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In Situ Bioremediation in Mixed-Culture Inoculated Biological Permeable Reactive Barrier Systems

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Cr(VI) reducing bacteria was isolated from dried sludge collected from sand drying beds at a local wastewater treatment plant in Brits (South Africa). The plant received high periodic loadings of Cr(VI) contaminated effluent from an abandoned chrome processing foundry within the chrome mining town of Brits. The isolated bacteria were tolerant to high Cr(VI) loadings with significant chromium removal activity at loadings up to 80 $mg \cdot L^{-1}$ under continuous flow conditions. The active species in the sludge culture were determined to be predominated by *Bacillus thirungiensis*, *Bacillus cereus*, *Lycinobacillus sphaerococcus*. The isolated consortium culture was introduced as a biocatalyst in a microbial permeable bioreactive barrier for treatment of Cr(VI) contaminated water through soil medium. When tested in continuous flow bench-scale systems, the steady-state condition was attained after the reactor's operation for 25 days. Time course concentration profiles in batch tests fitted well with first- and second-order exponential rate equations yielding first-order rate constants in the range of 0.615 h^{-1} and 0.0532 $L \cdot mg^{-1} \cdot h^{-1}$ for Cr(VI) loadings ranging from 50 to 400 mg/L. The laboratory scale studies showed that the biological permeable reactive barrier technology using indigenous microbes has potential application for hexavalent chromium remediation in contaminated environments. Finally, the technology using bacteria in-situ shows that Cr(VI) can be remediated in the environment using a passive system at a low cost with minimum intervention.

1. Introduction

In the aquatic environment, chromium mainly exists as trivalent chromium, Cr(III), and hexavalent chromium, Cr(VI). Cr(VI) is highly soluble in water and can easily cross the membranes of cells (Yang et al., 2021). Cr(VI) is considered to be about a thousand times more toxic than Cr(III) (Bedemo et al., 2016). it is carcinogenic and mutagenic (Chen and Gu, 2005. The maximum contaminant level for Cr(VI) in domestic water supplies was set to be 0.01 mg/L (EPA, 2023).

On the other hand, the reduced form of chromium, Cr(III) is 1000 times less toxic than Cr(VI) and known to be an essential micronutrient required for- metabolism of carbohydrates in mammals. Cr(VI) is highly toxic due to its high oxidation potential, mobility, and permeability, while Cr(III) has a lower toxicity due to its low solubility and mobility in neutral and alkaline conditions (Ma et al., 2022). A low concentration of Cr(III) is an essential nutrient for human beings to better uptake protein, fat, and sugar (Ma et al., 2022).

Numerous techniques have been investigated for the removal of Cr(VI) from wastewater that include chemical precipitation (Lee et al., 2006), reverse osmosis (Yang et al., 2020), ion exchange (Chen and Gu, 2005) and lime coagulation (Yang et al., 2020). These methods have been reported to be costly, require high energy inputs or large quantities of reagents, and can create other forms of waste (Lee et al., 2022). Furthermore, they do not achieve complete heavy metal removal due to secondary pollution (Yang et al., 2020). The bioreduction of toxic Cr(VI) to less toxic Cr(III) using microbial organisms is an attractive alternative as it is a promising and cost-effective approach for Cr(VI) remediation (Kholisa and Chirwa, 2021).

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Permeable reactive barriers were previously studies using chemical reagents such as iron or specially isolated microbial communities with the capability of converting toxic compounds to environmentally compatible forms (Puls and Powell, 1997). Biological permeable reactive subsurface barriers (BPRBs) can be also be created by introducing competent organisms in the subsurface designed to intercept a contaminant plume (Molokwane et al., 2013).
In this study, Cr(VI) reducing microbial consortium collected from a local wastewater treatment plant from Brits (North West Province, South Africa) was used a the reactive barrier material in laboratory-scale PBRBs. A novel Mechanistic Diffusion-Reaction model representing fundamentals of dispersion and mass conservation was created to predict the performance of a BPRBs simulated in the laboratory using a controlled laboratory pilot reactor.

