# Effective bioreduction of hexavalent chromium–contaminated water in fixed-film bioreactors

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## ABSTRACT

Hexavalent chromium ($Cr^{6+}$) contamination from a dolomite stone mine in Limpopo Province, South Africa, has resulted in extensive groundwater contamination. In order to circumvent any further negative environmental impact at this site, an effective and sustainable treatment strategy for the removal of up to 6.49 mg/ℓ $Cr^{6+}$ from the groundwater was developed. Laboratory-scale, continuous up-flow bioreactors were constructed to evaluate reduction of $Cr^{6+}$, with a residence time of 24 h, an efficiency porosity of 44% and a flow rate of 1.5 mℓ/min. Stoichiometrically balancing terminal electron acceptors in the feed water with a selected electron donor, directed reactor balance for complete $Cr^{6+}$ reduction. The microbial community shifted in relative dominance during operation to establish an optimal metal-reducing community, including *Enterobacter cloacae*, *Flavobacterium* sp. and *Ralstonia* sp., which achieved 100% reduction. Evaluation after reactor termination with SEM-EDX and XRD confirmed the establishment of biofilm on the reactor matrix, as well as trivalent chromium ($Cr^{3+}$) precipitation within the reactor. Due to gravitational force, high concentrations of $Cr^{3+}$ were found in the bottom third of the reactor. Based on the results from the laboratory investigation, a 24 000 ℓ fixed-film pilot bioreactor was designed and constructed at this site. Influent flow rates, electron donor injection and automated sampling were remotely controlled by a programmable logic controller (PLC). Similar to the laboratory column study, steady state conditions could be achieved and successful $Cr^{6+}$ reduction was evident. This is the first up-scaled, effective demonstration of a biological chromium(VI) bioremediation system in South Africa.

**Keywords:** Bioreduction, fixed-film reactor, hexavalent chromium, microbial diversity

## INTRODUCTION

The study source site is located in the Limpopo Province of South Africa at a facility that actively mines and processes marble and dolomite stone as a graded aggregate for landscaping and construction purposes. Unrelated historic land-use activities resulted in ground and surface water contamination with hexavalent chromium ($Cr^{6+}$). Despite efforts to prevent migration of site wastes to the surrounding environment, leaching of $Cr^{6+}$ into the groundwater has occurred over the 80-year history of the site. Over a 5-year sampling period, measured $Cr^{6+}$ concentrations in site groundwater ranged between 2.30 and 6.49 mg/ℓ.

Hexavalent chromium is classified as a SABS 0228 danger group 8(II) compound leading to birth defects and reproductive impairment (Arlauskas et al. 1985; DWAF, 1998; Kanojia et al., 1998). It can cross the cellular membrane via sulphate transporters and, once inside the cell, can generate reactive oxygen species (ROS), implicated as mutagens and carcinogens, from the cyclic single electron transfer between $Cr^{6+}$, molecular oxygen and $Cr^{5+}$ (Ackerley et al., 2004). Trivalent chromium, on the other hand, is considered to be relatively innocuous and usually occurs as insoluble organic and inorganic complexes due to its strong Lewis acid nature (Barceloux, 1999).

The preferred technology for treatment of $Cr^{6+}$ in South Africa is chemical reduction with ferrous sulphate or sodium sulphite, followed by co-disposal and landfilling of residues (DWAF, 1998). However, chemical treatment of $Cr^{6+}$ requires high concentrations of reagents, generates environmentally hazardous by-products and is generally more effective at higher concentrations (Szpyrkowicz et al., 2001). The potential for application of bioreduction of $Cr^{6+}$ as an alternative remediation strategy has been highlighted in several publications; however, this is the first attempt at a systematic study aimed at an industrial-scale bioreactor with larger volumes (Thacker et al., 2006; Amoosegar et al., 2007).

Biological reduction of $Cr^{6+}$ with indigenous bacteria in semi-passive systems can be an environmentally sound alternative or complementary technology to active chemical treatment technologies, but at comparatively lower lifecycle costs with less specialised labour requirements. Bacterial reduction of $Cr^{6+}$ to its less toxic form is a complex phenomenon and can proceed under both aerobic and anaerobic conditions utilising membrane or cytoplasmic proteins that may or may not require the presence of co-factors (Opperman and Van Heerden, 2007). Dissimilatory chromate-reducing bacteria utilise $Cr^{6+}$ as terminal electron acceptor, ultimately precipitating it as insoluble chromium hydroxides, demonstrating the potential for remediation (Desai et al., 2008).

