Studies on heavy metal resistance of bacterial isolates from a former uranium mining area

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Once again, what appears to us in the mystical guise of pure science and objective knowledge about nature turns out, underneath, to be political, economic, and social ideology.

—R. C. Lewontin

Contents

1.1 Metals in the environment

"When you create a mine there are two things you can't avoid: a hole in the ground and a dump for waste rock." As simple as this comment of Charles Park in a novel by John McPhee (McPhee, 1971) sounds, as severe is the consequence. The surface of the Earth is affected by mining operations with an area of 240.000 square kilometres (Furrer, 2002). The inevitably injurious effects on the biosphere, not only within the mining sites but across stretched regions in the surrounding as well, are hard to foresee and to estimate. Long-term effects, the delay of effects, and the dimension of the affected areas are only some of the crucial factors determining alteration and destruction of biotopes. Biotopes are rarely protected by geo- or pedological barriers from the intrusion of pollutants; on the contrary, they maintain an intense interconnection with the mining site itself. The lack of spatial and temporal separation from the site leads to ecological disturbances. Most important is the transmission of pollutants like heavy metals from waste piles and pits with the waterpath which can be noxious to microbes, plants, animals and human beings. The unearthing of geological formations with its subsequent scarcely preventable weathering and chemical alteration of minerals can cause the generation of acidic seepage waters, which trickle through soil habitats and are distributed vertically and horizontally into microbial habitats. Microbes, however, play the key role in mineralization of biological compounds, especially biopolymers like, e.g., lignocellulose and chitin by decomposing (McCarthy and Williams, 1992; de Boer et al., 1999). Thus, they are essential for the global biogeochemical cycling of elements. Perturbations of this particular type of habitat by infiltration of metals can have enormous effects on the biosphere.

According to Ross (1994), the anthropogenic sources of metal contamination can be divided into five main groups: (1) metalliferous mining and smelting, (2) industry, (3) atmospheric deposition, (4) agriculture, and (5) waste disposal. Worldwide, there is an increasing market for raw materials causing intensified mining activities. Use and dispersion of metals has assumed enormous proportions during the last century, and the behaviour of metals in the environment is therefore a matter of rising concern (Nriagu, 1990). The society as profiteer of mining products has to accept responsibility for minimizing the impact of mining operations on the biosphere, for the development of methods to protect biotopes, and for the remediation of contaminated areas.

1.2 Metallomorphic microbial habitats

1.2.1 Habitat characterization

The most characteristic feature of microbial habitats is the great variability of environmental parameters like, e.g., temperature or nutrient availability over short

distances. Many basic requirements of heterogeneous microorganisms are satisfied. In ecological terms, the microbial habitat consists of a multiplicity of niches. The microbial community, then, can be composed of diverse taxa with different nutritional demands within a small microenvironment. 'Every microbe can be found everywhere' and 'the environment selects' are the two seemingly contradictory hypotheses still discussed (Martiny et al., 2006). For the habitats of mining areas it is a clear mutual influence: microbes in soil are not only affected by their environment, but they also control particular soil parameter, directly and indirectly. Growth and metabolism can lead to changes in pH, Eh, and ionic strength of the soil. For example, excretion of organic acids leads to a pH decline and thereupon to higher mobility of heavy metals. This process of metal mobilization, in turn, determines the species composition within the habitat to a great extent. The microflora, again, strongly participates in processes like decomposing soil constituents as well as particle aggregation and influences soil texture and availability of nutrients for plants (Krasilnikov, 1961). This means that the food web in the soil is constituted to a high degree by microbes, which (1) produce substances that change the microenvironment by, e.g., solubilization of minerals and subsequent rock breakdown (Cole, 1979), (2) modify the soil structure by, e.g., production of extracellular polysaccharides (Hepper, 1975), and (3) influence the biogeochemical cycling of elements, e.g., sulphur (Schippers et al., 1996). The impairment of the biological activity of soils due to metal loading leads basically to a reduction in decomposition and turnover rates of organic matter (Babich and Stotzky, 1985). Ultimately, this interference can cause a reduction in primary production (Tyler, 1972).

For the availability of nutrients in the microbial habitat the intimate contact between water and soil is of utmost importance. Not only is the distribution of nutrients determined by the waterpath, the availability of trace elements and toxic metals is so as well. The bioavailability of metals in the habitat is influenced by the constitution of the soil matrix, climatic conditions, microbial activity, and especially the water flow. The metals contained in soil minerals are released into the soil solution as a result of weathering processes. Among the many parameters that govern the behaviour of a metal in the soil, the hard-soft character of a metal is not to underestimate as it determines the ligand preferences of the metal (Ahrland, 1968). The ligand preference, in turn, affects the distribution and speciation of the metal, thereby influencing the organisms of the habitat (Nieboer and Richardson, 1980). Biologically essential metals, like nickel, are hard or semi-hard, i.e. they prefer oxygen ligands and usually form ionic bonds with the ligands (Hughes and Poole, 1989). On the other hand, many toxic metals, e.g., cadmium, are soft. These metals, often associated with environmental pollution, have a higher affinity for nitrogen and sulphur containing ligands and form bonds of covalent character.

The cause for the frequently widely dispersed metal loading of habitats in mining areas has been found in the formation of acid mine drainage (AMD). The run-off from mining heaps of active and abandoned mines can reach pH values as low as pH 2. The microbes mainly

responsible for the formation of AMD are metabolically active even below pH 2 (Rawlings, 2002). If the chemical and microbial processes in the exposed overburden are set into motion once, AMD formation is hard to control again and can last for incalculably long times. Chemical and biological oxidation of the abundant mineral pyrite (FeS_2) takes place after the unearthing of pyrite containing rock formations and results in an acidification of the dump material (Colmer, 1947). AMD has a typical orange or ocherous appearance which is due to the iron hydroxide that is formed during the oxidation. The iron hydroxide precipitates as sludge, coating the bottoms of streams and canals (Fig. 1, 2 and 3). Under acidic conditions, most heavy metals are leached from the dump waste and are subsequently transported as AMD in streamwaters, if they are not collected. Conditions required for the generation of AMD are: (1) contact with the atmospheric oxygen, (2) an aqueous environment, (3) and the occurrence of iron oxidizing, acidophilic bacteria.

Figure 1: AMD formation at a former uranium mining site in Thuringia. Photo: S. Senitz.

Figure 2: Accumulated AMD with typically precipitated iron hydroxide. Photo: S. Senitz

Figure 3: AMD collected in canals and pumped to a treatment plant. Photo: S. Senitz.

Iron oxidizing bacteria like members of the genera *Thiobacillus*, *Leptospirillum* and *Ferroplasma* use Fe^{2+} as electron donor to satisfy their energetic demands (Fig. 4). But due to the high energy demand for autotrophic life – supply of reducing power for CO_2 fixation – the energetic yield of the Fe^{2+} to Fe^{3+} oxidation is relatively scarce for the overall energy requirement of the cell. To satisfy the energy demand and to maintain the vital functions of the cell, the substrate turnover has to be high. The formation of one gram biomass (dryweight) requires the oxidation of an amount of about 55 gram Fe^{2+}. Fe^{3+}, in turn, oxidizes pyrite in a fast autocatalytic mechanism in the presence of water under generation of protons which lead to a pH decrease. In the overall reaction, the part of the abiotic oxidation of iron is comparatively slow under acidic conditions. But due to the regeneration of the ferrous ion as electron donor for the bacteria, the change of iron from

(a) Abiotic process, slow oxidation rate, initiator reaction, acidification of the site:

$$FeS_2 + 7/2\ O_2 + H_2O \rightarrow Fe^{2+} + 2\ SO_4^{2-} + 2\ H^+$$

(b) Biotic process, energy-yielding reaction of iron-oxidizing bacteria, high turnover:

$$4\ Fe^{2+} + O_2 + 4\ H^+ \rightarrow 4\ Fe^{3+} + 2\ H_2O$$

(c) Abiotic process, fast, autocatalytic mechanism, regenerates electron donor for (b):

$$FeS_2 + 14\ Fe^{3+} + 8\ H_2O \rightarrow 15\ Fe^{2+} + 2\ SO_4^{2-} + 16\ H^+$$

Figure 4: Equations of AMD formation.

the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state enters a propagation cycle, and acidification accelerates (Singer and Stumm, 1970).

Formation of AMD is both a problem of active mining and of abandoned mines. The high number of abandoned mines worldwide poses a threat to the potable water protection areas. Generation of AMD is hard to avoid, because pyrite is the most common sulphide mineral and pyrite containing excavated matter is the result of worldwide operating metal and coal mining. It is a global issue, affecting not only countries where mining activities take place, but also neighbouring countries, whose environments may be adversely affected by migrating pollution. There are several options to reduce the rise of AMD. Access of oxygen to the dump material can be prevented by water saturation of the sulphidic material. In some cases the mining operations can be performed in the absence of water. In many remediation sites the dump material is sealed with watertight substrates. Liming of the dump material supports neutralization of acidic seepage waters. However, there is no perfect barrier to separate the reaction components and therefore the resulting AMD has to be collected in reservoirs (Fig. 5). Precaution and permanent monitoring are of utmost importance for the protection of nearby biotopes (Fig. 6). In some cases the AMD treatment can comprise the recovery and recycling of precious metals by using biomass material as biosorbent (Volesky, 2001). There are terrains known for generation of acidic drainage with the same chemical and microbial processes, but not initiated by human intervention. This natural type of alteration of sulphidic rocks, for example, in Rio Tinto, Spain, is considered as acid rock drainage (ARD). The high acidity of both, AMD and ARD, and the high amounts of dissolved heavy metals generally lead to an extreme toxicity to most organisms (Pentreath, 1994). Nevertheless, there are microbes thriving even in this type of environment. The phylogenetic diversity of both, prokaryotes and eukaryotes dwelling in drainage influenced habitats can reach unexpected dimensions as has been shown, e.g., for the extremely acidic environments (pH 1.7–2.5) of the Rio Tinto (Zettler et al., 2003).

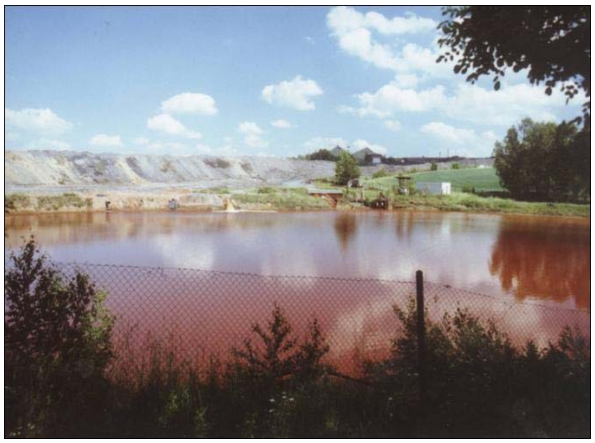

Figure 5: Drainage basin „Pohlteich“ at former waste rock dump „Nordhalde“, mining area Eastern Thuringia. Photo: L. Zeggel.

Figure 6: AMD run-off through a biotope inside a former uranium mining site, close to Ronneburg. Photo: L. Zeggel.

1.2.2 Examples of metallomorphic habitats

Microbes have to cope with high concentrations of different heavy metals in various kinds of habitats. For life under extreme conditions, habitats in areas in which mining is pursued are probably the most prominent (Fig. 7), but in terms of evolutionary time those habitats within naturally metalliferous biotopes are more influential on adaptation and expression of microbial resistance determinants. It has been found that bacteria isolated from serpentine soils have developed strong resistance mechanisms that seem to be fundamental for survival in worldwide occurring, naturally nickel enriched soils (Fig. 8). Serpentine soils are depleted in nutrients causing a remarkably low number of microbes of any physiological group (Lipmann, 1926). This soil type is characterized by deficiency in available phosphate, paucity in ammonia and lack of readily decomposable carbon source. The magnesium to calcium ratio is high. Furthermore, serpentine soils are not only enriched in nickel, but due to the mineral composition of the base rock they display elevated levels of chromium, cobalt and iron as well. Taking all the pedological facts in consideration, the occurrence of typical serpentinophytes (Prasad and de Oliveira Freitas, 1999) and a characteristic microbial community structure become understandable (Fig. 8). These soils can contain enormous amounts of various metals, as it was shown on, e.g., soil samples from Andaman with up to 8 g nickel, 4 g chromium and 150 g iron per kg dry soil (Pal et al., 2005). A multiple metal-resistance of resident microbes is prerequisite for the occupation of this ecological niche. Bacterial isolates of serpentine soils of Tuscany were investigated on their resistance pattern towards several heavy metals and the magnitude of resistance in relation to the distance from the typical serpentinophyte *Alyssum bertolonii*. A simultaneous resistance to a set of metals and highest resistance from isolates of the rhizosphere were found to be characteristic (Mengoni et al., 2001). It is known, that nickel hyperaccumulating plants as, e.g., *A. bertolonii* provide a niche for nickel resistant bacteria (Schlegel et al., 1991). An example for the adaptation of microbes to the soil substrate of

metallomorphic habitats has been presented and shows the influence of nickel on soil structure of neocaledonian soils (Hery et al., 2003). The extreme environments in serpentinized neocaledonian soils are microbiologically well investigated. Owing to the particular mineral composition and to the distribution in isolated patches serpentinized outcrops have been considered ecological islands (Kruckeberg, 1984).

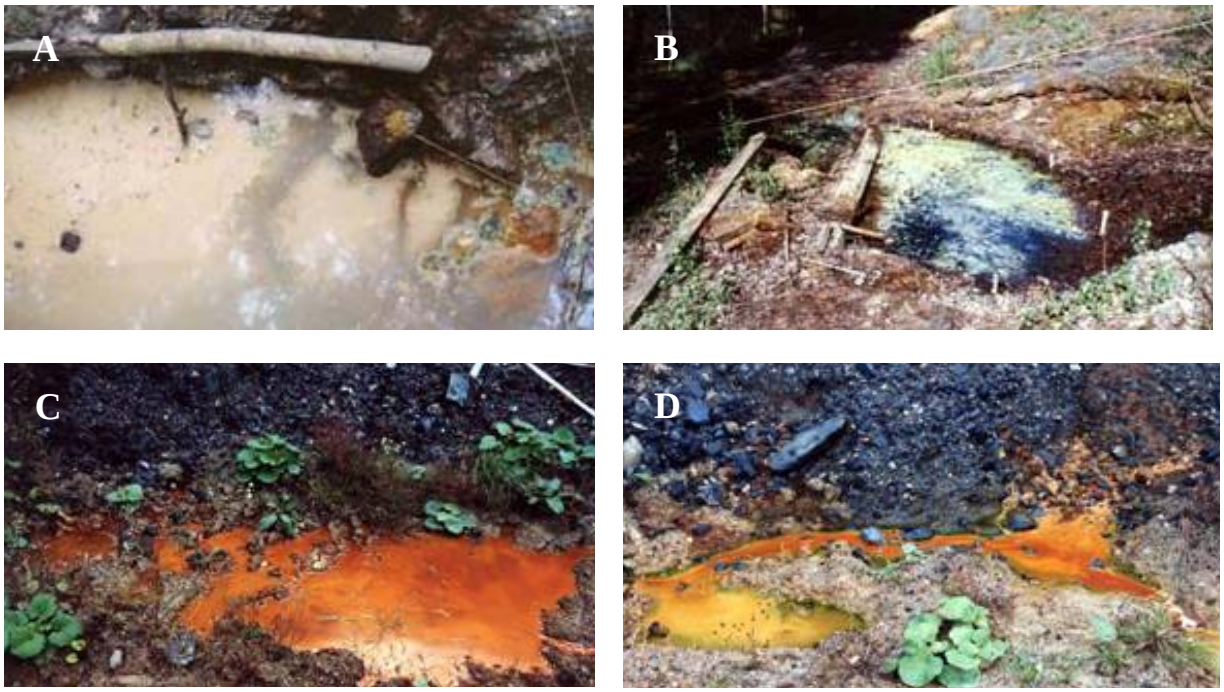

Figure 7: Metallomorphic microbial habitats of a former uranium mining area in Eastern Thuringia with elevated concentrations in iron, nickel and aluminium. A: Pool of seepage with high concentration of Al, B: Seepage discharge at base of a waste heap containing high concentration of Ni, C: AMD seeping through soil, D: AMD of a seepage discharge, note green layer of phototrophic growth. Photos: S. Senitz and L. Zeggel.

Anthropogenically created metalliferous habitats can be understood as ecological islands in a similar sense. Isolation of highly resistant organisms from anthropogenic metal rich habitats is not unusual. Publications on isolation of metal resistant bacteria from the sewage sludge of waste water treatment plants and metal-processing industry are numerous. Stoppel and colleagues isolated from a mineral oil emulsion tank a *Klebsiella oxytoca* strain resistant to 10 mM nickel (Stoppel et al., 1995). From a decantation tank of a zinc factory a strongly metal resistant *Alcaligenes xylosoxidans* strain could be isolated (Schmidt et al., 1991). This implies that ephemeral locations of technical facilities with a partially extremely high enrichment of metal containing compounds can act as metallomorphic microbial habitat and harbour bacteria with remarkable resistance attributes.

Figure 8: Serpentine biotope in Tuscany, Pieve Santo Stefano, Italy. Photo: L. Zeggel

If we continue to focus on time available for evolutionary adaptation, then the extreme habitats of mining areas can be positioned between long-lived metal containing outcrops (e.g., 40 million years of influence on adaptation in Neocaledonia) and short-lived, man-created metal contaminations of industrial installations. Microbial strains of various taxonomic categories have been isolated from metal mining sites (Schippers et al., 1995; Sprocati et al., 2006). Usually, studies on distribution and phylogenetic grouping involve the investigation of resistance mechanisms.

1.2.3 Aspects on methodologies for habitat description

There is only limited literature available on methods necessary for description of metallomorphic microbial habitats. In order to understand the role microbes play in mobilisation/immobilisation of metals in soils, it has to be differentiated between microbial activity and the effects of the soil matrix itself on mobility and sorption of metals. Rare earth elements (REE) are usually associated with the waste of metal mining. Minute amounts of REE are sufficient for detection and analysis. The fractionation of the chemically very similar REE, both in natural habitats and in microbial cultures under laboratory conditions, could be used as a tracer method to estimate predomination of geological or biological effects on metal mobility (Merten and Büchel, 2004; Merten et al., 2004). This tracer method indication can be used to estimate the proportion of physical sorption to microbial metal uptake. The pattern of the REE fractionation depends on the applied source which is in contact with the REE containing liquid. Different sources behave differently towards fractionation and could therefore help to explain their role in the metallomorphic habitat. Physical sorption leads to a gradual change of the REE pattern of seepage waters whereas microbial uptake fractionates the pattern due to higher affinity for particular REE.

1.3 Microbes dwelling in heavy metal enriched habitats

1.3.1 Microbe-metal-interactions

With 10^6-10^9 viable cells cm^{-3} bacteria are usually the most numerous organisms in soil (Lynch, 1988). Due to their small size, bacteria have a high surface to volume ratio and therefore provide a large contact area for interactions with the surrounding environment. Besides their occurrence in high numbers and their high surface to volume ratio it is the negative net charge of the cell envelope that makes these organisms prone to accumulate metal cations from the environment (Collins and Stotzky, 1992). Microbes can potentially accumulate metals either by a metabolism-independent, passive, or a metabolism-dependent, active process (Gadd, 1988). Thus, overall accumulation is determined by two characteristics of the cell: sorptivity of the cell envelope and capacity for uptake into the cytosol. Active uptake into the cytosol is usually slower than passive adsorption and is dependent on element-specific transport systems (Gadd, 1988). Passive adsorption is likely to be the dominant mechanism in metal accumulation, since scarcity of nutrients is the ground state for many natural environments in soils, and active uptake requires energy. Additionally, microbes probably lack highly specific uptake systems for most metals. The surface characteristics of the bacteria determine their metal-adsorption properties. The differences in cell wall construction of Gram-positive and Gram-negative bacteria have minor influence on the sorption behaviour of different metals (Jing et al., 2004). The bulk functional group chemistry of both classes of bacterial surfaces is similar, but particular single constituents of the cell envelope can have great importance for metal binding. For example, phosphoryl groups of lipopolysaccharides, carboxylic groups of teichoic and teichuronic acids, or capsule forming extracellular polymers influence the metal sorption of the cell envelope (Tab. 1). The interplay of metal mobilizing mechanisms and metal fixation forces is highly complex and dependent on a number of soil characteristics (Fig. 9).

Metals with no biological function are in general tolerated only in minute concentrations, whereas the essential metals with biological functions are usually tolerated in higher concentrations. They accomplish either metabolic functions as constituents of enzymes or meet structural demands as, e.g., in supporting the cell envelope. The concentration and the speciation of the metal determine whether it is useful or harmful to the bacterial cell. Homoeostasis is therefore essential and bacteria have developed a fine-tuned regulatory system of uptake, incorporation and excretion. Adverse effects of metals on the microbial cell are decreased decomposition of soil organic matter, reduced soil respiration, lower diversity, and decreased activity of several soil enzymes (Rühling and Tyler, 1973; Tyler, 1974). Depending on the external conditions microbial cells have developed mechanisms to cope with high concentrations of metals (Silver and Misra, 1988).

Table 1: Examples for metal-binding functional groups in bacterial surface components.

Functional group / Compound class	Location	Organism	Reference
Carboxyl	Lipopolysaccharide (LPS)	*Pseudomonas aeruginosa*	Langley and Beveridge, 1999
	Exopolymer	River sediment bacterial isolate	Geesey et al., 1988
	Peptidoglycan	*Escherichia coli*	Hoyle and Beveridge, 1984
	Peptidoglycan	*Bacillus subtilis*	Beveridge and Murray, 1980
Amine		*B. subtilis*	Beveridge and Murray, 1980
Amine / imidazole	Polypeptide	*Klebsiella pneumonia*	Möhl et al., 1988
Phytochelatins	Cysteine-rich peptides	*E. coli,* genetically engineered	Bae et al., 2000
Metallothioneins	Cysteine-rich peptides	*Ralstonia eutropha,* genetically engineered	Valls et al., 2000
Phosphoryl	Lipopolysaccharide (LPS)	*E. coli*	Ferris and Beveridge, 1986
	Teichoic acid	*B. subtilis*	Beveridge, 1981
	Phospholipids	Bacteria	Beveridge, 1989

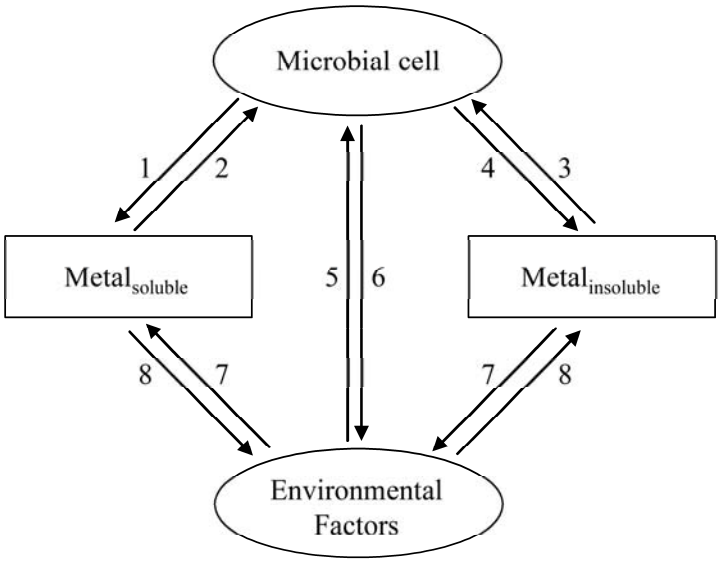

Figure 9: Model for the biogeochemical significance of metal transformation by microbial cells (modified after Gadd and Sayer, 2000). Microbial influence leads to changes in metal solubility which is influenced by environmental factors on these processes and on microbial growth, morphogenesis and physiology. The relative balance between the processes will depend on the environment, organisms, and interactions with other organisms including animals, plants and anthropogenic activities. 1, Metal solubilization by, e.g., heterotrophic leaching, siderophores, metabolite excretion including organic acids and H^+, redox reactions, methylation and biodegradation of organometal(loid)s. 2, Effects of soluble metal species on microbes and metal immobilization by biosorption, transport, intracellular sequestration and compartmentation, redox reactions, precipitations, and crystallization. 3, Effects of insoluble metal species on microbes, particulate adsorption, and entrapment by polysaccharide and/ or mycelial network. 4, Metal immobilization by, e.g., precipitation, crystallization, or reduction. 5, Influence of environmental factors, e.g., pH, O_2, CO_2, nutrients, salinity, toxic metals, and other pollutants, on microbial growth, metabolism, and morphogenesis. 6, Influence of microbial activities on the environment, e.g., alterations in pH, O_2, CO_2, and redox potential; depletion of nutrients; and enzyme and metabolite excretion. 7 and 8, Environmental factors which direct the equilibrium between soluble and insoluble metal species towards metal mobilization (step 7) or metal immobilization (step 8).

1.3.2 Survival strategies of heavy metal resistant bacteria

A great number of heavy metal resistant bacteria as, e.g., *Cupriavidus metallidurans* is known to possess efflux transporters that excrete toxic or over-concentrated metals (Nies, 1995; 2003). This type of transporters is characterized by a low K_m value and can therefore keep the cytosolic metal concentration low. From genomic data mining, actinobacteria presumably can have cation efflux transporters, but they were not functionally identified yet. The ABC transporters of actinobacteria are, in contrast, well investigated and are responsible for antibiotic resistance. Some of the ABC transporters function as metal efflux pumps as well (Borges-Walmsley et al., 2003). Alternatively, the microbial cell can prevent itself from being intoxicated by the release of metal binding compounds into the extracellular surrounding. The metals are chelated outside the cell and thus blocked from entering the cell through the unspecific membrane transporters that otherwise would facilitate the influx. Membrane transport systems of the cell can not differentiate between the trace elements needed for metabolic actions and toxic metals that would – once inside the cell – interfere with, e.g., the phosphoryl groups of nucleic acids or the thiol groups of

proteins. Some fungal and bacterial organisms are able to keep metals outside the cell by the extracellularly active melanin (Fogarty and Tobin, 1996; Beausejour and Beaulieu, 2004). This secondary metabolite has powerful cation chelating properties through the anionic function such as the carboxyl, and the deprotonated hydroxyl groups (Riley, 1997). For the metal burden of soil habitats the interplay of biosolubilization and bioprecipitation is of great importance. Numerous soil microbes, for example the widespread fungus *Aspergillus niger*, solubilize metals by the release of organic acids, while others – or even the same microorganisms – immobilize metals through the excretion of compounds as, e.g., oxalates (White et al., 1997; Gadd, 1999). If toxic metals have entered the cell and can not be excreted by efflux transporters several organisms have developed a cytosolic sequestration mechanism for protection. It has been shown for many metal resistant organisms that internal inclusion bodies, like, e.g., polyphosphate granules (volutin) bind large amounts of metal cations (Gonzalez and Jensen, 1998). The cell envelope equips the cell with an additional metal resistance feature. The cell wall, in combination with the cell membrane, supports the sorption of metals and facilitates bioreduction as well. It has been shown that, for example, *Penicillium chrysogenum* has the capacity to reduce silver. After reduction the metallic silver precipitates at the cell wall (Gadd, 1996).

Thus it can be summarized (Fig. 10) that metal resistance of microbes is accomplished by intra- and extracellular mechanisms; metals can be excreted via efflux transport systems; sequestering compounds of the cytosol can bind and detoxify metals inside the cell; the release of chelators into the extracellular milieu leads to bound and fixed metals; the structure of the cell envelope is prone to bind large amounts of metals by sorption thus preventing influx.

The investigation of microbial resistance mechanisms towards heavy metals is essential for the potential applications of microorganisms in bioremediation. The understanding of the resistance phenomena at the molecular level is the prerequisite necessary for the biological treatment of solid mining waste and resulting effluents. The combination of genomic approaches with geochemical and hydrological models is the ultimate goal to accelerate bioremediation (Lovley, 2003).

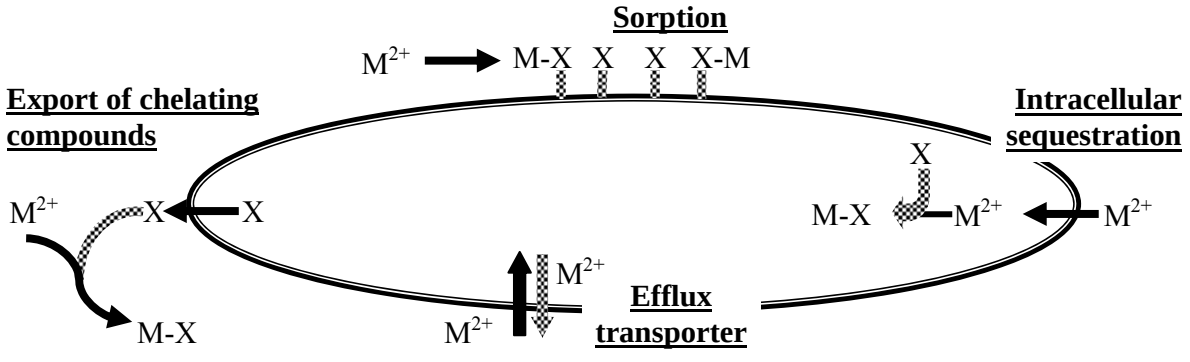

Figure 10: Overview of microbial resistance mechanisms. (X) – Cell constituents interacting with metal cations, (M) – Metal cation.

1.3.3 Search of microbes applicable to bioremediation processes

The average concentration of trace metals in soil solution – iron and manganese excepted – is usually within the range of 1 -100 ppm, depending on the abundance of the element in the lithosphere (Kabata-Pendias and Pendias, 2001a). Bacteria isolated from non-polluted environments are adapted to cope with concentrations within or below the micromolar range. However, for soil remediation with biological treatments, microbes adapted to far higher concentrations are required. Therefore, screening for microbes with high accumulation capacities and stable resistance characteristics is an inevitable part of any remediation strategy. Bioremediation is the use of living organisms to reduce, eliminate or immobilize environmental hazards resulting from accumulation of toxic chemicals and other hazardous wastes. The treatment is performed ex-situ or in-situ with solid and liquid waste (Gadd, 2000). The technology is based on the utilisation of naturally occurring (or genetically engineered) microorganisms or plants to transform organic and inorganic compounds. Often, biostimulation and bioaugmentation are components of a bioremediation strategy. Biostimulation utilizes the site-specific indigenous microorganisms. Nutrients or electron acceptors are added to the contaminated material. Bioaugmentation is the introduction of specific competent microbes to the local microbial population in order to increase the metabolic capacities needed for remediation (Gentry et al., 2004).

The processes of bioremediation are based on two premises: (1) the removal or detoxification of the pollutant and (2) the maintenance/improvement of soil fertility. The characteristics of the soil are not – or only to a limited extent – altered by chemical and mechanical measures. Phytoextraction applies selected plants which can take up a plant-specific set of metals as hyperaccumulators. These metals are subsequently enriched in the biomass and in comparison to excluder plants concentrated up to 1000 fold (Erdei et al., 2005). By phytoextraction, large quantities of the bioavailable metal fraction of the soil solution can be removed from the ground with the harvest, and are further processed for metal recycling. In comparison to conventional methods like soil excavation (*ex-situ* remediation), phytoextraction is time consuming, but cost effective and less labour-intensive. Interestingly, the role microbes play in phytoextraction is still underestimated. Often, the application of plants for bioremediation is restricted by the immature and poor soils of typical metal contaminated mining sites and mining affected grounds. High concentrations of heavy metals lead to retardation in growth and subsequently low yield during harvest whereas soils with lower metal concentrations do not warrant extraction. In both cases, the soil shows an unvaried load of the pollutant. For soils only weakly contaminated, another type of bioremediation can be applied. Biogenic barriers made of microbial biomass, so called biocurtains, are introduced into soil areas of contamination. The microbial cells act directly on the spot of contamination. Cost-effective biocurtains for the degradation and removal of halogenated hydrocarbons are already in use (Hyndman et

al., 2000). For metal pollution, contaminants are immobilized at (cell surface) or inside (cytosol) the cell. Thereby, heavy metals will be retained in the soil and the water path can be protected from metal pollution by blocking the contamination route. For this preventive measure the introduced microbes should: (1) be resistant towards the metals occurring in the habitat, (2) possess a high capacity for metal uptake or sorption, (3) be capable to adapt to the conditions of the habitat, (4) fix metals stably, in order to prevent a flush due to, e.g., a dying off in the cold season, and (5) allow biomonitoring. Literature on bioaugmentation using biocurtains for immobilization of heavy metal contaminants is, however, scarce.
In the presented thesis, soil samples of habitats from a former uranium mining area in Eastern Thuringia characterized by a diverse and sporadically high heavy metal load of up to 30 mM nickel in the dump material were selected for isolation of typical soil bacteria. Hyphal growth, spore formation on an aerial mycelium and adaptation to the oligotrophy of most soils are marked features of the group of actinobacteria. Among them, especially the genus *Streptomyces* is well investigated for secondary metabolite production, as best seen with the enormous medical relevance in antibiotic production (Vining, 1992). Geosmines, the substances causing the particular odour of many soils, are another type of the numerous classes of secondary metabolites. However, actinobacteria synthesize and excrete a multitude of other compounds as well. Their exoenzymes play an important role in degradation of biogenic polymers. This attribute of mineralization has a great impact on metal mobility in soil. A collection of over 100 strains isolated from metalliferous habitats during the work presented here were morphologically and physiologically characterized and screened for high metal resistance. The resistance towards single metals and metal cocktails in AMD was tested in plate and liquid culture. For this test, minimal medium with a minor tendency for complexation of metal cations was applied to approximate to the conditions of the habitat. The contamination of most of the investigated habitats with nickel and the clear correspondence between nickel contamination and a distinct nickel resistance of the isolate were the reason to emphasize the research topic on nickel in this thesis. Some of the isolates were resistant towards 10 mM nickel in liquid medium and could be adapted stepwise to grow at even higher concentrations. Investigation of these organisms aims at the understanding of the molecular mechanisms of nickel resistance and adaptation. This knowledge is essential for the application of bacteria in a biocurtain which is of interest in a bioremediation strategy.

1.4 Studies on heavy metal resistant bacteria with special regard to actinobacteria isolated from a former uranium mining area

The investigations were conducted in order to obtain a better knowledge of the interactions between metals that typically occur in areas strongly influenced by AMD and bacteria that have evolved an adaptation to the characteristics of mining areas for survival. The aim was to gain insight into the resistance mechanisms that make survival and growth of bacteria in

metalliferous habitats feasible. Most of the work has been performed on strains of actinobacteria, which is an immensely important group for soil ecosystems. This research is framed by a complex approach: How could metal resistant bacteria be exploited for bioremediative applications? The main questions addressed in this thesis therefore are:
(Roman numerals in parentheses indicate corresponding manuscript.)

1. How is the **distribution of resistance towards nickel** among members of the group of actinobacteria? Are isolates from naturally nickel-enriched serpentine soils evolutionary better adapted than strains isolated from a mining area? Can nickel resistant actinobacteria be found in non-contaminated soils? (I, III)

2. Can the **fractionation of rare earth element patterns** be used as a tool to study accumulation of heavy metals by different microbes? Is it possible to differentiate between adsorption and active uptake of heavy metals? Can this fractionation be used in field studies to distinguish between microbial activity and physical sorption on geological substrates? (II, V)

3. Which are the **impacts of heavy metals on the life cycle** of actinobacteria? Do actinobacteria display a pattern of resistance towards a variety of heavy metals? (III)

4. Which are the **elements preferably retained from AMD** by several prokaryotic and eukaryotic microbes, if growth is supported due to nutrient addition? (IV)

5. What influence has the high metal load of contaminated habitats on **survival and performance in AMD**? Do heavy metal resistant isolates from a former mining area survive incubation in AMD waters? Which are the elements preferably retained from the metal cocktail by sorption? Are metals biosorbed from AMD persistently bound to bacterial biomass? (IV, V)

6. How do **heavy metals influence the secondary metabolism** of actinobacteria? (VI)

7. Do the investigated streptomycetes possess the capability of **biomineralization** if treated with high concentrations of $NiCl_2$ x 6 H_2O? Is **biomineralization** correlated with heavy metal resistance of actinobacteria? (VII)

8. Where does the nickel go in the cells of a resistant mining isolate? How does this *Streptomyces* strain prevent itself from being intoxicated by nickel in a metalliferous habitat or when cultured in nickel supplemented media? (I, VI, VII)

2 Summary of manuscripts

I Adaptation to nickel tolerance of nickel resistance in streptomycetes isolated from contaminated and non-contaminated soil samples.

Götz Haferburg, André Schmidt, Martin Reinicke, Dirk Merten, Georg Büchel, Erika Kothe.

