Biocatalytic chromium(VI) reduction by bacterial culture grown in symbiotic autotrophy with fresh water microalgae

By

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ABSTRACT

Title: Biocatalytic Chromium(VI) Reduction by Bacterial Culture Grown in Symbiotic Autotrophy with Fresh Water Microalgae

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The ability to reduce Cr(VI) to Cr(III) has been discovered in multiple species of bacteria. This ability has manifested as either a detoxification strategy to ensure survival in Cr(VI) rich environments, or as a metabolic necessity due to other properties of the bacteria. Bacterial species that can reduce Cr(VI) to Cr(III), and can survive in Cr(VI) rich environments, and are called chromium reducing bacteria (CRB). In this study, several pure cultures of bacteria were isolated from sludge, wastewater and soil samples from a Cr(VI) contaminated site in Brits (South Africa). Colonies were tested individually for Cr(VI) tolerance and reduction capability. The locally isolated cultures proved successful in reducing Cr(VI) and were identified using 16S rRNA sequencing as *Escherichia coli*, *Citrobacter sedlakii*, and *Bacillus thermoamylovorans*. Cr(VI) reduction in bacteria is facilitated either by passive reaction systems of reaction such as chemical oxidation organic compounds or by enzymatic reactions catalysed by specially expressed Cr(VI) reducing enzymes biochemically classified as Cr(VI) reductases. Cr(VI) reduction in the presence of oxygen, although fast, does not yield energy for cell growth and metabolism, and is therefore cometabolic in nature. Cr(VI) under anaerobic conditions, on the other hand, is known to be dissimilatory in nature whereby Cr(VI) is used as the solo terminal electron sink in a process that yields energy for cell metabolism and growth. In this study Cr(VI) reduction experiments were conducted under aerobic conditions to simulate possible application in an open surface water body with algal growth.

The rationale for the investigation was to develop a self-sustained bioremediation process for Cr(VI) reduction where carbon sources are produced internally. Such a system could be energy efficient and carbon-negative in nature. Algae offer a solution to the problem since they produce organic carbon from CO_2 in the presence of sunlight as an energy source. Engineered algal cultivation has the benefit of not requiring diversion of agricultural land, as cultivation can take place in freshwater, marine, and brackish water environments. Additionally, algae cultivation can be used as a carbon sink to consume CO_2 emitted from specific industrial processes.

The freshwater algae used in this study were obtained from the Hartbeespoort Dam, an artificial reservoir located in Hartbeespoort (North West Province, South Africa). The algae were identified as *Chlorococcum Ellipsoideum* by 18S rRNA and 28S rRNA genotype fingerprinting followed by Basic Local Alignment Search Tool (BLAST) Search of the gene sequence in the National Center for Biotechnology Information (NCBI) database. Control algal cultures, *Chlamydomonas reinhardtii* and *Tetradesmus obliquus*, were purchased from the Culture Collection of Algae and Protozoa (CCAP) and China, respectively.

In the algae-CRB system, the Cr(VI) reduction process in the batch experiment was determined to be enzyme mediated with minimal adsorption taking place. In the batch experiments, complete reduction and removal of Cr(VI) from solution was achieved in less than 24 hours in batches loading with an initial concentration of up to 50 mg/L. At 100 mg/L initial Cr(VI) concentration, 92% of the Cr(VI) was reduced within 24 hours. The algal species tested in this study provided carbon sources for bacterial growth with a resultant Cr(VI) reduction even though the process was mostly sacrificial with respect to the survival of algae.

A biokinetic model was developed for the bacterial reduction of Cr(VI) in the algae-CRB system based on Michaelis-Menten or Monod model. Two apparent Cr(VI) reduction rates prevailed in the algae-CRB system, i.e., a rapid reduction rate, followed by a slow reduction rate. The kinetic parameters in the Cr(VI) reduction model was determined using the software AQUASIM 2.3. The predicted model was able to fit the experimental data well.

DECLARATION

I, Maria Magdalena Roestorff, declare that this dissertation entitled “Biocatalytic Cr(VI) Reduction by Cr(VI) Reducing Consortium of Bacteria combined with in cooperation with/in Symbiotic Autotrophy with Fresh Water Algae” which I hereby submit for a Master of Engineering degree with specialisation in Environmental Engineering at the University of Pretoria is my own work and has not been previously submitted by me for any degree at this or other tertiary institution.

I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

_____ _____

Maria M Roestorff Date

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SYMBOLS

k_1	Rapid Cr(VI) reduction constant (hr^{-1})
k_2	Gradual Cr(VI) reduction constant (hr^{-1})
K_c	Half velocity concentration (mg/L)
k_i	Reaction constant (hr^{-1})
K_i	Non-competitive inhibition constant (mg/L)
k_m	Maximum specific Cr(VI) reduction rate (hr^{-1})
OD	Optical density (absorbance)
R_c	Finite maximum Cr(VI) reduction capacity (mg Cr(VI) removed /mg bacterial cells inactivated)
t	Time (hr)
X	Total bacterial cell concentration (mg/L)
X_o	Initial viable cell concentration (mg/L)

ABBREVIATIONS

AAS	Atomic adsorption spectrophotometer
APHA	American public health agency
ASBR	Anaerobic Sequencing Batch Reactor
BBM	Bold Basel Media
BLAST	Basic Logical Alignment Search Tool
CFU	Colony forming units
ChrR	Cr(VI) reductase
CRB	Cr(VI) reducing bacteria
Cr	Chromium
Cr(III)	Trivalent Chromium
Cr(VI)	Hexavalent Chromium
DNA	Deoxyribonucleic Acid
DPC	1,5-diphenylcarbazide
LB	Luria Bettani
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
PC	Plate Count
pH	Potential Hydrogen
rRNA	Ribosomal Ribonucleic Acid
SEM	Scanning electron microscopy
SE	Standard error
WHO	World Health Organisation
UV	Ultraviolet

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CHAPTER 1 INTRODUCTION

1.1 Background

Chromium is commonly found in the natural environment as Cr(III). However, during various industrial processes Cr(VI) is produced via the oxidation of Cr(III). Anthropogenic activities from mining, processing, and applications of Cr have expanded in the past few decades releasing progressively more Cr(VI) which inevitably will become burdensome for life on earth. Often the Cr(VI) is incorrectly discarded. Toxic metal contamination, such as Cr(VI) pose a serious threat to the environment. The industrial activities that are frequently responsible for Cr(VI) pollution include chrome alloy production in steel industries, chrome electroplating, ceramic glazes, inhibition of water corrosion, cement dust, refractory bricks, wood preservation, pressure treated lumber, contaminated landfill, asbestos lining erosion, textile dyes, production of paints and pigments, anti-algae agents, antifreeze, glassmaking, pulp and paper production, and leather tanning (Shahid et al., 2017; Pradhan et al., 2017; Miretzky and Cirelli, 2010).

South Africa was one of the few countries that increased chromium mine production between 2016 and 2017 (USGS, 2017). South Africa has over 7.7 billion tons of chromite in the Bushveld Complex. 98% of the Chromite minerals imported by the USA, is imported from South Africa. South Africa is the world-leading producer of chromite, and ferrochromium ore. In this regards, South Africa plays a vital role in stainless-steel production, as chromium is known for its oxide-forming properties. The total chromite resources of the world are more than 12 billion tons, which should be more than adequate for future requirements for stainless-steel production (Shahid et al., 2017).

1.2 Environmental and Health Concerns

Cr(VI) is classified as a carcinogen according to the International Agency for Research on Cancer (IARC, 1987). A mechanism that contributes significantly to Cr(VI) toxicity is the fact that Cr(VI) can easily diffuse across the cell membrane and, the reduction of Cr(VI) inside cells give rise to free radicals that cause DNA alterations (Jobby et al., 2018). Cr(III) is considered a thousand times less toxic than Cr(VI) as it cannot be transported across the cellular membrane (Thatoi et al., 2014). Exposure to Cr(VI) can cause severe health

hazards on both flora and fauna. Metals such as chromium cannot be destroyed, however the metal can be removed from the environment or converted to a less hazardous form. Cr(VI) therefore, requires extensive monitoring and research to develop a remediation technique that can convert Cr(VI) into a less toxic form.

1.3 Treatment Methods

Conventional treatment of Cr(VI) includes both physical and chemical remediation methods. The most common detoxification method is the reduction of Cr(VI) to Cr(III) under acidic conditions (pH around 2.0) and subsequent hydroxide precipitation of Cr(III), as $Cr(OH)_3(s)$, by increasing the pH above 8 (Pradhan et al., 2017). Physical treatment relies on the physicochemical properties of Cr(VI) and includes techniques such as adsorption, ion exchange, membrane filtration, granular activated carbon, and soil washing and separation (Jobby et al., 2018; Pradhan et al., 2017). Chemical remediation methods involve in-situ addition of chemicals that are electron donors and that facilitate the reduction of Cr(VI) to Cr(III), such as Hydrogen sulphide (H_2S), sodium metabisulfite ($NaHSO_3$), Sodium dithionite ($Na_2S_2O_4$) ferrous sulphate ($FeSO_4$) (Jobby et al., 2018). Photocatalysis is also an effective reduction method to help mitigate Cr(VI) (Jobby et al., 2018). These methods, however, have relatively high operational cost, high energy consumption, and produce secondary pollutants. Most of these methods, especially adsorption, only take effect under high initial Cr(VI) concentrations (Jobby et al., 2018).

Recently biological detoxification techniques have received a fair amount of attention. Bioremediation pertains to the use of biological agents for remediation, and capitalising on their unique characteristics to remove or detoxify pollutants. Microorganism biological agents such as algae, bacteria, fungi, and associated enzymes, present practical methods for targeting a specific toxic compound in the environment (Thatoi et al., 2014). Microorganisms adapt different strategies to survive in toxic environments, such as biosorption, bioaccumulation, and biotransformation for the detoxification of a toxic compound into a less toxic form (Jobby et al., 2018; Jobby et al., 2016). Bioremediation methods are considered relatively economical and eco-friendly, as they have low cost and have a low waste generation (Bharagava and Mishra, 2018).

Microorganisms have been isolated that can reduce the toxic forms of toxic metals that occur in transuranic waste (TRU), into precipitable forms that are mobile (Lloyd 2003).

The resistance and detoxifications mechanisms used, involve specific biochemical pathways that can detoxify Cr(VI), such as the extracellular reduction of Cr(VI), and the efflux of chromate ions from cell cytoplasm (Viti et al., 2014). In recent years, chromate reductases have garnered interest among the researcher around the world due to the promise of using these processes for remediation of metals under energy saving and environmentally compatible conditions.

1.4 Microbial Cr(VI) Reduction

Microorganisms, in most cases, require organic carbon sources to reduce Cr(VI). A carbon source can be used as either an electron donor, or an energy source (He et al. 2009). The carbon source has, in the past, been provided in the form of simple sugars such as glucose or lactate. More complex carbon sources from commercial agar or broth, such as Luria-Bertani (LB), have been tested in separate experiments (Molokwane and Chirwa 2008). Molokwane and Chirwa (2010) used sawdust as a carbon source for Cr(VI) reduction. Smith et al. (2002) found that Cr(VI) reducing bacteria were able to utilise a range of carbon sources including: hydrogen plus carbon dioxide, propylene glycol, L-asparagine, D-xylose, acetate, ethanol, glycolate, sucrose, and glycerol. The feasibility for commercial bioremediation technology could be reduced if the available carbon sources are too expensive (Vidotti et al. 2014). The possibility of using algae, and algae metabolites, as carbon sources for the bacterial reduction of Cr(VI) is as of yet still unexplored.

On the other hand, photoautotrophic algae use CO_2 (a greenhouse gas) as the primary carbon source to produce biomass. Dvoretsky et al. (2017) has found that the cultural liquid of microalgae can be as the basis of the nutrient medium used in the cultivation of the bacteria *Lactobacillus casei* B-3241. Algae cells, as well as the extracellular and intracellular compounds produced by the algae, can possibly be utilised as a substrate for bacteria (Bruckner et al. 2008). Algae cell internal metabolites are released by rupturing the algae cell walls, causing numerous compounds to leak from the algae cells. These compounds include primary metabolites such as organic acids, amino acids, sugars, and sugar alcohols, as well as other compounds such as carbohydrates, proteins, and lipids (Cicci et al. 2017; Dong et al. 2016).

Previous researchers have found that algae and bacteria were able to detoxify pollutants in a synergistic manner. During photosynthesis algae produce O_2, which is required by the

aerobic bacteria, and the bacteria provides CO_2 and other stimulatory means to support the photoautotrophic algae. The algae-bacteria consortia based bioremediation processes, have the potential to form a system that is self-sustaining. Compared to conventional remediation technologies that have high costs and produce secondary pollutants, a self-sustaining system can have a smaller impact on the environment, and be cheaper (Fu et al., 2016).

1.5 Objectives of the Study

The main objective of this research is to determine if Cr(VI) reducing bacteria (CRB) can utilise algae metabolites as carbon sources. By eliminating the need for additional glucose, the bioremediation process can be rendered even more cost effective.

The sub-objectives for this research are:

- Isolate CRB and algae from local environments and identify each species.
- Test algae and bacteria resistance towards Cr(VI) toxicity.
- Investigate Cr(VI) reduction kinetics of indigenous Cr(VI) reducing bacteria.
- Investigate the algae's ability to decrease the total amount of chromium in the system through the adsorption of the reduced Cr(VI).
- Evaluate if the algae-bacteria system is a practical remediation alternative.
- Compare the ability of different algae to serve as a carbon source for CRB.
- Develop a kinetic model for the algae-bacteria system

1.6 Main Findings

The ability of different algae to produce, or serve as, a carbon source for locally isolated bacteria that were used to reduce Cr(VI) to Cr(III) was tested. The complete removal of Cr(VI) was achieved within 24 hours in batch experiments with initial concentrations of up to 50 mg/L of Cr(VI). 92% of the Cr(VI) could be removed within 24 hours from a system with an initial concentration of 100 mg/L of Cr(VI). All of the tested algae species were found to be very sensitive to Cr(VI) toxicity. The Cr(VI) inhibited algae growth as well as reducing the algae's chlorophyll *a* content and, thereby, the algae's ability to undergo photosynthesis was also diminished.

The utilisation of carbon sources that are produced by algae would improve the real world practicality of the investigated bioremediation process over the use of glucose as a carbon source. This demonstrates the potential to decontaminate Cr(VI) polluted sites in South Africa through the combination of various green algae and Cr(VI) reducing bacteria that has been isolated locally.

1.7 Dissertation Layout

This dissertation is subdivided into seven chapters. The different sections are summarised below:

Chapter 1 – The first chapter introduces the main topic of this study by discussing the background, environmental and health concerns, treatment methods, microbial Cr(VI) reduction, the objectives of the study, the main findings and the dissertation layout

Chapter 2 – In the literature study, previous work and advances published in the Cr(VI) bioremediation field is discussed as well as the methodology of this study. Cr(VI) treatment methods; chemical physical and biological techniques are also included in this chapter.

Chapter 3 – The materials and methods chapter describes the details of the microorganism isolation and identification. This chapter also explains the experimental setup and include the chemicals and reagents used in this study.

Chapter 4 – This chapter focuses on the bacterial reduction of Cr(VI) and the verification of the kinetic model.

Chapter 5 – This chapter explains and illustrates the interactions between the algae and bacteria, and the algae and Cr(VI).

Chapter 6 – This chapter discusses the results from the CRB-algae system and presents a kinetic model for the Cr(VI) reduction in the CRB-algae system.

Chapter 7 – Presents the conclusions from this study and recommendations for future experiments.

CHAPTER 2 LITERATURE STUDY

2.1 Sources and Prevalence of Chromium

Elemental chromium was first discovered in Siberian red lead ore, $PbCrO_4$, by the French chemist Louis-Nicolas Vauquelin in 1797 (Schweitzer and Pesterfield, 2010). In 1798 the same metal was found to cause the green colouration in a Peruvian emerald, and as a result chromium was named after the ancient Greek word chromos, meaning colour, which is appropriate as many chromium compounds are richly coloured (Schweitzer and Pesterfield, 2010; Sahoo et al., 2013; Lepora, 2006). Chromium is usually contained in ores and minerals found in the earth's crust (Lepora, 2006). There are approximately 40 known chromium ores, and of these, chromite has the most economic potential (Nriagu and Nieboer, 1998). South Africa has one of the largest chromite deposits (Nriagu and Nieboer, 1998; IETEG, 2005). Around 49 % of the world's chromite is produced in South Africa and poses a high risk of pollution in the regions where chromite is mined and refined. (IETEG, 2005). Chromium is mined from seams containing trace amounts of platinum group metals and other base metals. In South Africa, the country with the largest reserve of chrome ore, the main seams include: Chromite: $(Mg,Fe^{2+})(Cr,Al,Fe^{3+})_2O_4$ – the main component of Merensky Reef of the Bushveld Igneous Complex in South Africa (von Gruenewaldt and Hatton, 1987; Wagner, 1923); Barbertonite: $Mg_6Cr_2(CO_3)(OH)_{16}.4H_2O$ – the major component of the Eastern Bushveld Igneous Complex (Meli, 2009; Calas et al., 1984); and Nichromite: $(Ni,CoFe^{2+})(Cr,Fe^{3+},Al,)_2O_4$, which was discovered first in the Bon Accord nickel deposit in Barberton District, South Africa (Tredoux et al., 1989).

In Brazil, the wealthiest chrome ore seam is in the Campo Formoso layered intrusion which also contains chromite (Cramer et al., 2004; Boukili et al., 1984). Consequently, tailing dumps and process waste stockpiles at chrome mining and ferrochrome processing sites contain significant levels of other Platinum Group and Rare Earth Metals (Chirasha and Shoko, 2010; von Gruenewaldt and Hatton, 1986). Chromium in the tailing dumps can exist either as Cr(III) or Cr(VI) depending on the environmental conditions within the landfill. Interaction with other metals at different oxidation states can influence the stability of the oxidation state of the chromium species inside the waste dump and its ability to leach into the surrounding water bodies (Tiwary et al., 2005; Ma and Garbers-

Craig, 2006). In areas where the leachate water enters agricultural supply water, there is a high risk of contamination of food products and bioaccumulation into higher order organisms (Chirasha and Shoko, 2010).

Chromium occurs naturally in the environment, predominately as a mineral ore, and is also produced by anthropogenic sources where roughly 35 % of the released chromium is Cr(VI) (Kotas and Stasicka, 2000; IETEG, 2005). The most significant anthropogenic sources of Cr(VI) emissions are chrome plating, chemical manufacturing of Cr(VI), evaporation cooling towers, and chromite mining (IETEG, 2005; Cheng et al., 2014). Cr(VI) is also discharged into surface waters from leather tanning, stainless steel production, wood preservation, pigment production, electroplating, and electronic manufacturing facilities (Cheng et al., 2014;).

Sodium chromate (Na_2CrO_4) is produced when roasting chromite in the open air (Xu et al., 2006; IETEG, 2005). Sodium chromate is used as a corrosion inhibitor in the petroleum industry, as a dyeing auxiliary in the textile industry, and as a wood preservative (Nriagu and Nieboer, 1998). Sodium chromate can be converted to sodium dichromate, which is the precursor to most chromium products; both sodium chromate and sodium dichromate are oxidising agents used in the tanning of leather (Nriagu and Nieboer, 1998). Chromic acid is mostly used for cleaning glassware (Steward, 1980), and chromic oxide is typically used in pigment production (Darrie, 2001).

Cr(VI) is mostly found in the effluent wastewater from the metallurgical industry, refractory industry, metal finishing industry, and the colour pigments and corrosion inhibition pigments production industry (IETEG, 2005). Cr(III) is mostly found in the effluent wastewaters of tanneries, textile, and decorative plating industries (IETEG, 2005).

Chromium compounds have a high resistance to corrosion, which has led to chromium being used as a polish, and it was added to cooling water to inhibit corrosion in power plants in the past (Nriagu and Nieboer, 1998). Due to chromium's strength, hardness, and temperature resistance, it is used in the manufacturing of refractory furnace lining bricks (Nriagu and Nieboer, 1998).

Cr(VI) industries play a crucial economic role in South Africa (Cawthorn, 2010). Mining and industrial uses of Cr(VI), produce waste products that enter the environment and are often challenging to remediate. In such aqueous waste, Cr(VI) is present as either

dichromate ($Cr_2O_7^{2-}$) in acidic environments or as chromate (CrO_4^-) in alkaline environments (Srinath et al., 2002)

2.2 Chromium Chemistry

Chromium exists in nine different valence states ranging from –2 to +6 (Cainelli and Cardillo, 1984). Cr(III) and Cr(VI) are the most stable valence states and occur most frequently in neutral pH and temperature ranges (Cheung and Ji-Dong Gu, 2007; IETEG, 2005). Cr(VI) species in solution are most often soluble chromate (CrO_4^{2-}), hydrogen chromate ($HCrO_4^-$), chromic acid (H_2CrO_4), or dichromate ($Cr_2O_7^{2-}$) depending on the pH of the solution and redox potential (Rai et al., 1989). These Cr(VI) species are highly soluble in water and does not form insoluble precipitates, therefore direct precipitation is not possible (Nriagu and Nieboer, 1998).

Cr(III) usually forms stable complexes with organic and inorganic ligands at neutral pH. (Gutiérrez-Corona et al. 2016) The most probable Cr(III) species in solution are Cr^{3+}, $Cr(OH)_2^+$, CrO^+, $HCrO_2$ and CrO_2. Naturally occurring chromium most frequently occur in the form of Cr(III) (Jobby et al., 2017), which is stable in acidic conditions and mostly immobile in the environment as it has a strong affinity for negatively charged ions and colloids in soils, and produces hydrated compounds such as Cr(OH).

High Eh values prefer oxidising species such as H_2CrO_4. In relatively low pH conditions, the main aqueous Cr(III) species (Morrison and Murphy, 2010). At pH values higher than 7, the $HCrO_4^{2-}$ form exists, while between the pH range of 1 and 6, CrO_4^{2-} and $Cr_2O_7^{2-}$ ions are predominant. (El Nemr et al., 2015).

2.3 Chromium Toxicity

The toxicity level is related to the speciation of chromium and is primarily due to its high reactivity, ability to penetrate biological membranes, as well as its high oxidising capabilities. (Singh and Tripathi, 2007)

Cr(III) has been estimated to be a thousand less toxic than Cr(VI) due to the fact that Cr(III) is less mobile and cell membranes are impermeable to most Cr(III) complexes (Singh and Tripathi, 2007). Cr(III) is nutritionally required, in trace amounts, for natural carbohydrates and lipid metabolism. (Pechova and Pavlata, 2007) However at high

concentration Cr(III) can cause negative effects on cellular structures (Pradhan et al., 2017).

Cr(VI) is highly toxic to all living organisms, causes mutations in bacteria, and is mutagenic and carcinogenic to both humans and animals (Singh and Tripathi, 2007). Therefore, Cr(VI) is classified as an initiator of carcinogenesis. Cr(VI) is also responsible for causing congenital disabilities by accumulating in the placenta (Cheung and Gu, 2007). Due to the structural similarity of CrO_4^{2-} with phosphate ion (PO_4^{3-}) and sulphate ion (SO_4^{2-}), Cr(VI) can easily diffuse, via sulphate transport channels, across the cell membrane in prokaryotic and eukaryotic organisms (Arita and Costa 2011; Gutiérrez-Corona et al. 2016). Inside the cells Cr(VI) generates active Cr(V) or Cr(IV) intermediates, with free radicals and Cr(III) as the final product. The Cr(III) inside the cell interferes with DNA replication and causes mutagenesis. Among the effects that result in mutagenesis due to exposure to high levels of Cr(VI) are: oxidative DNA damage (Tsou et al. 1996), DNA–DNA interstrand crosslinks (Xu et al. 1996; Singh et al. 1998a; Bridgewater et al. 1994), Cr–DNA adducts (Singh et al. 1998b; Xu et al. 1994), single-strand breaks (Bridgewater et al. 1994), DNA–protein crosslinks (Xu et al. 1994), chromosomal aberrations (Wise et al. 1992), DNA polymerase arrest, RNA polymerase arrest (Bridgewater et al. 1994), and the inhibition of DNA transcription and translation (Pritchard et al. 2000). These damages can alter the function of cells leading to cancers in the liver, kidney and lungs developing. At high concentrations, Cr(VI) is acutely toxic with visible symptoms such as burn-like symptoms and skin shading.

Exposure to Cr(VI) usually occurs through inhalation, ingestion, or dermal contact (IETEG, 2005: 215). Other health risks associated with chromium exposure are allergic dermatitis, ulceration of the skin, irritation of the mucous membranes and nasal septum, renal tubular necrosis, eardrum perforation, lung carcinoma, and an increase in the risk of respiratory tract infections (Browning and Wise, 2017; Cheung and Gu, 2007). Ingestion of Cr(VI) compounds result in severe gastrointestinal disorders, haemorrhagic diathesis, convulsions and ultimately death (Baruthio 1992).

The recommended acceptable concentration of chromium in drinking water is between 50 µg/L and 100 µg/L (Cheung and Gu, 2007; Sarkar, 2002: 266). The Cr(VI) LD50 for oral toxicity in rats is between 50 mg/kg and 100 mg/kg, and the Cr(III) LD50 is between 1900 mg/kg and 3000 mg/kg (Dhal et al., 2013). Chromium can accumulate in living organisms,

which is why the World Health Organization (WHO) recommends that the maximum acceptable concentration of chromium is 0.05 mg/L in drinking water, and 1 mg/L in wastewater (WHO 2004; US EPA 1998).

2.3.1 Cr(VI) Toxicity on Plants and Microalgae

Cr(VI) is usually directly emitted from industrial sources into the atmosphere, soil, or aquatic systems (IETEG, 2005). Cr(VI) can also be released into the environment through accidental spills, illegal disposal, and inappropriate storage (Latimer, 2015). Cr(VI) is extremely persistent, soluble, mobile, and bioavailable in soil and aquatic environments (Latimer, 2015).

The impact of Cr(VI) on the environment depends mostly on the Cr(VI) concentration. Cr(VI) can have moderate to high acute toxic effects on plants that may cause death or low growth rates (Latimer, 2015; Zeng et al., 2011). Cr(VI) usually enters plants through carriers via the root system, and can impede germination by damaging the plant's root system (Shanker et al., 2005), reduced root growth, stem growth and leaf development was also observed (Tiwari et al., 2009). Cr(VI) exposure are known to have a negative impact on photosynthesis in terms of electron transport, CO_2 fixation, and photophosphorylation. Cr(VI) exposure causes a decrease in total chlorophyll and carotenoids have been well-established (Rodriguez et al., 2012).

Scorching and necrosis of vegetation were observed near a chrome-plating factory in Switzerland (National Research Council, 1974). Accidental spillage of $Na_2Cr_2O_7$ occurred in Japan in 1969 and caused nearby rice plants to display dark spots. Cr(VI) is detrimental to plant growth, photosynthesis, and enzyme activities. (Shanker et al., 2005; Davies et al., 2002). For example, Cr(VI) exposure at 5-20 mg/L levels has been shown to prevent germination of monocotyledonous plant seeds.

Toxic effects of Cr(VI) on microorganisms such as algae include growth, respiration and photosynthesis inhibition. Cr(VI) can degrade the electron transfer system, thereby impairing electron transfer between Photosystem II (PSII) and Photosystem I (PSI) (Khalida et al., 2012) as well as causing the destruction of the PSII reaction centre of *Chlorella pyrenoidosa* (Hörcsik et al., 2007).

Cr(VI) also induced alterations in the cytoskeleton of the algae which may be involved in the loss of motility. Cr(VI) had a phototoxicity effect where the algae that were grown in

the dark was less affected by the Cr(VI) than algae grown in the light. Different algae species displayed different retention of Cr(VI); green algae retain more Cr than red or brown algae (Cervantes et al. 2001).

The toxicity of Cr(VI) has also been traced to the reactive intermediates (ROS). *Chlamydomonas reinhardtii* exposed to Cr(VI) tends to generate ROS, which can attack thylakoid membrane lipids (mainly unsaturated fatty acids). The peroxyl-radical chain reactions were initiated which can destroy membranes and indirectly can damage the structural pigment-protein complexes located in chloroplast membranes (Kumar et al., 2014). Rodríguez et al., (2007) reported that the complete pheophinitization of the chlorophylls occurred including the modification of the carotenoids.

Cr(VI), as opposed to Cr(III), can be leached out of the soil and enter groundwater, then enter aquifers and migrate to surface waters (IETEG, 2005). Cr(VI) is very toxic to aquatic organisms and can also have long-term chronic effects on the aquatic environment (Latimer, 2015). It is clear that Cr(VI) has adverse effects on humans, animals, and the environment, and therefore it is critical to find a clean and low-cost method to treat and remove Cr(VI) from the environment.

2.4 Treatment Technologies for Cr(VI)

Cr(VI) treatment technologies employ either toxicity reduction methods, destruction and removal methods, or containment methods (Mulligan et al., 2001). An effective treatment method requires an understanding of both Cr(VI) and the properties of the associated pollution site. (Wuana and Okieinmen, 2011) Some of these properties include the Cr(VI) compound's molecular structure, and the site's pH level.

2.4.1 Chemical and Physical Remediation Processes

Chemical remediation processes include and reduction at low pH followed by precipitation as chromium hydroxide ($Cr(OH)_3(s)$) at a higher pH. The reduction reaction requires considerable amounts of energy to overcome the activation energy, which is caused by the difference in electric potential between the two states (Rita and Ravisankar, 2014). Iron(II) chloride or iron(II) sulphate are frequently used as electron donors during the chemical reduction reaction and produces a large amount of solid waste such as ferric hydroxide (Jardine et al., 1999). Other electron donors include elemental Fe, manganese oxide,

methane and reduced organics such as humic acids, fulvic acids, and amino acids. The reduction reaction is accelerated at a pH below 3 (Jardine et al. 1999). After reduction, additional reagents such as sodium hydroxide or calcium hydroxide slurry are then added to precipitate the Cr(III) (Barrera-Díaz et al., 2012). The chemical reduction-and-precipitation method is becoming less appealing for Cr(VI) pollution treatment as it uses expensive chemical reductants, ineffective to treat dilute Cr(VI) solution and it generates large volumes of residual toxic sludge which requires specialised storage (Cabatingan et al. 2001).

Physical remediation processes focus more on the removal of Cr(VI) from the environment, whereas chemical and biochemical remediation processes convert Cr(VI) to the less toxic Cr(III) form (Palmer and Wittbrodt, 1991). The physical remediation processes utilise techniques such as ion exchange (resin adsorption) activated carbon adsorption, composite ceramic adsorption, carbon nanofibers adsorption, membrane filtration (reverse osmosis), photocatalysis, electrolysis, electrocoagulation (Rita and Ravisankar, 2014; Kaprara et al., 2013).

Ion exchange is a physical treatment process through which ions of a given species are displaced from an insoluble exchange material, by ions of a different species in a solution (Suthersan and McDonough, 1996). This process has a high removal efficiency, with fast kinetics, and is designed to recover Cr(III) (Kaprara et al., 2013).

Adsorption is a treatment process where Cr(VI) is adsorbed onto the surface of an adsorbent. The adsorption mechanism can include electrostatic attraction, ion exchange, chelation, chemisorption and complexation processes (Mohan and Pittman 2006) An advantage of this process is that the kinetics of Cr(VI) adsorption is relatively fast (Madhavi et al., 2013). Many studies have found that anionic Cr(VI) species (CrO_4^{2-} and $HCrO_4^-$) is adsorbed onto positively charges surfaces at low pH values. Adsorption of Cr(VI) is a function of pH, and only below a pH value of 3 are the adsorption rate adequately high. The drawback of adsorption processes is the chemical addition as a pre-treatment to lower pH (Kera et al. 2017).

