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RESEARCH ARTICLE

Molecular binding of Eu^{III}/Cm^{III} by *Stenotrophomonas bentonitica* and its impact on the safety of future geodisposal of radioactive waste

Miguel A. Ruiz-Fresneda[1,*], Margarita Lopez-Fernandez[1,*,#], Marcos F. Martínez-Moreno[1], Andrea Cherkouk[2], Yon Ju-Nam[3], Jesús J. Ojeda[3], Henry Moll[2], Mohamed L. Merroun[1]

[1]Department of Microbiology, University of Granada, Granada, Spain

[2]Institute of Resource Ecology, Helmholtz-Zentrum Dresden-Rossendorf e.V., Dresden, Germany

[3]Systems and Process Engineering Centre, College of Engineering, Swansea University, Swansea, UK

[*]Corresponding author/s: Miguel Angel Ruiz-Fresneda. Email: mafres@ugr.es; Margarita Lopez-Fernandez. Email: margaritalopez@ugr.es

[#]Present address: Institute of Resource Ecology, Helmholtz-Zentrum Dresden-Rossendorf e.V., Dresden, Germany

1. Abstract

Microbial communities occurring in reference materials for artificial barriers (*e.g.* bentonites) in future deep geological repositories of radioactive waste can influence the migration behavior of radionuclides such as curium (Cm^{III}). This study investigates the molecular interactions between Cm^{III} and its inactive analogue europium (Eu^{III}) with the indigenous bentonite bacterium *Stenotrophomonas bentonitica* at environmentally relevant concentrations. Potentiometric studies showed a remarkable high concentration of phosphates at the bacterial cell wall compared to other bacteria, revealing the great potential of *S. bentonitica* for metal binding. Infrared spectroscopy (ATR-FTIR) and X-ray photoelectron spectroscopy (XPS) confirmed the role of phosphates and carboxylate groups from the cell envelope in the bioassociation of Eu^{III}. Additionally, time-resolved laser-induced fluorescence spectroscopy (TRLFS) identified phosphoryl and carboxyl groups from bacterial envelopes, among other released complexing agents, to be involved in the Eu^{III} and Cm^{III} coordination. The ability of this bacterium to form a biofilm at the surface of bentonites allow them to immobilize trivalent lanthanide and actinides in the environment.

Keywords: europium, curium, bacterial speciation, mobility, geodisposal

2. Introduction

The safe disposal of radioactive waste is crucial to ensure the safety of future generations, as well as for the biosphere. The implementation of deep geological repositories (DGRs) is planned in the near future for the disposal of high level (HLW) and long-lived radioactive wastes, which are the most hazardous since they contain larger radionuclide concentrations and longer lived radionuclides.[1] DGR is a multi-barrier system to deposit radioactive waste, mainly generated by nuclear industry. A DGR option is to encapsulate the nuclear waste in metal containers (steel, iron, copper, etc.) surrounded by compacted bentonites, considered as geotechnical barriers, and emplace them in a stable geological formation at about 500-1000 m depth.[2] A high microbial diversity in bentonite clay formations from Almeria (Spain), considered as reference material of engineered barriers for repositories, has been previously reported.[3,4] Several studies have evidenced the impact of microbial processes on the corrosion of metal containers (steel, iron, copper, etc.), which could lead to the release of radionuclides to the surrounding environment.[5] Microbial processes also seem to play a crucial role controlling the speciation and mobility of radionuclides present in radioactive wastes, such as uranium (U) and curium (Cm).[3,4,6] Therefore, understanding the migration behavior and the environmental fate of radionuclides influenced by microorganisms will be essential for the risk assessment of repositories. Cm is a highly toxic radionuclide as indicated by the high α activity of some isotopes, such as ^{247}Cm (half-life: 1.6 x 10^7 years) and ^{248}Cm (half-life: 3.5 x 10^6 years) present in nuclear spent fuel.[7,8] Cm is a representative of trivalent actinides (An^{III}), which exhibits excellent luminescence properties that make it suitable for direct speciation studies at environmentally relevant metal concentration.[9] Similarly, europium (Eu) has been

studied as an inactive analogue of An^{III}, also providing excellent luminescence properties.[10]

Among other mechanisms, microbes can interact with actinides and lanthanides through the biosorption at cell surfaces.[11] A number of functional groups (*e.g.* carboxyl, phosphoryl) on microbial surfaces have been described to be effective for actinide complexation.[12,13] Cm^{III} and Eu^{III} form strong complexes with phosphoryl and carboxyl sites of the bacterial cell wall of *Sporomusa* sp. MT-2.99 and *Pseudomonas fluorescens.*[12,14] Recently, Yeasts and Archaea have also been investigated for their ability to complex An^{III} (*e.g.* Cm) and trivalent lanthanides (Ln^{III}) (*e.g.* Eu) through carboxyl and phosphate groups.[15,16] In addition, biofilm formation by microorganisms has to be considered, as it could lead to the immobilization of bioabsorbed radionuclides within the DGR system and consequently, could affect their integrity.

Since cell surfaces play a major role in the complexation of Cm^{III} and Eu^{III}, different spectroscopic and microscopic techniques can be used to investigate the contribution of functional groups and the corresponding mechanisms involved in the biosorption of these elements. Attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy, X-ray photoelectron spectroscopy (XPS), and time-resolved laser-induced fluorescence spectroscopy (TRLFS) are useful spectroscopic tools to determine the chemical speciation of these elements at environmentally relevant conditions. Potentiometric titrations have been used to determine types and abundance of active metal binding sites at the cell surface.[17,18] While a multidisciplinary approach combining different microscopic, spectroscopic, and potentiometric titration based methods is usually applied to investigate the interactions of U, as hexavalent actinide, with microbes,[13,19] the microbial interactions with Cm and Eu have only covered the use of TRLFS and potentiometric techniques.[12,14]

Spanish bentonite clays (Almeria, Spain) have shown to be excellent and suitable reference material of engineered barriers for DGRs due to their physico-chemical properties (low permeability, plasticity, high swelling pressure, thermal conductivity, etc.).[4] From these clays, *Stenotrophomonas bentonitica* has been isolated and well characterised,[20] and shown to influence the chemical speciation and mobility of other elements present in radioactive waste such as selenite (Se^{IV}) and U^{VI}.[4,21] However, the interactions between Eu^{III}/Cm^{III} and *S. bentonitica* have never been described before. For all mentioned above, the use of this strain as a model bentonite bacterial strain to investigate the impact of bentonite microbial population in the speciation of radionuclides within the concept of DGR is novel and could provide interesting results with regard to the biological, chemical and physical analysis that are currently undergoing to evaluate the DGR safety.

The present work studies the effect of *S. bentonitica* on the environmental fate of Eu^{III} and Cm^{III} under aerobic and anaerobic conditions, analogous to those expected in the geodisposal of radioactive waste. For this purpose, a combination of spectroscopic (ATR-FTIR, XPS, TRLFS) and microscopic (STEM-HAADF: Scanning Transmission Electron Microscopy-High Angle Annular Dark Field) techniques have been employed. This study will provide new insights on the influence of bentonite bacterial isolates in the immobilization of An^{III} within the concept of radioactive waste disposal, and will be useful to compare with other studies using elements such as Se and U. The safety of the DGR system have been well studied from a geological, chemical, and physical point of view, but not many studies have been conducted on the influence of microbiology. Therefore, this work is crucial to better understand how microbes can affect the safety of the disposal of such residues, which is a major environmental problem nowadays.

3. Materials and Methods

Experimental procedures related to preparation of Eu^{III} and Cm^{III} stock solutions, potentiometric titration of cell surfaces of *S. bentonitica* treated with Eu^{III}, Eu^{III} biosorption experiments, TRLFS experimental setup, and STEM-HAADF analysis are provided in the Supporting Information. Due to the hazardous nature and difficult handling of Cm^{III}, proper safety precautions and methodologies were employed in this study.

