CHAPTER 3

MATERIALS AND METHODS

3.1 Source of Cr(VI) Reducing Organisms

Four different environments were identified as possible sources of Cr(VI) reducing cultures: (1) soil from a contaminated site, (2) influent to a sewage treatment plant receiving high loadings of Cr(VI), (3) activated sludge from aeration tanks and (4) dried sludge from sand drying beds at the same treatment plant. The measured background Cr(VI) concentration in the influent and mixed liquor from the treatment plant was 2.45 and 2.63 mg/L, respectively, and the Cr(VI) content in dried sludge was 25.44 g/m^3 at the time of sampling. Higher values of the reduced form of total Cr were expected in the mixed liquor and dry sludge due to the presence of Cr(VI) reducing bacteria.

The bacteria from the different sources was incubated for 96 hours in LB broth at initial concentrations of 20, 50, 100, 150, 200, 300, 400 and 600 mg Cr(VI)/L under aerobic conditions. The temperature was maintained at an average of 30 ± 2^oC and the pH at 7. The existence of Cr(VI) reducing bacteria in the samples from different sources was indicated by high removal rates observed in the cultures as shown in Table 3-1 (Molokwane *et al*, 2008). All cultures in the screening tests above were incubated for 96 hours.

The cultures from contaminated soil yielded the lowest Cr(VI) removal rate. Soil bacteria reduced Cr(VI) as much as observed in an earlier studies by Chirwa and Wang (1997a, b), however, this performance was much lower compared to the performance of bacteria from dried sludge. Soil cultures were able to reduce 91.5 %

of Cr(VI) at an initial of 20 mg/L, and the Cr(VI) reduction ability significantly decreased with increasing initial concentration.

Cr(VI) reduction ability of cultures obtained from mixed liquor also decreased with increasing initial Cr(VI) concentration in the test batches. The best performance was observed in the cultures from dried sludge with complete Cr(VI) reduction observed at initial Cr(VI) concentrations up to 200 mg/L. At an initial concentration of 300 mg/L the dried sludge culture reduced 99.2 % after incubation for 96 hours.

Table 3-1: Percentage Cr(VI) reduction in cultures from different sources after 96 hours of incubation at different initial Cr(VI) concentration.

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

- no experiment conducted at this concentration

The high performance in the dried sludge cultures was attributed to the long period of contact between bacteria and Cr(VI) during the wastewater treatment process – from the influent to the final clarifiers. The presence of a wide variety of carbon sources and nutrients in the system was expected to support a wider biodiversity of bacterial species. In all cultures studied, there was no Cr(VI) reduction observed at an initial

concentration of 600 mg/L. Based on those results, bacteria from the sand drying beds was chosen the rest of the studies.

3.2 Mineral Media

The main kinetic experiments were conducted using Basal mineral medium (BMM) prepared by adding (in grams per litre of distilled water): 0.535 NH_4Cl, 10.744 $NaHPO_4$, 2.722 KH_2PO_4, 0.114 Na_2SO_4, 0.049 $MgSO_4$, 0.554 $CaCl_2$, 0.695 $FeSO_4$, 0.0013 $ZnCl_2$, 0.00341 $CuCl_2$, 0.00103 NaBr, 0.00121 Na_2MoO_4, 0.00198 $MnCl_2$, 0.00166 KI, 0.00124 H_3BO_3, 0.00238 $CoCl_2$, and 0.00128 $NiCl_2$. The medium was then sterilised by autoclaving at 121^{o}C for 15 minutes. The cells were initially cultivated in nutrient broth (NB) and Luria-Bettani (LB) broth. Plate count (PC) agar and Luria-Bettani (LB) agar were used for colony development (Merck, Germany). All broth and media were prepared by dissolving the recommended amounts of media powder in 1 L of distilled water and autoclaved at 121^{o}C for 15 minutes. Agar media were cooled to 45^{o}C before use. All salts unless stated otherwise were obtained from Sigma-Aldrich (St Louis, USA).

3.3 Culture Isolation

Pure cultures were prepared by depositing 1 mL of a serially diluted sample on LB agar followed by incubation at 30^{o}C to develop separate identifiable colonies. Individual colonies were transferred using a heat-sterilised wire loop into 100 mL sterile LB broth spiked with 75 mg Cr(VI)/L. Loop-fulls from individual colonies were used to inoculate fresh media containing 150 mg Cr(VI)/L. The cells were allowed to grow – colonies were grown again for the third time from serially diluted batches grown from individual colonies. Cultures from the third isolation were washed and used in the detailed Cr(VI) reduction rate analysis using BMM as

experimental media. Cr(VI) reducing colonies were selected by observing complete Cr(VI) reduction after incubation for 72 hours. The selected colonies were stored at 4^{o}C in test-tube slant cultures or agar-plate streaks.

3.4 Gram Stain Analysis

Gram stain analysis was conducted following the Hucker Method (APHA, 2005). 1 mL of culture from 24 hour cultivated cells was spread on microscopy slide and dried over a flame. The slide was then immersed in crystal violet then air-dried for 1 minute. The fixed cells on the slide were then washed gently and directly by running water for seconds. The slide was then immersed in iodine mordant for 1 minute, then again gently and directly washed under a tap water stream for 2 seconds. Afterwards, the slide was immersed in Safranin solution for 30 seconds and gently washed under tap water stream for 2 seconds. The slide was then immersed in 95% vol/vol ethanol for 5 seconds, and then gently washed under a stream of tap water for 2 seconds. The slide was then dried with absorbent paper. Finally, 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. Cells were then differentiated by the colour observed: black-violet for Gram-positive; and red-pink for Gram-negative cells.

3.5 Microbial Culture Characterisation

Phylogenetic characterization of cells was performed on individual colonies of bacteria from the 7th-10th tube in the serial dilution preparation. LB and PC agar was used for colony development. In preparation for the 16S rRNA sequence identification, the colonies were first classified based on morphology. Seven different

morphologies were identified for the aerobic cultures. These were streaked on nutrient agar followed by incubation at 37°C for 18 hours.

Culture purification and 16S rRNA sequencing were performed at the Department of Microbiology, University of Pretoria where the identification was done. At 99% identity match, the results indicated the predominance of four aerobe phenotypes. Genomic DNA was extracted from the pure cultures using a DNeasy tissue kit (QIAGEN Ltd, West Sussex, UK) as per manufacturer's instructions. The 16S rRNA genes of isolates were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using primers pA and pH1 (Primer pA corresponds to position 8-27; Primer pH to position 1541-1522 of the 16S gene) (Coenye *et al*., 1999). An internal primer pD was used for sequencing (corresponding to position 519-536 of the 16S gene). The resulting sequences were matched to genes for known bacteria in the GenBank using a basic BLAST search of the National Centre for Biotechnology Information (NCBI, Bethesda, MD).

3.5.1 Aerobic Culture

Partial sequences of 16S rRNA genes showed the predominance of the *Bacillus* groups – *Bacillus cereus* ATCC 10987, *Bacillus cereus* 213 16S, *Bacillus thuringiensis* serovar finitimus, *Bacillus mycoides* – and two *Microbacterium* species – *Microbacterium foliorum* and *Microbacterium sp*. S15-M4 (Table 3-2).

The observed microbial composition of the startup culture was consistent with microbial culture observed in activated sludge systems with Cr(VI) reducing bacteria (Francisco *et al*., 2002; Camargo *et al*., 2003; Faisal and Hasnain, 2006). In the studies by Camargo and others (2003), the Cr(VI) reducing species *Lysinibacillus* sp., *Bacillus* K1, *Bacillus cereus*, and *Bacillus thirungiensis* were identified as

predominant species in activated sludge cultures. A phylogenetic tree was constructed for the species from purified cultures grown under aerobic conditions based on a basic BLAST search of rRNA sequences in the NCBI database (Figure 3-1).

Table 3-2: Partial sequencing of aerobic CRB isolated from Brits dry sludge grown in solution containing 100 mg/L.

Pure culture	**Partial 16S ID[a]**	**%Identity**
X1	*Bacillus* cereus strain 213 16S, *Bacillus thuringiensis* 16S	99
X2	*Bacillus* sp. ZZ2 16s, *Bacillus cereus* ATCC 10987, B. *thuringiensis* strain Al Hakam	99
X3	*Bacillus sp*. 32-661 16s, *Bacillus cereus* strain 16S	99
X4	*Bacillus mycoides* strain BGSC 6A13 16S, *Bacillus thuringiensis serovar finitimus* strain BGSC 4B2 16S	99
X5	*B. mycoides* strain BGSC 6A13 16S, B. *thuringiensis serovar finitimus* strain BGSC 4B2 16S	99
X6	*B. mycoides* strain BGSC 6A13 16S, B. *thuringiensis serovar finitimus* strain BGSC 4B2 16S	99
X7	*Microbacterium* sp. S15-M4, *Microbacterium foliorum*	99

[a] S ID¼ 16 Svedburg rRNA Identity of partial sequences (16 Svedburg unit ribosomal Ribo-Nucleic-Acid Identity).

