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MICROBIAL CR(VI) REDUCTION IN INDIGENOUS CULTURES OF BACTERIA: CHARACTERIZATION AND MODELLING

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MICROBIAL CR(VI) REDUCTION IN INDIGENOUS CULTURE OF BACTERIA: CHARACTERIZATION AND MODELLING

By

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SYNOPSIS

South Africa currently faces multiple Cr(VI) contamination problems which are unsuccessfully remediated using available technologies. Cr(VI) is highly toxic, carcinogenic and mutagenic in nature and it is exclusively released through anthropogenic activities. A new treatment approach is proposed using locally isolated Cr(VI) reducing species of bacteria. This method is envisioned to be economical and ecologically friendly.

Indigenous chromium(VI) reducing bacteria (CRBs) were isolated from a dried sludge consortium collected in the Brits Wastewater Treatment Plant, North-West Province (South Africa). Characterisation using 16S rRNA fingerprinting followed by taxonomic studies revealed a wide diversity of CRBs isolated under anaerobic conditions than under aerobic conditions. The consortium was determined to be predominantly gram-positive. The Cr(VI) reducing component of the culture was determined to be predominantly facultative, consisting predominantly of *Bacillus sp.*, i.e. *B. cereus, B. thuringiensis* and *B. mycoides*.

Batch experiments under both aerobic and anaerobic conditions showed a high Cr(VI) reducing performance under relatively high initial Cr(VI) concentrations. The reduction rate using this culture was 3 to 8 times higher than reduction rates reported in bacteria previously isolated and studied in North America and Europe. The culture performed best as a consortium with the different species operating cooperatively. The bacteria were acclimated to Cr(VI) toxicity through the long period of contact during the activated sludge treatment process at the source.

A Monod like model was used to evaluate the rate of Cr(VI) reduction over a wide range of initial Cr(VI) concentrations. The model revealed that Cr(VI) reduction in the consortium culture followed quasi-first order kinetics with a Cr(VI) inhibitor term as a second exponential: $C = C_0 \,.\, \exp\,[-p \,.\, \exp\,(-q \,.\, C_0\,) \,.\, t]$. The parameter p and q for the semi-empirical first order model were statistically accurate with R^2 values greater than 94% for all data ranges evaluated. Previous studies were not able to pick the variability of Monod coefficients, k_{mc} and K_c, since at narrow ranges of initial Cr(VI) concentrations, the impact of the chromium toxicity variability was insignificant.

This study demonstrates the potential of a biological approach using locally isolated Cr(VI) reducing bacteria to decontaminate Cr(VI) polluted sites in South Africa.

DECLARATION

I, MELI Kakonge, declare that the dissertation, which I hereby submit for the MSc: Applied Science: Water Utilisation degree at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

MELI Kakonge

April 2009

ACKNOWLEDGEMENT

Samuel Smiles said: "*Progress however, of the best kind, is comparatively slow. Great results cannot be achieved at once; and we must be satisfied to advance in life as we walk, step by step*." This is another step of my life but more in research with a lot of contributions from the following individuals:

a) The first to whom I am grateful is our creator: GOD; "God of Science", who gave me the opportunity to realize this dissertation. GLORY BE TO HIM.
b) I am thankful to my supervisor, Professor Evans Martin Nkhalambayausi-CHIRWA for his excellent guidance, patience, support, encouragement, finally his commitment to high-quality results.
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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF NOMENCLATURES

AA	Atomic Absorbtion
BRB	Biological Reactive Barrier
C	concentration
CEC	Cationic Exchange Capacity
CFU	Colony Forming Units
ChrR	Chromosome R
COD	Chemical Oxygen Demand
Cr(III)	Chromium 3
Cr(VI)	Chromium 6
CRB	Chromium(VI) Reducing Bacteria
[Cr(VI)]	Chromium 6 concentration
Cyt	Cytochrome
^{o}C	Celsius degree
DO	Disolved Oxygen
DOC	Dissolved Organic Carbon
DNA	Deoxyribonucleic acid
D. Vulgaris	*Desulfovibrio vulgaris*
E. coli	*Escherichia coli*
Eh	Potential-redox
g	Gravitational force
hrs	Hours
LB	Lauria Bettani
mg/L	Milligram per Liter
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
P. fluorescens	*Pseudomonas fluorescens*
Ps. fluorescens	
ppm	Part per Million
pH	Potential Hydrogen
PRB	Permeable Reactive Barriers
Rc	Cr(VI) reduction capacity

Redox	Reduction–oxidation
rRNA	Ribosomal Ribonucleic acid
rpm	Rotation per minutes
SA	South Africa
SBMR	Sequencing Batch Membrane Reactor
SDC	Sodium dichromate
SRB	Sulphate Reducing Bacteria
t	*Time*
T	*Temperature*
TDS	Total Dissolved Solids
TOC	Total Organic Carbon
TSS	Total Suspended Solids
U.S. EPA	United State of America Environment Protection Agency
X	Viable cells

CHAPTER 1: INTRODUCTION

1.1 Introduction

This report is the presentation of our study: "*Microbial Cr(VI) reduction in indigenous culture of bacteria: Characterization and modelling*". The study was focused on the isolation, identification and characterization of Chromium(VI) reducing bacteria (CRB) in South Africa (SA). The study also elucidated the kinetic model for the microbial reduction of Cr(VI). This study is a preliminary investigation for further work on metal bio-decontamination in SA, where this efficient and environmental friendly technology is still unexplored.

Cr(VI) is extremely toxic and carcinogenic in its ionic form. It causes dermatitis, renal tubular necrosis, DNA damage or mutation and many other harmful consequences in mammals including humans (Flessel, 1979; NAS, 1974 Gibb *et al.* 2000; Caglieri *et al.*, 2006). It also reduces seed germination in plants (Anon, 1974; Towill *et al.*, 1978). The release of Cr(VI) in the environment can only occur by anthropogenic activities such as electroplating, leather-tanning processes, wood preservation, chromite ore processing and metal-finishing industries (Cohen and Costa, 2000; Chuan and Liu, 1996; Lawson, 1997; Karar *et al.*, 2006).

Many Cr(VI) polluting sites in South Africa have been reported, such as *i.e.* at Brits in the North West province of South Africa (Appendix Figure A-2), Mpumalanga Coal Mines, and Durban Municipality in Kwazulu Natal. Cr(VI) is a concern in the country since incorrect and inadequate attempt of physico-chemical remediation treatment was unsuccessful (DWAF, 2006). In spite of all advantages offered by bioremediation as economical and ecologically friendly (Viamajala *et al.*, 2002), this technique is unexplored in the country. This study will investigate the new approach in remediation of Cr(VI) pollution in South Africa.

This report provides a summary of our achievement and recommendations for uncharted areas in the process of microbial Cr(VI) reduction. It is subdivided in 6

sections: objectives of the study, outline of dissertation, methodology, problems encountered and solutions, summary of results obtained and further work.

1.2 Objective of the Study

The primary objective of the study was to investigate Cr(VI) reduction in a local indigenous cultures of Cr(VI) reducing isolates of bacteria and to see whether these cultures can be applied in bioremediation of effluents from local industries.

The specifics aims of the study were:

- To identify potential CRB sources for efficient Cr(VI) reduction in South Africa.
- To investigate the kinetics of Cr(VI) reduction in indigenous CRB consortium grown aerobically and anaerobically over a wide range of initial Cr(VI) concentrations.
- To characterize the consortium by isolation and identification of CRB species using the 16s rRNA method, followed by designation of taxonomies based on rRNA Blast search results.
- To determine the Cr(VI) reduction pathway in purified cultures from the indigenous culture consortium.
- To compare performance of the new isolate with species reported earlier in literature.

1.3 Outline of Dissertation

The outline of this research is subdivided in four main parts:

- *Literature review*: It contains background information for the study and record of recent developments in the field of bioremediation of Cr(VI). The information is focused on fate of chromium in the environment, remediation options, Chromium(VI) reducing microorganisms and Biological Cr(VI) reduction pathways.
- *Material and methods*: It details a reference of all methods used during this study.
- *Culture characterization*: In this chapter, CRB contained in the dried sludge was identified and classified based on colony morphologies, cell wall structure and DNA gene sequences. A phylogenetic tree was then constructed and a

comparative analysis was conducted against other known Cr(VI) reducing bacteria from other research groups.

– *Kinetic study*: The Monod like model was used to evaluate the rate of Cr(VI) reduction over a wide range of initial Cr(VI) concentrations. This study was performed in batch under anaerobic and aerobic conditions and results are presented in the section on the kinetics and modelling.

1.4 Methodology

The first step in the methodology used in the study was to collect information related to the study objective. This information was collected from previous research contained in scientific books or scientific journals. Secondly, standards methods or modified standards methods were used to set up and to run different experiments. Thirdly, results obtained were evaluated and analyzed. Results were also compared to information collected. And finally a conclusion of mains achievements was highlighted and recommendations suggested.

1.5 Summary of Results Obtained

CRBs isolated from the dried sludge collected at the Brits Wastewater treatment plant (SA) possess potential to be used in Cr(VI) biological remediation technology. The treatment plant's influent contain high Cr(VI) concentration above the maximum acceptable concentration. These indigenous CRB are the most important by its ability of growing in highly concentrated Cr(VI) solution. They can as well reduce highly concentrated Cr(VI) solution at shorter incubation time. The consortium is a gram positive dominant. Seven aerobes and eighteen facultative anaerobes were isolated from the consortium and identified using 16s rRNA methods. These isolates were adjoined to existing relatives in a phylogenetic tree in which relationships between these isolates and existing ones were shown. A synergetic metabolism of different CRBs which constitutes the consortium was elucidated. Finally a modified first order reduction rate was established and had been revealed to be depend on the initial Cr(VI) concentration and time. This reduction rate followed a semi-empirical modified first order equation.

1.6 Further work

Further research is required to develop metal bio-decontamination in SA. Results obtained in the study are sufficient to be used in the development and design of bio-reactors. The development and design of bio-reactors are fields to be explored in further research based on actual results. Further work can also be required to study individual species from the consortium in detail. And finally a kinetic study in continuous flow is suggested. For example, studies using microorganisms column could be used to simulate Cr(VI) reduction in aquifer, media which is indispensable for *in-situ* bioremediation.

1.7 Conclusion

Finally, it can be concluded that at the end of this study, all objectives established were achieved and the following pages describe each step. Results obtained are sufficient to promote bio-decontamination of Cr(VI) in South Africa, where Cr(VI) pollution is of great concern.

CHAPTER 2: LITERATURE REVIEW

2.1 Chromium in the Environment

2.1.1 Chromium in Nature

The word chromium is derived from the Greek word "χρωμα" ("*chroma*") which means colour. Chromium (Cr) is the seventh most abundant element in the earth's crust. The element Cr was discovered by Nicolasa-Louis Vauquelin in 1797. This element was discovered later than other metals because of its relatively low concentration in the earth's crust – detected at approximately 100 ppm in chromium rich rocks. Additionally, chromium does not appear terrestrially as a native metal, but is strongly bonded to minerals in which it occurs.

In nature chromium occurs in more than fifty different ores. The following are some examples:

- Barbertonite: $Mg_6Cr_2(CO_3)(OH)_{16}.4H_2O$
- Brezinaite: Cr_3S_4
- Chromite: $(Mg,Fe^{2+})(Cr,Al,Fe^{3+})_2O_4$
- Chromatite: $CaCrO_4$
- Nichromite: $(Ni,CoFe^{2+})(Cr,Fe^{3+},Al,)_2O_4$

The most important form metallurgically and economically is chromite. It is the most prevalent deposit in the world with the largest reserves located in South Africa's north-eastern region. Figure 2-1 shows the distribution of chromium sources based on the market value of total chrome ore exported in 2006 (Papp, 2006). The figure shows that 38% of world chromite ore and 30% of world ferrochromium are produced in South Africa with an associated high risk of pollution in the region where it is mined. Chromium only exists in a combined form, not at zero valence, in nature. Figure 2-2 illustrates which compounds can change their valence by natural oxidation-reduction activity through the natural chrome cycle.

Both the oxidation and reduction of Cr(VI) occurs naturally in geologic and aquatic environments as can be seen in Figure 2-3 which describes the natural redox cycle of Cr(III) and Cr(VI) species. Cr(VI) reduction through Cr(V) has been observed in

photochemical reactions whereas direct Cr(VI) reduction to Cr(III) is known to occur naturally in biological systems. The oxidation and reduction of chromium in soil depends on the soil structure and the redox conditions of the soil. Studies conducted to investigate the effect of the adsorption of chromate and Cr(VI) onto the clay sand mixture showed that clay is a suitable adsorbent for chromate due to its high cationic exchange capacity (CEC) and its strong binding capability (Ajmal *et al.*, 1984). Chromium speciation in groundwater is affected by the redox potential and pH conditions (Figure 2-4).

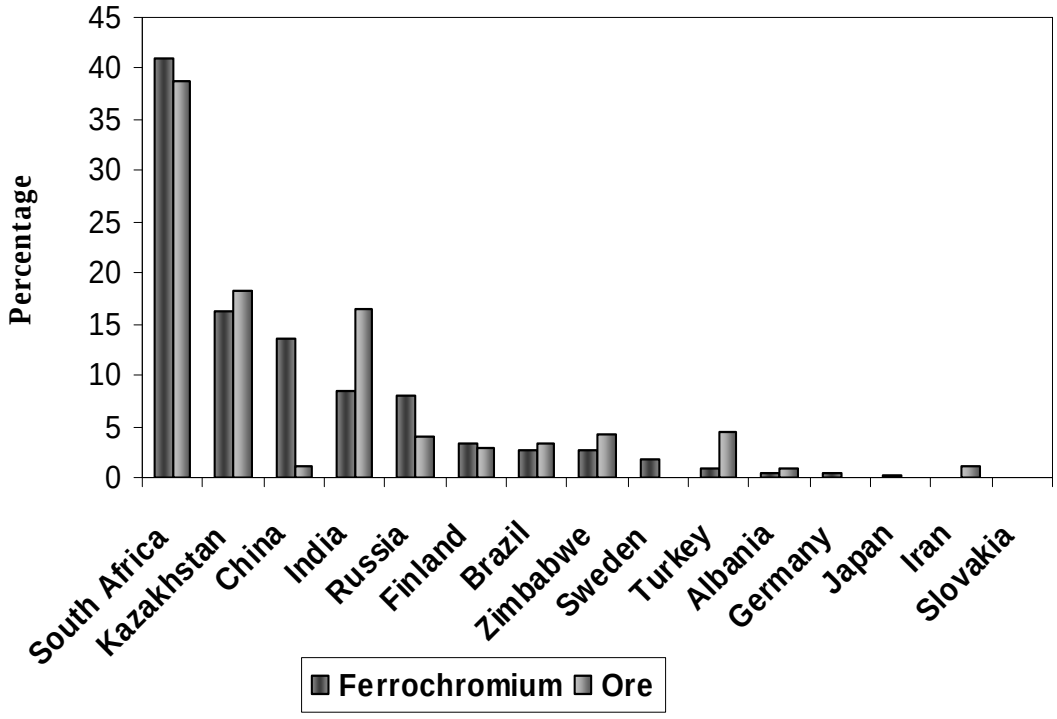


Figure 2-1: Percentage of ferrochromium and chromite ore produced worldwide (Papp, 2006)

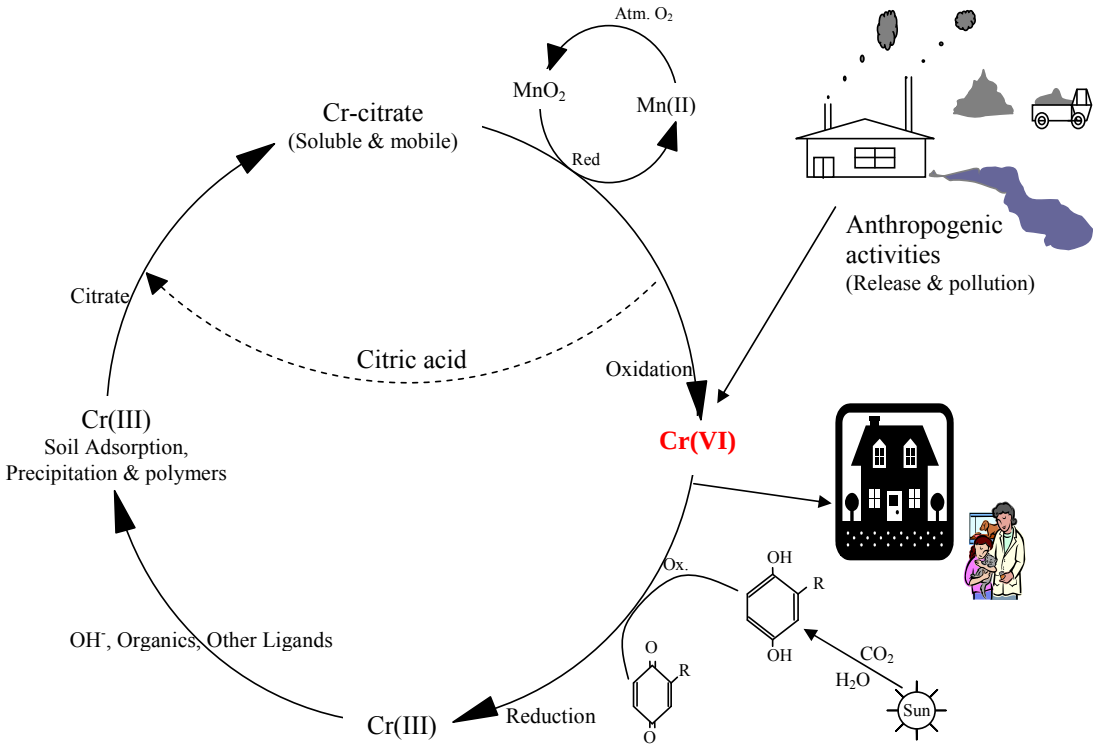


Figure 2-2: The natural Chrome cycle (Bartlett, 1991).

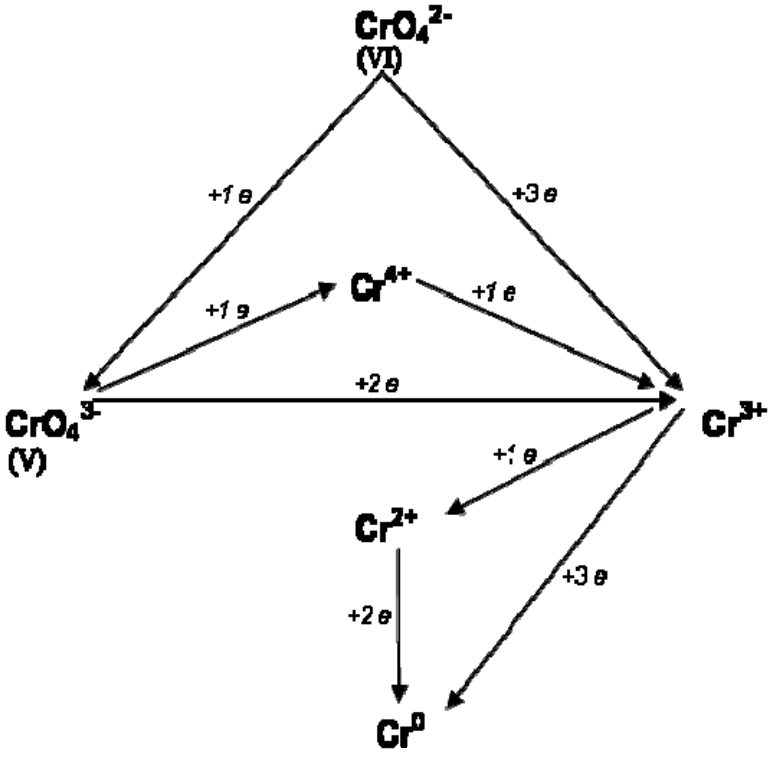


Figure 2-3: Chromium (VI) reduction pathways showing Cr(III) as the predominant equilibrium state.

2.1.2 Physical-Chemical Properties

Chromium is the twenty-fourth element with an atomic weigh of 51.966. It occurs naturally in nine valence states ranging from –2 to +6. Of these nine oxidation states, Cr(III) and Cr(VI) occur most frequently and are of major environmental significance since they are stable in the natural environment (Emsely, 1989).

Cr(III) compounds are more stable and less mobile, which results in greater partitioning in soil (Barnhart, 1997b; Bartlett and Kimble, 1976a,b). Cr(III) exists as cationic species Cr^{3+} at pH 3.5 to 6.0. Cr(VI) however, is highly reactive, is a strong oxidising agent, and exists only in oxygenated species. The equilibria of the Cr(VI) oxygenated species favours extremely high solubility and is pH dependent (Bartlett and Kimble, 1976a,b; Bartlett, 1991; Nieboer and Jusys, 1988). Equations 1-3 (below) show the equilibria of the protonated oxyanions of chromate $HCrO_4^-$ and H_2CrO_4 under acidifying conditions, as an example.

$$H_2CrO_4 \rightleftharpoons H^+ + HCrO_4^- \qquad K_{a1}=10^{0.6} \qquad (2\text{-}1)$$

$$HCrO_4^- \rightleftharpoons H^+ + CrO_4^{2-} \qquad K_{a2}=10^{-5.9} \qquad (2\text{-}2)$$

$H_2CrO_4^-$ is a strong oxidizing agent which is a dominant chromium species at extremely low pH below -0.6 (Cotton and Wilkinson, 1980). Monohydrogen chromate, $HCrO_4^-$, exists between the pH values of 1 to 6. CrO_4^{2-} predominates at or above pH 6. The $Cr_2O_7^{-2}$ dichromate ion is formed by the dimerization of $HCrO_4^-$ ion in Cr(VI) concentrations above 10^{-2} M (Sharma, 2002).

$$Cr_2O_7^{2-} + H_2O \rightleftharpoons 2HCrO_4 \qquad K=10^{-2.2} \qquad (2\text{-}3)$$

Figure 2-4 shows the predominant zone of Cr(III) and Cr(VI) existence in nature at different redox potential (Eh) and pH levels. This figure explains the predominance of both Cr(III) and Cr(VI) in natural conditions and their solubility in water. The change in chromium valences correlates with the variation of pH and redox potential

characteristics of the surrounding environment; Cr(VI) and Cr(III) forms are the most common in nature as shown earlier in Figures 2-3 and 2-4.

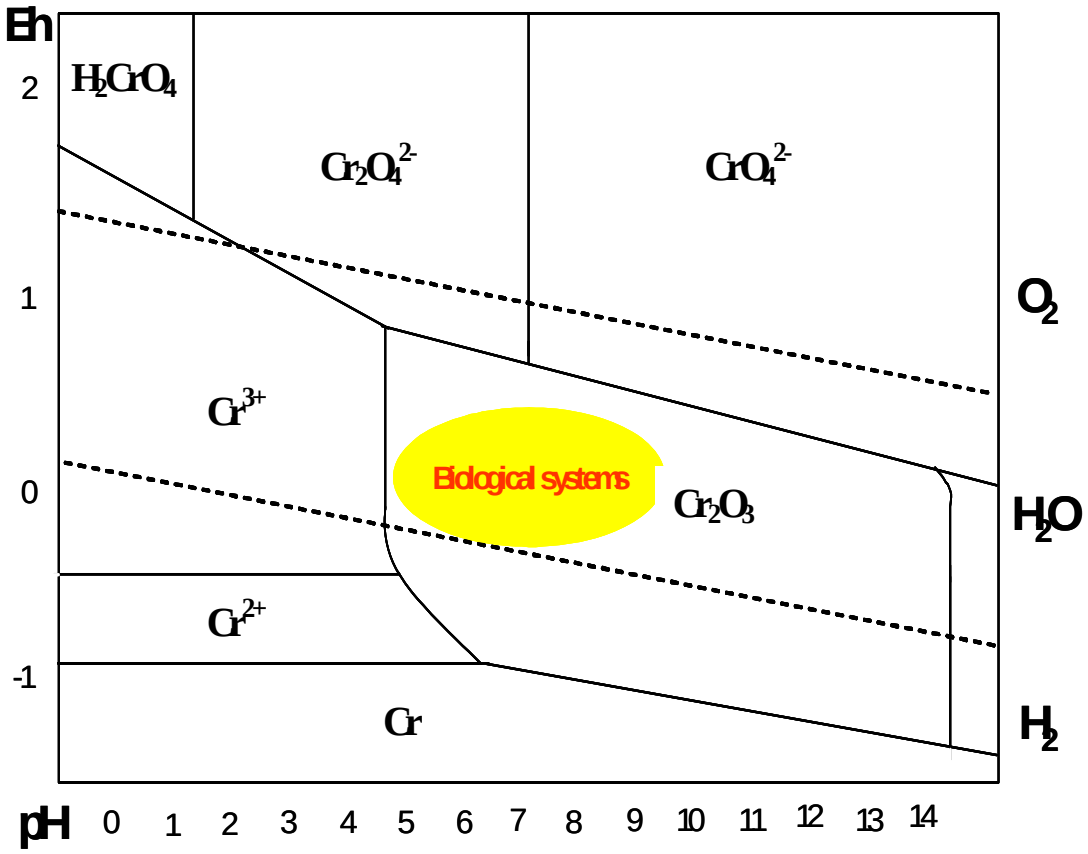


Figure 2-4: Eh-pH diagram for chromium-water systems at standard state conditions. Adapted from Pourbaix (1974) and Barnhart (1997)

The stability of trivalent chromium is due to its lower affinity for oxides and hydroxide ions. Cr(III) is known to form numerous complexes with both organic and inorganic ligands. Complexation species of Cr(III) tend to be more stable in solution because they are inert. The main aqueous Cr(III) species include Cr^{3+}, $Cr(OH)^{2+}$, $Cr(OH)_3$, and $Cr(OH)_4^-$.

For solutions containing trivalent chromium, the Cr^{3+} ionic species predominate at pH<3.6 (Francoise and Bourg, 1991), whereas $Cr(OH)_4^-$ predominates at pH>11.5 (Rai *et al.*; 1987). In slightly acidic to alkaline solutions, ionic Cr(III) species precipitate as an amorphous $Cr(OH)_3(aq)$ (Rai *et al.*, 1987) or as a solid mixture (Fe,

Cr)$(OH)_3$ in the presence of Fe(III) (Francoise and Bourg, 1991). Cr^{3+} can also be chelated to organic molecules that are adsorbed onto mineral surfaces (James and Bartlett, 1983 (a); James and Bartlett, 1983 (b)). However, Cr(VI) compounds CrO_4^{2-}, $HCrO_4^-$, $Cr_2O_7^{2-}$ are highly reactive and mobile in surface sediments because they are not strongly adsorbed onto soil particle surfaces (Sharma, 2002).

