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Biotreatment of Cr(VI) contaminated waters by sulphate reducing bacteria fed with ethanol

F. Pagnanelli [a], C. Cruz Viggi [a,*], A. Cibati [a], D. Uccelletti [b], L. Toro [a], C. Palleschi [b]

[a] Department of Chemistry, Sapienza University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy
[b] Department of Biology and Biotechnology, Sapienza University of Rome, P.le A. Moro 5, 00185 Rome, Italy

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ABSTRACT

Biological treatment of Cr(VI) contaminated waters was performed in fixed bed reactors inoculated with SRB (sulphate-reducing bacteria) growing on ethanol. Treatment efficiency was evaluated by checking chemical abatement of Cr(VI) and by ecotoxicological tests using the nematode *Caenorhabditis elegans*. A preliminary comparison between ethanol and lactate was performed, denoting that using ethanol, the same values of final sulphate abatement were obtained. In addition ethanol showed to be a substrate more competitive than lactate in kinetic terms. Fixed bed column reactors were continuously fed with a solution containing sulphates ($3\,g\,L^{-1}$), ethanol ($1.5\,g\,L^{-1}$) and Cr(VI) ($50\,mg\,L^{-1}$). At steady state the column inoculated with SRB removed $65 \pm 5\%$ of sulphate and $95 \pm 5\%$ of chromium. Bioactive removal mechanisms predominated over biosorption. Diminution of Cr(VI) toxicity was assessed by using the nematode *C. elegans* as a test organism showing that the survival of nematodes was 20% in the presence of the untreated influent and raised up to 53% when the nematodes were exposed to the treated effluent.

1. Introduction

Sulphate-reducing bacteria (SRB) are anaerobes that use sulphate as final electron acceptor for the oxidation of various organic compounds (electron donors) [1].

Dissimilatory sulphate reduction results in alkalinity generation (reaction (1)) and metal precipitation as sulphide (reaction (2)):

$$SO_4^{2-} + 2CH_2O + 2H^+ \rightarrow H_2S + 2H_2CO_3 \quad (1)$$

$$Me^{2+} + H_2S \rightarrow MeS^{+}\downarrow + 2H^+ \quad (2)$$

Until recently, the use of SRB was limited to *ex situ* treatment in sulphidogenic bioreactors, but latterly attention has been placed on their application in *in situ* passive systems, such as artificial wetlands and Permeable Reactive Barriers (PRBs) [2–4].

The choice of electron donors is a central point in the treatment of metal-bearing wastewater by sulphate reduction processes. Selection of a suitable carbon source/electron donor is based on its degradability, cost and availability [5,6]. Low-molecular-weight organic compounds are generally used as electron donors by SRB [1]. Among these, lactate is widely used in lab-scale experiments, but its application in large scale would imply too high operative costs [7,8]. In literature, organic substances including wastes such as sewage sludge, wood chips, animal manure, vegetal compost, and other agricultural wastes are usually employed as alternative electron donors and carbon sources for SRB growth [9–13]. Nevertheless the heterogeneity of wastes, the reduced biodegradability of some of them (especially lignocellulosic materials) [13], the secondary release of COD and pathogens and the presence of side mechanisms hardly quantifiable in pollution removal (i.e. adsorption) [9] could hamper the applicability of such materials.

Therefore other organic substrates have been tested even in liquid form such as carboxylic acids and alcohols [6]. Among these ones, ethanol is an attractive electron donor due to its ease of availability, its low cost and because it can be obtained also from renewable sources [14,15]. Efficient sulphate reduction by SRB growing on ethanol as carbon source was observed by different authors [7,8,11,16].

Stoichiometry of ethanol oxidation by SRB can be represented by the following equation:

$$2C_2H_5OH + SO_4^{2-} \rightarrow 2CH_3COO^- + HS^- + H^+ + 2H_2O \quad (3)$$

Ethanol oxidation is usually incomplete and lead to acetate formation [8]. Acetate can be also used as an electron donor and carbon source in the sulphate reduction process:

$$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^- \quad (4)$$

* Corresponding author. Tel.: +39 06 49913333; fax: +39 06 490631.
E-mail addresses: francesca.pagnanelli@uniroma1.it (F. Pagnanelli), carolina.cruzviggi@uniroma1.it (C. Cruz Viggi), alessio.cibati@uniroma1.it (A. Cibati), daniela.uccelletti@uniroma1.it (D. Uccelletti), luigi.toro@uniroma1.it (L. Toro), claudio.palleschi@uniroma1.it (C. Palleschi).

