Science of the Total Environment 955 (2024) 177210

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Uranium (VI) reduction by an iron-reducing *Desulfitobacterium* species as single cells and in artificial multispecies bio-aggregates

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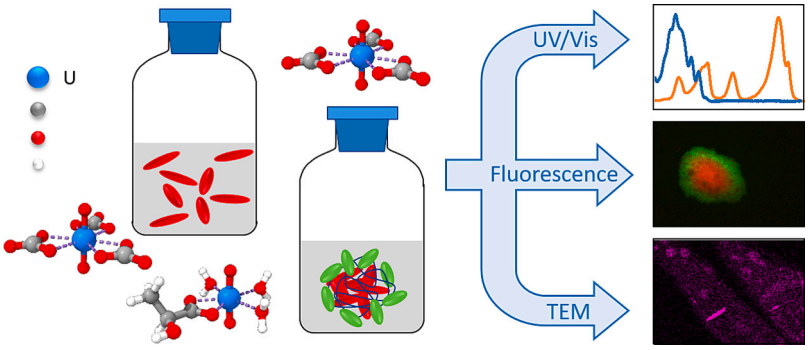
HIGHLIGHTS

- Investigation of speciation-dependent microbial U(VI) reduction by an iron-reducing bacterium.
- Reduction of U(VI)-carbonate but not U (VI)-lactate complex.
- First evidence of U(VI) reduction by artificial multispecies bio-aggregates.
- Rearrangement of cells within the artificial biofilm under aerobic conditions.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Yifeng Zhang

Keywords:
Microbial uranium(VI) reduction
Iron-reducing bacteria
Speciation-dependent reduction
Artificial biofilms

ABSTRACT

Microbial U(VI) reduction plays a major role in new bioremediation strategies for radionuclide-contaminated environments and can potentially affect the safe disposal of high-level radioactive waste in a deep geological repository. *Desulfitobacterium* sp. G1-2, isolated from a bentonite sample, was used to investigate its potential to reduce U(VI) in different background electrolytes: bicarbonate buffer, where a uranyl(VI)-carbonate complex predominates, and synthetic Opalinus Clay pore water, where a uranyl(VI)-lactate complex occurs, as confirmed by time-resolved laser-induced fluorescence spectroscopic measurements. While *Desulfitobacterium* sp. G1-2 rapidly removed almost all U from the supernatants in bicarbonate buffer, only a low amount of U was removed in Opalinus Clay pore water. UV/Vis measurements suggest a speciation-dependent reduction by the microorganism. Scanning transmission electron microscopy coupled with energy-dispersive X-ray spectroscopy revealed the formation of two different U-containing nanoparticles inside the cells. In a subsequent step, artificial multispecies bio-aggregates were formed using derivatized polyelectrolytes with cells of *D*esulfitobacterium sp. G1-2 and *Cobetia marina* DSM 50416 to assess their potential for U(VI) reduction under aerobic and anaerobic

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https://doi.org/10.1016/j.scitotenv.2024.177210
Received 16 July 2024; Received in revised form 22 October 2024; Accepted 23 October 2024
Available online 2 November 2024

conditions. These findings provide new perspectives on microbial U(VI) reduction and contribute to the development of a safety concept for high-level radioactive waste repositories, as well as to new bioremediation strategies.

1. Introduction

Decades of nuclear activities have resulted in widespread environmental pollution, posing significant environmental and human health hazard (Yan and Luo, 2015). Areas associated with mining, milling, ore processing, and U enrichment are particularly affected, exhibiting elevated levels of U and other radioactive substances (Gavrilescu et al., 2009; Li and Zhang, 2012; Todorov, 2006). This contamination requires remediation efforts to reduce radiation exposure to acceptable levels. Additionally, since the 1950s, nuclear power plants have been in use for commercial energy generation (*Nuclear Power Reactors in the World-REFERENCE DATA SERIES No. 2*, 2020). The safe disposal of the resulting high-level radioactive waste, predominantly consisting of U, requires extensive research on radionuclide behavior in the environment for a comprehensive safety analysis.

Microbial U bioremediation stands at the forefront of innovative environmental cleanup strategies, harnessing the unique metabolic capabilities of microorganisms to mitigate U contamination in various environments (Lakaniemi et al., 2019; You et al., 2021). While a wide range of approaches, including physical, chemical, and biological methods, have been employed to address environmental contamination caused by U (Gavrilescu et al., 2009), microbial U bioremediation offers several advantages over conventional remediation methods. These include its potential for in-situ application, minimal environmental disturbance, absence of secondary pollution, and relatively low cost (Lopez-Fernandez et al., 2021; Selvakumar et al., 2018). A key factor controlling these processes is the distinct redox chemistry of this radionuclide. Under naturally occurring redox conditions, U is predominantly found in two oxidation states: hexavalent U(VI) and tetravalent U(IV). Additionally, each redox state exhibits different solution behaviors, affecting their environmental mobility. U(VI) typically forms water-soluble compounds, thereby exhibiting higher mobility than U(IV), which often forms less soluble compounds (Grenthe et al., 2008). Certain microorganisms, particularly iron- and sulfate-reducing bacteria, enzymatically convert U(VI) to U(IV) through a process known as microbial U reduction (Wall and Krumholz, 2006). This process not only immobilizes U but also reduces its toxicity and environmental mobility, thereby mitigating the risk of groundwater contamination and environmental exposure. In addition to microbial biotransformation of U, other interaction mechanisms such as biosorption, bioaccumultion, or biomineralization can play a role in immobilizing U (Jones et al., 2015; Lloyd and Macaskie, 2002; Selenska-Pobell and Merroun, 2010).

In this study, we aimed to investigate the reduction behavior of the iron-reducing microorganism *Desulfitobacterium* sp. G1-2, which was isolated from a bentonite sample. Bentonite is considered as a potential backfill material in a final repository for high-level radioactive waste, particularly in clay rock and crystalline rock formations (Fachinger et al., 2006; Pusch, 2009, 1992). Therefore, this microorganism could play a crucial role in the direct surrounding of a nuclear repository and may interact with U and other radionuclides that could potentially be released during a worst case scenario, when water enters the repository. The reduction capacity of different other *Desulfitobacterium* spp. in a bicarbonate-buffered medium with pyruvate or H_2 gas as electron donors (depending on the strain) was previously investigated by Fletcher et al. (2010). Their results demonstrated a complete reduction of U(VI) to U(IV) within three to nine days, depending on the strain used. Our objective was to examine whether the isolate *Desulfitobacterium* sp. G1-2 is capable of reducing U(VI) under different experimental conditions to better simulate environmental conditions.