A fixed-film bioreactor is a treatment process which employs a porous media with an attached biofilm through which contaminated water is passed and treated. The main functions of the media are to provide attachment sites for the biofilm, a hydraulic network for conveying flow through the system as well as minerals for biofilm development. The biofilm helps to create a physical environment within the bioreactor

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which encourages chemical transformation of certain soluble pollutants. Insoluble particulates are readily adsorbed to the biofilm surface and sequestered in the biomass. Similarly, Orandi et al. (2012) demonstrated the removal of a number of pollutants from acid mine drainage contaminated water using algal-microbial biofilm.

The environmental condition within the bioreactor that is of most interest for metals treatment in natural waters is the redox potential (ORP/Eh). For $Cr^{6+}$ treatment, the microbes in the reactor are used to drive the environment to a reducing state while maintaining a relatively neutral pH. At the desired redox potential (≈ –200 mV) and with the preferred consortium dominating the biofilm, desirable physical and chemical reactions can occur. In the present study, this is achieved by introducing the inoculum to the media matrix, carefully regulating their metabolic activity by controlling the available carbon source and stoichiometric balancing with the electron acceptors as well as controlling the hydraulic retention time.

## MATERIALS AND METHODS

### Source water and electron donor

Source water was collected in batches from the site, transported to the laboratory and stored at 4°C in 25 ℓ carboys. Each batch was analysed for key chemical constituents at the Institute for Groundwater Studies, University of the Free State. Food-grade citric acid was used as an electron donor that is readily available. The citric acid was analysed for the presence of impurities or inhibitory substances by HPLC on an Aminex 87 H column at 60°C using 0.01 N $H_2SO_4$ at 0.6 mℓ/min as mobile phase (Schneider et al., 1987).

### Up-flow column set-up and operation

The bioreactor was constructed from 110 mm polyvinyl chloride (PVC) pipe with threaded end caps, taps and appropriate silicon tubing. The bioreactor was packed with dolomite stone to infer uniformly-graded and packed material, with selected hydraulic properties. The media bed was hydrated with water and the reactor drainage volume determined. The pore volume (PV), static- (Eq. (1)) and efficiency porosity (Eq. (2)) was determined by performing a tracer test with 5 mM NaCl.

$$\text{Static porosity} = \text{drainage volume/empty bed volume} \quad (1)$$

$$\text{Efficiency porosity} = \text{tracer peak volume/empty bed volume} \quad (2)$$

The bioreactor was seeded with a 5% (v/v) solution of biome collected from a groundwater seep at the site that contained a metabolically diverse consortium of bacteria able to reduce a range of substances, including $NO_3^-$, $Fe^{3+}$, $Cr^{6+}$ and $SO_4^{2-}$. Influent water, containing 500 mg/ℓ citric acid adjusted to pH 6 with 1 M NaOH, was introduced to the base of the bioreactor using a Watson Marlow Bredel S520 peristaltic pump at a 24-h hydraulic retention time (HRT). Effluent water was characterised during the operation of the bioreactor, while the redox potential (ORP/Eh), pH, electrical conductivity (EC), temperature (HI 9828 pH/ORP/EC/DO probe, Hanna Instruments Inc, Woonsocket, RI, USA), bacterial cell counts (Porter and Feig, 1980) and $Cr^{6+}$ concentrations (Urone, 1955) were monitored. Once reducing conditions were established within the bioreactor, the electron donor was incrementally lowered until the minimum donor requirement was determined empirically.

### Bioreactor termination

After continuous operation for 6 months (representing approximately 775 ℓ of contaminated water having flowed through the reactor), the bioreactor was drained in timed 500 mℓ increments and each drained fraction recorded to correspond with a specific section of the column. The bioreactor was cut open lengthwise and matrix samples collected for microscopic examination, including scanning electron microscopy (SEM), X-ray fluorescence- and energy dispersive X-ray spectroscopy analyses.

