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1 **Bio-precipitation of uranium by two bacterial isolates recovered from extreme**
2 **environments as estimated by potentiometric titration, TEM and X-ray absorption**
3 **spectroscopic analyses**

4
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1 **ABSTRACT**

2 This work describes the mechanisms of uranium biomineralization at acidic conditions by
3 *Bacillus sphaericus* JG-7B and *Sphingomonas* sp. S15-S1 both recovered from extreme
4 environments. The U-bacterial interaction experiments were performed at low pH values (2.0-
5 4.5) where the uranium aqueous speciation is dominated by highly mobile uranyl ions. X-ray
6 absorption spectroscopy (XAS) showed that the cells of the studied strains precipitated
7 uranium at pH 3.0 and 4.5 as a uranium phosphate mineral phase belonging to the meta-
8 autunite group. Transmission electron microscopic (TEM) analyses showed strain-specific
9 localization of the uranium precipitates. In the case of *B. sphaericus* JG-7B, the U(VI)
10 precipitate was bound to the cell wall. Whereas for *Sphingomonas* sp. S15-S1, the U(VI)
11 precipitates were observed both on the cell surface and intracellularly. The observed U(VI)
12 biomineralization was associated with the activity of indigenous acid phosphatase detected at
13 these pH values in the absence of an organic phosphate substrate. The biomineralization of
14 uranium was not observed at pH 2.0, and U(VI) formed complexes with organophosphate
15 ligands from the cells. This study increases the number of bacterial strains that have been
16 demonstrated to precipitate uranium phosphates at acidic conditions via the activity of acid
17 phosphatase.

18
19 *Keywords:* Uranium biomineralization; potentiometric titration; XAS; TEM/EDX; acid
20 phosphatase

1. Introduction

Uranium is a long-lived, naturally occurring radionuclide that is an ecological contaminant and a human health hazard. The main sources of U pollution include mining activities, manufacture of nuclear weapons, nuclear energy production, and storage of radioactive wastes [1]. In Eastern Germany, the majority of U mining activities were concentrated in Saxony and Thuringia and produced 220.000 metric tons of U starting from 1946 until 1991[2]. These operations ended due to financial and political causes resulting in the accumulation of abandoned contaminated mine works with a subsurface void volume greater than 10^8 m³. In addition, 5×10^8 tons of radioactive mining waste was spread over 3000 piles and 20 tailings in densely populated areas [2]. These U contaminated sites need a long-term stewardship in addition to remediation.

The microbial based remediation of environmental metal pollution offers an efficient and cheap alternative to the commonly used physicochemical approaches, such as chemical precipitation and osmosis [3]. The most developed microbial remediation techniques are biosorption and biomineralization. Biosorption is constrained by the bioavailability of metal-binding sites [4], however biomineralization mechanisms are less limited. Hence, biomineralization continues to be one of the more promising technologies for metal removal from highly diluted solutions.

The bacterial remediation of uranium in anoxic environments has been previously documented by work mainly focused on the enzymatic reduction of soluble, highly mobile U(VI) to a less toxic, solid U(IV) oxide (uraninite) species [5-8]. Uraninite has low solubility and is very stable in reducing environments. However in oxidizing water, it readily dissolves to form aqueous uranyl complexes [9]. In contrast to anaerobic microbially-induced reductive precipitation, biomineralization takes place under aerobic conditions. Therefore, biomineralization is being considered as a potential remediation strategy for radionuclides in

1 oxygenated subsurface zones and contaminated ground water [10-12].

2 Previous studies have described the use of indigenous and genetically introduced
3 recombinant acidic phosphatases for the biomineralization of uranium and its removal from
4 aqueous solutions as uranium phosphate mineral phases [13-15]. However, these studies used
5 an exogenous organic phosphate substrate (e.g. glycerol 2-phosphate) to liberate the inorganic
6 phosphate groups which are responsible for the precipitation of the radionuclide. To date, no
7 study has been performed on the bioprecipitation of U by acid phosphatase from naturally
8 occurring microbes in the absence of an external organic phosphate source. The novelty of
9 this work resides in the fact that the bacterial strains used here are capable of releasing and
10 hydrolyzing stored phosphorus as a consequence of increased cell wall permeability and
11 indigenous acid phosphatase activity.

12 In this study, we have used U L_{III}-edge X-ray absorption spectroscopy (XAS), TEM/EDX
13 and potentiometric titration to examine the ability of these bacterial strains, isolated from
14 extreme habitats, to bioprecipitate U(VI) from solution under acidic conditions. XAS was
15 used as a suitable tool for the determination of the local coordination of radionuclides in
16 biological systems and for fine atomic characterization of the precipitated amorphous U
17 complexes [16-20].

18 19 **2. Materials and methods**

20 21 *2.1. Bacteria, isolation media and culture conditions*

22
23 The two bacterial strains used in this work were isolated from two different extreme
24 habitats. The *Sphingomonas sp.* S15-S1 strain was isolated from a ground water sample;
25 collected at a depth of 290 to 324 meters below the surface from the S15 monitoring well,
26 located near the nuclear waste repository site Tomsk-7 in Siberia, Russia [21]. The *B.*

1 *sphaericus* JG-7B strain was isolated from acidic sediment (pH 4.5) of a uranium mining
2 waste pile near the town of Johanngeorgenstadt, Germany [22]. Both strains were isolated
3 using an enrichment culture. An oligotrophic R2A medium was used for the isolation of
4 *Sphingomonas* sp. S15-S1 [23]. In the case of *B. sphaericus* JG-7B, a nutrient broth (NB)
5 medium (8 g L⁻¹, pH 7.0) was employed. Details of the molecular characterization for the
6 isolated species, the acid phosphatase enzymatic assay and colorimetric determination of
7 phosphates are presented in the supporting information.

9 2.2. Potentiometric titration studies

11 Potentiometric titrations were carried out to determine the characteristic functional groups
12 present on the bacterial surfaces [24]. All titrations were performed using a Metrohm Titrino
13 718 STAT automatic titrator (Metrohm, UK) at 25 °C. The temperature was kept constant and
14 continuously monitored during the titration. The titrator was set to only add successive acid or
15 base once a drift equal or less than 5 mV min⁻¹ was achieved.

16 An amount of live bacteria equivalent to 0.05-0.1 g of dry biomass (washed four times
17 with NaClO₄) was suspended in a vessel with 25 mL of 0.05, 0.1 or 0.5 M NaClO₄, and then
18 purged with N₂ for 1h to remove dissolved CO₂. A positive N₂ gas pressure was maintained
19 during the titration. The suspension was titrated with 0.1 M HCl to pH 3.5 followed by 0.1 M
20 NaOH to pH 10.0. To test the reversibility of the protonation-deprotonation behavior, the
21 suspension was back-titrated with 0.1 M HCl from pH 10.0 to 3.5. The HCl and NaOH were
22 previously calibrated against primary standards.

23 To calculate the acidity constant (p*K*_a) values and the corresponding total concentration of
24 the binding sites for the two strains, *Sphingomonas* sp. S15-S1 and *B. sphaericus* JG-7B, data
25 from five replicates of each titration curve were fitted using the program Prototit 2.1 [25].
26 Variations in the experimental results are reported as the average ± standard deviation.

2.3. Experimental procedure for XAS sample preparation

Bacterial cells grown to the late-exponential phase were harvested by centrifugation at 15.000 x g for 20 min at 4 °C and washed three times with 0.1 M NaClO₄ to remove the interfering ingredients of the growth medium. The pellet was suspended in 10 mL of a previously filtered 0.5 mM UO₂(NO₃)₂ · 6 H₂O solution prepared at three pH values (2.0, 3.0 and 4.5). The samples were shaken for 48 h in an orbital shaker (Gallenkamp, London, UK) at 200 rpm. The cells were harvested by centrifugation after being in contact with the uranium solution and washed with 0.1 M NaClO₄. The cell pellets were powdered after being dried in a vacuum incubator at 30 °C for 24 h. Experimental details for the XAS measurements can be found in the supporting information.