3.1. Bacterial strain and growth conditions

The bacterial strain used was isolated from bentonite clay formations recovered from Almeria (Spain),[4] and was recently described as a novel species named *Stenotrophomonas bentonitica* BII-R7^{T}.[22] The cells were grown aerobically in Luria-Bertani (LB) broth medium (tryptone 10 g/L, yeast extract 5 g/L and NaCl 10 g/L, pH 7.0 ± 0.2) at 28 ºC under agitation (180 rpm).

3.2. Attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy

S. bentonitica cells were suspended in a 30 µM Eu^{III} chloride solution ($EuCl_3 \cdot 6H_2O$) under aerobic conditions at pH 6. After 48 hours, the samples were collected by centrifugation (2700 x g; 10 min) and washed with 0.1 M $NaClO_4$. Finally the samples were lyophilized according to standard protocols.[17,23] Bacterial cells without addition of Eu^{III} were employed as controls.

ATR-FTIR measurements were performed on a Perkin Elmer Spectrum Two spectrometer, equipped with an ATR accessory, consisting of a diamond crystal at a fixed angle of 45º. 32 scans with spectral resolution 4 cm^{-1} and wavenumber range from

4000 to 400 cm^{-1} were collected for each sample. All measurements were performed in triplicate.

3.3. X-ray photoelectron spectroscopy (XPS)

Eu^{III}-treated cells of *S. bentonitica* were prepared as described in the section 1.3 of the Supporting Information. The obtained powder was mounted on standard sample studs using double-sided adhesive tape. Non-treated cells were prepared and used as controls. XPS measurements were made on a KRATOS SUPRA Photoelectron Spectrometer at 10 KV and 20 mA using a monochromatic Al Kα X-ray source (1486.6 eV). The take-off angle was fixed at 90º. On each sample the data were collected from three randomly selected locations, and the area corresponding to each acquisition was 400 µm in diameter. Each analysis consisted of a wide survey scan (pass energy 160 eV, 1.0 eV step size) and high-resolution scan (pass energy 20 eV, 0.1 eV step size) for component speciation. All experiments were conducted in triplicate. The binding energies of the peaks were determined using the C_{1s} peak at 284.5 eV. The software CasaXPS 2.3.17 was used to fit the XPS spectra peaks.[24]

3.4. Time-resolved laser-induced fluorescence spectroscopy (TRLFS) analyses

TRLFS measurements were performed in order to determine Eu^{III}/Cm^{III} species involved in interactions with the bacterial cells. Cells of *S. bentonitica* were brought into contact with 30 µM Eu^{III} both aerobically and anaerobically and with 0.3 µM Cm^{III} anaerobically, and collected as indicated in section 1.3 of the Supporting Information. The inactivity and hence easy handling of Eu^{III} allowed the TRLFS studies under both respiring conditions. The Cm^{III} experiments were performed anaerobically in a glove box in order to exclude carbonate complexation of Cm^{III} and for radiation protection

issues. The obtained pellets were washed and subsequently re-suspended in 5 mL of 0.1 M $NaClO_4$ for analysis by TRLFS. For Eu^{III}, the pH was kept constant at 6, while varying the incubation time (1, 24 and 48 h). For Cm^{III}, a pH dependent spectroscopic titration (pH 2.33 to 8.04) was carried out.

4. Results and discussion

4.1. Potentiometric titration studies

The potentiometric titrations curves of *S. bentonitica* BII-R7 before and after Eu^{III} exposure are presented in Figure S1. The concentration of deprotonated sites is standardized per mass of dry biomass (mol g^{-1}), and calculated according to Fein et al.[25] To calculate the acidity constants and the total concentration of each binding site, data from the titrations curves were fitted using ProtoFit 2.1 rev1,[26] using a Non-Electrostatic Model (NEM). It has been demonstrated that electrostatic treatments, such as diffuse layer and triple layer electrostatic models to titration data, greatly over-predict the effect of ionic strength on bacterial surface protonation reactions, resulting in poorer fits and more variability in stability constants than non-electrostatic models.[27,28]

The titrated bacterial suspensions exhibited a protonation-deprotonation behaviour over the whole pH range studied (Figure S1). The shape of the titrations curves obtained suggested the presence of functional groups with close acid-base pK_a values, showing that although some small variability could be perceived in each set of the same bacterial sample, essentially reproducible results were obtained (the variation between the titration curves was below 6% of $[H^+]_{exchanged}$ between pH 3.5 and 10.0). Although a small hysteresis could be observed between acid and base titrations at the same ionic

strength, results from reverse titrations did not vary strongly and suggested a reversible proton adsorption/desorption reaction.

Table S1 summarizes the pK_a values for *S. bentonitica* before and after Eu^{III} exposure. The calculated values were 4.97 ± 0.08 and 4.78 ± 0.06 for pK_1, 6.88 ± 0.02 and 6.75 ± 0.13 for pK_2, and 9.43 ± 0.02 and 9.48 ± 0.11 for pK_3. The obtained pK_a values are representative of carboxylic groups for pK_1, phosphate groups for pK_2 and amine and hydroxyl groups for pK_3.[17,18,25,29–32]

The existence of pH zero proton charge (pH_{zpc}) indicated that *S. bentonitica* developed a positive net charge at low pH values, indicating the presence of at least one positively ionising, plausibly amino group. Models which only include negatively ionising groups such as carboxyl, phosphoryl and hydroxyl groups could not develop a net positive charge at low pH.[33] The pH_{zpc} around 5.7 also indicated that the cells were negatively charged at neutral pH = 7 and electrostatic attraction with positive-charged mineral surfaces or metals is favourable.

The surface site densities obtained using ProtoFit are also presented in Table S1. The pK_a values for bacterial samples with and without Eu^{III} were comparable, indicating similar concentration of the active functional groups on the cell wall. One exception was found, the concentrations corresponding to phosphate groups (C_2) was significantly lower for *S. bentonitica* cells exposed to Eu^{III}. This could suggest a strong affinity of Eu^{III} to phosphate sites, making them inaccessible to the protonation/deprotonation reaction. The considerable high concentration of phosphate groups at the surface of *S. bentonitica* (10.78 ± 0.31 x 10^{-4} mol/g) comparing with other bacterial species such as *Sporomusa* sp. MT-2.99 (5.30 ± 0.8 x 10^{-4} mol/g), *Sphingomonas* sp. S15-S1 (3.16 ± 0.56 x 10^{-4} mol/g), or *B. sphaericus* JG-7B (2.19 ± 0.25 x 10^{-4} mol/g)[12,34] (Table S1) pointed out the potentially high metal-binding ability of *S. bentonitica*.

The results of potentiometric titration experiments indicated that the cell surface groups capable for metal binding sites could involve carboxyl groups (pK around 3-5), phosphate groups (pK around 6-7), and hydroxyl and amine groups (pK > 8). These findings are in agreement with previous studies on bacterial surfaces.[17,35,36] Liu et al.[36] demonstrated the role of carboxyl, phosphoryl, and amino functional groups of *Synechococcus* sp. PCC 7002 cells as metal surface ligands by means of potentiometric titrations. In the case of Eu^{III} and Cm^{III}, their sorption on the cell envelope of *Sporomusa* sp. MT-2.99, *B. subtilis* and *P. fluorescens* can be due to their coordination with carboxyl and phosphate groups.[12,14,37,38] Consequently, phosphate and carboxyl groups of *S. bentonitica* might be expected to be involved in the binding of Eu^{III}. However, the potentiometric results only showed phosphate groups as the main potential binding sites in the pH range studied due to their high surface concentration. It is probable that the extent of the carboxyl group involvement in the Eu^{III} binding is either too small to be detected by titration methods, or the sorption/desorption of Eu^{III} by the carboxylic groups is reversible at low pH (possible exchange between Eu(III) and protons at low pH for the carboxylate groups).

