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1 Peptidoglycan as major binding motif for Uranium bioassociation on

2 Magnetospirillum magneticum AMB-1 in contaminated waters

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19 20

21 Abstract

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23 The U(VI) bioassociation on Magnetospirillum magneticum AMB-1 cells was investigated using 24 a multidisciplinary approach combining wet chemistry, microscopy, and spectroscopy methods to 25 provide deeper insight into the interaction of U(VI) with bioligands of Gram-negative bacteria for 26 a better molecular understanding. Our findings suggest that the cell wall plays a prominent role in 27 the bioassociation of U(VI). In time-dependent bioassociation studies, up to 95 % of the initial 28 U(VI) was removed from the suspension and probably bound on the cell wall within the first hours 29 due to the high removal capacity of predominantly alive Magnetospirillum magneticum AMB-1 30 cells. PARAFAC analysis of TRLFS data highlights that peptidoglycan is the most important 31 ligand involved, showing a stable immobilization of U(VI) over a wide pH range with the formation 32 of three characteristic species. In addition, *in-situ* ATR FT-IR reveals the predominant strong 33 binding to carboxylic functionalities. At higher pH polynuclear species seem to play an important 34 role. This comprehensive molecular study may initiate in future new remediation strategies on 35 effective immobilization of U(VI). In combination with the magnetic properties of the bacteria, a 36 simple technical water purification process could be realized not only for U(VI), but probably also for other heavy metals. 37

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40 1. Introduction

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42 Weathering and leaching of geogenic deposits as well as their industrial use can lead to high local 43 contamination of soils and waters with heavy metals and radionuclides. The resulting pollution of 44 the environment is a worldwide problem with serious consequences for the environment and local 45 populations. A particular challenge here is the remediation of radionuclide-polluted waters. Since chemical treatments are often associated with high costs and toxic waste, research into alternative 46 47 ways of purifying water has been ongoing for several years. One possibility here is the development 48 of customized nanomaterials for the removal of radionuclides. Examples include carbon nanotubes 49 as potential carriers of pollutants (Schierz and Zänker, 2009), hydroxylated vanadium carbide 50 $V_2C(OH)_2$ MXene nanosheets (Zhang et al., 2017), metal-organic frameworks with abundant 51 functional groups and tailorable structure (Li, J. et al., 2018), porous microcubes composed of fine 52 Fe₂O₃ nanoparticles (Li, X. et al., 2018), and MXenes, consisting of transition metal nitrides, 53 carbides and carbonitrides (Yu et al., 2022), to name just a few. Nanomaterials could have great 54 potential in water treatment. However, so far the practical application of these materials is still 55 difficult due to the complicated product process and the high costs. Another possibility is the use 56 of microorganisms for bioremediation purposes and the removal of heavy metals (Gadd, 2008; 57 Lesmana et al., 2009; Young et al., 2009). Several mechanisms of interactions of microorganisms 58 with radionuclides are known, like biosorption on functional groups of the cell surface (Lloyd and 59 Macaskie, 2002; Kelly et al., 2002; Merroun et al., 2005; Ojeda et al., 2008), bioaccumulation, 60 where the metal is taken up into the cell (Suzuki and Banfield, 2004; Brookshaw et al., 2012), the 61 enzymatic reduction of metals, which is called bioreduction (Beyenal et al., 2004; Wu et al., 2006), 62 and biomineralization, where radionuclides can precipitate with microbial generated ligands, *e.g.*, phosphate, sulphide or carbonate (Merroun et al., 2011; Macaskie et al., 2016). The investigated 63 64 microorganisms included Gram-negative and Gram-positive bacteria as well as fungi.

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The Gram-negative bacteria include, among others, magnetotactic bacteria (Bazylinski and Frankel, 2004). According to the current state of research, only a few studies on magnetotactic bacteria exist, although magnetotactic bacteria are widely distributed in aquatic environments, in sediments of freshwater, brackish, marine, and hypersaline habitats, where they make up an important part of the bacterial biomass (Lefèvre and Bazylinski, 2013). The abundance of the

71 magnetotactic bacteria has been determined by several researchers. Monteil et al. (2021) report 72 about a large abundance up to 5.8×10^5 cells mL⁻¹ of porewater in the sediments of Lake Pavin (France). In previous studies, the abundance was even found to be up to 10^7 cells mL⁻¹ in sediments 73 of aquatic systems (Spring et al., 1993; Flies et al., 2005; Jogler et al., 2009), so that already Spring 74 75 et al., (1993) assigned a dominating role to these bacteria in the microbial ecology in his studies of 76 Lake Chiemsee sediments. Magnetotactic bacteria belong to the family of Rhodospirilliaceae and 77 are associated with the Alpha-, Gamma-, and Deltaproteobacteria classes of the Proteobacteria 78 phylum and with the Nitrospirae phylum (Amann et al., 2007). They vary greatly in shape, existing 79 in spiral, vibrio, rod, or even coccoid forms (Lefèvre and Bazylinski, 2013). The special feature of 80 magnetotactic bacteria is that they synthesize intracellular magnetic mineral crystals, the so-called 81 magnetosomes (Balkwill et al., 1980), which are protected by a lipid bilayer membrane about 3-4 82 nm thickness. The magnetic crystals can be made of iron oxide magnetite (Fe₃O₄) (Frankel et al., 83 1983) or iron sulfide greigite (Fe₃S₄) (Mann et al., 1990). Some cells are able to form both types 84 of mineral (Bazylinski et al., 1993). The magnetosomes are responsible that magnetotactic bacteria 85 orient in the presence of external Earth's magnetic field lines (Blakemore, 1975) and actively swim 86 by the means of their flagellar rotation toward a preferred oxygen concentration (Frankel et al., 87 1997). They are either microaerophiles, anaerobes, or both and are generally found at the oxic-88 anoxic interface and the anoxic regions of the habitat (Bazylinski et al., 2013).

