Investigations on indigenous microorganisms isolated from a former uranium mine and their interaction mechanisms with uranium - a possible bioremediation study

Dissertation

To Fulfill the
Requirements for the Degree of
"doctor rerum naturalium" (Dr. rer. nat.)

Submitted to the Council of the Faculty
of Biological Sciences
of the Friedrich Schiller University Jena

by Dipl.-Biol. Ulrike Gerber

born on 16.11.1986 in Suhl

Reviewer

Reviewer 1: Prof. Dr. Erika Kothe (FSU Jena)

Reviewer 2: Prof. Dr. Georg Büchel (FSU Jena)

External reviewer: Prof. Dr. Thorsten Stumpf (TU Dresden, HZDR)

Day of defense

Monday, 7 January 2019

Beginnings are usually scary, and endings are usually sad, but its everything in between that makes it all worth living.

Bob Marley

Contents

Abstract

Heavy metal and radionuclide contaminations of soil and water, resulting from former mining and milling industries, represent a serious problem worldwide. Due to the fact that heavy metals and radionuclides, like uranium, are not biodegradable, their remediation is of great concern. Remediation of such contaminated sites is often laborious and cost-intensive, requiring the need of technical facilities and high amounts of chemicals. In this thesis, investigations were performed to find alternatives for the currently used conventional remediation and waste water treatment at the site of the former uranium mine in Königstein (Saxony, Germany). As a concept of remediation, the administrating company of the former uranium mining sites in Eastern Germany, Wismut GmbH, started a controlled flooding of the underground mine in Königstein in 2001. As a consequence of the former leaching process, started in the 1980s, with sulfuric acid to mobilize the uranium within the sandstone, the underground and flooding water displays the characteristics of an AMD (acid mine drainage) site. Despite the harsh conditions prevalent within the flooding water a high microbial diversity could be detected. Microorganisms display abilities to interact with metals or radionuclides in various ways. They are able to mobilize or immobilize them. The usage of microorganisms or plants for decontamination of metal- or radionuclide-polluted soils and waters is called bioremediation. The restoration of such highly contaminated sites using bioremediation requires vital microbial cells, which are able to survive within these harsh environmental conditions. Against this background, in this thesis indigenous microorganisms were isolated from the flooding water and investigated for their ability removing uranium from surrounding solutions and their tolerance against heavy metals, in particular uranium.

The bacterial strain *A. facilis*, identified by DNA-based methods within the flooding water, was investigated with regard to its interaction mechanisms with uranium. The results demonstrate that the gram-negative betaproteobacterium is able to remove high amounts of uranium (130 $\mathrm{mg\,U/g\,dbm}$) by passive biosorption and active bioaccumulation. Uranium is either sorbed to the outer membrane or actively taken up into the cell. Inside the

cytoplasm it was observed that the removed uranium is associated with polyphosphate granules. In addition, the uranium tolerance tests displayed, that the bacterial cells are able to grow in the presence of uranium concentrations similar to those present in the flooding water (MIC 0.2 mM). In further investigations, structural information on uranium immobilized by the bacterial cells was generated. The spectroscopic analyses showed, that the uranium immobilization on functional groups at the outer membrane of *A. facilis* is dependent on the incubation time. During the first hours uranium is mainly bound on lipopolysaccharides suggesting the formation of uranyl-phosphoryl species. With increasing incubation time, uranium seemed to be bound to carboxylic groups of the peptidoglycan layer in addition to the phosphoryl groups.

One indigenous strain, directly isolated from the flooding water using culture-dependent methods, represents the yeast KS5 (*R. toruloides*). The investigations on this natural occurring isolate revealed high uranium tolerances (MIC 6 mM), indicating the development of adaption mechanisms. Furthermore, the fungal strain has demonstrated a high efficiency of uranium removal (350 $\mathrm{mg\,U/g\,dbm}$). TEM studies identified both underlying interaction mechanisms, bioaccumulation and biosorption. Nevertheless, active bioaccumulation seems to be the prominent process involved in the uranium immobilization. Inside the cytoplasm, uranium is associated in lipid granules resulting in the formation of meta-autunite minerals.

Culture-independent methods could identify the presence and activity of anaerobic microorganisms within the flooding water of the former uranium mine. Sulfate (SRB) and iron reducing bacteria (IRB) are known for their ability to reduce uranium(VI) into uranium(IV). For that reason, investigations on microbial uranium(VI) reduction, using the flooding water as background medium, were performed. As carbon source and electron donor, 10 mM glycerol, were directly added to the flooding water. After six weeks of incubation at 30 °C a complete reduction to uranium(IV) was detected using XANES und UV-vis spectroscopy. By 16S rDNA gene analyses the bacterial diversity was identified. The majority of the found species are IRB (> 40 %). Thus, the microbial uranium(VI) reduction is mediated by these bacteria.

Finally, the obtained results of the microbial uranium(VI) reduction occurring directly within the flooding water could be transferred to a pilot plant with a volume of 100 L. The laboratory scale experiments were successfully repeated and verified. Thus, it was shown that the microbial reduction of uranium(VI), by adding only 10 mM glycerol to the flooding water, could be used in future applications for *in situ* for bioremediation approaches at the site of the former uranium mine Königstein.

In conclusion, this study shows the importance of investigating uranium interaction mechanisms of natural occurring microorganisms in conjunction with bioremediation of contaminated sites. This thesis contributes to the development of bioremediation approaches for the treatment of metal and radionuclide contaminated sites resulting from former mining industry.

Kurzfassung

Schwermetall- und Radionuklidkontaminationen von Böden und Gewässern sind zum Großteil das Ergebnis ehemaliger Bergbau- und Metallgewinnungsindustrien und stellen weltweit ein ernstzunehmendes Problem dar. Aufgrund der Tatsache, dass Schwermetalle und Radionuklide, wie Uran, nicht durch biologische oder andere Prozesse abbaubar sind, ist deren Sanierung von großer Bedeutung. Durch den hohen technischen Aufwand und den Einsatz großer Mengen an Chemikalien, ist die Sanierung ehemaliger Minen oft aufwendig und mit hohen Kosten verbunden. Im Rahmen dieser Arbeit wurden Untersuchungen durchgeführt, um auf dem Gelände der ehemaligen Uranmine in Königstein (Sachsen, Deutschland) Alternativen für die verwendete konventionelle Flutungswasserbehandlung zu finden. Als Sanierungskonzept startete die Betreibergesellschaft der ehemaligen Uranabbaugebiete in Ostdeutschland, die Wismut GmbH, im Jahr 2001 eine kontrollierte Flutung der Mine in Königstein. Aufgrund des sinkenden Urangehaltes im Sandstein wurde Mitte der 1980er Jahre zur Gewinnung von Uran mit Schwefelsäure versetztes Wasser zur Laugung eingesetzt. Auf diese Weise konnte das Uran, aber auch andere Metalle, mobilisiert und somit in Lösung gebracht werden. Noch heute weist das Flutungswasser aus diesem Grund einen niedrigen pH und hohe Metallkonzentrationen auf und ähnelt somit typischen AMD (acid mine drainage)-Standorten. Trotz der vorherrschenden harschen Bedingungen im Flutungswasser konnte eine hohe mikrobielle Diversität durch kulturunabhängige Methoden nachgewiesen werden. Durch vorangegangene Studien konnte gezeigt werden, dass Mikroorgansimen verschiedene Prozesse entwickelt haben, um mit Metallen oder Radionukliden zu interagieren. Speziell angepasste Mikroorganismen können so in der Lage sein, Metalle oder auch Radionuklide zu mobilisieren, aber auch zu immobilisieren. Ein alternatives Konzept, welches Mikroorganismen oder Pflanzen zur Sanierung von Metall- oder Radionuklid- kontaminierten Böden und Gewässern verwendet, stellt die Biosanierung dar. Vor diesem Hintergrund wurden in der vorliegenden Arbeit natürlich vorkommende Mikroorganismen aus dem Flutwasser isoliert und auf ihre Fähigkeit hin untersucht, Uran aus der umgebenden Lösung zu entfernen. In weiteren Experimenten wurde ihre

Toleranz gegenüber Schwermetallen, insbesondere Uran, getestet.
Das Bakterium *A. facilis*, welches mittels DNA-basierter Methoden im Flutungswasser identifiziert werden konnte, wurde zunächst auf seine Wechselwirkungen mit Uran untersucht. Die Ergebnisse zeigen, dass dieses gramnegative Betaproteobakterium in der Lage ist, große Mengen an Uran durch passive Biosorption und aktive Bioakkumulation zu immobilisieren (130 mg U/g dbm). Mit Hilfe von ortsauflösenden Messmethoden, wie TEM (Transmission Elektronen Mikroskopie), wurde Uran an der äußeren Membran und innerhalb der Zelle detektiert. Im Zytoplasmas liegt Uran mit Polyphosphatkörnern assoziiert vor. Darüber hinaus konnten Toleranztests belegen, dass *A. facilis* in der Lage ist, in Gegenwart von Uran zu wachsen (MIC 0.2 mM), die denen im Flutungswasser der ehemaligen Uranmine entsprechen. Um detaillierte Informationen auf molekularer Ebene zu erhalten, wurden in weiteren Untersuchungen die strukturellen Informationen, des durch die Bakterienzellen immobilisierten Urans, aufgeklärt. Mit Hilfe von spektroskopischen Analysen konnte gezeigt werden, dass Uran, in Abhängigkeit von der Inkubationszeit an unterschiedliche funktionelle Gruppen der äußeren Membran von *A. facilis* gebunden wird. Während der ersten Stunden ist Uran hauptsächlich an Lipopolysacchariden lokalisiert. Dies wurde durch die Identifizierung von Uranylphosphoryl-spezies nachgewiesen. Im weiteren Verlauf der Inkubation wurde festgestellt, dass Uran an Carboxylgruppen der Peptidoglykanschicht sorbiert ist. Ein weiterer untersuchter natürlich vorkommender Mikroorganismus, der mit kulturabhängigen Methoden direkt aus dem Flutwasser isoliert wurde, repräsentiert die Hefe KS5 (*R. toruloides*). Die Ergebnisse zeigen, dass das Isolat eine hohe Toleranz gegenüber Uran aufweist (MIC 6 mM). Darüber hinaus ist KS5 in der Lage, relativ hohe Mengen an Uran zu immobilisieren und somit aus der umgebenden Lösung zu entfernen (350 mg U/g dbm). Mit Hilfe von TEM-Studien konnten die beiden Interaktionsmechanismen, Bioakkumulation und Biosorption, identifiziert werden. Anders als bei *A. facilis* zeigen die Ergebnisse, dass bei KS5 die aktive Bioakkumulation den dominierenden Prozess bei der Uranimmobilisierung darstellt. Innerhalb des Zytoplasmas der Hefezellen ist Uran mit Lipidgranula assoziiert.
Weitere Experimente auf der Grundlage von DNA- und RNA-basierten Methoden konnten sowohl das Vorhandensein, als auch die Aktivität von anaeroben Mikroorganismen im Flutwasser der ehemaligen Uranmine nachweisen. Insbesondere die anaeroben Sulfat- (SRB) und Eisen-reduzierenden Bakterien (IRB) sind für ihre Fähigkeit bekannt, Uran(VI) zu Uran(IV) zu reduzieren. Aus diesem Grund wurden Untersuchungen zur möglichen mikrobiellen Reduktion von Uran(VI) unter Verwendung des

Flutungswassers und der darin vorhandenen Mikroorganismen durchgeführt. Zur Induzierung der mikrobiellen Aktivität wurde als Kohlenstoffquelle und Elektronendonor 10 mM Glycerin direkt in das Flutungswasser gegeben. Nach sechs Wochen Inkubation bei 30 °C wurde mittels XANES- und UV-vis-Untersuchungen eine vollständige Reduktion zu Uran(IV) nachgewiesen. Die Ermittlung der bakteriellen Diversität nach sechs Wochen Inkubation ergab, dass es sich bei der Mehrheit der identifizierten Bakterien um IRB handelt (> 40 %). Somit zeigt sich, dass die mikrobielle Uran(VI)- Reduktion hauptsächlich durch die Anwesenheit von IRB hervorgerufen wird.
Schlussendlich konnten die erhaltenden Erkenntnisse der mikrobiellen Uran(VI)- Reduktion in eine Pilotanlage mit einem Volumen von 100 L überführt werden. Die Ergebnisse dieser Arbeit zeigen, dass die Ergebnisse auf industrielle Maßstäbe übertragbar sind. Damit konnte gezeigt werden, dass die mikrobielle Reduktion von Uran(VI) allein durch die Zugabe von 10 mM Glycerin bei zukünftigen Anwendungen als *in situ* Biosanierungsapplikationen auf dem Gelände der ehemaligen Uranmine Königstein genutzt werden könnte.
Zusammenfassend ergibt sich, dass im Rahmen dieser Arbeit die Wechselwirkungsmechanismen zwischen natürlich vorkommenden Mikroorganismen und Uran im Detail beschrieben und neue Zusammenhänge zwischen aktivem und inaktivem Stoffwechsel der Mikroorganismen gezeigt werden konnten. Somit können diese einen wertvollen Beitrag zur Entwicklung von Biosanierungsansätzen für die Behandlung von Metall- und Radionuklid-kontaminierten Standorten aus der ehemaligen Bergbauindustrie leisten.

1 Introduction

1.1 Uranium mining activities in Germany and the former uranium mine Königstein

Due to the industrial mining and milling of uranium ores, one big challenge of this century is the management of radioactive waste and the protection of humans and the environment from its chemical and radiological impacts. Large areas of the environment were processed for nuclear energy and weapon production since the 1940s [1]. In Eastern Germany, hundreds of millions of cubic meters waste rock materials and tailings were left behind after the cessation of uranium mining [2]. A total of 231,000 metric tons of uranium were mined, making East Germany the fourth-largest producer of uranium worldwide [3]. Since 1991 the Wismut GmbH is responsible to carry out the closure of the former uranium mining sites in Eastern Germany, including their environmental remediation and clean-up of the complete surface areas [4]. These former mining sites are Ronneburg, Schlema, Königstein, Pöhla and Dresden Gittersee, with Ronneburg in Thuringia and Schlema in Saxony being the largest and most famous uranium mining locations [4].

Between 1960 and 1990, approximately 18,000 metric tons of uranium were mined at the former uranium mine in Königstein. The former mining site is located in Saxony close to Dresden (Figure 1.1). The site covers an area of approximately 6.5 km^2 and comprises four mine levels. The ore body is located in the 4th aquifer of the sandstone rock formation (Figure 1.2). It is situated in an ecologically sensitive area, due to the fact that the 3rd aquifer located above the ore body serves as an important water reservoir for the surrounding cities and therefore is environmentally very sensitive. During the first two decades, uranium was recovered using conventional mining methods, by blasting operations of the underground and transport of the rocks to surface, where is was further processed [5]. From 1984 onwards, uranium was mined by underground block leaching using sulfuric acid (2-3 g/L H_2SO_4), due to the decreasing uranium content within the sandstone [6]. As a result, the decommissioned mine displays high levels of pollutants,

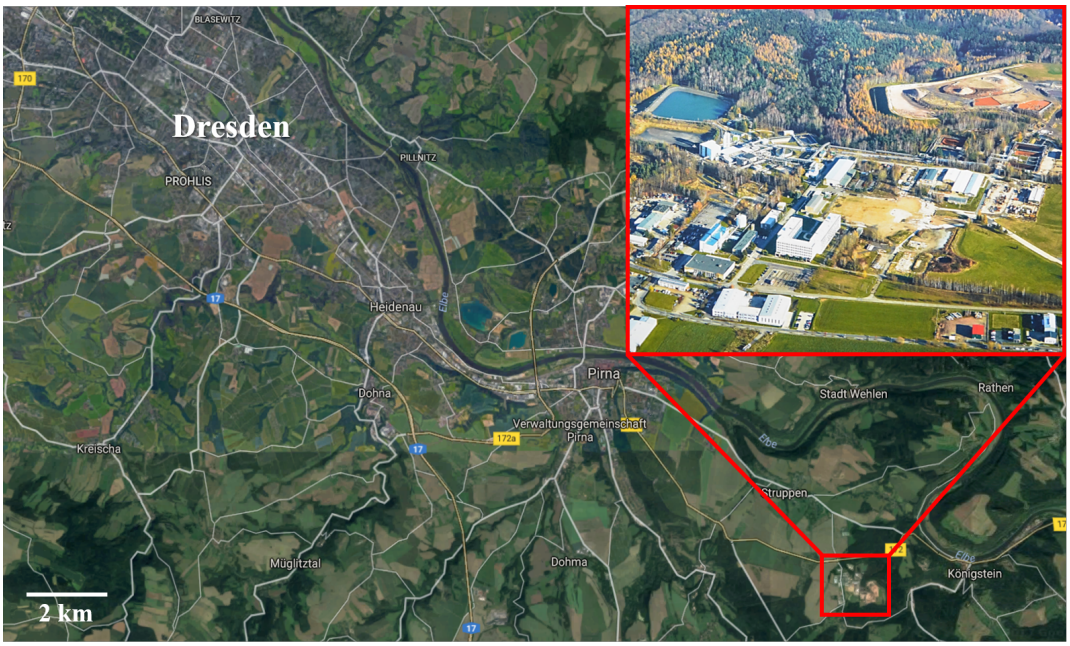


Figure 1.1: Geographic localization of the former uranium mining site Königstein (adapted after maps.google.de [access 03.11.2017 10:00]), with overview (red square) of the former mining site (picture library Wismut GmbH)

such as sulfate, heavy metals, and naturally occurring radionuclides (uranium and radium).

Since 2001, the former uranium mine is being remediated by controlled flooding up to a water level of 139.5 m above sea level [7]. The concept of the controlled flooding process was developed to avoid contamination of the overlaying aquifers, which is facilitated by the presence of the north fault (Figure 1.2 gray shaded area), a disturbance in the underground rock formation. Therefore, the flooding water is drained off, collected and pumped to the surface where it is purified in a laborious water treatment plant (Figure 1.1 overview red box) [8]. The controlled flooding will lead to a reduction of the pollutant concentrations, to the restoration of hydraulic conditions to near pre-mining settings, and finally prevent the migration of contaminations to nearby aquifers [9]. But, estimates of the Wismut GmbH suggest that, the treatment of the flooding water will be necessary for at least two to three decades from now.

1.2 Chemical composition of the fooding water in Königstein

As a consequence of the leaching with sulfuric acid, the flooding water in Königstein still displays a low pH of 2.8 to 3.0. The acidic conditions cause the mobilization of metals from the host rock, including uranium. With a uranium concentration between 8 mg/L and 13 mg/L, the flooding water displays a 10,000 times higher concentration as compared to surface waters [10] (limit for drinking water in Germany 0.01 mg/L). Not only the concentration of uranium is increased, but also the sulfate and iron contents. Together, these characteristics give rise to typical acid mine drainage (AMD) conditions. The formation of AMD is a consequence of sulfide-bearing materials, which are exposed to oxygen and water. The production often occurs in iron sulfide-aggregated rocks. However, the process of AMD formation may also occur naturally, mining industry can promote AMD generation simply through increasing the quantity of sulfides expose [11]. AMD sites display a high risk to contaminate surface, groundwater, and soil, since some effluents generated by the metal mining industry contain large quantities of toxic substances, such and heavy metals, which have serious human health and ecological implications [12,13]. For that reason, it is an important task to remediate these contaminated former mining sites and prevent the pollution of the surrounding environment. The technical and chemical effort of conventional water treatment, associated with high costs, leads to a significant interest in alternative approaches [14]. A detailed table with all important parameters and chemical compounds within the flooding water is shown in Chapter 3.4 Table 1. Within the flooding water not only radionuclides like uranium and radium are present in high concentrations, also iron, manganese, arsenic and other metals occur in high amounts [16].

Aside from metals and ions, the total organic carbon (TOC) represents a major factor for microbial activity. Carbon compounds like humic matter, organic acids, carbohydrates or aminoamides represent important energy sources. Carbon can be present in different forms, soluble, particular, organic, or inorganic as CO_2, HCO_3^- or CO_3^{2-} [17]. Within the flooding water a low concentration of around 1.0 mg/L TOC was detected and thus it is one of the limiting factors for microbial life and activity in the underground of the former mining site.

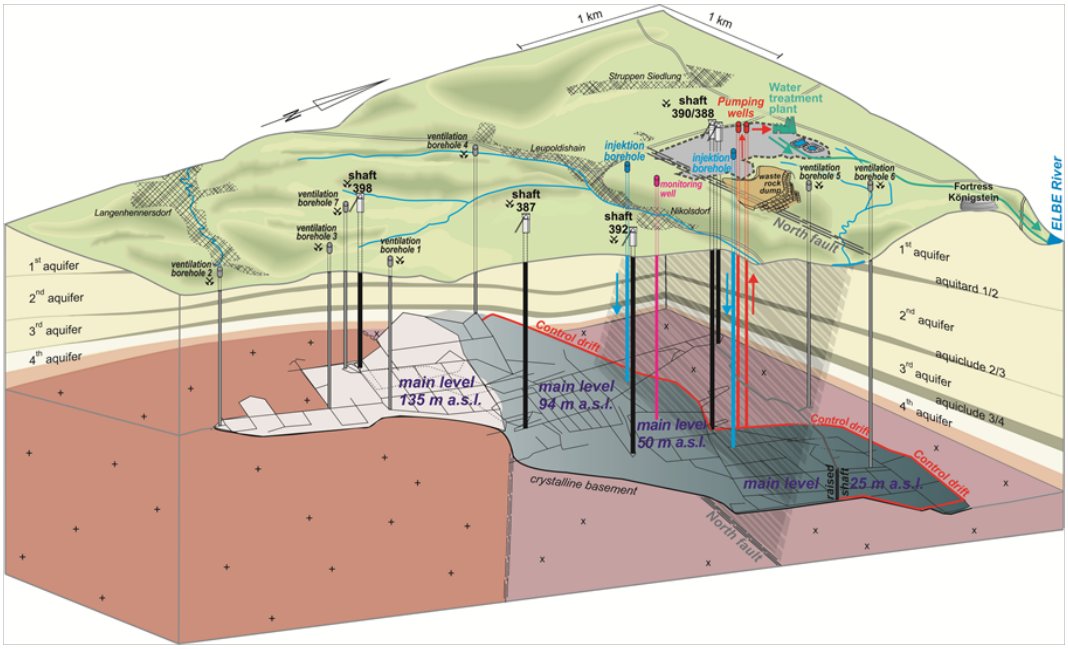


Figure 1.2: 3D geological view of the Königstein mining area (from [15]). The north fault is shown as grey shaded area.

1.3 Uranium

With the atomic number of 92 uranium belongs to the actinides, which are defined as elements with atomic number between 90 and 103. These elements subsequently fill up the 5f electron shell. The special property of actinides is their missing of stable nuclides, by meaning all isotopes of these elements are radioactive. Some isotopes occur as primordial nuclides (e.g.^{235}U and ^{238}U). Due to their long half-life (> 10^8 years), they represent the composition of the earth since their evolution. Elements with an atomic number higher than uranium, transuranium elements, are not naturally occurring in the environment, they arise for example by neutron capture within reactors. Uranium is a heavy metal with the atomic mass for natural uranium of 238.03 g/mol. In nature, uranium occurs as three of its 23 known isotopes, ^{234}U (0.005 %), ^{235}U (0.72 %), and ^{238}U (99.27 %) [18]. The half-life of these uranium isotopes ranges from 2.5×10^5 to 4.5×10^9 years [19]. Due to its properties as a 'light' actinide, uranium exhibits a variety of stable oxidation states in solution. Based on the delocalization of the 5f electrons, the 5f orbital is overlapped with the 6d orbital [20]. The electron configuration of uranium is [Rn]$5f^3 6d^1 7s^2$.

The naturally occurring isotopes of uranium are α-emitters, which decay to radioactive daughters. At the end of the decay chain of ^{238}U, the non-radioactive ^{206}Pb occurs [21]. As the 49th most abundant chemical element in the Earth's crust and with a concentra-

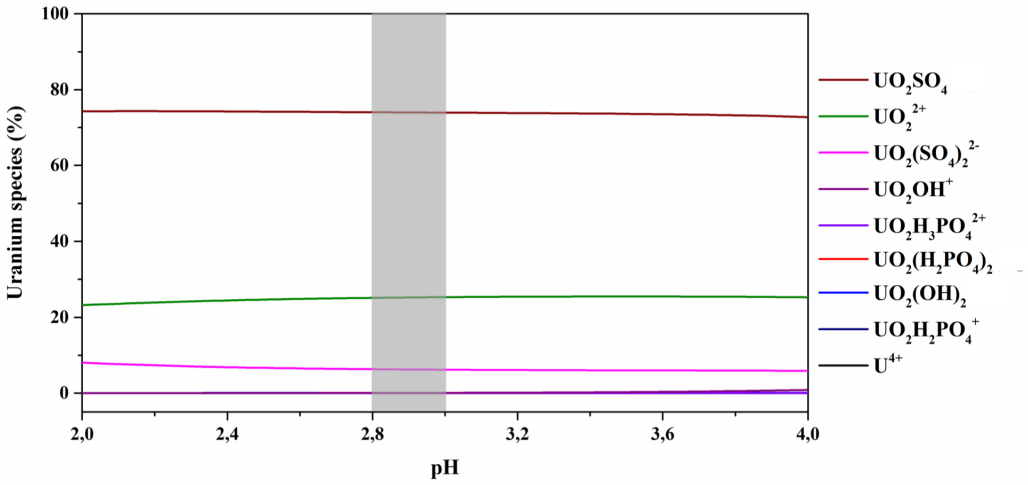


Figure 1.3: Speciation of uranium within the flooding water of the former uranium mine, under environmental conditions at a pH range of 2-4, grey area displays the prevalent pH conditions of the flooding water.

tion of $1 - 10\ \mu g/g$, uranium is not rare. By weathering processes, it can be transported to groundwater and other water systems. Nevertheless, the concentration of uranium in surface waters is low ($10\ \mu g/L$) [22]. The toxicity of the heavy metal was investigated by several further studies. The chemical toxicity of soluble uranium compounds can even surpass the potential radiotoxic effects, under special circumstances [23].

The uranium transport behavior in nature is dependent on its chemical speciation. The oxidation states, in which it can occur, are +2, +3, +4, +5, and +6 [24, 25]. However, the oxidations states +2, +3, and +5 are unstable at environmental conditions. In nature, U(VI) and U(IV) are most frequent, though U(IV) is less soluble and usually forms the oxide mineral uraninite (UO_2). In contrast, U(VI) commonly forms soluble and sometimes highly mobile complexes. The oxidation states U(V) and U(VI) are able to form actinyl ions, so called uranyl ions, $[O{=}U{=}O]^{+/2+}$. In solution, uranium exhibits a complex redox behavior due to the different redox potential of different redox pairs (e.g. U(VI)/U(V) = 0.088; U(VI)/U(IV) = 0.267; U(IV)/U(III) = -0.553 at 298.15 K in water) [26, 27].

In addition, the pH value strongly influences the interaction mechanisms of uranium with the environment. The solubility can be altered by pH-dependent hydrolysis reactions, and thus can influence the sorption of uranium to inorganic matter [28]. Furthermore, the ionic strength as well as the type and concentration of inorganic ligands, and the prevalent redox potential play a crucial role for the speciation of uranium in natural aquatic systems. Besides the mentioned chemical factors, also biotic parameters

influence uranium mobility in natural environments. In particular, the bioavailability of uranium, meaning its ability to interact with indigenous microorganisms, depends on its concentration and physicochemical speciation. Evidence from previous studies suggest that UO_2^{2+} and UO_2OH^+ are the most bioavailable forms of uranium(VI) [29].
Within the flooding water of Königstein, at acidic pH value and under oxidizing conditions uranium mainly occurs as dissolved mobile uranium(VI) species, UO_2SO_4 (~73 %), UO_2^{2+} (~22 %), and $UO_2(SO_4)_2^{2-}$ (~10 %) (Figure 1.3). In addition to theoretical calculations, TRLFS studies confirmed these findings and displayed the highly mobile speciation form UO_2SO_4 [30]. However, in minor concentrations $UO_2(SO_4)_2^{2-}$ and UO_2OH^+ are present in the flooding water as well.

1.4 Metal-microbe interactions

As mentioned, indigenous microbial communities in the flooding water are able to interact with radionuclides, such as uranium, and other metals in multiple ways (Figure 1.4). For example, studies demonstrated that the binding to the surface of microbial cells may be more efficient than binding to surrounding inorganic components under specific conditions [31–33]. This ability of microbial cells to form metal complexes is primarily based on two facts: the usually high number of metal binding ligands, e.g. peptidoglycan, lipopolysaccharides, proteins and glycolipids, and the high surface-to-volume ratio. The chemical binding of metals to the surfaces of microbes will be affected by processes like ion exchange, complexation, adsorption, and electrostatic interactions [34, 35]. The present ligands on the surface of microbes include negatively charged, functional groups, like phosphate, carboxyl, hydroxyl, amino and thiol groups. The mentioned passive and rapid process, biosorption, is simply based on physical adsorption or chemical sorption [36, 37]. The process is only controlled by pH dependent protonation, and thus, is independent of the cell metabolism. Besides this well described passive biosorption, metabolism-dependent processes can also alter the migration behavior of metals and radionuclides. Active processes such as biotransformation, biomineralization, bioaccumulation, and complexation by microbially-generated compounds, can have mobilizing and immobilizing effects on uranium and other metals. The term bioaccumulation, in general, describes an interaction mechanism, whereby metals are taken up in an active process using metal transporters, located within the cell walls of the microorganisms. Compared to the fast process of biosorption, this interaction will generally be slower. So far, no specific transporters for uranium were identified which lead to the speculation For

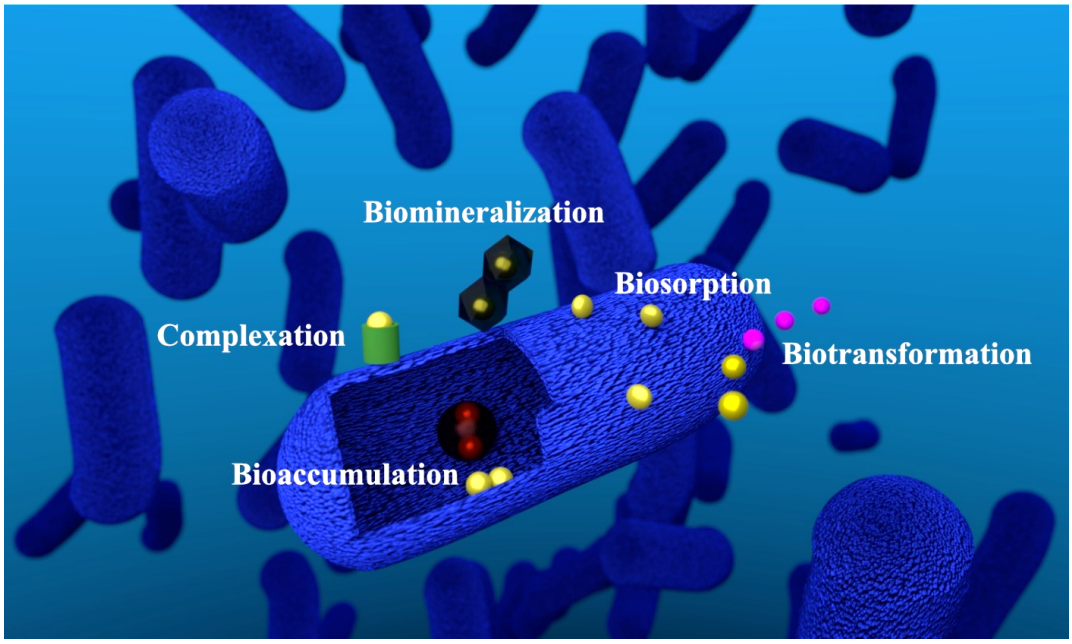


Figure 1.4: Interaction mechanisms of microorganisms and metals. Simplified model of a microbial cell interacting with uranium. Red structure within the cytoplasm shows uranium associated with polyphosphate granules.

uranium that it could be taken up faulty by other transporters, due to a mix up with essential ions like calcium [29,38]. As a consequence, the uranium uptake mechanism into cells is still not completely understood. However, recent studies assume that the uptake of uranium is metabolism-independent, and the transport occurs as a consequence of an increased permeability of the cell membrane due to a toxic stress reaction [39]. Nevertheless, the results in this thesis demonstrate, that active processes could be involved and that an active metabolism of the cells is required for uptake of uranium within the cells (Chapters 3.1 and 3.3). In addition, studies on bioaccumulation of uranium by bacterial cells have shown, that it was found associated in polyphosphate granules (Figure 1.4) [40].

Previous investigations revealed that these phosphate inclusions are important for the intracellular storage of divalent cations [37,41].

Another interaction mechanism which influences the uranium transport behavior in the environment is the mineralization of metals and radionuclides by organic or inorganic compounds released from microorganisms [42]. Biomineralization is distinguished between uranium-binding ligands that reduce its solubility and those that enhance solubility and mobility. This interaction mechanism includes the precipitation of mineral complexes, resulting from the release of microbial inorganic ligands, such as phosphates, carbonates and sulfides (Eqs. (1.1)–(1.3)) [38]. Another pathway for biologically-induced

precipitation is the release of reducing ligands. Previous studies identified microorganisms, which are able to release orthophosphate (inorganic phosphate, PO_4^{3-}) resulting from the activity of several enzymes, summarized as phosphatases. This enzyme activities are well described for a large variety of aerobic and anaerobic bacteria and for some archaea [43–50].

$$HPO_4^{2-} + M^{2+} \longrightarrow MHPO_4 \tag{1.1}$$

$$CO_4^{2+} + M^{2+} \longrightarrow MCO_3 \tag{1.2}$$

$$H_2S + M^{2+} \longrightarrow MS \tag{1.3}$$

Specific microorganisms also produce organic compounds, that affect the mobility of uranium. One example is the release of humic substances resulting from the microbial degradation of complex organic matter. These complexes are very resistant to further biodegradation and consequently accumulate in nature [51]. These humic substances can be differentiated by acidity and chemical composition, resulting in humin (insoluble fraction), humic acid (soluble under alkaline conditions), and fulvic acid (soluble at all pH values) [52]. Humic and fulvic acids in particular are known to interact with uranium and thus influence its migration behavior in nature [53–59]. Nevertheless, humic substances could also be involved in the reduction of uranium(VI). They serve as terminal electron acceptors in microbial respiratory pathways and may subsequently donate these electrons to uranium(VI) [60, 61].

The production of bioligands is another interaction mechanism between microorganisms and metals. Well described compounds, which can interact with metals in highly efficient ways, are siderophores. Microorganisms usually form these chelating agents in case of iron deficiency. Siderophores increase the iron solubility due to their complexation with functional groups, in particular hydroxamate and catechol groups [62]. However, the binding of siderophores is typically not highly specific. Consequently, they can also increase the solubility of other metals and radionuclides, which leads to an increase in bioavailability. For example, the siderophores pyoverdine and desferrioxamin-B are known to enhance the mobility of uranium [63, 64].

Microbe-mediated oxidation and reduction processes, which lead to a chemical modification of metals and radionuclides caused by metabolic activity, are called biotransformation. The oxidation of uranium under aerobic conditions was demonstrated for several acidophilic microorganisms, such as the bacterial strain *Acidithiobacillus ferrooxidans* [65] and the two archaeal species *Sulfolobusmetallicus*

and *Metallosphaerasedula* [66, 67]. In contrast to the oxidation of uranium, the reduction takes place under anaerobic conditions. Thereby, anaerobic microorganisms transfer electrons from an electron donor to uranium(VI), and thus reduce it to uranium(IV). It is assumed that the reduction of uranium(VI) proceeds by a single-electron transfer to uranium(V), followed by disproportionation [68]. At circumneutral conditions, uranium(VI)/(IV) displays a similar redox-couple compared to Fe(III)/(II), thus iron-reducing bacteria are able to respire uranium(VI) as an alternative electron acceptor [69]. Eqs. (1.4) and (1.5) show the reaction of Fe(III) and uranium(VI) under anaerobic conditions, while acetate serves as an electron donor in the other half-cell reaction [69]:

$$CH_3COO^- + 8\,Fe(III) + 4\,H_2O \longrightarrow 8\,Fe(II) + 2\,HCO_3^- + 9\,H^+ \tag{1.4}$$

$$CH_3COO^- + 4\,U(VI) + 4\,H_2O \longrightarrow 4\,U(IV) + 2\,HCO_3^- + 9\,H^+ \tag{1.5}$$

Other groups of microorganisms are also able to reduce uranium(VI), including sulfate-reducing bacteria [70], fermentative bacteria [71], acido-tolerant bacteria [72], and myxobacteria [73]. Furthermore, a uranium reduction was demonstrated at high temperatures (about 100 °C), for *Pyrobaculumislandicum*, a hyperthermophilic archaeon [74]. Most of these microorganisms use the reduction of uranium(VI) to gain energy for growth, while others do not gain energy [75]. The speciation of the reduced uranium is frequently found to be uraninite (UO_2) [70, 76]. Notably, abiotic uranium reduction by Fe(II) minerals [77–81] and additionally by biominerals [82–84] is also possible. However, under ambient environmental conditions, the majority of the studies suggest a direct enzymatic reduction as the dominant mechanism mediating uranium(VI) reduction [85, 86].

In conclusion, the clear separation between the mentioned processes is not trivial and they will frequently be interconnected, e.g. in the formation of UO_2 through biotic reduction. It is, however, obvious, that microbial processes have a significant impact on the behavior of radionuclides across a wide range of environments and will be important in processing contaminated sites [87].

1.5 Bioremediation and field studies

Due to the reason, that toxic metals, in particular uranium, are not metabolic degradable, their remediation depends on an approach which decreases their bioavailability [88]. The initial step may be the increasing contaminant mobility for extraction or the im-

mobilization of the metal using sequestration, complexation, or change in speciation to reduce solubility [71]. All these mentioned mechanisms are exhibited in the microbial repertoire. To intelligently design practical applications and accurately predict long-term behavior, the mechanisms must be understood on the molecular level [88]. The need for developing bioremediation approaches is mainly due to the high costs, the technological limitations, and the lack of specificity of the present available physicochemical methods. Furthermore, the invasive physical clean-up, which includes excavation, transport, and disposal of wastes, as well as pumping and treatment of the flooding water could have drastic influences on the indigenous biodiversity and thus, could even increase human health risks [89]. For that reason, *in situ* bioremediation approaches could be advantageous, as they are expected to be cost-effective, more specific and environmental-friendly. Moreover, previous studies could demonstrate, that the recovery of metals using biological approaches is highly efficient, enabling the treatment of sites with low uranium concentrations which are not amenable to chemical methods [90].
For bioremediation approaches, it seems to be necessary to establish procedures based on metabolically active microorganisms. Therefore, industrial applications are primarily determined by the ability of the microorganisms to maintain and survive the radiation exposure and the chemical toxicity of uranium. On that account, highly tolerant strains would be beneficial. An example for a well-studied and eligible strain is *Deinococcus radiodurans*, which is able to maintain 5000 Gray [91]. The toxicity of actinides should not impede bioremediation approaches based on active and living microorganisms. The strains investigated in the present work, for example, displays high tolerances against uranium up to concentrations of 6.0 mM for *Rhodosporidium toruloides* (Chapter 3.3). Hence, it is obvious that the growth and metabolism of indigenous strains within the flooding water of Königstein is in principle possible at uranium-polluted sites.
The main focus for active microbial bioremediation applications of uranium-contaminated sites is based on uranium immobilization by precipitation caused by uranium(VI) reduction. The microbial reduction of highly soluble uranium(VI) into less soluble uranium(IV) minerals has been studied, as it promises a high potential. Encouraging microcosm experiments of polluted sediments from the inactive "Midnite mine" in Stevens County (WA, USA) and contaminated soils from "DOE NABIR field research center site" in Oak Ridge (TN, USA) were performed. The addition of organic substrates lead to the stimulation of microbial uranium(VI) reduction at both sites [92, 93]. The injection of ethanol stimulated the activity of indigenous microorganisms, as a consequence of which uranium(VI) levels were drastically reduced from about 50 mg/L to

less than 30 µg/L within two years. Subsequent studies on the microbial diversity revealed a high abundance of metal reducing bacteria such as *Geobacter*, *Shewanella* and *Desolfovibrio* [94].

Another example of successful bioremediation was conducted at a former uranium ore processing facility, "Old Rifle" (CO, USA). Here, acetate was injected into the subsurface, resulting in a decrease of uranium(VI) concentration within 50 days from initial values of 0.4 and 1.4 µM to less than 0.18 µM [95]. Also in this case, the microbial composition was determined, which displayed in particular *Geobacter* species as dominant representatives, indicating that they are key players in the uranium(VI) reduction [96].

Nevertheless, it has to be mentioned, that the successful bioremediation strongly depends on the surrounding conditions. For instance, in the presence of oxygen and rather high nitrate concentrations under highly acidic conditions, the uranium(VI) reduction was suppressed in microcosm experiments with sediments from the FRC aquifer (Oak Ridge, TN, USA) [97].

Besides the microbial uranium(VI) reduction under anoxic conditions, one alternative approach at oxygenated conditions could be the precipitation of uranium in the form of insoluble inorganic compounds. Previous studies investigated the bioprecipitation of uranium by the activity of non-specific phosphatases, which are expressed by a large variety of aerobic and anaerobic bacteria [45, 98–100]. It is well-described, that these enzymes release inorganic orthophosphate form organic phosphate compounds. The released orthophosphate interacts with uranium and causes the precipitation of inorganic uranyl phosphate minerals which are formed in the surrounding aqueous system or directly on the cell surface of the microbes. Studies on different *Pseudomonas* strains, which were able to release sufficient amounts of orthophosphate, demonstrated the applicability of this interaction mechanism as a possible bioremediation approach. The release of orthophosphate was stimulated by the addition of an organic phosphate source and simultaneous overexpression of phosphatase genes [101].

Furthermore, studies were performed to investigate the possible application of biosorption for bioremediation approaches. Their outcome suggests that the availability of uranium-binding sites may limit biosorption as use for biotechnological processes. Moreover, the insufficient stability and specificity of biosorbents resulted in little progress for industrial application [102]. For that reason, the authors concluded that the commercial application of biosorption is rather limited, and therefore is likely to be used in approaches as a supporting process [103]. However, the process of biosorption is substantially faster compared with direct bioreduction. On that account, several previous

studies on biosorption have shown their potential use within these approaches [103–105]. In conclusion, all these case studies show that the use of microorganisms for remediation applications depend on the surrounding conditions and thus, it seems to be necessary to develop adjusted solutions. Nevertheless, many field studies could prove the applicability of *in situ* bioremediation approaches.

1.6 Microbial diversity within the flooding water of Königstein

Even though AMD waters are known to be toxic the majority of microorganisms [106, 107], they can contain highly-specialized diverse microbial life [108]. Despite the harsh conditions in the flooding water of the former uranium mine Königstein, investigations on the microbial diversity displayed a high number of metabolically active microorganisms [109]. As a consequence of the controlled flooding, the diversity in the underground changes drastically. Previous investigations during the beginning of the flooding process have shown a poor biodiversity at so far unflooded shafts. In the bacterial community mainly, betaproteobacteria (> 60 %) were detected, which were dominated by the species of *Ferrovum myxofaciens*. Besides this obligate chemolithotrophic iron- oxidizing bacteria, also the sulfur-oxidizing bacterium, *Acidithiobacillus ferrooxidans*, could be identified in lower abundances. In addition, also in minor amounts iron-reducing bacteria such as *Acidocella* spp. and *Acidiphilum* spp. were detected [109]. In addition, eukaryotes could also be identified in the microbial community. Five classes of metabolic active microorganisms were found: Heteroblosea, Fungi, Opithokonta, Cerozoa, and Ciliophora. However, the majority of the obtained sequences (88.5 %) were identified as unclassified eukaryotes [109].

In contrast, after about ten years in the ongoing flooding process the bacterial diversity was dominated by alpha-, beta-, and gammaproteobacteria, mainly by the phylogenetic groups of Nitrospira, Firmicutes, Acidobacteria, and TM7. The dominating iron oxidizing bacterium (IOB) *F. myxofaciens* could not be detected and was assumed to be displaced by *Acidithiobacillus* spp. The bacterial diversity is still dominated by iron oxidizing and reducing bacteria, as well as sulfur oxidizing bacteria. Furthermore, by culture- dependent approaches sulfate reducing bacteria were found and identified, with the major species *Desulfosporosinus* spp. In addition to bacteria, also archaea were detected. The dominating group of this domain was Thermoplasmata. In addition to bacteria and archaea, also eukaryotes were investigated. Interestingly, compared to the

conditions before the flooding process, less eukaryotic diversity and also differences in the composition was found in the flooding water [109].
Investigations on the microbial diversity within the flooding water of the former uranium mine Königstein, as well as other previous studies on AMD sites demonstrate that environmental factors influence the microbial communities tremendously. Thus, pH, temperature, concentration of dissolved metals and other solutions, total organic carbon, and dissolved oxygen shape the AMD associated microbial diversity [110]. AMD sites triggered by anthropogenic mining activities represent a significant environmental problem. The associated microbiome is restricted to almost a few abundant taxa of specialized archaea and bacteria. Such habitats comprise unique microorganisms with novel metabolic functions selected to deal with the harsh conditions [110].

1.7 Aims of the study

The challenge of this thesis was to gain new insights in the microbial interactions of natural occurring organisms with uranium(VI). In particular, the objective was to understand the impact of indigenous microorganisms within the flooding water of the former uranium mine Königstein on the migration behaviour of uranium(VI) to investigate possible strategies on *in situ* bioremediation approaches. The gained knowledge could be used to develop new setups to improve the existing waste water treatment plant and to eventually replace the conventional treatment using *in situ* bioremediation.
Therefore, investigations on natural occurring microorganisms isolated from the flooding water with uranium(VI) were performed. The existing knowledge of the interaction mechanisms between indigenous microorganisms and uranium(VI) is limited. Several investigations on well-studied model organisms were performed, but the underlying molecular mechanisms are still not completely understood. For that reason, experiments performed in this thesis should help to answer the following questions:

1. Are indigenous microorganisms adapted towards high heavy metals concentrations, compared to strains isolated from non-contaminated sites?

2. How do strains isolated from flooding water interact with uranium(VI) and which interaction mechanisms took place?

3. Are natural occurring microorganisms able to remove high amounts of uranium(VI) from surrounding solutions, and furthermore are they suitable candidates for *in situ* bioremediation approaches?

4. Is the usage of indigenous microorganisms for bioremediation strategies advantageous, and why could they be a better alternative compared to well-studied model organisms?

5. How is the metabolism of microbes involved in the uranium removal capacity and is it necessary for possible bioremediation processes to work with living microorganisms?

6. Where is the uranium located when associated with cells and which functional groups are involved on these immobilization process?

7. Despite oxidizing conditions within the flooding water, are anaerobic microorganisms active and are they able to interact with uranium by redox reactions?

8. Are the obtained results in this thesis transferable to industrial scale applications, to prove the applicability for on-site bioremediation by using indigenous microorganisms?

2 Summary of the Manuscripts

2.1 Combined use of flow cytometry and microscopy to study the interactions between the gram-negative betaproteobacterium *Acidovorax facilis* and uranium(VI)

Gerber U, Zirnstein I, Krawczyk-Bärsch E, Lünsdorf H, Arnold T, Merroun ML (2016) **J Hazard Mater 317:127-134. doi: 10.1016/j.jhazmat.2016.05.062.**

In this manuscript the interaction mechanisms of the betaproteobacterium *A. facilis* with uranium(VI) were investigated. This strain was detected within the flooding water of the former uranium mine Königstein by culture-independent methods. *A. facilis* was studied to figure out the possibility of its use for *in situ* bioremediation approaches. The cells show a fast and effective capacity to remove uranium from solution and the strain has a high tolerance for uranium, withstanding concentrations of up to 0.1 mM. Thus, our results could demonstrate, that the investigated bacterial strain *A. facilis* could be a suitable candidate for *in situ* bioremediation of the flooding water in Königstein as well as for other contaminated waters.

Contribution of the authors

Ulrike Gerber:	concept and design of all experiments, performed all experiments, evaluation of the data, preparation of the manuscript, **overall own contribution: 80 %**
Isabel Zirnstein:	phylogenetic calculations
Evelyn Krawczyk-Bärsch:	supervision of the project, discussion of the results, corrections of the manuscript
Heinrich Lünsdorf:	TEM sample preparation and TEM analyses, corrections of the manuscript
Thuro Arnold:	co-supervision of the project, corrections of the manuscript
Mohamed L. Merroun:	discussion of the results, supervision of the project, corrections of the manuscript

2.2 Multidisciplinary characterization of U(VI) sequestration by *Acidovorax facilis* for bioremediation purposes

Krawczyk-Bärsch E, Gerber U, Müller K, Moll H, Rossberg A, Steudtner R, Merroun ML (2017)
J Hazard Mater 347:233–241. doi.org/10.1016/j.jhazmat.2017.12.030.

By multidisciplinary characterization combining aqueous chemistry, TEM, EXAFS, TRLFS and ATR FT-IR the interaction mechanisms of *A. facilis* with uranium(VI) were studied on the molecular level. The results from kinetic batch experiments demonstrate that uranium is bound preferentially to phosphoryl and carboxyl functionally groups of the outer membrane. The findings obtained from this study contribute to a better understanding of the fate and transport of uranium within contaminated environments and improve the possibility to use this bacterial strain for future bioremediation applications.

Contribution of the authors

Ulrike Gerber:	concept and design of experiments, laboratory work, evaluation of the data, co-preparation of the manuscript, **overall own contribution: 50 %**
Evelyn Krawczyk-Blärsch:	supervision of the project, discussion of the results, preparation of the manuscript
Katharina Müller:	evaluation of the *in situ* ATR FT-IR data
Henry Moll:	evaluation of the EXAFS data
André Rossberg:	evaluation of the EXAFS data
Robin Steudtner:	evaluation of the TRLFS data
Mohamed L. Merroun:	performed TEM analyses, discussion of the results, supervision of the project, corrections of the manuscript

2.3 Metabolism-dependent bioaccumulation of uranium by *Rhodosporidium toruloides* isolated from the flooding water of a former uranium mine

Gerber U, Hübner R, Rossberg A, Krawczyk-Bärsch E, Merroun ML
Submitted to PLOS ONE (29/03/2018), first revision (16/05/2018)

The aim of this study is to investigate the indigenous strain KS5 (*R. toruloides*) isolated from the flooding water of the former uranium mine Königstein and its interaction mechanism with uranium. Tolerance tests demonstrate that the isolated yeast exhibits high tolerance towards uranium and chromium, in contrast to a tested reference strain. These findings indicate, the strain has developed adaption mechanisms as a response to the surrounding conditions, i.e. high concentration of uranium and other heavy metals. In addition, the uranium removal capacity was studied and revealed a high uranium immobilization capacity. In temperature-dependent experiments, a metabolism-dependent uranium interaction could be demonstrated. TEM analyses identified the main interaction mechanisms of active bioaccumulation. The investigations revealed that KS5 could be used for bioremediation approaches due to its high tolerance und removal capacity of uranium.

Contribution of the authors

Ulrike Gerber:	concept and design of all experiments, laboratory and field work, preparation of TEM samples, evaluation of the data, preparation of the manuscript, **overall own contribution: 80 %**
René Hübner:	performed TEM analyses, corrections of the manuscript
André Rossberg:	evaluation of the EXAFS data
Evelyn Krawczyk-Bärsch:	supervision of the project, discussion of the results, correction of the manuscript
Mohamed L. Merroun:	performed TEM (elemental distribution) analyses, discussion of the results, co-supervision of the project, corrections of the manuscript

2.4 Microbial mediated uranium(VI) reduction within the flooding water of a former uranium mine - a possible bioremediation approach

Gerber U, Schäfer S, Röder G, Lehmann S, Zirnstein I, Krawczyk-Bärsch E, Rossberg A

In preparation for submission

In this manuscript, the interaction mechanisms of anaerobic microorganisms with uranium(VI) directly within the flooding water were studied. To stimulate the metabolic activity of indigenous anaerobic microorganisms, glycerol as a carbon source and possible electron donor was added to the flooding water. During six weeks of incubation, reduction of uranium(VI) to uranium(IV) by microorganisms could be induced. The transformation of the highly soluble uranium(VI) into the less soluble uranium(IV) driven by microbial activity could be the basis of bioremediation processes directly on site of contaminated environments. Anaerobic microorganisms present within the flooding water of the former uranium mine Königstein showed fast and efficient reduction of uranium(VI). To verify the obtained results performed in lab scale experiments a pilot plant was designed. The results could be confirmed and show the high potential to use these specialized anaerobic microbes for *in situ* bioremediation.

Contribution of the authors

Ulrike Gerber:	concept and design of all experiments, laboratory and field work, evaluation of the data, preparation of the manuscript, **overall own contribution: 80 %**
Sebastian Schäfer:	laboratory and field work (pilot plant)
Grit Röder:	laboratory and field work
Susanne Lehmann:	UV-vis spectroscopy
Isabel Zirnstein:	culture-independent analyses of the microbial diversity
Evelyn Krawczyk-Bärsch:	supervision of the project, discussion of the results
André Rossberg:	XANES data evaluation, discussion of the XANES results

3 Manuscripts

3.1 Combined use of flow cytometry and microscopy to study the interactions between the gram-negative betaproteobacterium *Acidovorax facilis* and uranium(VI)

Gerber U, Zirnstein I, Krawczyk-Bärsch E, Lünsdorf H, Arnold T, Merroun ML (2016)
J Hazard Mater 347:233–241. doi.org/10.1016/j.jhazmat.2017.12.030

Journal of Hazardous Materials 317 (2016) 127–134

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Combined use of flow cytometry and microscopy to study the interactions between the gram-negative betaproteobacterium *Acidovorax facilis* and uranium(VI)

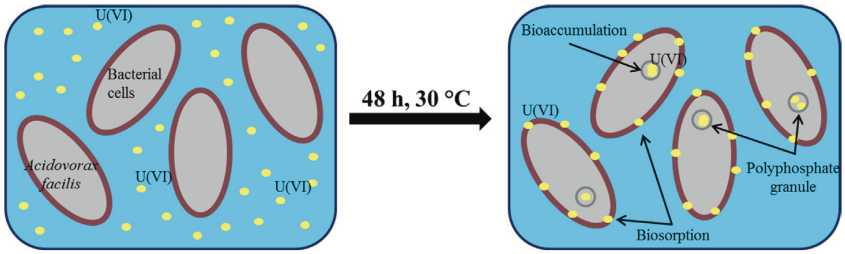
U. Gerber[a,*], I. Zirnstein[b], E. Krawczyk-Bärsch[a], H. Lünsdorf[c], T. Arnold[a], M.L. Merroun[d]

[a] Helmholtz-Zentrum Dresden-Rossendorf, Institute of Resource Ecology, P.O. Box 510119, 01314 Dresden, Germany
[b] Research Institute of Leather and Plastic Sheeting (FILK) gGmbH, Meissner Ring 1-5, 09599 Freiberg, Germany
[c] Helmholtz Centre for Infection Research, Central Facility for Microscopy, Inhoffenstr. 7, D-38124 Braunschweig, Germany
[d] University of Granada, Department of Microbiology, Campus Fuentenueva, E-18071 Granada, Spain

HIGHLIGHTS

- *Acidovorax facilis* is able to remove 130 mg U/g dry biomass from solution.
- Kinetically temperature-dependent uranium removal was studied.
- Cell viability and metabolic activity was tested by flow cytometry.
- Uranium was removed by active biosorption and passive bioaccumulation.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 13 April 2016
Received in revised form 17 May 2016
Accepted 19 May 2016
Available online 20 May 2016

Keywords:
Acidovorax facilis
AMD
Uranium
In situ bioremediation
Flow cytometry
Metal tolerance

ABSTRACT

The former uranium mine Königstein (Saxony, Germany) is currently in the process of remediation by means of controlled underground flooding. Nevertheless, the flooding water has to be cleaned up by a conventional wastewater treatment plant. In this study, the uranium(VI) removal and tolerance mechanisms of the gram-negative betaproteobacterium *Acidovorax facilis* were investigated by a multidisciplinary approach combining wet chemistry, flow cytometry, and microscopy. The kinetics of uranium removal and the corresponding mechanisms were investigated. The results showed a biphasic process of uranium removal characterized by a first phase where 95% of uranium was removed within the first 8 h followed by a second phase that reached equilibrium after 24 h. The bacterial cells displayed a total uranium removal capacity of 130 mg U/g dry biomass. The removal of uranium was also temperature-dependent, indicating that metabolic activity heavily influenced bacterial interactions with uranium. TEM analyses showed biosorption on the cell surface and intracellular accumulation of uranium. Uranium tolerance tests showed that *A. facilis* was able to withstand concentrations up to 0.1 mM. This work demonstrates that *A. facilis* is a suitable candidate for *in situ* bioremediation of flooding water in Königstein as well as for other contaminated waste waters.

