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Biological Remediation of Chromium (VI) in Aquifer Media Columns

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Hexavalent chromium (Cr(VI)) is highly soluble in water, but is widely used in industrial sectors for many purposes. The effluent from these industrial activities is often released to the environment posing a threat to aquatic life and to humans. Conventional methods of treating Cr(VI) often require the use of pump and treat methods followed by the use of harmful toxic chemicals that are hard to dispose of and usually are expensive. The study explores reduction of Cr(VI) to Cr(III) using biological means (a popular issue of bioremediation of contaminated aquifer) in columns; control with aquifer media, system 1 inoculated with CRB (chromium reducing bacteria) dried sludge, system 2 further amended with saw dust and system 3 amended with vegetative material from target site as carbon source. CRB are essential and were inoculated in the columns (system 1-3) as dried sludge, previously isolated (*Enterococcus casseliflavus*, *Pseudomonas putida*, *C.istrobacter sedlakii*, *Enterobacter cloacae* and *Enterobacter hormaechei*) and tested in a batch system. The column reactors were run for 60 days at concentrations 40, 60 and 80 mg/L. Culture isolates in the effluent of the reactors were isolated and identified. Complete reduction was observed from all columns under different concentrations, with some failures at certain periods before quasi steady state (determined after 40 d). At 40 mg/L more than 95 % of Cr(VI) was reduced across the spectrum. System 3 best reduced Cr(VI) compared to other treatment column reactors at high concentrations of 80 mg/L. System 2 carbon source didn't enhance Cr(VI) reduction compared to Syetem 1 with no carbon material. Algae growth was observed in the columns after operating for 40 d at 40 mg/L. Using 18s rRNA the dominant algae was identified as *Chlamydomonas debaryana*. As Cr(VI) concentration was increased, both CRB and the algae were assumed to be in synergy as Cr(VI) was reduced at influent concentration as high as 80 mg/L (double the concentration from the site). Further investigation was done in the study to identify Cr(VI) reducing potential by algae. The use of vegetative material from the target site in the presence of algae proved to enhance Cr(VI) reduction by CRB even though saw dust did not perform as expected. This method has potential to be used in Cr polluted sites in South Africa with careful application.

1. Introduction

Chromium is most important for its use in a variety of metallurgical, refractory and chemical processes. In industrial activities it is used for production of steels and alloys, metal plating and tanneries (Roestorff and Chirwa, 2018). Cr is one of the top 20 contaminants on the Superfund priority list of hazardous substances (Gome et al., 2012) and in the environment exists mainly in two forms: (Cr(III)), which readily forms the insoluble and less mobile species $Cr(OH)_3$ in water and (Cr(VI)), which exists as soluble and mobile oxyanions, chromate and dichromate (CrO_4^{2-} or $Cr_2O_7^{2-}$) (Meli, 2009). Cr(VI) is a human carcinogen and large quantities are discharged into the environment, mainly to soils and ground water, owing to improper disposal, leakage and its high mobile nature (Gomes et al., 2012). Cr(III) is less toxic and desirable as an essential nutrient in trace amounts for carbohydrates and lipid metabolism and is used as a nutritional supplement (weight loss agent) by athletes (Kaimbi and Chirwa, 2015). Several governments such as the Environmental Protection Agency (EPA) in the USA have identified Cr(VI) as a type A carcinogen and mandated a stringent discharge limit of 0.05 mg/L to protect surface waters from contamination (Qian et al., 2006).
South Africa holds one of the highest Cr reserve in the world due to the excessive steel production and one of its largest chromium ore production situated in the North West Province (Di Palma et al., 2012). Cr(VI) can be

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reduced chemically by reducing agents or biologically via microbial conversion of Cr(VI) to Cr(III) (Qian et al., 2016). Chemical treatments are expensive as they are either energy intensive or require large quantities of expensive chemical; reagents and they also generate large quantities of toxic sludge that is difficult to dispose (Mtimunye and Chirwa, 2013).

