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Área de Especialização: Biotecnologia Ambiental

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Abstract

The objective of the research described in this thesis was the identification and characterization of anaerobic bacterial communities with high metal resistance and ability for metal removal, thus with potential for application in bioremediation processes.

A sulphate-reducing bacteria (SRB) consortium resistant to high concentrations of heavy metals (Fe, Cu, Zn), similar to those typically present in acid mine drainage (AMD), was obtained from a wastewater treatment plant. Moreover, this consortium showed ability to use wine wastes as carbon and electron source. The phylogenetic analysis of the *dsr* gene sequence revealed that this consortium contains species of SRB affiliated to *Desulfovibrio fructosovorans, Desulfovibrio aminophilus* and *Desulfovibrio desulfuricans*. Wine wastes as carbon source for SRB activity were applied with success in a bioremediation process for the treatment of artificial AMD. TGGE fingerprinting and phylogenetic analysis showed that the composition of the community in the bioreactor fed with wine wastes remained stable during the whole time of operation and its bacterial diversity was higher than the community in the bioreactor fed with ethanol.

Several microbial communities were investigated for their ability to remove uranium (VI) and additionally the impact of U(VI) on SRB communities was explored. Although the original communities were mainly composed by SRB, after uranium exposure these bacteria were not detected in the communities. The highest efficiency for U(VI) removal was observed with a consortium from a soil collected in Monchique thermal place. Moreover this community also showed ability to remove Cr(VI). However when U(VI) was replaced by Cr(VI) several differences in the structure of the bacterial community were observed. The mechanism of U(VI) removal by this consortium was also investigated and was found that U(VI) removal occurred by enzymatic reduction and bioaccumulation. Phylogenetic analysis of 16S rRNA showed that this community was mainly composed by bacteria closely related to *Sporotalea* genus and *Rhodocyclaceae* family.

Keywords: Bacterial communities, Bioremediation, AMD, Uranium (VI), Chromium (VI)

Resumo

O principal objectivo do presente trabalho foi identificar e caracterizar comunidades de bactérias anaeróbias resistentes a metais tóxicos e com capacidade para os remover, consequentemente, com elevado potencial para serem aplicadas em processos de biorremediação de águas e efluentes contaminados com metais, nomeadamente águas ácidas de mina (AMD), e efluentes contendo urânio ou crómio.

As AMD possuem um pH muito baixo e contêm normalmente elevadas concentrações de metais pesados e sulfato. Assim, o tratamento biológico com bactérias redutoras de sulfato (BSR) tem sido considerado uma alternativa promissora para a descontaminação de AMD. As BSR são consideradas importantes membros da comunidade microbiana, sendo interessantes tanto ao nível económico como ambiental e biotecnológico. Estas bactérias utilizam o sulfato como aceitador de electrões na degradação da matéria orgânica, produzindo sulfureto de hidrogénio. O sulfureto de hidrogénio gerado reage com determinados metais pesados, tais como ferro, cobre e zinco, produzindo sulfuretos metálicos que são bastante insolúveis. Assim, através da utilização das BSR num processo de biorremediação para o tratamento da AMD consegue-se eliminar simultaneamente os sulfatos e metais pesados. Para o desenvolvimento de um processo eficiente de biorremediação da AMD são necessárias BSR resistentes às concentrações de metais pesados que normalmente estão presentes nestas águas. Os metais pesados são normalmente tóxicos para os microorganismos inclusive para as BSR. A toxicidade dos metais pesados deve-se ao facto destes terem capacidade de se ligarem às células através da substituição dos iões essenciais e de bloquearem os grupos funcionais de moléculas importantes, como por exemplo as enzimas. Assim, os metais podem provocar a lise celular e a inactivação das enzimas. Deste modo é necessário pesquisar BSR resistentes aos metais pesados que se pretendem eliminar.

Neste trabalho conseguiu-se isolar um consórcio bacteriano contendo BSR com uma elevada resistência a metais pesados (Fe, Cu e Zn) a partir de lamas de uma estação de tratamento de águas residuais. Este consórcio mostrou suportar as concentrações destes metais que normalmente se encontram presentes nas AMD. A análise filogenética revelou que este consórcio bacteriano continha espécies de BSR afiliadas com *Desulfovibrio*

desulfuricans e *Desulfobulbus rhabdoformis.* Observou-se também que este consórcio bacteriano utilizava tanto o etanol como o lactato como fonte de carbono e de electrões na redução do sulfato. Visto que o lactato é uma fonte de carbono mais dispendiosa do que o etanol, assim é preferível num processo de biorremediação usar o etanol para promover a actividade destas BSR. Normalmente as AMD não possuem elevadas concentrações de compostos que possam funcionar como fonte de carbono e de electrões para as BSR, pelo que é necessário adicionar uma fonte externa, de forma a promover a actividade bacteriana. Assim, foi investigada a possibilidade de usar resíduos de indústrias alimentares (indústrias vinícolas e queijarias) como fonte de carbono e de electrões para as BSR, visto que estes resíduos são largamente produzidos em Portugal. Observou-se que os resíduos de uma indústria vinícola (que contém etanol) podem ser usados eficientemente pelas BSR como fonte de carbono e de electrões, desde que misturados com lama de mármore, que também é um resíduo. A lama de mármore funcionou como tampão e agente neutralizante. Verificou-se que o consórcio bacteriano com capacidade para usar resíduos vinícolas como fonte de carbono e de electrões era constituído por espécies de *Desulfovibrio fructosovorans, Desulfovibrio aminophilus* e *Desulfovibrio desulfuricans*. Posteriormente, investigou-se a aplicação dos resíduos vinícolas como fonte de carbono e de electrões para a actividade das BSR num processo de biorremediação para o tratamento da AMD. Verificou-se, tal como nos estudos anteriores, que estes resíduos promoveram uma eficiente actividade das BSR e consequentemente o sistema de biorremediação apresentou uma eficiente performance no tratamento da AMD, conseguindo produzir água tratada com características, em termos de pH, sulfato e metais (Fe, Cu e Zn), que cumprem a legislação em vigor para água de rega.

Para além da eficiência do processo de biorremediação da AMD usando resíduos vinícolas como fonte de carbono e de nutrientes foi também estudada a dinâmica das populações bacterianas no sistema de biorremediação, através da técnica de electroforese em gel com gradiente de temperatura (TGGE) e da análise filogenética. Procedeu-se também à comparação da estrutura da população bacteriana desenvolvida no biorreactor alimentado com resíduos vinícolas com a estrutura desenvolvida no biorreactor alimentado com etanol. O perfil de TGGE e a análise filogenética mostraram que a composição da comunidade bacteriana no biorreactor alimentado com resíduos vinícolas permaneceu estável durante todo tratamento e que possui uma maior diversidade bacteriana comparativamente com o

biorreactor alimentado com etanol. Através da análise filogenética das bandas do TGGE observou-se que a comunidade bacteriana no biorreactor alimentado com resíduos vinícolas era constituída por bactérias afiliadas com os géneros *Desulfovibrio*, *Clostridium*, *Citrobacter* e *Cronobacter* e com a ordem Bacteroidales, enquanto a comunidade dominante desenvolvida no biorreactor alimentado com etanol era composta por bactérias pertencentes somente ao género *Desulfovibrio*. A presença de diferentes grupos bacterianos no biorreactor alimentado com resíduos vinícolas sugere que talvez ocorra uma interacção sinérgica entre as diferentes populações bacterianas. Esta sinergia poderá ser a razão pela qual as BSR conseguem utilizar o resíduo vinícola, que é um substrato orgânico complexo, como fonte de carbono e de electrões na redução do sulfato.

Algumas águas subterrâneas e superficiais estão contaminadas com urânio. Esta contaminação poderá ser o resultado de processos naturais, actividades mineiras ou descargas de material nuclear para o ambiente. As técnicas convencionais aplicadas na remoção de urânio de soluções aquosas são normalmente baseadas em processos físico-químicos, como por exemplo precipitação através da adição de carbonatos. No entanto estas técnicas têm elevados custos associados e limitações técnicas.

Actualmente a biorremediação é considerada como uma potencial alternativa aos métodos convencionais de remoção de urânio, uma vez que possui varias vantagens tais como baixos custos operativos e uma elevada eficiência no tratamento de efluentes com concentrações baixas de urânio.

Durante as últimas duas décadas tem-se vindo a descobrir que diversos grupos de microrganismos possuem capacidade para remover urânio de soluções aquosas. As BSR são um exemplo desses microrganismos. Algumas destas bactérias tem capacidade para reduzir enzimaticamente o urânio (VI) a urânio (IV) que é bastante insolúvel, ao contrário do urânio (VI). Consequentemente as BSR são consideradas como potenciais candidatas para serem aplicadas em processos de biorremediação de águas e efluentes contaminados com urânio. Assim, investigou-se a capacidade de várias comunidades de BSR para removerem urânio (VI) de soluções aquosas. Adicionalmente, investigou-se o efeito do urânio (VI) na estrutura das comunidades bacterianas através da análise por TGGE. Conseguiu-se obter consórcios com capacidade para remover urânio (VI) a partir das comunidades de BSR, tendo-se verificado no entanto uma drástica alteração na composição das comunidades bacterianas durante a exposição ao urânio (VI). Surpreendentemente as

BSR, que eram o grupo de bactérias que predominavam nas culturas originais, não foram detectadas nas comunidades com capacidade para remover urânio. Este resultado salienta a necessidade de monitorizar as populações dominantes durante os estudos de bio-remoção. Através da análise filogenética das bandas do TGGE observou-se que os consórcios bacterianos com habilidade para remover urânio eram constituídos por bactérias afiliadas com o género *Clostridium* e com as famílias *Caulobacteraceae* e *Rhodocyclaceae.* Assim estas bactérias possuem potencial para serem aplicadas em processos de biorremediação de águas e efluentes contaminados com urânio.

Visto que o urânio (VI) e o crómio (VI) possuem algumas semelhanças nomeadamente o mesmo estado de oxidação da forma mais solúvel e a possibilidade de serem reduzidos por via biológica originando estados de oxidação insolúveis, U(IV) e Cr(III), foi investigada a possibilidade das comunidades bacterianas com capacidade para removerem o U(VI) terem também capacidade de remover o Cr(VI). Para atingir este objectivo estudou-se a capacidade de remoção de U(VI) por comunidades bacterianas obtidas a partir de diversas amostras ambientais, umas contaminadas com urânio e outras não contaminadas. Posteriormente as comunidades que demonstraram capacidade para remover U(VI) foram testadas também para a remoção de Cr(VI). Também se comparou a estrutura das comunidades bacterianas com capacidade de remover urânio (VI) com a das comunidades bacterianas que demonstraram capacidade para remover crómio (VI). Esta comparação foi realizada através da análise por TGGE. Verificou-se que de todas as comunidades bacterianas testadas, somente três é que mostraram habilidade para remover urânio (VI): uma proveniente de amostras de solo das termas de Monchique, outra proveniente de lamas da uma zona húmida da mina da Urgeiriça e outra proveniente de sedimentos da mina da Urgeiriça. A maior eficiência de remoção de ambos os metais foi observada com um consórcio bacteriano obtido a partir de amostras de solo das termas de Monchique. Este consoórcio conseguiu remover 91% de U(VI) de uma solução que continha 22 mg/L e 99% de Cr(VI) de uma solução que continha 13 mg/L de Cr(VI). Este estudo demonstrou que as comunidades com capacidade para remover urânio (VI) também possuem capacidade para remover crómio (VI). No entanto observaram-se diversas diferenças na estrutura das comunidades quando o urânio (VI) foi substituído por crómio (VI). Através da análise filogenética das bandas do TGGE, verificou-se que os consórcios bacterianos com capacidade para remover urânio eram maioritariamente constituídos por bactérias afiliadas

com o género *Clostridium* e com a família *Rhodocyclaceae*, enquanto que as comunidades estabelecidas na presença do crómio (VI) eram maioritariamente compostas por bactérias pertencentes ao género *Clostridium* e às famílias *Rhodocyclaceae* e *Enterobacteriaceae*. Este último grupo foi somente detectado nas comunidades com capacidade de remover o crómio (VI). A presença de bactérias nunca reportadas como possuindo capacidade para remover estes metais, como é o caso das bactérias pertencente às famílias *Rhodocyclaceae* e *Enterobacteriaceae*, foi uma importante descoberta encorajando a exploração das potencialidades destes microorganismos para fins de biorremediação. Uma vez que estas comunidades são constituídas por espécies bacterianas pouco ou nada exploradas em termos de biorremediação, o mecanismo envolvido na remoção de urânio (VI) não está ainda esclarecido. Tem sido demonstrado que os microrganismos possuem diversos mecanismos de remoção de metais como por exemplo acumulação do metal no interior das células, adsorção à superfície celular e alteração do estado de oxidação do metal, tornando-o menos solúvel. Este último mecanismo é normalmente realizado por intermédio de enzimas existentes nas células. Assim, para obter conhecimentos sobre a remoção de urânio (VI) pelas comunidades bacterianas descobertas anteriormente, procedeu-se ao estudo do mecanismo envolvido na remoção de urânio (VI) de duas das comunidades bacterianas: uma proveniente de um local não contaminado (termas de Monchique) e outra proveniente de uma mina de urânio (mina da Urgeiriça). A remoção de U(VI) foi testada na presença de células vivas, células mortas e produtos produzidos extracelularmente pelas bactérias. Em ambos os consórcios observou-se somente remoção de U(VI) na presença de células vivas e verificou-se, através da análise de difracção por raio X, a presença de U(IV) no precipitado produzido biologicamente. Através da análise das células bacterianas por microscopia electrónica de transmissão (TEM) observou-se a presença de precipitados densos maioritariamente na região periplasmática das células de ambos os consórcios e precipitados arredondados no citoplasma de algumas células do consórcio proveniente das amostras de solo das termas de Monchique. Estes resultados sugerem que a remoção de urânio (VI) pelo consórcio bacteriano proveniente de amostras de solo das termas de Monchique ocorreu por redução enzimática e por bioacumulação, enquanto que a redução enzimática provavelmente foi o único mecanismo envolvido na remoção de urânio (VI) pelo consórcio proveniente de sedimentos da mina da Urgeiriça. Os resultados da análise por espectrometria de infravermelho por transformada de Fourier (FTIR) sugeriram que

depois da redução do urânio (VI), o metal poderá ter-se ligado aos grupos carboxílicos, fosfato e amida existentes nas células bacterianas. A análise filogenética, baseada na sequenciação do gene 16S rRNA, mostrou que a comunidade proveniente de amostras de solo das termas de Monchique era maioritariamente constituída por bactérias afiliadas com o género *Sporotalea* e com a família *Rhodocyclaceae*, enquanto que a comunidade proveniente de sedimentos da mina da Urgeiriça era maioritariamente composta por bactérias afiliadas com o género *Clostridium* e também com a família *Rhodocyclaceae*.

Tendo em conta que existe uma grande diversidade de microrganismos na natureza é de grande importância pesquisar e caracterizar comunidades bacterianas resistentes a metais e com capacidade para os remover. Com este trabalho conseguiu-se demonstrar que é possível obter consórcios bacterianos a partir de amostras ambientais com potencialidades para serem aplicados em processos de biorremediação de águas e efluentes contaminados com metais. Conclui-se também que a exposição das comunidades a diferentes metais promove o crescimento de diferentes populações bacterianas, o que enfatiza a necessidade de, para além de monitorizar a eficiência de remoção dos metais, monitorizar as populações bacterianas dominantes envolvidas nos estudos de biorremediação. A identificação das comunidades bacterianas, bem como o conhecimento sobre as interacções entre as bactérias e os metais poderão contribuir para o desenvolvimento de estratégias de biorremediação mais eficientes.

Palavras-chave: Comunidades bacterianas, Biorremediação, AMD, Urânio (VI), Crómio (VI)

“O valor das coisas não está no tempo em que elas duram,
mas na intensidade com que acontecem.
Por isso existem momentos inesquecíveis,
coisas inexplicáveis e pessoas incomparáveis"

(Fernando Pessoa)

Contents

Chapter 1

General Introduction

1. Introduction

Metals constitute about 75% of the known elements. They are distributed all over the earth (Raab and Feldmann, 2003; Gadd 2010) and are usually classified in the following three categories: toxic metals (such as Cr, Zn, Cu, Ni, Cd, As, etc.), precious metals (such as Pd, Pt, Ag, Au, etc.) and radionuclides (such as U, Th, Ra, Am, etc.) (Bishop, 2002; Wang and Chen, 2006). Metals are a kind of resource that is becoming rarer. However, due to their increasing application, the heavy metals pollution has accordingly become one of the most serious environmental problems.

Current and past mining activities are considered as the principal sources of metal contamination in soils and waters (Johnson and Hallberg, 2005). The mining industry produces acidic wastewaters containing high concentrations of sulphate, heavy metals and metalloids (such as arsenic) (Nagpal *et al.*, 2000; Garcia *et al*., 2001; Johnson and Hallberg, 2005). Such waters, known as acid mine drainage (AMD), are considered one of the major environmental problems faced by the mining industries (Chockalingam and Subramanian, 2009). Uranium mining activities have also resulted in the generation of large amounts of wastes containing different heavy metals and radionuclides with severe impact in the environment (Lloyd and Macaskie, 2000). Moreover, with the rapid development of several industries (including energy and fuel production, fertilizer and pesticide, electroplating, photography, dyes, textile), wastes containing metals are directly or indirectly discharged into environment (Barnhart, 1997; Bishop, 2002; Wang and Chen, 2006). Heavy metal contamination represents a severe environmental concern owing to their hazardous impact, causing serious damage to human health, biodiversity and ecosystems (Navarro *et al.*, 2008).

Physicochemical methods, such as chemical neutralization followed by hydroxide or carbonate precipitation of metals, solvent extraction, ion exchange processes, adsorption on activated carbon and membrane technologies have been conventionally employed for heavy metals removal from industrial wastewaters (Lanouette, 1977; Shokes and Moller, 1999; Burgess and Stuetz, 2002; Wang and Chen, 2006). However, these methods have significant disadvantages, such as incomplete metal removal, cost (high reagent and/or energy requirements) generation of toxic sludge or other waste pollutants, and they are not

selective enough to allow the recovery of heavy metals present in the effluent (Chubar *et al.*, 2004; Saeed *et al.*, 2005; Chen *et al.*, 2008). For theses reasons, research and development focused on the search of better decontamination methods and new technologies has been intensive.

Bioremediation strategies based on the use of microorganisms are considered a potential alternative and an economically attractive approach that offers several advantages over the traditional techniques, such as low operating costs, minimum production of disposable sludge volume and high efficiency in detoxifying very dilute effluents (Valls and de Lorenzo, 2002). However, heavy metals are generally toxic for microorganisms, due to substitution of essential ions on cellular sites, and blockage of functional groups of important molecules such as enzymes (Gonzalez-Silva *et al.*, 2009). This results in denaturation and inactivation of enzymes and disruption of cell organelle membrane integrity (Sani *et al.*, 2001; Cabrera *et al.*, 2006). In order to develop an efficient bioremediation process, selection of the most efficient metal-resistant bacteria is required

The present study focuses in the identification and characterization of anaerobic bacterial communities with high metal resistance and ability for metal removal, thus with potential for application in bioremediation processes for treatment of wastewaters containing metals, namely AMD, wastewaters containing uranium and industrial effluents contaminated with chromium.

2. Wastewaters containing metals

2.1. Acid mine drainage

Acid mine drainage (AMD), also known as acid rock drainage (ARD), results from mining activities where pyritic minerals have been exposed to water and oxygen (Roman *et al.*, 2008). During mining exploration oxygen and water was introduced into the deep geological environment leading to the oxidation of minerals (Christensen *et al.*, 1996; Banks *et al.*, 1997). Oxidation also occurs when reduced minerals (mainly sulphide) are brought to the surface and deposited in heaps (Banks *et al.*, 1997). The oxidation of sulphide minerals leads to the formation of acidic wastewaters containing high

concentrations of sulphate and metallic ions, known as AMD (Kaksonen and Puhakka, 2007; Costa *et al.*, 2008; Pérez-López *et al.*, 2008). Metals concentration in AMD is determinate by the composition of the minerals. The low concentration of organic matter (below to 20 mg/L) presents a problem for bioremediation of these wastewaters (Johnson and Hallberg 2003).

AMD is currently one of the most widespread forms of pollution worldwide (Tabak *et al.*, 2003, Pinto and Silva, 2005). This problem is also very important in Portugal where extensive sulphide mining activities played an important role from pre-historic until recent times. Nevertheless, in the last decades practically all the mining activities have been suspended and as a consequence several mine sites have been left untreated (Oliveira *et al.*, 2000). Portugal has about 175 abandoned mine sites and 14% of the 85 abandoned mines studied by Oliveira *et al.*, in 2002, were found to generate AMD and/or to pose a high degree of environmental risk. Examples of such source of pollution are mines of S. Domingos (Fig. 1.1), Aljustrel, Caveira and Lousal (Pinto and Silva, 2005).

Fig. 1.1 São Domingos mine: open pit lake (left) and AMD (right). (Photos: Mónica Martins).

The abandoned copper mine of São Domingos, located in Mértola (Beja District) is one of the most emblematic Portuguese mining sites in the Iberian Pyrite Belt (IPB). The beginning of mining exploitation in the area dated back to the pre-Roman period, remaining in activity until 1966, when it was definitely closed. The intense mining activity is reflected by the presence of huge volumes of AMD (Pérez-López *et al.*, 2008). The AMD from S. Domingos is highly acidic (pH around 2) and is characterised by high concentrations of heavy metals, mainly Fe (500 mg/L), Cu (50 mg/L), Zn (110 mg/L) and sulphate (3100 mg/L) (Costa and Duarte, 2005; Costa *et al.*, 2008). These characteristics make this AMD

an excellent candidate for the application of a treatment based in the SRB activity for metal precipitation through biologic sulphate reduction.

2.2. Wastewaters containing radionuclides

Uranium mining and mineral processing for production of nuclear power have resulted in the generation of significant amounts of wastes containing radionuclides (mainly uranium) with severe impact on environment (Gorby and Lovley, 1992; Choudhary and Sar, 2009). Due to the use of sulphuric or nitric acid as extractants in the operation, these wastewaters are generally characterised by very low pH, high levels of uranium, heavy metals, sulphate and nitrate (Yi *et al.*, 2007).

Portugal was particularly rich in uranium mines (e.g. Urgeiriça, Cunha Baixa, Quinta do Bispo, Vale de Abrutiga) (Pinto and Silva, 2005). In the last century 4370 tons of U_3O_8, radio salts and about 13 millions tons of different kinds of wastes were produced in Portugal (Nero *et al.*, 2003). Underground and open pit mining were the main extraction techniques used, as well as *in situ*-leaching to recover uranium from the poorest ore (Nero *et al.*, 2003). Wastewaters produced by *in situ*-leaching process still persist in all of the areas where uranium mining occurred, even after the exploration has ceased (Pereira *et al.*, 2008). The abandoned uranium mine of Urgeiriça located in Canas de Senhorim (Viseu district, North Portugal) is an example of such source of contamination. Urgeiriça mine exploration began in 1913 for radium extraction and this activity was maintained until 1944. After this year, this mine was exclusively dedicated to the production of uranium (Madruga *et al.*, 2001). The mine exploration has been stopped in the decade of 90.

Radionuclides like uranium are of particular concern due to their high toxicity and long half lives. Uranium can be characterised as a heavy, ductile and slightly paramagnetic metal and is widely dispersed in the earths crust, rocks and soils at the level of about 2-4 ppm by weight (Gavrilescu *et al.*, 2009). Uranium is in fact more abundant than gold, silver, mercury or cadmium (Gavrilescu *et al.*, 2009).

Natural uranium exists in three different forms (isotopes): ^{238}U, ^{235}U, and ^{234}U, in relative abundances of 99.27 %, 0.72 %, and 0.0055 %, respectively (Eisenbud and Gesell, 1997). Uranium can exist in the +3, +4, +5 and +6 oxidation states, however only U(IV) and

U(VI) are stable in aqueous solution. Uranium exists in solution predominantly as U (VI) in the form of divalent oxocomplex (UO_2^{2+}) and as soluble carbonate complexes (Gavrilescu *et al.*, 2009).

Uranium (IV) is very insoluble forming uraninite (UO_2), on the other hand uranium (VI) is much more soluble and mobile (Gavrilescu *et al.*, 2009). Therefore the reduction of U(VI) to U(IV) by some microorganisms has been viewed as a potential mechanism for sequestration of environmental uranium contamination (Wall and Krumholz, 2006).

2.3. Wastewaters containing chromium

Chromium is one of the metals most widely used in the industry. Consequently, large amounts of chromium are discharged into the environment (Remoundaki *et al.*, 2007) due to inadequate waste treatment (Chen and Hao, 1998). Leather tanning, electroplating, metallurgy, petroleum refining, textile manufacturing and metal finishing are the major sources of chromium discharge (Barnhart, 1997; Remoundaki *et al.*, 2007).

Textile manufacturing is considered as the most polluting of all industrial sectors (Khelifi *et al.*, 2009). In fact, several pollutants are present in textile effluents such as dyes, heavy metals (e.g. chromium) and sulphate salts (Cetin *et al.*, 2008). The chromium complex in textile dyes is formed through the chemical reaction between Cr_2O_3 and a variety of azo organic compounds (Cetin *et al.*, 2008). The structure of these chromium complexes is very stable and hard to destroy (Delee *et al.*, 1998). In the automotive sector, hexavalent chromium is used in surface treatment in order to ensure anti-corrosion protection of metallic components in steel or aluminum, friction facilitation and decoration (Remoundaki *et al.*, 2007).

The aqueous solubility of chromium and its toxicity are strongly dependent on its oxidation state (Chardin *et al.*, 2002). Chromium exists in a number of oxidation states being the most stable and common forms the trivalent, Cr(III), and the hexavalent, Cr(VI), (Remoundaki *et al.*, 2007; Thacker *et al.*, 2007). Cr(VI) is highly soluble and thus, mobile and bio-available in aquatic systems (Chung *et al.*, 2006). Cr(VI) is known as carcinogenic and mutagenic, being actively transported into cells via the anion transport pathway (Stearns et al., 1995; Flores and Pérez 1999; Shi *et al.*, 1999). In contrast, Cr(III) has relatively low toxicity and tends to form insoluble and strong complexes with hydroxides at

neutral pH (Rai *et al.*, 1989; Palmer and Wittbrodt, 1991). Biotransformation of Cr(VI) to non toxic Cr(III) by microorganisms offers an economical and eco-friendly option for treatment of waters contaminated with chromium (Pal *et al.*, 2005).

3. Biologic sulphate reduction applied in the treatment of wastewaters containing metals

The role of SRB in acid mine waters was first explored by Colmer and Hinkle (1947) and since then microbial remediation of sulphate and metal-containing wastewater was applied in several passive and active processes (Tuttle *et al.*, 1969). Due to combined removal of metals and sulphate, the biological treatment with SRB is considered the most promising alternative for the treatment of several types of industrial wastewaters, namely AMD (Johnson and Hallberg 2005; Neculita *et al.* 2007).

3.1. Diversity of sulphate-reducing bacteria

SRB constitute a diverse group of prokaryotes phylogenetically and metabolically versatile and may represent the first respiring microorganisms with subsequent role in the biochemistry of the various environments (Barton and Fauque, 2009). Until the early 1980s it was thought that sulphate reducers played only a minor part in the carbon cycle. However, through the research carried by Fritz Widdel (1980), it was discovered that SRB are the main players in anaerobic carbon cycling (Muyzer and Stams, 2008). SRB have successfully adapted to almost all the ecosystems of the planet and consequently they are widespread in anoxic habitats such as marine sediments, hydrothermal vents and hydrocarbon seeps (Muyzer and Stams, 2008; Barton and Fauque, 2009). SRB can also be found in habitats with extreme pH values such as mining wastewaters, where pH can be as low as 2 and in soda lakes, where pH can be as high as 10 (Muyzer and Stams, 2008). They are also present in aquifers and in engineered systems, such as anaerobic wastewater treatment plants (Muyzer and Stams, 2008; Barton and Fauque, 2009).

Based in analysis of 16S rRNA gene sequences, the known SRB can be grouped into seven phylogenetic groups: five within the bacteria and two within the Archaea (Table 1.1).

Table 1.1 Phylogeny of sulphate-reducing prokaryotes (Castro *et al.*, 2000; Garrity *et al.*, 2003; Meyer and Kuever, 2007; Muyzer and Stams, 2008).

Phylum	Class	Family	Genus
Proteobacteria	Delta-proteobacteria	Desulfovibrionaceae	*Desulfovibrio*
		Syntrophobacteraceae	*Desulfacinum;*
			Desurforhabdus
			Desulfovirga
			Thermodesulforhabdus
		Syntrophaceae	*Desulfomonile*
			Desulfobacca
		Desulfobacteraceae	*Desulfobacterium*
			Desulfobacter
			Desulfobacula
			Desulfobotulus
			Desulfocella
			Desulfococcus
			Desulfofrigus
			Desulfonema
			Desulfofaba
			Desulforegula
			Desulfosarcina
			Desulfospira
			Desulfotignum
		Desulfobulbaceae	*Desulfobulbus*
			Desulfocapsa
			Desulforhopalus
			Desulfofustis
			Desulfortalea
		Desulfarculaceae	*Desurfarculus*
		Desulfonatronumaceae	*Desulfonatronum*
		Desulfomicrobiaceae	*Desulfomicrobium*
			Desulfocaldus
		Desulfohalobiaceae	*Desulfohalobium*
			Desulfonatronovibrio
			Desulfonauticus
			Desulfothermus
Firmicutes	Clostridia	Peptococcaceae	*Desulfotomaculum*
			Desulfosporosinus
			Desulfosporomusa
Thermo-desulfobacteria	Thermo-desulfobacteria	Thermo-desulfobacteriaceae	*Thermodesulfobacterium*
			Thermodesulfatator
Nitrospira	Nitrospira	Nitrospiraceae	*Thermodesulfovibrio*
Unclassified	Unclassified	Thermodesulfobiaceae	*Thermodesulfobium*
Euryarchaeota	Archaeoglobi	Archaeoglobaceae	*Archaeoglobus*
Crenarchaeota	Thermoprotei	Thermoproteaceae	*Thermocladium*
			Caldivirga

All of the SRB groups are characterised by their use of sulphate as a terminal electron acceptor during anaerobic respiration. Most of the sulphate reducers belong to Deltaproteobacteria. The majority of SRB of this class are mesophilic, however *Desulfacinum* and *Thermodesulforhabdus* are thermophilic (Castro *et al.*, 2000). The class Clostridia contains SRB affiliated to *Desulfosporomusa*, *Desulfosporosinus* and *Desulfotomaculum*. This bacterial group includes the only SRB known to form heat-resistant endospores (Castro *et al.*, 2000) and all are Gram-positive. Some species of *Desulfotomaculum* are thermophilic, although their optimal growth temperatures are lower than those of thermophilic Gram-negative (*Desulfacinum* and *Thermodesulforhabdus* genera) and archaeal sulphate reducers (Castro *et al.*, 2000). The Termodesulfobacteriaceae, Nitrospiraceae and Thermodesulfobiaceae families only contain thermophilic SRB. Within Archaea, at present only three genera of sulphate reducers are known: *Archaeoglobus*, *Thermocladium* and *Caldivirga* genera. The archaeal sulphate reducers exhibit optimal growth temperatures above 80ºC, however they can survive at lower temperatures (Castro *et al.*, 2000).

3.2. Sulphate reduction and metals precipitation

3.2.1 Assimilatory and dissmilatory sulphate reduction

Several microorganisms reduce sulphate in the small amounts required for the synthesis of cellular material. This small-scale reduction of sulphate has been known by assimilatory sulphate reduction (Peck, 1961). In assimilatory sulphate reduction, sulphide is not usually produced in detectable amounts from sulphate. A minor group of microorganisms have the ability to reduce sulphate in great excess of nutritional requirements and thus massive amounts of sulphide are produced (Peck, 1961). This large-scale reduction of sulphate to sulphide has been identified by dissimilatory sulphate reduction. In dissimilatory sulphate reduction (also called sulphate respiration) microorganisms use sulphate as an external electron acceptor in the oxidation of energy substrates resulting in the production of sulphide (Peck, 1961; Barton and Fauque, 2009). The major group of microorganisms known to be dissimilatory sulphate reducers are SRB. The pathway of dissimilatory and

assimilatory sulphate reduction is described in Fig. 1.2 (Peck, 1961; Postgate, 1984; Madigan *et al.*, 2003; Barton and Fauque, 2009).

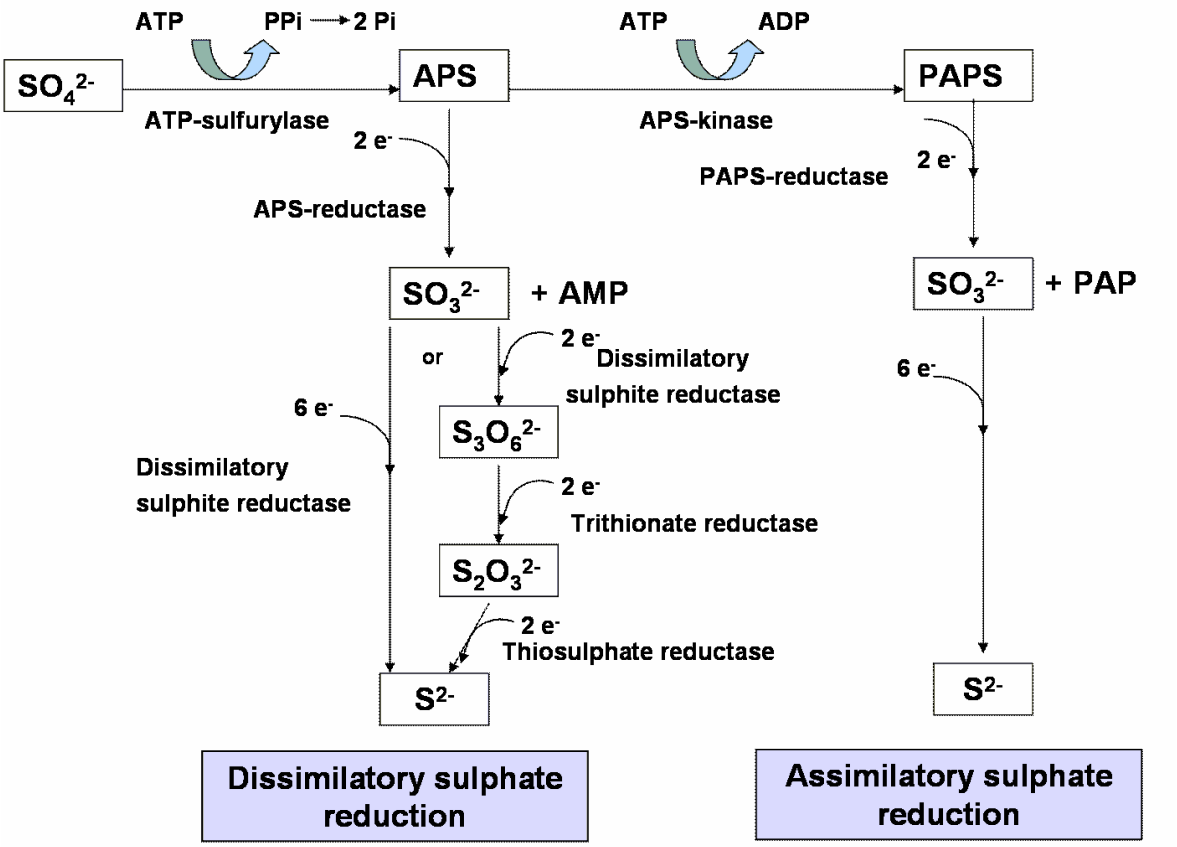

Fig. 1.2 Dissimilatory and assimilatory sulphate reduction: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine 5´-phosphosulphate (APS), inorganic pyrophosphate (PPi), adenosine monophosphate (AMP), phosphoadenosine 5´-phosphosulphate (PAPS) and phosphoadenosine 5´-phosphate (PAP). (Adapted from Postgate, 1984; Madigan *et al.*, 2003)

The first step of assimilatory and dissimilatory sulphate reduction is the activation of the sulphate ion. Sulphate is the unique electron acceptor that must be activated before it can be reduced. This activation is made by means adenosine triphosphate (ATP). The enzyme ATP-sulfurylase catalyzes the binding of sulphate to phosphate group of ATP molecule, forming adenosine 5´-phosphosulphate (APS) and inorganic pyrophosphate (PPi) (Peck, 1961; Postgate, 1984; Madigan *et al.*, 2003; Barton and Fauque, 2009). APS structure is

similar to ATP but two of the phosphate groups of ATP are replaced by a sulphate group (Postgate, 1984). The formation of PPi is thermodynamically unfavourable, therefore the reaction needs to be pulled to completion. This last reaction was carried by an inorganic pyrophosphatase (pyrophosphate phosphohydrolase) which hydrolyzes PPi forming phosphate (Peck, 1961; Madigan *et al.*, 2003; Barton and Fauque, 2009).

In dissimilatory sulphate reduction APS is reduced directly to sulphite (SO_3^{2-}) with the release of adenosine monophosphate (AMP). This reaction is catalyzed by APS-reductase and it is the first redox reaction. In assimilatory reduction, another phosphate group is added to APS forming phosphoadenosine 5´-phosphosulphate (PAPS) in the presence of the enzyme APS-kinase. Then PAPS is reduced to sulphite, by PAPS reductase, with the release of phosphoadenosine 5´-phosphate (PAP) (Peck, 1961; Postgate, 1984; Madigan *et al.*, 2003; Barton and Fauque, 2009).

The six-electron reduction of sulphite to sulphide must compensate the energy investment of sulphate activation and yield additional ATP for growth (Barton and Fauque, 2009). The mechanism of sulphite reduction to sulphide is somewhat controversial and two different metabolic pathways have been proposed (Fig. 1.2) (Postgate, 1984; Madigan *et al.*, 2003; Barton and Fauque, 2009):

1) Sulphite is reduced to sulphide in one step, catalyzed by dissimilatory sulphite reductase (DSR) without the formation of free intermediates.
2) Sulphite is reduced to sulphide in three steps using two free intermediates: trithionate ($S_3O_6^{2-}$) and thiosulphate ($S_2O_3^{2-}$).

In the dissimilatory sulphate reduction, sulphide is excreted into the environment whereas in assimilatory reduction, the sulphide formed is immediately converted into organic sulphur compounds, such as amino acids (Madigan *et al.*, 2003).

The resulting sulphide from dissimilatory sulphate reduction may dissociate according to the environmental conditions. The following equilibrium equations describe this dissociation (Johnson and Hallberg, 2005):

$$H_2S \leftrightarrow HS^- + H^+ \qquad pKa_1=6.9 \quad (25ºC) \qquad (1.1)$$

$$HS^- \leftrightarrow S^{2-} + H^+ \qquad pKa_2=12.9 \quad (25ºC) \qquad (1.2)$$

In the optimum pH for SRB activity (around 7) H_2S and HS^- are the predominant sulphide forms.

3.2.2 Metal precipitation by biologic sulphide

SRB are anaerobic microorganisms that use sulphate as a terminal electron acceptor for the degradation of organic compounds, resulting in the production of hydrogen sulphide and alkalinity (Kaksonen and Puhakka, 2007; Muyzer and Stams, 2008; Qiu *et al.*, 2009). Biologic hydrogen sulphide reacts additionally with certain metals dissolved in contaminated waters forming insoluble precipitates (White *et al.* 2003, Costa and Duarte 2005, Vega-López *et al.* 2007) and, as a result, the concentrations of sulphate and dissolved metals are reduced. The biological transformation process is described in the following reactions, where CH_2O represents the electron donor and M^{2+} metals, such as Zn^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+}, Hg^{2+}, Pb^{2+}or Cd^{2+} (Kaksonen and Puhakka, 2007; Qiu *et al.*, 2009):

$$2\ CH_2O + SO_4^{2-} \rightarrow H_2S\ (g) + 2\ HCO_3^- \qquad (1.3)$$

$$H_2S + M^{2+} \rightarrow MS\ (s) + 2H^+ \qquad (1.4)$$

$$HCO_3^- + H^+ \rightarrow CO_2\ (g) + H_2O \qquad (1.5)$$

The potential advantages of metal sulphide precipitation include production of lower sludge volume and lower solubility of the metal sulphides generated compared with the corresponding hydroxides or carbonates (Table 1.2) (Bayrakdar *et al.*, 2009; Gonzalez-Silva *et al.*, 2009). Moreover, a high degree of selective metal precipitation is possible with sulphide, as opposed to hydroxide precipitation (Fig. 1.3) (Huisman *et al.*, 2006). Optimal pH values for precipitation of several metals have been suggested by Hammack *et al.* (1994), Govind *et al.* (1997) and Tabak *et al.* (2003): copper can be precipitated as CuS at extremely low pH values ($pH \leq 1.0$) without precipitation of other metals, whereas ZnS precipitates at pH values between 2 and 5. Moreover, Fe(II) does not precipitate as FeS until pH reaches values above 4.5. Thus, a selective precipitation of metal sulphides by pH control can be an additional advantage.

Table 1.2 Solubility product constants of several metals with hydroxide, carbonate and sulphide ions, K_{sp}, at 25ºC (Sillen and Martell, 1964).

Metal	K_{sp}		
	OH^-	CO_3^{2-}	S^{2-}
Cu^{2+}	2.0×10^{-19}	1.3×10^{-10}	1.0×10^{-36}
Fe^{2+}	2.0×10^{-15}	5.0×10^{-11}	1.0×10^{-18}
Zn^{2+}	5.0×10^{-17}	1.0×10^{-7}	1.0×10^{-23}
Cd^{2+}	2.0×10^{-14}	2.5×10^{-14}	1.0×10^{-28}
Pb^{2+}	4.0×10^{-15}	1.6×10^{-13}	1.0×10^{-28}
Ni^{2+}	2.0×10^{-16}	1.3×10^{-7}	1.0×10^{-22}

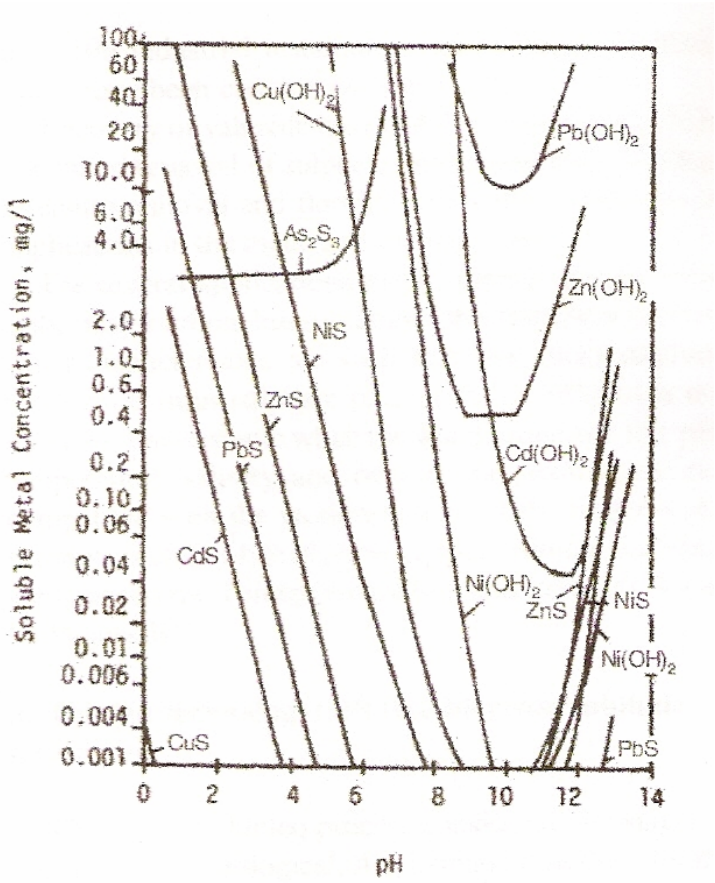

Fig. 1.3 Solubility of metal hydroxides and sulphides as function of pH (Huisman *et al.*, 2006).

3.3. Electron donor and carbon source for sulphate-reducing bacteria

SRB are known to utilise hydrogen and a number of simple organic compounds such as carboxylic acids (e.g. formate, lactate and acetate) or alcohols (e.g. ethanol and methanol) as energy sources (Table 1.3) (Liamleam and Annachatre, 2007). Oxidation of organic compounds can be incomplete with acetate as a by-product or complete, leading to the final production of carbon dioxide (Postgate, 1984; Muyzer and Stams, 2008). SRB that degrade organic compounds completely normally also use acetate as carbon and electron source (Postgate, 1984; Muyzer and Stams, 2008).

Table 1.3 Sulphate reducing reactions with different electron donors. Gibbs free energy changes were calculated from Thauer *et al.* (1977).

Substrate	Equation	ΔG^o (kJ/reaction)
Hydrogen	$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$	-151.9
Lactate	$2CH_3CHOHCOO^- + SO_4^{2-} \rightarrow 2CH_3COO^- + HS^- + 2HCO_3^- + H^+$	-159.6
Formate	$4HCOO^- + SO_4^{2-} + H^+ \rightarrow HS^- + 4HCO_3^-$	-146.7
Acetate	$CH_3COO^- + SO_4^{2-} \rightarrow HS^- + 2HCO_3^-$	-47.3
Ethanol	$2CH_3CH_2OH + SO_4^{2-} \rightarrow 2CH_3COO^- + HS^- + 2H_2O + H^+$	-132.7
Methanol	$4CH_3OH + 3SO_4^{2-} \rightarrow 4HCO_3^- + 3HS^- + 4H_2O + H^+$	-361.7

Thermodynamically, sulphidogenesis with hydrogen as electron source is more favourable than with acetate or formate and would be a relatively inexpensive substrate (Lens *et al.* 2003; Fedorovich *et al.* 2000; Nagpal *et al.* 2000. However, engineering and safety requirements are needed at a commercial scale (Huisman *et al*. 2006; Kaksonen and Puhakka, 2007) which largely contribute to increase the investment and operating costs of the process. Most SRB that use hydrogen as electron donor are able to grow on formate (Liamleam and Annachatre, 2007).

