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Biotic and Abiotic Biostimulation for the Reduction of Hexavalent Chromium in Contaminated Aquifers

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Abstract: Hexavalent chromium is a carcinogenic heavy metal that needs to be removed effectively from polluted aquifers in order to protect public health and the environment. This work aims to evaluate the reduction of Cr(VI) to Cr(III) in a contaminated aquifer through the stimulation of indigenous microbial communities with the addition of reductive agents. Soil-column experiments were conducted in the absence of oxygen and at hexavalent chromium (Cr(VI)) groundwater concentrations in the 1000–2000 μg/L range. Two carbon sources (molasses and EVO) and one iron electron donor ($FeSO_4 \cdot 7H_2O$) were used as ways to stimulate the metabolism and proliferation of Cr(VI) reducing bacteria in-situ. The obtained results indicate that microbial anaerobic respiration and electron transfer can be fundamental to alleviate polluted groundwater from hazardous Cr(VI). The addition of organic electron donors increased significantly Cr(VI) reduction rates in comparison to natural soil attenuation rates. Furthermore, a combination of organic carbon and iron electron donors led to a longer life span of the remediation process and thus increased total Cr(VI) removal. This is the first study to investigate biotic and abiotic Cr(VI) removal by conducting experiments with natural soil and by applying biostimulation to modify the natural existing microbial communities.

Keywords: hexavalent chromium; anaerobic chromium reduction; biostimulation; organic and inorganic electron donors

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1. Introduction

Chromium (Cr) is a heavy metal that occurs in soils, sediments and groundwater through geogenic and anthropogenic sources. The main natural sources of Cr in the environment are ophiolithic and serpentine rocks, as well as their weathering products [1,2]. Anthropogenic Cr is mainly related to industrial processes, such as energy production, tanneries, ore-processing facilities, industrial metal processing, wood preservation, and to a lesser degree, agricultural activities [2–7]. Chromium compounds are usually found in two oxidation states. Trivalent chromium (Cr^{+3}) is a micronutrient used in some cases as a dietary supplement [8–11] and a stable form of Cr, that has relatively low toxicity, forms insoluble substances, and is not able to cross cell membranes [12]. In contrast, hexavalent chromium (CrO_4^{2-}, $Cr_2O_7^{2-}$) is highly soluble, toxic and a well-known carcinogen [13,14], that has been designated by the USEPA as one of seventeen chemicals posing the greatest threat to human health [15]. The World Health Organisation has set a limit of 50μg/L for total Cr in drinking water [16]. However, in several parts of the world reported values for geogenic Cr(VI) in aquifers can exceed that limit by tenths of μg/L, while anthropogenic Cr(VI) concentrations can exceed 10,000 μg/L, such as in the Asopos river basin in Greece [17–21]. Consequently, it is important to establish adequate treatment methods to rapidly reduce Cr(VI) to Cr(III) in Cr-contaminated aquifers in order to protect the environment and public health.

Traditional technologies for chromium removal from groundwater, such as adsorption, chemical reduction and precipitation, electrocoagulation and ion exchange, are rapid, effective and applicable to highly contaminated sites [22,23]. However, these techniques also present several disadvantages such as high capital and operational cost, production of hazardous by products, etc. [3,24–26]. On the contrary, biological reduction of hexavalent chromium is a cost effective, environmentally friendly and sustainable method of remediating polluted groundwater in situ, which can be achieved by stimulating the indigenous microbial populations in soils and/or groundwater through the addition of various carbon sources [26–28].

Biostimulation may exert a two-fold positive effect on Cr(VI) bioremediation efficiency. Carbon addition causes an increase in growth rates of indigenous bacteria that are able to directly reduce Cr(VI) biologically, through the production of chromate reductase [29–32] and the development of anaerobic conditions that result in the production of Fe^{+2} by iron reducing bacteria, which chemically reduces Cr(VI) to Cr(III) [33–35]. Thus by cycling minor amounts of iron, a significant amount of Cr(VI) could potentially be reduced to Cr(III) [36–39]. Therefore, stimulation of anaerobic processes in situ can be an effective treatment alternative for Cr(VI)-polluted waters and has the potential for widespread application.

Several organic electron donors (acetate, glucose, lactate, yeast, etc.) have been tested for their potential to enhance Cr(VI) bioreduction [40,41] and for their suitability for industrial scale application. Molasses and emulsified vegetable oil (EVO) are two organic substrates with different properties, that have recently gained ground as a means to remediate contaminated aquifers in situ. Molasses is a waste by product of sugarcane refining that contains sugars (sucrose, glucose and fructose) and small amounts of polyphenols and vitamins [42]. It is a low cost substance that at acidic pH can chemically reduce Cr(VI) to Cr(III), but at alkaline pH acts as a readily available carbon and energy source for microbes [43,44]. One major drawback is that it is rapidly biodegraded, thus requiring frequent injections to aquifers and increasing the operation costs [45,46]. In contrast, EVO are slowly soluble substrates, that ferment to acetate and hydrogen [47], thus providing longevity to the remediation process. However, their colloidal nature affects their mobility in porous materials [48,49] and thus their effective distribution. Moreover, laboratory studies have shown that the presence of EVO decreases microbial richness and diversity [50]. In view of the above, the objective of this study was to assess the influence of two carbon electron donors (molasses and EVO) on potential in-situ microbial Cr(VI) reduction to Cr(III) and to investigate Cr(VI) reduction by a coupled biotic–abiotic pathway in the presence of iron reducing bacteria. In order to simulate closely natural conditions, we conducted column experiments with soil samples collected from the deep aquifer in the Asopos river basin region, while Cr(VI) groundwater concentrations were chosen in the 1000–2000 μg/L range to indicate the average pollution levels in this area. The ultimate goal of this particular study was to be able to propose a comprehensive, environmentally friendly and cost-effective way to remediate polluted aquifers in situ.