1. Introduction

The former uranium mine of the WISMUT GmbH near Königstein (Saxony, Germany) was one of the biggest uranium mines in Eastern-Germany. Here, uranium was leached from the sand-

* Corresponding author.
E-mail address: u.gerber@hzdr.de (U. Gerber).

http://dx.doi.org/10.1016/j.jhazmat.2016.05.062
0304-3894/

stone using sulfuric acid. Since 2001, controlled flooding of the mine has been implemented for remediation purposes [1]. Due to the leaching process the flooding water is characterized by a low pH of 2.9 and high concentrations of uranium (13 mg/L) and other heavy metals [2]. Because of the potential risk of uranium mobilization from the flooding waters into neighboring aquifers, particularly the aquifer utilized by the nearby city of Pirna, only half of the mine has been flooded. By doing so, the flooding water can be pumped to the surface and purified by a conventional and intensive wastewater treatment plant [3]. Notably, the flooding water also contains a high concentration of sulfate originating from the sulfuric acid leaching, which has resulted in the formation of acid mine drainage (AMD). AMD is a major problem that often occurs in the mining industry. AMD waters are characterized by high sulfate concentrations and high metal content at low pH values. Despite the fact that it is known to be toxic to aquatic organisms [4,5] uranium plays a major role in the ecosystem of the Königstein flooding water [2]. In bulk solutions, uranium predominantly occurs as aqueous species, such as highly mobile UO_2SO_4-species that has been identified in the AMD flooding water [2]. It is an important task to remove the uranium and further associated toxic metals from AMD waters to prevent environmental pollution. The technical effort and high cost of conventional metal remediation techniques based on chemical applications [6] leads to a non-negligible interest for alternative treatments.

The transport of uranium is governed by the contrasting chemistry of U(IV) and U(VI). U(VI) generally forms mobile aqueous complexes with carbonate and hydroxide, while U(IV) precipitates as the highly insoluble mineral uraninite [7]. The highly soluble form UO_2SO_4 that is present in the flooding water in Königstein could easily migrate into the groundwater, and thus could contaminate drinking water. Nevertheless, migration processes are strongly influenced by abiotic factors such as ions, pH, and minerals [8–11]. Furthermore, microbial interactions can influence the mobility of radionuclides and their migration behavior as well.

AMD waters are known to contain abundant microbial life [12,13]. Indigenous microorganisms could be used for bioremediation purposes since they are known to interact with metals and radionuclides in a variety of ways: (i) by biosorption where the metal is passively sorbed onto functional groups of the cell surface [14,15], (ii) by bioaccumulation where the metal is actively taken up into the cell [16,17,18], (iii) by biotransformation where the metal will be reduced or oxidized by the microorganism [19,20,16], or (iiii) by biomineralization [21,22]. Hereby, microorganisms could change the speciation of radionuclides and influence their mobility.

In this study, the gram-negative betaproteobacterium *Acidovorax facilis* (*A. facilis*, former name *Pseudomonas facilis*) [23] was used to investigate the interactions between uranium(VI) as a representative microorganism that would be found in the flooding water of Königstein. *A. facilis* is ubiquitously distributed in the nature and a common soil microorganism. The bacterial cells are rod-shaped and at in vitro growth 2 μm long. It belongs to the family of Comamonadaceae [24] and grows aerobically. Flow cytometry studies were used to analyze the cell viability together with the metabolic activity of bacterial cells brought in contact with uranium. The multiparameter flow cytometry has become a powerful tool over the last 20 years [25]. This technique allows a fast and single-cell analysis. Furthermore, the interactions of *A. facilis* with uranium were investigated for the first time and results emphasize a significant potential of uranium removal in dependence with the metabolism. In order to obtain detailed information about local deposit of removed uranium by *A. facilis*, ultrastructural analysis by transmission electron microscopy (TEM) in combination with electron energy-loss spectroscopy (EELS) was performed. These results provide new insight on the interaction of bacterial cells with uranium, and consequently, the potential to use bacterial cells for *in situ* bioremediation of contaminated AMD waters.

2. Methods and material

2.1. Water sampling, cultivation of acidophilies

Samples (5 L) of flooding water from the uranium mine Königstein (Germany) were taken for the cultivation of aerobic, acidophilic microorganisms. The transport took place at 4 °C and maintained at temperature until further processing (at the latest 24 h). For isolation of iron-and sulfur-oxidizing bacteria, 100 μL of the sampled mine water were streaked onto solid FeTSB medium [26] and incubated at 28 °C for up to 4 weeks. Bacterial colonies were then divided into those that were ferric iron encrusted (i.e., iron-oxidizing bacteria) and those that were not (i.e., acidophilic heterotrophs). Purification of cultures was achieved by repeated plating and single-colony isolation.

2.2. Organism and culture conditions

The strain of *Acidovorax facilis* was kindly provided by the Centre for Environmental Research – UFZ in Leipzig (Germany), Department of Environmental Microbiology (Antonis Chatzinotas). The cells were grown in Nutrient Broth medium (NB) (Peptone 5.0 g/L and Beef Extract 3.0 g/L, pH 7.0 ± 0.2, Sifin, Berlin, Germany) at 30 °C and 120 rpm on a rotary shaker (Thermoshake, EA2, Gerhardt, Königswinter, Germany) overnight.

2.3. Determination of minimal inhibitory concentration (MIC) of uranium for bacterial growth

For the uranium tolerance test, cells were grown in NB overnight at 30 °C and 130 rpm in 500 mL flasks. Cells were washed twice in aqueous 0.9% NaCl. A cell suspension of 100 μL with an $OD_{600\,nm}$ of 0.5 were plated onto solid agar plates containing NB medium 1:5 diluted with the adjusted metal concentrations from 0.01 mM to 1.0 mM uranium, which was added as $UO_2(NO_3)_2$. The plates were incubated for 48 h at 30 °C. The minimal inhibitory concentration (MIC), which is defined as the lowest concentration to prevent bacterial growth and colony formation, was determined in triplicates.

2.4. Uranium interaction experiments – removal capacity for uranium

For uranium interaction experiments, cells were grown in NB medium overnight at 30 °C at 130 rpm till an OD_{600nm} of 2.0 was reached. The cells were washed two times with sterilized tap water with a pH adjusted to 5.0. Washed cells were diluted to an OD_{600nm} of 1.0 (12.0 ± 1.0 mg dry weight/mL) with sterilized tap water at pH 5.0. A solution of $UO_2(NO_3)_2$ (0.1 M) was added to a final concentration of 0.05 mM and 0.1 mM, respectively. For temperature dependent experiments at 4 °C and 30 °C, cells were washed and suspended in sterilized tap water pH 5.0 that was acclimated to 4 °C or 30 °C. Cell suspensions were incubated at the selected temperature for 48 h and 130 rpm. All experiments were carried out in triplicates. After distinct times (5 min, 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, 8 h, 24 h, 30 h, 48 h), samples were taken to determine residual uranium concentrations in the supernatant. These samples were centrifuged for 5 min at 13,000 rpm at room temperature (Centrifuge 5415R, Eppendorf AG, Hamburg, Germany) and the supernatant was taken for Inductively Coupled Plasma Mass Spectrometry measurements (ICP-MS) using an ELAN 9000 type ICP-MS spectrometer (Perkin Elmer, Überlingen, Germany). Passive adsorption experiments of uranium onto dead cells were performed using cultures grown overnight that were immediately autoclaved for 20 min at 121 °C

and 1 bar. Autoclaved cells were assayed using the same method as living cells as is described above. The amount of removed uranium from the solution was normalized to the dry biomass after drying the cell pellet for 24 h at 100 °C in an oven (Memmert UE500, Schwabach, Germany).

2.5. Energy-filtered transmission electron microscopy (EF-TEM) and electron energy-loss spectroscopy (EELS)

When uranium interaction experiments were performed the supernatant was removed and the cell pellet was washed twice with 20 mM Hepes at pH 7.2 for 5 min at 6000 rpm (Centrifuge 5804R, Eppendorf AG, Hamburg, Germany). Subsequently, they were fixed at 1% (vol/vol) Glutardialdehyde from 50% (v/v) stock (Carl Roth, Karlsruhe, Germany). Sample preparation with minor modifications was done according to [27]. After immobilization in 1% (w/v) aqueous low melting agarose (Life Technologies Inc., Darmstadt, Germany), cells were dehydrated by an ethanol series (10, 30, 50, 70, 90, 100% for 2 min on ice; 100% for 15 min at room temperature) followed by ERL-resin impregnation and polymerization [28]. Ultrathin sections of 35–40 nm were cut with a diamond knife (EMS, Munic, Germany) and analyzed with an in-column energy-filter TEM (LIBRA 120plus, Zeiss, Oberkochen, Germany) at nominal magnifications of ×4000–×50,000, and an energy setting as is described in Ref. [27].

2.6. Live-dead staining

After the uranium interaction experiments, cells were harvested by centrifugation at 13,000 rpm for 5 min and subsequently washed with 0.9% NaCl solution. Afterwards, the cells were stained with the LIVE/DEAD® BacLighlyt™ Bacterial Viability Kit (Molecular Probes, Inc., Eugene, OR, USA) with propidium iodide and SYTO®9 following the user instructions (https://tools.thermofisher.com/content/sfs/manuals/mp07007.pdf). The fluorescent dye propidium iodide can only enter cells with injured/impaired cell membranes, thus staining dead cells in red. In contrast, SYTO®9 can diffuse through intact cell membranes into the cells staining them green (living cells). Application of both dyes allows the discrimination of dead and living cells. To 330 μL cell suspension, 1 μL of the staining solution was added. After incubation at ambient temperature for 10 min in the dark, the cells were washed again to remove the unbounded stains. The washed and stained cells were diluted in 330 μL 0.9% NaCl. For the microscopically investigations, an Olympus light microscope-BX-61 (Olympus Europa Holding GmbH, Hamburg, Germany), combined with BX-UCB (control box) and U-RFL-T (power supply for the 100 W mercury lamp) was used. Fluorescence microscopy at wavelengths between 420 and 460 nm was performed using a super-wide band filter mirror unit (U-MSWB, Olympus Europa Holding GmbH, Hamburg, Germany).

2.7. Flow cytometry studies

An inoculum of an overnight culture was added to 1:5 diluted NB medium either without uranium as a control or containing a uranium concentration of 0.05 mM or 0.1 mM. The cells were grown for 48 h at 30 °C and 130 rpm. All experiments were done in triplicates. After incubation, the cells were harvested by centrifugation at 8000 rpm (Centrifuge 5804R, Eppendorf AG, Hamburg, Germany) for 10 min and washed twice with 35 °C warmed Phosphate Buffered Saline (PBS). Afterwards, the cells were immediately dissolved in PBS to approximately 10^6 cells/mL. An aliquot of the cells were incubated for 45 min at 80 °C as the 'dead' control. The cell viability test was done with propidium iodide (PI) and fluorescein diacetate (FDA). The dyes were added to a final concentration of 2 μL/mL for PI and 20 μL/mL FDA. To measure metabolic activity as a response of the membrane potential, 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) was added with a final concentration of 10 μM. The cell suspension with the different staining was incubated 15 min in the dark at ambient temperature. Microbial suspensions incubated in the presence of both stains (PI and FDA) simultaneously were analyzed by flow cytometry for non-viable/dead (red) and viable (green). Samples were analyzed using a FACSCantoII cytometer Becton Dickinson (San Jose Palo Alto, California), equipped with three lasers: 488 nm blue, 620 nm red, and 405 nm violet.

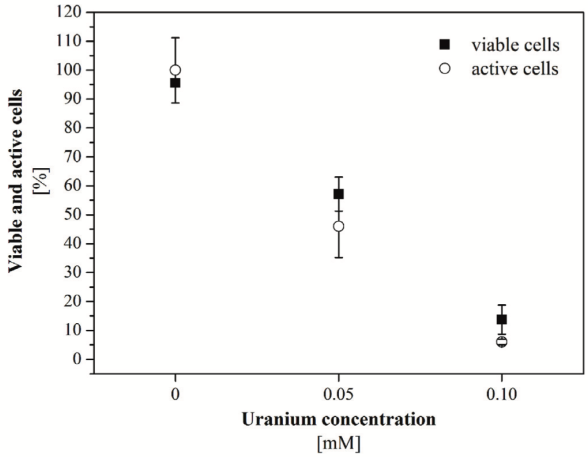


Fig. 1. Cell viability and metabolic activity. Percentage of viable cells after cell viability test (squares) stained with PI and FDA. Percentage of active cells (circles) after metabolic activity test stained with $DiOC_6$.

3. Results

3.1. Uranium tolerance of A. facilis

3.1.1. Determination of minimal inhibitory concentrations of uranium in solid media

To evaluate the uranium tolerance levels of *A. facilis*, solid agar plates with increasing metal concentrations were inoculated and examined for cell growth and formation of colonies. The results obtained indicated that the cells were able to grow at up to 0.1 mM uranium (maximal tolerated concentration, MTC). The Minimal inhibitory concentration (MIC) for the growth of this bacterial strain was 0.2 mM.

3.1.2. Cell viability and metabolic activity

The uranium tolerance of *A. facilis* was also studied by flow cytometry using the live-dead staining approach (cell viability); test were conducted with PI and FDA together with $DiOC_6$ (metabolic activity) that stains active cells. The cell viability test is based on the use of a kit containing these two stains, both, which stain nucleic acids. FDA stains viable cells, and thus is able to enter all cells, whereas PI enters only cells with damaged membranes, i.e. dead cells.

The percentages of viable and active cells of the bacterial population treated with uranium concentrations ranging from 0.05 to 0.1 mM during 48 h are displayed in Fig. 1 (histograms see Fig. 2). In absence of uranium, 95.6% of the cells were viable (Fig. 2b) and 100% of the cells were active stained. For comparison, the dead control exhibited 100% dead cells (Fig. 2a). The results of the cell viability test showed a decrease in function with increasing uranium concentration. At 0.05 mM of uranium, 57% of the cells (Fig. 2c) exhibited intact cell membranes and thus were viable. At 0.1 mM, cytotoxic concentration had been reached, as 86.3% of total cells

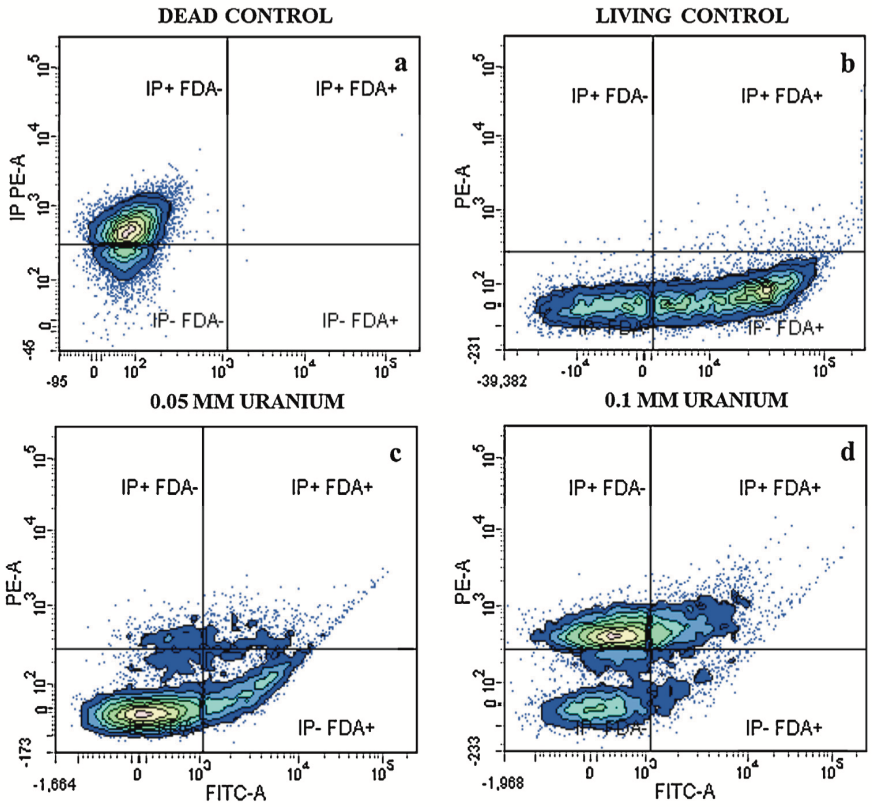


Fig. 2. Histograms of cell viability. Dead control (a), living control without uranium (b), with 0.05 mM uranium (c) and with 0.1 mM uranium (d). Cells were stained with FDA and PI. IP+ FDA- (cells positive for PI, and negative for FDA, non-viable cells), IP+ FDA+ (cells positive for both stains, non-viable cells), IP- FDA- (cells negative for both stains, unstained cells), IP- FDA+ (cells positive for FDA, viable cells).

(Fig. 2d) were not viable. To examine the effect of uranium on the metabolic activity of the bacterial cells the fluorescent dye $DiOC_6$ was used. This dye shows fluorescence as a function of the membrane potential and can be used as a test of metabolic activity, where only viable cells should exhibit an intact electrochemical membrane potential [29,30]. The results of the oxidative stress response (Fig. 1, circles) were comparable to the cell viability test. At a uranium concentration of 0.05 mM, 46% of the cells were active, whereas at 0.1 mM, only 6% of the cells were stained active. This indicates that with increasing uranium concentration the metabolic activity of the cells decreases.

Compared with the results of cell viability, the metabolic activity displays nearly the same results, and thus correlates significantly. The results of the flow cytometry showed *A. facilis* to be quite tolerant against a uranium concentration of 0.05 mM. At twice the uranium concentration, fewer of the total cells were viable.

3.2. Uranium interactions

3.2.1. Uranium removal by A. facilis

In Fig. 3a, the uranium removal capacity at 0.05 mM (filled circles) and 0.1 mM (empty squares) of living bacterial cells is presented. The equilibrium of uranium removal was reached after the first 8 h. The uranium removal occurred quite rapidly, and at both concentrations metabolically active cells were able to remove nearly 100% of the initial uranium concentration from the solution after 24 h incubation time. At an initial dry biomass (DBM) of 12 ± 1 mg, the cells of *A. facilis* removed around 45 mg and 100 mg of uranium at a metal concentration of 0.05 mM and 0.1 mM, respectively. After 24 h, maximum removal capacity by bacterial cells was reached and no changes were visible.

Uranium removal within the first 8 h indicates a fast, and therefore likely passive process. In order to determine the active uptake of uranium, the removal capability was additionally tested with dead cells at initial uranium concentrations of 0.05 mM and 0.1 mM, respectively. An overnight culture was autoclaved and dead cells were subsequently tested using the same method as the living cells of *A. facilis*. Fig. 3b shows similar removal capacity of dead cells in comparison to that of living cells. However, maximum removal was reached after 5 min incubation, which was remarkably faster than observed for the removal of uranium and not the case for the living cells. Experiments with dead cells revealed no time-dependent component for the removal of uranium, which was not observed for living cells. During the 48 h period of incubation, there was no remobilization of cell-bound uranium observed.

To test the effect of temperature on the removal capacity of uranium by *A. facilis*, two different temperatures, i.e. 4 °C and 30 °C, were chosen (Fig. 3c). The results show a distinct temperature-dependence of uranium removal. At 30 °C (filled squares) after an incubation time of 48 h, cells removed about 100 mg U/g DBM, whereas at 4 °C (empty squares) only 65 mg U/g DBM were removed by the cells. At 2 h of incubation, the first noticeable differences were detectable. Cells removed higher amounts of uranium (40 mg/g DBM) at a temperature of 30 °C relative to experiments performed at 4 °C.

To determine the uranium removal capacity as a function of the dry biomass, different amounts of dry masses were tested (Fig. 3d). Results indicated that with decreasing dry mass the ura-

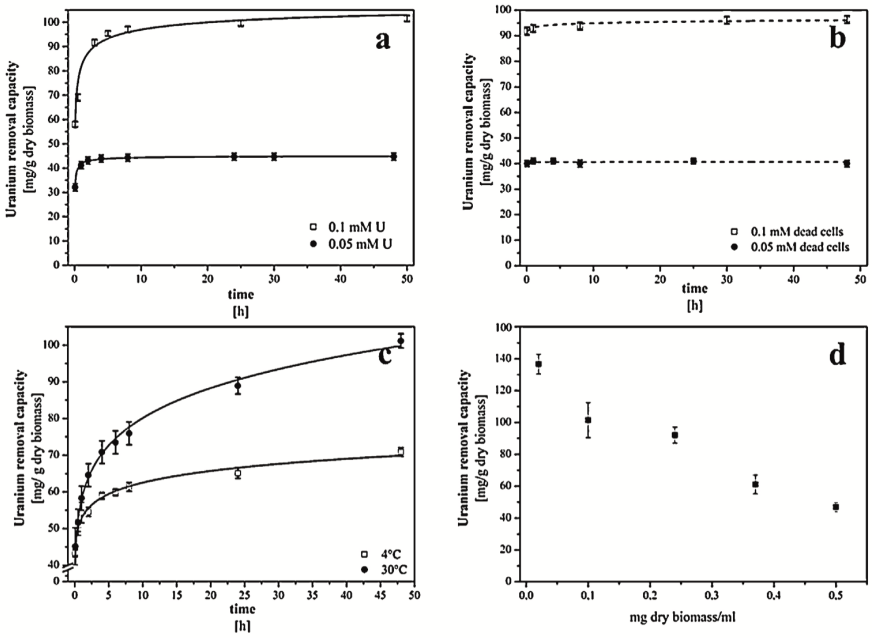


Fig. 3. Uranium removal capacity of *A. facilis*. Uranium removal capacity of living cells at 0.05 and 0.1 mM uranium (a), with dead cells at 0.05 and 0.1 mM uranium (b), and with living cells at 4 °C and 30 °C at 0.1 mM uranium (c). Uranium removal capacity as a function of the dry biomass (d) at 0.1 mM uranium.

nium removal capacity per mg DBM was increased. Maximum removal was reached at 0.02 mg DBM/mL with a uranium removal capacity of about 140 mg U/g dry biomass. These data show that the amount of uranium removed from the solution by *A. facilis* strongly depends on the dry biomass concentration.

After uranium removal experiments, the cells were tested for their cell viability by live-dead staining (staining with PI and Syto®9). Fluorescence light microscopy (Fig. 4) showed high numbers of viable or living cells after 48 h incubation. Compared to control samples (Fig. 4a), cells that were incubated at a uranium concentration of 0.05 mM showed nearly the same number of viable cells (Fig. 4b). At an initial uranium concentration of 0.1 mM, (Fig. 4c) fewer cells were stained non-viable. Nevertheless, the majority of cells were viable after 48 h with both 0.05 mM and 0.1 mM uranium. Finally, when extending the uranium removal period, cells remained viable and were able to tolerate both uranium concentrations.

3.2.2. In situ *localization of the immobilized uranium by EF-TEM and EELS*

Immediately following the immobilization experiments, cells were harvested, washed and fixed with glutardialdehyde for electron microscopic analysis in order to localize uranium at the subcellular level. Results showed uranium was accumulated within the cytoplasm and bound to the outer membrane of *A. facilis*. During the removal of uranium, two sorption sites with low elemental load were observable: (i) at polyphosphate granules within the cytoplasm, and (ii) at the cell periphery, i.e., the outer membrane (Fig. 5b, d). Parallel EELS analysis (Fig. 5c) identified uranium as a constituent element based on the U-$O_{4,5}$ ionization edge ($O_{4,5}$ = 93.5 eV) [31]. In addition, ionization edges of $PL_{2,3}$ were obtained (Fig. 5c) and the corresponding energy-loss near-edge structure (ELNES) fingerprint [32] clearly indicated the presence of phosphate-phosphorus. The encircled area in Fig. 5b indicates the measuring area during wide range parallel EELS (WR-PEELS) registration (Fig. 5c). Besides the electron dense polyphosphate inclusion, which exhibits a uranium load (Fig. 5d), the elemental map showed further electron-dense matter deposited on the cell surface, i.e., the outer membrane (Fig. 5d). In contrast to cytoplasmic PPGs, no characteristic P-$L_{2,3}$ phosphorus could recognized on the cell surface (Fig. 5e), which means that the phosphorous content of the outer membrane, i.e., the phospholipids, was rather low and beyond the detection limit.

4. Discussion

This work describes the interaction mechanisms between uranium and *A. facilis* using a multidisciplinary approach combining microbiological, cell biology, and microscopic techniques. *A. facilis* is a common soil bacterium spread worldwide and is not fastidious concerning a carbon source [23,33]. Based on sequence homology (99%) of isolated microorganisms from the flooding water in Königstein, *A. facilis* is closely related and belongs to the class of betaproteobacteria (Supplementary information S1). Previous results have shown that bacteria belonging to this class were the major part of metabolic active microorganisms of the flooding water [34]. Thus, members of this class are able to live and survive under these extreme environmental conditions, i.e., acidic pH of 2.9 and high uranium concentration of 13 mg/L. Because of these characteristics and the ease in handling, *A. facilis* was chosen for this study.

The ability to remove uranium from the solution was tested at two initial concentrations at a pH-value of 5.0. At both uranium concentrations 0.05 mM and 0.1 mM, *A. facilis* was able to nearly remove all soluble uranium. In total, the bacterial cells were capable to remove around 130 mg U/g DBM. This removal capacity can be compared with other microorganisms such as *Paenibacillus* sp. JG-TB8, which was recovered from a soil sample of the uranium mining waste pile “Haberland” (Johanngeorgenstadt, Saxony, Germany), that displayed a uranium removal capacity of 138 mg U/g BDM (at pH 4.5) [35]. However, *A. facilis*, a bacterium not native to uranium-contaminated environments, was able to remove nearly the same

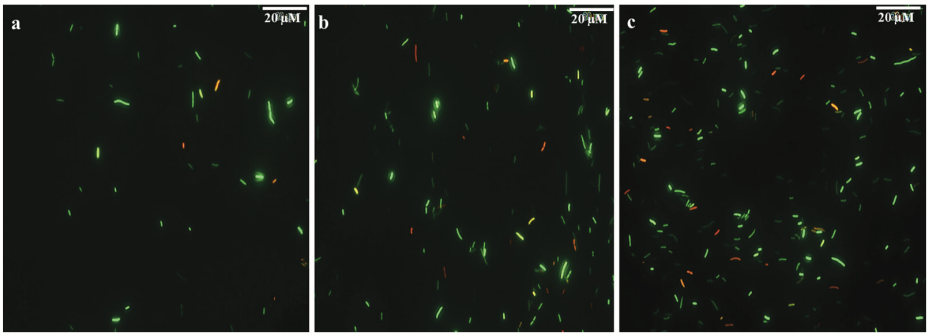


Fig. 4. Live-dead staining of *A. facilis* cells after uranium interaction experiments. Control samples without uranium, back ground solution tap water pH 5.0 (a), cells with 0.05 mM uranium (b), and 0.1 mM uranium (c). Washed cells were stained with PI (red fluorescence, non-viable cells) and SYTO9 (green fluorescence, viable cells).

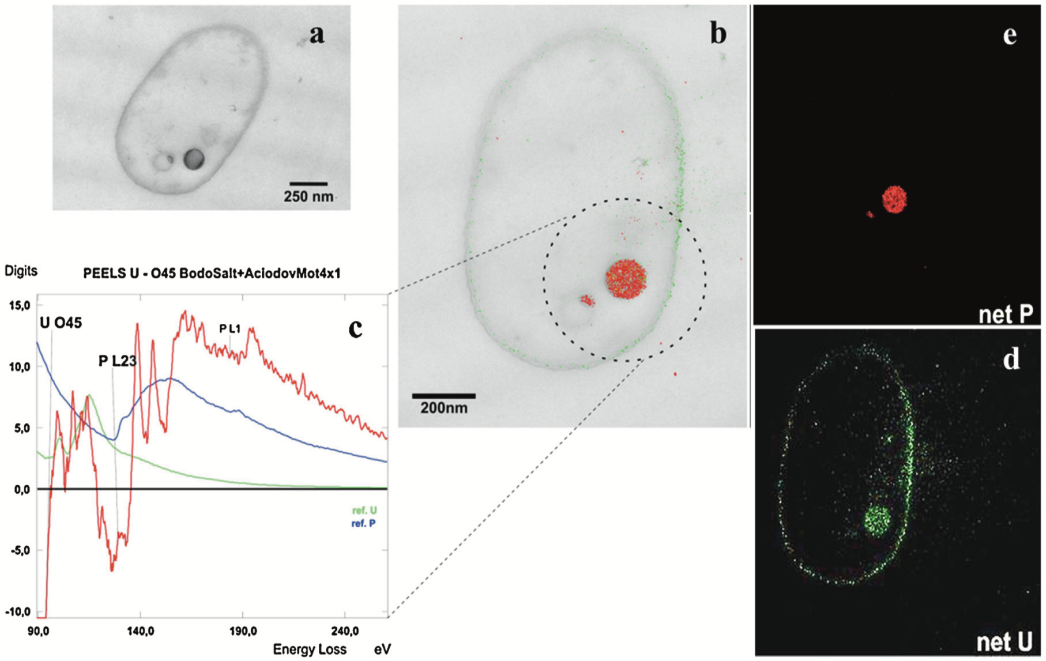


Fig. 5. EF-TEM analysis of an ultrathin-sectioned *A. facilis* sample after uranium removal. Survey view of a typical bacterial cell with an electron dense polyphosphate granule (a), superposition of phosphorous (red) and uranium (green) elemental maps on the bacterial motif (b), WR-PEELS of the measuring area (red graph) (c), encircled in (b); reference spectra of U (green graph) and P (blue graph) are shown and ionization edges are indicated. Net U-O45 map (d), net P-L23 map (e).

amounts of uranium as those of the indigenous isolates. Similarly, the gram-negative bacterium *Pseudomonas fluorescens*, which was isolated from a granitic rock aquifer at the Aspö Hard Rock Laboratory (Schweden), showed a uranium binding capacity at pH 6.0 of 124 mg U/g DBM at an initial uranium concentration of 24 mg/L [36].

The kinetic studies indicated that the removal of uranium was a two phasic process: (i) a fast phase during the first 8 h most likely associated to metabolically independent biosorption, and (ii) a slow phase where uranium was accumulated intracellularly by metabolically dependent mechanisms. The fast biosorption of uranium on the cell surface could be explained by the removal capacity of alive and dead cells, which showed no differences. Similar results have also been reported in [37]. The temperature-dependent bioaccumulation and removal capacity of uranium by *A. facilis* might be explained by differences in metabolic activity at these temperatures. The bacterial cells removed higher amounts of uranium at 30 °C compared to 4 °C. The process of biosorption is described by a metabolism-independent sorption of uranium on carboxyl, amine, hydroxyl, phosphate, and sulfhydryl groups of the negatively charged cell membrane [38]. The process is rapid and will be not affected by temperature [39]. In contrast, the gram-negative bacterium *Citrobacter freudii* showed a fairly steady uranium adsorption capacity of 48 mg U/g DBM in temperature ranges of 25–54 °C [40]. Thus, *C. freudii* exhibit no temperature-dependent uranium removal possibly due to the high initial concentration of uranium (150 mg/L). [40] performed no investigation on the cell viability or metabolic state of the bacterial cells, which could be useful to understand the interaction mechanisms of bacterial cells and uranium. The mechanism of intracellular bioaccumulation of actinides is poorly understood. Nevertheless, previous studies assumed an active, yet metabolism-independent transport of uranium into the cell [41]. Thus, it is possible metabolically active cells are likely to remove higher amounts of uranium at lower initial concentrations by active processes like bioaccumulation.

The results of the cell viability and metabolic activity revealed in a decrease of active cells with increasing uranium concentration. This could be explained due to the chemical and radiological toxicity of uranium. Compared to copper and nickel uranium is 20–40 times more toxic [42]. Nevertheless, the toxicity of uranium is primarily derived from the chemical toxicity rather than from its radiological [43].

Flow cytometry and CFU test results revealed that *A. facilis* could tolerate concentrations up to 0.05 mM uranium in solution and 0.1 mM uranium on solid medium, respectively. Compared to indigenous microorganisms, the uranium tolerance of *A. facilis* was relatively moderate. Natural isolates from ore deposit in northeast India have displayed tolerances up to 4.0 mM [44]. Additionally, bacteria isolated from a Spanish clay deposit have shown tolerates up to 6.0 mM uranium [45]. This suggests that microorganisms that have been exposed to high uranium concentrations in the environment have become better adapted. Additionally, flow cytometry was used for the first time to investigate the uranium tolerance of *A. facilis* in liquid medium, which proved to be a fast and quantitatively reliable method for these tests. In addition, the results from live-dead staining after uranium removal experiments revealed similar findings; the majority of the cells were stained viable. Therefore, the cells were able to tolerate both uranium concentrations as well as survive in sterilized tap water with pH 5.0 without any carbon supplement. The measured amount of TOC (total organic carbon) within the flooding water of 1.1 mg/L [34] is similar to tap water with 1.7 mg/L. Despite the low content in both waters, *A. facilis* has demonstrated that it could survive, and thus remove the uranium from the contaminated water *in situ*. Therefore, *A. facilis* could be used as a biofilter to remove uranium from the contaminated water under these conditions. Similar applications were described for bacterial biofilms that were responsible for the degradation of organic contaminants [46].

The results of the TEM investigations (incubation at 30 °C) verified the two processes predicted by the temperature-dependent uranium removal experiments. The element mapping of the ultra-thin section clearly showed biosorption of uranium onto the cell membrane as well as intracellular accumulation of uranium. Intracellular accumulated uranium was found at low loading concentrations within cytoplasmic polyphosphate inclusions, whereas the concentration of uranium bound to the cell membrane was much higher. Previous studies have shown that phosphorylic or carboxylic groups that compose the cell membrane bind uranium, and the immobilization at these sites seems to be species-specific [47,17,48,15,49,50,18]. However, with this study it was not possible to detect the phosphorylic groups of the cell membrane due to the detection limit of TEM/EELS studies. Further investigations should be performed in order to get detailed information about uranium binding to the cell membrane of *A. facilis*. Within the cytoplasm, uranium was found associated with polyphosphate granules, which are known to be important stores of divalent cations [51,15], that act in detoxification of intracellular uranium [18]. Previous studies showed that *Arthrobacter* only accumulated uranium intracellularly. Here TEM-EDX studies revealed that uranium was associated with polyphosphate granules similar to cells of *A. facilis* [18]. In contrast to [52], who assumed an energy-independent diffusion system, the accumulation of uranium seems to be partially energy-dependent by *A. facilis*. Formation of polyphosphate granules (PPGs) as intracellular electron-dense inclusions is generally observed in bacteria, but is also known from microorganisms isolated from uranium mine wastes piles [53]. Literature explains the formation of PPGs as the cells' response to heavy metal stress caused by the release of inorganic phosphate ($H_2PO_4^-$) from the cellular polyphosphate and subsequent precipitation of uranium as uranyl phosphate ($UO_2[H_2PO_4]_2$) [47]. These results clearly reveal that *A. facilis* was able to remove high amounts of uranium from solution within 48 h by two different processes: passive biosorption and active bioaccumulation.

5. Conclusion

The present study describes the interactions of the betaproteobacterium *A. facilis* with uranium. It has been demonstrated that *A. facilis* is capable of removing high concentrations of uranium in solution (130 mg U/g dry biomass), and furthermore, able to tolerate uranium concentrations consistent with those found in the flooding water of Königstein. Flow cytometry has been used to investigate the uranium tolerance of the bacterium *A. facilis*. Compared to the CFU test on solid agar plates, *A. facilis* tolerates half of the uranium concentration in liquid medium. Nevertheless, live-dead staining after uranium removal experiments revealed that the majority of the cells were viable. Furthermore, the results of the investigations with uranium and *A. facilis* showed that removal occurred via different mechanisms. According to TEM observations, uranium was passively bound to the outer membrane of the bacterium and actively taken up into the cytoplasm. Temperature-dependent removal experiments showed that *A. facilis* removed higher amounts of uranium at higher temperatures. In conclusion, uranium removal by *A. facilis* is fast, efficient, and thus appropriate for *in situ* bioremediation of the flooding water from the former uranium mine Königstein. The chemical remediation of the Königstein flooding water is expensive and a time consuming process, which will probably have to be run the next 100 years. The *in situ* bioremediation by microorganisms could reduce the costs and efforts dramatically.

Acknowledgement

This work was supported by the Bundesministerium für Bildung und Forschung (BMBF) grand n° 02NUK030 F (TransAqua). This work was funded also by the ERDF Grants, CGL-2012-36505, CGL2014-59616-R (80% finding by FEDER) (Ministerio de Ciencia e Innovación). The authors are grateful to E. V. Johnstone for his grammatical corrections of this manuscript, the Wismut GmbH for allowing access to the sampling site, S. Röder for assistance in water sampling, S. Gurlit and S. Schubert for ICP-MS measurements. The skillful work of electron microscopic sample preparation by I. Kristen (HZI, Braunschweig) is gratefully acknowledged. We thank J. Lazuen-Alcon (Flow Cytometry Service, Centro de Instrumentación Científica University of Granada, Spain) for his help with flow cytometry measurements.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2016.05.062.

References

[1] J.S.A.U. Zimmermann, On Uranium Mining and Hydogeology II, in: B. Merkel, C. Helling (Eds.), Sven von Loga, Freiberg, Germany, 1998, pp. 140–151.
[2] T. Arnold, N. Baumann, E. Krawczyk-Barsch, S. Brockmann, U. Zimmermann, U. Jenk, S. Weiss, Identification of the uranium speciation in an underground acid mine drainage environment, Geochim. Cosmochim. Acta 75 (2011) 2200–2212.
[3] J.S.H. Märten, D. Seeliger, K. Sommer, Uranium removal from flood water at königstein mine, in: B.P.-F.B.J. Merkel, C. Wolkersdorfer (Eds.), Uranium in the Aquatic Environment, Springer, 2002, pp. 763–768.
[4] P. Barton, The acid mine drainage, sulfur in the environment, in: J.O. Nriagu (Ed.), Ecological Impacts, Part II, Wiley-Interscience, New York, 1987, pp. 313–358.
[5] M. Kelly, Mining and the Freshwater Environment, Elsevier Applied Science, London, 1988.

[6] J.R. Lloyd, D.R. Lovley, L.E. Macaskie, Biotechnological application of metal-reducing microorganisms, Adv. Appl. Microbiol. 53 (2003) 85–128.
[7] J.L. Nyman, T.L. Marsh, M.A. Ginder-Vogel, M. Gentile, S. Fendorf, C. Criddle, Heterogeneous response to biostimulation for U(VI) reduction in replicated sediment microcosms, Biodegradation 17 (2006) 303–316.
[8] T. Arnold, T. Zorn, H. Zanker, G. Bernhard, H. Nitsche, Sorption behavior of U(VI) on phyllite: experiments and modeling, J. Contam. Hydrol. 47 (2001) 219–231.
[9] M.O. Barnett, P.M. Jardine, S.C. Brooks, H.M. Selim, Adsorption and transport of uranium(VI) in subsurface media, Soil Sci. Soc. Am. J. 64 (2000) 908–917.
[10] M.C. Duff, J.U. Coughlin, D.B. Hunter, Uranium co-precipitation with iron oxide minerals, Geochim. Cosmochim. Acta 66 (2002) 3533–3547.
[11] M.H. Baik, S.P. Hyun, W.J. Cho, P.S. Hahn, Contribution of minerals to the sorption of U(VI) on granite, Radiochim. Acta 92 (2004) 663–669.
[12] D.K. Nordstrom, G. Southam, Geomicrobiology of sulfide mineral oxidation, in: J.F. Banfield, K.H. Nealson (Eds.), Geomicrobiology: Interactions Between Microbes and Minerals, Reviews in Mineralogy, Mineralogical Society of America, Washington, D.C, 1997, pp. 361–390.
[13] B.J. Baker, G.W. Tyson, L. Goosherst, J.F. Banfield, Insights into the diversity of eukaryotes in acid mine drainage biofilm communities, App. Environ. Microbiol. 75 (2009) 2192–2199.
[14] J.R. Lloyd, Bioremediation of radionuclide-containing wastwaters, in: D.R. Loveley (Ed.), Environmental Microbe-Metal Interactions, ASM Press, Washington D.C, 2000, pp. 277–327.
[15] M.L. Merroun, J. Raff, A. Rossberg, C. Hennig, T. Reich, S. Selenska-Pobell, Complexation of uranium by cells and s-layer sheets of *Bacillus sphaericus* JG-A12, Appl. Environ. Microbiol. 71 (2005) 5532–5543.
[16] D.R. Brookshaw, R.A.D. Pattrick, J.R. Lloyd, D.J. Vaughan, Microbial effects on mineral-radionuclide interactions and radionuclide solid-phase capture processes, Mineral. Mag. 76 (2012) 777–806.
[17] M.L. Merroun, C. Hennig, A. Rossberg, G. Geipel, T. Reich, S. Selenska-Pobell, Molecular and atomic analysis of uranium complexes formed by three eco-types of *Acidithiobacillus ferrooxidans*, Biochem. Soc. Trans. 30 (2002) 669–672.
[18] Y. Suzuki, J.F. Banfield, Resistance to and accumulation of, uranium by bacteria from a uranium-contaminated site, Geomicrobiol. J. 21 (2004) 113–121.
[19] D.R. Lovley, E.J.P. Phillips, Y.A. Gorby, E.R. Landa, Microbial reduction of uranium, Nature 350 (1991) 413–416.
[20] D.R. Lovley, E.J. Phillips, Reduction of uranium by *Desulfovibrio desulfuricans*, Appl. Environ. Microbiol. 58 (1992) 850–856.
[21] L.E. Macaskie, K.M. Bonthrone, P. Yong, D.T. Goddard, Enzymically mediated bioprecipitation of uranium by a *Citrobacter* sp.: a concerted role for exocellular lipopolysaccharide and associated phosphatase in biomineral formation, Microbiology-UK 146 (2016) 1855–1867.
[22] M.L. Merroun, M. Nedelkova, J.J. Ojeda, T. Reitz, M.L. Fernandez, J.M. Arias, M. Romero-Gonzalez, S. Selenska-Pobell, Bio-precipitation of uranium by two bacterial isolates recovered from extreme environments as estimated by potentiometric titration, TEM and X–ray absorption spectroscopic analyses, J. Hazard. Mater. 197 (2011) 1–10.
[23] A. Schatz, C. Bovell, Growth and hydrogenase activity of a new bacterium, *Hydrogenomonas facilis*, J. Bacteriol. 63 (1952) 87–98.
[24] A. Willems, E. Falsen, B. Pot, E. Jantzen, B. Hoste, P. Vandamme, M. Gillis, K. Kersters, J. Deley, Acidovorax, a new genus for *Pseudomonas facilis, Pseudomonas delafieldii, e-falsen (ef) group 13, ef group 16, and several clinical isolates*, with the species *Acidovorax facilis comb-nov, Acidovorax delafieldii comb-nov, and Acidovorax temperans sp-nov*, Int. J. Syst. Bacteriol. 40 (1990) 384–398.
[25] M. Berney, F. Hammes, F. Bosshard, H.U. Weilenmann, T. Egli, Assessment and interpretation of bacterial viability by using the live/dead baclight kit in combination with flow cytometry, Appl. Environ. Microbiol. 73 (2007) 3283–3290.
[26] D.B. Johnson, J.H.M. Macvicar, S. Rolfe, A new solid medium for the isolation and enumeration of *Thiobacillus ferrooxidans* and acidophilic heterotrophic bacteria, J. Microbiol. Methods 7 (1987) 9–18.
[27] H. Lunsdorf, C. Strompl, A.M. Osborn, A. Bennasar, E.R. Moore, W.R. Abraham, K.N. Timmis, Approach to analyze interactions of microorganisms hydrophobic substrates, and soil colloids leading to formation of composite biofilms, and to study initial events in microbiogeological processes, Methods Enzymol. 336 (2001) 317–331.
[28] A.R. Spurr, A low-viscosity epoxy resin embedding medium for electron microscopy, J. Ultra Mol. Struct. R. 26 (1969) 31–43.
[29] B.P. Tracy, S.M. Gaida, E.T. Papoutsakis, Flow cytometry for bacteria: enabling metabolic engineering, synthetic biology and the elucidation of complex phenotypes, Curr. Opin. Biotech. 21 (2010) 85–99.
[30] T.H. Nielsen, O.R. Sjoholm, J. Sorensen, Multiple physiological states of a *Pseudomonas fluorescens dr54* biocontrol inoculant monitored by a new flow cytometry protocol, FEMS Microbiol. Ecol. 67 (2009) 479–490.
[31] I. Reimer, U. Zepke, J. Moesch, S. Schulze-Hillert, M. Ross-Messemer, W. Probst, Eelspectroscopy: A Reference Handbook of Standard Data for Identification and Interpretation of Electron Energy Loss Spectra and for Generation of Electron Spectroscopy Images, Institute of Physics, University of Münster, Germany, and Carl Zeiss, Electron Optics Division, Oberkochen, Germany, 1992.
[32] F. Hofer, P. Golob, New examples for near-edge fine-structures in electron-energy loss spectroscopy, Ultramicroscopy 21 (1987) 379–383.
[33] J.E. Gavagan, R. DiCosimo, A. Eisenberg, S.K. Fager, P.W. Folsom, E.C. Hann, K.J. Schneider, R.D. Fallon, A gram-negative bacterium producing a heat-stable nitrilase highly active on aliphatic dinitriles, Appl. Microbiol. Biotechnol. 52 (1999) 654–659.
[34] I. Zirnstein, Charakterisierung der dikrobiellen biozönose im sauren grubenwasser des ehemaligen uranbergwerks königstein, in: Dresden, Institut für Ressourcenökologie, TU, Dresden, 2015.
[35] T. Reitz, A. Rossberg, A. Barkleit, S. Selenska-Pobell, M.L. Merroun, Decrease of U(VI) immobilization capability of the facultative anaerobic strain *Paenibacillus* sp. JG-TB8 under anoxic conditions due to strongly reduced phosphatase activity, PLoS One 9 (8) (2014).
[36] L. Lütke, H. Moll, G. Bernhard, Insights into the uranium(VI) speciation with *Pseudomonas fluorescens* on a molecular level, Dalton Trans. 41 (2012) 13370–13378.
[37] J.R. Lloyd, L.E. Macaskie, Bioremediation of radionuclide-containing wastewaters, in: D.R. Loveley (Ed.), Environmental Microbe-Metal Interactions, ASM Press, Washington, D.C, 2000, pp. 277–327.
[38] F.G. Ferris, T.J. Beveridge, Site specificity of metallic ion binding in *Escherichia coli k-12* lipopolysaccharide, Can. J. Microbiol. 32 (1986) 52–55.
[39] B. Volesky, Z.R. Holan, Biosorption of heavy metals, Biotechnol. Progr. 11 (3) (1995) 235–250.
[40] S. Xie, J. Yang, C. Chen, X. Zhang, Q. Wang, C. Zhang, Study on biosorption kinetics and thermodynamics of uranium by *Citrobacter freudii*, J. Environ. Radioact. 99 (2008) 126–133.
[41] Y. Suzuki, J.F. Banfield, Geomicrobiology of uranium, Rev. Mineral. 38 (1999) 393–432.
[42] L.G. Leduc, G.D. Ferroni, J.T. Trevors, Resistance to heavy metals in different strains of *Thiobacillus ferrooxidans*, World J. Microbiol. Biotechnol. 13 (1997) 453–455.
[43] H.L. Ehrlich, Geomicrobiology, in: M. Dekker (Ed.), 3rd ed., New York, 1996, pp. 276–294.
[44] R. Kumar, M. Nongkhlaw, C. Acharya, S.R. Joshi, Uranium (U)-tolerant bacterial diversity from U ore deposit of domiasiat in north-east india and its prospective utilisation in bioremediation, Microbes Environ. 28 (2013) 33–41.
[45] M. Lopez-Fernandez, O. Fernandez-Sanfrancisco, A. Moreno-Garcia, I. Martin-Sanchez, I. Sanchez-Castro, M.L. Merroun, Microbial communities in bentonite formations and their interactions with uranium, Appl. Geochem. 49 (2014) 77–86.
[46] X.S. Huang, H. Wu, Biodegradation of styrene vapor in a biofilter, Appl. Mechanics Mater. I 275–277 (Pts 1–3) (2013) 2329–2332.
[47] A.J. Francis, J.B. Gillow, C.J. Dodge, R. Harris, T.J. Beveridge, H.W. Papenguth, Uranium association with halophilic and non-halophilic bacteria and archaea, Radiochim. Acta 92 (2004) 481–488.
[48] M.L. Merroun, C. Hennig, A. Rossberg, T. Reich, S. Selenska-Pobell, Characterization of U(VI) *Acidithiobacillus ferrooxidans* complexes using EXAFS transmission electron microscopy, and energy-dispersive X-ray analysis, Radiochim. Acta 91 (2003) 583–591.
[49] M.L. Merroun, S. Selenska-Pobell, Bacterial interactions with uranium: an environmental perspective, J. Contam. Hydrol. 102 (2008) 285–295.
[50] N. Renninger, R. Knopp, H. Nitsche, D.S. Clark, J.D. Keasling, Uranyl precipitation by *Pseudomonas aeruginosa* via controlled polyphosphate metabolism, Appl. Environ. Microbiol. 70 (2004) 7404–7412.
[51] G.M. Roomans, Localization of divalent-cations in phosphate-rich cytoplasmic granules in yeast, Physiol. Plantarum 48 (1980) 47–50.
[52] G.W. Strandberg, S.E. Shumate, J.R. Parrott, Microbial-cells as biosorbents for heavy-metals – accumulation of uranium by *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa*, Appl. Environ. Microbiol. 41 (1981) 237–245.
[53] M.L. Merroun, M. Nedelkova, A. Rossberg, C. Hennig, S. Selenska-Pobell, Interaction mechanisms of bacterial strains isolated from extreme habitats with uranium, Radiochim. Acta 94 (2006) 723–729.

Supplementary Information

Combined use of flow cytometry and microscopy to study the interactions between the gram-negative betaproteobacterium *Acidovorax facilis* and uranium(VI)

U. Gerber[1], I. Zirnstein[2], E. Krawczyk-Bärsch[1], H. Lünsdorf[3], T. Arnold[1], M. L. Merroun[4]

[1] Helmholtz-Zentrum Dresden - Rossendorf, Institute of Resource Ecology, Bautzner Landstraße 400, 01328 Dresden, Germany

[2] Research Institute of Leather and Plastic Sheeting (FILK) gGmbH, Meissner Ring 1-5, 09599 Freiberg, Germany

[3] Helmholtz Centre for Infection Research, Central Facility for Microscopy, Inhoffenstr. 7, D-38124 Braunschweig, Germany

[4] University of Granada, Department of Microbiology, Campus Fuentenueva, E-18071 Granada, Spain

PCR amplification of 16S rDNA gene fragments and sequencing

To perform colony PCR, colonies grown on agar plates were picked using a sterile pipette tip and were suspended in 100 µl of nuclease-free water followed by incubation at 95 °C for 5 min. An aliquot of this colony suspension was then added to each PCR reaction. The colony suspension (1-3 µl) was used in a 25 µl amplification assay with gene-specific primers. For the amplification of the 16S rDNA, the primer combination 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used. A Taq DNA Polymerase (5 U/µl, Promega, Mannheim, Germany) was used to catalyse the PCR using the following cycling conditions: initial denaturing at 94 °C for 12 min, followed by 30 cycles of denaturing at 94 °C for 1 min, annealing at 54 °C for 45 s, and extension at 72 °C for 90 s, finalized by a 20 min elongation at 72 °C. PCR products were purified with the innuPREP-PCR pure Kit (Analytik Jena, Jena, Germany), and eluted in double-distilled water. Purified PCR products were sequenced by GATC (GATC Biotech AG, Konstanz, Germany). The retrieved 16S rDNA sequences were compared with sequences available in the non-redundant nucleotide database of the National Center or Biotechnology database (http://www.ncbi.nlm.nih.gov) using BLASTN and the ribosomal database project (RDP, `http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp`).

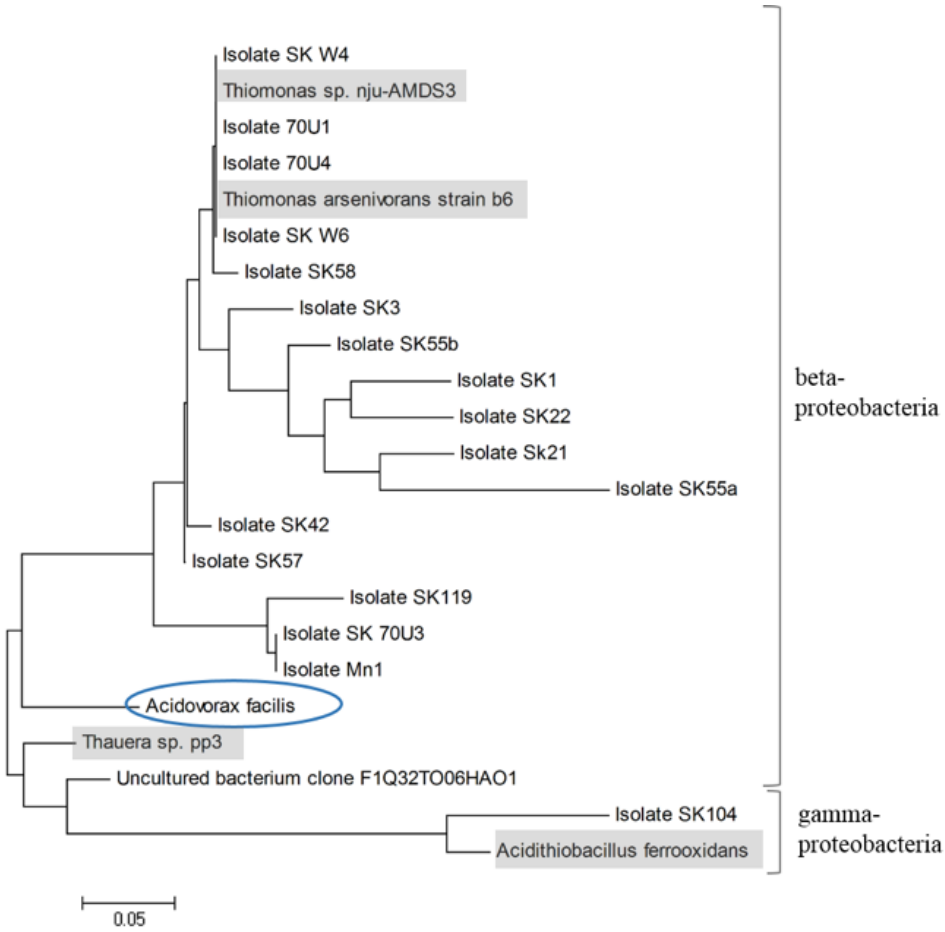


Figure 1: Phylogenetic tree based on minimum-evolution analysis of 16S ribosomal RNAs using neighbor-joining evolutionary distance model. Sequences retrieved from the acidic uranium contaminated mine water of the former uranium mine Königstein and were denoted as "Isolate". The reference organism *A. facilis* was added to compare the relationship to the isolates. The scale bar corresponds to a distance of 5 substitutions per 100 nucleotide positions.

