CHAPTER 1

INTRODUCTION

1.1 Background

Hexavalent chromium is one of the toxic heavy metals with high mobility in soil and groundwater which can produce harmful effects on organisms including humans. Hexavalent chromium [Cr(VI)] compounds are used in a wide variety of commercial processes such as chromite ore processing, electroplating, and leather-tanning processes, among others (Chuan and Liu, 1996; Lawson, 1997). The unregulated disposal of chromium containing effluents has led to the contamination of soil, aquatic sediments, and surface and groundwater environments.

Most of the contaminated sites around the world are treated using abiotic processes implemented with pump-and-treat or dig-and-treat methods that require follow up precipitation or immobilisation steps (Cifuentes *et al*., 1996; Quintana *et al*., 2001). Among the latest of the findings is the probability of using biological reduction methods for the treatment of hexavalent chromium-containing wastes (Donat and Guruchet, 2003, Rege *et al*., 1997; Rajwade and Paknikar, 1997; Mel Lytle *et al*., 1998; Salunkhe *et al*., 1998).

In situ bioremediation technology using permeable reactive barriers is a relatively a new application, commonly not been implemented much but slowly finding use for the remediation of organic pollutants (Borden *et al*., 1997); Rasmussen *et al*., 2002; Wilkin *et al*., 2003; Carsten *et al*., 2004). Studies using zero valent iron to reduce and precipitate Cr(VI) have been assessed in both batch and column tests (Blowes & Ptacek, 1992; Powell *et al.,* 1995). However there has been minimum research on

bioremediation of heavy metals including Cr(VI) by means of PRB (permeable reactive barrier) using microorganisms. The process of cleaning up metals is not a straight forward one since metals cannot be destroyed; they are only transformed from one oxidation state to another as it is in the case with hexavalent chromium.

Bioremediation processes are considered a better alternative to physical-chemical treatment technologies since they do not introduce any foreign material into the ecosystem. They also do not involve further transportation of toxic material which may lead to more spillage in transit. Nevertheless, there is still a need for final removal of reduced metal or oxidised states trapped in the media.

The current research evaluates a methodology that could offer an opportunity for on site treatment of the contaminant using chromium reducing bacteria in the form of a permeable reactive barrier. This could minimise the disadvantages and negative impacts experienced with physical-chemical processes. This technology will later be tested at a pilot site around the abandoned refinery found in Brits. The Cr (VI) at the contaminated site estimated at 4,050 kg Cr(VI) will take approximately 30 years to flush out using the currently employed pump-and-treat method.

1.2 Unique Methods

Consortium cultures were characterised using 16S rRNA genomic fingerprinting. In suspended growth batch cultures gram-positive *Bacillus* genera predominated under aerobic conditions with a small composition of the gram-negative *Microbacterium* sp. More biodiversity was observed in anaerobic cultures.

Phylogenetic characterization of cells was also performed on individual colonies of bacteria from cultures grown anaerobically from soil samples extracted from the microcosms at the beginning and end of the experiment. Genomic DNA was extracted

from the pure cultures using a DNeasy tissue kit (QIAGEN Ltd, West Sussex, UK). The 16S rRNA genes of isolates were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) as described by Coenye *et al.* (1999). Internal primers complimentary to base-pair 519–536 of the 16S gene were used for amplification and sequencing.

The significant part of the current investigation was to establish an *in situ* bioremediation method which could be tried in the pilot study at the site under investigation. This involved operation of a bench-scale mesocosm with a barrier inoculated with a consortium of cultures collected from a local waste water treatment plant. The study demonstrates the potential of *in situ* inoculation as a method of establishing a permeable reactive barrier with minimum engineering work and with no construction required. The laboratory studies were conducted at concentrations of 40 mg/L and 50 mg/L representing aquifer conditions at the target barrier location approximately 200m from the hot spots of contamination.

1.3 Objectives

The main objective of this exercise is to evaluate the prospect of Cr(VI) pollution containment in groundwater aquifers at a site. The proposed methodology could offer a more sustainable alternative to the current pump-and-treat method. Task was undertaken in the in the following order to achieve the main objective:

- Evaluation of the performance of cultures and individual species in the source organism.
- Investigation of microbial culture dynamics during operation of simulated microbial barriers.
- Development and evaluation of a predictive dispersion-reaction for Cr(VI) removal in microbial barrier.

1.4 Main findings

In packed column microcosm reactors, approximately 95% Cr(VI) removal was achieved by live cultures of bacteria from sludge. Experimental results from packed laboratory mesocosm experiment have shown that 50 mg/L of hexavalent chromium was reduced by more than 85% after the feed solution migrated through the microbial barrier. It was also evident that after exposing microorganisms to hexavalent chromium, there was a shift in bacterial composition showing adaptability of the inoculum culture.

CHAPTER 2

LITERATURE STUDY

2.1 Chromium Sources

Chromium (atomic number 24, atomic weight 51.996 g/mole) was discovered by a French chemist Louis Vauquelin in 1797. Vauquelin gave the element the Greek name 'χρωμα' ('*chroma*') which means colour due to the many different colours found in its compounds (Mohana and Pittman Jr, 2006). The gemstones 'emerald' and 'ruby' owe their colors to traces of chromium in the matrix. Chromium is the earth's twenty-first most abundant element detected at a concentration of approximately 122 mg per kg of earths crust. Among the transitional metals, it is the sixth most abundant element. Notably, chromium does not occur in nature in pure elemental form, but is rather bonded in complex mineral forms.

Chromium occurs in nature predominantly in the trivalent form (Cr(III)) mostly as chromite ($FeOCr_2O_3$) and crocoite ($PbCrO_4$) in granitic rocks, serpentine rocks, and coal (Hintze, 1930; Merian, 1984). Small amounts of chromium in the hexavalent state (Cr(VI)) occur in silicate rich groundwater associated with Tertiary and Quaternary Alluvium filled basins.

Continuous hydrolysis of silicates in the old alluvial sediments raises the pH of the water causing oxidation of Cr(III) to Cr(VI) (Robertson, 1975). Cr(VI) is also released into the atmosphere from forest fires, burning of coal, volcanic eruptions, automobile exhaust, and combustion of chromium containing materials (Merian, 1984; Xing and Okrent, 1993). The elemental form Cr(0) is also possible although it oxidizes quickly upon exposure to air.

The thin oxide layer so formed is impermeable to oxygen thus protects the rest of the metal against further oxidation. This property is utilized for protection of other metals by electroplating. The other oxidation states of chromium (-2, +4, and +5) only appear transitionally under controlled laboratory conditions.

