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Uranium removal from complex mining waters by alginate beads doped with cells of *Stenotrophomonas* sp. Br8: Novel perspectives for metal bioremediation

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ABSTRACT

Uranium-containing effluents generated by nuclear energy industry must be efficiently remediated before release to the environment. Currently, numerous microbial-based strategies are being developed for this purpose. In particular, the bacterial strain *Stenotrophomonas* sp. Br8, isolated from U mill tailings porewaters, has been already shown to efficiently precipitate U(VI) as stable U phosphates mediated by phosphatase activity. However, the upscaling of this strategy should overcome some constraints regarding cell exposure to harsh environmental conditions. In the present study, the immobilization of Br8 biomass in an inorganic matrix was optimized to provide protection to the cells as well as to make the process more convenient for real-scale utilization. The use of biocompatible, highly porous alginate beads for Br8 cells immobilization resulted the best alternative when investigating by a multidisciplinary approach (High-Angle Annular Dark-Field Scanning Transmission Electron Microscopy (HAADF-STEM), Environmental Scanning Electron Microscopy (ESEM), Fourier Transform Infrared Spectroscopy with Attenuated Total Reflectance, etc.) several consolidated entrapment methods. This biomaterial was applied to complex real U mining porewaters (containing 47 mg/L U) in presence of an organic phosphate source (glycerol-2-phosphate) to produce reactive free orthophosphates through Br8 phosphatase activity. Uranium immobilization rates around 98 % were observed after one cycle of 72 h. In terms of U removal ability as a function of biomass, Br8-doped alginate beads were determined to remove up to 1199.5 mg U/g dry biomass over two treatment cycles. Additionally, optimized conditions for storing Br8-doped beads and for a correct application were assessed. Results for U accumulation kinetics and HAADF-STEM/ESEM analyses revealed that U removal by the immobilized cells is a biphasic process combining a first passive U sorption onto bead and/or cell surfaces and a second slow active biomineralization. This work provides new practical insights into the biological and physico-chemical parameters governing a high-efficient U bioremediation process based on the phosphatase activity of immobilized bacterial cells when applied to complex mining waters under laboratory conditions.

1. Introduction

Uranium (U) mining as well as nuclear plants/reactors generate a large volume of hazardous wastewaters containing U and other heavy metals. To prevent potential contamination of nearby water bodies with highly soluble U compounds and subsequent U introduction into the trophic chain, these effluents must be efficiently treated. Conventional physicochemical-based methodologies for processing heavy metals

Abbreviations: Uranium, (U); Glycerol-2-phosphate, (G2P); Experimental techniques: High-Angle Annular Dark-Field - Scanning Transmission Electron Microscopy, (HAADF-STEM); Environmental Scanning Electron Microscopy, (ESEM); Fourier Transform Infrared Spectroscopy with Attenuated Total Reflectance, (FTIR-ATR).

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polluted wastewaters are known to be inherently costly at economical and environmental level, as well as inefficient at low heavy metals concentrations (Birungi and Chirwa, 2015; Jaafari and Yaghmaeian, 2019). In contrast, biological technologies emerge as an attractive approach for metal remediation mainly due to its low environmental impact and high efficiency at rather low heavy metals concentration (Das and Osborne, 2018). Numerous recent studies exhibited the great prospects of applying selected microbes to decontaminate heavy metals polluted waters through different interaction mechanisms (reviewed in Soni et al., 2019). In the case of U, main processes reported to be involved in microbial-based bioremediation strategies are biosorption (Acharya et al., 2009; Akhtar et al., 2009), bioprecipitation (Martinez et al., 2007; Nedelkova et al., 2007; Choudhary and Sar, 2011; Merroun et al., 2011; Pinel-Cabello et al., 2021) and enzymatic reduction (Lovley and Phillips, 1992; Williams et al., 2013). Recently, it was demonstrated the ability of the bacterial strain *Stenotrophomonas* sp. Br8 to precipitate U(VI) as long-term stable U phosphates mediated by inorganic phosphates which are generated via phosphatase enzymes under different environmental conditions relevant for U contaminated waters (pH, temperature, etc.) (Sánchez-Castro et al., 2020). This microbial-driven U immobilization procedure is considered particularly attractive (Benzerara et al., 2011) in comparison with other extensively reported biological methods like the bioreduction of U(VI) to U(IV) showing stability issues of the resulting compounds (e.g. Bargar et al., 2008).

In order to make this Br8-based U(VI) biomineralization process (Sánchez-Castro et al., 2020) applicable at field scale, several issues like cell exposure to harsh environmental conditions (Prabhakaran et al., 2016) or potential up-scaling drawbacks should be overcome. In addition, the use of cells in planktonic state may result in microbial activity reduction because of cell washout in continuously operated reactors (Baskaran and Nemati, 2006). In this sense, the immobilization of microorganisms is a procedure known to provide a series of advantages in different biotechnological applications such as bio-fuel production or wastewaters purification (Ranganathan et al., 2008; Covarrubias et al., 2012). The micro-environments created within these immobilization matrices can improve the preservation of cell integrity under limiting conditions as well as provide specific properties to microbes (Bouabidi et al., 2019). Many other practical reasons such as easy transportation and handling, the possibility to use it repeatedly and easy separation/recuperation of cells and elements accumulated in the matrix (Dianawati et al., 2016; Shi et al., 2018) make this alternative more efficient at multiple levels (e.g. operational, economical), and thus, convenient for real-scale application.

A great variety of biomass immobilization technologies have been proposed in the last decades (Dzionek et al., 2016). Those based on the immobilization of microbial biomass in the supporting material through the use of different chemical agents are the most commonly used (Bouabidi et al., 2019). In this way, methods such as adsorption (including ionic and covalent binding to a solid support), cell cross-linking to obtain the formation of stable cellular aggregates and mainly, biomass entrapment in a polymeric matrix have been already tested with satisfactory results (Bouabidi et al., 2019). In relation to this last method, different natural (mainly isolated from algae such as agar, chitosan, carrageenan, cellulose or alginate salt) and synthetic polymers (e.g. polyacrylamide, poly(carbamoyl) sulphonate, silica, polyethylene glycol, polyurethane or polyvinyl alcohol) showing specific features are proposed depending on the nature of the biomass to be immobilized (Wang et al., 2010; Kumari et al., 2017; Kiran et al., 2018). To enhance the effectiveness of the immobilization process for a particular biotic agent, and therefore the application performance of the immobilized-biomass system, an optimization process by assessing supporting materials with different characteristics seems essential. Some desirable features to apply this type of biomaterials in water treatment are hydrophilicity, inertness towards any kind of external bioactivity, biocompatibility, resistance to biotic and abiotic harmful agents and low production costs (Brodelius and Mosbach, 1987).

