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# Bromate removal by anaerobic bacterial community: Mechanism and phylogenetic characterization

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ABSTRACT

A highly bromate resistant bacterial community and with ability for bromate removal was obtained from a sulphate-reducing bacteria enrichment consortium. This community was able to remove 96% of bromate and 99% of sulphate from an aqueous solution containing 40 μM bromate and 10 mM sulphate. Moreover, 93% of bromate was removed in the absence of sulphate. Under this condition bromate was reduced stoichiometrically to bromide. However, in the presence of sulphate only 88% of bromate was reduced to bromide. Although, bromate removal was not affected by the absence of sulphate, this anion promoted a modification on the structure of the bacterial community. Phylogenetic analysis of 16S rRNA gene showed that the community grown in the presence of bromate and sulphate was mainly composed by bacteria closely to *Clostridium* and *Citrobacter* genera, while the community grown in the absence of sulphate was predominantly composed by *Clostridium* genus. It is the first time that *Clostridium* and *Citrobacter* genera are reported as having bromate removal ability. Furthermore, bromate removal by the consortium predominantly composed by *Clostridium* and *Citrobacter* genera occurred by enzymatic reduction and by extracellular metabolic products, while the enzymatic process was the only mechanism involved in bromate removal by the consortium mainly composed by *Clostridium* genus.



## 1. Introduction

Bromate ($BrO_3^-$) is mainly formed in drinking water, when ozonation is adopted to treat bromide ($Br^-$) contained in water [1]. Ozone is commonly used for organic pollutant oxidation in water treatment [2]. Bromate formation occurs via a molecular ozone pathway by ozonation of bromide to hypobromide (Eqs. (1) and (2)) and indirectly via a free radical pathway [1]

$$O_3 + Br^- \rightarrow O_2 + BrO^- \quad (1)$$

$$2O_3 + BrO^- \rightarrow 2O_2 + BrO_3^- \quad (2)$$

The presence of bromate in drinking water is a matter of concern since this anion is considered as a possible human carcinogen [3]. Bromate causes renal cell tumors in rats [4,5] and male mice [5] that were fed aqueous bromate. Following these evidences of carcinogenicity, bromate was classified as a Group 2B carcinogen by the World Health Organization (WHO). A maximum allowed contaminant concentration of 10 μg/L was imposed for bromate by the European Union [6] and the U.S. Environmental Protection Agency [7], while the WHO set a provisional guideline value of 25 μg/L [8]. Thus, the removal of bromate from drinking water is nowadays a matter of special interest.

Bromate is a very stable anion, characterized by a high solubility and low reactivity. This pollutant cannot be removed by traditional water treatment methods such as filtration, chlorination or lime softening [1]. New methods have been considerate for its removal, such as membrane processes, ultraviolet irradiation and photocatalytic decomposition [1]. However, many of these methods have disadvantages, for instance, high energy requirements and generation of concentrated brines that require further treatment or disposal [9]. Therefore, the search for novel technologies has lately been encouraged. Bioremediation strategies based on the use of microorganisms have been considered a potential alternative. The ability to reduce bromate to the bromide ($Br^-$), which is considered relatively innocuous [10] has been demonstrated for some mixed and pure cultures of denitrifying bacteria like *Pseudomonas* spp. [10–13]. Reduction of bromate by indigenous groundwater

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microbial populations and anaerobic mixed microbial cultures was also reported [14,15]. Moreover, bromate bio-reduction has also been observed on biologically active carbon (BAC) filters [16,17].

