CHEMICAL ENGINEERING TRANSACTIONS
VOL. 76, 2019
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Petar S. Varbanov, Timothy G. Walmsley, Jiří J. Klemeš, Panos Seferlis

ISBN 978-88-95608-73-0; **ISSN** 2283-9216
DOI: 10.3303/CET1976218

The Cr(VI) Bioremediation Potential of *Chlamydomonas Debaryana*

Maria M. Roestorff*, Nondumiso C. Mbonambi, Evans M.N. Chirwa

Water Utilization and Environmental Engineering Division, Department of Chemical Engineering, University of Pretoria, Pretoria 0002, South Africa
Maria.roestorff@gmail.com

Chromium (Cr) is an essential industrial component and has many applications in industrial manufacturing. Anthropogenic activities can lead to the release of hexavalent chromium [Cr(VI)] as well as trivalent chromium [Cr(III)]. Cr(VI) is 1,000 times more toxic than Cr(III), and very detrimental to most organisms even at low concentrations. *Chlamydomonas debaryana*, a freshwater algae, appeared to be Cr(VI) resistant and was able to survive in Cr(VI) concentrations of up to 80 mg/L for an extended period in packed aquifer media columns. This micro-green algae strain was isolated alongside Cr(VI) reducing bacteria (CRB) at a Cr(VI) contaminated site in South Africa. In this study, the growth response of *C. debaryana* in the presence of Cr(VI) was compared to that of *Chlamydomonas reinhardtii*, to assess the Cr(VI) tolerance of *C. debaryana.* At 50 mg/L Cr(VI) concentration the maximum chlorophyll *a* increase was 25.3 % for *C. debaryana* and 34.2 % for *C. reinhardtii.* However, after 3.5 d the chlorophyll *a* content decreased only 38.1 % for *C. debaryana* compared to a 100 % decrease for *C. reinhardtii.* Indicating that *C. debaryana* is only slightly more resistant than *C. reinhardtii*. Zero Cr(VI) removal was observed with the algae: *C. debaryana* could not reduce Cr(VI) to Cr(III) or adsorb Cr(VI) anions from the solution. *C. debaryana* as well as *C. reinhardtii*, algae cells are inhibited when directly exposed to Cr(VI) in continuously mixed batch reactors. *C. debaryana* might be better suited to use in cooperation with CRB for Cr(VI) treatment in a biofilm reactor. This research can lead to an improved treatment set up for Cr(VI) pollution that requires fewer chemical and energy inputs and produces less secondary waste.

1. Introduction

In the environment, Cr mostly exists in the Cr(III) valence state. Cr is used in industrial processes such as mining and refining of ore, alloy production, wood treatments, paints and pigments production, anti-algae agents, and leather tanning. These anthropogenic activities are responsible for the release of Cr(VI) into the environment, as Cr(VI) is frequently discarded incorrectly (Tshuto et al., 2017). Cr(VI) is very toxic and classified as a carcinogen (IARC, 1987). Cr(VI) can easily be transported across cell membranes and, inside cells, Cr(VI) is reduced to Cr(V) and Cr(III) thereby releasing free radicals that can cause DNA alteration and damage. (Jobby et al., 2018). Cr(III) is less mobile and cannot diffuse across cell membranes, and is therefore considered less toxic than Cr(VI) (Kaimbi and Chirwa, 2015). It is pertinent to treat Cr(VI)-contaminated soil and drinking water to ensure that the Cr(VI) levels are within permissible limits. Cr(VI) treatment methods usually include detoxification by reducing Cr(VI) to non-toxic Cr(III) or removing Cr(VI) entirely from the solution via adsorption. The most common Cr(VI) treatment is the reduction of Cr(VI) to Cr(III) at a pH 2.0 followed by precipitation of Cr(III), as $Cr(OH)_3(s)$, at a pH above 8 (Pradhan et al., 2017). Additional conventional treatments include photocatalysis, adsorption, and ion exchange (Jobby et al., 2018). These treatment methods are, however, associated with high cost, high energy requirements, high chemical usage, and produces significant amounts of secondary pollutants (Busto et al., 2016).

