

**Presence of uranium(V) during uranium(VI) reduction by  
Desulfosporosinus hippei DSM 8344T**

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1 Presence of uranium(V) during uranium(VI) reduction by *Desul-*  
2 *fosporosinus hippei* DSM 8344<sup>T</sup>

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18 Key words:

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20 uranium, membrane vesicles

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26

27 **Abstract**

28 Microbial U(VI) reduction influences uranium mobility in contaminated subsurface envi-  
29 ronments and can affect the disposal of high-level radioactive waste by transforming the  
30 water-soluble U(VI) to less mobile U(IV). The reduction of U(VI) by the sulfate-reducing  
31 bacterium *Desulfosporosinus hippei* DSM 8344<sup>T</sup>, a close phylogenetic relative to naturally  
32 occurring microorganism present in clay rock and bentonite, was investigated. *D. hippei*  
33 DSM 8344<sup>T</sup> showed a relatively fast removal of uranium from the supernatants in artificial  
34 Opalinus Clay pore water, but no removal in 30 mM bicarbonate solution. Combined spe-  
35 ciation calculations and luminescence spectroscopic investigations showed the depend-  
36 ence of U(VI) reduction on the initial U(VI) species. Scanning transmission electron mi-  
37 croscopy coupled with energy-dispersive X-ray spectroscopy showed uranium-contain-  
38 ing aggregates on the cell surface and some membrane vesicles. By combining different  
39 spectroscopic techniques, including UV/Vis spectroscopy, as well as uranium M<sub>4</sub>-edge X-  
40 ray absorption near-edge structure recorded in high-energy-resolution fluorescence-de-  
41 tection mode and extended X-ray absorption fine structure analysis, the partial reduction  
42 of U(VI) could be verified, whereby the formed U(IV) product has an unknown structure.  
43 Furthermore, the U M<sub>4</sub> HERFD-XANES showed the presence of U(V) during the process.  
44 These findings offer new insights into U(VI) reduction by sulfate-reducing bacteria and  
45 contribute to a comprehensive safety concept for a repository for high-level radioactive  
46 waste.

## 47 **1 Introduction**

48 Clay formations are potential host rocks for the long-term storage of high-level radioac-  
49 tive waste in deep geological repositories.<sup>[1,2]</sup> A multi-barrier system is favored to isolate  
50 the waste from the biosphere, consisting of the technical (container with the waste), the  
51 geotechnical (sealing and back-filling material, *e.g.* compacted bentonite) and the geolog-  
52 ical barrier (host rock).<sup>[3,4]</sup> Different influencing factors have to be taken into account to  
53 ensure the long-term safety of such a repository. One of them is the presence of natural  
54 microbial communities in these environments which can have an influence on the waste,  
55 *e.g.* in a worst-case scenario, if water enters the repository. As a result, microorganisms  
56 can interact with the released radionuclides and can influence their mobility by different  
57 processes, *e.g.* sorption, accumulation, change in the speciation or reduction/oxidation.<sup>[5-  
58 7]</sup> In the context of these investigations, particular attention will be paid to the change in  
59 oxidation states, especially with respect to uranium, which represents the largest fraction  
60 of the spent nuclear fuel.

61 Various studies showed that sulfate-reducing microorganisms, especially *Desulfosporosi-*  
62 *nus* species, occur in different clay formations, as well as in bentonite.<sup>[8,9]</sup> An important  
63 representative of the genus is *Desulfosporosinus hippei* DSM 8344<sup>T</sup>. This bacterium was  
64 originally isolated from permafrost soil and identified as *Desulfotomaculum orientis* by  
65 Vainshtain *et al.*<sup>[10,11]</sup> *Desulfotomaculum orientis* was reclassified as *Desulfosporosinus ori-*  
66 *entis* by Stackebrandt *et al.*, 1997.<sup>[12]</sup> Later on, the genetic and phenotypic attributes of  
67 *Desulfosporosinus hippei* DSM 8344<sup>T</sup> were investigated and recognized as representing a  
68 distinct and novel species within the genus *Desulfosporosinus* (Vatsurina *et al.*, 2008).<sup>[13]</sup>  
69 It has been known for some time that various microorganisms can reduce uranium and  
70 other metals. In 1991, Lovley *et al.* demonstrated for the first time the reduction of U(VI)  
71 to less soluble U(IV) by the Fe(III)-reducing microorganisms *Geobacter metallireducens*  
72 and *Shewanella oneidensis*.<sup>[14]</sup> These bacteria can generate energy for anaerobic growth  
73 by reducing U(VI). In addition, various sulfate-reducing microorganisms, for example  
74 *Desulfotomaculum reducens*,<sup>[15]</sup> *Desulfovibrio desulfuricans*<sup>[16]</sup> and *Desulfovibrio vul-*  
75 *garis*,<sup>[17]</sup> as well as different other *Desulfovibrio* species,<sup>[17-19]</sup> are also capable of convert-  
76 ing U(VI) to the insoluble U(IV). But not all of them can grow based on energy gain from  
77 U(VI) reduction only. U(VI) reduction by the sulfate-reducing bacterium *Desulfotomacu-*  
78 *lum reducens* MI-1 was investigated via transcriptomics by Junier *et al.*<sup>[20]</sup> They found the

79 upregulation of genes encoding for proteins involved in respiration, suggesting that elec-  
80 trons were shuttled to the electron transport chain, which points to the reduction of U(VI)  
81 as a metabolic process. Furthermore, genes involved in *c*-type cytochrome biogenesis  
82 were upregulated. Additionally, spores of this sulfate-reducer are capable of reducing  
83 U(VI) under certain circumstances.<sup>[21]</sup> Another study showed, that a certain uranium iso-  
84 topic fractionation is induced by U(VI) reduction by different microbial strains in contrast  
85 to chemical uranium reduction.<sup>[22]</sup> In 2004, Suzuki *et al.* investigated the U(VI) reduction  
86 by *Desulfosporosinus* species for the first time.<sup>[23,24]</sup> The process was studied using *Desul-*  
87 *fosporosinus orientis* DSM 765 and *Desulfosporosinus* sp. P3 at a pH of 7 and a U(VI) as well  
88 as lactate concentration of 1 mM. A visible reduction of U(VI) by the formation of black-  
89 brown precipitates was verified in this study by X-ray absorption near-edge structure  
90 (XANES) spectroscopy. It was also shown that these *Desulfosporosinus* species differ sig-  
91 nificantly from other U(VI)-reducing microorganisms in one particular respect. They were  
92 not able to reduce U(VI) in bicarbonate solution. This buffer is preferentially used to study  
93 U(VI) reduction by various microorganisms, not only for sulfate-reducers.<sup>[14,16-18,25,26]</sup>  
94 Furthermore, *Desulfosporosinus* spp. do not contain cytochrome *c*<sub>3</sub>,<sup>[27]</sup> which has been  
95 shown to play an important role, but is not the only pathway for U(VI) reduction by sul-  
96 fate-reducing microorganisms as *e.g.* *Desulfovibrio*.<sup>[19,28]</sup> Nevertheless, the process of  
97 U(VI) reduction by sulfate reducers is not yet completely understood. Therefore, the aim  
98 of this study was to get more insights into the occurring interaction mechanisms of the  
99 sulfate-reducing microorganism *D. hippei* DSM 8344<sup>T</sup> with U(VI) using multiple micro-  
100 scopic and spectroscopic techniques. In order to mimic as closely as possible the natural  
101 conditions in a repository for high-level radioactive waste in clay rock, artificial Opalinus  
102 Clay pore water solution<sup>[29]</sup> was employed as a background electrolyte for the reduction  
103 experiments, and lower U(VI) concentrations as previously studied were applied. Thus,  
104 this work differs significantly from previous reduction studies in which bicarbonate or  
105 other buffers were primarily used. As a comparison, some of the studies presented herein  
106 were also performed in bicarbonate buffer. The U(IV) formed during reduction is less mo-  
107 bile than the higher oxidation state +VI because U(IV) often forms water-insoluble com-  
108 pounds. Different studies characterized the biogenic products of the U(VI) reduction for  
109 various anaerobic microbial genera, *e.g.* *Shewanella*, *Geobacter*, *Desulfovibrio*, and also  
110 *Desulfosporosinus*, and found uraninite as the main product of the process.<sup>[24,30-32]</sup> In con-  
111 trast to this, Bernier-Latmani *et al.* showed the formation of different U(IV) products in

112 dependence on different experimental conditions and used microbial strains (*e.g. She-*  
113 *wanella oneidensis*, *Clostridium acetobutylicum*, and *Desulfotomaculum reducens*).<sup>[26]</sup> In  
114 this case, non-uraninite products including different U(IV)-orthophosphates were found  
115 in addition to uraninite. Therefore, structural characterization of the reduction products  
116 remains a crucial part of an overall reduction study. Furthermore, uranyl(V) seems to play  
117 an important role in the reduction process by iron-reducing bacteria, as previously stud-  
118 ied for *Shewanella oneidensis* MR1 and *Geobacter sulfurreducens*, as well.<sup>[33,34]</sup> L<sub>3</sub>- and M<sub>4</sub>-  
119 edge XANES, as well as fluorescence spectroscopy suggest a one-electron transfer as a  
120 mechanism for the reduction process.<sup>[7,33,34]</sup> While other actinyl(V) species, specifically  
121 plutonyl(V) and neptunyl(V), are of certain environmental significance,<sup>[34-36]</sup> the ura-  
122 nyl(V) cation is reported to be relatively unstable due to the occurring disproportionation  
123 to U(IV) and uranyl(VI).<sup>[37]</sup> In contrast to this, recent studies showed the possibility of  
124 stabilizing this oxidation state in the presence of different ligands<sup>[37-43]</sup> or in iron-bearing  
125 phases.<sup>[44-49]</sup> Furthermore, Vettese *et al.* reported a certain stabilization of U(V) during  
126 the reduction by *Shewanella oneidensis* MR1.<sup>[33]</sup> X-ray absorption spectroscopic tech-  
127 niques verified the presence of up to 30% U(V) even after about 120 h.

128 In this study, we investigated the U(VI) reduction in artificial Opalinus Clay pore water  
129 solution by *Desulfosporosinus hippei* DSM 8344<sup>T</sup>, an important representative of the mi-  
130 crobial communities in clay rock and bentonite. Therefore, a unique combination of dif-  
131 ferent spectroscopic and microscopic techniques was used to get more information about  
132 the ongoing processes. The presence of different uranium oxidation states in the samples  
133 was verified using UV/Vis spectroscopy, as well as uranium M<sub>4</sub>-edge high-energy-resolu-  
134 tion fluorescence-detected X-ray absorption near-edge structure (HERFD-XANES spec-  
135 troscopy).<sup>[50,51]</sup> Furthermore, new information about the chemical surroundings of the  
136 formed uranium reduction products were provided via EXAFS. Time-resolved laser-in-  
137 duced fluorescence spectroscopy (TRLFS) provided more information about the U(VI)  
138 speciation in the supernatants. Moreover, different microscopic techniques such as fluo-  
139 rescence microscopy and transmission electron microscopy (TEM) showed the cell via-  
140 bility during the experiment and the uranium localization in the cells. Only by combining  
141 these different methods, it is possible to draw a picture of the ongoing processes during  
142 the U(VI) reduction. The obtained information will contribute to a comprehensive safety  
143 assessment considering microbiological influences for the selection of a final disposal site  
144 in clay rock as well as for the use of bentonite as a possible sealing and backfill material.