Published in: Simposio Internacional de Biotecnologia (SIB) – II Simposio Italiano-Argentino de Bacterias Lacticas. ISBN: 987-21607-0-8. San Miguel de Tucumán, 2004.

The **distribution of resistance towards nickel** was studied using 100 streptomycetes strains isolated from naturally nickel containing soils (serpentine sites of Tuscany, Italy) and anthropogenically contaminated areas (mining sites and processing plants in Thuringia, Germany and S.M. de Tucumán, Argentina). In a control screening, isolates of non-contaminated soils were tested on resistance. Each isolate of every habitat was grouped into one of the four classes: (1) high resistance (growth on at least 10 mM Ni); (2) resistance (growth on 5 mM Ni); (3) tolerance (growth on 0.5 mM Ni); (4) sensitivity (no nickel supplementation tolerated). Strains isolated from serpentinite soils as well as from a mining site showed 11 % resistance to 10 mM $NiCl_2$. Two strains of the 37 isolates originating from non-contaminated control soils were able to grow on up to 5 mM $NiCl_2$. All strains of all contaminated sites were at least nickel tolerant, whereas 25 % of the isolates from the control sites were sensitive.

II Rare earth element patterns: A tool for understanding processes in remediation of acid mine drainage.

Dirk Merten, Jörn Geletneky, Hans Bergmann, Götz Haferburg, Erika Kothe, Georg Büchel.

Published in: Geochemistry / Chemie der Erde 65 (2005) S1, 97–114.

The **fractionation of rare earth element patterns** in acid mine drainage was studied comparing geogenic and biogenic substrates in their retention capacity. The use of the differences in the fractionation patterns is evaluated as a probable tool to monitor remediation at a former mining site. A selection of 15 diverse, metal resistant, microbial strains (fungi, single-celled bacteria, filamentous bacteria) originating from a mining site was treated with acid mine drainage. 11 of the 15 strains were able to grow under these harsh conditions. The resulting patterns of fractionation were compared with the reference (distribution of single concentrations of rare earth elements in the pure acid mine drainage) and plotted. One fungal strain displayed a significant alteration of the rare earth element pattern after incubation.

III Heavy metal resistance mechanisms in actinobacteria for survival in AMD contaminated soils.

André Schmidt, Götz Haferburg, Manuel Siñeriz, Dirk Merten, Georg Büchel, Erika Kothe.

Published in: Geochemistry / Chemie der Erde 65 (2005) S1, 131–144.

The influence of heavy metals on soil respiration and distribution of resistance as well as the **impacts of heavy metals on the life cycle of actinobacteria** were studied in metal contaminated habitats of a former uranium mining area. A correlation of the high metal load of the habitat with low soil respiration, a diminished number of colony forming units and an increased level of resistance along a gradient of contamination with acid mine drainage has been found. Effects of various heavy metals on isolates of the group actinobacteria are demonstrated. The loss of capacity for sporulation, changes in cell pigmentation and the release of chelating compounds are discussed in their ecological consequence.

IV Microbes adapted to acid mine drainage as source for strains active in retention of aluminum or uranium.

Götz Haferburg, Martin Reinicke, Dirk Merten, Georg Büchel, Erika Kothe.

Published in: Journal of geochemical exploration 92 (2007), 196-204.

A selection of heterogeneous microorganisms originating from a collection of metal resistant mining isolates was studied on the **elements preferably retained from AMD**. The extraction and retention of solved metals including rare earth elements and radioisotopes from AMD was investigated using ICP-MS analysis of the supernatant. Most of the strains could grow in minimal medium containing AMD diluted by half, representing the stressful conditions of the former mining site a few meters downstream of the entry point of the AMD. Two strains of filamentous growth (one *Streptomyces* isolate and a fungus) were noticeable in retention of uranium. A single-celled bacterium showed promising capacity for aluminum retention.

V Biosorption capacity of metal tolerant microbial isolates from a former uranium mining area and their impact on changes in rare earth element patterns in acid mine drainage.

Götz Haferburg, Dirk Merten, Georg Büchel, Erika Kothe.

Intended for publication in: Journal of basic microbiology.

Three representatives of a metalliferous soil habitat from a mining site were studied on **survival and performance in AMD** during a time course of four weeks. The isolates, belonging to the genera *Bacillus*, *Micrococcus* and *Streptomyces* were incubated in pristine AMD (pH 2.9; high concentrations of Al, Co, Cu, Mn, Ni, U; collected close to the microbial sampling spot) and tested after one hour, one week and four weeks on survival, biosorption and fractionation of rare earth elements. All strains survived the four weeks of incubation and were able to grow after the treatment. After an initial strong biosorptive activity the release of the investigated metals from the biomass was observed. The implications on a probable bioremediation strategy are discussed.

VI Shifts in secondary metabolism of metal tolerant actinobacteria under conditions of heavy metal stress.

Götz Haferburg, Ingrid Groth, Erika Kothe, Isabel Sattler.

Intended for publication in: Journal of biotechnology.

Ten different actinobacteria, members of the genera *Streptomyces*, *Kitasatospora* and *Lentzea*, isolated from metal-contaminated and non-contaminated environments were tested, if **heavy metals influence the secondary metabolism** and change the pattern of produced secondary metabolites. The heavy metal resistant isolates were cultured in two different media (complex and minimal media), both metal-supplemented and metal-free. Biomass and culture filtrates were solvent extracted after separation. Both fractions were separated using thin-layer chromatography (TLC). A set of various detection agents was applied to identify different substance groups. Additionally, the crude extracts were tested in an agar diffusion assay on antimicrobial activity. It could be demonstrated that metals added to the medium strongly influence the secondary metabolism. Some of the strains displayed a strong antimicrobial activity only after growth in presence of the metal. Prominent bands appearing in some of the crude extracts of the metal treated strains were detected through TLC.

VII "Ni-struvite" – a presumably new biomineral generated by a nickel resistant *Streptomyces acidiscabies* strain.

Götz Haferburg, Gert Klöß, Werner Schmitz, and Erika Kothe.

Intended for publication in: Biometals.

The capability of **biomineralization** of *Streptomyces acidiscabies* E13 isolated from a soil sample of a former uranium mining area has been studied in solid and liquid culture supplemented with high concentrations of nickel. It was noticed that colonies of *S. acidiscabies* E13 grown in various solid media were surrounded by, and interspersed with, crystals of a greenish appearance after three weeks of incubation. Microscopic observation showed crystals attached to the mycelial pellets of standing cultures grown in complex and minimal medium. Abiotic crystal formation due to crystal growth at the hyphae, acting as condensation nucleus, was excluded thus showing active biomineralization. The chemical composition of the bioliths was determined by electron probe microanalysis. The formation of the mineral $Ni(NH_4)(PO_4)\bullet 6H_2O$, preliminarily named nickel-struvite, is discussed as a possible resistance mechanism of the highly nickel resistant mining isolate.

3 Manuscipts

3.1 Adaptation to nickel tolerance of nickel resistance in streptomycetes isolated from contaminated and non-contaminated soil samples.

Adaptation to nickel tolerance of nickel resistance in streptomycetes isolated from contaminated and non-contaminated soil samples

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Abstract

Nickel tolerance was compared to nickel resistance in 100 streptomycete strains isolated from nickel containing and non-contaminated habitats. Strains isolated from serpentinite soils naturally rich in nickel as well as from a uranium mining site showed 11 % resistance to 10 mM $NiCl_2$. Non-contaminated control soils did show resistance in only one isolate out of 75. A comparatively similar tolerance towards 0.5 mM $NiCl_2$ of 100 % was observed at all contaminated sites while 75 % were tolerant at control sites.

Molecular mechanisms of high nickel resistance included: 1.) The effect of nickel efflux was observed by measuring nickel in the culture supernatant. A gene for a high affinity nickel transporter could be shown by PCR. 2.) Sequestration leading to higher concentrations in the biomass as compared to the media concentrations was attributed to extracellular adsorption and uptake into living cells which was determined by comparison of cultures with growing cells versus poisoned cultures that cannot contribute to the effect of nickel adsorption by intracellular mechanisms.

An extracellular substance induced by nickel addition to the culture was shown in thin layer chromatography. 3.) Intracellular nickel accumulation was visualized by TEM micrographs. Proteome analysis could show induced and repressed proteins by 2D gel electrophoresis.

Introduction

The aerobic soil microflora consists of several different taxa of bacteria in addition to fungi, nematodes and protozoa. One major group of soil bacteria represents the filamentous, Gram-positive actinobacteria including the streptomycetes which can provide up to 20 % of the aerobic soil bacteria population in arable land. This group is noticeable for the odor of tilled soil which is attributed to geosmin, a secondary metabolite of streptomycetes, and the group is renowned for the capacity to produce antibiotics. At the same time an active secondary metabolism enables the bacteria to cope with different stress factors, among those heavy metal ions in the substrate which are toxic and are as a secondary effect leading by the Fenton reaction to the production of oxygen radicals, another stress factor.

Adaptive responses towards heavy metal stress thus should involve detoxification of oxygen radicals and indeed it could be shown that over-expression of superoxide dismutase in both the bacterium *Escherichia coli* and the baker's yeast *Saccharomyces cerevisiae* allow higher heavy metal tolerance. In addition, any mechanism sequestering the heavy metal by binding to organic molecules could lead to detoxification and hence higher tolerance levels. The adsorption of generally positively charged heavy metal cations to the cells walls of Gram-positive bacteria has been established and cell walls of *Bacillus* species are used for heavy metal containing water treatment on an economic scale (Beveridge and Murray, 1976; Doyle et al., 1980).

However, in addition to biophysical and biochemical parameters of soil microbe cell walls the active uptake of radioisotopes has been known at least since the Tchernobyl fall-out to occur in basidiomycete fungi which led to extremely high enrichment of cesium in fruitbodies sampled from contaminated areas (Sugiyama et al., 2000). If heavy metals are taken up into the cells, resistance factors have to be present in order to protect the cells from the toxic effects. Heavy metal resistance has been investigated largely in Gram-negatives, e.g. *Ralstonia eutropha* or *R. metallidurans*. In the cases investigated, efflux transporters have been identified for heavy metal resistance.

Investigation of such adaptive mechanisms can be performed comparing sites of different evolutionary time scales such as sites with geogenic, long-term heavy metal pollution and sites with anthropogenic, comparatively short-term pollution to control sites without pollution. This could be done for different heavy metals. Nickel has been chosen for this investigation, because of the high level of nickel in most arable land. In addition, it is an essential as well as toxic element, depending on concentrations (McIlveen, W. D. and Negusanti, J. J., 1994). Approximately 40 % of arable areas in Germany are almost reaching the contents of 50 µg/kg which is defined as the preventive level where remediation measures are to be taken. Thus, the impact for farming is especially high. In addition, nickel is both essential and highly toxic which has led to specific homeostasis regulatory systems in living organisms. This basis for adaptation is thus especially well prone to see alterations within reasonable times.

One site with geogenic high contents of nickel and therefore very long-term pollution is soil that has formed on serpentinite in Tuscany, Italy. Here, levels of approx. 1200 to 2000 ppm nickel are found (Mengoni et al.,2001). Stoppel and Schlegel (1995) found strong homologies between the nickel resistance determinants present in anthropogenically nickel-percolated ecosystems and the nickel resistance

determinants isolated from serpentinite soils. Owing to the extreme mineral composition and to the distribution in isolated patches, these outcrops have been considered as ecological islands (Kruckeberg, 1984) and represent a powerful agent for the evolution of colonizing organisms (Mengoni et al., 2001).

The former uranium mining site Wismut in Eastern Germany represents an ideal outdoor laboratory to investigate adaptation of microorganisms to anthropogenic pollution with heavy metals. Uranium mining from this site has made the German Democratic Republic the third largest producer of uranium world-wide with 210,000 tons of uranium produced during the years 1949 through 1990. After re-unification of Germany the uranium mining was stopped and the Wismut GmbH was founded to remediate the site financed from the German Federal Government. The strategy chosen for remediation was to replace the waste rock material into the former open mining pit. The water table, which was pumped to produce a depression cone during mine operations, is allowed to rise again, re-establishing anoxic conditions in saturated zones. This will prevent further oxidation of the pyrite-rich material and prevent further production of AMD waters. However, seepage waters that resulted from leaching of the former waste rock piles by acid mine drainage have infiltrated adjacent soils and surface waters. They contain large amounts of nickel (up to 20 ppm water-extractable nickel from soil adjacent to a contaminated creek), cadmium, copper, chromium, rare earth elements and other heavy metals (Merten et al., 2004). The mixed pollution is a challenge for microorganisms that have to cope with multiple pollutants at the same time. This should allow for specific adaptive responses to the mixture of heavy metals available for the past 40 years at the field site, the former uranium mining site at Ronneburg, Eastern Thuringia, Germany.

A third polluted site was chosen near Tucuman, Argentina, where sugar mill and mining effluents led to contamination of the waterways including Rio Hondo dam with

high cadmium and nickel concentrations. Control sites were chosen within the city of Jena, in the Botanical garden and a park, the Paradies. The comparison of streptomycete isolates from these three sites was chosen to elucidate adaptive mechanisms for nickel tolerance and resistance acquired by evolution on a cocktail of heavy metals.

Material and Methods

Isolation of streptomycetes

Soil samples were taken from Pieve Santo Stefano, Tuscany, Italy, from the former mining area Wismut near Ronneburg, Germany at selected spots according to high concentration of heavy metals. Samples from polluted areas in Argentina were taken from an effluent channel of a copper filter plant, province of Tucuman, a gamexane-enriched soil and an artificial lake comprising several inflows of sugar cane industry in Las Termas province of Santiago del Estero. Control soil samples were taken in the Botanical Garden of the University of Jena, in a forest site and a public park (Paradies) of Jena, Germany.

Air dried, ground soil samples were heat treated at 80°C for 2 h and 1 g was suspended in 9 ml *A. dest.* After 2 h shaking, supernatant was plated on soil extract medium (modified after Thiemann et al., 1968) 150 g garden soil were extracted over night in 600 ml tap water by stirring, the supernatant centrifuged for 10 min at 4,000 rpm, and the clear supernatant was adjusted to 1 l, adding 1.8 % (w/v) agar for solid media) at dilutions 10^{-4} to 10^{-6}. The plates were incubated for 5 days at 28°C, colonies were microscopically analyzed and transferred to starch casein medium containing streak plates.

Cultivation

Streptomycete strain cultivation was performed on starch casein medium (10 g/l starch, 1 g/l casein dissolved in 0.3 M NaOH, 0.5 g/l K_2HPO_4, 15 g/l agar, pH 7.0–7.5). For growth with heavy metals, minimal medium was used (0.5 g/l asparagine, 0.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4$, 0.01 g/l $FeSO_4$, 10 g/l glucose and 18 g/l agar for solid media). For nickel supplementation a stock solution of $NiCl_2$ x $6H_2O$ was used. Minimal medium has to be used in resistance determination order to minimize complexation of the heavy metal ions. Tryptic Soy Broth medium (TSB) was used for cultures investigated on extracellular metabolite excretion. 30 g TSB dissolved in 1 L *A. dest.*

Nickel retention

Strains *S. acidiscabies* E13, *Streptomyces* spec. Tosca3 and *S. coelicolor* DSM 40783 were grown on 50 ml minimal medium supplemented with 0.1 mM $NiCl_2$ for 7 days from 50 µl spore suspension used as inoculum. ICP-MS analysis was used to measure residual nickel in supernatant of cultures.

Thin layer chromatography

Cultures grown on TSB medium with or without nickel addition were used to dry 10 ml supernatant which were then extracted over night with 5 ml MeOH. Aliquots were spotted on silica-gel 60 F_{254} plates (Merck), developed in MeOH/H_20/acetic acid (1:1:0.005), nickel detection was performed using dimethyl-glyoxime (1% in EtOH) (Feigl and Kulka, 1924).

Gel filtration chromatography

Streptomyces acidiscabies E13 was grown in 50 ml minimal medium supplemented with 0.3 mM $NiCl_2$. Mycelium was harvested, washed and resuspended in 5 ml polyvidon buffer (108 ml 50 mM K_2HPO_4 and 42 ml KH_2PO_4 were mixed; in this solution 15.4 mg dithioerythritol and 2 g polyvinyl pyrrolidon were dissolved and adjusted with the stock solution to 100 ml). Cell debris after French press was centrifuged at 140.000 x g (Beckman LE-70 Ultracentrifuge) and 1 ml of cytosol solution fractionated on Sephadex G50 by HPLC (XK 26/100, Amersham Biosciences). Nickel detection was performed by ICP-MS and protein by UV photometry (Uvicord SII, Amersham Biosciences).

Results and Discussion

Isolation of 100 streptomycetes

For comparison of resistance levels 100 strains were analyzed. The strains were isolated (examples: Fig. 1) from 4 different sources: serpentinite soil (Tuscany, Italy) for naturally nickel rich environments, soil sample from a former uranium mining site (Wismut near Ronneburg, Germany) as example of 40 years of anthropogenic heavy metal pollution, a site with organic and heavy metal pollution from gold mining and suger mills in a drainage ditch in northern Argentinia (near Tucuman, Argentinia) and control samples from sites in Jena (Botanical Garden and Paradies, Jena). From the Tuscany soil samples 33 streptomycetes were isolated from Wismut samples 20 strains and from Argentinia 10 strains. The control samples yielded 37 isolates.

Fig. 1: Strains of streptomycetes isolated from Wismut soil.

Nickel resistance of the isolates

All isolated streptomycete strains were grown on solid minimal media containing no nickel addition and with 0.5 and 5 mM $NiCl_2$. Growth on 0.5 mM $NiCl_2$ was considered tolerant and strains growing on 5 mM $NiCl_2$ were classified resistant and subsequently tested for maximal resistance. The 33 strains isolated from naturally heavy metal rich Tuscany serpentinite soil all were tolerant and a high percentage showed a higher degree of tolerance towards intermediate levels of 2 mM nickel with 43 % (16 strains). Within this group resistance was observed with 4 strains or 11 %. 2 strains were able to grow even on 10 mM $NiCl_2$ containing minimal media and were considered highly nickel resistant (Fig. 2). In some cases, altered growth pattern, lack of aerial mycelium formation or altered formation of diffusible stains were seen. This has been reported earlier for many cases (Raytapadar, S., Datta, R. and Paul, A. K., 1995).

The 20 strains from the anthropogenically contaminated mining site Wismut were all tolerant (100 %), but only one strain (5 %) showed extremely high resistance of up to 20 mM $NiCl_2$ while 25 % were resistant to 5 mM nickel addition to the media and 2 strains grew on 10 mM nickel containing plates. The 10 strains of Argentina which were isolated from sites of multiple contamination including heavy metals as well as organic and lindene, were all tolerant (100 %), and 6 strains (60 %) showed

resistance towards 5 mM $NiCl_2$. Two of these were able to grow at more than 10 mM $NiCl_2$. Among the 37 strains from control samples 27 (73 %) were tolerant of 0.5 % $NiCl_2$. Among those only two strains (5.4 %) were able to grow on up to 5 mM $NiCl_2$.

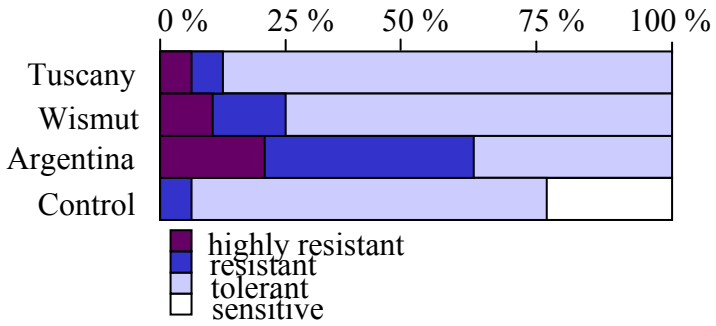

Fig. 2: Nickel resistance levels of isolated strains from contaminanted and non-contaminated soil samples. Sensitive strains did not grow on 0.2 mM nickel containing plates, 0.5 mM nickel tolerance and 5 mM nickel resistance were compared to highly resistant strains growing in the presence of 10 mM or more nickel. Percentage of strains from all isolates of a site are given.

The results show that nickel tolerance is widely distributed. This might reflect the high proportion of arable land that shows nickel concentrations just around the threshold levels of 50 ppm (or 0.75 mM). The potential allergenic nature of nickel has led to legal actions which dropped the allowed nickel concentrations in water to 20 ppm which is equivalent to 0.3 mM. Our study showed that the soil microflora generally is able to tolerate these levels of nickel in soil and must have evolved mechanisms that protect the cells from damage by reactive oxygen species produced from radical reactions of the heavy metals, from protein and DNA damage and from acidification.

However, resistance levels were found higher in samples from contaminated areas corresponding somewhat with the levels of contamination, with Tuscany soil (11 %), Wismut samples (25 %) and Argentina samples (60% resistance). Partially, the resistance level is higher than the concentrations observed in the soil. This might be

indicative of heterogenous nickel concentrations which locally may change, especially if microhabitats for bacteria are considered which include organic debris which is used as energy and carbon source. Locally, the pH also may differ which greatly influences the availability of heavy metals in the water phase which is prerequisite for the uptake into cells. In the Argentina samples, anthropogenic pollution has prevailed for the past 50 years, but concentrations of heavy metals are so high that only resistant strains are able to survive and reproduce. The fact, that only 10 strains could be isolated from these soil and sediment samples shows that here a low vitality rate can be assumed for growing streptomycetes, even though the isolation procedure should not be used for a live count of streptomycetes in soil since differences in microflora community structure is prone to greatly influence the picture of isolated strains. However, the small number of isolates can be taken as indication of low streptomycete biomass, awaiting other methods for *in situ* detection of strains like fluorescence *in situ* hybridization. The natural soil rich in heavy metals from Tuscany, Italy, showed a higher proportion of resistant strains as compared to the control sites which is also true for the Wismut site. The doubled resistance rate of 11 % (as compared to 5 %) resistant strains at geogenically contaminated sites indicate a selective pressure that has led to selection of strains carrying resistance or tolerance genes. Thus, resistance mechanisms are investigated further.

Nickel efflux

The effect of a nickel efflux transporter would lead to no change in extracellular nickel concentration although resistance of the strains is seen. This phenomenon could be observed with *S. acidiscabies* E13 (Fig. 3). The influx cannot be avoided as transport of essential nickel must be allowed. In addition, more unspecific transporters for other elements like magnesium or calcium allow nickel and other heavy metals to enter the

cell. Specific, high affinity efflux transporter can then allow removal of the toxic quantities of heavy metals detoxifying the cells. First results for such a transporter system in *S. acidiscabies* E13 have been found (Amoroso et al., 2000).

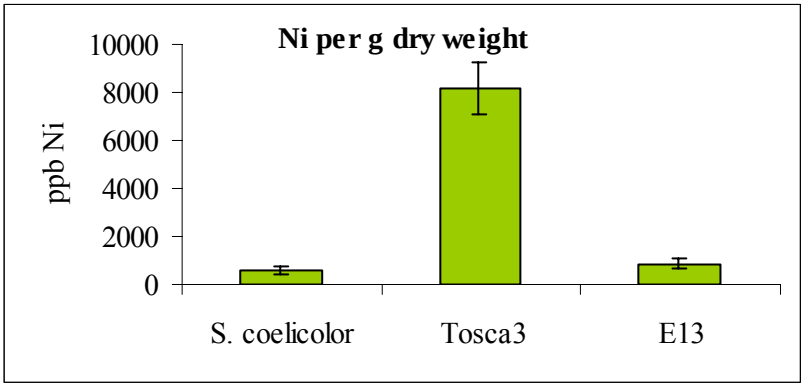

Fig. 3: Nickel uptake from media is high with strain *S. spec.* Tosca 3 while *S. acidiscabies* E13 shows low uptake indicating an efflux system for resistance.

Efflux as resistance mechanisms are known especially with gram-negative bacteria, where the genes responsible for efflux are different from the uptake transporters needed to gain enough of the essential metal (for review: Eitinger and Mandrand-Berthelot, 2000).

Extracellular nickel sequestration

Another possibility for detoxification would be extracellular sequestration by compounds excreted from the cells. This could include binding of heavy metals to the cell wall of the Gram-positive bacteria. Indeed, this was shown with technological application already using *Bacillus shpaericus* in Biocer ceramics as exchange material (Raff et al., 2003). However, more and different heavy metal sequestering

agents can be identified. Thin layer chromatography was used to identify two substances which show high nickel binding capacity from culture supernatant (Fig. 4).

Fig. 4: Nickel sequestering compounds exuded into the medium. Thin layer chromatography of medium after growth of nickel resistant strain *S. acidiscabies* E13 from Wismut soil in media lacking (left) and containing 1 mM nickel (right). The chromatogram was stained with nickel specific dimethyl-glyoxime giving a red stain. The uppermost, brown-colored band is assumed to be melanin.

Intracellular nickel accumulation

Especially promising for future application would be the identification of intracellular nickel binding proteins. A first investigation could show that 75 % of intracellular nickel as determined by ICP-MS is eluted from a gel chromatography column at low protein concentrations (Fig. 5). In this case, only the cytosolic fraction of E13 was used which shows, albeit an export system for nickel is assumed to be present, still 5 to 30-fold elevated intracellular nickel concentrations as compared to a sensitive strain, *S. coelicolor* A3(2). The putative nickel-binding protein is small with a mass of below 25 kDa. This might hint at metallothionein or phytochelatin compounds. The identification of putative intracellular nickel-sequestering proteins is a step towards understanding nickel resistance in streptomycetes. Future research will be directed at the identification of proteins specifically expressed under nickel induction by 2-dimensional gel electrophoresis.

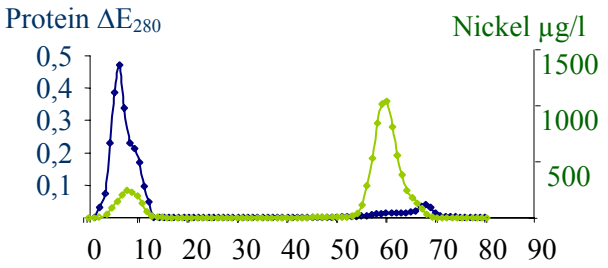

Fig. 5: Gel chromatography using Sephadex G-50 of cytosolic components from *S. acidiscabies* E13. Nickel concentration in each fraction (1-82) was measured and protein content estimated by absorption at 280 nm.

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3.2 Rare earth element patterns: A tool for understanding processes in remediation of acid mine drainage.

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Rare earth element patterns: A tool for understanding processes in remediation of acid mine drainage

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Abstract

The distribution of rare earth elements (REE) was applied to study processes in remediation of acid mine drainage (AMD). The concentration of total REE is up to $3\,mg\,l^{-1}$ in AMD. Normalization of REE concentrations in seepage and surface waters to Post-Archean Australian Shale (PAAS) shows strong enrichment of heavy REE. For the case of the studied AMD REE patterns are representative for the seepage location. Therefore, they can be used to identify (former) waste rock dumps as a source of valley sediment contamination.

Results from percolation experiments and LA-ICP-MS measurements show that REE patterns of seepage water samples do not reflect the total REE pattern of the source rocks but are preferentially eluted from Silurian "Ockerkalk". Along a flow path of the creek Gessenbach, REEs decrease in concentration and fractionate due to coprecipitation with Fe phases. REE patterns are also applied to the study of sorption/uptake of heavy metals by biomass. A strain with high potential for remediation purposes could be identified by incubating microbial strains isolated from the investigation area directly in AMD.

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Finally, for a contaminated soil with acidic pH and thus high availability of heavy metals to plants the transfer of heavy metals from soil to plants was studied by the use of REE patterns. For different herbaceous plants and trees different REE patterns were observed although the corresponding soil was identical. Concentrations of REEs are always higher in leaves than in shoots/branches. However, within one species REE patterns are quite similar for leaves and the corresponding shoots/branches.

Keywords: Rare earth elements; Acid mine drainage; Remediation processes; Bioremediation

1. Introduction

The Ronneburg and Seelingstädt mining district in Eastern Thuringia (Germany) was one of the largest uranium mining and processing sites worldwide. More than 113 kt of U originated from open pit and underground mining operations. While in the northern part of the area near Ronneburg uranium was excavated by underground and open pit operations until 1990, in the southern part near Seelingstädt extensive uranium mining operations closed in 1967. Since 1960 the ore was milled and processed by acid and soda-alkaline leaching and the former open pits were used as tailing ponds.

The excavated rocks in the northern part of the mining area mainly consist of black shales, metabasaltic rocks and carbonates of Ordovician to Devonian age, containing up to 7 wt% sulfides, 5–9 wt% organic carbon, 30–60 ppm uranium (locally up to 5000 ppm) and a series of trace elements including rare earth elements (REE: La–Lu). By the end of 1990, the mining legacy consisted of 16 waste rock dumps with about 200 Mio m^3 of partly acid generating waste rock. In the southern part of the area the ore was found in seams of sand and siltstones, dolomites and conglomerates of Younger Paleozoic ages (Zechstein). The average uranium content was 0.07–1 wt%.

At the uranium mining site near Ronneburg strata-bound structure-controlled ore bodies, containing sulfur-bearing minerals (pyrite, markasite), were exposed at the surface. The resulting series of oxidation and hydrolysis reactions are known as acid mine drainage (AMD). The reactions are controlled by internal factors, such as surface size/grain size of the sulfide minerals and external factors, such as availability of oxygen or ferrous iron, pH, temperature and water content (Evangelou and Zhang, 1995; Nordstrom, 1982; Singer and Stumm, 1970). At low pH the reactions are strongly catalyzed by microorganisms, particulary *Acidi Thiobacillus ferrooxidans* (Alpers and Blowes, 1994).

Starting in 1990, the remediation projects (carried out by WISMUT GmbH) are mainly aimed at reducing the radiological and chemical exposure to the public and the environment. Remediation actions taken in the northern area are passive flooding of the underground mine shafts (Gatzweiler et al., 1997) and backfilling the former waste rock dumps into the open pit mine of Lichtenberg. In the southern part, the remediation is focused on in-situ decommissioning of the tailing ponds by dewatering and covering the milling sites. Since only some of the dumps had been covered – allowing precipitation to enter into the uncovered dumps – minor

pollution is still seen from present and former sites of heaps. Due to efficient pyrite oxidation within the dump material, low pH seepage water still leaves the dumps bases (AMD). The drainage waters are sources of heavy metal contamination in soil and water. They contain radioactive heavy metals such as uranium and a multitude of non-radioactive heavy metals including rare earth elements. The precipitation of secondary minerals of iron and sulfate (jarosite, schwertmannite, gypsum) as AMD educts causes coprecipitation of heavy metals. For understanding the behavior of heavy metals along the migration path in an AMD influenced system, several studies of surface water and groundwater were performed (Alpers and Blowes, 1994; Nordstrom, 1982).

Since large areas with minor heavy metal contamination are still present, inexpensive remediation strategies are desirable. Bioremediation processes are promising due to their simple application and reasonable price. Plants are often applied to extract heavy metals from contaminated soil (Ouyang, 2002; Chen et al., 2004; Singh et al., 2003; Dushenkov, 2003). Also, microbes are well known to play important roles in the remediation of heavy metals and radionuclides from surface waters (Ouyang, 2002). The adsorption and uptake of heavy metals by fungi has been monitored in some detail after the Chernobyl accident (Baeza et al., 2000; Gaso et al., 1998; Kirchner and Daillant, 1998). However, the processes involved in bioremediation are only partly understood.

REE (La–Lu) show smooth, but continuous variations in chemical behavior as a function of their atomic number. After standardization to Post-Archean Australian Shale (PAAS), Taylor and McLennan, 1985) they can be used as tracers in ground- and surface water (Johannesson and Lyons, 2001). Furthermore, they are well suited to study processes such as dissolution, sorption, complexation (Astrom, 2001), coprecipitation (Byrne and Kim, 1993) and especially water-rock interaction (Worrall and Pearson, 2001). They are also well suited to study transfer of heavy metals between soil and plants (Raju and Raju, 2000; Miekeley et al., 1994; Shan et al., 2003) and the sorption/uptake of heavy metals by microbes (Merten et al., 2004; Andrès et al., 2003; Philip et al., 2000; Yoshida et al., 2004). REE patterns are used in this work to identify seepage water of (former) waste rock dumps as a source of contamination of valley sediment. Furthermore, fractionation of REE is used to study processes important for the generation and remediation of AMD such as (partial) dissolution of minerals, coprecipitation and sorption/uptake of heavy metals by plants and microbes. Some of the these processes can be explained by controlled experiments in the laboratory.

2. Investigation area

2.1. Geology

According to Dahlkamp (1993) the uranium deposit of Ronneburg (Fig. 1) is a strata-controlled structure-bound deposit type. It consists of uranium concentrations

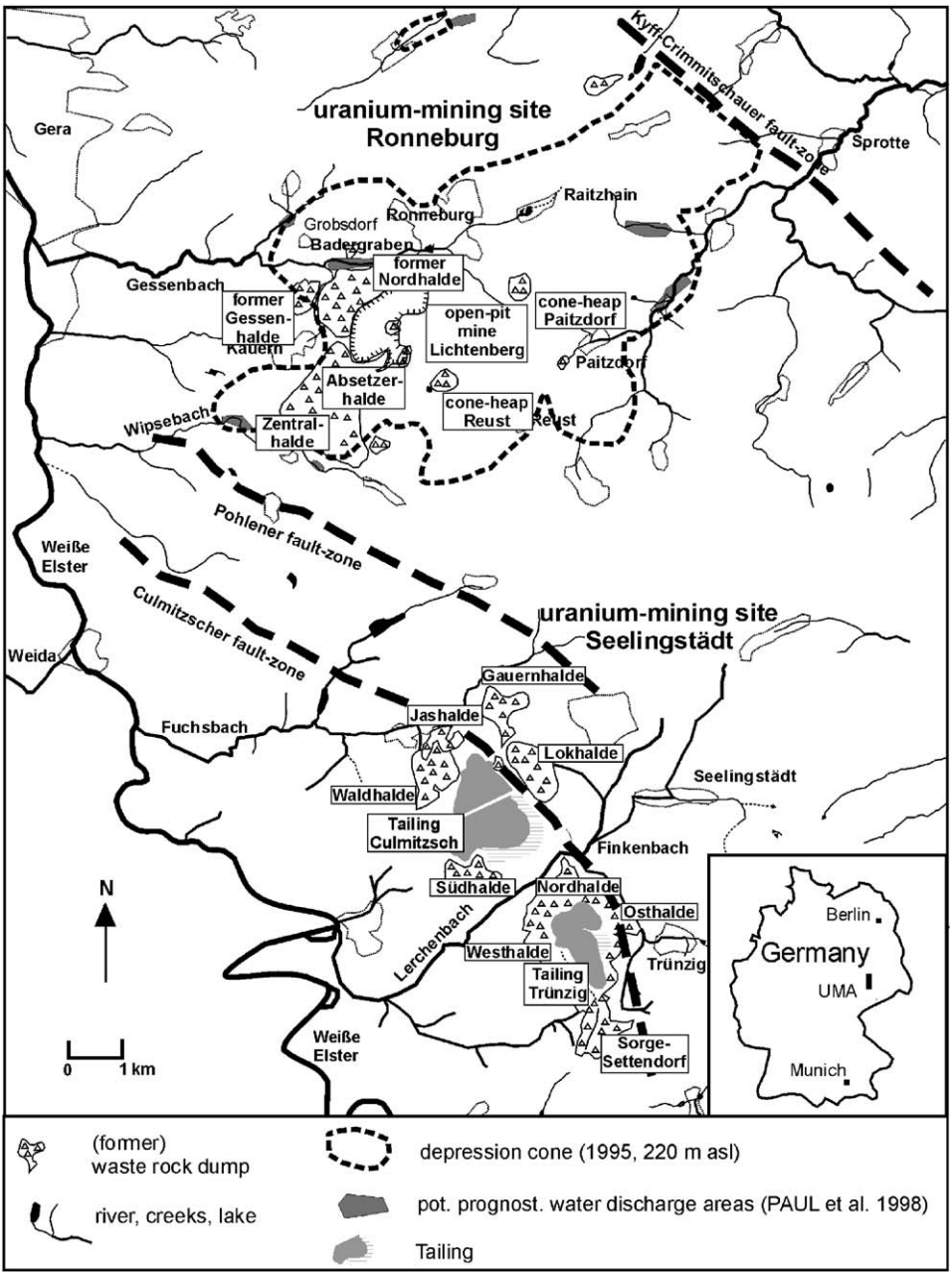

Fig. 1. The former uranium mining site of Ronneburg and Seelingstädt, Germany (1950–1990) (UMA: uranium mining area Ronneburg and Seelingstädt).

in small-scale brittle structures which form stockworks within or immediately adjacent to carbonaceous, pyritic black shales. The Paleozoic host rocks mainly consist of argillaceous and siliceous black shales with intercalated dolomitic and phosporite nodules (Silurian ''Graptolithenschiefer''). The main black shale horizon lies below Ordovician carbonaceous sandy shales and overlies Silurian carbonate rocks (''Ockerkalk''). Some Devonian metabasaltic dikes and sills cut the metasedimentary rocks (Dahlkamp, 1993). The intensively folded and faulted, incompetent and competent rocks have high-density small-scale brittle structures (fissures, joints, faults). Permian and Tertiary supergene oxidation processes associated with mobilization and precipitation of trace elements created an oxidation and cementation zone. The irregular distribution and size of the ore bodies are controlled by major and minor faults. The uranium ore cropped out near the surface in the southern part and down to 1000 m depth in the northern part of the Ronneburg mining district.

