REMOBILIZATION OF TRIVALENT CHROMIUM AND THE REGENERATION OF IN SITU PERMEABLE REACTIVE BARRIERS DURING OPERATION

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ABSTRACT

Title: Remobilization of Trivalent Chromium and the Regeneration of *In situ* Permeable Reactive Barriers during Operation

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Chromium exists largely in two oxidation states, namely hexavalent chromium (Cr(VI)) which is carcinogenic, mutagenic to living organisms including humans and trivalent chromium (Cr(III)) which is known to be 1000 times less toxic than Cr(VI). It is therefore desirable in most cases to reduce Cr(VI) to Cr(III). Various studies have been conducted on the Cr(VI) reduction process either *in situ* or *ex situ*. However *in situ* bioremediation using permeable reactive barrier system appears as a potential and attractive technology compared to other *in situ* technologies. This study was conducted to evaluate the reduction of Cr(VI) to Cr(III) in the short term and regeneration of the biological reactive barrier to achieve continuous long term operation. It was observed from the study that the chromium hydroxide $Cr(OH)_3(s)$ precipitated and thus affected the porosity and hydraulic conductivity of the barrier system. It was therefore proposed to implement a regeneration process involving remobilization of precipitated $Cr(OH)_3$ using a dilute acid (0.1% HCl) and recover Cr(III) by electrokinetics.

Lowering the pH in the reactor introduced harsh conditions which necessitated the evaluation of a possible culture shift during the regeneration phase. Microbial culture composition during bioremediation and after soil washing was evaluated using a 16S rRNA finger printing method. The microbial barrier was initially inoculated with indigenous bacterial species from dried sludge. The results presented in the phylogenic tree diagrams confirm that, after microbial barrier system operation, the well-known Cr(VI) reducers *Bacillus mycoides*,

Lysinibacillus fusiformis and *Micrococcus lylae* were the predominant species in the microbial community of the barrier.

The microbial barrier system successfully achieved near complete removal of Cr(VI), whereby approximately 75% Cr(VI) removal was achieved within 63 days of operation. The formation of $Cr(OH)_3(s)$ was observed in the second week of operation. After 4 weeks of operating the mesocosm under soil washing with 0.1% HCl and electrokinetics remediation with a DC voltage of 50-150 V an increase in total chromium (73%) was observed suggesting that the trapped chromium species in the mesocosm was effectively remobilized with the assumption that Cr(III) had attached to the cathode forming a white-yellow precipitate layer around the cathode. Additionally more than 95% Cr(VI) was transformed to lower toxicity Cr(III) during electrokinetics and soil washing remediation. However, one of the limitations of electrokinetics is near anode focusing effect whereby a layer of precipitate is formed around the anode that lead to the reduction of efficiency of the technology.

Keywords: Hexavalent chromium, microbial chromate reduction, trivalent chromium, remobilization, permeable reactive barriers in situ remediation, soil washing, electrokinetics.

DECLARATION

I Lapaka Albertina Kaimbi, declare that the thesis which I hereby submit for Master of Science: Applied Science (Environmental Technology) degree at the University of Pretoria is my own work and has not been previously submitted by me for any other degree at this or other institutions.

_____

Lapaka A Kaimbi

This ……. day of ……. 2014

Dedicated to

Kondjela and Eunice Kaimbi

My beloved parents whose prayers and love strengthens me,

my generous aunt

Saara Haipinge

A strong woman I look up to

and;

Ndapandula, Tutala, Kulaumone, Ndatilaomwene and Linekeela

my sisters and blessings from God,

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AAS	Atomic adsorption spectrophotometer
APHA	American Public Health Agency
AR	Analytical reagent
BLAST	Basic Logical Alignment Search Tool
BOD	Biological oxygen demand
BTEX	Benzene, toluene, ethylbenzene, and xylenes
CEC	Cation exchange capacity
ChrR	Cr(VI) reductase
Cr	Chromium
Cr(III)	Trivalent Chromium
$Cr(OH)_3$	Cr(III) hydroxide
Cr(VI)	Hexavalent chromium
CRB	Cr(VI) reducing bacteria
DC	Direct current
DNA	Deoxyribonucleic acid
DWAF	Department of Water Affairs and Forestry
Eh	Reduction potential
FADH	Flavin adenine dinucleotide
HCl	Hydrochloric acid
HDPE	High-density polyethylene
IAEA	International Atomic Energy Agency
LB	Luria Bertani
MSM	Mineral salt medium
NADH	Nicotinamide adenine dicheotide
NADPH	Nicotinamide adenine dicheotide phosphate

PAH	Polycyclic aromatic hydrocarbons
PC	Plate Count
pH	Potential hydrogen
ppm	Parts per million
PVC	Polyvinyl chloride
Rrna	Ribosomal Ribonucleic acid
SOS	Save our souls
US EPA	United States Environmental Protection Agency
VB	Vogel Bonner

NOMENCLATURE

A	Cross-sectional area (m^2)
C	Cr(VI) concentration at time, t (mg/L)
C	State variable (mg/L)
C_r	Cr(VI) toxicity threshold concentration (mg/L)
E	Electric field strength (Vm^{-1})
K_C	Half velocity constant (mg/L)
k_d	Cell death rate (T^{-1})
k_e	Coefficient of electro-osmotic permeability ($m^2V^{-1}s^{-1}$)
K_i	Inhibition coefficient (mg/L)
k_m	Maximum specific Cr(VI) reduction rate (T^{-1})
q_A	Electro-osmotic flow rate (m^3s^{-1})
R_c	Cr(VI) reduction capacity coefficient (MM^{-1})
t	Time (T)
u_m	Ionic migration velocity (ms^{-1})
v	Ionic mobility ($m^2s^{-1}V^{-1}$)
X	Biomass concentration at time, t (mg/L)
X_o	Initial biomass concentration (mg/L)

CHAPTER 1

INTRODUCTION

1.1 Background

Chromium the element was first isolated by the French chemist Nicolas-Louis Vauquelin in 1797 from a sample of a very beautiful orange-red material (Jacobs and Testa, 2005). Chromium in the environment exists mainly in two forms: trivalent chromium (Cr(III)) which readily forms the insoluble and less mobile species, $Cr(OH)_3(s)$ in water (Zayed and Terry, 2003), and hexavalent chromium (Cr(VI)), which exists as the soluble and mobile oxyanions, chromate and dichromate (CrO_4^{2-}or $Cr_2O_7^{2-}$). Hexavalent chromium is very toxic and carcinogenic such that it is listed as a Class A carcinogen by the U.S EPA (Federal Register, 2004). In biological systems, hexavalent chromium acts as a carcinogen, mutagen and teratogen. Chromium concentrations as low as 0.5 mg/L in solution and 5 mg/L in soils can inhibit seed germination in grassy plants (Panda and Sarkar, 2012). Trivalent chromium on the other hand is non-toxic to living organisms as it is necessary in animal nutrition (Vincent and Love, 2012). In recent studies chromium has been popular as a nutritional supplement, weight loss agent and used in muscle development agents. Trivalent chromium is needed in mammals for carbohydrates and lipid metabolism (Vincent, 2004). Among the main exporters of chromite ore in the world are South Africa, Kazakhstan and Zimbabwe. Exports from these countries account for 97% of the world wide chromite ore production (Bachmann *et al*., 2010).

Cr(VI) is discharged into the environment from industrial processes such as paint and pigment production, leather tanning, wood preservation, rubber and steel production thereby causing serious pollution. In South Africa, large scale pollution of groundwater and surface

water bodies have been attributed to illegal discharge from abandoned mines and chrome refineries (DWAF, 2005).

As mentioned above, Cr(VI) is highly toxic and its discharge is discouraged or disallowed in most countries. The allowable concentration for exposure to natural ecosystems is 0.05 mg /L (Federal Register, 2004). The common remediation strategies for Cr(VI) involves its reduction to the trivalent state (Cr(III)) followed by immobilization by precipitation and/or absorption to substrates. Trivalent chromium mobility can be decreased by adsorption to clays and oxide minerals below pH 5. At pH values below 5, formation of $Cr(OH)_3(s)$ occurs.

Chinthamreddy and Reddy (1998) indicated three strategies for possible remediation of chromium contaminated soils. The first strategy being using excavations and landfills to remove contaminated soils, the second strategy employing ex-situ process such as landfarming or *in-situ* process like soil flushing and bioventing. The third strategy involving reduction of chromium(VI) to chromium(III) by using Fe^{2+} or chromium reducing bacteria (CRB). Earlier, researchers observed Cr(VI) reduction activity in microbial species such as *Escherichia coli* (Shen and Wang, 1993), *Agrobacterium radiobacter* (Masood and Malik, 2011), *Pseudomonas fluorenscens* (Shen and Wang, 1994), *Enterobacter cloacae* (Sethuraman and Balasubramanian, 2010), *Micrococcus roseus* (Mirsha *et al*., 2010) and *Pseudomonas putida* (Kaimbi and Chirwa, 2013). The studies showed the potential of indigenous microbial cultures as catalysts for reduction of Cr(VI) from wastewater and Cr(VI) contaminated soil environment to Cr(III).

In other studies, metal contaminated soils were treated using techniques such as soil washing, excavation, solidification and stabilization. Recent developments in the remediation of chromium contaminated soil include the evaluation of Cr(VI) immobilization using biological permeable reactive barriers. Biological remediation barriers have been used more

successfully in treating toxic organic compounds in water. Gibert and co-workers (2007) successfully removed Polycyclic Aromatic Hydrocarbons (PAHs) and BTEX compounds with a biological sequential reactive barrier.

It was found that some chromium reducing bacteria such as *Bacillus cereus* are capable of forming a precipitate chromium hydroxide which is immobile and clogs the aquatic pores spaces of the barrier and reduces the hydraulic loading of the barrier (Molokwane, 2010). This formation leads to the decrease in the reduction of chromium in the contaminated groundwater and it increases the costs of running a barrier as the clogged barrier needs to be replaced and this exercise is not cost effective.

Soil washing has been developed and tested in the remediation of chromium using extracting agents such as acids, neutral salts and chelating agents. Isoyama and Wada (2006) previously used hydrochloric acid to effectively remove chromium and lead from contaminated soils. This study describes the use of soil washing with a mineral acid to remobilize $Cr(OH)_3$ at the same time regenerating the barrier and subsequently collecting the mobile Cr(III) at the cathode under the influence of an electrokinetics potential. Soil washing is found to be economically feasible and easy to carry out according to Mann, (1999). Studies on the combination of bioremediation and acidification through soil washing studies are fairly new, however, by combining these two technologies, some of the limitations experienced with each technology can be mediated and thus increasing the efficiency of the system as a whole.

1.2 Research Objective

The primary objective of the study was to evaluate the effectiveness of hydrochloric acid in the removal of trivalent chromium from contaminated soil and the regeneration of the biological reactive barrier. In order to achieve the main objective, different experimental tasks were conducted on the Cr(VI) reduction process, i.e:

- Evaluation of Cr(VI) reduction in aquifer mesocosm reactor with 50 mg/L Cr(VI) feed concentration;
- Observation of $Cr(OH)_3$ formation in the reactor;
- Evaluation of the effectiveness of 0.1% HCl in remobilizing $Cr(OH)_3$ precipitate and recovering Cr(III); and
- Evaluation of the recovery of Cr(III) at the cathode using electrokinetics.

1.3 Main Findings

The study was divided into a two phase process. In the first process, bioremediation was conducted at the initial Cr(VI) feed concentration of 50 mg/L whereby 75% of Cr(VI) removal was achieved after 63 days of operation by live culture of bacteria from sludge. It was observed that $Cr(OH)_3$ started forming in the reactor after 2 weeks of operation and the precipitate clogged the pores of the barrier and this led to the decrease in the Cr(VI) reduction capacity of the microorganisms in the barrier. The second phase of the study consisted of soil washing with 0.1% HCl to remobilize $Cr(OH)_3$ and 73% total chromium was achieved after flushing the reactor with acid. Electrokinetics was used to attract Cr(III) in the reactor to the cathode. 95% Cr(VI) was transformed to a lower toxicity of Cr(III) during electrokinetics and soil washing remediation. This suggests that the trapped chromium species on the barrier in the mesocosm were remobilized. The acidification of the soil led to a shift in the microbial community which showed the adaptability of the inoculums culture.

1.4 Significance of Study

Bioremediation of Cr(VI) in biological permeable reactive barriers (BPRBs) using a local culture inoculum was proposed by Molokwane, (2010). The technology was desirable as it could achieve hexavalent chromium reduction and immobilization of chromium species

around a contaminated site. The problem is that the reduced chromium species exist as the amorphous $Cr(OH)_3(s)$ a precipitate that could eventually block the pores of the permeable reactive barrier and limit water flow.

After operating a microbial barrier for some time, it will be necessary to regenerate the barrier zone by remobilizing the chromium hydroxide that has accumulated over time. Lowering the pH could result in the remobilization of Cr(III) and high spike of pollution to the receiving aquifer. This could be prevented by a pump-and-treat method (very expensive) that is why in this study electrokinetics was proposed as an extraction method for Cr(III) at the cathode. The extracted Cr(III) could then be in industrial processes such as feed stream chrome plating or for ornamental applications.

1.5 Organization of Dissertation

The outline of the dissertation is subdivided into six main parts:

Literature Review: This section contains background information on studies of current and previous developments on hexavalent chromium reduction processes. The information is focused on the biochemistry of chromium in the environment, recovery methods of hexavalent chromium and trivalent chromium, bioremediation of hexavalent chromium.

Material and Methods: It details a reference of all the methods used during this study.

Culture Characterization: In this section CRB contained in the dried sludge was identified and classified based on colony morphologies, cell wall structure and DNA gene sequence. The phylogenetic tree was then constructed and a comparative analysis was conducted against known hexavalent chromium reducing bacteria from other research groups.

Kinetic Study: The Monod model was used to evaluate the rate of hexavalent chromium reduction over a wide range of initial chromium concentrations. This study was performed in batch under anaerobic and aerobic conditions and results are presented in the section on the kinetics and modeling.

Cr(VI) Reduction Studies: Contains the evaluated performance of the mesocosm studies.

Cr(III) Recovery : In this chapter soil washing with hydrochloric acid to remobilize $Cr(OH)_3$ was studied and trivalent chromium was then recovered at the cathode during electrokinetics.

CHAPTER 2

LITERATURE REVIEW

2.1 Chromium Biochemistry in the Environment

In the environment, chromium can exist in valence states ranging from -2 to +6. However, the valance states +3 and +6 are the most common under ambient environmental conditions (Reddy *et al.*, 1997). The divalent and quintavalent forms are unstable in most compounds as they are easily oxidized to the trivalent and hexavalent forms by oxygen in air. Cr(VI) is a strong oxidizing agent as it reacts with a wide range of reducing compounds in the environment to form Cr(III) (Patterson *et al.*, 1997). Cr(VI) occurs most frequently in its oxyanionic (oxygen combined) form either as chromate (CrO_4^{2-}) or as dichromate (Cr_2O^{2-}) (Cervantes and Campos-Garcia, 2007). The oxyanionic forms speciates into the acidic species $H_nCr_xO_y^{-(2-n)}$, where *x* and *y* are 1 and 4 for chromate, 2 and 7 for dichromate, and *n* increases towards the value of 2 as the pH drops. The resulting forms of aqueous chromate are the mono-, bi- and hydrogen chromate – $HCr_xO_y^{(-)}$, $Cr_xO_y^{2-}$ and $H_2Cr_xO_y$, respectively, depending on the pH of the water.

Cr(VI) is known to be carcinogenic, mutagenic and teratogenic in biological systems (De Flora, 2000). In plants, concentrations as low as 0.5 ppm in the pore water and 5ppm in soils results in the inhibition of seed germination in cereal plants (Panda and Sarkar, 2012). Cr(III) on the other hand, exists in cationic or complexed hydroxyl forms $Cr(OH)^{2+}$, $Cr(OH)_3$, $Cr(OH)^{4-}$and $Cr(OH)_5^{2-}$ depending on the pH of the solution(Virkutye *et al.*, 2002). Notably, Cr(III) is not toxic to living organisms as it is necessary in animal nutrition (Saha *et al.*, 2011). Cr(III) is an essential nutrient for mammals as it is used in dietary supplements to maintain normal glucose, fatty acid and cholesterol metabolism (Hu and Deming, 2005;

Rossouw, 2009). In humans, exposure to Cr(VI) as high as 10 ppm causes kidney and liver failure, and can negatively affect the immune system (Costa, 1997).

Unlike other metals, Cr(VI) can combine with oxygen to form water soluble, negatively charged oxyanions such as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) which adsorb to positively charged species in contrast to cationic metal species. Therefore, hexavalent chromium species are not strongly sorbed in many soils under alkaline to slightly acidic conditions. Thus they can be very mobile in subsurface environment moving at the same rate as groundwater.

Several species have been demonstrated to catalyze the reduction of Cr(VI) to Cr(III) under natural ambient conditions (Table 2-1 and Table 2-2).The following is a list of mechanisms that microorganisms use for Cr(VI) resistance:

- Cr(VI) resistance is plasmid-associated phenomenon (Bopp *et al*., 1983; Bopp and Ehrlich, 1988; Chen and Hao, 1998).
- Countering chromate-induced oxidative stress by activating enzymes involved in reactive oxygen species (ROS) scavenging (catalase, superoxide dimutase) (Ackerley *et al*., 2004; Molokwane, 2010).
- Modification of sulphate transport (Brown *et al*., 2006; Hu *et al*., 2005; Thompson *et al*., 2007; Molokwane, 2010).
- Specialized repair of DNA damage by SOS response enzymes (RecA, RecG, RuvAB) (Hu *et al.,* 2005; Llagostera *et al*., 1986; Miranda *et al*., 2005; Molokwane, 2010).
- Regulation of iron intake, which may serve to sequester iron in order to prevent the generation of highly reactive hydroxyl radicals via the Fenton reaction (Brown *et al*., 2006; Molokwane, 2010).

- Extracellular reduction of Cr(VI) to Cr(III), which reacts with lipopolysaccharide ligands (functional groups) on the cell surface (Flemming *et al*., 1990; McLean *et al*., 1990; Snyder *et al*., 1978; Molokwane, 2010).

Table 2-1: Gram-positive microbial species that reduce Cr(VI) to Cr(III)

Name of Species	**Isolation Conditions/ Carbon Source**	**References**
Arthrobacter sp.	Anaerobic/ Vogel-Bonner broth, nutrient broth medium	Megharaj *et al*., (2003)
Bacillus firmus	Anaerobic/ PYG Broth	Sau *et al*., (2008)
Bacillus megaterium TKW3	Aerobic/ nutrient broth-medium salt medium-glucose, maltose and mannitol	Cheung *et al*., (2006)
Bacillus mycoides	Aerobic/ Luria Bertani broth	Molokwane and Chirwa, (2009)
Bacillus sp.	Aerobic/ Nutrient medium	Liu *et al*., (2006)
Bacillus thuringiensis	Aerobic/ Luria Bertani broth	Molokwane and Chirwa, (2009)
Lysinibacillus sphaericus	Aerobic/ Luria Bertani broth	Molokwane and Chirwa, (2009)
Microbacterium sp.	Anaerobic/ Luria-Bertani broth	Humphries and Macaskie, (2002)
Micrococcus sp.	Aerobic/ TGY Broth	Sultan and Hasnaim, (2005)
Pediococcus pentosaceus	Anaerobes/ Luria-Bertani broth, MRS broth	Illias *et al.,* (2011)
Staphylococcus aureus	Facultative anaerobic/ Luria-Bertani broth or nutrient broth	Illias *et al*., (2011)

Table 2-2: Gram-negative microbial population that reduce Cr(VI) to Cr(III)

Name of Species	Isolation Conditions/ C-Source	References
Achromobacter sp. StrainCh1	Anaerobic/ Luria broth; glucose-lactate	Zhu *et al.*, (2008)
Acinetobacter sp.	Anaerobic/ Luria-Bertani-broth, nutrient broth medium	Panda and Sarkar, (2012)
Aeromonas sp.	Facultative anaerobes/ nutrient broth medium	Panda and Sarkar, (2012)
Desulfovibrio vulgaris	Anaerobic/ Luria-Bertani Broth	Humphries and Macaskie, (2002)
Enterobacter aerogenes	Anaerobic/ Nutrient broth	Panda and Sarkar, (2012)
Enterobacter cloacae H01	Anaerobic/ KSC medium-Sodium acetate	Ohtake *et al.*, (1990)
Escherichia coli ATCC 33456	Aerobic-Anaerobic/ Nutrient broth medium; glucose, acetate, propionate, glycerol, glycine and Luria-Bertani Broth	Bae *et al.*, (2000)
Ochrobacterium anthrapi	Anaerobic/ nutrient broth medium, acetate	Fransico *et al*., (2002)
Pantoeaagglomerans SP1	Anaerobic/ acetate	Francis *et al*., (2000)
Providencia sp.	Aerobic-Anaerobic/ Luria broth (tryptone-yeast extract)	Thacker *et al*., (2006)
Pseudomonas aeruoginosa	Aerobic/ Nutrient broth or Luria broth	Wang and Xiao, (1995)
Pseudomonas putida	Anaerobic/ Luria-Bertani	Kaimbi and Chirwa, (2013)
Pseudomonas sp.	Anaerobic/ Vogel-Bonner broth	Mclean and Beveridge, (2001)
Thiobacillus thioparus	Anaerobic/ nutrient broth medium	Donati *et al.*, (2003)

In other species, this process is cometabolic since chromium serves as a terminal electron acceptor, and during its reduction large amounts of protons are consumed which results in elevation of the background pH (Chirwa and Wang, 2000). However, during metabolic Cr(VI) reduction energy is derived from the Cr(VI) reduction process. In the latter process, Cr(VI) serves as a terminal electron acceptor in the membrane electron-transport respiratory pathway, a process resulting in energy conservation for growth and cell maintenance (Lovley and Phillips, 1994).

