Prokaryotic microorganisms in uranium mining waste piles and their interactions with uranium and other heavy metals

Von der Fakultät für Chemie und Physik

der Technischen Universität Bergakademie Freiberg

genehmigte

DISSERTATION

Zur Erlangung des akademischen Grades

Doctor rerum naturalium

Dr. rer. nat.

vorgelegt

von Dipl. Geoökol. Andrea Geißler

geboren am 10.10.1978 in Freital

Gutachter: Prof. Dr. Michael Schlömann, Freiberg

Prof. Dr. Karsten Pedersen, Göteborg, Schweden

Dr. Sonja Selenska-Pobell, Dresden

Tag der Verleihung: 11.05.2007

Table of contents

CHAPTER 5

INTERACTIONS OF TWO *ARTHROBACTER* STRAINS ISOLATED FROM THE URANIUM MINING WASTE PILE HABERLAND WITH Pb AND U

Introduction

Migration of radionuclides from the radioactive waste repository sites, as well as from uranium mill tailings and mining piles, is of serious environmental concern. About 231000 tons of uranium were produced in Eastern Germany from 1946 to 1990 (Meinrath *et al*., 2003). More than 5 x 10^8 tons of radioactive wastes, a total of 3000 piles and about 20 tailings had to be remediated or decontaminated (Beleites, 1992). The fate and the transport of uranium are governed by the contrasting chemistry of U(IV) and U(VI). U(VI) generally forms soluble, and thus mobile, complexes with carbonate and hydroxide, while U(IV) precipitates as the highly insoluble mineral uraninite (Nyman *et al*., 2006). Abiotic factors such as ions and minerals strongly influence the migration process of uranium (Barnett *et al*., 2000; Arnold *et al*., 2001; Duff *et al*., 2002; Baik *et al*., 2004). In addition, microbial processes can influence the mobility of heavy metals and radionuclides and, thereby, their migration behaviour (Francis, 1998; Lloyd & Lovley, 2001; Merroun & Selenska-Pobell, 2001; Lloyd & Macaskie, 2002; Selenska-Pobell, 2002; Merroun *et al*., 2003, 2005, 2006; Suzuki *et al*., 2003, 2005; Kalinowski *et al*., 2004; Lloyd & Renshaw, 2005; Lloyd *et al*., 2005; Pedersen, 2005). These processes can act metal immobilising or mobilising and involve biotransformations as oxidation (DiSpirito & Tuovinen, 1982; Beller, 2005) and reduction (Lovley *et al*., 1991, 1993a; Lloyd, 2003; Khijniak *et al*., 2005; Wu *et al*., 2006), biosorption by cell surface polymers (Selenska-Pobell *et al*., 1999; Raff *et al*., 2003; Beveridge, 2005; Merroun *et al*., 2005), uptake of metals inside the cells (McLean & Beveridge, 2001; Merroun *et al*., 2003; Francis *et al*., 2004; Suzuki & Banfield, 2004), metal precipitation and generation of minerals (Macaskie *et al*., 2000; Merroun *et al*., 2006; Nedelkova *et al*., 2006) and chelation by siderophores and other microbial compounds (Kalinowski *et al*., 2004; Pedersen, 2005) (Fig. 1).

To better understand the microbial mechanisms which influence the radionuclide migration and in order to establish bioremediation strategies for the contaminated sites, information on the distribution and the activities of the microorganisms in these extreme habitats are required. Because more than 99% of the microorganisms in the environment are uncultivable by using standard cultivation techniques, culture-independent molecular approaches based on analyses of 16S rRNA genes were used to explore microbial diversity in nature (Pace, 1997; Pedersen, 1997; Hugenholtz, 2002; Selenska-Pobell, 2002; Torsvik & Øvreås, 2002).

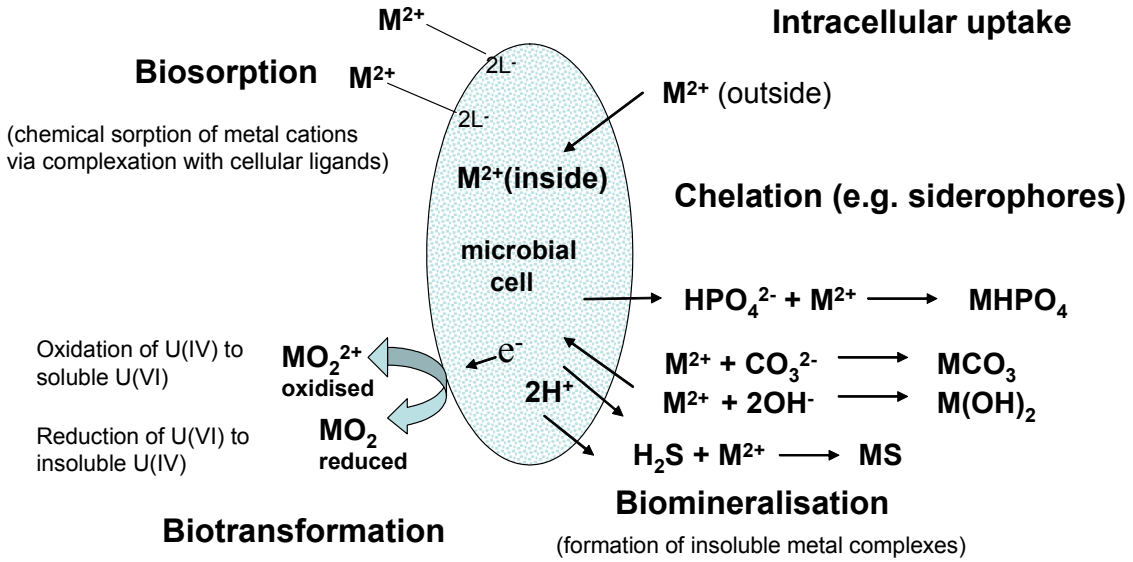


Fig. 1. Mechanisms of radionuclide-microbe interactions changed after Lloyd & Macaskie (2002).