The uranium deposits of Seelingstädt (Culmitzsch, Trünzig) are located in the Culmitzsch halfgraben which is part of the Pohlener and Culmitzscher fault zones (Fig. 1). These structures are local sections of the Gera–Jachymov system. The uranium ores are hosted in sediments which were accumulated in marginal areas of the German Zechstein basin. These clastic, partly clay-rich sediments with high organic content are separated by an unconformity from underlying Ordovician shales (''Phycodenschiefer''). Sulfide uranium mineralization is coupled to organic and clay-rich material. However, the exact primary and post-sedimentary processes during mineral formation are still under debate (Lange et al., 1998, Tonndorf, 1994).

2.2. Hydrogeological setting

One of the most important drainage systems at Ronneburg mining site is the catchment area of the creek ''Gessenbach''. The valley ''Gessental'' is located between the cities of Ronneburg and Gera. In its eastern part near the city of Ronneburg it is influenced by two (former) waste rock dumps called Gessenhalde and Nordhalde. The former ore leaching dump Gessenhalde was transported to the open pit mine in 1992–1995. Between 1998 and 2003, 27 Mio m^3 of waste rock from the dump Nordhalde was relocated in the Lichtenberg open pit mine. The waste rock dumps partly consisted of acid generating material. Before and during relocation highly mineralized and acidic seepage water was drained into the upper section of the adjacent creeks and Quarternary sediments. These waters provide information on the preferred groundwater and surface water flow paths (Geletneky et al., 2002).

The Gessenbach creek flows northward, turning westward after confluence with the Badergraben (Fig. 2). The Gessental valley will be one of the main discharge areas of mine water in the post-flooding period (Paul et al., 1998). It is located above the former underground mine and due to its orographic position between 240 and 280 m above sea level, it is the lowest valley of the area. Finally, the outcrop of Silurian metasedimentary rocks with higher hydraulic conductivity allows flooding water to reach the surface in the near future.

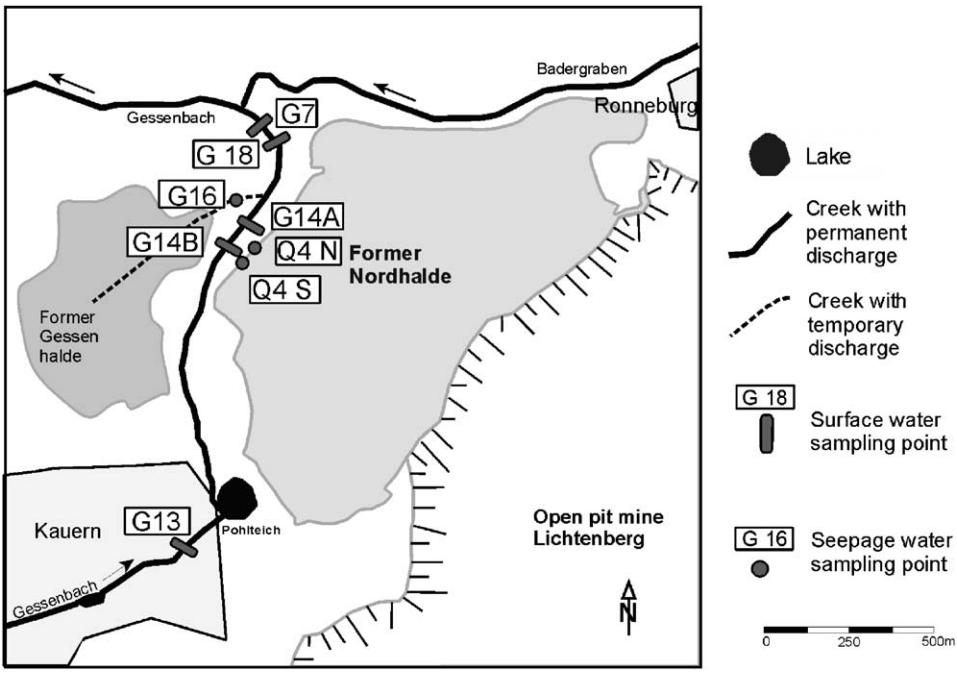

Fig. 2. Sampling points of seepage water and surface water around the waste rock dumps "Nordhalde" and "Gessenhalde".

Fig. 2 shows a detailed view of the northern investigation area and the sampling locations where seepage water and surface water samples were obtained in 1998–2002.

3. Material and methods

3.1. Water sampling procedure

Water samples (100 ml each) were collected at the western boundary of the former waste rock dump "Nordhalde" (still being present during investigation time), at the northeastern boundary of the former waste rock dump "Gessenhalde" and along the Gessenbach creek (Fig. 2). The water samples were filtered in the field using glass fibre prefilters (Millipore, Germany) and cellulose acetate filters (Sartorius, Germany) with a pore size of 0.45 μm. The pH, electrical conductivity, redox potential and temperature of the unfiltered samples were measured in the field using portable instruments pH320, LF320 and an external thermocouple (WTW Wissenschaftlich Technische Werkstätten, Germany). One hundred microliters of nitric acid (65%, Baker Ultrex, USA) was added to 50 ml of the water samples for stabilization. Another 50 ml of the samples remained unacidified for determination of anions by ion chromatography, photometry and titration. The samples were transferred to the laboratory the same day and stored at 6 °C until analysis.

3.2. Isolation of microbes and direct incubation of AMD

For incubation experiments 15 microbial strains were isolated from metal-enriched soil habitats from locations near G16 sampling point (Fig. 2). The strains were assorted in single celled bacteria, filamentous actinobacteria and fungi, five strains each. For isolation of bacteria, actinobacteria, and fungi, 1 g of air dried, ground soil samples (heat treated for isolation of actinobacteria at 80 °C for 2 h) was suspended in 9 ml distilled water. After 2 h of shaking, supernatant was plated in aliquots of dilutions from 10^{-4} to 10^{-6}. For isolation of soil bacteria, Standard I medium (Merck, Darmstadt, Germany) supplemented with half-concentrated seepage water was used. The seepage water was sampled on 07–11–2001 from sampling point G16. It was sterilized seperately in order to prevent precipitation of compounds after adjusting pH to 6, sterile filtration with 0.2 μm cellulose acetate filters (Sartorius, Germany) and mixing with the medium. Actinobacteria were plated on soil extract medium (modified after Thiemann and Beretta, 1968) containing half-concentrated seepage water. For soil extract medium, 150 g garden soil was extracted overnight in 400 ml tap water by stirring, the supernatant was centrifuged for 10 min at 4000 rpm, and the clear supernatant was adjusted to 500 ml by adding 3.6% (w/v) agar before autoclaving. For isolation of soil fungi, CYM medium (2 g l^{-1} trypticase pepton, 2 g l^{-1} yeast extract, 20 g l^{-1} glucose, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.5 g l^{-1} KH_2PO_4, 1.0 g l^{-1} K_2HPO_4, 18 g l^{-1} agar; Schwalb and Miles, 1967) supplemented with seepage water, was used. The plates were incubated for 7 days at 28°C and the colonies were microscopically analyzed and bacteria, actinobacteria and fungi were subsequently transferred to standard I (bacteria), starch casein medium (actinobacteria) (10 g l^{-1} starch, 1.0 g l^{-1} casein dissolved in 0.3 M NaOH, 0.5 g l^{-1} K_2HPO_4 and 16 g l^{-1} agar, pH 7.0–7.5) and CYM (fungi), respectively.

The strains were grown in minimal medium (0.5 g l^{-1} L-asparagine, 0.5 g l^{-1} K_2HPO_4, 0.2 g l^{-1} $MgSO_4.7H_2O$, 0.01 g l^{-1} $FeSO_4.7H_2O$, 10 g l^{-1} glucose, Amoroso et al., 2000) to late logarithmic growth phase, and inoculated after three steps of washing with distilled water 1:2000 into 10 ml minimal medium/half-concentrated seepage water G16 (sampled on 07–11–2001) and incubated for 7 days at 28°C.

Eleven of the 15 selected microbial strains were able to grow under these conditions in liquid culture and were used for the investigation of REE fractionation. After incubation the samples were centrifuged at 4000 rpm and 4°C (Beckman, Palo Alto, USA) and filtered twice including a 0.2 μm cellulose acetate sterile filter (Sartorius, Göttingen, Germany). The clear supernatant was analyzed for REE content.

3.3. Analysis of aqueous samples

Main cations (Na, K, Ca, Mg, Fe) were determined using Atomic Absorption Spectrometry (AAS5, Analytik Jena, Germany), whereas Cl^-, SO_4^{2-}, PO_4^{3-}, NO_3^- and NO_2^- were analyzed by ion chromatography (DX-120, Dionex, USA). HCO_3^- was analyzed by titration (Titrino, Metrohm, Germany) and Si by photometry (DR 4000U, Hach, Germany). Trace element concentrations (Al, Cd, Cr, Cu, Mn, Ni, U, and Zn and REE) were determined using a PQ3 (VG Elemental, UK) ICP mass spectrometer equipped with S-option interface (Brenner et al., 1999). A Perimax 12 peristaltic pump (Spetec, Germany) was applied to ensure a constant sample solution delivery rate of 0.4 ml min^{-1} to a concentric MicroMist nebulizer (Glass Expansion, Australia). The spray chamber used was a water-cooled Impact Bead chamber (VG Elemental, UK) maintained at a temperature of 4 °C. The instrumental parameters were optimized for high sensitivity for the mass range covered by the REE accompanied by low molecule ion levels using a tune solution containing 1 μg l^{-1} Ce. For the determination of REE

and other trace elements in the water samples external calibration was performed with blank solutions and multi-element stock solutions (Spex, Germany). Standard solutions contained each REE in concentrations of 0.1, 1, 2.5, 5 and 10 $\mu g l^{-1}$, whereas Al, Cd, Cr, Cu, Mn, Ni, U, and Zn were contained in concentrations of 1, 5, 10, 100, and 250 $\mu g l^{-1}$. The samples were diluted 1/10 to 1/100 corresponding to their total dissolved solids content. Blank solutions, standard solutions and samples each contained 10 $\mu g l^{-1}$ Be, Ga, Ru, Re and Tl (Merck, Germany) and 20 $\mu g l^{-1}$ Se (Merck, Germany) as internal standards for drift correction. All samples were made up to a final volume of 10 ml in polypropylene vessels (Greiner, Germany) with deionized water of a resistance of 18.1 $M\Omega\,cm^{-1}$ (PureLab Plus, USF Elga, Germany). The samples were stabilized by a final concentration of 2% (v/v) of nitric acid of Ultrapure grade (Baker Ultrex, USA).

In ICP-MS, middle and heavy REE are interfered by oxide and hydroxide molecule ions of Ba and the light REE. Therefore, interference correction was applied where necessary. For multi-isotopic REE the less interfered isotope was used. The analytical procedure is described in detail in Merten and Büchel (2004).

3.4. Analysis of solid samples

Batch experiments using deionized water were performed using a dynamic batch setup (UIT, Germany) for samples of Silurian "Ockerkalk" (sample 1124), a fine-grained alluvial sediment (18/80 M1) and a coarse-grained one (19/19), all sampled from boreholes and digging pits in the Gessental valley. The samples were air-dried and milled to <2 mm. Samples of 50 g were kept between 100 μm polyethylene filter plates and 500 ml deionized water was pumped in circuit at a flow-rate of 3.4–4 ml min^{-1}. Samples for analyses were taken after 2 days for the alluvial sediments and 4 days, for the Silurian "Ockerkalk". The samples were filtered (0.45 μm) and subsequently acidified to pH <2 by the addition of ultrapure HNO_3 (Baker, USA).

For measurements of REE content in solid samples the ICP-MS instrument was coupled to a Microprobe II (Merchantek, USA) Nd:YAG laser ablation (LA) system operating at a wavelength of 266 nm. Sensitivity was optimized by ablating a certified glass standard NIST SRM 612 (NIST, USA). The rocks were powdered to <63 μm and glass beads were formed by fusing 0.4 g of the sample with 4 g of a mixture of 66.6% di-Litetraborate and 33.3% di-Limetaborate (Spectromelt A12, Merck, Germany). The fused glass beads are homogeneous with respect to the rare earth elements as shown by spatially resolved analysis (data not shown). REE are calibrated using certified standard reference glasses NIST SRM 610, NIST SRM 612 and NIST SRM 614 (NIST, USA) and Ba as an internal standard. Concentrations for REE and Ba in the reference materials were taken from Horn et al. (1997) and Pearce et al. (1996). The concentrations of Ba within the samples as necessary for internal standardization were obtained by X-ray fluorescence (PW2400, Philips, The Netherlands).

Samples of plants were obtained from the former dump "Sorge Settendorf" located about 6 km south-west of the community of Seelingstädt (Thuringia, Germany, Fig. 1). This location is featured by significant contamination with heavy metals due to former mining activities, acidic soil pH and therefore high availibility of heavy metals to plants. Two plant species from the herb layer (*Geum urbanum* and *Geranium robertianum*) and three tree species (*Betula pendula, Popolus balsamifera* and *Robinia pseudo-acacia*) were sampled. For the herbs, leaves and shoots were analyzed separately, whereas for the trees 1-year-old branches and leaves were sampled independently. The samples were rinsed with deionized water and approximately 0.2 g of leafs, shoots and branches were digested separately by microwave-assisted pressure

digestion (Kürner, Germany) using 2 ml HNO_3. After dissolution, the samples were adjusted to 15 ml. The samples were further diluted 1/20 for analysis by ICP-MS.

4. Results and discussion

4.1. Surface and seepage water at Ronneburg mining site

The seepage water from the (former) dumps can be characterized as Mg–Ca–SO_4^{2-}-type, being typical for AMD. Seepage water G16 had a pH between 3 and 5.5, total dissolved solids (TDS) in the range of 4–5 $g\,l^{-1}$ and high redox potentials of typically 400–500 mV. The seepage waters in the western part of the Nordhalde (Q4 in Fig. 2) were again highly mineralized (TDS about 10 $g\,l^{-1}$), had an even lower pH (2–3.6) and also high redox potentials. Table 1 gives examples of physicochemical parameters for the investigated samples. All AMD waters had high concentrations of Ca, Mg, Mn, SO_4^{2-}, Si, Cu, Zn, Ni, Cd, Cr, U and REE in common. The main differences in concentrations of dissolved elements between seepage water G16 and Q4 are found for Al and Fe. Whereas concentrations of about 2.5 $g\,l^{-1}$ Fe were obtained for Q4, it was only 2–100 $mg\,l^{-1}$ for G16, typically around 10 $mg\,l^{-1}$. Al was generally found to be $> 100\,mg\,l^{-1}$ for sampling site Q4, however, it was down to 6 $mg\,l^{-1}$ for sampling location G16, depending on pH. Hydrogeochemistry between 1998 and 2002 showed only minor variation in chemical composition of AMD Q4 (Fig. 3) with the only exception of slightly varying Mg contents.

For seepage water G16, REE concentrations were quite variable, ranging between 300 and 3000 $\mu g\,l^{-1}$ in the sampling period between June 2001 and June 2002. Most samples had total REE concentrations of around 700 $\mu g\,l^{-1}$. The total REE concentration in seepage water Q4 N varied between 1600 and 3000 $\mu g\,l^{-1}$ in 1999–2001. Such high REE concentrations as obtained for the two investigated seepage locations are only found in AMD waters. After normalizing REE

Table 1. Physicochemistry and rare earth element concentrations of surface water and seepage water sampled from the Gessenbach creek and from dumps Gessenhalde and Nordhalde.

Sample	Date	pH	El. conductivity ($\mu S\,cm^{-1}$)	Redox potential (mV)	T(°C)	Σ Rare earth elements ($\mu g\,l^{-1}$)
G16	07–11–2001	4.88	6470	480	6.4	712
Q4 S	24–10–2000	3.55	11,900	440	12.2	2490
Q4 N	26–06–2001	2.77	11,900	580	16.2	2200
G13	26–06–2001	7.82	1970	140	18.2	< 0.1
G14B	26–06–2001	3.41	11,200	480	14.5	1845
G14A	26–06–2001	5.94	3100	310	17.2	67
G18	26–06–2001	5.70	2850	290	13.9	41
G7	26–06–2001	4.51	2700	490	15.5	41

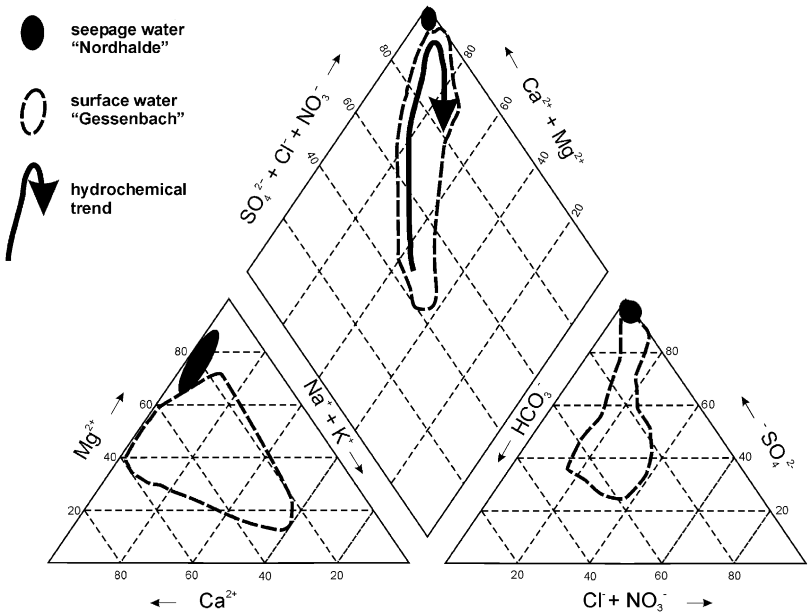

Fig. 3. Hydrogeochemistry of seepage water samples Q4 (black), and surface water sampled along the Gessenbach creek (area surrounded by dotted lines) for the time period 1998–2002. The arrow indicates changes in hydrogeochemistry of the surface water along the flow path of the Gessenbach creek.

concentrations in seepage waters to PAAS enrichment of middle REE (Sm–Dy) and especially of heavy (Ho–Lu) REE as compared to the light ones (La–Nd) was observed. For the seepage water of the Nordhalde (Q4) – sampled over a period of 2 years – the shale normalized (SN) REE patterns are similar, although the concentration differs (Fig. 4). Thus, for AMD with low pH of 2–3.6 REE patterns are independent of REE concentrations. For sample Q4 N the enrichment of heavy REE over the light ones expressed as $(Lu/La)_{SN}$ is calculated to be 42 ± 2 ($N = 4$). It is interesting to note that the REE patterns show variations in the range of only a few % relative standard deviation and thus within the analytical error, although the sampling period covers about 2 years. This has also been shown for two other seepage water sampling sites of the Nordhalde sampled between June and September 2001 (Merten and Büchel, 2004). It was also reported by Verplanck et al. (2004) for sulfate-rich AMD that REE do not fractionate at pH below 5.1. Thus, under acidic conditions, REE can be used as conservative tracers and REE patterns provide a fingerprint of AMD from waste rock dumps contaminating surrounding valley sediments, creeks and groundwater.

The hydrochemistry of the surface water at the northern investigation area is influenced by the seepage water from the (former) dumps and by the groundwater depression cone of the underground mine. To investigate the impact of the seepage water of the dumps on the Gessenbach creek, sampling was performed before (G13),

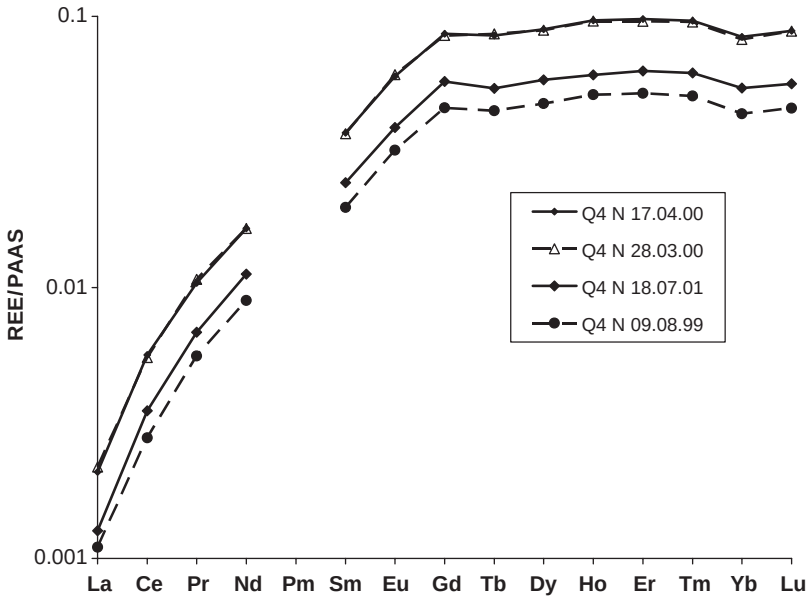

Fig. 4. PAAS-normalized REE patterns of seepage water sampling site Q4 N(orth) sampled over a period of about 2 years.

during (G14A, G14B) and after the passage of dump Nordhalde (G18, G7) (Fig. 2). Before the creek passed the dump the headwaters were strongly influenced by untreated municipal sewage from Kauern. The pH of the water is variable but mostly alkaline (7.5–9). TDS (1.5–2 $g\,l^{-1}$) is low compared to the seepage water. When the Gessenbach creek passed the area of the Nordhalde and Gessenhalde the water was enriched in TDS (elevated concentrations of Fe, Ca, Mg, Al, Mn, SO_4^{2-}, Si, Cu, Zn, Ni, Cd, Cr, U and REE), although discharge measurements in the Gessenbach creek showed that the amount of seepage water flowing direcly into the creek was low (0.5–1 $l\,s^{-1}$). Some of the components were transported as conservative tracers (Mg, Ca, Na, K, Cl, PO_4^{3-}), while others were prone to reactive transport (Fe, Mn, Si, Cu, Ni, REE). The influence of the AMD on the surface water is demonstrated by the variation of the hydrochemical composition (Fig. 3). The surface water changed from an alkaline Ca–Mg–SO_4^{2-}–HCO_3^- type to an acidic Mg–Ca–SO_4^{2-} type when passing the dump. The pH of the surface waters was varying between 9 and 3.4 depending on the amount of AMD entering the creek. After the dump passage the surface water chemistry changed quickly in flow direction due to mixing, dilution and coprecipitation back to a Ca–Mg–SO_4^{2-}–HCO_3^- type with low metal contents. Iron-rich ochreus precipitates with high contents of heavy metals temporally occured in the creek. Hydrochemical modeling showed that these phases mainly consist of poorly crystalline Fe-SO_4 minerals (e.g. ferrihydrite, schwertmannite), Al-(hydr)oxides, and jarosites. Samples of the precipitates collected from the bed of the creek also showed enrichment in heavy metals (Geletneky et al., 2002).

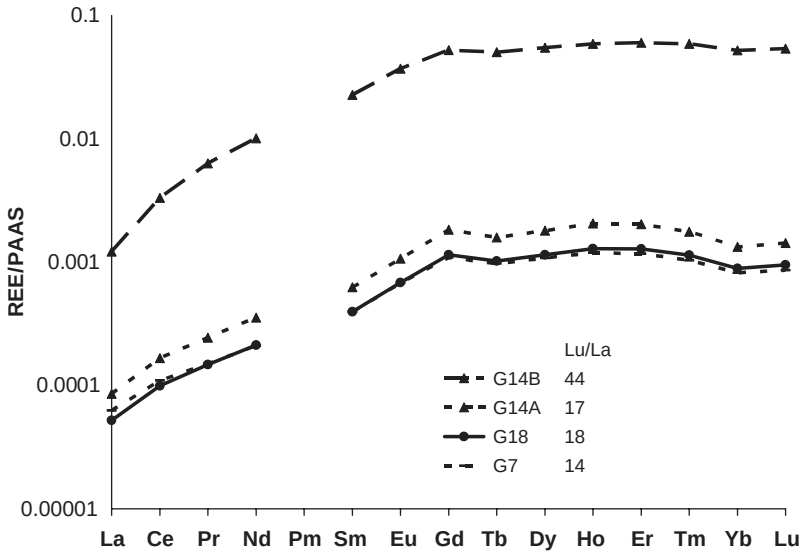

Fig. 5. REE concentrations normalized to PAAS with enrichments in middle and heavy REE along a flowpath in the Gessenbach creek. Along the flowpath both total concentrations and enrichment of heavy REE (see Lu/La coefficient) as compared to the light REE decrease.

At surface water sampling location G13, REE concentrations were below the detection limits (Table 1). For the other sampling points in the surface water (G14A, G14B, G18, G7) REE patterns quite similar to the ones of the AMD were observed (Fig. 5). This represents a diffuse inflow of REE-rich seepage of the Nordhalde dump into the creek. The absolute concentrations of REE in the creek were orders of magnitude lower than in seepage water, due to mixing with creek water (see Table 1). Shale normalized coefficients Lu/La_{SN} show a significant decrease with increasing distance from the dump (Fig. 5). The reason for the observed fractionation of REE patterns along the creek was investigated more thoroughly. For a sample of the seepage water Q4 S with a Lu/La_{SN} relation significantly different from those of Q4 N (28 compared to 42 ± 2) the initial pH of 3.6 was changed to 5.5 and 7.0 by the addition of NaOH. As can be seen from Fig. 6 the concentration of the REE decreases with increasing pH and heavy REE are enriched as compared to the light ones. With Fe being present at concentrations of about $2.5\,g\,l^{-1}$ in Q4 N and observed Fe precipitates along the Gessenbach creek, REE are fractionated due to preferential coprecipitation of heavy REE with amorphous Fe-hydroxides. Coprecipitation of REEs by Fe(oxy)hydroxides is well known and frequently applied for preconcentration of REEs from aqueous solutions (Johannesson et al., 1996, Piepgras et al., 1979). Further, fractionation of REE during coprecipitation by amorphous Fe(oxy)hydroxides has been reported (Verplanck et al., 2004; Bau, 1999; Gammons et al., 2003). Thus, it is possible to investigate reactive transport of heavy metals by the use of REE patterns.

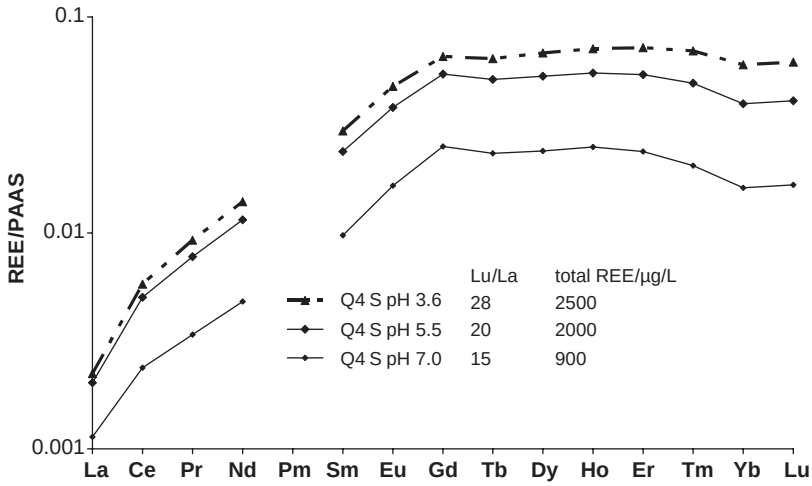

Fig. 6. PAAS-normalized REE patterns for seepage water sample Q4 S(outh) for the initial pH of 3.6 and for pHs of 5.5 and 7 after addition of NaOH. Both a decrease in total concentration of REE and in enrichment of heavy REE as compared to the light ones is observed on increasing pH.

The REE patterns as obtained after direct incubation of AMD G16 with different microbial strains are displayed in Fig. 7. As can be seen from Fig. 7 most of the investigated strains did not alter the REE pattern of the seepage water sample G16. Again, an enrichment in heavy REE was obtained ($Lu/La_{SN} = 13.7 \pm 0.3$). The enrichment is significantly different from the one obtained for samples Q4 N. However, there is one fungal strain (F1) that significantly alters the REE pattern after incubation ($Lu/La_{SN} = 11.9$). This gives an important hint to the remediation abilities of this strain since fractionation among REE was either due to sorption to biomass, due to bioprecipitation caused by the metabolism or due to active uptake into biomass. In this way, REE patterns can be used for a first survey of the abilities of microbes in heavy metal retention. The identified strain should be investigated more thoroughly by other methods in order to better understand the processes involved in sorption/uptake/precipitation of heavy metals.

4.2. Analysis of solid samples

Both the concentrations and the REE patterns of the tested whole rocks as obtained by LA-ICP-MS measurements on fused glass beads are quite similar (Fig. 8). The REE patterns are slightly enriched in middle REE as often observed for phosphate bound REEs (Hannigan and Sholkovitz, 2001). By spatially resolved LA-ICP-MS analysis in thin sections of Ordovician and Silurian shales (e.g. ''Kieselschiefer'') from the Ronneburg uranium mining site, Fischer (2002) has shown that REE are bound to phosphate-rich phases, like phosporite (nodules) and/or monazite. In deeper parts of the Ronneburg uranium mine, where oxidation

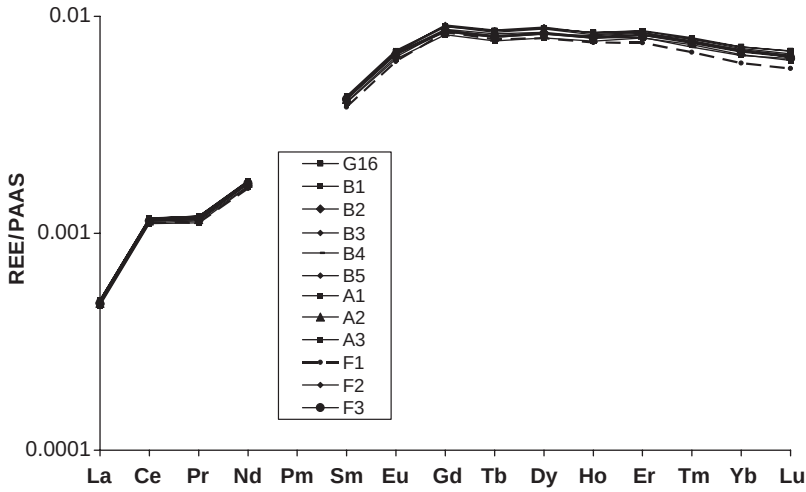

Fig. 7. PAAS-normalized REE patterns of AMD G16 incubated with actinobacteria (A), bacteria (B) and fungi (F), isolated from the Gessental area.

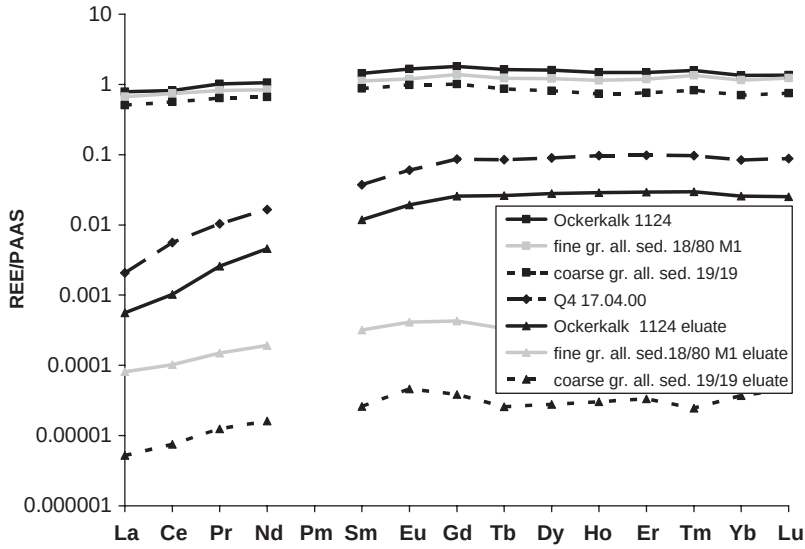

Fig. 8. PAAS-normalized REE patterns of different lithotypes from the Ronneburg mining district as obtained by LA-ICP-MS from fused glass beads (squares) and REE patterns as obtained by sequential leaching (triangles) of Silurian Ockerkalk and fine and coarse grained alluvial sediments. The REE pattern of seepage water sample Q4 North obtained on 17-04-2000 is shown for comparison.

processes were limited and sulfide phases occur REE elements were bound to carbonates. In the oxidation zone of the deposit and in waste rock environment the carbonate content is low compared to phosphate phases, which are more resistant

against low-pH water. Since the shales (e.g., Silurian "Ockerkalk" and "Graptolithenschiefer") are rich in phosporite nodules it is very probable that REE are bound to phosphorites in the investigated lithologies. REE patterns of the investigated whole rocks are rather flat compared to the ones of the seepage water (Fig. 8), and REE patterns of the seepage water did not reflect the REE distribution of the source rocks which is a very common occurence for limestone.

However, the aqueous leachates of the investigated lithologies differ in both concentrations and REE patterns (Fig. 8). For Silurian "Ockerkalk" the REE concentrations were higher by a factor of more than 100 than for the coarse-grained alluvial sediment. All leachates showed an enrichment of heavy REE like the seepage water samples. However, the enrichment and the overall patterns are quite different for the three samples investigated. Only the REE pattern of the Silurian "Ockerkalk" is similar to the pattern observed in seepage water Q4 N (Fig. 8). Thus, it seems very probable that precipitation infiltrating the dump material leads to leaching from Silurian "Ockerkalk" generating this special REE pattern in seepage water.

4.3. Uptake of REE by plants

As can be seen for *Geum urbanum* and *Geranium robertianum* sampled from waste rock dump Sorge Settendorf REE concentrations are quite variable for different herb species and also the REE patterns are slightly different (Fig. 9). For each single herb species, however, the REE distribution was virtually identical, while REE concentration was higher in leaves than in shoots.

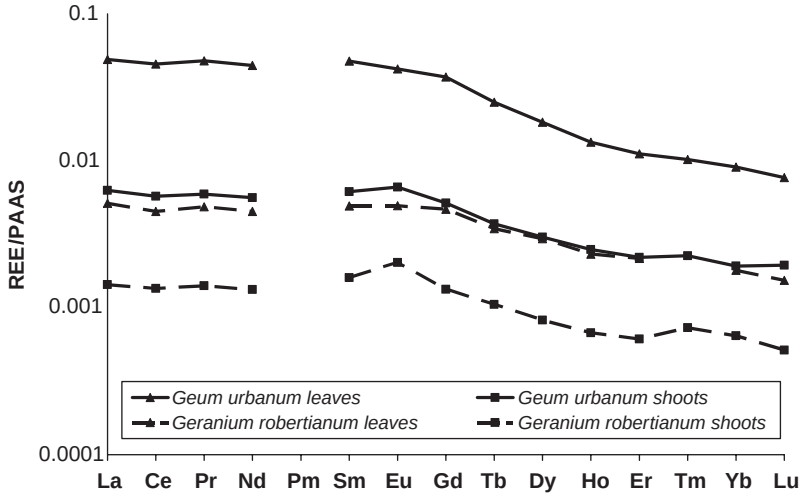

Fig. 9. PAAS-normalized REE patterns in leaves and shoots of plant species grown at the dump site Sorge Settendorf.

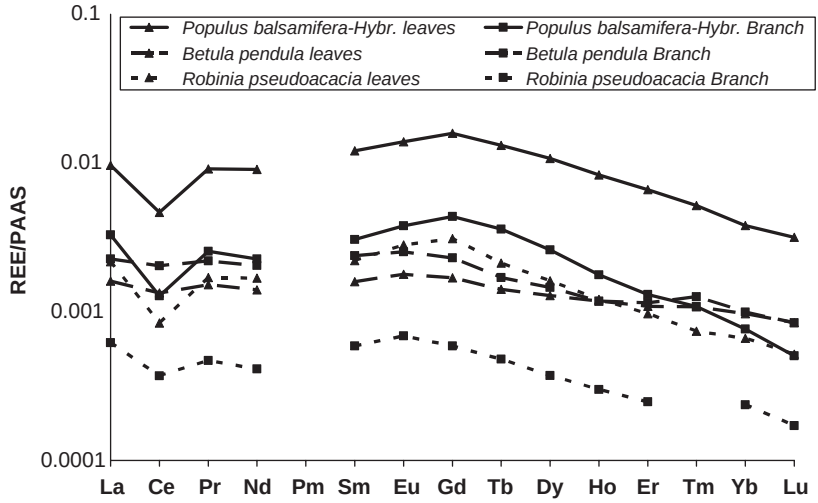

Fig. 10. REE patterns normalized to PAAS in tree species grown at the dump site Sorge Settendorf.