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In the present work ethanol was tested as carbon and electron source for SRB in the treatment of hexavalent chromium [Cr(VI)] contaminated waters. This metal was chosen as target metal due to its high toxicity and because its conventional treatment is a two-step process: Cr(VI) reduction and the subsequent Cr(III) precipitation [17]. The use of SRB, creating potential redox and pH conditions suitable for chromium abatement, could integrate both steps into one.

The use of ethanol as electron donor for SRB for the treatment of Cr(VI) contaminated waters represents a novelty aspect. In fact, to our knowledge, the treatment of Cr(VI) has been performed only using lactate and organic wastewaters as electron donors for SRB [17,18].

In the literature the bioassessment of metal-bearing waters toxicity was performed using different test-organism systems such as microorganisms, daphnids, shrimps, fishes and plants [9]. These studies generally investigated toxicity effects associated with water samples collected in different polluted sites and did not involve bioassessment of treated effluents. Nevertheless, toxicity tests on column effluents (as a simulation of full scale permeable reactive barriers) can give preliminary information about the potential impact of treated effluents in the environment [19].

In this paper treated effluents were monitored for their toxicity by using *Caenorhabditis elegans* (*C. elegans*), an abundant free-living nematode in soil ecosystems which plays a key role in decomposition and recycling of nutrients [20]. This small nematode is usually found in the fluid phase of terrestrial habitats and, therefore, in direct contact with soil contaminants. It is the first multicellular organism whose genome has been completely sequenced [21], resulting in the amazing observation of a fairly high level of conservation with respect to the vertebrate genome sequences. In addition, due to the small size (~1.5 mm) and easy manipulation, this nematode has been extensively characterized and its developmental processes and behaviour can be easily monitored under a simple stereoscope. Because of these properties several toxicity tests using growth, reproduction and survival of nematodes as toxic endpoints have been developed for ecological risk assessment in soil [22–25].

This work aimed to investigate the performances of ethanol as electron donor in a continuous fixed bed column reactor filled with an inert material and inoculated with SRB for the treatment of Cr(VI) contaminated waters. Experimental data obtained from this system (resembling fluidodynamic conditions inside real PRBs) allowed to validate the treatment efficiency and to obtain kinetic parameters for the preliminary estimate of barrier thickness.

Novelty aspects of the present study are the use of ethanol as an electron donor in SRB for the treatment of Cr(VI), the comparison of equilibrium and kinetic characteristics of sulphate abatement obtained in batch and column reactors, the isolation of a bioactive mechanism strictly related to SRB activity from the contribution of sorbing properties of the inert material used as column filling, and the bioassessment of the process effectiveness by ecotoxicological in vivo tests.

2. Materials and methods

2.1. Sulphate-reducing bacteria (SRB)

SRB inoculum was a consortium, kindly provided by the research group of Professor Groudev (Department of Engineering Geoecology, University of Mining and Geology, Sofia, Bulgaria); the sample was collected in the Curilo mine district located near Sophia [26].

Bacteria used in batch experiments were cultivated in closed serum vials using standard procedures for SRB [1]. C medium, prepared in anaerobic conditions by supplying nitrogen, was used for bacterial growth: KH_2PO_4 0.5 g L^{-1}; NH_4Cl 1 g L^{-1}; Na_2SO_4 4.5 g L^{-1}; $CaCl_2{\cdot}6H_2O$ 0.06 g L^{-1}; $MgSO_4{\cdot}7H_2O$ 0.06 g L^{-1}; sodium lactate 6 g L^{-1}; yeast extract 1 g L^{-1}; $FeSO_4{\cdot}7H_2O$ 0.004 g L^{-1}; sodium citrate$\cdot 2H_2O$ 0.3 g L^{-1}. Na_2S (0.5 g L^{-1}) was added to C medium in order to create and maintain a reducing environment, necessary for SRB growth. Usually 20 mL of bacteria inoculum were added to 80 mL of C medium. All vials were incubated at room temperature (25 °C) under shaking conditions (150 rpm).

Table 1
Experimental design of factorial tests.