2.4. Sample Preparation for TEM/EDX Analyses

Bacterial cells grown to the late-exponential phase were harvested by centrifugation at 15.000 x g for 15 min at 4 °C and washed twice using 0.1 M NaClO₄ to remove interferences from the growth medium. The pellet was then suspended in 10 mL UO₂(NO₃)₂ solution (0.5 mM, pH 2.0, 3.0 and 4.5) and incubated for 48 h. The metal-treated cells were harvested by centrifugation and washed with 0.1 M NaClO₄ to remove any excess of uranium solution. The TEM sample preparation was carried out as described in Merroun et al. [17]. The samples were examined using a high-resolution Philips CM 200 transmission electron microscope at an acceleration voltage of 200 kV under standard operating conditions with the liquid nitrogen anti-contaminator in place. Energy-dispersive X-ray (EDX) analysis, which provides elemental information via the analysis of X-ray emissions caused by a high-energy electron

1 beam, was also performed at 200 kV using a spot size of 70 Å and a live counting time of 200
2 s.

3. Results

3.1. Phylogenetic affiliation of the bacterial isolates

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6
7
8 The phylogenetic affiliation of the two bacterial isolates studied in this work, based on
9 their 16S rRNA gene analysis, is shown in Fig. 1. The results indicated that the strain *B.*
10 *sphaericus* JG-7B was affiliated with two other strains of *B. sphaericus* (JG-A12 and DSM28)
11 with a 100% of 16S rRNA gene identity. The strain JG-A12 was enriched from a soil sample
12 of the same uranium mining waste pile (near Johanngeorgenstadt, Saxony, Germany) and was
13 demonstrated to have an unusually high capability to bind U(VI) reversibly [17, 26]. The
14 strain DSM28 is the type strain of *B. sphaericus* deposited at the German collection of
15 microorganisms (DSMZ, Braunschweig, Germany). The 16S rRNA gene sequence of the
16 isolate S15-S1 is related to *S. yabuuchiae* A1-18 isolated from the space laboratory Mir [27].
17 Direct molecular and cultivation dependent approaches have demonstrated that representatives
18 of *Bacillus* and *Sphingomonas* strains, similar to those described here, were also found in
19 extreme habitats including those contaminated with radionuclides and heavy metals. Strains of
20 *Bacillus* species have been recovered from uranium contaminated sites with acidic pH values
21 [26] and high concentrations of inorganic contaminants [16]. Representatives of
22 *Sphingomonas* have also been identified as major components of biofilm populations formed
23 in naturally nickel-rich river water [28]. These bacteria lack special growth requirements and
24 grow easily in nutrient limited environments [29-30].

3.2. Potentiometric Titration studies

The potentiometric titration curves of *Sphingomonas* sp. S15-S1 and *B. sphaericus* JG-7B are presented in Figs. 2A and 2B. The concentration of deprotonated sites is standardized per mass of dry biomass (mol g^{-1}) and calculated according to Fein et al. [31] as follows:

$$[\text{H}^+]_{\text{consumed/released}} = (C_a - C_b - [\text{H}^+] + [\text{OH}^-]) / m_b$$

where m_b is the biomass concentration in the suspension (g L^{-1}), C_a and C_b are the concentrations of acid and base added at each step of a titration, and $[\text{H}^+]$ and $[\text{OH}^-]$ represent molar species concentrations of H^+ or OH^- . In order to calculate the acidity constants and the total concentration of each binding site, the titration curve data was fitted using ProtoFit 2.1 [24] using a Non-Electrostatic Model (NEM).

The titrated bacterial suspensions exhibited a protonation-deprotonation behavior over the whole pH range studied (Figs. 2A and 2B). No evidence of saturation was found with respect to proton adsorption. This indicates that, even at pH 3.5, full protonation of the functional groups on the cell wall was not achieved. For all titration curves, the protonation/deprotonation process was reversible.

Figs. 3A and 3B show a comparison of the titration data at different ionic strengths. An intersection point around pH 5.8 and 5.5 can be seen for *Sphingomonas* sp. S15-S1 and *B. sphaericus* JG-7B, respectively. These values were set as the experimental pH of zero proton charge (pH_{zpc}), and are in agreement to the values predicted by ProtoFit 2.1 (see Table 1). There is an ionic strength effect over the pH range studied (Figs. 3A and 3B). However, these effects are weak when compared to the experimental errors associated with potentiometric titrations of bacteria [31-33].

Table 1 summarizes the pK_a values for *Sphingomonas* sp. S15-S1 and *B. sphaericus* JG-7B. The calculated values are 4.27 ± 0.45 and 4.37 ± 0.27 for pK_1 , 7.03 ± 0.86 and 6.37 ± 0.31

1 for pK_2 and 9.92 ± 0.32 and 9.95 ± 0.16 for pK_3 , for *Sphingomonas* sp. S15-S1 and *B.*
2 *sphaericus* JG-7B respectively. The obtained pK_a values are representative of carboxylic
3 groups for pK_1 , phosphate groups for pK_2 and amine and hydroxyl groups for pK_3 [34-36].

4 The surface site densities obtained using ProtoFit are also presented in Table 1. The pK_a
5 values for both bacterial strains are comparable indicating similar concentration of the active
6 functional groups on the cell wall. However, the concentration corresponding to
7 amine/hydroxyl groups (C_3) is slightly higher for *Sphingomonas* sp. S15-S1.

9 3.3. X-ray Absorption spectroscopy

10
11 A visual comparison of the XANES fingerprints for the reference samples U(VI) and
12 U(IV) indicated the presence of U(VI) in the six samples studied in this work (Figs. 4A and
13 4B). The presence of U(VI) in the XANES spectra is evidenced by the display of a
14 characteristic shoulder at 17.188 eV, which is consistent with the U(VI) oxidation state [37].

15 The uranium L_{III} -edge EXAFS spectra and their corresponding Fourier transforms (FT)
16 for the uranium species formed at pH 2.0, 3.0 and 4.5 by the cells of *Sphingomonas* sp. S15-
17 S1 and *B. sphaericus* JG-7B are presented in Figs. 5A and 5B. The FT represents a pseudo-
18 radial distribution function of the uranium near-neighbor environment.

19 The FT of the EXAFS spectra of the samples at pH 2.0, 3.0 and 4.5 show five to six
20 significant peaks (Figs. 5A and B). Tables 2 and 3 show the quantitative fit results (distances
21 are phase shift corrected). The adsorbed U(VI) observed has the common linear trans-dioxo
22 structure: two axial oxygen atoms at a radial distance of $1.76 - 1.79 \pm 0.02 \text{ \AA}$, and an
23 equatorial shell of 4 to 5 oxygen atoms at $2.27 - 2.36 \pm 0.02 \text{ \AA}$. As evident from the results
24 presented in Tables 2 and 3, the coordination numbers, bond distances and Debye-Waller
25 factors of the U- O_{eq1} shell are affected by the pH of the uranium solution. A four-fold
26 coordination of uranium to ligands of the bacterial cells ($N \sim 4$ and $R = 2.27 \pm 0.02 \text{ \AA}$) was

1 observed on the EXAFS spectra of both bacterial strains at pH 4.5 and in that of the JG-7B at
2 pH 3. The lower Debye-Waller factors obtained ($0.0040-0.0046 \text{ \AA}^2$), indicated the absence of
3 a disorder in $U-O_{eq1}$ distances contributing to the EXAFS signal. However, the higher Debye-
4 Waller factor of the EXAFS spectrum of S15-S1 at pH 3 (0.0132 \AA^2) indicated that there is
5 probably a wide spread of $U-O_{eq1}$ distances with an average value of $2.32 \pm 0.02 \text{ \AA}$. The
6 samples incubated at pH 2.0, showed the presence of a five-fold uranium coordination ($N \sim 5$
7 and $R = 2.34-2.36 \pm 0.02 \text{ \AA}$). The $U-O_{eq1}$ bond distance is within the range of previously
8 reported values for phosphate bound to uranyl [16-21].

