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Research paper

Microbial community changes induced by uranyl nitrate in bentonite clay microcosms

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ABSTRACT

Deep geological repository (DGR) is one of the internationally accepted options to dispose radioactive wastes. Bentonite formations from Almeria, Spain, were selected as reference material for artificial barriers for the future Spanish repository. However, the safety of this long-term disposal could be compromised not only by physicochemical factors but also by microbial processes. The highly radioactive waste must be safely stored at least for 100,000 years for the radioactivity to decrease to similar levels to those of natural uranium. To simulate a scenario where the mobilization of radionuclides from the repository to the host formations may occur, long-term microcosms were studied. After being exposed to uranyl nitrate for 5 months, the response of the bentonite microbial community to the addition of this radionuclide was evaluated. High throughput 16S rRNA gene sequencing revealed that the structure of the microbial community after the uranyl nitrate treatment differs to that of the control microcosms. The microbial diversity was dominated by Firmicutes and Proteobacteria. Moreover, after the uranyl nitrate treatment OTUs annotated as *Paracoccus* and *Bacillus* were highly enriched. The mineralogy of bentonites was not affected by the uranyl nitrate treatment as was demonstrated by X-ray diffraction analysis. In addition, the study of uranium-bacteria interaction revealed the ability of isolates to biomineralize uranium as uranium phosphate mineral phases. Thus, the changes induced by the release of uranium in the microbial population may also affect the mobility of this radionuclide, making it less mobile and therefore less harmful for this environment.

1. Introduction

Management of nuclear waste is a serious environmental problem all over the world. A long-term disposal scenario is required for the safe storage of these hazardous wastes over hundreds of thousands of years for the radiotoxicity to decrease to levels similar to those of natural uranium and its products (Hedin, 1999). Since the 1970s, worldwide efforts have been focused on finding a safe and sustainable disposal concept for highly radioactive waste. The use of deep geological repositories (DGR) has been internationally proposed as the safest option for the disposal of these hazardous materials (IAEA, 2003). The general DGR concept is based on a multi-barrier storage system that entails encapsulating the waste in corrosion-resistant metal containers (first barrier), surrounded by a bentonite buffer (second barrier), and buried deeply within a stable geological formation (third barrier) (Ojovan and Lee, 2013). Clay formations are one of the candidate host rocks proposed for high-level nuclear waste repository. These environments have been extensively studied in Europe (Lopez-Fernandez et al., 2014; ONDRAF/NIRAS, 2001; Pedersen et al., 2017; Stroes-Gascoyne et al., 2007; Wouters et al., 2013). Additionally, bentonite clays are also a suitable material for the second barrier. Within this context, bentonite clays from Almeria, Spain, were selected as Spanish reference material for the engineering barrier after an extensive characterization of their mineralogical, geochemical and technological properties (Villar et al., 2006). However, over the last decades, the presence of microorganisms several kilometers below the surface has been demonstrated (Gold, 1992; McMahon and Parnell, 2014). Thus, safety of this long-term geological disposal could be compromised by physical and chemical factors, but also by biogeochemical activity of either indigenous or microorganisms introduced during the construction of the repository

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(Meleshyn, 2011; Stroes-Gascoyne and West, 1997). The bentonite barrier performs important functions in maintaining the integrity of the metal canisters isolating the spent fuel; containing, preventing, and retarding the dispersion of radionuclides; and acting as a buffer against rock movements (SKB, 2010). Therefore, it is crucial to study the microbial population in the proposed bentonite buffer engineering barrier as microorganisms could impact the stability of the repository.

Previous studies have extensively investigated the microbial diversity in different types of commercially available bentonites such as Asha, Calcigel and Wyoming MX-80 (Bengtsson and Pedersen, 2017; Svensson et al., 2011) or Opalinus Clay (Leupin et al., 2017). The microbial diversity has also been studied in these Spanish reference bentonite clay formations (Lopez-Fernandez et al., 2015; Lopez-Fernandez et al., 2014). Microorganisms mainly affect the geochemistry of clays through three different mechanisms: reduction or dissolution of the structural clay minerals, e.g. Fe(III) (Pentráková et al., 2013); alteration of mineral surfaces by the production of siderophores and small-organic acids; and formation of biofilm on the clay mineral surface (Meleshyn, 2011). In addition, microbes are able to control the speciation and mobility of radionuclides (Newsome et al., 2014) through processes such as biosorption to the cell surface (Lloyd and Macaskie, 2000; Merroun et al., 2005), intracellular accumulation (Brookshaw et al., 2012; Suzuki and Banfield, 2004), biomineralization (Macaskie et al., 2000; Merroun et al., 2011), or biotransformations (Brookshaw et al., 2012; Lovley et al., 1993). Therefore, microbial processes occurring in the bentonite clay engineering barrier might play an important role in the mobility and migration of radionuclides in these environments.

There are several studies describing microorganisms influencing the speciation of radionuclides to be stored within a DGR, such as uranium (Lopez-Fernandez et al., 2014; Lütke et al., 2013; Lütke et al., 2012; Merroun et al., 2011), through a biomineralization process resulting in the formation of U(VI) phosphate mineral phases probably due to the activity of acid or alkaline phosphatases. However, these studies were conducted using bacterial pure cultures that did not simulate the natural conditions of the DGR of nuclear wastes. Nevertheless, there are some works studying the effect of uranium addition in the microbial diversity of soil microcosms (Geissler et al., 2009), dune sand microcosms (Handley-Sidhu et al., 2009) and sediment microcosms (Salome et al., 2013). But, the mentioned studies were not focused on clays considered as engineering barriers for DGR of radioactive wastes. Nevertheless, it is important to consider the specific scenarios where uranium may be mobilized from the underground repository to the environment. Therefore, this work aimed to 1) investigate the mineralogical and microbial changes induced by the addition of uranyl nitrate to microcosms elaborated with bentonite clay samples from Almeria; and 2) study the role of uranyl-treated bentonite bacterial isolates in the mobility of uranium.

2. Materials and methods

2.1. Sample collection and description

Bentonite clay samples were collected from two different locations of Almeria, Spain. Sample called BI was collected from El Cortijo de Archidona and sample BII was taken from El Toril. Afterwards, samples were transported on ice to the laboratory and processed immediately. Geochemical and mineralogical characterization of these two different clay samples was previously described in Lopez-Fernandez et al. (2014).

2.2. Preparation of clay microcosms

Microcosms were prepared in sterile Petri dishes using 20 g of dry crushed bentonite clay soil from El Cortijo de Archidona (BI-A, BI-B and BI-C) and from El Toril (BII-A, BII-B and BII-C). All microcosms were treated with an appropriate volume (20 ml for bentonite BI and 16 ml for bentonite BII) of the corresponding solution to saturate the bentonite. The solutions were gently and homogenously added to the microcosms, then, the microcosms were tightly closed to keep them moist over the incubation time. Sodium nitrate and uranyl nitrate solutions were prepare by adding 151 ppm of $NaNO_3$ and 300 ppm of $UO_2(NO_3)_2$, respectively, and sterilized by filtration (pore size 0.22 μm). Microcosms called BI-A and BII-A were treated with sterile MilliQ water (water controls). BI-B and BII-B microcosms were nitrate-controls, treated with a sodium nitrate solution; and microcosms BI-C and BII-C were treated with a uranyl nitrate solution and considered as uranyl-treated samples (Table 1). Water-controls (-A) were included in this study to test the effect of the long term incubation under the same experimental conditions. Nitrate-controls (-B) were added to investigate the nitrate effect on the bacterial diversity since uranyl ion was added as uranyl nitrate. The pH of the microcosms was measured as described in Lopez-Fernandez et al. (2015). The pool of microcosms was incubated at room temperature in darkness under oxic conditions, for five months.