To the best of our knowledge this study is the first to investigate biotic and abiotic Cr(VI) removal by conducting experiments with natural soil and by applying biostimulation to modify the existing natural occurring bacteria. There are indeed some studies [50–52] that have studied Cr removal in column studies, however these studies employed bioaugmentation and were conducted using sand columns. It should be underlined that biaugmentation acceptance at least in Europe is low due to time-consuming permits and regulations that require detailed risk assessment studies to prevent significant perturbations to the environment and contamination of native flora at a site [53].

2. Materials and Methods

2.1. Descpription of Study Area

The study area is located at the Asopos River Basin in the Region of Sterea Ellada, Greece. Asopos River Basin accounts for approximately 20% of the total national industrial

production and experience significant pressures on both the quantity related to increased water abstraction and lowering the respective water levels, and quality related to pollution from point and diffusive sources. The surface and groundwater systems of the Asopos River Basin present high concentrations of chromium and hexavalent chromium both in surface waters and groundwater, a situation that has generated considerable public concern. The groundwater is used mostly for irrigation purposes, and to a lesser extent for drinking water supply. This research work stemmed from a LIFE project CHARM (http://www.charm-life.gr, accessed on 26 November 2021) that aimed to develop and apply remediation technologies and policy measures to protect groundwater from hexavalent Cr pollution in Asopos river basin. During the project a "hot spot" was identified in a heavily industrialized area at Inofyta in the Asopos river basin and a groundwater monitored program was carried out. For the hot spot identified, new boreholes were designed and properly constructed in strategically selected locations to monitor Cr(VI) pollution. The results confirmed the high Cr(VI) groundwater pollution mainly at Inofyta with Cr(VI) concentrations within the 6–10,103 μg/L range.

Soil samples used for the column experiments were collected from 28–28.5 m deep, transported to the lab and stored at 4 °C. The soil main properties and components were determined with XRF analysis and are presented in Table 1. The soil was sandy clay loam and its main components were CaO (30.03%) and SiO_2 (19.04%). Cr and Ni were the main metals present (2195 mg/kg and 1082 mg/kg respectively), which was expected, as the soil originated from a heavily contaminated industrialized area. The soil pH was 8.31.

Table 1. Properties and characteristics of the soil used in the column experiments.

Main Geochemical Properties	
Texture	58.8% Sand 15.5% Silt 25.8% Clay
Particle density (ρp, g/cm^3)	2.55
Specific surface area (m^2/g)	42.08
pH	8.31
ORP (mV)	214.7
NP (g $CaCO_3$/kg)	583.4
NP (mol $CaCO_3$/kg)	5.83
Loss on ignition (%)	26.72
Total carbon C (%)	7.02
Organic C (%)	0.16
Main Components (%)	
CaO	30.03
SiO_2	19.04
MgO	9.50
Fe_2O_3	5.08
Al_2O_3	1.20
Na_2O	0.64
MnO	0.13
Trace elements (mg/kg)	
Cr	2195
Ni	1082
Co	118
Ba	45
Cu	21.1
Cd	3.1
Hg	2.5
As	<20
Pb	<9

2.2. Description of Lab-Scale Column System

Experiments were carried out using two soil-filled up-flow columns made of cell cast acrylic material (plexiglass). The internal diameter of each column was 50 mm and the length 300 mm. Cr(VI) solutions were kept in two 5 L anaerobic containers under an inert nitrogen atmosphere and a two head peristaltic pump (Shenchen LabM6, Baoding Shenchen Precision Pump Co., Ltd, Baoding, China) was used to connect the reactors with the columns. The soil columns were operating under saturated conditions with their pores entirely filled with water. The effluent of each column was recirculated into the container, in order to better simulate the conditions of an aquifer and its flow velocity. Flow was set at 1 L per day resulting to an average pore velocity of approximately 0.91 m/day. In order to avoid algae growth, the columns and the containers were wrapped with aluminum foil to prevent sun light penetration. The experimental set-up is shown in Figure 1. It should be underlined that the two columns were operated under identical conditions.

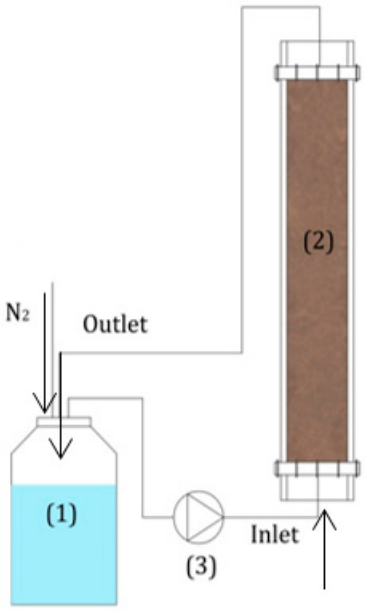


Figure 1. Scheme of the column lab-scale system: (1) 5 L Container with Cr(VI) solution, (2) Column filled with soil, (3) Peristaltic pump.

2.2.1. Soil Pretreatment and Filling Procedure of Columns

Prior to the commencement of the experiments, soil was air-dried and the soil agglomerates were broken down by gently tapping with a hammer. The experiments were conducted using the soil fraction (<2 mm) as the work sample. The soil was placed manually in the columns and gently vibrated at several stages to ensure uniform packing. The detailed properties of the columns are given in Table 2.

Table 2. Column properties and operating conditions.