9 Adding an oxygen shell at a distance of $R = 2.82-2.87 \pm 0.02 \text{ \AA}$ improved significantly the
10 fit for all samples. The fitted distance between uranium and oxygen atoms is not related to
11 direct bonding but has been previously interpreted as scattering contributions from
12 neighboring ligand shells known as “short contacts” in crystallography [16,21].

13 The fifth FT peak observed at $R+\Delta \sim 3 \text{ \AA}$ (radial distance $R = 3.59 - 3.62 \text{ \AA}$), is the result
14 of a back-scattering from phosphorus atoms. This distance is typical for a mono-dentate
15 coordination of U(VI) by phosphate [16-21].

16 The EXAFS spectra of the U-treated bacterial cell samples at pH 4.5 and that of JG-7B
17 treated at pH 3.0 are comparable to the spectra of meta-autunite. All spectra showed similar
18 features and distances for $U-O_{eq}$, $U-P$ and $U-U$. These findings suggest the precipitation of an
19 inorganic m-autunite-like uranyl phosphate by the bacterial cells.

20 The EXAFS spectra of the samples treated at pH 2.0 showed close similarities to that of
21 organic phosphate ligands complexed with U such as fructose 1,6-phosphate (see Fig. 5) [38].
22 At pH 3, the EXAFS spectrum of S15-S1 may consist of both uranyl phases (organic and
23 inorganic phosphates complexes). This suggestion is supported by the high Debye-Waller
24 factor value of the $U-O_{eq1}$ bond distance (Table 3).

3.4. Cellular localization of the bound U(VI) by TEM

The TEM of *B. sphaericus* JG-7B cells exposed to U solution (0.5 mM, pH 4.5) (Fig. 6A) revealed the presence of electron-dense accumulations on the cell surface. There was no intracellular accumulation of U observed in these samples. The EDX spectrum derived from these deposits (Fig. 6B) indicated that their chemical composition was mainly oxygen (O), phosphorus (P), and uranium (U). In the case of *Sphingomonas* sp. S15-S1, samples prepared at pH 2.0 showed very small amount of U bound to bacterial cell surfaces of both isolates (data not shown). Similarly to *B. sphaericus*, the presence of U associated with intracellular space was not observed. At pH 3.0 and 4.5, *Sphingomonas* sp. S15-S1 cells accumulated U in the form of precipitates at the cell membrane and intracellular accumulation of U was also observed in the form of electron dense granules (Figs. 7A-E). EDX analysis of the intracellular and cell wall deposits of the thin sections of the Figs. 7A-B shown in Figs. 7F-G, respectively, indicated the presence of phosphorus and U.

3.5. Acidic phosphatase activity and determination of inorganic phosphate

The measured acidic phosphatase (APase) activity of JG-7B and S15-S1 cell suspension at pH 2.0, 3.0 and 4.5 are presented in Fig. 8. In addition, the APase activity was also calculated for heat-killed cells.

The results indicate that the enzymatic activity is highly strain-specific. The cells of the strain S15-S1 showed activities 20 orders of magnitude higher than those of the strain JG-7B. In both cases, the phosphatase activity increases with increasing pH and reaches an optimum value at pH 4.5. Acidic phosphatase activity (within the experimental errors) was neither detected in the suspensions of heat-killed cells, nor in cells incubated in NaClO₄ at pH 2.0 for both strains.

1 The concentration of orthophosphate in the supernatants of the cells of the two strains
2 incubated for 48 h at pH 4.5 in 0.1 M NaClO₄ (control sample) and in 0.5 mM uranium
3 solution (U treated samples) are shown in Fig. 9. As evident from this figure, the amount of
4 the orthophosphate liberated by the control samples is two to three orders of magnitude higher
5 than the samples containing U.

6 7 **4. Discussion**

8
9 In the present work, a complex methodological approach involving a combination of
10 surface chemistry, transmission electron microscopy and advanced solid state speciation
11 techniques were used to characterize uranium biomineralization mechanisms. Bacterial strains
12 isolated from two different extreme environments were studied under acidic and aerobic
13 conditions. The studied strains were: the Gram-positive strain *B. sphaericus* JG-7B, cultivated
14 from a uranium mining waste sediment in Germany; and the Gram-negative
15 Alphaproteobacterial strain *Sphingomonas* sp. S15-S1, recovered from ground water extracted
16 from the S15 deep monitoring well of the Siberian radioactive waste subsurface depository
17 Tomsk-7 in Russia. In these extreme environments, bacteria may interact efficiently with
18 these inorganic contaminants through different mechanisms such as intracellular accumulation
19 [16], precipitation [39,40] and biosorption at the cell surfaces [17].

20 21 *4.1. Potentiometric Titration studies*

22
23 Bacterial cell walls contain a variety of functional groups that provide metal binding sites,
24 such as hydroxyl, phosphoryl, amino, and carboxylate groups. These functional groups can
25 protonate or deprotonate when interacting with their immediate surroundings and as a result
26 the cell walls develop a net pH-dependent charge [24,32,34,41-42]. Therefore, knowledge of

1 the cell surface properties is crucial to understand the interaction mechanisms between
2 bacteria and surrounding metals. The concentration and characteristics of proton active
3 carboxylic, hydroxyl, phosphate, phosphodiester, and amine groups on the cell surfaces play
4 an important role in this respect, as they are responsible for surface binding ability [36]. The
5 results of potentiometric titration experiments on the studied *Sphingomonas* sp. and *B.*
6 *sphaericus* strains indicated that the cell surface groups capable for metal binding are sites
7 involving carboxyl groups ($pK < 4.27-4.37$), sites involving phosphate groups ($pK \approx 7$), and
8 sites involving hydroxyl and amine groups ($pK > 8$). These findings are in agreement with
9 previous studies on bacterial surfaces [31, 42]. Haas et al. [42] showed that in the presence of
10 U(VI), sorption is accounted for by using two separate adsorption reactions that form the
11 surface complexes $>COO-UO_2^+$ and $>PO_4H-UO_2(OH)_2$. This mechanism indicates that
12 phosphate and carboxyl groups are expected to be involved in the binding of this radionuclide.
13 However, XAS studies demonstrated that for the investigated bacterial strains, phosphate
14 groups are the main binding sites for uranium in the pH range studied. The extent of the
15 carboxyl group involvement in the uranium binding is probably too insignificant to be
16 detected by titration methods.

17 The titration studies also showed that the surface site density of *B. sphaericus* JG-7B is
18 similar to those found for *B. subtilis* by Chatellier and Fortin [43], except for the lower value
19 obtained for phosphate groups on *B. sphaericus*. Cox et al. [44] reported that the presence of
20 a thick peptidoglycan layer on Gram positive bacteria seems to be responsible for a higher
21 binding capacity for metals. The *Sphingomonas* sp. and *B. sphaericus* JG-7B strains studied in
22 this work showed site densities of the same order of magnitude, suggesting a similar potential
23 capacity to bind metals.

24 4.2. X-ray Absorption spectroscopy molecular scale analysis of U/bacteria complexes

1 The speciation of U associated with cells under the studied conditions is a pH dependent
2 process. At pH 4.5, EXAFS analysis indicated that cells of the S15-S1 and JG-7B strains
3 precipitated uranium as a meta-autunite mineral-like phase since the local coordination of U in
4 these samples matches that of meta-autunite. In addition, evidence from the acidic
5 phosphatase studies indicated the presence of phosphatase enzyme in the cell suspensions.
6 These findings suggest a direct link on the precipitation of U by liberation of inorganic
7 phosphates in solution. The amount of the orthophosphate in the supernatant of U treated
8 samples of S15-S1 and JG-7B decreased by 2 to 3 orders of magnitude in comparison to that
9 of the control sample, indicating that these ions are scavenged for the precipitation of U in
10 solution. The implication of acid phosphatase activity in the precipitation of U is supported
11 by the fact that at pH 2 no enzymatic activity was detected and therefore uranium
12 biomineralization did not occur. Several studies reported the role played by this enzyme in the
13 liberation of inorganic phosphates that precipitate uranium [16,39,45,46]. *Citrobacter* sp. was
14 shown to accumulate heavy deposits of uranyl phosphate at the cell surface after
15 enzymatically liberating phosphate ligands *via* the activity of a phosphatase, resulting in
16 biomineralization of NaUO_2PO_4 and/or $\text{H}_2\text{UO}_2\text{PO}_4$ [46]. Indigenous acidic phosphatase
17 activity in naturally occurring strains of the genera *Bacillus* and *Rahnella* isolated from
18 radionuclide- and metal-contaminated soils has been recently demonstrated to be involved in
19 the precipitation of uranium [15]. This is due to the liberation of orthophosphate from the
20 added glycerol-3-phosphate as a source of organic phosphates.