The main aim of the present work is to optimize a suitable U immobilization procedure based in the use of *Stenotrophomonas* sp. Br8, a strain isolated from U-mining porewaters, which in planktonic form efficiently precipitates soluble species of U(VI) in chemically stable U-phosphate mineral phases with a structure similar to that of meta-autunite (Sánchez-Castro et al., 2020). For this purpose, several immobilization strategies were tested and final products were characterized at multiple levels (matrix physico-chemical properties, cells distribution and viability, etc.). Biomass immobilized under optimized conditions was applied to perform batch-scale experiments for U removal from mining porewaters containing glycerol-2-phosphate (G2P; source of organic phosphates) amended to produce reactive free orthophosphates through Br8 phosphatase activity. So far, few works have demonstrated the applicability of immobilized bacteria to successfully treat real complex U-containing mining porewaters. The present study is essential to determine optimal application conditions to scale-up the proposed low-cost removal process. Moreover, this type of studies is clearly necessary as a preliminary step towards the field application of innovative technologies for heavy metals bioremediation.

2. Materials and methods

Experimental procedures related to the study of the effect of incubation time/biomass concentration/biomaterial storage as well as microscopic analyses (HAADF-STEM/EDAX and ESEM) are provided as Supplementary Material.

2.1. Bacterial strain Br8 and optimization of a biomass immobilization procedure

The bacterial strain *Stenotrophomonas* sp. Br8 was isolated from U mill tailings porewaters in the vicinity of former mining sites in France (Sánchez-Castro et al., 2017). This strain showed great U removal potential as planktonic cells under different conditions (Sánchez-Castro et al., 2020). Cells of this bacterial isolate were employed to assess and enhance previously described biomass immobilization procedures for the decontamination of U polluted waters.

Stenotrophomonas sp. Br8 cells were grown in LB broth (casein peptone 10 g/L, yeast extract 5 g/L, pH 7.2; Scharlau Chemie, SA) at 28 °C under shaking at 160 rpm for 24 h. Subsequently, mid-exponential growth phase cells were recovered by centrifugation (10,000×*g* for 5 min at 4 °C) and washed twice with 0.9 % NaCl solution. Afterwards, the washed biomass was immobilized similarly as indicated in the different reference protocols considered (stated below), but using always the same biomass concentration (~$1.5{\cdot}10^7$ CFU/g biomaterial) to make comparable at biotic level all biomaterials generated. Well-established entrapment methods were used as baseline to optimize Br8 cells immobilization. Firstly, the Na-alginate entrapment technique by Smidsrød and Skjakbraek (1990) is based on the formation of beads by dripping through a syringe a mixture of cells and an aqueous solution of Na^+ alginate into a solution containing Ca^{2+} ions. The second method used as reference produces sol−gel ceramics by gelling and drying a mixture of biomass and aqueous silica nanosols composed of tetraethyl orthosilicate (Raff et al., 2003; Soltmann et al., 2003). And finally, alternative approaches combining both methodologies stated above were also considered to develop this work (Pannier et al., 2014; Perullini et al., 2014).

Resulting Br8-doped biomaterials (biocer, beads, etc.) were investigated at multiple levels using a multidisciplinary approach combining microscopy, spectroscopy, etc. Thus, external and internal morphology and cell distribution within the matrix were characterized using environmental scanning electron microscopy (ESEM, model FEI Quanta 650 FEG, Thermofisher-FEI) analysis as described below. Presence and identification of major types of functional groups at surface level were determined by Fourier Transform Infrared Spectroscopy with Attenuated Total Reflectance (FTIR-ATR). The analyses were performed with a

Jasco 6200 spectrometer, with a total of 32 scans per measure and a resolution of 0.25 cm^{-1} in the mid-infrared region (4000–400 cm^{-1}). Abiotic matrices corresponding to each method applied were prepared for comparison and for the evaluation of the effect of biomass in the immobilization support at structural level (mechanical stability, porosity, etc.).

2.2. Chemical characterization and U speciation in the studied mining water

Real U mining porewaters, under the monitoring of ORANO Mining, were used as incubation medium in all cases. Geochemical characterization and aqueous uranium speciation in this mining water, named COMI_79, was already given in Reiller and Descostes (2020). Uranium concentration was estimated in 47.4 mg/L and U(VI) was mainly found under $Ca_nUO_2(CO_3)_3^{4-2n}$ aqueous species. pH of the mining water was 7.75. Porewater samples received were analyzed in our laboratory and characteristics previously reported were confirmed (data not shown). This mine water was chosen as its composition is characteristic of U mining context, that is to say high U content and relatively high ionic strength.

2.3. Uranium removal batch experiments

Batch U removal experiments applying the previously optimized *Stenotrophomonas* sp. Br8-doped biomaterial were performed using 100 mL acid-washed glass Erlenmeyer flasks filled with 25 mL of non-filtered COMI_79 mining water as incubation medium. G2P (Sigma Aldrich) was selected as phosphate source (5 mM). Flasks including: a) cell-free beads; b) cell-free G2P-containing system; or c) phosphate-free system, were assayed as controls to validate the results obtained. To distinguish between the role of the bacterial strain Br8 and that of naturally occurring microbes in the mining water used as incubation medium, 0.22 μm filter-sterilized COMI_79 mining water was also evaluated. Moreover, to investigate the potential reusability of the biomaterial for U removal, two 48-h-incubation cycles were assayed by using the same biomaterial but changing mining water solution between both cycles. Incubation was conducted at 28 °C for 48–72 h and under continuous shaking (165 rpm) in all cases.

