NOTE TO USERS

Investigation of the role of electron mediators and siderophores on the concurrent biodegradation of naphthalene and reduction of hexavalent chromium

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Abstract

The concurrent biodegradation of polycyclic aromatic hydrocarbons (PAHs) and transformation of heavy metals by redox reactions has been demonstrated by several studies. The mechanisms and reactions that result in heavy metal transformation in such systems is, however, poorly understood. This study explored the role of metabolic intermediates, metabolic end-products, and microbial chelators generated by a PAH-degrading microorganism, *Pseudomonas putida* ATCC 17484, in reducing or transforming Cr(VI). This study investigates the role of 1,2-naphthoquinone to serve as potential redox mediators in the reduction of Cr(VI) to Cr(III). Laboratory experiments were conducted to evaluate the extent of Cr(VI) reduction in the presence of various concentrations of the naphthalene biodegradation by-product 1,2-naphthoquinone, and the metabolic intermediate 1,2-dihydroxynaphthalene. Rapid, but limited reduction of Cr(VI) was observed in the presence of 1,2-dihydroxynaphthalene. The effect of siderophore Desferrioxamine B (DFB), a common chelator produced by the soil microorganisms, on the biodegradation of naphthalene was also investigated. Synthesized DFB was added directly into the test systems, and resulted in instantaneous binding to chromium (VI). The chelated complex remained stable for a period of 4 days even when subjected to acidic conditions.

Résumé

La co-décontamination des hydrocarbures aromatiques polycycliques avec des métaux lourds à l'aide des bactéries fût prouvée possible antérieurement. Cependant, les mécanismes et les réactions qui mènent aux transformations des métaux lourds demeurent obscures. Cette étude explora le rôle des métabolites intermédiaires, des produits finaux métaboliques, et des sidérophores générés par le microorganisme, *Pseudomonas putida* ATCC 17484, dans la réduction ou transformation du chrome (VI). Cette étude enquêta sur le rôle potentiel du 1,2-naphthoquinone comme médiateur de réactions réduction-oxidation dans la réduction du chrome (VI) au chrome (III) Des expériences furent effectuées pour évaluer l'ampleur de la réduction du chrome (VI) avec la présence de diverses concentrations du sous-produit de la biodégradation du naphthalène, le 1,2-naphthoquinone ainsi que le métabolite intermédiaire 1,2-dihydroxynaphthalène. La réduction du chrome (VI) s'avéra rapide mais limité avec la présence de 1,2-dihydroxynaphthalène. L'effet du sidérophore Desferrioxamine B, un chélateur de métal communément secrété par des bactéries terrestres, sur la réduction du chrome (VI) et la biodégradation du naphthalène fût également étudié. Avec l'ajout du sidérophore dans le système, la rapide formation d'un complexe sidérophore-chrome fût observée. Le nouveau complexe demeura stable durant les quatre jours d'observation et même lorsque soumis à des conditions acides.

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Table of Contents

List of Tables

List of Figures

1.0 Introduction

At many industrial sites and hazardous-waste sites, the soil and groundwater is contaminated with many pollutants that are known to be hazardous and toxic. A major factor hindering clean-up attempts at such sites is the co-occurrence of organic compounds and heavy metals, so-called mixed waste sites. Approximately 49% of all hazardous waste sites surveyed were contaminated with both organic and metal pollutants (PRC Environmental Management Inc., 1997). Organic pollutants co-occurring with metal contaminants are commonly found at major industrial sites, disposal sites for contaminated soils and sediments, as well as at many brownfield sites. Many of these sites are contaminated with two major pollutant classes: polycyclic aromatic hydrocarbons (PAHs) and heavy metals (Fischer et al., 1999). These pollutants are often present together at sites where prolonged activities related to various industrial operations such as petroleum processing, mining, wood treatment with creosote, and chemical (inks, paints etc.) manufacturing have occurred (Mueller et al., 1989; Luthy et al., 1994; Kong et al., 1995). Both PAHs and heavy metals cause significant adverse effects on the environment and on human health and are listed as priority pollutant in Canada, U.S. and most other countries. To date, research on remediation technologies as well as practice has focused on either organic compounds or metals but not on mixed-waste sites (NRC, 1994).

The most common heavy metals found in contaminated sites are: lead, chromium, arsenic, zinc, cadmium, copper, and mercury (Fischer et al., 1999). The chemical

speciation of the heavy metal influences its mobility, solubility, and toxicity. The speciation of the metals depends upon the source of the metal waste, and the soil chemistry. The soil chemistry comprises of pH, Eh (redox potential), ion exchange capacity, and the concentration of organic matter capable of chelating with the metal. Traditional treatment technologies for heavy metal-contaminated sites include excavation and off-site disposal, soil-washing with chelating agents, and immobilization of the metals in cementitious materials. Recently in-situ reduction of chromates by zero valent iron in permeable barriers has been proposed (Blowes et al., 1999). However, none of the above options can address remediation of co-occurring organic compounds.

Promising approaches for mixed-waste sites are phytoremediation and electrokinetic remediation (Lye et al., 2003). Although phytoremedation may have significant potential for rhizosphere bioremediation of PAHs and concurrent phytoaccumulation of heavy metals, it can only be used for near ground-surface contamination (Pivetz, 2001). Electrokinetic remediation is another promising technology but has limitations in that only very soluble contaminants are effectively extracted above ground (Sandia National Laboratories, 1997). Since PAH compounds have low aqueous solubility, their removal will be slow and extracted contaminants require further treatment.

Bioremediation technologies are generally cost-effective, but until recently have not been considered for mixed-waste sites because it is perceived that the presence of metal co-contaminants will inhibit biodegradation of organic pollutants, making

bioremediation ineffective (Shen and Wang, 1995b). Furthermore, the potential for transformation of heavy metals by microorganisms has been only recently understood (Lovley, 1993).

One of the challenges in employing bioremediation is providing the right conditions in the field where microorganisms could be metabolically active and facilitate the desired reactions. A thorough understanding of the processes involved in biodegradation of PAHs and transformation of heavy metals, and of the inter-relationships between various processes is required for development of efficient bioremediation strategies.

This study explored the role of quinonic compounds produced during the biodegradation of naphthalene, a PAH compound, in the reduction of the toxic and highly soluble Cr(VI) to its relatively less toxic and insoluble form Cr (III). Al-Hakak (2000) observed that the addition of quinonic compounds can significantly increase the biodegradation rate of naphthalene. Rau et al. (2002) have shown that the biodegradation of sulfonated azo compounds, nitro aromatic compounds, and hexachloroethane is accelerated by the presence of quinonic compounds.

The study also investigated the role of siderophores in PAH biodegradation and Cr(VI) removal. Siderophores are low molecular weight high-affinity ferric iron chelators that are synthesized and secreted by many microorganisms in response to iron deprivation (John et al., 2001). Thus siderophores chelate various valence state metals and serve as an

agent for metal transport for microorganisms (Hernlem et al., 1999; Kraemer et al., 2002). The use of siderophore-producing microorganisms for degradation of organic compounds in the presence of heavy metals have not been reported to date, and has the potential to be a unique approach for the bioremediation of organic compounds at mixed waste sites. Furthermore, the ability of a siderphore to chelate hexavalent state chromium was investigated.

1.1 Chromium in the environment

Chromium is a heavy metal since its density (7.14 g/cm^3) is higher than the 5 g/cm^3 benchmark (Weast, 1984). It is a metal used in electroplating, wood treatment, leather tanning, pigments manufacture and cooling tower treatment for corrosion control (Palmer and Wittbrodt, 1991). Chromium can enter drinking water sources through discharges from industries, leaching from hazardous waste sites, and from erosion of natural deposits (Barnhart, 1997). Chromium is also used as a dye in both textile and glass industry (Palmer and Wittbrodt, 1991). The most common forms of chromium in the environment are chromium (III) and chromium (VI) (Evanko and Dzombak, 1997). Like most metals, chromium is not found as free metal in nature, but rather as compounds like chromium hydroxide $CrOH^{2+}$, precipitate $Cr(OH)_3$, chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$) forms (Schmieman et al., 2000). Chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$) are the major species of hexavalent chromium found in the environment (Schmieman et al., 2000). Sources of chromium contamination include electroplating process waste and the release of the wood preservative mixture of chromate, copper, and arsenate (CCA) into the environment (Mueller et al., 1989). In the environment,

chromium's chemical form is determined by pH and the redox conditions (Evanko and Dzombak, 1997). Nevertheless, in the environment chromium can be found in both species, its hexavalent and its trivalent less toxic form depending upon the environment's condition. Each form has unique characteristics. The hexavalent form of chromium is toxic, and may cause cancer if moderate respiratory intake of Cr(VI) occurs or if significant oral intake occurs (Kong et al., 1995). The U.S. Environmental Protection Agency (USEPA) has classified chromium (VI) as a human carcinogen by inhalation. In 1991, USEPA reviewed the existing chromium standard, and raised the Maximum Contaminant Level (MCL) from 0.05 mg/L (1975 Interim Drinking Water Standard) to 0.1 mg/L as total chromium, based on its decision that hexavalent chromium was not as carcinogenic by ingestion (USEPA, 1998).

The solubility of the Cr(VI) compounds is variable, but is generally higher than 100 g/L at pH 7 (Cross, 1997); it is quite high compared to its trivalent less toxic form (at pH 7.0, the solubility is about 0.1 mg/L) (Cross, 1997). This causes hexavalent chromium to have a greater potential for groundwater contamination. While in its trivalent form, its solubility is reduced and is also less toxic (Schmieman et al., 2000). Thus reduction to the trivalent form constitutes a feasible option of remediating contaminated soils if the reaction is not reversible. In other words, it is best to ensure that the trivalent chromium will not be oxidized back to its initial hexavalent form.

1.2 PAHs in the environment

PAH molecules are made up of multiple fused benzene rings and are carcinogenic and mutagenic. Incomplete oxidation during combustion of carbon-containing materials

leads to the formation of PAHs (Masters, 1997). The persistence of a PAH depends directly upon the number of fused benzene rings it has. A higher molecular weight means a higher number of fused benzene rings for the PAH, and a longer time required to break the rings (Cerniglia, 1992). Benzo[a]pyrene, a PAH with five fused benzene rings, is well known for its ability to cause lung and kidney cancer (Masters, 1997). While naphthalene, the PAH studied here, is composed of two fused benzene rings and can potentially cause haemolytic anaemia if ingested or exposed acutely (De Flora, 2000; Faust, 1993).

PAHs have mainly entered into the environment due to careless petroleum manufacturing and related industries of wood preservation practices (Fischer et al., 1999). Other sources include waste incinerators, coal and oil power plants, and smeltering of metals, and motor vehicle exhaust (Fischer et al., 1999). In groundwater contaminated with PAHs, a partitioning between the aquifer and PAHs occurs because of the limited aqueous solubility and the hydrophobic nature of PAHs (Masters, 1997). As a result the PAHs lay floating atop or beneath the aqueous phase like a nonaqueous phase liquid (NAPL) and dissolves slowly, but continuously into the aqueous phase. Most PAHs are dense NAPLs with a few exceptions.

1.3 Research objectives

The main objective of this study is to attempt to increase the rate of biotransformation of naphthalene and the reduction of hexavalent chromium when both are found as co-contaminants. Two chemicals were utilized: 1,2-naphthoquinone, from

the quinone family, and Desferrioxamine B (DFB), a siderophore. 1,2-naphthoquinone was selected as the quinone to be used, because it is a metabolic by product from the mineralization of naphthalene. So an attempt was made to understand the possible role of naphthoquinone in the biosystem and to identify its effect on the biotransformation system. The second part of the study focused on the second chemical used DFB. An attempt was made to explore the effect of the siderophore towards the microorganism culture, the chelation with the chromium metal, and towards the whole biotransformation system. The ultimate objective is to see if the biotransformation rate will increase when those chemicals are added to the system.

Similar to the approach taken by Rau et al. (2000) dealing with the quinoid remediators in the azo dye study, the current research adopts a similar experiment scheme to assess the reduction potential of the quinone, but under aerobic conditions and replacing the nitroaromatic compound with chromium (VI) and naphthalene. Thus, the first part of this study deals with the investigation of the following hypothesis (see Figure 2): NADH produced by the bacteria is able to rapidly reduce the quinone to hydroquinone, and which in turn, reduces the hexavalent chromium to its trivalent form (Lovley, 1993; Schmieman et al., 2000; Shen and Wang, 1994a, 1994b). Another goal of this study is to evaluate whether the Desferrioxamine B siderophore's ligand will be able to bond with hexavalent chromium metal and whether the chelated complex is recognized, uptaken, and used by the *Pseudomonas putida* bacteria. To sum it all up, the main research objectives are to understand the role and the influence of each compound

in the biodegradation mixture. The biodegradation profile of naphthalene with the addition of these two chemicals is also explored.

Although quinone mediators have already been deeply investigated in azo dyes degradation studies, no known research has dealt with aerobic conditions and chromium as the compound to be reduced. Since the present research leans on the reduction of heavy metal under aerobic conditions, oxygen will be in competition with the quinone for the role of terminal electron acceptor. It is interesting to see how well the quinone would behave in an aerobic environment. A secondary objective is to obtain the best scheme for optimized rate of biodegradation with the said additives and suggest a range of concentration the chemicals that should be used. To end this research, an attempt to clarify the profile of naphthalene mineralization under systems containing 1,2-dihydroxynaphthalene, and siderophore was done.

1.4 Approach and scope of the study

The study has been divided in two main parts: the first deals with the investigation of the effect of 1,2-naphthoquinone on chromium reduction, and the second tackles the effect of siderophore on the chromium bioremediated reduction process. Both parts utilized biometer flasks containing aqueous solutions of naphthalene dissolved in HMN, chromate, mineral salts, bacteria strain *Pseudomonas putida* ATCC 17484, and other chemicals of interest. Heptamethylnonane (HMN) is used, because it provides a uniform naphthalene rate of dissolution. Naphthalene is continuously released from the NAPL (HMN) to the aqueous phase as it was degraded by the bacteria.

For the quinone study, it has been hypothesized that first the NADH reduces the quinone (Rau et al., 2002; Saffarini et al., 2002; Schwarzenbach et al., 1990). Since NADH is not readily available in bulk quantity (the bacteria produces too little), the experiment has been conducted via chemical reduction of quinone via manual addition; thereby simulating the reducing effect of bacterial produced NADH. HPLC analyses were used to assess the concentrations of 1,2-naphthoquinones, 1,2-dihydroxynaphthalene, and NADH. While radiolabelled (^{14}C naphthalene) and the diphenycarbazide methods were used to monitor the mineralization of naphthalene and the concentrations of Cr(VI) left in solution, respectively.

For the siderophore studies, it has been thought that its addition to the biodegradation system might enhance the rate of reduction of chromium. An investigation into whether the siderophore desferrioxamine B (DFB) can bind with hexavalent chromium and form a stable complex under aerobic condition was performed. The siderophore employed in this study is Desferrioxamine B (DFB), because it is ubiquitous in nature and has been employed by many different studies as a model siderophore (Hernlem et al., 1996; Birus et al., 1987; Monzyk et al., 1982; Tufano et al., 1981). Colorimetric (diphenylcarbazide) method is employed to assess the concentration of the remaining Cr(VI) in solution. Optical density at wavelength 540 nm was used to determine the effect of various chemicals on its growth behaviour.

The final goal was to look at mineralization of naphthalene in batch reactors containing DFB and 1,2-naphthoquinone. Radiolabelled tracing methods were employed to assess the naphthalene mineralization profile.

1.5 Organization of thesis

The literature review, materials and methods, research, and analysis of results are presented in the next three chapters. In Chapter 2 an overview of quinone chemistry and properties is first described, followed by its usage in microbial transformation of pollutants. A description of the Cr(VI) reduction mechanisms, naphthalene biodegradation, and concurrent biodegradation and reduction of naphthalene and Cr(VI) are given afterwards. The chapter ends with a presentation on siderophore chemistry, siderophore usage in decontamination of heavy metals, the uptake and fate mechanisms of siderophore, and an estimation of the stability constant for DFB-Cr(VI) complex. In Chapter 3, a thorough description of the chemical reagents used, the preparation of the chemical stocks and experimental procedures and analytical methods (including calibration curves) is presented. Chapter 4 discusses the observed rate of Cr(VI) reduction when 1,2-naphthoquinone, 1,2-dihydroxynaphthalene, and DFB was used in concurrent treatment of Cr(VI) and naphthalene. The quinone-hydroquinone interconversion issue is also addressed. The conclusions and suggested directions for future research are discussed in Chapter 5.

2.0 Literature Review

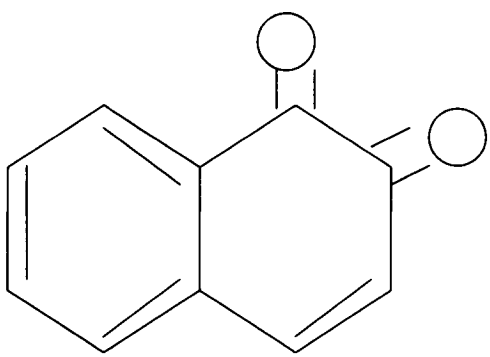
2.1 Quinone chemistry and microbial transformation of pollutants

Recent research has focused on the ability of quinone to act as an electron mediator in the reduction of polyhalogenated hydrocarbons. Quinones are di-ketones with the structure O=C-(C=C)n-C=O (Patai and Rappoport, 1988). They are derived from aromatic compounds so that the two carbonyl groups may be in different rings (Morton, 1965). The oxidation-reduction potentials of quinones are important in many biochemical systems. Because of their flexible tendency with electrons, quinone can oxidize compounds with a lower potential or reduce compounds with a higher potential (Ullmann, 1991). Quinones can be found naturally in living organisms, animals, insects, vegetation and fungi (Morton, 1965). Experiments have been performed with naturally occurring quinones such as juglone (from leaves of walnut tree, dormant male catkin buds, green shells), to research whether these chemicals can act as an electron mediators (Patai and Rappoport, 1988; Rau et al., 2002;Van Der Zee et al. 2003;Yamazaki et al., 1999). The results were positive in the degradation of azo dyes, nitro aromatic compounds and hexachloroethan (Rau et al., 2002). From these experiments, it has been concluded that only quinones with a redox potential lower than -50 mV are effective in the biodegradation of aromatic compounds (amaranth -250 mV). The redox potential of 1,2-naphthoquinone is equal to 0.135 V at a neutral pH (Morton, 1965). The standard redox potential of redox mediators should meet certain level to provide sufficient reaction rates and to be competitive with oxygen. The redox potential of hexavalent chromium, quinone, NADH, and oxygen are respectively 1.35 V, 0.135 V, -0.320 V, and 1.22 V

(Rau et al., 2002; Scifinder). So NADH can reduce the quinone which in turn can reduce Cr(VI) or give up its electron to oxygen. It is expected that oxygen will be competing with Cr(VI) for the electrons.

It has also been found that biodegradation of naphthalene may lead to 1,2-naphthoquinone metabolite (Ghoshal and Luthy, 1998). Furthermore, another study (Al-Hakak, 2000) showed the formation of 1,2-dihydroxynaphthalene when 1,2-naphthoquinone is left to dissolve in water. These results may hint that 1,2-naphthoquinone has the ability to mediate electrons by acting as electron acceptors or donors depending upon the system's redox conditions. 1,2-dihydroxynaphthalene may be the reduced form of quinone, the hydroquinone (Schwarzenbach et al., 1993).