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90 Due to the magnetic properties of the magnetosomes, potential applications in microbiology, 91 biophysics, biochemistry, nanotechnology and also biomedicine are known, *e.g.*, immobilization 92 of biomolecule, drug delivery, targeted and controlled delivery of anticancer agents for tumor 93 therapy (Jacob et al., 2016; Afkhami et al., 2011; Mokrani et al., 2010). So far, a potential 94 environmental application for magnetotactic bacteria in water purification processes was not yet 95 implemented, although the use of the magnetic properties of these bacteria could play a major role 96 in water treatment by removing heavy metal-loaded bacteria from water using simple technical 97 means. Presumably it can be justified by the fact that so far only a few studies on the sorption of 98 heavy metals by magnetotactic bacteria are known to date. In the past, the uptake of Pu by magnetotactic bacteria was shown by adding purifed Pu, consisting of mixed α (²³⁸Pu, ²³⁹Pu, ²⁴⁰Pu) 99 and β (²⁴¹Pu) emitters to a culture of magnetotactic bacteria (Bahaj et al., 1998). In a study from 100 101 Bahaj et al. (1994) the effect of heavy metals (Al, Cd, Co, Cu, Fe, Mg, Mn, Pb, Zn) at different 102 concentrations (1 ppm, 10 ppm, 100 ppm) were measured on the motility of magnetotactic 103 bacteria. In somewhat recent studies more detailed data are given by Wang et al. (2011). The 104 removal efficiency of Ag(I) and Cu(II) by Magnetospirillum gryphiswaldense MSR-1 strain was 105 mentioned to be 91 % and 62 %, respectively when using a wet weight biomass of 10 g/L and an 106 initial concentration of 80 mg/L Ag(I) and Cu(II) at a pH of 4.0 and 5.0, respectively. In studies 107 with the magnetotactic bacterial isolate *Stenotrophomonas* sp. it was shown, that Au(III) was 108 removed from the contaminated water with a high biosorption capacity of 506, 369 and 308 mg 109 Au(III) per g dry weight biomass at the initial pH values of 2.0, 7.0 and 12.0, respectively (Song et 110 al., 2008). In recent studies, Pseudomonas aeruginosa Kb1, which was found to synthesis 111 intracellular magnetosomes, was able to remove 99.4 % and 70 % of Cd and Pb at initial 112 concentration of 4 ppm (Kabary et al., 2017). Jayaraman et al. (2021) showed that the strains of 113 Stenotrophomonas maltophilia, Pseudomonas aeruginosa and Achromobacter xylosoxidans are 114 able to tolerate higher concentration of Mn, Zn, Cu and Cr ranging from 20, 30, 50, 70, 90 and 115 150 mg. The studies were performed on modified nutrient agar medium using MnCl₂, ZnSO₄, 116 CuSO₄·5H₂O and K₂Cr₂O₇. Although these studies already indicate, that magnetotactic bacteria 117 could be a promising biosorbent for heavy metals, detailed information about binding properties, 118 molecular identification of the bioassociation process, and statements on the formed species are 119 missing. In the presented study, these issues are addressed using a multidisciplinary approach that 120 includes wet chemistry, microscopy, and spectroscopy. The highly sensitive *crvo*-time resolved 121 laser-induced fluorescence spectroscopy was used in combination with parallel factor analysis. 122 This new type of evaluation offers the advantage over the previous spectroscopic method to obtain 123 individual spectra of metal-ligand species from a sum spectrum. In-situ attenuated total reflection 124 Fourier transform infrared spectroscopy can furthermore contribute to the molecular identification 125 of the bioassociation process. These information are helpful for a better understanding of the 126 mechanisms involved in the interaction of magnetotactic bacteria with heavy metals and could 127 contribute to the development of remediation strategies of contaminated waters.

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Our studies focused on uranium (U) since, to our knowledge, there are no published studies to date showing the interaction of U with magnetotactic bacteria, although there should be great interest in it, since the existence of U in waters and sediments causes hazardousness to the environment and humans. The entry of U into the human body via the food chain, by inhalation of dusts, and through water can lead to serious illness or even death (Lloyd and Macaskie, 2002). 134 It is known from previous studies that U often binds to the cell wall of Gram-negative and Gram-135 positive bacteria by interacting with ligands in the cell wall such as phosphate, hydroxyl, carboxyl 136 and amino groups (Lloyd and Macaskie, 2002; Kelly et al., 2002; Ojeda et al., 2008; Merroun et 137 al., 2008; Krawczyk-Bärsch et al., 2018; Hufton et al., 2021). The cell wall of Gram-negative 138 bacteria is mainly made up of a thin peptidoglycan layer, which is rich in carboxylate groups. A 139 lipid/protein bilayer forms the outer membrane, including lipopolysaccharides with their phosphate 140 groups (Bäuerlein, 2003). In our studies, peptidoglycan, lipopolysaccharide and several other 141 ligands of the cell wall were used as reference and measured for possible binding sites of U to the 142 cell wall.

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145 **2. Materials and methods**

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147 2.1. Cultivation
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The strain of Magnetospirillum magneticum AMB-1 was kindly provided by the Molecular and 149 150 Environmental Microbiology Department of the Institute of Biosciences and Biotechnologies from 151 CEA Cadarache in France. The cells were grown in 200 mL flasks in 1.5 mM MagMin medium 152 containing Wolfe's mineral solution, Wolfe's vitamin elixir and iron-malate after Komeili et al. 153 (2004), modified by CEA Cadarache with the addition of iron malate (1:2000) and vitamin elixir 154 (1:2000). Before the cells were transferred to the flasks, the sealed flasks, filled with 98 mL of 155 MagMin medium (pH 6.9), were gassed with N₂/O₂ in a 98 % / 2 % ratio for 20 minutes to create 156 a low oxygen medium with a low oxygen gas atmosphere. Subsequently, 50 µL vitamin solution 157 and 50 µL iron malate solution were added to each flask. For inoculation of Magnetospirillum 158 magneticum AMB-1 cells, 2 mL of an inoculum were added to the medium. During the incubation, 159 the flasks were kept at 30 °C on a rotary shaker (Rotamax 120, Heidolph Instruments) at 80 rpm. 160 The best growth conditions and the highest optical density at 600 nm of 0.4 was achieved using 161 UV/Vis spectrometry (Specord 50 Plus, Analytik Jena, Germany) after an incubation of 24 h. 162 Evidence that the bacteria synthesize magnetosomes was provided by placing a small magnet close 163 to the flasks. After a few hours, an accumulation of bacteria formed (Supplementary Fig. 1).