The sequences were aligned to the closest phylogenetic relatives by using CLUSTALW version 1.7 [1]. Neighbour-joining trees were calculated by using MEGA 4 software [2].

Isolated bacteria from mining water

In this study, a high number of betaproteobacteria strains were isolated using FeTSB medium [3], a nutrient broth containing 0.25 g/L as organic carbon suitable for the cultivation and enrichment of heterotrophic and mixotrophic iron-oxidizing bacteria. The isolates (SK W4, 70U1, etc.) were phylogenetically affiliated to the genus Thiomonas (Fig. 1). FeTSB culture media with different potassium tetrathionate concentrations selected the growth of autotrophic iron-oxidizing bacterial strains related to Acidithiobacillus ferrooxidans. Based on dominance of isolated betaproteobacteria from earlier Königstein uranium mine water experiments, *A. facilis* was selected for further

investigations. Although *A. facilis* was not isolated from the flooding water, it has been well studied and is closely related to the betaproteobacteria that were identified by pyrosequencing, making it an ideal reference organism. Additionally, RNA studies with following pyrosequencing showed the dominance of metabolic active betaproteobacteria in the mine water [4].

References

[1] J. D. Thompson, D. G. Higgins and T. J. Gibson, *„CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice.“*, Nucleic Acids Res. **22**, 4673 (1994).

[2] K. Tamura, J. Dudley, M. Nei and S. Kumar, *„MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0“*, Mol. Biol. Evol. **24**, 1596 (2007).

[3] D. Johnson, J. H. Macvicar and S. Rolfe, *„A new solid medium for the isolation and enumeration of Thiobacillus ferrooxidans and acidophilic heterotrophic bacteria“*, J. Microbiol. Methods **7**, 9 (1987).

[4] I. Zirnstein, *„Charakterisierung der Mikroorganismen im sauren Grubenwasser des ehemaligen Uranbergwerks Königstein“*, PhD thesis, Technische Universität Dresden (2015).

3.2 Multidisciplinary characterization of U(VI) sequestration by *Acidovorax facilis* for bioremediation purposes

Krawczyk-Bärsch E, Gerber U, Müller K, Moll H, Rossberg A, Steudtner R, Merroun ML (2017)
J Hazard Mater 347: 233–241. https://doi.org/10.1016/j.jhazmat.2017.12.030

Journal of Hazardous Materials 347 (2018) 233–241

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Multidisciplinary characterization of U(VI) sequestration by *Acidovorax facilis* for bioremediation purposes

E. Krawczyk-Bärsch [a,*], U. Gerber [a], K. Müller [a], H. Moll [a], A. Rossberg [a], R. Steudtner [a], M.L. Merroun [b]

[a] Helmholtz-Zentrum Dresden-Rossendorf, Institute of Resource Ecology, Bautzner Landstraße 400, D-01328 Dresden, Germany
[b] University of Granada, Department of Microbiology, Campus Fuentenueva, E-18071 Granada, Spain

HIGHLIGHTS

- First kinetic studies on U removal by *A. facilis*.
- U is mainly bound to phosphate groups of LPS within the first hour.
- Subsequent U removal on carboxyl groups of PGN.
- *A. facilis* as suitable candidate for bioremediation purposes.

ARTICLE INFO

Article history:
Received 11 July 2017
Received in revised form 4 December 2017
Accepted 10 December 2017
Available online 28 December 2017

Keywords:
Uranium
Acidovorax facilis
Spectroscopy
Bioremediation

ABSTRACT

The contamination of the environment by U may affect plant life and consequently may have an impact on animal and human health. The present work describes U(VI) sequestration by *Acidovorax facilis* using a multidisciplinary approach combining wet chemistry, transmission electron microscopy, and spectroscopy methods (*e.g.* cryo-time resolved laser-induced fluorescence spectroscopy, extended X-ray absorption fine structure spectroscopy, and *in-situ* attenuated total reflection Fourier transform infrared spectroscopy). This bacterial strain is widely distributed in nature including U-contaminated sites. In kinetic batch experiments cells of *A. facilis* were contacted for 5 min to 48 h with 0.1 mM U(VI). The results show that the local coordination of U species associated with the cells depends upon time contact. U is bound mainly to phosphate groups of lipopolysaccharide (LPS) at the outer membrane within the first hour. And, that both, phosphoryl and carboxyl functionality groups of LPS and peptidoglycan of *A. facilis* cells may effectuate the removal of high U amounts from solution at 24–48 h of incubation. It is clearly demonstrated that *A. facilis* may play an important role in predicting the transport behaviour of U in the environment and that the results will contribute to the improvement of bioremediation methods of U-contaminated sites.

1. Introduction

The contamination of the environment by U-containing waste from mining and extraction as well as of reactor operation and fuel reprocessing is a world-wide problem. U is a naturally occurring radionuclide, which in enriched concentrations represents a major health hazard. It is well known that its transport behaviour strongly depends on its oxidation state. U typically occurs in nature in the oxidation state IV or VI. U(IV) is present in the precipitated form, *e.g.*, as the highly insoluble mineral uraninite under acid and anaerobic conditions [1]. In contrast, U(VI) is much more soluble and occurs predominantly as mobile aqueous species with carbonate, hydroxide or sulphate [2,3]. Arnold [4] reported that uranium speciation in acid mine drainage (AMD) flooding water of the former U mine Königstein (Saxony/Germany) is dominated by highly mobile, soluble and toxic UO_2SO_4 species. The conventional metal remediation of this underground U mine is realized by controlled flooding [5] and a following long-lasting water treatment. Hence, alternative treatments are needed, which may reduce the efforts. During the last decades, researchers are focusing their works on the application of bioremediation strategies to reduce the levels of inorganic contaminants, including U, in the environment. These strategies are based on the ability of microorganisms to interact with heavy metals through different mechanisms influencing

* Corresponding author.
E-mail address: E.Krawczyk-Baersch@hzdr.de (E. Krawczyk-Bärsch).

https://doi.org/10.1016/j.jhazmat.2017.12.030
0304-3894/

the transport and migration of radionuclides in the environment. They can be distinguished as biosorption, bioaccumulation, biotransformation, biomineralization, and redox reactions [6], with potential for the substantial retention of radionuclides. Biosorption can be summarized as the sorption and accumulation of trace elements to the surface of microbial cells [7]. Bioaccumulation is a process which requires energy. Microbes have evolved energy dependent uptake systems for physiologically important metals. Radionuclides can precipitate with microbial generated ligands, *e.g.* phosphate, sulphide or carbonate as biomineralization [7]. Under anaerobic conditions, many microorganisms may catalyze the microbial transformation [8,9] of U(VI) to sparingly soluble and immobile U(IV), including Fe(III)-reducing bacteria, such as *Shewanella* spp. and *Geobacter* spp. [10,11,12]; sulfate-reducing bacteria, such as *Desulfovibrio* spp. [13,14,15,16] and *Desulfosporosinus* spp. [17,18].

In the present work we focused on the gram-negative bacterium *Acidovorax facilis* [19] for interaction experiments with U(VI). Species of the genus *Acidovorax* were widely found in U-contaminated sites. For instance, *Acidovorax* spp. was identified in the sediments of a former U-mining district [20] and in U mine tailings [21]. Whereas *Acidovorax* spp. is often described as a part of a stable U(VI)-reducing microbial community in a complex anaerobic ecosystem of contaminated sediments [22,1,23], *Acidovorax facilis* is known as a facultative aerobic, chemoorganotrophic bacterium ubiquitously distributed in the nature including pristine soils [24] and U-contaminated sites [25]. Sequences closely related to the species *A. facilis* and *Dechloromonas agitata* were detected by the authors in 14% of the clone library of subsurface sediments contaminated with U(VI) and nitrate during biostimulation. A recent study [26] demonstrated that *A. facilis* is a suitable candidate for *in-situ* bioremediation of U- contaminated waste waters, *e.g.* AMD water. U(VI) removal and tolerance mechanisms of *A. facilis* were described by the authors by using a combination of flow cytometry and microscopy methods. The authors reported a high capacity of this bacterium to remove U from aqueous solutions up to 130 mg U/g dry mass by biosorption and bioaccumulation processes. However, no U solid state characterization studies were conducted on the association of U with this bacterial species.

Based on the results obtained by [26], the present study focuses on the molecular scale structural characterization of the uranyl species associated with the cell biomass of *A. facilis*. The results of microscopic and spectroscopic methods, *i.e.* high-angle annular dark field scanning transmission electron microscope (HAADF-STEM), cryo-time resolved laser-induced fluorescence spectroscopy (cryo-TRLFS), extended X-ray absorption fine structure (EXAFS) spectroscopy and *in-situ* attenuated total reflection Fourier transform infrared (ATR FT-IR) spectroscopy will improve our understanding of the mechanisms of microbial response to radionuclides on a molecular level.

2. Material and methods

2.1. Bacterial growth and cultivation conditions

For our studies the strain of *A. facilis* was kindly provided by the Centre for Environmental Research (UFZ in Leipzig, Germany). The cells were grown in nutrient broth medium (NB) (Peptone 5.0 g/L and Beef Extract 3.0 g/L, pH 7.0 ± 0.2, Sifin, Berlin, Germany) at 30 °C until reaching the stationary phase and 120 rpm on a rotary shaker overnight. Subsequently, the cells were washed and diluted to an OD_{600nm} of 1.0 (12.0 ± 1.0 mg dry weight/mL) with sterilized tap water at pH 5.0 and used for each experimental method.

2.2. Sample preparation for STEM/HAADF/EDX analysis

Cells of *A. facilis*, previously contacted for 20 min with $UO_2(NO_3)_2$ solution (0.1 mM, pH 5) under sterile conditions, were recovered by centrifugation (6000 rpm, 5 min). The supernatant was removed and the cell pellet was washed twice with 20 mM Hepes at pH 7.2. Subsequently, the pellet was fixed at 1% (vol/vol) Glutardialdehyde from 50% (vol/vol) stock solution. After immobilization in 1% (w/v) aqueous low melting agarose, cells were dehydrated by an ethanol series (10, 30, 50, 70, 90, 100% for 2 min on ice; 100% for 15 min at room temperature) followed by ERL-resin impregnation and polymerization [27]. Ultrathin sections of 35–40 nm were examined under a HAADF-STEM (FEI TITAN G2 80-300) for high resolution studies. TEM specimen holders were cleaned by plasma prior to STEM analysis to minimize contamination. The STEM is equipped with HAADF detector and EDAX energy dispersive X-ray (EDX).

2.3. cryo-TRLFS measurements

In preparation for the measurements the *A. facilis* cells were washed two times and then re-suspended in sterilized tap water with pH 5.0. A stock solution of $UO_2(NO_3)_2$ was added to adjust to a U(VI) concentration of 0.1 mM in the cell suspension. The experiments were performed at pH 5, under aerobic conditions and at 30 °C on a rotary shaker at 130 rpm. After 5 min, 1 h, 16 h and 24 h the samples were taken, centrifuged for 5 min at 13,200 rpm. The supernatants were used for determination of U concentration by means of inductively coupled plasma spectrometry (ICP-MS). The pellets were re-suspended in sterilized tap water (pH 5). Each sample was shock frozen in a plastic cuvette by N_2 and stored at −80 °C. In addition to all measurements reference blank samples of sterilized tap water (pH 5) with 0.1 mM U(VI) and the cell suspension without U(VI) was investigated. The U(VI) luminescence at 153 K was measured after excitation with laser pulses at 266 nm (Minilite high-energy solid-state laser; Continuum) and average pulse energy of 300 μJ. The emission of the samples was recorded using an iHR550 spectrograph (HORIBA Jobin Yvon) and an ICCD camera (HORIBA Jobin Yvon) in the 370.0–670.0 nm wavelength ranges by averaging 100 laser pulses and using a gate time of 2000 μs. Data were analyzed using Origin software, version 9.0 (OriginLab Corporation). For further details on the experimental cryo-TRLFS setup, see [28].

2.4. EXAFS analysis

The bacterial sample was taken after the U interaction experiments, where *A. facilis* were exposed to 0.1 mM U for 48 h (30 °C, pH 5) and ultra-centrifuged for 1 h at 187.000 × *g*. The pellet was filled into a polyethylene sample holder. The holder was heat sealed at 300 °C by using a soldering iron and immediately frozen in liquid nitrogen. EXAFS measurements were carried out on the Rossendorf Beamline BM20 at the European Synchrotron Radiation Facility (ESRF) [29]. The bacterial sample was measured at 15 K in a closed-cycle He cryostat in fluorescence mode using a 13-element Ge solid-state detector, while the reference samples meta-autunite, $Ca(UO_2)_2(PO_4)_2{\cdot}6\,H_2O$ [30], and the aqueous U(VI)-hydrate and U(VI)-tricarboxylate complex [31], where measured at room temperature in transmission mode using ionization chambers. The incident photon energy was tuned with a water-cooled Si(111) double-crystal monochromator in channel cut mode (5–35 keV). For energy calibration of the sample spectra, the K-edge spectrum of a Y metal foil (first inflection point at 17,038 eV) was recorded simultaneously. The ionization energy, E_0, of the uranium L_{III}-edge was defined as E_0 = 17,185 eV. The software package EXAFSPACK [32] was used for dead-time correction of the 13

fluorescence channels, energy calibration, averaging of the multiple sample scans, extraction of the EXAFS signal, and for the shell fitting. For the shell fit of meta-autunite theoretical backscattering phase and amplitude functions were calculated with the FEFF8 code [33] using a 128 atom cluster having the atomic positions from $Ca(UO_2)_2(PO_4)_2{\cdot}6\,H_2O$ [30]. For the shell fit of the other samples a synthetic structural model described in [34] was used. For all shell fits the 4-legged multiple scattering (MS) path U-O_{ax1}-U-O_{ax2} was included in the model calculations, while the coordination number (N) was kept constant at two and the radial distance (R) and the Debye-Waller factor (σ^2) were linked to twice the R and σ^2 of the single-scattering (SS) path U-O_{ax}, respectively [35]. The phosphate interaction was modelled with a U-P SS path, the 3-legged and the 4-legged MS path U-O_{eq}-P and U-O_{eq}-P-O_{eq}, respectively. For the 3-legged and the 4-legged MS path N was linked to twice and once the N of the U-P SS path, respectively, while the effective path length of the MS paths where linked to R of the U-P SS path according the structural model. The σ^2 of the MS paths were linked to once the σ^2 U-P SS path. In the case of the bacterial sample for all paths which includes the phosphate interaction σ^2 was kept constant at $\sigma^2 = 0.0072$ Å^2 as found for m-autunite. The amplitude reduction factor, $S_0{}^2$, was held constant at 0.9 for all fits.

We used linear-combination (LC) fit to estimate the relative fractions of interacting ligands, like water molecules, phosphate and carboxylic groups.

2.5. In-situ *ATR FT-IR spectroscopy*

For monitoring and complementary molecular identification of the U(VI) sorption process on *A. facilis* cells and distinct cell components, *in-situ* ATR FT-IR spectroscopy with a sub-minute time resolution was performed. *A. facilis* cells were treated to obtain cell components, *i.e.* the outer membrane containing lipopolysaccharide (LPS), proteins and phospholipids. For this purpose, the *A. facilis* cell suspension was sonicated for 5 min by room temperature. The pellet was washed three times with autoclaved H_2O and harvested by centrifugation (10.000 rpm, 10 min), respectively. For comparison, a sample with living *A. facilis* cells was used. Polyphosphate granules (PPGs), which were separated from *A. facilis* cells using the description for isolation of PPGs after [36], were incubated in tap water at pH 5. PPGs are often found within the cytoplasm of bacteria cells as a storage compartment for phosphorus compounds. They are characterized by their high content of P [39]. Infrared spectra were measured from 1800 to 800 cm^{-1} on a Bruker Vertex 80/v vacuum spectrometer equipped with a Mercury Cadmium Telluride detector. Spectral resolution was 4 cm^{-1}, and spectra were averaged over 256 scans. A horizontal diamond crystal with nine internal reflections (DURA SamplIR II, Smiths Inc.) was used. Further details on the experimental ATR FT-IR spectroscopy setup are compiled in [37]. The performance of *in-situ* sorption experiments requires a thin microbial cell film prepared directly on the surface of the ATR diamond crystal as stationary phase. This was accomplished by pipetting 5 μL of respective suspended *A. facilis* samples of living cells, outer membrane components or PPGs on the ATR crystal and subsequent drying with a gentle stream of N_2. Flow-through measurements at a rate of 100 μL min^{-1} were performed using a flow cell (V = 200 μL). First, the film was flushed with a blank solution (sterilized tap water, pH 5) for 60 min conditioning. In a second step, a 10 μM U(VI) solution (sterilized tap water, pH 5) was rinsed for sorption during the next 120 min. Finally, the U loaded cell film was flushed again with the blank solution (120 min) in order to gain more information on the reversibility of the sorbed species.

The applied principle of reaction-induced difference spectroscopy allows the detection of very small absorption changes provoked by the sorption process in comparison to the very strong absorbing background, *i.e.*, water, *A. facilis* cell film. Further details on the calculation of difference spectra are given in [37,38].

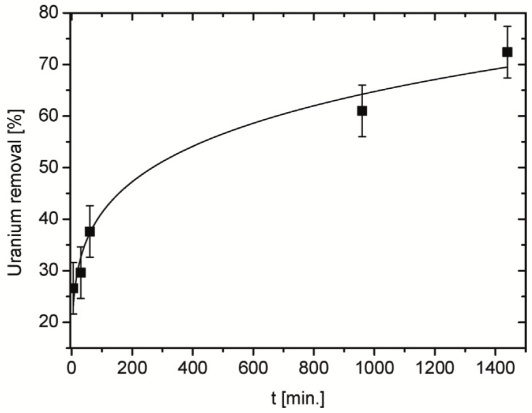


Fig. 1. U removal by living *A. facilis* cells exposed to 0.1 mM U(VI).

3. Results and discussion

3.1. *U(VI) sequestration by* A. facilis

For cryo-TRLFS measurements the samples were centrifuged and the supernatant were analysed by ICP-MS. As shown in Fig. 1a removal of U by *A. facilis* cells was already detectable during the first minutes. After 5 min already 26.60% of the initial U is removed from the solution. After 1 h an uptake of 37.60% was determined. The uranium removal capacity of *A. facilis* cells at 0.1 mM U was determined in mg U per g dry biomass (DBM) and showed, that 21 mg U was removed during the first 5 min of the experiments. After incubation time of 1 h the cells removed about 30 mg U/g DBM. After 24 h the equilibrium was reached and the bacterial cells displayed a total U removal capacity of 58 mg U/g DBM. In [26] the fast sequestration of U(VI) by *A. facilis* within the first 8 h was interpreted as a passive process. U was passively bound to the outer membrane of the bacterium, most likely associated to a metabolically independent biosorption process. In a later stage, after 16 h and 24 h, an uptake of 61.00% and 72.40%, respectively was determined as a result of an active process (bioaccumulation) as described in [26], where the metal is actively taken up into the cell. Analysis of the samples used for EXAFS were taken after 48 h showing a removal of U of 95.10%. Live-dead staining studies of *A. facilis* cells showed a majority of living biomass after 48 h incubation with 0.1 mM U [26]. It was shown, that living cells are responsible for the sequestration of U by passive biosorption during the first hours of incubation. Living cells are also responsible for an active bioaccumulation during a later stage. In contrast, the U sequestration by dead biomass is fast. The authors showed that the maximum of U removal was already reached after 5 min incubation by passive biosorption.

3.2. *STEM/HAADF analysis*

For STEM/HAAD/EDX studies several ultrathin sections of the *A. facilis* samples loaded with 0.1 mM uranium for 20 min were prepared. In Fig. 2a some *A. facilis* cells are shown with spherical inclusion bodies, which are characterized by an increased electron density. These are PPGs, which are characterized by their high content of P [39]. Elemental distribution analysis of U (Fig. 2b) and P (Fig. 2c) were performed over *A. facilis* cells and clearly indicates that uranium is entirely present in the cell membrane and only with minor amounts in the PPGs. EDX analysis clearly indicate, that, com-

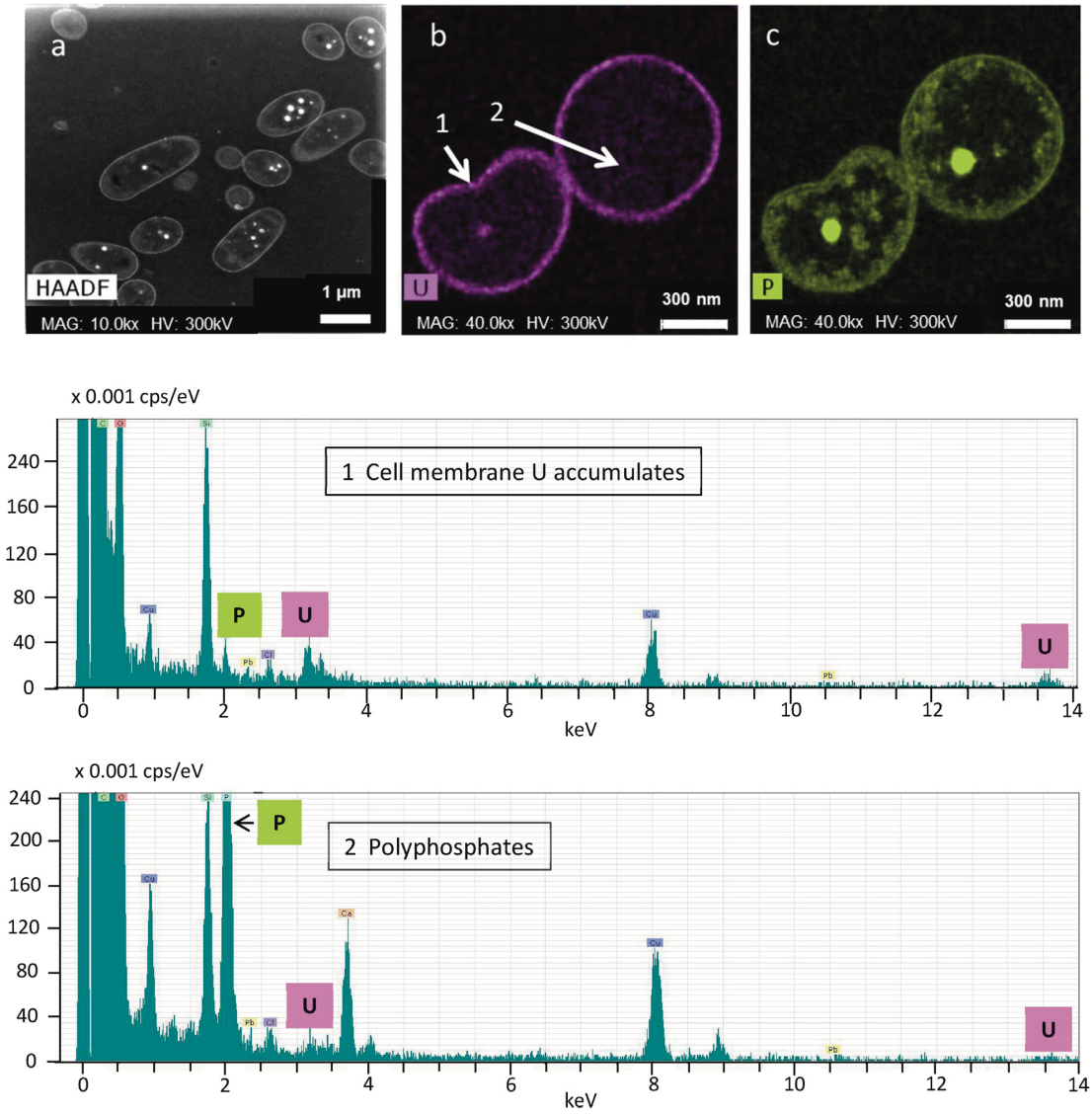


Fig. 2. STEM/HAADF images of *A. facilis* cells and PPGs with high electron density (a). EDX analysis indicate high intensity peaks of U as cell membrane U accumulates (1) compared to low peaks in PPGs (2), confirmed by distribution analysis of U (b) and P (c) in *A. facilis* cells incubated for 20 min.

pared to the cell membrane, the concentration of U is much lower or not detectable in the cytoplasmic PPGs. Consequently, the removal of U by *A. facilis* during the first 20 min of incubation is limited to the outer membrane of the cells, whereas an uptake of U into the cytoplasm and in PPGs, respectively results from a subsequent active process as described in [26].

3.3. cryo-TRLFS

The measured emission spectra of the U complexes formed by the cells of *A. facilis* at 0.1 mM U concentration and incubation time of 5 min, 1 h, 16 h and 24 h are characterized by five emission bands. As shown in the luminescence spectrum (Fig. 3A) the luminescence intensities increase with incubation time, which is correlated with the time-dependent removal of U(VI) by *A. facilis* cells as described above (3.1). In addition, a shift of the uranyl(VI) band positions to lower wavelengths ($\Delta = -2.8$ nm) is clearly indicated. This shift can be interpreted as a change of the uranyl-speciation in *A. facilis* cells over the incubation time. In detail, the spectra of the samples which were incubated with U for 5 min and 1 h are characterized by similar peak maxima at 480.7, 499.7, 520.1 and 545.1 nm ± 0.5 nm. Compared to the band positions of the uranyl-lipopolysaccharide-complex (R-O-PO_3-UO_2) luminescence spectrum at pH 4 [40], there is a good agreement with only small deviations (Table 1). It seems that UO_2^{2+} is bound on LPS at the beginning of the incubation. LPS is known as a major component of the outer membrane of gram-negative bacteria. The main binding sites for metal ions are carboxyl, phosphoryl, hydroxyl and amino groups, located in the LPS [41]. However, studies have shown that the LPS uranyl phosphoryl coordination is dominating with a very strong phosphoryl binding of uranyl to LPS [40]. Concerning our studies we consequently suggest a formation of uranyl-phosphoryl species on *A. facilis* cells during the first hour of incubation with U.

Table 1
Luminescence band positions of *A. facilis* cells exposed to 0.1 mM U compared to Band positions of reference spectra of Uranyl-lipopolysaccharide-complex [40] and Uranyl-peptidoglycan-complex [42].

Samples	Incubation time/h	Luminescence emission bands/nm				Ref.
A. facilis pH 5.0	0.01	480.7	499.7	520.1	545.1	This work
A. facilis pH 5.0	24	479.3	495.5	517.3	541.4	This work
LPS – R-O-PO_3-UO_2 pH 4.0	24	481.5	498.1	519.6	542.9	[40]
PGN – $(R\text{-}COO)_2$-UO_2 pH 5.6	24	481.6	498.1	518.0	539.0	[42]

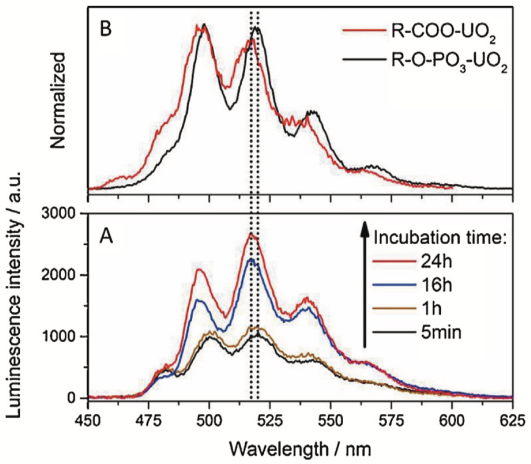


Fig. 3. Luminescence spectra of *A. facilis* cells exposed to 0.1 mM U after different incubation times (Fig. 3A) at 30 °C. The spectra are compared to reference spectra of uranyl-LPS-complex [40] and uranyl-PGN-complex [42] (Fig. 3B).

The spectra of the samples, which were measured after 16 h and 24 h of incubation time with U, are characterized by uranyl(VI) band positions, which are shifted to lower wavelengths (Fig. 3A). The peak maxima are determined at 479.3, 495.5, 517.3 and 541.4 nm ± 0.5 nm and show similarities to reference spectra of the 1:2 uranyl carboxyl complex ($[R\text{-}COO]_2$-UO_2) of uranyl-peptidoglycan at pH 5.6 [42] as shown in Table 1 and in Fig. 3B. The relatively thick (20–80 nm) peptidoglycan (PGN) layer is the main component of the cell wall of gram-negative bacteria [43]. Basically the PGN can consist of two sugar derivatives, *N*-acetyglucos-amine and *N*-actylmuramic acid, which are bonded together with a short stem of four amino acids [44]. As shown in [45], the carboxylate groups of the PGN have been implicated as main sites of metal binding including U [46]. The luminescence spectra obtained by TRLFS in our studies after 16 h and 24 h of incubation with U are indicating that UO_2^{2+} was bound on carboxyl functionality groups of the PGN. However, the determined spectra and luminescence characteristics definitely refer to the formation of more than one uranyl-species. It has to be taken into account that a uranyl phosphoryl coordination is additionally formed which could be consisted of a uranyl-LPS-complex.

3.4. EXAFS analysis

Fig. 4 shows the shell fit of the raw k^3-weighted EXAFS spectra of the bacterial sample incubated with U for 48 h and the selected reference samples, while the corresponding structural parameters are listed in Table 2. For U(VI)-hydrate and meta-autunite the determined structural parameter match well the data supplied by the literature (Table 2), hence showing that the used shell fit model is appropriate. In the case of the bacterial sample a common U-O_{ax} distance of 1.76 Å and a U-O_{eq} distance of 2.35 Å is measured which is situated between the distances of 2.28 Å and 2.40 Å as observed for meta-autunite and U(VI)-hydrate, respectively. The coordination number of O_{eq} 3.8 which could point out that U(VI) is 4-fold coordinated. However, in consideration of the error in the determination of N (20%) in the case of U(VI) interactions with structurally complicated biological systems, coexisting structurally different U(VI) complexes might be expected which could have 4-fold, 5-fold and 6-fold coordination. The shell fit of such spectral mixtures will result in EXAFS structural parameter which reflects the average of all contributing U(VI) complexes. At 3.58 Å a U-P interaction is observed with a coordination number of N_P = 1.4 (Table 2). The U-P distance agrees within the common error in determination of interatomic distances probed by EXAFS (0.01–0.02 Å; [47]). In comparison with the U-P distance determined for meta-autunite ($R_{U\text{-}P}$ = 3.60 Å, Table 2), it indicates that the bacterial phosphate groups are monodentately coordinated. If in average a 4-fold up to a 5-fold coordination is present, and with N_P = 1.4, at least 2–3 O_{eq} atoms must be supplied from other ligands than phosphate groups. Moreover, the U-O_{eq} distance of the additionally coordinated ligands must be much longer than the U-O_{eq} distance of 2.28 Å, as observed for coordinated phosphate groups, to reach the average U-O_{eq} distance of 2.35 Å. The presence of O_{eq} in at least two different U-O_{eq} distances is further evidenced by the high σ^2 of ~0.01 $Å^2$ (Table 2). Beside the phosphate groups the most probable candidates of ligands who supply U-O_{eq} distances longer than 2.28 Å are coordinated water molecules, with $R_{U\text{-}Oeq}$ = 2.40 Å, and bidentately coordinated carboxylic groups with $R_{U\text{-}Oeq}$ = 2.47 Å (U(VI)-hydrate and U(VI)-triscarboxylate), respectively (Table 2). Two LC fits of the EXAFS spectrum of the bacterial sample are shown in Fig. 4 by introducing all three references ((1) in Fig. 4) and by using only U(VI)-hydrate and meta-autunite ((2) in Fig. 4) as references. Obviously all spectral features as marked in the Fourier-transform (FT) in Fig. 4 (features (1)–(4)) are well reproduced by the spectral contributions from 43% U(VI)-hydrate, 37% meta-autunite and 12% of U(VI)-triscarboxylate with a standard deviation (SD) between the experimental spectrum and the LC fit of SD = 0.24. Note that the spectral feature at 2.4 Å in the FT (feature (3), Fig. 4), which is visible in all FT's of the measured EXAFS spectra (Fig. 4) is also reproduced. The origin of this feature could be explained by carbon atoms in a U-C distance of 2.9 Å stemming from bidentately coordinated carboxylic groups. In the case of the bacterial sample the shell fit results in 1.9 C at 2.90 Å. The nature of this small FT peak was extensively discussed in [48] and it was observed that a shell fit overestimates the number of C atoms by ~1 atom, hence pointing out that the feature is an artefact. Consequently, and by introducing only 55% U(VI)-hydrate and 36% meta-autunite (Fig. 4) as references the LC fit leads to the same good description (SD = 0.26) of all spectral features as observed by introducing all three references (SD = 0.24). In accordance with the shell fit of U(VI)-hydrate and meta-autunite an overestimation of 1.3 C–1.4 C atoms can be assumed, so that for the bacterial sample the expected number of carbon atoms would be N_C < 0.5, hence likely undetectable with a shell fit. Note that the LC fit is not influenced by the error introduced by assumptions, which are made for the calculation of the scattering functions by FEFF and for the shell fit. However, as a conservative estimate of the error in determination of the fractions by the LC fit the deviation of the total sum of the fractions from 100% can be taken. For both LC fits

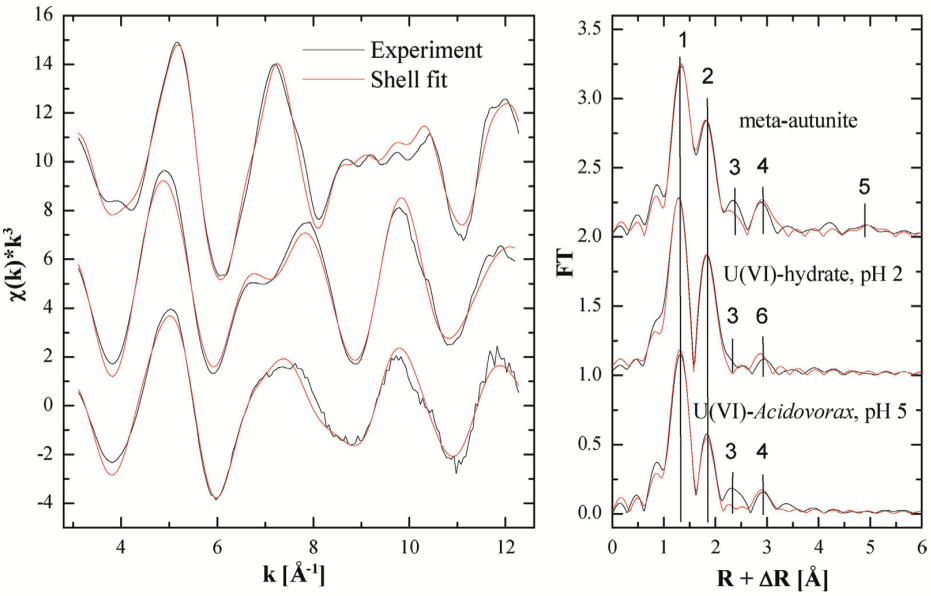


Fig. 4. U L_{III}-edge k^3-weighted EXAFS spectra (left) and the corresponding Fourier transforms (right) of U(VI)-*A. facilis* and two reference uranyl compounds. Experimental data (black) with shell fit (red). Spectral features 1–6 correspond to: (1) – O_{ax}, (2) – O_{eq}, (3) – artefact, (4) – SS U-P, MS U-O_{ax1}-U-O_{ax2}, MS U-O_{eq}-P-O_{eq}, MS U-O_{eq}-P, (5) – S U-U, (6) - MS U-O_{ax1}-U-O_{ax2}. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 2
Summary of selected structural parameter based on shell fit analysis of the EXAFS data.

Sample	Shell	N[a]	R (Å)[b]	σ^2 (Å^2)[c]	ΔE_0 (eV)[d]
U(VI)-Acidovorax	U=O	2[f]	1.76	0.0016	2.9
pH 5.0	U-O_{eq}	3.8	2.35	0.0105	
[U(VI)]0 = 0.1 mM	U-P	1.4	3.58	0.0072[f]	
	U-O_{eq}-P	/2.8	/3.67	0.0072[f]	
	U-O_{eq}-P-O_{eq}	/1.4	/3.77	0.0072[f]	
U(VI)-hydrate	U=O	2[f]	1.76 (1.76)[g]	0.0016 (0.0013)[g]	2.8
pH 2.0	U-O_{eq}	5[f] (5.0)[g]	2.40 (2.40)[g]	0.0070 (0.0066)[g]	
[U(VI)] = 50 mM					
meta-autunite	U=O	2[f]	1.78 (1.78)[e]	0.0024	1.3
	U-O_{eq}	4[f]	2.28 (2.32)[e]	0.0036	
	U-P	4[f]	3.60 (3.59)[e]	0.0072	
	U-O_{eq}-P	/8	/3.70	0.0072	
	U-O_{eq}-P-O_{eq}	/4	/3.79	0.0072	
	U-U	/4	5.24 (5.23)[e]	0.0114	
U(VI)-triscarboxylate [31]	U=O	2[f]	1.78	0.0014	4.9
	U-O_{eq}	5.8	2.47	0.0071	
	U-C	3.3	2.88	0.0042[f]	
	U-C_{dis}	/3.3	4.36	0.00645[f]	
U(VI)-fructose-6-phosphate [49]	U=O	2[f]	1.77	0.0014	1.0
	U-$O_{eq\,1}$	5.2	2.32	0.0120	
	U-$O_{eq\,2}$	1.6	2.88	0.004	
U(VI)-lipopolysaccharide [50]	U=O	2[f]	1.79	0.0034	1.8
	U-$O_{eq\,1}$	2.7	2.33	0.0043	
U:P = 5 : 3, pH = 3.9	U-$O_{eq\,2}$	0.9	2.52	0.0043	

/parameter linked proportional to the parameter in the row above.
C_{dis} distal carbon atom of bidentately coordinated carboxylic group.
[a] Errors in coordination numbers (N) are ±25%.
[b] Errors in radial distance (R) are ±0.02 Å.
[c] Debye-Waller factor (σ^2).
[d] Shift in threshold energy (ΔE_0).
[e] Bond distances based on the XRD analysis from meta-Autunite published by [30].
[f] Fixed parameter.
[g] Bond distances and Debye-Waller factors published by [57].

the sum of the fractions is only 90% which lead to an estimated error of 10% so that the number bidentately coordinated carboxylic groups in the bacterial sample could be given as 0.4 ± 0.3. In consequence of the relative high error, we conclude that no or only a small fractional amount of U(VI) is bound to carboxylic groups. The number of coordinated water molecules (N_{Owater}) determined by the LC fit with three and two references is N_{Owater} = 2.7 ± 0.5 and N_{Owater} = 2.2 ± 0.5, respectively, whereas in average 1.5 ± 0.4 monodentately coordinated phosphate groups are obtained for both LC fits which is in line with the shell fitted number of phosphate

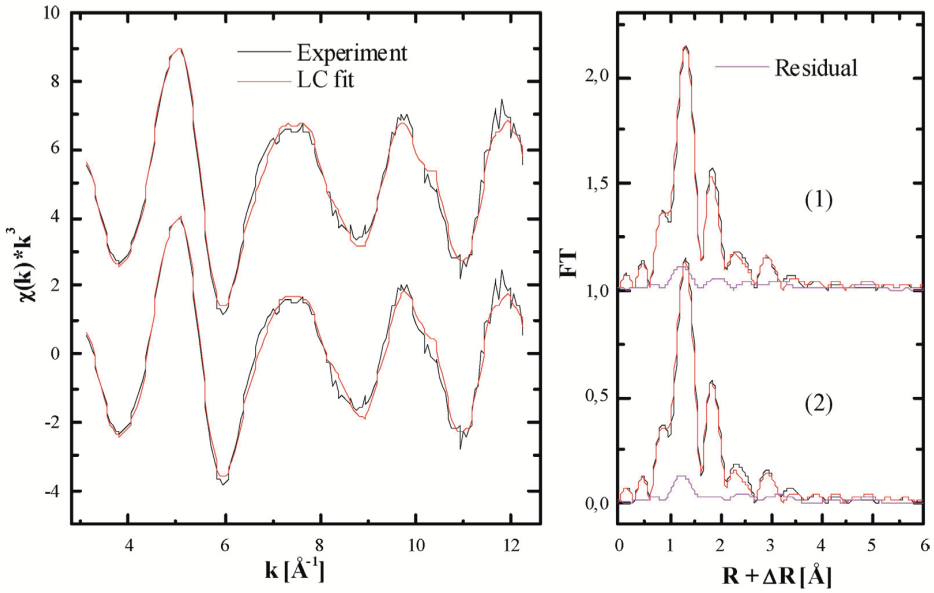


Fig. 5. U L_{III}-edge k^3-weighted EXAFS spectra (left) and corresponding Fourier transforms (right) of U(VI)-*A. facilis*. LC fits (red) of experimental data (black) by using two different models, including reference spectra: (1) U(VI)-hydrate, meta-autunite, aqueous U(VI)-triscarboxylate complex, (2) U(VI)-hydrate and meta-autunite. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

groups of $N_P = 1.4$ (Table 2). The total coordination number of O_{eq} is $N_{Oeq} = 4.2 \pm 0.4$ and $N_{Oeq} = 4.4 \pm 0.4$ for the LC fit with two and three references, respectively.

Similar spectral features and a relative short average U-O_{eq} bond length of 2.35 Å where observed for the U(VI) interaction with sugar phosphates, like fructose-6-phosphate, and for the U(VI) interaction with lipopolysaccharide (LPS) (U:P = 5:3 at pH 3.9) where a binding of the U(VI) *via* organic phosphate groups in a monodentate fashion was proposed [49,50] (Table 2). Moreover, the measured structural parameter of the bacterial sample (Table 2) are in close agreement with those observed in the U(VI)-*C. algae* system [34] for which also a monodentate U-P interaction was proposed.

Meta-autunite could be the result of a biomineralization process triggered by *A. facilis* cells. As described in the literature [51,52,53] microbial cells are able to induce biomineralization processes in the presence of U(VI) by secreting phosphate. By comparing the measured EXAFS oscillation of the bacterial sample with those of meta-autunite we can exclude this hypothesis (Fig. 5). Moreover, the FT shows no U-U interaction at 4.9 Å (feature (5), Fig. 5) which would be an indication for the formation of meta-autunite due to a biomineralization processes. Hence, a biosorption process of U(VI) onto functional groups of the cell envelope might dominate.

3.5. In-situ *ATR FT-IR spectroscopy*

For complementary molecular and kinetic information with a sub-minute time resolution the U(VI) sorption process on *A. facilis* cells, cell membrane and PPGs were investigated and selected spectra are given in Fig. 6. Two spectra are calculated for the conditioning step (Fig. 6A, red traces). Small positive bands during 30 min of conditioning likely demonstrate the adaption of surface functionalities to certain solution conditions, such as pH 5 during the equilibration of the system. The absence of any bands in the spectrum obtained at the end of prolonged conditioning reflects the stability of *A. facilis* cell film on the ATR crystal which is indispensable for the detection of sorbed species during the following sorption process. Same stabilities were found for films prepared of cell membrane and PPGs.

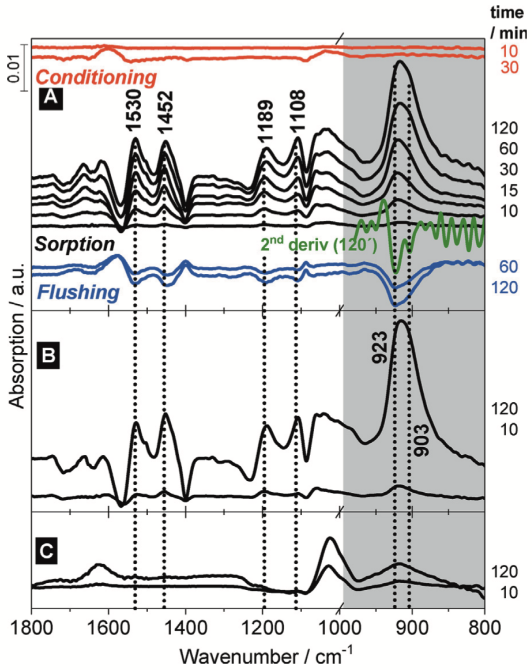


Fig. 6. *In-situ* ATR FT-IR difference spectra of U(VI) sorption on *A. facilis* cells and cell components. (A) Cells: stable cell film on the ATR crystal (red). Cells: recorded at different times after induction of U(VI) (black). Cells: show reversibility (blue); green trace: exact band position values, the second-derivative; (B) cell membrane; (C) PPG; in (B) and (C) only sorption spectra obtained after 10 and 120 min after induction of U(VI) are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

The ATR FT-IR difference spectra calculated between the conditioning and at several time steps after induction of U(VI) sorption show well resolved absorption bands from 1550 to 1400 cm^{-1} and from 1200 to 1100 cm^{-1} as well as below 950 cm^{-1} with increas-

Table 3
Tentative assignment of bands observed in difference spectra of in-situ ATR FT-IR sorption experiments.

Peak position (cm^{-1})	Tentative assignment to vibrational mode [54,55,50,56,58]
1530	ν_{as} (COO^-)
1452	ν_s (COO^-)
1189	ν_{as} (P-O)
1108	ν_{as} (P-O)
1022 (broad)	ν_{as} (P-O) inorganic phosphate in PPG
923	ν_3 (UO_2) coordinated to carboxyl groups
918 (broad)	ν_3 (UO_2) coordinated to inorganic phosphate in PPG
903	ν_3 (UO_2) coordinated to phosphoryl groups

ing intensities upon time reflecting U(VI) accumulation on the cell surface (Fig. 6A, black traces). The band at ~914 cm^{-1} originates from the asymmetric stretching mode of the uranyl moiety, $\nu_3(UO_2)$. A second-derivation analysis revealed two local peak maxima at 923 and 903 cm^{-1}, suggesting the presence of two uranyl species (green: 120 min sorption spectrum). From a previous IR spectroscopic study, similar frequencies of $\nu_3(UO_2)$ mode were observed and were assigned to a coordination of uranyl to carboxylate and phosphoryl groups, respectively. This is in perfect agreement with the spectral changes in the frequency range of carboxylate (1530–1430 cm^{-1}) and phosphoryl (1200–1110 cm^{-1}) groups (Table 3) [54,56,50]. A closer look to the $\nu_3(UO_2)$ band exhibits slight shifting within the time-resolved data, suggesting that the ratio between carboxylate and phosphoryl bound U(VI) may change upon accumulation on the cell. The experiment was repeated using only the separated *A. facilis* cell membrane as stationary film on the ATR crystal. The spectra and derived information are equal to the experiment with *A. facilis* cells (Fig. 6B), in according to STEM/HAADF results showing that the majority of U(VI) is bound to functionalities at the cell membrane (Fig. 2). Subsequent to sorption, the U loaded cell film was flushed again with blank solution. The difference spectra obtained at 10 and 120 min (Fig. 6A, blue traces) show negative bands at equal frequencies to those observed during the sorption stage but much reduced in intensity. In addition, the band of $\nu_3(UO_2)$ is little distorted. The spectra reflect a reversibility of the U(VI) association whereby only a low amount of U(VI) was released within 120 min, possibly to a higher extent by carboxylate functionalities. Indicating a strong interaction, most of the bound uranium presumably remained on the cells. The sorption spectra obtained from the PPG *in-situ* experiment are given in Fig. 6C, showing two broad bands at 1025 and 915 cm^1 very similar to spectral data obtained from inorganic uranium-phosphate complexation in solution and as precipitate and as sorption complex on mineral interfaces [57]. To a certain proportion these features may also contribute to the sorption spectra of the *A. facilis* cell and cell membrane, explaining the broad undefined band at ~1025 cm^1.

In summary, the *in-situ* ATR FT-IR spectroscopic measurements clearly show the formation of two dominating species involved in the U(VI) association reactions on *A. facilis* cells: U(VI) bound to carboxylate groups and phosphoryl groups of the outer membrane identified by spectral fingerprint of modes related to U(VI) and the functionalities. The *in-situ* ATR FT-IR studies on isolated PPGs showed bands similar to spectral data obtained from inorganic uranium-phosphate complexation, which could not be determined in ATR FT-IR spectra of living *A. facilis* cells due to the detection limit of the method. This indicates that PPGs may play a subordinated role during the early stage of U incubation by *A. facilis* cells. Even in STEM/HAAD/EDX studies it was shown that, compared to the cell membrane, the concentration of U is much lower or not detectable in the cytoplasmic PPGs.

4. Conclusions

This work characterizes the U(VI) sequestration by *A. facilis* using a multidisciplinary approach combining spectroscopic and microscopic techniques. The local coordination of U associated with the cells of *A. facilis* depends upon time incubation. U biosorption by outer membrane LPS containing phosphoryl residues was observed within the first hours of contact between the cells and U. By increasing the incubation time up to 24 h the implication of carboxyl groups within the cell wall PGN was proved by TRLFS and EXAFS analysis in addition to phosphoryl groups. In addition to these functional groups located at the cell surfaces, U is coordinated also, but with low degree, to phosphoryl groups of the intracellular PPG as was indicated by STEM analysis. This study showed that different cell compartments play a major role in the sequestration of U. Our findings contribute to a better understanding of the mechanisms of microbial response to U and demonstrate that *A. facilis* may play an important role in predicting the fate and transport of U in uranium-contaminated sites by being a suitable candidate for bioremediation purposes.

Acknowledgements

This work was supported by the Bundesministerium für Bildung und Forschung (BMBF) grand No. 02NUK030 F (TransAqua). We acknowledge the technical support of Maria del Mar Abad of the Centro de Instrumentacion Cientifica (University Granada) for STEM/HAADF analysis. Karsten Heimś skilful work on *in-situ* ATR FT-IR spectroscopy is gratefully acknowledged. Sabrina Gurlit and Stefanie Schubert are thanked for ICP-MS measurements.

References

[1] J.L. Nyman, T.L. Marsh, M.A. Ginder-Vogel, M. Gentile, S. Fendorf, C. Criddle, Heterogeneous response to biostimulation for U(VI) reduction in replicated sediment microcosms, Biodegradation 17 (2006) 303–316.
[2] S. Sachs, V. Brendler, G. Geipel, Uranium(VI) complexation by humic acid under neutral pH conditions studied by laser-induced fluorescence spectroscopy, Radiochim. Acta 95 (2007) 103–110.
[3] H. Moll, T. Reich, C. Hennig, A. Rossberg, Z. Szabó, I. Grenthe, Solution coordination chemistry of uranium in the binary UO_2^{2+}-SO_4^{2-} and the ternary UO_2^{2+}-SO_4^{2-} OH^-–system, Radiochim. Acta 88 (2000) 559–566.
[4] T. Arnold, N. Baumann, E. Krawczyk-Barsch, S. Brockmann, U. Zimmermann, U. Jenk, S. Weiss, Identification of the uranium speciation in an underground acid mine drainage environment, Geochim. Cosmochim. Acta 75 (2011) 2200–2212.
[5] Umweltbericht der Wismut GmbH, 2015, Chemnitz.
[6] J.R. Lloyd, L.E. Macaskie, Biochemical basis of microbe-radionuclide interactions, in: M.J. Kieth-Roach, F.R. Livens (Eds.), Interactions of Microorganisms with Radionuclides, Elsevier, Amsterdam, 2002, pp. 313–342.
[7] K. Pedersen, Microorganisms and their influence on radionuclide migration in igneous rock environments, J. Nucl. Radiochem. Sci. 6 (1) (2005) 11–15.
[8] D.R. Lovley, E.J.P. Phillips, Y. Gorby, E.R. Landa, Microbial reduction of uranium, Nature 350 (1991) 413–415.
[9] R. Ganesh, K.G. Robinson, G.D. Reed, G.S. Sayler, Reduction of hexavalent uranium from organic complex by sulfate- and iron-reducing bacteria, Appl. Environ. Microbiol. 63 (1997) 4385–4391.
[10] D.R. Lovley, S.J. Giovannoni, D.C. White, J.E. Champine, E.J. Phillips, Y.A. Gorby, S. Goodwin, *Geobacter metallireducens* gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals, Arch. Microbiol. 159 (4) (1993) 336–344.
[11] J.K. Fredrickson, J.M. Zachara, D.W. Kennedy, M.C. Duff, Y.A. Gorby, S.W. Li, K.M. Krupka, Reduction of U(VI) in goethite (R-FeOOH) suspensions by a dissimilatory metal reducing bacterium, Geochim. Cosmochim. Acta 64 (2000) 3085–3098.
[12] D.E. Holmes, K.T. Finneran, R.A. O'Neil, D.R. Lovley, Enrichment of members of the family *Geobacteraceae* associated with stimulation of dissimilatory metal reduction in uranium contaminated aquifer sediments, Appl. Environ. Microbiol. 68 (2002) 2300–2306.
[13] D.R. Lovley, E.J.P. Phillips, Reduction of uranium by Desulfovibrio desulfuricans, Appl. Environ. Microbiol. 58 (1992) 850–856.
[14] R. Ganesh, K.G. Robinson, L. Chu, D. Kucsmas, G.D. Reed, Reductive precipitation of uranium by *Desulfovibrio desulfuricans*: evaluation of cocontaminant effects and selective removal, Water Res. 33 (1999) 3447–3458.