2.2 Chromium Occurrence in the Environment

2.2.1 Chromium in the Aquatic Systems

Chromium enters the aquatic system through natural sources such as wet precipitation, weathering of rocks and run-off from terrestrial systems. Trivalent chromium species $Cr^{3+}$are prevalent at pH lower than 3.6, $Cr(OH)^{2+}$ is prevalent at the pH values from 2 to 6.3, $Cr(OH)_3(s)$ is prevalent in the aquatic system at the pH value from 6.3 to 11.5. $Cr(OH)^-_4$ is the main aqueous Cr(III) species at the pH value greater than 11.5 (Gheju, 2011). Other hydroxyl chromium complexes such as $Cr_2(OH)_2^{4+}$, $Cr_3(OH)_4^{5+}$ and $Cr(OH)_6^{6+}$ have been measured in natural systems (Gheju, 2011). The most predominant hexavalent forms in aquatic systems are $HCrO_4^-$ and CrO_4^{2-} depending on the pH in the environment. In aquatic solutions the equilibrium between protons, water molecules and the hexavalent chromium species are as follows:

$$H_2CrO_4 + H_2O \leftrightarrow HCrO_4^- + H_3O^+ \quad (2\text{-}1)$$

$$HCrO_4^- + H_2O \leftrightarrow CrO_4^{2-} + H_3O^+ \quad (2\text{-}2)$$

At pH between 2 and 6 the $HCrO_4^-$oxyanion can diminerize to form the oxyanion of dichromate ($Cr_2O_7^{2-}$):

$$2HCrO_4^- \leftrightarrow Cr_2O_7^{2-} + H_2O \quad (2\text{-}3)$$

In the aquatic environment, Cr(III) and Cr(VI) distribution is regulated by oxide-reduction reactions. A Eh-pH diagram (Figure 2-1) shows the hydrolysis speciation and valance states of chromium over a wide range of pH and Eh values. Eh–pH diagram shows the thermodynamic stability areas of chromium species in an aqueous solution (Ponou *et al.*, 2011).Trivalent chromium is the most stable under reduced conditions, and at the pH of 4 and 8 trivalent species are domination the first or second hydrolysis product (Fendorf, 1995).

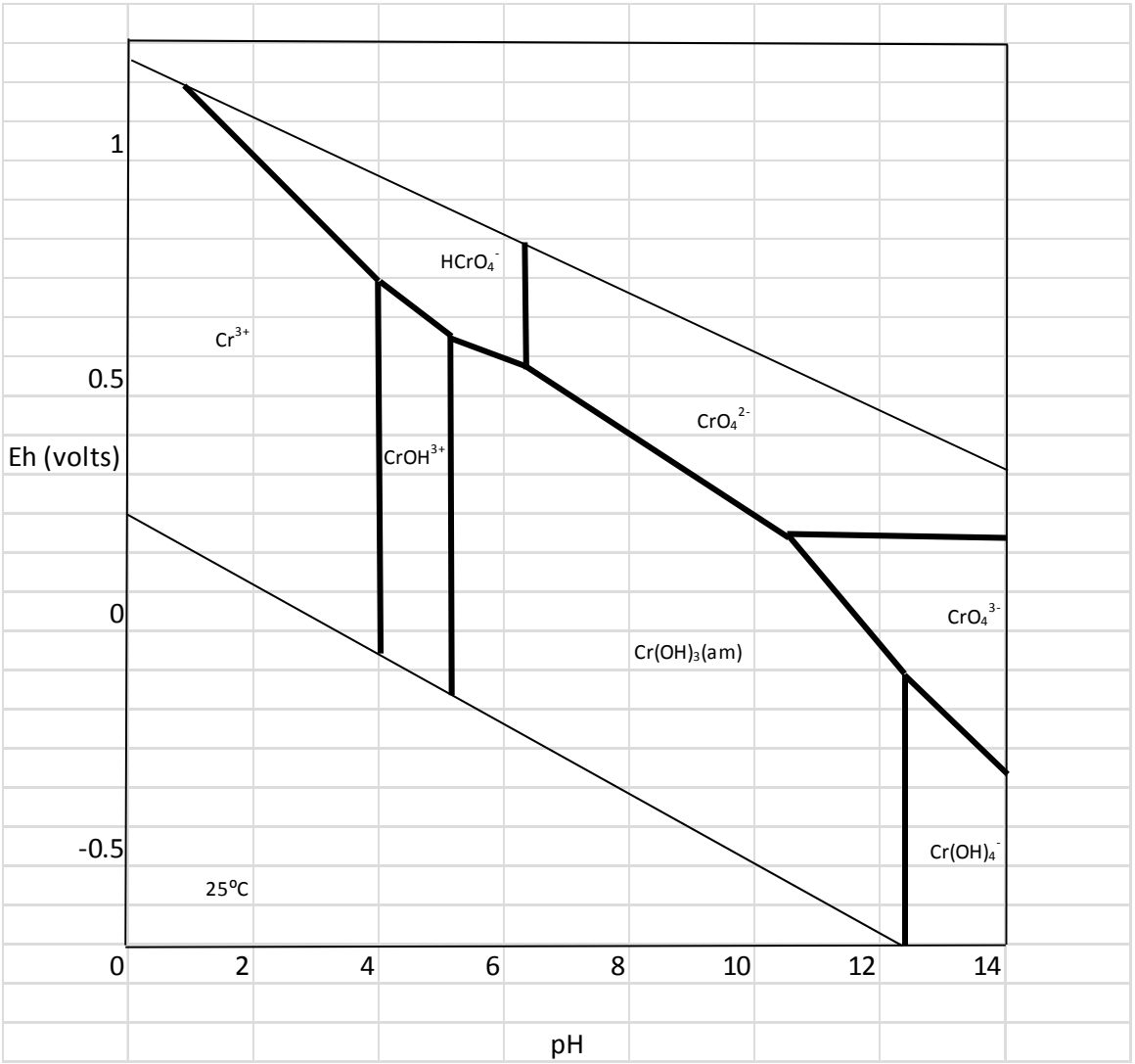

Figure 2-1: Illustration of the Eh-pH diagram for chromium (Bartlett, 1991)

2.2.2 Chromium in Soils and Sediments

In rocks and soils, chromium is weathered from minerals, and it is predominately present in the Cr(III) state that is sorbed on hydroxides (Kimbrough *et al.*, 1999). Compared to trivalent chromium concentration in the soils and sediments, hexavalent chromium rarely naturally occurs in the environment and thus its presence indicates contamination from anthropogenic activities (Kimbrough *et al,*. 1999). These anthropogenic sources can be atmospheric depositions and other sources are fallout and washout of atmospheric chromium containing particles (Kotaś and Stasicka, 2000).

The most dominant chromium in soils depends on the soil pH, soils with pH<4 contain $Cr(H_2O)_6^{3+}$. The presence of other metals such as iron, vanadium, sulfides and other organic matter in the soil aid in the transformation of Cr(VI) to Cr(III) or vice versa. The processes that occur during the transformation of Cr(VI) to Cr(III) in the soil include oxidation, reduction, sorption, precipitation and dissolution. With reference to organic matter influence, Losi and co-workers (1994) reported that the organic matter content, the bioactivity, and the oxygen status may affect the reducing capacity in Cr(VI) contaminated soil. According to Xu *et al.*, (2011), MnO_2 is able to oxidize Cr(III) to Cr(VI) in the soil. Chromate may be sorbed onto the soil, iron aluminum oxides and hydroxides prior to reduction (Kimbrough *et al.*, 1999). However, in some cases sorption and reduction may occur simultaneously. Trivalent chromium that is not sorbed is hydrolyzed to hydroxide and precipitated in the aqueous phase of soils and sediments.

2.2.3 Chromium in the Atmosphere

Anthropogenic activities account for 60-70% chromium in the atmosphere (Kotaś and Stasicka, 2000) and the remaining 30-40% is from natural sources (Seigneur and

Constantinou, 1995). The natural sources include volcanic eruptions and erosions of soil and rocks.

Anthropogenic activities such as wood preservation, leather tanning and metal plating contribute the most chromium in the form of Cr(VI) in the atmosphere (Shanker *et al*., 2005). The presence of chromium in an area at a particular time is due to the industrial processes, proximity to sources, the amount of chromium released and the meteorological factors. In the atmosphere, chromium is transported in the form of solids and liquids due to its high boiling points (2676°C) or in the form of aerosols. Chromium released into the atmosphere is either particle-bound or dissolved in droplets.

2.3 Toxicity to Microorganisms

Toxicity of chromium in the cell occurs due to the transitional release of OH radicals during the reduction of Cr(VI) to lower oxidation states (Cervantes *et al*., 2001). According to DeLeo and Ehrlich (1994), Cr(VI) inhibits enzyme activity and it 'poisons' cells non-specifically by blocking essential groups, displaying essential metal ions and modifying the conformations of the biological molecules or induce mutations.

During the reduction of Cr(VI) to Cr(III) a transient species Cr(V) is formed. Cr(V) complexes formed from Cr(VI) reduction by physiological reducing agents such as $FADH_2$, NAD(P)H, glutathione, and several pentose may react with added H_2O_2 and generate significant amounts of •OH radicals(Cervantes *et al*., 2001. The DNA phosphate groups affects the replication transcription and lead to mutagenesis due to intracellular Cr(III) (Aiyar *et al*., 1991). Mutagenic effects of chromium on microorganisms results in cell elongation, cell enlargement, inhibited cell division which in turn leads to the inhibition of cell growth (Coleman and Paran, 1983; Mtimunye, 2011).

2.4 Microbial Chromium Reduction

Cr(VI) is the most prevalent species of chromium and it is a known contaminant; several studies have been conducted that evaluate the removal or the immobilization of hexavalent chromium from the environment by reducing it to a less toxic Cr(III). Microbial reduction of organic wastes was found to be oxidized to carbon dioxide and water as a result of biological oxygen demand (BOD) and chemical oxygen demand (COD) removal by microbial activities (Chen and Hao, 1998). The mechanism by which microbes remove Cr(VI) in the environment include sorption, uptake, precipitation and valence state change. Bacteria may protect themselves from toxic substances in the environment by transforming toxic compounds through oxidation, reduction, or methylation into more volatile, less toxic, or readily precipitating forms (Chirwa and Wang, 1997).

Previously, Luli and co-workers (1983) isolated 89 species of bacteria from metal contaminated river sediments, 42 of which showed resistant to 100 mg/L Cr(VI). Recently, Meli (2009) identified Cr(VI) reducing organisms isolated from a dried activated sludge from the Wastewater treatment plant in Brits (South Africa). These organisms achieved 100% removal of Cr(VI) from cultures with initial concentration up to 200 mg/L in 48 hours (Meli, 2009).

2.5 Permeable Reactive Barrier (PRB)

Permeable reactive subsurface barrier is defined as an emplacement of reactive materials in the subsurface designed to intercept a contaminant plume, provide a flow path through the reactive media, and transform the contaminant(s) into environmentally acceptable forms and attain remediation concentration goals down gradient of the barrier (Puls and Powell, 1997). The processes that occur during the interaction of the pollutants and the reactive materials can be classified into three categories: degradation which occurs through either chemical or

biological reactions that leads to the degradation or decomposition of contaminants into harmless compounds; sorption whereby the contaminants are immobilized with the reaction zone by adsorption or complex formation. The final category is precipitation; contaminants within the reaction zone are immobilized by the formation of insoluble compounds (Roehl *et al*., 2005). Over the past two decades permeable reactive barriers have been developed to treat groundwater contaminated by organic and inorganic constituents (Gibert *et al*., 2011)

Permeable reactive barriers (PRBs) are an emerging alternative to traditional pump and treat systems for groundwater remediation (Gibert *et al*., 2011). They are typically used to treat inorganic contaminants like lead, arsenic, chromium, cadmium and molybdenum. One approach is by using a semipermeable reactive membrane placed in the flow path of a plume of a contaminant. During chromium reduction reactive medium either organic compounds or microbes transform Cr(VI) to Cr(III) by immobilizing Cr(VI) to a less toxic form (Basu and Johnson, 2012).The barriers should be designed in a way that the contaminant remains immobilized within the aquifer or it can be retrieved with the reactive material following treatment (Molokwane, 2010).

2.5.1 Chemical Reactive Barrier

Chemical reactive barriers are designed to utilize chemical reactive compounds to transform contaminants to less toxic or less mobile forms such as Cr(VI) to Cr(III). Zero valent iron has traditionally been used in PRB to treat contaminants that need reducing (Cantrell *et al*., 1995; Gu *et al*., 1998; McMahon *et al*., 1999; Das, 2002). Blowes and Ptacek (1992) have reported that elemental iron (Fe^0) rapidly reduces Cr(VI) compared to pyrite (FeS_2) and siderite ($FeCO_3$). The following reaction sequence details the reduction of Cr(VI) to Cr(III) by Fe^0:

$$Cr(VI)(aq) + 3Fe(II)(aq) \rightarrow Cr(III)(aq) + 3Fe(III)(aq) \quad (2\text{-}4)$$

$$xCr(III) + (1\text{-}x)Fe(III) + 3H_2O \rightarrow (Cr_xFe_{1\text{-}x})(OH)_3(s) + 3H^+ \quad (2\text{-}5)$$

Where x may vary from 0 to 1 (Patterson *et al.*, 1997). The precipitate $(Cr,Fe)(OH)_3(s)$ formed in the above reaction could eventually block the pores of the barrier thereby imposing a physical constraint on the system.

2.5.2 Biological Permeable Reactive Barrier

Biological reaction involves the utilization of naturally occurring microorganisms for the degradation of toxic contaminants (Thiruvenkatachari *et al.*, 2008). In biological barriers, aerobic and anaerobic microbes are used however aerobic reduction is preferred over anaerobic reduction as aerobic metabolism generates more energy for the cells that leads to higher metabolic rates and higher cell growths.

Biological permeable reactive barriers (BPRB) have been used more successfully in treating toxic organic compounds in water which can be degraded completely to water and carbon dioxide using specially selected organisms (Liu *et al.*, 2006). Attempt on heavy metal removal using BPRB have also been made (Pagnarelli *et al.*, 2009). Microbial reduction of Cr(VI) often results in consumption of large amounts of proton as reducing equivalents which results in the elevation of the background pH. The increased pH facilitates the precipitation of the reduced chromium as chromium hydroxide that can be illustrated as follows (Brock and Madigan, 1991; Zakaria *et al.*, 2007; Mtimunye, 2011):

$$CrO_4^{2-} + 8H^+ + 3e^- \xrightarrow{CRB} Cr^{3+} + 4H_2O \xrightarrow{Neutral\ pH} Cr(OH)_3 + 3H^+ + H_2O \quad (2\text{-}6)$$

The CRB represent Cr(VI) reducing bacteria or enzyme. The CrO_4^{2-} accepts three electrons to be reduced to Cr(III).

Under anaerobic conditions acetate which is an electron donor in this case leads to the following Cr(VI) reduction:

$$3CH_3COO^- + 8CrO_4^{2-} + 17H_2O \xrightarrow{CRB} 8Cr(OH)_3(s) + 6HCO_3^- + 13OH^- \quad (2\text{-}7)$$

There is potential to treat chromium *in situ* using the Cr(VI) reducing organisms. However, the problem associated with such an *in situ* process is mainly the accumulation of the precipitated forms that could in return affect the permeability of the barrier.

2.6 Theory of $Cr(OH)_3$ Formation

Chromium reducing bacteria such as *Bacillus* has been reported to reduce Cr(VI) at the cell surface or by bacterial metabolic products such as H_2S. The results from the study by Rahman *et al.*, (2009) demonstrated extracellular Cr(VI) reduction which was confirmed by the presence of insoluble chromium hydroxide that accumulated around the cell. Furthermore, Daulton and co-workers (2007) also reported the presence of $Cr(OH)_3$ both on the cell surface of the *Shewanella oeidensis* cells and on the medium after Cr(VI) reduction. The accumulation of the Cr(III) precipitate on the cell surface may serve as a proactive mechanism of bacterial cells against shock loadings of Cr(VI). The following reaction illustrate the precipitation of Cr(III) species at near neutral pH (pH>6.5) (Wu *et al.*, 2008):

$$Cr^{3+} + H_2O \leftrightarrow Cr(OH)^{2+} + H^+ \qquad pK = 4.0 \quad (2\text{-}8)$$

$$Cr(OH)^{2+} + H_2O \leftrightarrow Cr(OH)_2^+ + H^+ \qquad pK = 5.7 \quad (2\text{-}9)$$

$$Cr(OH)_2^+ + H_2O \leftrightarrow Cr(OH)_3 + H^+ \qquad pK = 8.3 \quad (2\text{-}10)$$

2.7 Recovery Methods of Chromium

2.7.1 Hexavalent Chromium Recovery Methods

Several methods have been used for the recovery of chromium from water and soil mediums. For example, the leather tanning process, up to 60-70% of the Cr(VI) in the raw chemical can be consumed by the leather leaving behind as much as 30-40% in the solids and liquid waste. The chromium remaining is converted to Cr(III) which is removed by precipitation using magnesium oxide (Esmaeili *et al.*, 2005). In other applications, Chmielewski *et al.* (1997) investigated the feasibility of recovery of chromium and copper ions in wastewater from the electroplating industry using a combination of electrochemical oxidation and ion exchange. Furthermore, separation investigations were conducted at the laboratory scale by Rengaraj *et al*., (2003) where Cr(VI) and Cr(III) were recovered from water using strongly basic resin and a weakly acidic resin, respectively. From the equilibrium analysis of different resins, it was demonstrated that chelating agents can be engineered onto resins to achieve target efficiencies in recovering chromium. In all cases, the uptake of chromium was affected by pH, temperature, initial metal concentration and contact time.

2.7.2 Trivalent Chromium Recovery Methods

Trivalent chromium is generally less toxic in water than Cr(VI) and it readily forms the hydroxide precipitate $Cr(OH)_3(s)$ (Narayani and Vidya, 2012). In large quantities Cr(III) can have adverse effects on the environment as it can be re-oxidized to Cr(VI) and therefore Cr(III) needs to be removed. There are several methods used to remove and recover Cr(III) from the environment. These methods include solvent extraction and precipitation, Liu *et al.,* (2001) used a spheroidal cellulose adsorbent to adsorb Cr(III) from aqueous solutions and the absorbed Cr(III) in the form of $Cr(OH)_3$ was treated with 1.2 M HCl which recovered up to 86.8% of Cr(III).

2.8 Remediation Strategies

Chromium can be treated from the aquatic phase using physical-chemical treatment methods such as adsorption, ion exchange, pump and treat remediation and electrochemical mobilization.

2.8.1 Adsorption Techniques

Adsorption is described as a process where molecules are concentrated on the surface of the sorbent. Chitosan, industrial waste, activated carbon, biological material and zeolites have been used as sorbents for removal of Cr(VI) from wastewater, of the five listed sorbents, activated carbon is the most studied adsorption source (Mohan and Pittman, 2006). The large surface area of granular activated carbon (GAC) (area in the range 500-1500 m^2/g carbon), makes it a first choice in water treatment systems (Chingombe *et al*., 2005; Owlad *et al*., 2009). The advantage of using adsorption as a treatment method is that it is flexible in design and operation, it is economical, it produces high quality treated effluent (Hu *et al,* 2011) making it very effective and the absorbent can be regenerated.