During the last decade, a number of studies investigated microbial communities in radionuclide contaminated environments by using the 16S rRNA gene retrieval, in order to determine the phylogenetic placement of the microorganisms that are inhabiting these environments (Pedersen *et al*., 1996; Crozier *et al*., 1999; Selenska-Pobell *et al*., 2001; Selenska-Pobell, 2002; Selenska-Pobell *et al*., 2002; Suzuki *et al*., 2003, 2005; Fredrickson *et al*., 2004; Satchanska *et al*., 2004; Fields *et al*., 2005; Radeva & Selenska-Pobell, 2005; Satchanska & Selenska-Pobell, 2005).

Differences in the composition of the bacterial communities in contaminated environments were observed, which are site-specific and possibly connected to their different grade of contamination, to their different geographic and geologic origin as well as to their site history (Selenska-Pobell *et al*., 2002; Geissler, 2003). *Alphaproteobacteria* and *Acidobacteria* were found to be predominant applying the 16S rRNA gene retrieval with different primer sets in soil samples collected from different depths of the uranium mining waste pile Haberland, located near the town of Johanngeorgenstadt in Germany (Selenska-Pobell *et al*., 2002; Selenska-Pobell, 2002; Geissler, 2003; Satchanska *et al*., 2004). In soil samples from the uranium mill tailings Gittersee/Coschütz in Germany no *Acidobacteria* were identified and the *Alphaproteobacteria* were not predominant (Selenska-Pobell *et al*., 2002; Satchanska & Selenska-Pobell, 2005). Instead, the number of *Gammaproteobacteria* especially of *Pseudomonas* spp. as well as the number of representatives of the *Bacteroidetes* phylum was extremely high in these samples (Selenska-Pobell *et al*., 2002; Satchanska & Selenska-Pobell, 2005). *Gammaproteobacteria* were also predominant in a soil sample from the uranium

depository site Gunnison, Colorado, USA (Selenska-Pobell *et al*., 2002; Geissler, 2003). In contrast, the composition of the bacterial community in a soil sample from the uranium mill tailings Shiprock, New Mexico, USA was extremely complex and Gram-positive bacteria, especially *Bacillus* spp., green non-sulphur bacteria, and *Gammaproteobacteria* were found to be predominant (Selenska-Pobell *et al*., 2002; Geissler, 2003). Bacterial diversity was also investigated in water samples collected from several uranium mining wastes (Radeva & Selenska-Pobell, 2005). The analysis of the 16S rRNA gene retrieval revealed that *Nitrospina*-like bacteria are predominant in the uranium mill tailings Schlema/Alberoda, Germany, whereas *Pseudomonas* spp. and *Frateuria* spp. from *Gammaproteobacteria* were predominant in the uranium mill tailings Shiprock, New Mexico, USA (Radeva & Selenska-Pobell, 2005). Similarly to the soil samples, *Proteobacteria* and representatives of the *Bacteroidetes* phylum were also found in waters of the uranium mill tailings Gittersee/Coschütz (Radeva & Selenska-Pobell, 2005). However, in the Steinsee Deponie 1 in Germany mainly *Acinetobacter* spp. were identified (Radeva & Selenska-Pobell, 2005).

The bacterial community structure analyses based on 16S rRNA gene clone libraries of groundwater from the Field Research Centre (FRC), which is located at the Y-12 complex within the Security Oak Ridge National Laboratory in Oak Ridge, Tennessee, USA, demonstrated high diversity at the background site (Fields *et al*., 2005). However, three other studied acidic groundwater samples from the FRC site, with high concentrations of nitrate, nickel, aluminium, and uranium, had a decreased diversity and the majority of the clones were closely related to *Azoarcus* spp. and *Pseudomonas* spp. (Fields *et al*., 2005). From the same site, but from another sampling point, a large variety of 16S rRNA gene sequences was retrieved including species from the *Alpha*-, *Beta*-, *Delta*-, and *Gammaproteobacteria* as well as Gram-positive species with low- and high-G+C content (North *et al*., 2004). This part of the site was contaminated with uranium and nitrate as well as with other radionuclides such as plutonium, technetium, other heavy metals and a variety of organic contaminants (North *et al*., 2004).

In order to determine if the inhabiting microorganisms are capable of carrying out the processes of interest for bioremediation, functional genes or enzyme analyses were employed as well. In addition to the 16S rRNA gene retrieval, culture-independent approaches targeting specific metabolic key enzymes were used to obtain information about the activities and ecological impact of functional bacterial groups that are responsible for certain processes. The investigation of sulphate-reducing bacteria (SRB) in contaminated groundwaters from the uranium mill tailings disposal site at Shiprock, New Mexico, USA revealed a remarkable