Chromium is mainly extracted from the earth as one of the many chromium ores. About fifty ores have so far been identified, including the following abundant types:

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

The most mined ore is ferric chromite, $FeCr_2O_4$, mainly found in South Africa. The chromite ore reserve in South Africa represents approximately 72% of the earth's identified sources. Other countries with exploitable chromium ore reserves include Russia, Zimbabwe, Finland, India, Kazakihstan, the Philippines, and Brazil (Figure 2-1).

2.2 Chromium Uses and Pollution

Chromium has been used extensively in industrial processes such as leather tanning, electroplating, negative and film making, paints and pigments, and wood preservation (Stern, 1982; Beszedits, 1988). Additionally, chromium has been used as a metallurgical additive in alloys (such as stainless steel) and metal ceramics. Chromium plating has been widely used to give steel a polished silvery mirror coating. The radiant metal is now used in metallurgy to impart corrosion resistance. Its ornamental uses include the production of emerald green (glass) and synthetic

rubies. Due to the its heat resistant properties chromium is included in brick molds and nuclear reactor vessels (Namasivayam and Yamuna, 1995; Dakiky *et al*, 2002).

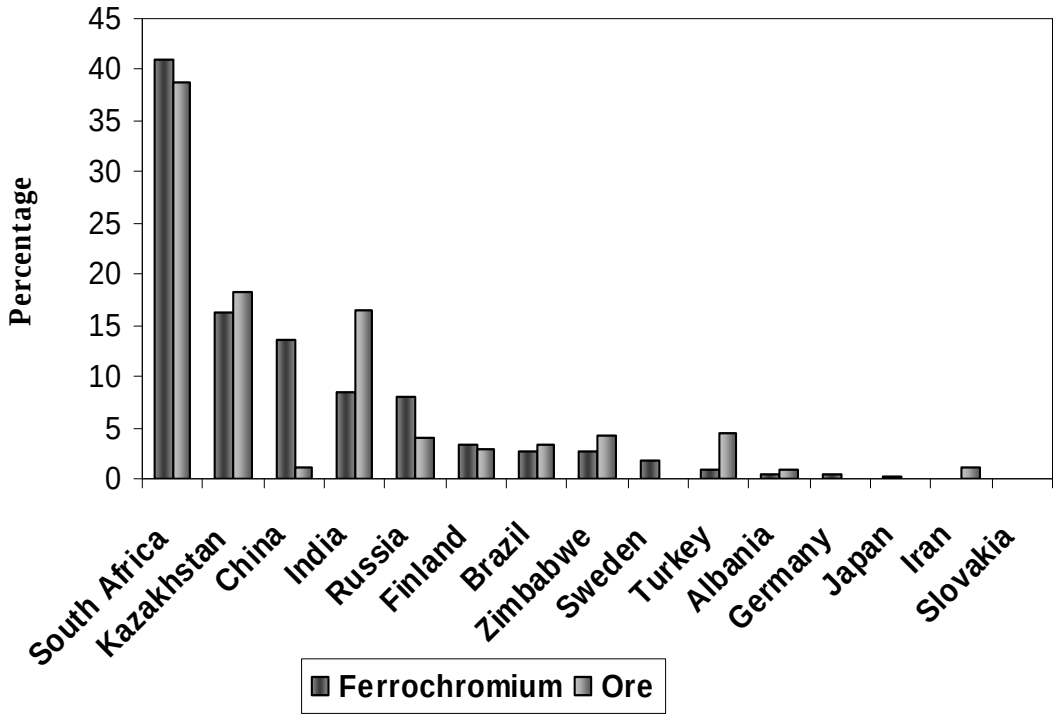

Figure 2-1: Percentage of ferrochromium and chromite ore produced worldwide (Papp, 2006).

Through the above and many other industrial uses, a large amount of chromium (4,500 kg/d) is discharged into the environment making it the most voluminous metallic pollutant on earth (U.S.EPA, 1978). Almost all chromium inputs to the natural systems originate from human activities. Only 0.001% is attributed to natural geologic processes (Merian,1984).

Chromium from the anthropogenic sources is discharged into the environment mainly as hexavalent chromium [Cr(VI)]. Cr(VI) — unlike Cr(III) — is a severe contaminant with high solubility and mobility in aquatic systems. Cr(VI) is a known carcinogen classified by the U.S.EPA as a Group A human carcinogen based on its chronic and

subchronic effects (Federal Register, 2004). It is for this reason that most remediation efforts target the removal of Cr(VI) primarily.

2.3 Environmental and Health Effects

Hexavalent and trivalent chromium compounds differ in their health and environmental effects. Cr(VI) is toxic, carcinogenic and mutagenic to animals as well as humans and is associated with decreased plant growth and changes in plant morphology (Rosko *et al*., 1977, Silverberg, *et al*., 1977). The biotoxicity of Cr(VI) is largely due to its high reactivity, its ability to penetrate biological membranes as well as its high oxidizing capabilities (NAS, 1974). The natural intracellular Cr(VI) reduction pathway may involve an acceptance of electrons from organic electron donors such as NAD(P)H resulting in the formation of the transitory Cr(V) state (Horitsu *et al*., 1990).

In humans and other mammals, acute exposure to Cr(VI) produces several health risks including allergic dermatitis, ulceration of the skin, irritation of the mucous membranes, nasal septum, renal tubular necrosis, and increase risk of respiratory tract infections. Super-active ionisation of water may result in the formation of the free radical ($OH^{\bullet}$) which in turn results in excessive DNA damage (Flessel, 1979). Chronic exposure results in carcinogenesis and teratogenesis (abortions and premature still births) in mammals. Due to these and other observed toxic effects, the World Health Organisation (WHO) has set the maximum acceptable concentration of chromium in drinking water to 0.05 mg/L (50 µg/L) (Kiilunen, 1994; Lu and Yang, 1995; ACGIH, 2004).

In contrast, trivalent chromium Cr(III) is relatively less toxic, less mobile, and even essential to human glucidic metabolism, contributing to the glucose tolerance factor necessary for insulin-regulated metabolism (Nriagu and Nieboer,1998; Fendorf *et al*., 2000; Mertz, 1981). Ingestion of small to moderate amounts of trivalent chromium is thus essential to human metabolism.