### Microbial diversity

Microbial diversity shifts were studied during the bioreactor operation with daily sample (1 mℓ) collection. Samples were analysed corresponding to selected redox potential and electron donor concentration changes during the operation of the column bioreactor. The centrifuged cell pellets from each sample were re-suspended in 50 µℓ $ddH_2O$ and used as a template for amplification of a ≈200 bp fragment of the 16S rDNA with universal bacterial primers 341F-GC (Muyzer et al., 1993) and 517R (Reysenbach et al., 1992), to analyse shifts in the microbial community. Amplification products were separated on an 8% (w/v) polyacrylamide gel with a urea/formamide denaturing gradient ranging from 30% to 60%. Electrophoresis was performed in 1X TAE buffer, pH 8.0 at a constant voltage of 200 V at 60°C for 3 h. Gels were stained with SYBR Gold (0.05%) (Life Technologies, Carlsbad, CA, USA) and viewed with a UV transilluminator (Bio-Rad Laboratories, Hercules, CA, USA).

The terminated bioreactor community was analysed by filtering the water samples corresponding to the different fractions through 0.22 µm polyethersulfone (PES) membrane filters. Genomic DNA was extracted using the NucleoSpin® soil kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. Partial 16S rDNA fragments (≈600 bp) were amplified from the gDNA extracts using universal bacterial primers 341F-GC and 907RM (Muyzer et al., 1993), resulting in larger DNA fragments for identification of the bacteria present in the microbial community. The amplification products were separated on a 7% (w/v) polyacrylamide gel as described above. Predominant bands were excised and re-amplified using the primers 341F and 907RM (Muyzer et al., 1993). The amplification products were purified using the Biospin Gel Extraction kit (Bio Flux Corporation, Tokyo, Japan). Sequencing of the rDNA fragments was performed using the ABI BigDye Terminator v3.1 Ready Reaction Sequencing kit (Life Technologies, Carlsbad, CA, USA). The sequences were submitted to the National Center for Biotechnology Information (NCBI).

### Construction and operation of a pilot fixed-film bioreactor

Following the laboratory up-flow column bioreactor study, a pilot-scale treatment system was designed, constructed, operated and monitored to evaluate this technology for inclusion in the overall site remediation programme. The proposed treatment approach is similar to an in-situ bioremedial system developed by Geosyntec Consultants, USA. A concrete containment structure of 15.2 m × 4.28 m × 0.85 m was constructed at a $Cr^{6+}$ contaminated site. The containment structure was lined with a heavy-duty polyvinyl liner installed between two layers of geotextile material. Thereafter, the containment structure was filled with dolomite stone resulting in a media porosity of 44%. Sampling ports (5) were imbedded in the reactor matrix media


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**TABLE 1**
**Calculated stoichiometric electron donor demand**

| Electron acceptor | Balanced redox reaction | Concentration (mg/ℓ) | Donor demand (mg/ℓ) |
|---|---|---|---|
| Oxygen | $2C_6H_8O_7 + 9O_2 \rightarrow 8H_2O + 12CO_2$ | 8.00 | 10.67 |
| Nitrate | $5C_6H_8O_7 + 18NO_3^- + 18H^+ \rightarrow 29H_2O + 30CO_2 + 9N_2$ | 60.75 ± 7.80 | 52.29 ± 6.72 |
| Chromate | $C_6H_8O_7 + 6CrO_3 + 5H_2O \rightarrow 6Cr(OH)_3 + 6CO_2$ | 5.97 ± 0.52 | 7.65 ± 0.67 |
| Sulphur | $4C_6H_8O_7 + 9SO_4^- + 18H^+ \rightarrow 16H_2O + 24CO_2 + 9H_2S$ | 15.00 | 13.33 |
| Total | | | 83.94 ± 7.39 |