Although sulphate-reducing bacteria (SRB) have been intensively explored for the treatment of several wastewaters, bromate removal by this bacterial group was never reported. SRB are anaerobic microorganisms that use sulphate as a terminal electron acceptor for the degradation of organic compounds, resulting in the production of sulphide [18]. In addition, SRB can use other electron acceptors for growth and can ferment substrates in the absence of inorganic electron acceptors [19]. The reduction of nitrate and nitrite to ammonium by SRB has already been reported [20]. Furthermore, some metals (Fe(III), U(VI), Cr(VI), and As(VI)) and organic compounds (fumarate, dimethysulphoxide and monochlorobenzoate) can be used as terminal electron acceptors by SRB [19]. The terminal electron acceptors versatility turns these microorganisms potential candidates for bromate removal. Therefore, the ability for bromate removal from aqueous solution by a sulphate-reducing bacteria enrichment consortium was investigated for the first time. The phylogenetic diversity of microorganisms that participate in bromate removal and the mechanism involved were also elucidated.

## 2. Material and methods

### 2.1. Microorganisms and growth conditions

The anaerobic bacterial community used in the present study was selected from previous works and containing the SRB species *Desulfovibrio desulfuricans* and *Desulfobulbus rhabdoformis* [21]. This consortium was obtained from sludge sample from a municipal waste water treatment plant located in Montenegro, Faro, in southern Portugal. Stock cultures were maintained in Postgate B medium [22], at room temperature (±21 °C) in anaerobic conditions. Subsequently, the bacterial mixed culture was grown in bromate test medium (BTM) which contains 0.5 g/L $(NH_4)_2HPO_4$, 0.5 g/L $K_2HPO_4$, 0.06 g/L $CaSO_4{\cdot}2H_2O$, 0.05 g/L yeast extract, 0.06 g/L $MgSO_4{\cdot}7H_2O$, 1 g/L $Na_2SO_4$ and 5 g/L sodium lactate. The culture was sub-cultured every 3 weeks using 10% (v/v) inoculum and the bacterial growth was monitored by weekly determination of pH, Eh and sulphate concentration.

### 2.2. Batch experiments

The assays were performed in batch under anaerobic conditions, using the bromate test medium (BTM) previously described (pH = 7.0). All experiments were performed in duplicate using glass bottles (120 or 35 mL) containing 100 or 30 mL of BTM and 10% (v/v) of inoculum. The bacterial cells obtained previously were harvested by centrifugation at 4000 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. The bottles were sealed with butyl rubber stoppers and aluminium crimp seals and incubated at room temperature (21 ± 1 °C).

#### 2.2.1. Effect of bromate on sulphate reduction

The effect of bromate on sulphate reduction was studied using the 120 mL glass bottles containing 100 mL BTM, previously described, supplemented with bromate as potassium bromate ($KBrO_3$) at concentrations ranging from 7.8 to 39 μM. A test without bromate was done as positive control. 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.

#### 2.2.2. Bromate removal assays

The ability of bromate removal by the enriched SRB culture was studied in the presence (10 mM) and in the absence of sulphate using 120 mL glass bottles containing 100 mL of BTM supplemented with 40 μM bromate. 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.

Bromate bio-removal by heat-killed cells and extracellular metabolic products was also explored using 35 mL glass bottles. Bacterial cells (30 mL) were harvested by centrifugation at 4000 rpm for 10 min and washed with growth medium. The cells were killed by autoclaving (121 °C, 30 min) and added to bottles containing BTM supplemented with 35 μM bromate. For the study of bromate removal by extracellular metabolic products, bromate (35 μM) was added to 30 mL of cell-free medium obtained from the bacterial cultures. The medium was filtered with a 0.2 μm hydrophilic polyestersulfone membrane (Machererey-Nagel) to remove cells and purged with nitrogen gas [23]. Furthermore, abiotic reduction of bromate by sulphide (12, 23 and 50 μM) was investigated using 120 mL glass bottles containing 100 mL BTM supplemented with 35 μM bromate. Sulphide was added as sodium sulphide anhydrous ($Na_2S$).