After incubation, 5-mL aliquots of the mining water used as incubation solution from all replicates were centrifuged at 10000 g for 10 min at 4 °C, and supernatants were stored for further analysis. Recovered biomaterials were washed twice with 0.9 % NaCl to remove the interfering elements of the incubating solution before analyzing. Uranium removal efficiency was evaluated by estimating the precipitation of dissolved U(VI) under defined experimental conditions. Chemical composition variations in the incubation mining water solution were determined through ICP-MS.

All treatments were conducted in triplicate and all subsequent analyses utilized all replicates data for statistical analysis.

2.4. Multilevel batch experiments evaluation

2.4.1. Determination of inorganic phosphates (IP) released

The IP concentration in solution after the incubation period was determined by means of the "ammonium-molybdate method" (Murphy and Riley, 1962). This technique is based in the reaction of the orthophosphate ions with ammonium-molybdate in acidic solution forming phosphomolybdic acid. Upon reduction with ascorbic acid, this compound produces a blue complex whose intensity is quantified spectrophotometrically at 850 nm.

2.4.2. Quantification of uranium removal ratio

Concentration of U(VI) in solution was determined spectrophotometrically by using the Arsenazo III method (Jauberty et al., 2013). In brief, 1 mL of a disodium salt of 2,7-bis(2-arsenophenylazo)-1, 8-dihydroxynaphthalene-3,6-disulfonic acid (also called Arsenazo-III) solution was mixed with 250 μL of supernatant and absorbance was measured at 651 nm after 30 s incubation. Reagent was prepared by dissolving 70 mg of Arsenazo-III into 1 l of 3 M $HClO_4$. Uranium concentrations were determined by comparison with a scale of uranyl nitrate accounting for 1–15 mg/L. The percentage of U removed from solution was calculated by the difference between the initial and final U concentrations.

2.4.3. Recovered biomaterial evaluation

Mechanical stability of the beads was assessed taking into account the morphology intactness using optical microscope and scanning transmission electron microscope (STEM) as described below. Viability of the immobilized biomass after incubations was evaluated by grinding surface-sterilized biomaterial and culturing it in LB plates for 48–72 h at 28 °C.

2.5. Statistical analysis

Data in this manuscript are presented as averages and standard deviations (SD) for at least three independent replicates per experimental condition tested. Analyses of variance (ANOVA) were performed together with the post hoc Tukey's test to detect significant differences among all treatment means. Significant differences ($p < 0.05$) among treatments after ANOVA and Tuckey's test ($n = 3$) were represented by different letters. All statistical analyses and data visualization were done using GraphPad Prism 8.0.2 (GraphPad Software, San Diego, California USA) and R Project software (R Core Team, 2020).

3. Results

3.1. Selection and optimization of the biomass immobilization method

Methods based on the use of tetraethyl orthosilicate (Raff et al., 2003; Soltmann et al., 2003) and Na-alginate (Smidsrød and Skjakbraek, 1990) as inorganic supports, as well as modified versions of other enhanced approaches (Pannier et al., 2014; Perullini et al., 2014), were implemented with Br8 cells. Multiple parameters like mechanical and chemical stability, identity of functional groups at surface level, biocompatibility, and external/internal morphology (e.g. porosity, cell distribution) were evaluated in comparison with control abiotic immobilization matrices. Taking into account these results (illustrated in Table S1 and Figs. S1–S7 of the Supporting Information (SI)), an optimized immobilization procedure, based on that described by Smidsrød and Skjakbraek (1990) using Na-alginate as inorganic support, was developed. Modifications of the original protocol, aimed to enhance long-term stability of the biomaterial produced, which in turn would lead to decrease the economic costs of the U removal process proposed. The main modifications were related to increase the concentration of the aqueous stock solution of Na-alginate (5 % instead of 2–4%) and the mixing proportion of Na-alginate solution to concentrated washed-cells solution (4:1 instead 1:1) to achieve the desired cells dosage and a final alginate concentration of 4 %. In addition, concentration of $CaCl_2$–based solidifying solution (increased from 0.02-0.1 M–0.3 M) and incubation time in this hardening solution (2–3 h instead 5–30 min) were modified in relation to the original method (Smidsrød and Skjakbraek, 1990) as they yielded better stability.

3.2. Characterization of the biomass immobilization system

The enhanced immobilization procedure resulted in the formation of beads with a size of about 1.5–2 mm as it is shown in Fig. 1.

FTIR-ATR spectroscopy results (Fig. S1) are provided as Supplementary Material.

By microscopic observation (ESEM and HAADF-STEM), the structure of the inner section of the bead was shown to contain numerous macro-

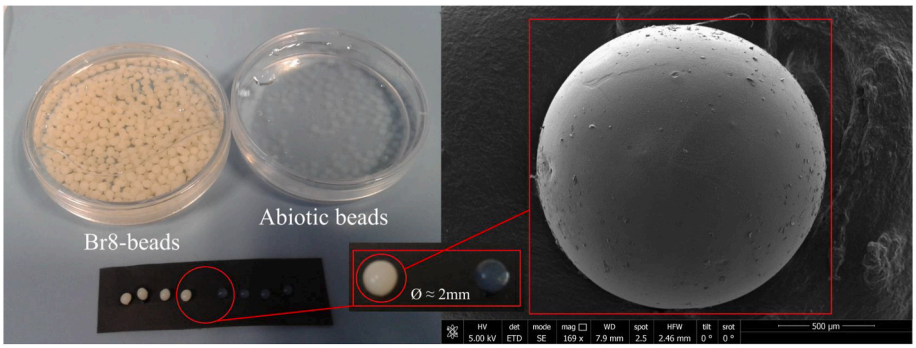

Fig. 1. Alginate beads, with and without *Stenotrophomonas* sp. Br8 cells, generated with the enhanced method based in the protocol of Smidsrød and Skjakbraek (1990). Right micrograph was obtained with Environmental Scanning Electron Microscope FEI Quanta-400.

and micro-pores suggesting a good ratio surface/volume and high diffusion rates (Figs. S2–S7). In addition, cells entrapped within the beads were confirmed to be abundant and homogenously distributed (Figs. S2–S7). Although the beads demonstrated very high mechanical stability since no relevant alterations were observed through optical microscope after 120-days incubation in saline solution under agitation, a Na-citrate solution (50 mM) dissolved them after 30–60 min. This fact is the main weakness of alginate beads since they are widely known to be chemically unstable in the presence of calcium chelators such as phosphate, lactate or citrate (Ching et al., 2017).

