Biodegradation (2012) 23:693–703
DOI 10.1007/s10532-012-9545-x

ORIGINAL PAPER

Aluminum and sulphate removal by a highly Al-resistant dissimilatory sulphate-reducing bacteria community

Mónica Martins · Rita Taborda · Gonçalo Silva · Ana Assunção · António Pedro Matos · Maria Clara Costa

Received: 15 July 2011 / Accepted: 8 February 2012 / Published online: 25 February 2012

Abstract A highly Al-resistant dissimilatory sulphate-reducing bacteria community was isolated from sludge of the wetland of Urgeiriça mine (community W). This community showed excellent sulphate removal at the presence of Al^{3+}. After 27 days of incubation, 73, 86 and 81% of sulphate was removed in the presence of 0.48, 0.90 and 1.30 mM of Al^{3+}, respectively. Moreover, Al^{3+} was simultaneously removed: 55, 85 and 78% of metal was removed in the presence of 0.48, 0.90 and 1.30 mM of Al^{3+}, respectively. The dissociation of aluminium-lactate soluble complexes due to lactate consumption by dissimilatory sulphate-reducing bacteria can be responsible for aluminum removal, which probably precipitates as insoluble aluminium hydroxide. Phylogenetic analysis of 16S rRNA gene showed that this community was mainly composed by bacteria closely related to *Desulfovibrio desulfuricans*. However, bacteria affiliated to *Proteus* and *Ralstonia* were also present in the community.

Keywords Aluminum · Bioremediation · Dissimilatory sulphate-reducing bacteria · Sulphate

Electronic supplementary material The online version of this article (doi:10.1007/s10532-012-9545-x) contains supplementary material, which is available to authorized users.

M. Martins · R. Taborda · A. Assunção · M. C. Costa (✉)
Departamento de Química e Farmácia, Faculdade de Ciências e de Tecnologia, Centro de Ciências do Mar (CCMAR), Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal
e-mail: mcorada@ualg.pt

G. Silva
Faculdade de Ciências e de Tecnologia, Centro de Biodiversidade, Genómica Integrativa e Funcional (BioFIG), Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

A. P. Matos
Serviço de Anatomia Patológica, Hospital Curry Cabral, Campo Grande, Lisboa, Portugal

A. P. Matos
Faculdade de Ciências, Centro de Estudos do Ambiente e do Mar (CESAM)–Polo de Lisboa, Universidade de Lisboa, Campo Grande, Lisboa 1749-016, Portugal

Introduction

Aluminum (Al) is the most abundant metal in the earth crust, representing 8.3% of weight, and the third most abundant element after oxygen (45.5%) and silicon (25.7%) (Fischer et al. 2002). Nevertheless, it is one of the few elements without biological function. Moreover, aluminum toxicity has been demonstrated in several organisms. The growth of *Escherichia coli* was inhibited by millimolar Al concentrations (Guida et al. 1991) and micromolar concentrations showed an inhibitory effect in the growth of *Anabena cylindrical* (Petterson et al. 1985). Histopathological findings demonstrated aluminum neurotoxicity to humans (Corain et al. 1996) and its toxic effects (encephalopathy,

osteomalacia and microcytic anemia) are well known into patients with chronic renal failure (D'Haese and De Broe 1994; Yokel 2004). Furthermore, aluminum is considered to be one of the risk factors for Alzheimer's disease (Jansson 2005).

Due to aluminum insolubility at neutral pHs (Hem 1970; Brown and Sadler 1989), this metal rarely occurs naturally in water at concentrations greater than a few tenths of a milligram per liter (Earle and Callaghan 1989). However, in drinking water a major part of the Al may be present as soluble monomeric Al associated to inorganic and organic ligands (Reiber et al. 1995; Earle and Callaghan 1989), which can be the reason of serious health problems with even mortal consequences. That was the case of Camelford and Évora water pollution incidents, involved the accidental contamination of the drinking water supply to the town of Camelford, Cornwall, England with 20 tons of aluminum sulphate in 1988 (Rowland et al. 1990), and the excess of aluminum in the water used in dialysis in the hospital of Évora, Alentejo, Portugal, in 1993, which caused the death of 20 patients (Araújo 1995).

Although aluminum is not usually considered as a major environmental pollutant it is present in several environmental pollutants namely industrial waste waters from mining activities, metal cleaning and metal processing and automobile industries (Ojumu et al. 2006; Blight and Ralph 2008), mainly due to the acidity of those solutions.

Several methods have been used for metal removal including precipitation, oxidation/reduction, ionic exchange, filtration, electrochemical process, membrane separation and evaporation. Most of these methods are ineffective or excessively expensive when metal concentrations are lower than 100 mg/L (Ahluwalia and Goyal 2007). Therefore, the search for novel technologies has recently been encouraged.

Bioremediation strategies based on the use of microorganisms have been considered a potential alternative. Although dissimilatory sulphate-reducing bacteria (DSRB) have been intensively explored for the treatment of metals containing wastewaters (Sheoran et al. 2010), only a few researches have been done on aluminum toxicity in DSRB (Ammonette et al. 2003). Therefore, the effect of aluminum (III) on the growth and activity of DSRB consortia isolated from several environmental sources was investigated in the present work. The phylogenetic characterization of Al-resistant bacterial cultures and the mechanism involved in the aluminum (III) removal were also explored.

Materials and methods

Microorganisms and growth conditions

The bacterial communities used in these experiments were obtained from semisolid environmental samples collected in Portugal: sludge from the impoundment of the former municipal waste water treatment plant of Montenegro, located in Faro, in South (sample B), activated sludge from an aeration tank of the waste water treatment plant of the leather industry of Alcanena in Central Portugal (sample T) and sludge from the wetland of Urgeiriça mine in North (sample W).