Parameter	Soil Column	
	I	II
Soil weight, M (g)	649	670
Column internal diameter, d (mm)	50	50
Column height, L (mm)	300	300
Bed Volume, BV (cm^3)	589	589
Dry bulk density, ρ_p (g/cm^3)	1.102	1.137
Porosity, θ	0.568	0.554
Pore Volume, Vpv (cm^3)	334.54	326.30
Filter	Glass wool	Glass wool

2.2.2. Preparation of Experimental Solutions

To perform the experiments, three distinctive aqueous solutions were prepared. The organic load solution was prepared by dissolving 0.2 g concentrated molasses (Thermo Fischer Scientific, Waltham, MA, USA) and 0.8 g of concentrated EVO (JRW Bioremediation, L.L.C., Lenexa, KS, USA) to 1L of filtered groundwater in order to achieve a total COD of approximately 1600 mg/L. Properties and characteristics of molasses and EVO are presented in Table 3. Molasses and EVO were added as a mixture in order to provide immediate stimulation to the microbial community and enable microbial diversity. For the ferrous iron solution, 312 mg of ferrous sulfate heptahydrate (Thermo Fischer Scientific) was dissolved in 1 L distilled water which was previously pre-treated with nitrogen gas to remove dissolved oxygen. The columns were fed with a $K_2Cr_2O_7$ solution (Sigma-Aldrich, St. Louis, MO, USA) that was prepared with filtered groundwater, in which hexavalent chromium was added, at an initial concentration of 1000–2000 ppb. All groundwater used in the experiments was from the National Technical University of Athens campus water supply network.

Table 3. Properties and characteristics of molasses and EVO used in the experiments.

Properties	Molasses	Emulsified Vegetable Oil
Molecular Formula	$C_6H_{12}NNaO_3S$	CH_3-CHOH-COONa
Appearance/Physical state	Dark brown liquid	Clear to light yellow liquid
Components	-	60% Lactate/40% Water
Density	1.4 g/mL	-
Viscosity	-	100cP (at 20 °C)
Molecular weight	201.22 g/mol	112.07 g/mol
pH	5.1	6.0–8.5

2.3. Experimental Methods

2.3.1. Start-Up Protocol

In order to achieve soil enrichment with native microorganisms, each column was saturated with unfiltered groundwater from the National Technical University of Athens campus water supply network and incubated under anaerobic conditions for 14 days.

2.3.2. Control Experiment

After the start-up period, both columns were supplied for two weeks via recirculation with a 4L Cr(VI) solution in order to evaluate the reductive capacity of each soil column without the addition of any reducing agent.

2.3.3. Bio-Stimulation Experiment

To evaluate biologically induced Cr(VI) removal, an aqueous solution that contained the necessary organic load for the biological processes was recirculated in the two columns for 2 days. The experiment then proceeded by continuously recycling Cr(VI) solution without the addition of any reducing agent, that had an initial volume of 4 L and a Cr(VI) concentration of 1000 μg/L. During each run the initial Cr(VI) concentration was equal to approximately 1 mg/L and each run was carried out till Cr(VI) was completely removed from the feeding solution. This part of the experiment continued until the columns' Cr(VI) reducing capabilities decreased significantly. Samples from the inlet and the outlet of the columns were analysed frequently and the results were recorded for further processing.

2.3.4. Biotic-Abiotic Cr(VI) Removal Experiment

The objective of this experimental series was to evaluate the potential reduction of Fe(III) to Fe(II) (Fe(II) regeneration) with the addition of organic carbon sources and to study a coupled biological and chemical Cr(VI) removal pathway. In order to accomplish this, each column was supplied at first with 1 L of the ferrous iron solution for one day. After the 1 day feeding period, contaminated groundwater containing 1000 μg/L Cr(VI), was

introduced in the columns without e addition of any reducing agent and the outlet of the column was returned in the feed tank and then recycled back to the column continuously. This part of the experiment continued until no further Cr(VI) removal was reported. The experiment proceeded by supplying the columns with the organic donors solution, as was described in Section 2.2.2. The experiment continued by recirculating 4 L of groundwater with 1000–2000 ppb hexavalent chromium concentration. The reductive capacity of both columns was re-evaluated by collecting samples frequently from the inlet and the outlet of the columns.

2.4. Analytical Procedures

Samples were collected frequently from inlet and outlet of the columns and were analyzed for pH, DO, total and hexavalent chromium, total and soluble COD and ferrous iron Fe(II). The parameters of pH and DO were determined by a WTW™ MultiLine™ 3410 Portable Digital Multiparameter (Fisher Scientific, Göteborg, Sweden). Throughout the experiments, pH did not exhibit any significant changes and was approximately 8.37 ± 0.11, while DO was determined in order to verify the anaerobic conditions in the column systems. Total chromium was determined by a LCS313 Hach cuvette test [54]. Chromate in solution was measured colorimetrically using the USEPA 7196a method (1,5-diphenylcarbazide method) [55]. COD was determined by USEPA approved standard method 5220 D [56]. Ferrous iron Fe(II) was determined by the 1,10-phenanthroline method [57].

Soil permeability was determined by applying the constant head permeability test based on the principle of Darcy's Law [58]. Permeability was calculated as follows:

$$K = V \times L/(A \times \Delta H \times t), \quad (1)$$

where K: coefficient of permeability (cm/s), V: collected volume of water (mL), ΔH: head difference (cm), L: length of soil sample (cm), t: time required to collect V volume (s) and A: cross sectional of the soil sample (cm^2).

The test was carried out on soil samples with A = 19.63 cm^2, ΔH = 104.5 cm and length L = 30 cm (compacted at dry bulk density equal to 1.102 g/cm^3 and 1.137 g/cm^3 respectively for Columns I and II). Measurements were repeated three times. Soil permeability was measured immediately before the experiments commenced and after they concluded, with the exception of the biotic-abiotic experiment, where it was also evaluated immediately after the addition of ferrous iron.

In order to identify the different groups of microbes that participated in Cr(VI) reduction, FISH method was employed according to the protocol described by Mamais et al. [59] and Panousi et al. [60]. Liquid samples were collected immediately after the Control experiment and after the biotic-abiotic Cr(VI) removal experiment. The probes used, as well as the formamide and NaCl concentrations are shown in Table 4. Microscopic observations were carried out at 1000× magnification utilising an epifluorescence microscope (E50i, Nikon Instruments Inc., Melville, NY, USA). The results were estimated as ratios of target probe/total DAPI stained cells.

Table 4. Probes used for FISH analysis.

