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Prospecting bacterial consortia from a geothermal site for metals biotransformation

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Abstract Biomats that flourished in a fumarole located on the geothermal site *Los Azufres* (Mexico) were used as inocula to select aerobic and sulfate-reducing bacteria consortia for studying their capacity to reduce hexavalent chromium [Cr(VI)], aiming to use these consortia in biotransformation technologies. The sample site is characterized by slightly warm (nearly 27 °C), acid (pH 3) and about hypoxic (1.8 mg L^{-1} of dissolved oxygen) conditions. Four culture systems (2 aerobic and 2 anaerobic) were investigated, including their enzymatic activity, capacity to produce biofilms, and an analysis of the total bacterial populations. For the anaerobic condition (using sulfate and sulfur as electron acceptors), four pH values (from 2 to 8) and four carbon sources (pyruvate, glycerol, Na-lactate and Na-acetate) were probed. Significant biological Cr(VI) removal was observed for all the pH values probed, particularly during the first 12 h, being more effective at the most acid conditions. At a pH value of 4 and using pyruvate as carbon source, 100 mg L^{-1} of Cr(VI) were completely depleted in less than 12 h, while the use of Na-lactate was less effective but still reasonable. These results indicate that sulfate-reducing bacteria consortia from geothermal sites like the one studied here are capable of biotransforming Cr(VI) and have the potential to provide metal bioremediation technologies.

Keywords Sulfate-reducing-bacteria-consortia · Acidophiles · Exopolysaccharides · Biofilms · Hexavalent-chromium · Bioprocesses

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Introduction

The *Los Azufres* geothermal site, which is located in Michoacán state (Mexico) and is part of the Trans-mexican Volcanic Belt, has been exploited as a geothermal power source since the 1950's. During this period, some scientific studies focusing on

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geological and geophysical aspects or intending to evaluate the environmental impact caused by this exploitation have been performed (e.g. Birkle and Merkel 2000; Barragán et al. 2005).

More recently, the bacterial diversity of biomats and a mud lake of the *Los Azufres* Spa, a local thermal resort, was investigated using molecular approaches (Brito et al. 2014). That study revealed that biomats flourish in warm streams, colonized by populations of *Rhodoblastus, Methylocella, Chlorobaculum, Chlorobium, Chlorella, Thiomonas, Desulfobacterium, Thiobacillus, Desulfatirhabadium, Thermodesulfobium* and *Thermoanaerobacter* genera. This confirmed the potential of the site to host extremophile microorganisms, especially some related to sulfur- and sulfate-reducing bacteria (SRB). Also, among the natural extreme conditions of the site (high temperatures, acid pH, oscillating level of dissolved oxygen), a high concentration of metals was found which, with the detected capacity of some microorganisms to grow forming biofilms, bring different possibilities for bioprospection and the development of metal transformation technologies.

Biomats or biofilms are sessile microbial communities found in different aquatic systems (oceans, lakes, rivers, hot springs, drainage areas, etc) (Tazaki 1999; Shutherland 2001). These communities are protected within a polysaccharide matrix, promoting physical and physiological interactions among the microorganisms, bringing mutual benefits and favoring the survival during environmental stress periods (Decho 2000; Singh et al. 2006).

Sulfur and sulfate reducers are omnipresent in anoxic habitats, while they are of great interest because they can be used to remediate metal contaminated sites due to their ability to reduce metal oxides (Muyzer and Stams 2008; Liu et al. 2002; Lovley 1995). One example of such application of the SRB is the proposal by Dar et al. (2007), who described a bioprocess for the removal of heavy metals (cadmium, cobalt, copper, iron, nickel and zinc), where the sulfide produced by the SRB reacts with the metals and co-precipitate with them. After precipitation the heavy metals can be recovered and reused. Kieu et al. (2011) showed that the SRB can also directly reduce hexavalent chromium [Cr(VI)] under sulfate-rich anaerobic conditions. Nevertheless, most of the described bioprocesses using SRB are related only to the metals co-precipitation as metal-sulfides.

The bad management of industrial wastes generates plenty of environmental issues, some of them very difficult to mitigate such as those involving metals and metalloids contamination. In Guanajuato state (Mexico), a large amount of industrial residues from the processing of chromine resulted in groundwater pollution with Cr(VI) (Armienta et al. 1993). Due to its high toxicity (Losi et al. 1994; Katz and Salem 1993), it is urgent to find a feasible and nature friendly process to remediate this problem, which, unfortunately, also occurs in many other parts of the planet.

Some recent works have searched for and studied SRB bacterial consortia able to reduce the Cr(VI) in the medium (e.g., Jin et al. 2017; Shi et al. 2019; Zheng et al. 2019), although all of them have used activated sludges as inocula. The aim of the present study was to obtain bacterial consortia from a site in which metals and metalloids occur naturally from geological input, and verify their potential to mitigate chromium contamination. For this, we probed different culture media where a inoculum from biofilms, developed on a warm and acid stream of *Los Azufres* geothermal site (Mexico), could grow. We also studied the behavior of these consortia under different pH values, using distinct carbon sources, all using the Cr(VI) as a model of contaminant and stress agent.

Materials and methods

Sampling site and physico-chemical characterization of the samples

White microbial mat samples were collected from the *Los Azufres* Spa (19° 46′ 51.7″ N and 100° 39′ 23.6″ W), located in *Los Azufres* geothermal field (Fig. 1). The samples for cultivating the consortia probing different culture media were obtained on December 10, 2010, while the samples for studying the behavior of the consortia under different pH values and using distinct carbon sources were collected on November 16, 2013. All samples were taken around noon.