21 In the present study, no organic phosphate sources were added to the metal-bacteria
22 mixture. At this stage of investigation, the origin of the phosphates which precipitate U is still
23 unknown. One possible source of organic phosphate is the lysis of dead cells. [The presence of
24 dead cells in the treated samples was demonstrated earlier in our previous study by using
25 TEM \[21\]. In the same work we have shown also by using live/dead staining approach that
26 the percentage of dead cells represents 40% of the cell population.](#) In uranium contaminated

1 sites, dead cells of U-sensitive microbial populations will liberate significant quantities of
2 biopolymers able to precipitate uranium after lysis [47-48]. These phosphorylated biological
3 components could be used as substrates for acidic phosphatase activity, precipitating uranium.
4 Similar results were found with microbial strains *Myxococcus xanthus* [39] and *Sulfolobus*
5 *acidocaldarius* [49] which precipitated uranium as an m-autunite-like phase due to the
6 activity of indigenous acidic phosphatase expressed in uranium treated cell suspensions
7 without an external organic phosphate source. The biomineralization of uranium by bacteria
8 based on indigenous or introduced recombinant alkaline phosphatase has been proposed as
9 appropriate technology for the treatment of alkaline waters containing uranium [50].

10 At pH 3, the speciation of U associated with cells of the two strains is dominated by the
11 uranium phosphate mineral phase. Uranium organic phosphate species were found on samples
12 of JG-7B cells. In the case of strain S15-S, the uranium speciation is a mixture of U organic
13 phosphate species and U inorganic phosphates mineral phases. The mixture of these two U
14 phases is evidenced by the high Debye-Waller factor of the U-O_{eq1} shell, estimated to be
15 0.0132 Å² by EXAFS spectroscopy.

16 17 4.3. TEM cellular localization of U precipitates

18
19 The presence of precipitated uranium at cell level is strain-specific and affected by the
20 uranium solution pH as demonstrated by TEM analysis. *B. sphaericus* JG-7B cells
21 precipitated uranium at the cell wall, which consists of peptidoglycan and S-layer protein. The
22 latter envelope component may play a crucial role in the protection of the cells of this
23 bacterium against uranium toxicity. These results are in agreement with those found for a
24 different *B. sphaericus* strain, where the accumulated uranium was only localized at the cell
25 envelope [16]. In the case of *Sphingomonas* sp., microscopic observations of cells exposed to
26 uranium solution showed electron-dense precipitates at the cell envelope and in some cases

1 also within the cells. The intracellular accumulation occurs in two different forms, as electron
2 dense granules and as needle-like fibrils. These findings reveal that Gram-negative and Gram-
3 positive bacteria accumulate uranium via different mechanisms. These differences could be
4 explained by variations on the structural composition of their cell walls.

6 *4.4 Environmental implication of U biomineralization at acidic conditions*

8 The remediation of polluted sites by conventional methods, such as excavation and pump
9 and treat, can be costly and disruptive. Therefore *in situ* remediation strategies, based on the
10 stimulation of the growth of microbial strains with acid phosphatase activity such as the ones
11 reported here (*Sphingomonas* sp. and *Bacillus* sp.), could be considered as a potential strategy
12 for decontamination of such environments. The high acid phosphatase activity demonstrated
13 for these strains makes them ideal candidates to use on bioremediation of uranium
14 contaminated sites. The natural habitats of these bacteria are characterized by extremely
15 complex biogeochemistry (low pH values, high concentrations of heavy metals) and high
16 microbial diversity and activity. **Despite these complexities, these bacterial strains are capable
17 to produce inorganic phosphate species in sufficient quantities to remove at least 70% (and in
18 some cases nearly 100%) of U(VI) from solution via phosphate-associated precipitates.** These
19 characteristics add extra features to the biomineralization process widening the possibilities of
20 using this technique as a remediation route for radionuclides in the environment.

22 **5. Conclusions**

24 This study demonstrates that inorganic phosphate responsible for uranium
25 biomineralization at pH 4.5 and 3 is liberated by the cells of *Sphingomonas* sp. and *B.*
26 *sphaericus* strains, as a consequence of both increased cell wall permeability and the APase

1 activity of the strains. The impact of uranium precipitation at acidic and aerobic conditions by
2 bacteria isolated from different extreme environments must be acknowledged in attempts to
3 fully understand actinide cycling and dispersal in the environment. This work supports
4 previous findings that uranium bioremediation could be achieved via the biomineralization of
5 U(VI) in phosphate minerals as a complementary process to the more widely investigated
6 U(VI) bioreduction.

8 **Acknowledgments**

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FIGURES LEGEND

Fig. 1. 16S rDNA based affiliation of the studied bacterial isolates (given in bold), recovered from extreme environments.

Fig. 2. Potentiometric titration data for cell suspensions of *Sphingomonas* sp. S15-S1 (A) and *B. sphaericus* JG-7B (B) compared with the 0.1M NaClO₄ electrolyte. Closed symbols correspond to the forward titration data and open symbols correspond to back titration.

Fig. 3. Potentiometric titration data for cell suspensions of *Sphingomonas* sp. S15-S1 (A) and *B. sphaericus* JG-7B (B) at different electrolyte concentrations (0.05, 0.1 and 0.5M NaClO₄).

Fig. 4. Normalized uranium L_{III}-edge XANES spectra of 0.04 M U(IV) in 1 M HClO₄, 0.04 M U(VI) in 1 M HClO₄, U(VI)-treated cells of JG-7B and S15-S1 isolates at pH 2, 3 and 4.5. The spectra were normalized to equal intensity at 17230 eV.

Fig. 5. Uranium L_{III}-edge k^3 -weighted EXAFS spectra (left) and the corresponding Fourier transforms (FT) (right) of uranium complexes formed by the cells of bacterial isolates at pH values 2, 3 (A) and 4.5 (B) and reference compounds (m-autunite and U-fructose(1,6) phosphate).

Fig. 6. Transmission electron micrographs (A), coupled with Energy Dispersive X-ray spectrum (B), of a thin section of *B. sphaericus* JG-7B treated with uranium at pH 4.5. The metal accumulated is localized on the cell surface.

Fig. 7. Transmission electron micrographs of thin sections of *Sphingomonas* sp. S15-S1 treated with uranium at pH 4.5 (A, B) and 3 (C, D, E). Energy Dispersive X-ray spectra of intracellular (F) and cell wall (G) U precipitates showed by heads of B and D, respectively.

Fig. 8. Acidic phosphatase activity of cells of *Sphingomonas* sp. S15-S1 and *B. sphaericus* JG-7B incubated in 0.1 M NaClO₄ for 48 h at pH 2, 3 and 4.5, and that of heat killed cells.

Fig. 9. Concentrations of ortho-phosphates liberated in the supernatant by cells of *Sphingomonas* sp. S15-S1 and *B. sphaericus* JG-7B -incubated in 0.1 M NaClO₄ (control sample) and -treated with 0.5 mM U at pH 4.5.