### 2.3. Analytical methods

Periodically, samples from cultures were collected using a syringe and optical density at 600 nm (OD600) was measured in each sample. The samples were analyzed after centrifugation at 4000 rpm for 5 min. Redox potential and pH were determined using a pH/E Meter (GLP 21, Crison). Sulphate and sulphide concentrations were quantified by UV/visible spectrophotometry (Hach-Lange DR2800 spectrometer) using the method of SulfaVer®4 and Methylene Blue Method (Hach-Lange, Dusseldorf, Germany), respectively. Bromate removal and bromide production were monitored by a DX-120 ion chromatograph with conductivity detector (reference DS4 Detection Stabilizer) equipped with pre-column IonPac AG HC 4–50 mm, column IonPAc AS9HC 4–250 mm and a suppressor ASRS 300 mm. The ion chromatograph, as well as the mentioned components, is from Dionex brand. The analysis was performed with $Na_2CO_3$ (9 mM) as mobile phase; at a flow rate of 1 mL/min.

### 2.4. Molecular characterization

#### 2.4.1. 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. [21]. 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′) [24]. The primers were purchased from Thermo Fischer Scientific. The reaction mixture used for PCR amplification contained 31.75 μL of sterilized 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 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.™ Gel Extraction Kit (Omega Bio-tech, USA). The purified products were ligated into the cloning vector pGEM®-T

Easy according to the manufacturer's instruction (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 expected DNA insert were saved at −20 °C.

#### 2.4.2. 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 e 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: JF749220 to JF749226. Phylogenetic trees were constructed using MEGA 4 and the Neighborhood-Joining algorithm was applied [25,26].

## 3. Results

### 3.1. Effect of initial bromate concentration on sulphate removal

Fig. 1 shows the influence of different bromate concentrations on sulphate removal rate of a SRB enriched community. The sulphate removal rates were determined using the following equation, where Co is the initial sulphate concentration, Cf is the sulphate concentration after 7 days of incubation and IT is the incubation time, which is 7 days:

$$\text{Sulphate removal rate} = \frac{\text{Co} - \text{Cf}}{\text{IT}} \times 100 \quad (3)$$

It was observed that sulphate removal was not affected by the presence of bromate, even when the bacterial consortium was grown in the presence of 39 μM of bromate. Independently of bromate concentration, the sulphate removal rate was 2.01 ± 0.13 mmol/L d.

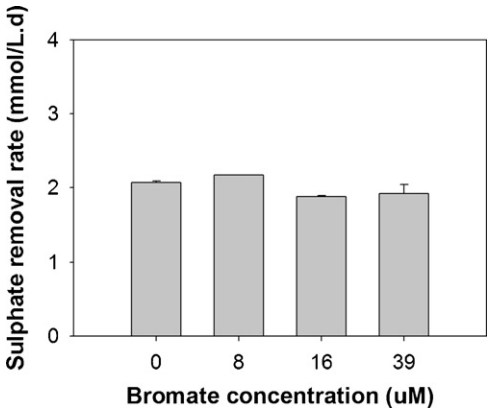


**Fig. 1.** Effect of the initial bromate concentration on sulphate removal rate (pH 7.6; 15.6 ± 1.1 mM of initial sulphate concentration). Data are the average of duplicates and error bars indicate the standard deviations of the average values.

### 3.2. Bromate and sulphate removal

Bromate removal by the bacterial consortium in the presence and absence of sulphate is shown in Fig. 2. An efficient bromate removal was observed in both conditions. After 3 days of incubation, the percentage of bromate removal was 96% and 90%, in the presence (Fig. 2a) and absence of sulphate (Fig. 2b), respectively. In the absence of sulphate, bromate removal increased during the incubation time achieving 93% in the end of the experiment.

Moreover, sulphate and bromate were simultaneously removed (Fig. 2a). After 7 days of incubation, 99% of sulphate was removed. Sulphate and bromate removal were not observed in the abiotic controls.

During bromate bio-removal studies, pH and redox potential were measured. Fig. 3 shows the variation of pH and redox potential in a medium with 40 μM bromate in the presence of 10 mM sulphate and in the absence of sulphate, in a period of 7 days. The pH of the medium supplemented with bromate in the presence of sulphate increased during all the experiment, achieving a value of 7.4 in 7th day of incubation time. The increase of pH was accompanied with a decrease of redox potential reaching the lowest value (−298 mV) in 7th day of incubation time.