2. Materials and Methods

2.1 Culture and Medium

Luria Bertani (LB) Broth (Sigma-Aldrich) was used as a growth medium to grow fresh bacteria from the sludge. This medium was freshly prepared before every experiment to avoid contamination that would occur if a large batch was made and stored in the cool room. To prepare this medium, 25 g of LB Broth powder was added to 1000 mL of distilled water (Kholisa and Chirwa, 2021). LB Agar (Invitrogen) was used for colony development of the bacteria from the sludge. This was used to prepare agar plates with bacteria to be sent to the Microbiology Department at the University of Pretoria for analysis. To prepare this medium, 40 g of LB Agar powder was added to 1000 mL of distilled water (Kholisa and Chirwa, 2021). The mineral salt medium (MSM) was meant to be used for the experiments after the bacteria had been freshly grown using LB Broth. This was freshly prepared before every experiment to avoid contamination. The purpose of the MSM was to provide enough minerals to keep the bacteria alive, but only enough to maintain their current state. In other words, MSM does not promote the growth of bacteria. According to Kholisa and Chirwa (2021), 2.12 g of K_2HPO_4 (GlassWorld, Johannesburg, South Africa), 2.12 g of KH2PO4 (GlassWorld), 2 g of NaCl (GlassWorld), 1 g of MgSO4.7H2O, 0.1 g of CaCl2 (Fluka), 4 g of KNO_3 (GlassWorld) and 5 g of glucose (GlassWorld) as a carbon source needed to be added to 1000 mL of distilled water to prepare this medium.

2.2 Reactor Configuration and Operation

The Cr(VI) remediation experiments were conducted in two horizontal flow tanks with the dimensions 820 x 170 x 200 mm (L x B x H) were constructed using 5 mm thick transparent Perspex sheets (Evonik Rohm GmbH, Essen, Germany) as shown in Figure 1, with duplicate samples collected from each sample port for analysis.

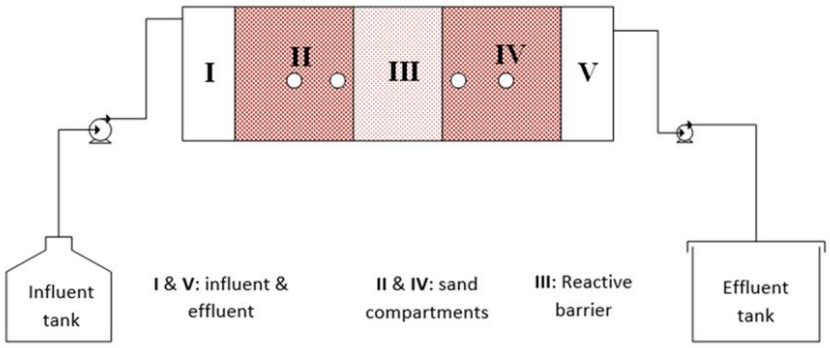

Figure 1: Horizontal flow reactor system configuration for testing the microbial barrier performance.

Each reactor consisted of five compartments, i.e., influent and effluent reservoirs I and V (100 mm × 170 mm × 200 mm) each, non-reactive soil zones II and IV (230 mm × 170 mm × 200 mm) each, and the microbial inoculated barrier zone III (150 mm × 170 mm × 200 mm). The non-reactive compartments were filled with acid treated soil to serve as controls. The middle compartment was filled with a mixture of dried sludge and treated soil with a mass ratio of 70% to 30%. The reactors were operated as plug-flow systems with four sampling ports along the length. The first sampling point was placed 115 mm from the influent reservoir, the second and third were 10 mm before and after the microbial barrier and the fourth was placed 495 mm from the influent reservoir. The flow was delivered using a Watson-Marlow 120U peristaltic pump.

2.3 Cr(VI)/Cr(III) Concentration Measurement

1 mL samples were collected overtime and centrifuged using 2 mL Eppendorf centrifuge tubes at 6000 rpm for 15 min in a Minispin® Microcentrifuge (Eppendorf, Hamburg, Germany) to separate the suspended cells from the solution. The cell-free supernatant was then extracted using a pipette without re-suspending the cells and was used for Cr(VI) analysis. Then 0.2 mL of the extracted supernatant was added to a 10 mL volumetric flask followed by the addition of 1 mL (1N) H_2SO_4 solution for digestion of the sample. The flask was filled with distilled water to the 10 mL mark. 0.2 mL of 1,5-DPC solution was then added to yield a purple colour. UV/Vis Spectrophotometer (WPA Lightwave II) from Labotech, South Africa was used for measuring the absorbance of the mixture at 540 nm wavelength, across a 10 mm light path. The intensity of the purple colour was proportional to the Cr(VI) concentration in the sample (APHA/AWWA/WEF, 2023). Varian AA - 1275 Series Atomic Adsorption Spectrophotometer (AAS) (Varian, Palo Alto, CA (USA)) equipped with a 3 mA chromium hollow cathode lamp was used to measure the total Cr at a wavelength of 359.9 nm. The AAS was calibrated before total Cr analysis using 1-5 $mg{\cdot}L^{-1}$ Cr(VI) concentration prepared from the Cr(VI) stock solution. Cr(III) was determined as the difference between total Cr and Cr(VI) concentration.