Acetate is a key intermediate in the breakdown of organic substances in anaerobic processes and can be used as an electron donor and carbon source by some SRB. However,

low biomass yield is achieved with this substrate (Kaksonen and Puhakka, 2007; Liamleam and Annachatre, 2007). Lactate is the carbon source most widely used by SRB (Postgate 1984; Barnes 1998; El Bayoumy *et al.*, 1999). Although lactate promotes high biomass yield and high alkalinity production, this substrate would be too expensive for a large scale process (Kaksonen and Puhakka, 2007; Liamleam and Annachatre, 2007). Methanol is of particular interest as electron donor because it is readily available and cost effective (Kaksonen and Puhakka, 2007; Liamleam and Annachatre, 2007). Methanol can be directly used by SRB or indirectly used via the involvement of other anaerobic microorganisms. The growth of SRB with methanol is slow and methanogens can compete for it with SRB under mesophilic conditions (Kaksonen and Puhakka, 2007; Liamleam and Annachatre, 2007). According to the literature, ethanol seems to be the most cost effective substrate (Huisman *et al*., 2006; Tsukamoto *et al*., 2004). However, a draw-back of using this carbon source is a rather low growth rate of SRB (Kaksonen and Puhakka, 2007; Liamleam and Annachatre, 2007). Furthermore, the incomplete oxidation of ethanol does not produce alkalinity and leads to acetate accumulation (Kaksonen and Puhakka, 2007; Liamleam and Annachatre, 2007).

Several natural sources of organic materials serving as electron donors and carbon sources have been already investigated. Zagury *et al.*, (2006) investigated several carbon sources for biological sulphate reduction in the treatment of AMD: maple wood chips, leaf compost, conifer compost and poultry manure. The author demonstrated that a mixture of organic materials was most effective compared with single natural organic substrates. In the study of Chang *et al.* (2000) oak chips, spent mushroom compost and organic rich soil were tested as electron donors for SRB. Results showed that spent mushroom compost is better electron donor compared to oak chips and organic rich soil (Chang *et al.*, 2000). Boshoff *et al.* (2004) showed that tannery effluent can be used as a carbon and electron source by SRB in the treatment of AMD. Gibert *et al.* (2004) showed that sheep manure was the best substrate for SRB followed by poultry manure and oak leaf. The efficacy of lignocellulose as a carbon source for sulphate reduction has been recently demonstrated by Roman *et al*. (2008).

The selection of the carbon source depends to a great extent on the degradability of the organic substrate and on the composition of the bacterial community. Normally, complex organic compounds are not direct substrates for SRB (Liamleam and Annachhatre, 2007;

Muyzer and Stams, 2008). Therefore, SRB are dependent on other microorganisms that degrade these substrates and ferment them to products that can be used as carbon source by SRB (Liamleam and Annachhatre, 2007; Muyzer and Stams, 2008) (Fig. 1.4). The syntrophic relationships established between various microorganisms allowed the degradation of complex molecules, such as glucose, and the use of the corresponding degradation products by SRB (Zhao *et al.*, 2010). Moreover, *Clostridium* sp. has been reported as playing an important role in fermenting molasses and glucose, and as cooperating with SRB for sulphate reduction (Bruggemann and Gottschalk, 2009). The involvement of *Lactobacilli*, other fermentative bacteria, in molasses fermentation was also reported when this substrate was provided as electron donor for sulphate reduction (Maree *et al.*, 1986). The co-existence of SRB and fermentative bacteria may be the key factor for the utilization of complex organic substrates as carbon sources for sulphate reduction.

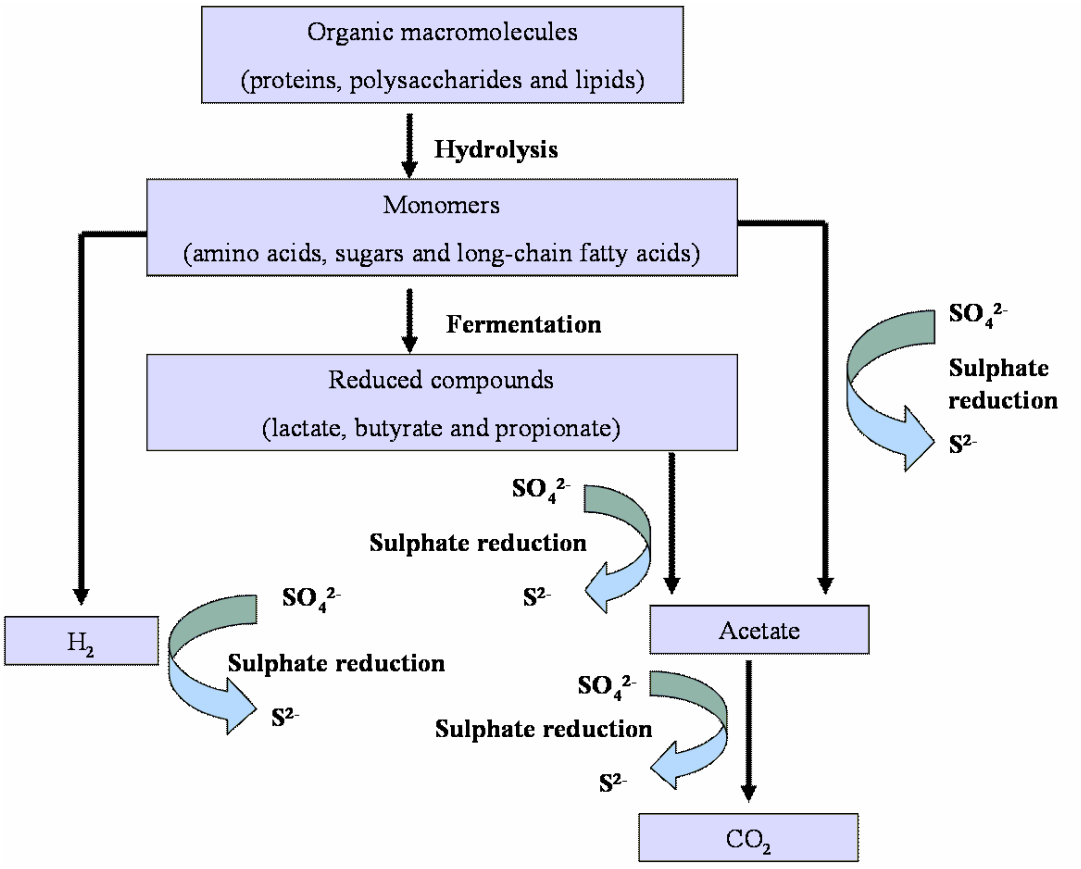

Fig. 1.4 Schematic representation of anaerobic microbial degradation of organic compounds in the presence of sulphate (adapted from Muyzer and Stams, 2008).

3.4. Sulphate-reducing based processes

The bioremediation processes can be categorized into two types: passive and active treatments systems (Jonhnson and Hallberg 2005; Kaksonen and Puhakka, 2007).

3.4.1. Passive biologic treatments

Passive applications for treatment of groundwater contaminated with metals include the enhancement of the microbial activity in groundwater aquifers through substrate injection or permeable reactive barriers (Kaksonen and Puhakka, 2007) (Fig. 1.5a and b). Permeable reactive barriers consist of zones of reactive material installed across the flow path of contaminated groundwater (Richardson and Nicklow, 2002). SRB reduces sulphate from water by using the electron donors present in the barrier (Kaksonen and Puhakka, 2007). This process generates alkalinity and precipitation of metals as sulphides. The success of this type of treatment depends on the selection of the material that composes the barrier. Gravel can be mixed with organic material to increase the permeability and limestone may be added to provide additional alkalinity (Waybrant *et al.*, 1998; Amos and Younger, 2003).

Infiltration beds and wetland systems are passive treatments that can be applied for surface contaminated waters (Kaksonen and Puhakka, 2007) (Fig. 1.5c and d). Infiltration beds are used for treating contaminated surface waters in a similar manner as reactive barriers are used for groundwater. The bed contains organic materials that support the growth and activity of sulphate reducers and is covered with an impermeable liner that helps to create anaerobic conditions (Jonhnson and Hallberg 2005; Kaksonen and Puhakka, 2007) (Fig. 1.5c).

Wetlands have been recognized for several years as low cost systems to treat metal containing wastewaters (Jonhnson and Hallberg 2005; Kaksonen and Puhakka, 2007). These systems have been applied for the removal of sulphate, metals and radionuclides from mine waters (Jonhnson and Hallberg 2005; Kaksonen and Puhakka, 2007). Wetlands can be classified as aerobic and anaerobic. The major objective of the aerobic wetland systems is to enhance the oxidation and hydrolysis reaction of metals and to retain the resulting metal precipitates (Jonhnson and Hallberg 2005; Kaksonen and Puhakka, 2007). The hydrolysis of metals produces acidity and consequently this type of system is generally

constructed to treat waters that are alkaline. Macrophytes are normally planted in aerobic wetlands for aesthetic reasons as well as to regulate water flow and to filter and stabilise the metal precipitates (Jonhnson and Hallberg 2005; Kaksonen and Puhakka, 2007).

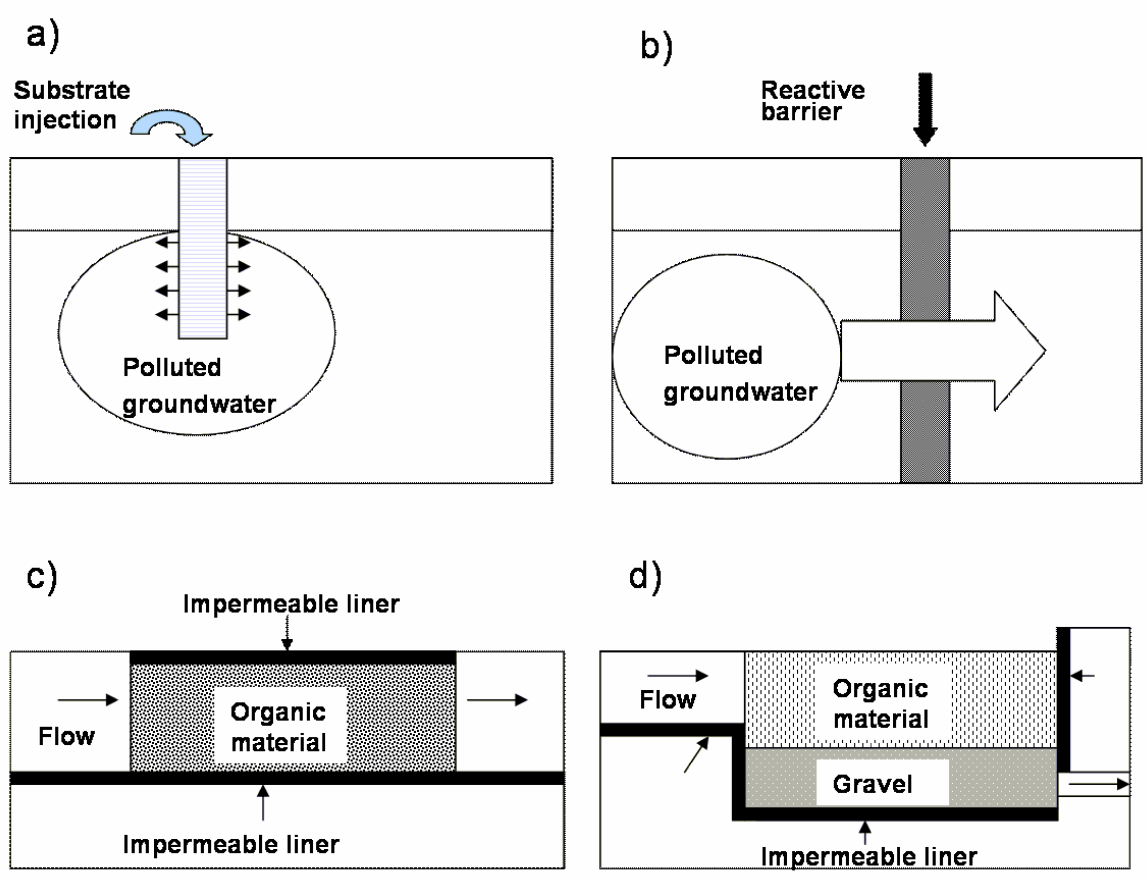

Fig. 1.5 Passive treatment based in sulphate reduction, for AMD: a) substrate injection, b) permeable reactive barriers, c) infiltration beds and d) anaerobic wetland systems (adapted from Kaksonen and Puhakka, 2007).

Wetlands supplement with submerged organic substrate are called anaerobic wetland or compost bioreactors (Fig. 1.5d). These organic substrates stimulate microbial activity. The biologic reactions that occur in anaerobic wetlands generate alkalinity and biogenic sulphide and therefore may be used to treat wastewaters that are acidic and metal rich (Jonhnson and Hallberg 2005; Kaksonen and Puhakka, 2007). Macrophytes can be used in anaerobic wetlands but only for aesthetic reasons. Penetrating plant roots may cause the

ingress of oxygen into anaerobic zones which is unfavourable to reductive processes (Jonhnson and Hallberg 2005).

Passive biological treatment approaches offer low-cost and minimal maintenance solutions for treating metal containing wastewaters, and thus they are also suitable for remote mining areas. However the required area may be large, the metal recovery is difficult, and the control and predictability of the process poor due to seasonal variations (Kaksonen and Puhakka, 2007). To minimize the weaknesses of passive treatment, SRB can be selectively enriched and their activity used in more controlled bioreactors (active treatments) (Kaksonen and Puhakka, 2007).

3.4.2. Active biologic treatments

Numerous reactor designs for biological sulphate reduction have been reported, such as batch reactors, sequencing reactors, fluidized-bed reactors, up-flow anaerobic packed bed reactors and membrane bioreactors (see review of Kaksonen and Puhakka, 2007).

Biological sulphate reduction and metal precipitation can be applied in single or separated unit processes where the metals are precipitated prior to the biological step by recycling either sulphide containing water or H_2S containing gas (Fig. 1.6).

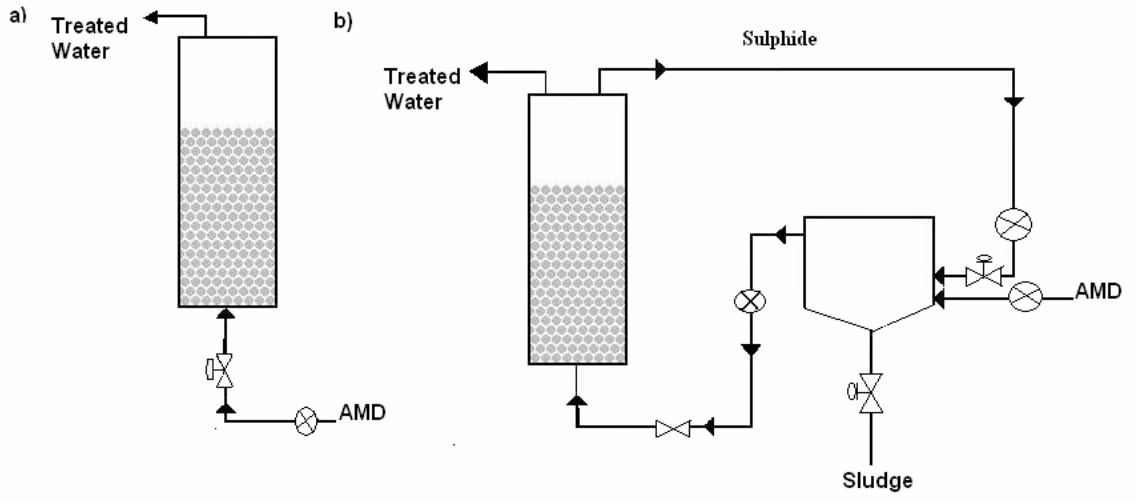

Fig. 1.6 Active treatment based in sulphate reduction, for AMD: a) single-stage process (up-flow anaerobic packed bed reactor), b) two-stage process with sulphide recirculation (up-flow anaerobic packed bed reactor with a settler).

The single-stage treatment process is a low-cost solution for AMD decontamination, but it may not be viable if the wastewater is very acidic or contains high concentrations of heavy metals. Many single-stage treatment systems have utilized alkaline materials to generate additional alkalinity. Barros *et al.* (2009) showed that the incorporation of a solid waste material (calcite tailing) in the bioreactor provides adequate conditions for simultaneous removal of metals and sulphate from AMD.

Active systems where metals precipitate prior to the biological step allow a selective precipitation of metal sulphides which can be an additional advantage (Tabak *et al.*, 2003; Huisman *et al.,* 2006). By controlling the pH and sulphide concentration, metals can be selectively recovered from a multi-metal waste as a pure metal-sulphide based on their different solubilities (Fig. 1.3) (Tabak *et al.*, 2003; Huisman *et al.,* 2006). Tabak *et al.*, (2003) developed a two stage process for metals separation from AMD using sulphide and hydroxide precipitation. In a recent study, Sahinkaya *et al.* (2009) showed the efficiency of selective precipitation of copper and zinc in an anaerobic baffled reactor. Moreover, Bijmans *et al.* (2009) showed selective recovery of nickel over iron in a gas lift bioreactor. At industrial stage, Thioteq technology is an example of full-scale process which uses sulphate reduction and metal precipitation in separated unit processes (Huisman *et al.*, 2006). This technology has been developed by Paques BV and has been applied with success in treatment of metal and mining industry wastewaters (Huisman *et al.*, 2006).

4. Biologic mechanisms applied in metal removal

Metals can be classified into essential and toxic elements for each organism. Iron, copper, zinc, calcium, manganese, magnesium, sodium and potassium are metals that are essential for most organisms (Raab and Feldmann, 2003). However, essential metals can be toxic when their concentration is higher than that required for metabolism, at this point the metal can act as inhibitor of metabolic pathways by strongly binding to enzymes, or by forming unwanted radicals or less stable reaction-products and therefore wasting energy (Raab and Feldmann, 2003). Therefore, microorganisms have developed a whole range of mechanisms to deal with unfavorable environmental conditions, such as the presence of higher concentration of metals, allowing their survival in extremely harsh conditions like

soils and waters contaminated with metals (Raab and Feldmann, 2003). In addition, these microbe-metal interactions have played an important role in the solubility and mobility of metals in soil in waters (Lovley and Coates, 1997). The distribution of metals between solid, soluble and volatile species was thought for a long time to result purely from geological processes. The knowledge about the influence of microorganisms on this distribution grew over the last decades (Raab and Feldmann, 2003). Some microorganisms act as geochemical agents promoting precipitation, transformations or dissolutions of minerals (Lovley and Coates, 1997; Raab and Feldmann, 2003). For instance microbes can dissolve metal ions, for example iron from pyrite, and convert them into soluble species. This mechanism can help in the leaching of these contaminants from soils (Lovley and Coates, 1997; Raab and Feldmann, 2003). Moreover, microorganisms can also operate in the opposite way by changing the redox state of metals, converting them to insoluble or volatile species (e.g. Hg^{2+} can be enzimatically reduced to volatile Hg^{0}) (Lovley and Coates, 1997; Raab and Feldmann, 2003; Gadd, 2010). The adsorption and accumulation of metals by microbial biomass can also prevent further migration of metals (Lovley and Coates, 1997; Raab and Feldmann, 2003). These biologic processes are considered as important as the physicochemical reactions that occur in the environment (Raab and Feldmann, 2003). Nowadays these biologic mechanisms were explored in order to developed efficient processes for treatment of soils and waters contaminated with metals.

4.1 Biosorption and bioaccumulation of metals

Biosorption and bioaccumulation have been demonstrated to possess good potential to replace conventional methods for the removal of metals (Volesky and Holan, 1995; Malik, 2004). The differences between biosorption and bioaccumulation processes are listed in Table 1.4. Biosorption is a metabolic passive process by which metals are bound to the surface of cells walls and occurs even with dead biomass (Lovley and Coates, 1997; Chojnacka, 2010). This process is very similar to conventional adsorption or ion-exchange except that the sorbent is biologic (Lovley and Coates, 1997; Chojnacka, 2010). Bioaccumulation is a metabolic active process which is performed by living cells (Vijayaraghavan and Yun, 2008; Chojnacka, 2010). In the bioaccumulation process the metals accumulate inside the cells, and this process occurs in two stages: the first, faster, is

identical to biosorption, and the second stage, which is slower, includes metal transport into the cells (mainly via energy active transport systems) (Aksu and Dönmez, 2000; Chojnacka, 2010). Inside the cells the metals precipitate or are transformed into other species by oxidation or reduction (Wang and Chen, 2006; Yilmazer and Saracoglu, 2009).

Table 1.4 Comparison between biosorption and bioaccumulation (Vijayaraghavan and Yun, 2008; Chojnacka, 2010).

Features	**Biosorption**	**Bioaccumulation**
Mechanism	Metals are bound to cellular surface	Metals are bound to cellular surface and accumulate inside of the cells
Biomass	Not alive	Alive
Cost	Usually low	High, the process involves living cells, therefore costs with cell maintenance are required
Selectivity	Poor	Better than biosorption
Versatility	Good. The binding sites can accommodate several metals	Not very flexible. The process is affected by high metals concentration
Rate of removal	Fast	Slow
Metals affinity	High	Depends on the toxicity of the metal
Metal recovery	High, with proper selection of elutant.	Even if possible, the biomass cannot be used for next cycle
Regeneration and Reuse	High	Very limited since most of the metals are intracellularly accumulated

With the bioaccumulation process it is possible to reach lower residual concentration of metal in solution. Part of the metals is transported into the cell releasing the binding sites present in surface, therefore additional amount of metals can be bound, increasing the removal rate (Chojnacka, 2010). On the other hand, bioaccumulation is a more complex process with more costs associated, since this process requires nutrients for cell maintenance (Vijayaraghavan and Yun, 2008; Chojnacka, 2010).

Biosorption might be an important natural process for metals concentration in soils and contaminated aquifers. Furthermore a barrier of microorganisms with biosorptive abilities could be applied in order to remove metals from groundwater (Lovley and Coates, 1997). The biosorption process can effectively sequester dissolved metal ions from dilute complex solutions with high efficiency and quickly (Norton *et al*., 2004; Orhan *et al*., 2006).

For a long time the mechanism of biosorption was unknown. Several different processes were thought to contribute to what was observed as biosorption: surface complexation and precipitation, physical adsorption and ion exchange (Wang and Chen 2006; Wang and Chen 2009). Recently the dominating role of the ion exchange process was confirmed (Chojnacka, 2010). Therefore the pH is the operation condition that most strongly affects the efficiency of this process; pH determines the protonation or deprotonation of metal ions binding sites and thus influence the availability of the binding sites to metal cations (Chojnacka, 2010). Generally the process of biosorption can be described as biological ion exchange with binding groups present on the surface of cell walls such as hydroxyl, carboxyl, phosphoryl and amino groups (Jiang *et al.*, 2004; Acharya *et al.*, 2009).

The first major challenge for metal removal by bioaccumulation and biosorption is to select the most promising biomass. The biosorbents should have high metal-binding capacity (Wang and Chen, 2009). In addition, the bioaccumulating organisms should be resistant to high concentrations of metals and they should not have mechanisms which protect from excessive accumulation inside the cell (Deng and Wilson, 2001; Kocberber and Dönmez, 2007). They should have mechanisms of intracellular binding, for instance synthesis of low molecular weight proteins, such as metallothioneins, which bind the metals in order to exclude them from metabolic processes. These proteins are frequently synthesized as the response to the presence of metal ions in the growth medium (Martin-Gonzalez *et al*., 2006). Moreover some bacteria belonging to genera *Bacillus, Acinetobacter, Pseudomonas* and SRB, can produce extracellular polymeric substances (EPS) (Beech and Cheung, 1995; Pirog, 1997; Flemming and Wingender, 2001; Guibaud *et al*., 2005; Ueshima *et al.*, 2008). EPS are produced during microorganisms growth and their composition depends on the strain and the culture conditions. However, they generally contain high molecular weight compounds such as proteins, polysaccharides, glycoproteins and lipopolysaccharides, which possess a substantial quantity of anionic functional groups (carboxylic, phosphoric,

amino, and hydroxyl groups) contributing for metal adsorption to the biomass (Flemming and Wingender, 2001; Guibaud *et al*., 2005; Ueshima *et al.*, 2008).

Several microorganisms have been investigated for their metal binding ability under several conditions (see reviews by Whang and Chen (2006), Vijayaraghavan and Yun (2008) and Whang and Chen (2009)). Moreover the process of biosorption has been recently commercialized and BIO-CLAIM® is one of the biosorbents available on the market. This product consists of bacteria of the genus *Bacillus* treated with caustic soda and immobilized in polyethyleneimine (PEI) beads and glutaraldehyde (Chojnacka, 2010).

4.2. Metals reduction and precipitation

Microorganisms can remove a number of metals from the environment by reducing them to a lower redox state (Lovley and Coates, 1997). With this approach the mobility and toxicity decrease for several metals for instance U(VI) to U(IV) and Cr(VI) to Cr(III) (Wall and Krumholz, 2006; Cheung and Gu, 2007; Gadd, 2010). Metals reduction can be a detoxification mechanism or a metabolic process in which metals are used as electron acceptors in anaerobic respiration (dissimilatory metal reduction) (Lovley 1993; Slobodkin, 2005). Different physiological groups of bacteria are responsible for dissimilatory metal reduction such as SRB, thermophilic microorganisms, Fe(III)-reducing bacteria and fermentative bacteria (Slobodkin, 2005; Wall and Krumholz, 2006; Cheung and Gu, 2007; Mohapatra *et al.*, 2010). Moreover, in recent years, attention has been focused on the application of this approach for recovery of platinum group metals. Lloyd *et al.* (1998) reported that Pd(II) was reduced to Pd(0) on the surface of *Desulfovibrio desulfuricans* with hydrogen as electron donor. The enzyme implicated in this reaction was a hydrogenase (Lloyd *et al.*, 1998). Yong *et al.* (2002) demonstrated the application of this approach to the recovery of Pd(0) from industrial wastes containing soluble Pd(II). Recently the reduction of Rh(III) and Pt(IV) by a mixed consortium of SRB was demonstrated by Ngwenya and Whiteley (2006) and Rashamuse and Whiteley (2007), respectively. Moreover, it was discovered that two different hydrogenase enzymes were involved in the bioreduction of platinum (IV) into platinum (0): First platinum (IV) was reduced to platinum (II) by oxygen-sensitive novel cytoplasmic hydrogenase and second platinum (II) ion was reduced

to platinum (0) by another two-electron bioreduction involving an oxygen-tolerant/protected periplasmic hydrogenase (Riddin *et al.*, 2009).

4.2.1. Dissimilatory reduction of radionuclides

It was been reported that several microorganisms are able to enzymatically reduce radionuclides (Mohapatra *et al.*, 2010). For instance, the ability of SRB (Lloyd *et al.*, 1998; De Luca *et al.*, 2001), *Thermoterrabacterium ferrireducens*, *Tepidibacter thalassicus* (Chernyh *et al.*, 2007) and *Clostridium* sp. (Francis *et al.*, 2002) for reduction of Tc(VII) was demonstrated. The enzymatic reduction of Pu (IV) to Pu (III) has been documented for *Bacillus* sp., *Clostridium* sp., *G. metallireducens* and *S. oneidensis* (Russin *et al.*, 1994; Boukhalfa *et al.*, 2007; Francis *et al.*, 2007).

Geobacter metallireducens and *Shewanella putrefaciens* were the first bacteria found to use U(VI) as a terminal electron acceptor (Lovley *et al.*, 1991). In 1992, Lovley and Phillips, demonstrated that bacteria belonging to the genus *Desulfovibrio* were also capable of U(VI) reduction. Since the pioneer work of Lovley and co-workers in the early ninety's (Lovley *et al.*, 1991; Lovley and Phillips, 1992) a number of bacterial genera responsible for the reduction of uranium (VI) have been described: *Clostridium* (Francis *et al.*, 1994; Gao and Francis, 2008), *Cellulomonas* (Sani *et al.*, 2002), *Desulfotomaculum* (Tebo and Obraztsova, 1998), *Thermoanaerobacter* (Slobodkin, 2005).

The dissimilatory reduction of U(VI) is achieved by the enzyme uranium reductase in the presence of hydrogen (equation 1.6) or organic compounds (equation 1.7) (Mohapatra *et al.*, 2010). Upon reduction the highly soluble and mobile U(VI) is converted to insoluble U(IV), which then precipitates from aqueous solutions.

$$UO_2^{2+}{}_{(aq)} + H_2 \rightarrow UO_{2\,(s)} + 2H^+ \qquad (1.6)$$

$$2UO_2^{2+}{}_{(aq)} + CH_2O + H_2O \rightarrow 2UO_{2\,(s)} + CO_2 + 4H^+ \qquad (1.7)$$

Beside the dissimilatory U(VI) reduction, SRB can reduce uranium (VI) by indirect pathway due to the production of H_2S during dissimilatory sulphate reduction. Hua *et al.* (2006) suggested that the reaction stoichiometry which best represents U(VI) reduction by hydrogen sulphide in anaerobic conditions could be the following equation:

$$UO_2^{2+}{}_{(aq)} + HS^- \rightarrow UO_{2\,(s)} + S^0 + H^+ \qquad (1.8)$$

The reduction of U(VI) to U(IV) requires two electrons, however the mechanism of this microbial electron delivery has not yet been conclusively determined for any dissimilatory metal reducing bacteria (DMRB) (Wall and Krumholz, 2006).

A few uranium reductases has been purified and characterized from bacteria belonging to genera *Geobacter*, *Desulfovibrio* and *Shewanella* (Lovley *et al.*, 1993; Wade and DiChristina, 2000; Lloyd *et al.*, 2003; Bencheikh-Latmani *et al.*, 2005). Biochemical characterization of the uranium reductase of *G. sulfurreducens* indicates that it is a periplasmic cytochrome c_7 (PpcA) with a molecular weight of 9.6-kDa. In vitro studies indicate that in *Desulfovibrio vulgaris* the uranium reductase activity with hydrogen as electron donor is present in the soluble fraction and requires cytochrome c_3 and a perisplasmic hydrogenase (Wade and DiChristina, 2000; Wall and Krumholz, 2006). The U(VI) reduction in *S. putrefaciens* strain 200 occurred by outer membrane c-type cytochrome (Wade and DiChristina, 2000; Wall and Krumholz, 2006). By assays of mutants of *S. oneidensis* MR-1 several proteins, including one involved in menaquinone biosynthesis (MenC), periplasmic cytochrome (MtrA), outher membrane protein (MtrB), cytochrome localized in the cytoplasmic membrane (CymA), were shown to be need for optimal U(VI) reduction (Bencheikh-Lantmani *et al.*, 2005).

4.2.2. Dissimilatory chromium (VI) reduction

The microbial reduction of Cr(VI) to Cr(III) has been one of the most widely studied forms of metal bioremediation (Lovley and Coates, 1997). Microbial Cr(VI) reduction was first reported in the late 1970s when Romanenko and Korenkov (1977) observed the Cr(VI) reduction by *Pseudomonas* spp. grown under anaerobic conditions. Since then a variety of genera of bacteria were found with ability for Cr(VI) reduction under varying conditions: *Thermoanaerobacter* (Roh *et al.*, 2002), *Desulfovibrio* (Lovley, 1995; Goulhen *et al.*, 2006), *Desulfomicrobium* (Michel *et al.*, 2001, Chardin *et al.*, 2002), *Geobacter* (Lovley *et al.*, 1993) and *Shewanella* (Myers *et al.*, 2000).

Cr(VI) serves as a terminal electron acceptor in the membrane electron-transport respiration pathway, a process that results in energy conservation for growth and cell maintenance

(Horitsu *et al.*, 1987; Lovley and Phillips, 1994). Cr(VI) reduction by microorganisms consumes large amounts of protons which results in the increase of the background pH. The increase of pH facilitates the precipitation of the reduced chromium as chromium hydroxide as shown in equation 1.9 (Molokwane *et al.*, 2008):

$$CrO_4^{2-}{}_{(aq)} + 8H^+ \rightarrow Cr^{3+} + 3H_2O \rightarrow Cr(OH)_{3\ (s)} + 3H^+ \qquad (1.9)$$

SRB have been extensively studied for reduction of metals, including Cr(VI) (Lovley, 1995; Michel *et al.*, 2001; Chardin *et al.*, 2002; Goulhen *et al.*, 2006). Equation 1.10 illustrates biologic Cr(VI) reduction by SRB using lactate as a carbon source and electron donor (Chardin *et al.*, 2002):

$$CH_3CHOHCOO^- + Cr_2O_7^{2-}{}_{(aq)} + 4H^+ \rightarrow CH_3COO^- + CO_2 + 2\ Cr(OH)_{3\ (s)} \qquad (1.10)$$

In addition, SRB can also indirectly promote Cr(VI) reduction by sulphide production which can abiotically reduce Cr(VI) (Lovley, 1995).

Periplasmatic and membrane associated enzymes were found to mediate the process of Cr(VI) reduction in anaerobic conditions (Cheung and Gu, 2007). The cytochrome proteins in out membrane are frequently involved in the electron transport from electron donor to electron acceptor hexavalent chromium (Cheung and Gu, 2007; Hong and Gu, 2009). The enzymatic reduction of Cr(VI) in *Desulfovibrio vulgaris* was found to involve a soluble c_3 cytochrome (Lovley, 1995) while in *Desulfomicrobium norvegicum* a hydrogenase and a *c*-type cytochrome catalyzed Cr(VI) reduction (Michel *et al.*, 2001; Chardin *et al.*, 2002).

Treatment based in the metals bio-reduction is an environmentally friendly method because it avoids the use of chemicals and the reactions occur at ambient temperature (Renshaw *et al.*, 2007; Mohapatra *et al.*, 2010). Therefore dissimilatory reduction of U(VI) and Cr(VI) is a potential mechanism for removing these metals from contaminated environments.

5. Scope and organization of this thesis

The main goal of the research described in this thesis was to identify and characterize anaerobic communities with high metal resistance and ability for metal removal by different mechanisms. Moreover, the microbe-metal interactions were also explored. The organization of the objectives of this thesis is shown in Fig.1.7.

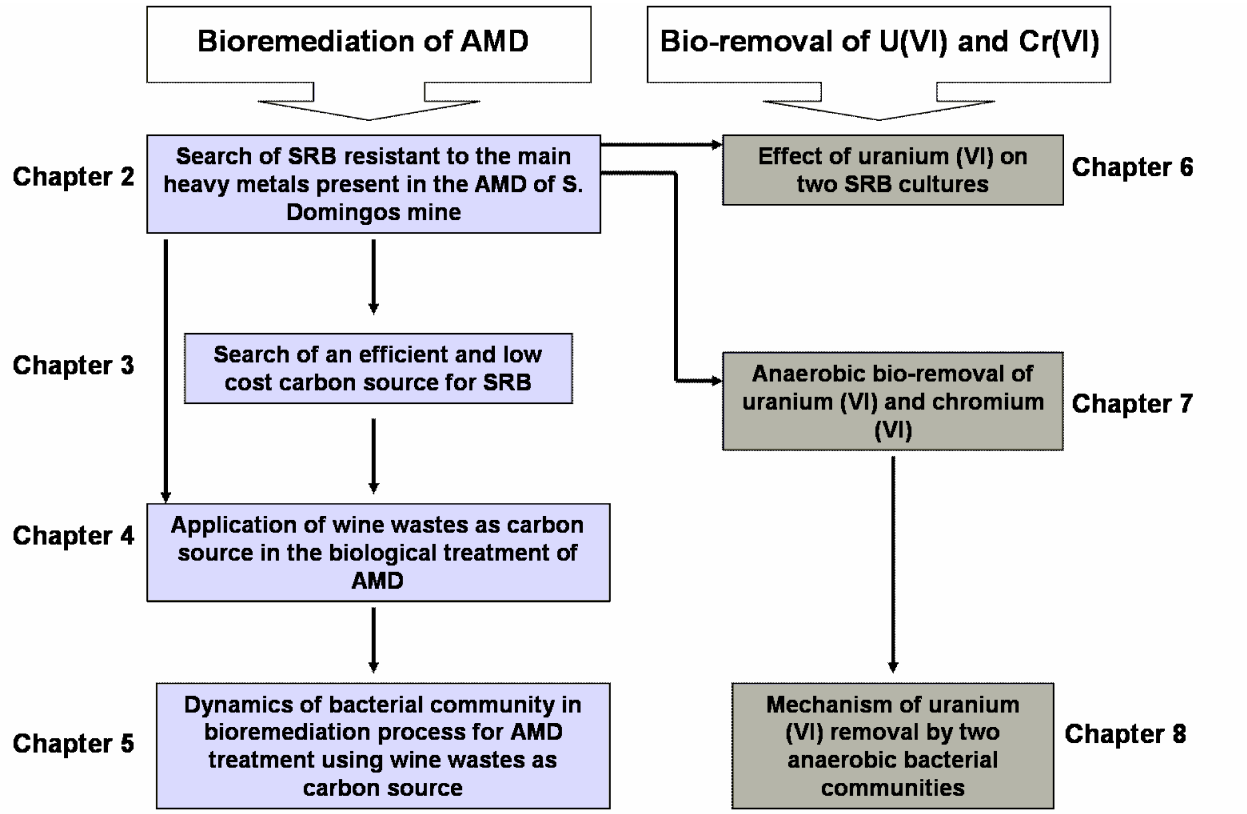

Fig. 1.7 Schematic organization showing the objectives and the respective chapters.

In **Chapter 2** the tolerance of SRB consortia from several environmental sources to the main heavy metals (Fe, Zn and Cu) present in the AMD of S. Domingos mine is compared. Generally, AMD is deficient in carbon sources/electron donors and thus, an external addition is required to achieve sulphate reduction. Therefore, the choice of carbon source for SRB activity can be the key-point to ensure high performance, long-term efficiency and not less important the economical viability of the treatment. Thus, **Chapter 3** is devoted to the study of the possibility of using two wastes from food industry as sources of carbon

compounds to promote sulphate reduction by the SRB consortium selected in Chapter 2. In **Chapter 4** the application of the bacterial consortium and the wastes from food industry, previously selected in Chapters 2 and 3 respectively, in a bioremediation process for AMD treatment is studied. The dynamic of the bacterial populations in the bioremediation system applied to the AMD treatment using wine wastes as carbon source was elucidated by temperature gradient gel electrophoresis (TGGE) analysis and is presented in **Chapter 5**.

The U(VI) reduction ability of SRB has been demonstrated (Lloyd *et al.*, 1998; De Luca *et al.*, 2001), consequently SRB are thought to have a high bioremediation potential for uranium-contaminated waters. Therefore, in **Chapter 6**, besides the assessment of the efficiency of uranium bio-removal by two SRB enrichment consortia, the analysis of the community structural shifts in these consortia, due to the presence of uranium is also presented. Considering some similarities between uranium and chromium, namely the same oxidation state of the most soluble form (VI) and the possible bio-reduction to insoluble oxidation states, U(IV) and Cr(III), the investigation concerning if the anaerobic bacterial communities able to remove uranium (VI) are also effective for chromium (VI) removal is presented in **Chapter 7**. The structure of the bacterial communities with ability for uranium (VI) and chromium (VI) bio-removal is also compared in this chapter. The mechanism involved in uranium (VI) removal from aqueous solution by two anaerobic bacterial consortia (selected in the Chapter 7) is presented in **Chapter 8**. Moreover, the molecular identification of the two consortia is also included.

6. References

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Chapter 2

Characterization and activity studies of highly heavy metal resistant sulphate-reducing bacteria to be used in acid mine drainage decontamination

Abstract

Biological treatment with sulphate-reducing bacteria (SRB) has been considered as the most promising alternative for acid mine drainage (AMD) decontamination. Normally, these waste waters contain high concentrations of sulphate and heavy metals, so the search for SRB highly resistant to metals is extremely important for the development of a bioremediation technology. A SRB consortium resistant to high concentrations of heavy metals (Fe, Cu and Zn), similar to those typically present in AMD, was obtained among several environmental samples, from a wastewater treatment plant. The phylogenetic analysis of the *dsr* gene sequence revealed that this consortium contains species of SRB affiliated to *Desulfovibrio desulfuricans* and *Desulfobulbus rhabdoformis.* The results show that the presence of usually lethal concentrations of Fe (400 mg/L), Zn (150 mg/L) and Cu (80 mg/L) is not toxic for the sulphate-reduction bacteria present in this sample. As a consequence, a very good efficiency in terms of sulphate reduction and metals removal was obtained. Both ethanol and lactate can be used by this inoculum as carbon source. With the other samples tested sulphate reduction was inhibited by the presence of copper and zinc.

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1. Introduction

Sulphate-reducing bacteria (SRB) are important members of microbial communities with economic, environmental and biotechnological interest. SRB have the ability to reduce sulphate to sulphide and this sulphide reacts with certain metals dissolved, such as copper, iron and zinc, forming insoluble precipitates (Benedetto *et al.*, 2005). Anaerobic reduction of sulphate by SRB has been reported to be used for the treatment of a variety of sulphate-containing industrial effluents (Gibert *et al.*, 2004; Tsukamoto *et al*., 2004; Icgen and Harrison, 2006), being mining wastewaters, rich in heavy metals, one of the most relevant examples. However, the use of SRB for these applications has generally one important limitation: lack of bacterial resistance to metals.

Heavy metals are generally toxic for microorganisms, including SRB, due to substitution of essential ions on cellular sites, and blockage of functional groups of important molecules such as enzymes. This results in denaturation and inactivation of enzymes and disruption of cell organelle membrane integrity (Sani *et al.*, 2001; Cabrera *et al.*, 2006). It has been reported that toxic concentrations of heavy metals for SRB range from a few ppm to as much as 100 ppm (Sani *et al.*, 2001; Cabrera *et al.*, 2006). The metal resistance of SRB varies with the species. Different organisms exhibit diverse responses to toxic ions, which confer them a certain tolerance to metals. They have a number of specific resistance mechanisms, such as sequestration or transformation to other chemical species (Valls and De Lorenzo, 2002).

The search for SRB resistant to metals is very important for the development of efficient bioremediation processes based on the use of these bacteria.

The purpose of this work was to compare the tolerance of SRB consortia from several environmental sources to the most concentrated heavy metals (Fe, Zn and Cu) present in acid mine drainage (AMD) of S. Domingos mine (an abandoned copper mine in Southeast Portugal). The AMD from S. Domingos pit lake is highly acidic (pH around 2) and characterised by high concentrations of heavy metals mainly Fe (500 mg/L), Cu (50 mg/L), Zn (110 mg/L) and sulphate (3100 mg/L) (Costa and Duarte, 2005; Costa *et al*., 2008).

2. Materials and methods

2.1. Sampling and chemical characterization

The search for SRB was done by using environmental samples collected in the Provinces of Algarve and Alentejo, South Portugal. In Algarve, soil samples from Monchique thermal place, sediments from Formosa estuary and sludge from two waste water treatment plants, located in Montenegro and in Estói, were collected. Sediments from the mining area of S. Domingos (Alentejo) were also collected in two places: Corta (near the top of the pit lake) and near the stream of Chança, that crosses S. Domingos mine. The samples were identified by the names of the places where they were collected. The use of natural sources has advantages over the use of pure bacterial cultures: they contain bacterial consortia that facilitate the development of reducing conditions and they are also more easily available (Gibert *et al*., 2002).

Multielemental analysis of the environmental samples collected (Table 2.1) was carried out by Total Reflection X-Ray Fluorescence (TXRF) using an EXTRA-IIA (Atomika Instruments) spectrometer. Previous to instrumental analysis, samples were submitted to microwave acid digestion in closed Teflon® Parr® bombs, using *aqua regia*, HF and gallium as internal standard (Barreiros *et al.*, 2001).

2.2. SRB enumeration

SRB populations were enumerated by the three-tube Most Probable Number (MPN) assay with serial dilutions in Postgate E medium (Postgate 1984). Essays were performed in triplicate. The MPN tubes were incubated at room temperature (21±1ºC) for 5 days.

Table 2.1 Elemental composition of the environmental samples used for SRB search. Values for the metals tested in the present study are in bold.

Element	Concentration (g/kg)					
	Monchique	**Montenegro**	**Corta**	**Formosa**	**Estói**	**Chança**
K	30	7.6	14	11	2.3	14
Ca	4.8	43	4.7	12	77	0.52
Ti	6.8	2.8	5.3	2.2	1.5	3.8
V	0.178	0.052	0.147	0.068	0.052	0.099
Cr	<0.03	0.040	0.065	0.024	0.029	0.054
Mn	0.83	0.13	<0.03	0.075	0.18	<0.03
Fe	**24**	**16**	**73**	**15**	**8.4**	**61**
Ni	<0.01	0.018	<0.01	<0.01	0.022	<0.01
Cu	**0.009**	**0.10**	**2.0**	**0.009**	**0.25**	**0.72**
Zn	**0.077**	**0.430**	**0.99**	**0.061**	**1.2**	**0.55**
As	<0.01	<0.01	1.0	0.011	<0.01	1.6
Se	<0.007	<0.007	<0.007	<0.007	<0.007	<0.007
Rb	0.25	0.068	0.098	0.085	0.017	0.074
Sr	0.82	0.118	0.145	0.079	0.36	0.094
Cd	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Sn	<0.04	<0.04	3.2	<0.04	<0.04	0.16
Sb	<0.04	<0.04	0.36	<0.04	<0.04	0.19
Ba	0.7	0.4	0.6	<0.2	0.4	0.5
Pb	0.020	0.08	6.0	0.019	0.10	4.6

2.3 Batch experiments

Experiments were performed in batch and in anaerobic conditions. All experiments were performed in duplicate using 120 ml glass bottles containing 100 ml of growth medium with pH around 7. Oxygen diffusion was eliminated by 10 ml of sterile liquid paraffin. After inoculation, the bottles were sealed with butyl rubber stoppers and aluminium crimp seals and incubated at room temperature (21±1ºC).