145 Additionally, this study provides new findings in the field of bioremediation of contami-  
146 nated anoxic environments.<sup>[52-56]</sup>

147

## 148 **2 Materials and methods**

### 149 **2.1 Cultivation**

150 *D. hippei* DSM 8344<sup>T</sup> was purchased from the Leibniz Institute DSMZ – German Collection  
151 of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The strain was  
152 cultivated in modified DSM 641 medium containing per L: 1 g NH<sub>4</sub>Cl, 2 g Na<sub>2</sub>SO<sub>4</sub>, 1 g  
153 Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> x 5 H<sub>2</sub>O, 1 g MgSO<sub>4</sub> x 7 H<sub>2</sub>O, 0.1 g CaCl<sub>2</sub> x 2 H<sub>2</sub>O, 0.5 g KH<sub>2</sub>PO<sub>4</sub>, 2 g NaHCO<sub>3</sub>, 2.5 g  
154 NaC<sub>3</sub>H<sub>5</sub>O<sub>3</sub>, 1 g yeast extract, 1 mL trace element solution, 1 mL selenite-tungstate solution,  
155 and 25 mg Na<sub>2</sub>S. The trace element solution contains per L: 1.5 g FeCl<sub>2</sub> x 4 H<sub>2</sub>O dissolved  
156 in 10 mL HCl (25%), 70 mg ZnCl<sub>2</sub>, 100 mg MnCl<sub>2</sub> x 4 H<sub>2</sub>O, 6 mg H<sub>3</sub>BO<sub>3</sub>, 190 mg  
157 CoCl<sub>2</sub> x 6 H<sub>2</sub>O, 2 mg CuCl<sub>2</sub> x 6 H<sub>2</sub>O, 24 mg NiCl<sub>2</sub> x 6 H<sub>2</sub>O, and 36 mg Na<sub>2</sub>MoO<sub>4</sub> x 2 H<sub>2</sub>O. The  
158 selenite-tungstate solution consists of 0.5 g NaOH, 3 mg Na<sub>2</sub>SeO<sub>3</sub> x 5 H<sub>2</sub>O, and 4 mg  
159 Na<sub>2</sub>WO<sub>4</sub> x 2 H<sub>2</sub>O per liter. Both stock solutions were autoclaved (20 min at 120 °C) for  
160 storage.

161 All components of the medium except the sodium sulfide were dissolved in deionized wa-  
162 ter. Afterwards, the medium was fumigated for 45 min with a gas mixture of N<sub>2</sub>:CO<sub>2</sub>  
163 (80:20), because it contains bicarbonate. After autoclaving, the medium was completed  
164 by adding a sterile (autoclaved as well) anoxic stock solution of Na<sub>2</sub>S. The cultivation was  
165 done at 30 °C in the dark. Cells were harvested in the mid exponential growth phase  
166 (OD<sub>600</sub> of about 0.08 – 0.1 after 42 – 48 h of growth, corresponding to cell numbers of  
167 6x10<sup>5</sup> – 8x10<sup>5</sup> cells/mL) by anaerobic centrifugation at 10,000 x g and 18 °C for 10 min.  
168 For further experiments, cells were washed with anoxic sterile artificial Opalinus Clay  
169 pore water solution at pH 5.5 (see 2.2.1) once and resuspended in an appropriate volume  
170 of the same solution to obtain a stock suspension with an OD<sub>600</sub> of 2.5. The optical density  
171 of the cell suspension was measured with a Specord® 50 Plus UV/VIS spectrometer from  
172 Analytik Jena at a wavelength of 600 nm.

173

174

## 175 **2.2 Uranium(VI) concentrations in the supernatants**

### 176 **2.2.1 Preparation of artificial Opalinus Clay pore water**

177 For the reduction experiments, artificial Opalinus Clay pore water was used as back-  
178 ground electrolyte. Its composition was determined by Wersin *et al.*<sup>[29]</sup> The pore water  
179 has the following composition per L: 12.6 g NaCl, 3.8 g CaCl<sub>2</sub> x 2 H<sub>2</sub>O, 2.0 g Na<sub>2</sub>SO<sub>4</sub>, 120 mg  
180 KCl, 3.5 g MgCl<sub>2</sub> x 6 H<sub>2</sub>O, 130 mg SrCl<sub>2</sub> x 6 H<sub>2</sub>O, and 40 mg NaHCO<sub>3</sub> (molar concentrations  
181 see Table S1). In addition to the natural components 22 mg Na<sub>2</sub>S were added to maintain  
182 strict anaerobic conditions. For preparation, all components except for the sodium sulfide  
183 were dissolved in deionized water and gasified for 45 min with a gas mixture containing  
184 N<sub>2</sub>:CO<sub>2</sub> (80:20). The pore water was transferred into an anaerobic box with N<sub>2</sub>-atmos-  
185 phere (MB200 B, M. Braun, Munich, Germany), and sodium sulfide was added to the solu-  
186 tion as solid compound inside the anaerobic box. The final solution was filled into serum  
187 bottles sealed with butyl plugs. The bottles were brought outside the anaerobic chamber,  
188 and the atmosphere above the pore water was exchanged for a mixture of 80% nitrogen  
189 and 20% carbon dioxide. Afterwards, the artificial Opalinus Clay pore water was auto-  
190 claved for 20 min at 121 °C and stored at 4 °C until usage.

191

### 192 **2.2.2 Batch experiments**

193 For the U(VI) reduction experiments, the anoxic U(VI) solutions in artificial Opalinus Clay  
194 pore water containing either 100 or 500 µM U(VI) and 10 mM sodium lactate at a pH of  
195 5.5 were prepared. If not otherwise stated, the sample volume was 20 mL in 50-mL sealed  
196 serum bottles. As electron donor lactate was added for the investigation of a potential  
197 reduction of U(VI) to U(IV). Lactate and other organic matter occur in lower concentra-  
198 tions in natural Opalinus Clay pore water.<sup>[57]</sup> Nevertheless, in these experiments long-  
199 term processes present in nature were simulated. Experimentally, however, only much  
200 shorter periods can be investigated. Therefore, higher lactate concentrations were used  
201 to speed up the processes. Furthermore, such lactate concentrations had been applied be-  
202 fore for different kind of U(VI) reduction experiments.<sup>[19,33]</sup>

203 U(VI) solutions in 30 mM bicarbonate buffer were prepared in the same way using only  
204 500 µM U(VI) as initial concentration. The pH value of the buffer was around 6.8. The



205 U(VI) stock solution (0.1 M U(VI) in 0.5 M HCl) used for these experimental solutions was  
206 prepared as previously described.<sup>[58]</sup>

207 Afterwards, an appropriate amount of the washed cell suspension (in artificial Opalinus  
208 Clay pore water or bicarbonate buffer) was added to the solution to achieve an OD<sub>600</sub> in  
209 the samples of 0.1 (dry bio mass (DBM) = 0.044 mg/mL, cell numbers: 8x10<sup>5</sup> cells/mL).  
210 Reference samples with heat-killed cells were prepared via boiling of the cell suspension  
211 at 99 °C for 5 min (1 mL aliquots). Viability of the cells was checked by live/dead staining  
212 (see 2.4 and Fig S1). Furthermore, an experiment with incubation in the dark was carried  
213 out, as well, to exclude the influence of a possible light-mediated U(VI) reduction by lac-  
214 tate. Stability of the U(VI) solution was proven by measuring U concentrations in a cell-  
215 free blank solution over time (see Fig S2)

216 Suspensions were incubated at room temperature in an anaerobic chamber, and samples  
217 were taken between 0 h and one week. To determine the U concentration by inductively  
218 coupled plasma mass spectrometry (ICP-MS), as well as to determine the sulfate and lac-  
219 tate concentrations, samples were centrifuged for 5 min at 10,000 x g and 18 °C in the an-  
220 aerobic glove box. Subsequently, 1 mL of the supernatants was acidified with 10 µL of  
221 concentrated nitric acid (69%) and the U concentration were determined via ICP-MS as  
222 described previously.<sup>[59]</sup> To determine the sulfate concentration, 1 mL of the supernatants  
223 were taken and examined by ion chromatography (integrated ion chromatography sys-  
224 tem, Thermo Scientific). The lactate concentrations were determined with a sample vol-  
225 ume of 250 µl using high pressure liquid chromatography (HPLC system Agilent 1200  
226 with a G1315B 1200 diode array detector). Concentrations were calculated using a cali-  
227 bration curve in the range between 0 and 20 mM. Except when otherwise stated, experi-  
228 ments were performed in triplicate. Not every analytical method was performed in every  
229 experiment.

230

### 231 **2.3 Time-resolved laser-induced fluorescence spectroscopy**

232 Time-resolved laser-induced fluorescence spectroscopic (TRLFS) measurements of the  
233 supernatants were used to investigate the speciation of U(VI) (100 µM) in the superna-  
234 tants. Samples for TRLFS were taken after 2 h, 4 h, 24 h, and 48 h of incubation time, ex-  
235 cept when otherwise stated. For sample preparation, 1 mL of each supernatant (after cen-  
236 trifugation at 18 °C and 10,000 x g for 5 min) was transferred into a semi-micro UV/Vis

237 cuvette in the anaerobic glovebox. Furthermore, a blank solution without cells was pre-  
238 pared, as well.

239 To enable an assignment of the recorded and evaluated spectra to different chemical spe-  
240 cies, a lactate reference is necessary. Therefore, a solution of artificial Opalinus Clay pore  
241 water containing 100  $\mu\text{M}$  U(VI) and 10 mM sodium lactate at a pH of 5.5 was prepared.

242 All samples, as well as the blank solution and the reference, were frozen in liquid nitrogen  
243 and stored under  $-80\text{ }^{\circ}\text{C}$  until measurement. Subsequent measurements and data evalua-  
244 tion were carried out according to Bader *et al.*<sup>[60]</sup> To minimize the quenching effect of the  
245 chloride anions (originating from the artificial Opalinus Clay pore water) on the U(VI) lu-  
246 minescence, the measurements were performed at a temperature of 150 K.<sup>[61]</sup>

247

#### 248 **2.4 Verification of cell viability**

249 For fluorescence microscopy imaging of uranium-incubated cells, 1 mL of the suspensions  
250 were taken and centrifuged anaerobically at  $18\text{ }^{\circ}\text{C}$  and  $10,000\text{ x g}$  for 5 min. Cells were  
251 resuspended in a small amount of anoxic artificial Opalinus Clay pore water. Staining was  
252 performed with a LIVE/DEAD<sup>®</sup> BacLight<sup>™</sup> Bacterial Viability Kit (Thermo Fisher Scien-  
253 tific, Waltham, MA, USA) according to the manufacturer's instructions under anaerobic  
254 conditions. Cover glasses were sealed with transparent nail polish to avoid oxygen intake  
255 during image capture outside the glovebox. Fluorescence was excited by light with wave-  
256 lengths of 420 and 460 nm. Therefore, filters Cy3 and FITC were applied and images were  
257 taken by using a phase-contrast microscope Olympus BX-61 (Olympus Europa Holding  
258 GmbH, Hamburg, Germany) with support of the imaging software "CellSense Dimension  
259 1.11.

