KINETIC STUDIES OF CR(VI) REDUCTION IN AN INDIGENOUS MIXED CULTURE OF BACTERIA IN THE PRESENCE OF AS(III)

by

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ABSTRACT

Title: Kinetic Studies of Cr(VI) reduction in an indigenous mixed culture of bacteria in the presence of As(III)

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An indigenous mixed culture of bacteria collected from a Wastewater Treatment Plant (Brits, North West Province, South Africa), biocatalytically reduced Cr(VI) in the presence of As(III). Both the reduced chromium (Cr(III)) and the oxidised arsenic (As(V)) readily form amorphous hydroxides that can be easily separated or precipitated from the aqueous phase as part of the treatment process. Treatment of Cr(VI) and As(III) before disposal of wastewater is critical since both compounds are known to be carcinogenic and mutagenic at very low concentrations, and acutely toxic at high concentrations.

Batch experiments were conducted to evaluate the rate of Cr(VI) reduction under anaerobic condition in the presence of its co-contaminant As(III) typically found in the groundwater and mining effluent. Results showed near complete Cr(VI) reduction under initial Cr(VI) concentrations up to 70 mg/L in a batch amended with 20 mg/L As(III). However, increasing Cr(VI) concentrations up to 100 mg/L resulted in the inhibition of Cr(VI) reduction activity.

Further investigation was conducted in a batch reactor amended with 70 mg/L Cr(VI) concentration at different As(III) concentrations ranging from 5-70 mg/L to evaluate the effect of varying As(III) concentration on Cr(VI) reduction efficiency. Results showed that Cr(VI) reduction efficiency increased as As(III) concentrations increased from 5-40 mg/L. However, further increase in As(III) concentration up to 50 mg/L resulted in incomplete Cr(VI) reduction and decrease in Cr(VI) reduction efficiency. These results suggest that the rate of Cr(VI) reduction depends on the redox reaction of As(III) and As(V) with Cr(VI). Moreover, the inhibitory effect observed at high Cr(VI) and As(III) concentration may also

be attributed to the dual toxicity effect of Cr(VI) and As(III) on microbial cell. From the above batch kinetic studies lethal concentration of Cr(VI) and As(III) for these strains was evaluated and established.

Initial evaluation of the bacteria using 16S rRNA partial sequence method showed that cells in the mixed culture comprised predominantly of the Gram-positive species: *Staphylococcus sp., Enterobacter sp.,* and *Bacillus sp.* The biokinetic parameters of these strains were estimated using a non-competitive inhibition model with a computer programme for simulation of the Aquatic System "AQUASIM 2.0".

Microbial reduction of Cr(VI) in the presence of As(III) was further investigated in continuous-flow bioreactors (biofilm reactor) under varying Cr(VI) loading rates. The reactor achieved Cr(VI) removal efficiency of more than 96 % in the first three phases of continuous operation at lower Cr(VI) concentration ranging from 20-50 mg/L. However, 20 % decrease in Cr(VI) removal efficiency was observed as Cr(VI) concentration increase up to 100 mg/L. The reactor was able to recover from Cr(VI) and As(III) overloading phase after establishing the resilient nature of the microorganism. Similarly to the batch reactor studies the overall performance of the reactor also demonstrated that the presence of As(III) greatly enhance Cr(VI) reduction in a bioreactor. This was evident by near complete removal of Cr(VI) concentration up to 50 mg/L. The basic mass balance expressions on Cr(VI) along with the non-competitive inhibition model were used to estimate the biokinetic parameters in the continuous flow bioreactor system.

Cr(VI) reduction efficiency along the longitudinal column was also evaluated in this study. Results showed that Cr(VI) efficiency increased as Cr(VI) concentration travels along the longitudinal column. Other important factors such as oxygen and pH during biological Cr(VI) reduction in the presence of As(III) oxidation were also evaluated.

Keywords: Cr(VI) reduction, redox biocatalytic cycle, arsenic, anaerobic condition, biodetoxification.

DECLARATION

I Igboamalu Tony Ebuka, affirm that the thesis which I hereby submit for a Master of Engineering in Chemical Engineering degree at the University of Pretoria is my own work and has not been previously submitted by me for any degree at this or other institutions.

IGBOAMALU TONY EBUKA	29/ 08/2014
	Date

Dedicated to my family

Chief Igboamalu Francis Chukwudi

A hardworking, loving, dedicated brother who contributed financially,

Dr Igboamalu Christian Chukwudi

and;

Friends who have impacted positively in my life.

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LIST OF ABBREVIATIONS

AA	Activated Alumina
AAS	Atomic adsorption spectrophotometer
APHA	American public health agency
ASBR	Anaerobic Sequencing Batch Reactor
BLAST	Basic Logical Alignment Search Tool
BMM	Basal Mineral Medium
CFU	Colony forming units
ChrR	Cr(VI) reductase
CRB	Cr(VI) reducing bacteria
DMA	Dimethyl arsenic Acid
EPS	Exopolly Saccharides
GAC	Granular activated carbon
HRT	Hydraulic retention time
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
PFR	Plug flow reactor
RBC	Rotary Bed Contactor
SEM	Scanning electron microscopy
TMA	Trimethyl arsenic Acid
WHO	World Health Organisation

LIST OF SYMBOLS

A	cross-sectional area of a reactor column (L^2)
A_f	biofilm surface area (L^2)
C	Cr(VI) concentration at time, t (ML^{-3})
C_1	state variable (ML^{-3})
C_b	Cr(VI) concentration in the bulk flow (ML^{-3})
C_o	initial Cr(VI) concentration (ML^{-3})
C_r	Cr(VI) toxicity threshold concentration (ML^{-3})
C_S	Cr(VI) concentration at the surface (ML^{-3})
D_w	dispersion coefficient (L^2T^{-1})
j_{flux}	mass transport rate (LT^{-1})
j_c	Cr(VI) flux rate ($ML^{-2}T^{-1}$)
k	limiting constant (ML^{-3})
k_d	cell death rate (T^{-1})
k_{ad}	adsorption rate coefficient (T^{-1})
K	half velocity constant (ML^{-3})
K_i	inhibition coefficient (ML^{-3})
u_max	maximum specific Cr(VI) reduction rate(T^{-1})
L	length of the reactor (L)
L_w	stagnant film thickness (L)
Q	inflow rate ($L^{-3}T^{-1}$)
q_c	adsorption rate ($ML^{-3}T^{-1}$)
r_c	Cr(VI) reduction rate ($ML^{-3}T^{-1}$)
K_c	Cr(VI) reduction capacity coefficient (MM^{-1})
t	time (T)
u	flow velocity (LT^{-1})
V	volume of the reactor (L^3)
X	biomass concentration at time, t (ML^{-3})
X_o	initial biomass conc. (ML^{-3})
ΔV	differential volume (L^3)
ρ_c	medium density (ML^{-3})
ΔG	gibbs free energy (KJ/mol)

CHAPTER ONE

INTRODUCTION

1.1 Background

Environmental pollution associated with toxic metal ions (metalloids) is a legacy left mainly by industrial activities. Effluent from gold and antimony mining, textile production, leather tanning, electroplating, paint and pigment manufacturing and wood processing industries contain considerable amounts of toxic metal ions (Marcovecchio *et al.,* 2007; Bailar, 1997). Among these are metalloid ions of Cr(VI), As(III), and other toxic inorganic contaminants such as cyanide, selenium and uranium (Sami *et al*., 2003). Chromium (Cr) exists in the environment mostly in the hexavalent form (Cr(VI)) or trivalent form (Cr(III)) (Cheunga *et al.,* 2007; Zyed and Terry, 2003). In mining processes, Cr(VI) may be discharged together with its co-pollutant, As(III) and cyanide. Treatment of Cr(VI) and As(III) is critical before final disposal of the effluents since both compounds are known to be carcinogenic and mutagenic to living organism at low exposure conditions, and acutely toxic at high concentrations (Federal Register, 2004; Katz, *et al.,* 1994). In addition, inorganic Cr(VI) and its co-pollutant (As(III)) have been associated with high incidents of skin, lung, bladder and liver cancer (De Flora, 2000; Costa M, 1997).

South Africa is among the top coal, gold, and precious metal producing nations in the world, accounting for about 70-90 % of the world viable chromite reserve (Bachmann *et al.,* 2010; Mintek, 2004). Recent data shows that about 4.4 million tons/yr Cr is currently produced from fourteen separate ferrochrome smelter plants in South Africa (Beukes *et al.,* 2012). Improper disposal of Cr(VI) containing waste from these industries, and its subsequent mobility in groundwater aquifers is a subject of great concern. Contamination of groundwater aquifers has resulted in elevated levels of Cr(VI) and its co-pollutant (As(III)). The high level of these compounds in groundwater renders it unsuitable for human consumption. The removal of compounds containing Cr(VI) and As(III) involves redox processes whereby Cr(VI) is reduced to Cr(III), and oxidation of As(III) to As(V) that precipitate easily as hydroxide complexes (Aniruddha and Wang, 2010; Sun *et al*., 2010; Sun *et al*., 2008).

Pump and treat method, involving chemical processes such as pH adjustment is one of the current method employed for remediation of Cr(VI) contaminated sites (Beukes *et al*., 2000). The problem associate with these methods is that they are energy intensive, and generate harmful sludge that is difficult to dispose (Molokwane *et al*., 2008). Bio-detoxification or remediation of Cr(VI) may be considered as an alternative to physical and chemical processes, as it can be achieved under natural pH and redox conditions, and therefore less environmental intensive (Li *et al*., 2007).

Several studies have demonstrated the effect of facultative microbes in oxidizing As(III) to As(V) using nitrate (NO_3^-) or chlorate (ClO_3^-) as terminal electron acceptors while conserving energy for cell growth (Sun *et al*., 2008; Sun *et al.,* 2010; Aniruddha and Wang, 2010*)*. By exploring the biocatalytic redox reaction taking place in Equation 1.4, chromate (CrO_4^-) can be considered a possible alternative oxidant for the reaction.

$$As^{3+} \rightarrow 2e^- + As^{5+} \quad (1.1)$$

$$Cr^{6+} + e^- \rightarrow Cr^{5+} \text{ (intermediate product formed as a result of cell redox cycle)} \quad (1.2)$$

$$Cr^{5+} + 2e^- \rightarrow Cr^{3+} \quad (1.3)$$

Overall red-ox reaction; from combining Equation 1.1 and 1.3, gives

$$As^{3+} + Cr^{5+} \rightarrow As^{5+} + Cr^{3+} (\Delta G = -256 \text{ kJ/mol}) \quad (1.4)$$

Equation above shows the individual stages of As(III) oxidation, and step wise reduction of Cr(VI). Equations 1.2 occur as a result of redox cycle in the microbial cell, generating an intermediate product Cr(V) (Cervantes *et al.,* 2001; Suziki *et al.,* 1992). From biocatalytic redox reactions Equation 1.4, As(III) donate $2e^-$, and oxidized to As(V), while Cr(V) an intermediate product formed, on the other hand accept $2e^-$, and reduced to Cr(III). Based on bioenergetics consideration, the reaction is feasible as indicated a highly exothermic reaction Equation 1.4, releasing a reasonable amount of energy for cell growth and metabolism. However, the reaction is feasible since only two electrons are required for Cr(V) reduction. So far, only few studies have been done on simultaneous Cr(VI) reduction and As(III)

oxidation by micro-organism. Bachate *et al.,* (2013) reported simultaneous Cr(VI) reduction and As(III) oxidation by *Bacillus firmus* TE7.

In this present study, kinetic studies of Cr(VI) reduction in the presence of As(III), in indigenous mixed culture of bacteria collected from a Wastewater Treatment Plant (Brits North West South Africa), was explored to evaluate the inhibitory effect of As(III) on Cr(VI) reduction. The experiment was conducted under anaerobic condition in batch and continuous flow bioreactors. The feasibility of co-contaminant oxidation of As(III) during biological Cr(VI) reduction was also evaluated.

1.2 Aim and objectives

The aim and objectives of this study was to evaluate the inhibitory effect of As(III) during Cr(VI) reduction in an indigenous mixed culture of bacteria. In order to achieve this objective, the experimental tasks which were subdivided into four parts were undertaken to achieve the following:

- To evaluate Cr(VI) reduction kinetic in a batch assay over a wide range of Cr(VI) concentrations in the presence of As(III).
- To evaluate Cr(VI) reduction in a continuous bioreactor under anaerobic condition over a range of Cr(VI) feed concentrations in the presence of As(III).
- To evaluate the microbial changes in the bioreactor, as well as morphological characterization using 16S rRNA partial sequence method.
- To estimate bio-kinetic parameters of the strains for Cr(VI) reduction in the presence of As(III) using 'AQUASIM 2.0 and Octave 3.0'

1.3 Methodology

The methodology of this present work was based on previous studies on Cr(VI) and its co-pollutant (As(III)). Previously, it was established that microorganisms exposed to toxic metals/metalloid ions developed diverse resistance mechanisms to tolerate the toxicity of toxic metal ions (Bachate *et al*., 2013). These resistance mechanisms involve specific

biochemical pathways that can alter chemical properties of toxic metal ions, resulting in their detoxification (Silver and Phung, 2005). Several studies have been reported on biological reduction of Cr(VI) and oxidation of As(III) (Molokwane *et al.,* 2008; Zakaria *et al.,* 2007). Information from these studies was used to establish the theory of the present study.

1.4 Outline of dissertation

The outline of this dissertation is listed as follows:

- Chapter 1 described the background information, and the objective of this thesis.
- Chapter 2 review current and previous studies on chromium and arsenic.
- Chapter 3 described materials and methods used in this study.
- Chapter 4 present experimental results and interpretation.
- Chapter 5 described Cr(VI) reduction using a non-competitive inhibition model.
- Chapter 6 present thesis conclusion and future work required.

1.5 Research significance

South Africa is a huge industrial producer of ore and other metalloids. This can be correlated to high environmental pollutants in aqueous environment. During steel and chromate production a considerably amounts of wastes are formed, which can be toxic, hence making the treatment of ferrochrome waste materials necessary. However, remediation strategies of this are of paramount important. According to the Beukes et al. (2012), the remediation strategy in South African ferrochrome industries for waste treatment involves Cr(VI) reduction with ferrous iron. This treatment strategy is inefficient, since it is not cost effective, and may produce harmful sludge. Exploring bioremediation of Cr(VI) and its co-pollutant containing waste using mixed culture of facultative anaerobes from local environment. It can be established that bioremediation treatment strategy could be perceived as an alternative to South African ferrochrome producer's treatment strategy, since it is cost effective and generate less harmful sludge.

CHAPTER TWO

LITERATURE STUDY

2.4 Chemistry and toxicity of chromium (Cr) and its co-pollutant Arsenic (As)

2.1.1 Chromium (Cr)

Chromium (Cr) (atomic number: 24 and atomic weight: 51.9961) is a group VI member of the periodic table and classified as a transition metal (Wackett *et al*., 2004). Chromium is the seventh most abundant element in the earth's crust with an average concentration of 100 mg/kg (Oliveira, 2012). It exists in different oxidation states ranging from (-2) to (+6) (Zayed and Terry, 2003). Among chromium oxidation states, only chromium (+3) and (+6) are the most stable under natural pH and temperature condition (Shupak, 1991). However, the existence and transformation of this metal/metalloid is controlled by physiochemical processes such as; oxidization and reduction reaction, electrochemical potentials and pH, precipitation or adsorption process, and solubility (Kimbrough, *et al.,* 1999). In aqueous state, the existence of chromium species is dependent on the pH of the aqueous solution. Cr(III) predominate at pH less than 3.5, trivalent chromium hydroxyl species ($Cr(OH)^{2+}$, $Cr(OH)_2^{+}$, $Cr(OH)_3$, and $Cr(OH)_4^{-}$) predominate at pH greater than 3.5, while Cr(VI) (CrO_4^{2-}) on the other hand, predominate at or above pH of 6 (Barnhart, 1997). However, equilibrium equation (2.1-2.3) illustrate the existence of Cr(VI) species in aqueous solution, where ($HCrO_4^{-}$) exist at pH values of 1 to 6 (Park *et al*., 2005). The dichromate ion ($Cr_2O_7^{2-}$) is formed by dimerization of $2HCrO_4^{-}$ in Cr(VI) concentration above 10^{-2} (Sharma, 2002).

$$H_2CrO_4 \Leftrightarrow HCrO_4^{-} + H^{+} \qquad K_{a1}=10^{0.6} \qquad (2.1)$$

$$HCrO_4^{-} \Leftrightarrow CrO_4^{2-} + H^{+} \qquad K_{a2}=10^{-5.6} \qquad (2.2)$$

$$Cr_2O_7^{2-} + H_2O \Leftrightarrow 2HCrO_4^{-} \qquad K_{a3}=10^{-2.2} \qquad (2.3)$$

Chromium toxicity depends intensely on its speciation, since different species exert different effects on animals, microbes and humans. Chromium is known for environmental health problem after exposure of organism to moderate to high concentrations (Sharma *et al.,* 1995).

The toxicity is attributed to high solubility, mobility and bioavailability, of the hexavalent state (James, 2002). On ingestion or inhalation of Cr(VI) contaminated water or air; nose, throat, and lungs irritation, kidney and liver cancer or even death has been reported (Barceloux, 1999). Additionally, Cr(VI) undergoes a redox cycle in the cell, to regenerate Cr(V) which produces a reactive oxygen species that easily combined with DNA-protein complex, modifying the cell DNA structure (Cervantes *et al.,* 2001). Finally, it changes the structure of soil microbial communities, thereby reducing microbial activities (Turpeinen *et al.,* 2004). However, due to the toxic effect of chromium, the maximum regulatory standard of Cr(VI) and total chromium for drinking water, surface water and soil was set at 0 and 50 µg/L, 50 and 100 µg/L, and 250 µg/L respectively (Environmental Quebec, 1999). The first step in remediation of chromium often involves reduction of all hexavalent species to trivalent state followed by extraction through precipitation. This conversion is beneficial since Cr(III) is about 1000 times less toxic than Cr(VI) (Sharma *et al.,* 1995; Petrilli and Flora, 1997).

The biochemistry of Cr species has been demonstrated by many researchers (Molokwane *et al.,* 2008; Suzuki *et al.,* 1992). Suzuki *et al., (*1992), reported that NADH in the cell protoplasm can serve as an electron donor in stepwise reduction of Cr(VI) to intermediate Cr(V), which accepts two electrons from the same co-enzyme to yield Cr(III) as shown in Equation (2.4 and 2.5). It has been suggested that the energy generated from Equation (2.5), can facilitate the microbial cell growth and metabolism (Wang and Shen, 1995; Suzuki *et al*., 1992).

$$Cr^{6+} + \{NADH\}\ e^{-} \rightarrow Cr^{5+} \quad (2.4)$$

$$Cr^{5+} + 2e^{-} \rightarrow Cr^{3+} + \text{Energy} \quad (2.5)$$

2.1.2 Chromium co-pollutant (As)

Arsenic (atomic number: 33 and atomic weight 74.9216), a co-pollutant of chromium contaminated site is a group (V) member of the periodic table and also classified as a transition metal (Wackett *et al.,* 2004). Arsenic is the twentieth most abundant element in the earth's crust (Bhumbla and Keefer, 1994). As a transition metal (metalloid), mostly exists as arsenite (As(III)) and arsenate (As(V)) (Smedley and Kinniburgh, 2002). The oxidation state

of this metalloid determines its toxicity. Arsenic like chromium is also known to cause environment and health problems to human and living organisms (Singh *et al.,* 2008). Similar to Cr(VI), health impacts such as skin, liver lung, bladder and kidney cancer has been reported on ingestion or inhalation of As(III) contaminated water or air (Smith *et al.,* 1992). However, because of its high toxicity, the maximum contamination level of arsenic in drinking water was set at a much lower level of 10 µg/L (USEPA, 2001).

Similarly, aqueous speciation of arsenic species shows that arsenate specie H_3AsO_4 predominantly dominate at pH ≤ 2.2, whereas arsenite species predominate at pH as follows ($H_3AsO_3^o$ at pH ≤ 9.2, $H_2AsO_3^-$ ≥ 9.2 and $HAsO_3^{2-}$ ≥ 12.3) (Wagman *et al*., 1968; Ferguson and Gavis, 1972). Equilibrium Equation (2.6-2.7) shows that $H_2AsO_3^-$ anion is dominant in basic or slightly acidic solution whiles the $HAsO_3^{2-}$dominate in basic solution (Wagman *et al.,* 1968).

$$H_3AsO_3^o \Leftrightarrow H_2AsO_3^- + H^+ \qquad K_{a4} = 10^{-9.2} \qquad (2.6)$$

$$H_2AsO_3^- \Leftrightarrow HAsO_3^{2-} + H^+ \qquad K_{a5} = 10^{-12.3} \qquad (2.7)$$

The biochemistry of arsenic species has also been demonstrated by researchers (Wang *et al*., 2013; Sun *et al.,* 2010; Aniruddha and Wang, 2010; Sun *et al*., 2008). Aniruddha and Wang, (2010) reported that As(III) can be oxidised to As(V) in the presence of oxidizing agent by donating 2 electrons, while generating a considerable amount of energy for cell growth and metabolism Equation (2.8).

$$As^{3+} \rightarrow As^{5+} + 2e^- + \text{energy} \qquad (2.8)$$

2.4 Occurrence and source of chromium and its co-pollutant arsenic

2.2.1 Chromium

Chromium compounds are found in the environment from natural sources in the form of ore, in the hexavalent state. Free chromium in the form of chromate mainly originated from industrial activities (WHO, 1988; Merian, 1984). Naturally, chromite is the most prevalent form in the environment. It consists of two main refined products such as: ferrochromium and metallic chromium (Westbrook, 1983; Hartford, 1983). Secondly, lead chromate (as crocoite)

and potassium dichromate (as lopezite) are known to occur naturally in the environment (IARC, 1990). Industrial activities such as mining and smelting, and leaching of soluble Cr(VI) compounds from wastes such as mine tailings, waste rock, dust and slag piles are the major source of chromium in the environment (Barceloux, 1999). Figure (2.1) describes the total input of chromium in the environment with metal use being the highest chromium input followed by rock weathering and coal combustion (Merian, 1984).

Chromium is found in all matters such as rock, air, water and soil (Kimbrough, *et al.,* 1999). In the rocks, the most important mineral deposit of chromium is chromite (Mg, Fe^{2+})(Cr, Al, Fe^{3+})$_2O_4$ which is rarely pure (Kimbrough, *et al.,* 1999). The concentration of chromium in rocks varies from an average of 5 mg/kg (range of 2-60 mg/kg) in granitic rocks, to an average 1800 mg/kg (range of 1100-3400 mg/kg) in ultrabasic and serpentine rocks (US NAS, 1974b). Chromium is present in most soils in its trivalent form, although Cr(VI) can occur under oxidizing conditions (ATSDR, 2008a). In the USA, the geometric mean concentration of total chromium was 37.0 mg/kg (range of 1.0-2000 mg/kg) based on 1319 samples collected in contaminated soils (ATSDR, 2000), whereas in 173 Canadian sites, chromium soil concentration ranges from (10-100 mg/kg) (d.w.) (CEPA 1994c). The concentration of chromium in uncontaminated waters is extremely low (< 1 µg/L or < 0.02 µmol/L) (CEPA 1994c). Anthropogenic activities (e.g. electroplating, leather tanning) and leaching of wastewater (e.g. from sites such as landfills) may cause contamination of the drinking-water (EVM, 2002). In the air, chromium is usually introduced through forest fires, volcanic eruptions, combustion and industrial emissions. Cr(VI) is reported to account for approximately one third of the 2700-2900 tons of chromium emitted to the atmosphere annually in the USA (ATSDR, 2008a). Based on USA data collected from 2106 monitoring stations during 1977-1984, the arithmetic mean concentrations of total chromium in the ambient air (urban, suburban, and rural) were in the range of 0.005-0.525 µg/m^3 (ATSDR, 2000).

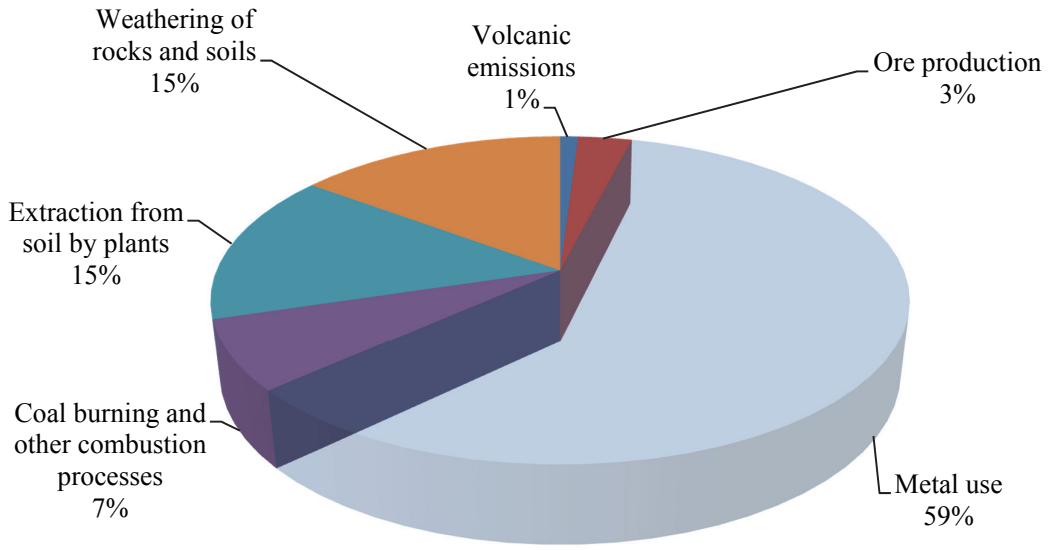


Figure 2.1: Total input of chromium in the environment (Mirian 1984)

2.2.2 Chromium co-pollutants

Arsenic mostly occurs as a result of rock weathering and volcanic activities (Rhine *et al.,* 2006). Examples of natural occurring arsenic bearing minerals (rocks) include: arsenian pyrite ($Fe(AsS)_2$), realgar (AsS), arsenopyrite (FeAsS), and orpiment (AsS_3) (Nordstrom, 2002). Arsenic concentration in igneous, metamorphic and sedimentary rock has been reported ranging from (1.5-18) mg/kg (Smedley and Kinniburgh, 2002; Webster, 1999). Anthropogenic activities such as smelter slag, coal combustion, run-off from mine tailing, hide tanning waste, pigment production, paint and dye and pesticides are the major source of arsenic contamination in the ground water, and soil sediments (Bhumbla, 1994).

Arsenic concentration in soils generally ranges between 5-10 mg/kg (Boyle and Jonasson, 1973). The arsenic content in soils is generally governed by principal factors such as climate, organic and inorganic component of the soil, and redox potential respectively (Aniruddha and Wang, 2010). In natural waters, arsenic is generally present at very low concentration. In fresh water, the concentration of arsenic generally varies between 0.15-0.45 µg/L (Leonard 1991). However, Smedley and co-workers (1996) reported arsenic concentration in the range of (100-5000) µg /L in unpolluted fresh waters located in areas of sulfide mineralization and mining. In sea water the concentration of arsenic varies between 0.09-24 µg/L (Leonard 1991).

Environmental cycling of chromium and its co-pollutants

As mentioned earlier, anthropogenic source such as coal burning, mining operation and smelting, and others are the major sources of these metalloids in the environment. However, microbes play an important role in the cycle of these metalloids (Cr and As) in the environment. Figure (2.2) below summarise chromium cycle in the environment. The cycle consists of chromium from rocks and soil carried by water, animal and human to water. Another cycle consists of airborne chromium from natural sources, such as fires, and from the chromate industry. This cycle also contains some hexavalent chromium, with by-products going into the water and air, but a very significant portion goes into the repository, the ocean, where it ends up as sediment on the ocean floor (WHO, 1988).

As(III) on other hand can be released from arsenate laden sediments by arsenate respiring bacteria leading to arsenic contamination of the ground water (Oremland and Stolz, 2003). These microbes generally use As(V) as a terminal electron acceptor in the anaerobic respiration process (Oremland and Stolz, 2003). The released As(III) can be further oxidized to As(V) by certain bacteria via detoxification mechanism or utilize the energy released during the oxidation process for cellular growth (Stolz *et al*., 2006). As(V) as a result of the oxidation process may be converted to water or lipid soluble organic compounds such as methylarsonic acid or dimethylarsinic acid (DMA),trimethylated arsenic derivatives (TMA), arsenocholine, arsenobetaine, arsenosugars, and arsenolipids by marine organisms such as phytoplankton, algae, crustaceans, mollusks, and fish (Knowles and Benson, 1983). The arsenic geocycle is completed with the conversion of arsenobetaine back into inorganic arsenic species as a result of microbial metabolism (Dembitsky and Levitsky, 2004). Figure (2.3) illustrate the possible processes in biogeochemical cycling of arsenic in the environment.