Bioremediation utilises biological organisms for remediation purposes and has recently become popular. Microorganisms such as algae, bacteria, yeast, fungi, protozoa, or plants can target a specific toxic compound (Kaimbi and Chirwa, 2015). Bioremediation is associated with low operation costs and produces less secondary pollution than conventional treatment methods. Thus, contributing to its popularity (Bharagava and Mishra, 2018).

Paper Received: 25/03/2019; Revised: 21/06/2019; Accepted: 24/06/2019
Please cite this article as: Roestorff M.M., Mbonambi N.C., Chirwa E.M.N., 2019, The Cr(VI) Bioremediation Potential of Chlamydomonas Debaryana, Chemical Engineering Transactions, 76, 1303-1308 DOI:10.3303/CET1976218

Lately, a wide range of microorganisms, predominately bacteria, have been isolated and identified that are Cr(VI) resistant and can reduce Cr(VI) to Cr(III) (Lloyd, 2003). Researchers from the University of Pretoria have identified more than 6 bacterial species that were able to successfully reduce Cr(VI) to Cr(III, including: *Bacillus thermoamylovorans, Citrobacter sedlakii, Escherichia coli* (Roestorff and Chirwa, 2018)*, Bacillus cereus*, *Staphylococcus sp., Enterobacter sp.* (Igboamalu and Chirwa, 2016).
Only a few algae species have been identified that is Cr(VI) resistant and algae that can reduce Cr(VI) to Cr(III) are very rare. Cr(VI) resistant *Chlorella spp*. were isolated by Rehman and Shakoori (2001) from a tannery and reported that the algae were able to grow in the presence of Cr(VI). *Chlorella spp*. were also able to reduce Cr(VI) from a concentration of 12 µg/mL. Yewalkar et al. (2007) also isolated *Chlorella spp*. from a paper-pulp disposal dump. Yewalkar et al. (2007) observed that the Cr(VI) reduction was higher in the light than in darkness. The addition of carbon sources stimulated Cr(VI) reduction.
Most algae species are susceptible to Cr(VI) toxicity; *Chlorococcum Ellipsoideum* and *C. reinhardtii* were reportedly exceptionally adversely affected by Cr(VI) even at low concentrations (Roestorff and Chirwa, 2019). Rodríguez et al. (2007) suggested that *C. reinhardtii* can be used as an indicator of Cr(VI) pollution as *C. reinhardtii* has a low tolerance level for Cr(VI). Therefore isolating an algae species that is Cr(VI) resistant is unique.
Algae can use sunlight, with CO_2 as its primary carbon source, to produce biomass which allows cultivation to be inexpensive. Algae contribute to CO_2 sequestering and are found worldwide. Thus, algae are low-cost, eco-friendly, and ubiquitous. Algae is frequently used in bioremediation applications as a biosorbent as both living, and dead, algae biomass can remove Cr(VI) (Joutey et al., 2015). Biosorption can occur either with or without metal transformation (Birungi and Chirwa, 2014). However, maximum biosorption is observed in acidic conditions (Deng et al., 2009). As a result, additional chemicals are needed to lower the pH of the solution. The experiments in this study were carried out at neutral pH. The objective of this study is to determine if *C. debaryana* is Cr(VI) resistant by observing the algae's growth response in the presence of Cr(VI). The growth response is also compared to that of *C. reinhardtii* under the same conditions. This study also aims to gain insight into how *C. debaryana* survived in the aquifer media columns. *C. debaryana* were also tested in its ability to remove Cr(VI) by either reduction of Cr(VI) to Cr(III) or by biosorption.