One-electron oxidation of hydroquinones or reduction of quinones results in the formation of semiquinones; they can exist as radical anions ($Q^{\cdot-}$) or neutral radicals ($QH^{\cdot}$) (Morton, 1965). These radicals are often the intermediates in redox reactions. Semiquinones can be generated from the reduction of quinones by electron abstraction from oxygen species or other inorganic ions and by electron transfer.



Chemical Formula: $C_{10}H_6O_2$

E' (Redox Potential) = 0.135 V

Quinone(Q), Hydroquinone(QH_2), Semiquinone ($QH^{\cdot}$) relation

$QH \leftrightarrows Q^{-} + H^{+}$ ----- pK_1

$QH_2 \leftrightarrows QH^{-} + H^{+}$ ----- pK_2

$QH^{-} \leftrightarrows Q^{2-} + H^{+}$ ----- pK_3

Formation of semiquinone

$Q + QH_2 \leftrightarrows 2QH^{\cdot}$

Figure 1. Molecular structure of 1,2-naphthoquinone and various informations on quinone. Most quinone species undergo two successive one-electron reduction steps.

Naphthoquinones are used for fumarate, trimethylanmine oxide, and dimethyl sulfoxide respiration by the *Escherichia coli* strain (Saffarini et al., 2002). This principle is applicable to other bacteria species, but the quinone used may not be necessarily naphthoquinone. In most cases, quinones transfer electrons from dehydrogenases to the terminal reductases (Saffarini et al., 2002).

Quinones are very electrophylic, cyclic dicarbonyl compounds (Morton, 1965; Patai and Rappoport, 1988; Perry, 1997). By the addition of two electrons and two protons, quinones are converted into a hydroquinone which possesses a positive electron affinity (Patai and Rappoport, 1988). Benzoquinone and ubiquinone are commonly found quinones in the environment where biological reactions occur (Schmid, 1996). These chemicals serve as redox mediators in many chemical reactions (Schwarzenbach et al., 1993). Quinones are commonly used as oxidizing agents or electron acceptors (Patai and Rappoport, 1988). However, quinones may also function as electron donors if they are first reduced to hydroquinone that can give up the acquired electrons if the redox

environmental conditions favour such a reaction (Schwarzenbach et al., 1993). The hydroquinone reverts back to quinone once it loses its electrons (Schwarzenbach et al., 1993; Rau et al., 2002; Perry, 1997).

Some quinones are found to be toxic to microorganisms or do not have an adequate redox potential, and thus are not useful to mediate the biotransformation (Rau et al., 2002). Therefore more research is needed to determine which types of quinones are fit to suit the role of electron mediators in biodegradation processes of various contaminants.

Quinones may aid concurrent biotransformation of organic compound and metals because of their hydroquinone-quinone interconversion characteristics (Keck et al., 1997; Rau et al., 2002; Schmid, 1996). When Cr(VI) is present, little mineralization of naphthalene occurs (Al-Hakak, 2000). However once most Cr(VI) are reduced by the bacteria to Cr(III), naphthalene mineralization activity starts increasing and bacterial growth resumes (Al-Hakak, 2000). The quinone interconversion, which occurs rapidly and reversibly, may help accelerate the electron transfer from the nicotinamide adenine dinucleotide reduced (NADH) to the Cr(VI), thus aiding the bacteria to get rid of the inhibiting products.

Hydroquinone-quinone interconversion reactions are found in humans and other organisms to obtain energy from food by oxidation or electron transfer (Schmid, 1996). This redox reaction occurs in a multistep process. An example of this is the cyclic redox

process in the biotransformation of chromium and naphthalene by *Pseudomonas putida* as summarized in Figure 2. Generally, bacteria excrete reductase enzymes to break down the pollutant to degrade the carbonaceous substrate (Cerniglia, 1992). This process often requires a long time for many recalcitrant pollutant compounds. However, this study deals with a co-contaminants, so there is a competition between the PAH and Cr(VI) to react with reductase. Since Cr(VI) shows more affinity toward reductase than naphthalene does, it is reduced first (Al-Hakak, 2000). The addition of quinone facilitates electron transfer from the NADH to the metal Cr(VI). Therefore, quinones serve as a key shuttle in electron transfer during the reduction of chromium (Fultz and Durst, 1982). When nearly all Cr(VI) has been reduced to Cr(III), biodegradation of naphthalene resumes.

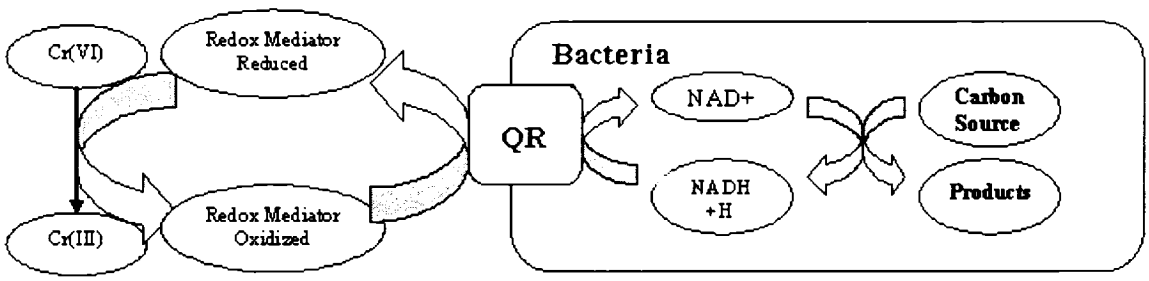


Figure 2. Proposed mechanism for the redox mediator dependent reduction of Cr(VI) by Pseudomonas putida. Bacteria secretes NADH. NADH reduces quinone at QR to hydroquinone. The reduced redox mediator (hydroquinone) reacts with Cr(VI). Cr(VI) is reduced to Cr(III) and the hydroquinone reverts back to its oxidized form the quinone.

2.2 Reduction of Cr(VI) coupled with Organic Pollutants

There are numerous types of bacterial strains that are capable of reducing chromium. These strains either operate in aerobic or anaerobic condition or both. It was observed that under aerobic conditions, NADH and endogenous cell reserves may serve

as the electron source, while in anaerobic conditions, cytochromes from the bacteria's membrane were involved in the Cr(VI) reduction (Schwarzenbach et al., 1993). Shen and Wang (1994) studied enzymatic Cr(VI) reduction by *Escherichia coli* ATCC 33456 using various electron donors such as glucose, acetate, propionate, glycerol, and glycine. Different conditions, such as varying the initial cell concentration, pH, and temperature, were also assessed and found to profoundly affect the rate of Cr(VI) reduction. Ishibashi et al. (1990) studied the Cr(VI) reduction in *Pseudomonas putida* PRS2000 and observed that chromate reductase activity was associated with the soluble protein fraction.

The reduction of hexavalent chromium to its trivalent state consists of at least two stages, since evidence has shown that the intermediate states of chromium such as Cr(V) have been detected (Suzuki et al. 1992). Suzuki et al. (1992) suggested two possible sample pathways for chromate reduction depending upon the type of bacteria. For example, Cr(VI) reduction with *Pseudomonas sp.* G-1 would follow path B and employ NADH (Figure 3). A simultaneous Cr(VI) reduction and phenol degradation with a co-culture consisting of *Escherichia coli* ATCC 33456 and *Pseudomonas putida* DMP-1 would follow path A (Figure 3).

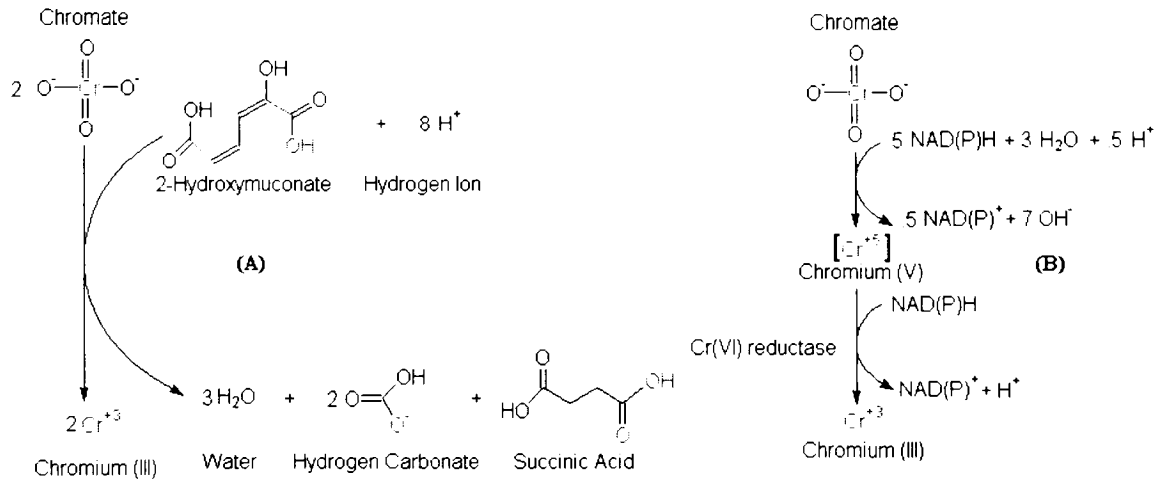


Figure 3. Proposed pathway. (A) Pathway for direct reduction of chromium from hexavalent to its trivalent state by *Pseudomonas sp.* G-1. (B) *Escherichia coli* ATCC 33456 pathway for multi-reductive steps which leads to the intermediate formation of Cr(V). Adapted from Suzuki et al., (1992) and Dommer (2003).

The electron donor responsible for the reduction of hexavalent chromium differs for aerobic and anaerobic bacteria. With aerobic bacteria, Cr(VI) reduction is carried out by the reductase enzymes associated with the soluble proteins with the help of NADH, NADPH as the electron donors, and the flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) as an electron carrier component (Lovley, 1993; Dommer, 2003; Paustian, 2000; Wang and Shen, 1995). In the case of limiting electron donors, the bacteria may use its endogenous electron reservoir (Wang and Shen, 1995). The anaerobic bacteria chromium reduction mechanisms are associated with the respiratory chain of the bacteria in the membrane area (Wang and Shen, 1995; Paustian, 2000). The electrons flow from high potential to low potential electron carriers is as depicted in Figure 4. Charged particles cannot pass through the membrane directly, so they require some special protein such as cytochrome to transport them inside. The terminal electron acceptor in this case would be Cr(VI).

Quinones also have a role in the respiratory chain of the bacteria since they mediate electron transport in cytochrome b and c or d depending upon the bacteria strain (Shen and Wang, 1993). The rate at which the quinones are reduced and substrates (pollutants) degraded, depends upon the availability of electron donors which depends upon the level of microbial metabolic activity. Some factors affecting the amount of electron donors includes: bacterial density and growth state, presence of other chemicals, pH, and temperature to name a few (Shen and Wang, 1995a). Figure 4 shows the electron mediating role of the quinones inside the membrane of a typical bacteria. NADH first donates it's electrons to NADH reductase. Then NADH reductase transfers its protons and electrons to flavoprotein and which in turn reduces a non-heme iron protein and release the protons outside the cell. The free electrons and protons (from cytoplasm) are transferred to the quinone. The quinone then finally gives its electrons and protons to the cytochrome. Each quinone donates its electrons and protons to cytochrome b. The first electron/proton pair takes a high potential path, passing through the iron-sulfur protein (ISP) onto cytochrome c_1. The first reduction causes a conformational shift in the protein such that the second electron takes a low potential path ending up on the second heme group of cytochrome b. Oxidation of a second quinone results in a fully reduced heme that can then donate both pairs of electrons, and two protons from the cytoplasm back to one of the quinones (Paustian, 2000).

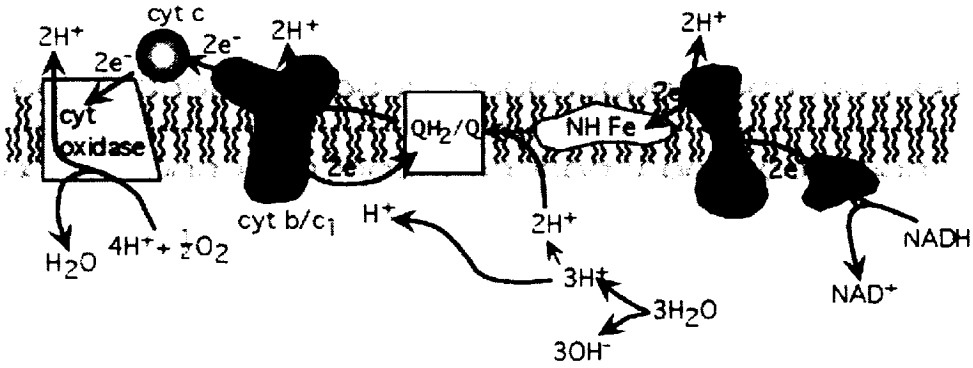


Figure 4. Arrangement of the electron transport system in the membrane of a typical bacteria. Note: NADH = nicotinamide adenine dinucleotide reduced, FP = flavoprotein, NH Fe= non-heme iron, Q = quinone, cyt = cytochrome, and ISP = iron-sulfur protein. Source: Paustian (2000).

Many bacteria were observed to degrade naphthalene such as *Pseudomonas putida* G7, *genera Aeromonas*, *Alcaligenes*, *Cyanobacter*, *Flavobacterium* (Luthy et al., 1994). The general biodegradation pathway of naphthalene is shown in Figure 5. The enzymatic action proceeds via the dioxygenase pathway in the bacterial degradation of aromatic compounds (Eaton and Chapman, 1992). Naphthalene first reacts with naphthalene-1,2-dioxygenase to form naphthalene dihydrodiol. The latter undergoes 1,2-dihydroxy-1,2-naphthalene dehydrogenase and form 1,2-dihydroxynaphthalene. This metabolite undergoes further reductase reactions until the metabolic end-product catechol is formed (Ghoshal and Luthy, 1998; Zeng et al., 2004). Since naphthalene's structure consists of two fused benzene rings, the simplest PAH compound, its rate of biodegradation is faster than other PAHs containing more than two fused rings (Cerniglia, 1992).

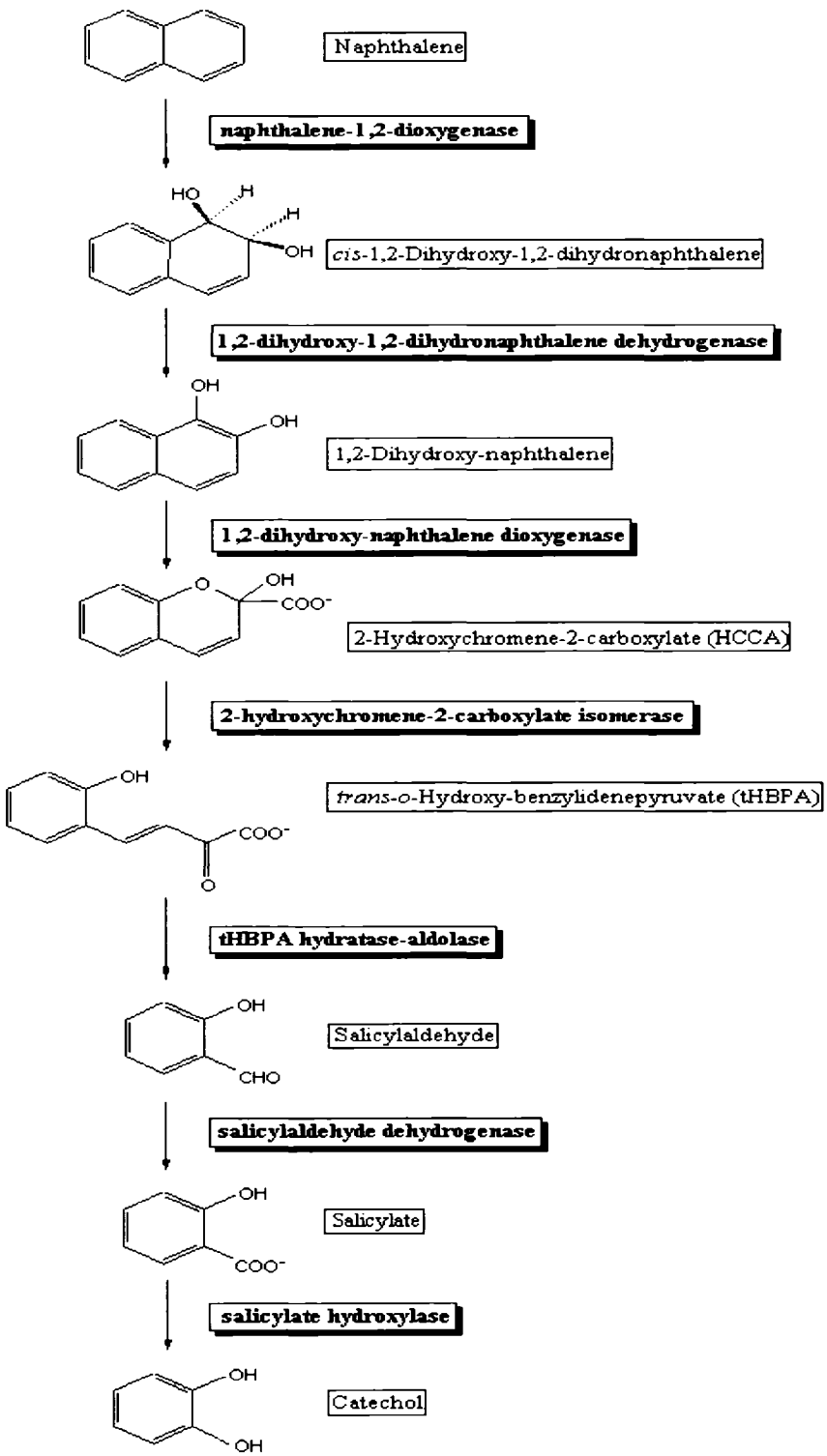


Figure 5. Naphthalene degradation pathway. Adapted from Zeng et al. (2004).

Concurrent metal cation reduction with naphthalene degradation has been done before (Al-Hakak, 2000; Malakul et al., 1998). Al-Hakak observed that during the concurrent degradation of naphthalene and reduction of Cr(VI), little mineralization of naphthalene and little bacteria growth occurred, while the system showed remaining traces of Cr(VI). Once most of the Cr(VI) have been reduced, the mineralization of naphthalene gradually picked up and the bacteria's growth profile returned to normal. For example, with an initial Cr(VI) concentration of 6.3 mg/L, it took nearly 400 hours before any significant mineralization activity was seen (Al-Hakak, 2000). With a lower initial Cr(VI) concentration (2.1 mg/L), the time required to observe significant mineralization was less than 50 hours (Al-Hakak, 2000). The maximum Cr(VI) concentration that was tolerated by *Pseudomonas Putida* ATCC 17484 was 6.3 mg/L during biodegradation of naphthalene, but the time required for complete Cr(VI) reduction was longer than 550 hours compared to 40 hours for Cr(VI) at a concentration of 2.1 mg/L (Al-Hakak, 2000). Furthermore, Al-Hakak (2000) observed that some traces of hexavalent chromium were detected inside the bacteria from sonication experiments of cells grown in the presence of naphthalene, and Cr(VI). Thus, there is a possible accumulation of the Cr(VI) inside the cells.