4.2. Eu^{III} removal capacity of *S. bentonitica* over time

These studies were carried out to estimate the Eu^{III} removal capacity of *S. bentonitica* with increasing time under aerobic and anaerobic conditions. The maximum amount of Eu^{III} removal was 12.9 ± 0.11 mg of Eu/g of dry biomass after 96 h of aerobic incubation (Figure S2). This amount corresponds to a 54 ± 0.44 % of Eu^{III} removed from the total amount of Eu in the solution. Higher values were obtained by Bader et al. (2019)[16] in their bioassociation kinetics studies with the halophilic archaeon *Halobacterium noricense* DSM15987^{T} by using the same Eu^{III} initial concentration (30

245 μM). They found that around 73% of Eu^{III} was removed after 1 week of incubation.
246 Under anaerobic conditions, the maximum amount of Eu^{III} removal was 6.06 ± 0.25 mg
247 of Eu/g of dry biomass after 18 h incubation (Figure S2), which corresponds to a 31.2 ±
248 1.3 % of Eu^{III} removal. The Eu^{III} removal improved by increasing contact time of
249 incubation until equilibrium was attained under both conditions. However, these results
250 clearly showed that *S. bentonitica* cells have a higher removal capacity under aerobic
251 conditions. This could be a consequence of the more stressful anoxic conditions,
252 probably affecting the bacterial interaction process.
253 These results suggested that the interaction was mediated not only by biosorption, since
254 this mechanism is generally defined as a quick process ocurring up to a few hours.[39]
255 More specifically, the Eu^{III} removal studies showed that time-dependent Eu interaction
256 with the cells could be a biphasic process. First, a rapid phase where 12.5 ± 0.73 and
257 13.9 ± 1 % of Eu^{III} removal was achieved (aerobically and anaerobically, respectively)
258 within the first 2 h (Figure S2). This fast phase is usually associated to metabolic
259 independent biosorption mechanisms. Secondly, a slow phase seems to occur, where
260 Eu^{III} accumulation process seems to reach equilibrium after 24 h. This phase could be
261 controled by metabolically dependent interaction mechanisms such as intracellular
262 accumulation or bioprecipitation, among others.
263
264 4.3. Characterization of Eu^{III}-*S. bentonitica* interactions using ATR-FTIR
265 Figure 1 shows the ATR-FTIR spectra obtained from *S. bentonitica* after 48 h
266 incubation with 30 μM Eu^{III} solution. The observed infrared bands confirmed the
267 presence of proteins, lipids, polysaccharides, and polyphosphate groups. The functional
268 groups assigned to the infrared bands and the corresponding frequencies for the
269 bacterial cells are summarised in Table S2.

The region between 3000 and 2800 cm^{-1} exhibited the typical C-H stretching vibrations (ν_{C-H}) corresponding to the CH_3 and $>CH_2$ functional groups present in the fatty acids and lipids, and the O-H stretching band (ν_{O-H}) corresponding to the presence of hydroxyl groups in bacterial cells. Complementary information could be found at the region between 1800 and 750 cm^{-1}, where vibrations of C-H, $>CH_2$ and $-CH_3$ groups, amides, carbonyl groups and polysaccharides were observed. The peaks observed at 1308 and 1455 cm^{-1} could be attributed to the bending of $-CH_3$ and $>CH_2$ of proteins (δ_{CH2}, δ_{CH3}), and the signals at 1635 and 1535 cm^{-1} corresponded to the amide I and II bands, respectively. The amide I band was due to the stretching C=O ($\nu_{C=O}$) of amides associated with proteins and the amide II band was actually a combination of bending N-H (δ_{N-H}) of amides and contributions from stretching C-N (ν_{C-N}) groups. The peak at 1455 cm^{-1} also concealed the amine III group. The peak around 1404 cm^{-1} was due to the symmetric stretching C-O of carboxylate groups ($\nu_{sym\ COO^-}$), and the peak corresponding to the asymmetric stretching vibration ($\nu_{asym\ COO^-}$) was concealed by the amide II band at 1535 cm^{-1}. A small shoulder around 1745 cm^{-1} was a combination of two peaks: a signal corresponding to the vibrational C=O stretching ($\nu_{C=O}$) of carboxylic acids at 1747 cm^{-1} and another peak corresponding to the stretching C=O of ester functional groups from membrane lipids and fatty acids at 1730 cm^{-1}.[17,30,31,40] The double bond stretching of >P=O of general phosphoryl groups and phosphodiester of nucleic acids could be observed at 1240 cm^{-1}. The stretching of P=O groups of polyphosphate products, nucleic acid phosphodiester and phosphorylated proteins can be found around 1070 cm^{-1}, and the peak at 933 cm^{-1} showed the asymmetric O-P-O stretching modes.[30,31,40]

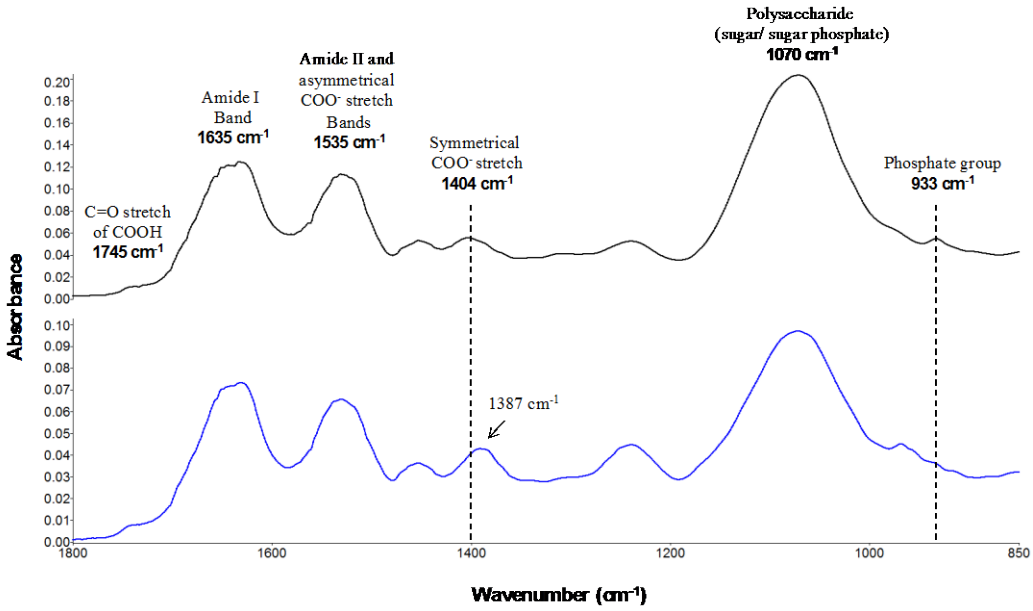


Figure 1. Comparison between the ATR-FTIR spectra for *S. bentonitica* cell suspensions in 0.1 M $NaClO_4$ (electrolyte) solution only (top, in black color) and in 30 µM Eu^{III} solution + electrolyte (bottom, in blue color) after 48 hours.