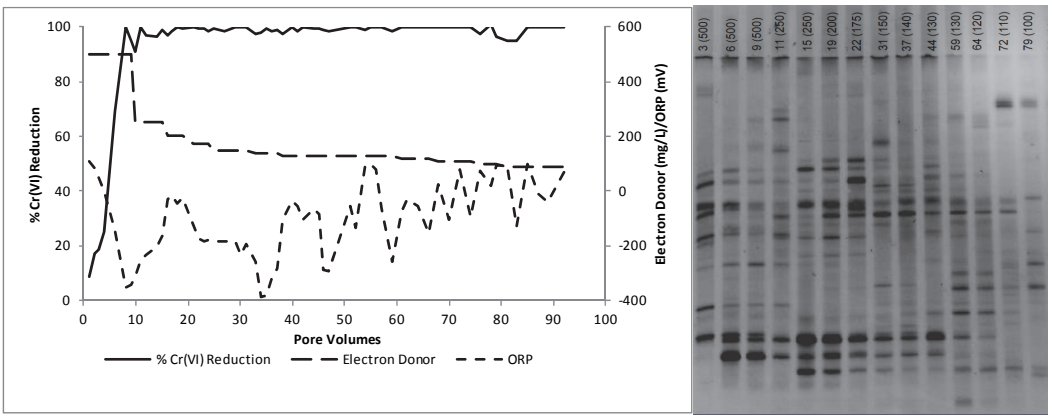


***Figure 1***
*A: Start-up and operation of the $Cr^{6+}$ reducing bioreactor; B: Denaturing gradient gel electrophoresis (DGGE) of selected pore volumes during the electron donor weaning period. (Pore volumes are indicated numerically, while electron donor concentrations (in mg/ℓ) are bracketed).*

for manual as well as automated sampling and parameter monitoring. A PVC influent manifold system was installed (a third of the reactor height) at the front end of the reactor to distribute the influent water uniformly across the vertical cross-section of the reactor matrix. A PVC discharge manifold was installed in the back-end of the reactor at half the height of the reactor wall, running across the width of the reactor. The effluent was collected into a sump that leads to an aeration cascade.

As in the case of the laboratory column bioreactors, citric acid was used as the electron donor and a stock solution was prepared in a 500 ℓ holding tank from where it was injected into the influent line via a dosing pump (Grundfos DDI 209, Grundfos Holdings A/S, Bjerringbro, Denmark) controlled by a programmable logic controller (PLC); (Siemens AG, Munich, Germany).

The reactor source water was relayed from an elevated cement distribution dam through gravitational feed providing 167 kPa of static pressure, which is sufficient for a constant source water flow rate to the reactor. An electronic flow control valve (Type 3003, Bürkert Fluid Control Systems, Ingelfingen, Germany) with a flow rate meter (Type 8032, Bürkert Fluid Control Systems) was installed in the influent line to regulate the influent water flow into the reactor.

The bioreactor was monitored by an automated PLC system consisting of a sampling pump (TeknaEvo 500, Seko Spa, Rieti, Italy) and valved manifold feeding into a redox potential sensor (Orbisint CPS12, Endress + Hauser, Reinach, Switzerland) connected to a controller box (Liquiline CM442, Endress + Hauser). Data on reactor water level and influent feed rate was collected continuously and redox potential measurements taken at 6-h intervals. Data was logged into a CSV file, backed up on the PLC itself and sent to the off-site operator via an internet connection.

## RESULTS AND DISCUSSION

### Laboratory-scale bioreactor

A bioreactor was packed with dolomite stone collected from the site to achieve a uniformly packed matrix of 43% and 44% static- and efficiency-porosity, respectively, as inferred from symmetrical conservative tracer curves and peak electrical conductivity measurements. High pressure liquid chromatography analysis of the citric acid purchased from the local vendor indicated that the compound was highly similar to analytical grade citric acid with regards to chromatographic retention time, purity and concentration. Chemical analysis of the source water demonstrated that during the course of a 2-year sampling period, the water composition remained fairly uniform. The major terminal electron acceptors in addition to chromate were nitrate (60.75 ± 7.80 mg/ℓ) and sulphate (402.40 ± 33.03 mg/ℓ), with low concentrations of other metals such as iron and manganese. By stoichiometrically balancing terminal electron acceptors present in the feed water with the potential electron donor, the theoretical amount of citric acid required for complete chromate bioreduction could be calculated (Table 1).