Table 1
Bentonite and microcosm samples description: treatments, pH and incubation time.

Sample	Treatment	ppm	pH	Incubation time
BI	–	–	9.08	0 month
BI-A	Water	–	9.03	5 months
BI-B	$NaNO_3$	151	8.58	5 months
BI-C	$UO_2(NO_3)_2$	300	7.32	5 months
BII	–	–	7.86	0 month
BII-A	Water	–	7.82	5 months
BII-B	$NaNO_3$	151	7.61	5 months
BII-C	$UO_2(NO_3)_2$	300	6.73	5 months

2.3. X-ray diffraction analysis

The mineralogy of the bentonite clay microcosms used in this work was studied by X-Ray diffraction (XRD) before and after the different treatments and 5 months incubation time. The mineralogy before the treatments and incubation is considered time 0 and was earlier reported in Lopez-Fernandez et al. (2014). For the analysis after the incubation time, the diffractometer used was a Bruker D8 Advance instrument with Bruker Linxeye detector, Cu Kα radiation (λ = 1.5406 Å), a 2θ explored area of 5 to 70°, and a goniometer speed of 0.02° 2θ s^{-1}. XRD goniometer calibration was performed using a silicon standard. Powder samples were placed in zero-background silicon sample holders.

2.4. DNA extraction and Illumina sequencing

To study the microbial diversity of the bentonite clay microcosms total DNA was extracted in triplicates using the Fast Prep DNA extraction protocol described in Vilchez-Vargas et al. (2013), with some modifications. Briefly, microcosm samples (1 g) were mixed with 200 mg of glass beads and 1000 μl of lysis buffer (Tris/HCl (100 mM, pH 8.0), supplemented with 100 mM EDTA, 100 mM NaCl, 1% (wt/vol) polyvinylpyrrolidone and 2% (wt/vol) sodium dodecyl sulfate). Cells were lysed in a Fast Prep-24 instrument (40 s, 6 m s^{-1}), two times. Samples were centrifuged at 14000*g* for 5 min. Supernatants were collected in new tubes and pellets were dissolved into 1000 μl of MilliQ water and disrupt another two times (40 s, 6 m s^{-1}). This extra step was included to detach all cells from clay particles. After that, samples were centrifuged at 14000*g* for 5 min. All supernatants were washed with one volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v), pH 7. Then, samples were centrifuged at 14000*g* for 1 min and the aqueous phase was washed with one volume of chloroform. After centrifugation, nucleic acids present in the aqueous phase were precipitated with one volume of ice-cold isopropanol and 1:10 volume of 3 M sodium acetate. Finally, total DNA was washed with 80% ethanol and the pellet was dissolved into 100 μl of MilliQ water and purified

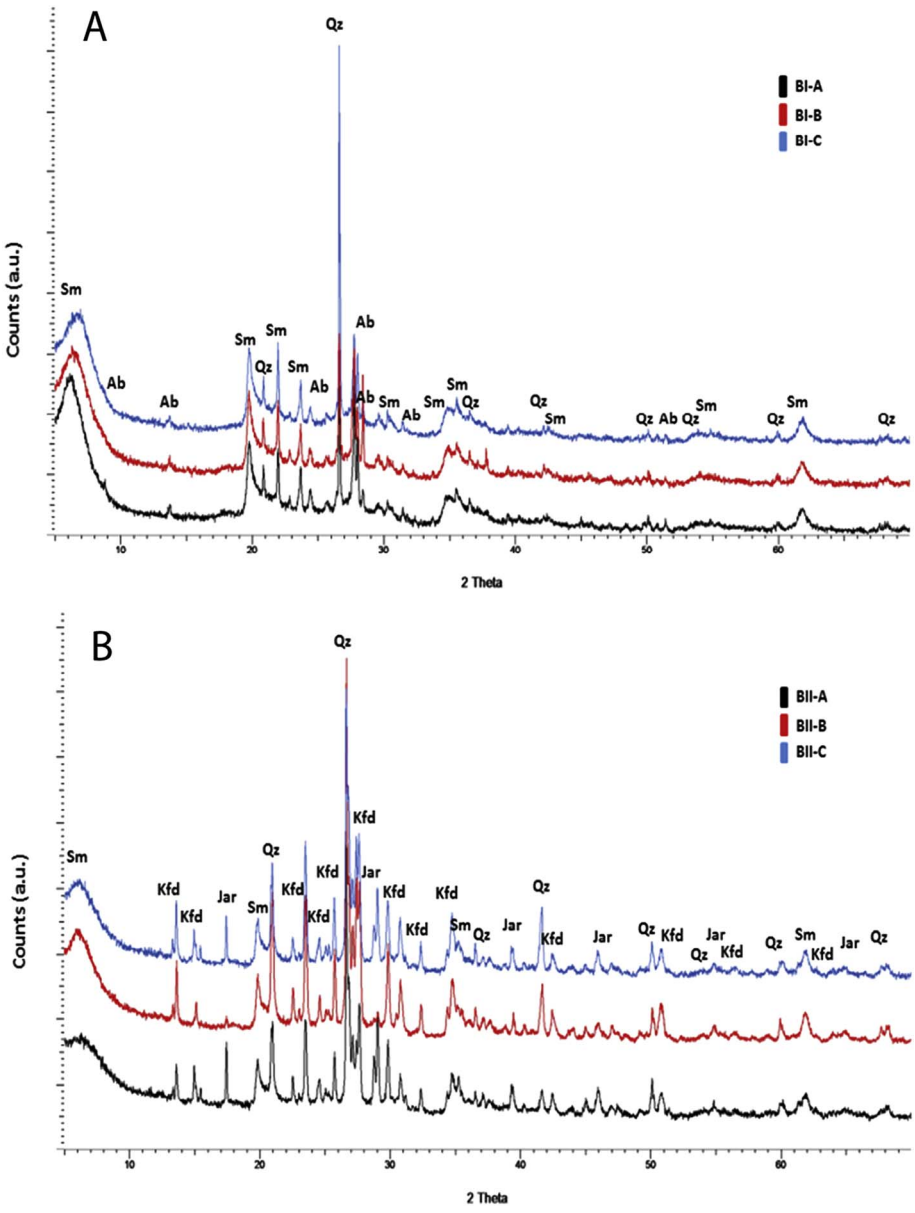


Fig. 1. XRD patterns of BI (A) and BII (B) microcosm samples treated with: -A: water, -B: Sodium nitrate, -C: Uranyl nitrate. Sm: smectite; Ab: plagioclase-feldspar (albite); Kfd: potassium-feldspar; Jar: jarosite; Qz: quartz.

with Wizard® DNA Clean-Up System (Promega). The quality and quantity of the extracted DNA was analyzed on 1% agarose gels and sequenced by Illumina following the procedure of Camarinha-Silva et al. (2014). Hypervariable region V5-V6 of the 16S rRNA gene was amplified by using universal primers based on 16S-$_{807f}$ and 16S-$_{1050r}$ (Bohorquez et al., 2012), as described in Lopez-Fernandez et al. (2015). Libraries were sent for paired-end sequencing on a MiSeq System Sequencer (Illumina, CA, USA), obtaining sequences of about 250 nucleotide length. The well-established UPARSE pipeline was used to process the sequences and cluster operational taxonomic units (OTUs) (Edgar, 2013). OTUs were then annotated against the SILVA Incremental Aligner (SINA) (Quast et al., 2013) and finally analyzed in Explicet 2.10.5 (Robertson et al., 2013). Further analyses and environmental indices calculations (Shannon diversity index (H), Shannon eveness index (E), Simpson index (D) and Chao-1) were performed in Explicet 2.10.5 (Robertson et al., 2013). The indices were calculated based on richness/evenness/species data from each sample (BI and BII). PCA was included to extract the important information from the dataset of the six microcosms, by displaying the pattern of similarity of the different samples as points in maps.