The physico-chemical parameters, such as temperature, pH, conductivity and dissolved oxygen (DO), were measured *in situ* using specific probes (Conductronic PC 18 pHmeter, J.T. Baker®, and Sensionion 6, Hach®). The nutrients ($P–PO_4^{3-}$, $N–NO_3^-$ and N–

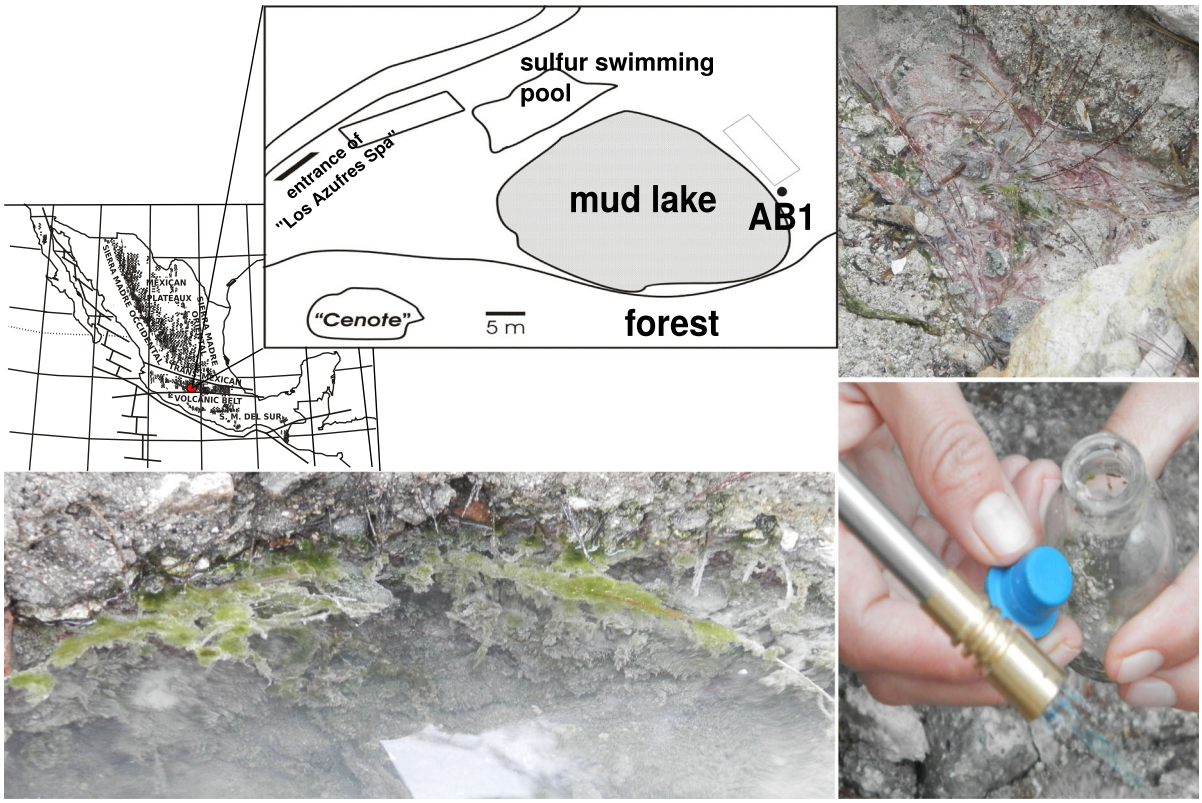

Fig. 1 Location of *Los Azufres* geothermal field (red point; 19° 46′ 51.7″ N and 100° 39′ 23.6″ W), inside the Transmexican Volcanic Belt. The zoom in first plane shows the SPA *Los Azufres*, where the AB1 sample site is annotated to the right in the map. The photos top right, bottom right and bottom left show, respectively, details of the thermal vent, the sample collected and the biomats

NH_4^+) were measured with a Hach® kit, following manufacturer's instructions.

For the microbiological sampling, sterile spoons were used and the samples were stored in sterile conical tubes™, for molecular and chemical analysis, or in penicillin sterile bottles, for enrichments. The samples were stored at − 20 °C for molecular analysis and at 4 °C for enrichments and chemical analyses. Water of the sample site was also collected for preparing the culture mineral media (as described below).

Bacterial consortia retrieving

All the culture media were prepared using water from the site. The water was sterilized in three autoclaving steps of 2 h each (with 24 h intervals) and, before use, it was filtered (on 0.22 µm Millex®GP Millipore membrane). This water constitutes the basic minimum mineral medium (BMM).

Four conditions were probed: 2 aerobic, varying the carbon sources, and 2 anaerobic, changing the electron acceptors. Before the enrichments, the BMM was autoclaved again (for 20 min at 121 °C) but, for the anaerobic media, penicillin bottles stoppered with blue butyl rubber impermeable to gases were used and immediately degassed by bubbling with a N_2 gas during 10 min. Then, all media received a vitamin solution (1 mL L^{-1}) and a metal trace solution (1 mL L^{-1}). For the aerobic systems the media were then supplemented with glucose (0.5 M) or sucrose (0.3 M), named **Glu** and **Suc** respectively, to work as carbon sources and electron donors. For the anaerobic conditions, the media were supplemented with a solution of mixed carbon sources (pyruvate, Na-lactate, glycerol and Na-acetate), using 10 mM of each one (Guyoneaud et al. 1996), and enriched with 1 mM of

Fe_2SO_4 or elemental sulfur, as electron acceptors, named respectively **Sul** and **Azu**. The use of a mix of carbon sources (to work also as electron donors) in this case was intended to broaden the range of prospected SRB to different metabolisms.