2.1.3 Anthropogenic Sources

Most chromium compounds are produced and used at a low pH. Sodium chromate, sodium dichromate, chromic acid and basic chromium sulphate are produced between pH 3 to 4, and are used in the same range in wood treatment and leather tanning. On the other hand, chromic oxide is used in the production of stainless steel at pH 5 to 6 (Barnhart, 1997a).

The metal chromium at zero valence and its alloy compounds are used in different industrial process, such as electroplating, leather tanning, ore and petroleum refining, textile manufacturing, wood preservation, inorganic chemicals and pulp production. It is also used as an additive during pigment and paint production. Cr compounds are also added to cooling water to inhibit corrosion in power plants as well as in other metal finishing industries such as aluminium anodizing, metal cleaning, pre-plating, and electroplating operations (NRC, 1974; Wang and Xiao, 1995; Patterson, 1985; Cohen and Costa, 2000). Some of the above sources are illustrated in Figure 2-5.

Chrome compounds have high corrosion resistance; they are therefore widely used as a high polish on surfaces and household water faucets. Strength, hardness and temperature resistance justifies chrome's use in manufacturing of refractory bricks used in furnace lining.

In South Africa, chromium plays a major role in industries and in the economy; since its end products offer several economic advantages. However, waste products from these processes are often difficult to treat. The waste may be introduced into the environment through industrial effluents or accidental spills.

2.1.4 Health Impacts

The need to treat Cr(VI) in waste streams arises from observed deleterious impacts upon contact and exposure through inhalation, ingestion or dermal contact. The biotoxicity of chromate is largely due to its high reactivity, its ability to penetrate biological membranes as well as its high oxidizing capabilities (NAS, 1974).

Chromate compounds are known to cause mutations in bacteria and transformations in mammalian cells. Chromium(VI) is classified as an initiator of carcinogenesis on the basis of its genetic toxicity (Figure 2-6). The natural intracellular Cr(VI) reduction occurs by successively accepting one electron, which generates Cr(V). As by-product, super-active ionisation of water results in free radical ($OH^{\bullet}$) formation that results in DNA damage (Flessel, 1979). Exposure to Cr(VI) produces other health risks in mammals including humans. The deleterious effects resulting from exposure to Cr(VI) include allergic dermatitis, ulceration of the skin, irritation of the mucous membranes, nasal septum, renal tubular necrosis, and increase risk of respiratory tract infections. Due to these and other observed toxic effects, the World Health Organisation (WHO) has set the maximum acceptable concentration of chromium in drinking water to 0.05 mg/L (50 µg/L) (Kiilunen, 1994; Lu and Yang, 1995; ACGIH, 2004).

Recent studies have suggested a link between exposure to Cr(VI) and several forms of DNA damage. For example, exposure to chromate was shown to induce sister chromatid exchanges and chromosome aberrations in cultured human skin fibroblasts (MacRae *et al*., 1979); human lymphocytes (Douglas *et al*., 1980); Chinese hamster ovary cells (Majone and Levis, 1979). Furthermore, chromate enhanced the transformation of Syrian hamster embryo cells by *Simian adenovirus* SA7 at non-toxic doses (Casto *et al*., 1979). Some of the pathways of Cr(VI) into a leaving cell that result in DNA damage are shown in Figure 2-6 below.

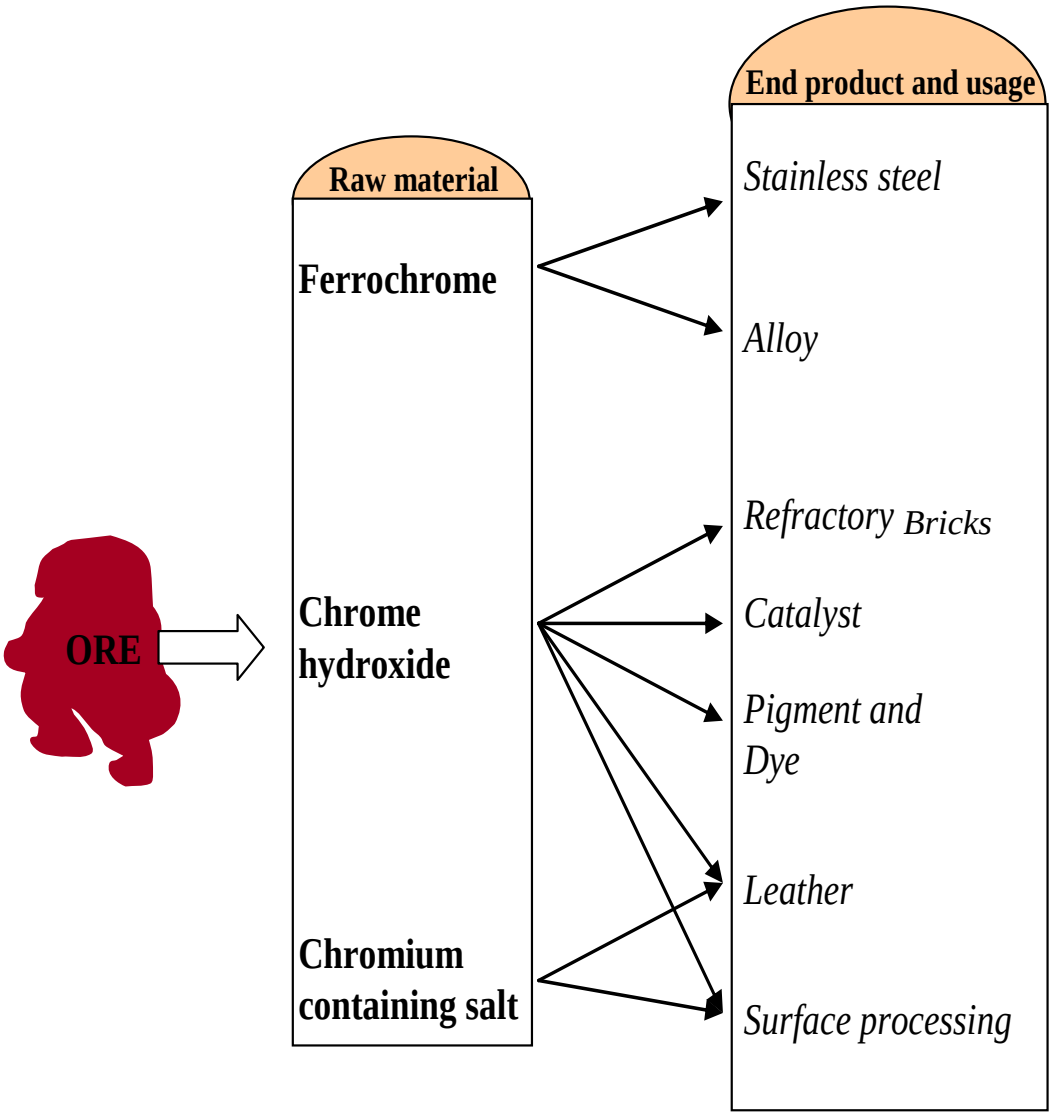


Figure 2-5: Chrome process final product and use.

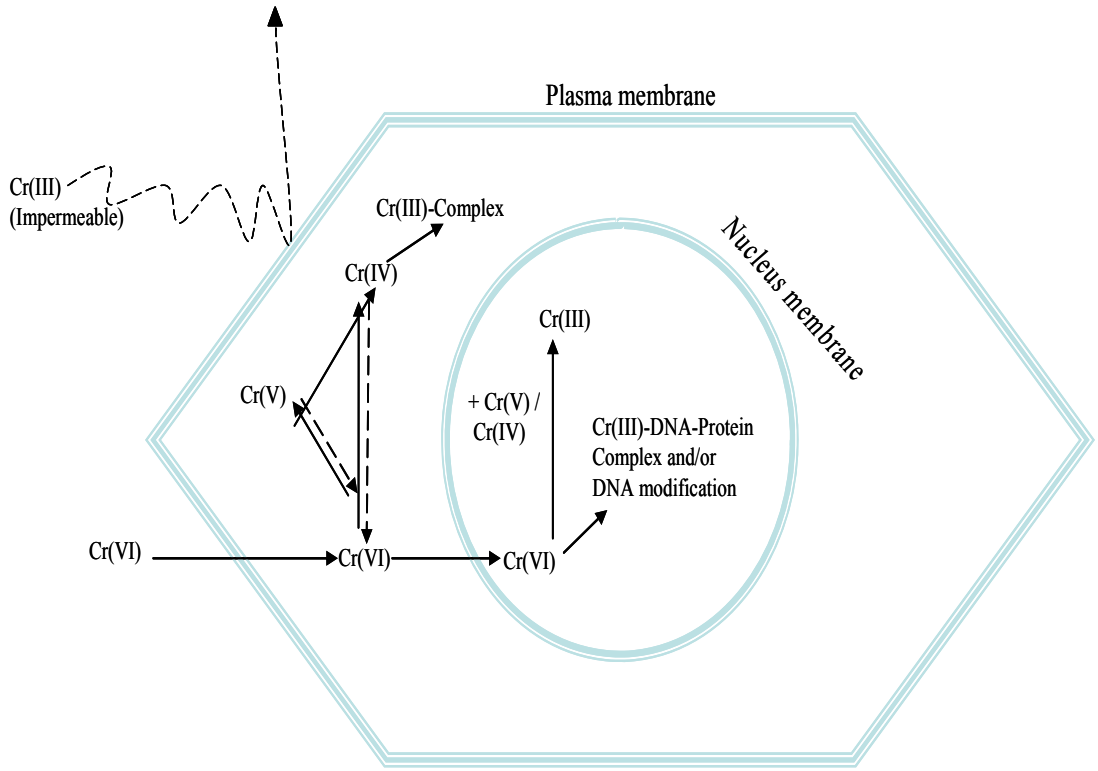


Figure 2-6: Schematic diagram of the toxicity and mutagenicity of Cr(VI) (Cheung *et al.*, 2007)

2.1.5 Impacts on the environment

Cr(VI) negatively impacts the environment due to its high solubility, mobility and reactivity. Soils and groundwater environments are the most susceptible to Cr(VI) contamination from spills, illegal disposal and unprotected stock piles of newly processes chromium products. The pollution problems are exacerbated by the following properties of Cr(VI):

- Cr(VI) is extremely soluble and mobile in water. This results in high uptake rate in animal and plant cells.
- Cr(VI) is highly accessible to living organisms through multiple routes of entry such as ingestion, dermal contact, inhalation, and absorption (in the case of plants and root systems).

- Cr(VI) reduces seed germination in plant – can result to up to 20% reduction of seed germination in plants (Anon, 1974; Towill *et al*., 1978). This is suspected to be due to root damage.
- Other studies indicated retardation of growth, photosynthesis, and enzyme activities in algae due to the presence of Cr(VI) at concentration as low as 10 ppm (Rosko *et al*., 1977, Silverberg, *et al*., 1977; Sharma, 2002).

2.2 Remediation Options

2.2.1 Physical and Chemical Treatment

Physical/chemical remediation processes such as ion exchange, precipitation, and reverse osmosis have been used to remove Cr(VI) from effluents, mainly as end of the pipe solutions (Patterson, 1985). For contaminated surfaces, most commonly applied technologies include excavation and removal of contaminated material which be sent to a landfill while the site is subjected to a pump-and-treat process to limit the spread of pollutant. The treated water is pumped back into the aquifer dilute or flush the pollutant (Nyer, 1992; Watts, 1998). Unfortunately, this technique presents the disadvantages of producing chemical residues that are harmful to the environment. The above method does not address the final waste disposal problem, and is generally expensive due to high operational costs (Patterson *et al.*, 1985; Viamajala *et al*., 2002 (a); Zahir, 1996).

2.2.2 Biological Treatment (Bioremediation)

The term "bioremediation" has been used to describe the process of using microorganisms to degrade or remove hazardous pollutants from the environment (Glazer and Nikaido, 1995). Even though Cr(VI) can be reduced by algae or plants, in soil, bacteria has been demonstrated to be most efficient (Cervantes *et al*., 2001; Basu *et al*., 1997; Ganguli and Tripathi, 2002; Francisco *et al*., 2002). Microbial applications have been tested directly for Cr(VI) removal in industrial effluents and groundwater using bioreactors but has not been fully tested *in-situ*.

Information on Chromium(VI) Reducing Bacteria (CRB) comes from studies conducted using cultures collected from tannery effluents, tannery waste, ponds and environments receiving tannery or electroplating effluents. Most of these CRBs, exhibit resistance to Cr(VI) under high exposure conditions up to 300 mg/L (Bopp and Ehrlich, 1988; Branco *et al.*, 2004; Camargo *et al.*, 2003; Ramírez-Ramírez *et al.*, 2004). CRBs are potentially useful for bioremediation of Cr(VI) contaminated sites. The advantage of using bacteria for Cr(VI) reduction is that they do not require high energy input. Additionally, there are no significant chemical by-products during treatment and may employ native, non-invasive strains of bacteria. The latter off-sets the concerns over possible introduction of alien species with unforeseeable damaging impacts to the native environment.

Bioremediation may be applied either as an *ex-situ* or *in-situ* process, each technique having its own advantages and disadvantages. Of the two processes *in-situ* bioremediation presents more advantages than the *ex-situ* process. The *in-situ* process is characterised by low installation costs as it may be implemented without extensive drilling or excavation costs. Additionally, *in-situ* bioremememediation circumvents the operation costs associated with continuous pumping and mechanical maintenance in *ex-situ* systems.

2.2.3 Microbial Processes and Bio-Engineering

In practice the application of microbial processes is limited by the strength of the waste and the presence of inhibitory co-contaminants in the waste. If the concentration of Cr(VI) is high, significant viable cell loss occurs. The existence of toxic co-contaminants such as toxic aromatic compounds and heavy metals may severely inhibit Cr(VI) reduction. Therefore, many authors made an effort to develop appropriate biological reactor systems to effectively detoxify Cr(VI) in both aerobic and anaerobic, batch and continuous flow processes in the presence of or together with other toxic organic pollutants (Chirwa and Wang, 2001; Mazerski *et al.*, 1994; Shen and Wang, 1995).

Most studies on biological reduction of Cr(VI) have been conducted in batch reactors using pure cultures (Wang and Xiao, 1995; Shakoori *et al.*, 2000; Megharaj *et al.*,

2003). In addition to these studies, Blowes (1992) and others describe batch experiments aimed at assessing the feasibility of biological reduction of Cr(VI) and the potential for the removal of Cr(VI) in porous reactive walls (Blowes, 1992; Blowes *et al.*, 1997; Jambor *et al.*, 2005). Concurrently DeLeo and Ehrlich (1994) revealed the high performance of hexavalent chromium reduction in shaken batch cultures of *Pseudomonas fluorescens* LB300 as well as in immobilized batch and continuous flow reactions. These experiments were performed under aerobic conditions and neutral pH using citrate as carbon source with initial Cr(VI) concentrations of 314.0, 200.0 and 112.5 mg Cr(VI)/L. *P. fluorescens* LB300 reduced 81% of Cr(VI) in 147 h in stationary culture and 80% in 122 h in shaken culture with insignificant uptake into the cells.

At industrial scale, the feasibility of biological treatment of high strength tannery wastewater was investigated using a Sequencing Batch Membrane Bioreactor (SBMB) system (Yamamoto *et al*., 1991). Yamamoto's system consisted of a hollow fibre microfiltration unit in a SBMB for solid-liquid separation. The aim of the SBMB was to enhance sludge concentration in the reactor for stable removal of organic matter and heavy metals. The observed removal efficiency for COD in the effluent was 93.7 to 96.3% and 95.4 to 97.7% for total Cr.

2.2.4 *In-Situ* Bioremediation

In-situ bioremediation consists of placing a permanent, semi-permanent, or replaceable treatment unit down-gradient of the contaminated source as shown in Figure 2-7. The barrier is placed across the groundwater flow path of a contaminant plume and it acts as a treatment wall. The intercepted contaminant material is either immobilized or transformed to a less toxic compound within the PRB while allowing the groundwater to continue to flow naturally. These biological barriers are designed to intercept and treat contaminant plumes but future action is required to remove the contaminant sources.

Remediation is mainly focused on chemical treatment and only 2% of all decontamination sites use microorganisms based on the 2009 data. This represents an

underutilisation of the technology in spite of the obvious advantages such as environmentally friendliness and low operational cost. The application of *in-situ* bioremediation has been primarily applied for the attenuation and removal biodegradable organic pollutants (Morgan and Watkinson, 1989; Farhadian *et al*,, 2008; Atlas, 1995). Microbial reactive barriers have been only applied on the experimental scale for the treatment of toxic metals such as Cr(VI). For example, a biological reactive barrier was studied for Cr(VI) decontamination using the yeast *Saccaromyces cerevisiae* by Krauter *et al.* (1996).

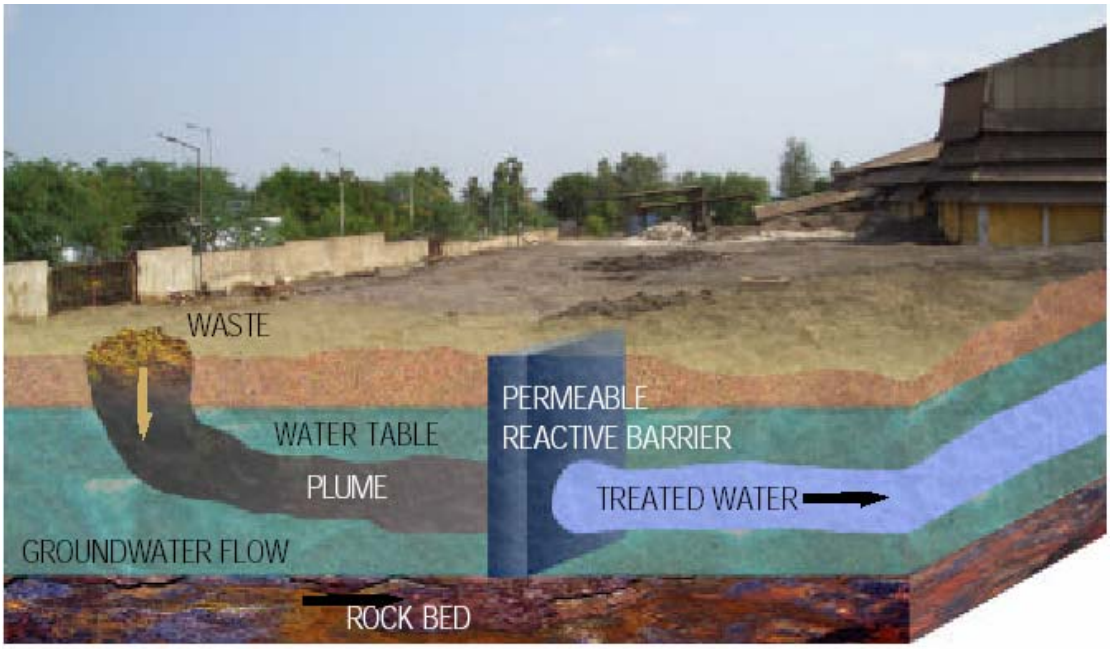


Figure 2-7: Design of typical PRB (Sovani, 2005)

2.2.5 *Ex-Situ* Bioremediation

This is typically a pump-and-treat methodology in which the pollutant is removed from the contaminated subsurface and treated on the surface in bioreactors. The contaminant is then stored or disposed of in a controlled way while the water is returned to the subsurface. This method offers one advantage over the *in-situ* bioremediation which is the removal of metal species from the aquifer material which might have otherwise precipitated and accumulated in the media during *in*-situ bioremediation. (U.S. EPA, 1997a; U.S. EPA, 1996b; U.S. EPA, 1998; U.S. EPA, 2000; Suthersan, 1997).

2.3 Chromium(VI) Reduction Microbiology

2.3.1 Cr(VI) Reducing Organisms

Chromium(VI) is toxic to biological systems due to its strong oxidizing potential that can damage cells (Kotas and Stasicka, 2000). However, some microorganisms in the presence or absence of oxygen can reduce the toxic form of Cr(VI) to its trivalent form (Polti *et al.*, 2007; Francisco *et al.*, 2002). These microorganisms are known as chromium reducing bacteria (CRB). It was demonstrated that among CRB, gram-positive CRB are significantly tolerant to Cr(VI) toxicity at relatively high concentration, while gram-negative CRB are more sensitive to Cr(VI) (Coleman, 1988; Ross *et al*., 1981).

Since Romanenko and Korenkov isolated the first anaerobic CRB (the *Pseudomonas strain*) from sewage sludge in 1977, many other CRB have been found (Table 2-1). Initially, researchers focussed on facultative microorganisms such as *Pseudomonas dechromaticans*, *Pseudomonas chromatophila* and *Aeromonas dechromatica*. A series of other diverse microorganisms were later isolated including sulphate-reducing bacteria (SRB) such as *Desulfovibrio vulgaris* (Lovley and Philips, 1994) and Fe(III)-reducing bacteria such as and *Thiobacillus ferrooxidans* (QuiIntana *et al*., 2001).

Cr(VI) reduction in the sulphate reducing bacteria was determined to be partially due to the production of and subsequent reaction with the sulphide species (S^{2-} and HS^{-}). In *Desulfotomaculum reducens* MI-1, Cr(VI) was utilised as the sole electron acceptor

(Tebo and Obraztsova, 1998). This capability was also reported in another uncharacterised SRB consortium by Cheung and Gu (2003).

2.3.2 Aerobic Cultures

Studies conducted using various species of microorganisms have shown that some species isolated from Cr(VI) contaminated environments are capable of reducing Cr(VI) to Cr(III). Most of these microorganisms show remarkable resistance to Cr(VI) toxicity through cellular level mutations and other community level survival mechanisms. Shown below (Table 2.1) are one of the most studied Cr(VI) Reducing Aerobic isolates of bacteria.

The Cr(VI) reduction activity under aerobic condition is generally associated with soluble proteins that use NADH as an electron donor either as a requirement for growth (Ishibashi *et al.*, 1990) or for enhanced activity (Horitsu *et al.*, 1987) as shown in following equation:

$$2\cdot CrO_4^{2-} + 13\cdot H^+ + 3\cdot NADH \rightarrow 2\cdot Cr^{3+} + 3\cdot NAD^+ + 8\cdot H_2O \qquad (2\text{-}4)$$

$$\Delta G^{o'} = -917.71 \text{ kJ/mol}$$

Organisms may also reduce Cr(VI) under anaerobic conditions via the mediation of either a soluble reductase, a membrane-bound reductase or both (Wang and Shen 1995).

2.3.3 Anaerobic Cultures

In subsurface conditions at the contaminated site may be severely oxygen depleted due to lack aeration in laminar flow groundwater systems. Under anaerobic conditions microorganisms reduce Cr(VI) via the mediation of either a soluble reductase, a membrane-bound reductase or both (Wang and Shen 1995; Capone *et al.*, 1983; Miller *et al.*,1989; Robertson, 1975; Cheung *et al.*, 2006). Characteristic Cr(VI) reducing organisms isolated under anaerobic conditions are shown in Table 2-1.

Table 2-1: Known Cr(VI) reducing bacteria reported in literature.

Name of Species	**Isolation Conditions/ C-Sources**	**References**
Achromobacter sp. StrainCh1	Anaerobic / Luria Broth; glucose-lactate	Zhu *et al.*, 2008
Agrobacterium radiobacter EPS-916	Aerobic-Anaerobic / glucose-mineral salts medium	Llovera *et al.*, 1993
Alcaligenes eutrophus	Aerobic / sodium gluconate	Nies and Silver, 1989
Bacillus megaterium TKW3	Aerobic / nutrient broth-minimal salt medium-glucose, maltose, and mannitol	Cheung *et al.*, 2006
Bacillus sp.	Aerobic/ Vogel-Bonner (VB) broth-citric acid; D-glucose	Chirwa and Wang, 1997;
Bacillus sp. ES 29	Aerobic / Luria-Bertani (LB) medium	Camargo *et al.*, 2003
Bacillus subtilis	Anaerobic / Minimal medium - trisodium citrate and dehydrate glucose	Carlos *et al.*, 1998
Deinococcus radiodurans R1	Anaerobic / Basal Medium-Lactate-Acetate-Pyruvate-Succinate-Ethanol-L-lactate, and D-lactate	Frederickson *et al.*, 2000
Enterobacter cloacae HO1 *strain*	Anaerobic / KSC medium-Sodium acetate	Wang *et al., 1989(a)*
Escherichia coli ATCC33456	Aerobic-Anaerobic / Nutrient broth medium; glucose, acetate, propionate, glycerol and glycine	Shen and Wang, 1994
Ochrobactrum sp.	Aerobic / glucose	Zhiguo *et al.*, 2009
Pantoea agglomerans SP1	Anaerobic / acetate	Francis *et al.*, 2000
Providencia sp.	Aerobic-Anaerobic / Luria broth (tryptone-yeast extract)	Thacker *et al.,* 2006
Pseudomonas aeruginosa	Aerobic / Nutrient broth or Luria broth	Aguilera *et al.*, 2004
Pseudomonas fluorescens	Aerobic-Anaerobic / Glucose-Acetate-Pyruvate-Lactate-Succinate	Bopp *et al.*, 1983; Ohtake *et al.*, 1987
Pseudomonas fluorescens strain LB300	Aerobic / Vogel-Bonner broth	Bopp and Ehrlich, 1988
Pseudomonas putida MK1	Anaerobic / Luria-Bertani -citric acid-Tris-acetic acid	Park *et al.*, 2000
Pseudomonas sp	Aerobic / Peptone-glucose; chemostat	Gopalan and Veeramani, 1994
Pseudomonas spp.	Anaerobic / Vogel-Bonner (VB)- D-glucose	Mclean and Beveridge, 2001
Shewanella alga (BrYMT) ATCC 55627	Aerobic-Anaerobic / M9 broth- Glucose	Guha *et al.*, 2001
Shewanella putrefaciens MR-1	Anaerobic / lactate- fumarate	Myers *et al.*, 2000

Some of the organisms do not require organic carbon sources as energy sources and electron donors. Some of these utilise CO_2 and HCO_3^- as carbon sources as shown in the following equation:

$$HCO_3^- + CrO_4^{2-} + 9{\cdot}H^+ + 3{\cdot}NADH \rightarrow Cr^{3+} + 3{\cdot}NAD^+ + \frac{3}{2}{\cdot}H_2 + 5{\cdot}H_2O + CO_2 \qquad (2\text{-}5)$$

$$\Delta G^{o'} = -\ 740.69\ \text{kJ/mol}$$

an energy intensive reaction involving the consumption of NADH and ATP where Cr(VI) is reduced at the expense of cellular growth and maintenance.

2.3.4 Consortium Cultures (Natural Systems)

A consortium is a mixture of bacteria collected from the natural environment. Usually a contaminated site is chosen as the ideal source for the particularly useful consortium. Consortia provide a closer picture of what really happens in the environment where microorganisms do not live in pure cultures. Consortium communities of bacteria can degrade a wide variety of pollutants in the environment due to the diversity of metabolic pathways in the community (Sharma, 2002).