2.5. Phosphatase activity of the microcosm samples

It has been previously described that acid and alkaline phosphatase are key enzymes involved in the precipitation of uranium (Beazley et al., 2011; Kulkarni et al., 2013; Newsome et al., 2015). In this study, total acid and alkaline phosphatase activities were measured according to the method described in Vilchez et al. (2007), using 1 g of bentonite microcosm suspended in 10 ml of sterile distilled water. Samples were measured in triplicate.

2.6. Microbial isolation and molecular characterization of isolates

Bacterial strains were isolated and characterized from the microcosm samples using different culture media (LB, R2A and CYP), as previously described in Lopez-Fernandez et al. (2014). Eukaryotic strains were isolated as described above, but they were characterized by rDNA sequencing. Concretely, ITS fragment was performed using the DNA-barcodes established for fungi (Schoch et al., 2012). Comparison of assembled rDNA ITS sequence was performed with GenBank and MycoID databases. The multiple sequence alignment program BioEdit (Hall, 1999) was used for sequence alignment. Phylogenetic trees were

generated by using MEGA software (Tamura et al., 2007) based on the results of the maximum likelihood algorithm with distance analysis (Tamura-Nei corrections). The nucleotide sequences reported here were submitted to the EMBL Nucleotide Sequence Database under accession numbers LT548955 - LT548979.

2.7. Sample preparation for HAADF-STEM/EDX analyses

To determine the ability of the isolated strains to precipitate uranium as uranium phosphates scanning transmission electron microscopic analysis was performed. Thus, bacterial cultures were prepared in LB medium (160 rpm/28 °C) and harvested at exponential growth. Then, cells were treated with 1 mM of uranyl nitrate dissolved in 0.1 M $NaClO_4$, pH 7 for 48 h (160 rpm, 28 °C). TEM thin samples were prepared according to the method described in Merroun et al. (2005) and examined under the high-angle annular dark field scanning transmission electron microscope (HAADF-STEM) FEI TITAN G2 80–300. The high resolution STEM is equipped with HAADF detector and EDAX energy dispersive X-ray.

3. Results

3.1. Mineralogical characterization

A detailed characterization of the geochemical and mineralogical composition of the natural bentonite clays (considered as time 0 in this study) was described in Lopez-Fernandez et al. (2014). This characterization revealed that the mineralogy of the studied clay samples BI and BII was dominated by montmorillonite in both cases (84 and 71%, respectively). Sample BI contained plagioclase-feldspar and quartz, while sample BII was composed of K-feldspar, quartz and jarosite, an iron sulfate mineral phase (Table S1 and Fig. S1). After the different treatments and long term incubation, XRD analyses of the microcosm samples revealed that the mineralogy of all microcosms was dominated by montmorillonite. Plagioclase-feldspar albite and quartz were detected in microcosms BI, while K-feldspar sanidine, jarosite and quartz were measured in BII microcosms (Fig. 1). The mineralogical data (time 0 and time 5) showed the stability of these bentonite clay formations. The mineralogy of the bentonite clay microcosms, after the treatment and long-time incubation was very similar to that of the natural bentonite samples at time 0.

3.2. Diversity of the microcosm communities

Sequencing information and the annotated Operational Taxonomic Units (OTU) with relative abundance are shown in Tables S2, S3 and S4. Concretely, 1531 and 2629 phylotypes were separated and classified for microcosms BI and BII, respectively. In total, 22 phyla were classified, concretely 19 of them belonging to bacterial phyla such as Acidobacteria, Actinobacteria, Armatimonadetes, Bacteroidetes, candidate division BRC1, candidate division WPS-1, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Firmicutes, Fusobacteria, Gemmatimonadetes, Nitrospirae, Planctomycetes, Proteobacteria, Spirochaetes, Synergistetes, Thermodesulfobacteria and Verrucomicrobia; and 3 of them belonging to Archaea: Crenarchaeota, Euryarchaeota and Thaumarchaeota. Phyla such as Proteobacteria, Bacteroidetes and Actinobacteria were previously described as the most abundant bacterial phyla in natural bentonite clay formations (Lopez-Fernandez et al., 2015). Principal component analysis (PCA) of the phylotype abundance matrices showed a clear separation between the different treatments in BI and BII samples (Fig. 2). A major fraction of the dominant phylotypes clustered together in both samples, but especially in sample BII, showing a strong correlation to the non-treated control.

The microbial diversity was dominated by Firmicutes and Proteobacteria, being the main phyla identified in both samples BI and BII. In the case of sample BI, the diversity of microcosm BI-A, the non-treated control, as assessed by the Shannon index (H) was the highest of all microcosms ($H < 6$; Table S5). It was mainly dominated by Firmicutes (≈ 91%), followed by Alphaproteobacteria representing ≈ 4.6% of the community (Fig. 3). Particularly, OTU-1221, annotated as *Bacillus* was one of the most dominant, in comparison with the rest of the OTUs, representing > 17% of the population (Fig. 4). In addition, other three OTUs were highly represented: OTU-1242 (≈ 8%), OTU-1318 (≈ 8%) and OTU-1286 (≈ 7%), annotated as *Oxobacter*, *Tumebacillus* and *Bacillus*, respectively (Table S3). The microbial diversity in BI-B microcosm, the nitrate-control, was lower and slightly different to that of BI-A microcosm ($H \approx 5$; Table S5). Firmicutes was still dominating the community with a slight decrease (≈ 77%), but Betaproteobacteria and Alphaproteobacteria made up to 13 and 8%, respectively. The dominant OTUs in sample BI-A were decreased comparing to sample BI-B. However, OTU-1242 was highly enriched (≈ 22%, Fig. 4). Moreover, other OTUs as OTU-1551, -1214, -1370, -1285 and -1221, annotated as *Ammoniphilus*, *Ralstonia*, *Paeniporosarcina*, *Sphingomonas* and *Bacillus*, respectively, were also enriched (Table S3). Finally, comparing the uranyl nitrate-treated BI-C microcosm to both control samples (BI-A and BI-B), some differences were observed in the structure of the microbial population, probably due to the uranyl treatment. In BI-C microcosm the diversity was the lowest of all BI samples ($H \approx 4.7$; Table S5). The dominant phylum in sample BI-C was Firmicutes (≈ 71%). Alphaproteobacteria class was increased up to 27%, while Betaproteobacteria was strongly reduced (≈ 0.2%, Fig. 3). In addition, OTU-1221 and OTU-1286, both annotated as *Bacillus* were decreased in comparison with BI-A (Table S4). Nevertheless, these OTUs and OTU-1242 (annotated as *Oxobacter*) were slightly enriched compared to that of sample BI-B. On the other hand, OTU-2569 (Fig. 4), annotated as *Paracoccus*, was highly enriched (≈ 25%).