Table 1: Deprotonation constants and surface site concentrations for *Sphingomonas* sp.S15-S1 and *B. sphaericus* JG-7B as calculated by ProtoFit 2.1

	pK_1	pK_2	pK_3	C_1 ($\times 10^{-4}$ mol/g)	C_2 ($\times 10^{-4}$ mol/g)	C_3 ($\times 10^{-4}$ mol/g)	pH_{zpc}
<i>Sphingomonas</i> sp. S15-S1	4.27±0.45	7.03±0.86	9.92±0.32	4.91±1.04	3.16±0.56	9.24±2.97	5.75±0.54
<i>B. sphaericus</i> JG-7B	4.37±0.27	6.37±0.31	9.95±0.16	4.70±0.55	2.19±0.25	4.56±0.77	5.55±0.28

Table 2. Structural parameters of the uranium complexes formed by the bacterial isolates at pH 2 and 3

Sample	Shell	N ^a	R(Å) ^b	σ^2 (Å ²) ^c	ΔE (eV)
					-12.40
JG-7B pH2	U-O _{ax}	2 ^d	1.77	0.0024	
	U-O _{eq1}	4.4(4)	2.36	0.0071	
	U- O _{eq2}	0.8(2)	2.86	0.0038 ^d	
	U-P	2.6(4)	3.62	0.0027	
	U- O _{eq1} -P (MS)	5.2	3.74	0.0027	
JG-7B pH 3	U-O _{ax}	2 ^d	1.79	0.0024	-12.60
	U-O _{eq1}	4.2(3)	2.27	0.0052	
	U- O _{eq2}	1.0(2)	2.87	0.0038 ^d	
	U-P	3.2(5)	3.60	0.0040	
	U- O _{eq1} -P (MS)	6.4	3.72	0.0040	
	U-U	3.2(6)	5.21	0.0080 ^d	
S15-S1 pH 2	U-O _{ax}	2 ^d	1.77	0.0025	-13.40
	U-O _{eq1}	4.8(5)	2.34	0.0105	
	U- O _{eq2}	0.8(2)	2.86	0.0038 ^d	
	U-P	1.5(3)	3.60	0.0010	
	U- O _{eq1} -P (MS)	3.0	3.73	0.0010	
S15-S1 pH 3	U-O _{ax}	2 ^d	1.76	0.0040	-15.50
	U-O _{eq1}	4.6(6)	2.32	0.0132	
	U- O _{eq2}	0.7(3)	2.82	0.0038 ^d	
	U-P	1.9(5)	3.59	0.0034	
	U- O _{eq1} -P (MS)	3.8	3.72	0.0034	

a: Errors in coordination numbers are $\pm 25\%$, and standard deviations, as estimated by EXAFSPAK are given in parentheses.

b: errors in distance are ± 0.02 Å.

c: Debye-Waller factor.

d: value fixed for calculation.

Table 3. Structural parameters of the uranium complexes formed by the bacterial isolates at pH 4.5

Sample	Shell	N ^a	R(Å) ^b	σ^2 (Å ²) ^c	ΔE (eV)
S15-S1	U-O _{ax}	2 ^d	1.77	0.0021	-13.54
	U-O _{eq1}	4.0(3)	2.27	0.0082	
	U- O _{eq2}	0.8(1)	2.86	0.0038 ^d	
	U-P	1.8(2)	3.59	0.0040 ^d	
	U- O _{eq1} -P (MS)	3.6	3.71	0.0040 ^d	
	U-U	1.9(6)	5.19	0.0080 ^d	
JG-7B	U-O _{ax}	2 ^d	1.79	0.0024	-12.40
	U-O _{eq1}	4.4(2)	2.27	0.0046	
	U- O _{eq2}	1.0(2)	2.85	0.0038 ^d	
	U-P	3.2(5)	3.61	0.0050	
	U- O _{eq1} -P (MS)	6.4	3.72	0.0050	
	U-U	2.4(6)	5.20	0.0080 ^d	

a: Errors in coordination numbers are $\pm 25\%$, and standard deviations, as estimated by EXAFSPAK are given in parentheses.

b: errors in distance are ± 0.02 Å.

c: Debye-Waller factor.

d: value fixed for calculation.

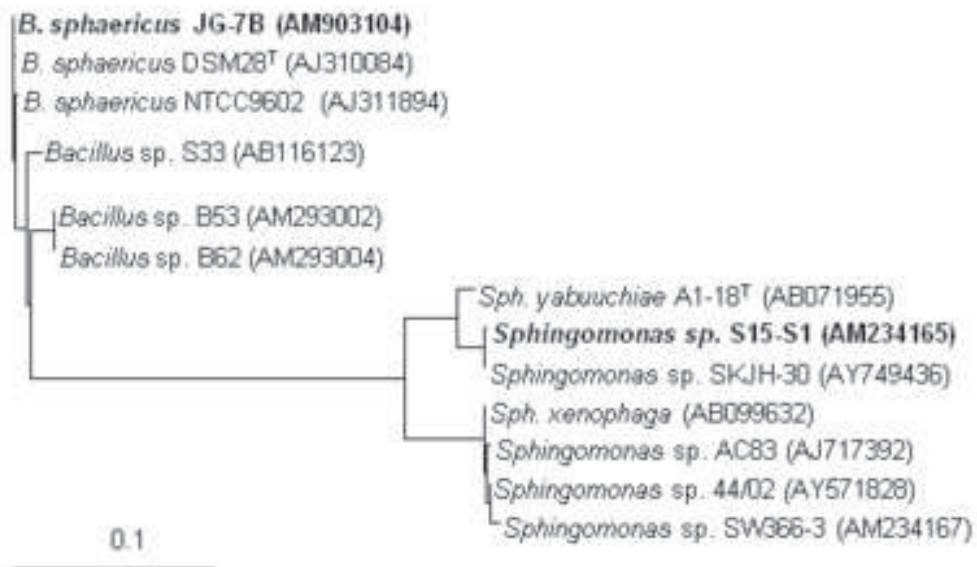


FIG. 1

Figure(s)