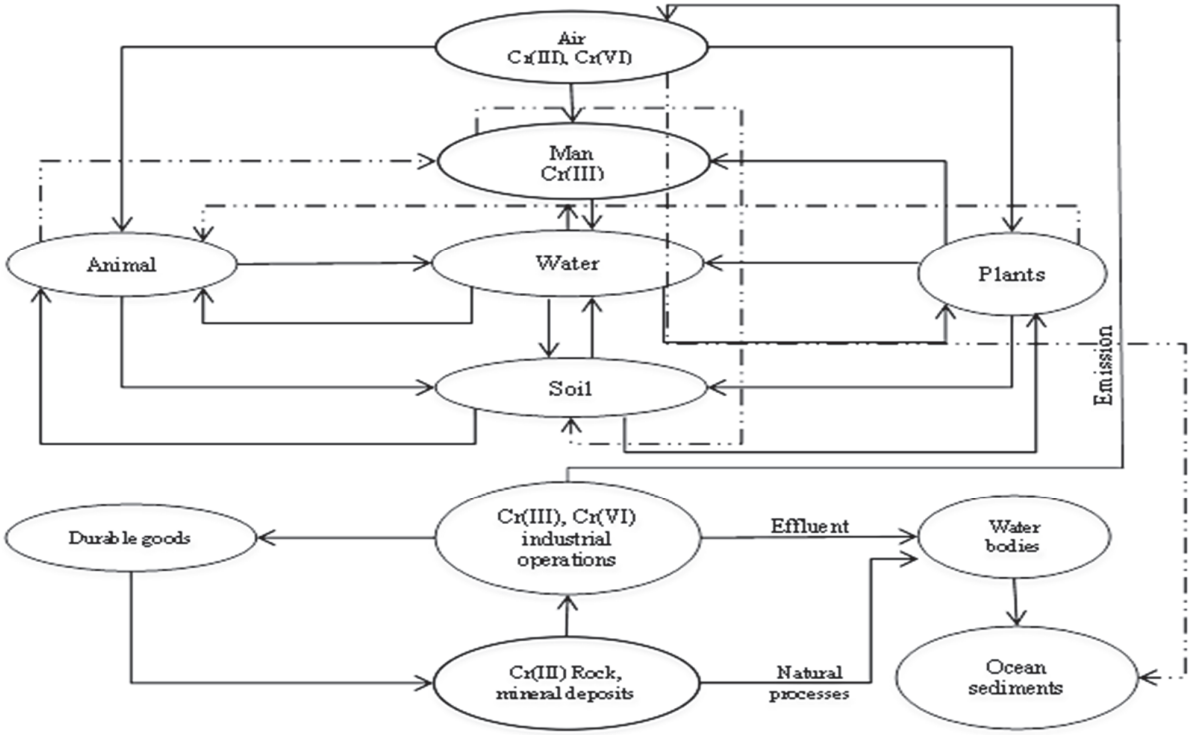


Figure 2.2: Environmental cycling of chromium (Modified WHO 1988)

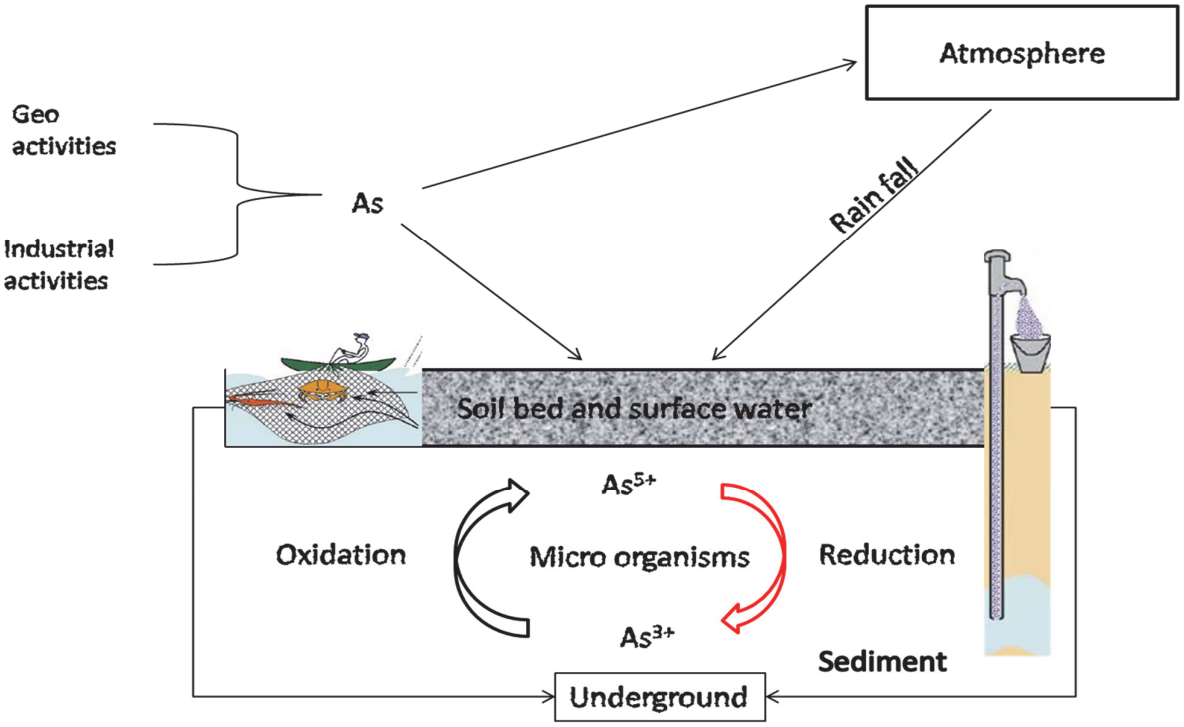


Figure 2.3: Possible processes in biogeochemical cycling of arsenic

2.4 Chromium production and industrial uses

Chromium ore is mined in many countries, but more than 90 % of chromite comes from South Africa, Kazakhstan, India, Brazil, Finland, Turkey and Zimbabwe (Hoffmann *et al.,* 2002). South Africa is among the largest chrome ore production in the world, accounting for about 44 % chrome ore production as shown in Figure 2.4 (Mintek, 2004; Barhart, 1997). Based on 2007 statistics, the South African ferrochrome smelting industry produces approximately 46 % of the global production volume of ferrochrome (FeCr), such being in the form of Charge chrome (typically containing 48-54 % Cr) (ICDA, 2008). However, there are currently fourteen separate FeCr smelter plants in South Africa, with a combined production capacity of 4.4 million tons/year (Beukes *et al*., 2012).

Industrial use of chromium started from chromite mining typically ferrous chromite, and its demand for different forms of chromium has continued to increase through the last decades (Kimbrough, *et al.,* 1999). Chromium minerals such as crocoite ($PbCrO_4$) are too rare to be of profitable value as chromium ores (Klein and Hurlbut, 1999). Chromite on the other hand is one of the first minerals separated from a cooling magma, and is usually associated with ultrabasic rocks such as peridotites and serpentines, etc. (Klein and Hurlbut, 1999). Most industrial use of chrome includes; stainless steel production, pigment production, electroplating, leather tanneries, fungicides production and wood preservation, and as a catalyst in the synthesis of organic chemicals etc. (Sandvik, 2004; Lipscher, 2004; Katz and Salem 1994; Barnhart, 1997). Steel industries are the major use of chromium; where steel in form of iron and alloy is mixed with about 12 % chromium to produce a non-corrosive stainless steel (Sandvik, 2004; Brown, 1995). Among the metal used the metal processing industries contribute up to 77 % followed by chemical (16 %) and refractory industries (12 %) as shown in Figure (2.5).

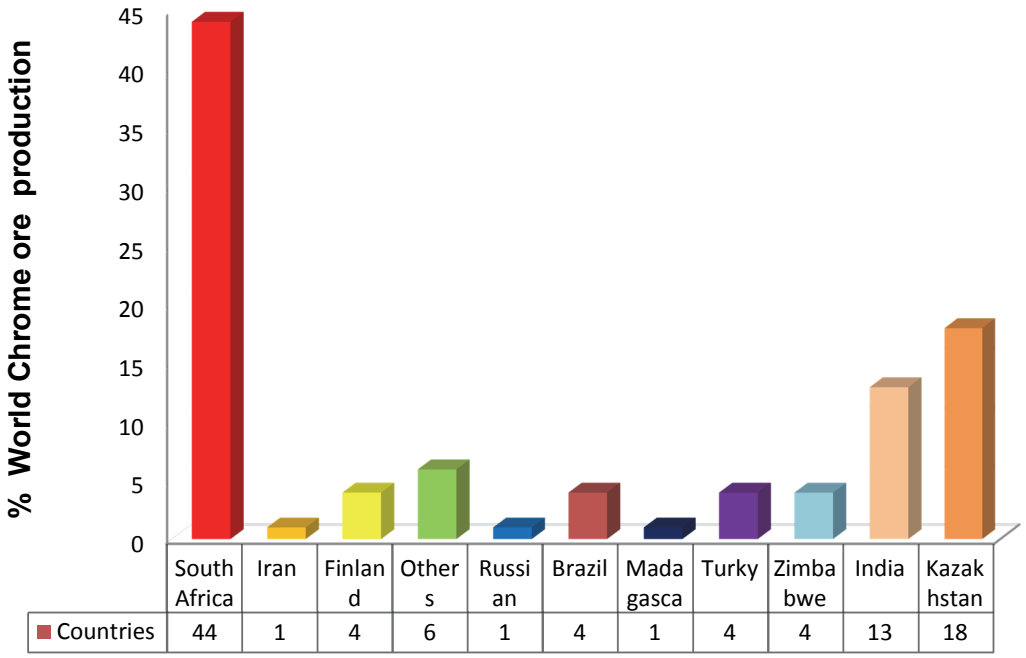


Figure 2.4: World production of chrome ore (Armitage, 2002)

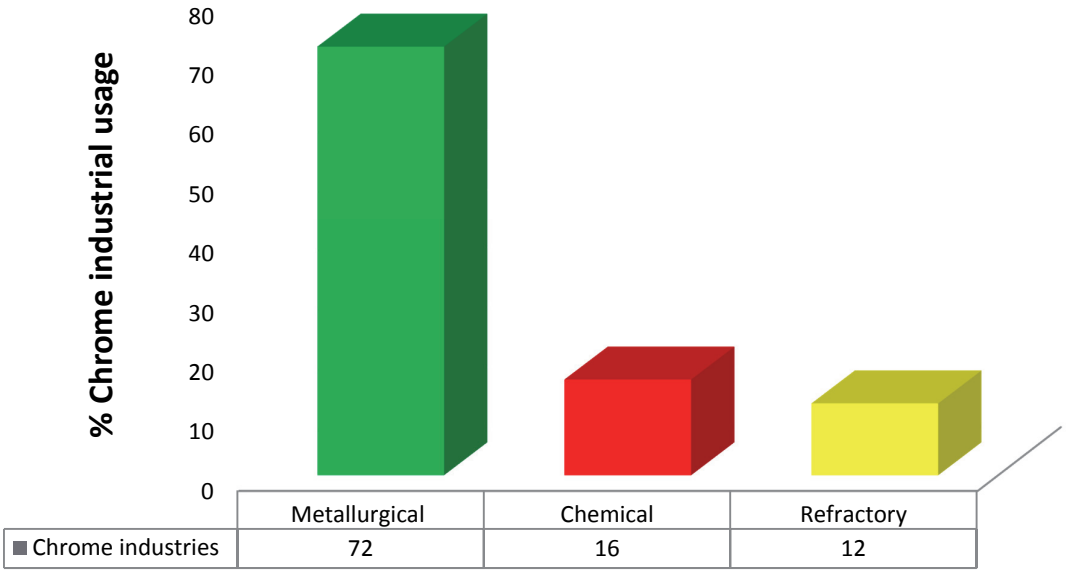


Figure 2.5: Industrial usage of chromium (Papp, 1999)

2.5 Removal techniques of chromium and its co-pollutant arsenic

The existing technologies for treatment of toxic metals (metalloids) are based on remediation by reduction or oxidation. Remediation by reduction or oxidation can be applied as ex-situ or in-situ process. Ex-situ remediation process is a strategy where contaminated site is excavated and transported off-site for treatment, while in-situ process on the other hand is a strategy where contaminated site is treated on-site. However, owing to high costs of transport, landfill space and pumping attributed to ex-situ process, in-situ process is seems to be more attractive than ex-situ process (Hawley *et al*., 2004). Several techniques have been explored in the past. These techniques include; ion exchange, adsorption process, membrane process, chemical treatment process and bioremediation process.

2.5.1 Ion exchange

Ion exchange is a physical treatment technology where ion with a high affinity for the resin material of the ion exchange column replaces an ion with a lower affinity that was previously bound to the column resin. Ion exchange resins has been reportedly capable of removing Cr(VI) and As(III) to a concentration less than the detection limit (Hawley *et al*., 2004; Clifford *et al*., 2003). However, the problem associated with this technology is its high cost of resin regeneration and complexity in operation. Also, pre-oxidation step for conversion of As(III) to As(V) is required for efficient As(III) removal (Johnston and Heijnen, 2001).

2.5.2 Adsorption

Adsorption is a physical/chemical process whereby the target metal ions present in the contaminated water are adsorbed onto the surface of the adsorbents (Hawley *et al*., 2004). Granular Activated Carbon (GAC) has been reportedly used to remove Cr(VI) and As(III) from wastewater. Secondly, Activated Alumina (AA) has also reportedly used for the removal of As(III) from wastewater (Clifford, 1999). However, during on-site GAC or AA regeneration, adsorbed chromium or arsenic would be released as Cr(VI) or As(III), creating a second waste stream that would require further treatment (Hawley *et al*., 2004).

2.5.3 Membrane process

Membranes such as microfiltration, ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) are generally selective barriers allowing the passage of certain constituents with the rejection or exclusion of others in the water (USEPA 2000; Johnston and Heijnen, 2001). They have been reportedly used in water treatment to remove Cr(VI) and As(III) from wastewater. Cr(VI) ions are too small to be removed by microfiltration or ultrafiltration membranes, unless a pre-treatment is performed to complex the Cr(VI) or As(III) by larger molecules (Hawley *et al*., 2004; USEPA 2000; Johnston and Heijnen 2001).

2.5.4 Chemical treatment process

Cr(VI) and As(III) removal has been reportedly achieved by conventional chemical treatment process through pH adjustment or precipitation (Rhine *et al*. 2006; Clifford 1993). Precipitation or pH adjustment involves the use of acid and base to remove Cr(VI) or As(III) as precipitate. Unfortunately, the cost of setting up the required equipment and operation processes are expensively high for a large-scale treatment.

2.5.5 Bioremediation process

Bioremediation process involves the application of micro-organism to reduce or oxidise metals or metalloids. Over the years, microorganisms have evolved mechanisms to remediate both metals and metalloids contaminants from water and wastewater. The special ability of this microorganism is usually demonstrated by changes in the redox states of the corresponding metals / metalloids or by adsorption onto its surface. The net result of both the processes leads to the reduction in the mobility of these contaminants in the environment (Mtimuye, 2011).

2.6 Bioremediation processes for a contaminated site

Bioremediation processes for treatment of a contaminated site has been widely explored, and it provides a potential for mitigation of toxic metals in the environment. Hence, the understanding of microbial reduction gives a promising step towards mitigation of environmental pollution and toxicity. The principal application of the bioremediation

processes are subdivided into three major categories such as; biosorption, biological reduction and biological oxidation.

2.7 Bioremediation of chromium and its co-pollutant arsenic

2.7.1 Microbial reduction of Cr(VI) to Cr(III)

Most microorganisms in the presence or absence of oxygen can detoxify Cr(VI) to Cr(III). These microorganisms are known as chromium reducing bacteria (CRB) (Kakonge, 2009). Chromium reducing bacteria has been investigated in a wide array of bacterial strains under both aerobic and anaerobic conditions by several researchers (Molokwane *et al.,* 2008; Zakaria *et al.,* 2007; Cheung and Ji-Dong, 2006; Chirwa and Wang, 2000). Early observation on Cr(VI) reduction and phenol degradation under both anaerobic and aerobic conditions showed that *P.putida* and *E.coli* are capable of simultaneously degrading phenol and reducing Cr(VI) in the contaminated environment (Chirwa and Wang, 2000).

Microbial reduction of Cr(VI) to Cr(III) can be direct enzymatic reduction or indirect reduction under anaerobic and aerobic condition (Molokwane, 2010; Yang *et al.,* 2009; Guha *et al.,* 2000; Sedlak and Chan, 1997; Pettine *et al.,* 1994). Under anaerobic condition, it was reported that Cr(VI) reduction is attributed to energy yielding dissimilatory respiratory process, in which Cr(VI) serves as a terminal electron acceptor. In addition, it may also be attributed with soluble reductase, a membrane bound with possibility of involving hydrogenase or cytochrome (Michel *et al.,* 2001). Equation (2.9) described anaerobic reduction of Cr(VI) to Cr(III), using acetate as electron donor.

$$3CH_3COO^- + 8CrO_4^{2-} + 17H_20 \xrightarrow{CRB} 8Cr(OH)_{3(s)} + 6HCO^{3-} + 13OH^- + \text{energy} \quad (2.9)$$

2.7.2 Microbial oxidation of As(III) to As(V)

Microbial oxidation of As(III) to As(V) was first observed in certain microorganisms, in cattle-dipping tanks (Green, 1918). A number of microorganisms capable of oxidizing As(III) to As(V) under both aerobic and anaerobic conditions have been investigated (Aniruddha and Wang, 2010; Sun *et al.,* 2010; Sun *et al*., 2008). The first heterotrophic As(III) oxidizing bacteria was described by (Green 1918), whereas an autotrophic As(III) oxidizing strain,

Pseudomonas arsenitoxidans, was first reported in 1981(Ilialetdinov and Abdrashitova 1981). Heterotrophic As(III) oxidation may represent a detoxification reaction on the cell's cytoplasmic (inner) membrane, whereas autotrophic As(III) oxidation releases energy that is used for CO_2 fixation and cell growth under both aerobic and anaerobic conditions (Santini *et al.*, 2000; Anderson *et al*., 1992).

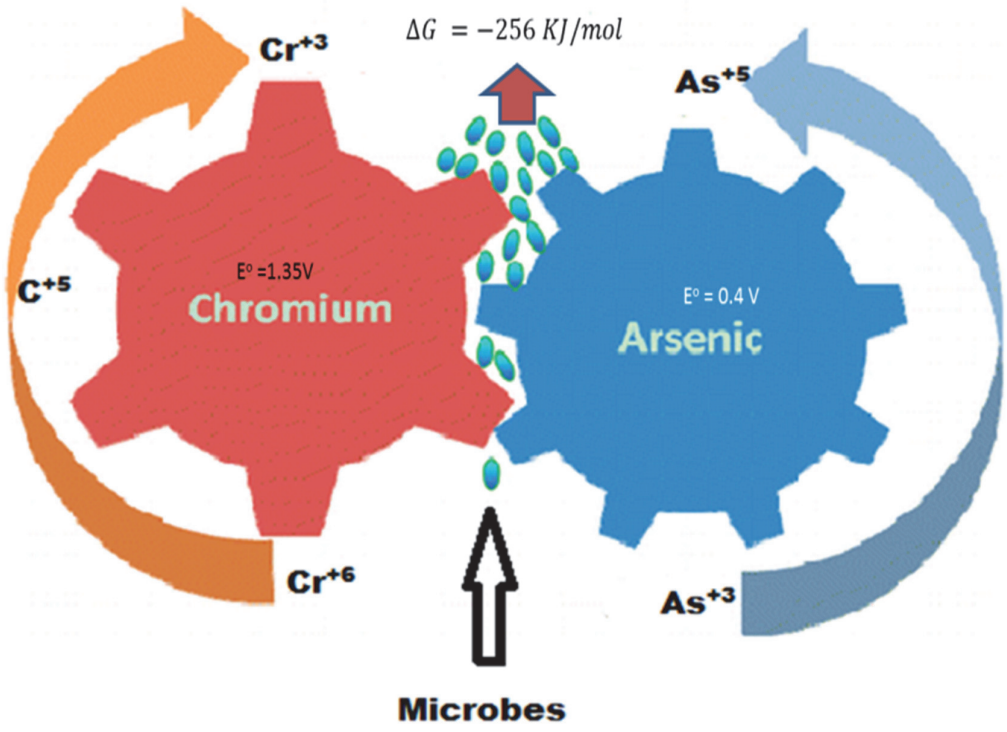


Figure 2.7: Mechanism of Cr(VI) reduction linked to As(III) oxidation

2.7.3 Cr(VI) reduction linked to As(III) oxidation

Figure 2.7 above described the mechanism of Cr(VI) reduction linked to As(III) oxidation catalysed by facultative microbes. Aniruddha and Wang (2010) reported that about 256 KJ/mol energy is generated during oxidation of As(III) to As(V). Further studies showed that about 467.95KJ of energy could be generated in the process (Wang, 2013). Recently, studies on Cr(VI) reductions linked to As(III) oxidation have been explored. According to Wang (2013), Cr(VI) reduction linked to As(III) oxidation was greatly accelerates by the addition of H_2O_2. This process was enhanced at acidic pH. Further studies under aerobic condition shows that *Bacillus firmus* TE7 strain could completely reduce reduced 15 mg/L Cr(VI) in the

presence of 50 mg/L of As(III), although the study reported that Cr(VI) reduction was not linked to As(III) oxidation (Batchate *et al*., 2013).

2.8 Bioremediation applications

Wide applications of Cr(VI) bioremediation have been studied, either in a batch process or continuous flow process. In these applications, a variety of organic substrates in combination with basal mineral medium has been utilized. However, micro-organisms can be employed as suspended cell or attached cell. In most applications, complete reduction of Cr(VI) concentrations ranging typically from 5 to 150 mg/L was achieved at various time intervals. The application of Cr(VI) bioremediation can be categorised into batch process and continuous flow process.

2.8.1 Cr(VI) Bioremediation with batch system

Cr(VI) reduction in a batch system is employed as suspended or attached growth system. In a batch process, micro-organism is placed in liquid suspension by appropriate mixing techniques or grown on a media. Since 1977, when biological Cr(VI) reduction was first reported by Romanenko and Koren'kov, numerous authors have published on biological chromate reduction. A variety of micro-organisms, including bacteria and fungus, have been identified to be able to reduce Cr(VI), hence this reduction is agreed to be enzymatic. Most batch studies have been aimed at optimizing physical conditions, establishing the biochemical mechanisms involved and analyzing kinetic potential (Caravelli and Zaritzky, 2009).

Successful Cr(VI) reduction with microbes in a batch assay under several conditions have been severally reported by many researchers (Wang *et al*., 2000; Mazerski *et al*., 1994; Shen and Wang, 1994a). Most of this studies were done under aerobic condition, however only few studies were done under anaerobic condition. Shen and Wang (1993 and 1994) reported a high Cr(VI) reduction under anaerobic condition. Molokwane et al. (2008) on the other hand, reported high Cr(VI) reduction, where anaerobic condition was achieved by purging 99 % of pure nitrogen gas. In order to explore previous Cr(VI) reduction in a batch studies, Table (2.1) bellow summarized some the previous batch investigation on biological Cr(VI) reduction.

Table 2.1: Summary of batch studies on bacterial Cr(VI) reduction conditions (Modified Slabbert, 2010).

Micro organism	**Substrate**	**Reduction Conc. (mg/L)**	**Oxygen**	**Tempe rature**	**Reference**
Agrobacterium radiobacter	Resting cells	25.5	Aerobic	10-40	Llovera *et al.* (1993)
Arthrobacter aurescens	Resting cells	26	Anaerobic	10-40	Llovera *et al.* (1993)
Arthrobacter aurescens	VB broth	50	Aerobic	10	Horton *et al.(2006)*
Arthrobacter sp	Glucose	40	Aerobic	40	Córdoba *et al.,* (2008)
Bacillus sp.	Glucose	25	Aerobic	25	Wang & Xiao (1995)
Bacillus sphaericus	VB broth	20	Aerobic	20	Pal & Paul (2004)
Bacillus subtilis	Glucose-MSM	25	Aerobic	30	Garbisu *et al.*(1998)
Desulfovibrio vulgaris	Resting cells	2600	Anaerobic	30	Lovley & Phillips (1994)
Escherichia coli	Glucose	291	Aerobic	35	Shen & Wang (1993)
Esche richia coli	Glucose	30	Aerobic	10-50	Shen & Wang (1993)
Escherichia coli	Glucose	40	Anaerobic	10-50	Shen & Wang (1994)
Enterobacter cloacae	KSC medium	210	Anaerobic	30	Ohtake, *et al.*,(1990)
Leucobacter sp.	LB broth	1700	Aerobic	15-42	Zhu *et al.* (2008)
Pseudomonas	Glucose and LB broth	22	Aerobic	20	McLean *et al.*,(2001)
Pseudomonas ambigua	Nutrient broth	150	Aerobic	20	Horitsu *et al.* (1987)
Pseudomonas fluorescens	Glucose and benzoate	25	Aerobic	30	Shen & Wang (1995)
Sphaerotilus natans	Glucose-MSM	20	Aerobic	20	Caravelli, *et al.,* (2008)
Streptomyces griseus	Broth II medium	50	Aerobic	20-50	Laxman & More (2002)
Consortium (18 species)	LB-broth	150	Anaerobic	30	Molokwane *et al.*(2008)

Recently, studies on Cr(VI) reduction in a batch assay, with consortium culture isolated from Wastewater Treatment Plant, (North West Province South Africa) demonstrated that consortium culture outperforms individual pure culture, which is attributed to diversification of different microbes (Molokwane *et al*., 2008).

2.8.2 Cr(VI) Bioremediation with continuous flow system

Most continuous systems were designed as attached growth (biofilm system); were consortium or pure cultures responsible for bioremediation are grown on the media. Media typically used in attached growth system includes; soil, rocks, gravel, plastic beads, and glass beads etc. Continuous flow system can be operated in anaerobic or aerobic condition. Due to its advantage over batch system, continuous flow reactors have been used for treatment of high effluent Cr(VI) containing waste. However, many types of anaerobic reactors exist such as; up flow anaerobic sludge blanket reactor, expanded granular sludge blanket reactor, multiplate reactor, anaerobic filter, fixed-film reactor, down flow fixed-film reactor, fluidized bed reactor, anaerobic ponds, anaerobic sequencing batch reactor (ASBR), two-phase digestion and up flow fixed- film reactor (Mulligan, 2002).

Biofilm studies in continuous flow systems have been demonstrated by many researches (Mtimuye, 2011; Slabbert, 2010; Molokwane *et al.,* 2009; Nicolella *et al.,* 2000; Stoodley *et al.,* 1999; Chirwa and Wang 1997). According to Stoodley et al. (1999) and Nicolella et al. (2000), biomass limitation improves culture flexibility and allows high specific biomass retention which increases volumetric yield in a continuous flow system. Chirwa and Wang (1997), reported biofilm flexibility by observing remediation after Cr(VI) overloading. This illustrates that attached growth systems enable higher volumetric reduction rates than suspended growth system. However, higher Cr(VI) removal was observed in the biofilm system than in the suspended system, and this is attributed to culture adaptation and mass transport resistance across the attached biofilm layer (Wang and Chirwa, 2001). This suggests that the exposure of Cr(VI) toxicity to bacterial cells decreases with an increase in biofilm depth. In addition, continuous system is preferred than batch system since it is commercially applicable, allows easier handling and operation (Ahmad *et al*., 2010).

2.9 Biofilm theory and structure

Environmental microbiologist has long recognised that complex bacteria communities are responsible for driving the biochemical that maintains the biosphere (Davey *et al*., 2000). Moreover, it becomes clear that these natural assemblages of bacteria function as a cooperative consortium, in a relatively complex and coordinate manner (Costerton *et al*., 1995). For the purpose of this study biofilms are defined as an assemblages of microorganism or communities of bacteria attached to a solid substratum and embedded in a "glycocalyx" matrix consisting of self-excreted exopolysaccharides (EPS) (Aniruddha and Wang, 2010).

EPS consists of polysaccharides, proteins, glycoproteins, glycolipids, and in some cases, certain amounts of extracellular DNA (e- DNA) (Flemming *et al*., 2007). It is one of the key components of the biofilm matrix, because it mediates the process of adhesion between the bacterium and the attachment surface (Donlan and Costerton, 2002). According to Watnick and Kolter (1999), the biofilms of single species are formed in several multiple steps. These steps resulting from the association between the bacterium and the attachment surface and other microorganisms already present on the surface finally leads to the formation of the three-dimensional biofilm matrix (Watnick and Kolter, 2000).