3. Results and Discussion

3.1 Microbial Culture Characterisation

The sludge C bacteria consortium was chosen for characterization due to its high performance. The bacterial isolates were identified based on 16S rDNA gene sequencing analyses and were carried out by the Microbiology Department, at the University of Pretoria to identify bacterial communities present after the sludge had been exposed to 100 mg/L Cr(VI). BLASTN analysis of the bacterial isolates X1, X2, X3, X4, X5, X6 and X7 are presented in Table 1 and shows four predominant species under aerobic conditions. The sequence for X1 was 99% similar to that of *Bacillus cereus* 213 16S and *Bacillus thuringiensis* strains. X2 and X3 isolates produced similar results and showed close association with *B. cereus* ATCC 10987, *Bacillus sp*. ZZ2 16s, *B. thuringiensis* str. *Al Hakam* having a 99% identity. While X4, X5 and X6 were in close association with *B. mycoides* strain BGSC 6A13 16S, *B. thuringiensis serovar finitimus* strain BGSC 4B2 16S strains. The sequence for X7 was 99% similar to that of *Microbacterium sp.* S15-M4 and *Microbacterium foliorum*. A phylogenetic tree was constructed for the species from purified cultures grown under aerobic conditions based on a basic BLAST search of rRNA sequences in the NCBI database (Figure 2).

Table 1: Sludge Cr(VI)-Reducing Bacteria strain characterisation using 16S rRNA

Blast Results	Pure Isolates							ID Index
	X1	X2	X3	X4	X5	X6	X7	
B. cereus ATCC 10987		√	√					0.99
B. thuringiensis serovar finitimus strain BGSC 4B2 16S				√	√	√		0.99
B. thuringiensis str. Al Hakam		√	√					0.99
Bacillus cereus strain 213 16S	√							0.99
Bacillus mycoides strain BGSC 6A13 16S				√	√	√		0.99
Bacillus sp. ZZ2 16S		√	√					0.99
Bacillus thuringiensis 16S	√							0.99
Microbacterium foliorum							√	0.99
Microbacterium sp. S15-M4							√	0.99

3.2 Reactor Performance

Two reactors were operated at hydraulic loading of 200 $mL{\cdot}h^{-1}$ and were fed with distilled water for 14 days to saturate the reactors, remove air space between the pores and acclimatize the bacteria. The data is presented in Figure 3. The control reactor compartments were packed with treated soil in order to study the abiotic effect on the Cr(VI) removal and was fed with a Cr(VI) concentration of 40 $mg{\cdot}L^{-1}$ as shown in Figure 3(a). It can be seen that effluent Cr(VI) concentration in the control reactor gradually increased until day 6. After day 6, the reactor reached a steady state as the influent Cr(VI) concentration was the same as effluent. This run took into consideration the effects of sorption regardless of the solution chemistry (Tadeo-jalife et al., 2021).

In the BPRB reactor, compartments 2 and 4 were packed with clean soil while compartment 3 was packed with 70% and 30% soil-sludge mixture. After the saturation phase which lasted 14 days, the BPRB reactor was fed with distilled water containing Cr(VI) concentration of 40 $mg{\cdot}L^{-1}$ and the results are shown in Figure 3(b). The 20 cm barrier had a hydraulic retention time of 8 h. After feeding the reactor with Cr(VI) for 30 days, no Cr(VI)

was detected in the effluent for this period. This indicated that the Cr(VI) removal was 100% in the BPRB. Cr(VI) concentration was then increased by 20 mg/L and continued to operate at 60 mg/L Cr(VI) concentration for another 30 days. Cr(VI) concentration was not detected in the effluent, showing 100% efficiency. On day 47, 4.11 mg·L^{-1} of Cr(VI) concentration was first detected and the following day (48) Cr(VI) was 100% removed. From day 52, a Cr(VI) concentration of 2.2 mg·L^{-1} was noticed and a sharp increase in Cr(VI) concentration from 2.7 mg·L^{-1} to 20.9 mg·L^{-1} in the effluent from day 54 to day 57.

Cr(VI) concentration in the effluent continued to increase up to 23.1 mg·L^{-1} in day 61 which is equivalent to 38% Cr(VI) removal. This increase in Cr(VI) concentration in the effluent was attributed to the depletion of the carbon source from the sludge. Microorganisms utilize a variety of organic carbon sources, either as an energy source or as an electron donor to facilitate Cr(VI) bioreduction (Han et al., 2021). Han et al. (2021) further explained that organic carbon sources play an important role in enhancing Cr(VI) bioreduction by the stimulation of microorganisms for providing more electron donors.