260

#### 261 **2.5 Transmission electron microscopy**

262 Transmission electron microscopy (TEM) was used to get more information about the lo-  
263 calization of uranium on/in the cells. To enable this, thin sections of the uranium-incu-  
264 bated cells were prepared. Two U(VI) concentrations of 100 and 500  $\mu\text{M}$  and two incuba-  
265 tion times (4 h and 24 h) were investigated, as well as a reference without uranium incu-  
266 bation also after 24 h. TEM specimen preparation was performed as previously described

267 by Völkner *et al.*, with the modification that the *en-bloc* staining with uranyl acetate was  
268 omitted.<sup>[62,63]</sup> In particular, the cells were incubated for the respective times as described  
269 before (see 2.2.2). Afterwards, samples were centrifuged (18 °C and 10,000 x g for 5 min)  
270 and washed in anoxic 0.1 M sodium cacodylate buffer (pH 7.2). The cell pellet was resus-  
271 pended in 3 mL of an anoxic fixation buffer (0.1 M sodium cacodylate at pH 7.2, containing  
272 2% glutaraldehyde), subsequently. Further processing was done aerobically at the CRTD  
273 (Center for Regenerative Therapies Dresden). Semi-thin sections were cut with a Leica  
274 UC6 ultra microtome and stained with toluidine blue/borax to identify potential regions  
275 of interest, followed by ultrathin sectioning using a diamond knife. The ultrathin sections  
276 were collected on carbon-coated slot grids.

277 Bright-field and high-resolution transmission electron microscopy (TEM) images were  
278 recorded with an image-C<sub>s</sub>-corrected Titan 80-300 microscope (Field Electron and Ion  
279 Company (FEI), Eindhoven, The Netherlands) operated at an accelerating voltage of  
280 300 kV. Furthermore, high-angle annular dark-field scanning transmission electron mi-  
281 croscopy (HAADF-STEM) imaging and spectrum imaging analysis based on energy-dis-  
282 persive X-ray spectroscopy (EDXS) were performed with a Talos F200X microscope  
283 equipped with a high-brightness X-FEG electron source and a Super-X EDX detector sys-  
284 tem at an accelerating voltage of 200 kV (FEI). Prior to each (S)TEM analysis, the ultrathin  
285 section mounted in a high-visibility low-background holder was placed for 2 s into a  
286 Model 1020 Plasma Cleaner (Fischione, Export, PA, USA) to remove potential contamina-  
287 tion.

288

## 289 **2.6 UV/Vis spectroscopy**

290 To clearly give proof of the formed U(IV), UV/Vis measurements of the dissolved cell pel-  
291 lets were carried out. Therefore, a total of 60 mL (triplicates of 20 mL sample volume,  
292 DBM = 2.64 mg,  $4.8 \times 10^7$  cells) of the uranium-incubated cell suspension was centrifuged  
293 (at 18 °C and 10,000 x g for 10 min) at the respective time points as well as heat-killed cell  
294 samples incubated with U(VI) for one week.

295 Afterwards, the collected cell pellet was dissolved in 4 mL of anoxic 5 M HCl. Meanwhile,  
296 the sample was shaken for one hour on a shaking plate at 120 rpm. The suspension was  
297 centrifuged at 18 °C and 10,000 x g for 10 min, and the supernatant was transferred into  
298 a quartz glass cuvette. To calculate the ratio between U(VI) and U(IV) afterwards, the total

299 uranium concentrations were determined by ICP-MS measurements for each sample. All  
300 work was carried out anaerobically. A Cary 5G UV-Vis-NIR spectro-photometer from Var-  
301 ian was used for the UV/Vis measurement, and the spectrum was recorded in the spectral  
302 range between 200 and 800 nm with a minimum step width of 0.1 nm. U(VI) and U(IV)  
303 reference spectra were prepared with a concentration of 1 mM uranium in the corre-  
304 sponding oxidation state in 5 M HCl. Both the calculations and the results for all time  
305 points are given in the supporting information.

306

## 307 **2.7 High-energy-resolution fluorescence-detected X-ray absorption near-edge** 308 **structure spectroscopy (HERFD-XANES) and extended X-ray absorption fine struc-** 309 **ture (EXAFS) methods**

310 For extended X-ray absorption fine structure (EXAFS) and high-energy-resolution fluo-  
311 rescence-detected X-ray absorption near-edge structure (HERFD-XANES) spectroscopy,  
312 samples were incubated with 100  $\mu\text{M}$  U(VI) for different time points (4 h, 24 h, 48 h, and  
313 168 h). In this case, suspensions with a doubled optical density of 0.2 ( $1.6 \times 10^6$  cells/mL)  
314 were used to get more biomass for the measurements. The influence of a higher biomass  
315 on the reduction process was determined in advance and no major differences occurred  
316 (see Fig S4). Due to the low biomass used in the experiments compared to the relatively  
317 high sample volumes required for the X-ray absorption spectroscopic measurements,  
318 larger volumes of uranium-incubated cell suspension were employed for these experi-  
319 ments. Overall, 250 mL of the U(VI)-incubated cell suspensions (quintuplets of 50 mL in  
320 100-mL serum bottles) were used per time point containing a DBM of 22 mg cells ( $4 \times 10^7$   
321 cells). Incubation took place in quintuplicates containing 50 mL cell suspension each. The  
322 expected removal of uranium in the supernatants was proven by measurement of the ura-  
323 nium concentrations in each sample via ICP-MS. Cell pellets were collected after certain  
324 incubation times by centrifugation at  $10.000 \times g$  at  $18^\circ\text{C}$  for 10 min gathered in a single  
325 sample vial and washed with artificial Opalinus Clay pore water solution. Afterwards, for  
326 EXAFS measurements, the cell pellet was transferred as wet paste in a 3-mm-thick poly-  
327 ethylene sample holder double-confined with 13 micron Kapton tape and polyethylene.  
328 For HERFD-XANES measurements, the cell pellet was transferred into a sample carrier  
329 with round recess of 1 mm depth single-confined with 13 micron Kapton tape only, to  
330 allow low-energy  $M_4$  edge X-ray beams to reach the sample. The uranium  $M_4$  HERFD-

331 XANES measurements<sup>[50,51,64]</sup> and the uranium L<sub>3</sub> transmission EXAFS measurements<sup>[65]</sup>  
332 were performed at beamline BM20<sup>[66]</sup> at the European Synchrotron Radiation Facility  
333 (ESRF) in Grenoble. For XANES measurements, the incident energy was selected using the  
334 111 reflection from a double Si crystal monochromator. XANES spectra were measured in  
335 high-energy-resolution fluorescence-detected (HERFD) mode using an X-ray emission  
336 spectrometer.<sup>[67]</sup> The sample, analyzer crystal, and photon detector (silicon drift detector)  
337 were arranged in a vertical Rowland geometry. The uranium HERFD spectra at the M<sub>4</sub>  
338 edge were obtained by recording the maximum intensity of the uranium M<sub>b</sub> emission line  
339 (~3337 eV) as a function of the incident energy. The emission energy was selected using  
340 the 220 reflection of five spherically bent striped Si crystal analyzers (with 0.5-m bending  
341 radius) aligned at 75° Bragg angle. The intensity was normalized to the incident flux. The  
342 paths of the incident and emitted X-rays through air were minimized in order to avoid  
343 losses in intensity due to absorption. Data was collected under cryo conditions with cryo-  
344 ostream on a sample. All samples have been tested for short-term beam damage, since in  
345 some cases, X-ray may induce the reduction of many elements. First, an extended time  
346 scan (>2 min with 0.1 s exposure time per step) at the maximum of the U M<sub>4</sub> edge white  
347 line was performed before data collection, to monitor any long-term variations in the fluo-  
348 rescence signal. Later several fast HERFD scans (total counting time is less than 10 s) in  
349 the short energy range were performed and compared with all HERFD data collected per  
350 sample. Based on that procedure, the estimated X-ray exposure time can be derived for  
351 each sample. However, we didn't find any evidence of the spectral change caused by X-ray  
352 exposure.

353 EXAFS measurements were performed as previously described,<sup>[60]</sup> but under cryogenic  
354 conditions (15 K by using a closed-cycled He-cryostat). The ionization potential at the ura-  
355 nium L<sub>3</sub> edge was set arbitrarily to 17185 eV. EXAFSPAK was used for the data treatment  
356 such as energy calibration, averaging of multiple sample scans, correction for the X-ray  
357 absorbing background, isolation of the EXAFS signal, and the shell fit.<sup>[68]</sup> The ab-initio scat-  
358 tering code FEFF8.20<sup>[69]</sup> was used for the calculation of the scattering phase- and ampli-  
359 tude functions based on a structural model of a trimeric U(VI)-tartrate complex and a  
360 modified structure of the mineral ningyoite (Figs S17, S18).<sup>[70]</sup> Assuming the presence of  
361 coexisting U(IV), U(V), and U(VI) species, iterative target transformation factor analysis  
362 (ITFA)<sup>[71]</sup> was applied for the mathematical decomposition of the originating spectral  
363 mixtures into the spectra of the pure uranium species, i.e. components, and their fractions

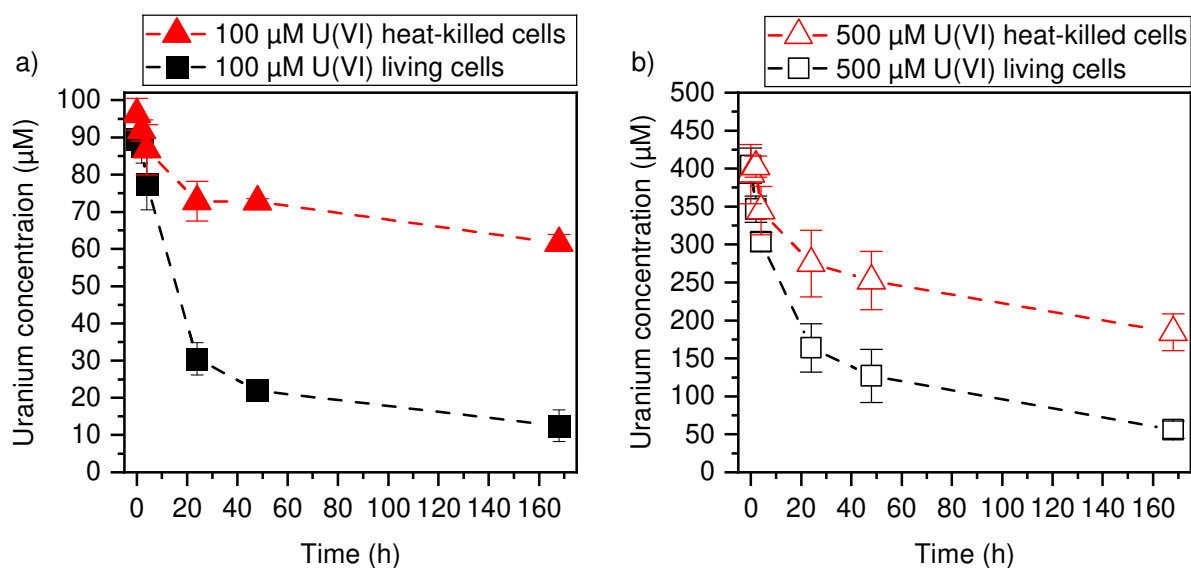
364 in HERFD-XANES and EXAFS data. Furthermore, target factor analysis (TFA)<sup>[60,72,73]</sup> was  
365 applied for the chemical identification of the uranium species by using 81 EXAFS refer-  
366 ence spectra from various organic and inorganic chemical systems with uranium in the  
367 oxidation states IV, V, and VI (Figs S19, S20).