Reducing technologies of Cr(VI) to Cr(II) using microbes have been a popular topic. Microorganisms (CRB) have developed various resistant mechanisms towards Cr(VI) toxicity in the environment of exposure (Molokwane and Chirwa, 2009). Microbial conversions include resistant mechanisms involving specific biochemical pathways that can alter chemical properties of the toxic metal (Igboamalu and Chirwa, 2016). Microorganisms such as *Bacillus sp. Bacillus sphaericus, Pseudomonas sp. Pseudomonas aeruginosa, Exiguobacterium sp.* and *Escherichia coli* have been isolated from chromium-contaminated areas for the biodegradation of Cr(VI) to Cr(III). The potential of these CRB strains for reduction of Cr(VI) to less toxic Cr(III) under normal conditions has been previously successfully demonstrated (Mohapatra et al., 2017). The bacterial strains usually require an organic carbon source, as either an energy source or as an electron donor. These carbon sources can be in a form of microalgae (photoautotrophic microorganism that use CO_2 as its main carbon source). Stated by Roestorff and Chirwa (2018), the cells of microalgae can be used as the basis of the nutrient medium for bacteria as the algae cells die off in the systems (due to exceeded Cr(VI) tolerant) the ruptured cell walls produce nutrients such as ammonium salts, amino acids, B vitamins, cobalt, copper, manganese, molybdenum, iron, zinc, iodine and other trace elements.

In this study, Cr(VI) resistance bacteria were isolated from dried sludge known to be loaded with varying Cr(VI) concentrations These bacteria facilitated the reduction of Cr(VI) to Cr(III) in continuous column system with varying treatments. Algae together with vegetative source from the target site increased the potential bioreactors at high Cr(VI) concentrations.

2. Materials and methods

2.1 Bacterial culture and growth medium

Cr(VI) reducing cultures were isolated from Britz Wastewater Treatment Works Dried Sludge (North West Province, South Africa), that is exposed to periodic loading of Cr(VI) from the nearby chrome ore refining plant. Bacterial cultures were isolated from the dried sludge by adding 0.5 g to 400 mL sterile Luria-Bertani (LB) broth (25 g in 1 L). LB broth containing sludge was incubated at 37 °C for 24 h. Agar plates were inoculated with 1 mL samples and the colonies were sub-cultured using differential techniques (exhibited colours and morphologies) and incubated at 30 °C for 24 h. Each of colonies were then tested for their Cr(VI) reducing capabilities on a Basal Mineral Medium (BMM) with an addition of 5g per L of Glucose (Roestorff and Chirwa, 2018). The flasks containing 400 mL BBM, were spiked with 50, 100, 200 and 400 mg/L and were incubated at 37 °C under continuous shaking. Samples were taken under time intervals for a period of 24 h. Characterization was done by using 16S rRNA genotype fingerprint method and identified on Table 1.

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

Pure Culture	Identification ID	%Query cover	Accession Number
X1	*Enterococcus casseliflavus*	100	CP032739
X2	*Pseudomonas putida*	99	CP016634
X3	*Citrobacter sedlakii*	99	KP861248
X4	*Enterobacter cloacae*	100	MG576153
X5	*Enterobacter hormaechei*	99	AJ853889

2.2 Algae cultivation and growth medium

Algae started growing from the sides of the columns after 40 d of 40 mg/L Cr(VI) concentration loading. The algae were then cultured using a 3 by 1 Nitrogen, Bold Basal Media with Vitamins (3N-BBM+V) in 250 mL Erlenmeyer flasks kept under algal light; Osram L 36W/77 Floura at 20 - 23 °C (Birungi and Chirwa, 2017) for 14 d with continuous shaking. Samples centrifuged at 6,000 rpm for 10 min under 4 °C to retrieve cells. BBM agar plates were streaked with algal growth and incubated under light for 14 d. Characterization was done by using 18S rRNA.

2.3 Reagents and analytical methods

Cr(VI) stock solution of 1,000 mg/L was prepared as stock solution (3.74 g of pure $K_2Cr_2O_4$ was dissolved in 1 L of distilled water). DPC was 0.5 g of 1,5 diphenylcarbozide in 100 mL of HPCL grade acetone in an amber bottle. Sodium chloride solution was 1.85 g (0.85 %) in 100 mL distilled water and autoclaved at 121 °C for 15 min. Cr measured by UV-Vis spectrophotometer (WPA, Light Wave II, Labotech, South Africa) operated at a

wavelength of 540 nm after acidification of 0.2 mL sample with 1 mL of 1 N H_2SO_4 and dilution with ultra-pure water to 10 mL followed by reaction with 1.5 diphenyl carbazide to produce a purple colour. Cr(III) was determined as the reduction of Cr(VI) from the initial absorbance.