2.4 Chemical Properties

Chromium can achieve nine oxidation states ranging from -2 to +6. Two of these, +3 and +6, are the stable forms found in the environment. The tetravalent [Cr(IV)] and pentavalent [Cr(V)] quickly reduces to Cr(III) and oxidizes to Cr(VI), respectively, in the presence of reducing or oxidising agents. Among all the oxidation states, Cr(III) is the most stable, it resides in the lowest energy trough among the oxidation states. The negative standard potential (E^o) of the Cr(III)/Cr(II) metal ion couple signifies that Cr(II) is readily oxidized to Cr(III), and Cr(II) species are stable only in the absence of any oxidant (anaerobic conditions) (Kotas and Stasicka, 2000).

In the aquatic environment, the redox potential of the medium affects the oxidation state of chromium where as the pH affects its complexation with anionic forms including the hydroxyl ion (OH^-) (Figure 2-2). This figure shows the predorminance of the insoluble form [$Cr(OH)_3(s)$] in the pH range 5.5-10.5 under natural redox conditions (E_h ranging from -0.4 +0.6V). This correlates with the area where the majority of biological reactions occur. Figure 2-2 is adapted from Ball and Nordstrom (1998); Richard and Bourg (1991); Nieboer and Jusys (1988) and Rai *et al.* (1987, 1989).

The presence of Cr(III), its concentration and its forms in a given compartment of the environment is dependent on different chemical and physical processes, such as

hydrolysis, complexation, redox reactions and adsorption. In the absence of complexing agents, other than H_2O or OH^-, Cr(III) exists as a hexa-aquachromium(3+) and its hydrolysis products (Figure 2-2) (Rai *et al*., 1987).

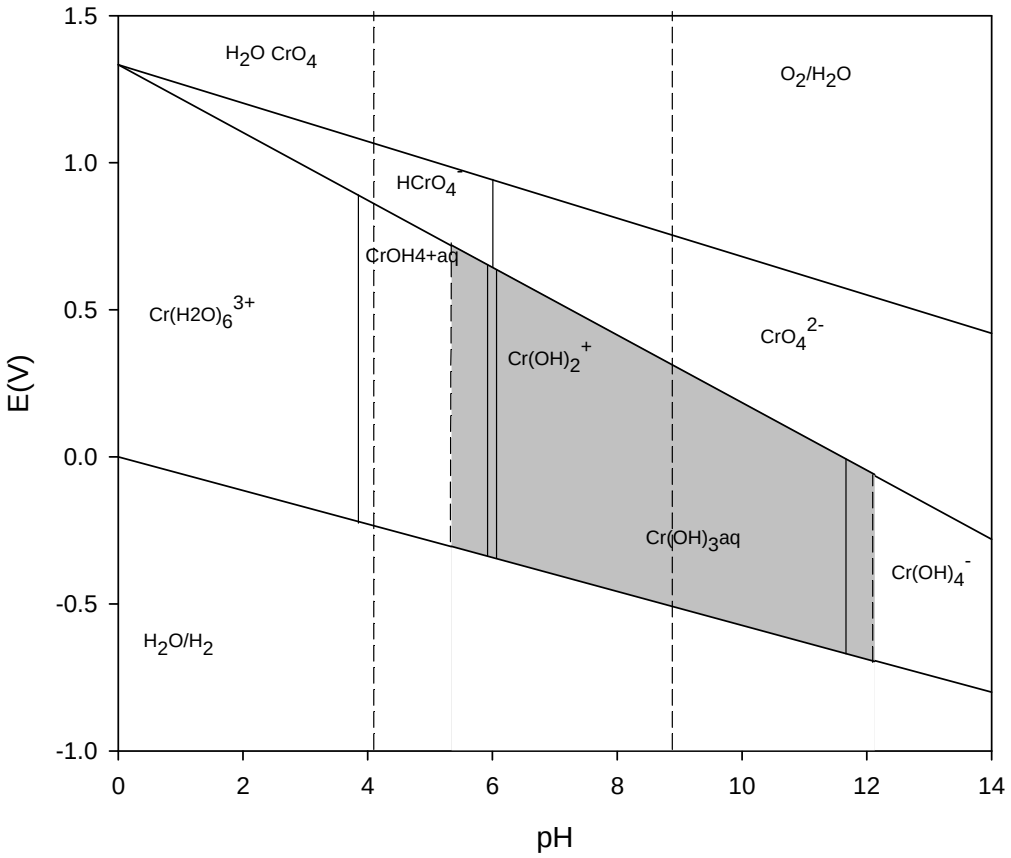

Figure 2-2: A simplifed Pourbaix diagram for chromium (Cr) species dominating in diluted aerated aqueous solutions in the absence of any complexing agents, other than H_2O or OH^- (Adapted from Ball and Nordstrom (1998)).

Cr(VI), on the other hand, exists mainly in the oxyanionic forms: chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$). Cr(VI) is highly reactive, is a strong oxidising agent, and exists only in oxygenated species. The equilibria of the Cr(VI) oxygenated species favours extremely high solubility and is pH dependent (Nieboer and Jusys, 1988).

Equations 2-1 to 2-3 (below) show the equilibria of the protonated oxyanions of chromate $HCrO_4^-$ and H_2CrO_4 under acidifying conditions, as an example.

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

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

$H_2CrO_4^-$ is a strong oxidizing agent which is a dominant chromium species at extremely low pH below -0.6 (Cotton and Wilkinson, 1980). Monohydrogen chromate, $HCrO_4^-$, predominates between the pH values of 1 to 6. CrO_4^{2-} predominates at or above pH 6. The $Cr_2O_7^{-2}$ dichromate ion is formed by the dimerization of $HCrO^-_4$ ion in Cr(VI) concentrations above 10^{-2} M (Sharma, 2002).

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

2.5 Pollution Remediation Strategies

2.5.1 Physical-Chemical Treatment Methods

Cr(VI) is transformed to Cr(III) at low pH through the following reduction-oxidation (redox) reaction:

$$Cr_2O_7^{2-} + 14H^+ + 6e^- \rightarrow 2Cr^{3+} + 7H_2O + 1.33v\ (E^0) \qquad (2\text{-}4)$$

(Garrel and Christ, 1965). Because of the difference in electric potential between the two states, substantial amounts of energy are needed to oxidize Cr(III) to Cr(VI). It is therefore practical to assume that spontaneous oxidation of Cr(III) to Cr(VI) never occurs in natural aquatic systems at ambient pH and temperature.

The redox reaction of Cr(VI) to Cr(III) requires the presence of another redox couple to donate the three necessary electrons. Sets of common Cr(VI) reducing couples in

natural waters include H_2O/O_2, Mn(II)/Mn(IV), NO_2^-/NO_3^-, Fe(II)/Fe(III), S^{2-}/SO_4^{2-}, and CH_4/CO_2 (Morel and Hering, 1993; Richard and Bourg, 1991). Once reduced to Cr(III), chromium forms a creamy white precipitate, chromic hydroxide, and other soluble chromic complexes. Chromic hydroxide, $Cr(OH)_3(s)$, is the predominant reduced chromium species under neutral and alkaline conditions (Ottinger *et al.*, 1973).