For the investigated trees the results are comparable (Fig. 10). For each single tree species REE patterns are quite identical, whereas for different species fractionation is clearly observed although the trees were grown on the same soil. For *Populus* species and *Robinia* species negative Ce anomalies are observed (Fig. 10). Negative Ce anomalies were observed for different plant species, however, the origin is not yet clear (Fu et al., 2001, Wyttenbach et al., 1998, Xu et al., 2003).

The REE concentrations were highest for the *Populus* species and lowest for the *Robinia* species. Since all species had been growing on the same soil the differences in REE concentrations reflect different uptake of REE by different species (different transfer factors). In this way REE patterns can contribute to the study of heavy metal transfer between soil and plants.

5. Conclusions

It was shown that the identification of waste rock dumps as contaminant sources can be performed using shale-normalized REE patterns of seepage water. It is also possible to follow flow paths of contamination in surface water by using REE patterns. Both the chemical composition and the REE distribution in the surface water were influenced by acidic seepage water, which originated from waste rock dumps. The REE patterns in the water samples did not reflect the source rock pattern as was demonstrated by laser ablation ICP-MS experiments. Percolation of different lithotypes as seen by batch experiments shows that the unique heavy REE enriched patterns were most likely due to preferential leaching especially from Silurian "Ockerkalk". Furthermore, it was shown that REE patterns are also useful

to study processes important in remediation actions such as reactive transport and transfer of heavy metals from soil to different microbial or plant species. Some of the investigated processes could be verified using controlled laboratory experiments. Thus, REE patterns are a usefool tool in monitoring and studying processes involved in generation and remediation of AMD.

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3.3 Heavy metal resistance mechanisms in actinobacteria for survival in AMD contaminated soils.

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Heavy metal resistance mechanisms in actinobacteria for survival in AMD contaminated soils

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Abstract

A site in the former uranium mining area of Eastern Thuringia near Ronneburg was investigated with regard to effects of acid mine drainage (AMD) on reactive transport and bioattenuation. Processes involved in this attenuation might include physico-chemical reactions in reactive transport as well as activities of microorganisms for bioattenuation. In order to test the influence of the soil microbes, a mapping was carried out including both hydrogeochemical and microbiological parameters.

Mapping of contamination was performed along the banks of a creek in a 900 m stretch in 50 m steps by hydrogeochemical analysis of water extracts of soil samples, while general microbial activity was scored by examining soil respiration. The soil samples with high heavy metal load did show low soil respiration as a parameter for microbial activity and plating revealed minimal counts for spore producing bacteria at these contaminated locations. Actinobacteria strains isolated from adjacent locations revealed high levels of resistance as well as high numbers of resistant strains. Specific responses in actinobacteria were investigated after isolation from each of the 18 measuring points along the creek. Specific adaptation strategies and high yields of (intra)cellular heavy metal retention could be seen. Several

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strategies for coping with the high heavy metal contents are further discussed and genes for proteins expressed specifically under high nickel concentration were identified by two-dimensional gel electrophoresis.

Keywords: AMD; Actinobacteria; Streptomycetes; Heavy metal resistance; Bioremediation; Soil extract; Bioavailable heavy metals; Proteome analysis; Molecular biology

1. Introduction

Bacteria in soil consist of different taxa, among them aerobic, spore forming, Gram-positive bacteria of the *Bacillus* and actinobacteria groups. Spore formation is thought to be an adaptation to the differing conditions in soil with changing water contents due to dryness and rain (Vobis, 1997). Actinobacteria including streptomycetes are known to be prominent representing up to 20% of the aerobic soil bacteria population in arable land, and their strong secondary metabolism makes them good candidates for identification of components altering soil chemistry. This capacity of formation of secondary metabolites is exemplified by production of geosmin, the compound responsible for the odor of tilled soil, as well as by production of antibiotics (Kieser et al., 2000). Other products of secondary metabolism may enable the bacteria to cope with stress factors including toxic levels of heavy metals (So et al., 2000).

While many heavy metals are essential micronutrients since they are incorporated into enzymes and cofactors (Fe, Zn, Mn, Co, Cu, Ni, V, Mo) they still are toxic in high concentrations because of adversary binding to enzymes and DNA, and by production of oxygen radicals through the Fenton reaction (Lopez-Maury et al., 2002). Therefore, the organisms must maintain a homeostasis within the cell that keeps the reactive heavy metals at an optimal, sub-toxic level. Resistance factors may allow them to maintain intracellular low levels of heavy metals or intracellular fractionation of the metal in non-harmful complexes (Eitinger and Mandrand-Berthelot, 2000). Thus, adapted microbial populations are prone to show higher resistance to heavy metals as compared to populations of non-contaminated sites.

Adaptive responses towards heavy metal stress may involve detoxification of oxygen radicals, as has been shown for superoxide dismutase overproducing strains of the bacterium *Escherichia coli* which are tolerant to higher heavy metal concentrations (Geslin et al., 2001). Streptomycetes are known to possess two superoxide dismutases: one iron- and one nickel-containing enzyme which are regulated by nickel (Kim et al., 1998a, b). Other resistance mechanisms include sequestration of heavy metals and adsorption of generally positively charged heavy metal cations to the cells walls. The cell walls of the Gram-positive bacterium *Bacillus sphaericus* are commercially used for water treatment (Beveridge and Murray, 1976; Doyle et al., 1980; Raff et al., 2003). In addition, active uptake of radioisotopes has been known at least since the Chernobyl fall-out for basidiomycete fungi which led to extremely high enrichment of cesium in fruitbodies

(Haselwandter, 1978; Giovani et al., 2004). Heavy metal resistance has been investigated largely in Gram-negative bacteria, especially *Ralstonia metallidurans* where efflux transporters could be identified (Mergeay et al., 2003; Nies, 2003).

The former uranium mining site Wismut in Eastern Germany shows great promise to investigate adaptation of microorganisms to anthropogenic pollution with heavy metals. Uranium mining from this site has made the German Democratic Republic the third largest producer of uranium world-wide with more than 210,000 tons of uranium produced during the years 1949 through 1990 (Henningsen and Katzung, 2002). After German re-unification uranium mining stopped and remediation of the area was started by placing the waste rock material in the former open pit. The water table is allowed to rise, thereby re-establishing anoxic conditions in saturated zones. This will prevent further oxidation of the pyrite-rich material and prevent further production of acid mine drainage (AMD) waters. However, seepage waters that resulted from leaching of the former waste rock piles by AMD have infiltrated adjacent soils and surface waters. They contain large amounts of heavy metals including nickel, cadmium, copper, chromium and rare earth elements (Geletneky and Büchel, 2002; Russe et al., 1993).

A detailed hydrogeochemical and microbiological mapping of the surface waters was performed in order to establish the basis for the investigation of specific adaptive responses of microorganisms which in the future might lead to the development of advanced bioattenuation products. Nickel was investigated in particular for cellular adaptive responses in actinobacteria since mechanisms for coping with high nickel contents in the environment have already been analyzed in other microbes (McIlveen and Negusanti, 1994).

2. Material and methods

2.1. Soil sample collection

Soil samples were collected at 50 m intervals from the bank of the creek Gessenbach in a 900 m flow path after merging of the Badergraben with the Gessenbach. Control soils were taken in unpolluted sites within the city limits of Jena, Germany. In each case, mixed soil samples were used to even out heterogeneity which might persist on a very small scale.

Sterile Falcon tubes (50 ml) were punched into the soil at the bank of the creek approximately 15 cm above water level on 19 December 2003, for isolation of soil microbes. Soil for element analyses and soil parameters was collected from the same site in plastic bags. The place was weeded before sample collection if necessary and large roots were removed. The samples were immediately brought to the laboratory and dried (for abiotic tests) or stored in the cold (4 °C; for microbiological measurements).

2.2. Determination of pH, contamination, soil moisture and respiration

The pH was measured in a mixture of 10 g dried soil which was ground with a mortar and pestle. The sieved (1 mm) soil was incubated with 25 ml $CaCl_2$ (10 mM), thoroughly mixed and measured after 1 h (pH meter Hydrus 300, Fisherbrand) (Alef, 1991).

Watery extracts were analyzed using inductively coupled plasma mass spectrometry (ICP-MS, PQ3-S, Thermo Elemental, Winsford, UK). The analyses included U, Al, a series of transition elements (Cd, Co, Cu, Mn, Ni), and alkaline earth elements (Mg, Sr).

Soil respiration was measured from incubation of 20 g soil of native moisture by capturing evolving CO_2 in NaOH and subsequent titration of phenolphthalein–NaOH with HCl (Alef, 1991). Thus, the amount of CO_2 produced could be calculated. The soil was then dried to constant weight and soil moisture and production of CO_2 per gram dry weight was calculated.

2.3. Isolation and growth of microbes

Air dried, ground soil samples were heat-treated at 80 °C for 45 min and suspended in *Aqua dest.* The emulsion was then plated on minimal medium (0.5 g/l L-asparagine, 0.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4$, 0.01 g/l $FeSO_4$, 10 g/l D-glucose, 16 g/l agar). The plates were incubated for 5 days at 28 °C, colonies were microscopically analyzed and transferred to starch casein medium (10 g/l starch, 1 g/l casein dissolved in 0.3 M NaOH, 0.5 g/l K_2HPO_4, 15 g/l agar, pH 7.0–7.5). For supplementation sterile filtered stock solutions of the heavy metal salts ($NiCl_2$, $CoCl_2$, $CuSO_4$, $MnSO_4$, $Cd(NO_3)_2$) were used. Minimal medium has to be used in resistance determination in order to minimize complexation of the heavy metal ions. *Streptomyces coelicolor* A3(2) (DSM 40783) and *Streptomyces acidiscabies* E13 (Amoroso et al., 2000) were grown on minimal media. For two-dimensional (2D) gel electrophoresis the liquid growth media were supplied with 0.1 and 0.3 mM $NiCl_2$.

2.4. Proteome analyses

The comparison of proteins expressed in a cell has become possible by high-resolution protein gel electrophoresis. At the same time, the identification of proteins extracted from a gel is possible for any organism, for which the entire genome encoding the proteins has been sequenced. If two gels are obtained from cultures grown under different conditions like metal stress versus normal media, proteins with higher or lower expression can be identified giving the differential expression pattern under the conditions used. 2D-gel electrophoresis (Lottspeich and Zorbas, 1998) was performed for identification of proteins regulated under heavy metal stress. First dimension separated isoelectric points, second dimension for size in SDS containing gels. Cytosol was prepared using polyvinylpyrrolidone (15.4 mg dithioerythritol and 2 g polyvinylpyrrolidon *ad* 100 ml with potassium buffer: 50 mM; 108 ml K_2HPO_4, 42 ml KH_2PO_4) as buffer for French Press (SLM Instruments) cell disruption followed by centrifugation at 140,000*g* to remove membrane and cell wall fragments. The cytosolic proteins were precipitated (20% trichloracetic acid, 50% acetone, 20 mM dithiothreitol) for 30 min at −20 °C, incubated for 2 h at 4 °C and centrifuged at 11,000 rpm. After two steps of washing with acetone the pellet was dried (Speed Vac) and redissolved in rehydration buffer (8 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT). After ultracentrifugation at 75,000*g*, 500 μg protein (Bradford, 1976) were used for each IEF strip (Immobiline Dry Strip, 24 cm, pH 3–10, Amersham Biosciences) in 500 μl rehydration buffer adding 3 μl 4% bromphenol blue. IEF was performed at 150 V (2 h), 300 V (2 h), 600 V (1.5 h), 1200 V (1 h), 2400 V (1 h), and 3500 V (17 h) and 30/80 V, 16 h in 10% acrylamide (Rotiphorese, Roth) for the second dimension using IPGPHOR II and Ettan DALTwelve (Amersham Biosciences).

Analysis of the spots cut from the gel and extraction with acetonitrile and drying was performed by trypsin digestion and electron spray ionization mass spectroscopy (ESI-MS; Lottspeich and Zorbas, 1998). The system LCQ Deca XP (Thermo) was used with the *S.*

coelicolor genome (NCBI database) after reversed phase HPLC for purification for mass detection and in silico analysis with Sequest 3.1.

3. Results

3.1. Characterization of the site

Soil samples were taken from the bank of creek Gessenbach near Ronneburg in Thuringia, Germany (Fig. 1). The distance between merging of the Badergraben with the Gessenbach and the next road connecting Kauern and Ronneburg of approx. 900 m was sampled every 50 m. At the first location in flow direction, P0, contamination was assumed to be highest due to passage of two former dump sites which release AMD waters. For control, five independent soil samples were taken from uncontaminated sites within the city of Jena in the Saale river valley.

The pH did not show the expected gradient of low pH at measuring point P0 with lowest pH increasing to point P18, but rather indicated additional, so far unknown, AMD influence. Additional acidification was observed at locations P14 and P16 (Fig. 2), while most of the other samples show circum-neutral pH comparable to control soils (sample K, Fig. 2). This interpretation was confirmed by analyses of heavy metals and other elements (Fig. 3). The effluence could be localized at two sources at point P14 and close to P16, with salt incrustations observed at the water sources.

While some elements show clearly high enrichment (Al, Cd, Co, Cu, Mn, Ni, U, Zn), other elements proved to be of no clear ecotoxicological risk at the concentrations found (As, Pb, Sr) or can be viewed as markers for flow paths rather than influencing microbial life (Ba, most likely as $BaSO_4$). The data provided are measurements of supernatant of roughly five-fold diluted soil (10 g soil plus 45 ml *A. dest.*). This represents a simple water extract which is equivalent to the lowest portion of bioavailable metals in the soil. Thus, the ecotoxicological risk is rather understated here.

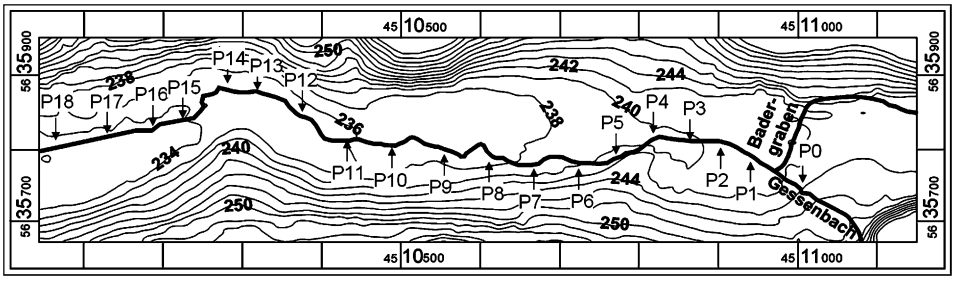


Fig. 1. Map of the area investigated with sites for soil sample collection along the bank of creek Gessenbach near Ronneburg in Thuringia, Germany (from the topographic map of Thuringia, Landesvermessungsamt, 1999).

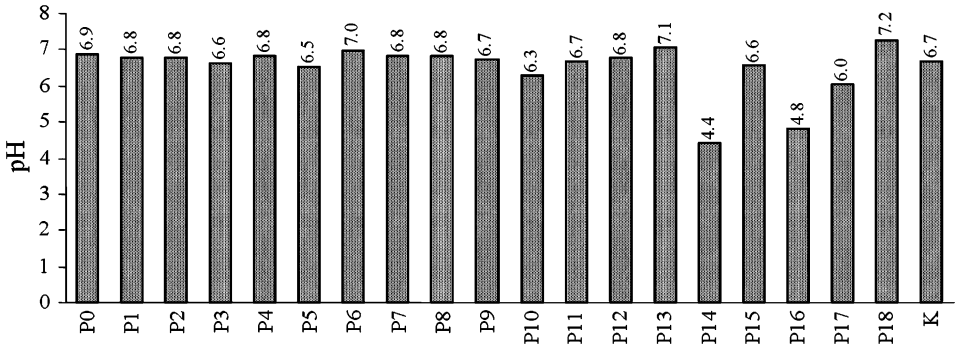


Fig. 2. Determination of soil pH at sampling sites P0 through P18 along the Gessenbach. A control site (K) is included ($n = 5$).

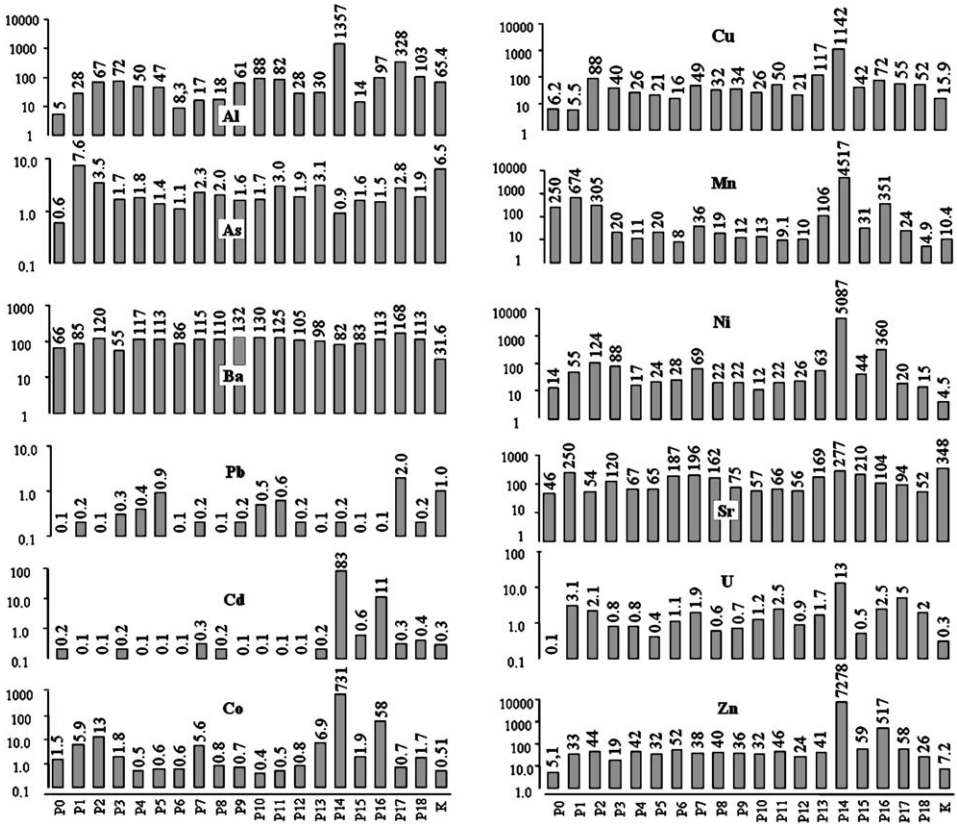


Fig. 3. Heavy metals contents of watery soil extracts at sampling sites P0 through P18 along the Gessenbach (ppb). A control site (K) is included ($n = 5$).

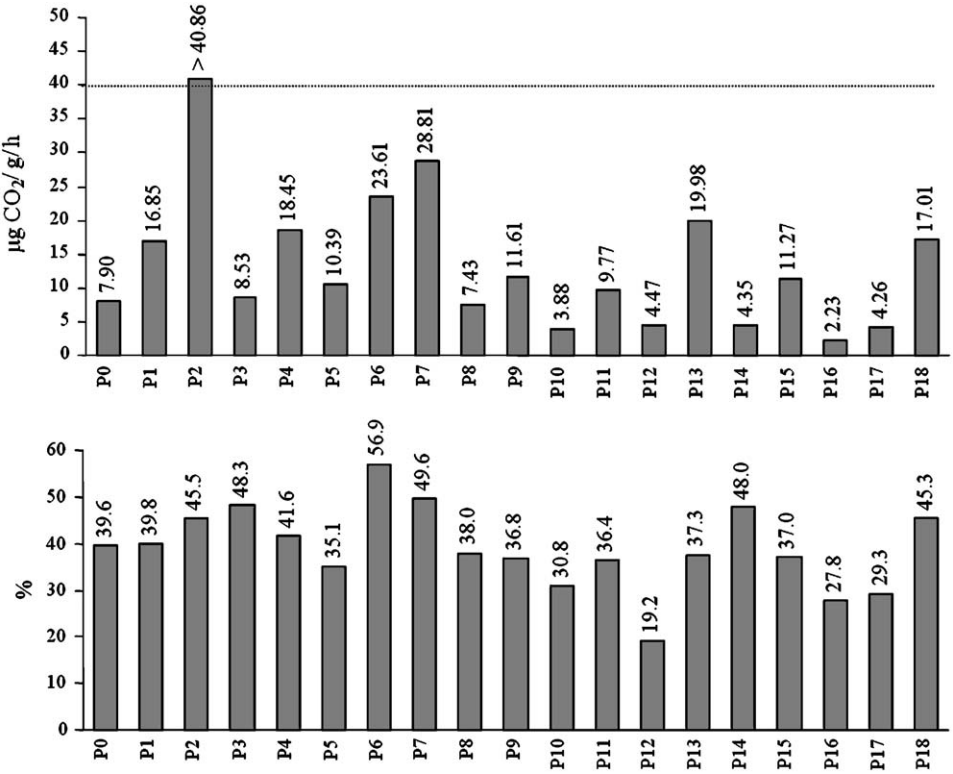


Fig. 4. Soil respiration (above) and soil moisture (below) at sampling points P0 through P18 along the Gessenbach. Soil respiration of control sites is indicated by a dotted line.

Soil respiration as a parameter for microbiological activities was examined at every sampling point (Fig. 4), since Cd, Co, Cu, Ni and at high concentrations Zn are known to threaten microbial life. While in control soil samples, respiration of about 40 µg CO_2 released per hour per gram dry weight of soil was found, five samples did show respiration below 5 µg CO_2 per hour per gram dry weight of soil. These included the two sites with high contamination, P14 and P16, but also encompassed P10, P12 and P17 for which contamination was intermediate. One of these sampling points, P12, did show very low soil moisture (19.2%, while all other samples had around 30–50%) which might explain low soil respiration for this site. Another site, P6, was very wet (57%) but did still show soil respiration at expected activities.

3.2. Isolation of spore forming bacteria

Colony forming units (cfu) for hyphal bacteria of the genus *Streptomyces* and other filamentous actinobacteria were analyzed in the samples after heat-inactivating

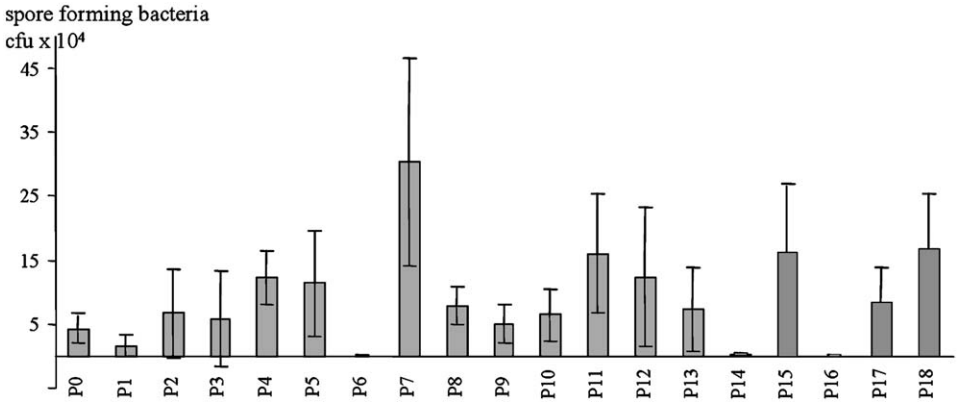


Fig. 5. Colony forming units (cfu) of bacteria on minimal media. cfu from heat resistant spores (80 °C).

vegetative cells. The plate counts clearly showed the toxic influence of contaminated sites as P14 and P16 had less than 10,000 cfu/g dry mass of soil (Fig. 5). Only one other sampling point (P6) showed such low levels of actinobacteria spore contents. In this case, the high water content of over 50% soil moisture can be assumed to cause the effect since actinobacteria are dependent on well-aerated dry habitats.

These isolation plates did not contain metals and thus allowed to determine the number of strains present in the sample. In order to assess the amount of strains which show adaptation to the contaminated environment, an isolation strategy was used including one heavy metal (Ni) into the plates. Subsequently heavy metal resistance towards an array of heavy metals was determined.

3.3. Heavy metal resistance

The soil samples were also used for isolation of heavy metal resistant strains on plates containing 5 mM $NiCl_2$ (Table 1). While soils samples P0, P1, P3, P6, P14, P15 and P16 yielded no nickel resistant colony, varying amounts of nickel resistant strains could be isolated from the other soil samples (0.01–29% compared to cfu on plates without nickel). In the five control soil samples, only six strains could be isolated on plates containing nickel which represents only 5.3×10^{-5} to 2.8×10^{-4}% of the colonies found on plates lacking the heavy metal.

Four of the newly isolated strains and four control strains were tested more precisely for their heavy metal resistance. Plates containing the salt were inoculated with strains P4-3, P8-3, P9-9 and P17-6 from sampling points P4, P8, P9 and P17, respectively. These sampling points were comparable in their heavy metal content but slightly differ in contamination for single metals. Ni, Co, Cu and Mn were especially of interest since the contents are high. Control strains included *S. coelicolor* A3(2), since this is the genetically best-defined streptomycete with a known genome sequence which should not carry heavy metal resistances, as well as three

Table 1. Heavy metal resistance of spore forming actinobacteria colonies isolated with or without nickel

	cfu[a]/g−Ni	cfu/g + Ni	% resistant
P0	40988 ± 21485	0	—
P1	16676 ± 16438	0	—
P2	67046 ± 68668	19444	29.00
P3	57907 ± 74873	0	—
P4	122564 ± 41272	47	0.04
P5	115103 ± 81454	1024	0.89
P6	362 ± 724	0	—
P7	303758 ± 162874	1990	0.66
P8	78078 ± 28132	13367	17.12
P9	50607 ± 29837	52	0.10
P10	64393 ± 40614	2632	4.00
P11	160041 ± 93036	12	0.01
P12	123669 ± 108325	29	0.03
P13	73294 ± 65786	41	0.06
P14	2159 ± 2267	0	—
P15	162605 ± 107319	0	—
P16	616 ± 633	0	—
P17	83404 ± 54314	53	0.06
P18	166840 ± 86042	1895	1.14

[a]cfu/g dry soil are given.

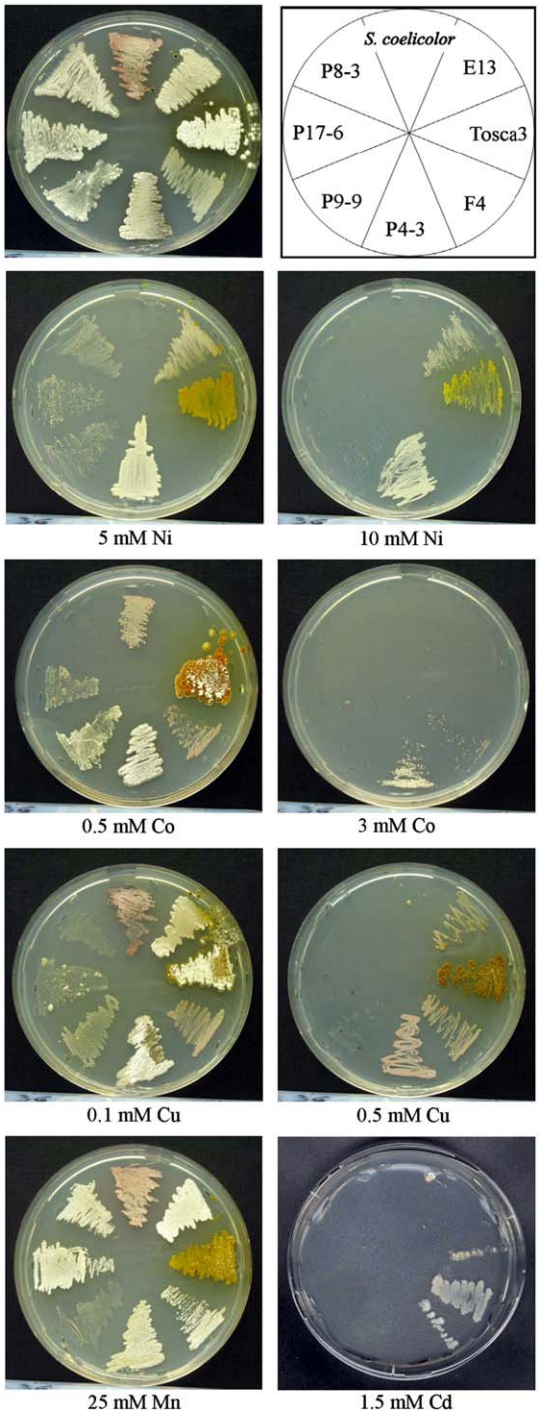
strains identified in previous studies which are known to be resistant to Ni (E13, Tosca3) or Cd (F4).

As can be seen from the plate assays (Fig. 6), strain P4-3 is highly nickel resistant. In contrast to other isolates, growth occurs still on 10 mM $NiCl_2$ (equivalent to 600 ppm) containing plates. In addition, this strain handles 3 mM cobalt well and is able to form spores on plates containing 2 mM $CoCl_2$.

Strain P8-3 is the most sensitive of the tested strains against cobalt and copper, while strain P9-9 is manganese sensitive which is especially obvious if comparing spore formation (no spores even on 5 mM $MnSO_4$).

Isolate 17-6 shows release of a diffusible substance which allows growth of strain P8-3 to form spores on manganese containing minimal medium just at the border line between both strains (Fig. 6). A similar phenotype, excreting chelating substances, could be observed for F4 on Cd containing media. This already showed the ability to form chelating, extracellular compounds.

Another feature associated with high heavy metal concentrations is the loss of capacity to form spores. Since this was observed here for the first time, the strategy for isolation of hyperresistant strains had to be reconsidered. During the first round of isolation, the soil samples were incubated at 80 °C before plating in order to reduce growth of vegetative soil bacteria. However, subsequently a new isolation protocol was followed by streaking soil samples directly on plates containing 15 mM


S. coelicolor
P8-3
E13
P17-6
Tosca3
P9-9
F4
P4-3
5 mM Ni
10 mM Ni
0.5 mM Co
3 mM Co
0.1 mM Cu
0.5 mM Cu
25 mM Mn
1.5 mM Cd

$NiCl_2$. Indeed, with this isolation strategy six hyperresistant strains could be isolated from sampling points P13 through P18, growing with good growth rates on plates containing 0.3% or 3000 ppm nickel. Further analyses will be necessary to identify these actinobacteria to the genus and species level and to determine the maximum concentrations of heavy metals endured in solid and liquid minimal media.

3.4. Proteome analyses

Other resistance mechanisms could involve intracellular sequestration. To identify intracellular proteins which are involved in heavy metal resistance, 2D gel electrophoresis was performed. Using *S. coelicolor* A3(2) was essential since its genome sequence allows identification of separated and extracted spots from the gel by ESI-MS and in silico comparison of fragments to fragments predicted for the annotated genes from the entire genome. In addition, first investigations with *S. acidiscabies* E13 were performed in order to assess the possibility to use a related, but heavy metal-resistant strain for analyses of the proteome.

First, the control strain *S. coelicolor* was grown with or without nickel in the medium and 2D gel electrophoresis (Fig. 7) indicated induction of some proteins. Of the proteins repressed by nickel, four proteins encoded on the actinorhodin biosythesis gene cluster were identified while among the proteins induced with $NiCl_2$, three ribosomal proteins (S8, S9 and L29) were identified, one protein could not be identified and the remaining two showed homology to a putative protein Lsr2 and a putative transcription factor of the *tetR*-like family (spot C6).

Thus, we could show that this approach is feasible for future, more thorough proteome analyses. However, the use of a strain that is heavy metal resistant promised more interesting results. Therefore it was investigated whether the use of *S. acidiscabies* E13 would allow the in silico comparison with the known genome of *S. coelicolor* for identification of heavy metal regulated proteins. In comparison, gels run from cells grown with and without nickel showed a substantial shift in physiology (Fig. 8). From the gels, eight spots were cut and analyzed. All could be identified in silico. From the three spots repressed under nickel, one served as an internal standard since the iron containing superoxide dismutase *sodF* is known to be repressed under nickel in streptomycetes. From the five spots induced in nickel grown cultures, three are of interest: induction of glycerin-3-phosphate dehydrogenase (spot E6) and fructose-1,6-bisphosphate aldolase (spot E7) were shown. This was unexpected as enzymes involved in glycolysis are generally assumed to be constitutively expressed. However, since more intracellular storage components could be visualized by electron microscopy (data not shown) in cells grown with nickel, enhanced glycolysis might be linked to biosynthesis of storage components. The third protein of interest is again, as seen with *S. coelicolor*, a homolog to the

Fig. 6. Plate assays for heavy metal resistance. Four strains from the isolation campaign and four control strains are shown without or with indicated concentrations of heavy metals.

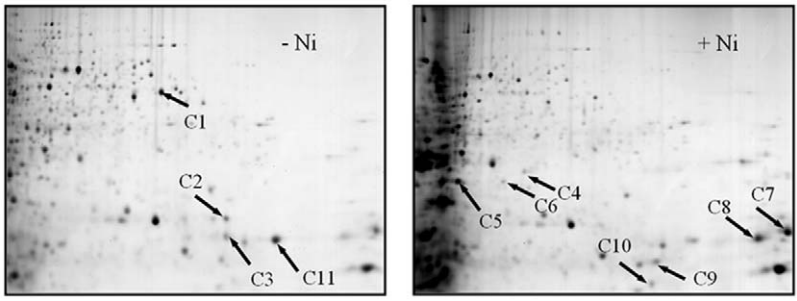


Fig. 7. Two-dimensional gel electrophoresis of the control strain *Streptomyces coelicolor* A3(2) grown without or with 0.1 mM $NiCl_2$ in the medium. The identified, differentially expressed protein spots C1 through C11 were used for identification of proteins.

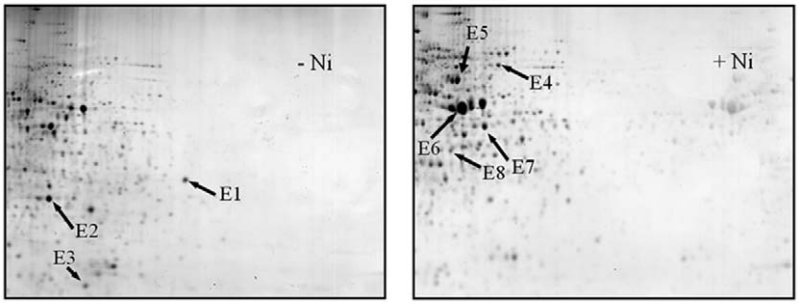


Fig. 8. Two-dimensional gel electrophoresis of the control strain *Streptomyces acidiscabies* E13 grown without or with 0.3 mM $NiCl_2$ in the medium. The identified, differentially expressed protein spots E1 through E8 were used for identification of proteins.

tetR-like transcription factor (spot E8). Further analysis defining the proteome answer in *S. acidiscabies* on heavy metal stress therefore is possible using 2D gel electrophoresis and ESI-MS.

4. Discussion

The field site was characterized and two new sites of AMD influence on the Gessenbach could be determined by investigating soil samples along the creek's banks. It could be shown that adaptation has occurred within the past 40 years in that the amount of strains with the capacity to withstand high concentrations of heavy metals are enriched in soils which have been influenced by AMD during this period of time. The percentage of heavy metal resistant strains is significantly higher than in comparable control soils.

Four strains were examined in particular and different mechanisms can be assumed for protection of the cells from heavy metals. Strain P4-3 is capable of growth under high cobalt and nickel concentrations without loosing the capacity to form spores at least on 0.5 mM Co. Since the loss of spore formation is due to altered secondary metabolism within the cell, it might be argued that the cells are able to exclude the detrimental heavy metals. This indicates an efflux transport system which has been investigated for Gram-negative bacteria where co-resistance against cobalt and nickel has been reported (Nies, 2003).

Strain P17-6 could be shown to produce a compound which is diffusible and provides a protection to some other strains (P8-3 could form spores at the side facing P17-6, while strain P9-9 did not profit from that same compound). Such a substance might become interesting in the future if water treatment is developed making use of biological complexation agents. A similar observation was made with one other strain, F4, which shows cadmium resistance and did produce diffusible compound(s) allowing sensitive strains to grow adjacent to the producer on cadmium. Diffusible components have been described for other heavy metal resistant bacteria (Raytapadar et al., 1995). Previous analyses had shown the ability to take up heavy metals (Merten et al., 2004).