Target Microorganisms	Oligonucleotide Probe	Sequence (5′ to 3′)	Formamide Concentration (%*v*/*v*)	Reference
Most Bacteria	EUB338	GCT GCC TCC CGT AGG AGT	35	[61]
Most Archaea	ARCH915	GTG CTC CCC CGC CAA TTC CT	35	[62]
Methanosarcina & Methanosaeta spp.	MSMX860	GGC TCG CTT CAC GGC TTC CCT	45	[63]
Gammaproteobacteria	GAM42a	GCC TTC CCA CAT CGT TT	35	[64]
Deltaproteobacteria	DELTA495a	AGT TAG CCG GTG CTT CCT	35	[65]
Desulfobacteraceae & Syntrophobacteraceae	DSBAC357	CCA TTG CGC AAA ATT CCT CAC	35	[66]
Acetobacterium spp	AW	GGC TAT TCC TTT CCA TAG GG	30	[67]
Geobacter spp.	GEO3A	CCG CAA CAC CTA GTA CTC ATC	30	[68]
Desulfovibrio spp.	SRB687	TAC GGA TTT CAC TCC T	15	[69]

2.5. Data Calculation

The experimental data from the inlet of both columns were plotted in graphs with the values $\ln(C_t/C_{initial})$ versus time, in order to evaluate the first order equation constant ($k_{Cr(VI)}$), describing Cr(VI) removal:

$$\ln\left(\frac{C_t}{C_{initial}}\right) = -k \times t, \quad (2)$$

$$k = k_{Cr(VI)} \times \left(\frac{V_{soil}}{V_{liquid}}\right), \quad (3)$$

where $C_{initial}$: initial Cr(VI) concentration at the start of the experiments (mg/L), C_t: Cr(VI) concentration over time remaining in the recycling solution (mg/L), t: time/the experimental day at each run, k: slope of the linear trendline produced on the graphs, $k_{Cr(VI)}$: the first order Cr(VI) removal constant (d^{-1}), V_{soil}/V_{liquid}: volume ratio between the soil and the water that was treated.

The parameter V_{soil}/V_{liquid} was employed to represent the fact that at higher soils to water ratios the water comes in contact with a higher amount of soil that contains the added reducing agents and therefore is expected to remove Cr(VI) at a higher rate. In a saturated aquifer the V_{soil}/V_{liquid} can be calculated based on the porosity by the following equation:

$$\left(V_{soil}/V_{liquid}\right) = ((1-\Theta)/\Theta), \quad (4)$$

where Θ is the porosity of the soil.

The graphs used for the determination of the $k_{Cr(VI)}$ constant are provided in Supplementary Material (Figures S1–S8).

The biotic experiments were conducted at room temperature at 17 °C ± 2, whereas the rest were carried out at 25 °C ± 2. The reduction rates from the biotic experiments were corrected at 25 °C ± 2 using the Arrhenius equation.

$$k = A \times \exp[-E_a/(R \times T)], \quad (5)$$

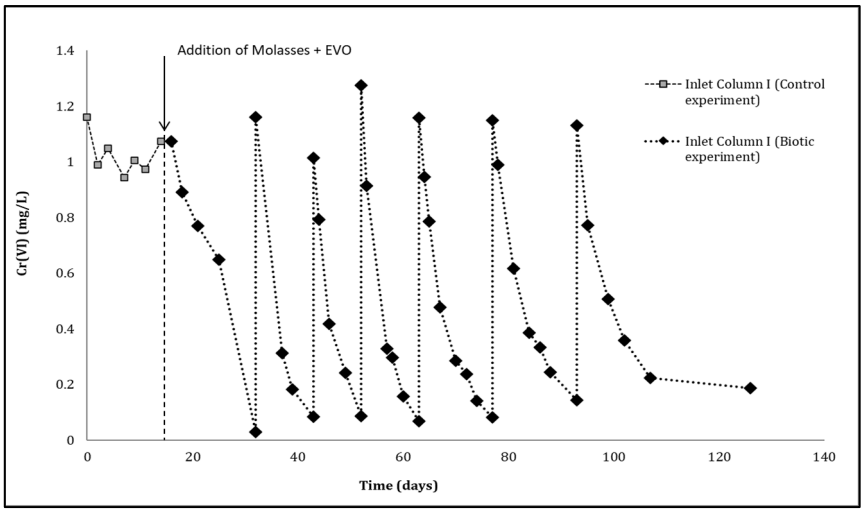
where k: Cr(VI) removal rate (d^{-1}), A: a frequency factor (d^{-1}), E_a: activation energy of the process (J/mol), R: the ideal gas constant (8.314 J/mol/K), T: temperature (Kelvin).

Values for the correction were used from studies for chromium bioreduction under anaerobic conditions [59,70,71].

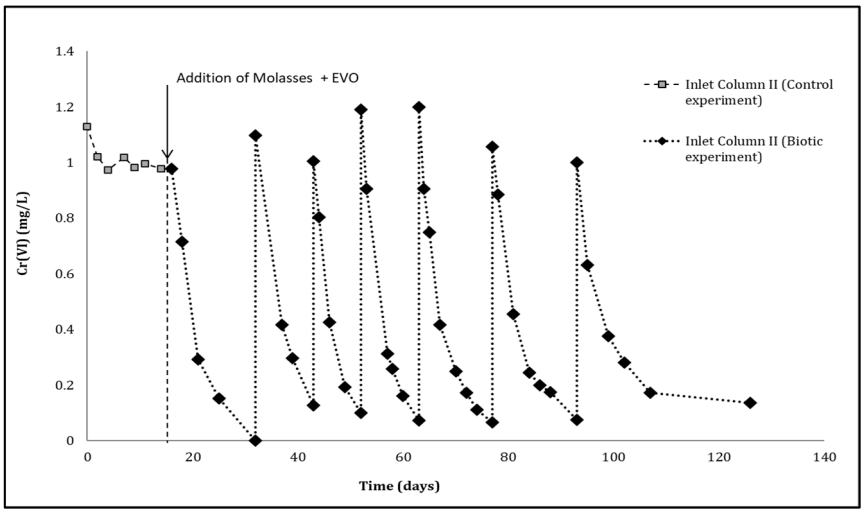
3. Results and Discussion

3.1. Natural Soil Capacity Experiment (Control)

During the control experiments, the overall natural Cr(VI) removal capacity of the soil was very low (approximately 1.12 ± 0.21 mg (Cr(VI)/kg soil). As observed in Figure 2, the inlet Cr(VI) concentrations remained close to the spiked concentration throughout the experiment. The $k_{Cr(VI)}$ removal rate was approximately 0.16 ± 0.05 d^{-1}. Other soil column laboratory studies carried out with sterile and non-sterile soils have reported similarly low Cr(VI) removal rates [50,72].



