Cr(VI) Mediated Hydrolysis of Algae Cell Walls to Release TOC for Enhanced Biotransformation of Cr(VI) by a culture of Cr(VI) Reducing Bacteria

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Abstract

Hexavalent chromium [Cr(VI)], the most toxic form of chromium, is frequently released into the environment from anthropogenic sources. Cr(VI) mainly occurs in the oxyanionic forms, CrO_4^{2-} and $Cr_2O_7^{2-}$. It is highly oxidative and carcinogenic under chronic and subchronic exposure conditions. Conventionally, Cr(VI) pollution is remediated by reducing Cr(VI) to Cr(IIII). Cr(III) is naturally less toxic than Cr(VI) and is a 1000 times less mobile in the aquatic phase than Cr(VI). Biological reduction and detoxification of Cr(VI) are viewed as the most ecologically friendly process for remediation of Cr(VI) pollution. However, fast reduction of Cr(VI) mainly occurs under aerobic conditions in the presence of organic carbon sources. In the current research, freshwater algae are utilized as a carbon source for Cr(VI) reduction with using symbiotic bacterial cultures. The algal species, *Chlamydomonas reinhardtii* and *Chlorococcum ellipsoideum*, were tested in their ability to serve as or produce a carbon source for locally isolated bacteria to achieve reduction of Cr(VI) to Cr(III). Batch experiments were conducted under aerobic conditions at different concentrations of Cr(VI) to determine the kinetics of the biological reduction reaction. In the batch experiments, complete removal of up to 50 mg L^{-1} of initial Cr(VI) concentration was achieved within 24 hours. At 100 mg L^{-1} initial Cr(VI) concentration the system could remove 92% of the Cr(VI). Algae was found to be very sensitive to Cr(VI) toxicity. The Cr(VI) inhibited the algae growth and reduced the chlorophyll *a* content and by extension the algae's ability to undergo photosynthesis.

Keywords: phytoremediation, freshwater algae, Cr(VI) reduction, bioremediation, batch kinetic.

Introduction

Chromium (Cr) occurs in environment mainly either as Cr(III) in ores and oxides or Cr(VI) from anthropogenic sources (Miranda et al. 2012). These two oxidation states affect the aquatic environment quite differently. For example, Cr(VI) is readily taken up by cells through the anionic transport system (Arita and Costa 2011). Inside the cells Cr(VI) generates active Cr(V) and/or Cr(IV) intermediates, 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; Macfie et al. 2009), single-strand breaks (Bridgewater et al. 1994), DNA–protein crosslinks (Macfie et al. 2009), chromosomal aberrations (Wise et al. 1992), DNA polymerase arrest, RNA polymerase arrest (Bridgewater et al. 1994) and inhibition of transcription and translation (Pritchard et al. 2000). At high concentration, Cr(VI) is acutely toxic with visible symptoms such as burn-like symptoms and skin shading. Reduction of Cr(VI) to Cr(III) is an important step in the decontamination of Cr(VI) in aqueous bodies (Yewalkar et al. 2007).

Cr can accumulate in living organisms, which is why the World Health Organization (WHO) recommends that the maximum acceptable concentration level of Cr in drinking water is 0.05 mg L^{-1} and in wastewater it is 1 mg L^{-1} (WHO 2004; US EPA 1998). The hexavalent form of chromium [Cr(VI)] is extremely mobile and therefore poses a high risk to organisms in water and utilizing water contaminated with Cr(VI) (Barrera-Díaz et al. 2012). Cr(VI) is conventionally treated using physicochemical methods such as adsorption, ion exchange, and chemical reduction followed by precipitation as chromium hydroxide [$Cr(OH)_3(s)$] at pH > 8.0. The current conventional physical-chemical methods tend to leave behind chemical byproducts which render the methods unsustainable in the long term (Miranda et al. 2012). Experience at sites where pump-and-treat remediation of Cr-contaminated groundwater is underway suggests that, although it is feasible to remove high levels of Cr from the subsurface, it becomes more difficult to remove the remaining Cr as concentration decreases (EPA-Odessa 2005). The shortcomings in the

chemical and physical-chemical processes have generated increased interest in technologies that are able to reduce Cr(VI) to Cr(III) through the use of microorganisms.

Microorganisms exposed to toxic organic and metallic species have been known to develop diverse resistance mechanisms which can enable them to withstand or neutralize the source of toxicity. Biological treatment of toxic metals is based on the principle of emulating the naturally occurring processes to convert and derive energy from potentially toxic elements (Igboamalu and Chirwa 2016; Thatoi et al. 2014). In the past three billion years of existence, microorganisms have evolved mechanisms to survive in hostile environments and to adapt to changes in the environment (Bush 2003). Environmental engineers around the world have undertaken to find ways to tap into the mysteries of nature by diligently studying the action of microorganisms as they adapt to extreme conditions.

One of the most conserved mechanisms in the living cell is the biochemical pathway for electron-transport through the cytoplasmic membrane to conserve energy through the oxidation of an electron donor and reduction of an electron acceptor such as oxygen. This process has been conserved over billions of years, such that, to this day, all life on earth depends on variants of this pathway (Bush 2003; Thomas et al. 1985; Nealson 1999; Kalckar 1974). Most biochemical processes for degradation and/or detoxification of compounds are linked to the above process.

Lately, microorganisms have been isolated that are capable of reducing the toxic forms of heavy metal and transitional metal elements, that occur in transuranic waste (TRU), into less mobile precipitable forms (Lloyd 2003). The resistance and detoxifications mechanisms involve specific biochemical pathways that can detoxify Cr(VI), such as the efflux of chromate ions from the cell cytoplasm and the extracellular reduction of Cr(VI) (Viti et al., 2014). Bacteria species that can tolerate Cr(VI) toxicity and can reduce Cr(VI) to Cr(III), are called chromium reducing bacteria (CRB) (Molokwane et al. 2008).

Microorganisms usually need an organic carbon source to reduce Cr(VI). The carbon source can be used as either an energy source or as an electron donor (Zhiguo et al. 2009). In the past, the carbon source was provided in the form of simple sugars such as glucose or lactate. More complex carbon sources from commercial broth or agar sources, such as

Luria-Bertani (LB), were also tested in separate experiments (Molokwane et al. 2008). Smith et al. (2002) found that CRB can utilize a variety of carbon sources including; acetate, D-xylose, ethanol, hydrogen plus carbon dioxide, glycerol, glycolate, L-asparagine, propylene glycol, and sucrose. The cost of the carbon source can conceivably limit the commercial application of the bioremediation technology (Vidotti et al. 2014).

The possibility of using algae and algae metabolites as a carbon source for Cr(VI) bacterial reduction is considered in this study. 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). Raptured algae cells 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).

This study aims to determine whether locally isolated CRB can utilize carbon sources produced by green freshwater algae species for growth and sustenance of Cr(VI) reducing bacteria. The effects of Cr(VI) toxicity on algae and the interactions between the algae and the bacteria were investigated. SEM analysis was used to determine the extent of damage caused by Cr(VI) to the algal cells. A kinetic model was derived to describe the unique processes that occur during bacterial Cr(VI) reduction while utilizing algae as a carbon source.

Materials and Methods

Isolation of Cr(VI) reducing bacteria

The Cr(VI) reducing bacteria was collected from the sand drying beds at the Brits Wastewater Treatment Works located in the North West province in South Africa. The bacteria were isolated from the sludge samples using the following enrichment technique: (1) 5 g of the collected sludge samples was inoculated in 100 mL of sterilized Miller's LB broth (purchased from ThermoFisher Scientific) amended with 100 mg L^{-1} of Cr(VI) (Molokwane et al. 2008); (2) the isolated cultures were incubated for 24 hours at 30 °C in a Rotary Environmental Shaker at 120 rpm (Labotec, Gauteng, South Africa); (3)

cultures were grown in 250 mL Erlenmeyer flasks plugged with sterilized cotton wool to allow for aeration and to filter out floating organisms in the air.

Pure cultures of bacteria were prepared by depositing 1 mL of serially diluted sample from the 7th to the 10th tube in the Petri dishes containing LB agar using the spread method and continual streak plating. The plates were then incubated for 24 hours at 30 °C to develop separately identifiable colonies. The pure colonies were identified by carrying out 16S ribosomal RNA gene (rRNA) sequencing. The pure bacterial strains were stored in a screw cap tube with 20% glycerol and 80% LB broth at -70 °C. Proper storage is essential to retain the bacteria's chromium reducing capabilities over a long period. Genomic DNA was extracted from the pure cultures using a DNeasy tissue kit (QIAGEN Ltd, West Sussex, UK) as per manufacturer's instructions. The 16S rRNA genes of isolates were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using primers pA and pH1 (Primer pA corresponds to position 8-27; Primer pH to position 1541-1522 of the 16S gene) (Coenye et al. 1999). An internal primer pD was used for sequencing (corresponding to position 519-536 of the 16S gene). The resulting sequences were matched to known bacteria in the GenBank using a basic BLAST search of the National Centre for Biotechnology Information (NCBI, Bethesda, MD). Phylogenetic tree diagrams were then constructed using the neighbor-joining method. Confidence in the tree topology was determined by bootstrap analysis based on 100 re-sampling (Felsenstein, 1985) and is shown in the supplementary material. The isolated bacteria were identified as *Escherichia coli*, *Bacillus thermoamylovorans*, and *Citrobacter sedlakii*.

Source of algae

Chlamydomonas reinhardtii (11/32a) algal species was purchased from the Culture Collection of Algae and Protozoa (CCAP). Wild-type algal samples were obtained from the freshwater Hartbeespoort Dam in South Africa. Sub-cultures of the algal species were preserved for long-term use by suspension in glycerol and deep-freeze storage at -70 ^{o}C. The rest of the algal samples were cultivated for a week as described in the algal growth media section below before the samples were isolated using the streak plating method. The plates that contained separate pure colonies were identified by carrying out 18S rRNA and 28S rRNA sequencing. To amplify the 18S rRNA gene, the forward primer,

SR1, and the reverse primer, SR12, were used (Nakayama et al. 1996; see supplementary material). To amplify the 28S rRNA gene, the forward primer, 28S-568F, and the reverse primer, 28S-803R, were used (Marande et al. 2009; see supplementary material). Polymerase chain reaction (PCR) amplification was carried out in a DNA Thermal Cycler (Gene Amp PCR System 2400 - Perkin Elmer). The resulting sequences were matched to genes for known algal species in the GenBank using BLAST. The algal species of *Chlorococcum Ellipsoideum* were found to have 97% sequence identities with the collected samples.