The ATR-FTIR spectra showed a shift in the band attributed to symmetric stretching of carboxylate groups (around 1404 cm^{-1}) to lower frequencies, when compared to the spectra of the cells in background electrolyte (Figure 1). Extensive studies made on metal complexes of carboxylic acids have established an empirical correlation between the position of the symmetric stretching ($\nu_{sym\ COO^-}$) and asymmetric stretching ($\nu_{asym\ COO}$) of carboxylate groups and the difference in frequency between them ($\Delta\nu$). The values of $\Delta\nu$ descend in the follow order: $\Delta\nu_{unidentate} > \Delta\nu_{brinding} \sim \Delta\nu_{free\ ionic} > \Delta\nu_{chelate(bidentate)}$.[41–44] Chu et al.[41] and Deacon and Phillips,[44] after careful examinations of IR spectra of many acetates with known X-ray crystal structures, arrived at the conclusion that: i) for unidentates complexes, $\Delta\nu > 200$ cm^{-1} and the position of $\nu_{sym\ COO^-}$ is generally shifted to lower frequencies; ii) for bidentate chelating complexes, $\Delta\nu < 100$ cm^{-1} and the position of $\nu_{sym\ COO^-}$ is shifted to higher frequencies, whereas $\nu_{asym\ COO^-}$ is shifted to

lower frequencies; and iii) for bidentate bridging complexes, $\Delta\nu \sim 160$ cm^{-1} and the position of $\nu_{sym\ COO^-}$ and $\nu_{asym\ COO^-}$ can shift in either direction.[41] The symmetric stretching ($\nu_{sym\ COO^-}$) band for *S. bentonitica* in contact with Eu^{III} shifted to lower frequencies by ~15 cm^{-1}, but, as can be observed in Figure 1 and Table S2, the asymmetric stretching (ν_{asym} COO^-) of carboxylate groups was hidden by the amide II band, and therefore it is difficult to determine if there was a shift in this band to higher or lower frequencies. Based purely on the position of $\nu_{sym\ COO^-}$ shifting to lower frequencies, the carboxyl functional groups could form unidentate complexes with the Eu^{III} metals. If the asymmetric stretching ($\nu_{asym\ COO^-}$) of carboxylate groups (hidden by the amide II band) did not shift, then $\Delta\nu$ would be around 150 cm^{-1}, suggesting that the carboxyl functional groups arising from the macromolecules of the cell wall of the bacterial cells could form bidentate bridging complexes with the Eu^{III} metals. However, further studies would be needed as there is no evidence of the frequency of the asymmetric $\nu(COO^-)$ mode. EXAFS analysis could provide more detailed information about the local coordination of Eu associated with these cells, but it falls beyond the main scope of this study. This would provide more unequivocal indications of the ability of bentonite-isolated bacteria to interact with Eu in a unidentate or bidentate bridging mode. Nevertheless, these results provide further verification that carboxyl functional groups from the macromolecues of the bacterial cells are responsible in forming organo-metallic complexes with the Eu^{III} metals, as also reported by the potentiometric and luminiscence results.

In addition, the ATR-FTIR spectra indicated that phospholipids might also be involved in the cell-metal complexation. The lower intensity of the band found at 933 cm^{-1} of Eu^{III}-treated cells compare with Eu^{III}-untreated cells suggests phosphate groups as candidates for Eu^{III} complexation.[23]

335 4.4. Characterization of Eu^{III}-*S. bentonitica* interactions using XPS.

336 This method was applied to determine the local coordination of Eu^{III} at the cell surface
337 of *S. bentonitica* approximately up to 5 nm.[45] The elemental composition of the *S.*
338 *bentonitica* surface, resulting from integrating the C_{1s}, O_{1s}, N_{1s} and P_{2p} from the wide
339 scan spectrum can be seen in Figure 2A-D. Sodium and chlorine were also detected as
340 samples were washed with 0.1 M $NaClO_4$. Eu was detected in the bacterial sample in
341 contact with a 30 µM Eu^{III} solution for 48 hours. Nitrogen appeared at a binding energy
342 of 399.99 eV, attributable to amine or amide groups of proteins.[23,46–49] Phosphorus was
343 found at a binding energy of 133.99 eV, and can be attributed to phosphate groups.[47–49]
344 The presence of amine groups from proteins and phosphate groups based on the binding
345 energies of N_{1s} and P_{2p} are in agreement with the results from potentiometric titrations
346 (pK_a = 6.8 and pK_a = 9.4) and the FTIR spectra (adsorption bands at 1635 cm^{-1}, 1535
347 cm^{-1}, and 933 cm^{-1}).

348 XPS peaks corresponding to Eu_{3d} were also analysed at high resolution to assess the
349 nature of the Eu^{III} complex and shown in Figure 2D. The local coordination of Eu
350 associated to the cells of the studied strain, observed at 1135 eV, is similar to that of Eu-
351 acetate as was described by Mercier et al.[50] This suggests that carboxyl groups
352 containing cell wall molecules like glutamic acid of peptidoglycan are involved in the
353 Eu binding. Previous studies showed the role of carboxyl groups from glutamic and
354 aspartic acid present in proteins of the S-layer of *B. sphaericus* in the complexation of
355 uranium and palladium.[51,52] Therefore, carboxyl groups of the glutamic acid of the
356 peptidoglycan (PG) layer of *S. bentonitica* could be involved in the interaction of Eu^{III}.

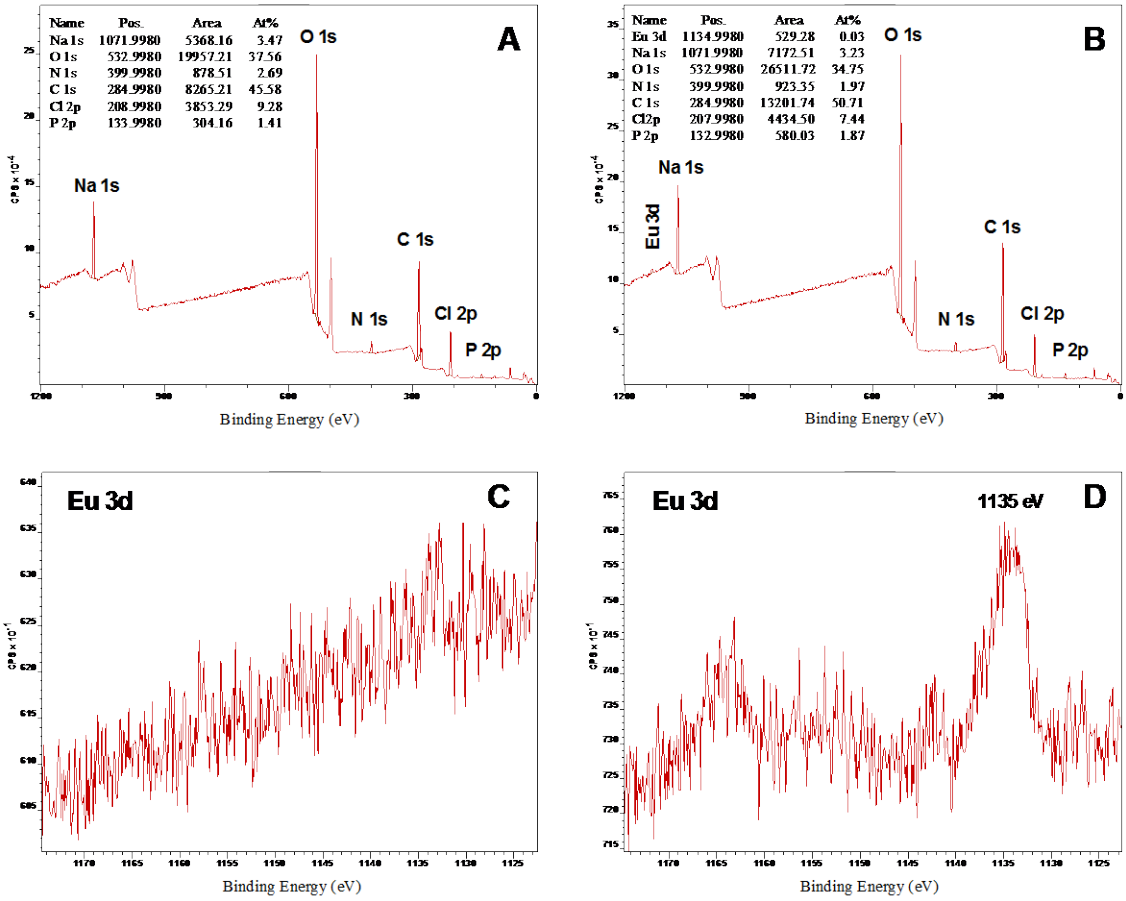


Figure 2. XPS spectra of *S. bentonitica* in absence (A, C) and presence of 30 µM Eu^{III} (B, D). High-resolution spectra of the region belonging to Eu 3d (C and D).