(**a**)



(**b**)

Figure 2. Cr(VI) profiles in the inlet of Columns I (**a**) and II (**b**) during the control and bio-enhancement experiments.

3.2. Influence of Organic Electron Donors in Cr(VI) Bio-Reduction

Cr(VI) biotic removal capacity of both columns was evaluated by feeding with Cr(VI) contaminated groundwater for 110 days following the protocol described in Section 2.3.3. Overall, seven experimental runs were conducted during this period. During each run the initial Cr(VI) concentration was equal to approximately 1000 μg/L and each run was carried out until at least 90% of Cr(VI) concentration was removed from solution. The total COD retained in the columns from the 2-day feeding period, the total amount of Cr(VI) reduced and the total biotic Cr(VI) removal are shown in Table 5. Comparing the experimental

results from the two columns, it is shown that both exhibited the same behaviour. During each run, as illustrated in Figure 2, the biostimulated microbial community grown in the soil matrix was able to completely remove Cr(VI) from the feed solution in approximately twelve days (runs 2–6). Excluding the last run, where the organic e^- donor was completely depleted, the biotic Cr(VI) removal rates ranged from 1.74 to 4.35 d^{-1}, while the majority of them was in the 3–4 d^{-1} range (36%). Comparing these results with the results from the Control experiment, biostimulation was achieved and removal rates increased up to 20 times.

Table 5. Experimental results from the biotic experiment.

Experiment	Parameter	Soil Column	
		I	II
Biotic experiment	Retained COD (mg COD/kg soil)	1834	1798
	Duration of experiment (days)	110	110
	No of experimental runs	7	7
	Mass of reduced Cr(VI) (mg)	22.41	21.06
	Total Cr(VI) removal (mg Cr(VI)/kg soil)	34.53	31.44
	Soil permeability reduction (End of experiment) (%)	44.92	63.32

Addition of single organic electron donors such as molasses or EVO has been evaluated in several laboratory scale studies [40,44,45,49–51,73]. Results from previous studies are in agreement with the findings of this work and indicate that both carbon sources are able to support complete Cr(VI) reduction even at high initial Cr(VI) concentrations, that far exceed pollution levels in the environment.

3.3. Biotic–Abiotic Cr(VI) Removal

In order to examine the hypothesis that the addition of carbon sources in the presence of iron results in the development of reducing conditions and the stimulation of iron reducing bacteria that leads to a constant production of Fe(II), columns experiments were carried out as described in Section 2.3.4. The experiments were conducted in two phases: during the 1st phase only abiotic Cr(VI) removal was evaluated while during the 2nd phase combined abiotic–biotic Cr(VI) removal was studied.

3.3.1. Abiotic Cr(VI) Removal

Initially both columns were fed with 1 L of ferrous iron solution (concentration of 50 mg/L Fe^{+2}) for 1 day and Cr(VI) contaminated groundwater for 45 days. As shown in Figure 3a,b ferrous sulfate addition resulted in Cr(VI) removal from groundwater by Cr(VI) reduction, adsorption and co-precipitation. However the efficiency of the abiotic removal was very limited and was approximately 0.77–2.52 mg Cr(VI), because a large portion of Fe(II) added was passivated decreasing the overall performance and longevity of the process. The results also indicated that the reductive capability of column I was slightly better than column II, although that difference was not significant.