Examples of Cr(VI) reducing chemical agents are iron sulfide (FeS) and pyrite (FeS_2). Iron sulfide (FeS) is ubiquitous in reducing environments such as saturated soils, sediments, and sludge zones of secondary clarifiers in sewage treatment plants. Cr(VI) reduction by iron sulfides leaves a complex precipitate in solution:

$$Cr(VI)_{(aq)} + 3Fe(II)_{(aq)} \rightarrow Cr(III)_{(aq)} + 3Fe(III)_{(aq)} \quad (2\text{-}5)$$

$$xCr(III) + (1\text{-}x)Fe(III) + 3H_2O \rightarrow (Cr_xFe_{1-x})(OH)_{3(s)} + 3H^+ \quad (2\text{-}6)$$

where x may vary from 0 to 1 (Patterson *et al.*, 1997; Eary and Rai, 1988). The precipitate $(Cr_xFe_{1-x})(OH)_3(s)$ is innocuous and unaesthetic, and therefore must be removed from treated water before discharging into the environment. In practice, the removal of byproducts of Cr(VI) reduction such as the Fe-OH complexes may be very difficult and expensive. The final process may require a system operated at low pH ranges (<2.0) for the removal of Fe-OH compounds followed by operation at a much higher pH range (8-9.5) for the removal of the Cr(III) precipitate ($CrOH_3(s)$) (Eary and Rai, 1988).

Chemical treatment can be performed *ex situ* or *in situ*. However, *in situ* chemical agents must be carefully selected so that they do not further contaminate the treatment area. The primary problem associated with chemical treatment is the nonspecific

nature of the chemical reagents. Oxidizing/reducing agents added to the matrix to treat one metal could transform other metals in the system into mobile and more toxic forms (NAS, 1974). Additionally, the long-term stability of reaction products is of concern since changes in soil and water chemistry might create conditions where the detoxified forms are reversed back to toxic forms.

In the case of groundwater, the conventional chemical reduction–precipitation technique has been extensively used involving a two-step process as described above in Equations 2-5 and 2-6 (Mukhopadhyay *et al*, 2007). Due to the cost of pumping and risk of re-introducing undesirable byproducts during *ex situ* treatment, more effort is directed towards less expensive and less environmentally intrusive *in situ* treatment technologies.

2.5.2 Chemical Reactive Barriers

Several types of treatment walls have been studied to attenuate the movement of metals in groundwater at contaminated sites. Trench materials that have been investigated include zeolite, hydroxyapatite, elemental iron, and limestone (Vidic and Pohland, 1996). Elemental iron has been tested for chromium (VI) reduction and other inorganic contaminants (Powell *et al*., 1995) and limestone for lead precipitation and adsorption (Evanko and Dzombak, 1997)

Permeable reactive barriers are an emerging alternative to traditional pump-and-treat systems for groundwater remediation. Such barriers are typically constructed from highly impermeable emplacements of materials such as grouts, slurries, or sheet pilings to form a subsurface “wall.” Permeable reactive barriers are created by intercepting a plume of contaminated groundwater with a permeable reactive material.

The properties of the reactive material are selected to promote the attenuation of the contaminant through degradation, precipitation, adsorption or reduction into a sparingly soluble phase. Reactive mixtures for the attenuation of inorganic species are designed to maintain their permeability as secondary precipitates accumulate. The barrier should also be designed in such a way that the contaminant remains immobilized within the aquifer or can be retrieved with the reactive material following treatment.

A wide range of reaction mechanisms can be employed to remove both negatively charged and positively charged contaminants from flowing groundwater. These include adsorption of inorganic anions and cations (Morrison and Spangler, 1993), simple precipitation (McMurty and Elton, 1995), adsorptive precipitation (Baker *et al*, 1997), reductive precipitation (Blowes and Ptacek, 1992), and biologically mediated transformations (Waybrant *et al*, 1995; Robertson and Cherry, 1995; Benner *et al*,1997).

So far, permeable reactive barriers have been evaluated for the treatment of inorganic contaminants in groundwater, including As, Cd, Cr, Cu, Hg, Fe, Mn, Mo, Ni, Pb, Se, Te, U, V, NO_3, PO_4, and SO_4. Smalls scale field studies have indicated the potential for treatment of Cd, Cr, Cu, Fe, Ni, Pb, NO_3, PO_4, and SO_4. Permeable reactive barriers have been used in full-scale installations for the treatment of hexavalent chromium and a range of dissolved constituents including nitrate and phosphate (Blowes *et al*, 1998; Blowes and Ptacek, 1992; Powell *et al*, 1995; McRae *et al*, 1997). Specific application for Cr(VI) removal was tested at the US Coast Guard Support Centre (1996) and the Hanford site (1997) where Fe^0 was used in the reactive barrier material to treat sodium dichromate (US EPA, 2002).

2.5.3 Physical-Chemical Permeable Reactive Barriers: Design Concept

There are two conventional designs of permeable reactive barriers (PRBs), the continuous trench permeable reactive barrier (CT-PRB) and the funnel-and-gate system (FGS) (Figure 2-3 A and B). The continuous trench PRB does not contain any structures, therefore the contaminant plume flows through the treatment zone using the natural hydraulic gradient. This PRB, which is perpendicular to groundwater flow direction, needs to be slightly larger than the cross sectional area of the contaminated