4.5. TRLFS characterization of Eu^{III}/Cm^{III} interaction with *S. bentonitica*

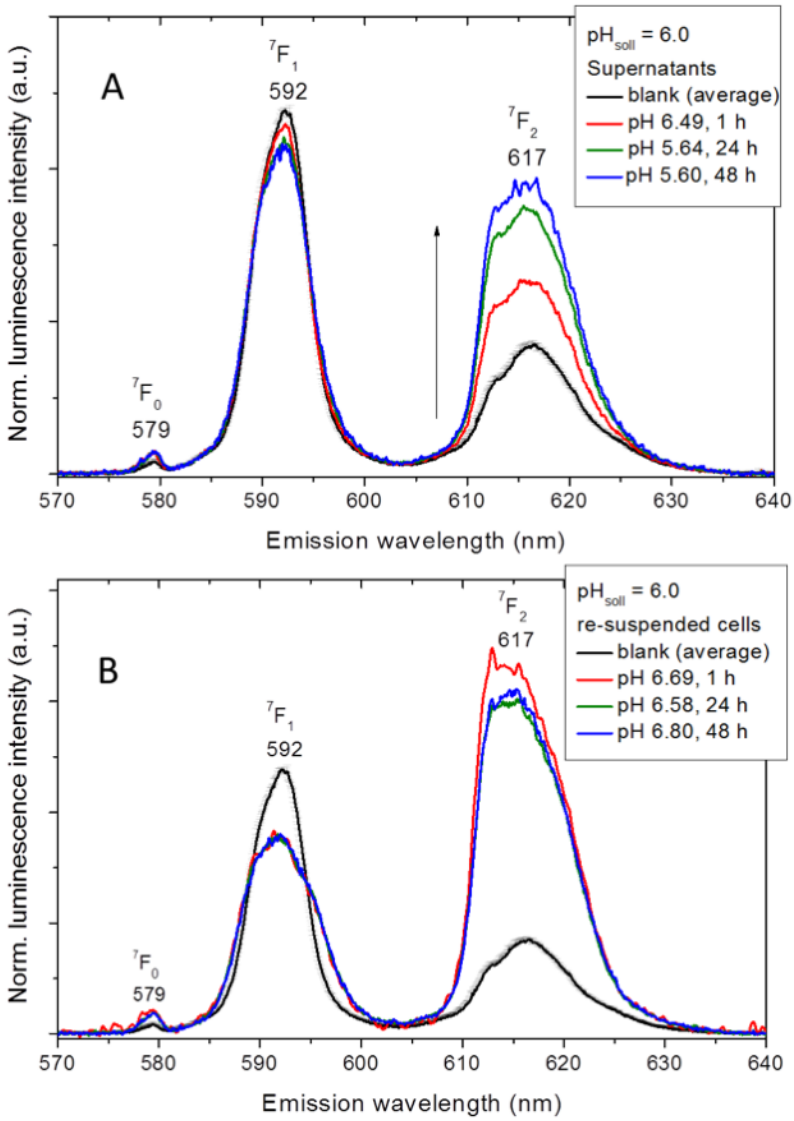
Potentiometric titrations, ATR-FTIR, and XPS studies showed the involvement of phosphate and carboxyl groups in the coordination of Eu^{III} by the *S. bentonitica* cells. In addition, Eu^{III} and Cm^{III} were used as luminescence probes to investigate Cm^{III}/Eu^{III} binding on *S. bentonitica* based on changes of the intrinsic luminescence properties due to microbial interaction. The studies with Cm^{III}, radioactive analogue of Eu^{III}, were carried out at much lower concentrations relevant to environmental conditions (0.3 µM).

370 *4.5.1. Europium*

371 The luminescence spectra depicted in Figure 3 showed the interaction of Eu^{III} with *S.*
372 *bentonitica* through typical changes as result of cell addition after 1, 24 and 48 h of
373 anaerobic incubation at pH 6 in both, supernatants and re-suspended cells. This
374 suggested the complexation of Eu^{III} with extracellular released complexing agents and
375 bacterial surface functional groups, respectively. In the supernatant and the re-
376 suspended cells the $^7F_0$ transition appeared at 579 nm with a slightly higher intensity
377 than in the blank sample. This pointed to a different symmetry around the Eu^{III} center
378 compared to the blank spectrum and is a further argument for interaction process of
379 Eu^{III} with the cells. The luminescence spectrum of Eu^{III} aqua ion (blank) is
380 characterized by emission bands at 585-600 nm (magnetic dipole transition $^5D_0 \rightarrow {}^7F_1$)
381 and 610-630 nm (hypersensitive transition $^5D_0 \rightarrow {}^7F_2$). An increased intensity of the
382 hypersensitive $^7F_2$ transition at 617 nm moving from blank via supernatant to the re-
383 suspended cells was discovered.

384 In the supernatants, there was a systematic increase in the $^7F_2$ transition as a function of
385 the incubation time (Figure 3A). This could indicate an increase in the release of
386 complexing agents from the cells at a longer incubation time. Total organic carbon
387 (TOC) content of the supernatant samples increased after 24 h of incubation (Figure
388 S3), suggesting the release of complexing substances from the cells. These results are in
389 agreement with the Eu^{III} removal studies (section 4.2), in which the amount of Eu
390 adsorbed increased with the incubation time. In the re-suspended cells, there was a fast
391 rise in the intensity of the $^7F_2$ transition after an incubation time of 1 h. Then, no
392 systematic changes in the spectra, as a function of the incubation time, were observed
393 (Figure 3B).

394



395

396 **Figure 3.** Luminescence emission spectra of 30 µM Eu^{III} measured for the supernatants
397 after separating the *S. bentonitica* cells (0.2 g/L) (A) and the re-suspended cells (B)
398 under anaerobic conditions at pH 6 and different incubation times (1, 24 and 48 hours)
399 in 1 M $NaClO_4$.

400

401 In supernatants and re-suspended cells, Eu^{III} appeared in two different coordination
402 environments. The short-lived component in supernatants was measured at 117, 129 and
403 133 µs after 1, 24, and 48 h, respectively (Table 1). These lifetimes indicated a similar

coordination environment after 24 and 48 h. Those Eu^{III}-species containing approximately eight water molecules and one binding site will be filled by functionalities of the released substances. The luminescence lifetime of 114 ± 5 µs corresponding to 8.8 ± 0.5 coordinated water molecules found in the blank was characteristic to the Eu^{3+} ion. The short lifetime of 117 µs found in the supernatant after 1 h of incubation indicated the presence of the Eu^{III} ion. The longer lifetimes, 387 to 500 µs, could indicate an interaction of Eu^{III} with released substances from the cells independently from the incubation time. In this second type of Eu^{III} complex only up to 1 to 2 water molecules remained. In the case of re-suspended cells, a bi-exponential luminescence decay was measured indicating two coordination environments of Eu^{III}. The short-lived component showed luminescence lifetimes between 144 and 225 µs (7 and 4 coordinated water molecules, respectively), whereas the long-lived component varied between 477 and 609 µs (2 and 1 coordinated water molecules, respectively). In a first approximation, similar Eu^{III} species were formed for the short-lived component of the supernatant and re-suspended cells. In the same way, the long-lived component of both supernatant and re-suspended cells suggested a similar coordination environment but different from the one found for the short-lived component.