2.8.2 Ion Exchange

During ion exchange synthetic or natural ion exchange resins are used to exchange cations with metals in wastewater (Fu and Wang, 2011). The ion exchange resins release and exchange ions that are of the same charge in a chemically equivalent amount with the ions in wastewater. Usually the resins have either strongly acidic groups like sulfonic acid groups (SO_3H) or weakly acid groups like carboxylic acids (-COOH) attached to them and they act as exchangeable ions (Fu and Wang, 2011). The exchange occurs when wastewater with heavy metals passes through a cation column, an exchange of metal ions and hydrogen ions occurs on the resin (Fu and Wang, 2011). Certain variables affect the uptake of heavy metals such as initial metal concentration, contact time and pH (Gode and Pehlivan, 2006)

Ion exchange resins have shown to hold great potential in the removal of heavy metals from water and industrial wastewater (Mukherje *et al.,* 2012). The main advantage of using ion exchange over chemical precipitation of chromium is the recovery of high metal value, selectivity, less sludge volume is produced and metals recovered by ion exchange meet the strict discharge specifications (Rengaraj *et al*., 2001), this was achieved in a study conducted by Tiravanti *et al.* (1997) on the recovery and removal of Cr(III). They found that the sludge was reduced by 80% compared to other traditional treatment methods and the cost for sludge treatment and disposal was lowered.

Ion exchange is an attractive treatment method for wastewater containing Cr(VI) especially from electroplating and metal finishing process as they contain low Cr(VI) concentrations and low pH value (Xing *et al*., 2007). Studies by Galan *et al.,* (2005) found that more than 90% reduction was achieved through ion exchange and the eluate was pure water that could be reused thus not resulting in further pollution.

2.8.3 Pump and Treat Remediation

Pump and treat method is one of the widely used remediation technologies for contaminated groundwater (Palmer and Fish, 1992). The technology removes contaminated groundwater by pumping it to the surface and treating it in a treatment facility above ground (Higgins and Olson, 2009). This is done to maintain gradient control and as to prevent the contaminant from migrating (US EPA, 1988; Palmer and Wittbodt, 1991). The drawbacks of pump and treat is that it fails to attend to the source of the contamination in the vadose zone and it also creates the problem of lowering the water table therefore leaving contamination in the new vadose zone (Mtimunye, 2011). Mackay and Cherry (1989) have concluded that pump and treat method would work best as a management tool to prevent the spread of contamination by hydraulic manipulation of the aquifer continuation of contaminant migration, instead of it

being used as a permanent aquifer cleanup method as its limitations causes it to be ineffective for permanent cleanup.

2.8.4 Chemical Precipitation

Soluble metallic ions are transformed into insoluble precipitate during chemical precipitation by adding precipitation agents such as hydrogen sulphide and magnesium oxide followed by separation of the insoluble compounds from clean water in clarifiers (Hyman and Dupont, 2001). The major precipitation processes used include alkaline (hydroxide) precipitation and sulfide precipitation. The main step in the processes consists of the following (Hyman and Dupont, 2001):

1. Addition of an alkaline agent or sulfide, plus addition of coagulants and flash mixing.
2. Addition of flocculants and slow mixing to promote particle growth.
3. Settling of the particles.

Hydroxide precipitation uses alkaline agents to raise the pH to a point where the metal exhibits minimum solubility (Suthersan, 1996) and it causes dissolved metals such as lead, copper, zinc and chromium to precipitate as hydroxides. Hydroxide precipitation is more widely used due to its simplicity, it is inexpensive and it is easy to control the pH (Huisman *et al*., 2006). Sodium hydroxide and lime are used as precipitation agents; however additional chemical in the form of coagulants are added during the process to enhance removal of heavy metals from wastewater (Fu and Wang, 2011). The drawbacks of hydroxide precipitation are that the process produces large volumes of sludge and disposing of this sludge can increase the costs (Kongsricharoern and Polprasert, 1996).

Sulfide precipitation is an effective alternative to hydroxide precipitation for the removal of heavy metals from groundwater (Suthersan, 1996). The addition of sulfide in the form of

sodium sulfide (Na_2S) or sodium hydrosulfide (NaHS) induces precipitation of dissolved metals as metal sulfide. Soluble ferrous sulfide (FeS) slurry can also be used (Suthersan, 1996). The treatment is effective for metals including zinc and cadmium. Sulfide precipitation has more benefits over hydroxide precipitation in the quantity of sludge generated and the sludge may easily be reprocessed to recover the metal, (Fu and Wang, 2011). However, the addition of sulfide into the environment may lead to possible toxicity due to the excess hydrogen sulfide (H_2S) production. In a study by Fu and Wang, (2011) reduction of Cr(VI) to Cr(III) was achieved by using $Ca(OH)_2$ and Na(OH) whereby more than 90% removal was observed (Fu and Wang, 2011).

2.8.5 Soil Washing

Soil washing involves the separation of pollutants from the soil matrix by solubilizing them in a washing solution (Moutsatsou *et al*., 2006). This process is a form of *ex situ* remediation. The technology has been applied for remediation of heavy metal contaminated soils (Gitipour *et al*., 2011).

The soil washing process can utilize either physical separation, chemical extraction or the combination of both (Gitipour *et al.,* 2011). Physical separation involves separating the contaminated soil into small volumes according to its physical characteristics. The soil samples are then treated with chemical reagent like acids or chelating agents which solubilize the metal contaminant found in the form of a precipitate (Dermont *et al*., 2008). In this particular study, the focus was on chemical extraction. The two basic approaches used in soil washing are: immobilizing the heavy metals to minimize their migration and secondly, the transfer of soil-bound heavy metals to the liquid phase by desorption and solubilization (Gitipour *et al*., 2011). The removal of contaminants with chemical agents depends on the following factors: soil geochemistry, the metal contamination characteristics, the dosage and

chemistry of the reagent and the processing conditions (Dermont *et al*., 2008). These factors are important in deciding which chemical reagent to use as one reagent might remove one species of a metal while its interaction with the other species in the soil might lead to further pollution in the environment such as chromium species' presence with other organic matter in the soil. Soil washing has been found to be an effective alternative to solidification/stabilization and landfilling from the economic and environmental point (Gitipour *et al.,* 2011). The soil properties that affect the efficiency of soil washing are cation exchange capacity, organic matter, soil moisture content and the soil pH. Soil washing is easier and more efficient when the soil organic matter is high as high organic matter load obstruct soil washing (Abumaizar and Khan, 1996). Organic matter in the soil occurs in two forms, humus and non-humus substances and in the presence of metal ions, the ions bound to the organic matter by two processes – complexation and chelation. When this occurs an organimetallic ring makes the complexation to become highly stable and these results in the reduction of the mobility of the metal and the metal will not easily be ionized by any acid during acid washing and thus result in a low metal removal efficiency during soil washing (Abumaizar and Khan. 1996).

A study by Isomaya and Wade (2006) found that dilute HCl was able to effectively remove Cr(V) compared to Cr(III) that was gradually being oxidized to Cr(VI) in the presence of manganese oxide. They found that HCl is not efficient when there is a combination of the cationic and anionic forms if Cr species mainly because cationic metals stabilizes as the soil pH is increased and the opposite happens for anionic metals (Isoyama and Wada, 2006). Additionally soil washing has its drawbacks, it poses potential hazard to the environment and this should be controlled (Abumaizer and Khan, 1996). The treated soil may contain the solubilized metal and it will remain in the treated soil (Page and Page, 2002).

2.8.6 Electrokinetics

The first electrokinetics phenomenon was observed at the beginning of the nineteenth century when Reuss (1808) (Walls, 2010) applied a direct current to a clay-water. However, experimenting with electro-osmotic velocity of fluid and the zeta potential under an imposed electric gradient was first proposed by the two scientists called Helmholtz and Smoluchowski (1879) as quoted in Walls, (2010). The technology was demonstrated to be successful in removing large quantities of heavy metals from the soil by electric fields in laboratory studies. The technology involves the application of low voltage direct current through electrodes that are placed across a section of contaminated ground and the charge moves the contaminant. The principle of the technology is an electric current is used to mobilize ions. In order for electrokinetics remediation to be carried out, the pore fluid should be present as it has the following functions: conducting the electrical field, transporting species that are injected, controlling and modify the electrode reactions.

Ions are transported by electromigration, electro-osmosis, electrophoresis and other modes of transport like diffusion. Electromigration of ionic species is defined as the movement of ions in the pore fluid of the soil under the influence of an electric current, cations move toward the cathode and anions move towards the anode. Cationic and anionic contaminants are both removed by electromigration. Compared to electro-osmosis in terms of cation contaminant migration, electromigration has been reported to have greater charge of ionic species present, field strength and ionic concentration which influences electromigration during electrokinetics (Page and Page, 2002). The movement is described by the following of equation:

$$u_m = vE \qquad (2\text{-}11)$$

where u_m is the velocity of an ion, and v is ionic mobility (Page and Page, 2002). Factors such as concentration, ionic charge and temperature affect the electrical conductivity of the solution and in turn this is related to the ionic mobility (Page and Page 2002).

The second transport mechanism is electro-osmosis which is the movement of pore water under an electrical potential difference from the anode to the cathode. This process is affected by the soil porosity, zeta potential of the soil medium. It occurs due to the drag interaction between the bulk of the liquid in the pore and a thin layer of charged fluid next to the pore wall. The ions move under the action of the electric field in a direction parallel (Probstein and Hicks, 1993; Reddy and Parupudi, 1997). The electro-osmotic flow rate depends on the balance between the electrical force on the liquid and the surface of the soil particles (Page and Page, 2002). Electro-osmosis has been found to be effective in removing cation at low concentrations. The flow rate of electro-osmotic in the soil is described by Darcy's law for hydraulic flow (Page and Page, 2002):

$$q_A = -k_e EA \qquad (2\text{-}12)$$

where k_e is the coefficient of electro-osmotic permeability (or conductivity), E is the electric field strength or negative potential gradient, and A is the total cross-sectional area normal to the flow direction.

Electrophoresis refers to the transport of charged particles under the influence of an electric current, these charged particles colloids in soil-liquid mixture and it is an important mechanism in remediation of sludge. The drawback of this mechanism is that in compact systems like clay soil, movement of contaminants is restrained. However electrophoresis is important in remediating colloids that have contaminants adsorbed to them (Pamukcu and Wittle, 1992; Reddy and Parupudi, 1997).

Other mechanisms that are involved in electrokinetics are diffusion which plays a significant role in cationic and anionic contaminant transport, advection which moves soil moisture or groundwater due to hydraulic forces and finally convection which is responsible for the movement of soil moisture or groundwater due to buoyancy forces. Trivalent chromium migrates towards the cathode due to electromigration and CrO_4^{2-} and Cl^- migrates towards the anode due to electromigration and the negative charged colloids move due to electrophoresis.

The electrolysis reaction at the electrodes generates hydrogen ions (H^+) and oxygen gas at the anode and hydroxyl ions (OH^-) and hydrogen gas at the cathode. The oxygen gas produced at the anode and the hydrogen gas produced at the cathode escape out of the soil. The hydrogen ions in the anode attempt to migrate through the soil toward the cathode, whereas the hydroxyl ions in the cathode attempt to migrate through the soil towards the anode. The degree at which the H^+ and OH^- ions migrate depends on the buffering capacity of the soil. An acid front is produced at the anode and at the cathode a base front is produced and these two fronts move towards opposite directions (Page and Page 2002) have found that the acid front moves faster than the base front due to the fact that the mobility of H^+ exceeds that of $OH^-$and electro-osmotic flow is generally towards the cathode.

During electrokinetics the pH of the soil becomes acidic with the reading at the anode dropping to around 2 and the pH at the cathode increases to above 10. The rate of acid and base production depends on the current density (Castillo *et al.,* 2012). Precipitated hydroxides occur at the point where the pH change occurs as the solubility of metal ions is at a minimum. In order to enhance the electrodes and reduce the pH at the cathode and increase the pH at the anode an alkaline solution needs to be added at the anodic compartment and an acid solution needs to be added to the cathode compartment.

According to Acar *et al.*,(1995) the application of electric current has the following effects:

1. It produces an acid in the anode compartment that is transported across the soil and desorbs contaminants from the surface of soil particles.
2. It initiates electromigration of species available in the pore fluid and those introduced at the electrodes.
3. It establishes an electric potential difference which may lead to electroosmosis generated flushing of different species.

In order to remove the contaminants from the soil by electrokinetics, the contaminants should exist in pore water in dissolved ionic form so that they are transported to either the cathode or anode. In a study by Reddy and Chinthamreddy (2003b) they found that in order to improve the performance of electrokinetics the following can be done: changing the operating conditions such as switching the electrodes, prolonging the processing time and increasing electric gradient or by controlling the reservoir fluid pH.

Trivalent chromium behaves differently under electrokinetics in different soil types. In a study conducted by (Reddy and Chinthamreddy, 1999) they found that during the electrokinetics of both Cr(III) and Cr(VI), the pH near the anode decreased to a value between 2 and 3 and at the cathode the pH increased between 11 and 12 due to the high pH at the cathode a precipitate of chromium hydroxide was formed that clogged the pore space of the cathodic base front. The limitation of this process is the near anode focusing effect which results in the formation of a precipitate layer block around the anode resulting in the reduction of efficiency with time (Shen *et al.*, 2007; Li *et al.*, 2011).

The two most common occurring valence states of chromium trivalent exists in the form of cationic hydroxides such as $Cr(OH)_3$ which will migrate towards the cathode during electrokinetics remediation (Figure 2-3). However, chromium(VI) exists as CrO_4^{2-} at high pH and as $HCrO_4^-$ at low pH and other forms of oxyanions such as CrO_4^{2-}which migrate towards

the anode, however it is adsorbed by the soil in the low pH regions and it stops the complete removal of Cr (VI) from the soil. Electrokinetics is highly dependent on the acidic condition which favors the re-solubilization of heavy precipitated metals contaminants into the solution phase which makes it easier to transport; this can be done by acidification.

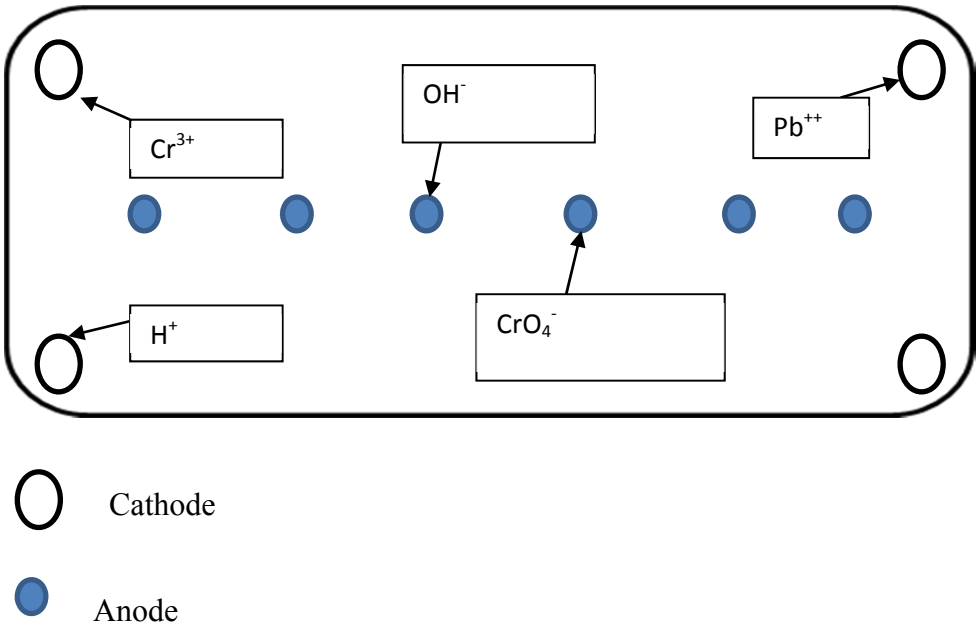

Figure 2-2: Movement of ionic species under electokinetics

CHAPTER 3

METHODS AND MATERIALS

3.1 Microbial Cultures and Media

3.1.1 Source of Cr(VI) Reducing Organisms

Cr(VI) reducing bacteria were sourced from dried sludge from the sand drying beds at the Brits Wastewater Treatment Works (North West Province, South Africa). The Cr(VI) content in the dried sludge at the time of sampling was measured at 25.44 mg/L (Molokwane, 2010). Bacteria sourced from the above environment was thus expected to be resistant to Cr(VI) toxicity. Organisms from the sludge were cultured by adding 0.2 g of sludge to 400 mL sterile Luria-Bertani broth (LB) prepared as described in 3.1.2 below, spiked with 50 to 75 mg/L Cr(VI) to select for Cr(VI) reducing organisms followed by incubation under continuous shaking for 24 hours. 1 mL samples from the inoculums culture were plated on agar plates and the colonies which formed were subcultured and tested for Cr(VI) reducing capability individually.

3.1.2 Media for Culture Enrichment and Isolation

The media used for culture enrichment and isolation were Luria-Bertani (LB) broth, Luria-Bertani (LB) agar and Plate count (PC) agar (Merck, Johannesburg, South Africa) amended with 50-75 mg/L Cr(VI) to ensure selection for Cr(VI) resistant organisms. The growth media was prepared as per manufacturer's instructions. The different broth and agar media were prepared by dissolving (in 1 L distilled water) 25 g powder of Luria-Bertani (LB) broth, 43 g powder in 1 L for LB agar and 23 g powder in 1 L for PC agar. The solutions were sterilized by autoclaving for 15 minutes at 121˚C. PC and LB agar were allowed to cool and then aseptically poured into agar plate.

3.1.3 Vogel Bonner Broth (VB)

Vogel Bonner (VB) broth concentrate was prepared by adding 50 g K_2HPO_4 (di-potassium phosphate); 17.5 g $Na(NH_4)HPO_4 \cdot 4H_2O$ (Sodium ammonium phosphate); 1g $MgSO_4 \cdot 7H_2O$ (magnesium sulfate), and 10 g of citric acid into 100 mL distilled water. Glucose concentrate was made up by adding 50 g of D-glucose powder in 100 mL of distilled water. The VB concentrate and glucose concentrate were sterilized by autoclaving at 121 ˚C for 15 minutes. The media growth was prepared from the above mentioned concentrates by adding 10 mL of VB concentrate and 10 mL of the glucose concentrate to 500 mL of autoclaved distilled water.

3.2 Chemical Reagents

3.2.1 Chemicals

Sulphuric acid solution (37 N) was prepared by dissolving 2.77 mL of acid in a 100 mL of distilled water. The acid was used in the analysis of Cr(VI) reduction. Sodium chloride solution (0.85% NaCl) was prepared by dissolving 0.85 g of sodium chloride salt in 100 mL distilled water. The solution was autoclaved at 121°C for 15 minutes. Hydrochloric acid that was used to solubilize the precipitate was prepared by diluting 1L of 37 N HCl into 10 L of distilled water. All the chemicals were of analytical grade obtained from Sigma Aldrich, Johannesburg, South Africa.

3.2.2 Cr(VI) Stock Solution

1000 mg/L of Cr(VI) stock solution was prepared by dissolving 3.76 g of 99% purity K_2CrO_4 (Analytical Grade) into 1 L of distilled water. The 1000 mg/L concentration of the Cr(VI) stock solution was used to prepare the desirable Cr(VI) concentration throughout the experimental studies.

A linear graph/calibration curve with the regression of 99% was obtained by diluting the stock solution with distilled water to give the desired final Cr(VI) concentration (0.1, 0.2, 0.3, 0.4, 0.6, 0.8 mg/L). The linearized standard curve was generated by plotting the absorbance of the known Cr(VI) concentrations at the wavelength of 540 nm (refer to 3.5). The generated standard curve was then used to estimate the unknown Cr(VI) concentration in the sample.

3.3 Characterization of Microbial Species

3.3.1 Gram Staining

For gram staining the Hucker method was used (APHA, 2005). A 1 mL bacteria sample from 24 hour cultivated cells was spread on a film and dried over a flame. The heat-fixed film was then immersed in crystal violet and then left to air-dry for 1 min. The film was gently and directly washed in a tap water stream for 2 sec. The film was then immersed in iodine mordant for 1 min and gently washed in a tap water stream for 2 sec. The slide was then immersed in Safranin solution for 30 sec and gently washed under tap water stream for 2 sec. The slide was then immersed in 95% vol/vol ethanol for 5 sec, and then gently washed under a stream of tap water for 2 sec. The slide was then dried with absorbent paper, the 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. The cells were differentiated by the color observed: black-violet for Gram-positive; and red-pink for gram-negative cells.