Several studies have been conducted to investigate and understand the complex structure of the EPS and its components (Flemming *et al*., 2007). However, the most widely accepted theory is the creation of the microenvironment, which helps to counter severe pH changes in the bulk liquid, and also to resist toxic substances from entering the biofilm matrix. In addition, the close spacing of the cells in the matrix was important for effective transport of essential nutrients across the cells (Rittmann, 2001).

Other studies on biofilms shows that biofilms are not simply organism containing slime layers on the surface, instead biofilm represent the biological systems with a high level of organisms where bacteria structured, coordinated, and functional communities (O'Toole *et al*., 2001). Secondly, biofilms has been found positioned onto surface in various mechanisms. The most common mechanism is the flagellar motility and different methods of surface translocation, including twitching, gliding, darting and sliding (Davey *et al*., 2000). Other mechanism includes the synthesis of cellulose, thereby forming a fibrous pellicle that places cells aids near air water interface. In addition, some species have magnetosomes

(intracellular structure consisting of a crystal magnetic mineral) surrounded by a membrane that cause the cells to passively align with the earth's geomagnetic filed, thereby restricting lateral excursion (Davey *et al*., 2000).

In the applications of biofilm, packed bed reactors are the most common type of biofilm reactors; the cells are usually attached to a stationary medium, and are generally used for aerobic and anaerobic treatment of wastewater (Rittman, 2001). Fluidized bed and RBC reactors are another kind of biofilm reactors which are commonly employed for wastewater treatment. The cell in the fluidized bed reactors are immobilized, and kept in the suspension under a high effluent recycle flow rate (Aniruddha and Wang, 2010). The biggest advantage of the packed bed reactor over the other reactors is the capacity to withstand higher substrate loading rate due to the presence of strong attachment force between the cells and the surface (Aniruddha and Wang, 2010). For the purpose this study, packed bad reactor was used for Cr(VI) reduction investigation in the presence of As(III).

2.10 Summary

The co-existence of Cr(VI) and As(III) suggests a redox cycle, which provides a potential for simultaneous bioremediation of these metals/metalloids in a single close system. Generally, remediation of these metalloids involves reduction of Cr(VI) to Cr(III) and oxidation of As(III) to As(V), which are often treated separately. Till date bioremediation of Cr(VI) together with As(III) has not been achieved in a single system. However, this study explores bioremediation of Cr(VI) and As(III) in a single system. Detoxification of these metalloids will be achieved by combining biocatalytic reduction of chromium Equation (2.5) and biocatalytic oxidation of Arsenic in Equation (2.8).

Generally, despite the knowledge of the persistence of Cr(VI) and its co-pollutants in the environment, and the hazards associated with its exposure, high mining and other industrial activities in South Africa have resulted to the contamination of ground water and sediments. For example, Brits Wastewater Treatment Work, (North West Province South Africa) has been reported containing 2.45 mg/L, 2.63 mg/L and 25.44 g/m^2 of Cr(VI) in the effluent, mixed liquor and dried sludge respectively. This is found to be above Cr(VI) maximum regulatory standard of 50-100 μg/L (Molokwane *et al*., 2008). This problem may be exacerbated by wrongfully decommissioned or abandoned mining operations.

To date, only few studies has been done on simultaneous Cr(VI) reduction and As(III) oxidation (Wang, 2013; Batchate *et al*., 2013). In the study by Wang (2013) As(III) oxidation was facilitated by the presence of oxygen containing compound in the system which induced aerobic conditions in the system. Batchate et al. (2013) also conducted studies on simultaneous Cr(VI) reduction and As(III) oxidation with *Bacillus firmus* strain TE7 under aerobic condition. In the present study simultaneous Cr(VI) reduction and As(III) oxidation was evaluated under anaerobic condition. The evaluation of Cr(VI) reduction and As(III) oxidation under oxygen stressed conditions was attributed to the observed stability of As(III) released from laden sediments, a zone which has less or no oxygen by arsenate respiring bacteria (Oremland and Stolz, 2006).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Source of micro-organism

Dried sludge from sand drying beds at the Brits Wastewater Treatment Works (North West Province, South Africa) was used as inoculum for the mixed culture of bacteria used. The treatment plant is located nearby an abandoned chrome processing facility, which periodically received flows from it and discharged high level of Cr(VI). It was reported that Cr(VI) concentration of the treatment plant's effluent, mixed liquor and dried sludge were 2.45 mg/L, 2.63 mg/L and 25.44 g/m^2, respectively (Molokwane *et al.*,2008). Microorganisms at such site were expected to resist high Cr(VI) concentration, and growth possibility is based on their acclimatization to Cr(VI) toxicity. Dried sludge from different locations of sand drying bed labeled (*Sx$_1$, Ss$_1$, Ss$_3$, Ss$_4$, Ss$_5$*) were collected with sterilized plastic bottles and stored at 4^{o}C.

3.2 Culture inoculation

0.2 g of sludge from different sludge samples (*Sx$_1$, Ss$_1$, Ss$_3$, Ss$_4$, Ss$_5$*) of sand drying bed stored at 4^{o}C, was inoculated in 100 mL of sterile nutrient broth, amended with 30 mg/L of Cr(VI) and 20 mg/L As(III) respectively for 24 h. Batches were incubated under shaking at 120 rpm in a rotary Environmental Shaker (Labotech, Gauteng, South Africa) at 30±0.2 ^{o}C. The inoculant was amended with Cr(VI) and As(III) to acclimatise the culture in the sludge samples. However, anaerobic cultures were grown in 100 mL serum bottle purged with 99.9 % of nitrogen gas for about 5-10 min. The bottle was then closed with silicone rubber and aluminum stoppers. All media were autoclave (HICLAVE HV-50 Hivayama South Africa) for 5 min at 121^{o}C before use.

3.3 Culture isolation

From spread method (Molokwane *et al*.,2008), cultures were isolated by depositing 1 mL of serially diluted sample from the 7th to the 10th test tubes using a pipette into Petri dishes containing sterilized nutrient agar. The nutrient agar was then incubated for about 24 h at 30±0.2^{o}C, in order to develop separate identifiable colonies. After 24 h of incubation, individual colonies from the latter agar plates were then transferred into new sterilized agar plates with a sterile wire loop based on their colour and morphology. Subsequently, the plates were then incubated for 24 h at 30±0.2^{o}C. The isolated cultures were then stored as pure stock solution at -70 ^{o}C.

3.4 Culture storage

20 mL of sterile glycerol (20 %, v/v) was added to 80 mL bacteria culture. The mixture was checked to evenly dispersed glycerol before transferring into 2 mL screw cap tube. Subsequently, the transferred samples in 2 mL screw cap tubes were stored at -70 ^{o}C for further use. For each experimental run, the frozen cultures were melted for about 10-20 minutes, then streaked on the surface of the sterilized nutrient agar plate using sterile inoculating loop. The nutrient agar plates were then incubated for about 24 h at 30±0.2 ^{o}C in an insulated incubator room.

3.5 16S rRNA partial sequence analysis

Phylogenetic characterization of cells was performed on individual colonies of bacteria from the 7th and 10th tube in the serial dilution preparation using a method described by (Molokwane *et al*., 2008). 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). An internal primer pD was used for sequencing (corresponding to position 519-536 of the 16S gene). The resulting sequences were matched to known bacteria in the GenBank using a basic BLAST search of the National Centre for Biotechnology Information (NCBI, Bethesda, MD).

3.6 Microbial analysis

In a 100 mL serum bottles containing nutrient broth, isolated cultures were grown anaerobically for 24 h. Cells were harvested after inoculated for 24 h by centrifuging for 10 min at 6000 rpm and 4^{o}C. The supernatant was decanted and the remaining pellet was wash three times in a sterile saline solution (0.85 % NaCℓ). During each wash, cells were suspended in the saline solution, centrifuged at 6000 rpm for 10 minutes and the pellet re-suspended in clear saline. The extent of sludge samples to reduce Cr(VI) in the presence of As(III) was conducted in a sterilized 100 mL serum bottles containing BMM, amended with 30 mg/L of Cr(VI) and 20 mg/L of As(III) respectively under anaerobic condition. Subsequently, individual pure isolates were also evaluated for individual Cr(VI) in a 100 mL serum bottle containing BMM, with the same initial concentration of Cr(VI) and As(III). Anaerobic condition was achieved by purging 99.99 % of N_2 gas in the serum bottle containing the sludge samples and harvested cells for 10 min to sweep any residual oxygen before sealing with silicon stoppers and aluminum seals.

3.7 Batch experiment

3.7.1 Cr(VI) reduction at different concentrations in the presence As(III)

In 100 mL sterilized bottles containing BMM, the harvested cells was re-suspended before adding Cr(VI) and As(III), to give a desired concentration. Cr(VI) and As(III) stock solution were added to give a final concentration of 50-500 mg/L and 20 mg/L respectively. Subsequently, the 100 mL bottles containing the harvested cells were purge with N_2 gas for before sealing with silicon stoppers and aluminum seals. The experiments were conducted at 30±0.2^{o}C over time at 120 rpm on the orbital shaker (Labotec, Gauteng, South Africa). Prior to inoculating the bottles with harvested cell, 1 mL of the sample was initially withdrawn from the serum bottle to determine the absorbance of Cr(VI) before introducing the cells in each serum bottle. The samples withdrawn in serum bottles over time were centrifuged using a 2 mL Eppendorf tube at 6000 rpm for 10 min in a Minispin® Microcentrifuge (Eppendorf, Hambury, Germany) and the supernatant was used for Cr(VI) reduction analysis.

3.7.2 Cr(VI) reduction at different As(III) concentrations

Again, in 100 mL sterilized bottles containing BMM, the harvested cells was re-suspended before adding Cr(VI) and As(III), to give a desired concentration. Cr(VI) and As(III) stock solution were added to give a final concentration of 70 mg Cr(VI)/L and 5-70 mg As(III)/L respectively. Subsequently, the 100 mL bottles containing the harvested cells were purge with N_2 gas for 10 min to sweep any residual oxygen before sealing with silicon stoppers and aluminum seals. The experiments were conducted at 30±0.2^{o}C over time at 120 rpm on the orbital shaker (Labotec, Gauteng, South Africa). Prior to inoculating the 100 mL bottles with harvested cell, 1 mL of the sample was initially withdrawn from the serum bottle to determine the absorbance of Cr(VI) before introducing the cells in each serum bottle. The samples withdrawn in serum bottles over time were centrifuged using a 2 mL Eppendorf tube at 6000 rpm for 10 min in a Minispin® Microcentrifuge (Eppendorf, Hambury, Germany) and the supernatant was used for Cr(VI) reduction analysis.

3.7.3 Abiotic experiment

In 100 mL serum bottle containing BMM, amended with the desired stock solution of Cr(VI) and As(III) concentrations with no suspension of the harvested cells were used to determine the abiotic Cr(VI) reduction in presence of As(III). The samples withdrawn in serum bottles over time were centrifuged using a 2 mL Eppendorf tube at 6000 rpm for 10 min in a Minispin® Microcentrifuge (Eppendorf, Hambury, Germany) and the supernatant was used for Cr(VI) reduction analysis.

3.7.4 Cr(VI) reduction linked to As(III) oxidation

Cr(VI) reduction linked to As(III) oxidation experiments was tested in 100 mL serum bottles containing BMM under anaerobic condition. The serum bottles were inoculated with harvested cells, amended with Cr(VI) and As(III) stock solution to give a desired concentration of 70 mg/L Cr(VI), 5-40 mg/L As(III), as well as 5 g of glucose. The experiment was conducted in three different batches, and each batch was purged with 99.9 % of N_2 gas. The first batch of the experiment consist of Cr(VI) and As(III) concentration, the second batch consist of Cr(VI) concentration and glucose, and the third batch consist of Cr(VI) concentration supplemented with bicarbonate, serving as the main experimental

control. The samples withdrawn in serum bottles over time were centrifuged using a 2 mL Eppendorf tube at 6000 rpm for 10 min in a Minispin® Microcentrifuge (Eppendorf, Hambury, Germany) and the supernatant was used for Cr(VI) reduction analysis

3.7.5 Total biomass

Total biomass was determine by re-suspending pellet in 1 mL of distilled water and filtered through a pre-weight Whatman filter paper No.1. The filter with the microorganism was dried in the oven at 60 ^{o}C to get a constant weight. The difference between the dried filter paper with the cells and the empty filter paper was considered as biomass (VSS).

3.7.6 Viable cell concentration

Viable cell concentration was determined using the serial dilution spread plate method. 1 mL of suspended cell solution was diluted serially into 9 mL NaCl solution (18.5 g/L) contained in ten test tubes. Test tubes were sterilised just before use with 99 % purity ethanol and then rinsed with distilled water. One millilitre of suspended cell solution was transferred from test tubes 8th, 9th and 10th to three Petri dishes with agar-medium. The agar-medium was a mixture of Plate Count Agar and LB Agar (Merck) dissolved collectively into tap water at half the recommended concentration each; 11.5 g/L and 22.5 g/L were dissolved, respectively. The agar-medium was autoclaved at 121°C for 20 min before use. The suspended cell solutions were spread onto the agar-medium, then the Petri dishes were turned upside-down and were incubated overnight in a temperature controlled incubator at 30±3 ^{o}C. The cell colonies on each plate were counted and the geometric mean between the three plates is reported as colony forming units (CFU) per milliliter of sampled solution

3.8 Analytical methods

3.8.1 Sampling

2 mL samples were collected from the effluent stream into Eppendorf-type centrifuge tubes at various intervals of experiment. The samples were centrifuged at 6000 rpm, 2000 g (Hermle GmbH Z100 M mini-centrifuge) for 15 min to remove the cells as pellets at the bottom of the

tubes. The cell free supernatant used for analytical procedures was extracted from the centrifuge tubes with a pipette without re-suspending the separated cells.

3.8.2 Cr(VI) measurement

Cr(VI) was measured using the UV/Vis spectrophotometer at 540 nm wavelength (WPA, light wave II, Labotech, South Africa). The experiment was carried after digestion of sample with 1 mL of 1N H_2SO_4, followed by distilled water up to mark and reaction with 0.2 mL of 1, 5- diphenyl carbazide (DPC) in a 10 mL volumetric flask. The presence of Cr(VI) in the sample was visualized by the change of colour after adding DPC (APHA, 2005).

3.8.3 Total Cr measurement

10 mL of the sample was reacted with 1 mL 1N H_2SO_4 to dissolve chromium hydroxide precipitates, and to extract adsorbed Cr(VI) for total Cr analysis. The samples were determined in the Varian AA-1275 Series Flame Atomic Adsorption Spectrophotometer (AAS) (Varian, Palo Alto, CA (USA)) equipped with a 3 mA and chromium hollow cathode lamp at 359.9 nm wavelength.

3.9 Reagents

3.9.1 Chemicals

Sodium arsenite ($NaAsO_4$, 99.9 % purity), di-potassium chromate (K_2CrO_4, 99 % purity), H_2SO_4 (99.9 % purity), 1, 5 - diphenyl carbazide (99 % purity), (0.85 %) NaCl. All chemicals were purchased from Merk (South Africa).

3.9.2 Standard solutions

Cr(VI) stock solution (1000 mg/L) was prepared by dissolving 3.74 g of 99 % pure K_2CrO_4 (Analytical grade) in 1 L deionised water, whereas As(III) 1000 mg/L stock solution was purchase from Merck South Africa. These stock solutions were used throughout the experiments to serve as Cr(VI) and As(III) sources. The standard solutions of Cr(VI) were prepared from the Cr(VI) stock solutions in a 10 mL volumetric flask by diluting certain volume of Cr(VI) stock solution with distilled water to give desirable final concentrations of

(0, 1, 2, 3, 4, 6 and 8) mg/L. From these data points (absorbance against concentration) a linear graph or calibration curve with the regression of 99.95 % was obtained Appendix (D).

3.9.3 DPC solution

Diphenyl carbozide (Merck, South Africa) solution was prepared for Cr(VI) reduction analyses by dissolving 0.5 g of 1, 5 diphenylcarbozide in 100 mL of HPCL grade acetone and was stored in a brown bottle covered with a foil.

3.10 Growth media

3.10.1 Basal mineral media

Basal mineral medium (BMM) was prepared by dissolving: 10 mM NH_4l, 30 mM Na_2HPO_4, 20 mM KH_2PO_4, 0.8 mM Na_2SO_4, 0.2 mM $MgSO_4$, 50 µM $CaCl_2$, 25 µM $FeSO_4$, 0.1 µM $ZnCl_2$, 0.2 µM $CuCl_2$, 0.1 µM NaBr, 0.05 µM Na_2MoO_2, 0.1 µM $MnCl_2$, 0.1 µM KI, 0.2 µM H_3BO_3, 0.1 µM $CoCl_2$, and 0.1 µM $NiCl_2$ into 1 L of distilled water, amended with 0.6 g of bicarbonate, and 5 g of glucose respectively. The prepared medium was sterilized before use by autoclaving at 121°C at 115 kg/cm^2 for 15 min.

3.10.2 Commercial broth and agar

Luria-Bettani (LB) broth, Luria-Bettani (LB) agar, and Soy broth (Merck, Johannesburg, South Africa) was prepared by respectively dissolving 25 g, 45 g, and 23 g in 1000 mL of distilled water. The LB and PC agar media were cooled at room temperature after sterilization at 121°C at 115 kg/cm^2 for 15 min and then dispensed into petri dishes to form agar plates for colony development.

3.11 Continuous flow reactor experiment

3.11.1 Reactor set-up

The continuous flow reactor (packed bed reactor) was constructed from a Pyrex glass column (height: 40±0.01 cm, internal diameter: 6.0±0.01 cm) packed with 4480, 5 mm spherical Pyrex glass beads (Fisher Scientific Co, Pittsburgh, PA), Figure(3.1). The total external

surface area of the glass beads available for cell attachment is 88000 mm^2, in the packed bed reactor volume of 1131.43 cm^3 and surface area of 28.29 cm^2. Prior to assembling, the components of the pumps, control valves and the connecting tubing were autoclaved at 121°C for 15 min. Subsequently, the interior of the reactor was rinsed in 95 % ethanol and dried.

For a working reactor volume of 1131.43 cm^3, distilled water was used to pre-calibrate peristaltic pumps used in order to achieve the desired volumetric flow rate Table 3.1. The reactor was operated in an up-flow mode to ensure near completely submerged condition. The reactor was design to operate continuously at hydraulic retention time of 14.4 h under volumetric feed flow 0.0131 cm^3/s. The reactor consists of sample ports of same diameter, and 2 L influent and effluent tanks Figure 3.1.

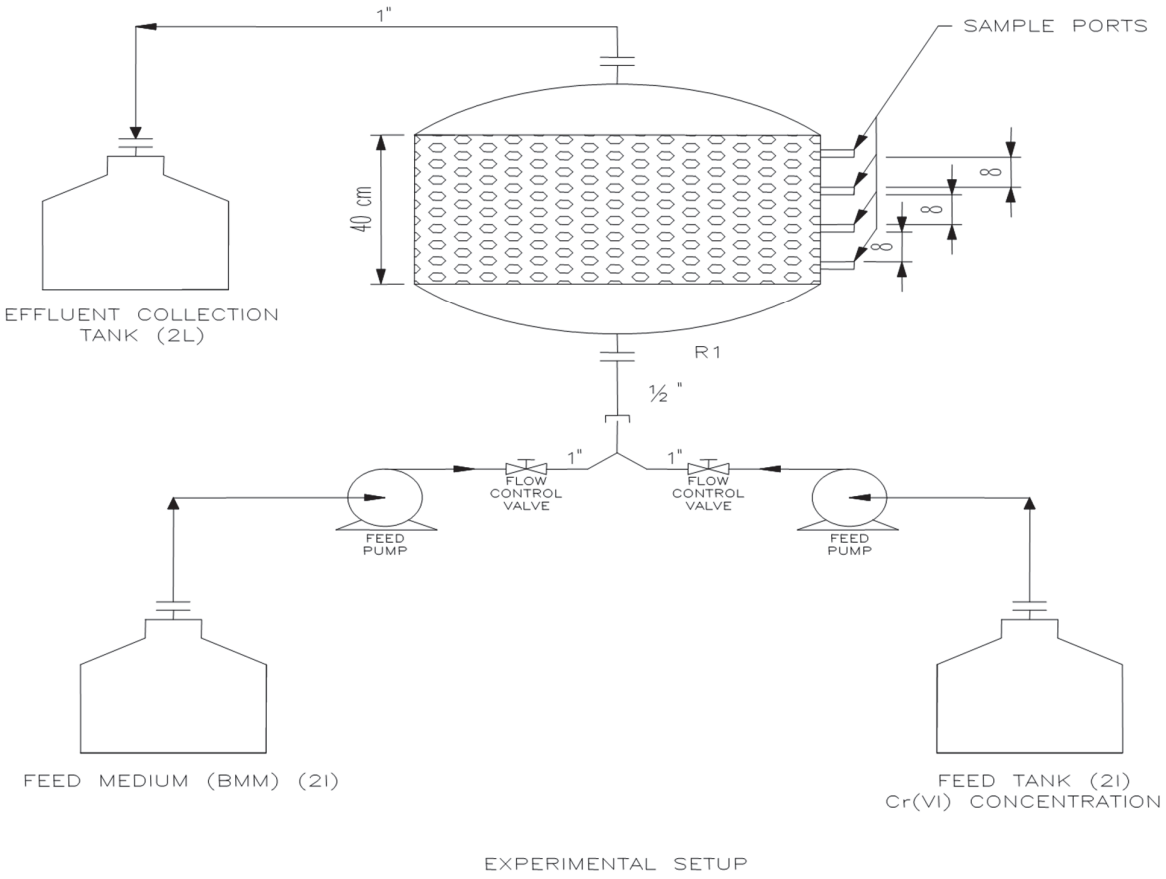


Fig 3.1: Continuous flow reactor set up

Table 3.1 Reactor design and operational parameters

Reactor design parameters	Units	Value
Height	cm	40
Internal diameter	cm	6
Diameter per glass bead	mm	5
Reactor surface area	cm^2	28.29
Reactor volume	cm^3	1131.43
Surface area per bead	mm^2	19.64
Total surface area of the beads	mm^2	88000
Volumetric flow rate	cm^3/s	0.0131
Porosity	%	60
Hydraulic retention time	h	14.4
Room temperature	^oC	30±0.2

3.11.2 Start-up culture

Stored harvested cells used in the batch experiment were incubated for 24 h. Subsequently, the cells were centrifuged at 6000 rpm (2820 g) for 10 min, and then thoroughly mixed with a basal mineral medium before fed into the reactor.

3.11.3 Reactor start-up

The reactor was inoculated with 40 mL overnight grown anaerobic mixed culture, mixed with LB broth medium, and incubated for 24 h at 30±0.2 ^oC. After 24 h incubation, the reactor was operated under anaerobic condition for more than 14 days until visible cell attachment was observed on the glass beads and column of the reactor. At this stage, glass beads were collected from four different locations for scan electron microscopic analysis. However, once a biofilm was clearly established on the glass beads, the reactor was then operated under influent Cr(VI) and As(III) concentration at volumetric flow rate 0.0131 cm^3/s and HRT of 14.4 h.

3.11.4 Cr(VI) reduction in the presence of As(III)

The continuous flow biofilm reactor, Cr(VI) reduction was evaluated in the presence of As(III). The reactor was loaded with Cr(VI) concentration ranging from 20-100 mg/L in the presence of 40 mg/L As(III) for a hydraulic retention time of 14.4 h. The reactor was operated for 120 days under anaerobic condition. Biological growth in the feed solution and tubes was minimized by close monitoring and periodical replacement. In addition, the optimum operating conditions were maintained in the reactor by frequent monitoring of dissolved oxygen and pH, in order to achieve the desired dissolved oxygen and pH. From the sampling ports, samples were withdrawn over time and centrifuged using a 2 mL Eppendorf tube at 6000 rpm for 10 min in a Minispin® Microcentrifuge (Eppendorf, Hambury, Germany) and the supernatant was used for Cr(VI) reduction analysis.

3.11.5 Steady-state determination

For each phase of experimental run, the reactor was continuously operated for at least for more than 14 days to ensure steady-state condition before changing the Cr(VI) loading rate. The time taken by a completely mixed reactor to reach 95 % of its steady-state concentration is at least three to four times the HRT. In the present study, the operation periods ranged from 14-28 times the HRTs and thus satisfying the steady-state assumptions for the entire operational phases. Secondly, steady state was also predicted when the effluent Cr(VI) concentration remain constant over a consecutive period of time.

3.12 Scanning electron microscopy

Scanning electron microscopy was done subsequent to reactor culture startup, in order to examine biofilm growth, and establish the existence of biofilm on the glass beads. A sample of glass bead was removed from four locations in the biofilm reactor in order to have randomly selected sample. The beads used were grinded to create a rough surface area for microbial attachment. However, the procedure used the achieve scan electron microscopic study is listed below.

The procedure is as follow:

- Fixing the biomass with 2.5 % glutaraldehyde dissolved in 0.075 M phosphate buffer (pH = 7.4-7.6) for 30 minutes.

- Rinsing 3 times for 5 min each time with phosphate buffer.

- Fixing with 0.25 % aqueous osmium tetroxide 3 times for 5 min each time (in a fume hood).

- Rinsing 3 times with distilled water (in the fume hood).

- Dehydrating with 20, 50, 70, 90 and 99 % ethanol for 5 min at a time.

- Drying twice for 15 min at a time with hexamethyldisilazane.

- Evaporating hexamethyldisilazane from the particles under atmospheric conditions for approximately 30 min.

- Attaching particles to carbon tape which in turn was fixed to an aluminum support.

- Covering in gold under argon plasma.

3.13 Dissolved oxygen determination

Dissolved oxygen content of the reactor was measured using DO meter (LD0101 Hatch South Africa). DO profile of the system was measured when the system has reached a steady state condition. DO meter was calibrated with standard buffers of 4 and 7 and disinfected by 95 % ethanol before use.

3.14 pH determination

pH of the effluents was measured using pH meter (PHC101 Hatch South Africa). pH meter was calibrated with standard buffers of 4 and 7 and disinfected by 95 % ethanol before use.

CHAPTER FOUR

EXPERIMENTAL RESULT AND DISCUSSION

4.1 Preliminary studies

4.1.1 Microbial analysis

Prior to experiment, microbial performance in a dried sludge from Wastewater Treatment Works (North West province South Africa) was investigated for Cr(VI) reduction in the presence of As(III) under anaerobic condition. The essence of this experiment was to evaluate the performance, and resistivity of the sludge samples for Cr(VI) reduction in the presence of As(III), which usually co-exist with Cr(VI) in the mine waste. In various dried sludge samples (*Sx1, Ss1, Ss3, Ss4,* and *Ss5*) from different locations in the drying bed, was amended Cr(VI) and As(III) concentration of 30 mg/L and 20 mg/L respectively. However, these dried sludge samples used has been reported containing 25.44 g/m^2 Cr(VI) concentration (Molokwane *et al*., 2008). It is assumed that microorganism at such site can tolerate high Cr(VI) concentration.

Results showed a near complete Cr(VI) reduction within 2 hours of incubation, signifying the capacity of these samples to reduce Cr(VI) in the presence of As(III) Figure (4.1). In addition, individual pure isolates (*X1, S1, S3, S4,* and *S5*) from sludge samples were also investigated. The existence of Cr(VI) reducing bacteria and As(III) resistant, were indicated by the improved removal rates as shown in the Figure (4.2). It was observed that highest removal rate was achieved in isolates *S3*, followed by *S4* and *S1*, when compared to *X1, S2* and *S5* etc. However, high removal efficiency of these isolates were attributed to its acclimatization to Cr(VI) and As(III) toxicity, suggesting that these microbes are resistant to Cr(VI) and As(III) toxicity. Examples of these comparative anaerobes are shown in Table 4.1, with *Staphylococcus sp* (*S1, X1,* and *S5*), *Enterobacter sp*, (*S3*), *Bacillus sp* (*S4*), as predominant species. Subsequently, individual isolates were mixed in order to make a consortium or mixed culture of Cr(VI) reducing and As(III) resistant anaerobes; after establishing the fact that these isolates can indeed reduce Cr(VI) in the presence of As(III). Further experiments were conducted to investigate the extent of reducing Cr(VI) in a consortium or mixed culture, and compare the result with individual performance of the selected isolates.