The bioreactor achieved ≈100% chromate reduction after 7 pore volumes (PV); (expressed as a percentage of chromate in the influent feed solution) with redox potential readings indicating anoxic conditions within the bioreactor (Fig. 1). As the amount of electron donor in the influent water was gradually lowered, chromate reduction was maintained at >95% (Fig. 1). The microbial community profiling by denaturing gradient gel electrophoresis (DGGE) during the period of electron donor weaning shows evidence of the biological activity in the bioreactor, as a highly adaptive bacterial community with shifts in

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dominance occurs (Fig. 1).

The bacterial community at PV 3 was diverse, showing various dominant bacterial species, as indicated by the band intensity; however, during this time there was <20% chromate-reducing activity in the bioreactor, indicating that the community was able to tolerate the chromate, but was not efficient at reducing it to trivalent chromium. A shift in the bacterial community occurred from PV 6, coinciding with an increase in reduction potential and increase in chromate reduction (≈70%), thereby providing crucial evidence of biological chromate reduction as all parameters were kept constant during this period. Adaptations in the microbial community composition continued as the electron donor concentration was gradually decreased, which also corresponded with the fluctuations in the redox potential. An example of this is PVs 11 (–225 mV) and 15 (–165 mV/), both corresponding to an electron donor concentration of 250 mg/ℓ and ≈100% chromate reduction (Fig. 1). Pore volume 11 represents a snapshot of the microbial community shortly after lowering the electron donor concentration, while PV 15 represents the bacterial community adapted to this electron donor concentration. A similar adaptive trend can be seen with PVs 44 (–66 mV) and 59 (–256 mV) (Fig. 1).

The minimum concentration of electron donor (90 mg/ℓ) at these operational conditions (24 h and hydraulic retention time; 5.97 ± 0.52 mg/ℓ [$Cr^{6+}$]) corresponded well with the calculated electron donor demand of 83.94 ± 7.39 (Table 1). The small difference between the empirical- and calculated electron donor demand values can be attributed to the complex nature of the system, with many factors such as metabolic flux and contributions from other metals (iron and manganese) that were not taken into account during electron donor requirement calculations. In terms of the microbial community, operating the bioreactor at the lower limit of electron donor feed represents a stressed environment, which is reflected by the adaptations and changes in the bacterial community profile PVs 64, 72 and 79 (Fig. 1). The initial intention was the removal of the $Cr^{6+}$ from the water; however, after treatment it was evident that the water complied with Class 2 drinking water (SANS, 2011). This is due to the reduction potential favouring specific metabolic activities; the reductant (citric acid) transfers electrons to the lowest unoccupied electron level, before transferring electrons to higher unoccupied electron levels, such as nitrate (Stumm and Morgan, 1996). If this balance favours excess electrons added to the system as a result of the stoichiometric balancing to include a portion of the available sulphate, electrons are transferred to all lower unoccupied electron levels, which include oxygen, nitrate, manganese, iron and chromate, resulting in their subsequent reduction. Thus, as an additional benefit, potential contaminants such as nitrates and sulphates are also removed from the water.

After bioreactor termination, a green precipitate was clearly visible in the bottom third of the bioreactor, which corresponds to increased levels of chromium as determined by XRF and EDX analyses (Fig. 2 and 3). Since this bioreactor was operated in upflow mode with the electron donor arrival at the bottom, the maximal nutrients would be at an inlet location near the bottom of the reactor. As a result, maximal activity in a form of a green precipitate was observed with the light microscopy.

During the operation of the bioreactor, there were no appreciable changes in the matrix porosity, indicating that operation of the bioreactor under these conditions did not present a significant clogging potential. Light microscopy of the matrix material showed discrete patches of biofilm with little potential for pore clogging, as would be the case if continuous slime films were present. Biofilm formation on the matrix surface was also clearly demonstrated by SEM imaging (Fig. 3), as shown by Marsili et al. (2007).