3.3.2 General characterization of isolated cultures using 16S rRNA

The phylogenetic characterization of cells was then performed by homogenizing the 1 gram of the soil sample in 9 mL ringer solution. The cultures in the 7^{th} - 10^{th} test tube in the serial dilution preparation were streaked on LB agar plate and incubated at 30 ± 7˚C for 18 hours. The fingerprint method was used to obtain DNA sequence of pure isolated cultures in order

to find out the 16S rRNA (16 Svedburg unit ribosomal Ribo-Nucleic-Acid). The first step in 16S rRNA sequence identification is the classification of the colonies based on morphology. For the mesocosm study ten different morphologies were identified. These cultures were streaked onto nutrient agar followed by incubation at 33 ± 7˚C for 18 hours.

A DNeasy tissue kit (QIAGEN Ltd, West Sussex, UK) was used to determine the genomic DNA extracted from the pure cultures according to the instructions of the manufacturer. The 16S rRNA genes of the 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). An internal primer pD was used for sequencing (corresponding to position 519-536 of the 16S gene). The resulting sequences were deposited in the Genbank to be compared to known bacteria using a basic BLAST tool search of the National Centre for Biotechnology Information (NCBI, Bethseda, MD).

3.4 Batch Reactor Studies

3.4.1 Anaerobic Cr(VI) Reduction Experiments

The dried sludge culture cells were grown anaerobically in a 1 L Erlenmeyer flask containing 400 mL LB broth for a period of 24 hours. Cells were then collected under anaerobic conditions by centrifuging at 6000 rpm at 4˚C for 10 min. The supernatant was decanted and the remaining pellet was washed three times in a sterile saline solution (0.85% NaCl) under an anaerobic glove bag purged with 99% N_2 gas. 100 mL serum bottles were used to conduct anaerobic Cr(VI) reduction experiment by adding Cr(VI) stock solution into the VB broth to give the desirable final Cr(VI) concentration of 30 mg/L, 50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L and 400 mg/L. Before the serum bottles were inoculated with viable cells, 1 mL of the sample was withdrawn from each serum bottle at various Cr(VI) concentrations to determine the absorbance of Cr(VI) prior to inoculation. The harvested cells were then transferred into

100 mL serum bottles under an anaerobic glove bag. The bottles were then purged with 99% N_2 gas for 10 minutes to expel any oxygen gas before sealing with silicone rubber stopper and aluminium seals. The serum bottles were incubated at 30 ± 2˚C with continuous shaking on a lateral shaker (Labotec, Gauteng, South Africa) at 120 rpm. Cr(VI) reduction was monitored by withdrawing 1mL of the sample hourly using a sterile syringe. The withdrawn samples were then centrifuged using a 2 mL Eppendorftube at 6000 rpm for 10 minutes in a Minispin® Microcentrifuge (Eppendorf, Hamburg, Germany) before Cr(VI) analysis, this was done to remove suspended cells.

3.4.2 Aerobic Cr(VI) Reduction Experiments

The dried sludge culture from Brits was used to conduct the aerobic batch studies. The cells were grown aerobically for 24 hours in a 1 L Erlenmeyer flask containing 400 mL of LB broth. The cells were collected by centrifuging at 6000 rpm at 4˚C for 10 minutes. The supernatant was decanted and the remaining pellet was washed three times in a sterile saline solution (0.85% NaCl) while centrifuging. Cr(VI) reduction experiments were conducted in a 250 mL Erlenmeyer flask by adding Cr(VI) stock solution into 100 mL VB broth to give the desired effective final concentration of 30, 50, 75, 100, 200 and 400 mg/L. Before inoculating the flasks with harvested cells, 1 mL of the sample was extracted from the flask to determine the absorbance of Cr(VI) before adding the cells in each flask. The 250 mL Erlenmeyer flasks containing the viable cells were plugged with cotton wool to allow aeration at the same time filtering away microorganisms from the air and then incubated at 30 ± 2˚C while continuous shaking on a lateral shaker (Labotec, Gauteng, South Africa) at 120 rpm. To monitor Cr(VI) reduction, 1 mL of the sample was withdrawn every hour for the first 15 hours and then centrifuged using a 2 mL eppendorf tubes at 6000 rpm for 10 minutes and the supernatant was used for Cr(VI) concentration analysis.

3.5 Cr(VI) Analysis

For Cr(VI) reduction analysis a UV/VIS spectrophotometer (WPA, Light Wave II and Lebotech, South Africa) was used. The measurement of Cr(VI) reduction was carried out using the following procedure: In a 10 mL volumetric flask, 0.2 mL of the sample was acidified with 1 mL 1N H_2SO_4, filled to 10 mL level with distilled water, followed by 0.02 mL of 1,5 diphenylcarbazide (DPC). The mixture was left standing for 3 minutes to allow the development of the violet-purple color. The fully developed sample was then measured for absorbance at the wavelength of 540 nm in a calibrated instrument.

3.6 Total Cr Analysis

Total Cr was measured at a wavelength of 359.9 nm using a Varian AA-1275 Series Flame Atomic Adsorption Spectrophotometer (AAS) (Varian, Palo Alto, CA) equipped with a 3 mA chromium hollow cathode lamp. The 10 mL of the sample was digested with concentrated 1 mL of nitric acid (HNO_3) before analysis. Cr(III) was determined as the difference between total Cr and Cr(VI) concentration. AAS was calibrated prior to total Cr analysis using 1-5 mg/L Cr(VI) concentration prepared from the Cr(VI) stock solution and 2.5% nitric acid.

3.7 Continuous Flow System Studies

3.7.1 Reactor Setup

Design of Mesocosm System

The design of the mesocosm system was adapted from the system design used by Molokwane (2010). A simulation of the reactive barrier mesocosm in the form of an open tank with the dimensions 123 × 52 × 50 cm (L × B × H) was constructed from Plexiglass ® (Evonik Rohm GmbH, Essen, Germany) and reinforced by steel bars (Figure 3-1). Aquifer medium from the target site was compacted to a compaction consistent with the ground conditions. During the packing process nine sampling ports of 30 cm in length and 11 mm diameter glass tubing

were inserted in the aquifer medium. Sample ports were strategically placed to capture the longitudinal concentration across the continuous flow reactor.

The ports were all placed horizontally at the same length in the aquifer material; in order to evaluate Cr(VI) concentration profile at the same depth. Three sampling ports were placed upstream of the barrier to evaluate the conditions of chromium before the water enters the barrier zone (Z1). The remaining six sampling ports represented by zone (Z2) and zone 3 (Z3) were placed after the barrier to assess the performance of the barrier.

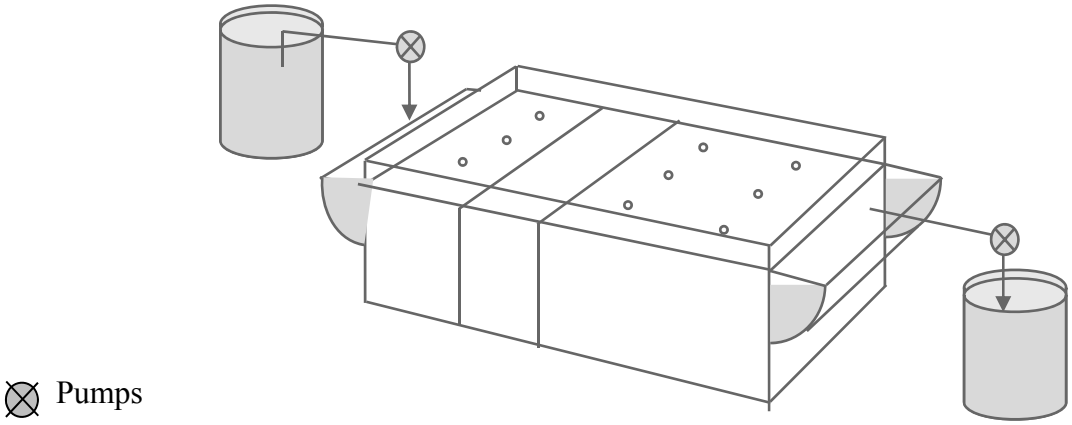

Figure 3-1: Mesocosm reactor setup using soil collected from Cr(VI) contaminated site in Brits, North West.

3.7.2 Start-up culture

A consortium culture from the dried sludge was mixed with the soil and packed in the microbial reactive barriers. Characterization of the bacteria in the sludge was done and a phylogenetic tree was produced.

3.8 Remobilization Procedure

3.8.1 Design of Electrokinetic System

The electrokinetic system was set up in the reactor as depicted in Figure 3-2. The experiment apparatus consisted of four principle parts: soil cell; three graphite electrodes compartments; three hollow PVC tubes and power supply. Two of the electrodes were cathodes and one was

an anode. The electrodes were placed in the PVC tubes that firmly placed in the soil. The PVC tubes measuring 50 cm in length and a diameter of 4 cm had holes drilled on the sides, to allow uniform contaminant flow. The tubes were enclosed in HDPE monofilament nets this was to prevent soil particles from flowing into the electrode compartment. The electrodes were connected to a Variable power supply with a DC rectifier. A 10 amps circuit breaker was installed into the power supply for as a safety precaution and during the experiment the temperature, current and voltage were monitored.

3.8.2 Soil Washing Reagent Preparation

For metal extraction 0.1% HCl was used to remove the maximum chromium accumulated. A low costing commercial HCl (Merck) was used as the extracting agent with the following characteristics: 37% purity and 1.19 kg/L density.

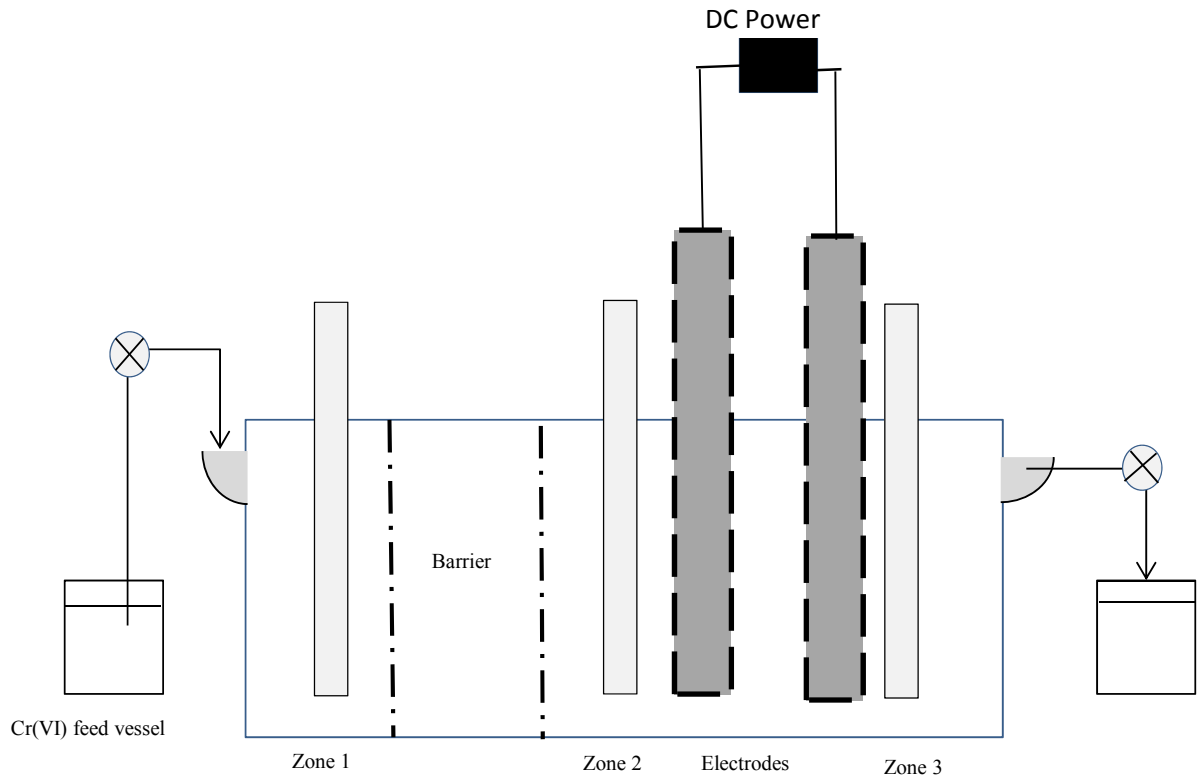

Figure 3-2: Mesocosm reactor with electrokinetics compartment.

3.8.3 Sample Analysis

Total chromium determination: 1 ml of the sample was added to 2 ml of nitric acid in a 10 ml flask and diluted with distilled water to the mark. Total chromium analysis was done using an AAS.

Trivalent chromium determination: the content of Cr(III)was calculated by subtracting Cr(VI) from total chromium.

3.8.4 Procedure for Soil Washing

The remobilization technique used in this experiment was soil washing by using a single agent- 0.1 M HCl. Soil washing involves the separation of contaminants that are sorbed on fine soil particles with liquid. 0.1 M HCl was made up in a 10 L bucket and this was fed into the reactor from the influent tray. The acid was allowed to flow through the system for three weeks, during this time the reading of Cr(VI) and total chromium was still being carried out. Soil properties were to be investigated before soil washing was carried out as these properties influence the efficiency of soil washing. The soil analyses were conducted by the Department of Plant Production and Soil Science, University of Pretoria.

3.9 Soil Analysis

3.9.1 Soil pH

The pH of the soil was determined in a 1:2,5 soil/water ratio suspension on a mass basis. A pH meter reading reproducible to 0.05 pH units was calibrated with commercially available buffer solutions, pH = 4.0; 7.0 and 8.0. The drift in the pH meter was compensated by re-calibrating the meter hourly. To determine the pH, 10 grams of dried soil was placed in a glass beaker with 25 mL de-ionised water and the content was stirred for 5 seconds with a glass rod. After 50 min the mixture was allowed to stand for 10 minutes and the pH was

determined with the electrodes positioned in the supernatant. The results are reported as pH (H_2O) (Table 3-2).

3.9.2 Extractable Cations: Ammonium Acetate

The nutrient status in the soil is represented by extractable cations Ca^{2+}, Mg^{2+}, K^+ and Na^+. 1 mol dm^{-3} reagent of NH_4OAc solution, was used and it was prepared by diluting 57 ml glacial acetic acid (99,5% AR) with de-ionised water to a volume of 500 mL. 69 mL of concentrated ammonia solution was added to the diluted solution of acetic acid. The solution was mixed well and diluted to about 900 mL with de-ionised water and the pH was adjusted by adding acetic acid or ammonia solution. 5 ± 0.05 g of air dried soil was added to 50 cm^3 NH_4OAc solutions that is cooled at 20 ± 2°C in the extraction bottle. The bottle was then shaken horizontally on a reciprocation shaker at 180 oscillations per minute for 30 minutes. The liquid is filtered rapidly through a Buchner funnel with suction, the filtrate was collected but the first few drops are discarded. The filtrate is further re-filtered if the extract is not clear. The analysis of cations K^+, Ca^{2+}, Mg^{2+} and Na^+ in the filtrate were determined by either flame emission or atomic absorption spectroscopy where applicable.

Ammonium acetate extractable cations = $\frac{b \times 50}{5}$ mg/kg^{-1}

Where:

b = mg dm^{-3} of Ca, Mg, Na and K in the extract

3.9.3 Cation Exchange Capacity (CEC)

Introduction

Ammonium acetate solution (1 mol dm^{-3}) serves as extractant for exchangeable and water soluble cations (Schollenberger and Simon, 1945). The maximum exchange occurs in a few minutes.

In the presence of free lime and gypsum the most questionable cations extracted with this method are Ca^{2+} and Mg^{2+}. In the case of soils containing free lime or gypsum, this method should not be used if accurate results for exchangeable Ca^{2+} and Mg^{2+}or CEC are required. The level of extractable potassium may increase on drying of some soils. However, soil samples can be extracted in a moist state.

The water soluble cations are determined separately in soils containing significant quantities (resistance < 460 ohms) of soluble salts. These are subtracted from extractable cations to obtain the exchangeable cations.

After the exchange complex has been saturated with the index cation, the adsorbed cation and the small amount of solution entrained by the soil after centrifuging can be directly displace by another salt solution, such as potassium chloride. Ammonia is separated by steam distillation (Bremner and Keeney, 1965) and is taken as equal to the CEC of the soil.

Efficiency of Method

This method is recommended by the Soil Science Society of America because it is highly repeatable, precise and a direct measure of a soil's CEC. The method detection limit is approximately 1.0 c mol kg^{-1} (or meq/100 vgm on a dry soil basis) and it is generally reproducible with ± 10%. Testing was run in three replicates so as to achieve 95% confidence.

Reagents

The following reagents where used in the analysis of CEC:

Ammomium acetate, 1 mol dm^{-3}, pH 7: 114 cm³ glacial acetic acid (AR) was diluted with de-ionised water to about 1 dm³. In a separate flask 138 cm³ concentrated ammonia solution was diluted with de-ionised water to a volume of 1 980 cm³ and the pH was adjusted to 7 by adding more ammonia solution if necessary. The final solution was made up to 2 dm³ with de-ionised water.

Ammonium acetate, 0.1mol dm^{-3}: 1 mol dm^{-3} solution was diluted ten times with de-ionised water.

Potassium chloride, 1 mol dm^{-3}: 74.4 g KCl (AR) was diluted in 1 dm³ de-ionised water.

Lanthanum chloride solution: 9.4 g La_2O_3 was dissolved in 500 cm³ de-ionised water in a 1 dm³ flask. While swirling slowly, 40 cm³ was added to concentrated hydrochloric acid. The solution was mixed well to dissolve oxide, before mixing with de-ionised water and filtering.

Boric acid indicator solution: 20 g boric acid (AR) was dissolved in about 700 cm³ of hot water and the solution was transferred into a 1 dm³ volumetric flask containing 200 cm³ ethanol (95%) and 20 cm³ mixed indicator solution was prepared by dissolving 0.330 g bromocresol green and 0.165 g methyl red in 500 cm³ ethanol (95%). After mixing the contents of the flask, about 0.05 cm³ of 1 mol dm^{-3} NaOH was carefully added until the indicator colour changed from pink to pale green.

Sulphuric acid: Standardized 0.05 mol dm^{-3}

Procedure

Cation Exchange Capacity Extraction

10 ± 0.1 g air-dried soil sample was placed in a 100 cm³ centrifuge tube with a stopper and the mass of the tube and soil was determined (X_1g). 50 cm³ 1 mol dm^{-3} ammonium acetate solution was added to the tube and the tube was shaken horizontally for 60 minutes. The sample was removed from the shaker and left overnight. The next day the tube was centrifuged at 2 000 to 5 000 rpm for 10 minutes to obtain a clear supernatant solution and the supernatant liquid was decanted into a 100 cm³ volumetric flask, without losing any soil.

Again 50 cm³ of 1 mol dm^{-3} ammonium acetate solution was added to the soil and the tube was shaken well by hand to ensure that the soil has dispersed properly (a vortex mixer was used if necessary). The tubes were then placed on the shaker for 30 minutes, centrifuged and the clear solution was decanted into the same 100 cm³ volumetric flask. The solution was made up to volume with ammonium acetate solution, the final solution was filtered and the solution was kept for the determination of Ca, Mg, Na and K (solution **A**).

50 cm³ of 0.1 mol dm^{-3} ammonium acetate solution was added to the soil in the centrifuge tube. The solution was shaken for 30 minutes ensuring that the soil has dispersed properly. The solution was centrifuged as before and the clear supernatant solution was decanted into a plastic storing bottle for the determination of NH_4^+ in the occluded solution (solution **B**). The mass of the centrifuged tube was determined plus soil and occluded solution (X_2g).

Finally 50 cm³ of KCl solution (1 mol dm^{-3}) was added to the soil in the centrifuge tube, the solution was shaken for 30 minutes as described. The tube was centrifuged and the supernatant solution was decanted into a 200 cm³ volumetric flask. The procedure was repeated with a second aliquot of 50 cm³ of KCl solution ensuring that the soil has dispersed

properly. The volumetric flask was filled to volume with 1 mol dm^{-3}KCl solution (solution **C**).

Cation Exchange Capacity Determination

10 cm³ of boric acid indicator solution was added to a 100 cm³ Erlenmeyer flask marked to indicate a volume of 50 cm³. The flask was placed under the discharge of the condenser of the stream distillation apparatus. In separate distillations, 5 cm³ of the ammonium acetate (solution **B)** or 10 cm³ of the KCl extract (solution **C**) was pipette into the distillation flasks. The volume was increased to about 20 cm³ with de-ionised water and 1 teaspoon (2.5 cm³) heavy MgO was added through a dry funnel into the bulb of the flask.

The distillation flasks were connected without delay to the steam generator and distilled to a volume of ± 50 cm³ into the flask containing the boric acid indicator. Distillation was stopped by opening the stopcock of the steam by-pass tube and then removed the distillation flask. The exit tube of the condenser was rinsed. NH_4^+ was determined by titrating with 0.05 mol dm^{-3} sulphuric acid. The colour change at the end point is from green to a permanent faint pink.