Bacteria were cultured under anaerobic conditions at room temperature (21 ± 1°C) using 120 mL glass bottles. The medium was purged with nitrogen gas to achieve an anaerobic environment prior to inoculation. After inoculation, 10 mL of sterile liquid paraffin was added in order to maintain an airtight seal. The first sulphate-reducing bacteria 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 (0.01 g/L). This redox indicator reveals a pink coloration in oxidant conditions while in reductive condition is colourless. Subsequently, the bacterial cultures were grown and maintained in minimal medium (Martins et al. 2010), 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. This minimal medium was used in order to avoid chemical removal of aluminum. The culture was sub-cultured every 3 weeks using 10% (v/v) of inoculum. The growth of sulphate-reducing bacteria (SRB) was monitored by weekly determination of pH, Eh and sulphate concentration.

Sulphate and aluminum (III) removal assays

The assays were performed in batch under anaerobic conditions, using the minimal medium previously described with pH 6.4 ± 0.1. All experiments were performed in duplicate using 120 mL glass bottles containing 100 mL of medium and 10% (v/v) of inoculum. The bacterial cells obtained previously

were harvested by centrifugation at 4,000 rpm for 10 min, washed with growth medium and transferred to the bottles containing the medium to be tested. 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 aluminum crimp seals and incubated at room temperature (21 ± 1°C).

The effect of aluminum (III) on the dissimilatory sulphate reduction was studied using the minimal medium (containing 20 mM of sulphate), supplemented with aluminum (III) as aluminum chloride ($AlCl_3$) at a concentration ranging from 0.48 to 1.30 mM. The ability of aluminum (III) removal by the enriched SRB culture was also studied in the absence of sulphate. For each experiment an abiotic control was carried out in parallel. The abiotic controls were prepared in the same way as the biotic tests, but without inoculum addition. Furthermore, abiotic reduction of aluminum (III) with sulphide (0.27, 0.80, 1.28 and 2.85 mM) was investigated using medium supplemented with 0.80 mM aluminum (III). Sulphide was added as anhydrous sodium sulphide (Na_2S).

Analytical methods

Periodically, 5 mL samples were collected using a syringe and centrifuged at 4,000 rpm for 5 min. Redox potential and pH were determined using a pH/E Meter (GLP 21, Crison). Sulphate and aluminum (III) concentrations were quantified by UV/visible spectrophotometry (Hach-Lange DR2800 spectrometer) using the method of SulfaVer®4 and AluVer®3 (Hach-Lange, Dusseldorf, Germany), respectively. Optical density at 600 nm (OD_{600}) was measured (Hach-Lange DR2800 spectrometer) in each sample. The lactate consumption and acetate production were monitored by High Performance Liquid Chromatography (Merck-Hitachi system, L-5000 LC controller, 655A UV monitor, 655A-11 liquid chromatograph), equipped with RP-18(5 µm) LiChrospher® column (25 × 0.40 cm^2, Merck). The analysis was performed with NaH_2PO_4 (20 mM, pH 2.7) as mobile phase, at a flow rate of 0.5 mL/min.

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

The precipitates generated during the bio-removal process were analysed by X-Ray powder diffraction (XRD) and by transmission electron microscopy coupled with an energy dispersive spectrometer (TEM-EDS). Samples were dried under vacuum at room temperature prior to XRD analysis using a PANalytical X'Pert Pro powder difractommeter operating at 45 kV and 40 mA, with CuKα radiation filtered by Ni. 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 fresh bacterial cells exposed to aluminum (III) were prepared for TEM by fixation with glutaraldehyde 3% followed by dehydration and embedding in Epon-Araldite (Glauert 1975). Thin sections (79–90 nm) without staining were used for detection of electron-dense precipitates. The precipitates were analyzed at 200 kV using an EDAX EDS detector.

Molecular characterization

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

Total genomic DNA was extracted after harvesting cells by centrifugation at 4,000 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 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 Go-Taq®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 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min and completed with an extension period of 5 min at 72°C. 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 DH5α* 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 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min and completed with an extension period of 5 min at 72°C. The PCR products were analyzed by electrophoresis in 1% (w/v) agarose gel and TAE buffer and the clones containing the expected DNA insert were saved at −20°C.

Restriction fragment length polymorphism analysis (RFLP) of 16S rRNA gene and phylogenetic analysis

RFLP analysis of the previously amplified 16S rRNA gene was performed using the restriction enzymes *HhaI* and *MspI* (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). Sequence identification was performed by use of the BLASTN facility of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences obtained in this study have the following accession numbers: JF733466–JF733470. Phylogenetic trees were constructed using MEGA 4 and the neighborhood-joining algorithm was applied (Saitou and Nei 1987; Studier and Keppler 1988).

Results

Effect of initial Al^{3+} concentration on dissimilatory sulphate reduction

The dissimilatory sulphate reduction profile and growth of DSRB communities, from sludge from the waste water treatment plants of Montenegro (B) and of the leather industry (T) and from sludge from the wetland of Urgeiriça mine (W), in the absence and in presence of three different concentrations of Al^{3+}, is shown in Fig. 1. The DSRB cultures B, T and W were able to reduce 82, 60 and 88% of sulphate, respectively, in 18 days of incubation in the absence of metal (Fig. 1a). Although consortium T showed the lowest dissimilatory sulphate reduction, its growth was fast and it reached OD_{600} values similar to those of culture W, which exhibited the highest dissimilatory sulphate reduction. Bacterial cultures T and W reached OD_{600} values of 0.37 and 0.31 after 4 and 7 days of incubation, respectively. These days of incubation correspond to the end of the exponential phase. The growth of consortium B was slower than that observed with the other two and the maximum OD_{600} value (0.29) was achieved after 14 days of incubation.

All DSRB consortia showed ability to reduce sulphate in the presence of Al^{3+}. However, bacterial growth was affected by the presence of this metal. The presence of Al^{3+} in the medium promoted an extension of the lag phase and a decrease of bacterial growth (Fig. 1).

In the presence of 0.48 mM of Al^{3+} (Fig. 1b), all the consortia presented similar behaviour in terms of dissimilatory sulphate reduction. However, at the end of the experiment the concentration of sulphate removed by inoculum T was higher comparatively with the other inocula, contrarily to what was observed in the absence of Al^{3+} (Fig. 1a). After 27 days of incubation, 92% of sulphate was removed by consortium T, while 73 and 53% of sulphate were removed by consortia W and B, respectively, in the presence of 0.48 mM of Al^{3+} (Fig. 1b). These results are in accordance with bacterial growth, since the growth of consortium T was faster comparatively to the other cultures, achieving the maximum OD_{600} value (0.23) after 13 days of incubation. The OD_{600} values achieved by cultures T and B were 0.14 and 0.13, respectively, after the same time of incubation.