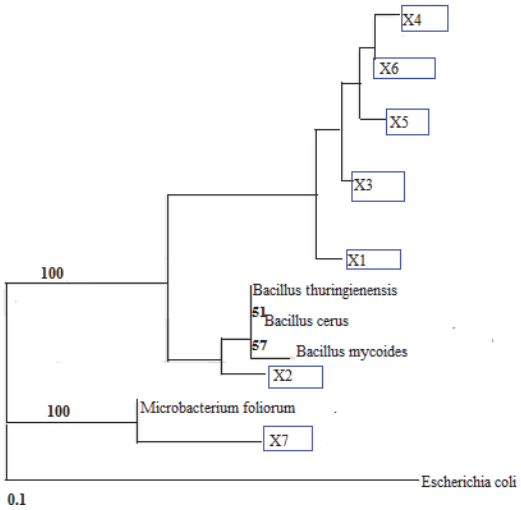

Figure 2: Phylogenetic tree constructed by neighbour-joining algorithm based on the partial 16S rRNA gene sequences and 1000 bootstrap replicates, showing the microbial diversity of Cr(VI) reducing consortium from Sludge C under aerobic conditions

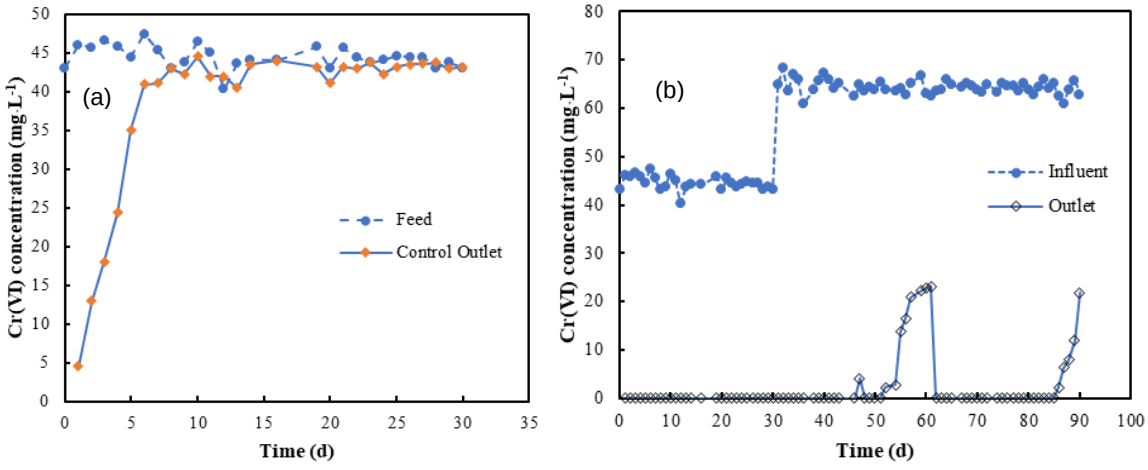

Figure 3: Phylogenetic tree constructed by neighbour-joining algorithm based on the partial 16S rRNA gene sequences and 1000 bootstrap replicates, showing the microbial diversity of Cr(VI) reducing consortium from Sludge C under aerobic conditions. The values reported were averages from (n = 2) each sample port and each sampling event. The standard error <0.1% for the spectrometric method was too small to appear on the plots.

3.3 Mass Transport Advection-Reaction Model

The mass transport of all dissolved species across the boundary layer (L_w) into a soil particle is due to the random thermal motion of molecules at temperatures above absolute zero. Mass transfer within the attached cell layer follows *Fick's law* of diffusion. The mass transfer is a function of external mass transfer resistance (k_L) across the heterogeneous-film interface area into the bulk liquid as described by Equation 1.

$$-V\frac{dC}{dt} = k_L A_f \frac{dC}{dx} = \frac{k_L}{L_w} A_f \left(C_b - C_s\right) = -J_c A_f \tag{1}$$

where: k_L is the dispersion coefficient of Cr(VI) in water (L^2T^{-1}); L_w is the heterogeneous film thickness (L); A_f is the biofilm surface area (L^2); C_b is the bulk liquid Cr(VI) concentration at time, t ((ML^{-3}); C_s is the liquid-biofilm interface Cr(VI) concentration (ML^{-3}). The full derivation of the model is based on the PhD thesis of Molowane (2010). The Advection-Reaction model combined with non-competitive inhibited Cr(VI) reduction rate kinetics fitted well to the experimental data as shown in Figure 4. The model was fitted using the numerical software AQUASIM 2.3 (EAWAG Dübbendorf, Switzerland).