Algae growth media

The algal strain was cultured axenically in the modified recipe of Bold Basal Media (BBM) with 3-fold nitrogen and vitamins (3N-BBM+V) medium (CCAP 2015). Cultures were grown in sterilized and continuously stirred 1 L Erlenmeyer flasks plugged with sterilized cotton wool 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 when 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 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 supernatant were used in the Cr(VI) reduction batch experiments.

Standard solutions and chemicals

Cr(VI) stock solution (1,000 mg L^{-1}) was prepared by dissolving 3.74 g of 99% pure K_2CrO_4 (Analytical grade) in 1 L distilled water. This stock solution was used as the source of Cr(VI) in the batch experiments. Diphenylcarbazide (DPC) solution was prepared for Cr(VI) analysis by dissolving 0.5 g of 1,5 diphenylcarbazide in 100 mL of HPLC grade acetone and was stored in a brown bottle covered with a foil (APHA 2005). All chemicals used were of analytical grade obtained from Sigma Aldrich, Johannesburg, South Africa.

Cr(VI) analysis

Cr(VI) concentration was determined colorimetrically using UV/vis spectrophotometer (WPA, LightWave II, and Labotech, South Africa) at a wavelength of 540 nm (10 mm light path). 0.1 mL of a sample containing Cr(VI) was acidified with 1mL of 1N H_2SO_4, diluted with distilled water up to 10 mL and then 0.2 mL of 1,5 DPC was added (APHA, 2005). A red-violet purple color forms with a specific intensity depending Cr(VI) concentration which can be measured using the calibrated UV/vis spectrophotometer.

Bacterial Biomass

The bacterial viable cell concentration was determined using the serial dilution spread plate method. 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. 0.1 mL of suspended cell solution was transferred from test tubes 6th, 7th and 8th to three Petri dishes with plate count (PC) agar. The diluted cell solutions were spread onto the PC agar. The Petri dishes were incubated upside down for 18-24 hours in a temperature-controlled incubator at 30±2 °C. After incubation the colonies on each plate were counted and multiplied by a dilution factor, the mean between the three plates is reported as colony forming units (CFU) per milliliter of sample.

Algal Biomass

Algal cells were grown in 3N-BBM+V until dry biomass reached 1.5 g L^{-1}. The algae cells were then harvested and re-suspended in 3N-BBM+V with or without bacteria at different Cr(VI) concentrations. 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. After incubation, the optical density of the extracted liquid 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 with the following formula (Liang et al. 2013):

$$\text{chlorophyll A (mg L}^{-1}) = 11.64\ OD_{663\,nm} + 2.16\ OD_{645\,nm} + 0.1\ OD_{630\,nm} \quad (1)$$

Batch Experiments

The algae and bacteria cells were cultivated separately as described, and then harvested and re-suspended together in sterilized 50 mL Erlenmeyer flasks with 3N-BBM+V and amended with Cr(VI) to give different initial concentrations of Cr(VI). The pH of the solution was adjusted to 7.5. The flasks were placed in a 120-rpm orbital shaker at 30 ±2 °C in the dark. Samples for Cr(VI) analysis and biomass analysis were taken 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. The flasks were plugged with cotton to allow for aeration. All the batch experiments used the same dry biomass concentration, i.e., 3,500 mg L^{-1} bacteria and 1,500 mg L^{-1} algae and were carried out in triplicates. The mean values are reported in the results section.

Scanning Electron Microscopy

The bacterial and algal cells were studied using a Scanning Electron Microscope (Zeiss Ultra PLUS FEG SEM). The wet samples were prepared using conventional chemical methods before embedding for SEM (Glauert and Reid, 1975). The samples were first rinsed three times in 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 and rinsed three times in distilled water. Each sample was then dehydrated in ethanol (30%, 50%, 70%, 90%, and 100%) for 10 minutes. The samples were dried overnight, mount on the stub and carbon coated. After sample preparation, the SEM was used to obtain images at different magnification before and after the batch experiment.

Kinetic Model

The data from the kinetic studies of the bacteria and algae combined system were simulated using the Computer Program for the Identification and Simulation of Aquatic Systems AQUASIM 2.01 (AQUASIM™, EAWAG, Dübendorf, Switzerland). The modified Monod model (shown in equation 2) derived by Shen and Wang (1994) is valid in experiments in which only one carbon source, typically glucose, is abundantly

available. However, algae would provide numerous carbon sources (polysaccharides, fatty acids, and protein) that can be utilized at different rates by the CRB. As suggested by Fujie et al. (1994), the Cr(VI) reduction rate is proportional to the substrate uptake rate. The kinetic parameters in equation 1 are; the cell concentration, *X*, Cr(VI) concentration, *C*, the maximum reduction rate, k_m, and the half velocity constant, K_c. The K_c and k_m parameters describe a unique enzyme-substrate relationship. Therefore, multiple carbon sources would have multiple k_m and K_c values.

$$\frac{dC}{dt} = \frac{-k_m C}{K_c + C} X \tag{2}$$

Although in reality the algae would provide more than two types of carbon sources, however for simplicity, the kinetic model is divided up into two parts: carbon sources that are utilized rapidly, S_{fast}, and carbon source that is utilized at a slow rate, S_{slow}, or are slowly released from the ruptured algal cells. The simplified fast and slow reactions are expressed in equation 3 and 4.

$$S_{fast} + Cr(VI) \xrightarrow{bacteria} Cr(III) \tag{3}$$

$$S_{slow} + Cr(VI) \xrightarrow{bacteria} Cr(III) \tag{4}$$

The first reaction, equation 3, can be described by a first order reaction (Kc >> rapidly consumable substrate) as shown by the differential equation 5. The extent of the Cr(VI) reduction from equation 3 is limited by the amount of substrate that can be taken up rapidly. Thus, once the S_{fast} is depleted the Cr(VI) reduction rate only depends on the S_{slow} consumption rate and this transition can be seen as a 'kink' in the Cr(VI) reduction curves.

$$\frac{dS_{fast}}{dt} = -k_1 S_{fast} X \tag{5}$$

The reaction in equation 4 is described by a zero-order reaction (K_c << slow consumable substrate) as shown by the differential equation 6. Although non-completive inhibition arises as the kinetics are directly affected by an increase in initial Cr(VI) concentration.

$$\frac{dS_{slow}}{dt} = -\frac{k_2}{1 + Cr(VI)_0 / K_i} X \tag{6}$$

The Cr(VI) concentration is a function of the fast and slow substrate consumption in the batch experiment as expressed in equation 7.

$$Cr(VI)_0 - Cr(VI) = \left(S_{0,fast} - S_{fast}\right) + \left(S_{0,slow} - S_{slow}\right) \quad (7)$$

The concentration of viable cells remaining is expressed in equation 8. The reduction capacity of the cells, R_c, is proportional to the amount of Cr(VI) removed divided by the number of cells that inactivated. The concentration of the cells was too high to allow for the production of new cells; therefore, the cell growth kinetics can be ignored in the present case.

$$X = X_0 - \frac{Cr(VI)_0 - Cr(VI)}{R_c} \quad (8)$$

Results

Effect of Cr(VI) on algae

The chloroplast in the algal cells that were exposed to Cr(VI) did not retain its green pigmentation, and the algal cells became completely discolored. Figures 1 and 2 show the chlorophyll *a* content in the cells that were exposed to increasing concentrations of Cr(VI) in an algal growth medium with a pH of 7.5 and a temperature of 25 °C. In the first two days, all the algae experienced the lag phase: there was no significant decrease in chlorophyll *a* content in the experiments with Cr(VI) present. After the second day, the algae without Cr(VI) started to grow exponentially. The algae with Cr(VI) present barely entered the exponential growth phase before the chlorophyll *a* content started to decrease. The chlorophyll *a* content in the *Chlorococcum ellipsoideum* algal cells, when exposed to 5 mg L^{-1} of Cr(VI), is 2.38 mg L^{-1} after two days, which is higher compared to the 0.99 mg L^{-1} chlorophyll *a* content that remained in the algal cells when exposed to 50 mg L^{-1} Cr(VI). When exposing the *Chlamydomonas reinhardtii* algal cells to 5 mg L^{-1} of Cr(VI) for two days the chlorophyll *a* concentration is 4.07 mg L^{-1}, which is higher than the 0.28 mg L^{-1} chlorophyll *a* concentration that remains after exposing the same algal cells to 50 mg L^{-1} Cr(VI) for two days. The *Chlamydomonas reinhardtii* algal cells grew at a higher rate than the *Chlorococcum ellipsoideum* independent of Cr(VI) presence; however, the chlorophyll *a* content in *Chlamydomonas reinhardtii* decreased at a faster rate than in

Chlorococcum ellipsoideum in the presence of high Cr(VI) concentration. Higher Cr(VI) concentration in the solution caused the chlorophyll *a* content of the algal cells to decreases at a faster rate.

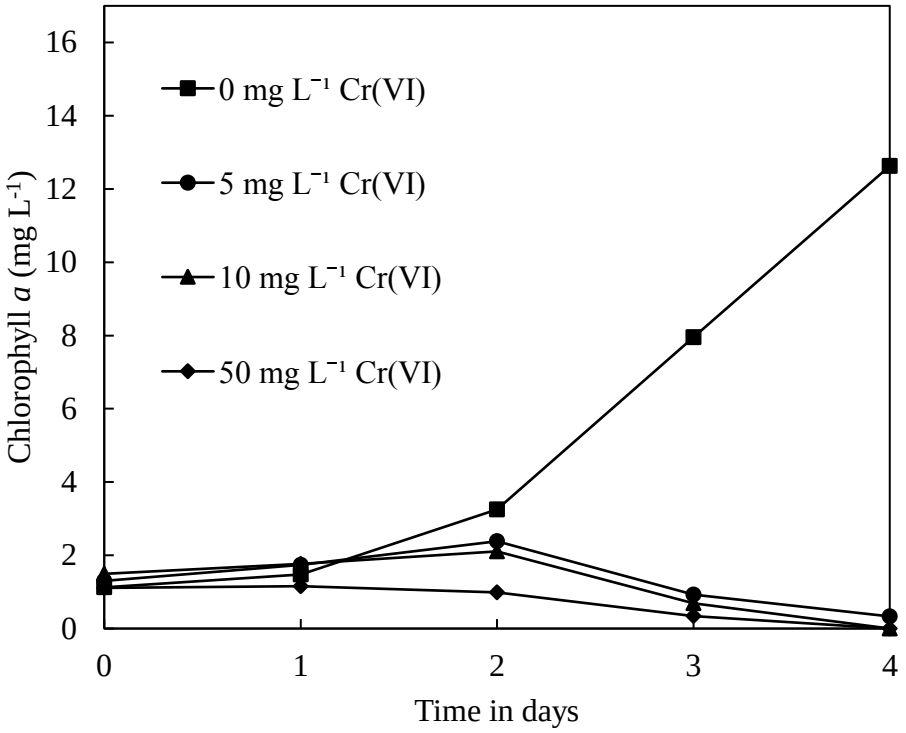

Figure 1: Chlorophyll *a* content (mg L^{-1}) of *Chlorococcum ellipsoideum* cells in BBM exposed to different Cr(VI) concentrations: 5 mg L^{-1} Cr(VI) (filled circles), 10 mg L^{-1} (filled triangles), 50 mg L^{-1} (filled diamonds) and the control (filled squares) where the algal cells were in BBM without Cr(VI).