368

### 369 3 Results & discussion

#### 370 3.1 Uranium concentration in the supernatants

371 A time-dependent U(VI) reduction experiment in artificial Opalinus Clay pore water was  
372 performed to find out whether *D. hippei* DSM 8344<sup>T</sup> is capable of reducing U(VI). Two  
373 different initial concentrations of 100 and 500  $\mu\text{M}$  U(VI) were used to investigate the oc-  
374 ccurring processes. Fig 1 shows the concentration of uranium in the supernatants in de-  
375 pendence on the incubation time.



376

377 Fig 1. Uranium concentration in the supernatants of the batch experiments with living and heat-killed cells of *D. hippei*  
378 DSM 8344<sup>T</sup> with an initial U(VI) concentration of a) 100  $\mu\text{M}$  and b) 500  $\mu\text{M}$  (artificial Opalinus Clay pore water, pH 5.5,  
379 10 mM lactate, DBM = 0.044 mg/mL,  $8 \times 10^5$  cells/mL).

380 A decrease in uranium concentration in the supernatants of the experiments with living  
381 cells is visible with increasing incubation times for both concentrations. At the lower  
382 U(VI) concentration of 100  $\mu\text{M}$ , within 48 h, around 80% of the uranium were removed  
383 from the supernatants, after one week almost 90%. The reduction experiment with  
384 500  $\mu\text{M}$  U(VI) also showed an almost complete removal of the uranium from the superna-  
385 tants. Within 4 h, 40% were already removed, and after one week, only 10% of the initial

386 uranium was still detectable. At both concentrations, black-brown precipitates occurred  
387 after certain incubation times, suggesting the reduction of soluble U(VI) (Fig S5). Stability  
388 of the pH values of the samples during the experiment were checked for the initial U(VI)  
389 concentration of 100  $\mu\text{M}$  (Fig S6). A comparison with other *Desulfosporosinus* spp. shows  
390 a similar, only slightly faster removal of uranium from the supernatants. In case of the  
391 strains P3 and DSM 765, the process is almost completed after 24 h at a slightly higher pH  
392 value of 7, 1 mM uranyl(VI) acetate, and 1 mM lactate.<sup>[23,24]</sup> However, similar time frames  
393 for the uranium removal were also observed by iron-reducers as *Geobacter* spp. and *She-*  
394 *wanella* spp.<sup>[14,33,74]</sup> Furthermore, experiments with heat-killed cells were performed. At  
395 100  $\mu\text{M}$  U(VI), 35% of the uranium was removed from the supernatant after one week,  
396 probably due to different association processes, e.g. biosorption to functional groups on  
397 the cell surface.<sup>[5]</sup> The heat treatment promotes cell breakup, which provides more bind-  
398 ing sites for uranium association. Therefore, dead cells generally bind more uranium in  
399 contrast to living cells.<sup>[75,76]</sup> In addition, it could be that the heat treatment did not com-  
400 pletely kill all the cells. In this case, the surviving cells can contribute to the decrease of  
401 the uranium concentration in the supernatant. However, the removal of U is significantly  
402 higher in the living cell experiment compared to the experiment with the heat-killed cells.  
403 Therefore, the living cells contribute through their activity to the higher removal of ura-  
404 nium in the living-cell experiments. The experiment with heat-killed cells at the higher  
405 initial U(VI) concentration shows a relatively high removal of uranium from the superna-  
406 tants. However, the proportion of the removed uranium is still not as high as in the exper-  
407 iment with the living cells. In this case, a partial precipitation of U(VI) during the experi-  
408 ment due to the higher initial concentration could also contribute to the increased values  
409 of U removal from the supernatants.

410 For further investigation of the occurring processes, lactate and sulfate concentrations  
411 were determined during the bioreduction experiments (Fig S7). The initial sulfate con-  
412 centration in Opalinus Clay pore water solution is about 14 mM. Until 48 h, the sulfate  
413 concentration remains almost constant. Only after one week, a slight decrease is observed,  
414 probably due to a reduced bioavailability of the U(VI). Therefore, this microorganism  
415 seems to reduce U(VI) prior to sulfate. These results are also in good agreement with the  
416 redox potentials of both ions (pH 7:  $E(\text{UO}_2^{2+}/\text{U}^{4+}) = 0.12 \text{ V}$ ,  $E(\text{S}^{2-}/\text{SO}_4^{2-}) = -0.2 \text{ V}$ ).<sup>[77,78]</sup> The  
417 higher reduction rate of sulfate in the experiment with 500  $\mu\text{M}$  U(VI) (Fig S7) could be due  
418 to an increased co-precipitation of U(VI) and U(IV). In this case, the bioavailability of U(VI)

419 would decrease, which would favor sulfate reduction. Furthermore, no decrease in sulfate  
420 concentrations was detected in the heat-killed-cell experiment.

421 With help of HPLC measurements, changes in the lactate concentration over time were  
422 determined (see Fig S7b). Lactate can serve as an electron donor for the reduction of U(VI)  
423 and is itself oxidized to acetate. Therefore, lactate concentrations should decrease over  
424 time. Evaluation of the HPLC data indicates a decrease, but only small amounts of the  
425 lactate are consumed. A reason for this could be the huge excess of lactate in the samples  
426 in comparison to the proportions of uranium. Furthermore, for every molecule of lactate,  
427 which is oxidized, two uranyl(VI) ions can be reduced according to stoichiometry. The  
428 formation of acetate could also be determined via this method. An acetate peak was visible  
429 via HPLC measurements, but the amount was too low to be quantified.

430 U(VI) reduction experiments under anaerobic conditions are often performed in a bicar-  
431 bonate-buffered medium to stabilize U(VI) in solution.<sup>[14,16-18,25,26,33]</sup> *D. hippei* DSM 8344<sup>T</sup>  
432 was also investigated regarding its capability of reducing U(VI) in 30 mM bicarbonate  
433 buffer in the presence of lactate (Fig S8). In this case, no dark precipitates and no decrease  
434 in uranium concentration in the supernatants occurred. Therefore, we assume that *D.*  
435 *hippei* DSM 8344<sup>T</sup> cannot reduce U(VI) in bicarbonate buffer, where the 1:3-uranyl(VI)-  
436 carbonate complex is the dominant species (Fig S9a). This is also in good agreement with  
437 former findings of Suzuki *et al.*, who investigated the reduction of U(VI) by other *Desul-*  
438 *fosporosinus* spp.<sup>[24]</sup> These strains were also not capable of reducing U(VI) in the presence  
439 of bicarbonate. The present uranyl(VI)-carbonate complex apparently cannot be reduced  
440 by this bacterial genus. Potential explanations could be, as already determined by Suzuki  
441 *et al.*, that these microorganisms do not contain *c*-type cytochromes <sup>[13]</sup>, which seems to  
442 play an important role for the U(VI) reduction by other bacteria.<sup>[19,28]</sup> In addition, it was  
443 described that some *Desulfosporosinus* spp. can grow using bicarbonate as the sole elec-  
444 tron acceptor in combination with lactate as an electron donor during homoacetogenic  
445 fermentation.<sup>[79]</sup> In this case, this energy metabolism would be preferred by the microor-  
446 ganisms, because the microorganisms will gain more energy by this metabolism.

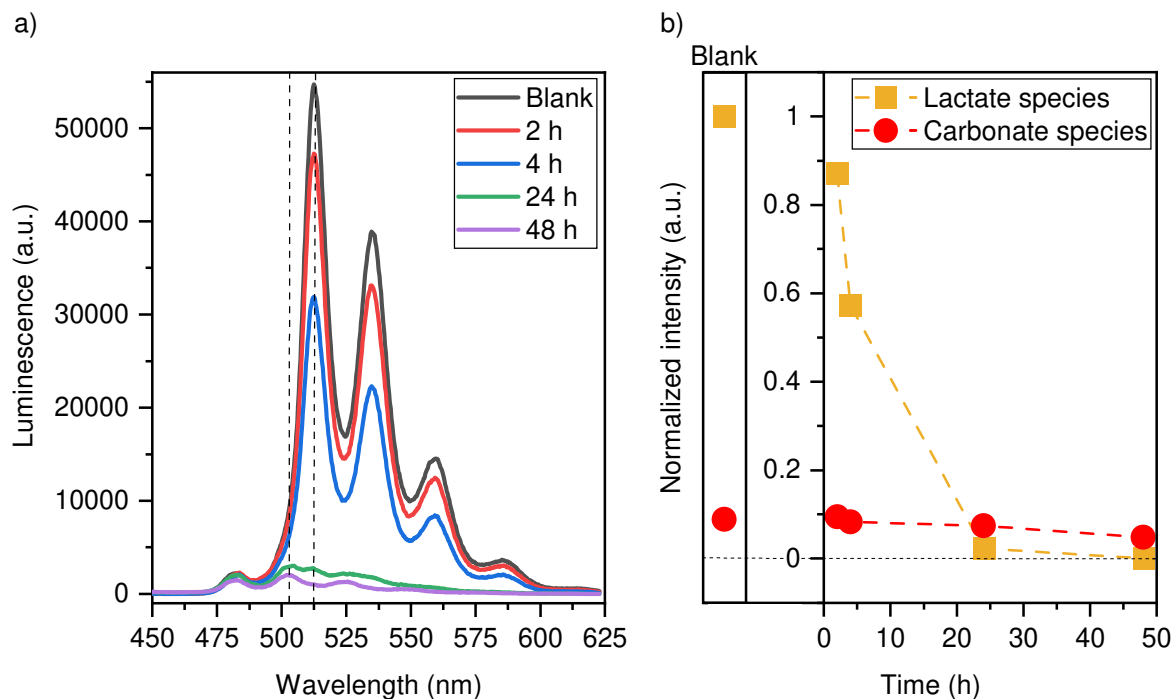
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### 448 **3.2 Fluorescence spectroscopic studies of the supernatants**

449 To get more information about the U(VI) speciation in the supernatants, time-resolved  
450 laser-induced fluorescence spectra were recorded at different time points of the batch



451 experiment. The U(VI) emission spectra of the supernatants after different times of incu-  
452 bation with cells of the anaerobic bacterium *D. hippei* DSM 8344<sup>T</sup> are shown in Fig 2a.



453  
454 Fig 2. a) Emission spectra of the supernatants after different incubation times (2 h – 48 h) with *Desulfosporosinus hippei*  
455 DSM 8344<sup>T</sup> in artificial Opalinus Clay pore water ([U(VI)<sub>initial</sub>] = 100 μM; [Lactate] = 10 mM; excitation wavelength =  
456 266 nm); b) distribution of the U(VI) species in the supernatants as a function of the incubation time determined by  
457 parallel factor analysis (PARAFAC)<sup>[80]</sup> (orange = uranyl(VI)-lactate complex, red = 1:1-uranyl(VI)-carbonate complex).