[15] R.B. Payne, D.M. Gentry, B.J. Rapp-Giles, L. Casalot, J.D. Wall, Uranium reduction by *Desulfovibrio desulfuricans* strain G20 and a cytochrome c3 mutant, Appl. Environ. Microbiol. 68 (2002) 3129–3132.
[16] H. Beyenal, R.K. Sani, B.M. Peyton, A.C. Dohnalkova, J.E. Amonette, Z. Lewandowski, Uranium immobilization by sulfate-reducing biofilms, Environ. Sci. Technol. 38 (2004) 2067–2074.
[17] Y. Suzuki, S.D. Kelly, K.M. Kemner, J.F. Banfied, Nanometersize products of uranium bioreduction, Nature 419 (2002) 134.
[18] Y. Suzuki, S.D. Kelly, K.M. Kemner, J.F. Banfied, Microbial populations stimulated for hexavalent uranium reduction in uranium mine sediment, Appl. Environ. Microbiol. 69 (2003) 1337–1346.
[19] A. Schatz, C. Bovell, Growth and hydrogenase activity of a new bacterium, *Hydrogenomonas facilis*, J. Bacteriol. 63 (1952) 87–98.
[20] M. Fabisch, F. Beulig, D.M. Akob, K. Küsel, Surprising abundance of *Gallionella*-related iron oxidizers in creek sediments at pH 4.4 or at high heavy metal concentrations, Front. Microbiol. 4 (390) (2013) 1–12.
[21] V.F. Bondici, J.R. Lawrence, N.H. Khan, J.E. Hill, E. Yergeau, G.M. Wolfaardt, J. Warner, D.R. Korber, Microbial communities in low permeability, high pH uranium mine tailings: characterization and potential effects, J. Appl. Microbiol. 114 (2013) 1671–1686.
[22] W.-M. Wu, J. Carley, T. Gentry, M.A. Ginder-Vogel, M. Fienen, T. Mehlhorn, H. Yan, S. Caroll, M.N. Pace, J. Nyman, J. Luo, M. Gentile, M.W. Fields, R.F. Hickey, B. Gu, D. Watson, O.A. Cirpka, J. Zhou, S. Fendorf, P.K. Kitanidis, P.M. Jardine, C.S. Criddle, Pilot-scale in situ bioremedation of uranium in a highly contaminated aquifer. 2. Reduction of U(VI) and geochemical control of U(VI) bioavailability, Environ. Sci. Technol. 40 (2006) 3986–3995.
[23] E. Cardenas, W.-M. Wu, M.B. Leigh, J. Carley, S. Carroll, T. Gentry, J. Luo, D. Watson, B. Gu, M. Ginder-Vogel, P.K. Kitanidis, P.M. Jardine, J. Zhou, C.S. Criddle, T.L. Marsh, J.M. Tiedje, Microbial communities in contaminated sediments, associated with bioremediation of uranium to submicromolar levels, Appl. Environ. Microbiol. 74 (12) (2008) 3718–3729.
[24] A. Willems, M. Gillis, Genus M II, Acidovorax Willems, Falsen, Pot, Jantzen, Hoste, Vandamme, Gillis, Kersters and De Ley 1990, 394vp, in: D.J. Brenner, N.R. Krieg, J.T. Staley, G.M. Garrity (Eds.), Bergey's Manual of Systematic Bacteriology, Springer, New York, 2005, pp. 696–703.
[25] N.N. North, S.L. Dollhopf, L. Petrie, J.D. Istok, D.L. Balkwill, J.E. Kostka, Change in bacterial community structure during in situ biostimulation of subsurface sediment contaminated with uranium and nitrate, Appl. Environ. Microbiol. 70 (8) (2004) 4911–4920.
[26] U. Gerber, I. Zirnstein, E. Krawczyk-Bärsch, H. Lünsdorf, T. Arnold, M.L. Merroun, Combined use of flow cytometry and microscopy to study the interactions between the gram-negative betaproteobacterium *Acidovorax facilis* and uranium(VI), J. Hazard. Mater. 317 (2016) 127–134.
[27] A.R. Spurr, A low-viscosity epoxy resin embedding medium for electronmicroscopy, J. Ultrastruct. Res. 26 (1969) 31–43.
[28] R. Steudtner, S. Sachs, K. Schmeide, V. Brendler, G. Bernhard, Ternary uranium(VI) carbonato humate complex studied by cryo-TRLFS, Radiochim. Acta 99 (2011) 687–692.
[29] W. Matz, N. Schell, G. Bernhard, F. Prokert, T. Reich, J. Claußner, W. Oehme, R. Schlenk, S. Dienel, H. Funke, F. Eichhorn, M. Betzl, D. Pröhl, U. Strauch, G. Hüttig, H. Krug, W. Neumann, V. Brendler, P. Reichel, M.A. Denecke, H. Nitschea, ROBL – a CRG beamline for radiochemistry and materials research at the ESRF, J. Synchrotron Radiat. 6 (6) (1999) 1076–1085.
[30] E.S. Makarov, V.I. Ivanov, The crystalline structure of $Ca(UO_2)_2(PO_4)_2{\cdot}6H_2O$ meta-autunite, Doklady Akademii Nauk SSSR 132 (3) (1960) 673–676.
[31] C. Lucks, A. Rossberg, S. Tsushima, H. Foerstendorf, A.C. Scheinost, G. Bernhard, Aqueous uranium(VI) complexes with acetic and succinic acid: speciation and structure revisited, Inorg. Chem. 51 (2012) 12288–12300.
[32] G.N. George, I.J. Pickering, EXAFSPAK: A Suite of Computer Program for Analysis of X-ray Absorption Spectra, Stanford Synchrotron Radiation Laboratory, Stanford, CA, 1995.
[33] A.L. Ankudinov, B. Ravel, J.J. Rehr, S.D. Conradson, Real-space multiple-scattering calculation and interpretation of x-ray-absorption near-edge structure, Phys. Rev. B 58 (1998) 7565.
[34] M. Vogel, A. Günther, A. Rossberg, B. Li, G. Bernhard, J. Raff, Biosorption of U(VI) by the green algae *Chlorella vulgaris* in dependence of pH and cell activity, Sci. Total Environ. 409 (2010) 384–395.
[35] E.A. Hudson, P.G. Allen, L.J. Terminello, M.A. Denecke, T. Reich, Polarized X-ray-absorption spectroscopy of uranyl ion: comparison of experiment and theory, Phys. Rev. B 54 (1996) 156–165.
[36] I.S. Kulaev, Biochemistry of inorganic polyphosphates, Rev. Physiol. Biochem. Pharmacol. 73 (1975) 131–157.
[37] K. Müller, H. Foerstendorf, T. Meusel, V. Brendler, G. Lefèvre, M.J. Comarmond, T.E. Payne, Sorption of U(VI) at the TiO_2–water interface: an *in-situ* vibrational spectroscopic study, Geochim. Cosmochim. Acta 76 (2012) 191–205.
[38] K. Müller, H. Foerstendorf, V. Brendler, A. Rossberg, K. Stolze, A. Gröschel, The surface reactions of U(VI) on gamma-Al_2O3 – *in-situ* spectroscopic evaluation of the transition from sorption complexation to surface precipitation, Chem. Geol. 357 (2013) 75–84.
[39] R. Docampo, Acidocalcisomes and polyphosphate granules. Microbiol monogr (1), in: J.M. Shively: Inclusions in Prokaryotes, Springer-Verlag Berlin Heidelberg, 2006.
[40] A. Barkleit, H. Moll, G. Bernhard, Interaction of uranium(VI) with lipopolysaccharide, Dalton Trans. (2008) 2879–2886.
[41] T.J. Beveridge, R.J. Doyle, Metal Ion and Bacteria, John Wiley & Sons, Inc., New York, N.Y, 1989.
[42] A. Barkleit, H. Moll, G. Bernhard, Complexation of uranium(VI) with peptidoglycan, Dalton Trans. (2009) 5379–5385.
[43] V.R.F. Matias, T.J. Beveridge, Native cell wall organization shown by cryo-electron microscopy confirms the existence of a periplasmic space in *Staphylococcus aureus*, J. Bacteriol. 188 (2006) 1011–1021.
[44] A.D. Warth, J.L. Strominger, Structure of the peptidoglycan from vegetative cell walls of *Bacillus subtilis*, Biochemistry 10 (1971) 4349–4358.
[45] B.D. Hoyle, T.J. Beveridge, Metal binding by the peptidoglycan sacculus of *Escherichia coli* K-12, Can. J. Microbiol. 30 (1984) 204–211.
[46] M.L. Merroun, J. Raff, A. Rossberg, C. Hennig, T. Reich, S. Selenska-Pobell, Complexation of uranium by cells and S-layer sheets of *Bacillus sphaericus* JG-A12, Appl. Environ. Microbiol. 71 (2005) 5532–5543.
[47] G.G. Li, F. Bridges, C.H. Booth, X-ray-absorption fine-structure standards: a comparison of experiment and theory, Phys. Rev. B 52 (9) (1995) 6332–6348.
[48] A. Rossberg, K.-U. Ulrich, S. Weiss, S. Tsushima, T. Hiemstra, A.C. Scheinost, Identification of uranyl surface complexes on ferrihydrite: advanced EXAFS data analysis and CD-MUSIC modeling, Environ. Sci. Technol. 43 (5) (2009) 1400–1406.
[49] A. Koban, G. Geipel, A. Rossberg, G. Bernhard, Uranium(VI) complexes with sugar phosphates in aqueous solution, Radiochim. Acta 92 (2004) 903–908.
[50] A. Barkleit, H. Foerstendorf, B. Li, A. Rossberg, H. Moll, G. Bernhard, Coordination of uranium(VI) with functional groups of bacterial lipopolysaccharide studies by EXAFS and FT-IR spectroscopy, Dalton Trans. 40 (2011) 9868–9876.
[51] M. Merroun, M. Nedelkova, A. Rossberg, C. Hennig, S. Selenska-Pobell, Interaction mechanisms of bacterial strains isolated from extreme habitats with uranium, Radiochim. Acta 94 (2006) 723–729.
[52] F. Jroundi, M.L. Merroun, J.M. Arias, A. Rossberg, S. Selenska-Pobell, M.T. Gonzalez-Munoz, Spectroscopic and microscopic characterization of uranium biomineralization in *Myxococcus xanthus*, Geomicrobiol. J. 24 (2007) 441–449.
[53] E. Krawczyk-Bärsch, H. Lünsdorf, K. Pedersen, T. Arnold, F. Bok, R. Steudtner, A. Lehtinen, V. Brendler, Immobilization of uranium in biofilm microorganisms exposed to groundwater seeps over granitic rock tunnel walls in Olkiluoto, Finland, Geochim. Cosmochim. Acta 96 (2012) 94–104.
[54] B. Li, J. Raff, A. Barkleit, G. Bernhard, H. Foerstendorf, Complexation of U(VI) with highly phosphorylated protein, phosvitin a vibrational spectroscopic approach, J. Inorg. Biochem. 104 (7) (2010) 718–725.
[55] A. Barkleit, H. Foerstendorf, K. Heim, S. Sachs, G. Bernhard, Complex formation of uranium(VI) with l-phenylalanine and 3-phenylpropionic acid studied by attenuated total reflection Fourier transform infrared spectroscopy, Appl. Spectrosc. 62 (7) (2008) 798–802.
[56] M.J. Comarmond, R. Steudtner, M. Stockmann, K. Heim, K. Müller, V. Brendler, H. Foerstendorf, The sorption processes of U(VI) onto SiO_2 in the presence of phosphate: from binary surface species to precipitation, Environ. Sci. Technol. 50 (21) (2016) 11610–11618.
[57] H. Moll, G. Geipel, T. Reich, G. Bernhard, T. Fanghänel, I. Grenthe, Uranyl(VI) complexes with alpha-substituted carboxylic acids in aqueous solution, Radiochim. Acta 91 (2003) 11–20.
[58] W. Jiang, A. Saxena, B. Song, B.B. Ward, T.J. Beveridge, S.C.B. Myneni, Langmuir 20 (2004) 11433–11442.

3.3 Metabolism-dependent bioaccumulation of uranium by *Rhodosporidium toruloides* isolated from the flooding water of a former uranium mine

Gerber U, Hübner R, Rossberg A, Krawczyk-Bärsch E, Merroun ML

Submitted to PLOS ONE (29/03/2018), first revision (16/05/2018)

Metabolism-dependent bioaccumulation of uranium by *Rhodosporidium toruloides* isolated from the flooding water of a former uranium mine

Ulrike Gerber[1,*,#], René Hübner[2], André Rossberg[1,3], Evelyn Krawczyk-Bärsch[1], Mohamed L. Merroun[4]

[1] Helmholtz-Zentrum Dresden - Rossendorf, Institute of Resource Ecology, Bautzner Landstraße 400, 01328 Dresden, Germany

[2] Helmholtz-Zentrum Dresden - Rossendorf, Institute of Ion Beam Physics and Materials Research, Bautzner Landstraße 400, 01328 Dresden, Germany

[3] Rossendorf Beamline at ESRF - The European Synchrotron, CS40220, 38043 Grenoble Cedex 9, France

[4] University of Granada, Department of Microbiology, Campus Fuentenueva, E-18071 Granada, Spain

[#] Present Address: Faculty of Natural Sciences, Brandenburg University of Technology Cottbus-Senftenberg, Universitätsplatz 1, 01968 Senftenberg, Germany

[*] Corresponding author
E-Mail: gerberu@b-tu.de (UG)
Telephone: +49 3573 85 974
Fax: +49 3573 85 909

Abstract

Remediation of former uranium mining sites represents one of the biggest challenges worldwide that have to be solved in this century. During the last years, the search of alternative strategies involving environmentally sustainable treatments has started. Bioremediation, the use of microorganisms to clean up polluted sites in the environment, is considered one the best alternative. By means of culture-dependent methods, we isolated an indigenous yeast strain, KS5 (*Rhodosporidium toruloides*), directly from the flooding water of a former uranium mining site and investigated its interactions with uranium. Our results highlight distinct adaptive mechanisms towards high uranium concentrations on the one hand, and complex interaction mechanisms on the other. The cells of the strain KS5 exhibited high uranium tolerance being able to grow up

to 6 mM, and also high ability to accumulate this radionuclide (350 mg uranium/g drybiomass, 48 hours). The removal of uranium by KS5 displays a temperature- and cell viability-dependent process, indicating that metabolic activity could be involved. By STEM (Scanning transmission electron microscopy) investigations we observed that uranium was removed by two mechanisms, active bioaccumulation and inactive biosorption. This study highlights the potential of KS5 as a representative of indigenous species within the flooding water of a former uranium mine which may play a key role in bioremediation of uranium contaminated sites.

Introduction

Heavy metal pollution of the environment is one of the biggest problems today due to its great impact on the surface and groundwater, and even in the catchment areas of drinking water [1]. In particular, uranium contamination is a result of former mining activities, by weathering of uranium containing minerals or by accidental release to the environment [2, 3]. In Germany, the former uranium mine Königstein was one of the smallest mining sites in Eastern-Germany. Uranium was recovered from the underground sandstone by leaching with sulfuric acid [4]. The mining activity resulted in the release of about 2 million of cubic meters of acid pore water, which contained high amounts of uranium. Due to the stop of uranium mining in Germany, the underground is actually in the process of remediation and since 2001, the mine is flooded in a controlled way. Nevertheless, the flooding water still contains relatively high concentrations of uranium (8 mg/L) and other heavy metals. Additionally, the flooding water displays a low pH (around 3) as a consequence of the leaching process [5]. For this reason, the flooding water is treated by an elaborate chemical wastewater treatment plant. In contrast to other former uranium mines in Germany, Königstein is unique in the remediation effort, due to disturbances in the underground rock formation, it is not possible to flood the mine completely, otherwise nearby aquifers would be potentially contaminated with uranium. Therefore, the flooding water is pumped to the surface (pump-and-treat technique) and is treated by chemical precipitation and ion exchange [6].

Within natural environments the mobility and bioavailability of uranium depends strongly on its speciation and physicochemical form [7]. Particularly in acidic (pH < 5) and non-reducing conditions the free uranyl ion (UO_2^{2+}) predominates the uranium speciation [8]. Previous studies showed that uranium mainly occurs in the bulk solution as highly soluble UO_2SO_4-species within the flooding water of the former uranium mine [9]. Besides abiotic factors such as pH, redox potential, dissolved organic and inorganic ligands, and the presence of solid particulates [5, 10, 11] [5,10,11], biological processes could have a significant impact on the migration of radionuclides. They can affect their mobility by a variety of interaction mechanisms, such as biosorption on functional groups of the cell-surface, bioaccumulation within the cell, biotransformation (reduc-

tion or oxidation of the radionuclide), and biomineralization [2, 12–19].

Despite harsh environmental conditions generated in the mining process, such as high uranium and sulfate concentrations, and low pH, leading to acid mine drainage (AMD), microorganisms are able to survive and even display metabolic activity in these environments [20]. In addition, culture-independent studies of the microbial diversity within the flooding water of the former uranium mine Königstein revealed a diverse community of microbial life [21, 22]. Therefore, microorganisms should be taken into consideration to investigate possible interaction mechanisms which could be helpful for biological approaches concerning bioremediation.

Investigations on the influence of yeast cells on actinides, like uranium are still in its infancy [23]. Much is known about the interaction of model organisms, such as *Saccharomyces cerevisiae* and *Escherichia coli*, with heavy metals and radionuclides [24–26]. It seems to be important to consider microorganisms from ecological niches, like the former uranium mining site Königstein, for the use in metal bioremediation approaches, since conventional technologies, such as chemical precipitation and ion exchange, are cost-intensive and often inefficient for metals at low concentrations [27–29]. Therefore, the present study aimed to describe the possible role of microorganisms that were isolated from their natural uranium-contaminated habitats as a potential alternative to conventional chemical remediation strategies, due to their adaptive tolerances and possible immobilization ability of radionuclides. We investigated an isolated yeast, KS5, which was identified as *Rhodosporidium toruloides*. *R. toruloides* (syn. *Rhodotorula gracilis*) belongs to the division of Basidiomycota and is an oleaginous yeast [30]. Species of *Rhodosporidium* were isolated from heavy metal contaminated soil in former studies. Heavy metal tolerance tests with this strain displayed high tolerances against several metals [31]. In addition to the high tolerances, *R. toruloides* offers many other biotechnological opportunities as an alternative yeast model compared to *S. cerevisiae*, which lacks several biochemical features [30]. Furthermore, we investigated the uranium removal capacity of the indigenous yeast KS5 and its tolerance to selected heavy metals. More precisely, different influencing factors on the uranium removal capacity of KS5 were examined, such as temperature, initial dry biomass and viability of the yeast cells. To obtain a detailed and closer look on the localization of the removed uranium on the cell membrane and possibly inside the cells, high-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM) and energy-dispersive X-ray spectroscopy (EDXS) were performed. The structural parameters of the uranium complexes formed by the strain were studied using EXAFS (extended X-ray absorption fine structure spectroscopy) spectroscopy. In addition, we compared the metal tolerances of the isolated strain KS5 with the reference strain DSM 10134 to investigate natural adaption mechanisms against heavy metals. The results of our investigations provide new insights on the interaction of indigenous yeast cells with uranium, and consequently, a possible use of microbial cells for *in situ* bioremediation.

Methods and Materials

Water sampling, microbial isolation and cultivation conditions

Samples (1 L) of flooding water from the former uranium mine in Königstein (Saxony, Germany) were taken into sterile glass bottles. The water was stored at 4 °C until further processing. For isolation of aerobic fungal microorganisms, 500 µL of the flooding water was plated onto solid SDA (Sabouraud Dextrose Agar) medium (Peptone 5.0 g/L, Casein Peptone 5.0 g/L, Glucose 40.0 g/L, 15.0 g/L Agar-Agar, pH 6.5 $\pm$ 0.1) [32]. The plates were incubated at 30 °C for five days. After appearing of single colonies, they were picked and transferred into liquid SDA medium to obtain pure cultures. The purity of the cultures was tested by plating again onto solid plates and by PCR analysis. *R. toruloides* DSM 10134 was obtained from the DSMZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany).

Molecular characterization, amplification of rRNA ITS gene fragments and sequencing

For molecular identification of the isolated microorganisms, cells were grown in liquid SDA medium for 48 hours at 30 °C and 130 rpm. Two mL of the solution were sampled and the DNA was extracted using alkaline lysis [33]. The rRNA ITS gene fragments which are the DNA-barcode established for fungi [34] were amplified using the primer pair ITS1 and ITS4 [35]. PCR was performed as described in White *et al.*, 1990. Before sequencing the amplified products, the DNA was purified using the innuPREP-PCR pure Kit (Analytik Jena, Jena, Germany), and eluted in double-distilled water according the instructions of the manufacturer. The purified PCR products were sequenced by GATC (GATC Biotech AG, Konstanz, Germany). The retrieved rRNA ITS sequences were compared with sequences available in the non-redundant nucleotide database of the National Center or Biotechnology database (`http://www.ncbi.nlm.nih.gov`) using BLASTN and the ribosomal database project.

Heavy metal tolerance (determination of MIC) and use of different carbon sources

For heavy metal tolerance tests with uranium, chromium, zinc, cadmium, and copper, yeast cells (KS5 and DSM 10134) were grown for 48 hours in liquid SDA medium at 30 °C and 130 rpm. Subsequently, cells were washed twice in 0.9 % NaCl solution to remove the residual ingredient medium. The microbial suspension with an initial OD_{600nm} of 5.0 was 1:500 diluted and 100 µL were plated onto solid agar plates containing SDA 1:5 diluted with adjusted metal concentration

(cadmium, copper and chromium 0.1 - 5.0 mM; zinc and uranium 0.1 - 10.0 mM). The plates were incubated for 48 hours at 30 °C. The minimal inhibitory concentration (MIC) was determined in triplicates. In addition, tolerance test with uranium in liquid medium was determined using the isolate KS5. SDA medium 1:5 diluted was adjusted with different uranium concentrations (0.05, 0.1, 0.2 mM) and was inoculated with 50 µL of a 48 hours old culture. After distinct time points, samples were taken to analyze the OD_{600nm}. The incubation took place at 30 °C and 130 rpm in triplicates. The resulting growth curves were plotted with logarithmic scale to calculate the growth rate $\mu = \frac{\ln x_t - \ln t_0}{t - t_0}$ and doubling time $t_d = \frac{\ln 2}{\mu}$ within the linear exponential phase. To investigate the use of different carbon sources, KS5 and DSM 10134 were pre-cultured in liquid SDA medium as described above and washed two times with 0.9 % NaCl solution. The washed cells were diluted to an OD_{600nm} of 0.1. 50 µL solution from the diluted and washed cell suspension was inoculated in 50 mL minimal-salt-medium (MSM, 0.2 g/L KH_2PO_4, 0.6 g/L K_2HPO_4, 0.5 g/L $(NH_4)_2SO_4$, 0.1 g/L $MgSO_4 \cdot 7\,H_2O$, 0.01 g/L $CaCl_2$, 0.005 g/L $FeCl_2$, 0.01 g/L $MnCl_2$) which was supplemented with 1 % of different carbon sources (lactate, maltose, mannose, fructose, glucose, sucrose, xylose, acetate, oxalic acid, glycerol, ethanol, galactose). The cells were incubated at 30 °C and 130 rpm for 72 hours. The experiments were carried out in triplicates. Finally, to investigate the growth with the different carbon sources, the OD_{600nm} was measured.

Determination of uranium removal capacity

To test the ability to remove uranium from the surrounding solution, yeast cells of KS5 were grown in liquid SDA medium for 48 hours at 30 °C and 130 rpm. Afterwards cells were washed three times with sterilized tap water pH 5.0. The washed yeast cells were suspended in the background solution (sterilized tap water pH 5.0) to an initial OD_{600nm} of 1.0 (6.5 mg/mL $\pm$ 0.5 mg/mL). Subsequently, uranium as $UO_2(NO_3)_2$ was added to reach a final concentration of 0.1 mM. To investigate the temperature-dependent uranium removal capacity, the yeast cells were washed with an acclimated background solution at 4 °C or 30 °C. The cell suspensions were incubated at selected temperatures for 48 hours. During the incubation, samples were taken at distinct time points (5 min, 0.5 h, 1 h, 1.5 h, 2 h, 4 h, 6 h, 8 h, 26 h, 48 h) to determine the residual uranium concentration within the supernatant. All experiments were carried out in triplicates. The samples were centrifuged for 5 min at 13,000 rpm at RT (Centrifuge 5415R, Eppendorf AG, Hamburg, Germany) and the acidified supernatant was analyzed with Inductively Coupled Plasma Mass Spectrometry measurements (ICP-MS) using an ELAN 9000 type ICP-MS spectrometer (Perkin Elmer, Überlingen, Germany). To study the uranium removal capacity of dead cells, cultures grown for 48 h were immediately autoclaved for 30 min at 121 °C and 1 bar for 30 min. The autoclaved cells were assayed same as it is described above. The amount of removed uranium from the solution was normalized to the dry biomass after drying the cell

pellet for 24 h at 100 °C in an oven (Memmert UE500, Schwabach, Germany). To investigate the uranium removal capacity dependent on the initial dry biomass (dbm), different weights of biomass were applied. The cells were incubated with 0.1 mM uranium for 48 hours at 30 °C and 130 rpm. Afterwards the cells were harvested and dried as described above.

Effect of uranium on cellular viability using flow cytometry

To investigate the effect of uranium on the cell, uranium-treated cells were stained with fluorescent dyes and analyzed by flow cytometry techniques. Therefore, an inoculum of a pre-grown culture of KS5 and DSM 10134 was added to 1:5 diluted SDA medium either without uranium as a control or containing a uranium concentration of 0.1 mM. The cells were incubated for 24 hours at 30 °C and 130 rpm. Afterwards, the cells were harvested by centrifugation at 8,000 rpm (Centrifuge 5804R, Eppendorf AG, Hamburg, Germany) for 10 minutes and washed twice with Phosphate Buffered Saline (PBS). Subsequently, the cells were dissolved in PBS to approximately 106 cells/mL. As the "dead" control an aliquot of the cells were incubated for 45 min at 80 °C. The cell viability test was performed with propidium iodide (PI) (stain dead cells) and fluorescein diacetate (FDA) (stain alive cells). The fluorescent dyes were added to a final concentration of 2 µL/mL for PI and 20 µL/mL FDA. The cell suspension was incubated for 15 min in the dark at ambient temperature. After the incubation with the two dyes both strains were analyzed by flow cytometry using a FACSCantoII cytometer Becton Dickinson (San Jose Palo Alto, California) available at the "Centro de Instrumentación Científica" of the University of Granada, equipped with three lasers: 488 nm blue, 620 nm red, and 405 nm violet. All experiments were done in triplicates.

Transmission electron microscopy (TEM) and Energy-dispersive X-ray spectroscopy (EDXS)

Immediately after the uranium removal experiments, the cells were harvested by centrifugation for 10 min at 6,000 rpm (Centrifuge 5804R, Eppendorf AG, Hamburg, Germany) at room temperature to remove the supernatant. The cell pellet was washed twice with sterilized tap water at pH 5.0 added with 0.2 % glucose. Subsequently, the cells were fixed with 2.5 % (vol/vol) glutardialdehyde from 50 % (vol/vol) stock (Carl Roth, Karlsruhe, Germany) over night at 4 celsius. After fixation, the cells were transferred in 4 % (w/v) aqueous low-melting agarose (Life Technologies Inc., Darmstadt, Germany), and after cooling, dehydrated by an ethanol series (25, 50, 75, 95 % for 10 min; 100 % for 2 h; 100 % over night at RT), followed by ERL-resin impregnation and polymerization. Sample preparation with minor modifications was done according to the user manual [36]. Ultrathin sections of 100 to 200 nm were cut with a diamond knife (EMS, Munic, Germany) and transferred onto carbon-coated Cu grids (lacey carbon on

200 mesh Cu (S166-2), Plano GmbH, Wetzlar, Germany). TEM investigations were done with an image Cs-corrected Titan 80-300 microscope (FEI) operated at an accelerating voltage of 300 kV. Qualitative atomic number contrast images were obtained by high-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM). Employing a Li-drifted silicon detector (EDAX) in STEM mode, energy-dispersive X- ray spectroscopy (EDXS) measurements were performed for qualitative chemical analysis. For elemental distribution analysis samples were examined in HAADF-STEM mode with Titan G2 80-300 microscope (FEI) at "Centro de Instrumentación Científica" at the University of Granada, Spain. Prior to each STEM analysis, the specimen holder was plasma-cleaned to minimize contamination.

Extended X-ray absorption fine structure spectroscopy (EXAFS)

In order to obtain information about the structure of the formed uranium complexes at molecular scale, EXAFS analyses were performed. After uranium immobilization experiments with 0.1 mM uranium, contacted with the yeast cells for 48 h and at 30 °C (described above) the cells were ultra-centrifuged (Ultracentrifuge Optima XL100K, Rotor: SW 32Ti; Beckman Coulter, USA) for 1 h at 187 000 $\times$ g. The supernatant was removed and the resulting cell pellet was placed into polyethylene sample holders. The sample holders were sealed, frozen, and stored in liquid nitrogen until the x-ray absorption measurements. The measurements were carried out at the Rossendorf Beamline BM20 at the European Synchrotron Radiation Facility (ESRF) [37]. The yeast cells were measured at 15 K in a closed-cycle He-cryostat in order to reduce thermal noise and to avoid radiation-induced redox reactions of uranium during the measurements.
A water-cooled Si(111) double-crystal monochromator in channel cut mode (5-35 keV) was used to monochromatize the incoming synchrotron X-ray. In dependence of the uranium amount the spectra were collected in fluorescence mode or in transmission mode using ionization chambers. A reference sample, meta-autunite $Ca(UO_2)_2(PO_4)_2 \cdot 6\,H_2O$ [38], was measured at room temperature in transmission mode [39]. The K-edge spectrum of an yttrium metal foil (first inflection point at 17038 eV) was recorded simultaneously for energy calibration of the sample spectra. E_0, the ionization energy, of the uranium L_{III}-edge was defined as the maximum of the second derivative of the averaged spectra. Eight scans in fluorescence mode were collected of the yeast cells incubated with 0.1 mM uranium. The fluorescence spectra were corrected for the detector dead time and subsequently averaged. The spectra were analyzed using the data analysis programs Sixpack/SamView (Version 0.59) (Webb 2005) and WinXAS (version 3.11) [40].

Results and discussion

Phylogenetic affiliation, morphological characterization and utilization of different carbon sources of KS5

Culture dependent methods based on the use of SDA medium resulted in the isolation of different microbial strains. SDA medium was developed and used for enrichment of fungal stains from environmental and clinical samples [31]. Single colonies obtained were used for the enrichment of pure cultures indigenous within the flooding water of Königstein. On SDA agar medium, the isolate KS5 displayed red round and shiny colonies (see Supplementary Material). The phylogenetic affiliation of the microbial isolate based on ITS rRNA gene analysis displayed high similarity to *R. toruloides* (strain JZ-9, 99 % identity and 100 % query cover). These yeast cells are known for their production of lipid related molecules, including biodiesel, adhesives, and high-value nutritional oils [41–47]. In addition, *R. toruloides* is able to utilize a wide variety of carbohydrates derived from plant biomass, including xylose and cellobiose [47–49]. To investigate the isolated strain in more detail, studies on the metabolic versatility regarding the use of different carbon sources were performed. In addition, the reference strain DSM 10134 was investigated in the same way. These experiments were performed in order to find a suitable carbon source to grow the isolated strain KS5 directly within the flooding water for *in situ* bioremediation approaches. The results (Table 3.1) displayed slight differences for the carbon sources mannose, acetate and ethanol. KS5 displays better growth on mannose and acetate, compared to DSM 10134. Notably, KS5 shows the ability to grow in the presence of xylose, in contrast to DSM 10134 which was not able to metabolize this sugar. The fermentation of xylose to ethanol by yeasts was well studied by several investigations and could be a useful process for the production of bioethanol [50–53]. Nevertheless, the results show that the uranium bioremediation potential of the isolated strain KS5 could be enhanced by the ability to use different sugars like maltose, fructose, mannose and sucrose.

Impact of uranium and selected heavy metals on microbial growth

To investigate the impact of uranium on the microbial growth, 1:5 diluted SDA medium with increasing metal concentrations was used. The growth curves of KS5 incubated with two different uranium concentrations are displayed in Fig. 1. It is clearly visible that the curves with uranium (0.05 and 0.1 mM) are clearly shifted compared to the control (without uranium). The lag-phase was longer which might originate from a possible adaption of the cells to uranium. Based on the growth curves, we calculated the growth rate μ and doubling time t_d for all three approaches (Table 3.2). Compared to the uranium-free control, a decrease of the growth rates μ with increasing metal concentrations was observed. At the highest uranium concentration of 0.1 mM, the growth

rate is more than half times smaller compared to that of the control. Furthermore, with increasing uranium concentrations also the doubling times increased. At 0.1 mM, the doubling time is more than twice higher. Nevertheless, the yeast cells are able to grow up to a uranium concentration of 0.1 mM in liquid SDA medium. Furthermore, the uranium tolerance of KS5 in comparison to the reference strain DSM 10134 was studied using flow cytometry. The cells were grown in 1:5 diluted SDA medium supplemented with 0.1 mM uranium. After an incubation time of 24 h the cells were stained with PI and FDA. The percentage distribution of viable and dead cells with and without uranium is displayed in Fig. 2. In the absence of uranium more than 80 % of the KS5 and all the DSM 10134 cells displayed viability. At a uranium concentration of 0.1 mM the two microbial strains tolerate this metal in different ways. The cell viability was reduced to 60 % and 10 % for KS5 and DSM 10134, respectively. The high uranium tolerance of KS5 could be explained by adaption of this strain to uranium in its natural habitat contaminated with uranium. To survive within the flooding water, containing high amounts of uranium, KS5 has to develop adaption mechanisms. Previous studies have shown that microorganisms, which were isolated from contaminated sites, display the ability to tolerate relatively higher concentrations of heavy metals [54–56]. These natural occurring microbes could be promising candidates for their use in the bioremediation of these inorganic contaminants [31]. In addition, Sakamoto *et al.*, 2012 [57] concluded that several genes of *S. cerevisiae* are involved in uranium tolerance. Phosphate transporter genes were observed to be responsible to contribute to uranium tolerance and furthermore, cell surface proteins contributed to the uranium accumulation [57]. Further investigations with the isolated strain KS5 should be performed to identify possible gene responsible for the high

Table 3.1: Growth on different carbon sources of KS5 and DSM 10134

Carbon source	KS5	DSM 10134
lactate	+	+
maltose	++	++
mannose	++	+
fructose	++	++
glucose	+	+
sucrose	++	++
xylose	+/-	-
acetate	+	+/-
oxalic acid	-	-
glycerol	+	+
ethanol	+/-	+
galactose	+/-	+/-

The two strains were grown in liquid mineral-salt-medium which was added with 1 % carbon source. Growth was determined by measuring of the OD_{600nm}. ++ good growth (OD > 0.4), + growth (OD 0.2-0.39), +/- less growth (OD < 0.2), - no growth (OD = 0.0), (n=3).

uranium tolerance. To evaluate the tolerance against selected heavy metals (Cr, Cu, Cd, Zn) and uranium, KS5 was grown on solid agar plates with increasing concentrations of metals. To compare possible adaption mechanisms of the indigenous yeast KS5 the reference strain, DSM 10134, was investigated in the same way. The results of the heavy metal tolerances (Table 3.3) showed clear differences between the two strains. The investigated uranium tolerance on solid agar plates revealed a six-fold higher tolerance of the natural isolate (6.0 mM) compared to the reference strain (1.0 mM). Previous studies using other isolated yeasts from Spanish clay deposit showed similar results. Two isolated strains from bentonite samples exhibit tolerances up to 10 mM for uranium [58]. In addition, also for chromium KS5 showed a distinct higher tolerance than the reference DSM 10134. An isolated strain of *Rhodosporidium* sp. from metallurgical wastes displayed a similar high resistance to chromium [59]. However, for copper, cadmium and zinc we could not see obvious differences between the natural isolate and the reference strain. Our investigations on uranium and selected heavy metals tolerance revealed a possible evolutionary adaption of KS5 to the surrounding environmental conditions. Compared to the reference strain, DMS 10134, the indigenous yeast exhibits tremendously high tolerances of uranium and chromium. To identify the molecular mechanisms behind these high tolerances gene expression analysis should be performed. Nevertheless, the results display the ability of KS5 to survive high concentrations of uranium as well as other heavy metals and thus, this indigenous yeast could play an important role of *in situ* bioremediation approaches of contaminated sites.

Uranium removal capacity of KS5

In order to determine the influence of KS5 on the immobilization capacity of uranium at natural conditions, uranium removal studies were performed. Uranium was removed rapidly from the surrounding solution within 24 h. After 48 h incubation, metal binding saturation by the cells was reached. The uranium removal capacity of the strain KS5 is a temperature-dependent process as was indicated in Fig. 3a. The cells were able to remove around 150 mg U/g dry biomass (dbm) from the surrounding solution at 30 °C, whereas at 4 °C the cells removed only around 75 mg U/g dbm, respectively. Experiments with heat-killed cells incubated at the same temperatures revealed that dead cells remove lower amounts of uranium compared to living yeast cells. Nevertheless, the amount of removed uranium of around 60 mg U/g dbm is almost equal to the amount for cells incubated at 4 °C. Furthermore, regarding to the percentage removal of uranium (Fig. 3b), living cells of KS5 removed nearly 100 % of soluble uranium from solution.

Table 3.2: Growth rate and doubling time of KS5 growing with different uranium concentrations

	Control	0.05 mM	0.1 mM
Growth rate μ [h^{-1}]	0.19	0.13	0.08
Doubling time t_d [h]	3.73	5.55	8.56

In contrast, only 40 % of uranium was removed by heat killed cells. These findings imply that uranium removal by KS5 cells is metabolism-dependent. Moreover, the temperature-dependent experiments indicate that the uranium removal is based on different interaction processes, probably passive biosorption and active bioaccumulation. In addition, the less amount of removed uranium by dead cells of KS5 could prove the occurrence of metabolically-dependent processes. The process of biosorption is rapid and will be not affected by temperature due to the metabolism-independent sorption of uranium on negatively charged groups of the cell membrane [60, 61]. In contrast, the mechanism of intracellular bioaccumulation of actinides especially uranium is poorly understood. However, previous studies assumed an active transport of uranium into the cells [62]. The same temperature-dependent and thus possibly metabolism-dependent process was observed by uranium interaction experiments on *A. facilis* [63]. Similar to KS5, the bacterial cells removed lower amounts of uranium at lower temperatures. Compared to other yeast cells, like *S. cerevisiae*, KS5 could remove distinct higher amounts of uranium from the surrounding solution [64]. Although the authors used lower concentrations of the metal, the studied strain removed only 40 % of the initial concentration, which correspondents to a uranium removal capacity of 17 mg U/g dbm. In addition, they investigated the removal capacity of living and dead cells for comparison purpose. On the contrary to our findings *S. cerevisiae* accumulated higher amounts of uranium onto dead cells [64]. Indicating again that active mechanisms, such as bioaccumulation, could be responsible for the uranium removal by living cells of KS5 further to the passive process of biosorption. STEM analyses (results discussed below) support this assumption. Fig. 4 shows the effect of biomass concentration of KS5, ranging from 0.05 to 0.24 mg/mL on uranium removal capacity. The results reveal that the uranium binding capacity of the isolate KS5 decrease with increasing the biomass concentration from 0.05 to 0.1 mg/mL, accumulating up to 350 and 175 mg U/g dbm, respectively. Afterwards, the equilibrium of uranium removal capacity was reached at around 150 mg U/g dbm and almost the complete amount of dissolved uranium was removed by the yeast cells. In addition, the removal capacity dependent on dry biomass displays the same result as the kinetic studies mentioned above. The ability to remove uranium was different at two tested temperatures. Cells, which were incubated at 4 °C removed much less uranium compared to cells at 30 °C. Even at the lowest initial dry biomass only around 80 mg U/g dbm was removed by the yeast cells. Compared with other fungal strains, KS5 displays a high capacity of uranium removal. All observed *Rhizopus* strains displayed a ca-

Table 3.3: MIC (mM) for KS5 and DSM 10134 on SDA medium 1:5 diluted

	Uranium	Chromium	Copper	Cadmium	Zinc
KS5	6.0	5.0	0.4	< 0.1	1.0
DSM 10134	1.0	1.0	0.3	< 0.1	0.7

MIC: concentration at which no growth occurred; n=3.

pacity between 180 and 260 mg U/g dbm [65]. In contrast to bacterial cell such as *Paenibacillus* sp. JG-TB8, which was recovered from a soil sample of another uranium mining site (Johanngeorgenstadt, Germany) and displayed a uranium removal capacity of 138 mg U/g dbm (at pH 4.5) [66], KS5 shows a higher capacity. Compared to the model organism *S. cerevisiae*, which displayed maximum biosorption quantity of 102 mg U/g dbm [67], KS5 was able to remove more than twice. The biosorption of heavy metals especially uranium by yeast cells was shown in previous studies and confirmed our investigations of actively intracellular uptake [62, 68].

Localization of removed uranium by TEM studies

Transmission electron microscopy analyses were performed to investigate the cellular localization of uranium accumulated by the cells of KS5, and to elucidate the possible uranium interaction mechanisms with this yeast. The temperature-dependent uranium removal capacity tests, conducted at 4 °C and 30 °C , suggest the implication of two possible interaction mechanisms, namely passive biosorption and active bioaccumulation. Fig. 5a shows a STEM image of KS5 incubated at 30 °C together with two EDX spectra obtained for two regions of metal accumulates (Figs. 5b and 5c) localized within the cells. Intracellular uranium is detected in the form of phosphorous-containing needle-like structures which are localized at the plasmatic membrane, as well as at the outer membrane of the nucleolus. Additionally, uranium is associated in lipid granules localized within the cytoplasm. Several studies showed that *R. toruloides* is known for overproduction of lipids and pigments [44, 69–71] and for the formation of lipid droplets which serve as energy reservoir [72]. To investigate the possible binding sites of uranium, further element distribution analyses were performed (Figs. 6a-d). The results clearly indicated the common presence of uranium together with phosphorus (needle-like structures) (Fig. 6b). In contrast, when cells incubated at 4 °C (Fig. S2), it is clearly visible that, considerably less uranium amount was immobilized by the cells. Uranium is localized only at the outer membrane and is not taken up into the cell. Due to the fact, that only cells which were incubated at 30 °C display intracellular accumulated uranium whereas cells incubated at 4 °C showed no uranium inside the cells, it could be assumed that uranium is actively accumulated. In comparison to other yeast strains, the same needle-like fibrils were observed [64]. In contrast to our results, however, the authors could not detect any uranium accumulates inside the yeast cells. Furthermore, Strandberg *et al.* 1981 [73] suggested no metabolisms-dependent uranium interaction mechanism by *S. cerevisiae* which seems to be in disagreement with the finding reported in the present work, where the cells of the strain KS5 interact with uranium in a metabolisms-dependent way due to the temperature-dependent uranium immobilization capacity. Other studies on naturally isolated yeast cells from Spanish clay deposits confirmed a similar uranium immobilization behavior [58]. Uranium was precipitated on the outer cell surface as well as intracellularly. In addition, also needle-like structures of the immobilized uranium could be observed and were identified as uranyl-phosphate

minerals with a structure similar to H-autunite [58]. Resulting from the TEM investigations, we confirmed our previous results and our hypothesis that uranium was immobilized by two different mechanisms, by active bioaccumulation and by passive biosorption.

Speciation of removed uranium by EXAFS analysis

EXAFS measurements were performed to determine and identify the structure of uranium immobilized by the isolated strain KS5. Fig. 7 displays the k^3-weighted EXAFS spectra and their corresponding Fourier transforms (FT) of the yeast cells contacted with 0.1 mM uranium at 30 °C for 48 h and of the meta-autunite reference. The FT signal of the uranium (U) interaction (Fig. 7, U_1) at 4.8 Å (not corrected for phase shift) was Fourier filtered in the R-interval of 4.53 Å - 5.18 Å for both samples. When comparing the signatures in the FT of the uranium solid formed upon contact with the KS5 strain (red traces) with the reference material meta-autunite (black traces), strong similarities can be seen, pointing toward the formation of a meta-autunite-like biomineral under the influence of the yeast cells. A strong indication of meta-autunite formation as a consequence of the biomineralization processes is the detected U-P interaction at R+ΔR = 2.9 Å and the U-U interactions at R+ΔR = 4.8 Å (U_1) and at R+ΔR = 6.8 Å (U_2) (Fig. 7). Moreover, the Fourier filtered U_1 signals are in phase (Fig. 7, left), showing that the radial U-U_1 distance is the same for both samples. Though, the presence of other uranium species in minor contributions cannot completely be excluded, since the spectrum of the yeast sample does not match exactly with that of meta-autunite. According to the TEM studies, uranium was mainly removed by bioaccumulation within the cytoplasm and boned via protonated phosphoryl containing groups. The formation of meta-autunite, as a response of uranium interaction with microorganisms, was mentioned by several further studies [19, 74, 75]. However, few studies with *R. toruloides* and meta-autunite formation are known. The removal of uranium by yeast cells and its resulting immobilization by formation of uranium minerals may play an important role in bioremediation of uranium contaminated sites due to their stability for long time periods [76].

Conclusion

Our present study describes the interaction mechanisms of KS5 with uranium(VI) and its tolerance to selected heavy metals. Uranium removal studies and TEM analyses revealed that the cells of the strain interact with uranium through a temperature-dependent process. For yeast cells incubated at 30 °C, intracellular uranium accumulates as needle-like structures were detected in the cytoplasm and also within lipid-granules, which might be a consequence of different detoxification mechanisms. Our findings confirm, that natural occurring microorganisms may play an important role in predicting the transport and fate of uranium at contaminated sites which could be used for *in situ* bioremediation.

Acknowledgments

This work was supported by the Bundesministerium für Bildung und Forschung (BMBF) grant № 02NUK030F (TransAqua). Further support took place by the ERDF-co-financed Grants CGL2012-36505 and 315 CGL2014-59616R (Ministerio de Ciencia e Innovación, Spain: 80 % funded by FEDER. The authors are grateful to the Wismut GmbH for allowing access to the sampling site, S. Röder for assistance in water sampling, S. Gurlit and S. Schubert for ICP-MS measurements, S. Weiß for his assistance during preparation of EXAFS samples, N. Huittinen for her support by the interpretation of the EXAFS data, and T. Schneider for his grammatical correction of this manuscript. We thank J. Lazuen-Alcon (Flow Cytometry Service, Centro de Instrumentación Científica University of Granada, Spain) for his help with flow cytometry measurements.

References

[1] M. Gavrilescu, L. V. Pavel and I. Cretescu, *„Characterization and remediation of soils contaminated with uranium“*, J Hazard Mater **163**, 475 (2009).

[2] J. R. Lloyd and L. E. Macaskie, *„Bioremediation of Radionuclide-Containing Wastewaters“*, in *Environmental Microbe-Metal Interactions*, P. 277–327 (2000).

[3] A. Meinrath, P. Schneider and G. Meinrath, *„Uranium ores and depleted uranium in the environment, with a reference to uranium in the biosphere from the Erzgebirge/Sachsen, Germany“*, J Environ Radioactiv **64**, 175 (2003).

[4] J. Schreyer and U. Zimmermann, in *Uranium, Mining and Hydrogeology II*, Ed. B. Merkel and C. Helling. Sven von Loga, Köln (1998).

[5] T. Arnold, T. Zorn, H. Zänker, G. Bernhard and H. Nitsche, in *J. Contam. Hydrol.*, Vol. 47, P. 219–231 (2001).

[6] K. Märten, JSH, Seeliger, D, Sommer, *„Uranium removal from flood water at Königstein mine“*, in *Uranium in the Aquatic Environment*, Ed. C. Merkel, B, Wolkersdorfer, P. 763–768, Springer (2002).

[7] S. J. Markich, *„Uranium Speciation and Bioavailability in Aquatic Systems: An Overview“*, Sci. World J. **2**, 707 (2002).

[8] W. M. Murphy and E. L. Shock, *„Environmental aqueous geochemistry of actinides.“*, Rev. Mineral. Geochem. **38**, 221 (1999).

[9] T. Arnold, N. Baumann, E. Krawczyk-Bärsch, S. Brockmann, U. Zimmermann, U. Jenk and S. Weiß, *„Identification of the uranium speciation in an underground acid mine drainage environment“*, Geochim. Cosmochim. Acta **75**, 2200 (2011).

[10] M. Barnett, P. Jardine, S. Brooks and H. Selim, *„Adsorption and Transport of Uranium(VI) in Subsurface Media“*, Soil Sci Soc Am J **64**, 908 (2000).

[11] M. C. Duff, J. U. Coughlin and D. B. Hunter, *„Uranium co-precipitation with iron oxide minerals“*, Geochim. Cosmochim. Acta (2002).

[12] M. L. Merroun, J. Raff, A. Rossberg, C. Hennig, T. Reich and S. Selenska-Pobell, *„Complexation of uranium by cells and S-layer sheets of Bacillus sphaericus JG-A12“*, Appl Environ Microb **71**, 5532 (2005).

[13] D. R. Brookshaw, R. A. D. Pattrick, J. R. Lloyd and D. J. Vaughan, *„Microbial effects on mineral-radionuclide interactions and radionuclide solid-phase capture processes“*, Mineral. Mag. **76**, 777 (2012).

[14] M. L. Merroun, C. Hennig, A. Rossberg, G. Geipel, T. Reich and S. Selenska-Pobell, *„Molecular and atomic analysis of uranium complexes formed by three eco-types of Acidithiobacillus ferrooxidans“*, Biochem Soc T **30**, 669 (2001).

[15] Y. Suzuki and J. F. Banfield, *„Resistance to, and accumulation of, uranium by bacteria from a uranium-contaminated site“*, Geomicrobiol J **21**, 113 (2004).

[16] D. R. Lovley, E. J. P. Phillips, Y. A. Gorby and E. R. Landa, *„Microbial reduction of uranium“*, Nature **350**, 413 (1991).

[17] D. R. Lovley and E. J. P. Phillips, *„Reduction of uranium by Desulfovibrio desulfuricans“*, Appl Environ Microb **58**, 850 (1992).

[18] L. E. Macaskie, D. T. Goddard, K. M. Bonthrone and P. Yong, *„Enzymically mediated bioprecipitation of uranium by a Citrobacter sp.: a concerted role for exocellular lipopolysaccharide and associated phosphatase in biomineral formation“*, Microbiology **146**, 1855 (2000).

[19] M. L. Merroun, M. Nedelkova, J. J. Ojeda, T. Reitz, M. L. Fernández, J. M. Arias, M. Romero-González and S. Selenska-Pobell, *„Bio-precipitation of uranium by two bacterial isolates recovered from extreme environments as estimated by potentiometric titration, TEM and X-ray absorption spectroscopic analyses“*, J Hazard Mater **197**, 1 (2011).

[20] B. J. Baker and J. F. Banfield, *„Microbial communities in acid mine drainage“*, FEMS Microbiol. Ecol. **44**, 139 (2003).

[21] I. Zirnstein, T. Arnold, E. Krawczyk-Bärsch, U. Jenk, G. Bernhard and I. Röske, *„Eukaryotic life in biofilms formed in a uranium mine“*, MicrobiologyOpen **1**, 83 (2012).

[22] I. Zirnstein, *„Charakterisierung der Mikroorganismen im sauren Grubenwasser des ehemaligen Uranbergwerks Königstein“*, PhD thesis, Technische Universität Dresden (2015).

[23] C. Acharya, P. Chandwadkar and C. Nayak, *„Unusual versatility of the filamentous, diazotrophic cyanobacterium Anabaena torulosa revealed for its survival during prolonged uranium exposure“*, Appl Environ Microb **83** (2017).

[24] N. B. Omar, M. L. Merroun, M. T. González-Muñoz and J. M. Arias, *„Brewery yeast as a biosorbent for uranium.“*, J Appl Microbiol **81**, 283 (1996).

[25] T. Wang, X. Zheng, X. Wang, X. Lu and Y. Shen, *„Different biosorption mechanisms of Uranium(VI) by live and heat-killed Saccharomyces cerevisiae under environmentally relevant conditions“*, J Environ Radioactiv **167**, 92 (2016).

[26] S. a. Churchill, J. V. Walters and P. F. Churchill, *„Sorption of Heavy Metals by Prepared Bacterial Cell Surfaces“*, J. Environ. Eng. **121**, 706 (1995).

[27] M. Spinti, H. Zhuang and E. M. Trujillo, *„Evaluation of Immobilized Biomass Beads for Removing Heavy Metals from Wastewaters“*, Water Environ Res **67**, 943 (1995).

[28] J. L. Zhou, *„Zn biosorption by Rhizopus arrhizus and other fungi“*, Appl Microbiol Biot **51**, 686 (1999).

[29] S. Zafar, F. Aqil and I. Ahmad, *„Metal tolerance and biosorption potential of filamentous fungi isolated from metal contaminated agricultural soil“*, Bioresource Technol **98**, 2557 (2007).

[30] S. Kumar, H. Kushwaha, A. K. Bachhawat, G. P. S. Raghava and K. Ganesan, *„Genome sequence of the oleaginous red yeast Rhodosporidium toruloides MTCC 457“*, Eukaryot Cell **11**, 1083 (2012).

[31] A. J. Muñoz, E. Ruiz, H. Abriouel, A. Gálvez, L. Ezzouhri, K. Lairini and F. Espínola, *„Heavy metal tolerance of microorganisms isolated from wastewaters: Identification and evaluation of its potential for biosorption“*, Chem Eng J **210**, 325 (2012).

[32] N. Rijal, *„Sabouraud Dextrose Agar (SDA): Principle, composition, uses and colony morphology“*, Microbeonline P. 2013–2016 (2015).

[33] Y. L. Tsai and B. H. Olson, *„Rapid method for direct extraction of DNA from soil and sediments“*, Appl Environ Microb **57**, 1070 (1991).

[34] C. L. Schoch, K. a. Seifert, S. Huhndorf, V. Robert, J. L. Spouge, C. A. Levesque and W. Chen, *„From the Cover: Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi“*, P Natl Acad Sci Usa **109**, 6241 (2012).

[35] T. White, T. Bruns, S. Lee and J. Taylor, *„Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics“*, P. 315–322, Academic Press, San Diego (1990).

[36] Sigma Aldrich, *„Spurr Low -Viscosity Embedding Kit“*, Technical report, St. Louis MO, USA ().

[37] W. Matz, N. Schell, G. Bernhard, F. Prokert, T. Reich, J. Claußner, W. Oehme, R. Schlenk, S. Dienel, H. Funke, F. Eichhorn, M. Betzl, D. Pröhl, U. Strauch, G. Hüttig, H. Krug, W. Neumann, V. Brendler, P. Reichel, M. a. Denecke and H. Nitsche, *„ROBL – a CRG beamline for radiochemistry and materials research at the ESRF“*, J Synchrotron Radiat **6**, 1076 (1999).

[38] E. Makarov and V. Ivanov, *„The crystalline structure of Ca(UO2)2(PO4)2 x 6H2O metaautunite“*, Doklady Akad. Nauk SSsR **132(3)**, 673 (1960).

[39] C. Hennig, P. J. Panak, T. Reich, A. Roßberg, J. Raff, S. Selenska-Pobell, G. Bernhard, H. Nitsche, W. Matz, J. J. Bucher, P. J. Panak and H. Nitsche, *„EXAFS investigation of uranium(VI) complexes formed at Bacillus cereus and Bacillus sphaericus surfaces“*, Radiochim Acta **89**, 625 (2001).

[40] T. Ressler, *„WinXAS: a program for X-ray absorption spectroscopy data analysis under MS-Windows.“*, J Synchrotron Radiat **5**, 118 (1998).

[41] A. Beopoulos, J. Verbeke, F. Bordes, M. Guicherd, M. Bressy, A. Marty and J. M. Nicaud, *„Metabolic engineering for ricinoleic acid production in the oleaginous yeast Yarrowia lipolytica“*, Appl Microbiol Biot **98**, 251 (2014).

[42] J. Blazeck, A. Hill, L. Liu, R. Knight, J. Miller, A. Pan, P. Otoupal and H. S. Alper, *„Harnessing Yarrowia lipolytica lipogenesis to create a platform for lipid and biofuel production.“*, Nat Commun **5**, 3131 (2014).

[43] F. Matthäus, M. Ketelhot, M. Gatter and G. Barth, *„Production of Lycopene in the non-carotenoid producing yeast Yarrowia lipolytica.“*, Appl Environ Microb **80**, 1660 (2013).

[44] C. Ratledge and J. P. Wynn, *„The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms“*, Adv Appl Microbiol **51**, 1 (2002).

[45] C. D. Rutter, S. Zhang and C. V. Rao, *„Engineering Yarrowia lipolytica for production of medium-chain fatty acids“*, Appl Microbiol Biot **99**, 7359 (2015).

[46] Z. Xue, P. L. Sharpe, S.-P. Hong, N. S. Yadav, D. Xie, D. R. Short, H. G. Damude, R. a. Rupert, J. E. Seip, J. Wang, D. W. Pollak, M. W. Bostick, M. D. Bosak, D. J. Macool, D. H. Hollerbach, H. Zhang, D. M. Arcilla, S. a. Bledsoe, K. Croker, E. F. McCord, B. D. Tyreus,

E. N. Jackson and Q. Zhu, *„Production of omega-3 eicosapentaenoic acid by metabolic engineering of Yarrowia lipolytica“*, Nat Biotechnol **31**, 734 (2013).

[47] S. Zhang, J. M. Skerker, C. D. Rutter, M. J. Maurer, A. P. Arkin and C. V. Rao, *„Engineering Rhodosporidium toruloides for increased lipid production“*, Biotechnol Bioeng **113**, 1056 (2016).

[48] R. R. Bommareddy, W. Sabra, G. Maheshwari and A.-P. Zeng, *„Metabolic network analysis and experimental study of lipid production in Rhodosporidium toruloides grown on single and mixed substrates.“*, Microb Cell Fact **14**, 36 (2015).