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166 2.2. U bioassociation experiments with varying biomass concentration

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168 For each experiment cells were grown in 200 mL flasks in MagMin medium for 24 h at 30 °C on 169 a rotary shaker at 80 rpm. To reach a defined biomass concentration, a different number of flasks 170 was used, concentrated and the optical density at 600 nm (OD_{600nm}) was controlled. This resulted 171 in a biomass concentration of 89.00, 77.04, 49.62 and 21.45 mg dry biomass (DBM) with an 172 OD_{600nm} of 2.32, 1.64, 1.02 and 0.45, respectively. The cells were centrifuged at 7.870 x g for 10 173 minutes (Centrifuge Avanti J-20 CP, Beckman Coulter, Country?), suspended and washed twice 174 in sterilized tap water at pH 6.5. The washed cells were suspended again for each experiment in 175 100 mL sterilized tap water at pH 6.5. Subsequently, 100 µL of a 0.1 M UO₂(NO₃)₂ stock solution 176 were added to each experiment to reach a final U(VI) concentration of 0.1 mM. During the 177 incubation on a rotary shaker (Rotamax 120, Heidolph Instruments) at 30 °C, 1 mL of each cell 178 suspension was collected three times at distinct time points (5, 15, 30 and 45 min, as well as after 179 1, 2, 3, 4, 5, 6, 24 and 25 h). Each sample was centrifuged at 13.225 x g for 1 minute (Centrifuge 180 5415R, Eppendorf AG, Hamburg, Germany). The supernatants were sampled and acidified with 181 HNO₃ for Inductively Coupled Plasma Mass Spectrometry (ICP-MS) measurements using an 182 ELAN 9000 type ICP-MS spectrometer (Perkin Elmer, Überlingen, Germany) to determine the 183 residual U(VI) concentration within the supernatant.

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186 2.3. U bioassociation experiments with different pH

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188 For the experiments a high biomass concentration was chosen (74.3-82.8 mg DBM) for each 189 experiment, which was determined in prior by an OD_{600nm} in the range of 1.60–2.44. The cells were 190 centrifuged, suspended and washed twice in sterilized tap water at defined pH related to each 191 experiment (3.5, 4.5, 5.5, 6.5 and 7.5). The washed cells were suspended again for each experiment 192 in 101 mL sterilized tap water at the defined pH and treated as described in the previous experiment. 193 1 mL was taken for OD_{600nm} measurements. To reach a final U(VI) concentration of 0.1 mM, 194 100 µL of a 0.1 M UO₂(NO₃)₂ stock solution was added to each experiment. 1 mL of each cell 195 suspension was taken three times at distinct time points (5, 15, 30 and 45 min, 1, 2, 3, 4, 5, 6 and 196 24 h) during the incubation, centrifuged, acidified and used for ICP-MS measurements to

determine the U(VI) concentration. The cell pellets were dried for 25 h at 100°C in an oven (Memmert UE500, Schwabach, Germany) and the weight were determined. Together with the residual biomass, which was taken from the flask at the end of the experiments, the weight of the biomass was used for the calculation of the uranium removal capacity dependent on the dry biomass by normalizing the amount of removed U(VI) from the solution to the dry biomass.

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204 2.4. Live-dead staining

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206 During the U(VI) interaction experiments, samples were taken at the beginning of the experiments 207 and after 1, 3, 6 and 24 hours to check the viability of *Magnetospirillum magneticum* AMB-1 cells 208 depending on the U(VI) incubation time, but also on the pH. The cells were harvested by 209 centrifugation at 13.225 x g (Centrifuge 5415R, Eppendorf AG, Hamburg, Germany) for 1 min., 210 the supernatants discarded and the pellets resuspended in 200 μ L of tap water. A volume of 0.3 μ L of a Svto[®]9/propidium iodide mixture (vol/vol) (LIVE/DEADTM BacLightTM Bacterial Viability 211 212 Kit, InvitrogenTM) was added to the cell suspensions and incubated in the dark for 15 min. The green fluorescent dye SYTO[®]9 can diffuse through intact cell walls into the cells. The red 213 214 fluorescent dye propidium iodide can only enter dead cells through their defect cell walls. The cells 215 were washed again to remove the unbounded stains. The washed and stained cells were diluted in 216 330 µL 0.9% NaCl. The cell viability was observed using a confocal laser scanning microscope 217 (Leica CLSM, Leica Microsystems, Wetzlar, Germany).

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220 2.5. (Scanning) transmission electron microscopy ((S)TEM) and Energy-dispersive X-ray
 221 spectroscopy (EDXS)

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For (S)TEM/EDXS studies, U(VI) interaction experiments were performed using cell suspensions with the same optical density at pH 6.5. An initial U(VI) concentration of 0.1 mM was adjusted and the sample was stored on a rotary shaker at 30°C. After 5 hours, the U(VI) incubation was stopped. At the end of the experiment, the cells were harvested by centrifugation for 10 minutes at 7.870 x g (Centrifuge5804R, Eppendorf AG, Hamburg, Germany) at 30 °C. The supernatant was 228 removed and the cell pellet was washed twice with sterilized tap water at the defined pH. 229 Subsequently, the cells were fixed with 2.5 % (vol/vol) glutardialdehyde from a 25 % (vol/vol) 230 stock solution (Serva, Heidelberg, Germany) and stored at 4 °C. A reference sample without U was 231 treated the same way. The fixed samples were prepared for transmission electron microscopy 232 studies at the Center for Regenerative Therapies Dresden (CRTD) of the Technical University 233 Dresden (Germany). In particular, ultrathin sections of < 100 nm were placed on carbon-coated Cu 234 grids (200 mesh, Plano GmbH, Wetzlar, Germany). Bright-field transmission electron microscopy 235 (TEM) images were recorded with an image-C_s-corrected Titan 80-300 microscope (Field Electron 236 and Ion Company (FEI), Eindhoven, The Netherlands) operated at an accelerating voltage of 237 300 kV. Furthermore, high-angle annular dark-field scanning transmission electron microscopy 238 (HAADF-STEM) imaging and spectrum imaging analysis based on energy-dispersive X-ray 239 spectroscopy (EDXS) were performed with a Talos F200X (Thermo Fischer Scientific) microscope 240 equipped with a high-brightness X-FEG electron source and a Super-X EDX detector system at an 241 accelerating voltage of 200 kV (FEI). Prior to (S)TEM analysis, the specimen mounted in a high-242 visibility low-background holder was placed for 2 s into a Model 1020 Plasma Cleaner (Fischione, 243 Export, PA, USA) to remove potential contamination.