2. Materials and methods

2.1 Algae isolation and identification

The algae used in this study were isolated from aquifer media columns (shown in Figure 1) that were used to detoxify Cr(VI) at pilot scale. The soil was collected from a Cr(VI) contaminated site and was used as packing in the columns. The assumption is that the collected soil contained algae. In the columns, the algae grew rapidly as the column is clear and allowed enough sunlight through. The room in which the column was located had windows. The algae continued to grow as a biofilm on the sides of the columns even when exposed to an increasing amount of Cr(VI) concentration.
About 5 g of soil containing possible algae samples were removed from the columns and suspended in Erlenmeyer flasks containing 400 mL sterile 3-fold Nitrogen, Bold Basal Media with Vitamins (3N-BBM+V) and a pH of 6.7. Plankton nets were also used to cover the algae in the Erlenmeyer flasks. The Erlenmeyer flasks containing the algae were continuously mixed under the required light conditions (Osram L 36W/77 Floura) at 25 ℃ for 7 d. After the initial cultivation period, the algae were isolated using streak plating on 3N-BBM+V agar in Petri dishes. The plates were placed upside down under the algal lamps until the algal colonies formed. Repeated streak plating was done to ensure that the algae were free from bacterial contamination. The algae identification was done at Inqaba Biotechnical Industries (Pty) Ltd.

Figure 1: Aquifer media columns, the green biofilm was positively identified as algae.

The plates that contained separate pure colonies were identified by carrying out 18S rRNA sequencing. The resulting sequences were matched to known genes for algal species in the GenBank using BLAST. The algal species of *Chlamydomonas debaryana* were found to have 97 % sequence identities with the collected samples.

2.2 Algae cultivation

The algae were cultured in sterilised 3N-BBM+V media (CCAP, 2015) and continuously mixed, closed with planktonic nets, under the required algal light conditions roughly at 60 µmol photons/m^2 s (Osram L 36W/77 Floura) at 25 °C. After 14 d, the optical density at 700 nm of the culture solution reached 1.05. The algae cells were harvested and centrifuged for 10 min at 6,000 rpm in a Sorvall Lynx 6000 (Thermo scientific, Stockholm, Sweden).

2.3 Chlorophyll an assay

The chlorophyll assays were done by withdrawing 2 mL samples from the experiments in which the algal cells were exposed to Cr(VI). The 2 mL samples were centrifuged in a Minispin Microcentrifuge (Eppendorf, Hamburg, Germany) at 6,000 rpm for 10 min; the supernatant was discarded. The algal cells were ground up in 2 mL of 90 % acetone solution and incubated at 4 °C for 24 h in darkness. After incubation, the samples were centrifuged again to remove the chlorophyll *of* free cells. All of the chlorophyll *a* has been extracted from the cells, leaving behind the white algal cells. The optical density of the supernatant was measured at 630 nm, 645 nm and 663 nm with a spectrophotometer (WPA, LightWave II, and Labotech, South Africa). The chlorophyll content was calculated with Eq(1) (Liang et al., 2013):

$$\text{chlorophyll } a\ (\text{mg/L}) = 11.64\text{OD}_{663\text{ nm}} + 2.16\ \text{OD}_{645\text{ nm}} + 0.1\text{OD}_{630\text{ nm}} \quad (1)$$

The chlorophyll content from *C. debaryana* was compared to that of *C. reinhardtii,* a non-Cr(VI)-resistant algal species. The chlorophyll *a* data of *C. reinhardtii* was gathered from Roestorff and Chirwa (2019).

2.4 Analytical method

Cr(VI) was measured using the UV/Vis spectrophotometer (WPA, light wave II, Labotech, South Africa) at a wavelength of λ = 540 nm (10 mm light path). The appearance of a purple colour after acidification with 1N H_2SO_4 and adding 1,5-diphenyl carbazide indicates the presence of Cr(VI) in the sample (APHA, 2005). The intensity of the purple colour is proportional to the Cr(VI) concentration.

2.5 Batch experiments

The harvested algal cells were re-suspended in 50 mL Erlenmeyer flasks containing sterilised 3N-BBM+V (pH of 7) and Cr(VI) to give the desired concentration. The initial concentration of Cr(VI) was varied between 5, 10, 50 mg/L. The algae were also allowed to grow without Cr(VI) as a control. All the experiments were conducted at 25 °C, at 120 rpm on the orbital shaker (Labotec, Gauteng, South Africa). The samples, taken every 12 h, were centrifuged using in 2 mL Eppendorf tube at 6,000 rpm for 10 min in a Minispin® Microcentrifuge (Eppendorf, Hambury, Germany) and the supernatant was used for Cr(VI) analysis. The algal cell pellet that formed at the bottom of the Eppendorf tube was used for chlorophyll assays. The algal activity in the experiments was represented by the changes in the chlorophyll content.

