL was achieved. Measurements were taken at $t = 0$ days, and then the bottles were placed on shakers rotating at 130 rpm in a 35°C.

3.5 Sampling and Analysis:

1.5 mL samples were periodically taken from each bottle for analysis. Fe(II) concentrations were measured using the ferrozine method adapted from Lovley (Lovley, 1987). 50 µL of sample was added 950 µL of 0.5 N HCl solution and digested for 1 hour in the dark. 100 µL of digested sample was then added to 1 mL of a ferrozine solution containing 1 g/L ferrozine in 50 mM HEPES (Fisher) buffer. The color was allowed to develop for 30 minutes in the dark before absorbance was measured using a UV-Vis spectrophotometer (Hewlett-Packard 8543) at 562 nm and compared to standard curves generated with known Fe(II) concentrations. Fe(II) stock solutions were prepared using ferrous ethylenediammonium sulfate ($C_2H_{10}N_2O_4S{\cdot}FeSO_4{\cdot}4H_2O$) (Fluka). Total Fe was quantified by digesting 50 µL of sample in 950 µL of a 0.25 M $NH_4OH \cdot HCl$ in 0.25 M HCl solution for 1 hour to reduce all Fe(III) to Fe(II). 100 µL of digested sample was then added to 1 mL of the ferrozine solution and allowed to stand for 30 minutes prior to measuring absorbance at 562 nm. Fe(III) concentrations were calculated as the difference between total Fe and Fe(II) concentrations.

Cr(VI) was measured using the diphenylcarbazide method (Bartlett, 1976). 100 µL of unfiltered sample was added to 400 µL of pH=1.0 nano-pure water. 200 µL of the acidified sample was added to 1800 µL of an s-diphenylcarbazide solution prepared by adding 25 mL of nano-pure water to a Hach Cr(VI) reagent pillow containing s-diphenylcarbazide. The color was allowed to develop for 30 minutes before absorbance at 540 nm was measured. Absorbance values were converted to concentration values by comparison with standard curves generated from absorbance values of known Cr(VI) concentrations. Cr(VI) stock solutions were prepared using potassium dichromate (K_2CrO_4) (Sigma). The sensitivity of the diphenylcarbazide method is reported to be 0.1 µM (Bartlett, 1976). Total Cr was measured by diluting 500 µL of sample in 4500 µL of .5 M HNO_3 and measuring using an inductively coupled mass spectrometer (ICP-MS) (Model 4500, Agilent). Cr(III) concentrations were calculated as the difference between total Cr and Cr(VI) concentrations.

Protein concentrations were measured using a procedure based on the Coomassie assay (Bradford, 1976). Samples were centrifuged at 12,000 rpm for 10 min, then washed with substrate free medium. After a second washing, samples were sonicated for 3 min. at 30% amplitude using a Branson sonicator (model # 102C). 500 µL of sonicated sample was then added to 500 µL of Coomassie dye (Pierce) and measured at 595 nm and compared with standard curves generated from known protein concentrations. Standards of known protein concentration were made using bovine serum albumin (BSA) (Pierce). The exact procedure, as prepared by Catherine Albaugh, can be found in Appendix 1.

SEM images were collected using a Hitachi S-570 scanning electron microscope coupled to a digital camera (Quartz Imaging). Appendix 2 details the fixation procedure used for this purpose.

3.6 Cell number to mg-protein conversion

Although protein concentrations were measured in this study, many studies report results in terms of actual cell numbers. To properly compare the results of this study with the results of such studies, it was necessary to find a suitable conversion factor to convert cell numbers into protein mass. Viamajala used a value (calculated by Peyton, 1992) of 7.3×10^{12} cells/g dry cell weight in his studies with *Shewanella oneidensis* MR-1 (Viamajala, 2003). This value can be coupled to the results of a study which found that dry cells are approximately 50% carbon (Bratbak, 1984), assuming that cell carbon and protein contents are equivalent (Zubkov, 1999). Given these values and assumptions, a conversion factor of 1.5×10^{10} cells/mg-protein can be calculated.

3.7 Calculation of kinetic parameters: Growth experiments

Three parameters were calculated to mathematically represent the growth experiments: doubling time (t_d, days), maximum growth rate (μ_{max}, day $^{-1}$), and substrate yield (Y, mg-protein/mM-metal). Curves representing protein accumulation as a function of time was used to calculate t_d and specific growth rate. The portions of the curves corresponding to the exponential growth phase were fit in Microsoft EXCEL using linear regression after taking the natural logarithm of the protein concentrations. The specific

growth rate was calculated as the slope of this line. The maximum specific growth rate was calculated using the Monod equation:

$$\mu = \frac{\mu_{\max} M}{K_S + M} \quad \text{Eqn. 3.7.1}$$

Where M = the concentration of the appropriate metal (mM), μ = the specific growth rate (day^{-1}), μ_{max} = the maximum growth rate (day^{-1}), and K_s is the half-saturation constant (mM). During exponential growth, $M >> K_S$, simplifying Eqn. 3.7.1 such that $\mu = \mu_{max}$. The doubling time is related to the maximum specific growth rate according to the following expression:

$$t_d = \frac{\ln 2}{\mu_{\max}} \quad \text{Eqn. 3.7.2}$$

The substrate yield (Y) is equal to the rate of change of protein mass with respect to metal concentration, according to the following expression:

$$Y = \frac{dP}{dM} \quad \text{Eqn 3.7.3}$$

Equation 3.7.3 can be expanded as follows:

$$Y = \frac{\frac{dP}{dt}}{\frac{dM}{dt}} \quad \text{Eqn. 3.7.4}$$

The derivatives were calculated by fitting the linear portions of the appropriate graphs using liner regression in EXCEL. Y could then was then calculated as the quotient of the two slopes.

3.8 Calculation of kinetic parameters: Non-growth experiments

The Monod equation was used to calculate the parameters dictating metal reduction in non-growth media and a constant cell concentration, unless zero-order kinetics provided statistically identical results, in which case zero-order kinetics, which assumes a constant reduction rate equal to the maximum specific reduction rate, was used. Zero-order kinetics described all reduction on lactate, and Fe(III) reduction on acetate. For these experiments, metal concentration versus time curves were fit to a line using liner regression in EXCEL, with the slope of this line equaling the maximum specific reduction rate.

All other experiments were described using the Monod equation. To solve for μ_{max} (mM metal/(mg-protein·day) and K_S (mM metal), the Monod expression must first be linearized as follows:

$$\mu = \frac{\mu_{max} M}{K_S + M}$$

$$\frac{1}{\mu} = \frac{K_S + M}{\mu_{\max} M} = \frac{K_s}{\mu_{\max} M} + \frac{1}{\mu_{\max}}$$

$$\frac{1}{\mu} = \left(\frac{K_s}{\mu_{\max}} \right) \frac{1}{M} + \frac{1}{\mu_{\max}} \qquad \text{Eqn. 3.8.1}$$

Plotting Eqn. 3.8.1, known as a Lineweaver-Burke plot, will therefore produce a straight line with a y-intercept equal to the reciprocal of the maximum reduction rate, and a slope equal to the half-saturation constant divided by the maximum reduction rate (Fogler, 2001).

3.9 Statistical analysis

Metal reduction experiments were done in duplicate. Averaged values were used as data points, with error bars representing one standard deviation of the two measurements. The average distance between the data points and the corresponding curves generated by liner regression in EXCEL were calculated, squared, and added to the square of the standard deviation values mentioned above. The square root of this value was used to represent the error of the kinetic parameters reported in the results section.

CHAPTER 4

GROWTH EXPERIMENTS USING CONSORTIA TAKEN FROM SEDIMENT OF MONOMOLIMNION LAYER OF SOAP LAKE

4.1 Characterization

Contrary to expectation, the SEM images of the consortia (sec 4.2) revealed the presence of organisms which, based on their size, do not resemble bacteria but rather more closely resemble fungi. The large amount of uncertainty regarding the nature of the organism prevented further characterization via 16S rRNA sequencing.

4.2 SEM images

SEM imaging revealed some interesting features of the microorganisms present in the growth media. Filamentous, worm-like organisms, possibly fungi, were observed, ranging in length from 0.5 – 1 mm (Fig 4.2.1), and in thickness from 0.01 - 0.025 mm (Fig 4.2.2). Upon magnification, small, oblong openings approximately 5 – 10 μm in width and 25 – 30 μm in length were seen (Fig 4.2.3).

Figure 4.2.1.
SEM image depicting overall structure of microorganism taken from monomolimnion layer of Soap Lake. Based on the scale on the lower left, this organism is over 0.5 mm long.

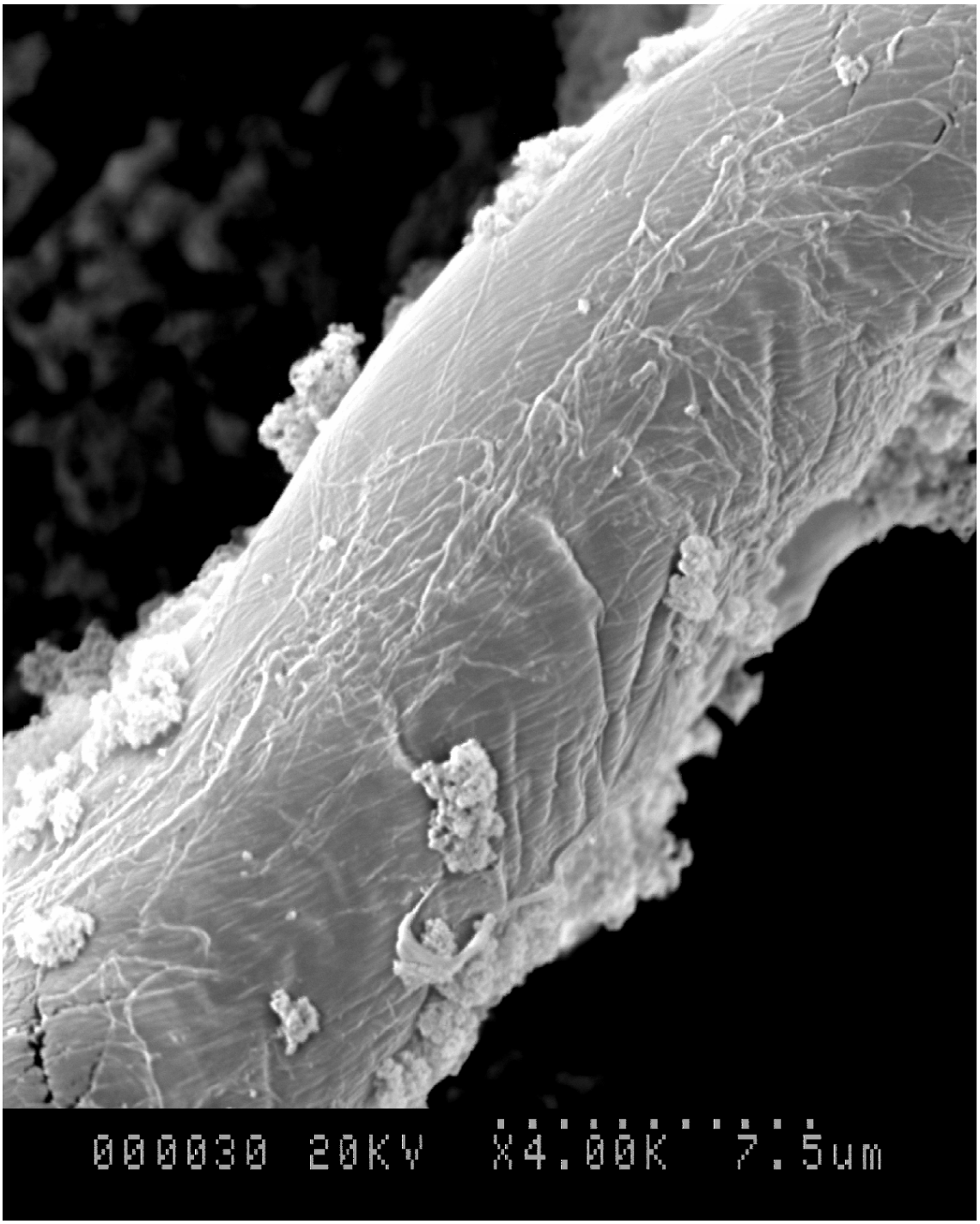

Figure 4.2.2.
SEM image showing close-up of mid-section of microorganism taken from monomolimnion layer of Soap Lake. The thickness of the organism exceeds 0.01 mm.