Membrane filtration utilises a semi-permeable barrier between two phases, that could restrict the movement of Cr(VI). The types of membranes used are classified as ultrafiltration, nanofiltration, and reverse osmosis (Madhavi et al., 2013). Membrane

filtration processes require a high capital investment, as well as high maintenance costs (Kaprara et al., 2013)

Electrochemical reduction is another physical treatment method; it uses two electrodes namely a cathode and an anode. At high currents Cr (VI) is reduced directly at the cathode and at low currents, the Cr (VI) is converted to Cr (III) through reduction by additional Fe(II) ions (Mukhopadhyay et al., 2007).

Most physical and chemical treatment processes have disadvantages such as high operational and maintenance cost, require large amounts of additional chemicals, the production of secondary pollution, high energy consumption, or a combination of these disadvantages. A cost-effective approach is needed for remediating Cr(VI) contamination. Bioremediation is a promising remediation technology option which could lead to a smaller amount of secondary pollution and should be inexpensive (Rita and Ravisankar, 2014). Table 2.1 shows all the disadvantage and advantage for different Cr(VI) treatment methods (Owlad et al., 2009).

Table 2.1: Advantages and disadvantages of different Cr(VI) removal methods

Treatment method	Advantages	Disadvantages
Chemical reduction	High removal efficiency Fast kinetics	High activation energy Acid conditions Generates residual sludge Expensive chemical reductants Ineffective to treat dilute concentration Not environmentally friendly
Ion exchange	High removal efficiency Fast kinetics Effective to treat dilute concentration Low maintenance	Regeneration or replacement of resins required Optimal under acid conditions
Adsorption	Fast kinetics No secondary pollution Inexpensive sorbents	Ineffective to treat dilute concentration Optimal under acid conditions Limited sorption–desorption cycles
Electrochemical reduction	High selectivity Low cost	Generates residual sludge Optimal under acid conditions
Bioremediation	High removal efficiency Inexpensive microorganisms Neutral pH No secondary pollution Effective to treat dilute concentration Low maintenance Effective to treat dilute concentration	Slow kinetics Nutrient and carbon source requirements

2.4.2 Biological Remediation Processes

Bioremediation is a treatment process where living organisms are used to destroy, or render harmless, a toxic contaminant (Vidali, 2001). Usually naturally occurring microorganisms such as bacteria, yeast, algae, fungi, protozoa, or plants are used for bioremediation (Vidali, 2001; Zahoor and Rehman, 2008). Some of the biological remediation processes include biotransformation, biosorption, and bioaccumulation (Whiteley and Lee, 2006). These processes are overlapping with subtle differences.

Biotransformation

As the name suggests, the biotransformation processes result in the transformation of a toxic substance into a less toxic substance (Krishna and Philip, 2005). Microbes are able to adapt and thrive in adverse conditions, such as high acidity, alkalinity, toxicity, and temperatures (Lowe et al., 1993). These microbes develop a biological resistance or tolerance against specific hazardous contaminants in the environment (Sinha et al., 2009; Brannan, 2006). This tolerance has evolved from continual exposure to toxic compounds and allows the organism to withstand or neutralise the pollutant. Biotransformation can be achieved either by direct enzymatic reduction or indirectly by producing complexes with metabolites (such as H2S or Fe(II)) (Thatoi et al., 2014). A wide range of microbes has been identified to have Cr(VI) tolerance, resistance, and even reducing ability (Chirwa and Molokwane, 2011). Biotransformation is based on the advanced, and well conserved, membrane electron transport respiratory apparatus within the microbe (Chirwa and Molokwane, 2011).

Bioaccumulation

The bioaccumulation process is similar to the biosorption process, except it is an active process that requires energy from the microbes or plants involved (Abbas et al., 2014). Bioaccumulation consists of metal ion attachment or uptake processes which are metabolically dependent and require living biomass, whereas biosorption is metabolic-independent and mostly uses non-living biomass (Bilal et al. 2018). Metal bioaccumulation onto a microorganism cell surface consists of an initial rapid adsorption. The subsequent phase is a slower metabolism-dependent transport of the metal ion into

the cell cytoplasm through the cell membrane with the aid of transporter proteins, and is bioaccumulated inside the cell (Sinha et al., 2009; Jobby et al. 2018).

Biosorption

Biosorption involves the removal of a toxic substance by utilising the adsorptive properties of microbes, plants or dead biomass to transfer the substance from aqueous solutions to a more appropriate medium (Sinha et al., 2009). Dead biomass has powerful adsorption properties without the constraints of living organisms, which can only survive in certain conditions and need nutrients for growth. Active functional groups on the cell wall of the dead biomass can adsorb toxic metal which can be quickly recovered by altering the medium conditions. Biosorption can occur either with or without transformation (Birungi and Chirwa, 2014)

Biosorption is a reversible process that consists of both adsorption and desorption. There are a few factors that affect this process, including the pH, initial metal concentration, ionic strength, and temperature of the solution (Das et al., 2008). pH plays a vital role in biosorption; it affects both the chemistry of the solvent, in which the metal ions are present, as well as the chemistry of the cell wall of the sorbent.

There are a few different types of low cost biosorbents such as seaweed, agricultural waste, cane molasses, maize stocks, wood chips, grass, maize tassels, yeast, fungi, bacteria, and algae. The chemical groups on the biosorbents that contribute to the binding to the metal ion include hydroxyl, carbonyl, carboxyl, sulfhydryl, thoether, sulfonate, amine, imine, amide, imidazol, phosphonate and phosphodiester (Mohan and Pittman 2006; Volesky 1987). Microalgae were found to better biosorbents, specifically brown algae (Volesky, 1994; Wang and Chen, 2009; Sen and Dastidar, 2010).

Benefits and Limitations of Bioremediation

Bioremediation is limited by the initial concentration of Cr(VI) as it can deactivate certain microbes and cause cell loss at high concentrations (Krishna and Philip, 2005; Wang and Shen, 1995). Bioremediation does not require a high-energy input, does not produce a significant amount of chemical by-product during treatment, and can employ native, non-invasive microbes if available (Rita and Ravisankar, 2014; Chirwa and Molokwane, 2011).

Indigenous bacteria not only result in better remediation performance but will also be safer to use in the local environment because they will not alter the existing microbial communities (Chirwa and Molokwane, 2011: 89). Microorganisms usually need an organic carbon source to be able to reduce Cr(VI). The carbon source can be used either as an energy source, or as an electron donor (He et al. 2009). The cost of the carbon source can conceivably limit the commercial application of bioremediation technology (Vidotti et al. 2014).

2.5 Bacterial Cr(VI) Detoxifying Mechanisms

2.5.1 Bacterial resistance

The resistance and detoxifications mechanisms involve specific biochemical pathways that can detoxify Cr(VI), such as extracellular reduction, adsorption, DNA methylation, cellular accumulation, intracellular reduction, precipitation and efflux mechanisms (Viti et al. 2014; Pradhan et al. 2017).

Decreasing the Cr(VI) uptake into the cells can be an effective defensive strategy. Cr(VI) is usually present in the environment as chromate (CrO_4^{2-}) which has a similar structure to sulphate (SO_4^{2-}) and phosphate (PO_4^{3-}). As a result, Cr(VI) is readily taken up by eukaryotic and bacterial cells through the sulphate or phosphate transport system (Karthik et al., 2017). The transport of chromate can be limited if the chromosome-encoded sulphate uptake pathway in bacteria is mutated (Ramirez-Diaz et al., 2008). The chromate ion transporter protein ChrA is also responsible for the transport of Cr(VI). The ChrA protein, also known as the chromate efflux transporter protein, decreases chromate accumulation inside the bacterial cell and is crucial for chromate resistance in bacterial species (Cervantes et al., 1990). O_2^-)

Certain bacteria have the ability to produce enzymes can that repair damaged DNA. Inside the bacterial cells Cr(VI) is readily reduced to Cr(III) through various enzymatic or non-enzymatic activities. As a result, reactive oxygen species (ROS) are produced which can lead to DNA damage as discussed in section 2.3. As a response, enzymes that can repair DNA (RecA, RecG and RuvB), as well as enzymes that are ROS scavengers, are produced by the bacterial cell. (Hu et al., 2005; Ahemad, 2014).

Bacterial reduction, especially extracellular reduction, can also be considered as an effective Cr(VI) resistance mechanism (Dhal et al., 2013). Although the Cr(VI) reducing ability was found in both sensitive and resistant bacterial strains (Ahemad, 2014)

2.5.2 Bacterial Cr(VI) Reduction Mechanisms

Bacteria obtain energy for metabolism by participating in several redox reactions. Bacteria have evolved the ability to mediate various oxidation-reduction couplings to conserve energy, which created a pathway for transforming toxic substances as electron donors or electron sinks (Diaz, 2004; Boopathy, 2000). Cr(VI) may be reduced either as a response to Cr(VI) toxicity or because of a physiological need to conserve energy in the cell through a dissimilatory pathway reaction (Joutey, 2015).

Bacterial Cr(VI) reduction can either be enzymatic or non-enzymatic. A CRB cell deliberately produces Cr(VI) reducing enzymes also known as reductases, when Cr(VI) is detected in the solution. Therefore, these processes are highly regulated (Extracellular Reduction). During enzymatic Cr(VI) reduction the reaction is catalysed by either soluble cytosolic proteins or insoluble cell membrane enzymes. Bacterial chromate reductases include ChrR, LpDH, NemA, NfsA, YieF, cytochromes and hydrogenases; these enzymes can function under both aerobic and anaerobic conditions (Gutierrez-Corona et al., 2016). The enzymatic Cr(VI) reduction mechanism is discussed below. The complex interaction between the bacteria cell and chromate are depicted in Figure 2.1 (Chai et al., 2019).

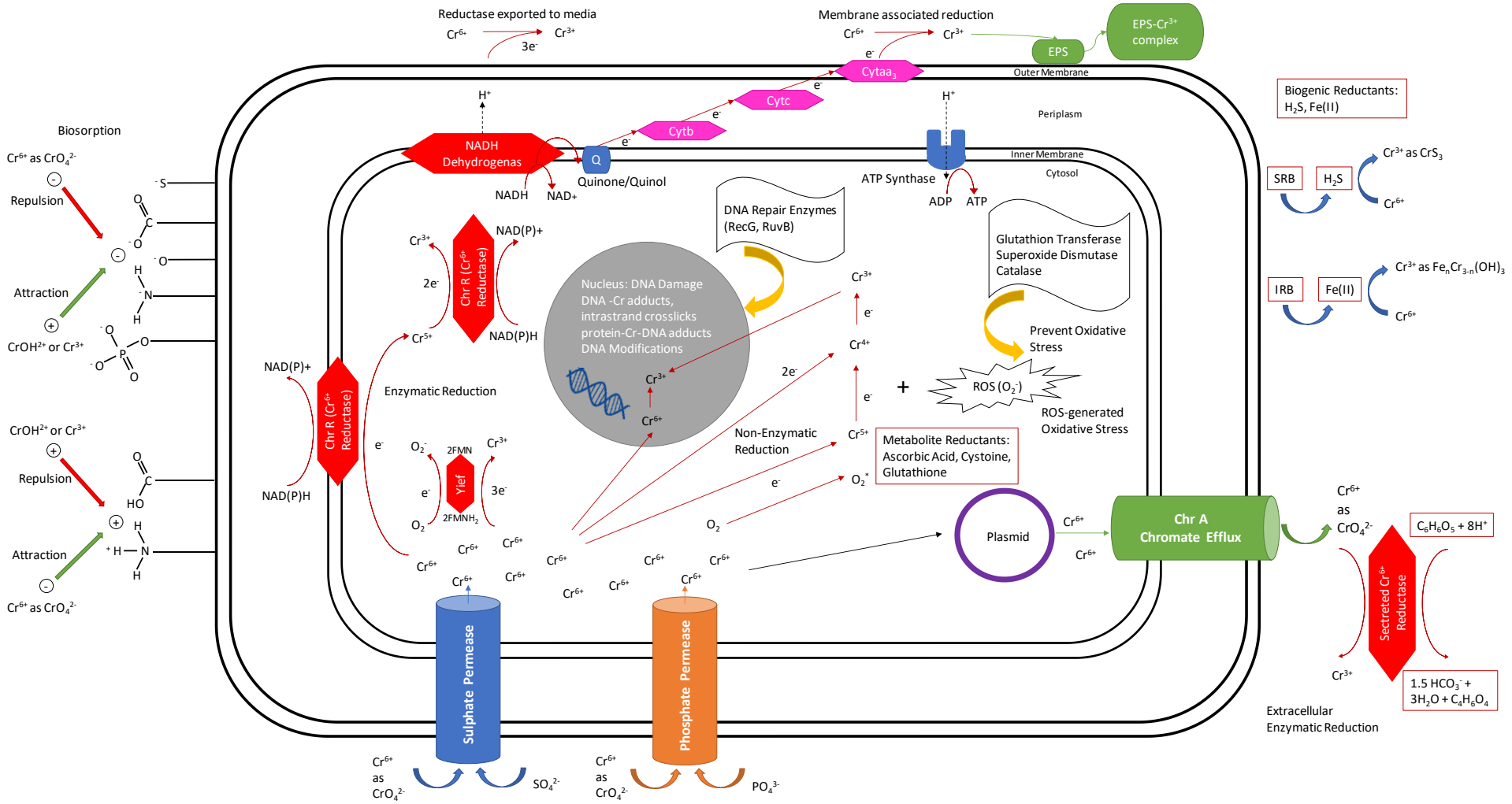

Figure 2.1: Resistance and Cr(VI) reduction mechanisms of CRB.

A wide variety of bacteria are known to have evolved a biochemical pathway that can reduce Cr(VI) to Cr(III) either aerobically or anaerobically (Chirwa and Wang, 2001). The genes that are responsible for Cr(VI) reduction can be either plasmid-borne or located on the chromosomal DNA (Thatoi et al.,2014).

Anaerobic

During anaerobic reduction, Cr(VI) serves as the terminal electron acceptor. Anaerobic reduction of Cr(VI) is usually a membrane bound process. Cr(VI) reduction by bacteria leads to high consumption of protons as reducing equivalents, which will increase the background pH. The increased pH could lead to the precipitation of the reduced Cr as chromium hydroxide, $Cr(OH)_3(s)$ or hydrated oxide ($Cr_2O{\cdot}H_2O$). Equations 2.1 and 2.2 below show Cr(VI) reduction with CRB, where CrO_4^{2-} needs to accept three electrons. Equation 2.3 shows a reaction under anaerobic conditions using acetic acid as a carbon source and electron donor (Chirwa and Molokwane, 2011; Singh et al., 2011)

$$CrO_4^{2-} + 8H^+ + 6e^- \xrightarrow{\text{Bacteria}} Cr^{3+} + 4H_2O \qquad 2.1$$

$$Cr^{3+} + 4H_2O \xrightarrow{\text{neutral pH}} Cr(OH)_3(s) + 3H^+ + H_2O \qquad 2.2$$

$$3CH_3OO^- + 4HCrO_4^- + 4CrO_4^{2-} + 33H^+ \rightarrow 8Cr^{3+} + 6HCO_3^- + 20H_2O \qquad 2.3$$

Sulphate and iron reducing bacteria can produce Fe(II) and H_2S as metabolites under anaerobic conditions. Biogenic Fe(II) and H_2S can also reduce Cr(VI) to Cr(III) (Gutierrez-Corona et al., 2016).

Aerobic

Under aerobic conditions the bacteria use a carbon substrate as the electron donor and oxygen as the electron acceptor. Aerobic reduction is a cometabolic process where bacteria do not gain energy from the reduction process. Instead, the contaminant is reduced via a side reaction (Thatoi et al., 2014). The enzymes responsible for the reduction of Cr(VI) to Cr(III) require NAD(P)H as a co-factor. A two step reaction have been proposed: first Cr(VI) accepts one electron from one molecule of NADH to generates Cr(V) as an intermediate (Equation 2.4), and then Cr(V) accepts two electrons to form Cr(III) (Equation 2.5). This reaction frequently occurs inside the bacterial cell.

$$Cr^{6+} + e^{-} \rightarrow Cr^{5+} \qquad 2.4$$

$$Cr^{5+} + 2e^{-} \rightarrow Cr^{3+} \qquad 2.5$$

CRBs can operate under a wide range of conditions. Both gram-positive and gram-negative CRBs have been identified, although it has been suggested that gram-positive CRBs are more resistant than gram-negative CRBs (Dermou et al., 2005).

Soluble Reductase Reduction

Soluble reductases are associated with aerobic reduction. Soluble reductases can participate in both extracellular and intracellular reduction. Cytosolic proteins are soluble chromate reductases such as flavin reductases, nitrate reductases, flavin proteins and ferric reductases. (Thatoi et al., 2014). YieF, a soluble chromate reductase, is unique as it facilitates the direct reduction of Cr(VI) to Cr(III) through a four-electron transfer shuttle, in which three electrons are consumed during the reduction of Cr(VI), and the other electron is transferred to oxygen. Cr(VI) reduction mediated by YieF generates very little ROS inside the bacterial cell (Park et al., 2002).

The soluble reductase is produced either through necessity or for enhanced activity by the bacterial cell to reduce Cr(VI) to Cr(III), NADH serves as the electron donor in the cytoplasm. Other carbon substrates, such as glucose, can also serve as an electron donor.

However soluble reductases have been found to mediate the Cr(VI) reduction process under aerobic conditions (Thatoi et al., 2014).

Membrane Bound Reduction

Anaerobic Cr(VI) reduction involving membrane-associated reductases such as flavin reductase, cytochromes and hydrogenases, have been observed by Romanenko and Koren'kov (1977). Membrane bound reductase can reduce Cr(VI) extracellularly by using electron shuttling compounds coupled to cytoplasmic membrane reduction and the flux of protons through ATP-synthase. The proton flux and production of ATP requires energy for use in cellular metabolism (Chirwa and Molokwane, 2011).

As stated in section 2.5.2, during, membrane-associated reductase activity, Cr(VI) acts as a terminal electron acceptor during anaerobic bacterial respiration (Horitsu et al., 1987; Chirwa and Molokwane, 2011). NADH, NADPH or formate can serve as the electron donors for the reductase. In some cases, the CRB even utilised H_2 as an electron donor. The reduced chromate is known to precipitate as insoluble $Cr(OH)_3$ on the bacterial cell surface. Thus, such a reduction mechanism protects cells further from Cr(VI) toxicity (Thatoi et al., 2014)

Enzymatic Intracellular Reduction

During intracellular reduction, Cr(VI) is reduced in the cytosol via cytoplasmic soluble reductase enzymes. Intracellular reduction depends on Cr(VI) interaction and biosorption as well as the membrane transport systems available to transport Cr(VI) into the bacterial cells. Reduced Cr(VI) species would be difficult to remove from inside the cells if the cell membranes remained intact.

Enzymatic Extracellular Reduction

Cr(VI) reducing enzymes/ reductases are produced purposely by the bacterial and excrete into the media to catalyse the reduction of Cr(VI) to Cr(III). Since protein excretion is an energy intensive process, the production of these enzymes only occurs Cr(VI) is detected in the solution and therefore highly regulated.

Extracellular Cr(VI) reduction via reductase has been confirmed through a mass balance of Cr(VI). The extracellular reduction of Cr(VI) is believed to be responsible for around 90 % of the reduced Cr(III) species to be found in the media. (Shen and Wang, 1993; Chirwa and Wang, 1997; Smith and Gadd, 2000).

This process is favourable as the cell does not require a transport system to transfer Cr(VI) into the cell and excrete Cr(III) back into the media. Thus, the extracellular reduction mechanism protects the cell from DNA damage. This mechanism will also allow for easy separation of the cells from the spent media (Chirwa and Molokwane, 2011).

The NADH-dehydrogenase reaction, shown in Equation 2.6 is expected to predominate under aerobic conditions.

$$2CrO_4^{2-} + 13H^+ + 3NADH \rightarrow 2Cr^{3+} + 3NAD^+ + 8H_2O \qquad 2.6$$

Priester et al. (2006) found that chromate reductases are released into the solution after the bacteria cell (*P. putida*) burst open. Thereby reducing Cr (VI) to Cr (III) extracellularly with cytoplasmatic reductases as a result of cell lysis.

Non-Enzymatic Reduction

Nonenzymatic reduction mechanism is closely related to the intracellular mechanism in terms of Cr(VI) transportation into the cell. Nonenzymatic reduction involves several components of these cells' protoplasm such as amino acids, organic acids, NADH (NADPH in some species), flavoproteins, and other hemeproteins that readily reduce Cr(VI) (Chirwa and Molokwane, 2011; Ackerley et al., 2004). Therefore, the cytoplasm fraction of a disrupted cell could be able to reduce Cr(VI). The cytoplasmic enzymic reduction is not a metabolic process, but cellular one-electron reducers cause its partial reduction. Partial reduction will directly affect the cell since such a reaction will produce unstable Cr(V) which is a harmful reactive-oxygen species (ROS) that can cause DNA damage (Joutey, 2015).

Bacteria Consortium Communities

Consortium communities of bacteria can also be used to degrade Cr(VI). Consortium communities have a rich diversity of metabolic pathways which allows the degradation of various toxic heavy metals. The microbes in a consortium are more completive and, as a result, are more likely to survive than isolated microbe strains (Joutey, 2015).

Table 2.2 illustrates the whole range of aerobic CRBs with the various carbon sources used during reduction. Most of the bacteria were isolated from a Cr(VI) contaminated site. Table 2.2 also shows the diversity in carbon sources and electron donors, which proves that bioremediation would be possible in various contaminated environments (Chirwa and Molokwane, 2011).

Table 2.2: Cr(VI) reducing bacteria isolated by various authors.

Name of Species	Carbon Sources	References
Acinetobacter calcoaciticus	Luria Bertani medium	Samantaray and Mishra, 2012
Agrobacterium radiobacter EPS-916	Glucose-mineral salts medium	Llovera *et al*., 1993
Arthrobacter sp.	M9 broth- Glucose	Megharaj et al., 2003
Bacillus cereus	Luria Bertani broth	Tanu et al., 2016
Bacillus megaterium TKW3	Nutrient broth-minimal salt medium-glucose, maltose, and mannitol	Cheung and Gu., 2007
Bacillus sp.	Vogel-Bonner (VB) broth-citric acid; D-glucose	Chirwa and Wang, 1997
Bacillus sp.	M9 broth- Glucose	Megharaj et al., 2003
Bacillus sp. ES 29	Luria-Bertani (LB) medium	Camargo *et al.*, 2003
Bacillus drentesis	Luria Betani Broth	Molokwane and Chirwa, 2009
Bacillus thuringiensis	Luria Betani Broth	Molokwane and Chirwa, 2009
Bravibacterium sp. CrT-12	Nutrient Broth	Faisal and Hasnain, 2004
Corynebacterium hoagii ChrB20	TRIS-minimal medium plus gluconate 0.2%	Viti et al., 2003
Corynebacterium paurometabolum SKPD 1204	Vogel Bonner broth	Dey and Paul, 2016
Escherichia coli ATCC 33456	Glucose, acetate, propionate, glycerol and glycine	Shen and Wang, 1994
Enterobacter sp.	Luria Betani Broth	Molokwane and Chirwa, 2009

Table 2.2: Cr(VI) reducing bacteria (continued…)

Name of Species	Carbon Sources	References
Halomonas spp.	Acetate-soil	Lara et al., 2017
Lysinibacilus sphaericus	Luria Betani Broth	Molokwane and Chirwa, 2009
Lysinibacillus fusiformis ZC1	R2A medium	He et al., 2011
Nesterenkonia sp. MF2	Nutrient broth medium	Amoozegar et al., 2007
Ochrobactrum intermedium	DeLeo and Ehrlich (DE) medium	Batool et al., 2012
Ochrobactrum sp.	Glucose	He *et al.*, 2009
Pannonibacter phragmitetus	Lactose, fructose, glucose, pyruvate, citrate, formate, lactate, NADPH and NADH	Shi et al., 2012
Pseudomonas fluorescens	Glucose-Acetate-Pyruvate-Lactate-Succinate	Bopp *et al.*, 1983
Pseudomonas fluorescens LB300	Vogel-Bonner broth	Bopp and Ehrlich, 1988
Pseudomonas mendocina	Tryptic soy broth	Dogan et al., 2014
Pseudomonas sp.	Nutrient Broth	Wani and Ayoola, 2015
Pseudochrobactrum saccharolyticum LY10	Modified Luria-Bertani media;	Long et al., 2013
Providencia sp.	Luria broth (tryptone-yeast extract)	Thacker *et al.*, 2006

Table 2.2: Cr(VI) reducing bacteria (continued…)

Name of Species	Carbon Sources	References
Rhodococcus erythopolis		Banerjee et al., 2017
Shewanella alga (BrYMT) ATCC 55627	M9 broth- Glucose	Guha *et al.*, 2001
Shewanella putrefaciens MR-1	Lactate- fumarate	Myers *et al.*, 2000
Sporosarcina saromensi	216LB medium	Ran et al., 2016
Stenorophomonas maltophilia	Feather protein hydrolysate and peptone	Bhange et al., 2016
Streptomyces violaceoruber	Starch-Casein agar (SC) medium,	Chen et al., 2014
Vigribacillus sp.	Luria-Bertani (LB) medium	Mishra et al., 2012

2.6 Algae Cr(VI) Removing Mechanisms

2.6.1 Algae

Algae are divided into two main groups: macroalgae and microalgae. Examples of macroalgae include seaweed or multicellular plants growing in freshwater or saltwater (McHugh, 2003). Microalgae are unicellular microscopic organisms that can be found in both freshwater and saltwater. The most commonly found microalgae are diatoms, golden algae and green algae (Ahmad et al., 2016: 209).

The term algae refer to a large and diverse assemblage of eukaryotic organisms that carry out oxygenic photosynthesis that oxidizes water to molecular oxygen. (Davis et al., 2003; Wang and Chen, 2009). Photosynthesis is facilitated by chlorophyll, a pigment that gives algae its green colour. Chlorophyll *a* ($C_{55}H_{72}MgN_4O_5$) and chlorophyll *b* ($C_{55}H_{70}MgN_4O_6$) helps to transfer energy from sunlight to biochemical energy for the algae. Autotrophic algae use CO_2 (a greenhouse gas) as the primary carbon source to produce biomass. Algae also produce extracellular and intracellular compounds. These cellular compounds, as well as the physical algae cell, can be used as a substrate for the bacteria (Bruckner et al. 2008). Ruptured algae cells can leak numerous compounds such as lipids, proteins, carbohydrates, and primary metabolites such as sugars, sugar alcohols, amino acids, and organic acids (Cicci et al. 2017; Dong et al. 2016).

Biosorption utilises algal biomass to remove toxic substances. Algae is a low-cost and environmentally friendly biomass alternative with high sorption capacity and the potential for recovery. Algae are found nearly everywhere, and both living as well as dead algae can remove Cr(VI) (Birungi and Chirwa, 2014; Joutey et al., 2015). Utilising living algae in toxic metal waste treatment could also improve the Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) whereas bacteria usually increase the BOD of the water (Das et al., 2017).

2.6.2 Algal Biosorption Treatment of Cr(VI)

Biosorption efficiency depends on the physical and chemical conditions of the solvent as well as the sorption capacity, affinity, and specificity of the algal sorbent (Davis et al., 2003). Metal ion affinity for ligands, as well as equilibrium, drives biosorption (Wang and Chen, 2009). The ionic charge on the metal ion and the algal species also influence

sorption capacity (Das et al., 2007). The higher the electronegativity of the metal ion, the higher the affinity of the algae is for the metal ion (Mehta et al., 2000). As stated before biosorption is an equilibrium process and therefore if the initial metal concentration is increased, it will improve adsorption capacity until the active sites are saturated (Wang and Chen, 2009). Temperature does not profoundly affect the biosorption process between the range of 20°C and 35°C (Das et al., 2007).

Cr(III) exist as cationic species (Cr^{3+} and $CrOH^{2+}$) while Cr(VI) exist as anionic species (CrO_4^{2-} and $HCrO_4^-$). The binding behaviours of the two Cr species will consequently be very different depending on the pH (Murphy et al. 2008). Cr(VI) sorption occurs between a pH range of 1 and 3, in which Cr(III) cannot be sorbed due to proton competition (Pagnanelli et al. 2013). At pH above 3.5, dissociation of sulphonate and carboxyl functional groups increases the negative charge on the algal biomass; as a result, anionic Cr(VI) are repelled, and cationic Cr(III) are attracted to the biomass, thus improving the metal binding capacity (Murphy et al. 2008).

The four important biosorption mechanisms of Cr(VI) include anionic adsorption, adsorption-coupled reduction, anionic and cationic adsorption, and reduction and anionic adsorption (Saha and Orvig 2010).

- The *anionic adsorption mechanism*: Cr(VI) anions bind with the cationic functional groups through electrostatic attraction. This process occurs at low pH values.
- The *adsorption-coupled reduction mechanism*: complete reduction of Cr(VI) to Cr(III) by the biomass under acidic conditions, followed by the partial adsorption of Cr(III) onto the biomass.
- The *anionic and cationic adsorption mechanism*: incomplete reduction of Cr(IV) occurs, followed by adsorption of both anionic Cr(VI) and cationic Cr(III) onto the biomass.
- The *reduction and anionic adsorption mechanism*: incomplete reduction of Cr(VI) occurs at low pH, followed by adsorption of only Cr(VI) anions to the cationic functional groups on the biomass while Cr(III) cations remain unbound in the solution. This mechanism is prevalent as the maximum adsorption of Cr(III) does not occur under very acidic conditions (Saha and Orvig 2010).

The adsorption process usually involves the binding of Cr(VI) or Cr(III) ions, either with covalent bonds, van der Waals forces, or electrostatically, onto the cell surface of the algae via interactions between Cr(VI) or Cr(III) and the functional groups on the cell surface. This step occurs rapidly and can be a combination of coordination, chelation, complexation, ion exchange, physical adsorption, or inorganic microprecipitation (Das et al., 2007). The metal ions can penetrate the cell membrane, and intracellular bioaccumulation or biotransformation of Cr(VI) occurs. This step is an active metabolic process and, as such, occurs at a much slower rate (Sen and Dastidar 2010; Joutey et al., 2015).

The functional groups present on the cell wall depend on the algae type. Weak acidic carboxyl groups, R-COOH, on algal cell walls are the most abundant functional groups and offer several active sites where most of the ion exchange occurs (Kratochvil and Volesky, 1998).

Chlorophyta (green algae), phaeophyta (brown algae) and rhodophyta (red algae) are some of the different types of algae. The difference in colour can be attributed to the additional pigments present in the algae (Wang and Chen, 2009). Brown algae seem to have the best metal reducing the ability, which can be attributed to the dominant carboxyl and sulphate functional groups.

Red algae can also be used in the biosorption process. Sulphated polysaccharides, made of galanctanes, are essential for red algae biosorption. The removal of Cr(VI) by the red algae, *Cyanidium caldarium*, occurs by cell surface precipitation of metal-sulphide microcrystals.

Green algae have several functional groups such as amino, carboxyl, sulphate, and hydroxyl (Romera et al., 2007). Green algae, however, retain more Cr(VI) than brown or red algae (Cervantes et al., 2001).

Algae species that can remove Cr(VI) include Filamentous alga *Cladophora* (green algae) which rapidly removed 72 % of the initial chromium in 15 minutes (Vymazal, 1990). In an experiment conducted by (Brady et al., 1994) Scenedesmus, Selenastrum and Chlorella removed between 38 % and 99 % of the Cr(III), but only between 18 % and 22 % of the Cr(VI). For *Chlorella* and *Cladophora* biosorption of Cr(VI) took place first, followed by the bioreduction of Cr(VI) and biosorption of Cr(III) onto the algal biomass. (Joutey et al., 2015). Immobilised cells of *Chlorella vulgaris* removed 34% of chromium in a packed

bed reactor, and *Scenedesmus acutus* removed 31% (Travieso et al., 1999; Cervantes et al., 2001).