For the two remaining strains, either intra- or extracellular binding of the heavy metals can be expected.

The observation that spore production ceases on media containing heavy metals led to a refined isolation procedure and indeed, so far six hyperresistant strains were identified. Their capacity to grow on solid media containing 0.3% $NiCl_2$ or 50 mM of nickel is a feature that makes them especially interesting in future studies.

The first, initial proteome analysis regarding soil bacteria and their heavy metal resistance could show that this approach is valuable for determining cellular responses and adaptive mechanisms in actinobacteria, especially within the genus *Streptomyces* since genome data are available and allow identification of up-regulated proteins from 2D gel electrophoresis. The analysis of a *tetR*-like transcription factor seems especially promising for future identification of a whole cascade of genes under transcriptional control by this protein. Such findings would allow addressing regulatory cascades in heavy metal resistance of soil microbes.

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3.4 Microbes adapted to acid mine drainage as source for strains active in retention of aluminum or uranium.

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Microbes adapted to acid mine drainage as source for strains active in retention of aluminum or uranium

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Abstract

The use of microorganisms for the extraction of contaminants like solved metals from drainage or surface waters was investigated using strains adapted to a polluted environment at a former uranium mining site near Kauern, Eastern Thuringia, Germany. Soil respiration data showed increasing variation indicating stress response and hence need for adaptation. Thus, isolation of single strains was performed for more detailed analyses. Of the isolated fungi and bacteria (single-celled bacteria as well as filamentous actinobacteria), 15 were grown in mine drainage waters in order to test their capacity to retain (heavy) metals including rare earth elements and radioisotopes. Out of the 15 strains (respectively 5 single-celled bacteria, actinobacteria and fungi), 11 strains could grow in media containing acid mine drainage waters diluted by half which is representative of the conditions a few meters downstream of the entry point of the acid mine drainage. Two strains showed promising capacity for aluminum or uranium retention. Using rare earth elements as tracers, selective biosorption or uptake of heavy rare earth elements was prominent in one sample, a fungal isolate. The actinobacterial strains also showed capacity for bioremediation of contaminated seepage waters. Different reactions to single elements varying between all isolates indicate biologically controlled transport processes because such strong fractionation would not be expected from physico-chemical adsorption processes.

Keywords: Heavy metals; Rare earth elements; Retention; Bioremediation; Bacteria; Fungi

1. Introduction

Anthropogenic use of the landscape has led to the excavation of large amounts of rocks for ore and coal mining. The excavated waste rocks often contain large amounts of sulfur which is bound, in many instances, in pyrite (FeS_2). The oxidation of pyrite under oxic conditions in waste rock piles leads to substantial release of sulfate and to a decrease in pH. With the acidic pH of the seepage waters, most heavy metals are leached as they are soluble under acidic conditions. The process is microbiologically driven by the activity of bacteria like *Acidithiobacillus ferrooxidans* which gains its energy from the proton gradient across the cellular membrane and subsequent iron oxidation.

$$2Fe^{2+} + 0.5O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O$$

The Fe^{3+} is able to oxidize pyrite, at the same time releasing protons. The sum equation also takes into account

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chemically driven reactions which, however, are forced by the removal of reactants:

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+$$

Thus the overall activity is dependent on the presence of microorganisms which speed up the process up to 1,000,000 times. With the cycling of Fe^{2+}/Fe^{3+} a net release of two protons is associated driving the acidification of the environment. This process is known as acid mine drainage (AMD) and leads to acceleration of weathering as compared to abiotic alteration processes. Leaching of heavy metals under acidic conditions leads to contamination of water ways through seepage waters into the surface waters of creeks and streams (see also: Lovley and Coates, 1997; Rawlings, 2002). In order to protect ground water and drinking water wells, the AMD waters have to be pumped and treated chemically which will continuously cost large sums of money.

One area which is prone to see such problems is the former uranium mining area in Eastern Germany (Lange, 1995; Geletneky et al., 2002). Uranium mining from this site has made the German Democratic Republic the third largest producer of uranium worldwide with over 210,000 tons of uranium produced during the years 1949 through 1990. After re-unification of Germany, the uranium mining was stopped and the Wismut GmbH was founded to remediate the site as financed by the German Federal Government. The strategy chosen for remediation was to replace the waste rock material into the former open mining pit. The water table, which was pumped to produce a depression cone during mine operations, was allowed to rise again, re-establishing anoxic conditions in saturated zones. This is thought to prevent further oxidation of the pyrite-rich material and prevent further production of AMD waters.

This procedure will minimize necessary interventions in the future. However, it cannot be expected that the remaining sites of former heaps are entirely free of heavy metal contamination. Rather, a low but widespread contamination is to be expected. The surface waters from these sites will have to be treated in order to prevent contamination of river systems downstream. Land-use of the sites will have to be restricted unless a system can be established that can remediate the soils on-site. Wetlands have been propagated as low-maintenance strategies for the remediation of contaminated waters (Banks et al., 1997; Younger, 2000). For soil remediation, the use of phytoextraction has been widely and controversially discussed (Ouyang, 2002). Since plants take up the nutrients as well as heavy metals with the water from the soil, the bioavailability of heavy metals – which is determined at large by the microbial communities – greatly influences this process. At the same time, soil biophysical and chemical parameters are determined by a wide range of soil microbes. Organic carbon is released by heterotrophic microorganisms as CO_2 to the atmosphere contributing to the greenhouse effect, and soil microbes contribute to solubilization as well as fixation of heavy metals, thereby determining the availability of heavy metals to plant root systems (Cervantes et al., 2001).

One major group of soil microbes represents the filamentous, Gram-positive actinobacteria including the streptomycetes. This group is noticeable for the odor of tilled soil which is attributed to geosmins, a group of secondary metabolites of streptomycetes. Another group of important soil microbes by their numbers are the Gram-negative proteobacteria followed by soil fungi and other Gram-positives including bacilli. The three groups of filamentous Gram-positives, single-celled Gram-positives and Gram-negatives, and soil fungi will influence soil differentially. They have different metabolic activities and they present different surfaces to the environment with different capacity to adsorb heavy metals (Beveridge, 1989; Beveridge et al., 1997; Gabriel et al., 2001). The adsorption of heavy metal cations to the cells walls of Gram-positive bacteria has been established (Beveridge and Murray, 1976; Doyle et al., 1980) and cell walls of *Bacillus* species are used for heavy metal-containing water treatment on an economic scale, and other biopolymers have been proposed for technical use (Gutnick and Bach, 2000).

However, in addition to the biophysical and biochemical parameters of soil microbe cell walls, the active uptake of radioisotopes has been known at least since the Chernobyl fall-out to occur in basidiomycete fungi which led to extremely high enrichment of cesium in fruit-bodies sampled from contaminated areas (Baeza et al., 2000; Sugiyama et al., 2000). If heavy metals are taken up into the cells, resistance factors have to be present in order to protect the cells from the toxic effects (Cooksey, 1994). Heavy metal resistance has been investigated largely in Gram-negatives, e.g. *Ralstonia eutropha* or *R. metallidurans* (Nies, 1992; Stanton et al., 2000). In the cases investigated, efflux transporters have been identified for heavy metal resistance which would make this group less prone to act as biosorption or bioremediating organisms (Nies, 2000).

Here, we proceed to test Gram-positive as well as Gram-negative bacteria and fungi for their capacity to survive on metal containing acid mine drainage waters and their potential use for bioremediation. The aim of this investigation was to test potential bio-immobilization by

microbes for future application in water treatment or inoculation of soil, where even temporary uptake into microbes will help to lower peaks of wash-out and stabilize plant growth by reducing the plant-available concentrations (see also Lovley and Lloyd, 2000).

2. Material and methods

2.1. Collection of AMD waters

Water was collected at a seepage site located on the former Gessen dump in the community of Kauern near Ronneburg in Eastern Thuringia, Germany (Fig. 1). The drainage water used in this study was sampled on 7.11.2001 at measuring point G16. The water samples (3 L) were filtered in the field using glass fibre prefilters (Millipore, Eschborn, Germany) and cellulose acetate filters (Sartorius, Göttingen, Germany) with a pore size of 0.45 μm. The pH of 4.88, electrical conductivity 6479 μS/cm, redox potential 480 mV, and temperature 6.4 °C of the unfiltered samples were measured directly in the field (WTW, Weilheim, Germany). The samples were transferred to the laboratory the same day and stored at 4 °C until use. The water samples were filtered in the lab using a 0.2 μm cellulose acetate filter (Sartorius, Göttingen, Germany) before inoculation.

2.2. Geochemical analyses

The samples were analyzed hydrogeochemically using atomic absorption spectrometry (AAS, AAS 5EA and AAS 5FL, Carl Zeiss, Jena, Germany), inductively coupled plasma mass spectrometry (ICP–MS, PQ3-S, Thermo Elemental, Winsford, UK), titration (Titrino 716 DMS, Metrohm, Filderstadt, Germany) and spectrophotometry (DR/4000U, Hach, Loveland, USA). The analyses included U, Y, rare earth elements (REE), Al, a series of transition elements (Cd, Co, Cu, Fe, Mn, Ni), alkali (K, Na) and alkaline earth elements (Ca, Mg, Sr), as well as Cl^-, SO_4^{2-}, PO_4^{3-} and nitrogen compounds (NH_4^+, NO_3^-).

After incubation and prior to geochemical analysis, the samples were centrifuged at 6000 rpm and 4 °C (Beckman, Palo Alto, USA) and filtered twice, including a 0.2 μm sterile filter (Sartorius, Göttingen, Germany).

2.3. Isolation and growth of microbes

For isolation of bacteria, actinobacteria and fungi ground soil samples (heat treated for isolation of actinobacteria at 80 °C for 2 h) were suspended (1 g in 9 mL distilled water) and after 2 h of shaking supernatant was plated in 100 μL aliquots of dilutions from 10^{-4} to 10^{-6} on solid media.

For isolation of soil bacteria, standard I medium (Merck, Darmstadt, Germany) supplemented with seepage water diluted by half was used. The seepage water (from measuring point G16) was sterilized separately in order to prevent precipitation of compounds after adjusting pH to 6 and sterile filtration (0.2 μm pore size) and mixed with the medium.

Actinobacteria were plated on soil extract medium containing seepage water diluted by half (modified after Thiemann et al., 1968). For soil extract, 150 g garden soil were extracted over night in 400 mL tap water by stirring, the supernatant centrifuged for 10 min at 4000 rpm, and the clear supernatant adjusted to 500 mL, adding 3.6% (w/v) agar before autoclaving.

For the isolation of soil fungi CYM medium (2 g/L trypticase peptone, 2 g/L yeast extract, 20 g/L glucose, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L KH_2PO_4, 1.0 g/L K_2HPO_4, 18 g/L agar; Schwalb and Miles, 1967) supplemented with seepage water (50%) was used.

The plates were incubated for 5 days at 28 °C, colonies were microscopically analyzed and bacteria, actinobacteria and fungi were subsequently transferred to standard I, starch casein medium (10 g/L starch, 1 g/L casein dissolved in 0.3 M NaOH, 0.5 g/L K_2HPO_4, 15 g/L agar, pH 7.0–7.5) and CYM, respectively.

2.4. Incubation of mine waters

The strains were grown in minimal medium (0.5 g/L asparagine, 0.5 g/L K_2HPO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $FeSO_4 \cdot 7\ H_2O$, 10 g/L glucose; Amoroso et al., 2000) to late logarithmic growth phase, and inoculated after three steps of washing with distilled water 1:2000 into 10 mL minimal medium/seepage water (1:1) and incubated for 7 days at 28 °C. The supernatant was analyzed geochemically for anion and cation contents.

2.5. Microscopy and staining

Microscopical observation was performed using a Zeiss Axioplan 2 fluorescence microscope. Direct cell counts were obtained after staining with DAPI (4-6-diamidino-2-phenylindol; Raju, 1982).

2.6. Soil respiration

The soil pH was measured following the protocol of Schinner et al. (1993). Soil respiration was determined by titration using 40 μL of 0.1% phenolphthalein in 60%

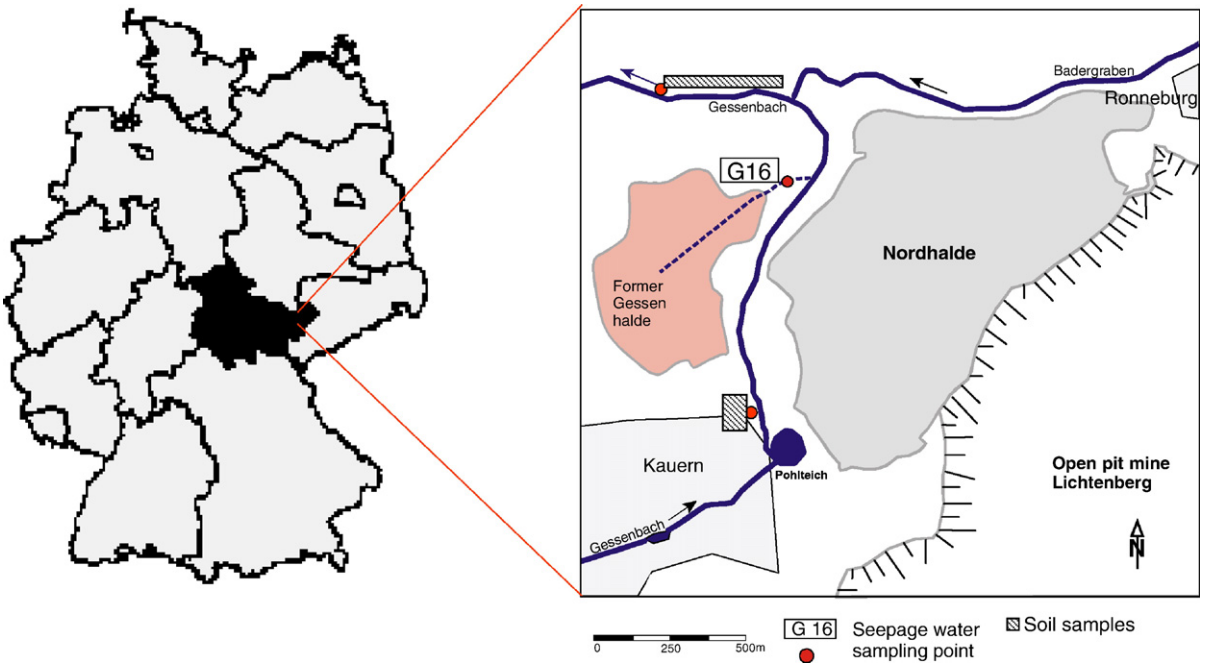

Fig. 1. Map of the investigated area including sampling point G16 used to collect AMD waters at Kauern near Ronneburg in Eastern Thuringia, Germany.

ethanol (Schinner et al., 1993) in three replicates for each measurement. For substrate-induced respiration, 0.3% glucose or 0.2% yeast extract was added. For determination of microbial biomass, the soil samples were autoclaved and re-inoculated with 10% of non-sterilized soil such that the biomass of the dead cells can be used as energy, carbon and nitrogen source. The control would be the non-inoculated, sterilized soil to test for abiotic CO_2 production.

3. Results

3.1. Characterization of seepage waters

At the former uranium mining site (Fig. 1) one heap that has been used for acidic leaching has been removed early in the remediation process. This former Gessen heap site is located within the area that has no connection to the natural groundwater yet because of mining-related pumping. The entire surface waters are captured by the Gessen Creek which makes the measuring of influent waters easy since the contributing waters are easy to determine and to control. One point that allowed access to Gessen site drainage waters is the surface water measuring point G16 which was surveyed for some time. The waters show low pH (pH 4.88) and high heavy metal concentrations as expected for such a site (Table 1). Iron (hydro)oxide precipitation was visible before filtering. Especially the rare earth elements (REE) show a specific signature in these waters,

Table 1
Concentrations of heavy metals and solutes in G16 water (sampled 7.11.2001) before (G16) and after (G16 sterilized) autoclaving

Analyte		G16	S.D.	G16 sterilized	S.D.
Cd	µg/L	44	1	47.16	0.02
Sr	µg/L	732	7	763	3
U	µg/L	634	6	681	5
Y	µg/L	612	13	643	1
Al	mg/L	47.4	0.8	51.0	0.2
NH_4^+	mg/L	0.03	0.01	0.12	0.01
Ca	mg/L	721	18	754	16
Co	mg/L	2.02	0.01	2.12	0.04
Cu	mg/L	1.18	0.01	1.25	0.01
Fe	mg/L	0.1	0.02	6.1	0.2
K	mg/L	2.4	0.1	2.5	0.1
Mg	mg/L	923	2	966	2
Mn	mg/L	71.7	0.6	74.9	0.9
Na	mg/L	33.2	0.5	34.9	0.1
Ni	mg/L	11.4	0.1	11.9	0.2
Cl^-	mg/L	246	2	261	1
NO_3^-	mg/L	22	0.4	23	1
PO_4^{3-}	mg/L	0.10	0.01	0.05	0.01
SO_4^{2-}	mg/L	5250	100	5465	160
∑REE	µg/L	712	7	748	6

Error levels (1σ) are given for two replicates.

which allows source/sink determinations. Drainage water G16 can be used as a source to determine microbial extraction of REE as well as other metals and for the differentiation between mechanisms of retention such as adsorption and bioaccumulation, since only active uptake into the cells will show clear fractionation between different elements.

3.2. Microbes in contaminated soils

Soil samples from five sites close to the sampling point of drainage waters, G16, were analyzed for soil respiration and substrate induced respiration. For comparison, a site in the Gessen Creek Valley, approximately 500 m upstream, which shows low contamination in soil and surface waters was used. The control site, however, still shows anthropogenic influence with some elevated levels of contaminants while the contaminated site was high in copper (11-fold increase over control), lead (9-fold), chromium (3-fold), zinc (55-fold), arsenic (8-fold) and uranium (45-fold) (Table 2).

While the control showed soil respiration of 44 ± 3 μg CO_2 per hour per gram dry weight with two independent measurements from each of four sampling points at pH 7.0 to 7.5, the contaminated sites showed low soil respiration with highly variable results for the 5 sampling points (33 ± 20 μg CO_2 per hour per gram dry weight with four measurements for each sample) at pH 5.5 to 6.8. This indicates high variability which can be attributed to differences in actual contamination and resulting effects of stress on variability (Fig. 2). While substrate induced soil respiration showed carbon-source limitation at the non-contaminated control site in an agriculturally used area, the soil respiration at the contaminated sites was neither carbon nor nitrogen inducible which points to inhibition of soil respiration at the contaminated sites. Determination of microbial biomass for the non-contaminated control sites was 3 ± 0.9 μg CO_2 per hour per gram dry weight resulting from biological degradation of biomass in the samples. The contaminated site, on the other hand, showed highly divergent abiotic production of CO_2 (with 0.5–4 μg CO_2 per hour per gram dry weight) and lower microbial biomass with 1.1 ± 1.7 μg CO_2 per hour per gram dry weight. In the control soil abiotic CO_2 production was low and inoculation resulted in an 8-fold increase in CO_2 production while contaminated samples showed only an 1.5-fold increase in CO_2 production after inoculation. This again could show the effect of toxicity in the contaminated soil samples.

Numbers of bacterial cells were determined by staining with DAPI. For the non-contaminated control site approximately 6×10^7 cells/g soil were found compared to 5×10^5 cells/g in contaminated soil. Cultivation on different media allowed isolation of bacteria, spore forming bacteria and fungi. From the contaminated samples, a typical isolation protocol would yield 8×10^4 bacteria and 2×10^3 fungi including yeasts and 2×10^2 filamentous fungi while from the control samples 1.3×10^7 colony-forming units (cfu) were obtained. Both numbers are in accordance with approximately 10% of cultured isolates as opposed to direct observation of cells. To obtain isolates single colonies were restreaked on the respective media and analyzed for growth on heavy metal containing plates.

3.3. Growth on seepage waters

In a first experiment the capacity of bacteria and fungi to grow on heavy metal-contaminated media was compared. Since the seepage waters contain a mixture of contaminants, this water was used instead of testing single contaminants which would allow single resistance mechanisms to lead to detection of resistant strains which are then unable to grow with other contaminants present as found in the seepage waters. The strains were supposed to retain heavy metals including uranium and rare earth elements (REE) from the water phase and therefore must be able to cope with a mixture of environmental stress conditions.

The first criterion for selection of isolated strains was their ability to grow on plates containing the seepage waters of the sampling site G16 at half maximal concentrations. The media were buffered such that influence of pH was eliminated from this test and resulting growth was solely dependent on resistance to the

Table 2
Selected contaminant concentrations in soil samples at two locations along Gessen Creek, 500 m before (control site, mean of 5 samples) and after (contaminated samples, mean of 4 samples) passage of waste rock mining heaps

	Al (mg/kg)	As (mg/kg)	Cd (mg/kg)	Co (mg/kg)	Cr (mg/kg)	Cu (mg/kg)	Pb (mg/kg)	U (mg/kg)	Zn (mg/kg)
Control site	38	18	2	50	36	1110	19	20	360
Contaminated samples	460	136	3	44	100	12,310	174	900	19,720

Data represent the concentrations obtained in a mixed sample.

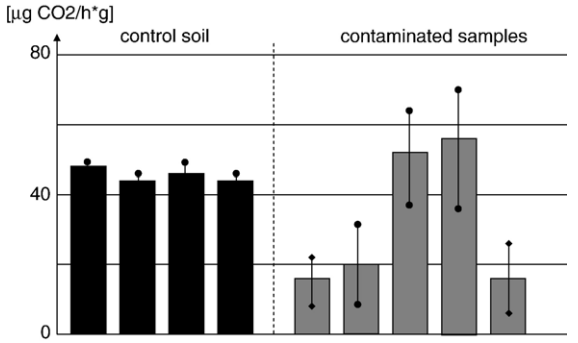

Fig. 2. Soil respiration in 4 non-contaminated control soil samples 500 m upstream and five samples within 50 m around sampling point G16 ($n=8$).

available toxic metals. This resembles natural conditions some 10 m downstream from the sampling site due to mixing with creek water. For this analysis the respective plates were prepared and diluted soil extracts were plated as before. While in plates without seepage waters the control soil samples showed colony-forming numbers of approx. 10^7, the seepage water containing plates gave rise to 3×10^5 cfu. In the contaminated soil samples without seepage water 8×10^4 cfu were obtained while seepage water containing plates gave rise to 1.7×10^4 cfu. This is a good indication for higher resistance of the microbial population at the contaminated site with 21% resistant colony forming units, while the control site showed 3% resistance to half-maximal concentration of seepage water G16.

Selected strains were isolated from the seepage water-containing plates and from these, five bacterial, five actinobacterial and five fungal strains were further analyzed by incubation experiments using mine water. The selected strains were tested for growth in liquid media containing the half-maximal concentrations of G16 seepage waters. Heavy metals in liquid media generally are more toxic as opposed to surface cultivation since the sequestration of heavy metals in the vicinity of a growing colony may permit local growth which is not observed in shaking cultures where the local concentrations cannot be minimized by secretion of chelating substances. After 16 days of growth two actinobacterial strains and two fungal strains did not show growth. The remaining 11 culture media were analyzed for their heavy metal contents.

3.4. Extraction of heavy metals from seepage waters

Reduction of REE in the culture supernatants was tested (Table 3). While the overall contents were only slightly reduced, one fungal strain, F1, and one streptomycete, A3, were seen with the lowest contents of remaining REE in the culture media. The actual degree of retention varied, but in every instance, one of the two strains did show the overall lowest amount of REE left over in the supernatant. The fungal strain F1 was especially interesting since fractionation between different REE could be observed which is seen in the division of light/medium REE (La/Sm) and light/heavy REE (La/

Table 3
REE concentrations in G16 water containing media supernatant (medium) after incubation for 7 days at 28 °C (incubated) with respect to the capacity of 11 selected bacterial and fungal isolates (B1, B2, B3, B4, B5; A1, A2, A3; F1, F2, F3) to extract REE from the medium

	^{139}La (µg/L)	^{140}Ce (µg/L)	^{147}Sm (µg/L)	^{151}Eu (µg/L)	^{160}Gd (µg/L)	^{163}Dy (µg/L)	^{175}Lu (m/L)	La/Sm	La/Lu
Medium	18.0	89	22.9	7.1	40.4	39.3	2.7	0.78	6.4
Incubated	18.4	91	23.2	7.3	41.6	41.3	3.0	0.80	6.1
Bacteria:									
B1	18.8	93	23.8	7.5	42.0	41.2	2.9	0.79	6.3
B2	18.1	90	23.0	7.2	40.6	39.4	2.8	0.79	6.4
B3	18.8	93	23.2	7.3	42.4	41.8	2.8	0.80	6.6
B4	17.9	89	22.3	7.0	40.1	39.1	2.7	0.79	6.4
B5	18.6	92	23.2	7.3	39.7	38.9	2.8	0.80	6.6
Actinobacteria:									
A1	18.8	93	23.5	7.4	41.9	41.0	2.8	0.80	6.5
A2	18.1	90	22.9	7.2	39.5	38.0	2.8	0.79	6.3
A3	**17.7**	**88**	22.2	6.9	**38.3**	37.2	2.7	0.79	6.5
Fungi:									
F1	18.4	89	**21.1**	**6.6**	39.3	**36.8**	**2.4**	**0.87**	**7.4**
F2	18.4	92	23.7	7.4	40.0	39.2	2.9	0.78	6.3
F3	18.1	90	23.0	7.2	39.5	38.8	2.8	0.79	6.4

Lowest remaining concentrations are indicated in bold. The fractionation between REE is indicated by the relative extraction of La *vs.* Sm and La *vs.* Lu, respectively. Measurements are obtained in duplicate; the statistical variance is below 1% of each measurement.

Table 4
Concentrations of metals in G16 water containing media supernatant (medium) after incubation for 7 days at 28 °C (incubated) with respect to the capacity of 11 selected bacterial and fungal isolates (B1, B2, B3, B4, B5, A1, A2, A3, F1, F2, F3) to extract elements from the medium

	^{27}Al (μg/L)	^{53}Cr (μg/L)	^{55}Mn (μg/L)	^{59}Co (μg/L)	^{60}Ni (μg/L)	^{63}Cu (μg/L)	^{66}Zn (μg/L)	^{86}Sr (μg/L)	^{111}Cd (μg/L)	^{208}Pb (μg/L)	^{238}U (μg/L)
Medium	22,770	4.983	35,080	1008	5648	636.8	1497	356.2	20.5	2.255	582.2
Incubated	23,770	5.081	37,100	1100	6041	693.9	1595	346.2	22.25	9.754	612.2
Bacteria:											
B1	24,410	5.376	36,650	1080	5967	625.6	1646	362.3	22.26	4.343	536.7
B2	23,220	4.952	34,810	1033	5701	678	1574	358.1	20.87	5.375	542.3
B3	23,500	5.406	36,340	1080	6002	675.5	1574	370.5	21.44	3.404	496.5
B4	22,960	5.04	34,890	1019	5695	622	1555	358.8	20.46	3.605	564
B5	**18,710**	5.27	37,660	1125	6207	652.9	1524	368.9	22.02	12.22	469.1
Actinobacteria:											
A1	23,780	5.472	36,610	1081	5988	675.7	1588	372.4	22.26	9.04	474.2
A2	22,070	4.918	36,990	1080	5960	688.5	1598	364.4	20.94	4.419	**301.4**
A3	22,940	5.499	36,430	1084	6021	635.1	1683	362.2	20.47	5.348	445.8
Fungi:											
F1	22,360	5.253	36,600	1076	5952	602.2	1520	372.3	21.83	4.593	**319.5**
F2	23,130	5.416	36,400	1081	5958	667.1	1592	366.6	21.63	5.695	539.8
F3	20,960	5.106	35,780	1057	5911	648	1577	356.6	20.62	2.337	548.8

Lowest Al and U concentrations are indicated in bold. Measurements are obtained in duplicate; the statistical variance is below 1% of each measurement.

Lu). Both isolates (F1 and A3) capable to retain lanthanides from the water phase were also active in retaining the actinide U from the supernatant (Table 4). One actinobacterial strain, A2, was even more active in removing uranium from the water phase as compared to strain A3 with 50% removal, while the two remaining fungal isolates could not exceed the capacity shown by fungal isolate F3 which reduced the uranium content by more than 40%. Single-celled bacterial strains showed no significant reduction of uranium but one bacterial isolate was able to remove approx. 20% of the aluminum from the solution (Table 4). In some cases, e.g. for Ce with strains B1, A1 and F2, there is an indication for enhanced biomobilization which can be explained by production of chelating substances.

4. Discussion

The former uranium mining site represents an ideal outdoor laboratory to investigate adaptation of microorganisms to anthropogenic pollution with heavy metals (Merten et al., 2004). The mixed pollution is a challenge for microorganisms that have to cope with multiple pollutants at the same time.

Soil samples close to the sampling point of drainage waters, G16, were analyzed for soil respiration and substrate-induced respiration. For comparison, an agriculturally used site with geogenic and residual contamination in the Gessen Creek Valley was analyzed. Soil respiration at the contaminated site showed a high divergence which may indicate variability of actual contamination, or the induction of variability upon stress that is generally seen in disturbed ecosystems (Stanton et al., 2000). Carbon-source limitation was found at the non-contaminated control site while no nitrogen limitation was detectable. The area is fertilized for agricultural use which explains the lack of nitrogen limitation. However, the lack of substrate inducibility of soil respiration and low microbial biomass at the contaminated sites is indicative of toxic effects in these samples which is easily explained by the heavy metal contents. The lower contents of cells in both staining of samples and cultivation from the samples corroborate these findings even if not directly comparable to the soil respiration data since not all strains are expected to be growing in culture.

From the samples, single-celled Gram-positive and Gram-negative bacteria, filamentous bacteria of the genus *Streptomyces* and filamentous fungi were isolated. While in the control soil samples only 3% of the isolates showed resistance to the (heavy) metal containing media made from seepage water G16, in the contaminated area the number of resistant strains was higher with 21% tolerant strains. From the selected 15 isolates, 11 were able to grow in liquid media containing G16 water. Since growth in liquid media is generally more sensitive to heavy metals, the ability of 73% to withstand these harsh conditions is remarkable. The high level of nickel resistance was previously shown in a strain isolated from a site nearby to be linked to a high

affinity nickel exporter proteins (Amoroso et al., 2000). Other resistance mechanisms are also plausible and are currently under investigation.

We proceeded to test retention of REE from the culture supernatant by growth of the selected 11 microorganisms (see also Merten et al., 2004). One fungal strain (F1) and one streptomycete (A3) could be identified with highest retention potential for REEs.

One indication of active transport in the strains identified as opposed to mere binding to the cell wall is a fractionation of REE, specifically the retention of some and relative refusal of others. Since the rare earth elements are chemically related, such a behavior would hint at active mechanisms that are able to discriminate between different ions. Strain F1, which showed the best retention capacity, showed a relative higher uptake of heavy rare earth elements, which led to the idea that the underlying process is an active transport through the biological membrane coupled to intracellular storage. This can be seen from the quotients of light rare earth element (La) to medium (Sm) or light (La) to high molecular weight rare earth element (Lu) retention. For the fungal strain these values are higher than for the other microbes. We therefore conclude, that in addition to possible binding to the cell wall and changing the physico-chemical parameters during growth, fungal isolate F1 is able to actively take up rare earth elements.

The retention of REE was shown to provide some indication for retention of U as well, but as seen with the Al retaining strain, A2, testing for each of the metals of interest is important so as not to miss possible application. The strains identified here are providing a good starting point for application in the field, since bioremediation using biomass produced in the seepage water-containing creek waters or contaminated soil is feasible. It is certain that the cells did survive and obtain enough energy to allow active transport.

5. Conclusions

It could be shown that the contamination resulting from uranium mining activities for the past 40 years has led to an adapted microflora which in several aspects deviates from a normal soil profile microflora. The effect of pH as well as metal contamination has been shown to lead to stress response with high variation in some parameters like soil respiration. Toxicity to many strains was seen as the most likely reason for inhibition of respiration activity since both C and N source addition did not improve respiration while abiotic CO_2 production was markedly enhanced in the disturbed ecosystem.

From the contaminated environment, strains could be isolated which were then screened for their capability to remove metals like aluminum, which is present in very high amounts in the seepage waters infiltrating soil and surface waters, as well as uranium as an example of toxic heavy metals. This finding can be used for future remediation strategies which are designed to prevent contamination of the water ways, e.g. by placing hyphal mats in contaminated surface waters for metal removal. In addition, remediation of soil on former heap sites could also make use of strains capable of retaining metals. The resulting stimulation of plant growth by trapping metals in biomass reduces the actual level of bioavailable metals (like aluminum or uranium for the strains isolated) at least temporarily. This would be a prerequisite for phytostabilization and future land-use.

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3.5 Biosorption capacity of metal tolerant microbial isolates from a former uranium mining area and their impact on changes in rare earth element patterns in acid mine drainage.

Biosorption capacity of metal tolerant microbial isolates from a former uranium mining area and their impact on changes in rare earth element patterns in acid mine drainage

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Abstract

The concentration of metals in microbial habitats influenced by mining operations can reach enormous concentrations. Worldwide, much emphasis is placed on the research of resistance and biosorptive capacities of microorganisms suitable for bioremediation purposes. Using a collection of isolates from a former uranium mining area in Eastern Thuringia, Germany this study presents three Gram-positive bacterial strains with distinct metal tolerances. These strains were identified as members of the genera *Bacillus*, *Micrococcus* and *Streptomyces*. Acid mine drainage (AMD) originating from the same mining area is characterized by high metal concentrations of a broad range of elements and a very low pH. It was analyzed and used as incubation solution. The sorption of rare earth elements (REE), aluminum, cobalt, copper, manganese, nickel, strontium, and uranium through selected strains was studied during a time course of four weeks. Biosorption was investigated after one hour, one week and four weeks by analyzing the concentrations of metals in supernatant and biomass. Additionally, dead biomass was investigated after four weeks of incubation. The maximum of metal extraction was reached after one week. Up to 80% of both Al and Cu, and more than 60% of U was shown to be removed from the supernatant. High concentrations of metals could be bound to the biomass, as for example 2.2 mg/g U. The strains could survive four weeks of incubation. Distinct and different patterns of rare earth elements of the inoculated and non-inoculated AMD water were observed. Changes in REE patterns hint at different binding types of heavy metals regarding incubation time and metabolic activity of the cells.

Key words: Biosorption, Heavy Metals, Rare earth elements, Acid mine drainage, *Bacillus*, *Streptomyces*, *Micrococcus*

Introduction

Habitats that have developed as a consequence of mining activities are often characterized by extreme environmental conditions such as low pH, sparse nutrients, high metal content and intense salt load. Acid mine drainage (AMD) is the metal-rich effluent formed by chemical reactions between water and rocks containing sulphur-bearing minerals. Microbes adapted to this habitat mediate the chemical reactions (Colmer and Hinkel, 1947). Typically, AMD in a mining area leaks at dispersed locations and contains high concentrations of heavy metals. Different strategies have been devised for

bioremediation of former mining sites that carry metals in concentrations making ex-situ decontamination less attractive for ecological and economical reasons (Lovley and Lloyd, 2000, Lovley, 2003). Often the metal content of the surface-mined land is still high enough to compromise land-use (e.g., agriculture or tourism). Plant extraction of metals is dependent on time required by the plant for adaptation and the supply of nutrients through fertilization. The introduction of microbial populations can support bioremediation under such circumstances (Lucy et al., 2004). Biomobilization can be accomplished by microbes that mobilize metals due to their metabolic activity in combination with appropriate plants which take up higher loads and subsequently can be removed by harvest. The prerequisite for bioremediation strategies is the investigation of the microbial populations of the contaminated site regarding resistance, biosorption capacity, abundance and fitness. Thompson et al. (2005) argue that for application it is not sufficient to have only strains selected with the fitting bioremediation relevant genes, but among other features, survival and competition in the habitat have to meet the demands.

However, microorganisms can also be applied in a remediation strategy alone. The application of microorganisms is characterized by the advantage of a high surface to volume ratio and a negative surface charge of the cell wall as reactive matrix. In many environments microbial cells are able to bind large amounts of heavy metal cations (Lovley and Coates, 1997). The autochthonous microbial flora or artificially introduced strains can be used to minimize the discharge of metals into the water path. Metals are bound at or inside the microbial cell, thus temporarily locking them and reducing their bioavailability. Consequently, the metal uptake into the crop is (at least partially) prevented.