2.3 Siderophore chemistry and bacterial uptake of siderophore

Siderophores are small molecules with a molecular weight of 400 to 2000 Da (Albrecht-Gary et al., 1994). They are produced in iron deficient conditions by microorganisms to bind and bring external iron into the cells via a high affinity system (Albrecht-Gary et al., 1994). Siderophore has the ability to alter the mobility of some

metals such as iron, plutonium, and uranium (Hernlem et al., 1999; John et al., 2001). Through the stages of evolution, microorganisms have developed ways to sequester fundamental nutrients such as in iron in nutrient deprived environments (Albrecht-Gary et al., 1994; Faraldo-Gomez and Sansom, 2003; Tufano and Raymond, 1981). There are approximately ten orders of magnitude discrepancy between the average Fe ($\sim 10^{-17}$ *M*) concentration in natural aquatic systems and the microorganisms' metabolic requirement ($\sim 10^{-7}$ *M*) in aerobic conditions (Hersman et al., 2000). This chelation of iron with certain siderophores alters the effective solubility of the metal. For example, iron hydroxide is usually insoluble, but once complexed, it is highly soluble, and stable (Braun et al., 1998). Moreover, past and ongoing research points toward these organic ligands as the cause of enhanced mobility of transuranic radionuclides in subsurface environments (Kraemer et al., 2002). Thus siderophore are produced with the goal of facilitating the transport of metallic ions essential to the growth and metabolism of the microorganisms in nutrient deprived environment (Albrecht-Gary et al., 1994; Faraldo-Gomez and Sansom, 2003; Tufano and Raymond, 1981).

Both marine and soil bacteria have been shown to secrete or use siderophores for complexing iron, zinc, and other trace metals that are used by the microorganisms to build enzymes that catalyze biochemical reactions at key points in the nitrogen and carbon cycles (Butler, 1998; Boopathi and Rao, 1999). Several Pseudomonas strains such as *Pseudomonas Putida* type A and *Pseudomonas mendocina* can utilize siderophores, particularly pseudobactin (pyoverdin) (Faraldo-Gomez and Sansom, 2003; Maurice and Hersman, 2002, Boopathi et al., 1999; Khalil-Rizvi et al., 1997).

The coordination chemistry of chromium-siderophore complexes has not been explored in much detail (Bergeron et al., 2001; Schalk, et al. 2002). The molecular structure/stability of siderophore seems to be pH dependent (Birus et al., 1987). It is not known if the coordination chemistry of the organic ligands with the chromium metal are similar to those of Fe(III).

Once the siderophore is excreted outside the bacteria, it chelates with a free metal (e.g., iron) cation and forms an inert soluble complex often, usually detectable to the type of microorganisms that secreted it (Butler, 1998). Some microorganisms are able to detect and uptake the complex for metabolism despite its inability to secrete the chelator molecule, because they possess the corresponding protein receptor responsible for the detection and uptake along its membrane (John et al., 2001). The complete mechanism of introducing the siderophore-metal complex into the cytoplasm inside the bacteria remains unclear (Faraldo-Gomez and Sansom, 2003). The introductory pathway of the complex varies from one strain to another (Stintzi et al., 2000; Tufano and Raymond, 1981). Some bacteria such as *Pseudomons aeruginosa* have protein receptors present along their membrane that are responsible for the detection and uptake of the siderophore complex (Schalk et al., 2002). Some uncomplexed siderophores have been found in abundance along its membrane surface. It was also found that when the complexed siderophore metal came into contact with the receptors, the layer of iron free-siderophore gave way and formed a passageway for the complex to enter the bacteria (Faraldo-Gomez and Sansom, 2003). The specific chelation reaction and the transport mechanism of the complex into the bacteria (recognition, opening/creation of channels) and the subsequent

fate of the complex (e.g., dissociation/lysis of the complex, reduction / transformation of the metal), may be different in various bacterial strains (Tufano and Raymond, 1981; Schalk et al., 2002; Birus et al., 1987; Monzyk and Crumbliss, 1982; Faraldo-Gomez and Sansom, 2003).

Siderophores are in fact binding ligands. There are two groups of siderophores: hydroxamates, and catecholates (Faraldo-Gomez and Sansom, 2003). Hydroxamates use hydroxamic acids while catecholates contain catechol rings and are known for their chelating ability (Albrecht-Gary et al., 1994). Most commonly found natural siderophores are hexadendate ligands; they are either composed of three bidendate chelating groups which can be either three catechol groups; or three hydroxamate groups (Boukhalfa et al., 2000; Kraemer et al., 2002). Bidentate ligands have two points of attachment to the metal center and occupy two coordination sites as shown in Figure 6 (Toreki, 2003). A chelating ligand, involves a polydendate ligand that forms a ring that includes the metal.

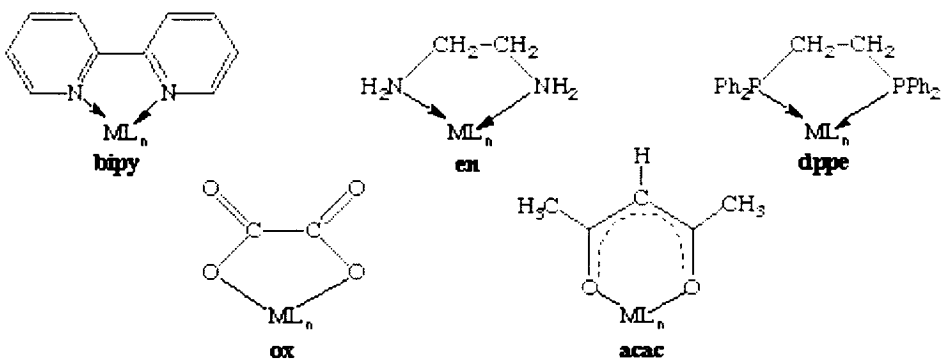


Figure 6. Several Examples of Bidendate ligands: 2,2'-bipyridine (bipy), ethylenediamine (en), diphenylphosphinoethane (dppe), acetylacetonate (acac), and oxalate (ox) Source: Toreki (2004).

The siderophore DFB (desferrioxamine B), which is produced by *streptomyces pilosus*, is a linear trihydroxamic acid whose basic structural feature is alternating units of 1-amino-5-(hydroxyamino) pentane and succinic acid (Figure 7) (Spasojevic et al., 1999; Boukhalfa et al., 2000; Tufano and Raymond, 1981; Birus et al., 1987). Research indicates that certain bacteria can profit from this specific siderophore (Kraemer et al., 2002; John et al., 2001; Moncyk and Crumbliss, 1982). Although, some bacteria such as *Salmonella typhimurium* do not secrete siderophore itself, they are able to uptake and use it as an iron transport agent (Stintzi et al., 2000). Desferrioxamine B is part of the hydroxamate group, having a bidendate ligand (Monzyk and Crumbliss, 1982). When they chelate with a metal, they form an extremely stable five-membered ring by coordination of a central metal ion through the two oxygen atoms (Figure 7) (Tufano and Raymond, 1981). Complexation is accompanied by simultaneous dissociation of one proton per coordinated hydroxamate group (Birus et al., 1999).

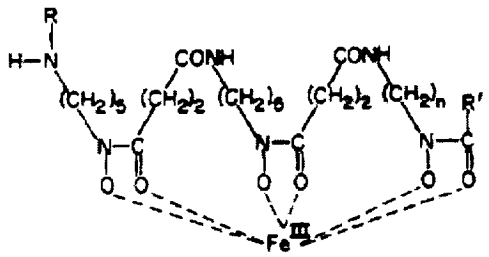


Figure 7. Desferrioxamine B chelated with Iron III, it is a linear trihydroxamic acid. Its basic structural feature is alternating units of 1-amino-5-(hydroxamino)pentane and succinic acid. Source: Tufano and Raymond (1981).

There are many different uptake mechanisms for siderophore-mediated microbial metal transport (Tufano and Raymond, 1981). The first type of uptake mechanism involves release of iron in the proximity of the cell membrane. The released metal is bound anew to another siderophore located in the membrane, which completes the

transport into the cell (*Rhodotorula pilimanae* uses this) (Tufano and Raymond, 1981). The second mechanism is the uptake of the metal-siderophore complex followed by intracellular iron release and re-excretion of the free ligand (see recycling of siderophore ligand in Figure 8 by *Ustilago sphaerogena*) (Tufano and Raymond, 1981). The third mechanism involves transport of the intact metal complex, but once inside the cell, the metal is released through chemical degradation of the siderophore ligand (*enterobactin*) (Tufano and Raymond, 1981). Gram negative bacteria usually use the first mechanism, also called the shuttle transport mechanism (Figure 9).

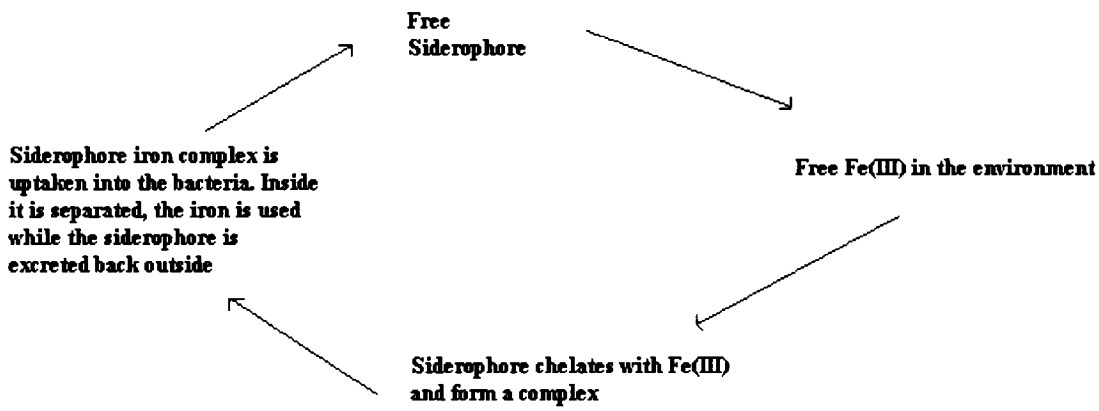


Figure 8. Cycle of siderophore. Free siderophore ligands binds with free Fe(III) to form a complex molecule. As a result of this, its mobility is enhanced, and thus eventually is transported into the bacteria. Once inside, the molecule dissociates, the metal is used by the microorganism and the siderophore ligand is excreted back outside (Schalk et al., 2002; Stintzi et al., 2000).

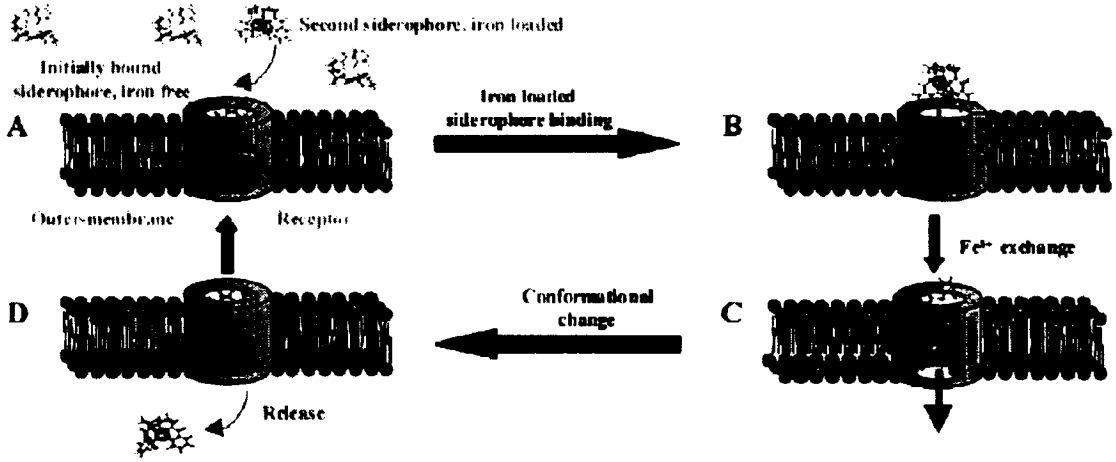


Figure 9. Siderophore shuttle transport mechanism. When an iron loaded siderophore approaches the vicinity of the membrane, a protein receptor detects it and iron exchange from the initial siderophore carrier to the secondary one occurs. The initial siderophore is released back outside, while a passageway is formed to allow the secondary siderophore to enter the membrane. Source: Stintzi et al. (2000).

Faraldo-Gomez and Sansom (2003) described the acquisition of siderophores in gram-negative bacteria. These types of bacteria use the first siderophore uptake mechanism described earlier. Once the siderophore-metal complex approaches the vicinity of the outer-membrane region of the cell, high-affinity receptors are then supplied with energy by the protein complex (Ton system) and are able to uptake the ligand (Faraldo-Gomez and Sansom, 2003; Schalk et al., 2002). The interaction with the Ton complex induces structural changes in the receptors, which translocates the siderophore from the cytoplasmic membrane into the periplasm (Faraldo-Gomez and Sansom, 2003). Once in the periplasm, siderophores are sequestered by periplasmic-binding proteins (PBP's), which traverse the peptidoglycan and deliver the complex to ATP-dependent transporters that reside in the cytoplasmic membrane (Faraldo-Gomez and Sansom, 2003).

The stability of the complex is pH dependent (Boukhalfa et al., 2000; Albrecht-Gary et al., 1994). This factor is crucial into determining the stability and kinetics of dissociation of the complex molecule. The kinetics of dissociation of the siderophore-metal complex depend on the coordination of the binding sites of both siderophore and the metal in question (Schalk et al., 2002; Monzyk and Crumbliss, 1982; Birus et al., 1987). In other words, if the metal must adapt its coordination pattern to fit the siderophore ligand (tetrahedral to octahedral, for example), the rate of formation and dissociation is greatly lowered (Winkelmann, 2002; Tufano and Raymond, 1981). Another factor to take into consideration is the shape of the siderophore: linear or cyclic. Cyclic siderophores usually take longer to dissociate from the metal, because of their circular structure and higher stability (Winkelmann, 2002). The exterior bonds must first be broken before the chelating agent can make contact with the metal at the center of the molecule (Spasojevic et al., 1999). The stepwise dissociation mechanism for ferrioxamine B is shown in Figure 10. The dissociation of a hydroxamate chelate group occurs initially at the N-O atomic bond (at the protonated amine end of the molecule), followed by carbonyl-oxygen atom dissociation from the inner coordination shell of iron(III) (Monzyk and Crumbliss, 1982; Birus et al., 1999).

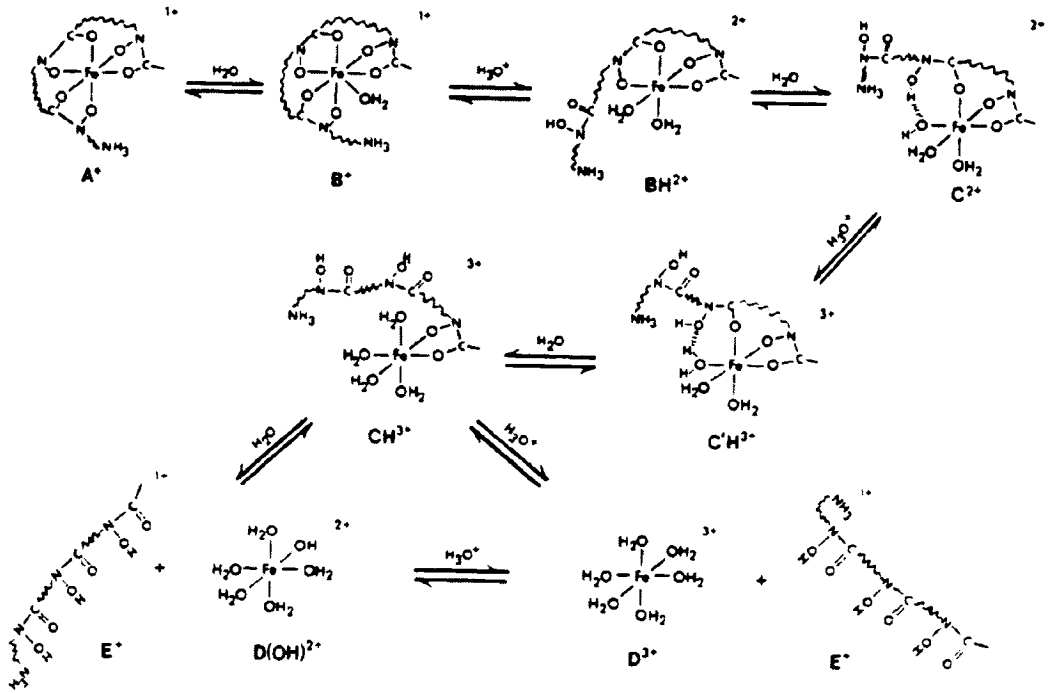


Figure 10 Stepwise Dissociation of Fe(III) from Ferrioxamine B in Aqueous Acid. Source: Monzyk et al. (1982).

In gram-negative bacteria, iron release is usually done so by hydrolysis or by reduction of Fe^{3+} to Fe^{2+} (Birus et al., 1987; Monzyk and Crumbliss, 1981; Boukhalfa et al., 2000). A complete discussion about the ligand dissociation of iron-DFB is found in Boukhalfa et al. (2000). The chemical reduction weakens the bound between the metal and the siderophore (Faraldo-Gomez and Sansom, 2003). At physiological conditions, many iron siderophore complexes have redox potentials that are non favourable to biological reducing agents such as NADH. However, under mildly acidic conditions, the redox potential of some iron-hydroxamate siderophores increases significantly, so that reduction by the reducing agents may be thermodynamically possible (Spasojevic et al., 1999). The dissociation kinetics of chromium complex may involve more than one intermediate step if any bacterial-induced dissociation is possible. Fortunately, the

kinetics of chelation is very rapid. A more in depth and future study should be performed with empirical equations describing each rate.

To determine the feasibility of the chelation reaction and the possible uptake of the complex, an estimate of the stability of the DFB-Cr(VI) complex must first be calculated. Hernlem et al. (1999) have demonstrated several correlations for predicting the metal affinities by using the ratio (z/r) of charge/ionic radius, and the metal of interest's first hydrolysis constant. Unfortunately, the study does not deal with chromium. Nevertheless, by using the same approach, the formation constant for DFB and Cr (VI) was found to be higher than 35, while for DFB and Cr(III), the formation constant is about 25. Table 1 summarizes the computed stability constant for Cr(III) and Cr(VI) using the approaches suggested by Hernlem et al. (1999). The stability constants of Cr(III) and Cr(VI) are computed using the correlation via logK1 as shown on Figure 11, while Figure 12 shows the stability constants of Cr(III) and Cr(VI) employing the correlation via z/r approach. Therefore, the DFB-Cr (VI) complex should be more stable than the DFB-Cr(III) complex, but competition between them will still be present. With such a high number, the complex may be too stable to be dissociated (Schalk et al., 2002). Even if the complex is uptaken successfully, the microorganisms may not be able to separate the molecules.