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246 2.6. Cryo-time resolved laser-induced fluorescence spectroscopy (cryo-TRLFS)

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248 The cryo-TRLFS method was chosen due to the high sensitivity toward U(VI) complex formation 249 in aqueous solutions (Moulin et al. 1990, 1995). For our experiments cultures of Magnetospirillum 250 magneticum AMB-1 with an optical density of 1.6 at 600 nm were taken after 24 h of incubation 251 and washed twice using sterilized tap water at defined pHs (3.5, 4.5, 5.5, 6.5, 7.5). Each washed 252 cell suspension at the defined pH was resuspended in sterilized tap water and transferred into 4 253 flasks. A 0.1 M stock solution of $UO_2(NO_3)_2$ was added to the flasks to adjust an initial U(VI) 254 concentration of 0.1 mM in the cell suspension. The flasks were stored at 30 °C on a rotary shaker 255 (Rotamax 120, Heidolph Instruments). After 0.5, 2, 5 and 24 h samples were taken and centrifuged 256 for 5 min at 7870 x g (Centrifuge5804R, Eppendorf AG, Hamburg, Germany). A volume of 1 mL 257 of each supernatant was used for determination of the U concentration by means of ICP-MS. For 258 cryo-TRLFS measurements each pellet was washed twice in sterilized tap water at the defined pH, 259 and transferred into a UV cuvette, shock frozen by N₂ and stored at -80 °C. In preparation of the

260 cryo-TRLFS measurements, the pellets were transferred just before into a cupper holder. At 153 K 261 the U(VI) luminescence was measured after excitation with laser pulses at 266 nm (Minilite high-262 energy solid-state laser; Continuum) and average pulse energy of 300 µJ. The emission of the 263 samples was recorded using an iHR550 spectrograph (HORIBA Jobin Yvon) and an ICCD camera 264 (HORIBA Jobin Yvon) in the 370.0-670.0 nm wavelength ranges by averaging 100 laser pulses 265 and using a gate time of 2000 µs. The measurements were performed from 0.1 µs to 12.503 µs to 266 ensure that the lifetimes of all species present were detected. The specific separation of the sum 267 spectra obtained from the measurements into individual spectra was performed using parallel factor 268 analysis (PARAFAC). For more information see Drobot et al. (2015). Important ligands, e.g., 269 peptidoglycan (PGN) from Bacillus subtilis (Sigma Aldrich), lipopolysaccharide (LPS) from 270 Pseudomonas aeruginosa (Sigma Aldrich), L-rhamnose (Sigma Aldrich), D-(+) galactose (Sigma 271 Aldrich), D-(+) mannose (Sigma Aldrich) were used as reference ligands and measured for 272 comparison and interpretation of possible binding sites of U(VI) to the cell wall. For this, each 273 ligand was dissolved in 2 mL of sterile tap water at pH of 3.5, 4.5, 5.5, 6.5 and 7.5 with a ligand 274 excess of 1:20. A defined volume of a 0.1 M UO₂(NO₃)₂ stock solution was added to reach a final 275 U concentration of 0.1 mM. The samples were transferred into a UV cuvette, shock frozen by N₂, 276 stored at -80°C and used for cryo-TRLFS measurements.

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279 2.7. In-situ attenuated total reflection Fourier-transform infrared spectroscopy (ATR FT-IR)

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281 For molecular identification of the U(VI) bioassociation process of Magnetospirillum magneticum 282 AMB-1 cells, *in-situ* ATR FT-IR spectroscopy with a sub-minute time resolution was performed. 283 On a Bruker Vertex 80/v vacuum spectrometer equipped with a Mercury Cadmium Telluride 284 detector infrared spectra were measured from 4000 to 400 cm⁻¹. Spectral resolution was 4 cm⁻¹, 285 and spectra were averaged over 256 scans. A horizontal diamond crystal with nine internal 286 reflections (DURA SamplIR II, Smiths Inc.) was used. Further details on the experimental ATR 287 FT-IR spectroscopic setup are compiled in Müller et al. (2012). In our experiments a suspension 288 of Magnetospirillum magneticum AMB-1 cells after 24 hours of incubation was centrifuged at 289 7870 x g for 10 minutes, washed twice with autoclaved tap water (at pH 4.5 and 6.5) and 290 resuspended in 100 µL tap water at the same pH. A thin cell film was prepared directly on the 291 surface of the ATR diamond crystal as stationary phase by pipetting twice 1 µL of the sample on 292 the ATR crystal and subsequent drying with a gentle stream of N₂. The cell film was flushed using 293 a flow cell with a blank solution (sterilized tap water, pH 4.5 and 6.5) with a flow rate of 294 0.1 mL min⁻¹ for 60 min for conditioning. Subsequently, a 0.01 mM uranylchloride solution (in 295 sterilized tap water, pH 4.5 and 6.5) was rinsed over the cell film. Difference spectra are calculated 296 at several times during the U(VI) bioassociation process. After 120 min of bioassociation, 297 equilibrium was reached and no further U(VI) accumulation or change of the cell film was observed 298 within the spectral data. Then, the U(VI)-loaded cell film was flushed again with sterilized tap 299 water for 60 min for controlling the possible reversibility of the sorbed species. Further details on 300 the calculation of difference spectra are given in Müller et al. (2013).