Extreme habitats, such as those influenced by mining activities, can be colonized only by microbes adequately adapted. Conditions of the habitat, and subsequently the nutritional state of the microbes, strongly influence the toxic effects of metals on bacteria. The selection pressure within the harsh habitat leads to either adaptation of the autochthonous microbial population, or invasion and enforcement of a zymogenous population that can occupy this niche (Thompson et al., 2005). Bacterial adaptation requires a high genetic flexibility including gene amplification as reviewed by Romero and Palacios (1997). A number of metal resistant bacteria has been isolated from various naturally occurring and anthropogenically created extreme habitats such as a metal decantation tank of a zinc factory in Liege, Belgium (Schmidt et al., 1991), material of waste heaps from a former uranium mining area (Amoroso et al., 2000), and serpentinite derived soils in New Caledonia (Park et al 2003) and Tuscany (Mengoni et al., 2001).

For remediaton purposes, however, it is very important to know more about the mechanisms of interactions between microbes and metals in order to differentiate between the processes of temporary sorption and a more sustainable binding by uptake into the biomass. In this publication, the biosorption capacities of metal tolerant isolates selected from a strain collection derived from a former uranium mining area in Eastern Thuringia, Germany were analyzed and compared. The focus is on time dependent changes of rare earth element (REE) patterns of AMD during incubation indicating

processes such as biosorption, bioprecipitation, and bioaccumulation. REE (La-Lu) show smooth but continuous variations in chemical behaviour as a function of their atomic number. When normalising the concentration of REE and other metals in the solution before and after incubation, this can be taken as a value for recovery. Additionally, the fractionation of REE can be studied. Fractionation of REE can be used to investigate processes such as sorption/uptake of metals by microbes (Andres et al. 2000, Andrès et al. 2003, Aruguete et al. 1998; Brantley et al. 2001, Merten et al. 2004, 2005; Philip et al. 2000) and uptake of metals by terrestrial (Tyler and Olsson 2001, Ozaki and Enamoto 2001) and aquatic plants (Weltje et al., 2002) since they affect the single REE in a distinct way. Binding of REE is affected by their physicochemical characteristics, especially by the ionic radius and thus leading to fractionation in bioremediation processes.

Material and methods

1. Sampling of AMD and hydrochemical analyses

AMD was taken from the former Gessenhalde leaching heap drainage site located in the former uranium mining area, Eastern Thuringia, Germany, between the communities of Kauern and Ronneburg (see Fig. 1). About 4 L of AMD were sampled on 14-06 2002. pH, redox potential, electrical conductivity, and temperature of the unfiltered samples were measured directly in the field (WTW, Weilheim, Germany). The samples were sterile filtered (0.2 µm cellulose acetate filter, Sartorius, Göttingen, Germany) and an aliquot of 50 mL was acidified to pH $\leq$ 2 with HNO_3 (subboiled quality) for analysis of cations. The pH of an aliquot of 200 mL of the AMD was adjusted from pH 2.8 to 6 by addition of NaOH. All solutions were analyzed hydrochemically using inductively coupled plasma mass spectrometry (ICP-MS, PQ3-S, Thermo Elemental, Winsford, UK). The analyses included Al, Co, Cu, Mn, Ni, Sr, U, and REE. The sterile filtered AMD was analyzed for its dissolved organic carbon (DOC) concentration (DIMATOC, Dimatec, Germany) and for its concentrations of Cl^- and SO_4^{2-} by ion chromatography (DX 600, Dionex, Idstein, Germany).

2. Isolation of strains

22 soil samples (5 g each) from the upper 5 cm were collected at a former uranium mining site in Eastern Thuringia, Germany (Fig.1). The sampling locations included (former) dumps, drainage trenchs, creek sediments, sites affected by secondary mineral precipitations and seeping sites and thus reflect the diversity of contamination within the investigation area. The soil samples were air dried, ground, resuspended in 0.9% NaCl-solution and agitated for 1h. 100 µL aliquots of 10^{-3} to 10^{-5} dilutions were plated on soil extract agar (Thiemann et al., 1968). After 5 days of incubation at 28°C, precultures were obtained on minimal medium (0.5 g/L asparagine, 0.5 g/L K_2HPO_4, 0.2 g/L $MgSO_4$, 0.01 g/L $FeSO_4$, 10 g/L glucose, 15 g/L agar; Amoroso et al. 2000) or casein yeast medium for fungi (2 g/L trypticase pepton, 2 g/L yeast extract, 20 g/L glucose, 0.5 g/L $MgSO_4 \times 7\ H_2O$, 0.5 g/L KH_2PO_4, 1.0 g/L K_2HPO_4, 18 g/L agar; Schwalb and Miles 1967).

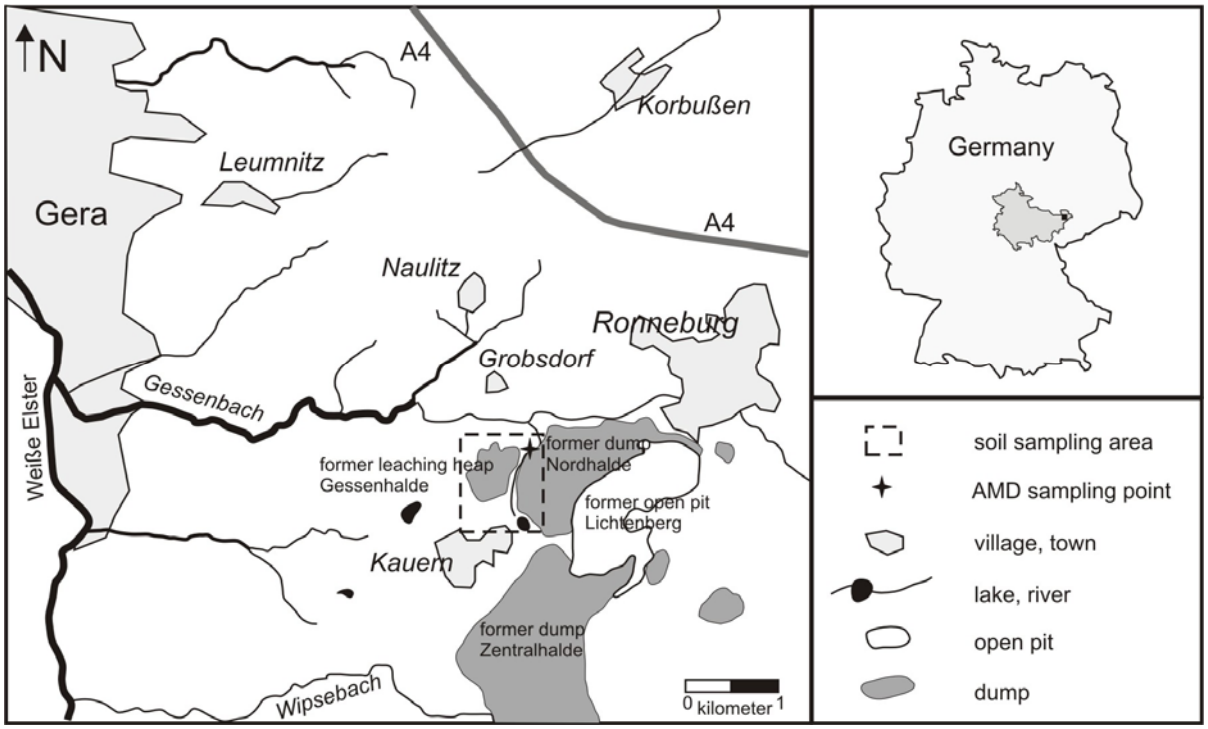

Fig. 1: Former uranium mining area Ronneburg, Germany with sampling points for AMD and soil samples

3. Screening of a strain collection for biosorptive isolates

40 isolated strains were tested in minimal medium containing AMD diluted by half with a pH adjusted to 6. Two of the 40 strains were not able to grow in AMD solid medium. The remaining 38 strains were cultured in 50 mL shaking flasks. 50 µL of a preculture grown in minimal medium overnight at 28°C without AMD were used as inoculum. The cultures were incubated 7 d at 28°C in half-concentrated AMD at pH 6. Removal of metals from supernatant was determined by ICP-MS and compared to the non-inoculated sample. Based on the results of the screening, three strains displaying both high potential of biosorption in AMD, and good growth were selected for further investigations.

4. Phenotypical characterization and taxonomy of selected isolates

For taxonomic grouping, the three strains selected from the screening have been classified on the genus level. Genomic DNA was isolated following the CTAB method (Kieser et al., 2000) and used for PCR with 16S rDNA specific primers (TPU1 AGAGTTTGATCMTGGCTCAG and RTU3 GWATTACCGCGGCKGCTG, Choi et al., 1994). The amplified 500 bp fragments were cloned and sequenced (JenaGen, Jena, Germany). Blast analyses were performed to identify similarities to database entries (NCBI).

5. Test on salt and metal tolerance

Minimal medium was supplemented with 0.5, 1, 5, 7.5, 10 or 15 % (w/v) NaCl or K_2SO_4 in solid and liquid medium. The metals Al ($AlCl_3$ x 6 H_2O), Co ($CoCl_2$ x 6 H_2O), Cu ($CuSO_4$ x 5 H_2O), La ($La(NO_3)_3$ x 6 H_2O), Mn ($MnCl_2$ x 4 H_2O), Ni ($NiCl_2$ x 6 H_2O), Sr ($SrCl_2$ x 6 H_2O) and U ($UO_2(NO_3)_2$ x 6 H_2O) that occur in this AMD in elevated concentrations (Merten et al., 2005) were tested separately in solid minimal medium at pH 4.5. Sterile filtered metal solution was added after autoclaving and before solidification. The final concentration of each metal in the plates was 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 mM. For La and U concentrations 0.02 and 0.05 mM were tested additionally. Growth after incubation for 7 days at 28°C was compared to growth on non-supplemented media.

6. Biosorption assay

Three selected strains were grown in Standard I (Merck, Darmstadt, Germany) to late logarithmic growth phase, separated from the nutrient solution and washed 3 times in sterile, distilled water. The wet weight of the inocula was determined. The cell pellet of each strain was resuspended in 50 mL 0.9% NaCl solution and inoculated in homogeneous 5 mL cell suspension aliquots in 100 mL sterile, undiluted AMD at pH of 2.8 (two replicates each). 500 mL Erlenmayer flasks were used for incubation experiments. Samples were incubated for 1 hour, 1 week and 4 weeks separately. Additionally, autoclaved biomass was used for four weeks of incubation. After incubation, the samples were centrifuged at 4000 rpm and 4°C (Beckman, Palo Alto, USA). The supernatant was filtered through a 0.2 µm sterile filter (Sartorius, Göttingen, Germany). Redox potential, pH (pH 320, WTW, Germany) as well as metal concentrations were measured. Independently, an aliquot was sampled from the liquid for the test for survival by plating 100 µL on Standard I.

The cell pellets were washed one time in 20 mL deionized water and then again in 20 mL 0.1 M EDTA pH 8.0. After washing, the cell pellets were dried at 80°C until weight-constancy, weighted and subsequently dissolved in a microwave assisted pressure digestion system (Mars 5, CEM, Germany). Supernatant, biomass, and EDTA rinse solutions were analyzed for metal content by ICP-MS, whereas the solution obtained after rinsing with deionized water was discarded.

Results

1. Hydrochemical analysis of AMD

The pH of the collected AMD was 2.8 and a redox potential of 560 mV was measured in the field. Electrical conductivity was 11.600 µS/cm. The AMD contained 11.5 g/L SO_4^{2-} and 0.3 g/L Cl^-. The main cations are Al and Mn, both with 230 mg/L, Ni concentration was 41.4 mg/L. Co (10 mg/L), Cu (6.5 mg/L), U (5 mg/L), and Sr (0.4 mg/L) were measured at elevated concentrations, total REE content was about 3.2 mg/L and DOC was 2.0 mg/L.

2. Strain isolation on half-concentrated AMD

On media containing half-concentrated AMD at a pH of 6.0, 40 strains could be isolated from 22 soil samples collected within a former uranium mining area. Aside from 3 fungal isolates, 19 actinobacteria and 18 single-celled bacterial isolates were obtained.

3. Screening of a strain collection for biosorptive isolates

38 out of the 40 isolates were able to grow in plate and shaking flask cultures containing minimal medium supplemented with half-concentrated AMD at a pH of 6. Strains W-12, W-20 and W-28 showed high abilities to reduce the metal concentration in the supernatant. Strain W-12 was able to reduce the concentrations of U by 66%, Al, Mn, Co, Ni, Cu each by 50% and Sr by 25%. Strain W-20 removed the concentrations of Al and Cu by 80%, and U by 45%. Strain W-28 was able to reduce the concentrations of U by 45%, Cu by 40%, Ni by 20% and Co by 15%.

4. Description of strains selected for biosorption assay

Strain W-12 shows the typical growth characteristics of streptomycetes with mycelial colony formation. It has been described as *Streptomyces acidiscabies* and was denoted E13 (Amoroso et al. 2000). Strain W-20 is a coccoid, tetrad-forming bacterium with a capsule if grown on medium Standard I, visualized after staining with Chinese ink. *Blast* search revealed that the sequenced 467 bp amplicon of the 16 S rDNA is identical with *Micrococcus luteus*, strain Ballarat. Strain W-28 is a long, rod shaped bacillus, forming cell chains and endospores. After *Blast* search, the 498 bp amplicon matched with the genus *Bacillus*.

5. Salt and metal tolerance

The three strains could grow in the presence of 1% NaCl in liquid and solid culture (minimal medium), but none of them tolerated 5% NaCl. Good growth has been observed in plate culture for strains *S. acidiscabies* W-12 and *Bacillus spec.* W-28 in the presence of 15% K_2SO_4, whereas strain *M. luteus* W-20 grew well only up to 5% K_2SO_4. In liquid culture, *M. luteus* W-20 and *Bacillus spec.* W-28 were able to grow in the presence of 15% K_2SO_4. Growth of strain *S. acidiscabies* W-12 could be observed up to 7.5 %. Strain *M. luteus* W-20 was not able to grow in the presence of any of the tested metals at a concentration of 0.1 mM (Tab.1). *Bacillus spec.* W-28 could not grow in the presence of Cu, La, Ni or U at concentrations higher than 0.1 mM, and tolerated concentrations of 1 mM of Al and Co and 20 mM Mn and Sr (higher concentrations were not tested). In the presence of Al and Co, the formation of a brownish pigment, which did not occur in the absence of metal supplementation, has been observed for strain *Bacillus spec.* W-28. *S. acidiscabies* W-12 could not tolerate U in concentration higher than 0.05 mM, but could grow in presence of La and Cu until 0.2 mM. This strain was able to tolerate Al up to a concentration of 1 mM, Co 2 mM, Ni 5 mM and Mn and Sr at least up to 20 mM.

Table1: Maximum of tolerated metal concentration and concentration of metals in AMD [mmol/L]

Strain	Al	Co	Cu	La	Mn	Ni	Sr	U
S. acidiscabies W-12	1.0	2.0	0.2	0.2	20	5	20	0.05
M. luteus W-20	< 0.1	< 0.1	< 0.1	0.05	< 0.1	< 0.1	< 0.1	0.02
Bacillus spec. W-28	1.0	1.0	< 0.1	0.05	20	0.5	20	0.05
AMD	8.5	0.17	0.1	0.001	4.2	0.7	0.005	0.02

6. Biosorption

Wet weight of inocula was determined to be 1.117 g for strain W-12, 0.841 g for strain W-20 and 0.818 g for strain W-28. As regards physicochemistry of the supernatant for all three investigated strains the same trend is observed. Starting with a pH of 2.8, only slight variations were observed during incubation. After one hour of incubation, pH is virtually the same as prior to incubation (ranging from 2.8 for *M. luteus* W-20 to 2.9 for *Bacillus spec.* W-28 and *S. acidiscabies* W-12). Reproducibility of pH as derived from the two replicates is excellent. Deviation calculated as the absolute standard deviation is 0.08 pH units at the most. After one week of incubation, pH is slightly higher ranging between 2.9 and 3.0, again showing high reproducibility between replicates. After four weeks of incubation the trend of slightly increased pH is confirmed; pH ranged from 2.9 to 3.1. For four weeks of incubation of *Bacillus spec.* W-28 one of the replicates showed a higher pH of 3.4.

Redox potential was measured to be 560 mV before incubation and therefore indicated oxidizing conditions. Redox potential shows a decrease with incubation time. After one hour of incubation, redox potential has decreased to 450 - 480 mV. After one week of incubation redox potential decreased to 420 - 450 mV. Finally, after four weeks of incubation a redox potential of 380 to 420 mV was observed. In all measurements, slightly higher values of redox potential were observed for *S. acidiscabies* W-12 and the lowest for *Bacillus spec.* W-28. For all three strains and all incubation times, investigated redox potential between replicates for same experimental conditions differed by less than 20 mV.

Survival of the three investigated strains in AMD could be shown for over four weeks since all strains could be regrown. Fig. 2 shows the recovery (ratio of concentration in the supernatant after incubation to the initial content) of Al, Mn, Co, Ni, Cu, Sr and U from the supernatant after incubation with the investigated strains for one hour, one week and four weeks respectively. Error bars indicate the deviations in concentration for two replicates under same experimental conditions.

For all three strains the same trend is observed. As regards the influence of incubation time it can be seen that the recovery of the investigated elements is highest for one hour and four week incubation

with living biomass. Typically, the recovery of the investigated elements is in the range of 40-60% with the exception of U and Sr. U generally shows lower recovery of only 20-30%, whereas Sr shows highest recovery of 70-90%. Except for Sr, recovery is lowest and ranges between 10 and 30% for one week incubation. Sr is showing similar recovery in supernatant for all incubation times using living biomass. Incubation for four weeks with dead biomass leads to concentrations in the supernatant that are lower compared to those after four weeks incubation with living cells.

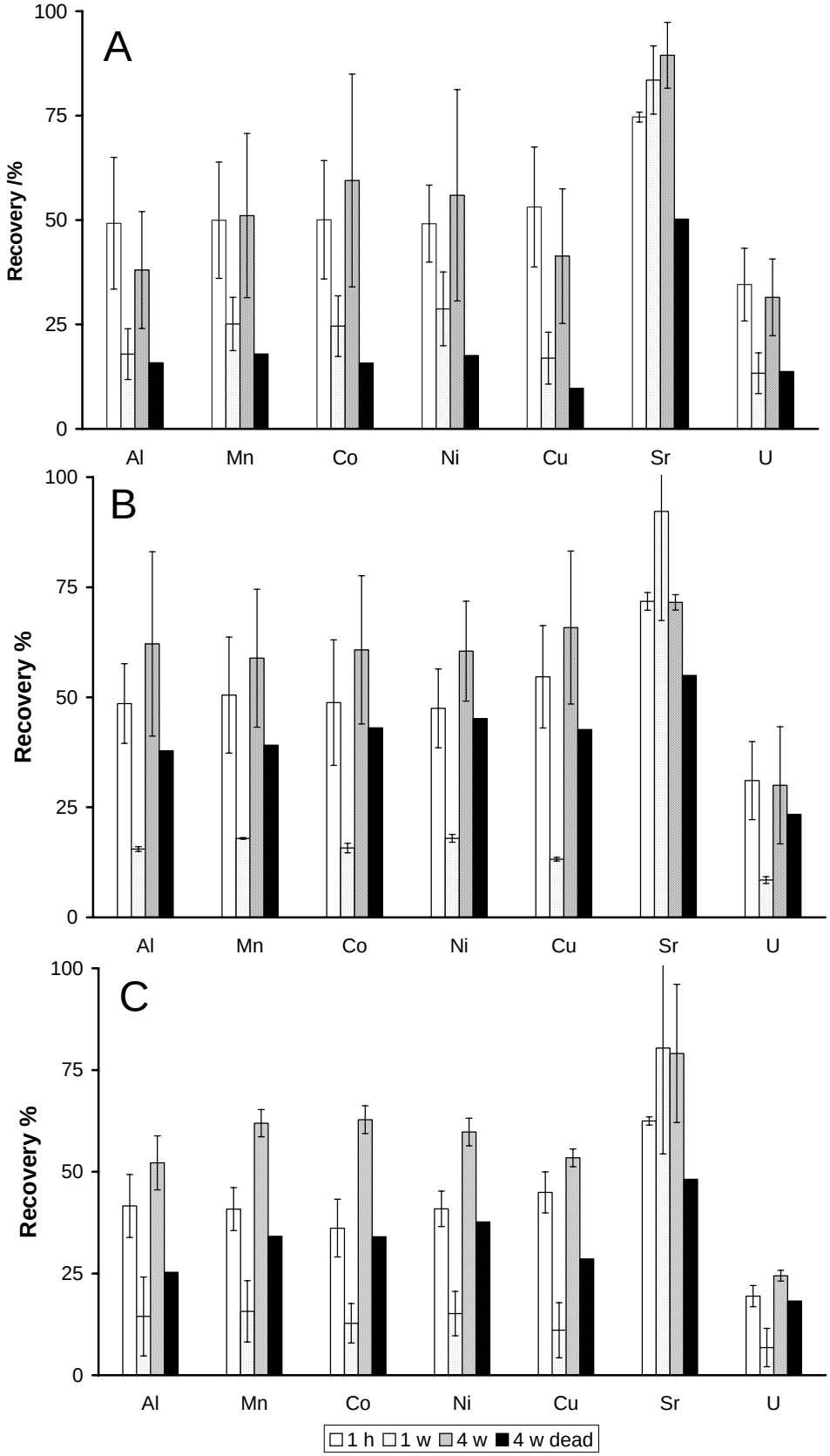

Fig. 2: Recovery of Al, Mn, Co, Ni, Cu, Sr and U from supernatant after incubation of *S. acidiscabies* W-12 (A), *M. luteus* W-20 (B) and *Bacillus spec.* W-28 (C) in AMD for 1 hour, 1 week and 4 weeks with living and for 4 weeks with dead biomass. Error bars represent absolute standard deviation of two replicates for the case of incubation with living biomass.

For all three investigated strains the recovery of REE from aqueous solution is similar and ranges from 40 to 60 % for the sum of all REEs (Tab. 2). However, recovery is significantly different for individual REEs. The recovery was highest for Ce and Pr (40-80%) and lowest for the heavy REE. Thus, strong fractionation among REE was observed (Fig. 3). For all strains recovery of REE is comparable to the other investigated metals. Recovery increases from one hour incubation to one week incubation and then strongly decreases between one week and four week incubation. After four weeks of incubation the concentrations of REE in the aqueous solution are higher than after one hour. After four weeks recovery of elements from solutions is lower when dead biomass was used. Although total concentrations of REE differ significantly even for replicates, the patterns of fractionation are identical for the same experimental conditions. For all three strains REE patterns are comparable for incubation times of one hour and four weeks using both living and autoclaved biomass. For one week incubation, however the REE patterns look significantly different.

Tab. 2: Concentration of REE in the supernatant, REE recovery of *S. acidiscabies* W-12, *M. luteus* W-20, *Bacillus spec.* W-28 after incubation in AMD for one hour (1 h), one week (1 w) and four weeks (4 w); Autoclaved culture measured after four weeks (dead), Standard deviation (SD) was derived from two parallels; Sum REE: Total concentration of rare earth elements.

Sample	Sum REE [µg/L]	SD	Recovery %
AMD	3154		
W-12 (1 h)	1809	88	57
W-12 (1 w)	1713	43	54
W-12 (4 w)	1997	267	63
W-12 (4 w dead)	1295		41
W-20 (1 h)	1794	30	57
W-20 (1 w)	1606	213	51
W-20 (4 w)	1880	239	60
W-20 (4 w dead)	1409		45
W-28 (1 h)	1637	78	52
W-28 (1 w)	1477	379	47
W-28 (4 w)	2041	344	65
W-28 (4 w dead)	1194		38

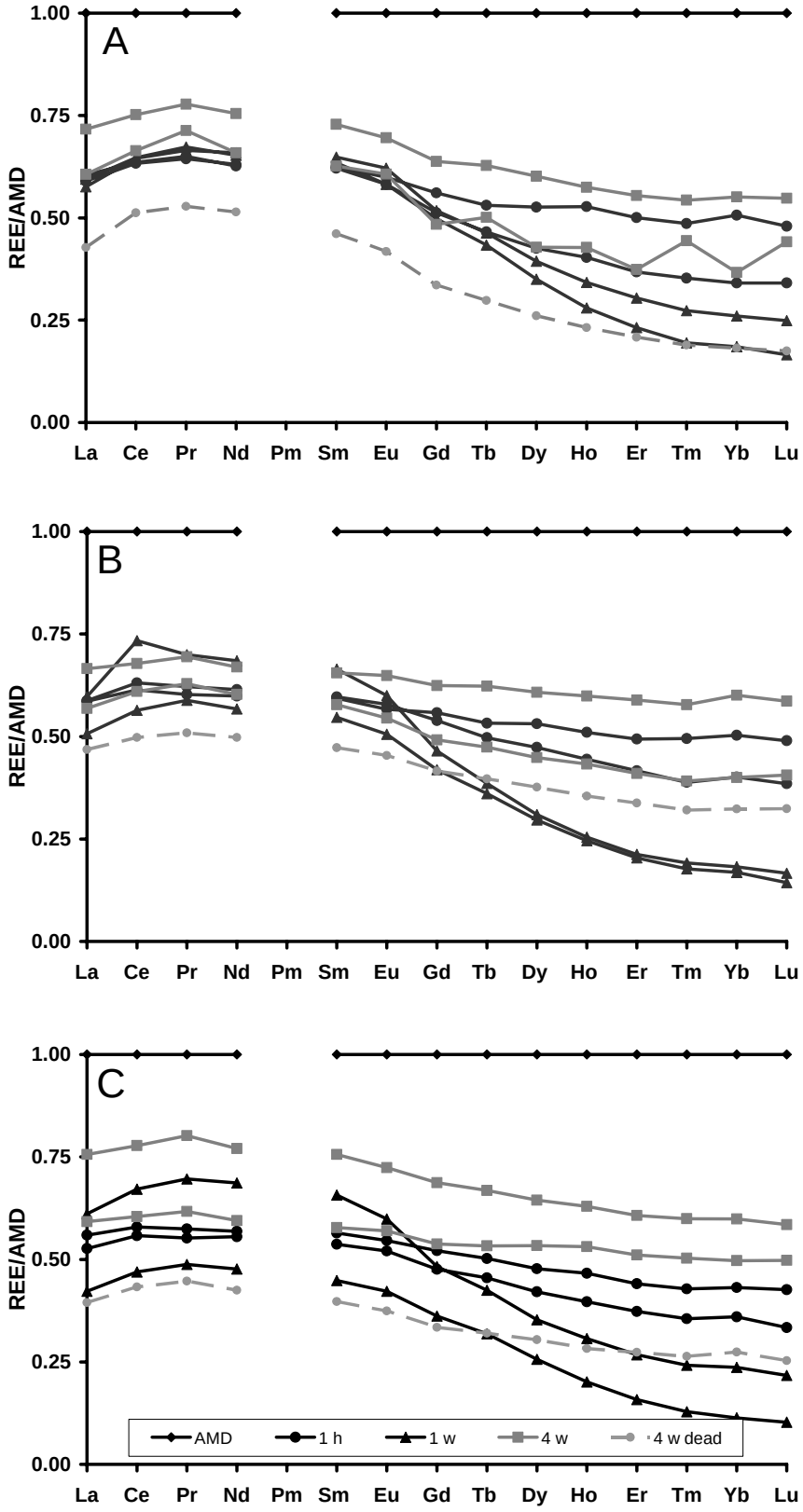

Fig. 3: Concentrations of REEs after incubation of AMD for one hour, one week, and four weeks, respectively with *S. acidiscabies* W-12 (A), *M. luteus* W-20 (B) and *Bacillus spec.* W-28 (C) after normalisation to the corresponding concentrations in AMD without incubation.

Desorption of metals from biomass by 0.1 M EDTA solution (results not shown) displays similar characteristics as for the remaining biomass. High concentrations associated with the biomass were obtained for Al (up to 30 mg/g), Mn (up to 0.5 mg/g) and U (up to 2.2 mg/g). For Sr the lowest concentrations attached to the biomass were measured (about 2 µg/g). The investigated strains did not display strong differences in the accumulation of single metals (Fig. 4). For U and especially Al it was observed that the highest accumulation was found for four week incubation with dead biomass. For strain W-12 *S. acidiscabies* it was observed that for all investigated elements except for Sr and U the concentration of metals associated with the biomass is lowest for the one week incubation experiments. For strains W-20 and W-28, most of the elements display similar sorption after one hour and one week. This is also true when comparing sorption after one week and four weeks using living biomass except for Mn and Cu. For the latter elements significantly higher concentrations bound to the biomass were obtained after four weeks of incubation.

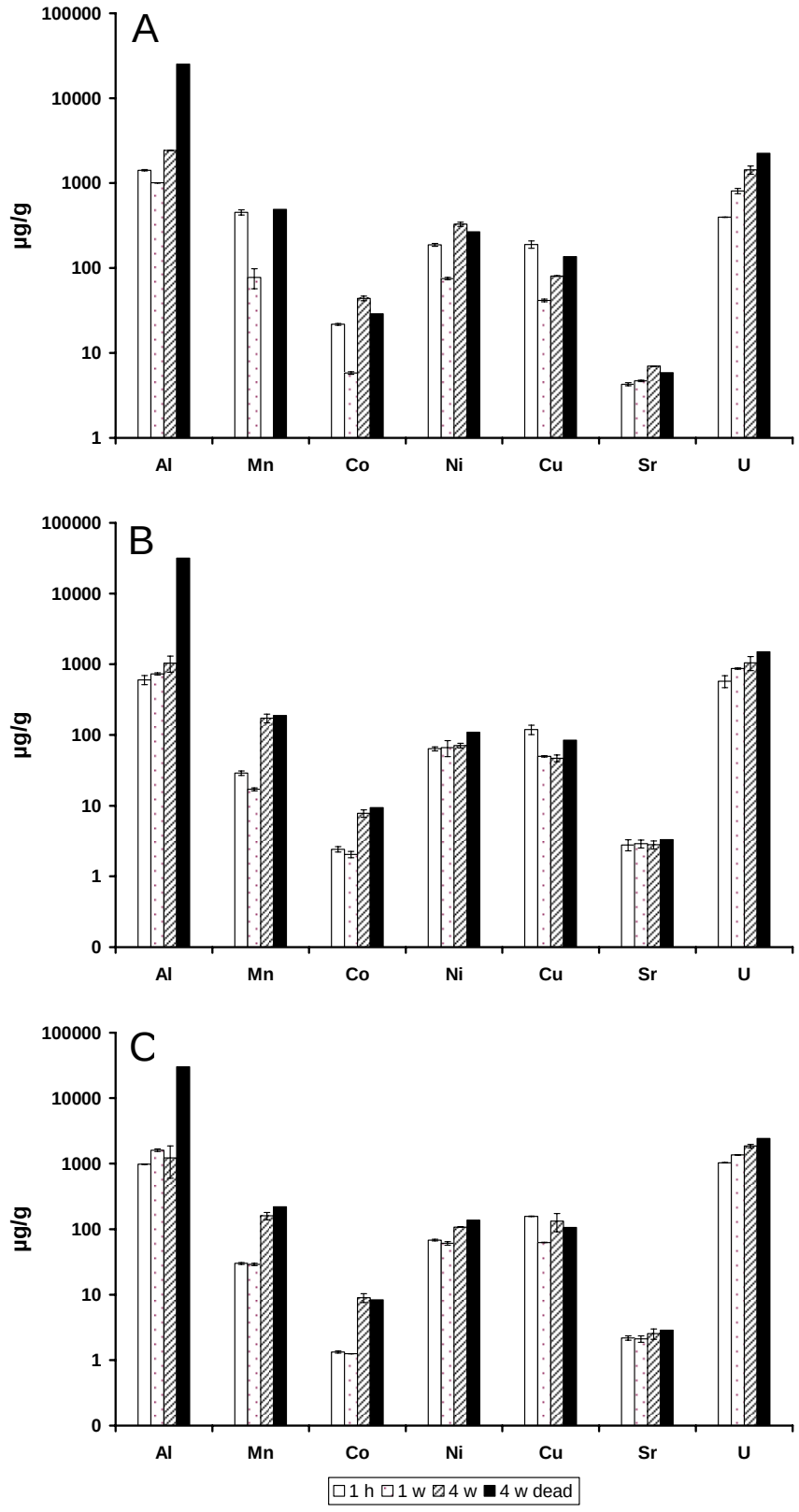

Fig. 4: Concentration of Al, Mn, Co, Ni, Cu, Sr and U in in µg/g dry weight biomass of *S. acidiscabies* W-12 (A), *M. luteus* W-20 (B) and *Bacillus spec.* W-28 (C) after incubation in AMD for one hour, one week and four weeks with living or dead biomass, respectively. Error bars represent absolute standard deviation of two replicates.

All investigated strains display the same sorption characteristics for REE (Fig. 5). In any case concentrations of REE bound to biomass are lowest for one hour and one week incubation and highest for four week incubation with dead biomass. For investigation of REE fractionation the concentrations obtained for the REE in digested biomass are normalized to the concentrations in the water used for incubation experiments. For incubation with living biomass the REE patterns are flat from La to Ho and show a slight to steep increase from Ho to Lu. The increase in concentration of heavy REE bound to biomass is most pronounced for *Micrococcus luteus* (W-20) and *Bacillus spec.* (W-28) and here especially for the four week incubation. For strain *S. acidiscabies* (W-12) only minor fractionation is observed. For incubation with dead biomass, however concave REE patterns were obtained (Fig. 5). Additionally to an enrichment of heavy REE also an enrichment of light REE was observed. This is again most pronounced for *Micrococcus luteus* (W-20) and *Bacillus spec.* (W-28). For the EDTA rinse solutions, however also for incubation with dead biomass rather flat REE patterns from La to Ho were obtained (results not shown).

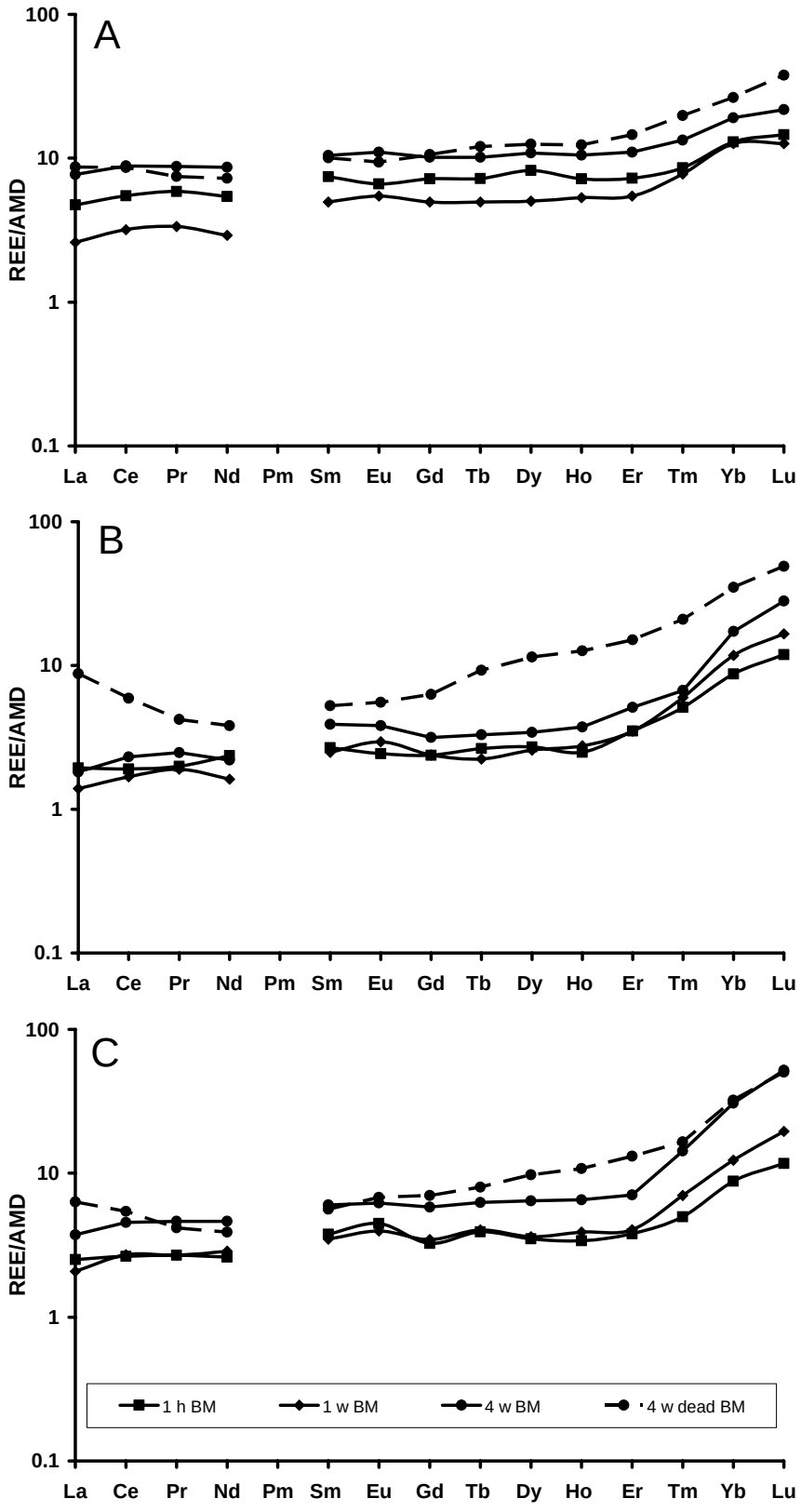

Fig. 5: Concentrations of REEs in biomass (µg/g dry weight) after one hour, one week and four weeks incubation of *S. acidiscabies* W-12 (A), *M. luteus* W-20 (B), *Bacillus spec.* W-28 (C) with living and dead biomass respectively, after normalization to the corresponding concentrations in AMD without incubation.