When the concentration of Al^{3+} was increased to 0.90 mM, the concentration of sulphate removal after 27 days of incubation by consortia T and W was similar, although an extended lag phase was observed with inoculum T (Fig. 1c). The lowest sulphate removal was observed in the presence of inoculum B. After 27 days of incubation, 86, 83 and 68% of sulphate was removed from the medium by consortia W, T and B, respectively. These results are in agreement with bacterial growth. In fact, the growth

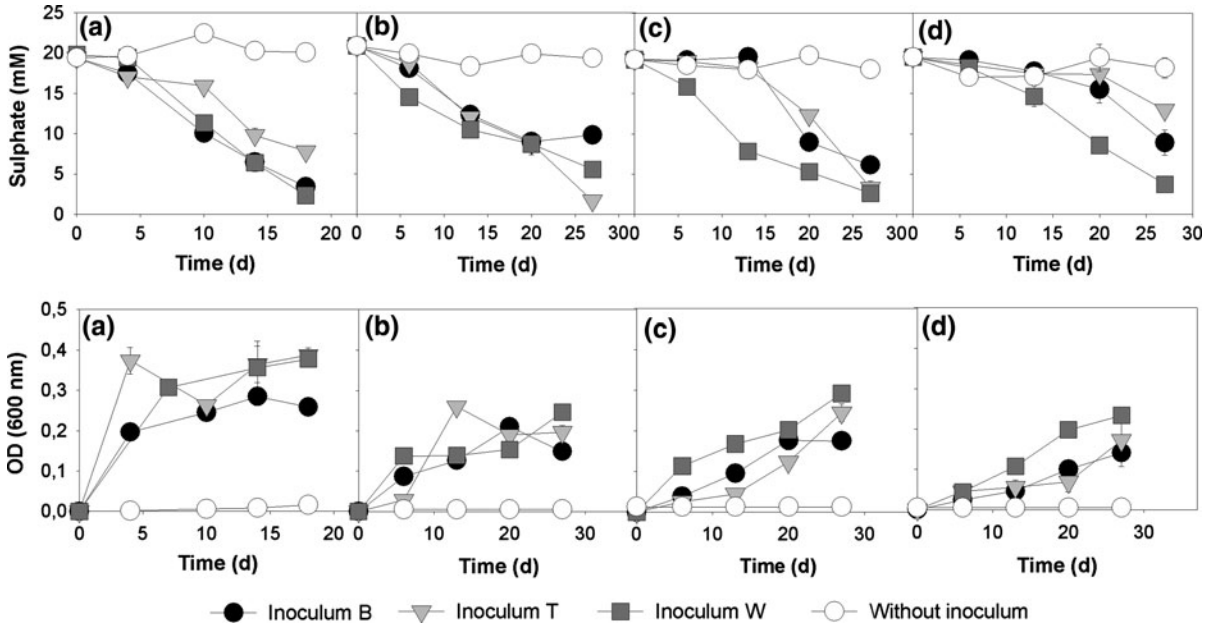

Fig. 1 Effect of Al^{3+} on dissimilatory sulphate reduction and growth of bacterial consortia from waste water treatment plant of Montenegro (B), waste water treatment plant of leather industry in Alcanena (T) and wetland of Urgeiriça mine (W): absence of Al^{3+} (**a**), 0.48 mM Al^{3+} (**b**), 0.90 mM Al^{3+} (**c**) and 1.30 mM Al^{3+} (**d**). Data are the average of duplicates and *error bars* indicate the standard error of the average values

of inoculum W was faster comparatively to the other cultures. Nevertheless, consortia W and T reached similar OD_{600} values in the end of the experiment.

The efficiency of sulphate removal by inocula T and B was significantly affected by the presence of 1.30 mM of Al^{3+} in the medium (Fig. 1d). On the other hand, sulphate removal by consortium W seems not be affected by the increase of metal concentration. Actually, this bacterial consortium showed the best performance of dissimilatory sulphate reduction. After 27 days of incubation, 81, 54 and 34% of sulphate was removed from the medium containing 1.30 mM of Al^{3+}, by bacterial communities W, B and T, respectively. Moreover, in this condition the highest bacterial growth was observed in culture W, reaching the maximum OD_{600} value (0.23) in the end of the experiment (Fig. 1d). The OD_{600} values obtained in consortia T and B were 0.17 and 0.14, respectively, after the same incubation time.

No significant growth and sulphate removal were observed in all abiotic sets (Fig. 1).

An increase of pH (from 6.4 to 7.5) was observed in all cases at the same time that sulphate was removed from the medium (data not shown). Moreover, Eh showed a decreasing tendency, reaching values of -345 ± 15 mV in the end the experiments. In abiotic sets the pH and Eh values were maintained at 6.4 ± 0.1 and 60 ± 10 mV, respectively.

Al^{3+} removal assays

Al^{3+} and sulphate removal by DSRB of consortium W was investigated in the presence of the three metal concentrations mentioned in the previous section and 20 mM sulphate (Fig. 2). The removal of Al^{3+} was observed for all metal concentrations. After 27 days of incubation, 55, 85 and 78% of metal was removed by consortium W in the presence of 0.48, 0.90 and 1.30 mM of Al^{3+}, respectively. Moreover, sulphate and Al^{3+} were simultaneously removed. After 27 days of incubation, 73, 86 and 81% of sulphate was removed. In the abiotic sets (without inoculum) no relevant removal of sulphate and Al^{3+} was observed.

The profile of Al^{3+} removal during dissimilatory sulphate reduction by DSRB of consortium W in the presence of lactate as carbon source is shown in Fig. 3a. It was observed that dissimilatory sulphate reduction occurred at same time that lactate was consumed, resulting in the production of acetate. In addition, Al^{3+} was simultaneously removed. After 27 days of

incubation, 20 mM of sulphate and 0.72 mM of Al^{3+} were removed from the medium containing 22 and 0.84 mM of sulphate and Al^{3+}, respectively.