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303 **3. Results**

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305 3.1. U(VI) bioassociation of Magnetospirillum magneticum AMB-1

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307 The U(VI) bioassociation of *Magnetospirillum magneticum* AMB-1 cells was first determined by 308 time-dependent experiments, using different amounts of biomass (89.00, 77.04, 49.62 and 309 21.45 mg DBM) and an initial U(VI) concentration of 0.1 mM. The experiments were performed 310 at pH 6.5, each with an incubation time of 25 h. The results showed a fast increase of the U(VI) 311 bioassociation during the first hour for all biomass concentrations (Fig. 1). Equilibrium conditions 312 were already reached in the experiment with the highest biomass concentration after 1 h. After 24 h 313 at the latest, a steady state has been established in all other experiments. The removal efficiency of 314 U(VI) by Magnetospirillum magneticum AMB-1 cells appears to be very effective, independent of 315 the biomass concentration. As shown in Fig. 1, between 85.7 and 95.5 % of the initial U(VI) was 316 removed from the suspensions, except the experiment with the lowest biomass. Here, only 55.8 % 317 of the U(VI) was bound.

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In further U(VI) bioassociation experiments a defined biomass concentration, each in a range from
74.3–82.8 mg DBM was chosen at different pH (3.5, 4.5, 5.5, 6.5, 7.5). These experiments provide

321 evidence of a pH dependent bioassociation. It is shown in Fig. 2, that the most U is bound at pH 6.5. 322 The U(VI) bioassociation, measured in mg U/g DBM, shows the highest value of 30.17 mg U/g323 DBM after 24 h. All other pH values included, 25.2–27.6 mg U were absorbed per 1g DBM. It is 324 a fast bioassociation that takes place in the first hour. Already 77.5 % of the U is removed from the 325 suspension at pH 6.5. After 24 h, at the end of our experiment, 95 % of the initial U concentration 326 is bound to Magnetospirillum magneticum AMB-1 cells. A slower bioassociation at the beginning 327 of the experiment could be detected for acidic pH. At pH 3.5 there are only 46.7 % U(VI) bound 328 in the first hour. Nevertheless, at the end of the experiment 86.7 % of U(VI) were removed from 329 the suspension, even indicating an efficient removal of U(VI) by Magnetospirillum magneticum 330 AMB-1 cells at low pH.

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- 333 *3.2. Cell viability by live-dead staining*
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335 During the U(VI) removal experiments, which were performed with an initial U(VI) concentration 336 of 0.1 mM at pH 3.5–7.5 the cells were tested for their cell viability by live-dead staining (Syto®9 337 and propidium iodide) using CLSM. The results show that high numbers of viable or living cells 338 were detected after 1 h incubation, with the exception of pH 3.5, where individual agglomerates of 339 cells were visible (Supplementary Fig. 2). Compared to samples after 3 h of incubation, the AMB-340 1 cells showed nearly the same number of viable cells. After 24 h the majority of cells were viable 341 with the exception of the cells, which were handled in tap water at a pH of 3.5 and also at a pH of 342 7.5. At acidic and basic pH the cells have only a very limited viability after 24 h. Agglomeration 343 of dead cells are already determined at the beginning of the experiment at pH 3.5.

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346 3.3. Localization of U by (S)TEM/EDXS analysis

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For (S)TEM/EDXS studies, ultrathin sections of *Magnetospirillum magneticum* AMB-1 cells loaded with 0.1 mM U for 5 h at pH 6.5 were prepared. The bright-field TEM image in Fig. 3a shows a typically elongated *Magnetospirillum magneticum* AMB-1 cell that has formed up to five magnetosomes, which are visible as black dots. EDXS analysis clearly confirms that they exclusively consist of Fe (Fig. 3b). Furthermore, element distribution analysis of P (Fig. 3c) and U
(Fig. 3d) clearly indicates that U is predominantly located in the cell wall.

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356 *3.4. Determination of U species from cryo-TRLFS using PARAFAC analysis*

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358 After the incubation of Magnetospirillum magneticum AMB-1 cells with 0.1 mM U(VI) at different 359 pH (3.5–7.5) and different contact times (0.5, 2, 5 and 24 h), the cells were washed, centrifuged 360 and used for *cryo*-TRLFS measurements. A subsequent analysis of all emission spectra from the 361 TRLFS data set by PARAFAC extracted the single component spectra of five U(VI) species, which 362 were probably formed during the U(VI) bioassociation of Magnetospirillum magneticum AMB-1 363 cells (Fig. 4b, c, d, f, g). The single component spectra are characterized by different emission 364 bands. For the identification and assignment of the extracted spectra to possible binding sites of U 365 on the cells, reference spectra of potential ligands loaded with U(VI) were measured. Since EDXS 366 elemental distribution analysis clearly indicate that U(VI) is predominantly bound in the cell wall, 367 important ligands, e.g., peptidoglycan (PGN), lipopolysaccharide (LPS), L-rhamnose, D-(+) 368 galactose and D-(+) mannose were considered as possible complexants for U(VI) on 369 Magnetospirillum magneticum AMB-1 cell walls. The luminescence properties have shown that 370 there is no correspondence with the extracted single component spectra of U species formed on 371 Magnetospirillum magneticum AMB-1 cells with LPS, L-rhamnose, D-(+) galactose and D-(+) 372 mannose (Supplementary Fig. 3). However, the situation is completely different concerning PGN. 373 In Fig. 4b, c, d three extracted single component spectra (species 1-3) are shown together with the 374 appropriate reference spectra of U loaded PGN from Bacillus subtilis. Peak positions and ratios of 375 the U associated species indicate a very good match with the three reference spectra of U-PGN 376 (Tab. 1). Since the studies were performed in the pH range from 3.5 to 7.5, statements on the pH 377 dependent formation of the U species with PGN are significant. As shown in the relative 378 luminescence intensity distribution of the PARAFAC extracted species (1-5) versus pH and time 379 (Fig. 4e), species (1) is mainly significant in the acidic pH range and is rather negligible in the neutral and basic pH range. Species (2) dominates the bioassociation of U(VI) in the mentioned 380 381 broad pH range, with a dominance at pH 5.5 being emphasized. Species (3), on the other hand, 382 only gains significance in the basic pH range. The detected properties of the species are also proved 383 by studies performed on the U-PGN references of the relative luminescence intensity distribution

of the extracted U-PGN species. Fig. 4a shows the significance of the U-PGN species as a function
of pH by PARAFAC analysis, which clarifies the distribution of the U-PGN species in the pH range
from 3.5 to 7.5.