4.1.2 Individual culture versus reconstitute consortium under anaerobic condition

Figure 4.3 compares reconstituted consortium or mixed culture and selected individual pure isolates at Cr(VI) and As(III) concentration of 50 mg/L and 40 mg/L. Individual pure isolates *S3*, S4 and *S1* were selected because of their high individual Cr(VI) reduction efficiency achieved in the presence of As(III). Result showed near complete Cr(VI) reduction achieved by reconstituted consortium after 20 h of incubation, compare to other individual isolates which achieved near complete Cr(VI) reduction after 75 h of incubation. However, individual isolate did not achieve the same level of Cr(VI) reduction rate as the reconstitute culture. A similar trend was observed when Cr(VI) concentration was increased to 70 mg/L Figure (4.3). Reconstituted consortium or mixed achieved near complete Cr(VI) reduction after 45 h of incubation, whereas an incomplete reduction were observed in other individual pure isolates. These suggest that micro-organism existing as a community possess a significant stability and metabolic capabilities. These findings are in agreement with previous observations; where reconstitute culture outperform individual pure culture (Molokwane *et al*., 2008). Synergism could be the reason why individual cultures could not perform when compare to mixed culture consortium (Molokwane *et al*., 2008). A control system was also evaluated to determine the extent of abiotic Cr(VI) reduction. Abiotic result showed that the removal of Cr(VI) in the absence of biomass was negligible within the tested time interval Figure (4.5 and 4.6).

4.2 Batch Experiment

4.2.1 Cr(VI) reduction at different concentrations in the presence As(III)

Cr(VI) reduction experiment in a batch experiment, under varying Cr(VI) concentration ranging from 50-500 mg/L using harvested and concentrated cells were investigated. The harvested cells used in this experiment were grown anaerobically, in the presence of As(III), and initially tested for Cr(VI) reduction as described previously in this study. Experiment was conducted to investigate the performance of reconstituted consortium culture for Cr(VI) reduction in the presence of As(III) under different Cr(VI) concentrations. However, the typical Cr(VI) tolerance concentrations in which cell biological activity ceased was also investigated. All Cr(VI) removal experiments were performed at room 30±0.2 ^{o}C and neutral pH under anaerobic condition.

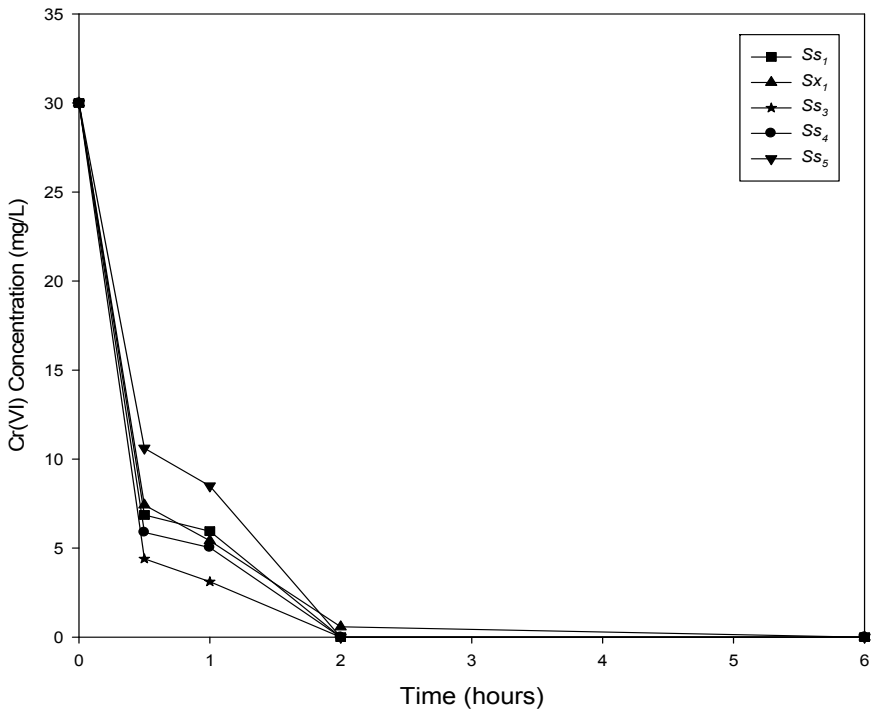


Figure 4.1: Cr(VI) reduction of sludge samples in the presence of 20 mg/L As(III)

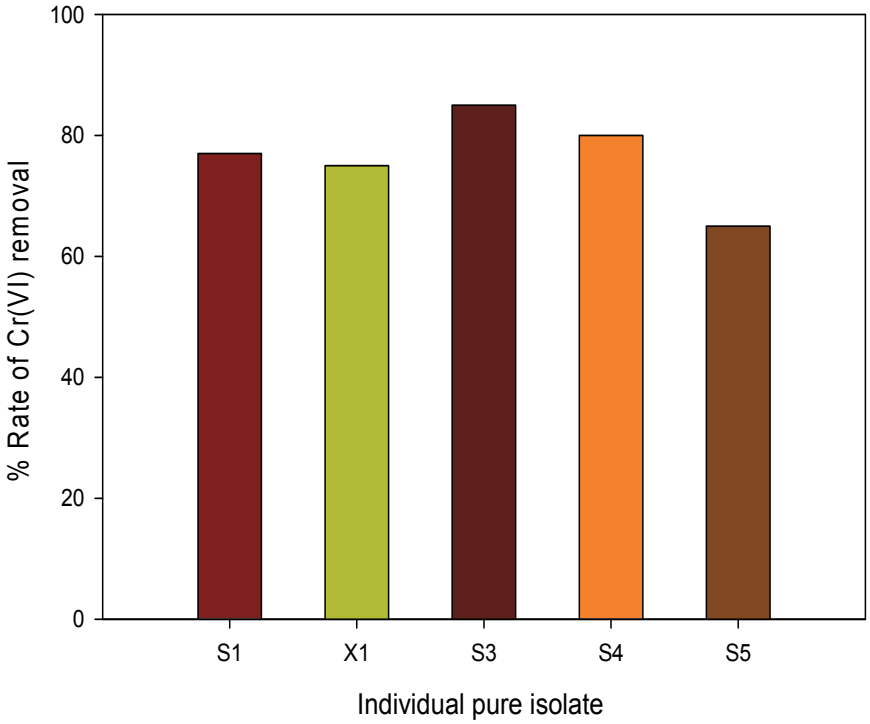


Figure 4.2: Cr(VI)reduction efficiency of individual isolates sourced from dried sludge

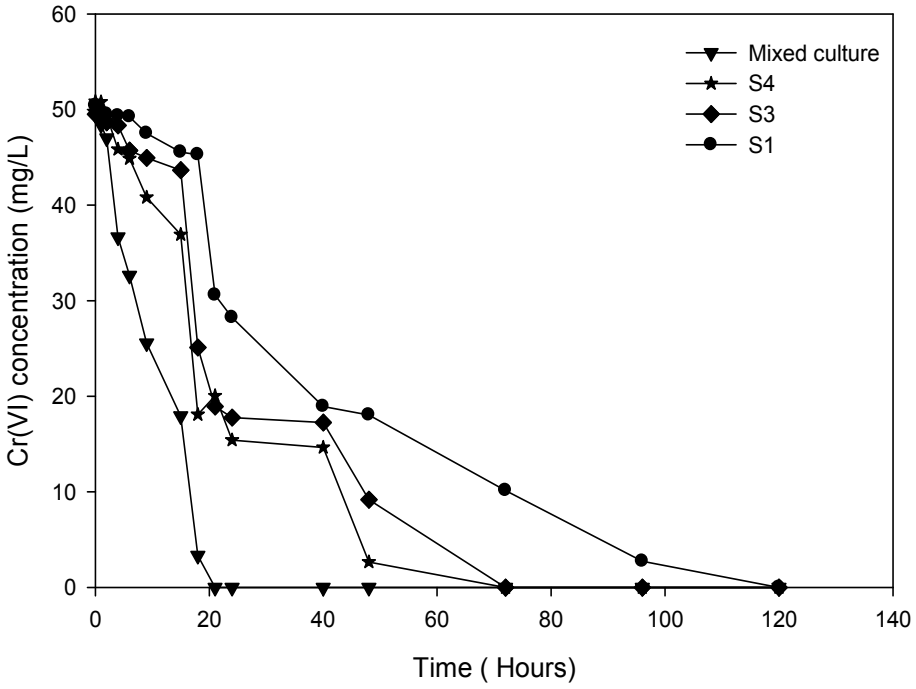


Figure 4.3: reconstituted consortium (mixed) culture (*X1, S1, S3, S4, and S5)* versus selected individual pure isolates at 50 mg/L

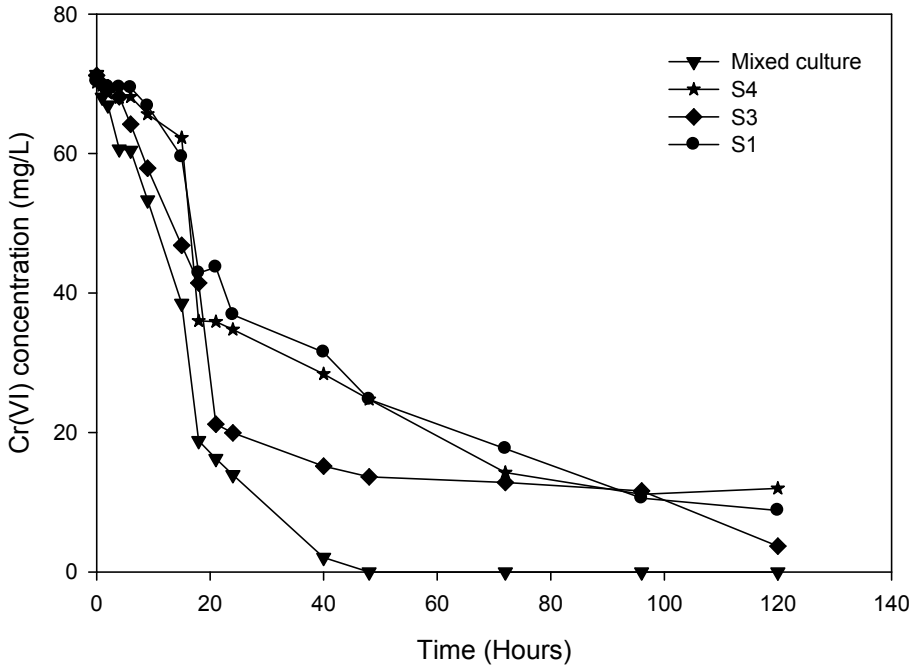


Figure 4.4: reconstituted consortium (mixed) culture (*X1, S1, S3, S4, and S5)* versus selected individual pure isolates at 70 mg/L

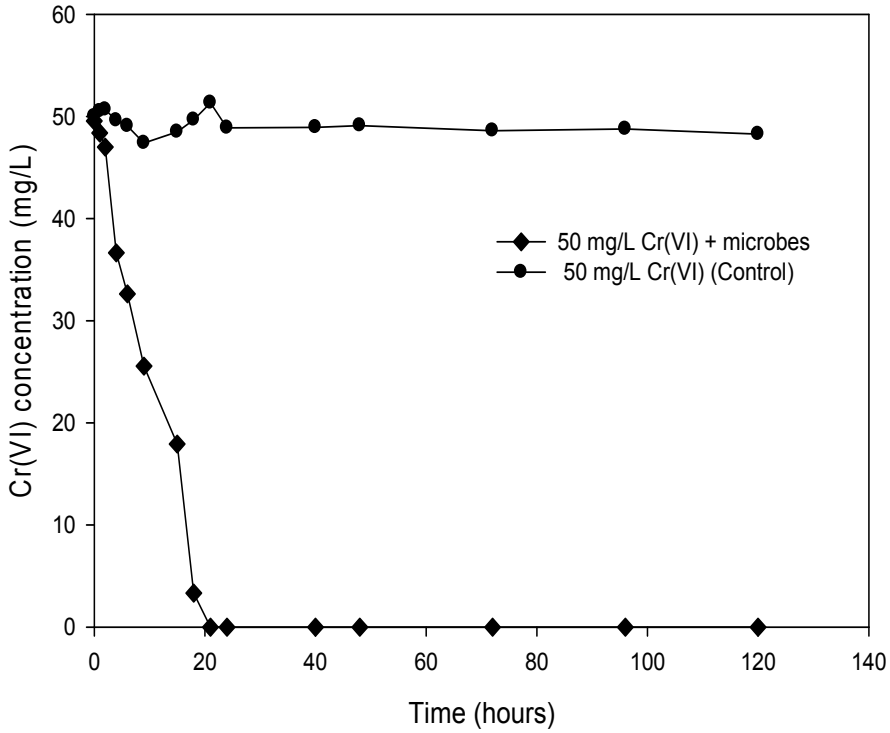


Figure 4.5: Abiotic Cr(VI) reduction at 50 mg/L

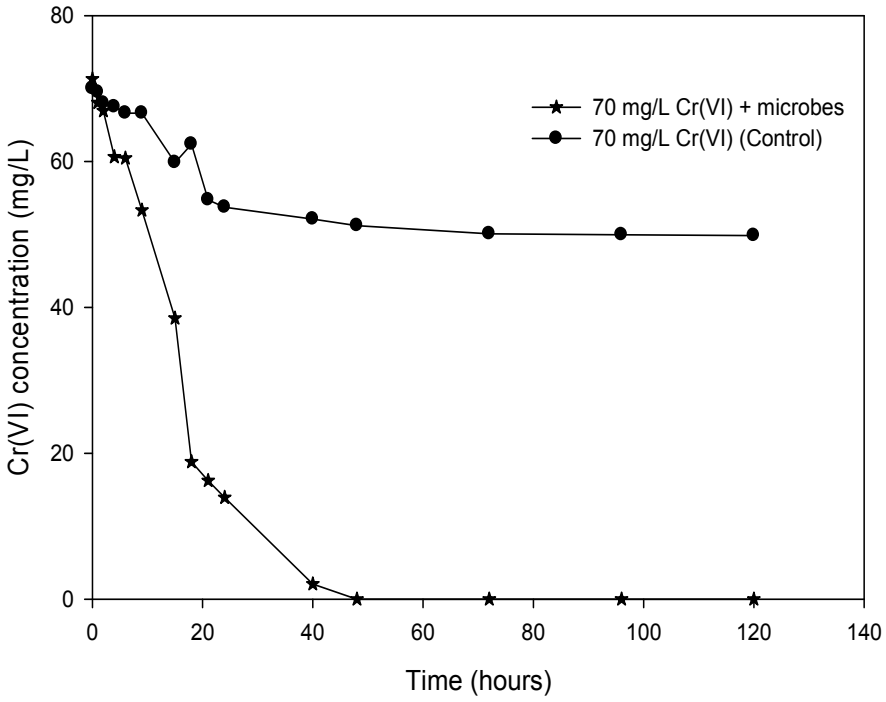


Figure 4.6: Abiotic Cr(VI) reduction at 70 mg/L

Result showed that reconstitute consortium or mixed culture achieved near complete Cr(VI) reduction at initial lower concentration 50-70 mg/L within 48 h of incubation in the presence of As(III). However, increasing Cr(VI) concentration up to 100-500 mg/L showed an incomplete Cr(VI) reduction Figure (4.7). Above 350 mg/L Cr(VI) concentration, low or negligible reduction rate was observed. In addition, Cr(VI) reduction efficiency was evaluated in different batch experiments (C1, C2, C3, C4, C5, and C6). However each batch experiment represent different Cr(VI) concentrations ranging from 50-500 mg/L. Result showed that Cr(VI) reduction efficiency decreases as Cr(VI) concentration increases in the presence of As(III). For example, Cr(VI) reduction efficiency of 90, 80, 50, 33 and 3 % was observed at 50, 70, 100, 200, and 350 mg/L Cr(VI) (Figure 4.8). A negligible or no reduction was observed at 500 mg/L Figure (4.8). These suggests that the cells could resist high Cr(VI) concentration above 200 mg/L, however, the typical Cr(VI) tolerance for this cell occurs at 350 mg/L in presence of As(III). The resistance level of these cells towards Cr(VI) in the presence of As(III) is high when compare other previous studies (Molokwane *et al*, 2000). Additionally, the observed inhibition effect was as a result of high Cr(VI) and As(III) concentration, which correlate to Cr(VI) and As(III) toxicity to microbes. This observation is in agreement with previous studies of Cr(VI) reduction in a batch assay were the optimum Cr(VI) tolerance was found at 500 mg/L (Molokwane *et al*, 2000).

4.2.2 Cr(VI) reduction at different As(III) concentrations

Subsequently, the effect of different As(III) concentration on Cr(VI) reduction was investigated. Cr(VI) concentration of 70 mg/L was investigated in a batch experiment at different As(III) concentration ranging from 5-70 mg/L. Previously it was observed that the reconstituted consortium or mixed culture used in the experiment achieved a near complete Cr(VI) reduction at initial Cr(VI) reduction of 70 mg/L in the presence of 20 mg/L. Despite the toxicity of Cr(VI) and As(III), a successful reduction was achieved at higher concentrations under anaerobic condition. Result showed that a near complete Cr(VI) reduction was achieved after 48 h of incubation, when the batch experiment was amended with As(III) concentration ranging from 5-40 mg/L. Consequently, increasing As(III) concentration up to 50 mg/L, an incomplete Cr(VI) reduction was observed (Figure 4.9). The incomplete Cr(VI) reduction observed was as a result of toxic effect of Cr(VI) and As(III) on microbial cell.

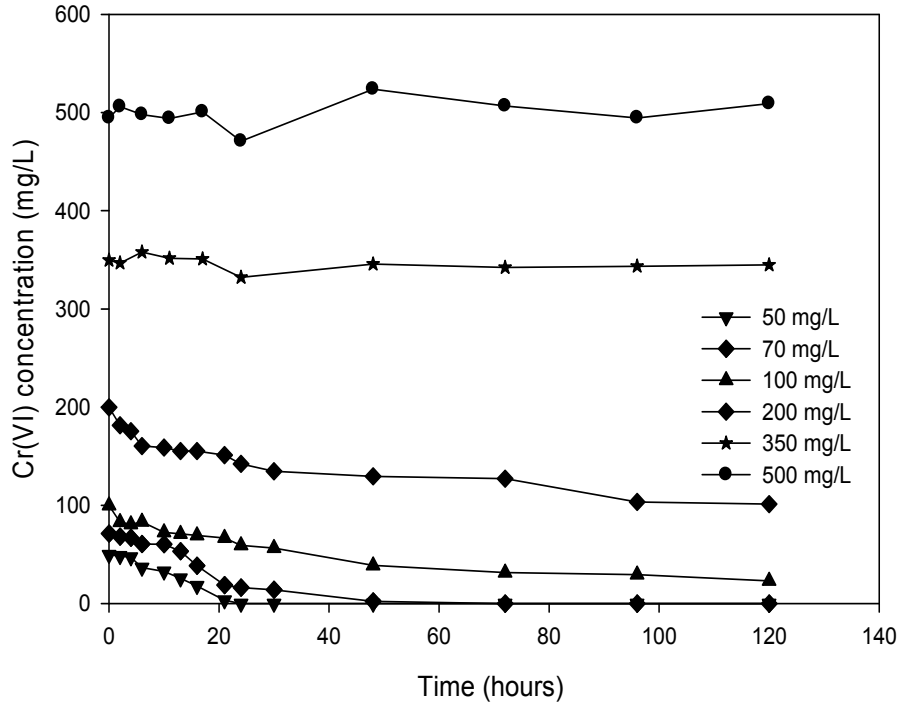


Figure 4.7: Performance evaluation of reconstitute consortium culture at different Cr(VI) concentration ranging from (50-500) mg/L

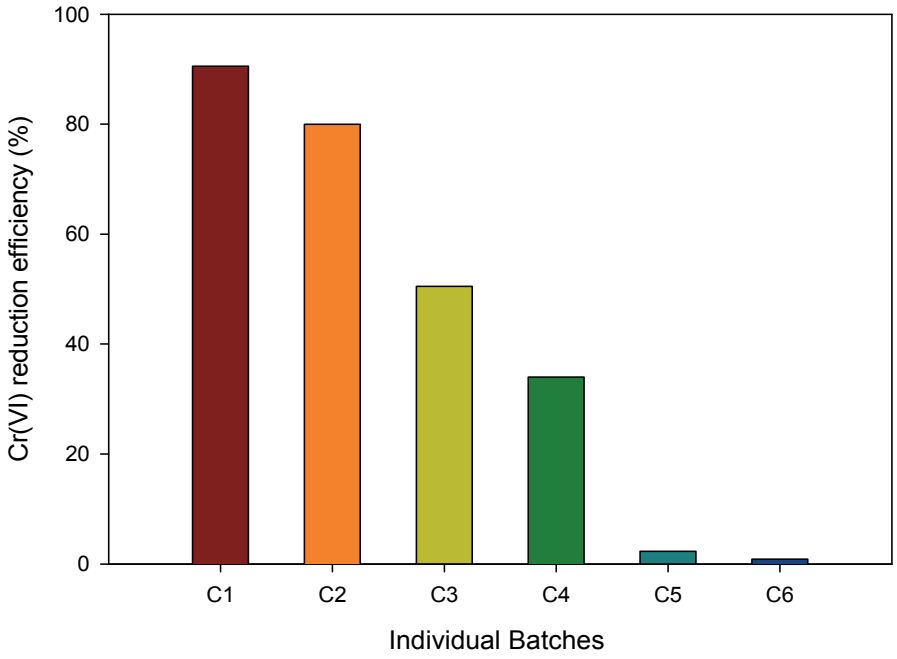


Figure 4.8: Cumulative Cr(VI) reduction efficiency at different concentration ranging from (50-500) mg/L

In addition, Cr(VI) reduction efficiency was evaluated after 10, 24 and 48 h of incubation. Result showed that the Cr(VI) reduction efficiency increases as As(III) concentration increases from 5-40 mg/L. At much higher As(III) concentration 50-70 mg/L, a decrease in Cr(VI) reduction efficiency was observed Figure 4.10. For instance, after 10 h of incubation, Cr(VI) reduction efficiency was about 72, 75, 80 , 88, 89, and 91 % at 5, 10,15, 25, 30, and 40 mg/L As(III). At high As(III) concentrations of 50, 60, and 70 mg/L, the observed Cr(VI) reduction efficiency was 67, 58, and 56 % respectively. A similar trend was observed after 24 and 48 h of incubation. For example, after 24 h of incubation, Cr(VI) reduction efficiency was about, 86, 88, 97, 98, and 99 % at 5, 10, 15, 25, 30, and 40 mg/L As(III), whereas at 50, 60, and 70 mg/L As(III) concentration, 81, 67, and 64 % Cr(VI) reduction efficiency was observed. These suggests that the efficiency of Cr(VI) reduction increases as the time of incubation increases; however, this could be as a result of cell resistivity and growth.

Again, overall cumulative Cr(VI) reduction efficiency depicted similar trend of Cr(VI) in different batch experiments (B1, B2, B3, B4, B5, B6, B7, B8 and B9) investigated. These batches represent different As(III) concentration ranging from 5-70 mg/L. Result showed that high reduction efficiency was achieved as As(III) concentration increases from 5-40 mg/L, and decreases above 50 mg/L Figure (4.11). For instance, Cr(VI) reduction efficiency of about 68, 78, 81, 84, 89, and 91 % was observed at lower As(III) concentrations; 5, 10, 15, 25, 30, and 40 mg/L respectively, whereas 71, 61, and 58 % was observed at high As(III) concentrations of 50, 60 and 70 mg/L respectively. These suggests the microbial growth was enhanced as As(III) concentration increase from 5 to 40 mg/L, and the inhibitory effect observed at higher As(III) concentration above 40 mg /L was a result of toxicity effect of Cr(VI) and As(III) to microbial cells. Therefore, it could be established that As(III) concentration ranging from 5-40 mg/L facilitates Cr(VI) reduction, instead of acting as an inhibitor. However, the optimum tolerance for these cells could be found at 70 mg/L and 40 mg/L of Cr(VI) and As(III). These observations are in agreement with previous studies where Cr(VI) reduction was simultaneously achieved with As(III) oxidation (Bachate *et al.,* 2013). The present studies achieve high Cr(VI) reduction efficiency at high Cr(VI) and As(III) concentrations, and assumed linking Cr(VI) reduction to As(III) oxidation. Moreover, a control system was also examined in order to determine the extent of a biotic Cr(VI) reduction. Only, about 2 % decrease in Cr(VI) concentration was observed in abiotic control over time which may be attributed to the reaction between Cr(VI) and As(III) (Figure 4.9).

This suggests that abiotic Cr(VI) removal in the absence of biomass was negligible within the tested time interval.

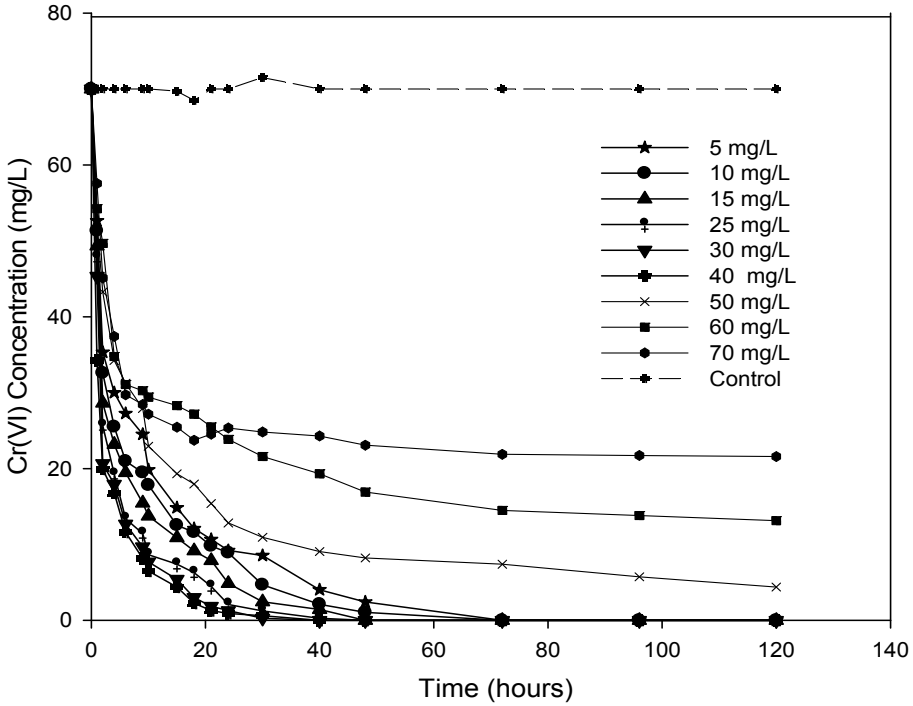


Figure 4.9: Cr(VI) reduction at different As(III) concentrations at 70 mg/L Cr(VI) under anaerobic condition

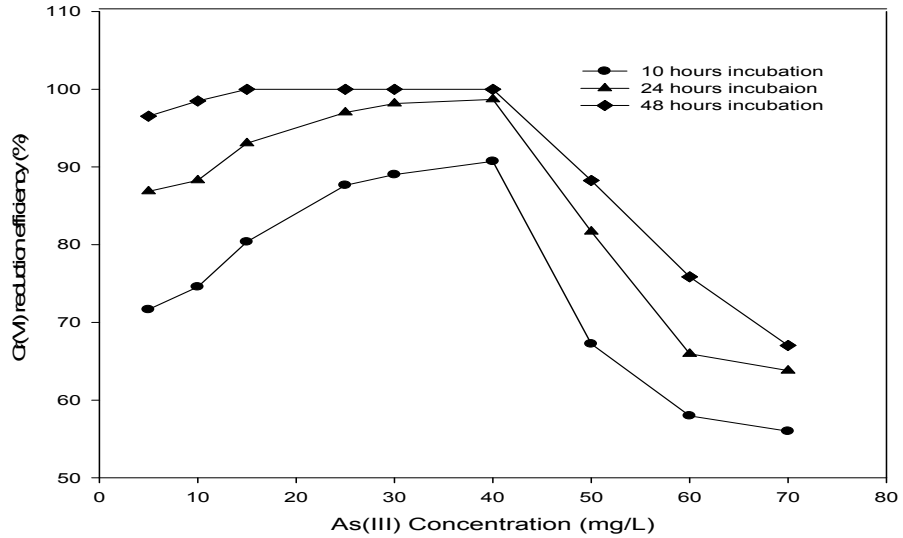


Figure 4.10: Cr(VI) reduction efficiency in the presence of As(III) at different incubation time

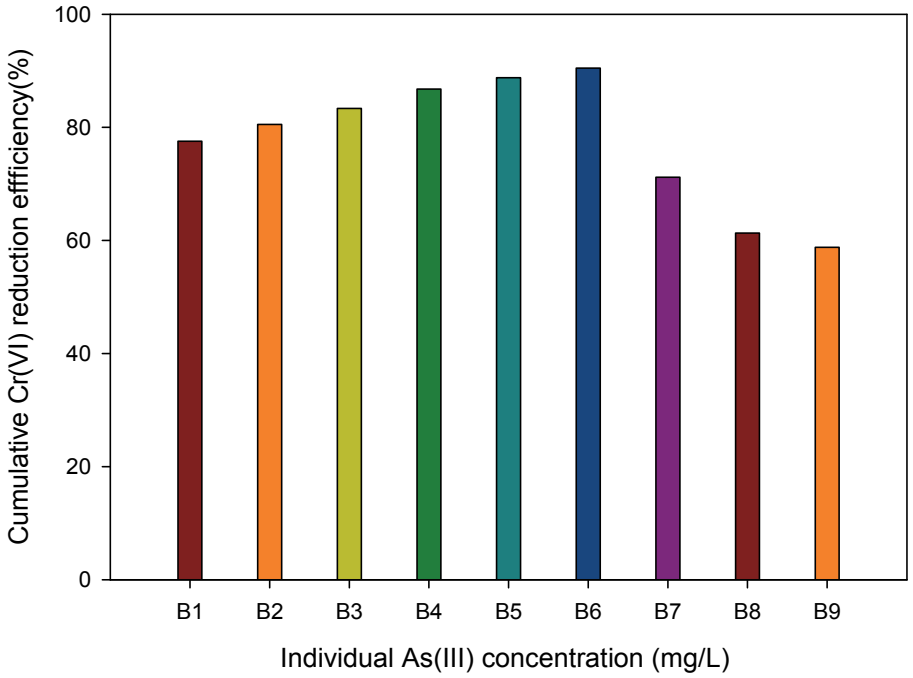


Figure 4.11: Cumulative Cr(VI) reduction efficiency in the presence of As(III) concentration ranging from (5-70) mg/L

In the course of the experimental, the tendency of As(III) to serve as an electron source for Cr(VI) reduction was evaluated in a mineral medium amended with 70 mg/L , 5-40 mg/L and 5 g of Cr(VI), As(III) and glucose respectively. This experiment was conducted to investigate if As(III), an inorganic compound could indeed act as an electron donor for Cr(VI) reduction. In the experiment, it was observed that Cr(VI) reduction began at about 2 h of incubation, and was completed within 48 h incubation in As(III) and Cr(VI), glucose and Cr(VI) batches, and required about 96 h to complete in Cr(VI) alone supplemented with bicarbonate. However, glucose was chosen as alternative electron source in the experiment because it has been established as an electron source for Cr(VI) reduction (Molokwane *et al*., 2008). Cr(VI) reduction efficiency in the presence of As(III), glucose and absence of As(III) in the mineral medium was compared after 6 h of incubation.