Test name	Factors		Operating conditions	
	A Ethanol	B Sulphate	Ethanol (g L^{-1})	Sulphate (g L^{-1})
1	–	–	1.5	1.5
a	+	–	3	1.5
b	–	+	1.5	3
ab	+	+	3	3

2.2. SRB growth in batch tests

Glass serum vials (120 mL) were used for all the experiments. 80 mL of Postgate C medium or modified C medium (ethanol instead of lactate) were added in the vials for batch tests with lactate and ethanol, respectively. The concentration of ethanol in C medium was 3 g L^{-1}, in order to obtain the same molar concentration of lactate (about 0.065 mol L^{-1}). Subsequently the vials were sealed and 20 mL inoculum of bacteria cultivated in C medium were added by a sterile syringe through the sampling port. All experiments were conducted at room temperature under shaking condition. pH, E_h, SO_4^{2-} and H_2S production were monitored for 40 days (a sample of 2 mL every three days). pH and E_h were determined by pH-meter CRISON GLP22, sulphate concentration was determined by a turbidimetric method [10] and H_2S production was checked by lead acetate paper. Each test was performed in duplicate and average values were reported.

The effect of ethanol and sulphate concentration was investigated performing batch growth tests arranged according to a full factorial design with 2 factors (A: ethanol; B: sulphate) taken at two levels. The standard order of this 2^2 factorial design is reported in Table 1. All batch tests were run simultaneously by using the same initial inoculum.

Batch tests were performed in serum vials like those previously described, filled with 80 mL of modified C medium (without lactate and with ethanol) and 20 mL of inoculum. Biomass growth was monitored in the different operating conditions by measuring pH, E_h, SO_4^{2-} and H_2S production for 30 days.

Experimental data were statistically analyzed by analysis of variances (ANOVA) with 95% of significance [27].

2.3. Cr(VI) removal in batch tests

Mechanisms of Cr(VI) removal were investigated in batch tests with ethanol as carbon source for SRB growth. Batch tests were performed in glass serum vials as above. Four batch tests with different medium composition were carried out:

- Test 1: 80 mL of modified C medium (ethanol 1.5 g L^{-1}) + 20 mL of SRB inoculum;
- Test 2: 100 mL of modified C medium;
- Test 3: 100 mL of modified C medium (without Na_2S);
- Test 4: 100 mL of distilled water (blank).

Samples were prepared as those previously described in Section 2.2, with the addition of metal spikes during SRB growth. 5 mL

of a Cr(VI) stock solution ($1\,g\,L^{-1}$) were added to vials in order to have an initial metal concentration of about $50\,mg\,L^{-1}$ (1 mM). Every five days a liquid sample was collected (5 mL) for the determination of the residual metal concentration; each time 5 mL of metal-bearing stock solution were added to the vials in order to maintain a constant total volume and in order to have an increase of metal concentration. Collected samples were filtered and H_2S production, pH, E_h, SO_4^{2-} and Cr(VI) and total soluble Cr concentrations were determined. Each test was performed in duplicate. Total soluble Cr was determined by an Inductively Coupled Plasma Optical Emission Spectrometer (Varian Vista-MPX CCD Simultaneous, ICP-OES). Cr(VI) was determined by the diphenylcarbazide assay [17].

2.4. Cr(VI) removal in fixed bed reactors

Column tests were performed using two fixed bed columns (one inoculated by SRB) (height 1 m; diameter 0.2 m; column volume, $V = 6.65 \times 10^{-3}\,m^3$) made of Plexiglas with 10 equally distant sampling ports along the axial length, numbered from the bottom to the top of the column. Both columns were packed with perlite (pore volume $V_0 = 3.5$ L). Columns were continuously fed ($F = 0.5\,mL\,min^{-1}$) with a solution containing sulphate ($3\,g\,L^{-1}$) and ethanol ($1.5\,g\,L^{-1}$). After biomass acclimatization, Cr(VI) ($50\,mg\,L^{-1}$) was also fed.

pH and E_h values of the influent solution were 6.7 ± 0.1 and 200 ± 20 mV, respectively. Samples from three different sampling ports (1, 5 and 9 at the bottom, at the centre and at the top of the column, respectively) were analyzed for pH, E_h and residual amounts of sulphates and chromium.

2.5. Nematode culture and determination of lifespan and progeny

The *C. elegans* strain SEK-1 (kindly provided by the *C. elegans* Genetics Centre, Minnesota) was used for the experiments and maintained on nematode growth medium (NGM) supplemented with *Escherichia coli* (*E. coli*) strain OP50. The NGM plates containing the solution before and after the column treatment were prepared under sterile conditions and ten-fold diluted in sterile distilled water as indicated.