The growth media used was modified Postgate B medium (Postgate 1984). The modifications of the medium composition for individual experiments are described below.

2.3.1. Study of efficiency of sulphate reduction by SRB populations from the collected samples

The growth medium used was supplemented with resazurin as redox indicator (0.03 g/L). The inoculation was carried out using 5 g of each of the samples previously mentioned (sediment, sludge or soil).

Further tests were carried out using bacterial cells collected from the previous study. The bacterial cells were harvested by centrifugation, washed and transferred to the test solutions.

2.3.2. Effect of heavy metals on sulphate reduction

The growth media contained lactate (6 g/L) as carbon and energy source, sulphate (3.5 g/L) and resazurin as redox indicator (0.03 g/L). Different experiments were carried out with 0.4 g/L or 0.8 g/L of iron only, or 0.4 g/L of iron, 0.08 g/L of copper and 0.15 g/L of zinc in the mixture. The metal salts used for the study were $FeSO_4.7H_2O$, $ZnSO_4.7H_2O$ and $CuSO_4.5H_2O$. A test without metals was done as control.

2.3.3. Effect of carbon source on sulphate reduction

The growth media contained a carbon and energy source (6 g/L) and resazurin as redox indicator (0.03 g/L). Lactose and ethanol were the carbon sources selected, considering the great availability of these compounds in by-products of the cheese and winery Portuguese industries and the eventual future utilization of these wastes as carbon sources. Lactate is the carbon source most widely used by SRB (Postgate, 1984; Barnes, 1998) and was also used as control.

2.4. Sampling and analytical methods

Periodically, 5 mL samples were collected using a syringe, and filtered using a 0.2 µm hydrophilic polyestersulfone membrane (Machererey-Nagel). Redox potential, pH, soluble concentrations of lactate, lactose, ethanol, acetate, sulphate and heavy metals were measured in each sample. Redox potential and pH were measured immediately after sample collection and filtration using a pH/E Meter (GLP 21, Crison). A high performance liquid chromatograph (Beckman), equipped with a polyspher® OAHY column (30cm × 0.65cm,

Merck) and a RI detector, was used for lactate, lactose, ethanol and acetate analysis. Sulphate concentration was measured by a UV-VIS spectrophotometer (Hach-Lange DR2800), using the sulfaVer4 method (Hach-Lange). For determination of the dissolved metals (Fe, Cu and Zn) the filtered samples were acidified with nitric acid and analysed by Atomic Absorption Spectroscopy (AAS) using a Shimadzu, AA-680 model spectrometer.
An optical microscope equipped with a digital camera (Leica D C300FX) was used to visualise the bacteria present in the inoculum of Montenegro. Cells were centrifuged (10 min at 4000 rpm) and washed with sterile distilled water prior to Gram staining.

2.5. Molecular characterization

2.5.1. Extraction of DNA

Total genomic DNA was extracted from cell cultures grown on modified Postgate B media. The cells were harvested from 20 mL of cell culture by centrifugation at 4000 rpm for 10 min and twice washed with chilled deionised water. DNA extraction was carried out by the following method: 300 µL of sodium dodecyl sulphate (SDS) lysis mixture [500 mM Tris-HCl pH 8, 3% (w/v) SDS, 100 mM NaCl] and 300 µL of phosphate buffer pH 8 were added, followed by a freeze-thaw treatment (three cycles consisting of 1 min in liquid N_2 followed by 5 min in a 37ºC water bath). After cellular lysis, 300 µL of chloroform-isoamyl alcohol (24:1) were added. The solution obtained was centrifuged at 13000 rpm, for 10 min. After precipitation with isopropanol at -20ºC, for 20 min, DNA was resuspended in 35 µL H_2O. Nucleic acid extraction was evaluated on a 1% (w/v) agarose gel electrophoresis in Tris-Acetate-EDTA (TAE) buffer.

2.5.2. PCR amplification of *dsr* gene

PCR was conducted in a total volume of 50 µL. Community dissimilatory sulphite reductase (*dsr*) genes were amplified using the primers DSR1F and DSR4R (Wagner et *al.*, 1998; Chang *et al.*, 2001; Zagury *et al.*, 2006), which amplify a 1.9 Kb fragment. The primers were purchased from Thermo Fischer Scientific. The reaction mixture used for PCR amplification contained 30.75 µL of H_2O, 1 µL of each primer (10 pmol/µL), 1 µL of dNTP`s (10 mM), 5 µL of $MgCl_2$ (25 mM), 10 µL of 5×Go Taq® buffer (Promega, Madison, USA), 0.25 µL of GoTaq®DNA polymerase (Promega, Madison, USA), and 1

µL of DNA. The DNA of a strain of *Desulfovibrio* subsp. and of *Escherichia coli* was used as positive and as negative control, respectively. PCR amplification was carried out in a thermocycler (T1, Biometra). Thermal cycling was carried out by using an initial denaturation step of 94ºC for 3 min, followed by 30 cycles of 94ºC for 1 min, 56ºC for 1 min and 72ºC for 2 min and completed with an extension period of 5 min at 72ºC. The PCR products were separated in a 1% (w/v) agarose gel in TAE Buffer.

2.5.3. Cloning of *dsr* gene and RFLP analysis

PCR products were purified (E.Z.N.A.^{TM} Gel Extration Kit, Omega) and ligated into the cloning vector pGEM®-T Easy followed by transformation into *E. coli* DH5-alpha competent host cells, according to the manufacturer's instructions (Promega, Madison, USA). All the white colonies were amplified by direct PCR with the DSR1F and DSR4R primers according to the conditions described above.

Restriction fragment length (RFLP) analyses were done using the restriction enzymes H*haI* and H*aeIII* (Promega). Fragments of the digested PCR products were separated in a 2% (w/v) agarose gel in TAE Buffer.

2.5.4. Sequencing and phylogenetic analysis

Representative clones from each digestion pattern were selected for sequencing at CCMAR (Centro de Ciências do Mar, Universidade do Algarve). The *dsr* gene inserted in plasmids was amplified using the primers DSR1F and DSR4R, according to the conditions described above. PCR products were purified (E.Z.N.A.^{TM} Gel Extration Kit, Omega) and sequenced. Sequence identification was performed by use of the BLASTN facility of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Cladograms were constructed using MEGA version 4 (Tamura *et al.*, 2007) and the Neighbour-Joining algorithm was applied (Saitou and Nei, 1987; Studier and Keppler, 1988).

3. Results

3.1. Determination of sulphate-reducing bacteria population

Large number of SRB was observed in the sludge coming from the waste water treatment plants of Montenegro and Estói where 1.8 $\times 10^7$ CFU/g and 5.3 $\times 10^6$ CFU/g respectively, were found (Fig. 2.1). In the soil samples of Monchique thermal place, the number of SRB reached was 2.3 $\times 10^4$ CFU/g, while in the sediments of Formosa estuary 5 $\times 10^3$ CFU/g were found. In both samples from the mining area (Corta and Chança) SRB were not detected.

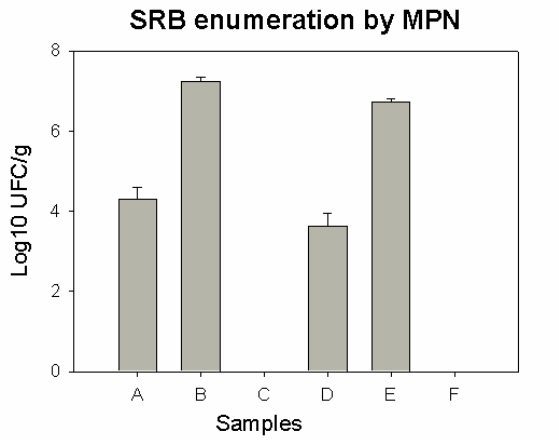

Fig. 2.1 SRB enumeration in samples collected. Samples: A- Monchique, B- Montenegro, C- Corta, D- Formosa, E- Estói and F- Chança. Data are the average of triplicates and error bars indicate the standard deviations.

The efficiency of sulphate reduction by SRB existing in the samples collected was investigated. After 42 days of study, the most efficient sulphate reduction was verified with the bacteria present in the samples from Montenegro (99.5%) and Estói (93.0%). The bacteria from Monchique presented a sulphate reduction efficiency of 64.7 % and bacteria from Formosa 33.5 %. The sulphate reduction was not observed in the samples of the mining area (Corta and Chança).

The following studies were performed using the inocula of Montenegro, Estói and Monchique, which presented the highest efficiency in terms of sulphate reduction (higher than 50 %) in the batch tests.

3.2. Effect of heavy metals on sulphate reduction

In these experiments the efficiency of biological sulphate reduction in the presence of two different concentrations of iron and in the presence of iron, copper and zinc was investigated. The effect of those metals on sulphate reduction is shown in Fig. 2.2.

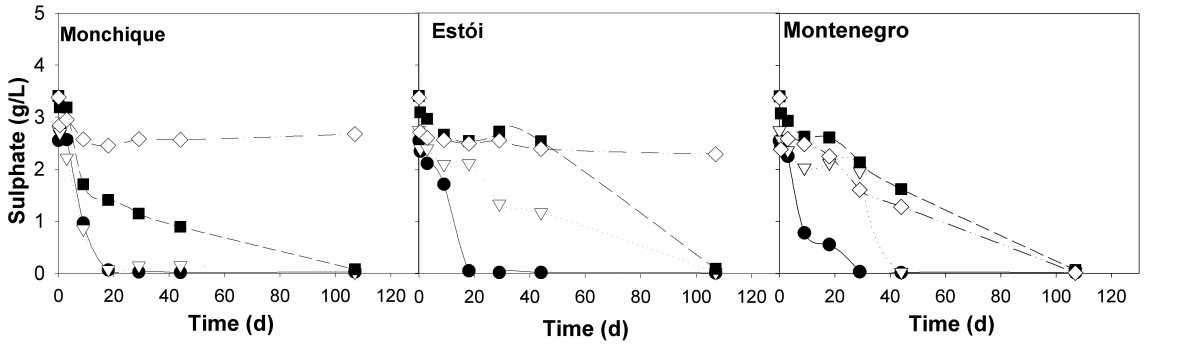

Fig. 2.2 Evolution of sulphate reduction by SRB in the different test: without metals (●), 0.4 g/L Fe (▽), 0.8 g/L Fe (■) and mix test with Fe, Cu and Zn (◊). Data are the mean of duplicates and error bars are smaller than the symbols, therefore not shown.

The presence of iron in the medium affected the rate of sulphate reduction, and this effect also depended of iron concentration. The only exception was observed with the sample of Monchique, for which 0.4 g/L of iron in the medium did not influence the efficiency of sulphate reduction as a function of time: both in the absence and in the presence of that concentration of iron, sulphate was completely reduced after 20 days of experiment. When the concentration of iron in the medium was increased to 0.8 g/L, sulphate concentration was reduced to less than half the initial value within 20 days.

Was observed that the presence of zinc and copper in the medium inhibited sulphate reduction by SRB in the samples of Monchique and Estói (Fig. 2.2). However, this did not happen with the SRB from Montenegro. In fact, after 30 days of experiment sulphate

concentration decreased about 50 %, although the lag time was increased. Complete sulphate reduction was achieved after 110 days, showing that the toxic effect of these metals did not prevent sulphate reduction by this community.

The removal of these metals is an indirect consequence of biological activity, so a larger extent of reduction of sulphate to sulphide results in larger efficiency of metal removal.

The rate of decrease in concentration of copper in the medium was faster than that of zinc or iron. As shown in Fig. 2.3, with 80 mg/l Cu as initial concentration, this metal was completely removed from the medium within 15 days with all the inoculum sources. In the case of zinc, the concentration decreased from 150 mg/L to 2.0 mg/L within 18 days, with SRB from Montenegro. With the other two inocula (Estói and Monchique), zinc was almost completely removed but after a much longer time (40 days).

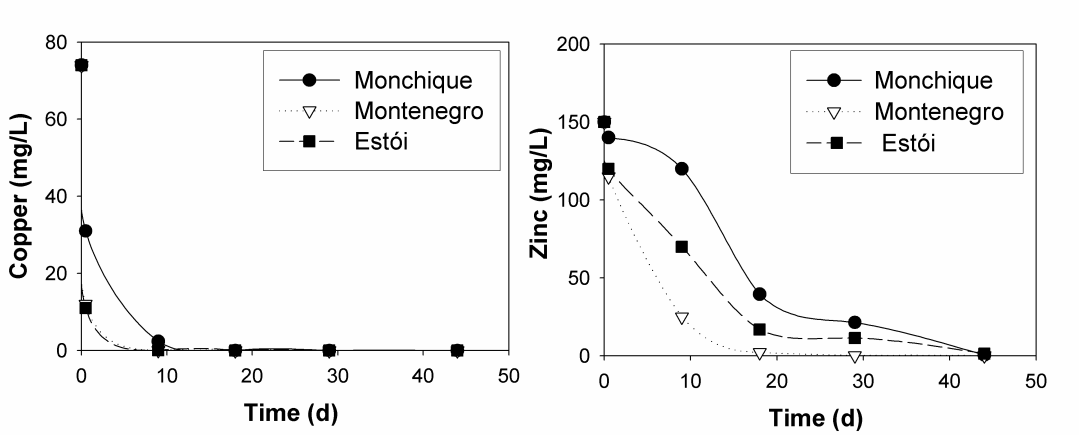

Fig. 2.3 Evolution of copper and zinc concentrations in the media as a function of time. Data are the mean of duplicates and error bars are smaller than the symbols, therefore not shown.

The removal of iron takes longer: this metal was significantly removed within 44 days (from 400 mg/L to 58 mg/L) with SRB from Montenegro, independently of the presence of zinc and copper in the medium (Fig. 2.4).

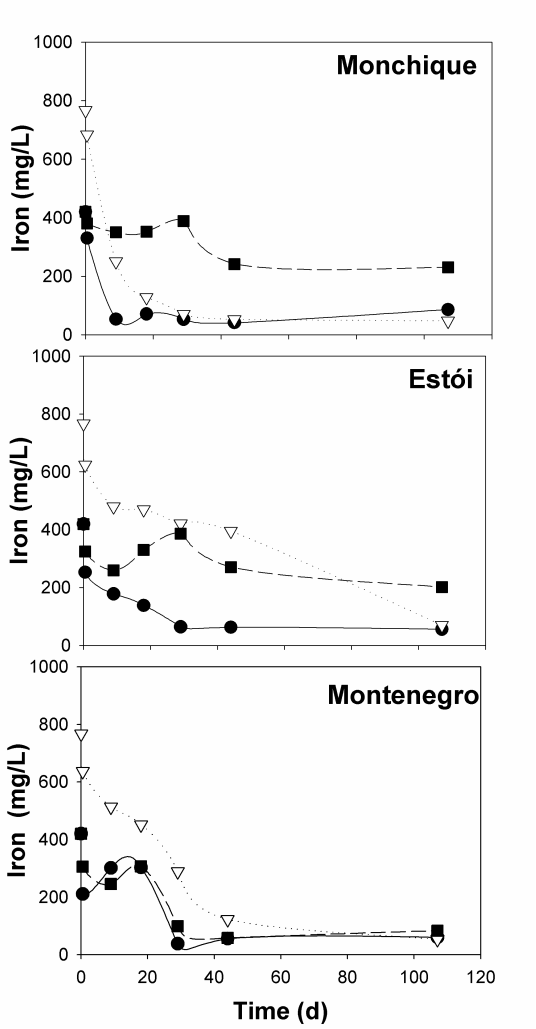

Fig. 2.4 Evolution of iron concentration in the media as a function of time for the different test: 0.4 g/L Fe (●), 0.8 g/L Fe (▽) and mix test with Fe, Cu and Zn (■). Data are the mean of duplicates and error bars are smaller than the symbols, therefore not shown.

According to the results obtained the sample of Montenegro contains the SRB most resistant to the metals under study. This inoculum is constituted mainly by Gram-negative bacilli and some cocci (Fig. 2.5).

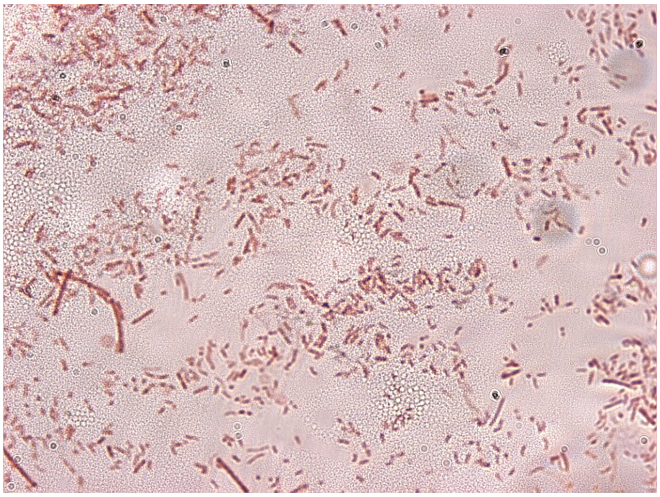

Fig. 2.5 Photomicrograph of bacteria present in the inoculum of Montenegro. Amplification of 1000X.

3.3. Effect of carbon source on sulphate reduction

Experiments were conducted to compare the sulphate reduction profile in the presence of the three carbon sources mentioned (Fig. 2.6). The most efficient sulphate reduction was generally observed with lactate for all inocula. Lactate was totally consumed in the first days of experiment at the same time as acetate production was observed.

Efficient sulphate reduction by SRB with ethanol as carbon source was only observed with the inocula from both wastewater treatment plants (Montenegro and Estói). Although the concentration of ethanol has decreased in the sample of Monchique, no sulphate reduction was observed.

In the presence of lactose, a more complex organic molecule, no sulphate reduction was observed independently of the inocula. However, lactose consumption was detected.

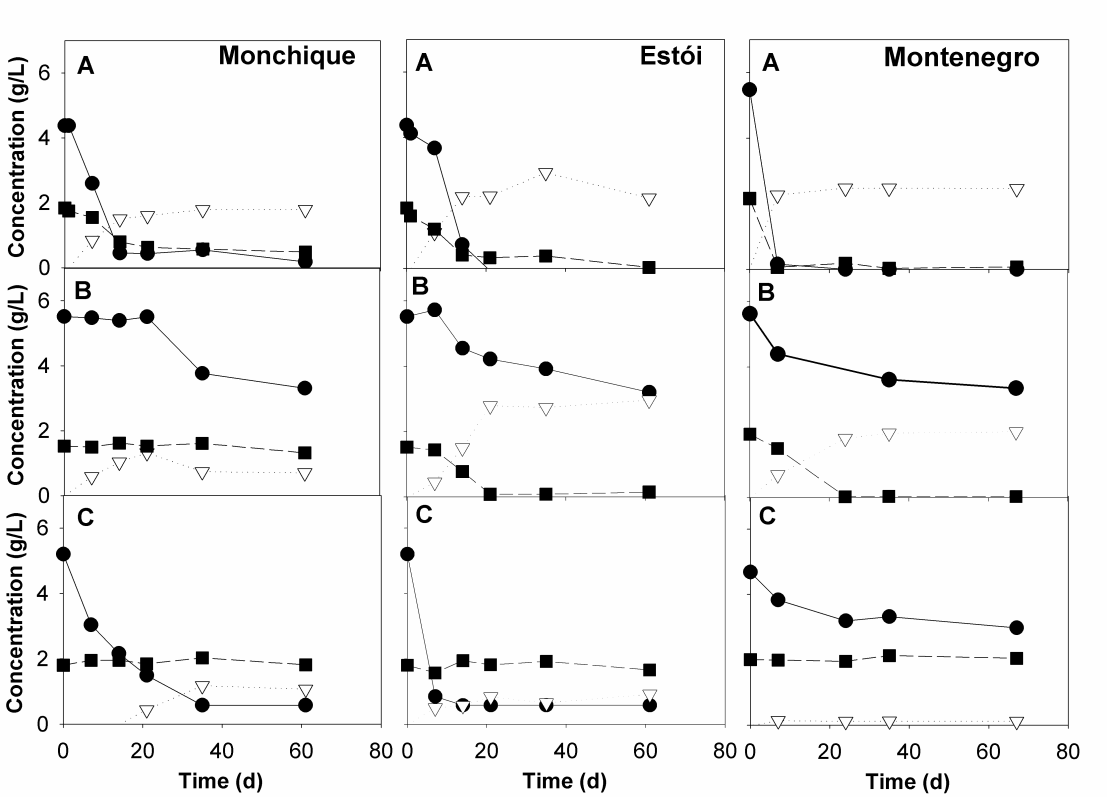

Fig. 2.6 Evolution of lactate, acetate, ethanol, lactose and sulphate concentrations in the presence of lactate (A), ethanol (B) and lactose (C) as carbon source on sulphate reduction by SRB. Data are the mean of duplicates and error bars are smaller than the symbols, therefore not shown. Symbols: (●) carbon source, (▽) acetate and (■) sulphates.

3.4. RFLP and Sequence Analysis

The dissimilatory sulphite reductase gene (*dsr*) was used to elucidate the composition of the SRB consortium of each of the different samples investigated. To achieve this goal, the primer pair DSR1F/DSR4R (Wagner *et al.*, 1998; Chang *et al.*, 2001; Castro *et al.*, 2002) which amplifies a 1.9 Kb *dsr* gene fragment was used. These primers have shown to be a powerful tool on SRB diversity studies where the phylogenetic analyses were based either on restriction analysis of the cloned fragment or sequencing the cloned 1.9 Kb *dsr* fragment (Baker *et al.*, 2003; Liu *et al.*, 2003; Bahr *et al.*, 2005).

A total of forty three clones were obtained: eight from the samples of Monchique (Monc), eleven from Estói (E) and twenty four clones from Montenegro (Mont) samples. The combination of the RFLP patterns from both enzymes produced four patterns for

Monchique samples, five for Estói and seven for Montenegro samples. The sequences obtained for the *dsr* gene from these clones were analysed and equally produced four major clusters (Fig. 2.7).

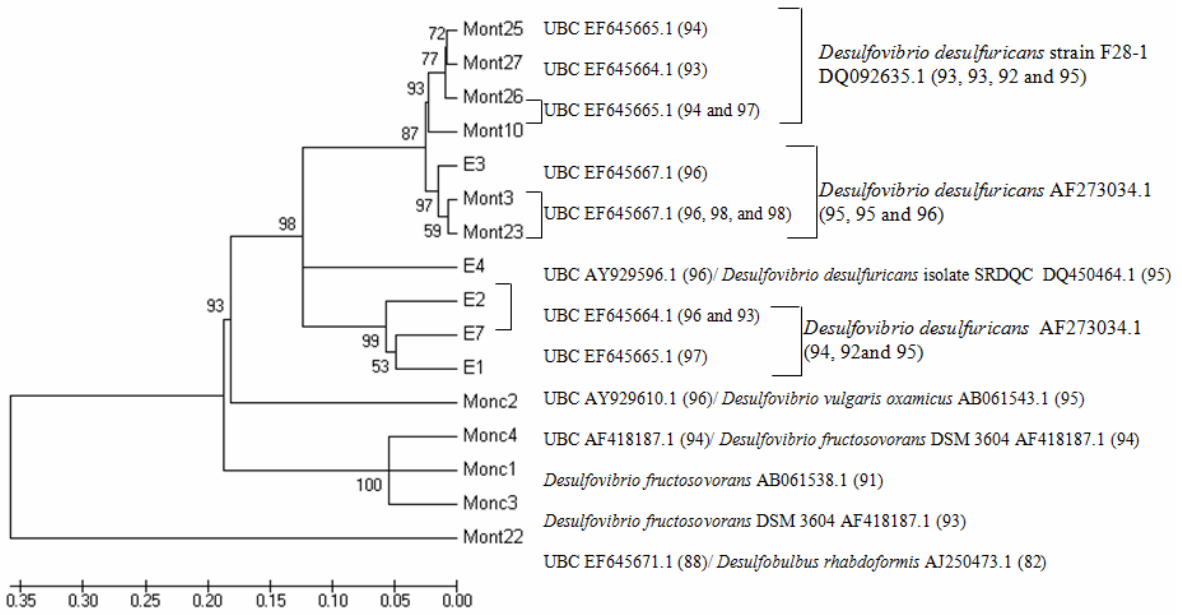

Fig. 2.7 Cladogram constructed for the selected clones from Monchique (Monc), Estói (E) and Montenegro (Mont) samples using *dsr* gene sequence. The Neighborhood-Joining algorithm was used. *Bootstrap values* are indicated on branches. Following the clone name the most closely related species and the most related cultured species are indicated. The percentage of similarity is indicated in brackets. UBC – uncultured bacterium clone.

The sequences obtained were registered in the GenBank (EU189153 – EU189184). The first cluster is formed by Mont25, Mont26, Mont27 and Mont10 clones and the sequence analysis revealed that these clones share similarity with *Desulfovibrio desulfuricans* strain F28-1 as the most closely related cultured species. The second cluster is constituted by the clones Mont3, Mont23 and E3 and the cultured species most closely related to them is *Desulfovibrio desulfuricans* AF273034.1. The remaining clones from Estói constitute a third group, in which E4 is the most distant. By sequence analysis the E1 clone is affiliated to uncultured bacterium clone NTUA-1A-DSR14 EF645665.1, whereas the clones E2 and E7 share similarity to uncultured bacterium clone NTUA-1A-DSR1 EF645664.1. The sequence of clone E4 is affiliated to uncultured SRB clone GranDSR2 and the cultured

Desulfovibrio desulfuricans isolate SRDQC. The cluster constituted by Monc1, Monc3 and Monc4 clones showed similarity with the cultured *Desulfovibrio fructosovorans,* namely the sequence of Monc 1 is close to *D. fructosovorans* AB061538.1 and Monc 3 and Monc 4 are related to *D. fructosovorans* DSM 3604 AF418187.1. Regarding the clone Monc 2, it is phylogenetically affiliated with *Desulfovibrio vulgaris oxamicus*. The sequence analysis of the clone Mont 22 revealed affiliation to uncultured bacterium clone, NTUA-1A-DSR1 EF645664.1 and to cultured *Desulfobulbus rhabdoformis* AJ250473.1.

4. Discussion

Regarding the diversity of samples it was expected to obtain diverse bacterial communities, in order to increase the probability of finding metals resistant SRB suitable to be used with the aim of AMD bioremediation. The highest efficiency of sulphate reduction by SRB was observed in sludge samples from the wastewater treatment plants of Montenegro and Estói, where larger numbers of SRB were detected. The presence of SRB was not observed in the samples from the mining area (Corta and Chança). Accordingly, sulphate reduction was not detected either. A possible reason for this result is the high acidity of these sediments, which do not allow SRB survival. In the remaining experiments the pH value was maintained at about 7, much higher than the pH values of the media with Corta or Chança samples, which decreased from 6.7 to 3.7 and 4.5, respectively, immediately after addition of the solid samples. The difficulty to grow SRB in acid medium has already been mentioned in the literature by Garcia *et al.* (2001).

In this study the SRB existing in the consortium from Monchique demonstrate a certain tolerance for iron. This behaviour can eventually be due to the fact that among the three samples tested, the sample from Monchique is the one that presents the highest iron content (Table 2.1) and therefore its SRB are probably more adapted to that metal. Our results show that the presence of zinc (150 mg/L) and copper (80 mg/L) significantly inhibited the activity of SRB present in the samples of Monchique and Estói. It has been reported that the characteristic toxic concentration of zinc for SRB is 13 to 40 mg/L (Hao *et al.*, 1994; Poulson *et al.*, 1997; Utgicar *et al.*, 2001). It has been mentioned that SRB are sensitive to copper, with no growth observed at 2 mg/L (Sani *et al.*, 2001) or 10 mg/L (Azabou *et al.*,

2007) of this metal. In the present study, even though the concentration of both elements was higher than those previously reported (Table 2.2), they did not stop sulphate reduction by SRB from Montenegro. The difference in behaviour can be due to a higher natural tolerance to these metals of the inoculum of Montenegro compared to the others and suggests different phylogenetic affinities.

Table 2.2 Toxic concentrations of metals reported by other authors.

Metal	Toxic concentrations (mg/L)	SRB cultures	Reference
Cu (II)	>10	*Desulfomicrobium* sp.	Azabou *et al.*, 2007
	9	*Desulfovibrio vulgaris*	Cabrera *et al.*, 2006
	2	*Desulfovibrio desulfuricans* G20	Sani *et al.*, 2001
	12	Mixed culture	Utgikar *et al.*, 2001
	4-20	Mixed culture	Hao *et al.*, 1994
Zn (II)	>125	*Desulfomicrobium* sp.	Azabou *et al.*, 2007
	20	*Desulfovibrio vulgaris*	Cabrera *et al.*, 2006
	20	Mixed culture	Utgikar *et al.*, 2001
	13	*Desulfovibrio desulfuricans*	Poulson *et al.*, 1997
	25-40	Mixed culture	Hao *et al.*, 1994
Fe (III)	>60	*Desulfomicrobium* sp.	Azabou *et al.*, 2007

In this study, the removal of metals was also evaluated. It was observed that copper was the first element to be removed, followed by zinc and then iron. This result was in accordance with the literature (Christensen *et al.*, 1996) and can be explained by the solubilities of CuS, ZnS and FeS, which are respectively 5.83×10^{-18} mg/L, 2.31×10^{-7} mg/L and 3.43×10^{-5} mg/L (De Vegt *et al.*, 1998; Johnson and Hallberg, 2005). Hence, copper needs the least amount of sulphide to precipitate, while iron needs the highest. In most cases, although the yield of sulphate reduction was more than enough to precipitate completely all the metals in the solution, the precipitation of iron was never quantitative. This is probably due to the fact that produced H_2S easily escapes as a gas during sampling being some of it not accessible to the dissolved metals.

Due to near neutral pH of the media, some metals precipitation as (oxy)hydroxides probably occurs, as already reported by Zagury *et al.* (2006). Therefore the re-dissolution of

$Fe(OH)_3$ in reducing conditions (Johnson and Hallberg, 2005; Zagury *et al.*, 2006), may also be responsible for incomplete iron precipitation.

The phylogenetic analysis showed that Montenegro is the most diverse sample, having species closely related to two different sequences of *Desulfovibrio desulfuricans* (AF273034.1 and F28-1 DQ092635.1) and one related to another SRB genus *Desulfobulbus rhabdoformis*, a genus not found in the other samples. This fact is particularly relevant due to the excellent performance of the Montenegro SRB consortium in the presence of iron, copper and zinc compared with the other inocula and considering their ability to use ethanol as carbon source. D. *rhabdoformis* was recently identified on bioreactors working in the presence of ethanol as carbon and energy source (Dar *et al.*, 2007). In addition, its ability to use a significant range of substrates, namely propionate, lactate, pyruvate, malate and fumarate is known (Lien *et al.*, 1998). The Monchique consortium includes species affiliated to *Desulfovibrio fructosovorans*, which is known to differ from all other described *Desulfovibrio* species by the ability to use fructose (Ollivier *et al.*, 1988).

The SRB consortium of Monchique samples also includes a sulphate and nitrate reducing bacterium *Desulfovibrio vulgaris oxamicus*, that was reclassified by Lopez-Cortés *et al.* (2006) as *D. oxamicus* sp. nov. comb. nov. The type strain (DSM 1925^T) of *D. oxamicus* is known to be able to oxidize incompletely lactate and ethanol to acetate (Lopez-Cortés *et al.*, 2006). Thus, the presence of this species, together with other than SRB in the consortium, may explain the slight decrease of ethanol, accompanied by an ineffective sulphate reduction.

The SRB inoculum from Estói samples is affiliated mainly with the cultured species of *Desulfovibrio desulfuricans*, which showed a similar performance to Montenegro SRB consortium in terms of sulphate reduction, in the presence of ethanol. The occurrence of bacteria affiliated to *D. desulfuricans* in both samples of Montenegro and Estói is not surprising, as both are from wastewater treatment plants whereas Monchique inoculum was collected from a thermal place.

The results of this study emphasise that the composition of the inoculum can be determinant in the performance of sulphate reducing systems for the treatment of acid mine drainage. Moreover was observed the sulphate reduction was affected by the type of carbon source used. Most efficient sulphate reduction was observed with lactate. When lactose was

added as carbon source no sulphate reduction was detected, probably due to the complexity of this molecule. However, lactose consumption was detected which can be due to the presence of bacteria other than SRB in the consortium, which are able to use this carbon source in their metabolism. The preference of SRB for simple organic molecules, like lactate or ethanol, instead of complex molecules such as lactose was previously reported in the literature (Barnes, 1998; Gibert *et al.*, 2004; Tsukamoto *et al.*, 2004; Zagury *et al.*, 2006).

5. Conclusions

A SRB consortium resistant to high concentrations of heavy metals (Fe, Cu and Zn) was isolated from Montenegro wastewater treatment plant. Thus, the Montenegro consortium, constituted by SRB affiliated to *Desulfovibrio desulfuricans* and *Desulfobulbus rhabdoformis*, seems to be the more suitable for an application to the treatment of AMD containing sulphate and metals (at least Fe, Zn and Cu). An efficient sulphate reduction by this consortium was observed with lactate and ethanol as carbon sources. In addition, the ability of the highly metals tolerant SRB consortium to use ethanol as carbon source is a promising result considering an eventual utilization of ethanol rich wastes, which are easily available in Portugal. The Montenegro consortium will be used to inoculate a sulphate-reducing bioreactor for the decontamination of AMD from S. Domingos mine.

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Chapter 3

Biological sulphate reduction using food industry wastes as carbon sources

Abstract

Biological treatment with dissimilatory sulphate-reducing bacteria has been considered as the most promising alternative for decontamination of sulphate rich effluents. These wastewaters are usually deficient in electron donors and require their external addition to achieve complete sulphate reduction. The aim of the present study was to investigate the possibility of using food industry wastes (a waste from the wine industry and cheese whey) as carbon sources for dissimilatory sulphate-reducing bacteria. The results show that these wastes can be efficiently used by these bacteria provided that calcite tailing is present as a neutralizing and buffer material. A 95 and 50% sulphate reduction was achieved within 20 days of experiment by a consortium of dissimilatory sulphate-reducing bacteria grown on media containing waste from the wine industry or cheese whey respectively. Identification of the dissimilatory sulphate-reducing bacteria community using the *dsr* gene revealed the presence of the species *Desulfovibrio fructosovorans, Desulfovibrio aminophilus* and *Desulfovibrio desulfuricans*. The findings of the present study emphasises the potential of using wastes from the wine industry as carbon source for dissimilatory sulphate-reducing bacteria, combined with calcite tailing, in the development of cost effective and environmentally friendly bioremediation processes.

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1. Introduction

In recent years several bioremediation processes based on the use of dissimilatory sulphate-reducing bacteria (DSRB) have been developed for the treatment of acid mine drainage (AMD) (Neculita *et al.*, 2007; Johnson and Hallberg, 2005; Tabak and Govind, 2003; Steed *et al.*, 2000) or other sulphate-rich effluents (Burgess and Stuetz, 2002; Lima *et al.*, 2001). DSRB use sulphate as electron acceptor and an energy rich carbon source as electron donor (Pfenning *et al.*, 1981), generating sulphide. This sulphide reacts additionally with certain metals dissolved in contaminated waters, such as copper, iron or zinc, forming insoluble precipitates (Vega-López *et al.*, 2007; Costa and Duarte, 2005; White *et al.*, 2003) and, as a result, the concentrations of sulphate and dissolved metals are reduced.

Considering that sulphate reduction is an energy intensive process (Barnes, 1998), a considerable amount of an energy-rich reductant is required. Consequently, the choice of the carbon source has an important effect on the efficiency and economical viability of the bioremediation technologies based on the use of these bacteria.

DSRB are known to utilise simple organic compounds such as carboxylic acids or alcohols (Widdel and Bak, 1992; White, 1995) as carbon and energy sources. Lactate is the carbon source most widely used by DSRB in laboratory culture conditions (Barnes, 1998; Postgate 1984; El Bayoumy *et al.*, 1999). However lactate would be too expensive for a large scale process.

Hydrogen can also be used as an energy source by some DSRB (Lens *et al.*, 2003; Fedorovich *et al.*, 2000; Nagpal *et al.*, 2000). Although hydrogen would be a relatively inexpensive substrate, this was deemed not to be an acceptable energy source because of engineering and safety requirements at a commercial scale (Huisman *et al*., 2006). According to the literature, ethanol seems to be the most cost effective substrate (Huisman *et al*., 2006; Tsukamoto *et al*., 2004).

Several natural sources of organic materials serving as electron donors and carbon sources have been already investigated: molasses, bagasse, sewage sludge, leaf mulch, wood chips, animal manure, vegetal compost, sawdust, mushroom compost, whey, and other agricultural wastes (Coetser *et al*., 2006; Costa and Duarte, 2005; Annachhatre and Suktrakoolvait, 2001; Waybrant *et al*., 1998; Christensen *et al*., 1996; Hammack *et al*.,

1994; Dvorak *et al*., 1992). The selection of the carbon source depends to a great extent on the degradability of the organic substrate.

The purpose of this work was to investigate the possibility of using two wastes from food industry as sources of carbon compounds to promote sulphate reduction by DSRB. The two wastes were selected from wine and cheese industries, since they are produced in Portugal in large amounts and widespread geographic locations and consequently are easily available at zero or negative cost. Tens of thousands of m^3 of both wine industry wastes and cheese whey are produced yearly in the continental Portuguese territory.

The search for efficient, low cost and largely available carbon sources (preferably wastes) for DSRB to be used in bioremediation processes for the treatment of sulphate rich effluents is of outmost importance. In addition, to use wastes in such processes is relevant from an environmental point of view, since it reduces the problematic of their disposal and promoting their biodegradation contributes to decrease pollutant release to the environment.

2. Materials and methods

2.1. Source and cultivation of DSRB community

The community of DSRB utilised in these experiments was obtained from a sludge sample from a wastewater treatment plant, located in Montenegro, Faro, in southern Portugal. This consortium was previously selected in **Chapter 2**. The bacterial community was grown and maintained in Postgate B medium (Postgate, 1984), at room temperature in anaerobic conditions. The bacteria were harvested by centrifugation, washed with Postgate B medium, and transferred to the batch solutions.

2.2. Composition of industrial food wastes used as carbon source

The wastes used in the experiments as carbon sources came from the cheese and wine Portuguese industries. Cheese whey was analysed using a MILKO-SCAN spectrophotometer and is mainly composed by fat (1.75%), proteins (0.30%), lactose (4.52%) and total solids (6.37%). Lactate was not detected by HPLC analysis. The waste

from the wine industry was collected prior to the bottling stage and contains 53.5 g/L ethanol, measured by HPLC. Both wastes were stored at 4ºC.

2.3. Composition of calcite tailing

The calcite tailing used in the experiments as a neutralizing and buffer material is the residue from a marble stone cutting and polishing industry. The presence of crystalline phases was assessed by X-ray diffraction, using a Bruker AXS-D8 Advance diffractometer with Cu Kα radiation and step of 0.02 º/s. The EVA code was used for the identification of the peaks and phase analysis. This material is mainly composed of magnesium calcite (~89%), quartz (~11%) and traces of illite mineral. Previous studies (data not shown) show that no biological sulphate reduction occurs in the presence of calcite tailing without addition of a suitable electron source.

2.4. Batch experiments

The growth experiments were carried out in duplicate using 120 mL glass bottles containing 100 mL of Postgate B medium (Postgate, 1984) with the following modifications: 6 g/L of a carbon and energy source compound, 2 g/L sulphate and resazurin as a redox indicator (0.03 g/L). Oxygen diffusion was eliminated by adding 10 mL of sterile liquid paraffin. The inoculum size used was 5% (v/v), with most probable number (MPN) of DSRB of $1.8x10^6$ CFU/mL. After inoculation, the bottles were sealed with butyl rubber stoppers and aluminium crimp seals and incubated at room temperature (21±1ºC), in anaerobic conditions.

Several carbon sources were tested: cheese whey, waste from the wine industry, lactate, lactose and ethanol. A test without carbon source was carried out as control. Batch tests were also carried as described, but the media were supplemented with 2 g of calcite tailing.

2.5. Statistical analysis

Each set of experiments was carried out in duplicate. The data were subject to one-way ANOVA. All differences were considered to be statistically significant if $P < 0.05$.

2.6. Sampling and analytic methods

5 mL samples were periodically collected, using a syringe, and filtered using a 0.2 µm hydrophilic polyestersulfone membrane (Machererey-Nagel). Redox potencial and pH were determined using a pH/E Meter GLP 21, Crison. High performance liquid chromatograph (Beckman) equipped with a polyspher® OAHY column (30 cm × 0.65 cm, Merck) and a refractive index detector, was used for soluble lactate, lactose, ethanol and acetate analysis. The analysis was performed with sulphuric acid (H_2SO_4 1.4 mM) as eluent, at a flow rate of 0.5 mL/min. The compounds were identified by their retention times in comparison to standards: lactate (11.347 min) as sodium lactate, ethanol (17.123 min), acetate (13.636 min) as sodium acetate.3-hydrate and lactose (6.859 min) monohydrate. Sulphate concentration was quantified by UV/visible spectrophotometry at 450 nm (Hach-Lange DR2800 spectrometer) using the method of sulfaVer4 (Hach-Lange), (Susuki *et al.,* 2003).

2.7. Molecular characterization of DSRB community

2.7.1. Extraction of DNA

Total genomic DNA was extracted from cell cultures grown on modified Postgate B media. The cells were harvested from 20 mL of cell culture by centrifugation at 4000 rpm for 10 min and twice washed with chilled deionised water. DNA extraction was carried out by the following method: 300 µL of sodium dodecyl sulphate (SDS) lysis mixture [500 mM Tris-HCl pH 8, 3% (w/v) SDS, 100 mM NaCl] and 300 µL of phosphate buffer pH 8 were added, followed by a freeze-thaw treatment (three cycles consisting of 1 min in liquid N_2 followed by 5 min in a 37ºC water bath). After cellular lysis, 300 µL of chloroform-isoamyl alcohol (24:1) were added. The solution obtained was centrifuged at 13000 rpm, for 10 min. After precipitation with isopropanol at -20ºC, for 20 min, DNA was resuspended in 35

µL H_2O. Nucleic acid extraction was evaluated on a 1% (w/v) agarose gel electrophoresis in Tris-Acetate-EDTA (TAE) buffer.

2.7.2. PCR amplification of *dsr* gene

PCR was conducted in a total volume of 50 µL. Community dissimilatory sulphite reductase (*dsr*) genes were amplified using the primers DSR1F (5'-ACC CAC TGG AAG CAC G-3') and DSR4R (5'-GTG TAG CAG TTA CCG CA-3') (Wagner *et al*., 1998; Chang *et al*., 2001; Castro *et al*., 2002), which amplify a 1.9 Kb fragment. The primers were purchased from Thermo Fischer Scientific. The reaction mixture used for PCR amplification contained 30.75 µL of H_2O, 1 µL of each primer (10 pmol/µL), 1 µL of dNTP`s (10 mM), 5 µL of $MgCl_2$ (25 mM), 10 µL of 5×Go Taq® buffer (Promega, Madison, USA), 0.25 µL of GoTaq®DNA polymerase (Promega, Madison, USA), and 1 µL of DNA. The DNA of a strain of *Desulfovibrio* subsp. was used as positive control and of *Escherichia coli* as a negative control. PCR amplification was carried out in a thermocycler (T1, Biometra). Thermal cycling was carried out by using an initial denaturation step of 94ºC for 3 min, followed by 30 cycles of 94ºC for 1 min, 56ºC for 1 min and 72ºC for 2 min and completed with an extension period of 5 min at 72ºC. The PCR products were separated in a 1% (w/v) agarose gel in TAE Buffer.

2.7.3. Cloning of *dsr* gene and restriction fragment length polymorphisms (RFLP) analysis

PCR products were purified (E.Z.N.A.^{TM} Gel Extration Kit, Omega) and ligated into the cloning vector pGEM®-T Easy (an insert vector ratio of 3:1) with T4 ligase enzyme followed by transformation into *E. coli* DH5-alpha competent host cells, according to the manufacturer's instructions (Promega, Madison, USA). All the white colonies were amplified by direct PCR with the DSR1F and DSR4R primers according to the conditions described above.

Restriction fragment length (RFLP) analyses were done using the restriction enzymes H*haI* and H*aeIII* (Promega). Fragments of the digested PCR products were separated in a 2% (w/v) TAE agarose gel.

2.7.4. Sequencing and phylogenetic analysis

Representative plasmids from each digestion pattern were selected for sequencing at CCMAR (Centro de Ciências do Mar, Universidade do Algarve). The dsr gene inserted in plasmids was amplified using the primers DSR1F and DSR4R, according to the conditions described above. PCR products were purified (E.Z.N.A.^{TM} Gel Extration Kit, Omega) and sequenced. Sequence identification was performed by using the BLASTN facility of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Cladograms were constructed using MEGA version 4 (Tamura *et al.*, 2007) and the Neighbour-Joining algorithm was applied (Saitou and Nei, 1987; Studier and Keppler, 1988).