Table 1. Formation constant of chromium complex

Product	Z/R (A^{o-1})	Log K_f via Z/R correlation	Log K_1 Baes et al. (1976)	Log K_f via K_1 correlation
Cr(III)	3/0.755	20	10.0	25
Cr(VI)	6/0.40 or 6/0.58	67.5	14.83	37

Note:

Z = charge,

R = Ionic radius (Angstrom),

Log K_f = formation constant,

Log K_1 = first hydrolysis constant

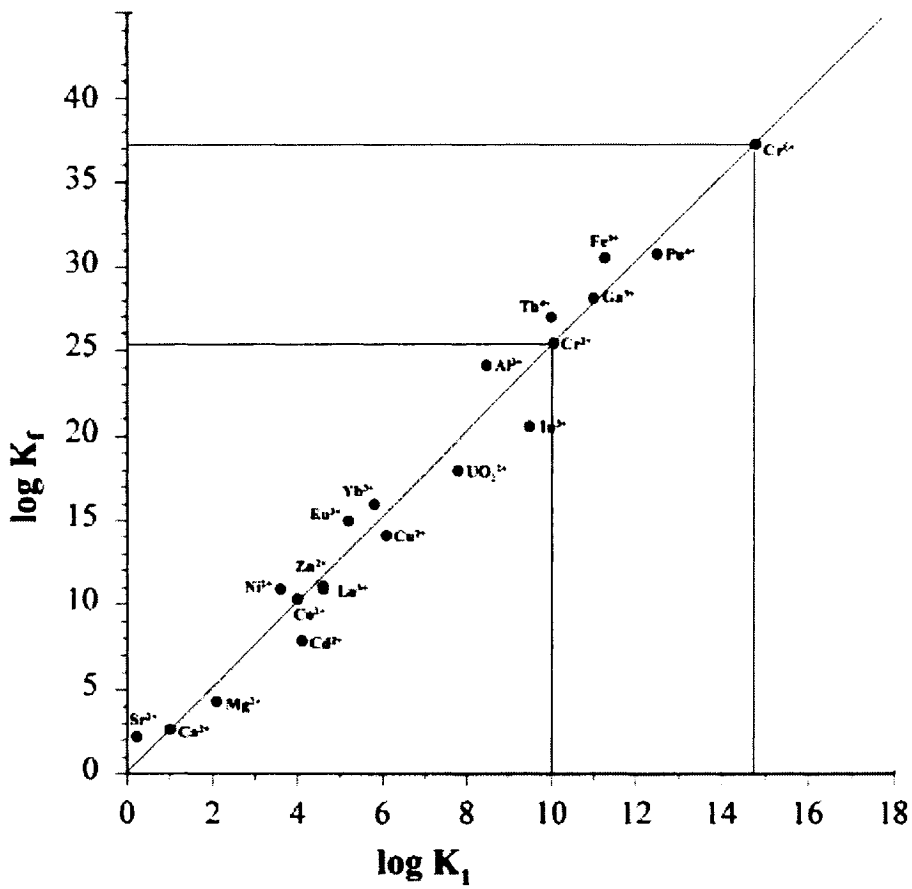


Figure 11. Correlation via logK1 approach. Adapted from Hernlem et al. (1999).

Hernlem et al., 1999 determined that the stability constant of the DFB-Fe(III) complex is near 30.6. The ionic radius information for chromium was obtained from Winter (2003). The radius for Cr(VI) has two possible values depending upon its atomic

coordination (6-coordinate, octahedral or 4-coordinate, tetrahedral) (Winter, 2003). The lower value of 0.4 is used to determine the lower bound of the stability constant.

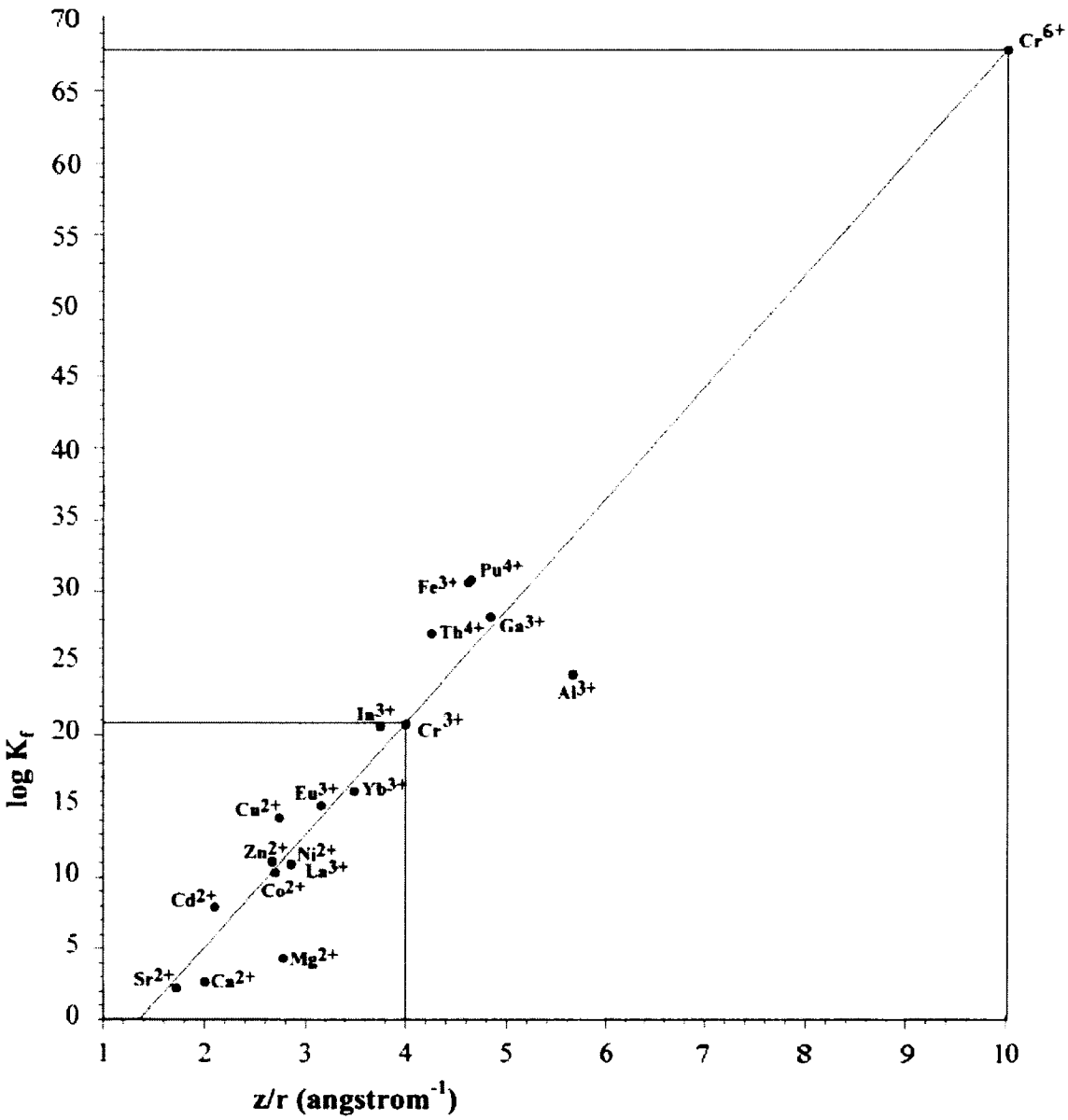


Figure 12. Correlation via z/r approach. Adapted from Hernlem et al. (1999).

Knowing that DFB-Cr(III and VI) have high stability constants, it is safe to assume that the complex will not dissociate easily and its mobility can be enhanced in most conditions. To dissociate the complex, bacteria may reduce the Cr(VI) to Cr(III) since the molecule is less tightly bound in the trivalent state if reduction of chromium is possible while in the complex form. Metal mobility can still be inhibited when chelated molecules come into contact with certain surfaces due to adsorption. For example, when DFB-Cu, DFB-Zn, or DFB-Cd comes in contact with montmorillonite. The adsorption inhibits the mobility of these molecules (Neubauer, et al., 2000).

3.0 Material & Methods

3.1 Chemical Reagents

Naphthalene crystals (99% purity), 2,2,4,4,6,8,8-heptamethylnonane (HMN), and 1,2-naphthoquinone (97% purity) were purchased from Aldrich Chemicals (Milwaukee, WI). β-NADH reduced dipotassium salt (98% purity), desferrioxamine mesylate salt (95% purity) (DFB), NaOH (98%), and 1,2-dihydroxynaphthalene were all purchased from Sigma-Aldrich (St-Louis, MO). Diphenylcarbazide was purchased from Anachemia (Montreal, Qc), *Pseudomonas putida* ATCC 17484 was purchased from the American Type Culture Collection. Water used in the experiments was deionized filtered water (Millipore). Potassium chromate was purchased from Fisher Scientific (Fair Lawn, NJ). The organic scintillation liquid was purchased from Beckman Coulter (Fullerton, CA).

3.2 Chemical stock preparation

The diphenylcarbazide reagent was prepared by dissolving 0.25 g of diphenylcarbazide in 50 ml acetone, this reagent was stored for short periods of time in a bottle wrapped with brown paper and refrigerated at 4°C to avoid exposure to light. The solution was discarded when left for a long period of inactivity. NaOH solution (2 N) was prepared by dissolving 40g of NaOH with 500 ml of filtered deionized water.

Phosphate stock solution was prepared by dissolving 2.125g of KH_2PO_4, 5.425g of K_2HPO_4, and 4.4 g of Na_2HPO_4 in 250 ml deionized water. NH_4Cl stock solution was prepared by dissolving 10.625 g NH_4Cl in 250 ml deionized water. Ca stock, $MgSO_4$

stock, and $FeCl_3$ stock were prepared as follow respectively: dissolve 2.75 g in 100 ml deionized water, dissolve 2.25 g in 100 ml deionized water, and dissolve 0.025 g in 100 ml deionized water. These three solutions were sterilized by filtering them through a 0.45 micron membrane instead of autoclaving to avoid precipitations of these salts. To prepare 1 L of MSM, 20 ml of phosphate stock solution and 20 ml of NH_4Cl stock solution were added to a 1L autoclavable bottle. The bottle was filled with deionized filtered water up to the 1L mark. The bottle was autoclaved at 120 °C for 40 minutes in the liquid cycle. It was then left on the bench to cool down. Then 1 ml of each of the following sterilized solutions were added: $CaCl_2$, $MgSO_4 \cdot 7H_2O$, and $FeCl_3 \cdot 6H_2O$.

Other stock solutions were prepared as follows: 41.7 mg of potassium chromate K_2CrO_4 in 100 ml MSM, 0.25 g diphenylcarbazide in 50 ml acetone, 0.10 g of 1,2-naphthoquinone in 50 ml methanol, and 0.10 g of 1,2-dihydroxynaphthalene also in 50 ml methanol. Further dilutions from these stock solutions employed deionized water. To provide naphthalene continuously to the aqueous phase, a NAPL interface, naphthalene crystals were dissolved in HMN. Naphthalene crystals (0.63 g) were dissolved in 9 ml HMN to have a concentration of 70 mg/ml.

3.3 Bacterial Strain and Cultivation Conditions

The *Pseudomonas putida* ATCC 17484 culture was initially revived from frozen stock in glycerol solution at –80°C. A small amount of the frozen stock was placed in 50 ml MSM containing 10-15 mg naphthalene crystals using sterile methods. The flask was then left to incubate in a rotary shaker at 150 rpm and at a constant temperature of 25°C. Its growth was monitored spectrophotometrically at a wavelength of 600 nm (Ulttrospec

2000, Pharmacia Biotech). When sufficient growth was observed (OD~0.50), five ml of the culture was transferred into fresh culture flask. This was repeated twice before using them for an experiment in order to revive their metabolism and to remove any remaining glycerol. Every week, a fresh bacterial culture was started in order to maintain their activity.

3.4 Bacterial Enumeration Techniques

The pour plate technique was used to enumerate the number of live bacterial cells in solution (Standard Methods, 1998). Noble agar plates were used and prepared by dissolving 15 g of Noble Agar in MSM solution. It was then autoclaved and allowed to cool down. Approximately 15 ml of the solution was poured per plate. Unused plates were sealed with parafilm and rapidly placed in the refrigerator. Serial dilutions were used where the bacterial count was too high. The enumeration procedure was done under sterile environment. Naphthalene was supplied as the carbon source by placing 10 mg of naphthalene crystals to the plate cover, and placing the plates inverted (lid down) so that the volatilized naphthalene could be diffused into the agar. After three days of incubation in the constant temperature room (25 ^{o}C), the number of colonies of bacteria were counted and reported as colony forming units per ml (cfu/ml).

3.5 Cr(VI) Reduction Experiments

Diminishing the toxicity of Cr(VI) by reducing it to Cr(III) constitutes the ideal solution, but may take a long period of time. A set of experiments was performed to investigate the effect of several chemicals that may influence the reduction rate of Cr(VI). Flasks (125 ml) served as batch reactors. The flasks and stoppers were first sterilized and

then filled with 50 ml of freshly prepared MSM. The following scenarios were investigated: the Cr(VI) reduction by bacteria, the Cr(VI) reduction by NADH, the Cr(VI) reduction by naphthoquinone and bacteria, the Cr(VI) abiotic reduction by dihydroxynaphthalene, and the Cr(VI) reduction with bacteria and 1,2-dihydroxynaphthalene (Table 2).

Table 2. Description of Cr(VI) reduction experiments

Label	Content	Cr(VI) (mg/L)	Naphthalene-HMN added from stock
A	Bacteria (O. D. 0.964)	3.45	0.7 ml
B	Bacteria (O. D. 0.964), 1,2-napthoquinone (0.24 mg/l)	3.45	0.7 ml
C	Bacteria (O. D. 0.687)	6.84	0.7 ml
D	Bacteria (O. D. 0687), 1,2-naphthoquinone (0.1 mg/l)	6.84	0.7 ml
E	Bacteria (O. D. 0.639), 1,2-dihydroxynaphthalene (0.2 g/l)	3.27	0.7 ml
F	Bacteria (O. D.0.778)	3.22	0.7 ml
G	NADH (50 mg/l)	3.75	0
H	1,2-dihydroxynaphthalene (12 mg/l)	6	0 ml
I	Cr(VI) only	6	0 ml

At predetermined time intervals, generally every 24 hours, a sample was taken and analysed for Cr(VI) absorbance with the diphenylcarbazide test which enabled to establish the time profile of Cr(VI) concentration and see how the reduction rate compared with the control. However, when the Cr(VI) reduction kinetics was found to be too fast, the experiment was repeated, and the sampling time is adjusted accordingly.

For abiotic experiments, NADH was used to simulate the bacterial reductase enzymes (Table 2, label H). The amount of naphthalene added when employing bacteria

was the same in all flasks (0.7 ml naphthalene in HMN (70 mg/ml HMN). Two set of experiments consisted of determining the effect of quinone compound and its related dihydroxynaphthalene chemical on hexavalent chromium (Table 2, label B, D, E, and H). The controls are performed as described in Table 2, label A, C, F, and I. A duplicate of each sets of experiments were performed.

3.6 Siderophore Experiment

It was not known from the literature if the bacteria used in this study were able to uptake the siderophore desferrioxamine B. DFB is a common siderophore encountered among soil and marine bacteria (Guan and Kamino, 2001; Butler, 1998). A batch reactor test was conducted by initially adding DFB and MSM, before inoculating the bacteria and adding HMN-naphthalene. Since the MSM contained Fe(III), it was expected that the siderophore would first react with it. If the bacteria lacked an appropriate receptor, then an inhibition in the bacteria's growth rate would be observed due to its inability to recognize the complex and would then starved for iron deficiency. A competitive chelating experiment between siderophores and simple organic chelators showed that hydroxamate siderophores may inhibit solubilization of metals such as uranium and plutonium by other chelators (Ruggiero et al., 2001). To counter the highly specificity of some siderophores, some bacteria have developed a multifunctional receptor that can uptake any type of siderophore-chelated compound (Stintzi et al., 2000). The siderophore added to the system is assumed to be completely chelated with the free iron(III). So if the bacteria are capable of uptaking this DFB-metal complex, then the experiment's results

should show an accelerated growth of the bacteria or at least comparable to the normal growth without addition of siderophore as iron is readily available.

If the bacteria was found to be able to uptake the siderophore-iron complex, that would mean it has the appropriate protein receptor. Further research is needed to prove that it can uptake siderophore-chromium complex though due to the different nature of the metals involved. Continuously mixed batch reactor experiments performed with Cr(VI), siderophore and a control system of bacteria is required to determine the effect of siderophores. At several time intervals, samples were taken, centrifuged and the supernatant as well as the bacterial pellets were analyzed for Cr (VI). HPLC analysis was used to detect the presence of the complex and the diphenylcarbazide test was used to measure free Cr(VI).

3.7 Naphthalene Mineralization Experiments

By lowering the toxicity of Cr(VI) faster with the addition of 1,2-naphthoquinone or DFB, the bacteria should be able to mineralize naphthalene at a faster rate. A 125 ml biometer flask equipped with a side arm was used to perform biodegradation experiments that required monitoring of CO_2 production by mineralization of napthalene. For other biodegradation experiments that do not require monitoring of CO_2 formation, a 125 ml flask was used instead.

The biometer flasks contained 50 ml of MSM. Five ml of 2 N NaOH was placed in the side arm to trap any carbon produced by the bacteria. The stoppers were wrapped in aluminum foil to prevent sorption of naphthalene volatilized from the HMN.

Furthermore, parafilm was wrapped around the stoppers to ensure the systems were well sealed. Biodegradations were commenced by inoculating with 5 ml of the bacterial culture (OD^{600} ~ 0.4). Flasks were mounted on a shaker at 175 rpm placed inside a walk-in, constant temperature room maintained at 25 ± 1°C.

Radiolabelled tracer techniques were used to evaluate naphthalene mineralization rates in solutions containing various treatments such as Cr(VI), naphthoquinone, and siderophore. A 100 μCi of radiolabelled naphthalene was dissolved in 1 ml methanol to make the stock solution of ^{14}C naphthalene. The solution was stored at 4°C when not in use. In most experiments, 630 mg of pure, unlabelled naphthalene crystals were dissolved in 9 ml HMN to achieve a concentration of 9%. The naphthalene stock solution was mixed and left to warm up to room temperature until all crystals were dissolved. A specific volume of ^{14}C labelled naphthalene was injected to the stock so that approximately 10^6 dpm of radioactivity was associated with 0.7 ml HMN-naphthalene mixture, which was the volume added to each flask. Before starting an experiment, the activity of the radiolabelled solution was measured by extracting 10μL and adding it to 5 ml of Beckman Coulter scintillation liquid. The concentration of naphthahelene was determined using a liquid scintillation counter (Beckman LS 5000 TD). Each biometer flask contained initially 50 ml MSM, 0.7 ml of HMN-naphthalene mixture containing ^{14}C and unlabelled naphthalene, as well as the chemicals 1,2-dihydroxynaphthalene or DFB and 5 ml of fresh NaOH in the sidearm. Biodegradation was commenced by the addition of 5 ml of inoculation.

Three controls were performed: one without bacteria (D) to estimate the natural mineralization of naphthalene without the aid of microorganisms; another without chromium (E) to estimate its detrimental effect on mineralization of naphthalene by bacteria, and a last one without 1,2-dihydroxynaphthalene or DFB (C) which served as the standard benchmark for comparison. The biodegradation tests were performed as shown in Table 3.