[49] S. Lane, S. Zhang, N. Wei, C. Rao and Y. S. Jin, *„Development and physiological characterization of cellobiose-consuming Yarrowia lipolytica“*, Biotechnol Bioeng **112**, 1012 (2015).

[50] H. Schneider, P. Y. Wang, Y. K. Chan and R. Maleszka, *„Conversion of D-Xylose into Ethanol by the Yeast Pachysolen-Tannophilus“*, Biotechnol Lett **3**, 89 (1981).

[51] T. W. Jeffries, *„Conversion of xylose to ethanol under aerobic conditions by Candida tropicalis“*, Biotechnol Lett **3**, 213 (1981).

[52] P. J. Slininger, R. J. Bothast, J. E. Van Cauwenberge and C. P. Kurtzman, *„Conversion of D-xylose to ethanol by the yeast Pachysolen tannophilus“*, Biotechnol Bioeng **24**, 371 (1982).

[53] A. Matsushika, H. Inoue, T. Kodaki and S. Sawayama, *„Ethanol production from xylose in engineered Saccharomyces cerevisiae strains: Current state and perspectives“*, Appl Microbiol Biot **84**, 37 (2009).

[54] G. Massaccesi, M. C. Romero, M. C. Cazau and A. M. Bucsinszky, *„Cadmium removal capacities of filamentous soil fungi isolated from industrially polluted sediments, in La Plata (Argentina)“*, World J. Microbiol. Biotechnol. **18**, 817 (2002).

[55] A. Malik, *„Metal bioremediation through growing cells“*, Environment International **30**, 261 (2004).

[56] L. Ezzouhri, E. Castro, M. Moya, F. Espinola and K. Lairini, *„Heavy metal tolerance of filamentous fungi isolated from polluted sites in Tangier, Morocco“*, Afr J Microbiol Res **3**, 35 (2009).

[57] H. Sakamoto, F, Nankawa, T, Ohnuki, T, Fujii, T, Iefuji, *„Yeast Genes Involved in Uranium Tolerance and Uranium Accumulation: A Functional Screening Using the Nonessential Gene Deletion Collection“*, Geomicrobiol J **29**, 470 (2012).

[58] M. López-Fernández, O. Fernández-Sanfrancisco, A. Moreno-García, I. Martín-Sánchez, I. Sánchez-Castro and M. L. Merroun, *„Microbial communities in bentonite formations and their interactions with uranium“*, Appl. Geochem. **49**, 77 (2014).

[59] M. Pepi and F. Baldi, *„Modulation of chromium(VI) toxicity by organic and inorganic sulfur species in yeasts from industrial wastes“*, Biometals **5**, 179 (1992).

[60] F. G. Ferris and T. J. Beveridge, *„Site specificity of metallic ion binding in Escherichia coli K-12 lipopolysaccharide.“*, Can J Microbiol **32**, 52 (1986).

[61] B. Volesky and Z. R. Holan, *„Biosorption of heavy metals“*, Biotechnol. Progr. **11**, 235 (1995).

[62] Y. Suzuki and J. F. Banfield, *„Geomicrobiology of uranium“*, Rev. Mineral. Geochem. **38 (1)**, 393 (1999).

[63] U. Gerber, I. Zirnstein, E. Krawczyk-Bärsch, H. Lünsdorf, T. Arnold and M. L. Merroun, *„Combined use of flow cytometry and microscopy to study the interactions between the*

gram-negative betaproteobacterium Acidovorax facilis and uranium(VI)", J Hazard Mater **317**, 127 (2016).

[64] X. Lu, X. jiao Zhou and T. shan Wang, *„Mechanism of uranium(VI) uptake by Saccharomyces cerevisiae under environmentally relevant conditions: Batch, HRTEM, and FTIR studies"*, J Hazard Mater **262**, 297 (2013).

[65] M. E. Treen-Sears, B. Volesky and R. J. Neufeld, *„Ion exchange/complexation of the uranyl ion by Rhizopus biosorbent"*, Biotechnol Bioeng **26**, 1323 (1984).

[66] B. Allard, H. Boren, C. Pettersson and G. Zhang, *„Degradation of humic substances by UV irradiation"*, Environ Int **20**, 97 (1994).

[67] M. Liu, F. Dong, X. Yan, W. Zeng, L. Hou and X. Pang, *„Biosorption of uranium by Saccharomyces cerevisiae and surface interactions under culture conditions"*, Bioresource Technol **101**, 8573 (2010).

[68] A. Griffiths, H.B. and Greenwood, *„The Concentric Bodies of Lichenized Fungi"*, in *Arch. Mikrobiol.*, P. 285–302, Springer Verlag, 87. Ed. (1972).

[69] Y. Li, Z. K. Zhao and F. Bai, *„High-density cultivation of oleaginous yeast Rhodosporidium toruloides Y4 in fed-batch culture"*, Enzyme Microb Tech **41**, 312 (2007).

[70] S. Wu, C. Hu, G. Jin, X. Zhao and Z. K. Zhao, *„Phosphate-limitation mediated lipid production by Rhodosporidium toruloides"*, Bioresource Technol **101**, 6124 (2010).

[71] P. Buzzini, M. Innocenti, B. Turchetti, D. Libkind, M. van Broock and N. Mulinacci, *„Carotenoid profiles of yeasts belonging to the genera Rhodotorula, Rhodosporidium, Sporobolomyces, and Sporidiobolus."*, Can J Microbiol **53**, 1024 (2007).

[72] Z. Zhu, Y. Ding, Z. Gong, L. Yang, S. Zhang, C. Zhang, X. Lin, H. Shen, H. Zou, Z. Xie, F. Yang, X. Zhao, P. Liu and Z. K. Zhaoa, *„Dynamics of the lipid droplet proteome of the oleaginous yeast Rhodosporidium toruloides"*, Eukaryot Cell **14**, 252 (2015).

[73] G. W. Strandberg, S. E. Shumate and J. R. Parrott, *„Microbial cells as biosorbents for heavy metals: Accumulation of uranium by Saccharomyces cerevisiae and Pseudomonas aeruginosa"*, Appl Environ Microb **41**, 237 (1981).

[74] E. Krawczyk-Bärsch, L. Lütke, H. Moll, F. Bok, R. Steudtner and A. Rossberg, *„A spectroscopic study on U(VI) biomineralization in cultivated Pseudomonas fluorescens biofilms isolated from granitic aquifers"*, Environ Sci Pollut R **22**, 4555 (2015).

[75] F. Jroundi, M. L. Merroun, J. M. Arias, A. Rossberg, S. Selenska-Pobell and M. T. González-Muñoz, *„Spectroscopic and microscopic characterization of uranium biomineralization in Myxococcus xanthus"*, Geomicrobiol J **24**, 441 (2007).

[76] J. L. Jerden and A. K. Sinha, *„Phosphate based immobilization of uranium in an oxidizing bedrock aquifer"*, Appl. Geochem. **18**, 823 (2003).

Figures

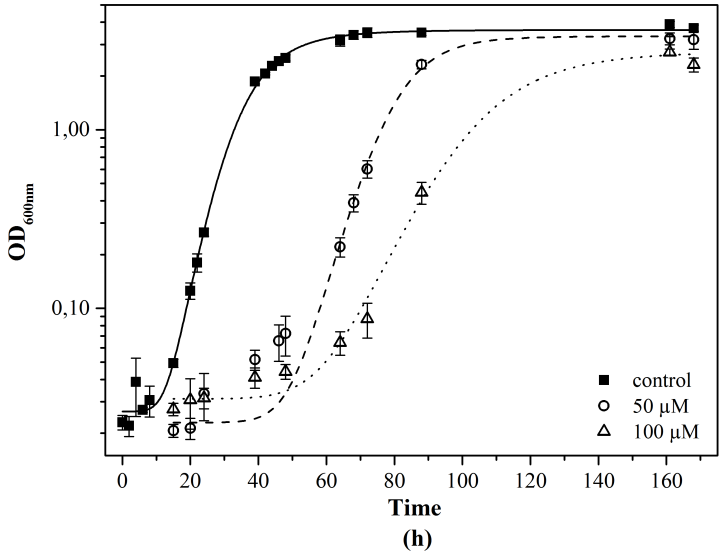


Figure 1: Growth curves of KS5 incubated with different uranium concentrations, the cells were grown in liquid SDA 1:5, uranium was added in different concentrations, squares no uranium, circles 0.05 mM uranium and triangle 0.1 mM uranium, incubation for 172 h at 30 °C; n=3, error bars indicate standard deviation.

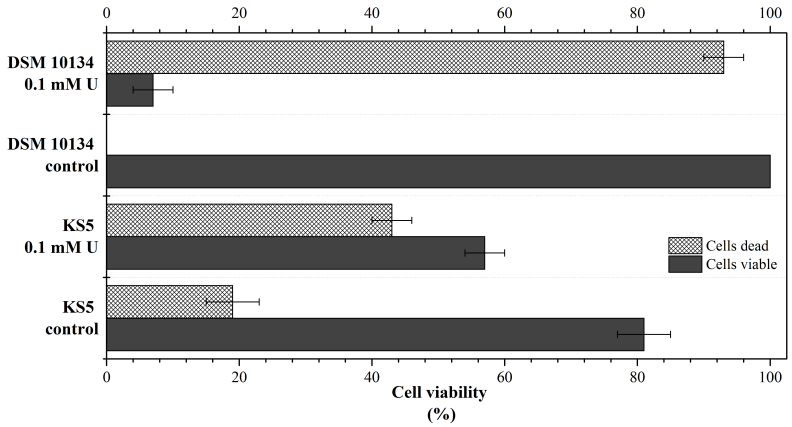


Figure 2: Percentage distribution of viable and dead cells using flow cytometry, cells of KS5 and DSM 10134 were incubated with 0.1 mM uranium or without (control) in 1:5 diluted SDA medium for 24 h at 30 °C. Cells were stained with FDA and PI for cell viability test. Viable (living cells) are displayed in dark grey and dead cells in grey pattern; n=3, error bars indicate standard deviation.

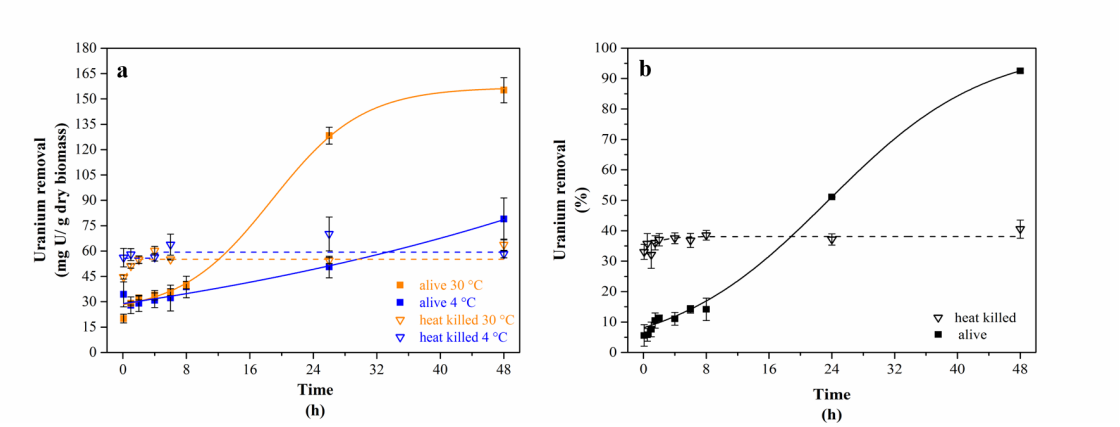


Figure 3: Temperature-dependent uranium removal capacity of KS5 (a) Uranium removal relating to dry biomass, orange curves show incubations at 30 °C and blue curves at 4 °C, filled scares display living cells (solid lines) and open triangles display heat killed cells (dashed lines); (b) Percentage uranium removal of living cell (solid line and filled scares) and heat killed cells (dashed line and open triangles) at 30 °C; initial uranium concentration 0.1 mM; background solution tap water pH 5.0; n=3, error bars indicate standard deviation.

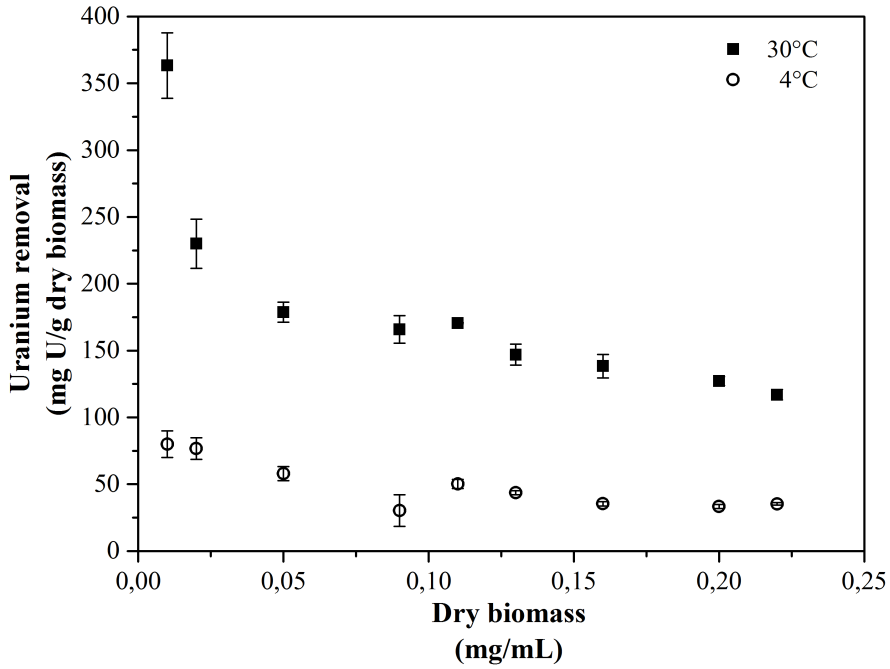


Figure 4: Uranium removal capacity by KS5 as function of the initial dry biomass, initial uranium concentration 0.1 mM, background tap water pH 5.0, incubation time 48 h at 30 °C (filled squares) and 4 °C (open circles); n=3, error bars indicate standard deviation.

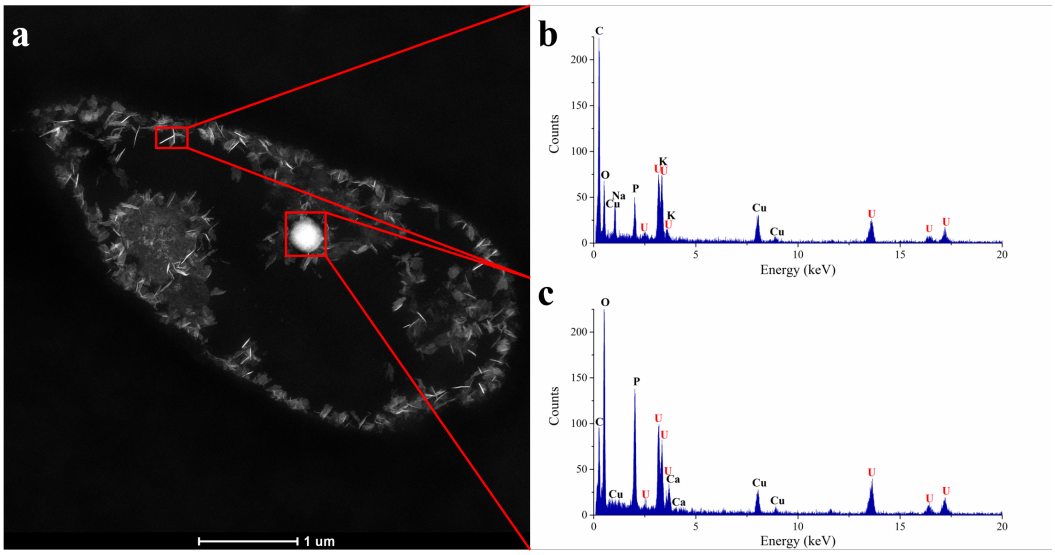


Figure 5: STEM-EDXS analysis of an ultrathin-sectioned KS5 sample after uranium removal experiments at 30 °C for 48 h (a) HAADF-STEM micrograph together with (b) EDX spectra of a needle-like structure localized at the inner cytoplasm-membrane, and (c) of immobilized uranium localized within lipid granules. The characteristic peaks of copper in the EDX spectra are caused by fluorescence excitation of the TEM support grid.

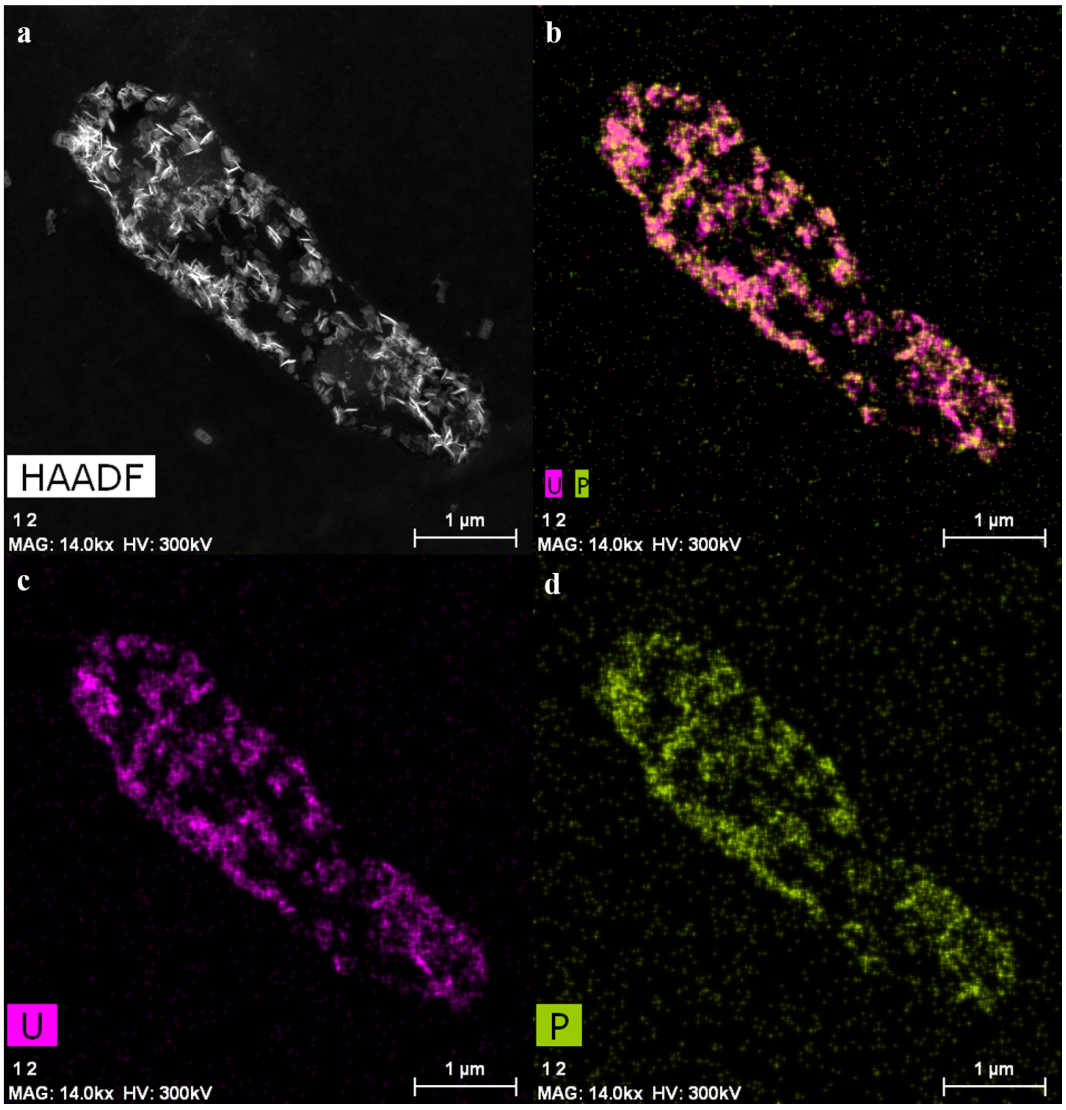


Figure 6: HAADF-STEM analysis of an ultrathin-sectioned KS5 sample after uranium removal experiments at 30 °C for 48 h (a) HAADF-STEM micrograph together with distribution analysis of (b) uranium (purple) and phosphorus (green), (c) uranium, and (d) phosphorus.

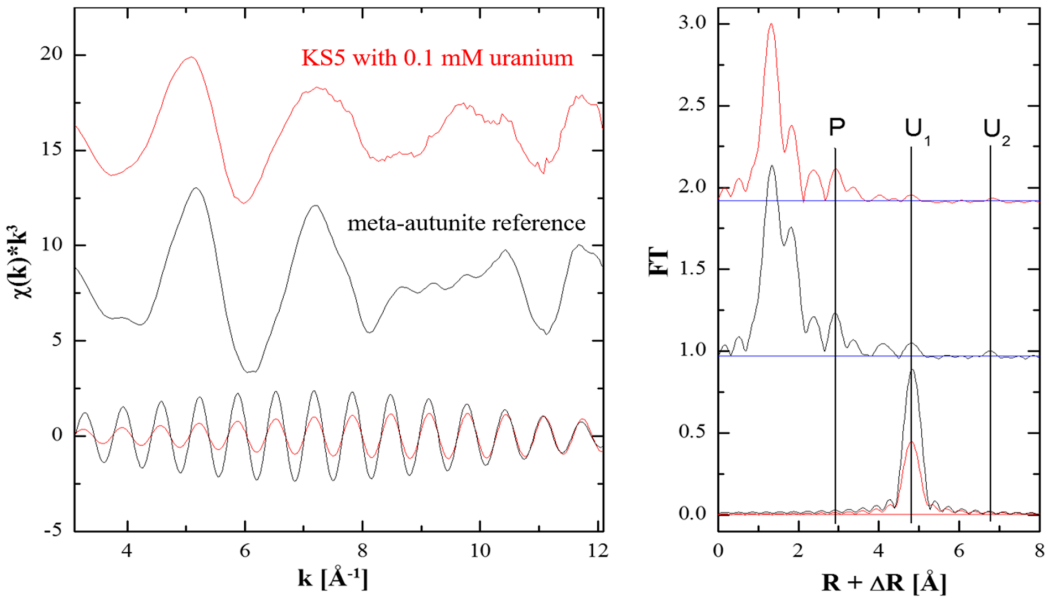


Figure 7: ULIII EXAFS spectra (left) and corresponding Fourier transform (FT) (right), KS5 was incubated with 0.1 mM uranium at 30 °C for 48 h (shown in red), The reference meta-autunite (shown in black) together with the Fourier filtered uranium interaction at 4.8 Å (bottom). The noise level (background) was determined from the FT peak magnitude in the 15-20 Å R-region, where no significant signal from the sample itself is expected (blue line).

Supplementary Information

Metabolism-dependent bioaccumulation of uranium by *Rhodosporidium toruloides* isolated from the flooding water of a former uranium mine

Ulrike Gerber[1,*,#], René Hübner[2], André Rossberg[1,3], Evelyn Krawczyk-Bärsch[1], Mohamed L. Merroun[4]

[1] Helmholtz-Zentrum Dresden - Rossendorf, Institute of Resource Ecology, Bautzner Landstraße 400, 01328 Dresden, Germany

[2] Helmholtz-Zentrum Dresden - Rossendorf, Institute of Ion Beam Physics and Materials Research, Bautzner Landstraße 400, 01328 Dresden, Germany

[3] Rossendorf Beamline at ESRF - The European Synchrotron, CS40220, 38043 Grenoble Cedex 9, France

[4] University of Granada, Department of Microbiology, Campus Fuentenueva, E-18071 Granada, Spain

[#] Present Address: Faculty of Natural Sciences, Brandenburg University of Technology Cottbus-Senftenberg, Universitätsplatz 1, 01968 Senftenberg, Germany

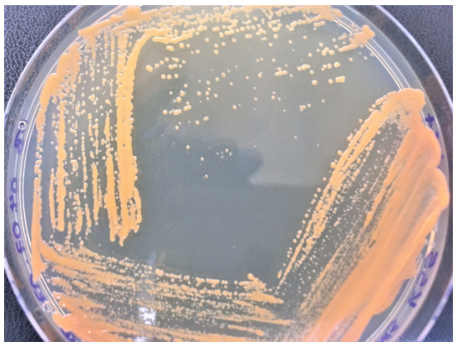


Figure S1: Red colonies of KS5, singe colonies appearing on solid SDA agar plates, incubation at 30 °C for 48 hours.

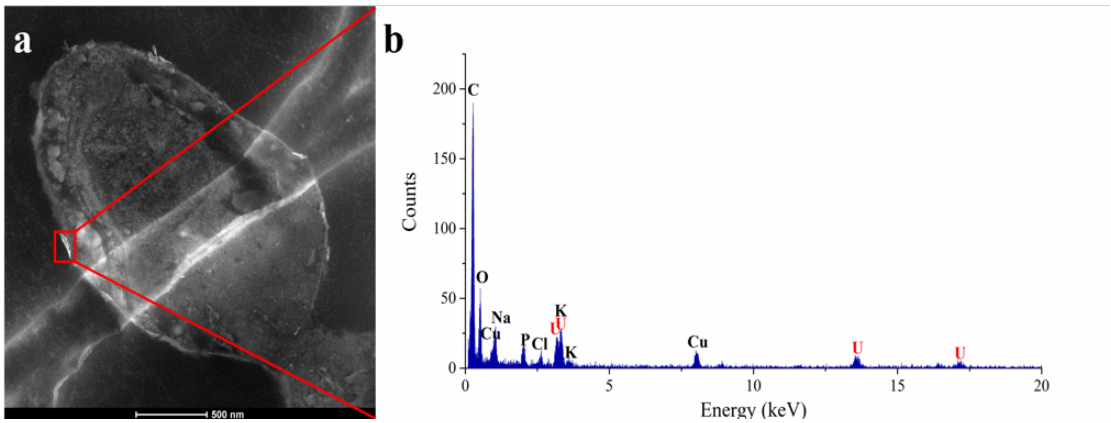


Figure S2: STEM-EDXS analysis of an ultrathin-sectioned KS5 sample after uranium removal experiments at 4°C for 48 h (a) HAADF-STEM micrograph together with EDX spectrum (b) of a needle-like structure localized at the outer cytoplasm membrane.

3.4 Microbial mediated uranium(VI) reduction within the flooding water of a former uranium mine - a possible bioremediation approach

Gerber U, Schäfer S, Röder G, Lehmann S, Zirnstein I, Krawczyk-Bärsch E, Rossberg A

In preparation for submission

Microbial mediated uranium(VI) reduction within the flooding water of a former uranium mine - a possible bioremediation approach

Ulrike Gerber[1,*,#], Sebastian Schäfer[1,2], Grit Röder[3], Isabel Zirnstein[1,†], Susanne Lehmann[1] Evelyn Krawczyk-Bärsch[1], André Rossberg[1]

[1] Helmholtz-Zentrum Dresden - Rossendorf, Institute of Resource Ecology, Bautzner Landstraße 400, 01328 Dresden, Germany

[2] Technical University Dresden, Faculty of Biology, Institute of Microbiology, Chair of Molecular Biotechnology, 01062 Dresden, Germany

[3] Interdisciplinary Ecological Center, TU Bergakademie Freiberg, Leipziger Strasse 29, 09599 Freiberg, Germany

[#] Present Address: Faculty of Natural Sciences, Brandenburg University of Technology Cottbus-Senftenberg, Universitätsplatz 1, 01968 Senftenberg, Germany

[†] Present Address: Research Institute of Leather and Plastic Sheeting (FILK) gGmbH, Meissner Ring 1-5, 09599 Freiberg, Germany

[*] Corresponding author
E-Mail: gerberu@b-tu.de (UG)
Telephone: +49 3573 85 974
Fax: +49 3573 85 909

Abstract

This study investigated the sustainability of microbial mediated uranium(VI) reduction in the flooding water of a former uranium mining site located in Germany, for possible applications of bioremediation. Presently, the site of the former uranium mine is remediated by an intensive and time-consuming waste water treatment, due to the fact that the underground is flooded in a controlled way. The task for the next years is to find alternative approaches which combine cost efficiency, environmental friendliness and minimizing time effort. We examined the ability of anaerobic bacteria, natural occurring within the flooding water, for their uranium(VI) reduction capability. Therefore, the electron donor and carbon source glycerol was added, to stimulate microbial growth. During six weeks of incubation, analytical techniques, like measurement of

redox potential, pH, uranium redox state, and iron and sulfate concentration, were performed. A distinct decrease of the redox potential was observed after six weeks of incubation time. In combination with XANES (X-ray absorption near edge structure) a complete reduction of uranium(VI) to uranium(IV) was detected. Furthermore, the underlying molecular mechanisms of the microbial uranium(VI) reduction and the bacterial diversity after six weeks incubation were investigated. To verify the applicability for industrial scale bioremediation approaches, a pilot plant was designed and the obtained results from laboratory scale experiments were successfully repeated. We could demonstrate that the results achieved in the laboratory scale samples are transferrable to industrial scale applications and thus, could be used for potential *in situ* bioremediation approaches of former uranium mining sites.

Introduction

The presence of high levels of uranium at contaminated sites, resulting from former uranium mining activities in the last century, represent a major concern due to its potential for mobilization and dissolution in the surrounding environment. Studies demonstrated that toxic radionuclides have migrated into groundwater for decades, which leads to a serious threat to the environment and public health [1]. Consequently, the main objective in the last years is the remediation of heavy metal contaminated sites. However, clean-up approaches for these areas are projected to be expensive and to last many decades. One of numerous former uranium mining sites in Germany represents the uranium mine in Königstein, which is currently in the remediation process. Therefore, the underground of the mine is flooded in a controlled way and the flooding water is pumped to the surface. In following steps, the flooding water is treated by a conventional chemical waste water treatment plant [2]. Traditional remediation methods of groundwater by pump-and-treat, followed by ion exchange, seems to be elaborated and cost-prohibitive. For that reason, *in situ* bioremediation could be a possible alternative, by using indigenous microorganisms to clean-up contaminated sites [3]. Moreover, previous studies demonstrated that bioremediation provides an efficient, cost-effective, and environmental-friendly technology [4].

As a consequence of the leaching process with sulfuric acid in the last years of uranium mining activity, the flooding water still displays a low pH (~ 3.0) and contains high concentrations of sulfate (~ 1.0 g/L). In addition to the high uranium concentration present within the flooding water (8 mg/L), also other heavy metals display high concentration levels. Taken as a whole, these conditions characterize this site as an acid mine drainage (AMD). AMD sites often occur in the mining industry and represent a major problem, that poses an additional risk to the environment due to the fact that they contain elevated concentrations of metals and metalloids. The major cause of the development of AMD sites is the accelerated oxidation of iron pyrite (FeS_2 and sulphidic minerals through the contact with oxygen and water, as a result of the mining processes [5].

However, AMD waters are known for their abundant microbial diversity [6, 7]. The microorganisms inhabiting such harsh environments are extremophile microorganisms and known for their specific characteristics. Thus, they can be efficient in removing radioactive pollutants from the environment and therefore, could be used for industrial processes, bioremediation, and discovery of new biosorbents [8]. Microorganisms are able to interact with metals and radionuclides, like uranium, in various ways. Several studies were performed investigating immobilization of metals by biosorption and bioaccumulation [9, 10]. Biosorption is defined as a passive process, whereby the metal is bound on functional groups of the outer membrane of microorganisms. It is independent of metabolic activity and therefore, nutrients are not required [11]. In contrast, bioaccumulation is considered to be an active process. The metal is taken up into the cytoplasm of the cells. In addition, also biomineralization is a known active process. Due to enzymatically mediated mechanisms, uranium is transformed to insoluble non-oxide minerals, usually metal phosphates [12, 13]. However, uranium(VI) could additionally serve as an electron acceptor for redox reactions of certain anaerobic metal and sulfate reducing bacteria (MRB/SRB) [3, 14–16]. The reduction of uranium is defined as the process, by which microorganisms chemically reduce uranium from the highly soluble oxidation state VI by a two-electron process to the less soluble oxidation state IV. Consequently, this fact leads to the general hypothesis, that uranium reduction displays a high potential for bioremediation or immobilization strategies [17]. The stimulation of microbial activity to fix aqueous uranium(VI) into insoluble minerals *in situ* seems to provide a suitable approach, due to its relatively inexpensive and non-destructive characteristics [18]. In contrast, uranium(IV) can serve as an electron donor with nitrate as electron acceptor [19]. Both described processes are called biotransformation [3, 20, 21].

Moreover, the transport properties of uranium in natural environments are governed by contrasting chemistry behavior of the both oxidation states uranium(VI) and uranium(IV). In solutions and under oxidizing conditions uranium(VI) generally forms highly mobile complexes with carbonate and hydroxide. On the contrary, uranium(IV) precipitates in solution under anoxic conditions are highly insoluble, and thus immobile minerals, such as uraninite [21]. Nevertheless, the uranium mobility dependents on other factors as well, like pH and prevalent minerals [22–25].

In this study, we investigated the capability of anaerobic microorganisms, naturally occurring within the flooding water, for the uranium(VI) reduction. Previous studies investigating the microbial diversity in the flooding water, revealed the presence of anaerobic MRB, like sulfate (SRB) and iron reducing bacteria (IRB) [26]. To stimulate the microbial activity, we added 10 mM glycerol to the flooding water and incubated the samples over six weeks. During incubation time, the redox potential was periodically measured, to investigate microbial induced redox reactions. Furthermore, samples were analyzed using XANES (X-ray absorption near edge structure) spectroscopy to determine the redox state of uranium. Beside uranium, also the reduction of iron and sulfate were investigated to obtain a detailed view of the microbial mediated redox reactions and furthermore, to understand which MRB are mediators for the microbial uranium(VI) reduc-

tion. Therefore, additional analyses based on 16S rDNA were performed to examine the bacterial diversity within the flooding water after six weeks incubation time and successful uranium(VI) reduction. We were able to induce a microbial uranium(VI) reduction within the flooding water in laboratory scale experiments (1 L) by adding the electron donor glycerol. With the obtained knowledge, a pilot plant (100 L) was designed to confirm these findings and to test the applicability of microbial mediated uranium(VI) reduction, by adding glycerol to the flooding water, in industrial scale applications. The investigated microbial uranium(VI) reduction in the flooding water of the former uranium mining site in Königstein could be used as *in situ* bioremediation to support the conventional water treatment, and in the future replace the elaborated chemical remediation techniques.

Methods and Materials

Sampling site

The Königstein mine is an inactive uranium mine located in Saxony (Germany). The investigated area represents a very special case due to its localization in an ecological sensitive and dense populated area (50°54'54.1"N 14°01'42.0"E). The ore body is located in the 4th sandstone aquifer. However, the overlaying 3rd aquifer serves as an important water reservoir for the nearby cities. A contamination of this environmental sensitive aquifer would have tremendous consequences for the drinking water in this area

Sampling campaign

Flooding water samples were sampled directly from the borehole at the Königstein site into sterile 1 L glass serum bottles. The bottles were directly sealed with butyl rubbers and aluminum caps. Immediately after the sampling campaign, the water within the sealed serum bottles was sterile gasified with N_2. Until further applications, the water samples were stored at 4 °C in the dark. For the realization of the pilot plant, 100 L flooding water were sampled into sterile 10 L plastic canisters. The samples were immediately transferred to the laboratory and were filled into the pilot plant. Afterwards the 100 L flooding water were gasified with N_2. The lid and all other vents were sealed airtight.

Uranium(VI) reduction experiments

To investigate the possible uranium(VI) reduction, 10 mM glycerol (0.92 g/L) as electron donor and carbon source was added sterile and under anoxic conditions to the flooding water samples. Afterwards the samples were incubated for six weeks at 30 °C in the dark. The control samples

were either incubated without the addition of glycerol or as sterile control, whereby the flooding water was immediately sterile filtrated through a 0.2 µm sterile filter. Subsequently, the sterile samples were treated like described above. Three times a week samples were taken within an anaerobic chamber to measure the redox potential (WTW SenTix® ORP Xylem Analytics Germany Sales GmbH & Co. KG, Weilheim, Germany) and the pH value (WTW SenTix® Mic, Xylem Analytics Germany Sales GmbH & Co. KG, Weilheim, Germany). In addition, once a week a sample was prepared for XANES analyses, as describes below.
In addition, to examine a possible uranium(VI) reduction in field scale, 100 L flooding water within the pilot plant were treated in same way, except for the incubation temperature. The pilot plant was incubated at room temperature (20 °C) over six weeks. The monitoring of pH, temperature and redox potential took place by an on-line measurement system. The electrodes were directly installed into the lid of the pilot plant. Furthermore, samples were regularly taken, using a N_2-flushed sterile syringe, to examine the redox state of uranium with UV-vis spectroscopy (Lambda 750 Uv/vis/NIR Spectrophotometer, PerkinElmer, USA), the Fe(II) and moreover, the Fe(II) concentration and sulfate concentration.

DNA extraction, amplification, and phylogenetic analysis

For the investigation of the microbial diversity of the laboratory scale experiments (1 L), two samples were pooled, respectively. The DNA extraction, amplification and purification of the 16S rDNA gene was performed by Blue Biolabs GmbH (Germany). In addition, the sequencing and data evaluation were completed by the same company.
In comparison, to investigate the microbial diversity of the pilot plant (100 L) after six weeks incubation, the DNA was extracted and purified using the alkine lyse method [27]. Therefore, the water sample was sterile filtered (0.2 µm), and the remaining biomass from the filter was washed and suspended with 0.05 M oxalic acid. The extracted DNA was purified and concentrated with DNA Clean & Concentrator-5 Kit (Zymo Research, USA). Subsequently, a PCR (polymerase chain reaction) was performed for 16S rDNA gene amplification. The obtained PCR product was purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA). Cloning and transformation of the PCR product were performed using TOPO TA Cloning Kit for Sequencing (Thermo Fisher Scientific Inc., USA). By colony PCR, positives clones were selected and PCR products were sequenced by GATC Biotech AG (Eurofins Genomics, Germany). The obtained sequence information was identified by the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI).

XANES (X-ray absorption near edge structure) measurements and data evaluation

To investigate the oxidation state of uranium within the samples, incubated for six weeks, XANES measurements were performed. Therefore, every week a sample (1 L) was centrifuged for 20 min at 20 000 rpm at room temperature. The obtained pellet was resuspended in 15 mL of the supernatant and ultra-centrifuged for 1 h at 187 000 × g. The formed pellet was filled into a polyethylene sample holder. The sample holder was hot sealed and immediately frozen in liquid nitrogen. All steps were performed under anoxic conditions to avoid uranium reoxidations with oxygen. In addition, reference samples of uranium(VI) and uranium(IV) were prepared with the flooding water as background solution. XANES measurements were carried out at the Rossendorf Beamline BM20 located at the European Synchrotron Radiation Facility (ESRF) [28]. The samples were measured at 15 K in a closed-cycle helium cryostat in fluorescence mode using a 13-element germanium solid-state detector. The X-ray beam was monochromatized by a silicon (111) double-crystal monochromator in channel cut mode (5-35 keV). For energy calibration of the sample spectra, the K-edge spectrum of an yttrium metal foil (first inflection point at 17038 eV) was recorded simultaneously. The ionization energy, E_0, of the uranium L_{III}-edge was defined as E_0 = 17185 eV [28].
The average oxidation state of uranium in the flooding water samples was determined by fitting the XANES region by a linear combination of standard spectra, obtained from the two references samples of uranium(IV) and uranium(VI) using ITFA (iterative target factor analyses) [29].

Analytical techniques

After determination of the pH, the samples were acidified and investigated regarding their chemical composition. For inorganic elements, inductively coupled plasma spectroscopy (ICP-MS) (ELAN 9000 type ICP-MS spectrometer, Perkin Elmer, Überlingen, Germany) and atomic absorption spectroscopy (AAS) (Perkin Elmer 4100 AAS, Überlingen, Germany) were used. Anion concentration was determined by Ion Chromatography (IC-system 732/733, Metrohm, Filderstadt, Germany). In addition, the total organic carbon (TOC) was analyzed with HT1300-TOC (Analytik Jena, Jena, Germany). The concentrations of sulfate and iron(II) were measured by precipitation of sulfate with barium chloride according to Kolmert *et al.* [30] and by a ferrozine assay according to Viollier *et al.* [31] (spectrophotometer µQuant™ of Bio-Tek Instruments, Inc., USA). All dilutions were prepared with two-times distilled water. All samples were determined in triplicates.

Development of a pilot plant for industrial scale applications

To transfer the obtained results from laboratory scale to industrial applications, a pilot plant with a total capacity of 100 L was designed (Fig. 1). A glass tank with 330.0 to 405.0 mm diameter and 880.0 mm height (glass wall thickness 20 mm) was manufactured by Marcel Hellbach (Glasbläserei Hellbach, Brand-Erbisdorf, Germany). The lid (545.0 mm diameter, 13.0 mm thickness) was designed removable. For on-line measurements of pH, redox potential, temperature and for sampling, devices were placed in the lid. The pilot plant was located in the laboratory without additional heating and exclusion of light. Two independent runs of the pilot plant with freshly sampled flooding water were performed, respectively.

Results and discussion

Characteristics of the flooding water samples

Prior to the investigations of a possible microbial uranium(VI) reduction, the flooding water samples were characterized using different analytical techniques (Tab. 1). In comparison with previous results [26, 32], the pH value shows no drastic changes during the last years of remediation. As a consequence of the *in situ* leaching with sulfuric acid the pH is acidic (2.8 - 3.0). Due to the ongoing flooding process, oxidizing conditions are prevalent. The redox potential (600 mV - 670 mV) and the oxygen concentration (2.3 mg/L - 3.7 mg/L) displayed no changes since 2011. However, the underground of the former mine is not completely mixed, for that reason one cannot rule out the possibility of areas which could present reducing conditions. During the last years, the metal and trace element concentrations fluctuate considerably. Until 2011, the concentrations are still increasing tremendously for almost all elements. This phenomenon could be explained by additional flooding steps of underground areas (in 2010). Nevertheless, due to the constantly flooding with fresh water during the last years the overall concentrations are decreasing, except for lead. However, in particular the uranium concentration with 8 mg/L is still high and represents the highest risk for possible contamination of the surrounding environment. Together with high concentrations of sulfate (1470 mg/L), iron (56.2 mg/L - 112.0 mg/L), low pH value and other heavy metals, all the mentioned parameters represent a typical acid mine drainage (AMD) site [33].

For microbial activity and metabolism, the total organic carbon (TOC) represents the limiting factor, which is low within the flooding water (1.1 mg/L). AMD sites usually contain a low organic carbon concentration and thus, the addition of extra electron donors is required. At acidic conditions the inhibitory effect of potential substrates should be taken into consideration [34]. Therefore, to stimulate the microbial activity for possible redox reactions and specifically, for uranium(VI) reduction, the electron donor glycerol was added to the flooding water samples.

Previous studies could demonstrate that at acidic conditions the reduction of metals, as well as sulfate, could be affected by different factors [34]. For instance, organic acids could act inhibitory at acidic conditions. The toxicity of these is dependent on their dissociation constants thus, at pH 3.0 half of the concentration of lactate acid would be present in the acid form (undissociated), which is able to diffuse into the cell. Inside the cytoplasm protons would be released, which cause a lowering of the intracellular pH value. Consequently, the protons have to be actively pumped out of the cell [34]. Therefore, glycerol, as well as other non-ionic substrates, like hydrogen, sugars and other alcohols, represents a suitable and cost-effective electron donor, which are preferred at acidic conditions [35–37]. Several SRB, like *Desulfovibrio* spp. and *Desulfosporosinus* spp., were reported to grow with glycerol as carbon and energy source [34, 38–42].

Microbial uranium(VI) reduction

To investigate the possible uranium(VI) reduction ability of the indigenous anaerobic microorganisms within the flooding water, the electron donor and carbon source glycerol was added. The flooding water samples were therefore flushed with N_2 and incubated at 30 °C to achieve

Table 1: Physical-chemical Parameters of the flooding water. Alteration of the parameters prevalent in the flooding water during remediation over six years.

	2011 [26]	2013 [26]	2015	2017
pH	3.3	3.0	2.8 - 2.9	2.8 – 2.9
Redox potential [mV]	600	640	670	650
O_2 [mg/L]	2.3	3.0	3.7	n.d.
Temperature [°C]	13.5	13.8	13.0	14.0
Na [mg/L]	32.8	108.3	91.3	45.6
SO_4^{2-} [mg/L]	489.8	973.1	1470.0	n.d.
U [mg/L]	6.1	7.8	14.0	8.0
TOC [mg/L]	0.8	0.3	1.1	1.1
Fe [mg/L]	91.6	56.2	122.0	62.5
K [mg/L]	12.5	8.8	7.7	8.6
Mg [mg/L]	8.0	15.4	31.8	18.5
Ca [mg/L]	91.6	246.5	304.0	207.0
Mn [mg/L]	2.5	7.2	8.9	8.4
Cd [µg/L]	19.5	73.4	112.0	62.1
Co [µg/L]	114.7	181.0	292.0	n.d.
Cr [µg/L]	16.3	35.5	75.0	n.d.
Cu [µg/L]	6.9	41.0	86.0	75.8
Pb [µg/L]	79.2	304.0	654.0	917
Ni [µg/L]	242.5	454.1	617.0	384
Zn [mg/L]	3.5	5.7	16.2	9.2

n.d. not determined

reducing and anaerobic conditions. To verify the adjusting reducing conditions, the redox potential was measured. In Fig. 2 the changes of the redox potential during six weeks of incubation time is displayed. A distinct decrease of the redox potential from initially 670 mV to 560 mV was detected after 14 days. In comparison, the sterile control without bacteria, but added with glycerol, displayed no change. After 35 days the equilibrium of around 230 mV was reached. Notably, the sterile control displayed no change during the whole incubation time, consequently the addition of glycerol has no influence on the decrease of the redox potential and the acquired reducing conditions. Within six weeks of incubation time, an absolute change of 440 mV was detected. Theoretical calculations of the uranium speciation and redox state (data not shown) using the experimental conditions (including ion concentrations, pH value, redox potential, and temperature) indicated a uranium reduction. At the detected redox potential of 230 mV after six weeks incubation, uranium(IV) is theoretically predicted.

On this basis, XANES spectroscopy was performed. Therefore, anoxic flooding water samples were prepared and investigated regarding their uranium redox states (Fig. 3). After five weeks of incubation time at 30 °C with 10 mM glycerol, a distinct shift of the spectrum was detected (Fig 3, grey area). Furthermore, compared to the uranium(VI) reference sample, the 'typical' uranium(VI) shoulder disappeared (Fig. 3, arrow). Thus, with XANES spectroscopy a microbial mediated uranium(VI) reduction after five weeks was demonstrated. To quantify the prevalent uranium(VI) and uranium(IV) concentrations during six weeks of incubation time, ITFA (iterative target factor analyses) were performed [29]. By comparison of the obtained spectra with the two prepared references samples, it was possible to determine the relative uranium oxidation state concentrations (Fig. 4). As shown in Fig. 4, after five weeks of incubation approximately 50 % of uranium(VI) was reduced to uranium(IV), indicating the microbial reduction of uranium. During the previous four weeks no change of the oxidation state VI could be detected. Nevertheless, after six weeks, uranium(VI) was completely reduced to uranium(IV). In conclusion, by combining the analyses of the redox potential, theoretical calculations and XANES spectroscopy, a microbial mediated uranium(VI) reduction was demonstrated by simply adding 10 mM glycerol.

Until now only few studies were performed on microorganisms capable of reducing uranium(VI), using cultivation-dependent techniques in natural environments. Furthermore, in contrast to our investigations, they were conducted in sediment slurries under controlled laboratory conditions. Investigations on anaerobic MRB or SRB that carry out respiration in acidic environments is still in its infancy, though the above describes impact of acidic pH on microbial redox reactions [43]. However, in this study we were able to demonstrate the capability of indigenous anaerobic bacteria to use glycerol as electron acceptor for the microbial mediated uranium(VI) reduction. The concept of anaerobic indigenous bacteria, present in the groundwater of a uranium-contaminated site at the Bear Creek Valley (TN, USA), has been tested in laboratory scale experiments. Numerous results using controlled conditions in laboratory experiments displayed the feasibility of

microbial uranium(VI) reduction [44–48].
Moreover, during six weeks of incubation time the sulfate and Fe(II) concentrations were measured to investigate the microbial activity of SRB and IRB within the flooding water (Fig. S1). In association with the analysis of the bacterial diversity using 16S rDNA, the activity of bacteria should be investigated to detect their possible influence on the uranium(VI) reduction. IRB represented the major group, which were identified for their capability of dissimilatory uranium(VI) reduction [49–51]. In subsurface environments, IRB bacteria are likely to outcompete SRB, due to the fact that Fe(II) is usually a much more abundant electron acceptor than sulfate [43, 50, 51]. Therefore, IRB are thought to have a high bioremediation potential in uranium-contaminated subsurface sediments [43]. In accordance to this, during six weeks of incubation the Fe(II) concentration displayed a slight increase, possibly due to the activity of IRB within the flooding water (Fig. S1, black symbols and curve). However, the analyzed sulfate concentration revealed no changes, by meaning no increase could be detected during the whole incubation time (Fig. S1, blue symbols and curve).

Applicability of microbial uranium(VI) in pilot plant scale

The promising results of the microbial uranium(VI) reduction obtained in laboratory scale experiments (1 L) were scaled up and transferred to a pilot plant with a total volume of 100 L (Fig. 1). Similar to the previous experiments, 10 mM glycerol were directly added to the with N_2 flushed and anoxic incubated flooding water. By using an on-line measurement system pH value, redox potential and temperature were examined. The approach was incubated for six weeks at RT. Two independent runs, each time using fresh flooding water samples, were performed. Due to the fact, that both approaches displayed slight deviations, the results were considered independently, and therefore no mean values were calculated.
In contrast to the results obtained from the laboratory scale experiments, the redox potential displayed a faster decrease and furthermore, a lower equilibrium of around 100 mV after 14 days (Fig. 5a and b) in both approaches. However, during the first incubation of the flooding water in the pilot plant a drastic increase of the redox potential at day 14 was detected (Fig. 5a, grey area). This abrupt change could be explained by an accidental invasion of oxygen. Nevertheless, within the following days the redox potential reached the same equilibrium compared to the second run. While incubation at RT, the initial temperature of the flooding water (14-15 °C) increased to nearly 22 °C in the first days and no drastic changes during six weeks of incubation time was observed (Fig. S2, black curves). However, the pH showed distinct changes from initially 2.6/2.8 to 2.4/2.2 (Fig. S2, blue curves). The results obtained from the first run of the pilot plant approach displayed the highest variations during the incubation time (Fig. S2, open circles). The pH value decreased drastically during the second week. Subsequently. the pH increased until the initial value. This observed deviation of the pH is in accordance to the change of the redox potential of

the first run, thus the possible invasion of oxygen in the flooding water of the pilot plant could be an explanation for these differences. In addition, the Fe(II) and sulfate concentrations were investigated (Fig 5b-c, e-f). In accordance to the experiments from laboratory scale samples, the Fe(II) concentration revealed a distinct increase (Fig. 5e). As a consequence of the observed drastic change of the redox potential during the first run at day 14, the Fe(II) concentration increasd, too (Fig. 5b). The oxygen invasion could lead to a Fe(II) reoxidation. Moreover, after 26-28 days of incubation time, the sulfate concentration decreased slightly (Fig. 5c and f). Thus, in contrast to the laboratory scale approaches, a possible microbial mediated sulfate reduction could be detected in the samples of the pilot plant.

To confirm the microbial uranium(VI) reduction in the pilot plant, UV-vis analysis of both runs were performed. The two runs revealed the same findings. The results displayed an increase of the uranium(IV) concentration after 21 days (Fig. S3b). During the first 2.5 weeks only uranium(VI) was detected by UV-vis spectroscopy (Fig. S3a). Kirishima *et al.* [52] investigated the luminescence properties of uranium(IV) in solution and demonstrated characteristic maxima of uranium(IV) spectra, using UV-vis spectroscopy. One typical maximum of uranium(IV) displayed at around 650 nm. Compared to the spectra of the samples from the pilot plant approaches starting from day 21, this typical maximum could be detected. Thus, in accordance to the reference samples from previous investigations, uranium(IV) was obviously identified. Although, it has to be mentioned, that due to the absorption maximum of glycerol between 400-500 nm, which was described previously, the typical maxima of uranium(VI) could not clear distinguished [53]. Therefore, a low uranium(VI) concentration, within the samples after three weeks of incubation time, cannot completely ruled out. Using XANES spectroscopy to examine the laboratory scale samples, it was shown that uranium(VI) was completely reduced to uranium(IV). Further investigations should be performed, using the same method, to quantify the uranium(IV) concentration in the flooding water samples from the pilot plant after six weeks of incubation.

In summary, two independent runs of the pilot plant were performed and revealed in the same findings compared to the results of the laboratory scale experiments. Only slight differences were shown. However, we demonstrated a microbial mediated Fe(II) reduction followed by a uranium(VI) reduction. Moreover, due to the lower redox potential, compared to the laboratory scale samples, a microbial sulfate reduction during the last week was shown. Thus, the up-scaling experiments in pilot plant approaches (100 L) verified the industrial applicability of the microbial mediated uranium(VI) reduction in the flooding water of the former uranium mine Königstein for possible bioremediation methods.

Bacterial diversity after uranium(VI) reduction

To investigate the microorganism responsible for uranium(VI) reduction, 16Sr DNA analyses were performed. Compared to previous studies of the bacterial diversity prevalent in the flooding

water of the former uranium mine, the bacterial community displayed distinct changes (Fig. 6). However, in both samples the majority of the bacteria were identified belonging to the class of gammaproteobacteria. In addition, also the quantity of unclassified bacteria revealed in similar numbers. But besides that, the bacterial diversity changes drastically. The investigation of the flooding water before incubation displayed that the majority of the identified species, belonging to gammaproteobacteria, were *Acidithiobacillus* spp. [26]. Studies on the diversity of bacteria at AMD sites demonstrated that *Acidithiobacillus* species represents a dominating group in such harsh environments [54, 55]. Notably, *A. ferrooxidans* displays an important species, which is involved in the degradation of mineral ores and furthermore, the oxidation of iron at AMD sites. Thus, *A. ferrooxidans* is effecting the development of AMD sites [56]. Consequently, the major occurrence of these bacteria is in good accordance to previous investigations, as well as the diversity study before the microbial uranium(VI) reduction within the flooding water. The majority of the identified species after six weeks incubation, belonging to the class of gammaproteobacteria, were represented by *Halomonas* spp. (39 %). Although *Halomonas* species are known for their tolerance to alkaline conditions, previous studies demonstrated their ability to reduce Fe(III) and Cr(VI) [57]. Within the first weeks of incubation an Fe(III) reduction was detected, explaining the occurrence of IRB like *Halomonas* spp.. In addition, the IRB *Acidiphilium* spp. could be identified, which belong to the class of alphaproteobacteria. Cultivated under anoxic conditions, these bacteria are able to reduce Fe(III) to Fe(II) [58, 59]. Furthermore, previous investigation about the ability of Fe(III) reduction displayed, that *Acidiphilium* is able to reduce insoluble Fe(III) into soluble Fe(II) at a redox potential of 770 mV, which equals the prevalent potential in the flooding water before six weeks of incubation [50]. To the best of our knowledge, no studies which described the ability of this identified IRB to reduce uranium(VI) are published. Due to their majority of the bacterial diversity after six weeks incubation and in addition the knowledge that IRB are able for uranium(VI) reduction, it could be assumed that *Halomonas* spp. and *Acidiphilium* spp., are responsible for the microbial mediated uranium(VI) reduction within the flooding water samples. However, further studies should be performed to proof this hypothesis and to investigate their ability to reduce uranium(VI) in more detail.