458 The experiment was performed in artificial Opalinus Clay pore water with an initial U(VI)  
459 concentration of 100 μM. Already the initial spectra show a decrease in the luminescence  
460 intensity with time, which is in good agreement with the decreasing U(VI) concentrations  
461 in the samples. Overall, two species occur in the spectra, with both species showing ap-  
462 proximately the same intensity after 24 h (Fig. 2a). Furthermore, there is no significant  
463 difference between the blank spectrum of the initial U(VI) solution and the spectra after  
464 up to 4 hours. Prior to the exposure of cells, the speciation of U(VI) in artificial Opalinus  
465 Clay pore water with 10 mM sodium lactate was modeled, and a uranyl(VI)-lactate com-  
466 plex was found to be the dominant species under these conditions (pH 5.5). Furthermore,  
467 a uranyl(VI)-carbonate complex was determined to a lesser extent. The results of this  
468 modeling can be found in the supporting information (Fig S9b, c) and provide insights into  
469 the U(VI) speciation in the supernatants for the initial solution (blank).

470 It is not possible to draw direct conclusions from the emission spectra about the different  
 471 involved U(VI) species in the supernatants, because of the partial superposition of the sin-  
 472 gular-component spectra. Therefore, a deconvolution of the spectra with the help of the  
 473 mathematical method called parallel factor analysis (PARAFAC) was carried out.<sup>[80]</sup> The  
 474 spectra of two different species could be extracted in this way (Fig S10), which is already  
 475 in good agreement with the previous calculations. With the help of several reference spec-  
 476 tra from previously determined species, an assignment to two different uranyl(VI) com-  
 477 pounds was possible. The band positions of the extracted spectra and references are  
 478 shown in Table 1. The first extracted spectrum shows a high agreement with a uranyl(VI)-  
 479 lactate complex. The reference was prepared and measured under the same conditions as  
 480 the samples. Both, spectral decomposition and band positions are in good agreement. The  
 481 formation of this complex is favored at a pH of 5.5 because of the high excess of lactate in  
 482 the samples. The second spectrum also shows a very good agreement with the reference  
 483 of the 1:1-uranyl-carbonate complex originating from sodium bicarbonate in the Opalinus  
 484 clay pore water solution and the gasification with a mixture of 20% carbon dioxide and  
 485 80% nitrogen to get the solutions anaerobic. All in all, both calculated species could be  
 486 confirmed experimentally with luminescence spectroscopy.

487 Table 1. Assignment of the band positions of the extracted time-resolved laser-induced fluorescence spectra (Fig S10).  
 488 Extraction of the spectra was performed using PARAFAC<sup>[80]</sup>.

	<b>Band position (nm)</b>					<b>Reference</b>
<b>Spectrum 1</b>	512.5	534.7	559.4	585.7	614.6	This work
<b>Uranyl(VI)-lactate</b>	511.8	534.0	558.1	584.9	614.0	This work
<b>Spectrum 2</b>	482.3	503.3	524.5	547.9	575.0	This work
<b>UO<sub>2</sub>CO<sub>3</sub></b>	482.0	502.9	525.6	549.2	575.1	[58]

489  
 490 The species distribution (Fig 2b) shows that the uranyl(VI)-lactate species is the major  
 491 component in the initial solution. During the reduction process, the species distribution  
 492 shows a decrease of the uranyl(VI)-lactate species with time. In contrast to this, the pro-  
 493 portion of the carbonate complex remains almost constant. This can be attributed to the  
 494 assumption that this genus cannot reduce the uranyl(VI)-carbonate species.<sup>[24]</sup> This  
 495 would be in good agreement with the non-changing values of U(VI) concentrations in the  
 496 supernatants of the bicarbonate experiment and shows the influence of the initial U(VI)  
 497 species on U(VI) reduction by this microorganism.

498 In addition to these results, a luminescence spectroscopic experiment with more frequent  
499 sampling showed an interesting behavior of the luminescence intensities over time, espe-  
500 cially during the first hours of the bioreduction process. Although the emission intensity  
501 of uranyl(VI) underwent a general decrease, the rate of this decline is not consistent (Fig  
502 S11). Instead, the luminescence intensities decrease and increase several times. An ini-  
503 tially sharp decrease of the intensity is followed by a subsequently partial increase. This  
504 distinctive 'saw tooth' pattern was already observed in other bioreduction processes, *e.g.*  
505 in the reduction of U(VI) by *Geobacter sulfurreducens* and *Shewanella oneidensis*.<sup>[7,33]</sup>  
506 These studies explained the observed pattern by the formation of uranyl(V) as an inter-  
507 mediate uranium species during the reduction. Therefore, time-resolved luminescence  
508 spectroscopy could give an indication of the occurrence of U(V) during reduction by sul-  
509 fate-reducing microorganisms.

510

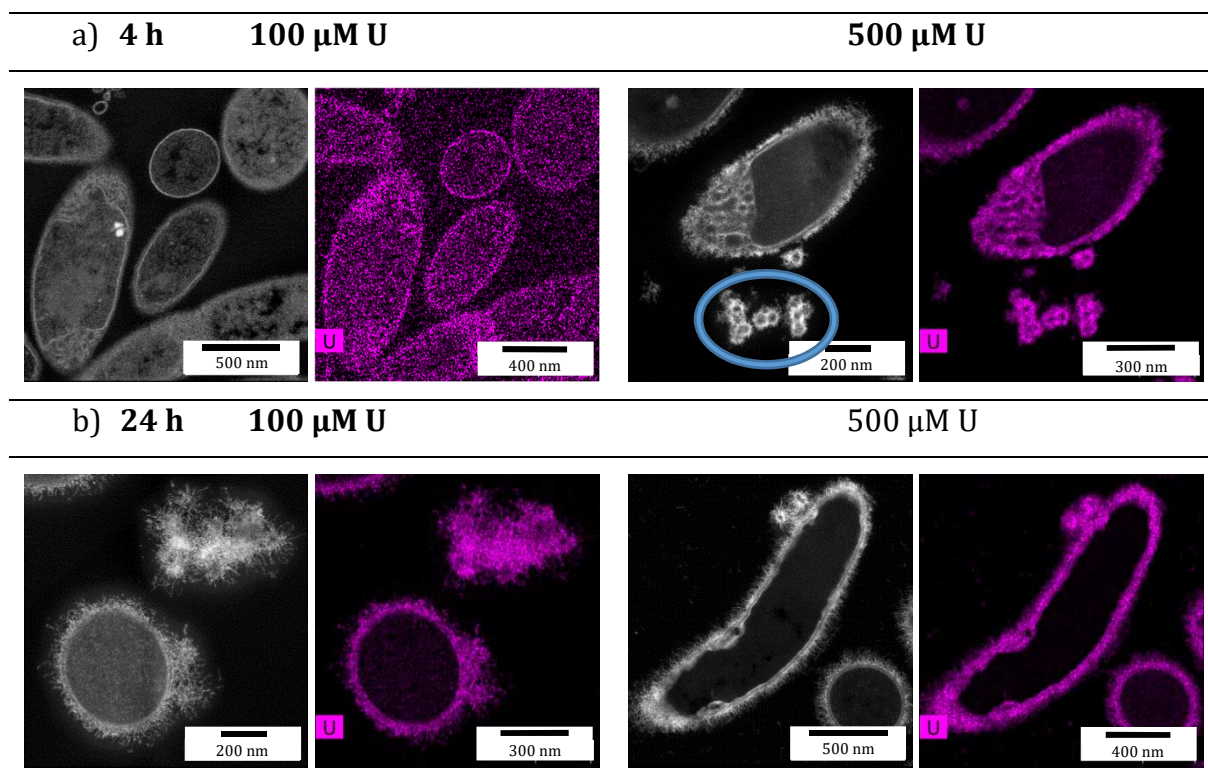
### 511 **3.3 Microscopic investigations**

512 Live/dead staining of the cells was carried out at different incubation times. The images  
513 show an increased agglomeration of the cells with the incubation time at both concentra-  
514 tions (see Fig S12). In addition, the proportion of the dead cells increases, what becomes  
515 clear from the yellowish color of the agglomerates indicating a mixture of living (green)  
516 and dead (red) cells. In contrast to this, those agglomerates do not occur in the cell blank  
517 without U(VI) incubation, not even after one week. The live/dead images at both concen-  
518 trations do not differ significantly from each other. At the higher U(VI) concentration of  
519 500  $\mu\text{M}$ , the agglomerates are slightly bigger than those in the experiment with an initial  
520 U(VI) concentration of 100  $\mu\text{M}$ .

521 The localization of uranium in/on the cells was investigated by scanning transmission  
522 electron microscopy (STEM) analyses of ultrathin sectioned samples of the U(VI)-incu-  
523 bated cells. In particular, atomic-number-contrast HAADF-STEM imaging was combined  
524 with spectrum imaging analysis based on energy-dispersive X-ray spectroscopy (EDXS).  
525 Fig 3 shows the resulting images and U distribution maps for two U(VI) concentrations  
526 (100  $\mu\text{M}$  and 500  $\mu\text{M}$ ) and two incubation times (4 h and 24 h).

527

528



529 Fig 3. Representative HAADF-STEM images (left) and corresponding U element distribution (right) of ultrathin sec-  
530 tioned samples of *Desulfosporosinus hippei* DSM 8344<sup>T</sup> cells treated with uranium ( $[\text{U(VI)}]_{\text{initial}} = 100/500 \mu\text{M}$ ) for a) 4 h  
531 and b) 24 h. The blue ellipse highlights possibly released membrane vesicles.

532 The amount of cell-associated uranium increases with time and concentration, which is in  
533 good agreement with the decreasing uranium concentrations in the supernatants (see  
534 Fig 1). Uranium-containing aggregates are mainly present on the cell surface. These partly  
535 have the shape of small needles (for higher magnification see Fig S13). High-resolution  
536 TEM imaging coupled with fast Fourier transform analysis for *D. hippei* DSM 8344<sup>T</sup> after  
537 incubation with 500  $\mu\text{M}$  U(VI) for 24 h showed the aggregates to be of amorphous struc-  
538 ture (Fig S14). Especially at the lower concentration and with shorter incubation times,  
539 uranium is located also inside the cells, almost evenly spread (Fig 3a). As can be seen in  
540 the images (blue ellipse, Fig 3a), there is an indication for the release of membrane vesi-  
541 cles from the cell surface. This could be a possible defense mechanism of *D. hippei* DSM  
542 8344<sup>T</sup> to mitigate cell encrustation and has already been reported for other microorgan-  
543 isms, e.g. *Shewanella oneidensis* MR-1 or *Geobacter sulfurreducens*.<sup>[81,82]</sup>