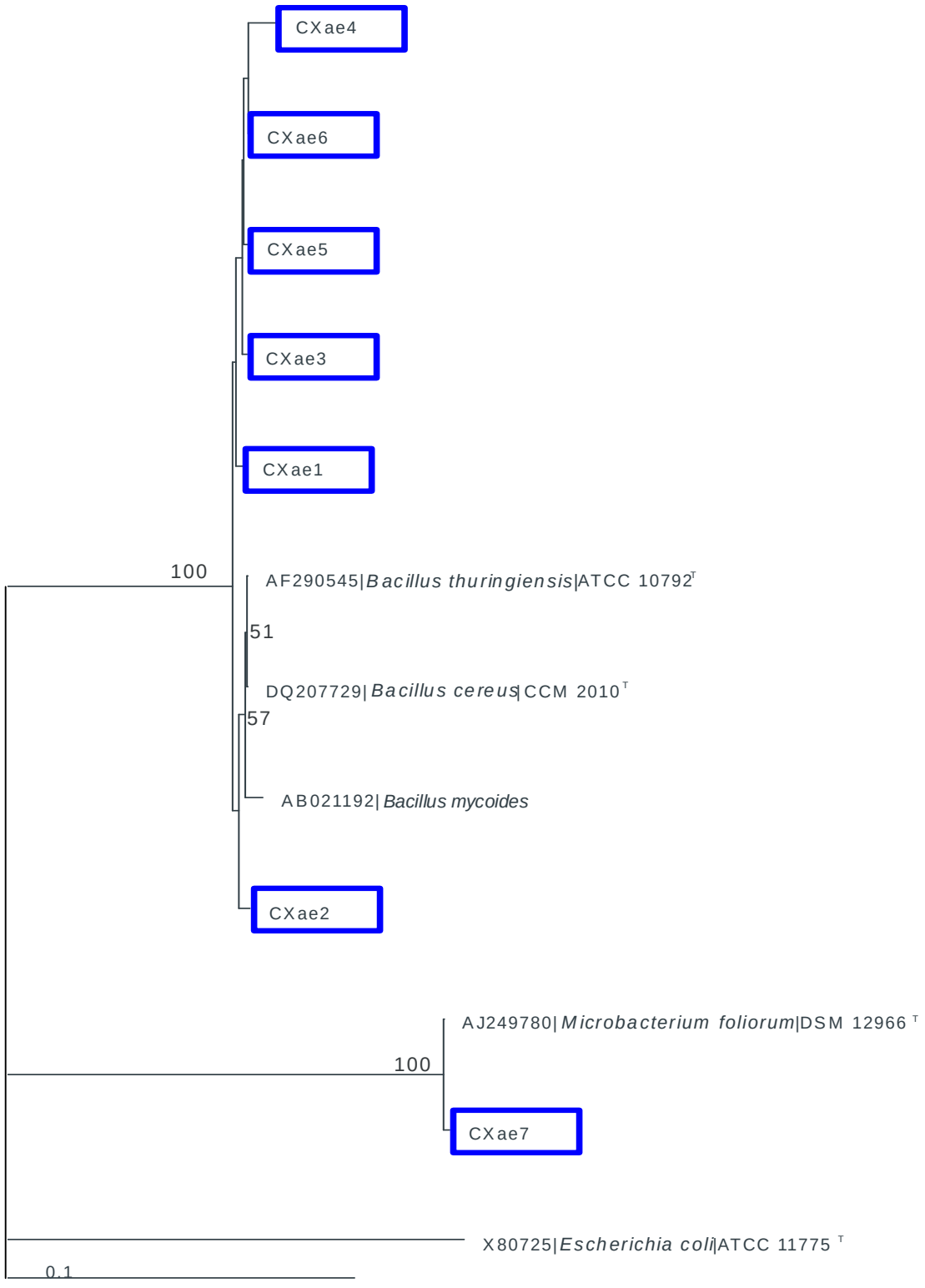

Figure 3-1: Phylogenetic tree of species from Brits dry sludge reflecting microbial diversity under aerobic conditions.

3.5.2 Anaerobic culture

Anaerobic bacteria was isolated from dry sludge following the same procedure described for aerobic cultures, modified by maintaining anaerobic conditions by purging reactors with nitrogen and sealing in serum bottles. All transfers were conducted in an anaerobic glove bag purged with nitrogen. The cultures were isolated under 100, 150 and 200 mg Cr(VI)/L. Eighteen different morphologies were identified from anaerobic cultures (Figure 3-2). Some of the bacteria were unculturable but produced a fingerprint during 16S rRNA analysis. Some were cultured but were marked as unidentified. Only 11 colonies from the anaerobic cultures were partially identified and seven colonies could not be amplified for partial gene sequencing.

Results indicated the predominance of eighteen anaerobic phenotypes ten of which were positively identified as shown in Table (3-3). Partial sequences of 16S rRNA matched the seven *Bacillus* groups:

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

The genetic relationship between species based on the BLAST search is shown in Figure 3-2. The anaerobic data showed a wider microbial diversity probably due to the partially anaerobic conditions in the aeration tanks at the Wastewater Treatment Plant from which the bacteria were originally collected.

Table 3-3: Characteristics of pure cultures and nearest matches based on the BLAST analysis of 16S rRNA partial sequences.

[Cr(VI)] mg/L	Sample No	Colour	Blast result	% Identity
100	X2	Off-white	*Enterococcus avium*, *Enterococcus pseudoavium*	99
	X3	Cream	Uncultured *Bacterium* clone Y2, *Acinetobacter* sp. ANT9054	97
	X6a	Yellow	*Arthrobacter* sp. Sphe3, Uncultured soil *Bacterium* clone TA12	93,94
150	X6b	Yellow	Arthrobacter sp. AK-1	99
	X7	Cream and yellow rings	*Bacillus drentensis*, *Bacillus drentensis*	96,97
	X10	Light brown	*Oceanobacillus* sp. JPLAK1, *Virgibacillus necropolis*	99,98
	X11	Off-white	*Enterococcus faecium* strain R0026, *Rumen bacterium* R4-4	99
	X12	Coral	*Paenibacillus pabuli*, *Paenibacillus xylanilyticus* strain XIL14	99
200	X15	Cream	[*Brevibacterium*] *frigoritolerans*, *Bacillus* sp. R21S	99
	X17	Cream	Uncultured *bacterium*, *Bacillus* sp. BS19	93

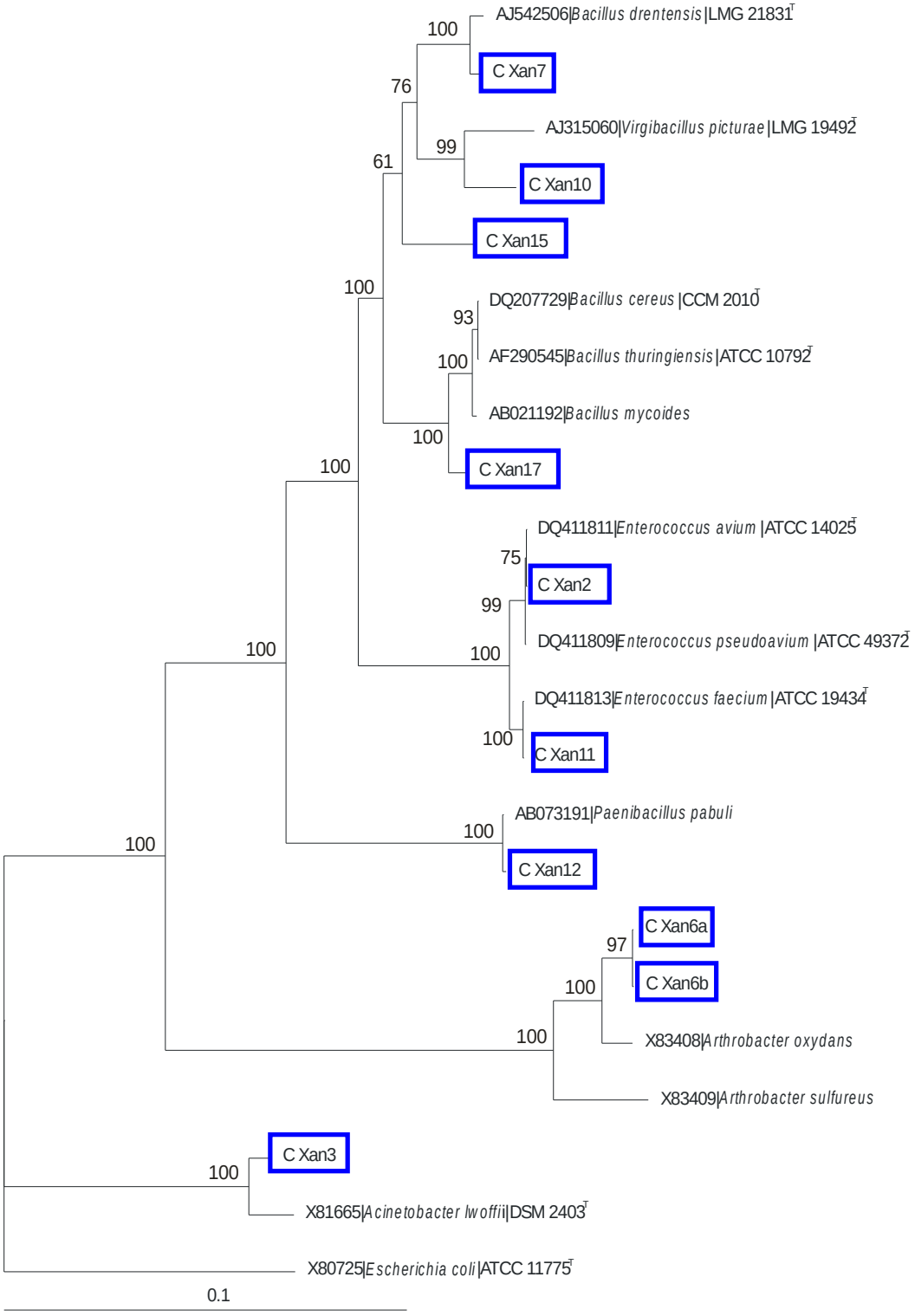

Figure 3-2: Phylogenetic tree of species from Brits dry sludge reflecting microbial diversity under anaerobic conditions.