Lifespan was determined according to [9]. Worms were allowed to lay eggs at 16 °C, and all progeny was observed daily for 72 h and counted with a Zeiss Axiovert 25 microscope (Thornwood, NY). Numbers of progeny (±standard deviation) were calculated from at least 10 individual animals along 72 h of exposition and repeated three times.

The software package JMP IN5.1.2J (SAS Institute Inc.) was used for statistical analyses. The means and standard errors of the life spans or the progeny for animals in each group were calculated and the significant differences in each nematode population were analyzed by the Kaplan–Meier method [28].

3. Results and discussion

3.1. SRB growth in batch tests

Sulphate reduction by SRB was preliminary tested in batch tests using liquid C medium containing lactate, the substrate conventionally used for SRB growth and maintenance. Biomass activity was evaluated by checking H_2S release and sulphate diminution along time (Fig. 1).

H_2S formation was observed in all batch experiments confirming that sulphate abatement was related to SRB metabolism. An abatement of sulphate of $65 \pm 2\%$ in 40 days was observed with a steep decrease during the first three days, followed by a slower

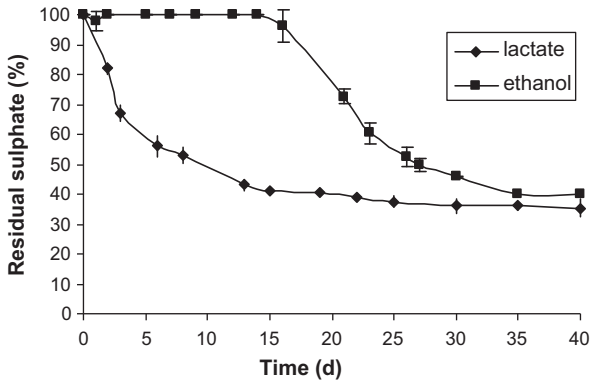

Fig. 1. Residual sulphates during time for SRB batch growth using lactate and ethanol.

removal. Results of sulphate reduction and operating conditions of pH and E_h (pH = 7.6 ± 0.4; $E_h = -330 \pm 20$ mV) obtained using C medium, can be taken as representative of optimal growth and performances of the SRB inoculum (optimal pH and E_h values for SRB growth are in the range of 7–8.5 and −150 to −350 mV, respectively) [1].

Data of batch experiments using ethanol are reported in Fig. 1. A long lag phase with no sulphate reduction, lasted about 15 days was observed, then a rapid removal of sulphate occurred until the 35th day. Final sulphate reduction ($60 \pm 5\%$ on 40th day) and operative conditions of pH and E_h (pH = 7.8 ± 0.1 and E_h -425 ± 5 mV) confirmed the SRB capacity of growing using ethanol [29].

Data from batch experiments, neglecting the initial lag phase, were modelled by a first-order degradation rate for sulphate [9], which gives a good representation of experimental data. A degradation rate constant of $0.0012 \pm 0.0002\,h^{-1}$ was obtained for batch tests with C medium (lactate), and $0.0025 \pm 0.0005\,h^{-1}$ for modified C medium (with ethanol). Even though similar final sulphate abatements were obtained using lactate and ethanol, ethanol degradation rate was higher than that of lactate. Then ethanol is not only suitable for SRB growth but also seems to be more promptly oxidized than lactate.

Preliminary optimization of SRB growth with ethanol, necessary for the following column experiments, was obtained by batch tests performed according to a full factorial design with 2 factors (A: ethanol concentration; B: sulphate concentration) taken at two levels (Table 1).

Fig. 2 shows residual sulphates during time in all the investigated conditions (described in Table 1). The significance of the effect of each factor on sulphate removal on 30th day was determined by

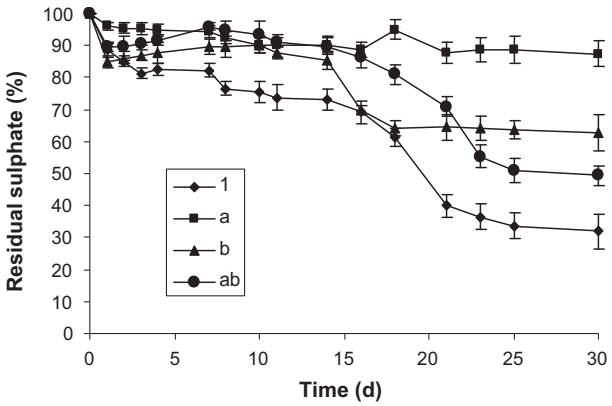

Fig. 2. Residual sulphates during time for SRB batch growth in the factorial experimentation.