In the absence of sulphate, an increase of pH of the medium (7.0–7.5) was observed in the first three days of the experiment. However, after this time the pH decreased to 6.9 in the end of

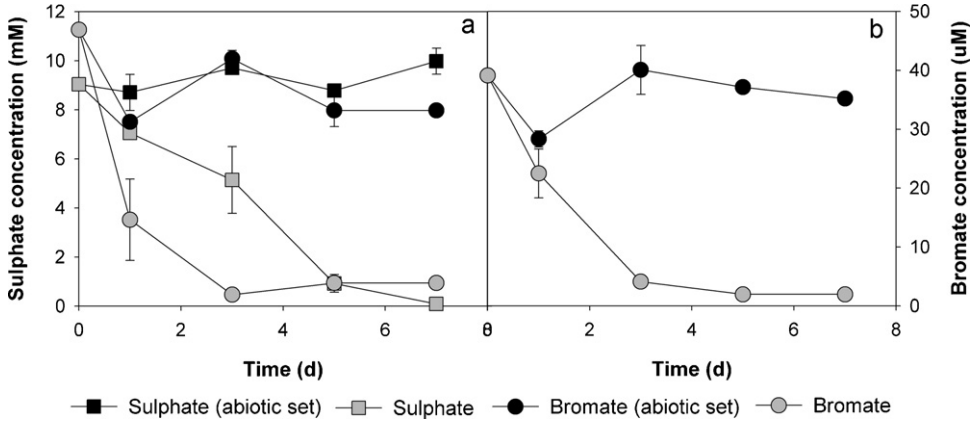


**Fig. 2.** Bromate and sulphate removal from the medium with 40 μM bromate in the presence of 9 mM sulphate (a) and in the absence of sulphate (b) (pH 7.1). Data are the average of duplicates and error bars indicate the standard deviations of the average values.

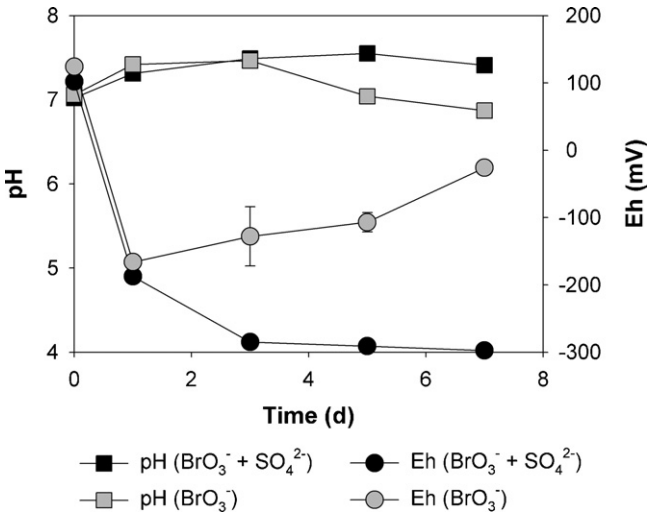


**Fig. 3.** pH and Eh profile during bromate bio-removal studies from the medium with 40 μM bromate in the presence of 10 mM sulphate and in the absence of sulphate (pH 7.1). Data are the average of duplicates and error bars indicate the standard deviations of the average values.