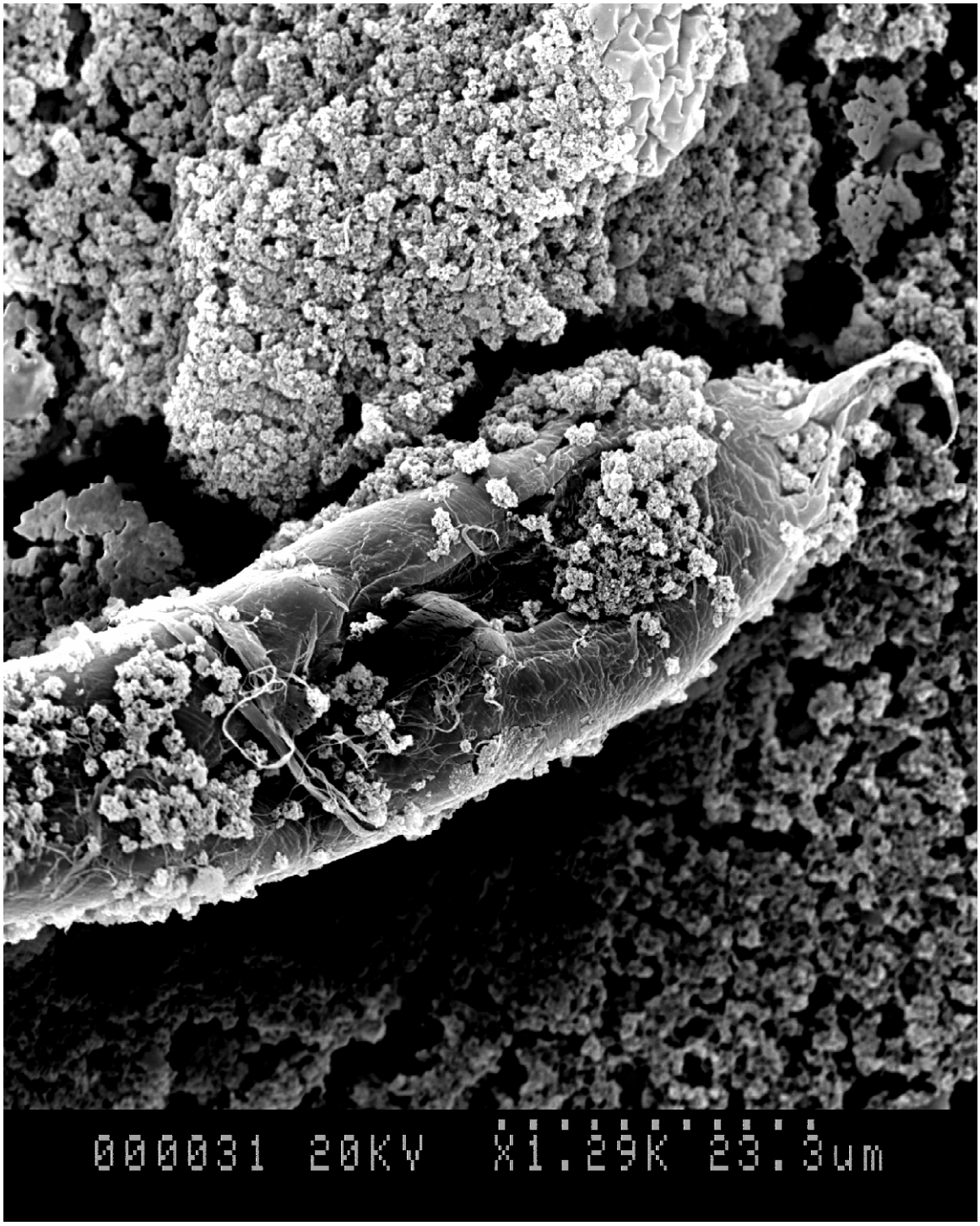

Figure 4.2.3.
SEM image showing close-up of opening found at one end of microorganism taken from monomolimnion layer of Soap Lake. Residual iron can be seen in backdrop and covering organism.

4. 3 Fe(III) and Cr(VI) reduction, and Cr(VI) reduction in the presence of Fe(III)

To measure Fe(III) and Cr(VI) reduction, anaerobic growth experiments were carried out using either lactate or acetate as carbon sources. Chromium(VI) reduction was also tested in the presence of Fe(III) to test the electron shuttling potential of Fe(III). Regardless of substrate, no significant Cr(VI) reduction took place over a 25 day period either in the presence or absence of Fe(III) (data not shown). Metal-free and heat-killed cell control experiments also showed no Cr(VI) reduction (data not shown). However, as shown in Figures 4.3.1 - 4.3.4, significant amounts of Fe(III) was reduced when either acetate or lactate were used as carbon sources. Error bars represent one standard deviation of experiments done in duplicate. Hollow data points correspond to abiotic control experiments performed in parallel.

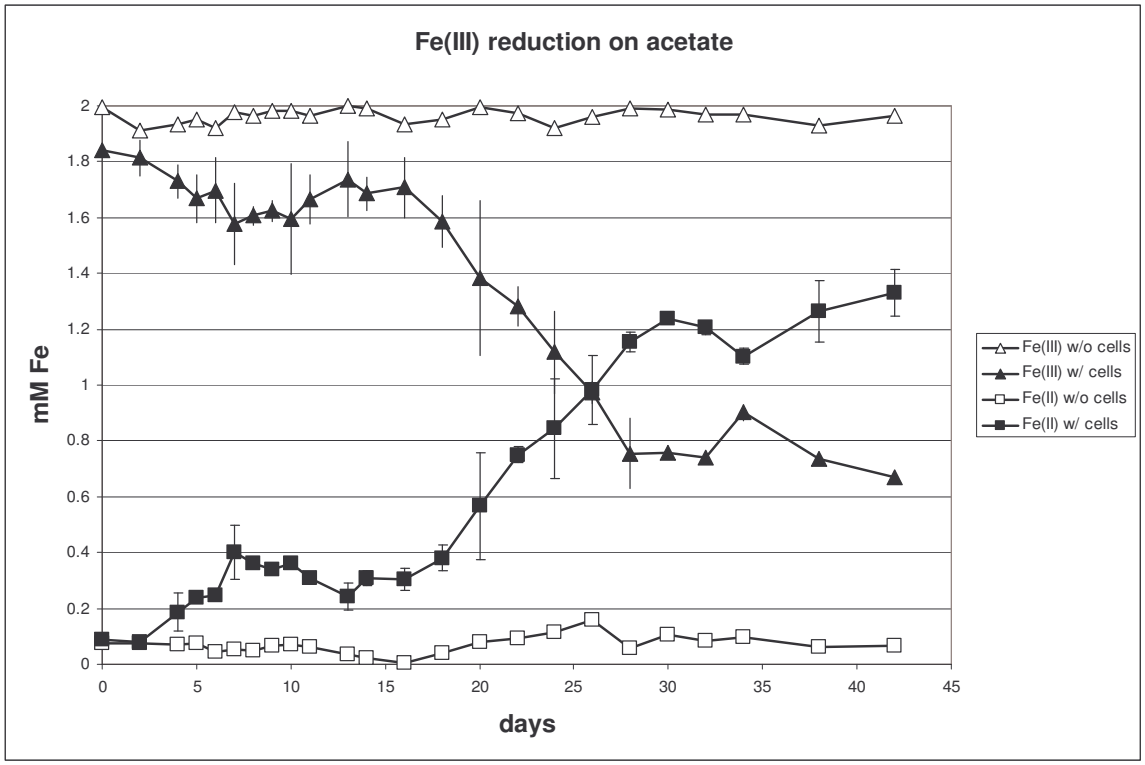

Figure 4.3.1. Fe(III) reduction and Fe(II) accumulation when acetate served as carbon source. After 42 days of inoculation, less than 70% of the initial 2mM Fe(III) was reduced.