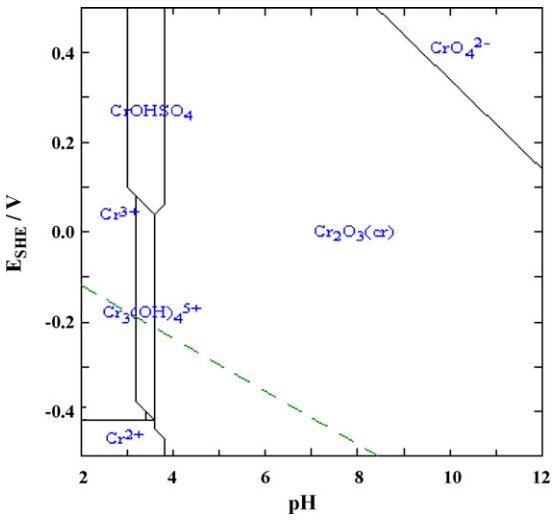

Fig. 3. Chromium speciation in solution in the operative conditions of bioprecipitation tests (Cr = 7.5 mmol L^{-1}; SO_4^{2-} = 31 mmol L^{-1}; T = 25 °C).

ANOVA. The statistically significant effects (95%) were the variation of ethanol concentration (whose increase had a negative effect on sulphate abatement) and the interaction sulphate–ethanol (with a negative effect on sulphate abatement). The negative effect of ethanol concentration is probably due to the incomplete oxidation of ethanol to acetate which can have an inhibiting effect on biomass.

The variation of ethanol and sulphate concentration did not affect significantly pH and E_h trends (data not reported here): in all the investigated conditions pH and E_h values were in the range of 7.5–8 and −350 to −400 mV, respectively.

Based on ANOVA of the results presented in Fig. 2, column experiments were then performed using the lowest investigated concentration of ethanol (1.5 g L^{-1}), while the standard concentration of C medium was chosen for sulphate (3 g L^{-1}). In fact further increase of sulphate concentration did not significantly affected the final sulphate removal.

3.2. Mechanisms of chromium abatement in batch tests

Chromium (as chromate) was chosen as target metal for abatement experiments due to different reasons:

- Cr(VI) is highly toxic: mutagenic, teratogenic and carcinogenic [18];
- the conventional treatment of Cr(VI) polluted waters is a two step process (Cr(VI) reduction and Cr(III) precipitation) while using SRB Cr(VI) removal can be achieved in one step of biotreatment;
- Cr(VI) is highly soluble and only after reduction can precipitate: this make easier the identification of removal mechanisms.

Mechanisms of Cr(VI) removal were investigated in batch tests with ethanol. In these test conditions, Cr removal can be due to two main mechanisms: bioprecipitation (chromium precipitation due to SRB activity) and chemical precipitation (chromium precipitation due to the reducing conditions generated by C medium compounds and especially Na_2S).

The studies performed with Cr(VI) bioaccumulating microorganisms have shown that microbial Cr(VI) removal from solutions typically included the following stages: the binding of Cr to cell surface, the translocation of chromium into the cell and the reduction of Cr(VI) to Cr(III) [30].

Chromium speciation in solution was checked by a dedicated software for chemical equilibrium modelling [31] (Fig. 3). These simulations showed that, in the pH and E_h conditions generated by

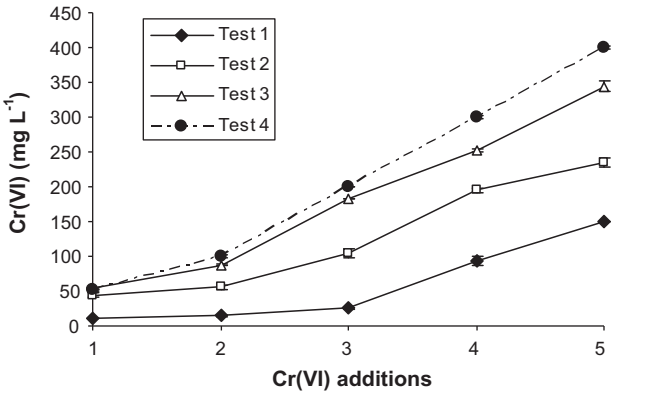

Fig. 4. Chromium abatement versus chromium additions in batch tests for the different test conditions.