By comparing our lifetime results with literature data, phosphoryl and carboxyl groups present on bacterial cell envelopes and bacterial released substances seem to play an important role in the Eu^{III} coordination sites characterized by, for instance, their individual luminescence lifetimes, probably in form of R-O-PO_3-Eu^{2+} (R-O-PO_3H-Eu^{2+} under acidic pH conditions) and R-COO-Eu^{2+} as revealed by previous studies (Table 1).[12,53] Specifically, the Eu^{III}-*S. bentonitica* complexes seem to have similar properties as the surface species R-O-PO_3H-Eu^{2+}observed on cell envelopes of *Sporomusa* sp. MT-2.99 as revealed by the long lifetimes.[12] The coordination site characterized by

429 short lifetimes seem to interact with Eu^{III} with similar properties as the surface species
430 R-COO-Eu^{2+} observed on cell envelopes of *Sporomusa* sp. MT-2.99 and *P.*
431 *fluorescence.*[12] It is important to note that the results presented here were very similar to
432 those obtained aerobically and have comparable significance (Figure S4).
433

434 **Table 1.** Spectroscopic properties obtained from the Eu^{III}-*S. bentonitica* system at pH 6
435 using different incubation times and other relevant model systems.

Sample	**$R_{E/M}$**	**Lifetime (µs)**	**Proposed species**	**Reference**
Eu^{III} control	0.50 ± 0.05	114 ± 5	Eu^{3+}	This work
Supernatants				This work
Eu^{III}-*S. bentonitica*				
1 h incubation	0.9	117; 387	Eu^{3+}; phosphoryl sites	
24 h incubation	1.2	129; 490	Carboxyl; phosphoryl	
48 h incubation	1.3	133; 500	Carboxyl; phosphoryl	
Cells				This work
Eu^{III}-*S. bentonitica*				

1 h incubation	2.3	144; 477	Carboxyl; phosphoryl sites	
24 h incubation	2.1	174; 561	Carboxyl; phosphoryl	
48 h incubation	2.1	225; 609	Carboxyl; phosphoryl	
Eu^{III}-*Sporomusa* sp. MT-2.99	3.3	170	R-COO-Eu^{2+}	Moll et al.[12]
	1.8	515	R-O-PO_3H-Eu^{2+}	
Eu^{III}-*Bacillus subtilis*		230	Carboxyl sites	Markai et al.[54]
		730	Phosphoryl sites	
Eu^{III}-*Pseudomonas aeruginosa*		98-254	Carboxyl sites	Texier et al.[53]
		534-677	Phosphoryl sites	

4.5.2. Curium

The chemical speciation of Cm^{III} with *S. bentonitica* cells was studied at trace (0.3 μM) Cm^{III} concentrations by TRLFS. These measurements were conducted assuming that the influence of the luminescence properties of the microbial Cm^{III}-species dominates over the influence of soluble Cm^{III}-species with, for instance, released complexing agents.

The pH-dependent spectroscopic Cm^{III} speciation in the cell suspensions is shown in Figure 4.

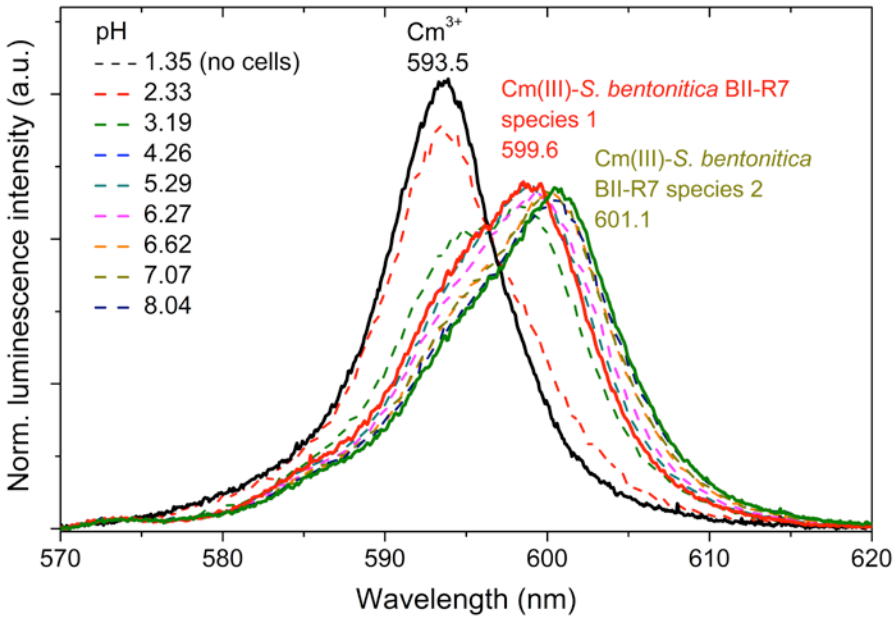


Figure 4. Luminescence emission spectra of 0.3 µM Cm^{III} in 0.1M $NaClO_4$ measured as a function of pH at a fixed biomass concentration of 0.2 $g_{dry\ weight}$/L.

From the dependencies found in the TRLFS spectra, it can be concluded that there are two coordination environments of Cm^{III} due to interactions with functional groups of the cell surface and possibly with released complexing agents. Thus, the Hypspec analysis of the pH dependent emission spectra measurements revealed two Cm^{III} bacterial species (Figure 4). Cm^{III}-*S. bentonitica* species 1 was characterized by an emission maximum at 599.6 nm while Cm^{III}-*S. bentonitica* species 2 showed a more red shifted emission maximum at 601.1 nm. The extracted single component spectra of both species are shown in Figure 4. TRLFS of the supernatants and the Cm^{III} loaded biomass after washing with 0.1M $NaClO_4$ showed that 73% of the detected Cm^{III} luminescence intensity remained in solution at pH 8.04, while, only 23% was associated to the biomass. This evidence indicated that a complexation of Cm^{III} by substances released

from the cells was occurring. In all samples containing cells, a bi-exponential luminescence decay was detected (Table S3). At pH 3.2 the short lifetime of 71 µs points to uncomplexed Cm^{3+}. Between pH 4 and 8 both lifetimes amounted to 120 ± 8 and 290 ± 23 µs corresponding to 5 and 2 coordinated water molecules, respectively. By comparing our results with the ones reported in literature, a close agreement was found to the study of Lopez-Fernandez et al.[15] The long lifetime and the corresponding emission maximum matches with Cm^{III} interactions with microbial phosphoryl sites, whereas the short lifetime can be attributed to carboxyl interactions of Cm^{III}.

4.6. Cellular localization of Eu^{III} by STEM-HAADF (Scanning Transmission Electron Microscopy-High Angle Annular Dark Field).

STEM-HAADF micrographs of thin sections of *S. bentonitica* cells exposed to Eu^{III} revealed the presence of electron-dense accumulations, mainly at the cell surface (Figure 5A-D) under both aerobic and anaerobic conditions. In addition, very few extracellular (Figure 5A-D) and intracellular (Figure 5D) accumulations were observed. EDX analysis (Figure 5E-F) and element-distribution mapping (Figure 6) of these accumulations indicated a main composition of Eu and P. The detection of P in the EDX analysis of the Eu^{III} precipitates also confirmed the key role of functional groups containing phosphorus in their interaction with Eu^{III}. These results showed biosorption of Eu^{III} as the main interaction mechanism with the cells of *S. bentonitica*. However, the presence of few extracellular and intracellular Eu^{III} precipitates indicated that the interaction is not only mediated by biosorption, thus other processes, such as bioaccumulation and bioprecipitation, could also occur. This matches very well with the Eu^{III} removal studies (section 4.2.) suggesting the implication of other interaction mechanisms.