3.3. Batch studies of U removal using Br8 immobilized biomass

The interaction mechanisms occurring when contacting Br8 immobilized cells with U-containing mining waters (COMI_79) and optimal application conditions for U removal were investigated. A series of batch assays considering the effect of biomass-water contact time, biomass concentration and beads storage conditions were conducted. Additionally, resulting precipitates were characterized by HAADF-STEM and ESEM microscopic observations.

3.3.1. Contact time effect: kinetics study

The immobilized cells obtained using the modified procedure described above were characterized for its potential in the removal of U from real mining waters. Br8-doped beads efficiency to precipitate solved U(VI) in presence of G2P increased gradually with longer contact times (from 0.08 h to 72 h), reaching a highest value of 97.8 % at the end of the incubation (72 h) (Fig. 2a). Planktonic Br8 cells provoked the U immobilization in a shorter time, although the final removal rate is quite similar than that of the immobilized cells at final incubation time (72 h; Fig. 2a). Release of inorganic phosphates resulted slower for the Br8-beads in comparison to planktonic cells (Fig. 2b). This fact is probably explained by the easier accessibility of Br8 phosphatases to the amended G2P in planktonic cells than in embedded ones.

Control experiments confirmed the results presented above (Fig. 3). For instance, cell-free beads (abiotic control beads) in presence of G2P could remove less than 4 % of the initial U concentration. Contrary to our results, some studies described a significant contribution of cell-free alginate beads in the removal of uranium from dilute aqueous solutions at different optimized conditions (Gok and Aytas, 2009; Kulkarni et al., 2013; Yu et al., 2017). In the case of exogenous phosphate-free treatments, no significant U removal was evidenced (Fig. 3). When using filter-sterilized COMI_79 mining water no relevant changes were observed in comparison with non-filtered water, confirming that indigenous microbial populations inhabiting this mining water have no relevant role on the U removal process (Fig. 3).

The U removal ability of the immobilized Br8 cells over two treatment cycles was determined to be up to 1199.5 mg U/g dry biomass (637.5 and 562 mgU/g dry biomass for the first and second cycle, respectively). Residual U concentration increased approximately 10 % after the second treatment cycle, exhibiting a final removal rate of 87.4 %. This data clearly revealed that immobilized Br8 beads can be reused at least for two cycles achieving high U removal rates. Application of additional cycles with the same batch of beads would suppose a significant increase in the total U precipitation capacity of immobilized Br8 biomass, although our data suggested a decrease in removal efficiency

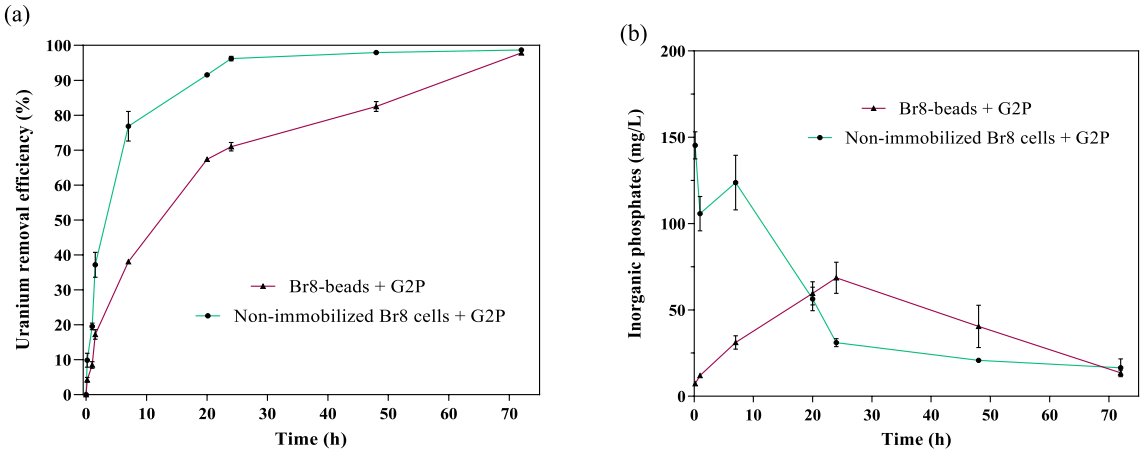

Fig. 2. Uranium removal efficiency (%) kinetics (a) and inorganic phosphates in solution (mg/L) detected (b) during incubation (28 °C; 165 rpm) of natural COMI_79 mining water (initial U concentration 47.4 mg/L; pH 7.75; amended with 5 mM G2P) in presence of 4 % Na-alginate beads doped with *Stenotrophomonas* sp. Br8 cells (~1.5·107 CFU/g bead). Flasks with non-immobilized Br8 cells were used as control treatment. Data are showed as the mean ± SD of at least three independent measurements. For some points, the error bars are shorter than the height of the symbol.

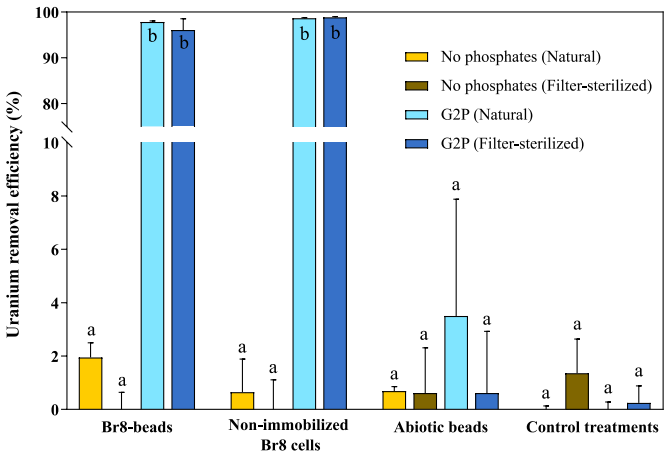

Fig. 3. Uranium removal efficiency (%) after incubation (72 h; 28 °C; 165 rpm) of natural and filter-sterilized COMI_79 mining water (initial U concentration 47.4 mg/L; pH 7.75; amended either with 5 mM G2P or without amended phosphates) in presence of 4 % Na-alginate beads doped with *Stenotrophomonas* sp. Br8 cells (~1.5·107 CFU/g bead). Flasks with non-immobilized Br8 cells, abiotic beads, and without Br8 cells were used as control treatments. Data are showed as the mean ± SD of at least three independent measurements.

after each cycle in agreement with recent surveys (e.g. Shi et al., 2018). Conversely, other studies reported no detrimental effects on heavy metals removal rates after multiple applications of the beads (Kiran et al., 2018).