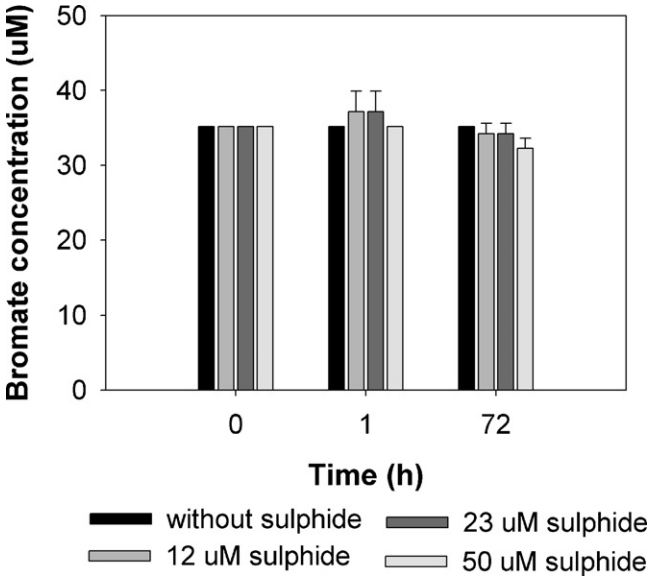


**Fig. 5.** Bromate removal from medium with 35 μM bromate by different concentrations of sulphide (pH 7.9 ± 0.1). Data are the average of duplicates and error bars indicate the standard deviations of the average values.

incubation time. The profile of redox potential in this experimental condition shows that this parameter decreased in the first day of the experiment (from +124 to −166 mV), but after this time increased reaching a value of −26 mV.

Fig. 4 shows bromate removal by live cells, heat-killed cells and extracellular metabolic products in the presence (Fig. 4a) and in the absence of sulphate (Fig. 4b). The highest bromate removal from the medium was observed with live cells, independently of the presence or absence of sulphate. After 5 days of incubation, 43 μM of bromate was removed from the medium containing 47 μM of bromate and 10 mM sulphate, while 38 μM of bromate was removed from the medium containing 40 μM of bromate in the absence of sulphate. At the same time 38 μM of bromide was detected in both conditions, which indicates that 88% of bromate removed was reduced to bromide in the presence of sulphate, while all bromate removed was reduced to bromide in the absence of sulphate. These results could indicate that other mechanism besides bromate reduction to bromide is involved in bromate removal in the presence of sulphate. Bromate removal by extracellular metabolic products was not observed in the medium without sulphate, while in the medium with sulphate the extracellular products showed ability for bromate removal. 44% of bromate was removed immediately after the addition of extracellular products to the medium containing 35 mM of bromate and 72% of bromate was removed until the end of the experiment. The existence of bromide was not detected in the presence these extracellular products. The removal of bromate by the extracellular products could explain the difference between the values of bromate removal (43 μM) and bromide production (38 μM) by the bacterial community in the presence of sulphate. In the presence of heat-killed cells, no relevant bromate removal was observed from the medium containing 35 μM of bromate in the presence and in the absence of sulphate.

The ability of sulphide to reduce bromate was studied in abiotic batch tests utilizing different sulphide concentrations (0, 12, 23, 50 μM) (Fig. 5). However, bromate was not removed by any sulphide concentrations.

### 3.3. Phylogenetic analysis

The molecular identification of the bacterial community with ability for bromate removal in the presence and absence of sulphate was performed in order to investigate eventual shifts in the