SRB activity (pH 7–8.5; E_h −150 to −350 mV), Cr(VI) was reduced to Cr(III) and precipitated as Cr_2O_3. Indeed reducing conditions of C medium, due to Na_2S addition, could also cause the partial removal of chromium as Cr_2O_3 or $Cr(OH)_3$ even without SRB.

Four batch tests with different medium compositions were carried out (Section 2.3) in order to isolate the contribution of each component on metal removal (bioprecipitation and chemical precipitation).

These batch tests were performed by adding metal spikes during SRB growth thus resembling the way pollutants impact on the PRB.

In test conditions Cr(VI) was promptly reduced to Cr(III) and was not detected by the diphenylcarbazide method [17]. Therefore the total soluble chromium concentration determined by ICP can be completely attributable to Cr(III) ions.

In Table 2 the average values of sulphate reduction, the amount of chromium removed, pH and E_h values on 20th day of growth were reported, denoting the effect of C medium composition on sulphate and Cr removal.

In Fig. 4 chromium concentration in solution versus metal additions for the different investigated conditions was reported. The trend of test 4 showed the absence of Cr precipitation in water during the different additions.

The highest metal removal was observed in the batch test with SRB (test 1) where Cr removal could be attributable to both bioprecipitation and chemical precipitation.

In test 2 (having the same operative conditions of test 1 except for SRB absence) metal abatement was mainly due to chemical precipitation as Cr_2O_3.

In test 3 (having the same operative conditions of test 2 except for Na_2S absence) metal abatement was very low; such metal removal was probably due to interactions between chromium and C medium components.

For each test condition, metal abatement was estimated as the integral removal of Cr (q_i, mmol) calculated for the final time of the experiment (20 days):

$$q_i = \int (C_b - C)dt \quad (5)$$

where C_b (mmol) is chromium concentration after each addition of metal stock solution and C (mmol) is the residual concentration measured in the suspension five days after each addition (see Section 2.3).

Integral metal removals (mmol) determined for each test condition (see Table 2) were used to estimate Cr abatement (%) due to the different operating mechanisms. In particular the amount of chromium removed by bioprecipitation was 39 ± 3%, the amount removed by chemical precipitation due to Na_2S presence was 27 ± 2% and the amount removed by speciation phenomena was

Table 2
Average values of sulphate abatement (%), chromium removed (mmol), pH and E_h and operating mechanisms in the removal in the different batch tests.

Test	Composition	pH	E_h (mV)	SO_4^{2-} abatement (%)	Cr removed (mmol)	Operating mechanisms
1	Modified C medium + SRB	9.4 ± 0.2	−300 ± 20	39 ± 3	0.27	Bioprecipitation Chemical precipitation
2	Modified C medium	9.4 ± 0.3	−250 ± 10	8 ± 2	0.19	Chemical precipitation
3	Modified C medium (without Na_2S)	5.3 ± 0.1	200 ± 10	0	0.14	Chemical precipitation
4	Distilled water (blank)	5.0 ± 0.2	250 ± 10	–	–	–

21 ± 3%. The 13 ± 1% of added chromium remained not removed. Therefore, in batch test conditions with SRB growing on ethanol, Cr removal is due to both biological mechanisms and chemical precipitation. This discrimination between the different removal mechanisms is necessary in order to avoid an overestimation of the sulphate-bioreduction capacity of the system, and then misleading results in the following scale-up and design phases.

3.3. Column experiments

Column experiments were performed using two identical fixed bed columns, one inoculated by SRB and the other used as blank, in order to isolate side mechanisms (such as adsorption or chemical precipitation) different from bioreduction/bioprecipitation.

The efficiency of column reactors was monitored for pH, E_h and residual amounts of sulphates and chromium at three different outputs of the column (Table 3).

The volume of treated effluent was expressed as bed volume $B_V = V/V_0$, where V is the treated volume and V_0 is the pore volume of the column.

Bacterial growth in the inoculated column was clearly evidenced by the gradual change of colour of the filling due to the formation of black precipitates of FeS (Fe is present in C medium as $FeSO_4 \cdot 7H_2O$).