3.6 Cr (VI) Reduction Experiments

3.6.1 Aerobic Batch Experiments

Aerobic Cr(VI) reduction experiments were conducted in 250 mL Erlenmeyer flasks containing 100 mL sterile BMM, using cells harvested after 24 hours incubation in nutrient broth. The cells were washed twice by centrifugation at 6000 rpm (2817.36 g) for 10 minutes in a Hermle 2323 centrifuge (Hermle Laboratories, Wehingen, Germany). After centrifugation, the cells were re-suspended in a sterile solution of 0.085% NaCl for each wash. The cells were then finally re-suspended in the sterile BMM after the final wash- concentrated by a ratio of 4:1. Cr(VI) of a known concentration was added to the media marking the beginning of the Cr(VI) reduction experiment. The batches were covered with cotton plugs during incubation to allow aeration while filtering away microorganisms from the air.

Cr(VI) concentration in the range of 50 to 400 mg/L was added and the solution was incubated under shaking at 30 $\pm 2^{o}$C. 1 mL samples were withdrawn at time intervals determined by the observed rate of Cr(VI) removal. The samples were centrifuged at 6000 rpm (2817.36 g) for 10 minutes in the Hermle 2323 centrifuge (Hermle Laboratories) to remove suspended cells before analysis.

3.6.2 Anaerobic Batch Culture Experiments

Anaerobic batch experiments were conducted in 100 mL serum bottles using cells harvested after 24 hours incubation under anaerobic conditions. The cells were transferred under an anaerobic glove bag purged with 99.99% N_2 gas. Cells were concentrated to a 4:1 ratio, and washed twice in a sterile solution of 0.085% NaCl before adding Cr(VI) as described earlier for aerobic batches.

The bottles were purged with nitrogen gas (99.99%) for 10 min to expel any residual oxygen before sealing with silicon stoppers and aluminium seals. After sealing, the cultures were incubated at 30±1^{o}C for 7 days. 1 mL samples were withdrawn using a sterile syringe at time intervals determined by the observed rate of Cr(VI) removal. The samples were centrifuged at 6000 rpm (2817.36 g) for 10 minutes in a Hermle 2323 centrifuge (Hermle Laboratories) to remove suspended cells before analysis. Headspace gases were sampled by syringe and analysed by gas chromatography.

3.6.3 Microcosm Reactor Studies

Microcosm cores collected from target environments were set up in the laboratory as packed-bed reactors. The cores were encased in 22-30 cm long and 6 cm diameter polyvinyl chloride (PVC) or Plexiglas® (Evonik Röhm GmbH, Essen, Germany) columns and operated under a constant hydraulic head of Cr(VI) contaminated water (Figure 3-3). A peristaltic pump was used to maintain the feed level in the reservoir. Cr(VI) concentration in the range 40-50 mg/L was used in the experiment representing the Cr(VI) concentration at one of the target sites (Brits, North West Province).

Conditions tested in the microcosm studies included the effect of (1) abiotic processes – adsorption and chemical reduction by elements in the soil, (2) natural bacteria from the soil, (3) inoculation with live sludge cultures, and (4) adding a natural carbon source on Cr(VI) reduction. The natural carbon source used was saw dust to simulate the carbon sources leached from the overlying vegetation above the ground.

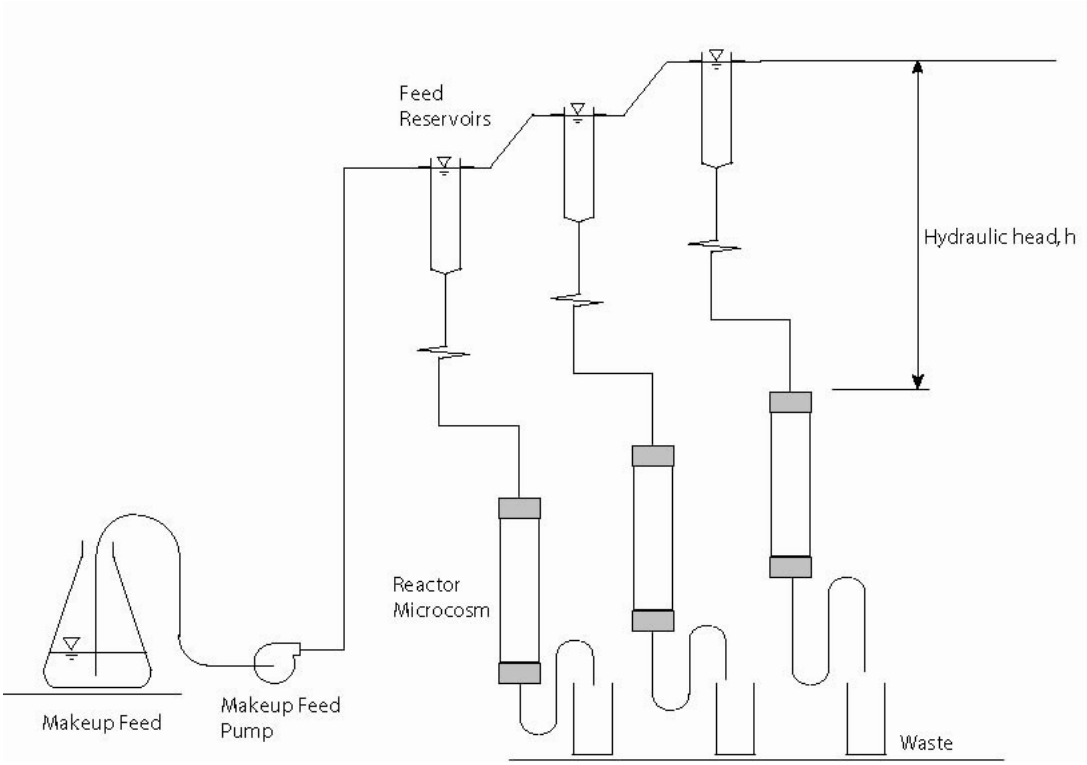

Figure 3-3: Experimental set-up for gravity fed microcosm.

3.6.4 Mesocosm Reactor Studies

An open top tank of (in cm) 123 × 52 × 50 (L×B×H) was constructed from Plexiglas® (Evonik Röhm GmbH, Essen, Germany) reinforced by steel bars as shown in Figure 3-4. The reactor was filled with aquifer medium compacted by hand to a compaction consistent with the ground conditions. Fourteen sample ports of 11 mm diameter and up to 47 cm in length, (M-level being 22cm and L-level 47cm) glass tubing were inserted during placement of the aquifer material. Sample ports were strategically placed to capture the longitudinal and vertical concentration profiles and the concentration drop across the microbial barrier. Two sets of sample ports were placed before the reactive barrier in order to capture the conditions before the chromium had gone through the reactive barrier. The rest of sampling ports were placed after the barrier to evaluate the performance of the barrier. This test was run at a chromium (VI) concentration of 50 mg/L.

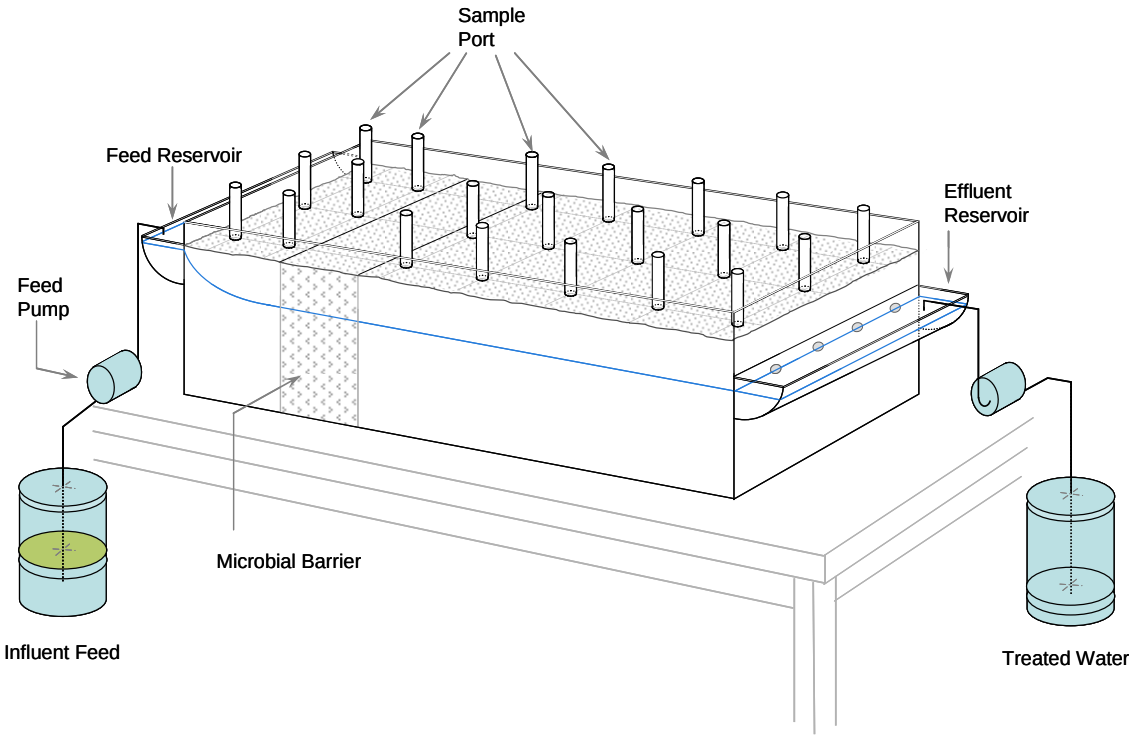

Figure 3-4: Mesocosm reactor setup using the rock media from the Cr(VI) contaminated site in Brits (North West).