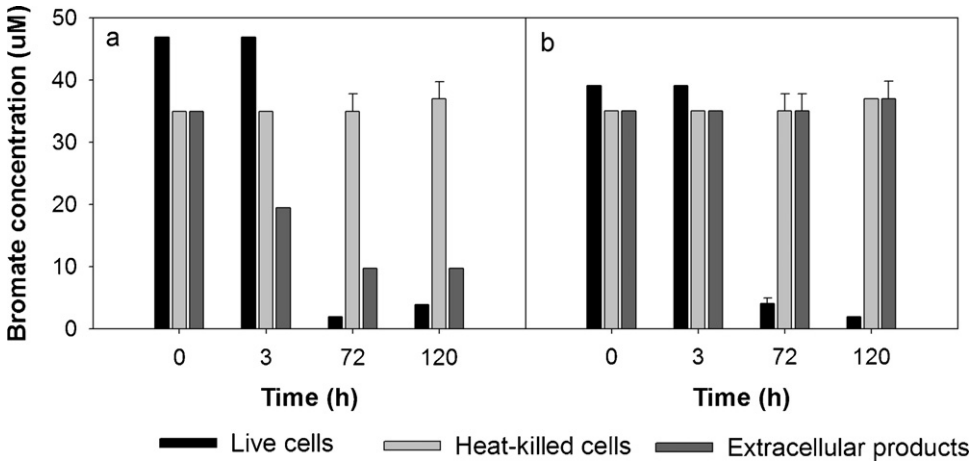


**Fig. 4.** Bromate removal from the medium with bromate, in the presence of 10 mM sulphate (a) and in the absence of sulphate (b), by cells (live and heat-killed) and by extracellular metabolic products (pH 7.3 ± 0.3). Data are the average of duplicates and error bars indicate the standard deviations of the average values.