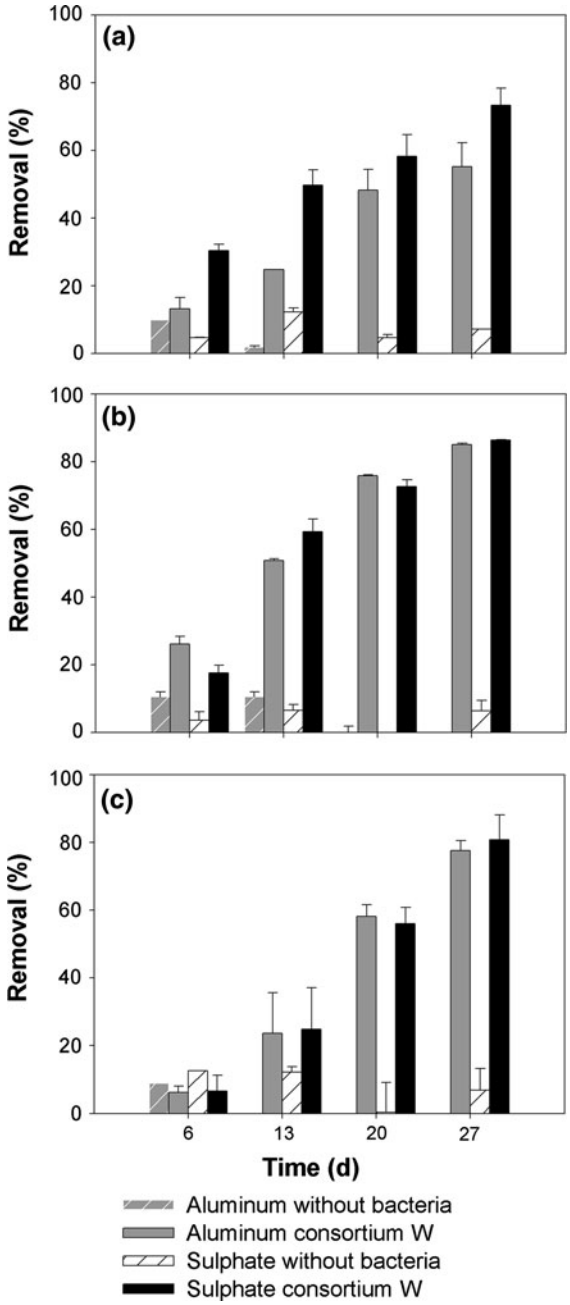

Fig. 2 Al^{3+} and sulphate removal by DSRB consortium from the wetland of Urgeiriça mine (W) in the presence of sulphate (20 mM) and different concentrations of Al^{3+}: 0.48 mM (**a**), 0.90 mM (**b**) and 1.30 mM (**c**). Data are the average of duplicates and *error bars* indicate the standard error of the average values

Furthermore, 37 mM of lactate was consumed and 15 mM of acetate was produced during the same time of incubation. An increase of pH (from 6.3 to 7.6) was observed at the same time that sulphate was removed from the medium (data not shown). In the abiotic control, the concentrations of sulphate, Al^{3+} and lactate did not changed during all the experiment (Fig. 3b).

Al^{3+} removal by the same inoculum in the absence of sulphate was also investigated. Nevertheless, no Al^{3+} removal was detected in this condition (data not shown).

The eventual removal of aluminum from the solution as a consequence of reduction by the biological generated sulphide was tested. Thus, the abiotic reaction of Al^{3+} (0.80 mM) with sulphide added as Na_2S was investigated (Fig. 4). Among the several sulphide concentrations studied (0.27, 0.80, 1.28 and 2.85 mM), an aluminum removal of 24% was only observed in the presence of 2.85 mM of sulphide after 3 days.

Microscopic analysis of DSRB community and precipitates analysis

The photomicrograph of the bacterial consortium W in the presence of aluminum and sulphate after Gram staining (Fig. 5a) shows that the community was mainly composed by large and very long bacilli. Moreover, TEM analysis (Fig. 5b) shows the presence of dense precipitates only outside of bacterial cells and the EDS coupled to TEM confirmed the presence of aluminum in the precipitate. (Fig. 5c). Other elements such as carbon and copper, were also detected. The presence of copper could be originated from the supporting grid, and carbon from the EPON-ARALDITE section, since they were also present in background areas. The XRD analysis showed that the precipitates resulting from the biological activity in the presence of aluminum were composed by amorphous material.

Phylogenetic analysis

The molecular identification of the aluminum resistant DSRB community isolated from the wetland of Urgeiriça mine (consortium W) was performed in order to establish the relationships between bacterial groups and aluminum resistance.

A total of 41 recombinant colonies were recovered and approximately 1.4 kb fragment of bacterial 16S rRNA gene was amplified and used for RFLP analysis.

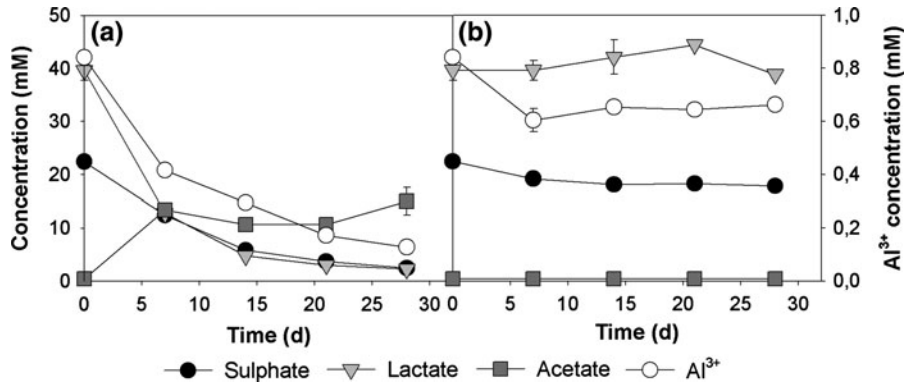

Fig. 3 Profile of Al^{3+} removal, dissimilatory sulphate reduction and consumption of carbon source by the bacterial consortium from the wetland of Urgeiriça mine (**a**) and in the abiotic control (**b**). Data are the average of duplicates and *error bars* indicate the standard error of the average values