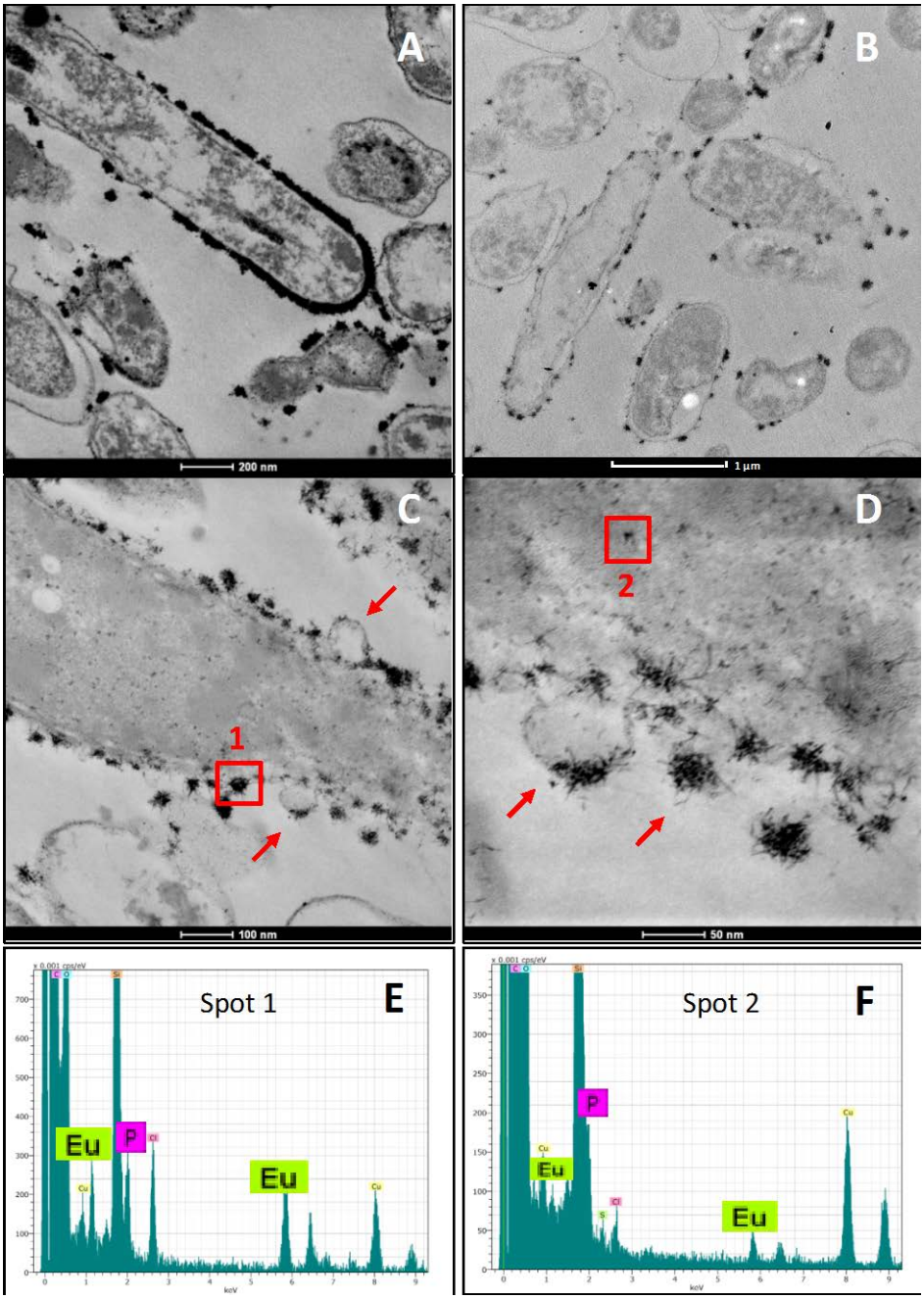


Figure 5. STEM-HAADF micrographs showing electron-dense accumulations at the cell surface, extracellular, and intracellularly under aerobic (A-B) and anaerobic conditions (C-D). EDX analysis (E-F) confirming the Eu and P composition of the accumulations. The formation of vesicles by *S. bentonitica* cells is indicated by arrows (C-D). Scale bars: 200 nm (A), 1µm (B), 100 nm (C), 50 nm (D).

The formation of outer membrane vesicles (OMVs) by *S. bentonitica cells* exposed to Eu^{III} was observed in the STEM-HAADF micrographs (Figure 5C-D). The production of OMVs by Gram-negative bacteria plays a prominent role in cell protection against hostile environments.[55,56] Morever, it represents a mechanism to alleviate stress through the packaging and release of stress-products.[57] Therefore, the vesicle formation mechanism of *S. bentonitica* cells could be involved in their Eu^{III} tolerance. In addition, the detection of extracellular precipitates could be a consequence of the release of intracellular accumulates through the formation of vesicles. However, further investigations are needed to confirm this hypothesis. On the other side, intracellullar accumulation could be a consequence of a passive process associated to damage of the cell membrane permeability, since Eu do not play any biological function and the cells do not have a specific transport system for the uptake of this element. The explanation of how and why elements such as Eu are accumulated in the cytoplasm of some microorganisms remains unknown.

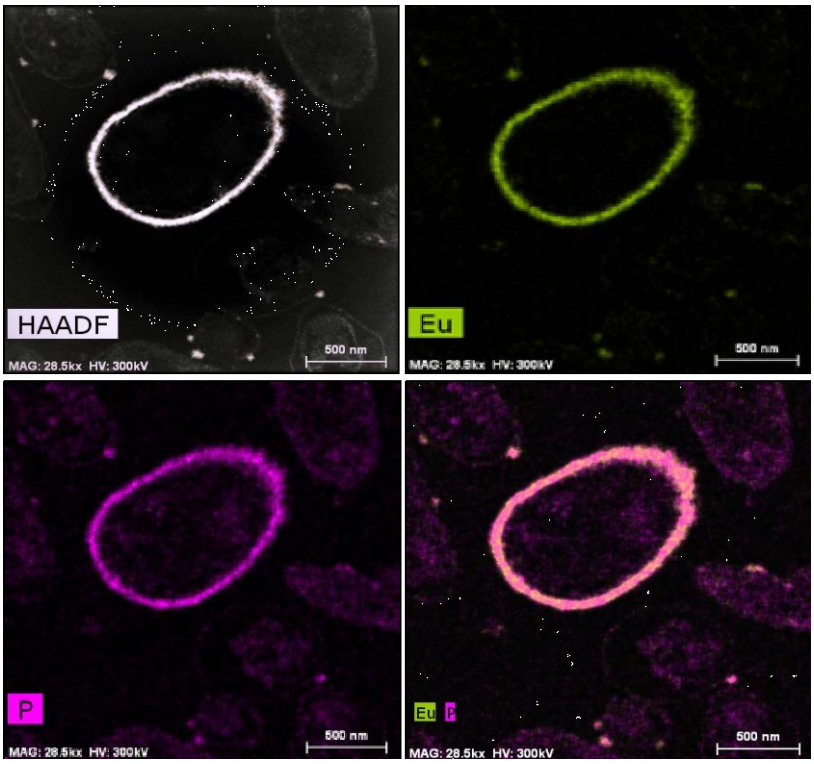


Figure 6. STEM-HAADF micrographs of thin sections showing the adsorption of Eu^{III} on a *S. bentonitica* cell after 48 h in contact with 30 µM Eu^{III} solution. Scale bars: 500 nm.