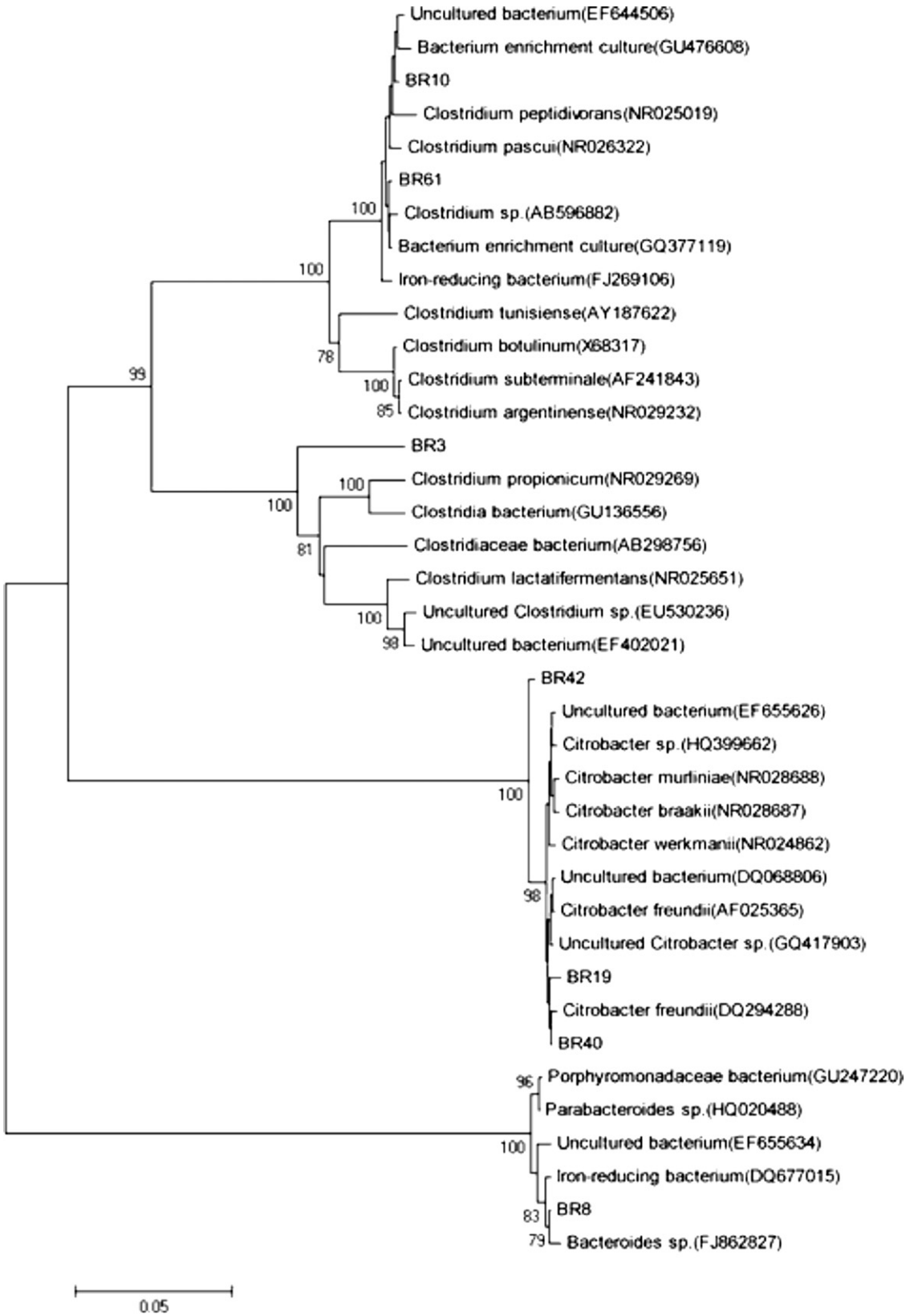


**Fig. 6.** Phylogenetic tree obtained with 16S rRNA sequences, corresponding to the clones representative of each restriction profile and to the most closely related ones retrieved from BLAST search. Phylogeny was inferred using the Neighborhood-Joining algorithm analysis of aligned 16S rRNA fragments. *Bootstrap values* are indicated on branches. Access numbers of GenBank sequences are indicated in figure.

consortia due to the presence of sulphate and to establish the relationships between bacterial groups and bromate removal.

All recombinant colonies (50) were recovered and approximately 1.4 kb fragment of bacterial 16S rRNA gene was amplified and used for RFLP analysis. Seven RFLP groups (Br3, Br8, Br10, Br19, Br40, Br42 and Br61 as representative clones) were originated from mixed culture grown in the presence of bromate and sulphate. The community grown in the presence of bromate without sulphate originated three RFLP groups, which were also originated from culture grown with bromate and sulphate (Br3, Br8 and Br42 as representative clones). Phylogenetic analysis of the representative clones allowed the identification of the corresponding sequences (Fig. 6).

Both communities were composed by bacteria belonging to three phylogenetic groups: *Clostridium*, *Citrobacter* and *Bacteroides*. However, the presence of sulphate affected the ratio of each group. Most of clone sequences from the community with ability for bromate removal in the absence of sulphate were closely related to *Clostridium* (90%). Most of clone sequences from the community with ability for bromate removal in the presence of sulphate were also closely related to *Clostridium* (58%). However, other dominant bacterial group was also detected in this community: 39% of clones were affiliated to *Citrobacter* genus. Clones with sequences closely related to *Citrobacter* were also present in the community grown in the presence of bromate without sulphate (5%).

Phylogenetic analysis also showed that 5% and 3% of clone sequences from the community grown in the presence of bromate without sulphate and with sulphate, respectively, were closely related to *Bacteroides*.

## 4. Discussion

In order to develop efficient bioremediation strategies to treat bromate wastewaters it is essential to search bromate resistant bacterial with ability for its removal and elucidate the mechanism responsible for bromate bio-removal. In this paper, the ability for bromate removal by a SRB enriched community was investigated. This community has been showed an excellent metal resistance [21] and consequently could be a potential candidate for bromate removal. Before bromate exposure, the SRB mixed culture showed excellent sulphate reducing performance. When bromate was added to the medium sulphate reduction was not affected. The sulphate reduction rate was maintained near 2 mmol/L d even in the presence of the highest bromate concentration tested (39 μM). Beside the high bromate resistance, this bacterial community showed ability to remove simultaneously bromate and sulphate and consequently bromate and sulphate removal can be considered compatible processes. 92% of bromate and 99% of sulphate were removed after 7 days. This result is an important finding, since to our knowledge the simultaneous removal of both sulphate and bromate was never reported. Moreover, bromate removal was also observed in the absence of sulphate. This suggests that the removal of bromate is not related to sulphate reduction.