diversity among the dissimilatory sulphite reductase (DSR) gene fragments, including sequences from *Deltaproteobacteria*, Gram-positive organisms, and the *Nitrospira* division (Chang *et al*., 2001). Dominance of ammonia-oxidising *Betaproteobacteria* was demonstrated by using both the 16S rRNA gene and ammonia monooxygenase gene (*amo*A) retrievals in the samples collected from the same site (Ivanova *et al*., 2000). Nitrate-contaminated groundwater samples from the already mentioned FRC site in Oak Ridge, Tennessee, USA were analysed for nitrite reductase gene (*nir*K and *nir*S) diversity (Yan *et al*., 2003). The results indicated that the contaminated groundwater contained novel, not previously described *nir*K and *nir*S sequences. The functional diversity of both genes changed in relation to contaminant gradients, but the *nir*K and the *nir*S functional diversity were differently affected by particular contamination fluctuations (Yan *et al*., 2003). Nitrate is a common cocontaminant in uranium mining wastes (Finneran *et al*., 2002; Istok *et al*., 2004; North *et al*., 2004) and can strongly influence the mobility of uranium. It was demonstrated that nitrate inhibits U(VI) bioreduction in acetate-amended aquifer sediments and that both U(VI) and Fe(III) were reduced concurrently only when nitrate was depleted (Finneran *et al*., 2002). The addition of nitrate can also result in reoxidation of U(IV) (Finneran *et al*., 2002; Senko *et al*., 2002; Istok *et al*., 2004). Nitrate-dependent U(IV) oxidation often proceeds through Fe(III) (Finneran *et al*., 2002; Senko *et al*., 2005a, 2005b). Fe(III) can be produced by nitrate-dependent Fe(II)-oxidising microorganisms (Straub *et al*., 1996, 2004; Straub & Buchholz-Cleven, 1998; Lack *et al*., 2002). Also possible is the oxidation of Fe(II) by nitrite, which accumulates during dissimilatory nitrate reduction (Senko *et al*., 2002, 2005a). Recently, it was demonstrated that the chemolithoautotrophic bacterium *Thiobacillus denitrificans* oxidatively dissolves synthetic and biogenic U(IV) oxides (uraninite) in a nitrate-dependent reaction under strictly anaerobic conditions (Beller, 2005). The reduction of nitrate to nitrite can be catalysed by three distinct nitrate reductases classes NAS, NAR and NAP in prokaryotes (Richardson *et al*., 2001). The membrane-bound nitrate reductase NAR was used as a molecular marker to target nitrate-reducing communities in different environments (Philippot *et al*., 2002; Chèneby *et al*., 2003; Deiglmayr *et al*., 2004; Mounier *et al*., 2004; Héry *et al*., 2005), but not yet in uranium mining wastes.

For developing cost-effective *in situ* bioremediation technologies, the microbial reduction of U(VI) has been intensively studied (Lovley *et al*., 1991, 1993a, 1993b). To reveal the occurrence of and pathways for U(VI) reduction in aquatic sediments containing naturally produced organic matter, sediments from the inactive open pit uranium mine Midnite, located in Stevens County, eastern Washington, were incubated for 30 days at room temperature

under anaerobic conditions and analysed (Suzuki *et al*., 2005). X-ray absorption near-edge structure (XANES) spectroscopy revealed that U(VI) was reduced to U(IV) in these sediments, which contained nitrate, Fe(III), and sulphate. The latter had also been reduced during the experiment (Suzuki *et al*., 2005). Analysis of the sediment particles and the microbial cells by scanning and transmission electron microscopy coupled with elemental analysis by energy dispersive spectroscopy revealed that the reduced uranium was concentrated at microbial cell surfaces. Because the U(IV) was not associated with framboidal pyrite or nanometre-scale iron sulphides, the authors suggested that U(VI) was reduced by the enzymatic activities of the microorganisms (Suzuki *et al*., 2005). Microbial populations in the original sediment were analysed by amplification and sequencing of the 16S rRNA and dissimilatory sulphite reductase genes, which demonstrated that organisms belonging to the families *Geobacteraceae* and *Desulfovibrionaceae* were present. Because cultivated members of these lineages are also able to reduce U(VI), the authors suggestion of biological U(VI) reduction was confirmed (Suzuki *et al*., 2005). In addition to the bacterial diversity, archaeal diversity was also investigated in these uranium contaminated sediments. Archaeal 16S rRNA gene sequences representing *Methanobacteria* of *Euryarchaeota* were found to be predominant (Suzuki *et al*., 2005). Methan-producing *Archaea* (*Methanobacterium subterraneum*) were also isolated from deep granitic groundwater from the Äspö hard rock laboratory located in the vicinity of the Simpevarp nuclear power plant north of Oskarshamm, South-East Sweden (Kotelnikova *et al*., 1998). Only a few additional studies investigated the archaeal diversity in heavy metal contaminated environments (Takai *et al*., 2001; Stein *et al*., 2002). Recently, it was demonstrated that the hyperthermophilic crenarchaeon *Pyrobaculum islandicum* is able to reduce U(VI) to the insoluble U(IV) mineral uraninite at ca. 100 °C (Kashefi & Lovley, 2000).

Different studies were performed by addition of nutrients to uranium contaminated groundwaters and soils to increase the number and activity of indigenous microorganisms prospective for bioremediation (Holmes *et al*., 2002; Anderson *et al*., 2003; Nevin *et al*., 2003; Suzuki *et al*., 2003; North *et al*., 2004; Brodie *et al*., 2006; Nyman *et al*., 2006). Changes in the microbial community were observed when U(VI) reduction was stimulated by addition of acetate in sediments from three different uranium-contaminated sites in the floodplain of the San Juan River in Shiprock, New Mexico, USA. The treatment resulted in a dramatic enrichment of microorganisms of the family *Geobacteraceae,* which are known as U(VI)- and Fe(III)-reducing microorganisms (Holmes *et al*., 2002). *Deltaproteobacteria*, including *Anaeromyxobacter dehalogenans*-related and also several *Geobacter*-related

species, were stimulated in acidic subsurface from the FRC in Oak Ridge, Tennessee, USA as well as by pH neutralisation and during *in situ* biostimulation with glucose or ethanol (North *et al*., 2004). These results were supported by the investigation of Fe(III)-reducing enrichment cultures initiated from sediments of the same site possessing low pH values as well as high uranium and nitrate contaminations (Petrie *et al*., 2003). These cultures were predominated by different Fe(III)-reducing *Anaeromyxobacter* spp., and by Gram-positive organisms previously not known to reduce Fe(III), such as *Paenibacillus* spp. and *Brevibacillus* spp. (Petrie *et al*., 2003).