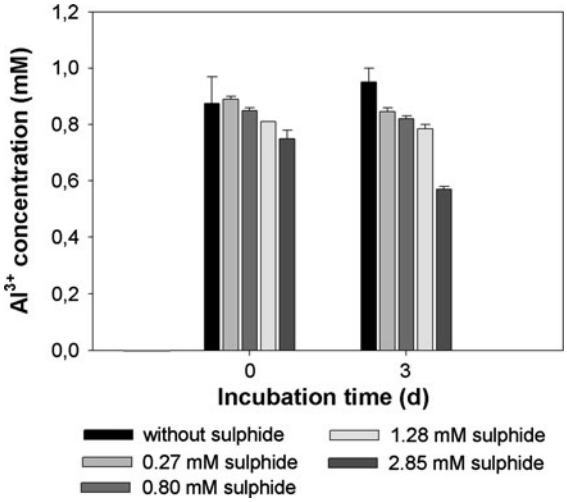

Fig. 4 Al^{3+} removal from the medium in the presence of different concentrations of sulphide. Data are the average of duplicates and *error bars* indicate the standard error of the average values

Five RFLP groups (W5, W27, W51, W60 and W87 as representative clones) were originated from the mixed culture grown in the presence of aluminum and sulphate (Fig. 6a). Phylogenetic analysis of the representative clones allowed the identification of the corresponding sequences (Fig. 6b). Most of clone sequences (90%) were closely related to *Desulfovibrio desulfuricans*. Moreover, bacterial affiliated to *Proteus* (5%) and *Ralstonia* (5%) were detected in this community.

Discussion

DSRB have been intensively explored for the treatment of several metals containing wastewaters namely acid mine drainage (Johnson and Hallberg 2005; Neculita et al. 2007; Sheoran et al. 2010). Although aluminum is usually present in this type of wastes, the effect of this metal on DSRB activity has been poorly investigated. In order to develop efficient bioremediation strategies to treat aluminum containing wastewaters it is essential to search aluminum resistant communities. In this paper, the effect of Al^{3+} on the growth and activity of DSRB consortia from several environmental sources was explored. Moreover, the mechanism involved in aluminum bio-removal by DSRB was investigated.

The distinction between pH and aluminum effect on bacterial toxicity is very difficult, since this metal is only soluble in aqueous solutions at pH below 6 or higher than 8 (Scancar and Milacic 2006). Due to the low solubility of Al^{3+} at the neutral pH values, usually favourable for DSRB growth and activity (Costa et al. 2009), lactate was used in the present study not only as a carbon source but also as an organic ligand that allows maintaining this metal soluble in the nutrient medium. In fact, aluminum lactate ($AlLac_3$) is largely used in toxicology experimentation because it is very soluble in water (Zatta et al. 1998; Amonette et al. 2003) and the solubility is even maintained at neutral pH (Amonette et al. 2003).

Among the bacterial communities studied, the DSRB community obtained from sludge of the wetland of Urgeiriça mine (W) showed the highest resistance to Al^{3+}. 81, 54 and 34% of sulphate was removed by communities W, B and T, respectively, in the presence of the highest concentration of Al^{3+} tested (1.30 mM). The dissimilatory sulphate reduction by community W in the absence of Al^{3+} (88%) is similar to the observed in the presence of 1.30 mM of Al^{3+}, contrarily to what was observed with the other

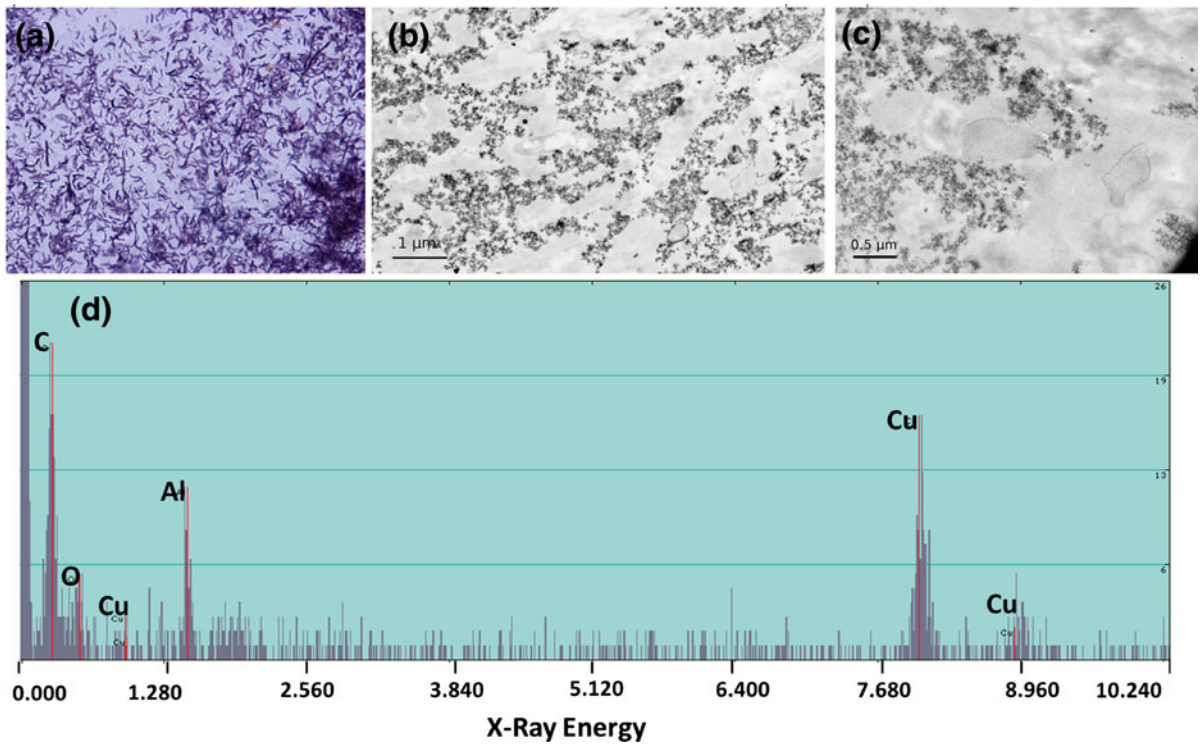

Fig. 5 Photomicrographs of bacterial consortium W in the presence of Al^{3+} (1.30 mM) and sulphate (20 mM): optical microscopy after Gram staining with amplification of 1,000× (**a**), TEM of cells sections without staining (**b** and **c**) and EDS spectrum of the precipitate (**d**)