The microbial diversity of BII bentonite clay microcosm differed to that of the BI microcosms, as it was expected due to the distinct geochemical and mineralogical characteristics of these two bentonites at time 0 (Lopez-Fernandez et al., 2014). The diversity assessed by the Shannon index was lower than in samples BI (Table S5). In the case of sample BII, water control microcosm called BII-A, diversity ($H \approx 3$) was completely dominated by phylum Firmicutes (≈ 98%), as shown in Fig. 3. Bacillales was the dominant Firmicutes class mainly represented by OTU-2211 (≈ 41%), annotated as *Anaerobacillus*, along with OTU-1372, -1091 and -1196, annotated as *Bacillus*, *Brevibacillus* and *Bacillus*, respectively (Fig. 4). The sodium nitrate treatment affected the diversity of the microcosm BII-B, where the diversity was strongly reduced to $H \approx 0.3$ (Table S5). The presence of Firmicutes decreased to ≈ 0.8%, while Betaproteobacteria (≈ 99%) became the most represented class. A single OTU (OTU-1214, annotated as *Ralstonia*) was dominating the community (≈ 96%). OTU-1214, was only enriched in microcosm BII-B (Fig. 4 and Table S4), reinforcing the previously described influence of the sodium nitrate treatment for *Ralstonia* genus (Dalsing and Allen, 2014). In microcosm BII-C OTU-1196, annotated as *Bacillus*, was highly enriched (≈ 91%) compared to microcosms BII-A and BII-B, where it was almost not detected (Fig. 4 and Table S4).

3.3. Acid and alkaline phosphatase enzymatic activity

The measured activity of the acid phosphatase was increased (but not significantly) in microcosm BII-C, compared to both controls (BII-A and BII-B) (Fig. S2). In sample BI no evidence of any change in the acid phosphatase activity was measured. No significant differences were observed for the alkaline phosphatase activity, in the microcosm samples (Fig. S2).

3.4. Culture dependent microbial diversity

Phylogenetic trees of the microbial strains isolated from the bentonite clay microcosms studied are shown in Fig. 5. Phylogenetic

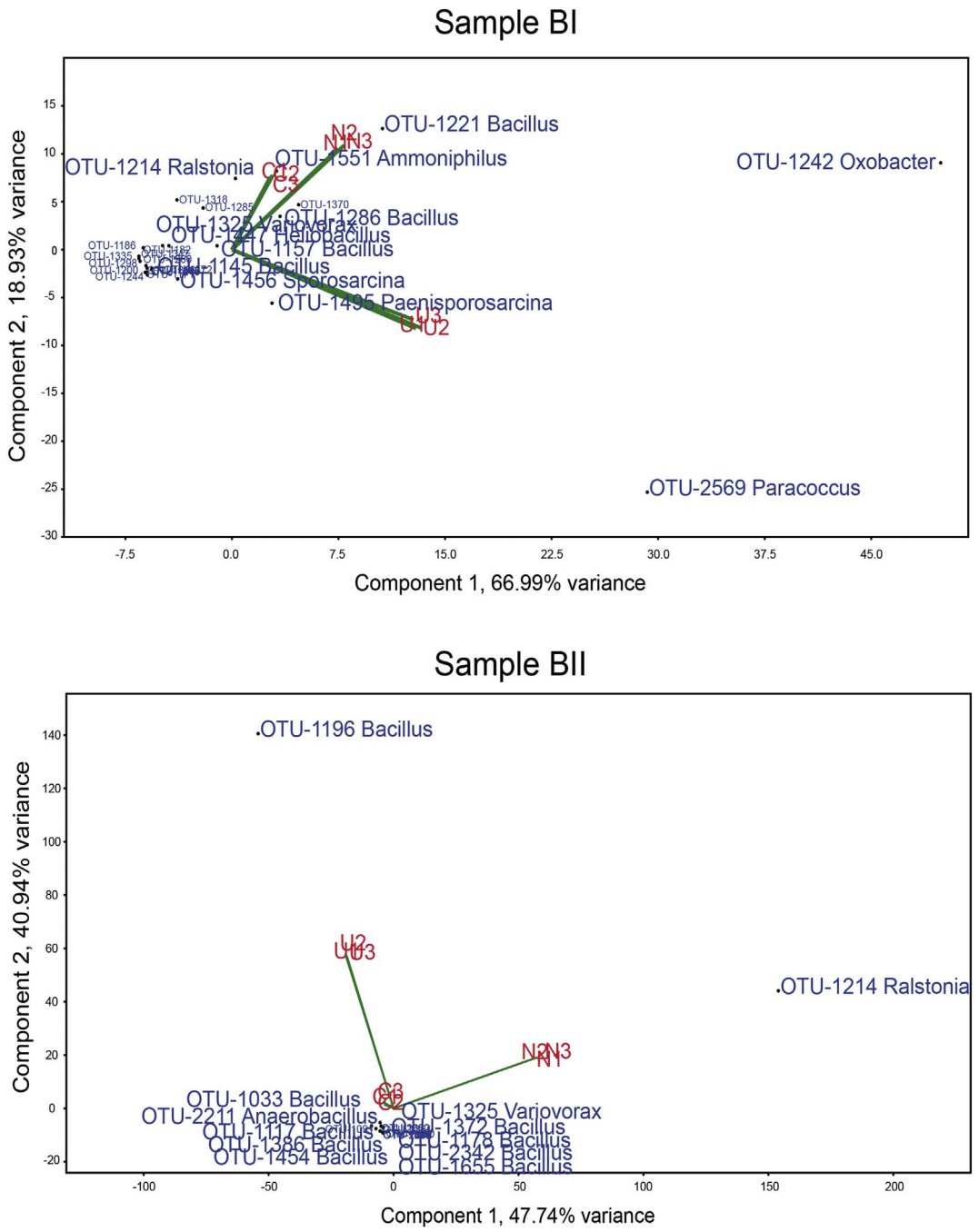


Fig. 2. Principal component analysis (PCA) plot comparing the microbial community structure of the different microcosms. C1: BI-A microcosm, N1: BI-B microcosm, U1: BI-C microcosm, C2: BII-A microcosm, N2: BII-B microcosm and U2: BII-C microcosm.

affiliation is based on 16S rRNA/ITS rDNA gene analysis. By using different culture media (LB, R2A and CYP) 9 and 16 microbial strains were isolated from BI and BII microcosms, respectively. BI isolates were dominated by phylum Firmicutes, concretely by *Bacillus* spp. Strains belonging to division Basidiomycota and phylum Actinobacteria were also isolated from microcosms BI. A total of 5 strains were isolated from the uranyl nitrate treated BI microcosm, belonging to *Bacillus* spp. (3 strains) and to *Rhodotorula mucilaginosa* (2 strains). Microbial diversity of microcosms BII was represented by phyla Proteobacteria, Actinobacteria and Firmicutes. Proteobacteria was the dominant phylum, represented by Alpha-, Beta- and Gammaproteobacteria classes. In the case of microcosm BII treated with uranyl nitrate only 3 strains were isolated BII-C1, BII-C3 and BII-C4 affiliated to *Microbacterium*, *Bacillus* and *Erwinia* species respectively.