Table 3. Biodegradation experiments description

Label	Content	Cr(VI) (mg/L)	Naphthalene-HMN added from stock
A	Dihydroxy. 0.25 mg/L	3.65	0.7 ml
B	DFB 0.25 g/L	2.0	0.7 ml
C	Control bacteria	3.65	0.7 ml
D	Control no bacteria	3.65	0.7 ml
E	Control bacteria	0	0.7 ml

Radiolabelled CO_2 was measured by sampling 0.5 ml of NaOH from the side arm with a syringe needle and adding it to 5 ml of scintillation liquid placed in scintillation vials. After each sampling, the remaining NaOH was carefully removed and fresh 5 ml NaOH was replaced in the side arm. The scintillation vials were placed in the dark, overnight, before reading the activity count on the Beckman LS 5000 TD liquid scintillation counter. At the end of a naphthalene mineralization experiment, a mass balance for ^{14}C was calculated, the radioactivities remaining in the NAPL and aqueous phases was determined. To determine the activity left in the aqueous phase, 0.5 ml was withdrawn to measure radioactivities remaining in solution. For the NAPL phase, a piece of parafilm was first weighed, and then immersed in the NAPL. The contaminated parafilm was weighed again. The difference in weight equaled the weight of NAPL. Knowing the molecular weight of the NAPL, it was possible to determine the activity

left. The radioactivity on the parafilm was analyzed in the LSC by introducing it into a scintillation vial filled with 5 ml of scintillation liquid.

3.8 Analytical Methods

The diphenylcarbazide test measures the absorbance of a treated sample with the aid of a spectrophotometer and then using a standard curve to evaluate the concentration of hexavalent chromium. The treatment consists of filtering the sample to separate the bacteria and other solid components, then acidifying the solution to a pH between 1 ± 0.3 using 1N sulphuric acid. The wavelength used to monitor the absorbance was 540 nm.

Using the diphenylcarbazide method, the following calibration curve (Figure 13) was obtained by scanning several samples of Cr(VI) solution concentration at 540 nm. MSM was measured as the background. Samples that showed concentrations of Cr(VI) higher than 6.0 mg/L were diluted since the absorbance was no longer linear with concentration (Standard Methods, 1998).

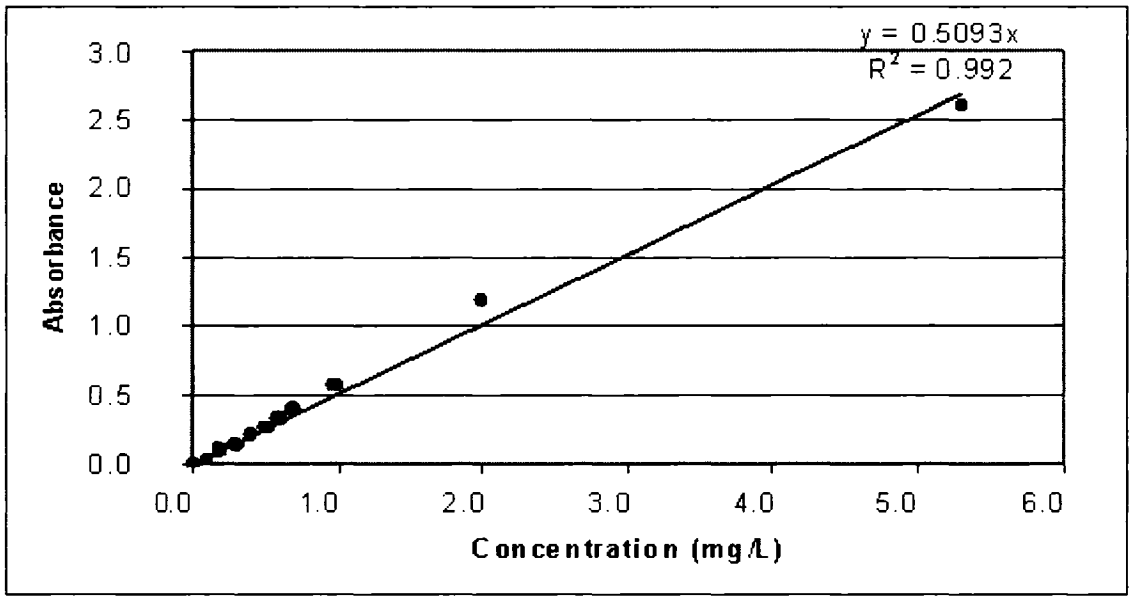


Figure 13. Calibration curve for Cr(VI) using the spectrometric analysis of the diphenylcarbazide method.

For the HPLC analysis, the wavelengths used were: 220, 248, 254, and 275 nm (Chiavari et al., 1982; Poulsen et al., 1990). They are found to be the best to monitor the chemicals used in this study, namely naphthalene, 1,2-naphthoquinone, and 1,2-dihydroxynaphthalene. Calibration curves for 1,2-dihydroxynaphthalene, 1,2-naphthoquinone, and naphthalene have been obtained as shown in Figures 14, 15, and 16, respectively. Aqueous samples were first centrifuged at 4000 rpm for 15 minutes at a temperature of 20°C to remove any solids. Then 1 ml of the sample to be analysed was diluted with 9 ml of HPLC grade methanol. The carrier solvents used were deionized filtered water and acetonitrile. The flow rate was 0.5 ml/minute, and the volume injected was 5 microliters. Calibration samples were first injected to determine the retention time, and the wavelength for optimal detection was employed. Each sample was analyzed for 15 minutes. Fresh stock solution standard was prepared for every time an experiment was performed after a long period of time.

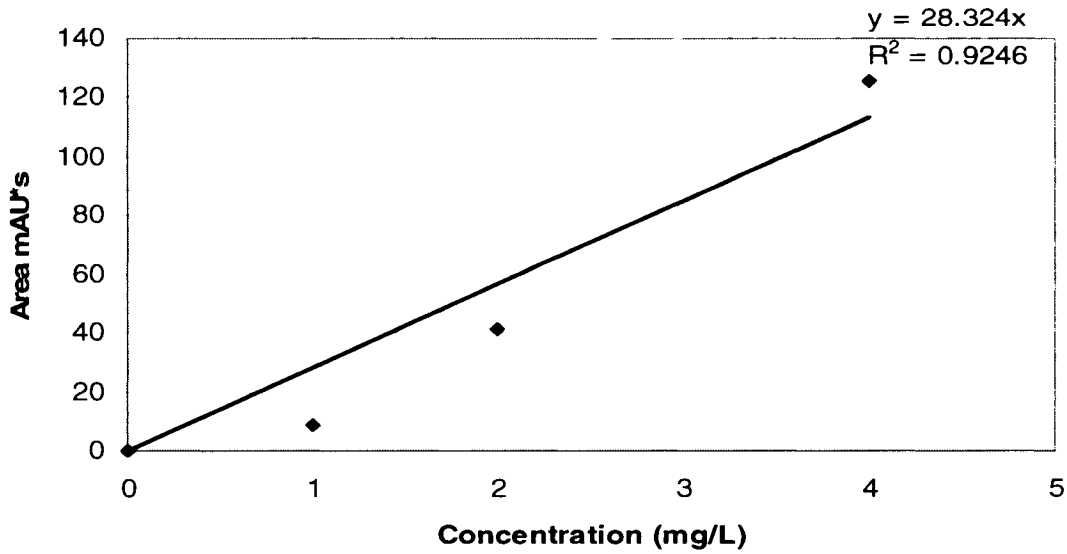


Figure 14. Calibration curve for 1,2-dihydroxynaphthalene using the HPLC, analysis at 248 nm.

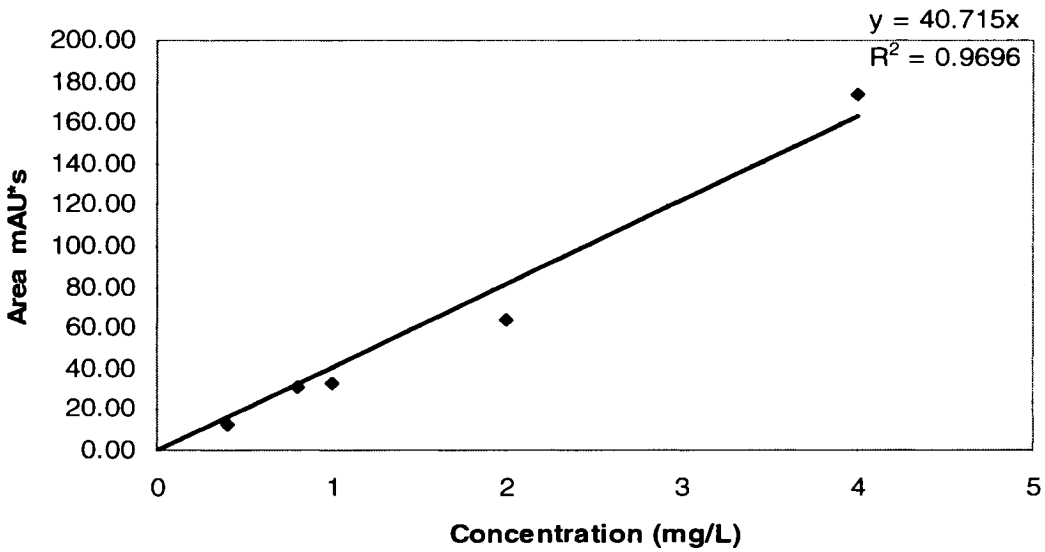


Figure 15. Calibration curve for 1,2-naphthoquinone using the HPLC, analysis at 248 nm.

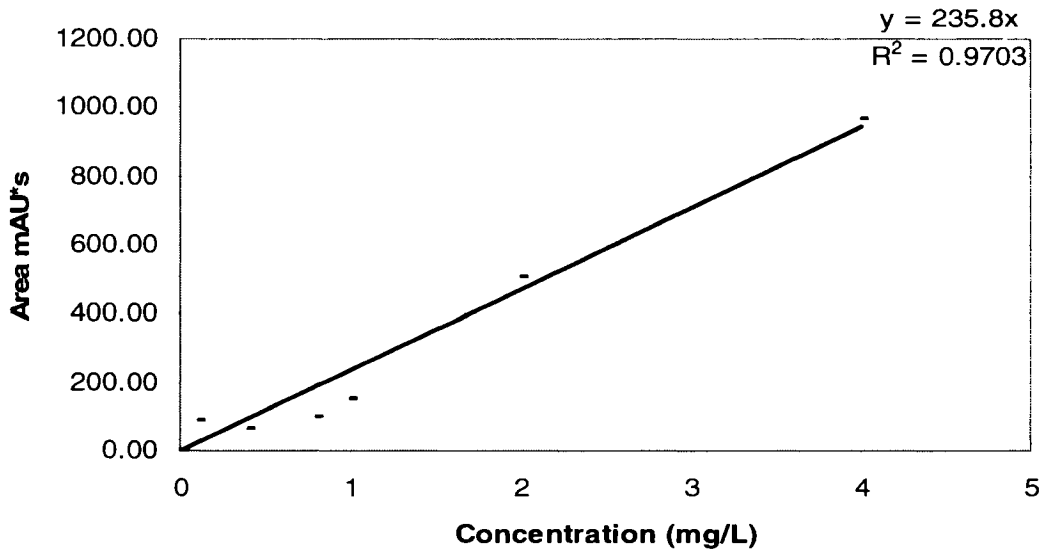


Figure 16. Calibration curve for naphthalene using the HPLC, analysis at 220 nm.

3.9 Statistical Analysis

Duplicates and sometimes triplicates samples were taken in all experiments. The mean of the duplicates or triplicates were plotted in various graphs. When duplicates of each concentration were not used, the whole experiment was repeated at least once. The standard deviation was employed to plot the error bars.

4.0 Results & Discussion

4.1 Growth of bacteria

The growth pattern with time of the strain ATCC 17484 in MSM with naphthalene, as a premature or an aging culture will have considerably different end results, compared to using a healthy metabolically active one. In Figure 17, the optical density at wavelength 600 nm of the culture was monitored for over 300 hrs (culture revived from frozen stock). It grew all along, until 250 hrs when net growth ceased. By then, the optical density has already reached 1.8. The bacteria's optimum condition occurs when its optical density reaches around 0.4-1 since it has just emerged from its exponential growth phase and is about to enter the linear growth (Figure 17). The culture's metabolism is most active at that point and its secretion of reductase enzymes responsible for Cr(VI) reduction is maximized. The results obtained were significantly different compared to those reported by Duetz et al. (2000). Their *Pseudomonas putida* culture reached its peak optical density within 24 hrs. However the carbon source used was glucose and intense aeration was applied in that study.

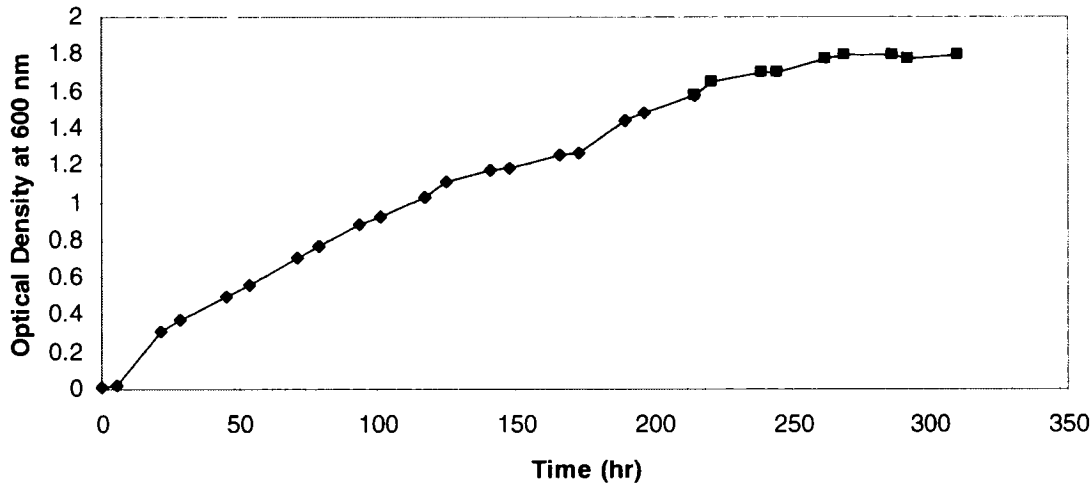


Figure 17. Growth of *Pseudomonas putida* ATCC 17484 in the presence of naphthalene at 25°C.

In a separate experiment, the inhibitive effect of 1,2-dihydroxynaphthalene was verified. The optical density was monitored over 48 hrs, and the results showed little difference between a system with 1,2-dihydroxynaphthalene and one without. In fact, with the addition of 1,2-dihydroxynaphthalene, the bacteria's growth rate was slightly faster (results not shown).

4.2 The Effect of 1,2-naphthoquinone on Cr (VI) reduction

The rate of Cr(VI) reduction was investigated when 1,2-naphthoquinone was added. The quinone should behave as a redox mediator and facilitate electron transport from the electron donor to Cr(VI). Batch reactor tests were performed in pairs. Each pair consisted of a control reactor and a reactor which contained 1,2-naphthoquinone.

Figure 18 and 19 show the time profile of Cr(VI) reduction in biometer flasks containing the bacteria, MSM, 0.7 ml HMN-naphthalene and different concentrations of

Cr(VI) and 1,2-naphthoquinone. The difference between a reactor with quinone, and a reactor without quinone was negligible. The graph even showed that reactors without quinone achieved slightly more reduction. Since 1,2-naphthoquinone is known to inhibit bacterial metabolism (Murphy and Stone, 1955; Ghoshal and Luthy, 1998), the Cr(VI) reduction rates were expected to be reduced by 1,2-naphthoquinone. One pair of reactors used an initial bacteria concentration of OD^{600}~0.687 and a Cr(VI) concentration of 6.84 mg/L, this reactor only achieved 60% Cr(VI) reduction over 125 hours. Suspecting that the toxicity of Cr(VI), a second batch of reactors employed a lower Cr(VI) concentration (3.45 mg/L) and a higher cell density (OD^{600}~0.964). The difference between a system containing 1,2-naphthoquinone and one without was negligible once more, but a higher percentage of Cr(VI) reduction was obtained. Shen et al. (1994) observed that a higher cell density increased the rate of reduction provided that the culture was still within the exponential or linear growth stage. Because the Cr(VI) concentration employed was not kept constant, these set of experiments only demonstrates that a higher cell density along with a smaller dose of Cr(VI) leads to a higher percentage of Cr(VI) reduced.

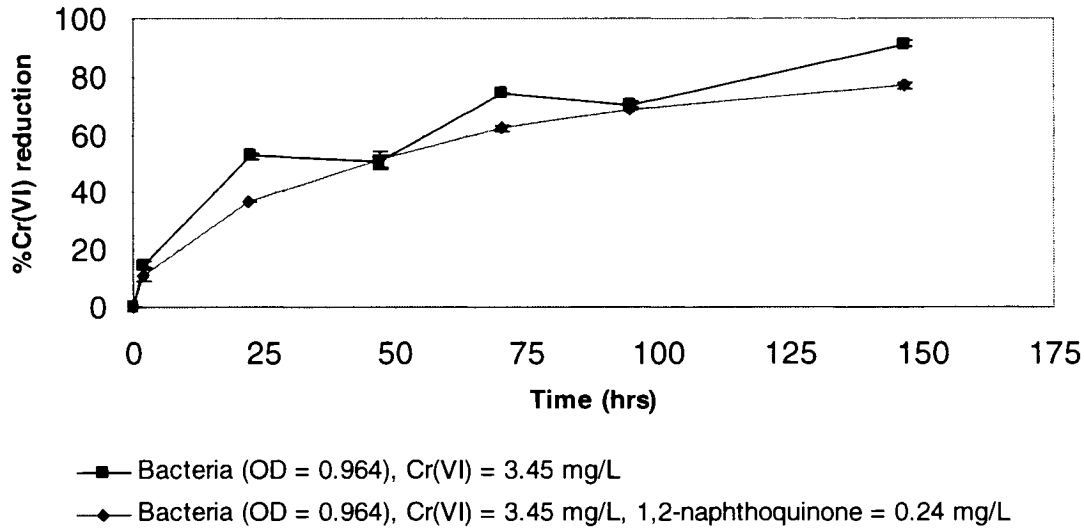


Figure 18. Effect of 1,2-naphthoquinone on Cr(VI) reduction in the presence of bacterial cells (ATCC 17484) with 3.45 mg/L Cr(VI) and 0.24 mg/L 1,2-naphthoquinone.