3. Results and discussions

3.1 Chlorophyll an assay in batch experiments

The chlorophyll *a* content of the algae is linked to the ability of the algae to undergo photosynthesis. The chlorophyll *a* content of the algal cells in the experiments indicates the overall algae cell health and is also related to the biomass (Hong et al., 2016). Figure 2 shows the chlorophyll content of algal cells that were exposed to different Cr(VI) concentrations. In the first day, the algae were in lag growth phase before entering the exponential growth phase. In the control experiment, it can be observed that *C. debaryana* has a very rapid growth rate. After the first 2 d, the algae in the control experiment entered the stationary phase and maintained a constant chlorophyll *a* concentration for the remainder of the experiment period.

It is clear that the algae growth was inhibited by the Cr(VI). After 2 d the chlorophyll *a* content steadily decreased in the experiments in which the algae were exposed to Cr(VI). The effect of the Cr(VI) on *C. debaryana* is harmful, and it would appear that these algae are not very Cr(VI) resistant. At Cr(VI) concentrations of 5 and 10 mg/L, the algae were able to grow in the first 24 h, and at 19 h the chlorophyll content increased by 56 % and 59 % respectively for the investigated concentrations. The maximum biomass increase of 64.7 % was observed at 43 h for the 5 mg/L Cr(VI) experiments. At 50 mg/L of Cr(VI) the algae were more notably inhibited; the chlorophyll *a* content only increased by 25.3 %.

These experiments demonstrate that the algae cells which are directly exposed to Cr(VI), in a mixed batch reactor, are adversely affected. The algae in the column reactors (Figure 1) that were exposed to Cr(VI), but still managed to grow, were likely protected by a biofilm in the soil. The CRB that are present in the soil can also limit the Cr(VI) concentration that reaches the algae om the wall of the column, by reducing Cr(VI) to Cr(III).

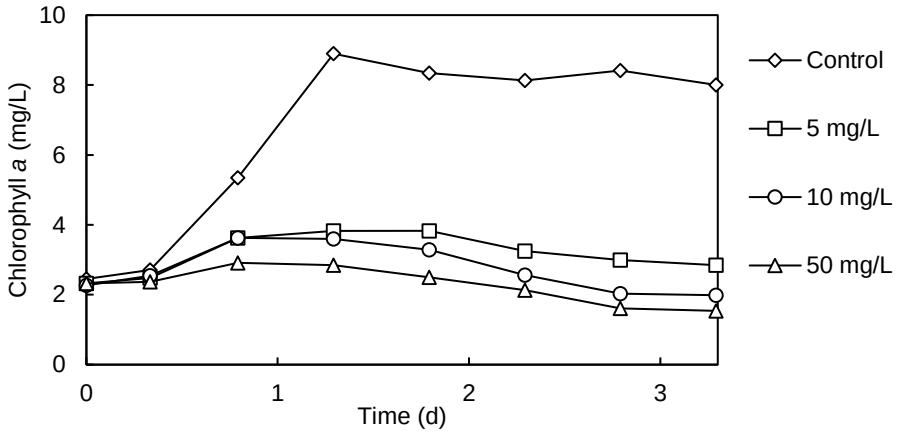

Figure 2: Chlorophyll a content (mg/L) of C. debaryana cells in 3N-BBM+V exposed to different Cr(VI) concentrations. Under algal light; Osram L 36W/77 Floura at 25 °C and neutral pH.

3.2 Cr(VI) resistance comparison between *C. debaryana* and *C. reinhardtii*

The chlorophyll *a* content of *C. debaryana* that was exposed to Cr(VI) is compared to *C. reinhardtii* (Roestorff and Chirwa, 2019) to determine if *C. debaryana* is more resistant than other algal species. The two algae species had different growth patterns. During the first day, the *C. debaryana* algae had a faster growth rate than the *C. reinhardtii* algae, but the *C. reinhardtii* algae achieved a higher chlorophyll *a* concentration at the end of the control experiments (shown in Figure 3a). *C. debaryana* did not grow to a high cell concentration as compared with *C. reinhardtii*. The *C. debaryana* algae maintained a constant chlorophyll *a* concentration for a longer period than the *C. reinhardtii* algae.