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388 In addition to the PGN species two more species should be mentioned. Species (4) shows four 389 characteristic maxima of the luminescence emission peaks (Fig. 4f) and a dominance in the pH 390 range from 3.5–4.5 (Fig. 4e). The emission peaks are comparable to literature data of reference 391 spectra. These are spectra of molecules containing carboxylic acids. A study on the interaction of 392 UO2²⁺ with malonate by using TRLFS and a metal concentration relevant for environmental aquatic 393 systems in a 0.1M perchloric media at pH 4 shows comparable data (Brachmann et al., 2002). The position of the emission bands of $UO_2C_3H_2O_4^{0}_{(aq)}$ are in good agreement with those of species (4). 394 395 Thus, we conclude the formation of a malonate-like species at acidic pH during the bioassociation 396 of U(VI) on Magnetospirillum magneticum AMB-1 cells.

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The PARAFAC extracted single component spectra of species (5) is characterized by a broad emission band. Similar spectra are known from the literature as an unidentified species (Bader et al., 2018). In *Magnetospirillum magneticum* AMB-1 cells this species exists mainly at $pH \ge 6.5$. In this pH range it may be the dominant U species bound on *Magnetospirillum magneticum* AMB-1 cells and thus have to be considered as an important species in the bioassociation process at higher pH.

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406 3.5. Analysis of U(VI) coordination by in-situ ATR FT-IR

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In-situ ATR FT-IR spectroscopy allows to discriminate which functional groups are responsible for U(VI) complexation at microorganism interfaces, *e.g.*, carboxylate, phosphoryl, amino or even hydroxyl groups (Barkleit et al., 2008; Barkleit et al., 2011; Comarmond et al., 2016; Jiang et al., 2004; Li et al., 2010; Theodorakopoulos et al., 2015; Krawczyk-Bärsch et al., 2018; Hilpmann et al., 2022). The experimental procedure involves the preparation of a cell film as stationary phase directly on the ATR crystal, which is then rinsed with aqueous background and U(VI) solutions at identical pH and ionic strength. In the meantime the cell film is continuously monitored by 415 recording single beam spectra in the sub-minute range. The calculation of difference spectra 416 extracts only changes of the cell film that are associated with U(VI) interaction, constant spectral 417 parts are not given. The time-resolved ATR FT-IR spectra at pH 4.5 and 6.5 are comparatively 418 presented in Fig. 6. First, the cell film is equilibrated to aqueous sample conditions, e.g., pH 4.5 419 and 6.5 by flushing a blank solution. The red traces in Fig. 5 show only weak absorption bands 420 during last 30 minutes of conditioning that can be attributed to cell interactions in contact with 421 water. This is a measure of quality for the stability of the cell film and hence, prerequisite for the 422 in-situ ATR FT-IR experiment. The black spectra are difference spectra calculated at different 423 times of U(VI) accumulation, ranging from 5 to 120 minutes. At both pH conditions significant 424 spectral changes are observed. The increasing intensities of the bands upon U(VI) contact time suggests metal accumulation at the cell surface. The bands below at 940 cm⁻¹ are attributed to the 425 426 asymmetric stretching vibrational mode of the uranyl(VI) moiety, v₃(UO₂). Generally, the frequency of this mode is observed at 961 cm⁻¹ for the fully hydrated ion in strong acidic aqueous 427 428 solution (Quilès and Burneau, 2000). Upon complexation in solution and at biogeochemical 429 interface it is shifted to lower frequencies (Müller et al., 2008; Kretzschmar et al., 2021; Hilpmann 430 et al., 2022).

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432 At pH 4.5, $v_3(UO_2)$ is observed at 925 cm⁻¹, characteristic for complexation to carboxylate groups 433 (Fig. 5a). This is in accordance with the spectral signature, we observe at higher wavenumbers. The bands at 1453 cm⁻¹ and 1535 cm⁻¹ can be assigned to the symmetrical $v_s(COO^-)$ and 434 435 asymmetrical $v_{as}(COO^{-})$ stretching vibrations of the carboxylate group (Barkleit et al., 2008; 436 Theodorakopoulos et al., 2015; Krawczyk-Bärsch et al., 2018). The spectral region, where the 437 asymmetric v_{as}(PO) stretching modes are observed, shows also intense peaks at 1197 and 1106 cm⁻¹, suggesting that also phosphoryl functional groups are involved in U(VI) binding. 438 439 However, since the symmetry of the $v_3(UO_2)$ band hints strongly to the predominance of only one 440 surface species and phosphoryl binding is characterized by a stronger shift of $v_3(UO_2)$ to 905 cm⁻¹, 441 this binding motif can be ruled out. With regard to the time-resolved data, the $v_3(UO_2)$ band shows 442 a fast bioassociation of U during the first minutes. After 60 min sorption still takes place, but to a 443 less extent, possibly due to mostly occupied binding sites. These findings agree very well with the 444 kinetic batch results (Fig. 1, 2).

446 At pH 6.5, the spectra offer some significant differences (Fig. 5b). First, it must be noted that the 447 overall intensity is considerably reduced in comparison to lower pH. Second, the spectral signature 448 has changed, the $v_3(UO_2)$ mode is now characterized as an asymmetric-shaped broad band. Here, a multiple species set can be derived from 2nd derivative analysis of the time-resolved data. At 449 15 min of U(VI) bioassociation, three local peak maxima at 923, 911 und 896 cm⁻¹ are extracted 450 451 (data not shown). With ongoing bioaccumulation, the v₃(UO₂) mode is distinctly shifted to wavenumbers below 900 cm⁻¹ and contributions from 923 and 911 cm⁻¹ become less important. 452 453 One may interpret this spectral data set as a combination of U(VI) bioassociated with carboxyl as 454 well as phosphoryl functionalities, as this was observed for certain microorganisms (Bader et al., 455 2017; Krawczyk-Bärsch et al., 2018). The vibrational modes of the ligands $v_{as s}(COO^{-})$ and $v_{as}(PO)$ 456 would support this. However, the appearance of $v_3(UO_2)$ mode is different in comparison to these 457 previous works, where we clearly observed only two contributions, (1) the coordination to carboxylic groups with local band maximum at 925 cm^{-1} and (2) to the phosphorylic groups at 458 905 cm⁻¹. Local band maxima below 900 cm⁻¹ were not observed so far. Unfortunately, the 459 460 information given by (S)TEM/EDXS elemental distribution analysis, where phosphorous 461 compounds together with U are detected in the cell wall, do not bring further insight on the binding 462 motif. Therefore, an alternative interpretation of the IR data set becomes necessary. In a previous 463 study of the complexation of U(VI) with citrate, a hydroxy-tricarboxylic acid, we observed in the 464 experimental IR data a similar shifting of the $v_3(UO_2)$ mode with increasing complex nuclearity 465 and changing speciation from binary U(VI)-CA to ternary complexes involving also hydoxides (Kretzschmar et al., 2021). Spectral shifts were observed from 961 down to 879 cm⁻¹ with changing 466 467 absorption properties of the different complexes.