Although, previous investigations revealed in the presence and activity of SRB in the flooding water, within the laboratory scale samples (1 L) they could not be detected, using 16S rDNA investigations. However, the bacterial diversity of the pilot plant sample (100 L) displayed the occurrence of SRB. Yet, it was not possible to obtain enough sequence information to get an entire overview about the bacterial diversity after six weeks of the pilot plant approaches (data not shown). Nevertheless, the results revealed that the majority of the sequences were identified as *A. ferrooxidans* (60 %), followed by *Desulfovibrio* spp. and *Acidocella* spp.. Despite the low bacterial diversity information, all obtained sequences were associated with SRB and IRB/IOB, which is in good agreement to the results of the laboratory scale experiments.

One explanation for the non-successful stimulation of SRB within the laboratory scale samples

could be the differences of the redox potential. A microbial sulfate reduction at 230 mV is rather unlikely, due the fact that sulfate reduction mediated by microorganisms was investigated in previous studies at redox potentials at -220 mV [60]. Therefore, SRB were rather not metabolic active and thus, could not be detected using 16S rDNA analyses. However, besides the differences of the bacterial diversity of both approaches, the results of the pilot plant confirmed the microbial mediated uranium(VI) reduction in up-scaling setups. In conclusion, this findings suggest the applicability of microbial uranium(VI) reduction for industrial bioremediation approaches on site of the former uranium mine Königstein.

Conclusion

With the obtained results from this study, we could clearly demonstrate the capability of anaerobic microorganisms inhabiting the flooding water to reduce uranium(VI). During an incubation time of six weeks the prevalent uranium(VI) was completely reduced to uranium(IV), only by the addition of 10 mM glycerol. In addition, we could also demonstrate a microbial induced Fe(III) and sulfate reduction. In combination with investigations on the bacterial diversity after six weeks of incubation, bacteria were identified, which might be responsible for the observed redox reduction processes. The results revealed that the bacterial diversity is dominated of IRB and SRB. Concluding, these bacteria, naturally occurring within the flooding water, seems to be responsible for the uranium(VI) reduction. However, the bacterial communities of the laboratory scale approaches compared to the pilot plant sample differs drastically. Thus, further experiments should be performed to investigate the microbial community within the flooding water after uranium(VI) reduction. Moreover, the results obtained from laboratory scale experiments could successfully transferred and verified in industrial scale applications within a pilot plant. The microbial mediated reduction of uranium(VI) could be used for possible bioremediation approaches, which would be cost-effective and less time-consuming, than the conventional pump-and-treat process. With the obtained results, we could confirm previous studies which assumed the applicability of microbial mediated uranium(VI) reduction for *in situ* bioremediation approaches. However, detailed investigations should be performed on the formed uranium(IV) complexes and their stability at long-time conditions.

Acknowledgments

This work was supported by the Bundesministerium für Bildung und Forschung (BMBF) grant № 02NUK030F (TransAqua). The authors are grateful to the Wismut GmbH for allowing access to the sampling site, in particular S. Röder for assistance in water sampling. We thank S. Gurlit and C. Eckardt for ICP-MS and AAS measurements, S. Weiß for his assistance during preparation of XANES samples, T. Zimmermann and R. Steudtner for UV-vis analyses and T. Schneider for his grammatical correction of this manuscript.

References

[1] K. H. Williams, J. R. Bargar, J. R. Lloyd and D. R. Lovley, *„Bioremediation of uranium-contaminated groundwater: A systems approach to subsurface biogeochemistry“*, Curr. Opin. Biotechnol. **24**, 489 (2013).

[2] U. Jenk, K. Nindel and U. Zimmermann, in *Uranium, Mining and Hydrogeology*, Ed. B. J. Merkel and A. Hasche-Berger, P. 431–436. Springer Verlag (2008).

[3] D. R. Lovley, E. J. P. Phillips, Y. A. Gorby and E. R. Landa, *„Microbial reduction of uranium“*, Nature **350**, 413 (1991).

[4] M. L. Merroun, M. Nedelkova, J. J. Ojeda, T. Reitz, M. L. Fernández, J. M. Arias, M. Romero-González and S. Selenska-Pobell, *„Bio-precipitation of uranium by two bacterial isolates recovered from extreme environments as estimated by potentiometric titration, TEM and X-ray absorption spectroscopic analyses“*, J. Hazard. Mater. **197**, 1 (2011).

[5] D. B. Johnson and K. B. Hallberg, *„Acid mine drainage remediation options: A review“*, Sci. Total Environ. **338**, 3 (2005).

[6] B. J. Baker, G. W. Tyson, L. Goosherst and J. F. Banfield, *„Insights into the diversity of eukaryotes in acid mine drainage biofilm communities“*, Appl Environ Microb (2009).

[7] J. R. Lloyd and L. E. Macaskie, *„Bioremediation of Radionuclide-Containing Wastewaters“*, in *Environmental Microbe-Metal Interactions*, P. 277–327 (2000).

[8] H. Shimura, K. Itoh, A. Sugiyama, S. Ichijo, M. Ichijo, F. Furuya, Y. Nakamura, K. Kitahara, K. Kobayashi, Y. Yukawa and T. Kobayashi, *„Absorption of Radionuclides from the Fukushima Nuclear Accident by a Novel Algal Strain“*, PLoS One **7** (2012).

[9] P. Kotrba, L. Dolecková, V. de Lorenzo and T. Ruml, *„Enhanced bioaccumulation of heavy metal ions by bacterial cells due to surface display of short metal binding peptides.“*, Appl Environ Microb **65**, 1092 (1999).

[10] M. D. Mullen, D. C. Wolf, F. G. Ferris, T. J. Beveridge, C. A. Flemming and G. W. Bailey, *„Bacterial sorption of heavy metals“*, Appl Environ Microb **55**, 3143 (1989).

[11] B. Volesky, *„Biosorption and me“*, Water Res. **41**, 4017 (2007).

[12] K. R. Salome, M. J. Beazley, S. M. Webb, P. A. Sobecky and M. Taillefert, *„Biomineralization of U(VI) phosphate promoted by microbially-mediated phytate hydrolysis in contaminated soils“*, Geochim. Cosmochim. Acta (2017).

[13] Y. Suzuki and J. F. Banfield, *„Resistance to, and accumulation of, uranium by bacteria from a uranium-contaminated site“*, Geomicrobiol. J. **21**, 113 (2004).

[14] A. J. Francis, C. J. Dodge, F. Lu, G. P. Halada and C. R. Clayton, *„XPS and XANES Studies of Uranium Reduction by Clostridium sp“*, Environ. Sci. Technol. **28**, 636 (1994).

[15] D. R. Lovley, P. K. Widman, J. C. Woodward and E. J. P. Phillips, *„Reduction of uranium by cytochrome c3 of Desulfovibrio vulgaris“*, Appl Environ Microb **59**, 3572 (1993).

[16] Y. Suzuki, S. D. Kelly, K. M. Kemner and J. F. Banfield, *„Direct microbial reduction and subsequent preservation of uranium in natural near-surface sediment“*, Appl Environ Microb **71**, 1790 (2005).

[17] E. L.-W. Majumder and J. D. Wall, *„Uranium Bio-Transformations: Chemical or Biological Processes?“*, Open J Inorg Chem (2017).

[18] L. Newsome, K. Morris, D. Trivedi, N. Atherton and J. R. Lloyd, *„Microbial reduction of uranium(VI) in sediments of different lithologies collected from Sellafield“*, Appl. Geochem. **51**, 55 (2014).

[19] K. T. Finneran, R. T. Anderson, K. P. Nevin and D. R. Lovley, *„Potential for Bioremediation of Uranium-Contaminated Aquifers with Microbial U(VI) Reduction“*, Soil Sediment Contam **11**, 339 (2002).

[20] D. R. Brookshaw, R. A. D. Pattrick, J. R. Lloyd and D. J. Vaughan, *„Microbial effects on mineral-radionuclide interactions and radionuclide solid-phase capture processes“*, Mineral. Mag. **76**, 777 (2012).

[21] D. R. Lovley and E. J. P. Phillips, *„Reduction of uranium by Desulfovibrio desulfuricans“*, Appl Environ Microb **58**, 850 (1992).

[22] T. Arnold, T. Zorn, H. Zänker, G. Bernhard and H. Nitsche, *„Sorption behavior of U(VI) on phyllite: Experiments and modeling“*, J. Contam. Hydrol. **47**, 219 (2001).

[23] M. H. Baik, S. P. Hyun, W. J. Cho and P. S. Hahn, *„Contribution of minerals to the sorption of U(VI) on granite“*, Radiochim. Acta **92** (2004).

[24] M. Barnett, P. Jardine, S. Brooks and H. Selim, *„Adsorption and Transport of Uranium(VI) in Subsurface Media“*, Soil Sci. Soc. Am. J. **64**, 908 (2000).

[25] M. C. Duff, J. U. Coughlin and D. B. Hunter, *„Uranium co-precipitation with iron oxide minerals“*, Geochim. Cosmochim. Acta (2002).

[26] I. Zirnstein, *„Charakterisierung der Mikroorganismen im sauren Grubenwasser des ehemaligen Uranbergwerks Königstein“*, PhD thesis, Technische Universität Dresden (2015).

[27] H. C. Bimboim and J. Doly, *„A rapid alkaline extraction procedure for screening recombinant plasmid DNA“*, Nucleic Acids Res. (1979).

[28] W. Matz, N. Schell, G. Bernhard, F. Prokert, T. Reich, J. Claußner, W. Oehme, R. Schlenk, S. Dienel, H. Funke, F. Eichhorn, M. Betzl, D. Pröhl, U. Strauch, G. Hüttig, H. Krug, W. Neumann, V. Brendler, P. Reichel, M. a. Denecke and H. Nitsche, *„ROBL – a CRG beamline for radiochemistry and materials research at the ESRF“*, J. Synchrotron Radiat. **6**, 1076 (1999).

[29] A. Roßberg, T. Reich and G. Bernhard, *„Complexation of uranium(VI) with protocatechuic acid-application of iterative transformation factor analysis to EXAFS spectroscopy“*, Anal. Bioanal. Chem. (2003).

[30] Å. Kolmert, P. Wikström and K. B. Hallberg, *„A fast and simple turbidimetric method for the determination of sulfate in sulfate-reducing bacterial cultures“*, J Microbiol Meth **41**, 179 (2000).

[31] E. Viollier, P. W. Inglett, K. Hunter, a. N. Roychoudhury and P. Van Cappellen, *„The Ferrozine Method Revisited: Fe (II)/Fe (III) Determination in Natural Waters“*, Appl. Geochem. **15**, 785 (2000).

[32] T. Arnold, N. Baumann, E. Krawczyk-Bärsch, S. Brockmann, U. Zimmermann, U. Jenk and S. Weiß, *„Identification of the uranium speciation in an underground acid mine drainage environment“*, Geochim. Cosmochim. Acta **75**, 2200 (2011).

[33] D. B. Johnson, *„Chemical and microbiological characteristics of mineral spoils and draiangewaters at abandoned coal and metalmines“*, Water Air Soil Pollut **3**, 47 (2003).

[34] I. Sánchez-Andrea, J. L. Sanz, M. F. M. Bijmans and A. J. M. Stams, *„Sulfate reduction at low pH to remediate acid mine drainage“*, J. Hazard. Mater. **269**, 98 (2014).

[35] M. F. M. Bijmans, E. de Vries, C.-H. Yang, C. J. N. Buisman, P. N. L. Lens and M. Dopson, *„Sulfate reduction at pH 4.0 for treatment of process and wastewaters“*, Biotechnol. Progr. P. NA–NA (2010).

[36] M. Koschorreck, *„Microbial sulphate reduction at a low pH“*, FEMS Microbiol Ecol **64**, 329 (2008).

[37] I. Ňancucheo and D. B. Johnson, *„Selective removal of transition metals from acidic mine waters by novel consortia of acidophilic sulfidogenic bacteria“*, Microb. Biotechnol. **5**, 34 (2011).

[38] D. Alazard, M. Joseph, F. Battaglia-Brunet, J. L. Cayol and B. Ollivier, *„Desulfosporosinus acidiphilus sp. nov.: A moderately acidophilic sulfate-reducing bacterium isolated from acid mining drainage sediments“*, Extremophiles (2010).

[39] D. R. Kremer and T. A. Hansen, *„Glycerol and dihydroxyacetone dissimilation in Desulfovibrio strains“*, Arch. Microbiol. **147**, 249 (1987).

[40] H. J. Nanninga and J. C. Gottschal, *„Properties of Desulfovibrio carbinolicus sp. nov. and Other Sulfate-Reducing Bacteria Isolated from an Anaerobic-Purification Plant.“*, Appl. Environ. Microbiol. **53**, 802 (1987).

[41] A.-I. Qatibi, A. Bories and J.-L. Garcia, *„Sulfate reduction and anaerobic glycerol degradation by a mixed microbial culture“*, Curr. Microbiol. **22**, 47 (1991).

[42] S. C. Santos, M. G. Liebensteiner, A. H. van Gelder, M. R. Dimitrov, P. F. Almeida, C. M. Quintella, A. J. M. Stams and I. Sánchez-Andrea, *„Bacterial glycerol oxidation coupled to sulfate reduction at neutral and acidic pH“*, J. Gen. Appl. Microbiol. **64**, 1 (2018).

[43] L. Petrie, N. N. North, S. L. Dollhopf, D. L. Balkwill and J. E. Kostka, *„Enumeration and Characterization of Iron(III)-Reducing Microbial Communities from Acidic Subsurface Sediments Contaminated with Uranium(VI)“*, Appl. Environ. Microbiol. **69**, 7467 (2003).

[44] D. A. Elias, L. R. Krumholz, D. Wong, P. E. Long and J. M. Suflita, *„Characterization of Microbial Activities and U Reduction in a Shallow Aquifer Contaminated by Uranium Mill Tailings“*, Microb. Ecol. **46**, 83 (2003).

[45] B. Gu, W. M. Wu, M. A. Ginder-Vogel, H. Yan, M. W. Fields, J. Zhou, S. Fendorf, C. S. Criddle and P. M. Jardine, *„Bioreduction of uranium in a contaminated soil column“*, Environ. Sci. Technol. **39**, 4841 (2005).

[46] I. Ortiz-Bernad, R. T. Anderson, H. A. Vrionis and D. R. Lovley, *„Resistance of Solid-Phase U(VI) to Microbial Reduction during In Situ Bioremediation of Uranium-Contaminated Groundwater“*, Appl Environ Microbiol **70**, 7558 (2004).

[47] J. M. Senko, J. D. Istok, J. M. Suflita and L. R. Krumholz, *„In-situ evidence for uranium immobilization and remobilization“*, Environ. Sci. Technol. **36**, 1491 (2002).

[48] J. L. Uhrie, J. I. Drever, P. J. Colberg and C. C. Nesbitt, *„In situ immobilization of heavy metals associated with uranium leach mines by bacterial sulfate reduction“*, Hydrometallurgy **43**, 231 (1996).

[49] D. R. Lovley, *„Bioremediation of organic and metal contaminants with dissimilatory metal reduction“*, J Ind Microbiol **14**, 85 (1995).

[50] D. R. Lovley, D. E. Holmes and K. P. Nevin, *„Dissimilatory Fe(III) and Mn(IV) reduction“*, Adv. Microb. Physiol. (2004).

[51] D. R. Lovley and R. T. Anderson, *„Influence of dissimilatory metal reduction on fate of organic and metal contaminants in the subsurface“*, Hydrogeology Journal **8**, 77 (2000).

[52] A. Kirishima, T. Kimura, R. Nagaishi and O. Tochiyama, *„Luminescence properties of tetravalent uranium in aqueous solution“*, Radiochim. Acta **92** (2004).

[53] M. Nanda, Z. Yuan, W. Qin, M. Poirie and X. Chunboa, *„Purication of Crude Glycerol using Acidi cation: Effects of Acid Types and Product Characterization“*, Austin Chem Eng. **1(1)**, 1 (2014).

[54] K. Duquesne, S. Lebrun, C. Casiot, O. Bruneel, J. C. Personné, M. Leblanc, F. Elbaz-Poulichet, G. Morin and V. Bonnefoy, *„Immobilization of Arsenite and Ferric Iron by Acidithiobacillus ferrooxidans and Its Relevance to Acid Mine Drainage“*, Appl. Environ. Microbiol. **69**, 6165 (2003).

[55] D. Leduc, L. G. Leduc and G. D. Ferroni, *„Quantification of bacterial populations indigenous to acidic drainage streams“*, Water Air Soil Pollut **135**, 1 (2002).

[56] D. K. Nordstrom and G. Southam, *„Geomicrobiology of sulfide mineral oxidation“*, in *Geomicrobiology: interactions between microbes and minerals*, Ed. J. F. Banfield and K. H. Nealson, P. 361–390, Mineralogical Society of America, Washington, DC (1997).

[57] M. R. VanEngelen, B. M. Peyton, M. R. Mormile and H. C. Pinkart, *„Fe(III), Cr(VI), and Fe(III) mediated Cr(VI) reduction in alkaline media using a Halomonas isolate from Soap Lake, Washington“*, Biodegradation (2008).

[58] T. A. M. Bridge and D. B. Johnson, *„Reduction of soluble iron and reductive dissolution of ferric iron- containing minerals by moderately thermophilic iron-oxidizing bacteria“*, Appl Environ Microb (1998).

[59] K. Küsel, T. Dorsch, G. Acker and E. Stackebrandt, *„Microbial reduction of Fe(III) in acidic sediments: Isolation of Acidiphilium cryptum JF-5 capable of coupling the reduction of fe(III) to the oxidation of glucose“*, Appl Environ Microb (1999).

[60] J. D. Wall and L. R. Krumholz, *„Uranium Reduction“*, Annu. Rev. Microbiol. **60**, 149 (2006).

Figures

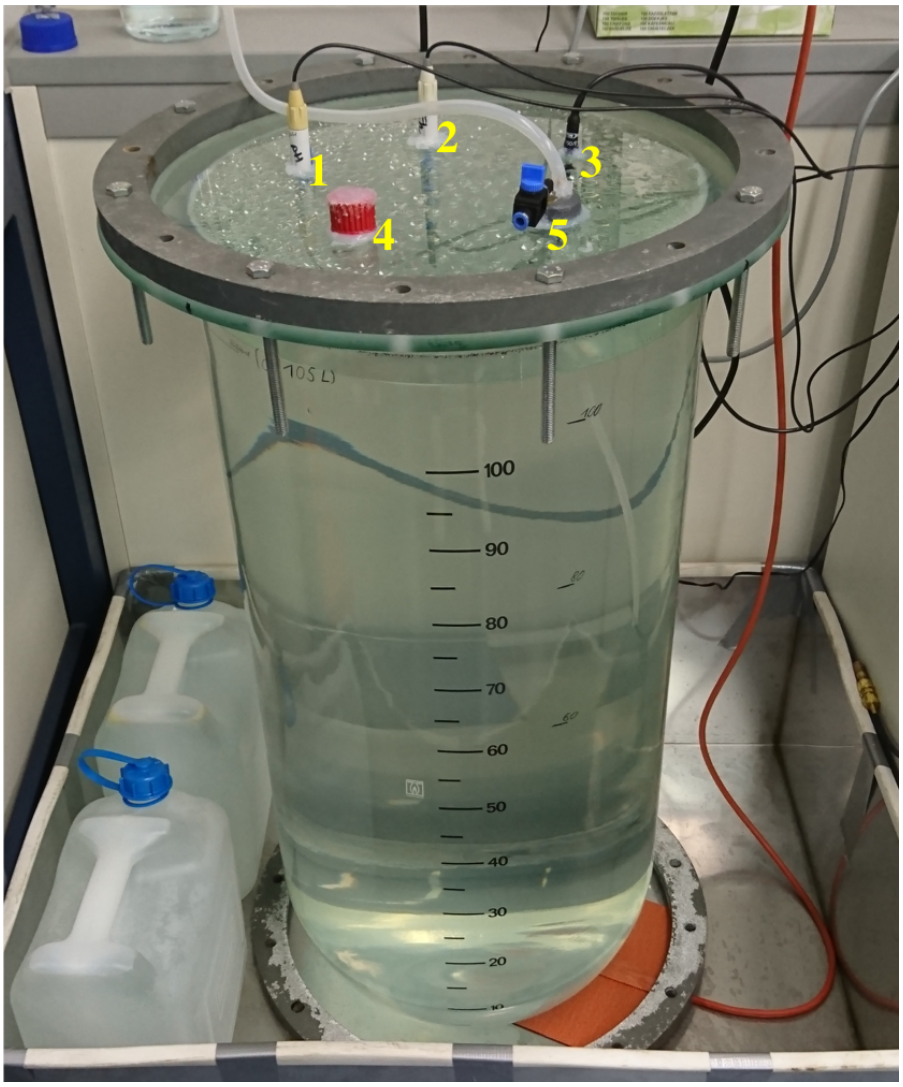


Figure 1: Pilot plant. Flooding water (100 L) was collected and N_2-flushed, 10 mM glycerol were added, incubation for six weeks at room temperature. For on line measurement, devices were placed on the lid, (1) pH electrode, (2) redox electrode, (3) temperature sensor, (4) device for anaerobic samplings by syringe, (5) vent for N_2 flushing.

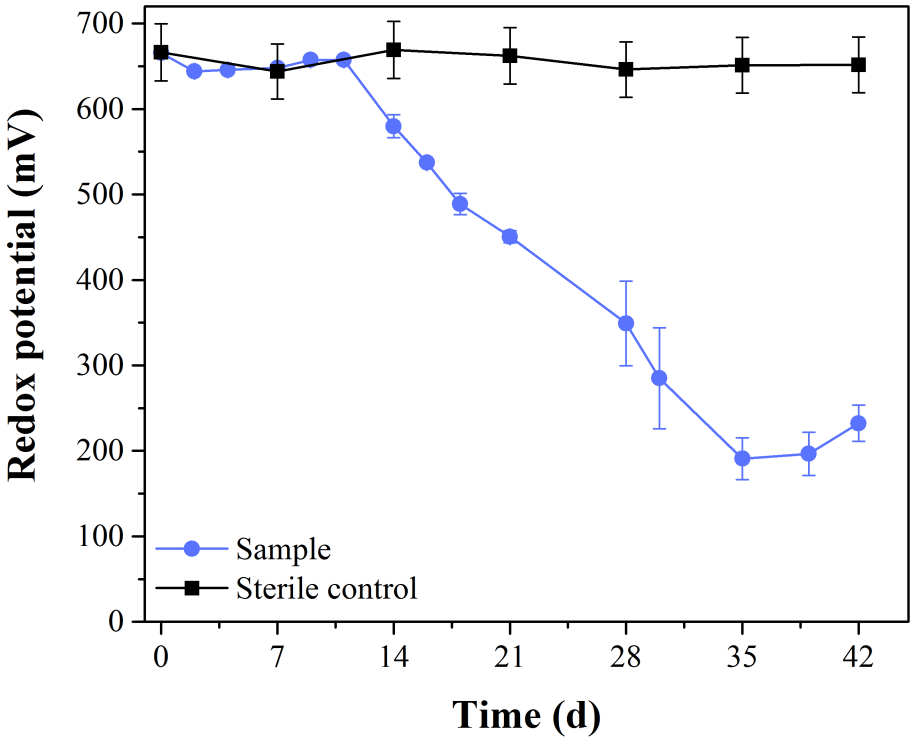


Figure 2: Redox potential of flooding water samples incubated over 6 weeks. Flooding water was collected and N_2-flushed to achieve anoxic conditions. As carbon source 10 mM glycerol was added and the samples were incubated at 30 °C for six weeks in the dark. As sterile control, flooding water was sterile-filtered and supplemented with 10 mM glycerol. Error bars indicate standard deviation, n=3.

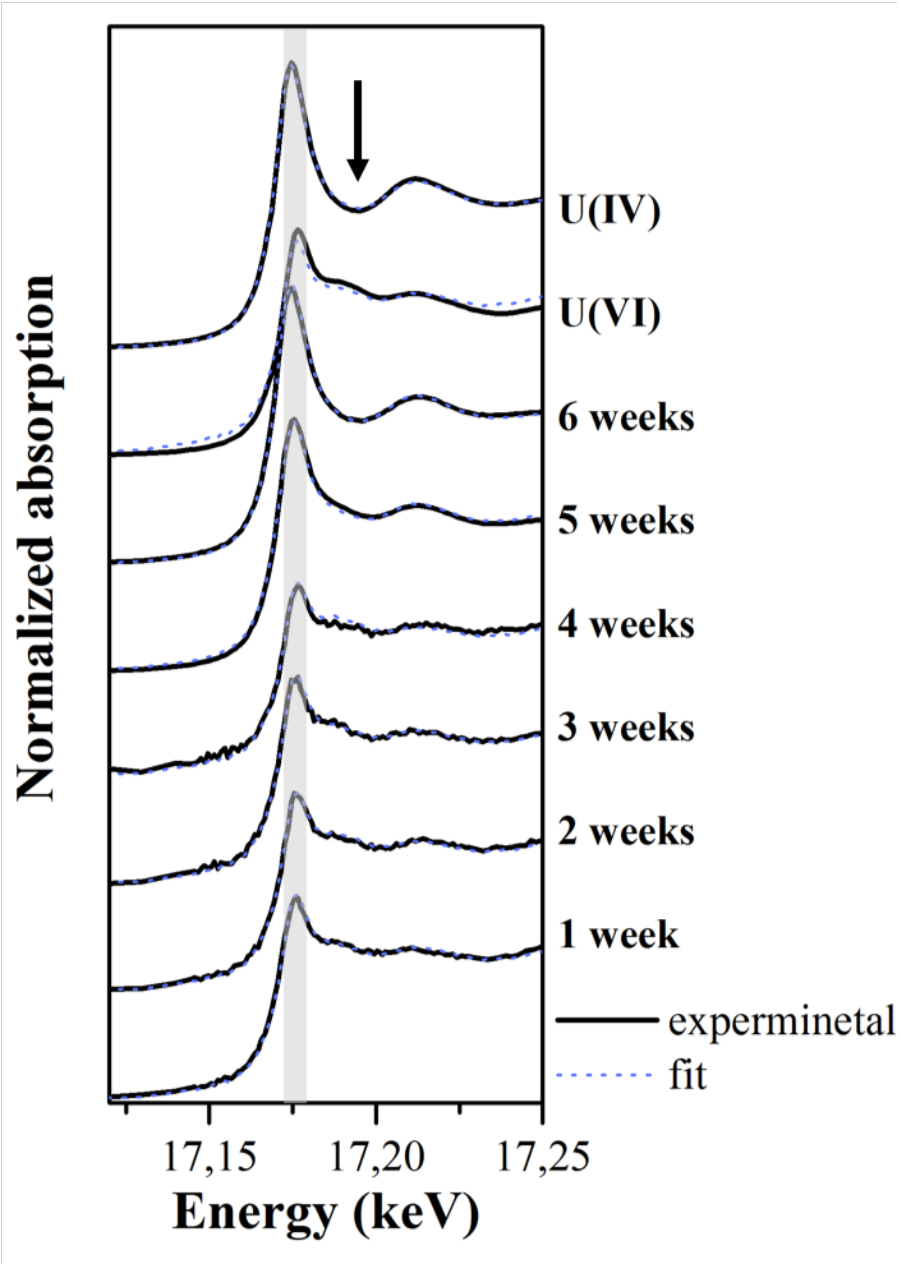


Figure 3: XANES measurements of anoxic flooding water samples. Samples were incubated at 30 °C for six weeks in the dark, 10 mM glycerol was added, samples were taken once a week. As references for uranium(VI) and uranium(IV) samples were prepared with the flooding water a background solution. Grey area displays the shift of the spectra, and the black arrow indicates the disappearing of the typical uranium(VI) shoulder of the uranium(IV) spectra.

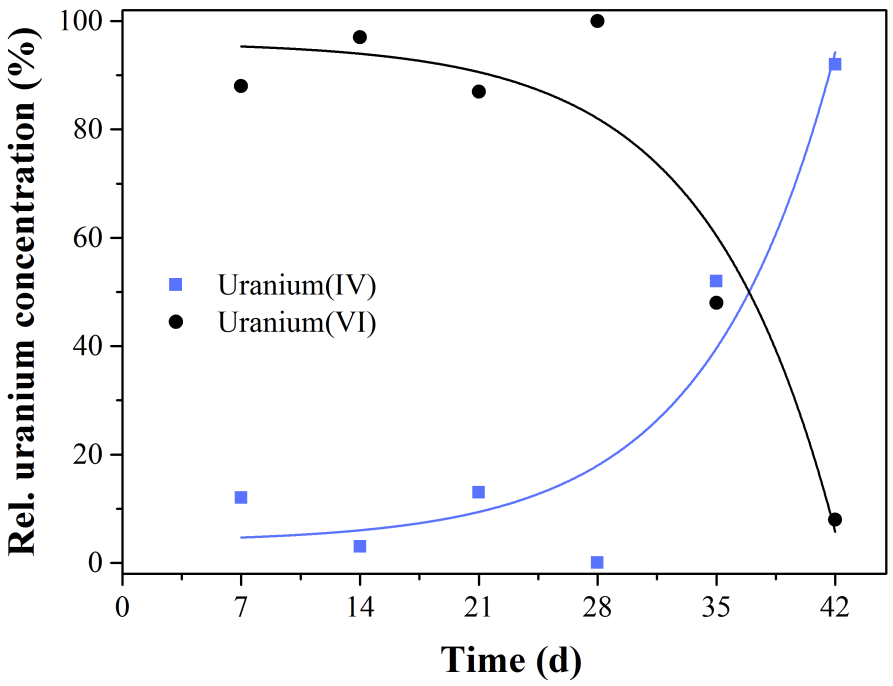


Figure 4: Relative uranium concentration of the anoxic flooding water samples calculated by ITFA. Samples were incubated at 30 °C for six weeks in the dark, 10 mM glycerol was added, samples were taken every week.

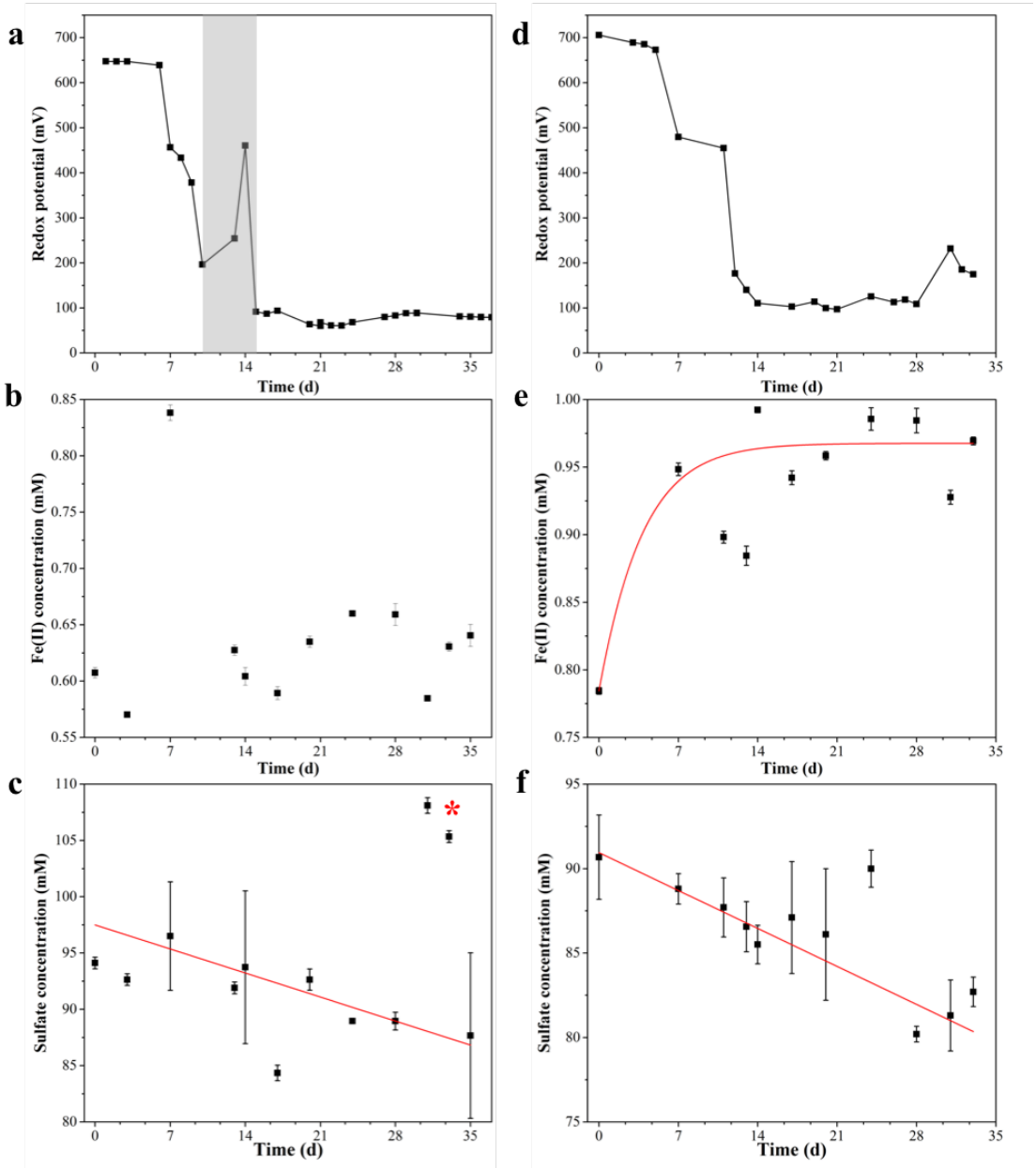


Figure 5: Redox potential, Fe(II) and sulfate concentration during six weeks of incubation time of the pilot plant samples (100 L). Plots (a-c) display the results of the first run of the pilot plant, (d-f) display the second run. Grey marked area in (a) shows a distinct increase of the redox potential, possibly due to an oxygen invasion. The red curves represent a guide to the eye. The red star in (c) indicates two possible outliers of the measured sulfate concentration, which were excluded from the linear fitting.

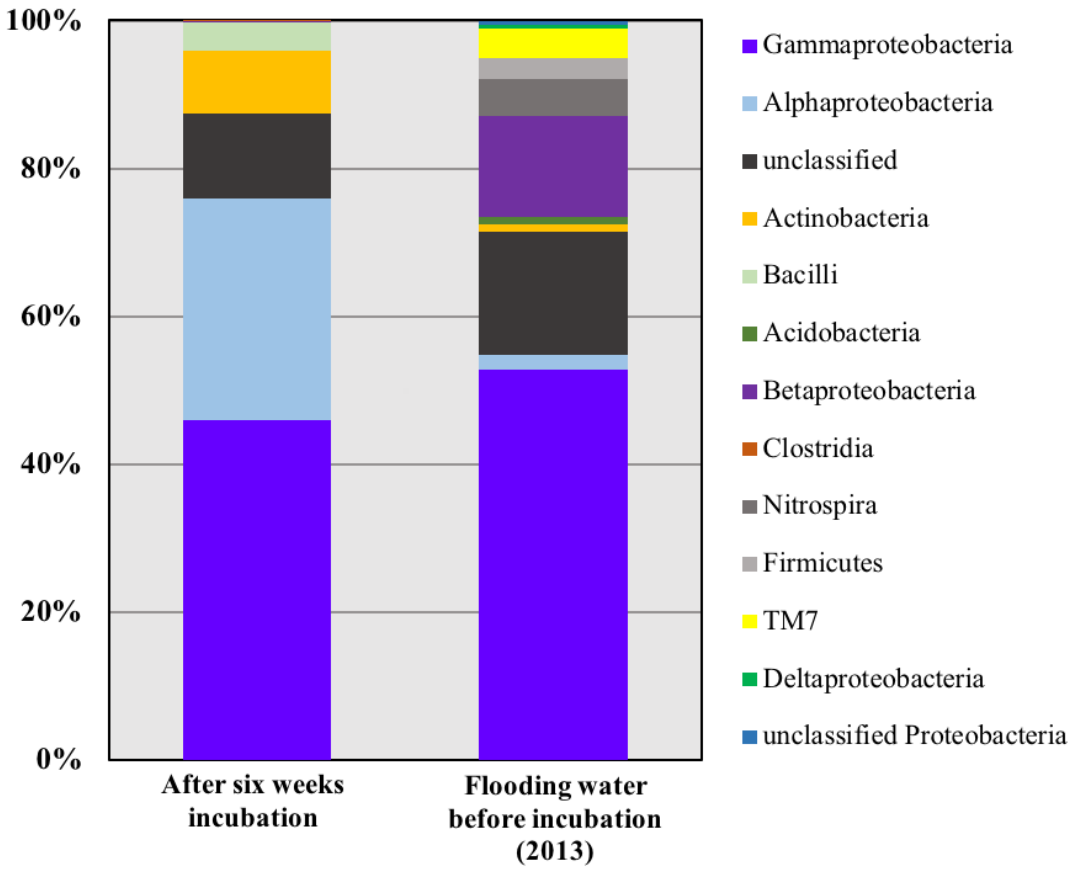


Figure 6: Bacterial diversity of the laboratory scale experiments (1 L) after six weeks incubation and before. Determined using 16S rDNA gene analysis, diversity before incubation (2013) changed after [26].

Supplementary

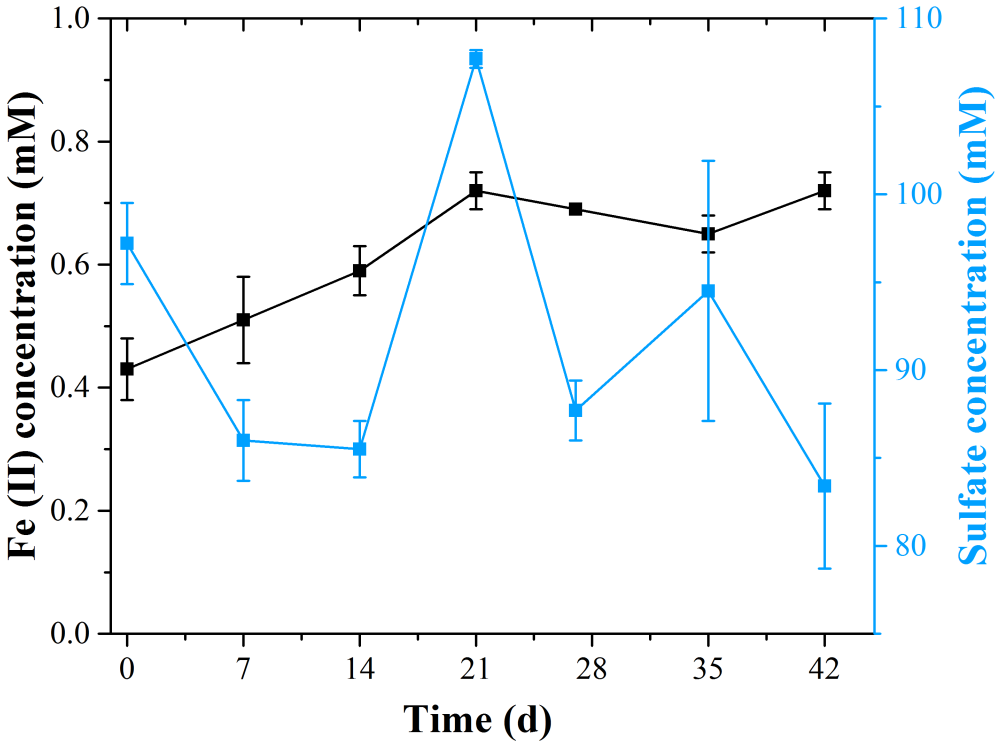


Figure S1: Fe(II) (black symbols) and sulfate (blue symbols) concentration during six weeks of incubation time within the laboratory scale experiments (1 L). Samples were incubated at 30 °C for six weeks in the dark, 10 mM glycerol was added, samples were taken every week.

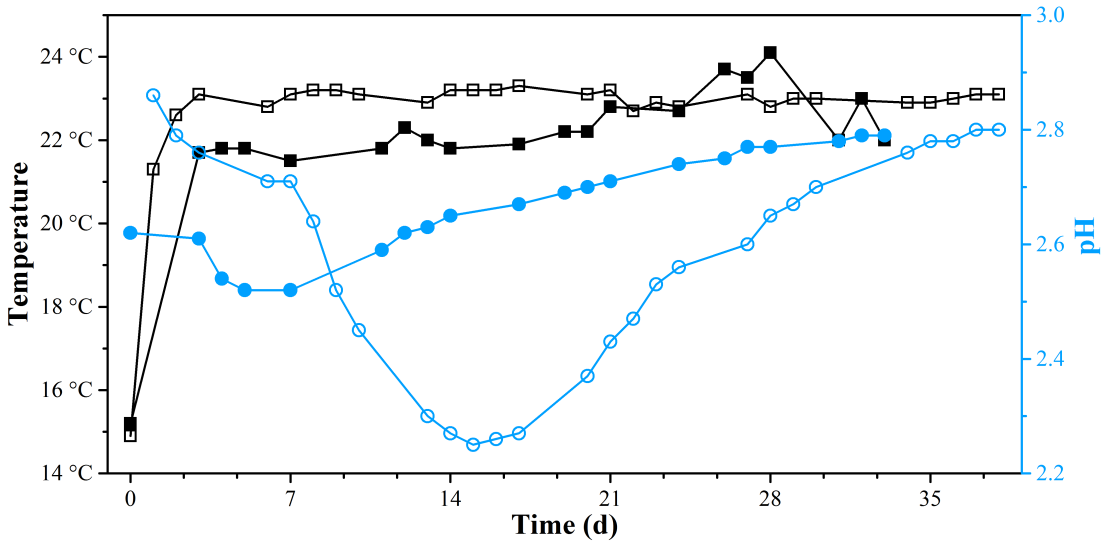


Figure S2: Temperature and pH value of the flooding water samples obtained from the pilot plant. In black the progression of the temperature during six weeks is displayed. The blue curves show the changes of the pH value. Open symbols represent the first run, and closed symbols the second run.

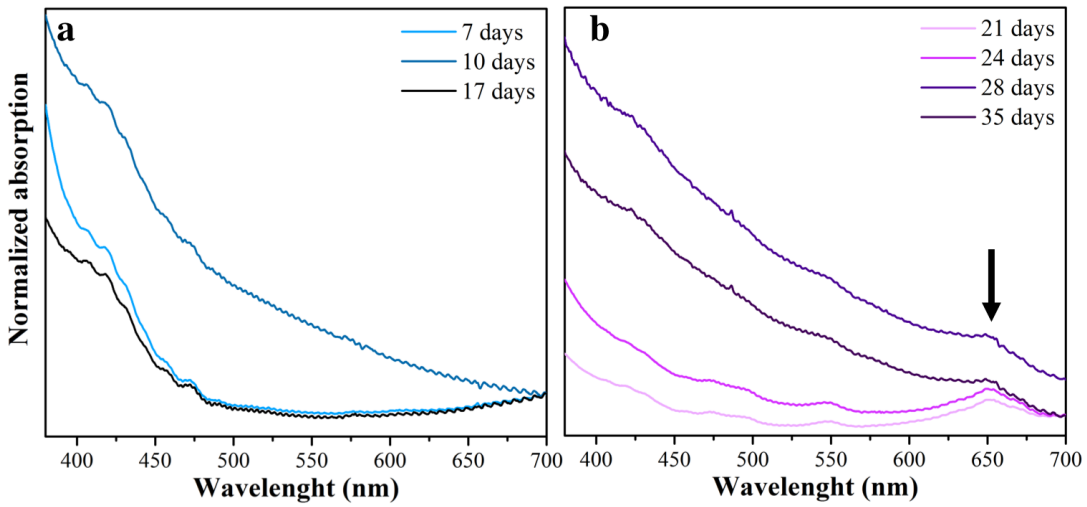


Figure S3: UV-vis spectra of the flooding water samples obtained from the pilot plant. Spectra till day 17 in (a) displayed no uranium(IV) concentration, in (b) the spectra after three weeks of incubation time with detected uranium(IV) (black arrow) are displayed.

4 Discussion

Bioremediation represents a cost-effective and sustainable alternative for the treatment of heavy metal-contaminated sites at large scale. This emerging strategy uses microorganisms to remove or immobilize metals and radionuclides from contaminated environments [111]. In addition, also the use of green plants to decontaminate polluted sites, known as phytoremediation, seems to be promising for the removal of metals [112, 113]. The most attractive advantage of bioremediation is the reduced impact on natural ecosystems, by using indigenous microorganisms [114]. Microorganisms play an important role in the maintenance of ecosystems as they are known to be capable of rapid adjustment towards environmental changes. Furthermore, they are considered to be the first life forms, and thus they are versatile and adaptive to various challenging environmental conditions. Microorganisms are omnipresent and play a major role in regulating biogeochemical cycles within extreme environmental conditions such as acidic lakes, hydrothermal vents, and bottoms of deep oceans [115]. In addition, they are able to produce diverse enzymes which could be applied for the removal of contaminants, by direct destruction or through transformation into lesser toxic intermediates [116]. Microorganisms display many characteristics which make them suitable for bioremediation applications. Some bacteria for example, are able to develop unique properties, like the synthesis of bioactive compounds, biofilm formation or production of biosurfactans [117,118]. Previous studies could show, that bacteria inhabitant within heavy metal and radionuclide contaminated sites are well adapted to these harsh conditions. Consequently, they play a major role in biogeochemical cycling of toxic metals and may influence their mobility and toxicity [40, 119].

As a consequence of former mining and milling industry, but also by occasional accidents at nuclear facilities, such as the casualty 1986 in Chernobyl, radionuclides are released to the environment, which result in a possible health risk. It is well described, that exposure to radionuclides causes acute health effects to humans. Studies demonstrated, that long-term exposure leads to an elevated risk of kidney damage, leukopenia and leukemia, particularly by the intake of contaminated drinking water [120, 121]. Moreover, early symptoms of high doses could include vomiting, nausea and headache. Increased exposure may result in weakness, fever, hair loss and dizziness, but ultimately death. In addition, radionuclide exposure to fetuses could have effects on a cellular level, which can result in smaller head or brain size, poorly formed eyes, abnormal growth and mental retardation [122–124]. In conclusion, this shows the importance of the elimination of radionuclides, such as uranium, from the environment. Therefore, microbial trans-

formations of heavy metals and radionuclides are a vital part within natural ecosystem processes and could have beneficial impacts for our human community [111].
The results described in this thesis could demonstrate that all investigated strains display different mechanisms to interact with uranium(VI). Resulting from that, the ability to interact with uranium(VI) and decrease its mobility within surrounding solutions is dependent on the individual organism. In addition, it was clearly demonstrated that indigenous microorganisms influence the migration behavior of uranium(VI) in tremendous ways. To predict the fate of uranium in the environment it is important to have knowledge about the indigenous microbial diversity and their possible impact on its solubility. Moreover, the assessment of microbial populations isolated from uranium-contaminated sites not only enables us to gain better insights in their role of metal and radionuclide biogeochemistry, but also allows to evaluate their potential to design effective bioremediation approaches.

4.1 Uranium removal capability and interaction mechanisms of the betaproteobacterium *A. facilis*

The investigated strain *A. facilis* represents a common soil bacterium which is spread worldwide and which is not fastidious to special carbon sources [125, 126]. Previous studies could show that this species represents a major part of metabolic active microorganisms within the flooding water of the former uranium mining site Königstein [109]. Due to its ability to grow at acidic conditions (pH 2.9) and at high uranium concentrations (8-13 mg/L) this strain was examined for its capability to remove uranium from surrounding solutions.
The results of the batch sorption experiments revealed, that the bacterial cells were capable to remove 130 mg U/g dbm, which corresponds to a removal capacity of almost 100 % [127]. Compared to an isolated strain from a potential site for radioactive waste disposal in China, namely *Bacillus* sp. removing about 60 % of total uranium, *A. facilis* is more efficient in uranium removing [128]. However, other investigated gram-negative bacterial strains could remove similar amounts of uranium [129–131].
Further experiments were performed to examine the tolerance of *A. facilis* to grow in the presence of uranium. Interestingly, despite the fact that the bacterial strain was not directly isolated from the flooding water of the former uranium mine and thus, the bacterial cells were not naturally exposed to high uranium concentrations, the strain exhibits a high tolerance against this radionuclide. The investigations revealed a MIC for uranium of 0.2 mM which equals 23.8 mg/L. Compared to the current uranium concentration of 8 mg/L within the flooding water, the bacterial strain is able to tolerate nearly a threefold higher concentration and therefore, would be able to resist the harsh conditions at the former uranium mining site in Königstein. Nevertheless, the uranium tolerance test was performed on solid agar plates. Previous investigations assume that

due to different conditions of diffusion, complexation and bioavailability of the metal, higher tolerances are reported on solid media, in comparison to liquid media, and in addition in rich media, in comparison to minimal media [132, 133]. In conclusion, the revealed values of heavy metal tolerances of the investigated microorganisms vary with type and composition of the used media. Thus, it is often difficult to compare obtained results with previous studies due to the usage of different conditions [40, 134].

To investigate the uranium tolerance of the bacterial cells in more detail, cell viability and metabolic activity of *A. facilis* growing in the presence of uranium were performed using flow cytometry. The cells were grown in liquid NB medium 1:5 diluted to avoid the precipitation of uranium. After an incubation time of 48 h at 30 °C the cells were stained with different fluorescent dyes to examine the viability and activity after uranium exposure. In contrast to the tolerance test on solid medium, these investigations revealed that a concentration of 0.05 mM reduced the activity and viability of the cells by nearly 50 %. Furthermore, at the highest concentration of 0.1 mM uranium 86.3 % of the total cells were not viable and only 6 % of the cells were active [127]. Thus, in liquid medium the cells were able to tolerate only half of the uranium concentration tested on solid medium, which could be explained by different conditions as described above, including bioavailability, diffusion and complexation of uranium in solution. In conclusions, with both investigations it was demonstrated that the tolerance of metals depends on different factors like mentioned above. The results obtained from this study revealed in different uranium concentrations for the tolerance of *A. facilis*. Thus, to study the ability of microorganisms to tolerate heavy metals and radionuclides, like uranium, different methods should be combined to obtain complete insights rather than misinterpretations.

Furthermore, TEM analysis were performed to examine the localization of the removed uranium by the bacterial cells. This method allows the visualization of uranium and other elements on a cellular level. In contrast, the visualization using SEM analyses is limited since this method is surface specific. By embedding and cutting the samples in ultra-thin sections it is possible to visualize the inside of the cells including sub-cellular structures. The TEM based investigation the of interaction mechanisms of *A. facilis* with uranium showed both, heavy metal uptake into the cytoplasm and immobilization on the outer cell membrane. Active bioaccumulation and passive biosorption could by identified as mechanisms of uranium removal from solution. Furthermore, uranium was localized inside the cell in association with polyphosphate granules, which are often found in the cytoplasm of bacteria as storage compartments for phosphorous compounds [135]. Microorganisms are capable to precipitate uranium by the degradation of intracellular polyphosphate granules resulting in the release or efflux of phosphate [136]. However, *A. facilis* interacts with uranium not by the release of phosphate to the surrounding solution (data not shown), uranium is immobilized inside the cytoplasmic polyphosphate inclusions. Similar observations were made by strains isolated from uranium waste piles [137]. In summary, an immobilization of uranium by microorganisms and by means of phosphates is not limited to

phosphate release or efflux, but may also occur via an uptake into the cell and binding uranium to phosphorus-rich compartments inside the cytoplasm. The investigation of *A. facilis* demonstrated that the described interaction mechanisms do not only occur as isolated processes, but occasionally super-impose with other processes and are thus sometimes difficult to recognize. In this case study, the bacterial cells were able to remove the uranium by biosorption on the outer cell membrane and by bioaccumulation within the cytoplasm. During the accumulation, uranium was immobilized by mineralization associated with phosphate in polyphosphate granules.
Moreover, detailed investigations on the molecular structure of cell-associated uranyl species (with *A. facilis*) may contribute to a better understanding of the response of gram-negative bacteria to uranium. This work demonstrates, that uranium is associated to the bacterial cell by the formation of different distinct species, like bound to carboxyl-groups and phosphoryl-groups, and furthermore to polyphosphate granules which are located within the cytoplasm. But not only the in depth described structural information of the uranium speciation, also the kinetically investigations during the removal experiments led to interesting findings. Within the first hours, uranium is mainly bound to the outer membrane of the bacterial cells and therefore, associated with lipopolysaccharides. Subsequently, within the following hours of incubation time, the association pattern change and it is indicated that uranium is additionally bound to carboxylic groups, corresponding to the peptidoglycan. Consequently, uranium immobilization by *A. facilis* represents a complex time-dependent process [138]. The majority of previous studies investigated the endpoint of removal experiments not considering transitional interaction mechanisms in the meantime [139–141]. With the kinetically investigations about the interactions of *A. facilis* with uranium this missing gap could be closed and furthermore, could demonstrate that interaction patterns changes during incubation time. It is important to develop a complete process understanding to predict the fate of radionuclides, like uranium, in the environment and consequently, promising remediation strategies could be developed [36, 142].
In order to obtain information about the prevalent oxidation state after uranium interaction experiments, the XANES region of the obtained X-ray absorption spectra were analyzed. The investigations clearly demonstrate that the removed and immobilized uranium is still prevalent as uranium(VI) and was not reduced to uranium(IV) (data not shown). In addition, uranium(VI) immobilization experiments with *A. facilis* were performed under anaerobic conditions (data not shown), due to the fact that previous studies displayed a uranium(VI) reduction by *Acidovorax* species. For instance, investigations in a long-term experiment (more than 2 years) to evaluate the *in situ* reduction of uranium(VI) at a highly uranium-contaminated site in Oak Ridge (TN, USA), proved the presence of *Acidovorax* spp. [143]. In addition, *Acidovorax* sp. was detected in a community of several metal reducing bacteria in microcosm experiments within sediment samples from the FRC Oak Ridge (TN, USA), to be capable of uranium(VI) reduction [144]. It was shown, that the presence of this denitrifier could contribute to the removal of competing electron acceptors and ensure the stability of the reduced uranium(IV) [145]. However, to stim-

ulate the microbial reduction of uranium(VI) in these previous studies ethanol and lactate were added as carbon source and electron donor, while the experiments performed in this thesis only used sterilized tap water without adding any carbon source or electron donor. This fact might explain the not-detectable uranium(VI) reduction by *A. facilis*. In addition, uranium(VI) reduction was detected within microcosm experiments, by meaning of a community composed of different microorganisms. Thus, the observed uranium(VI) reduction was therefore catalyzed by a community of different microbes. Which could be explaining that no uranium(VI) reduction took place, by performing experiments using a pure culture of *A. facilis*. Further experiments under oxygen-free conditions and by adding, for instance ethanol, should be performed to examine the physiological ability of *A. facilis* pure cultures to reduce uranium(VI).
In conclusion, to investigate the uranium removal capacity, the localization of the removed uranium, the ability to grow in the presence of uranium and furthermore the speciation of uranium associated with the bacterial cells, different methods were used and could deliver detailed informations about the mechanisms how *A. facilis* interacts with uranium(VI). The findings presented in this thesis contribute to a better understanding of microbial interactions with uranium and demonstrate that the investigated betaproteobacterium may play an important role for the prediction of the mobility and fate of uranium in the environment, as well as in contaminated sites, such as the former uranium mine Königstein. Together with the obtained fundamental understanding from this study and the ability of *A. facilis* to immobilize uranium fast and efficient, this bacterial strain could be appropriated for *in situ* bioremediation approaches.