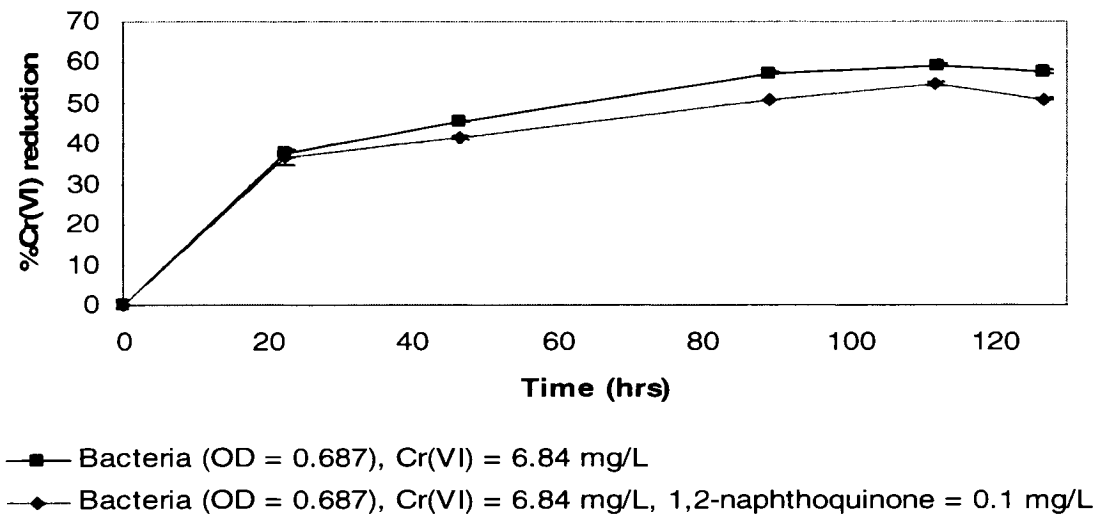


Figure 19. Effect of 1,2-naphthoquinone on Cr(VI) reduction in the presence of bacterial cells (ATCC 17484) with 6.84 mg/L Cr(VI) and 0.1 mg/L 1,2-naphthoquinone.

The obtained Cr(VI) reduction rate and percentage were comparable to those reported by Shen and Wang (1993), as well as Al-Hakak (2000) for systems where no quinone was added. The time required for complete chromium reduction in a reactor with

1,2-naphthoquinone was found to be at least an order of magnitude greater than those obtained by Al-Hakak (2000). A 75% Cr(VI) reduction within an hour of reaction with an initial Cr(VI) concentration as high as 8.0 mg/L was observed by Al-Hakak (2000). The highest reduction rates that have been observed in this study within an hour using similar 1,2-naphthoquinone concentration and bacteria concentration is a little lower than 10% with a chromium concentration under 8.0 mg/L. Using higher concentrations of 1,2-naphthoquinone and bacteria, and concentration of Cr(VI) lower than those used by Al-Hakak (2001) did not produce Cr(VI) reduction rates in the range reported. This is in agreement with Rau et al. (2002) who reported that 1,4-naphthoquinone was unsuitable as a redox mediator for anaerobic treatment of azo dyes, because of its positive redox potential.

The expected increase in the rate of reduction due to the addition of 1,2-naphthoquinone was not observed in this study; thus, the quinone was unable to mediate the electron transfer. The reduction of Cr(VI) with redox mediators such as quinones requires a continuous supply or an excess of electron donors as in the proposed pathway in Figure 2. If the bacteria are not metabolically active, NADH and other enzyme production is low, and little Cr(VI) reduction can be expected.

4.3 The Effect of 1,2-dihydroxynaphthalene on Cr (VI) reduction

Since no increase in the Cr(VI) reduction rate was detected in the experiments employing 1,2-naphthoquinone, the focus of the study shifted to another chemical 1,2-

dihydroxynaphthalene. 1,2-dihydroxynaphthalene was suspected of being a potential reductant and closely related to 1,2-naphthoquinone. The compound 1,2-dihydroxynaphthalene was found to increase the reduction activity of amaranth dye (Keck et al., 1997). Following the same goal, the objective is to investigate whether or not adding this chemical to the reactor would increase Cr(VI) reduction.

To verify if 1,2-dihydroxynaphthalene could reduce Cr(VI) in the absence of bacteria, abiotic tests were performed with batch reactors containing MSM, Cr(VI), and 1,2-dihydroxynaphthalene. The control reactor consisted only of MSM, and Cr(VI). The other reactors contained MSM, Cr(VI), and 1,2-dihydroxynaphthalene. The Cr(VI) concentration decreased to a negligible extent in the control. Using the diphenylcarbazide method to monitor the metal concentration with time, there was modest, but significant Cr(VI) reduction as shown in Figure 20. After 20 hours, the Cr(VI) concentration decreased when 1,2-dihydroxynaphthalene was employed. An initial 1,2-dihydroxynaphthalene concentration of 12 mg/L reduced Cr(VI) from 5.9 mg/L to around 5.5 mg/L. There was no significant difference between the control and the reactor containing a lower concentration of 1,2-dihydroxynaphthalene (4 mg/L, results not shown). Thus, this experiment revealed that 1,2-dihydroxynaphthalene could abiotically reduce chromate. Aside from Cr(VI), and amaranth dyes, this chemical may potentially be used to reduce other target pollutants.

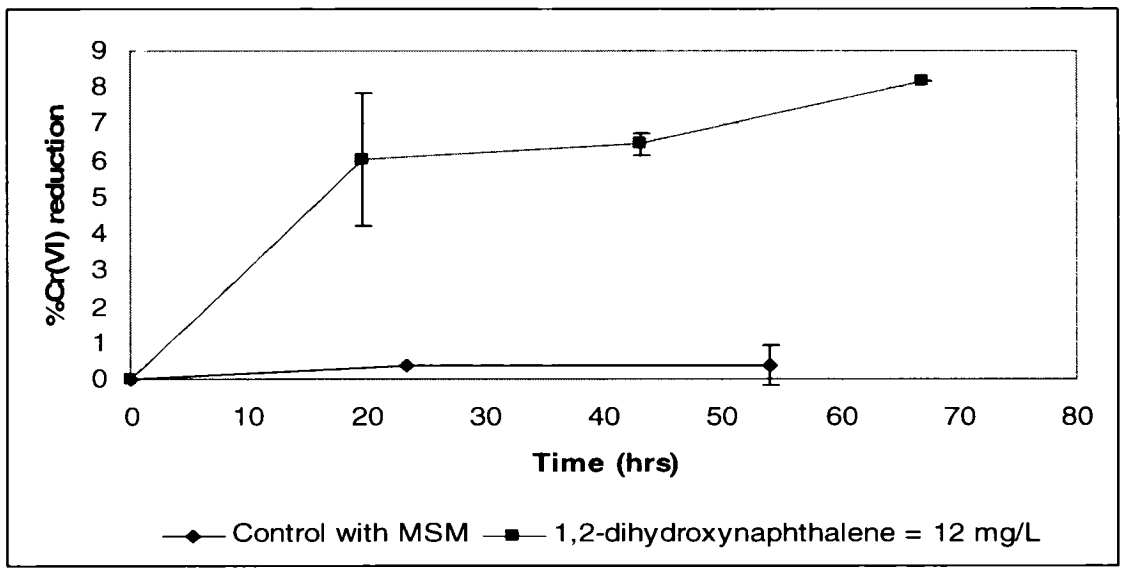


Figure 20. Effect of 1,2-dihydroxynaphthalene on Cr(VI) concentration in an abiotic system with initial Cr(VI) concentration ~6 mg/L.

The kinetics of Cr(VI) reduction by 1,2-dihydroxynaphthalene seemed rapid within the first 20 hours (Figure 20). Because the sampling time interval was too large, the curves did not represent reality. The experiment was repeated using the same concentration of 1,2-dihydroxynaphthalene (12 mg/L) and an initial Cr(VI) concentration of 6.36 mg/L, but the sampling was taken over a shorter time interval. The time profile of Cr(VI) concentration with shorter sampling interval is shown in Figure 21. Within 0.2 hr, around 9% of the Cr(VI) was already reduced. This indicated that Cr(VI) reduction rate was rapid when 1,2-dihydroxynaphthalene was added to the reactor.

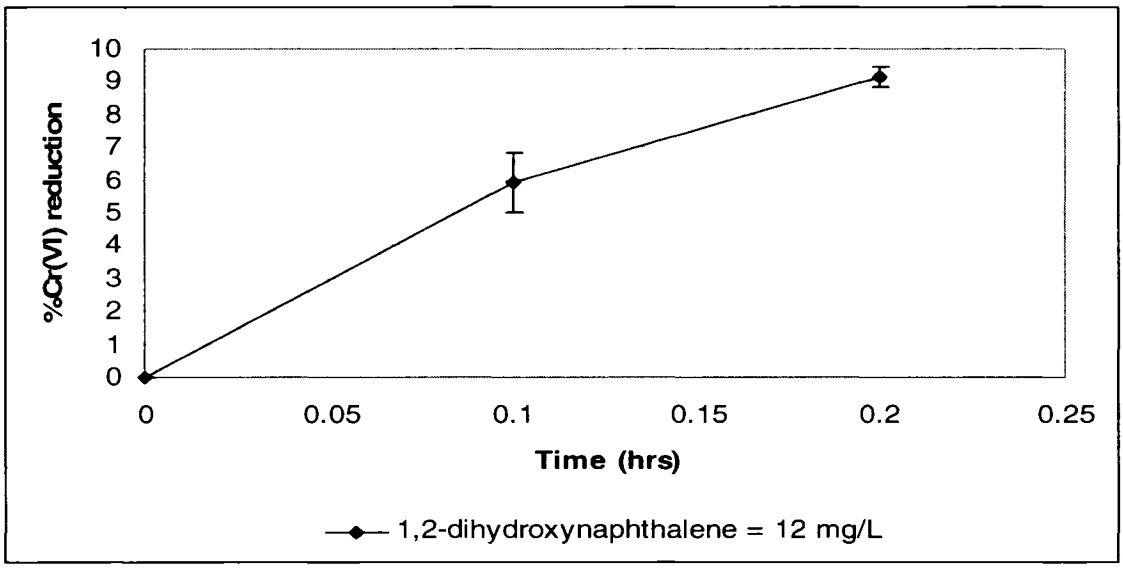


Figure 21. Effect of 1,2-dihydroxynaphthalene (12 mg/L) on Cr(VI) concentration (~6 mg/L) in an abiotic system with shorter sampling time interval.

At 4 mg/L and 12 mg/L, the effects by 1,2-dihydroxynaphthalene were questionable, so a higher concentration (0.2 g/L) of 1,2-dihydroxynaphthalene was employed. The batch reactors contained bacteria, Cr(VI), and MSM. Figure 22 demonstrated that the more 1,2-dihydroxynaphthalene was used, the more hexavalent chromium reduced. The difference in percentage of Cr(VI) reduction between the control and the reactor containing 1,2-dihydroxynaphthalene almost reached 80% after 2.5 hours. If sufficient 1,2-dihydroxynaphthalene was added to the system, it would be able to carry on the reduction of Cr(VI) abiotically up to a 100% Cr(VI) reduction.

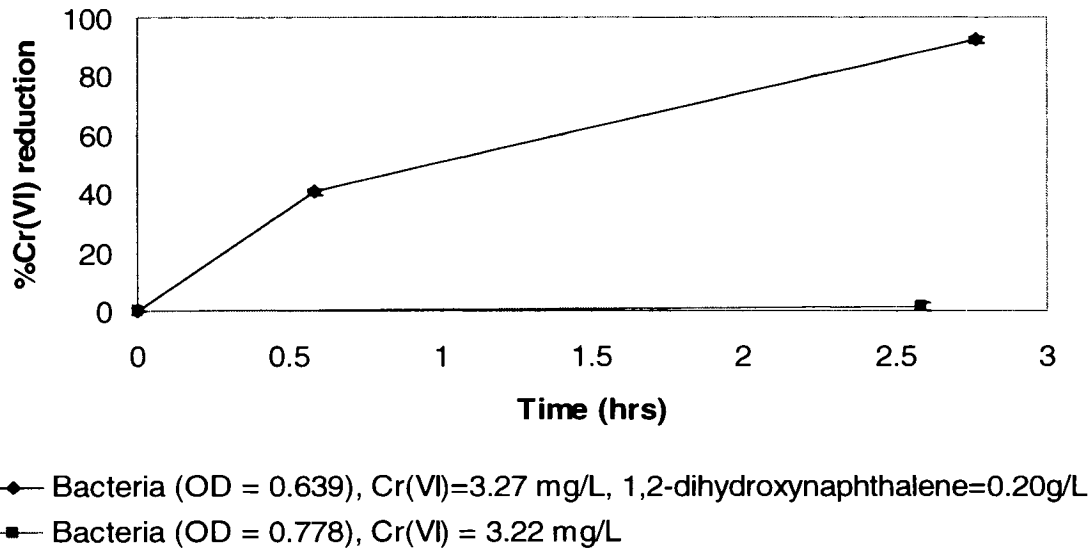


Figure 22 Time Profile of Cr(VI) concentration in a system with high concentration of 1,2-dihydroxynaphthalene.

The compound 1,2-dihydroxynaphthalene was found to be able to abiotically reduce Cr(VI). However, in situations when the concentration of 1,2-dihydroxynaphthalene employed is insufficient to reduce all the Cr(VI), it was not known if the Cr(VI) reduction rate by the bacteria would be influenced. The concentration profile of chromate with time was assessed in a system where a low concentration of 1,2-dihydroxynaphthalene was initially added to reduce the toxicity of Cr(VI) towards the microorganisms. The biometer flasks initially contained bacteria with an OD^{600} of 0.778, Cr(VI)~3.22 mg/L, and dihydroxynaphthalene~12 mg/L. The control was maintained without 1,2-dihydroxynaphthalene.

Unfortunately the rapid reduction of Cr(VI) could not be sustained by the microorganisms as shown in Figure 23. Once all the 1,2-dihydroxynaphthalene reacted

with Cr(VI) after the initial few moments, the Cr(VI) reduction rate resembled those of regular system without 1,2-dihydroxynaphthalene. On the other hand, past research indicated success in sustained rapid reduction of the pollutant (Curtis et al., 1994; Keck et al., 1997; Rau et al., 2002). In fact, Keck et al. (1997), even managed to obtain a ten fold increase of the reduction of sulfonated azo dye amaranth. A concentration of 12 mg/L of 1,2-dihydroxynaphthalene resulted in a 20% Cr(VI) reduction within the first hour of reaction, and a 60% Cr(VI) reduction in 40 hrs. An estimated 15% Cr(VI) reduction difference was seen throughout the experiment between the control, and the reactor employing 1,2-dihydroxynaphthalene.

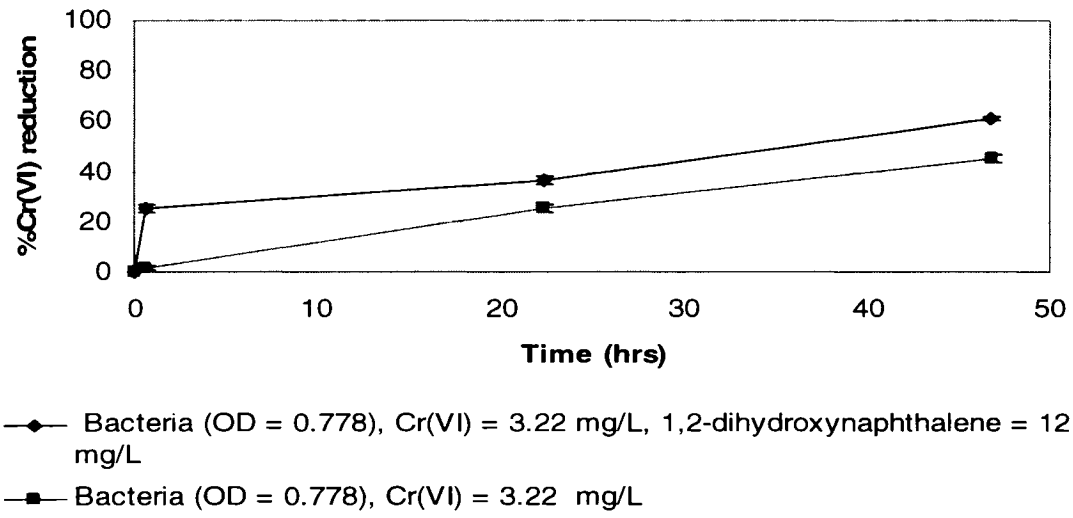


Figure 23. Time Profile of Cr(VI) concentration in systems with and without 1,2-dihydroxynaphthalene

4.4 The interconversion between 1,2-naphthoquinone and 1,2-dihydroxynaphthalene

Past studies have shown that during the mineralization of naphthalene, 1,2-naphthoquinone and 1,2-dihydroxynaphthalene were detected (Ghoshal and Luthy, 1998; Al-Hakak, 2001; Keck et al., 1997). The compound 1,2-dihydroxynaphthalene was proven to be the reducing agent and not 1,2-naphthoquinone. The next objective was to find a link between 1,2-dihydroxynaphthalene and 1,2-naphthoquinone under aerobic conditions. The interconversion scheme between quinone (1,2-naphthoquinone) and its suspected hydroquinone (1,2-dihydroxynaphthalene) was explored.

NADH was used as an electron donor to simulate the bacterial reductase. When NADH was added to 1,2-naphthoquinone, it was suspected that 1,2-dihydroxynaphthalene would form inside the aerobic batch reactor. HPLC analysis was used to identify the presence of 1,2-naphthoquinone and 1,2-dihydroxynaphthalene.

Three HPLC analysis were performed: one on a solution of 4 mg/L of 1,2-naphthoquinone only, another on a solution of 4 mg/L of 1,2-dihydroxynaphthalene only, and one last on a solution where NADH was added to 1,2-dihydroxynapthalene. Both NADH and 1,2-naphthoquinone were initially dissolved in HPLC-grade methanol and then introduced into the MSM. The initial concentration in this particular batch reactor was 0.208 g/L NADH and 4 mg/L 1,2-naphthoquinone. The sample was taken after 5 min. of reaction. The samples were first centrifuged before being analysed on the HPLC.

Figure 24 shows the result of the analysis. At 275 nm, the formation of 1,2-dihydroxynaphthalene was clearly evident. The optimal retention time for 1,2-

naphthoquinone and 1,2-dihydroxynaphthalene was found to be 1.34 and 0.912 min, respectively. In Figure 24 (B) the two peaks were observed at 0.93 and 1.34 min. Thus, 1,2-dihydroxynaphthalene was effectively produced when 1,2-naphthoquinone came into contact with an electron donor.

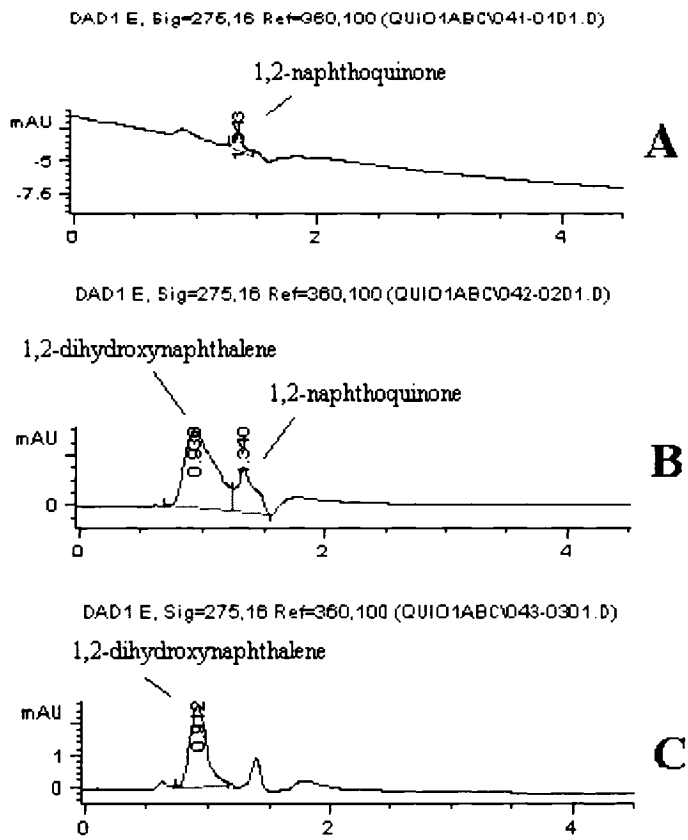


Figure 24. HPLC analyses result. A) spectra of 4 mg/L 1,2-naphthoquinone only. B) spectra of 4 mg/L 1,2-naphthoquinone with 0.208 g/L NADH. C) spectra of 4 mg/L 1,2-dihydroxynaphthalene only.