Many studies on microbial U(VI) reduction often overlook the initial speciation of U(VI). Typically, a bicarbonate-buffered system is used in experimental setups, where a uranyl(VI)-carbonate complex predominates (Hilpmann et al., 2023). Previous research revealed that the sulfate-reducing bacterium *Desulfosporosinus hippei* DSM 8344^T exhibits a speciation-dependent U(VI) reduction behavior (Hilpmann et al., 2023). In this case, experiments were conducted both in bicarbonate buffer and artificial Opalinus Clay pore water (Wersin et al., 2011). In the latter, a uranyl(VI)-lactate complex represents the primary U(VI) species in solution, introduced by the addition of lactate as an electron donor. *Desulfosporosinus hippei* DSM 8344^T could only reduce the lactate species and not the carbonate complex. Earlier research on speciation-dependent U(VI) reduction by diverse iron-reducing *Shewanella* strains primarily concentrated on the impact of various metal cations such as Mg^{2+} or Ca^{2+}, as well as the addition of dissolved organic carbon (DOC) to the reduction solutions, but overlooked the influence of the ligand itself (Belli et al., 2015; Neiss et al., 2007; Ulrich et al., 2011). Consequently, in this study, we aimed to investigate whether *Desulfitobacterium* sp. G1-2 similarly demonstrates a speciation-dependent reduction of U(VI).

Employing a unique combination of microscopic and spectroscopic techniques, we examined the processes at a molecular level. Time-resolved laser-induced luminescence spectroscopy (TRLFS) was used to analyze the initial U(VI) speciation in the samples, while UV/Vis spectroscopy on various samples confirmed the reduction of U(VI). Additionally, (scanning) transmission electron microscopy ((S)TEM) coupled with energy-dispersive X-ray spectroscopy (EDXS) provided insights into the U distribution and the formation of U particles within the bacterial cells. Only through the integration of these diverse methods can we gain a comprehensive understanding of the mechanisms involved in U(VI) reduction.

With the insights gained from pure planktonic culture experiments, we explored the potential controlled application of the uranium-bioreducers using the innovative approach of artificial bio-aggregates, that provide tailor-made solutions to remediation challenges (Deev et al., 2021; Rijavec et al., 2019). This approach involves encapsulating negatively charged bacterial cells with acetylated polyethyleneimine (Ac-PEI) to induce a charge-driven self-organization into artificial biofilms/bio-aggregates, even though the used microorganisms do not form a biofilm by themselves. Subsequently, bacterial cells from a second genus are added, adhering to the positively charged surface of the encapsulated cells to create artificial biofilms with various core-shell-structures. Our objective was to combine the reduction capacities of iron-reducing bacteria, exemplified by *Desulfitobacterium* sp. G1-2, with the aerobic marine inhabitant *Cobetia marina* DSM 50416, to investigate the potential for U(VI) reduction under both aerobic and anaerobic conditions. The immobilization of anaerobic U(VI)-reducing bacteria within artificial biofilms in this fashion could enhance various bioremediation applications by simplifying the separation of immobilized U(IV) from solution as it associates with the biofilm.

The formation of the artificial multispecies bio-aggregates was monitored using fluorescence microscopy. Additionally, determinations of U concentrations in the supernatants and UV/Vis spectroscopic investigations were conducted to observe a potential U(VI) reduction by the aggregates under both aerobic and anaerobic conditions.

These findings offer new insights into microbial U(VI) reduction by iron-reducing bacteria and will contribute to new U bioremediation approaches, offering sustainable and efficient solutions for addressing U contamination in diverse environmental settings. Furthermore, the study contributes valuable insights to the development of a comprehensive safeguard concept for a final repository for high-level

radioactive waste in both clay rock and crystalline rock.

2. Materials and methods

2.1. Phylogenetic characterization and cultivation

2.1.1. *Desulfitobacterium sp. G1-2*

The used bacterial strain G1-2, was isolated from a bentonite sample of the Dismantling Section 60 called B-C-60 from the Full-scale High Level Waste Engineered Barriers Experiment Dismantling Project (FEBEX-DP) performed at the Grimsel Underground Research Laboratory (URL) (Bengtsson et al., 2017). In this project, the coupled thermal-hydraulic-mechanical (THM) processes in the barrier systems of the host rock granite were investigated. Therefore, a heating element was used in an "*in situ*" test, which simulates the heat generated by radioactive decay, and the heating element is surrounded by bentonite blocks and sensors. DNA was isolated from a bacterial culture of the isolate G1-2 with the DNeasy PowerSoil Kit (QIAGEN) according to the manufacturer's instructions. However, some minor modifications were made: 250 μL of culture was used instead of 250 mg of soil, steps 7 and 10 were performed on ice, for step 19, 40 μL of sterile, DNA-free water (LiChrosolv Water; Merck KGaA, Germany) was used instead of 100 μL of C6, and all samples were incubated for 2 min at room temperature. The 16S rRNA gene was amplified from the extracted DNA, sequenced and analyzed as described before (Bachran et al., 2019). For the amplification, the primers 16S-7f (10 μM, 5′-A AGA STT TGA TYN TGG CTC AG-3′) (MWG Biotech AG, Germany) and 16S-1513r (10 μM, 5′-TAC GGY TAC CTT GTT ACG ACT T-3′) were used. The reaction tubes were placed in a T-Professional Thermocycler (Analytik Jena/Biometra) and heated up to 95 °C for 3 min, followed by 5 cycles with 90 s at 95 °C, 40 s at 59 °C, and 90 s at 72 °C (the annealing temperature was lowered by 1 °C during each cycle), followed by 25 cycles of 90 s at 95 °C, 40 s at 55 °C, and 90 s at 72 °C. The final step included 20 min at 72 °C. Sequencing of the purified PCR product was performed at Eurofins Genomics (GATC Service) with the primers 16S-1513r (10 μM), 16S-802r (10 μM, 5′-TAC CAG GGT ATC TAA TC-3′), and 16S-342rev. (10 μM, 5′-CCC ACT GCT GCC TCC CGT AG-3′) according to their instructions.

The sequence of the amplified 16S rRNA gene from the bacterial strain G1-2 can be found in the supporting information. The comparison of the sequence with other sequences present in the database was done with the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) (Madden, 2013). The results showed that the bacterial strain G1-2 belongs to genus *Desulfitobacterium*, because its 16S rRNA gene sequence was 96.87 % similar to the 16S rRNA gene sequence of *Desulfitobacterium dichloroeliminans* LMG P-21439 (CP003344), 96.38 % similar to the 16S rRNA gene sequence of *Desulfitobacterium hafniense* DCB-2 (NR_122068), and 96.23 % similar to the sequence of *Desulfitobacterium metallireducens* 853-15 (NR_025125).