Consortium studies explain how the activity of specific CRB may be influenced by the presence of other organisms; such as synergetic activities or bacterial competition. Synergism can be illustrated by the investigation made by Chirwa and Wang, (2000) in a study where the interaction between *E. coli* and phenol-degraders in a consortium demonstrated a high phenol-degrader/*E. coli* ratio resulted in a high rate of Cr(VI) reduction. Chirwa and Wang concluded that *E. coli* utilized metabolites formed from phenol degradation as electron donors for Cr(VI) reduction. This kind of system was also demonstrated by Guan *et al*. (1993a, b) in their study on a CRB and a consortium of denitrifying bacteria. They concluded that the sorption of Cr(VI) and its reduction are linked to the metabolic process.

2.3.5 Cr(VI) Reduction in Extreme Environments

Extremophiles have also been found that reduce Cr(VI) using the enzymatic process described above for anaerobic cultures. One example is the Cr(VI) reducing, radiation-resistant *Deinococcus radiodurans* R1 (Fredrickson *et al*., 2000; Brim *et al.*, 2006), a close relative of the *Archae bacterium – Thermo-anaerobacter ethanolicus* – isolated from deep subsurface sediments (Roh *et al*., 2002). Another culture, the *Pyrobaculum islandicum* (Kashefi and Lovley, 2000) reduces Cr(VI) at high temperatures, and the *Thermus scotoductus strain* SA-01, a thermophile isolated from groundwater from a South African gold mine at a depth of 3.2 km, has also been shown to reduce a variety of heavy metals including Cr(VI), cultured anaerobically under resting cell conditions using lactate as an electron donor and recently in aerobic conditions (Kieft *et al*., 1999; Möller and van Heerden, 2006, Opperman and van Heerden, 2007).

2.4 Biological Chromium(VI) Reduction Pathways

2.4.1 Intracellular Processes

Earlier studies on microbial Cr(VI) reduction indicated that bacteria such as *E.coli* ATCC 33456, *D vulgaris*, *P. putida* PRS2000 and *P. ambigua* G-1 produced a membrane associate reductase capable of catalysing the reduction of Cr(VI) to Cr(III). Experimentation with the supernatant samples of cell extracts yielded the same amount of Cr(VI) reduction as in living cells. These studies also revealed that, in some cases, Cr(VI) reduction was a cometabolic process induced by the reductase (Shen and Wang, 1993; Lovley and Phillips, 1994; Ishibashi *et al*., 1990; Horitsu *et al*., 1987; Suzuki *et al*., 1992).

In intracellular processes, Cr(VI) is reduced in the cytosol using cytoplasmic soluble reductase enzymes. These enzymes play an intermediate role between associated biological electron donors. The electron donors implicated in a Cr(VI) reduction are NADH and NADPH; they are active within a wide range of temperatures from 40 to 70°C and pH 6 to 9 under laboratory conditions. These ranges contain natural

physiological intracellular parameters, 37°C and pH 7 (Suzuki *et al.*, 1992; Bopp *et al.*, 1983; Cervantes and Silver, 1992).

During the experimentation with intracellular Cr(VI) reduction, Cr(III) could not be removed from the cells as long as the cell membrane remained intact (Aaseth *et al.*, 1982). Most of these bacteria are more resistant to Cr(VI) toxicity and are generally gram-negative as demonstrated by other studies on CRB such as *Pseudomonas aeruginosa, Pseudomonas sp.* CRB5, *E. coli* ATCC 33456, *Rhodobacter sphaeroides, Alcaligenes Enterobacter*, and *Pseudomonas flourescens* LB300. However, an exception was found in *Bacillus subtilis,* a gram-positive bacterium (Horitsu *et al.*, 1983; Ohtake *et al.*,1990).

At the molecular level, Cr(VI) reduction was shown to be encoded on the *ChrR* which produces the reductase ChrR. This enzyme catalyses the transfer of electrons from NADH, NADPH and other endogenous electron reserves to Cr(VI) (Cervante *et al.*, 1990; Alvarez, 1999; Appenroth *et al.*, 2000). Cr(VI) may be reduced either as a response to Cr(VI) toxicity or as a result of a physiological need to conserve energy in the cell through a dissimilatory pathway reaction.

In recent years, more gram-positive Cr(VI) reducing bacteria have been identified such as *Bacillus cereus* (Camargo *et al.*, 2003; Faisal and Hasnain, 2006), *Bacillus thuringiensis* (Camargo *et al.*, 2003) and a variety of sulphate reducing bacteria (SRB) (Fude *et al.*, 1994).

2.4.2 Extra-Cellular Processes

Biofilms can influence removal of metal species through adsorption to extra-cellular polymeric substances and cellular excretions. It was observed while working with cell extracts that Cr(VI) could be released into the supernatant from the cytoplasma in the form of $Cr(OH)_3(aq)$. Formation of $Cr(OH)_3(aq)$ under the higher pH intracellular environment is expected and represents a physiological reaction which protects cells by forming a barrier from Cr(VI) toxicity and confers a low cell membrane permeability to Cr(VI) (Chen and Hao, 1998).

In the year 2000, Smith and Gadd established the extra-cellular Cr(VI) reduction pathways in SRB through a mass balance in which 90 % of the reduced Cr was detected in the supernatant. More recently, Yewalkar and co-workers (2007) established the distribution of Cr(VI) using a radioisotope of Cr [^{51}Cr(VI)] and four species of unicellular green algae collected from disposal sites of paper-pulp and electroplating industries in the medium. In the above study, Yewalkar *et al*. detected 10-19% of the total radioactivity inside the cells, 1-2% was attached to the cell wall and the remaining radioactivity was situated in the supernatant. This confirms the extracellular Cr(VI) reduction theory. In this case, only living cells could reduce Cr(VI).

2.4.3 Membrane Associated Cr(VI) Reduction

It has been suggested that Cr(VI) reduction may take place through the membrane electron chain respiratory pathway (Figure 2-8). It is also suggested that the reductase may be exported to the medium and that Cr(VI) is reduced in the external environment. This theory was investigated by Shen and Wang in 1993 using mass balance studies of chromium inside cells and in the supernatant in which Cr(VI) reduction pathways in and outside the cells were investigated using the gram-negative bacteria – *E. coli* ATCC 33456. In the latter study, Cr(III) formed from Cr(VI) reduction was probably attached to the surface of cells since Cr(III) is generally excluded from entering the cells. Approximately 70% of the total chromium remained in the supernatant and 30% was probably attached to the surfaces of the cells. *E. coli* ATCC 33456 membranes are associated with soluble reductase to catalyse Cr(VI) reduction in the cytoplasmic fraction of ruptured cells.

In the membrane-associated Cr(VI) reduction, a constitutive enzyme mediates the transfer of electrons from intercellular electron donors such as NADH and NADPH to Cr(VI) as the terminal electron acceptor. In this model, electron transport to the reductase is mediated by the transmembrane proton pumps such as NADH-dehydrogenase and the cytrochromes a to c_3. For this reason, inhibitors of electron flow through the electron transport and oxidative phosphorylation pathway, such as

azide and cyanide, also block electron transfer to Cr(VI). This type of Cr(VI) reduction has been observed in *P. maltophilia* O-2 and *Bacillus megaterium* TKW3 (Cheung and Gu, 2007; McClean and Beveridge, 2001) and in *Shewanella oneidensis* MR-1 (Myers *et al.*, 2000).

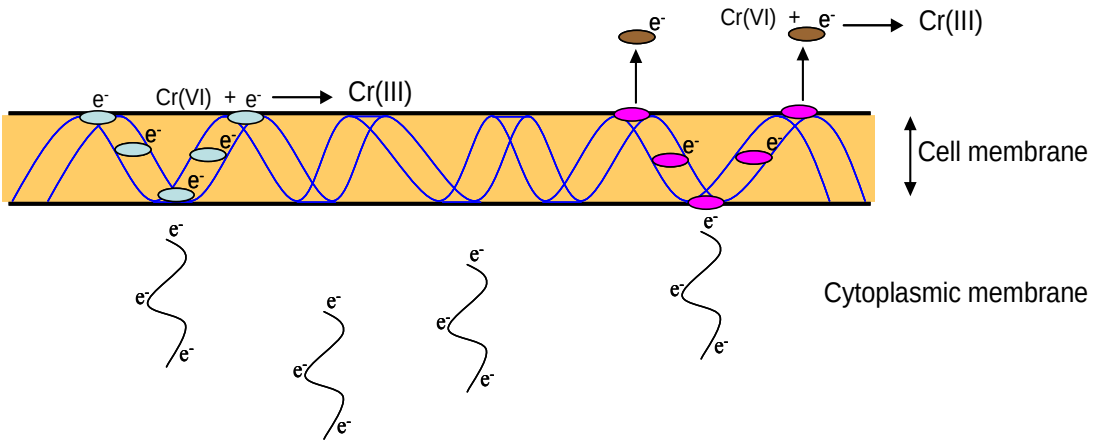


Figure 2-8: Cr(VI) reduction in respiratory chain involving trans-membrane protein.

2.5 Summary

From this literature survey, it can be concluded that microbial Cr(VI) reduction has been investigated by several research groups from different research backgrounds since the late 20th century. The intensity of the research in both extent and depth shows the deepening concern over extant Cr(VI) pollution problems around the world. In spite of this wide research, problems of Cr(VI) pollution still remain, mainly affecting soil and groundwater environments. Direct application of biological systems for treatment of Cr(VI) are still rare. There is therefore an urgent need to develop and deploy environmentally friendly technologies to remediate contaminated sites and to prevent the spreading of the pollutions to adjacent aquifers and surface water resources especially in SA, the leading producer of chromite ore. The high levels of Cr(VI) contamination resulting from chrome mining and ore processing activities require the development of appropriate treatment technologies and management of Cr(VI) pollution.

The other significant finding during the literature study is that, although research on the bioremediation of Cr(VI) has been conducted widely in USA and around the world, this effort has not been pursed in South Africa. This study represents the first in-depth effort to evaluate the microbial Cr(VI) reduction strategy for bioremediation of Cr(VI) contaminated sites under natural conditions.

The following chapters will demonstrate the feasibility of the biological treatment process using a consortium of bacteria sourced locally. This is followed by characterisation of the species, testing the species capability individually, and development of a predictive semi-empirical model that helps elucidate the enzymatic Cr(VI) reduction mechanism utilised by the bacteria.

CHAPTER 3: MATERIALS AND METHODS

3.1 Source of C(VI) Reducing Bacteria

Cr(VI) reducing bacteria (CRB) cultures were sourced from two different sampling sites in Brits (Republic of South Africa). The first sampling point was at an abandoned Chrome processing facility, commissioned as early as 1996. Sampling from this site was based on the fact that the soil bacteria at this facility is expected to be exposed to high levels of Cr(VI), thus may be acclimatized to Cr(VI) toxicity. From this site, contaminated soil samples were collected in sterile containers, and stored between 0 and 4ºC. The other sampling site was the Brits Wastewater Treatment Works. This treatment plant receives periodic flows from the abandoned Ferro-Chrome processing facility containing high levels of Cr(VI) as sodium dichromate. From this site, samples were collected from three different points: influent sewage, activated sludge (mixed liquor) and dried sludge collected from sand drying beds. All these samples were also kept refrigerated between 0 and 4^{o}C.

3.2 Chemical Reagents and Apparatus

Di-potassium chromate (K_2CrO_4, 99 % purity), was purchased from Sachem (Krugersdorp, South Africa); H_2SO_4 (99.99 % purity), 1,5-diphenyl carbazide (99 % purity) and NaCl were purchased from Merck (South Africa); Luria Bertani (LB) broth, Luria Bertani (agar) agar and Plate Count (PC) agar were purchased from Sigma-Aldrich (USA). All other analytical grade reagents used were purchased from either Merck or Sigma-Aldrich (South Africa). Other resources were the UV/Vis spectrophotometer (WPA, Light Wave II, Labotech, South Africa); the centrifuge (Hermle Z323) from Hermle Laboratories (Germany); the lateral environmental shaker, (Labcon SPL-MP 15, Labcon Laboratory Services, South Africa); the ZEISS Axioscop II microscope (Carl-Zeiss, oberkochen, Germany) and the Varian AA – 1275 Series Flame Atomic Adsorption Spectrophotometer (AAS) (Varian, Palo Alto, CA (USA)).

3.3 Preparation of Cr(VI) Stock and Standard Solution

A Cr(VI) stock solution was made by dissolving 3.773 g of K_2CrO_4 in 1L of distilled water to make a solution of 1000 mg Cr(VI) per litre. This solution was used for all the experiments as Cr(VI) source. Cr(VI) standard solutions were prepared from the Cr(VI) stock solution by diluting with distilled water to give the desired final Cr(VI) concentration (0, 0.5, 1, 2, 3 and 4 mg/L). A linearized Cr(VI) standard curve was generated by plotting the absorbance (at 540 nm) of each Cr(VI) standard solution against mass/10.2 mL. Standard curve for Cr(VI) measured demonstrated high degree of accuracy with R^2 = 99.5% for a composite data set from predetermined points. This standard curve was used for estimating Cr(VI) concentration [Appendix Figure A-1].

3.4 Growth Media Preparation

LB broth, PC agar and LB agar were prepared according to manufacture's instructions, by dissolving 30 mg, 23 mg and 37 mg, respectively in 1 L distilled water. The solutions were then separately sterilized by autoclaving for 15 minutes at 121 ^oC (2 bar) in a Tomin autoclave (Durawell Co. Ltd). Before use, LB Broth was cooled to room temperature. PC agar and LB agar were cooled to 40 ^oC temperature and then aseptically poured into agar plates.

3.5 Analytical Procedures

3.5.1 Cr(VI) and Total Cr Concentration Determination

Prior to Cr(VI) analysis, the samples were centrifuged for 10 minutes at 6000 rpm (2820 x g) to separate the dissolved fraction of Cr(VI) from the cells. Cr(VI) concentration was measured using the UV/Vis spectrophotometer. A 0.2 mL sample was acidified with 1 mL of 1N H_2SO_4 then diluted with distilled water to make up to 10 mL. 0.2 mL of 1,5-diphenyl carbazide was then add in the solution which produces a purple colour in the presence of Cr(VI). The absorbance was measured at a wavelength of 540 nm in a 10 mm light path (APHA, 2005).

To determine total Cr, the sample withdrawn from the reactor was diluted to give a final Cr concentration of 5 mg/L in 100 mL deionised water, followed by the addition of 0.9 mL of 30% H_2O_2. The absorbance of the mixture was measured at a wavelength of 359.9 nm using the AAS equipped with a 3 mA chromium hollow cathode lamp. Cr(III) was determined as the difference between total Cr and Cr(VI) concentration.

3.5.2 Biomass Weight Determination

A 5 mL sample of Cr(VI) reducing bacteria grown in LB broth containing 75 mg Cr(VI) /L was withdrawn by sterile pipette after 24 hrs of incubation at 33^{o}C (± 2). The sample was centrifuged for 10 minutes (2820 x g) and the supernatant was decanted. The wet biomass pellet was placed in a pre-weighed sintered glass. The wet biomass was dried in the oven at 105 ± 2^{o}C and weighed. The drying and weighing process was repeated until a constant dry weight was obtained. The dry weight of the biomass in 5 mL was calculated as the difference in weight between that of the sintered glass plus biomass and that of the empty sintered glass. The dry weight of the biomass per litre was obtained by extrapolation from the 5 mL volume. The biomass concentration values determined ranged from 3876 to 4382 mg/L. These biomass concentrations are on the higher side of the typical values observed in mixed liquor concentration of activated sludge (Metcalf and Eddy, 2003), thus the change of the dry mass during should be insignificant. This value was used for all computation during the determination of Monod parameters.

3.6 Abiotic Controls

Killed culture cells and azide exposed cultures were used to determine the extent of abiotic Cr(VI) reduction in the batch experiments. Overnight grown cells were heat-killed by autoclaving at 121^{o}C for 30 minutes. Another set of overnight grown cells was subjected to azide toxity by incubating the cells in a broth consisting of 0.1% azide solution using sodium azide (NaN_3) (Ginestet *et al*., 1998). Both cultures cells were collected by centrifugation 2820 x g for 10 minutes, and were washed twice in a

sterile saline solution (0.85% NaCl) followed by centrifugation 6000 rpm (2820 x g) for 10 minutes. The collected pellets were used in different batch experiments.

3.7 Bacteria Screening

3.7.1 Comparative Cr(IV) Reduction at Different Initial Concentrations

Cr(IV) reduction experiments were conducted in 100 mL Erlenmeyer flasks covered with cotton plugs during incubation to allow aeration while filtering away microorganisms from the air. CRB culture samples from the different sources; soil, sewage influent, activated (liquid) sludge and dried sludge were transferred into 100 mL sterile LB broth spiked with Cr(VI) at an initial concentration range of 20 - 600 mg/L. The cells were allowed to grow in the presence of Cr(VI) for 96 hours. Samples (1 mL) were withdrawn at the end of the growth period, and the residual Cr(VI) concentration in the cell suspensions was measured. Prior to Cr(VI) analysis, the samples were centrifuged at 6000 rpm (2820 g) for 10 minutes in a Hermle 2323 centrifuge (Hermle Laboratories, Germany) to remove suspended cells from the liquid.

3.7.2 CRB Isolation and Growth in the Presence of Cr(VI)

CRB cultures from soil samples as well as CRB cultures obtained from sewage influent, activated sludge (mixed liquor) and dried sludge where separately grown in 100 mL sterile LB broth spiked with 75 mg Cr(VI) /L for 24 hours at 33 ±2 ^{o}C in an incubator. Cultures were grown in suspension by agitation at 120 rpm using a Labcon SPL-MP 15 Lateral Shaker. Pure cultures were prepared by depositing 1 mL of a serially diluted sample from the different sources on PC agar, followed by incubation for 48 hours at 33 ±2 ^{o}C to develop separate identifiable colonies. Individual colonies based on their colour and morphology were transferred using a heat-sterilised wire loop into 100 mL sterile LB broth spiked with 75 mg Cr(VI) /L in the first stage. The cells were allowed to grow in the presence of Cr(VI) for 72 hours. The residual Cr(VI) in the cell suspensions exposed to 75 mg Cr(VI) /L was measured at the end of the growth period (72 hours). 1 mL of a serially diluted samples from each of the individual colonies grown in LB broth containing 75 mg Cr(VI) /L were deposited on

PC agar, followed by incubation for 48 hours at 33 ±2 ^{o}C to develop separate identifiable colonies. Individual colonies obtained were then transferred using a heat-sterilised wire loop into 100 mL sterile LB broth spiked with a higher concentration (150 mg/L) of Cr(VI) for the second screening stage. The residual Cr(IV) concentration was monitored at 24, 48 and 96 hours.

3.8 CRB Isolation and Culture Characterization

CRB cultures obtained from dried sludge where grown aerobically in 250 ml flasks containing 100 mL sterile LB broth spiked with 100 mg Cr(VI) /L for 24 hours at 33 ±2 ^{o}C in an incubator. Cultures were grown in suspension by agitation at 120 rpm using a Labcon SPL-MP 15 Lateral Shaker (Labcon Laboratory Services, South Africa). Pure cultures were prepared by depositing 1 mL of a serially diluted sample from different batches on PC agar and LB agar for colony development. After incubating for 48 hours at 33 ±2 ^{o}C, cells developed separate identifiable colonies based on their colour and morphology. Facultative anaerobic bacteria were isolated from dry sludge using the same procedure as described for aerobic cultures, except that cell were grown in anaerobic environment as described above. All transfers were conducted in an anaerobic glove box purged with nitrogen. The cultures were isolated under 100, 150 and 200 mg Cr(VI)/L.

The phylogenetic characterization of cells was performed on individual colonies of bacteria from the 7th-10th tube in the serial dilution preparation. In preparation for the 16S rRNA (16 Svedburg unit *ribosomal* Ribo-Nucleic-Acid) sequence identification, the colonies were first classified based on morphology. Seven different morphologies were identified from the aerobic cultures and 17 morphologies from the facultative anaerobic cultures. These were streaked on nutrient agar followed by incubation at 33 ±2 ^{o}C for 18 hours.

Genomic DNA was extracted from the pure cultures using a DNeasy tissue kit (QIAGEN Ltd, West Sussex, UK) as per manufacturer's instructions. The 16S rRNA genes of isolates were amplified by a reverse transcriptase-polymerase chain reaction (RT-PCR) using primers pA and pH1 (Primer pA corresponds to position 8-27;

Primer pH to position 1541-1522 of the 16S gene) (Coenye *et al*., 1999). An internal primer pD was used for sequencing (corresponding to position 519-536 of the 16S gene). The resulting sequences were compared to known bacteria in the GenBank using a basic BLAST search of the National Centre for Biotechnology Information (NCBI, Bethesda, MD).

The 16S rRNA is used to established microbial species interrelationships based on proximity of genetic phyla. This method is thus valuable as a tool for studying microbial diversity and interdependence of species in a consortium (Patel, 2001; Stackebrandt, *et al.*, 2002; Wisotzkey *et al.*, 1992).

3.9 Gram Staining

Gram staining method used, followed the Hucker method (APHA, 2005). 1 mL of bacteria (previously grown in LB broth for 24 in incubator) was spread on film and dried on flam. Heat-fixed film was then immersed in crystal violet then air-dried for 1 minute. The film was then gently and directly washed on tap water stream for 2 seconds. The film was then immersed in iodine mordant for 1 minute, then again gently and directly washed on tap water stream for 2 seconds. Afterwards, the slide was immersed in Safranin solution for 30 seconds and gently washed under tap water stream for 2 seconds. The slide was then immersed in 95% vol/vol ethanol for 5 seconds, and then gently washed under a stream of tap water for 2 seconds. Afterwards, the slide was dried with absorbent paper. Finally bacteria on the slide were photographed using a ZEISS Axioscop II microscope (Carl-Zeiss, oberkochen, Germany) equipped with a 100*/1.30 oil PLAN-NEOFLUAR objective. Cells were then differentiated by the colour observed: black-violet for Gram-positive; and red-pink for Gram-negative cells.

3.10 Cell Free Extract and Membrane Fragments

Pure cultures isolated in this study were grown in 500 mL for 24 hours in sterile LB broth. The cells were then harvested by centrifugation at 6000 rpm (2820 g) for 10 min. Pellets formed at the bottom of the centrifuge tubes were washed two times with

sterile 0.85% NaCl solution. The washed pellets were re-suspended at 2-3 gram wet weight per 10 mL sterile 0.85% NaCl. Re-suspended cells were disrupted by a 3 mm diameter microtip mounted to the Ultra High Intensity Ultrasonic Processor 500 watt model (VCX 500, Sonics & Materials, Inc., Newtown, CT). The tubes containing concentrated cells were placed inside an ice container to avoid overheating during sonication. The tip was previously cleaned with ethanol and dried thoroughly before use. Cells were sonicated in 4 cycles of 15 min with 5 min rests between cycles. Disrupted cells were centrifuged at 1200 rpm (11270 g) for 20 min to remove undisrupted cells and large pieces of cellular debris. Two 100 mg/L Cr(VI) batches were prepared, one containing undisrupted cells and cellular fragmented membranes and the other supernatant and cytoplasmic materials. Cr(VI) reduction was then monitored and compared with Cr(VI) reduction rates in intact cells. All experiments were conducted in duplicates.

3.11 Kinetic Experiments

3.11.1 Aerobic Cr(VI) Reduction

CRB contained in dried sludge were grown aerobically in 1L Erlenmeyer flasks containing 500 mL LB Broth at 33 ±2 ^{o}C for 24 hours. Cells were collected by centrifugation 2820 x g for 10 minutes, and were washed twice in a sterile saline solution (0.85% NaCl) followed by centrifugation 6000 rpm (2820 x g) for 10 minutes. Cr(VI) reduction experiments were conducted in 250 mL Erlenmeyer flasks covered with cotton plugs to allow aeration while filtering away microorganisms from the air. Harvested cells were first re-suspended in 100 mL LB broth at 5:1 concentration ratio before adding Cr(VI) to give the desired concentration. Cr(VI) stock solution was then added in the suspension to give a final concentration in the range of 50 to 600 mg/L. The suspension was incubated at 33 ±2 ^{o}C with continuous shaking on a lateral shaker at 120 rpm. To monitor Cr(VI) reduction, 1 mL samples were withdrawn at timed intervals. The samples were centrifuged 6000 rpm (2820 x g) for 10 minutes and the supernatant was used for Cr(VI) concentration analysis. Viable cell count was also determined during the incubation period to determine the toxicity of Cr(VI). All experiments were conducted in duplicates.

3.11.2 Anaerobic Cr(VI) Reduction

Anaerobic Cr(VI) reduction experiments were conducted in 100 mL serum bottles using harvested cells after 24 hours incubation under anaerobic conditions. The cells were transferred under an anaerobic glove bag purged with 99.99% N_2 gas. Cells were previously washed twice in a sterile solution of 0.085% NaCl, and then concentrated to a 5:1 ratio before running the reduction experiment. The bottles were purged with nitrogen gas (99.99%) for 10 min to expel any oxygen gas before sealing with silicon stoppers and aluminium seals. After sealing the cultures were incubated at 33 ±2 ^{o}C for 7 days. 1 mL samples were withdrawn using a sterile syringe at time intervals to monitor Cr(VI) reduction. The samples were centrifuged at 6000 rpm (2820 x g) for 10 minutes in a Hermle 2323 centrifuge (Hermle Laboratories, Wehigen, Germany) to remove suspended cells before analysis. All experiments were conducted in duplicates.

CHAPTER 4: CULTURE CHARACTERIZATION

4.1 The Characterization Paradigm

The initial characterisation of the bacterial composition was based on colony morphology, Gram status, 16S rRNA genetic makeup, and taxonomy (Kılı *et al.*, 2007, Francisco *et al.*, 2002). In order to identify the Cr(VI) reducing species, batches inoculated with the consortium from activated sludge were grown under 150 mg Cr(VI)/L. This served to eliminate most of the Cr(VI) sensitive organisms from the culture. Samples from the Cr(VI) reducing cultures were drawn from the batches after incubation for 96 hours. Some of the samples were treated with Gram staining reagents and viewed under a microscope. Another sample was serially diluted and spread on LB and plate count agar to develop colonies. The morphology of the colonies was then used to further determine the predominant species in the Cr(VI) challenged consortium. The logical process followed during the characterisation procedure is shown in Figure 4-1.