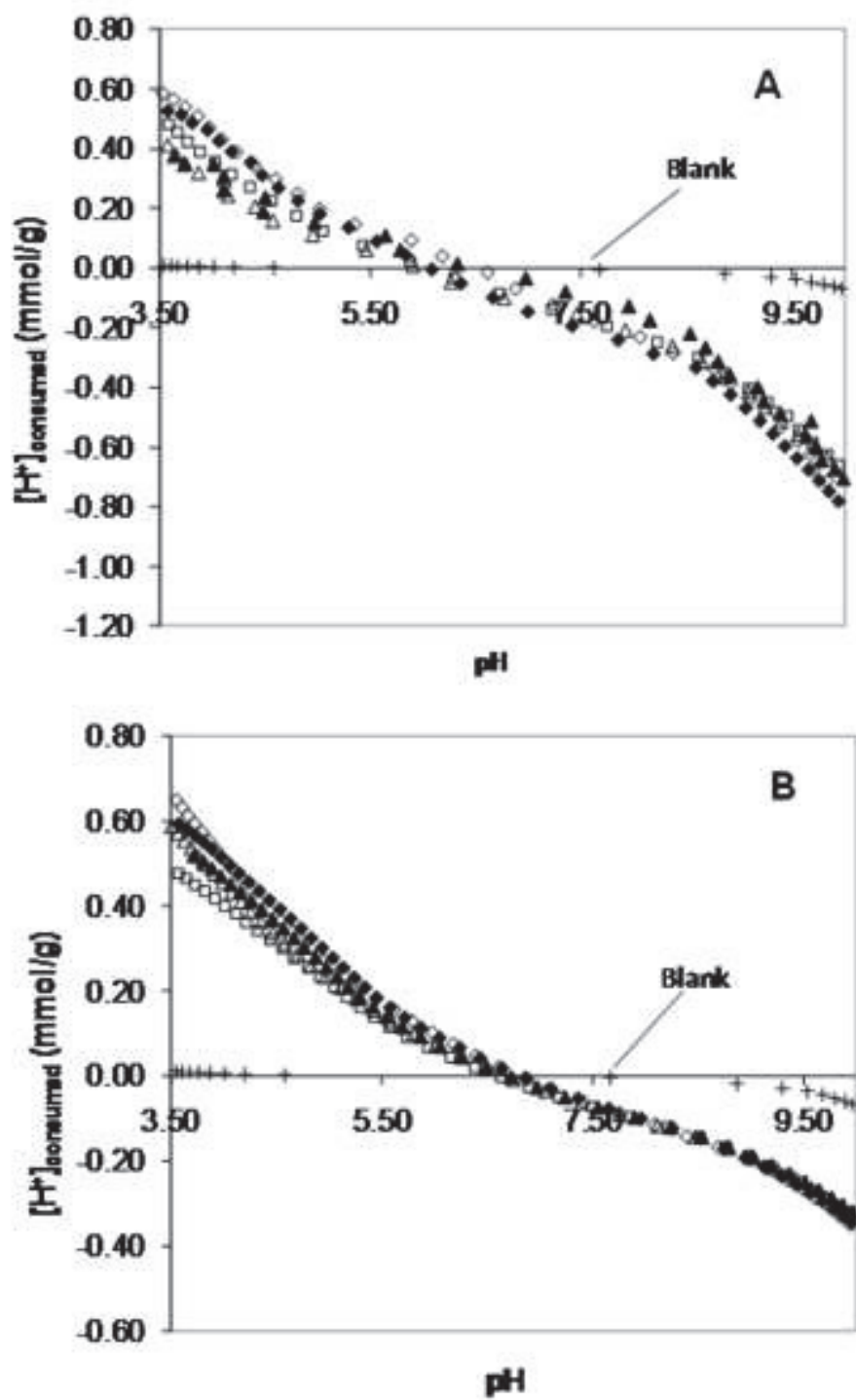


FIG. 2

Figure(s)

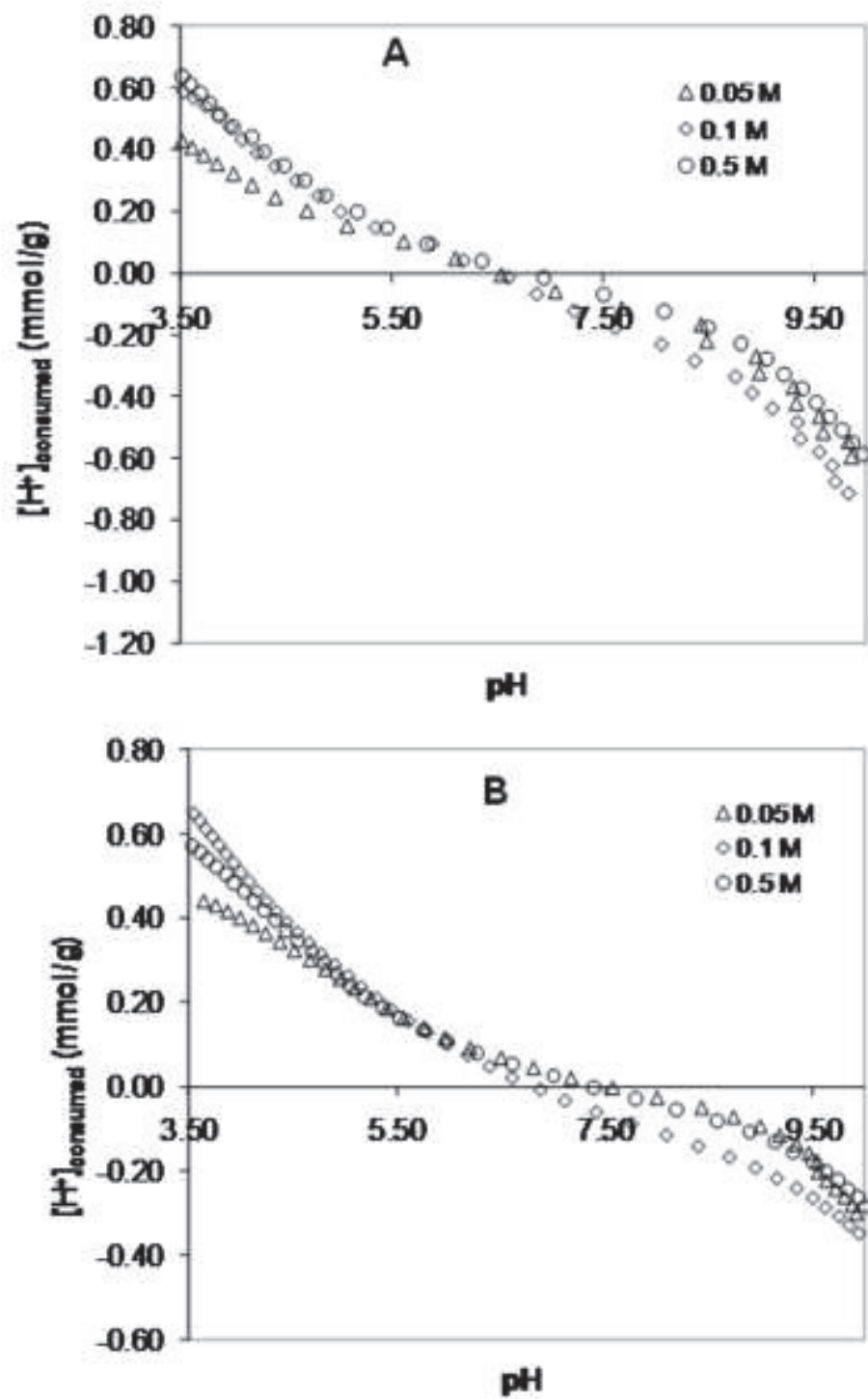


FIG. 3

Figure(s)

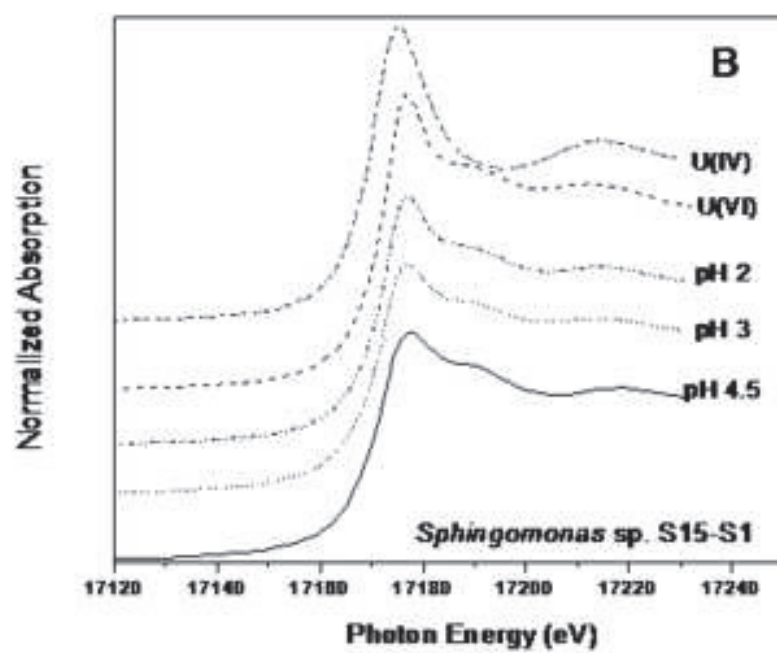
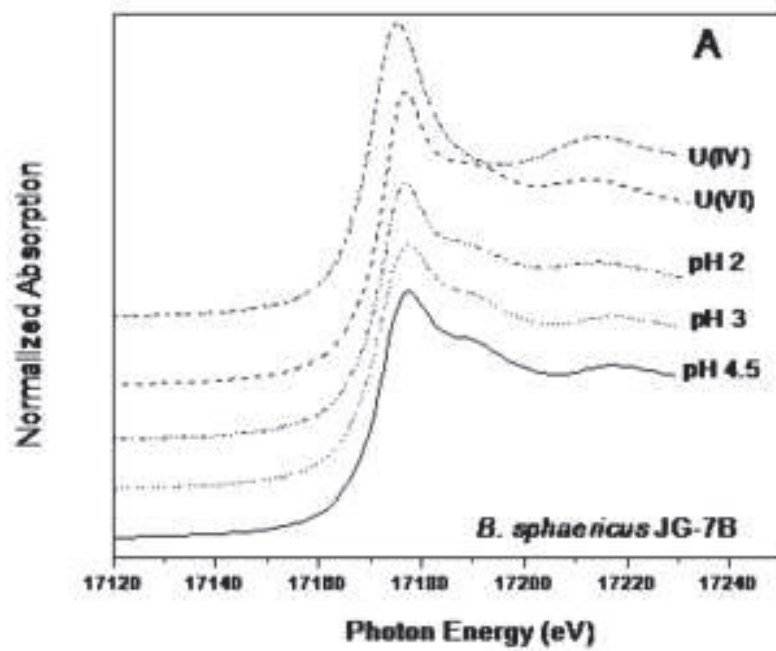