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469 As a last step of the *in-situ* ATR FT-IR experiment, the U(VI) loaded cell film is again flushed 470 with the blank solution at the respective pH (blue traces in Fig. 5). Thus, the reversibility of the 471 bioassociation process is given by the difference spectra calculated at distinct times after changing 472 aqueous solutions. Please note the differences in absolute intensities upon comparing the sorption 473 (black) and flushing IR spectra (blue). At pH 4.5 the spectral features at both stages are very similar 474 and the reversibility seems to be very low. That means, the coordination of U(VI) to carboxylic 475 groups at pH 4.5 is very strong and only few quantities are removed from the cell walls by simply 476 flushing the blank solution. Again, the situation is different at pH 6.5. From the set of coordinating species only those exhibiting the $v_3(UO_2)$ mode mostly red shifted to 899 cm⁻¹ is reversible. From 477

478 comparing the relative intensities, it seems that the assigned polynuclear species is quantitatively479 removed from the cells upon flushing a blank solution at the same pH.

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482 4. Discussion

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484 The interaction of U(VI) with *Magnetospirillum magneticum* AMB-1 cells is characterized by a 485 fast pH dependent bioassociation that takes place in the first hours of incubation due to the removal 486 capacity of predominantly alive cells. Up to 95 % of the initial U(VI) concentration was bound to 487 the cells, which tolerate a U(VI) concentration of 0.1 mM, but are not able to survive permanently 488 at acidic and basic pH. Similar results, which were received under similar experimental conditions, 489 were reported in Gerber et al. (2016). Batch sorption and flow cytometry studies using the live-490 dead staining approach confirmed that metabolically active cells of a Gram-negative Acidovorax 491 facilis strain were able to remove nearly 100 % of the initial U(VI) concentration from the solution 492 in the first hours of incubation with 0.05 mM and 0.1 mM U(VI) at pH 5. Also Hufton et al. (2021) 493 mentioned the highest U(VI) binding to cell wall components in Gram-negative bacteria at pH 5.5. 494 A high sorption capacity was also determined by the Gram-positive bacterium Brachybacterium 495 sp. G1 (Hilpmann et al., 2022), which was explained by the high number of carboxylate groups 496 within the PGN layer of the cell wall (Bäuerlein, 2003). In the previously performed infrared 497 spectroscopic studies on Brachybacterium sp. G1 by Bader et al. (2018) it has already been shown 498 that carboxylate groups of the cell wall were dominantly involved in the U(VI) bioassociation. It 499 seems that high sorption capacities of U(VI) could be found both in both Gram-positive and in 500 Gram-negative bacteria, although the structure of the cell walls differs. In Gram-positive bacteria 501 the cell wall components are mainly PGN (Hufton et al., 2021), while only a thin, 4 nm thick, PGN 502 layer is found in the cell walls of Gram-negative bacteria (Bäuerlein, 2003). In addition to the PGN 503 layer the Gram-negative cell wall also contains an outer membrane, which consists of 504 phospholipids and lipopolysaccharides, representing potential binding sites for the predominant positively charged UO2²⁺ species. Involvement of phospholipids and lipopolysaccharides in the 505 506 bioassociation would therefore be expected and has been demonstrated in previous studies on 507 Gram-negative bacteria (Kelly et al., 2002; Merroun et al., 2005; Llorens et al., 2012; Luetke et al., 508 2012; Krawczyk-Bärsch et al., 2018). In contrast, in the presented work, it is clearly shown that 509 phosphoryl sites could be excluded as major binding site for U(VI). The data obtained by ATR FT-

510 IR spectroscopy provide evidence for the formation of strong complexation of U(VI) with 511 carboxylic functionalities at pH 4.5 during the first minutes of incubation. The spectroscopic proof 512 of a fast U(VI) bioassociation agrees very well with the kinetic batch results. TRLFS studies in 513 combination with PARAFAC confirm the complexation of U(VI) to carboxylate groups as 514 described by ATR FT-IR. The comparison of three PARAFAC extracted single component spectra 515 with the appropriate reference spectra of U(VI) loaded PGN from Bacillus subtilis showed an 516 excellent match and emphasizes that PGN is the most important ligand in the bioassociation of 517 U(VI) to Magnetospirillum magneticum AMB-1 cells. A pH dependent study indicates furthermore 518 that the complexation of U(VI) with PGN even takes place in the investigated pH range from 3.5-519 7.5. In previous potentiometric titration and TRLFS studies of Barkleit et al. (2009) on the interaction of UO2²⁺ with PGN reference samples, three different U-PGN complexes were 520 521 identified as well. However, we must take into account that the previous studies were not conducted 522 under the same conditions as our studies. All solutions in the previous studies were prepared with 523 carbonate-free deionized water with an ionic strength of 0.1 M by adding stock solutions from NaClO₄·H₂O. Also, UO₂²⁺ was added as UO₂(ClO₄)₂·6H₂O and all samples were measured at room 524 525 temperature. To obtain comparable data from the reference studies to our experiments with 526 Magnetospirillum magneticum AMB-1 cells, tap water was used. High ionic strength was thus 527 omitted in order to provide the bacteria with a natural environment in which they can survive. For the same reason, care was also taken to use the added UO_2^{2+} in form of $UO_2(NO_3)_2$ stock solution. 528 529 The most important difference, however, is that in the studies of Barkleit et al. (2009) mixed spectra 530 were presented, whereas in our investigations with the help of PARAFAC single spectra of U-PGN 531 could be shown. Nevertheless, the results of Barkleit et al. (2009) are indicating possible 532 complexations due to the observed luminescence properties. Following these studies, species U-PGN 1 was determined in our studies at acidic pH. According to the previous studies, it could be 533 534 assigned to the 1:1 uranyl carboxyl PGN complex R-COO-UO₂⁺ (glutamic acid or diaminopimelic 535 acid), which appears between pH 3 and 5. U-PGN 2 could be assigned to the 1:2 uranyl carboxyl 536 chelate complex (R-COO)₂-UO₂, which dominates around pH 5.6 (Barkleit et al. (2009). In the 537 neutral pH range the U-PGN 3 species is dominating. This U-PGN species might be the uranyl 538 chelate complex with the mixed coordination (R–COO–UO₂⁽⁺⁾–Ai–R) (Ai = NH₂ or O⁻). 539