Desulfitobacterium sp. G1-2 was cultivated in modified DSMZ medium 720 ("720. Desulfitobacterium hafniense medium", 2024) containing per L: 1 g NH_4Cl, 0.1 g NaCl, 0.1 g $MgCl_2 \cdot 6\ H_2O$, 0.05 g $CaCl_2 \cdot 2\ H_2O$, 0.3 g K_2HPO_4, 1 g yeast extract, 2.6 g $NaHCO_3$, 2.5 g Na-pyruvate, 1.25 g $Na_2S_2O_3 \cdot 5\ H_2O$, 1 mL trace element solution, 1 mL selenite-tungstate solution, and 1 mL vitamin solution. The composition and preparation of the trace element and the selenite-tungstate solutions were described previously (Hilpmann et al., 2023). The vitamin solution contains per L (10×): 20 mg biotin, 20 mg folic acid, 100 mg pyridoxine hydrochloride, 50 mg thiamine HCl, 50 mg riboflavin, 50 mg nicotinic acid, 50 mg calcium D-(+)-pantothenate, 1 mg vitamin B_{12}, 50 mg p-aminobenzoic acid, and 50 mg (DL)-alpha-lipoic acid. The vitamin solution was filter-sterilized, fumigated with N_2 gas for anaerobization and kept in the fridge for storage.

All components of the medium except the Na-pyruvate, the $Na_2S_2O_3 \cdot 5\ H_2O$, and the vitamin solution were dissolved in deionized water. Afterwards, the medium was sparged for 45 min with a gas mixture of N_2:CO_2 (80:20), because it contains bicarbonate. Inside an anaerobic chamber, the pH value of the medium was set to 7.1. After autoclaving, the medium was completed by adding the vitamin solution and anoxic (sparging with N_2), filter-sterilized solutions of Na-pyruvate and $Na_2S_2O_3 \cdot 5\ H_2O$. The cultivation was done at 30 °C in the dark. Cells were harvested in the mid-exponential growth phase (OD_{600} of about 0.14–0.16 after 42–48 h of growth, corresponding to cell numbers of 4×10^6–6×10^6 cells/mL) by anaerobic centrifugation (6000 x*g* for 7 min). For further experiments, cells were washed with anoxic sterile Opalinus Clay pore water solution at pH 5.5 or 30 mM bicarbonate buffer (see Section 2.2.1) once and resuspended in an appropriate volume of the same solution to obtain a stock suspension with an OD_{600} of 2.5. The optical density of the cell suspensions were measured with a Specord® 50 Plus UV/VIS spectrometer from Analytik Jena at a wavelength of 600 nm.

2.1.2. *Cobetia marina DSM 50416*

Cobetia marina DSM 50416 was purchased from the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany) and cultivated in modified DSMZ medium 79 ("79. Leucothrix medium", 2024), which contains per L: 10 g tryptone, 24 g NaCl, 11 g $MgCl_2 \cdot 6\ H_2O$, 4 g Na_2SO_4, 1.34 g $CaCl_2 \cdot 2\ H_2O$, 0.7 g KCl, 0.1 g KBr, 0.03 g H_3BO_3, 2.14 mg $NaSiO_3$, 0.04 g $SrCl_2 \cdot 6\ H_2O$, 3 mg NaF, 2 mg NH_4NO_3, and 1.2 mg $FeCl_3 \cdot 4\ H_2O$.

All components except the $CaCl_2 \cdot 2\ H_2O$ and the NaF were dissolved in deionized water. Afterwards, the pH of the medium was set to 7.8. After autoclaving, the remaining components were added separately as filter-sterilized solutions to complete the medium. The cultivation was done at room temperature on a shaker at 90 rpm. Cells were harvested in the mid-exponential growth phase (OD_{600} of about 7.4–7.6 after 24 h of growth) by centrifugation at 10,000 x*g* for 10 min. For further experiments, cells were washed twice in sterile 0.9 % NaCl solution and resuspended in an appropriate volume to obtain a stock suspension with an OD_{600} of 1.0.

2.1.3. *Formation of artificial multispecies bio-aggregates*

For U(VI) reduction experiments by artificial multispecies bio-aggregates, cells of *Desulfitobacterium* sp. G1-2 and *C. marina* DSM 50416 were used. The sample preparation was done in an anaerobic chamber and followed the method of electrostatic modification of bacterial cells (Rybkin et al., 2019) and the further co-aggregation of cells of these two species was done using the approach described by Deev et al. (2021). The cells of *Desulfitobacterium* sp. G1-2 and *C. marina* DSM 50416 were resuspended in anoxic 0.9 % NaCl solution to achieve an OD_{600} of the suspensions of 1. Subsequently, the cell suspension of *C. marina* DSM 50416 was combined with a 0.25 % acetylated polyethyleneimine (Ac-PEI) solution in a 1:1 volume ratio. The PEI derivatization, as per the method by Deev et al. (2021), was conducted to minimize its toxicity to the cells. The mixture was incubated for 5 min with occasional mixing on a vortex mixer. The Ac-PEI coated cells, now featuring a positively charged surface, were centrifuged at 1500 x*g* for 5 min. Following decantation of the supernatant, the cells were washed once and resuspended in half of the volume of 0.9 % NaCl. Subsequently, an equal volume of a cell suspension of *Desulfitobacterium* sp. G1-2 (naturally exhibiting a negatively charged surface) in anoxic 0.9 % NaCl was added, and the mixture was incubated for 40 min. The resulting multispecies bio-aggregates were then transferred into DSMZ 720 medium and incubated for 72 h at 30 °C under aerobic conditions.

To visualize the formation of artificial multispecies bio-aggregates and the growth of the stained cells within them, a second batch was created in a similar manner, using stained cells of *Desulfitobacterium* sp. G1-2 and *C. marina* DSM 50416. Syto® 9 (green-fluorescent, *Desulfitobacterium* sp. G1-2) and Syto® 59 (red, *C. marina* DSM 50416) dyes from Thermo Fisher Scientific, Waltham, MA, USA were utilized for staining. Following various incubation times, images of the aggregates were captured using fluorescence microscopy with a phase-contrast microscope, Olympus BX-61 (Olympus Europa Holding GmbH, Hamburg,

Germany), supported by the imaging software "CellSense Dimension 1.11". The appropriate wavelength for dye excitation was achieved using Cy3 and FITC filters.

2.2. Uranium(VI) reduction batch experiments

2.2.1. Pure culture

For U(VI) reduction experiments by cells of *Desulfitobacterium* sp. G1-2, two different background electrolytes were used. Anoxic 100 μM U (VI) solutions in artificial Opalinus Clay pore water, whose composition was determined by Wersin et al., and in 30 mM bicarbonate buffer were prepared as previously described with the exception that sodium sulfide was omitted in the preparation of the pore water solution (Hilpmann et al., 2023; Wersin et al., 2011). Afterwards, an appropriate amount of the washed cell suspension (in artificial Opalinus Clay pore water or bicarbonate buffer) was added to the solutions to achieve an OD_{600} of 0.1 in the samples (cell numbers of around 3×10^6 cells/mL). Control samples with heat-killed cells were prepared via boiling the cell suspension at 99 °C for 5 min (1 mL aliquots). Incubation and sampling was performed as described elsewhere (Hilpmann et al., 2023).