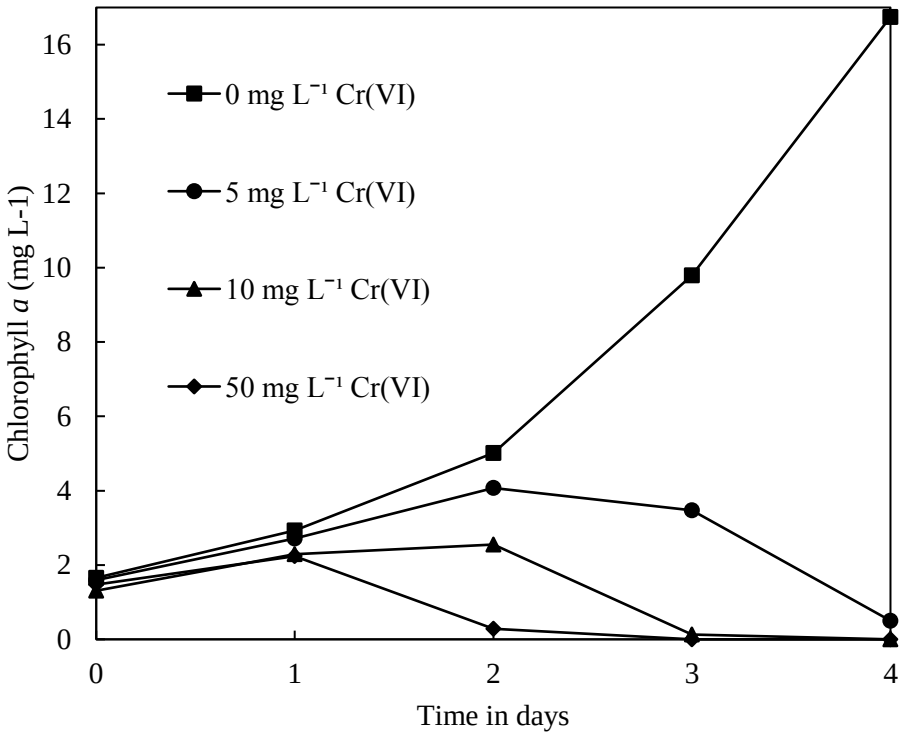

Figure 2: Chlorophyll *a* content (mg L^{-1}) of *Chlamydomonas reinhardtii* cells in BBM exposed to different Cr(VI) concentrations: 5 mg L^{-1} Cr(VI) (filled circles), 10 mg L^{-1} (filled triangles), 50 mg L^{-1} (filled diamonds) and the control (filled squares) where the algal cells were in BBM without Cr(VI).

Both the *Chlamydomonas reinhardtii* and *Chlorococcum ellipsoideum* algae cells did not have a significant effect on the Cr(VI) concentration in the solution. In the first hour a spike in the Cr(VI) concentration appeared, for both algal species, as the Cr(III), which is up to 25% of the total Cr in the solution, was oxidized to Cr(VI). After the initial spike, the Cr(VI) concentration remained constant as shown in Figure 3. There was no significant Cr(VI) adsorption onto the algal cell wall under the experimental conditions, with a pH of 7.5. However, repeating the same experiments at a pH below 2 found that up to 65% of Cr(VI) was removed.

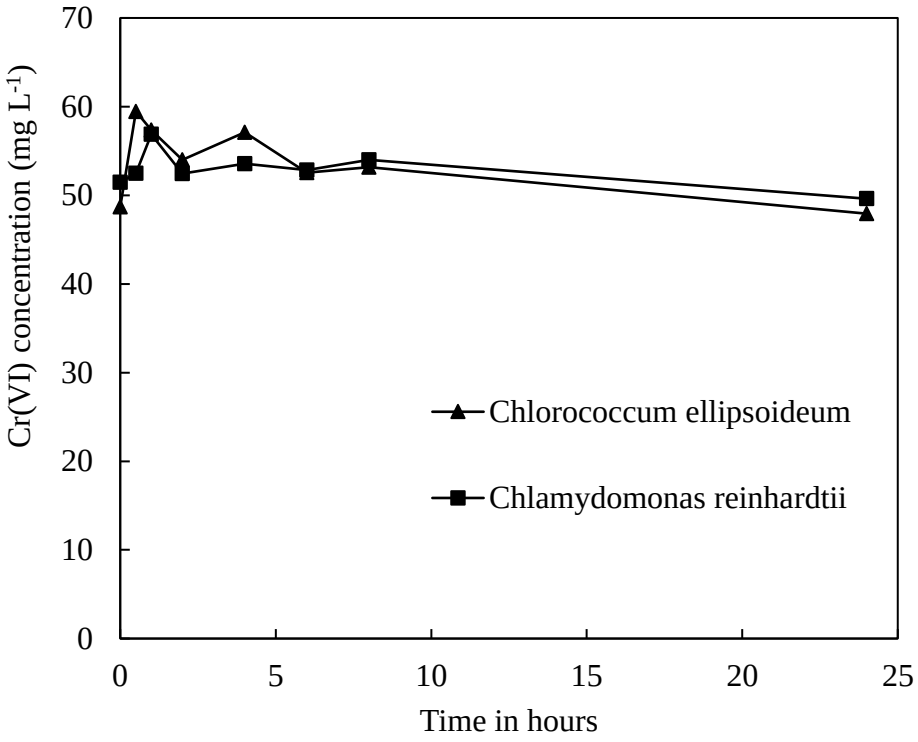

Figure 3: Cr(VI) concentration in batch experiments with just the different algae species (no bacteria present): *Chlamydomonas reinhardtii* (filled squares) and *Chlorococcum ellipsoideum* (filled triangles).

Interactions between algae and bacteria

Figure 4 shows the chlorophyll *a* content of the algae that were inoculated with CRB. The chlorophyll *a* content in both algal species (*Chlamydomonas reinhardtii* and *Chlorococcum ellipsoideum*) depleted within 4 days, which indicates a decline in algal biomass. Both algal species reacted similarly to the presence of the CRB. The high concentration of both algal and bacterial biomass and the ratio of CRB to algae cells in the experiment did not allow for a symbiotic relationship (Pell et al., 2017). In the experiments, the pH was raised from 7.5 to 8.8 within 24 hours, which corresponds to the growth phase of the algae as can be seen in Figure 4. After the first day, the pH remained constant as the chlorophyll *a* content began to decrease rapidly.

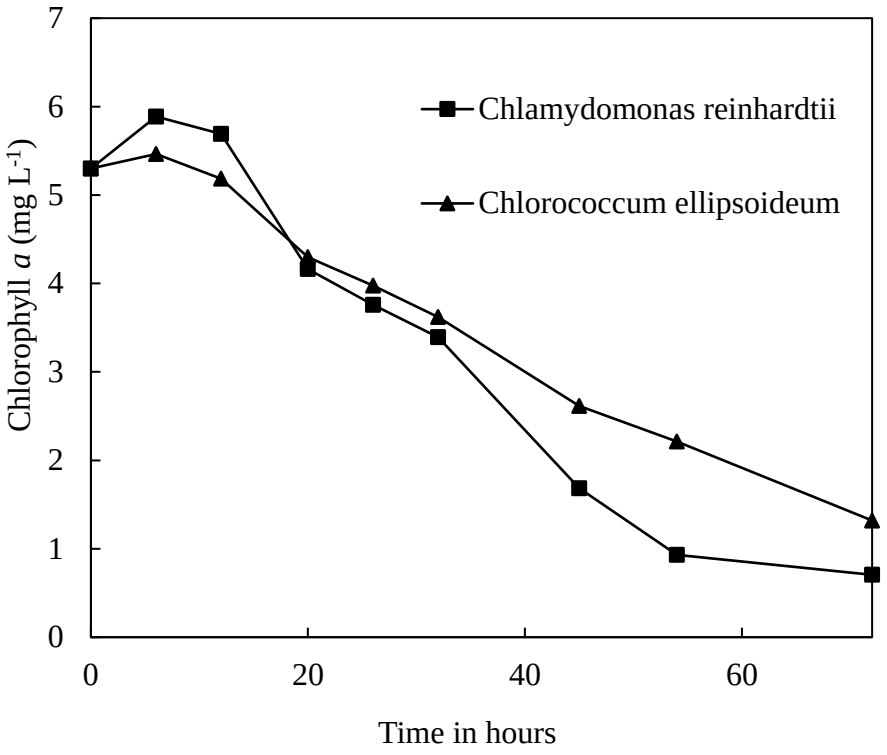

Figure 4: Chlorophyll *a* content in the system that combined the bacteria consortium with the different algae species: *Chlamydomonas reinhardtii* (filled squares) and *Chlorococcum ellipsoideum* (filled triangles).

Figure 5 and 6 shows the impact that the two algae species had on the CRB. The experiment was carried out at 0 and 50 mg L^{-1} of initial Cr(VI) concentration. Without Cr(VI) present the CRB was able to grow up to 12 hours. At 12 hours the initial bacterial CFU count increased by 60% and 58% for the *Chlorococcum ellipsoideum* and *Chlamydomonas reinhardtii* systems, after which the CFU count decreases steadily. In the experiments with Cr(VI) present (50 mg L^{-1} Cr(VI) initial concentration) a decrease in the CFU count occurs just after 4 hours. This indicates that the Cr(VI) also inhibits CRB growth as all the energy goes toward detoxifying the immediate environment instead of growth. The two algae species performed similarly as potential carbon sources. Without Cr(VI) present *Chlamydomonas reinhardtii* as a carbon source resulted in a slower decline in the bacterial CFU count.