3.3.2. *Microscopic characterization of U immobilization process*

HAADF-STEM and ESEM analyses showed that Br8-doped beads incubated for 72 h in G2P-amended COMI_79 water displayed apparently intact macro-morphological properties besides complex nature of the treated water (Reiller and Descostes, 2020). STEM micrographs of thin sections of the Br8-doped beads and ESEM images revealed the presence of needle-like U precipitates distributed at surface and inner areas of the beads (Fig. 4 and S8-S9). Uranium precipitates within the beads were mainly found around the cells, what is likely caused by the localization of phosphatases at this level (Kulkarni et al., 2016; Chandwadkar et al., 2018). No intracellular U precipitates were observed as it was also reported for U treated planktonic cells (Sánchez-Castro et al., 2020). Cell-free beads (abiotic control) showed no significant U precipitation (data not shown) confirming the key role of Br8 in the U removal process described above. Further microscopic and spectroscopic analyses revealed U and P as main elements composing resulting precipitates (Fig. 5), what suggests formation of biogenic uranium phosphates of amorphous nature as indicated by XRD analysis (Fig. S10).

3.3.3. *Effect of the biomass concentration on U removal*

The effect of biomass dosage and number of beads used per mL of tested mining water was investigated. As stated in Materials and Methods section, simple dosage will be considered ~$1.5{\cdot}10^7$ CFU/g biomaterial. Use of 1 or 2 beads per mL with simple cell dosage or 1 bead per mL with double cell dosage showed all similar U precipitation rates over 90 % (Fig. 6a) after 72 h incubation. The use of 0.5 Br8-beads per mL with simple cell dosage reduced the U removal efficiency in 15–20 %, reaching a maximum value of 77 %. In the same trend, the release of inorganic phosphates was similar in all cases except for treatment using 0.5 beads (simple dosage) per mL which resulted in a lower orthophosphates release (Fig. 6b).

3.3.4. *Effect of the immobilized biomass storage conditions*

To enhance the applicability of the proposed biomaterial, different storage conditions (temperature, time and physical state) were tested before using it and investigating their impact on its U removal performance. At U immobilization level, no significant differences were found after storing non-freeze-dried beads at 4 °C for 7 d, 90 d and 300 d, as well as after storing freeze-dried beads at 25 °C for 90 d, reaching in all cases values between 90 and 95 % after 72 h (Fig. 7a). However, non-freeze-dried beads stored at 25 °C for 90 d evidenced a dramatic decrease to removal values under 3 %, what may be explained by a low cell viability which produced a poor release of inorganic phosphates (Fig. 7b). Although non-freeze-dried beads conserved for 300 d (4 °C) and freeze-dried beads after rehydration showed some alterations in their external morphology, these changes seemed not to affect their performance as stated above (Fig. 7).

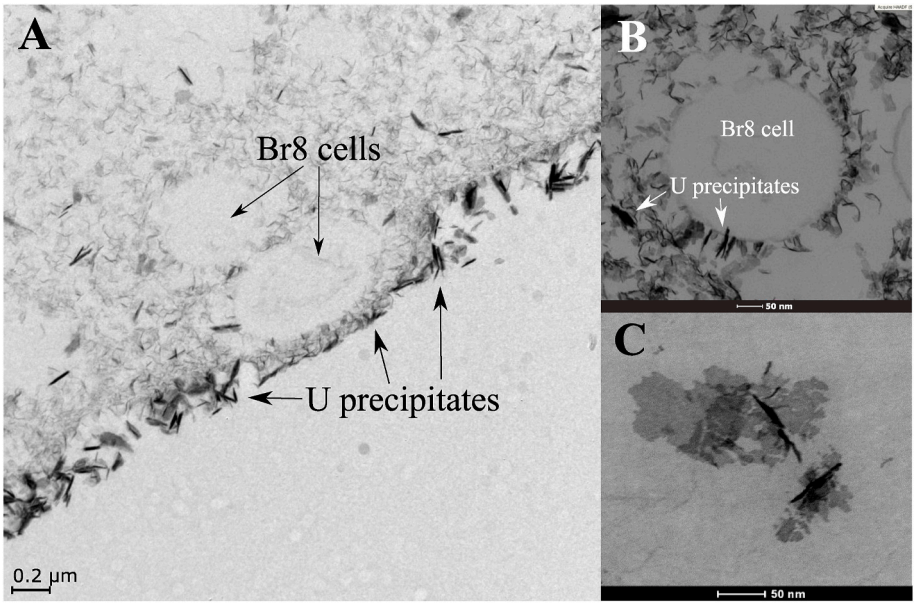

Fig. 4. HAADF-STEM micrographs of thin sections of 4 % Na-alginate beads doped with *Stenotrophomonas* sp. Br8 cells (~1.5·107 CFU/g bead) recovered after incubation (72 h; 28 °C; 165 rpm) in natural COMI_79 mining water (initial U concentration 47.4 mg/L; pH 7.75; amended with 5 mM G2P). Image A shows the edge of the bead. Image B focused in a Br8 cell immobilized within the bead and image C shows a zoomed-in view of U-phosphate precipitates.