2.2.2. Artificial multispecies bio-aggregates

For U(VI) reduction experiments with artificial multispecies bio-aggregates, the suspensions with the formed aggregates (see Section 2.1.3) were centrifuged (2.500 xg, 3 min), and resuspended in one-eighth of the volume used during bio-aggregate preparation. This adjustment was necessary to achieve a sufficient biomass concentration due to a partial loss of cell material during aggregate preparation. Further sample preparation and sampling followed the previously described methods (see Section 2.2.1) in both anaerobic and aerobic bicarbonate buffer containing 100 μM U(VI) and 10 mM sodium lactate with a prolonged incubation time of up to 15 days. Since specifying an OD_{600} was no longer possible due to the aggregate formation, the protein concentration of the samples was determined to compare the individual samples. Proteins were extracted from the initial aggregate suspension using 0.26 g of 0.1 mm glass beads per mL sample volume in an ultrasonic homogenizer Bead Ruptor 24 (OMNI International, GE, USA) with a velocity of 4.2 m/s for 1 min. This process was repeated twice with 30-seconds pauses. The samples were then centrifuged at 13,000 xg for 9 min, and protein concentrations in the supernatants were determined using the Qubit® Protein Assay Kit (Invitrogen, ORE, USA) in combination with the Qubit® 2.0 fluorometer, following the manufacturer's instructions, yielding always in a value of 2.8 ± 0.1 μg of extracted proteins per mL of sample.

2.3. Time-resolved laser-induced fluorescence spectroscopy

Time-resolved laser-induced fluorescence spectroscopic (TRLFS) measurements of the supernatants were used to investigate the speciation of U(VI) in both the bicarbonate buffer and the artificial Opalinus Clay pore water. Samples for TRLFS were taken from the blank solution without cells and after 48 h of incubation for comparison purposes. The measurements followed the procedures previously described (Hilpmann et al., 2023).

2.4. Transmission electron microscopy

Localization of U at the single-cell level was achieved through (scanning) transmission electron microscopy ((S)TEM) coupled with energy-dispersive X-ray spectroscopy (EDXS) of ultrathin sectioned samples from *Desulfitobacterium* sp. G1-2 cells incubated with U in artificial Opalinus Clay pore water for 48 h. Details of sample preparation and image acquisition can be found in a prior publication (Hilpmann et al., 2023).

2.5. UV/Vis spectroscopy

To unequivocally confirm the presence of U(IV), UV/Vis measurements were conducted on the dissolved cell pellets of *Desulfitobacterium* sp. G1-2 and the dissolved multispecies bio-aggregates. Details regarding preparation of samples and reference solutions, as well as measurement specifications are provided elsewhere (Hilpmann et al., 2023). All samples were incubated with U for one week prior to the measurements. The experiments in pure culture included investigations of samples with living cells as well as heat-killed cells.

3. Results & discussion

3.1. Uranium(VI) bioreduction by Desulfitobacterium sp. G1-2 in pure culture

3.1.1. Aqueous U(VI) speciation in the supernatants

Previous studies indicated differences in the speciation of U(VI) in bicarbonate buffer and artificial Opalinus Clay pore water (Hilpmann et al., 2023), which were used as background electrolytes for the microbial U(VI) reduction experiments. In Opalinus Clay pore water at the investigated pH of 5.5, a uranyl(VI)-lactate complex was identified as the dominant species, whereas in bicarbonate buffer, the 1:3-uranly (VI)-carbonate complex played a major role. To confirm the initial speciation of U(VI) in the supernatants of the current experiments, time-resolved laser-induced fluorescence spectra were recorded from the initial blank solutions without cell incubation of both the 30 mM bicarbonate buffer and artificial Opalinus Clay pore water. This step was essential to establish the experimental setup for investigating the potential speciation-dependency of microbial U(VI) reduction by *Desulfitobacterium* sp. G1-2. Fig. 1 displays the resulting emission spectra.

The comparison of band positions confirmed the differences in the initial U(VI) speciation between the two background electrolytes, which is crucial for the subsequent investigations. The spectra of U(VI) in the bicarbonate buffer closely match those of a uranyl(VI)-carbonate complex. Conversely, in the artificial Opalinus Clay pore water, the band positions and intensities align well with the reference spectrum of a uranyl(VI)-lactate complex. Minor deviations may arise due to the potential presence of smaller proportions of other U(VI) complexes in the solutions, precluding the recording of single spectra during the measurements. These findings are consistent with previous luminescence measurements and speciation calculations (Hilpmann et al., 2023).

To assess potential changes in speciation over time, TRLFS measurements were conducted on samples after 48 h of incubation, as well. No significant alterations were observed, indicating that the presence of cells had minimal to no influence on the speciation in solution. Spectra obtained after 48 h are provided in the supporting information (Fig. S1).

3.1.2. Uranium concentrations in the supernatant

Time-dependent U(VI) reduction experiments were conducted to investigate the ability of *Desulfitobacterium* sp. G1-2 to reduce U(VI). A reduction of U(VI) can be indicated by decreasing U concentrations in the supernatants due to the reduced solubility of U(IV) (Fletcher et al., 2010; Junier et al., 2010; Lovley et al., 1991; Vettese et al., 2020). The experiments were performed in two different background electrolytes: artificial Opalinus Clay pore water and 30 mM bicarbonate buffer. TRLFS studies to verify the different speciation of U(VI) in both background electrolytes are detailed above. Earlier research demonstrated that the sulfate-reducing bacterium *Desulfosporosinus hippei* DSM 8344^T exhibited a speciation-dependent reduction of U(VI), being able to reduce the uranyl(VI)-lactate complex but not the carbonate complex (Hilpmann et al., 2023). The current study aimed to investigate whether *Desulfitobacterium* sp. G1-2, an iron-reducing bacterium, shows differences in U(VI) reduction behavior depending on the initial U(VI) speciation, as well. Fig. 2 shows the concentrations of U in the supernatants in dependence as a function of the incubation time for both

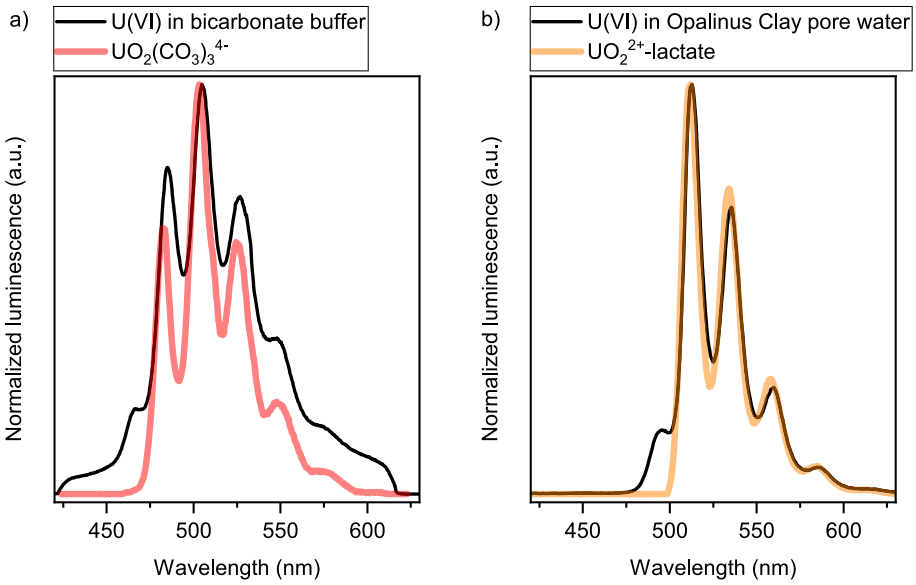

Fig. 1. Emission spectra of the U(VI) blank solutions without cell incubation of a) 30 mM bicarbonate buffer and b) artificial Opalinus Clay pore water in comparison with the reference spectra of a) a 1:3-uranyl(VI)-carbonate complex and b) a uranyl(VI)-lactate complex.