3. Results

3.1. Lactate as carbon source

The profile of sulphate reduction by DSRB in the presence of lactate as carbon source is shown in Fig. 3.1. In the first 7 days of the experiment sulphate was completely reduced and lactate was totally consumed. At the same time acetate production by DSRB was observed. Without carbon source sulphate concentration was always near 2 g/L. The pH values were close to 7 during all the experiment.

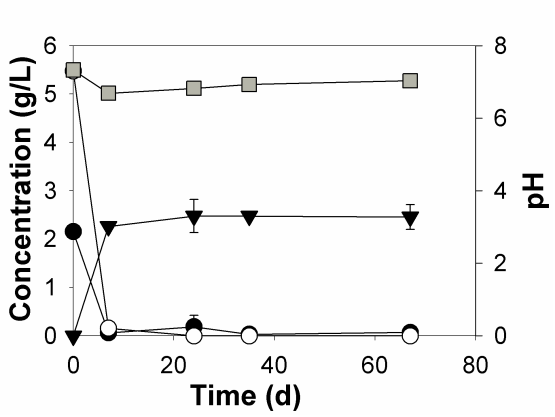

Fig. 3.1 Profile of sulphate reduction and consumption of carbon source by bacterial consortium using lactate as carbon source without calcite tailing. Data are the average of duplicates and error bars indicate the standard deviations of the average values. Symbols: (●) sulphate, (○) lactate, (▼) acetate and (■) pH.

3.2. Lactose and cheese whey as carbon source

Sulphate reduction was not detected when lactose was utilised as carbon source (Fig. 3.2a). A similar behaviour was observed when cheese whey was utilised (Fig. 3.2b). However, lactose consumption was verified in both cases (around 33%). The production of lactate (0.96 g/L) was observed in the presence of cheese whey.

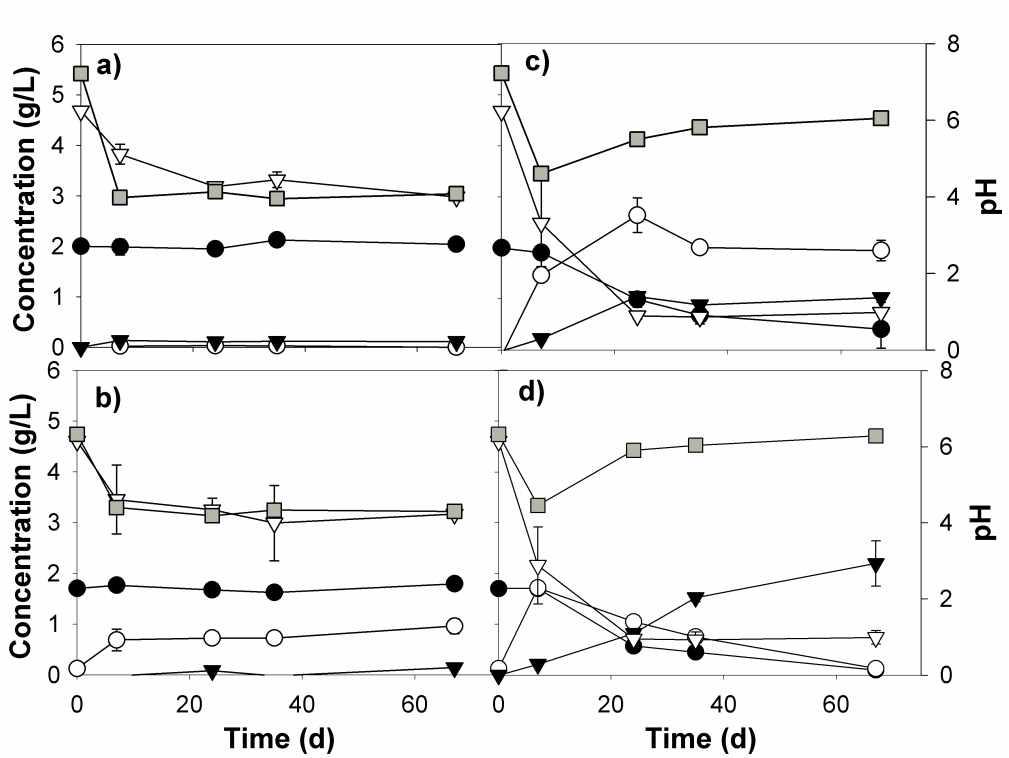

Fig. 3.2 Profile of sulphate reduction and consumption of carbon source by bacterial consortium using two lactose-based carbon sources: a) lactose, b) cheese whey, c) lactose with calcite tailing and d) cheese whey with calcite tailing. Data are the average of duplicates and error bars indicate the standard deviations of the average values. Symbols: (●) sulphate, (○) lactate, (▼) acetate, (▽) lactose and (■) pH.

When the media containing lactose and cheese whey were supplemented with calcite tailing, efficient sulphate reduction was achieved (Fig. 3.2c and 3.2d): 80% and 94% sulphate reduction was obtained respectively, at the end of the experiment. In addition, higher consumption of lactose and higher production of lactate and acetate were observed in both cases, in comparison with the experiments performed without calcite tailing. It was observed that the pH of the media containing lactose and cheese whey in the absence of

calcite tailing decreased from 7 to values below 4 during the experiments. When these carbon sources were supplemented with calcite tailing the pH increased from 4 to 6 and thereafter it was maintained near this value until the end of the experiments.

3.3. Ethanol and waste from the wine industry as carbon source

The sulphate reduction profile with ethanol as carbon source is shown in Fig. 3.3a. The pH of the solution when ethanol was used as carbon source does not decrease to values lower than 5, not compromising DSRB growth and activity. This behavior, already observed in previous studies, justified that no calcite tailing needed to be added.

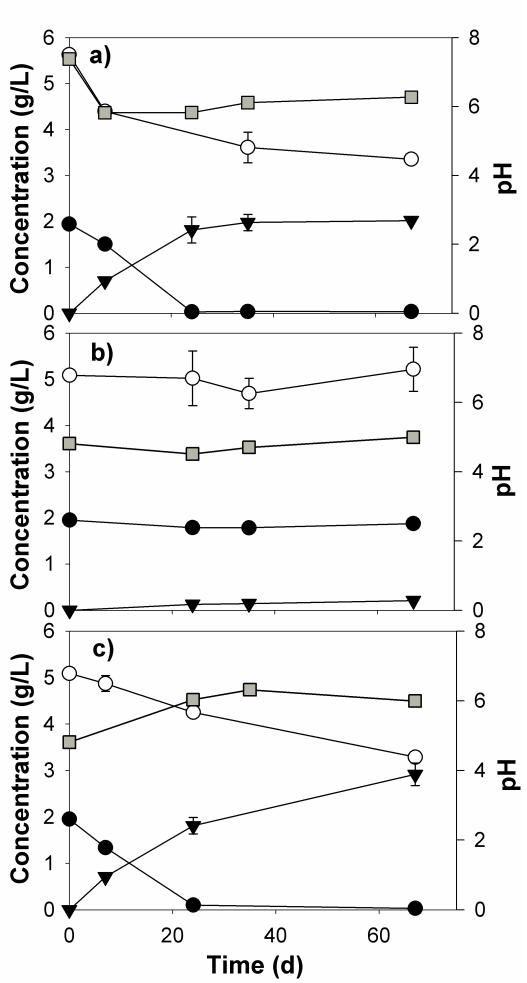

Fig. 3.3 Profile of sulphate reduction and consumption of carbon source by bacterial consortium using two ethanol-based carbon sources: a) ethanol, b) wastes of winery industry and c) wastes of winery industry with calcite tailing. Data are the average of duplicates and error bars indicate the standard deviations of the average values. Symbols: (●) sulphates, (○) ethanol (▼) acetate and (■) pH.

High sulphate reduction was observed when pure ethanol was used as carbon source. 98.5% reduction of sulphate was observed in the first 20 days. For this reduction the consortium of DSRB used 1.8 mmol ethanol per mmol sulphate reduced and 1.6 mmol acetate was produced.

When the waste from the wine industry was used by itself as carbon source sulphate were not reduced and ethanol was not consumed by DSRB (Fig. 3.3b). However, in the presence of calcite tailing (Fig. 3.3c) efficient sulphate reduction was achieved. After 20 days 95% sulphate reduction was observed. Ethanol consumption and acetate production were also observed. For each mmol sulphate reduced, 0.94 mmol ethanol was consumed and 1.6 mmol acetate was produced. When the waste from the wine industry was used in the absence of calcite tailing the pH of the medium presented values near 4.7 during all the experiment. On the other hand, when calcite tailing was present the pH increased to values close to 6, similar to what was observed in the experiment with ethanol.

3.4. Phylogenetic analysis of DSRB community

The dissimilatory sulphite reductase gene (*dsr*) was used to elucidate the composition of the DSRB consortium grown with waste from the wine industry as carbon source.

A total of sixteen clones were obtained and all of these clones were subjected to RFLP analysis. Eight different patterns were obtained. The representative clones from each pattern were selected for sequencing and these sequences were submitted to the GenBank (accession numbers: EU552471 to EU552486).

In the cladogram obtained for the *dsr* gene sequence of the selected clones three major clusters were identified (Fig. 3.4). The first one is composed of two clones affiliated to *Desulfovibrio desulfuricans*: *Desulfovibrio desulfuricans* isolate SRDQC (accession number DQ450464.1) and *Desulfovibrio desulfuricans* strain F28-1 (accession number DQ092635.1). The second group contains clones similar to *Desulfovibrio fructosovorans* (accession number AB061538.1). The third assemblage consists of clones with affiliation to *Desulfovibrio aminophilus* strain DSM 12254 (accession number AY626029.1).

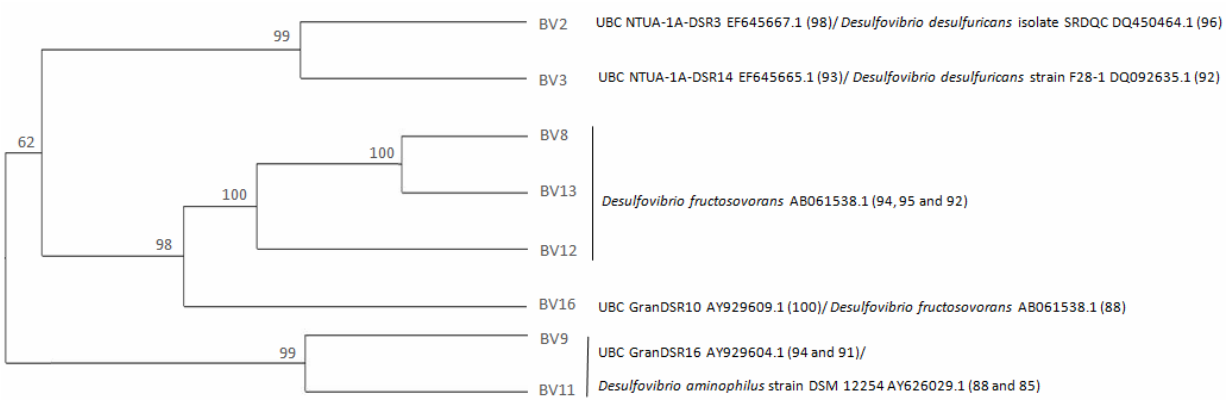

Fig. 3.4 Cladogram constructed for the selected clones using *dsr* gene sequence. Partial weighted combined-data cladogram of relationships of *dsr* gene sequences for the selected dissimilatory sulphate-reducing bacteria clones. The Neighbour-Joining algorithm was used. *Bootstrap values* are indicated on branches. Following the clone name the most closely related species and/or the most related cultured species are indicated. The percentage of similarity is indicated in brackets. UBC - uncultured bacterium clone.

4. Discussion

This study shows, for the first time, that a waste from the wine industry can be used by DSRB present in a bacterial consortium, provided that a neutralising and buffer material is present. In this case calcite tailing, another waste material, was used. According to *dsr* gene analysis, the DSRB consortium is constituted by members of the species *Desulfovibrio fructosovorans*, *Desulfovibrio aminophilus* and *Desulfovibrio desulfuricans*. The dominance of the *Desulfovibrio* genus was observed, which is consistent with literature data mentioning its predominance in wastewater treatment plants (Santegoeds *et al*., 1998; Baena *et al*., 1998; Dar *et al*., 2005). The genus *Desulfovibrio* represents a group of Gram-negative sulphate reducers in which all species oxidise their substrates incompletely to acetate (Widdel and Bak, 1992).

The increased efficiency of sulphate reduction by DSRB observed when the food industry wastes were supplemented with calcite tailing can be explained by the increased pH. The difficulty to grow DSRB in media with low pH has already been mentioned previously (Garcia *et al*., 2001; Benedetto *et al*., 2005). According to the literature, specific conditions such as an anaerobic environment, a redox potential around -200 mV and pH values above 5 must be met to enable sulphate-reducing activity (Cohen, 2006). When the medium was

supplemented with calcite tailing, due to its neutralising and buffer capacities, the pH was close to 6 or higher during all the experiment. Therefore, good conditions for DSRB activity were achieved, allowing sulphate reduction and consumption of the carbon source.

Sulphate reduction observed in the presence of lactose and in the presence of cheese whey (both supplemented with calcite tailing) was slower compared to sulphate reduction in the presence of lactate, ethanol or waste from the wine industry, the last one supplemented with calcite tailing. When lactose was supplemented with calcite tailing, approximately 20 days were needed to consume half of the added sulphate concentration, while with lactate and ethanol near complete sulphate reduction occurred within 7 and 20 days, respectively. The complexity of lactose molecule, in comparison with ethanol or lactate, may explain this result. In fact, few bacterial species, and among them no DSRB, are able to metabolize lactose, thus it required more time for its degradation.

When lactose or cheese whey was provided as electron donors for sulphate reduction the pH of the medium decreased and lactose was consumed. However, no sulphate reduction was observed. These results can be explained by the presence in the bacterial consortium of bacteria other than DSRB with ability to use lactose in their metabolism. The production of lactate observed in Fig. 3.2b can result from the activity of lactic bacteria present in the cheese whey.

When lactose and cheese whey were supplemented with calcite tailing, production of lactate was observed suggesting that lactose can be used by other bacteria present in the consortium, such as lactic bacteria, producing lactate that may be further utilized by DSRB and converted to acetate. This indirect path was already reported when molasses were used as carbon source (Maree *et al.*, 1986).

Consumption of the acetate produced, by DSRB, mentioned by several authors (Barnes, 1998; Dar *et al*., 2007), was not observed. This probably indicates the absence of acetate utilising bacteria in this particular community.

When waste from the wine industry was used as carbon source in the presence of calcite tailing, lower ethanol consumption (0.94 mmol/mmol sulphate reduced) was observed compared with pure ethanol (1.8 mmol/mmol sulphate reduced). This is probably due to the fact that the waste from the wine industry may contain other nutrients that may be used as carbon source. *Desulfovibrio fructosovorans* is known to differ from all other described *Desulfovibrio* species by its ability to use fructose (Olliver *et al*., 1988) and *Desulfovibrio*

aminophilus has been reported to be able to use amino acids as carbon and energy sources (Hernandez-Eugenio *et al.*, 2000). The waste from the wine industry usually contains amino acids and fructose in significant amounts, so it is possible that these can be consumed by the DSRB consortium. This in turn explains the reduced amount of ethanol consumed per mmol sulphate. According to the literature (Waybrant *et al*., 1998; Zagury *et al*., 2006), materials containing multiple organic substrates or mixtures are most effective in promoting sulphate reduction compared to those containing a single organic substrate.

The use of waste from the wine industry as carbon source for sulphate reduction by DSRB is promising in contrast to other wastes. When conifer sawdust and composted spruce chips were used no sulphate reduction was observed (Zagury *et al*., 2006). Waybrant *et al*. (1998) achieved sulphate reduction only when sheep manure plus calcite and sand were mixed with other organic constituents.

5. Conclusions

Taking into account the results achieved the waste from the wine industry in the presence of calcite tailing seems to be promising as carbon source to promote DSRB activity. Cheese whey in the presence of calcite tailing can also be used as carbon source for biological sulphate reduction, but the process is considerably slower.

Their efficiency as carbon sources is only revealed when those wastes are supplemented with calcite tailing that acts as a neutralizing and buffer material, achieving suitable pH conditions for DSRB activity.

The possibility of using food industry wastes, particularly the wastes from wine industry, to promote an efficient sulphate reduction is an important finding. By this way these wastes can be reutilised in bioremediation processes based on DSRB for the treatment of sulphate rich effluents, with both environmental and economical benefits.

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Chapter 4

Wine wastes as carbon source for biological treatment of acid mine drainage

Abstract

Possible use of wine wastes containing ethanol as carbon and energy source for sulphate-reducing bacteria (SRB) growth and activity in the treatment of acid mine drainage (AMD) is studied for the first time. The experiments were performed using anaerobic down flow packed bed reactors in semi-continuous systems. The results show that efficient neutralization and high sulphate removal (> 90%) were attained with the use of wine wastes as substrate allowing the production of effluents with concentrations below the required local legislation for irrigation waters. This is only possible provided that the AMD and wine wastes are contacted with calcite tailing, a waste material that neutralizes and provides buffer capacity to the medium. The removal of metals using wine wastes as carbon source was 61-91% for Fe and 97% for both Zn and Cu. The lower removal of iron, when wine waste is used instead of ethanol, may be due to the presence of iron-chelating compounds in the waste, which prevent the formation of iron sulphide, and partial unavailability of sulphide because of re-oxidation to elemental sulphur. However, that did not affect significantly the quality of the effluent for irrigation. This work demonstrates that wine wastes are a potential alternative to traditional SRB substrates. This finding has direct implication to sustainable operation of SRB bioreactors for AMD treatment.

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1. Introduction

Biological treatment with sulphate-reducing bacteria (SRB) has been considered the most promising alternative for the treatment of several types of industrial wastewaters, namely acid mine drainage (AMD) (Johnson and Hallberg 2005; Neculita *et al.*, 2007).

Generally, AMD is deficient in carbon sources/electron donors and thus, an external addition is required to achieve sulphate reduction. Therefore, the choice of carbon source for SRB activity can be the key-point to ensure high performance, long-term efficiency and economical viability of the treatment. Selection of a suitable carbon source and electron donor for biological sulphate reduction is based on three factors: degradability of the carbon source and hence its capacity to allow complete sulphate reduction by SRB, its cost per unit of sulphate converted to sulphide and its availability (van Houten *et al.*, 1994; Liamleam and Annachhatre, 2007).

Until now diverse organic wastes, namely sewage, animal manure, vegetal compost, wood chips, sawdust, sugar, stillage from ethanol distilleries, were used as carbon sources and electron donors for sulphate reduction (Amos and Younger, 2003; Frömmichen *et al.*, 2003; Gibert *et al.*, 2004; Costa and Duarte, 2005; Zagury *et al.*, 2006; Gonçalves *et al.*, 2007). However, most of them, due to their complexity, are not easily degraded (Zagury *et al.*, 2006). Thus, there is an increased concern in testing new organic substrates for SRB, especially those that are at the same time cheap and widely available. The main focus of this research was to study the removal efficiency of sulphate and main metals present in a synthetic AMD using wine waste as carbon source.

In previous studies performed in batch using nutrient medium showed that some communities of SRB have the ability to use wine wastes as carbon source under specific conditions (Chapter 3). In the present work wine wastes are applied for the first time as carbon and electron source for SRB in the treatment of AMD, using a down-flow anaerobic packed bed reactor (DAPB). This organic waste was selected because it is produced in large amounts in most Portuguese regions, as well as in other wine producing countries, and consequently, can be easily available at zero or even at negative cost, which can be determinant for the economy of the bioremediation process.

2. Materials and methods

2.1. Inoculum

A mixed culture containing the SRB species: *Desulfovibrio fructosovorans, Desulfovibrio aminophilus* and *Desulfovibrio desulfuricans,* was used as inoculum. This consortium was previously selected in batch studies (Martins *et al.*, 2009). The bacterial community was grown and maintained in modified Postgate B medium (Postgate, 1984) (lactate was replaced by with wine wastes supplemented with calcite tailing), in anaerobic conditions at room temperature. Bacterial cells were harvested by centrifugation (4000 rpm, 10 min) and transferred to the bioreactors.

2.2. Calcite tailing and wine wastes composition

The waste from the wine industry was collected prior to the bottling stage and its composition was similar to that of diluted red wine. Ethanol concentration was 53.5 g/L, and pH was 3.8-3.9.

Calcite tailing is the residue from a marble stone cutting and polishing industry and was used as a neutralizing and buffer material. X-ray diffraction (XRD) showed that its main composition is magnesium calcite (~ 89%), quartz (~ 11%) and traces of illite. Previous studies (data not shown) showed that no biological sulphate reduction occurs in the presence of calcite tailing without addition of electron source.

2.3. AMD composition

Synthetic AMD similar to S. Domingos pit lake mine water was used, containing about 2.5 g/L sulphate, 550 mg/L Fe, 175 mg/L Zn and 70 mg/L Cu, pH = 2.5, (Costa and Duarte, 2005).

Pro-analysis $FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$ and $CuSO_4 \cdot 5H_2O$ were used. Na_2SO_4 and H_2SO_4 were used as additional source of sulphate and to acidify the final solution, respectively.

2.4. Bioreactor characterization and experimental description

Two laboratory scale DAPBs were used for AMD treatment. The experiments were performed using glass column bioreactors (inner diameter 5.5 cm, height 35 cm) at room temperature (21±1 ºC) for about 184 d for bioreactor I (fed with ethanol) and 226 d for bioreactor II (fed with wine wastes).

The DAPBs were packed in two stages: first ~ 700 g of coarse sand (0.3-0.5 cm) and 30 mL of inoculum were added to each reactor. This layer was filled with modified Postgate B medium (Postgate, 1984), containing ethanol (in bioreactor I) or wine wastes (in bioreactor II) as carbon source. The bioreactors were operated in batch conditions for about 55 d to promote bacterial growth. Subsequently, 90 g of a second packing layer, consisting of a mixture of 2:1 (w/w) of coarse sand and calcite tailing (0.7-1 cm) was placed on the top. This layer was filled with 30 mL of synthetic AMD, starting the treatment.

The experiment was performed in a semi-continuous system with the influent fed to the top of the column and the effluent gravity collected at the bottom. The daily volumes of added AMD and collected effluent were the same (50 mL), corresponding to a hydraulic retention time of 8 d, considering 400 mL as working volume of each bioreactor.

Different concentrations and addition regimes of each carbon source were used (Table 4.1). An initial high concentration of ethanol (70 g/L) was provided in bioreactor I to guarantee efficient process start-up.

2.5. Analytical methods

Periodically, samples were collected from each bioreactor with a syringe via a side port at the base of the column and filtered through 11 µm filter paper. Redox potential and pH were measured using a pH/Eh Meter (GLP 21, Crison). Sulphate concentration was measured by a UV-VIS spectrophotometer (Hach-Lange DR2800), using the sulfaVer4 method (Hach-Lange). High performance liquid chromatograph (Beckman), equipped with a polyspher OAHY column (30 cm × 0.65 cm, Merck) and a Refractive Index detector, was used for ethanol and acetate analysis. Heavy metals (Fe, Cu and Zn) were measured by flame atomic absorption spectroscopy using a Shimadzu AA-680 model spectrometer. For

each sample, three aliquots were considered and the results were critically treated and only accepted if a reasonable standard deviation (< 10%) was achieved.

Table 4.1 Carbon source feeding regime.

Bioreactor I - fed with ethanol			Bioreactor II - fed with wine wastes		
Operation time (d)	Volume added	Ethanol in the influent (g/L)	Operation time (d)	Volume added[d]	Ethanol in the influent (g/L)
0 to 120	1 mL[a] weekly	15.3	0 to 91	30 mL weekly	20.3
121 to 184	3 mL[b] daily[c]	3.40	92 to 128	20 mL weekly	15.4
			129 to 149	10 mL weekly	9.0
			150 to 226	2 mL daily[c]	2.0

a) Volume added from a 780 g/L ethanol solution
b) Volume added from a 60 g/L ethanol solution
c) Five days a week
d) Volume of wine wastes added. The wine wastes contain 53.5 g/L of ethanol

SRB populations were enumerated by the three-tube Most Probable Number (MPN) assay with serial dilutions in modified Postgate E medium (Postgate, 1984) with ethanol as carbon source. Essays were performed in triplicate. MPN tubes were incubated at room temperature (21± 1ºC) for 5 d.

To evaluate the formed precipitates, micro-morphology and elemental composition, scanning electron microscopy and energy dispersive X-ray spectrometry (EDS) were carried out using a JEOL JSM-7001F scanning electron microscope, coupled with an OXFORD X-ray energy dispersive spectrometer, with a Si(Li) detector. The samples were coated with a thin, conductive gold film.

Presence of crystalline phases was assessed by XRD, using a Bruker AXS-D8 Advance diffractometer with Cu Kα radiation. The Diffrac^{Plus} EVA software was used for peak identification and phase analysis.

3. Results and discussion

The efficiency of the processes was assessed through determination of pH, Eh, sulphate, metals (Fe, Zn, Cu), ethanol and acetate concentrations and SRB numbers in the effluent.

3.1. pH and ORP

In bioreactor I (Fig. 4.1) AMD neutralisation (to pH 6.0-7.5) occurred during all the experiment mainly due to calcite tailing placed on the top of the column, providing optimal pH for SRB growth and activity (Cohen, 2006). In bioreactor II, calcite tailing also promoted neutralisation. However, a slight decrease in effluent pH was observed throughout the experiment (Fig. 4.1), with non-optimal values for SRB activity (< 5.5) being observed after day 150. Besides the wine waste itself being slightly acidic, this can be due to metabolisation of other compounds present, with production of acidic sub-products, either by bacteria inoculated to the reactor or others originally present in that waste.

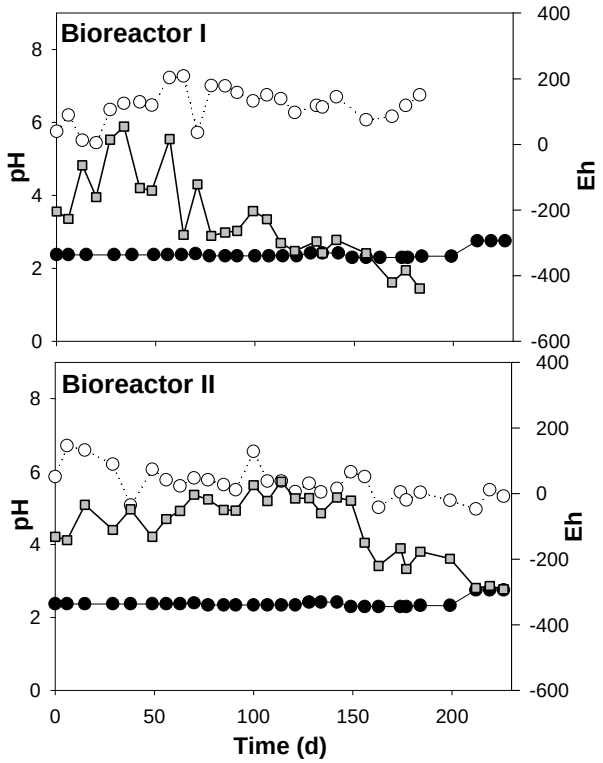

Fig. 4.1 Time course of pH and Eh in bioreactors I and II: pH in AMD (●), pH in effluent from bioreactor (○) and Eh in effluent from bioreactor (■).

pH values achieved in the effluents were generally within the range of maximum recommended values (MRV) for irrigation waters imposed by Portuguese legislation (6.5-8.4), and were always within the range of the maximum admitted values (MAV) for this parameter (4.5-9.0).

Eh in bioreactor I showed a decreasing tendency, reaching values near -400 mV in the end of the experiment. In bioreactor II, Eh varied from -150 to 28 mV and only after day 150 a decrease down to near -300 mV was observed. In both cases daily addition of carbon source promoted the decrease of Eh, which is associated with good SRB activity.

3.2. Biological sulphate reduction

Since the beginning of the experiment and independently of used carbon source, high degrees of sulphate reduction (> 90%) were generally achieved (Fig. 4.2).

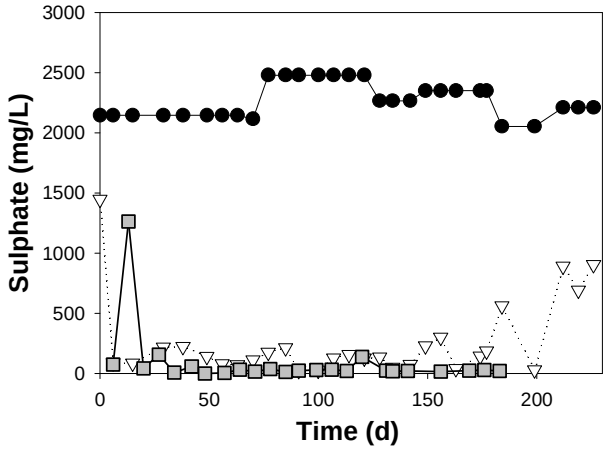

Fig. 4.2 Sulphate reduction performance of bioreactors I and II during the operation time: sulphate concentration in AMD (●), in effluent from bioreactor I (■) in effluent from bioreactor II (▽).

Sulphate concentration in the effluent of bioreactor I was lower than 30 mg/L throughout the entire experiment, which corresponds to above 98% removal. In bioreactor II, sulphate reduction was above 90%, until day 184, corresponding to concentrations in the effluent below 200 mg/L. After that sulphate concentration increased gradually up to 905 mg/L in day 226. This increase only occurred when effluent pH came below 5.5, becoming non-

optimal for SRB activity. Hence, the results emphasise that biological sulphate reduction is limited by pH. Except for the last three samples collected from bioreactor II, sulphate concentration in the effluent is below the MRV of Portuguese legislation (575 mg/L).

3.3. Metals removal

In bioreactor I, the metals were almost completely removed (Fig. 4.3), since the concentrations of Fe, Cu and Zn in the effluents were always below 9, 3 and 4 mg/L, respectively (see removal percentages in Table 4.2).

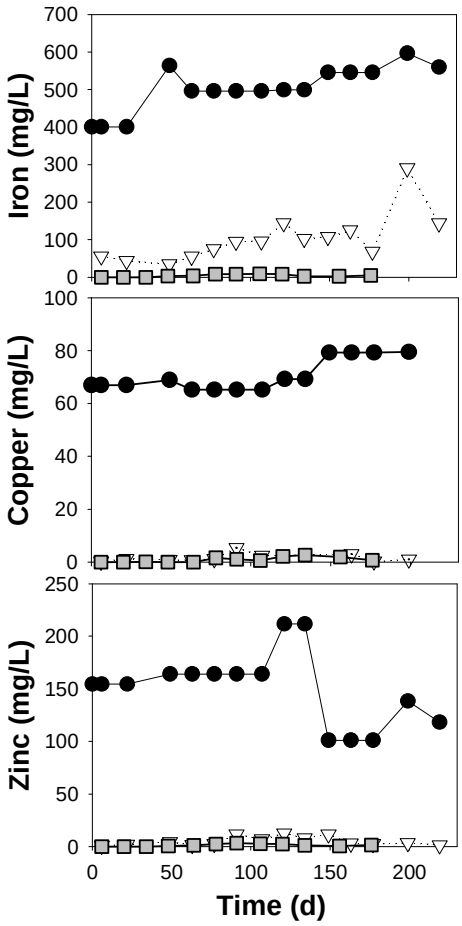

Fig. 4.3 Metals removal performance of bioreactor I and II during the operation time: metals concentration in AMD (●), in effluent from bioreactor I (■) and in effluent from bioreactor II (▽).

Table 4.2 Performance of bioreactors.

Bioreactor	pH		Ions concentration		Performance parameters	
	Feed	Final	Feed (mg/L)	Effluent (mg/L)	Removal (%)	Removal rate (mg/Ld)
I	2.4	6.5-7.5	SO_4^{2-} ± 2300	30	98	283
			Fe ± 530	9	98	65
			Cu ± 71	3	95	9
			Zn ± 154	4	97	19
II	2.4	6.7- 5.0	SO_4^{2-} ± 2300	200-905	91-61	263-174
			Fe ± 500	35-290	91-42	58-26
			Cu ± 70	2	97	9
			Zn ± 151	5	97	18

In bioreactor II the highest percentages of iron removal, between 85 and 91%, were obtained in the first 63 d of the experiment. After that time Fe concentration in the effluent shows a tendency to increase, reaching a maximum value of 290 mg/L at day 199. Taking into account that Portuguese legislation does not impose a MAV for iron concentration in irrigation water, the use of the effluent for that purpose is not compromised. For Cu, values lower than 5 mg/L (MAV) were always achieved, while Zn concentration in the effluent was generally below 10 mg/L (the MAV), which corresponds to high percentages of removal (Table 4.2).

Both processes are efficient for the removal of metals, although lower concentrations of iron were achieved in the effluent of bioreactor I. Considering that the amount of sulphate reduced should be enough to give the same extent of precipitation of metals in both reactors, a possible cause for this difference in behaviour could be the accumulation in bioreactor II of substances either originally present in the wine wastes or resulting from the activity of bacteria present in those wastes. The action of those compounds could be formation of soluble iron complexes, or prevention of precipitate particles aggregation, remaining these in colloidal suspension, thus explaining the results obtained. In fact, the iron-chelating properties of phenolic acids, usually present in wine, are documented (Andjelkovic *et al.*, 2006).

3.4. SRB quantification and ethanol consumption

As expected, ethanol, used in bioreactor I as an electron donor for sulphate reduction, was suitable for SRB growth and activity (Nagpal *et al.*, 2000; Kaksonen *et al.*, 2006; Zagury *et al.*, 2006). In comparison, use of wine wastes was also effective. In both bioreactors ethanol was consumed and acetate was produced (Fig. 4.4).

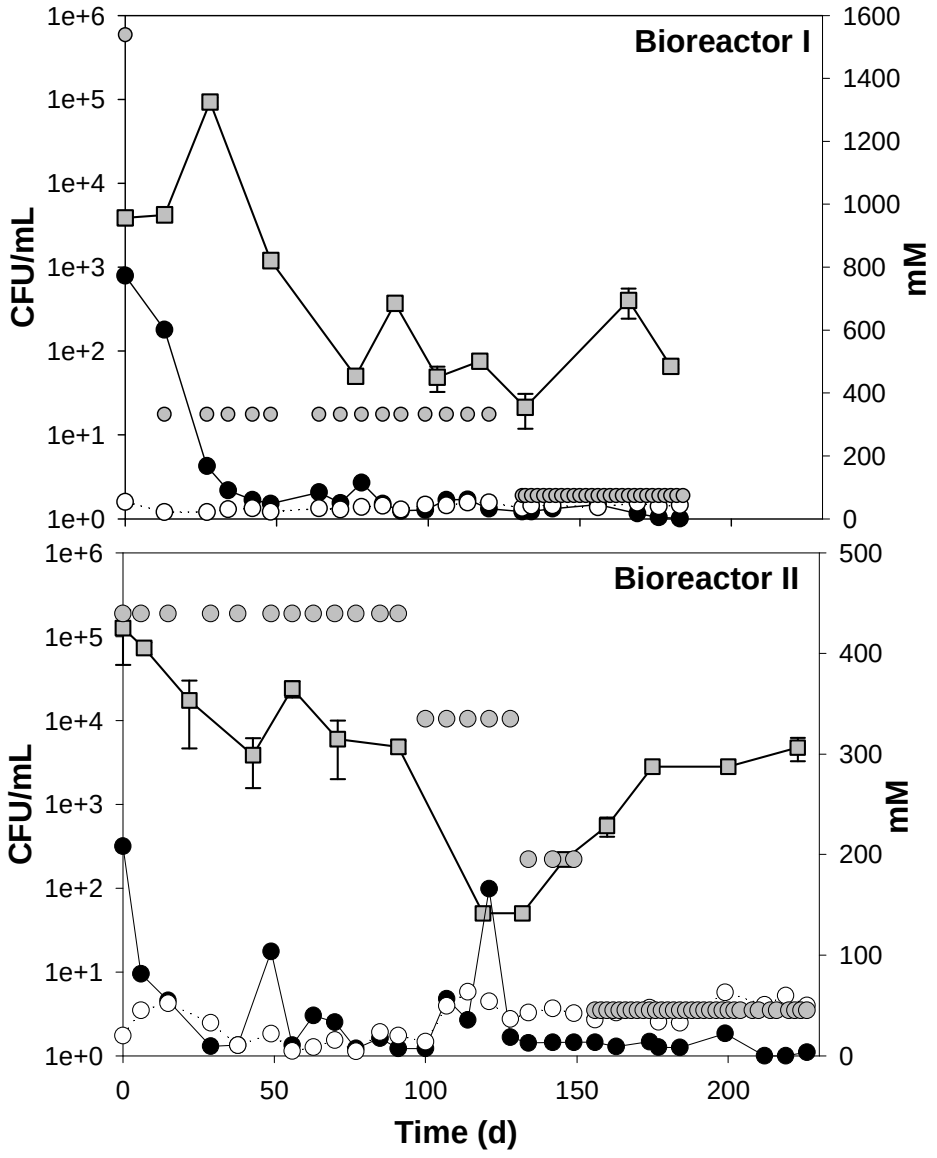

Fig. 4.4 Performance of SRB consortium in bioreactor I and II during the operation time: ethanol concentration in influent (●) and in effluent from bioreactor (●), acetate concentration in effluent from bioreactor (○) and SRB number in bioreactor (■).

Use of high initial concentration of ethanol (70 g/L) in bioreactor I is determinant to achieve a considerable MPN ($9x10^4$ CFU/mL), similar to that existing at start-up with bioreactor II (Fig. 4.4). This guarantees efficient start-up of the process. Similar growth profiles of SRB were obtained in both bioreactors (Fig. 4.4), since a decrease of MPN was observed in both cases until about day 130. Hence, the decrease of MPN in bioreactor II is not related with toxicity of the wine wastes, and seems not to affect sulphate reduction. Change to a daily carbon source addition regime (Table 4.1) seems to enhance SRB growth (Fig. 4.4) and helps to keep redox potential low (Fig. 4.1).

Simultaneously with the increase of MPN, it is possible to observe higher consumption of ethanol and formation of acetate than in the first part of the experiment. Presence of acetate in the effluents is consistent with the use of *Desulfovibrio*, which according to the literature (Liamleam and Annachhatre, 2007; Muyzer and Stams, 2008) does not use this compound as carbon source. Incomplete ethanol oxidation was also observed by Koschorreck *et al.*, (2002). However, complete oxidation of ethanol to CO_2 using SRBs alone was reported using cultures of *D. desulfuricans* (Napgal *et al.*, 2000), one of the species which is present in the used inoculum.

Decrease of ethanol concentration in bioreactor II did not show detrimental effect on the activity of SRB, which was enough to bring sulphate concentration below the MRV for irrigation water (575 mg/L), one of our targets.

The observed ratios ethanol consumption/sulphate consumption ($EtOH/SO_4^{2-}$) and acetate formation/sulphate consumption ($AcOH/SO_4^{-2}$) were calculated for the periods of nearly steady state behaviour of the bioreactors.

Thus, for bioreactor I between days 131 and 183, the $EtOH/SO_4^{2-}$ molar ratio was 2.30, and the $AcOH/SO_4^{2-}$ molar ratio was 1.78. This stoichiometry is quite close to that of the redox reaction between sulphate and ethanol to produce sulphide and acetate (eq. 4.1), again showing incomplete use of the carbon source:

$$SO_4^{2-} + 2\ C_2H_5OH \rightarrow S^{2-} + 2\ CH_3COOH + 2H_2O \qquad (4.1)$$

The differences observed to this stoichiometry (slightly higher consumption of ethanol, and lower formation of acetate) can be explained by other processes taking place in the system,

such as formation of ethanol oxidation subproducts other than acetate, or ethanol consumption by bacteria other that SRB that certainly are present.

The same calculations applied to bioreactor II show that between days 158 and 200, the $EtOH/SO_4^{2-}$ molar ratio was 1.60, and the $AcOH/SO_4^{2-}$ molar ratio was 2.08. Again these values are close to those expected from Eq. 4.1, showing that incomplete oxidation of ethanol is also dominant in this system. The main difference is the lower consumption of ethanol. This indicates that other compounds present in the wine waste are used to reduce sulphate. In fact, wine is known to have significant amounts of metabolisable organic compounds, such as carbohydrates, organic acids and aminoacids. This is also consistent with our finding that *D. fructosovorans*, known for its ability to use fructose-containing carbohydrates, is one of the species that thrives when using wine wastes as carbon source (Martins *et al.*, 2009).

3.5. Precipitates analysis

XRD patterns exhibit the characteristic peaks of column matrix materials (coarse sand and calcite tailing), in which the major crystalline phases are quartz, illite and calcite. Particularly interesting is that, in both cases, no metal sulphides were observed by XRD, which, together with a swelling background, is indicative that the metals precipitate essentially in amorphous phases. Crystalline iron carbonates (siderite) were also found probably due to reaction of iron with the carbonates from calcite or with CO_2 originated from ethanol oxidation. The observed yellowish coating of calcite granules corroborates this hypothesis of Fe precipitation due to neutralisation. Only for bioreactor II, where wine was used as carbon source, molecular sulphur was identified as one of the major components of the precipitates. Elemental sulphur probably results from re-oxidation of biologically generated sulphide. In fact, according to the Pourbaix diagram for sulphur (Pourbaix, 1974), the combination of pH and Eh values in the effluent of this bioreactor (Fig. 4.1) was thermodynamically favourable for the formation of elemental sulphur between days 63 and 149. This also helps to explain incomplete iron removal in this bioreactor, because re-oxidation makes a lower amount of sulphide available for metal precipitation.

Scanning electron micrographs show that, in both cases, precipitates are composed of small flake-like microparticles with variable dimension (Fig. 4.5). EDS spectra presented in the same figure show that the precipitates are mainly composed by iron and sulphur with copper and zinc as secondary elements, which is consistent with precipitation of those metals as sulphides. The semi-quantitative analyses of the EDS spectra show that the amount of iron is two to three times lower in the precipitates from bioreactor II, which is consistent with incomplete precipitation of this metal when using wine waste.

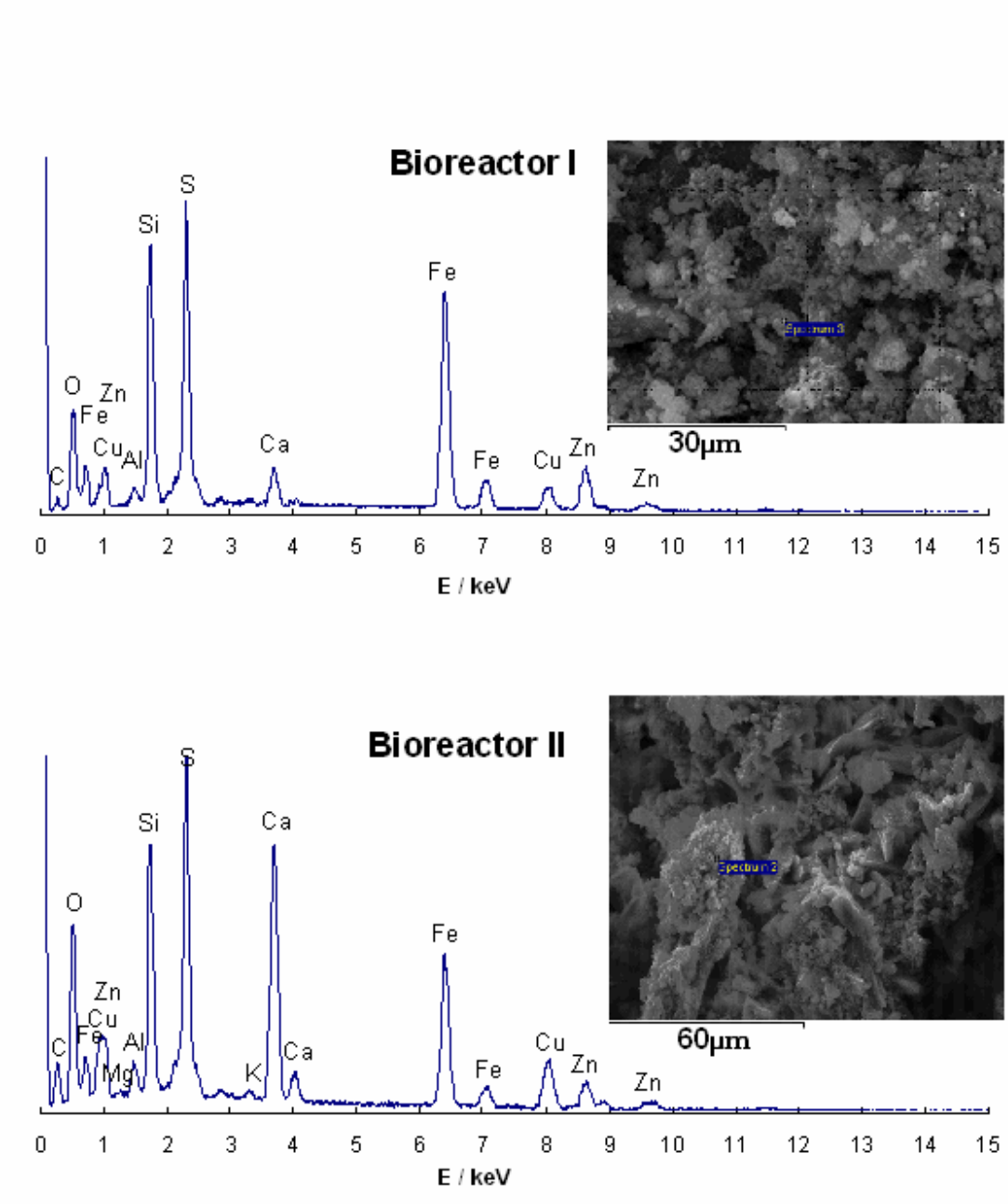

Fig. 4.5 EDS spectra and SEM micrographs of the precipitates from bioreactors I and II.