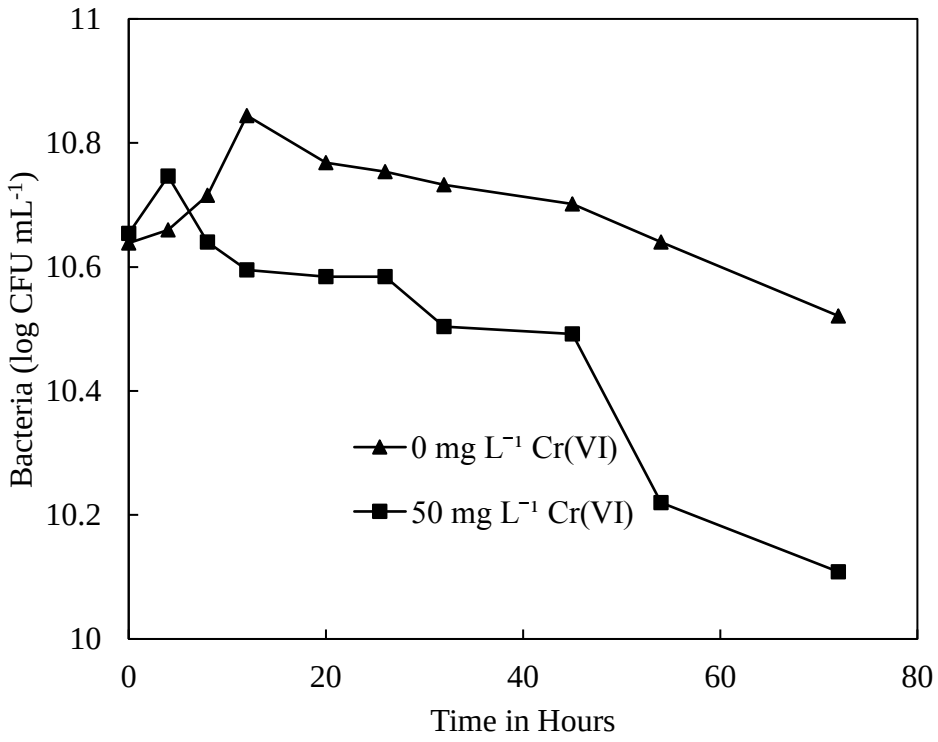

Figure 5: Bacterial CFU count (log CFU mg L^{-1}) in which *Chlorococcum ellipsoideum* cells were used as a potential carbon source at with (50 mg L^{-1} initial Cr(VI) concentration, filled squares) or without Cr(VI) (filled triangles).

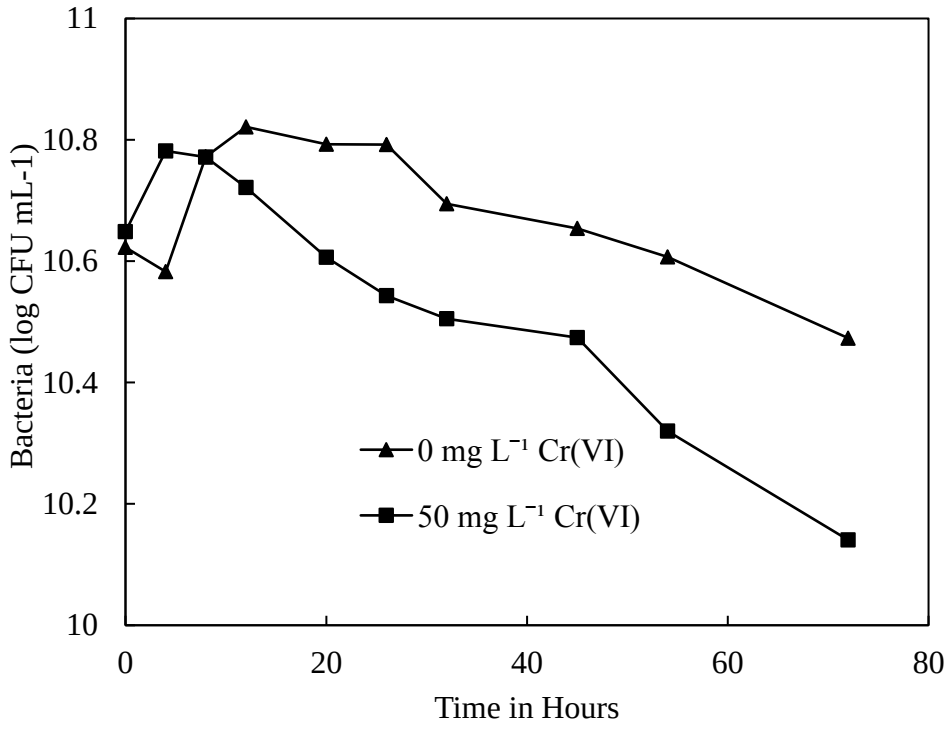

Figure 6: Bacterial CFU count (log CFU mg L^{-1}) in which *Chlamydomonas reinhardtii* cells were used as a potential carbon source at with (50 mg L^{-1} initial Cr(VI) concentration, filled squares) or without Cr(VI) (filled triangles).

Scanning electronic microscopy (SEM) images of the CRB-algae interaction at different Cr(VI) concentrations (0, 50 and 100 mg L^{-1} Cr(VI)) are shown in Figure 7. Figure 7 a shows a *Chlorococcum ellipsoideum* algae cell with a consortium of bacteria after 1 hour exposure (no Cr(VI)). The algae cell is plump and still intact. The CRB was found attached to the algal cell surface. Figure 7 b also shows a plump algae cell with bacteria attached to a damaged part of the cell wall after several hours of exposure. It was unclear if the bacteria were responsible for the damage. Figure 7 c shows how the bacteria are attached to the algal cell wall. The CRB use filamentous bridges, which are flexible fibers resembling pili, to attach itself onto the algae cell wall. These fibers can be used to increase interactions between the CRB and the host algal cells (Gardiner et al. 2014). Figure 7 d shows an algae cell after 24 hours of exposure to 50 mg L^{-1} Cr(VI). The cell wall was shriveled up and lost structural integrity.

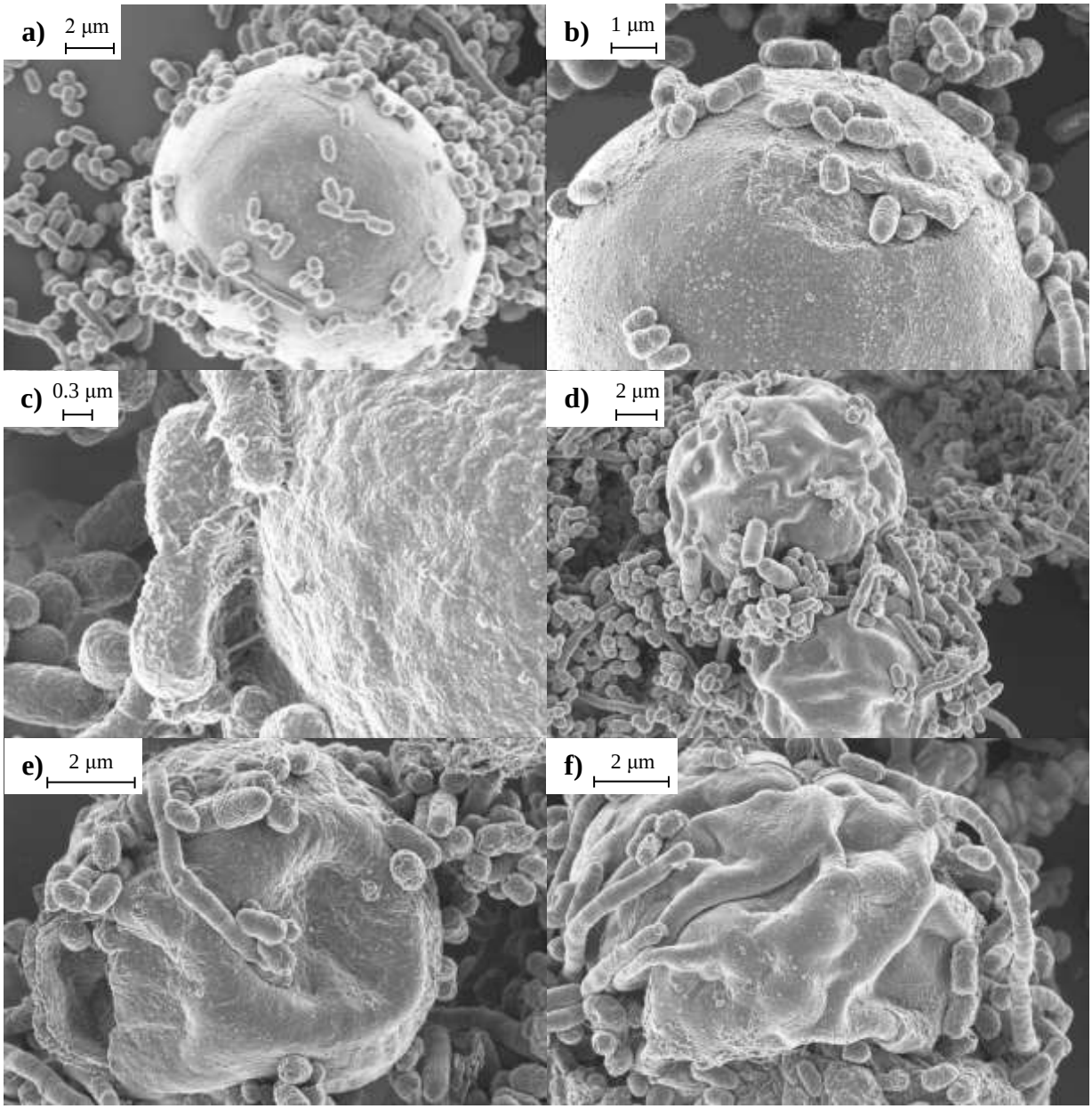

Figure 7: SEM images bacteria and *Chlorococcum ellipsoideum* cells a), b) and c) without Cr(VI), d) 50 mg L^{-1} Cr(VI), e) and f) 100 mg L^{-1} Cr(VI).

Figure 7 e shows an algae cell after 24 hours of exposure to 100 mg L^{-1} Cr(VI). The algal cell wall ruptured and, the CRB was seen inside the ruptured algal cell. Some internal cell cytoplasmic debris were also seen in Figure 7 e. The area surrounding the lysed algal cell has high microbial activity. The photosynthate carbon produced by the algal cell was released as dissolved or colloidal material which could be metabolized by the surrounding

CRB (Cole et al. 1982). Figure 7 f also shows the algal cell after exposure to 100 mg L^{-1} Cr(VI) after 24 hours. Compared to the algal cells that were exposed to 50 mg L^{-1} Cr(VI), the cells were even more shriveled up when exposed to 100 mg L^{-1}. This leads to the conclusion that a higher Cr(VI) concentration is more detrimental to the algal cell. The overall effect of the combination of parasitic CRB and toxic Cr(VI) has a very negative overall effect on the algae.