Microbial diversity analysis of the established stable community within the different fractions of the bioreactor showed a number of uncultured bacteria, which indicates the novelty in the biodiversity present in the water fractions from the chromium-reducing bioreactor (Table 2). In addition, various sequences were related to bacteria able to reduce a variety of elements and compounds, including (i) nitrate, such as *Chloroflexi* sp. (Kawaichi et al., 2013), *Flavobacterium* sp. (Park et al., 2006), *Acidobacteria* sp. (Ward et al., 2009), *Ralstonia* sp. (Coenye et al., 1999; Chen et al., 2001) and *Enterobacter cloacae* (Losi and

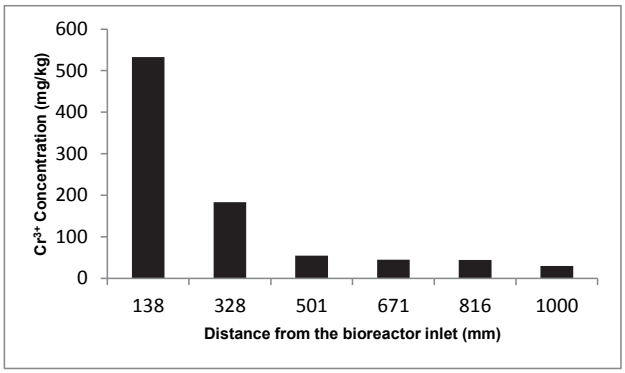


***Figure 2***
*Precipitated chromium on the bioreactor matrix fractions after termination*

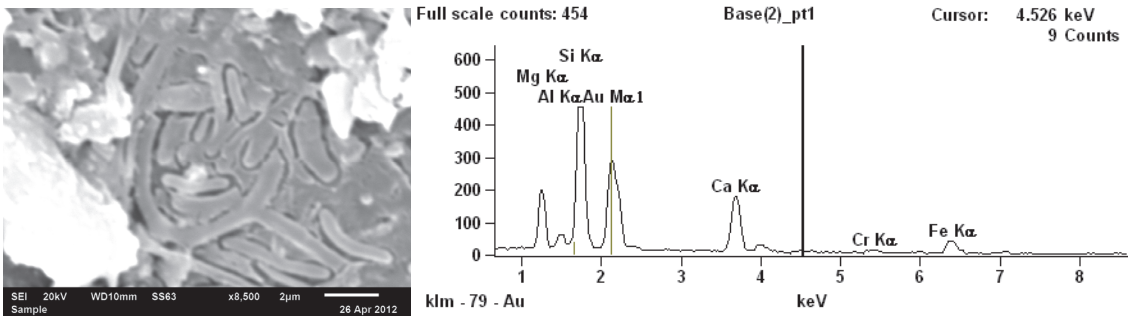


***Figure 3***
*SEM and EDX analyses confirmed biofilm formation and chromium precipitation on the matrix surface*

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| TABLE 2<br>Sequencing results retrieved from the BLASTN algorithm for the chromium bioreactor water fractions | | |
|---|---|---|
| **Accession number** | **BLASTN sequence alignment** | **Maximum identity** |
| JF508351.1 | Uncultured *Chloroflexi* bacterium clone AlertH04 16S ribosomal RNA gene, partial sequence | 98% |
| HM062452.1 | Uncultured *Acidobacteria* bacterium clone KBS_T1_R4_149264_b9 16S ribosomal RNA gene, partial sequence | 99% |
| KF453773.1 | *Enterobacter cloacae* strain JUNL-10 16S ribosomal RNA gene, partial sequence | 99% |
| KC914541.1 | *Ralstonia* sp. CPO 4.0037 16S ribosomal RNA gene, partial sequence | 99% |
| JX066803.1 | *Flavobacterium* sp. BX12 16S ribosomal RNA gene, partial sequence | 97% |
| EF188662.1 | Uncultured alpha proteobacterium clone 2030 16S ribosomal RNA gene, partial sequence | 99% |