Cation exchange capacity (CEC) Calculations

$CEC = (T_1 \times 20) - (X_2 - X_1) \times 0{,}2 \times T_2$ cmol(+) kg^{-1}

Where: T_1 = titration value for KCl solution

T_2 = titration value for ammonium acetate solution

X_1 = mass of tube plus soil (g)

X_2 = mass of tube plus occluded solution (g)

3.9.4 Organic Matter Determination

Purpose

This test is performed to determine the organic content of soils. The organic content is the ratio, expressed as a percentage, of the mass of organic matter in a given mass of soil of the dry soil solids.

Significance

Organic matter influences many of the physical, chemical and biological properties of soils. Some of the properties influenced by organic matter include soil structure, soil compressibility and shear strength. In addition, it also affects the water holding capacity, nutrient contributions, biological activity, and water and air infiltration rates.

Efficiency of Method

In order to achieve 95% confidence of the measured value, three replicates of the sample was tested. The method has a detection limit of approximately 0.10% and is generally reproducible with ± 20%.

Equipment

Muffle furnace, balance, porcelain dish, spatula and tongs

Test Procedure

1) The mass of an empty, clean, and dry porcelain dish was determined (M_P)
2) The entire or part of oven-dried test specimen was placed in the porcelain dish and the dish was weighed and the mass of the dish and soil specimen was recorded (M_{PDS}).
3) The dish was placed in a muffle furnace and the temperature was gradually increased to 440˚C. The specimen was left in the furnace overnight.

4) The porcelain dish was removed carefully using tongs, and it was allowed to cool to room temperature. The mass of the dish containing the ash (burned soil) was determined and recorded (M_{PA}).

Data Analysis:

1) Determine the mass of the dry soil.

$$M_D = M_{PDS} - M_P$$

2) Determine the mass of the ash (burned) soil

$$M_A = M_{PA} - M_P$$

3) Determine the mass of organic matter

$$M_O = M_D - M_A$$

4) Determine the organic matter content

$$\left(OM = \frac{MO}{MD} x\ 100\right)$$

Standard Reference

ASTM D 2974 – Standard Test Methods for Moisture, Ash, and organic Matter of Peat and Organic Soils.

3.9.5 Particle Size Distribution

Soil particles are discrete units comprising the solid phase of the soil. They generally cluster together as aggregates, but can be separated from one another by chemical and mechanical means. Particles have diverse composition and structure and generally differ from one another in both size and shape. The method described will apply only to the inorganic particles, typically single crystalline fragments. The particle size distribution of a soil expresses the proportions of the various sizes of particles it contains. The methods of fraction and particles size analysis described are limited to sieving and sedimentation procedures.

Particle size classes used for describing soil are given in Table 3-1

Reagents

Hydrogen peroxide (H_2O_2): 30-35 (%) volume percent

Procedure

Coarse fraction (>2 mm)

- Spread the entire field sample on a large sheet of paper or plastic and leave to air dry. Determine the mass of the sample, then after gently crushing the sample in a porcelain mortar and pass the sample through a 2 mm sieve. If fine earth adheres to the larger particles, wash the coarse material with water.

Table 3-1: Particle size class of the soil

Class	Diameter (mm)	Method of separation
Gravel	>2	Sieve
Coarse sand	2.0 – 0.5	Sieve
Medium sand	0.5 – 0.25	Sieve
Fine sand	0.25 – 0.106	Sieve
Very fine sand	0.106 – 0.05	Sieve
Coarse silt	0.05 – 0.02	Sedimentation
Fine silt	0.02 – 0.002	Sedimentation
Clay	<0.002	Sedimentation

Fine soil (≤ 2 mm)

- Firstly the mass of the dried washed >2 mm particle is determined and expressed as a percentage of the entire sample.

- The mass of the representative air-dried sample that is ≤ 2 mm is determined (10 g for clays, 20 g for loams, 40 g sandy loams and 80 g for sands).

Calculation

A = mass (g) of sand fraction on sieve

B = mass (g) of pipetted coarse silt plus fine silt

C = mass (g) of pipetted fine silt plus clay

D = mass (g) of pipetted clay

E = mass correction of dispersing agent (0.01 g)

F = mass (g) of pretreated oven dry total sample

G = mass (g) of residual silt and clay that passed through the 0.053 mm sieve

Sand fractions:

Percentage of sieved sand fractions = $\frac{\text{Ax } 100}{\text{F}}$

Silt and clay fractions:

Percent coarse silt = $\frac{(\text{B}-\text{C})\text{x}1000\text{x}100}{\text{Fx } 25} + \frac{\text{Gx } 100}{\text{F}}$

Percent fine silt = $\frac{(\text{C}-\text{D})\text{x } 1000 \text{ x } 100}{\text{Fx}25}$

Percent clay = $\frac{(\text{D}-\text{E}) \text{ x } 1000 \text{ x } 100}{\text{F x } 25}$

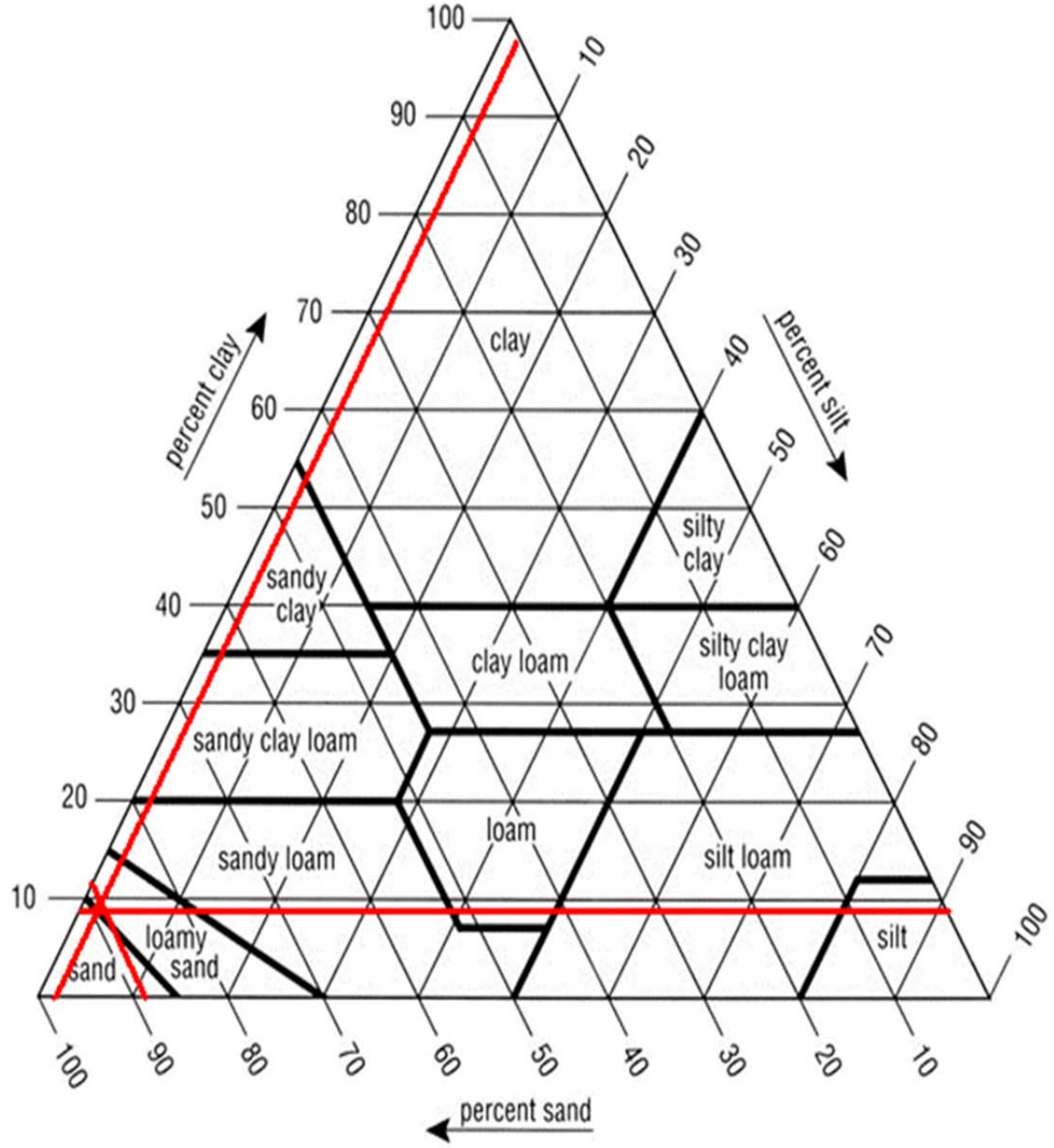

Figure 3-3: Textual triangle for soil classification

The results obtained from the particle size soil analysis were used to determine the soil classification using a textual triangle. The soil used in the experiment was found to be loamy sand soil.

Table 3-2: Soil Properties of aquifer material

pH	**8.0**
Cation (mg/kg)	
• Calcium	2978
• Potassium	17
• Magnesium	386.5
• Sodium	10
• Phosphorus	0.8
Particle size	
• Coarse sand	86.4%
• Silt	1.95%
• Clay	8.1%
Cation Exchange Capacity	22.34 $cmol.kg^{-1}$
Organic carbon	4.38 %
Porosity	0.38

CHAPTER 4

CR(VI) REDUCTION BATCH KINETICS STUDIES

4.1 Preliminary Studies

The batch experiments were conducted under aerobic and anaerobic conditions. Results from anaerobic and aerobic batches showed complete removal of Cr(VI) from solutions in reactors with initial concentrations of 30, 50, 75 mg/L and 100 mg/L. The results showed that higher rates of Cr(VI) removal were possible under anaerobic conditions. However, the microbial culture performed better at higher concentrations compared to anaerobic cultures at higher concentrations. The removal was associated with Cr(VI) reduction since a precipitate accumulated with time in the batches. Results were validated by analyzing total Cr and drawing a mass balance on the Cr species at the end of the experiment. The results obtained from both experiments were used later in the development of batch kinetic model.

4.2 Batch Cr(VI) Reduction Kinetics

4.2.1 Batch Cr(VI) reduction under Aerobic Conditions

Preliminary studies under aerobic conditions were conducted at varying initial Cr(VI) concentrations of 30 – 400 mg/L. The range 30 – 400 mg/L was selected for the batch studies to determine the optimum concentration for Cr(VI) reduction rate. Earlier studies suggest that Cr(VI) reduction rate decreases with increasing initial Cr(VI) concentration due to toxicity effect of Cr(VI) on cells (Meli, 2009; Mtimunye and Chirwa, 2014).

Figure 4-1 shows that Cr(VI) reduction trends in aerobic batches in which, at the concentrations of 30 mg/L, 50 mg/L and 75 mg/L, more than 89% of Cr(VI) was removed after incubation for 16 hours. When the experiment was terminated at 55 hours, 70% was reduced at an initial concentration of 200 mg/L and only 32% Cr(VI) reduction was observed

from initial concentration of 400 mg/L. Cr(VI) reduction and the rate of reduction decreased with an increase in initial Cr(VI) concentration. The results suggest that increased Cr(VI) toxicity affected the reduction capacity of cells which resulted in a reduced Cr(VI) reduction rate of high Cr(VI) loadings. These results confirm earlier observed results in batch studies by Wang and Shen (1997); Molokwane *et al*., (2008); and Mtimunye (2011).

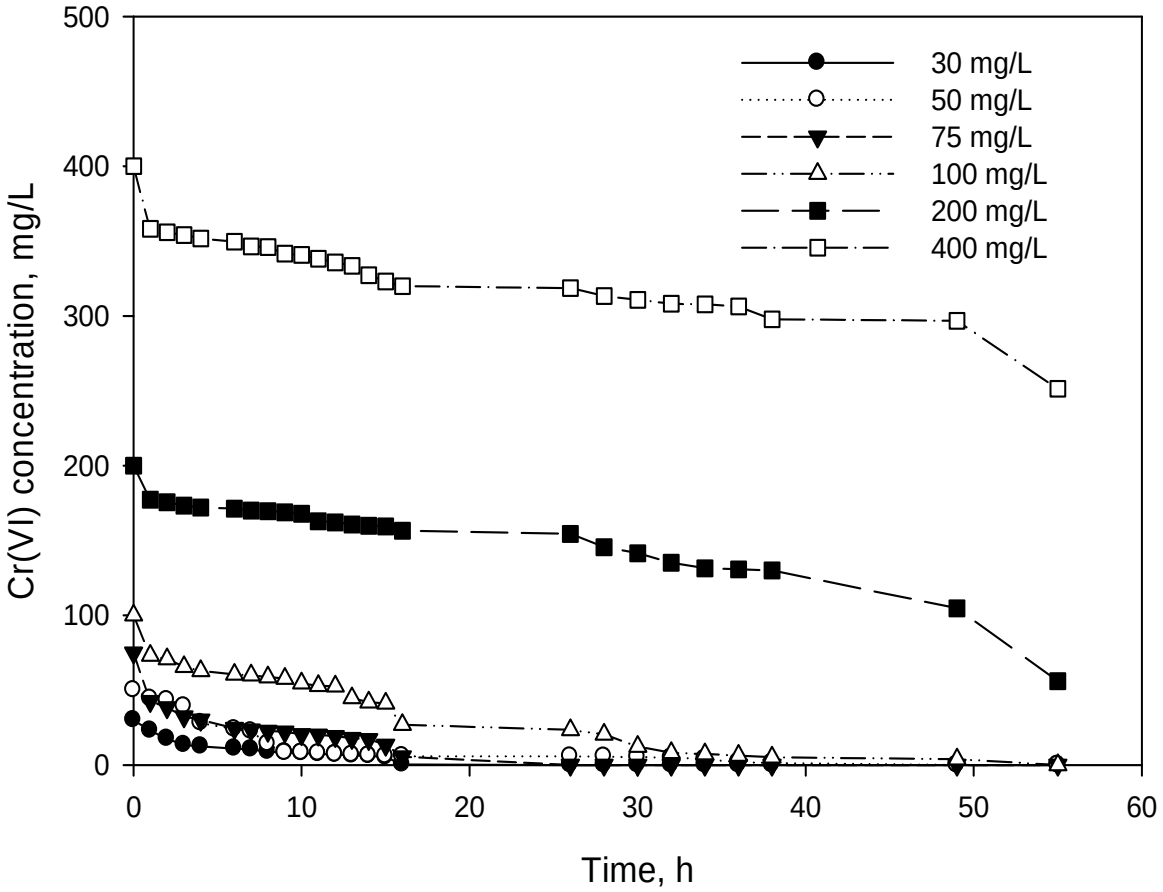

Figure 4-1: Batch Cr(VI) reduction in aerobic conditions (30-400 mg/L)

4.2.2 Batch Cr(VI) Reduction under Anaerobic Conditions

In situ bioremediation processes such as the one simulated in this study are normally closed systems. Oxygen supply in underground systems may be very limited. In the absence of oxygen, Cr(VI) may act as an electron sink during oxidation of organic compounds (Lovley and Phillips, 1994). Earlier experiments by Chirwa and Wang (2000) using anaerobic cultures

of *Escherichia coli* ATCC 33456 showed that indeed Cr(VI) may serve as an electron sink to derive energy that could be used for cell growth. Higher Cr(VI) reduction rates were evident in the anaerobic batches investigated in the range of initial Cr(VI) concentrations at the temperature of 30±2˚C and pH 7±0.2 (Figure 4-2). The results in Figure 4-2 show that at lower concentrations (19 – 94.33 mg/L), complete reduction of Cr(VI) was observed. Complete reduction at the initial concentration of 19 mg/L was achieved within 2 hours a much shorter period, compared to 15 hours in corresponding aerobic batches. Cr(VI) reduction at 200 mg/L showed incomplete reduction after 55 hours of incubation and less than 48% of Cr(VI) was reduced at the concentration of 400 mg/L as only 16% Cr(VI) reduction was recorded.

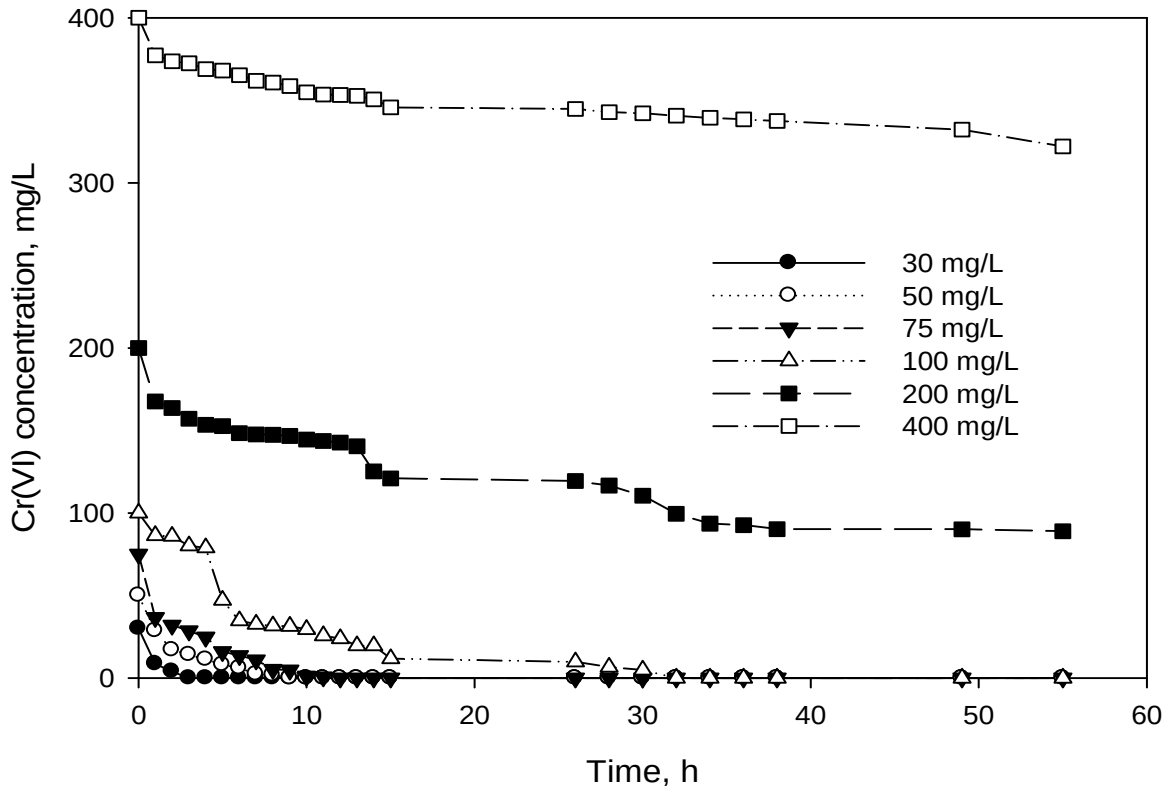

Figure 4-2: Batch Cr(VI) reduction in anaerobic conditions (30-400 mg/L)

The inhibitive effect of Cr(VI) was also observed in the anaerobic batches with a decreased Cr(VI) reduction capacity in cells resulting in incomplete Cr(VI) reductions in cultures at 200 mg/L and 400 mg/L initial Cr(VI) concentration.

The overall performance of the batch study under anaerobic condition showed better performance over aerobic Cr(VI) reduction. Similar findings were observed by Bopp and Ehrlich, (1988); Wang *et al.,* (1989); Srinath *et al.,* (2002). The microbial species used in anaerobic and aerobic batch studies were from the same source. However, from the results obtained it can be concluded that the microbial cells favored an oxygen depleted environment thus concluding that the cultures were facultative anaerobes leading to their predominant performance over reduction under aerobic conditions.

4.3 Kinetic Modeling Theory: Enzyme Kinetics

Enzymatic reduction of chromate by chromium reducing bacteria can occur under aerobic and anaerobic conditions (Pal *et al*., 2005). Wang *et al.* (1989) found that Cr(VI) reduction under anaerobic conditions is caused by respiratory chain system of the cell membrane. In the absence of oxygen Cr(VI) serves as an electron terminal acceptor in the respiratory chain for large electron donors like hydrogen NAD(P)H, fat and protein (Wang, 2000; Cheung and Gu, 2007). Bopp and Ehrlich (1988) found that constructive enzymes mediate the transfer of electrons from NADH to chromate. Under anaerobic conditions, reduction of Cr(VI) is catalyzed by membrane-bound or soluble reductase which receives electrons from the cytochrome system (Wang and Shen, 1995).