Benefits and Limitations of Biosorption

Biosorption is a relatively cheap process with high efficiency, minimal secondary pollution production, and the biosorbents can be reused. Biosorption that utilises dead biomass does not require additional nutrients and presents the opportunity for chromium recovery. Algae use light as an energy source, which is much cheaper than organic carbon substrates which are needed by bacteria (Modak and Natarajan, 1995; Das et al., 2007). To achieve the maximum biosorption results the optimisation of parameters (such as pH, temperature, agitation time, adsorbent concentration, adsorbent dose, initial chromium concentration, contact time) are required (Jobby et al. 2018).

In order to reuse the biosorbent, the metal must be first removed, which can be achieved by altering the metal valence state (Das et al., 2007). A possible solution would be to alter the chromium valence state from Cr(VI) to Cr(III) with biotransformation processes utilising bacteria, before bioadsorbing the Cr(III). Certain algal biosorbents are more challenging to regenerate for reuse (Miretzky and Cirelli, 2010).

2.6.3 Cr(VI) Resistant Algae

In order to remove Cr(VI) from the environment, the living algae must be tolerant of, or resistant against, Cr(VI) toxicity. The blue-green algae, *Nostoc* displayed Cr(VI) resistance capabilities. *Nostoc* was able to survive in a soil persistently contaminated by Cr(VI). The Cr(VI) pollution was from a nearby leather tannery (Sinha et al., 2009). The algal species *Oscillatoria*, *Phormidium*, *Scenedesmus*, and *Pandorina* also found to be resistant towards Cr(VI) (Sudhakar et al., 1991).

2.6.4 Algal Cr(VI) Reduction

Only a few algae species have been identified and isolated that can directly reduce Cr(VI) to Cr(III). The pathway of Cr(VI) reduction in algae species is not yet understood. Rehman and Shakoori (2001) isolated Cr(VI) resistant *Chlorella spp*. from a tannery in Pakistan.

Chlorella spp. were able to grow in Bold basal medium spiked with Cr(VI) in the form of $K_2Cr_2O_7$. These algae species were also reported to have the ability to reduce Cr(VI) from an initial concentration of 12 µg/mL.

Another Cr(VI) resistant *Chlorella spp*. algae species were isolated and identified by Yewalkar et al. (2007) from a paper-pulp disposal dump in India. This *Chlorella spp*. species could also reduce Cr(VI). Yewalkar et al., (2007) suggest that Cr(VI) reduction was stimulated by light as well as the addition of reduced-carbon compounds such as acetate, glucose, and malate. *Chlorella, Oscillatoria* and *Zoogloea* were also reported to have the ability to reduce Cr(VI) (Kamaludeen et al. 2003). Photochemical reduction of Cr(VI) is the proposed mechanism for Cr(VI) reduction by *Chlorella valguris* that was isolated by Das et al. (2017)

Marine macro-alga *Sargassum cymosum* has been used as an electron donor for the reduction of Cr(VI) by de Souza et al. (2016). The algal biomass was oxidised and served as a natural cation exchanger for the chromium sequestration. *Sargassum cymosum* were able to reduce the 3.0 mM of Cr(VI). Hackbarth et al. (2016) used brown macroalga *Pelvetia canaliculata* as a natural electron donor for the reduction of Cr(VI) in acidic electroplating wastewater.

2.7 Combined Algae and Bacteria systems

In the environment algae and bacteria co-exist and often interact with other microorganisms. Algae and bacteria can engage in a symbiotic relationship, and these interactions can be beneficial to both species. Numerous studies have investigated the symbiotic relationship between bacteria and algae. (Ramanan et al. 2016; Guo and Tong 2014).

Algae can release a variety of organic substances, composing of proteins, lipids and nucleic acids. These substances can serve as substrates for bacterial growth (Abed et al., 2010). The use of algae combined with bacteria to treat wastewater has been investigated by Muñoz and Guieysse (2006). One of the advantages of this treatment method is that the algae produce the O_2 required by the aerobic bacteria to mineralise organic pollutants and the oxygen is a crucial electron acceptor, the algae, in turn, the uses the CO_2 released during the mineralisation process (Muñoz et al., 2005). Kazamia et al. (2012) found

another example of a bacteria-algae mutualistic relationship, in which heterotrophic bacteria delivers Vitamin B12 whenever required to *Chlamydomonas reinhardtii*. Cho et al., 2015 found algae that supplied fixed organic carbon to an artificial consortium of mutualistic bacteria, mostly belonging to plant growth promoting bacteria, and the bacteria, in return, supply dissolved inorganic carbon and low molecular organic carbon for algal consumption.

Fu et al., 2016 suggest that a consortium of algae and bacteria can work synergistically to detoxify both organic and inorganic pollutants. Subashchandrabose et al. (2011) stated that an algae-bacteria combined system could produce metabolites or by-products that are commercially valuable. However, certain factors can cause a shift from mutualism to parasitism in the algae-bacteria relationship. Still a deeper understanding of the algal and bacterial interactions is needed to formulate practical solutions for bioremediation processes.

CHAPTER 3 MATERIALS AND METHODS

3.1 Chemicals Reagents

3.1.1 Cr(VI) Standard solutions

Cr(VI) stock solution (1000 mg/L) was prepared by dissolving 3.74 g of di-potassium chromate (K_2CrO_4, 99 % purity) in 1 L deionised water. This stock solution served as the main source of Cr(VI) during all the experiments.

3.1.2 DPC solution

Diphenyl carbozide 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.

3.1.3 Chemicals

Sodium chloride solution (0.85% NaCl) was prepared by dissolving 1.85 g of NaCl in 100 mL deionised water and autoclaving the solution. Sulphuric acid solution (H_2SO_4) (1N) was was prepared by dissolving 6.9 mL of acid in 100 mL of deionised water. The acetone for algal assays was prepared by diluting the acetone with deionised water to 90%. Hydrochloric acid, sodium hydroxide, nitric acid, D-glucose and all the chemicals were purchased from Merk (South Africa) or Sigma Aldrich.

3.2 Growth media

3.2.1 Bacterial growth media

Bacterial Luria-Bettani (LB) broth, Luria-Bettani (LB) agar, and plate count (PC) agar (Merck, Johannesburg, South Africa) was prepared as per the manufacturer's instructions. The broth and agar were prepared by dissolving 25 g LB broth powder in 1 L deionised water, 45 g LB agar powder in deionised water, and 23 g PC agar powder in deionised

water. The LB and PC agar media were cooled at room temperature after autoclaving at 121°C at 115.

3.2.2 Bacteria Cultivation Before Batch Experiments

Before the batch experiments, the three pure isolated bacteria were cultivated aerobically in 400 mL of sterile LB broth for 24 hours at 30±2 ℃ in 1 L Erlenmeyer flasks. The flasks containing the inoculant was stoppered with cotton wool and placed in a shaking incubator shaking at 100 rpm. After 24 hours the cells were harvested and centrifuged for 10 min at 12,500 g (6,000 rpm) in a Sorvall Lynx 6000 (Thermo scientific, Stockholm, Sweden). The supernatant was removed, and the remaining pellet was washed three times in a sterile 0.85% NaCl solution to ensure that no carbon source from the LB broth remained with the cells. During each wash, the bacterial cells were suspended in the sterile 0.85% NaCl solution using a vortex mixer (rotated at 1000 rpm up to homogeneous suspension) and then centrifuged again at 6000 rpm for 10 minutes. The washed pure bacterial cells were then mixed homogeneously to form a consortium and added to the batch experiments using a sterile spatula. The total dry bacterial biomass weight was determined by drying the bacteria cells in an oven at 60 °C overnight on a watch glass. The difference between the watch glass with the cells and the empty watch glass was considered as biomass. The dry bacterial biomass concentration for each batch experiment was ±3.5 g/L.

3.2.3 Algal growth media

The algal strain was cultured axenically in the BBM and the modified recipe of BBM with 3-fold nitrogen and vitamins (3N-BBM+V) (CCAP 2015). The media was prepared by firstly preparing the stock solutions of trace elements and vitamins as shown in Table 3.1 and Table 3.2. All stocks were stored at 4°C in the cold room. After all the stocks solutions were added, the final volume was then adjusted to 1L with distilled water, and the pH adjusted to 6.6 and the media was autoclaved. The algae were found to have the highest growth rate in 3N-BBM+V. Therefore 3N-BBM+V was used for batch experiments, and BBM was only used for the initial isolation of the algae.

3.2.4 Algae Cultivation Before Batch Experiments

Cultures were grown in sterilised and continuously stirred 1 L Erlenmeyer flasks, closed with planktonic nets, under the required algal light conditions roughly at 60 µmol photons/m^2 s^1 (Osram L 36W/77 Floura) at 25 °C. After 14 days optical density at 650 nm of the culture solution reached 1.8 which corresponds roughly to a dry algae weight of 1.5 g/L (Kothari 2012). The dry algal biomass weight was determined by drying the algae in an oven at 60 °C overnight on a watch glass. The difference between the watch glass with the cells and the empty watch glass was considered as biomass. The algae cells were harvested and centrifuged for 10 min at 12,500 g (6,000 rpm) in a Sorvall Lynx 6000 (Thermo scientific, Stockholm, Sweden). The supernatant was further centrifuged five times until it was completely clear and cell-free. Both the algal cells and the cell-free spent supernatant were used in the Cr(VI) reduction batch experiments.

3.3 Source of Cr(VI) Reducing Organisms

Researchers in the water utilisation and environmental engineering group at the University of Pretoria have found that there is a wide variety of bacteria that are able to reduce Cr(VI) located at the Brits Wastewater Treatment Works in the North West province in South Africa. These bacteria have evolved these exceptional capabilities to adapt to and colonise, the noxious metal-polluted environments due to long-term exposure to elevated Cr(VI) concentrations.

Other researchers also collected bacteria that can reduce Cr(VI) from various tanneries around the world (Megharaj et al., 2003; Viti et al., 2003; Srinath et al., 2002; Shakoori at al., 2000). As these tanneries are engaged in the chrome tanning process, they release untreated effluent that contains Cr(VI) into the environment. (Srinath et al., 2002). Sludge samples were also collected from the effluent of a tannery in South Africa, although the Cr(VI) content in the effluent was found to be insignificant. All the soil samples were collected in sterile containers and stored in a cold room between 0 °C and 4 °C.

Table 3.1: Contents of BBM per 1 L from CCAP

Stock solutions		
(1) NaNO3	10 g	Add to 400 mL distilled water
(2) $CaCl_2 \cdot 2H_2O$	3 g	
(3) $MgSO_4 \cdot 7H_2O$	1 g	
(4) K_2HPO_4	3 g	
(5) KH_2PO_4	7 g	
(6) NaCl	1 g	
(7) Trace element solution	(Autoclave to dissolve)	
$ZnSO_4 \cdot 7H_2O$	8.82 g	Add to 1 L of distilled water
$MnCl_2 \cdot 4H_2O$	1.44 g	
MoO_3	0.71 g	
$CuSO_4 \cdot 5H_2O$	1.57 g	
$Co(NO)_3 \cdot 6H_2O$	0.49 g	
(8) H_3BO_3	11.42	
(9) EDTA	50 g	Add to 1 L distilled water
KOH	31 g	
(10) $FeSO_4 \cdot 7H_2O$	4.98 g	Add to 1 L distilled water
H_2SO_4 (conc)	1 mL	
Medium		
Stock solution 1 - 6	10 mL each	Add to 1 L of distilled water
Stock solution 7 - 10	1 mL each	

Table 3.2: Contents of 3N-BBM+V per 1 L from CCAP

Compound	Stock solution (g in 1 L water)	For 1 L of final media
$NaNO_3$	25 g	30 mL
$CaCl_2 \cdot 2H_2O$	2.5 g	10 mL
$MgSO_4 \cdot 7H_2O$	7.5 g	10 mL
K_2HPO_4	7.5 g	10 mL
KH_2PO_4	17.5 g	10 mL
NaCl	2.5 g	10 mL
Trace element solution	(see below)	6 mL
Vitamin B1	(see below)	1 mL
Vitamin B12	(see below)	1 mL
Trace element stock solution		
$Na_2EDTA \cdot 2H_2O$	0.75 g	Add to 1 L of distilled water
$FeCl_3 \cdot 6H_2O$	97 mg	
$MnCl_2 \cdot 4H_2O$	41 mg	
ZnCl2	5 mg	
$CoCl_2 \cdot 6H_2O$	2 mg	
$Na_2MoO_4 \cdot 2H_2O$	4 mg	
Vitamin B1 Thiamine hydrochloride	0.12 g	Add to 100 mL distilled water
Vitamin B12 Cyanocobalamin	0.1 g	Add to 100 mL distilled water

3.4 Isolation of Cr(VI) Reducing Bacteria

The pure cultures of bacteria were isolated from sludge samples collected from the sand dry beds at the Brits Wastewater Treatment Works. The different 5 g soil and sludge samples containing bacteria were added to 100 mL bacterial growth media, LB broth, in 250 mL Erlenmeyer flasks. The broth was prepared by adding 30 g LB powder to 1 L of distilled water. After the pH was checked to be around 7 the LB broth was sterilised by autoclaving for 15 min at 120 °C. The inoculant was spiked with Cr(VI) which allowed only Cr(VI) resistant bacteria to grow. The final concentration of Cr(VI) in the solution was 100 mg/L. The Erlenmeyer flasks were plugged with cotton wool to allow got aeration while filtering out floating microorganisms from the air. The bacteria from the sludge/soil were cultivated for 24 hours in an orbital shaker incubator, shaking at 120 rpm. From trial and error, a shorter cultivation period allowed for the isolation of the bacteria with the best Cr(VI) reducing capabilities. The temperature was controlled at an average of 32 ± 2 °C.

The high growth rate of bacteria indicated the existence of Cr(VI) resistant bacteria in the samples, and the presence of Cr(VI) reducing bacteria was signified by a decrease in the Cr(VI) concentration in the solution. The cultures from the Brits samples were able to completely reduce all the Cr(VI) that was used to spike the LB broth within 24 hours. The cultures from the tannery samples were not able to remove significant amounts of the Cr(VI). However, both cultures were able to able to grow up to high cell concentration (of CFU/mL) in the presence of 100 mg/L of Cr(VI). Indicating that the tannery sludge did possibly contain Cr(VI) resistant bacteria. Nevertheless, only the cultures from brits were used for future experiments.

Serial dilutions and streak plating were used to isolate the pure bacteria cultures. Firstly, 1 mL of the cultivated solution was serially diluted into sterile tubes containing 9 mL of sterile 0.85 % NaCl solution. After the serial dilutions were completed, 0.1 mL from the 6th to the 10th tubes were deposited into the Petri dishes containing sterile LB agar using the spread method. The plates were incubated for about 24 hours at 32 ± 2 °C to develop separately identifiable colonies. The Petri dishes were inverted during incubation. The individual colonies were transferred with a sterile wire loop to two LB agar plates each. In preparation for the identification of the bacterial species, the colonies were first classified based on morphology. Three different morphologies were identified for the Brits

cultures and two for the tannery cultures. Interesting to note is that the three different bacteria species from the Brits sludge sample had vastly different smells.

Immediately after the pure colonies formed, a small amount of each individual bacteria (potential species) was picked up by a sterile wire loop and transferred to sterile LB broth. The plates were then sent for identification. The LB broth with the inoculant was incubated for 24 hours in an orbital shaker incubator shaking at 120 rpm. After the incubation period, the LB broth containing the bacteria strains were mixed with glycerol in a ratio of 1:4. The pure bacterial strains were stored in 2 mL screw cap tube with 20 % glycerol and 80 % LB broth at -70 °C. Proper storage is essential to retain the bacteria's Cr(VI) reducing capabilities over a long period. Before each batch experiment, a screw cap tube was allowed to defrost, and the bacteria strains were inoculated in LB broth.

3.5 Bacterial Culture Characterisation

Phylogenetic characterisation of the bacterial colonies on LB agar plates was performed. The 16S rRNA sequencing and identification were carried out at the Department of Microbiology, University of Pretoria. Genomic DNA was extracted from the pure cultures using a DNeasy tissue kit (QIAGEN Ltd, West Sussex, UK) according to the instructions from the manufacturer. The 16S rRNA genes of isolates were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using primers pA and pH1 (Primer pA corresponds to position 8-27; Primer pH to position 1541-1522 of the 16S gene) (Coenye *et al*., 1999). An internal primer pD was used for sequencing (corresponding to position 519-536 of the 16S gene). The resulting sequences were matched to genes for known bacteria in the GenBank using a basic BLAST search of the National Centre for Biotechnology Information in the United States of America (NCBI, Bethesda, MD). Phylogenetic tree diagrams were then constructed using the neighbour-joining method. Confidence in the tree topology was determined by bootstrap analysis based on 100 re-samplings (Felsenstein, 1985) and is shown in Figure 3.1 to Figure 3.3. The isolated bacteria from the Brits samples showed about 99 % sequence identity with *Escherichia coli*, *Bacillus thermoamylovorans*, and *Citrobacter sedlakii*. The samples from the tannery were identified as *Bacillus subtilis* and *Bacillus mojavensis* (trees not shown).

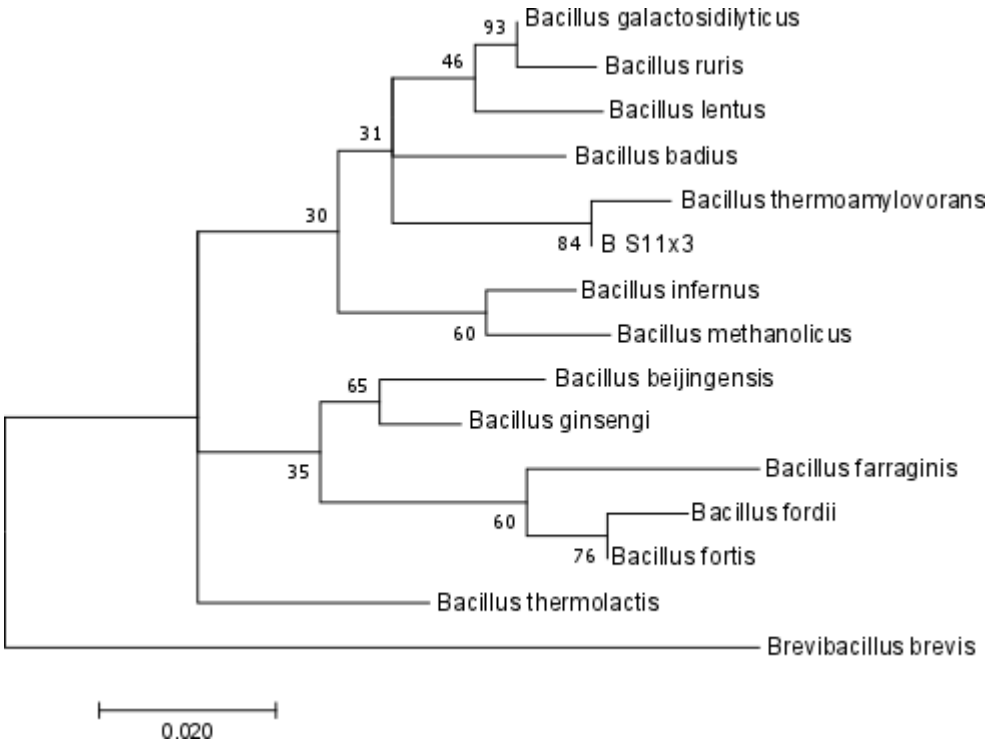

Figure 3.1: Phylogenetic tree for the *Bacillus thermoamylovorans* bacteria strain that was used in the batch experiments, derived from the 16S rRNA gene sequence.

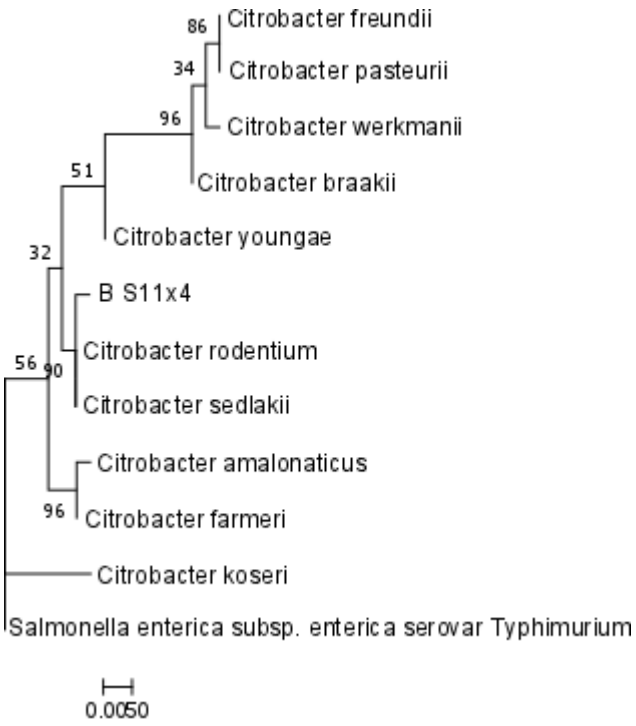

Figure 3.2: Phylogenetic tree for the *Citrobacter sedlaki* bacteria strain that was used in the batch experiments, derived from the 16S rRNA gene sequence.

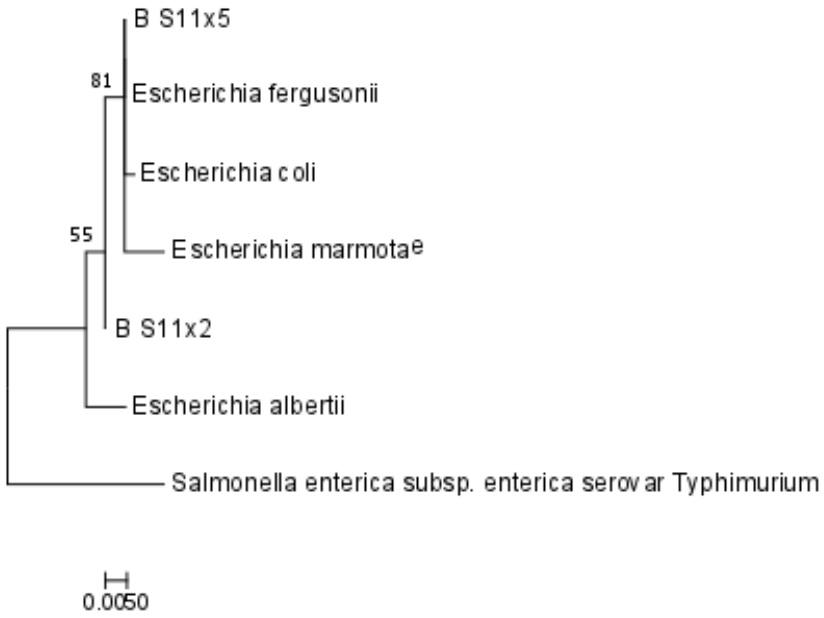

Figure 3.3: Phylogenetic tree for the *Escherichia coli* bacteria stain that was used in the batch experiments, derived from the 16S rRNA gene sequence.

3.6 Source of Algae

The algae were isolated from the Hartbeespoort Dam in the North West province of South Africa. About 10 litres of dam water was obtained in sterile buckets. Plankton nets (30 and 120 µm) were used to scoop out potential algae cells from the dam water. The cells were suspended in Erlenmeyer flasks containing sterile Bold Basel Media (BBM) with a pH of 6.7. The preparation of BBM is shown in Table 3.1. The plankton nets were also used to cover the Erlenmeyer flasks and filter the air as the cultivation period of algae is longer than for bacteria, and cotton wool is not suitable for long periods. The Erlenmeyer flasks containing the algae were placed on magnetic stirrers under required light conditions (Osram L 36W/77 Floura) for seven days. *Chlamydomonas reinhardtii* algal species that was purchased from the Culture Collection of Algae and Protozoa (CCAP) was used to compare with the locally isolated algal species as a potential carbon source.

3.7 Algal Culture Isolation

After the algae cells were cultivated for seven days, 12 mL samples were taken and centrifuged at 6000 rpm for 5 min in a Sorvall Lynx 6000 (Thermo scientific, Stockholm, Sweden). The supernatant was decanted, and the cells were suspended in sterile 0.85 % NaCl solution and centrifuged again. The algal cells were isolated using streak plating on BBM agar in Petri dishes. The agar was prepared by adding 9 g of bacterial agar to 1 L of BBM solution. The agar was autoclaved at 120 ℃ for 15 min and left to cool down. After the agar has cooled down the BBM agar is poured into Petri dishes and left for one day to solidify completely before transferring the algae cells. The plates were inverted and placed under the Osram L 36W/77 Floura lamps at 20 – 25 ℃ until the algal colonies were clearly visible. Repeated streaking was done to obtain single colonies free from contamination. The clear plated algae were sent to Inqaba Biotechnical Industries (Pty) Ltd for identification.

3.8 Algae Culture Identification

The algae were separated into plates that contained pure colonies, which were then identified by carrying out 18S rRNA and 28S rRNA sequencing. The forward primer SR1, and the reverse primer SR12, were used to amplify the 18S rRNA gene (Shown in Table 3.3). The forward primer 28S-568F, and the reverse primer 28S-803R, were used to amplify the 28S rRNA gene (shown in and Table 3.4). Polymerase chain reaction (PCR) amplification was carried out in a DNA Thermal Cycler (Gene Amp PCR System 2400 - Perkin Elmer). Confidence in the tree topology is shown in Figure 3.4. The resulting sequences were matched to known genes for algal species in the GenBank using BLAST. The algal species of *Chlorococcum Ellipsoideum* were found to have 97% sequence identities with the collected samples.

Table 3.3: Primers that were used for 18S rRNA sequencing.

Name of Primer	Sequences (5' to 3')	Region	References
SR1	TACCTGGTTGATCCTGCCAG	1-20 (F)	Nakayama et al. 1996
SR12	CCTTCCGCAGGTTCACCTAC	1727-1746 (R)	Nakayama et al. 1996

Table 3.4: Primers that were used for 28S rRNA sequencing.

Name of Primer	Sequences (5' to 3')	Region	References
28S-568F	TTGAAACACGGACCAAGGAG	~568 (F)	Marande et al. 2009
28S-803R	ACTTCGGAGGGAACCAGCTA	~803 (R)	Marande et al. 2009

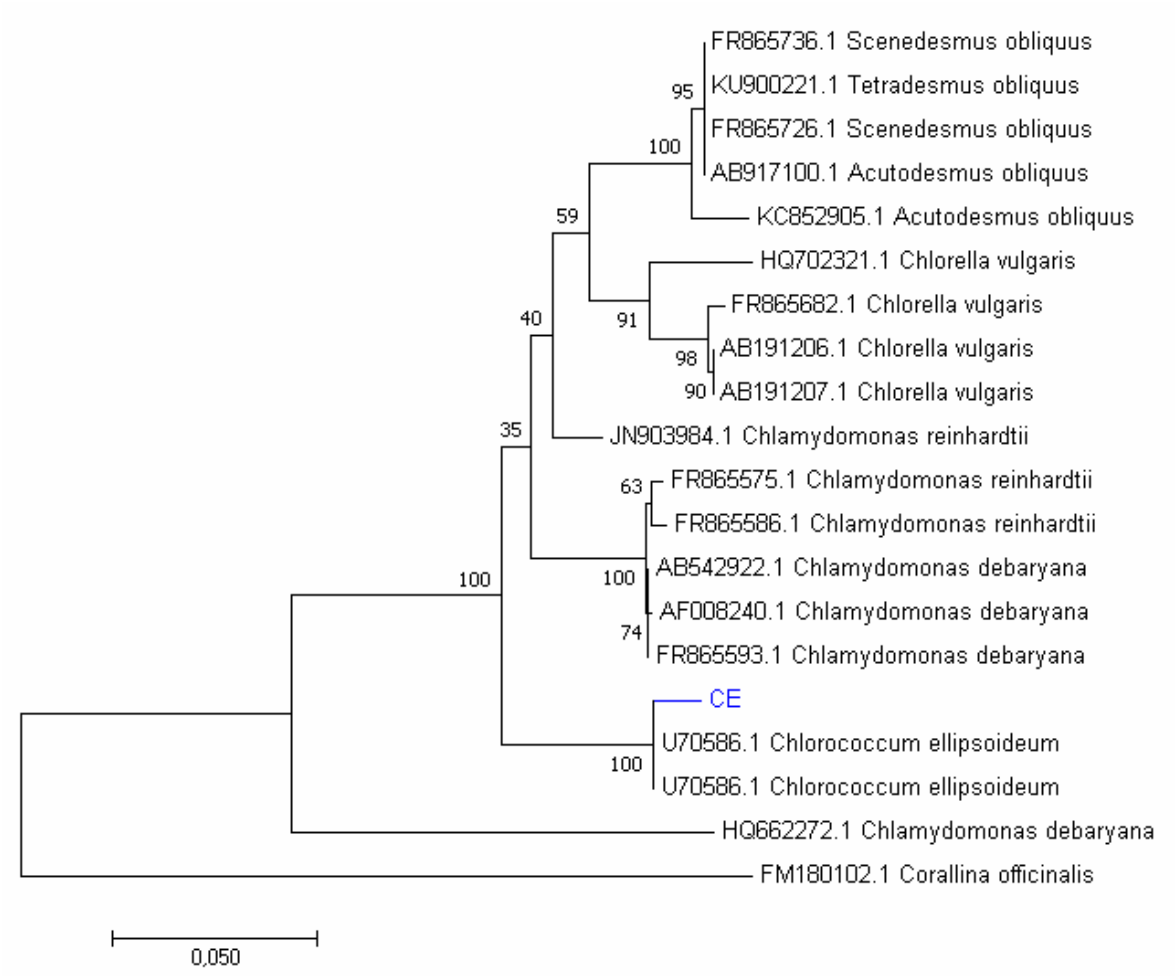

Figure 3.4: Phylogenetic tree for the *Chlorococcum Ellipsoideum* bacteria stain that was used in the batch experiments, derived from the 18S and 26S rRNA gene sequence.

3.9 Cr (VI) Reduction Experiments

In the first set of experiments, only the CRB were used. The bacterial cells were harvested after 24 hours incubation in LB broth. The cells were washed thrice by centrifugation at 6000 rpm (2,415 g) for 10 minutes in a Hermle 2323 centrifuge (Hermle Laboratories, Wehingen, Germany). The bacterial cells were suspended in 3N-BBM+V for consistency, and the bacteria could also benefit from the nutrients in 3N-BBM+V. The aerobic Cr(VI) reduction experiments were conducted in 100 mL Erlenmeyer flasks containing 50 mL sterile 3N-BBM+V. A known concentration of Cr(VI) was added to the flasks marking the beginning of the Cr(VI) reduction experiment. Glucose ($C_6H_{12}O_6$) was used as a carbon source in specific control experiments. The concentration of the glucose was made up to

be 0.05 g/L. In other control experiments, cell-free spent algae media were also used instead of fresh 3N-BBM+V and glucose.

For the main experiments the algae and bacteria cells were cultivated separately as described in the previous section, and then harvested and re-suspended together in sterilised 50 mL Erlenmeyer flasks with 3N-BBM+V and amended with Cr(VI) to give different initial concentrations of Cr(VI) (0, 30, 50, 75, 100, 150 and 200 mg/L) (Kaimbi and Chirwa, 2015). The pH of the solution was adjusted to 7.5, and the flasks were plugged with cotton during incubation to allow aeration while filtering away microorganisms from the air. The batches were placed in a 120-rpm orbital shaker at 30 ±2 °C in the dark. Samples for Cr(VI), total chromium and biomass analysis were taken into Eppendorf-type centrifuge tubes at predetermined time intervals. The samples were centrifuged in a Minispin Microcentrifuge (Eppendorf, Hamburg, Germany) at 2,415 g (6,000 rpm) for 10 min and the supernatant was used for Cr(VI) analysis. All the main batch experiments used the same dry biomass concentration, i.e., 3,500 mg/L bacteria and 1,500 mg/L algae and were carried out in triplicates. The mean values are reported in the results section.