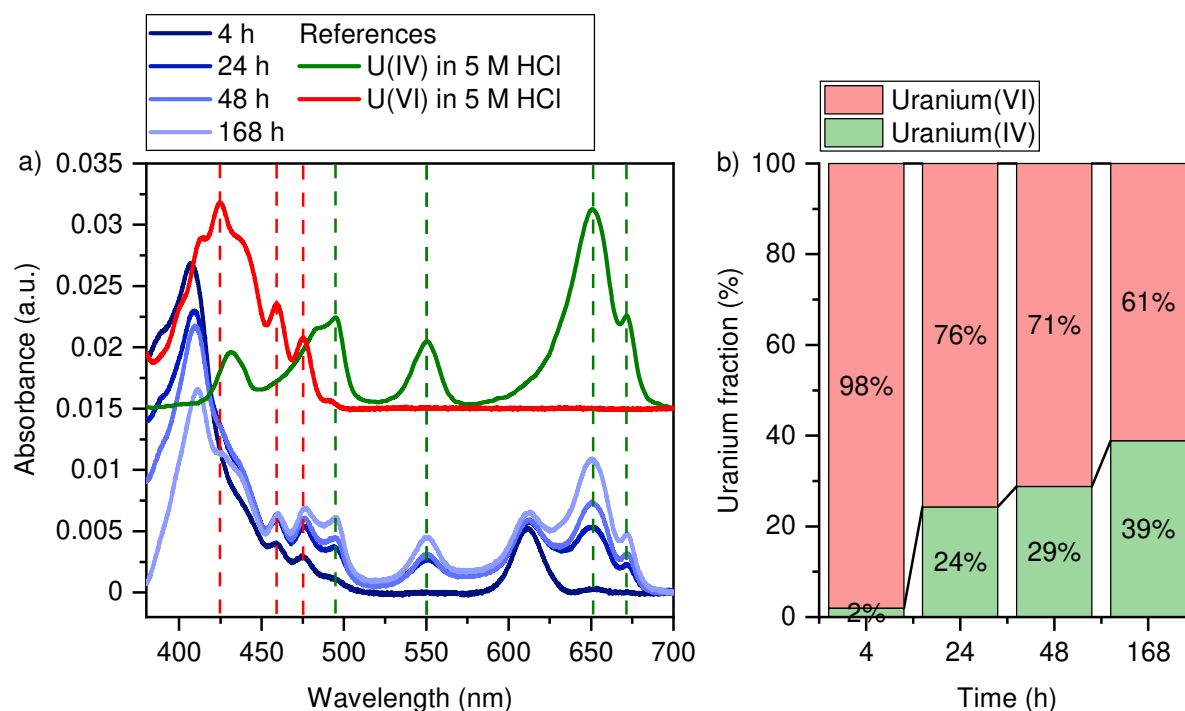
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546

### 547 3.4 UV/Vis spectroscopy

548 The formation of U(IV) was proven by UV/Vis studies of the dissolved cell pellets. Fig 4a  
549 shows the UV/Vis spectra of the dissolved cell pellets after different incubation times in  
550 comparison with the reference spectra of U(VI) and U(IV).



551  
552 Fig 4. a) UV/Vis spectra of the dissolved cell pellets after different incubation times in comparison with normalized  
553 reference spectra of U(IV) and U(VI). b) Calculated proportions (see supporting information) of U(IV) and U(VI) under  
554 consideration of the corresponding extinction coefficients of both oxidation states ( $[U(VI)_{initial}] = 100 \mu M$ ).

555 A comparison of the band positions provides clear proof of the formed U(IV). At the char-  
556 acteristic band at around 650 nm, we observe an increase in the proportion of U(IV) with  
557 time, which becomes visible in the spectra. In the spectral region between 400 and  
558 500 nm, also bands of U(VI) are still detectable. These partly overlap with those of U(IV).  
559 This suggests that not all of the U(VI) is reduced to the lower oxidation state in the cell  
560 pellets, and therefore the fraction of removed uranium from the supernatants is not en-  
561 tirely U(IV), not even after one week. Consequently, the occurring process seems to be  
562 based on a combination of association with the cells and reduction. The band at a wave-  
563 length of 610 nm is caused by residual cell components, because a blank spectrum of a  
564 dissolved cell pellet without U(VI) incubation also shows this feature (Fig S15). The band  
565 at 410 nm, which partly overlaps with bands of U(VI), can provide further indication of  
566 intermediately occurring U(V) during the reduction process, which disproportionates to  
567 U(VI) and U(IV). Nagai *et al.* investigated the absorption properties of U(V) in molten

568 NaCl-2CsCl.<sup>[83]</sup> They showed that U(V) exhibit an intense absorption band at this wave-  
569 length. Further data evaluation regarding this oxidation state were not performed be-  
570 cause of the huge differences in the chemical surroundings, affecting different physical  
571 quantities, *e.g.* the molar attenuation coefficient. It would be possible, however, that the  
572 proportion of U(VI) in the samples is lower, since the proportion of the possibly occurring  
573 U(V) would have to be subtracted. Therefore, to get the exact values of the proportions of  
574 U(VI) and U(IV) in the cell pellets at every time point, calculations considering the molar  
575 extinction coefficients were carried out.

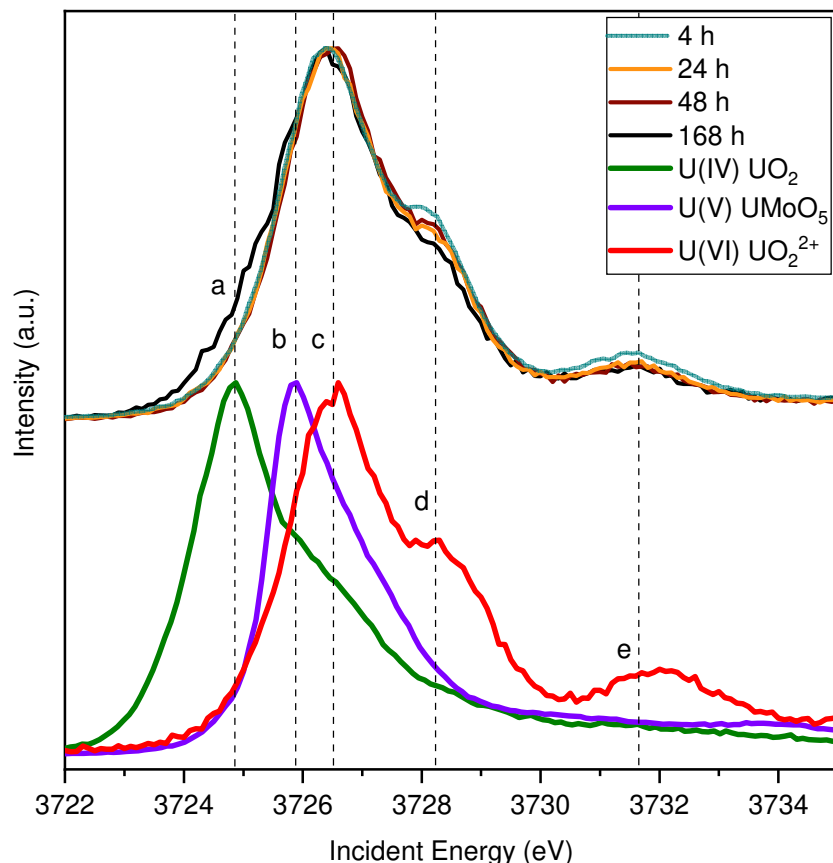
576 As can be seen in Fig 4b, the proportion of U(IV) in the samples increases continuously  
577 with time. After 4 h, only a very small proportion of the U(VI) is reduced. Only 2% of U(IV)  
578 are present in the samples. But already after 24 h, round about a quarter of the U(VI) is  
579 reduced to U(IV) in the cell pellets. This value is further increasing until one week, where  
580 about 40% are reduced. However, when comparing the percentages of the different oxi-  
581 dation states over time, the different proportions of cell-associated uranium must also be  
582 taken into account. Especially after 4 h, only 20% of the uranium are removed from the  
583 supernatant. After 24 h, however, the proportions do not change much. The UV/Vis ex-  
584 periment with heat-killed cells only shows a minor reduction of U(VI) under these condi-  
585 tions (Fig S15). This can be due to a partly reduction, possibly caused by remaining living  
586 cells after heat treatment or by a light-mediated reaction with lactate<sup>[84,85]</sup>. However, the  
587 intensity of the uranium(IV) band at 650 nm is much weaker than in the experiment with  
588 the living cells. Furthermore, no differences in the reduction behavior could be observed  
589 in the experiments with incubation in the dark (data not shown). Therefore, reduction of  
590 U(VI) by lactate seems to have only a minor or no influence on the experiments.

591

### 592 **3.5 High-energy-resolution fluorescence-detected X-ray absorption near-edge** 593 **structure spectroscopy (HERFD-XANES)**

594 The electronic structure of the uranium system has been investigated by HERFD-XANES  
595 measured at the uranium M<sub>4</sub> edge, which probes directly the f-orbitals through 3d<sub>3/2</sub> →  
596 5f<sub>5/2</sub> electronic transitions.<sup>[50,51,64]</sup> The appearance of the main HERFD transitions at dif-  
597 ferent incident energy in the X-ray spectroscopy process is generally attributed to the  
598 change of the oxidation state, which produce the chemical shift of the detected 3d-5f tran-

599 sitions.<sup>[50]</sup> Fig 5 shows the HERFD-XANES spectra of the cell pellets after different incuba-  
 600 tion times in comparison with the reference spectra of U(VI), U(V),<sup>[86]</sup> and U(IV). Refer-  
 601 ence spectra for U(VI) and U(IV) were measured simultaneously with the samples.



602  
 603 Fig 5. Uranium M<sub>4</sub> HERFD XANES data recorded on the cell pellets after different incubation times compared with the  
 604 reference spectra of U(VI) as uranyl(VI) nitrate, U(V) as UMoO<sub>5</sub>,<sup>[86]</sup> and U(IV) as UO<sub>2</sub>. Dashed lines in a–c indicate the  
 605 white line energy positions for U(IV), U(V), and U(VI) valence states, respectively; d and e indicate post-edge feature for  
 606 the uranyl(VI) structure ([U(VI)<sub>initial</sub>] = 100 μM).

607 Figure 5 shows that spectra recorded on cell pellets after the 4 h, 24 h, 48 h, and 168 h  
 608 incubation time are different. First of all, the intensity of the first post-edge feature  
 609 (marked d) after the white line (marked c) is gradually going down upon increasing incu-  
 610 bation times. The second post-edge feature (marked e) has the same tendency. Generally,  
 611 the hexavalent uranium M<sub>4</sub> HERFD shows three features (marked c, d, e)<sup>[51]</sup> and reflect  
 612 the f density of states for the specific atomic orbital. Data recorded on cell pellets after  
 613 different incubation times show that U(VI) is the dominant uranium oxidation state for all  
 614 samples, but some tiny differences, as explained above, are still noticeable. Moreover, the  
 615 uranium spectrum recorded for a sample with 168 h incubation time is much broader

616 than other spectra and there is also a shoulder on the left side from the main edge transi-  
617 tions. The appearance of the shoulder at the low-energy side is generally attributed to the  
618 formation of lower oxidation states – the formation of the U(IV) or/and U(V). In order to  
619 extract the exact contributions of U(IV), U(V), and U(VI), we used the ITFA package.<sup>[71]</sup> As  
620 input files, we used the UO<sub>2</sub> as U(IV), the uranyl(VI) as U(VI), and U(V) has been taken as  
621 uranate in UMoO<sub>5</sub> published by Pan *et al.*<sup>[86]</sup> ITFA-extracted eigenvectors of the HERFD-  
622 XANES data, isolated single-component spectra and the reproduced U M<sub>4</sub> HERFD spectra,  
623 as well as differences between experimental and reproduced spectra are shown in Fig  
624 S16. The results of the ITFA analysis are presented in Table 2 and provide first evidence  
625 of the presence of U(V) in the microbial reduction not only by the non-cytochrome-c con-  
626 taining bacteria *D. hippei* DSM 8344<sup>T</sup>, but also by sulfate-reducing bacteria in general. The  
627 proportion of this oxidation state remains almost constant over the investigated time  
628 frame, which indicates a stabilization by the chemical surrounding as previously de-  
629 scribed.<sup>[37-49]</sup> Furthermore, these findings verify the above-mentioned indications of this  
630 oxidation state via UV/Vis and luminescence spectroscopy.