3.5. Cellular location of uranium accumulates

To determine the ability of the dominant isolated microbial strains from the uranyl-treated microcosms to biomineralize this radioanuclide as U phosphates, a combination of STEM-HAADF was applied. STEM micrographs of thin sections of isolate *Bacillus* sp. BII-C3 (U: 1 mM, incubation time: 48 h) are shown in Fig. 6. Electron-dense precipitates were observed within the extracellular space. EDX element-distribution map from these U precipitates showed that they are mainly composed of U and P (Fig. 6). The copper (Cu) peak resulted from the copper grid used to support the specimen. The presence of the silicon (Si) peak can be attributed to impurities in the culture medium and/or from the glass material of the flasks in which the cells were grown.

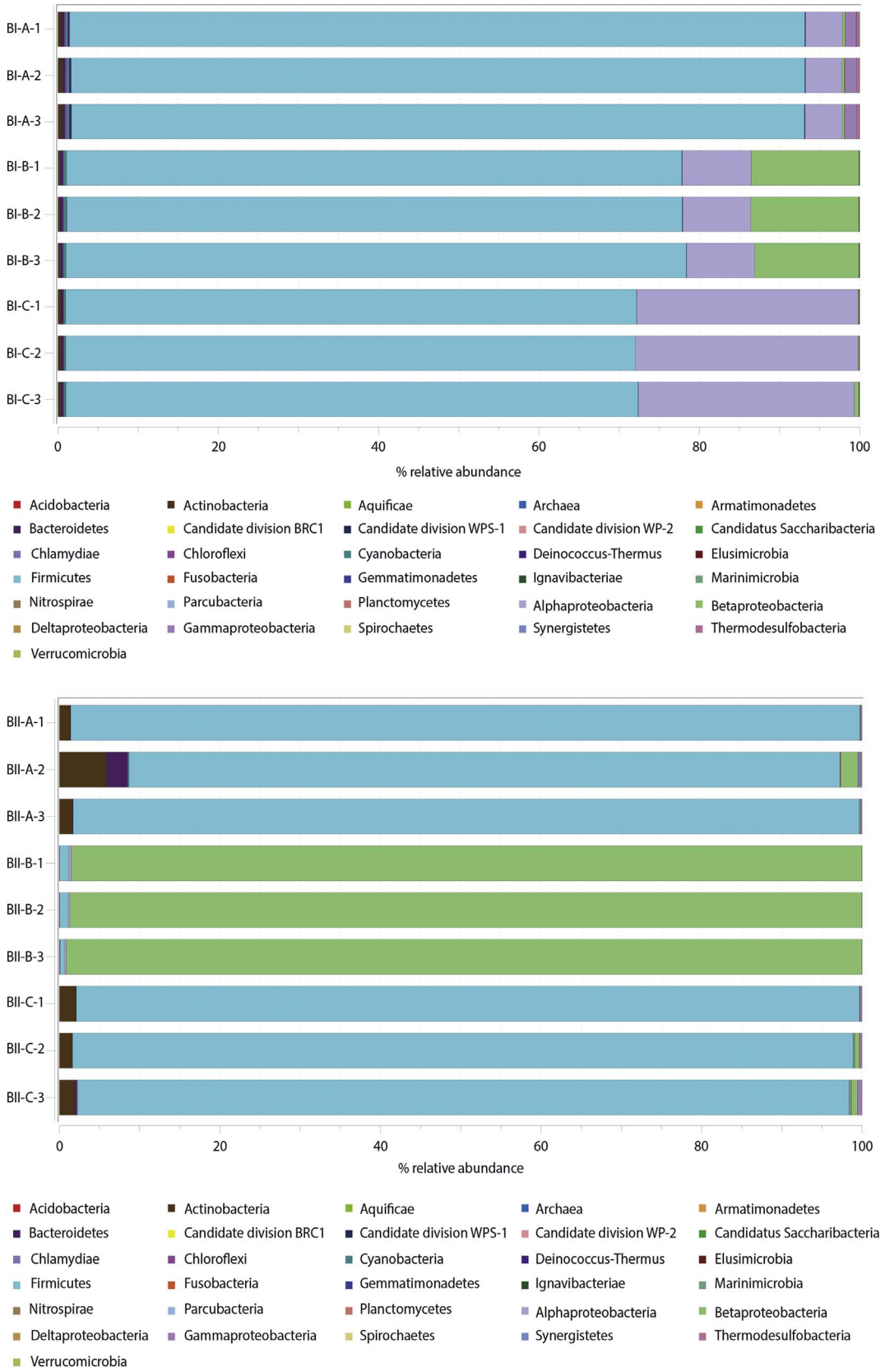
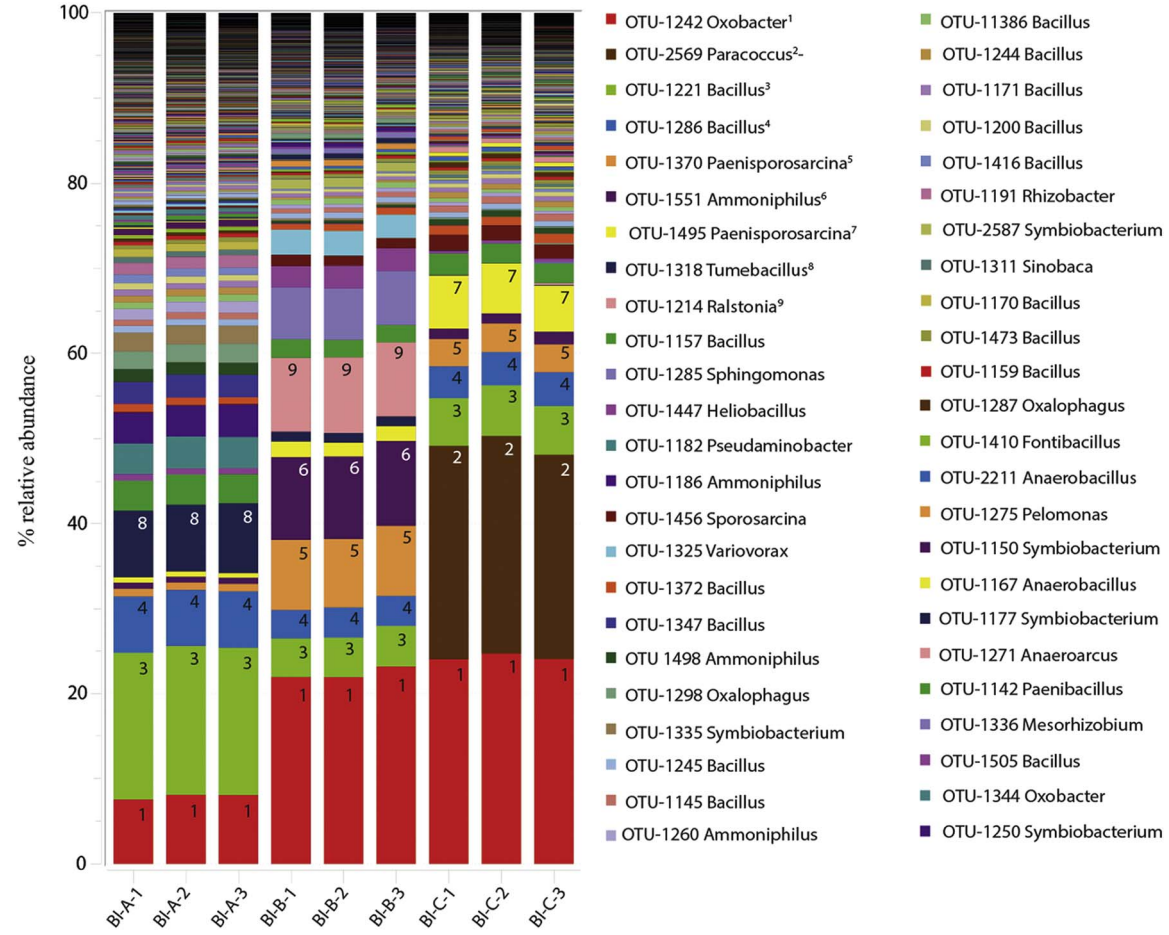


Fig. 3. Taxonomic composition of the microbial communities of BI and BII microcosm samples, represented at phylum/class level.