3.7 Analytical Methods

3.7.1 Elemental Analysis

Metallic elements in microcosm media were characterised using Induction Coupled Plasma Mass Spectrometry (ICP-MS) in the Soil Sciences Laboratory at the University of Pretoria. This was done to reveal sources of possible interference and levels of background chromium in the samples. Mass concentrations of elements with the most significant presence in the samples are shown in Table 3-4. Of significant note are the concentrations of total iron (Fe), aluminium (Al), and calcium which are expected to produce interference in the UV/Vis spectrophotometric measurements for Cr(VI) (APHA, 2005). However the observed Fe concentration level did not contribute significantly to the coloration of the samples.

Table 3-4 : Trace metal concentrations.

Element	Symbol	Mass concentration, µg/kg
Aluminium	Al	4003
Calcium	Ca	2868
Potassium	K	282
Magnesium	Mg	542
Sodium	Na	248
Phosphorous	P	1046
Manganese	Mn	543
Chromium	Cr	50
Copper	Cu	13
Iron	Fe	15145
Sulphur	S	143
Zinc	Zn	367

3.7.2 Cr(VI) and Total Cr

Cr(VI) Measurement

Cr(VI) was measured using a UV/VIS spectrophotometer (WPA, Light Wave II, Labotech, South Africa). The measurement was carried out at a wavelength of 540 nm (10 mm light path) after acidification of 0.2ml samples with 1N H_2SO_4 and reaction with 1,5-diphenyl carbazide to produce a purple colour (APHA, 2005).

Total Chromium Measurement

Total Cr was measured at a wavelength of 359.9 nm using a Varian AA – 1275 Series Atomic Adsorption Spectrophotometer (AAS) (Varian, Palo Alto, CA (USA)) equipped with a 3 mA chromium hollow cathode lamp. Cr(III) was determined as the difference between total Cr and Cr(VI) concentration.

3.7.3 Viable Biomass Analysis

Viable cells in batch experiments were determined using the pour plate method and colony counts as described in the Standard Methods for the Examination of Water and Wastewater (APHA, 2005), with the colonies grown on Luria Betani (LB) and Plate Count (PC) agar. Colonies were developed by platting 1 mL samples serially diluted samples (1 mL transferred into 9 mL sterile 0.85% NaCl solution) onto Petri dishes followed by incubation at 30^{o}C for 24 hours. The Petri dishes were inverted during incubation. The number of cells was reported as colony forming units (CFU) per 100 mL of water.

For the mesocosm study (Figure 3-4), soil samples were extracted from barrier section within the mesocosm reactor. Suspended biomass from the soil samples was cultured on LB agar for heterotrophic culture growth. The numbers of colonies counted gave a representation of biomass density in the soil column. Total biomass concentration was estimated using a correlation analysis between viable cells as CFU and total biomass (mg/L) during the log growth phase when over 95% percent of the cells were expected to be viable(Molokwane *et al*., 2008).

3.7.4 Total Biomass (Suspended Cells)

Five (5) mL of LB broth containing grown cells was withdrawn by sterile pipette after 24 hrs of incubation at 30^{o}C and filtered through a washed dried and weighed sintered glass (tare weight). The sintered glass and wet biomass was dried in the oven at 105^{o}C, cooled in a desiccator and weighed. The drying, cooling and weighing was carried out until a constant dry weight was obtained. The dry weight of the biomass in 5 mL was calculated as the difference in weight between the tare weight of the

sintered glass and the dry weight of sample + sintered glass. The dry weight of the biomass (mg/L) was obtained by multiplying the obtained mass (mg) by a factor of 200. There are 200 samples × 5 mL samples in 1 litre.

3.7.5 Cr(VI) Reduction Activity

The Cr(VI) reduction capacity of the cells was determined as the amount of Cr(VI) reduced per amount of viable cells inactivated during incubation (Shen and Wang, 1994):

$$R_c = \frac{C_o - C}{X_o - X} \tag{3-1}$$

where R_c = Cr(VI) reduction capacity (mg Cr(VI) removed /mg cells inactivated), C_o = initial Cr(VI) concentration (mg/L), C = Cr(VI) concentration at a time of incubation t, X_o = initial viable cell concentration (mg/L), and X = viable cell concentration (mg/L) at any time t. A viable cell conversion factor of 1.833×10^{-10} mg/cell was used to convert cell count (CFU) to the mass concentration (determined from a standard curve of cell mass versus cell counts performed during the log growth phase). A near linear relationship for the linearised relationship of cell mass versus colony count with the *Pearson's Regression Coefficient*, $R^2 = 0.997$ was obtained.

CHAPTER 4

MICROBIAL CR(VI) REDUCTION KINETIC STUDIES

4.1 Modelling Methodology

The problem of evaluating fundamental processes in the biologically mediated Cr(VI) reduction in soil media was simplified by studying individual processes first. The reaction rate kinetics in the system is time dependent, thus is best evaluated in batch reactor systems. Time series (batch) experiments were first conducted on the isolated culture from the Cr(VI) contaminated environment. The effect of Cr(VI) on the removal rate was evaluated at different initial Cr(VI) concentrations, 50-400 mg/L, and the results were later used to evaluate the effects of Cr(VI) toxicity on the Cr(VI) reduction rate.

4.2 Mixed Culture Performance (Batch)

4.2.1 Biotic versus Abiotic Cr(VI) Reduction

Abiotic Cr(VI) reduction activity was evaluated by conducting experiments at 100 mg Cr(VI)/L with heat killed and azide inhibited cultures (Figure 4-1). A live cell culture control showed best performance with near complete Cr(VI) removal at 22.5 hours. There was significant decrease in Cr(VI) reduction activity due heat inactivation of the cells.

Only 30% Cr(VI) removal was observed in heat-killed cultures after incubation for 22.5 hours, a much lower removal value than that observed in the live consortium. The 30% removal may be due to Cr(VI) reductase released into the medium from heat-lysed cells and regrowth of cells that escaped destruction by heat. An azide inhibited culture indicated partial inactivation of cells with an observed Cr(VI)

reduction potential of the oxygen stressed culture. Approximately 50% Cr(VI) was removed in the azide inhibited cultures whereas 18% of Cr(VI) was reduced from cell free control experiments.

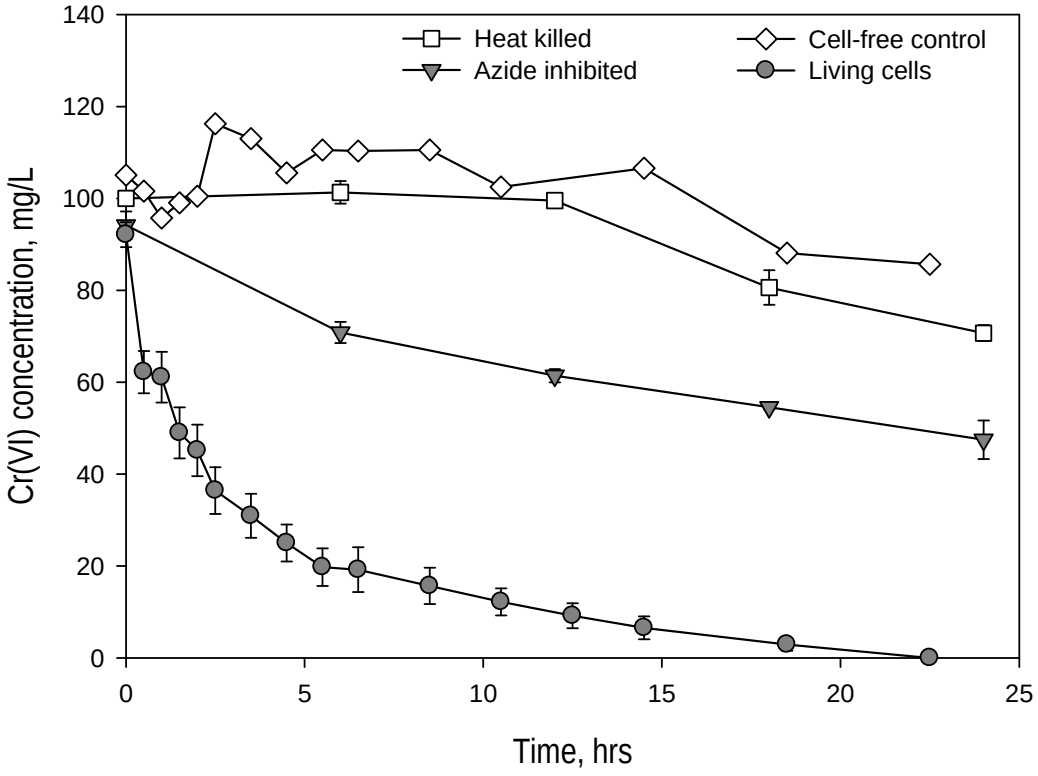

Figure 4-1: Evaluation of abiotic Cr(VI) reduction in heat-killed and azide inhibited cells (inoculated with 5×10^4 CFU/mL before incubation).