Result showed that Cr(VI) reduction efficiency increased as As(III) concentration increases, and subsequently attained the same reduction efficiency as when it is amended with glucose. At 5 mg/L As(III), Cr(VI) reduction efficiency was observed at 61 % compare to glucose

which is 98 % Figure 4.12. Subsequent increases in Cr(VI) reduction efficiency was observed when the system was amended with 10 mg/L, 25 mg/L, 30 mg/L and 40 mg/L As(III) concentration Figure 4.13-4.16. For example, Cr(VI) reduction efficiency after 6 h of incubation was about 70, 72, 80, 84 % at 10 mg/L, 15 mg/L, 25 mg/L, and 40 mg/L As(III) respectively. These suggests that Cr(VI) reduction was mediated by electron transfer from oxidation of As(III) to As(V), catalysed by membrane bound arsenite oxidase enzyme (Bachate, 2013). In the present study, As(III) concentrations were not quantified, and therefore, more studies is required to evaluate As(III) oxidation. Additionally, these results demonstrates the possibility of reducing Cr(VI) using As(III) as an alternative electron source. Control experiment showed about 35 % Cr(VI) reduction efficiency. The decrease in Cr(VI) concentration can be ascribed to the presence of bicarbonate which was supplemented in the mineral medium (Wang 2010).

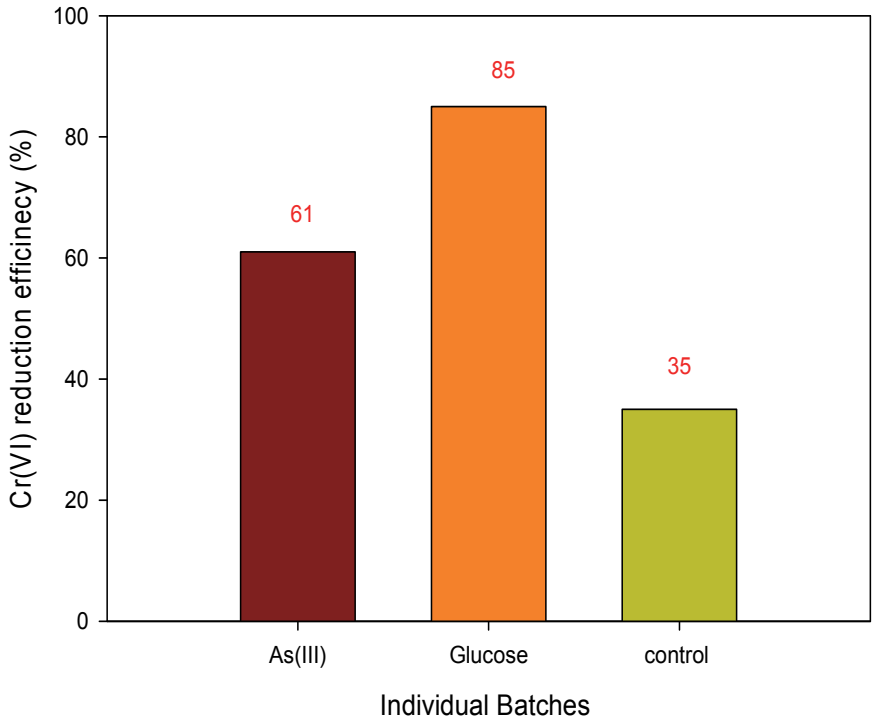


Figure 4.12: Cr(VI) reduction efficiency in the presence of 5 mg/L As(III) and 5 g of glucose

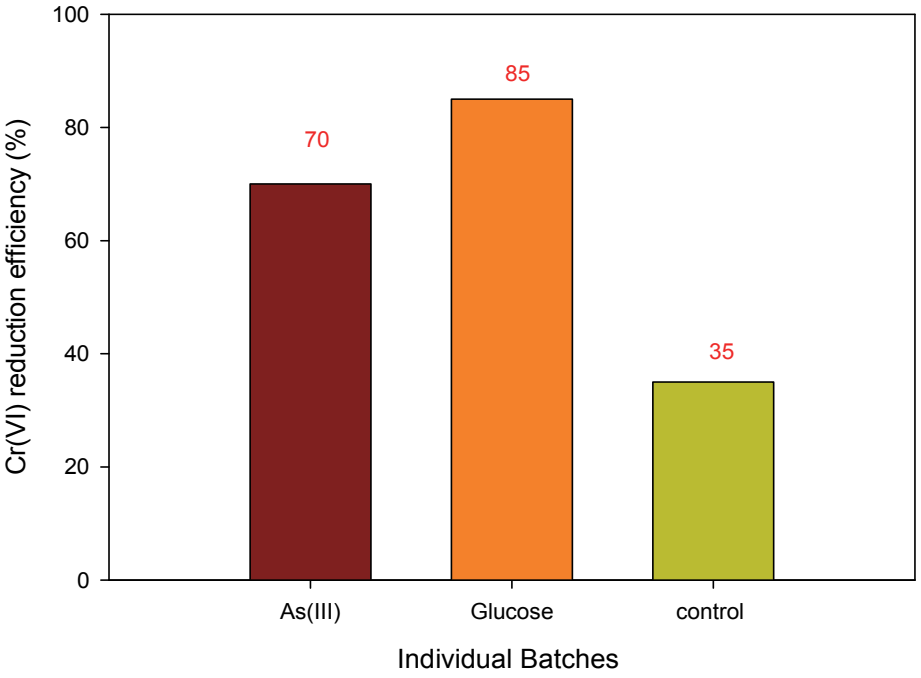


Figure 4.13: Cr(VI) reduction efficiency in the presence of 10 mg/L As(III) and 5 g of glucose

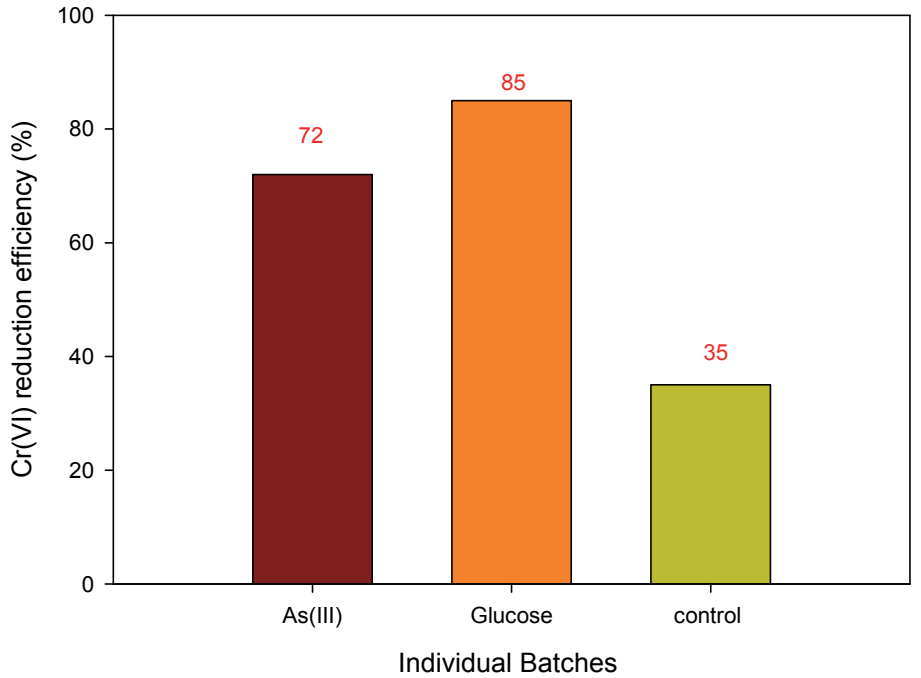


Figure 4.14: Cr(VI) reduction efficiency in the presence of 15 mg/L As(III) and 5 g of glucose

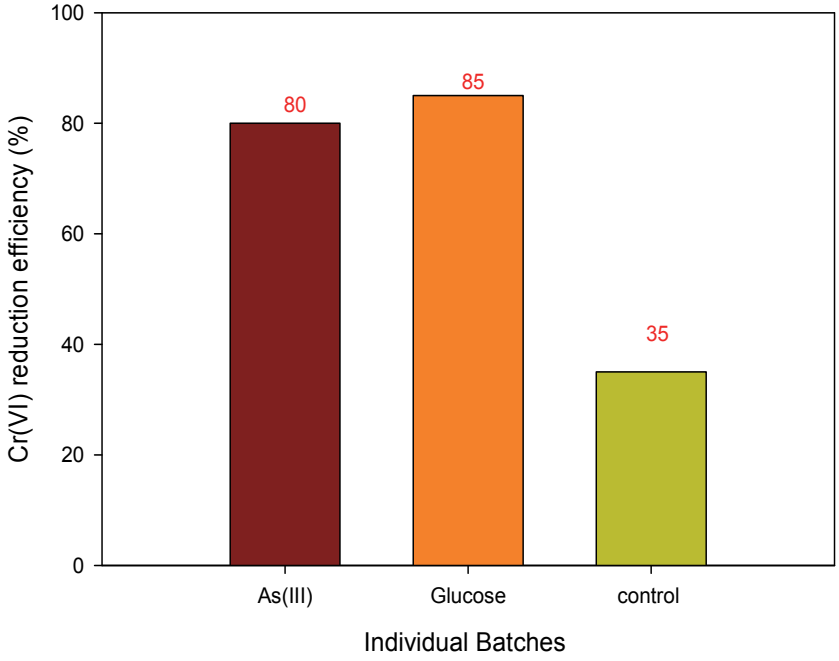


Figure 4.15: Cr(VI) reduction efficiency in the presence of 25 mg/L As(III) and 5 g of glucose

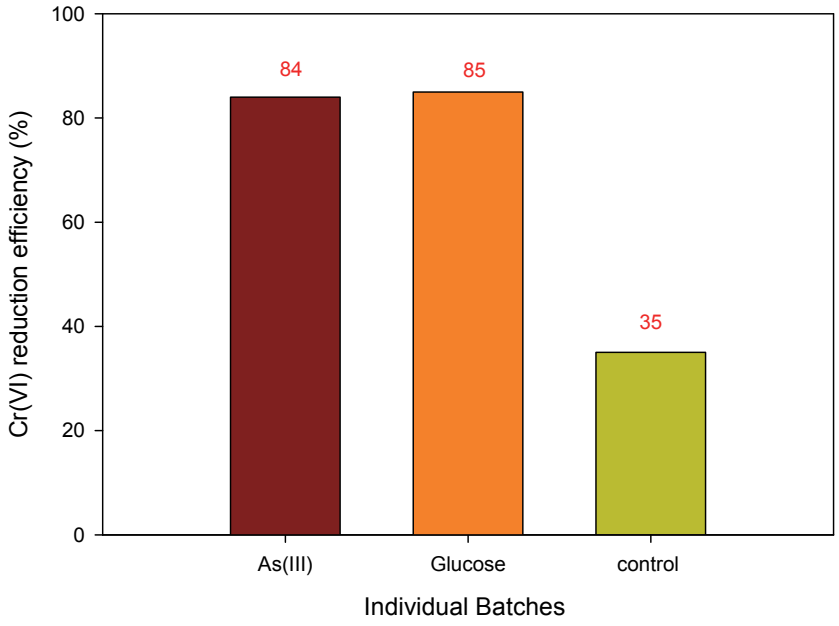


Figure 4.16: Cr(VI) reduction efficiency in the presence of 40 mg/L As(III) and 5 g of glucose

4.3 16S rRNA partial sequence analysis

Phylogenetic characterization of cell was performed on individual colonies of bacteria isolated from Wastewater treatment Works Brits South Africa. The strains were identified by 16S rDNA sequencing, and it showed about 99.9 % sequence identity with *Bacilli, Entrobacteria*, and *staphylococcus species* (Table 4.1). The resulting sequences were matched to known bacteria in the GenBank using a basic BLAST search of the National Centre for Biotechnology Information (NCBI, Bethesda, MD).

Results show 11 possible Cr(VI) reducing anaerobes present in the enriched culture. The phylogenetic analysis of 16S rDNA of the strains showed it was closely related to *Bacilli thuringiensis* and *cereus, Entrobacteria amniqenus and staphylococcus saprophyticus* and others (Figure 4.17). However, the consortium composition obtained in this study differs from the composition initially reported. Only *Bacilli* and *Entrobacteria species* present in this investigation are CRB initially reported by Molokwane et al. (2008). The difference in microbial composition is attributed to the present of As(III) in the experiment, suggesting that these microbes are chromium reducing and arsenic resistant bacteria.

Table 4.1: Bacterial consortium analysis results indicating best matches

Sample name	**Blast results**	**% Similarity**
S1	*Staphylococcus sp.*	100%
	Staphylococcus haemolyticus	100%
	Uncultures bacterium	100%
S3	*Enterobacter sp.*	99%
	Enterobacter amniqenus	99%
S4	*Bacillus cereus*	100%
	Bacillus thuringiensis	100%
S5	*Staphylococcus sp.*	100%
	Staphylococcus saprophyticus	100%
X1	*Staphylococcus sp.*	99%
	Staphylococcus epidermidis	99%

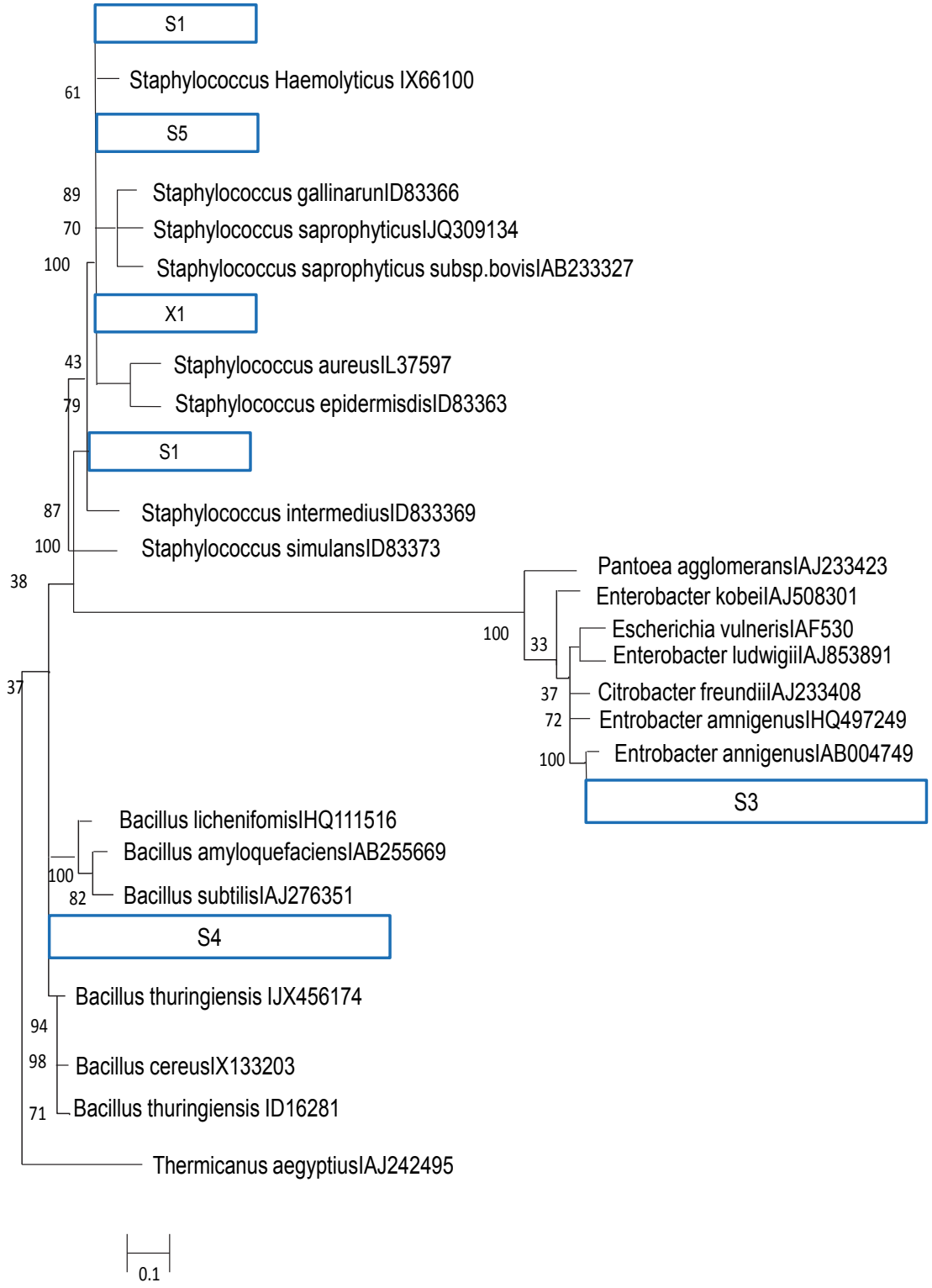
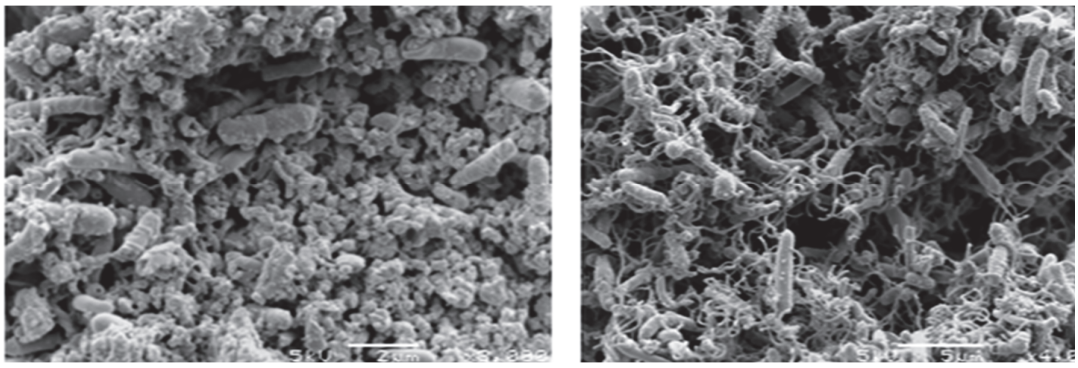


Figure 4.17: Phylogenetic tree of persistent bacterial cells in inoculated batch reactor after operation derived from the 16S rRNA gene sequence. Possible Cr(VI) reducing species in the presence of As(III) were detectable including, *Enterobacter species*, and *Bacillus species.*

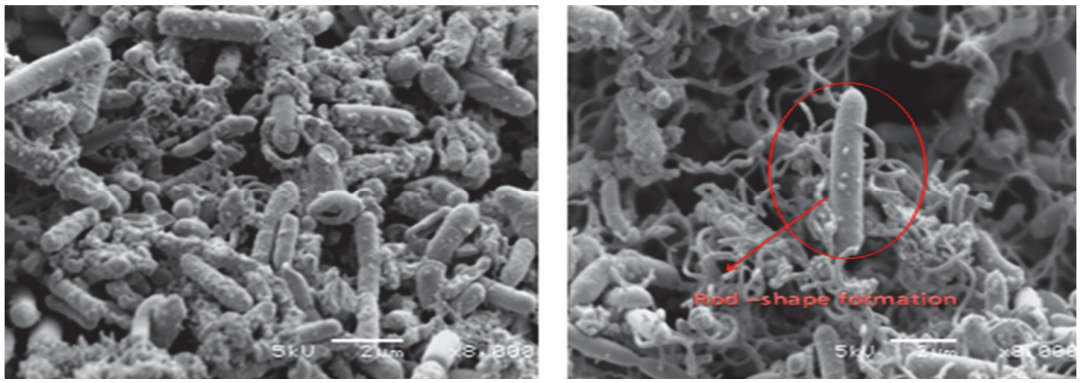
4.4 Continuous flow reactor experiment

4.4.1 Biomass characteristics

Biofilm growth on the glass beads in the continuous flow reactor was examined under electron scan microscope. The purpose of this investigation was to establish the existence of biofilm on the glass beads. The glass beads used in this study were grinded to create a rough surface area for microbial attachment. Samples of glass beads were collected from four different locations. Morphological observation showed a dense population of microbial growth or biofilm growth on the glass beads (Figure 4.18). However, the observed evidence of biofilm growth on the glass beads suggest that Cr(VI) feed content in the presence of As(III) was indeed reduced by biofilm attached on the glass beads.



Location: 1 and 2



Location: 3 and 4

Fig 4.18 : SEM photographs of a crevice at different magnifications showing biofilm attachment on the beads collected at four different locations.

4.4.2 Cr(VI) reduction in the presence of As(III) - Reactor Performance

Continuous biofilm reactor was investigated for Cr(VI) removal under anaerobic condition in the presence of As(III). The reactor was inoculated with a mixed culture of Cr(VI) reducing anaerobes prior to experimental start up, operated continuously for a period of 120 days over a range of influent Cr(VI) concentration of 20-100 mg/L, at hydraulic retention time of 14.4 h, volumetric flow rate (0.0131 m^3/s) and temperature (30±2°C). The essence of this investigation was to evaluate the inhibitory effect of As(III) on Cr(VI) reduction, in a continuous biofilm reactor. Successful Cr(VI) reduction was achieved over 120 successive days of continuous operation. The steady state results of continuously operated reactor was summarized in (Table 4.2), and the data in (Figure 4.19) showed the influent and effluent Cr(VI) concentration of the reactor throughout the operational stages. The different stages (I, II, III, IV, IV, V, VI, VII, VIII) marked on (Figure 4.19) correspond to changes in inlet Cr(VI) concentration. The stages of experimental run are also listed in (Table 4.2). However, the reactor steady states were assumed when Cr(VI) outlet concentrations remained constant for at least three hydraulic retention times.

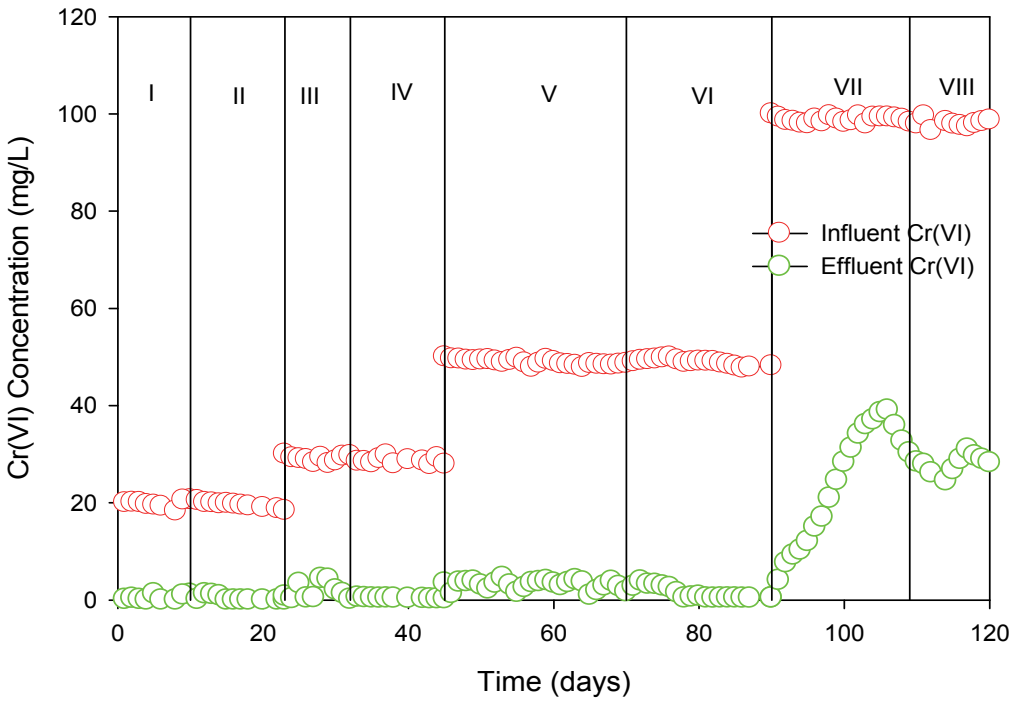


Figure 4.19: Cr(VI) reduction in a continuous flow (biofilm) reactor in the presence of 40 mg/L As(III) at different Cr(VI) concentration of (20-100) mg/L

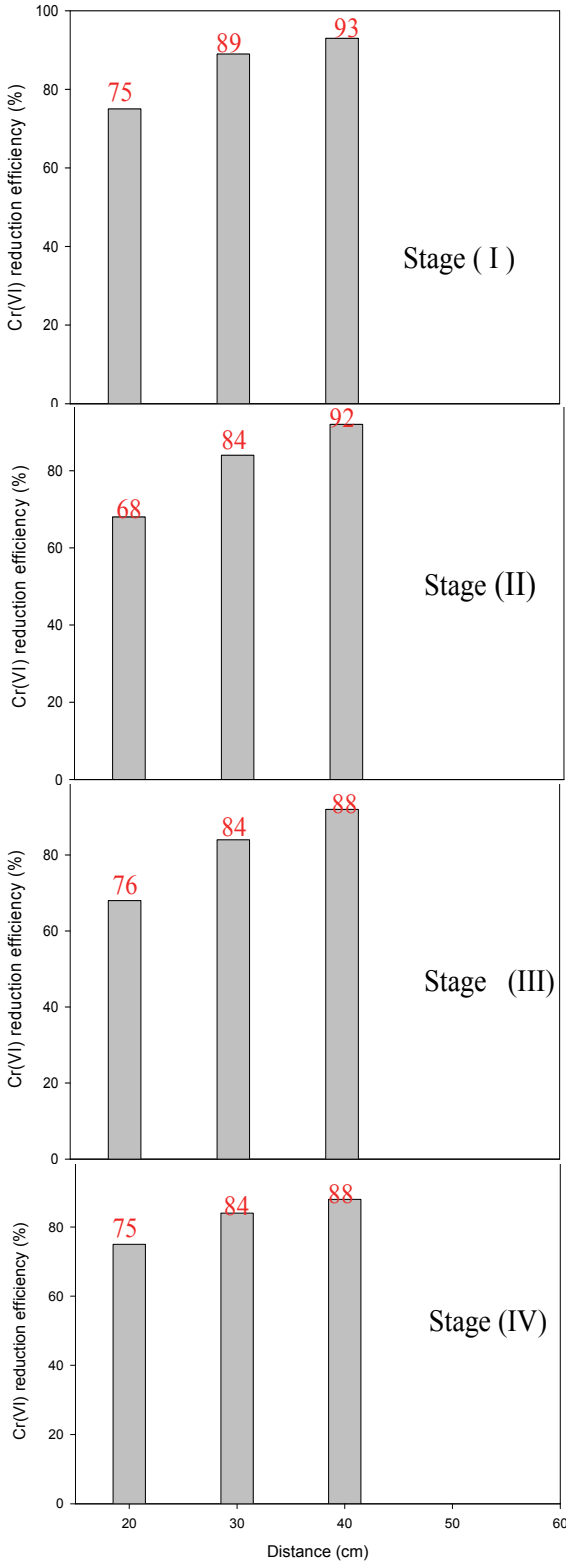


Figure 4.20a: Cr(VI) reduction efficiency across the longitudinal column at different stages of operation