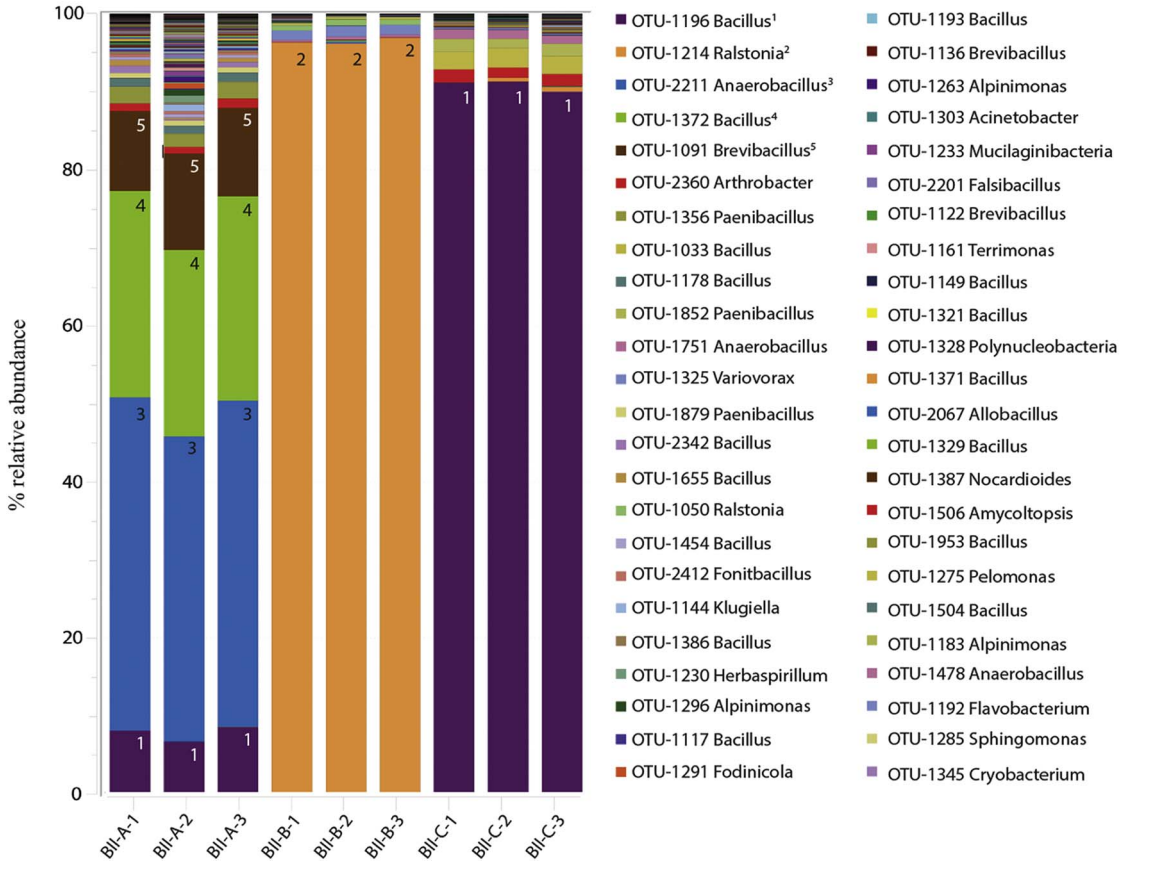


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Fig. 4. Structure of the microbial community of the BI and BII microcosm samples, represented only by dominant OTUs annotated to the least common ancestor. Major OTUs are labelled with a superscript for clarification.

4. Discussion

Bentonite clay formations were selected as one of the best candidate reference material for the engineering barrier in the planned Spanish DGR. Thus, a deep knowledge of microbial diversity and the influence of radionuclides in the structure and composition of natural microbial communities would be helpful to get a better understanding of this process. In addition, detailed studies on the effect of microbial processes in the transformation of bentonite clays should be considered. In this work, the changes in the microbial diversity after long-term uranyl nitrate treatment are described under oxic conditions. Oxygen will be trapped within the repository as result of the drilling process and ventilation galleries required for the construction and operation of the DGR. Under in situ conditions in the repository, several chemical and microbial processes may contribute to the consumption of this oxygen. For example, microbial induced reactions near the bentonite/host rock interface (Puigdomenech et al., 2001), corrosion of the copper canister, and inorganic reactions with minerals in the bentonite (SKB, 2006). Some studies showed that the timescale for oxygen depletion ranged between few years and hundred years (Carlsson and Muurinen, 2006; Wersin et al., 1994). Thus, oxic conditions should be also considered to assess the safety disposal of radioactive wastes.

The pH conditions in the DGR are also a key factor for the radionuclide interactions with microorganisms. In repository environments the pH of groundwater and clay pore water is buffered above 7 (Pedersen et al., 2017). However, the acidification of these environments could lead to the solubilization of minerals produced by the activity of indigenous microbes, which may affect the biogeochemical cycles of the stored radioactive waste. In this study the addition of the different treatments was not considerably affecting the pH of the microcosms. The microbial diversity in the water control microcosms BI-A and BII-A was higher than in the sodium nitrate and uranyl nitrate treated microcosms, probably because they were non-treated samples. The results shown in this work were compared to those of the microbial diversity and mineralogy of the same bentonite clay samples at time 0 (previously described in Lopez-Fernandez et al. (2015; 2014). The mentioned study revealed the predominance of phylum Bacteroidetes (49%) in the case of bentonite BI and a clear dominance of Betaproteobacteria class in bentonite BII (98%). Moreover, the mineralogy of these bentonites was stable over time before and after the treatments (Figs. 1 and S1). It has been described that stressing factors such as heavy metals or high salinity can influenced the technical parameters of the bentonites (Dutta and Mishra, 2016; Kolaříková and Hanus, 2008). Therefore, the addition of the uranyl nitrate to the bentonite clays might have an effect in the mineralogy of the bentonite samples. However, the uranyl nitrate treatment had no effect in the physical parameters of the bentonite (unpublished data).