Recently, interesting results were published from replicate batch microcosms containing contaminated sediment collected from a well within FRC in Oak Ridge, Tennessee, USA, added with an inoculum from a pilot-scale fluidised bed reactor representing the inoculum in the field experiment and then supplemented with ethanol and uranyl acetate (Nyman *et al*., 2006). After an initial reduction of nitrate, both sulphate and soluble U(VI) concentrations decreased, resulting in U(IV) formation, which was confirmed by XANES spectroscopy. Denitrifying organisms related to *Acidovorax* were predominant as demonstrated by terminal restriction fragment length polymorphism (T-RLFP) and cloning (Nyman *et al*., 2006). Interestingly, *Acidovorax* isolates from the inoculum were also shown to reduce U(VI). However, in some microcosms, the soluble U(VI) concentration increased after longer incubations due to reoxidation that was explained by the authors to be a result of the microbial and/or mineralogical heterogeneity among the samples (Nyman *et al*., 2006).

Uranium-contaminated sediment and water collected from the inactive uranium mine Midnite, located in Stevens County in eastern Washington, USA, were incubated anaerobically with a mixture of different organic substances (lactate, acetate, ethanol, benzoic acids, glucose, yeast extract and peptone) (Suzuki *et al*., 2003). The removal of U(VI) from the solution within one month was observed after this biostimulation. Bacterial community analysis, based on the 16S rRNA gene retrieval, revealed that the natural microbial populations were shifted from microaerophilic *Proteobacteria* to anaerobic low-G+C Gram-positive sporeforming bacteria, belonging to *Desulfosporosinus* spp. and *Clostridium* spp. (Suzuki *et al*., 2003). In the highly saline uranium-contaminated aquifer sediments at the uranium mill tailings site Shiprock, New Mexico, USA an enrichment of both *Desulfosporosinus* spp. and *Pseudomonas* spp. was observed by the addition of acetate, which was associated with the removal of U(VI) from the groundwater (Nevin *et al*., 2003).

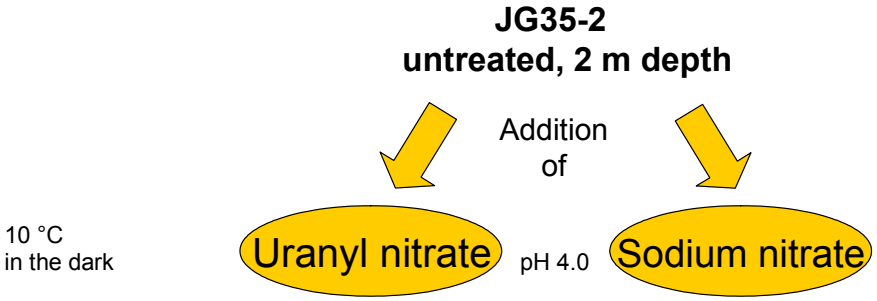
At the Uranium Mill Tailings Remedial Action site (UMTRA) in Rifle, Colorado, an *in situ* biostimulation with acetate was monitored over three months (Anderson *et al*., 2003). In this

case, the decrease in soluble U(VI) within 50 days was associated with an increase of Fe(II) and an enrichment of *Geobacter* spp. This indicated that the U(VI)-reducing microorganisms have the potential to immobilise uranium *in situ*. However, after 50 days of acetate injection, U(VI) began to increase within many of the field wells. These changes, after 50 days of injection, were accompanied by loss of sulphate from the groundwater and accumulation of sulphide. The composition of the microbial community was changed as well and sulphate reducers became predominant (Anderson *et al*., 2003). These results suggest that the long-term stability of reduced U(IV) is a major point for a successful bioremediation.

To monitor the long-term stability of bioreduced U(IV), flow through column incubations for more than 500 days, using soil from another area of the uranium contaminated FRC at Oak Ridge, Tennessee were performed by adding Na-lactate (Wan *et al*., 2005; Brodie *et al*., 2006). In this case, U(VI) reduction and immobilisation was successful within the first 100 days, followed by reoxidation and remobilisation of U(IV) under continuous reducing conditions (Wan *et al*., 2005). In order to determine if members of the microbial community are involved in the U(IV) reoxidation, a high-density oligonucleotide microarray-based approach was applied by Brodie *et al*. (2006). The amplicons of known metal-reducing bacteria, such as *Geothrix fermentans* and those within *Geobacteraceae*, were abundant during U(VI) reduction and U(IV) reoxidation. On the basis of these results, the authors suggest that observed reoxidation of uranium under reducing conditions occurred despite elevated microbial activity and the consistent presence of metal-reducing bacteria (Brodie *et al*., 2006). The terminal electron acceptor for U(IV) oxidation was not identified but the authors hypothesised that either Fe(III) or Mn(IV) were the most likely candidates (Wan *et al*., 2005; Brodie *et al*., 2006).