Batch results

Figure 8 and 9 shows the reduction of Cr(VI) in which CRB utilizes carbon sources provided by the algae. The batch experiments were carried out at 25 ^{o}C and a pH of 7.5. Both *Chlorococcum ellipsoideum* (Figure 8) and *Chlamydomonas reinhardtii* (Figure 9) were able to serve as a carbon source to facilitate the bacterial reduction of Cr(VI). In the batch studies complete bacterial reduction of 50 mg L^{-1} initial Cr(VI) concentration was achieved within 24 hours for both the algal carbon sources. *Chlamydomonas reinhardtii* and *Chlorococcum ellipsoideum* as carbon sources respectively allowed for 98% and 92% bacterial Cr(VI) reduction within 24 hours for a solution with an initial concentration of 100 mg L^{-1} Cr(VI). At a higher initial Cr(VI) concentration the total amount of Cr(VI) reduced decreases. It has been found that the reduction process is inhibited at higher initial Cr(VI) concentrations (Chen and Hao 1998). Increasing the CRB biomass density did not increase the reduction percentage. At 150 mg L^{-1} Cr(VI) and 200 mg L^{-1} Cr(VI), the inhibition increased, and the process could not achieve more than 46.0% and 49.4% Cr(VI) reduction respectively, utilizing *Chlorococcum ellipsoideum* and *Chlamydomonas reinhardtii*.

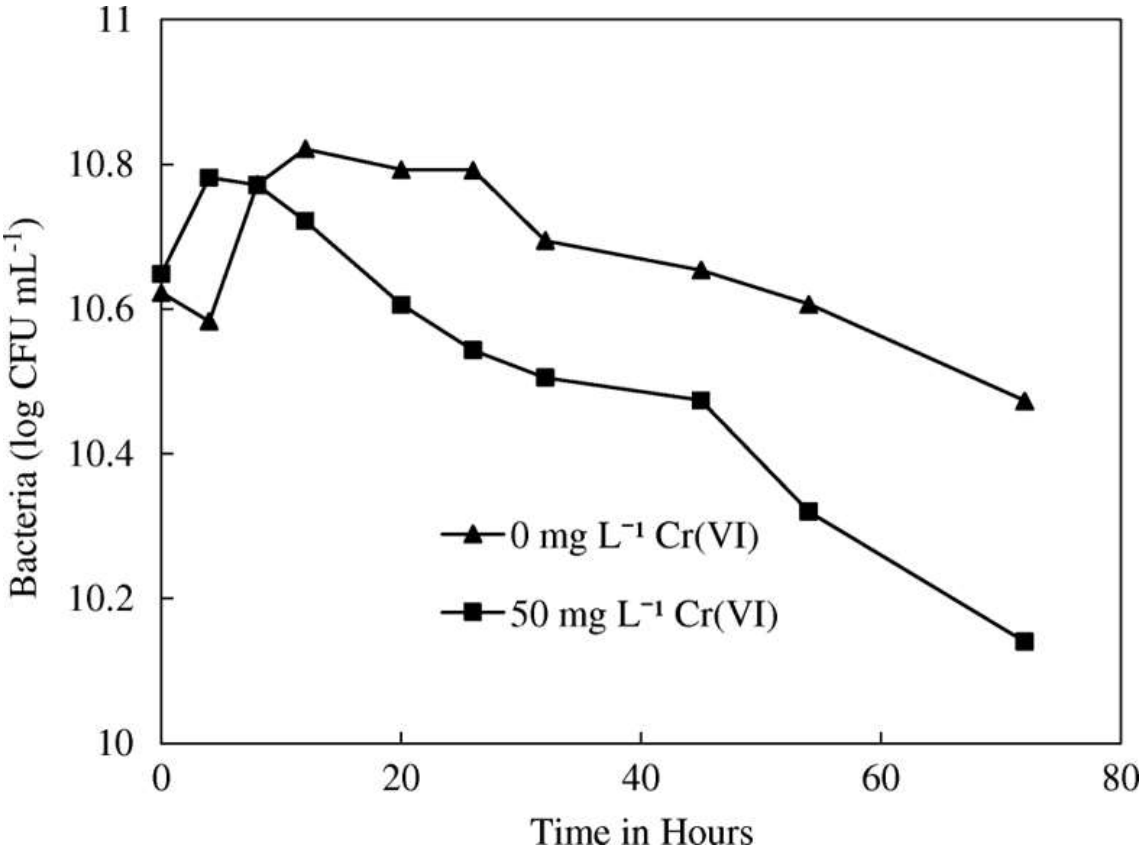

Figure 8: Experimental and model validation for *Chlorococcum ellipsoideum* as carbon source in the *bacterial Cr(VI) reduction at different initial Cr(VI) concentration: 200 mg L^{-1} Cr(VI) (filled squares), 150 mg L^{-1} Cr(VI) (filled circles), 100 mg L^{-1} Cr(VI) (filled triangles) and 50 mg L^{-1} Cr(VI) (filled diamonds).*

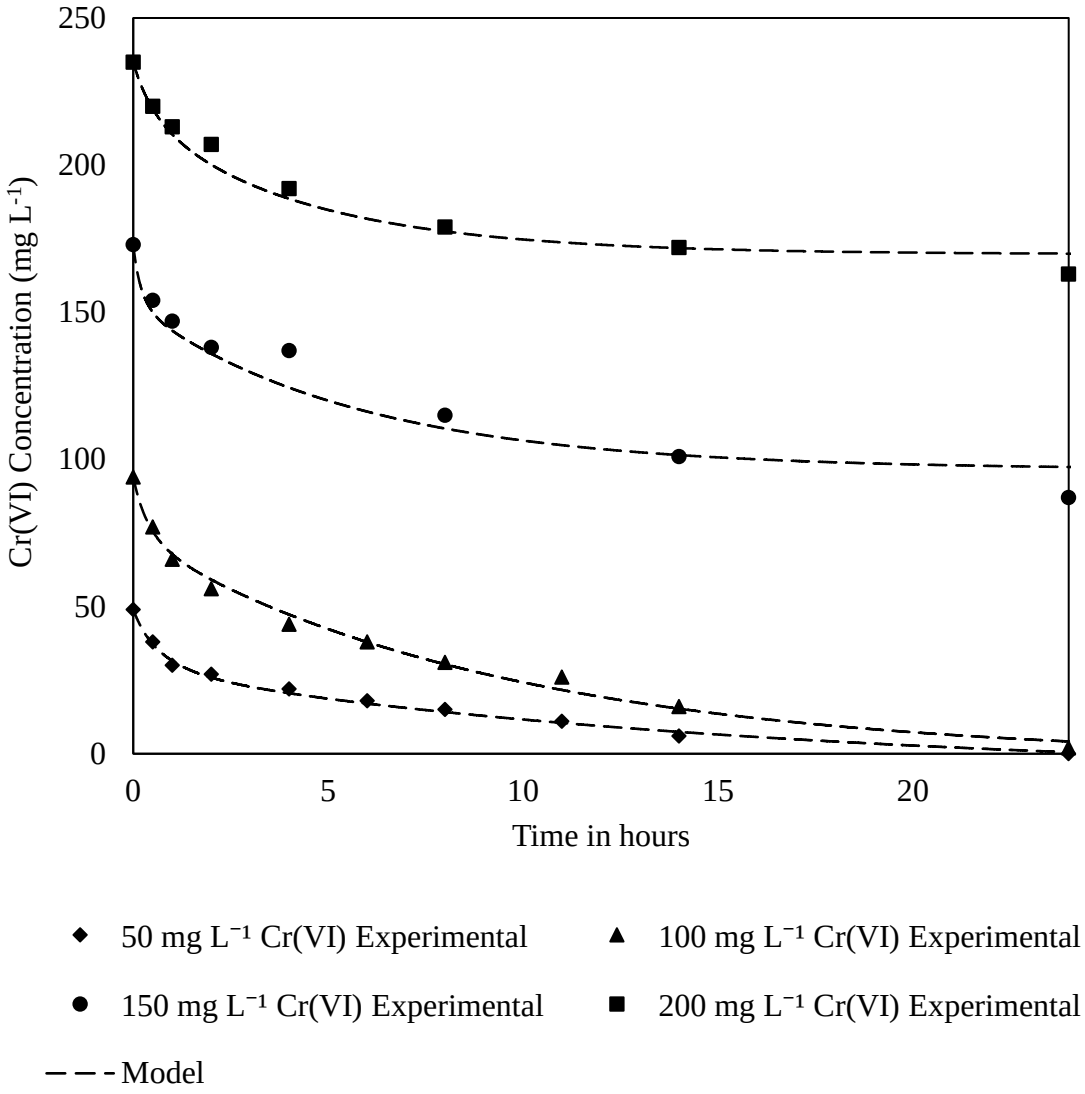

Figure 9: Experimental and model validation for *Chlamydomonas reinhardtii* as carbon source in the bacterial Cr(VI) reduction at different initial Cr(VI) concentration: 200 mg L^{-1} Cr(VI) (filled squares), 150 mg L^{-1} Cr(VI) (filled circles), 100 mg L^{-1} Cr(VI) (filled triangles) and 50 mg L^{-1} Cr(VI) (filled diamonds).

Figure 10 shows the comparison of different carbon sources used for bacterial reduction of Cr(VI). In the experiment in which no carbon sources are available in the media, the total amount of Cr(VI) removed was just 30% after 24 hours. This indicates the importance of carbon sources during the reduction process. In Figure 10, it can be seen that in the presence of a carbon source, that is either oxidizable or fermentable (such as glucose), the percentage Cr(VI) reduction is very high. The metabolic link was

established by the fact that in the absence of a carbon source only 30% of the Cr(VI) was removed, compared to 100% when glucose was added.