Another set of control experiments was carried out to investigate the interaction between algae and Cr(VI) (without bacteria). The algae were cultivated and harvested and suspended in 3N-BBM+V with different initial concentrations of Cr(VI) (0, 5, 10, and 50 mg/L). The experimental conditions were similar to algal cultivation conditions. The pH was adjusted to 7.5, and the temperature was around 20 ±2 °C under algal light conditions to see if the algae were able to absorb the Cr(VI) and how the Cr(VI) influence the algae health. The pH of the batch experiments was measured using pH meter (PHC101 Hatch South Africa) at various time intervals.

3.10 Analytical Methods

3.10.1 Cr(VI) Measurement

Cr(VI) concentration was determined colourimetrically using a UV/VIS spectrophotometer (WPA, LightWave II, Labotech, South Africa). The measurement was carried out at a wavelength of 540 nm (10 mm light path). 0.1 mL of the cell-free sample containing Cr(VI) was acidified with 1 mL of 1N H_2SO_4 and diluted with distilled water up to 10 mL. The 10 mL solution was then reacted with 0.2 mL of 1,5 diphenyl carbazide.

A red-violet purple colour forms with a specific intensity depending Cr(VI) concentration which can be measured using the calibrated UV/vis spectrophotometer (APHA, 2005). The standard solutions of Cr(VI) were prepared at different concentrations 5, 10, 30, 50, 80 and 120 mg/L. These standard solutions were used to obtain a calibration curve with the regression of 99 % (absorbance against concentration).

3.10.2 Total Chromium Measurement

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

3.10.3 Scanning Electron Microscopy

The bacterial and algal cells were studied using a Scanning Electron Microscope (Zeiss Ultra PLUS FEG SEM) to determine the interactions between the bacteria and algae as well as the influence of Cr(VI) on the cells. Samples were taken at the beginning of the experiments and after several hours of exposure. The wet samples were prepared using conventional chemical methods before embedding for SEM (Glauert and Reid, 1975). The samples were first fixed with 2.5% glutaraldehyde dissolved in 0.075 M phosphate buffer (pH 7.4-7.6) for 30 minutes. The cells were then rinsed three times in the 0.075 M phosphate buffer for 10 minutes. The buffer was used to protect the biological sample from becoming acidic. The clean cells were then fixed in 0.5% aqueous osmium tetroxide for 2 hours in a fume hood and rinsed three times in distilled water. Each sample was then dehydrated in increasing concentration of ethanol (30%, 50%, 70%, 90%, and 100%) for 10 minutes at each concentration. The samples were dried overnight, mounted on the stub and carbon coated. After sample preparation, the SEM was used to obtain images at different magnifications before and after the experiment.

3.10.4 Total Organic Carbon Analysis

The total organic carbon (TOC) produced by the algae was measured with the Model TOC-VWP Shimadzu TOC Analyzer with ASI-V 101 autosampler (Shimadzu Corporation, Kyoto, Japan) After the algae growth phase the cells were harvested for reduction experiments by centrifugation at 6000 rpm (2,415 g) for 10 minutes in a Hermle 2323 centrifuge (Hermle Laboratories, Wehingen, Germany). The supernatant was centrifuged five times to ensure all the algal cell was removed. The supernatant was further filtered with Millipore syringe filters. The supernatant was transferred to 40 mL sample vials and analysed. The TOC Analyzer was calibrated by preparing standard sucrose solutions (0-100 mg/L carbon content)

3.11 Biomass Analysis

3.11.1 Bacteria Biomass analysis

The viable bacterial cells in the experiments were determined using the pour and spread plate method, and the colony counts were done as described in APHA (2005), with the colonies growing on Plate Count (PC) agar. 1 mL of suspended cell solution samples were withdrawn from the batch experiments at regular time intervals and was diluted serially into 9 mL of 0.85% NaCl solution contained in eight test tubes. Colonies were developed by platting 1 mL samples serially diluted samples from the 6th, 7th and 8th tubes onto three Petri dishes. The diluted cell solutions were spread onto the PC agar. The Petri dishes were incubated upside down at 30±2 °C for 18-24 hours in a temperature-controlled incubator. After incubation the colonies on each plate were counted and multiplied by a dilution factor. Colony forming units (CFU) per millilitre of sample was reported as the mean between the three plates. The benefit of this technique is that it only takes into account the bacterial colonies as the algae growth rate is much slower than the bacteria and only form colonies after a week.

3.11.2 Algal Biomass

Algal cells were grown in 3N-BBM+V until a dry biomass of 1.5 g/L was reached. The algae cells were then harvested and re-suspended in 3N-BBM+V at different Cr(VI)

concentrations with or without bacteria. For assay of chlorophyll *a* content of the algal cells, 2 mL samples were taken from experiments were the algal cells were exposed to Cr(VI) and/or bacteria. The 2 mL samples were centrifuged in a Minispin Microcentrifuge (Eppendorf, Hamburg, Germany) at 2,415 g (6,000 rpm) for 10 min, and the supernatant was discarded. After being washed with ultrapure water three times, algal cells were ground up in 10 mL of 90% acetone solution and incubated at 4 °C for 24 h in darkness in the cold room. After incubation, the sample solution centrifuged again at 2,415 g (6,000 rpm) for 10 min to remove the chlorophyll *a* free cells (the cells are now colourless). The optical density of the supernatant was measured at 630 nm, 645 nm and 663 nm with a spectrophotometer (WPA, LightWave II, and Labotech, South Africa). The content of chlorophyll *a* was calculated as shown in Equation 3.1 (Liang et al. 2013): The benefit of this technique is that it only takes into account the chlorophyll *a* from the algae cells; the bacteria cells do not influence the reading as would be the case if only the turbidity were measured.

$$\text{chlorophyll } a\,(\text{mg}/L) = 11.64\,OD_{663\,nm} + 2.16\,OD_{645\,nm} + 0.1\,OD_{630\,nm} \qquad 3.1$$

CHAPTER 4 BACTERIAL CR(VI) REDUCTION

4.1 Performance of Single Pure Stains of CRB

Preliminary experiments were done to assess the different bacteria species' abilities to reduce Cr(VI). Figure 4.1 shows how *Citrobacter sedlakii* reduces Cr(VI) at various initial concentrations. *Citrobacter sedlakii* was able to completely reduce Cr(VI) at initial concentrations below 30 mg/L. At higher Cr(VI) concentrations the reduction capacity of the bacterial cells is either overwhelmed, or the bacteria cells are inhibited by the high Cr(VI) concentration. After 24 hours the *Citrobacter sedlakii* were able to reduce 82 % of the 100 mg/L initial Cr(VI) concentration. It is also unclear why at 100 mg/L Cr(VI), which is the highest initial Cr(VI) concentration that the bacteria were able to reduce, exponential reduction continued for up to 7 hours, whereas at an initial concentration of 75 mg/L Cr(VI) the bacteria was only able to reduce exponentially for up to 6 hours. No studies have isolated or utilised *Citrobacter sedlakii* bacterial species for Cr(VI) reduction. In these preliminary experiments, glucose was used as the sole carbon source.

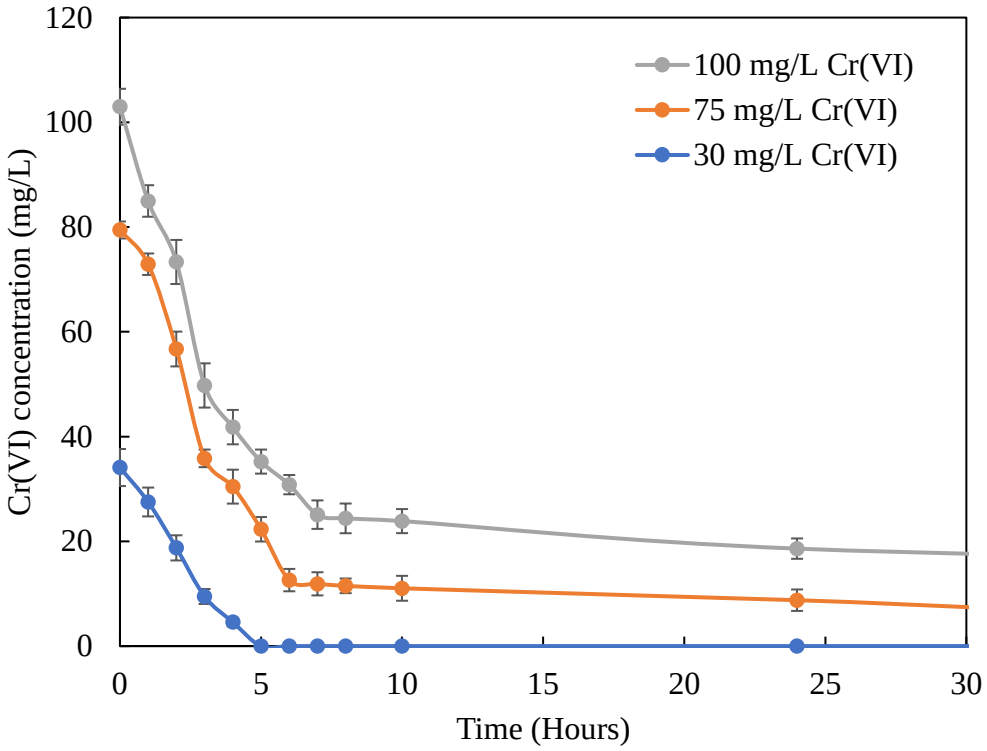

Figure 4.1: Cr(VI) reduction in by *Citrobacter sedlakii* utilising glucose as a carbon source at 30 ± 2 ℃ and neutral pH at various initial Cr(VI) concentrations (mg/L). (Mean ± SE; n = 3)

Figure 4.2 shows the reduction of Cr(VI) by *Escherichia coli*. Out of the three isolated bacteria species, it seems that Cr(VI) reduction capabilities of the *Escherichia coli* bacterial species were best. *Escherichia coli* were able to reduce 97 % of a 100 mg/L initial Cr(VI) concentration. As with *Citrobacter sedlakii*, the highest rate reduction occurred in the first 7 hours and *Escherichia coli* was also able to completely reduce the 30 mg/L initial Cr(VI) concentration. Many other studies have reported on the reduction capabilities of *Escherichia coli* (Barak et al., 2006; Shen and Wang, 1994; Liu et al., 2010). Different *Escherichia coli* strains have been found with many different enzymes that are responsible for the Cr(VI) reduction capabilities. YieF, a dimeric flavoprotein that was isolated from *Escherichia coli*, reduces Cr(VI) to Cr(III). This enzyme can directly reduce Cr(VI) to Cr(III) through a four-electron transfer process, in which three electrons are consumed in reducing Cr(VI), and the fourth electron is transferred to oxygen (Park et al., 2001; Ackerley et al., 2004). Other enzymes isolated from *Escherichia coli* that are responsible

for Cr(VI) reduction include; ChrR (Eswaramoorthy et al., 2012), NemA (Robins et al., 2013), AzoR (Robins et al., 2013), NfsA (Ackerley et al., 2004), and KefF (Prosser et al., 2010). All these enzymes have different mechanisms with regards to Cr(VI) reduction.

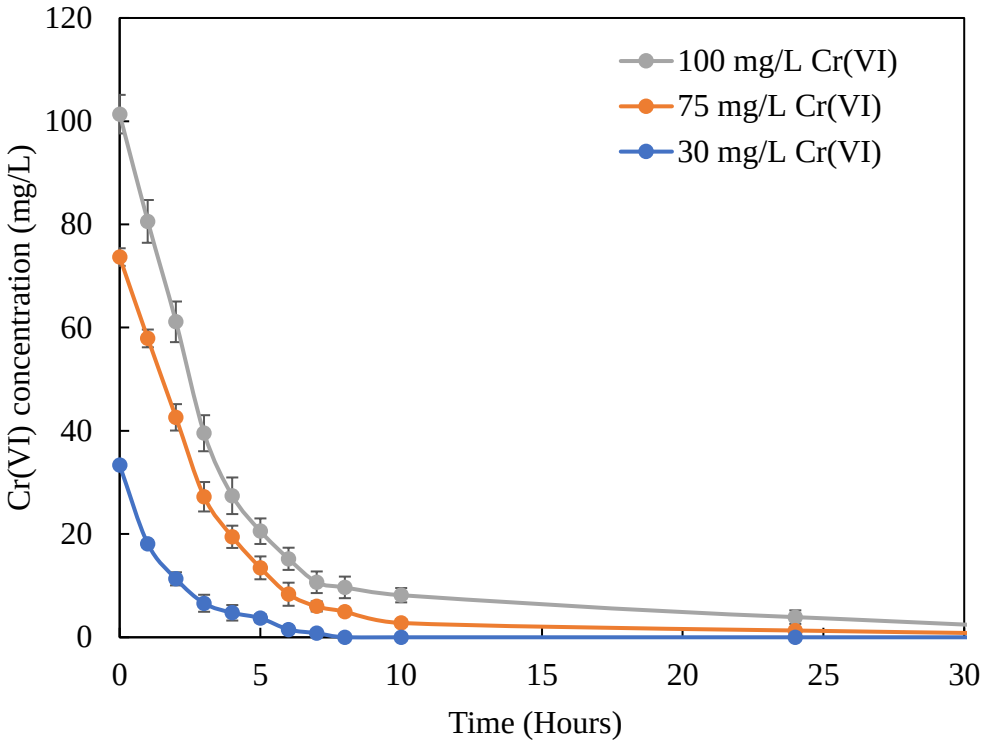

Figure 4.2: Cr(VI) reduction by *Escherichia coli* utilising glucose as a carbon source at 30 ± 2 ℃ and neutral pH at various initial Cr(VI) concentrations (mg/L). (Mean ± SE; n = 3)

Bacillus thermoamylovorans had the worst performance out of the three bacteria species as can be seen in Figure 4.3. *Bacillus thermoamylovorans* was only able to reduce 62 % of 100 mg/L initial Cr(VI) concentration and 78 % of 30 mg/L initial Cr(VI) concentration. Slobodkina et al., (2007) reported having isolated *Bacillus thermoamylovorans* SKC1 which were also capable of reducing Cr(VI) as well as Selenite Tellurite and Iron (III). *Bacillus thermoamylovorans* is a thermophilic bacterium and Slobodkina et al., (2007) found that the optimum temperature for Cr(VI) reduction was at 50 ℃. The experiments in this study were carried out at 30 ± 2 ℃ which could have been too low for *Bacillus thermoamylovorans*.

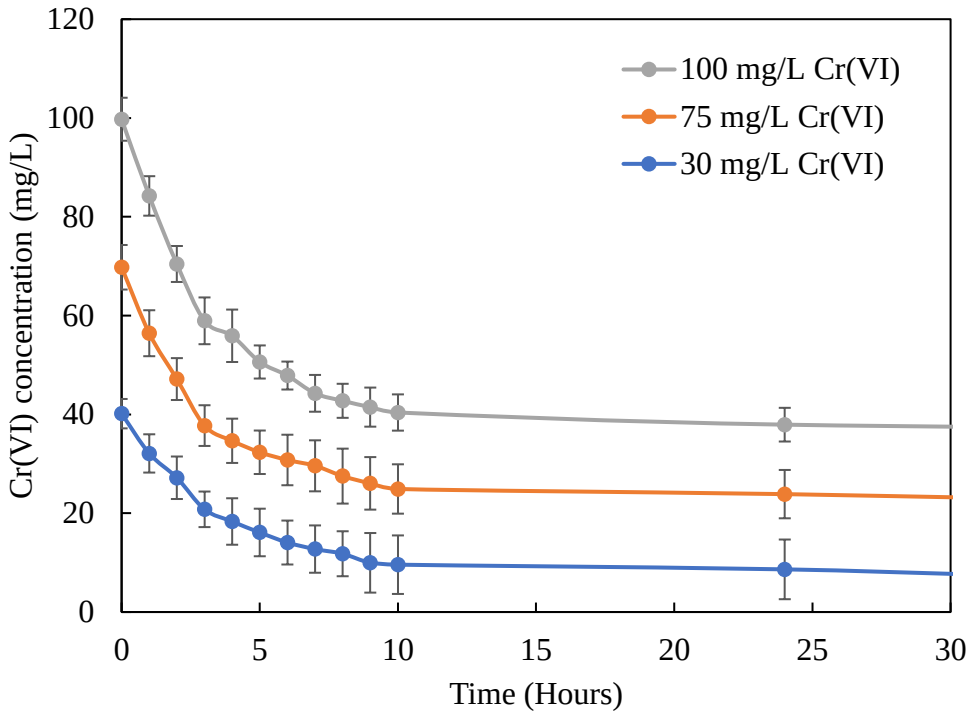

Figure 4.3: Cr(VI) reduction by *Bacillus thermoamylovorans* utilising glucose as a carbon source at 30 ± 2 ℃ and neutral pH at various initial Cr(VI) concentrations (mg/L). (Mean ± SE; n = 3)

4.2 Performance of Mixed CRB

The performance of the mixed culture was also evaluated, and the results are shown in Figure 4.4. Again, glucose was used as a carbon source for the CRB. After 7 hours all the Cr(VI) in the solution was reduced entirely even at 100 mg/L initial Cr(VI) concentration. As expected the mixed culture of CRB resulted in better and higher reduction rates than the single pure strains under the same conditions. The assumption is that the different CRB species were cooperatively reducing the Cr(VI). The results from this study in this regard are consistent with results from numerous researchers at the University of Pretoria (Molokwane et al., 2008).

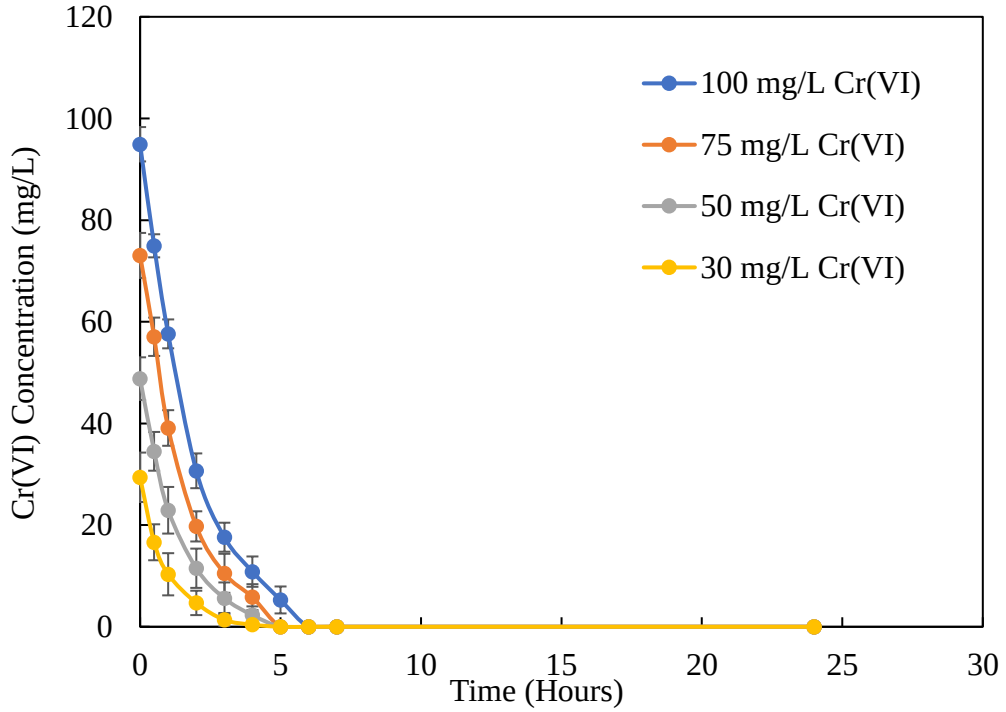

Figure 4.4: Cr(VI) reduction by the mixed culture of CRB utilising glucose as a carbon source at 30 ± 2 °C and neutral pH at various initial Cr(VI) concentrations (mg/L). (Mean ± SE; n = 3)

In nature bacteria normally exits as a consortium in which bacteria survive symbiotically. At the Cr(VI) contaminated site in Brits where the CRB were collected and isolated, the individual bacteria evolved their survival and detoxification mechanisms alongside other

bacteria. As such it makes sense to use the isolated bacteria as a reconstructed consortium and that the consortium would result in better Cr(VI) reduction. A mixed culture of CRB can more effectively emulate the natural processes that occur in the presence of Cr(VI) pollution. From the results, it would appear that the mixed culture of CRB has a synergetic metabolism. In the environment, consortium communities of bacteria have very diverse metabolic pathways which allow them to degrade a wide variety of pollutants. Another example of symbiosis between different bacteria species is illustrated by Chirwa and Wang (2001). In this study, *Pseudomonas putida* and *Escherichia coli* were cocultured in a fixed film bioreactor. Chirwa and Wang (2001) found that Pseudomonas putida released organic acid by-products during phenol degradation which the Cr(VI)-reducer, Escherichia coli, utilised for growth and Cr(VI) reduction.

Kaimbi and Chirwa (2015) achieved similar results under anearobic conditions. Using a mixed culture of CRBs isolated from Brits Wastewater Treatment Plan, Kaimbi and Chirwa (2015) were able to completely reduce an initial Cr(VI) consentration of 30 mg/L in 5 hours, which is similar to the results shown in Figure 4.4.

4.3 Modelling Enzymatic Cr(VI) Reduction

Shen and Wang (1994) developed a model based on the Monod or Michaelis-Menten model to describe the enzymatic reduction of Cr(VI) by bacteria in batch systems. A similar model was used to describe the Cr(VI) reduction kinetics in the preliminary experiments of this study in which glucose was used as a carbon source (without algae). Shen and Wang (1997) derived the model for pure bacterial cultures, however, in this study, the model was applied to describe the Cr(VI) reductase activity of a mixed culture of bacteria. Therefore, the Cr(VI) reduction effect of the whole culture is represented by the sum of all the individual enzymatic Cr(VI) reduction actions of each of the three bacteria species.

The CRB produce both soluble and membrane-associated enzymes (reductase) that mediate the process of Cr(VI) reduction (Chirwa and Molokwane, 2011:81). The soluble reductase uses NADH as the electron donor either by necessity (Horitsu et al., 1987) or for maximum activity (Ishibash et al., 1990). The enzymes serve as a catalyst for the Cr(VI) reduction reaction. The assumption is that enzymes are only produced by the

bacteria if Cr(VI) is detected; these enzymes help to transfer electrons to the Cr(VI). Another assumption is that the enzymes have 1 or 2 transfer sites that can only be used once.

In this model Cr(VI) reversibly associates with the enzymes, E, in the first step and the resulting enzyme-Cr(VI) complex allows the transfer of electrons. The transitional enzyme-Cr complex breaks down and releases the deactivated enzymes and Cr(III). This process is depicted in Equation 4.1.

$$E + \mathrm{Cr(VI)} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E^{*}\mathrm{Cr(VI)} \xrightarrow{k_{cat}} E + \mathrm{Cr(III)} \qquad 4.1$$

E represents the Cr(VI) reductase enzyme for the mixed bacteria culture as a whole. The reaction constants are symbolised by k_1, k_{-1} and k_{cat}. The reduction rate of Cr(VI) is equal to the formation of Cr(III) which is described by a first order reaction as shown in Equation 4.2. However, the enzyme-Cr(VI) complex term, $[E^{*}\mathrm{Cr(VI)}]$, is considered unmeasurable and must be replaced by experimentally defined, independent variables.

$$\frac{d\mathrm{Cr(III)}}{dt} = -\frac{d\mathrm{Cr(VI)}}{dt} = k_{cat}[E^{*}\mathrm{Cr(VI)}] \qquad 4.2$$

The rate of formation and consumption of the $[E^{*}\mathrm{Cr(VI)}]$ complex is described in Equation 4.3. At steady state, if the reaction velocity remains constant, the $[E^{*}\mathrm{Cr(VI)}]$ complexes are being consumed and formed at the same rate, and Equation 4.4 is equal to zero.

$$\frac{dE^{*}\mathrm{Cr(VI)}}{dt} = k_1[E][\mathrm{Cr(VI)}] - k_{-1}[E^{*}\mathrm{Cr(VI)}] - k_{cat}[E^{*}\mathrm{Cr(VI)}] \qquad 4.3$$

$$0 = k_1[E][\mathrm{Cr(VI)}] - k_{-1}[E^{*}\mathrm{Cr(VI)}] - k_{cat}[E^{*}\mathrm{Cr(VI)}] \qquad 4.4$$

All the kinetic constants in Equation 4.4 can be grouped, and a new constant can be defined, namely K_C, as shown in Equations 4.5 and 4.6.

$$\frac{k_{\_1} + k_{cat}}{k_1} = \frac{[E][\mathrm{Cr(VI)}]}{[E^*\mathrm{Cr(VI)}]} \quad 4.5$$

$$K_C = \frac{k_{\_1} + k_{cat}}{k_1} \quad 4.6$$

When considering the conservation of mass, the total enzymes in the batch reaction can be expressed as shown in Equations 4.7 and 4.8.

$$[E]_{total} = [E] + [E^*\mathrm{Cr(VI)}] \quad 4.7$$

$$[E] = [E]_{total} - [E^*\mathrm{Cr(VI)}] \quad 4.8$$

Equations 4.5 and 4.8 can be substituted into Equation 4.6 to give Equation 4.9.

$$K_C = \frac{[E]_{total} - [E^*\mathrm{Cr(VI)}])[\mathrm{Cr(VI)}]}{[E^*\mathrm{Cr(VI)}]} \quad 4.9$$

The $[E^*Cr(VI)]$ term can be isolated on the left side thus producing Equation 4.10.

$$[E^*\mathrm{Cr(VI)}] = \frac{[E]_{total}[\mathrm{Cr(VI)}]}{K_C + [\mathrm{Cr(VI)}]} \quad 4.10$$

Now Equation 4.10 can be substituted into Equation 4.2 to give Equation 4.11, also k_{cat} is usually defined as k_m.

$$\frac{d\mathrm{Cr(VI)}}{dt} = \frac{-k_m[\mathrm{Cr(VI)}]}{K_C + [\mathrm{Cr(VI)}]}[E]_{total} \quad 4.11$$

In Equation 4.11, k_m is the maximum specific Cr(VI) reduction rate per hour, and K_C is the half velocity concentration (mg/L). The amount of Cr(VI) reductase, $[E]_{total}$, produced by the mixed culture of bacterial cells in the batch experiments are proportional to the viable bacterial cell concentration (Molokwane, 2010). Therefore, the enzyme, E, can be replaced by the total bacterial cell biomass term X to give Equation 4.12.

$$\frac{d\mathrm{Cr(VI)}}{dt} = \frac{-k_m[\mathrm{Cr(VI)}]}{K_C + [\mathrm{Cr(VI)}]}X \qquad 4.12$$

The degree of Cr(VI) reduction in the batch experiments depends on the number of bacterial cells that are available and able to produce Cr(VI) reductase enzymes. Each cell has a theoretical Cr(VI) reduction capacity. The amount of Cr(VI) reduced is also proportional to the number of bacterial cells that are inactivated by Cr(VI).

In Equation 4.13 $Cr(VI)_0$ is the initial Cr(VI) concentration (mg/L), X_0 is the initial biomass concentration (mg/L), $Cr(VI)$ is the Cr(VI) concentration (mg/L) at time *t*, and R_c is the finite maximum Cr(VI) reducing capacity (mg/mg). Equation 4.13 can be substituted into Equation 4.12 to give Equation 4.14.

$$X = X_0 - \frac{\mathrm{Cr(VI)}_0 - \mathrm{Cr(VI)}}{R_c} \qquad 4.13$$

$$\frac{d\mathrm{Cr(VI)}}{dt} = \frac{-k_m[\mathrm{Cr(VI)}]}{K_C + [\mathrm{Cr(VI)}]}\left(X_0 - \frac{\mathrm{Cr(VI)}_0 - \mathrm{Cr(VI)}}{R_c}\right) \qquad 4.14$$

The derived model shown in Equation 4.14 was simulated using AQUASIM (Reichert, 1998). The results showed that the model fit the results well as shown in Figure 4.5, and the model parameters are given in Table 4.1.

Table 4.1: Kinetic parameters for the bacteria glucose batch experiments.

Parameter	k_m(hr^{-1})	K_C (mg/L)	R_c (mg/mg)	χ^2
Value	0.084	627.28	0.6	16

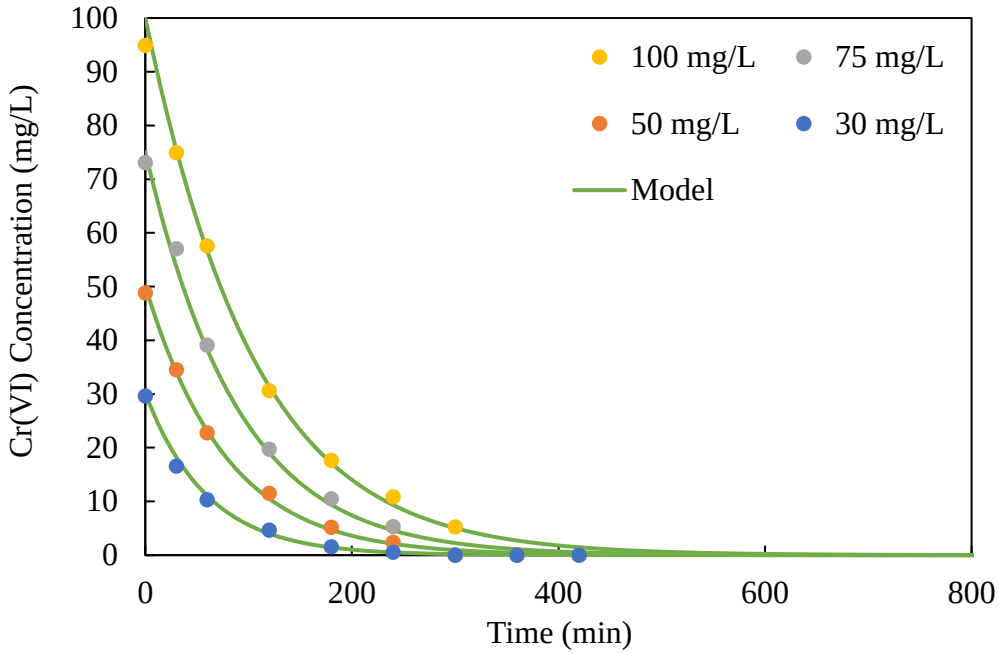

Figure 4.5: Modified Monod model fitted to the experimental data of the Cr(VI) reduction by the mixed culture of CRB utilising glucose as a carbon source.

4.4 Mixed Culture of CRB without a Carbon Source

Figure 4.6 shows the results of the control experiments, where only the CRB was used in a Cr(VI) solution without a carbon source. The CRB could only achieve 30% Cr(VI) removal from an initial concentration of 100 mg/L Cr(VI). This shows the importance of a carbon source during Cr(VI) reduction. The CRB was harvested while still in an exponential growth phase and were washed three times to ensure the removal of all the growth media. The bacteria were still alive in these experiments which means they are still somewhat metabolically active and could have still produced enzymes to combat the Cr(VI) toxicity. However, some of the Cr(VI) removal could have occurred through nonmetabolic related processes such as the adsorption of Cr(VI) onto the bacterial cells followed by intracellular reduction to Cr(III) by cytoplasmic enzymes (Chirwa and Molokwane 2011) or reduction by functional groups on the bacterial cell surface (Kang et al. 2007).