The four cultures were prepared each one containing 50 mL of the specific medium and 2 mL of inoculum (the biomat resuspended), at a neutral pH. They were pre-incubated at room temperature for 15 days. A subculturing was, then, performed: 2 mL of this first culture were incubated in fresh medium (20 mL) for 30 days. At the end of this period, each culture was analyzed for: (i) enzymatic activity, (ii) bacterial diversity by terminal-restriction fragment length polymorphism (T-RFLP) and (iii) determination of the ability to develop biofilms.

Enzymatic activity

The enzymatic activity was inferred by enzymatic hydrolysis of fluorescein diacetate (FDA) to fluorescein, following the methodology described by Stubberfield and Shaw (1996). This is a non-specific reaction that represents esterase activity, which involves proteases, esterases and lipases (Medzon and Brady 1969).

FDA was also measured for the original sample, as well as the classical Colony-forming Unit (CFU, Greenberg et al. 1992) and Most Probable Number (MPN, Alexander 1982) of viable microorganisms.

Bacterial diversity: DNA extraction and T-RFLP

The total DNA of each enrichment was extracted (2 tubes of 250 mg of sample) using PowerSoil® DNA kit (MoBio Laboratories). DNA quality was verified by electrophoresis in a 1% agarose gel in Tris-Acetate-EDTA buffer. DNA solutions were stored at −20 °C until further analysis.

The T-RFLP analysis of 16S rRNA gene was performed according to Brito et al. (2014) (see, also, Brito et al. 2006). First the 16S rRNA gene was amplified by PCR using the primers 8F (5′-AGA GTT-TGA TCC TGG CTCA G-3′), labeled with carboxi-fluorescein (FAM Lane 1991, and 907R (5′-GCC CCC GTC AAT TCM TTT RAG TTT-3′), unlabeled (Lane et al. 1985), in a PTC 200 Thermo-cycler (MJ research). The PCR products, purified using GFX PCR DNA purification kit (Amersham), were digested with 3 U of restriction enzymes *Hae*III or *Hin*P1I at 37 °C for 3 h. Then, 50 ng of digested DNA were mixed with 20 µL of deionised formamide and 0.5 µL of TAMRA® ladder (Applied Biosystem®) and denatured (94 °C for 5 min and chilled on ice). The length of the terminal restriction fragments (T-RFs) was determined by capillary electrophoresis on an ABI prism 310 (Applied Biosystem®), and the T-RFLP profiles were analyzed using GeneScan Software (Applied Biosystem®). Data sets were normalized and T-RFs representing less than 1% of total fluorescence were removed (Hewson and Fuhrman 2006). Statistical analyses were carried out with MVSP software (Multi-Variate Statistical Package 3.1, Kovach Computing Services, UK). The T-RFLP analysis provides only limited information about the presence and absence of the main populations, grouped in the T-RFs (e.g. Caretta and Brito 2011). This approach was used to follow the behavior (richness of populations) as response of different experimental conditions.

Exopolysaccharide quantification

At the end of incubation, after taking the aliquotes for the analysis of enzymatic activity, T-RFLP and chromium degradation (next sub-section), all remnant of each enrichment was used to infer the ability to develop biofilms through the exopolysaccharides (EPS) production. EPS was also determined on the original biofilms.

The EPS was extracted using the method described by Liu and Fang (2002); briefly: the samples were lyophilized and weighted for estimating the cellular fraction (% cel_biomass g^{-1} dry weight). To recover the EPS fraction weakly adhered to biomass, samples were centrifuged (15,000 rpm for 25 min) and the supernatant was reserved. To recover EPS strongly adhered to biomass the pellet was re-extracted with NaCl 0.35 M (incubated during 3 h at 80 °C), and centrifuged (15,000 rpm for 25 min). Both supernatants (weakly and strongly adhered to biomass) were joined and lyophilized. The total amount of EPS extracted was measured by weight after lyophilization (% EPS g^{-1} dry weight).

Cr(VI) and total chromium analyses

After pre-culture, the four enrichments received 0.2 mM of Cr(VI), in the respective amount of $K_2Cr_2O_7$, for investigating the effect of the different culture media in the capability of the bacterial consortium to diminish this metal. Enrichments were prepared in triplicates. Sterile controls, that is, culture media without inocula, were prepared following the same protocol. For aerobic enrichments, aliquotes were taken at 1, 2, 6, 10 and 15 days of incubation for measuring Cr(VI), while for anaerobic enrichments, aliquotes were taken at 24, 38, 41, 42, 46 and 91 h, 6, 10 and 15 days.

To evaluate the amount of Cr(VI) in each sample, 0.1 mL of enrichment was diluted in 4.8 mL Milli-Q® water containing 0.1 mL of H_2SO_4 5 M and 0.05 mL of 1,5-diphenylcarbazide 0.02 M, incubated for 10 min at room temperature. The absorbance was measured at λ 540 nm (spectrophotometer Hach DR 5000™) (Norma Mexicana 2001). A standard curve with linear relationship between absorbance and $K_2Cr_2O_7$ was performed for Cr(VI) quantification. Measurements were also done in triplicates. Detection limit for Cr(VI) concentration was around 1 mg L^{-1}. To eliminate any metal contamination the vessels were previously washed with concentrated HNO_3. Also, only analytical grade reagents were used.

Total chromium was determined as described by Malm et al. (1989), at the Eduardo Penna Franca Radioisotopes Laboratory (LREPF-IBCCF-UFRJ, Rio de Janeiro, Brazil). In brief, the solid samples are first lyophilized and crushed, while the liquid samples, after centrifuged (8000 rpm for 5 min), are processed directly. Then, they are digested with concentrated HNO_3 and HFl (Merck P.A.), for 18 h at 120 °C, evaporated and, after, receive 10 mL of 0.1 N HCl. The samples were analyzed using a Varian spectrophotometer (AA240FS, Fast Sequential Atomic Absorption Spectrometer, USA). Blanks were run throughout the analyses to check for any contamination; measurements were done in triplicate; and certified analytical grade reference samples were used for calibration. The detection limit was calculated using the formula: $(3 \times S_b)/X_b$, where S_b is the standard deviation of 6 measurements of the blank and X_b is the mean of the angular coefficient of the calibration curve (Silva and Alves 2006), resulting in 0.01 mg kg^{-1}.