The Gram-staining exercise provided a primary classification based on the cell wall structure – the results give two basic cell wall configurations based on the content of the compound peptidoglycan, i.e., Gram-positive and Gram-negative bacteria (Gram, 1884). From earlier studies it is shown that Cr(VI) Reducing Bacteria (CRB) can either be Gram-positive or Gram-negative (Camargo, 2005; Kılı *et al.*, 2007). Gram-positive bacteria are more resistant to Cr(VI) toxicity than Gram-negative bacteria due to the presence of a thick *peptidoglycan* layer comprising 50-90% of cell wall in the Gram-positive cultures (Ross *et al.*, 1981).

After colony morphology and gram-staining classifications the 16s rRNA identification and classification was conducted. 16s rRNA is one of the most recent developments in the microbial culture characterisation. This method is more accurate in determining species compositions of culture communities than the classification based on morphology. For example, using the 16s rRNA method, Camargo (2005) isolated and identified ten new CRB out of 16 CRB from soil samples obtained from Brazil and the United State of America. The accuracy of the method is enhanced if

applied together with more advanced genetic separation techniques such as the Denatured Gradient Gel Electropholysis (DGGE) for culture communities.

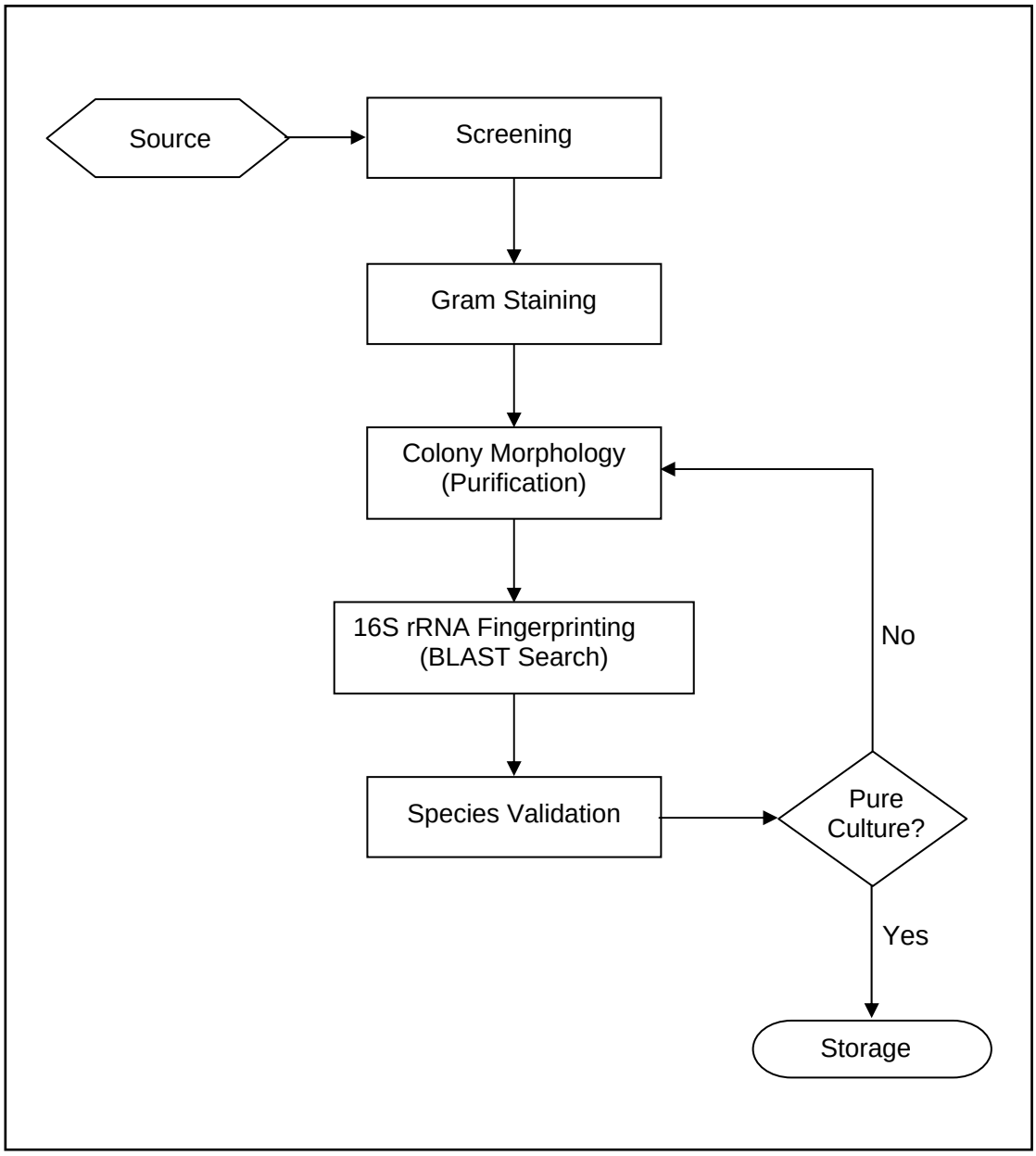


Figure 4-1: The paradigm of microbial culture characterisation in mixed- and consortium culture systems.

The CRB isolated by other researchers using the 16s rRNA method formed close matches with the *Bacillus* genera. Examples of CRB from literature include *Bacillus cereus*, *Arthrobacter bacterium,* and *Bacillus thuringiensis* (Francisco *et al.*, 2002) Recently, Kili *et al.* (2007) identified Cr(VI) reducing activities in three gram

negative species – *Ochrobactrium sp.*, *Salmonella enterica* and *Pseudomonas aeruginosa* – using the 16S rRNA method. The 16s rRNA fingerprinting method is able to pick species that are unculturable using traditional solid agar colony development methods (Amann *et al.*, 1995; Rondon *et al.*, 2000).

4.2 CRB Source Identification and Screening

4.2.1 Comparative Analysis of Cr(VI) Reduction by Cultures from Different Sources

Four different sources were identified as possible sources of CRB: (1) soil from a contaminated site, (2) influent to a sewage treatment plant, (3) activated sludge from aeration tanks and (4) dried sludge from sand drying beds. The measured background Cr(VI) concentration in the influent and mixed liquor from the treatment plant was 2.45 and 2.63 mg/L, respectively, and the Cr(VI) content in dried sludge was 25.44 g/m^3 at the time of sampling. Higher values of the reduced form of total Cr were expected in the mixed liquor and dry sludge due to the presence of Cr(VI) reducing bacteria. The existence of chromium (VI) reducing bacteria in the samples from different sources was indicated by high removal rates observed in the cultures as shown in Table 4-1. All cultures were inoculated for 96 hours in the screening test.

The cultures from contaminated soil yielded the lowest Cr(VI) removal rate. Soil bacteria reduce Cr(VI) as much as observed in Chirwa and Wang (1997) study, but the performance was insignificant and the weakest compared to dried sludge cultures, mixed liquor cultures and sewage cultures. Soil cultures were able to reduce 91.5 % of Cr(VI) at an initial of 20 mg/L, and the Cr(VI) reduction ability significantly decreased with an increase in initial concentration.

The Cr(VI) reduction ability of cultures obtained from mixed liquor also decreased with an increase in initial Cr(VI) concentration. However, complete Cr(VI) reduction was observed at initial Cr(VI) concentrations of up to 100 mg/L. Complete Cr(VI) reduction in cultures from sewage influent was observed at initial Cr(VI) concentrations of up to 50 mg/L and thereafter Cr(VI) reduction decreased with increasing concentration.

The highest Cr(VI) reduction performance was observed in cultures from dried sludge, where complete Cr(VI) reduction was observed at initial Cr(VI) concentrations up to 200 mg/L. At an initial concentration of 300 mg/L Cr(VI), the dried sludge culture reduced 99.2 % after incubation for 96 hours. In all cultures studied, there was no Cr(VI) reduction observed at an initial concentration of 600 mg/L. These cultures were able to completely reduce more than the 2mM initial Cr(VI) concentration reported in most studies (Francisco *et al.,* 2002; Opperman and Heerden; 2007; Chirwa and Wang, 1997; Wang and Shen, 1997), regardless of the source. The high performance in the dried sludge was attributed to the long period of contact with Cr(VI) from the initial wastewater treatment stage to the final stage in the treatment plant at Brits. The presence of a wide diversity of carbon sources and nutrients in the system sustained a wider biodiversity in the system. The results in Table 4-1, inform on the choice of the culture for detailed analysis later in the study.

Table 4-1: Percentage Cr(VI reduction in cultures from different sources after 96 hours of incubation at different initial Cr(VI) concentration

Sources of CRB	**Initial Cr(VI) concentration (mg/L)**						
	20	**50**	**100**	**150**	**200**	**300**	**600**
Dried sludge cultures	100 %	100 %	100 %	100 %	100 %	99.2 %	0 %
Mixed liquor cultures	100 %	100 %	100 %	93.6 %	67.6 %	60.3 %	0 %
Sewage cultures	100 %	100 %	-	74.2 %	-	14.1 %	0 %
Soil cultures	91.5 %	76.0 %	-	29.9 %	-	7.45 %	0 %

- no experiment conducted at this concentration

4.2.2 CRB Screening

4.2.2.1 First Isolation

Based on the colour and morphology of the individual colonies obtained on the PC agar plates, five different types of colonies were identified and were labelled as "T" (Table 4-2). The first type (T1), was white and longish, the second type (T2) white and spherical, the third (T3) cream coloured and spherical, the fourth (T4) yellow and the last type (T5) was pink. All the obtained colonies grew on the agar surface, except for the first type which was embedded in agar. Only T1 and T2 were identified on plates from influent sewage cultures. All five types were present on plates from mixed liquor activated sludge cultures. Apart from T5, the rest of the colony types were present on plates from dried sludge cultures as well.

When each of the types of colonies were grown in LB broth containing 75 mg/L Cr(VI) and inoculated for 72 hours, a high Cr(VI) reduction performance of 99.23, 94.96 and 94.2% was observed with T4 (99.23%) from mixed liquor, followed by T4 (94.96%) and T2 (94.2%) both from dried sludge (Table 4-3). However, no reduction was observed in T3 and T1, and a partial reduction in T2 and T5 cultures from mixed liquor. In types from sewage, a 58 and 56 % Cr(VI) reduction was observed in T1 and T2, respectively. Screened colonies from dried sludge resulted in the best reduction. The worst case for dried sludge was as high as 49.32% observed in T1.

Results in Table 4-2 and 4-3 show that dried sludge contain more CRB than the mixed liquor. The mixed liquor in the Table, shows a wide diversity of species, but apart from T4, the other showed low performance (T2 and T5), and insignificant results (T1 and T3). For these results, it was concluded that T1 and T3 in the mixed liquor cultures were just Cr(VI) resistant bacteria.

Table 4-2: Types of first screened colonies from Sewage, Mixed liquor and Dried Sludge

Type	Sewage (SWG)	Mixed-Liquor (ML)	Dried Sludge (SD)
T1 (White longish, embedded in agar)	YES	YES	YES
T2 (White spherical, on agar surface)	YES	YES	YES
T3 (Cream, on agar surface)	-	YES	YES
T4 (Yellow, on agar surface)	-	YES	YES
T5 (Pink, on agar surface)	-	YES	-

-: means no colonies observed

Table 4-3: Percentage Cr(VI) reduction by different types of colonies from Sewage, Mixed liquor and Dried Sludge at an initial Cr(VI) concentration of 75 mg/L.

Type	Sewage (SWG)	Mixed Liquor (ML)	Dried Sludge (SD)
T1	58.63	0	49.32
T2	56.58	32.56	94.96
T3	-	0	82.65
T4	-	99.23	94.02
T5	-	35.47	-

- = no colonies observed, 0 = no reduction observed

4.2.2.2 Second Isolation

Each colony type from influent sewage, mixed liquor and dried sludge was grown for the second time in LB broth containing 75 mg/L of Cr(VI) for 24 hours, followed by growth on PC agar. Sub-types of each colony were observed; except for sewage types where no growth was observed in the LB broth. Based on the same physical properties as previously (colour and morphology), the sub-types morphology from each colony were labelled as: "A" white longish, "B" white spherical, "C" cream coloured and spherical, "D" yellow and the last "E" pink (Table 4-4).

Results obtained revealed the predominance of white colonies in all screened types from both mixed liquor and dried sludge. More diversity was observed in mixed liquor (16 sub-types) than in dried sludge (10 sub-types) under aerobic conditions. In this second screening experiment the presence of pink coloured colonies was observed in T1 plates from dried sludge, whereas no pink colonies were observed in the first dried sludge screening experiment (Table 4-2) and no sub-type D (yellow colonies) were present in the dried sludge. Sub-type A was present in all dried sludge types while subtype B was present in all mixed liquor.

Table 4-4: Sub-types of second screened colonies from Mixed liquor and Dried Sludge.

Source	**1st screened type**	**A** (w-l)	**B** (w-s)	**C** (cream)	**D** (yellow)	**E** (pink)
MIXED	ML T1	YES	YES	YES	-	-
LIQUOR	ML T2	YES	YES	YES	-	YES
	ML T3	YES	YES	YES	YES	-
	ML T4	YES	YES	-	-	-
	ML T5	-	YES	YES	YES	-
DRIED	SD T1	YES	-	-	-	YES
SLUDGE	SD T2	YES	YES	YES	-	-
	SD T3	YES	YES	-	-	-
	SD T4	YES	YES	YES	-	-

- : no colonies observed; w-l: white longish; w-s: white spherical

Each of the sub-types was then re-suspended in a more concentrated and toxic media containing 150 mg/L Cr(VI) for 96 hours. Figures 4-2A, B, C and D show the fraction of 150 mg/L Cr(VI) remaining in solution for each individual dried sludge sub-type after 96 hours incubation time. Best performance was observed in SD T2A, SD T2C, SD T3B and SD T4A which reduced 99.75 %, 99.55 %, 97 %, and 99.6 % of Cr(VI), respectively. The lowest was SD T1A which reduced only 60.8%.

Out of the 16 sub-types from mixed liquor, only 5 sub-types reduced more than 90% of the initial 150 mg/L Cr(VI) after 96 hours (Figures 4-3A, B, C, D and E). These 5 sub-types [4 sub-types from T2 (ML T2A, B, C and E) and one from T4 (ML T4B)] reduced 97.5 %, 92.0 % 99.6 %, 99.6% and 99.6 %, respectively. ML T4A reduced 89.4 % of the initial Cr(VI) concentration. Sub-types from T3 from mixed liquor resulted in a Cr(VI) reduction in the range of 51.5 to 69.6 %, whereas sub-types from ML T1 and ML T5 resulted in a partial reduction. However, ML T5D cultures resulted in a 78.1% Cr(VI) reduction.

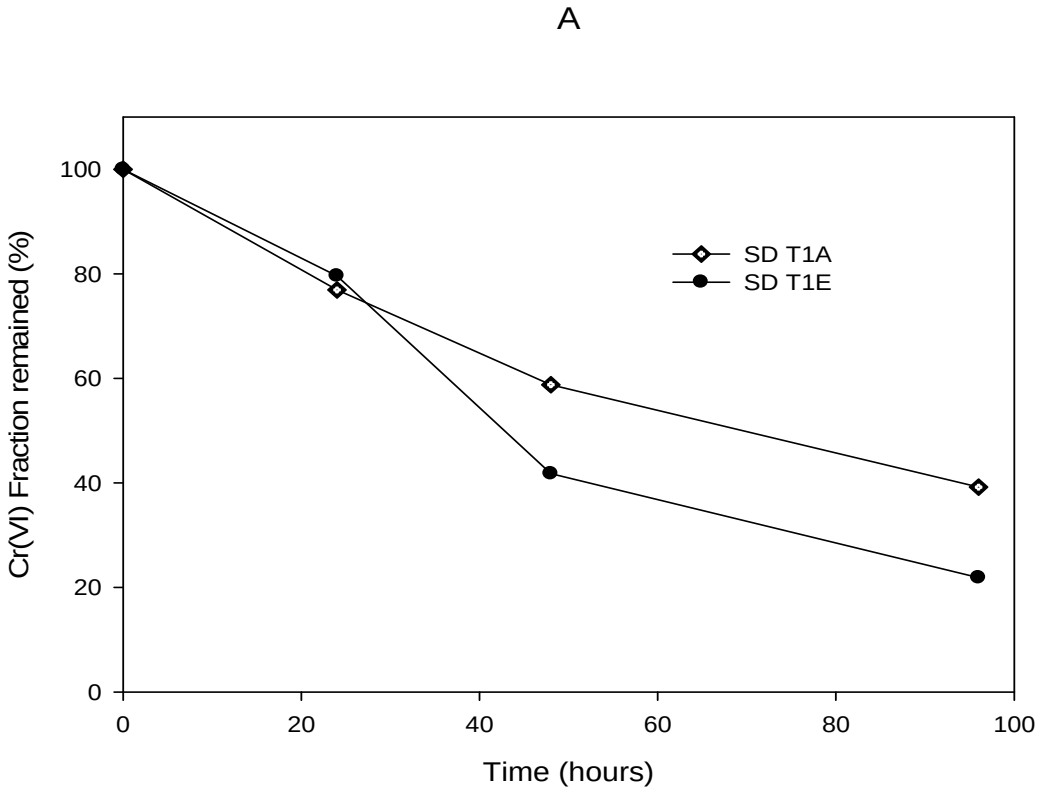


Figure 4-2A: Fraction of Cr(VI) remained after reduction by different dried sludge sub-type screened

B

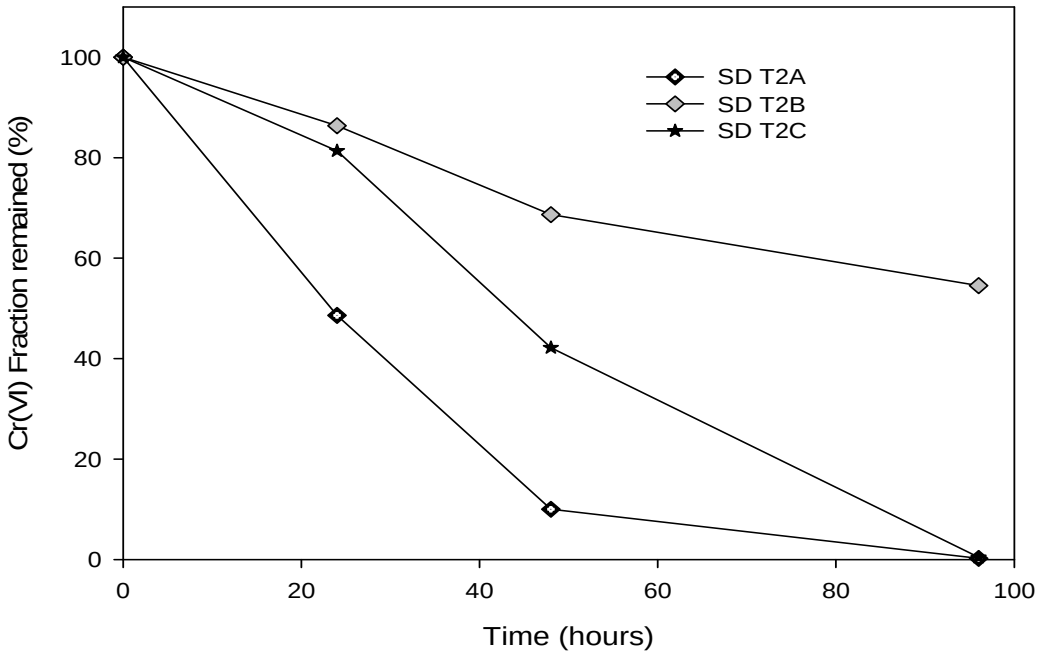


Figure 4-2B: Fraction of Cr(VI) remained after reduction by different dried sludge sub-type screened

C

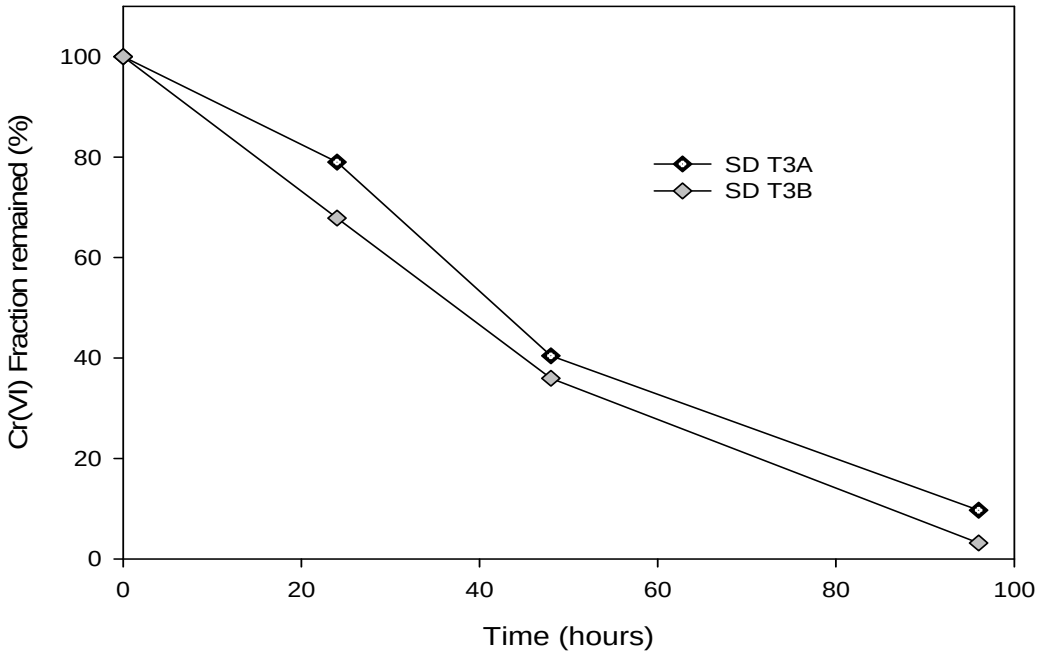


Figure 4-2C: Fraction of Cr(VI) remained after reduction by different dried sludge sub-type screened.

D

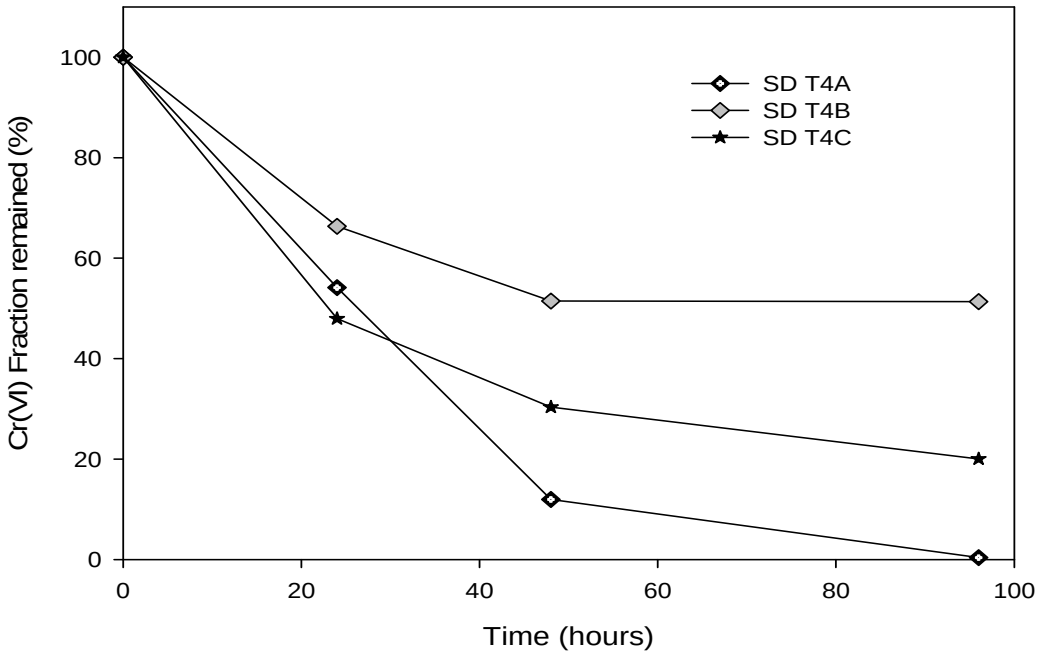


Figure 4-2D: Fraction of Cr(VI) remained after reduction by different dried sludge sub-type screened.

A

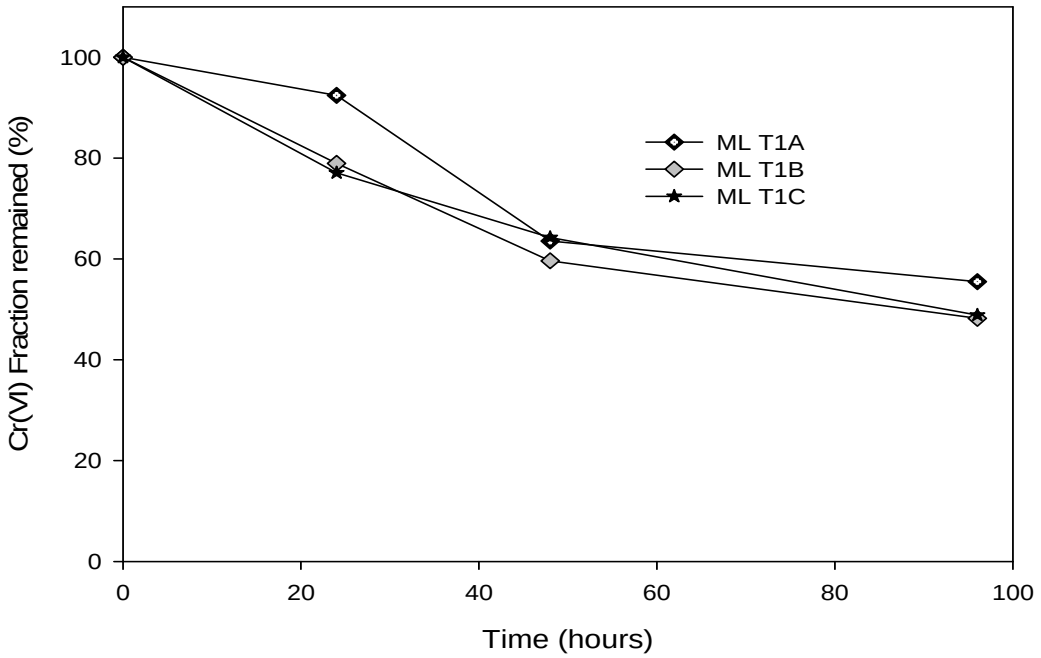


Figure 4-3A: Fraction of Cr(VI) remained after reduction by different mixed liquor sub-type screened.