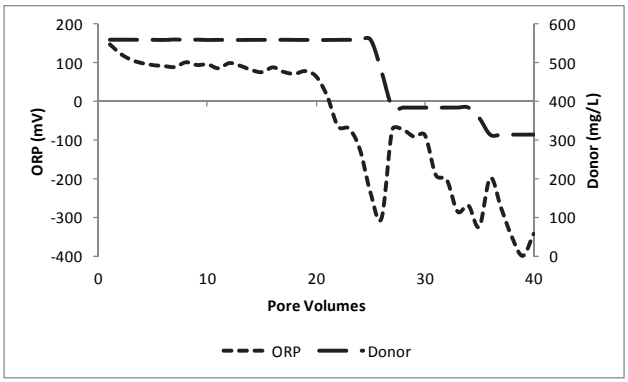


***Figure 4***
*ORP and electron donor profiles during the first 40 pore volumes after bioreactor commissioning*

Frankenberger, 1997); (ii) nitrite, such *Flavobacterium* sp. (Park et al., 2006), *Acidobacteria* sp. (Ward et al., 2009) and *Ralstonia* sp. (Coenye et al., 1999); (iii) iron(III), such as *Chloroflexi* sp. (Kawaichi et al., 2013) and *Acidobacteria* sp. (Blothe et al., 2008); (iv) chrome(VI), such as *Enterobacter cloacae* (Wang et al., 1989; Wang et al., 1990; Rege et al., 1997); (v) selenium oxyanions, such as *Enterobacter cloacae* (Watts et al., 2003; Losi and Frankenberger, 1997; Ridley et al., 2006; Yee et al., 2007) and *Ralstonia* sp. (Valls et al., 2000; Sarret et al., 2005); and (vi) arsenic, such as *Flavobacterium* sp. (Macur et al., 2004).

### On-site pilot bioreactor

The 24 000 ℓ fixed-film pilot bioreactor was successfully operated at a hexavalent chromium-contaminated site. The redox potential and electron donor profiles during the first 40 days after bioreactor commissioning are illustrated in Fig. 4. The bioreactor was started up using 550 mg/ℓ citric acid as electron donor to promote the formation of biofilm on the matrix of the bioreactor (Fig. 4). The redox potential within the reactor reached anoxic conditions after 22 PVs (Fig. 4), indicating the successful formation of the biofilm on the matrix of the reactor. The electron donor was gradually decreased during the following 18 PV's to 315 mg/ℓ (Fig. 4), resulting in a further decrease of the redox potential to –400 mV.

After 22 PVs >95% chromate reduction was achieved as a result of the anoxic conditions within the bioreactor, which indicates the biological activity responsible for the hexavalent chromium reduction. Similar to the laboratory column study, steady-state conditions could be achieved, resulting in maintaining the reduction of chromium(VI) (>99%) and nitrate (>80%) and therefore facilitating an added benefit by supplying water which could be used for sanitary purposes on site. This is the first up-scaled, effective demonstration of a start-up $Cr^{6+}$ bioremediation system in South Africa.

## CONCLUSIONS

The design of the up-flow bioreactor can be applied to a myriad of metal (and other) potential contaminants commonly found in aqueous environments by effective management of the redox potential and electron donor/acceptor balancing to direct bacterial biofilm development and steady-state activity. The presence of a suitably adapted microbial community effective for chromium reduction is essential for this system. Microbes are extremely adaptable due to their high cell turnover and corresponding high evolutionary rate, and the potential for horizontal gene transfer through plasmids and transposons, therefore, it is highly probable that an appropriate microbial community will be present in any historically contaminated environment. Given the potential for accessing the appropriate microbial community at sites where the indigenous bacterial community has been exposed and adapted to contamination, this system may be used as a model for other chromium-contaminated sites. This contaminant has been present for 80 years in this specific environment, allowing indigenous bacteria to adapt to the chromium and be used as effective biocatalysts for chromium reduction. During this project it became clear that scaling up from laboratory reactors to an on-site pilot reactor presented various challenges. The start-up of the pilot reactor required a higher concentration of electron donor and took a longer time for the reactor to reach anoxic conditions. During electron donor weaning of the laboratory reactors it was possible to decrease the electron donor to the theoretical electron donor demand without decreasing the hexavalent chromium reduction potential; however, this balance is more difficult to achieve for the pilot reactor where factors on site can influence stoichiometry, e.g., blasting with ammonium nitrate, rainfall, etc. Once the electron donor concentration in the pilot reactor was decreased to below approximately double the theoretical electron donor demand, the hexavalent chromium reduction potential decreased. Research results presented in this paper provide the basis for a low-cost, and low-maintenance strategy for the biological treatment of hexavalent chromium-contaminated water.

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