4.2 The isolated yeast KS5 *(R. toruloides)* and its interaction mechanisms with uranium(VI)

To isolate and investigate fungal strains from the flooding water, by using culture-dependent methods, a special medium was needed. Therefore, SDA medium contains a high amount of glucose and is commonly used for fungal cultures [146]. After incubation on solid agar plates containing SDA medium the appearance of single colonies could be observed. The hereby obtained isolates were transferred to fresh liquid medium and phylogenetic affiliations by ITS rDNA gene analysis were performed. Due to its ability to grow in the presence of high uranium concentration the isolated strain KS5, identified as *Rhodosporidium toruloides* (also known as *Rhodotorula toruloides*) [147], was chosen to further investigate its interaction with uranium(VI) and to identify dominant interaction mechanisms. The morphological and physiological characterizations displayed typical yeast cell properties, which were in accordance to the phylogenetic results. Interestingly, *R. toruloides* is an oleaginous yeast with great biotechnological potential, due to its ability to accumulate up to 70 % lipids based on the dry biomass and moreover, regarding to its carotenoid biosynthesis [148].

To investigate the physiologically properties of this basidiomycete in more detail the utilization of different carbon sources was tested. In addition, to identify special capabilities as a result of the surrounding conditions present in the flooding water, a reference strain (DSM 10134 *R. toruloides*), was investigated. In agreement with previous studies the isolated strain KS5 as well as the reference strain DSM 10134 were able to utilize most of the tested carbon sources. However, both strains displayed less growth in the presence of galactose, which was also described before [148]. Despite the different conditions present at the isolating sites of the investigated strains, no drastic differences could be revealed. Only for the tested carbon source xylose differences were detectable. KS5 showed less growth, whereas DSM 10134 was not able to utilize the wood sugar. Several studies could demonstrate that the utilization of xylose to ethanol represents a useful process for the production of bioethanol performed by several yeast cells [149–151]. The question arises, why does the isolated strain KS5 exhibit the ability to metabolize xylose in contrast to the reference strain? One explanation could be the remaings of the former uranium mining industry. To underpin the shafts, wooden beams were used and, despite the ongoing flooding, they were left behind. As a consequence of the low TOC within the flooding water microorganisms have to develop adaption mechanisms in form of using alternative carbon sources. Thus, KS5 may have developed adaption mechanisms to survive at the present environmental conditions by using xylose as alternative carbon source. Nevertheless, further investigations should be performed to examine this hypothesis in detail, for instance by gene expression analyses to study possible involved genes responsible for xylose utilization using both strains.

Apart from the physiologically investigations on the isolated strain KS5, its ability to tolerate and even to grow in the presence of uranium were tested. Additionally, the interaction of KS5 with uranium was investigated to examine its potential for bioremediation approaches. Therefore, different methods were used, like growth behavior in liquid medium supplemented with different uranium concentrations and tolerance tests on solid medium containing uranium and other selected heavy metals. To investigate the uranium tolerance in liquid medium growth rate and doubling time were examined, in combination with flow cytometry to observe cell viability. The results clearly demonstrate that the isolated yeast is able to tolerate high uranium concentrations and furthermore, is able to grow in its presence. The tolerance test on solid agar plates revealed a MIC for uranium of 6.0 mM, which correlates to a concentration of 1.4 g/L. Compared to the prevalent uranium concentration within the flooding water of 8 mg/L, KS5 is able to tolerate a 175-fold higher uranium concentration. This high MIC results were coherent with finding from previous investigations, indicating that the occurrence of heavy metal tolerant strains increases with the increase of heavy metal or radionuclide concentrations at contaminated sites [152, 153]. Next to the high tolerances of uranium, KS5 also displayed a high tolerance against chromium (MIC 5.0 mM). The ability to grow in the presence of high chromium contents was demonstrated by a former study on *Rhodosporidium* sp. isolated from a metallurgical waste site [154]. This

again represents the adaption ability of the examined yeast cell to tolerate high concentrations of heavy metals, as well as radionuclides. To investigate the adaption mechanism of KS5 further analysis using gene expression should be performed. Interestingly, the tolerance against several heavy metals and radionuclides is not the only useful ability which was encountered for *Rhodosporidium* sp.. Previous investigations could demonstrate its utilization of organic sulfur for the removal from fossil fuels to increase their quality, which is also known as biocleaning [155]. In conclusion, with these first results it could be shown that KS5 represents a useful microorganism in many biotechnological applications regarding remediation of contaminated sites, as well as biocleaning.

However, to investigate the suitability of KS5 in possible bioremediation approaches at uranium-contaminated sites, like the former uranium mine in Königstein, it was necessary to study its uranium removal and immobilization capacity. Therefore, tests were performed to examine the removal of uranium from solution. The results obtained from this thesis could demonstrate a uranium removal of 350 mg U/g dbm (90 %) at the lowest tested dry biomass, representing the maximum capacity to remove uranium from solution. Compared to other fungal strains, KS5 could display an impressive uranium removal capacity. Previous studies on *Pleurotus ostreatus* revealed a maximum biosorption capacity for uranium of about 20 mg U/g dbm [156]. However, the observed strains of *Rhizopus* showed a removal capacity between 180 and 260 mg U/g dbm, which represents a high amount of immobilized uranium as well [157]. Nevertheless, KS5 seems to exhibit a high capacity even for fungi. Compared with the bacterial strains *Arthrobacte*r sp. and *Streptomyces longwoodensis* which display a uranium removal capacity of 600 and 440 mg U/g dbm [158, 159], gram-positive bacteria seem to be able to remove higher amounts. Nevertheless, it has to be considered, that the previous performed studies sometimes did not distinguish between immobilization of uranium by biosorption or bioaccumulation. However, the tremendous higher uranium removal by the bacterial strains could be a result of the high surface-to-volume ratio of bacterial cells. The large surface area of bacteria permits on the one hand the efficient uptake of nutrient and the release of metabolic waste products, and on the other hand the interaction with mobile metal fractions of the environment.

With the present results it could only be assumed that the isolated strain KS5 is able to tolerate high concentrations and remove high amounts of uranium. In order to investigate the underlying interaction mechanisms further investigations were performed, using TEM and EXAFS analyses. The obtained results clearly revealed the interaction mechanisms bioaccumulation, biosorption and biomineralization with uranium. In contrast to the examined bacterium *A. facilis*, where biosorption was identifies as the dominating process, nearly the whole mount of uranium was removed by bioaccumulation into the cytoplasm and on the inner cytoplasm membrane of the yeast cells. In addition, uranium could be detected inside lipid granules. The findings obtained from this thesis revealed an interesting cascade of metabolic response employed by the cells of naturally occurring yeast cells to mitigate high uranium concentrations and to survive over

an extended period of uranium exposure. The results could show that uranium was localized within the cytoplasm as well as concentrated in granules corresponding to lipid bodies. This compartmentalization of uranium within lipid granules in *R. toruloides* during 48 h of uranium exposure made uranium less available to the cells and consequently minimize its toxicity. Previous studies with cyanobacteria could demonstrate similar findings [160]. Uranium was localized in polyphosphate inclusion bodies, like described above for *A. facilis*. These kind of detoxification mechanisms such as bioaccumulation and "self-protection" play a key role in the migration behavior of radionuclides within the environment. So far, the mechanisms behind the uranium uptake within the cells are not completely understood. Uranium displays no known biological function compared with other heavy metals, and previous studies suggested that uranium may be taken up into the cells as a consequence of increased membrane permeability, which could be caused by its toxicity [39]. However, the findings obtained from the experiments performed in this thesis could demonstrate an active uptake mechanism of uranium within the cytoplasm of the yeast cells. The results revealed different underlying interaction mechanisms at two tested temperatures. Thus, only at a temperature of 30 °C uranium could be detected within the cells of KS5. On the contrary, at 4 °C no uranium was localized inside the cells and furthermore, the removal capacity was drastically reduced. By applying two different temperatures at uranium removal experiments it could be demonstrated, that active mechanisms are involved and furthermore, the uranium uptake by the cells is metabolism-dependent.

To investigate the speciation of the immobilized uranium at a molecular level EXAFS analysis was performed. The resulting spectrum could verify the formation of mineral-like structures, possibly meta-autunite. In accordance to the needle-like structures within the cytoplasm of the yeast cells, detected using TEM analyses, uranium was mainly bound via protonated phosphoryl groups. The formation of meta-autunite minerals by microorganisms as a response of uranium exposure was mentioned by previous studies [161–163] and seems to be an effective detoxification mechanism against uranium. Furthermore, the uranium sequestration as insoluble biominerals represents a promising technique for *in situ* bioremediation approaches, particularly at sites where bioreduction could be unfeasible due to the possible risk of reoxidation [87]. The main advantage of the formation of biominerals like meta-autunite is that the end-products are reported to be insoluble and do not undergo redox changes. Thus, the microbial formation of minerals, probably meta-autunite, by the isolated strain KS5 could represent a promising result to develop new bioremediation approaches. KS5 represents a perfect candidate for further investigations in industrial scale applications, due to its characteristics to persist high uranium concentrations, to remove high amounts of uranium within a short time, and its ability to form insoluble uranium minerals.

4.3 Microbial uranium(VI) reduction - most suitable capability for bioremediation approaches?

The investigations on anaerobic microorganisms within the flooding water of the former uranium mine Königstein demonstrated, that they are able to reduce uranium(VI) to uranium(IV) in efficient manner. Despite the prevalent oxidizing conditions, the obtained results revealed that anaerobic microorganisms survive within the flooding water and are even metabolically active. By adding 10 mM glycerol as an energy source and electron donor, the microbial reduction was stimulated. The dissimilatory uranium(VI) reduction under anaerobic conditions was first described by Lovley *et al.* [69], who reported that *Geobacter metallireducens* and *Shewanella oneidensis* are able to conserve energy for growth via the reduction of uranium(VI). These results could show that some specialized bacteria are able to reduce uranium(VI), whereby the identified species were mainly related to IRB and SRB [69, 70, 73, 164]. However, the identified bacteria including *Clostridium* sp. and *Desulfovibrio vulgaris* also reduce uranium(VI), but are unable to conserve energy for their growth resulting from this transformation [165, 166]. Investigations on the anaerobic microbial diversity in the flooding water of the former uranium mine Königstein at the beginning of the controlled flooding process using culture-independent methods revealed in the presence of aSRB [167]. Additionally, after the ongoing flooding process (2014) culture-dependent approaches were applied to investigate the most probable number (MPN) of aSRB (after [168, 169]) resulting in a probable cell number of 0.21-1.5 MPN/mL of metabolically active heterotrophic aSRB [109]. The same investigations were performed two years later and could confirm the previous results but showed an increase in the most probable cell number to 4.8 MPN/mL. Due to the changing conditions in the underground resulting from the ongoing flooding process this slight aberration could be explained. Furthermore, the underground of the mine is not completely mixed and areas with anoxic conditions are quite likely. In such areas, aSRB could be enriched, explaining the minor increase in the cell number. In conclusion, different previous investigations identified the presence of aSRB and in addition their metabolically activity within the flooding water of the former uranium mine Königstein. For that reason, studies in this thesis were performed to investigate the possible microbial uranium(VI) reduction ability by anaerobic bacteria present within the flooding water.

First, to stimulate microbial uranium(VI) reduction it was necessary to figure out the most suitable carbon source and electron donor. At acid conditions, like predominating within the flooding water of the former uranium mine, the microbial reduction of uranium(VI) and other compounds can be affected by different factors. Thus, the proton concentration represents a major key player. Since the pH scale is a logarithmic scale, at pH 4 1.000 times more protons are present compared to pH 7. This causes a diffusion pressure towards the cell membrane of microbes, consequently much more protons diffuse through it. By active pumping processes, these protons have to be

pumped out of the cell, resulting in the destruction of the proton motive force. For that reason, at low pH microorganisms need to invest additional energy to maintain a higher internal pH. Consequently, less energy is available for growth [170, 171]. Only if this extra needed energy can be compensated, microbial growth can be achieved. Furthermore, carbon sources in form of organic acids can act inhibitory at acid conditions. The toxicity is dependent on their dissociation constants since different concentrations of the protonated form would be present at different pH values. As an example, the pK_a of lactic acid is 3.08, therefore at pH 3 half of the lactate would be present as protonated species. At acid conditions the undissociated organic acid form is able to diffuse into the cell. Due to the higher pH inside the cell, the acid dissociates, releases protons and consequently lowers the internal pH. Additionally, protons have to be pumped out and this again implies an energy loss. For that reason, previous studies on the isolation of aSRB within acidic environments failed using lactate as an electron donor, which represents the most common substrate at neutral conditions. Subsequent studies suggest, that non-ionic substrates like glycerol, hydrogen, alcohols or sugars are more convenient [169, 170, 172–174]. In conclusion, for the enrichment of acidophilic SRB and IRB within the flooding water the non-ionic electron donor glycerol was chosen.

To investigate a possible microbial reduction of uranium(VI) 10 mM glycerol were added directly to the flooding water, which was previously flushed with nitrogen to eliminate remaining oxygen. Afterwards, the flooding water samples were incubated for six weeks at 30 °C. By regular measurements a decrease of the redox potential could be detected from initially 670 mV to 230 mV. Theoretical calculations of the prevalent uranium speciation (Figure 4.1) demonstrated a change in the redox state from uranium(VI) to uranium(IV). However, by applying the geochemical calculations only the available thermodynamic constants (respective formation constants) of inorganic chemical parameters at 25 °C, like cations and anions, were considered [26], meaning, neither the addition of 10 mM glycerol nor the possible production of microbial metabolites were included, due to the lake of respective formation constants. In addition, no kinetic process was included. Consequently, the resulting diagram may not completely display the complex composition prevalent within the flooding water. However, the received diagram represents a simplified system to gain a rough estimation about the dominant uranium species after six weeks of incubation. In agreement with previous studies, the prevalent uranium species within the flooding water of the former uranium mine Königstein could be identified as the highly soluble uranium sulfate (UO_2SO_4 , Figure 4.1 square) [30]. Furthermore, the authors suggested, as a consequence of the ongoing flooding process, an increase of the pH value, due to dilution processes, and a decrease of the redox potential. Thus, the speciation of uranium would change to a solid uranium(IV) phase, either at pH values above 4.2 or at a redox potential below 300 mV [30].

The obvious decrease of the redox potential after six weeks would represent, in comparison with the theoretically calculations, a change in the redox state of uranium (Figure 4.1 circle). To verify the first results further methods, like XANES and UV-vis measurements, were used.

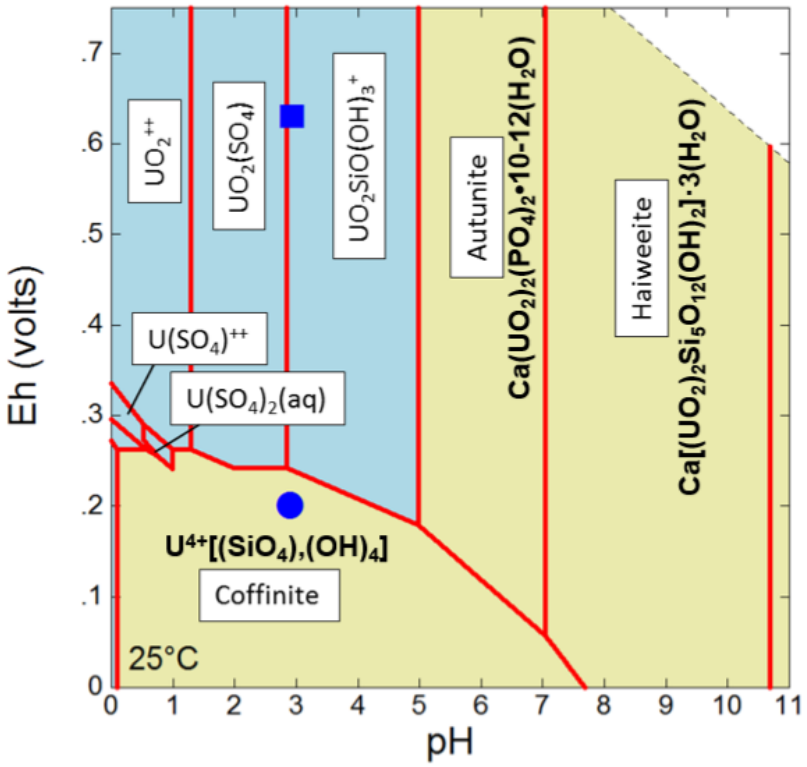


Figure 4.1: pH-Eh predominance area diagram for uranium at 25 °C using the mean elemental concentrations of the flooding water (see Table 1, Chapter 3.4). Theoretical geochemical calculation of the prevalent uranium species was performed using geochemical speciation code "Geochemist's Workbench" Version 8.0.8/ACT2. Yellow areas represent solid uranium phases whereas the blue areas designate aqueous species, the square represents the initially condition at 670 mM, the circle represents the conditions after 6 weeks at a redox potential of 230 mM.

In accordance with the geochemical calculations, a microbial uranium(VI) reduction was identified. XANES spectroscopy was used to determine the oxidation state of uranium and could display an increase of uranium(IV) after five weeks. After six weeks only uranium(IV) could be detected. However, uranium was not reduced within the control samples without microorganisms or glycerol (data not shown), indicating that the addition of 10 mM glycerol in combination with anaerobic conditions resulted in a complete reduction of uranium(VI) induced by active microorganisms prevalent within the flooding water. In agreement with previous studies, the microbial uranium(VI) reduction induced by adding an electron donor within environmental samples in laboratory experiments and also *in situ* was described and seems to be a promising state-of-the-art technique for applications in the field [175–180].

Apart from the determination of the oxidation state of uranium, also iron and sulfate were investigated. During six weeks of incubation, different anaerobic biogeochemical processes could be detected. According to the change of the redox potential, next to a uranium reduction also iron

was reduced. By measuring the Fe(II) concentration during six weeks of incubation a slight increase could be detected, thus Fe(III), prevalent within the flooding water, was microbial reduced. The kinetic investigations revealed the Fe(III) reduction took place within the first three weeks. Compared with the measured redox potential and the resulting standard redox potentials these results are in good agreement (Table 1). During the first weeks and relatively high redox potentials the microbial induced Fe(III) reduction is the dominating process. Afterwards, and by a drastically decrease of the redox potential the dominating redox reaction is the microbial uranium(VI) reduction. However, contrary to the results of the Fe(II) and uranium(VI) reduction no significant change of the sulfate concentration could be detected. In accordance to the standard redox potential (Table 1) a microbial sulfate reduction at 230 mV is rather unlikely. With the addition of 10 mM glycerol to the flooding water it is possible to induce a microbial uranium(VI) reduction. However, to understand the underlying processes in detail it is necessary to have knowledge about the microbial diversity responsible for the reduction of uranium. Therefore, DNA was isolated from samples incubated for six weeks and analyzed with regard to the phylogenetic composition of their microbial communities. The results clearly indicate the dominance of IRB which also explain the initial Fe(III) reduction followed by the uranium(VI) reduction. By 16S rDNA gene analysis more than 40 % of the bacteria were identified as IRB. In minor amounts (< 1%) also IOB were identified, belonging to genus *Acidithiobacillus*, which were found by previous studies within the flooding water. This IOB was detected using culture-independent as well as culture-dependent methods, indicating that these bacteria are metabolically active [109]. Furthermore, *Acidithiobacillus* represents an ubiquitous inhabitant of AMD sites, and is often the dominating group [182, 183]. As a consequence of the changing conditions after flushing with nitrogen and the addition of glycerol, IOB represent after six weeks incubation only a minority of the bacterial community. Nevertheless, the investigations of the bacterial diversity showed no occurrence of SRB, in accordance with the results of sulfate concentration during the six weeks of incubation. However, in contrast to that, by culture-dependent methods they could be detected [109]. In summary, by analytical investigations of iron and sulfate reduction, together with molecular methods of the bacterial diversity, it could be assumed that the microbial uranium(VI) reduction within the flooding water was mediated only by the activity of IRB.

The reasons for this non-successful stimulation and detection of SRB could be the not suitable reducing conditions (high redox potential), thus SRB were not metabolic active and consequently, the cell number was too low for detection. For the culture-dependent determination of SRB

Table 1: Caption

	Chemical equation	Standard redox potential (mV)
Iron	$Fe^{3+} + e^- \longrightarrow Fe^{2+}$	+770 [181]
Uranium	$U_2^{2+} + 2\,e^- \longrightarrow UO_2$	-42 to +86 [88]
Sulfate	$SO_4^{2-} + 8\,e^- + 10\,H^+ \longrightarrow H_2S + 4\,H_2O$	-220 [88]

within the flooding water a special designed medium was used [109]. However, in this study only glycerol was added to stimulate anaerobic reducing bacteria. Furthermore, to achieve reducing conditions (the redox potential of the medium must be below -150 mV) in the prepared medium reductants like sodium sulfide or sodium thioglycolate, with sodium ascorbate, were added [184]. During the uranium(VI) reduction experiments, performed in this study, no additional reductants were added to avoid uranium(VI) reduction by these substances. The presence of metals as cofactors for several enzymes of SRB is also an important factor. For instance, nickel and selenium are required for their hydrogenase activity. In addition, iron and calcium represent essential elements for microbial metal reduction [184]. Nevertheless, the missing of essential metals may not be the reason for the non-successful stimulation of SRB, due to the fact that the flooding water contains high concentrations of the mentioned metals. Although, previous studies demonstrated that glycerol represents a suitable electron donor and carbon source for the enrichment of SRB under acidic conditions, other substrates should be taken into consideration. Another explanation could be the added concentration of glycerol (10 mM), which could be the limiting factor. Despite previous tests (data not shown), which demonstrated high levels of glycerol after six weeks of incubation time, the concentration could be too low after the metabolic activity and uranium(VI) reduction of IRB. On the contrary, the chosen glycerol concentration could also be too high and could act inhibiting for the metabolic activity of SRB. Also, the incubation time or chosen temperature could be a limiting factor for SRB. So far, the non-successful stimulation of SRB within the flooding water during uranium(VI) reduction experiments seems to be unclear. However, also without the presence of SRB the microbial mediated uranium(VI) reduction by IRB was successful.

In conclusion, results obtained by the use of geochemical methods and molecular biology demonstrate that the indigenous microorganisms within the flooding water of the former uranium mine Königstein display the ability to utilize glycerol as an electron donor and uranium(VI) as an electron acceptor. The addition of glycerol stimulates the microbial reduction capability of uranium(VI) directly, by serving as electron donor for both Fe(III) and uranium(VI) reduction. The microbial induced uranium(VI) reduction is therefore the result of the metabolic activity of IRB. Based on the successful stimulation of microbial uranium(VI) reduction in laboratory scale experiments, a pilot plant for industrial scale applications was designed, to verify the applicability to use this bioremediation approach directly on site of the former uranium mine Königstein. For this purpose, 100 L flooding water were used and treated in the same way like the 1 L samples. Similar to the laboratory scale experiments, samples were examined with regard to a possible uranium(VI) reduction. An online measurement system was applied to obtain real-time information about pH value, redox potential and temperature. Moreover, Fe(II) and sulfate concentration were measured and with molecular genetic methods the microbial diversity was determined after six weeks. Two independent runs of the pilot plant revealed in nearly similar results. During the incubation time of six weeks a distinct decrease of the redox potential was

detectable. In comparison to the laboratory scale experiments (1 L), a more drastic decrease to about 100 mV was measured, which implies a difference of more than 130 mV. In addition, UV-vis measurements were performed to obtain detailed information about the oxidation state of uranium present within the flooding water. A distinct uranium(IV) concentration could be detected after 21 days of incubation. Compared to the experiments performed in laboratory scale the microbial uranium(VI) reduction took place earlier. In laboratory scale experiments, uranium(IV) could be measured after 35 days, by meaning two weeks later compared to the pilot plant. In summary, in consideration of the redox potential and the oxidation state of uranium, reducing conditions were faster adjusted in the 100 L approach of the pilot plant.

Furthermore, similar to the laboratory scale experiments the Fe(II) and sulfate concentration was investigated. However, the first run could not detected a distinct Fe(II) reduction, due to a possible oxygen invasion during the first weeks, the second run confirmed the results from the previous experiments in laboratory scale. Despite these non-successful determination of Fe(II) by analytical methods, pictures of the flooding water, incubated in the pilot plant, revealed an obvious change of the color during six weeks of incubation time (Figure A1). After filling and flushing with N_2 the color of the water turned from colorless to brownish-orange (Figure A1, 3 h), which indicates a oxidation of Fe(II) to insoluble Fe(III) participates. Starting from day 7 the color disappeared and the flooding water displayed colorless like initially. In conclusion to these observations, during the first three weeks a slight but significant increase of Fe(II) could be analytical detected at the second run of the pilot plant. Moreover, in contrast to the previous studies with 1 L flooding water, a slight decrease of sulfate was observable during the last days of the incubation, indicating a microbial sulfate reduction. In conclusion, during the first days of incubation a slight but distinct Fe(III) to Fe(II) reduction was demonstrated, followed by uranium(VI) reduction induced at day 21 and a sulfate reduction after day 30 (Figure 4.2). With the obtained results from this study, it is possible to understand the synergy of the underlying microbial reduction processes and furthermore, to predict the fate of metals, especially uranium, in the environment under reducing conditions. As a consequence of the availability of different metals and ions within the flooding water of the former uranium mine in Königstein, it is not surprising that several reactions will take place simultaneously. However, for possible bioremediation approaches this obtained knowledge could help to predict processes more specifically and furthermore, to demonstrate that other supportive redox reactions might take place, resulting in a decrease of other soluble metals and increase of pH values.

In addition, the bacterial diversity was investigated using 16S rDNA gene analysis. However, it was not possible to obtain enough sequence information to get an entire overview about the bacterial diversity after six weeks within the approach of the pilot plant. Only ten sequences could be obtained and were compared to the NCBI database. The majority of these sequences were identified as *A. ferrooxidans* (60 %), followed by *Desulfovibrio* spp. and *Acidocella* spp.. Two sequences could be associated with the phylum Firmicutes. Despite only a few sequences

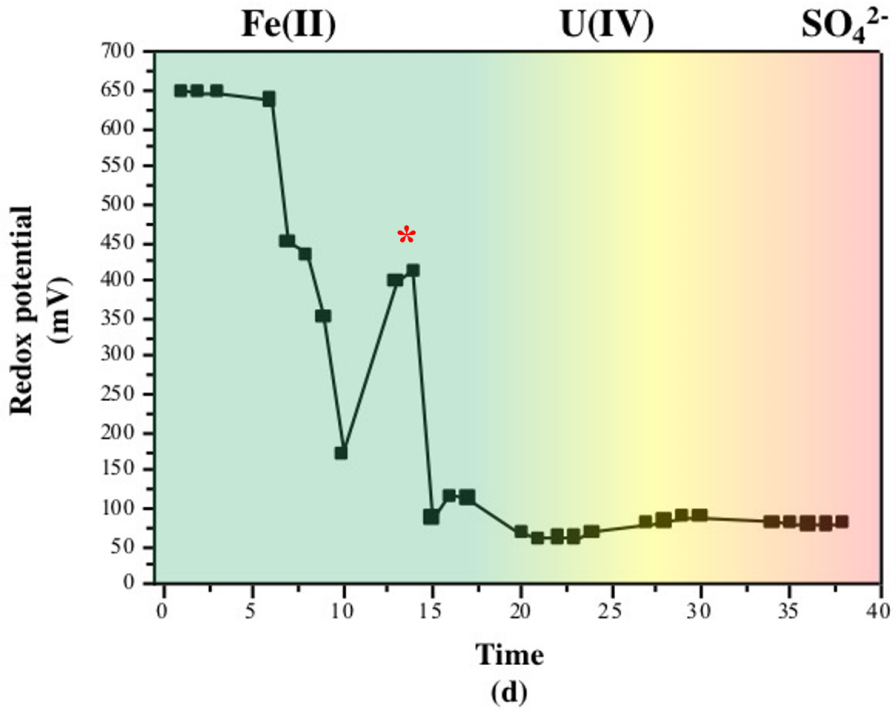


Figure 4.2: Measured redox potential of the pilot plant during six weeks of incubation. Colored areas display the microbial redox reaction taken place while incubation, green - iron reduction, yellow - uranium reduction and red - sulfate reduction. Red star indicates a distinct increase of the redox potential, possibly caused by an oxygen invasion.

could be obtained, all were associated with either IRB/IOB or SRB, which is in accordance to the results to the laboratory scale experiments.

Previous studies demonstrated the ability of *A. ferrooxidans* to reduce metals [185]. These bacteria are able under aerobic conditions to oxidize elemental sulphur to sulphuric acid [186]. Moreover, *A. ferrooxidans* could be identified as a major part of the consortium found at AMD sites and it could be shown that these species are tolerant to uranium [187, 188]. In addition, *A. ferrooxidans* can grow under anaerobic conditions using Fe(III) as final acceptor and sulphur as substrate [186]. More than a half of the identified sequences could be associated with this species, which is also known to reduce uranium(VI) [186]. Thus, these bacteria could be one of the species which are responsible for the detected microbial Fe(II) and uranium(VI) reduction. Interestingly, previous investigations of the microbial diversity within the flooding water identified *A. ferrooxidans* as a dominating group, which is in good accordance of these findings [109]. In addition, the heterotrophic *Acidocella* spp. represent IRB, which are known to be extremely acidophilic. A well-studied strain of *A. aromatica* (PFBC) is able to catalyzes Fe(III) reduction under micro-aerobic and anaerobic conditions [189]. Furthermore, experiments demonstrated the ability of chromium reduction and immobilization by *A. aromatica* [190]. Experiments performed to investigate the metal resistance of *Acidocella* strains revealed high tolerances of

zinc, nickel, copper, and cadmium [191]. In comparison with previous studies on the bacterial diversity of the flooding water in Königstein using culture-independent and culture-dependent methods, the species of *Acidocella* spp. was detected as well [109]. Until now, no uranium interaction mechanisms of *Acidocella* spp. are described. However, with this study it was shown that this species could be involved in microbial uranium(VI) reduction within the flooding water. Further investigations should be performed to examine the possible uranium interaction mechanisms of the *Acidocella* species occurring in the flooding water of the former uranium mining site Königstein. The in addition identified species *Desulfovibrio* was described by Lovley and Phillips [70] for its uranium(VI) reduction capability. The authors highlighted that the enzymatically uranium(VI) reduction was mediated by the cytochrome c_3. Moreover, the enzyme could by successfully cloned and expressed in two other strains, suggesting that the ability to reduce uranium(VI) could be genetically combined with other metabolic useful properties, like the ability to degrade organic contaminants and denitrification [192]. The previously obtained results demonstrated, that *Desulfovibrio* species are able to reduce uranium(VI) under anaerobic conditions and thus, they could be responsible for the microbial mediated uranium(VI) reduction of flooding water of the pilot plant. Two sequences could be identified with high similarity to the phylum of Firmicutes. By investigations using culture-dependent methods, the species *Desulfosporosinus* could be enriched from the flooding water samples. A well-defined medium for acidophilic SRB [168] was used and incubated with several dilutions of the flooding water (Figure A2). Microscopically investigations displayed bacterial cells containing spores (Figure A3). Previous investigations using similar conditions and 16S rDNA analyses demonstrated the presence of the spore-forming bacterium *D. acidophilus* [109]. This bacterium represents an obligate anaerobic, spore-forming, acidophilic SRB, which was isolated previously from an AMD site [169]. In addition, the SRB bacterium *D. reducens* was described for its ability to reduce metals, like Mn(IV), Fe(III), and Cr(VI). Even uranium(VI) was reduced to uranium(IV) [193]. Thus, the previous detected and described bacterium *Desulfosporosinus* spp. could be, within a consortium of several bacteria, responsible for the microbial uranium(VI) reduction within the flooding water samples of the pilot plant approaches. Despite the differences of the microbial diversity detected within the laboratory scale experiments and the pilot plant approaches, mainly MRB were identified. The obtained results indicate a combined uranium(VI) reduction, mediated by several species, which seems to be reasonable due to the relative high microbial diversity within the flooding water of the former uranium mine.

In conclusion, the results from the pilot plant experiments confirm the microbial reduction of uranium(VI) under anoxic conditions, only by adding of 10 mM glycerol, in up-scaling setups. These findings suggest the applicability of microbial uranium(VI) reduction at the site of the former uranium mine Königstein as a preferred bioremediation approach. Due to the usage of the cost-effective electron donor glycerol and the fast bioreduction process in just six weeks, the microbial-mediated uranium(VI) reduction represents a suitable alternative, which could replace

the traditional waste water treatment. Nevertheless, further experiments should be performed to support this hypothesis and to repeat the experiments in industrial scale applications (> 100 L). In addition, the microbial diversity should be investigated in more detail, to obtain a complete overview of the microorganism, responsible the for uranium(VI) reduction.

4.4 Possible applications for *in situ* bioremediation at the former uranium mining site Königstein

Resulting from the obtained findings within this thesis, different options could be possible to remediate the former uranium mining site by using microorganisms. To select the most suitable process it has to be determined which approach provides a low-cost treatment, at a high efficiency and minimizing the volume of chemical and/ or biological sludge to be handled [104].
The bacterial strain *A. facilis* and the yeast *R. toruloides* were able to remove high amounts of uranium by the two identified interaction mechanisms, biosorption and bioaccumulation. *A. facilis* was identified by culture-independent, DNA-based, methods. However, the investigated strain was not directly isolated from the flooding water. Therefore, the bacterial cells have to be pre-cultured in large scale procedures to obtain high amounts of biomass. In additional steps the cells have to be separated from the growth medium and transferred to the flooding water to remove the soluble uranium. Thus, the use of *A. facilis* for *in situ* bioremediation could display further challenges, including additional facilities for the growth of the bacterial cells.
Besides, the major interaction mechanism of *A. facilis* with uranium is biosorption. Most researchers define biosorption as a passive and metabolically-independent process [103, 105, 194] which can be performed by dead biomass or cell fragments with the advantages of easy and safe handling and preparation. However, biosorption can also be performed by living cells as a passive uptake or metabolically-independent adsorption via surface complexation onto cell walls or outer membranes [105, 194]. The biosorption of metals and radionuclides by microorganisms can be affected by different factors, like solution pH, ionic strength, initial pollutant concentration, other pollutants or competitive ions, the biosorbent itself, temperature and the speed of agitation. The most important factor affecting biosorption represents the chemistry of the biosorbents themselves and their availability of metal-binding sites, the activity of functional groups and the competition with coexisting ions within the solution [34]. For instance, an increase of the surrounding pH value enhances the removal of cationic metals, but reduces the removal of anionic metals [104]. Among the biotechnological applicability, a number of proposed biosorption processes have been patented for commercial application. Fomina *et al.*, 2014 [104] published a full list of patents related to biosorption from 1973 till 2011. However, despite the numerous results which could be obtained over decades of research, most biosorption processes are still at the laboratory scale. Reasons for this could be a poor understanding of the underlying mechanisms,

the kinetics and thermodynamics of the process [104]. On the other hand, previous studies suggested an insufficient specificity and stability of biosorbents, which cause the little progress in industrial scale applications [102]. In conclusion, the potential use of *A. facilis* for bioremediation approaches at the former uranium mining site Königstein is rather low and will likely be used only as supporting process in remediation application in the future, as a consequence of the lacking knowledge of the underlying mechanisms of biosorption of metals and radionuclides on microbial surfaces and their potential stability.

Due to the mentioned uncertainties, many established bioremediation approaches are based on active processes, conducted by living cells. Compared to the direct reduction of uranium, which would lead to an immobilization, the process of biosorption is subsequently faster. However, the uranium-polluted flooding water is poor in biomass concentration, due to the high toxicity of uranium and low TOC content. Therefore, biosorption alone may not be sufficient to bioremediate uranium from polluted sites, unless the biomass content is increased [111].

R. toruloides (KS5), in contrast, was directly isolated and experiments displayed that this strain is able to grow directly within the flooding water (data not shown) only by adding a carbon source in the form of sugar. As a consequence, no additional industrial processing with special growth media or growth reactors would be necessary to obtain large amounts of biomass for the removal of uranium directly from the flooding water. Furthermore, the uranium tolerance test displayed that the yeast cells are resistant to the prevalent uranium concentrations, and could tolerate even higher uranium concentrations. The uranium removal by KS5 cells is fast and efficient, nearly the whole amount of soluble uranium in solutions was immobilized during 48 h incubation. In contrast to the bacterial cells of *A. facilis*, the predominant interaction mechanism of the yeast cells with uranium is bioaccumulation, the active uptake within the cells. Also the removal efficiency of KS5 compared to the bacterial cells of *A. facilis* is tremendously higher. As described above, preferentially used bioremediation approaches are based on active interaction mechanisms. Thus, the isolated strain KS5 could represent a candidate to investigate its applicability in larger scale experiments, like pilot plants. Furthermore, uranium is immobilized as minerals associated with phosphate, which exhibit a low solubility and remain stable under changing conditions. Further experiments should be performed such as growing the yeast cells directly within the flooding water and examine its uranium removal ability at the prevalent conditions.

However, the stimulation of anaerobic microorganisms to reduce aqueous uranium(VI) into insoluble minerals *in situ* may provide a cost-effective and non-invasive alternative to remediate radionuclide-contaminated sites. The employment of bioreduction, in particular, appears promising, with the suitable applicability in the field [87]. No additional technical applications to separate the uranium-bearing microorganisms from solution would be necessary and furthermore, the elaborated pump-and-treat technique could be discontinued. The idea was to stimulate microbial growth and activity directly within the flooding water and to use the underground itself as sediment basin for metal sludge formed by precipitation of uranium as well as sulfide and other

metals. This *in situ* treatment using anaerobic microorganisms is a possible low-cost and low maintenance concept for acid mine water processing. Thus, costs for sludge handling could be minimized or eliminated and no additional heavy metal polluted waste would occur. Since the reduction of sulfate could be detected during the last days of incubation time, a longer incubation would possibly lead to a complete sulfate reduction and consequently an increase of the pH value. Thus the high sulfate concentration could be removed from the AMD flooding water with a concomitant increase of the acid pH [195], consequently less chemicals would be necessary for the water treatment at the site of the former uranium mine in Königstein.
The results obtained in this thesis, indicate that the isolated strain KS5, but also anaerobic microorganisms indigenous within the flooding water of the former uranium mine Königstein, may have a crucial role in the bioremediation of uranium at this investigated site and should be taken into consideration for alternative strategies. However, the investigations on the anaerobic microbial uranium(VI) reduction could be transferred to industrial scale applications, and thus represent the most suitable and best characterized approach concerning applicability for *in situ* bioremediation processes at the site of the former uranium mine Königstein.

4.5 Conclusion and scientific relevance

This thesis provides new insights about the interaction of indigenous microorganisms form uranium-contaminated environments. It was shown that the fate and transport behavior of uranium within the environment and at the former uranium mining site strongly depends on the presence and metabolic activity of natural occurring microorganisms. Furthermore, the results demonstrate that the investigated strains *A. facilis* and *R. toruloides* are able to remove high amounts of uranium from surrounding solutions by passive biosorption and active bioaccumulation. In addition, a correlation between microorganisms isolated from heavy metals polluted sites, and high tolerances against them were highlighted. With the obtained results from experiments with anaerobic microorganisms and their potential of uranium(VI) reduction, a pilot plant was developed to verify the previous findings. The results from laboratory scale experiments were successfully transferred and simultaneously confirmed by experiments in larger scale applications, indicating that with the provided knowledge *in situ* bioremediation approaches could be applicable in the future. By comparison of the possible advantages and disadvantages of the investigated interaction mechanisms, it was shown that bioremediation using anaerobic MRB for the reduction of uranium(VI) seems to be the most suitable method.
With this thesis, strategies are presented for possible alternative concepts which could support the conventional and elaborated water treatment of former uranium mining sites, or in future steps to replace them. However, it has to be taken into consideration to prevent or minimize the generation of uranium-contaminated AMD sites at the beginning. By meaning, when there

is the risk of AMD generation, the first option should be avoiding the formation itself [170]. Johnson and Hallberg outlined in their review [196], "prevention is better than the cure", and described approaches, which could be used to prevent or minimize the generation of mine drainage waters. Summarizing, as long as the activity of acidophilic microorganisms increases the formation of AMD sites, technologies should be used, to avoid either oxygen, water or both from contacting the ore, which could elude their microbial activity [197–199]. While metals are mined by conventional methods, which result in the formation of acidic waters mobilizing heavy metals like uranium, extensive waste water treatment processes have to be performed. Therefore, bioremediation represents a promising problem-solving approach.

The obtained results from this thesis were published in international scientific journals and presented at international conferences. Therefore, they are available to a broad community interested in alternative approaches for the remediation of heavy metal polluted sites, like the former uranium mine Königstein. On that account, these findings should be taken into consideration for future remediation applications.

5 Outlook

Although the revealed results could give detailed and unique insights about the interaction mechanisms of the investigated microbial strains with uranium(VI), some open questions still remain. Until now, the uptake process of uranium inside the cells is unknown. With the obtained results from this thesis it could be demonstrated, that the bioaccumulation of uranium seems to be an active mechanism, by meaning it is dependent on the microbial metabolisms. Nevertheless, transporters for uranium-uptake are unidentified. For that reason, investigation should be performed to identify possible transport-proteins which may be responsible for the uptake of uranium. In addition, gene expression analyses could be used to gain information about possibly involved genes. Therefore, this identified genes could be transformed and expressed in reference strains or other microorganisms to investigate the resulting uranium removal efficiency. Consequently, such genetically modified microbes could be used to understand the uptake mechanism of uranium on the one hand, and they could be used for bioremediation approaches at other contaminated sites on the other hand. However, the use of genetically modified microorganisms within the environment is difficult, thus indigenous strains should be preferred. The strain KS5 (*R. toruloides*), which was well described within this study, should be examined in further experiments using directly the flooding water. In further steps the obtained results from laboratory scale could be transferred to industrial scale applications to design a pilot plant, placed directly on site, possibly connected with the borehole.

Moreover, in these studies only a few microorganisms were investigated regarding their interactions with uranium(VI). With *A. facilis* and *R. toruloides* exclusively pure cultures of these strains were used for the experiments. However, in nature microbes occur in communities composed of a large variety of different bacteria, eukaryotes and archaea. Thus, it seems to be necessary to perform microcosm experiments using environmental samples to achieve similar conditions to those found within the flooding water. By using anaerobic microorganisms for uranium(VI) reduction directly within the flooding water these conditions were almost achieved. Nevertheless, working under oxygen-free conditions needs further treatment of the water and a special design of reactors or pilot plants.

The microbial community after anaerobic incubation over six weeks was analyzed using 16S rDNA gene analyses of two pooled samples. Thus, not the complete diversity, including bacteria, archaea and eukarya, could be displayed. Consequently, further investigation should be performed to obtain a detailed and closer view of the microbial community within the flooding

water samples. In addition, the same investigations should be implemented using samples from the pilot plant to compare both experimental approaches in detail. Thus, the microbial diversity could be compared to obtain results about possible differences between the two different approaches, for instance changes due to the up-scaling process. Furthermore, to gain a detailed process understanding a quantification of the microbial biodiversity should be performed, to know which microorganisms are mainly responsible for the microbial uranium(VI) reduction. Therefore, 16S/18S rRNA gene analyses in combination with meta-transcriptome analyses could be used to identify the dominating metabolic active microorganisms. Furthermore, meta-proteomic analyses could be performed to identify proteins involved in uranium stress response to clarify the underlying mechanisms of the microbial uranium(VI) reduction.

Glycerol represents a cost-effective and non-toxic polyhydric alcohol, which is industrially used in food- and pharmaceutical industry. Nevertheless, other similar carbon sources should be taken into consideration to stimulate the microbial uranium(VI) reduction. Therefore, further experiments with alternative carbon sources, like ethanol and sugar derivates, should be performed to investigate their efficiency in stimulation of microorganisms responsible for metal-/radionuclide reduction.

Another important area for further investigations is the determination of the stability or longevity of the bioreduced uranium(IV) complexes/minerals, particular if the environmental conditions changes. For instance, re-oxidation processes of uranium(IV) are well described and known to be catalyzed by oxygen, nitrate, Fe(III) minerals, oxides, organic ligands and bicarbonate [145, 200–208]. At the moment only half of the underground mine is flooded, meaning there are large surfaces contacting the water with the surrounding atmosphere. Consequently, within the flooded underground the prevalent redox processes are still oxidizing and oxygen could re-oxidize the microbial reduced uranium(IV). Previous studies using glycerol phosphate as electron donor could demonstrate, that uranium(VI) was successfully reduced by microorganisms and precipitated as uranium phosphate minerals [209]. The precipitation of phosphate minerals is a promising alternative due to the high stability to oxidative changes and their longevity, which has been demonstrated in natural analog sites [49, 210–212]. Therefore, it could be promising to stimulate the microbial reduction of uranium(VI) within the flooding water by adding glycerol phosphate, which could enhance, under the prevalent conditions, the stability of the reduced uranium(IV). With XANES spectroscopy it was possible to determine the oxidation state of uranium within the samples, after stimulation of microbial activity by adding 10 mM glycerol. However, due to the low content of uranium in the natural samples, it was not possible to identify the speciation of the reduced uranium(IV). Further investigation on the formed uranium(IV) species should be performed to understand the microbial reduction of uranium(VI) on molecular level. In addition, the information about the species of uranium(IV) could help to predict the stability of the formed uranium(IV) complexes and to estimate their stability within the underground of the former uranium mine.

Bibliography

[1] J. F. Ahearne, *„Radioactive Waste: The Size of the Problem“*, Phys. Today **50**, 24 (1997).

[2] M. Hagen and A. T. Jakubick, *„Returning the WISMUT legacy to productive use“*, in *Uranium in the Environment - Mining Impact and Consequences*, P. 11–26 (2006).

[3] Wismut GmbH, *„http://www.wismut.de“* (25 Oct 2017]).

[4] M. Paul, E. Kreyßig, J. Meyer and U. Sporbert, *„Remediation effects of the WISMUT project to surface waters in the Elbe watershed: An overview“*, P. 435–450, Springer-Verlag Berlin Heidelberg (2008).

[5] Wismut GmbH, *„Sonderausgabe 25 Jahre Wismut GmbH“*, in *Dialog - Mitarbeiterzeitschrift der Wismut GmbH*, P. 10–17, 90. Ed. (2016).

[6] S. Eulenberger, U. Jenk and M. Paul, in *Mine Water and Circular Economy IMWA 2017*, Ed. C. Wolkersdorfer, L. Sartz, M. Sillanpää and A. Häkkinen, P. 296–302, Lappeenranta, Finland (2017).

[7] J. Schreyer and U. Zimmermann, in *Uranium, Mining and Hydrogeology II*, Ed. B. Merkel and C. Helling. Sven von Loga, Köln (1998).

[8] U. Jenk, K. Nindel and U. Zimmermann, in *Uranium, Mining and Hydrogeology*, Ed. B. J. Merkel and A. Hasche-Berger, P. 431–436. Springer Verlag (2008).

[9] J. Schreyer, U. Zimmermann and U. Jenk, in *Uranium in the Aquatic Environment*, Ed. B. J. Merkel, B. Planer-Friedrich and C. Wolkersdorfer, P. 755–762. Springer (2002).

[10] M. Birke, U. Rauch, H. Lorenz and R. Kringel, *„Distribution of uranium in German bottled and tap water“*, J. Geochem. Explor. **107**, 272 (2010).

[11] A. Akcil and S. Koldas, *„Acid Mine Drainage (AMD): causes, treatment and case studies“*, Journal of Cleaner Production **14**, 1139 (2006).

[12] A. Peppas, K. Komnitsas and I. Halikia, *„Use of organic covers for acid mine drainage control“*, Miner. Eng. **13**, 563 (2000).

[13] A. Azapagic, *„Developing a framework for sustainable development indicators for the mining and minerals industry“*, Journal of Cleaner Production **12**, 639 (2004).

[14] J. R. Lloyd, D. R. Lovley and L. E. Macaskie, *„Biotechnological Application of Metal-reducing Microorganisms“*, Adv. Appl. Microbiol. **53**, 85 (2003).

[15] U. Jenk, K. Nindel, C. Wedekind, M. Frenzel, R. Uebe, S. Eulenberger, T. Metschies, U. Terra and U. Gross, *„Bericht zur Flutung der Grube Königstein, Teilbereich I – 140 m NN - report number WIS K 358 (2014) Wismut GmbH“*, Technical report, Wismut GmbH (2014).

[16] M. Paul, J. Meyer, U. Jenk, D. Baacke, A. Schramm and T. Metschies, *„Mine Flooding and Water Management at Underground Uranium Mines two Decades after Decommissioning“*, P. 1081–1087, Golden, Colorado, USA (2013).

[17] D. Uhlmann and W. Horn, *„Hydrobiologie der Binnengewässer“*, Eugen Ulmer Verlag, Stuttgart (2001).

[18] C. Normand, G. Pfennig, J. Magill and R. Dreher, *„Mapping the nuclear landscape: 50 years of the Karlsruher Nuklidkarte“*, J Radioanal Nucl Ch **282**, 395 (2009).

[19] A. Holleman and E. Wiberg, *„Lehrbuch der Anorganischen Chemie“*, Vol. 53 (1995).

[20] R. D. Dos Reis, L. S. Veiga, C. A. Escanhoela, J. C. Lang, Y. Joly, F. G. Gandra, D. Haskel and N. M. Souza-Neto, *„Unraveling 5f-6d hybridization in uranium compounds via spin-resolved L-edge spectroscopy“*, Nat Commun **8** (2017).

[21] N. Mitchell, D. Pérez-Sánchez and M. C. Thorne, *„A review of the behaviour of U-238 series radionuclides in soils and plants“*, J. Radiol. Prot. **33**, R17 (2013).

[22] A. F. Holleman and E. Wiberg, *„Lehrbuch der Anorganischen Chemie“*, Vol. 102, Walter de Gruyter Berlin, New York (1995).

[23] J. L. Domingo, *„Reproductive and developmental toxicity of natural and depleted uranium: A review“*, Adv Exp Med Biol **15**, 603 (2001).

[24] D. R. Lide, *„Uranium“*, CRC Press, 74. Ed. (1994).

[25] M. R. MacDonald, M. E. Fieser, J. E. Bates, J. W. Ziller, F. Furche and W. J. Evans, *„Identification of the +2 oxidation state for uranium in a crystalline molecular complex, [K(2.2.2-Cryptand)][(C5H4SiMe 3)3U]“*, J Am Chem Soc **135**, 13310 (2013).

[26] R. Guillaumont, F. Mompean and O. N. E. Agency, *„Update on the Chemical Thermodynamics of Uranium, Neptunium, Plutonium, Americium and Technetium“*, Vol. 5 of *Chemical thermodynamics*, Elsevier (2003).

[27] I. Grenthe, J. Fuger, R. J. Lemire, A. B. Muller, H. Wanner and I. Forest, *„Chemical Thermodynamics of Uranium“*, OECD-NEA **1**, 715 (1992).

[28] G. R. Choppin and B. E. Stout, *„Actinide behavior in natural waters“*, Sci. Total Environ. **83**, 203 (1989).

[29] S. J. Markich, *„Uranium Speciation and Bioavailability in Aquatic Systems: An Overview“*, Sci. World J. **2**, 707 (2002).

[30] T. Arnold, N. Baumann, E. Krawczyk-Bärsch, S. Brockmann, U. Zimmermann, U. Jenk and S. Weiß, *„Identification of the uranium speciation in an underground acid mine drainage environment“*, Geochim. Cosmochim. Acta **75**, 2200 (2011).

[31] R. Bencheikh-Latmani, J. O. Leckie and J. R. Bargar, *„Fate of uranyl in a quaternary system composed of uranyl, citrate, goethite, and Pseudomonas fluorescens“*, Environ. Sci. Technol. **37**, 3555 (2003).

[32] T. Ohnuki, T. Yoshida, T. Ozaki, M. Samadfam, N. Kozai, K. Yubuta, T. Mitsugashira, T. Kasama and A. J. Francis, *„Interactions of uranium with bacteria and kaolinite clay“*, Chem. Geol. **220**, 237 (2005).

[33] S. G. Walker, C. A. Flemming, F. G. Ferris, T. J. Beveridge and G. W. Bailey, *„Physicochemical interaction of Escherichia coli cell envelopes and Bacillus subtilis cell walls with two clays and ability of the composite to immobilize heavy metals from solution“*, Appl Environ Microb **55**, 2976 (1989).

[34] K. Vijayaraghavan and Y. S. Yun, *„Bacterial biosorbents and biosorption“*, Biotechnol. Adv. **26**, 266 (2008).

[35] F. Veglio' and F. Beolchini, *„Removal of metals by biosorption: a review“*, Hydrometallurgy **44**, 301 (1997).

[36] J. R. Lloyd and L. E. Macaskie, *„Bioremediation of Radionuclide-Containing Wastewaters“*, in *Environmental Microbe-Metal Interactions*, P. 277–327 (2000).

[37] M. L. Merroun, J. Raff, A. Rossberg, C. Hennig, T. Reich and S. Selenska-Pobell, *„Complexation of uranium by cells and S-layer sheets of Bacillus sphaericus JG-A12“*, Appl Environ Microb **71**, 5532 (2005).

[38] J. R. Lloyd and L. E. Macaskie, *„Biochemical basis of microbe-radionuclide interactions“*, in *Radioactivity in the Environment*, Ed. M. J. Keith-Roach and F. R. Livens, Vol. 2, P. 313–342, Elsevier Science Ldt, Oxford, UK (2002).

[39] Suzuki, Y. and Banfield, J.F., *„Geomicrobiology of uranium“*, in *Reviews in Mineralogy*, Ed. R. Burns, P., Finch, P. 393–432, Mineralogical Society of America, Washington, DC (1999).

[40] Y. Suzuki and J. F. Banfield, *„Resistance to, and accumulation of, uranium by bacteria from a uranium-contaminated site“*, Geomicrobiol. J. **21**, 113 (2004).

[41] Roomans, Godfried M., *„Localization of divalent cations in phosphate-rich cytoplasmic granules in yeast“*, Physiol. Plant. **48**, 47 (1980).

[42] A. Francis, *„Biotransformation of uranium and other actinides in radioactive wastes“*, J Alloy Compd **271-273**, 78 (1998).

[43] E. Dassa, M. Cahu, B. Desjoyaux-Cherel and P. L. Boquet, *„The acid phosphatase with optimum pH of 2.5 of Escherichia coli. Physiological and biochemical study“*, J. Biol. Chem. **257**, 6669 (1982).

[44] N. Kurosawa, K. Fukuda, Y. H. Itoh and T. Horiuchi, *„Partial purification and characterization of thermostable acid phosphatase from thermoacidophilic archaeon Sulfolobus acidocaldarius“*, Curr Microbiol **40**, 57 (2000).

[45] R. K. Porschen and E. H. Spaulding, *„Phosphatase activity of anaerobic organisms.“*, Appl Microbiol **27**, 744 (1974).

[46] N. Renninger, R. Knopp, H. Nitsche, D. S. Clark and J. D. Keasling, *„Uranyl precipitation by Pseudomonas aeruginosa via controlled polyphosphate metabolism“*, Appl Environ Microb **70**, 7404 (2004).

[47] M. J. Beazley, R. J. Martinez, P. A. Sobecky, S. M. Webb and M. Taillefert, *„Uranium biomineralization as a result of bacterial phosphatase activity: Insights from bacterial isolates from a contaminated subsurface“*, Environ. Sci. Technol. **41**, 5701 (2007).

[48] L. E. Macaskie, D. T. Goddard, K. M. Bonthrone and P. Yong, *„Enzymically mediated bioprecipitation of uranium by a Citrobacter sp.: a concerted role for exocellular lipopolysaccharide and associated phosphatase in biomineral formation“*, Microbiology **146**, 1855 (2000).

[49] J. L. Jerden and A. K. Sinha, *„Phosphate based immobilization of uranium in an oxidizing bedrock aquifer“*, Appl. Geochem. **18**, 823 (2003).

[50] A. Geissler, M. Merroun, G. Geipel, H. Reuther and S. Selenska-Pobell, *„Biogeochemical changes induced in uranium mining waste pile samples by uranyl nitrate treatments under anaerobic conditions“*, Geobiology **7**, 282 (2009).