The structure of the bacterial communities with ability for bromate removal in the presence and in the absence of sulphate was determined, in order to investigate if the removal of bromate in both conditions was performed by the same consortium. The phylogenetic analysis showed that the bacterial community developed in the medium with bromate and sulphate was mainly composed by bacteria closely related to *Clostridium* and *Citrobacter* genera, while the community grown in the absence of sulphate was predominantly composed by *Clostridium* spp. These results showed that the structure of the bacterial communities was affected by the presence/absence of sulphate, although bromate removal has been similar in both cases. Though the original community was composed by SRB [21], this group of bacteria was not detected in both bromate-removal consortia, although sulphate removal has been observed. These results strongly suggest that SRB are not responsible for bromate removal and for sulphate removal. In fact, it has already been demonstrated that modifications in bacterial growth conditions can imply drastic modifications on bacterial population [27].

*Clostridium* species are not able to reduce sulphate to sulphide [28]. However, *Citrobacter* spp. are considered sulphate reducing microorganisms, which do not belong to the traditional SRB group [29]. Therefore, the presence of this bacterial group can explain sulphate removal. The presence of *Citrobacter* spp. in mixed SRB cultures has already been reported [30,31]. Furthermore, the potential of *Citrobacter* species for bioremediation of wastewater contaminated with dyes was yet demonstrated [32]. However, the ability for bromate removal by this genus was never reported.

The mechanism of bromate removal by both bacterial communities (one grown in the presence of bromate and sulphate and the other grown with bromate) was also investigated. Bromate removal was tested with live cells, heated-killed cells, extracellular metabolic products and by abiotic reduction by sulphide.

Abiotic reduction of bromate by sulphide was not observed in the conditions of the present study, though it has been demonstrated that sulphide can be an effective bromate reducing agent [9].

Bromate removal by the bacterial community grown without sulphate, which was mainly composed by *Clostridium* spp., was only observed with live cells. The lack of bromate removal by extracellular products and heat-killed cells suggests that only viable cells can be responsible for bromate removal from the solution. Moreover, bromate was totally reduced to bromide by this bacterial community. These results demonstrated that a mechanism of enzymatic reduction is probably involved in bromate removal by the community composed by *Clostridium* spp. It has been reported that *Clostridium* species are able to enzymatically reduce some metals, namely Tc(VII) [33], Pu(IV) [34], Se(VI) [35] and U(VI) [36]. Hence, they are probably capable of reducing bromate to bromide.

Bromate removal by the community composed by *Clostridium* spp. and *Citrobacter* spp. (grown in the presence of bromate and sulphate) was also observed in the presence of live cells. Nevertheless, only 88% of bromate was reduced to bromide, suggesting that other mechanism than enzymatic reduction was involved in the removal of bromate. Contrarily to what was observed with the culture mainly composed by *Clostridium* species, the extracellular products excreted by this bacterial community showed ability for bromate removal. However, bromide was not detected in this condition. Bromate adsorption to the extracellular products may be responsible for bromate removal in the presence of these products. Therefore, two mechanisms could be involved in bromate removal in the presence of sulphate: enzymatic reduction of bromate to bromide by live cells and bromate removal due to the action of the extracellular products of bacteria. The removal of uranium by extracellular products of *Citrobacter* sp. was previously demonstrated by Macaskie et al. [37].

The presence of sulphate in the medium promoted modifications in the structure of the bacterial community having as a consequence the occurrence of different mechanisms of bromate removal.

## 5. Conclusions

In the present work, a bacterial community highly resistant to bromate and with ability for bromate removal was obtained from an enrichment SRB culture. This community is capable to remove simultaneously bromate and sulphate. Moreover, the ability to

remove bromate was maintained in the absence of sulphate. However, the structure of the bacterial community was affected by the presence of sulphate. In the presence of both bromate and sulphate the community was mainly composed of bacteria that was closely related to *Clostridium* and *Citrobacter*, and the bromate removal was done by enzymatic reduction and by extracellular metabolic products. On the other hand, the community grown in the absence of sulphate was predominantly composed by *Clostridium* spp. and the enzymatic process was the only mechanism involved in bromate removal. These results are relevant findings, since all these bacteria species were never reported as bromate-reducing-bacteria, or even bromate resistant. Therefore this work can be a contribution for the development of a biological process to solve the problematic presence of bromate in drinking water.

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