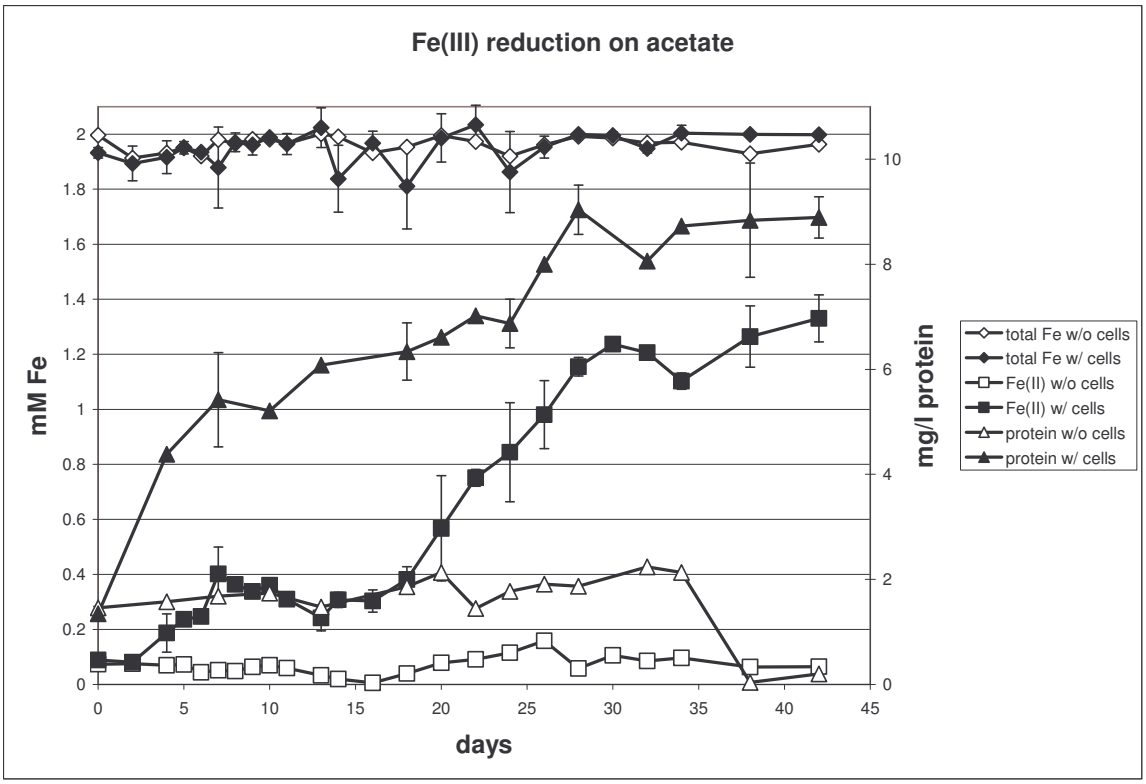

Figure 4.3.2. Fe(II) accumulation, total Fe, and protein accumulation when acetate served as carbon source. After 42 days, the protein concentration appeared to level out at a concentration near 9 mg/L.

Figures 4.3.1 and 4.3.2 depict data collected from the same Fe(III) reduction experiment, in which acetate was used as the electron donor. Figure 4.3.1 shows the reduction of Fe(III) and subsequent accumulation of Fe(II), and Figure 4.3.2 depicts the accumulation of Fe(II) and the concurrent accumulation of protein. After 42 days of inoculation, approximately 66.5% of the initial 2.0 mM of Fe(III) was reduced, with protein concentrations reaching a final concentration just below 9 mg/L. The large amount of time needed to reach these concentrations indicates poor growth conditions, probably associated with the use of such minimal substrate.

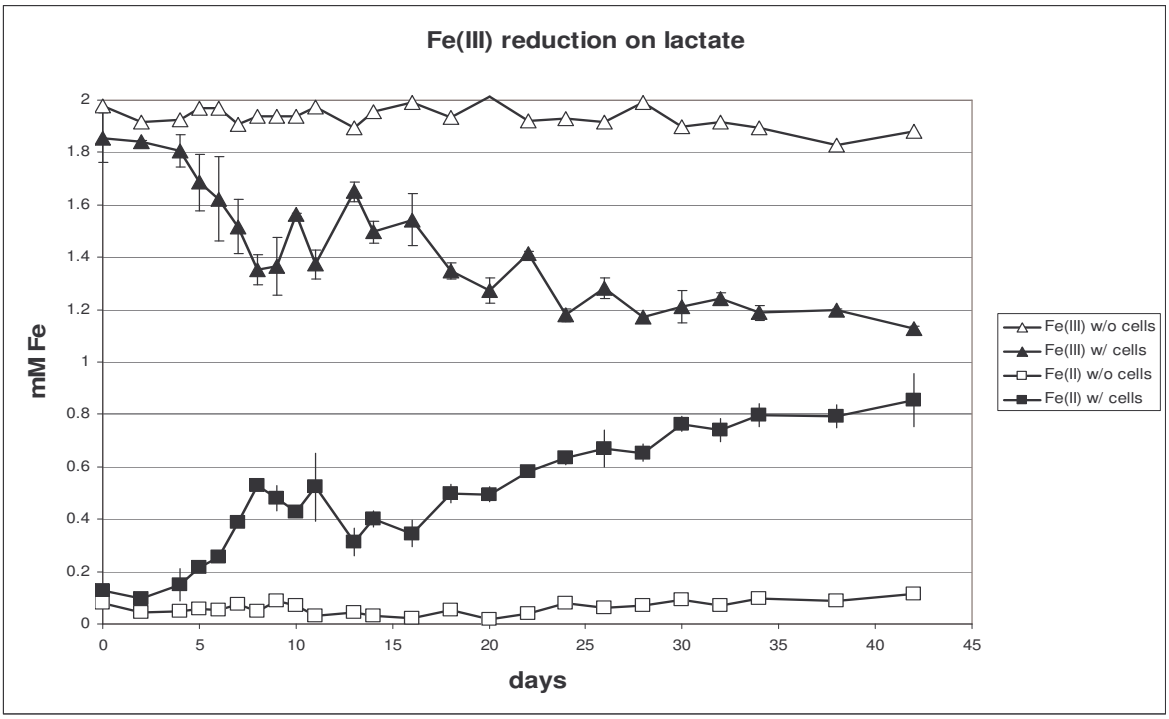

Figure 4.3.3. Fe(III) reduction and Fe(II) accumulation when lactate served as carbon source. Less than half of the original 2mM Fe(III) was reduced after 42 days.

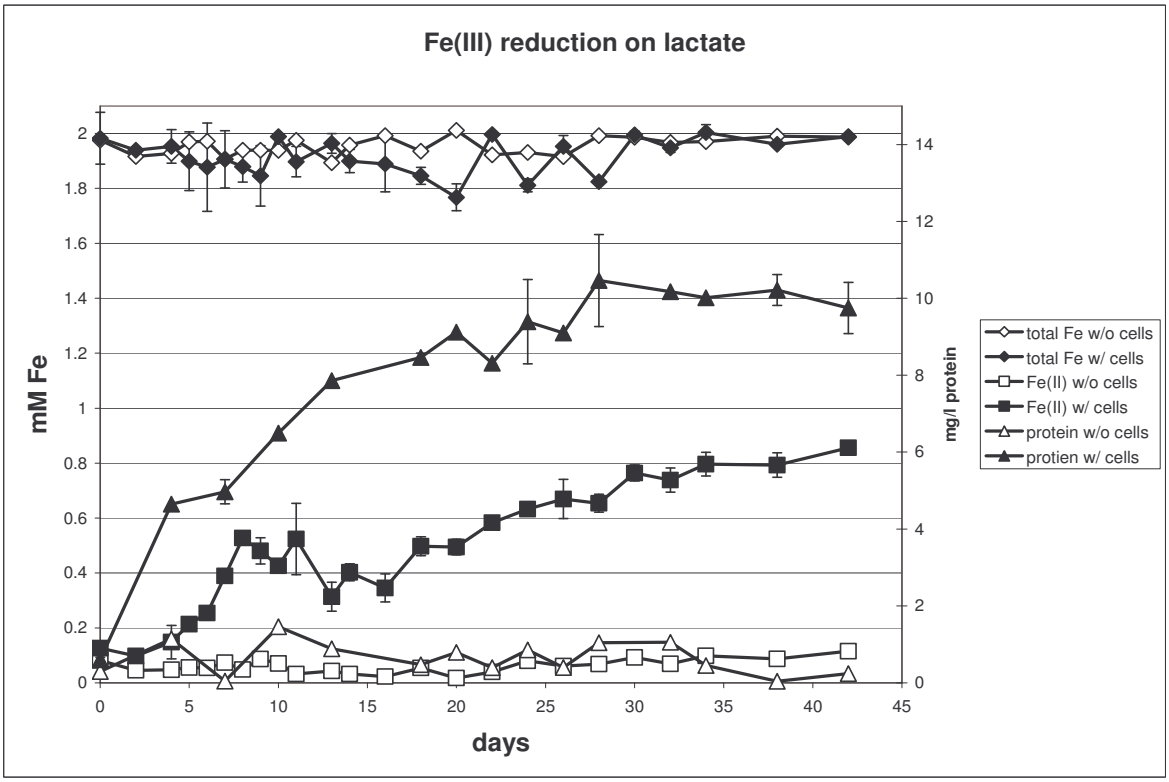

Figure 4.3.4. Fe(II) accumulation, total Fe, and protein accumulation when lactate served as carbon source. Protein concentrations reached a final value near 10 mg/L.

Over the 42 day period during which samples were taken, the mixed culture reduced 55% more Fe(III) when grown on acetate compared to lactate. Indeed, less than 43% of the initial 2.0 mM Fe(III) added to the media was reduced when lactate served as substrate, compared to the more than 65% of the Fe(III) that was reduced on acetate. Measured protein concentrations remained comparable over the duration of the experiment, with protein concentrations measuring on average 20% higher in the lactate system. Therefore, given equal protein concentrations, acetate served as a more effective substrate for Fe(III) reduction than lactate.

Since Fe(III) served as the limiting substance in both experiments, Fe(III) reduction was assumed to follow Monod kinetics. For this reason, the Monod expression was used to analyze the above data and calculate the kinetic parameters dictating the Fe(III) reduction process, as tabulated in Table 4.3.1. Comparison of the protein yields further reveals the inefficiency of lactate, for the purposes of Fe(III) reduction, compared to acetate. Normalizing against initial substrate concentration, which is necessary since unequal amounts of lactate and acetate were used, shows that the acetate system reduced more than 30% more Fe(III) per gram of initial substrate per mg protein.

Table 4.3.1. Kinetic parameters dictating the reduction of Fe(III) by consortia taken from monomolimnion layer of Soap Lake

Growth Substrate	Doubling time (days)	Protein yield (mg-protein/mM-Fe)	Max. Specific Growth Rate (day^{-1})
Lactate	14.4 ± 0.36	19.3 ± 2.9	0.048 ± 0.001
Acetate	28.4 ± 1.8	2.53 ± 0.13	0.0244 ± 0.002

CHAPTER 5

NON-GROWTH EXPERIMENTS USING ISOLATE TAKEN FROM THE MIXOLIMNION LAYER OF SOAP LAKE

5.1 Charaterization

Samples labeled as "mixo-isol" were prepared according to the protocol established by Laragen, Inc. (Appendix D) for 16S rRNA sequencing. Table 5.1.1 lists the closest genetic matches, and Figure 5.1.1 is a phylogenetic tree depicting how the closest matches are joined. Figure 5.1.2 is the actual consensus sequence reported by Laragen. According to the report, the isolate belongs to the genus *Halomonas* (Gram-negative, members of the $\gamma 3$ subdivision of the Proteobacteria), with *H. salina* and *H. ventosae* representing the closest genetic neighbors.

Table 5.1.1 The left column lists the closest genetic matches to the isolate. The right column lists the percent genetic similarity between the isolate and the bacterial species to which it was matched in the right column.