Effect of pH and carbon sources on Cr(VI) degradation

Following the results of the previous degradation experiment, we selected a mix of anaerobic **Sul** plus **Azu** culture media for probing the best conditions for Cr(VI) removal, both chemically (controls without microorganisms) and biologically (enrichments with microorganisms). Two experiments were performed after the new pre-culture: one using different pH values (2, 4, 6 and 8) and another using different combinations of carbon sources. All possible combinations of the four carbon sources, namely acetate (A), pyruvate (P), glycerol (G) and lactate (L), were probed: APGL, APG, APL, AGL, PGL, AP, AG, AL, PG, PL, GL, A, P, G, L. These last tests were done at pH 4.

Results and discussion

Sampling site

Based on the physical-chemical and chemical parameters measured in the water overlaying biomats (Table 1) the sample site can be classified as hypoxic (DO below 2 mg L^{-1}), acid (pH of 3) and oligotrophic. While the temperature at the sampling time was slightly warm (27.4 °C), the sample site is a thermal vent with varying temperature (27–36 °C) depending

Table 1 Physico-chemical and chemical analyses and biological activity

Parameter	Value
Temperature (°C)	27.4
pH	3
Conductivity (μS cm^{-1})	4
DO (mg L^{-1})	1.8
$P–PO_4^{3-}$ (mg L^{-1})	0.1
$N–NO_3^-$ (mg L^{-1})	0.3
$N–NH_4^+$ (mg L^{-1})	DL
FDA (μg min^{-1} g^{-1})	0.573
CFU (cel mL^{-1})	930
MPN (cel mL^{-1})	1.8×10^5

DL below the detection limit

on the output of the sulfur spring geyser. These conditions favored the flourishment of a white biomat, from which nearly 55% of its dry weight was related to EPS. Measured values for enzymatic activity and number of viable cells in the original sample are also presented in Table 1.

In a previous work, we determined high contents of heavy metals (Hg, Zn, Cr, Ni, Pb, Cu, Cd and Fe) on these biomats (Brito et al. 2014; Villegas-Negrete 2010). Also, by molecular approaches, we demonstrated that these biomats were inhabited primarily by Proteobacteria (60%) and Chlorobia (20%) classes that were implicated on the sulfur cycle (Villegas-Negrete 2010). We observed a dominance of *Rhodoblastus* genus, followed by *Methylocella* and *Chlorobaculum* related sequences and *Chlorobium*. But the presence of *Thiomonas, Desulfobacterium, Thermodesulfobium* and *Thermoanaerobacter* genera was also observed. The capability of the bacterial populations to grow forming biomats on environmental conditions and the metal concentrations detected could suggest some resistance to these elements. Based on this assumption, we tested four distinct media (aerobic and anaerobic) for prospecting these microorganisms and verifying their capability to diminish the Cr(VI) concentration in their culture medium.

Characteristics of the consortia

Results from T-RFLP analysis showed that all the enrichments presented low diversity, with 6 to 12, and 14 to 24 T-RFs, respectively for *Hin*P1I and *Hae*III restrictions (Fig. 2). Few T-RFs were highly dominant under aerobic experimental conditions: 219 and 290 for **Suc** enrichment (with *Hin*P1I and *Hae*III, respectively); and 225 (with *Hin*P1I), 218 and 230 (with *Hae*III), for **Glu** enrichment. This was also the case for anoxic conditions: T-RF 230 was dominant using *Hae*III restriction, whatever the electron acceptor, but the dominance varied a little for *Hin*P1I restriction (236 and 238 for **Azu** and **Sul** enrichments, respectively). Since the four profiles were found to be different, one can conclude that the supplement (**Glu** or **Suc**, for anaerobic enrichments, and **Sul** or **Azu**, for the anaerobic ones) is more effective in selecting the populations than the Cr(VI) itself.

Based on the esterase activity, bacterial growth was similar for the four conditions tested. The measured activity was of 0.57 μg min^{-1} g^{-1} under aerobic conditions (**Glu** and **Suc**) and of 0.39 and 0.30 μg min^{-1} g^{-1} for **Sul** and **Azu** conditions, respectively. However, the capability to form biofilms by all these bacterial consortia was weak, even on the aerobic systems using high contents of glucose or sucrose that could support the overproduction of EPS (Petronella and Jeroen 1999; Freitas et al. 2011; Donot et al. 2012). The capability to grow on biofilms may provide additional advantages for a particular bioprocess because the biofilms may protect microorganisms from xenobiotics (Singh et al. 2006). Apparently, the bacterial communities capable to grow on biofilms, such as the ones observed in situ, require a very specific set of environmental parameters, such as temperature, pH, dissolved oxygen and carbon sources, conditions that we could not achieve to reproduce in vitro on the tested cultures.

Results from EPS fraction analysis in the remnant of the enrichments, before the addition of Cr(VI), revealed that the aerobic **Glu** system showed the highest capability to produce EPS (98%), as expected. A still relatively high fraction of EPS was found for the anaerobic **Sul** enrichment (78%), while the aerobic **Suc** system presented an intermediate one (43%). The lowest capability to produce biofilms was presented by the anaerobic **Azu** condition, only 10%. This suggests a relation between the production of EPS and the energy budget of the involved reactions: while **Glu** and **Sul** can be used directly by the microorganisms, **Suc** and **Azu** need intermediate reactions, making their use more energetically expensive. The sucrose double-ring must be first broken into two monosacarid rings before being used as carbon source and electron donor. Similarly, the S^0 must be first transformed to SO_4^{2-} prior to be used as an electron acceptor (e.g., Poser et al. 2013).