Discussion

The water is oxidizing and characterized by the very low pH of 2.8 as well as high metal load which is typical for AMD (Johnson and Hallberg, 2005). The very low concentration of DOC (2 mg/L) found in the investigated water is insufficient to serve as adequate carbon source for the investigated bacteria. Under these unfavourable conditions no growth is expected. However, in the vicinity of drainage sites, pH rises quickly to near-neutral conditions, and metals are diluted and also partly (co)precipitated while nutrients are present in the soil habitat.

A screeening of metal tolerant strains from former mining areas can be used to study the influence of mining operations on the microbial flora of the soil. Sampling of microbes during different stages of remediation and varying sampling locations with different contamination patterns yielded in a strain collection of 40 strains.

The three selected strains are gram-positive, soil dwelling bacteria. Bacilli and streptomycetes occur in soil habitats almost exclusively, whereas micrococci additionally colonize several non-soil habitats. Members of these genera are easy to isolate due to their high abundance in soil. In contrast to the oligotrophy of *Streptomyces* and *Bacillus*, *Micrococcus* is considered rather copiotrophic. Each of the three groups is known and well studied for their behaviour under unfavourable environmental conditions (e.g. heat, dryness, metal burden) (Kalakoutskii and Agre, 1976, El-Helow et al., 2000, Kaprelyants et al., 1993).

Test on halotolerance is usually performed in NaCl containing media. With respect to NaCl, the investigated strains are neither halophilic, since their growth does not depend on a certain concentration of NaCl, nor are they noticeably halotolerant. But since sulphate salts are the osmotically most influential compounds in the investigated water the tests were expanded using K_2SO_4. Tolerance against sulphate is much higher than that against chloride. The higher sulphate versus NaCl tolerance corresponded to the higher sulphate content (11.5 g/L) compared to chloride load (0.3 g/L) of the AMD collected on the field site. The strains *S. acidiscabies* W-12 and *Bacillus spec.* W-28 could tolerate higher concentrations of K_2SO_4 (15%) than strain *M. luteus* W-20 (5%). The remarkable tolerance against sulphate could be based on the synthesis of sulphur containing metabolites or the influx of a compatible solute. Typically, glycine or betaine is enriched in the cytosol of Gram-positive bacteria in order to resist dehydration caused by high extracellular salt content. But since no growth on NaCl containing medium has been observed, another mode of action seems likely. Sulphate itself probably could act as internalized compatible solute with no seriously deleterious effect on cytosolic constituents.

Of the three investigated strains, *M. luteus* W-20 shows the lowest tolerance against metals, whereas *S. acidiscabies* W-12 displays the highest. The concentrations of all elements except for Al in AMD are lower than those found to be tolerated in the single metal solution tests for strains *S. acidiscabies* and *Bacillus spec.* For all investigated strains U, Cu and La possess the highest toxicity. U is both chemo- and radiotoxic to biota and causes, amongst others, DNA damage (Brugge et al., 2005). Copper is

known to belong to the group of metals that expose high toxicity towards many bacterial taxa, although essential as cofactor in tiny amounts (Ruggiero and Neu, 2005). The toxicity of REE to biota was investigated early (Muroma, 1958). With the growing use of REE in fertilizers (Volokh et al. 1990, Yuan et al. 2001) especially in China the interest on the toxicity of REE increased (Weltje et al. 2004). In most cases, a stimulating effect of low doses of REE was observed, whereas at higher concentrations REE were found to be toxic. Al, Co and Ni show intermediate toxicity, whereas the toxicity of Mn and Sr is low, except for *M. luteus* W-20 showing increased Mn sensitivity.

It is remarkable that all three strains could survive four weeks of incubation in AMD. Vegetative cells most likely would have been damaged irreversibly at pH 2.8 and the high concentration of metals during the four weeks of treatment.

It is assumed that survival of the strains was feasible by formation of dormant cells. Streptomyces forms exospores which are commonly present in soil habitats rather than vegetative stages. *Bacillus* is able to form endospores. Endospores of *Bacillus* spec. were microscopically observed in aliquots of the incubated AMD for all investigated times. *Micrococcus* can persist in a dormant state, which is characterized by cells which are viable, but not culturable (Kaprelyants and Kell, 1993). Strain *M. luteus* W-20 could survive the four weeks of incubation in water although displaying only weak tolerance towards metals in the plate test. It seems very likely that the water containing a cocktail of stressors caused the induction of a „survival metabolism“. The response to a certain metal probably shielded the strain from being intoxicated by others, which were not tolerated in the single metal tests. The mechanism of resuscitation from dormancy is well investigated for *Micrococcus* strains (Kaprelyants and Kell, 1993; Mukamolova et al., 1998). This characteristic capacity found in the genus *Micrococcus* might explain why *M. luteus* W-20 was able to survive and regain growth on non-toxic media after the AMD treatment.

Cell envelopes of microorganisms typically exhibit negative charges (Camesano and Logan 2000) and are therefore able to sequester cationic metals. The biosorption capacity of bacteria depends on a number of features concerning the cell architecture, chemical composition of cell wall and adjacent extracellular layers, and compounds like the extracellular polysaccharides of streptomycetes or the capsular structures of micrococci. The main functional groups contributing to the charges on bacteria are phosphate moieties, either in phosphodiester bridges as in teichoic acids or at the end of a polymer as in phospholipids, protein or peptidoglycan-associated carboxylic groups, polysaccharide-associated carboxylic groups, protonated phosphates, and peptidoglycan or protein-associated ammonium (Camesano and Logan 2000, Conn et al., 1987).

At low pH, the binding sites are mostly protonated and ion exchange is limited. The sorption of cations to biomass should lead to a desorption of H^+ from the sorptive matrix therefore resulting in a decrease of pH. Despite the low pH, the strains displayed a strong capability for the extraction of cations from the solution. Precipitation of hydroxides of Al, Mn, Fe, Ni etc. also should lead to a decrease in pH. The slightly increasing pH observed during incubation contradicts these assumptions

but could be due to cell lysis resulting in the release of neutral cytosolic constituents. The redox potential decreases with incubation time probably due to consumption of oyxgen during growth. It is interesting to note that the rather small deviations of both redox potential and pH between replicates for same experimental conditions cannot explain the rather strong deviations between element concentrations in the supernatant solutions after incubation. Thus, it seems very probable that the large differences in concentrations of metals are predominant due to biological and biochemical processes and not due to inorganic processes alone. Although the concentrations of the single REEs differ significantly between replicates, the REE patterns are quite similar for the same experimental conditions making the use of REE patterns advantageous to study binding and retention characteristics.

All three strains show similar biosorption behaviour regarding both the temporal course and the relative amount of recovered elements from the supernatant independent of the tolerance characteristics. Already after one hour of incubation high extraction of metals was observed as reflected by a recovery of about 50% of the supernatant. An adsorption time of only 15 minutes for reaching the adsorption equilibrium of copper and nickel has been reported for *Streptomyces coelicolor* A(3)2 (Öztürk et al., 2004). The fast sorption of uranium from solution has been observed for cells of *Streptomyces levoris* (Tsuruta, 2004).

The most interesting result of the biosorption experiment is the observation that extraction from the supernatant increases strongly between one hour and one week, and then reverses. After four weeks the extraction of elements is almost the same as after one hour showing no significant differences for the investigated strains. This shows that the process of sorption is at least partially reversible. The REE fractionation observed for all investigated strains after one week of incubation clearly hints at differences in binding characteristics for one week incubation compared to other incubation times using both living and dead biomass. It is assumed that passive sorption to cell walls occurs within the first hour of incubation. Later on, active uptake into cells or restructuring of cell walls in response to metal exposure is supposed to be the reason for higher extraction from the supernatant. With increasing incubation time, lysis of cells becomes more prominent and metals are released into the solution thus reverting the REE pattern to the one also observed with dead biomass incubation. It is supposed that several processes such as passive adsorption, active changing of physico-chemical conditions as well as metal uptake and chelation, e.g., by extracelluar polysaccharides occur simultaneously. While some of the vegetative cells are lysing others survive by dormancy.

From the REE patterns of the biomass after incubation it can be concluded that different Gram-positive bacteria show similar sorption characteristics pointing at similar chemical structures responsible for binding of metals. This is understandable since all investigated strains are Gram-positive bacteria. When using 0.1 M EDTA rinse solutions, mobilization of less strongly bound metals from the biomass is possible. Differences in REE patterns for four week incubation with dead cells using either EDTA or the biomass after washing hint at different binding characteristics. Dead

biomass shows distinct REE patterns compared to living biomass whereas for EDTA rinse solutions the patterns are comparable.

Although Al and Mn are contained in concentrations being about 100-fold higher than those of other metals like U, binding to biomass is comparable (about 1 mg/g). Thus, sorption behaviour does not depend on the absolute amount of metals that are present. Uranium generally shows higher biosorption which can be explained by the high positive charge (+VI) of the cation. The high biosorption could not be easily explained in case of the uranium speciation as UO_2^{2+} which is often found in aqueous systems. The speciation of the uranium in the AMD remains to be analyzed.

The fact that for strain *S. acidiscabies* W-12 the lowest accumulation of metals (except for Sr and U) was found for one week incubation it can be concluded that at this time the cells were still vital enough to exclude toxic metals by transport mechanisms. This effect is less pronounced for the strains *M. luteus* W-20 and *Bacillus spec.* W-28. However, for Mn and Co exclusion seems possible as can be seen from the comparison of the concentration of metals bound to the biomass after one hour and one week incubation on the one hand and the four week incubations on the other hand. Sr is contained in comparably low concentrations in AMD and thus exerts no severe toxic effects. In contrast, U may be bound too strong possibly explaining the different behaviour of the latter two elements.

The lower accumulation of REE after one hour and one week of incubation compared to the four week incubation shows that still after one week not all binding sites are saturated with metals indicating high potential for binding of cations.

The present work shows that aside of the passive biosorption seen with dead cells of Gram-positive bacteria also other processes involving growing cells are present which might be exploited for applications in bioremediation. To further characterize the molecular mechanisms at work, further research is required on metal induced processes in different Gram-positive bacteria.

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3.6 Shifts in secondary metabolism of metal tolerant actinobacteria under conditions of heavy metal stress.

Shifts in secondary metabolism of metal tolerant actinobacteria under conditions of heavy metal stress

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Abstract

Numerous microbial habitats are strongly influenced by elevated levels of heavy metals. This type of habitat has developed either due to ore mining and metal processing or by pedogenesis above metal rich base rocks. Most actinobacteria are soil-borne microbes with a remarkable capability for the synthesis of a broad variety of biologically active secondary metabolites, produced at a late stage of their life cycle. Heavy metals can cause either a decrease or inhibition of secondary metabolism in common fermentation media or a stimulation of production. To assay the influence of heavy metals on secondary metabolite patterns we carried out a chemical and biological screening with extracts of both, supernatant and mycelial biomass of 10 metal tolerant strains from metal contaminated and non-contaminated environments. Metabolite patterns of cultures grown in complex and minimal media were compared to nickel or cadmium spiked parallels. *Escherichia coli*, *Mycobacterium smegmatis*, *Staphylococcus aureus* and *Candida albicans* were used to test antibiotic effects. Extracts of some strains displayed intense antibiosis only if grown in the presence of a metal salt. In contrast to the widely held opinion of metals as obstacles in secondary metabolism it is hypothesized that metals can induce or enhance synthesis of possibly potent and medically relevant metabolites.

Key words: *Actinobacteria*, antibiosis, heavy metal, screening program, secondary metabolism

Introduction

During a particular stage in the life cycle synthesis of secondary metabolites, among them numerous antibiotics, occurs in prokaryotes, e.g., *Actinobacteria*, *Myxobacteria* and *Bacillus*. But also various eukaryotes like fungi, and a great number of plants possess the capability of a secondary metabolism. *Actinobacteria* contribute with two-thirds of the total the lion's share of the antibiotics producers. The total number of antibiotics is stated with 5000 (Demain and Fang, 2000), or more than four times the number (Berdy, 2005). The objective of screening programs for detection of bioactive compounds has switched from mainly antibiotic assays up to the 1990ies to other types of assays that led to new discoveries, without which "there would be a significant therapeutic deficit in several important clinical areas, such as neurodegenerative disease, cardiovascular disease, most solid tumors, and

immune-inflammatory disease" (Nisbet and Moore, 1997). Nevertheless, antibiotics still remain the largest market of naturally derived drugs with 67% sales in 2000 (Bull et al., 2000), even if the discoveries of microbial metabolites with non-antibiotic activities now exceed that of antibiotic compounds (Hill et al, 1998). To get access to new microbial metabolites, several strategies can be applied, e.g., development of selective isolation procedures for rare actinomycetes and less thoroughly analysed *Streptomyces* clusters (Sanglier et al., 1993) that are considered as promising producers. Analysis of 16 S rDNA in soil samples by help of taxon specific primers is used to anticipate the presence of desired groups before starting specific isolation procedures (Donadio, 2003). A comprehensive review on search strategies is given by Bull et al. (2000). Additionally, optimization of the fermentation medium's composition to increase the yield of secondary metabolites is in the focus of various studies (reviewed by Iwai and Omura, 1982).

However, the influence of metals in the fermentation medium as inductors or enhancers of secondary metabolism has not been studied systematically, yet. To gain an insight into the effects that heavy metals can have on secondary metabolite production, we tested 10 selected, metal tolerant actinobacteria strains from contaminated, uncontaminated and naturally metal enriched environments. The aim of this investigation was to compare secondary metabolite patterns and antibiosis activities of metal spiked with non-spiked cultures. Due to the possibility that secondary metabolism (e.g. melanogenesis) can be linked to the stress response of the organism it was expected to observe changed patterns of secondary metabolites.

Material and methods

1. Soil sample collection and isolation of actinobacteria

Three soil samples were collected at a former uranium mining site in Eastern Thuringia, Germany (foot and top at the re-vegetated waste heap "Stolzenberg" and periphery of the seepage water reservoir "Pohlteich" characterized by deposits of secondary minerals). One sample originated from the serpentinite rich soil of Pieve Santo Stefano, Tuscany, Italy. Two non metal-enriched soil samples were taken from a public garden (Paradies) and the dry grassland of Windknollen (Napoleonstein) of Jena, Thuringia, Germany. The soils were air dried, ground and resuspended in 0.9% NaCl-solution and agitated for 1h. 100 µL aliquots of 10^{-3} to 10^{-5} dilutions were plated on soil extract agar (Thiemann et al., 1968). After 5 days of incubation at 28°C, pure cultures were obtained on minimal medium for actinobacteria (0.5 g/l asparagine, 0.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4$, 0.01 g/l $FeSO_4$, 10 g/l glucose, 15 g/l agar; Amoroso et al., 2000).

2. Phenotypic characterization and taxonomy

In order to obtain taxonomic information, the selected strains were classified at least on the genus level. Genomic DNA was isolated following the CTAB method (Kieser et al., 2000) and used for PCR with 16S rDNA specific primers (TPU1 AGAGTTTGATCMTGGCTCAG and RTU3

GWATTACCGCGGCKGCTG, Choi et al., 1994). The amplified 500 bp fragments were cloned and sequenced (JenaGen, Jena, Germany). Blast analyses were performed to identify similarities to database entries (NCBI).

3. Test on metal tolerance

Strains were streaked on plates containing metal supplemented minimal medium. Growth was observed and estimated visually after 7 days of incubation at 28°C and compared with growth on non-supplemented minimal medium. The metals $NiCl_2$ x 6 H_2O and $CdCl_2$ x 6 H_2O were added as 0.2 µm sterile filtered solution after autoclavation and before solidification of the medium. The final concentration of each metal in the plate was 1, 5 and 10 mM.

4. Cultivation and preparation of crude extract

The strains were grown in plate culture on starch casein medium (10 g/L soluble starch, 1 g/L casamino acids, 0.5 g/L K_2HPO_4, 16 g/L agar) until sporulation (7 days at 28°C). Spores were harvested with 5 mL sterile 0.9 % (w/v) NaCl solution per plate. 1 mL spore suspension was used as inoculum for a first preculture, consisting of 10 mL minimal medium in 50 mL Erlenmeyer flasks. Cultures were incubated at 28°C for 2-4 days on a rotary shaker. These cultures were used as inoculum for the subsequent preculture in 500 mL Erlenmeyer flasks containing 100 mL minimal medium. Cultures were incubated for 48h at 28°C. 10 mL of the second preculture were used as inoculum for the main culture, which was either soy-mannite medium (20 g/L soy meal and 20 g/L mannite, pH adjusted to 7.2) or minimal medium. 500 mL Erlenmeyer flasks were used for 100 mL culture volume. For induction of stress response, heavy metals were added to give a final concentration of 0.3 mM $NiCl_2$ x $6H_20$ or 45 µM $CdCl_2$ x $6H_2O$ (only for cadmium resistant strain F-4).

After 5 days of incubation the culture broth was separated by centrifugation into supernatant and mycelial fraction in order to test cytosolic and excreted compounds independently. For the supernatant, solid phase extraction (Amberchrom 161c) was applied. Extracting solvent was methanol. Likewise, the mycelial fraction was extracted with methanol.

5. Chemical screening on TLC plates

Extracts of supernatant and mycelia were analysed by thin-layer chromatography. Two different running systems were applied; butanol : glacial acetic acid : water = 4:1:5 and chloroform : methanol = 9:1. For detection of chemical compounds of different substance groups, thin layer chromatograms were developed using UV light at wave length 254 nm and 366 nm as well as staining with anis aldehyde, Ehrlichs reagent and orcinol.

6. Bioassay

Extracts of supernatant and mycelia were tested for antibacterial and antifungal effects in an agar diffusion assay. *Staphylococcus aureus* / MRSA + chinolon-r 134/93, *Escherichia coli* SG 458, *Mycobacterium smegmatis* SG 987 and *Candida albicans* BMSY 212 were used as test organisms. To evaluate the antibiotic effect of the single extracts the diameter of the inhibitory zones was measured.

Results and discussion

1. Isolation of heavy metal tolerant actinobacteria

Three different soil types were used to identify actinobacteria with presumably different levels of heavy metal tolerance. While non-contaminated soils should be expected to yield only strains which by chance were deposited there, higher levels of resistance should be expected to be found in soils evolved on naturally metal rich ultramafic rock. Samples from covered mining heaps and the samples from highly contaminated AMD sites might be expected to show multiple and high level resistance towards heavy metals. We tested heavy metal tolerance towards two heavy metals, nickel and cadmium. The ten isolates with the most pronounced tolerance were chosen from over 100 isolates derived from the three environments. Five of the selected strains originated from the former uranium mining area, three from the ultramafic biotope on serpentine soil and two from uncontaminated habitats.

All strains displayed clear tolerance towards nickel, whereas only strain F-4 was able to grow in presence of 1.0 mM cadmium. 1.0 mM nickel was tolerated by four strains from the mining area, two strains from ultramafic soil and one strain from an uncontaminated habitat. 5 mM was tolerated by two strains, one from the mining area and one from uncontaminated soil. The strain Tosca-3, originating from the ultramafic soil in Tuscany, was capable of growing in presence of 10 mM nickel, which can be considered as a marked resistance. For the ten selected isolates, taxonomic determination was performed by 16S rDNA sequencing (Tab. 1).

Table 1: Taxonomy of metal tolerant strains and resistance concentrations, [a)]*S. tendae* formerly described as *S. rochei* and [b)]*L. waywayandensis* as *Saccharothrix waywayandensis*

Strain	Sampling location	Date of isolation	Taxon	Resistance
E-13	waste dump Stolzenberg	Nov. 1999	*Streptomyces acidiscabies*	5 mM Ni
F-4	waste dump Stolzenberg	Nov. 1999	*Streptomyces tendae*[a)]	1 mM Cd / Ni
PT-1	seepage pond, Pohlteich	Jan. 2003	*Streptomyces ciscaucasicus*	1 mM Ni
PT-5	seepage pond, Pohlteich	Jan. 2003	*Streptomyces sp.*	1 mM Ni
PT-13	seepage pond, Pohlteich	Jan. 2003	*Streptomyces aureus*	1 mM Ni
Tosca-2	Pieve San Stefano	Oct. 2002	*Streptomyces purpurascens*	1 mM Ni
Tosca-3	Pieve San Stefano	Oct. 2002	*Streptomyces lincolnensis*	10 mM Ni
Tosca-4	Pieve San Stefano	Oct. 2002	*Lentzea waywayandensis*[b)]	1 mM Ni
JE-12	Napoleonstein, grassland	Sep. 2002	*Kitasatospora sp.*	5 mM Ni
WiP-14	Paradies, public garden	Jan. 2003	*Streptomyces sp.*	1 mM Ni

2. Comparison of products from crude extract of supernatant versus mycelium

After 7 d of shaking flask culture at 28°C supernatant and biomass were separated by centrifugation and subsequently extracted. The extracts were used in a chemical and biological screening. The chemical screening is used as a cost-effective first screening procedure. To detect different chemical compounds, thin layer chromatograms were developed using UV light as well as staining techniques. Putative bioactive compounds were found both in the mycelium (e.g., strain F-4) or released into the culture broth (e.g., strain PT-1). Therefore, both fractions were used for further screening procedures.

3. Natural compounds produced on rich versus minimal medium

When strains are cultured solely in the usual fermentation media compounds might be overlooked. Strains grown in minimal medium showed indeed a clearly reduced number of extractable metabolites as well in the supernatant as in the mycelium. However, some of the crude extracts contained metabolites which seemed to be synthesized solely in minimal medium. Examples can be seen in anisaldehyde-detected TL chromatograms of strains JE-12, E-13 and Tosca-4 (Fig. 1). Hence we propose to use a minimal medium in addition to rich fermentation media for evaluation of secondary metabolite production.

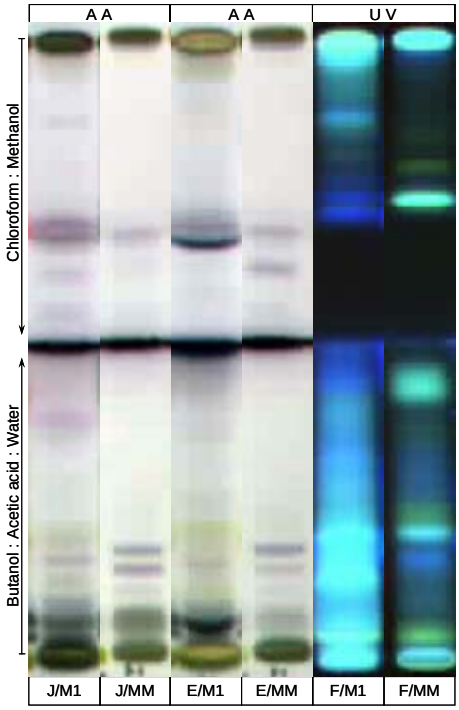

Figure 1: Extracts on TLC of strains JE-12 (J) - mycelial fraction, E-13 (E) - mycelial fraction and F-4 (F) - supernatant. Grown without metal addition in soy-mannite medium (M1) and minimal medium (MM) showing media dependency of metabolite production. Detection: anis aldehyde (AA) and UV-detection at 366nm (UV).

4. Heavy metal induction of secondary metabolites

In the chemical screening, heavy metals were found to induce production of secondary metabolites in complex as well as minimal media. Most of the metabolites are produced in both the metal free and metal containing media. Many bands of metabolites in TLC can be found only in metal free cultures, but there are bands of metabolites that only appear in metal spiked cultures, e.g. in extracts of mycelium of strain Tosca-3, grown in minimal medium (Fig. 2). Likewise, growth of strain Tosca-3 in soy-mannite medium supplemented with nickel displays particular substance bands. Mycelium of strain JE-12 displays a band in complex medium induced by metal supplementation (Fig. 3). Strain PT-1 in turn shows a band in the supernatant of a minimal medium grown culture, if metal was supplemented (Fig. 3). Strain PT-13 shows a prominent band in the extract of the supernatant after growth in complex medium (Fig. 3). The results indicate that for metal spiked cultures, no trend is to be observed: the unique metabolites are produced either only in minimal or in complex media. In addition, unique metabolites are excreted into the medium or can be found in the mycelium.

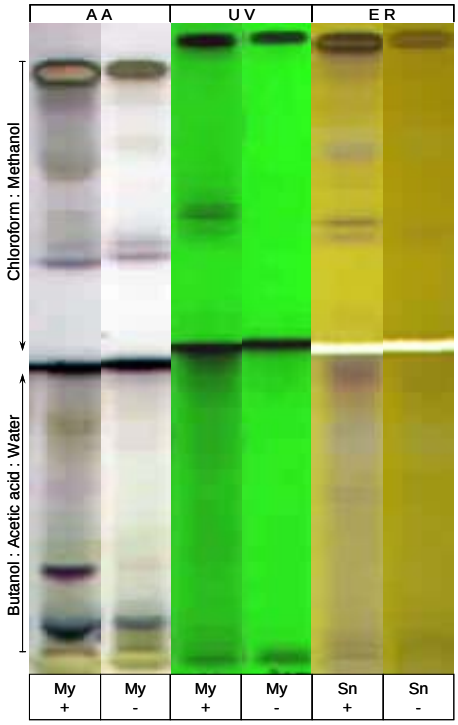

Figure 2: Extracts on TLC of the mycelial (My) and supernatant (Sn) fraction of strain Tosca-3 with (+) or without (-) nickel supplementation showing metal induced metabolites. Detection: anis aldehyde (AA), UV at 245 nm (UV) and Ehrlich's reagent (ER).

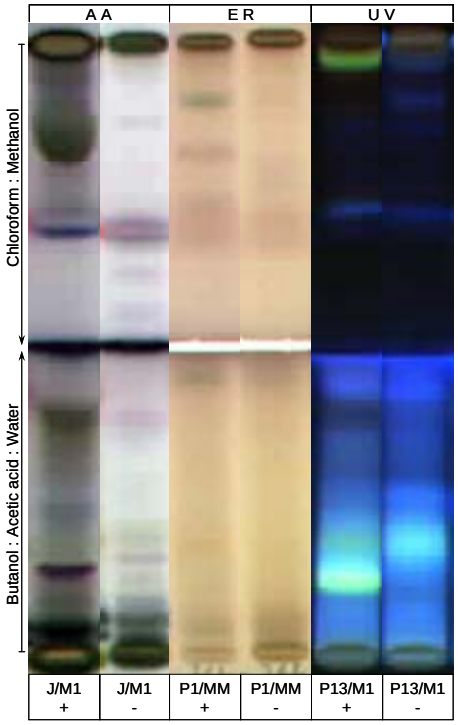

Figure 3: Extracts on TLC of strains JE-12 (J) - mycelial fraction, PT-1 (P1) - supernatant and PT-13 (P13) - mycelial fraction showing metal induced metabolites. Strains grown in either soy-mannite (M1) or minimal medium (MM) with (+) or without (-) nickel supplementation. Detection: anis aldehyde (AA), Ehrlich's reagent (ER) and UV at 366 nm (UV).

5. Antibiotic compounds

Advantageously, the activity of metabolites produced only at low concentrations can be detected in a biological screening (Tab. 2). Some of the crude extracts from metal spiked cultures showed biological activity towards several test organisms. Strains JE-12 and E-13 displayed activity only in the extracts of the metal grown cultures. This suggests a molecular mechanism of induction due to metal supply. It remains to test, if the compounds produced in these cultures are induced by or synthesized only in presence of metals. In the bioassay, the mycelial fraction of strain JE-12 as isolate of a non-contaminated soil shows a strong antibiosis towards test organism *M. smegmatis* only after growth in nickel supplemented soy-mannite medium and a moderate antibiosis against *S. aureus* only after growth in nickel supplemented minimal medium. There was no antibiosis detected in the non-spiked media. The same could be observed for the mycelial fraction of strain PT-1 as isolate of a mining area, which displayed a potent antibiosis towards *C. albicans* only after growth in nickel supplemented soy-mannite medium. The mycelial fraction of another isolate from the mining area, strain E-13, displayed antibiosis towards *S. aureus* and *M. smegmatis* after growth in nickel treated soy-mannite medium. Two of the three isolates from an ultramafic soil show effective antibiosis after growth in the presence of nickel. The supernatant fraction of strain Tosca-2 was able to inhibit *E. coli*, whereas the mycelial fraction of strain Tosca-3 was effective towards *S. aureus*. Hence, screening procedures should be re-evaluated for heavy metal induction even of strains, isolated from non-contaminated biotopes (like strain JE-12), and isolation of strains is proposed to include specifically heavy metal rich environments to yield heavy metal tolerant actinobacteria (like strains E-13 or Tosca-2, Tosca-3)

Table 2: Biological screening of extracts of supernatant (S) and mycelial fraction (M). blank – no inhibition, 1 – inhibition, 2 – moderate inhibition, 3 – strong inhibition, M1 – soy-mannite medium, MM – minimal medium

strain	main culture	*S. aureus* 134/94		*E. coli* 458		*M. smegmatis* 987		*C. albicans*	
		S	M	S	M	S	M	S	M
FSU-F4	M1+Cd		**3**				**3**		
	M1		**3**				**3**		
	MM+Cd								
	MM								
FSU-E13	M1+Ni		**2**				**3**		
	M1								
	MM+Ni								
	MM								
FSU-PT01	M1+Ni		**2**				**3**		**3**
	M1		**2**				**3**		
	MM+Ni								
	MM								
FSU-PT05	M1+Ni	**3**						**3**	
	M1	**3**					**1**	**2**	
	MM+Ni								
	MM	**2**	**2**	**3**			**1**		
FSU-PT13	M1+Ni						**3**		
	M1						**3**	**2**	
	MM+Ni								
	MM						**2**		
FSU-Tosca2	M1+Ni	**3**	**3**	**2**		**3**	**3**		
	M1	**3**	**3**			**3**	**3**		
	MM+Ni	**3**	**3**			**2**	**2**		
	MM	**3**	**3**			**3**	**3**		
FSU-Tosca3	M1+Ni	**3**	**3**				**3**		
	M1	**3**					**3**		
	MM+Ni		**3**						
	MM								
FSU-Tosca4	M1+Ni						**3**	**3**	
	M1						**3**	**3**	
	MM+Ni								
	MM								
FSU-JE12	M1+Ni						**3**		
	M1								
	MM+Ni		**2**						
	MM								
FSU-Wip14	M1+Ni	**3**		**1**		**2**		**2**	
	M1	**3**				**3**		**2**	
	MM+Ni						**2**		
	MM	**3**				**2**	**2**		

6. Metabolite-metal interactions

Many soil habitats in mining regions are enriched in heavy metals. What is the influence that excessive metal concentration in soil has on secondary metabolism? It can be hypothesized, that synthesis of metabolites which detoxify heavy metals by chelation is increased when metals are added to the fermentation medium of heavy metal tolerant strains. It has been shown that many antibiotics and other secondary metabolites, for example isatin, a metabolite of *S. albus*, can scavenge heavy

metals from the medium (Gräfe and Radics, 1986). The polyketide gamma-actinorhodin produced by *S. coelicolor* has been described as a chelator of iron (Coisne et al., 1999), and the secondary metabolite melanin has been shown to be involved in heavy metal tolerance in *S. scabies* (Beauséjour and Beaulieu, 2004). Besides that, raising the concentration of trace metals in fermentation media from usually 10^{-7} M by 10 - 100 fold is required for metabolite production (Iwai and Omura, 1982). The concentration of nickel that has been applied in the presented assay is with 3 x 10^{-4} clearly above threshold. Such high concentrations require tolerance as it is considered as inhibiting growth of sensitive strains. If under the conditions of heavy metal stress growth still occurs and metabolite production proceeds, than a stress response has been initiated, which very likely is based on a switch in metabolism. This very likely is based on a combination of effects caused by elevated concentrations of nickel. Both, the supply of nickel to Ni^{2+} dependent enzymatic steps and the cytosolic or extracellular chelation of nickel by metabolites is thought to play a key role in production of a specific set of secondary metabolites in nickel-spiked media.

Microorganisms adapted to life in hazardous environments are considered weak producers of antibiotics, reasoned to be due to the lack of competition within the microbial community of the habitat (Vining, 1990). With this study it is shown, that metal tolerant isolates of the group of *actinobacteria*, originating from highly metal enriched habitats of a former uranium mining site are capable of strong antibiosis towards various test organisms, even if extracted from soil and sediment samples of low cfu values (data not shown). Likewise, Sprocati and coworkers (2006) showed the influence of heavy metals on the metabolic profile of metal resistant, yet unidentified filamentous and other bacteria from an abondened mine. They showed that in presence of a heavy metal some of the 74 investigated substrates are utilized with higher oxidation rates (Sprocati et al., 2006). A metabolic shift due to growth in nickel or cadmium spiked cultures could consequently lead to formation of different secondary metabolites or metabolites in altered concentration.

Few reports on heavy metal treated plants show loss in the capacity to produce or accumulate secondary metabolites, as, e.g. observed in St. John's wort (*Hypericum perforatum* L.) grown in nickel supplemented soils (Murch, 2003). Examples for heavy metals that have an enhancing effect on production of secondary metabolites in plants, in contrast, are numerous. Cadmium, for example, can enhance the production of alkaloid secondary metabolites in perry-winkle (*Cataranthus roseus*) (Zheng, 2004) or phyllanthin metabolites in *Phyllanthus amarus* (Rai, 2005). It is not clear yet, if the same tendency is to be seen in actinobacteria.

A nickel adapted and highly nickel tolerant microflora has evolved in habitats that developed due to pedogenesis above ultramafic parent rocks, as shown by Mengoni et al. (2001) for an area in Tuscany, Italy, and for neocaledonian soils (Hery et al., 2003). This type of pedogenesis results in nickel rich serpentine soils with 1.2 – 2.0 mg g^{-1} nickel per soil dry weight (Mengoni et al., 2001). Here, we isolated the strain Tosca-3, displaying the most pronounced resistance (for calculating concentration of resistance see Duxbury, 1981 and Trevors et al., 1985), from the ultramafic soil sample of Tuscany.

There are only very few studies on antimicrobial characteristics of isolates originating from this type of soil. A strain of the new species *Streptomyces yatensis* isolated from a New-Caledonian ultramafic soil, revealed a remarkable spectrum of antimicrobial and antitumor activities (Saintpierre et al., 2003).

However, tolerance can be observed even in strains originating from uncontaminated soils as shown here. A shift of secondary metabolites under the influence of metals added has been observed with each type of isolate, originating from long adaptation on ultramafic soil (Tosca-2, Tosca-3), short-term adaptation (E-13), or non-contaminated areas (JE-12).

In contrast to our expectation to find the highest metal resistance among isolates of the mining area, strain Tosca-3, originating from serpentine soil of Tuscany, displays the most distinct resistance towards nickel. An explanation can be given by help of the concept of "ecological islands" by Kruckeberg (Kruckeberg, 1984; Lefèbvre and Vernet, 1990). The mineral composition of the serpentine soil with its extremely high concentrations of certain heavy metals like nickel and the time span for evolution of organisms dwelling in that habitat may lead to distinct adaptation.

The mining site had only 40 years of mining activity in the former uranium mining area for adaptive responses. Resistance of microbes in close contact with metal containing mining waste was less developed as compared to those in soil that was formed by pedogenesis above ultramafic parental rock for millions of years in this naturally occuring type of habitat.

The concentration of 45 µM Cd for stress induction of strain F-4 is considered high. For this element, no biological function has been shown. In contrast, the uptake of the element nickel with its essential function in a number of enzymes, like hydrogenase, urease and superoxide dismutase, has to be homoeostatically regulated. Therefore, the concentration for stress induction needs to be higher and has been chosen with 0.3 mM in the present study.

The genomes of *Streptomyces avermitilis* and *Streptomyces coelicolor* are predicted to encode at least 25 (Omura et al., 2001) and 22 (Bentley et al., 2002) different secondary metabolites, respectively. It is not clear under which culture conditions a certain pattern of secondary metabolites is produced. Supplementation of minimal or complex media with heavy metals as simulation of environmental conditions to which the strain might have adapted, could induce synthesis of hitherto undiscovered metabolites.