Halomonas salina	**98**
Halomonas ventosae	**98**
Halomonas alimentaria	**98**
Halomonas organivorans	**97**
Halomonas koreensis	**97**
Halomonas maura	**97**
Halomonas cupida	**97**
Halomonas subglaciescola	**96**
Halomonas hydrothermalis	**96**

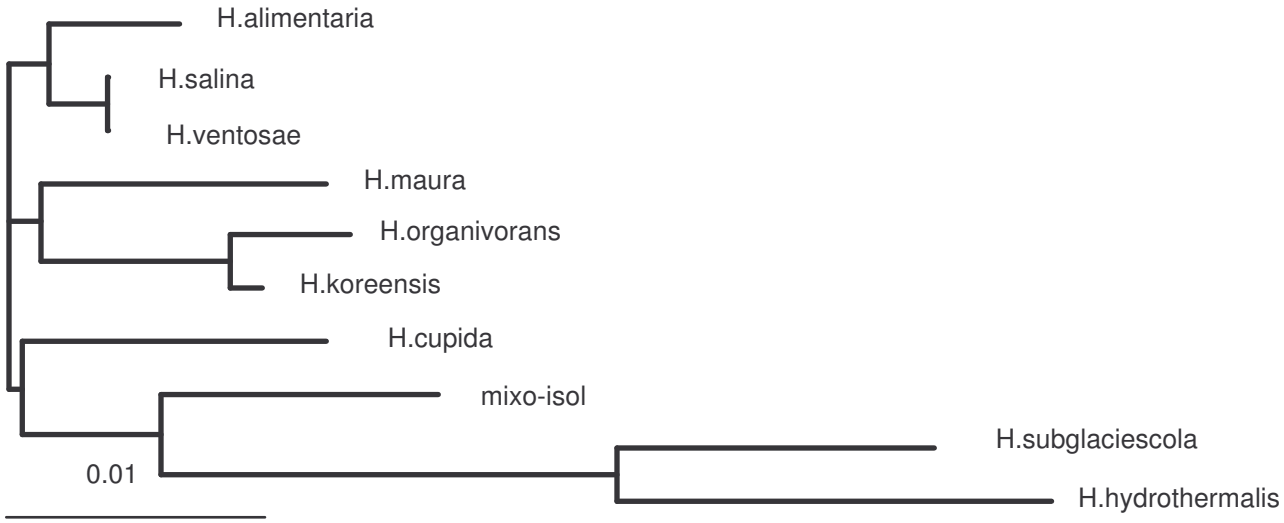

Figure 5.1.1 Phylogenetic tree depicting how the closest genetic matches are joined to the mixolimnion isolate, labeled as mixo-isol.

CAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGT
AGGCGGTcTGATAAGCCGGTTGTGAAAGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACTGT
CAGGCTAGAGTGCAGGAGAGGAAGGTAGAATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGG
GAGGAATACCAGTGGCGAAGGCGGCCTTCTGGACTGACACTGACGCTGAGGTGCGAAAGCGTGG
GTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGG
TCCTTGAGACCTTTGTGGCGCAGTTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAG
GTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATG
CAACGCGAAGAACCTTACCTACCCTTGACATCGTGCGAACTTTCCAGAGATGGATTGGTGCCCT
TGGGAACGCACAGACAGGTGCTGCACGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAG
TCCCGTAACGAGCGCAACCCTTGTCCTTATTTGCCAGCGGGTAATGCCGGGCACTCTAGGGAGA
CTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGAGTAGG
GCTACACACGTGCTACAATGGCCGGTACAAAGGGTTGCGAAACCGCGAGGTGGAGCTAATCCCG
AAAAGCCGGTCTCAGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTA
ATCG
Figure 5.1.2 16S rRNA sequence of mixolimnion isolate mixo-isol.

5.2 SEM images

SEM imaging revealed the presence of short rods approximately 1 - 2 μm in length in the non-growth media (Figures 5.2.1 and 5.2.2). The substance to which the isolates appear to be bound in Figure 5.2.1 went undetermined.

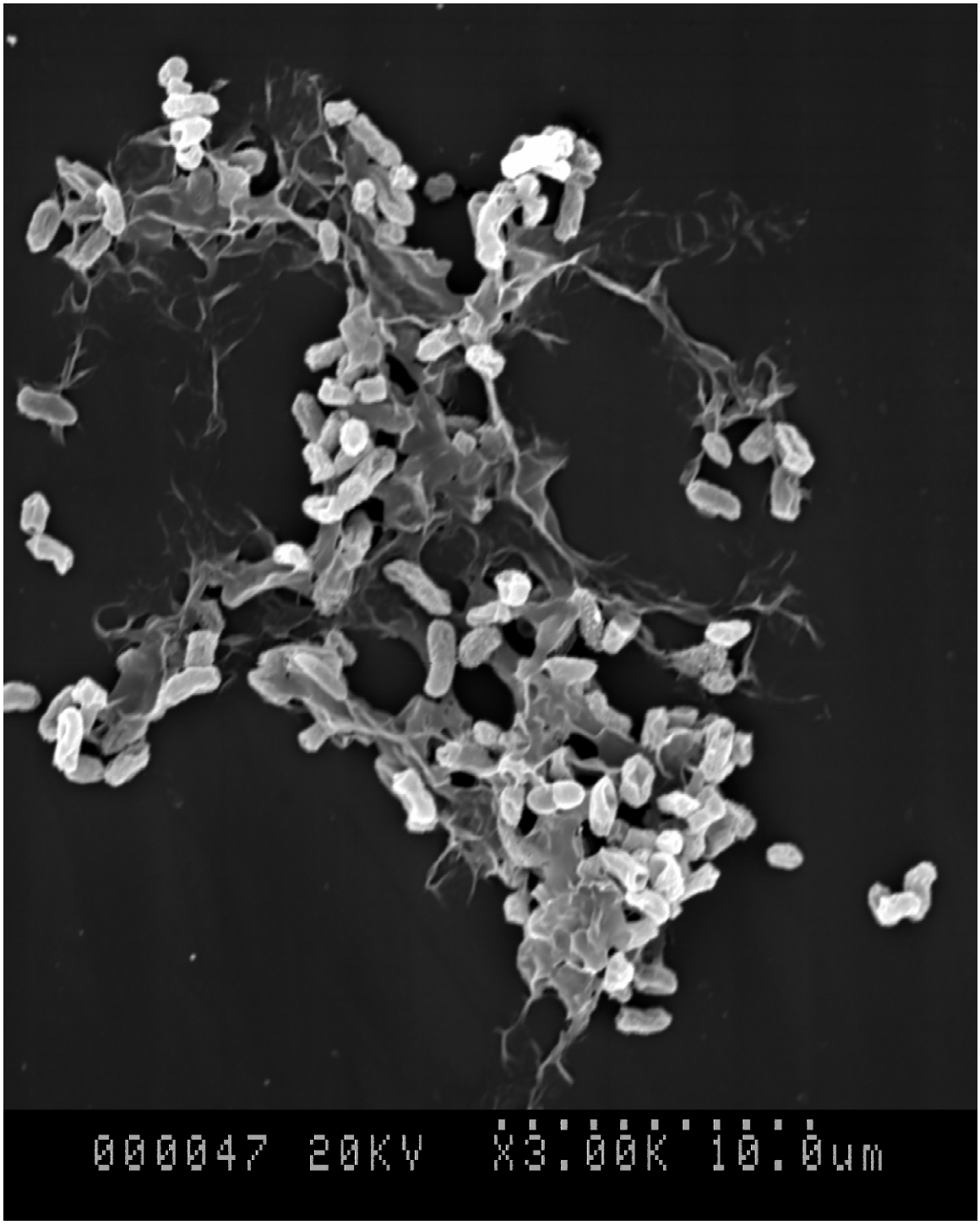

Figure 5.2.1.
SEM image depicting isolate taken from mixolimnion layer of Soap Lake. Isolates appear to be short rods approx. 1-2 μm in length, and attached to an undetermined substance.

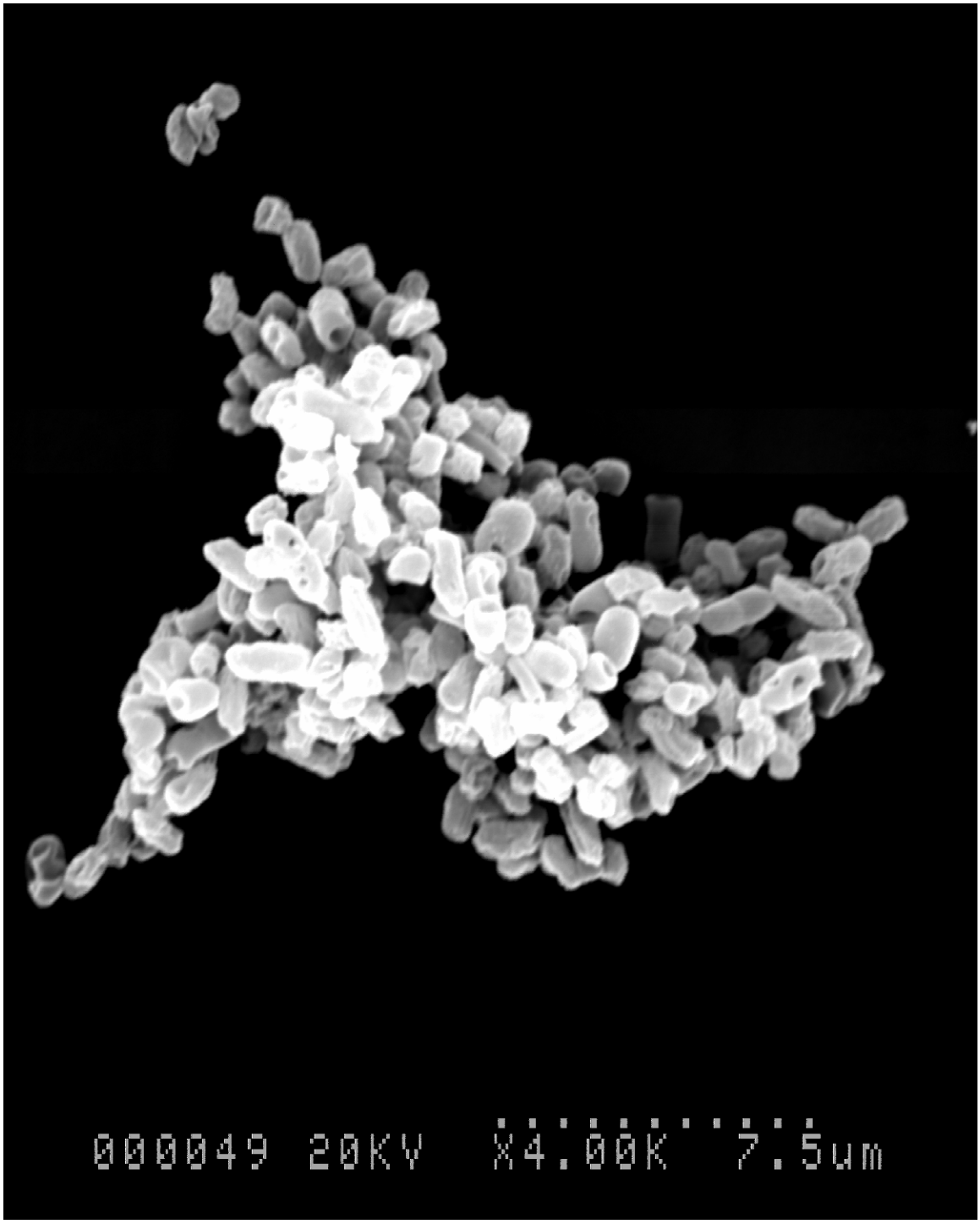

Figure 5.2.2.
Second SEM image depicting isolate taken from mixolimnion layer of Soap Lake.

