

Microbial Chromium (VI) Immobilization in Saturated Aquifer Columns Using Culture Inoculated Soil

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Chromium exists in two oxidation states in the environment namely; Cr(VI) which is highly toxic, carcinogenic and mutagenic in nature and Cr(III) which is less toxic, mobile and is required in trace elements with human diets. South Africa holds approximately 72 % of the currently documented chromium ore reserves. Areas where chromium is mined, experience severe Cr(VI) contamination due to poor treatments of Cr(VI) by products. Cr(VI) is exclusively released through anthropogenic activities into wastewaters. Biological reactors have been investigated in reducing chromium successfully. Bioremediation of Cr(VI) to Cr(III) has increasingly gained interest as it is seen as economic and ecological. In this study, *in situ* bioremediation of treating Cr(VI) aquifers is evaluated. Firstly, dried sludge containing indigenous chromium reducing bacteria (CRB) from a dried sludge was collected from Brits Wastewater Treatment Plant, North West Province (South Africa). Batch experiments by isolated bacteria were done under aerobic conditions. Cr(VI) reduction was recorded at 50, 100, 200 and 400 mg/L of Cr(VI) concentrations. *Pseudomonas putida* showed complete Cr(VI) reduction within 5 h of batch analysis. The continuous batch experiments were done using columns with five equally spaced sampling pots along the column length. Cr(VI) reduction profile was thus observed. At steady state there was complete reduction of Cr(VI) on all the columns including; sludge, saw dust and carbon source at 40 mg/L. The continuous batch system appeared to have certain amount of algae emerging after 30 d of operation, from the bottom of the column where there's reduced Cr(VI) concentration and so it was cultivated and characterized. The presence of algae may show a symbiosis relationship involving a complex relationship of detoxification survival of algae or a food source for bacteria. Cr(VI) reducing effects of the columns was further tested against higher Cr(VI) concentrations at 60 mg/L. Near complete Cr(VI) reduction was observed within a couple of days of operation at this concentration. This study demonstrates the potential of biological Cr(VI) reduction using Cr(VI) reducing bacteria that has been isolated from polluted sites in South Africa.

1. Introduction

Chromium (Cr) is derived from a Greek word called 'chroma', meaning color. A variety of colored compounds are formed in the presence of this metal. These include; $\text{Na}_2\text{Cr}_2\text{O}_7$ (bright orange), Cr_2O_3 , Zn and Pb salts of CrO_4^{2-} (green) (Zakari et al., 2010). The element chromium was first discovered by a French chemist Nicolas-louis Vauquelin in 1797 (Kaimbia and Chirwa, 2018). Chromium can be found mainly in two forms in the environment; trivalent chromium (Cr(III)) and hexavalent chromium (Cr(VI)). Cr(III) readily forms insoluble and less mobile species ($\text{Cr}(\text{OH})_3$) in water. On the other hand, Cr(VI) exists as a soluble and mobile oxyanions, chromate and dichromate (CrO_4^{2-} or $\text{Cr}_2\text{O}_7^{2-}$) (Meli, 2009). Cr(VI) is carcinogenic and mutagenic to living organisms and humans, exposure can lead to several health risks (Mthimunya, 2011). Furthermore, Cr may form free radicals (OH^*) in water which may lead to excess DNA damage (Molokwane, 2010). Due to these and other known toxic effects, Cr is classified as a type A carcinogen by the U.S EPA. Cr(VI) at a concentration as low as 0.5 mg/L in soil is toxic to plants (Oliveria, 2012). Cr(III), on the other hand, is not toxic even at a concentration as high as 600 mg/L. This reduced form of chromium is needed in mammals for carbohydrate and lipid metabolism. Furthermore, Cr(III) is used as nutritional supplement (weight loss agent) as chromium picolinate by athletes (Kaimbi and Chirwa, 2015).

Cr is one of the worlds most strategic and critical materials having a wide range of uses in metal and chemical industries. About 90% of the total ore production is used in metallurgical industries for steel, alloy, iron and nonferrous alloy production across the world. Other industries use it for leather tanning, electroplating and wood preservation as a pigment or oxidizing agent (Zakari et al., 2010). Thus Cr from the anthropogenic sources is discharged into the environment and wastewaters mainly as Cr(VI) resulting in serious environmental pollution (Kaimbia and Chirwa, 2018).