The fate of uranium in complex natural systems without the addition of organic substances is of great environmental importance in order to predict the potential risks of uranium migration within piles, tailings and depository sites and to prevent their spread out to groundwater flow. To our knowledge no experiments were performed including addition of uranyl nitrate to natural oligotrophic systems. For this purpose within the scope of this thesis, different microcosms experiments were performed to investigate the changes in the microbial community structure of a soil sample collected from the uranium mining waste pile Haberland after the addition of uranyl or sodium nitrate and incubations under aerobic or anaerobic conditions without supplementation with organic matter (Fig. 2). The work included the analysis of the bacterial as well as the archaeal diversity by using the 16S rRNA gene retrieval in the original untreated sample and in the treated samples (Fig. 2). Because uranium was

added in a form of uranyl nitrate to the soil samples and nitrate influences the mobility of uranium, the ability of the indigenous microorganisms to reduce nitrate was studied applying a culture-independent approach based on the nitrate reductase gene *nar*G as a functional marker. In addition, a spectroscopic method was used to determine the Fe(II)/Fe(III) ratio in the untreated sample and the uranyl nitrate treated samples incubated under anaerobic conditions.



Aeration conditions / Time of incubation	U content mg kg^{-1}	aerobic	anaerobic	aerobic	anaerobic	U content mg kg^{-1}
4 weeks	~100	JG35+U1	JG35+U4	JG35-K1	JG35-K4	26 - 40
14 weeks	~300	JG35+U2A	JG35+U3		JG35-K2	26 - 40
28 weeks	~300	JG35+U2B				

Fig. 2. Microcosms experiments performed.

Under aerobic conditions both treatments with uranyl and sodium nitrate resulted in the reduction of *Alphaproteobacteria* and *Acidobacteria* (Chapter 1), which were predominant in the untreated samples (Selenska-Pobell, 2002; Geissler, 2003; Satchanska *et al*., 2004). In the sub-sample treated with sodium nitrate for 4 weeks a strong proliferation of particular denitrifying and nitrate-reducing populations of *Arthrobacter* spp. and representatives of the *Bacteroidetes* phylum was observed. Stimulation of *Pseudomonas* spp. from *Gammaproteobacteria*, *Arthrobacter* spp. from *Actinobacteria* as well as of *Geobacter* spp. from *Deltaproteobacteria* was occurred in the parallel sub-sample incubated with uranyl nitrate for 4 weeks under the same conditions. However, by selective sequential extraction analyses and X-ray absorption spectroscopy no accumulation of U(IV) was observed in this sample even though the occurrence of some Fe(III)- and U(VI)-reducing *Geobacter* spp. (Geissler *et al*., 2005).

After a longer incubation for 14 weeks, even with higher amounts of uranyl nitrate different uranium-sensitive *Bacteroidetes* and alphaproteobacterial populations as well as

Arthrobacter spp. were stimulated. The strong proliferation of probably uranium sensitive representatives of the *Bacteroidetes* phylum indicated that at these late stages of the treatment the added U(VI) was no longer bioavailable. Moreover, it was noticed that after even longer incubation time of 28 weeks, of which half of the time was under anaerobic conditions, the bacterial community had a tendency to establish the initial structure of the natural sample JG35-2 (Chapter 1).

The treatment with uranyl or sodium nitrate under anaerobic conditions induced changes in the bacterial community structure as well, but as expected a proliferation of different bacterial groups occurred (Chapter 2). The anaerobic conditions correspond better to the natural conditions, because the sample studied was collected from a depth of 2 m. The addition of sodium nitrate and the incubation for 4 weeks resulted in a proliferation of representatives of *Firmicutes* and of *Betaproteobacteria*. By the treatment with uranyl nitrate under the same conditions only a small part of these populations was induced and established. Analogue to the untreated sample *Alphaproteobacteria* were predominant in the uranyl nitrate treated sample, although a shifting to probably uranium tolerant species was observed. In the latter sample *Gammaproteobacteria* especially *Rahnella* spp. were stimulated. Similarly to the sample treated with sodium nitrate for 4 weeks, *Betaproteobacteria* were stimulated after longer incubations for 14 weeks with sodium nitrate. However, after the addition of higher amounts of nitrate and a longer incubation for 14 weeks in contrast to the sample incubated for 4 weeks, the size of the *Firmicutes* was reduced and representatives of *Actinobacteria*, of the *Bacteroidetes* phylum as well as of *Gammaproteobacteria* were predominant. After the treatment with uranyl nitrate very diverse alphaproteobacterial populations and some uranium-sensitive betaproteobacterial and *Bacteroidetes* populations were established. The increased diversity, especially of U-sensitive populations in the latter sample indicated that at the late stages of the treatments the added U(VI) was no longer bioavailable. Interestingly, no or only a few representatives of *Deltaproteobacteria* were identified in the uranyl or sodium nitrate treated samples incubated under anaerobic conditions, whereas they represented the third predominant group in the untreated sample and most of them are known as sulphate- and metal-reducing bacteria (Lovley *et al*., 1991, 1993a, 1993b; Lovley & Phillips, 1992; Wu *et al*., 2006). Recently, biological U(VI) reduction was demonstrated for an increasing number of taxonomically diverse microorganisms such as *Clostridium* spp. (Francis *et al*., 1994; Suzuki *et al*., 2003), *Pseudomonas* sp. (McLean & Beveridge, 2001), *Desulfosporosinus* spp. (Suzuki *et al*., 2004), *Acidovorax* spp. (Nyman *et al*., 2006), *Salmonella subterranean* (Shelobolina *et al*., 2004), *Cellulomonas* spp. (Sani *et al*., 2002) and *Thermoanaerobacter*

spp. (Roh *et al*., 2002). For this reason we can not exclude U(VI) reduction in the studied uranyl nitrate treated samples.