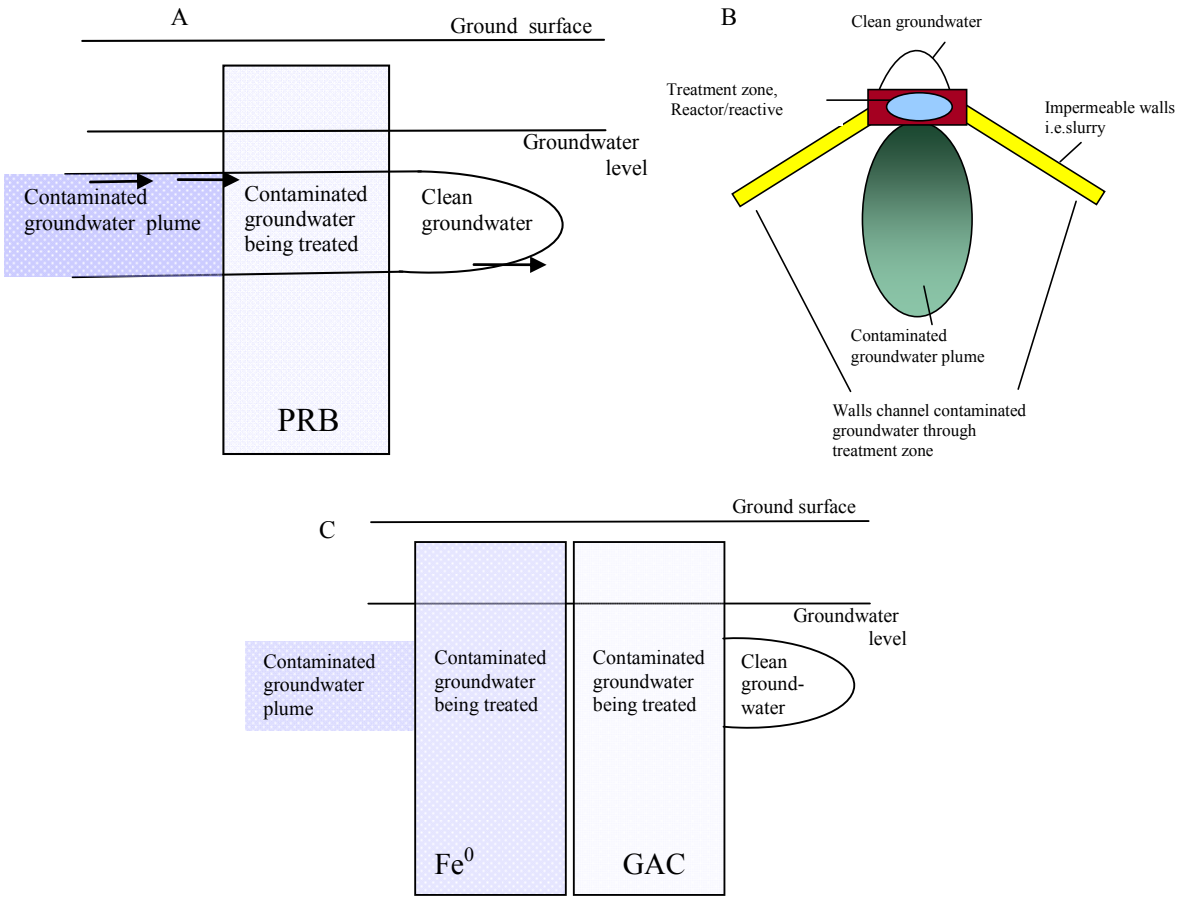

Figure 2-3: Conventional designs of permeable reactive barriers: (A) elevation view of a continuous trench or wall, (B) plan view of a funnel and gate, and (C) elevation view of a multi barrier.

groundwater in order to capture the contaminants in both vertical and horizontal directions (Gavaskar *et al*, 2000). The funnel-and-gate system is composed of impermeable walls and at least one reactive zone. The funnel structure could be sheet piles or slurry walls where the function of the funnel is to intercept the contaminated groundwater and lead it to the treatment zone. Phillips (2009) has elaborated on the designs of different reactive barriers, including mainly involving the thickness of the PRB to provide sufficient residence time for the contaminants within the treatment zone to be completely treated. Other complex designs have been tried including the multi-sequenced permeable reactive barriers (MS-PRBs) for multiple contaminants. MS-PRBs use multiple reactive materials in more than one reactive zone as shown in Figure 2-3C (Dries and Bastiaens, 2005).

2.5.4 Biological Permeable Reactive Barriers (BPRB)

These are PRBs specifically designed to utilise microorganisms in the treatment processes. A typical design comprises of a double-layer with an aeration zone followed by the bioremediation zone. One such system was evaluated against the removal of methyl-tert-butyl-ether (MTBE) contaminated groundwater (Figure 2-4) (Liu *et al*, 2006). The aeration in this case was achieved chemically by the oxidation of calcium peroxide (CaO_2) to release oxygen into the medium. Other growth nutrients were added to encourage the growth of MTBE degrading organisms in the second layer.

Notably, inorganic salts such as potassium dihydrogen phosphate (KH_2PO_4) and ammonium sulphate ($(NH_4)_2SO_4$) can act as buffers against pH changes caused by the oxidation of CaO_2 into carbonates (CO_3^{2-}). Thus, nutrients added in the second layer must include the phosphate buffer for the proper functioning of the barrier.

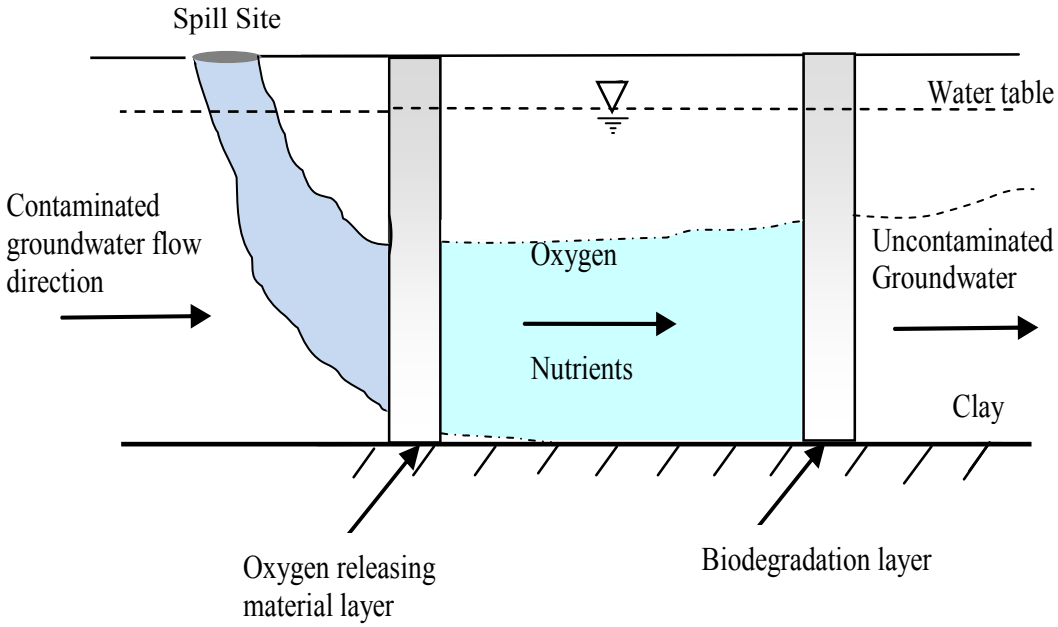

Figure 2-4: Schematic representation of a two layered biological barrier (adopted from Liu *et al*, 2006).