Considering the toxicity and the mobility of Cr(VI) in the environment, its removal or transformation to less toxic Cr(III) is essential (Mtimunye and Chirwa 2011). The conventional remediation strategies involve pumping the pollutant (Cr(VI)) from the contaminated sites/ aquifers, followed by *ex situ* treatment using physical/ chemical technologies such as; iron exchange, electrodepositing or chemical reduction to Cr(III) (Molokwane et al., 2008). Cr(III) is immobilized by precipitation and/or adsorption to substrates by using clay and oxide minerals below pH 5 and subsequently disposed as sludge (Kaimbi and Chirwa, 2018). The aforementioned treatments generate large volumes of sludge and dangerous gases involving harmful products that require careful further treatment and are difficult to dispose (Mishra et al. 2010). Furthermore, these methods are expensive, energy intensive and require large amounts of chemicals (Mthimunye and Chirwa, 2017).

Over the years, Cr(VI) remediation using microbial cultures with Cr(VI) toxic resistance has been increasingly gaining attention. Cr(VI) reduction capability in bacteria was first reported in the early 1970s where *Pseudomonas species* reduced Cr(VI) to Cr(III) under anaerobic conditions (Romanenko and Koren'kov, 1977). Through various studies, more bacterial strains of different genera have been isolated from chromium free or contaminated soils and identified *inter alia*; *Escherichia*, *Bacillus*, *Pseudomonas*, *Micrococcus* and *Staphylococcus* (Chirwa and Wang, 2000). This method is envisioned to be economically and ecologically friendly. In this study, Cr(VI) resistance bacteria were isolated from dried sludge of Cr contaminated site and used to facilitate the reduction of Cr(VI) to Cr(III) in a batch and continuous aquifer column system with varying treatments.

2. Materials and methods

2.1 Bacterial culture and growth medium

Dried sludge was obtained from Britz Wastewater Treatment Works (North West Province, South Africa), where it was exposed to periodic loading of Cr(VI) from a nearby chromium ore refining plant. The sampled sludge was stored in sterile plastics and kept in the fridge under 4 °C. Luria-Bertani (LB) broth was used to isolate bacterial cultures from the sludge by dissolving 0.5 g of sludge into 400 mL of broth. LB broth and agar were prepared by dissolving 25 g in 1 L and were autoclaved at 121 °C for 15 min. The broth containing sludge incubated at 37 °C for 24 h. Agar plates were inoculated with 1 mL samples from the broth containing growth culture and the colonies were sub-cultured using differential techniques (exhibited colours and morphologies) and incubated at 30 °C for 24 h. The process was done repeatedly in order to realize near pure culture of each identified colony. Each colonies were then tested for their Cr(VI) reducing capabilities on a Basal Mineral Medium (BMM) with an addition of 5 g/L of Glucose (Roestorff and Chirwa, 2018). For batch studies, the BBM used was inoculated with 50, 100, 200 and 400 mg/L, respectively. These were incubation at 37 °C under continuous shaking. Samples were collected using time intervals for a period of 24 h. Culture characterization was done using 16S rRNA. Identification of cultures was tabulated below:

Table 1: Culture identification of CRB from sludge using 16S rRNA

Pure culture	Species Identified	% Identity	Accession Number
X1	<i>Enterococcus casseliflavus</i>	100	CP032739
X2	<i>Pseudomonas putida</i>	99	CP016634
X3	<i>Citrobacter sedlakii</i>	99	KP861248
X4	<i>Enterobacter cloacae</i>	98	MG576153
X5	<i>Enterobacter hormaechei</i>	99	AJ853889

2.2 Algae cultivation and growth medium

Algae started growing from the sides of the columns after 30 d of 40 mg/L Cr(VI) concentration loading. To cultivate the algae a 3 by 1 Nitrogen, Bold Basal Media with Vitamins (3N-BBM+V) was prepared. To grow algal cells, 250 mL Erlenmeyer flasks were used which were kept under algal light; Osram L 36W/77 Flouora at 20-23 °C (Birungi and Chirwa, 2017) for 14 d with continuous shaking. To retrieve the cells, the samples were centrifuged at 6000 rpm for 10 min under 4 °C. The supernatant is repeatedly centrifuged for clear and free

cells. The algal cells were obtained by using BBM agar which was streaked with algal growth and incubated under light for 14 days. Characterization was done by using 18S rRNA.