4. Conclusions

This study shows that wine wastes can be used as carbon source by SRB in the treatment of AMD, since with the addition of 2.0 g/L of ethanol per day in the form of wine wastes these bacteria were able to efficiently remove 2.5 g/L of sulphate. In addition, with exception for iron, the extent of copper and zinc removal was enough to comply with the local legislation for irrigation water. The relatively high concentrations of iron in the effluent can be due to the prevention of iron sulphide precipitation by chelating or anti-coagulant substances present in the wine waste or partial re-oxidation of sulphide to elemental sulphur.

The incorporation of calcite tailing, not only to the top layer, but to all column matrix can eventually contribute to improve and extend process performance. That addition is needed to prevent pH decrease resulting from metabolisation of wine wastes or by the presence of acid substances existing in those wastes. Moreover, the adsorption capacity of the calcite material can also eventually contribute to retain compounds responsible for metals mobilization, thus improving iron removal efficiency.

Use of such a local and abundant waste as carbon source obtained at zero or even negative cost can be a determinant contribution to turn the decontamination of AMD by SRB technologies economically viable.

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Chapter 5

Dynamics of bacterial community in up-flow anaerobic packed bed system for acid mine drainage treatment using wine wastes as carbon source

Abstract

The dynamics of the bacterial populations in an up-flow anaerobic packed bed system (UAPB), applied in acid mine drainage treatment using wine wastes as carbon and nutrients source was elucidated by temperature gradient gel electrophoresis (TGGE) analysis. Moreover, TGGE fingerprints of the bacterial communities developed in a UAPB fed with wine wastes and a UAPB fed with pure ethanol were compared. TGGE fingerprinting and phylogenetic analysis showed that the composition of the community in the UAPB fed with wine wastes remained stable during whole time of operation and its bacterial diversity was higher. The bacterial community of the UAPB fed with wine wastes was composed by bacteria affiliated with *Desulfovibrio*, *Clostridium*, *Citrobacter* and *Cronobacter* genera and with Bacteroidales order, sp. The dominant community developed in the UAPB fed with ethanol was composed by bacteria affiliated with *Desulfovibrio* sp. The presence of several bacterial groups in the bioreactor fed with wine wastes suggests a synergistic interaction between the different populations. Syntrophic interaction may be the key factor for the utilization of wine wastes, a complex organic substrate, as carbon and electron source for sulphate reduction.

Submitted

1. Introduction

Biological treatment of sulphate and metal-containing wastewater using sulphate-reducing bacteria (SRB) is a viable option to conventional chemical treatment due to its lower cost and better sludge properties (Kaksonen *et al.*, 2003).

Generally, the content in carbon sources/electron donors of sulphate and metal-containing wastewater is very low (Johnson, 2000) and an external addition of these compounds is required to achieve sulphate reduction. The performance of the anaerobic bioremediation systems for liquid wastes treatment depends on the appropriate selection of an adapted microbial population and on its direct interactions with the available substrates (Sarti *et al.*, 2010). Therefore, several studies have been performed to find a cost effective carbon source/electron donor suitable for sulphate reduction. Various organic wastes including sewage sludge, animal manure and mushroom compost have already been tested as alternative carbon and electron sources for SRB (Liamleam and Annachhatre, 2007). However, little research work has been done concerning the structure of the microbial community involved in the degradation of those carbon sources.

Previous studies have shown that wine wastes can be used as carbon source by SRB for the biological treatment of acid mine drainage (AMD), indicating that this source is a potential alternative to traditional substrates (Costa *et al.*, 2009). The use of such an abundant waste without expenses in wine production countries, could really contribute to turn economically viable the decontamination of AMD or other metal-sulphate containing wastewaters using SRB based technologies.

In the present study, the dynamics of the bacterial populations involved in a bioremediation process for AMD treatment using wine wastes as carbon source was investigated by temperature gradient gel electrophoresis (TGGE) analysis of 16S rRNA gene. For this purpose two up-flow anaerobic packed bed systems (UAPB) were operated in parallel using two different carbon sources: one UAPB was fed with wine wastes (UAPB I) and the other was fed with ethanol (UAPB II), allowing the comparison of the bacterial community structure developed in each system. Additionally, it was investigated if the modifications introduced to the bioremediation process developed by Costa et *al.* (2009), namely reactor configuration (up-flow instead of down-flow system) and incorporation of calcite tailing in the bioreactor matrix, could improve the performance of the bioremediation process.

2. Materials and methods

2.1. Inoculum

A mixed culture containing SRB was obtained from a previous bioreactor fed with ethanol was used as inoculum (Costa *et al.*, 2009). The bacterial cells (30 mL) were harvested by centrifugation (4000 rpm, 10 min), washed and re-suspend in Postgate B medium (Postgate, 1984) and then used as inoculum.

2.2. Synthetic Acid Mine Drainage

Synthetic AMD, containing 2.5 g L^{-1} of SO_4^{2-}, 550 mg L^{-1} of Fe, 175 mg L^{-1} of Zn and 80 mg L^{-1} of Cu and pH = 2.5 was prepared. Pro-analysis $FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$ and $CuSO_4 \cdot 5H_2O$ were used as metals sources. Na_2SO_4 was used as additional source of sulphate and H_2SO_4 was added to acidify the final solution.

2.3. Calcite tailing and wine wastes composition

The calcite tailing used in the experiments as neutralising and buffer material was collected in a marble stone cutting and polishing industry. The calcite tailing characterization and its usefulness as neutralising agent in SRB based bioremediation processes has been described by Barros *et al.* (2009) and Martins *et al.* (2010). The waste from the red wine industry was collected prior to the bottling stage and presented an ethanol concentration of 53.5 g L^{-1}, and a pH of 3.8-3.9.

2.4. Experimental design

Two up-flow anaerobic packed bed systems (UAPB) were used to study the effect of the carbon source in the bacterial community in a bioreactor developed for the treatment of AMD. The bioremediation systems were carried out in lab-scale and operated in continuous system at room temperature (21ºC±1) for about 190 days. The experiments were performed

using glass reactors. Each UAPB was composed by two column reactors: a calcite tailing column (inner diameter 5.5 cm, height 15 cm) and an up-flow anaerobic packed bed reactor (inner diameter 5.5 cm, height 35 cm). The calcite tailing reactors were packed with a mixture of coarse sand and calcite tailing 2:1 (w/w) and were filled with synthetic AMD (87 mL). The anaerobic bioreactors were packed with 800 g of coarse sand and 30 mL of inoculum (2.0×10^3 CFU). Additionally, 5 g of calcite tailing was incorporated in the column matrix of the bioreactor fed with wine wastes. Each bioreactor was filled with 500 mL of modified Postgate B medium (Postgate, 1984) supplemented with resazurine as redox indicator (0.01g L^{-1}). Both UAPB operated in batch conditions for about 10 days to promote bacterial growth. After this initial time the treatment of synthetic AMD started and the systems were operated with 9 days of hydraulic residence time. One UAPB was fed with wine wastes (corresponding to 2.3 mmol of ethanol added daily) (UAPB I) and the other with ethanol (5.2 mmol of ethanol was added daily) (UAPB II). The amount of carbon source added per day was adjusted considering previous results that showed lower ethanol consumption when wine wastes were used as carbon source instead of pure ethanol (Martins *et al*., 2009). During 107^{th} and 149^{th} days, the double amount of wine wastes (corresponding 4.6 mmol ethanol d^{-1}) was added to the reactor in order to evaluate the impact of the excess of this carbon and nutrients source in the bacterial populations. After 149^{th} day the amount of wine wastes added per day was re-established.

2.5. Analytical methods

Samples from each reactor were weekly collected and filtered through 11 µm filter paper. Redox potential and pH were measured using a pH/Eh Meter (GLP 21, Crison), that make E correction to the standard hydrogen electrode. Sulphate concentration was measured by a UV-VIS spectrophotometer (Hach-Lange DR2800), using the sulfaVer4 method (Hach-Lange). Heavy metals were quantified by flame atomic absorption spectroscopy using a Shimadzu AA-680 model spectrometer.

SRB populations were enumerated by the three-tube Most Probable Number (MPN) assay in modified Postgate E medium (Postgate, 1984) with ethanol as carbon source, using 10 fold dilutions. MPN tubes were incubated at room temperature (21ºC±1) for 5 d.

2.6. Molecular characterization

2.6.1. Extraction of DNA and PCR Amplification of 16S rRNA gene fragments

Total genomic DNA was extracted after harvesting cells by centrifugation at 4000 rpm for 10 min using the method previously described by Martins *et al.* (2009).

Amplification of 16S rRNA gene fragments was performed using the primer pair 341F-GC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') / 534R (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer *et al.*, 1996). The primers were purchased from Thermo Fischer Scientific. The reaction mixture used for PCR amplification contained 31.75 µL of sterilised MiliQ water, 1 µL of each primer (10 pmol μL^{-1}), 1 µL of dNTP`s (10 mM), 4 µL of $MgCl_2$ (25 mM), 10 µL of 5×Go Taq® buffer (Promega, Madison, USA), 0.25 µL of GoTaq®DNA polymerase (Promega, Madison, USA), and 1 µL of DNA. PCR amplification was performed in a thermocycler (T1, Biometra, USA). Thermal cycling was carried out by using an initial denaturation step of 94ºC for 3 min, followed by 35 cycles of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 1 min and completed with an extension period of 3 min at 72ºC. The PCR products were analyzed by electrophoresis, in 1% (w/v) agarose gel and TAE Buffer.

2.6.2. TGGE analysis

PCR products, amplified with primers 341F-GC/534R, were resolved using a TGGE Maxi system (Biometra, USA), as specified by the manufacturer. Aliquots of each PCR product (5 µL) were electrophoresed in a gel containing 6% (w/v) acrylamide/bisacrylamide (39:1), 8 M urea, 2% (v/v) glycerol and 20% (v/v) formamide with a TAE 1X buffer system at a constant voltage of 150 V, for 20 hours, applying a thermal gradient of 42ºC to 53ºC. The gels were silver stained and scanned. Individual TGGE bands were excised from the gels, resuspended in 35 µL of TE 1X (10 mM Tris-HCl, 1mM EDTA) and stored at 4ºC. 3 µL of the supernatant was used for reamplification with the same primer pairs but without GC clamp. PCR products were purified using the Jetquick PCR Purification (Genomed GmbH, Lohner, Germany) and sequenced in CCMAR (Centro de Ciências do Mar, Universidade do Algarve). The sequences obtained in this study have the following accession numbers: HM214916 to HM214923.

2.6.3. Phylogenetic analysis

For phylogenetic analysis, sequence alignments were made with Clustal X (Thompson, 1997) and visually corrected. To estimate phylogenetic relationships the Bayesian Markov chain Monte Carlo (MCMC) method of phylogenetic inference (Huelsenbeck and Ronquist, 2001) was applied MrBayes software (Larget and Simon, 1999). This method allows estimation of the a posteriori probability that groups of taxa are monophyletic given the DNA alignment (i.e., the probability that corresponding bipartitions of the species set are present in the true unrooted tree including the given species). This Bayesian approach was repeated several times, using random starting trees and default starting values for the model parameters to test the reproducibility of the results.

3. Results and discussion

3.1. Performance of the bioremediation systems

Although, 5 to 30% sulphate present in AMD was removed in the calcite tailing columns, the major sulphate removal occurred in the biologic reactors independently from the carbon source added (Fig. 5.1a).

Similarly to a semi-continuous down-flow system used before (Costa et *al.*, 2009), the UAPB fed with wine wastes showed a good performance in sulphate removal. Until day 120 sulphate removal rate was above 90% (Fig. 5.1a). Between day 120 and 160, a decrease in sulphate removal rate was observed, achieving values near 50% in day 160. This result can be explained by the increase to the double of the amount of wine wastes added between days 107 and 149. The wine wastes could contain compounds that in large amounts might affected the SRB activity, or the excess of this carbon and nutrients source promoted changes in the bacterial community affecting its functional activity. The inhibitory effect caused by the increase of wine wastes in the SRB activity was supported by the fact that an increase of sulphate removal was observed again when the amount of the wine wastes added was re-established (Fig. 5.1a).

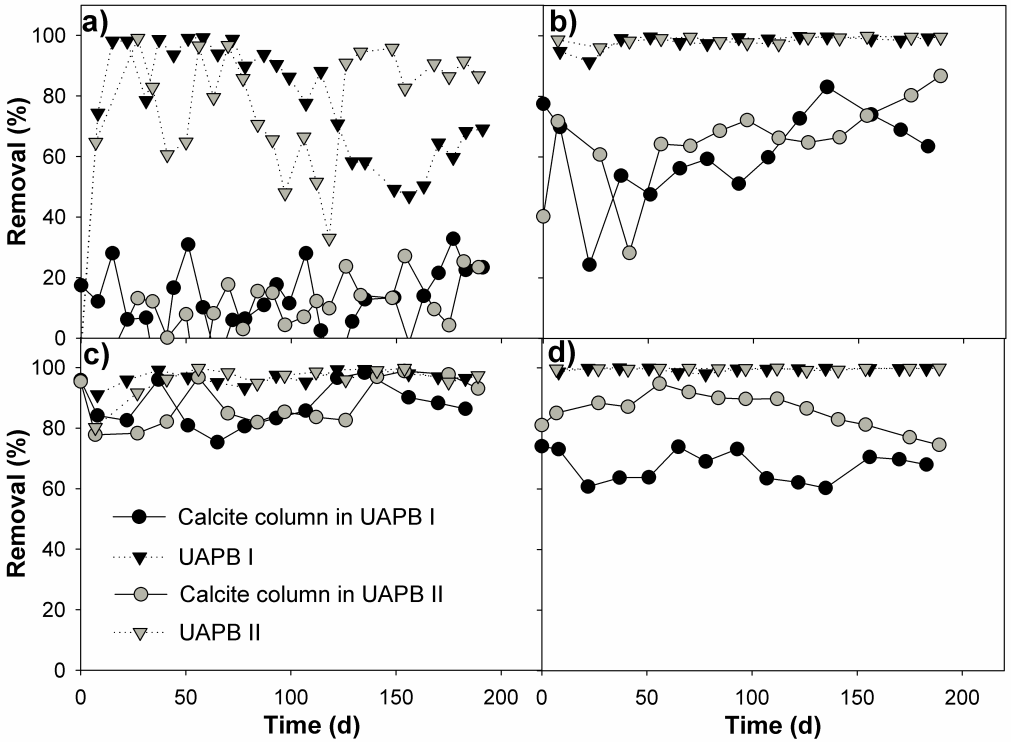

Fig. 5.1 Performance of UAPB I (fed with wine wastes) and II (fed with ethanol) during the operation time: a) sulphate, b) iron, c) copper and d) zinc removal.

The sulphate removal in the UAPB II ranged from 60 to 97% until day 97 (Fig. 5.1a). From this day, sulphate removal rate decreased until day 118, when it was observed the lowest removal rate (33%). This decrease occurred precisely when the pH of the effluent from the bioreactor also decreased. Between days 97 and 118 the pH was around 5.5 (data not shown), becoming non-optimal for SRB growth. However, during all the experiment the neutralization of AMD, from 2.5 to 6.9, was always observed in the calcite tailing column (data not shown). During seven days (day 112 to 118) 50 mL per day of Postgate B medium (Postgate, 1984) was added to the bioreactor in order to improve the SRB growth and recover the bioreactor activity. The effect of this action was observed after day 126: the pH in the bioreactor increased again for values around 6.5 and the normal function of the system was established. Sulphate removal efficiency was maintained above 85% from day 120 until the end of the experiment.

The greater amount of metals present in AMD was removed in the calcite tailing columns (Fig. 5.1b, c and d). The potential of calcite tailing for metals decontamination was already

reported (Barros *et al.*, 2009; Martins *et al.*, 2010). The removal of metal ions by the calcite tailing is due to pH increase, promoting metal precipitation (Gilbert *et al.*, 2005). In addition, the metals can also be adsorbed by the calcite material, which presents a high surface area (Barros *et al.*, 2009). High metal removal rates were achieved in both UAPB: over than 99.5% for all the three metals. The efficiency of iron removal in UAPB I was higher than reported by Costa *et al.* (2009), who described a maximum iron removal rate of 91%. This difference can be due to calcite tailing incorporation in the bioreactor matrix and to the type of system used: in the present study an up-flow system was applied, while Costa *et al*. (2009) used a down-flow system. Eventual iron-chelating compounds present in wine wastes, which prevent the formation of iron sulphide, could be adsorbed by the calcite tailing, therefore improving the iron removal rate. The iron-chelating properties of phenolic acids, usually present in wine, are documented (Andjelkovic *et al.*, 2006). Moreover, iron can also be adsorbed by calcite tailing.

These system modifications could contribute for its improvement and consequently for the development of a more efficient process for AMD decontamination, based in SRB activity and using wine wastes as carbon source.

3.2. Enumeration of SRB in the bioremediation systems

The number of cells estimated in UAPB I was always higher that in UAPB II during all the treatment, although both bioreactors were inoculated with the same number of SRB cells (Fig. 5.2). This difference ranged from 2-3 logs during the experiment. This result can be explained by the presence of compounds in wine wastes that can be used by SRB as alternative carbon sources. In fact, significant amounts of organic compounds, such as carbohydrates, organic acids and aminoacids are present in wine and probably in wine wastes. Furthermore, these compounds can also function as growth factors stimulating bacterial growth.

The estimation of SRB cells number also showed that the increase of the amount of wine wastes added per day to the reactor affected the sulphate reduction activity, but not the SRB growth (Fig. 5.2). Considering UAPB II, between the 95 and 124 days the number of SRB decreased, achieving values of 7×10^{1} CFU mL^{-1}. This reduction may explain the decrease of sulphate removal observed in the same period of time (Fig. 5.1a). The pH drop observed

between days 97 and 118 (data not shown) possibly promoted the decrease of the number of SRB in the bioreactor, causing the decrease of sulphate removal.

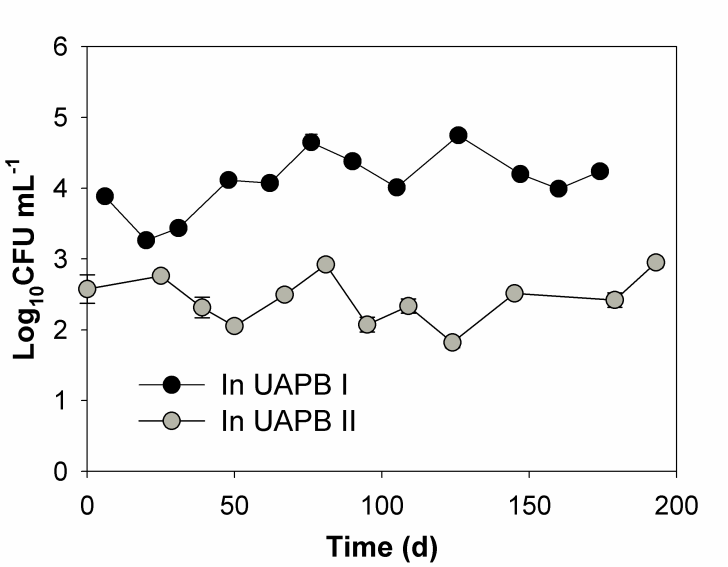

Fig. 5.2 Sulphate reducing bacteria number in UAPB I (fed with wine wastes) and II (fed with ethanol) during operation time. Data are the average of triplicates and error bars indicate the standard deviations of the average values.

3.3. 16S rRNA gene TGGE fingerprinting and phylogenetic analysis

Samples from UAPB I and II were taken at different times during the AMD treatment for TGGE fingerprinting of bacterial communities (Fig. 5.3). TGGE analysis revealed a low genetic diversity in the initial culture (Fig. 5.3, lane 0). The corresponding profile was composed by a predominant band (B1) and by other two bands presenting a lower intensity (B2 and B8). The low diversity observed in this initial community can probably be explained by the fact that the inoculum used in the present study was obtained from a previous bioremediation process used for AMD treatment (Costa *et al*., 2009). Since high degradation rates frequently generate communities with simple structure (Balcke *et al*., 2004; Lin *et al*., 2007), sulphate and metals present in the AMD probably promoted the growth of a few resistant species, selecting them as dominant populations.

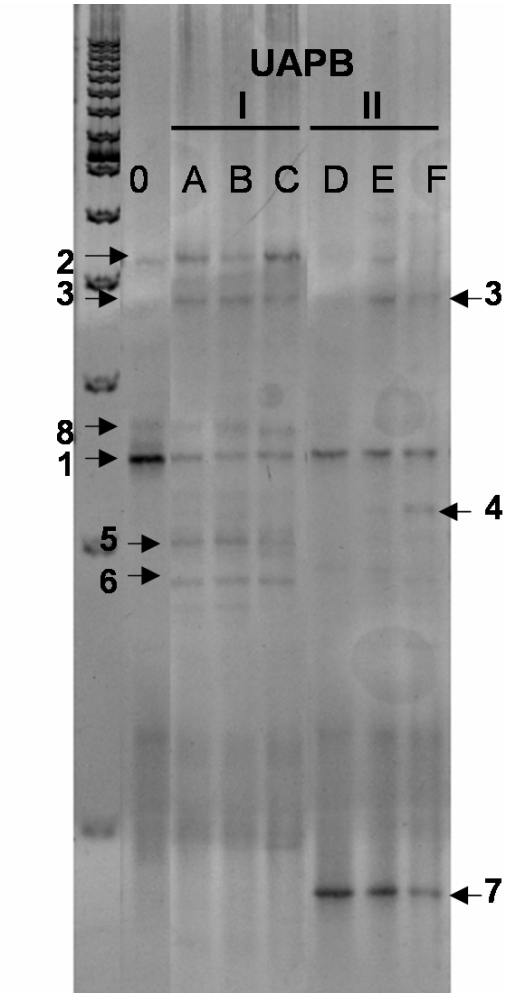

Fig. 5.3 16S rRNA gene TGGE profiles of bacteria community present in UAPB I (initial culture (0), after 80 days (A), 120 days (B) and 153 days (C) operation time) and in UAPB II (initial culture (0), 100 days (D), 140 days (E) and 170 days (F) operation time).

TGGE bands were re-amplified and sequenced in order to determine the composition of the microbial communities in UAPB I and II. Sequences were used for phylogenetic analysis in order to obtain a more accurate identification of bacteria present in each consortium (Fig. 5.4). The 16S rRNA gene sequence corresponding to the B1 was closely related to the genus *Desulfovibrio*. Sequence from Band 2 showed homology with members of the order Bacteroidales, even thought no sequences from cultured strains could be assigned to the bacteria represented by this band. The order Bacteroidales is composed by anaerobic, fermentative Gram-negative species (Dann *et al.*, 2009). Bacteria affiliated to Bacteroidales were already found in an artificial wetland system developed for the treatment of acidic,

iron and sulphate rich waste water derived from titanium mineral processing (Dann *et al.*, 2009). Band 8 was affiliated with Enterobacteriaceae family. Its closest relatives were from *Citrobacter* and *Cronobacter* genera. *Cronobacter* genus comprises Gram-negative, facultative anaerobic bacteria with ability to produce acids from several substrates such as glucose, sucrose and maltose (Iversen *et al*., 2007). Bacteria belonging to this genus do not produce hydrogen sulphide, in contrast with some strains of *Citrobacter* that have this ability (Iversen *et al*., 2007). The production of lactate, acetate and ethanol as by-products of glucose fermentation by *Citrobacter* sp. was reported (Hamilton *et al*., 2010). Considering that these products are suitable to support SRB growth as carbon and electron sources, the presence of these bacterial groups in the consortia is not unexpected. Furthermore, the potential of *Citrobacter* sp. for bioremediation of waste water contaminated with dyes was previously mentioned by Wang *et al*. (2009). Recently copper resistant *Citrobacter* specie with ability for sulphate reduction was also described (Qiu *et al*., 2009).

Although the initial community have changed in response to the UAPB I conditions, the corresponding TGGE fingerprints showed that the structure of the bacterial community stabilized during the first 80 days of operation and remained fairly constant during the remaining treatment (Fig. 5.3, lanes A, B and C). This observation is in accordance with the results reported for other bioremediation processes, in which has been observed the establishment of dominant populations and their maintenance after an initial period of adaptation to the environmental conditions (Gómez-Villalba *et al*., 2006; Qu *et al.*, 2009). The community structure of the bioreactor was maintained even when the amount of wine wastes was doubled. Therefore, it is possible to infer that this change only affected the SRB activity but not the community structure. Thus, the decrease of this activity can be due to an inhibitory effect in sulphate reduction resulting from high concentration of some compounds in the wine wastes.

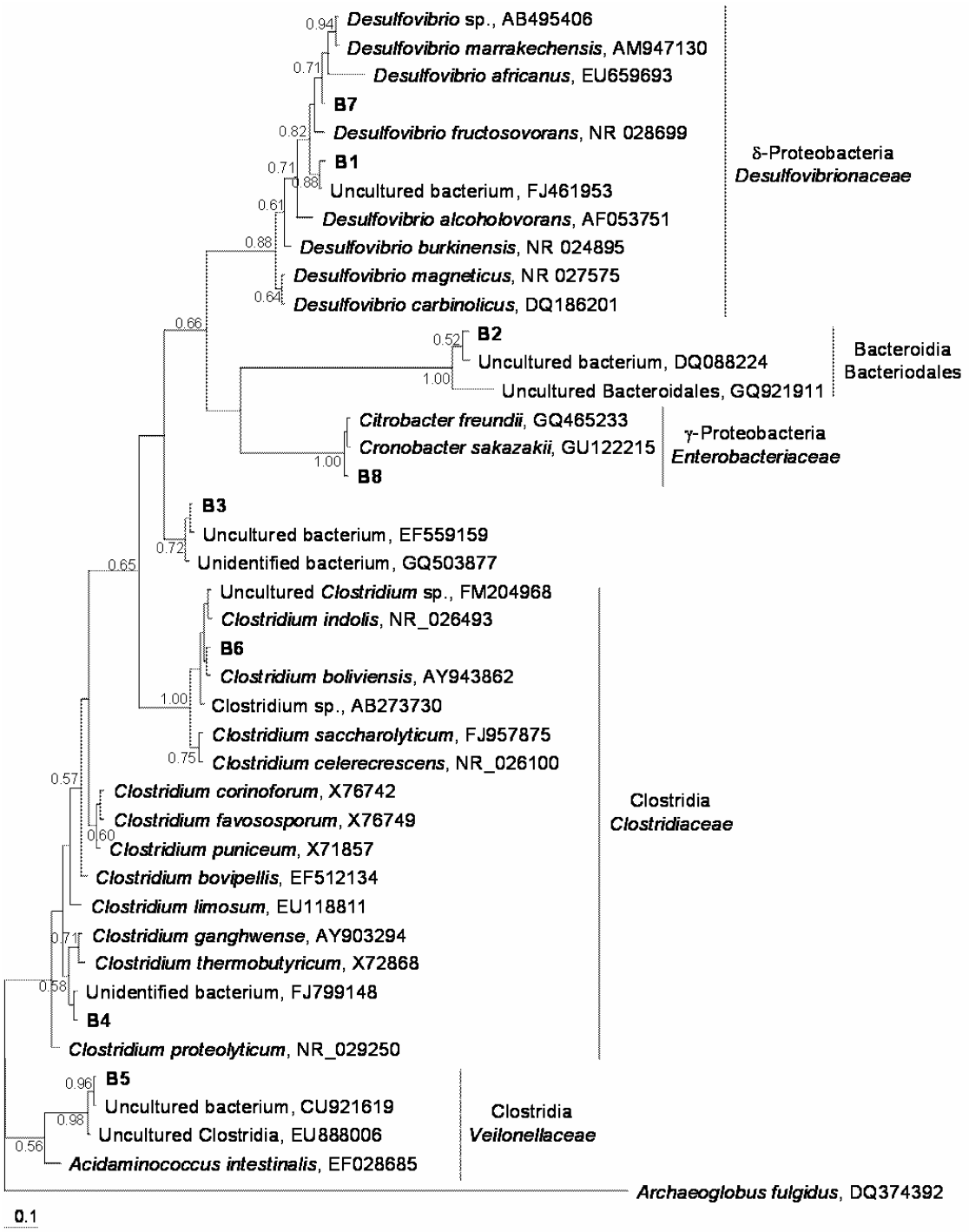

Fig. 5.4 Phylogenetic tree obtained with 16S rRNA partial sequences (196 nucleotide positions), corresponding to the reamplified TGGE bands and to the most closely related ones retrieved from BLAST search. Phylogeny was inferred using the Bayesian Markov Chain Monte Carlo analysis of aligned 16S rRNA fragments. *Archaeoglobus fulgidus*, a species from Archaea Domain was included to root the tree. Probability values associate to each node are showed. Access numbers of GeneBank sequences are indicated in the figure and names in bold face correspond to sequences determined in this work. The main bacterial groups detected in bacterial consortia are also indicated.

UAPB I TGGE profile was composed by the 3 bands present in the initial culture (B1, B2 and B8) and by three additional bands (B3, B5 and B6). Phylogenetic analysis of the corresponding sequences showed that the sequence of B3 was affiliated with an unidentified bacterium, but B5 and B6 were closely related to the genus *Clostridium*. This genus is composed by Gram-positive and spore-formed anaerobic bacteria, which are widely distributed in environment (Stackebrandt and Rainey, 1997; Bruggemann and Gottschalk, 2009). *Clostridium* species are unable to accomplish dissimilatory sulphate reduction and may present diverse morphologies, metabolic properties and nutritional requirements (Stackebrandt and Rainey, 1997). Although they are unable to perform dissimilatory sulphate reduction, some species can produce sulphide by sulphite reduction.

The co-existence of *Desulfovibrio* sp. and *Clostridium* sp. in bioremediation processes have been reported before (Boonchayaanant *et al.*, 2008). *Clostridium* spp. have been considered as playing an important role in molasses and glucose fermentation, cooperating with SRB for sulphate reduction (Bruggemann and Gottschalk, 2009).

TGGE fingerprinting analysis suggests that during the fist 100 days of system operation a core bacterial community was developed in UAPB II (Fig. 5.3, lane D). The community was only composed by bacterial species corresponding to two bands: B1, already present in the initial inoculum, and a new intense band (B7). Both were closely related to *Desulfovibrio* genus (Fig. 5.4). B7 was not observed in UAPB I, suggesting that the growth of this *Desulfovibrio* species was favoured under UAPB II operating conditions. In the subsequent 40 days (Fig. 5.3, lane E), a bacterial species affiliated with order Bacteroidales, already present in the initial culture (B2), was detected and a new band was also observed (B3). This band corresponded to an unidentified bacterium that was also present in UAPB I. These two bacteria were only detected in UAPB II after addition of Postgate B medium for system recovering. This change in the bacterial community profile could be caused by this additional source of nutrients. This hypothesis is reinforced by the observation that after the system recovery B2 was not detected and B3 was less intense (Fig. 5.3, lane F), probably due to the change in nutrients availability. At the end of AMD treatment (Fig. 5.3, lane F) the intensity of B7 decreased and a new weak band (B4) became visible. Phylogenetic analysis of B4 sequence revealed its affiliation with genus *Clostridium* (Fig. 5.4). The TGGE results of UAPB II suggest a dynamic structure of populations, although a core community is maintained all along the experiment.

Although both bioreactors were inoculated with the same bacterial community, TGGE fingerprints revealed clear differences between the bacterial consortia in UAPB I and II (Fig. 5.3). Since the two reactors were operated in similar conditions, with exception for the carbon and nutrients source added, the differences should be a consequence of the carbon and nutrients source fed to the system. Geets *et al*. (2005) and Zhao *et al*. (2010) have already reported that different carbon sources enriched different bacterial communities. The present results also suggest that the type of carbon source modulated the bacterial community responsible for AMD treatment.

TGGE fingerprinting and phylogenetic analysis showed that the bacterial diversity was higher in the UAPB I than that in UAPB II. The bacterial community developed with wine wastes (UAPB I) was composed by bacteria affiliated with *Desulfovibrio, Clostridium*, *Citrobacter* and *Cronobacter* genera and with Bacteroidales order. Although a more dynamics structure was observed for the community developed with ethanol (UAPB II), the core consortium was only composed by *Desulfovibrio* sp. Some of the 16S rRNA sequences of TGGE bands were related with sequences from uncultured bacteria with no identified close relative. This observation indicates that some microorganisms established during the bioremediation process are still unexplored.

The higher diversity observed for UAPB I community might be due to the high variety of available compounds in the wine wastes that can represent alternative carbon sources, nutrients and growth factors, stimulating the growth of different species. The presence of several bacterial groups in UAPB I suggests a synergistic interaction between SRB and fermentative bacteria. SRB are generally unable to use complex substrates as electron donors for sulphate reduction (Liamleam and Annachhatre, 2007). Usually these substrates are first converted to simple molecules by fermentative bacteria and then used by SRB (Liamleam and Annachhatre, 2007). The syntrophic relationships established between various functional groups allow the degradation of complexes molecules and the use the corresponding degradation products by SRB (Zhao *et al.*, 2010). The co-existence of SRB and fermentative bacteria may be the key factor for the utilization of wine wastes, a complex organic substrate, as carbon and nutrients source for sulphate reduction.

4. Conclusions

The present study demonstrated that the type of carbon source (wine wastes or ethanol) modulated the bacterial community responsible for the AMD bioremediation process based in the SRB activity. TGGE fingerprinting and phylogenetic analysis showed that the bacterial diversity was higher in the bioreactor fed with wine wastes than in the bioreactor fed with ethanol. The presence of SRB and fermentative bacteria (*Clostridium* sp., Bacteroidales order, *Citrobacter* sp. and *Cronobacter* sp.) in the bioreactor fed with wine wastes suggests a synergistic interaction between these bacterial groups, which can be the key factor for the use of such complex organic substrate, as carbon and electron source for sulphate reduction. In addition, the modification of the bioreactor configuration used in the bioremediation process (up-flow instead of down-flow system) and the incorporation of calcite tailing in the bioreactor matrix, contributed for the improvement of the system. The evaluation of the performance of the treatment together with the monitoring of the dynamics of the bacterial community is relevant topic for a better understanding the bioremediation process.

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Chapter 6

Effect of uranium (VI) on two sulphate-reducing bacteria cultures from a uranium mine site

Abstract

This work was conducted to assess the impact of uranium (VI) on sulphate-reducing bacteria (SRB) communities obtained from environmental samples collected on the Portuguese uranium mining area of Urgeiriça. Culture U was obtained from a sediment, while culture W was obtained from sludge from the wetland of that mine. Temperature gradient gel electrophoresis (TGGE) was used to monitor community changes under uranium stress conditions. TGGE profiles of *dsr*B gene fragment demonstrated that the initial cultures were composed of SRB species affiliated with *Desulfovibrio desulfuricans*, *Desulfovibrio vulgaris* and *Desulfomicrobium* spp. (sample U), and by species related to *Desulfovibrio desulfuricans* (sample W). A drastic change in SRB communities was observed as a result of uranium (VI) exposure. Surprisingly, SRB were not detected in the uranium removal communities. Such findings emphasize the need of monitoring the dominant populations during bio-removal studies. TGGE and phylogenetic analysis of the 16S rRNA gene fragment revealed that the uranium removal consortia are composed by strains affiliated to *Clostridium* genus, *Caulobacteraceae* and *Rhodocyclaceae* families. Therefore, these communities can be attractive candidates for environmental biotechnological applications associated to uranium removal.

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1. Introduction

Surface waters and groundwater may have undesirably high concentrations of dissolved uranium as the product of natural processes, from contamination resulting from uranium mining and processing activities or from the release of nuclear materials to the environment (Gorby and Lovley, 1992**).** Thus, the removal of uranium from aqueous solutions, especially from contaminated sources, is an important topic for the control of environmental pollution, and techniques for the removal of dissolved uranium from waters are needed for several environmental applications.

Given the high costs and the technical limitations of conventional metal remediation techniques, currently based on chemical approaches (Lloyd *et al*., 2003), there has been an unprecedented interest in the interactions of microorganisms with key radionuclides, e.g. uranium, in the hope of developing cost-effective bioremediation approaches for decontamination of waters, effluents and sediments impacted by nuclear waste (Lloyd *et al*., 2003). In this context, bioremediation is nowadays considered a potential alternative and an economically attractive strategy that offers several advantages over the traditional techniques, like low operative costs and high efficiency in detoxifying very dilute effluents.

Taking into account the diversity and the high abundance of microorganisms in the natural environment it is of high importance to identify and characterize microbial strains or communities with high metal resistance and ability for metal removal by different mechanisms (Kazy *et al*., 2009). Moreover, understanding and exploring the potential of microbe-metal interaction have gained recent interest due to their importance in various biotechnological applications like biosensors, biofuel, and most promisingly in microbe mediated nanomaterials synthesis (Mandal *et al*., 2006; Wu *et al*., 2008).

During the last two decades, many researchers have discovered that different groups of microorganisms, such as Actinomycetes (Gorab *et al*., 1991; Tsuruta, 2002) and other bacteria (Lovley *et al*., 1991; Tsuruta, 2002; Wall and Krumholz, 2006), yeasts (Strandberg, 1981; Tsuruta, 2002) and fungi (Galun *et al*., 1983; White and Gadds, 1990; Tsuruta, 2002), have the ability to remove uranium from aqueous media.

Since the pioneer work of Lovley and co-workers in the early ninety's (Lovley *et al*., 1991; Lovley and Phillips, 1992), a number of bacterial species including *Geobacter* spp. (Anderson *et al*., 2003), *Shewanella putrefaciens* (Lovley *et al*., 1991), and *Clostridium* sp.

(Francis *et al*., 1994) have been described for their ability to reduce uranium U(VI). The capacity to enzymatically reduce U(VI) has been demonstrated for sulphate-reducing bacteria (SRB) (Lovley and Phillips, 1992; Lovley *et al*., 1993b; Pietzsch *et al*., 1999). Upon reduction, the highly soluble and mobile U(VI) is converted to insoluble U(IV), which then precipitates from aqueous solutions. Using this mechanism, SRB are thought to have a high bioremediation potential in uranium-contaminated waters and effluents (Spear *et al*., 2000; Wall and Krumholz, 2006).

Although many studies have already been performed to investigate uranium removal by bacteria, most work has been conducted with pure cultures despite the widely demonstrated advantages of employing mixed cultures as opposed to pure cultures in bioremediation applications (White and Gadds, 1996; Mukred *et al.,* 2008). Those advantages over pure cultures include greater stability and increased metabolic capabilities, which can be linked to the effects of synergistic interactions among members of the association (Mukred *et al*., 2008). These characteristics enable the consortium to overcome limitations for the complete metabolization of toxic compounds (Davidson *et al.,* 1994). In addition, the majority of the studies that used mixed cultures for metal bioremediation do not take into account the possible shift in the bacterial community structure when metal ions are introduced to the media. In fact the dynamics of microbial populations capable of uranium removal are poorly understood and little is known about the modifications in the community structure and composition in response to changes in growth conditions, such as addition of metal ions. Thus, besides the assessment of the efficiency of uranium bio-removal and its relationship with microbial groups, the objectives of the present work included the analysis of community structural shifts in sulphate-reducing bacteria enrichment consortia, associated with the presence of uranium. For those purposes, temperature gradient gel electrophoresis (TGGE) was used since it is a powerful tool to characterize microbial communities and to monitor the spatial and temporal evolution dominant population (Etscheid and Riesner, 1998; Muyzer and Smalla, 1998).

2. Materials and methods

2.1 Enrichment of sulphate-reducing bacteria

The bacterial communities used in these experiments were obtained from sediments from the mining area of Urgeiriça (sample U) and sludge from the wetland of Urgeiriça mine (sample W). Urgeiriça mine, located in the north Portugal, was the most important uranium exploitation of this country.

Bacteria were cultured under anaerobic conditions at room temperature (21 ± 1ºC) using 120 mL glass bottles. The anaerobic conditions were achieved by purging the medium with nitrogen gas and by addition of 10 mL of sterile liquid paraffin.

The first SRB enrichment was carried out by addition of 5 g of each sample to 100 mL of Postgate B medium (Postgate, 1984) supplemented with resazurin as redox indicator (0.03 g L^{-1}). Subsequently, the bacterial cultures were grown and maintained in modified MTM medium (Sani *et al*., 2001), which contains 1 g L^{-1} NH_4Cl, 0.06 g L^{-1} $CaCl_2.6H_2O$, 0.05 g L^{-1} yeast extract, 1 g L^{-1} $MgSO_4.7H_2O$, 2 g L^{-1} Na_2SO_4 and 5 g L^{-1} sodium lactate. The culture was sub-cultured every 3 weeks using 10% (v/v) SRB inoculum. The growth of SRB was monitored by weekly determination of pH, Eh and sulphate concentration.

2.2. Uranium (VI) bio-removal experiments

The studies of U (VI) bio-removal were performed in batch under anaerobic conditions, using the growth MTM medium previously described, supplemented with uranium (VI) as uranyl acetate dihydrate. The bio-removal experiments are represented in the Fig. 6.1. For each experimental set an abiotic control was carried out simultaneously. For the biotic experiments, bacterial cells obtained previously were harvested by centrifugation at 4000 rpm for 10 min, washed with MTM medium and transferred to the bottles. All experiments were performed in duplicate using 120 mL glass bottles containing 100 mL of growth medium with pH~7 and 10% (v/v) of inoculum. The medium was purged with nitrogen gas to achieve an anaerobic environment prior to inoculation. After inoculation, oxygen diffusion was eliminated by adding 10 mL of sterile liquid paraffin. The bottles were sealed

with butyl rubber stoppers and aluminium crimp seals and incubated at room temperature (21 ± 1ºC). The abiotic control sets were prepared in the same way as the biotic tests, but without inoculum addition. The cultures were sub-cultured every 4 weeks. Samples of bacterial cultures were collected and frozen at -20ºC for nucleic acids extraction. Bacterial culture samples collected on day 21 of incubation were used for SRB enumeration. SRB were enumerated by the three-tube most-probable number (MPN) technique with 10-fold serial dilutions in Postgate E medium (Postgate, 1984). The experiment was performed in triplicate and the MPN tubes were incubated at room temperature for 5 days.

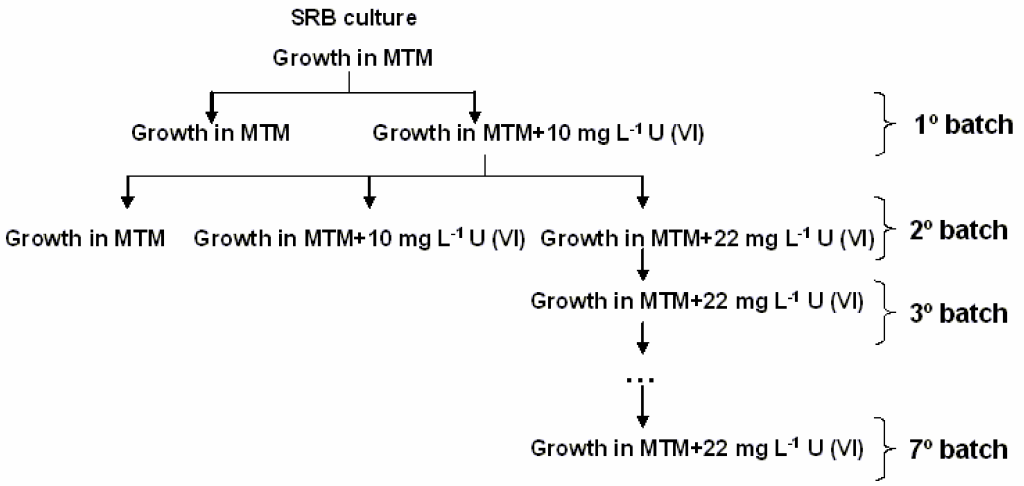

Fig. 6.1 Schematic representation of bio-removal experiments.

2.3. Analytical Methods

Periodically, 5 mL samples were collected using a syringe and centrifuged at 4000 rpm for 5 min. Redox potencial and pH were determined using a pH/E Meter (GLP 21, Crison). Sulphate concentration was quantified by UV/visible spectrophotometry at 450 nm (Hach-Lange DR2800 spectrometer) using the method of sulfaVer4 (Hach-Lange, Dusseldorf, Germany). Uranium was measured using the Arsenazo III (1,8-dihydroxynaphthalene3,6-disulphonic acid- 2,7-bisSTA(azo-2)-phenylarsonic acid) reagent (Sawin, 1961). Dissolved uranium was determined by mixing of 900 µL of sample with 300 µL of 0.5 M HCl, followed by the addition of 300 µL of Arsenazo III (0.1 % w/v). After 3 min, the purple-colour metal-arsenazo III complex was quantified spectrophotometrically at 652 nm. The

Arsenazo III solution was prepared by dissolving the reagent in 0.01 M HCl and in 10 % (v/v) ethanol.

2.4. Molecular Characterization

2.4.1. Extraction of DNA

Total genomic DNA was extracted after harvesting cells by centrifugation at 4000 rpm for 10 min. DNA extraction was carried out by the method described by Martins and co-authors (Martins *et al*., 2009). Briefly, total genomic DNA was extracted using the 300 µL of sodium dodecyl sulphate (SDS) lysis mixture (500mM TrisHCl pH 8, 3% (w/v) SDS, 100mM NaCl) and 300 µL of phosphate buffer pH 8, followed by a freeze-thaw treatment. After precipitation with isopropanol, DNA was re-suspended in 35 µl miliQ H_2O.