5. 3 Fe(III) and Cr(VI) reduction, and Cr(VI) reduction in the presence of Fe(III)

To measure Fe(III) and Cr(VI) reduction, anaerobic non-growth experiments were carried out using either lactate or acetate as carbon sources. Chromium(VI) reduction was also tested in the presence of Fe(III) to test the electron shuttling potential of Fe(III). Experiments using lactate each had constant protein concentrations of 19.3 +/- 0.87 mg-protein/L. Experiments using acetate each had constant protein concentrations of 80.1 +/- 10 mg-protein/L. The results of these experiments are shown in Figures 5.3.1 – 5.3.6. Error bars represent one standard deviation of experiments done in duplicate. No reduction was observed for metal-free and heat-killed cell control experiments (data not shown). Reduction rates are tabulated in Tables 5.3.1 and 5.3.2. Hollow data points correspond to abiotic control experiments performed in parallel.

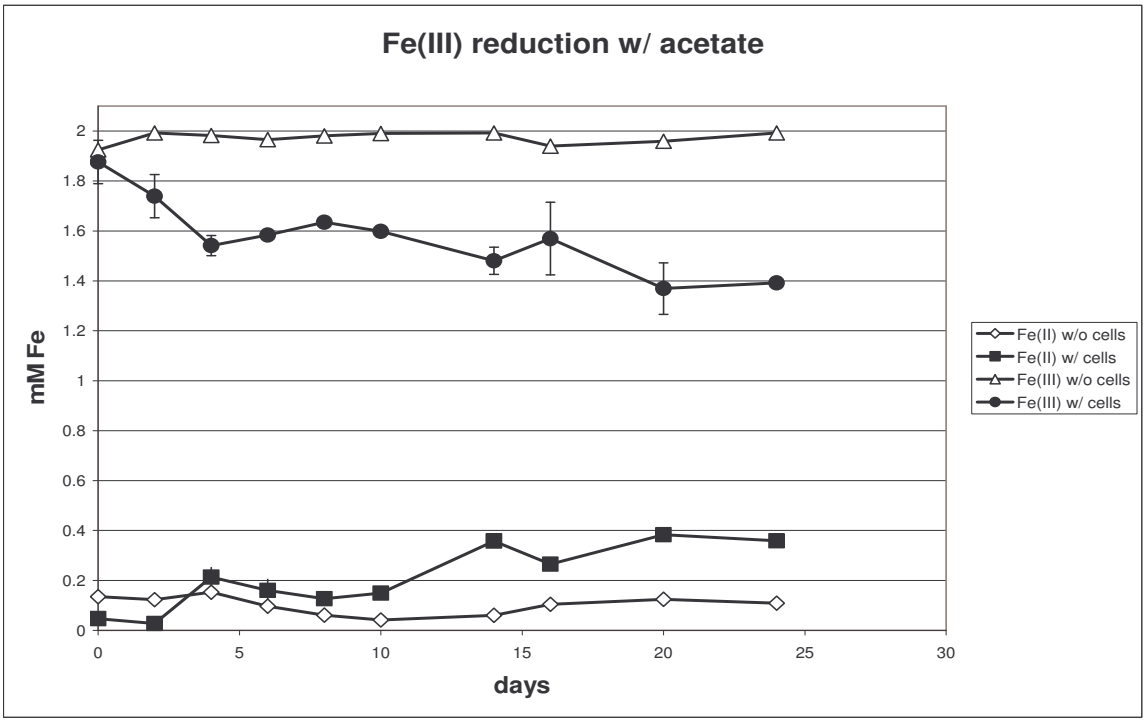

Figure 5.3.1. Fe(III) reduction and Fe(II) accumulation when acetate served as substrate, showing a relatively small amount of Fe(III) being reduced over a 24 day period.

Figure 5.3.1 depicts concurrent Fe(III) reduction and Fe(II) accumulation with 170 mM acetate serving as substrate. Over a 24 day period, a relatively small amount (less than 20%) of the initial 2.0 mM Fe(III) was reduced by the isolate, indicative of slow kinetic parameters, as tabulated in Table 5.3.1.

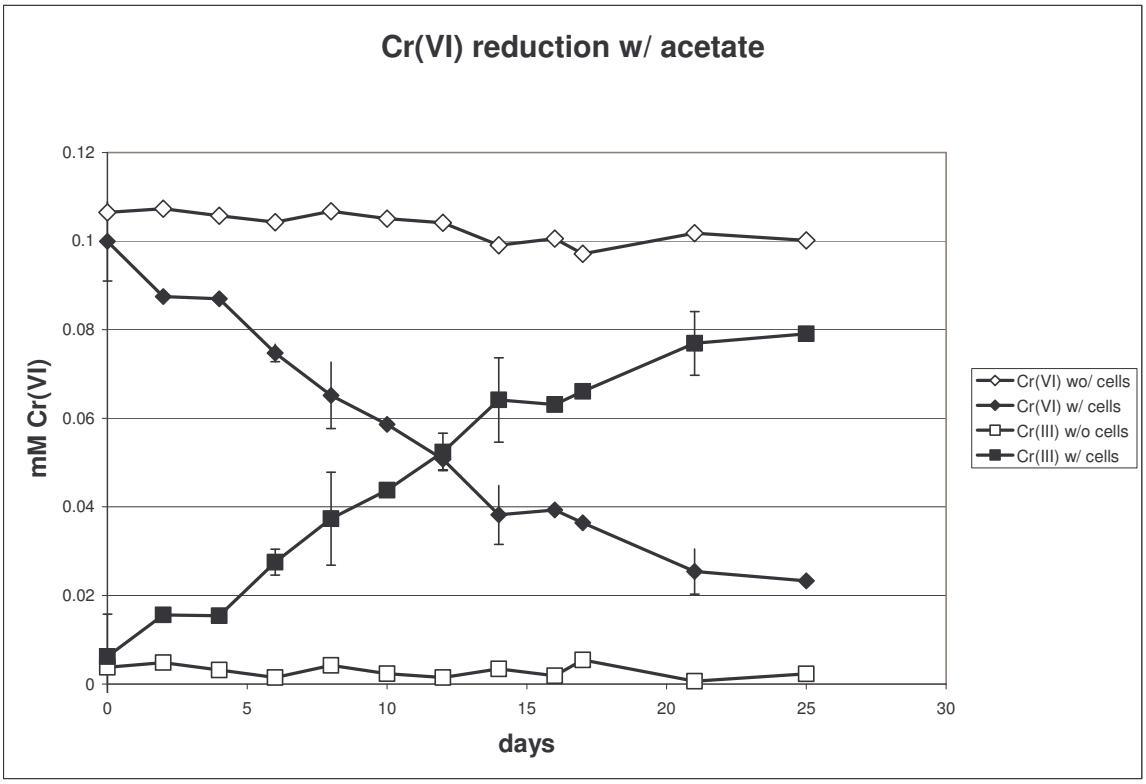

Figure 5.3.2. Cr(VI) reduction and Cr(III) accumulation when acetate served as substrate.

Figure 5.3.2 depicts Cr(VI) reduction and Cr(III) accumulation on acetate. After 25 days, most (> 75%) of the 0.1 mM Cr(VI) initially present was reduced to Cr(III). Although Cr(VI) reduction rates appear to be much faster than Fe(III) reduction rates, based on the appearance of Figure 5.3.2 compared to Figure 5.3.1, this is simply due to the difference in initial concentrations of the metals, leading to different scales on the

graphs. As seen in Table 5.3.1, maximum specific Fe(III) reduction rates and maximum specific Cr(VI) reduction are almost identical.

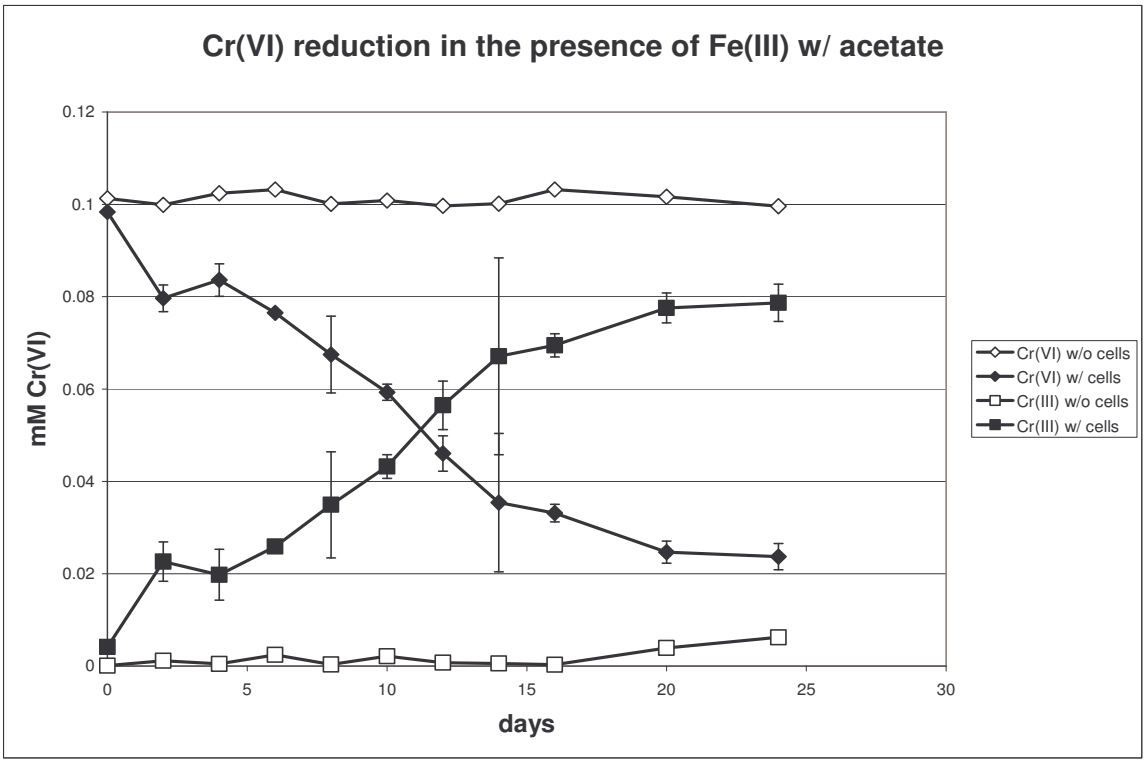

Figure 5.3.3. Cr(VI) reduction in the presence of Fe(III) when acetate served as substrate.