B

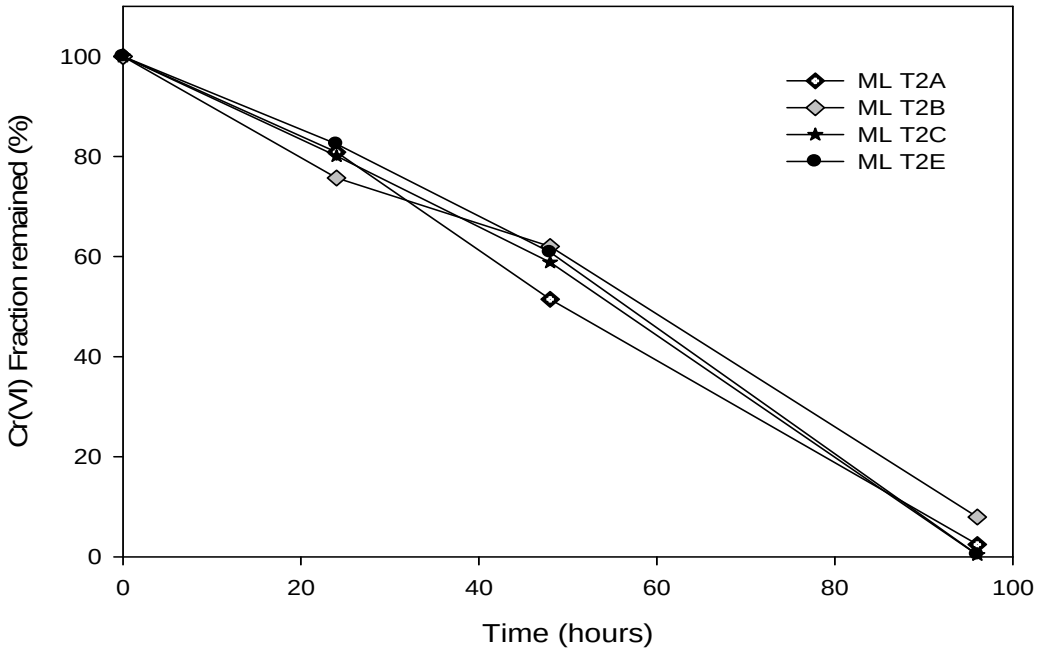


Figure 4-3B: Fraction of Cr(VI) remained after reduction by different mixed liquor sub-type screened.

C

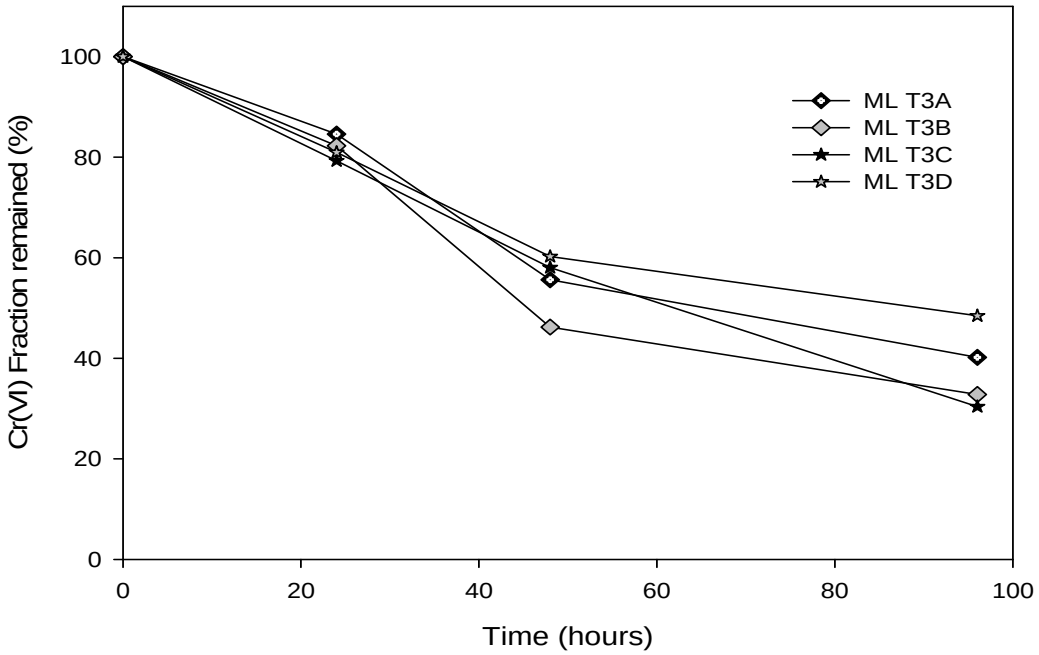


Figure 4-3C: Fraction of Cr(VI) remained after reduction by different mixed liquor sub-type screened.

D

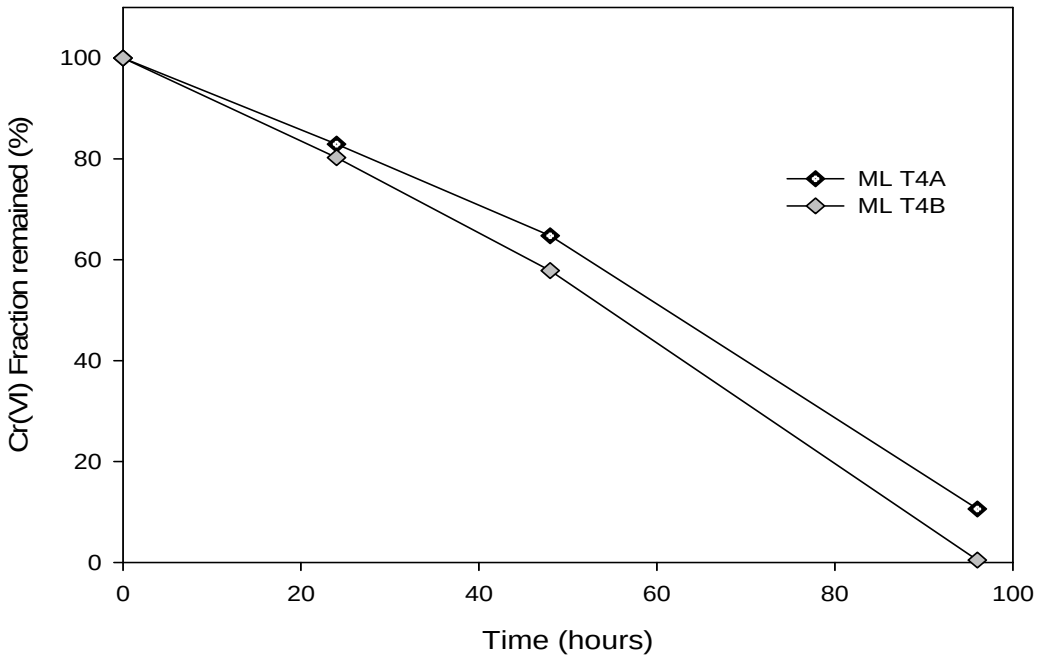


Figure 4-3D: Fraction of Cr(VI) remained after reduction by different mixed liquor sub-type screened.

E

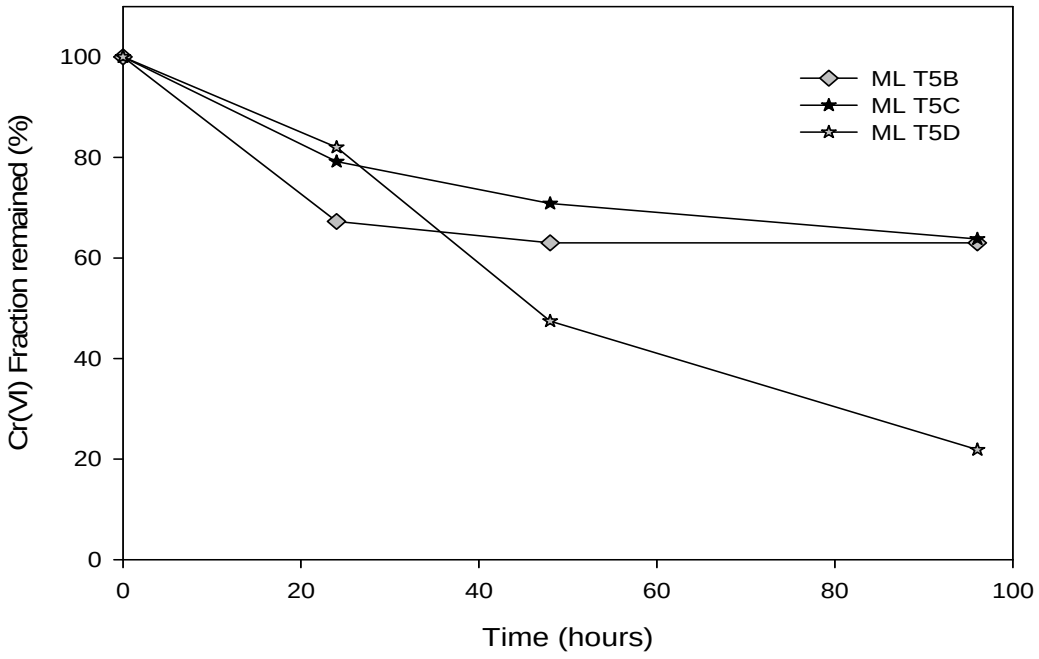


Figure 4-3E: Fraction of Cr(VI) remained after reduction by different mixed liquor sub-type screened.

4.3 Gram Staining Studies

4.3.1 Gram Staining of Aerobic Culture

The aerobic culture was predominated by the Gram-positive strains (Figure 4-4A). The culture showed a strong presence of rod-shaped Gram-positive cells which sometimes formed clusters suggesting the presence of flocs. This sugest that the Cr(VI) removal under aerobic conditions is carried out by Gram-positive species probably *Bacilli*.

4.3.2 Gram Staining of Anaerobic Culture

The anaerobic cultures showed predominant presence of rod-shaped Gram-negative cells (Figure 4-4B). The density of cells in the anaerobically grown cultures was much lower than that of the aerobic cultures which confirmed the observation by Ross *et al.* (1981) that Gram-negative cells are usually more susceptible to Cr(VI) toxicity than gram-positive bacteria – similar observations were also made by Srinath *et al.* (2002) and Muhammad and Shahinda (2005).

4.4 Classification of Bacteria in Aerobic Cultures

4.4.1 General Classification using 16S rRNA IDs

In preparation for the 16S rRNA sequence identification, the colonies were first classified based on morphology. These colonies were streaked on nutrient agar followed by incubating at 33°C (± 2) for 18 hours and 7colonies were obtained. 16S rRNA sequencing resulted in identification of 3 possible phenotypes, with 99% probability match (Table 4-5). Results indicated the predominance of the three aerobic phenotypes:

- the *Bacillus cereus* ATCC 10987, *Bacillus cereus* 213 16S,
- the *Bacillus thuringiensis* (*serovar finitimus*), *Bacillus mycoides*,
- and Microbacterium foliorum and Microbacterium sp. S15-M4.

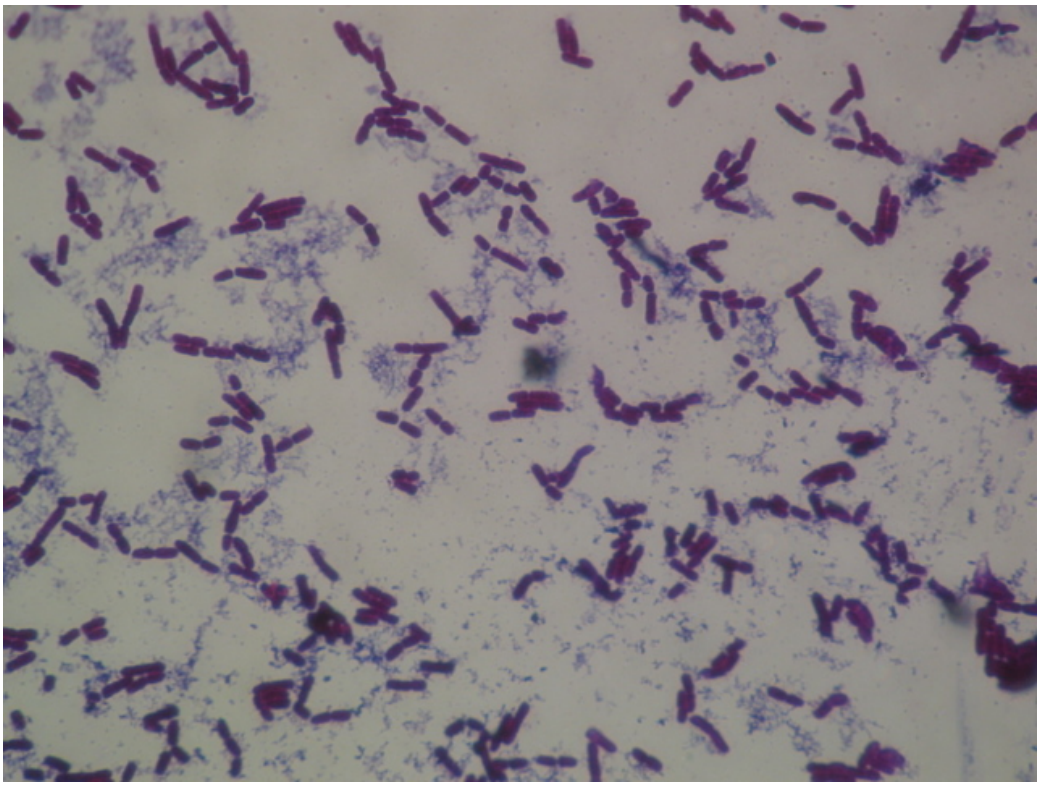


Figure 4-4A: Picture of Gram Staining Aerobic

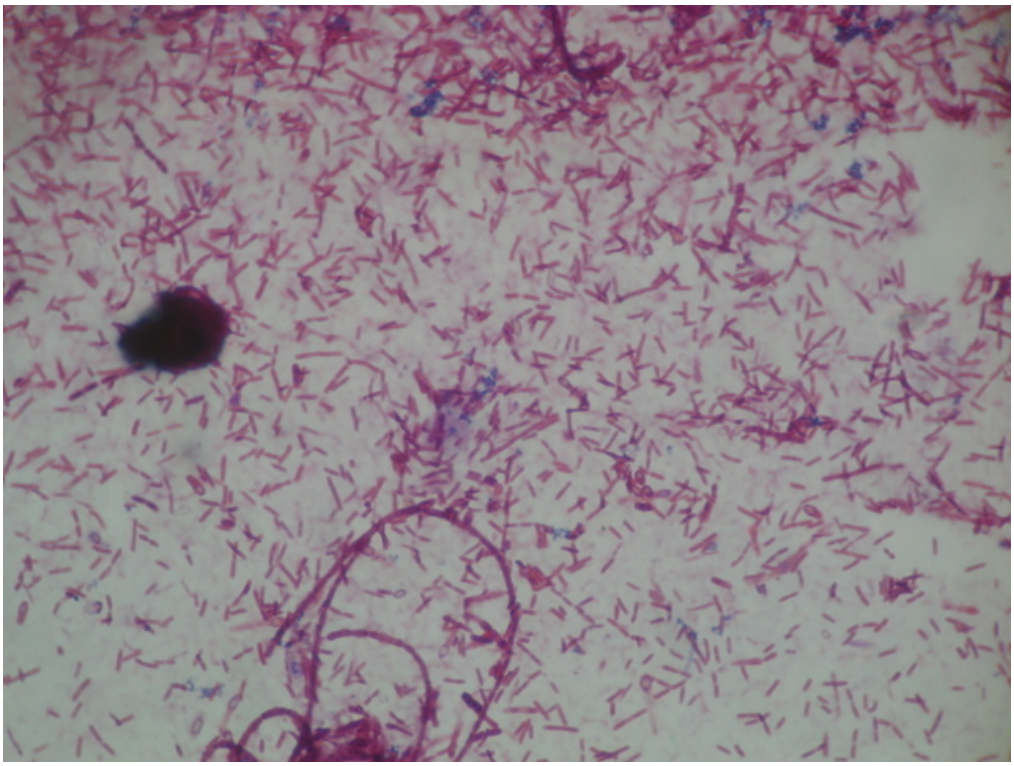


Figure 4-4A: Picture of Gram Staining Anaerobic

Table 4-5: Species identification by 16S rRNA in the aerobically grown culture

ISOLATED CRB	PARTIAL 16SID[1]	% Identity
X1 (CXae1)	*Bacillus cereus strain* 213 16S, *Bacillus thuringiensis* 16S	99
X2 (CXae2)	*Bacillus sp. ZZ2* 16s, *Bacillus cereus* ATCC 10987, *Bacillus thuringiensis* str. Al Hakam	99
X3 (CXae3)	*Bacillus sp.* 32-661 16s, *Bacillus cereus* strain 16S	99
X4 (CXae4); X5 (CXae5) and X6 (CXae6)	*Bacillus mycoides strain* BGSC 6A13 16S, *Bacillus thuringiensis* serovar finitimus strain BGSC 4B2 16S	99
X7 (CXae7)	*Microbacterium sp.* S15-M4, *Microbacterium foliorum*	99

[1]: 16 Svedburg rRNA Identity of partial sequences (16S ID)

4.4.2 Taxonomic Evaluation

A phylogenetic tree was constructed based on the blast results of the identified 16s sequences (Figure 4-5). The bacteria in the phylogenetic tree are divided into two groups: six of which belong to the *Bacillus* group and one to the *Microbacterium* group. Five of the *bacillus*: X1, X3, X4, X5 and X6 represented in the tree by (CXae1), (CXae3), (CXae4), (CXae5) and (CXae6), respectively, are associated with the reference strains AF2905451 |*Bacillus thuringiensis*| ATCC10792, DQ207729 |*Bacillus cereus*| CCM2010 and AB02 1192|*Bacillus mycoides*. The fifth, X2 (CXae2), though separated from these others mentioned above, is still associated with the *Bacillus* genera. The last, CXae7, aerobe CRB isolated from the dried sludge is unique and closely associated with the reference strain AJ249780| *Microbacterium foliorum*| D (Figure 4-5).

Among the reference strains observed in the study, some have been reported elsewhere as being capable of reducing Cr(VI) such as the *Bacillus thuringiensis* (Camargo *et al.*, 2003[b]; Sahin *et al.*, 2005); and *Bacillus cereus* (Camargo *et al.*, 2003[b]; Gvozdyak *et al*., 1986; Muhammad and Shahinda, 2005).

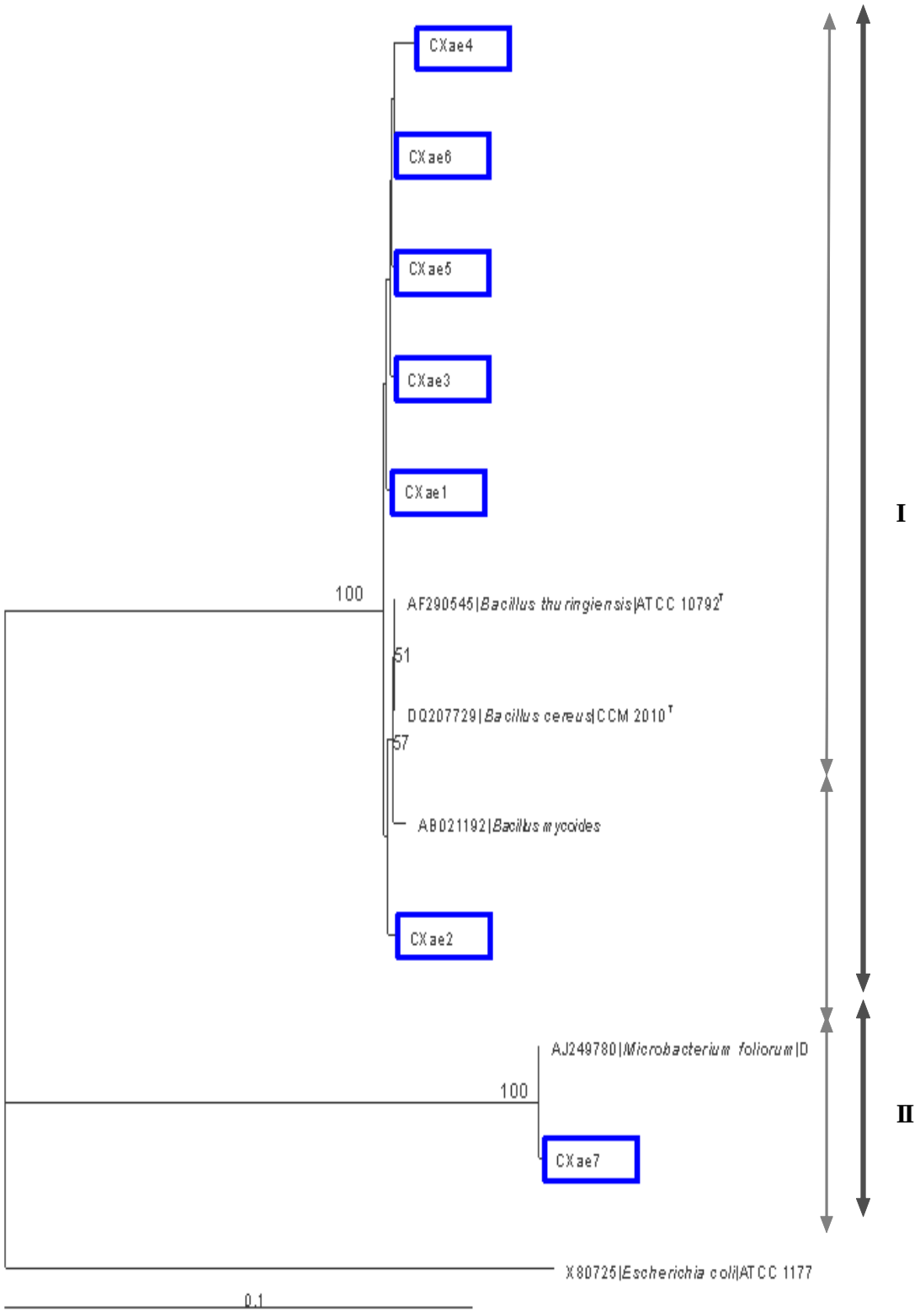


Figure 4-5: Phylogenetic tree of aerobe isolates cultures

4.5 Classification of Bacteria in Anaerobic Cultures

4.5.1 General Classification using 16S rRNA IDs

Eighteen different morphologies were identified in the anaerobically grown cultures using the 16s rRNA method, some of the bacteria were unculturable but left fingerprint during 16S rRNA analysis while some were culturable but unidentifiable. Only 10 colonies were partially identifiable by the blast result; 8 colonies could not be amplified for partial gene sequencing. Amplified cultures matched existing species in the gene bank with a percentage between 93% and 99%.

Results indicated the predominance of seven facultative anaerobic phenotypes on plates: light brown, off-white, cream, coral, yellow, yellow rings, orange (Table 4-6). The partial sequences of 16S rRNA matched *Bacillus* groups, *Microbacterium* groups, uncultured *bacterium* groups and *Enterococcus* groups:

- Seven phenotypes from the *Bacillus* groups – *Bacillus drentensis, Bacillus sp.* BS19, *Bacillus sp.* R21S, *Oceanobacillus sp.* JPLAk1, *Paenibacillus pabuli, Paenibacillus xylanilyticus strain* XIL14, *Virgibacillus necropolis*;
- Eight phenotypes from the *Microbacterium* group – *Acinetobacter sp.* ANT9054, *Arthrobacter sp.* AK-1, *Arthrobacter sp. Sphe*3, *[Brevibacterium] frigoritolerans, Rumen bacterium* R4-4;
- Three phenotypes from the uncultured *bacterium* group – uncultured *bacterium clone* Y2, Uncultured soil *bacterium clone* TA12; and
- Three phenotypes from the *Enterococcus* – *Enterococcus avium*, *Enterococcus faecium strain* R0026, *Enterococcus pseudoavium*.

4.5.2 Taxonomic Evaluation

A phylogenetic tree (Figure 4-6) was constructed using data from the ten amplified cultures previously generated from the BLAST search results: X2, X3, X6a, X6b X7, X10, X11, X12, X15 and X17 (Table 4-6) and labelled respectively as CXan2, CXan3, CXan6a, CXan6b, CXan7, CXan10, CXan11, CXan12, CXan15 and CXan17. These facultative anaerobe isolates produced five different groups:

- The first group represents the reference strain *Bacillus* and is subdivided into two subgroups: (1) CXan7, CXan10, and CXan15 and (2) CXan17. CXan7 is closely associated with the reference strain AJ542506 *Bacillus drentensis* LMG21831^T and CXan10 is closely associated with the reference strain AJ315060 *Virgibacillus* LMG 19492. CXan15 is closely associated with the strains mentioned above. CXan17 is closely associated with three reference strains: DQ207729 *Bacillus cereus* CCM 2010, AF290545 *Bacillus thuringiensis* ATCC 10792 and *Bacillus mycoides*.
- The second group representing the reference strain *Enterococcus* is subdivided into two subgroups: CXan2 in one and CXan11 in the other. CXan2 is closely associated with two reference strains DQ411811 *Enterococcus avium* ATCC 14025 and DQ411809 *Enterococcus pseudoavium* ATCC 49372. CXan11 is closely associated with the reference strain DQ411813 *Enterococcus faecium* ATCC 19434.
- The third group constitutes only isolate CXan12 which is closely associated with the reference strain AB073191 *Paenibacillus pabuli*.
- The fourth group which is composed of CXan6a and CXan6b is closely associated with the reference strains X83408 *Arthrobacter oxydans* and X83409 *Arthrobacter sulfureus*.
- The fifth and the last group consisted of one isolate, CXan3, which is closely associated with the reference strain X81665 *Acinetobacter lwoffii* DSM 2403.

The above data shows a wide biodiversity of microorganisms in the anaerobic cultures probably due to the partially anaerobic conditions in the aeration tanks at Brits where the cultures originated. It is of particular interest to note that the block associated with the isolate CXan17 in the anaerobic culture was exactly the same as the block associated with CXae2 and the other Cr(VI) reducing Bacilli in the aerobic cultures in Figure 4-5. This suggests that the Cr(VI) reducers in the system were facultatively anaerobic.