2.4 Reactor setup and reactor startup

The aquifer soil media (a structure of rock situated below ground surface that may have groundwater passing through it) was obtained from an abundant Cr ore site in the North West Province, South Africa. It was collected 3 m deep from the contaminated ground surface. Four 1 m long columns constructed from a polyvinyl chloride (Plexiglas) with five intermediate sample positions 18 cm distant were stored in the laboratory as continuous flow reactors. The effluent position was connected to a 25 L waste tank. Columns were packed with aquifer media from target site. Prior to closing the columns with a plastic cap, one was a control which only had aquifer media, System 1 was further inoculated with dried sludge, System 2 was further amended with an organic electron donor (saw dust), while System 3 was amended with carbon source also by the target site. Preparation of dried sludge to aquifer media was 3:5, while saw dust and vegetative material to aquifer media was 2:5, respectively. Preparation was done directly onto the column, with manual mixing. The flow rate of the influent contaminant was set at 260 mL/h (25, 000 mL lasted for 96 h). This was to archive lowest hydraulic lauding for the system. Each column was vertically connected to a 100 L reservoir which pumped 40, 60 and 80 mg/L Cr(VI) concentration respectively after 60 d. Prior experimental run, ultra-pure water was fed through each column in order to saturate with water.

3. Results and discussion

3.1 Batch studies (Bacterial and Algae independently)

Isolated CRB (Figure 1 to Figure 5) showed potential to reduce Cr(VI) from varying concentrations.