Interestingly, the enrichment of Firmicutes in microcosms BI-A and BII-A might be a consequence of the long term incubation, considering that the main population switched from Bacteroidetes in sample BI and Betaproteobacteria in sample BII (time 0) to Firmicutes in both samples (time 5 months). Moreover, at time 0 Firmicutes was slightly detected in the case of sample BI (0.2%) and not even detected in sample BII (Lopez-Fernandez et al., 2015). In addition, sodium nitrate- and uranyl nitrate-treatments might have an influence on the diversity since the presence of Firmicutes phylum was reduced, and Proteobacteria was

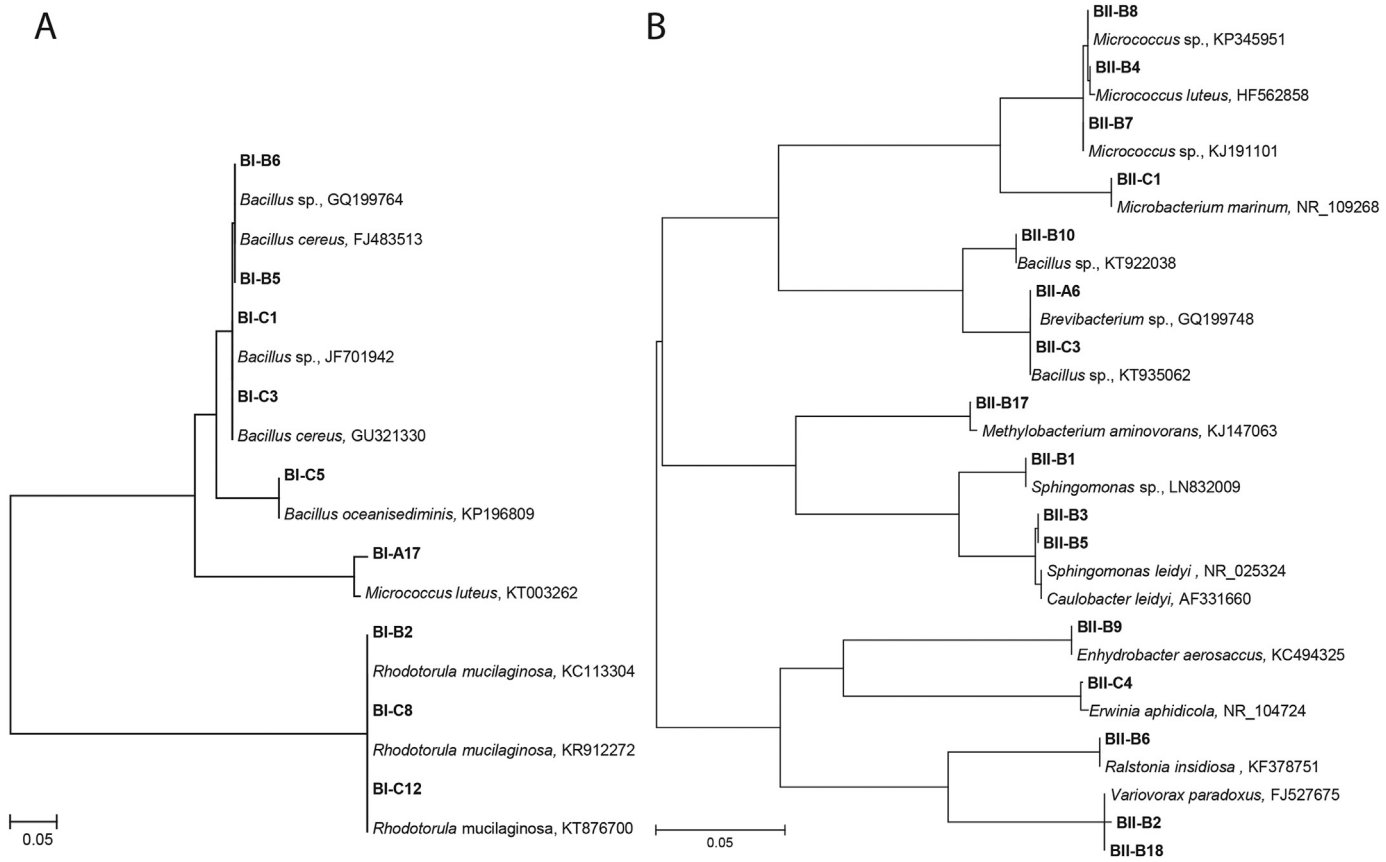

Fig. 5. 16S rRNA/18S rDNA gene based phylogenetic trees showing the diversity of the isolates from microcosms, obtained by the maximum likelihood algorithm (Tamura-Nei corrections). A. BI-microcosm phylogenetic tree. B. BII-microcosm phylogenetic tree.