Another documented application is the treatment of petrochemical pollutants (i.e., benzene, toluene, ethylbenzene, xylene and polyaromatic hydrocarbons), heavy metals (i.e., lead, arsenic etc), and cyanide in the system designed by Doherty *et al* (2006) using a modified ash system. The biological permeable reactive barriers (BPRB) system was implemented at an abandoned gas manufacturing plant after 150 years of operation.

Specific application of the biological permeable reactive barrier (BPRB) system for the removal of Cr(VI) in groundwater has not been attempted. This has been both due to the unavailability of microorganisms capable of growing under nutrient deficient conditions and lack of information on the fate of the reduced chromium species in the barrier.

2.6 Microbial Cr(VI) Reduction

2.6.1 Microbial Resistance to Cr(VI) Toxicity

Most micro-organisms are sensitive to Cr(VI), but some microbial species are resistant and can tolerate high levels of chromate. In bacteria, Cr(VI) resistance is mostly plasmid borne. Different resistance strategies have been identified, including:

- modification of sulphate transport (Brown *et al.*, 2006; Hu *et al.*, 2005; Thompson *et al.*, 2007);
- counteracting chromate-induced oxidative stress by activating enzymes involved in ROS scavenging (catalase, superoxide dismutase) (Ackerley *et al.*, 2006);
- specialised repair of DNA damage by SOS response enzymes (RecA, RecG, RuvAB) (Hu *et al.*, 2005; Llagostera *et al.*, 1986; Miranda *et al.*, 2005);
- regulation of iron uptake, 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); and
- 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).

In a few cases, Cr(VI) resistance was associated with the regulation of uptake mechanisms such as the sulphate uptake shuttle system. Because of its structural similarity to sulphate (SO_4^{2-}), CrO_4^{2-} in some species crosses the cell membrane via the sulphate transport system (Cervantes *et al.*, 2001). After crossing the membrane, CrO_4^{2-} is reduced to Cr^{3+} which interferes with DNA transcription resulting in increased mutagenesis. Additionally, Cr^{3+} may alter the structure and activity of enzymes by reacting with their carboxyl and thiol groups (Cervantes *et al.*, 2001).

Among the resistance mechanisms listed above, the extracellular reduction of Cr(VI) may be utilised in environmental engineering. Although the process is facilitated by bacteria for their own survival, this process can be used to lower the concentration of Cr(VI) in a contaminated environment using bacteria.

2.6.2 Diversity of Cr(VI) Reducing Microorganisms

Microbial Cr(VI) reduction was first reported in the late 1970s when Romanenko and Koren'Kov (1977) observed Cr(VI) reduction capability in *Pseudomonas* sp. grown under anaerobic conditions. Since then, several researchers have isolated new microorganisms that catalyse Cr(VI) reduction under varying conditions (Ackerley *et al*., 2004; Chirwa and Wang, 1997a; Ohtake *et al*. 1990; Ganguli and Tripathi, 2002; Suzuki *et al*., 1992; Ramírez-Ramírez *et al*., 2004; Baldi *et al*., 1990).

Lately, genetic sequences of 16S rDNA have been used to supplement the conventional methods of species identification and characterisation (Blackall *et al*.,1998; Molokwane *et al*., 2008; Molokwane and Chirwa, 2009). This allows identification of a wide range of organisms which are unculturable using the conventional solid agar culturing methods. It also helps uncover species that have not been identified before. The cumulative list of known Cr(VI) reducing bacteria and their growth conditions is shown in Table 2-1.

Table 2-1 illustrates a number of known chromium reducing bacteria. Most of the bacterial species were isolated from chromium (VI) contaminated environments (i.e. sediments, wastewater treatment plants, soil etc). Although earlier isolates grew mostly on aliphatic carbon sources, later studies have shown diversity in the preferred carbon sources and electron donors. For example, consortium cultures were shown to grow in the absence of organic carbon sources – utilising only bicarbonate (HCO_3^-) as the carbon source (Molokwane and Chirwa, 2009).

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

Name of Species	Isolation Conditions/ C-Sources	References
Achromobacter sp. StrainCh1	Anaerobic / Luria Broth; glucose-lactate	Zhu *et al.*, 2008
Agrobacterium radiobacter EPS-916	Aerobic-Anaerobic / glucose-mineral salts medium	Llovera *et al.*, 1993
Alcaligenes eutrophus	Aerobic / sodium gluconate	Nies and Silver, 1989
Bacillus megaterium TKW3	Aerobic / nutrient broth-minimal salt medium-glucose, maltose, and mannitol	Cheung *et al.*, 2006
Bacillus sp.	Aerobic/ Vogel-Bonner (VB) broth-citric acid; D-glucose	Chirwa and Wang, 1997;
Bacillus sp. ES 29	Aerobic / Luria-Bertani (LB) medium	Camargo *et al.*, 2003
Bacillus subtilis	Anaerobic / Minimal medium - trisodium citrate and dehydrate glucose	Carlos *et al.*, 1998
**Bacillus drentesis*	Aerobic/Luria Betani Broth	Molokwane and Chirwa, 2009
**Bacillus mycoides*	Aerobic/Luria Betani Broth	Molokwane and Chirwa, 2009
**Bacillus thuringiensis*	Aerobic/Luria Betani Broth	Molokwane and Chirwa, 2009
Deinococcus radiodurans R1	Anaerobic / Basal Medium-Lactate-Acetate-Pyruvate-Succinate-Ethanol-L-lactate, and D-lactate	Frederickson *et al.*, 2000
Enterobacter cloacae HO1 *strain*	Anaerobic / KSC medium-Sodium acetate	Wang *et al., 1989(a)*
Escherichia coli ATCC 33456	Aerobic-Anaerobic / Nutrient broth medium; glucose, acetate, propionate, glycerol and glycine	Shen and Wang, 1994b
**Enterobacter sp*	Aerobic/Luria Betani Broth	Molokwane and Chirwa, 2009
**Lysinibacilus sphaericus*	Aerobic/Luria Betani Broth	Molokwane and Chirwa, 2009
Ochrobactrum sp.	Aerobic / glucose	Zhiguo *et al.*, 2009
Pantoea agglomerans SP1	Anaerobic / acetate	Francis *et al.*, 2000
Pseudomonas fluorescens	Aerobic-Anaerobic / Glucose-Acetate-Pyruvate-Lactate-Succinate	Bopp *et al.*, 1983; Ohtake *et al.*, 1987
Pseudomonas fluorescens LB300	Aerobic / Vogel-Bonner broth	Bopp and Ehrlich, 1988
Pseudomonas putida MK1	Anaerobic / Luria-Bertani -citric acid-Tris-acetic acid	Park *et al.*, 2000
Pseudomonas sp	Aerobic / Peptone-glucose; chemostat	Gopalan and Veeramani, 1994
Pseudomonas spp.	Anaerobic / Vogel-Bonner (VB)- D-glucose	Mclean and Beveridge, 2001

Table 2-1: Known Cr(VI) reducing bacteria (Continued....)