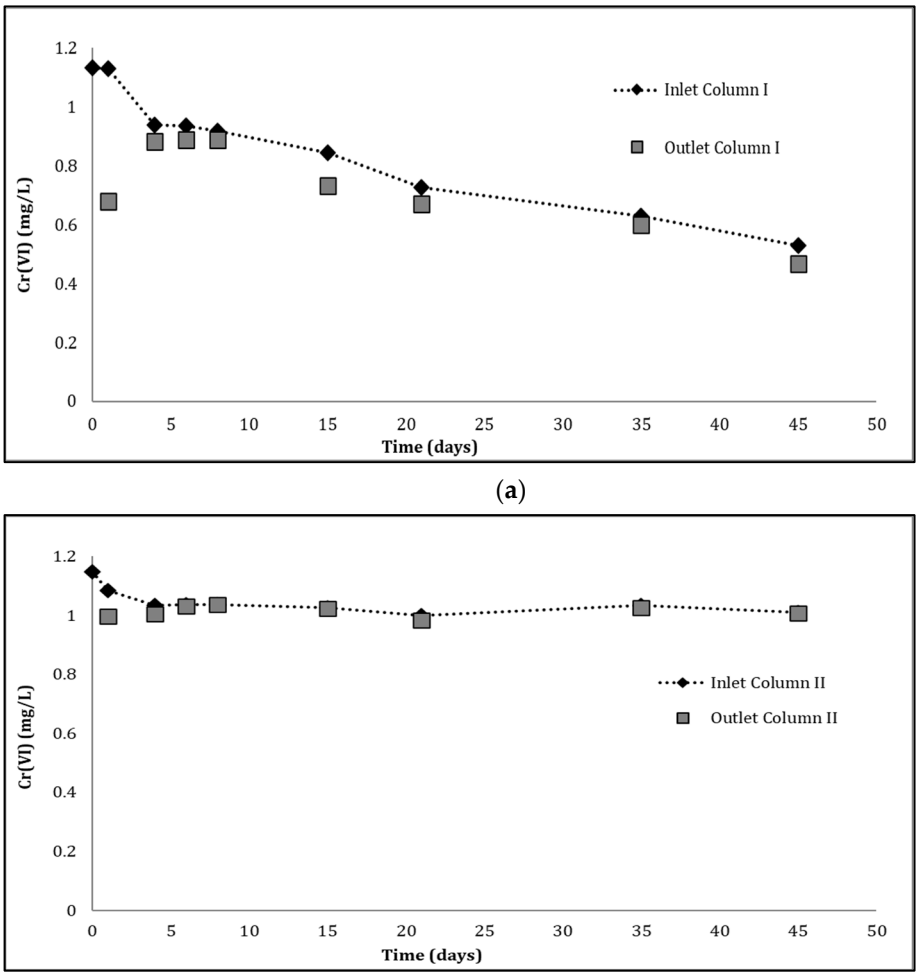


Figure 3. Cr(VI) profiles in the inlet and outlet of Columns I (**a**) and II (**b**) after the addition of ferrous iron.

Hexavalent chromium and ferrous iron react chemically as follows, producing non-toxic trivalent chromium:

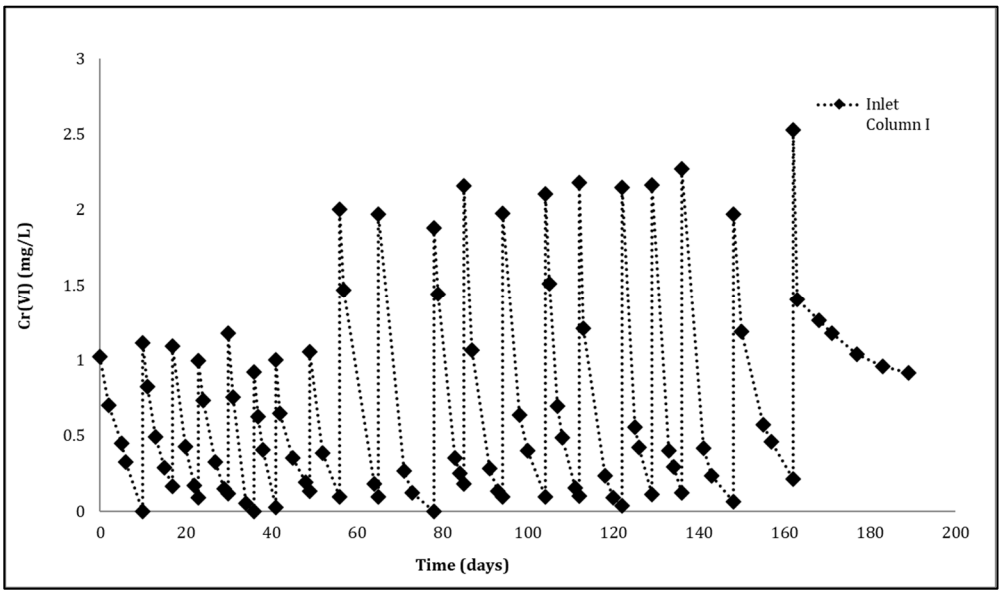
$$0.25\ CrO_4^{2-}(aq) + 0.75Fe^{2+}\ (aq) + 2H_2O \rightarrow Cr_{0.25}Fe_{0.75}(OH)_3(solid) + H^+ \quad (6)$$

Taking into consideration that the theoretical stoichiometric ratio of Cr(VI) removal is 0.31 gCr(VI)/gFe(II) added, it is obvious that most of Fe(II) added was unavailable for Cr(VI) reduction. The soil and groundwater pH (8.37 ± 0.11) and composition, favour the formation of iron containing solids, such as siderite ($FeCO_3$), ferrous sulfide (FeS), pyrite (FeS_2), and according to the data of this study, less than 16% of ferrous iron was soluble and thus effective. It is noted that no ferrous iron was detected in the inlet and outlet of the columns. Visual inspection of the soil in the column also showed that a brown-red solid was formed inside the soil column that reached less than 20% of the total column length, indicating the formation of $FeCO_3$ and that Fe(II) had limited mobility in this soil matrix.

3.3.2. Combined Biotic–Abiotic Cr(VI) Removal

Following the exhaustion of abiotic Cr(VI) removal the two columns were fed for two days with organic electron donors to study combined abiotic and biotic Cr(VI) removal. Figure 4 and Table 6 show the results from the two columns following addition of the organic electron donors. The columns were fed with Cr(VI) contaminated groundwater until the columns reductive capabilities were almost completely exhausted. During the

first eight runs, the initial Cr(VI) concentration was equal to approximately 1000 μg/L. Due to the significant Cr(VI) removal efficiency of both columns the remaining runs were conducted with an initial Cr(VI) concentration that was approximately 2000 μg/L. In Column I, total Cr(VI) removal was 26% higher in comparison to Column II and the experiment lasted for 30 more days. As shown in Figure 4a,b, both columns were able to completely remove Cr(VI) from the feed solution in approximately eight days. Furthermore, Cr(VI) concentration in column outlets was usually not detected. Excluding the last run, where the organic e^- donor was exhausted, Cr(VI) removal rates ranged from 2.44 to 7.38 d^{-1}, while the majority of them was between the 3–4 d^{-1} range (50%).