Table 4-6: CRB cultures isolated under anaerobic conditions

[CrVI)] used	Labelled specie	Colour on plates	Blast Result	% Identity
	X1	Light brown/cream	CNS/A	-
100 mg/L	X2 (CXan2)	Off-white	*Enterococcus avium*, *Enterococcus pseudoavium*	99
	X3 (CXan3)	Cream	Uncultured *bacterium clone* Y2, *Acinetobacter sp.* ANT9054	97
	X4	Coral	CNS/A	-
	X5	Yellow	CNS/A	-
	X6a (CXan6a)	Yellow	*Arthrobacter sp. Sphe*3, Uncultured soil *bacterium clone* TA12	93,94
	X6b (CXan6b)		*Arthrobacter sp.* AK-1	99
150 mg/L	X7 (CXan7)	Cream & Yellow rings	*Bacillus drentensis*, *Bacillus drentensis*	96, 97
	X8	Light brown	CNS/A	-
	X9	Light brown	CNS/A	
	X10 (CXan10)	Light brown	*Oceanobacillus sp.* JPLAk1, *Virgibacillus necropolis*	99,98
	X11 (CXan11)	Off-white	*Enterococcus faecium strain* R0026, *Rumen bacterium* R4-4	99
	X12 (CXan12)	Coral	*Paenibacillus pabuli*, *Paenibacillus xylanilyticus strain* XIL14	99
	X13	Yellow	CNS/A	-
	X14	Orange	CNS/A	-
200 mg/L	X15 (CXan15)	Cream	*[Brevibacterium] frigoritolerans*, *Bacillus sp.* R21S	99
	X16	Yellow	CNS/A	-
	X17 (CXan17)	Cream	Uncultured bacterium, *Bacillus sp.* BS19	93

CNS/A: Could not subculture/amplify

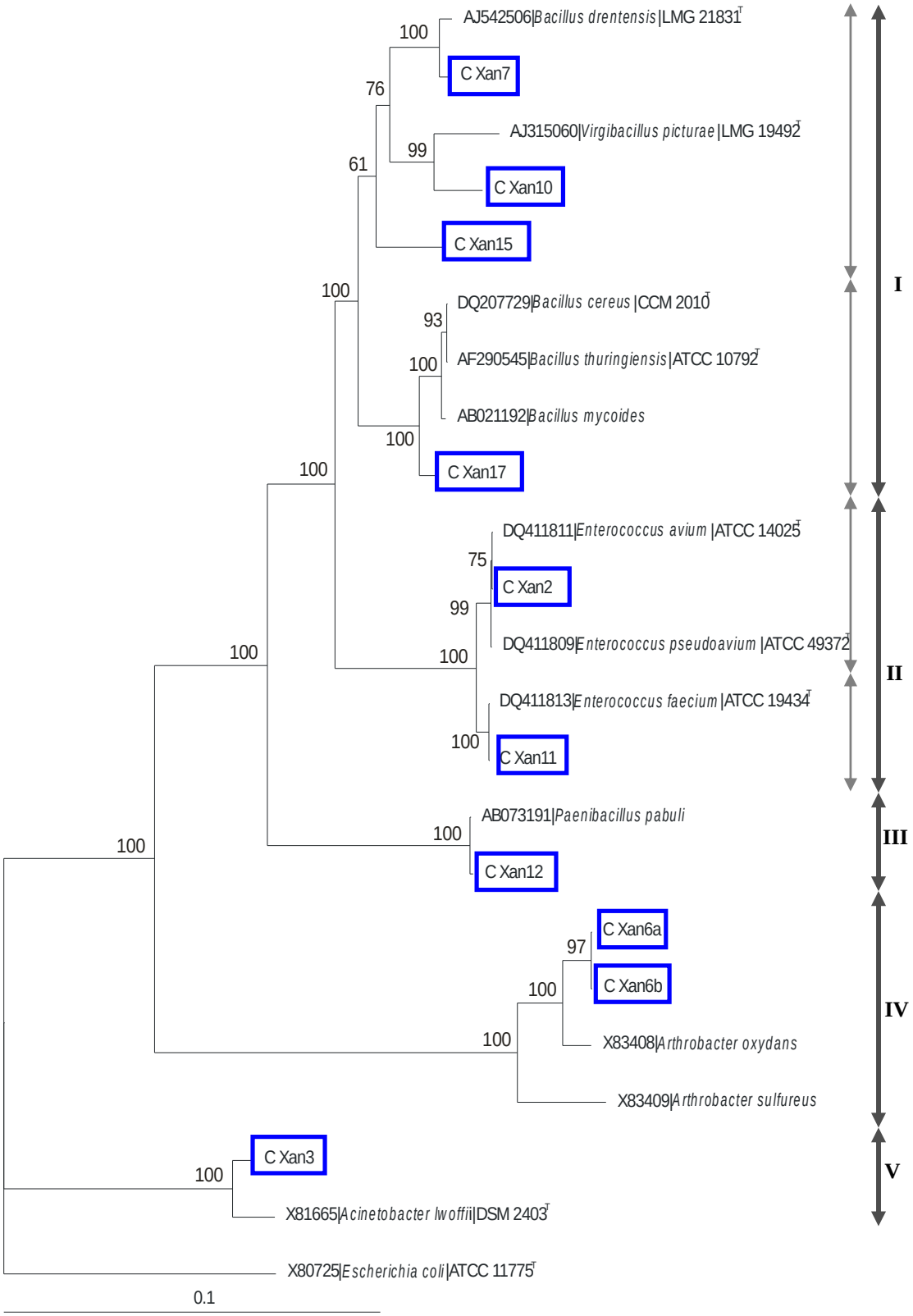


Figure 4-6: Phylogenetic tree of bacteria grown under anaerobic conditions indicating the presence of the identifiable isolates.

4.6 Performance Validation in Pure Cultures

4.6.1 Purified Cultures Versus Consortium

Cr(VI) reduction in pure cultures (X1–X7) was compared with the Cr(VI) reduction in the original consortium culture from the dried sludge. An example of the comparative data analysis is shown in Figure 4-7 and Figure 4-8 using all isolates. The preliminary analysis showed that the performance of different species matched that of the consortium culture at different times during incubation (Figure 4-7 and Figure 4-8). For example, Cr(VI) reduction rate in the culture X5 was approximately equivalent to that of the consortium culture during the first 40 hours of incubation after which, the removal rate was significantly slower. On the other hand, the Cr(VI) removal in the isolate X7 was equivalent to the consortium culture removal after 75 hours incubation. This shows that a fast performing culture may be more susceptible to Cr(VI) toxicity. Such a culture may act earlier in the presence of other more resilient slower performing cultures. This further indicates the importance of synergism for optimal performance of the culture as the main reason why individual cultures could not perform as well as the original mixed-culture consortium.

4.6.2 Performance Validation – Reconstituted Consortium

Further analysis was conducted using individual isolates to determine the Cr(VI) reduction rate in purified cultures. Cultures were reconstituted by inoculating the batches with minute amount of each pure culture isolate and harvesting after incubating for 24 hours. The cell numbers in all batches were determined to be approximately the same.

The data in Table 4-7 confirm that no species acting alone achieved the same level of Cr(VI) reduction rate as the original consortium. Additionally, reconstituted cultures, e.g., X1+ X2+ X3+ X4, performed better than individual cultures. For example, X5 and X6 acting alone achieved 70.0 and 67.7% Cr(VI) removal in 24 hours, but when grown together as a mixed-culture, they achieved 84.6% with the same time of incubation (24 hours). In the same table (Table 4-7), the fully reconstituted consortium from individual pure cultures (X1 + X2+ X3+ X4 + X5 + X6 + X7) showed 94.3% Cr(VI) removal after 24 hours and complete removal after 28.5 h (not

shown). This indicates that some synergistic process occurred that resulted in higher performance of the mixed-culture. This also validates the capability of the CRB from the dried sludge. One experiment conducted at an initial Cr(VI) concentration of 100 mg/L showed that addition of pure cultures X5 and X6 to the mixed-culture containing X1, X2, X3, and X4 improved the culture performance by 7.2% (i.e., 84.2–91.8 %) after incubation for 22.5 h. These experiments led us to the assumption that synergistic processes occurred between species that resulted in the higher performance of combined cultures.

4.6.3 Performance Evaluation (Comparison with Previous Isolated Cultures)

The dried sludge cultures from the Wastewater Treatment Works at Brits (NW) reduced Cr(VI) at higher concentrations and at a higher rate than known Cr(VI) reducing cultures including the pure cultures of *Bacillus sp*. [isolated from a Cr(VI) contaminated site in Newark (New Jersey) (Chirwa and Wang, 1997a)], *Pseudomonas fluorescens* LB300 [originally isolated from soil (Chirwa and Wang, 1997b)], and *Escherichia coli* ATCC 33456 [purchased (Wang and Shen, 1997)]. Comparison of Cr(VI) removal at 48 hours incubation for 90–120 mg Cr(VI)/L cultures shows Cr(VI) removal rate in indigenous sludge culture approximately 3, 8, and 8 times higher than values observed in *P. fluorescens* LB300 (Chirwa and Wang, 1997b; Bopp and Ehrlich, 1988), *Bacillus* sp. (Chirwa and Wang, 1997a) and *E. coli* ATCC 33456 (Wang and Shen, 1997), respectively (Table 4-7).

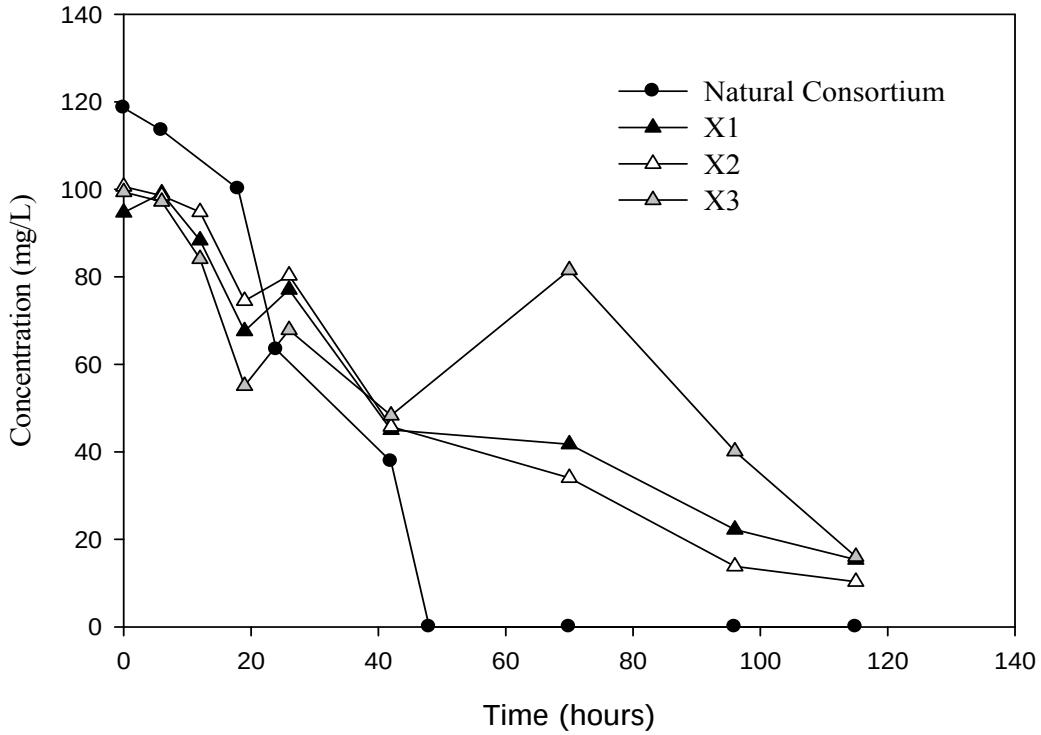


Figure 4-7: Cr(VI) reduction in growth conditions by aerobe isolates X1, X2, and X3.

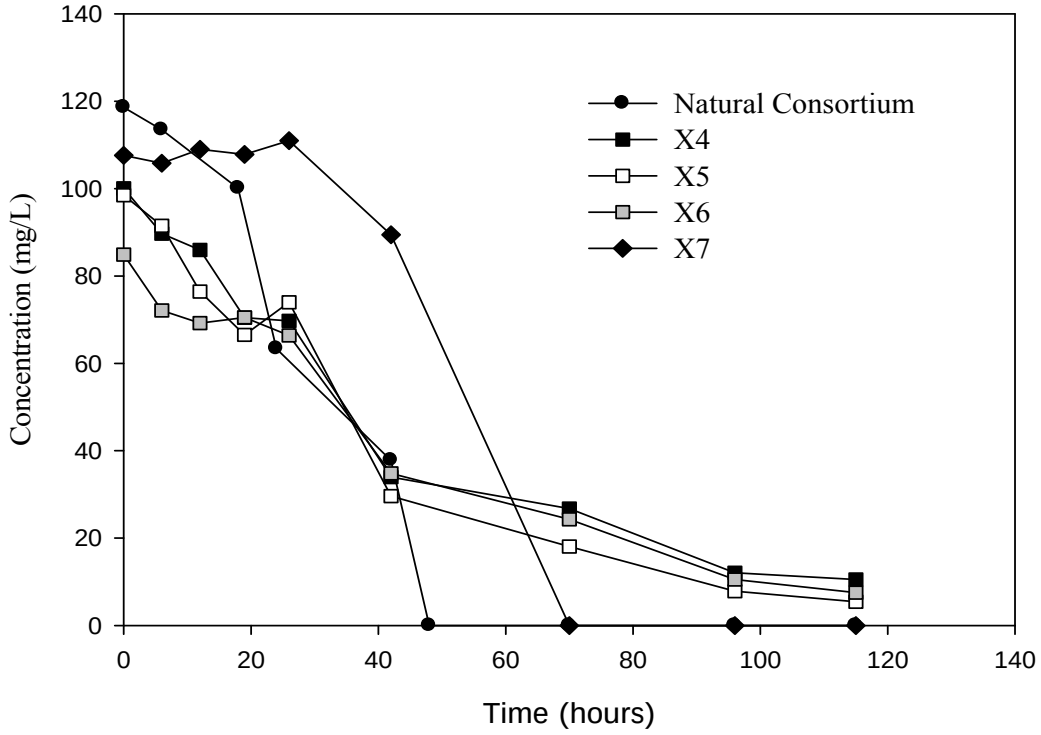


Figure 4-8: Cr(VI) reduction in growth conditions by aerobe isolates X4, X5, X6 and X7

Table 4-7: Comparison performance in different conditions

Culture Type	Time, h	[Cr(VI)]	%	References
Natural Brit's consortium	22.5	100[a]	100	This study
Natural Brit's consortium[b]	24.0	100[a]	97.9	"
Natural Brit's consortium[c]	24.0	99	67.1	"
X1	22.5	100[a]	56.8	"
X2	22.5	100[a]	61.9	"
X3	22.5	100[a]	59.7	"
X4	22.5	100[a]	64.4	"
X5	22.5	100[a]	69.98	"
X6	22.5	100[a]	67.7	"
X7	22.5	100[a]	38.1	"
X1 + X2 + X3 + X4[d]	22.5	100[a]	91.8	"
X5 + X6[d]	22.5	100[a]	84.6	"
Reconstituted[*]	22.5	100[a]	94.3	"
P. fluorescens LB300[3]	24	90	22.2	Chirwa and Wang, 1997
Bacillus sp.	24	94	7.5	Chirwa and Wang, 1997
E. coli ATCC 33456[c]	24	120	7.5	Wang and Shen, 1997
Bacillus sp.	66	40	100	Liu *et al.*, 2006

[*]: Reconstituted natural consortium: X1 + X2 + X3 + X4 + X5 + X6 + X7
[a]: 100 mg/L as a target concentration
[b]: in grown conditions
[c]: under anaerobic conditions
[d]: Recombination of isolates
[Cr(VI)] : Initial concentration(mg/L)
%: percentage reduced

4.7 Summary

The highest Cr(VI) reduction performance was observed in the cultures from the dried sludge. This consortium can grow in high Cr(VI) concentration with an high CRB diversity within it. Long period pre-exposure to Cr(VI) and the availability of nutrient. Sufficient nutrient to counter the toxicity effects may have resulted in the enrichment for Cr(VI) reducing cultures at the Brits Wastewater Treatment Plant.

Characterisation using 16S rRNA revealed a wide diversity of CRBs isolated under anaerobic conditions than under aerobic conditions. This was attributed to prevailing low dissolved oxygen conditions in the aeration tanks at Brits. One isolate under anaerobic conditions, X17, is associated with the same reference group (*Bacillus Cereus, Bacillus thuringiensis and Bacillus mycoides*) like all *Bacilli* detected in the aerobically grown cultures.

The CRBs isolated in this study reduced high Cr(VI) concentrations and at a shorter incubation time than all previously isolated bacteria except for the *Bacillus megaterium* and the *Bacillus circulans* (Srinath *et al.*, 2002). This performance is explained partly by the predominance of gram-positive bacteria in the cultures as explained by Ross *et al.* (1981) and Muhammad and Shahinda (2005); and by the synergism across species in the consortium as explained by high performance of reconstituted consortium cultures.

CHAPTER 5: KINETIC STUDIES AND MODELLING

5.1 Biochemical Pathway of Cr(VI) Reduction in Cells

Cr(VI) reduction in living cells is linked to the cellular metabolic process as illustrated in earlier studies by Wang *et al.* (1989), Ohtake *et al.* (1990), Shen and Wang (1993) and others. Shen and Wang (1993) showed that Cr(VI) was reduced using NADH as a solo electron donor. The reaction was assumed to be catalysed by the membrane bound NADH-dehydrogenase. Other researchers demonstrated the role of other transmembrane electron carriers such as cytochrome c_3 in the Cr(VI) reduction process (Lovley and Phillips, 1994). In this study, an attempt was made to validate the membrane Cr(VI) reduction pathway theory through experimentation with separated fractions of disrupted cells. The objective is to determine whether Cr(VI) reduction is carried out in the cytosol or outside.

In the pathway validation experiments, disrupted cells were centrifuged at 12000 rpm (11,300 × g). The cytosolic component containing soluble reductase remained in the supernatant and the membrane fraction formed a pellet at the bottom of the centrifuge tube. The pellet was then re-suspended and Cr(VI) reduction was tested for each fraction in the batch experiments as described earlier (Chapter 4). Results showed higher Cr(VI) reduction rate in all supernatant experiments than in the membrane fractions. Examples of the results are shown for the *Bacillus sp.* pure culture types X4 and X6 in Figures 5-1 and 5-2. The results from the rest of the experiments are summarised in Table 5-1.

The highest Cr(VI) reduction rate was observed in the supernatant of the isolates X4 (53.4 %) and X6 (50.6 %) after incubation for approximately 22.5 hours. In both cases, the difference in reduction rate was undistinguishable at the beginning of incubation until after 5 hours when the reduction in the supernatant batches proceeded faster than in the membrane fraction batches. Only the disrupted cells of the pure culture X3 showed no significant difference in reduction rate between the two fractions (20.2 % and 19.4 %, respectively) (Table 5-1).

Another conclusive observation in the experiment was that ruptured cells (non-centrifuged cell fragments) achieved higher Cr(VI) reduction rate than whole cells. This suggests the role of mass transport resistance either on the enzymes being produced in the cells or Cr(VI) entering the cells. However, the results on the actual mass balance on total versus added Cr(VI) reduction at the end of the experiments suggest that that all Cr(VI) reduced was found in the media, which serves as evidence that the Cr(VI) reductase may be secreted into the media by the bacteria (Table 5-2). This is consistent with earlier observations by Shen and Wang (1993) and others in which 97% of the Cr(VI) reduced in batch by the gram negative species *Escherichia coli* ATCC 33456 was recovered as Cr(III) in the medium.

Other researchers arrived at Cr(VI) reduction scenarios most of which support the above observations:

- Cheung and Gu (2007) observed that Cr(III) is generally excluded from entering the cells;
- Chen and Hao (1998) observed that Cr(III) is precipitated as $Cr(OH)_3$ and forms a barrier in a physiologic response to protect cells from Cr(VI) toxicity conferring a low cell membrane permeability to Cr(VI);
- Other researchers observed a high Cr(VI) reduction rate was observed in cell wall membranes of gram negative bacteria (20 to 35 % reduction) and significant Cr(VI) reduction by in extra-cellular processes.

The acceptable model so far is that electron donors from the cytosol pass electrons to transmembrane electron carriers and proton pumps which in turn pass electrons onto Cr(VI), directly, or through secreted membrane associated soluble reductase (Bopp and Ehrich, 1988; Ishibashi *et al.*, 1990).

Table 5-1: Percentage of Cr(VI) reduced in Cell extract and Cell membrane after 22.5 hours incubation time.

Sample Label	% Cr(VI) Reduction in Living Cells	% Cr(VI) Reduction in Supernatant	% Cr(VI) Reduction in Cells membrane	% Cr(VI) Reduction in Fractured Cells
X1	56.8	39.6	26.1	65.9
X2	61.9	30.6	23.5	54.1
X3	59.7	20.2	19.4	39.7
X4	64.4	53.4	32.0	85.4
X5	70.0	37.8	35.8	73.6
X6	67.7	50.6	33.5	84.1

Table 5-2: Mass balance analysis for added Cr(VI) and measured versus measured total Cr under aerobic conditions.

Target Conc. mg/L	Incubation Time, h	Cr(VI) Conc. mg/L	Total [Cr] mg/L	Recover Error %
50	4.5	43	52.0	+4.00
100	22.5	106	108.4	+8.37
150	40.5	151	154.5	+3.00
200	64.5	165	196.0	-1.98
300	211	288	297.7	-0.76
400	211	369	402.3	+0.58

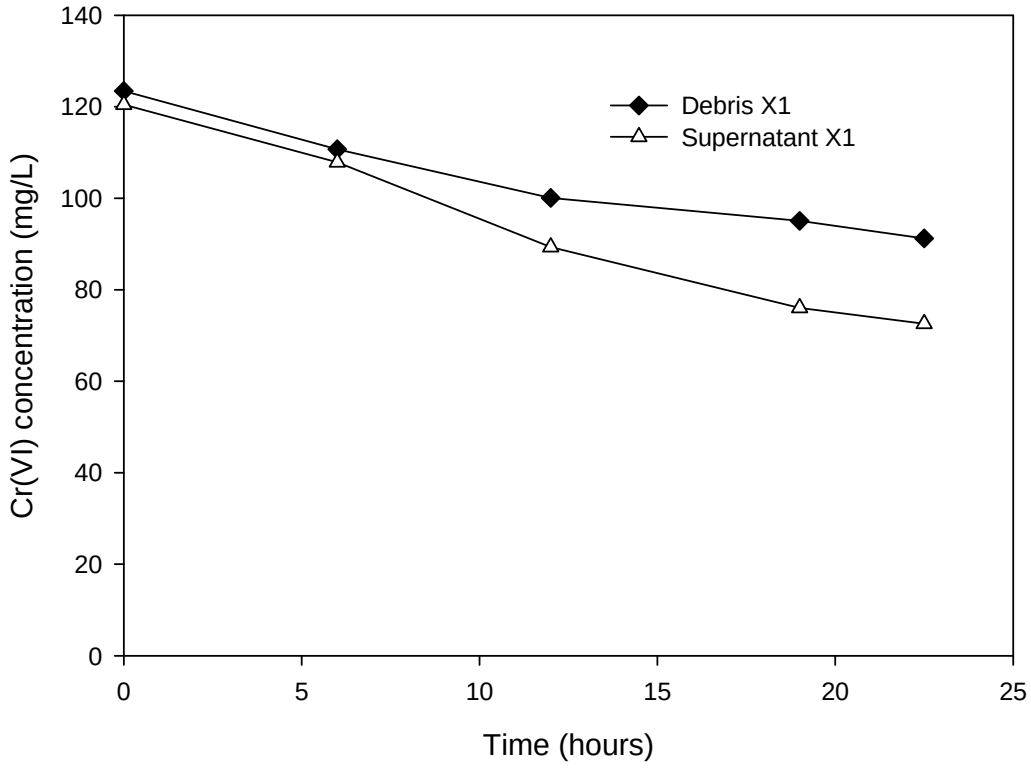


Figure 5-1: Cr(VI) reduction in isolates X1's cell extracts and cell walls

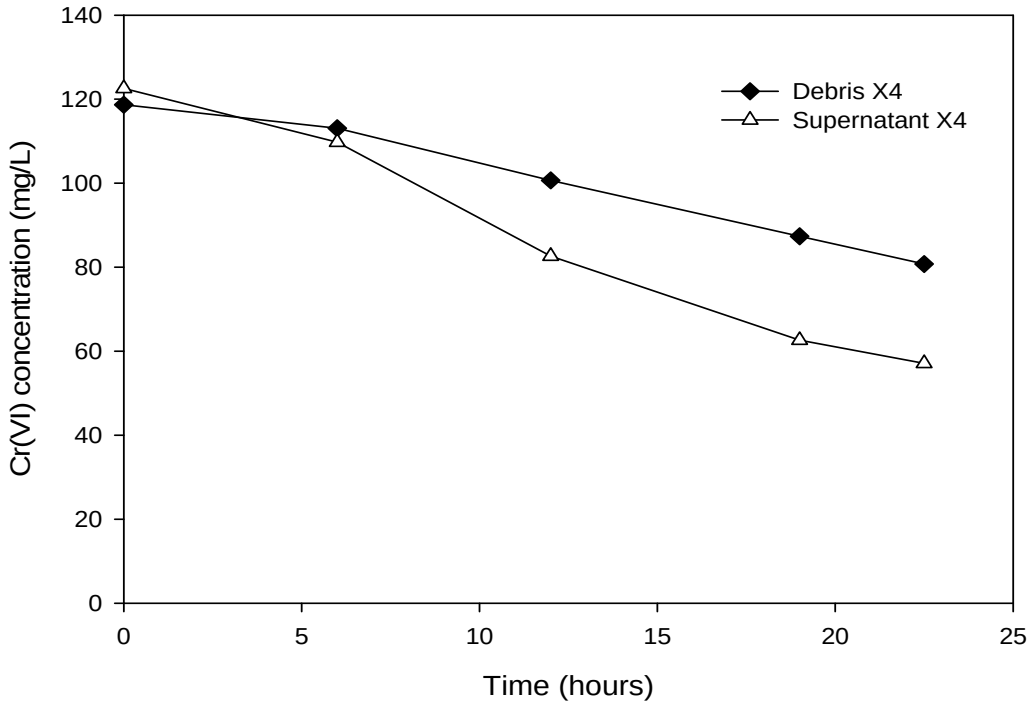


Figure 5-2: Cr(VI) reduction in isolates X4's cell extracts and cell walls

5.2 Kinetic Modelling Theory

The results on the biochemical process of Cr(VI) reduction in microbial cultures indicate that Cr(VI) reduction requires the physical presence of a soluble reductase or membrane associated reductase to mediate the transfer of electrons from membrane electron carriers to Cr(VI). For gram-negative bacteria, Shen and Wang (1993) suggests that Cr(VI) reduction may be catalyzed by a soluble reductase associated with the respiratory chain pathway. Under anaerobic conditions, a dissimilatory respiratory process was demonstrated in a number of cases involving the electron shuttles in the inner cell membrane and Cr(VI) or other toxic metal as the terminal electron sink. In one example, Lovley and Philips (1994) reported Cr(VI) reduction via the membrane electron transport system involving cytochrome-c_3 under anaerobic conditions (Lovley and Phillips, 1994). Energy production from dissimilatory Cr(VI) reduction with Cr(VI) as the sole electron sink has thus not been ruled out.