Name of Species	Isolation Conditions/ C-Sources	References
Providencia sp.	Aerobic-Anaerobic / Luria broth (tryptone-yeast extract)	Thacker *et al.*, 2006
Pseudomonas aeruginosa	Aerobic / Nutrient broth or Luria broth	Aguilera *et al.*, 2004
Shewanella alga (BrYMT) ATCC 55627	Aerobic-Anaerobic / M9 broth- Glucose	Guha *et al.*, 2001
Shewanella putrefaciens MR-1	Anaerobic / lactate- fumarate	Myers *et al.*, 2000

*Current study

2.6.3 Cr(VI) Reduction Pathways

Cr(VI) reduction has been demonstrated to be cometabolic (not participating in energy conservation) in certain species of bacteria, but is predominantly dissimilatory/respiratory under anaerobic conditions. 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 (Horitsu *et al*., 1987; Ishibashi *et al*,1990; Chirwa, 2005; Lovley and Phillips, 1994). In the dissimilatory/respiratory process, electrons are donated from the electron donor to Cr(VI) via nicotinamine di-hydrogen (NADH) (Suzuki *et al*., 1990; Chirwa and Wang, 1997a).

The dissimilatory nature of Cr(VI) reduction was demonstrated earlier in whole cell and disrupted cell experiments by Wang *et al.* (1990) in which reduced chromium was predominantly found in the medium and only less than 30% was released from disrupted cells of *Enterobacter cloacae* HO1. In 1993, Shen and Wang (1993) confirmed these results while working with the Cr(VI) reducing *Escherichia coli* ATCC 33456. In the latter experiment, only 10% of the reduced chromium was accumulated inside the cells.

Figure 2.5 illustrates the two common pathways for Cr(VI) reduction, the first one with Cr(VI) reduction involving the formation of the unstable intermediate Cr(V) (Suzuki *et al*., 1990), and the second depicting direct reduction from Cr(VI) to Cr(III) by a soluble or membrane associated reductase (Chirwa, 2001). The first pathway was observed under anaerobic conditions in *Pseudomonas* species where as the second is common under aerobic conditions mostly in *Bacilli*.

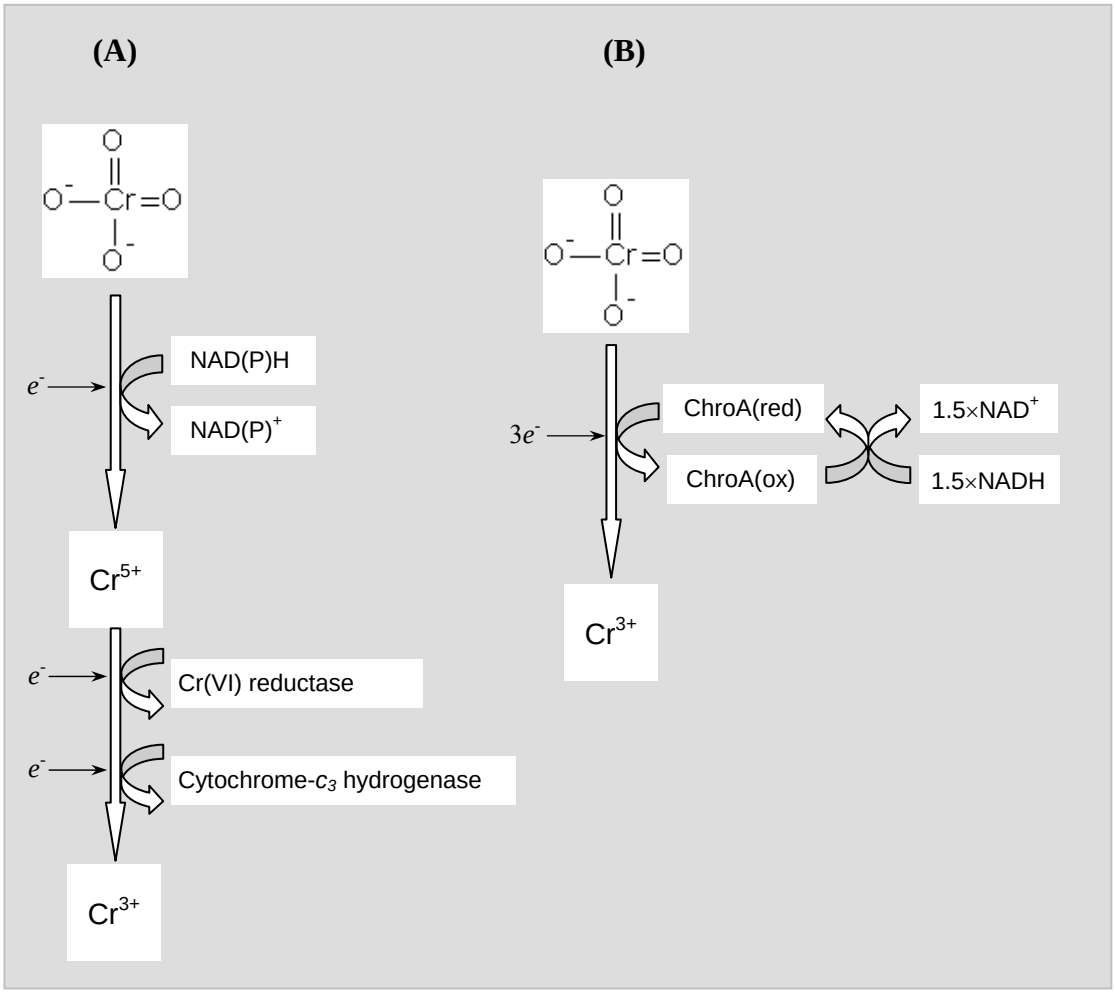

Figure 2-5: The two known Cr(VI) reduction pathways: (A) stepwise reduction via the unstable intermediate (Cr(V)) facilitated by NAD(P)H oxidation (Suzuki *et al*, 1990), and (B) direct reduction by a soluble reductase ChroA facilitated by the oxidation of NADH (Chirwa, 2001).