Removal of Cr(VI) by the bacterial consortia

To evaluate the capability of the consortia to diminish the Cr(VI) concentration on the enrichments over different culture media, we measured the amount of $K_2Cr_2O_7$ in different times as described in "Cr(VI) and total chromium analyses" section.

Already after 24 h, a significant decrease in the Cr(VI) concentration was observed: 39% for **Glu** system, 62% for **Sul** system, 68% for **Suc** system and

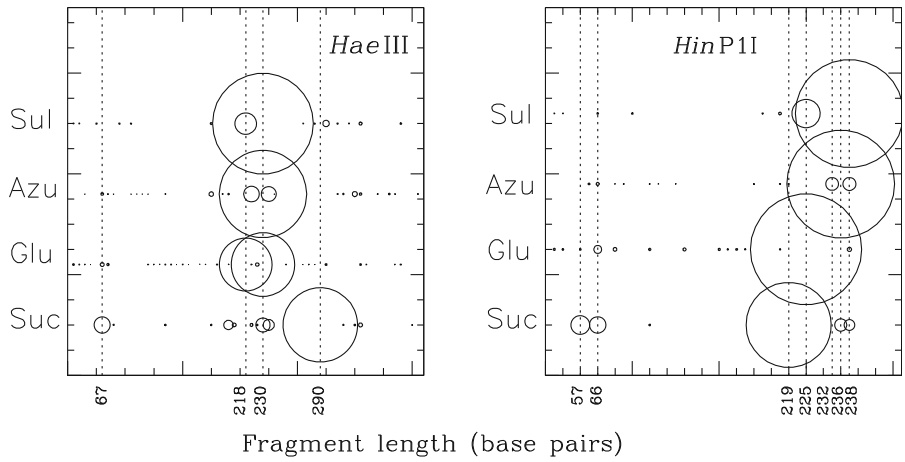

Fig. 2 Bacterial community profiles from T-RFLP for the four enrichments. The aerobic conditions are represented by the **Glu** and **Suc** systems, respectively with glucose and sucrose as carbon source, while the anaerobic enrichments had the same carbon sources (lactate, pyruvate, glycerol and acetate) but on different electron acceptors: SO_4^{2-} (**Sul** system) and S^0 (**Azu** system). The restriction was carried out with 2 enzymes: *Hae*III (left) and *Hin*P1I (right); the circle size is proportional to the relative abundance of T-RFs

75% for **Azu** system (Fig. 3). The calculated Cr(VI) diminution rates were 0.014 ± 0.002, 0.026 ± 0.004, 0.042 ± 0.003 and 0.080 ± 0.035 (mM h^{-1}) for **Glu**, **Sul**, **Suc** and **Azu** systems, respectively. Thus, the aerobic condition with glucose as carbon source showed the lowest efficiency to diminish the concentration of Cr(VI). Furthermore, this system needed nearly 360 h for a total dissipation of Cr(VI) from the media, while for the other systems this time was between 38 and 100 h. In general, the three other systems showed similar behavior (Fig. 3). This result is consistent with the previous one pointing the **Glu** as the most efficient to produce EPS. In the aerobic systems the cell respiration occurs through the organic matter oxidation mechanism, using O_2 as the electron acceptor and the **Glu** as the electron donor for the biosynthesis. In the same systems, the Cr(VI) can act as the electron acceptor, in a detoxification mechanism. Since both mechanisms have a very close redox potential (see Table 2), the way the cell will act depends on the whole set of conditions at that moment. In the case of **Glu** supplement, it seems that the microorganisms preferred to produce EPS instead of reducing the Cr(VI), the opposite way was chosen for the **Suc** supplement, with a dominance of detoxification. For the anaerobic systems the same options are available, although with the SO_4^{2-} or S^0 as the electron acceptors. Both in the **Sul** and **Azu** cases, the redox potential for the detoxification is much higher than the potential for the dissimilatory sulfate reduction and sulfo-oxidation, respectively, for all the four substrates used, giving priority to the Cr(VI) removal. For **Azu** system, the potential for sulfo-oxidation with all electron donors is even negative, consistent with their better performance to donate electrons for the Cr(VI) removal.

At the end of this experiment, the biomass fraction was separated from the culture medium in all systems, and the total chromium was determined on both fractions. For the **Glu**, **Sul** and **Azu** systems, less than 3% of the residual chromium was found in the liquid phase, implying that almost all (97%) the chromium was retained in the cellular fraction. For **Suc** system, around 80% of total chromium was found in the cellular fraction. Since the total chromium is almost completely composed by Cr(VI) and Cr(III) and the Cr(VI) was depleted from the media, we assume that the Cr(VI) in our systems has probably been adsorbed to the cells or transmembrane transported and, after, reduced intracellularly to Cr(III) and retained. The interaction between the living cells and the dissolved Cr(VI) has been studied by O'Brien et al. (2003)) and by Cheung and Gu (2007).