2.4.2. PCR Amplification of *dsrB* and 16S rRNA gene fragments

Amplification of 16S rRNA and *dsrB* gene (encoding the β-subunit of dissimilatory sulphite reductase) fragments was performed using the primer pairs 341F-GC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA
CGG GGG GCC TAC GGG AGG CAG CAG-3')/ 534R (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer *et al*., 1996) and DSRp2060F-GC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G CAA CAT CGT YCA YAC CCA GGG-3')/ DSR4R (5'-GTG TAG CAG TTA CCG CA-3') (Geets *et al*., 2006), respectively. The primers were purchased from Thermo Fischer Scientific. The reaction mixture used for PCR amplification contained 30.75 µL and 31.75 µL of sterilised MiliQ water for *dsrB* and 16S gene respectively, 1 µL of each primer (10 pmol/µL), 1 µL of dNTP`s (10 mM), 5 µL and 4 µL of $MgCl_2$ (25 mM) for *dsrB* and 16S gene respectively, 10 µL of 5×Go Taq® buffer (Promega, Madison, USA), 0.25 µL of GoTaq®DNA polymerase (Promega, Madison, USA), and 1 µL of DNA. PCR amplification was carried out in a thermocycler (T1, Biometra, USA). Thermal cycling was carried out by using an initial denaturation step of 94ºC for 3 min, followed by 35 cycles of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 1 min and completed with an extension period of 3 min at 72ºC. The PCR products were analyzed by electrophoresis, in 1% (w/v) agarose gel and TAE Buffer.

2.4.3. TGGE Analysis

PCR products, amplified with primers 341F-GC/534R and DSRp2060F-GC/DSR4R, were resolved using a TGGE Maxi system (Biometra, USA), as specified by the manufacturer. Aliquots of each PCR product (5 µL) were electrophoresed in a gel containing 6% (w/v) acrylamide/bisacrylamide (39:1), 8 M urea, 2% (v/v) Glycerol and 20% (v/v) formamide with a TAE 1X buffer system at a constant voltage of 150 V, for 20 hours, applying a thermal gradient of 42ºC to 53ºC. The gels were silver stained and scanned. Individual TGGE bands were excised from the gels, re-suspended in 35 µL of Tris-EDTA 1X (10 mM Tris-HCl, 1mM EDTA) and stored at 4ºC. Three µL of the supernatant was used for re-amplification with the same primer pairs but without GC clamp. PCR products were purified using the Jetquick PCR Purification (Genomed GmbH, Lohner, Germany) and sequenced by CCMAR (Centro de Ciências do Mar, Universidade do Algarve). Sequences obtained in this study have the following accession numbers: GQ404433 to GQ404447 for 16S rRNA gene and GQ404448 to GQ404464 for *dsrB*.

2.4.4. Phylogenetic analysis

For phylogenetic analysis, sequence alignments were made with Clustal X (Thompson, 1997) and visually corrected. To estimate phylogenetic relationships the Bayesian Markov chain Monte Carlo (MCMC) method of phylogenetic inference (Huelsenbeck and Ronquist, 2001) was applied as implemented in the computer program MrBayes (Larget and Simon, 1999). This method allows estimation of the a posteriori probability that groups of taxa are monophyletic given the DNA alignment (i.e., the probability that corresponding bipartitions of the species set are present in the true unrooted tree including the given species). This Bayesian approach was repeated several times, using random starting trees and default starting values for the model parameters to test the reproducibility of the results.

2.5. Statistical Analysis

The data were subject to analysis of variance (ANOVA) by SigmaStat 3.0 program. All differences were considered to be statistically significant for $P < 0.05$.

3. Results

The SRB cultures obtained from sediments from the mining area of Urgeiriça (U) and from sludge from the wetland of the same mine (W) were able to reduce more than 97% of sulphate in 14 days of incubation.

3.1. Uranium (VI) bio-removal

The performance of U and W bacterial consortia for uranium and sulphate removal during the sequential batch tests is shown in Fig. 6.2. In the initial batch with 10 mg L^{-1} of uranium (Fig. 6.2a), after 7 days of adaptation phase, uranium and sulphate were removed simultaneously by the bacterial community U. 89% of uranium and 92% of sulphate were removed in 25 days. Bacterial community W removed only uranium and 88% was removed in 17 days during the first batch. In the end of first batch with uranium the number of SRB in both cultures was 1.4×10^5 CFU mL^{-1}. In the batch without uranium the number of SRB in culture U was 2.0×10^5 CFU mL^{-1}, while in culture W was 3.9×10^5 CFU mL^{-1}.

In the second batch with ~10 mg L^{-1} uranium (Fig. 6.2b) it was observed that both consortia maintained the ability for uranium removal and presented a similar performance. However, it should be emphasized that bacterial community U lost the ability to remove sulphate, since no significant reduction of this anion was observed. The same result (no sulphate removal) was achieved when the medium contained only sulphate, without uranium and the behaviour of both communities was similar in the subsequent batch (data not shown). In all theses batch SRB were not detected by the MPN technique.

Figure 6.2c) shows uranium and sulphate removal when the concentration of uranium in the medium was increased to 22 mg L^{-1}. It was observed that uranium concentration was decreased to 7 mg L^{-1} within 20 days, although the lag time was increased. Sulphate reduction was not observed in both consortia. SRB were also not detected by the MPN technique, in these experiments.

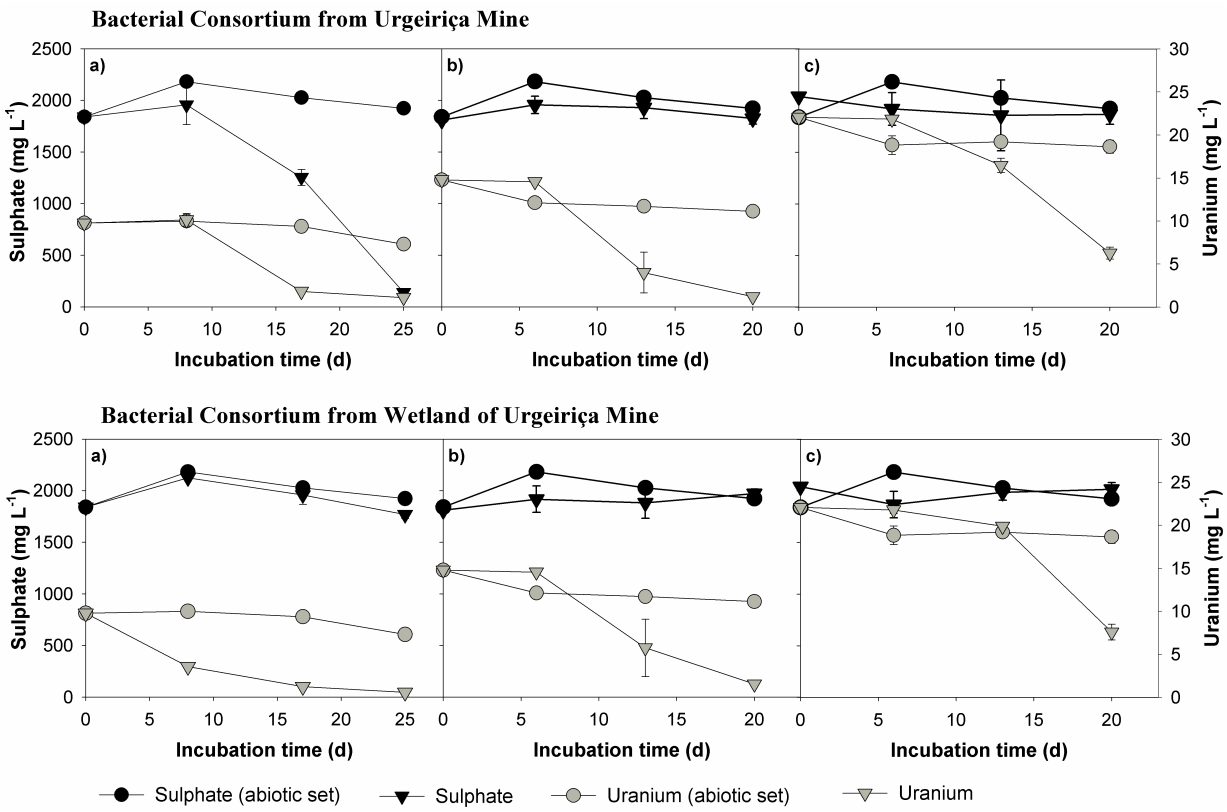

Fig. 6.2 Profile of sulphate and uranium removal by U and W bacterial consortia in sequential batch test: a) first batch with 10 mg L^{-1} uranium, b) second batch with 10 mg L^{-1} uranium and c) second batch with 22 mg L^{-1} uranium. Data are the average of duplicates and error bars indicate the standard deviations of the average values.

3.2. TGGE analysis of 16S rRNA gene during sequential batch tests

TGGE fingerprints based on 16S rRNA gene revealed clear changes in the original bacterial communities during the sequential incubations (Fig. 6.3a). In the initial profiles two predominant bands were observed in each community: bands B20 and B13 in community U (Fig. 6.3a), lane 0) and B29 and B13 in community W (Fig. 6.3a), lane 0). However, TGGE patterns showed that both bacterial consortia were modified after uranium addition and presented the same community structure: bands B20 and B29 disappeared and new bands, common to both consortia, were observed (B7, B10, B11 and B15) (Fig. 6.3a), lane I).

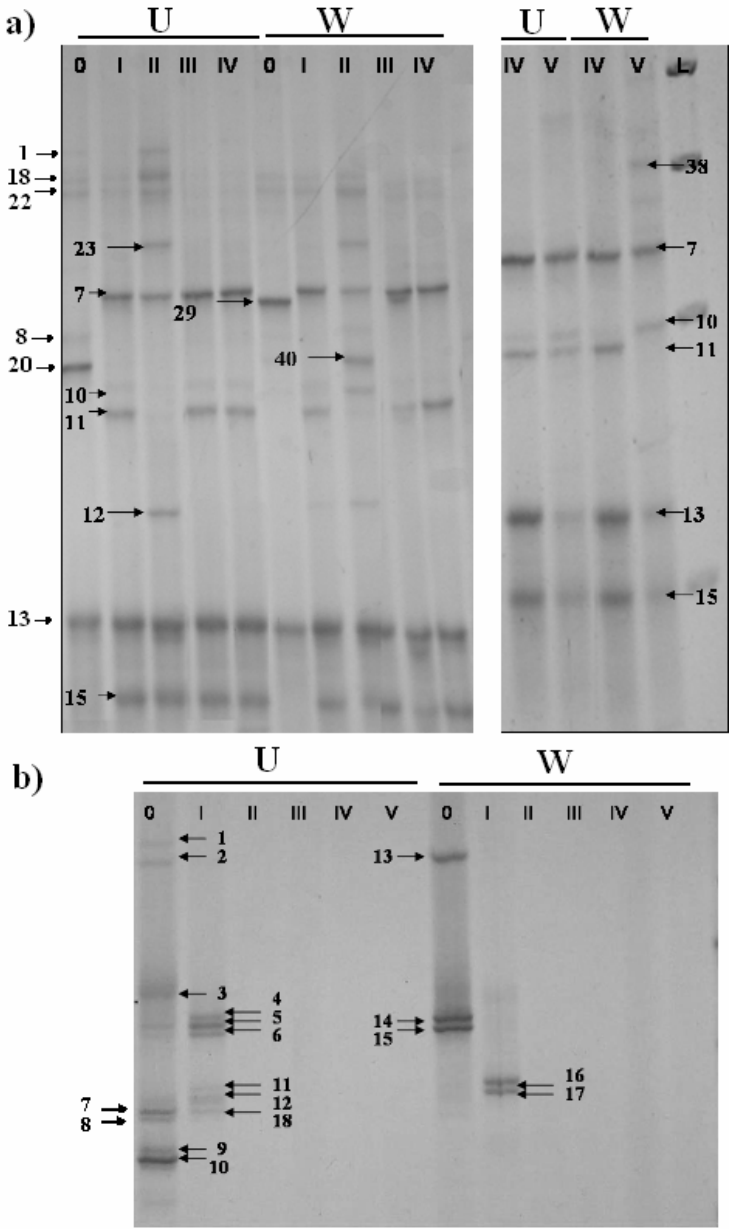

Fig. 6.3 TGGE profiles of 16S rRNA (a) and *dsrB* (b) gene fragments using DNA of U and W communities from batch experiments: beginning (0), 1º batch with 10 mg L^{-1} uranium + sulphate (I), 2º batch with sulphate (II), 2º batch with 10 mg L^{-1} uranium + sulphate (III), 2º batch with 22 mg L^{-1} uranium + sulphate (IV) and 7º batch with 22 mg L^{-1} uranium + sulphate (V).

Removal of uranium from the culture media led again to a community shift, but did not re-establish the initial consortia. Light bands in the original enrichment, such as B1, B18 and

B22 in community U and B40 and B22 in W, showed more intensity in the batch containing only sulphate and two new intense bands (B12 and B23) appeared in both consortia (Fig. 6.3a), lanes 0 and II). Moreover, band B11 was only observed in the presence of the metal. On the other hand, microorganisms corresponding to bands B1, B12, B23 and B40 were inhibited when uranium was added to the medium. B13 is the only band that was present in initial enrichment and maintained in all sequential mixed cultures. Inspection of TGGE fingerprints also revealed that increase of uranium concentration did not have any impact on the community structure and the corresponding profiles were maintained mainly stable after seven consecutive incubations with 22 mg L^{-1} of this heavy metal (Fig. 6.3a), lane IV and V). The exception was the emergence of one additional band in the 7^{th} batch (band B38) in W community.

3.3. TGGE analysis of dsrB gene fragments during sequential batch tests

As the initial consortia were obtained by successive culture enrichments directed for SRB selection, TGGE fingerprints of *dsrB* gene were also determined to monitor the SRB community during the successive batch incubations (Fig. 6.3b). In the original enrichment of U and W consortia 10 and 3 different bands were detected, respectively, but after uranium addition both SRB communities have changed. Some light bands observed in consortium U became predominant after first uranium exposure (Bdsr4, Bdsr5 and Bdsr6) and new bands were also observed (Bdsr 11, Bdsr 12 and Bdsr 18). In what concerns SRB consortium W, the changes were even more evident: the bands observed initially disappeared and two new bands became visible in the gel (Bdsr16 and Bdsr17). Unexpectedly, after the second batch with uranium addition it was not possible to detect SRB in both communities, as the amplification of *dsrB* gene from DNA extracts of these communities was attempted without success. After uranium exposition, the SRB communities could not be re-established when incubated in the absence of metal and presence of sulphate.

3.4. Phylogenetic analysis

Several TGGE bands were re-amplified and sequenced in order to determine the composition of the microbial communities. A total of 16 and 18 different bands were selected from 16S rRNA and *dsrB* TGGE gels, respectively. In order to obtain a more accurate identification of the bacteria present in each consortium in the consecutive batch cultures, sequences from TGGE bands and their closest BLAST relatives were used for phylogenetic analysis. The MCMC analysis of the 16S rRNA gene sequences revealed the presence of bacteria related to 6 phylogenetic main groups (Fig. 6.4): families *Rhodocyclaceae* of β-Proteobacteria subclass (B7, B13, B22 and B23); *Enterobacteriaceae* of γ-Proteobacteria subclass (B1, B12 and B18); *Caulobacteraceae* of α-Proteobacteria subclass (B10 and B38); genera *Desulfovibrio* of δ-Proteobacteria (B20 and B29); *Bacteroides* of Bacteroidia class (B40) and *Clostridium* of Clostridia class (B8, B11 and B15). Similarly, a phylogenetic approach was applied to *dsrB* TGGE band sequences in order to identify the SRB present in the mixed cultures (Fig. 6.5). Analysis of the phylogenetic tree obtained with *dsrB* gene sequences showed the presence of two SRB genera (Fig. 6.5): *Desulfovibro* and *Desulfomicrobium*. Most sequences were affiliated with *Desulfovibrio desulfuricans* (Bdsr3, Bdsr4, Bdsr5, Bdsr11, Bdsr13, Bdsr14, and Bdsr18) and *Desulfovibrio vulgaris* subsp. *vulgaris* (Bdsr6, Bdsr16 and 1 Bdsr7). Although the closest relative of Bdsr7, Bdsr8 and Bdsr12 is also *Desulfovibrio vulgaris* subsp. *vulgaris*, these band sequences constitute two independent *dsrB* phylogenetic groups. The remaining three bands present in the initial U consortium (Bdsr1, Bdsr2 and Bdsr9) were grouped in the same phylogenetic cluster, close related to an uncultured SRB and affiliated with the *Desulfomicrobium* group. Although Bdsr10 was the most intense band in the TGGE profile of U initial community, the BLAST search confirmed its chimerical origin.

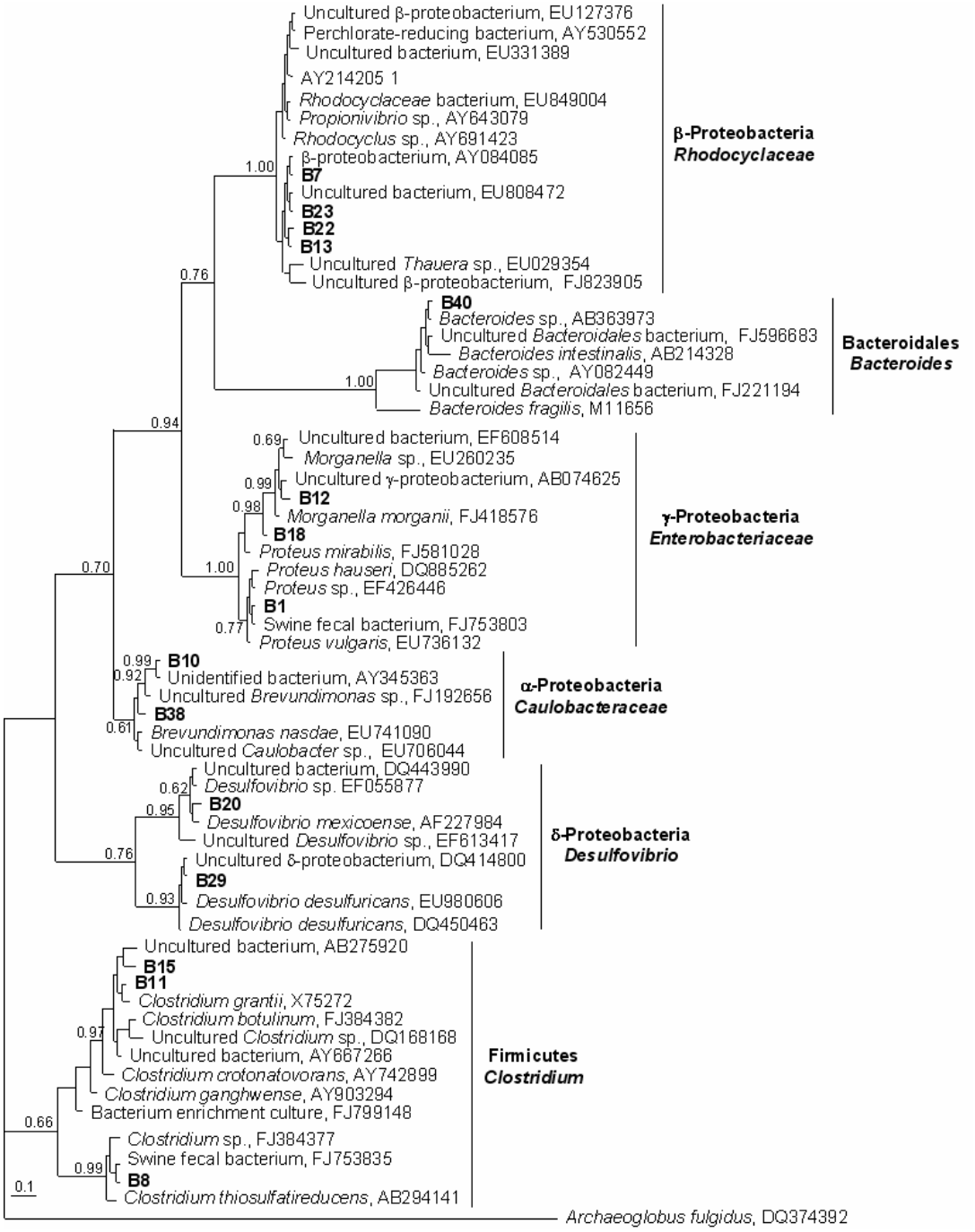

Fig. 6.4 Phylogenetic tree obtained with 16S rRNA partial sequences (196 nucleotide positions), corresponding to the reamplified TGGE bands and to the most closely related ones retrieved from BLAST search. Phylogeny was inferred using the Bayesian Markov Chain Monte Carlo analysis of aligned 16S rRNA fragments. *Archaeoglobus fulgidus*, a species from Archaea Domain was included to root the tree. Probability values associated to each node are showed. Access numbers of GenBank sequences are indicated in the figure and names in bold face correspond to sequences determined in this work. The main bacterial groups detected in bacterial consortia are also indicated.

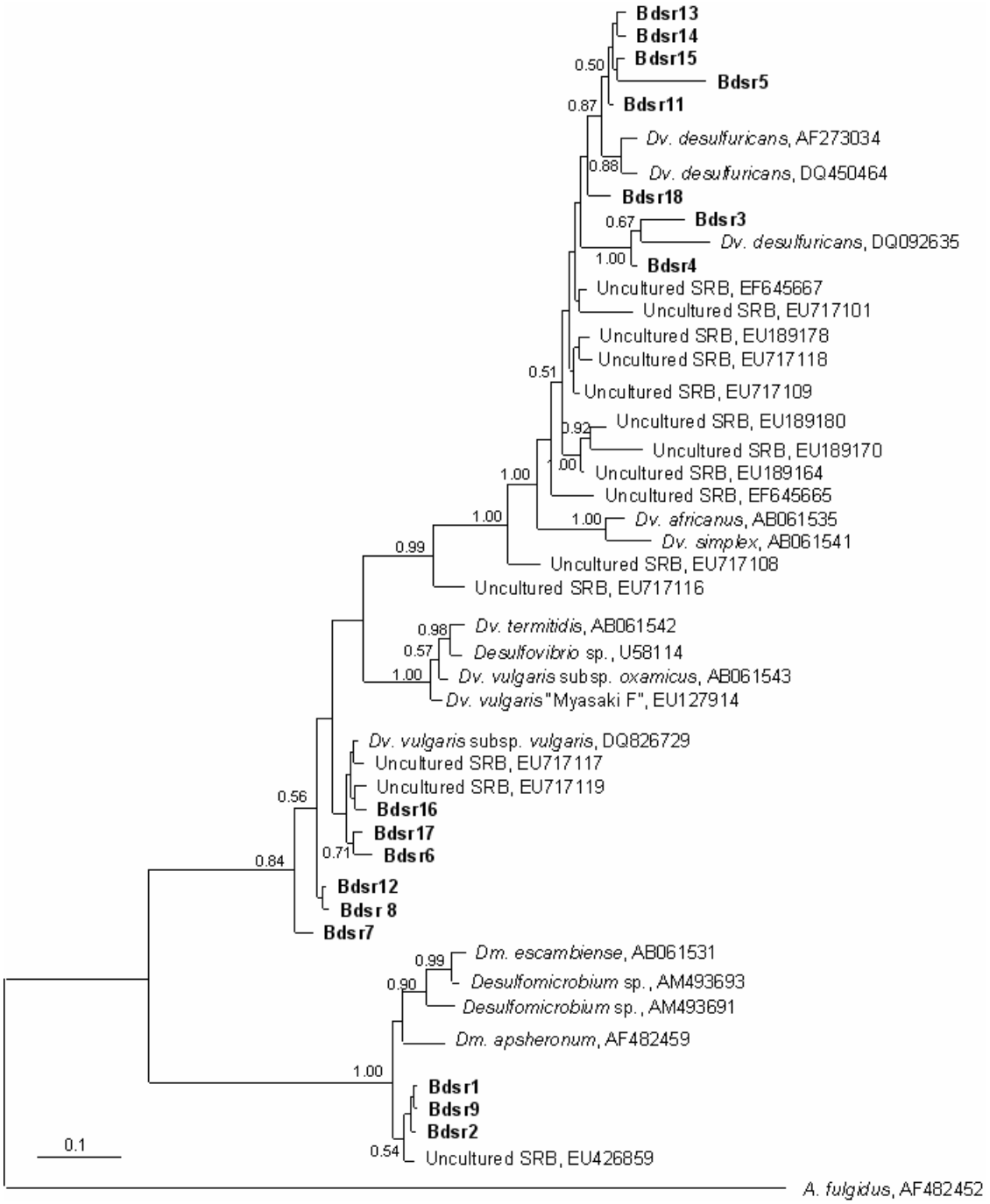

Fig. 6.5 Phylogenetic tree obtained with *dsrB* gene partial sequences (316 nucleotide positions), corresponding to the reamplified TGGE bands and to the most closely related ones retrieved from BLAST search. Phylogeny was inferred using the Bayesian Markov Chain Monte Carlo analysis of aligned *dsrB* fragments. *Archaeoglobus fulgidus*, a sulfate-reducing archaea, was included to root the tree. Probability values associated to each node are showed. Access numbers of GenBank sequences are indicated in the figure and names in bold face correspond to sequences determined in this work. *Dv.* – *Desulfovibrio*; *Dm.* – *Desulfomicrobium.*

4. Discussion

TGGE profiles of *dsrB* gene obtained from the initial enrichments demonstrated that the SRB mixed cultures were composed by species affiliated with *Desulfovibrio desulfuricans*, *Desulfovibrio vulgaris* and *Desulfomicrobium* spp. (sample U), as well as species related to *Desulfovibrio desulfuricans* (sample W). The presence of these species could support uranium removal, since the U (VI) reduction by strains of the genus *Desulfovibrio* has been described by several authors (Lovley and Phillips, 1992; Lovley *et al.*, 1993b; Ganesh *et al*., 1999; Spear *et al.*, 2000) and some *Desulfomicrobium* species have been reported as having the ability to enzymatically reduce Cr (VI) (Michel *et al*., 2001). Additionally, sulphate and uranium (VI) reduction are not considered incompatible processes. Spear and co-workers observed that SRB have the ability to reduce uranium (VI) and sulphate at the same time, and that the presence of sulphate even promotes a faster rate of uranium reduction (Spear *et al*., 2000). Preferential reduction of uranium (VI) under lactate limiting conditions was also reported for *Desulfovibrio desulfuricans* in the presence of sulphate (Ganesh *et al*., 1999).

Before uranium exposure, SRB mixed cultures of samples U and W showed excellent sulphate reducing performance. However, when uranium was added to the medium sulphate reduction was highly affected. In the presence of this metal no sulphate reduction was detected by bacterial community W and for bacterial community U it was only observed in the first batch with uranium. Uranium was removed in all cases. A slight decrease in the uranium concentration of the abiotic solutions was also observed. This fact, already mentioned in the literature (Spear *et al*., 1999), is related to the adsorption of the metal to the glass material.

The analysis of the TGGE fingerprints suggests that the structure of the bacterial community was also affected by the presence of uranium in the medium, particularly the structure of the SRB community. In fact, the SRB species identified in each initial community (corresponding to 16S rRNA gene TGGE bands 20 and 29) were not detected in the subsequent mixed cultures. Their detection in the first culture with uranium was only possible using a specific target gene as *dsrB*, as it allowed the selection of a minority population from the community.

TGGE and phylogenetic analysis of *dsrB* gene fragments confirmed the SRB community changes after uranium addition. *Desulfomicrobium* affiliated species where not detected in community U after the first batch culture with uranium and *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris* related strains were not the same that were present in the initial enrichment. In community W, the species related to *Desulfovibrio desulfuricans* detected in the initial culture were replaced by *Desulfovibrio vulgaris* after uranium addition. SRB became undetectable in all following cultures including those grown with sulphate in the absence of uranium, indicating that the SRB community shifts were irreversible. The absence of SRB in the mixed cultures is in agreement with the fact that sulphate reduction by both consortia was not observed. Fox *et al.* (2006) observed that SRB were not present in the community responsible by uranium (VI) reduction, although sulphate reduction was observed.

The results strongly suggest that SRB are not the bacteria responsible for uranium removal, contrarily to what was thought in the beginning of the experiments and to that is mentioned in the literature (Smith and Gadd, 2000; Rashamuse and Whiteley, 2007). This important finding clearly demonstrates the need for monitoring changes in bacterial populations when exposed to specific conditions as the presence of uranium or other metals.

The performance of uranium removal by the bacterial consortia was similar during the sequential culture tests and TGGE 16S rRNA gene profiles showed that the communities with ability for uranium removal had a similar structural composition. Both microbial structures were established on the first batch culture with uranium and remained stable during uranium exposition, even when this concentration was doubled. Phylogenetic identification of bacteria present in uranium resistant communities showed that species closely related to *Clostridium* spp. (bands B11 and B15), *Caulobacteraceae* family (band B10) and to *Rhodocyclaceae* family (bands B7 and B13), were predominant in U and W consortia. The isolation of the species present in the both consortia was carried out but without success, the failure in obtain isolates in anoxic conditions was also reported by VanEngelen *et al*. (2008). The failure in obtain isolates can be explained by synergistic interactions among members of the bacterial communities. Although the role in the uranium removal of each member that compose the communities was not known, these mixed cultures were able to remove uranium efficiently, suggesting an important role in the removal of this metal. In fact, it is widely demonstrated the advantages of employing mixed

cultures as opposed to pure cultures in bioremediation applications (White and Gadds, 1996; Mukred *et al.,* 2008).

Clostridium species are considered one of the major players in uranium (VI) reduction (Francis *et al.,* 1994; Susuki *et al.,* 2003; Gao and Francis, 2008) and their presence in mixed SRB cultures with ability for uranium removal was reported by Spear and co-workers (Spear *et al*., 2000). The relationship between *Clostridium* spp. present in both mixed cultures and uranium removal was also supported by TGGE analysis, as bands corresponding to these species (B11 and B15) were not visible when the metal was excluded from the enrichments. This phenomenon probably occurs because these *Clostridium* related species are not particular competitive and can only become predominant under environmental conditions that inhibit other bacteria. On the other hand, some members of *Rhodocyclaceae* family were detected in uranium contaminate mines (Susuki *et al*., 2003; Akob *et al.,* 2008). The *Rhodocyclaceae* family includes species of *Rhodocyclus*, *Thauera* and *Dechloromonas*. Some strains of *Rhodocyclus* spp. are known to participate in phosphorous removal (Zilles *et al.,* 2002) and others were also detected in a bioreactor developed to treat chromate waters (Battaglia-Brunet *et al*., 2007). Considering *Thauera* genus, one species is documented as being capable of selenate anaerobic respiration (Megan *et al*., 2003). *Dechloromonas* sp. was reported as being responsible for U(VI) reduction in microcosms (Akob *et al,* 2008). During the present work members of *Rhodocyclaceae* family were found as one of the predominant bacteria in a consortium with ability for uranium removal.

The two bacterial cultures used in this study, originated from different sources (one from sediments from the mining area of Urgeiriça (U) and other from sludge from the wetland of that mine (W)), were at the beginning composed of different bacteria. However, the presence of uranium in the media stimulated the growth of similar bacterial species that were maintained independently of the number of enrichments with uranium. This result is not surprising, since both samples were from similar environmental sources and because the bacterial enrichments were done in the same conditions, selecting the most uranium resistant bacteria. Thus, it can be expected that bacteria with ability for efficient uranium removal can be obtained from mixed cultures previously enriched for other physiologic groups as SRB, as a result of successive growth in media with that metal. The present work

indicates that the SRB community of both inocula probably did not possess mechanisms of resistance against this toxic metal.

5. Conclusions

The present work demonstrates that the structure of the community was drastically changed during the uranium bio-removal studies. The original communities were mainly composed by SRB, but after uranium exposure these bacteria were not detected in the communities. This result is an important finding emphasizing, besides the evaluation of the efficiency of uranium bio-removal, the importance of monitoring the microbial community structure during bioremediation processes. The bacterial consortia with ability for uranium removal, composed by strains affiliated to *Clostridium* genus, *Caulobacteraceae* and *Rhodocyclaceae* families, have potential to be used in new bioremediation processes.

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Chapter 7

Anaerobic bio-removal of uranium (VI) and chromium (VI): Comparison of microbial community structure

Abstract

Several microbial communities, obtained from uranium contaminated and non-contaminated samples, were investigated for their ability to remove uranium (VI) and the cultures capable for this removal were further assessed on their efficiency for chromium (VI) removal. The highest efficiency for removal of both metals was observed on a consortium from a non-contaminated soil collected in Monchique thermal place, which was capable to remove 91% of 22 mg/L U(VI) and 99% of 13 mg/L Cr(VI). This study revealed that uranium (VI) removing communities have also ability to remove chromium (VI), but when uranium (VI) was replaced by chromium (VI) several differences in the structure of all bacterial communities were observed. TGGE and phylogenetic analysis of 16S rRNA gene showed that the uranium (VI) removing bacterial consortia are mainly composed by members of *Rhodocyclaceae* family and *Clostridium* genus. On the other hand, bacteria from *Enterobacteriaceae* family were detected in the community with ability for chromium (VI) removal.

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1. Introduction

Uranium and chromium are elements of particular concern due to their toxicity and, in the case of uranium, also due to its radioactivity. Both are considered ecological and public health hazards (Viti *et al.*, 2003; Kazy *et al.*, 2009) and both are redox active elements, with oxidation states varying from 0 to +6, in the case of uranium, and -2 to +6 in the case of chromium. Uranium and chromium toxicity and mobility is highly dependent on their oxidation states, being the oxidation state +6 the most toxic and mobile for both elements. Uranium predominates in the liquid industrial wastes as salts of uranium (VI) (Gorby and Lovley, 1992), while chromium is usually present as chromate and dichromate (Cheung and Gu, 2007).

Uranium is essentially composed of the three radionuclides ^{238}U, ^{235}U, and ^{234}U, in relative abundances of 99.2745 %, 0.72 %, and 0.0055 %, respectively. Natural uranium is not considered a major radiological hazard due to the long half-lives of the radioisotopes. However, its chemical toxicity has been documented since the 1940s and its nephrotoxicity is well established (Craft *et al.*, 2004). Uranium compounds in +2 to +4 valence states are essentially insoluble (Gorby and Lovley, 1992; Wan *et al.*, 2006). However, *in vivo* soluble uranium is always hexavalent, regardless of the oxidation state of uranium compound taken up (Edison, 1994), being this form that is of toxicological importance.

Cr(VI), usually occurring as the highly soluble and toxic chromate anion, is reported as mutagenic, carcinogenic and teratogenic being approximately 100-fold more toxic than Cr(III) (Shen and Wang, 1995). Cr(III) is considered relatively innocuous because it is less soluble and does not permeate through eukaryotic and prokaryotic membranes (Francisco *et al.*, 2002).

It has been accepted for over a century that some microorganisms have the ability to change the oxidation state of metals. However, only in the past few decades researchers realized that these processes open up a window for new applications, including the remediation of metal-contaminated waters and soils. Removal of U(VI) and Cr(VI) from industrial waste, and eventual reuse, is essential taking into account their mobility and toxicity.

Since the pioneer work of Lovley and co-workers in the early ninety's (Lovley *et al.*, 1991; Lovley and Phillips, 1992), a number of bacterial species including mesophilic representatives of the genera *Shewanella* (Lovley *et al.*, 1991; Wade and DiChristina, 2000), *Clostridium* (Francis *et al.*, 1994) and *Geobacter* (Anderson *et al.*, 2003) have been described for their ability to reduce uranium (VI). The capacity to enzymatically reduce U(VI) has also been demonstrated for a range of Fe(III)-reducing bacteria and SRB (Lovley *et al.*, 1991; Gorby and Lovley, 1992; Lovley and Phillips, 1992). *Desulfotomaculum* species are examples of SRB known as coupling U(VI) reduction to growth, while *Desulfovibrio* spp. reduce U(VI), but do not obtain energy to support growth from this process (Okabe and Characklis, 1992; Lovley *et al.*, 1993; Tebo and Obraztsove, 1998; Wade and DiChristina, 2000; Pietzsch and Babael, 2003). A few thermophilic microorganisms, such as *Thermus scotoductus*, *Pyrobaculum islandicum*, and *Thermoanaerobacter* sp., have also been shown to enzimatically reduce U(VI) (Kieft *et al.*, 1999; Kashefi and Lovley, 2000; Roh *et al.*, 2002), but conservation of energy for growth during U(VI) reduction has not been demonstrated for any of these model organisms. After reduction, the highly soluble and mobile U(VI) is converted to insoluble U(IV), which can be then separated from aqueous solutions (Gorby and Lovley, 1992; Wan *et al.*, 2006).

A wide variety of bacteria have also been reported to reduce hexavalent chromium to the trivalent form under aerobic and anaerobic conditions, *e.g*. *Bacillus* sp. (Wang and Xiao, 1995), *Pseudomonas fluorescens* (Boop *et al.*, 1983), *Pseudomonas putida* (Ishibashi *et al.*, 1990), *Enterobacter cloacae* (Wang *et al.*, 1989) and sulphate-reducing bacteria (SRB) (Lovely and Phillips, 1994), but the enzymatic basis for chromate reduction has not been clarified (Thaker and Madamwar, 2005).

Considering some similarities between uranium and chromium, namely the same oxidation state of the most soluble form and the possible bio-reduction to insoluble oxidation states, U(IV) and Cr(III), the main objective of the present work was to investigate if the anaerobic bacterial communities able to remove uranium (VI) are also effective for chromium (VI) removal. The study was first conducted in the presence of sulphate in order to not exclude the SRB enrichment, since these bacteria are reported as having ability for U(VI) and Cr(VI) reduction (Lovley *et al.*, 1991; Lovely and Phillips,

1992; Lovley *et al.*, 1993; Lovely and Phillips, 1994; Tebo and Obraztsove, 1998; Wade and DiChristina, 2000). Moreover, sulphate is a common contaminant, usually present in metal contaminated waters and effluents, such as in mine waters resulting from uranium mining. Therefore, the bacterial communities also need to be sulphate resistant and eventually able for sulphate removal.

The structure of the bacterial communities with ability for uranium (VI) and chromium (VI) bio-removal was also compared, in order to investigate eventual shifts in the consortia due to the presence of a different metal ion and to establish the relationships between the bacterial groups and the metal removal. For those purposes, temperature gradient gel electrophoresis (TGGE) was used, as it is considered a powerful tool to monitor microbial communities under environmental changes. Furthermore, the phylogenetic analysis of TGGE band sequences allows the identification of the dominant populations in these communities.

2. Materials and Methods

2.1. Enrichment for uranium-removal bacteria

The anaerobic bacterial communities used in these experiments were obtained from environmental samples collected in several Portuguese locations. Soil samples from Monchique thermal place and sludge from two municipal waste water treatment plants (Montenegro and Estói) were collected in South. In North, sediments from the mining areas of Urgeiriça and Bica, as well as sludge from the wetland of Urgeiriça mine, were collected. Sludge from waste water treatment plant of the leather industry of Alcanena (central Portugal) was also collected. Cultures of sulphate-reducing bacteria previously obtained were also used (Martins *et al.*, 2009, **Chapter 2**).

Bacterial enrichments were performed in anaerobic conditions at room temperature (21± 1ºC) using 120 mL glass bottles. The anaerobic conditions were achieved by purging the medium with nitrogen gas and by addition of 10 mL of sterile liquid paraffin.

The enrichments were done to promote the growth of sulphate-reducing bacteria (SRB), as their ability to reduce U(VI) to U(IV) is recognised, making them good candidates for

bioremediation of uranium contaminated waters and effluents. The first bacterial enrichments were carried by adding 5 g of each sample to 100 mL of Postgate B medium (Postgate, 1984) supplemented with resazurin as redox indicator (0.03 g/L). Subsequently, the bacterial mixed cultures were grown in modified MTM medium (Sani *et al.*, 2001), which contains 1 g/L NH_4Cl, 0.06 g/L $CaCl_2.6H_2O$, 0.05 g/L yeast extract, 1 g/L $MgSO_4.7H_2O$, 2 g/L Na_2SO_4 and 5 g/L sodium lactate. The change of medium was carried out in order to avoid chemical removal of the metals. The enrichment of bacteria resistant to uranium and with potential for its removal was investigated by adding to MTM medium 10 mg/L of uranium (VI), as uranyl acetate dehydrate. Subsequently, the bacterial mixed cultures were maintained in modified MTM medium containing 10 mg/L of uranium (VI). The bacterial consortia were sub-cultured every 4 weeks using 10% (v/v) of inoculum.

2.2. Metal bio-removal experiments

The ability of uranium (VI) removal by the enriched cultures was studied in the presence and in the absence of sulphate. The capacity for chromium (VI) removal by uranium-removal bacterial was also investigated. Bio-removal experiments were performed in batch under anaerobic conditions. MTM medium supplemented with uranium (VI) as uranyl acetate dehydrate or with chromium (VI) as potassium dichromate was used for growth.

Different experiments were performed simultaneously, adding to the MTM medium containing 1800 mg/L of sulphate: i) 15 mg/L of uranium (VI), ii) 13 mg/L of chromium (VI), iii) 22 mg/L of uranium (VI) and iv) 25 mg/L of chromium (VI). A set of experiments in MTM medium without sulphate was also done with: v) 15 mg/L of uranium (VI), vi) 13 mg/L of chromium (VI) and vii) 22 mg/L of uranium (VI). For each experiment an abiotic set, without bacteria, was used as control. All experiments were carried out with medium and material sterilized by autoclaving.

For all the experiments, bacterial cells from uranium enrichments were harvested by centrifugation at 4000 rpm for 10 min, washed with MTM medium and transferred to the bottles containing the medium to be tested. All experiments were performed in duplicate

using 35 mL glass bottles containing 30 mL of growth medium with pH~7 and 10% (v/v) of inoculum. The medium was purged with nitrogen gas to achieve an anaerobic environment prior to inoculation. After inoculation, oxygen diffusion was eliminated by adding 5 mL of sterile liquid paraffin. The bottles were sealed with butyl rubber stoppers and aluminium crimp seals and incubated at room temperature (21±1ºC). Samples from the 25 d of incubation were collected and frozen at -20ºC for nucleic acids extraction.

2.3. Analytical methods

Culture samples were collected periodically using a syringe and centrifuged at 4000 rpm for 5 min. pH were determined using a pH/E Meter (GLP 21, Crison). Sulphate and chromium (VI) concentration were quantified by UV/visible spectrophotometry (Hach-Lange DR2800 spectrometer) using the method of SulfaVer4 and ChromaVer3 (Hach-Lange), respectively. Cr(VI) was determined by the 1,5-Diphenycarbohydrazide method (Lichtenstein and Allen, 1961) using a powder formulation called ChromaVer 3 of Hach-Lange. This reagent reacts with Cr(VI) giving a purple colour complex that was quantified spectrophotometrically at 540 nm. Dissolved uranium was quantified using the Arsenazo III (1,8-dihydroxynaphthalene3,6-disulphonic acid- 2,7-bisSTA(azo-2)-phenylarsonic acid) reagent (Sawin, 1961). The metal concentration was determined by mixing 900 μL of sample with 300 μL of 0.5 M HCl, followed by the addition of 300 μL of Arsenazo III (0.1 % w/v). After 3 min, the purple-colour metal-Arsenazo III complex was quantified spectrophotometrically at 652 nm. The Arsenazo III solution was prepared by dissolving the reagent in 0.01 M HCl and in 10 % (v/v) ethanol.

An optical microscope equipped with a digital camera (Leica D C300FX) was used to visualise the bacteria after Gram staining.

To evaluate the precipitates resulting from bio-removal process, micro-morphology and elemental composition analysis was carried out using a JEOL JSM-5410 scanning electron microscope (SEM), coupled with an OXFORD LINK energy dispersive spectrometer (EDS).

2.4. Molecular characterization

2.4.1. Extraction of DNA

Total genomic DNA was extracted after harvesting cells by centrifugation at 4000 rpm for 10 min. DNA extraction was carried out by the method described by Martins *et al*. (2009).

2.4.2. PCR Amplification of 16S gene fragments

Amplification of 16S rRNA gene fragments was performed using the primer pair 341F-GC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') / 534R (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer *et al.*, 1996). The primers were purchased from Thermo Fischer Scientific. The reaction mixture used for PCR amplification contained 31.75 µL of sterilised MiliQ water, 1 µL of each primer (10 pmol/µL), 1 µL of dNTP`s (10 mM), 4 µL of $MgCl_2$ (25 mM), 10 µL of 5×Go Taq® buffer (Promega, Madison, USA), 0.25 µL of GoTaq®DNA polymerase (Promega, Madison, USA), and 1 µL of DNA. PCR amplification was performed in a thermocycler (T1, Biometra, USA). Thermal cycling was carried out by using an initial denaturation step of 94ºC for 3 min, followed by 35 cycles of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 1 min and completed with an extension period of 3 min at 72ºC. The PCR products were analyzed by electrophoresis, in 1% (w/v) agarose gel and TAE Buffer.

2.4.3. TGGE analysis

PCR products, amplified with primers 341F-GC/534R, were resolved using a TGGE Maxi system (Biometra, USA), as specified by the manufacturer. Aliquots of each PCR product (5 µL) were electrophoresed in a gel containing 6% (w/v) acrylamide/bisacrylamide (39:1), 8 M urea, 2% (v/v) glycerol and 20% (v/v) formamide with a TAE 1X buffer system at a constant voltage of 150 V, for 20 hours, applying a thermal gradient of 42ºC to 53ºC. The gels were silver stained and scanned. Individual TGGE bands were excised from the gels, re-suspended in 35 µL of TE 1X (10 mM Tris-HCl, 1mM EDTA) and stored at 4ºC. 3 µL of the supematant was used for re-

amplification with the same primer pairs but without GC clamp. PCR products were purified using the Jetquick PCR Purification (Genomed GmbH, Lohner, Germany) and sequenced in CCMAR (Centro de Ciências do Mar, Universidade do Algarve). The sequences obtained in this study have the following accession numbers: GQ388248 to GQ388260.