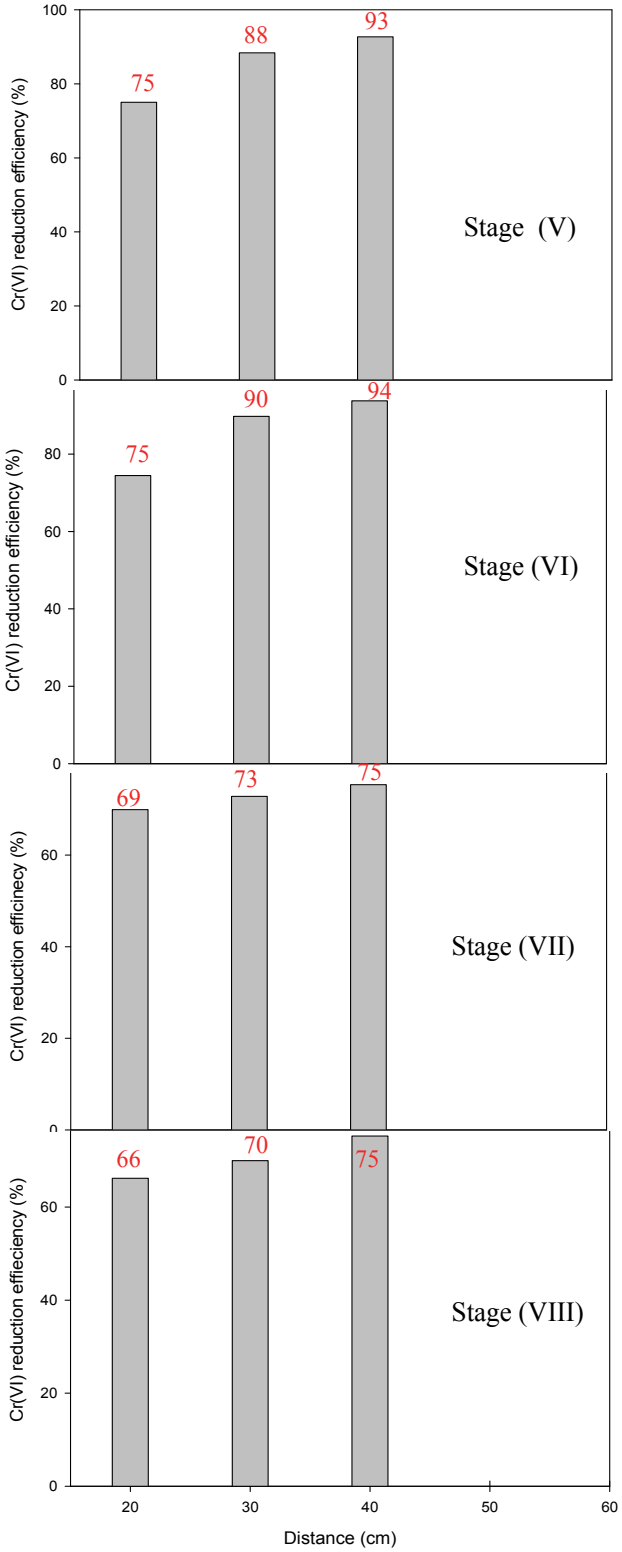


Figure 4.20b: Cr(VI) reduction efficiency across the longitudinal column at different stages of operation

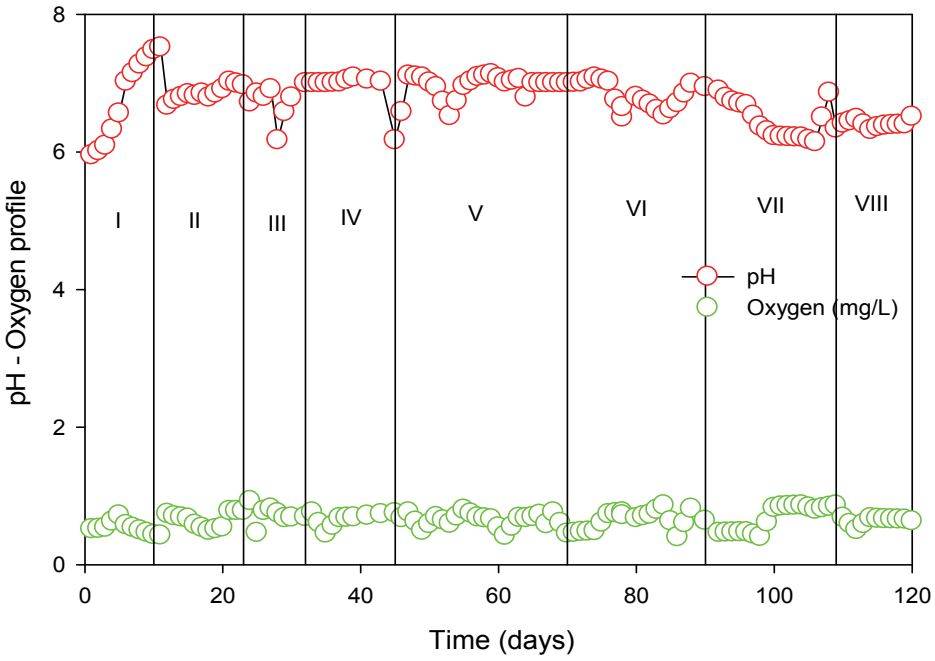


Figure 4.21: DO and pH profile of the continuous flow (biofilm) reactor throughout operation

Result showed a successful Cr(VI) reduction at initial lower concentration ranging from 20-50 mg/L, at Cr(VI) reduction rate ranging from 0.83-2 mg/L (Figure 4.19). About 95-97 % Cr(VI) removal efficiency was achieved in the first six stages of continuous operation Table (4.2). Increasing the reactor loading up to 100 mg/L at hydraulic retention time of 14.4 h, about 20 % decrease in removal efficiency was observed, given a removal efficiency of less than 70 % (< 70 %). A system robust was achieved when the influent Cr(VI) concentration was increased up to 50 mg/L, with high reduction efficiency approximately 97 % achieved after 60 days of continuous operation. This suggests that microbial growth or biomass increases as Cr(VI) concentration increases. Comparing Cr(VI) reduction efficiency at the first six stages of operation with seven and eight stages after 110 d of continuous operation, it could be establish that the efficiency of Cr(VI) reduction was inhibited, with 20 % drop in Cr(VI) reduction efficiency. This suggests that the reduction capacity of the cell is inhibited at this stage of operation. However, the observed inhibitory effect is attributed to the toxicity effect of Cr(VI) and As(III) to microbial cell.

Table 4.2: Steady State Performance of Continuous Flow (biofilm) reactor

Experimental Run/ stages	Duration day	HRT hours	Influent Cr(VI) mg/L	Effluent Cr(VI) mg/L	Cr(VI) Loading rate® mgl-1d-1	Cr(VI)Redn. rate® $mgl^{-1}d^{-1}$	Cr(VI) removal (%)	Total Cr conc. (mg/L)	pH	Reactor DO mg/L	O_2 uptake mg/cell mg
I¥1stage1	1 – 8	14.40	20.067	0.26	0.84	0.83	96.9	20.190	6.2	0.57	0.045
I¥1stage 2	10 – 21	14.40	21.220	0.36	0.88	0.87	97.1	20.068	7.3	0.58	0.045
II¥1stage 3	23 – 29	14.40	30.245	1.38	1.26	1.20	95.9	29.598	6.7	0.78	0.044
II¥1stage 4	32 – 43	14.40	30.304	0.87	1.26	1.22	96.7	30.259	7.0	0.61	0.044
III¥1stage 5	45 – 68	14.40	49.586	3.00	2.07	1.94	93.5	50.146	6.9	0.69	0.044
III¥1stage 6	70 – 88	14.40	50.336	1.64	2.10	2.03	96.7	52.358	6.8	0.70	0.044
IV¥1stage 7	90– 108	14.40	101.215	21.7	4.22	3.31	78.5	100.635	6.5	0.69	0.047
IV¥1stage 8	109- 120	14.40	100.025	22.44	4.17	3.23	70.8	101.081	6.3	0.62	0.044

Furthermore, comparing Cr(VI) reduction efficiency in the presence of As(III), in the continuous reactor with reduction efficiency previously observed in the batch experiment. About 60-75 % Cr(VI) reduction efficiency was achieved in the continuous reactor at Cr(VI) concentration of 100 mg/L (Table 4.2), whereas in batch system about 50 % Cr(VI) reduction efficiency was achieved (Figure 4.8). This suggests that the performance of continuous flow bioreactor system is better than that of batch reactors. The outperformance observed in the continuous flow biofilm reactor was attributed to biomass limitation in the continuous biofilm reactor, improving culture flexibility, and allowing high specific biomass retention time, thereby increases high volumetric yield (Nicolella *et al.*, 2000; Stoodley *et al.,* 1999). Moreover, culture adaption and mass transport resistance across the biofilm layer or cell exposure to toxicity also improved Cr(VI) reduction efficiency in a continuous biofilm reactor (Wang and Chirwa, 2001). These results are in agreement with previous research where high Cr(VI) removal rates were observed in a biofilm reactor than in a suspended reactor systems (Mtimuye, 2011; Molokwane *et al*., 2009; Chirwa and Wang, 1997).

Cr(VI) removal efficiency across the longitudinal reactor column was also evaluated. The essence of this investigation was to evaluate Cr(VI) profile along the reactor column in the presence of As(III). Mtimunye (2011) has previously conducted studies on Cr(VI) reduction in a column reactor under oxygen stressed conditions. It was reported from this study that the rate of Cr(VI) reduction in a column inoculated with CRB increases with the increasing length of the column. Similar result have been observed in this study as shown in (Figure 4.20 a) and (Figure 4.20 b). These figures demonstrate that at various Cr(VI) concentration ranging from 20-50 mg/L Cr(VI) reduction was increase across the length of the column at different stages of operation (I, II, III, IV, V, VI, VII, and VIII). For instance, Cr(VI) reduction efficiency at stage (I) and (II) along 20, 30 and 40 cm, was ≤ 75 %, ≤ 89 %, ≤ 93 % at the initial Cr(VI) feed concentration of 20mg/L. However, about 5 ± 0.2 % decrease in reduction efficiency was observed along the column at stage (III) and (IV). Increasing Cr(VI) feed concentration to 30 mg/L resulted into slight decline of Cr(VI) removal efficiency across the column as compared to Cr(VI) removal observed at Cr(VI) feed concentration of 20 mg/L. For instance at 20 , 30 , and 40 cm, Cr(VI) reduction efficiency at the operation of 30 mg Cr(VI)/L was ≤ 76 % , ≤ 84 %, and ≤ 88 %. Conversely, system robust was observed along the column at stage (V) and (VI) when Cr(VI) concentration was increase up to 50 mg/L, giving Cr(VI) reduction efficiency of ≤ 75 %, ≤ 90 % , ≤ 94 % at 20, 30 , and 40 cm. Increasing reactor loading up to 100 mg/L, a decrease in Cr(VI)

reduction efficiency was observed. For instance at 20, 30, and 40 cm, the observed Cr(VI) reduction efficiency was ≤ 69 %, ≤ 73 %, and ≤ 75 %. These suggests that the reduction of Cr(VI) across a vertical longitudinal reactor is directly proportional to height of the reactor, and inhibitory effect observed was attributed loss of cell reducing capacity , and toxicity of Cr(VI) and As(III) at high loading. These results are in agreement with those reported by previous studies on the performance of the bioreactor in reducing Cr(VI) along the reactor under shock loading conditions (Mtimuye, 2011).

During the course of the reactor operation, pH and oxygen concentration level of the reactor was monitored. This was done to maintain the required oxygen concentration level, and pH of the reactor for efficient Cr(VI) reduction. Oxygen and pH profile of the reactor at different stages of operation is shown in (Figure 4.21). Throughout the reactor operation, anaerobic condition was maintained at dissolved oxygen concentration (DO) levels ranging from (0.4-0.7) mg/L (Figure 4.22). Discrepancy observed might occur as a result of experimental errors at the point of sampling, and changes in consortium composition under different Cr(VI) loading. In addition, the pH of the reactors was observed at 7±0.2. However, pH of the reactor was maintain at near neutral point because of the presence of phosphate in the mineral medium serving as a buffer solution as well as nutrient for biofilm . This suggests that Cr(VI) was indeed removed at neutral pH facilitated by a mixed culture of facultative anaerobes. This observation is in agreement with previous studies where Cr(VI) was optimally reduced at pH of ≤ 7, under anaerobic condition (Molokwane *et al.,* 2009).

Throughout experimentation run, total chromium was accounted for with 95 % accuracy as measured by AAS and reported in Table 4.2. The result indicates that chromium was not absorbed into the biofilm. Secondly, evaluation of total biomass was performed at steady state condition. Results show that total biomass concentration was the same in all the experimental runs. In addition, viable cell count results showed that the concentration of living cells in reactor was in the range of (10^5-10^{10}) cfu/mL.

CHAPTER FIVE

Cr(VI) REDUCTION KINETIC USING NON-COMPETITIVE INHIBITION MODEL UNDER ANAEROBIC CONDITION

5.1 Cr(VI) Reduction kinetic

Biokinetic data achieved in this experiment were estimated using enzyme based model. The model was initially developed by integrating enzyme kinetics and Cr(VI) reduction capacity to validate toxic effect of Cr(VI). The reduction capacity designates the maximum amount of Cr(VI) that a batch culture can reduce, and the loss of Cr(VI) reduction capacity in the bacterial cultures may be associated with toxic effects of Cr(VI). This model was formerly used to describe Cr(VI) reduction in *E.coli ATCC* 333456, *Bacillus sp*., and consortium culture from Brits WWTP (Mtimuye, 2011; Molokwane *et al*., 2008; Chirwa and Wang, 2004; Wang and Sheng, 1997). In the contemporary study, optimum values of biokinetic parameters were estimated using a computer programme for simulation of the Aquatic System "AQUASIM 2.0".

5.2 Cr(VI) Reduction kinetic in the batch system

5.2.1 Model description

Cr(VI) reduction is facilitated by enzyme (ChrR) in the microbial cell membrane, and these enzymes reduce Cr(VI) while achieving other physiological functions (Viamajala 2003). Non-competitive model was predicted based on Monod equation, where enzyme activity is the driving force for Cr(VI) reduction (Shen and Wang, 1997). However, the activities of theses enzymes, having a net effect can be represented by a complex enzyme (E_T) (Shen and Wang, 1994b). The first principles of Monod equation is based on enzyme kinetic model, mathematically expressed as follows;

$$Cr(VI) + E \underset{k_b}{\overset{k_a}{\rightleftharpoons}} E^*Cr(III) \quad (5.1)$$

$$E^*Cr(III) \xrightarrow{k_e} E + Cr(III) \quad (5.2)$$

where; E = Enzyme, E* Cr(VI) = Enzyme Cr(VI) complex, k_a = rate constant for forward reaction, k_b= rate constant for the reverse reaction, k_e = rate constant for the third reaction.

Therefore, the enzyme rate equation from reaction 5.1 and 5.2 is expressed as follows:

Let 'C' represent Cr(VI) concentration,

$$\frac{dE}{dt} = k_a C E_T \tag{5.3}$$

$$\frac{dE^*}{dt} = k_a C E^* \tag{5.4}$$

$\therefore$

$$\frac{dE^*}{dt} = \frac{dC_r}{dt} = k_e E^* \tag{5.5}$$

Combining Equation 5.3, 5.4 and 5.5, gives the rate of E* formation represented as:

$$\frac{dE^*}{dt} = k_a C(E_T) - k_b(E^*) - k_e(E^*) \tag{5.6}$$

$$E_T = E - E^* \tag{5.7}$$

where; E^* = total complex and uncomplex enzyme

Combining Equation 5.6 and 5.7 to have:

$$\frac{dE^*}{dt} = k_a C(E - E^*) - k_b(E^*) - k_e(E^*) \tag{5.8}$$

At steady state condition, enzyme rate formation $\left(\frac{dE^*}{dt} = 0\right)$ approaches zero, Equation

5.8 become;

$$k_a C(E - E^*) - k_b(E^*) - k_e(E^*) = 0 \qquad (5.9)$$

Solving for E^*:

$$k_a C(E - E^*) = k_b(E^*) + k_e(E^*)$$

$$k_a CE - k_a CE^* = k_b(E^*) + k_e(E^*)$$

$$k_a CE = k_a CE^* + k_b(E^*) + k_e(E^*)$$

$$k_a CE = (k_a C + k_b + k_e)E^*$$

∴ 'E^*' can be simplified as; $E^* = \left(\frac{k_a CE}{k_a C + k_b + Ck_e}\right)$

$$E^* = \left(\frac{CE}{C + \left(\frac{k_b + k_e}{k_a}\right)}\right) \qquad (5.10)$$

From Equation 5.10, the rate of Cr(VI) reduction was predicted, and represented as

$$-\frac{dC}{dt} = \left(\frac{CE}{C + \left(\frac{k_b + k_e}{k_a}\right)}\right) \qquad (5.11)$$

Comparing Equation 5.11and 5.12:

$$-\frac{dC}{dt} = \left(\frac{u_{\_\max} C}{C + K}\right) X \quad \text{(Monod equation)} \qquad (5.12)$$

where; k_d is equivalent to μ_max = maximum specific Cr(VI) reduction rate (mg/L/h); E is equivalent to X = biomass concentration (mg/L): $\left(\frac{k_b + k_e}{k_a}\right)$ is equivalent to K (mg/L).

From Equation 5.12, it could be seen that the rate and the extent of Cr(VI) reduction in a bacterial system is proportional to the number of cells X in the system and the capacity of reduction.

∴, Biomass concentration X can represent as;

$$X = X_o - \left(\frac{C_o - C}{K\_c}\right) \tag{5.13}$$

where; C_o = initial Cr(VI) concentration (mg. /L); X_o = initial biomass concentration (mg/L); C = Cr(VI) concentration at a time‘t’ (mg/L); and K_c = maximum Cr(VI) reducing capacity (mg /mg).

Combining Equation 5.12 and 5.13, generate the predicted model equation for Cr(VI) reduction;

$$-\frac{dC}{dt} = \frac{u\_\max C}{C + K}\left(X_o - \left(\frac{C_o - C}{K\_c}\right)\right) \tag{5. 14}$$

where; μ_max = maximum specific Cr(VI) reduction rate (mg/L/hr); C_o = initial Cr(VI) concentration (mg/L); X_o = initial biomass concentration (mg/L); C = Cr(VI) concentration at a time‘t’ (mg/L); K_c = maximum Cr(VI) reducing capacity (mg/mg); and K = half velocity concentration (mg/L).

Under anaerobic condition, a non-competitive inhibition model which described Cr(VI) toxicity threshold was described in Equation 5.15. This equation was used as a predicted model to analysed Cr(VI) reduction data attained with the mixed culture in the batch system, in the presence of As(III). Simulation will be performed for best fit of the equation versus time curves to estimate the biokinetic parameter values μ_max, k, K, and K_c. In addition,

Kinetic parameters of these values were firstly estimated with initial guessed values, followed by simulation and optimization. Upper and lower constraints were set for each parameter to the exclusion of invalid parameter values. Whenever optimization converges or very close to constraint, the constraint was relaxed until the constraint did not force the model. The procedure was repeated until unique values lying away from the constraint but between set limits were found for each parameter (Chirwa and Wang, 2004).

$$-\frac{dC}{dt} = \frac{u_{\_\max} C}{k^{\left(1-\frac{C_r}{C_o}\right)}(C+K)}\left(X_o - \left(\frac{C_o - C}{K_{\_c}}\right)\right) \tag{5.15}$$

where, $\mu_{\_max}$ = maximum specific Cr(VI) reduction rate (mg/L/h); C_o = initial Cr(VI) concentration (mg/L); X_o = initial biomass concentration (mg/L); C = Cr(VI) concentration at a time 't'(mg/L); $K_{\_c}$ = maximum Cr(VI) reducing capacity (mg/mg); K = half velocity concentration (mg/L); Cr = Cr(VI) toxicity threshold concentration (mg/L); and k = limiting constant (mg/L).

5.2.2 Kinetic parameter estimation in the presence of As(III)

The model Equation (5.15) described Cr(VI) reduction in the presence of As(III) by mixed culture of facultative anaerobes was used to simulate Cr(VI) experimental data with initial guess values. Parameters $u_{\_max}$, K, k, and $K_{\_c}$ listed in Table 5.1 were obtained with curves of initial concentration 100 mg/L. Good fits between model simulation and experimental data were noted for all data set with initial concentration ranging from 50-200 mg/L Figure 5.1. Cr(VI) reduction capacity $K_{\_c}$ was indeed an evident in the batch studies as reduction capacity is directly proportional to initial Cr(VI) concentration. This implies that as $K_{\_c}$ increases, with increase in Cr(VI) concentration. The rate of Cr(VI) reduction by culture increases as concentration of Cr(VI) increases. Similar trend was observed in our model with Cr(VI) reduction in the presences of As(III)·In this model, $K_{\_c}$ increases as concentration significantly increases from 50-200 mg/L. The bio kinetic parameters obtained in our study are different of parameters initially reported with the same model. The differences might occur as a result of the presence of As(III) in our experiment.

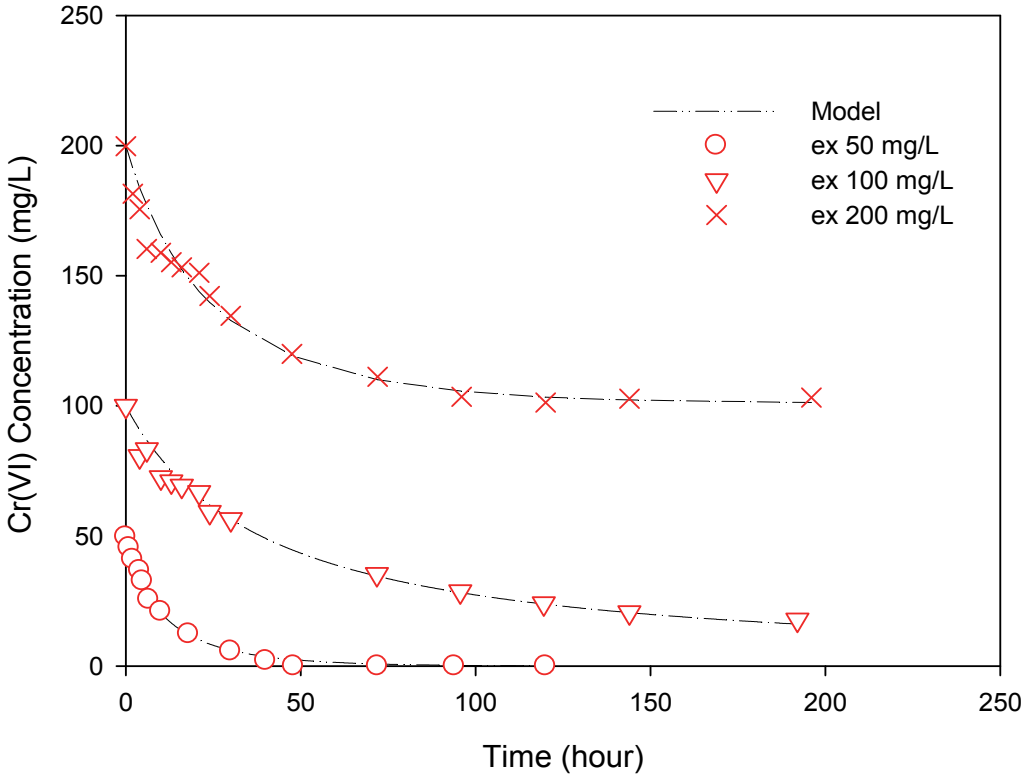


Figure 5.1:Cr(VI) reduction in the batch assay in the presence of As(III) for initial concentration of 50-200 mg/L

Table 5.1: Biokinetic parameter for Cr(VI) reduction in the presence of As(III) in the batch assay

Co(mg/L)	*K_c* (mg/mg)	*K* (mg/L)	*K* (mg/L)	*u_max* (hr^{-1})	X^2
50	0.986	732.301	4.317	0.6441	120.60
100	0.997	732.301	4.317	0.6441	225.62
200	1.012	732.301	4.317	0.6441	342.43

5.2.3 Sensitivity analysis

The sensitivity function of Cr(VI) concentration in the presence of As(III) under anaerobic condition with respect to u_{max}, K, k, K_c *and* Co were evaluated to equate the effect of these parameters on reduction process. It becomes evident from Figure (5.2) that u_{max}, K, k, and K_c *are* highly sensitive and dependant on Cr(VI) concentration (C).

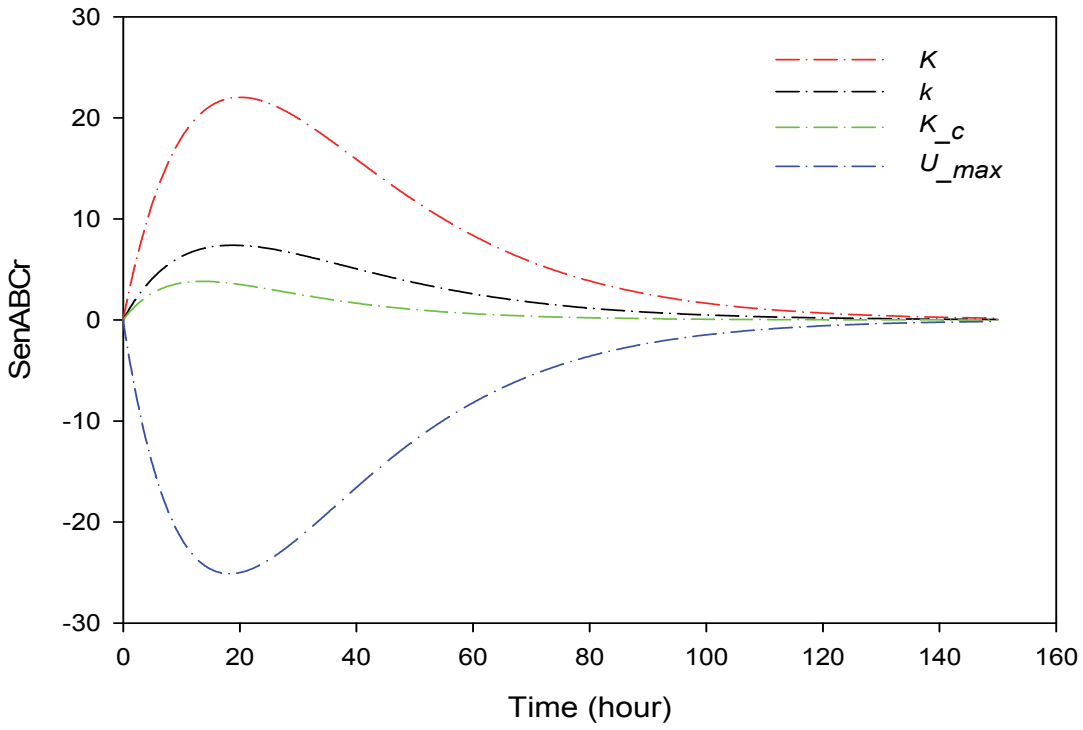


Figure 5.2: Time course sensitivity functions of Cr(VI) with respect to all the parameters in a batch system

From Figure (5.2) above, it was observed that these parameters were highly sensitive in the early hours of incubation, suggesting that cell Cr(VI) reduction activity was high during that period of incubation. However, the dependence of Cr(VI) concentration (C) on these parameters u_{max}, K, k, and K_c is different. The sensitivity of Cr(VI) concentration with respect to K, K_c and u_{max} increases from zero, reaches a maximum and then decreases again to zero (this is the behaviour of the absolute value of the sensitivity function, the negative sign indicates that Cr(VI) concentration 'C' decreases with increasing values of u_{max} whereas

the positive sign on the other hand indicates that Cr(VI) concentration 'C' increases with increase in K and K_c) with exclusion of k that halts constant after a certain point.