To simplify the model we suggest that a single representative enzyme reduces Cr(VI) as a result of the overall Cr(VI) biochemical reduction process in the consortium. The simplified Cr(VI) reduction equation can thus be written as follows:

$$\mathrm{Cr(VI)} + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E^{*}\cdot\mathrm{Cr(VI)} \xrightarrow{k_3} E + \mathrm{Cr(III)} \qquad (5\text{-}1)$$

where: E = Cr(VI) reductase as a bio-catalyst

$E^{*}\cdot$Cr(VI) = The transitional enzyme-Cr(VI) complex

k_1, k_2, and k_3 = reaction rate constants in the directions indicated by the arrows

If the Cr(VI) concentration is represented by C and the enzyme-Cr(VI) complex by E^{*}, and assuming that E^{*} forms and disappears spontaneously, the rate of Cr(VI) reduction will be equal to the Cr(III) formation. The overall rate of the reaction can then be represented by:

$$r = \frac{-d(C)}{dt} = \frac{d(Cr(III))}{dt} = k_3 \cdot E^{*} \qquad (5\text{-}2)$$

The rate formation of E^* can be described by:

$$\frac{d(E^*)}{dt} = k_1(E - E^*)(C) - k_2(E^*) - k_3(E^*) \quad (5\text{-}3)$$

In the above equation, steady state conditions can be assumed to prevail as long as E^* is formed and destroyed spontaneously such that $d(E^*)/dt \approx 0$. The mass balance represented by Equation 5-3 can thus be written as:

$$0 = k_1(E - E^*)(C) - k_2(E^*) - k_3(E^*) \quad (5\text{-}4)$$

After rearranging this Equation 5-4, E^* can be expressed as:

$$E^* = \frac{C \cdot E}{C + \frac{k_2 + k_3}{k_1}} \quad (5\text{-}5)$$

Thus, the Cr(VI) reduction rate in Equation 5-2 becomes:

$$r = \frac{-d(C)}{dt} = \frac{k_3 \cdot C \cdot E}{C + \frac{k_2 + k_3}{k_1}} \quad (5\text{-}6)$$

In this equation, k_1, k_2 and k_3 are constants. The groups of constants in Equation 5-6 can be replaced by meaningful symbols from enzyme kinetics as follows: $(k_2 + k_3)/k_1$ can be replaced by the half velocity concentration K_c (ML^{-3}), and k_3 can be replaced by the maximum specific Cr(VI) reduction rate coefficient k_{mc} (T^{-1}) such that:

$$r = \frac{-d(C)}{dt} = \frac{k_{mc} \cdot C \cdot E}{C + K_c} \quad (5\text{-}7)$$

For any amount of cells X, the amount of enzyme produced will be proportional to the cell concentration such that the enzyme E can be replaced by the total cell biomass term X if cells are harvested during the log growth phase. This gives a Monod type equation:

$$r = \frac{-d(C)}{dt} = \frac{k_{mc} \cdot C \cdot X}{C + K_c} \tag{5-8}$$

where C = Cr(VI) concentration at time t (ML^{-3})
k_{mc} = maximum specific Cr(VI) reduction rate coefficient (T^{-1})
K_c = half velocity constant (ML^{-3})
X = concentration of viable cells at time t (ML^{-3}), and
t = time (T)

A similar expression was derived previously by other researchers for Cr(VI) reduction in batch systems (Shen and Wang 1994; Mazierski, 1995; Schmieman *et al.*, 1998; Guha *et al.*, 2001; Li *et al.*, 2006; Shashidhar, 2007).

In this model only k_{mc} and K_c are the unknown parameters, and we can assume a stationary phase with respect to X as long as the experiment was performed under very high biomass concentration. Cells were concentrated in the ration 1/5, and screening performed in growth condition (Section 4.2, and Table 4-1) lead to the assumption that X is constant ($X = X^0$). To determine kinetic parameters, the analytical solution of Equation 5-8 was used. This was express as function of time as shown below.

$$t = \frac{K_c}{X^o \cdot k_{mc}} \ln\left(\frac{C_0}{C}\right) + \frac{1}{X^o \cdot k_{mc}}(C_0 - C) \tag{5-9}$$

where X^0 = initial biomass concentration (ML^{-3}) and C_0 = initial Cr(VI) concentration (ML^{-3}).

The parameters were estimated by optimisation of Equation 5-9 against batch experimental data using the Marquardt-Levenberg least sum of squares differences algorithm in SigmaPlot 9.0 (Systat Software, San Jose, CA). The model was validated by statistical analysis of the fit using the Pearson's Regression Coefficient (R), Normality, and Power Tests.

5.3 Parameter Evaluation Under Aerobic Conditions

5.3.1 Aerobic Experimental Data

Dried sludge cultures completely reduced Cr(VI) in a range of target initial Cr(VI) concentrations from 50 mg/L to 200 mg/L. In less than 6 hours, 50 mg/L Cr(VI) was completely reduced; 100 mg/L Cr(VI) was reduced in less than 24 hours; 150 mg/L was reduced in less than 45 hours and finally 200 mg/L Cr(VI) was completely reduced in less than 65 hours. Cr(VI) reduction activity was much slower in batches containing 300 and 400 mg/L Cr(VI) and no bacterial activity in batches containing 600 mg/L Cr(VI). Up to 94.0 % of Cr(VI) was reduced in solution containing 300 mg/L Cr(VI) after incubation for 115 hours. When the experimental time was extended to 211 hours, 98.3 % was reduced. In solution containing 400 mg/L, 51.7 % was reduced after 211 hours incubation period. Only 13.3 % was reduced from solution containing 600 mg/L Cr(VI). It can be suggested that, the high Cr(VI) toxicity at such concentration inhibits bacterial activity. In control batches (cell-free batches) Cr(VI) reduction was insignificant.

5.3.2 Determination of Kinetics Parameters Under Aerobic Conditions

Results obtained using SigmaPlot, show high values of maximum specific Cr(VI) reduction rate coefficient k_{mc} and half velocity constant K_c (Table 5-3). These results are statistical correct since the regression coefficients R are above 97 % and all R^2 are above 94 %. The Constant Variance Test and the Normality Test passed in all regression studied (i.e.: 50 to 200 mg/L).

However these parameters could not generate a unique representative model for all studied initial Cr(VI) concentrations. The results show that K_c values obtained from the non-linear regression were not constant and were shown to be much higher than C_0 values ($K_c >> C_o$) (Table 5-3).

According to Brey (1958) the reaction rate approaches first order (Equation 5-10) when the half velocity coefficient K_c is much greater than C_0. In fact, when K_c and k_{mc} values obtained are inserted and computed in the integrated Monod type equation

(Table 5-4), all numerical coefficients in the second part of equation are insignificant compared to numerical coefficients in the logarithmic part. They are 10^5 time less then logarithmic coefficients. If insignificant values are neglected, reduction rates are shown to correspond to a first order reduction equation (Equation 5-10).

$$C = C_0 e^{-k_{mi} \cdot t} \tag{5-10}$$

where k_{mi} is a representative first order Cr(VI) reduction rate coefficient.

It is shown that the parameter k_{mi} obtained by using the first order equation (Table 5-5) can not generate a unique representative model for all initial Cr(VI) concentrations . Even the average value could not provide an adequate model as presented previously by some studies at lower initial Cr(VI) concentrations (Hossain, 2006; Viamajala *et* al, 2002a; Viamajala *et al.,* 2002b; Viamajala *et al.*, 2003).

Similar results were obtained by Mazierski (1995), who observed a decrease in the maximum growth rate (μ_{max}) and an increase in the Monod constant (K_s). Mazierski observed a proportional increase in the value of μ_{max} for initial Cr(VI) concentrations in the range of 0.1 to 11 mg/L. Later, Dermou and Vayenas (2007) developed a semi-empirical equation using the dual-enzyme kinetic approach where all parameters values increased with increasing C_o. To model the Cr(VI) reduction rate, Dermou and Vayenas replaced these parameters (in the dual-enzyme model) by three different equations: a sigmoid, a decline exponential and a linear equation.

In this study, the k_{mi} values correlated exponentially with Cr(VI) initial concentration as shown in Figure 5-3 and k_{mi} values were shown to be dependent on the initial Cr(VI) concentration C_0. The relationship in the exponential graph (Figure 5-3) was used to derive the kinetic relationship between k_{mi} and C_0:

$$k_{mi} = 2.6168 \cdot \mathrm{e}^{-0.0211 \cdot C_0} \tag{5-11}$$

A generic equation was suggested for k_{mi} as shown in Equation 5-12 with two parameters p and q. The dimensions of p and q in the equation are respectively [1/Time] and [Volume/Mass].

$$k_{mi} = p \cdot e^{-q \cdot C_0} \qquad (5\text{-}12)$$

Table 5-3: Parameters obtained for Monod type equation under aerobic conditions.

Targeted	Observed C_0	Monod Parameters		Basic Statistic Test		
		K_c	k_{mc}	R	R^2	SEE**
50	43.4	71539.890	18.409	0.998	0.997	0.075
100	105.5	119305.410	6.610	0.983	0.967	1.288
150	152.9	721928.271	14.443	0.976	0.952	1.548
200	165.3	1002731.271	10.446	0.971	0.943	3.571

** *SEE*: Standard Error of Estimate

Table 5-4: Applied K_c and k_{mc} Parameter obtained, in the integrated Monod type equation under aerobic conditions.

Co Value mg/L	Kinetic equation showing parameters k_{mc} and K_c
43.4	$t = \{ 0.96887 * \ln(\frac{[Cr(VI)]_0}{[Cr(VI)]}) \} + \{ 1.35431E-05 * ([Cr(VI)]_0 - [Cr(VI)]) \}$
105.5	$t = \{ 4.499933 * \ln(\frac{[Cr(VI)]_0}{[Cr(VI)]}) \} + \{ 3.77178E-05 * ([Cr(VI)]_0 - [Cr(VI)]) \}$
152.9	$t = \{ 12.46189 * \ln(\frac{[Cr(VI)]_0}{[Cr(VI)]}) \} + \{ 1.7262E-05 * ([Cr(VI)]_0 - [Cr(VI)]) \}$
165.3	$t = \{ 23.93216 * \ln(\frac{[Cr(VI)]_0}{[Cr(VI)]}) \} + \{ 2.3867E-05 * ([Cr(VI)]_0 - [Cr(VI)]) \}$

When k_{mi} in Equation 5-10 is replaced by Equation 5-12, a unique modified first order kinetic model can be applied to the whole range of initial Cr(VI) concentrations. The final expression is shown in Equation 5-13. The parameters obtained are unique to the Brits dried sludge consortium. A validation of this mathematical model was performed and the model matched closely all observed experimental data (Figure 5-3 to Figure 5-5).

The exponential term in Equation 5-13 plays a role as a delay element when the initial concentration is increasing. Indeed, if the initial Cr(VI) concentration is increased, its toxicity effect on microorganisms increases which results in the inhibition of metabolic activities within the microorganisms. Since Cr(VI) reduction is metabolically linked, the increased Cr(VI) concentration in turn inhibits Cr(VI) reduction by the microorganisms.

$$C = C_0 \cdot e^{-p \cdot e^{(-q \cdot C_0)} \cdot t} \tag{5-13}$$

Table 5-5: Parameters obtained for the first order equation under aerobic conditions.

C_0	k_{mi}	R	Rsqr	Adj Rsqr	Normality Test	Constant Variance Test
43.4090	1.0426	0.9997	0.9995	0.9994	Passed (P = 0.8622)	Passed (P = 0.4907)
105.5275	0.3380	0.9659	0.9329	0.9281	Passed (P = 0.5809)	Passed (P = 0.8136)
139.2966	0.0917	0.9714	0.9435	0.9395	Passed (P = 0.5865)	Passed (P = 0.2616)
165.0000	0.0472	0.9686	0.9382	0.9346	Passed (P = 0.0802)	Passed (P = 0.9282)

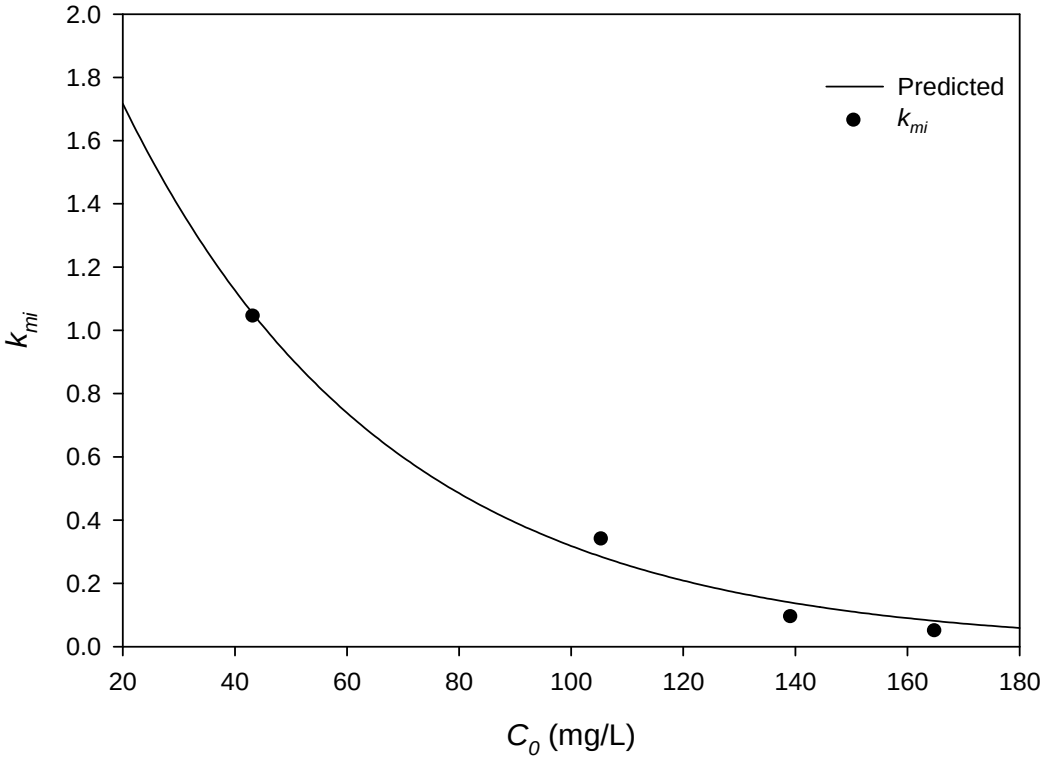


Figure 5-3: Correlation between obtained k_{mi} values and Cr(VI) initial concentration under aerobic conditions.

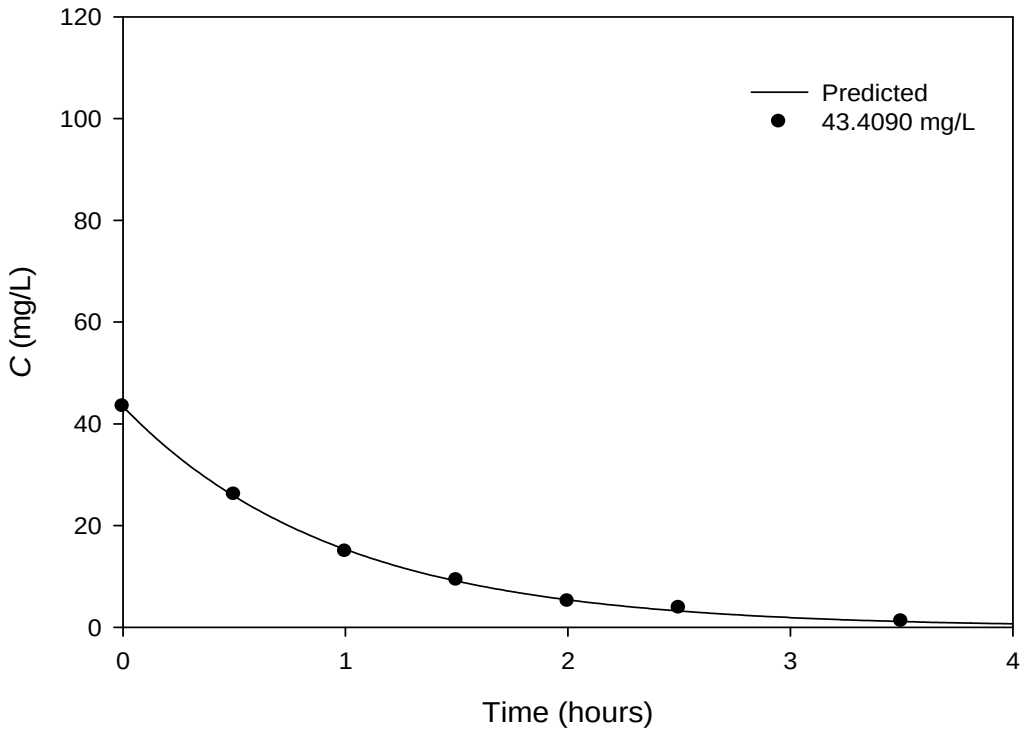


Figure 5-4: Model validation for C_0 = 43.41 mg/L under aerobic conditions.

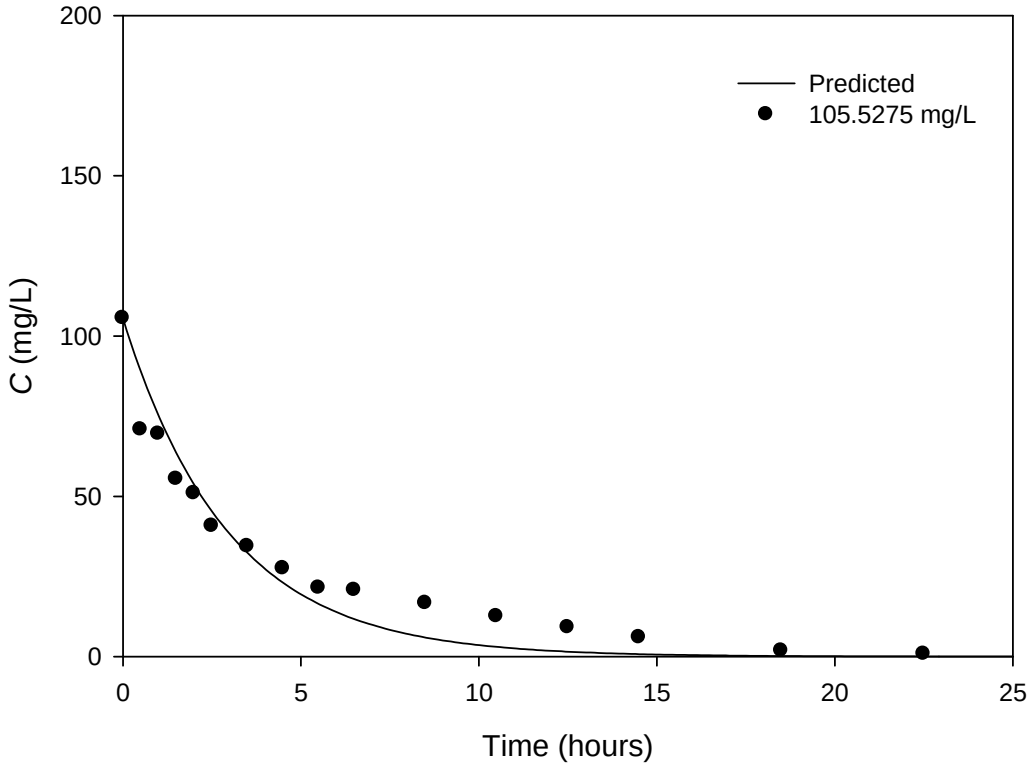


Figure 5-5: Model validation for C_0 = 105.53 mg/L under aerobic conditions.

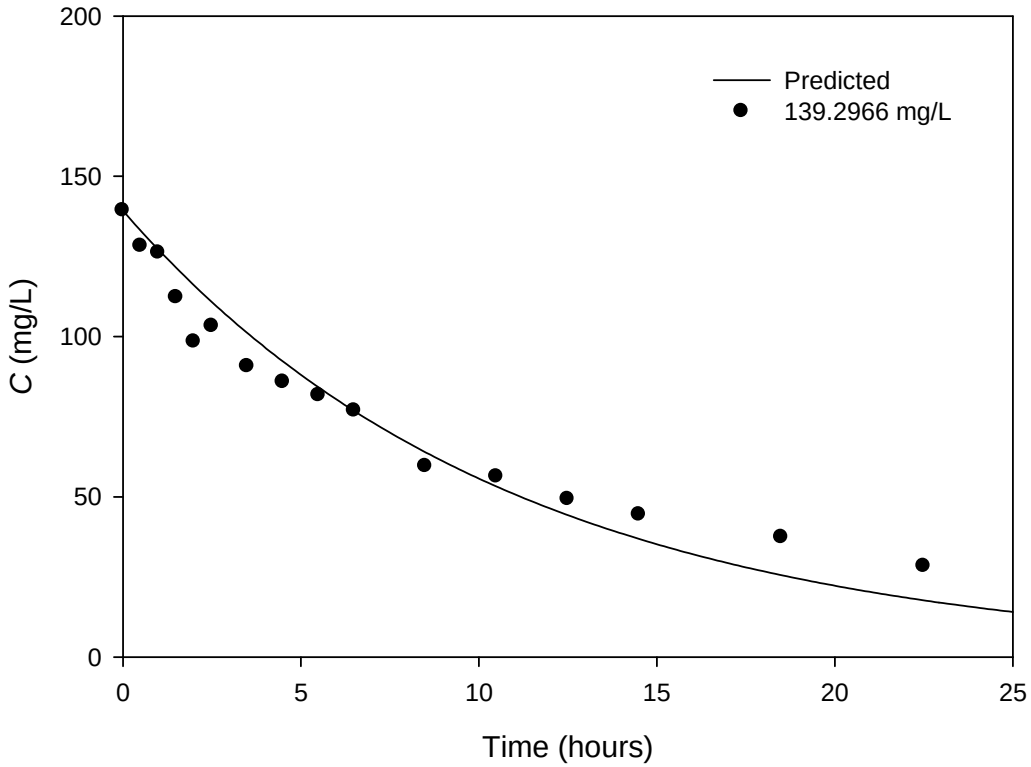


Figure 5-6: Model validation for C_0 = 152.9 mg/L under aerobic conditions.

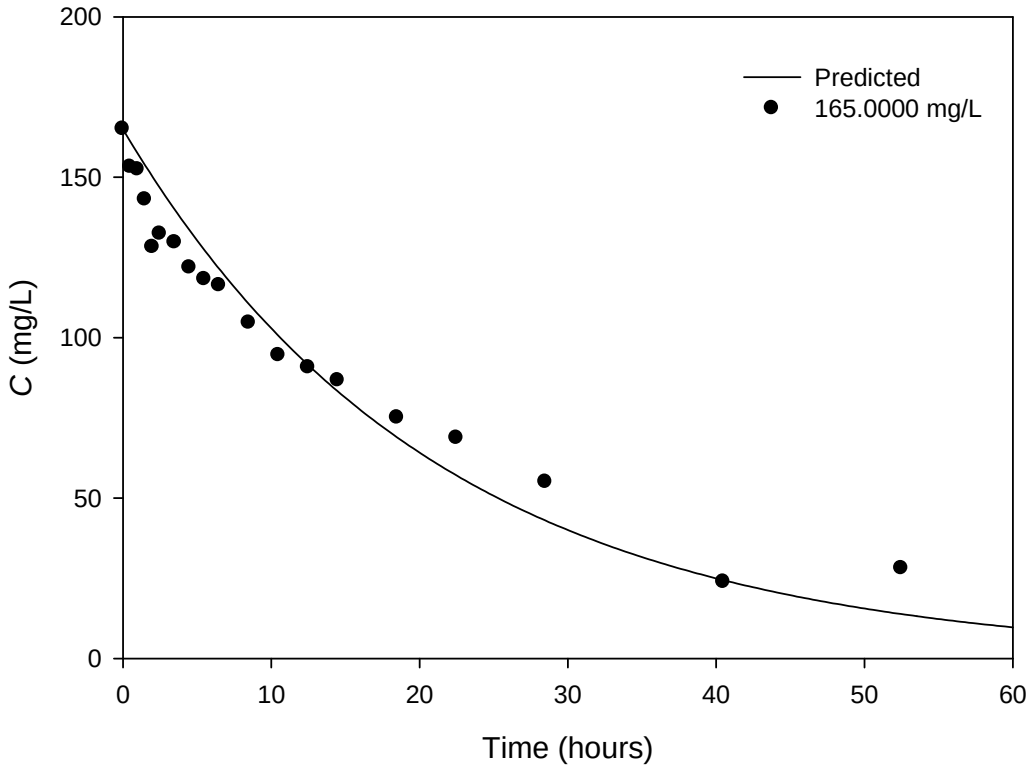


Figure 5-7: Model validation for C_0 = 165.31 mg/L under aerobic conditions.

5.4 Parameter Evaluation Under Anaerobic Conditions

5.4.1 Anaerobic Experimental Data

Cr(VI) reduction under anaerobic conditions was investigated due to its importance for certain applications such as bioremediation of sediment zones and groundwater environments; which are anoxic zones. The experiment under anaerobic conditions was conducted over a lower range of target initial concentrations (50-300 mg Cr(VI)/L) since slower growth was observed in the anaerobic cultures (Chirwa and Wang, 2000; Komori *et al.*, 1990; Myers *et al.*, 200). The reduction rate of Cr(VI) under anaerobic conditions was generally slower. Complete Cr(VI) reduction occurred in cultures with a lower initial Cr(VI) concentration of 150 mg/L after a longer incubation period (155 hours) than in aerobic cultures (less than 48 hours). Gas was also produced; probably carbon dioxide and methane. The amount of gas produced was lower at higher initial Cr(VI) concentration showing that gas production was an essential component of the metabolic process and that it was inhibited by Cr(VI).

The Cr(VI) reduction was only incomplete at a high initial Cr(VI) concentration of 200 mg/L – 50% removal under anaerobic conditions. Much lower Cr(VI) reduction occurred at the higher initial Cr(VI) concentrations of 300 and 400 mg/L in the anaerobic cultures.

5.4.2 Determination of Kinetic Parameters Under Anaerobic Conditions

Results obtained from anaerobic culture experiments were similar to those obtained under aerobic conditions. High values of the half velocity coefficient K_c were obtained. The value from fitting the Monod type equation to individual experiments yielded a good fit to experimental data with R values above 96% and R^2 values above 92%. As in the aerobic batch experiments, the Constant Variance Test and the Normality Test passed in all regressions studied except for data obtained at with the initial Cr(VI) concentration C_0 = 173 mg/L in which the Constant Variance failed.