From the four enrichments investigated here, we selected the anaerobic conditions (using S^0 and SO_4^{2-} as electron acceptors) to obtain SRB-consortia for probing the Cr(VI) remediation. The mix of both electron acceptors was intended to cover a larger range

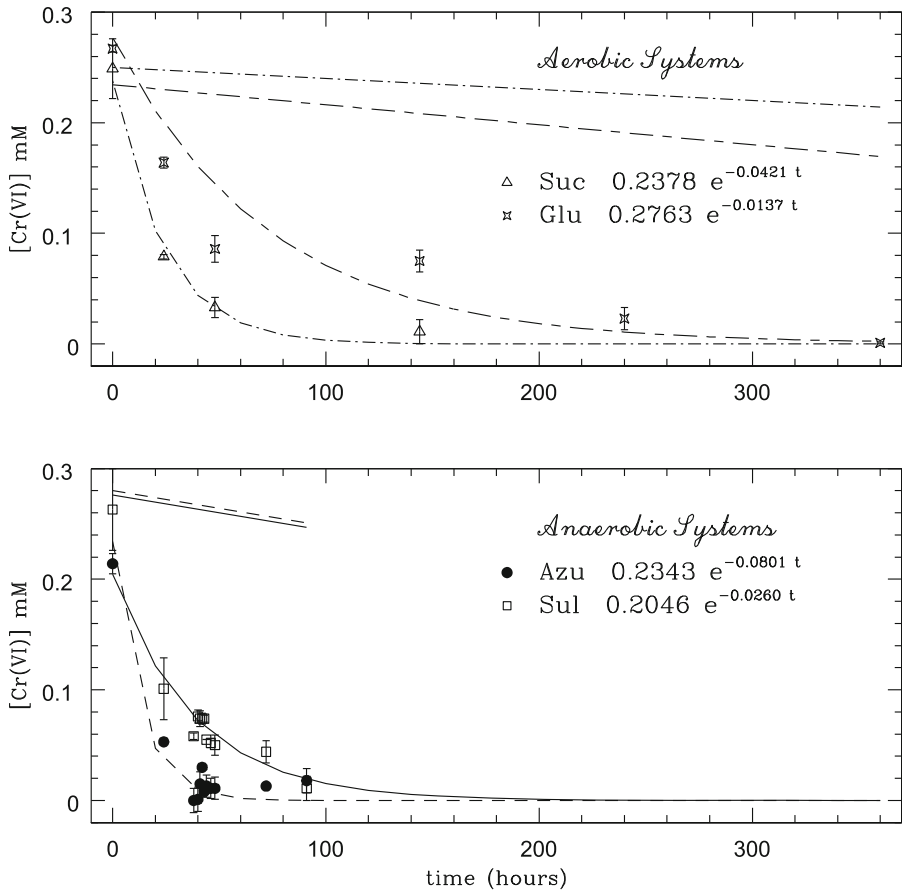

Fig. 3 Kinetic of Cr(VI) in the four systems tested. The aerobic (**Suc** and **Glu** systems) and anaerobic (**Azu** and **Sul** systems) conditions are the same as in Fig. 2. The fitting functions are shown top right of each panel: the starred crosses and long-short dashed line stand for **Glu** system, the open triangles and dot-dashed line for **Suc**, open squares and solid line for **Sul** and solid circles and dashed line for the **Azu**. The error bars correspond to the standard deviations for the triplicate values of each measurement. Since the pragmatic least square fits were exponential, for each system the factor multiplied by the time represents the respective decay respect to the initial value of Cr(VI) concentration (diminution rate). Lines without points are for the respective controls

of possibilities, since both **Azu** and **Sul** were the conditions with best rates of Cr(VI) removal. That is, with this new condition we expect to stimulate the growth of bacterial communities which are resistant to chromium and capable to reduce it quickly (with S^0) and, maybe, with some capability to produce EPS (with SO_4^{2-}).

pH effect on the Cr(VI) removal

After selecting the mixed anaerobic system for complementing our experiments, we measured the chemical and biological Cr(VI) removal under 4 distinct pH values. The results are shown in Fig. 4.

The chemical transformation of Cr(VI) to Cr(III) is known to be naturally favored under highly acid conditions (e.g. Daneshvar et al. 2002), and most of the industrial treatments applied for removing the Cr(VI) from contaminated effluents use this characteristic. Our results confirm this chemical drop, indicating that half of the Cr(VI) is naturally consumed after 15.9, 20.0, 27.3 and 45.3 h, respectively at pH values of 2, 4, 6 and 8.

Nevertheless, in all our pH tests the biological Cr(VI) removal was significantly more efficient than the chemical one. The most noticeable change occurs just after the addition of the Cr(VI): all tests received the same amount of this metal (100 mg L^{-1}), but the

Table 2 Reduction potential for the aerobic (glucose donating electrons to oxygen or to cromate) and anaerobic (acetate, glycerol, lactate and pyruvate donating electrons to sulfate, elemental sulfur or cromate) reactions