Enzymes facilitate the reduction of Cr(VI) and they are not substrate specific for Cr(VI) (Viamajala *et al,* 2003; Mtimunye, 2011). In order to make sense of the observed trends in Cr(VI) reduction in aerobic and anaerobic batches, Meli (2009) suggested the following:

1. Cr(VI) reduction is catalyzed by a single or dual-site enzyme.
2. The Cr(VI) reduction sites on the enzyme are non-renewable, such that new enzymes are required to be produced to reduce new load or continue reducing Cr(VI).
3. The enzyme is either regulated or induced, i.e., is produced when the cell is exposed to Cr(VI).
4. In a mixed culture system, several Cr(VI) reducing species of bacteria exist. However, the Cr(VI) reducing activity of the whole culture may be represented by a common effect, the sum of, or the highest of all the activities in all the Cr(VI) reducing species i.e. in the consortium.
5. The sum of, or the highest of the activities ΣE_i, may be represented by one representation enzyme, *E*.

Due to assumption 1 and the fact that there are different enzymes that act together and thus have a common effect on a consortium culture; this effect can be represented by one complex enzyme, *E*.

The enzymatic reaction can be represented as a single enzyme kinetic model (Meli, 2009):

$$\mathrm{Cr(VI)} + E \underset{k_1}{\overset{k_2}{\leftrightarrows}} E^{*}\mathrm{Cr(VI)} \xrightarrow{k_3} E + \mathrm{Cr(III)} \qquad (4\text{-}1)$$

Where: *E* = enzyme, *E**Cr(VI) = enzyme-Cr(VI) complex, k_1= rate constant for complex formulation, k_2 = rate constant for reserve complex formulation, k_3= rate constant for Cr(III) formulation. Directly from Equation (4-1):

Let Cr(VI) = C and *E**Cr(VI) = *E**

Therefore the rates of the above equation would be as follows:

$$\frac{dE^*}{dt}\Big|_{k=1} = k_1 C E_T \tag{4-2}$$

$$\frac{dE^*}{dt}\Big|_{k=2} = k_2 E^* \tag{4-3}$$

$$\frac{dE^*}{dt}\Big|_{k=3} = \frac{dCr(III)}{dt} = k_3 E^* \tag{4-4}$$

The rate formulation of E^* can be represented as:

$$\frac{dE^*}{dt} = k_1 C E_T - k_2(E^*) - k_3(E^*) \tag{4-5}$$

where: E_T (total complex and un-complex enzyme) = $E - E^*$

If the E*Cr(VI) reaction is quick, an equilibrium state can be stipulated with respect to the enzyme-Cr(VI) complex such that $\frac{dE^*}{dt} \approx$ approach a constant value as follows:

$$\frac{dE^*}{dt} = 0 = k_1 C_1(E - E^*) - k_2 E^* - k_3 E^* \tag{4-6}$$

Solving Equation (4-6) for E^* the following equation is obtained:

$$E^* = \frac{k_1 CE}{k_1 C + k_2 + k_3} = \frac{CE}{C + \frac{k_2 + k_3}{k_1}} \tag{4-7}$$

Then the Cr(VI) reduction rate becomes:

$$\frac{-dC}{dt} = \frac{k_3 CE}{C + \frac{k_2 + k_3}{k_1}} \tag{4-8}$$

Analogous to Monod kinetics, k_3 is analogous to maximum specific Cr(VI) reduction rate (k_m), E is analogous to biomass concentration (X) and $\frac{k_2+k_3}{k_1}$ is analogous to half saturation constant (K_c) (Chirwa and Wang, 2000).

$$\therefore \frac{-dC}{dt} = \frac{k_m C}{C + K_c} X \qquad (4\text{-}9)$$

Similar models were derived previously by other researchers such as Shen and Wang (1994), Mazierski (1995), Schmieman *et al*. (1998), and Guha *et al.* (2001). The following can be deduced from the model:

- The soluble reductase activity is the predominant mechanism of Cr(VI) reduction in bacterial systems.
- The model has appropriate expressions to cope with both toxicity and mutation effect of Cr(VI) during Cr(VI) reduction.
- The term R_c represents the capacity of reduction, the rate and extent of Cr(VI) reduction in bacterial system depends on the number of cells in the reactor.
- It can be concluded that the amount of Cr(VI) reduced under resting cells conditions will be proportional to the amount of cells inactivated by Cr(VI). The active biomass concentration is assumed to decrease with the increasing amount of Cr(VI)reduced due to toxicity (Shen and Wang, 1994; Mtimunye, 2011).

To represent the last statement in the model X (biomass concentration) in Equation 4-9:

$$X = X_0 - \frac{C_0 - C}{R_c} \qquad (4\text{-}10)$$

Integrating it into Equation (4-8):

$$-\frac{dC}{dt} = \frac{k_m C}{C + K_c}\left[X_0 - \frac{C_0 + C}{R_c}\right] \quad (4\text{-}11)$$

Where: k_m = maximum specific rate of Cr(VI) reduction (T^{-1}), K_c = half-velocity concentration (ML^{-3}), X_o = initial biomass concentration (ML^{-3}),C = Cr(VI) concentration at time, t (ML^{-3})**,**C_o= initial Cr(VI) concentration (ML^{-3}) and R_c= Cr(VI) reduction capacity of cells (MM^{-1}).

4.4 Parameter Determination

4.4.1 Parameter Estimation

The unknown kinetic parameters k_m, K_c, K, R_c and C_o were determined by performing a nonlinear regression analysis using the Computer Program for Identification and Simulation of Aquatic System (AQUASIM 2.0), (Riechert, 1998). For each parameter a search was carried out to estimate a range of values. Constrains were enforced to set upper and lower limits for each parameter this was done to remove nonsensical or invalid parameter values. Whenever optimization converged at/or very close to a constraint, the constraints were relaxed until the constraint no longer forced the model.

The aim was to obtain values that led to the best fit of the model. This was achieved by repeating the process until unique values lying within the constraints set between limits for each parameter were found. The least sum of squares between the observed and the modeled concentration was computed as follows for the function for parameter optimization:

$$\sigma^2 = \frac{1}{n-q}\sum_{i=1}^{i=n}(y_i - y)^2 \quad (4\text{-}12)$$

Where: σ = average deviation of model from the measured value, y_i = observed variables, y = simulated variables, n = number of observations and q = degrees of freedom representing the number of parameters being evaluated.

It was observed from the aerobic and anaerobic batch culture studies that the Cr(VI) reduction at lower concentration resulted in greater reduction compared to reduction at higher initial Cr(VI) concentration. It was for this reason that a non-competitive inhibition model that accounts for Cr(VI) toxicity threshold concentration was used instead of the enzymatic kinetic Cr(VI) reduction (Equation 4-10). However, Equation 4-10 could not describe Cr(VI) reduction under aerobic and anaerobic conditions as the kinetic process results showed that the high biodiversity of this species and slow growing culture are susceptible to toxic loading of Cr(VI). The non-competitive inhibition model used was as follows:

$$\frac{-dC}{dt} = \frac{k_m C}{K^{\left(1-\frac{C_r}{C_0}\right)}\left(K_c + C\right)}\left(X_0 - \frac{C_0 - C}{R_c}\right) \tag{4-13}$$

Where: k_m = maximum specific rate of Cr(VI) reduction (T^{-1}), K_c = half-velocity concentration (ML^{-3}), X_o = initial biomass concentration (ML^{-3}), C = Cr(VI) concentration (ML^{-3}) at time, t, C_o= initial Cr(VI) concentration (ML^{-3}), K = limiting constant (ML^{-3}) and R_c = Cr(VI) reduction capacity of cells (MM^{-1}).

4.4.2 Aerobic Batch Kinetics

The parameters were optimized simultaneously using 100 mg/L. The estimated parameters were used through the entire range of data and the results obtained were plotted against the measured data as shown in Figure 4-3. The model captured well the measured data; however there was a slight difficulty in fitting higher concentration of 100-400 mg/L, this could be due to the loss of viable biomass caused by the high Cr(VI) concentration (Table 4-1). However, it was observed that at higher concentrations, the microbial culture performed better than the anaerobic culture.

Table 4-1: Optimum kinetic parameter in aerobic batch cultures

Initial Feed Concentration (mg/L)	**Estimated concentration C_0 (mg/L)**	**K_c (mg/L)**	**$k_m(h^{-1})$**	**R_c(mg/mg)**	**X_o (mg/L)**	**χ^2**
30	23	999	0.997	0.9988	1079	41
50	49	999	0.997	0.9988	1241	379
75	49	999	0.997	0.9988	832	191
100	88	999	0.997	0.9988	500	1038
200	182	999	0.997	0.9842	500	3176
400	389	999	0.997	0.9681	500	1101

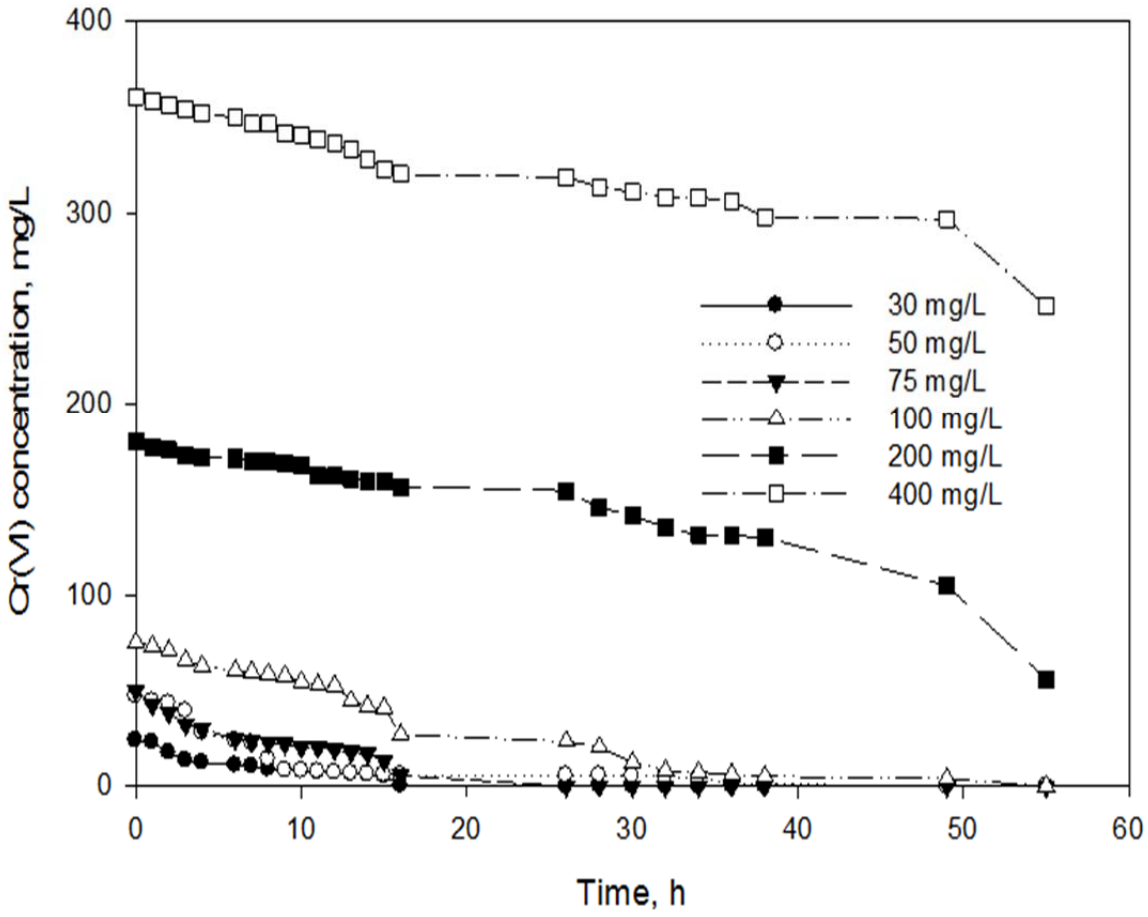

Figure 4-3: Aerobic batch culture model validation at 30-400 mg/L.

4.4.3 Anaerobic Batch Kinetics

Thermodynamically chromate reduction ($CrO_4^{2-} \rightarrow Cr^{3+}$) is favorable, Cr(VI) reduction to Cr(III) may be limited by reaction kinetics under physiological conditions (Glaze, 1990; Chirwa and Wang, 1999). However the kinetics of Cr(VI) reduction may be improved by coupling Cr(VI) reduction to other energy yielding reactions such as catabolism of organic compounds (Ishibashi *et al.*, 1990; Shen and Wang, 1995). During anaerobic Cr(VI) reduction Cr(VI) serves as a strong electron acceptor (Shen and Wang, 1994).

Equation 4-12 was used to determine the experimental data with initial Cr(VI) concentration of 30 mg/L and 100 mg/L were initially used to estimate the kinetic parameters k_m, K_c, R_c and

K. The validation of this model was performed that confirmed that the kinetic parameter values obtained at 30 mg/L simulated very well for low concentrations (50 and 75 mg/L), the kinetic parameters values obtained at 100 mg/L simulated data very well for higher concentration of 200 and 400 mg/L (Table 4-2).

Table 4-2: Optimum kinetic parameter in anaerobic batch cultures

Initial Feed Concentration (mg/L)	**Estimated concentration C_0 (mg/L)**	**K_c (mg/L)**	**$k_m(h^{-1})$**	**R_c(mg/mg)**	**X_o (mg/L)**	**χ^2**
30	19	11.6	0.221	0.964	752	1.5
50	48	11.6	0.221	0.964	532	66.3
75	64	11.6	0.221	0.964	523	225.6
100	90	2.36	0.118	0.105	888	1076.5
200	193	2.36	0.058	0.164	748	651.6
400	397	2.36	0.118	0.105	571	419.81

The model based on the parameters optimized in the 30 and 100 mg/L batch fitted well the rest of the experimental data as shown in Figure 4-4. Both the model predictions and experimental data indicated that the rate of Cr(VI) reduction decreased with continuous reduction of Cr (VI) and it finally ceased for cases where the initial Cr(VI) concentration exceeded 100 mg/L. The results indicate that R_c increase with the increase in concentration (Table 4-2). This is an indication that the bacterial species population decrease due to exposure of higher doses of Cr(VI) this may lead to the cell finite reduction capacity being reached due to Cr(VI) toxicity with cells.

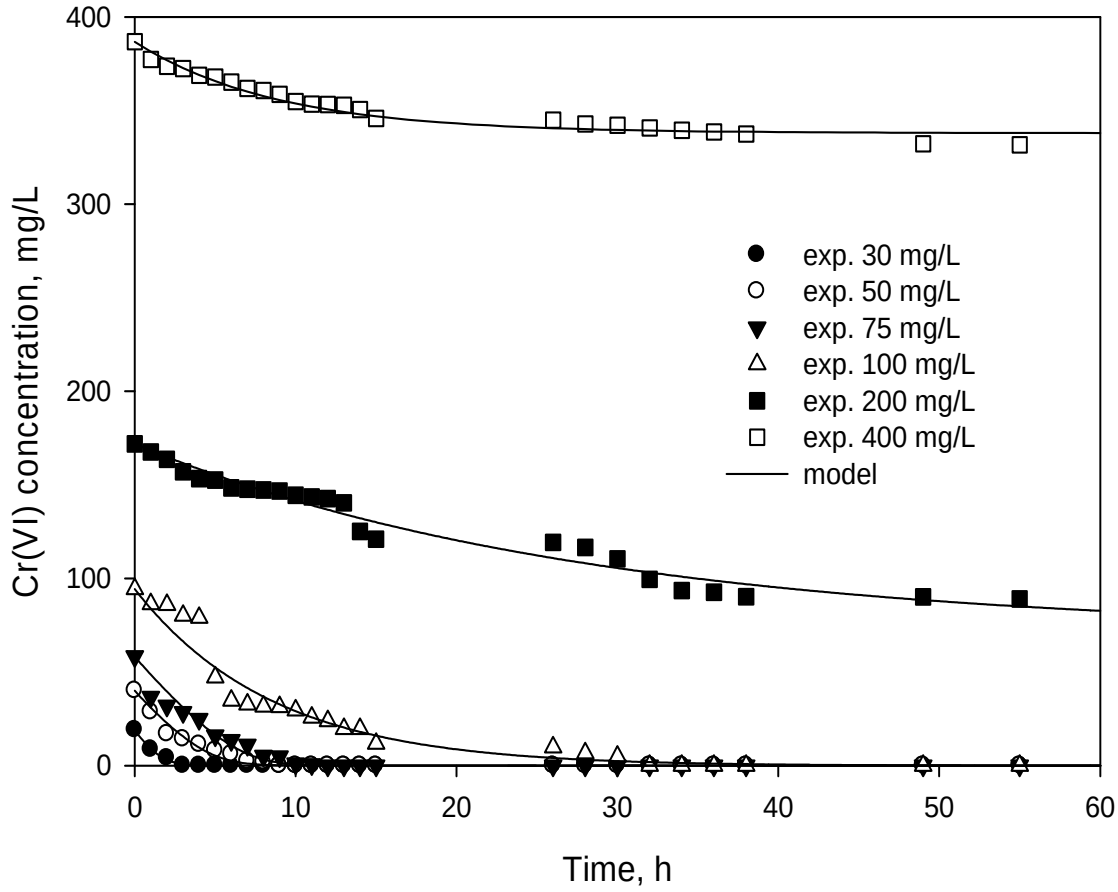

Figure 4-4: Anaerobic batch culture model validation at (30-400mg/L).

4.5 Sensitivity Analysis

Sensitivity analysis is used as it provides a systematic framework to study the accuracy and robustness of a mathematical model (Wang and Sheu, 2000). The sensitivity analysis under anaerobic and aerobic conditions is illustrated in Figure 4-5 and Figure 4-6, the time dependence of the sensitivity response curves for each kinetic parameter was done. For the aerobic culture, parameter sensitivity was conducted for the parameter K_c, K, R_c and k_m (Figure 4-5). The results show that the aerobic model was highly sensitive to K_c, K and k_m in the first 60 hours of incubation. The response was extremely high in the first 20 hours which indicates the period where the cell Cr(VI) reduction activity was high during that that period of incubation.

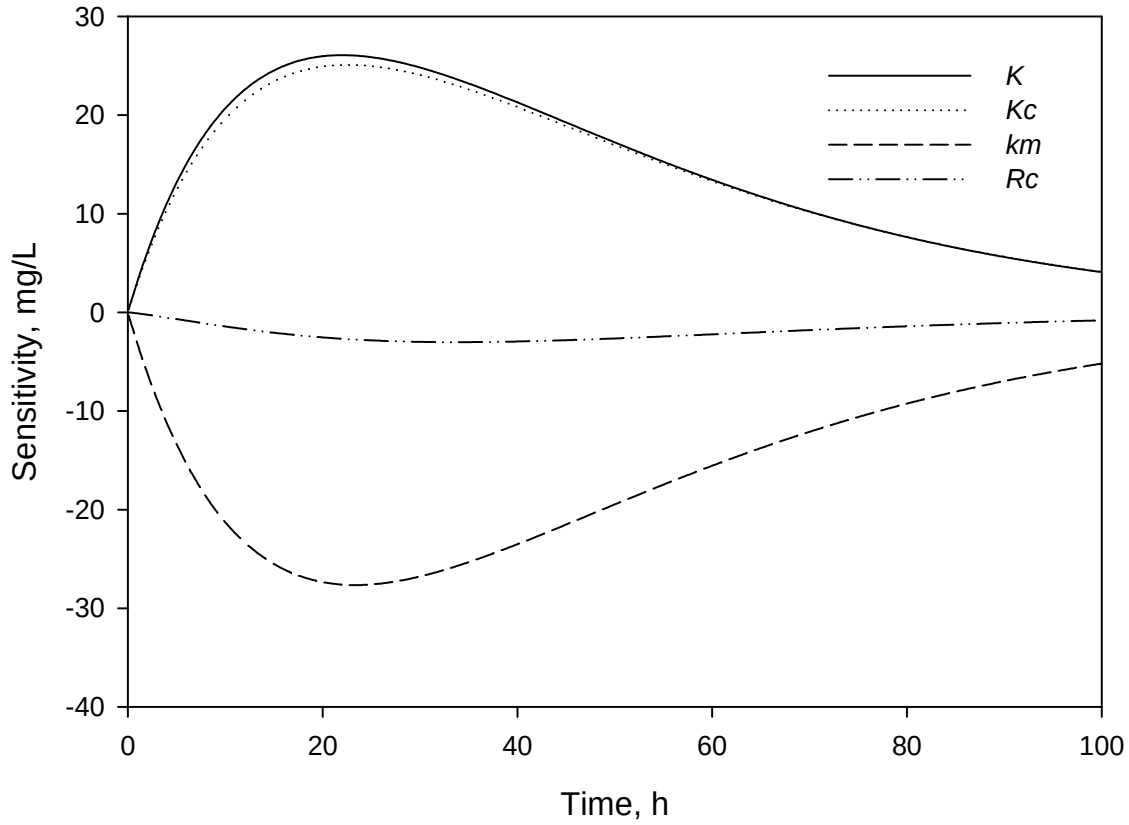

Figure 4-5: Aerobic sensitivity test at 100 mg/L

For anaerobic culture the following parameter k_m, R_c, K_c and K were evaluated. The results show that the anaerobic model was highly sensitive to minor adjustments in k_m, R_c and K in the first 40 hours of incubation (Figure 4-6). The response was extremely high in the first 10 hours of incubation which indicates that the Cr(VI) reduction rate activity was high during that period of incubation.