4.2.2 Cr(VI) reduction under aerobic conditions

Experimentation under varying initial Cr(VI) concentration of 50-400 mg/L in media with harvested and concentrated cells showed that the culture achieved complete Cr(VI) removal in batches under initial concentration up to 200 mg/L in less than 64.3 hours (2.7 days) (Figure 4-2). Up to 94% of Cr(VI) was removed at the initial concentration of 300 mg/L after incubation for 110 hours.

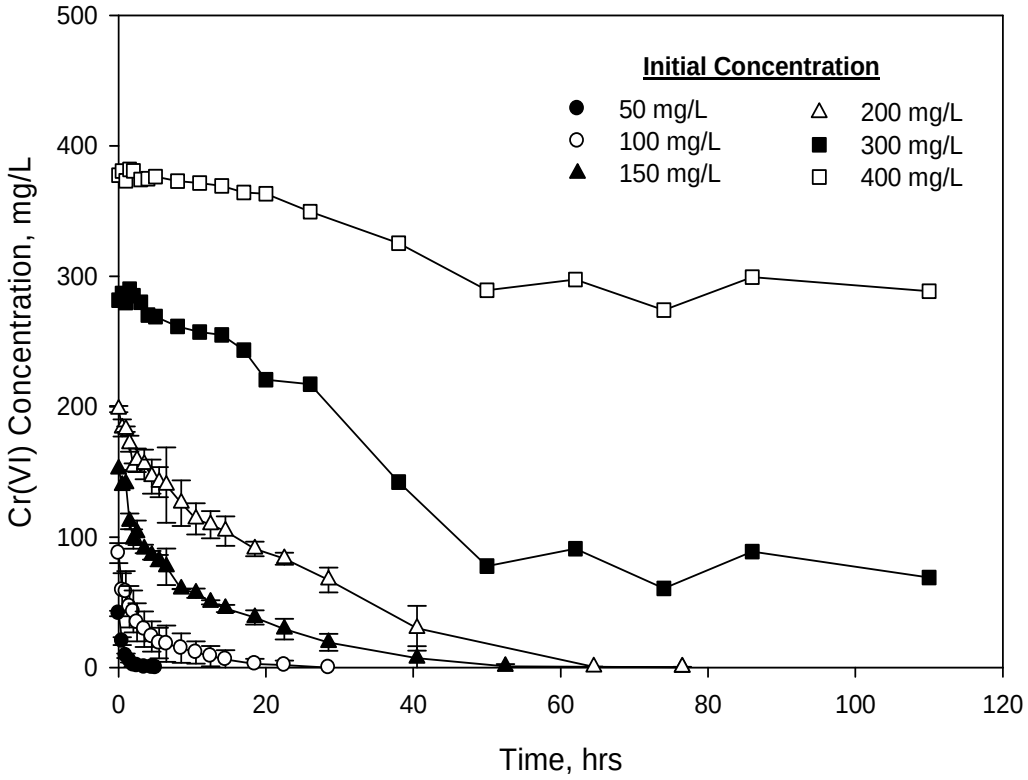

Figure 4-2: Aerobic culture experiment of Cr(VI) reduction in consortium from dried sludge grown at initial Cr(VI) concentrations ranging from 50 to 400 mg/L (resting cells: $5.2\pm2.1\times10^{9}$ CFU/mL).

Very little Cr(VI) was reduced at the highest concentration tested (400 mg/L). The loss of the capability to reduce Cr(VI) in cells under very high Cr(VI) loadings was directly correlated to the loss of cell viability. Viable cell concentration in the 400 mg/L batches decreased from $5.2\pm2.0\times10^{9}$ to $4.8\pm1.5\times10^{5}$ cells/mL after 22.5 hours incubation, a kill rate of 4.1 log, where as the kill rate at lower concentration of 100 mg/L was only 1.2 log ($6.1\pm1.8\times10^{9}$ to $3.81\pm1.5\times10^{8}$ cells/mL). The activation of cells was attributed to a combination of toxicity effects and the diversion of reducing equivalents away from metabolism and cell maintenance (Chirwa and Wang, 2000).

Based on the highest concentration completely removed, i.e., 200 mg/L batch, the value of the Cr(VI) reduction capacity coefficient (R_c) of 0.21 mg Cr(VI) reduced/mg

cells deactivated was determined. This value is much higher than the values previously reported in literature (Shen and Wang, 1994a, Nkhalambayausi-Chirwa and Wang, 2005).

4.2.3 Cr(VI) Reduction under Anaerobic Conditions

Cr(VI) reduction under anaerobic conditions has significant engineering implications since most of the environments where *in situ* bioremediation processes could occur are closed systems underneath the ground without any direct contact with the atmosphere. Additionally, anaerobic biological processes tend to be passive in nature without excessive energy input requirements for aeration. Anaerobic processes are the oldest and most versatile of all metabolic processes (Bush, 2003). In the absence of organic carbon sources, a range of autotrophic organisms are capable of utilising inorganic carbon sources such as carbon dioxide and carbonates from the rocks as raw materials for anabolic processes.

To evaluate Cr(VI) reduction under anaerobic conditions, batch experiments were conducted over a lower concentration range (50-300 mg Cr(VI)/L) at a temperature of 30^{o}C and pH of 7 ± 0.2. Due to observed slower growth in the anaerobic cultures, a lower Cr(VI) reduction capacity of the cells was expected, thus the experiments were conducted under lower initial concentrations than in the aerobic cultures. Complete Cr(VI) reduction occurred in cultures with a lower initial Cr(VI) concentration of 150 mg/L after a longer incubation period (155 hours) than in aerobic cultures (Figure 4-3). Cr(VI) reduction was incomplete at 200 mg/L initial Cr(VI) concentration after incubation for 130 hours (only 50% reduced). This was a much lower performance compared to the observed Cr(VI) reduction under the same concentration in aerobic cultures where 99.7% removal was achieved after 96 hours.

The lower Cr(VI) removal rates observed under anaerobic conditions were accompanied by lower Cr(VI) reduction capacity of the cells (R_c = 0.011427 g Cr(VI) reduced/g cells inactivated at 150 mg/L and 0.051697 g Cr(VI) reduced / g cells inactivated at 200 mg/L). The R_c value under anaerobic conditions was thus an order of magnitude lower than the value obtained from aerobically grown cultures from the same source.

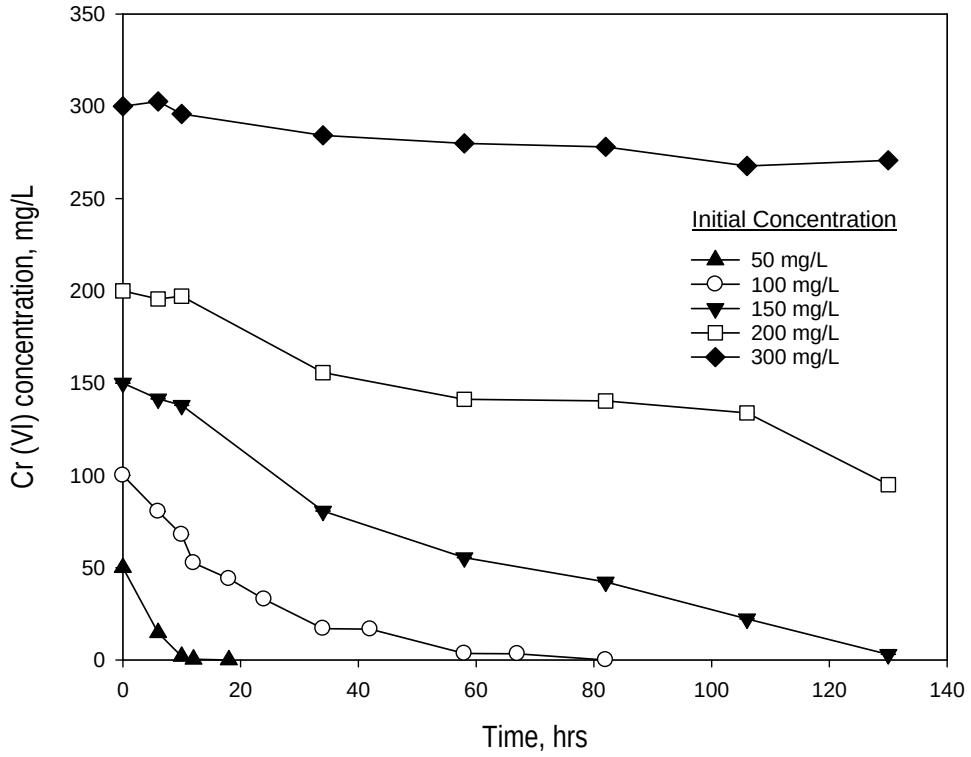

Figure 4-3: Anaerobic culture experiment of Cr(VI) reduction in consortium from dried sludge grown at initial Cr(VI) concentrations ranging from 50 to 300 mg/L (resting cells: 1.58±1.8 $\times 10^9$ CFU/mL).