[51] B. Allard, H. Boren, C. Pettersson and G. Zhang, *„Degradation of humic substances by UV irradiation“*, Environ. Int. **20**, 97 (1994).

[52] R. Sutton and G. Sposito, *„Molecular structure in soil humic substances: the new view.“*, Environ. Sci. Technol. **39**, 9009 (2005).

[53] J. J. Lenhart, S. E. Cabaniss, P. MacCarthy and B. D. Honeyman, *„Uranium(VI) complexation with citric, humic and fulvic acids“*, Radiochim. Acta **88**, 345 (2000).

[54] J. J. Lenhart and B. D. Honeyman, *„Uranium(VI) sorption to hematite in the presence of humic acid“*, Geochim. Cosmochim. Acta **63**, 2891 (1999).

[55] S. Sachs, V. Brendler and G. Geipel, *„Uranium(VI) complexation by humic acid under neutral pH conditions studied by laser-induced fluorescence spectroscopy“*, Radiochim. Acta **95**, 103 (2007).

[56] K. Schmeide, S. Sachs, M. Bubner, T. Reich, K. H. Heise and G. Bernhard, *„Interaction of uranium(VI) with various modified and unmodified natural and synthetic humic substances studied by EXAFS and FTIR spectroscopy“*, Inorg. Chim. Acta **351**, 133 (2003).

[57] P. M. Shanbhag and G. R. Choppin, *„Binding of uranyl by humic acid“*, J. Inorg. Nucl. Chem. **43**, 3369 (1981).

[58] M Wei and J Liao and N Liu and D Zhang and H Kang and Y Yand and Y Yand and J Jin, *„Interaction between uranium and humic acid (I): Adsorption behaviors of U(VI) in soil humic acids“*, Nucl. Sci. Tech. **18**, 287 (2007).

[59] W. C. Li, D. M. Victor and C. L. Chakrabarti, *„Effect of pH and Uranium Concentration on Interaction of Uranium(VI) and Uranium(IV) with Organic Ligands in Aqueous Solutions“*, Anal. Chem. **52**, 520 (1980).

[60] D. R. Lovley, J. L. Fraga, E. L. Blunt-Harris, L. A. Hayes, E. J. P. Phillips and J. D. Coates, *„Humic substances as a mediator for microbially catalyzed metal reduction“*, Acta Hydroch Hydrob **26**, 152 (1998).

[61] J. K. Fredrickson, H. M. Kostandarithes, S. W. Li, A. E. Plymale and M. J. Daly, *„Reduction of Fe(III), Cr(VI), U(VI), and Tc(VII) by Deinococcus radiodurans R1“*, Appl Environ Microb **66**, 2006 (2000).

[62] J. B. Neilands, *„Siderophores: Structure and function of microbial iron transport compounds“*, J. Biol. Chem. **270**, 26723 (1995).

[63] S. W. Frazier, R. Kretzschmar and S. M. Kraemer, *„Bacterial siderophores promote dissolution of UO2 under reducing conditions“*, Environ. Sci. Technol. **39**, 5709 (2005).

[64] B. E. Kalinowski, A. Oskarsson, Y. Albinsson, J. Arlinger, A. Ödegaard Jensen, T. Andlid and K. Pedersen, *„Microbial leaching of uranium and other trace elements from shale mine tailings at Ranstad“*, Geoderma **122**, 177 (2004).

[65] A. A. DiSpirito and O. H. Tuovinen, *„Uranous ion oxidation and carbon dioxide fixation by Thiobacillus ferrooxidans“*, Arch. Microbiol. **133**, 28 (1982).

[66] G. Huber and K. O. Stetter, *„Sulfolobus metallicus, sp. nov., a Novel Strictly Chemolithoautotrophic Thermophilic Archaeal Species of Metal-Mobilizers“*, Syst. Appl. Microbiol. **14**, 372 (1991).

[67] G. Huber, C. Spinnler, A. Gambacorta and K. O. Stetter, *„Metallosphaera sedula gen, and sp. nov. Represents a New Genus of Aerobic, Metal-Mobilizing, Thermoacidophilic Archaebacteria“*, Syst. Appl. Microbiol. **12**, 38 (1989).

[68] J. C. Renshaw, L. J. Butchins, F. R. Livens, I. May, J. M. Charnock and J. R. Lloyd, *„Bioreduction of uranium: Environmental implications of a pentavalent intermediate“*, Environ. Sci. Technol. **39**, 5657 (2005).

[69] D. R. Lovley, E. J. P. Phillips, Y. A. Gorby and E. R. Landa, *„Microbial reduction of uranium“*, Nature **350**, 413 (1991).

[70] D. R. Lovley and E. J. P. Phillips, *„Reduction of uranium by Desulfovibrio desulfuricans“*, Appl Environ Microb **58**, 850 (1992).

[71] A. J. Francis, C. J. Dodge, F. Lu, G. P. Halada and C. R. Clayton, *„XPS and XANES Studies of Uranium Reduction by Clostridium sp“*, Environ. Sci. Technol. **28**, 636 (1994).

[72] E. S. Shelobolina, S. A. Sullivan, K. R. O'Neill, K. P. Nevin and D. R. Lovley, *„Isolation, characterization, and U(VI)-reducing potential of a facultatively anaerobic, acid-resistant bacterium from low-pH, nitrate- and U(VI)-contaminated subsurface sediment and description of Salmonella subterranea sp. nov.“*, Appl Environ Microb **70**, 2959 (2004).

[73] Q. Wu, R. A. Sanford and F. E. Löffler, *„Uranium(VI) reduction by Anaeromyxobacter dehalogenans strain 2CP-C“*, Appl Environ Microb **72**, 3608 (2006).

[74] K. Kashefi and D. R. Lovley, *„Reduction of Fe(III), Mn(IV), and toxic metals at 100°C by Pyrobaculum islandicum“*, Appl Environ Microb **66**, 1050 (2000).

[75] M. L. Merroun and S. Selenska-Pobell, *„Bacterial interactions with uranium: An environmental perspective“*, J. Contam. Hydrol. **102**, 285 (2008).

[76] Y. Suzuki, S. D. Kelly, K. M. Kemner and J. F. Banfield, *„Radionuclide contamination: Nanometre-size products of uranium bioreduction“*, Nature **419**, 134 (2002).

[77] S. Regenspurg, D. Schild, T. Schäfer, F. Huber and M. E. Malmström, *„Removal of uranium(VI) from the aqueous phase by iron(II) minerals in presence of bicarbonate“*, Appl. Geochem. **24**, 1617 (2009).

[78] S. P. Hyun, J. A. Davis, K. Sun and K. F. Hayes, *„Uranium(VI) reduction by iron(II) monosulfide mackinawite“*, Environ. Sci. Technol. **46**, 3369 (2012).

[79] D. E. Latta, M. I. Boyanov, K. M. Kemner, E. J. O'Loughlin and M. M. Scherer, *„Abiotic reduction of uranium by Fe(II) in soil“*, Appl. Geochem. **27**, 1512 (2012).

[80] D. M. Singer, S. M. Chatman, E. S. Ilton, K. M. Rosso, J. F. Banfield and G. A. Waychunas, *„U(VI) Sorption and Reduction Kinetics on the Magnetite (111) Surface“*, Environ. Sci. Technol. **46**, 3821 (2012).

[81] P. M. Fox, J. A. Davis, R. Kukkadapu, D. M. Singer, J. Bargar and K. H. Williams, *„Abiotic U(VI) reduction by sorbed Fe(II) on natural sediments“*, Geochim. Cosmochim. Acta **117**, 266 (2013).

[82] E. J. O'Loughlin, S. D. Kelly and K. M. Kemner, *„XAFS investigation of the interactions of UVI with secondary mineralization products from the bioreduction of FeIII oxides“*, Environ. Sci. Technol. **44**, 1656 (2010).

[83] H. Veeramani, D. S. Alessi, E. I. Suvorova, J. S. Lezama-Pacheco, J. E. Stubbs, J. O. Sharp, U. Dippon, A. Kappler, J. R. Bargar and R. Bernier-Latmani, *„Products of abiotic U(VI) reduction by biogenic magnetite and vivianite“*, Geochim. Cosmochim. Acta **75**, 2512 (2011).

[84] H. Veeramani, A. C. Scheinost, N. Monsegue, N. P. Qafoku, R. Kukkadapu, M. Newville, A. Lanzirotti, A. Pruden, M. Murayama and M. F. Hochella, *„Abiotic reductive immobilization of U(VI) by biogenic mackinawite“*, Environ. Sci. Technol. **47**, 2361 (2013).

[85] J. R. Bargar, K. H. Williams, K. M. Campbell, P. E. Long, J. E. Stubbs, E. I. Suvorova, J. S. Lezama-Pacheco, D. S. Alessi, M. Stylo, S. M. Webb, J. A. Davis, D. E. Giammar, L. Y. Blue and R. Bernier-Latmani, *„Uranium redox transition pathways in acetate-amended sediments“*, Proc Natl Acad Sci. **110**, 4506 (2013).

[86] K. H. Williams, J. R. Bargar, J. R. Lloyd and D. R. Lovley, *„Bioremediation of uranium-contaminated groundwater: A systems approach to subsurface biogeochemistry“*, Curr. Opin. Biotechnol. **24**, 489 (2013).

[87] L. Newsome, K. Morris and J. R. Lloyd, *„The biogeochemistry and bioremediation of uranium and other priority radionuclides“*, Chem. Geol. **363**, 164 (2014).

[88] J. D. Wall and L. R. Krumholz, *„Uranium Reduction“*, Annu Rev Microbiol **60**, 149 (2006).

[89] F. W. Whicker, T. G. Hinton, M. M. MacDonell, J. E. Pinder and L. J. Habegger, *„Environment - Avoiding destructive remediation at DOE sites“*, Science **303**, 1615 (2004).

[90] D. Appukuttan, A. S. Rao and S. K. Apte, *„Engineering of Deinococcus radiodurans R1 for bioprecipitation of uranium from dilute nuclear waste“*, Appl Environ Microb **72**, 7873 (2006).

[91] M. M. Cox and J. R. Battista, *„Deinococcus radiodurans — the consummate survivor“*, Nat. Rev. Microbiol. **3**, 882 (2005).

[92] Y. Suzuki, S. D. Kelly, K. M. Kemner and J. F. Banfield, *„Direct microbial reduction and subsequent preservation of uranium in natural near-surface sediment“*, Appl Environ Microb **71**, 1790 (2005).

[93] B. Gu, W. M. Wu, M. A. Ginder-Vogel, H. Yan, M. W. Fields, J. Zhou, S. Fendorf, C. S. Criddle and P. M. Jardine, *„Bioreduction of uranium in a contaminated soil column“*, Environ. Sci. Technol. **39**, 4841 (2005).

[94] M. Xu, W.-M. Wu, L. Wu, Z. He, J. D. Van Nostrand, Y. Deng, J. Luo, J. Carley, M. Ginder-Vogel, T. J. Gentry, B. Gu, D. Watson, P. M. Jardine, T. L. Marsh, J. M. Tiedje, T. Hazen, C. S. Criddle and J. Zhou, *„Responses of microbial community functional structures to pilot-scale uranium in situ bioremediation“*, ISME J. **4**, 1060 (2010).

[95] R. T. Anderson, H. A. Vrionis, I. Ortiz-Bernad, C. T. Resch, P. E. Long, R. Dayvault, K. Karp, S. Marutzky, D. R. Metzler, A. Peacock, D. C. White, M. Lowe and D. R. Lovley, *„Stimulating the In Situ Activity of Geobacter Species to Remove Uranium from the Groundwater of a Uranium-Contaminated Aquifer“*, Appl Environ Microb **69**, 5884 (2003).

[96] C. Yun-Juan, P. E. Long, R. Geyer, A. D. Peacock, C. T. Resch, K. Sublette, S. Pfiffner, A. Smithgall, R. T. Anderson, H. A. Vrionis, J. R. Stephen, R. Dayvault, I. Ortiz-Bernad, D. R. Lovley and D. C. White, *„Microbial incorporation of 13C-labeled acetate at the field scale: Detection of microbes responsible for reduction of U(VI)“*, Environ. Sci. Technol. **39**, 9039 (2005).

[97] E. S. Shelobolina, K. O'Neill, K. T. Finneran, L. A. Hayes and D. R. Lovley, *„Potential for in situ bioremediation of a low-pH, high-nitrate uranium-contaminated groundwater“*, Soil Sediment Contam. **12**, 865 (2003).

[98] P. Nannipieri, L. Giagnoni, L. Landi and R. G. Enella, *„Role of phosphatase enzymes in soill“*, in *Phosphorus in Action: Biological processes in soil phosphorus cycling*, Ed. E. Bünemann, A. Oberson and E. Frossard, P. 215–243, Springer-Verlag Berlin Heidelberg (2011).

[99] L. E. Macaskie, K. M. Bonthrone and D. A. Rouch, *„Phosphatase-mediated heavy metal accumulation by a Citrobacter sp. and related enterobacteria“*, FEMS Microbiol. Lett. **121**, 141 (1994).

[100] G. Satta, G. Grazi, P. E. Varaldo and R. Fontana, *„Detection of bacterial phosphatase activity by means of an original and simple test.“*, J Clin Pathol **32**, 391 (1979).

[101] L. G. Powers, H. J. Mills, A. V. Palumbo, C. Zhang, K. Delaney and P. A. Sobecky, *„Introduction of a plasmid-encoded phoA gene for constitutive overproduction of alkaline phosphatase in three subsurface Pseudomonas isolates“*, FEMS Microbiol. Ecol. **41**, 115 (2002).

[102] B. Volesky, *„Biosorption and me“*, Water Res. **41**, 4017 (2007).

[103] G. M. Gadd, *„Biosorption: Critical review of scientific rationale, environmental importance and significance for pollution treatment“*, J. Chem. Technol. Biotechnol. **84**, 13 (2009).

[104] M. Fomina and G. M. Gadd, *„Biosorption: Current perspectives on concept, definition and application“*, Bioresource Technol **160**, 3 (2014).

[105] A. Malik, *„Metal bioremediation through growing cells“*, Environ. Int. **30**, 261 (2004).

[106] P. Barton, *„The acid mine drainage“*, in *Sulfur in the environmant, Part II: ecological impacts*, Ed. J. O. Nriagu, P. 313–358, Wiley-Interscience, New York (1978).

[107] M. Kelly, *„Mining and the freshwater environment“* (1988).

[108] D. K. Nordstrom and G. Southam, *„Geomicrobiology of sulfide mineral oxidation“*, in *Geomicrobiology: interactions between microbes and minerals*, Ed. J. F. Banfield and K. H. Nealson, P. 361–390, Mineralogical Society of America, Washington, DC (1997).

[109] I. Zirnstein, *„Charakterisierung der Mikroorganismen im sauren Grubenwasser des ehemaligen Uranbergwerks Königstein“*, PhD thesis, Technische Universität Dresden (2015).

[110] C. Méndez-García, A. I. Peláez, V. Mesa, J. Sánchez, O. V. Golyshina and M. Ferrer, *„Microbial diversity and metabolic networks in acid mine drainage habitats“*, Front. Microbiol. **6** (2015).

[111] D. Prakash, P. Gabani, A. K. Chandel, Z. Ronen and O. V. Singh, *„Bioremediation: A genuine technology to remediate radionuclides from the environment“*, Microb. Biotechnol. **6**, 349 (2013).

[112] S. D. Cunningham, W. R. Berti and J. W. Huang, *„Phytoremediation of contaminated soils“*, Trends Biotechnol. **13**, 393 (1995).

[113] S. D. Cunningham, J. R. Shann, D. E. Crowley and T. A. Anderson, *„Phytoremediation of Contaminated Water and Soil“*, in *Phytoremediation of Soil and Water Contaminants*, P. 2–17, American Chemical Society (1997).

[114] J. L. Zhang and C. L. Qiao, *„Novel approaches for remediation of pesticide pollutants“*, Int. J. Environ. Pollut. **18**, 423 (2002).

[115] F. Seigle-Murandi, P. Guiraud, J. Croize, E. Falsen and K. L. Eriksson, *„Bacteria Are Omnipresent on Phanerochaete chrysosporium Burdsall“*, Appl. Environ. Microbiol. **62**, 2477 (1996).

[116] H. R. Dash and S. Das, *„Bioremediation of mercury and the importance of bacterial mer genes“*, Int Biodeter Biodegr **75**, 207 (2012).

[117] N. Mangwani, H. R. Dash, A. Chauhan and S. Das, *„Bacterial quorum sensing: Functional features and potential applications in biotechnology“*, J. Mol. Microbiol. Biotechnol. **22**, 215 (2012).

[118] N. Mangwani, S. K. Shukla, T. S. Rao and S. Das, *„Calcium-mediated modulation of Pseudomonas mendocina NR802 biofilm influences the phenanthrene degradation“*, Colloids Surf., B **114**, 301 (2014).

[119] S. Choudhary and P. Sar, *„Real-time PCR based analysis of metal resistance genes in metal resistant Pseudomonas aeruginosa strain J007“*, J Basic Microb P. 1–10 (2015).

[120] M. Möhner, M. Lindtner, H. Otten and H. G. Gille, *„Leukemia and exposure to ionizing radiation among German uranium miners“*, Am. J. Ind. Med. **49**, 238 (2006).

[121] T. Liesch, S. Hinrichsen and N. Goldscheider, *„Uranium in groundwater - Fertilizers versus geogenic sources“*, Sci. Total Environ. **536**, 981 (2015).

[122] R. H. Nussbaum, *„The Chernobyl nuclear catastrophe: unacknowledged health detriment“*, Environ Health Persp **115**, A238 (2007).

[123] M. Al-Zoughool and D. Krewski, *„Health effects of radon: A review of the literature“*, Int. J. Radiat. Biol. **85**, 57 (2009).

[124] N. G. Bogutskaya, M. A. Zuykov, A. M. Naseka and E. B. Anderson, *„Normal axial skeleton structure in common roach Rutilus rutilus (Actinopterygii: Cyprinidae) and malfor-*

mations due to radiation contamination in the area of the Mayak (Chelyabinsk Province, Russia) nuclear plant“, J Fish Biol **79**, 991 (2011).

[125] A. Schatz and C. Bovell, *„Growth and hydrogenase activity of a new bacterium, Hydrogenomonas facilis.“*, J. Bacteriol. **63**, 87 (1952).

[126] J. E. Gavagan, R. DiCosimo, A. Eisenberg, S. K. Fager, P. W. Folsom, E. C. Hann, K. J. Schneider and R. D. Fallon, *„A gram-negative bacterium producing a heat-stable nitrilase highly active on aliphatic dinitriles“*, Appl Microbiol Biot **52**, 654 (1999).

[127] U. Gerber, I. Zirnstein, E. Krawczyk-Bärsch, H. Lünsdorf, T. Arnold and M. L. Merroun, *„Combined use of flow cytometry and microscopy to study the interactions between the gram-negative betaproteobacterium Acidovorax facilis and uranium(VI)“*, J Hazard Mater **317**, 127 (2016).

[128] X. Li, C. Ding, J. Liao, T. Lan, F. Li, D. Zhang, J. Yang, Y. Yang, S. Luo, J. Tang and N. Liu, *„Biosorption of uranium on Bacillus sp. dwc-2: Preliminary investigation on mechanism“*, J Environ Radioactiv **135**, 6 (2014).

[129] M. Z. Hu, J. M. Norman, B. D. Faison and M. E. Reeves, *„Biosorption of uranium by Pseudomonas aeruginosa strain CSU: Characterization and comparison studies“*, Biotechnol. Bioeng. **51**, 237 (1996).

[130] G. W. Strandberg, S. E. Shumate and J. R. Parrott, *„Microbial cells as biosorbents for heavy metals: Accumulation of uranium by Saccharomyces cerevisiae and Pseudomonas aeruginosa“*, Appl Environ Microb **41**, 237 (1981).

[131] A. A. DiSpirito, J. W. Talnagi and O. H. Tuovinen, *„Accumulation and cellular distribution of uranium in Thiobacillus ferrooxidans“*, Arch. Microbiol. **135**, 250 (1983).

[132] E. I. Yilmaz, *„Metal tolerance and biosorption capacity of Bacillus circulans strain EB1“*, Res. Microbiol. **154**, 409 (2003).

[133] R. Kumar, C. Acharya and S. R. Joshi, *„Isolation and analyses of uranium tolerant Serratia marcescens strains and their utilization for aerobic uranium U(VI) bioadsorption“*, J Microbiol **49**, 568 (2011).

[134] Z. Hanbo, D. Changqun, S. Qiyong, R. Weimin, S. Tao, C. Lizhong, Z. Zhiwei and H. Bin, *„Genetic and physiological diversity of phylogenetically and geographically distinct groups of Arthrobacter isolated from lead-zinc mine tailings“*, FEMS Microbiol. Ecol. **49**, 333 (2004).

[135] R. Docampo, *„Acidocalcisomes and Polyphosphate Granules“*, in *Inclusions in Prokaryotes*, Vol. 1, P. 53–70, Springer-Verlag, Berlin/Heidelberg (2006).

[136] J. W. van Groenestijn, G. J. Vlekke, D. M. Anink, M. H. Deinema and a. J. Zehnder, *„Role of Cations in Accumulation and Release of Phosphate by Acinetobacter Strain 210A.“*, Appl Environ Microb **54**, 2894 (1988).

[137] M. Merroun, M. Nedelkova, A. Rossberg, C. Hennig and S. Selenska-Pobell, *„Interaction mechanisms of bacterial strains isolated from extreme habitats with uranium“*, Radiochim. Acta **94**, 723 (2006).

[138] E. Krawczyk-Bärsch, U. Gerber, K. Müller, H. Moll, A. Rossberg, R. Steudtner and M. Merroun, *„Multidisciplinary characterization of U(VI) sequestration by Acidovorax facilis for bioremediation purposes“*, J Hazard Mater **347**, 233 (2018).

[139] T. Reitz, M. L. Merroun, A. Rossberg and S. Selenska-Pobell, *„Interactions of Sulfolobus acidocaldarius with uranium“*, Radiochim. Acta **98**, 249 (2010).

[140] M. Nedelkova, M. L. Merroun, A. Rossberg, C. Hennig and S. Selenska-Pobell, *„Microbacterium isolates from the vicinity of a radioactive waste depository and their interactions with uranium“*, FEMS Microbiol. Ecol. **59**, 694 (2007).

[141] M. Merroun, C. Hennig, A. Rossberg, T. Reich and S. Selenska-Pobell, *„Characterization of U(VI)-Acidithiobacillus ferrooxidans complexes using EXAFS, transmission electron microscopy, and energy-dispersive X-ray analysis“*, Radiochim. Acta **91**, 583 (2003).

[142] S. K. Kazy, S. F. D'Souza and P. Sar, *„Uranium and thorium sequestration by a Pseudomonas sp.: Mechanism and chemical characterization“*, J Hazard Mater **163**, 65 (2009).

[143] E. Cardenas, W. M. Wu, M. B. Leigh, J. Carley, S. Carroll, T. Gentry, J. Luo, D. Watson, B. Gu, M. Ginder-Vogel, P. K. Kitanidis, P. M. Jardine, J. Zhou, C. S. Criddle, T. L. Marsh and J. M. Tiedje, *„Microbial communities in contaminated sediments, associated with bioremediation of uranium to submicromolar levels“*, Appl Environ Microb **74**, 3718 (2008).

[144] J. L. Nyman, T. L. Marsh, M. A. Ginder-Vogel, M. Gentile, S. Fendorf and C. Criddle, *„Heterogeneous response to biostimulation for U(VI) reduction in replicated sediment microcosms“*, Biodegradation **17**, 303 (2006).

[145] J. M. Senko, J. D. Istok, J. M. Suflita and L. R. Krumholz, *„In-situ evidence for uranium immobilization and remobilization“*, Environ. Sci. Technol. **36**, 1491 (2002).

[146] S. Basu, C. Bose, N. Ojha, N. Das, J. Das, M. Pal and S. Khurana, *„Evolution of bacterial and fungal growth media“*, Bioinformation **11**, 182 (2015).

[147] Q. M. Wang, A. M. Yurkov, M. Göker, H. T. Lumbsch, S. D. Leavitt, M. Groenewald, B. Theelen, X. Z. Liu, T. Boekhout and F. Y. Bai, *„Phylogenetic classification of yeasts and related taxa within Pucciniomycotina“*, Stud Mycol **81**, 149 (2015).

[148] A. M. Johns, J. Love and S. J. Aves, *„Four inducible promoters for controlled gene expression in the oleaginous yeast Rhodotorula toruloides“*, Front. Microbiol. **7** (2016).

[149] L. d. F. Vilela, V. P. G. de Araujo, R. d. S. Paredes, E. P. d. S. Bon, F. A. G. Torres, B. C. Neves and E. C. A. Eleutherio, *„Enhanced xylose fermentation and ethanol production by engineered Saccharomyces cerevisiae strain“*, AMB Express **5**, 16 (2015).

[150] D. Moysés, V. Reis, J. Almeida, L. Moraes and F. Torres, *„Xylose Fermentation by Saccharomyces cerevisiae: Challenges and Prospects“*, Int J Mol Sci **17**, 207 (2016).

[151] S. H. M. Azhar, R. Abdulla, S. A. Jambo, H. Marbawi, J. A. Gansau, A. A. M. Faik and K. F. Rodrigues, *„Yeasts in sustainable bioethanol production: A review“*, Biochem Biophys Rep **10**, 52 (2017).

[152] T. M. Roane and S. T. Kellogg, *„Characterization of bacterial communities in heavy metal contaminated soils.“*, Can. J. Microbiol. **42**, 593 (1996).

[153] J. S. Angle, R. L. Chaney and D. Rhee, *„Bacterial resistance to heavy metals related to extractable and total metal concentrations in soil and media“*, Soil Biol. Biochem. **25**, 1443 (1993).

[154] M. Pepi and F. Baldi, *„Modulation of chromium(VI) toxicity by organic and inorganic sulfur species in yeasts from industrial wastes“*, Biometals **5**, 179 (1992).

[155] F. Baldi, M. Pepi and F. Fava, *„Growth of Rhodosporidium toruloides strain DBVPG 6662 on dibenzothiophene crystals and orimulsion“*, Appl Environ Microb **69**, 4689 (2003).

[156] C. Zhao, J. Liu, H. Tu, F. Li, X. Li, J. Yang, J. Liao, Y. Yang, N. Liu and Q. Sun, *„Characteristics of uranium biosorption from aqueous solutions on fungus Pleurotus ostreatus“*, Environ Sci Pollut R **23**, 24846 (2016).

[157] M. E. Treen-Sears, B. Volesky and R. J. Neufeld, *„Ion exchange/complexation of the uranyl ion by Rhizopus biosorbent“*, Biotechnol. Bioeng. **26**, 1323 (1984).

[158] T. Sakaguchi, *„Bioaccumulation of Uranium“*, Kyushu University Press, Hukuoka, Japan (1996).

[159] Z. Golab, B. Orlowska and R. W. Smith, *„Biosorption of lead and uranium by Streptomyces sp.“*, Water. Air. Soil Pollut. **60**, 99 (1991).

[160] C. Acharya, P. Chandwadkar and S. Apte, *„Interaction of uranium with a filamentous, heterocystous, nitrogen-fixing cyanobacterium, Anabaena torulosa“*, Bioresour. Technol. **116**, 290 (2012).

[161] M. L. Merroun, M. Nedelkova, J. J. Ojeda, T. Reitz, M. L. Fernández, J. M. Arias, M. Romero-González and S. Selenska-Pobell, *„Bio-precipitation of uranium by two bacterial isolates recovered from extreme environments as estimated by potentiometric titration, TEM and X-ray absorption spectroscopic analyses“*, J Hazard Mater **197**, 1 (2011).

[162] E. Krawczyk-Bärsch, L. Lütke, H. Moll, F. Bok, R. Steudtner and A. Rossberg, *„A spectroscopic study on U(VI) biomineralization in cultivated Pseudomonas fluorescens biofilms isolated from granitic aquifers“*, Environ Sci Pollut R **22**, 4555 (2015).

[163] F. Jroundi, M. L. Merroun, J. M. Arias, A. Rossberg, S. Selenska-Pobell and M. T. González-Muñoz, *„Spectroscopic and microscopic characterization of uranium biomineralization in Myxococcus xanthus“*, Geomicrobiol. J. **24**, 441 (2007).

[164] P. Zhou and B. Gu, *„Extraction of oxidized and reduced forms of uranium from contaminated soils: Effects of carbonate concentration and pH“*, Environ. Sci. Technol. **39**, 4435 (2005).

[165] A. J. Francis, *„Microbial transformations of radioactive wastes and environmental restoration through bioremediation“*, J Alloy Compd **213-214**, 226 (1994).

[166] J. R. Lloyd, A. N. Mabbett, D. R. Williams and L. E. Macaskie, *„Metal reduction by sulphate-reducing bacteria: Physiological diversity and metal specificity“*, Hydrometallurgy **59**, 327 (2001).

[167] J. Seifert, B. Erler, K. Seibt, N. Rohrbach, J. Arnold, M. Schlömann, S. Kassahun and U. Jenk, *„Characterization of the microbial diversity in the abandoned uranium mine Königstein“*, in *Uranium, Mining and Hydrogeology*, Ed. B. Merkel and A. Hasche-Berger, P. 733–742, Springer, Berlin (2008).

[168] A. Sen and B. Johnson, *„Acidophilic sulphate-reducing bacteria: candidates for bioremediation of acid mine drainage“*, in *Biohydrometallurgy and the Environment Toward the Mining of the 21st Century - Proceedings of the International Biohydrometallurgy Symposium*, P. 709–718, Elsevier (1999).

[169] D. Alazard, M. Joseph, F. Battaglia-Brunet, J. L. Cayol and B. Ollivier, *„Desulfosporosinus acidiphilus sp. nov.: A moderately acidophilic sulfate-reducing bacterium isolated from acid mining drainage sediments“*, Extremophiles (2010).

[170] I. Sánchez-Andrea, J. L. Sanz, M. F. M. Bijmans and A. J. M. Stams, *„Sulfate reduction at low pH to remediate acid mine drainage“*, J Hazard Mater **269**, 98 (2014).

[171] M. F. M. Bijmans, M. Dopson, T. W. T. Peeters, P. N. L. Lens and C. J. N. Buisman, *„Sulfate reduction at pH 5 in a high-rate membrane bioreactor: Reactor performance and microbial community analyses“*, J Microbiol Biotechn **19**, 698 (2009).

[172] I. Sánchez-Andrea, A. J. Stams, R. Amils and J. L. Sanz, *„Enrichment and isolation of acidophilic sulfate-reducing bacteria from Tinto River sediments“*, Environ Microbiol Rep **5**, 672 (2013).

[173] J. Meier, A. Piva and D. Fortin, *„Enrichment of sulfate-reducing bacteria and resulting mineral formation in media mimicking pore water metal ion concentrations and pH conditions of acidic pit lakes“*, FEMS Microbiol. Ecol. **79**, 69 (2012).

[174] D. B. Johnson, E. Jameson, O. Rowe, K. Wakeman and K. B. Hallberg, *„Sulfidogenesis at Low pH by Acidophilic Bacteria and its Potential for the Selective Recovery of Transition Metals from Mine Waters“*, in *Advanced Material Research*, Ed. E. R. Donati, M. R. Viera, E. L. Tavani, M. A. Giaveno, T. L. Lavalle and P. A. Chiacchiarini, Vol. 71-73, P. 693–696 (2009).

[175] M. J. Wilkins, F. R. Livens, D. J. Vaughan, I. Beadle and J. R. Lloyd, *„The influence of microbial redox cycling on radionuclide mobility in the subsurface at a low-level radioactive waste storage site“*, Geobiology **5**, 293 (2007).

[176] J. D. Begg, I. T. Burke, J. R. Lloyd, C. Boothman, S. Shaw, J. M. Charnock and K. Morris, *„Bioreduction behavior of U(VI) sorbed to sediments“*, Geomicrobiol. J. **28**, 160 (2011).

[177] G. T. Law, A. Geissler, I. T. Burke, F. R. Livens, J. R. Lloyd, J. M. McBeth and K. Morris, *„Uranium redox cycling in sediment and biomineral systems“*, Geomicrobiol. J. **28**, 497 (2011).

[178] J. D. Istok, J. M. Senko, L. R. Krumholz, D. Watson, M. A. Bogle, A. Peacock, Y. J. Chang and D. C. White, *„In Situ Bioreduction of Technetium and Uranium in a Nitrate-Contaminated Aquifer“*, Environ. Sci. Technol. **38**, 468 (2004).

[179] W. M. Wu, J. Carley, J. Luo, M. A. Ginder-Vogel, E. Cardenas, M. B. Leigh, C. Hwang, S. D. Kelly, C. Ruan, L. Wu, J. Van Nostrand, T. Gentry, K. Lowe, T. Mehlhorn, S. Carroll, W. Luo, M. W. Fields, B. Gu, D. Watson, K. M. Kemner, T. Marsh, J. Tiedje, J. Zhou, S. Fendorf, P. K. Kitanidis, P. M. Jardine and C. S. Criddle, *„In situ bioreduction of uranium (VI) to submicromolar levels and reoxidation by dissolved oxygen“*, Environ. Sci. Technol. **41**, 5716 (2007).

[180] K. H. Williams, P. E. Long, J. A. Davis, M. J. Wilkins, A. L. N'Guessan, C. I. Steefel, L. Yang, D. Newcomer, F. A. Spane, L. J. Kerkhof, L. Mcguinness, R. Dayvault and D. R. Lovley, *„Acetate availability and its influence on sustainable bioremediation of Uranium-contaminated groundwater“*, Geomicrobiol. J. **28**, 519 (2011).

[181] J. L. Slonczewski and J. W. Foster, *„Mikrobiologie“*, Springer Spektrum, 2. Ed. (2012).

[182] K. Duquesne, S. Lebrun, C. Casiot, O. Bruneel, J. C. Personné, M. Leblanc, F. Elbaz-Poulichet, G. Morin and V. Bonnefoy, *„Immobilization of Arsenite and Ferric Iron by Acidithiobacillus ferrooxidans and Its Relevance to Acid Mine Drainage“*, Appl. Environ. Microbiol. **69**, 6165 (2003).

[183] D. Leduc, L. G. Leduc and G. D. Ferroni, *„Quantification of bacterial populations indigenous to acidic drainage streams“*, Water Air Soil Pollut **135**, 1 (2002).

[184] L. L. Barton and F. A. Tomei, *„Characteristics and Activities of Sulfate-Reducing Bacteria“*, in *Sulfate-Reducing Bacteria*, P. 1–32, Springer US (1995).

[185] M. QuiIntana, G. Curutchet and E. Donati, *„Factors affecting chromium(VI) reduction by Thiobacillus ferrooxidans“*, Biochem. Eng. J. **9**, 11 (2001).

[186] R. Gargarello, D. D. Gregorio, H. Huck, J. F. Niello and G. Curutchet, *„Reduction of uranium(VI) by Acidithiobacillus thiooxidans and Acidithiobacillus ferrooxidans“*, Hydrometallurgy **104**, 529 (2010).

[187] Y. Suzuki, S. D. Kelly, K. M. Kemner and J. F. Banfield, *„Microbial populations stimulated for hexavalent uranium reduction in uranium mine sediment“*, Appl. Environ. Microbiol. **69**, 1337 (2003).

[188] O. H. Tuovinen and D. P. Kelly, *„Studies on the growth of Thiobacillus ferrooxidans - II. Toxicity of uranium to growing cultures and tolerance conferred by mutation, other metal cations and EDTA“*, Arch. Microbiol. **95**, 153 (1974).

[189] R. M. Jones, S. Hedrich and D. B. Johnson, *„Acidocella aromatica sp. nov.: An acidophilic heterotrophic alphaproteobacterium with unusual phenotypic traits“*, Extremophiles **17**, 841 (2013).

[190] Y. Masaki, T. Hirajima, K. Sasaki and N. Okibe, *„Bioreduction and immobilization of hexavalent chromium by the extremely acidophilic Fe(III)-reducing bacterium Acidocella aromatica strain PFBC“*, Extremophiles **19**, 495 (2015).

[191] S. Ghosh, N. R. Mahapatra and P. C. Banerjee, *„Metal resistance in Acidocella strains and plasmid-mediated transfer of this characteristic to Acidiphilium multivorum and Escherichia cell“*, Appl Environ Microb **63**, 4523 (1997).

[192] D. R. Lovley, P. K. Widman, J. C. Woodward and E. J. P. Phillips, *„Reduction of uranium by cytochrome c3 of Desulfovibrio vulgaris“*, Appl Environ Microb **59**, 3572 (1993).

[193] B. M. Tebo and A. Y. Obraztsova, *„Sulfate-reducing bacterium grows with Cr(VI), U(VI), Mn(IV), and Fe(III) as electron acceptors“*, Fems Microbiol Lett (1998).

[194] B. Volesky, *„Biosorption of Heavy Metals“*, CRC Press (1990).

[195] J. H. Tuttle, P. R. Dugan and C. I. Randles, *„Microbial sulfate reduction and its potential utility as an acid mine water pollution abatement procedure.“*, Appl Microbiol **17**, 297 (1969).

[196] D. B. Johnson and K. B. Hallberg, *„Acid mine drainage remediation options: A review“*, Sci. Total Environ. **338**, 3 (2005).

[197] L. M. A. B. S.-A. L., in *4th International Conference on Acid Rock Drainage*, P. 115–130, Vancouver, Canada (1997).

[198] S. D. B. S. W. G., in *4th International Conference on Acid Rock Drainage*, P. 1595, Vancouver, Canada (1997).

[199] L. M. B. C. M. J. I. E. S. R., in *5th Biennial Symposium of the Groundwater Division of the Geological Survey of South Africa Randberg*, P. 193–200, Transvaal, Pretoria (1989).

[200] J. K. Fredrickson, J. M. Zachara, D. W. Kennedy, C. Liu, M. C. Duff, D. B. Hunter and A. Dohnalkova, *„Influence of Mn oxides on the reduction of uranium(VI) by the metal-reducing bacterium Shewanella putrefaciens“*, Geochim. Cosmochim. Acta **66**, 3247 (2002).

[201] W. Luo and B. Gu, *„Dissolution of uranium-bearing minerals and mobilization of uranium by organic ligands in a biologically reduced sediment“*, Environ. Sci. Technol. **45**, 2994 (2011).

[202] R. K. Sani, B. M. Peyton, A. Dohnalkova and J. E. Amonette, *„Reoxidation of reduced uranium with iron(III) (Hydr)oxides under sulfate-reducing conditions“*, Environ. Sci. Technol. **39**, 2059 (2005).

[203] N. F. Spycher, M. Issarangkun, B. D. Stewart, S. Sevinç Şengör, E. Belding, T. R. Ginn, B. M. Peyton and R. K. Sani, *„Biogenic uraninite precipitation and its reoxidation by iron(III) (hydr)oxides: A reaction modeling approach“*, Geochim. Cosmochim. Acta **75**, 4426 (2011).

[204] J. Wan, T. K. Tokunaga, E. Brodie, Z. Wang, Z. Zheng, D. Herman, T. C. Hazen, M. K. Firestone and S. R. Sutton, *„Reoxidation of bioreduced uranium under reducing conditions“*, Environ. Sci. Technol. **39**, 6162 (2005).

[205] J. Wan, T. K. Tokunaga, Y. Kim, E. Brodie, R. Daly, T. C. Hazen and M. K. Firestone, *„Effects of organic carbon supply rates on uranium mobility in a previously bioreduced contaminated sediment“*, Environ. Sci. Technol. **42**, 7573 (2008).

[206] Z. Wang, S. W. Lee, P. Kapoor, B. M. Tebo and D. E. Giammar, *„Uraninite oxidation and dissolution induced by manganese oxide: A redox reaction between two insoluble minerals“*, Geochim. Cosmochim. Acta **100**, 24 (2013).

[207] W. M. Wu, J. Carley, M. Fienen, T. Mehlhorn, K. Lowe, J. Nyman, J. Luo, M. E. Gentile, R. Rajan, D. Wagner, R. F. Hickey, B. Gu, D. Watson, O. A. Cirpka, P. K. Kitanidis, P. M. Jardine and C. S. Criddle, *„Pilot-scale in situ bioremediation of uranium in a highly contaminated aquifer. 1. Conditioning of a treatment zone“*, Environ. Sci. Technol. **40**, 3978 (2006).

[208] L. Newsome, K. Morris, S. Shaw, D. Trivedi and J. R. Lloyd, *„The stability of microbially reduced U(IV); impact of residual electron donor and sediment ageing“*, Chem. Geol. **409**, 125 (2015).

[209] L. Newsome, K. Morris, D. Trivedi, A. Bewsher and J. R. Lloyd, *„Biostimulation by Glycerol Phosphate to Precipitate Recalcitrant Uranium(IV) Phosphate“*, Environ. Sci. Technol. **49**, 11070 (2015).

[210] R. Finch and T. Murakami, *„Systematics and paragenesis of uranium minerals“*, Rev. Mineral. Geochem. **38**, 91 (1999).

[211] K. A. Jensen, C. S. Palenik and R. C. Ewing, *„U6+ phases in the weathering zone of the Bangombé U-deposit: observed and predicted mineralogy“*, Radiochim Acta **90** (2002).

[212] A. J. Pinto, M. A. Gonçalves, C. Prazeres, J. M. Astilleros and M. J. Batista, *„Mineral replacement reactions in naturally occurring hydrated uranyl phosphates from the Tarabau deposit: Examples in the Cu-Ba uranyl phosphate system“*, Chem. Geol. **312-313**, 18 (2012).

Appendix

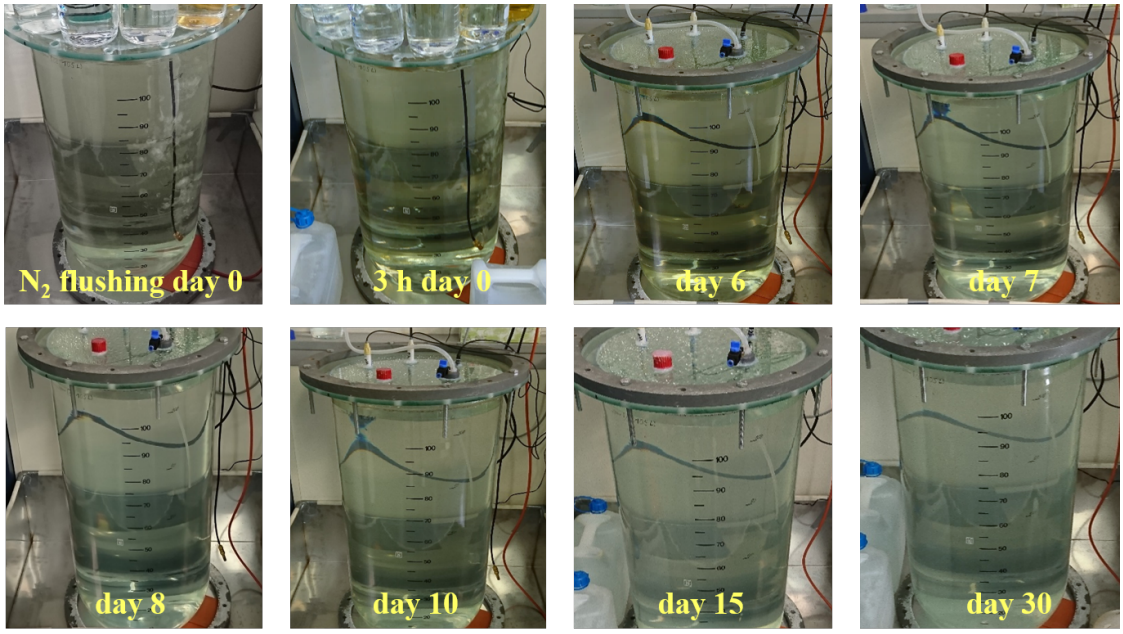


Figure A1: Color change of the flooding water in the pilot plant during 30 days of incubation time.

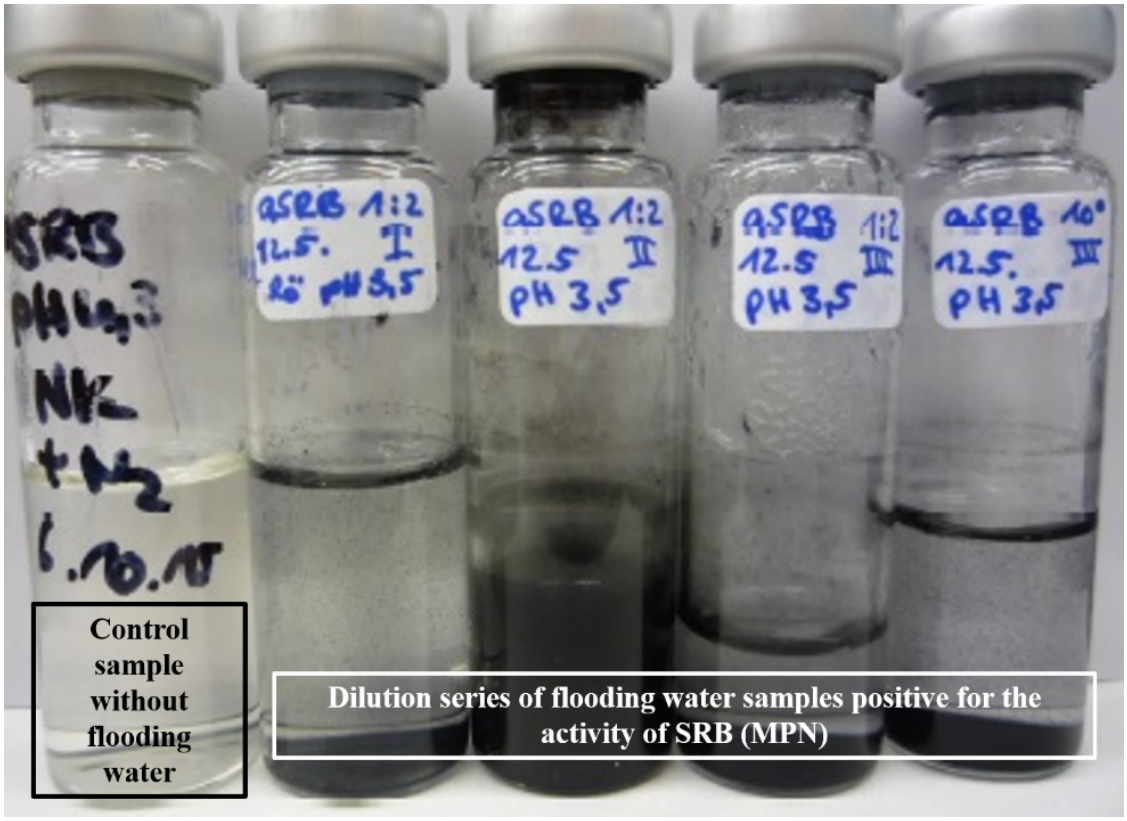


Figure A2: MPN approaches for determination of SRB. A dilution series of the flooding water samples were inoculated in aSRB medium [168]. Left in black control sample without flooding water, negative for SRB activity; right in white samples of the flooding water positive for SRB activity by the occurrence of black precipitation (FeS).

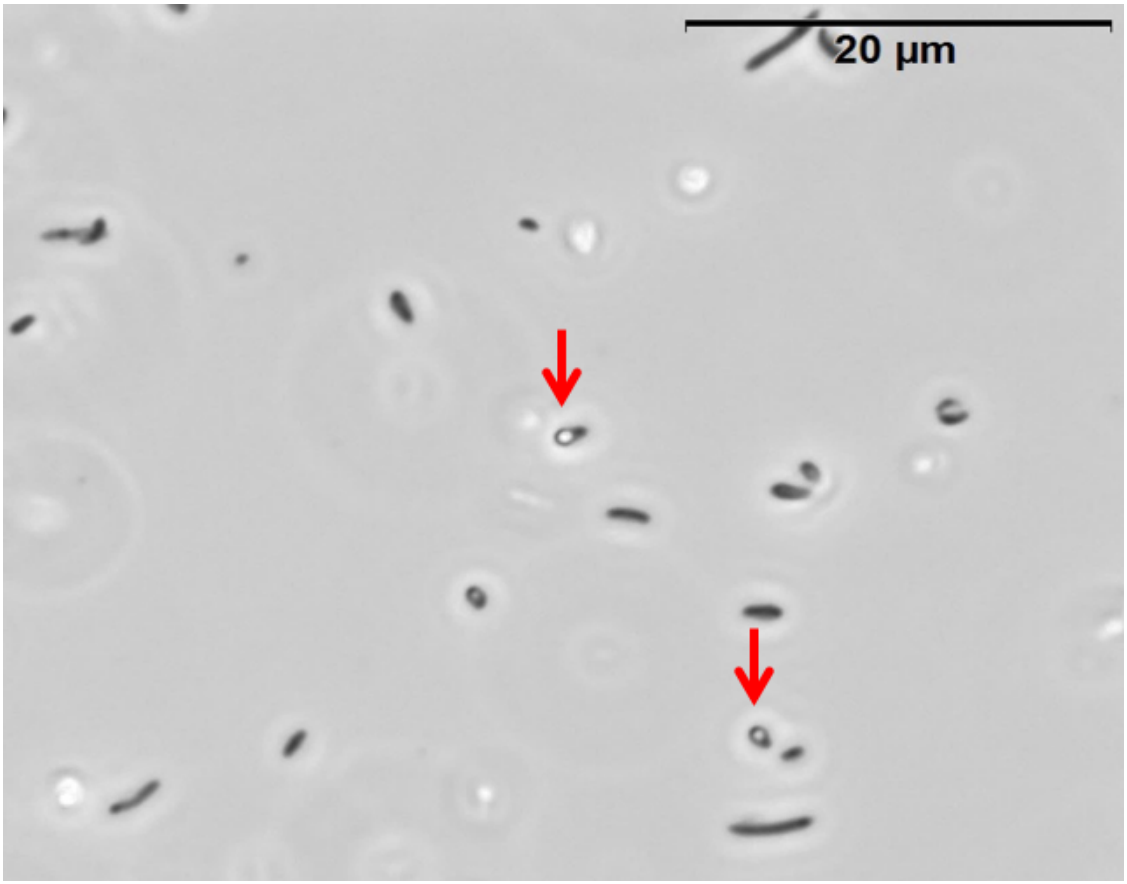


Figure A3: Light microscopy (phase-contrast) of spore-forming bacterial cells within the flooding water samples (1 L) after six weeks incubation at 30 °C, 10 mM glycerol added. Red arrows indicate the endospores.

Abbreviations

A. aromatica	*Acidocella aromatica*
A. ferrooxidans	*Acidothiobacillus ferrooxidans*
A. facilis	*Acidovorax facilis*
AMD	Acid mine drainage
aSRB	Acidophilic sulfate reducing bacteria
ATR FT-IR	Attenuated total reflection Fourier transform-infrared
BLAST	Basic local alignment search tool
CO	Colorado
D. acidophilus	*Desulfosporosinus acidophilus*
dbm	Dry biomass
DNA	Deoxyribonucleic acid
DSMZ	Leibniz-Institut Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH
e.g.	exempli gratia/for example
Eh	Redox potential
Eq.	Equation
Eqs.	Equations
et al.	*et alii*
EXAFS	Extended X-ray absorption fine structure
F. myxofaciens	Ferrovum myxofaciens
GmbH	Gesellschaft mit beschränkter Haftung
h	Hours
ICP-MS	Inductively coupled plasma mass spectrometry
IOB	Iron oxidizing bacteria
IRB	Iron reducing bacteria
ITS	Internal transcribed spacer
L	Liter

M	Molar
MIC	Minimal inhibitory concentration
min	Minutes
MPN	Most probable number
MRB	Metal reducing bacteria
MTC	Maximal tolerated concentration
NCBI	National Center for Biotechnology Information
OD	Optical density
P	Phosphorous
PCR	Polymerase chain reaction
pKa	Logarithmic acid dissociation constant
R. toruloides	*Rhodosporidium toruloides*
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
SDA	Sabouraud dextrose agar
SEM	Scanning electron microscopy
SRB	Sulfate reducing bacteria
TEM	Transmission electron microscopy
TN	Tennessee
TOC	Total organic carbon
TRLFS	Time resolved laser fluorescence spectroscopy
U	Uranium
USA	United States of America
v/v	Volume/volume
w/v	Weight/volume
WA	Washington
XANES	X-ray absorption near edge structure

Acknowledgement

First of all, I would like to thank the head of the Institute of Ressource Ecology, Prof. Dr. Thorsten Stumpf. I thank him for his supervision and to give me the possibility to investigate this really interesting topic. I know he was at the beginning a little bit skeptical about biological research, but nevertheless I hope with my findings I could convince him that Biology is also really important.

In addition, I would like to thank Prof. Dr. Erika Kothe of the FSU in Jena for the opportunity to be my reviewer. For her support with any questions which I had and her ideas in moments when I had no glue. Moreover, I would like to thank Prof. Dr. Georg Büchel for his willingness to be my second reviewer from the FSU. I would like to thank him for his kind words at every time we have met.

I also would like to thank my supervisor Dr. Evelyn Krawczyk-Bärsch. She supported me in every step and in every idea, I had in mind. Sometimes she was really hard to convince with my plans, but I hope at the end I could show her that other ways also reach in the same direction. I know to work with me was not always easy but she kept always calm. I took comfort from her in things concerning work and in addition also things concerning my personal life.

I would like to thank Dr. Corinna Gagell, Dr. Isabel Zirnstein and Dr. Juliane Schott for their support all the time. Unfortunately, we didn't had the time to work long time together, but nevertheless during the short time with you we built up a friendship which lasts till now.

Moreover, I would like to thank my both students, Grit Röder and Sebastian Schäfer. Grit, you were my first master student and it was a pleasure to work with you. I know the topic was not always easy and sometimes the scientific work was frustrating but you did an excellent job and I really liked to read your work. Sebastian, you performed a perfect work and I thank you for your independence. The work at the pilot plant was not always strait forward but you managed this challenge.

In addition, I thank all the people from the Institute of Ressource Ecology. In particular, I would like to mention the very nice atmosphere with my office colleagues Susanne Lehmann, Dr.

Stefan Hellebrandt, Sophia E. Hellebrandt, Manuel Eibl, and Henry Lösch. I really miss the coffee breaks with you. I also would like to thank Dr. Moritz Schmidt, Dr. Nina Huittinen and Robert Barthen for their proof-reading and for really good advises concerning this thesis and the manuscripts. Unfortunately, I can't mentioned all of the people, but I would like to mention the really good support from all the colleagues.

I would like to thank Prof. Dr. Mohamed L. Merroun from the University of Granada in Spain for providing me the opportunity of two laboratory exchanges to his group in Granada and for his support during this time and by supervising of the resulting manuscripts.

I thank the Bundesministerium für Bildung und Forschung (BMBF) grand №02NUK030F (TransAqua) for the financial support of this thesis. I am grateful to the Wismut GmbH for allowing access to the sampling site, in particular S. Röder for his assistance in several water samplings.

Last but not least, I would like to thank Tobias Schneider. We have met in a tough time of my life and together with you I could smile again. Thank you for your support and patience in every situation, thank you for your grammatical corrections of this work, and thank you for your love. Anne Hartenhauer, I would like to thank you in particular. For your friendly ear in every situation, for your honesty and for the time I could spent with you together. I thank my parents and the rest of my family for their support during my whole life and especially during my studies, although they were really skeptical concerning Biology. Thank you for everything!

Declaration/ Selbstständigkeitserklärung

Hereby I declare, that this present work was written by myself and no other than indicated sources were used. All text passages, which comply in word or sense other works, I have marked with references.
Hiermit erkläre ich, dass ich die vorliegende Arbeit eigenständig verfasst wurde und keine anderen als die angegebenen Hilfsmittel verwendet wurden. Alle Stellen, die anderen Werken in Wort oder Sinn entsprechen, habe ich mit Quellenangaben versehen.

Ulrike Gerber
Dresden, den 29.06.2018