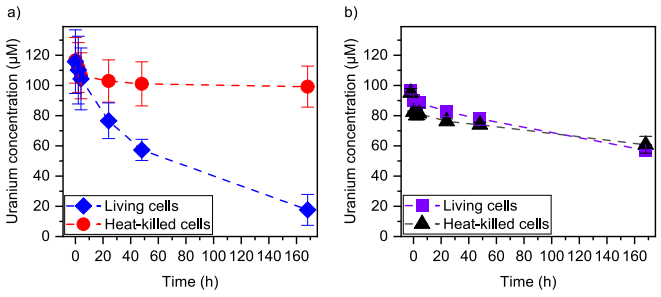

Fig. 2. Uranium concentrations in the supernatants of the batch experiments with living and heat-killed cells of *Desulfitobacterium* sp. G1-2 with an initial U (VI) concentration of 100 µM in a) 30 mM bicarbonate buffer and b) artificial Opalinus Clay pore water (10 mM lactate, 3×10^6 cells/mL).

background electrolytes.

The experiments in bicarbonate buffer with living cells showed a decrease of the U concentration in the supernatants with increasing incubation times. Within 48 h, nearly 60 % of the initial U was removed from the supernatants, and after one week (168 h), almost 90 % were removed. In comparison, the experiment with heat-killed cells also exhibited a decrease in U concentration, but to a much lower extend. In this case, after one week, only approximately 10 % of the U was removed. The partial decrease in the heat-killed cell experiment may be attributed to various bioassociation processes, such as biosorption of U to functional groups on the cell surface. This suggests that a metabolically active process could be responsible for the higher U removal observed in the living cell experiment. A comparison with the results of Fletcher et al. reveals that the removal time for *Desulfitobacterium* sp. G1-2 falls within in the same range as for other *Desulfitobacterium* spp. (Fletcher et al., 2010). However, in contrast to other iron-reducing bacteria, the removal time for *Desulfitobacterium* sp. G1-2 is relatively long. For instance, *Shewanella* spp. typically achieve almost complete removal of U from the supernatant within 24 h (Vettese et al., 2020).

In contrast, the experiments in artificial Opalinus Clay pore water show a similar trend for both living and heat-killed cells. Overall, only small proportions of U, 25 % after 48 h and nearly 40 % after one week of incubation, are removed. This indicates that probably no reduction is taking place, which would lead to higher amounts of U removal from the supernatant. Instead, other bioassociation processes are likely responsible for the lower proportions of removed U from the supernatant. Together with the luminescence spectroscopic measurements of the background electrolytes presented above, these results offer first evidence of a speciation-dependent U(VI) interaction process by *Desulfitobacterium* sp. G1-2.

Overall, the U concentrations in the supernatants give no clear indication of a reduction of U(VI). For this reason, UV/Vis spectra of the dissolved cell pellets were recorded, which are shown in the following section.

3.1.3. Determination of the uranium oxidation states

In order to ascertain if a reduction of U(VI) to U(IV) occurred in the samples during the uranium removal process UV/Vis spectroscopic measurements were conducted on the dissolved cell pellets (Fig. 3).

A comparison of the band positions provides clear evidence of the formation of U(IV) in the experiment with living cells in the bicarbonate-buffered system. Furthermore, no bands of U(VI) are observed in this sample, indicating that all the U associated with the cells has likely been reduced to U(IV). In contrast, in the experiment conducted in Opalinus Clay pore water, no U signal – neither U(VI) nor U(IV) – was detected. This suggests that, in this case, no significant reduction of U(VI)

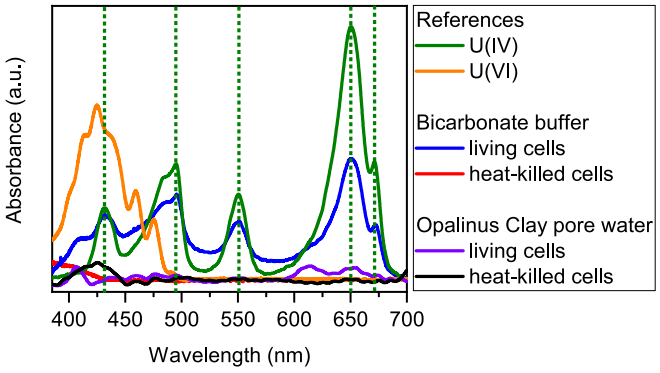

Fig. 3. UV/Vis spectra of the dissolved cell pellets of living and heat-killed cells of *Desulfitobacterium* sp. G1-2 after one week of incubation in 30 mM bicarbonate buffer and artificial Opalinus Clay pore water in comparison with normalized reference spectra of U(IV) and U(VI).

occurred. Additionally, the amounts of overall cell-associated U were so low that no signal of U(VI) could be detected either. Given the presence of different initial U(VI) complexes in both solutions, as revealed by speciation calculations and TRLFS investigations, it can be inferred that U(VI) reduction by the iron-reducing bacterium *Desulfitobacterium* sp. G1-2 is speciation-dependent. Specifically, cells of *Desulfitobacterium* sp. G1-2 demonstrated the ability to reduce the uranyl(VI)-carbonate complex present in bicarbonate buffer but not the uranyl(VI)-lactate complex, which is the primary species in Opalinus Clay pore water. Previous studies by Fletcher et al. determined a proportion of U(IV) of around 95 % via X-ray absorption near-edge structure (XANES) measurements for all investigated *Desulfitobacterium* strains, indicating an almost complete reduction of U(VI) in the samples (Fletcher et al., 2010). Due to the composition of the medium, a uranyl(VI)-carbonate complex should also be present as the initial species in these investigations.