4.2.4 Decisions from Observed Trends

In the above studies, a clear association was drawn between the Cr(VI) reduction rate and metabolic activity of the cells. Additionally, the experiments clearly show that Cr(VI) reduction facilitated by the cells was inhibited by high concentrations of

Cr(VI) in the medium. These observations are consistent with earlier studies by Shen and Wang (1994a), Wang and Shen (1997), Shen and Wang (1995), and Chirwa and Wang (1997a&b), in which high levels of Cr(VI) inhibited both the growth and Cr(VI) reducing activity in pure and mixed cultures of bacteria. These observations led us to develop the Cr(VI) reduction model based on the enzymatic Cr(VI) reduction kinetics as described in the following section.

4.3 Enzymatic Cr(VI) Reduction

The Cr(VI) reduction rate which forms part of the Cr(VI) removal regime in continuous-flow studies was derived based on the following assumptions:

1. That Cr(VI) reduction is catalysed by a single or dual-site enzyme.
2. That the enzyme is either regulated or induced, i.e., is produced when the cell is exposed to Cr(VI).
3. That 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).
4. In the mixed culture, it is assumed that 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.
5. The sum of or the highest of the activities, ΣE_i, may be represented by one representative enzyme, E.

The representative enzyme (E) is logically proportional to the viable cell concentration X as the only metabolically active component in the culture. Based on Assumption 1, the enzymatic reaction can be represented as:

$$\begin{array}{l} E + \mathrm{Cr(VI)} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E^{*} \cdot \mathrm{Cr(VI)} \xrightarrow{k_2} E + \mathrm{Cr(III)} \quad \text{(I)} \\ \qquad\qquad \downarrow \\ \qquad\qquad + \\ \mathrm{Cr(VI)} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{Cr(VI)} \cdot E^{*} \cdot \mathrm{Cr(VI)} \xrightarrow{k_4} \mathrm{Cr(III)} + E + \mathrm{Cr(III)} \quad \text{(II)} \end{array} \tag{4-1}$$

where E = the Cr(VI) reductase enzyme for the consortium which is proportional to biomass concentration X (ML^{-3}); E^{*} = the activated enzyme; E^{*}·Cr(VI) and Cr(VI)·E^{*}·Cr(VI) are the transitional enzyme-Cr(VI) complexes; and k_1, k_2, k_3, and k_4 are the reaction rate constants in the directions indicated by the arrows.

Previous studies have suggested that the reaction rate for the formation of the double Cr(VI) complex (k_3) is very slow compared to the decomposition reaction determined by k_2. Under these circumstances, the overall Cr(VI) reduction is simplified to:

$$r = \frac{-d(Cr(VI))}{dt} = \frac{d(Cr(III))}{dt} = k_3 \cdot E^{*}.Cr(VI) \tag{4-2}$$

Then the rate of formation of E^{*} can be described as follows:

$$\frac{d(E^{*})}{dt} = k_1(E - E^{*})(C) - k_2(E^{*}) - k_3(E^{*}) \tag{4-3}$$

Assuming steady-state conditions, E^{*} is formed and destroyed spontaneously such that:

$$d(E^{*})/dt \approx 0 \tag{4-4}$$

The mass balance represented by Equation 4-3 can be written as:

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

Then E^{*} can be expressed by:

$$E^* = \frac{C \cdot E}{C + \frac{k_2 + k_3}{k_1}} \tag{4-6}$$

And, the Cr(VI) reduction rate in Equation 4-3 becomes:

$$r = \frac{-d(C)}{dt} = \frac{k_3 \cdot C \cdot E}{C + \frac{k_2 + k_3}{k_1}} \tag{4-7}$$

where k_1, k_2 and k_3 are constants. The groups of constants in Equation 4-7 can be replaced by symbols from enzyme kinetics as follows: $(k_2 + k_3)/k_1$ can be replaced by the half velocity concentration K_c (ML^{-3}), and k_3 can be replaced by the maximum specific Cr(VI) reduction rate coefficient k_{mc} (T^{-1}) such that:

$$r = \frac{-d(C)}{dt} = \frac{k_{mc} \cdot C \cdot E}{C + K_c} \tag{4-8}$$

For any amount of live cells X, the amount of enzyme produced will be proportional to the viable cell concentration such that the enzyme E can be replaced by the cell biomass term X. This gives a Monod type equation (Shen and Wang,1994a):

$$r = \frac{-d(C)}{dt} = \frac{k_m \cdot C \cdot X}{C + K_c} \tag{4-9}$$

where C = Cr(VI) concentration at time t (ML^{-3})

k_{mc} = maximum specific Cr(VI) reduction rate coefficient (T^{-1})

K_c = half velocity constant (ML^{-3})

X = concentration of viable cells (ML^{-3}) at any time t (T)

Similar expressions were derived previously by other researchers for Cr(VI) reduction in batch systems (Shen and Wang, 1994a; Mazierski, 1995; Schmieman *et al.*, 1998;

Guha *et al*., 2001; Li *et al*., 2006).

4.4 Cr(VI) Reduction Capacity of Cells

The extent of Cr(VI) reduction in batch systems depends on the number of cells in the reactor and the Cr(VI) reduction capacity (R_c) per cell. When batch experiments are conducted using pre-concentrated washed cells, cell growth kinetics may be ignored as the concentration of cells is too high to allow production of new cells. The amount of Cr(VI) reduced under resting cell conditions will thus be proportional to amount of cells inactivated by Cr(VI):

$$C_o - C = R_c\left(X_o - X\right) \tag{4-10}$$

where X_o = initial active cell concentration (ML^{-3}),

X = active cell concentration (ML^{-3}) at time t (T),

C_o = initial Cr(VI) concentration (ML^{-3}), and

R_c = finite Cr(VI) reduction capacity (MM^{-1}).

This relationship is expressed in terms of the concentration of viable cells remaining in solution at any time t as follows:

$$X = X_o - \frac{C_o\text{-}C}{R_c} \tag{4-11}$$

R_c can be estimated from Cr(VI) reduced and initial cell concentration at high initial Cr(VI) concentration (when $C \neq 0$, and the rate of change, $dC/dt \cong 0$, at $t \rightarrow T_\infty$, where T_∞ represents a very long time of incubation) for the last highest batch in which Cr(VI) is completely removed:

$$R_c = \frac{C_o - C}{X_o} \tag{4-12}$$

Since the concentration of Cr(VI)-reductase (E_t) in whole cell cultures is proportional to viable cell concentration at time t, E_t in Equation 4-8 can be replaced with the term for active cell concentration (X) as represented in Equation 4-10 (Shen and Wang,1994a), such that:

$$-\frac{dC}{dt} = \frac{k_{mc} C}{K_c + C}\left(X_o - \frac{C_o - C}{R_c}\right) \tag{4-13}$$

4.5 Parameter Determination

4.5.1 Aerobic batch kinetics

Equation 4-13 was simulated initially using guessed values using the Computer Program for the Identification and Simulation of Aquatic Systems (AQUASIM 2.01) (Reichert, 1998). The results showed that the maximum reaction rate coefficient (k_{mc}) and half velocity concentration (K_c) was not constant over different Cr(VI) concentration ranges (Table 4-1). The results suggested non- competitive inhibition rate kinetics affected directly by the increase in initial Cr(VI) concentration. The amended kinetic model (Equation 4-14) produced near constant kinetic parameters at 95% confidence (Table 4-2).

Table 4-1: Optimisation of kinetic parameters using the cell inactivation only (Equation 4-13) under aerobic conditions.

Initial [Cr(VI)] (mg/L)	k_{mc} **(1/hr)**	K_c **(mg/L)**	R_c **(mg/mg)**	X_o **(mg/L)**
50	1.0797	835	0.0402	4500
100	0.3924	785	0.0776	4500
150	0.3909	2773	0.1435	3480
200	0.0046	3.549	0.1856	2000
300	0.2827	21432	10270.2	1460
400	0.0387	24677	0.0543	1250

$$-\frac{dC}{dt} = \left(\frac{k_m}{(1+C_o/K_I)}\right)\left(\frac{C}{K_c + C}\right)\left(X_o - \frac{C_o - C}{R_c}\right) \quad (4\text{-}14)$$

Table 4-2 shows the optimum parameters generated for each initial Cr(VI) loading condition using the non-competitive inhibition model. The parameter values determined using the revised model are reasonably constant thus this mechanisms was accepted under the aerobic conditions. The parameters optimized simultaneously using the 100 mg/L and 150 mg/L data were used to simulate the Cr(VI) concentration through the entire range of data and the results were plotted against measured data as shown in Figure 4-4. The model captured well the trend of data under all experimental conditions. Slight difficulty of fit was observed for the 400 mg/L data set mainly due to excessive loss of biomass not captured by the initial model. The highest range of uncertainty was observed in the data for the 300 mg/L mainly due to the instability in the measured values near time zero.