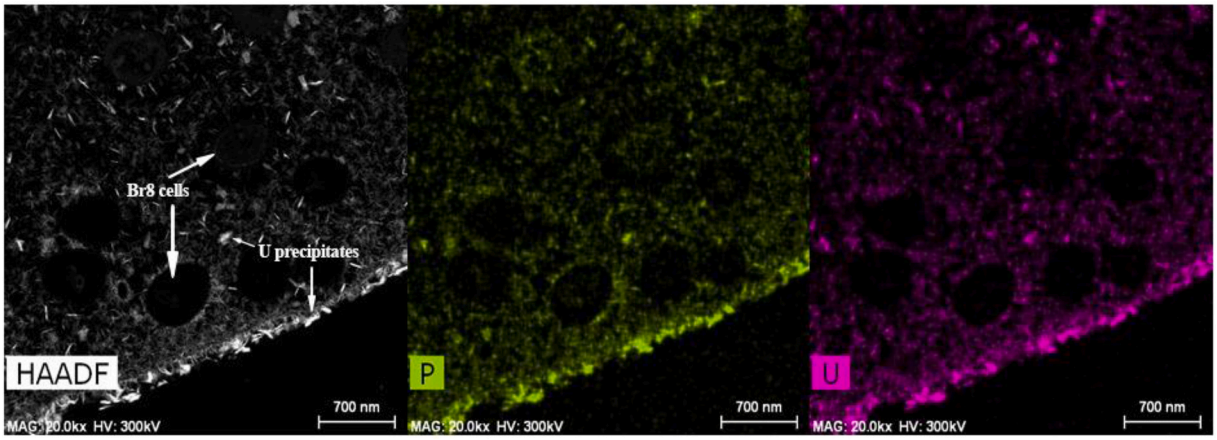

Fig. 5. EDX element-distribution maps for P and U and HAADF-STEM micrograph of a thin section showing U-phosphate precipitates in the inner part of a 4 % Na-alginate bead doped with *Stenotrophomonas* sp. Br8 cells (~1.5·107 CFU/g bead) recovered after incubation (72 h; 28 °C; 165 rpm) in natural COMI_79 mining water (initial U concentration 47.4 mg/L; pH 7.75; amended with 5 mM G2P).

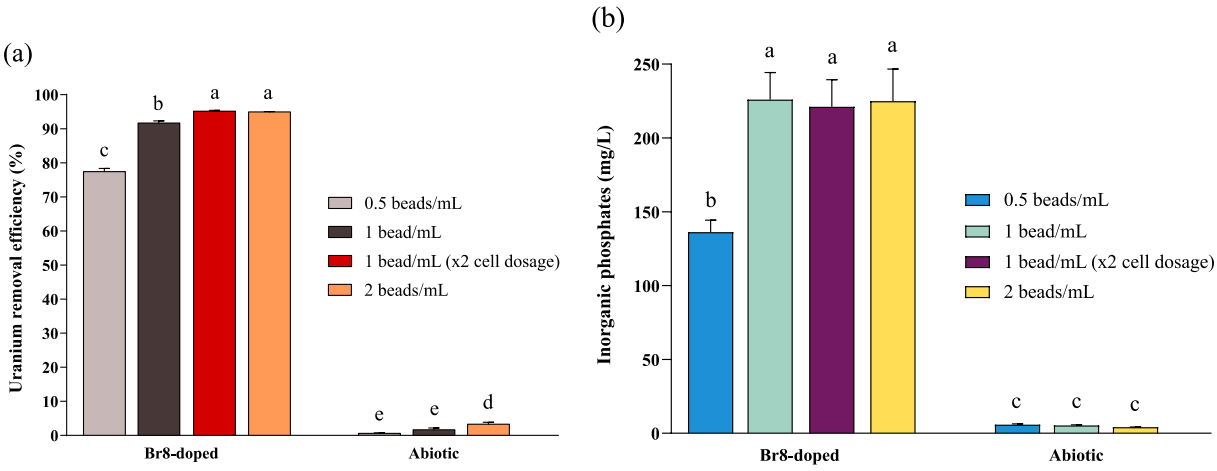

Fig. 6. Uranium removal efficiency (%) (a) and inorganic phosphates in solution (mg/L) detected (b) after incubation (72 h; 28 °C; 165 rpm) of natural COMI_79 mining water (initial U concentration 47.4 mg/L; pH 7.75; amended with 5 mM G2P) in presence of different number of 4 % Na-alginate beads doped with *Stenotrophomonas* sp. Br8 cells (~1.5·107 or ~3·107 CFU/g bead). Flasks with different number of abiotic beads were used as control treatments. Data are showed as the mean ± SD of at least three independent measurements. Different letters means significant differences ($p < 0.05$).

4. Discussion

4.1. Optimization of the immobilization process of Stenotrophomonas sp. Br8 bacterial biomass

Stenotrophomonas spp. are described for their high versatility and capacity for remediating a variety of metals like uranium (Merroun and Selenska-Pobell, 2008; Nazina et al., 2010; Islam and Sar, 2016), selenium (Ruiz-Fresneda et al., 2018, 2019, 2020), gold (Song et al., 2008), arsenic (Bahar et al., 2012), copper (Ghosh et al., 2020; Gopi et al., 2020), chromium (Ge and Ge, 2016; Aslam et al., 2020), zinc (Ge and Ge, 2016), nickel (Aslam et al., 2020; Ghosh et al., 2020), or lead (Aslam et al., 2020) through different mechanisms (e.g. biomineralization, bioreduction, bioaccumulation). In fact, the biomass of planktonic cells of the same *Stenotrophomonas* isolate used in this case (*Stenotrophomonas* sp. Br8) was previously characterized for its high U removal capacity in U nitrates solution amended with G2P at different metal concentrations (Sánchez-Castro et al., 2020). The present work goes a step beyond by evidencing their high capacity for efficiently immobilizing U from real U mining waters. For enhancing the applicability of this remediation strategy through protecting planktonic bacterial cells from toxicity provoked by high U (and other heavy metals) concentration and improving their stability, biomass immobilization through well-established entrapment protocols was evaluated.