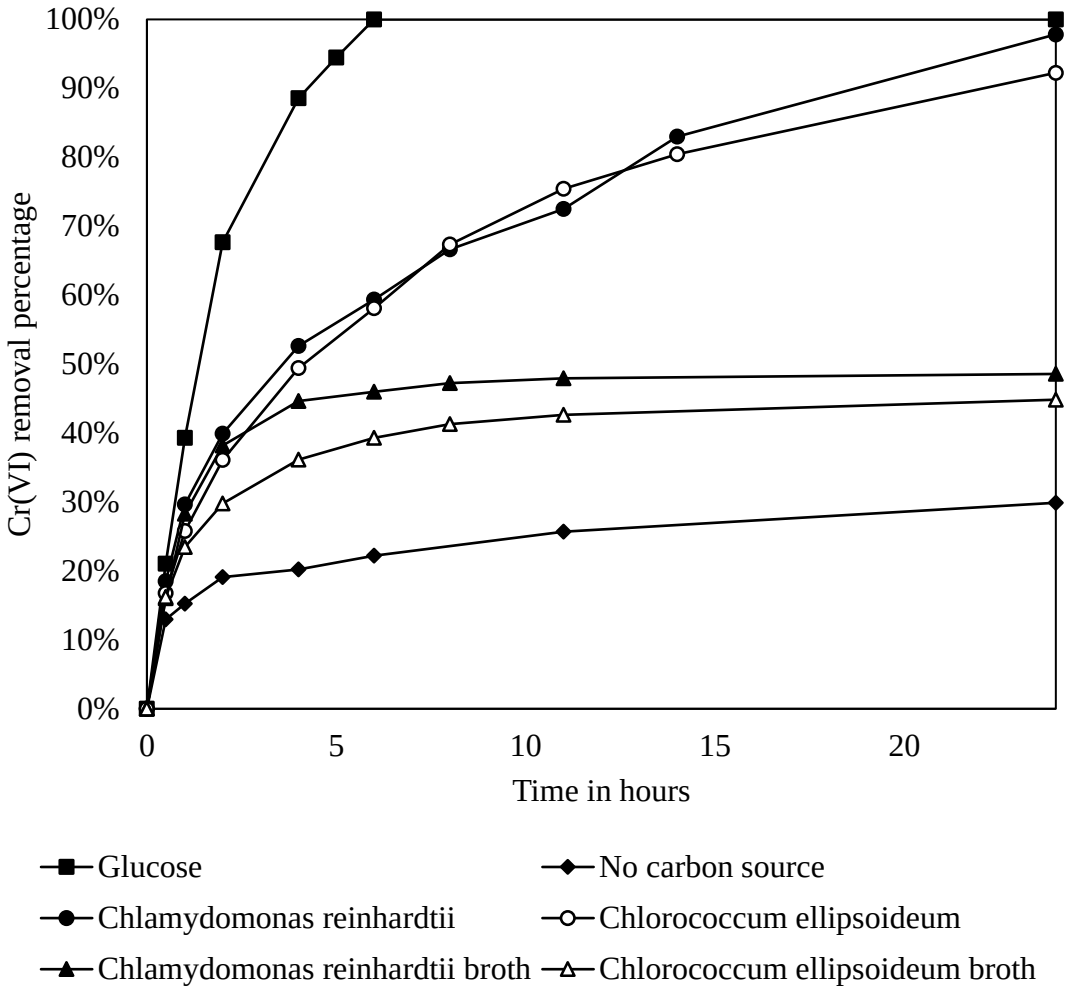

Figure 10: Comparison of different carbon sources utilized during the bacterial Cr(VI) reduction process: Glucose (filled squares), *Chlamydomonas reinhardtii* algal cells (filled circles), broth from *Chlamydomonas reinhardtii* cell free spent media (filled triangles), no carbon sources just 3N-BBM+V (filled diamond), *Chlorococcum ellipsoideum* algal cells (unfilled circles) and broth from *Chlorococcum ellipsoideum* cell free spent media. The initial Cr(VI) concentration is 100 mg L^{-1}.

Figure 10 also shows the curves where the algal broth, which is the cell-free spent media, was used to see if the CRB could utilize the extracellular products that were produced during the growth phase of both algal species. *Chlorococcum ellipsoideum* and *Chlamydomonas reinhardtii* cell-free spent media could respectively achieve 45% and

49% Cr(VI) reduction in 24 hours. Compared to the 30% Cr(VI) reduction without carbon sources, this suggests that 15%-19% Cr(VI) reduction was achieved via the utilization of extracellular products, assuming that the different processes could be superimposed. Which, in and of itself, is not very efficient but contributes to the overall process where algal cells are used as a carbon source for Cr(VI) reduction. Separating the algae from the media is an energy expensive process that is faced in producing biofuel from algae (Kleinová et al. 2012). Therefore, utilizing only extracellular products produced by algae is not feasible.

Table 1: Kinetic parameter estimation when Chlorococcum ellipsoideum is utilize as carbon source

C_o(mgL^{-1})	**50**	***100***	***150***	***200***
k_1 (hr^{-1})	0.0003	0.0003	0.0003	0.0003
k_2 (hr^{-1})	0.011	0.01	0.01	0.01
R_c (mg mg^{-1})	0.022	0.022	0.022	0.022
$S_{0,fast}$ (mg L^{-1})	28.45	28.45	28.45	28.45
X_0 (mg L^{-1})	2584.59	4801.21	3575.32	3054.03
K_i (mg L^{-1})	537.22	537.22	537.22	537.22
Chi^2	14.49	14.97	87.42	54.05

Kinetic model

The kinetics of the Cr(VI) reduction in the CRB, combined with algae system, was studied using the AQUASIM program. The experimental data were used for model optimization and simulation with a non-linear least square algorithm using equations (5 to 7). The parameters of the kinetic model that were determined with AQUASIM are listed in Table 1 and 2, and the simulated models are shown in Figure 8 and Figure 9. The experimental data fit very well with the derived model with most of the chi^2 value below 100. At lower concentrations in the range of 50 mg L^{-1} and 100 mg L^{-1} of Cr(VI), the model fitted better than at high concentrations of Cr(VI). The parameters obtained for both algal species

were very similar, the most significant difference was in the rate coefficient of the reaction of the rapidly consumed substrate (equation 3). The *Chlorococcum ellipsoideum* system has a smaller rate coefficient than the *Chlamydomonas reinhardtii* system.

Table 2: Kinetic parameter estimation when *Chlamydomonas reinhardtii* is utilize as carbon source

C_o(mgL^{-1})	**50**	***100***	***150***	***200***
k_1 (hr^{-1})	0.0006	0.0006	0.0006	0.0006
k_2 (hr^{-1})	0.015	0.015	0.015	0.015
R_c (mg mg^{-1})	0.021	0.021	0.021	0.021
$S_{0,fast}$ (mg L^{-1})	20.1	20.1	20.1	20.1
X_0 (mg L^{-1})	2697.71	4485.26	3582.07	3184.56
K_i (mg L^{-1})	499.89	499.89	499.89	499.89
Chi^2	10.47	50.63	302.76	100.42

Discussion

The results suggest that both *Chlamydomonas reinhardtii* and *Chlorococcum Ellipsoideum* are very sensitive toward Cr(VI) toxicity. Rodrıguez et al. (2007) also found that *Chlamydomonas reinhardtii* has a low tolerance level for Cr(VI) and has suggested that *Chlamydomonas reinhardtii* can be used as an indicator of Cr(VI) pollution. Limited information is available on the effect of Cr(VI) on *Chlorococcum Ellipsoideum*. Chlorophyll *a* content reflects the amount of algal biomass present (Liang et al. 2013). The decrease in chlorophyll *a* concentration could indicate that there was significant damage to the algal cell wall that allowed the chlorophyll *a* to leak out. Ünal et al. (2010) found high electric conductivity values when treating algal cells with 5 mM Cr(VI), which indicates injury to cell membranes. Arun et al. (2014) produced SEM images of saltwater algae, *D. salina* and *D. tertiolecta*, that were damaged by exposure to 40 ppm Cr(VI). A decrease in chlorophyll *a* content can cause inhibition of the photosynthesis processes

and the cell division processes (Volland et al. 2012). The chlorophyll *a* can also be converted to pheophytin as a response to the Cr(VI) toxicity (Rodríguez et al. 2007).
The two algae species did not have a large effect on the Cr(VI) concentration. Toxic metals disturb the oxidative balance of the algae; therefore, algae have developed a wide range of protective mechanisms to remove reactive oxygen species (ROS) before damage occurs on the sensitive parts of the cell (Hassoun et al. 1995). ROS such as hydrogen peroxide can oxidize Cr(III) to Cr(VI) (Xue et al., 2016), which can explain the initial spike in Figure 3. The results suggest that the Cr(VI) did not adsorb onto the algal cells. At the pH of the experiments, the negatively charged functional groups on the surface of the algal cell wall (carboxyl, phosphoryl, and hydroxyl) repelled the anionic forms ($HCrO_4^-$, CrO_4^{2-} and $Cr_2O_7^{2-}$) of Cr(VI) (Kratochvil et al. 1998). At a pH lower than 2.0, electrostatic attraction is present between the negatively charged Cr(VI) and the positively charged algae cell wall due to the presence of more functional groups carrying a positive charge (Murphy et al. 2008). Thus, pH can influence Cr(VI) biosorption by affecting the configuration of the active ion-exchange sites (Sibi 2016).

The CRB was also inhibited to an extent by the Cr(VI) toxicity. The bacterial cell concentration was already too high at the beginning of the experiments to allow for a significantly high growth rate or exponential growth. However, from Figure 5 and 6 it is clear that the bacterial CFU count did increase in the first few hours. After 12 hours the experiments without Cr(VI) showed a steady decrease in the CFU count, this phase corresponds to a pH value of 8. The algae consumed all the CO_2 in the solution which increases the pH of the solution. The higher pH can inhibit the bacteria growth and also contribute to the decrease in the CFU count. The easily accessible carbon sources available for the CRB to consume can also be depleted after 12 hours.

It can be concluded from the SEM results that a portion of the internal metabolites or photosynthate carbon leaked from the algal cells that were exposed to both Cr(VI) and parasitic CRB. This leaked substance could be potentially utilized as a carbon source by CRB allowing the latter to reduce the Cr(VI) to Cr(III).

Many bacteria are known to affect algae negatively(Dakhama et al. 1993). Algal cell lysis occurs in close proximity to bacteria, as part of the parasitic relationship between certain bacteria and algae (Ramanan et al. 2016). Toncheva-Panova and Ivanova (2000) found

that *Cytophaga sp*. *LR2* is responsible for lysis of the red microalga, *Rhodella reticulate*. The biological degradation of algae has been investigated by Ji et al. (2009) and found that *Bacillu spp.* and *Pseudomonas spp* were able to achieve a 60% chlorophyll *a* removal efficiency. Szymczak-Żyla et al. (2008) also found microorganism could degrade chlorophyll *a* content under aerobic conditions. The algae can defend against parasitic bacteria by raising the pH above 10, by consuming CO_2 faster than it can be replaced by bacterial respiration. The bacteria, which are not alkaliphilic organisms, would be severely inhibited by the high pH (Abdel-Raouf et al. 2012). Additionally, many studies have found that bacteria and algae can have a symbiotic relationship (Ramanan et al. 2016; Guo and Tong 2014). In the symbiotic relationship the algae would provide O_2 to heterotrophic aerobic bacteria, and in turn, the algae would utilize the CO_2 released from bacterial respiration (Munoz and Guieysse 2006).