631 Table 2. Fractions of U(VI), U(V), and U(IV) calculated by ITFA analysis.<sup>[71]</sup> Estimated error of the ITFA analysis is 2%.

Time (h)	U(VI) (%)	U(V) (%)	U(IV) (%)
<b>4</b>	74	25	1
<b>24</b>	67	31	2
<b>48</b>	72	27	1
<b>168</b>	60	30	10

632

633 We noticed that the estimated amount of different oxidation states from HERFD data is  
634 different from those extracted from UV/Vis. For example, for the 4h sample the U(VI) con-  
635 tribution was found to be 98% from UV/Vis versus 74% by HERFD. It can be related to  
636 the fact that in the UV/Vis experiments, the whole cell pellet is dissolved and measured,  
637 whereas in the HERFD-XANES studies, only a small spot of the sample is analyzed due to  
638 the limited dimension of the X-ray beam.<sup>[66]</sup> Nevertheless, the overall tendency of the  
639 U(IV) oxidation state over different incubation times is similar for UV/Vis and HERFD. In  
640 contrast to the iron-reducing microorganisms *Shewanella* and *Geobacter*, U(VI) reduction  
641 proceeds much more slowly in *D. hippei* DSM 8344<sup>T</sup>.<sup>[33,34]</sup> In case of *Shewanella oneidensis*,  
642 after 4.5 h, already 24–25% of the uranium was reduced to U(IV) in the cell pellets and

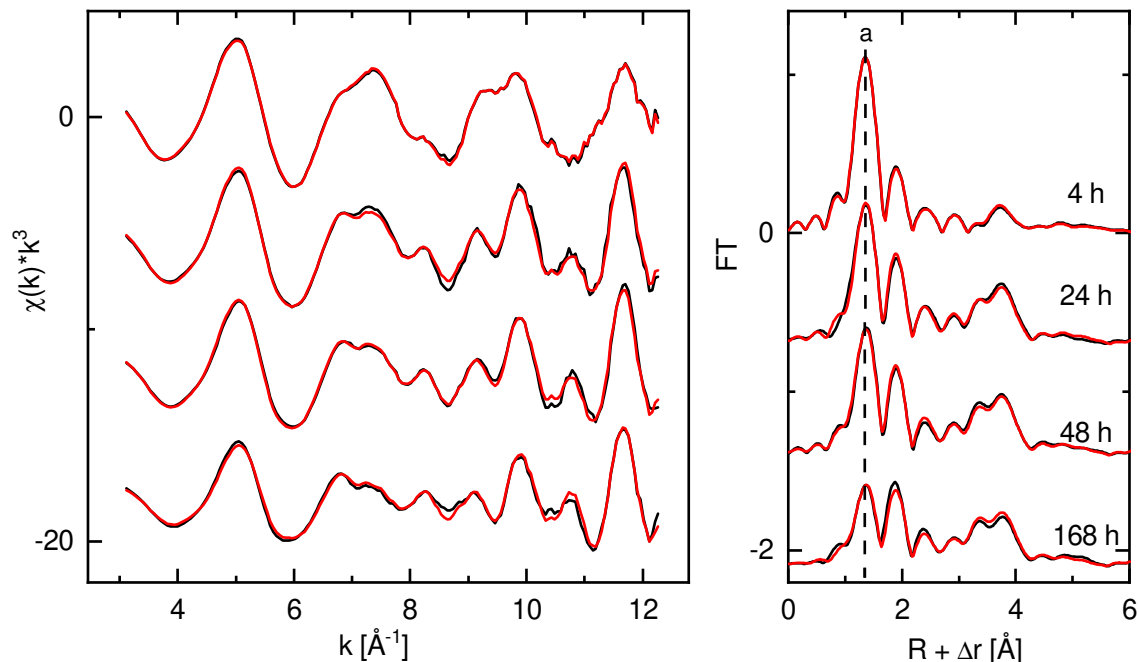


643 after 120 h, the proportion of U(IV) was approx. three quarters. Furthermore, also the  
644 proportion of U(V) during the reduction is higher for *Shewanella*. However, the pentava-  
645 lent oxidation state persists for longer incubation times in the reduction experiments with  
646 both microorganisms.<sup>[33]</sup> For *Geobacter*, the reduction process is even faster as deter-  
647 mined by L<sub>3</sub>-Edge EXAFS spectroscopy. In this case, after 24 h, all the uranium has been  
648 reduced to U(IV) and U(V) only occurs in samples after 4 h of incubation.<sup>[34]</sup>

649

### 650 3.6 Extended X-ray absorption fine structure (EXAFS)

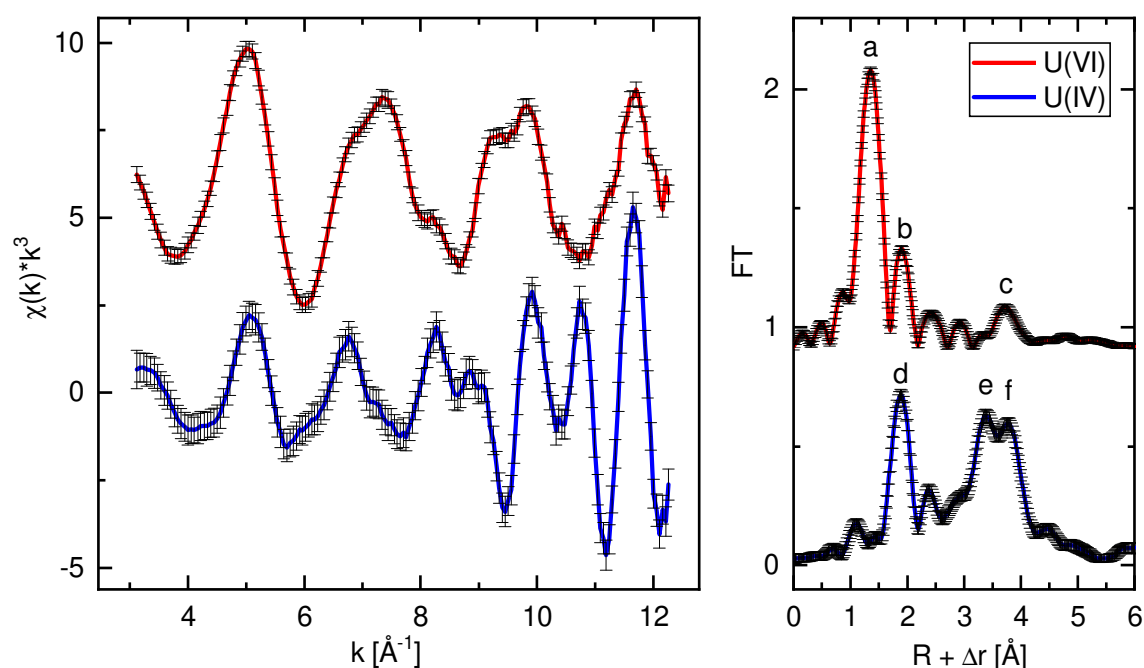
651 In order to estimate the number (n) and fractions of the coexisting uranium species and  
652 to isolate their spectra from the spectral mixtures, the EXAFS spectra of four samples at  
653 incubation times t = 4 h, 24 h, 48 h, and 168 h were analyzed with ITFA and TFA. As shown  
654 in Fig 6 the linear combinations of the ITFA-calculated first two eigenvectors reproduce  
655 the experimental spectra in high quality, hence only two spectral components are present  
656 and change their fractions as a function of the incubation time, thus determining the shape  
657 of the spectral mixtures.



658

659 Fig 6. Experimental (black) and ITFA-reproduced (red) uranium L<sub>3</sub> EXAFS spectra recorded on the cell pellets of *D.*  
660 *hippei* DSM8344<sup>T</sup> after incubation with uranium [100  $\mu\text{m}$ ] for different times (left) with corresponding Fourier trans-  
661 forms (FT) (right). FT peak of the axial oxygen atoms (a).

662 In a first attempt, the chemical origin of the two spectral components can be deduced by  
663 the inspection of the Fourier transform (FT) of the spectral mixtures. The systematic de-  
664 crease of the FT peak at 1.36 Å, coming from the axial oxygen atoms ( $O_{ax}$ ) of the U(VI) “yl”  
665 unit (Fig 6a)), points to an increase of the fraction of U(IV) with increasing incubation  
666 time, because for U(IV) no  $O_{ax}$  peak is expected in the FT. However, at the highest incuba-  
667 tion time, there is still a significant fraction of the U(VI) species present, as visible at the  
668  $O_{ax}$  FT peak (Fig 6, t = 168 h), so that the U(IV) species does not provide its pure experi-  
669 mental spectrum in the series. This is in good agreement with the UV/Vis and HERD-  
670 XANES data. Consequently, the spectral mixtures have to be mathematically decomposed  
671 so that the resulting spectra of the pure uranium species can be analyzed by the shell fit-  
672 ting approach. For the calculation of the fractions and spectra of the uranium species, ITFA  
673 needs at least  $n^2-n$  known fractions ( $n$  = number of spectral components) as constrains,  
674 hence two known fractions in the present case. However, only for the U(VI) species, we  
675 can assume 100% at t = 4 h, while no data are available for the U(IV) species. Fortunately,  
676 the fact that the  $O_{ax}$  signal can serve as an unequivocal and robust measure of the presence  
677 of U(VI) and that the fractions of a component can be calculated independently to the frac-  
678 tions of the remaining components by ITFA owes the possibility to calculate and isolate  
679 the fractions and spectra of the pure uranium species by a simple minimization strategy  
680 applied on the  $O_{ax}$  FT peak (see SI). As shown in Fig 7 and in comparison with the spectrum  
681 at t = 168 h (Fig 6), the  $O_{ax}$  FT peak vanishes substantially for the U(IV) species after the  
682 proposed ITFA treatment so that both isolated spectra refer to uranium species in their  
683 pure oxidation states for which a shell fit can be performed. The resulting fractions (Table  
684 3) deviate strongly from those observed by the HERFD measurements (Table 2) where  
685 U(V) was detected in addition to U(IV) and U(VI). However, the penetration depth of the  
686 X-rays at the U  $M_4$  and at the U  $L_3$  edge is different, which variates slightly the obtained  
687 results at the U  $L_3$  or U  $M_4$  edges. In addition, a lack of U(V) references for EXAFS, as well  
688 as the structural similarity of U(VI) and U(V), making it difficult to assign the isolated  
689 spectra to this oxidation state.



690

691 Fig 7. Uranium L<sub>3</sub> EXAFS spectra of the ITFA-isolated spectra of the isolated uranium species (left) with corresponding  
 692 Fourier transforms (FT) (right) and estimated standard deviations (black). FT peaks of the axial oxygen (O<sub>ax</sub>) (a), equa-  
 693 torial oxygen (O<sub>eq</sub>) of U(VI) (b), first oxygen shell of U(IV) (d), and uranium interactions (c, e, f).

694

695 Table 3. Fractions of U(VI) and U(IV) calculated by ITFA analysis (see SI).

Time (h)	U(VI) (%)	U(IV) (%)
4	100	0*
24	62(1)	38(1)
48	57(1)	43(1)
168	55(2)	45(2)

696 \* - fixed during ITFA procedure. Estimated standard deviations in parenthesis.