two communities tested. Although communities B and T showed ability to remove sulphate in the presence of 1.30 mM of Al^{3+}, the amount removed is considerably lower than in the absence of the metal (82 and 60% of sulphate was removed by B and T cultures, respectively) and the bacterial growth was also affected. The presence of Al^{3+} in the medium promoted an extension of the lag phase and a decrease of bacterial growth for all consortia. It was reported that the cellular yield of a number of acidophilic bacteria decreased with increase of aluminum concentration (Fischer et al. 2002). Metals can inhibit cell activity through a number of mechanisms, which include changing enzyme conformations, binding to transport sites and inducing the loss of membranes (Dopson et al. 2003). Aluminum toxicity is mainly based on its substitution for magnesium ions in biological reactions. The binding of Al ions to ATP is 10^7 fold stronger than that of Mg ions (MacDonald and Martin 1988).

The phylogenetic analysis of 16 s rRNA gene showed that the DSRB community W grown in the presence of the highest Al^{3+} concentration was mainly composed by bacteria closely related to *Desulfovibrio desulfuricans*, although bacteria affiliated to *Proteus* sp. and *Ralstonia* sp. were also detected. The genus *Desulfovibrio* represents a group of Gram-negative sulphate reducers in which all species oxidize their substrates incompletely to acetate, which cannot be oxidized further (Nagpal et al. 2000; Muyzer and Stams 2008). Although sulphate is the usual electron acceptor for the genus *Desulfovibrio*, some species have showed capacity to use metals as electron acceptors, namely Pd (II), U (VI) and Cr (VI) (Lloyd et al. 1998; Wall and Krumholz 2006; Goulhen et al. 2006). This ability can be applied to remove these metals from aqueous solutions, since insoluble oxidation states (Pd (0), U (IV) and Cr (III)) were originated after biologic reduction.

Since DSRB have been showed some terminal electron acceptor versatility, the ability of DSRB of community W to use Al^{3+} as electron acceptor, removing it consequently from the solution as Al^0, was investigated in the absence of sulphate. However, the

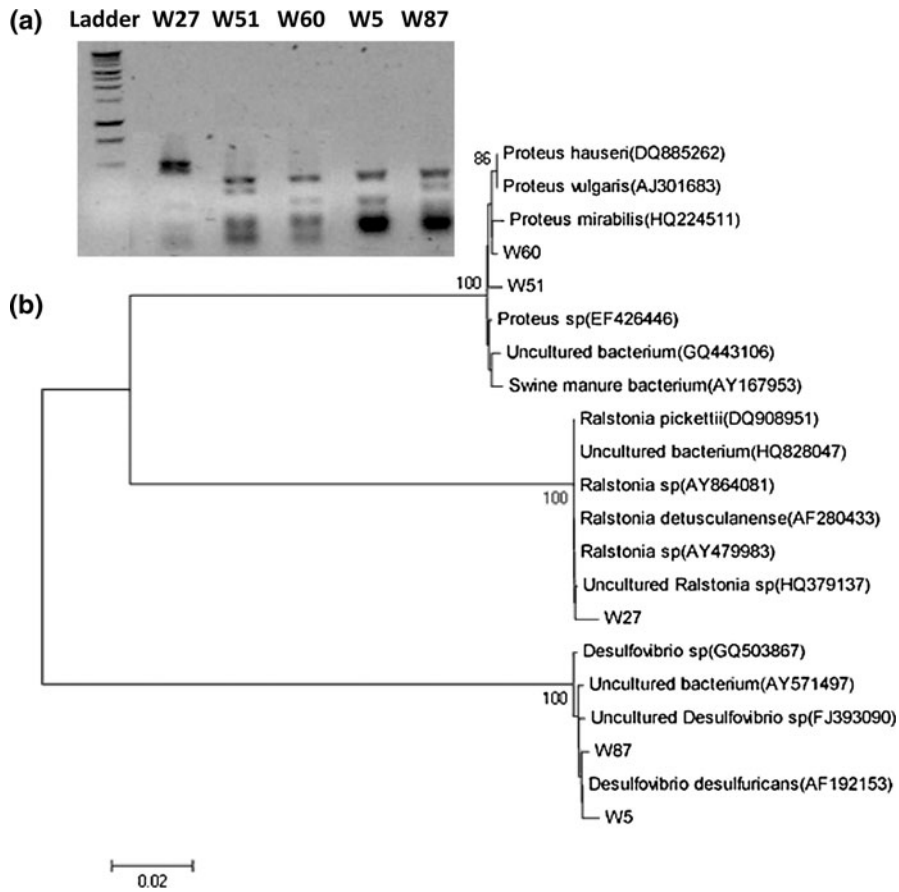

Fig. 6 RFLP band pattern of the representative clones from the bacterial community W (**a**) and phylogenetic tree obtained with 16S rRNA sequences, corresponding to the clones representative of each restriction profile and to the most closely related ones retrieved from BLAST search (**b**). Phylogeny was inferred using the neighborhood-joining algorithm analysis of aligned 16S rRNA fragments. *Bootstrap values* are indicated on branches. Access numbers of GenBank sequences are indicated in *brackets*

removal of Al^{3+} in this condition was not observed. Therefore, it is possible to infer that Al^{3+} was not a potential terminal electron acceptor for the DSRB of consortium W.