Figure 3b shows the chlorophyll *a* content of the algae exposed to 5 mg/L. Initially *C. debaryana* had a higher growth rate than *C. reinhardtii.* However, *C. reinhardtii* were able to increase 2.5 times and *C. debaryana* only increased 1.6 times. This may be due to the difference in the growth pattern. After the second day, the chlorophyll *a* content of the *C. debaryana* algae decreased at a slower rate than *C. reinhardtii.* Figure 4a and 4b shows the chlorophyll *a* content at 10 and 50 mg/L of Cr(VI). At the end of 3 d, the chlorophyll *a* content of *C. reinhardtii* was completely gone, whereas for *C. debaryana* the chlorophyll *a* content was above 1.5 mg/L. At higher Cr(VI) concentrations, *C. debaryana* appears slightly more resistant than *C. reinhardtii*.

Table 1 shows the comparison of the chlorophyll *a* content of *C. debaryana* and *C. reinhardtii* after 3.5 d. The chlorophyll *a* content decreased only 38.1 % for *C. debaryana* and 100 % for *C. reinhardtii*. *C. debaryana* were able to survive at a higher Cr(VI) concentration for a more extended period than *C. reinhardtii*.

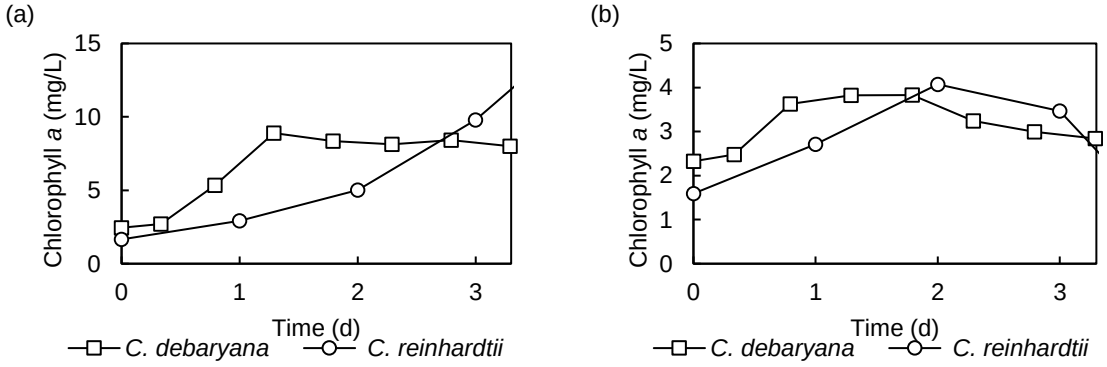

Figure 3: Chlorophyll a content of algae (a) without Cr(VI) exposure and (b) 5 mg/L Cr(VI) concentration

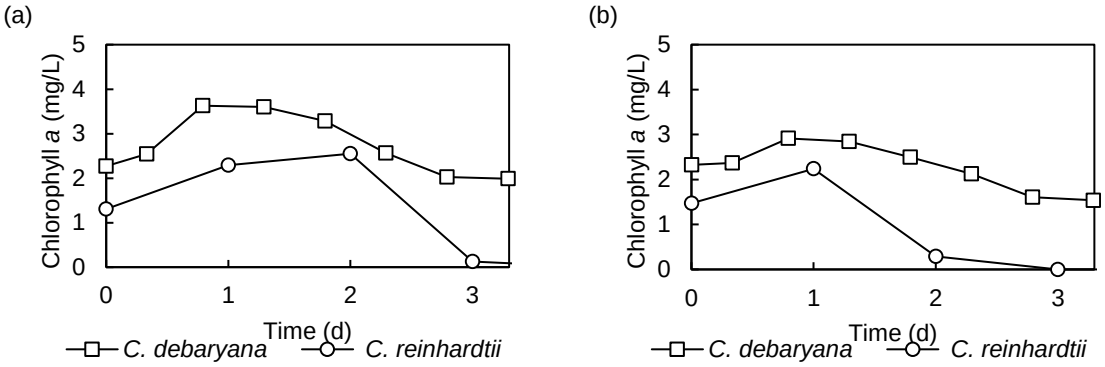

Figure 4: Chlorophyll a content of algae exposed to (a) 10 mg/L and (b) 50 mg/L Cr(VI) concentration

Table 1: Percentage change in chlorophyll a content of C. debaryana C. reinhardtii at different Cr(VI) concentration after 3.5 d.