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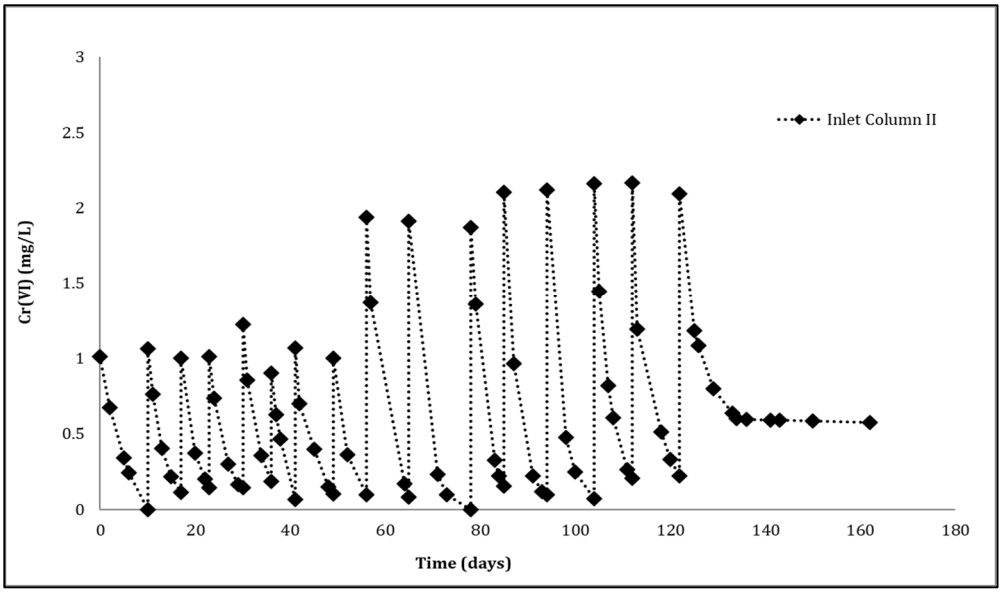

(**b**)

Figure 4. Cr(VI) profiles in the inlet of Columns I (**a**) and II (**b**) in the Biotic-Abiotic experiment after the addition of the carbon sources.

Table 6. Experimental results from the Biotic-Abiotic experiment.

Experiment	Parameter	Soil Column	
		I	II
Biotic-Abiotic experiment	Retained COD (mg COD/kg soil)	2354	2360
	Duration of experiment (days)	189	162
	No of experimental runs	20	16
	Mass of reduced Cr(VI) (mg)	82.8	63.2
	Total Cr(VI) removal (mg Cr(VI)/kg soil)	127.51	94.27
	Soil permeability reduction immediately after the addition of Fe^{+2} (%)	16.63	16.73
	Soil permeability reduction (End of experiment) (%)	89.91	94.33

FISH analysis (Figure 5) indicated a shift in the microbial community after the addition of the organic and inorganic electron donors. *Eubacteria* remained the predominant microorganism throughout the experiment, however, at the end of the experiment *Archaea* had increased from 5% to 35%, while *Methanosarcina* spp. and *Methanosaeta* spp. increased from 0.5% to 16%. This increase was apparently due to the establishment of anaerobic conditions. *Geobacter* spp., *Gammaproteobacteria* and *Deltaproteobacteria* remained minimal throughout the experiment since nitrates and sulfates were not present in the solution.

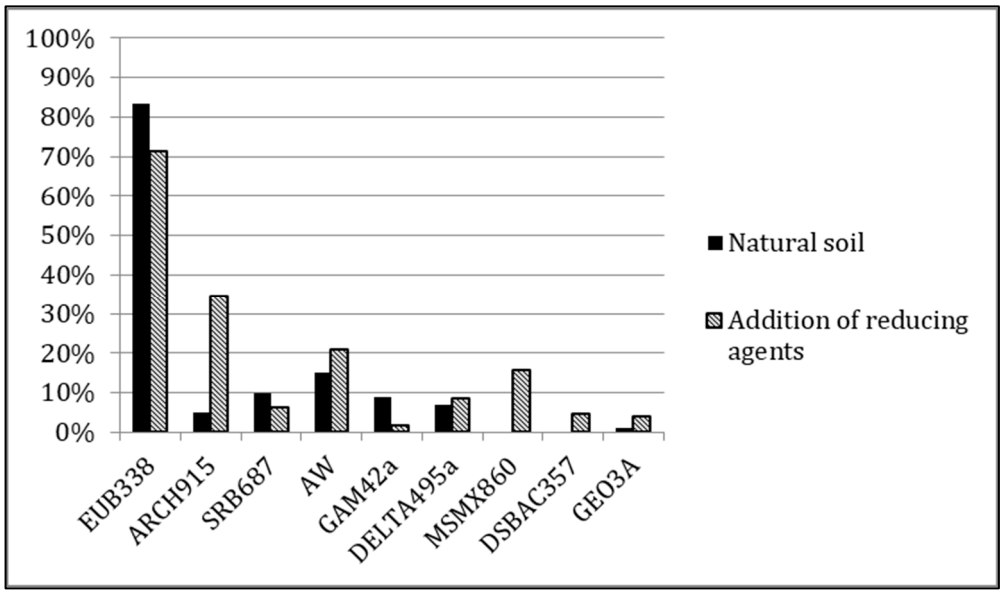

Figure 5. FISH microbial analysis results on natural soil and after the addition of the reducing agents (Molasses, EVO and $FeSO_4$).