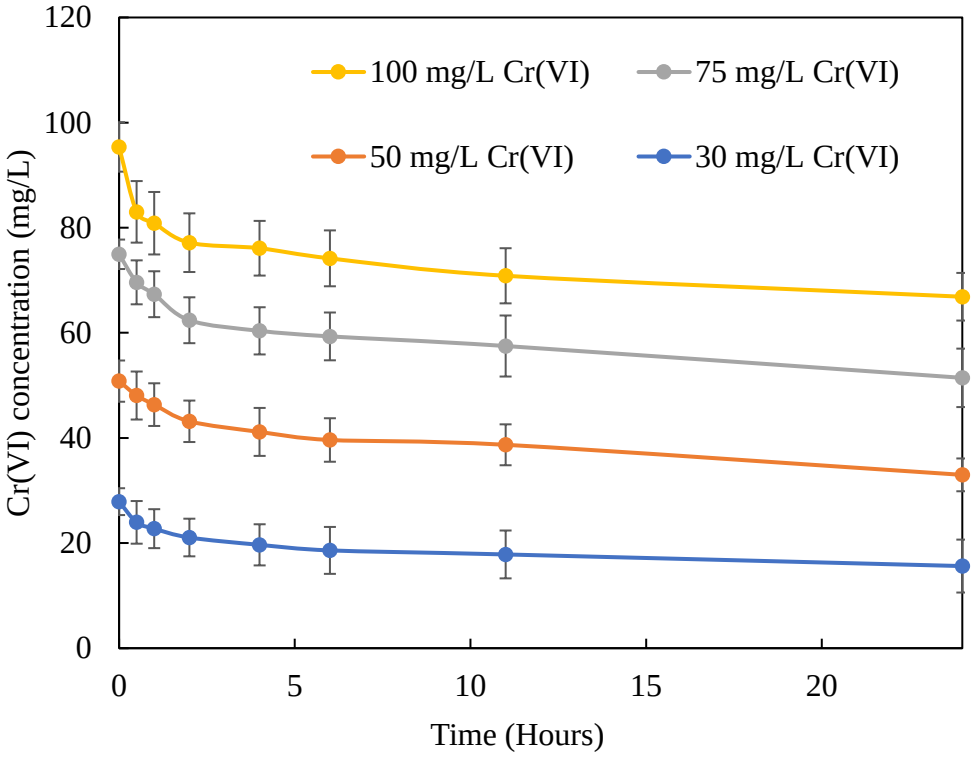

Figure 4.6: Cr(VI) concentration (mg/L) in the control experiment containing only a mixed culture of CRB. At 30 ± 2 ℃, neutral pH and at various initial Cr(VI) concentrations. (Mean ± SE; n = 3)

Molokwane (2010) was also only able to achieve 30 % Cr(VI) removal from an initial concentration of 100 mg/L Cr(VI) while using heat killed CRB cultures. Molokwane (2010) therefor achieved similar results to the results shown in Figure 4.6. Molokwane (2010) also concluded that an active metabolism is required to achieve Cr(VI) reduction.

The adsorption capacity of the bacterial cells depends on the pH of the solution as well as the functional groups on the cell surface. Park et al., (2007) has observed the reduction of Cr(VI) by functional groups on the bacterial cell surface. This mechanism is called "adsorption coupled reduction," and it allows the transfer of electrons from the surface functional groups to Cr(VI) anions. However, "adsorption coupled reduction" processes take place at a low pH (<3), and all the experiments in this study were carried out at a neutral pH level. Under acidic conditions, the potential for Cr(VI) to be reduced to Cr(III)

is significantly increased (Miretzky and Cirelli, 2010). At pH levels above 3.5, the carboxyl and amino functional groups on bacterial cells have a negative charge and repel the anionic Cr(VI) species (CrO_4^{2-}) (Ziagova et al. 2007)

Gram-positive bacteria, such as *Bacillus thermoamylovorans*, have functional groups that consist of teichoic and teichuronic acids, and peptidoglycans. These acids are responsible for the negative charge of the bacterial biomass at neutral pH and are protonated under acidic conditions. Anionic Cr(VI) species are repelled by these acids at neutral pH. Gram-negative bacteria, such as *Escherichia coli* and *Citrobacter sedlakii*, have fewer anionic functional groups, and the deprotonation of the metal binding sites on these bacteria occur at higher pH than for gram-positive bacteria, where anionic Cr(VI) compounds are repulsed (Ziagova et al. 2007). Most studies have found that acid conditions allowed for the optimal Cr(VI) adsorption by bacterial cells (Loukidou et al. 2004; Şahin and Öztürk 2005; Ozdemir et al., 2004). Although, the higher pH level of the batch experiments does not entirely eliminate the possibility that some adsorption could still have occurred but the total amount biosorption would be very limited.

Most CRB intentionally produce soluble or membrane-associated Cr(VI) reducing enzymes that are transported to outer membrane of the bacterial cell or directly into the Cr(VI) contaminated environment. Because protein excretion is an energy-intensive process, the CRB only generates Cr(VI) reducing enzymes when Cr(VI) is detected in the surrounding media (Chirwa and Molokwane 2011). Extracellular Cr(VI) reduction is preferable over intracellular Cr(VI) reduction as it decreases the possibility of internal DNA alteration and damage due to the presence of Chromate intermediates (Chirwa and Molokwane 2011). The electron transfer to Cr(VI) via the NADH dependent dehydrogenases, or cytochromes, is facilitated by the chromate reductases (Viti et al. 2014).

Extracellular reductases enzymes are not the only enzymes that are responsible for the reduction of Cr(VI) to Cr(III). For the reduction of Cr(VI) to Cr(III) intracellular cytoplasmic enzymes can also utilise cellular components such as hemeproteins, flavoproteins, NADH, and NADPH as electron donors. The structural similarity between the anionic Cr(VI) species, such as CrO_4^{2-}, and the sulphate anion (SO_4^{2-}), allows the Cr(VI) to be transported across biological membranes via active sulphate transporters in CRB (Gutiérrez-Corona et al. 2016) although this mechanism is not considered to

contribute significantly to the Cr(VI) reduction in the batch experiments. This mechanism can also contribute to internal DNA damage during the reduction of Cr(VI) to Cr(III).

It is impossible to completely rule out non-enzymatic mechanisms of Cr(VI) reduction. Although from the results shown in Figure 4.7, it is clear that these mechanisms play only a minimal role in the overall mechanism. If the CRB is not stored correctly (at -70 °C) as described in the materials and method section, the bacteria tend to "lose" their reduction capabilities after several months. The opposite effect of acclimatisation. These incorrectly stored but still active bacteria were not able to reduce significant amounts of Cr(VI) as shown in Figure 4.7, even with glucose as a carbon source. The suspected key difference between the correctly and incorrectly stored bacteria is the production of chromate reductase enzymes. Mtimunye and Chirwa (2014) also found that heat-killed bacterial cells were not able to reduce significant amounts of Cr(VI) and concluded that abiotic processes are negligible.

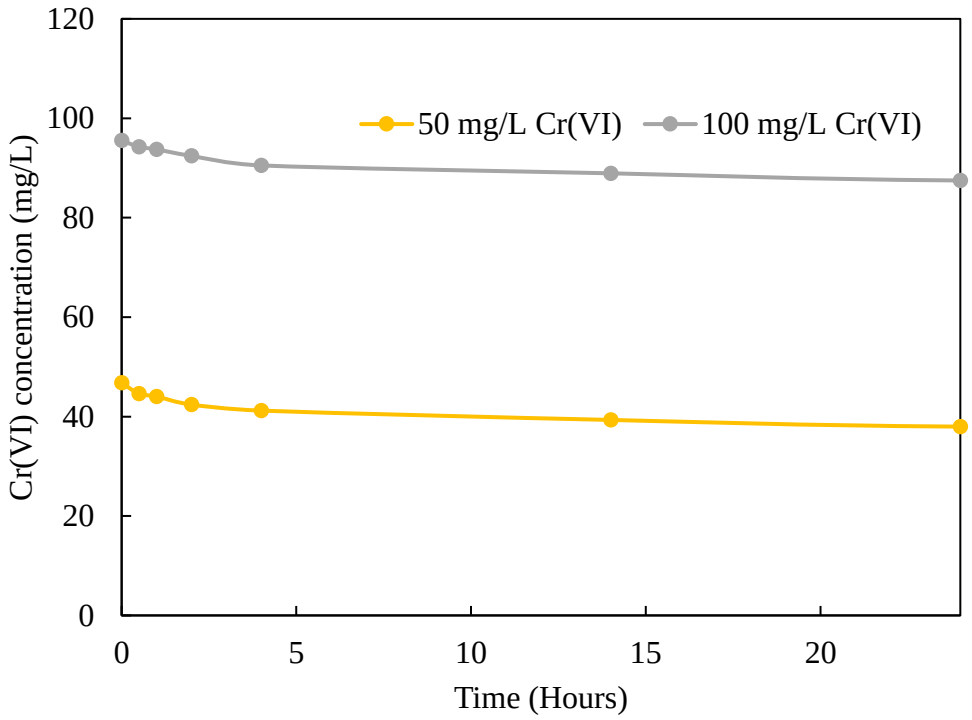

Figure 4.7: Cr(VI) concentration (mg/L) in the control experiment where the mixed culture of CRB lost their Cr(VI) reduction capabilities. At 30 ± 2 ℃, neutral pH and at various initial Cr(VI) concentrations.

4.5 Chapter Summary

The three isolated bacteria strains, *Escherichia coli*, *Bacillus thermoamylovorans*, and *Citrobacter sedlakii*, worked together in a synergistic manner. The consortium that consisted of these three bacteria strains performed better than each of the individual pure bacteria strains. In this chapter, it can be seen that a carbon source is essential for an effective bacterial reduction of Cr(VI). Glucose was used as a carbon source for the preliminary experiments. Correct bacterial storage is crucial to maintain capable CRB strains. The modified Monod model fitted the Cr(VI) reduction results very well.

Researchers that also used CRBs isolated from Brits Wastewater Treatment Plant achieved similar results to the results shown in this chapter.

CHAPTER 5 THE EFFECT OF CR(VI) AND CRB ON ALGAE

5.1 The Cr(VI) and Algae Control Batch Experiments.

5.1.1 The Impact of Cr(VI) on Algae

Chlorococcum ellipsoideum and *Chlamydomonas reinhardtii* were exposed directly to Cr(VI) anions firstly to evaluate the toxic effects the Cr(VI) has on the algae and to determine if the algae can adsorb the Cr(VI). The first noticeable effect the Cr(VI) had on the algae was the change in the algae colour. The green pigmentation of the chloroplast in the algae cells that were exposed to Cr(VI) was diminished, and after two days the algae cells became discoloured entirely.

The chlorophyll *a* content of the algal cells that were exposed to different concentrations of Cr(VI) are shown in Figure 5.1 and Figure 5.2. The algae were grown in the algal growth medium which was spiked with Cr(VI) at a pH of 7.5 and room temperature. Some of the algae were allowed to grow without Cr(VI) as a control. The chlorophyll *a* content gives an indication of the overall algae health and is also related to the algal biomass (Liang et al. 2013). In the first two days, both algae experience the lag phase: there was no change in chlorophyll *a* content in the batch experiments with or without Cr(VI). In the batch without Cr(VI) the algae entered the log growth phase after the second day, as the chlorophyll *a* content started to increase exponentially. *Chlamydomonas reinhardtii* cells had a higher growth rate than the *Chlorococcum ellipsoideum* cells. The algae exposed to Cr(VI) had barely entered the exponential growth phase before the decrease of the chlorophyll *a* content started.

The chlorophyll *a* content in the *Chlorococcum ellipsoideum* algal cells, when exposed to 5 mg/L of Cr(VI), is 2.38 mg/L after two days, which is higher than for the algae cells that were exposed to 50 mg/L Cr(VI) where the chlorophyll *a* content was only 0.99 mg/L. The chlorophyll *a* content in the *Chlamydomonas reinhardtii* algae cells that were exposed to 5 mg/L of Cr(VI) for two days, is 4.07 mg/L, which is higher than when the same algae cells were exposed to 50 mg/L Cr(VI) for two days in which case the chlorophyll *a* concentration was only 0.28 mg/L. The *Chlorococcum ellipsoideum* cells multiplied at a lower rate than the *Chlamydomonas reinhardtii* cells when exposed to the same initial Cr(VI) concentration. On the other hand, when exposed to the same initial Cr(VI)

concentration the chlorophyll *a* content in *Chlorococcum ellipsoideum* decreased at a higher rate than was the case for *Chlamydomonas reinhardtii*. In the batch experiments the chlorophyll *a* content of the algae cells decreased at a faster rate if the initial Cr(VI) concentration of the experiment was increased.

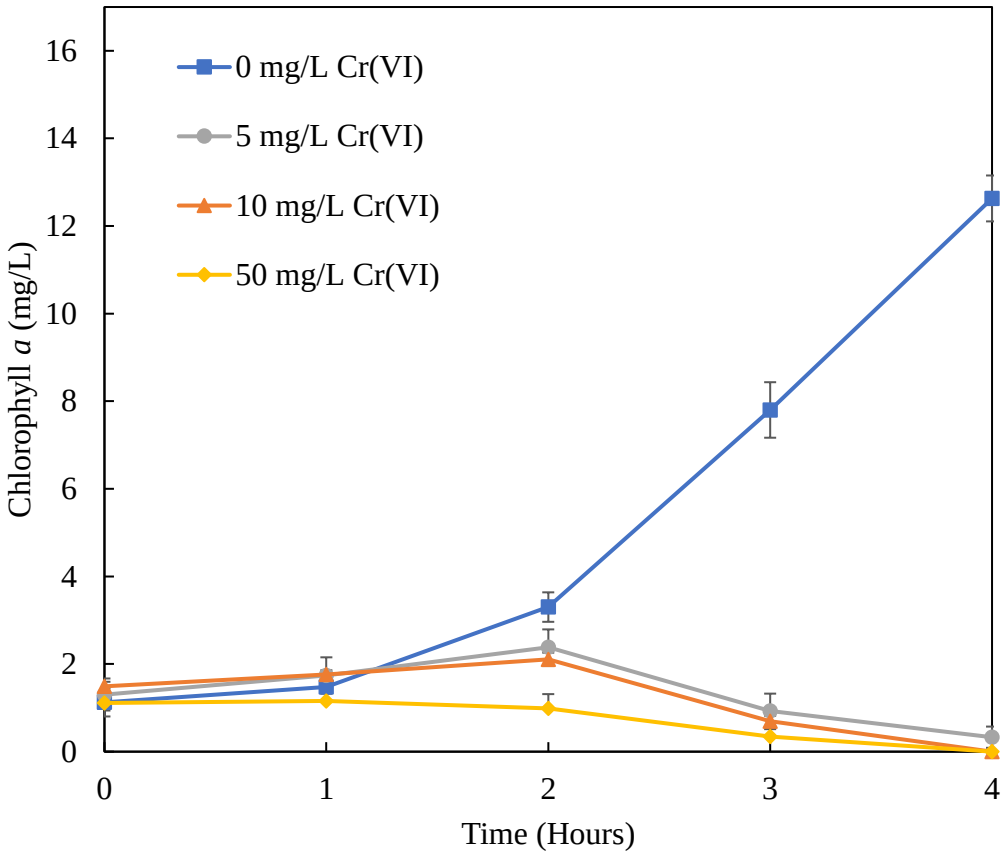

Figure 5.1: Chlorophyll *a* content (mg/L) of *Chlorococcum ellipsoideum* cells in 3N-BBM+V exposed to various Cr(VI) concentrations. Under algal light; Osram L 36W/77 Floura at 25 °C and neutral pH. (Mean ± SE; n = 3)

The results show that both the algae species are susceptible to Cr(VI) toxicity even at low Cr(VI) concentration. The higher the Cr(VI) concentration, the more the algae growth rate is inhibited. Rodrıguez et al. (2007) also found that *Chlamydomonas reinhardtii* has a low tolerance level for Cr(VI). The low Cr(VI) tolerance presents the opportunity to utilise the *Chlamydomonas reinhardtii* species as an indicator of Cr(VI) contamination in the environment. Very few studies have investigated the effect of Cr(VI) on *Chlorococcum Ellipsoideum*. The decrease in chlorophyll *a* content could indicate that the algae cell walls

were damaged by the Cr(VI), which allowed the chlorophyll *a* as well as other internal metabolites to leak out. Arun et al. (2014) produced SEM images of saltwater algae, *Dunaliella salina*, and *Dunaliella tertiolecta*, that were damaged by exposure to 40 ppm Cr(VI). A decrease in chlorophyll *a* content inside the algae cell can interfere with the photosynthesis process and the cell division processes of the algae (Volland et al. 2012). Photosynthesis is essential to produce energy for algae growth.

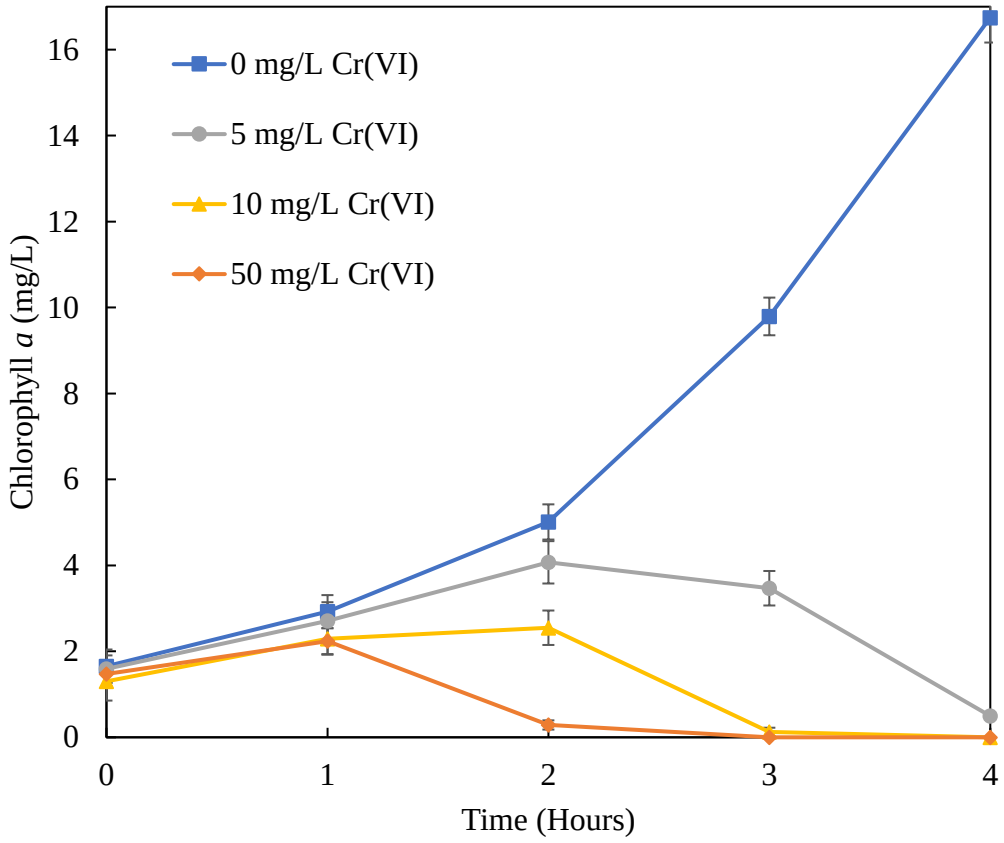

Figure 5.2: Chlorophyll *a* content (mg/L) of *Chlamydomonas reinhardtii* cells in 3N-BBM+V exposed to various Cr(VI) concentrations. Under algal light; Osram L 36W/77 Floura at 25 °C and neutral pH. (Mean ± SE; n = 3)

5.1.2 The Effects of the Microalgae on the Cr(VI) Concentration

Figure 5.3 shows the Cr(VI) concentration in batch experiments containing only the algal cells. The Cr(VI) concentration in the solution was not considerably affected by the algae cells; in the *Chlorococcum ellipsoideum* and *Chlamydomonas reinhardtii* batch experiments only a decrease of 1.6% and 3.6 % in the Cr(VI) concentration was observed. However, in the first few hours the Cr(VI) concentration oscillates and a sharp spike in the Cr(VI) concentration appears in the first hour. This phenomenon occurred in both *Chlorococcum ellipsoideum* and *Chlamydomonas reinhardtii* batch experiments. The likely explanation is that the Cr(III), which makes up 25% of the total chromium in the solution, was oxidised to Cr(VI). Toxic metals, such as Cr(VI), disrupt the oxidative balance of the algae cells; therefore, algae have developed a wide range of protective mechanisms that remove reactive oxygen species (ROS) before damage can occur to the sensitive parts of algae cells (Hassoun et al. 1995). ROS such as hydrogen peroxide (H_2O_2) can oxidise Cr(III) to Cr(V) and Cr(VI) (Xue et al., 2016), which can serve as a possible explanation for the initial spike in Figure 5.3.

After the oscilation and initial spike, the Cr(VI) concentration in the experiments remained constant as shown in Figure 5.3. Negligible Cr(VI) adsorption onto the algal cell wall occurred under experimental conditions with a neutral pH. The functional groups on the surface of the algal cell wall (carboxyl, phosphoryl, and hydroxyl) are negatively charged at a pH above 3.5 and repelled the anionic Cr(VI) forms ($HCrO_4^-$, CrO_4^{2-} and $Cr_2O_7^{2-}$) (Kratochvil et al. 1998). To verify this hypothesis the same experiments were repeated at a pH below 2. Under these acidic conditions it was found that up to 65% of Cr(VI) was removed. At a low pH, the algae cell wall is positively charged as more positively charged functional groups are present. Therefore the cells are electrostatically attracted to the negatively charged Cr(VI) anions (Murphy et al. 2008). pH affects the configuration of the active ion-exchange sites, and thus has a significant influence on Cr(VI) biosorption by algae (Sibi 2016).

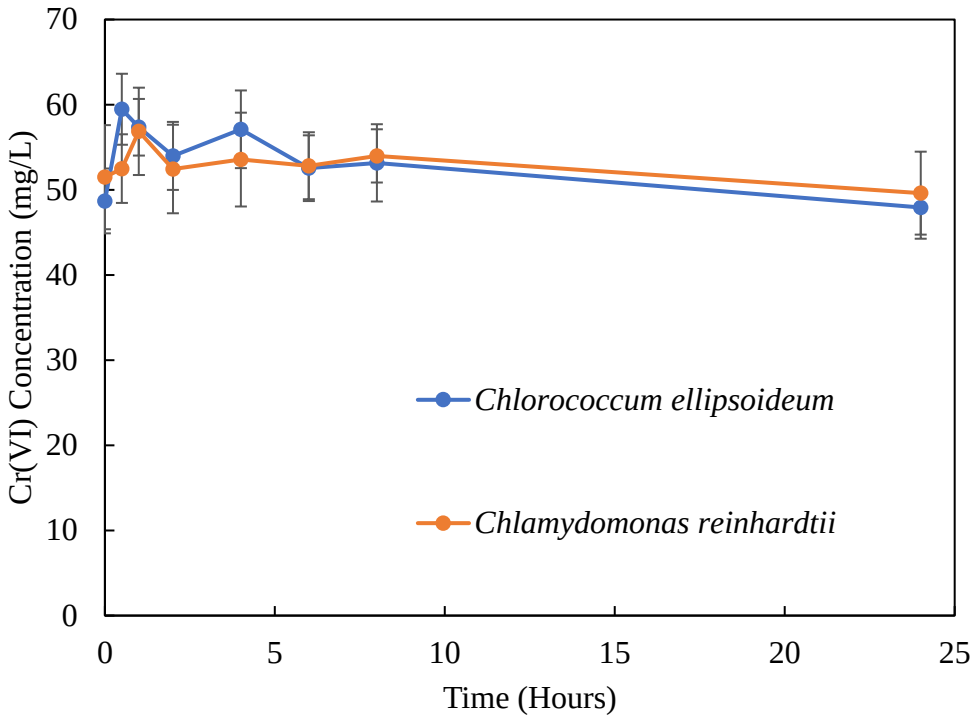

Figure 5.3: Cr(VI) concentration in the batch experiments with only algae present (without bacteria): *Chlamydomonas reinhardtii* and *Chlorococcum ellipsoideum* at 25 °C and neutral pH. (Mean ± SE; n = 3)

5.2 Interactions Between Algae and Bacteria

5.2.1 The Effect of CRB on the Algae

The effect of CRB on both algal species, *Chlorococcum ellipsoideum* and *Chlamydomonas reinhardtii*, are shown in Figure 5.4. Measuring the chlorophyll *a* content is an excellent technique to quantify the algal biomass in the presence of bacteria. The chlorophyll *a* content in both algae species were depleted within four days, which indicates a decline in algal biomass. It is clear that both algal species did not have a defence mechanism in place to counter the CRB affects. The biomass of the algae and CRB were very high in this batch experiments and could have led to competition for nutrients between the algae and CRB. The ratio of algae cells to CRB in the experiment could also have prohibited a symbiotic relationship to develop (Pell et al., 2017). Figure 5.4 also shows the pH in the experiments.

The pH was increased from a neutral pH to a pH of 8.8 in the first day, which corresponds to the algae's growth phase as can be observed in Figure 5.4. The algae consume the CO_2 in the solution which can be related to the increases the pH. The increase in pH can also be due to algal growth in general. When the chlorophyll *a* content began to decrease rapidly after the first day, the pH remained constant.

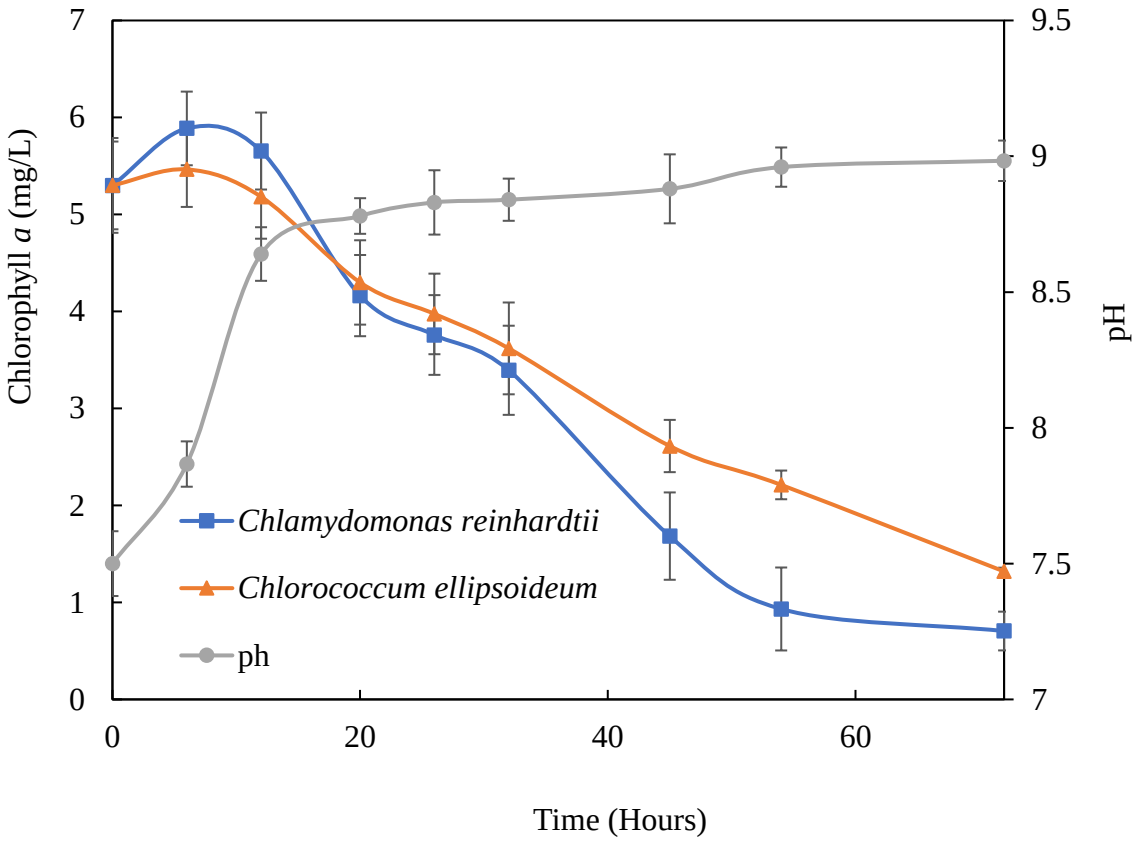

Figure 5.4: Chlorophyll *a* content in the batch experiments that combined the CRB with the two algae species: *Chlamydomonas reinhardtii* and *Chlorococcum ellipsoideum*. Under algal light; Osram L 36W/77 Floura at 25 °C and neutral pH. (Mean ± SE; n = 3)

Many bacteria species are well known to have adverse effects on algae (Dakhama et al. 1993). Algal cell lysis frequently occurs when the cells are in close proximity to bacteria, which is a factor of the parasitic relationship, and competition for nutrients, between bacteria and algae (Ramanan et al. 2016). Toncheva-Panova and Ivanova (2000) found

that the pathogenic bacterial strain, *Cytophaga sp.* LR2, is responsible for the cell lysis of the red microalga, *Rhodella reticulate*. *Cytophaga sp*. LR2 excreted slime to attached itself to the algal cells, followed by the production of a lytic enzyme to degrade the algae cell. Toncheva-Panova and Ivanova (2000) suggest that bacteria required some of the algal metabolites.

Shilo (1970) found that myxobacteria were responsible for the lysis of filamentous blue-green algae in fishponds. Ji et al. (2009) investigated the biological degradation of algae and found that algae lysing bacteria, *Pseudomonas spp*. and *Bacillu spp*. were able to achieve a chlorophyll *a* removal efficiency of 60%. *Bacillu spp*. secretes extracellular enzymes which dissolve the algae. Szymczak-Żyla et al. (2008) also found microbiota from the zooplankton gut could degrade chlorophyll *a* content under aerobic conditions. The algae can protect against parasitic bacteria by raising the pH above 10 during photosynthesis, by consuming CO_2 faster than it can be replaced by bacterial respiration. Algal treatment ponds have been used to disinfect (kill harmful bacteria) wastewater; at peak algal activity in the treatment ponds, the carbonate and bicarbonate ions react and provide more CO_2 for the algae, leaving an excess of hydroxyl ions, causing the pH of the water to rise above 9, which can kill bacteria. The bacteria, which are not alkaliphilic organisms, would be severely inhibited by the high pH (Abdel-Raouf et al. 2012).

Nutrient competition can also trigger parasitism between bacteria and algae. Competition for existing nutrients results in slower growth rates for bacteria, which allow bacteria to outcompete algae in the environment.

5.2.2 The Viable Bacterial Cell Count

Figure 5.5 and Figure 5.6 shows how the viability (CFU count) of the CRB changes in the presence of algae. Although the OD600 is commonly measured to represent the bacterial biomass, in this case the algae cells will influence the OD measurement, and therefore it was decided to rather use the CFU count as an indication of bacterial biomass. The batches were carried out with 50 mg/L of initial Cr(VI) concentration and without Cr(VI). In the presence of Cr(VI) the viability of the bacteria started to decrease 4 hours after the experiment began in both the *Chlorococcum ellipsoideum* and *Chlamydomonas reinhardtii* systems. This indicates that the Cr(VI) also inhibits the growth of CRB, which

redirects all its available energy toward detoxifying the immediate environment instead of using energy to grow. Other researchers have also observed this effect; Chirwa and Molokwane (2011) suggest that under aerobic conditions the reduction of Cr(VI) does not yield conserved energy for metabolism and that some of the electrons are diverted from biological activities and go toward Cr(VI) reduction. The Cr(VI) is reduced at the expense of metabolic activity in the cells. Thatoi et al., (2014) suggest that the oxidative stress caused by the Cr(VI) affects the viability of cells and the efficiency of Cr(VI) reduction. Even after all the Cr(VI) was reduced the viability of the CRB continued to decrease. Depleted nutrient sources and/ or the high pH of the solution could be responsible for this.