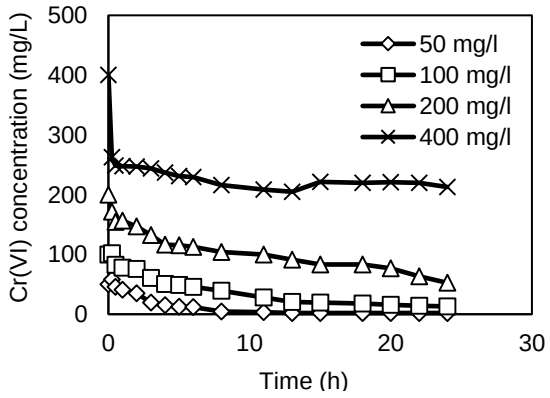

Figure 1: Cr(VI) reduction by Enterococcus casseliflavus

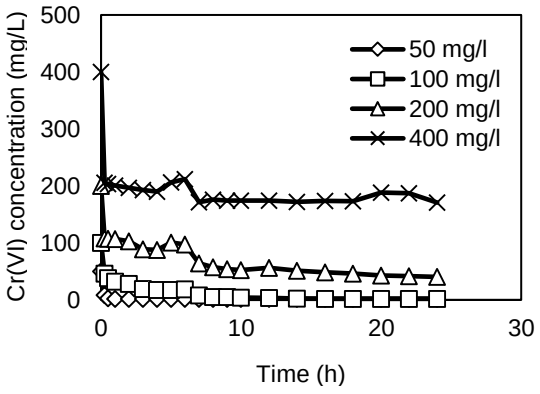

Figure 2: Cr(VI) reduction by Pseudomonas putida

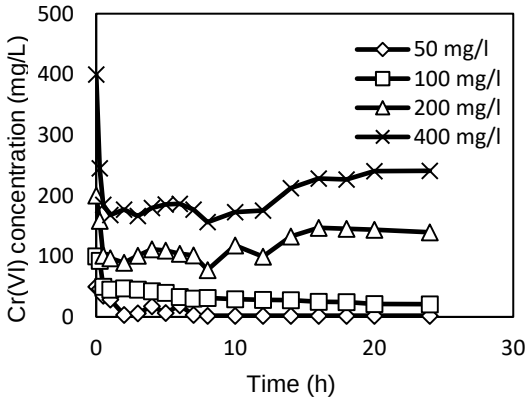

Figure 3: Cr(VI) reduction by Citrobacter sedlakii

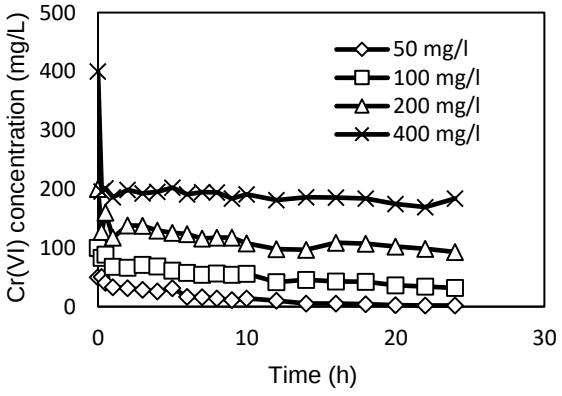

Figure 4: Cr(VI) reduction by Enterobacter cloacae

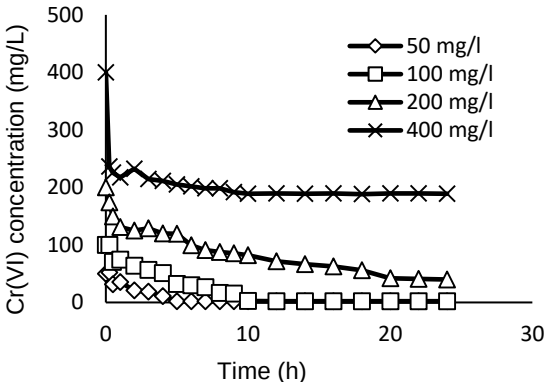

Figure 5: Cr(VI) reduction by Enterobacter hormaechei

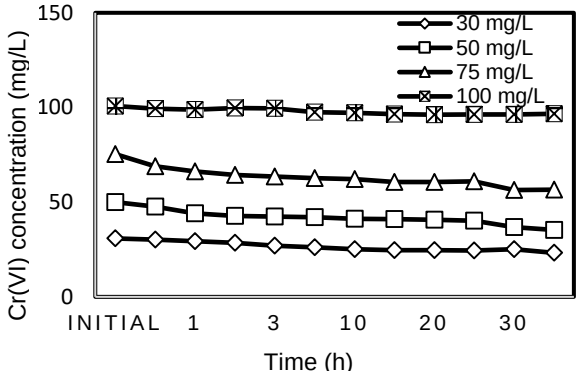

Figure 6: Cr(VI) reduction by isolated algae from the column reactors

The ability of these CRBs to reduce concentrations as high as 400 mg/L could be associated with their indigenous environment (Treatment Works) that receives periodic loading of Cr(VI) contaminant. These CRBs are already acclimatized the presence of Cr(VI) and reduce this contaminant better than alien microorganisms (bacteria). *Pseudomonas putida* (Figure 2) was the best performing culture from 50 mg/L to 400 mg/L, where 50 and 100 mg/L were reduced to 0 mg/L within 5 hours. *Citrobacter sedlakii* culture did not show good results in reducing Cr(VI) especially from 200 to 400 mg/L. All isolated cultures were able to reduce Cr(VI) concentration at 50 and 100 mg/L to 0 mg/L within 10 hours. Bacterial isolates from Sukinda mining area (Sukinda valley, the fourth most polluted place in the world) showed to tolerate Cr(VI) concentrations of 500, 600, 900 and 1,000 mg/L (Mishra et al., 2010).

Zhang et al. (2014), previously reported how organic carbon sources are required either in the form of energy or as an electron donor in order for a microorganism to reduce Cr(VI). The cost of carbon source (glucose and LB) is one of the most discussed drawbacks that presents the limitation of commercial application of the bioremediation pathway. The algae that were observed to be growing in the columns and could be acting as a carbon source for the bacteria were isolated and identified as *Chlamydomonas debaryana*. Its ability to reduce Cr(VI) was investigated using a batch study. A steady reduction of Cr(VI) was seen from 30 to 100 mg/L (Figure 6). The short operation time of the batch study did not reveal the great potential of *C. debaryana* in reducing Cr(VI), though at lower concentrations it seemed to be more effective. Algae metabolism is much slower and takes a while to enhance unlike bacteria (Roestorff and Chirwa, 2018).