Although aluminum was not removed in the absence of sulphate, an efficient aluminum removal was observed in the presence of this anion. Both species were removed simultaneously and the highest aluminum removal, observed in the presence of 0.90 mM of that metal (85%), also corresponded to the highest sulphate removal (86%). Hence, it is possible to conclude that aluminum removal was related to dissimilatory sulphate reduction. Consequently, several hypotheses were investigated in order to elucidate the mechanism of aluminum removal by community W.

Aluminum removal by sulphide produced by DSRB was one hypothesis that was evaluated. Sulphide is considered a strong reducing agent and the abiotic metal reduction by sulphide has been reported for several metals, namely U (VI) and Cr (VI) (Lovley 1995; Hua et al. 2006). Thus, the reduction of Al^{3+} by sulphide in the absence of bacteria was investigated in the presence of different sulphide concentrations. Among the several sulphide concentrations studied, an aluminum removal of 24% was only observed with 2.85 mM of sulphide. However, the concentration of aluminum removed was much lower than the concentration of metal removed in the presence of bacteria, showing that other mechanism than sulphide reduction is responsible for the disappearance of aluminium from solution.

DSRB utilise lactate as carbon and electron donor for dissimilatory sulphate reduction producing acetate and sulphide (Eq. 1) (Liamleam and Annachatre Liamleam and Annachhatre 2007).

$$2CH_3CHOHCOO^- + SO_4{}^{2-} \rightarrow 2CH_3COO^- + 2HCO_3{}^- + HS^- + H^+ \quad (1)$$

According to Fig. 3 it was observed that dissimilatory sulphate reduction occurred at same time that lactate was consumed, resulting in the production of

acetate. Moreover, Al^{3+} was simultaneously removed. After 27 days of incubation, 20 mM of sulphate and 0.72 mM of Al^{3+} were removed, while 37 mM of lactate was consumed and 15 mM of acetate was produced. Taking into account the stoichiometry of Eq. 1, the concentration of acetate produced was lower than half of the expected result. In addition, the bacterial community was mainly composed by *Desulfovibrio desulfuricans*, which cannot use acetate as carbon source (Muyzer and Stams 2008). However, the utilization of acetate as carbon source by some species that were also present in the community, such as *Ralstonia* sp. and *Proteus* sp., was reported (Miles and Miles 1951; Ampe et al. 1998). Therefore, the presence of other than DSRB bacterial groups, with ability to use acetate as carbon source, could explain the inconsistency between the observed acetate production and what should be expected based on Eq. 1.

Aluminum removal occurred during the dissimilatory sulphate reduction in which lactate was metabolised by the DSRB community producing acetate. Since aluminum was present in the solution as AlLac soluble complexes, the dissociation of those complexes due to lactate consumption may be responsible for aluminum removal, which probably precipitates as insoluble aluminium hydroxide, usually formed at neutral pHs (Mikutta et al. 2011) which was the pH of the medium during the aluminum removal (pH = 7.6). XRD and TEM-EDS analysis supported this hypothesis. TEM-EDS analysis showed that the precipitates resulting from the biological activity were only formed outside the bacterial cells and were mainly composed by aluminum. In addition, XRD analysis showed that the precipitates were composed by amorphous aggregates, which is consistent with the formation of aluminium hydroxide reported as amorphous (Amonette et al. 2003; Berkowitz et al. 2005; Mikutta et al. 2011). Therefore, the precipitation of aluminum as hydroxide after the lactate consumption could be the mechanism involved in Al removal by DSRB community.

Conclusions

A bacterial community isolated from sludge of the wetland of Urgeiriça mine (community W) showed high resistance to Al (III). Phylogenetic analysis of 16S rRNA gene showed that this community was mainly composed by bacteria closely related to *Desulfovibrio desulfuricans*. The dissimilatory sulphate reduction by community W in the absence of Al^{3+} was similar to the one observed in the presence of 1.30 mM of Al^{3+}. Beside sulphate removal, aluminum was also efficiently removed from the solution. Thus, this DSRB community can be a potential candidate to be used in biological treatment of waters contaminated with aluminum.

Acknowledgment The authors wish to thank Fundação para a Ciência e a Tecnologia (FCT) for funding this research through the PhD grant SFRH/BD/29677/2006.

References

Ahluwalia SS, Goyal D (2007) Microbial and plant derived biomass for removal of heavy metals from wastewater. Bioresour Technol 98(12):2243–2257

Amonette JE, Russel CK, Carosino KA, Robinson NL, Ho JT (2003) Toxicity of Al to *Desulfovibrio desulfuricans*. Appl Environ Microb 69:4057–4066

Ampe F, Léonard D, Lindley ND (1998) Repression of phenol catabolism by organic acids in *Ralstonia eutropha*. Appl Environ Microb 64:1–6

Araújo FO (1995) Effects of cyanobacteria on drinking water and human health: an epidemiologic study in Évora, Portugal. Assessing and managing health risks from drinking water contamination: approaches and applications. IAHS Publication, Oxfordshire, pp 101–110

Berkowitz J, Michael AA, Robert CG (2005) Laboratory investigation of aluminum solubility and solid-phase properties following alum treatment of lake waters. Water Res 39:3918–3928

Blight KR, DE Ralph (2008) Aluminum sulphate and potassium nitrate effects on batch culture of iron oxidising bacteria. Hydrometallurgy 92:130–134

Brown DJA, Sadler K (1989) Fish survival in acid waters. In: Morris R et al (eds) Acid toxicity and aquatic animals. Society for experimental biology seminar series: 34. Cambridge University Press, Cambridge, pp 31–44

Corain B, Bombi GG, Tapparo A, Perazzolo M, Zatta P (1996) Aluminium toxicity and metal speciation: established data and open questions. Coord Chem Rev 149:11–22

Costa MC, Santos ES, Barros RJ, Pires C, Martins M (2009) Wine wastes as carbon source for biological treatment of acid mine drainage. Chemosphere 75:831–836

D'Haese PC, De Broe ME (1994) In: Nicolini M, Zatta PF, Corain B (eds) Aluminium in chemistry biology and medicine: recent insights in the monitoring, diagnosis and treatment of aluminum-overload in dialysis patients, vol 2. Harwood Academic Publishers GmbH, Chur, pp 215–224