Simultaneous Cr(VI) reduction and phenol degradation was observed using Stenotrophomonas sp. isolated from tannery effluent contaminated soil. Bioreduction of Cr(VI) by *Pseudomonas stutzeri* L1 and *Acinetobacter baumannii* L2. Simultaneous Cr(VI) reduction and phenol degradation also occurred with an anaerobic consortium of bacteria. Aerobic chromate reduction was achieved with *Bacillus subtilis.*

The CRB cell viability (bacterial growth) increases up to 12 hours without Cr(VI) present. At the 12 hour mark, the initial bacterial CFU count has risen by 60% and 58% respectively for the *Chlorococcum ellipsoideum* and *Chlamydomonas reinhardtii* systems. The bacterial biomass concentration was much higher without Cr(VI) present which indicates that the CRB was also inhibited to an extent by the Cr(VI) toxicity. However, a particularly high growth rate or exponential growth was not achieved as the bacterial biomass concentration at the beginning of the batch experiments was already too high. Nonetheless, from Figure 5.5 and Figure 5.6, it is clear that the bacterial CFU count did increase in the first few hours.

Chirwa and Wang (2000) used E.coli as a CRB, however the E.coli were able to achieve a much higher growth rate than the growth rates of the CRBs shown in Figure 5.5 and Figure 5.6. The E.coli used by Chirwa and Wang (2000) were able to increase 1000 fold in biomass within 20 hours due to very low initial Cr(VI) concentrations at 2.5 mg/L and lower.

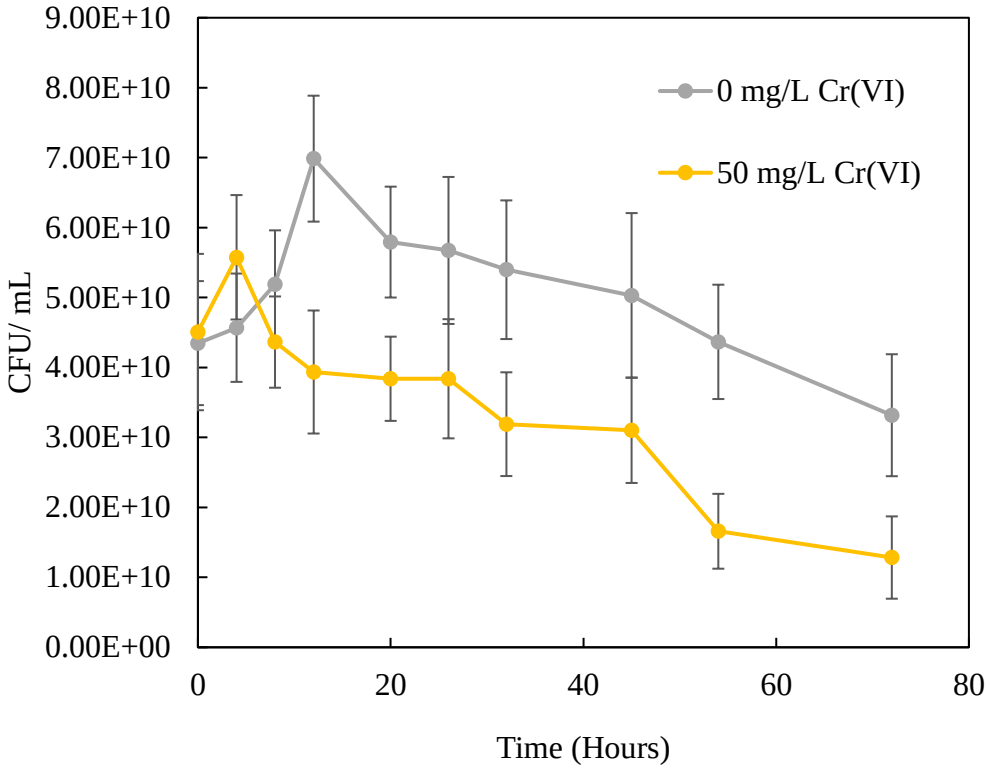

Figure 5.5: The CFU count of the CRB (log CFU mg/L) in which the algae cells of *Chlorococcum ellipsoideum* were used as a carbon source with 50 mg/L of initial Cr(VI) concentration, or without Cr(VI), at 30 ± 2 ℃ and neutral pH. (Mean ± SE; n = 3)

After 12 hours, the experiments devoid of Cr(VI) showed a steady decline in the CFU count, this phase corresponds to a pH value of 8 as can be observed in Figure 5.4. It is surmised that the algae consumed all the CO_2 in the solution which caused the pH of the solution to increase. The higher pH level can inhibit bacterial growth and also contribute to the decline of the CFU count. The carbon sources produced by the algae that can easily be consumed by the CRB have possibly been depleted after 12 hours. Certain algae and phytoplankton release substances such as antibiotics that can inhibit bacteria growth. Green microalgae, *Chlorella*, produces an antibiotic called chlorellin (Cole, 1982a).

The two algae species had similar performance as potential carbon sources. Without Cr(VI) present a slower decline in the bacterial CFU count was observed when *Chlamydomonas reinhardtii* was utilised as a carbon source when compared to *Chlorococcum ellipsoideum*. In the presence of Cr(VI), when *Chlamydomonas reinhardtii*

was utilised as a carbon source it also resulted in a higher CFU count at 4 hours than for *Chlorococcum ellipsoideum*. About a 26.4% increase from the initial CFU count was observed when *Chlorococcum ellipsoideum* was utilised as a carbon source, compared to only a 19.2% increase in CFU count for *Chlorococcum ellipsoideum*.

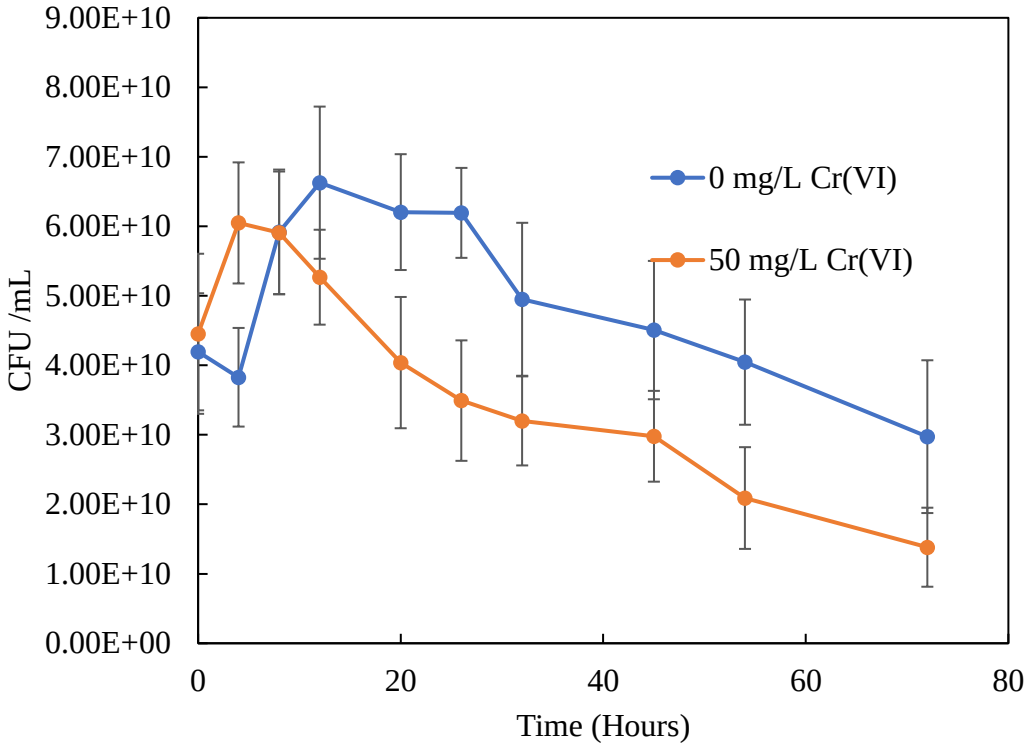

Figure 5.6: The CFU count of the CRB (log CFU mg/L) in which the algae cells of *Chlamydomonas reinhardtii* were used as a carbon source with 50 mg/L initial Cr(VI) concentration, or without Cr(VI), at 30 ± 2 ℃ and neutral pH. (Mean ± SE; n = 3)

5.3 SEM Results

Scanning electronic microscopy (SEM) images of the CRB-algae interaction after exposure to different initial Cr(VI) concentrations (0, 50 and 100 mg/L Cr(VI)) are shown in Figure 5.7 to Figure 5.12. The bacterial cells are the smaller cylindrical (rod-shaped) cells, and the algae cells are generally larger and spherical. Figure 5.7 shows a *Chlorococcum ellipsoideum* algae cell surrounded by CRB after 1 hour of exposure (without Cr(VI)). The algal cell is undamaged and plump. The CRB surrounds the algae cell surface. Figure 5.8 also shows a plump algae cell after several hours of exposure with bacteria attached to a damaged region of the algae cell wall. Figure 5.9 shows how the bacteria have attached to the algae cell wall. Filamentous bridges, i.e. flexible fibres resembling pili, are used by the CRB to affix itself onto the algal cell wall. These fibres can be used by the CRB to increase interactions with the algae cell host (Gardiner et al. 2014). Bacteria use cell-surface appendages such as pili and flagella to colonise many surfaces successfully. The binding of *Escherichia coli* to surfaces is promoted by the presence of both type 1 pili and the flagella.

Bacteria have been previously proven also to be effective in the control of harmful algal blooms (Gumbo et al., 2008). The bacteria cause cell lysis through multiple mechanisms: physical contact, entrapment, endoparasitism, and ectoparasitism. Entrapment involves the bacteria to surround the blue-green algae cell and to establish physical contact, followed by secretion of extracellular substances that cause damage to the cell wall, finally resulting in cell lysis and death. Endoparasitism involves the penetration of the blue-green algae by the bacteria, which causes the bacteria to have access to nutrients and accumulates inside cells. Caiola et al. (1984) found that Bdellovibrio-like bacteria were able to lyse *Microcystis aeruginosa* cells only after penetration.

Figure 5.10 shows an algae cell after 24 hours of exposure to 50 mg/L Cr(VI). The algae cell was no longer round and plum but has lost its structural integrity. The shrivelled-up algae cell was covered with bacteria. Most of the bacteria cells do not have any visible damage; however, in Figure 5.10 there appears to be damaged bacteria cell on the algae surface, although it could possibly just be algae cell debris. Other researchers have found that Cr(VI) have adverse impacts on bacteria cell. Pattanapipitpaisal et al., (2002) found that Cr(VI) promoted the loss of flagella in some Gram-positive strains and, lysis of some of the *Bacillus pumilus* cells. Upadhyay et al., (2017) found that high Cr(VI)

concentrations (e.g. 300 mg/L) initiate cell lysis in *Bacillus subtilis* MNU16, which might be due to the deposition of Cr(VI) in the cytoplasm and on the surface of the cells.

Figure 5.11 shows an algae cell after 24 hours of exposure to a solution with an initial concentration of 100 mg/L Cr(VI). The algal cell wall has ruptured, and the CRB can be seen inside the ruptured algae cell. Internal cell cytoplasmic debris can also be seen. There is high bacterial activity in the area surrounding the lysed algal cell. During the algal growth phase, it generated photosynthate carbon which is now released as dissolved or colloidal material. The surrounding CRB can presumably metabolise the photosynthate carbon (Cole et al. 1982b). Figure 5.12 also shows the algae cell after 24 hours exposure to a solution with an initial concentration of 100 mg/L Cr(VI). Compared to the algae cells that were exposed to a solution with an initial concentration of 50 mg/L Cr(VI), the cells in Figure 5.12 are even more shrivelled up. From these observations it follows that a higher initial Cr(VI) concentration is more detrimental to algae cell health.

At the onset of algal death, there is usually a substantial initial release of soluble materials from the algal cell (Cole, 1982a). In both laboratory experiments and the natural environment, it has been found that bacteria can metabolise organic matter from a dead algal cell (Iturriaga and Hoppe, 1977). Granting this occurrence is usually considered a result of close proximity between the cells. Parasitic bacteria have been found to be present when the algal cell wall sheaths, to facilitate cell wall degradation (Wang et al., 2010).

The SEM results indicate that a portion of the photosynthate carbon, and other internal metabolites, were leaked from the algae cells that were exposed to both parasitic CRB and Cr(VI). This leaked substance could potentially be utilised as a carbon source by CRB which allows for the reduction of Cr(VI) to Cr(III) by the CRB. The combination of toxic Cr(VI) with parasitic CRB, overall, has a very adverse impact on algal cell health.

5.4 Chapter Summary

In conclusion, it is clear that Cr(VI) inhibits algae growth severely whereas the CRB were only slightly inhibited by Cr(VI). CRB inhibition was confirmed by comparisons to low Cr(VI) concentration experiments conducted by Chirwa and Wang (2000). The bacteria have evolved resistance mechanisms against Cr(VI), but the algae have not developed such a mechanism, yet. The CRB also exhibits parasitic behaviour towards the algae cells. This means on the one side that the bacteria could forcefully receive the substrate from the algae that consequently would allow for the reduction of Cr(VI). However, on the other hand, the fact that both the Cr(VI) and CRB have such a huge negative impact on the algae mean it would be difficult to apply this system in a continuous and self-sustaining manner.

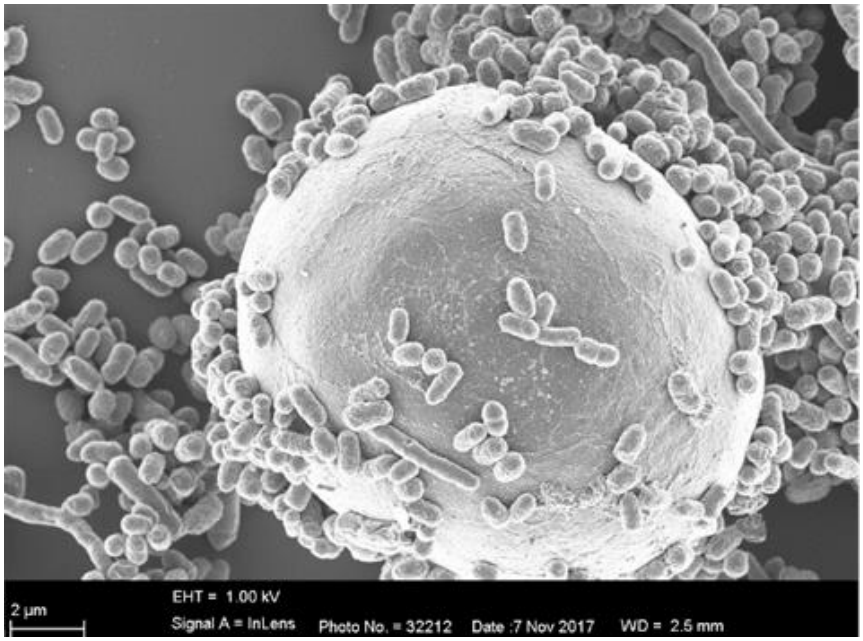

Figure 5.7: The SEM images of *Chlorococcum ellipsoideum* cells (large and round) together with CRB cells (small and cylindrical) without Cr(VI).

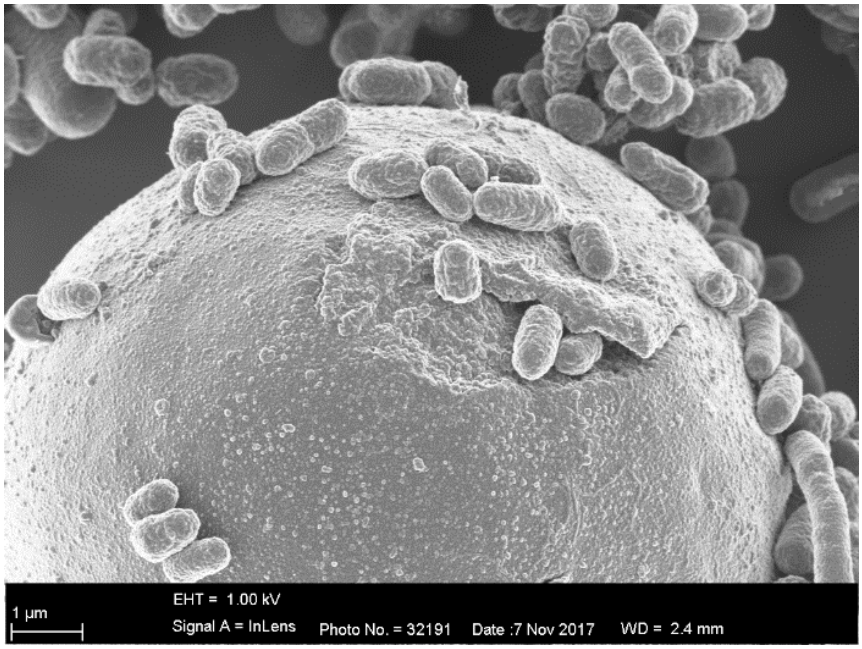

Figure 5.8: The SEM images of *Chlorococcum ellipsoideum* cells (large and round) together with CRB cells (small and cylindrical) without Cr(VI).

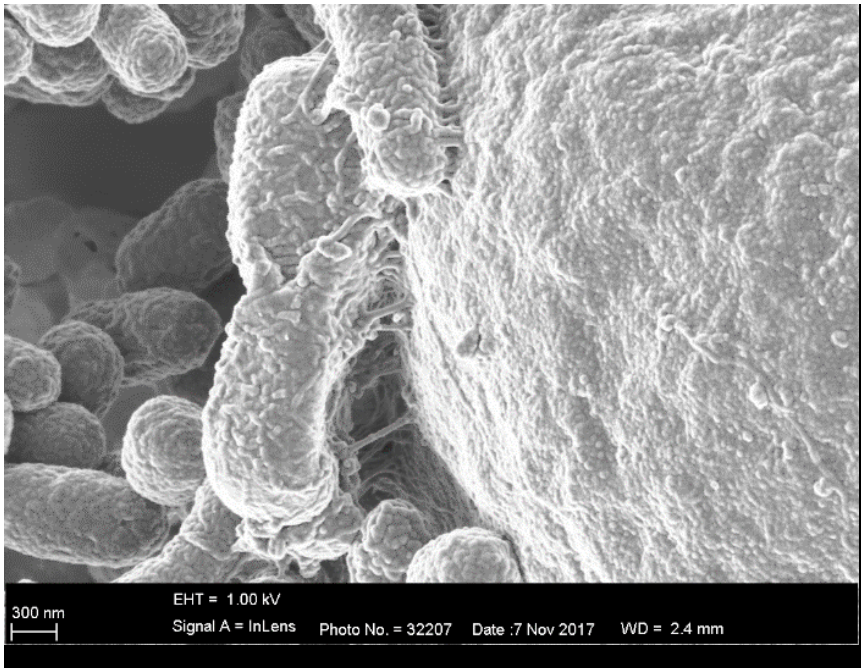

Figure 5.9: The SEM images of *Chlorococcum ellipsoideum* cells (large and round) together with CRB cells (small and cylindrical) without Cr(VI).

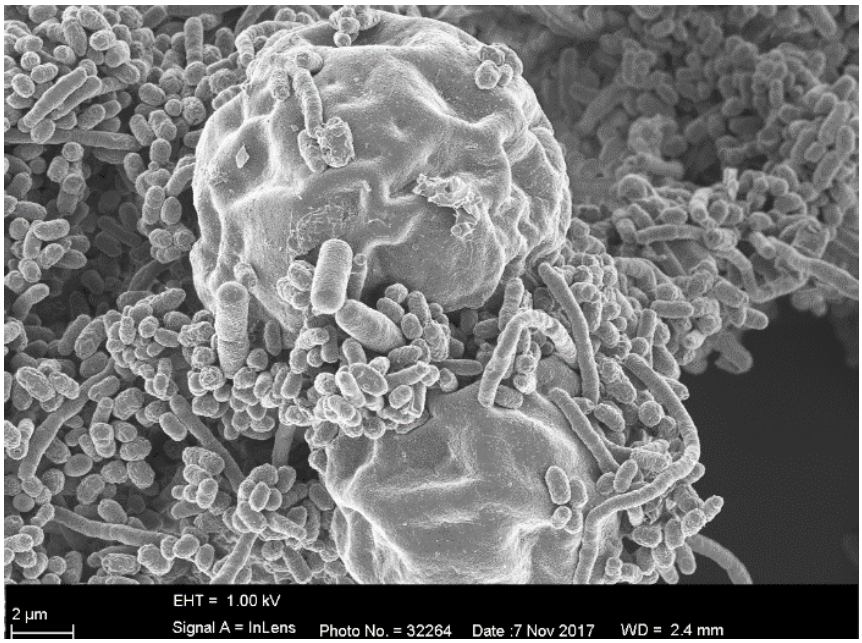

Figure 5.10: The SEM images of *Chlorococcum ellipsoideum* cells (large and round) together with CRB cells (small and cylindrical) exposed to 50 mg/L Cr(VI).

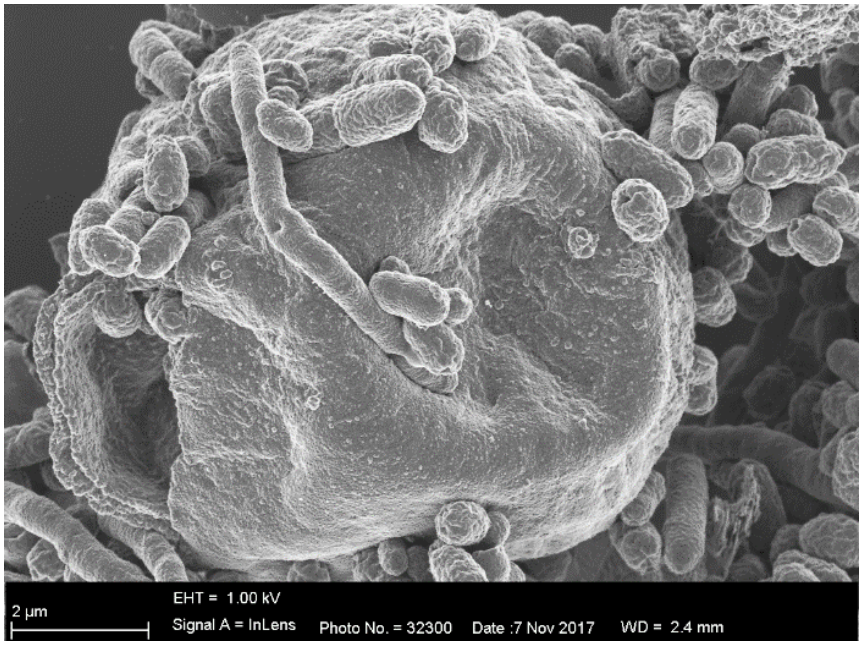

Figure 5.11: The SEM images of *Chlorococcum ellipsoideum* cells (large and round) together with CRB cells (small and cylindrical) exposed to 100 mg/L Cr(VI).

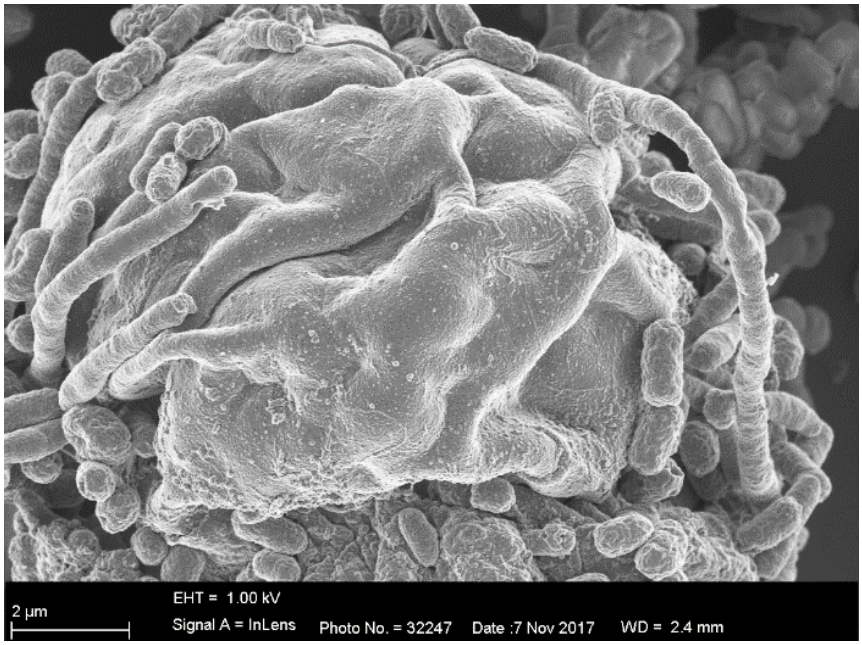

Figure 5.12: The SEM images of *Chlorococcum ellipsoideum* cells (large and round) together with CRB cells (small and cylindrical) exposed to 100 mg/L Cr(VI).

CHAPTER 6 Cr(VI) REDUCTION IN CRB-ALGAE SYSTEMS

6.1 Bacterial Cr(VI) Reduction in Spent Algae Media

In order to fully understand the processes that take place during the reduction of Cr(VI) in the CRB-algae systems, control experiments should first be conducted. To evaluate to what extent CRB can use the extracellular metabolites produced by the algae during Cr(VI) reduction, a control experiment was set up in which only the cell-free spent media from the algae was used with CRB. The algae cells were removed through multiple centrifugation steps as described in the materials and method section. The cell-free spent media is believed to contain the extracellular products that were produced by the algae during the algae growth phase. Figure 6.1and Figure 6.2 shows Cr(VI) concentration profiles in the control experiments of the CRB in a cell free spent algae media. After 24 hours in the experiments with an initial Cr(IV) concentration of 100 mg/L, the CRB in the *Chlamydomonas reinhardtii* and the *Chlorococcum ellipsoideum* cell-free spent media could achieve 49% and 45% Cr(VI) reduction respectively. Repeating these experiments with an initial concentration of 30 mg/L Cr(VI yielded 93 % Cr(VI) removal in both media. This indicates that algal metabolites were to some extent available for the CRB to utilise, however, the algal metabolites were not sufficient to allow for the complete reduction of Cr(VI) by the CRB in 24 hours.

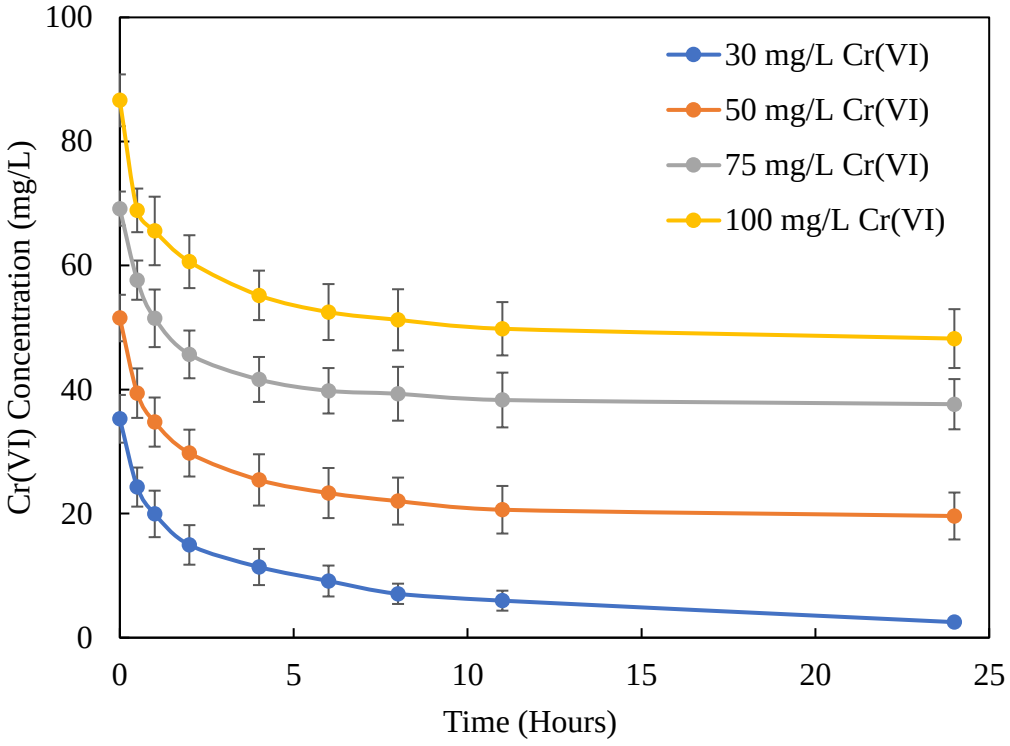

Figure 6.1: Cr(VI) concentration in a system containing CRB and *Chlorococcum ellipsoideum cell* free spent algae media. At 30 ± 2 °C, neutral pH and at different initial Cr(VI) concentrations. (Mean ± SE; n = 3)

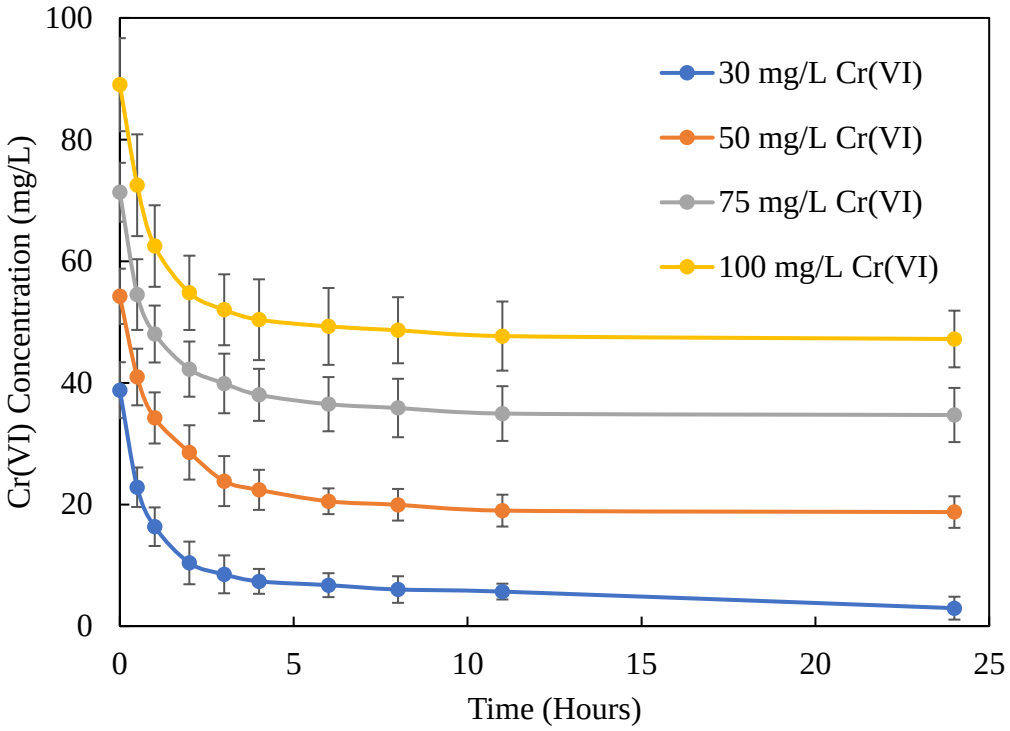

Figure 6.2: Cr(VI) concentration in a system containing CRB and Chlamydomonas reinhardtii cell-free spent media. At 30 ± 2 ℃, neutral pH and at different initial Cr(VI) concentrations. (Mean ± SE; n = 3)

The TOC of the cell-free spent algae media was measured using a TOC analyser to determine how much organic carbon were available for the CRB to utilise. TOC for *Chlorococcum ellipsoideum* and *Chlamydomonas reinhardtii* cell-free spent media were 46 and 62 mg/L TOC respectively. Living marine algae releases soluble compounds during growth (Cole, 1982a). *Chlamydomonas reinhardtii* releases a lytic factor (lytic enzyme) during mating that digests the cell wall, which allows the fusion of the gametes. The digestion of the cell wall also releases soluble carbohydrates (Cole, 1982a; Matsuda et al., 1985). The chemical composition of the released compounds is not known and is doubtlessly very complex. One of the molecules which are repeatedly found in extracellular excretions of the algae is Glycollate. Glycollate can be metabolised by bacteria (Cole, 1982a)

The algae cells release extracellular organic matter (EOM) through two processes: a diffusion process which is driven by equilibrium concentration between intra- and

extracellular compounds, and the irreversible degradation of the algae cell surface (Nguyen et al., 2005). The compounds that are released by the algae in the diffusion process have low to intermediate molecular weight. Glycolic and amino acids, produced in high concentrations inside the active algae cells, are lost by diffusion through the algae cell membrane (Nguyen et al., 2005). These compounds are relatively dominant during the exponential growth phase (Watt 1969). Watt 1969 found that healthy algae cells produced more EOM per unit biomass in the late exponential stage than algae with a declining population. Compounds that are produced by the algae cell surface degradation process usually have a higher molecular weight, as is the case with the produced polysaccharides that represent the leaching of the surface of senescent cells (Nguyen et al., 2005).