Table 4-2: Optimisation of the non-competitive inhibition model with cell inactivation (Equation 4-14) under aerobic conditions.

Initial [Cr(VI)] (mg/L)	k_{mc} **(1/hr)**	K_c **(mg/L)**	R_c **(mg/mg)**	K_I **(mg/L)**	X_o **(mg/L)**	Chi^2
50	0.0404	403.3	0.0665	145.1	4500	0.87965
100	0.0404	403.3	0.0665	145.1	4500	139.4847
150	0.0404	403.3	0.0665	145.1	3480	880.0954
200	0.0404	403.3	0.1730	145.1	2000	2082.352
300	0.0404	403.3	0.1730	145.1	1460	7646.5751
400	0.0113	551.9	0.1306	151.0	1250	2537.7485

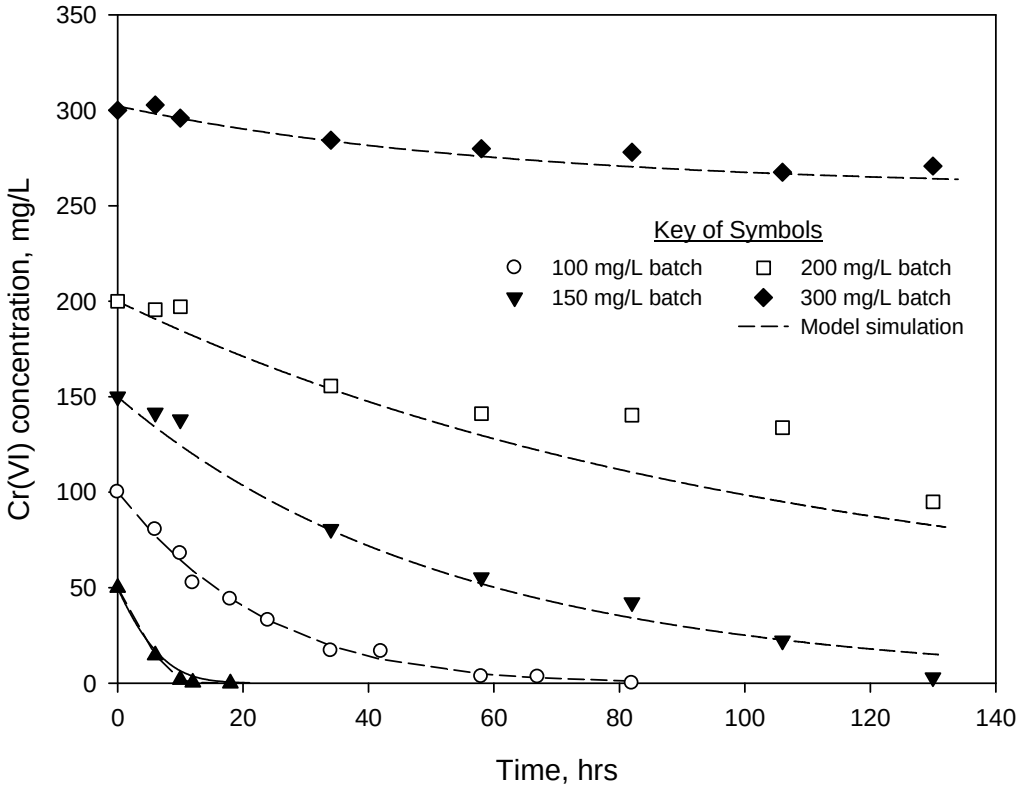

Figure 4-4: Aerobic Cr(VI) reduction at different concentration (modelled aerobic plots).

4.5.2 Anaerobic Batch Kinetics

Although chromate reduction ($CrO_4^{2-} \rightarrow Cr^{3+}$) is thermodynamically favourable, Cr(VI) reduction to Cr(III) may be limited by reaction kinetics under physiological conditions (Glaze, 1990). On the other hand, the kinetics of Cr(VI) reduction may be improved by coupling Cr(VI) reduction to other energy yielding reactions such as the catabolism of organic compounds and intermediates (Chirwa and Wang, 1997a, Shen and Wang, 1995a; Ishibashi *et al.*, 1990). Anaerobic Cr(VI) reduction can be carried out through energy yielding dissimilatory respiratory process in which Cr(VI) serves as a terminal electron sink (Lovley and Phillips, 1994).

In this study, Cr(VI) reduction under low initial Cr(VI) concentrations appeared to benefit from the presence of Cr(VI). In such a case, a toxicity threshold concentration

has to be reached before Cr(VI) inhibition becomes effective. The data in Table 4-3 shows that the equation derived for anaerobic conditions (Equation 4-14) could not describe the kinetics for Cr(VI) reduction under anaerobic conditions.

The model did not converge with respect to the inhibition parameter K_I under all tested conditions. Alternatively, a threshold of inhibition C_r was applied with a non-competitive inhibition model as shown in Equation 4-15a and b (below):

$$-\frac{dC}{dt} = \frac{k_m \cdot K^{-(C_0-C_r)/C_0} \cdot C}{(K_c + C)}\left(X_0 - \frac{C_0 - C}{R_c}\right) \qquad (4\text{-}15a)$$

For numerical formulation, the form presented below was used:

$$-\frac{dC}{dt} = \frac{k_m \cdot C}{K^{1-Cr/C_0} \cdot (K_c + C)}\left(X_0 - \frac{C_0 - C}{R_c}\right) \qquad (4\text{-}15b)$$

Table 4-3 shows that the parameters evaluated from different initial Cr(VI) concentration batches converged at decreasing values generally. This response shows that the non-competitive inhibition model is ill suited under these conditions. After introducing the threshold inhibition term, the maximum Cr(VI) reduction rate coefficient is stabilised. The model fits other data sets universally as shown by near constant values in Table 4-4. The target biomass concentration in the anaerobic cultures was 1800 mg/L. The optimum inhibition threshold concentration was determined to be between 95-105 mg/L.

Table 4-3: Optimisation of kinetic parameters under anaerobic conditions using non-competitive model with cell inactivation (Equation 4-14).

Initial [Cr(VI)] (mg/L)	k_{mc} (1/hr)	K_c (mg/L)	R_c (mg/mg)	K_I (mg/L)	X_o (mg/L)
50	0.0025	167.2	0.1804	1×10^9	--
100	0.0025	167.2	0.1804	1×10^9	4459.50
150	0.0025	167.2	0.2062	1×10^9	2098.64
200	0.0023	121.6	0.2318	1×10^9	883.65
300	0.0025	114.5	0.1473	1×10^9	281.56

Table 4-4: Optimisation of kinetic parameters under anaerobic conditions using the cell inactivation model with threshold inhibition (Equation 4-15b).

Initial [Cr(VI)] (mg/L)	k_{mc} (1/hr)	K_c (mg/L)	R_c (mg/mg)	K (mg/L)	X_o (mg/L)	Chi^2
50	0.0025	167.2	0.1027	9.964	4459	1.9387
100	0.0025	167.2	0.1027	9.964	4460	88.8598
150	0.0025	167.2	0.1027	9.964	2100	460.823
200	0.0025	167.2	0.1027	9.964	1000	685.1592
300	0.0025	167.2	0.0391	9.964	260	54.0873

The model based on model parameters optimised in the 100 mg/L batch fitted well the rest of the experimental data as shown in Figure 4-5. The data obeyed the proposed model at different concentrations except for the concentrations at lower levels, e.g., 50 mg/L, since the threshold was determined at 100 mg/L hence the 50 mg/L parameters are not shown. The more the bacteria were exposed to higher doses of chromium the more the population of the bacterial species decreased.