3.2 Bacterial culture in column

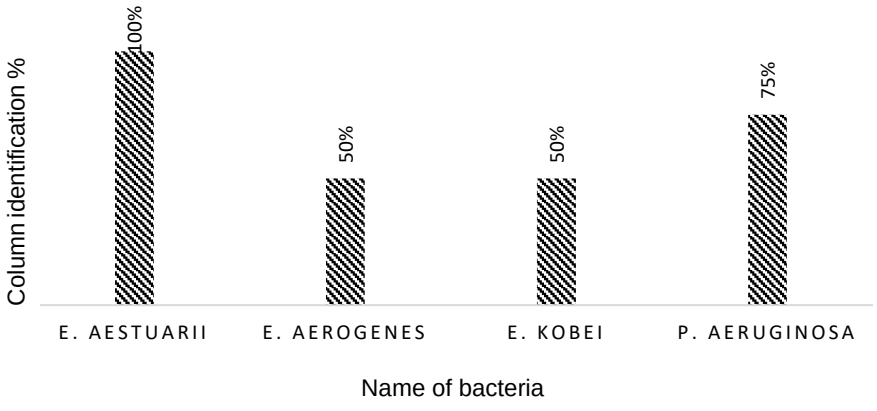

Figure 7: Bacterial cultures identified and identification percentages across all column reactor systems

Bacterial species were identified from the effluent of each bioreactor system. The cultures identified in the study proved to have Cr(VI) reducing abilities under different concentrations (40, 60 and 80 mg/L). These cultures were identified as; *Exiguobacterium aestuarii, Enterobacter aerogenes, Enterobacter kobei and Pseudomonas aeruginosa*. Some species were found to be present across all systems, having the ability to utilize the carbon

source from target site, foreign carbon source (saw dust) and simply surviving under the *in situ* bioremediation conditions on system 1 (*E. aestuarii* and *P. aeruginosa*) as seen on Figure 7. *Exiguobacterium* bacterium species, have been previously identified in Cr(VI) polluted sediments in China in a successful study of Cr(VI) bioremediation (Zhao et al., 2016). They exist in a wide variety of Cr(VI) contaminated environments and may be used as an effective bio indicator of Cr(VI) pollution in environmental monitoring (Mohapatra et al., 2017).