Figure 5.3.3 displays Cr(VI) reduction on acetate, and in the presence of 0.5 mM Fe(III). The purpose of this experiment was to test the enhancement of Cr(VI) reduction rates through the potential electron shuttling action of Fe(III). However, the Fe(III) did not enhance Cr(VI) reduction. An almost identical amount of Cr(VI) was reduced (approx. 76%) with or without Fe(III), and the specific reduction rates are statistically identical (Table 5.3.1). So similar are the reduction rates, test were performed to ensure that Fe(III) was added only to the correct serum bottles, with negative results. The large error bars at day 14 are the result of an unusually low Cr(VI) concentration measurement, probably due to experimental error, as the concentration recovered by the next day. Total

Fe and Fe(II) measurements remained constant (0.5 mM and 0 mM respectively) throughout the experiment (data not shown).

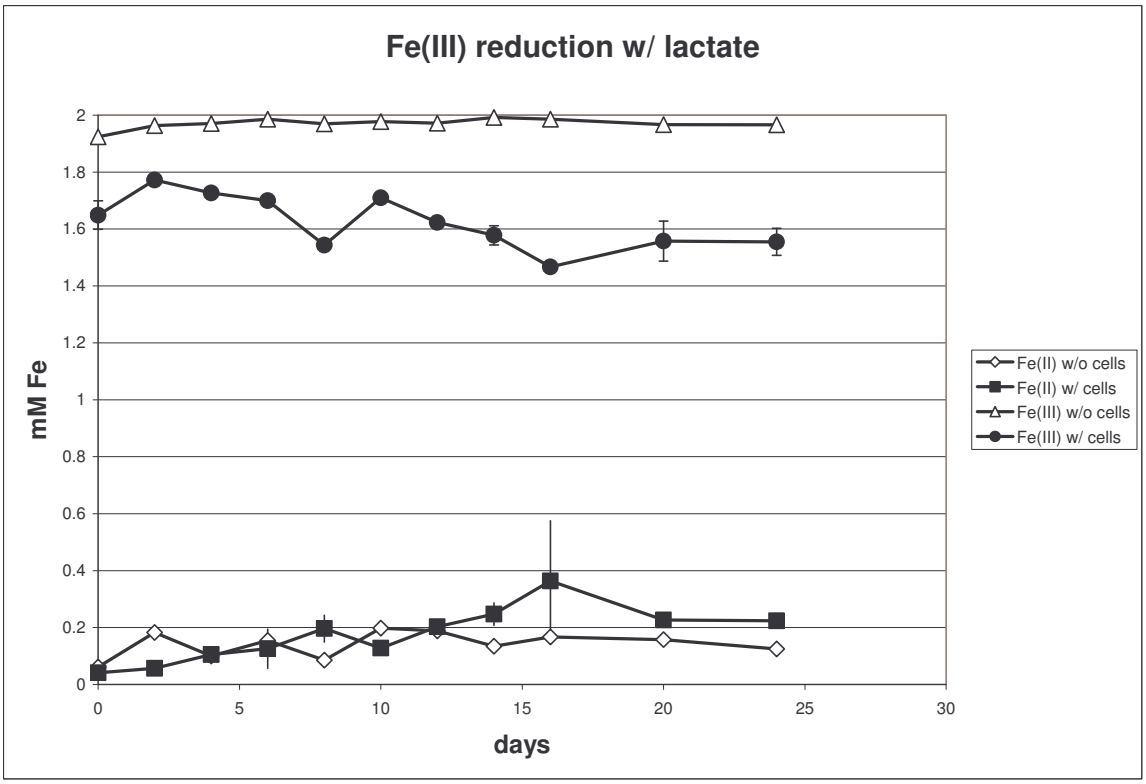

Figure 5.3.4. Fe(III) reduction and Fe(II) accumulation when lactate served as substrate.

After 24 days of inoculation, the isolate was only able to reduce about 11% of the initial 2.0 mM Fe(III) on 20 mM lactate. Although overall Fe(III) reduction rates appear slower on lactate than acetate, the reduced overall reduction rates is due to the differing cell concentrations between the two experiments. Specific Fe(III) reduction rates on lactate actually exceeded specific Fe(III) reduction rates on acetate by a factor of nearly 3 (Table 5.3.2).

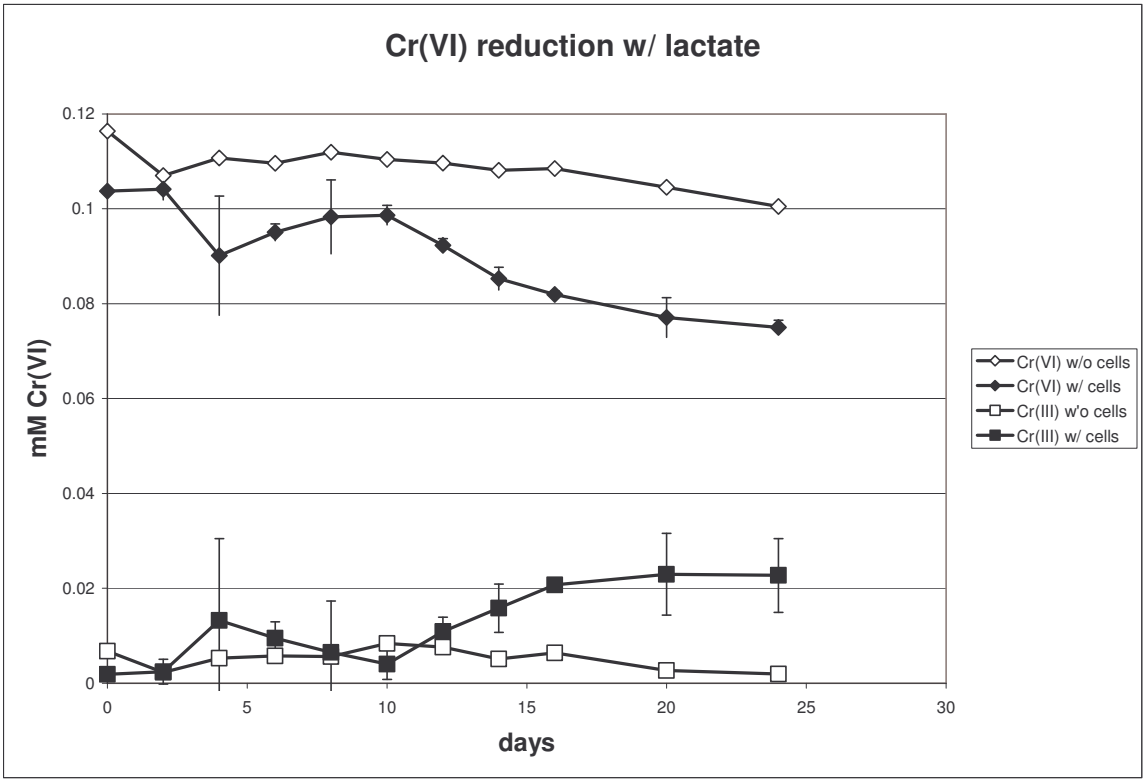

Figure 5.3.5. Cr(VI) reduction and Cr(III) accumulation when lactate served as substrate.

Figure 5.3.5 depicts Cr(VI) reduction on lactate over a 24 day period, during which time 26% of the initial 0.1mM Cr(VI) was reduced. Similar to Figures 5.3.1 and 5.3.2, Cr(VI) reduction rates only appear faster than Fe(III) reduction rates due to differing scales. Specific Fe(III) reduction rates exceeded Cr(VI) reduction rates by a factor of about 8 (Table 5.3.2).

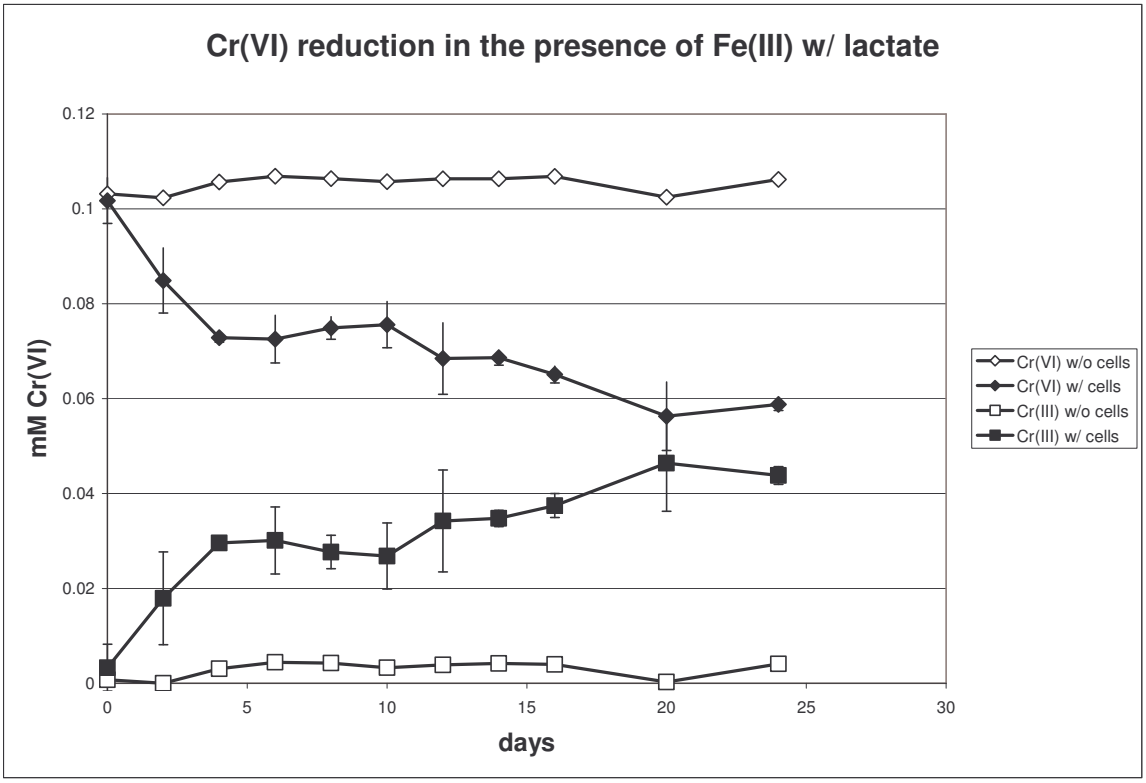

Figure 5.3.6. Cr(VI) reduction in the presence of Fe(III) when lactate served as substrate.