The half velocity coefficients obtained in the nonlinear regression were also extremely high ($K_c >> C_0$). It is therefore assumed that the reduction rate in anaerobic experiment will also follow first-order kinetics. The first order rate constant under anaerobic conditions varied exponentially with C_0 (Table5-6). An exponential regression fit for k_{mi} (Figure 5-8) yielded an exponential relationship with C_o as observed earlier in aerobic cultures. A validation of this mathematical model was performed and the model using the optimized p and q parameters for the anaerobic batches fitted all observed experimental data (Figure 5-9 to Figure 5-12). p and q values for anaerobic experiments were 1.4723 h^{-1} and 0.0334 L/mg, respectively (Table 5-7).

It was noticed that p value for aerobic experiments is higher than the value for anaerobic experiments (± double). However q value for aerobic experiments is lower than the anaerobic experiments value (Table 5-7).

Table 5-6: Parameter obtained for first order equation under anaerobic conditions.

C_0	k_{mi}	R	Rsqr	Adj Rsqr	Normality Test	Constant Variance Test
52	0.2634	0.9913	0.9827	0.9770	Passed (P = 0.6751)	Passed (P = 0.0500)
101	0.0482	0.9944	0.9889	0.9877	Passed (P = 0.7252)	Passed (P = 0.7755)
144	0.0180	0.9909	0.9819	0.9793	Passed (P = 0.7692)	Passed (P = 0.1534)
173	0.0050	0.9640	0.9294	0.9176	Passed (P = 0.9229)	Failed (P = 0.0149)

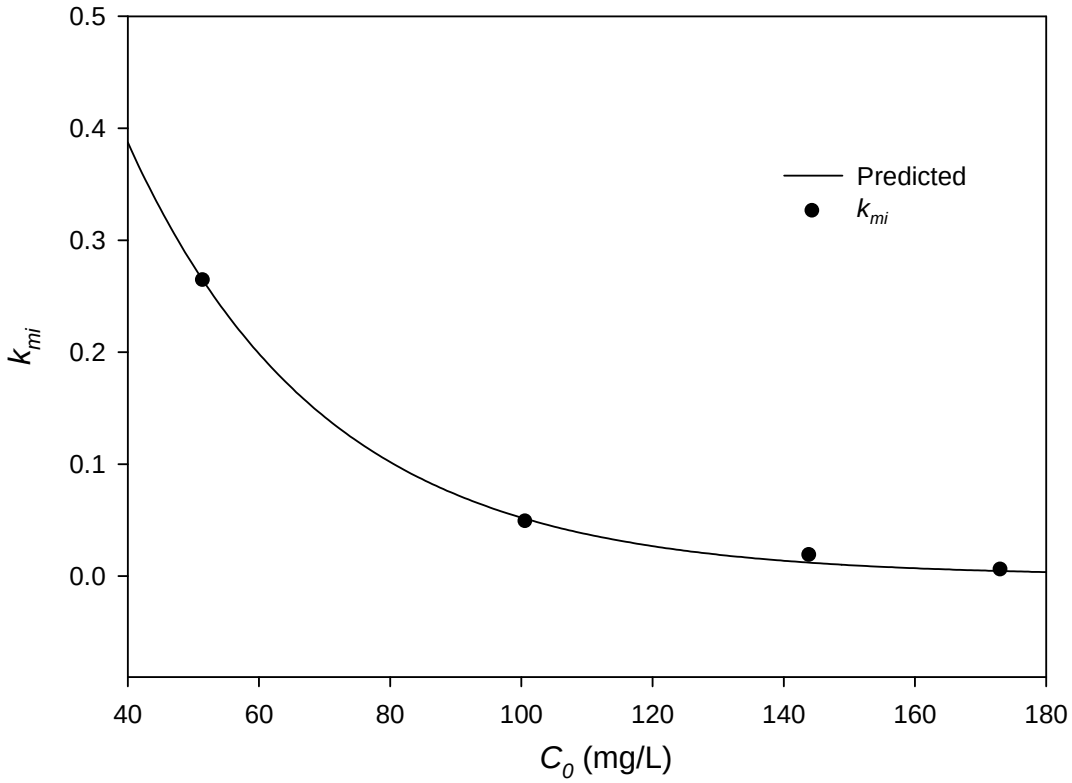


Figure 5-8: Correlation between obtained k_{mi} values and Cr(VI) initial concentration.

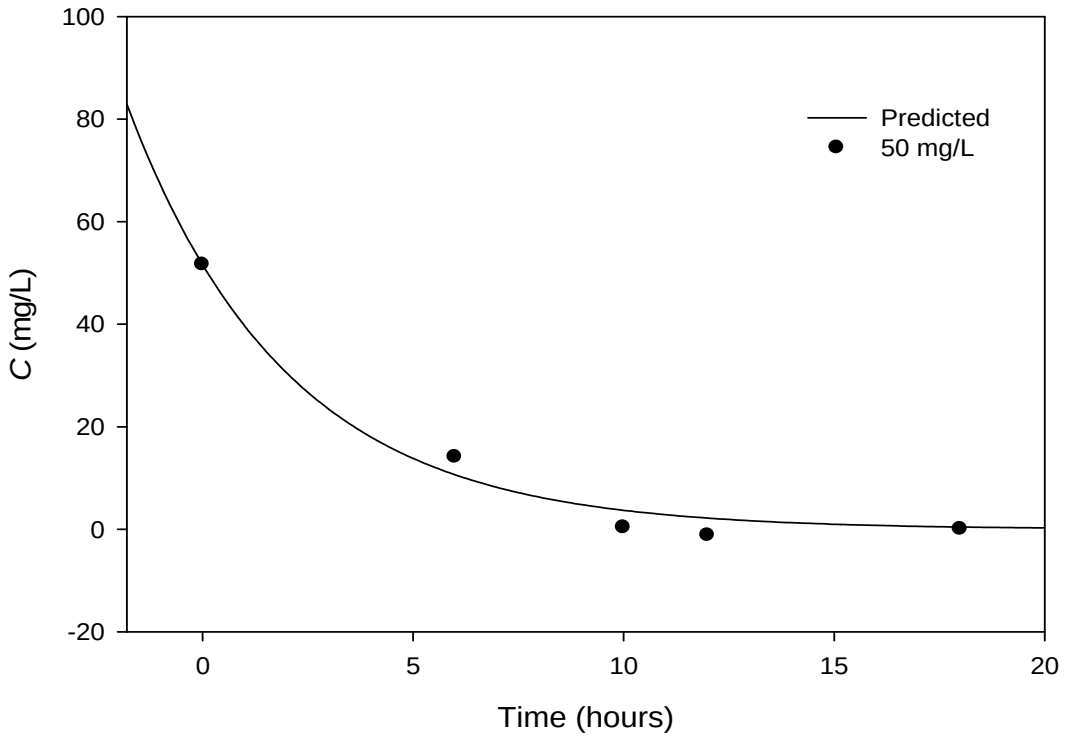


Figure 5-9: Model validation for C_0 = 50 mg/Lunder anaerobic conditions.

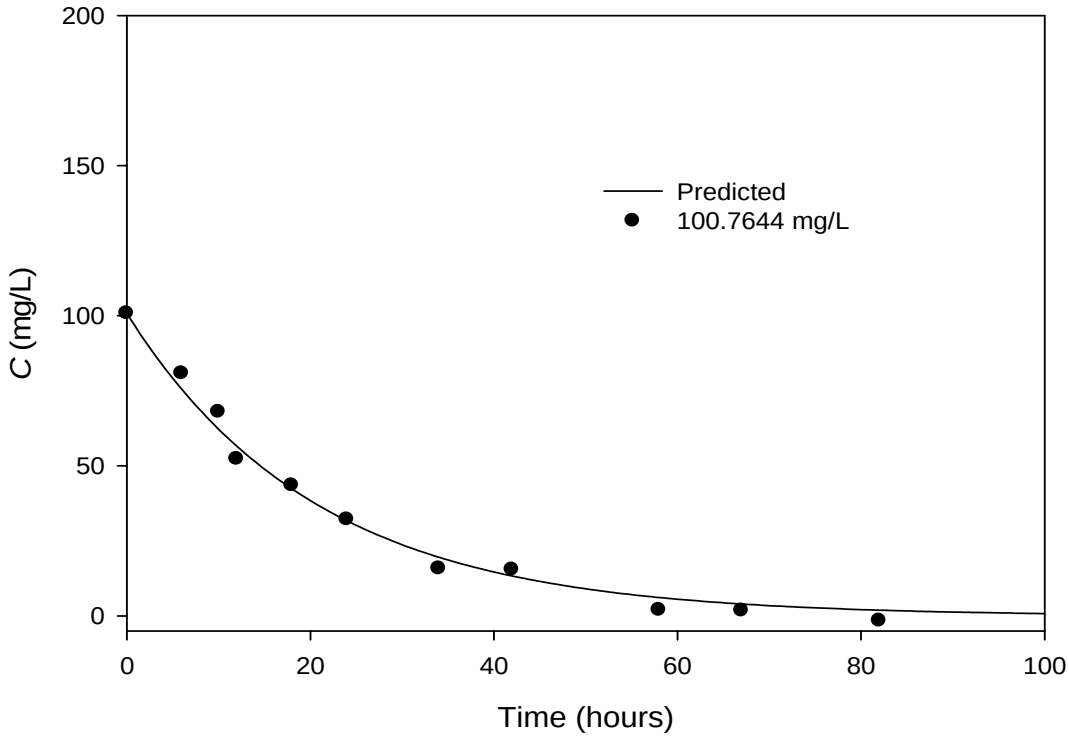


Figure 5-10: Model validation for C_0 = 100 mg/L under anaerobic conditions.

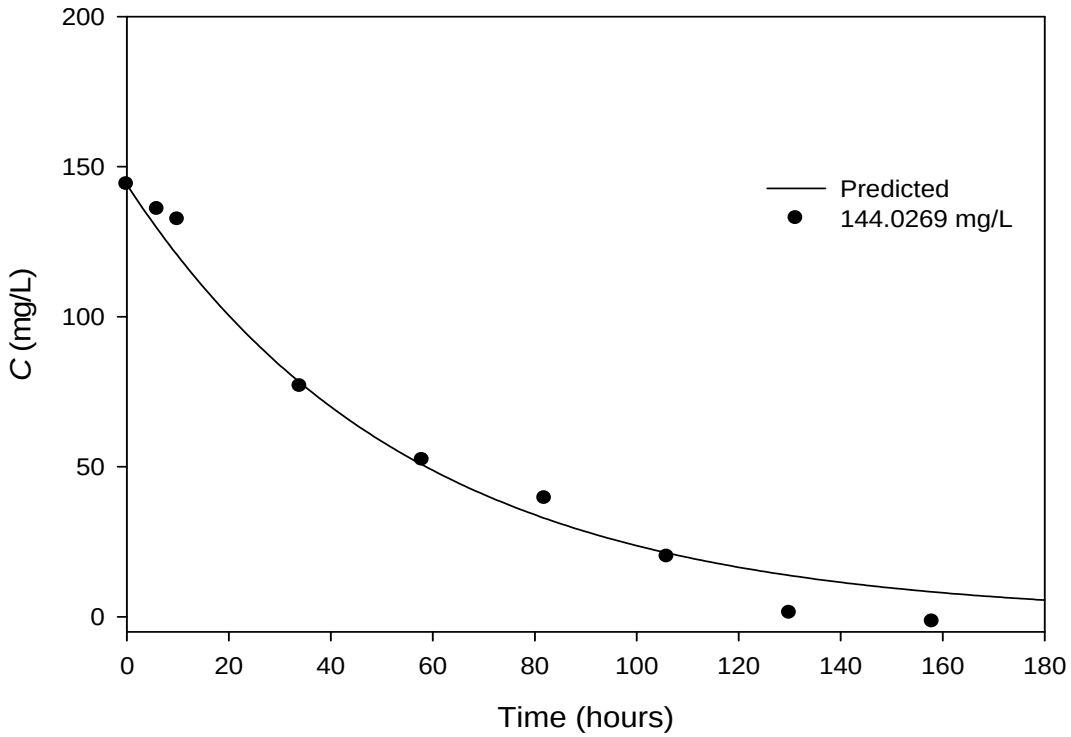


Figure 5-11: Model validation for C_0 = 144 mg/L under anaerobic conditions.

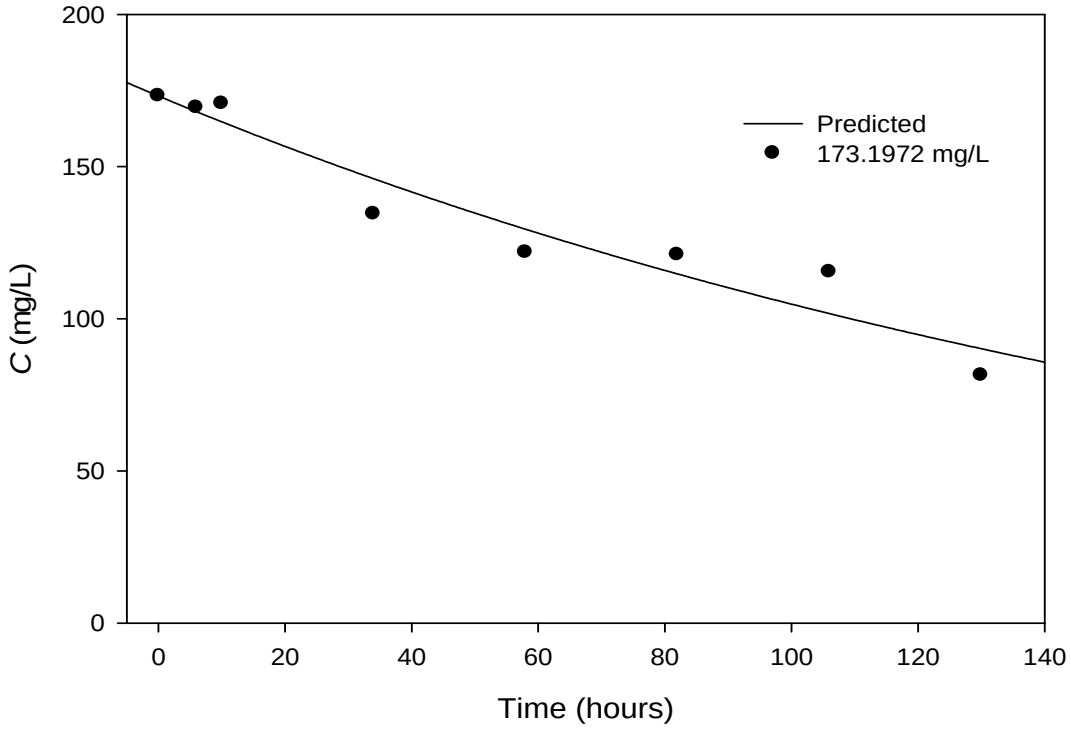


Figure 5-12: Model validation for C_0 = 173 mg/L under anaerobic conditions.

Table 5-7: Parameter obtained for the semi-empirical equation first order equation under aerobic and anaerobic conditions.

Experimental Conditions	***p*** **(T^{-1})**	***q*** **(L^3M^{-1})**	***R***	**Rsqr**	**Adj Rsqr**	**Standard Error of Estimate**	**Normality Test**
Aerobic	**2.6168**	**0.0211**	0.9949	0.9899	0.9849	0.0566	Passed (P = 0.7818)
Anaerobic	**1.4723**	**0.0334**	0.9995	0.9990	0.9985	0.0047	Passed (P = 0.8591)

5.5 Summary

In conclusion, it was confirm that the Cr(VI) reduction rate under aerobic and anaerobic conditions depended on the initial Cr(VI) concentration. Based on the Monod type equation, it was demonstrated that a modified first order equation with an empirical exponential solution provided the best fit to experimental data. The model is a semi-empirical equation that provides two parameters: p and q. The first is the rate constant and expressed per time (T^{-1}) and the second is C_0 dependent and expressed as volume/mass.

The second exponential expression plays a role as a delay element. When C_0 is increased, the metabolic activity in the bacteria is inhibited by the toxicity of Cr(VI) which slows down the Cr(VI) reduction. It is suggested therefore that the inhibition effect of Cr(VI) on cell metabolism increases exponentially with C_0.

The results differ from previous studies (Shen and Wang, 1994), where the studied range of initial Cr(VI) concentration was narrow (5.0-30.0 mg/L). In this study, Cr(VI) reduction was observed in wider range (50-300 mg/L), an order of magnitude higher than in the literature studies. We therefore suggest that previous studies were not able to pick the variability of Monod coefficients over a wide range of initial Cr(VI) concentrations since the impact of toxicity was insignificant at the narrow range.

Further studies are required to determine a mechanistic representation of the relationship between the parameters in the second exponential to the Cr(VI) reduction rate, i.e., the model must represent the actual cause of the dependence of k_{mi} on initial Cr(VI) concentration.

CHAPTER 6: SUMMARY AND CONCLUSION

The anthropogenic release of Cr(VI) is a concern in South Africa and around the world . there is an urgent need to develop biological treatment process for the Cr(VI) contaminated effluents from manufacturing and mining industries. The results from this study show the feasibility of such process using isolated high performing microorganism from Cr(VI) exposed environments.

Characterisation using 16S rRNA revealed a wide diversity of CRBs isolate under anaerobic conditions than under aerobic conditions. This can be explained by the sampling source of the culture were oxygen were not easily diffused in the dried bed sand. One isolate under anaerobic conditions, X17, is associated with the same reference *Bacillus* (*Bacillus Cereus, Bacillus thuringiensis and Bacillus mycoides*) like all the aerobe *Bacillus* isolates. This suggests that CRB found in the dried sludge are facultative anaerobic CRB. These CRBs reduce high Cr(VI) concentration and at a shorter incubation time than all previously isolated excepted for the *Bacillus megaterium* and the *Bacillus circulans* (Srinath *et al.*; 2002). This performance is explained in one hand by the fact that the consortium is gram-positive dominant; and in the other hand by the fact that the Cr(VI) reduction rate is the result of a synergetic metabolism of different CRB.

In this study, Cr(VI) reducing organisms were successfully isolated from local environments in the isolated cultures the highest Cr(VI) reduction performance was observed in cultures from the dried sludge. The culture performed best as a consortium with the different species operating cooperatively. The bacteria was acclimated to Cr(VI) toxicity through the long period of contact during the activated sludge treatment process at the source.

The main find and conclusion in this study can be enumerated as follow:

1. Cr(VI) reducing bacteria isolated in this study achieved 3 to 8 times higher Cr(VI) reduction rate than bacteria previously isolated and studied in North America and Europe.

2. High Cr(VI) reduction was achieved in aerobic cultures than in anaerobic cultures, may still higher than previous studies in anaerobic cultures.
3. Detailed taxonomic studies showed that the core Cr(VI) reducing *bacilli* group *B. thuringiensis*, *B. Cereus* and *B. mycoides* was preserved in both aerobic and anaerobic cultures, suggesting that the C(VI) reducing component of organisms is predominantly facultative. The difference in Cr(VI) reduction rates between the anaerobic and aerobic cultures may be predominantly due to differences in cell densities.
4. Cr(VI) reduction in the consortium culture followed quasi-first order kinetics with a Cr(VI) inhibitor term as a second exponential:

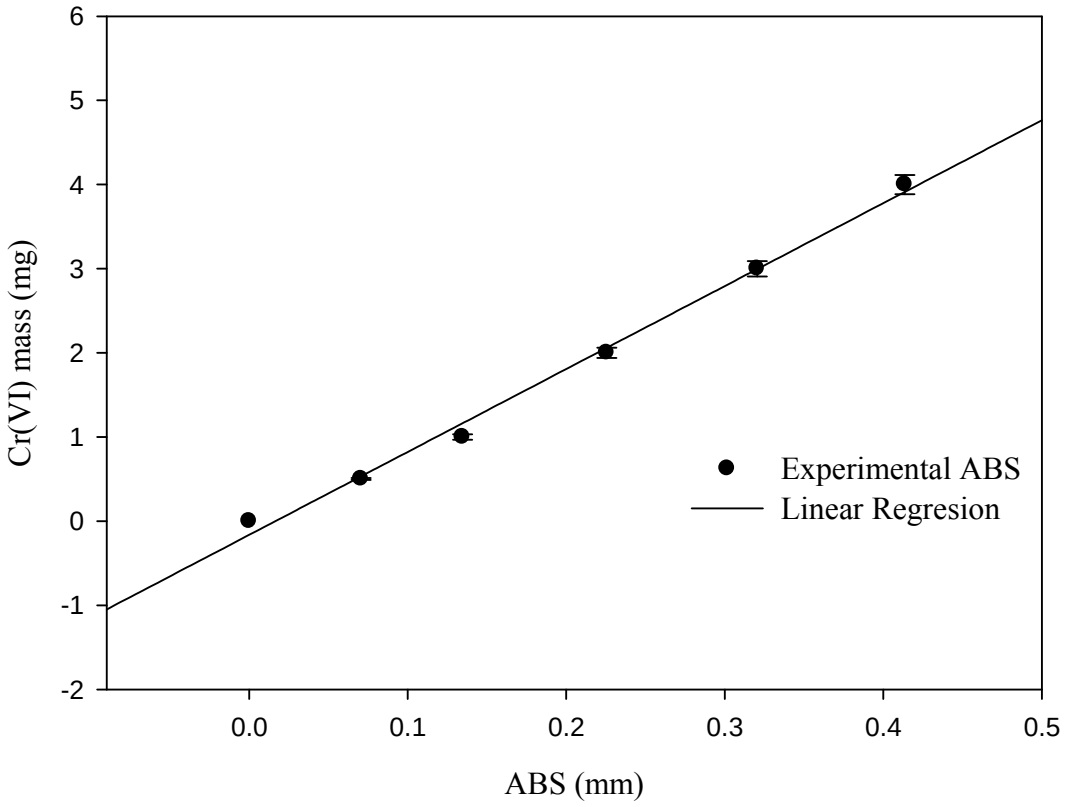
   $C = C_0 \,.\, \exp\,[-p \,.\, \exp\,(-q \,.\, C_0\,) \,.\, t]$

   The model fitted a wider range of influent Cr(VI) concentration data than previous models in which the Monod type coefficients k_{mc} and K_c were assumed to be constant for all ranges of data.
5. The parameter p and q for the semi-empirical first order model were statistically accurate with R^2 values greater than 94% for all data ranges evaluated. This confirms that the model develop in this study may be used in a wider range of initial Cr(VI) concentration $C_{0.}$

The study presents a detailed investigation on the use of natural bacterial consortia in the treatment of Cr(VI) contaminated wastewater. In order to achieve application of this technology, future research will be needed in the following areas:

- Research must be performed to find out which exact enzymes are involved in the Cr(VI) reduction process, what are the end products especially gas produced under both aerobic and anaerobic conditions; and what impact does the gas produced have on the Cr(VI) reduction process.
- Research is also required to determine a mechanistic representation of the relationship between the parameters in the second exponential to the Cr(VI) reduction rate, i.e., the model must represent the actual cause of the dependence of k_{mi} on initial Cr(VI) concentration.
- Finally, it is necessary to conduct studies in minimal medium, in continuous flow and in simulated aquifer necessary for *in-situ* bio-remediation.

APPENDIX A



Cr(VI) mass (mg) = 9.85 ABS - 0.163
R^2 = 99.5%

Figure A-1. A composite calibration curve for Cr(VI) determination using the UV/Vis Spectrophotometer. (n = 3, R^2 = 0.995).

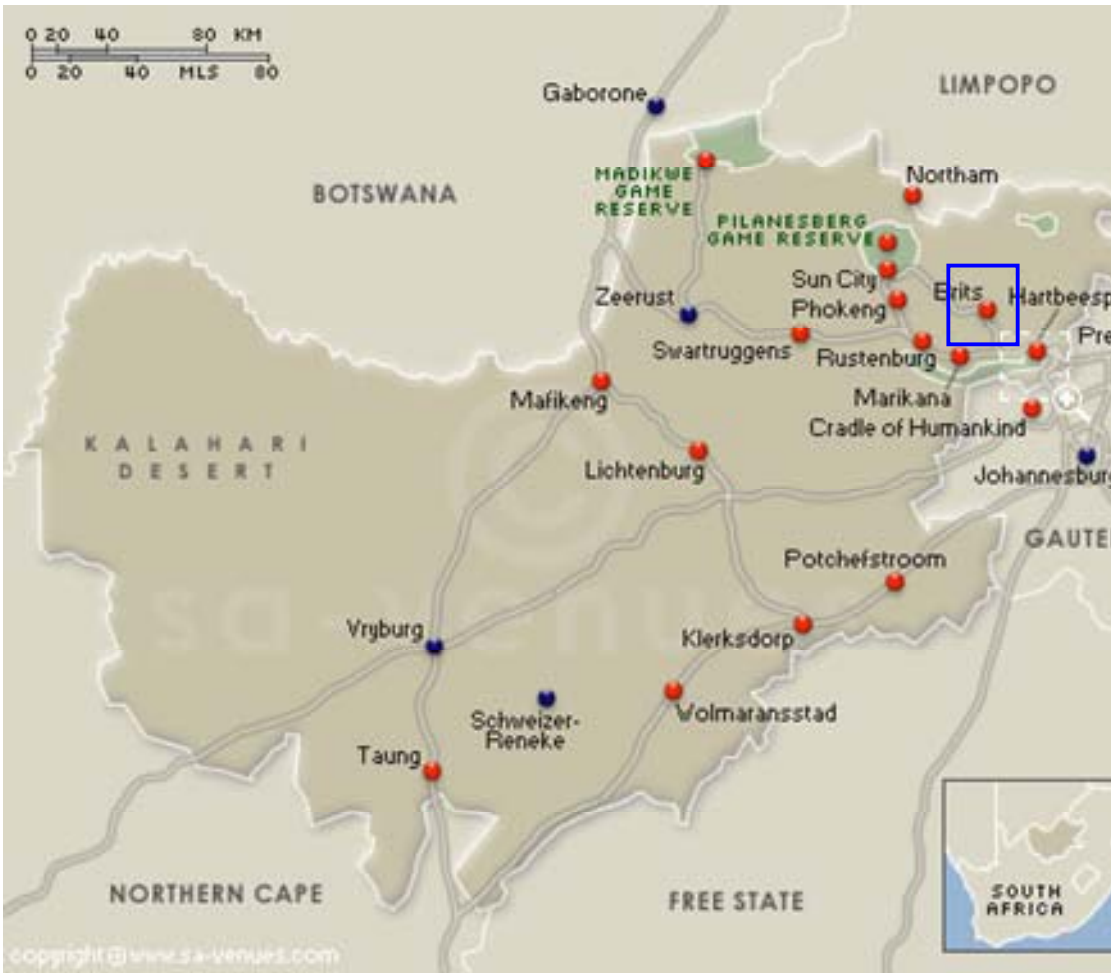


Figure A-2: Location of the abandoned chrome processing facility under cleanup operations in Brits, North West Province (SA).

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Intelligence plus character *plus wisdom*; that is the goal of true education

Dr. Martin Luther King Jr.