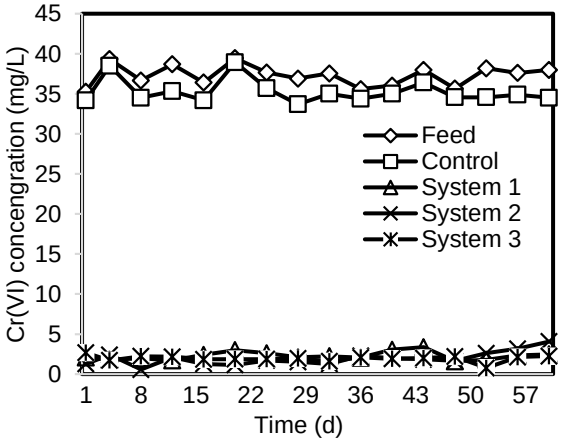

Figure 8: Cr(V) concentration reduction from 40 mg/L in the column

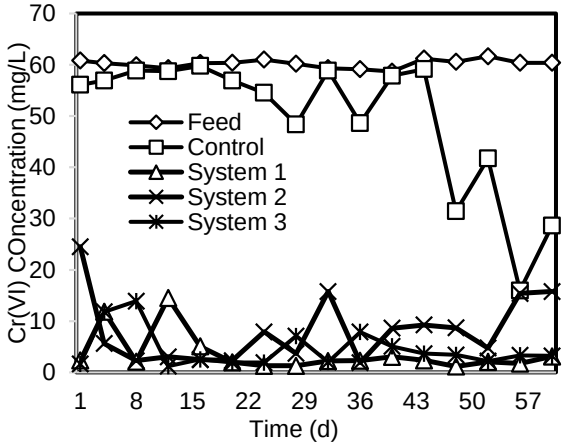

Figure 9: Cr(VI) concentration reduction from 60 mg/L in the column

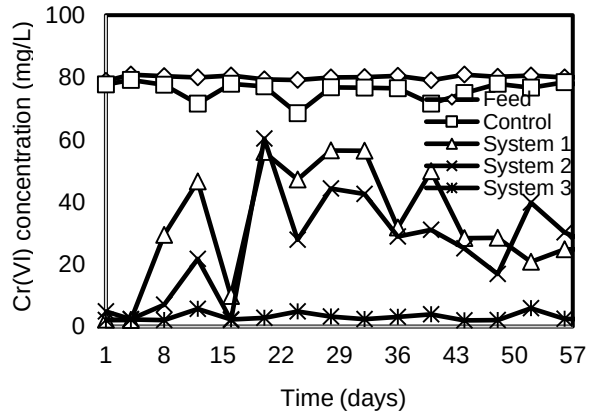

Figure 10: Cr(V) concentration reduction from 80 mg/L in the column

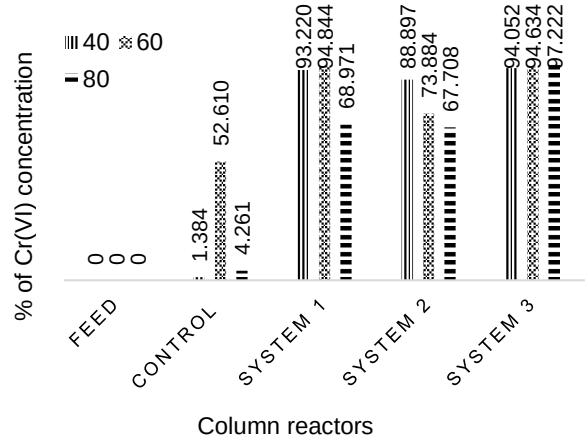

Figure 11: Percentage Cr(VI) concentration reduction

3.3 Bioreactor Cr(VI) reduction

Continuous batch reactor systems were running for a period of 60 d per concentration tested (40, 60 and 80 mg/L). The concentration at the target site was 40 mg/L as recorded by Molokwane and Chirwa (2009) and was used in this study as the lowest concentration studied. Steady Cr(VI) reduction was observed at 40 mg/L, with the lowest concentration of 0.582 mg/L recorded in 8 d. The feed and the control had similar trends at 40 and 80 mg/L, although the control showed reduction at 60 mg/L this was due to the soil assumed to be contaminated with Cr thus the presence of indigenous CRB is possible. Near complete Cr(VI) reduction has been previously recorded under 40 mg/L by Molokwane and Chirwa (2009), under low hydraulic loading. System 2 did not perform as well as system 3 (the best performing column reactor). In a study by Mthimunye and Chirwa (2013), at steady state no significant Cr(VI) reduction was examined between saw dust amended column and the column without saw dust. The vegetative material from the target site was bioavailable for the CRB than the saw dust (which was obtained from varying types of wood) that was not effectively utilized by CRB. The minute amounts of algae growth on the control resulted in Cr(VI) reduction to as low as 20 mg/L (Figure 4). At 80 mg/L System 3 showed the highest reduction of Cr(VI). The reduction is visible from System 1 and 3 from 60 mg/L to 20 mg/L. Reduction could be attributed to bacteria utilizing metabolites produced by the algae, or even the algae themselves as a source of carbon during the experiments (Roestorff and Chirwa, 2018). Further analysis of

results on Cr(VI) reduction was summarized by gathering Cr(VI) reduction percentage at assumed quasi-steady state for all tested reactors. System 3 had the highest reduction of Cr(VI) throughout all concentrations.

4. Conclusion

Successful Cr(VI) reduction was possible with bacterial cultures; *E.casseliflavus*, *P. putida*, *C. sedlakii*, *E. cloacae* and *E. hormaechei* according to batch studies. Bacterial isolates from the effluent of column reactors are associated with Cr(VI) reduction as previously reported. *Enterobacter* and *Pseudomonas* species were identified from dried sludge and also at the effluent showing adaptation capabilities and tolerance towards high Cr(VI) concentrations. The most efficient reactor was System 3 with Cr(VI) reduction from 80 mg/L to close to 0 mg/L. The presence of algae in the systems has increased the potential of this bioremediation technique. Thus this system has the potential to treat Cr ore contaminated plants without the use of heavy metals. However, proper application of this method is required. Appropriate parameters must be measured with the understanding of the contaminated area (also by-products of the system must be determined). Further research is to be done in the mechanisms (both bacteria and algae) used to achieve successful reduction and factors contributing to symbiotic relationships.

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