Dopson M, Baker-Austin C, Koppineedi PR, Bond PL (2003) Growth in sulfidic mineral environments: metal resistance mechanisms in acidophilic microorganisms. Microbiology 149:1959–1970

Earle J, Callaghan T (1989) Impacts of mine drainage on aquatic life, water uses, and man made structures In: Coal Mine Drainage Prediction and Pollution Prevention in Pennsylvania, chap 4, Department of Environmental Protection, Harrisburg, Pennsylvania, pp 1–10

Fischer J, Quentmeier A, Gansel S, Sabados V, Friedrich CG (2002) Inducible aluminum resistance of *Acidiphilium cryptum* and aluminum tolerance of other acidophilic bacteria. Arch Microbiol 178:554–558

Glauert AM (1975) Fixation, dehydration and embedding of biological specimens. In: Gauert AM (ed) Practical methods in electron microscopy, vol 3(1). Elsevier, Amsterdam, p 208

Goulhen F, Gloter A, Guyot F, Bruschi M (2006) Cr(VI) detoxification by *Desulfovibrio vulgaris* strain Hildenborough: microbe-metal interactions studies. Appl Microbiol Biotechnol 71:892–897

Guida L, Saidi Z, Hughes MN, Poole RK (1991) Aluminum toxicity and binding to *Escherichia coli*. Arch Microbiol 156:507–512

Hem JD (1970) Study and interpretation of the chemical characteristics of natural waters, 2nd edn, Water supply paper No 2254. United States Geological Survey, Washington, p 363

Hua B, Xu H, Terry J, Deng B (2006) Kinetics of uranium (VI) reduction by hydrogen sulfide in anoxic aqueous systems. Environ Sci Technol 40:4666–4671

Jansson ET (2005) Alzheimer disease is substantially preventable in the United States-review of risk factors, therapy, and the prospects for an expert software system. Med Hypotheses 64:960–967

Johnson DB, Hallberg KB (2005) Acid mine drainage remediation options: a review. Sci Total Environ 338:3–14

Liamleam W, Annachhatre AP (2007) Electron donors for biological sulfate reduction. Biotechnol Adv 25:452–463

Lloyd JR, Yong P, LE Macaskie (1998) Enzymatic recovery of elemental palladium by using sulphate-reducing bacteria. Appl Environ Microb 64:4607–4609

Lovley (1995) Bioremediation of organic and metal contaminations with dissimilatory metal reduction. J Ind Microbiol 14:85–93

MacDonald TL, Martin RB (1988) Aluminum in biological systems. Trends Biochem Sci 13:15–19

Martins M, Faleiro ML, Barros RJ, Veríssimo AR, Barreiros MA, Costa MC (2009) Characterization and activity studies of highly heavy metal resistant sulphate-reducing bacteria to be used in acid mine drainage treatment. J Hazard Mater 166:706–713

Martins M, Faleiro ML, Chaves S, Tenreiro R, Costa MC (2010) Effect of uranium (IV) on two sulfate-reducing bacteria cultures from a uranium mine site. Sci Total Environ 408: 2621–2628

Mikutta R, Zang U, Chorover J, Haumaier L, Kalbitz K (2011) Stabilization of extracellular polymeric substances (*Bacillus subtilis*) by adsorption to and coprecipitation with Al forms. Geochim Cosmochim Ac 75:3135–3154

Miles EM, Miles AA (1951) The Identity of *Proteus hydrophilus* Bergey et al. and *Proteus melanovogenes* Miles & Halnan, and their Relation to the Genus *Aeromonas* Kluyver & van Niel. J Gen Microbiol 5:298–306

Muyzer G, Stams AJM (2008) The ecology and biotechnology of sulphate-reducing bacteria. Nat Rev Microbiol 6:441–454

Nagpal S, Chuichulcherm S, Livingstone A, Peeva L (2000) Ethanol utilization by sulfate-reducing bacteria: an experimental and modeling study. Biotechnol Bioeng 16:533–543

Neculita CM, Zagury GJ, Bussiere B (2007) Passive treatment of acid mine drainage in bioreactors using sulfate-reducing bacteria: critical review and research needs. J Environ Qual 36:1–16

Ojumu TV, Petersen J, Searby GE, Hansford GS (2006) A review of rate equations proposed for microbial ferrous-iron oxidation with a view to application to heap bioleaching. Hydrometallurgy 83:21–28

Petterson A, Hällbom L, Bergmann B (1985) Physiological and structural responses of the cyanobacterium *Anabaena cylindrical* to aluminum. Physiol Plant 63:153–158

Postgate JR (ed) (1984) The sulphate-reducing bacteria. Cambridge University Press, Cambridge

Reiber S, Kukull W, Standish-Lee P (1995) J AWWA 87: 86–100

Rowland A, Grainger R, Smith RS, Hicks N, Hughes A (1990) Water contamination in north Cornwall: a retrospective cohort study into the acute and short-term effects of the aluminium sulphate incident in July 1988. J R Soc Health 110:166–172

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–525

Scancar J, Milacic R (2006) Aluminum speciation in environmental samples: a review. Anal Bioanal Chem 386:999–1012

Sheoran AS, Sheoran V, Choudhary RP (2010) Bioremediation of acid-rock drainage by sulphate-reducing prokaryotes: a review. Miner Eng 23(14):1073–1100

Studier JA, Keppler KJ (1988) A note on the neighbor-joining algorithm of saitou and neil. Mol Biol Evol 5:729–731

Suzuki Y, Kelly SD, Kemner KM, Banfield JF (2003) Microbial populations stimulated for hexavalent uranium reduction in uranium mine sediment. Appl Environ Microb 69: 1337–1346

Wall JD, Krumholz LR (2006) Uranium reduction. Annu Rev Microbiol 60:149–166

Yokel RA (2004) Aluminum. In: Merian E, Anke M, Ihnat M, Stoeppler M (eds) Elements and their compounds in the environment, occurrence, analysis and biological relevance, vol 2. Wiley-VCH, Weinheim, pp 635–658

Zatta P, Zambenedetti P, Milacic R (1998) Aluminum toxicity: the relevant role of the metal speciation. Analusis 26:72–76