540 The interpretation of time-resolved ATR FT-IR spectroscopy data obtained at pH 6.5 indicates 541 differences. Considerable shifts, when increasing U(VI) loading at the cell surface, hint to multiple

542 surface species. Initially, possibly mononuclear U(VI) species are predominating, whereas at pH 543 6.5, polynuclear species become more prominent. The interpretation of the TRLFS/PARAFAC 544 data also reflect the transition from mononuclear to polynulear U(VI) carboxylic coordination. In 545 Bader et al. (2018) similar spectra were described as a typical loss of fine structure of polynuclear 546 species, which they assigned to the 3:5 U(VI) hydroxo complex ($(UO_2)_3(OH)_5^+$). Wollenberg et al. 547 (2021) summarizes it more generally and assume that this polynuclear species could consist of 548 ligands in which amino, hydroxyl or sulphonyl groups could be important. In Magnetospirillum 549 magneticum AMB-1 cells they may become the dominant U(VI) species, which mainly exists at 550 $pH \ge 6.5$. In contrast to the data obtained at pH 4.5, ATR FT-IR spectroscopy data indicate that the 551 polynuclear species seems to be not as strongly coordinated and presents a higher reversibility 552 under the given experimental conditions. When using magnetotactic bacteria for remediation 553 purposes, this higher reversibility at $pH \ge 6.5$ will not be a disadvantage. In open systems there 554 will always be an inflow and thus a continuous flow of a high amount of bacteria.

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556 Even though magnetotactic bacteria show similar strong sorption capacities of heavy metals and 557 radionuclides as other Gram-negative or Gram-positive bacteria, there are some advantages that 558 make magnetotactic bacteria interesting for the use in bioremediation of contaminated waters: 559 i) Magnetotactic bacteria prefer to live planktonically (Lefèvre et al., 2013) and thereby provide an 560 ideally large surface area for interactions with metals. ii) They do not form biofilms, as is known 561 from many other bacteria such as *Bacillus* spp. (Zhong et al., 2021) or *Shewanella* sp. (Cao et al., 562 2011). Biofilm consists of multilayered cell clusters embedded in a matrix of exopolymeric 563 substance (EPS), which facilitate the adherence of microorganism. As shown in Zhong et al. 564 (2021), the EPS of these biofilms might also play a key role in U(VI) sequestration where U(VI) 565 could be bound through cellular phosphate, hydroxyl, carboxyl, and amide groups through 566 enzymatic bioprecipitation (Wei et al., 2017). However, biofilms are less suitable for flow-through 567 systems than planktonic bacteria due to the risk of clogging and sedimentation, and the need for a 568 carrier matrix which results in larger amounts of waste during disposal. The removal of heavy 569 metal-loaded biofilms thus seems difficult and cost intensive. Similar problems could also arise 570 with filament networks, e.g., fungal systems, although fungi are also known as very good 571 biosorbents for heavy metals and radionuclides due to the immobilization in the mycosphere, the 572 sorption to cell walls or the uptake into fungal cells (Baeza and Guillén, 2006; Harms et al., 2011; 573 Vázquez-Campos et al., 2015; Wollenberg et al., 2021). iii) Magnetotactic bacteria are unique 574 species of bacteria due to the presence of intracellular magnetic nanoparticles. With simple and 575 cheap technical means, the heavy metal-loaded magnetotactic bacteria could thus be removed very 576 easily from water with the help of magnets. In a laboratory study by Tanaka et al. (2016) it was 577 shown that over 80 % of bacteria were successfully recovered within 10 hours by magnetic force. 578 Even in our small microcosm experiments we could already show that this is already possible with 579 a small magnet.

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582 **5. Conclusions**

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584 The results obtained from our multidisciplinary approach combining microscopy and different 585 spectroscopic techniques to study the U(VI) biassociation on magnetotactic bacteria extend our 586 knowledge about the interactions of U(VI) with bioligands in the cell wall of Gram-negative 587 bacteria. In contrast to the previously characterized binding of U(VI) to carboxyl and phosphoryl 588 groups of Gram-negative bacteria, carboxylic functionalities now play the dominant role in the 589 bioassociation of U(VI) by Magnetospirillum magneticum AMB-1 showing a stable 590 immobilization of U(VI) over a wide pH range. The results presented highlight the dominant role 591 of the ligand peptidoglycan as main sorbent of U(VI) on the cell wall of Magnetospirillum 592 magneticum AMB-1 cells. Three characteristic peptidoglycan species were identified by use of 593 respective references. Especially with regards to the development of innovative bioremediation 594 strategies of contaminated water, the presented studies show clearly that Magnetospirillum 595 magneticum AMB-1 cells are suitable candidates. They can survive as planktonic cells both in a 596 wide pH range and with relatively high U(VI) concentrations of up to 0.1 mM, while effectively 597 and almost completely immobilizing U(VI). In combination with its magnetic properties, 598 Magnetospirillum magneticum AMB-1 cells offer many advantages for the development of various 599 bioassociation technologies not only for U(VI), but probably also for many other heavy metals.

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613	Appendix A. Supporting information
614	Supplementary data associated with this article can be found in the online version at:
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616	
617	References
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