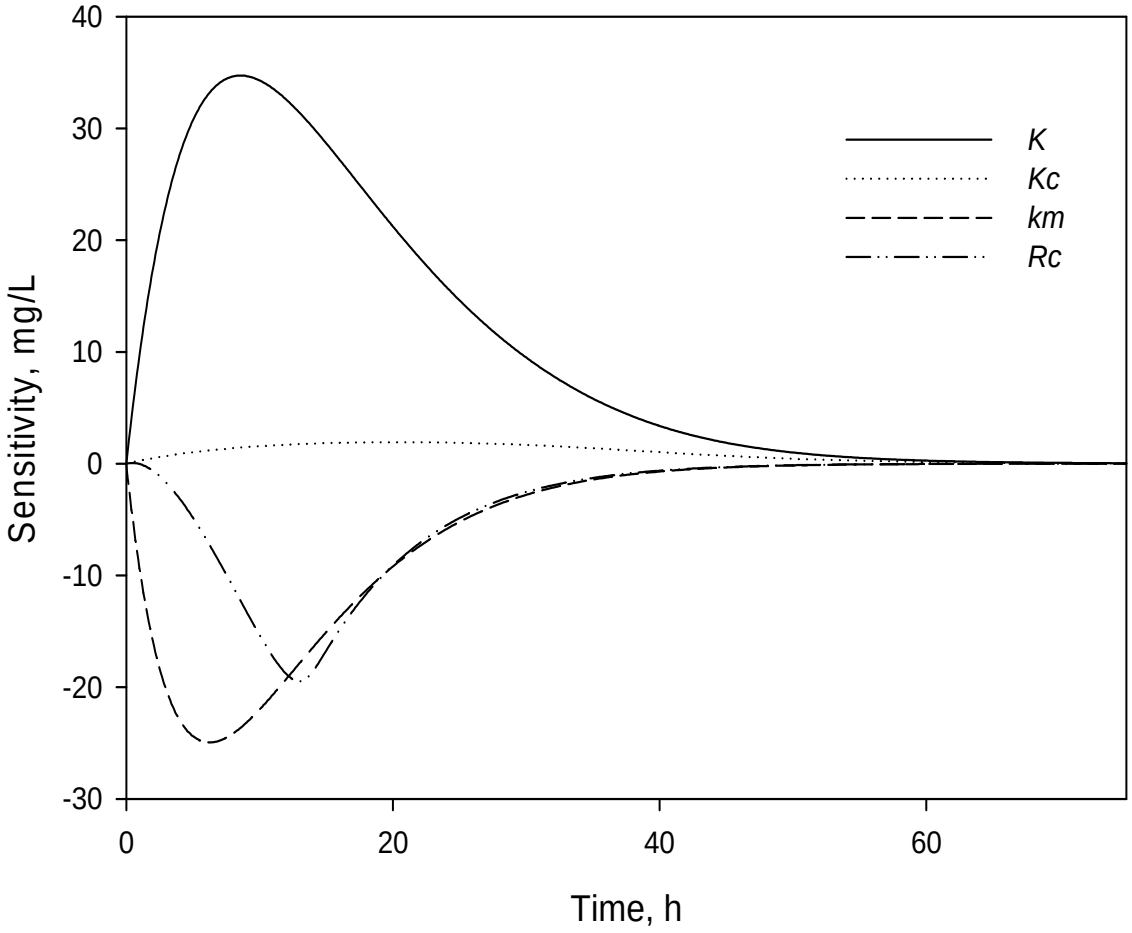

Figure 4-6: Anaerobic sensitivity test at 100 mg/L

4.6 Chapter Summary

The chapter describes the capability of a consortium of bacteria that was isolated from pure culture to reduce Cr(VI) to Cr (III). The batch studies were done to understand the Cr(VI) reduction capabilities of the microorganisms involved and the data obtained from both aerobic and anaerobic studies was further used in fitting kinetic models. A non-competitive inhibition model with cell inactivation was used to fit data obtained under aerobic and anaerobic conditions. The non-competitive inhibition model with Cr(VI) toxicity threshold best represented the aerobic and anaerobic culture with Cr(VI) toxicity threshold concentration of approximately 100 mg/L following mechanisms observed by Shen and Wang, (1995) and Molokwane, (2010).

The estimated parameters using the non-competitive inhibition model for the anaerobic experimental data were different from those obtained by Mtimunye (2011) and Molokwane (2010) who used the same model. The difference could be due to the cell age, the loss of cell (activity due to the storage conditions the sludge was stored), the initial biomass or due to the growth media used.

Sensitivity analysis for each kinetic parameter in the model was observed to be highly sensitive or affected by change in kinetic parameter (R_c, K and K_c). The sensitivity of R_c to the model under anaerobic condition was an indication that the cell reduction capacity was high during incubation. Anaerobic and aerobic models were found to be highly sensitive to changes in kinetic parameters (K_c, K and k_m).

CHAPTER 5

CR(VI) REDUCTION KINETIC STUDIES IN A CONTINUOUS FLOW REACTOR SYSTEM

5.1 Conceptual Basis of Mesocosm Studies

A microbial barrier system was constructed in the laboratory to simulate the behavior of Cr(VI) and to study how the system reduces species across strata in the open aquifer system of c contaminated site. The mesocosm study was the first step towards possible development of *in situ* bioremediation process for field testing at an actual Cr(VI) contaminated site.

In situ bioremediation of Cr(VI) in groundwater systems is a challenge as Cr(VI) is not destroyed but rather trapped in the aquifer matrix in its reduced state. Based on various environment conditions, the reduced Cr(III) trapped in the soil strata may remobilize back to its chemically toxic and mobile form and migrate down gradient to groundwater and surface water resources. In this study, the fate of Cr(VI) and its reduced species was investigated using a laboratory constructed microbial barrier system (mesocosm). The microbial barrier in the mesocosm was constructed using dried sludge cultures initially tested in batch system (Chapter 4).

The mesocosm study was conducted in a two-phase process. In the first phase, the toxic and mobile Cr(VI) was reduced to Cr(III) in the microbial barrier under near neutral pH which precipitates to $Cr(OH)_3$ near neutral pH. The second phase included the remobilizing of the accumulated $Cr(OH)_3$ in the system using a weak acid (0.1% HCl) to regenerate the biological barrier system. The latter method was implemented in order to effectively regenerate the barrier without removing the material from the system. The mesocosm reactor was operated under stressed nutrients conditions at the initial Cr(VI) feed concentration of 20

mg/L until steady state was achieved to acclimatize the culture which was 20 days. After 20 days, the Cr(VI) feed concentration was increased to 50 mg/L which is the current highest groundwater Cr(VI) concentration at the study site. After the bioremediation process operation, the barrier was regenerated without biogumentation.

5.2 Mesocosm Kinetic Studies

5.2.1 Cr(VI) Concentration Profile in a Control Zone

Cr(VI) reducing performance in an un-inoculated (control zone) Zone 1 in the mesocosm reactor was evaluated at the target initial feed concentration of 20 mg/L (Figure 5-1). The results show that Cr(VI) reduction in Zone 1 was insignificant throughout the operation indicating abiotic processes are negligible. Additionally, the tracer line showed a characteristic of exponential rise of Cr(VI) in the system also suggesting that the physico-chemical processes were insignificant over time.

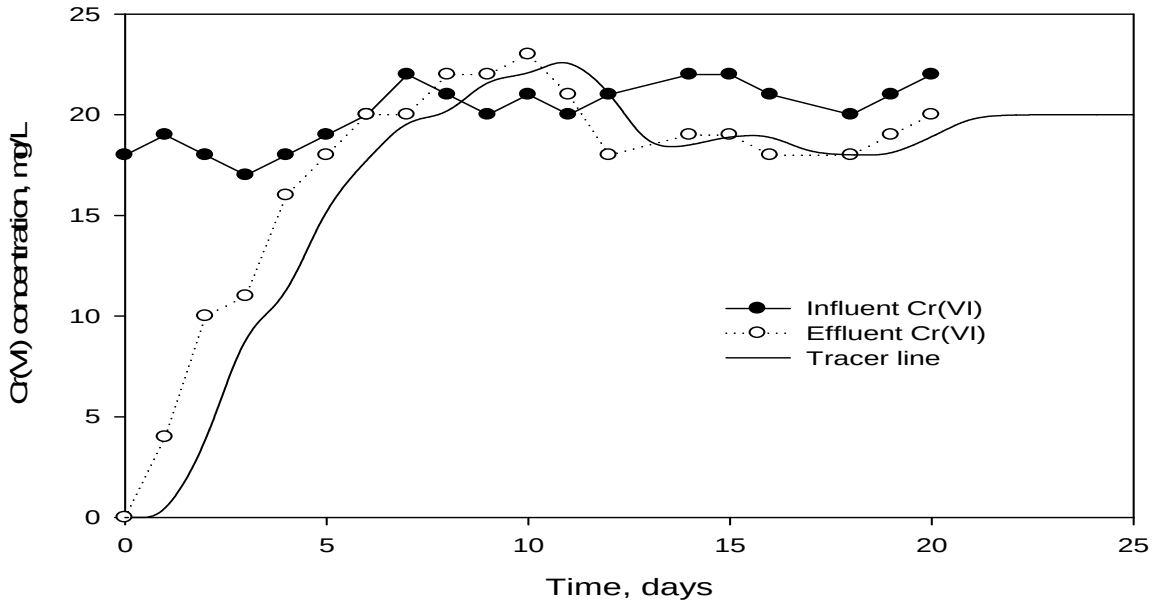

Figure 5-1: Performance on non-inoculated zone in the continuous flow reactor system which results in the exponential rise in the effluent Cr(VI).

5.2.2 Cr(VI) Reduction at Cr(VI) concentration of 20 mg/L

Experimentation on Cr(VI) reduction in the mesocosm reactor that was operated as a packed-bed reactor was initially conducted at the initial concentration of 20 mg/L under nutrient stressed conditions over time. Table 5-1 demonstrates that after 20 days of operation, Cr(VI) reduction rate was more pronounced in Zone 2 and Zone 3 (Zones allocated after microbial barrier) in the system. Complete Cr(VI) reduction was observed in Zone 2 and Zone 3 after operation which confirms the effectiveness of the consortium culture in reducing Cr(VI) under nutrient stressed conditions.

Table 5-1: Summary performance of Cr(VI) reduction after 20 days of operation at the initial Cr(VI) concentration of 20 mg/L

Zone	Cr(VI) effluent (mg/L)	Removal efficiency (%)
Zone 1	12	40
Zone 2	0	100
Zone 3	0	100

5.2.3 Cr(VI) Reduction at Higher Cr(VI) Concentration of 50 mg/L

The influent Cr(VI) concentration was not increased in the reactor until a steady-state in the Cr(VI) reduction at 20 mg/L was achieved. Figure 5-2 shows the average from each zone and the results shows that after increasing the Cr(VI) feed concentration, the concentration of Cr(VI) remained zero within the first 5 days of operation in Zone 3. The more pronounced Cr(VI) reduction rate observed in Zone 3 indicated that the consortium culture present in the reactor greatly enhanced Cr(VI) reduction. After 63 days of operation the removal efficiency of 75% was observed in Zone 3 demonstrating the effectiveness of the consortium culture in reducing Cr(VI) under shock loading conditions.

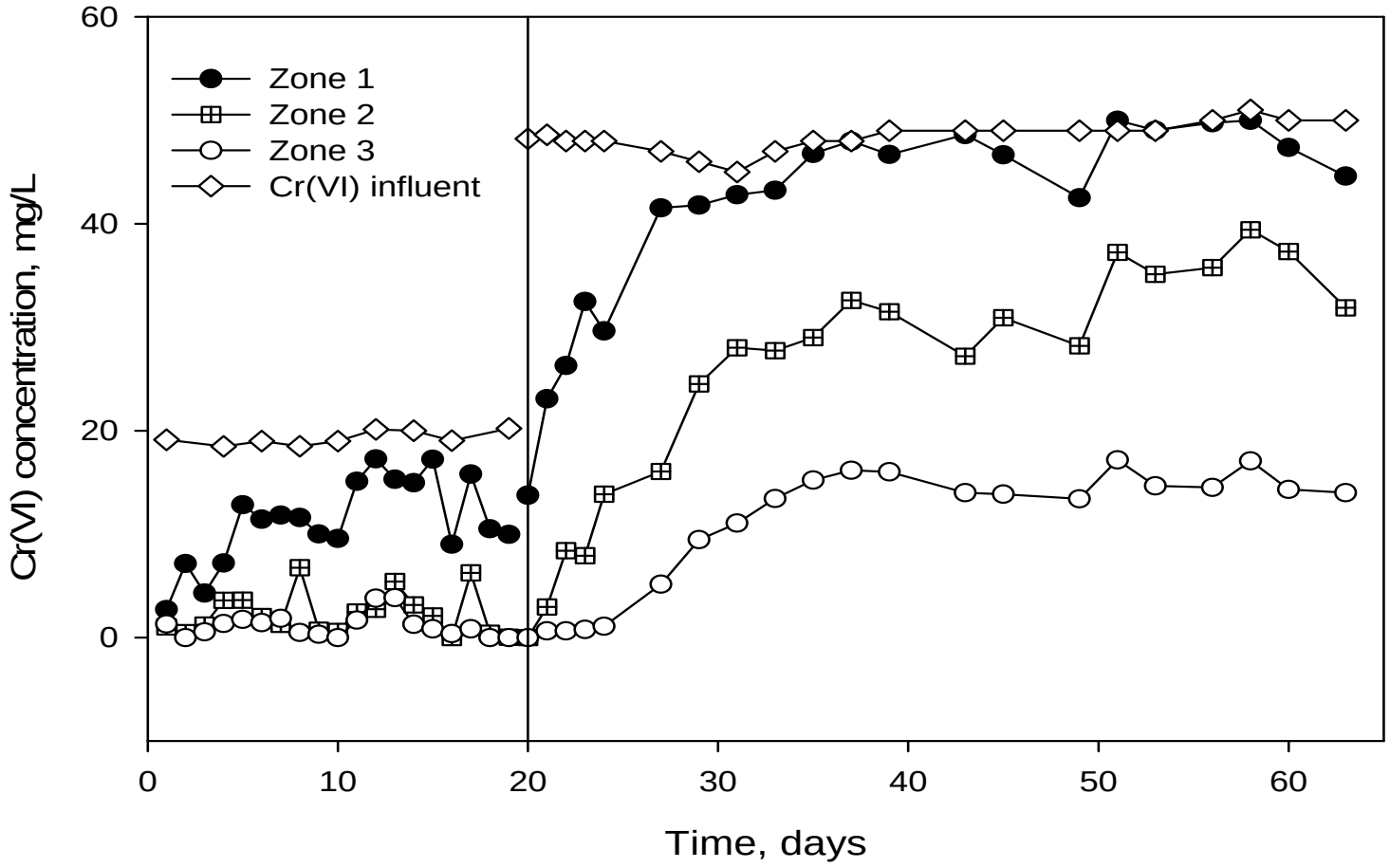

Figure 5-2: Performance of Cr(VI) reduction in Zone1, zone 2 and Zone 3 after 63 days of operation at the initial feed Cr(VI) concentration of 20 mg/L and 50 mg/L.

The decrease in Cr(VI) reduction observed over time in the reactor may be due to toxicity of Cr(VI) to cells, or it may be due to the presence of Cr(III) precipitate in the reactor which may decrease the capacity of the microbial barrier in reducing Cr(VI) by decreasing the porosity of the aquifer media over a long period of operation. Table 5-2 illustrates the summary performance of Cr(VI) after 63 days of operation at near neutral pH.

Table 5-2: Summary performance of Cr(VI) reduction after 63 days of operation at 50 mg/L

Zone	Cr(VI) effluent (mg/L)	Removal efficiency (%)
Zone 1	41.17	17.66
Zone 2	25.02	49.96
Zone 3	10.60	78.81

5.3 Remobilization of Cr(III) Precipitate in the Reactor

5.3.1 Conceptual Basis of Regeneration of the Barrier

Cr(VI) reduction was evaluated in a constructed mesocosm under shock loading conditions. After operating the reactor at the feed Cr(VI) concentration of 50 mg/L a quasi-steady state which was attributed to reduced capacity of the barrier to reduce Cr(VI) was achieved.

In this study the performance of the *in situ* bioremediation of Cr(VI) in the microbial barrier system is improved by remobilizing the Cr(III) precipitate present in the reactive microbial barrier using a weak acid (0.1% HCl).

To facilitate the process of barrier regeneration by recovering the reduced Cr species, the technology of electrokinetic was used to attract mobile Cr^{3+} species to the cathode (Reddy

and Chinthamreddy, 2003a). To recover significant amount of the precipitate chromium in the form of Cr^{3+} cathodes electrodes were placed in the last zone of the reactor (Zone 3). The efficiency of soil flushing with 0.1% HCl was evaluated by comparing total chromium achieved before soil acidification and total chromium observed after soil acidification. The efficiency of soil washing depends on the following factors: soil properties, the metal contamination characteristics, dosage and chemistry of the reagent and the processing conditions (Dermont *et al*., 2008). According to the soil classification triangle (Figure 3-3) the soil used in the soil was classified as loamy sand, with the pH of 8, CEC of 22.34 $cmol.kg^{-1}$, with an organic carbon load of 4.38% and soil porosity of 0.38 (Table 3-2). Low CEC and organic matter as soil properties contribute to the efficiency of soil flushing in practice. The shift in microbial community was also monitored and analyzed using 16S rRNA sequencing for microbial culture. This was done to determine the presence or absence of Cr(VI) reducing species identified prior and post acid addition.

5.3.2 Performance Evaluation of Barrier (Soil Washing)

After two weeks of flushing the microbial barrier system with 0.1% HCl, a yellow and white precipitate which was initially observed in the surface reactor was observed around the cathode. Similar results were observed by Reddy and Chinthamreddy, 2003a; Lu *et al.,* (2012) and Weng and Yuan, (2001) which indicate that most of the mobile Cr(III) species in the presence of 0.1% HCl was recovered at the cathode. To further confirm the efficiency of HCl in remobilizing the trapped chromium species in the system, total chromium species after acid addition was determined over time using the AAS, and then compared to total chromium prior to acid addition.

The increase in the total chromium (73%) was observed after flushing the reactor with the acid, suggesting that the trapped chromium species in the mesocosm were remobilized

(Figure 5-3). However, in this study no analysis was done to confirm that the precipitate observed at the cathode contained Cr(III) species. The assumption was that Cr(III) species were attached to the cathode based on the literature and the observation of change in total chromium of the effluent before and after acid addition.

High Cr(VI) removal observed in Figure 5-3 under acidic conditions indicate that the presence of the $Cr(OH)_3$ in the mesocosm influence the rate of Cr(VI) reduction in the soil strata after a long time of operation by clogging the pores of the soil in the system. Additionally, the results in Figure 5-3 also indicate that the presence of $Cr(OH)_3$ may be counterproductive to the bioremediation process as it can be re-oxidized to its toxic form Cr(VI), under various environmental conditions.

The following reaction occurs when HCl as an extraction agent is added to soil with $Cr(OH)_3$ precipitate:

$$Cr(OH)_3(s) + 3HCl(aq) \rightarrow CrCl_3(aq) + 3H_2O(l) \quad (5\text{-}4)$$

Equation 5-4 demonstrates that in the presence of HCl at low pH of 3.42, the $Cr(OH)_3$ in the system releases mobile Cr(III) species. This shows that low pH enhances the desorption that occurs when Cr(III) ions are exchanged for H^+ ions. The Cr(III) species bonds with negatively charged chloride ions which forms weak mobile complexes with chromium species ($CrCl_3$).