A speciation-dependent U(VI) reduction has already been observed for the sulfate-reducing bacterium *Desulfosporosinus hippei* DSM 8344^T (Hilpmann et al., 2023), where U(VI) reduction under the same conditions exhibited an opposite behavior: the cells reduced the lactate complex but not the carbonate complex. Previous studies on the speciation-dependent U(VI) reduction by various iron-reducing *Shewanella* strains predominantly focused on the influence of the presence of different metal cations like Mg^{2+} or Ca^{2+} or adding dissolved organic carbon (DIC) to the reduction solutions (Belli et al., 2015; Neiss et al., 2007; Ulrich et al., 2011). However, the explicit influence of the ligand itself had not been investigated extensively. One reason for the speciation-dependent U(VI) reduction can be that different enzymes are involved. For *Shewanella* and *Geobacter* species it is well known that c-type cytochromes play a role during U(VI) reduction (Marshall et al., 2006; Orellana et al., 2013; Shelobolina et al., 2007). However, it was shown that *Desulfitobacterium hafniense* DCB-2 contains some cytochrome *c* genes but only in a very limited number (Kim et al., 2012). The reduction of U(VI) as uranyl(VI)-carbonate was shown for all of these three different genera (*Shewanella*, *Geobacter* and *Desulfitobacterium*) indicating that perhaps cytochrome *c* is involved in the reduction of the uranyl(VI)-carbonate complex in all these three different genera. In contrast, no c-type cytochromes were observed in the strain *Desulfosporosinus hippei* DSM 8344 (Vatsurina et al., 2008), and this bacterium is not able to reduce uranyl(VI)-carbonate (Hilpmann et al., 2023). Therefore, the investigation of the reduction of uranyl(VI)-lactate by *Desulfosporosinus hippei* DSM 8344^T is currently under investigation with proteomic analysis. The involvement of different enzymes in the U(VI) reduction can have an influence on the reduction of different U(VI) species.

UV/Vis studies on the heat-killed cells incubated with U(VI) reveal the absence of significant amounts of U, regardless of its oxidation state, in both samples with bicarbonate buffer and artificial Opalinus Clay pore water. As anticipated, the heat-killed cells exhibit no observable reduction of U(VI). The amounts of cell-associated U were once again insufficient to get any UV/Vis signal in these cases.

3.1.4. Localization of uranium in the cells

The localization of U in or on the cells was investigated through (scanning) transmission electron microscopy ((S)TEM) analyses of ultrathin sectioned samples from U(VI)-incubated cells in bicarbonate buffer. Specifically, STEM imaging was coupled with spectrum imaging analysis based on energy-dispersive X-ray spectroscopy (EDXS). Fig. 4 shows a representative bright-field TEM image along with the EDXS-based distribution maps of U, P, Os, O, and S for the investigated initial U(VI) concentration of 100 μM and an incubation time of 48 h.

The recorded TEM image depicts the presence of nanoparticles with different shapes inside the cells (see Fig. 4a, blue ellipses). These include spherical nanoparticles with a diameter of up to 100 nm (see Fig. S2) and rod-shaped nanoparticles measuring around 150 nm in length. EDXS analysis confirmed the presence of U within both types of nanoparticles.

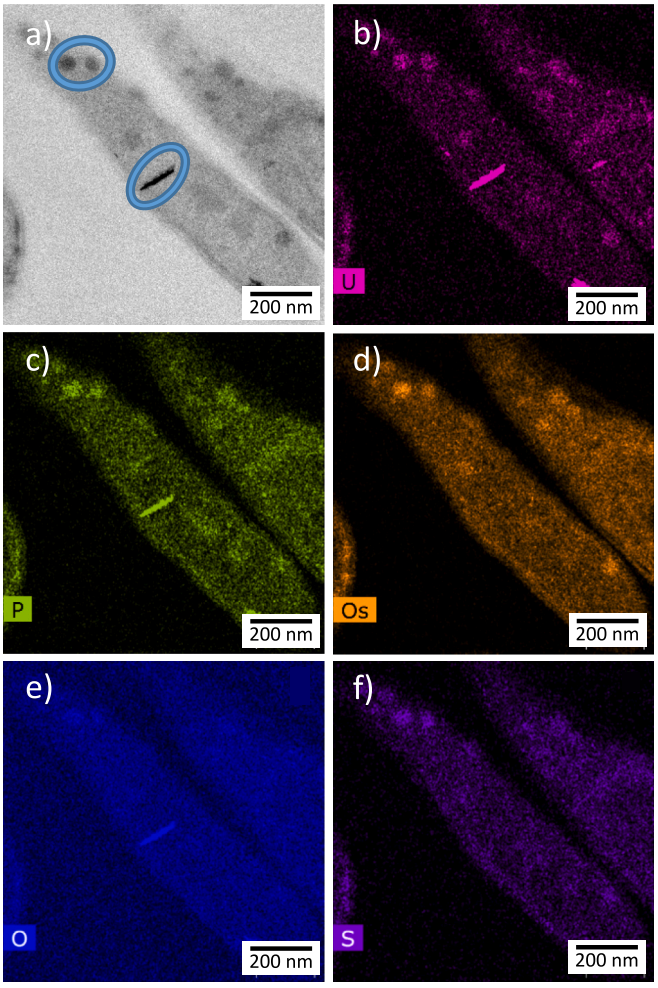

Fig. 4. Representative bright-field TEM image (a) and corresponding U (b), P (c), Os (d), O (e), and S (f) element distributions of an ultrathin sectioned sample of *Desulfitobacterium* sp. G1-2 cells treated with U ([U(*VI*)initial] = 100 μM) for 48 h. The blue ellipses highlight U-containing nanoparticles with different shapes inside the cells.

Notably, no U is detected outside the cells or on the cell surface, indicating a significant uptake of U within the cells. This suggests that U(VI) is taken up into the cells and subsequently reduced to U(IV).

Additionally, EDXS analysis reveals a co-localization of U and phosphorus (P) in the nanoparticles. The presence of P inside the cells may involve either inorganic phosphate or organic phosphoryl groups. The spherical nanoparticles also exhibit the accumulation of osmium (Os), introduced during the preparation of ultrathin sections to enhance image contrast. Os predominantly binds to organic cellular components like lipopolysaccharides, proteins, or amino acids, which are important building blocks for membranes and cell organelles (Zheng et al., 2020). This suggests that U is co-localized with organic phosphate compounds in the spherical nanoparticles. Previous studies by Alessi et al. already demonstrated the binding of U(IV) to organic phosphoryl groups, such as in lipopolysaccharides or phospholipids, during microbial U(VI) reduction by iron-reducing bacteria (Alessi et al., 2014). Additionally, sulfur (S) is co-localized in the nanoparticles as well, providing further evidence for the presence of an organic compound. In contrast, co-localization of Os is not observed for the rod-shaped nanoparticles, indicating that these particles likely consist of inorganic U-phosphate compounds. These could, for example, be compounds such as ningyoite (U(IV) phosphate), which was already found as a product of microbial U (VI) reduction processes (Bernier-Latmani et al., 2010; Lee et al., 2010). The rod-shaped nanoparticles also exhibit a stronger U signal, which

indicates that a higher U concentration is present. Another representative TEM-based study of *Desulfitobacterium* sp. G1-2 can be found in the supporting information (Fig. S2). These analyses provide the first microscopy images localizing U inside cells of a *Desulfitobacterium* sp. While Fletcher et al. did not utilize microscopy analyses in their investigations, extended X-ray absorption fine structure (EXAFS) measurements suggest the involvement of a U(IV)-phosphate species as a product of U(VI) reduction in their studies as well (Fletcher et al., 2010).