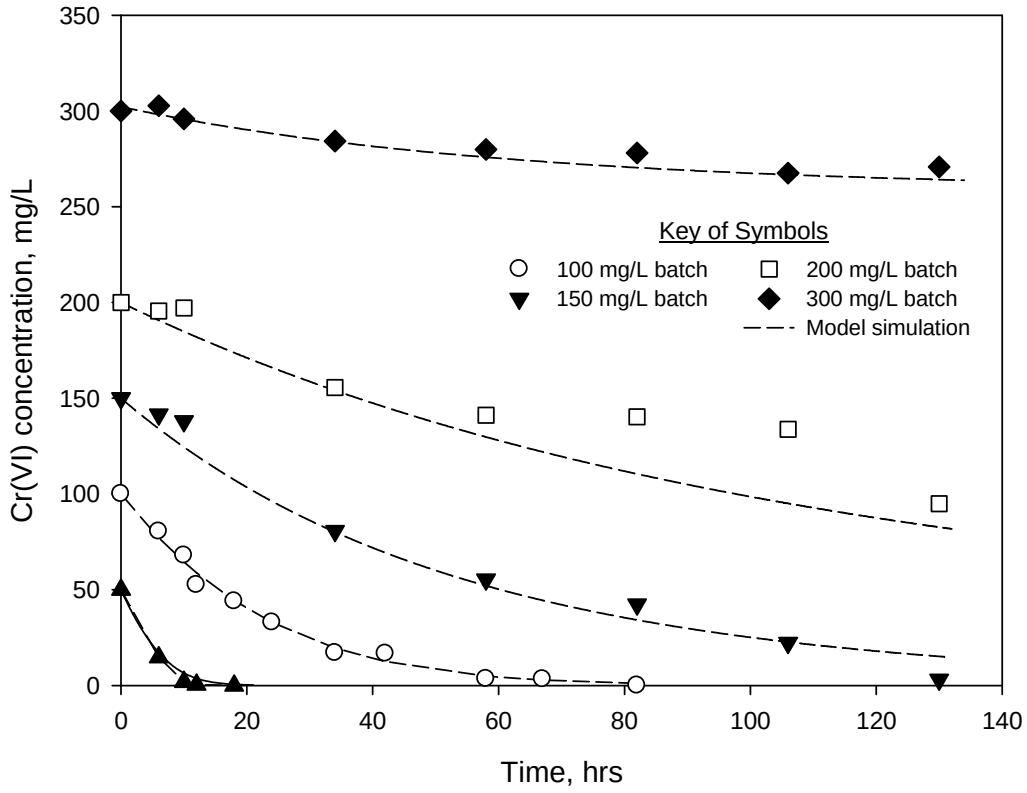

Figure 4-5: Anaerobic (threshold inhibition) chromium (VI) reduction at different concentration (modelled anaerobic plots).

4.6 Sensitivity Analysis

Sensitivity is a function used to compare the effect of different parameters on a common variable (Peter Reichert, 1998). Figures 4-6 and 4-7 illustrate the time dependence of the sensitivity response curves computed over a 1.0% variability of each kinetic parameter in the object function. For the aerobic culture, parameter sensitivity was conducted for the parameters k_{mc}, K_c, R_c, and K_I (Figure 4-6). The parameters k_{mc}, R_c, K_c and K were evaluated for the anaerobic culture. The parameter C_r was assigned and was expected to be strictly culture dependent.

The results show that the aerobic model was highly sensitive to minor adjustments in k_{mc}, K_c, and K_I in the first 20 hours of incubation. The response was highest at approximately 5 hours which indicates the period of highest activity. These results

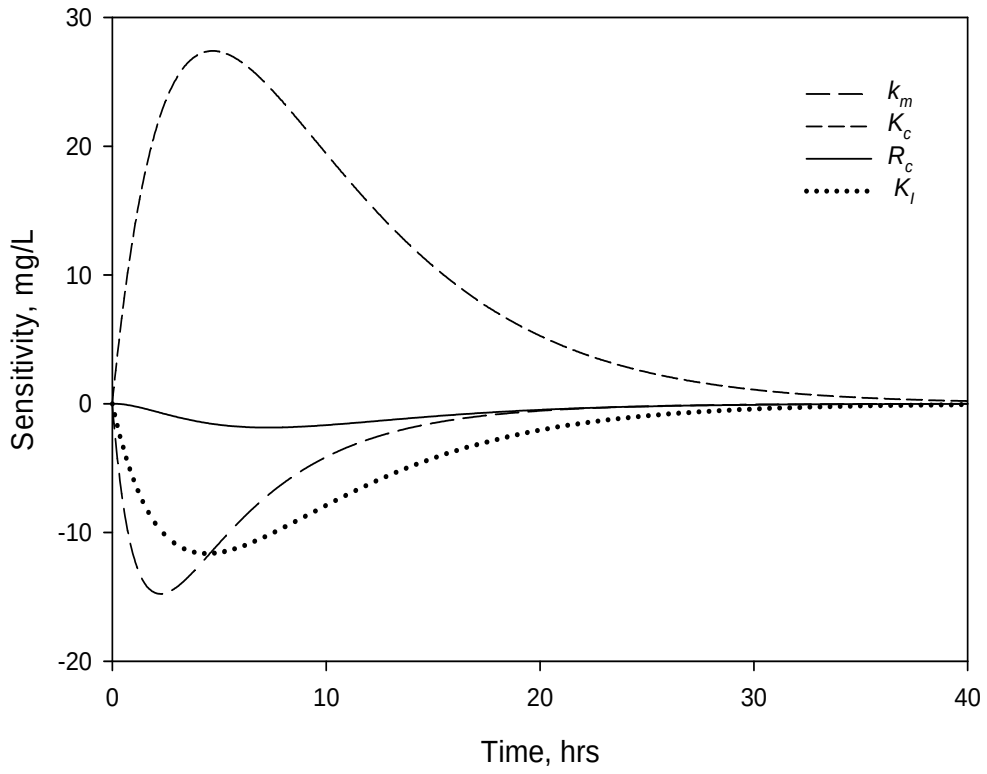

Figure 4-6: Aerobic sensitivity test at 100 mg/L.

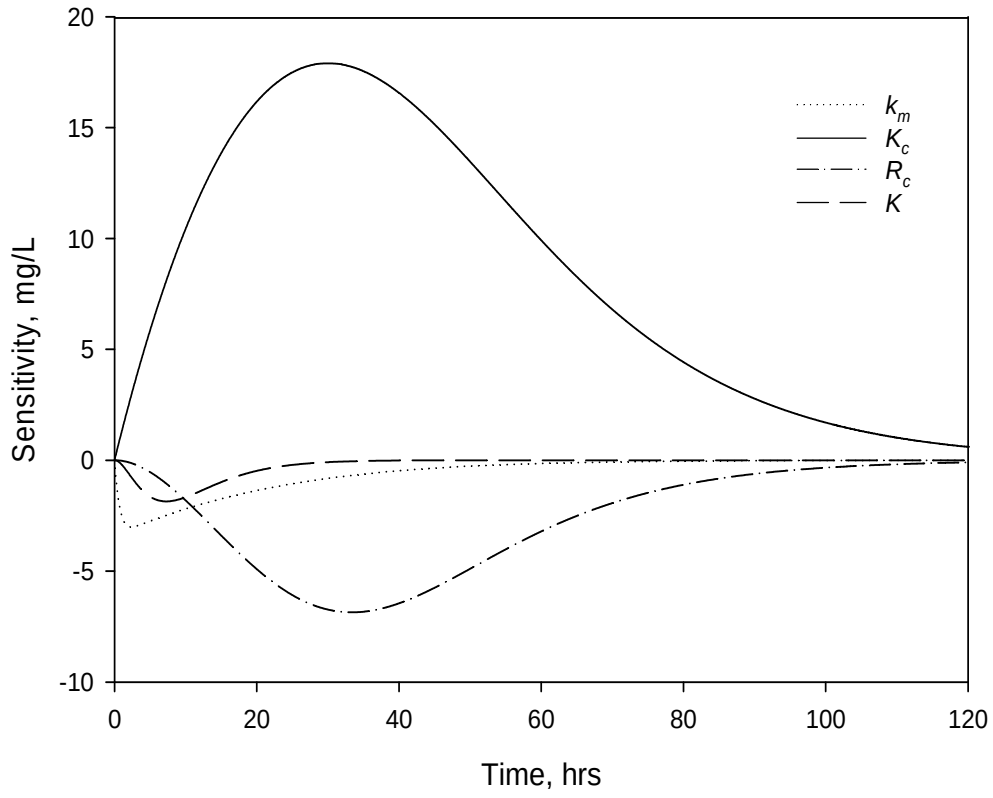

Figure 4-7: Anaerobic sensitivity test at 100 mg/L.

show that the kinetic parameters k_{mc}, K_c, and the inhibition parameter K_I will be very important in the scale up of the reactor.

Similarly, the anaerobic model was highly sensitive to the reaction rate coefficient k_{mc} and half velocity concentration K_c. Due to the low Cr(VI) reduction capacity in the anaerobic culture and higher sensitivity to toxicity, the Cr(VI) reduction capacity coefficient R_c is also significantly sensitive (Figure 4-7). The impact of the non-competitive parameter K was mild at 100 mg/L since this was just at the threshold when the presence of Cr(VI) is no longer beneficial to the metabolic process. The Sensitivity analysis highlights the fundamental difference between Cr(VI) reduction under anaerobic conditions at high metabolic rate and the Cr(VI) reduction under anaerobic conditions at slower metabolic rate. The complex interrelationship between the toxicity effects and Cr(VI) reduction capacity is illustrated by the behaviour of the R_c, K_I and K values under the two growth conditions.

4.7 Chapter Summary

The results showed that the performance of the bacteria fitted best the non-competitive inhibition model with cell inactivation under aerobic conditions. This suggests that the fast cell metabolism under aerobic conditions compensated for the competition for sites between the two available electron sinks – O_2 and Cr (VI). Under anaerobic conditions, the kinetic process was complex due to the high biodiversity of species and the slow growing culture that was more sensitive to toxic loading. Cr(VI) reduction under these conditions was competitively inhibited with cell inactivation. The competitive inhibition was effective above a threshold concentration of approximately 100 mg/L following a mechanism observed earlier by (Shen and Wang, 1995b). The threshold Cr(VI) toxicity level in this study was much higher than

reported by Wang and Shen (1995b) because non-toxic carbon sources similar to those found in the environment (mainly from decaying vegetation) were used.