FIG. 4

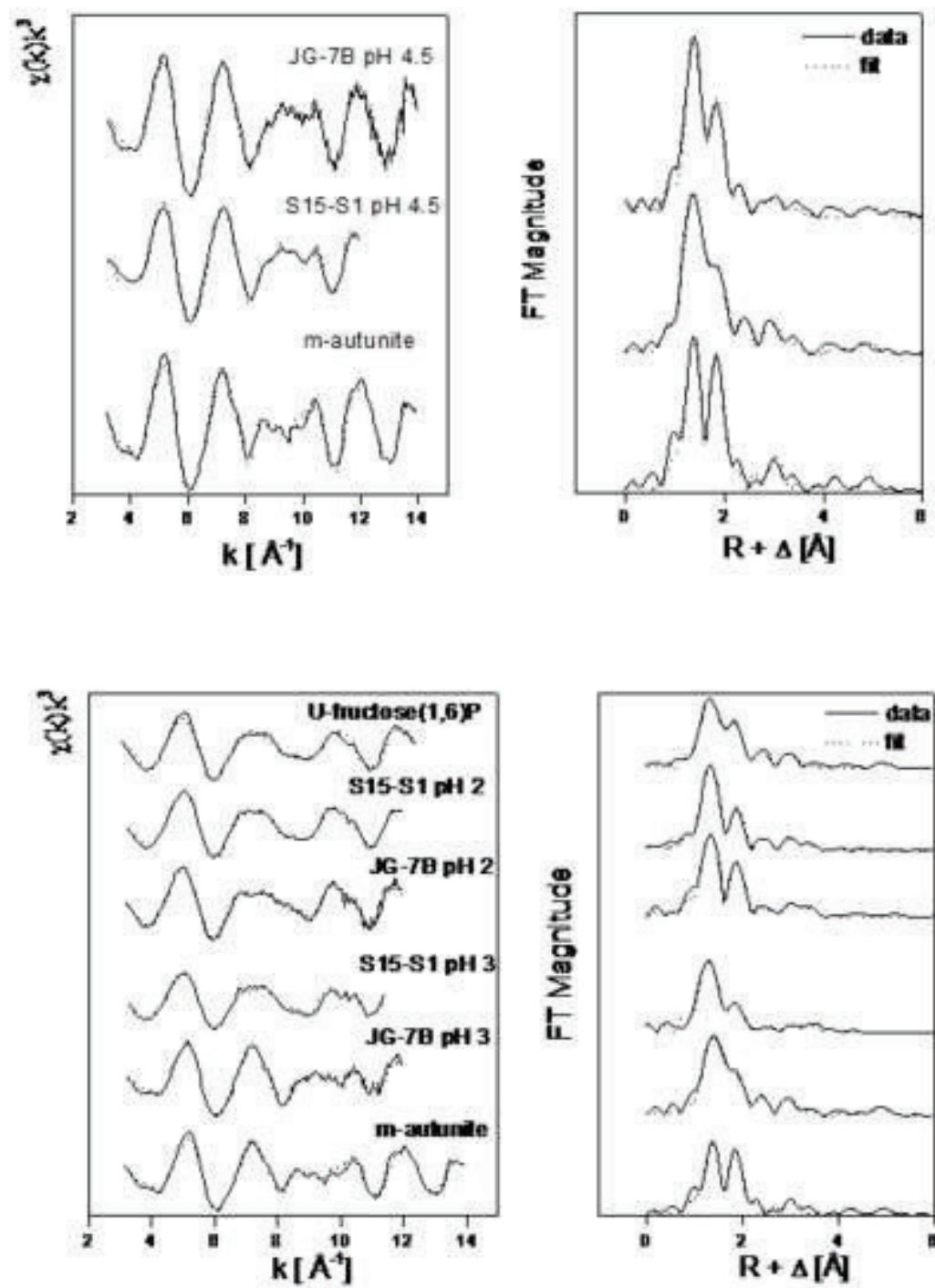


FIG. 5

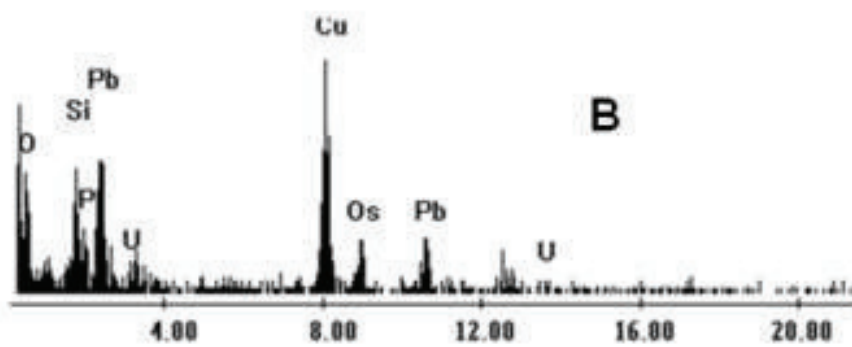
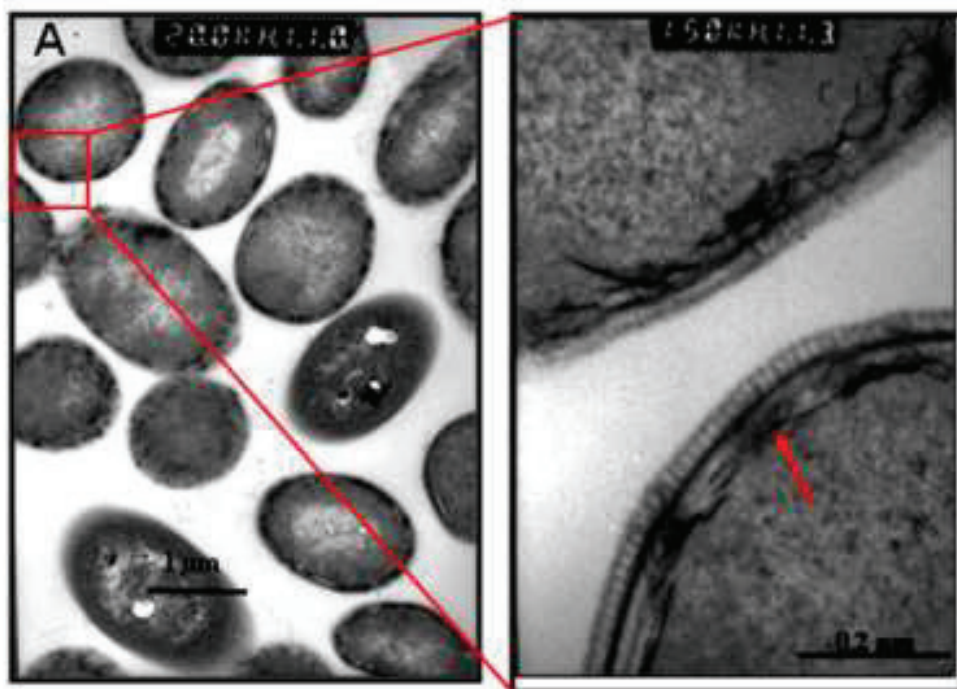