3.2. Formation of artificial multispecies bio-aggregates

Desulfitobacterium sp. G1-2 and *C. marina* DSM 50416 were co-aggregated in order to combine the reduction capacity of iron-reducing bacteria with an aerobic strain to investigate the potential for U(VI) reduction in artificial biofilms under aerobic and anaerobic conditions. The coating of microorganisms with derivatized polyelectrolytes and subsequent formation of artificial biofilms with various strains offer numerous new application possibilities.

Fluorescence microscopy was employed to examine the formation of artificial multispecies bio-aggregates. Fig. 5 shows the images acquired after different incubation times under aerobic conditions in DSM 720 medium. Aerobic conditions were chosen to observe the potential formation of anaerobic niches within the aggregates, where the reduction of U(VI) and other metals might occur.

At the beginning, red-stained cells of *C. marina* DSM 50416 are predominantly found within the aggregates, while green-stained cells of *Desulfitobacterium* sp. G1-2 are located on the aggregate surface. This distribution aligns with expectations, as the surface of *C. marina* DSM 50416 cells was coated with Ac-PEI, facilitating the subsequent attachment of *Desulfitobacterium* sp. G1-2 cells to the positively charged surface. However, aerobic conditions in the medium are suboptimal for the anaerobic bacterium, leading to a dynamic rearrangement of cells over time. As oxygen is consumed by *C. marina* DSM 50416 cells, *Desulfitobacterium* sp. G1-2 cells grow only inside the aggregates, where oxygen levels are low enough to sustain their growth. Consequently, the initially observed separation between cells of both species is rearranged and diminishes over time.

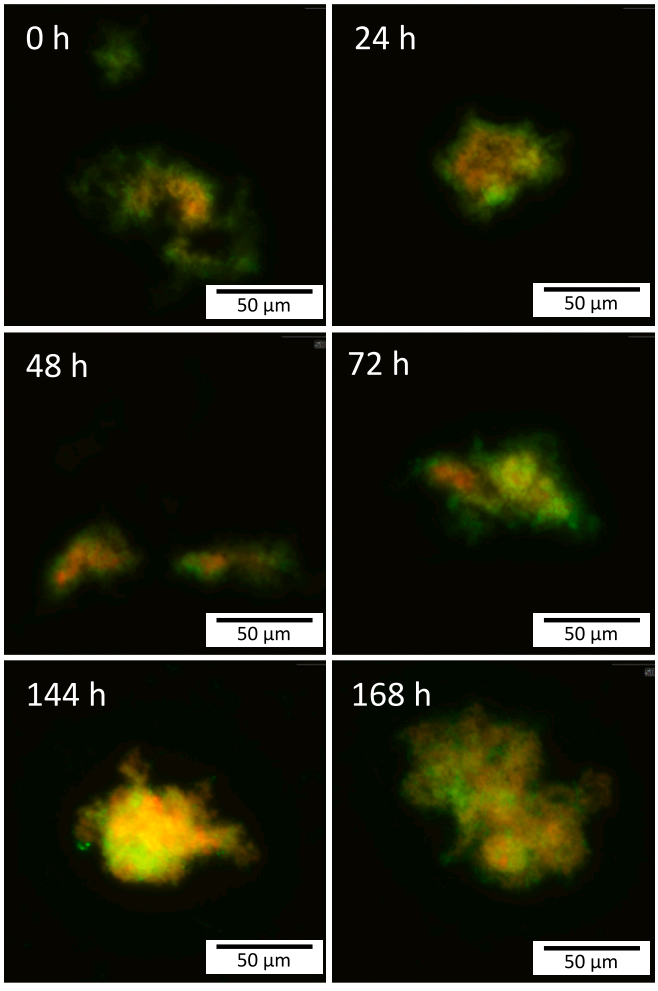

Fig. 5. Fluorescence microscopy images of artificial multispecies bio-aggregates of cells of *C. marina* DSM 50416 (stained red) and cells of *Desulfitobacterium* sp. G1-2 (stained green) after different incubation times under aerobic conditions in DSM 720 medium.

As the bacterial aggregates are suspended in an aerobic fermentative medium, conducive growth conditions are provided for the bacteria. Over time, the aerobic strain *C. marina* DSM 50416 exhibited notable growth in the aerobic medium, resulting in a visible increase in the size of the aggregates, particularly evident in the image captured after one week of incubation. Concurrently, *Desulfitobacterium* sp. G1-2 also demonstrated growth, albeit to a lesser extent. The growth of the anaerobic bacterial species under aerobic conditions may be attributed to a combination of factors, including the oxygen consumption by *C. marina* DSM 50416 and a certain degree of oxygen tolerance exhibited by the anaerobic bacterium. Previous studies on closely related strains, such as *Desulfitobacterium hafniense* DCB-2 and *Desulfitobacterium halogenans* JW/IU-DC1T, indicated their ability to survive in low oxygen concentrations (Madsen and Licht, 1992; Utkin et al., 1994). This high oxygen tolerance in *Desulfitobacterium* spp. is likely attributed to an alteration in the HydA subunit of the HUP2 gene, which is atypical for anaerobic bacteria (Kruse et al., 2017). This subunit, characterized by the presence of two additional cysteines in the binding motif for FeS, suggests the presence of an oxygen-tolerant hydrogenase, thereby explaining the survival of *Desulfitobacterium* spp. in low oxygen environments.(Kruse et al., 2017; Pandelia et al., 2012).

3.3. Uranium(VI) reduction by artificial multispecies bio-aggregates

As a subsequent step, for the first time, the reduction capacity of such multispecies artificial bio-aggregates, comprising cells of *C. marina* DSM 50416 and *Desulfitobacterium* sp. G1-2, was tested under both aerobic and anaerobic conditions. Fig. 6a shows the U concentrations in the supernatant in 30 mM bicarbonate buffer, with an initial U(VI) concentration of 100 µM.

In the experiment conducted under aerobic conditions, despite *Desulfitobacterium* sp. G1-2 cells were viable and they grew (Fig. 5), there was no observable decrease in the uranium concentration. This suggests that U(VI) reduction may not have occurred in this instance. It is possible that the aggregates used were too small to establish stable anaerobic niches for a sufficiently long period, where both U(VI) reduction and the stabilization of U(IV) could occur simultaneously. Alternatively, the extended incubation time of the aggregates in the DSMZ 720 medium under aerobic conditions might have led to the death of some *Desulfitobacterium* sp. G1-2 cells. Further investigations are warranted to gain a deeper understanding of these phenomena and a further development of the formation of the multispecies bioaggregates is needed especially in determining anaerobic conditions inside the aggregates as well as conditions to increase the stability of aggregates for longer time.

In contrast, in the experiment conducted under anaerobic conditions, a slight decrease in the U concentration in the supernatant was observed with increasing incubation times. After two weeks, approximately 15 % of the U had been removed, which again indicates an ongoing U reduction process.