2.4.4. Phylogenetic analysis

For phylogenetic analysis, sequence alignments were made with Clustal X (Thompson, 1997) and visually corrected. To estimate phylogenetic relationships the Bayesian Markov chain Monte Carlo (MCMC) method of phylogenetic inference (Huelsenbeck and Ronquist, 2001) was applied as implemented in the computer program MrBayes (Larget and Simon, 1999). This method allows estimation of the a posteriori probability that groups of taxa are monophyletic given the DNA alignment (i.e., the probability that corresponding bipartitions of the species set are present in the true unrooted tree including the given species). This Bayesian approach was repeated several times, using random starting trees and default starting values for the model parameters to test the reproducibility of the results.

2.5. Statistical analysis

The results of the experiments were subject to Two-way ANOVA by SigmaStat 3.0 program. All differences were considered to be statistically significant for $P < 0.05$.

3. Results

3.1. Enrichment of uranium-removal bacteria

The enrichment of uranium-removal bacteria was carried in medium with 10 mg/L of uranium and 1800 mg/L of sulphate. Among several bacterial communities obtained from the environmental samples tested and from previous studies (Martins *et al.*, 2009), only

the consortia from soil sample of Monchique thermal place (A), sediments from the mining area of Urgeiriça (U) and sludge from the wetland of Urgeiriça mine (W) showed resistance to uranium and capacity for uranium removal. During uranium enrichment these three consortia showed ability to remove approximately 85 % of uranium (VI), after 13 days of incubation (data not shown).

3.2. Bio-removal of metals

3.2.1. Uranium (VI) bio-removal

Anaerobic uranium removal by the previously selected bacterial consortia was studied in the presence of 15 and 22 mg/L of uranium (Fig. 7.1). The effect of sulphate in metal removal was also tested. In the experiment with 15 mg/L of uranium and 1800 mg/L of sulphate, all the three consortia showed an efficient uranium removal. The concentration of metal remaining in the medium after 13 days of incubation was 2, 4 and 6 mg/L for consortia A, U and W respectively. All the consortia maintained the ability to remove uranium when its concentration was increased to 22 mg/L, although an extended lag phase was observed. Bacterial communities A, U and W removed 20 mg/L, 16 mg/L and 15 mg/L of uranium, respectively, after 20 days of incubation (Fig. 7.1). No significant pH variation (7.0 to 7.2) was observed in either of the biotic or abiotic sets. A decrease of uranium concentration in the abiotic controls was observed and can be attributed to metal adsorption to the glass bottles used in the experiments, as already reported by other authors (Spear *et al.*, 1999). Sulphate reduction was not observed in all experimental sets (data not shown).

The efficiency of uranium (VI) removal by all consortia was not significantly affected by the absence of sulphate. After 13 days of incubation, 13, 10 and 9 mg/L of uranium was removed from the medium containing 15 mg/L U(VI), by consortia A, U and W, respectively (Fig. 7.1). In the sets containing 22 mg/L of uranium, the presence of sulphate also did not affect uranium removal by the bacterial consortia. However, a decrease of the lag phase was observed when consortia A and W were grown in medium without sulphate ($P<0.001$).

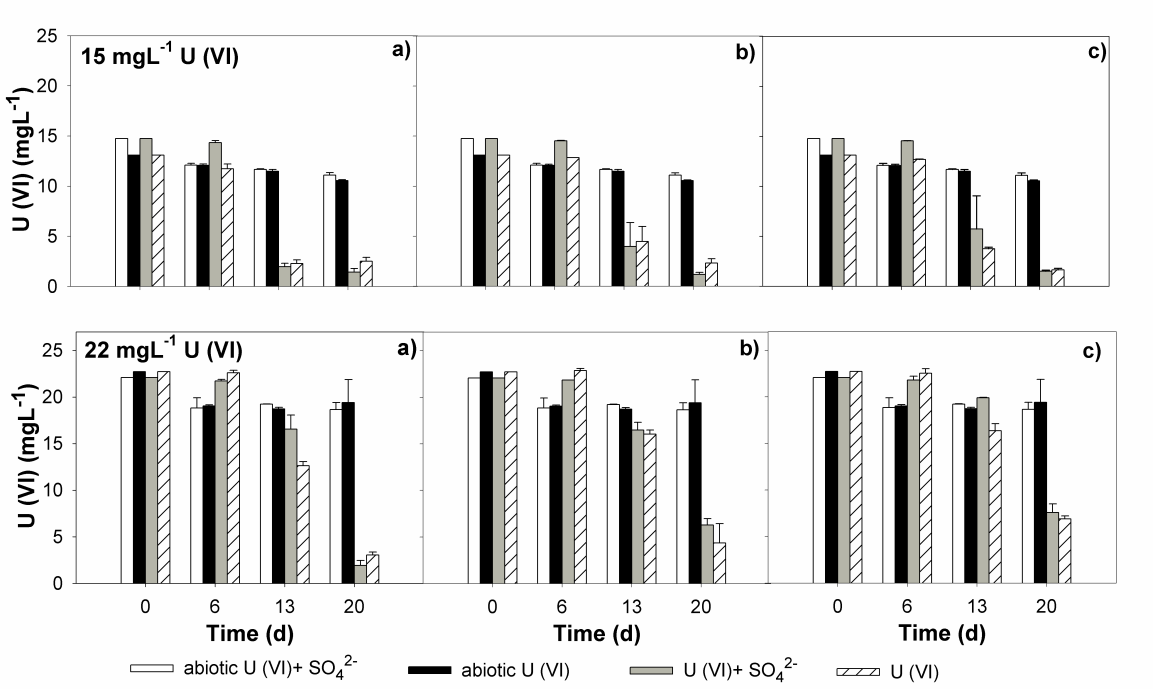

Fig. 7.1 Uranium (VI) removal profile by bacterial consortia A (a), U (b) and W (c). Values are averages ± standard deviations of the duplicates.

3.2.2. Chromium (VI) bio-removal

The ability for chromium (VI) removal by uranium-removal consortia was also investigated using 13 mg/L Cr(VI), both in the presence and absence of sulphate (Fig. 7.2). Consortium A showed the best performance for Cr(VI) removal in the presence of sulphate (12.9 mg/L was removed after 23 days). Consortia U and W only removed 5.7 mg/L and 8.6 mg/L of Cr(VI), respectively, after the same period of time. When the concentration of Cr(VI) in the medium was doubled, no removal of Cr(VI) was observed for any of the consortia (data not shown).

Although the performance of consortium A in Cr(VI) removal was not significantly affected by the absence of sulphate (P=0.111), the removal of this metal by U and W consortia decreased in the absence of this anion (Fig. 7.2). After 23 days of incubation 5.7 mg/L of Cr(VI) was removed by consortium U in the presence of sulphate and 12.6 mg/L in the absence of this anion. For consortium W, 8.6 mg/L and 11.2 mg/L of Cr(VI) was removed after the same period of time in the presence and in absence of sulphate,

respectively. No significant pH variation (6.9 to 7.1) was observed in either biotic or abiotic sets and sulphate reduction was not observed in all experimental sets (data not shown).

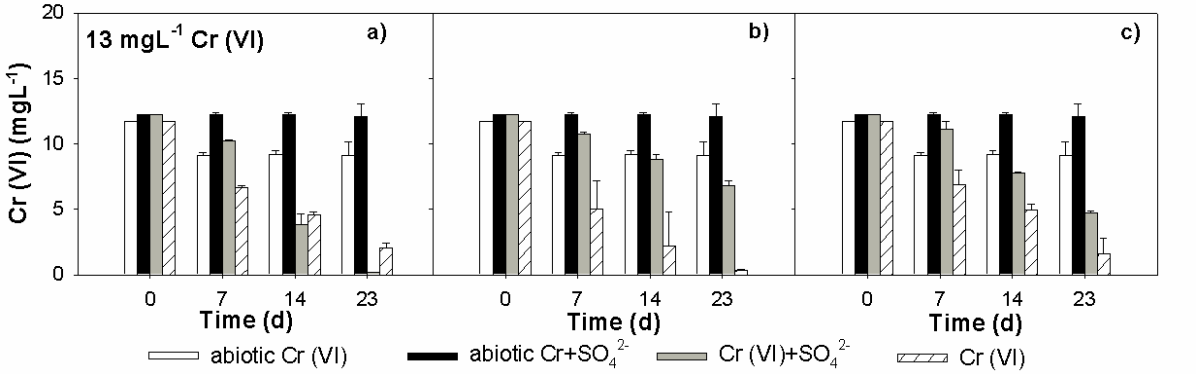

Fig. 7.2 Chromium (VI) removal profile by bacterial consortia A (a), U (b) and W (c). Values are averages ± standard deviations of the duplicates.

3.2.3. Microscopic analysis of bacterial consortia

Photomicrographs of bacterial consortia after U(VI) and Cr(VI) exposure show different cell morphologies in each situation (Fig. 7.3). In the presence of uranium (VI) (22 mg/L) and sulphate all bacterial consortia were mainly composed by large bacilli and cocci. However, when the consortia were exposed to 13 mg/L chromium (VI) the dominant population was constituted by large and very long bacilli.

3.2.4. Precipitates analysis

The precipitates formed during chromium and uranium removal were essentially composed by amorphous aggregates and precipitates with laminar form (Fig. 7.4). The EDS spectrum corresponding to the bio-removal of uranium shows that the precipitates were mainly composed by uranium, sulphur and phosphate (Fig. 7.4a). On the other hand, the precipitates resulting from the biological removal of chromium were mostly composed by chromium, sulphur, phosphate and chloride (Fig. 7.4b).

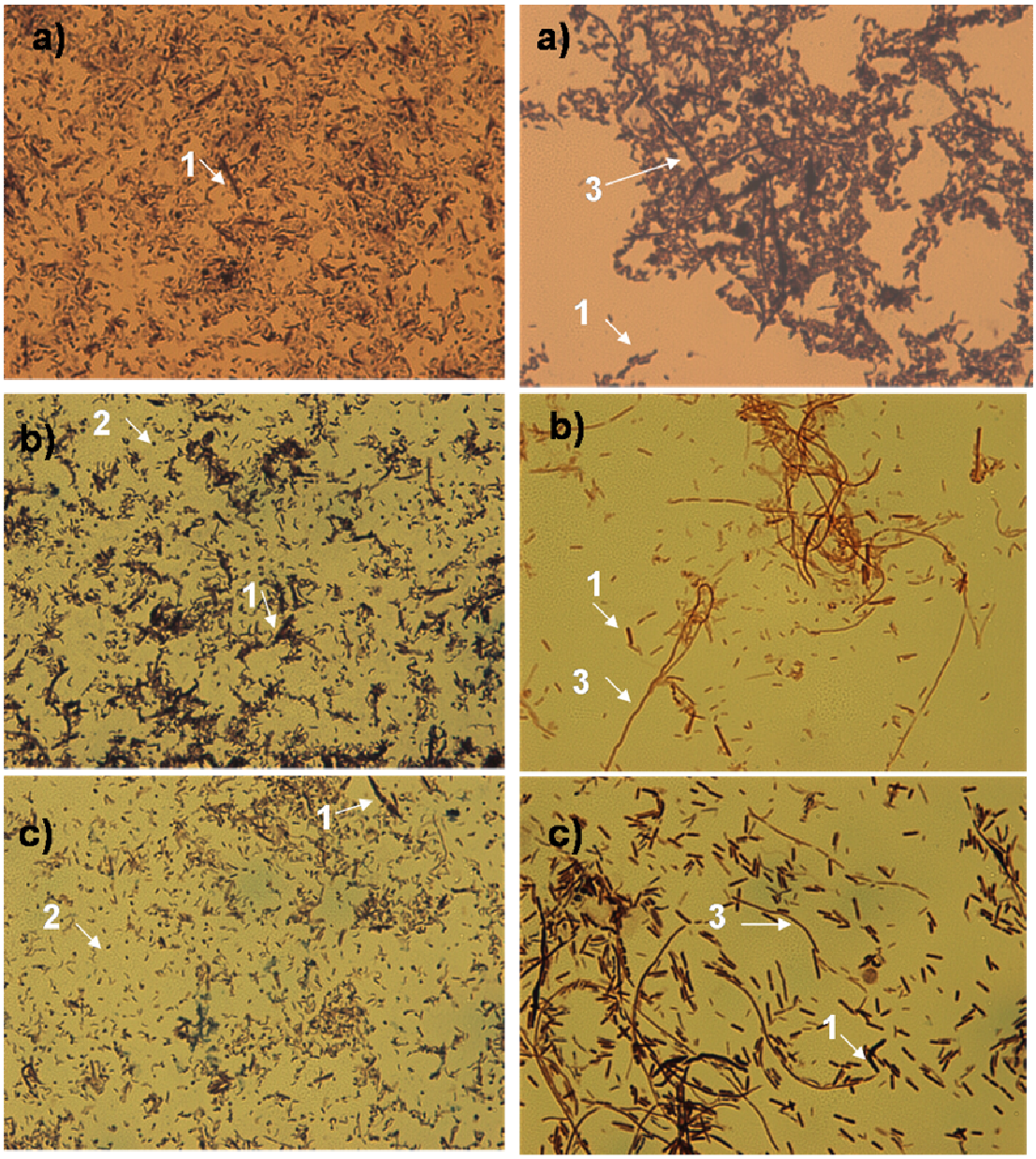

Fig. 7.3 Photomicrographs of bacterial consortia A (a), U (b) and W (c): in the presence of 22 mg/L uranium (VI) + sulphate (left) and in the presence of 13 mg/L chromium (VI) + sulphate (right). The different bacterial morphologies are indicated: 1- large bacilli, 2- cocci and 3 – very long bacilli. Amplification of 1000X.

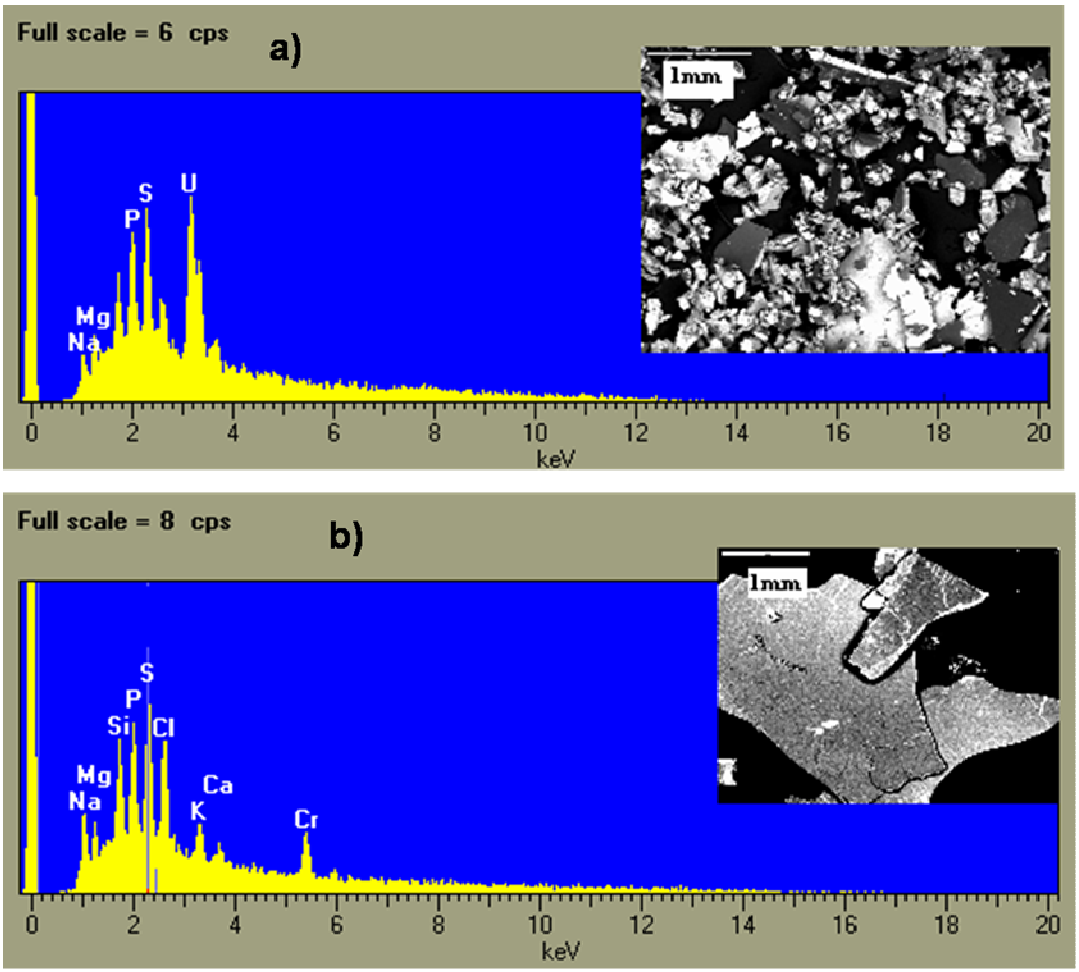

Fig. 7.4 EDS spectra and SEM micrographs of the precipitates formed in the presence of 22 mg/L uranium (VI) + sulphate (a) and in the presence of 13 mg/L chromium (VI) + sulphate (b).

3.3. TGGE and phylogenetic analysis of 16S rRNA gene

TGGE of 16S rRNA gene was applied to monitor possible changes in the bacterial communities during metal removal (Fig. 7.5). The A, U and W bacterial enrichments with uranium and sulphate revealed an identical TGGE profile. Four dominant bands were observed in the gel (B7, B11, B13 and B15) together with an additionally less intense band (B10) (Fig. 7.5, lanes 0).TGGE profile of the consortia was maintained stable independently of uranium concentration and presence of sulphate (Fig. 7.5, lanes I to IV). Reamplification and sequencing of TGGE bands allowed the identification of bacteria

present in uranium removal consortia. Phylogenetic analysis of band sequences (Fig. 7.6) revealed that the A, U and W communities with ability for uranium removal were affiliated with *Rhodocyclaceae* (B7, B13 and B24) and *Caulobacteraceae* families (B10), as well as with *Clostridium* spp. (B11 and B15).

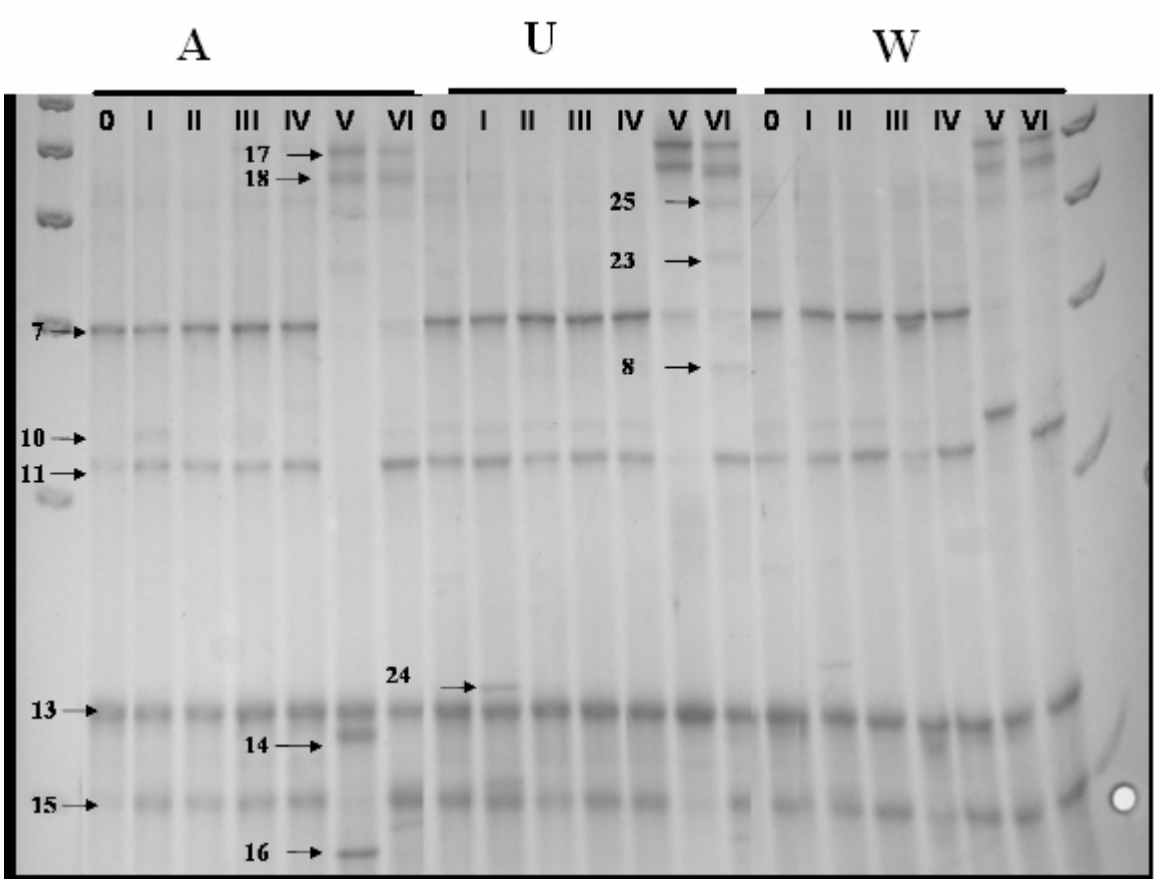

Fig. 7.5 TGGE profile of 16S rRNA gene fragments using DNA of A, U and W communities from batch experiments: beginning (0); 15 mg/L uranium (I), 15 mg/L uranium + sulphate (II), 22 mg/L uranium (III), 22 mg/L uranium + sulphate (IV), 13 mg/L chromium (V) and 13 mg/L chromium + sulphate (VI).

TGGE profiles showed that the replacement of uranium (VI) by chromium (VI) in the mixed cultures induced clear community changes in all samples (Fig. 7.5, lanes V). Two news bands (B17 and B18) were observed in all Cr(VI) enrichments and band B11 disappeared in all of them. Some particular changes also occurred in each consortium. Bands B10 and B15 were not observed for consortia A and U (Fig. 7.5, lanes V) and for

community A two dominant bands became visible in the gel (B14 and B16). Band B7 was only maintained in enrichment U. Finally, B10 became more intense in W community profile. Phylogenetic analysis (Fig. 7.6) revealed that all consortia established after Cr (VI) addition were composed by *Enterobacteriaceae* (B17 and B18) and *Rhodocyclaceae* members (B13). Additionally, community A included bacteria closely related with *Propionibacterium* genus (bands 14 and 16). Bacteria affiliated to *Clostridium* spp. (B15) and *Caulobacteraceae* members (B10) were also detected in consortium W.

Contrarily to what was observed in the uranium-removal communities, consortia responsible for chromium removal were affected by the presence of sulphate in the medium. In this condition, bands B11 and B15 (related with *Clostridium* spp.) became dominant again in the TGGE fingerprints. Bands B17, B18 (related with *Enterobacteriaceae* family) and B13 (affiliated to *Rhodocyclaceae* members) were maintained, but the B14 and B16 (corresponding to *Propionibacterium* sp.) present in community A and B10 (corresponding to *Caulobacteraceae* family) present in community W were absent. Community U also presented three new bands (B8, B23 and B25), related with *Clostridium* genus, *Rhodocyclaceae* and *Enterobacteriaceae* families.

4. Discussion

Several bacteria have potential to interact with metals, promoting their removal, and therefore offer interesting opportunities for biotechnological applications on water treatment. In the present work, several communities with ability for uranium (VI) removal were obtained from uranium contaminated and non-contaminated sediments. Considering the similarities between uranium and chromium, namely the same oxidation state of the most soluble form and the possible bio-reduction to insoluble oxidation states, the ability of these communities in chromium (VI) removal was also investigated. Furthermore, the structure of the bacterial communities with ability for uranium and chromium bio-removal was established, in order to investigate if the removal of each metal was performed by the same consortia or by different bacteria selected under different metal exposure.

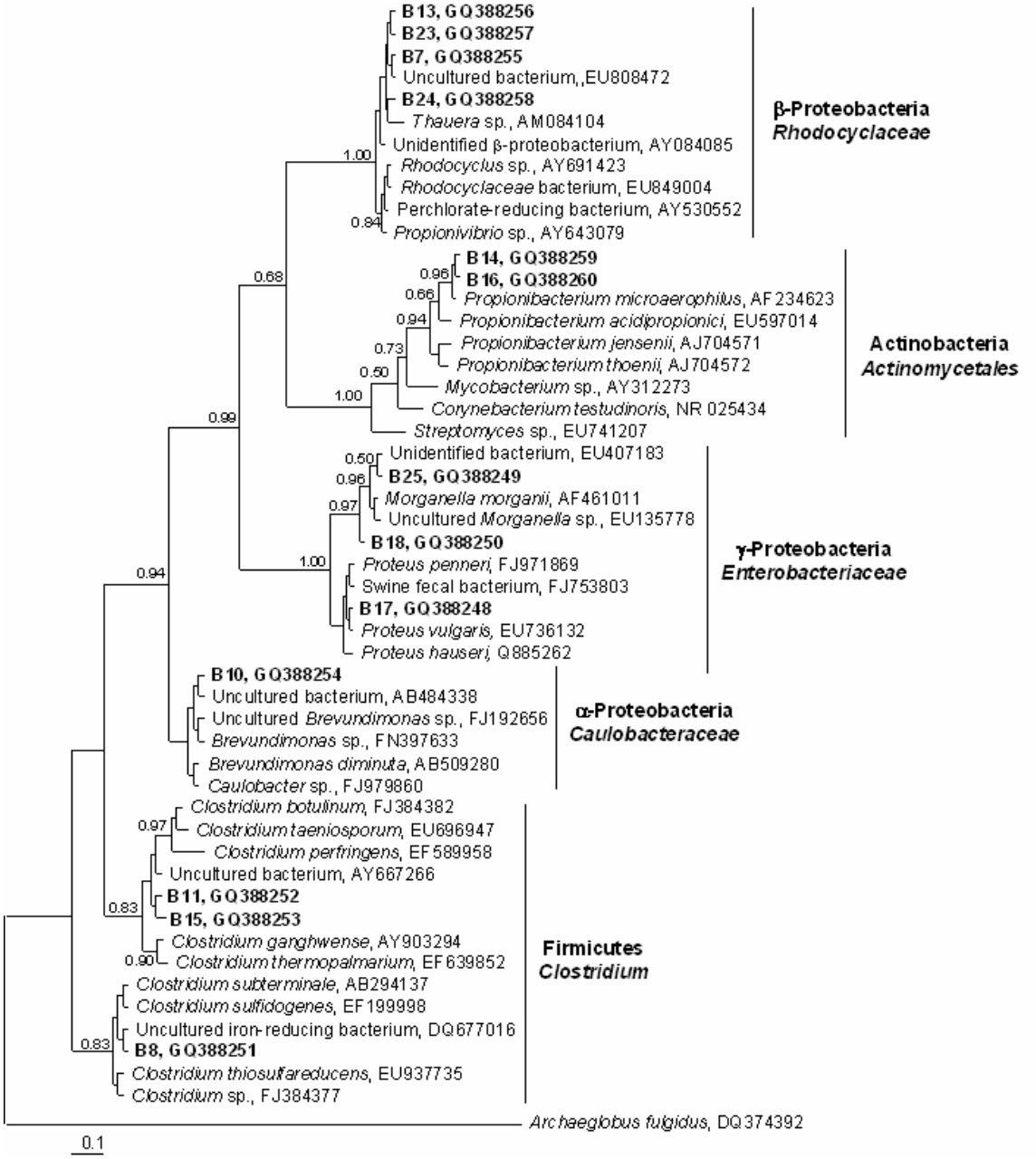

Fig. 7.6 Phylogenetic tree obtained with 16S rDNA partial sequences (196 nucleotide positions), corresponding to the reamplified TGGE bands and to the most closely related ones retrieved from BLAST search. Phylogeny was inferred using the Bayesian Markov Chain Monte Carlo analysis of aligned 16S rDNA fragments. *Archaeoglobus fulgidus,* a species from Archaea Domain was included to root the tree. Probability values associated to each node are showed. Access numbers of GenBank sequences are indicated in the figure and names in bold face correspond to sequences determined in this work. The main bacterial groups detected in bacterial consortia are also indicated.

Among the bacterial communities tested, only three were found to be uranium resistant: one from a non-contaminated sediment collected in Monchique thermal place (A), other from a sediment collected in uranium contaminated site (Urgeiriça mine) (U) and the last one from sludge of a wetland (W) located in the same mine site.

All these consortia showed potential for uranium removal from solutions containing 15 and 22 mg/L of U(VI) and also presented ability to remove Cr(VI) from solutions containing 13 mg/L of this metal. However, contrary to what was observed for U(VI) removal, the performance of Cr(VI) removal was affected when the concentration of this metal in the medium was doubled. These results are in accordance with previous reports (Kieft *et al.*, 1999), which refer that Cr(VI) is more toxic than U(VI) for most microorganisms and that the resistance to chromium may be induced by smaller increments in metal concentration. It has been reported (Boop and Echrlich, 1988; Wang *et al.*, 1989; Philips *et al.*, 1998; Jeyasingh anf Philip, 2005) that bacteria isolated from contaminated sites present higher resistance to toxic elements. Nonetheless, in this study the best performance for U(VI) and Cr(VI) removal was not achieved by consortia U and W (both recovered from contaminated sites), but by consortium A, recovered from an uncontaminated sediment. In approximately 20 days, 91% of 22 mg L^{-1} U(VI) and 99% of 13mg/L Cr(VI) were removed by this community and the removal rates did not change in presence of sulphate. The removal of this ion by U and W consortia was affected by sulphate presence, as Cr(VI) removal rates were increased in its absence.

SEM-EDS confirmed that both metals precipitated. This precipitation can eventually be associated to a previous reduction of Cr(VI) and U(VI) to insoluble oxidation states of Cr(III) and U(IV) (Wang *et al.*, 1989; Lovley *et al.*, 1991; Lovley and Phillips, 1992; Lovley and Phillips, 1994). SEM-EDS showed that the precipitates are also composed by S and P elements, suggesting that metals could be bond to the bacterial cells by phosphoryl and/or sulphydryl groups. It is known that microbial cell wall have anionic functional groups, such as carboxyl, sulphydryl, hydroxyl, phosphoryl and amide that show affinity for metal binding (Francis *et al.*, 2007; Acharya *et al.*, 2009). It was reported that uranium and plutonium were associated with phosphoryl group (Ohnuki *et al.*, 2005; Francis *et al.*, 2007). Metal reduction after binding to cell wall has also been reported for plutonium (Francis *et al.*, 2007). Thus, metal bio-removal by the consortia

under study may involve two possible mechanisms: simple metal binding to the functional groups present in the cell wall, or metal binding followed by change in the oxidation state of the metals.

Although the samples used for enrichment were from different sites, TGGE fingerprint analysis revealed that the three consortia (A, U and W) with ability to remove uranium have the same composition. This result indicates that the corresponding species are ubiquitous in these environments and under the same enrichment conditions the growth of the same bacteria was promoted. Even though the growth conditions were adjusted to select SRB, this group of bacteria was not detected in the uranium-removal consortia. The presence of uranium selected a microbial community containing bacteria related to *Rhodocyclaceae* and *Caulobacteraceae* families and *Clostridium* spp. *Clostridium* species are considered major players in uranium reduction. They can be found in soil, sediments and in low-level radioactive wastes (Lovley *et al.*, 1993; Susuki *et al.*, 2003; Gao and Francis, 2008). Although the mechanism of U(VI) reduction is known for some bacteria (e.g. *Desulfovibrio* sp. and *Geobacter* sp.) (Wall and Krumholz, 2006), the corresponding process in *Clostridium* spp. is not clear. Francis *et al.* (1994) have suggested that it can involve an enzymatic process. Considering Rhodocyclaceae members, they have been reported in uranium contaminated mines (Susuki *et al.*, 2003), and also in microbial populations stimulated for uranium removal (Akob *et al.*, 2008), but to date little is known about their role in uranium removal. TGGE profiles also showed that the community with ability to remove uranium was not affected by the absence of sulphate in the medium, which is in agreement with the fact that uranium (VI) removal occurs independently of sulphate presence.

When uranium (VI), was replaced by chromium (VI) several differences in the structure of all bacterial communities were observed. Microscopic analysis of the consortia supports these results as the cocci shaped cells highly decreased and were replaced by very long bacilli. The metal change probably induced the selection of Cr(VI) resistant bacteria, phylogenetically related with *Rhodocyclaceae* and *Enterobacteriaceae* families and *Clostridium* genus. Members of *Enterobacteriaceae* were not detected in the consortia with ability for U(VI) removal and were not previously associated to chromium bioremediation. The presence of bacteria resistant to Cr(VI) or as Cr(VI) reducing, never

reported before, may be explained by the existence of a possible mechanism of Cr(VI) resistance or removal, not yet explored. Furthermore, it was reported (Francisco *et al.*, 2002) that some members of *γ-Proteobacteria* were able to resist and reduce Cr(VI) present in the culture medium. Bacteria phylogenetically related to *Rhodocyclaceae* family were previously detected in a bioreactor used in the treatment of effluents containing Cr(VI) (Battaglia-Brunet *et al.*, 2007) and are known to participate in phosphorous removal (Zilles *et al.*, 2002).

Although the three bacterial communities grown in the presence of Cr(VI) and sulphate showed an identical TGGE profile, each of them was modified differently when sulphate was removed from the medium. *Clostridium* spp. were not detected in communities A and U in the absence of sulphate. In addition, species affiliated to *Propionibacterium* spp. were detected in the consortium A and members of *Caulobacteraceae* family were present in the consortium W when these communities were grown in the absence of sulphate. These findings indicate that the presence of sulphate probably inhibits the growth of these bacterial groups.

Combined analysis of TGGE fingerprints and data obtained from metal removal efficiency strongly suggests a direct relationship between the metal present in the media and established bacterial consortia.

5. Conclusions

Several communities with ability for uranium (VI) removal were obtained from uranium contaminated and non-contaminated samples. Unexpectedly, the highest efficiency of U(VI) and Cr(VI) removal was obtained with a consortium from a non-contaminated soil collected in Monchique thermal place (A).

The results obtained by TGGE analysis showed that the composition of the communities was influenced by the type of metal present in the medium. TGGE and phylogenetic analysis of 16S rRNA gene showed that members of *Rhodocyclaceae* family and *Clostridium* genus are predominant in the uranium removal communities, while the community established in the presence of Cr(VI) was mainly composed by members of *Rhodocyclaceae* and *Enterobacteriaceae* families and *Clostridium* genus. This change in

the bacterial community when uranium (VI) was replaced by chromium (VI) is a result of most importance, specially considering that these changes are usually not considered. The existence of bacteria never reported as U(VI) and Cr(VI) removing (such as *Rhodocyclaceae* and *Enterobacteriaceae* families) is also a relevant finding, encouraging the exploitation of microorganisms with new abilities that can be useful for bioremediation purposes.

Futures studies will be done to elucidate the mechanism involved in the metals removal by theses communities.

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Chapter 8

Mechanism of uranium (VI) removal by two anaerobic bacterial communities

Abstract

The mechanism of uranium (VI) removal by two anaerobic bacterial consortia, recovered from an uncontaminated site (consortium A) and other from an uranium mine (consortium U), was investigated. The highest efficiency of U (VI) removal by both consortia (97 %) occurred at room temperature and at pH 7.2. Furthermore, it was found that U (VI) removal by consortium A occurred by enzymatic reduction and bioaccumulation, while the enzymatic process was the only mechanism involved in metal removal by consortium U. FTIR analysis suggested that after U (VI) reduction, U (IV) could be bound to carboxyl, phosphate and amide groups of bacterial cells. Phylogenetic analysis of 16S rRNA showed that community A was mainly composed by bacteria closely related to *Sporotalea* genus and *Rhodocyclaceae* family, while community U was mainly composed by bacteria related to *Clostridium* genus and *Rhodocyclaceae* family.

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1. Introduction

Uranium mining and mineral processing for production of nuclear power have resulted in the generation of significant amounts of radioactive wastes with severe impact on environment (Choudhary and Sar 2009; Kazy *et al.*, 2009). Radionuclides like uranium are of particular concern due to their high toxicity and long half lives. Uranium exists as U (VI) in the form of divalent oxocomplex (UO_2^{2+}) in oxic aqueous systems (Gorby and Lovley, 1992; Fortin *et al*., 2007).

The conventional remediation processes of wastes containing uranium are highly expensive and ineffective particularly at low metal concentrations (Lloyd and Macaskie, 2000). Thus, the search of novel technologies is encouraged. Recently, bioremediation strategies based on the use of microorganisms are considered a potential alternative and an economically attractive strategy when compared with the traditional techniques (Choudhary and Sar 2009).

During the last two decades, many researchers have discovered that different groups of microorganisms, such as bacteria (Tsuruta, 2002; Wall and Krumholz, 2006), yeasts (Tsuruta, 2002) and fungi (White and Gadds, 1990; Tsuruta, 2002), have the ability to remove uranium from aqueous media. This ability was also observed for *Cystoseria indica*, a brown algae (Khani *et al.*, 2008). Although several studies described the ability of metals removal by diverse bacteria, reports focused on the mechanism of uranium removal were only recently available. Bacteria have been shown ability for uranium removal by several mechanisms such as adsorption (Acharya *et al.,* 2009) and accumulation inside the cells (Kazi *et al.,* 2009). In addition, some bacteria have showed the ability to reduce uranium (VI) (Wall and Krumholz, 2006; Gao and Francis, 2008). After reduction, the highly soluble and mobile U (VI) is converted to highly insoluble U (IV), which can be separated from aqueous solutions (Gorby and Lovley, 1992; Wall and Krumholz, 2006).

Although mixed bacterial cultures were frequently used in bioremediation strategies, only few studies about the mechanisms of metals removal by consortia are reported (Ngwenga and Whiteley, 2006; Rashamuse and Whiteley, 2007), and at our knowledge none of

them focus uranium removal. The advantages of employing mixed cultures as opposed to pure cultures in bioremediation applications are widely demonstrated (White and Gadds, 1996; Mukred *et al.*, 2008). Those advantages over pure cultures include greater stability and increased metabolic capabilities, which can be linked to the effects of synergistic interactions among members of the bacterial communities (Rashamuse and Whiteley, 2007; Mukred *et al*., 2008).

Taking into account the diversity of microorganisms it is of great importance to characterize metal resistant bacterial communities with ability for uranium removal, as well as to identify the mechanism or mechanisms involved in metal removal. Therefore, the mechanism involved in uranium (VI) removal from aqueous solution by two anaerobic bacterial consortia (one from an uncontaminated site and other from an uranium mine) was investigated for the first time. Moreover, the molecular identification of the two consortia was also performed. The identification of the bacterial community, as well the clarification of the process of metal-bacteria interaction can contribute to the development of an effective bioremediation strategy for uranium removal.

2. Materials and methods

2.1. Microorganisms and growth conditions

The bacterial communities used in the present study were obtained from sediments from the uranium mining area of Urgeiriça (sample U) and from soil from Monchique thermal place (sample A). Urgeiriça mine is located in the north Portugal and Monchique thermal place is located in the south Portugal. These consortia were selected from previous studies (Martins *et al.*, 2010). Stock cultures were maintained in modified MTM medium (Sani *et al*., 2001), which contains 1g/L NH_4Cl, 0.06 g/L $CaCl_2.6H_2O$, 0.05 g/L yeast extract, 1g/L $MgSO_4.7H_2O$, 2 g/L Na_2SO_4, 5g/L sodium lactate and 20 mg/L of uranium (VI), as uranyl acetate dehydrate. This medium was optimized in order to avoid chemical removal of uranium. The bacterial consortia were sub-cultured every 4 weeks using 10% (v/v) of inoculum and incubated at room temperature ($21 \pm 1^{o}C$).

2.2. Uranium (VI) bio-removal experiments

The studies of U (VI) bio-removal were performed in 35 mL glass bottles, in anaerobic conditions, using the MTM growth medium previously described, containing 22 mg/L of uranium (VI). Each set of experiments was carried out in duplicate. The medium was purged with nitrogen gas to achieve an anaerobic environment prior to inoculation. After inoculation, oxygen diffusion was eliminated by adding 5 mL of sterile liquid paraffin. The bottles were sealed with butyl rubber stoppers and aluminium crimp seals and incubated.

2.2.1. U (VI) removal by live cells in different conditions

The effect of pH and temperature on uranium (VI) removal was tested for both bacterial consortia in order to found the optimal conditions for uranium (VI) removal. The pH of the medium was adjusted to 4.6, 6.2 and 7.2 with 1M NaOH or HNO_3 and incubation was performed at room temperature (21±1ºC). For uranium (VI) bio-removal experiments at different temperatures, medium with pH 7.2 was used. Temperatures of 4ºC, 21ºC and 37ºC were tested.

Bacterial cells obtained previously were harvested by centrifugation at 4000 rpm for 10 min, washed with MTM medium, inoculated (10% v/v) in the bottles containing MTM medium with 22 mg/L U (VI) and incubated at the previously described conditions. For each experimental set an abiotic control (without bacteria) was carried out.

2.2.2. U (VI) removal by heat-killed cells

Bacterial cells (30 mL) collected after 20 days of incubation were harvested by centrifugation at 4000 rpm for 10 min and washed with MTM medium. The cells were killed by autoclaving (121ºC, 30 min) and added to bottles contained MTM medium with 22 mg/L U (VI) (pH=7.2).

2.2.3. U (VI) removal by extracellular metabolic products

U (VI) was added to 30 mL of cell-free medium obtained from the bacterial cultures after 20 days of growth. The medium was filtered with a 0.2 µm hydrophilic polyestersulfone membrane (Machererey-Nagel) to remove cells and purged with nitrogen gas.

2.3. TEM-EDS, FTIR and XRD analyses

The precipitates generated during the bio-removal process were characterized by X-ray powder diffraction (XRD). Transmission electron microscopy coupled with an energy dispersive spectrometer (TEM-EDS) and Fourier transform ***infrared*** (***FTIR***) spectroscopy were also used to assess possible metal-bacterial cells interactions. X-ray diffraction pattern of dried powder samples was performed using Bruker powder diffractometer (model D8 Advanced) using Cu-Kα radiation. The diffraction pattern was recorded from 3º to 60º (2θ) with a step length of 0.05º and time per step 20.0s. TEM-EDS (Hitachi H8100) was used to establish the localization of the metal precipitates in the cells and the elemental characterization of the metal deposits. Samples of bacterial cells exposed to uranium (VI) were prepared for TEM by fixation with glutaraldehyde 3% folowed by dehydration and embedding in Epon-Araldite (Glauert, 1975). Thin sections were studied without staining for detection of electron-dense precipitates. For FTIR spectroscopic analysis, samples of bacterial cultures exposed and not exposed to uranium (VI) were dried and blended with KBr. The FTIR spectra were recorded within the range 400-4000cm^{-1} in Bruker, Tensor 27 FTIR spectrophotometer.

2.4. Analytical methods

Periodically, samples from cultures were collected using a syringe. Optical density at 600 nm (OD600) and uranium (VI) were measured in each sample. Uranium was measured after centrifugation of samples at 4000 rpm for 5 min and quantified using the method described by Martins *et al.* (2010): 900 µL of sample was mixing with 300 µL of 0.5 M HCl, followed by the addition of 300 µL of Arsenazo III (0.1% w/v). After 3 min, the

purple-colour metal-arsenazo III complex was quantified spectrophotometrically at 652 nm. The Arsenazo III solution was prepared by dissolving the reagent in 0.01 M HCl and in 10 % (v/v) ethanol.

2.5. Molecular characterization

2.5.1 Extraction of DNA, PCR amplification and cloning of 16S rRNA gene

Total genomic DNA was extracted after harvesting cells by centrifugation at 4000 rpm for 10 min. DNA extraction was carried out as described by Martins *et al.* (2009).

Amplification of full-length 16S rRNA gene was performed using the primer pair 8F (5'-AGA GTT TGA TCC TGG CTC AG -3') / 1492R (5'- GGT TAC CTT GTT ACG ACT T -3') (Suzuki *et al*., 2003). The primers were purchased from Thermo Fisher Scientific. The reaction mixture used for PCR amplification contained 31.75 µL of sterilised MiliQ water, 1 µL of each primer (10 pmol/µL), 1 µL of dNTP`s (10 mM), 4 µL of $MgCl_2$ (25 mM), 10 µL of 5×Go Taq® buffer (Promega, Madison, USA), 0.25 µL of GoTaq®DNA polymerase (Promega, Madison, USA), and 1 µL of DNA. PCR amplification was performed in a thermocycler (T1, Biometra, USA). Thermal cycling was carried out by using an initial denaturation step of 94ºC for 3 min, followed by 30 cycles of 94ºC for 1 min, 60ºC for 1 min and 72ºC for 2 min and completed with an extension period of 5 min at 72ºC. The PCR products were analyzed by electrophoresis, in 1% (w/v) agarose gel and TAE buffer. The band with the proper size range (approximately 1.4 Kb) was excised and purified with E.Z.N.A.^{TM} Gel Extraction Kit (Omega).

The purified products were ligated into the cloning vector pGEM®-T Easy according to the manufacturer's instructions (Promega, Madison, USA), followed by transformation into *Escherichia coli* DH5*α* competent host cells. The white colonies were screened for inserts by amplification with a vector- specific primer set (Sp6 and T7). Thermal cycling was carried out by using an initial denaturation step of 94ºC for 3 min, followed by 30 cycles of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 2 min and completed with an extension period of 5 min at 72ºC. The PCR products were analyzed by electrophoresis, in 1% (w/v) agarose gel and TAE buffer and the clones containing expected DNA insert were saved at -20ºC.