2.3 Chemicals and reagents

Preparation of Cr(VI) stock solution was done by weighing 3.74 g of pure $K_2Cr_2O_4$ and dissolved in 1 L of distilled water. This standard solution was used throughout all experiments. Preparation of Diphenyl carbozide solution was done in an amber bottle by dissolving 0.5 g of 1,5 DPC in 100 mL of acetone (Merck, South Africa)

Sodium chloride (NaCl) solution (0.85 %) preparation was by dissolving 1.85 g of NaCl in 100 mL ultra-pure water and autoclaving at 121 °C for 15 min.

2.4 Analytical methods

Cr(VI) measurements were done using a UV-Vis spectrophotometer from Labotech, South Africa. During absorbance readings of Cr(VI), 540 nm wavelength was used. The method involved adding 0.1 mL sample into 10 mL volumetric flask, 1 ml of 1 N H_2SO_4 and taking it up to the 10 mL volume by using ultra-pure and finally 0.2 mL of 1.5 DPC to produce a purple colour. Cr(III) was identified as the reduced absorbance from initial reading.

2.5 Reactor setup and startup

Four columns in a form of Plexiglas (PVC glass) were used. These columns were 1 m long and filled with aquifer media from the target Cr(VI) contaminated site. Sample pots were bowed on each column; 18 cm distant from each other. A cover lid for the bottom of the column was used which had access for a pipe as effluent flow (25 L effluent tank). After the columns were packed with aquifer media, treatments were introduced and a control. System 1 had dried sludge containing cultures only, System 2 had dried sludge containing cultures and amended with saw dust and System 3 had dried sludge containing cultures with a vegetative carbon material retrieved by the target site. The four packed columns were capped on one end with shower caps that acted as an influent source. The columns were installed vertically on a steel gate by clapping. Each column was vertically connected to a 100 L reservoir in which the contaminant (Cr(VI)) was pumped into the system through the connecting tubes. Before start of the experiment, ultra-pure water was run through the system for saturation. Flow rates were measured and adjusted to establish the hydraulic retention time (HRT) of approximately 24 h in each reactor. The system was fed with 40 mg/L for 30 d followed by 60 mg/L for another 30 d.

3. Results and discussion

3.1 Bacterial culture composition

Samples were obtained from all columns for culture characterization. This was to identify bacterial communities present after the systems had been exposed to 40 mg/L and 60 mg/L

Table 2: Culture identification from column aquifer source exposed to Cr(VI) concentration

Pure culture	Species Identified	% Identity	Presence
Y1	<i>Exiguobacterium aestuarii</i>	100	System 1, 2 and 3
Y2	<i>Enterobacter aerogenes</i>	100	System 1 and 3
Y3	<i>Enterobacter kobei</i>	100	System 2 and 3
Y4	<i>Pseudomonas aeruginosa</i>	100	System 1 and 2

From preliminary characterization of cultures in Table 1, bacterial genera species such as *Enterobacter* and *Pseudomonas* formed part of the indigenous communities found in sludge that was previously exposed to Cr(VI) concentrations in the environment. Furthermore, the latter species survived 40 mg/L and 60 mg/L, facilitating Cr(VI) reduction to Cr(III). *Enterococcus* and *Citrobacter* species were not identified during Cr(VI) concentration loading to the columns, as shown on Table 2.

However, *Exiguobacterium aestuarii* reduced Cr(VI) concentration to Cr(III) in the presence of different carbon sources such as; saw dust and vegetative material. Bacterial cultures isolated from chromite polluted environments and known to be capable of reducing Cr(VI) have been previously reported by Mthimunya and Chirwa (2013) and identified as *Enterobacter* and *Pseudomonas* species.