The CRB and algae combined system could achieve up to 98% reduction of 100 mg L^{-1} initial Cr(VI) concentration within 24 hours through a combination of mostly metabolically dependent processes and a few metabolically independent processes. These results are good when compared to the system in which only Cr(VI) reducing *Chlorella spp*. were used by Yewalkar et al. (2007), that only achieved 97% reduction of an initial 3.12 mg L^{-1} Cr(VI) concentration in 72 hours. Both the above mentioned only-algae system and the CRB combined with algae system of this current study ultimately derived carbon by sequestering CO_2 from the surrounding environment.

Fujie et al. (1994) showed that the Cr(VI) reduction rate was proportional to the organic substrate uptake rate at a ratio of 0.6 mg of carbon to 1 mg Cr(VI) reduced. Hence, an inadequate carbon source can inhibit the Cr(VI) reduction at higher initial Cr(VI) concentration. It is likely that insufficient carbon sources were available for the CRB to utilize in the experiments that used 150 mg L^{-1} and 200 mg L^{-1} initial Cr(VI) concentrations. Taking the relationship between Cr(VI) reduction rate and substrate uptake rate into consideration, the shape of the curves in Figures 8 and 9 indicate how the carbon sources that are available are utilized. In the first two hours, the Cr(VI) concentration rapidly decreased, which could imply that the carbon sources were readily consumed. After the first two hours, the Cr(VI) concentration decreased steadily, which signifies that the carbon sources were consumed at a slower rate than before. The algae

cells produce various extracellular products: high molecular weight substances like polysaccharides, and also low molecular weight substances. These products can be utilized by CRB at different rates, depending on their bioavailability or degradability (Bell and Sakshaug 1980). Another possibility is that intracellular products, such as fatty acids and protein like substances (Grima et al. 2003), are released into the solution as the algal cells begin to rupture, and the cell rupture rate controls the intracellular product availability and, by extension, the Cr(VI) reduction rate. This also demonstrates how metabolically diverse the CRB are, and that they can reduce Cr(VI) to Cr(III) using various carbon sources.

Without carbon sources, the CRB could only achieve 30% Cr(VI) removal, which was probably due to adsorption onto the cells followed by intracellular reduction by cytoplasmic enzymes (Chirwa and Molokwane 2011) or reduction by functional groups on the bacterial cell surface (Kang et al. 2007). The adsorption capacity of the bacterial cells depends on the pH of the solution as well as the functional groups on the cell surface. Gram-positive bacteria, such as *Bacillus thermoamylovorans*, have functional groups consisting of peptidoglycans, teichoic and teichuronic acids. These acids contribute to the negative charge of the biomass at neutral pH and are protonated at low pH. Thus, repelling anionic Cr(VI) at neutral pH. Gram-negative bacteria, such as *Escherichia coli* and *Citrobacter sedlakii*, have fewer of these anionic functional groups, but deprotonation of the metal binding sites occurs as the pH increases which results in the repulsion of anionic Cr(VI) species (Ziagova et al. 2007). Most studies have found that a pH was below 3 allowed for the best Cr(VI) adsorption by bacterial cells (Loukidou et al. 2004; Şahin and Öztürk 2005; Ozdemir et al., 2004). Although, it is still plausible that some adsorption could occur at the neutral operational pH of the batch experiments. It is important to note that the bacteria were still alive during the experiment and still somewhat metabolically active and could have still produced extracellular enzymes to combat the Cr(VI) toxicity even without a carbon source.

Not only the extracellular reductases enzymes are responsible for the reduction of Cr(VI) to Cr(III), intracellular cytoplasmic enzymes can utilize cellular components (NADH, NADPH, flavoproteins, and hemeproteins) as electron donors to reduce Cr(VI) to Cr(III). Due to the structural similarity of Cr(VI) species, such as CrO_4^{2-}, with sulfate anion (SO_4^{2-}), the Cr(VI) can be transported across biological membranes via active sulfate

transporters in CRB (Gutiérrez-Corona et al. 2016). The cytoplasm portion from disrupted algal cells would also be able to reduce Cr(VI) to some extent. This reduction process does not need an active cellular metabolism but would be very harmful to the cell due to the production of ROS that could damage the DNA, since most of the intracellular proteins catalyze a one-electron reduction from Cr(VI) to Cr(V) (Chirwa and Molokwane 2011).

Certain CRB intentionally produce Cr(VI) reducing enzymes that are transported into the media to reduce Cr(VI) externally. The enzymes are only generated when Cr(VI) is detected in the surrounding media, as protein excretion is an energy-intensive process (Chirwa and Molokwane 2011). Extracellular Cr(VI) reduction is advantageous to the bacteria as it limits the DNA damages caused internally by Cr(VI) (Chirwa and Molokwane 2011). Another Cr(VI) reduction mechanism employs the membrane electron transport respiratory apparatus within the bacteria which uses Cr(VI) as either the electron donor or electron sink. NAD(P)H-dependent extracellular soluble reductases (membrane-associated enzymes) are produced purposely by the cell to reduce Cr(VI) (Ahemad 2014). The chromate reductase transfers electrons to Cr(VI) via the NADH dependent cytochromes (Viti et al. 2014). 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 metabolism as the mechanism of Cr(VI) detoxification.

In Figure 8 it is shown that glucose achieved the highest Cr(VI) reduction rate. Literature has found that among the different electron donors, glucose provided the highest Cr(VI) reduction compared to other electron donors (Barrera-Díaz et al. 2012). Previous studies have however also shown that any number of organic compounds might serve as an electron donor during the Cr(VI) reduction process. This counts toward the feasibility of using this process to treat Cr(VI) contamination (Chirwa and Molokwane 2011).

Conclusion

Successful Cr(VI) removal was achieved, at concentrations below 100 mg L^{-1} within 24 hours, for both algae species that were used as a carbon source for the CRB. The high removal was achieved through a combination of mostly metabolically dependent processes and a few metabolically independent processes. There was no difference in performance as a carbon source between the *Chlamydomonas reinhardtii* and

Chlorococcum ellipsoideum algae species. This indicates that it is possible for the CRB to use various carbon sources to achieve Cr(VI) reduction and presents the opportunity to eliminate the need to add glucose to the system.

Cr(VI) and the CRB had a negative effect on algal cell health and could have led to rupturing of algae cells. Experimental data showed a relatively good fit with the predicted model in AQUASIM. The practicality and self-sustaining nature of using algae and CRB in a continuous system must be evaluated and, if possible, pursued.

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References

Abdel-Raouf N, Al-Homaidan AA, Ibraheem IBM (2012) Microalgae and wastewater treatment. Saudi J Biol Sci 19(3):257-275.

Ahemad M (2014) Bacterial mechanisms for Cr (VI) resistance and reduction: an overview and recent advances. Folia Microbiol 59(4):321-332.

APHA (2005) Standard methods for the examination of water and wastewater. 21st Edition. By Clesceri LS, Greenberg AE, Eaton AD, American Public Health Association, American Water Works Association and Water Pollution Control Federation, Washington DC.

Arita A, Costa M (2011) Environmental agents and epigenetics. In Handbook of epigenetics 459-476.

Arun N, Singh DP (2014) Chromium (VI) induced oxidative stress in halotolerant alga Dunaliella salina and D. tertiolecta isolated from sambhar salt lake of Rajasthan (India). Cell Mol Biol (Noisy-le-Grand, France) 60(5):90-96.

Barrera-Díaz CE, Lugo-Lugo V, Bilyeu B (2012) A review of chemical, electrochemical and biological methods for aqueous Cr (VI) reduction. J Hazard Mater 223:1-12.

Bell WH, Sakshaug E (1980) Bacterial utilization of algal extracellular products. 2. A kinetic study of natural populations. Limnol Oceanogr 25(6):1021-1033.

Bridgewater LC, Manning FC, Patierno SR (1994) Base-specific arrest of in vitro DNA replication by carcinogenic chromium: relationship to DNA interstrand crosslinking. Carcinogenesis 15:2421-2427.

Bruckner CG, Bahulikar R, Rahalkar M, Schink B, Kroth PG (2008) Bacteria associated with benthic diatoms from Lake Constance: phylogeny and influences on diatom growth and secretion of extracellular polymeric substances. Appl Environ Microbiol 74(24):7740-7749.

Bush MB (2003) Ecology of a Changing Planet, 3rd Edition, Prentice Hall, New Jersey, USA.

CCAP (2015) The Culture Collection of Algae and Protozoa. Medium recipe for 3N-BBM+V (Bold Basal Medium with 3-fold Nitrogen and Vitamins; modified).

Chen JM, Hao OJ (1998) Microbial chromium (VI) reduction. Critical Reviews in Environ Sci Technol 28(3):219-251.

Chirwa, EM, Molokwane PE (2011) Biological Cr (VI) reduction: microbial diversity, kinetics and biotechnological solutions to pollution. In Biodiversity. InTech.

Cicci A, Sed G, Bravi M, (2017) Potential of choline chloride-based natural deep eutectic solvents (nades) in the extraction of microalgal metabolites. Chem Eng Trans 57:61-66.

Coenye T, Falsen E, Vancanneyt M, Hoste B, Govan JRW, Kersters K, Vandamme P (1999) Classification of Alcaligenes faecalis-like isolates from the environment and human clinical samples as Ralstonia gilardii sp. nov. Int J Syst Bacteriol 49:405-413.

Cole JJ, Likens GE, Strayer DL (1982) Photosynthetically produced dissolved organic carbon: an important carbon source for planktonic bacteria. Limnol Oceanogr 27: 1080-1090.

Dakhama A, De la Noüe J, Lavoie MC (1993) Isolation and identification of antialgal substances produced by Pseudomonas aeruginosa. J Appl Phycol 5(3):297-306.

Dong T, Knoshaug EP, Pienkos PT, Laurens LM (2016) Lipid recovery from wet oleaginous microbial biomass for biofuel production: a critical review. Appl energy 177:879-895.

EPA-Odessa (2005) Pump and Treat and In Situ Chemical Treatment of Contaminated Groundwater at the Odessa Chromium II South Plume Superfund Site Odessa, Ector County, Texas. U.S. Environmental Protection Agency.

EPA, US (1998) Toxicological review of hexavalent chromium. Washington, DC: United States of America.

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39(4):783-791.

Fujie K, Toda K, Ohtake H (1990) Bacterial reduction of toxic hexavalent chromium using a fed-batch culture of Enterobacter cloacae strain HO1. J Ferment Bioengr 69(6):365-367.

Gardiner M, Hoke DE, Egan S (2014) An ortholog of the Leptospira interrogans lipoprotein LipL32 aids in the colonization of Pseudoalteromonas tunicata to host surfaces. Front Microbiol 5:323.