The effects of SRB activity were also evident by observing E_h and pH values along the column length (Table 3). In fact E_h values were more negative in the inoculated column than in the blank one changing from the oxidant value of the feed (about 200 ± 20 mV) to the reducing one (outputs 1, 5 and 9) due to SRB activity. The reducing E_h values of the blank column could be originated by the anaerobiosis of the system. As for pH values, in the SRB column the incomplete oxidation of ethanol to acetate caused a slight decrease of pH (Table 3).

Steady state was reached after the treatment of about 4 bed volumes with a final average sulphate reduction of 65 ± 5% in the inoculated column, against 2 ± 1% of the blank one reached after 4 bed volumes (Table 3).

Thus, the sulphate abatement observed in the inoculated column (neglecting the first phase corresponding to 4 bed volumes) can be assigned to bioactive removal mechanisms.

At steady state the complete abatement of Cr(VI) was obtained in the inoculated column. Blank column, after the saturation of sorption sites of perlite (occurring in 7 BV), could not further retain the metal (Table 3).

Column tests with ethanol confirmed the ability of SRB of utilizing this substrate and thus removing sulphates and metals efficiently. Ethanol offers a valid alternative to conventional solid substrates, generally used as electron donors and carbon sources in biological PRB. In fact liquid substrates (such as ethanol) avoid the use of potentially harmful wastes as organic sources for SRB metabolism and also avoid adsorption phenomena which have a predominant contribution on contaminant removal with respect to bioprocesses in conventional systems using solid substrates.

Data from column experimentation were used in order to determine the sulphate removal rate in fluidodynamic conditions similar to those existing in real PRB.

Data were modelled assuming a first-order degradation rate for sulphate removal [9,32]. A degradation rate constant (k) of 0.013 ± 0.001 h^{-1} (R^2 = 0.9274) was obtained, denoting a significant improvement of SRB activity working in fluidodynamic conditions similar to those existing in real PRB with respect to batch conditions.

The kinetic analysis was also performed for chromium, being the adsorption capacity of column saturated towards it. Removal rate constant (k) for Cr was 0.065 h^{-1} (R^2 = 0.9899).

Residence times (τ) required to reduce the concentration below the specific maximum contaminant level (MCL) (250 mg L^{-1} for sulphate and 0.005 mg L^{-1} for Cr(VI)) were calculated: $\tau_{sulphate}$ = 9 d and τ_{Cr} = 2 d. It is therefore evident that, for the synthetic solution considered here, the barrier thickness must be designed using the sulphate residence time. A preliminary estimate of about 0.9 m barrier thickness was obtained by assuming a groundwater velocity of 0.1 m/d [33].

These findings can be compared with those obtained in a previous work [9], in which a fixed bed column filled with a solid mixture and inoculated by SRB was tested for the treatment of heavy metal contaminated waters. For this system at steady state a final sulphate abatement of 50 ± 10% and a degradation rate constant (k) of 0.015 ± 0.001 h^{-1} for sulphate removal were obtained [9], against an abatement of 65 ± 5% and a degradation rate constant (k) of 0.013 ± 0.001 h^{-1} for the column fed with ethanol.

Comparing these results, it can be concluded that ethanol showed to be a more competitive substrate for SRB growth than solid substrates, in the investigated operating conditions, both in stoichiometric and kinetic terms.

3.4. Bioassessment with C. elegans

A lethal toxicity test was conducted to investigate, from a biological point of view, the effectiveness of the proposed process in reducing the overall toxic potential in a synthetic waste. To this end we employed a SEK-1 mutant strain of *C. elegans*, already described as highly sensitive to heavy metals [9]. In the control experiments

Table 3
Average values of sulphate removal (%), chromium removal (%), pH and E_h of the two column systems (with SRB and blank).