Reaction[a]	Number of electrons	Reduction potential (V)
Aerobic		
$C_6H_12O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$	24	+ 1.241
$C_6H_12O_6 + 4Cr_2O_7^{2-} + 32H^+ \rightarrow 6CO_2 + 8Cr^{3+} + 22H_2O$	24	+ 1.372
Anaerobic		
$CH_3COO^- + SO_4^{2-} + 2H^+ \rightarrow 2CO_2 + HS^- + 2H_2O$	8	+ 0.177
$CH_3COO^- + S^0 + 2H_2O \rightarrow 2CO_2 + 4HS^- + 3H^+$	8	− 0.140
$3CH_3COO^- + 4Cr_2O_7^{2-} + 35H^+ \rightarrow 6CO_2 + 8Cr^{3+} + 22H_2O$	24	+ 1.285
$4CH_2OHCHOHCH_2OH + 7SO_4^{2-} + 7H^+ \rightarrow 12CO_2 + 7HS^- + 16H_2O$	56	+ 0.240
$CH_2OHCHOHCH_2OH + 7S^0 + 3H_2O \rightarrow 3CO_2 + 7HS^- + 7H^+$	14	− 0.077
$3CH_2OHCHOHCH_2OH + 7Cr_2O_7^{2-} + 56H^+ \rightarrow 9CO_2 + 14Cr^{3+} + 40H_2O$	42	+ 1.348
$2CH_3CHOHCOO^- + 3SO_4^{2-} + 5H^+ \rightarrow 6CO_2 + 3HS^- + 6H_2O$	24	+ 0.212
$CH_3CHOHCOO^- + 6S^0 + 3H_2O \rightarrow 3CO_2 + 6HS^- + 5H^+$	12	− 0.105
$CH_3CHOHCOO^- + 2Cr_2O_7^{2-} + 17H^+ \rightarrow 3CO_2 + 4Cr^{3+} + 11H_2O$	12	+ 1.320
$4CH_3COCOO^- + 5SO_4^{2-} + 9H^+ \rightarrow 12CO_2 + 5HS^- + 8H_2O$	40	+ 0.249
$CH_3COCOO^- + 5S^0 + 3H_2O \rightarrow 3CO_2 + 5HS^- + 4H^+$	10	− 0.068
$3CH_3COCOO^- + 5Cr_2O_7^{2-} + 43H^+ \rightarrow 9CO_2 + 10Cr^{3+} + 26H_2O$	30	+ 1.357

[a]Considering the complete oxidation of the electron donors

measured concentrations were between 20 and 70 mg L^{-1} (respectively for pH values 2 and 8). This is due to an immediate drop of the Cr(VI) by the microorganisms (probably by adsorption to cell walls) or by their products. Under the lowest pH value (2), in fact, we observed the highest decrease: most of the Cr(VI) was consumed between the addition and the measurement of this metal's concentration.

Three types of Cr(VI) removal can be considered biological (meaning "in the presence of microorganisms"): direct, when the contaminant is transported into the cells and bioreduced; by biosorption to the dead or living cell walls; and indirect, by the metabolites of active cells in culture medium (e.g., Alam and Ahmad 2011; Qian et al. 2017; Ma et al. 2019). From our previous results of total chromium in the liquid phase we can only discard the indirect biological removal (after the beginning and during the experiment), but we are not able to identify if one of the other two was predominant. However, it is clear that, although the selected consortium may resist from acid (pH 2) to slightly alkalines (pH 8) conditions, the biological Cr(VI) removal is more effective in the most acid ones. This adaptability of the microorganism consortia studied gives additional advantages for remediation bioprocesses because it enables to work effluents with a high range of pH with little impact on the active biomass. However, it is necessary to perform experiments using original effluents (not dicromate solution) to corroborate this hypothesis.

Carbon sources effect on the Cr(VI) removal

Finally, we carried out an experiment to observe the behavior of Cr(VI) removal with the use of different carbon sources. All enrichments were carried out at pH 4, intending to establish a compromise between the best conditions, which are the most acid ones, and the less expensive treatments that do not need a hard acidification of the effluent. The four carbon sources in the mix used for the first experiment (acetate, pyruvate, lactate and glycerol) were tested now also individually, in mix of two and in mix of three.

Almost all tests resulted in complete depletion of Cr(VI) after 24 h, except for acetate only, glycerol only and the mix of acetate and glycerol (Fig. 5). For these 3 enrichments, the Cr(VI) decrease was

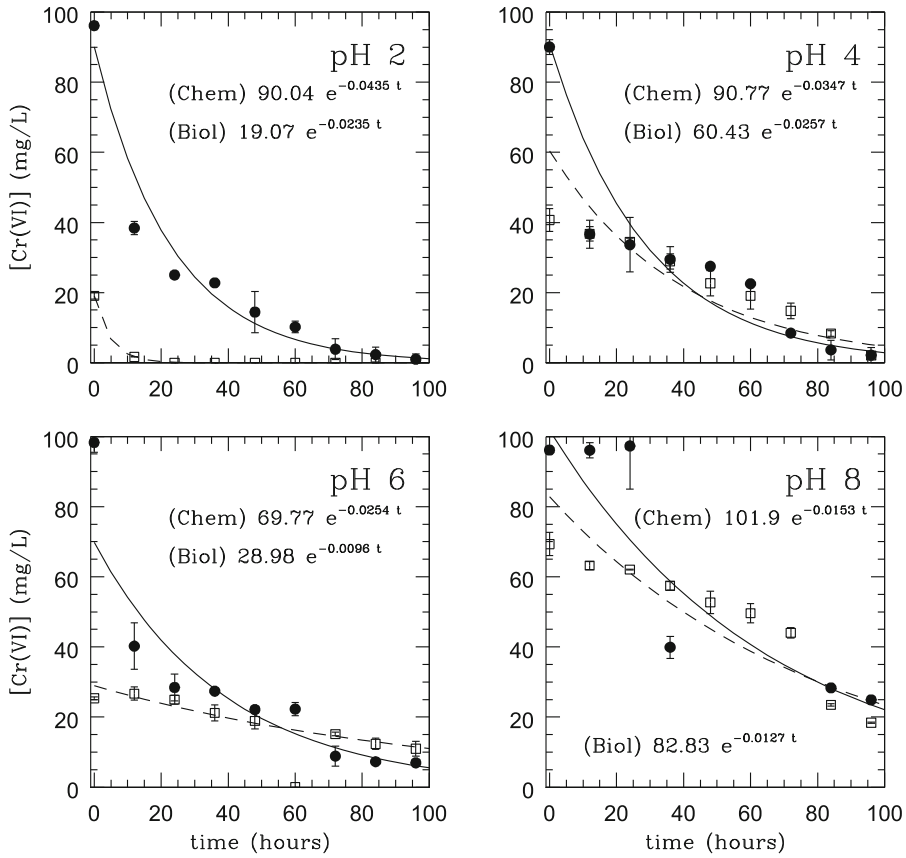

Fig. 4 Kinetic of Cr(VI) diminution at different pH values (2, 4, 6 and 8) under anaerobic condition (using lactate, pyruvate, acetate and glycerol as carbon sources, and SO_4^{2-} as electron acceptor, supplemented with S^0. The fitting functions are shown top right: the solid circles and lines represent the chemical reduction and the open squares and short-dashed lines, the biological reduction. The error bars correspond to the standard deviations for the triplicate values of each measurement