Glauert AM (1975) Fixation, dehydration and embedding of biological specimens. No. 04; QH327, G5.

Grima EM, Belarbi EH, Fernández FA, Medina AR, Chisti Y (2003) Recovery of microalgal biomass and metabolites: process options and economics. Biotechnol Adv 20(7-8):491-515.

Guo Z, Tong YW (2014) The interactions between Chlorella vulgaris and algal symbiotic bacteria under photoautotrophic and photoheterotrophic conditions. J Appl Phycol 26(3):1483-1492.

Gutiérrez-Corona JF, Romo-Rodríguez P, Santos-Escobar F, Espino-Saldana AE, Hernández-Escoto H (2016) Microbial interactions with chromium: basic biological processes and applications in environmental biotechnology. World J Microbiol Biotechnol 32(12):191.

Hassoun EA, Stohs SJ (1995) Chromium-induced production of reactive oxygen species, DNA single-strand breaks, nitric oxide production, and lactate dehydrogenase leakage in J774A. 1 cell cultures. J Biochem Mol Toxicol 10(6):315-321.

Igboamalu T, Chirwa E (2016) Kinetic study of Cr(vi) reduction in an indigenous mixed culture of bacteria in the presence of As(iii). Chem Eng Trans 49:439-444.

Ji RP, Lu XW, Li XN, Pu YP (2009) Biological degradation of algae and microcystins by microbial enrichment on artificial media. Ecol Eng 35(11):1584-1588.

Kalckar HM (1974) Origins of the concept oxidative phosphorylation. Mol Cell Biochem 5 (1-2):55-63.

Kang SY, Lee JU, Kim KW (2007) Biosorption of Cr (III) and Cr (VI) onto the cell surface of Pseudomonas aeruginosa. Biochem Eng J 36(1):54-58.

Kleinová A, Cvengrošová Z, Rimarčík J, Buzetzki E, Mikulec J, Cvengroš J (2012) Biofuels from algae. Procedia Eng 42:231-238.

Kothari R., Pathak VV, Kumar V, Singh DP (2012) Experimental study for growth potential of unicellular alga Chlorella pyrenoidosa on dairy waste water: an integrated approach for treatment and biofuel production. Bioresour Technol 116:466-470.

Kratochvil D, Pimentel P, Volesky B (1998) Removal of trivalent and hexavalent chromium by seaweed biosorbent. Environ Sci Technol 32(18):2693-2698.

Liang Z, Liu Y, Ge F, Xu Y, Tao N, Peng F, Wong M (2013) Efficiency assessment and pH effect in removing nitrogen and phosphorus by algae-bacteria combined system of *Chlorella vulgaris* and *Bacillus licheniformis*. Chemosphere 92(10):1383-1389.

Lloyd JR (2003) Microbial reduction of metals and radionuclides. FEMS Microbiol Rev 27:411-425.

Loukidou MX, Zouboulis AI, Karapantsios TD, Matis KA (2004) Equilibrium and kinetic modeling of chromium (VI) biosorption by Aeromonas caviae. Colloids Surf A Physicochem Eng Asp 242(1-3):93-104.

Macfie A, Hagan E, Zhitkovich A (2009) Mechanism of DNA− Protein cross-linking by chromium. Chem Res Toxicol 23(2):341-347.

Marande W, López- García P, Moreira D (2009) Eukaryotic diversity and phylogeny using small- and large- subunit ribosomal RNA genes from environmental samples. Environ Microbiol 11(12):3179-3188.

Miranda J, Krishnakumar G, Gonsalves R (2012) Cr6+ bioremediation efficiency of Oscillatoria laete-virens (Crouan & Crouan) Gomont and Oscillatoria trichoides Szafer: kinetics and equilibrium study. J Appl Phycol 24(6):1439-1454.

Molokwane PE, Nkhalambayausi-Chirwa EM, Meli KC (2008) Chromium (VI) reduction in activated sludge bacteria exposed to high chromium loading: Brits culture (South Africa), Water Res 42:4538-4548.

Munoz R, Guieysse B (2006) Algal–bacterial processes for the treatment of hazardous contaminants: a review. Water Res 40(15):2799-2815.

Murphy, V., Hughes, H., & McLoughlin, P. (2008). Comparative study of chromium biosorption by red, green and brown seaweed biomass. Chemosphere, 70(6), 1128-1134.

Nelson YM, Lion LW, Shuler ML, Ghiorse WC (1996) Modeling oligotrophic biofilm formation and lead adsorption to biofilm components. Environ Sci Technol 30(6):2027-2035.

Nakayama T, Watanabe S, Mitsui K, Uchida H, Inouye I (1996) The phylogenetic relationship between the Chlamydomonadales and Chlorococcales inferred from 18SrDNA sequence data. Phycological Res 44(1):47-55.

Ozdemir G, Ceyhan N, Ozturk T, Akirmak F, Cosar T (2004) Biosorption of chromium (VI), cadmium (II) and copper (II) by Pantoea sp. TEM18. Chem Eng J, 102(3):249-253.

Pell L, Löhn S, Weinberger G, Kuchta K, Hanelt D (2017) Mild disintegration methods of microalgae–bacteria flocs from wastewater treatment. J Appl Phycol 29(2):843-851.

Pritchard DE, Singh J, Carlisle DL, Patierno SR (2000) Cyclosporin A inhibits chromium(VI)-induced apoptosis and mitochondrial cytochrome c release and restores clonogenic survival in CHO cells. Carcinogenesis 21(11):2027–2033.

Ramanan R, Kim BH, Cho DH, Oh HM, Kim HS (2016) Algae–bacteria interactions: evolution, ecology and emerging applications. Biotechnol Adv 34(1):14-29.

Rodríguez MC, Barsanti L, Passarelli V, Evangelista V, Conforti V, Gualtieri, P (2007) Effects of chromium on photosynthetic and photoreceptive apparatus of the alga Chlamydomonas reinhardtii. Environ Res 105(2):234-239.

Şahin, Y, Öztürk A (2005) Biosorption of chromium (VI) ions from aqueous solution by the bacterium Bacillus thuringiensis. Process Biochem 40(5):1895-1901.

Shen H, Wang YT (1994) Modeling hexavalent chromium reduction in Escherichia coli ATCC 33456. Biotechnol Bioeng 43(4):293-300.

Sibi G (2016) Biosorption of chromium from electroplating and galvanizing industrial effluents under extreme conditions using Chlorella vulgaris. Green Energy & Environment 1(2):172-177.

Singh J, Bridgewater LC, Patierno SR (1998a) Differential sensitivity of chromium-mediated DNA interstrand crosslinks and DNA–protein crosslinks to disruption by alkali and EDTA. Toxicol Sci 45:72–76.

Singh J, McLean JA, Pritchard DE, Montaser A, Patierno SR (1998b) Sensitive quantitation of chromium-DNA adducts by inductively coupled plasma mass spectrometry with a direct injection high-efficiency nebulizer. Toxicol Sci 46:260–265.

Smith WA, Apel WA, Petersen JN, Peyton BM (2002) Effect of carbon and energy source on bacterial chromate reduction. Bioremediat J 6(3):205-215.

Szymczak-Żyla M, Kowalewska G, Louda JW (2008) The influence of microorganisms on chlorophyll a degradation in the marine environment. Limnol Oceanogr 53(2):851-862.

Thatoi H, Das S, Mishra J, Rath BP, Das N (2014) Bacterial chromate reductase, a potential enzyme for bioremediation of hexavalent chromium: a review. J Environ Manage 146:383-399.

Thomas PG, Quinn PJ, Williams WP (1985) The origin of photosystem-I mediated electron transport stimulation in heat-stressed chloroplasts. Planta 167(1):133-139.

Toncheva-Panova T, Ivanova J (2000) Influence of physiological factors on the lysis effect of Cytophaga on the red microalga Rhodella reticulata. J Appl Microbiol 88(2):358-363.

Tsou TC, Chen CL, Liu TY, Yang JL (1996) Induction of 8-hydroxydeoxyguanosine in DNA by chromium(III) plus hydrogen peroxide and its prevention by scavengers. Carcinogenesis 17:103–108.

Ünal D, Işik NO, Sukatar A (2010) Effects of Chromium VI stress on green alga Ulva lactuca (L.). Turk J Biol 34(2):119-124.

Vidotti A, Coelho R, Franco LM, Franco TT (2014) Miniaturized culture for heterotrophic microalgae using low cost carbon sources as a tool to isolate fast and economical strains. Chem Eng Trans 38:325-330.

Viti C, Marchi E, Decorosi F, Giovannetti L (2014) Molecular mechanisms of Cr (VI) resistance in bacteria and fungi. FEMS Microbiol Rev 38(4):633-659.

Volland S, Lütz C, Michalke B, Lütz-Meindl U (2012) Intracellular chromium localization and cell physiological response in the unicellular alga Micrasterias. Aquat Toxicol 109:59-69.

White TJ, Bruns T, Lee SJWT, Taylor JL (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications, 18(1):315-322.

Wise JP, Leonard JC, Patierno SR (1992) Clastogenicity of lead chromate particles in hamster and human cells. Mutat Res 278:69-79.

World Health Organization (2004) Guidelines for drinking-water quality: recommendations (Vol. 1). World Health Organization.

Xu J, Bubley GJ, Detrick B, Blankenship LJ, Patierno SR (1996) Chromium(VI) treatment of normal human lung cells results in guanine-specific DNA polymerase arrest, DNA–DNA cross-links and S-phase blockade of cell cycle. Carcinogenesis 17:1511-1517.

Xue Y, Jin W, Du H, Zheng S, Sun Z, Yan W, Zhang Y (2016) Electrochemical Cr (III) oxidation and mobilization by in situ generated reactive oxygen species in alkaline solution. J Electrochem Soc 163(8):684-689.

Yewalkar SN, Dhumal KN, Sainis JK (2007) Chromium (VI)-reducing Chlorella spp. isolated from disposal sites of paper-pulp and electroplating industry. J Appl Phycol 19(5):459-465.

Zhiguo H; Fengling G; Tao S, Yuehua H, Chao H (2009) Isolation and Characterization of a Cr(VI)-Reduction Ochrobactrum sp. strain CSCr-3 from Chromium Landfill. J Hazard Mater 163(2-3):869-873

Ziagova M, Dimitriadis G, Aslanidou D, Papaioannou X, Tzannetaki EL, Liakopoulou-Kyriakides M (2007) Comparative study of Cd (II) and Cr (VI) biosorption on Staphylococcus xylosus and Pseudomonas sp. in single and binary mixtures. Bioresour Technol 98(15):2859-2865.