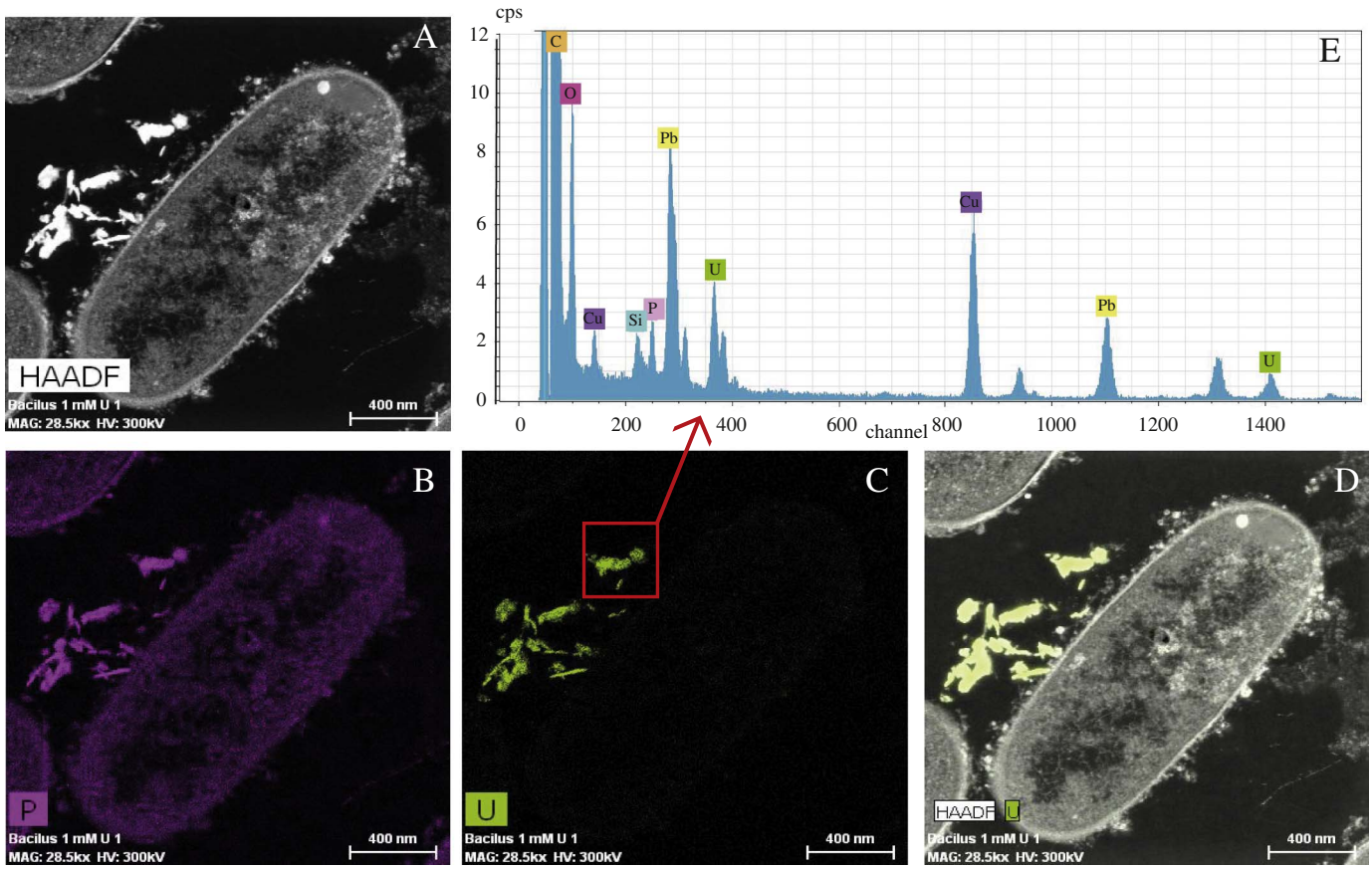

Fig. 6. Scanning transmission electron microscopy-high-angle annular dark-field imaging (STEM-HAADF) micrographs of thin sections of 1 mM U-treated cells of *Bacillus* sp. BII-C3 (A–D). EDX spectra of U precipitates located at the extracellular space (E).

increased, respectively.

After 5 months incubation of sample BI-A, the dominant OTU was annotated as *Bacillus*. This genus is described for including both, aerobic and facultative anaerobic bacterial species. So far, facultative anaerobic microorganisms are well adapted to cyclic changes in their oxygen tension, by switching their metabolism according to the presence or absence of oxygen (Reitz et al., 2014). The enrichment of *Oxobacter* after anaerobic incubation of soils has been recently described (Chen et al., 2016). Thus, the detection of a dominant OTU annotated as Oxobacter (8%) might be due to the oxygen consumption by *Bacillus* spp. in microcosm BI-A. In microcosm BII-A, after the incubation time, Firmicutes phylum was mainly represented by *Anaerobacillus*, *Bacillus*, *Brevibacillus* and *Paenibacillus*. In correlation with these results, BII-S2 strain, belonging to *Bacillus simplex*, was isolated through culture dependent techniques from bentonite clay BII (Lopez-Fernandez et al., 2014). Moreover, some other Firmicutes representatives were found in similar oligotrophic environments as the Spanish bentonite formations. For example, a facultative anaerobic *Paenibacillus* sp. was isolated from a uranium mining waste pile in Germany (Reitz et al., 2014), along with a facultative iron-reducing *Paenibacillus* sp. was recovered from subsurface sediment biofilms (Ahmed et al., 2012). A *Brevibacillus brevis*, isolated from cadmium contaminated soil, was described for the cadmium immobilization and its use for bioremediation (Vivas et al., 2005).

Several studies have examined the influence of sodium nitrate on the microbial population. For instance, Huang et al. (2012) demonstrated that the accumulation of nitrate in soil decreased the microbial diversity. However, Bougon et al. (2012) described that if the nitrate concentration is stable along the time, the nitrate reducing bacteria community do not vary. The influence of the sodium nitrate treatment in microcosms BI-B and BII-B influenced the bacterial population in a similar way. The increase of *Ralstonia* (OTU-1214) in both nitrate-treated microcosms BI-B and BII-B may be due to the ability of this genus to use nitrate as terminal electron acceptor, as it was described for the case of different *Ralstonia* species (Tiemeyer et al., 2007; Xiao et al., 2015). Finally, the presence of uranyl nitrate in sample BI-C might be strengthening the enrichment of *Paracoccus* (OTU-2569) since it was not even detected in samples BI-A and BI-B. *Paracoccus* has been identified in several environments but also in natural bentonites recovered from Almeria clay deposits (Lopez-Fernandez et al., 2015). This Alphaproteobacteria genus is one of the most metabolically versatile bacteria, described as chemoorganoheterotroph or facultative chemolitoautotroph. Due to the described versatile metabolism, these bacteria can play an important role in the cycling of elements in the environment. This means that they could be used for bioremediation strategies (Bartosik et al., 2003). The mineralogical composition of the microcosms after the incubation time was not affected by the microbial population (Fig. 1 and S1), even though some of the bacterial genera identified are known for taking part in the iron biogeochemical cycle, as for example *Ralstonia* or *Paracoccus*. In the microcosm BII-C, the specific case of OTU-1196, annotated as *Bacillus*, is remarkable as it was highly enriched in the presence of uranyl nitrate. Interestingly, strain BII-C3 affiliated to *Bacillus* sp. was isolated from sample BII-C, with a 94% of similarity to OTU-1196. STEM-HAADF analyses demonstrated that this isolated strain was able to precipitate uranium extracellularly as U phosphate mineral phases. However, attempts to demonstrate the presence of U phosphates in microcosms by means of Time Resolved Laser induced Fluorescence Spectroscopy (TRLFS) have been unsuccessful due to high background noise in the spectra (data not shown). Studies on the quantification of phosphatase activity of the strain BII-C3 in presence of U under DGR relevant conditions are in progress (data not shown). Species of *Bacillus* genus are able to interact efficiently with

uranium through different mechanisms, such as biomineralization or biosorption, affecting the biogeochemical cycle of this radionuclide (Li et al., 2014; Merroun et al., 2005, 2011). In addition, a *Bacillus* strain was described for its uranium biomineralization capacity, mediated by acid phosphatase activity (Merroun et al., 2011). Martinez et al. (2007) also described the uranium precipitation via phosphatase activity of naturally occurring *Bacillus* spp., isolated from an acidic subsurface. These characteristics can be of interest for the DGR of nuclear wastes, as well as for bioremediation of uranium contaminated sites. In addition, this might be the reason why OTUs annotated as *Bacillus* in this study were highly enriched after uranyl nitrate-long term incubation. Moreover, the capacity of the genus *Bacillus* to deal with uranium might be a survival mechanism of the cells to tolerate high concentrations of this radionuclide. This mechanism was previously described for the case of *B. subtilis*, showing almost linear growth at uranium concentration of 450 mg/l (Liao et al., 2013). Microbial U(VI)-phosphate precipitation has been identified as a promising strategy for bioremediation of uranium contaminated environments (Ray et al., 2011; Singh et al., 2010). However, microbial precipitation products might be re-solubilized under oxic conditions (Moon et al., 2007; Yi et al., 2007), or associated with pore-water colloids in natural environments (Wang et al., 2013) increasing the mobility of uranium. For this reason, it is of great interest to get a deeper knowledge of the metabolic potential of the microbial community in complex ecosystems.

5. Conclusions

This work describes the microbial community changes of bentonite clay microcosms induced by uranyl nitrate treatment and long term incubation under oxic conditions. The structure of the microbial community of the uranyl nitrate-treated microcosms (BI-C and BII-C) differs from that of the control microcosms (water- and sodium nitrate-treated). OTUs annotated as *Paracoccus* and *Bacillus* were highly enriched in microcosms -C, more likely due to the uranyl nitrate treatment. In addition, strain *Bacillus* sp. BII-C3 isolated from the uranyl nitrate-treated microcosm BII showed a high ability to precipitate uranium as U-phosphates. Further analyses are required to fulfill the knowledge about the metabolic potential of the microorganisms involved in the uranium bioprecipitation. The effects of uranium on the microbial diversity described in this work should be considered to evaluate the safety performance of the engineered barrier system for future DGR of radioactive wastes.

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Conflict of interest

The authors declare no conflicts of interest.

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