By using Mössbauer spectroscopy it was revealed that the amount of Fe(II) increased in the samples treated with uranyl nitrate for 4 and for 14 weeks under anaerobic conditions, which indicated the reduction of Fe(III). Reduction of Fe(III) to Fe(II) was observed associated with the reduction of U(VI) after biostimulation with acetate (Anderson *et al*., 2003) and by the incubation of uranium contaminated sediments under anaerobic conditions (Suzuki *et al*., 2005). The reduction of Fe(III) can occur abiotically with hydrogen sulphide or U(IV) as the reductant (Luther *et al*., 2001; Senko *et al*., 2005a) or biologically by Fe(III)-reducing bacteria. The electron donor for the Fe(III) reduction in the samples treated with uranyl nitrate studied in this work are not yet identified. Further studies are necessary to characterise the fate of U(VI) in the samples studied as well.

The results presented in this thesis demonstrated that the indigenous bacterial community of the uranium mining waste pile Haberland possesses a high potential to adapt to changing nitrate and U(VI) concentrations under aerobic and anaerobic conditions. Most of the cultured members of the lineages stimulated by the treatments are known to interact with uranium and nitrate. However, the full impact of the metabolic capabilities of the bacteria stimulated by the treatment on the behaviour of U(VI) remain unknown.

The second objective of the thesis was to study if the inhabiting microorganisms are capable to reduce nitrate. For this propose the nitrate-reducing bacterial community was studied in the untreated sample from the uranium mining waste pile Haberland as well as in the samples treated with uranyl or sodium nitrate and incubated for 4 weeks under aerobic or anaerobic conditions by using the nitrate reductase gene *nar*G as a functional marker (Chapter 3, Fig. 2). Most of the sequences retrieved from the untreated sample were closely related to environmental NarG sequences of not yet cultured bacteria described in previous studies (Chèneby *et al*., 2003; Deiglmayr *et al*., 2004; Mounier *et al*., 2004; Héry *et al*., 2005). They were distantly related to the NarG sequence of the alphaproteobacterium *Brucella suis*. Only two additional small groups of sequences clustered with the NarG of the betaproteobacterial species *Thiobacillus denitrificans* and *Polaromonas naphthalenivorans*. In the sample treated with uranyl nitrate and incubated under aerobic conditions for 4 weeks, no NarG sequence related to *Brucella suis* was identified. Instead, sequences related to the NarG of *Geobacter metallireducens*, *Chromobacterium violaceum*, *Pseudomonas fluorescens*, as well as of *Arthrobacter* spp. were detected. This result confirms the presence of *Pseudomonas* spp., *Geobacter* spp. and *Arthrobacter* spp. identified by the 16S rRNA gene retrieval in this

sample (Chapter 1). By the treatment with sodium nitrate under aerobic conditions most of the retrieved NarG sequences were closely related to the NarG of *Arthrobacter* sp. FB24 or formed novel clusters within the "*Brucella*"- and "*Polaromonas*"-like clusters. Interestingly, proliferation of *Arthrobacter* spp. was observed by the 16S rRNA gene retrieval as well (Chapter 1). In contrast, by the treatment with sodium nitrate under anaerobic conditions most of the NarG sequences retrieved were distantly related to NarG of *Bacillus* spp. and *Chromobacterium violaceum*. Representatives of *Firmicutes* and *Betaproteobacteria* were found to be predominant in this sample by using the 16S rRNA gene retrieval. However, no 16S rRNA gene sequences related to *Chromobacterium* were detected and the *Firmicutes* were represented by *Bacillus* spp., and *Clostridium* spp. (Chapter 2). The latter could not be identified by the *nar*G gene retrieval. The treatment with uranyl nitrate under anaerobic conditions induced mostly NarG sequences distantly related to the NarG of *Polaromonas* spp. or to the NarG of particular *Alpha*- and *Betaproteobacteria* found in the untreated sample. The results revealed that the nitrate-reducing community was influenced by the treatments dependent on the nitrate solution used as well as on the aeration conditions. It was demonstrated that in consensus to the 16S rRNA gene retrieval some of the bacteria stimulated by the treatments possess the membrane-bound nitrate reductase NAR and are capable of nitrate reduction.

The third objective of the thesis was to investigate the archaeal diversity in the samples studied (Chapter 4). To our knowledge this is the first analysis of an archaeal response to treatments with uranyl nitrate under aerobic or anaerobic conditions. The archaeal diversity in the untreated sample was estimated to be lower in comparison to the bacterial diversity. All the sequences retrieved were related to only a few lineages of mesophilic *Crenarchaeota* and no representatives of *Euryarchaeota* were identified. 64% of the clones retrieved from the untreated sample represented the crenarchaeal group 1.1a and 36% of them represented the crenarchaeal group 1.1b. Independent on the addition of uranyl or sodium nitrate and the incubations under aerobic or anaerobic conditions as well as on the time of incubation a reduction of the archaeal diversity in comparison to the untreated sample and a shifting to the mesophilic *Crenarchaeota* of group 1.1b was observed. Most of the 16S rRNA gene sequences, retrieved from the treated samples were closely related to the 16S rRNA gene sequence of the fosmid clone 54d9. The fosmid clone 54d9 is a 43 kb genomic fragment with a ribosomal 16S rRNA gene, which was identified by PCR screening of archaeal operon-encoding fragments in a 1.2 Gb large-insert environmental fosmid library prepared from a soil sample collected from the upper 10 cm layer of a sandy ecosystem called "Am Rotböhl" near