5.3 Cr(VI) reduction kinetic in the continuous flow system

5.3.1 Model description

Anaerobic batch studies were initially conducted in this study at various initial Cr(VI) concentration in the presence of As(III) prior to continuous-flow studies to assess the basics of each biological process at various time intervals. Advection and dispersion are known as the main modes of transport of Cr(VI) in the groundwater. However, reaction-microbial reduction in this study also significantly influences the fate and transport of Cr(VI) in a saturated porous media.

Under transient condition, a detailed mathematical model including a system of combined differential equations representing Cr(VI) reduction rate (r_c), mass transport rate (J_{flux}), adsorption rate (q_c) and dispersion was used in this study to simulate microbial Cr(VI) removal in the packed bed reactor system in the presence of As(III). However, the total mass balance of the reactor for modelling of fate and transport of Cr(VI) in the presence of As(III) is described below equation (5.16).

$$\frac{dC}{dt} = -r_{adv} - r_c \Delta V - J_{flux} - q_c \Delta V \tag{5.16}$$

Equation (5.16) is further described as follows:

r_{adv} is known as advection, which is defined as the transport of dissolved species of Cr(VI) along with bulk fluid flow. Mathematically represented as:

$$-\frac{d(CV)}{dt} = -r_{adv} = Q(C_o - C_e) \tag{5.17}$$

where; C_e = Effluent Cr(VI) concentration (mg/L); C_o = Influent Cr(VI) concentration (mg/L); V = Volume of the reactor (L), Q = Influent flow rate (L/s) ($Q = AU$); A = Cross sectional area of the (m^2); U =velocity of the flow (m/s)).

q_c is known as Adsorption defined as the rate at which Cr(VI) is transported and adsorbed in the biofilm of the reactor and also in the reaction taking place on the surface area. Mathematically, Cr(VI) removal by adsorption is represented as follows:

$$-\frac{dC}{dt} = -q_c = k_{ad}\left(C_{eq} - C\right) \tag{5.18}$$

where; k_{ad} = adsorption rate coefficient (S); C_{eq} = equilibrium concentration surface area mg/L; C = Cr(VI) concentration at any time (mg/L); q_c = rate of Cr(VI) removal by adsorption (S^{-1}).

J_{flux} is known as the contaminant flux across the stagnant layer to biofilm defined as a function of the contaminant dispersion coefficient and concentration. Mathematically, the flux through the attached cell is represented as follows;

$$-\frac{d(CV)}{dt} = -J_{flux} = \left(\frac{D_w C}{L_w}\right) A_f \tag{5.19}$$

Where: D_w = dispersion coefficient of Cr(VI) in water (m^2/h); C = bulk liquid Cr(VI) concentration (mg/L); L_w = thickness of the stagnant layer (m); A_f = biofilm surface area (m^2).

r_c is known as a non-competitive inhibition rate. It described Cr(VI) toxicity thresholds under anaerobic condition. Mathematical, it is represented as:

$$-\frac{dC}{dt} = -r_c \frac{u_{\_\max} C}{k^{\left(1-\frac{C_r}{C_o}\right)}(C+K)}\left(X_o - \left(\frac{C_o - C}{K_{\_c}}\right)\right) \tag{5.20}$$

Where; $\mu_{\_max}$ = maximum specific Cr(VI) reduction rate (mg/L/h); C_o = initial Cr(VI) concentration (mg/L); C = Cr(VI) concentration at a time 't'(mg/L); $K_{\_c}$ = maximum Cr(VI) reducing capacity (mg/mg); K = half velocity concentration (mg/L); C_r = Cr(VI) toxicity threshold concentration (mg/L); and k = limiting constant (mg/L); X_o = initial biomass concentration (mg/L).

The total mass balance Equation (5.16) was simulated using forth-order Runger-Kutta routine for solution of simultaneous ordinary and partial differential equations in a computer program for Identification and Simulation of Aquatic System (AQUASIM 2.0) (Reichart, 1998).

Moreover, kinetic parameters values were estimated with values from batch study, followed by simulation and optimization using "AQUASIM 2.0". Upper and lower constraints were set for each parameter to the exclusion of invalid parameter values. Whenever optimization converges or very close to constraint, the constraint was relaxed until the constraint did not force the model. The procedure was repeated until unique values lying away from the constraint but between set limits were found for each parameter (Chirwa and Wang, 2004).

Again, in the reactor the following Model assumptions were postulated:

- As(III) does not act as an inhibitor on microbial growth at 20 mg/L.
- The flow in the column is one dimensional.
- The porous media is homogenous.
- Cr(III) and As(V) generated due to biotransformation is precipitated and retained in the reactor.
- Suspended and attached microbes exist in the reactor.
- Temperature and pH are constant.

5.3.2 Cr(VI) removal kinetic at different concentration in the presence of As(III)

Cr(VI) removal kinetic in continuous biofilm reactor under transient condition was estimated at Cr(VI) concentrations of 20 and 100 mg/L in the presence of 20 mg/L As(III) (Figure 5.3 and 5.4). The optimum kinetic parameters summarized in (Table 5.2) shows that the dispersion coefficient when the reactor was fed with 20 mg/L Cr(VI) in the presence of As(III) is much higher than the one observed when the reactor was fed with Cr(VI) concentration of 100 mg/L. This suggests that the rate at which the contaminant disperses into the cell layer attached on the glass beads is influence at high Cr(VI) concentration of 100 mg/L. However, this could be attributed to the toxic effect of Cr(VI) and As(III) on microbial activities. Therefore, higher rates of Cr(VI) reduction observed at 20 mg/L could be attributed to higher dispersion rate in the column.

Table 5.2: Optimum kinetic parameter values obtained from continuous biofilm reactor

Parameter Symbol	Definition	Constrains [lower, upper]	Optimum value
Biological parameters			
C (mg/L)	State variable	constant	1×10^{-6}
C_{input} (mg/L)	Influent Cr(VI)	constant	20 – 100
C_r (mg/L)	Cr(VI) toxicity threshold	constant	50
K (mg/L)	Half velocity concentration	(0,1000)	688.9
K_c (hr^{-1})	Cr(VI) reduction capacity	(0,10)	0.04
K_d (hr^{-1})	Cell death rate coefficient	(0,1000)	0.0025
u_max (hr^{-1})	Specific reduction rate	(0,1)	0.095
u (h^{-1})	Biomass growth rate	(0,100)	0.021
Physical parameters			
D_w (m^2/s)	Dispersion coefficient	(0,100)	(4-52)
θ (%)	porosity	constant	0.6
α	alpha	constant	0.5
rho_s (kg/m^3)	Density of the medium	constant	2700
Q_{in} (L/hr)	Influent flow rate	constant	0.047
A (m^2)	Cross sectional area	constant	0.00283
Af (m^2)	Biofilm surface area	constant	0.0088

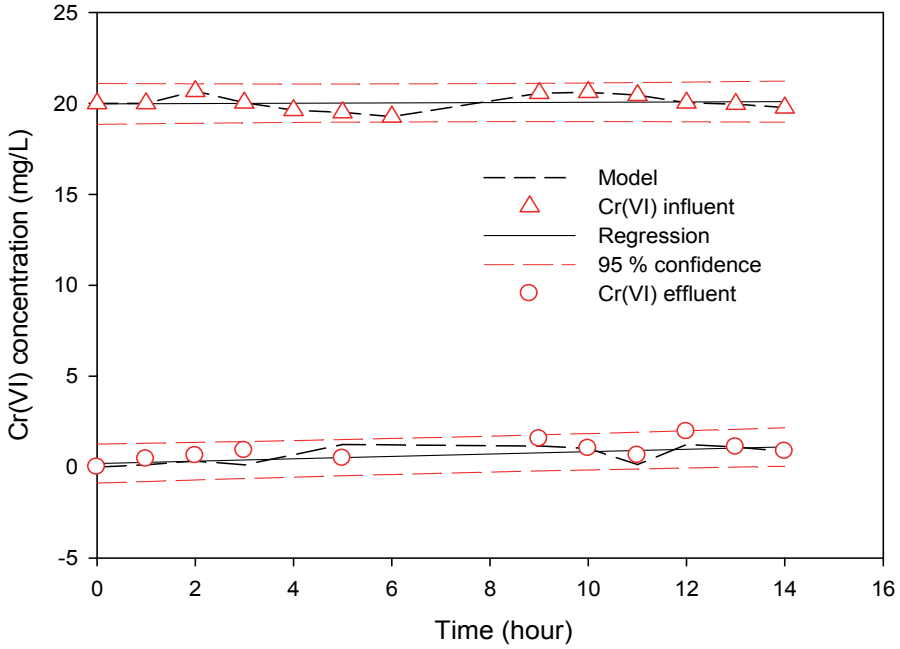


Figure 5.3: Simulation of Cr(VI) influent and effluent at 20 mg/L in in the presence of As(III)

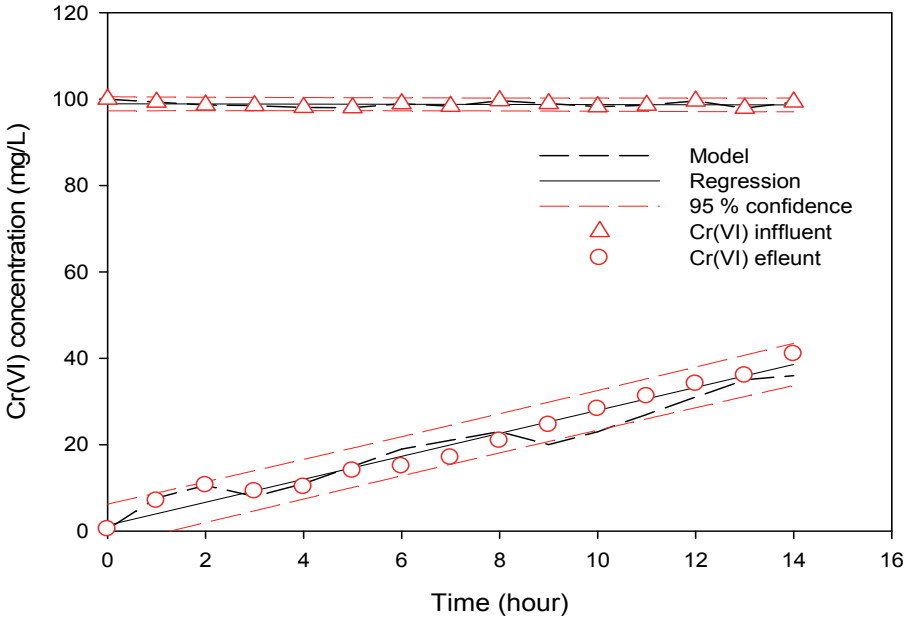


Figure 5.4: Simulation of Cr(VI) influent and effluent at 100 mg/L in in the presence of As(III)

5.4 Steady state spatial performance model

5.4.1 Model description

The steady state model for the continuous reactor for Cr(VI) reduction was modelled as a plug flow reactor using finite difference model. Second order differential equation representing both the flow characteristic and the predominant removal mechanism was used to stimulate Cr(VI) reduction in the continuous flow reactor. For an ideal plug flow, a perfect mixing in the radial dimension (uniform cross section concentration) and no mixing in the axial direction is assumed. The second order differential equation was derived after mass balance consideration in the PFR flow reactor Figure (5.5).

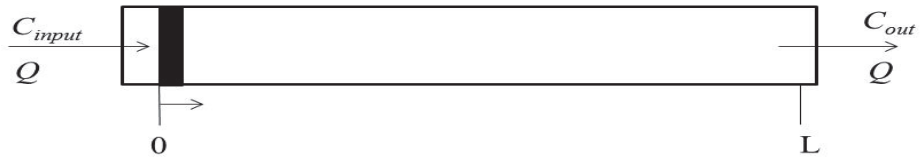


Figure 5.5: Mass balance model for PFR

For a time element *dt* and a volume element *Adx,* the generalized mole balance continuous equation on species (Cr(VI)) over a catalyst weight (W), and since the system is at steady state, the accumulation term zero in Equation (5.21).

$$QC\,(W) - QC\,(W+\Delta W) + (\Delta W)\,r = 0 \tag{5.21}$$

where: Q = in flow rate of Cr(VI) (MT^{-1}); W = $\partial A_f x$ = mass of the medium (M); and r = reaction rate ($ML^{-3}T^{-1}$); C = Cr(VI) concentration any time (ML^{-3}).

Dividing by ΔW and taking limit as $\Delta W \rightarrow 0$, Equation 5.21 becomes a differential form of mole balance for a plug flow reactor:

$$r = \frac{QdC}{A_f \partial dx} \tag{5.22}$$

According to Levenspiel, (1999), the rate of the microbial reactions that are subjected to reactant toxicity is giving as:

$$-r = kC\left(1-\frac{C}{C_r}\right)^n \tag{5.23}$$

where: r = reaction rate ($ML^{-3}T^{-1}$); k = reaction rate coefficient ($L^3.M^{-1}.T^{-1}$); C = effluent Cr(VI) concentration at any time (ML^{-3}); Cr = Cr(VI) toxicity concentration, (ML^{-3}); n = empirical dimensionless variable ($M^{-1}M^{-1}$).

Combining Equation 5.22 and 5.23, gives a second order differential equation:

$$-\frac{dC}{dx} = kC\left(\frac{A_f \partial}{Q}\right)\left(1-\frac{C}{C_r}\right)^n \tag{5.24}$$

where; C = effluent Cr(VI) concentration at any time (ML^{-3}), x = height of a reactor (L), k = reaction rate coefficient ($L^3.M^{-1}.T^{-1}$), ∂ = density of the medium (ML^{-3}), A_f = biofilm surface area (L^2), Q = inflow rate (L^3T^{-1}), C_r = Cr(VI) toxicity concentration, (ML^{-3}), n = empirical dimensionless variable ($M^{-1}M^{-1}$). N.B: n varies with the reactor environment.

5.4.2 Simulation of Cr(VI) reduction across the length of the reactor

Reactor performance under different Cr(VI) concentration was evaluated, and the slope of Cr(VI) concentration profiles across the reactor was defined by a second order ODE Equation 5.24. The plug flow model was tested with initial guess values to simulate length series data under various loading conditions in the reactor using the Computer Program for Solving Numerical Problems (Octave 3.0) as shown in Appendix B. The plug flow model described in this study under steady state condition for different loading conditions Equation 5.24 was used to simulate Cr(VI) effluent concentration in the continuous flow reactor in the presence of As(III). Cr(VI) experimental run of 20 mg/L was firstly tested with the model, and the optimum kinetic parameters obtained from the Cr(VI) feed concentration of 20 mg/L were used to simulate Cr(VI) effluent concentration at 30 and 50 mg/L. Figure (5.6-5.8) described the simulation of Cr(VI) effluent across the reactor length at different loading in the presence of As(III).

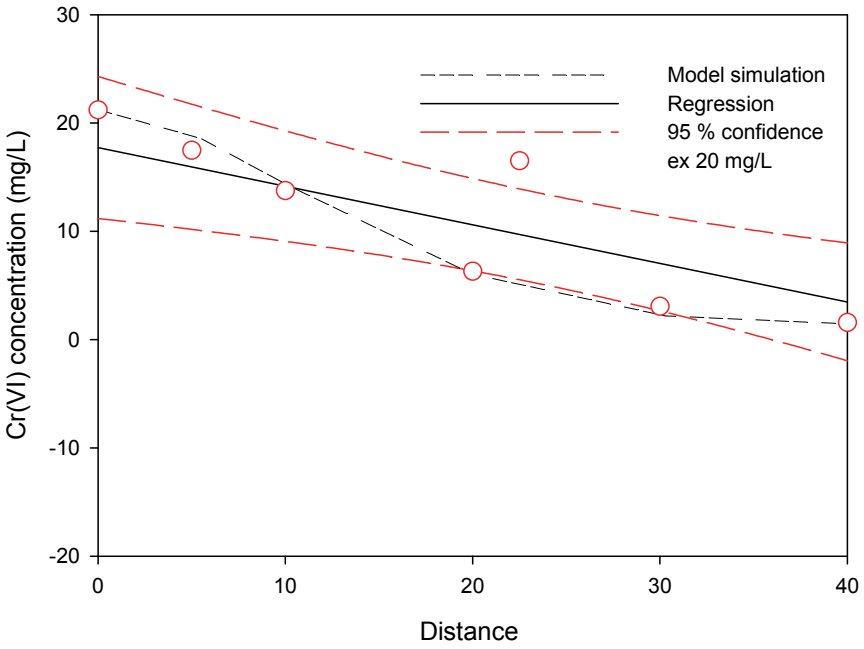


Figure 5.6: Simulation of Cr(VI) effluent at 20 mg/L over reactor length

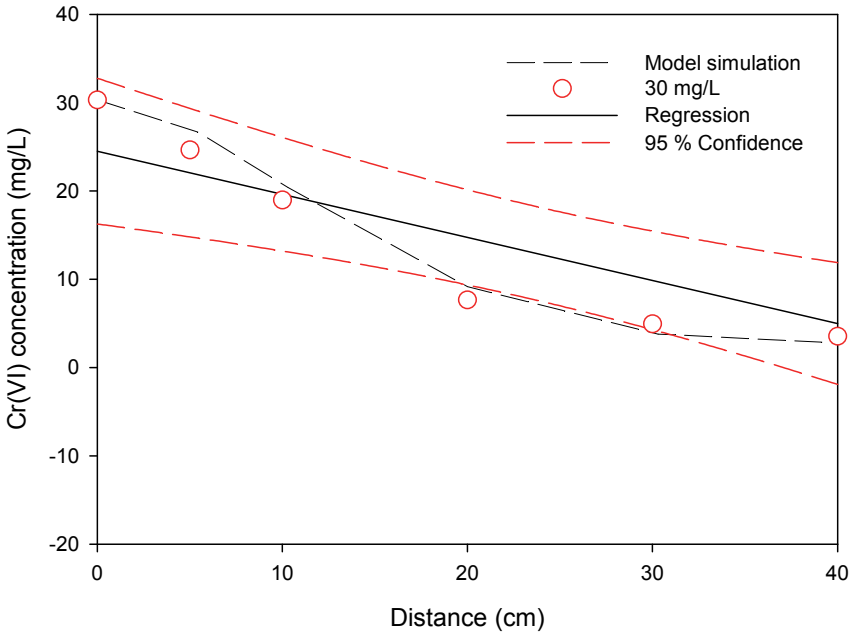


Figure 5.7: Simulation of Cr(VI) effluent at 30 mg/L over reactor length

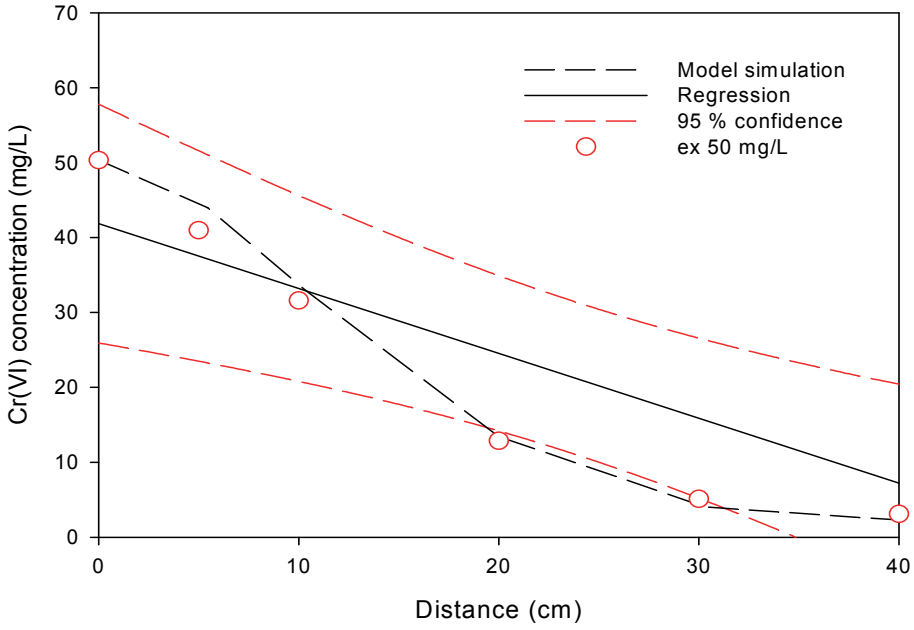


Figure 5.8: Simulation of Cr(VI) effluent at 50 mg/L over reactor length

However, optimum parameters obtained from this model fit in very well for Cr(VI) effluent concentration 30 mg/L and 50 mg/L. Table (5.3) shows the optimum parameter obtained with this model at different concentration. A little adjust was done on k value at 50 mg/L to achieve a good fit. The values of *n* and *K* achieved in study is different from the values previously achieved with the same model, however the difference might be attributed to the presence of As(III) in the reactor, thereby increasing microbial toxicity.

Table 5.3: Optimum kinetic parameter values for the biofilm at steady-state in the presence of As(III)

Effluent Cr(VI) concentration (mg/L)	k (L/mg/h)	n (mg/mg)
20	7.5×10^{-10}	4
30	7.5×10^{-10}	4
50	4.8×10^{-10}	4

5.5 Summary

Chapter 5 of this thesis evaluate a non-competitive inhibition model with Cr(VI) toxicity in a batch system where Cr(VI) reduction was achieved in the presence of As(III), using a mixed culture of facultative anaerobes. This model was chosen because it described the complexity of anaerobic processes and Cr(VI) toxicity threshold. It is believed that biological activities are the main mechanism for Cr(VI) reduction, and such enzymatic reduction of Cr(VI) was best described by the developed model.

The non-competitive inhibition model with Cr(VI) toxicity threshold successfully represent Cr(VI) in the presence of As(III), with Cr(VI) toxicity threshold concentration of 50 mg/L following the mechanism previously observed. The model predicted well with the experimental data at a wide range of Cr(VI) concentration 50, 100, and 200 mg/L. Cr(VI) reduction kinetics obtained in this study however, were indeed slightly different from those found in previous studies utilizing the same model (Mtimuye, 2011; Molokwane *et al.,* 2008). However, difference in the model results may be attributed to the presence of As(III) in the experimental studies, correlated to biomass concentration. Secondly, the sensitivity analysis of each biokinetic parameters (u_{max}, K, k, and K_c) obtained was slightly similar to parameters previously observed (Mtimuye, 2011). These values were seen very sensitive to the model. However, this indicates the reliability of a non-competitive inhibitory model with Cr(VI) toxicity threshold concentration in estimating Cr(VI) reduction under anaerobic condition. Therefore, the mathematical representations obtained from anaerobic batch modelling in this study was employ for simulation of Cr(VI) effluent in the continuous biofilm reactor study.

The model for a parked bed compartment at the steady state was determined by an ordinary differential equation as shown in (Equation 5.24). The parameter, n, which is defined as the empirical dimensionless variable in this model varies with the environmental conditions, as it is associated to the rate of Cr(VI) toxicity in each column experimental condition. The values of n was the same at different Cr(VI) loading suggesting that rate of Cr(VI) toxicity is much lower at concentration of 20-50 mg/L. However, higher the value of n slower the rate of Cr(VI) toxicity on cell. K values also found the same at 20 and 30 mg/L. A minor adjustment was done on K value at 50 mg/L in order to obtain a good fit.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Ferrochrome production processes in South Africa often generates a considerably amounts of Cr(VI) containing wastes and other toxic wastes As(III). The treatment of chromium-arsenic containing wastes before disposal is of utmost important because of its toxic effect. The treatment of theses metalloid involved reduction of Cr(VI) to less toxic Cr(III) and oxidation of As(III) to less toxic As(V). However, bioremediation of Cr(VI) and As(III) containing waste in an indigenous mixed culture from the local environment was explore in this studies.

From observation, 11 possible anaerobic species of Cr(VI) reducing bacteria in the presence of As(III) was isolated. These were successfully characterized using the 16S rRNA/DNA phenotype fingerprinting method. The indigenous mixed cultures isolated predominantly consist of the Gram-positive species: *Staphylococcus sp*., *Enterobacter sp*., and *Bacillus sp*, reveal high resistant to Cr(VI) and it co pollutant As(III). This suggests the capacity of this culture to detoxify Cr(VI) in the presence of its co pollutant As(III). However, to the best of my knowledge, this the first time Cr(VI) resistant or reducing bacteria was isolated from local environment in the presence of As(III).

Batch experimental studies were conducted under various Cr(VI) concentrations to estimate the effectiveness of indigenous mixed culture in reducing Cr(VI) in the presence of As(III). Near complete Cr(VI) reduction was achieved under initial Cr(VI) concentrations up to 70 mg/L when 20 mg/L of As(III) was amended in a batch reactor. However, increasing Cr(VI) concentrations up to 100 mg/L, inhibitory effect was observed. In a different batch experiment, 70 mg/L Cr(VI) concentration was investigated in the presence of As(III) concentration ranging from 5-70 mg/L. Near complete Cr(VI) reduction was observed when the system was amended up to 40 mg/L of As(III), with observed increased in reduction efficiency. However, increasing As(III) concentration up to 50 mg/L, an inhibitory effect was observed, with observed decrease in Cr(VI) reduction efficiency. Comparative studies in the, showed that in the presence of both As(III) and glucose as electron donor Cr(VI) reduction

Cr(VI) reduction was mainly achieved as a result of electron cycling between Cr(VI) and As(III). However, this might be correlated to Cr(VI) reduction linked to As(III) oxidation. Therefore further experimental studies were conducted in continuous-flow system for this purpose.

The possibility of using CCRB in a small scale pilot study for bioremediation of chromium-arsenic contaminated site was demonstrated by better performance of packed bed biofilm reactors inoculated with Cr(VI) reducing indigenous mixed culture from the local environment. The performance of the reactor was evaluated at different Cr(VI) loading. High removal efficiency was achieved as Cr(VI) concentration increases from 20-50 mg/L, while inhibitory effect was observed at high concentration above 100 mg/L. The inhibitory effects of Cr(VI) and As(III) on the Cr(VI) reducing bacteria in the reactor was demonstrated by a steady-state operation which was achieved after three to four days of continuous operation. Secondly, Cr(VI) reduction efficiency across the longitudinal reactor column showed that Cr(VI) reduction is proportional to the height of the column travelled.

Furthermore, AQUASIM 2.0 was used to simulate Cr(VI) reduction in the presence of As(III). Batch modeling results showed that the performance of Cr(VI) reducing culture fitted well the non-competitive model related with Cr(VI) toxicity threshold predicted under anaerobic conditions. Under transient condition, in the continuous reactor, the kinetic model with dispersion simulated well the packed bed column experimental data with a plug flow system. At a steady-state the plug flow model simulated well the experimental data at various Cr(VI) concentrations in the presence of As(III).

6.2 Recommendations

A successful Cr(VI) reduction in the presence of As(III) was observed in a mixed culture of facultative anaerobes from Brits North West South Africa. However, this study did not quantify As(III) concentration due to the limitation of As(III) measurement. Further studies are required to evaluate the simultaneous Cr(VI) reduction and As(III) oxidation in the mixed culture of facultative anaerobes from the study site. However, the tendency of this facultative anaerobes to biocatalytically reduce Cr(VI) in the presence of its co-pollutant As(III), afford a potential for simultaneous bioremediation of Cr(VI) and its co-pollutant As(III) in a chromium-arsenic contaminated site.