Column system	Output	SO_4^{2-} abatement (%)	Cr abatement (%)	pH	E_h (mV)
SRB	1	10 ± 5	40 ± 10	6.1 ± 0.5	−220 ± 20
	5	50 ± 10	80 ± 5	6.5 ± 0.3	−270 ± 30
	9	65 ± 5	95 ± 5	6.5 ± 0.3	−280 ± 20
Blank	1	5 ± 2	8 ± 2	6.7 ± 0.6	−140 ± 30
	5	5 ± 2	2 ± 3	6.8 ± 0.4	−110 ± 20
	9	2 ± 1	1 ± 2	6.9 ± 0.4	−100 ± 30

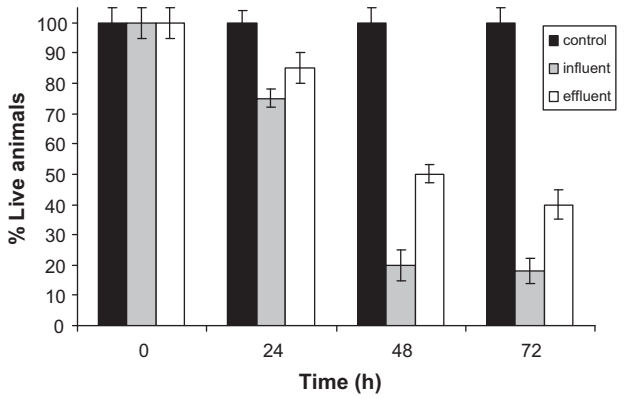

Fig. 5. Mean survivors of *C. elegans* SEK-1 animals seeded on 1:10 dilution of the hexavalent chromium at 16 °C before (grey bars) and after (white bars) the column inoculated with the SRB.

the animals were monitored by seeding them in a medium with the same ethanol and sulphate concentrations used inside the column but without the metal. These control animals did not show differences with respect to the ones seeded on standard NGM plates (not shown).

When the SEK-1 animals where monitored in the presence of the influent a strong reduction of the vitality was observed and only about 20% of the individuals were alive after 48 h, in agreement with the well known toxic effect of the hexavalent chromium (Fig. 5). A noticeable increase of the survivor number was instead obtained when the nematodes were surveyed in the presence of the column effluent: live individuals exceeded 50% of population after 48 h.

The ability to reproduce and the progeny size can be reasonably taken as a representative estimate of genotoxicity; the short and exhaustively described life cycle of *C. elegans* makes this nematode highly suited for such purposes. Therefore, the brood size of the animals exposed to the metal solution before and after the treatment was evaluated. The concentration of Cr(VI) in the influent drastically reduced the ability of the worms to reproduce; each individual was able to produce only one third of the progeny (92 ± 7) as compared to the control population (250 ± 10). After the column treatment, the nematodes exposed to the column effluent were able to produce an offspring (220 ± 12) having a size highly similar to that of the control unexposed animals. This supported that a substantial reduction in genotoxicity could be achieved by means of the proposed process.

This considerable improvement in survival of exposed animals should not induce to disregard that a significant acute toxicity is still present in the treated effluent. The individuation of the responsible compounds, either residual or even side-produced in the column, will deserve additional future investigation. On the other hand, the reproductive potential of the surviving individuals was highly similar to that of unexposed nematodes, thus confirming that the removal of genotoxicity was very effective. This means that the main risk associated with Cr(VI), i.e. genotoxicity and carcinogenicity, has been substantially removed.

It is worthwhile to notice that the bio-assay employing sizeable population of live individuals can efficiently address different aspects of bioremediation (acute toxicity versus genotoxicity) that are not amenable for analytical procedures.

4. Conclusions

In this paper ethanol was tested as carbon and electron source for SRB growth in the treatment of Cr(VI) contaminated waters. Batch tests denoted that using ethanol instead of lactate, comparable values of final sulphate abatement were obtained, but ethanol showed to be a substrate more competitive than lactate in kinetic terms. Two fixed bed column reactors were filled with inert material, one column was inoculated with SRB, while the other one was used as blank. Both columns were continuously fed by a synthetic solution containing sulphates, ethanol and Cr(VI). At steady state, inoculated column gave $65 \pm 5\%$ sulphate removal and $95 \pm 5\%$ chromium abatement, essentially due to bioactive removal mechanisms. Ethanol offers a valid alternative to conventional electron donors and carbon sources for SRB growth and SRB growing on this substrate are also able to remove Cr(VI). This aspect should be verified by a close economical analysis in view of full scale applications.

Kinetic estimates, obtained by contaminant profiles along the column, denoted significant improvement of SRB performances in fixed bed reactors operating in fluidodynamic conditions similar to those existing in real PRBs, with respect to batch conditions.

Reduction of Cr(VI) in vivo toxicity was assessed by using the nematode *C. elegans* as a test organism. The treatment effectiveness was supported by the increase of the survivor number of the animals treated with effluent with respect to the animals treated with the influent: 53% versus 20%, respectively.

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