Darmstadt, Germany (Treusch *et al*., 2005). The insert also encoded a homologue of a copper-containing nitrite reductase and two proteins related to subunits of ammonia monooxygenases (Treusch *et al*., 2005). Because of these results the authors suggested that the mesophilic *Crenarchaeota* might be capable of ammonia oxidation under aerobic and potentially also under anaerobic conditions (Treusch *et al*., 2005). On the basis of the close relatedness of our 16S rRNA sequences to the 16S rRNA gene found on the fosmid clone 54d9, a similar metabolism for the uncultured *Crenarchaeota* stimulated by the treatments performed in our study was suggested. Nitrate used in our treatments was most probably transformed to ammonium by nitrate reduction and denitrification or dissimilatory nitrate ammonification by the bacteria present in the samples. Indeed, the analysis of the bacterial diversity in the treated samples studied revealed a stimulation of nitrate-reducing, denitrifying and ammonifying populations (for instance *Geobacter* spp., *Pseudomonas* spp., *Arthrobacter* spp., and *Clostridium* spp.), which can supply the *Crenarchaeota* with different nitrogen oxide compounds (NO_2, NO) and ammonium (Chapters 1, 2, 3). Under anaerobic conditions, it is also possible that nitrite can be used as an electron acceptor and energy can be gained via nitrogen dioxide (NO_2)-dependent ammonia oxidation (Schmidt *et al*., 2004).

In line with the results of others (Vetriani *et al*., 1999; García-Martínez *&* Rodríguez-Valera, 2000; Benlloch *et al*., 2002), microdiversity among closely related archaeal 16S rRNA gene sequences was demonstrated in our samples. The microdiverse 16S rRNA genes might represent organismal genetic diversity, because up to date all cultured *Crenarchaeota* have only one rRNA operon (Vetriani *et al*., 1999; Ochsenreiter *et al*., 2003). The results presented in the thesis indicate dynamic and active archaeal populations that react to changes in environmental conditions in the studied uranium mining waste pile.

In addition to the description of the distribution of 16S rRNA and other gene sequences in the environment, it is necessary to have information on the physiology of pure microbial cultures related to the natural populations identified via molecular methods (Lovley, 2003). Thus, the fourth objective of the thesis was to isolate bacteria from the uranium mining waste pile and to study their interactions with uranium and other heavy metals (Chapter 5). A lot of efforts were put to culture microorganisms from radionuclide contaminated sites and to study their interactions with radionuclides and heavy metals (Selenska-Pobell *et al*., 1999; Selenska-Pobell, 2002; Benyehuda *et al*., 2003; Elias *et al*., 2003; Fredrickson *et al*., 2004; Nazina *et al*., 2004; Fields *et al*., 2005; Merroun *et al*., 2006; Nedelkova *et al*., 2006). The advantage of studying bacterial and archaeal cultures is that they can provide important information about the physiological and metabolical properties of the isolates. Lovley *et al*. (1991, 1993a)

demonstrated for the first time the dissimilatory U(VI) reduction by the Fe(III)-reducing bacteria *Geobacter metallireducens* and *Shewanella oneidensis* that can conserve energy for anaerobic growth via the reduction of U(VI). Other organisms as sulphate-reducing bacteria (*Desulfovibrio desulfuricans* (Lovley & Phillips, 1992), *Desulfovibrio vulgaris* (Lovley *et al*., 1993b), *Desulfosporosinus* sp. (Suzuki *et al*., 2004), *Clostridium* sp. ATCC 53464 (Francis *et al*., 1994), *Salmonella subterranean* (Shelobolina *et al*., 2004) and *Anaeromyxobacter dehalogenans* strain 2CP-C (Wu *et al*., 2006) are also able to reduce U(VI), however without conserving energy from this process. On the other hand, the oxidation of U(IV) to U(VI) by microorganisms was demonstrated (DiSpirito & Tuovinen, 1982; Beller, 2005).

The ability of different *Bacillus* spp. isolated from the uranium mining waste pile Haberland to remove toxic metals and uranium from solutions was also studied (Selenska-Pobell *et al*., 1999). The strain *Bacillus sphaericus* JG-A12 was used for construction of biological ceramics (biocers) via sol-gel immobilisation of its vegetative cells, spores or surface layer sheets. These biocers demonstrated high binding capacity of uranium and copper from contaminated waters (Raff *et al*., 2003).

Chemolithoautrophic bacteria, such as *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*, as well as heterotrophic bacteria such as *Desulfovibrio* spp., *Pseudomonas* spp. as well as strains related to *Agrobacterium*, *Rhizobium* and *Sphingomonas* were isolated from different uranium mining waste piles (Merroun & Selenska-Pobell, 2001; Selenska-Pobell *et al*., 2002; Selenska-Pobell, 2002; Merroun *et al*., 2003).

Bacteria were also isolated from groundwater samples collected in the vicinity of radioactive waste repositories. From the S15 deep-well monitoring site, located at the Siberan radioactive subsurface depository Tomsk-7, Russia, *Alphaproteobacteria* and *Actinobacteria* from the genus *Microbacterium* were isolated, which accumulate high amounts of uranium (Nedelkova *et al*., 2006). Sulphate-reducing bacteria as for instance *Desulfovibrio aespoeensis* were isolated from the granitic groundwater of south-eastern Sweden, where the Äspö hard rock laboratory (HRL) has been constructed to study the function of underground repositories for long-lived nuclear fuel waste (Motamedi & Pedersen, 1998)

From the Department of Energy (DOE) Hanford site Gram-positive bacteria closely related to *Arthrobacter* spp. were most common isolates among all samples, but other phyla with high G+C content including *Rhodococcus* and *Nocardia* were also presented (Fredrickson *et al*., 2004). From another study from the Hanford site also mostly high G+C Gram-positives (*Arthrobacter* spp.), low G+C Gram-positive bacteria (*Bacillus* spp. and *Staphylococcus* spp.) and different *Proteobacteria* (*Caulobacter* spp., *Sphingomonas* sp., *Variovorax* spp.,