3.4. Comparison between Biotic and Biotic-Abiotic Experiment

Figure 6 depicts Cr(VI) removal rates from the biotic and the biotic-abiotic experiments for each experimental cycle. Comparing the removal rates from the two experiments, it is observed that during the first four experimental runs, $k_{Cr(VI)}$ are similar for both experiments (3.90 ± 0.35 d^{-1} and 4.38 ± 0.63 d^{-1} respectively). However, after the 5th run they exhibited completely different behavior. In the biotic experiment, the removal rate declines gradually until the termination of the experiment on the 7th run where it reaches 0.64 ± 0.01 d^{-1}. On the other hand, the biotic-abiotic experiment lasted much longer, 16–20 runs, with the removal rates increasing considerably in the 5th and 6th run (5.42 ± 2.35 and 6.24 ± 1.61 respectively), while remaining in the 3–4 d^{-1} range for the whole duration of the biotic-abiotic experiment. Therefore, although the addition of Fe(II) combined with molasses and EVO appear not to exert a significant effect on the magnitude

of the Cr(VI) removal rate, it affects positively the longevity of the remediation process. Moreover, the combined abiotic and biotic removal resulted in a much longer life span that exceeded by approximately 48–72% the biotic removal. In addition, coupled biotic and abiotic process demonstrated a 67–73% increase in total Cr(VI) removal (mg Cr(VI)/kg soil). The experimental results illustrate that the addition of organic electron donors can lead to the regeneration of Fe(II) and the coupled abiotic and biotic processes can significantly improve Cr(VI) removal from groundwater. Wen et.al [50] have reported that the presence of EVO substrates improves microbial dissimilatory Fe^{+3} reduction, resulting in higher Fe^{+2} production. Furthermore, according to several studies [74–76], anaerobic microbial processes improve abiotic Cr(VI) reduction by Fe(II) by generating highly reactive minerals such as mackinawite, green rusts and magnetite, which may favour the removal of Cr(VI) by mechanisms such as adsorption, co-precipitation and/or reduction. It should be noted that the analyses performed during the course of the experiment demonstrated that Fe^{+2} concentration was less than 0.2 mg/L (detection limit) at both the inlet and the outlet of the columns, which is explained by (a) the limited mobility of iron in this particular soil matrix, (b) the fact that in the presence of Cr^{+6}, Fe(II) was oxidized to Fe(III) and (c) the potential creation of Cr-Fe complexes [51].

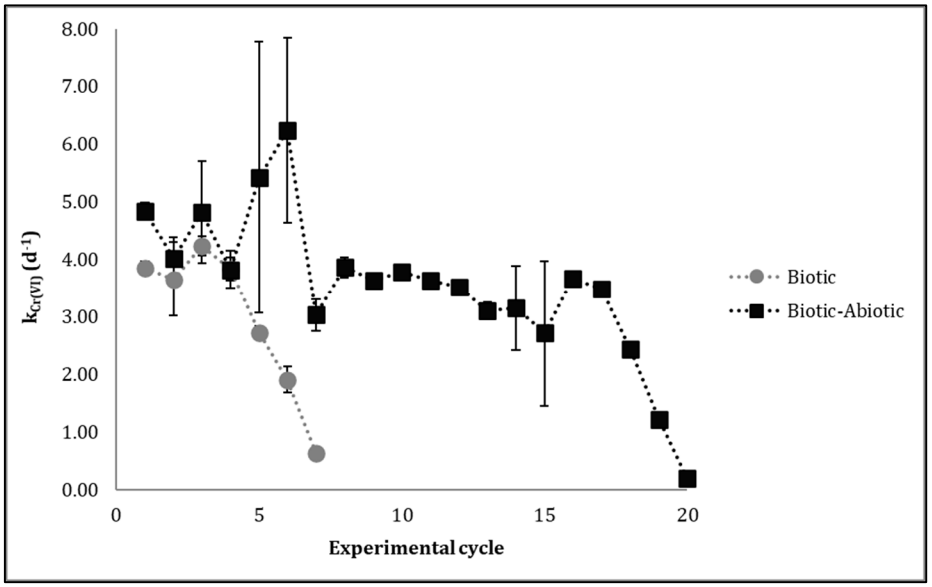

Figure 6. Time profiles of Cr(VI) reduction rates in the Biotic and Biotic-Abiotic experiments for each experimental cycle (error bars are equal to standard deviation).

It is also worth noting that, as shown in Tables 5 and 6, during the biotic-abiotic experiment soil permeability was reduced by 92.1 ± 3.1%, while in the biotic experiment only by 54.1 ± 13.0%. This difference in soil permeability reduction is attributed to physical clogging due to the addition of ferrous sulfate and the creation of insoluble Cr(III).

The presence of Cr(III) in the soil of both columns was confirmed with Graphite furnace atomic absorption analysis when the experiments were terminated. Total Cr concentration was 2339 ± 28 mg/kg, which is close to the expected concentration, if we take into consideration the initial concentration of Cr was 2195 mg/kg and the amount of Cr(VI) removal.

4. Conclusions

The aim of this study was to examine the potential microbial Cr(VI) reduction to Cr(III) by biostimulation through the addition of two carbon sources (molasses and EVO) and to assess the potential of Cr(VI) reduction by a coupled biotic–abiotic pathway. Cr(VI) conversion rates and total Cr(VI) removal were investigated in soil columns under anaerobic

conditions. The findings from the experiments could be summarized into the following conclusions.

- Natural soil attenuation in the Cr-contaminated aquifer studied was low and was significantly exceeded by the amount of Cr(VI) in contaminated groundwater;
- Addition of organic electron donors can increase Cr(VI) reduction rate up to 20 times in comparison to the natural soil capacity rates;
- Ferrous iron has low mobility in soil and groundwater with a pH close to 8 and is rendered passive quickly, leading to insignificant Cr(VI) removal;
- Combined biotic-abiotic Cr(VI) removal exhibited a longer-life span in the remediation of Cr(VI)-contaminated groundwater in comparison to biotic process.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w14010089/s1, Figure S1: Time profile of Cr(VI) during the Control experiment in Column I.; Figure S2: Time profiles of Cr(VI) during the biotic experiment in Column I.; Figure S3: Time profiles of Cr(VI) during runs 1–12 of the biotic-abiotic experiment in Column I.; Figure S4: Time profiles of Cr(VI) during runs 13–20 of the biotic-abiotic experiment in Column I.; Figure S5: Time profile of Cr(VI) during the control experiment in Column II.; Figure S6: Time profiles of Cr(VI) during the biotic experiment in Column II.; Figure S7: Time profiles of Cr(VI) during runs 1–12 of the biotic-abiotic experiment in Column II.; Figure S8: Time profiles of Cr(VI) during runs 13–16 of the biotic-abiotic experiment in Column II.

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