2.5.2. Restriction fragment length polymorphism analysis (RFLP) of 16S rRNA gene

RFLP analysis of the previously amplified 16S rRNA gene was performed using the restriction enzymes *Hha*I and *Msp*I (Promega) to search for similar rRNA gene clones. Fragments of the digested PCR products were separated in a 2% (w/v) TAE agarose gel. A representative clone from each digestion pattern was selected for sequencing. The 16S rRNA gene inserted in plasmids was amplified using the primers Sp6 and T7, according to the conditions described above. PCR products were purified using the Jetquick PCR Purification (Genomed GmbH, Lohner, Germany) and sequenced by CCMAR (Centro de Ciências do Mar, Universidade do Algarve). Sequences obtained in this study have the following accession numbers: GU255481 to GU255507.

2.5.3. Phylogenetic analysis

For phylogenetic analysis, sequence alignments were made with Clustal X (Thompson, 1997) and visually corrected. The Bayesian Markov chain Monte Carlo (MCMC) method of phylogenetic inference (Huelsenbeck and Ronquist, 2001) was applied to estimate phylogenetic relationships using MrBayes software (Larget and Simon, 1999). This method allows the estimation of the a *posteriori* probability that groups of taxa are monophyletic given the DNA alignment (i.e., the probability that corresponding bipartitions of the species set are present in the true unrooted tree including the given species). This Bayesian approach was repeated several times, using random starting trees and default starting values for the model parameters to test the reproducibility of the results.

3. Results

3.1. Effect of pH and temperature on U (VI) removal by bacterial communities

The influence of pH and temperature on the efficiency of U (VI) removal by bacterial communities from Monchique thermal place (A) and from Urgeiriça mine (U) was

studied (Fig. 8.1). Considering pH, it was observed that the metal removal and bacterial growth were affected by this factor (Fig. 8.1a). For both consortia, the U (VI) removal efficiency increased with pH increase. The best performance was observed at pH 7.2. At this pH value, both consortia achieved 97 % of U (VI) removal after 16 days of incubation, while at pH 6.2 only 70 % and 77 % of U (VI) was removed by consortia A and U, respectively. At pH 4.6 no relevant removal of U (VI) was observed. The decrease of U (VI) in the abiotic sets was 16 % at pH 4.6 and 22% at pH 6.2 and 7.2 on the end of the experiments (data not shown). Bacterial growth was not affected by pH in the same way as metal removal, as the growth of both cultures at pH 6.2 was higher than at pH 7.2.

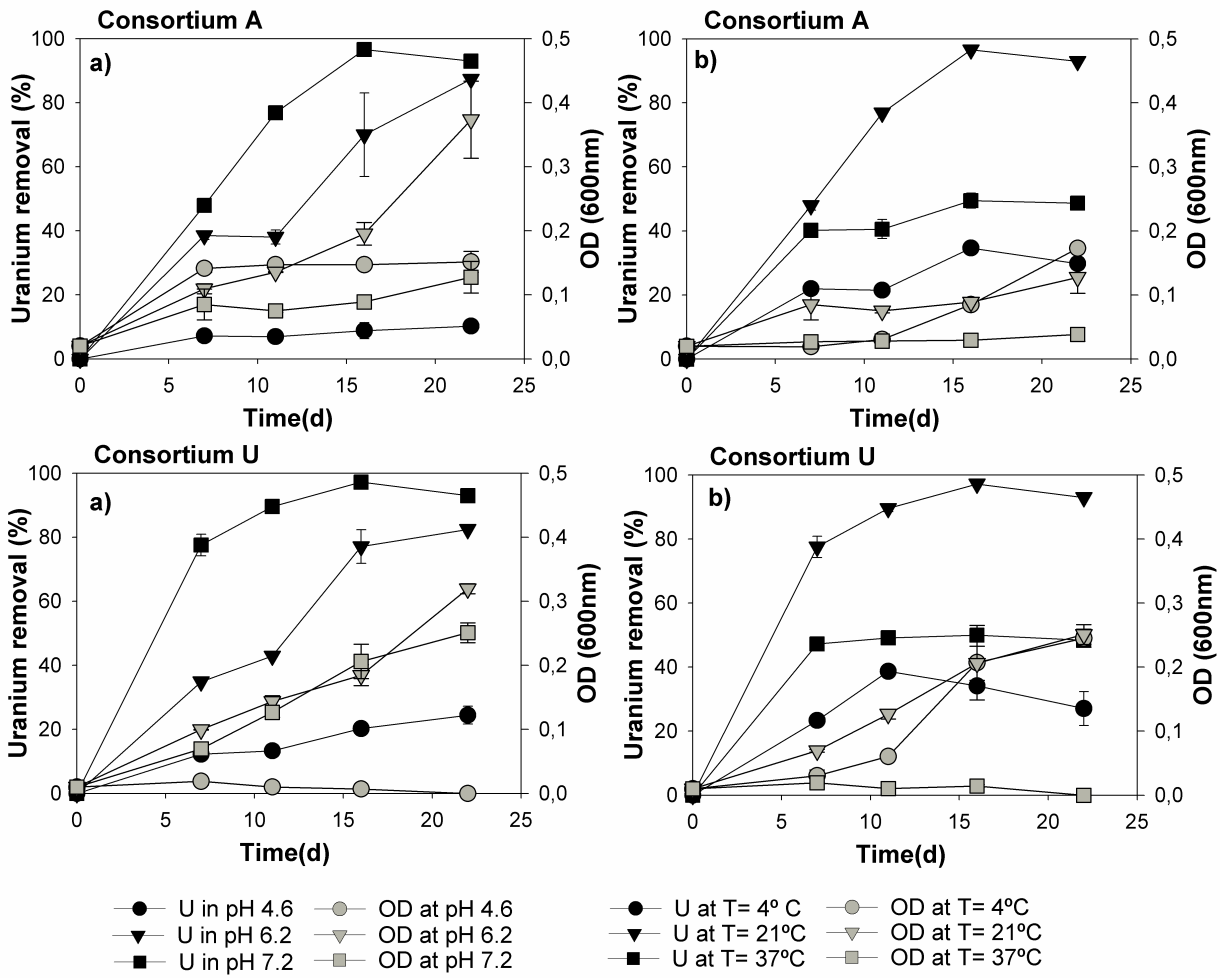

Fig. 8.1 Effect of pH (a) and incubation temperature (b) on uranium removal and growth of bacterial consortia from Monchique thermal place (A) and from Urgeiriça mine (U). Data are the average of duplicates and error bars indicate the standard deviations of the average values.

The effect of temperature incubation on U (VI) removal by bacterial consortia A and U is shown in Fig. 8.1b. Both cultures presented the highest U (VI) removal rate (97 % in 16 days) at room temperature (± 21ºC). At 37ºC only about 50 % U (VI) removal was achieved within the same period of time for both mixed cultures, but no relevant bacterial growth was detected. At 4ºC no obvious U (VI) removal was observed, though bacterial growth was observed in this case. Although the lag phase was higher, bacterial growth achieved similar OD_{600} values at 4ºC and 21ºC at the end of the experiment. The results of U (VI) decrease in the abiotic sets were 22 % at room temperature and 29 % at 4ºC and 37 ºC at the end of the experiments.

3.2. U (VI) bio-removal experiments

Fig. 8.2 shows uranium (VI) removal by live cells, heat-killed cells and extracellular metabolic products. The highest U (VI) removal from the medium was observed with live cells: 97 % of U (VI) was removed by both consortia in 16 days. However, no relevant uranium (VI) removal was observed with heat-killed cells, comparatively to the abiotic sets: 27 % for inoculum A and 11 % for inoculum U, while 22 % was achieved in the abiotic solutions. U (VI) removal by extracellular metabolic products was not detected.

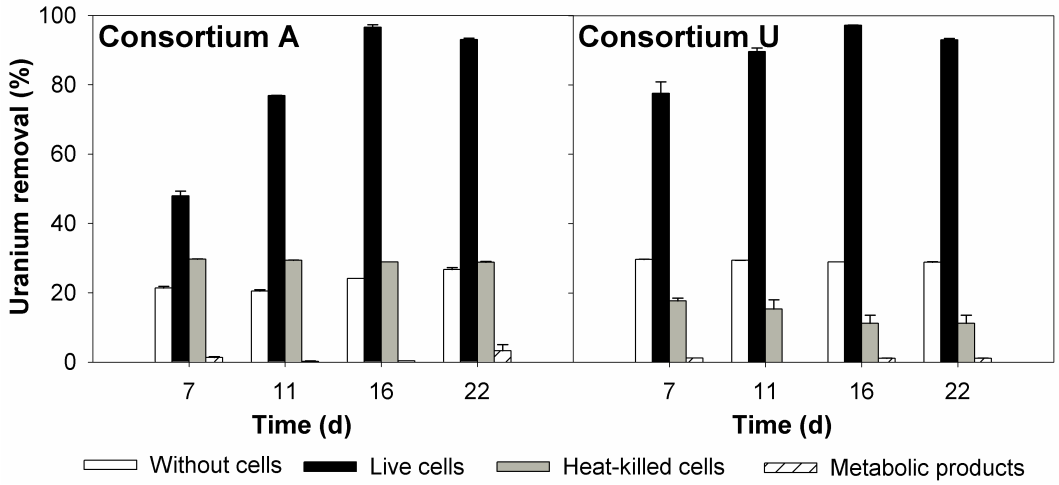

Fig. 8.2 Uranium removal from medium with 22 mg/L uranium by cells (live and heat-killed), and by extracellular metabolic products (pH 7.2; 21ºC). Data are the average of duplicates and error bars indicate the standard deviations of the average values.

3.3. X-ray powder diffraction analysis (XRD)

The black precipitate generated during the bio-removal experiments was composed by amorphous and crystallized material. Characterization of the mineral phase by XRD gave a spectrum that in accordance with PDF2 database is consistent with the presence of uranium oxide as U_3O_7 (Fig. 8.3).

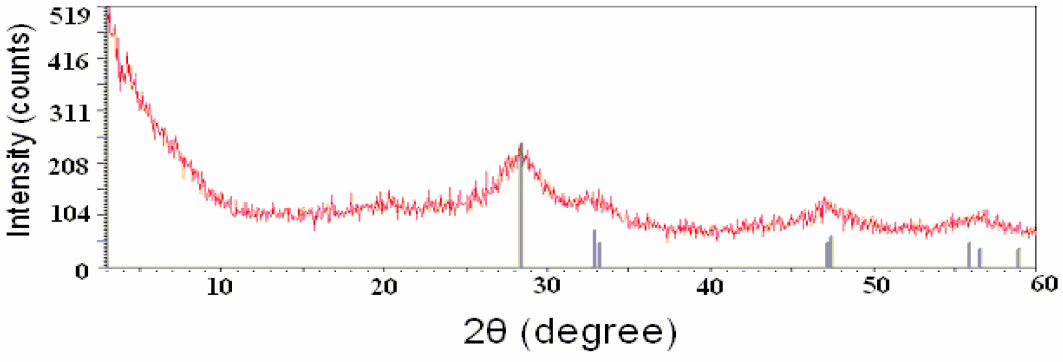

Fig. 8.3 X-ray diffraction spectrum of the precipitate formed during uranium bio-removal experiments. The vertical lines indicate the characteristic X-ray diffraction of U_3O_7.

3.4. Cellular localization of uranium

To establish the distribution and localization of uranium deposits in the cells, thin sections of cells exposed to uranium (VI) were viewed using TEM (Fig. 8.4). The cells of consortia A and U exhibited dense precipitates mainly within the periplasmic region (Fig. 8.4a, b, f, g and h). Fibrillar precipitates were observed in the capsule of some cells of consortium A (Fig. 8.4c and d) and round precipitates were also occasionally present in the cytoplasm of the cells of this consortium (Fig. 8.4e). Using EDS coupled to TEM for the elemental characterization of the metal deposits it was possible to confirm that the dense precipitates observed contained uranium (Fig. 8.4i). EDS spectrum also showed the presence of other elements such as P, Cu, Cl and Si. Some elements observed in the spectrum like Cu, Cl and Si, could be originated from external sources, since were also present in background areas and therefore represent elements from the supporting grid.

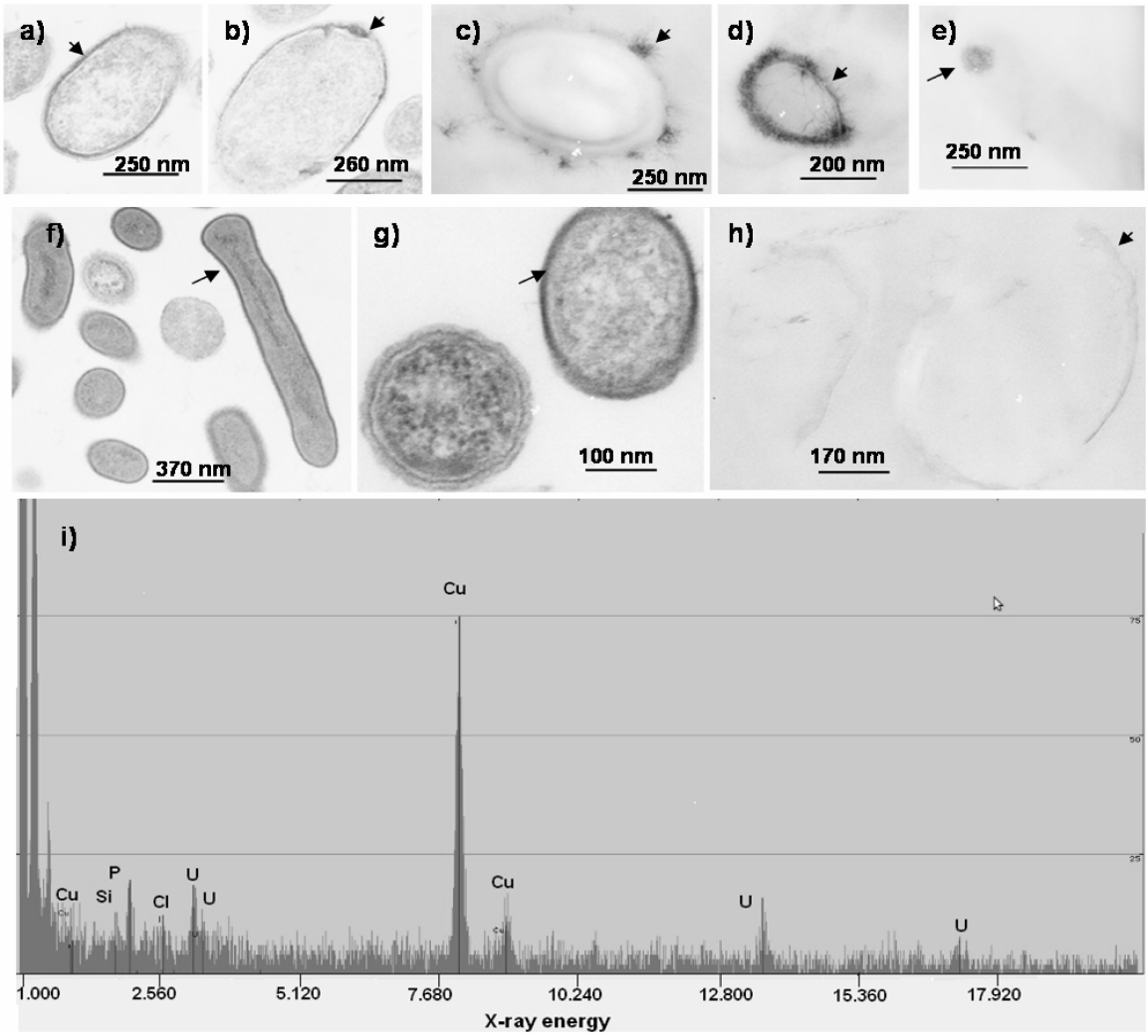

Fig. 8.4 TEM of thin sections (70-90 nm) of bacterial cells of inoculum A (a, b, c, d and e) and of inoculum U (f, g and h) after 20 days of growth with 22 mg/L U (VI): cells sections stained with uranyl acetate and lead citrate (a, b, f and g) and cells without staining (c, d, e and h). (→) Dense precipitates. EDS spectrum of precipitate (i).

3.5. FTIR spectroscopy

FTIR spectral analysis of control (metal-free) and uranium loaded cells allows some characteristic peaks to be assigned to the main functional groups present in the bacterial cells and to their role in metal binding process. Correspondences of the IR frequencies in the present study were based on known data from literature (Tellez *et al.*, 1995; Kamneva *et al.*, 1997; Pagnanelly *et al.*, 2000; Jiang *et al.*, 2004; Craciun *et al.*, 2005; Acharya *et*

al., 2009; Choudhary and Sar, 2009; Kazy *et al.*, 2009). The FTIR spectra from 400 to 4000 cm^{-1} of control cells (metal-free) and metal loaded cells are shown in Fig. 8.5.

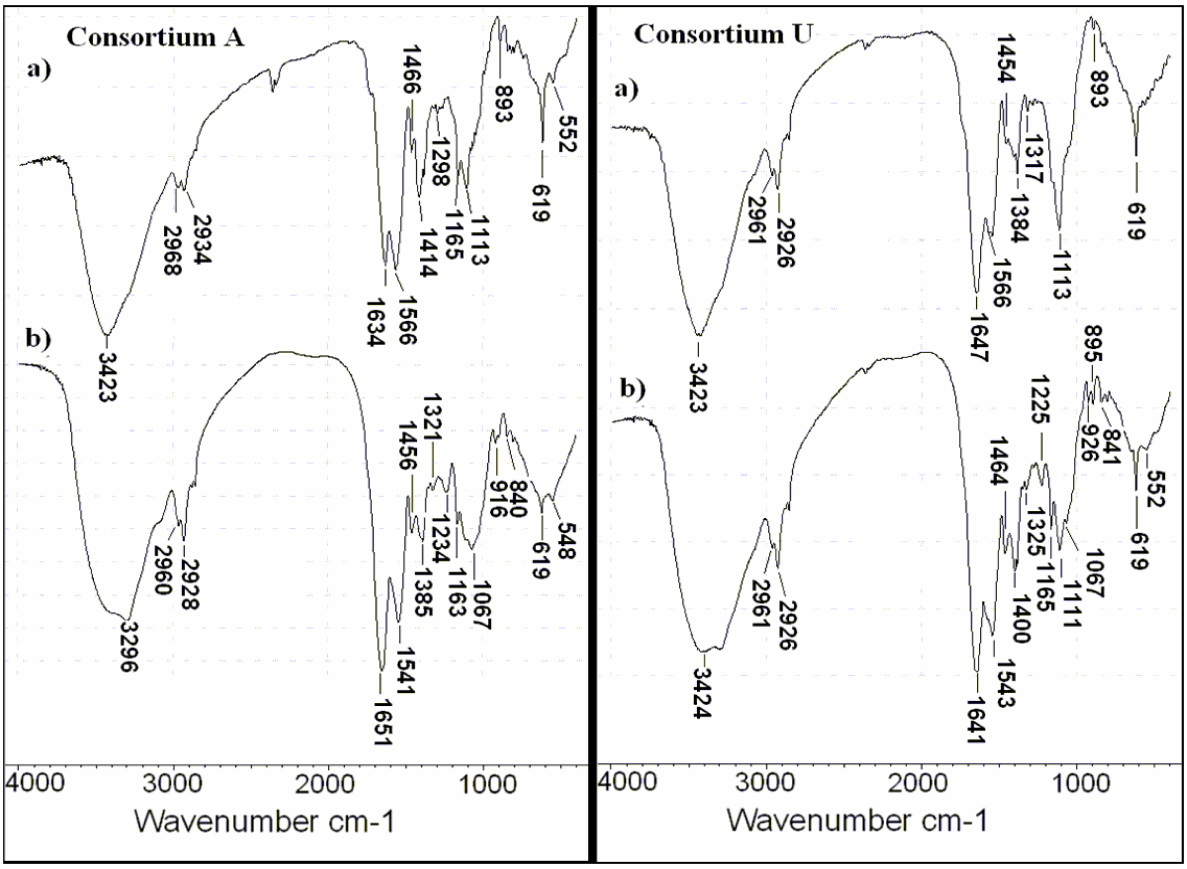

Fig. 8.5 FTIR spectra of bacterial cells of consortia obtained from Monchique thermal place (A) and from Urgeiriça mine (U): control cells (a) and metal loaded cells (b).

The spectra of control and metal loaded cells of consortia A and U showed a broad band between 3700 and 3000 cm^{-1} with a maximum around 3400 cm^{-1}. Bands corresponding to the N–H bond of amino groups, along with the O–H of the hydroxyl groups, usually lay in the region between 3800 and 3200 cm^{-1}.

All four spectra showed the presence of two peaks between 3000 and 2900 cm^{-1} which can be attributed to the asymmetric stretching of C–H bond of the $-CH_2$ groups combined with that of the $-CH_3$ groups.

The strong broad band between 1700 and 1470 cm^{-1} can be assigned to amide groups. The C=O stretching of amide (referred to as amide I) was observed at 1634 (consortium A) and 1647 cm^{-1} (consortium U) while the N-H bending (coupled to C-N stretching and referred to as amide II) appeared at 1566 cm^{-1} in both consortia. The spectrum of metal loaded cells (Fig. 8.5b) showed a shift of these bands to 1651 and 1541 cm^{-1} in consortium A and to 1641 and 1543 cm^{-1} in consortium U. Furthermore, a change in the relative intensities of the above bands was observed in cells of culture A.

In all spectra a peak around 1460 cm^{-1} was observed, which is characteristic of the scissoring motion of $–CH_2$ groups.

The peaks within 1400-1200 cm^{-1} are due to $COO^–$ symmetric stretching, non-ionized carboxylic groups and P=O stretching of the $C\text{-}PO_3^{2-}$ moiety. The peaks observed at 1298 or 1317 cm^{-1} in consortia A and U, respectively, are corresponding to stretching of non-ionized carboxylic groups (C-OH). The C-OH bands were shifted to 1321 (consortium A) and 1325 cm^{-1} (consortia U) after cell exposure to uranium (Fig. 8.5b). The peaks at 1234 and 1225 cm^{-1} observed in uranium loaded cells of consortia A and U, respectively, correspond to P=O stretching of the $C\text{-}PO_3^{2-}$ moiety.

A complex band was observed in the range 1200-950 cm^{-1}, which corresponds to C-O, C-C and C-H bonds in carbohydrates and alcohols, along with the symmetric and asymmetric stretching bands of PO_2^- and $P(OH)_2$ from phosphates. Two peaks at 1165 cm^{-1} and at 1113 cm^{-1} were observed in control cells A (Fig. 8.5a) and one peak at 1113 cm^{-1} in control cells U (Fig. 8.5a). In the spectra of metal loaded cells a broadening of the last band (at 1113 cm^{-1}) was evident, which results in the appearance of a new maximum at 1067 cm^{-1} in both spectra.

The absorption peaks at 893 cm^{-1} (control cells A and U) and 895 cm^{-1} in metal loaded cells may be attributed to substituted ethylenic system CH=CH groups.

In the spectra of metal loaded cells (Fig. 8.5b)new bands at 916 and 841 cm^{-1} were observed in cells A, and at 926 and 841 cm^{-1} in cells U.

A broad band between 700 and 400 cm^{-1}, with a maximum at 619 cm^{-1} was due to O-H bending. A change in this band shape in the metal loaded cells was observed. In metal loaded cells A, a band at 548 cm^{-1} and a band at 552 cm^{-1} in cells U were also observed.

3.6. Phylogenetic analysis

All recombinant colonies (87) were recovered and approximately 1.4 kb fragment of bacterial 16S rRNA gene was amplified and used for RFLP analysis. Sixteen RFLP groups were originated from mixed culture A and thirteen from mixed culture U. Phylogenetic analysis of the representative clones allowed the identification of the corresponding sequences (Fig. 8.6).

Most of clones sequences (59 %) from community A were closely related to *Sporotalea*, while majority of clones from community U were affiliated to *Clostridium* (53%). Clones with sequences closely related to *Clostridium* were also present in community A (2%). Both communities included members of *Rhodocyclaceae* family. Phylogenetic analysis also showed that sequences of 26 % of clones from community A and 32 % of clones from community U were closely related to *Rhodocyclus* or *Propionivibrio* and bacteria affiliated to *Brevundimonas* were also present in both communities (9 % and 3 % of clones from consortia A and U, respectively). Additionally, community A included bacteria closely related to *Pelosinus* (4 % of clones). Bacteria affiliated to *Sphaerochaeta* (3 %) and *Anaerofilo pentosovarans* (9 %) were also detected in community U.

4. Discussion

In previous studies (Martins *et al*., 2010) were discovered three bacterial communities with ability for uranium (VI) removal: one was recovered from a soil sample of an uncontaminated site (Monchique thermal place), while the other two were obtained from sediments collected in uranium mine site (Urgeiriça). Thus, in the present work, the mechanism of uranium (VI) removal from aqueous solutions by two of these cultures (one from an uncontaminated site and other from an uranium mine) was investigated for the first time.

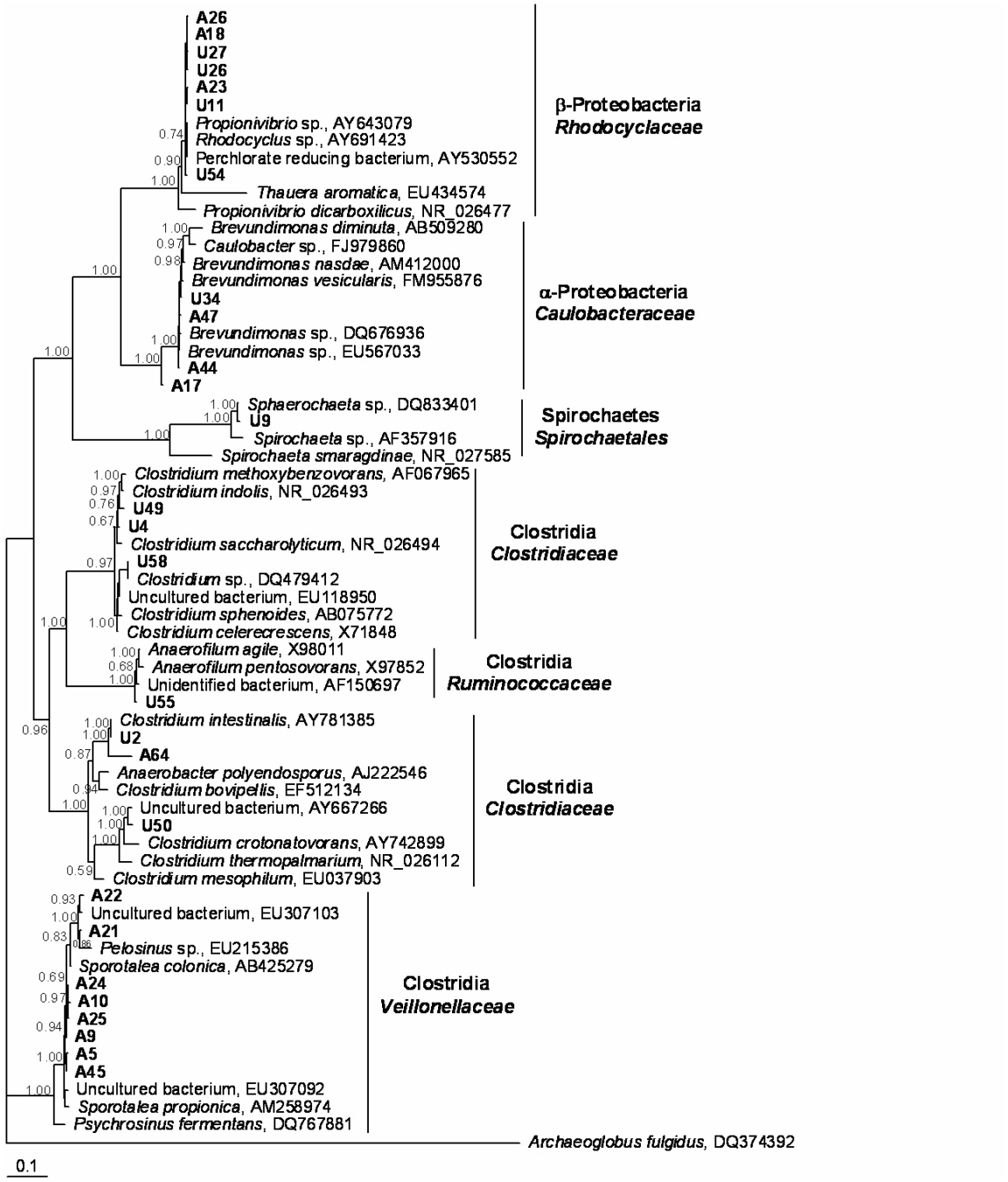

Fig. 8.6 Phylogenetic tree obtained with 16S rRNA sequences (1300 nucleotide positions), corresponding to the clones representative of each restriction profile and to the most closely related ones retrieved from BLAST search. Phylogeny was inferred using the Bayesian Markov Chain Monte Carlo analysis of aligned 16S rRNA fragments. *Archaeoglobus fulgidus*, a species from Archaea Domain was included to root the tree. Probability values associated to each node are showed. Access numbers of GenBank sequences are indicated in the figure and names in bold face correspond to sequences determined in this work. The main bacterial groups detected in bacterial consortia are also indicated.

The U (VI) removal was tested with live and heated-killed cells, as well with extracellular metabolic products. U (VI) removal by both communities was only observed in the presence of live cells. The lack of metal removal by extracellular products and heat-killed cells suggests that only viable cells can be responsible for uranium (VI) removal from the solution. A slight decrease in the uranium (VI) concentration in the abiotic solutions was also observed. This fact, already mentioned in the literature, is reported as related to the adsorption of the metal to the glass material (Spear *et al*., 1999; Acharya *et al*., 2009). This explanation was supported by the fact that no decrease of U (VI) was observed when plastic material was used (data not shown), even in the sets with extracellular metabolic products. The solution with extracellular metabolic products may contain other substances that can also be adsorb to the glass bottles, thus, competing with the metal to the adsorption sites.

X-ray diffraction analysis of the precipitate formed during the bio-removal experiments showed that it was mainly composed by uranium oxide as U_3O_7. The presence of U_3O_7 indicates that the mineral phase was composed by a mixture of uranium (VI) and (IV). This result can be explained by the slight oxidation of uranium (IV) due to oxygen exposition. The presence of U (IV) was also consistent with the black colour of the precipitate formed. Typically, U (VI) has an orange/yellow colour, while the precipitate of UO_2 shows a black/brown colour (Yu and Hanson, 1988; Magnuson *et al.*, 2006). The presence of U (IV) in the precipitate, together with the fact that uranium (VI) was only removed from solution in the presence of live cells, suggests a mechanism of enzymatic reduction, where U (VI) is converted to insoluble U (IV).

Many microorganisms affiliated with genera *Desulfovibrio*, *Geobacter*, *Tolumonas*, *Clostridium*, *Arthrobacter*, *Dechlomonas*, *Shewanella* and *Pseudomonas* can reduce U (VI) to U (IV) (Wall and Krumhols, 2006; Akob *et al*, 2008; Gao and Francis, 2008). *Clostridium* species are considered one of the major players in uranium (VI) reduction (Suzuki *et al.*, 2003; Gao and Francis, 2008) and bacteria affiliated to this genus were present in both consortia. Bacteria affiliated with *Pelosinus* were also found in community A and are reported as capable of reducing Fe (III) (Shelobolina *et al*., 2007). Considering that dissmilatory Fe (III)-reducing bacteria have already been reported as having the ability for U (VI) reduction (Gorby and Lovley, 1992), U (VI) reduction by

Pelosinus can not be excluded. Regarding *Rhodocyclaceae* members, they have been reported in uranium contaminated mines (Suzuki *et al*., 2003), and also in microbial populations stimulated for uranium removal (Akob *et al*., 2008), but to date the knowledge about their role on uranium removal is limited.

The maximum U (VI) removal by consortia A and U occurred at room temperature (± 21ºC) and with pH 7.2. However, at 4ºC bacterial growth was observed without significant uranium (VI) reduction. This result can be explained by the presence of different species in the consortia, which probably have different optimal growth conditions. Thus, this temperature (4ºC) may promote the growth of metal resistant bacteria, but without ability for uranium (VI) removal. Other explanation is that, since the enzymatic process is the dominant mechanism for uranium (VI) removal, at 4ºC the culture can grow but the enzymes responsible for metal reduction can not be expressed. It was reported that U (VI) reduction by dissmilatory Fe (III)-reducing bacteria (GS-15) (Gorby and Lovley, 1992) and Cr (VI) reduction by *Enterobacter cloacae* (Wang *et al*., 1990) were repressed at 4ºC.

It was also observed that uranium (VI) reduction increased with the increase of pH. The increase of pH can promote the optimal conditions for the expression of the enzymes responsible for uranium (VI) reduction. The influence of pH in metal reduction was also observed in previous studies concerning uranium (VI) reduction by *Clostridium* sp. (Gao and Francis, 2008) and platinum (IV) reduction by a sulphate-reducing bacteria consortium (Rashamuse and Whiteley, 2007).

Due the insoluble nature of U (IV), the site of its deposition in cell should give an indication of the reductase location. Hence, TEM was used to establish the distribution and localization of uranium deposits in the cells. TEM images showed the presence of dense precipitates mainly within the periplasmic region of cells of both consortia. This observation is consistent with other studies that reported the existence of reductases in the periplasmic region, in the outer membrane, or in both (Wall and Krumhols, 2006; Rashamuse and Whiteley, 2007). Fibrillar precipitates were also observed in the capsule of some cells of consortium A and round precipitates were also occasionally present in the cytoplasm. Ohnuki *et al.* (2005) have reported the presence of fibrillar precipitates contained uranium in cells of *Saccharomyces cerevisiae*. The presence of uranium

precipitates in the cytoplasm of some cells of inoculum A can be explained by the ability of some bacteria of this consortium to accumulate uranium inside the cells. Since the mixed culture was composed by diverse species of bacteria, the occurrence of more than one mechanism of uranium removal was expected.

Bacterial cells were also analyzed by energy dispersive spectrometer (EDS) coupled to TEM, allowing the confirmation of uranium presence in the dense precipitates. EDS could identify not only the elements present in the precipitate but also those of the microbial cells, such as phosphorous, which is an essential element in the bacterial cell wall (Kazy *et al.*, 2009).

The presence of dense uranium precipitates around bacterial cells, specially in consortium A, can be explain by the fact that bacteria are excellent nucleation sites for mineral formations (Rashamuse and Whiteley, 2007), due to the electronegative surface of functional groups such as carboxyl, hydroxyl, phosphoryl and amide groups (Jiang *et al.*, 2004; Acharya *et al.*, 2009). Therefore, after U (VI) reduction, the U (IV) ions could be bound to functional groups of bacterial cell surface. FTIR spectroscopic was applied to determine the functional groups of the bacterial cells that can be involved in U binding. According to Jiang *et al*. (2004), the FTIR spectra of Gram-positive and Gram-negative bacteria are similar. This is in accordance with the fact that no significant differences in the spectra of both consortia were observed.

The amide I absorption peak was mainly accounted by 3_{10}-helical secondary structure of proteins, although amino sugars (with N-acetyl/glucuronamide groups) from cell associated polysaccharides could also show an absorbance band in this region (Choudhary and Sar, 2009; Kazy *et al.*, 2009). The shift of the peaks of the amide groups after uranium exposition indicates a possible interaction of metal with cellular proteins. Furthermore, a change in the relative intensities of these bands was observed in consortium A. These changes in peak positions and relative intensities, most probably reflect some alteration in the secondary structure of cellular proteins from the predominant 3_{10}-helix to other possible conformation as a result of radionuclide sequestration (Kazy *et al.*, 2009). The shift of the C-OH bands of carboxylic groups (from 1298 cm^{-1} to 1321 cm^{-1} in consortium A and from 1317 cm^{-1} to 1325 cm^{-1} in consortium U) after uranium exposure and reduction could reflect the involvement of these groups in

metal binding. In fact, the strong role of carboxylic groups in radionuclide binding after sorption of U and Th by *Pseudomonas* sp. has already been reported by Kazy and co-workers (Kazy *et al*., 2009). Furthermore, the intense peak at 1234 and 1225 cm^{-1} observed in the uranium loaded samples A and U, respectively, is probably a result of uranium binding to the phosphate (Kazy *et al*., 2009). In the spectra of metal loaded cells, modifications were observed in the complex band in the range 1200-950 cm^{-1}, corresponding to C-O, C-C and C-H bonds in carbohydrates and alcohols, along with the symmetric and asymmetric stretching bands of PO_2^- and $P(OH)_2$ from phosphates (Jiang *et al*., 2004; Choudhary and Sar, 2009). The above groups, mostly belonging to various cellular components like phospholipids, nucleic acids, peptidoglycan, cell associated polysaccharides and peptides, are able to complex different metals (Pagnanelly *et al.*, 2000; Choudhary and Sar, 2009). Following metal exposition, a broadening of the band at 1113 cm^{-1} and a new peak at 1067 cm^{-1} were observed. Both changes may be attributed to U-O asymmetric stretching in U(IV) oxides (Craciu *et al.*, 2005) formed during the process. The change of band shape between 700 and 400 cm^{-1} observed in the metal loaded biomass comparatively with the control, could also be assigned to the presence of U–O bonds (Kazy *et al*., 2009).

The band observed at 916 (consortium A) or at 926 cm^{-1} (consortium U) in metal loaded cells may be ascribed to asymmetric stretching of U=O bonds, either in remaining UO_2^{2+} or in U(VI) complexes formed with bacteria functional groups. The peak at 840 cm^{-1} can correspond to symmetric stretching of the last one (Téllez *et al*., 1995; Kazy *et al.*, 2009). It was observed that the first band was stronger in a non-freshly prepared sample (data not shown) suggesting that oxygen exposition promotes metal oxidation, which results in an increment of the peak around 920 cm^{-1}. Finally, the band at 548 (consortium A) or 552 cm^{-1} (consortium U) can be attributed to symmetric stretching of weekly bonded oxygen ligands ($U–O_{ligand}$), both in U(IV) and U(VI) (Téllez *et al*., 1995; Kazy *et al.*, 2009). The overall spectral analysis indicates that carboxyl, amide and phosphate groups of bacterial cells are the dominant functional groups involved in bacteria-metal interaction. The involvement of phosphate groups is also in concordance with the presence of phosphorous in the EDS spectrum.

Uranium (VI) was only removed from solution in the presence of live cells and the presence of U (IV) in the precipitate was observed. These results together with the presence of dense precipitates mainly within the periplasmic region of cells of both consortia suggest a mechanism of enzymatic reduction by both consortia. Moreover, the presence of uranium precipitates in the cytoplasm of some cells of inoculum A suggests that this consortium could accumulate uranium inside the cells. Therefore, the present work showed that U (VI) removal by consortium A was carried by two mechanisms: enzymatic reduction and bioaccumulation, while the enzymatic process was the only mechanism involved in U (VI) removal by consortium U. The results also suggest that probably the process can be divided in two steps: first the enzymatic reduction of U (VI) to U (IV) occurs and then U (IV) binds to carboxyl, phosphate and amide groups of bacterial cells. These results are in accordance to those reported for plutonium removal by *Bacillus subtilis* (Ohnuki *et al*, 2007).

5. Conclusions

The present work demonstrated the ability of two anaerobic bacterial communities for U (VI) removal. Both communities were composed by several species of bacteria, a large number of them never reported as U (VI) reducing bacteria or even metal resistant. This is the case of *Sporotalea* sp., *Rhodocyclaceae* members, *Anaerofilo pentosovarans*, *Brevundimonas* sp., *Pelosinus* sp. and *Sphaerochaeta* sp. This result is a relevant finding, encouraging the exploitation of microorganisms with new abilities that can be useful for bioremediation purposes. Uranium is not known to be an essential component for biologic function and is toxic. Therefore, the discovery that the mechanism of U (VI) removal by these cultures occurs mainly through an enzymatic reduction opens a new research field for understanding the enzymes responsible for metal reduction.

6. References

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Chapter 9

Concluding remarks and future perspectives

Concluding remarks

The search of bacterial consortia highly resistant to metals is extremely important for the development of an efficient bioremediation technology. A SRB consortium resistant to high concentrations of heavy metals (Fe, Cu and Zn), similar to those typically present in AMD, was obtained among several environmental samples, from a wastewater treatment plant. In addition, the ability of this highly metals tolerant SRB consortium to use ethanol as carbon source is a promising result considering an eventual utilization of ethanol rich wastes, which are easily available in Portugal. Therefore, that consortium can be a potential candidate for a bioremediation process for the decontamination of AMD.

AMD are usually deficient in electron donors and require their external addition to achieve complete sulphate reduction. Considering that sulphate reduction is an energy intensive process, a considerable amount of an energy-rich reductant is required. Consequently, the choice of the carbon source has an important effect on the efficiency and economical viability of the bioremediation technologies based on the use of SRB. The search for efficient, low cost and largely available carbon sources (preferably wastes) for SRB to be used in bioremediation processes for the treatment of sulphate rich effluents is of outmost importance. Taking into account the results achieved, the waste from the wine industry in the presence of calcite tailing seems to be promising as carbon source to promote SRB activity. The possibility of using food industry wastes, particularly the wastes from wine industry, to promote an efficient sulphate reduction is an important finding. In this way these wastes can be reutilised in bioremediation processes based on SRB for the treatment of sulphate rich effluents, with both environmental and economical benefits.

The present study demonstrated that the type of carbon source (wine wastes or ethanol) modulated the bacterial community responsible for the AMD bioremediation process based one SRB activity. The bacterial diversity was higher in the bioreactor fed with wine wastes than in the bioreactor fed with ethanol. The presence of SRB and fermentative bacteria (*Clostridium* sp., Bacteroidales order, *Citrobacter* sp. and *Cronobacter* sp.) in

the bioreactor fed with wine wastes suggests a synergistic interaction between these bacterial groups, which can be the key factor for the use of such complex organic substrate, as carbon and electron source for sulphate reduction. The evaluation of the performance of the treatment together with the monitoring of the dynamics of the bacterial community is relevant topic for a better understanding the bioremediation process.

Several microbial communities were investigated for their ability to remove uranium (VI) and additionally the impact of U(VI) on SRB communities was explored. The present work demonstrates that the structure of the community was drastically changed during the uranium bio-removal studies. The original communities were mainly composed by SRB, but after uranium exposure these bacteria were not detected in the communities. Moreover, it was observed that the composition of the communities was influenced by the type of metal present in the medium. When U(VI) was replaced by Cr(VI) several differences in the structure of the bacterial community were observed. TGGE and phylogenetic analysis of 16S rRNA gene showed that members of *Rhodocyclaceae* family and *Clostridium* genus are predominant in the uranium removal communities, while the community established in the presence of Cr(VI) was mainly composed by members of *Rhodocyclaceae* and *Enterobacteriaceae* families and *Clostridium* genus. This change in the bacterial community when uranium (VI) was replaced by chromium (VI) is a result of most importance, specially considering that these changes are usually not considered. These results are important findings emphasizing, besides the evaluation of the efficiency of metal bio-removal, the importance of monitoring the microbial community structure during bioremediation processes.

The mechanism of uranium (VI) removal by two anaerobic bacterial consortia, one recovered from an uncontaminated site and other from a uranium mine was investigated. Both communities were composed by several species of bacteria, a large number of them never reported as U (VI) reducing bacteria or even metal resistant. This is the case of *Sporotalea* sp., *Rhodocyclaceae* members, *Anaerofilo pentosovarans, Brevundimonas* sp., *Pelosinus* sp. and *Sphaerochaeta* sp. This result is a relevant finding, encouraging the

exploitation of microorganisms with new abilities that can be useful for bioremediation purposes.

Future perspectives

Microorganisms have developed a whole range of mechanisms to deal with extremely harsh conditions such as the presence of higher concentration of heavy metals. Some microorganisms with high metal resistance were identified in my PhD research and some of them never reported as metal resistant. Therefore, I pretend to explore in the futures studies the molecular mechanisms responsible for metals resistance. The presence of heavy metals probability induces the expression of some genes coding proteins presumed to be involved in heavy metal resistance. Thus, I pretend to identify the putative heavy metal resistance genes in order to improve the knowledge about the molecular mechanisms involved in the metal resistance.

Valuable metals, such as gold, silver, palladium and platinum can be deposited in a reduced form or even in the form of zero-valent metals and since some microorganisms have demonstrated ability to remove metals from aqueous media by enzymatic reduction I also pretend to explore in the futures studies the potentialities of microbial species for recovery of precious metals. The recovery of these metals is interesting due to their high market prices along with various industrial applications. Since the availability of precious metals is very limited, the recovery of these metals from aqueous and waste solutions by biologic activity can be an economically attractive approach.

Appendixes

Acknowledgements

About the author

List of scientific publications

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About the author

Mónica Sofia Furtado Martins Neves was born on 9 March 1977 in Lagos, Portugal. In 2002, she completed the Biotechnological Engineering degree at University of Algarve. In the same year she initiated her career in scientific research working in metal extraction by organic solvents. Between 2003 and 2006 years, she worked in diverse investigation topics such as plants biochemistry in University of Algarve and population genetics in University of Madeira and in Escuela Politécnica Superior de Huesca (Spain). In 2006 she began working with the Environmental Technology group of CCMAR, University of Algarve in the ECOTEC project. The main objective of this project was the development of a bioremediation process, based in the sulphate-reducing bacteria activity, to treat acid mine drainage. In August 2006, she was granted a scholarship by Fundação para a Ciência e a Tecnologia to carry out her PhD research at the Environmental Technology group of CCMAR, University of Algarve, which resulted in this thesis.

List of scientific publications

Scientific Journals

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