Pseudomonas spp. and *Acinetobacter* spp.) were isolated. Similarly, the DOE`s Savannah River site (SRS) isolates were mostly high G+C Gram-positive bacteria (mainly *Arthrobacter* spp.) and *Proteobacteria* (*Comamonas* spp. and *Acinetobacter* spp.) (Benyehuda *et al*., 2003). In another study where cultivation-based methods were used to isolate aerobic heterotrophs present in radionuclide and heavy-metal contaminated subsurface soils at the FRC in Oak Ridge, Tennessee, USA the majority of the isolates (392 of 400) recovered were Gram-positive and belonged to the high-G+C-content genus *Arthrobacter* and low-G+C-content genus *Bacillus* as well (Martinez *et al*., 2006). The authors could demonstrate that the *Arthrobacter* strains exhibited the greatest tolerance to low pH and U toxicity. The type strain *A. nicotianae* could remove more than 80% of the uranyl ions from an aqueous solution at pH 4.0, which was suggested to be due to physico-chemical binding to the cell components (Tsuruta, 2002). Recently, it was demonstrated that *Arthrobacter* sp. S3 closely related to *Arthrobacter ilicis* is able to accumulate uranium intracellularly (Suzuki & Banfield, 2004). The ubiquity of *Arthrobacter* spp. in contaminated soils stimulated our research interest.

Two isolates JG37-Iso2 and JG37-Iso3 were recovered from the uranium mining waste pile Haberland, which 16S rRNA gene sequences affiliated with *Actinobacteria* from the genus *Arthrobacter* (Chapter 5). Interestingly, an increased number of 16S rRNA gene sequences closely related to the 16S rRNA gene sequences of these two *Arthrobacter* strains were identified in the samples treated with uranyl or sodium nitrate (Chapters 1, 2, 5). In this thesis, a combination of wet chemistry, spectroscopic, microscopic and microbiological methods was used to elucidate the tolerance of the two bacterial strains to lead and uranium. The two strains tolerate different amounts of lead. Lead sorption studies demonstrated that *Arthrobacter* sp. JG37-Iso3 accumulates up to 110 mg Pb g^{-1} dry biomass at pH 4.5, whereas *Arthrobacter* sp. JG37-Iso2 accumulates lower amounts of this heavy metal (up to 76 mg Pb g^{-1} dry biomass). The time course of Pb sorption by non-growing (resting) cells of the two *Arthrobacter* strains at pH 4.5 was studied as well. It was demonstrated that in the first two hours, only a small portion of the Pb was accumulated and then continuously more Pb was removed from the solution. This is an indication that more than one process is involved in the removal of lead from the solution by the non-growing cells. X-ray diffraction (XRD) analysis revealed that a lead phosphate phase pyromorphite ($Pb_5(PO_4)_3Cl$) was precipitated. Transmission electron micrographs (TEM) demonstrated that the precipitates of pyromorphite were localised mainly at the cell surface. These results are in congruence with the results of Templeton *et al*. (2003), who demonstrated by Extended X-ray Absorption Fine

Structure (EXAFS) spectroscopy and TEM observations that the enhanced Pb accumulation is due to the formation of nanoscale crystals of pyromorphite ($Pb_5(PO_4)_3OH$) adjacent to the outer-membrane of a fraction of the total population of *Burkholderia cepacia*. Life/Dead images from our lead-treated *Arthrobacter* cells demonstrated that after incubation for 48 h at pH 4.5 more than 50% of the cells are still alive.

Changes in the colour of the colonies from milky-white to brownish black were observed on low phosphate agar containing lead. X-ray diffraction analysis revealed that lead sulphide (PbS, galena) was formed by these isolates in this case, in contrast to the above mentioned pyromorphite formation by non-growing cells. At this stage of investigation, the origin of sulphide implicated in the precipitation of Pb is unknown.

In Chapter 5 the interactions of the two *Arthrobacter* strains JG37-Iso2 and JG37-Iso3 with uranium were studied as well. The time course of uranium sorption for both strains was similar to the sorption kinetics of lead, which was relatively slow. It was demonstrated that *Arthrobacter* sp. JG37-Iso3 accumulates higher amounts of uranium (up to 162 ± 6 mg U g^{-1} dry biomass) in contrast to *Arthrobacter* sp. JG37-Iso2 (up to 108 mg U g^{-1} dry biomass). TEM demonstrated that both strains studied are able to accumulate uranium intracellularly. These results are in line with the results of Suzuki and Banfield (2004), who demonstrated that *Arthrobacter* sp. S3, related to *Arthrobacter ilicis*, is able to accumulate uranium intracellularly in precipitates closely associated with polyphosphate granules. X-ray absorption spectroscopy (XAS) studies showed that U(VI) formed complexes with organically bound phosphates of the cells of the both *Arthrobacter* strains in a monodentate binding mode with an average bond distance between U and P of 3.60 ± 0.02 Å. The structural parameters of these uranium complexes are similar to those of the uranium complexes formed by fructose-6-phosphate (Koban *et al*., 2004). The results of the minimal inhibitory concentration for the growth of the isolates for nickel, chromium, copper, cadmium, silver, lead and uranium determined on low phosphate solid medium also revealed high heavy metal tolerance.

The results presented in Chapter 5 demonstrated that the growing cells of the strains precipitated Pb as galena (PbS), whereas in non-growing conditions pyromorphite ($Pb_5(PO_4)_3Cl$) phase was produced, alleviating probably the toxicity of Pb. Uranium was accumulated intracellularly by both strains as uranyl organic phosphate complexes. Our results indicated that these strains are interesting for the remediation of radioactive and mixed-wastes sites.