In this study, evaluation of parameters like mechanical/chemical stability, presence of certain functional groups, biocompatibility, and external/internal morphology demonstrated the high efficiency of alginate as inorganic matrix for Br8 cell immobilization. Despite its poor chemical stability in presence of Ca^{2+} chelators, this natural polymer is preferred over other materials for active cell immobilization, mainly because of its high biocompatibility, hydrophilicity, presence of carboxylic groups (which enhance heavy metal ion adsorption; Romera et al., 2007), low economical cost, easy availability and low-biodegradability under different conditions (Lozinsky and Plieva, 1998; Wani et al., 2016). Similarly, as proposed in the present work where alginate final concentration was increased to 4 % to gain stability, modifications of the original alginate beads production protocol (Smidsrød and Skjakbraek, 1990) were repeatedly reported aiming to enhance its performance (e.g. increasing stability; Kiran et al., 2018). Furthermore, some studies demonstrated higher efficiency in bioremediation by using immobilized cells in comparison to planktonic cells likely due to immobilization matrix sorption capacity as well as better conservation in the bacterial activity (Shi et al., 2018). Other advantages such as better storage stability and better reutilizing ability are also

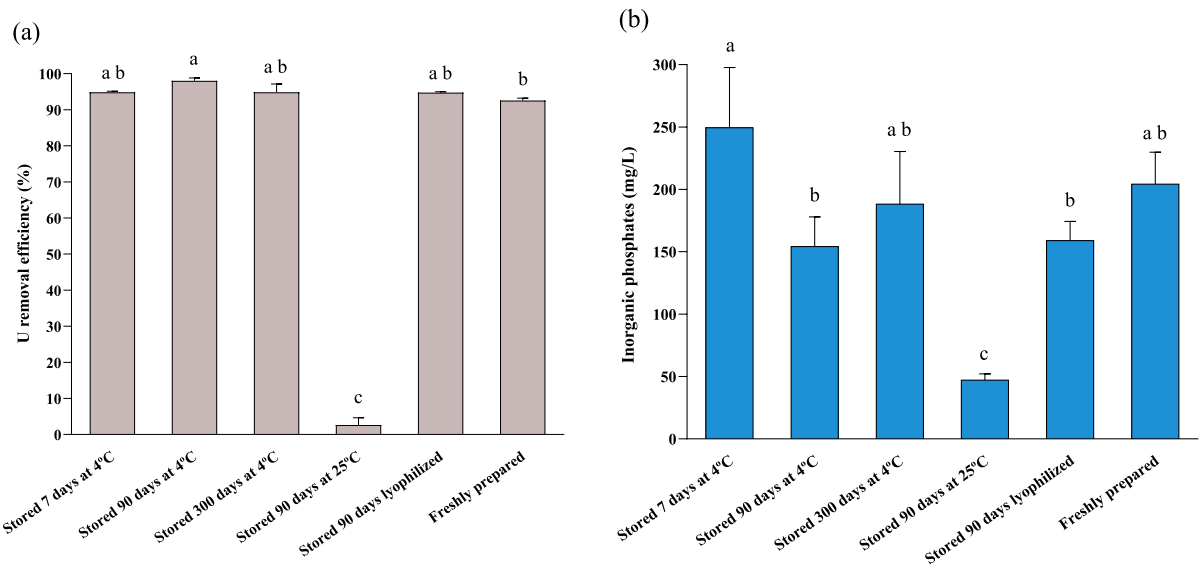

Fig. 7. Uranium removal efficiency (%) (a) and inorganic phosphates in solution (mg/L) detected (b) after incubation (72h; 28 °C; 165 rpm) of natural COMI_79 mining water (initial U concentration 47.4 mg/L; pH 7.75; amended with 5 mM G2P) in presence of 4 % Na-alginate beads doped with *Stenotrophomonas* sp. Br8 cells (~1.5·107 CFU/g bead) and stored under different conditions before application. Flasks with freshly prepared Br8-doped beads were used as control treatment. Data are showed as the mean ± SD of at least three independent measurements. Different letters means significant differences ($p < 0.05$).

addressed (Dianawati et al., 2016; Shi et al., 2018). For these reasons, alginate matrices doped with bacterial cells have been repeatedly investigated in the last years as efficient bioremediation agents of metals like lead (Zhang et al., 2020), chromium (Wu et al., 2019; El-Naggar et al., 2020), cadmium (Shi et al., 2018), uranium (Kulkarni et al., 2013), or copper and zinc simultaneously (Kiran et al., 2018). Moreover, cell-free alginate matrices have been also shown to produce comparable results (Yu et al., 2017).

4.2. Ability of Br8 immobilized biomass to remove U from mining water

In the present study we demonstrated the ability of immobilized cells, belonging to a strain of the genus *Stenotrophomonas*, for U immobilization. It was already reported multiple benefits of immobilizing cells from different strains of the genus in alginate beads or inert polyurethane foam to remediate colored textile wastewaters (Galai et al., 2010; Rajendran et al., 2015, respectively) or organic pollutants (Mukherjee and Roy, 2013), as well as being applied as biocontrol agents in agriculture (Ahmad et al., 2012). Likewise, catechol dioxygenases obtained from *Stenotrophomonas maltophilia* KB2 strain were immobilized in alginate hydrogels for enhancing the bioremediation and detoxification of xenobiotic-contaminated environments (Wojcieszyńska et al., 2012; Guzik et al., 2014). Regarding metals bioremediation, other microbes than *Stenotrophomonas* immobilized in alginate beads have been effectively used (Kiran et al., 2018; Leong and Chang, 2020). In particular, fungal cells (genus *Trichoderma*; Akhtar et al., 2007, 2009; and genus *Aspergillus*; Wang et al., 2010) immobilized in alginate beads have been successfully assessed to recover uranium from aqueous solutions. However, this is the first study describing the metal phosphate biomineralization as strategy to remove U from U contaminated water.