The TOC content of the cell-free spent algae is low compared to the available TOC content in the experiments in which glucose was used as the carbon source, which had a 200 mg/L TOC. The higher TOC content, and the fact that glucose is also more readily consumed by the CRB, can explain the lower reduction percentage achieved in these control experiments. Separating the algae cells from the growth media can be a problematic and energy expensive process, especially if the cells are not self-flocculating (Kleinová et al. 2012). Therefore, utilising only extracellular products produced by algae is not feasible.

6.2 Bacterial Cr(VI) Reduction Utilising Algae as a Carbon Source

Figure 6.3 and Figure 6.4 shows the reduction of Cr(VI) when both CRB and algae cells are present. The CRB has access to both the extracellular metabolites and the internal carbon sources provided by the algae cells. Based on the SEM results, it is very likely that internal metabolites have leaked out of the algae cells. The batch experiments were carried out at 25 °C, with a neutral pH, and without any glucose. Both *Chlorococcum ellipsoideum* and *Chlamydomonas reinhardtii* were able to successfully provide a carbon source for CRB to facilitate the reduction of Cr(VI).

In the batch experiments with an initial concentration of 50 mg/L Cr(VI), the total reduction Cr(VI) Cr(VI) was achieved within 24. In the batch experiments with an initial concentration of 100 mg/L Cr(VI), 92 % bacterial Cr(VI) reduction was achieved within 24 hours when *Chlorococcum ellipsoideum* was used as a carbon source, in contrast to the

98 % bacterial Cr(VI) reduction that was achieved when *Chlamydomonas reinhardtii* was used as a carbon source instead.

It is clear that the internal metabolites, and probably the physical algae cells, were utilised by the CRB. The shape of the curve in Figure 6.3 and Figure 6.4 can give some insight into how the reduction process proceeded. The decrease in the Cr(VI) concentration is thought to mainly be a result of metabolically dependent processes, although a few metabolically independent processes would also contribute.

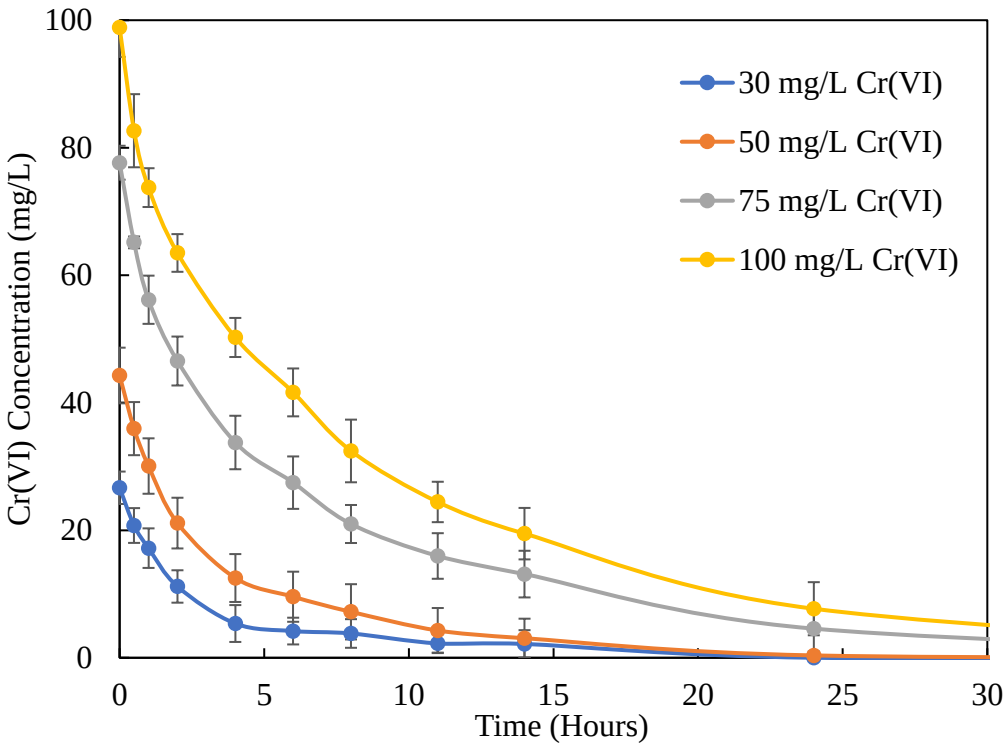

Figure 6.3: Cr(VI) concentration in a system containing CRB and algae: Chlorococcum ellipsoideum is used as a carbon source during the bacterial reduction experiments. At 30 ± 2 ℃, neutral pH and at different initial Cr(VI) concentrations. (Mean ± SE; n = 3)

Yewalkar et al. (2007) used *Chlorella spp*. to directly reduce an initial Cr(VI) concentration of 3.12 mg/L by 97% in 72 hours. Yewalkar et al. (2007) found that *Chlorella spp*. was able to grow in the 3.12 mg/L Cr(VI) environment. The CRB-algae system, investigated in the current study ultimately derived carbon by sequestering CO_2

from the environment, whereas Yewalkar et al. (2007) used acetate as a carbon source for the algae only system. Chirwa and Wang (2000) found that *E. coli* ATCC 33456 were able to utilised organic acid metabolites produced by phenol degraders in a consortium of anaerobic bacteria.

Molokwane (2010) were also able to utilise an alternative natural carbon source namely saw dust in mesocosm reactors in which Cr(VI) was treated. The saw dust was used to simulate the carbon sources leached from the overlying vegetation above the ground. The saw dust preformed well as a carbon source and were able to allow 70% Cr(VI) reduction in the reactor. The CRBs used in Molokwane's (2010) study was also isolated from the Brits wastewater treatment plant. Molokwane (2010) found that microcosm reactors with saw dust (alternative carbon source) performed better than the ones with no carbon source.

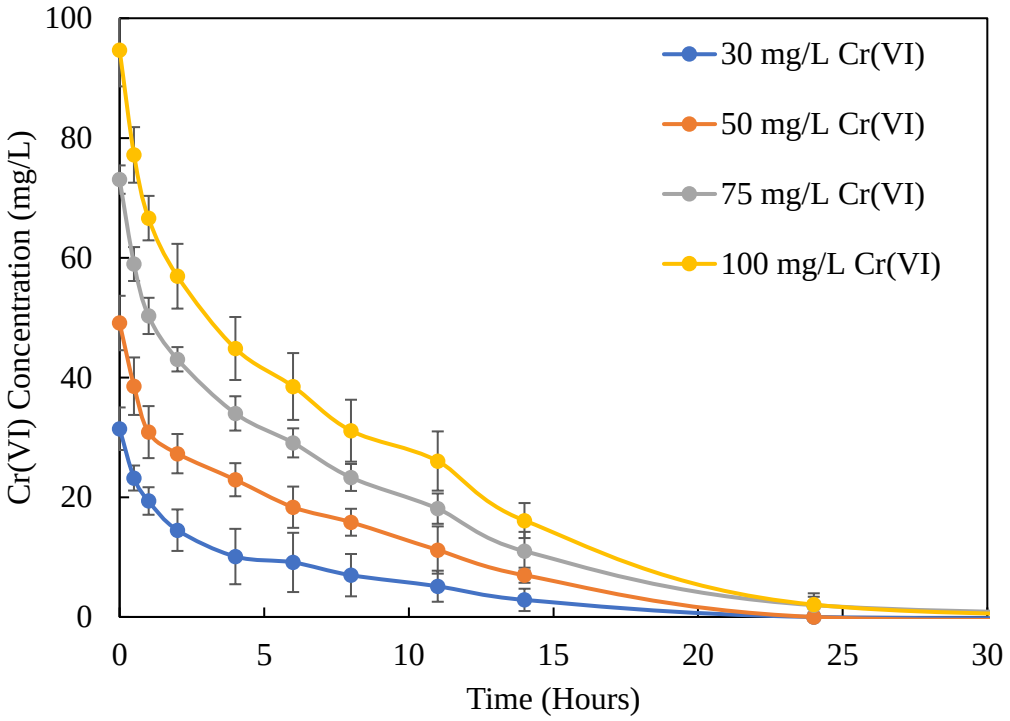

Figure 6.4: Cr(VI) concentration in a system containing CRB and algae: *Chlamydomonas reinhardtii* is used as a carbon source during the bacterial reduction experiments. At 30 ± 2 ℃, neutral pH and at different initial Cr(VI) concentrations. (Mean ± SE; n = 3)

6.3 Comparison of the Different Carbon Sources

A comparison between the different carbon sources should be made to ascertain if the algae-bacteria system is feasible for future decontaminations strategies. Figure 6.5 shows the bacterial Cr(VI) reduction percentage of a 100 mg/L initial Cr(VI) concentration with different carbon sources available – glucose, cell-free spent algae media, physical algae cells and without carbon sources. In the control experiments where no carbon sources are available for the CRB, the total amount of Cr(VI) removed was only 30% after 24 hours. This a very low Cr(VI) reduction percentage when compared with when carbon sources are available, which indicates the importance of carbon sources during the reduction process. The CRB cells are still active and could still generate chromate reductase (enzymes) which are ultimately responsible for the transfer of electrons, and thus the Cr(VI) reduction. Molokwane et al. (2008) found that only 30% Cr(VI) removal was achieved with heat-killed CRB after incubation and suggest that the decrease in Cr(VI) concentration was due to chromate reductases released into the solution from heat-lysed cells as well as from the surviving active cells.

The experiments in which glucose was used achieved the highest Cr(VI) reduction rate: 100% Cr(VI) reduction was achieved within 6 hours. Carbon sources that are either oxidizable or fermentable, such as glucose, resulting in the best Cr(VI) reduction results. Glucose specifically has been found in the literature to achieve the highest Cr(VI) reduction compared to other electron donors (Barrera-Díaz et al. 2012). It has, however, been shown in previous studies that any number of organic compounds may serve as an electron donor for the reduction of Cr(VI) to Cr(III) (Chirwa and Molokwane 2011). The fact that only 30% of the Cr(VI) removal was achieved in the absence of a carbon source, compared to 100% when glucose was present, establishes the metabolic link to Cr(VI) reduction. Algae cells are, however, a more cost-effective source of carbon than glucose.

Figure 6.5 also shows the Cr(VI) reduction for the cell-free spent algae media (broth). As discussed previously, within 24 hours *Chlamydomonas reinhardtii* and *Chlorococcum ellipsoideum* cell-free spent media could respectively achieve 49% and 45% Cr(VI) reduction. When compared to the 30% Cr(VI) reduction in the absence of carbon sources, this suggests that an extra 15%-19% Cr(VI) reduction was achieved through the utilisation of extracellular products present; assuming that the different processes could be

superimposed. Utilising cell free spent algae media is not very efficient, but contributes to the overall process where algal cells are used as a carbon source for Cr(VI) reduction.

The Cr(VI) reduction rate in the CRB-algae system is very different from the reduction rate in the CRB-glucose system. This shows how different the carbon sources are, and gives some indication of how the various carbon sources are utilised. Viti et al. (2014) also found that the reduction of Cr(VI) to Cr(III) is promoted by exploiting the reducing power generated by carbon metabolisation as the Cr(VI) detoxification mechanism.

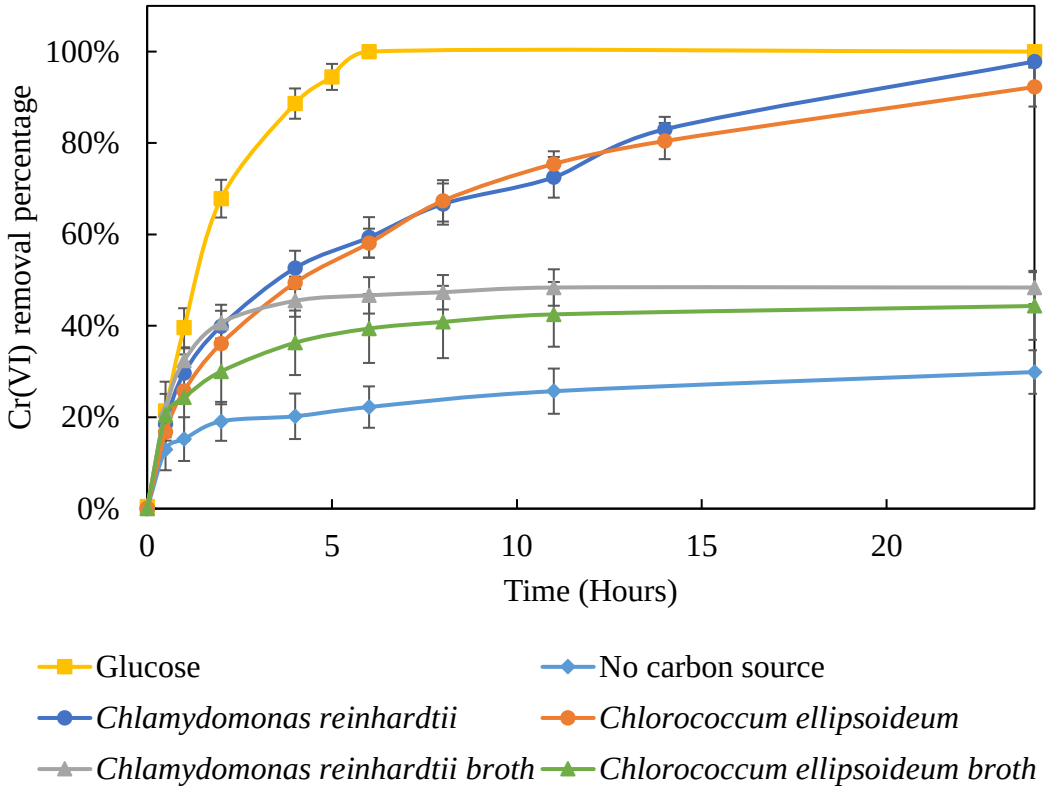

Figure 6.5: Comparison of the bacterial Cr(VI) reduction percentage of various carbon sources utilised by the CRB during the batch experiments. The initial Cr(VI) concentration is 100 mg/L. Carbon sources: Glucose, *Chlamydomonas reinhardtii* algal cells, broth from *Chlamydomonas reinhardtii* cell free spent media, no carbon sources only 3N-BBM+V, *Chlorococcum ellipsoideum* algal cells and broth from *Chlorococcum ellipsoideum* cell free spent media. (Mean ± SE; n = 3)

6.4 Total Chromium Concentration

The total chromium concentrations of the experiments for the different available carbon sources, *Chlorococcum ellipsoideum*, *Chlamydomonas reinhardtii*, and glucose, are shown in Figure 6.6. The total chromium concentration in the solution was initially higher than the Cr(VI) in the solution as there was already some Cr(III) present. The Cr(VI) concentration was 100 mg/L. The total chromium concentration had similar trends for all the carbon sources. There was a small decrease in the total chromium concentration in the first 8 hours, after which the total chromium concentration remained relatively constant. As discussed previously, Cr(VI) exists as anionic species (CrO_4^{2-} and $HCrO_4^-$) and, at neutral pH, the biomass also has a negative charge whereby the Cr(VI) is repelled.

In contrast to Cr(VI), Cr(III) exist as cationic species (Cr^{3+} and $CrOH^{2+}$) which would be attracted to the negatively charged biomass. At a pH above 3.5, dissociation of sulphonate and carboxyl functional groups increases the negative charge on the algal biomass, improving the Cr(III) binding capacity (Murphy et al.,2008). Therefore, although Cr(III) cations can be adsorbed onto the algal biomass, it would be challenging for Cr(VI) anions to be adsorbed as well. The decrease in the total chromium concentration could be caused by the adsorption of Cr(III) cations onto the bacterial and algal cell walls. The adsorbed Cr(III) could either have been present initially, or it could have been formed as the CRB reduced the Cr(VI).

Chirwa and Wang (2000) also observed the total Chromium concentration while using CRB. Chirwa and Wang (2000) found that the total Chromium concentration remained relatively constant during the Cr(VI) reduction experiments, with minimal biosorption taking place.

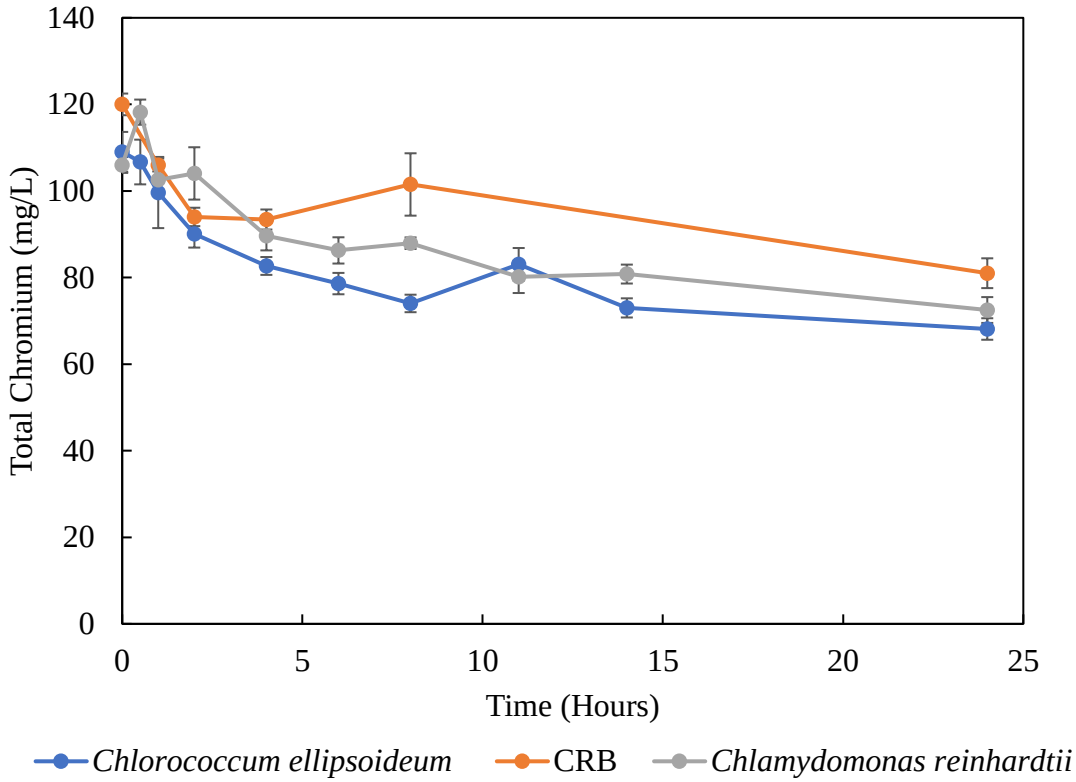

Figure 6.6: The total chromium concentration in the bacterial reduction experiments in which different carbon sources were available. At 30 ± 2 ℃, neutral pH and at 100 mg/L initial Cr(VI) concentrations. The Cr(III) concentration is the difference between the total chromium and Cr(VI) concentrations. (Mean ± SE; n = 3)

In the experiments where the algae cells are present, a more substantial decrease in total chromium concentration would be expected, because there is more biomass available (bacterial as well as algal biomass) to adsorb the Cr(III). However, this is not the case as the total chromium concentration decreased by 38%, 32% and 33% when *Chlorococcum ellipsoideum*, *Chlamydomonas reinhardtii*, and glucose were respectively used as carbon sources.

6.5 Influence of Different pH on Bacterial Reduction

pH is essential for any biological system. pH plays a significant role in the chemical reduction of Cr(VI) as well as the adsorption process. Figure 6.7 and Figure 6.8 shows the bacterial reduction of Cr(VI) for an initial concentration of 50 mg/L Cr(VI), with algae as carbon sources at different initial pH values. The CRB-algae system was susceptible to pH. The neutral pH of 7 resulted in the best and highest bioreduction results. The more acidic or alkaline the experimental conditions were, the less reduction took place. The lowest percentage of Cr(VI) reduction occurred at a pH of 4, which is unexpected as lower a pH increases the chemical reduction capacity, as well as the rate of adsorption onto biomass cells, of Cr(VI). For the bacterial reduction process, however, a low pH would inhibit the CRB.

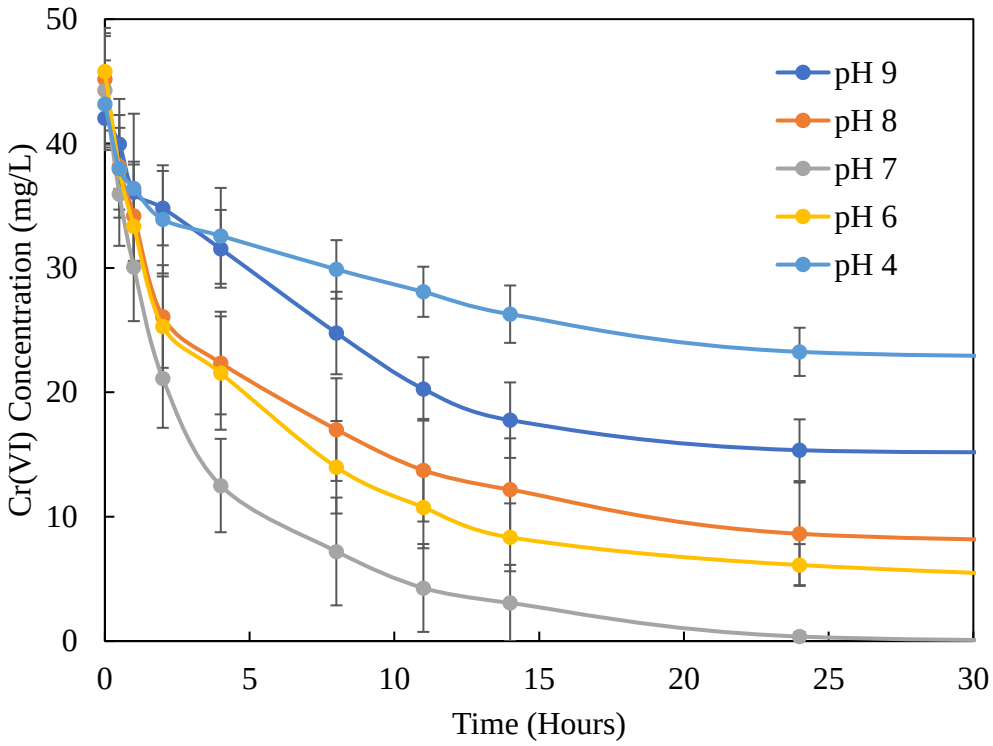

Figure 6.7: Cr(VI) concentration in a system containing CRB and algae. *Chlorococcum ellipsoideum* is used as a carbon source during the bacterial reduction experiments at various initial pH values. At 30 ± 2 °C and at 50 mg/L initial Cr(VI) concentrations. (Mean ± SE; n = 3)

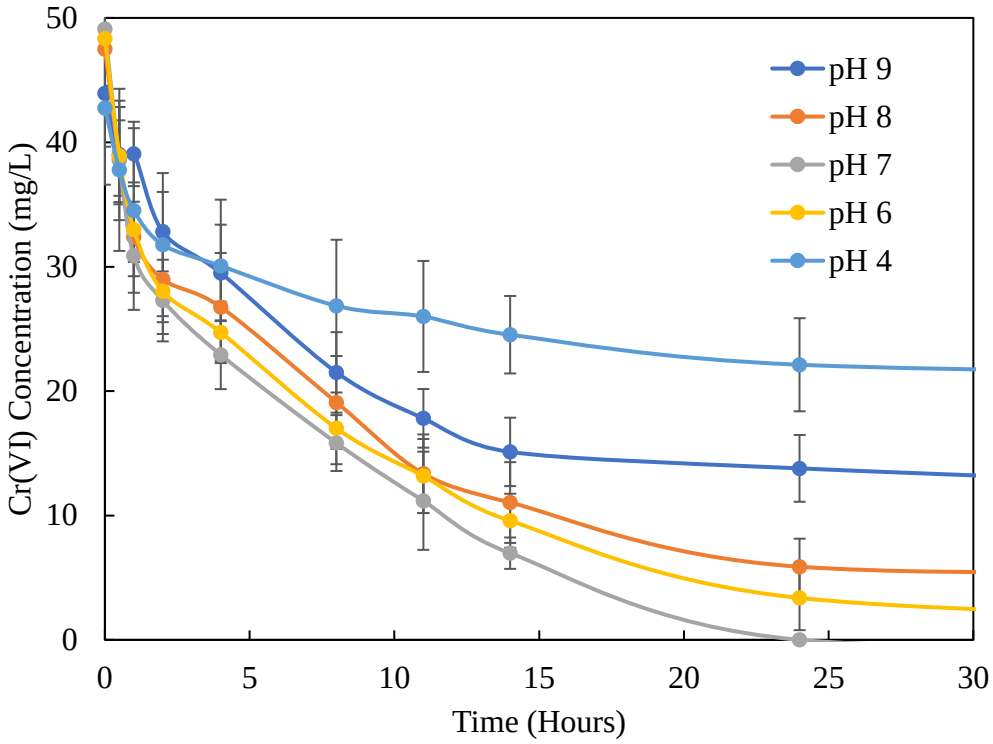

Figure 6.8: Cr(VI) concentration in a system containing CRB and algae. *Chlamydomonas reinhardtii* is used as a carbon source during the bacterial reduction experiments at various initial pH values. At 30 ± 2 ℃ and at 50 mg/L initial Cr(VI) concentrations. (Mean ± SE; n = 3)

Liu et al. (2006) tested the effect of pH on the Cr(VI) reduction capability of *Bacillus* sp. Liu et al. (2006) achieved similar results to the results shown in Figure 6.7 and Figure 6.8, except that for *Bacillus* sp. the optimal pH was found to be 9, as opposed to 7 being the optimal pH for the CRBs used in this study. Liu et al. (2006) found that a pH of 4 provided the lowest Cr(VI) reduction capability for *Bacillus* sp., which is the same as was found for the CRBs used in this study.

Wang and Xiao (1995) tested the effect of pH on the Cr(VI) reduction capability of *Bacillus* sp. and *Pseudomonas fluorescens* and found that the optimal pH for Cr(VI) reduction was 7, which is the same as the results shown in Figure 6.7 and Figure 6.8. Wang and Xiao (1995) found that a pH of 5 provided the lowest Cr(VI) reduction capability for *Bacillus* sp. and *Pseudomonas fluorescens*, which compares well with the results shown in Figure 6.7 and Figure 6.8.

Non-biological remediation studies have found that the best reduction and adsorption of Cr(VI) occurred at a low pH. Daneshvar et al. (2002) found that the highest efficiency of Cr(VI) reduction and adsorption occurred at a pH below 1. Many studies have found that the maximum adsorption of Cr(VI) occurred below a pH of 2 (Demirbas et al., 2004; Karthikeyan et al., 2005; Bishnoi et al., 2004; Kobya, 2004). Buerge and Hug (1997) determined that at a pH of 4 the reduction rate was minimal and that the reduction rate increased at the pH below 4.

Alkaliphilic *Bacillus subtilis* isolated by Mangaiyarkarasi et al. (2011) from a tannery was found to be able to grow and completely reduce Cr(VI) at an alkaline pH of 9. Cheng and Li (2009) found that the bioreduction was optimum at 37 °C and pH 8. Chung et al. (2006) observed that Cr(VI) bioreduction was sensitive to pH and that the optimum pH was near 7.0, however below 7.0 there is a sharp drop off, and above 7.0 there is a gradual decline in the reduction efficiency as the pH increases to 8.2. The results of this study appear to be consistent with the findings of Chung et al. (2006).

The fact that a neutral pH allowed for the best Cr(VI) reduction performance is very beneficial, especially compared with conventional treatment processes such as chemical reduction and adsorption. Conventional treatment processes require additional chemicals to alter the pH to the optimum pH. Adsorption specifically is driven by the initial Cr(VI) concentration, and thus it is not always effective in the treatment of contaminated sources with a lower Cr(VI) concentration, whereas bacterial reduction of Cr(VI) is very efficient at low concentrations. The extreme toxicity of Cr(VI) requires that it is best to remove all of the Cr(VI) from the polluted aqueous body.

6.6 The Kinetic Model for Cr(VI) Reduction in the CRB-Algae Systems

The kinetics of the Cr(VI) reduction in the combined CRB and algae system, was investigated using the AQUASIM program. The program was used to simulate the Cr(VI) reduction at different initial Cr(VI) concentrations: 50, 100, 150 and 200 mg/L. As seen in the batch experiments in this study, and corroborated in other studies, the reduction process facilitated by the bacterial cells were inhibited at high levels of Cr(VI) in the medium (Chen and Hao 1998). The inhibition was observed to be more noticeable at higher concentrations such as 150 mg/L Cr(VI) and 200 mg/L Cr(VI). In the 200 mg/L Cr(VI)

experiment a Cr(VI) reduction could not be achieved more than 46.0% and 49.4% respectively, utilising *Chlorococcum ellipsoideum* and *Chlamydomonas reinhardtii*. Wang and Shen (1997) made similar observations and, Chirwa and Wang (1997a and b), and in which high initial Cr(VI) concentrations inhibited both the bacterial growth as well as the Cr(VI) reducing capabilities of the CRB.

The process taking place in the system should be considered to understand the reduction kinetics. Based on the results from this current study, it is clear that a connection exists between the Cr(VI) reduction rate and the metabolic activity of the bacterial cells. The inhibition that occurred at high initial Cr(VI) concentration contributed to the development of the Cr(VI) reduction model based on the enzymatic Cr(VI) reduction kinetics as described in Chapter 4. However, the model derived in section 4.4 is inadequate and does not correctly describe the CRB-algal systems. Despite the fact that the same CRB are used in both chapters the key difference between the systems remains the availability of carbon sources.