Figure 5.3.6 displays Cr(VI) reduction in the presence of 0.5 mM Fe(III) with lactate as substrate. Once again, Cr(VI) reduction was not markedly enhanced by the presence of Fe(III). In the absence of Fe(III), 25% of the original 0.1 mM of Cr(VI) was reduced in the lactate containing media (Fig. 5.3.5), compared to 41% in the presence of Fe(III) (Fig. 5.3.6). Although this is a 64% increase, the specific reduction rates between the two were comparable (Table 5.3.2). Total Fe and Fe(II) measurements remained constant (0.5 mM and 0 mM respectively) throughout the experiment (data not shown).

Table 5.3.1.
Metal reduction rates when acetate served as substrate. Protein concentration had a constant value of 80.1 +/- 13% mg-protein/L.

*Tabulated rates = actual rates times 10^4

Metal Reduced	Fe(III)	Cr(VI)	Cr(VI) in presence of Fe(III)
Maximum reduction rate* ($mM\text{-}metal \cdot day^{-1} \cdot mg\text{-}protein^{-1}$)	1.8 ± 0.81	1.6 ± 0.24	1.25 ± 0.2
Half-saturation constant (mM)	NA	0.15 ± 0.01	0.10 ± 0.01

Table 5.3.2.
Metal reduction rates when lactate served as substrate. Protein concentration had a constant value of 19.3 +/- 4.5% mg-protein/L.

*Tabulated rates = actual rates times 10^4

Metal Reduced	Fe(III)	Cr(VI)	Cr(VI) in presence of Fe(III)
Maximum reduction rate* ($mM\text{-}metal \cdot day^{-1} \cdot mg\text{-}protein^{-1}$)	5.03 ± 1.5	$.622 \pm 0.04$	$.725 \pm 0.06$

In summary, metal reduction was observed in each set of trials, although complete reduction was not achieved in any of the experiments. Although on the same order of magnitude, specific reduction rates in lactate media exceeded specific reduction rates in acetate media. Percentage wise, Fe(III) underwent the least amount of reduction, regardless of substrate. Compared to Fe(III), a relatively greater amount of Cr(VI) was reduced, although specific reduction rates were slower. In addition, Cr(VI) reduction was not markedly enhanced by the presence of Fe(III).

CHAPTER 6

DICUSSION

6.1 Sample characterizations

Many species of *Halomonas* bacteria are known to exist (Mata, 2002) and have been isolated from soda lakes all over the world (Duckworth, 1996). However, no reports of *Halomonas* species isolation from Soap Lake samples currently exist in the literature, although *Halomonas campisalis* was isolated from a salt flat near Alkali Lake, north of Soap Lake (Mormile, 1999). In addition, no reports of metal reduction of Soap Lake isolates of any genera exist, thus adding to the overall contribution of this thesis to the understanding of the Soap Lake ecosystem and soda lakes in general, since culturing soda lake microorganisms is considered to be a major hurdle to the understanding of soda lake microbial communities (Jones, 1998).

If indeed the dominant organism in the Soap Lake consortium is a fungus, knowledge concerning the microbial communities of the lake could be greatly extended, since bacteria have remained the prime subject of investigation. In addition, alkaliphilic strains of the fungus *Aspergillus niger* have been used to study the ability of its acidic metabolic products to degrade the cement used in nuclear waste disposal (Perfettini, 1991). Iron reduction by fungal species has received wide attention since it is a crucial step in the degradation of wood by brown-rot fungi, the most common and destructive type of decay in wood structures in the northern hemisphere (Xu, 2001). However, little can be said about the results of the experiments using the consortium, and few, if any, comparisons with previously published data can be made until further characterization has been carried out.

6.2 Comparison with previously reported reduction rates

Table 6.2.1 contains a list of reported Fe(III), Cr(VI), and Fe(III) mediated Cr(VI) maximum specific reduction rates with acetate and lactate as substrates, and a comparison between these rates and the rates found in Table 5.3.1 and 5.3.2.

Table 6.2.1
*in mmol/(mg-protein*day)
[a]Values equal published rate/corresponding rates in Table 5.3.2.
[b]Assuming 1.5e10 cells is approximately 1 mg-protein.
[c]Assuming 1 mg dry cell weight is approximately .5 mg-protein

Metal	Substrate	Isolate	max. specific reduction rate*	reference	relative difference[a]
Fe(III)	acetate	*Geobacter metallireducens* GS-15	.462[a]	Liu, 2002	2.60E+03
	lactate	*Shewanella alga* BrY	133[a]		2.60E+05
		Shewanella putrefaciens CN32	1.13[a]		2.20E+03
		Shewanella oneidensis MR-1	204[a]		4.10E+05
Cr(VI)	acetate	*Microbacterium sp.* MP30	1.14E-03	Pattanapipitpaisal, 2001	7.1
		Pseudomonas stutzeri CMG462	7.20E-05	Badar, 2000	.45
		Pseudomonas stutzeri K2	1.34E-04		.83
		Pseudomonas stutzeri J3	7.68E-05		.48
	lactate	*Shewanella alga* BrY	1.69e-2[a]	Liu, 2002	270
		Shewanella putrefaciens CN32	6.63e-2[a]		1.10E+03
		Shewanella oneidensis MR-1	1.30e-2[a]		2.10E+02
Cr(VI) in the presence of Fe(III)	lactate	*Shewanella alga* BrY	7.68e-2[b]	Wielinga, 2001	610
		Shewanella alga BrY	.172[a]	Fendorf, 2000	1.4E+03

It is readily apparent that on average the reduction rates calculated as part of this study are slower than previously reported rates using different isolates. This is particularly true for Fe(III) reduction, which was found to be thousands of times slower than the reduction rates calculated by Liu et al. (2002). Less drastic are the reduction rates for Cr(VI) reduction, particularly Cr(VI) reduction rates when acetate is used as substrate. Pattanapipitpaisal et al. (2001) reported rates very comparable to those calculated in this study, and Badar et al. reported Cr(VI) reduction rates that were on average about half as fast as those found in this study (Badar, 2000). On lactate, and depending on the isolate, Liu et al. (2002) reported Cr(VI) reduction rates hundreds or thousands of times faster than reported here. Concerning Cr(VI) reduction in the presence of Fe(III), both Wielinga et al. (2001) and Fendorf et al. (2000) reported Cr(VI) reduction rates that were also thousands of times faster than reported here. Although not shown in Table 6.1.1, the K_S values corresponding to Cr(VI) reduction and Cr(VI) reduction in the presence of Fe(III) on acetate were very comparable to the K_S values calculated by Shen and Wang (1994) in their work with *Escherichia coli* ATCC 33456.

Although slower in each case, Cr(VI) reduction rates calculated in this study were within one order of magnitude of the corresponding Fe(III) reduction rates when lactate served as substrate, and were statistically equivalent when acetate served as substrate. This mirrors the behavior of *Shewanella putrefaciens* CN32, which, in a study performed by Liu, et al. (2002), was found to reduce Fe(III) less than 20 times faster than Cr(VI) (Table 6.1.1). This is compared to *Shewanella alga* BrY and *S. oneidensis* MR-1, which reduced Fe(III) faster than Cr(VI) with lactate as substrate by factors of 8,000 and 16,000, respectively.

Compared to Cr(VI) reduction rates reported by Liu, Wielinga et al. (2001) and Fendorf et at. (2000) found that Cr(VI) reduction by *Shewanella alga* BrY could be increased by an order of magnitude with the addition of Fe(III). Since Fe(III) tends to get reduced faster than Cr(VI), and since Fe(II) reduces Cr(VI) to Cr(III), Cr(VI) reduction rates can theoretically be enhanced through the electron shuttling action of the Fe(II). However, no such enhancement took place in this study, suggesting that in the presence of Cr(VI), Fe(III) reduction was not necessarily faster.

6.3 Potential for Bioremediation Purposes

According to the National Primary Drinking Water Standards established by the EPA, Cr(VI) concentrations are not to exceed 100 ppb. None of the trials led to the removal of enough Cr(VI) to comply with EPA standards, even after 24 or 25 days of inoculation. Overall reduction rates could be made more efficient using a variety of options. A substrate more conducive to bacterial growth and activity, such as tryptic soy broth, could be used, but this would add to the overall cost of the process (Pattanapipitpaisal, 2002). For example, only the purest and most expensive reagent grade acetate can approach the $100/kg one would have to pay for tryptic soy broth. A recent study demonstrated the impact of pH and salt concentrations on the activity of alkaliphilic bacteria, particularly *Halomonas campisalis* (Alva, 2003). Based on these results, optimizing the pH and salinity could potentially increase the metal reduction rates of the *Halomonas* species used in this study by one to two orders of magnitude. Increasing cell density does not necessarily represent a satisfactory solution either, since higher cell densities cannot be assumed capable of a directly proportional increase in

reduction capability, as was observed by Pattanapipitpaisal, et al. (2001). Further metal reduction studies using different cell concentrations would have to be carried out in order to determine how beneficial increased cell density would be for this process.

Experiments are also needed to further ascertain the fate of the Cr(VI) species after reduction to Cr(III). This is needed in order to determine whether or not easily removable hydroxide precipitates form, on which potential application to industrial wastewater treatment hinges. In addition, the fate of the Cr(VI) would need to be known if its behavior in the soil or groundwater, and subsequently its environmental mobility, is to be predicted.

CHAPTER 7

CONCLUSIONS

Metal reduction has been demonstrated in alkaline media using microorganisms taken from different portions of Soap Lake, Washington. From the mixolimnion layer of the lake a member of the bacterial genus *Halomonas* was isolated, with *H. salina* and *H. ventosae* representing the closest genetic neighbors. The isolate was able to anaerobically and under non-growth conditions couple the oxidation of either acetate or lactate to the reduction of Fe(III) and Cr(VI), and Cr(VI) in the presence of Fe(III). While Fe(III) reduction rates slow compared to previously reported rates, Cr(VI) reduction rates were comparable. The presence of Fe(III) was not found to enhance Cr(VI) reduction rates, contrary to findings of many who have found the electron shuttling action of Fe(III) beneficial in increasing reduction rates.

From the sediment of the monimolimnion layer of Soap Lake a consortium was enriched with Fe(III) reducing microorganisms, which upon inspection appear to be non-bacterial. The consortium was able to grow on either acetate or lactate, and couple the oxidation of these substrates to the reduction of Fe(III) under anaerobic conditions. The consortium was unable to grow on or reduce Cr(VI), whether Fe(III) was present or not.