To confirm these findings, UV/Vis studies of the dissolved aggregates were conducted. The resulting spectrum, compared with the reference spectrum of U(IV), is shown in Fig. 6b. However, two challenges emerged during the investigations. Firstly, the U concentrations in the UV/Vis samples were relatively low, leading to high levels of noise in the

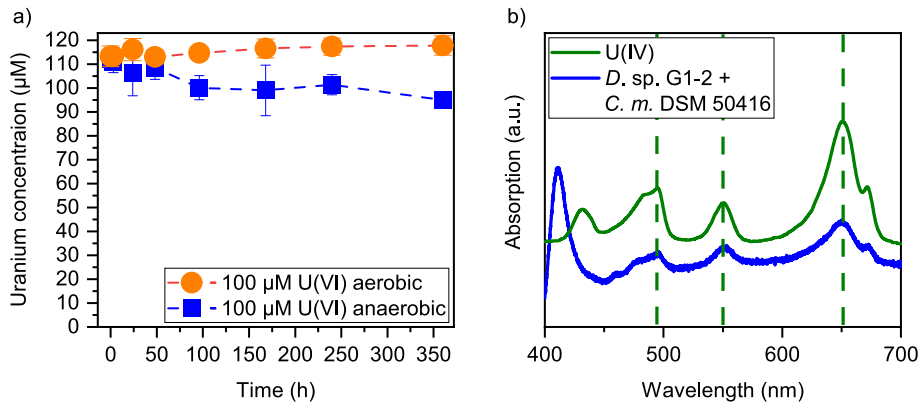

Fig. 6. a) Uranium concentrations in the supernatants of the batch experiments with artificial multispecies bio-aggregates of cells of *C. marina* DSM 50416 and *Desulfitobacterium* sp. G1-2 with an initial U(VI) concentration of 100 µM in 30 mM bicarbonate buffer; b) corresponding UV/Vis spectra of the dissolved aggregates after two weeks of anaerobic incubation in 30 mM bicarbonate buffer together with a normalized reference spectrum of U(VI).

signals. Secondly, the Ac-PEI or its degradation products exhibited a broad band in the UV/Vis spectrum. Consequently, background correction of the obtained spectrum was necessary, and quantification of the amount of reduced U was no longer feasible. Nevertheless, a spectrum with low intensities of the bands could be obtained. Comparison of the band positions indicated the presence of U(IV) in the samples, suggesting a partial reduction of U(VI) by the artificial bio-aggregates. Furthermore, no U(VI) could be detected, indicating that all the U associated with the aggregates had been reduced. The low amounts of reduced U(VI) could be attributed to factors such as the physiological state of the *Desulfitobacterium* sp. G1-2 cells after the previous incubation of the aggregates in the aerobic medium and in general lower amounts of cells in comparison to the experiments with pure cultures. Another explanation could be that *C. marina* DSM 50416 cells in the aggregates hinder *Desulfitobacterium* sp. G1-2 cells to access to some extent uranium. However, the experiments with bio-aggregates show that immobilization of anaerobic U(VI)-reducing bacteria within artificial biofilms could enhance various bioremediation applications by simplifying the separation of immobilized U(IV) from solution as it is associated with the bio-aggregates (increasing specific weight and size).

4. Conclusions

This study confirmed the speciation-dependent reduction of U(VI) by *Desulfitobacterium* sp. G1-2. TRLFS measurements verified the different U(VI) speciation in both background electrolytes. UV/Vis measurements of the dissolved cell pellets revealed an almost complete reduction of U (VI) within one week in 30 mM bicarbonate buffer, where a uranyl (VI)-carbonate complex is present. Conversely, experiments in artificial Opalinus Clay pore water, where a uranyl(VI)-lactate complex predominates, did not show a reduction of U(VI). The iron-reducing bacterium *Desulfitobacterium* sp. G1-2 thus showed an opposite behavior compared to the sulfate-reducing microorganism *Desulfosporosinus hippei* DSM 8344^T. Additional STEM-EDXS analyses revealed the formation of two-different types of U-containing nanoparticles inside the cells, each exhibiting a different shape and P source.

The second part of this study focused on the formation of artificial multispecies bio-aggregates comprising both aerobic and anaerobic bacteria to investigate U(VI) reduction under aerobic and anoxic conditions. Utilizing Ac-PEI, we successfully formed bio-aggregates with cells of *C. marina* DSM 50416 and *Desulfitobacterium* sp. G1-2. Fluorescence microscopy images were used to track aggregate formation, revealing a reorganization of cells over time. Subsequent U(VI) reduction experiments demonstrated only minimal removal of U under anoxic conditions and no removal of U under aerobic conditions. UV/Vis measurements confirmed the formation of a small proportion of U(IV) under anoxic conditions. This experiment shows that the formation of bio-aggregates can be a potential application for bioremediation, as it is easier to remove reduced U from solutions when the U(IV) is associated with the cells in bio-aggregates than in single cells.

This study highlights the importance of investigating the initial U(VI) speciation in bioreduction experiments. Such investigations are crucial for developing new bioremediation approaches and formulating comprehensive safety measures for high-level nuclear waste repositories in both clay rock and crystalline rock formations. Furthermore, the results emphasize the potential of employing artificial bio-aggregates in new strategies for the bioremediation of radionuclide-contaminated wastewater.

CRediT authorship contribution statement

Stephan Hilpmann: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Isabelle Jeschke:** Visualization, Investigation, Formal analysis, Data curation. **René Hübner:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis. **Dmitrii Deev:** Visualization, Methodology, Investigation. **Maja Zugan:** Investigation. **Tomaž Rijavec:** Validation, Supervision, Resources, Project administration, Funding acquisition. **Aleš Lapanje:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition. **Stefan Schymura:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Andrea Cherkouk:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors gratefully acknowledge the funding provided by the German Federal Ministry of Education and Research (BMBF) (Grant 02NUK053E) and the Helmholtz Association (Grant SO-093), as well as a partial funding by the Helmholtz Association, grant PIE-0007 (CROSSING). Furthermore, funding from the European Union's Horizon 2020 research and innovation program under the Grant Agreement No. 95237 (SurfBio), No. 826312 (GREENER), and Horizon Europe No. 101060211 (BIOSYSMO) are acknowledged. We also thank Sabrina Beutner and Stefanie Bachmann for multiple ICP-MS measurements, Jennifer Drozdowski and Sindy Kluge for their support in isolating and cultivating the bacterium *Desulfitobacterium* sp. G1-2. In addition, we

would like to thank Dr. Thomas Kurth and Susanne Kretzschmar from the Center for Regenerative Therapies Dresden (CRTD) for the preparation of the TEM specimens. Additionally, the use of the HZDR Ion Beam Center TEM facilities and the funding of TEM Talos by the German Federal Ministry of Education and Research (BMBF; grant No. 03SF0451) in the framework of HEMCP are acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.177210.

Data availability

Data will be made available on request.

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