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APPENDICE A

```
**********************************************************************
AQUASIM Version 2.0 (win/mfc) - Listing of System Definition

**********************************************************************
***
Date and time of listing:  01/19/2014 15:50:56
**********************************************************************
***
Variables
**********************************************************************
***
C:              Description:          Concentration
                Type:                 Dyn. Volume State Var.
                Unit:                 mg/l
                Relative Accuracy:    1e-006
                Absolute Accuracy:    1e-006
----------------------------------------------------------------------
---
Co:             Description:          Initial concentration
                Type:                 Formula Variable
                Unit:                 mg/L
                Expression:           100
----------------------------------------------------------------------
---
Cr:             Description:          Cr(VI) toxicity threshold
concentration
                Type:                 Formula Variable
                Unit:                 mg/L
                Expression:           50
----------------------------------------------------------------------
---
C_meas:         Description:          Cr(VI) measured
                Type:                 Real List Variable
                Unit:                 mg/L
                Argument:             T
                Standard Deviations:  global
                Rel. Stand. Deviat.:  0
                Abs. Stand. Deviat.:  1
                Minimum:              0
                Maximum:              1e+009
                Interpolation Method: linear interpolation
                Sensitivity Analysis: inactive
                Real Data Pairs (15 pairs):
                   0              99.933906
                   2              82.947786
                   4              80.634501
                   6              83.278255
                   10             72.504957
                   13             70.921235
                   16             9.2757880
                   21             66.822356
                   24             59.151269
```

30	56.442986
72	38.863186
96	31.460674
120	29.477859
144	23.000661
192	17.805684

K:	Description:	half velocity concentration
	Type:	Constant Variable
	Unit:	mg/L
	Value:	732.30073
	Standard Deviation:	1
	Minimum:	0
	Maximum:	1000
	Sensitivity Analysis:	inactive
	Parameter Estimation:	active

---

k:	Description:	limiting constant
	Type:	Constant Variable
	Unit:	mg/L
	Value:	4.3171613
	Standard Deviation:	1
	Minimum:	0
	Maximum:	100
	Sensitivity Analysis:	inactive
	Parameter Estimation:	active

K_c:	Description:	maximum Cr(VI) reducing capacity
	Type:	Constant Variable
	Unit:	mg Cr(VI)/mg cell
	Value:	0.99700
	Standard Deviation:	1
	Minimum:	0
	Maximum:	10
	Sensitivity Analysis:	active
	Parameter Estimation:	active

T:	Description:	Time
	Type:	Program Variable
	Unit:	hour
	Reference to:	Time

---

u_{max}:	Description:	maximum specific Cr(VI) reduction rate
	Type:	Constant Variable
	Unit:	mg/L/hr
	Value:	0.64404963
	Standard Deviation:	1
	Minimum:	0
	Maximum:	20

```
                Sensitivity Analysis: inactive
                Parameter Estimation: active
--------------------------------------------------------------------
---
Xo:              Description:          initial biomass concentration
                 Type:                 Formula Variable
                 Unit:                 mg/l
                 Expression:           100
********************************************************************
***

********************************************************************
**
Processes
********************************************************************
***
C_R:             Description:          Cr_reduction
                 Type:                 Dynamic Process
Rate:       ((k^(-1*(Co-Cr)/Co)))*u_max*C*(Xo-((Co-C/K_c)))/(K+C)
Stoichiometry:
                   Variable: Stoichiometric Coefficient
                   C: -1
********************************************************************
***
********************************************************************
***
Compartments
********************************************************************
***
Reactor1:        Description:          Batch
                 Type:                 Mixed Reactor Compartment
                 Compartment Index:    0
                 Active Variables:     C, Co, Cr, C_meas, K, k, K_c, t,
u_
                                       max, Xo
                 Active Processes:     C_R
                 Initial Conditions:
                   Variable (Zone): Initial Condition
                   C(Bulk Volume) : Co
                 Inflow:               0
                 Loadings:
                 Volume:               1
                 Accuracies:
                   Rel. Acc. Q:        0.001
                   Abs. Acc. Q:        0.001
                   Rel. Acc. V:        0.001
                   Abs. Acc. V:        0.001
********************************************************************
********************************************************************
******
Definitions of Calculations
********************************************************************
***
C_r:             Description:          Cr(IV) reduction
                 Calculation Number:   0
```

```
                Initial Time:          0
                Initial State:         given, made consistent
                Step Size:             0.1
                Num. Steps:            100
                Status:                active for simulation
                                       active for sensitivity analysis
************************************************************************
***
************************************************************************
***
Definitions of Parameter Estimation Calculations
************************************************************************
***
fit1:           Description:           Cr(VI) reduction
                Calculation Number:    0
                Initial Time:          0
                Initial State:         given, made consistent
                Status:                active
                Fit Targets:
                  Data : Variable (Compartment,Zone,Time/Space)
                  C_meas : C (Reactor1,Bulk Volume,0 rel.space)
************************************************************************
************************************************************************
******
Plot Definitions
************************************************************************
***
plot1:          Description:           Cr(VI) reduction
                Abscissa:              Time
                Title:                 Cr(VI) reduction
                Abscissa Label:        Time (hour)
                Ordinate Label:        Cr(VI) concentration (mg/L)
                Curves:
                  Type : Variable [CalcNum,Comp.,Zone,Time/Space]
                  Value : C [0,Reactor1,Bulk Volume,0]
                  Value : C_meas [0,Reactor1,Bulk Volume,0]
************************************************************************
************************************************************************
******
Calculation Parameters
************************************************************************
***
Numerical Parameters:    Maximum Int. Step Size:  1
                         Maximum Integrat. Order: 5
                         Number of Codiagonals:   1000
                         Maximum Number of Steps: 1000
------------------------------------------------------------------------
---
                         Fit Method:              simplex
                         Max. Number of Iterat.:  100
************************************************************************
************************************************************************
******
Calculated States
```

```
**********************************************************************
***
Calc. Num.  Num. States Comments
0           15          Range of Times: 0 - 192
```

****

```
**********************************************************************
***AQUASIM Version 2.0 (win/mfc) - Parameter Estimation File

**********************************************************************
***Date and time of listing:  01/19/2014 15:44:55


Number of parameters = 4
Number of data points = 15
Estimation method     = simplex


Parameters:
```

Name	Unit	Start	Minimum	Maximum
K	mg/L	650	0	1000
k	mg/L	1	0	100
K_c	mg Cr(VI)/mg cell	1	0	10
u_max	mg/L/hr	1	0	20

Calculations:

K [mg/L]	k [mg/L]	K_c [mg Cr(VI)/mg cell]	u_max [mg/L/hr]	Chi^2
650	1	1	1	12045.6
750	1	1	1	10396.5
650	11	1	1	1361.77
650	1	2	1	4560.66
650	1	1	3	26948.8
705.801	1.838	1.34341	0.59519	1380.36
729.24	3.16052	1.61286	0.746467	610.604
759.465	72.4756	2.25961	0.662331	12833.1
600.195	72.4756	2.25961	0.662331	10575.5
720.778	1.34499	1.16908	0.887139	6142.22
646.103	4.32433	1.74104	0.723499	439.906
600.195	72.4756	2.25961	0.662331	10575.5
716.402	97.5758	1.02482	0.58774	6910.55
668.308	1.5448	1.62794	0.851079	3043.52
701.484	84.3957	1.17182	0.655402	6301.96
677.03	2.12269	1.48579	0.792019	2110.77

693.614	8.31234	1.26145	0.695407	439.546
701.484	84.3957	1.17182	0.655402	6301.96
656.112	94.1314	1.34253	1.11013	3193.84
694.306	2.72939	1.34319	0.673454	1152.13
728.668	2.33851	2.57671	0.54842	1178.67
711.457	2.91617	1.88734	0.618349	450.132
699.869	7.182	1.94095	0.712461	1108.19
698.491	5.10975	1.75119	0.702293	508.469
639.608	7.7593	0.63571	0.628517	843.819
709.925	3.713	0.61852	0.713032	468.79
684.606	3.49834	0.46027	0.668457	605.865
695.098	4.58296	0.66907	0.693518	433.694
663.247	5.35684	0.59306	0.650542	468.016
675.777	4.82396	0.89936	0.665119	434.489
640.359	20.1074	0.90226	0.790046	899.805
695.522	3.72541	0.70101	0.653901	426.714
727.238	5.6938	0.87342	0.635235	456.285
669.014	4.60145	0.63338	0.69922	428.252
674.266	2.97507	0.92459	0.660317	568.414
688.921	5.75208	0.43154	0.686291	425.727
698.433	4.31893	0.60472	0.701385	429.443
681.178	4.39952	0.81148	0.67603	446.618
691.694	4.53569	0.92694	0.689062	426.406
673.94	4.79767	0.97963	0.663062	430.034
692.543	4.42949	0.99838	0.691397	426.53
713.239	4.40796	0.99656	0.66145	424.882
732.301	4.31716	0.99700	0.64405	425.623

Parameter estimation successfully finished (convergence criterion met)

K [mg/L]	k [mg/L]	K_c [mg Cr(VI)/mg cell]	u_max [mg/L/hr]

Estimated values of the parameters:

732.301	4.31716	0.99700	0.64405

Contribution of data series to Chi^2:

Calculation:	Data Series:	Chi^2 ini:	Chi^2 end:
fit1	C_meas	12045.6	225.623
		12045.6	225.623

Number of steps performed = 20
Number of simulations performed = 43

APPENDICE B

AQUASIM Version 2.0 (win/mfc) - Listing of System Definition

****
**
****
****
Variables
****
****

A:	Description:	Cross sectional area
	Type:	Formula Variable
	Unit:	m^2
	Expression:	0.00283

α:	Description:	Alpha
	Type:	Formula Variable
	Unit:	
	Expression:	0.5

A_f:	Description:	Biofilm surface area
	Type:	Formula Variable
	Unit:	m^2
	Expression:	0.0088

C:	Description:	Cr(VI) concentration
	Type:	Dyn. Volume State Var.
	Unit:	mg/L
	Relative Accuracy:	1e-006
	Absolute Accuracy:	1e-006

C_{in}:	Description:	Measured Cr(VI) influent
	Type:	Real List Variable
	Unit:	mg/L
	Argument:	T
	Standard Deviations:	global
	Rel. Stand. Deviat.:	0
	Abs. Stand. Deviat.:	1
	Minimum:	0
	Maximum:	1e+009
	Interpolation Method:	linear interpolation
	Sensitivity Analysis:	inactive
	Real Data Pairs (20 pairs):	
	0	20
	1	20
	2	20.672
	3	20.032
	4	19.624
	.	.

	.	.
	17	19.503
	18	19.304
	19	19.016
	20	18.768
	21	18.384

C_o:	Description:	Initial concentration
	Type:	Formula Variable
	Unit:	mg/L
	Expression:	20

C_{out}:	Description:	Cr(VI) measured effluent
	Type:	Real List Variable
	Unit:	mg/L
	Argument:	T
	Standard Deviations:	global
	Rel. Stand. Deviat.:	0
	Abs. Stand. Deviat.:	1
	Minimum:	0
	Maximum:	1e+009
	Interpolation Method:	linear interpolation
	Sensitivity Analysis:	inactive
	Real Data Pairs (18 pairs):	
	1	0.128
	2	0.3264
	3	0.1152
	5	1.248
	17	0
	18	0
	19	0
	20	0
	21	0

C_r:	Description:	Cr(VI) toxicity threshold
	Type:	Formula Variable
	Unit:	mg/L
	Expression:	50

$C_{\_alnum}$:	Description:	Calcnum
	Type:	Program Variable
	Unit:	h
	Reference to:	Calculation Number

$C_{\_crit}$:	Description:	Crit conc
	Type:	Formula Variable
	Unit:	mg/L
	Expression:	0.01

```
------------------------------------------------------------------------
---
C_in_1:         Description:          Time dependent inflow
concentration
                Type:                  Real List Variable
                Unit:                  mg/m^3
                Argument:              T
                Standard Deviations:   global
                Rel. Stand. Deviat.:   0
                Abs. Stand. Deviat.:   1
                Minimum:               0
                Maximum:               1e+009
                Interpolation Method: linear interpolation
                Sensitivity Analysis: inactive
                Real Data Pairs (4 pairs):
                   0               0
                   0.01            1
                   0.5             1
                   0.51            0
------------------------------------------------------------------------
---
D_w:            Description:          Dispersion coefficient
                Type:                  Constant Variable
                Unit:                  m^2/h
                Value:                 51.73166
                Standard Deviation:    1
                Minimum:               0
                Maximum:               100
                Sensitivity Analysis: inactive
                Parameter Estimation: active
------------------------------------------------------------------------
---
K:              Description:          half velocity concentration
                Type:                  Constant Variable
                Unit:                  mg/L
                Value:                 688.90043
                Standard Deviation:    1
                Minimum:               0
                Maximum:               1000
                Sensitivity Analysis: inactive
                Parameter Estimation: active
------------------------------------------------------------------------
---
k:              Description:          limiting constant
                Type:                  Constant Variable
                Unit:                  mg/L
                Value:                 11.091163
                Standard Deviation:    1
                Minimum:               0
                Maximum:               20
                Sensitivity Analysis: inactive
                Parameter Estimation: active
------------------------------------------------------------------------
---
```

K_1:	Description:	Relaxation rate constant for sorpti
		on of B
	Type:	Formula Variable
	Unit:	1/h
	Expression:	1000
K_d:	Description:	cells death rate
	Type:	Constant Variable
	Unit:	1/h
	Value:	0.0025
	Standard Deviation:	1
	Minimum:	0
	Maximum:	1000
	Sensitivity Analysis:	inactive
	Parameter Estimation:	inactive
K_f:	Description:	Freundlich coefficient
	Type:	Formula Variable
	Unit:	
	Expression:	0.00025
K_c:	Description:	maximum Cr(VI) reducing capacity
	Type:	Constant Variable
	Unit:	mg Cr(VI)/mg cell
	Value:	0.039561656
	Standard Deviation:	1
	Minimum:	0
	Maximum:	10
	Sensitivity Analysis:	inactive
	Parameter Estimation:	active
Q_{in}:	Description:	influent flow rate
	Type:	Formula Variable
	Unit:	L/hr
	Expression:	0.047
rho_s:	Description:	Density of the medium
	Type:	Formula Variable
	Unit:	kg/m^3
	Expression:	2700
S:	Description:	Adsorbed concentration
	Type:	Dyn. Volume State Var.
	Unit:	mg/kg
	Relative Accuracy:	1e-006
	Absolute Accuracy:	1e-006

```
Seq_0:          Description:          No sorption
                 Type:                 Formula Variable
                 Unit:                 mg/kg
                 Expression:           0
-----------------------------------------------------------------
---
Seq_langmuir:   Description:          Langmuir isotherm
                 Type:                 Formula Variable
                 Unit:                 mg/kg
                 Expression:           Smax*C/(K+C)
-----------------------------------------------------------------
---
Seq_lin:        Description:          Linear isotherm
                 Type:                 Formula Variable
                 Unit:                 mg/kg
                 Expression:           Kd*C
-----------------------------------------------------------------
----
Smax:           Description:          Maximum site density
                 Type:                 Formula Variable
                 Unit:                 mg/kg
                 Expression:           0.00029
-----------------------------------------------------------------
---
S_eq:           Description:          Isotherm
                 Type:                 Variable List Variable
                 Unit:                 mg/kg
                 Argument:             C_alnum
                 Interpolation Method: linear interpolation
                 Real-Variable Data Pairs (4 pairs):
                   0                Seq_0
                   1                Seq_lin
                   2                Seq_langmuir
                   3                Seq_langmuir
-----------------------------------------------------------------
---
S_eq_Freundlich:
                 Description:          Freundlich isotherm
                 Type:                 Formula Variable
                 Unit:                 mg/kg
                 Expression:            if C>C_crit then Kf*C^a else
Kf*C_
                                       crit^a*C/C_crit endif
-----------------------------------------------------------------
---
t:               Description:          time
                 Type:                 Program Variable
                 Unit:                 h
                 Reference to:         Time
-----------------------------------------------------------------
---
θ:              Description:          Porosity
                 Type:                 Formula Variable
                 Unit:
                 Expression:           0.6
```

```
------------------------------------------------------------------
---
u:              Description:          Specific biomass growth rate
                Type:                 Constant Variable
                Unit:                 1/h
                Value:                0.021
                Standard Deviation:   1
                Minimum:              0
                Maximum:              100
                Sensitivity Analysis: inactive
                Parameter Estimation: inactive
------------------------------------------------------------------
---
u_max:          Description:  maximum specific Cr(VI) reduction rate

                Type:                 Constant Variable
                Unit:                 mg/L/hr
                Value:                0.0947966
                Standard Deviation:   1
                Minimum:              0
                Maximum:              1
                Sensitivity Analysis: inactive
                Parameter Estimation: active
------------------------------------------------------------------
---
X:              Description:          Biomass concentration
                Type:                 Formula Variable
                Unit:                 mg/L
                Expression:           Xo*exp(-(u-Kd)*T)
------------------------------------------------------------------
---
Xo:             Description:          initial biomass concentration
                Type:                 Constant Variable
                Unit:                 mg/l
                Value:                180.12557
                Standard Deviation:   1
                Minimum:              0
                Maximum:              1000
                Sensitivity Analysis: inactive
                Parameter Estimation: inactive
******************************************************************
***
******************************************************************
****
Processes
******************************************************************
****
C_Reduction:    Description:          Cr_reduction
                Type:                 Dynamic Process
                Rate:                 ((k^(-1*(Co-
Cr)/Co)))*u_max*C*(Xo-(
                                      (Co-C/K_c)))/(K+C)
                Stoichiometry:
                  Variable: Stoichiometric Coefficient
                  C : -1
```

```
------------------------------------------------------------------
---
C_Sorption:    Description:          Cr(VI) sorption
               Type:                 Dynamic Process
               Rate:                 K1*(S_eq-S)
               Stoichiometry:
                 Variable: Stoichiometric Coefficient
                 C: -rho_s*(1-theta)/theta
                 S: 1
******************************************************************
***
******************************************************************
****
Compartments
******************************************************************
****
column:        Description:          packed bed column
               Type:                 Column Compartment
               Compartment Index:    0
               Active Variables:     C, S, A, a, A_f, Co, Cout, Cr,
C_al
                                     num, C_crit, C_in_1, D_w, K, k,
K1,
                                      Kd, Kf, K_c, Qin, rho_s,
Seq_0, Se
                                     q_langmuir, Seq_lin, Smax,
S_eq, S_
                                     eq_Freundlich, T, theta, u,
u_max,
                                     X, Xo
               Active Processes:     C_Reduction, C_Sorption
               Initial Conditions:
                 Variable(Zone) : Initial Condition
                 C(Advective Zone) : Cout
               Inflow:               Qin
               Loadings:
                 Variable: Loading
                 C: Qin*Cin
               Lateral Inflow:       0
               Start Coordinate:     0
               End Coordinate:       1
               Cross Section:        A
               Adv. Vol. Fract.:     theta
               Dispersion:           D_w
               Parallel Zones:
               Num. of Grid Pts:     52 (low resolution)
               Accuracies:
                 Rel. Acc. Q:        0.0001
                 Abs. Acc. Q:        1e-006
                 Rel. Acc. D:        1e-006
                 Abs. Acc. D:        1e-006
******************************************************************
***
******************************************************************
***
```

```
Definitions of Calculations
*****************************************************************
***
calc1:          Description:
                Calculation Number:    0
                Initial Time:          0
                Initial State:         given, made consistent
                Step Size:             0.1
                Num. Steps:            110
                Status:                active for simulation
                                       inactive for sensitivity
analysis
*****************************************************************
***
*****************************************************************
****
Definitions of Parameter Estimation Calculations
*****************************************************************
***
fit1:           Description:
                Calculation Number:    0
                Initial Time:          0
                Initial State:         given, made consistent
                Status:                active
                Fit Targets:
                  Data: Variable (Compartment, Zone, Time/Space)
                  Cout: C (column, Advective Zone,0)
*****************************************************************
***


*****************************************************************
***
Plot Definitions
*****************************************************************
***
Simulation:     Description:           Cr(VI) concentration
                Abscissa:              Time
                Title:                 mg/L
                Abscissa Label:        Time(h)
                Ordinate Label:        Concentration (mg/L)
                Curves:
                  Type: Variable [CalcNum, Comp.,Zone, Time/Space]
                  Value: C [0, column, Advective Zone, 0]
                  Value: Cout [0, column, Advective Zone, 0]
*****************************************************************
*****************************************************************
******
Calculation Parameters

*****************************************************************
***
Numerical Parameters:     Maximum Int. Step Size:  1
                          Maximum Integrat. Order: 5
                          Number of Codiagonals:   1000
```

```
                          Maximum Number of Steps: 1000
------------------------------------------------------------------------
---
                          Fit Method:              simplex
                          Max. Number of Iterat.:  100
************************************************************************
***
************************************************************************
***
Calculated States
************************************************************************
***
Calc. Num.  Num. States Comments
0           18          Range of Times: 0 - 21
************************************************************************
```

APPENDICE C

20 m/L Concentration

Model:

```
clear all;
close all;

Lraw=[0,5,10,20,30,40,60];
Craw= [21.22, 17.4925, 13.765, 6.31, 3.08, 1.59, 0.36];

Cr= 50;
Af= 8.8;
rho_s= 2700000;
Qin= 0.047;
k =0.00000000075;
n=4;
L= linspace (0, 60, 100) ;
Co= 21.22;
dCdL= @(C, L) (-k*C)*((Af*rho_s)/Qin)*((1-(C/Cr))^n) ;
[t,ci]=ode23(dCdL,L, Co) ;
plot(Lraw, Craw,'ro', L, ci(:,1),'black')
ylabel('Concentration (mg/L)')
xlabel('Distance (cm)')
legend('experimental values', 'model simulation')
```

30 mg/L Concentration

Model:

```
clear all;
close all;

Lraw=[0,5,10,20,30,40,60];
Craw= [30.304, 24.643, 18.982, 7.66, 4.95, 3.54, 0.87];

Cr= 50;
Af= 8.8;
rho_s= 2700000;
Qin= 0.047;
k =0.00000000075;
n=4;
L= linspace (0, 60, 100) ;
Co= 30.304;
dCdL= @(C, L) (-k*C)*((Af*rho_s)/Qin)*((1-(C/Cr))^n) ;
[t,ci]=ode23(dCdL,L, Co) ;
plot(Lraw, Craw,'ro', L, ci(:,1),'black')
ylabel('Concentration (mg/L)')
xlabel('Distance (cm)')
legend('experimental values', 'model simulation')
```

APPENDICE C Continued

50 mg/L Concentration

Model:

```
clear all;
close all;

Lraw=[0,5,10,20,30,40,60];
Craw= [50.336, 40.972, 31.608, 12.88, 5.12, 3.11, 1.64];

Cr= 50;
Af= 8.8 ;
rho_s= 2700000;
Qin= 0.047;
k =0.00000000048;
n=4;
L= linspace (0, 60, 100) ;
Co= 50.336;
dCdL= @(C, L) (-k*C)*((Af*rho_s)/Qin)*((1-(C/Cr))^n) ;
[t,ci]=ode23(dCdL,L, Co) ;
plot(Lraw, Craw,'ro', L, ci(:,1))
ylabel('Concentration (mg/L)')
xlabel('Distance (cm)')
legend('experimental values', 'model simulation')
```

APPENDICE D

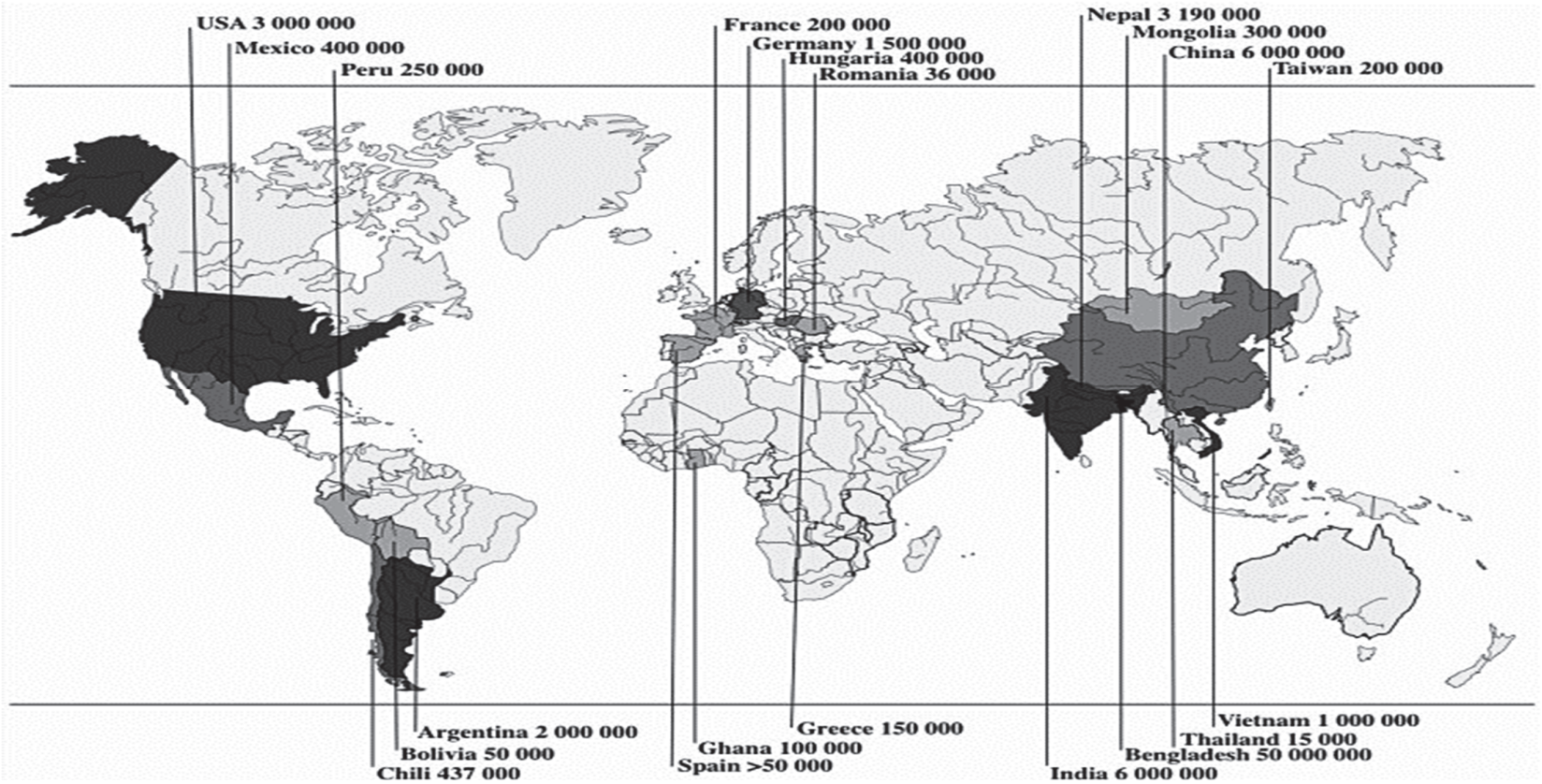

Figure 5.9: World map of populations at risk, based on the data currently available in the literature. The figures give the number of people whose daily water consumption includes arsenic levels (Didier *et al.,* 2009)

APPENDICE E

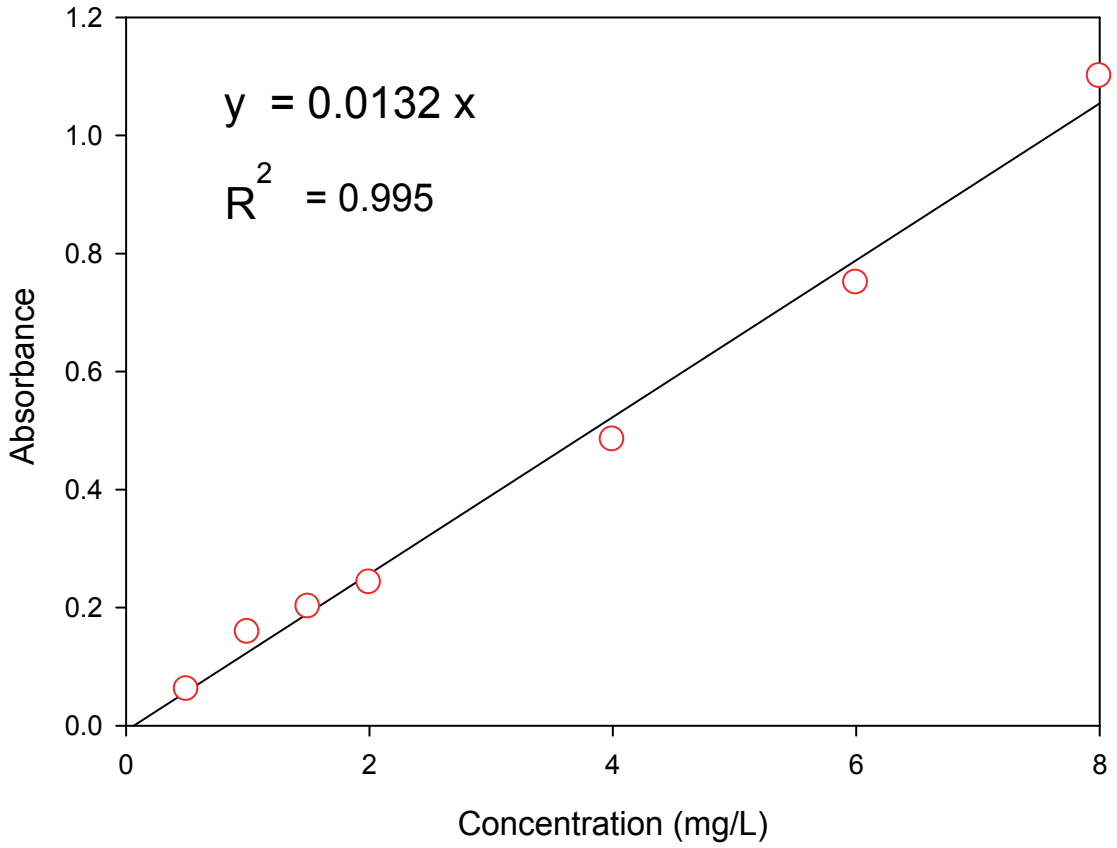

Figure 5.10 Concentration versus Absorbance a linear graph with regression of R^2 of 99.95%