In the second pathway (B), two electron moles are transferred per mole of NADH oxidised. The reductase ChroA was determined to be encoded by a chromosome borne gene *ChroA* which is activated by Cr(VI) in *Pseudomonas fluorescens* LB300. Cr(VI) reduction by microorganisms 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, $Cr(OH)_3(s)$ as shown Equations 2-7 and 2-8 below (Brock and Madigan, 1991; Zakaria *et al*., 2007).

$$CrO_4^{2-} + 8H^+ + 6e^- \xrightarrow[3e^-]{CRB} Cr^{3+} + 4H_2O \xrightarrow{neutralpH} Cr(OH)_3(s) + 3H^+ + H_2O \qquad (2\text{-}7)$$

$$3CH_3COO^- + 4HCrO_4^- + 4CrO_4^{2-} + 33H^+ \rightarrow 8Cr^{3+} + 6HCO_3^- + 20H_2O \qquad (2\text{-}8)$$

Equation 2-7 shows a generic Cr(VI) reduction reaction catalysed by Cr(VI) reducing bacteria whereas equation 2-8 shows Cr(VI) reduction with a simple carbon source as an electron donor. Other fatty acid byproducts of hydrolysis can also serve as electron donors for Cr(VI) reduction (Chirwa and Wang, 2000).

In other species, Cr(VI) reduction may occur as a fortuitous reaction in which redox-active biomolecules such as cytochromes transfer electrons to Cr non-specifically (Lovely and Phillips, 1994). Two species of bacteria, *Desulfotomaculum reducens* and *Pantoea agglomerans*, have been shown to obtain energy for growth using Cr(VI) as a respiratory electron acceptor while conserving energy from Cr(VI) reduction coupled to the oxidation of organic acids or H_2 (Francis *et al*., 2000; Tebo and Obraztsova, 1988). The above observations indicate that the presence of highly degradable substrates such as glucose, peptone, and tryptose is not always necessary to achieve biological Cr(VI) reduction. This indicates that the larger, energy rich molecules may be converted into simpler metabolites that are critical as carbon sources or intermediates for the cell's central metabolic system. This was demonstrated in experiments by Chirwa and Wang (2000) where Cr(VI) reducers (*E. coli* ATCC 33456) utilised organic acid metabolites produced by phenol degraders in an anaerobic consortium of bacteria.

2.7 Current and Future Biotechnology Solutions

2.7.1 Suspended Culture Systems

The first continuous-flow Cr(VI) reducing processes were investigated in suspended culture systems (Mazierski, 1994; Shen and Wang, 1994a; Wang *et al*., 2000). One common feature in all the above systems was that vigorous mixing was required to keep the systems homogenous. Cells in the suspended culture systems were susceptible to high Cr(VI) concentrations. Additionally the reactors needed to be recharged with new cells after shock loading due to excessive loss of biomass (Wang *et al*., 2000). During application on actual waste, it is often necessary to dilute the influent stream to lower the incoming toxicity levels to tolerable levels for the microorganisms (Ohtake *et al*., 1990). This results in large volumes of reactors to treat relatively low concentrations.

2.7.2 Attached Growth Systems

Biofilm systems have been used extensively in treatment processes due to the perceived resilience of microorganisms growing in films. In biological systems, up to 80% of the mass of the biofilm consists of exo-polysaccharide (EPS) matrix which offers mass transport resistance across the biofilm layer (Nelson *et al*, 1996). As a result, bacteria in the biofilm is exposed to a decreasing concentration profile with increasing depth. Other conditions may also vary resulting in the development of a complex community of microorganisms. For example, Nkhalambayausi-Chirwa and Wang, (2001) observed that spatial and physiological heterogeneity introduced within microbial communities by the formation of biofilm, enhanced Cr(VI) reduction by *E. coli* within the quasi-anaerobic interior of the biofilm while supporting maximum growth of *P. putida* along the more aerobic surface layers. This resulted in a self optimised system in which metabolites formed from phenol degradation in the

aerobic layer supported the growth of the Cr(VI) reducing species in the deeper layers of the biofilm.

2.7.3 *In Situ* Inoculation

Currently applications of *in situ* bioremediation emphasize the construction of a maintainable barrier system where the barrier material is either replaced occasionally or replenished by a reverse reaction. Both systems suffer from high cost and the high probability of producing toxic sludge. *In situ* inoculation as proposed in this study entails injecting an inoculum (mixed) culture of bacteria into the selected barrier zone and allow the microorganisms to grow and optimise in the new environment (Molokwane and Chirwa, 2009). This requires the presence of essential nutrients in the environment or in the waste stream to sustain the culture. The major advantage of this process is its low installation cost. The potential shortcoming is low degree of control with respect to the handling of products (Molokwane and Chirwa, 2010).

2.7.4 Bioaugumentation

The applications of the future will aim at modifying organisms already existing in the environment to treat waste by providing the organisms with the genetic information required to carry out the biotransformation process *in situ*. The genes could be shuttled into the native species through a soup of plasmids or transposons introduced into the environment either directly or through new microbial cultures (Top *et al*., 2006). Organisms are known to acquire genetic information from the environment when necessary, to deal with adverse conditions (Engo *et al*., 2002). The advantage is the avoidance of introducing alien species with possible unforeseeable detrimental effects to the native environment. Similarly to biological permeable reactive barriers, *in situ* bioaugumentation processes have only been tested for organic pollutants

(Jianlong *et al*, 2002). Applications on toxic metals including Cr(VI) face the same challenge of removal of the products from the aquifer.

2.8 Chapter Summary

New tools for isolation and characterising bacteria have enabled several research groups to identify a wider array of Cr(VI) reducing organisms recently. In spite of this new worth of knowledge, applications to actual environments and contaminant streams are still limited. One of the areas lagging behind is in the remediation of contaminated environments. Pump-and-treat processes currently applied at the sites have been ineffective and have generated large amounts of toxic sludge requiring further treatment and expensive disposal at landfill sites.

Construction and operation of permeable barriers has been evaluated for physical-chemical processes for treating inorganic pollutants and biological permeable reactive barriers in treating organic pollutants. The latter has not been used for Cr(VI) removal because of the difficulty of removing the reduced metal precipitate from the aquifer. The proposed lower cost inoculated barrier system which forms the basis for the microcosm and mesocosm studies in the following chapters. Although, the problem of dealing with the product (reduced metal precipitate) still exists, this technology could prevent the contamination of surrounding aquifer until the time when a permanent remedy such as a major excavation is achievable.