The advection term is linked to the non-competitive reaction rate kinetic term derived earlier by Molokwane (2010). In the reaction term, the rate of change is linked to Michaelis-Menten kinetic model with a non-competitive inhibition on the maximum Cr(VI) reduction rate kinetic (k_m) and cell deactivation at high Cr(VI) concentration above the Cr(VI) reduction capacity of cells (R_c). The Cr(VI) reduction rate term is therefore introduced as shown in Equation 2:

$$\frac{dC}{dt} = -\frac{k_m \cdot C}{K^{1-Cr/C_0} \cdot \left(K_c + C\right)}\left(X_0 - \frac{C_0 - C}{R_c}\right) = -r_c \tag{2}$$

where: k_m = maximum Cr(VI) reduction rate coefficient, d^{-1}, X_0 = initial viable cell concentration, $mg \cdot L^{-1}$, R_c = Cr(VI) reducing capacity of cells, $mg \cdot mg^{-1}$, K = non-competitive Cr(VI) reduction coefficient (dimensionless), and K_c = half-velocity coefficient, $mg \cdot L^{-1}$.

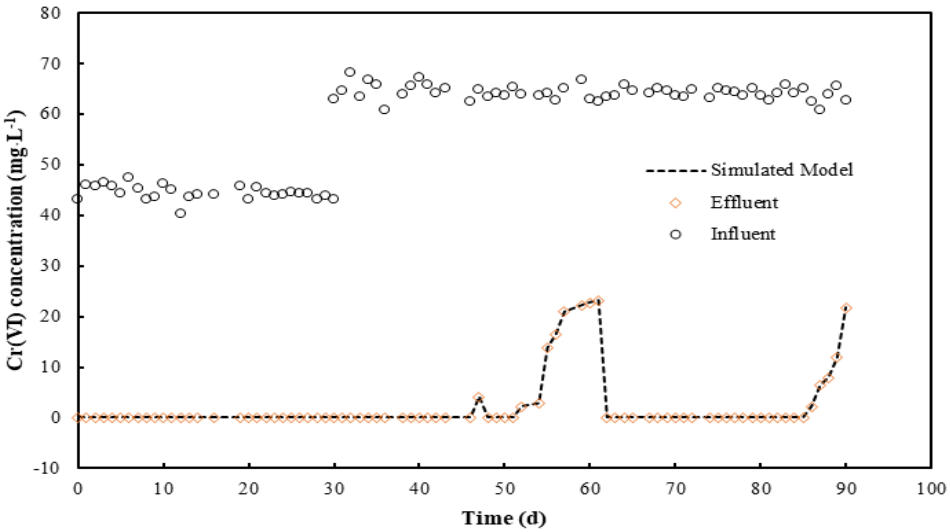

Figure 4: Simulation and optimization of influent and effluent Cr(VI) in the biological barrier

The developed model predicted the Cr(VI) effluent well under various Cr(VI) influent concentration loadings (40–60 $mg \cdot L^{-1}$) with 99.6% confidence. Despite the fact, that the effluent Cr(VI) concentration trend in the reactor was successfully traced by the model, adjustments would be needed to take into account the loss of working volume and decreasing flow rate due to the growth of biomass in the reactor. The model modification may result in a proper application in engineered biological systems for the treatment of groundwater with higher Cr(VI) concentrations and multiple toxic contaminants.

4. Conclusions

The study demonstrated that continuous self-replenishing Cr(VI) reduction can be achieved to prevent contamination from flow groundwater in pristine surrounding aquatic ecosystems. A consortium culture containing mainly *Bacilli*, i.e., *B. thirungiensis*, *B. Cereus*, and *L. sphaerococcus* was optimised in the laboratory and used to inoculate a Cr(VI) reducing permeable reactive barrier. The tested microbial barrier reduced up 95.9% of 80 $mg \cdot L^{-1}$ Cr(VI) concentration over a 1 day HRT for 90 day operational period. The results suggest that the biological permeable reactive barrier technology using indigenous bacterial strains has potential application for Cr(VI) remediation in contaminated environments. The study demonstrated the potential of using this technology to passively treated contaminated groundwater and avoiding high operation costs as is typically with pump-and-treat schemes. In an extended study from the current study, The effect of trying to enhance the performance by using a degradable carbon source (glucose) only resulted in increased acidic conditions from pH 6.91 to below 5.5. The findings show that Cr(VI) remediation can be achieved with natural organic materials (NOMs) found in soil and sludge without the need of adding an expensive purified carbon source.

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