3.2 Algae culture composition

The algae growth observed from the columns was isolated and identified as *Chlamydomonas debaryana*. This algae is able to grow in the presence of up to 60 mg/L Cr(VI) concentration loading in the column systems. The bacteria in the system could be providing the algae with CO₂ as a carbon source. Furthermore, the algae is also exposed to natural sunlight as it is able to thrive in the presence of high Cr(VI) toxicity. In turn, the algae could be used by bacteria as a growth medium. Cr is highly toxic to algae cells as it causes ruptured cell walls, leading to leaked lipids, proteins, carbohydrates, and primary metabolites such as sugars, sugar alcohols, amino acids, and organic acids (Cicci et al., 2017). The growth of *C. debaryana* on the system has facilitated Cr(VI) to Cr(III) shown in Figure 3 and 4.

3.3 Batch analysis

The analysis of identified cultures under different Cr(VI) concentrations operated for 24 h showed the ability of each culture to reduce Cr(VI) to Cr(III).

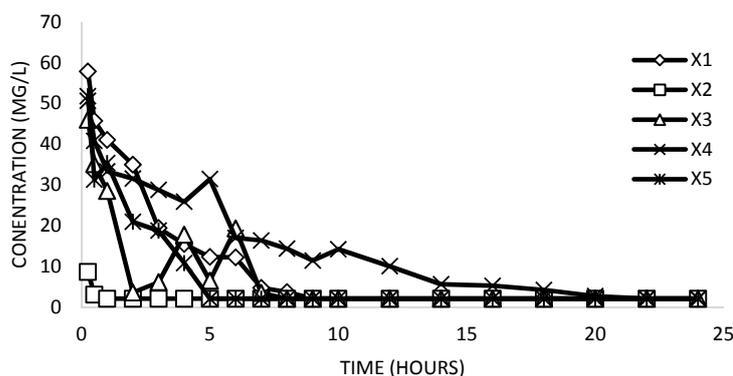


Figure 1: Batch analysis of identified Cr(VI) reducing bacteria at 50 mg/L of Cr(VI) concentration taken over a period of 24 h

Complete reduction by *Pseudomonas putida* and *Enterobacter hormaechei* cultures was observed just within 5 h of analysis as shown on figure 1. Complete Cr(VI) reduction has been previously recorded by Das et al. (2014) where the isolated Cr(VI) reducing bacteria (*Bacillus amyloliquefaciens*) showed total reduction of Cr(VI) to Cr(III) under 10,50 and 100 mg/L.

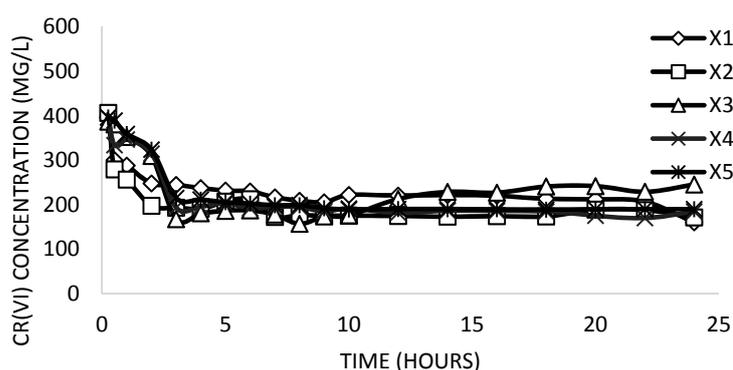


Figure 2: Batch analysis of identified Cr(VI) reducing bacteria at 400 mg/L of Cr(VI) concentration taken over a period of 24 h

Cr(VI) reducing bacteria have different resistant mechanisms to tolerate higher concentrations. Proposed Cr(VI) reduction mechanisms include; cytoplasmic enzymes (Cr(VI) reducing enzymes called reductase), membrane pathways (biochemical pathways) and biosorptive process (distribution of reactive functional groups) etc, (Zayed and Terry, 2013). Chromium reduction on Figure 2 show similar profiles amongst all bacterial cultures tested. More than 50 % Cr(VI) reduction was observed with no complete reduction. Between

0 – 5 h the bacterial resistant mechanisms are most active, as the tolerant levels decrease so does the reduction rate.