697 TFA can be used to identify spectra from different chemical reference systems whose lin-  
 698 ear combinations are suitable for the reproduction of spectral mixtures. Thus, TFA allows  
 699 the chemical identification of the uranium species. For this purpose, each reference spec-  
 700 trum (target) is subjected subsequently to the TFA procedure, while the SPOIL value<sup>[87]</sup>  
 701 measures for each target its suitability for the reproduction of the spectral mixtures. As  
 702 lower the SPOIL value as higher the probability that the subjected target spectrum refers  
 703 to a pure chemical species contained in the spectral mixtures. Here, we used about 81  
 704 EXAFS spectra from U(IV), U(V), and U(VI) systems with different inorganic and organic

705 ligands at various pH, concentrations, and temperatures (Figs S19, S20). Notably, if we  
706 exclude the formation of schoepite measured at room temperature (SPOIL = 2.25) and at  
707 15 K (SPOIL = 2.58), then only reference spectra of U(VI) with exclusively aqueous hy-  
708 droxy-carboxylic acids like lactate, tartrate, citrate, and malate form two groups of possi-  
709 ble references measured at room temperature (RT). In the first group, all acids between  
710  $6.6 \leq \text{pH} \leq 7$  with  $1.78 \leq \text{SPOIL} \leq 2.22$  are contained, while the second group consists  
711 of tartrate and lactate with SPOIL = 2.71 and 2.78 at pH 5.0 and 5.5, respectively. Moreo-  
712 ver, from a chemical point of view in the lower pH range, significant portions of polynu-  
713 clear dimeric and in the higher pH range, dominating trimeric U(VI) complexes are ex-  
714 pected for these hydroxyl-carboxylic acids.<sup>[88-92]</sup> However, U(VI) with lactate at pH 7 is  
715 the reference with the lowest SPOIL value of 1.78, hence the most probable U(VI) species  
716 in the system. In the case of the other oxidation states, the spectra with the lowest SPOIL  
717 value corresponds to colloidal U(IV)O<sub>2</sub> (SPOIL = 3.9) and U(V)-carbonate (SPOIL 13.9),  
718 respectively. Hence, both can be considered already as non-matching references. The shell  
719 fit of the best matching reference (U(VI) with lactate, pH 7) and of the ITFA-isolated spec-  
720 tra (Fig 7) is shown in Fig S21, while the EXAFS structural parameter are summarized in  
721 Table 4. In the case of the U(VI) species, the radial O<sub>ax</sub> distance (R<sub>Oax</sub>) matches R<sub>Oax</sub> ob-  
722 tained for U(VI)-lactate, while the radial distance of the equatorial (eq) oxygen atoms  
723 (R<sub>Oeq</sub>) is by 0.04 Å less, a value which exceeds the common absolute error in determina-  
724 tion of EXAFS radial distances of  $\pm 0.02$  Å.<sup>[93]</sup> Due to the vanishing thermally induced  
725 atomic disorder, a decrease of the Debye-Waller factor (DW) is expected for measure-  
726 ments at low temperatures,<sup>[94]</sup> thus we assumed for the U-U interaction a DW of 0.006 Å<sup>2</sup>  
727 and of 0.003 Å<sup>2</sup> for U(VI)-lactate and for the U(VI)/U(IV) species measured at RT and 15  
728 K, respectively. The fit of the uranium shell reveals a coordination number (CN) of one at  
729 3.88 Å and two at 3.83 Å for the U(VI) species and the U(VI)-lactate, respectively. Together  
730 with the common error in determination of CN of about 20%,<sup>[93]</sup> the gained structural pa-  
731 rameter (R, CN) deviates between the two spectra. However, these deviations are still rel-  
732 atively small, as visible in the overall agreement between the two spectra (Fig S21a and  
733 b). Note that especially for trimeric U(VI) species, the uranium core can be structurally  
734 different due to the presence or absence of a uranium-connecting central  $\mu_3\text{-O}$  atom<sup>[95-98]</sup>  
735 (Fig S17), which can lead to slight differences in the EXAFS structural parameter between  
736 the two isomeric forms. Furthermore, TFA yields from 81 chemically relevant systems the  
737 most probable references which are also the most reasonable from a chemical point of

738 view so that we conclude, together with the results of the luminescence spectroscopic  
739 studies (see above) the presence of a dimeric or trimeric U(VI)-lactate complex in the sys-  
740 tem.

741 In the case of the U(IV) species, the first shell oxygen peak is strongly reduced (Fig 7d)  
742 pointing to a strong structural disorder which deteriorates the shell fit if only one oxygen  
743 shell is included. Thus, the first shell needs to be split into at least two shells with  $R_{01} =$   
744  $2.36 \text{ \AA}$  and  $R_{02} = 2.61 \text{ \AA}$ , while for the fit, the total sum of their CNs are kept at  $\text{CN} = 8$ ,  
745 which was recently assumed for biogenic formed U(IV) species.<sup>[99,100]</sup> A carbon shell with  
746  $R_c = 2.89 \text{ \AA}$  is needed to reach a proper quality of the fit, while a phosphate group at  $3.06 \text{ \AA}$   
747 can be ruled out as proved by an F-test according to Downward *et al.* (see supporting in-  
748 formation)<sup>[101]</sup> Two uranium interactions (two up to three U at  $3.65 \text{ \AA}$  and at  $3.87 \text{ \AA}$ ) yield  
749 a good description of the degenerated peak between  $3.0\text{-}4.3 \text{ \AA}$  in the FT (Fig S21c)). This  
750 double peak in the Fourier transform differs significantly from other U(IV) compounds  
751 (Fig S19). According to the TFA analysis, no reference spectrum matches the U(IV) species  
752 and any attempts of fitting using a uraninite- or a ningyoite-like biogenic phosphate struc-  
753 ture<sup>[100]</sup> proved unsuccessful. Since these end products of uranium reduction by several  
754 environmentally relevant bacteria (Gram-positive and Gram-negative) and their spores  
755 can include a variety of U(IV) species, *e.g.* different phosphate compounds, various phos-  
756 phosphate references also showed no agreement with the recorded spectra (Fig S19).<sup>[25,26]</sup>  
757 However, in comparison to different other studies characterizing the biogenic products of  
758 the U(VI) reduction as  $\text{UO}_2$ ,<sup>[24,30-32]</sup> or ningyoite-like phosphates<sup>[100]</sup> in our study, another  
759 structure is formed. Furthermore, we cannot exclude that the isolated U(IV) spectrum  
760 consists of the sum of signals coming from multiple structurally different U(IV) species. In  
761 the special case, these species does not change their fractional ratios in the time series.  
762 Thus, we can give for the U(IV) species no reliable structural explanation at the moment.

763

764

765

766

767 Table 4. EXAFS shell fit structural parameter obtained for the best matching reference spectrum (U(VI)-lactate<sup>[92]</sup>) and  
 768 for the isolated spectra of the U(VI) and U(IV) species.

Path/sample	CN	r/Å	$\sigma^2/\text{Å}^2$	$\Delta E_0/\text{eV}$
<b>U(VI) with lactate at pH 7 at RT ([U] = 50mM, [lactate] = 0.5 M)</b>				
<b>U-O<sub>ax</sub></b>	2*	1.795(1)	0.0017(1)	4.0(3)
<b>MSO<sub>ax</sub></b>	2/	3.590/	0.0034/	4.0/
<b>U-O<sub>eq</sub></b>	5*	2.358(5)	0.0147(5)	4.0/
<b>U-U</b>	2.1(1)	3.826(4)	0.006*	4.0/
<b>U(VI) species at 15 K</b>				
<b>U-O<sub>ax</sub></b>	2*	1.796(2)	0.0020(1)	3.7(4)
<b>MSO<sub>ax</sub></b>	2/	3.592/	0.0040/	3.7/
<b>U-O<sub>eq</sub></b>	5*	2.321(6)	0.0191(8)	3.7/
<b>U-U</b>	0.95(8)	3.876(4)	0.003*	3.7/
<b>U(IV) species at 15 K</b>				
<b>U-O<sub>1</sub></b>	4.8(2)	2.360(3)	0.0054(4)	4.9(6)
<b>U-O<sub>2</sub></b>	3.2+	2.610(1)	0.0074/	4.9/
<b>U-C</b>	3.5(3)	2.891(7)	0.004*	4.9/
<b>U-U<sub>1</sub></b>	2.5(2)	3.648(4)	0.003*	4.9/
<b>U-U<sub>2</sub></b>	3.3(2)	3.870(4)	0.003*	4.9/

769 CN – coordination number, r – radial distance,  $\sigma^2$  – Debye-Waller factor,  $\Delta E_0$  - shift in energy threshold. Parameter fixed  
 770 (\*), linked (/), and linked to keep constant sum (+). Estimated standard deviations of the variable parameter as given  
 771 from EXAFSPAK in parenthesis. Amplitude reduction factor ( $S_0^2$ ) was set to  $S_0^2 = 0.9$ . In the case of U(VI) the twofold  
 772 degenerated 4-legged multiple scattering ( $MS_{O_{ax}}$ ) path, U-O<sub>ax(1)</sub>-U-O<sub>ax(2)</sub>-U, was included in the fit.

773

#### 774 4 Conclusions

775 A better understanding of the U(VI) reduction by the sulfate-reducing bacterium *D. hippei*  
 776 DSM 8344<sup>T</sup> is of high interest not only for the safe disposal of high-level radioactive waste  
 777 in clay rock, but also for different remediation approaches. In this study, we could verify  
 778 the reduction of U(VI) by this sulfate-reducing bacterium using different state-of-the-art  
 779 spectroscopic techniques (TRLFS, UV/Vis, HERFD-XANES and EXAFS). Together with var-  
 780 ious microscopic techniques, we were able to draw a more profound picture of the ongo-  
 781 ing processes. Reduction experiments with different media in combination with lumines-  
 782 cence spectroscopic investigations and speciation calculations showed the dependence of

783 the U(VI) reduction on the initial U(VI) species. The uranyl(VI)-carbonate species could  
784 not be reduced by the cells, but in contrast, the uranyl(VI)-lactate complex could be re-  
785 duced. In latter case, TEM-based analysis of the uranium-incubated cells showed ura-  
786 nium-containing aggregates on the cell surface and indicated the formation of membrane  
787 vesicles as a potential defense mechanism against cell encrustation. In connection with an  
788 increased amount of U(IV) over time determined by the different spectroscopic methods,  
789 a combined association-reduction process can be suggested as a possible interaction  
790 mechanism. Moreover, HERFD-XANES measurements verified the presence of U(V) dur-  
791 ing the experiment, proposing a single-electron transfer as a possible reduction mecha-  
792 nism for this sulfate-reducing genus. To our knowledge, this is the first proof of the occur-  
793 rence of U(V) during the U(VI) reduction by a sulfate-reducing microorganism. This study  
794 shows that the *Desulfosporosinus* species present in clay rock are able to reduce uranium  
795 and therefore immobilize it. The significant differences in interaction mechanisms com-  
796 pared to other microorganisms demonstrate the importance of studying the reduction  
797 behavior of bacteria of different genera. Furthermore, this study helps to better under-  
798 stand the complexity of redox processes in the environment, assists to close existing gaps  
799 in the field of bioremediation and provides new impulses for a comprehensive safeguards  
800 concept for a repository for high-level radioactive waste in clay rock.

801

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817



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