The relationship between the Cr(VI) reduction rate and the organic substrate uptake rate were investigated by Fujie et al. (1994). Fujie et al. (1994) observed a proportional relationship where for each 0.6 mg of organic carbon that was taken up 1 mg of Cr(VI) was reduced. From this, it follows that an inadequate carbon source can inhibit and limit Cr(VI) reduction at higher initial Cr(VI) concentrations. Therefore it is not only the high Cr(VI) level, but also insufficient carbon sources that are likely responsible for the low Cr(VI) reduction percentage at initial Cr(VI) concentrations of 150 mg/L and 200 mg/L.

The relationship between Cr(VI) reduction rate and substrate uptake rate is significant for the kinetic model and also explains the shape of the curves in Figure 6.3, Figure 6.4, Figure 6.9, and Figure 6.10. Thus the shape of the Cr(VI) reduction curves indicates how the bacteria utilise the present carbon sources. The Cr(VI) concentrations rapidly decreased in the first two hours of the experiments. The initial rapid decline implies that the carbon sources that could be readily consumed were entirely consumed in the first two hours. The Cr(VI) concentration decreased steadily after the first two hours which signifies that there were still carbon sources available in the solution, but these carbon sources were consumed at a slower rate. The algae are responsible for the provision of these carbon sources.

The algae cells produce various extracellular products: high molecular weight substances, like polysaccharides, as well as low molecular weight substances. These products can be

very distinct and, as a result, are utilised at different rates by CRB. The carbon uptake rate, and ultimately the Cr(VI) reduction rate, of the CRB, depends on the bioavailability or degradability of these compounds (Bell and Sakshaug 1980). This demonstrates how metabolically diverse the CRB are, and that they can reduce Cr(VI) to Cr(III) using various carbon sources

As shown in section 5 the algal cells are likely lysed by the Cr(VI) and the CRB. These lysed algal cells can release intracellular products, such as fatty acids and protein like substances (Grima et al. 2003). Therefore the Cr(VI) reduction rate of the system could be controlled by the rate at which the algal cells are lysed, which in turn controls the release of intracellular compounds. The cytoplasm portion from disrupted algal cells could theoretically reduce the Cr(VI) to some extent, although from section 5 it is apparent that this reduction process is minimal, and does not require an active cellular metabolism. This reduction would be very harmful to the cell, however, due to the production of the potentially DNA damaging ROS, since most of the intracellular proteins catalyse a reduction from Cr(VI) to Cr(V) (Chirwa and Molokwane 2011). In the experiments conducted in this study, the CRB provides the chromate reductase enzymes which will facilitate electron transport, and therefore non-metabolic reduction processes would not play a significant role.

The data from the batch experiments of the bacteria and algae combined system were used to simulate the kinetics of the system in the AQUASIM Computer Program for the Identification and Simulation of Aquatic Systems (AQUASIM, EAWAG, Dübendorf, Switzerland). The modified Monod model as derived by Shen and Wang (1994) is provided in Equation 4.12. This model is valid for experiments in which a single carbon source, usually glucose, is plentiful. Algae, however, would provide numerous carbon sources, such as proteins, fatty acids, and polysaccharides, that could possibly be utilised by the CRB. Certain carbon sources could be utilised faster than others. As suggested by Fujie et al. (1994), the Cr(VI) reduction rate is proportional to the substrate uptake rate. To recapitulate the kinetic parameters of Equation 4.12, Equation 6.1 is shown below. The maximum reduction rate (k_m) and the half velocity constant (K_c) parameters describe a unique enzyme-substrate relationship; therefore, multiple carbon sources would have multiple k_m and K_c values.

$$\frac{dC}{dt} = \frac{-k_m C}{K_c + C} X \qquad 6.1$$

The algae cells would provide multiple internal and external metabolites that can serve as carbon sources. However, in this study for simplicity two groups of carbon sources are considered. The kinetic model was divided up into two parts: carbon sources that are utilised rapidly (S_{fast}), and carbon sources that are either slowly released from the lysed algae cells, or utilised at a slow rate (S_{slow}). The simplified fast and slow reactions are expressed in Equation 6.2 and 6.3.

$$S_{fast} + Cr(VI) \xrightarrow{bacteria} Cr(III) \qquad 6.2$$

$$S_{slow} + Cr(VI) \xrightarrow{bacteria} Cr(III) \qquad 6.3$$

The first reaction, Equation 6.2, can be described by a first order reaction (The value of K_c is much larger than the value of rapidly consumable substrate) as shown by the differential Equation given in Equation 6.4. The extent of the reduction of Cr(VI), according to Equation 6.2, is limited by the amount of substrate that can rapidly be consumed. Thus, once the S_{fast} is depleted, the rate of the Cr(VI) reduction only depends on the S_{slow} consumption rate. This transition can be observe as a bend in the Cr(VI) reduction curves.

$$\frac{dS_{fast}}{dt} = -k_1 S_{fast} X \qquad 6.4$$

A zero-order reaction describes the reaction in Equation 6.3 (The value of K_c is smaller than the value of the gradual consumable substrate) as shown by the differential Equation in Equation 6.5. The assumptions are similar to the novel dual-enzyme kinetic model derived by Viamajala et al. (2003) for Chromate reduction in *Shewanella oneidensis* MR-1. Although non-competitive inhibition arises as the kinetics are directly affected by an increase in initial Cr(VI) concentration.

$$\frac{dS_{slow}}{dt} = -\frac{k_2}{1+Cr(VI)_0/K_i}X \qquad 6.5$$

According to Equation 6.6. the concentration of Cr(VI) is a function of both the fast and the slow substrate consumption rates in the batch experiments.

$$Cr(VI)_0 - Cr(VI) = \left(S_{0,\,fast} - S_{fast}\right) + \left(S_{0,slow} - S_{slow}\right) \qquad 6.6$$

Equation 6.7 gives the concentration of the remaining viable bacteria cells. The reduction capacity of the cells (R_c) is proportional to the amount of Cr(VI) removed, divided by the number of cells that have deactivated. In the present case the bacterial cell growth kinetics can be ignored, because the concentration of the bacteria cells was too high to allow for the production of new bacteria cells.

$$X = X_0 - \frac{Cr(VI)_0 - Cr(VI)}{R_c} \qquad 6.7$$

The experimental data were used for model optimisation and simulation with a non-linear least square algorithm according to Equations 6.4 to 6.7. The parameters of the kinetic model that were determined with AQUASIM are listed in Table 6.1 and Table 6.2, and the simulated models are shown in Figure 6.9 and Figure 6.10. The data from the experimnets fit the derived model with most of the chi^2 value below 100. At lower concentrations in the range of 50 mg/L and 100 mg/L of Cr(VI), the model fitted better than at higher concentrations of Cr(VI). The parameters obtained for the two algal species were very similar; the largest difference that was observed was in the rate coefficient of the reaction of the substrate that was rapidly consumed (Equation 6.4). The *Chlamydomonas reinhardtii* system had a larger rate coefficient than the *Chlorococcum ellipsoideum* system.

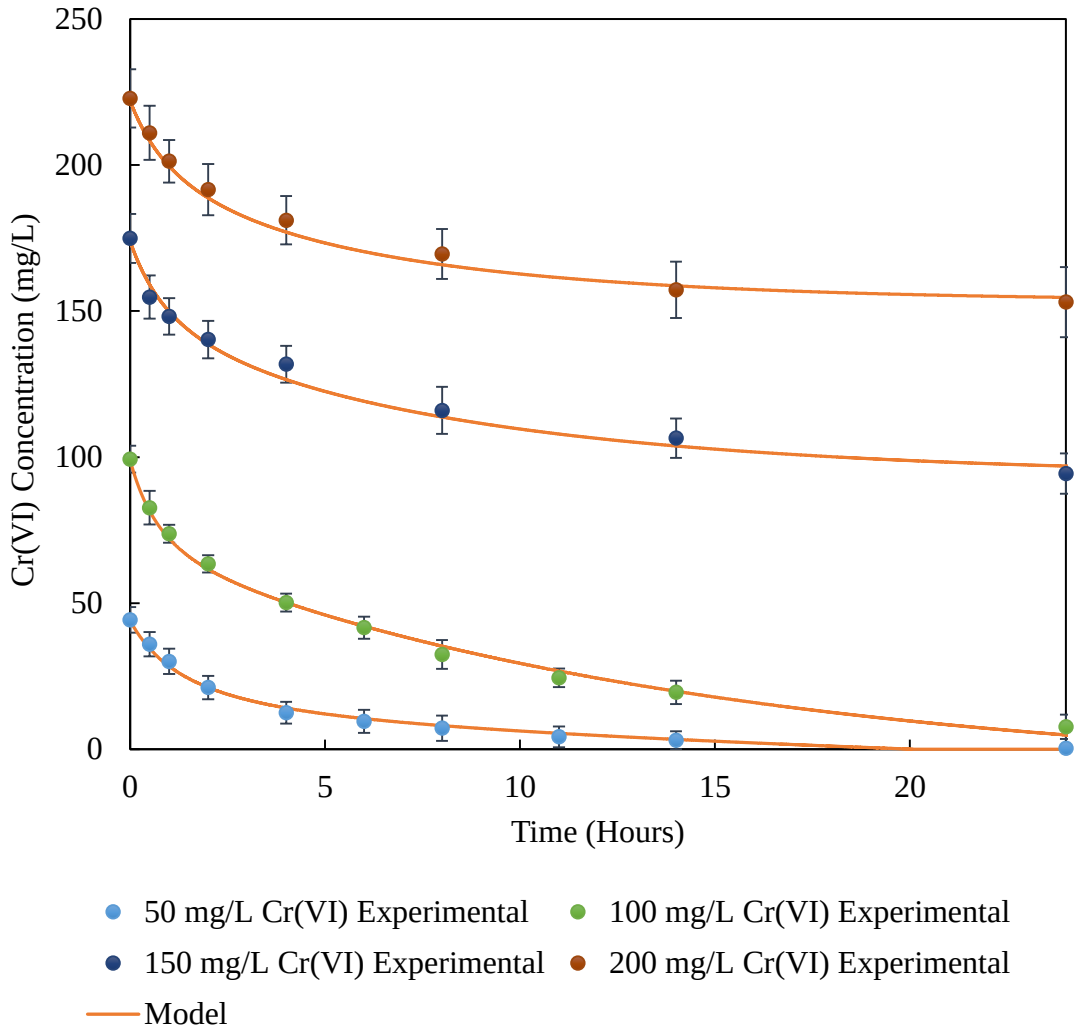

Figure 6.9: The derived kinetic model fitted to the batch experimental data of the bacterial Cr(VI) reduction utilising *Chlorococcum ellipsoideum* as a carbon source at various initial Cr(VI) concentration: 200 mg/L Cr(VI), 150 mg/L Cr(VI), 100 mg/L Cr(VI) and 50 mg/L Cr(VI). (Mean ± SE; n = 3).

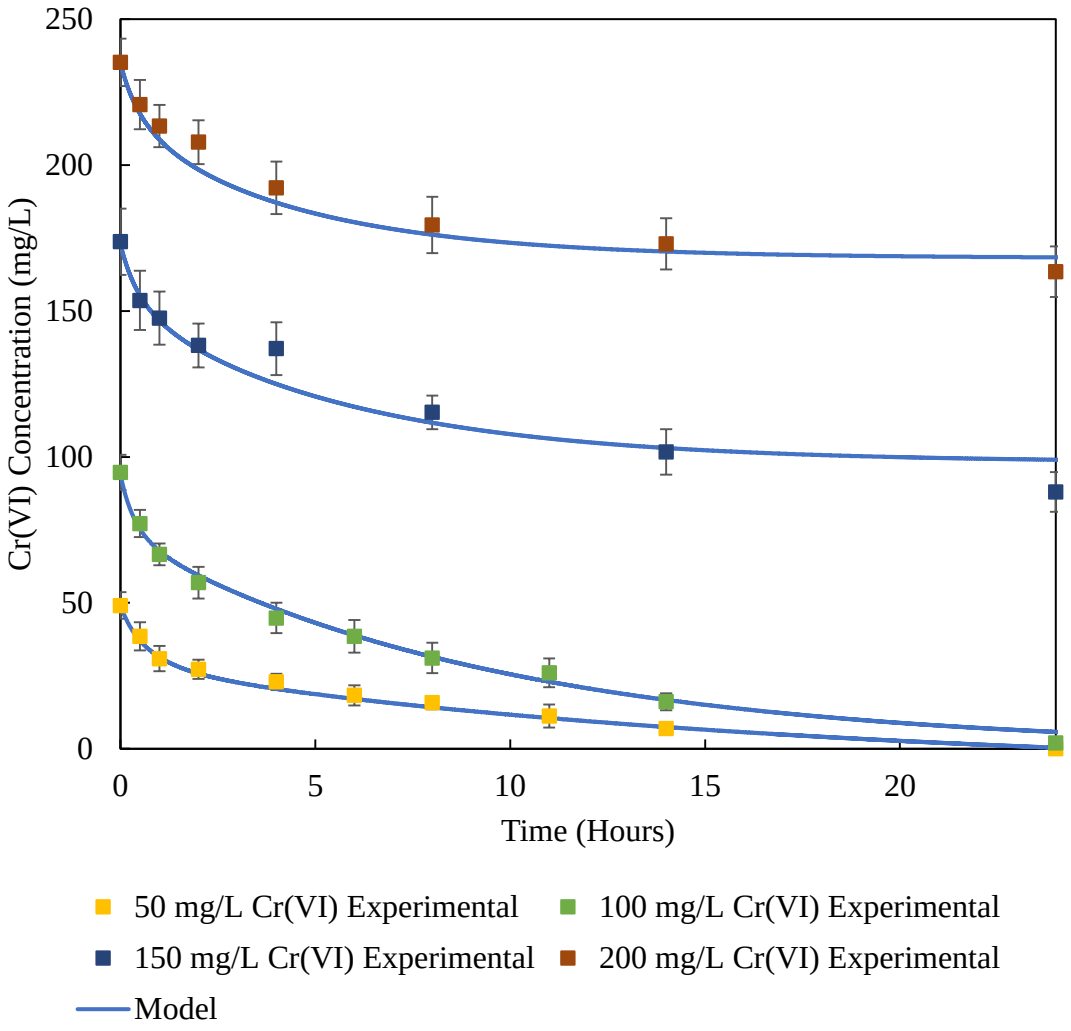

Figure 6.10: The derived kinetic model fitted to the batch experimental data of the bacterial Cr(VI) reduction utilising *Chlamydomonas reinhardtii* as a carbon source at various initial Cr(VI) concentration: 200 mg/L Cr(VI), 150 mg/L Cr(VI), 100 mg/L Cr(VI) and 50 mg/L Cr(VI). (Mean ± SE; n = 3)

Table 6.1: Kinetic parameter estimation for the derived kinetic model when *Chlorococcum ellipsoideum* is utilised as a carbon source.

C_o(mg/L)	50	100	150	200
k_1 (L/mg hr)	0.0003	0.0003	0.0003	0.0003
k_2 (1/hr)	0.0110	0.0100	0.0100	0.0100
R_c (mg/mg)	0.0225	0.0225	0.0225	0.0225
$S_{0,fast}$ (mg/L)	28.45	28.45	28.45	28.45
X_0 (mg/L)	2584.59	4801.21	3575.32	3054.03
K_i (mg/L)	537.22	537.22	537.22	537.22
Chi^2	14.49	14.97	87.42	54.05

Table 6.2: Kinetic parameter estimation for the derived kinetic model when *Chlamydomonas reinhardtii* is utilised as a carbon source.

C_o(mg/L)	50	100	150	200
k_1 (L/mg hr)	0.0006	0.0006	0.0006	0.0006
k_2 (1/hr)	0.0140	0.0140	0.0140	0.0140
R_c (mg/mg)	0.0210	0.0210	0.0210	0.0210
$S_{0,fast}$ (mg/L)	20.10	20.10	20.10	20.10
X_0 (mg/L)	2697.71	4485.26	3582.07	3184.56
K_i (mg/L)	499.89	499.89	499.89	499.89
Chi^2	10.47	50.63	302.76	100.42

6.7 Comparison of Different Algae as a Potential Carbon Source

To determine if there are an algae species that provided the most accessible carbon sources three different algae species were compared; *Chlorococcum ellipsoideum*, *Chlamydomonas reinhardtii*, and *Tetradesmus obliquus*. Figure 6.11 and Figure 6.12 compares the percentage of Cr(VI) reduction, after 14 and 24 hours, while utilising the different algae species and their metabolites as carbon sources. All three of the algae species proved to be satisfactory carbon sources for the bacterial reduction of Cr(VI). After 14 hours none of the experiments has reached complete Cr(VI) reduction. The *Tetradesmus obliquus* algae – CRB system resulted in the highest Cr(VI) reduction percentage for all the different initial concentrations. The *Chlorococcum ellipsoideum* and the *Chlamydomonas reinhardtii* systems performed very similarly. At higher initial Cr(VI) concentration the reduction process is slightly inhibited, as *Chlorococcum ellipsoideum* carbon sources could only allow for 80 % Cr(VI) reduction.

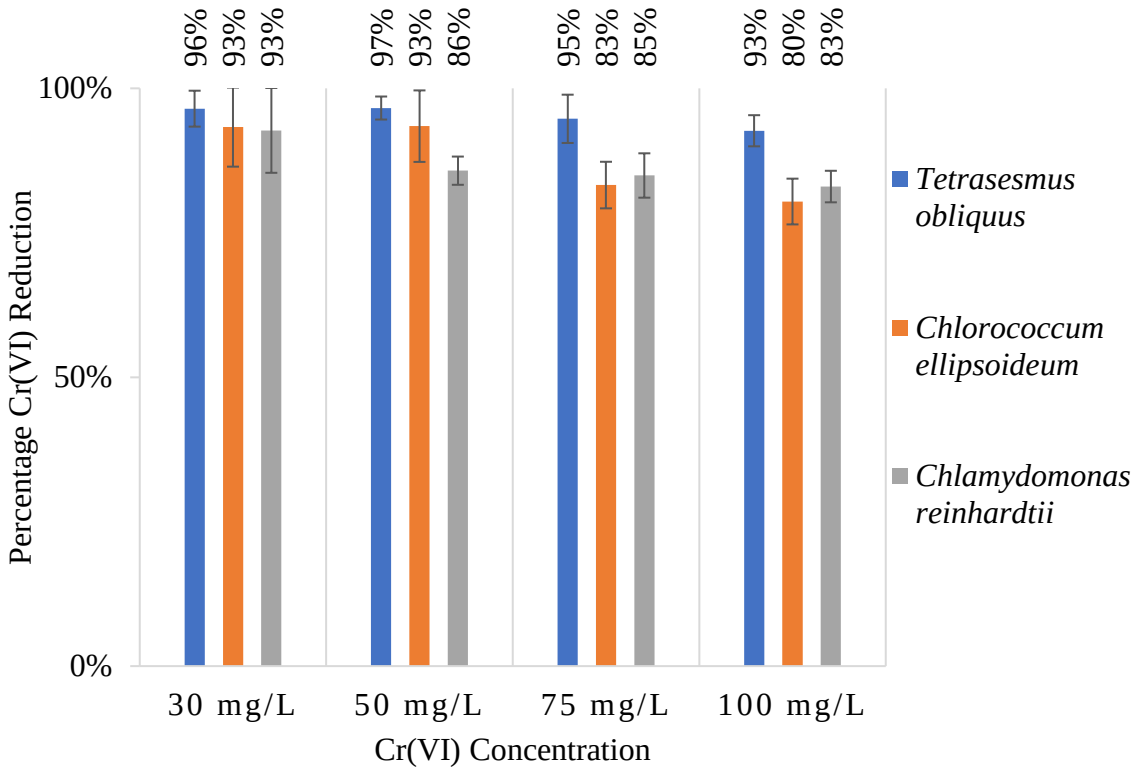

Figure 6.11: A comparison of the different algae species utilised as carbon sources for bacterial Cr(VI) reduction at various initial Cr(VI) concentrations after 14 hours. (Mean ± SE; n = 3)

After 24 hours the reduction reaction progresses further. At lower initial Cr(VI) concentrations, i.e. 30 mg/L, all three algae species allowed for the complete reduction of Cr(VI). A small inhibitory effect is also observed after 24 hours

Figure 6.12 indicates that *Tetradesmus obliquus* performed the best as a potential carbon source. This could be attributed to the fact that *Tetradesmus obliquus* produced more accessible intracellular and extracellular compounds for the CRB to consume. A drawback of *Tetradesmus obliquus* algae is that it has a low specific growth rate compared to *Chlorococcum ellipsoideum* which leads to more extended cultivation periods to attain the same dry biomass weight.

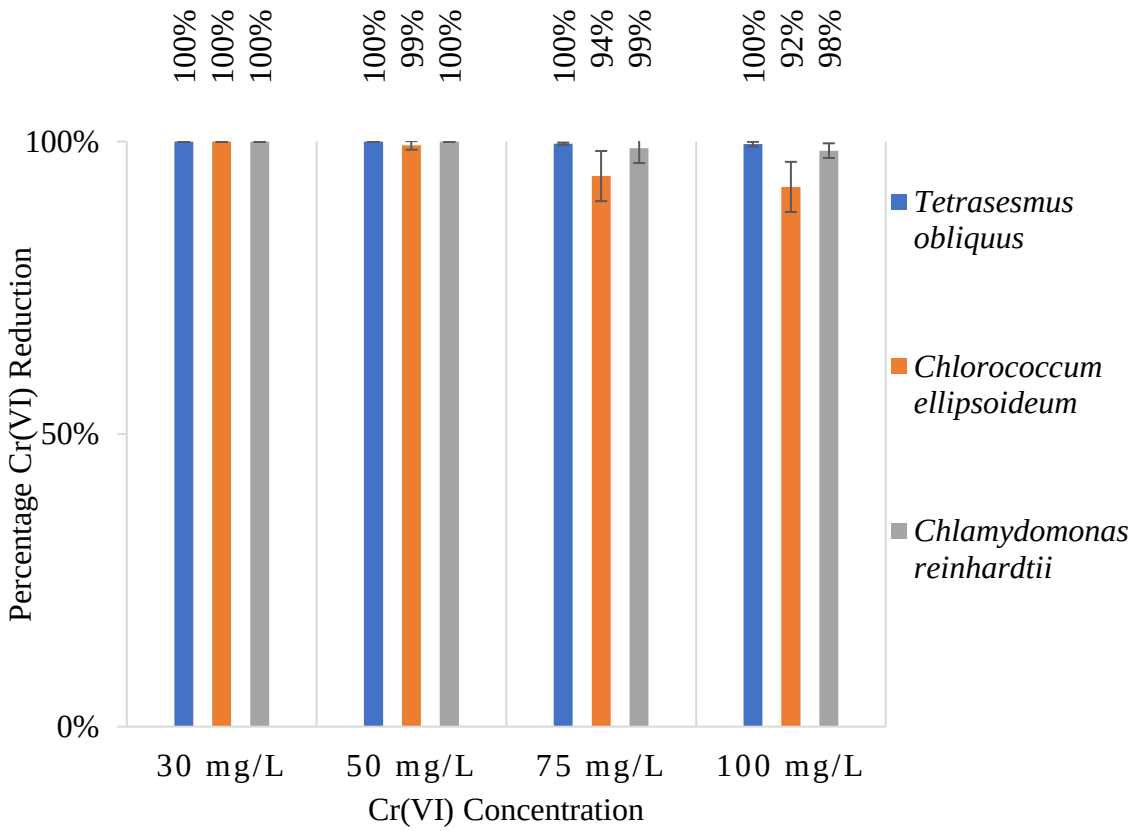

Figure 6.12: A comparison of the different algae species utilise as carbon sources for bacterial Cr(VI) reduction at various initial Cr(VI) concentrations after 24 hours. (Mean ± SE; n = 3)

Figure 6.13 also shows a comparison of the percentage Cr(VI) reduction between the different algal species utilised as carbon source by the CRBs during the batch experiments. Despite the fact that the carbon sources provided by the *Tetradesmus obliquus* algae species was able to achieve slightly better results, the three algae species performed very similarly. This indicates that the carbon sources produced by the different algae species were very comparable in nature and were utilised similarly by the CRBs.

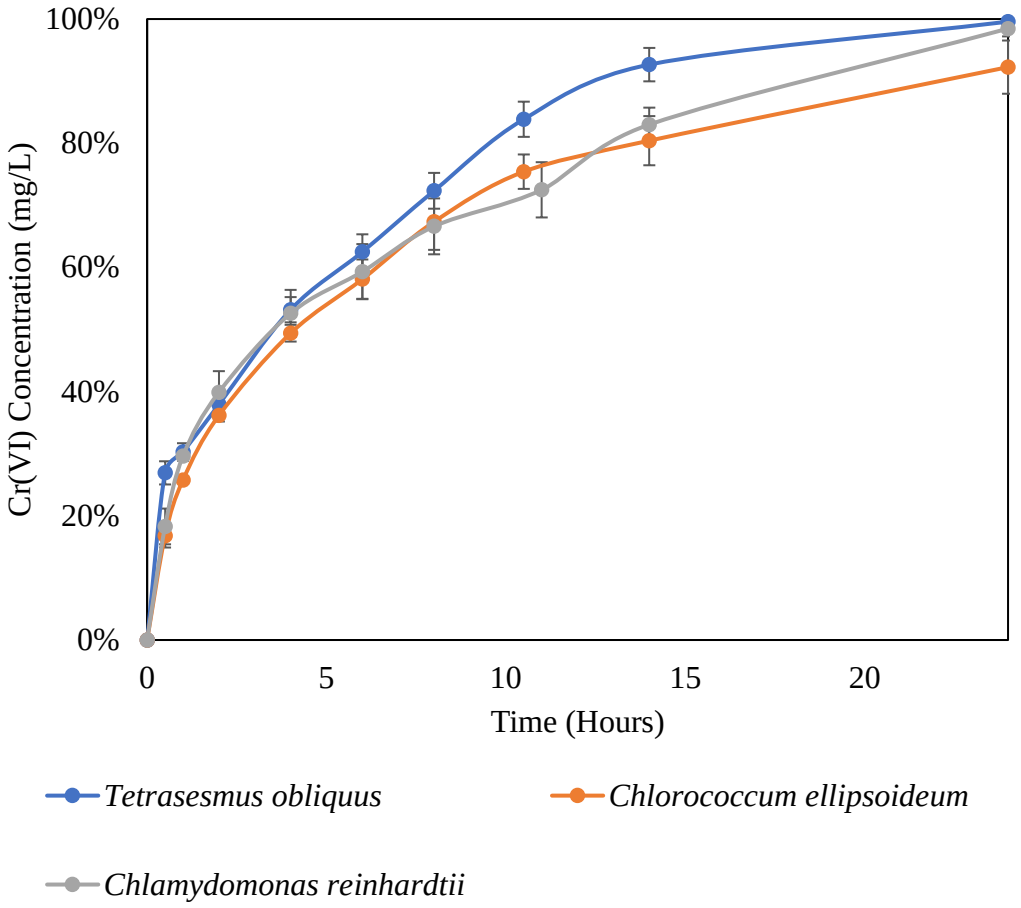

Figure 6.13: Comparison of the bacterial Cr(VI) reduction percentage of various carbon sources produced by different algae species utilised by the CRB during the batch experiments. At 30 ± 2 °C, neutral pH and at 100 mg/L initial Cr(VI) concentrations. (Mean ± SE; n = 3)

6.8 Chapter Summary

Cell free spent algae media is not sufficient to facilitate complete Cr(VI) reduction via the CRB, but there was more reduction than with no carbon sources. Therefore, it is clear that the CRB was able to utilise the extracellular carbon source provided by the algae.

In the experiments in which the algae cells were used as supplementary carbon sources in addition to the extracellular compounds, the Cr(VI) reduction rate was much higher, and at 50 mg/L the Cr(VI) was completely reduced to Cr(III) within 24 hours. A model was derived that fits the experimental data very well. Very high Cr(VI) concentrations inhibited the bacterial reduction process. The fact that the CRB was able to utilise different carbon sources demonstrates how metabolically diverse the CRB are.

It was found that during Cr(VI) reduction, the total chromium concentration remained relatively constant with minimal biosorption taking place. This confirmed the results presented by Chirwa and Wang (2000).

A neutral pH was found to be the best pH for bacterial Cr(VI) reduction. The effect of pH on the reduction of Cr(VI) by the CRBs used in this study compared well with the investigations into the effects of pH on bacterial Cr(VI) reduction done by Liu et al. (2006) and Wang and Xiao (1995). The only difference was that Liu et al. (2006) found that the optimal pH for *Bacillus* sp. was 9 instead of 7.

CHAPTER 7 CONCLUSION AND RECOMMENDATION

The locally isolated CRB and algae were used in Cr(VI) reduction batch experiments. The algae cells and metabolites served as a carbon source for the CRB. The batch experiments were carried out at the different initial Cr(VI) concentration ranging from 30 to 250 mg/L.

In the experiments with algae and CRB consortium, and initial Cr(VI) concentrations of 50 mg/L and 100 mg/L, total bacterial reduction of Cr(VI) was achieved within 24 hours. This high removal rate was achieved through a combination of mostly metabolically dependent processes and a few metabolically independent processes. In the experiments with initial Cr(VI) concentrations higher than 100 mg/L the Cr(IV) reduction capability of the CRB was inhibited.

A consortium consisting of algae and bacteria can potentially function in a synergistic manner to detoxify pollutants by forming a self-sustaining system. This outcome, however, was not achieved in this study. The algae used in this study were found to be very sensitive to Cr(VI) toxicity. The Cr(VI), together with the CRB, inhibited the algae growth and reduced the chlorophyll *a* content of the algae cell, and thereby, the algae's ability to undergo photosynthesis.

The algae appear to provide two main groups of carbon sources. The different carbon sources are utilised to varying rates by the CRB, which ultimately results in different Cr(VI) reduction rates: an initial rapid Cr(VI) reduction rate, followed by a slower Cr(VI) reduction rate.

A model, based on Michaelis-Menten/ Monod enzymes kinetics, was developed to describe the algal-CRB system. The model took into account that different carbon sources supplied by the algae resulted in different Cr(VI) reduction rates. The model was simulated in the AQUASIM (Computer Program for the Identification and Simulation of Aquatic Systems). The experimental data showed a relatively good fit with the predicted model in AQUASIM. Using the derived model, it was possible to predict the Cr(VI) reduction rate.

The CRB was able to use glucose and various algal metabolites as carbon sources, indicating a diverse metabolic pathway, which was probably prompted by the symbiosis between the different bacteria species. Metabolic diversity refers to the different metabolic strategies that organisms have evolved to obtain energy. Metabolic pathways evolved among prokaryotes before eukaryotes emerge, therefore bacteria can interact and coevolve

with changing physicochemical environmental conditions. The ability of the CRB to achieve Cr(VI) reduction through the use various carbon sources, presents an opportunity to reduce the need to add glucose to these types of bioremediation systems.

All of the evaluated algal species had similar performance when used as carbon sources for the CRB. Nonetheless, *Tetradesmus obliquus* appeared to perform marginally better as a carbon source than the other algae, *Chlorococcum ellipsoideum* and *Chlamydomonas reinhardtii*. Both the CRB, and the Cr(VI) had a severely detrimental effects on the health of algae cells, and could have caused the lysing of algal cells. Minimal adsorption of both Cr(VI) and Cr(III) was observed in the algae-CRB system, especially at the optimal pH for bacterial Cr(VI) reduction.

Additional investigations can be conducted by repeating the experiments in this study and introducing variations in the bacterial biomass or the temperature of the experiments, and the CO_2 levels can be monitored. Toxicity tests can also be conducted to identify the limit for algae growth inhibition by the presence of Cr(VI).

The self-sustainability and practicality of using a CRB-algae consortium in a continuous system must be evaluated. If practical, such a CRB-algae consortium can be used to continuously remediate Cr(VI) contamination of ground water and water bodies. These types of systems could present a cost-effective, sustainable, and environmentally friendly method for various industries to remediate their effluent before it is discarded.

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