CHAPTER 8
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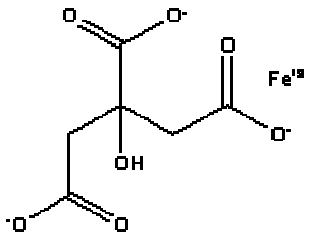
APPENDIX A

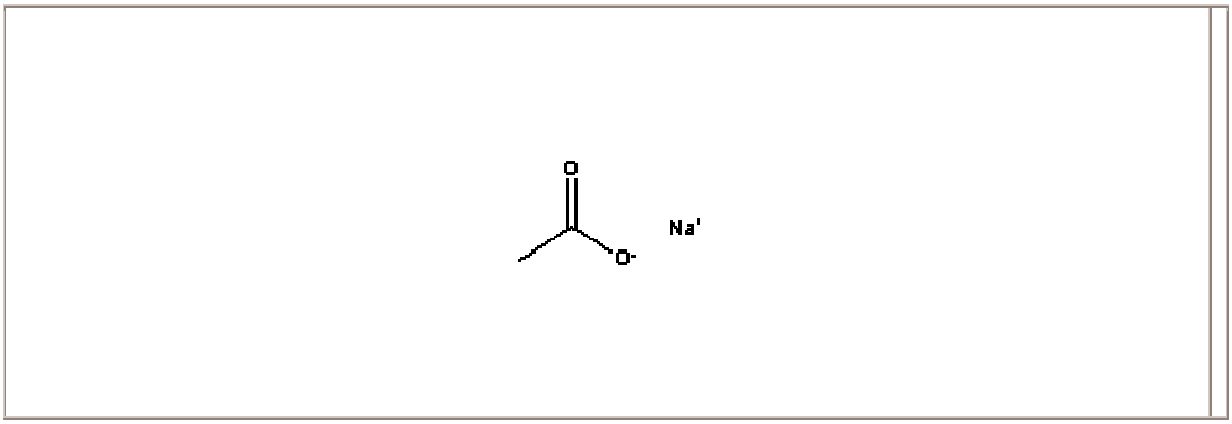
CHEMICAL STRUCTURES

ferric citrate, n-hydrate [2338-05-8]
Synonyms: 2-hydroxy-1,2,3-Propanetricarboxylic acid, iron salt; Iron Citrate; Ferric Citrate; Ferric citrate pentahydrate; Iron (III) citrate hydrate; ferric citrate, n-hydrate; Iron (III) citrate n-hydrate;



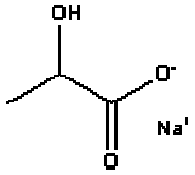
Formula	$C_6H_5FeO_7$	Molecular Weight	244.9463
CAS RN	2338-05-8	Melting Point (°C)	
ACX Number	X1002527-5	Boiling Point (°C)	
Density		Vapor Density	
Refractive Index		Vapor Pressure	
Evaporation Rate		Water Solubility	
Flash Point (°C)		EPA Code	
DOT Number		RTECS	
Comments	LIGHT SENSITIVE.		

Sodium acetate [127-09-3]
Synonyms: Sodium ethanoate; Sodium acetate; Acetic acid, sodium salt; Acetic acid, sodium salt (1:1);



Formula	$C_2H_3NaO_2$	Molecular Weight	82.03427
CAS RN	127-09-3	Melting Point (°C)	58
ACX Number	X1001489-6	Boiling Point (°C)	
Density	1.45	Vapor Density	
Refractive Index		Vapor Pressure	
Evaporation Rate		Water Solubility	
Flash Point (°C)		EPA Code	
DOT Number		RTECS	AJ4300010
Comments	HYGROSCOPIC.		

Sodium lactate [72-17-3]
Synonyms: Lacolin; Lactic acid, monosodium salt; Propanoic acid, 2-hydroxy-, monosodium salt; Sodium-DL-lactate; Sodium-DL-lactate, 60 wt% aqueous solution; Sodium lactate;



Formula	$C_3H_5NaO_3$	Molecular Weight	112.06047
CAS RN	72-17-3	Melting Point (°C)	
ACX Number	X1001037-2	Boiling Point (°C)	
Density	1.326	Vapor Density	
Refractive Index		Vapor Pressure	
Evaporation Rate		Water Solubility	
Flash Point (°C)		EPA Code	
DOT Number		RTECS	OD5680000
Comments			

APPENDIX B

PROCEDURE FOR PROTEIN ANALYSIS

(PREPARED BY CATHERINE ALBAUGH, 2005)

Protein Analysis Procedure

- Always use **Axygen** microcentrifuge tubes and pipette tips for protein analysis.
- Place a large beaker of water in the refrigerator to be used for a cold water bath later.
- Thaw the protein samples to be analyzed.
- Turn on the lamps for the UV-Vis, after ~15minutes take a blank measurement with water.
- The bottle of dye to be used (Coomassie Blue, kept in the refrigerator, only good for 1yr) should be gently inverted once to mix any settled material – do not shake the bottle.
- Pour out a little more than the amount needed (the number of samples x 2mL dye) into a beaker and let it come to room temperature.
- Once the samples have thawed, centrifuge for ~10minutes. Remove 0.9mL of the supernatant using a pipette with an Axygen pipette tip (taking care not to remove any of the biomass settled at the bottom. The supernatant can be disposed of.
- Add 0.9mL of pH-adjusted media to re-suspend the cells, shake up the tube and centrifuge a second time. Remove the supernatant again and replace with fresh media. (Use one pipette tip for removing the supernatant, and one for adding the media.)
- Place centrifuge tubes into a cold water bath and sonicate each tube for 1.5minute x 2. The sonicator should be set for 1minute at 30% (no temperature set). When each sample has been sonicated for a total of 3minutes, the cells should be broken apart so that the cell protein can be measured.

- Mark 7 large test tubes. Break open a vial of albumin which will be used as the protein standard. Compose the 7 solutions as directed using the albumin and pH-adjusted media. Make sure all solutions are well mixed before transferring anything to another tube.

Test Tube	Media	Albumin	Other
A (0.0mg/L)	4.00 mL	x	x
B (2.5mg/L)	1.50 mL	x	1.5 mL of C
C (5.0mg/L)	2.00 mL	x	2.0 mL of D
D (10mg/L)	2.50 mL	x	2.5 mL of F
E (15mg/L)	3.97 mL	30 µL	x
F (20mg/L)	4.95 mL	50 µL	x
G (25mg/L)	2.37 mL	30 µL	x

- Mark 3 small test tubes for each large one (21 altogether). Pipette 0.5mL of each concentration into each of the 3 small test tubes. Pipette 0.5mL of room temperature Coomassie Blue into each small tube and mix the solution well. Rinse the cuvette once with 1mL of **HPLC grade methanol**, and then rinse it twice with water between measurements. Take absorbance measurements for each small tube at 595nm. Record this absorbance data against the known concentration of each standard and construct a protein calibration curve.
- Label a small test tube for each sample. Pipette 0.5mL of each sample into the appropriate test tube (it is easiest to use the same pipette tip for transferring all samples – samples should be transferred in order of increasing protein concentration (i.e. increasing experiment time). Pipette 0.5mL of the room temperature Coomassie Blue into each test tube. The dye and the protein sample should always be at a 1:1 ratio.

- Beginning with the sample of smallest protein concentration (time zero), mix the test tube immediately before pouring sample into the cuvette, then measure the absorbance at 595nm.
- Dispose of the liquid in the beaker by pouring it into the waste container in the hood.
- Turn off the instrument lamps when finished.
- When calculating protein concentration from the calibration curve, make sure to correct for the absorbance at 0mg/L protein for all samples.

APPENDIX C

PROCEEDURE FOR SEM SAMPLE FIXATION

ELECTRON MICROSCOPY CENTER January, 2005
WASHINGTON STATE UNIVERSITY

Procedure for fixing and embedding animal tissue with SPURRs

Remember to label all vials with; sample name, fixative type, your name & date

Fixation:	2.5% Glutaraldehyde/2% Paraformaldehyde	
	in 0.1 M Phosphate or Cacodylate buffer	Overnight
Rinse:	0.1 M Phosphate or Cacodylate buffer	3x 10 mins ea
Optional:	Post fix: Osmium Tetroxide 1%	2h- overnight
Rinse:	0.1 M Buffer	2x 10 mins ea

Dehydration:	30% Ethanol	10 mins
	50% Ethanol	10 mins
	70% Ethanol	10 mins
	95% Ethanol	10 mins
	100% Ethanol	10 mins
	Ethanol: Acetone	
	1:1	10 mins
	100% Acetone	2x 10 mins ea

100%	HMDS
100%	HMDS

Air dry on filter paper or in a vial.

Gold coat using sputter coaters (Technics Hummer V, Anatech)

APPENDIX D

PROCEEDURE USED BY LARAGEN, INC. FOR 16S rRNA SEQUENCING

Sample requirements: Cultures should be grown in Terrific Broth in 96-well growth blocks or any 16 or 17 X 100 mm culture tubes. Growth blocks require 22 to 24 hours of incubation with 250 rpm shaking. Samples should be centrifuged, decanted and the pellets should be sent to us frozen.

Plasmid DNA Mini-prep for Fluorescent DNA Sequencing
(This protocol was originally obtained from Diane England and modified by Andy Blasband, ABI June 15, 1991.)

1. Pellet 1.5ml of culture grown in terrific broth (recommended) for 1 minute in a microcentrifuge. Remove all the supernatant by aspiration. If larger quantities of DNA are desired then spin down 3ml of culture.
2. Resuspend pellet in 200ul of GTE buffer (50mM Glucose, 25mM Tris pH 8.0, 10mM EDTA pH 8.0) by pipetting up and down.
3. Add 300ul of 0.2N NaOH/1% SDS buffer (freshly made), mix by tube inversion. Do Not Vortex . Incubate on ice for 5 minutes.
4. Add 300ul of 3.0M KOAc, pH 4.8 (3.0M NaOAc pH 4.8 also works). Mix by tube inversion. Do Not Vortex . Incubate on ice for 5 minutes.
5. Spin in microcentrifuge for 10 minutes at room temperature and transfer supernatant (approximately 700 to 750ul) to a new tube.
6. Add RNase A to a final concentration of 20ug/ml (approximately 2ul of 10 mg/ml stock). Incubate at 37C for 20 minutes.
7. Do two chloroform extractions using 400ul/extraction. (The tube capacity is too small do do equal volume extractions, half volume works fine.) Do not use phenol, use straight chloroform. Mix layers by shaking not vortexing. Spin extractions for 1 minute to separate phases and remove aqueous phase to a clean tube.
8. Precipitate DNA by adding an equal volume of isopropanol (700 to 750ul). Place tube on ice for 10 minutes.
9. Pellet DNA for 15 minutes at room temperature. Rinse pellet with 500ul of 70% ETOH. Pour off ETOH and dry the pellet in a speed vac.
10. Dissolve pellet in 32ul of dH2O. Add 8ul of 4M NaCl and 40ul of 13% PEG8000. Mix well and on ice for 20 minutes, longer incubations will improve yields.
11. Spin for 15 minutes at 4C. Remove supernatant (be careful as the pellet is translucent at this point) and rinse pellet with 500ul of 70% ETOH.
12. Pour off ETOH, dry pellet in a speed vac and resuspend in 20 to 30ul of water.

Terrific Broth
Add 100ml of a sterile solution of 0.17M KH2P04 and 0.72M K2HPO4 to 900ml of base broth. (Base broth = 12g of Bacto-tryptone, 24g of Bacto-yeast extract, 4ml of glycerol, q.v. to 900ml with dH2O and autoclave).