4.7. Environmental implications.

The safety of the DGR system have been well studied from a geological, chemical, and physical point of view but, very few works have investigated the impact of microbial processes in the safety of this disposal option. It is well known that microbe occurring in different DGR barriers, including bentonites, could affect the safety of a DGR through: 1) corrosion of metal containers, 2) transformation and alteration of bentonite minerals, 3) gas production, and 4) mobilization of radionuclides present in the system, such as curium, selenium, or uranium. Here we reported a clear effect of the activity of the bentonite bacterial isolate *S. bentonitica*, on the speciation and mobility of trivalent actinides such as Cm^{III} and its inactive analogue Eu^{III}.

A multidisciplinary approach combining microscopy, spectroscopy, and potentiometric titration based methods allowed us to provide new insights on the speciation of Cm and Eu asociated with bacterial strains (isolated from one of the most important arificial barriers, bentonites, of future DGR). The results obtained revealed that carboxyl and phosporyl groups from bacterial envelopes and other extracellularly released complexing agents seem to be involved in the interaction with Eu and Cm. Specifically, XPS analysis suggested that these carboxyl groups could arise from macromolecules located at the cell surface such as glutamic acids of the peptidoglycan layer, which could be involved in the complexation of Eu^{III}. In addition, ATR-FTIR suggested that the coordination of Eu^{III} with carboxyl groups from the bacterial cell wall could occur in a bidendate bridging mode. Finally, TEM analysis, in combination with the rest of the techniques, suggested that the Eu/Cm-bacteria interaction most probably occur through several microbial processes such as biosorption, intracellular accumulation, and biomineralization. The results here reported clearly suggested that *S. bentonitica* could influence the speciation and hence mobility of Eu and Cm, afeccting the safety of the DGR system.

Biosorption and bioaccumulation may enable the metal removal from contaminated aqueous solutions through the immobilization of bacterial biomass to inert supports,[58] which are nowadays receiving attention for bioremediation purposes. The immobilization of microorganisms in minerals from bentonites and other materials through the formation of biofilms could lead to the immobilization of bioadsorbed or bioaccumulated radionuclides. Indeed, genes coding for the formation of biofilms such as those involved in the formation of surface structures (*flhA, flhB, fliR, fliQ, fliP, fliN, fliM*) [59] or those encoding outer-membrane lipoproteins (*slp*) [60] have been reported to be

546 present in the genome of *S. bentonitica* [61] (GenBank accession number
547 MKCZ00000000). In addition, the production of flagella-like proteins by this bacterium
548 could be involved in the formation of biofilms. Clark et al.[62] demonstrated the role of
549 flagella-like filaments produced by *Desulfovibrio vulgaris* in the establishment and
550 maintenance of biofilms between cells and silica oxide surfaces. Therefore, *S.*
551 *bentonitica* could positively influence the safety of repositories by inducing the
552 immobilization of radionuclides through the biofilm formation.
553 In addition to biosorption and bioaccumulation, a long-term bioprecipitation process
554 could be involved as suggested by the extracellular Eu precipitates observed by STEM-
555 HAADF. Bioprecipitation basically leads to the inmobilization of radionuclides since it
556 is based on the conversion from soluble to insoluble forms through their precipitation
557 with released cell ligands (carbonates, phosphates, etc.).[11,63] From all mentioned above,
558 the present study could be really helpful to better understand how microbes affect the
559 safety of the disposal of radioactive residues, which is a global environmental concern
560 nowadays.
561

562 **5. Conflicts of interest**

563 The authors declare no competing financial interest.
564

565 **6. Acknowledgements**

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7. Supporting information

The supporting information includes 5 sections of methodology, 4 figures and 3 tables in a separate file.

1. Supplementary materials and methods

1.1. Preparation of Eu^{III} and Cm^{III} stock solutions

1.2. Potentiometric titration of cell surfaces of *S. bentonitica* treated with Eu^{III}

1.3. Eu^{III} biosorption experiments

1.4. TRLFS experimental setup

1.5. STEM-HAADF analysis

2. Supplementary figures and tables

Figure S1. Representation of the potentiometric titrations of *S. bentonitica* in 0.1 M $NaClO_4$ suspension (A) and in contact with 30 µM Eu^{III} solution (B) after 48 hours of incubation, compared with the background electrolyte. Closed symbols correspond to the forward titration data and open symbols correspond to back titration.

Figure S2. Time dependence in the Eu^{III} removal capacity of *S. bentonitica* cells under aerobic (A and B) and anaerobic (C and D) conditions. The Eu^{III} removal is expressed as mg of Eu per g of dry biomass (A and B) and percentage (B and D).

596 Figure S3. Total organic carbon as a function of time of supernatants obtained after
597 Eu^{III}-*S. bentonitica* interaction under anaerobic conditions.

598 Figure S4. Luminescence emission spectra of 30 µM Eu^{III} measured for the supernatants
599 after separating the *S. bentonitica* cells (0.2 g/L) and the re-suspended cells under
600 aerobic conditions at pH 6 and 24 h incubation in 1 M $NaClO_4$ (A). Spectroscopic
601 properties obtained from the Eu^{III}-*S. bentonitica* system (B).

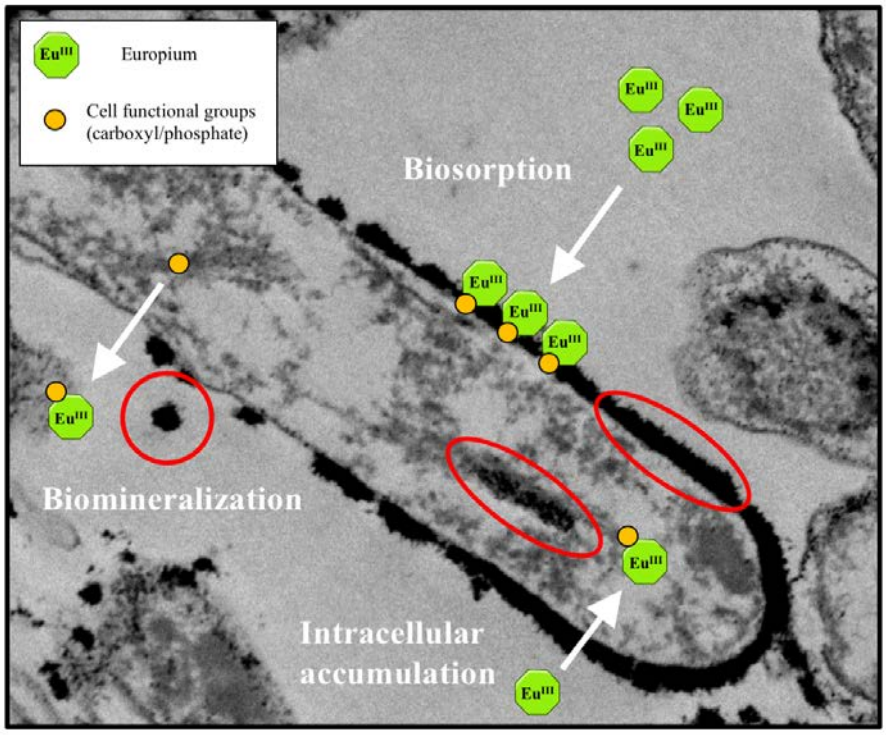
602 Table S1. Comparison of deprotonation constants and surface site concentrations for *S.*
603 *bentonitica* and other strains from different studies.

604 Table S2. Main infrared absorption bands of the bacterial cell functional groups

605 Table S3. Luminescence emission data of the Cm^{III}-*S. bentonitica* system including
606 those of relevant model systems for comparison.

607

608 **8. Table of content (TOC)/Abstract art**



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