3.4 Column reactor analysis

The column systems were exposed to 40 mg/L Cr(VI) concentration for a period of 30 d. Samples were taken every 2 d along the column (using sample pots) to observe Cr(VI) reduction trends in each system. This was done at steady state. System 3 showed the most reduction with up to 94 % at 90 cm (effluent). The rate of Cr(VI) reduction was observed to increase as the contaminant was gravitating along the column, near complete Cr(VI) removal was achieved at the final effluent (Mthimunya, 2011). The reduction rate of Cr(VI) reduction at quasi-steady state became steady for System 3 from 36 to 90 cm distance along the column as seen on figure 3.

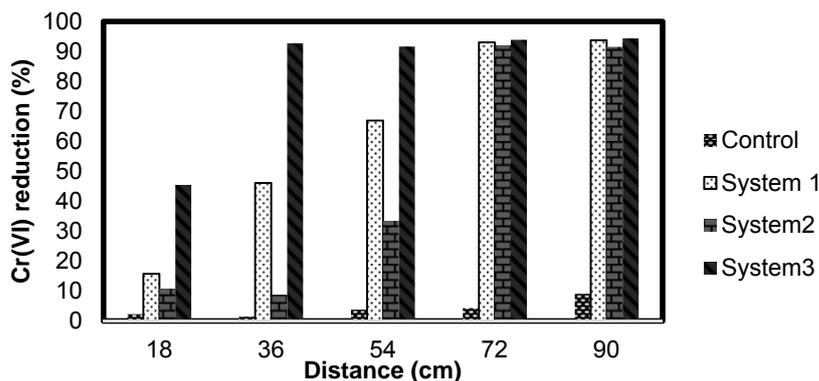


Figure 3: Cr(VI) 40 mg/L reduction percentage along the columns

The column systems were further exposed to 60 mg/L Cr(VI) concentration. Cr(VI) reduction rate did not increase as it would have been expected. Instead, some systems like System 2 showed increased reduction of Cr(VI) to Cr(III).

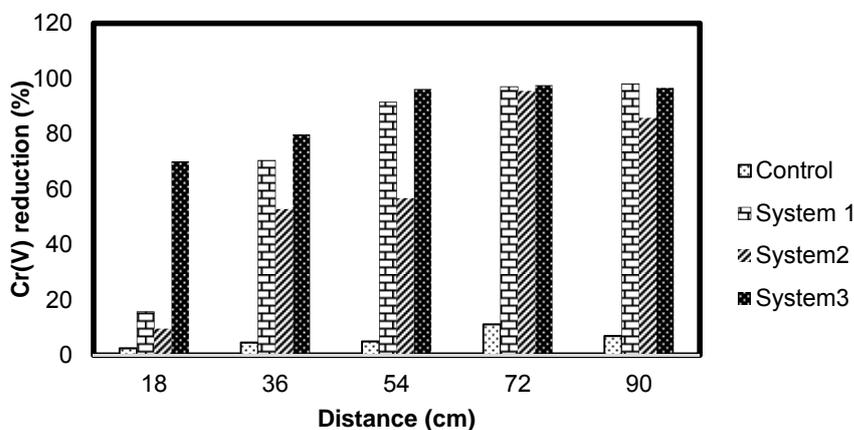


Figure 4: Cr(VI) 60 mg/L reduction percentage along the columns

System 1 and 2 showed to have about 50 % increase from 18 to 36 cm through the column due to the adaptation of Cr(VI) reducing bacteria. System 3 also out performed all the other systems along the column at 60 mg/L. The vegetative carbon source seemed to help bacteria reduce Cr(VI) concentration significantly, better than saw dust, which is usually used in order to avoid interferences and introduction of unnatural products into the system site (Molokwane and Chirwa, 2009).

4. Conclusion

Batch and column reactor analysis showed possible Cr(VI) reducing to Cr(III) by chromium resistant bacteria isolated near chromium ore refining plant. Almost complete Cr(VI) reduction was observed by bacteria even at a high concentration of 60 mg/L. The algae in the soil survived extremely harsh Cr(VI) conditions due to the presence of bacteria in the systems and the protection that is provided by the bacterial biofilm in the aquifer media. Cr(VI) reduction in system 3 showed to be the most effective as the carbon source was obtained from the area of assumed Cr resistance which provided favorable conditions for the bacteria to thrive in the *in situ* system. This study provides the basis upon which adequate bio-strategies can be developed and implemented for *in situ* treatment of Cr(VI) contaminated soils.

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