minimum. The systems with lactate only and with lactate mixed with acetate, glycerol and both were a little less effective than the mix of the four carbon sources, while all other systems with pyruvate were more effective, reaching depletion of Cr(VI) before 12 h.

This behavior of the Na-acetate and glycerol being less active to allow the Cr(VI) removal than Na-lactate and pyruvate was unexpected. As can be seen in Table 2, the four substrates have higher reduction potentials for donating electrons to cromate than to sulfate or elemental sulfur. Also, several sources in the literature have pointed that acetate, for instance, is an efficient electron donor to promote Cr(VI) reduction (e.g., Xu et al. 2011; Zheng et al. 2019). Again, there are probably other variables that define if the SRBs will prefer to detoxify the medium or to use the substrate as carbon source, including the specific bacterial strains in the consortia (e.g., Ma et al. 2019, fermentation products being less effective in promoting Cr(VI) reduction than glycolysis products (e.g., Bai et al. 2018, among others). Concerning the specific bacterial strains of our anaerobic consortium, it seems that the populations that ferment acetate (and the ones that metabolize glycerol) are not able to detoxify the medium when being selected alone due to the use of acetate (glycerol) as the only substrate.

Conclusion

Our results corroborate previous studies which propose that SRB are able to reduce Cr(VI) (Kieu et al. 2011; Singh et al. 2011; Pagnanelli et al. 2012; Qian et al. 2016). Both the use of sulfate and sulfur as electron acceptors and the production of a black

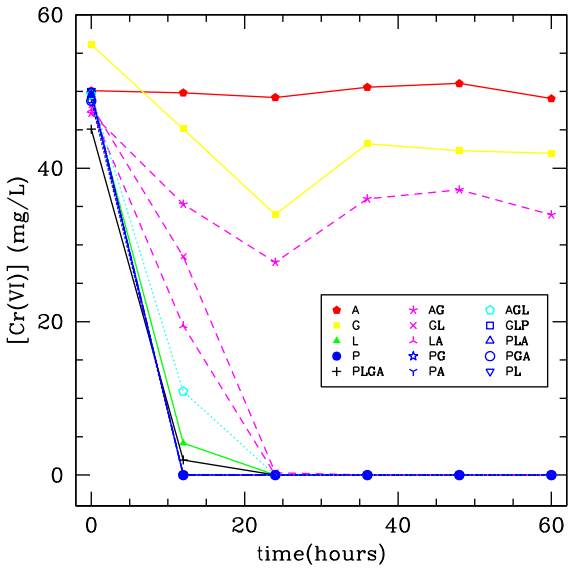

Fig. 5 Kinetic of the Cr(VI) diminution for the different combinations of carbon sources. The insert indicates the symbols used for each combination: blue lines and symbols represent the combinations that resulted more efficient than the original mix of the four carbon sources (symbols and lines are overlapping), while the others resulted less efficient. Error bars are not shown to avoid overcrowding

precipitate in our systems (in all probed pH values), confirm the presence of SRB in them. Unfortunately, it was not possible to amplify the 16S rRNA gene for all the enrichments and, thus, we could not compare their T-RFLP diversity profiles.

We could show that SRB consortia, retrieved from an extreme environment (acid and hypoxic), can deplete completely the Cr(VI) of their medium (*in vitro*) in less than 12 h, using conditions of slightly acid pH (around 4) and pyruvate as carbon source (alone or mixed with other carbon sources like lactate). This Cr(VI) is probably bioadsorbed by the SRB cell structure or bioreduced to Cr(III) internally. To confirm this assumption, we need to use more specific methodologies, such as SEM/TEM imaging coupled to a EDS, to identify exactly where the chromium is, and measurement of the H_2S biological production, to verify the extracellular Cr(VI) reduction; which we expect to do when the bioprocess is complete.

An important point of this work is that the obtained SRB-consortia, as expected, grow in acid conditions, an unusual ability of SRB in general, which prefer pH conditions near neutrality.

The potential application of microorganisms to mitigate the environmental contamination by metals has been subject of several scientific investigations (e.g. Gomes 2012). Contrary to the contamination by organic compounds, the metals are not biodegradable. Sometimes they are only transferred to the biomass (Gomes 2012), or transformed to a different less toxic oxidation state (Cheung and Gu 2007; Muyzer and Stams 2008), usually carried out by the same microorganisms.

To pass from this exploratory study to the development of a technology which applies these consortia to remediate real contaminated effluents one needs to: (i) prove the Cr(VI) removal under conditions of larger concentrations of this metal, different temperatures and salinity; (ii) prove if a larger biomass can affect the efficiency in reducing the Cr(VI); (iii) prove the stability of the consortia, that is, if they maintain their ability to diminish the Cr(VI) with the yield of new amounts of this metal; (iv) prove how this process behaves in larger periods of time. After that, it is necessary to study these SRB consortia in larger systems (such as between 500 to 1000 mL), using an industrial effluent contaminated by Cr(VI), to verify the stress caused on the bacterial community and if the efficiency to remove Cr(VI) observed here is maintained.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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