Another point that needs to be cleared is whether the microorganisms can mediate the 1,2-naphthoquinone and 1,2-dihydroxynaphthalene interconversion reaction without the aid of NADH addition. Thus a batch reactor was prepared with bacteria, 1,2-

naphthoquinone and naphthalene. The initial system contained 0.1 mg/l 1,2-naphthoquinone, 0.7 ml of HMN-naphthalene, and 5 ml of bacterial culture with an initial OD^{600} equal to 0.528. The batch reactor was incubated at a constant temperature of 25°C for 19.5 hrs before samples were taken for HPLC analysis to verify if 1,2-dihydroxynaphtalene was present in the system.

After 19.5 hours passed, 1 ml of sample was taken and diluted with 3 ml methanol and centrifuged before injecting into the HPLC. Figure 25 shows the result of the HPLC analysis where three peaks were obtained. The first one at 1.105 min. corresponds to 1,2-dihydroxynaphthalene (~0.912 min), the second one at 1.452 min. corresponds to 1,2-naphthoquinone (~1.432 min) and the third peak at 3.795 min. corresponds to naphthalene (~3.588 min). The variation of the retention time between the standards and this experiment was caused by a slight difference in the methanol media. Thus, analyses proved that 1,2-dihydroxynaphthalene could accumulate in a system containing bacteria, naphthalene, 1,2-naphthoquinone and exempt from major oxidant such as Cr(VI). However, it is suspected that the concentration of 1,2-dihydroxynaphthalene will decrease over time if left in contact with oxygen.

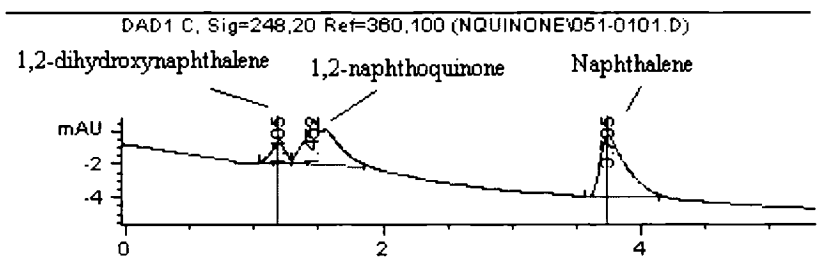


Figure 25 HPLC analysis result of a sample taken after 19.5 hrs of incubation time from a system containing bacteria, naphthalene, and 1,2-naphthoquinone.

Experiments investigating whether if 1,2-naphthoquinone and 1,2-dihydroxynaphthalene would form from the mineralization of naphthalene were not performed, because it is known from the study by Ghoshal and Luthy (1998) that these compounds would form under some conditions. They found that 1,2-dihydroxynaphthalene accumulates when enzymatic conversion of naphthalene to 1,2-dihydroxynaphthalene is faster than the subsequent transformation reactions. The 1,2-dihydroxynaphthalene may then be oxidized to 1,2-naphthoquinone if the residence time is sufficient (Ghoshal and Luthy, 1998). It was not known how 1,2-dihydroxynaphthalene, and 1,2-naphthoquinone would behave in a system containing bacteria, Cr(VI) and naphthalene. The following experiment consisted of monitoring 1,2-dihydroxynaphthalene and 1,2-naphthoquinone using the HPLC in a system with bacteria with an initial OD^{600} equal to 0.528, 20 mg/L naphthalene, 2.5 mg/L of 1,2-dihydroxynaphthalene and 1 mg/L Cr(VI). A reading was taken right at the start of the experiment and one more after 4 days of incubation, when the concentration of Cr(VI) was near zero as confirmed by the diphenylcarbazide test.

The initial reading (Figure 26) showed that the concentration of 1,2-dihydroxynaphthalene (1.189 min., 62.126 μAU°s) was 2.20 mg/L, the concentration of 1,2-naphthoquinone (1.367 min., 12.504 μAU°s) was 0.31 mg/L, and the naphthalene (3.569 min., 5099 μAU°s at 220 nm) concentration was 21.62 mg/L. This indicated that there was a rapid conversion of 1,2-dihydroxynaphthalene to 1,2-naphthoquinone. After 4 days of incubation, another reading (Figure 27) was taken. The new concentration of 1,2-

dihydroxynaphthalene (1.155 min., 37.14 μAU°s) was 1.31 mg/L, the concentration of 1,2-naphthoquinone (1.379 min., 8.44 μAU°s) was 0.21 mg/L, and naphthalene's (3.401 min., 1779.47 μAU°s at 220 nm) concentration dropped to 7.55 mg/L. Thus, some 1,2-dihydroxynaphthalene was converted to 1,2-naphthoquinone when it donated its electrons to chromate and about half remained in the system after the chromate has been completely reduced to Cr(III). The concentration of 1,2-naphthoquinone also decreased after all Cr(VI) had been reduced. The decrease of naphthalene concentration may be attributed to the mineralization activity by bacteria and volatilization loss.

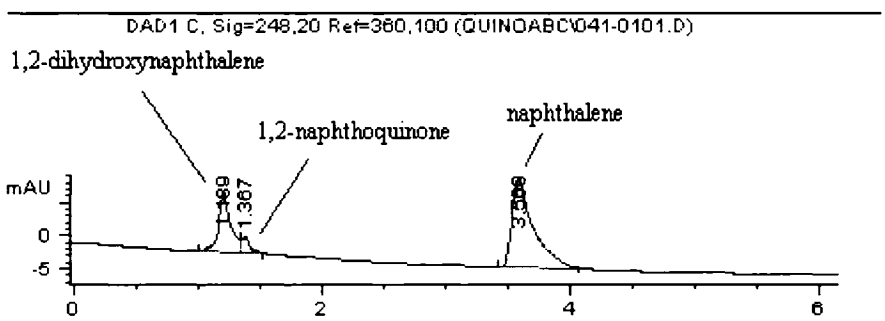


Figure 26. HPLC analysis at start of experiment of batch reactor with 1,2-dihydroxynaphthalene, Cr(VI), naphthalene, and bacteria.

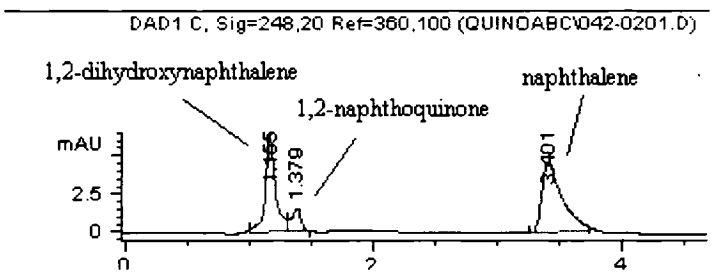


Figure 27. HPLC analysis at end of experiment when Cr(VI) ~ 0.

Another point to investigate is the kinetics of Cr(VI) reduction between systems containing 1,2-dihydroxynaphthalene and systems containing 1,2-naphthoquinone and NADH. It was not known how long it took a system to convert 1,2-naphthoquinone to 1,2-dihydroynaphthalene, and then finally reduce Cr(VI). A control containing only NADH and chromate was tested to determine if NADH reduced Cr(VI) similarly as 1,2-dihydroxynaphthalene.

Figure 28 shows that the Cr(VI) reduction kinetics, between the system containing NADH and Cr(VI) and the system containing NADH, 1,2-naphthoquinone and Cr(VI), cannot be compared due to their rapid reduction mechanisms. The control showed that NADH alone was able to reduce Cr(VI). By the time the samples have been treated for absorbance reading, the reduction reaction had already occurred. Thus, the relative Cr(VI) reduction kinetics between system employing 1,2-dihydroxynaphthalene and a system employing 1,2-naphthoquinone with a supply of electron donor (NADH) remains unknown. However it is certain that the Cr(VI) reduction reaction occurs rapidly in both cases.

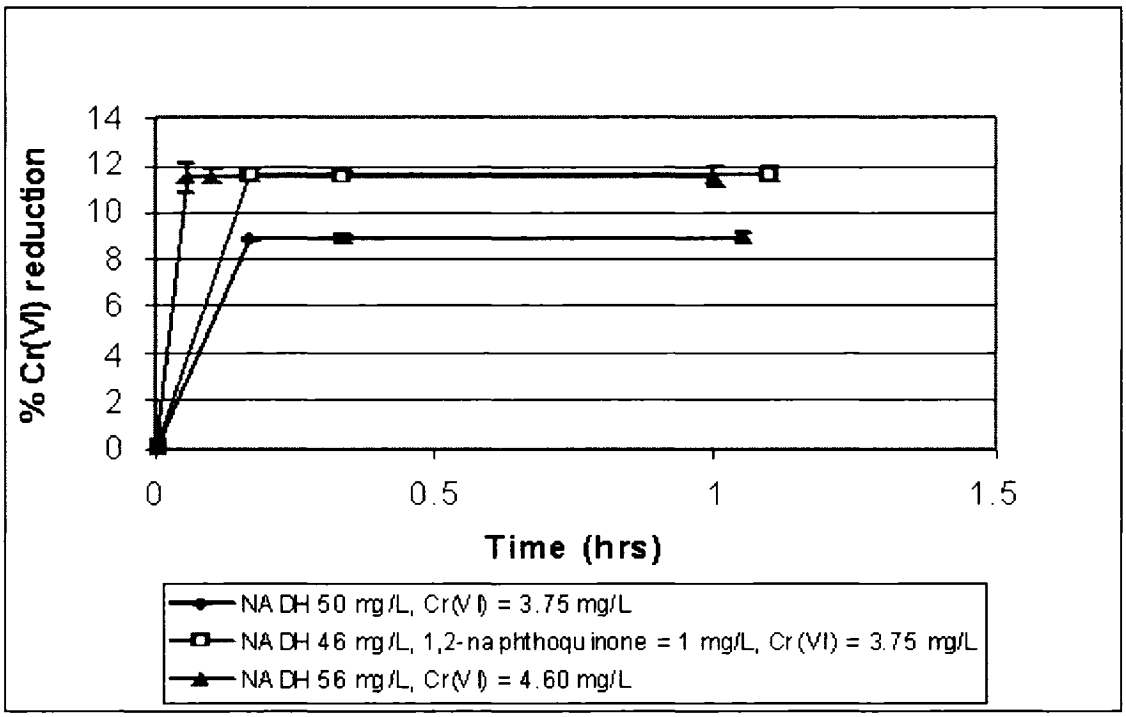


Figure 28. The effect of NADH on Cr(VI). NADH is able to reduce Cr(VI) by itself. The time required for the reduction is less than 0.3 hours.

Previous researchers indicated that the reduction occurs with the soluble protein reductase for aerobic bacteria and at the membrane for anaerobic bacteria and noted that the addition of quinone increased greatly the rate of reduction, but all these cases dealt with a constant or an excess of electron donor (Ishibashi et al., 1990). One of naphthalene metabolite's products, 1,2-dihydroxynaphthalene, was found to be able to reduce Cr(VI). This chemical can indeed reduce chromium (VI) abiotically. It is believed that the reduction occurs exclusively in the soluble phase, because of its unstable nature, 1,2-dihydroxynaphthalene would give out its electrons to the dissolved chromate before it has a chance to enter the bacteria while chromate can accumulate inside the bacteria.

4.5 The Effect of Siderophore on Cr(VI) reduction

This part of the research deals with the possible effect of the siderophore DFB on a biometer system containing Cr(VI) and naphthalene. It is not known if *Pseudomonas putida* ATCC 17484 produced or employed siderophore since there is no literature about this issue, although some *Pseudomonas* species are known to produce some (Schalk et al., 2002). The addition of DFB might be able to enhance the solubility of the Cr(VI) metal, favouring bacterial uptake, and Cr(VI) reduction. It was imperative to determine first whether the added siderophore had any effect on the growth of the bacteria, since the Cr(VI) reduction process depends greatly upon the metabolic activity of the cells and bacterial population size. The reactor flasks initially contained 50 ml MSM, 0.24 g/L DFB, and were inoculated with 5 ml of bacteria (OD^{600}~0.412).

From Figure 29, the siderophore significantly inhibited the growth of the *Pseudomonas putida* ATCC 17484. Other researchers had indicated that addition of siderophores promoted growth of the culture (Guan and Kamino, 2001; Sharma and Johri, 2003; Yamazaki et al., 1999). The growth phase has been retarded by as much as one-day time. In a system with only naphthalene and mineral salt media, the growth was linear and its absorbance reached 1.4 in about two days. The time required for a system containing DFB to reach a similar optical density of 1.4 was more than 3 days. The linear growth only started after 24 hours of incubation for a system with DFB. This might mean that the siderophore has chelated with the iron that was present in the mineral salt media, and was not immediately available to the *Pseudomonas*. The *Pseudomonas* strain took a period of time to adapt to the presence of DFB. The microorganisms would first use free

iron, and when free iron became rare, the need for iron triggered their metabolism to accept siderophore chelated compound. The entry mechanism is considered to employ a protein receptor embedded in the external membrane. Two other possible reasons for the growth inhibition might be the extra time required to separate the chelated compound inside the bacteria or the low concentration of free iron. Since most of the free iron is chelated, the bacteria might not be able to recognize the chelated compound, and thus rely on the remaining free irons for growth and activity, which would explain the extra time required to grow.

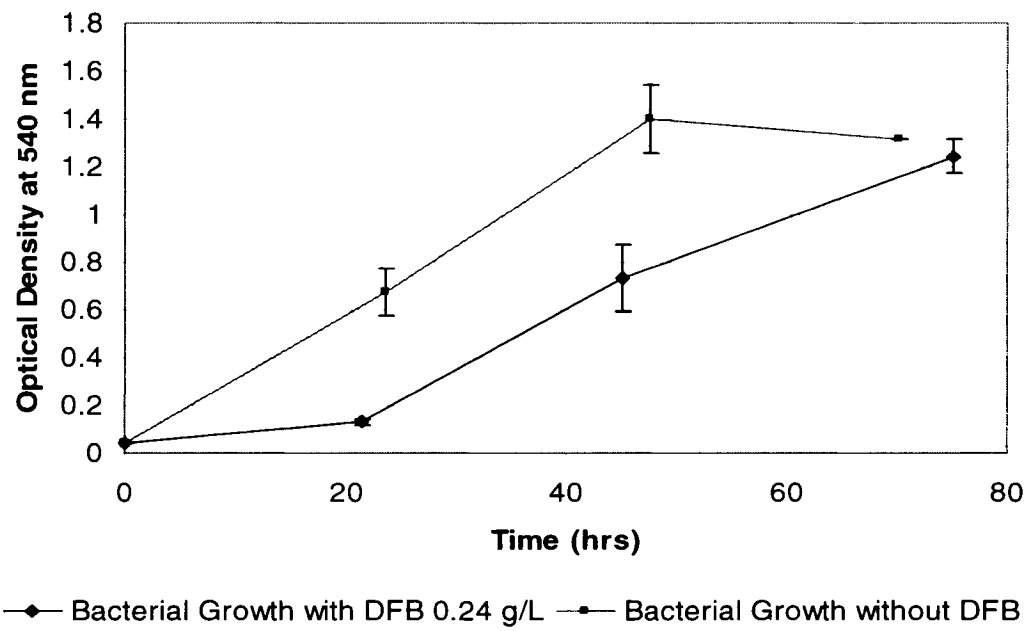


Figure 29. Time profile of the growth of bacteria with and without siderophore.

Another experiment consisted of verifying the removal of Cr(VI) from solution when DFB was present. The DFB experiments were performed similarly to those dealing with quinone and dihydroxynaphthalene, except that a desired amount of siderophore

(Desferrioxamine B) was added. The batch reactors initially contained 3.57 mg/L to 4.49 mg/L of Cr(VI), and 0.50 g/L to 0.54 g/L of DFB. The concentration of Cr(VI) was monitored with time. Figure 30 shows that the rate of decrease in Cr(VI) concentration was significantly different than the system without any addition of DFB. There was a substantial amount of Cr(VI) decrease within the first 24 hrs. After that time, no further Cr(VI) concentration change was observed, even when the solution was acidified to a pH of 1.0-2.0. Other researchers noticed a change in the properties of the siderophore-complex when the pH of the solution fluctuated (Spasojevic et al., 1999; Kraemer et al., 2002; Birus et al., 1987). After 48 hrs, no Cr(VI) was detected and the color of the solution turned from slightly turbid to a honey yellowish-brown color. It is possible that DFB reacted to Cr(VI) and formed a neutral complex that had a yellowish-brown color.

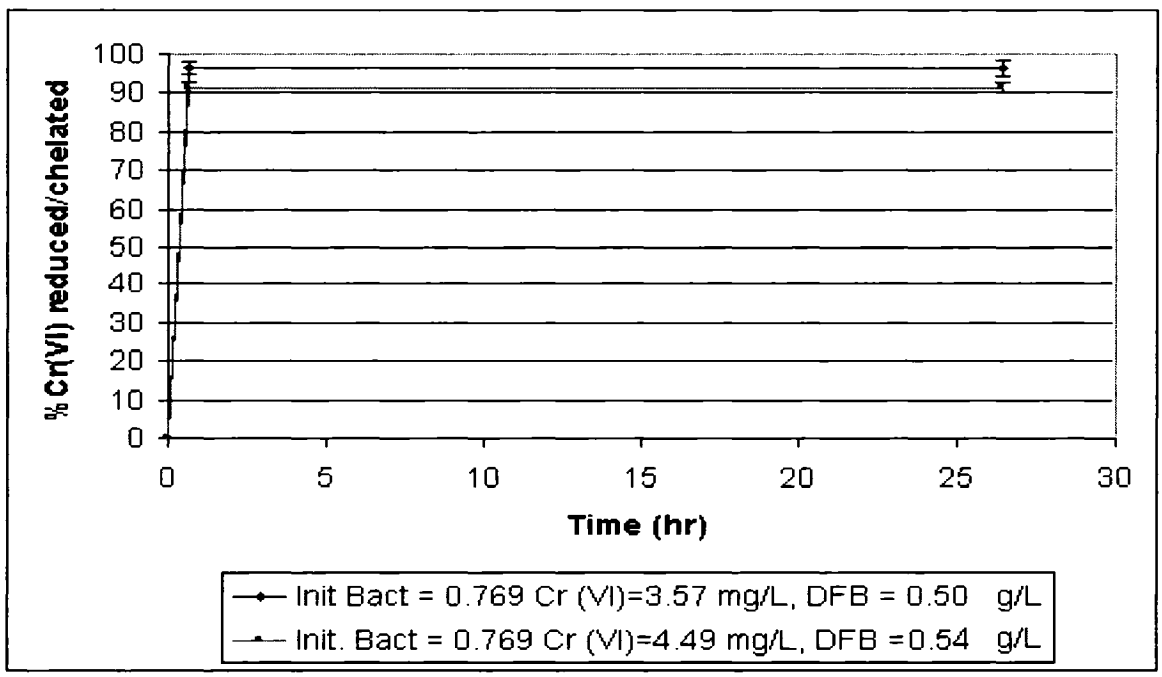


Figure 30. Effect of chelation by DFB on Cr(VI).