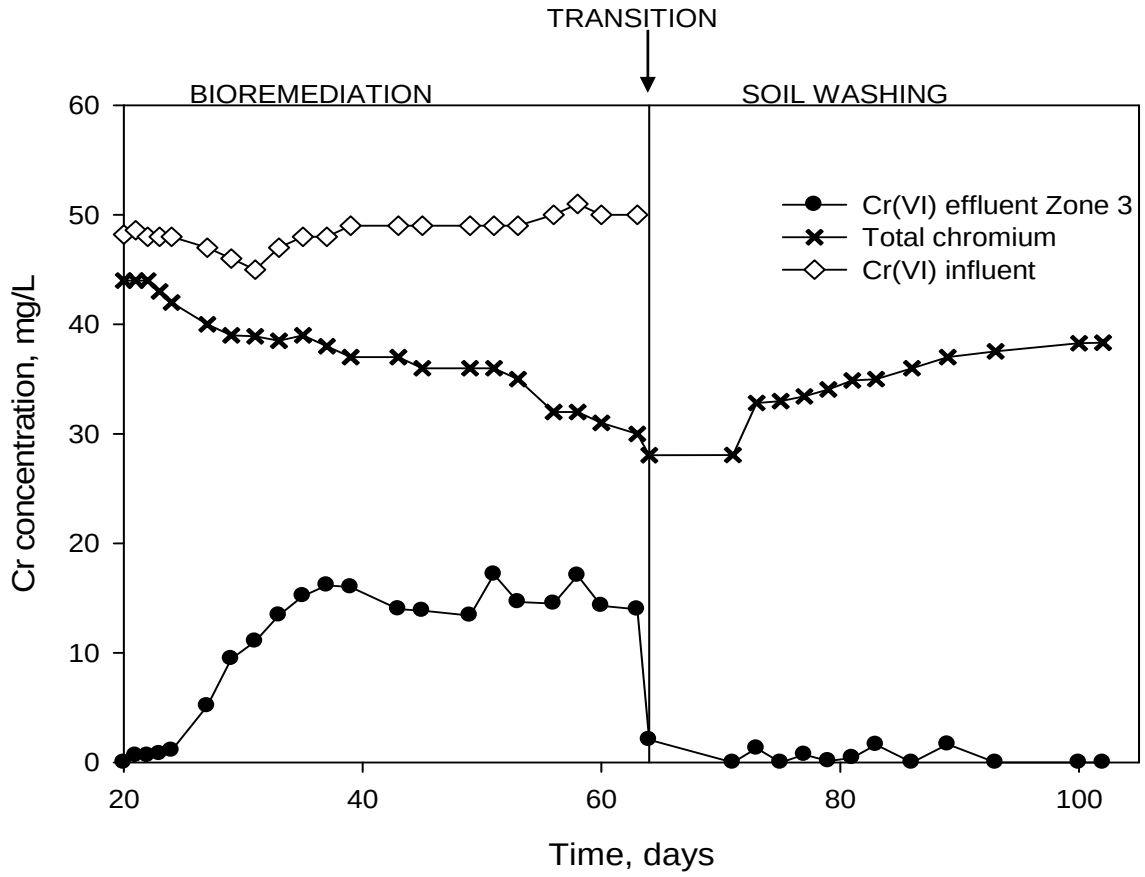

Figure 5-3: Measured total chromium and Cr(VI) in before soil and after soil washing experiment.

The overall performance of the mesocosm in reducing Cr(VI) to Cr(III) and regenerating the barrier is summarized in Table 5-3 below.

Table 5-3: Summary of Cr(VI) reduction performance prior washing the soil, at the Cr(VI) feed concentration of 50 mg/L.

BIOREMEDIATION				
Days	**Effluent Cr(VI) (mg/L)**	**Cr Removal (%)**	**Total Cr**	**Total Cr-Cr(VI)**
20	0	100	44.0	44.0
35	15.20	68.33	39.0	23.8
63	13.986	72	30.0	16.01

Table 5-4: Summary of barrier regeneration during soil flushing with hydrochloric acid.

SOIL FLUSHING			
Days	**Cr(VI) (mg/L)**	**Cr Removal (%)**	**Total Cr (mg/L)**
64	2.060	92.4	28.060
83	1.620	95.371	35.00
102	0	100	38.330

5.4 Microbial Culture Dynamic in Mesocosm

5.4.1 Characterization of Inoculated Culture during Bioremediation

Isolated culture from Brits, South Africa grown under anaerobic conditions. was used to inoculate the barrier.

After operating the reactor for 8 weeks, microbial culture dynamics were monitored using 16S rRNA fingerprinting method. The partial sequence of 16S rRNA matched the Bacillus groups – *Bacillus mycoides* and *Bacillus cereus, Eschericha hermannii, Enterobacter sp., Lysinibacullus fusiformis* and *Micococcus lylae.* Figure 5-4 to Figure 5-7, demonstrate the phylogenetic tree of the consortium culture used in the barrier system of this study. The cultures found in the reactor are known chromium reducing bacteria and are responsible for the reduction of Cr(VI) in the study.

5.4.2 Characterization of Inoculated Culture prior to the Addition of Acid

Figure 5-4: Phylogenetic tree showing isolate from 16S rRNA gene sequence

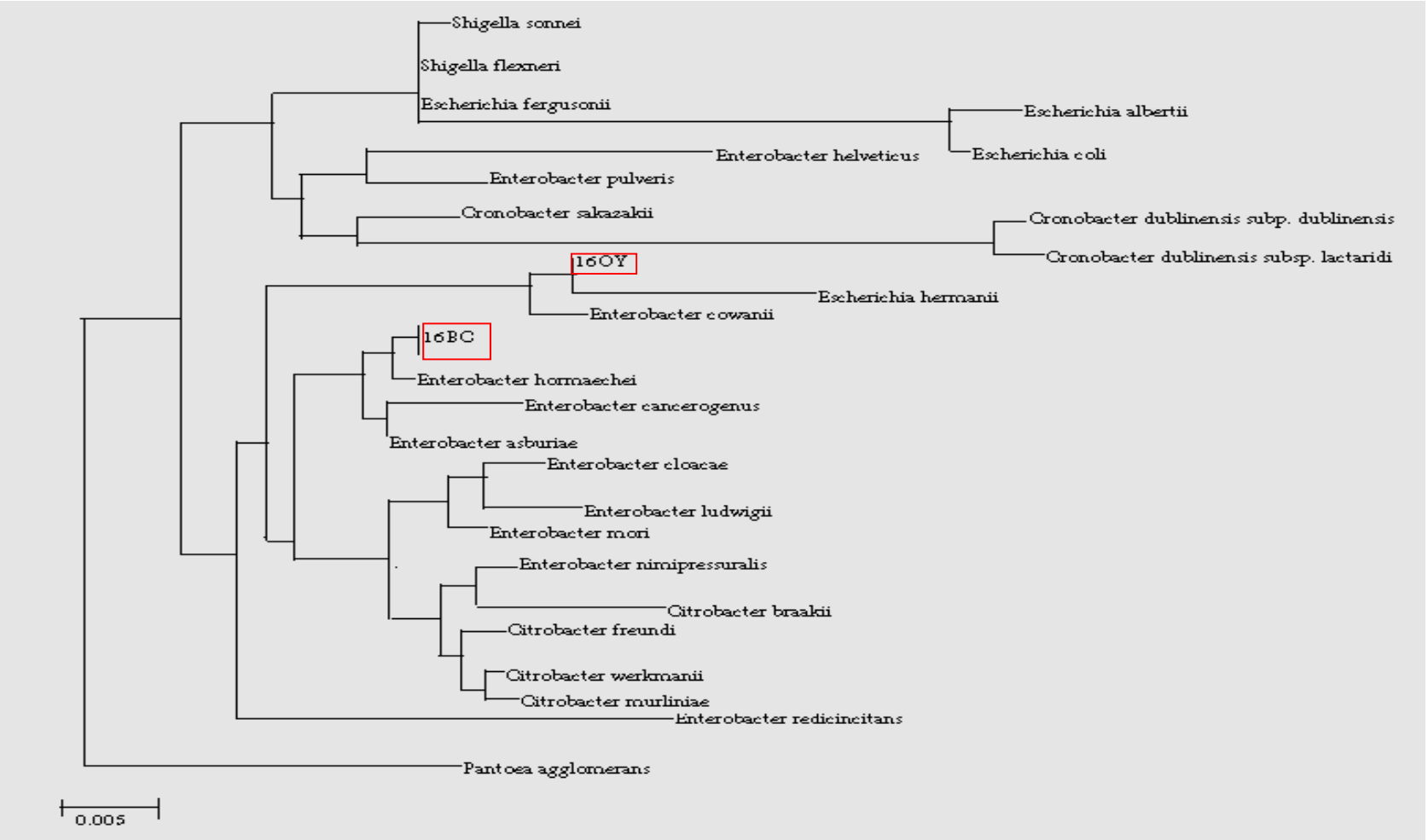

Figure 5-5: Phylogenetic tree showing the identities of the ***Enterobacter*** isolates inferred from 16S rRNA gene sequences of the consortium culture from the mesocosm reactor.

Figure 5-6: Phylogenetic tree showing the identities of the *Micrococcus sp*. isolates inferred from 16S rRNA gene sequences of the consortium culture from the microcosm reactor.

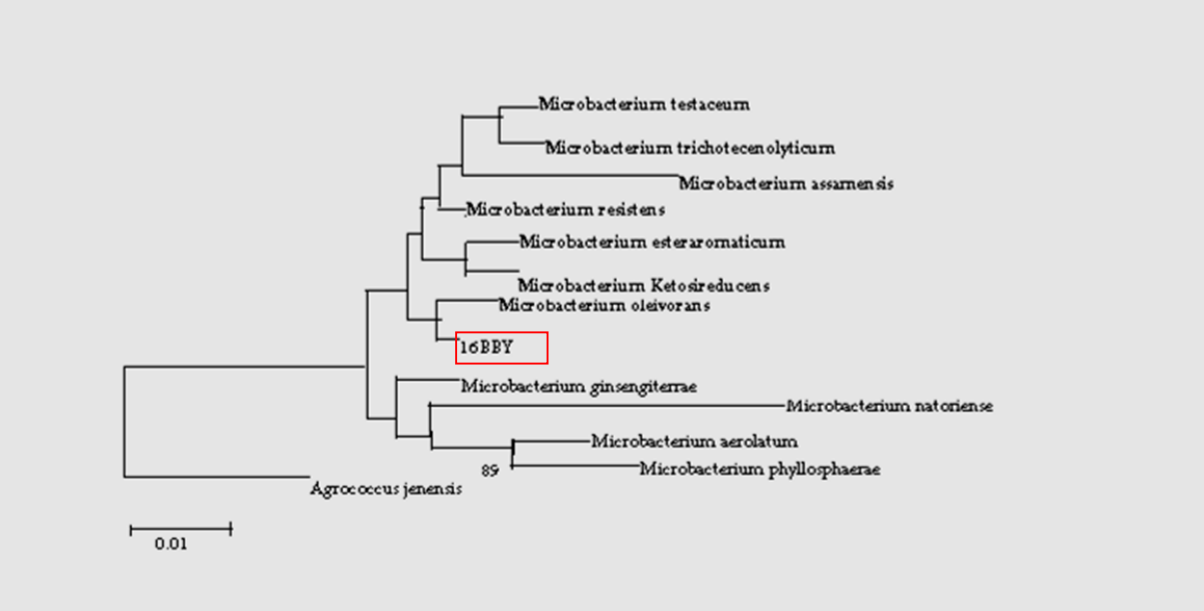

Figure 5-7: Phylogenetic tree showing the identities of the *Microbacterium sp.* isolates inferred from 16S rRNA gene sequences of the consortium culture from the microcosm reactor.

5.4.3 Characterization of Culture after Acidic Barrier Washing

Acidification is known to alter factors of the soil such as soil moisture, soil temperature and pH which are needed by microorganisms for survival. However, little is known about the specific impact of acidification on exposed microbial community during soil/barrier washing (Lear *et al.*, 2004). After operating the reactor under acidic conditions, it is expected that a microbial shift will occur. Microbial dynamics was monitored using the 16S rRNA fingerprinting method. It was observed in Table 5-5 that most of the species that were initially present in the barrier prior to acid addition were still present after acid washing, species such as *Bacillus sp*., *Lysinibacillus fusiformis* and *Micrococcus lylae* were present from the original culture. *Lysinibacillus fusiformis* is a well-known sludge bacteria (Molokwane, 2010) while *Bacillus sp*. are known to be chromium reducing bacteria (Wang and Shen, 1995).

The appearance of *Terribacillus saccharophilus,* and *Microbacterium sp.* indicates the microbial community shift in the reactor as it was not detected in the original sludge culture inoculated in the barrier. *Microbacterium sp.* is known to be resistant to chromium toxicity but rarely known as Cr(VI) reducer under anaerobic conditions (Rahman and Singh, 2014; Pattanapipitpaisal *et al*., 2001). The experiment was carried out under anaerobic conditions thus the presence of species that thrives under this conditions.

Table 5-5: Summary of shift in microbial community shift prior and after soil washing

Microbial Culture at prior soil washing	**16S rRNA**	**Microbial Culture after soil washing**	**16S rRNA**
Escherichia hermannii	100%	*Bacillussp*. or *Bacillus vietnamensis*	99%
Bacillus mycoides or *Bacillus cereus*	99%	*Bacillus firmus*	99%
Enterobacter sp.	99%	*Lysinibacillus fusiformis*	99%
Lysinibacillus fusiformis	99%	*Terribacillus saccharophilus*	99%
Micrococcus lylae	99%	*Microbacterium* sp.	100%
Bacillus sp.	*99%*	*Enterobacter* sp.	99%
		Bacillus mycoides or *Bacillus cereus*	98%

Additionally, little is known about *Terribacillus saccharophilusas*, a chromium reducing bacterium. The presence of this species in the reactor after acid addition confirms the effectiveness or the persistence of the species under shock loading. The shift in microbial community corresponds with the findings of Tabatabai and Olson (1985) that acidification of the soil changes the bacterial community within the soil.

5.5 Chapter Summary

The mesocosm study was conducted as the first step in the two phase process to remobilise trivalent chromium. The aim of the experiment was to evaluate the effectiveness of *in situ* bioremediation of Cr(VI) to Cr(III) which under near neutral pH forms a precipitate $Cr(OH)_3$.

The presence of $Cr(OH)_3$ precipitate in the microbial barrier system that simulates the fate of Cr(VI) in aquifer system resulted in the process of Cr(VI) bioremediation being counterproductive over time. To improve the effectiveness of Cr(VI) in situ bioremediation the Cr(III) precipitate which was trapped in the soil strata was remobilized by washing the microbial barrier with the weak acid. In this study the addition of weak acid in the system was effective as it resulted in barrier regeneration and improve Cr(VI) reduction rates in the system. The species present in the consortium culture were effective in reducing Cr(VI) at various initial Cr(VI) concentrations under nutrient stressed conditions.

Due to the acidification of the soil, a shift of the microbial community was expected and this was determined by carrying out a 16S rRNA sequencing which was done at the end of the experiment. Results showed *Lysinibacillus fusiformis*, *Enterobacter* sp., *Bacillus mycoides* and *Bacillus cereus* to be persistent in the reactor. The appearance of *Terribacillus saccharophilus, Enterobacter sp.* and *Microbacteriumsp* indicated that a microbial shift had occurred in the reactor. *Microbacterium sp* is a known CRB while *Terribacillus saccharophilus* has demonstrated to resist chromium toxicity.

CHAPTER 6

SUMMARY AND CONCLUSION

Biological reactive barrier as an *in situ* remediation technology is fast becoming a popular technology as it found to have more advantage over other remediation technologies that are currently in use such as pump and treat method.

Batch experiments at the initial Cr(VI) concentration of 30-400 mg/L in media with harvested indigenous culture were done under aerobic and anaerobic conditions. The results showed that complete removal of Cr(VI) was achieved under both conditions when the initial concentration were less than 100 mg/L after incubation for 55 hours. At the concentration of 30 mg/L, the anaerobic cultures removed 100% of Cr(VI) within 2 hours of incubation. However, at higher initial concentrations of 200-400 mg/L incomplete reduction was observed under both conditions. The anaerobic cultures showed faster Cr(VI) reduction rate compared to aerobic cultures.

AQUASIM 2.0 was used to simulate models and fit the experimental data obtained from the batch experiment. The results showed that the bacteria best fitted the non-competitive inhibition model with cell inactivation with the half velocity constant (K_c) parameter removed under aerobic conditions, under anaerobic conditions the performance of the bacteria fitted best a non-competitive inhibition model with Cr(VI) toxicity threshold concentration of about 100 mg/L.

The mesocosm experiment was carried out in two-phase process: the first process involves the use of chromium reducing bacterium to reduce Cr(VI) to Cr(III), whereby, indigenous cultures from dried sludge were packed into the barrier that consequently resulted in the formation of an

immobile precipitate $Cr(OH)_3$ under neutral pH. The second process involves the regeneration of the biological barrier with 0.1% HCl and the extraction of Cr(III) at the cathode during electrokinetics remediation.

The remediation experiment was carried at two concentrations of 20 mg/L and 50 mg/L. The initial Cr(VI) concentration feed of 20 mg/L was fed into the reactor until a steady state was achieved, then the influent Cr(VI) concentration was increased to 50 mg/L and after 63 days of operation. 75% removal of Cr(VI) was observed demonstrating the effectiveness of the consortium culture under shock loading conditions.

The $Cr(OH)_3$ formed in the reactor clogged the pores of the reactor, thereby affecting the porosity and hydraulic conductivity of the barrier system. It was observed that 0.1% HCl effectively remobilized the trapped chromium species in the system that led to a 73% increase in total chromium in the system after soil washing. However, it was believed that Cr(III) species were attracted to the cathodes and had formed a white-yellow layer around the cathode based on literature.

During the mesocosm experiment, 16S rRNA fingerprinting was conducted before and after acidification of the soil. The phylogenetic tree of cultures obtained during bioremediation showed the presence of CRB that were responsible for the Cr(VI) reduction observed. After soil washing, it was found that some bacterium namely *Bacillus sp.*, *Micrococcus lylae, Enterobater sp.* and *Lysinibacillus fusiformis* were persistent in the reactor and the appearance of *Terribacillus saccharophilus* indicated the microbial shift had occurred in the reactor. Based on the efficiency of 0.1% HCl, this combined remediation techniques can be optimized and applied to real soils in order to validate it as a large scale solution.

Recommendations

To achieve optimum application of this technology, future research will be required in the following areas:

- A method that analyses the species and the quantity of chromium species on the cathode must be developed.
- A method that accounts for biomass concentration profile must also be investigated.
- Further research needs to be conducted to determine the effects of acids on the microbial community during soil washing in a groundwater system.
- The surviving microbial community in the reactor after soil washing should be studied for their ability to reduce Cr(VI).

APPENDIX A

AQUASIM version 2.0

Variables

C:	Description:	Dissolved Cr(VI) concentration
	Type:	State Variable
	Unit:	mg/L
	Relative Accuracy:	1e-006
	Absolute Accuracy:	le-006

Cmeas	Description:	Measured Cr(VI)
	Type:	Real List Variable
	Unit:	mg/L
	Argument:	t
	Standard Deviations:	global
	Rel. Stand. Deviation:	0
	Abs. Stand. Deviation:	1

Minimum:	0
Maximum:	1e+009
Interpolation Method:	Linear interpolation
Sensitivity Analysis:	Inactive

Real Data Pairs (21 pairs):

Argument	Value
0	0
1	4
2	10
3	11
4	16
5	18
6	20
7	20
8	22
9	22
10	23
11	21
12	18
14	19
15	19

16	18
18	18
19	19
20	20

D:	Description:	Dispersion coefficient
	Type:	Formula Variable
	Unit:	m^2/d
	Expression:	0.1

Qin:	Description:	Flow rate
	Type:	Formula Variable
	Unit:	L/d
	Expression:	1

---

t:	Description:	time
	Type:	Program Variable
	Unit:	h
	Reference to:	Time

Compartments

Column:	Description:	
	Type:	Advective-Diffusive Compartment
	Compartment Index:	0
	Start Coordinate:	0
	End Coordinate:	1
	Cross sectional are:	
	Diffusion:	Without diffusion
	Num. Grid. Pts:	52
	Resolution:	high

	Active Variables;	C
	Active Processes:	
	Initial Conditions:	
	Input type:	Inlet input
	Water flow:	Qin
	Loading variable:	
	C:	Qin*Cmeas

Definitions of Calculations

Calc_1:	Description:	
	Calculation Number:	0
	Initial Time:	0
	Initial State:	given, made consistent
	Step Size:	0.01

Num Steps:	3000
Status:	active for simulation

Plot Definitions

Concentration plot:	Description:	Cr(VI) concentration
	Abscissa:	Time
	Title:	Cr(VI) Concentration
	Abscissa label:	Time [h]
	Ordinate Label:	Concentration [mg/L]

Curves:

Type: Variable [Calcnum, Comp, Zone, Time/Space]

Value: Cmeas [0, comp1, Water body, 0]

Value: C [0, comp1, Water body, 1]

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