FIG. 6

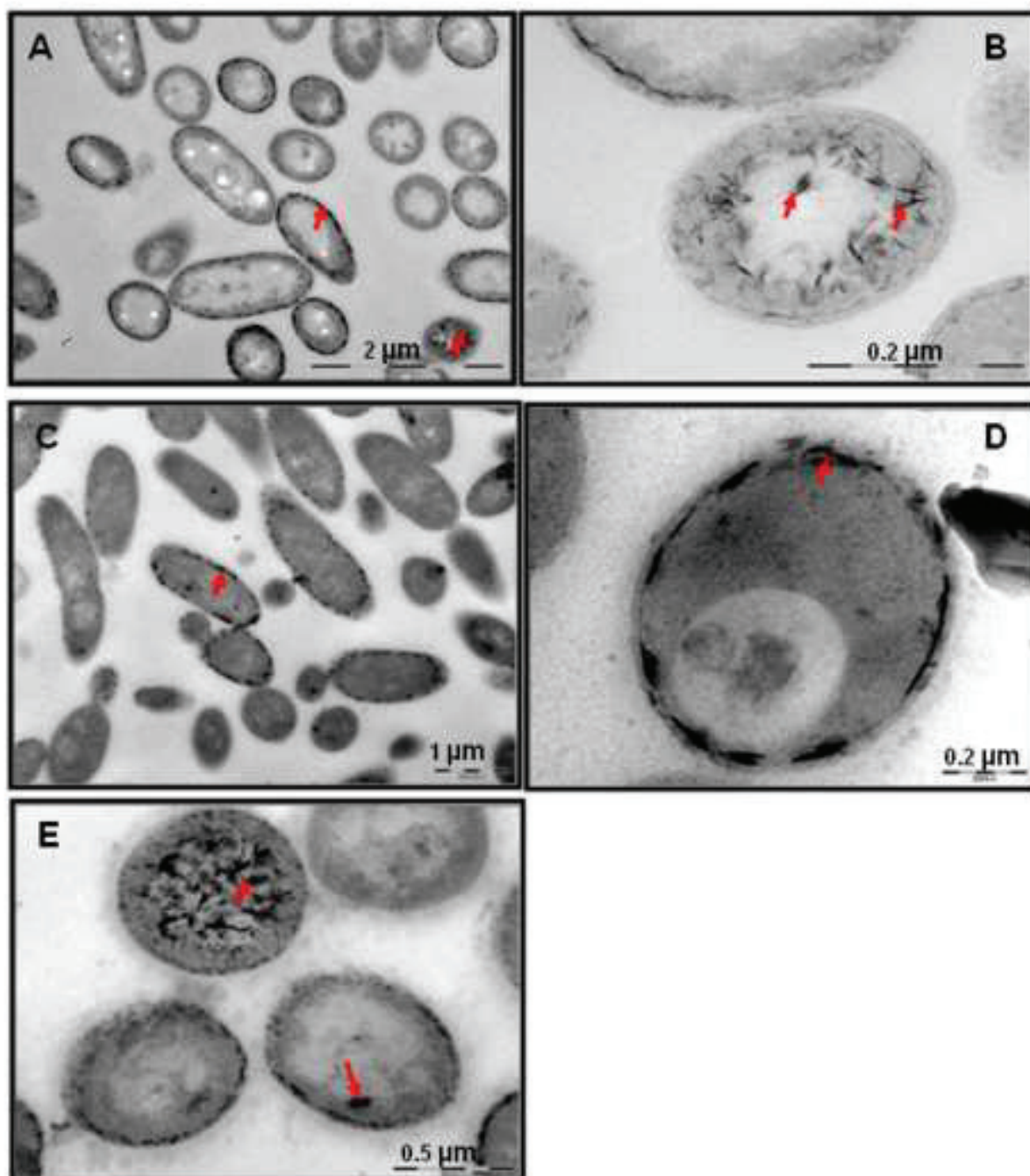


FIG. 7

Figure(s)

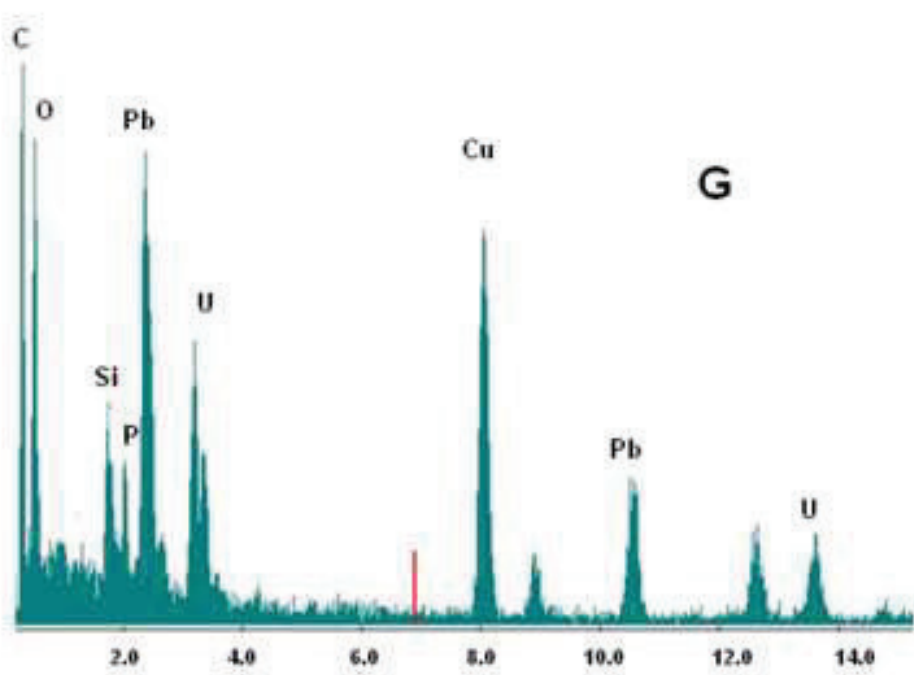
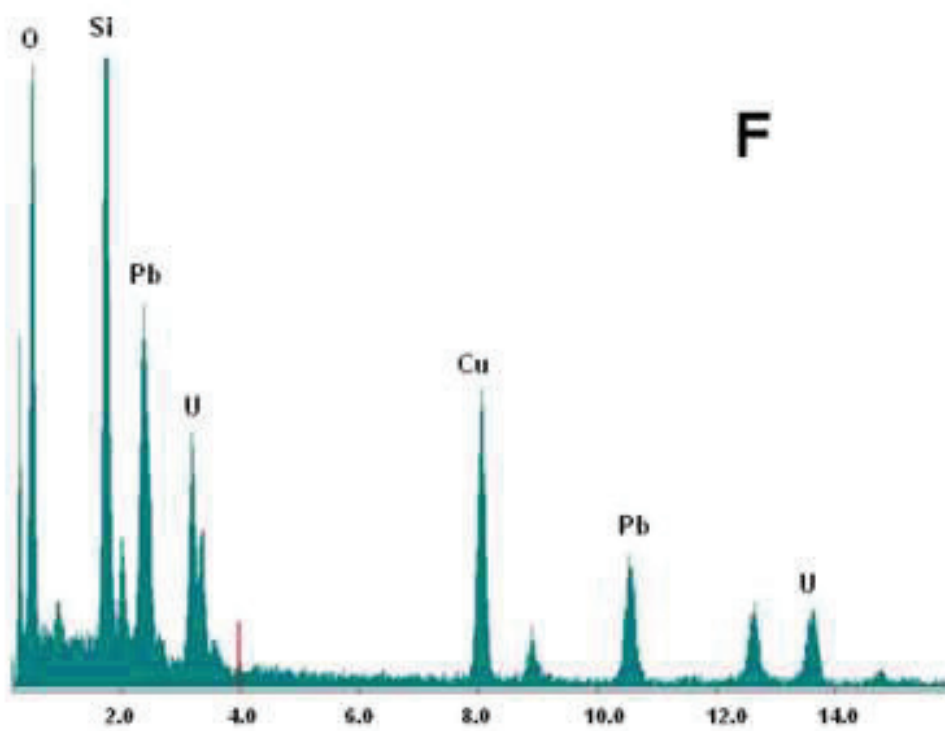


FIG. 7