Figure 30 shows that Desferrioxamine B alone can chelate with hexavalent chromium to a point that no Cr(VI) can be detected by the diphenylcarbazide test. The more DFB is added to the system, the more Cr(VI) is chelated. Additionally, the reaction kinetic is very fast. If a comparison is made between a system containing the usual bacteria, naphthalene, and Cr(VI) to a system with the same parameters except that we add siderophore, the total amount of Cr(VI) disappearance is greatly increased by as much as 30% as shown in Figure 31.

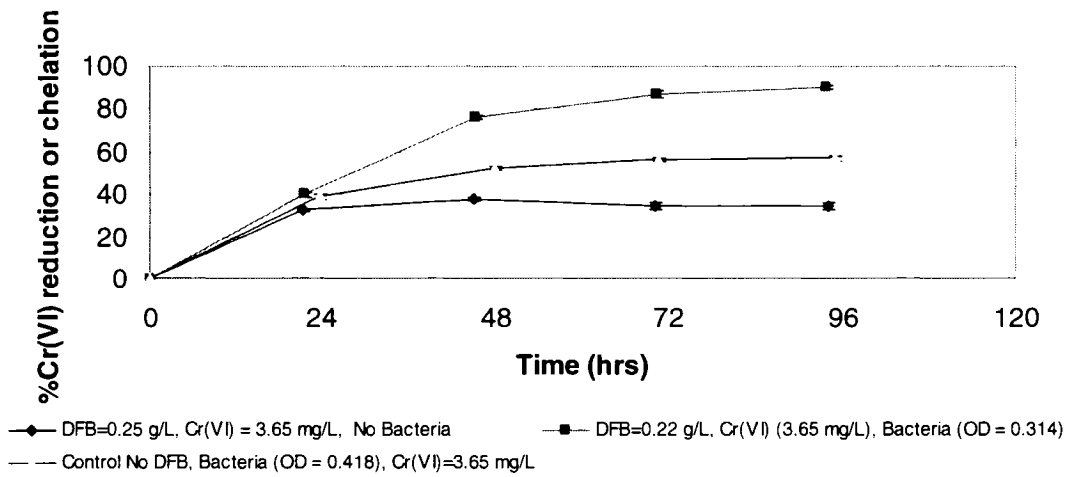


Figure 31. The effect of DFB on Cr(VI) reduction by bacteria

When DFB was added to a system containing bacteria, naphthalene, and chromate, the Cr(VI) concentration quickly decreased for the first 48 hours then slowed down considerably as shown in Figure 31. It is believed that Cr(VI) decrease within the initial 24 hours was caused by the chelation with DFB as shown by the control. Past the 24 hours mark, the microorganism was solely responsible for the Cr(VI) disappearance.

The reduction rate was greatly lowered due to the remaining chromate's toxicity toward bacteria.

4.6 The effect of 1,2-dihydroxynaphthalene and DFB on the mineralization of naphthalene

1,2-dihydroxynaphthalene and siderophore DFB showed signs that they could reduce or chelate with Cr(VI) leading to the hypothesis that the addition of those chemicals would surely increase the rate of mineralization of naphthalene in the presence of Cr(VI). The idea was that adding the 1,2-dihydroxynaphthalene or siderophore DFB would decrease the Cr(VI) concentration sufficiently so that the mineralization of naphthalene would pick up faster. However the experiments showed otherwise and are inconclusive. The results are shown in the Appendix.

5.0 Conclusion and Recommendations

5.1 Conclusions

It was found that the bacteria's metabolic activity at the moment of inoculation used is of utmost importance in system using 1,2-naphthoquinone as an additive to increase the mineralization rate of naphthalene and the reduction of Cr(VI). The metabolic activity of the bacteria clearly determines its ability to donate electrons via soluble reductase NADH. Following the electron mediator scheme discussed within this thesis, an excess of electron donors must be present to keep the cycle and the quinone-hydroquinone interconversion functioning. The Cr(VI) reduction experiments employing 1,2-naphthoquinone showed no significant difference from a system without that chemical. The formation of 1,2-dihydroxynaphthalene resulted from the reduction of 1,2-naphthoquinone by NADH addition. 1,2-dihydroxynaphthalene was found to be able to reduce Cr(VI) at rapid rate. The abiotic rate of Cr(VI) reduction by 1,2-dihydroxynaphthalene is proportional to the concentration employed. Once all the 1,2-dihydroxynaphthalene was converted back to 1,2-naphthoquinone, the rate of reduction resembles those from the control. The addition of the siderophore DFB inhibited the bacteria growth, but chelated rapidly with Cr(VI). The compound 1,2-dihydroxynaphthalene and DFB may be useful when dealing with a situation where the site is greatly contaminated with a high concentration of Cr(VI). These chemicals can be added to first decrease the Cr(VI) concentration down to a level where the bacteria would overcome the inhibition and start reducing activities. However, using DFB is problematic since it is not selective and can instead decrease the availability of iron to the

microorganism that do not have appropriate protein receptors and, as a result, inhibit its growth.

In the case of naphthalene biodegradation, a problem with the capture of ^{14}C, lead the study to inconclusive results. However, it is still believed that the addition of 1,2-dihydroxynaphthalene should increase the mineralization rate if the initial Cr(VI) concentration in the system is lower than 3.65 mg/L, but not for the case of siderophore. The addition of DFB to the system inhibits bacterial growth, thus it is expected that it will slow down the mineralization rate of naphthalene and the reduction of Cr(VI).

5.2 Recommendations

An area that needs to be researched is the affinity/selectivity of the DFB siderophore. It is not known how the siderophore will behave if exposed in the presence of other various metals and other bacterial strains. Will there be competition, and if so in favour of what? It was found that chelation with hexavalent chromium is rapid; indeed, it is almost instantaneous. The mechanism of the siderophore on chromium, and the bacteria remains obscure, and needs to be enlightened further. It is not known if *Pseudomonas putida* ATCC 17484 is able to uptake the DFB-metal complex. Another point of interest might be to evaluate the mobility of the newly formed siderophore-chromium complex compared to the free form of the metal. The idea of using redox mediators and siderophore to enhance the rate of biodegradation of naphthalene and the reduction of Cr(VI) remains feasible. Future studies should determine which type of

quinones and siderophores would be compatible with the simultaneous co-contaminant naphthalene and Cr(VI) decontamination.

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Appendix

A.1 Gibbs free energy calculations

Additional Gibbs free energy calculation to determine if 1,2-dihydroxynaphthalene changes to 1,2-naphthoquinone. Some quinone may be reduced to a hydroquinone radicals (semiquinone) form (see Figure 2). In Gibb's free energy calculations, the redox potential of quinone that should be used is the two-electron potential because of its availability in the literature for the relevant quinones rather than one-electron potentials. Using this value gives a good initial estimate when the data for single electron potential is not available (Rau et al., 2002).

$$5C_{10}H_8O_2 + 5O_2 \longleftrightarrow 5C_{10}H_6O_2 + 5H_2O_2 + e^- \quad \textbf{(1)}$$

Table 4. Gibbs free energy values taken from from sci-finder

ΔGfo (kJ/mole)	Compound
46.66	$C_{10}H_8O_2$ (1,2-dihydroxynaphthalene)
0	O_2
-27.599	$C_{10}H_6O_2$ (1,2-naphthoquinone)
-120.4	H_2O_2

Note for $C_{10}H_6O_2$,E'o = +0.143 @ pH 7.0 (Morton, p. 4 table 1)

$$\Delta G'_f = -nFE'_o = -2e^- \times \frac{96500 coulomb}{mol \cdot e^-} \times 0.143V \times \frac{1J}{1Vcoulomb} \times \frac{kJ}{1000J} = -27.599kJ$$

for $C_{10}H_8O_2$

$$\frac{[C_{10}H_6O_2]}{[C_{10}H_8O_2]} = \frac{Ka_1 Ka_2}{[H^+]^2}$$

pKa of $C_{10}H_8O_2$ =8.17±0.20 (Sci-Finder)

$$\Delta G'_r = -RT \ln K_{eq} = -8.314 \frac{J}{Kmole} * \frac{kJ}{1000J} * 298.15K \ln K_{eq}$$

$$pKa = -\log Ka = -2.303 \ln Ka$$

$$\Delta G'_r = 2.303 * 2.48 \frac{kJ}{mole} pKa = 46.66 \frac{kJ}{mole}$$

The Gibbs free energy for reaction (1) can now be calculated

$$\Delta G'_{reaction} = \sum \partial \Delta G' f_{(products)} - \sum \partial \Delta G' f_{(reactants)}$$

$$\Delta G'_{reaction} = 5[\Delta G' f_{C10H6O2}] + 10[\Delta G' f_{H+}] + 1[\Delta G' f_{e-}] - 5[\Delta G' f_{C10H8O2}]$$

$$\Delta G'_{reaction} = 5\left(-27.599 \frac{kJ}{mole}\right) + 10(0) + 1(0) - 5\left(46.66 \frac{kJ}{mole}\right) = -371.295 \frac{kJ}{mole}$$

Thus at constant temperature and pressure, this reaction is spontaneous (exergonic reaction); i.e 1,2-dihydroxynaphthalene changes to 1,2-naphthoquinone and gives 1 electron. Can the Cr(VI) use this e- to change into Cr(III)?

$$15C_{10}H_8O_2 + 15O_2 \longleftrightarrow 15C_{10}H_6O_2 + 15H_2O_2 + 3e^-$$

$$CrO_4^{2-} + 8H^+ + 3e^- \longleftrightarrow Cr^{3+} + 2H_2O$$

$$15C_{10}H_8O_2 + 15O_2 + CrO_4^{2-} + 8H^+ \longleftrightarrow 15C_{10}H_6O_2 + 15H_2O_2 + Cr^{3+} + 2H_2O$$

=-2882.245kJ/mole thus thermodynamically feasible by using Table 5's values.

Table 5. Gibbs free energy continued

ΔG'f (kJ/mole)	Compounds
46.6	$C_{10}H_8O_2$
0	O_2
-728	CrO_4^{2-}
0	H^+
-27.599	$C_{10}H_6O_2$
-120.4	H_2O_2
-237.18	H_2O
-216	Cr^{3+}

A.2 Mineralization of naphthalene

Biodegradation experiments of naphthalene were performed, but no significant mineralization was observed. The following scenarios were investigated: a pair of batch reactors contained 4 mg/L 1,2-dihydroxynaphthalene, 50 ml MSM, 0.5 ml HMN-naphthalene, and 3.65 mg/L Cr(VI); and another pair contained 50 ml MSM, 0.22 g/L DFB and 0.5 ml HMN-naphthalene. All the batch reactors were inoculated with bacteria OD^{600}~0.469.

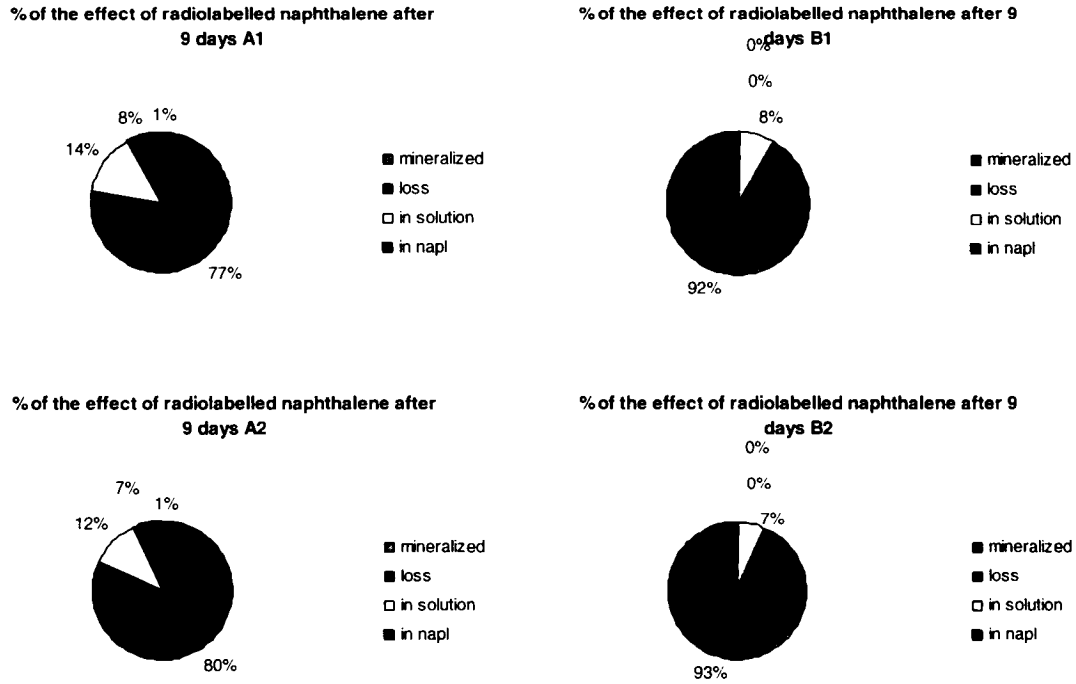

Figure 1 Distribution of the radioalabelled naphthalene after 9 days of incubation. System A1 and A2 contained 1,2-dihydroxynaphthalene, while system B1, and B2 contained DFB.

The addition of 1,2-dihydroxynaphthalene did not influence the rate of mineralization as first thought. In a previous research, it was determined that the maximum Cr(VI) concentration where naphthalene mineralization was detected is around 6.5 mg/L (Al-Hakak, 2000). Despite the fact that only 3.65 mg/L of Cr(VI) was present in each flask, there were no detectable traces of mineralization. An attempt to respike the sample with bacteria was done on the 4^{th} day when mineralization was still zero, but in vain. The persistence of the light yellow color in the solution indicated that Cr(VI) is still present. A sample was taken on the 6^{th} day and checked for optical density; as a result no bacteria growth occurred. Most ^{14}C remained in the NAPL and in the aqueous solution as shown in Figure 32.

Sections 4.3 and 4.5 showed that a significant concentration of Cr(VI) remained in the system when employing a low concentration of chemicals such as quinone, and siderophore. The remnant Cr(VI) might be the reason why little naphthalene mineralization occurred. Thus in conjunction to previous studies when Cr(VI) is present there is little or no mineralization of naphthalene occurs as well as bacteria growth. Little mineralization occurred even for the control containing no Cr(VI), however there was an unexplainable amount of loss of ^{14}C. It seems that in addition to the toxicity problem, there was a problem with the capture of ^{14}C in this study. According to previous studies, the mineralization of naphthalene can be completed nearly within 24 hrs of incubation. Passed 48 hours, there was very little mineralization activity left since most of the naphthalene has been degraded (Al-Hakak, 2000; Shen et al., 1994).

Suspicions arose when in section 4.5, the bacteria growth was hindered with the addition of siderophore. This clue predicted that adding DFB to a system containing MSM, HMN-naphthalene, and Cr(VI) will not increase the mineralization rate of naphthalene. It was confirmed in the experiment when desferrioxamine B was added to a biometer flask containing an initial Cr(VI) concentration of 3.65 mg/L and where radiolabelled naphthalene was monitored. Little or no mineralization occurred even after 4 days of incubation. The bacteria already had difficulty dealing with Cr(VI), with the addition of DFB, the bacteria growth was further inhibited since no increase in optical density was seen throughout the experiment. Instead of increasing the rate of biodegradation of naphthalene, it significantly decreased it. Thus, desferrioxamine B was

found to be not a suitable siderophore for *Pseudomonas putida* ATCC 17484. However, DFB does show promising future usage in the abiotic decontamination of Cr(VI).

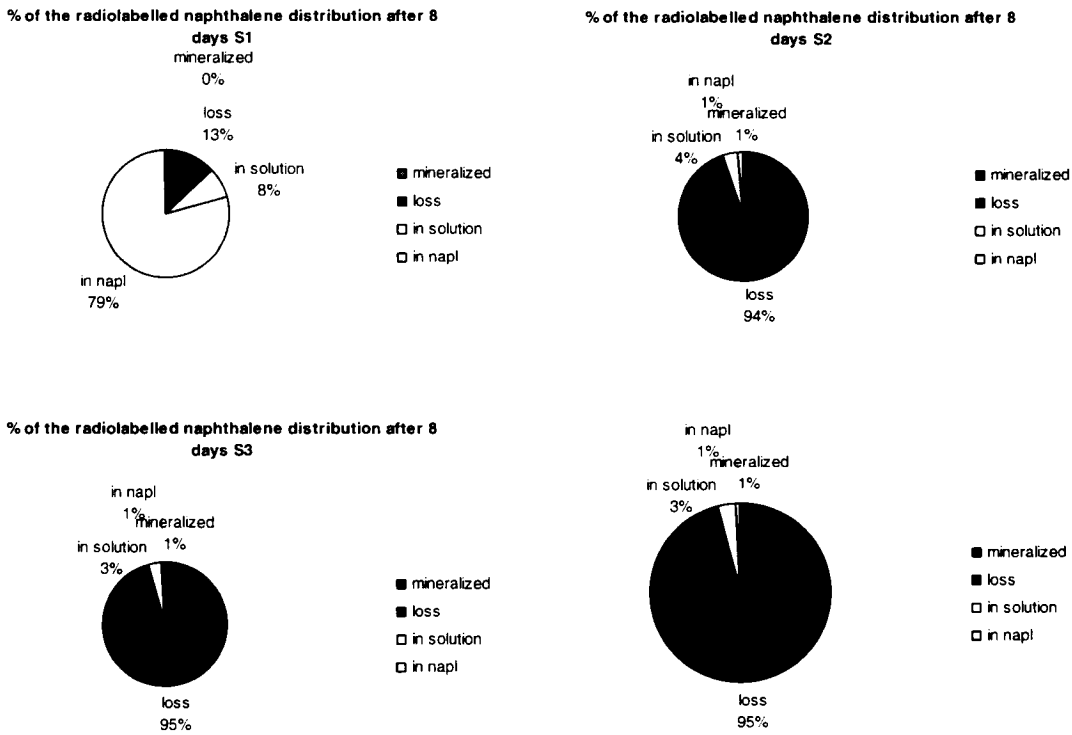

Figure 2 Distribution of the radioalabelled naphthalene after 9 days of incubation for controls. Every reactor contained MSM, bacteria OD~0.41, and naphthalene.

Since no traces (negligible) of mineralization occurred in the previous systems, then the control employed no Cr(VI). The reactors initially contained: 0.7 ml HMN-naphthalene, and 0.5 ml of bacteria (OD^{600}~0.41). Figure 33 shows the evident CO_2 capture problem. Since NaOH were spilled in the biometer flask, system S1 showed no bacteria growth, while for the other 3 reactors, bacteria growth was observed and mineralization of the naphthalene should have occurred too. However, the amount of ^{14}C trapped by the NaOH amounted to only 0.6%. As a result, it is safe to assume that the

true percentage of the naphthalene mineralized is close to the percentage of ^{14}C lost, meaning around 81.06%.

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