The fact that U immobilization process by Br8-doped alginate beads showed a first rapid metal removal phase (38 % in 7h) followed by a gradual slower phase where a maximum removal value (98 %) was achieved at 72 h, suggests that several interaction mechanisms are co-occurring in the system. During the first hours, passive mechanisms seem to mediate the immobilization of the uranium by sorption onto bead and/or cell surfaces, as observed in other studies (Gok and Aytas, 2009; Wang et al., 2010; Yu et al., 2017; Sánchez-Castro et al., 2020). Besides bacterial functional groups known to provide binding sites for U sorption (e.g. carboxyl and phosphoryl), other reactive groups detected on beads surface (e.g. hydroxyl and alkoxy groups) are likely participating (cell-free beads removed around 4 % of the total solved U; Fig. 3) in this first metabolism-independent phase (Yu et al., 2017). In a similar way as described in Sánchez-Castro et al. (2020), the rate of release of inorganic phosphates (mediated by phosphatase enzymes; Chandwadkar et al., 2018) by Br8 is presumably controlling the process kinetics of the subsequent U immobilization phase based on phosphate biomineralization active mechanisms. Cells' immobilization technology optimized in the present work seems not to affect phosphatase enzymes activity (97.8 % U removal after 72 h) although this kinetics study showed a delay in the process in comparison to planktonic Br8 cells application (Fig. 2).

As described in Sánchez-Castro et al. (2020), U precipitates within the beads were mainly localized around the cells, what is likely caused by the presence of phosphatases at this level (Kulkarni et al., 2016; Chandwadkar et al., 2018). No intracellular U precipitates were observed as it was also found for U treated planktonic cells (Sánchez-Castro et al., 2020), suggesting that cells remained viable during the U removal process. Elemental mapping (performed through STEM) and additional spectroscopic analyses indicated formation of biogenic U phosphates. These compounds which are known to be highly stable in comparison to other U precipitates showing chemical fragility such as U carbonate complexes (Duff et al., 2004), has been already reported for planktonic bacterial cells (Beazley et al., 2007, 2009, 2009; Choudhary and Sar, 2011; Povedano-Priego et al., 2019). Specifically, planktonic cells of the strain *Stenotrophomonas* sp. Br8 were already demonstrated to precipitate U as long-term stable U phosphate mineral phases with a structure similar to that of meta-autunite (Sánchez-Castro et al., 2020). In this study, cells from the same strain formed similar stable biogenic U phosphates after being immobilized within alginate beads. So far, most previous works using alginate beads (doped with microbes or not) for U immobilization are based on sorption mechanisms (Gok and Aytas, 2009; Wang et al., 2010; Yu et al., 2017) and then resulting in unstable U precipitates.

4.3. Novel uranium removal mechanism proposed and optimal application conditions to treat U mining waters

Considering all results presented above, the proposed U removal mechanism observed consist of a multi-step process starting with a first

fast sorption phase where a portion of the soluble U(VI) is bound mainly onto functional groups at the surface of the cells embedded within the alginate beads. Concomitantly, a Br8-phosphatase-mediated release of inorganic phosphates from amended G2P occurred. Then, these orthophosphates generated associate with bio-available and soluble uranyl ions in the mining water forming stable U phosphates phases.

In addition, key biological and physicochemical parameters like biomass concentration within the beads, storage conditions of the alginate beads before using, bead/water contact time, and amendment of an external organic phosphate source were assessed to enhance the applicability of this U removal process. Thus, use of 1 bead (containing approx. $1.8{\cdot}10^5$ CFU) per mL is estimated as the optimal amount to achieve an efficient U removal from COMI_79 mining waters. Alternatively, the use of the same number of cells distributed in a lower number of beads would reduce substantially the economic costs inherent to the immobilization process while offering comparable U removal efficiency rates. The possibility of storing this biomaterial under certain conditions (4 °C) for at least 300 days without compromising its U removal capacity, clearly enhance the relevance of the strategy proposed in this work. Previous studies reported a good conservation of viability in bacterial cells immobilized in similar inorganic matrices after 120d (Ontañon et al., 2017) and 180d (Brachkova et al., 2010; Zommere and Nikolajeva, 2017). However, to the best of our knowledge, no longer storage periods of time than 300d have been assessed before for bacterial cells immobilized in Na-alginate beads. Regarding freeze-drying process, it should be noted that it requires a sharp decrease in temperature which might have deadly effects on bacterial cells (Crittenden et al., 2006; Chávarri et al., 2010; Amine et al., 2014), mainly caused by water crystallization, protein denaturation or membranes injury (De Giulio et al., 2005). In this sense, the use of inorganic matrices for bacterial cells immobilization plays a protective role which may reduce the physiological deterioration, maintaining, at least partially, cells viability, metabolic potential and operational stability (Amine et al., 2014). Other key considerations to apply efficiently the strategy proposed is the amendment of an organic phosphates source like G2P or the incubation of the system for at least 72 h.

5. Conclusions

Uranium, particularly at high concentrations, is known to produce serious detrimental effects on bacterial diversity and activity. By embedding bacterial cells in an alginate matrix acting as a protective barrier, U cytotoxic effects can be reduced significantly through preventing direct contact between this hazardous agent and the cells. In this sense, it was developed an enhanced biomass immobilization method, based on previous consolidated protocols, resulting in biocompatible, highly porous Na-alginate beads. This biomaterial was thoroughly characterized and successfully applied, for one or multiple cycles, to immobilize soluble U from real mining wastewaters. Microscopic and spectroscopic characterization of the immobilized U solid phases confirmed formation of highly-stable U phosphates at bead surface level but also in their inner sections, associated to Br8 cells but never inside them. Chemical stability issues and potential Br8 cells leakage during removal process should be investigated by carrying out further studies focused on increasing the mechanical and chemical stability of the carrier material. Although U removal from non-natural U-containing solutions has received considerable attention, immobilization of U from real mining wastewaters remains relatively unexplored so far. The U precipitation as uranyl phosphate from real mining waters is of great scientific and environmental relevance but rather difficult on account of water chemical complexity. However, the process proposed in this study including an active enzymatic release of inorganic phosphates may overcome these difficulties and precipitate efficiently solved U(VI) and, likely, other heavy metals from these polluted wastewaters. In addition, key parameters like biomass dosage or biomaterial storage conditions were optimized for obtaining a cost-effective and applicable technology to remediate heavy-metals-containing water.

In conclusion, this lab-scale study revealed that U removal from complex mining waters employing alginate beads doped with cells of *Stenotrophomonas* sp. Br8 bacterial strain is a promising strategy even at high initial concentrations. Further research should focus on the scale-up of this highly efficient and low-cost process to develop eco-friendly heavy-metals-containing water treatment stations.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2021.113411.

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