Cr(VI) Concentration	*C. debaryana*	*C. reinhardtii*
5 mg/L	20.2 % increase ↑	24.9 % increase ↑
10 mg/L	20.4 % decrease ↓	95.2 % decrease ↓
50 mg/L	38.1 % decrease ↓	100 % decrease ↓

3.3 The Cr(VI) concentration in batch experiments with *C. debaryana*

Figure 5 shows the Cr(VI) concentration in the batch experiments with *C. debaryana* algae. The Cr(VI) concentration remained constant throughout the experiments. This suggests that the Cr(VI) were neither reduced nor adsorbed. It is most likely that *C. debaryana* could not reduce Cr(VI) due to a lack of chromate reductase (enzymes) and may also require a carbon source or electron donor to facilitate reduction. Biosorption could not occur, as the pH at which the experiments were carried out caused the negatively charged algal cells to repel the Cr(VI) anions (CrO_4^{2-}). On its own *C. debaryana* has limited Cr(VI) remediation potential. However together with CRB, a sustainable Cr(VI) removal system is possible.

During bacterial reduction of Cr(VI) to Cr(III) a carbon source is required to facilitate the reduction process. The possible presence of nutrients and nourishment in the soil, from which the algae were isolated, could also contribute to the successful survival of the algae. Therefore, the addition of a carbon source could stimulate the algae's Cr(VI) reduction capabilities.

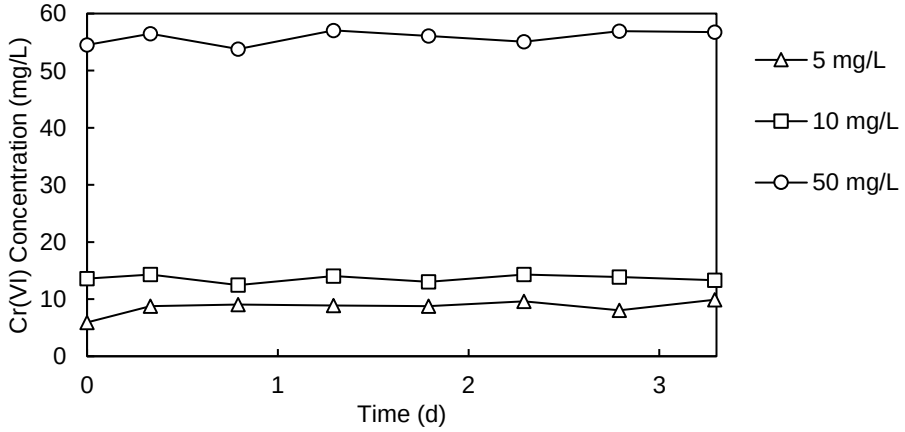

Figure 5: Cr(VI) concentration in batch experiments with C. debaryana.

4. Conclusions

Minimal Cr(VI) resistance was observed in the *C. debaryana* algae directly exposed to Cr(VI). *C. debaryana* was only slightly more Cr(VI) resistant than *C. reinhardtii. C. debaryana* was not able to remove the Cr(VI). In the batch experiments, the algae were directly exposed to Cr(VI) and had no physical barrier to shield from the toxicity. As a result, a decrease of 38.1 % was observed in the chlorophyll content after 3.5 d exposure to 50 mg/L of Cr(VI). This verifies how vital the algae biofilm layer is in the column reactors (Figure 1).
Most algae species can combine photoautotrophy and heterotrophy strategies to acquire nutrients from organic materials that are present in the soil. Therefore it is recommended for future research to repeat the experiments but with a carbon source, such as glucose, to re-evaluate the Cr(VI) resistance and reduction potential of *C. debaryana*.

Acknowledgements

This work is based on the research supported in part by the National Research Foundation of South Africa.

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