Synthesis of secondary metabolites of strains grown in minimal medium has to be compared with soy-mannite medium. Soy-mannite medium resembles in a high degree a typical fermentation medium for optimal product yield and can be considered as a (semi) rich medium. Minimal medium, however, reflects far better the nutritional situation in most natural habitats. Secondary metabolites produced under natural conditions, effective in microbial soil habitats should be screened using a medium that comes close to the conditions in the habitat in terms of, e.g., carbon supply, pH or concentration of trace elements. It has been shown that isolates from an anthropogenically heavy metal contaminated habitat (E-13), a naturally nickel enriched soil (Tosca-4) and a non-disturbed grassland soil (JE-12)

produce metabolite(s) in minimal medium that do not appear in the extracts of soy-mannite grown cells. This suggests that the capability of secondary metabolite production is not exhausted by testing the common fermentation media. Rather, minimal media can induce formation of secondary metabolites which otherwise would remain undiscovered. From an ecological perspective, secondary metabolites produced in minimal media are very likely the ones that play the dominant role in the soil habitat at the transition phase of the producer organism, when vegetative mycelium breaks down and "fatal attraction" (Shi and Zusman, 1993, Challis and Hopwood, 2003) starts to occur. In an extension, it might be speculated that a screening for characteristic secondary metabolite patterns should include shaking flask as well as solid fermentation in soil extract media as even more natural substrates.

The manifold and often opposed effects of different heavy metals on secondary metabolism have been studied in detail with *S. galbus*. Cadmium and chromium stimulated pigment production at lower concentrations, whereas nickel and mercury negatively affected the production (Raytapadar et al., 1995). In contrast to this finding, it was reported that nickel has a stimulatory effect on *S. rishiriensis* in producing the antibiotic coumermycin A1 (Claridge et al., 1966). The production of actinorhodin of *S. coelicolor* has been shown to be sensitive to mercury, cadmium, copper, nickel and lead, but can be slightly stimulated by chromium (Abbas and Edwards, 1990). From these aspects and based on the presented results of 10 strains of actinobacteria, we conclude that patterns of secondary metabolites are strongly changed upon addition of different heavy metals. The pattern depends on both, the kind of the metal added and the metal concentration. Isolates from both environments, non-contaminated and contaminated, can display similar responses, as could be shown for the biological activity of strains E-13 (isolate from contaminated site) and JE-12 (isolate from non-contaminated site). Interestingly, the two strains show biological activity only after growth in presence of nickel. The effect on secondary metabolism induced by metals is thought to be part of a stress response and can be exploited for the search of novel metabolites. It remains to be tested, if this activity is nickel specific or can be induced also by other metals to which the strains show tolerance. The strong biological activity of strain PT-1 towards *C. albicans* after growth on nickel supplemented medium seems to warrant further studies.

Conclusions

It has been demonstrated by the two independent procedures of biological and chemical screening that the secondary metabolite patterns of selected strains can vary under the influence of heavy metals added to the fermentation medium. Some metabolites are produced in raised concentration; others are produced solely in the presence of metal. Strains isolated from contaminated, as well as from non-contaminated habitats display an altered secondary metabolite pattern on TLC and a varied antibiotic activity in bioassays after growth in metal containing medium. This suggests that not only actinobacterial strains that are adapted to life with high metal concentration can be influenced in their secondary metabolism by metals, but isolates originating from undisturbed environments show similar responses. It certainly would be worthwhile to re-screen already established microbial strain

collections for novel metabolites produced under the influence of heavy metals. The background of the intensified synthesis of particular secondary metabolites very likely is the interplay of the metal dependency of many enzymatic synthesis steps, a metal chelating effect due to chemical properties and the influence on regulation of biosynthetic gene clusters in conjunction with the induction of transport systems by metal cations. The role of secondary metabolites as chelators warrants further investigation which will proceed with some of the ten assayed strains in the future. In addition, the identification of the newly detected antibiotic compounds will proceed from the current work.

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3.7 "Ni-struvite" – a presumably new biomineral generated by a nickel resistant *Streptomyces acidiscabies* strain.

"Ni-struvite" – a prospectively new biomineral generated by a nickel resistant *Streptomyces acidiscabies* strain

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Abstract

The mineral struvite is formed through biomineralization in soils, guano, putrescent matter and in sediments by the action of bacteria. A prospectively new mineral containing not magnesium but nickel in $Ni(NH_4)(PO_4)\cdot 6H_2O$ has been identified from the surface of colonies of a *Streptomyces acidiscabies* isolate resistant towards high nickel contents. The mineral was putatively named nickel struvite. The mineral formation is dependent on biological activity since non-viable bacterial cells are not capable to induce formation of Ni-struvite under identical conditions. The strain is able to induce formation of Ni-struvite upon desiccation of agar containing minimal or complex media when growing in the presence of elevated concentrations of $NiCl_2$ (8-15 mM). Formation of magnesium containing crystals was not observed although Mg^{2+} is present in the medium. Small crystals attached to the mycelial biomass of the strain have been microscopically found in liquid cultures of nickel supplemented minimal and complex media after two weeks of incubation. The capacity to induce biomineralization specific for nickel is interpreted as a resistance factor, allowing the soil bacterium to withstand high nickel concentrations in the natural habitat. The strain has been isolated from a former uranium mining site in Eastern Thuringia, Germany, where nickel concentrations of up to 2000 ppm (translating to appr. 30 mM) in the overburden dump are found as a result of former mining activities.

Key words: Struvite, nickel, biomineralization, bacteria, *Streptomyces*

Introduction

The heavy metal nickel interacts in various ways with the microbial cell. In low concentrations, i.e. nanomolar range, it plays an essential role in a variety of metabolic pathways. CO-dehydrogenase, hydrogenase, Ni-superoxide dismutase and urease are examples for enzymes that depend in their function on nickel. However, at high concentrations, i.e. the micro- to millimolar range, nickel represents a dangerous environmental noxa interfering with structural constituents and metabolic components of the cell. Nickel can enter the microbial cell via several uptake mechanisms, i.e. Mg^{2+} transport systems, cobalt transporters and specific high affinity Ni^{2+} transport systems (Watt and Ludden, 1999). In nickel-rich environments, like serpentine soils and ore mining sites, microorganisms

have strongly adapted to antagonize the ecotoxicological impact of nickel (Mengoni, et al., 2001; Amoroso et al., 2000). ATP depending nickel efflux transport systems that support the maintaining of a low intracellular nickel concentration are well studied for different bacteria (Nies, 1992). The Gram-negative bacterium *Hafnia alvei* 5-5 is known to be resistant up to 30 mM nickel due to a highly efficient efflux transport system (Park et al., 2004). The usually negatively charged cell envelope of Gram-positive and -negative bacteria participates in a basic tolerance by sorption of metal cations. Many of the functional groups of the bacterial surface that have an affinity to metal cations are well characterized (Jiang et al., 2004). Nickel can be bound to cell wall constituents, as, e.g., teichoic and teichuronic acids in Gram-positive bacteria (Beveridge, 1989). Thereby, the metal influx into the cell is impeded. Excessive nickel concentrations within the cell, on the other hand, can be detoxified by sequestering compounds like, phosphate bodies as shown for *Staphylococcus aureus* (Gonzalez and Jensen, 1998).

The bacterial tolerance and resistance mechanisms acting extracellularly are, in contrast, far less intensively studied. A number of bacteria is known to possess the capacity to precipitate constituents from solution (Bäuerlein, 2003). Although reported for a number of microbial strains to occur (Gadd, 1996; White et al., 1997), it has not been documented, whether bioprecipitation of metals can enhance the resistance characteristics of strains dwelling in metal enriched environments.

Streptomycetes are typically soil dwelling filamenous bacteria that have been reported to be isolated from metal rich soil and sediment habitats (Schmidt et al., 2005; Amoroso et al., 1998). The bacteria run through a complex life cycle, thereby changing the morphological and physical state. The physical state of streptomycetes in soil generally is spores rather than vegetative hyphae (Mayfield et al., 1972). A remarkable bioprecipitation of a prospectively new, nickel containing biomineral was observed during the investigation of a nickel resistant streptomycete derived from a former uranium mining site in Eastern Thuringia. Formation, chemical composition and structural characteristics of the biomineral were studied.

Material and methods

1. Cultivation of *Streptomyces acidiscabies* E13

A nickel resistant *Streptomyces* strain showing also co-resistance to other metals (e.g., chromium and zinc; Amoroso et al., 2000) was selected from a strain collection of approximately 100 bacterial isolates derived from metal contaminated soil and sediment samples of the former uranium mining area close to Ronneburg in Eastern Thuringia, Germany, for studies of nickel resistance. The strain has been previously described as *S. acidiscabies* E13 (Schmidt et al., 2005).

The strain *S. acidiscabies* E13 was cultured in liquid media or on plates containing (a) minimal medium (0.5 g/L asparagine, 0.5 g/L K_2HPO_4, 0.2 g/L $MgSO_4$, 0.01 g/L $FeSO_4$, 10 g/L glucose; Amoroso et al., 2000), (b) standard I (St I) medium (15 g/L peptone, 3 g/L yeast extract, 6 g/L NaCl, 15 g/L glucose; Merck-Darmstadt, Germany), (c) LB medium (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract; Sambrook and Russell, 2001) and (d) tryptic soy broth (TSB) medium (17 g/L

pancreatically digested casein, 3 g/L enzymatically hydrolysed soy meal, 2.5 g/L dextrose, 5 g/L NaCl, 2.5 g/L K_2HPO_4; Kieser et al., 2000), respectively. All media were prepared with distilled water and solid media contained 16 g/L. Media were autoclaved for 20 min at 121°C. After autoclavation and before pouring plates media were supplemented with a sterile filtered 0.5 M $NiCl_2$ stock solution to a final concentration of 0, 1.0, 2.5, 4.0, 5.0, 8.0, 10.0, 12.0 and 15.0 mM nickel. 100 mL Erlenmeyer flasks with 20 mL medium solution were used for liquid cultures. Nickel was supplemented after autoclaving.

2. Preparation of spore and mycelium suspensions

A spore suspension for inoculation was prepared by harvesting the mature spore layers of two well grown starch casein plates (starch casein medium consists of 10 g/L starch, 1.0 g/L casamino acids, 0.5 g/L K_2HPO_4, 16 g/L agar; Kieser et al., 2000) after seven days of incubation at 28°C. Spores were harvested with 20 mL distilled water and rinsed twice with the same volume. Finally, the spore suspension was filtered through a cotton wool sieve (Kieser et al., 2000).

For the preparation of dead biomass of strain *S. acidiscabies* E13 suspensions of spores and vegetative mycelium were used. 100 mL Erlenmeyer flasks containing 20 mL of liquid TSB medium were sterilized as described and inoculated with 0.5 mL of spore suspension. After incubation for five days at 28°C as shaking flask culture the mycelium was harvested by centrifugation and rinsed twice with 20 mL sterile distilled water. After rinsing, the vegetative mycelium was kept in 20 mL distilled water. Both suspensions (spore and mycelium) were subsequently autoclaved for 20 min at 121°C.

3. Biomineralization experiments

10 µL droplets of the vital spore suspension, the autoclaved spore suspension, and both, the pregrown, vital and the pregrown autoclaved mycelium suspensions of *S. acidiscabies* E13 were applied on plates as inocula. Droplets were spotted on solid minimal medium (MM) and complex media (St I, LB, and TSB) at each nickel concentration. Plates were incubated for three weeks at 28°C. Liquid cultures were inoculated with 0.5 mL spore or mycelium suspension and incubated on a rotary shaker (Infors, HT, Bottningen, 100 rpm) at 28°C for two weeks. Growth and crystal formation in the cultures were evaluated microscopically. A Zeiss Axioplan 2 fluorescence microscope was used for transmitted light-microscopy (TLM) and a Zeiss Stemi 2000C for incident light microscopy (ILM).

4. Determination of chemical composition of bioliths

The chemical composition of the grown bioliths was analyzed. Crystals were collected from the plate cultures and screened by electron microprobe analysis (EMPA) using a CAMECA SX 100. The qualitative phase analysis was performed by X-ray powder diffractometry using a SIEMENS D5000 with PSD scan and Cu-Kα source. The powder data set given by Negro and Stafferi was used as reference (JCPDS-PDF set 21-0034; (1967)). To calculate the powder diffractogram the structure refinement of Blachnik and coworkers was used (Blachnik et al., 1997). Differential thermogravimetry

measurement (DTG) was performed to obtain information about content of water and ammonium in the crystalline compound.

Results and discussion

1. Nickel-resistance

Strain *S. acidiscabies* is able to grow up to 10 mM nickel in MM (Schmidt et al., 2005) and 15 mM in complex media (St I, LB and TSB) as solid culture. In liquid culture the strain can tolerate 4 mM of nickel in MM and 8 mM in complex media. The amount of media ingredients with high binding affinity for metal cations varies between minimal and complex media. It is well understood how various media constituents can bind heavy metals, and thereby influence the interaction of metals with bacteria (Ramamoorthy and Kushner, 1975; Sterritt and Lester, 1980; Prahalad and Seenayya, 1988). However, differences in resistance concentrations between growth of *S. acidiscabies* E13 on minimal and several complex (rich) media are less distinct than expected. Showing still growth on at least 10 mM nickel (MM) in plate culture, the strain can be considered as remarkably resistant if compared with other resistant isolates (Hery et al., 2003; Pal et al., 2005). The well investigated metal resistant Gram-negative bacterium *Cupriavidus metallidurans* CH34, likewise isolated from a metal contaminated environment, is characterized by the possession of three nickel resistance determinants *cnr*, *nre* and *ncc*, respectively, mediating low (up to 3 mM), medium (up to 10 mM) and high (20-50 mM) resistance (Taghavi et al., 2001). The resistance of *C. metallidurans* is based on efficient efflux transport systems. The nickel resistance mediating transport systems have also been found in other Gram-negative bacteria, isolated from metal rich environments (Park et al., 2004). Due to the constitutive differences in the structure of the cell envelope it is believed that nickel resistance of the Gram-positive bacterium *S. acidiscabies* E13 is caused by resistance determinants other than solely metal efflux transport. Nevertheless, the degree of nickel resistance of *S. acidiscabies* E13 is comparable with the *nre*-determined resistance of *Cupriavidus metallidurans* CH34. The nickel resistance of *S. acidiscabies* E13 investigated so far, is composed of factors acting intra- and extracellularly (Amoroso et al., 2000; Schmidt et al., 2005).

2. Culture conditions for biomineralization

Crystals (up to 100 µm large) of greenish appearance – macroscopically visible after three weeks of incubation – were detected on colony surfaces of strain *S. acidiscabies* E13 grown on solid minimal and solid complex media (Fig. 1A and 1B). The crystals formed in MM are comparably small (Fig. 2B). Crystal formation also occurred on all complex media. TSB has been observed to be the medium characterized by fastest crystallization and highest yield of crystals. No crystals could be detected on plates inoculated with non-viable vegetative mycelium or autoclaved spore suspension, respectively. Therefore, the grown crystals are considered bioliths and were further characterized. Aliquots of liquid cultures were microscopically analyzed. Crystals attached to the mycelial pellets grown as liquid cultures could be observed after incubation for two weeks in TSB medium (Fig. 1C).

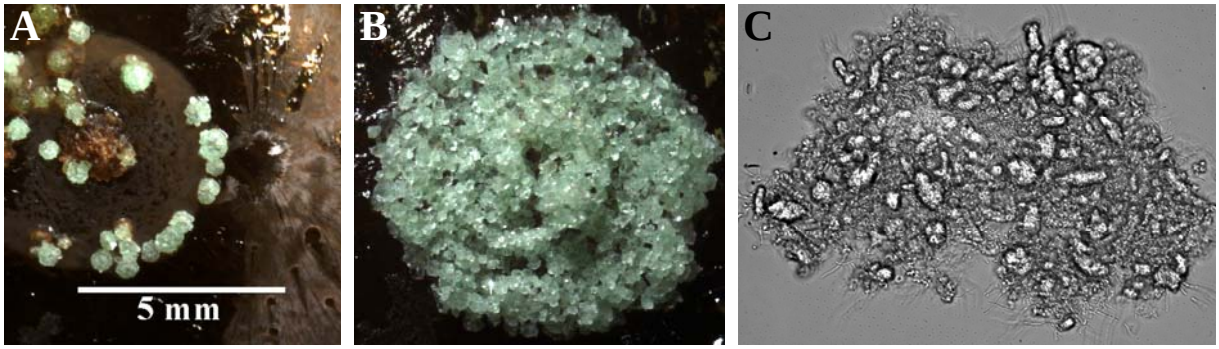

Figure 1: *S. acidiscabies* E13 grown on solid TSB supplemented with 15 mM $NiCl_2$ (A and B, TLM) showing formation of green crystals; (C, ILM) shows a mycelium of the wild type strain with attached crystals grown in liquid TSB containing 8 mM $NiCl_2$, diameter of a single hypha is appr. 1.0 µm

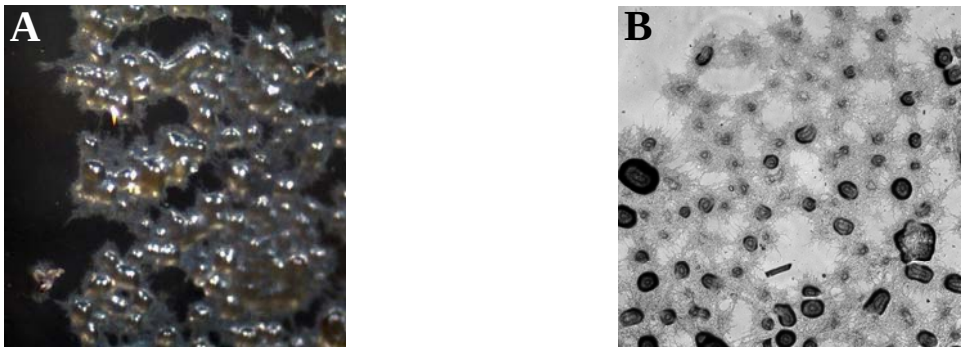

Figure 2: Periphery of *S. acidiscabies* colonies grown on solid TSB supplemented with 10 mM $NiCl_2$ (A) and solid MM supplemented with 5 mM $NiCl_2$ (B) showing crystal development after 3 weeks; both ILM

3. Chemical composition, crystallography and morphology of bioliths

The chemical analysis of the grown bioliths by EMPA revealed the presence of nickel and phosphorus. Ni-K_α, Ni-K_β, P-K_α, and P-K_β could be detected by LiF and PET crystals, respectively. Additionally, potassium and silicon could be detected in the dried pellicle. The light elements H, C, N and O were not recorded. The dried pellicle yields broad "vitreous peaks", peaks of hexahydrated ammonium nickel(II) phosphate and of halite. In the diffractogram of crystalline aggregates (Fig. 3) all strong peaks belong to $Ni(NH_4)(PO_4)\cdot 6H_2O$ with the only exception of the strongest halite peak. Significant differences between calculated and measured reflex intensities should be interpreted as an effect of cleavage during sample preparation.

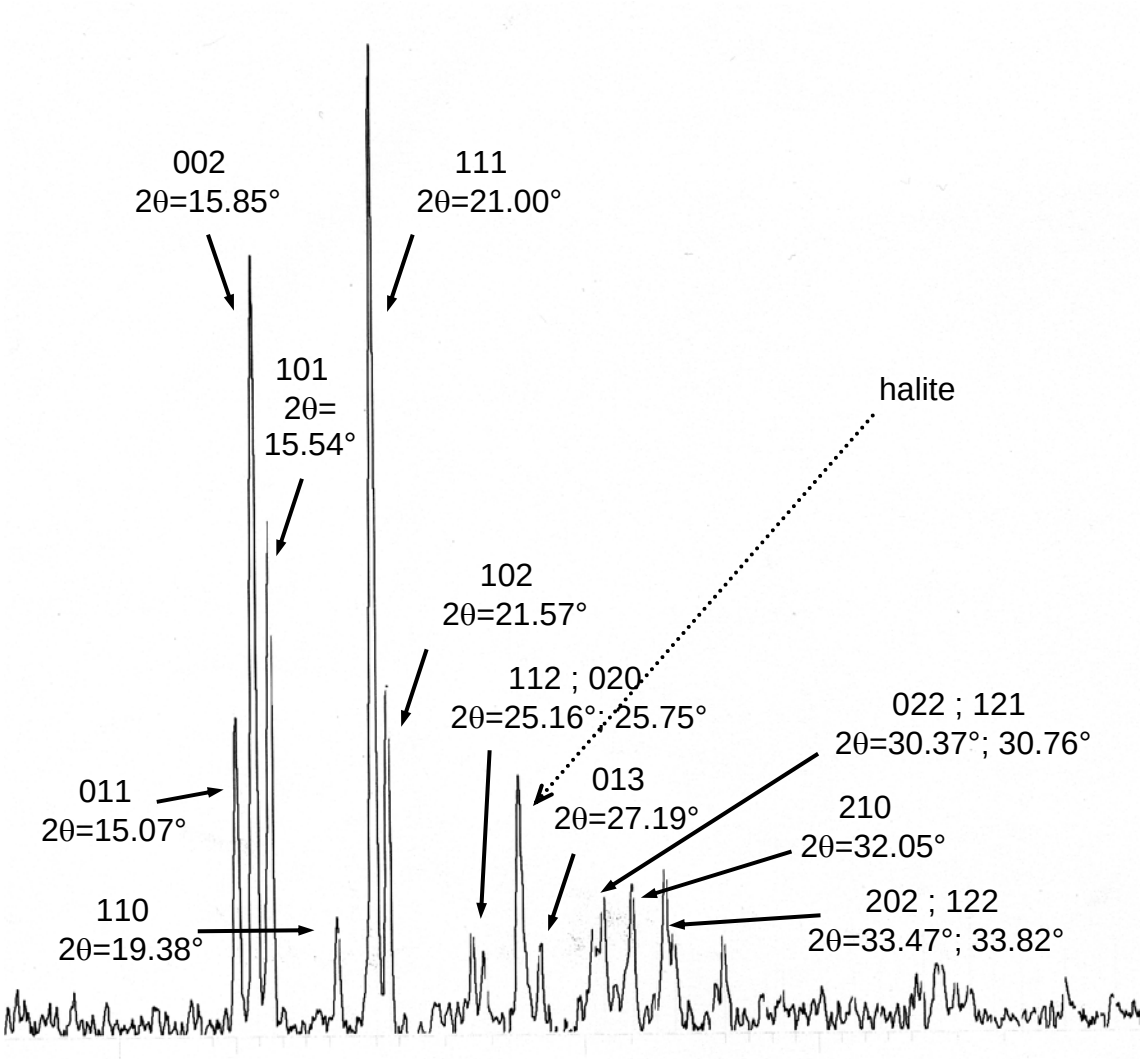

Figure 3: X-ray powder diffraction pattern of $Ni(NH_4)(PO_4)\cdot 6H_2O$ identified by PDF data set 21-0034.

In the crystal structure of struvite and "Ni-struvite", insular $Mg(OH_2)_6$ octahedra, PO_4^{3-} and NH_4^+ tetrahedra are linked by hydrogen bonds. Both compounds crystallize in the orthorhombic $Pmn2_1$ space group. The cell parameters of struvite, a=6.95 Å, b=6.14 Å, 11.22 Å, and "Ni-struvite", a=6.9032(8) Å, b=6.0907(5) Å, 11.1402(8) Å, are very similar (Goni et al., 1996; Strunz and Nickel, 2001). The thermal decomposition of $Ni(NH_4)(PO_4)\cdot 6H_2O$ starts at 80°C under a nitrogen atmosphere. The minimum of the DTG peak at 120°C corresponds to the mass loss of one ammonium and six water molecules (Δm=-45%) (Goni et al., 1996).

Because struvite and "Ni-struvite" are isotypes the crystal habits should be similar (Fig. 5). The crystals of struvite are a classic example of a hemimorphic development, possessing a perfect {001} cleavage. A review about detailed studies on struvite morphology has been published (Kostov and Kostov, 1999). The order of persistence of observed forms runs {00-1} → {010} → {101} → {012}

→ {110} → {10-1} → {01-1} → {001} → {011} → {01-2} → {11-1} → {113} → {111} → {021} → {103}. Forms like {103}, {013}, {021}, {102} and {113} are rare (Kleber and Kühn, 1957; Abbona and Boistelle, 1979 and 1985). Growing struvite crystals from gel, tabular to platy {101} crystals have been obtained at pH 6, wedge-shaped {110} crystals at pH 7 and prismatic crystals at pH 8 (Irusan et al., 1990). Furthermore the literature shows that the crystal habit of struvite is related to bacteria-induced and solution depending pH (Clapham et al., 1990; Suschka et al., 2005).

4. Biomineralization of struvite and Ni-struvite

The mineral struvite, $Mg(NH_4)(PO_4)\cdot 6H_2O$, was named already in 1845 after the Russian mineralogist Heinrich C.G. von Struve. Struvite is formed in deposits of guano, putrescent matter, in sediments or soils by microbial action. Struvite formation by microorganisms has been described already in the 19th century (Robinson, 1889). A number of soil inhabiting genera like *Azotobacter*, *Bacillus*, *Myxococcus*, and *Pseudomonas* has been studied on their potential to form struvite (Ben Omar, et al., 1998). It is also well known as a mineral in the human body as bladder and urinary concretions. In Germany it was found originally from Hamburg in a bed of peat underlying deposits of organic matter below an old church, and in cattle dung at Homburg v.d. Höhe. The minerals newberyite $Mg(PO_3OH)\cdot 3H_2O$, dittmarite $Mg(NH_4)(PO_4)\cdot H_2O$, and hannayite, $Mg_3(NH_4)_2(PO_3OH)_4\cdot 8H_2O$ occur associated with struvite. Struvite is also formed by incongruently dissolving of schertelite, $Mg(NH_4)_2(PO_3OH)_2\cdot 4H_2O$, in H_2O (Gaines et al., 1997).

The surface of an individual crystal is known to be a prime location for the adsorption of trace metals (Weiner and Dove, 2003). This might explain the great number of crystals covering the colonies of *S. acidiscabies* E13 in a multi-layered order in media containing $NiCl_2$ in high concentration (Fig. 4). Crystal formation occurs on media supplemented with 8 mM $NiCl_2$ or more, but not in presence of lower concentrations. This suggests a particular ratio of the components nickel, ammonium and phosphate essential as condition for crystallization. Phosphate is a medium ingredient sufficiently available in both types of media, whereas ammonium is believed to be released from the cell during incubation. Both media types are supplemented only with an organic nitrogen source, therefore a metabolic formation of ammonium seems likely. The pH in the media is buffered to near-neutral. As during growth bacteria acidify their media, medium acidification by *S. acidiscabies* E13 was tested. The strain shows only very weak acidification, leaving the conditions for biomineralization favorable for the crystallization of Ni-struvite.

Depending on the degree of biological control, biomineralization processes are generally divided in "biologically induced mineralization" (Lowenstam, 1981) and "biologically controlled mineralization" (Mann, 1983). It is not clear yet, if the formation of Ni-struvite should be classified as a process of the first or latter. If the cell surface of *S. acidiscabies* E13 hyphae only act as causative agent for the precipitation of Ni-struvite with limited control over type and habit of mineral deposits remains to be studied in detail. Bacteria that possess the capability to control mineralization bear the necessary information in the genome as has recently been shown for a gene cluster of *Bacillus subtilis* which is

involved in the biomineralization of calcium carbonate (Barabesi et al., 2007). The microbial precipitation of calcium carbonate is discussed as a probable detoxification mechanism to immobillize excess of extracellular calcium (Anderson et al., 1992). The intracellular calcium concentration must be maintained low to warrant the fundamental functions of this element essential in cellular regulation (Smith, 1995). In this regard *Streptomyces acidiscabies* E13 and other bacteria inhabiting nickel enriched habitats could have evolved a similar mechanism of precipitation to prevent cellular poisoning.

From point of view of materials science, the Ni analogue of struvite, $Ni(NH_4)(PO_4)\cdot 6H_2O$, is very interesting because of its magnetic properties. Generally, nickel(II) phosphates offer a considerable number of different structures. A systematic investigation of the reactions in water solution for the [$Ni^{2+}/H_3PO_4/NH_4OH$] system was carried out (Goni et al., 1996). The compounds were synthesized by adding $NiCl_2\cdot 6H_2O$ (0.004 $mol\cdot L^{-1}$) to different solutions of H_3PO_4 and NH_4OH at 90°. The presence of two phases, the yellow monohydrated and the green hexahydrated ammonium nickel(II) phosphate, was observed. At room temperature and pressure the solid-solution equilibrium favours the evolution of the hexahydrated compound which is thermodynamically more stable. Recrystallization is necessary to obtain single crystals that are adequate for X-ray diffraction. In former times nickel ammonium phosphate was of interest in forestry due to its potential use in wood preservation. The compound is an effective agent against wood destroying fungi and termites (Bateman and Baechler, 1959).

In the field of environmental mineralogy is currently a considerable interest in the precipitation of struvite to observe (Antakyali et al., 2005; Britton et al., 2005; Clapham et al., 1990; Rivadeneyra et al., 2006; Suschka et al., 2005; Ueno, 2004). The crystallization from sewage may be a potential route to recover phosphates for recycling in the form of fertiliser. The involved ammonium ions are generally present in waste waters and sewage sludge liquors in relatively high concentration (Suschka et al., 2005). The specific feature of the present case is the additional availability of nickel. Solidification, and subsequent immobilisation of that noxious heavy metal may benefit the recultivation and decontamination of former mining regions like the Ronneburg uranium deposit in Eastern Thuringia.

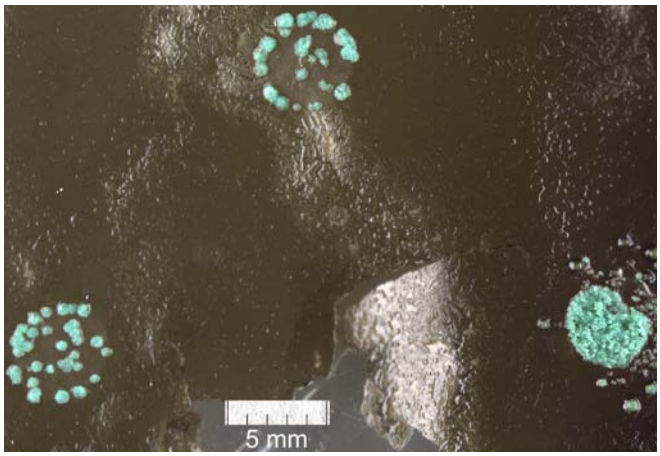

Figure 4: Crystalline aggregates of $Ni(NH_4)(PO_4)\cdot 6H_2O$ on the colony surface of *S. acidiscabies* E13 (ILM)

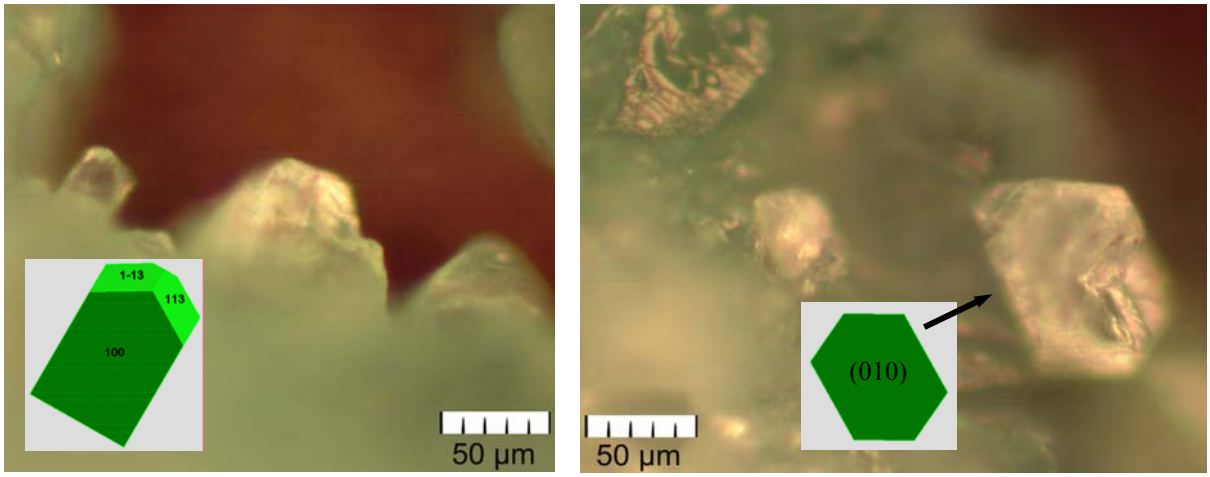

Figure 5: Habit of single crystals of $Ni(NH_4)(PO_4)\bullet 6H_2O$. The sketch in the left micrograph shows a possible habit formed by {00-1}, {100}, {010}, {113} and {001} faces. The sketch in the right micrograph illustrates the view direction parallel to [010] of an idealized struvite crystal formed by {010}, {100},{103} and {10-3} faces.

Conclusions

This is, to our knowledge, the first report on biomineralization of Ni-struvite. The occurrence of hexahydrated ammonium nickel(II) phosphate in nature may to be said to be probable. It should be formed under the same environmental conditions like struvite supplemented by a nickel source. The phosphate for crystallization is sufficient from the medium, whereas ammonium is very likely released from the cells during growth. Since biomineralization only occurs with the viable biomass, we can clearly associate biomineralization with physiological traits of the bacterium. This will enable future work on biomineralization of struvite-like substances. The broad heavy metal resistance of our bacterium will also facilitate the investigation of substitutions with other heavy metals. Hence, we have in our hands a system for the inducible biolith formation of struvites which could also be of importance for technological aspects such as waste water heavy metal cleansing.

Acknowledgement

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4 Discussion

4.1 Adaptation of microorganisms towards heavy metal resistance

Microbial life has conquered extremely hostile environments. It has been reported that habitats like AMD run-offs characterized by a pH of zero and a content of more than 100 g/L iron as well as the metals copper, arsenic, cadmium, zinc up to a range of tenths of grams per litre have been colonized by the archaeal iron-oxidizer *Ferroplasma* (Edwards et al., 2000). Colonization of this type of niche requires a high measure of adaptability.

Nevertheless, in a general view, the richness of bacterial diversity is yet unexplored to a very large extent. The difficulties in imitating the natural conditions that occur in the investigated niches and the duplication of niche characteristics on a laboratory-scale are the most challenging obstacles that have to be overcome for obtaining new taxa. The physiology of metal-adapted microorganisms determines the methodology that has to be applied for isolation and cultivation. There is a remarkable contradiction between the fact of more than 600 completely sequenced bacterial genomes (as at January 2007) and the numbers reported for yet undiscovered bacteria that range from 80% (Ward et al., 1990) to 99.9% (Davis, 1998). In order to expand our knowledge on microbial functions in an ecological context it seems worth-while to put more effort especially in the isolation of microorganisms from endangered and extreme environments like, e.g., mining sites and metallurgical plants. Well-adapted microbes isolated from these types of habitats can probably support remediation and are of great interest for strategies on environmental conservation. A lot of information on, for instance, taxonomy, resistance, and detoxifying mechanisms can be obtained from studies of total DNA/mRNA from the investigated environment. However, if a potential application on the cellular level should not be excluded, both, strain isolation and cultivation is an essential part of the analysis of microorganisms thriving in metalliferous habitats.

4.1.1 Impact of various heavy metals on morphology and physiology of single-celled bacteria and actinobacterial isolates

Heavy metals affect the microbial cell in various ways. On the macro- and microscopic level general changes in morphology, the disruption of the life cycle, increase or decrease of pigmentation are easy to observe and evaluate visually (Fig. 11). It has been shown that the impact of metals on the metabolism depends on the growth form. In consortia from mining sites the resistance towards different metals seem to be higher than for pure cultures (Sprocati et al., 2006). But over a year the species composition of an AMD site fluctuates seasonally, as has been shown for Iron Mountain, Richmond, USA (Edwards et al., 1999). Consequently, both approaches, pure culture analysis and research on consortia are important to gain knowledge for probable bioremediation applications.

Most of the strains discussed in the presented publications (I-VII) regarding their resistance, biosorption capacity, and metal-depending metabolic characteristics are isolates derived from a former uranium mining area in Eastern Thuringia, the so called Ronneburg district (Fig. 12). The mine field covers an area of 74 km^2 including 40 mine shafts and 3000 km mine drifts. Between 1952 and 1990 113.000 metric tonnes of uranium extracted from 154 million tonnes crude ore were produced in this district (Gatzweiler et al., 2001). 14 mine dumps with a total volume of 125 million m^3 of waste rock – containing considerable amounts of pyrite – are the major source for AMD generation. The AMD, in turn, determines the character of the habitats and their colonization by microorganisms through parameters like pH, metal content, ionic strength and redox potential. The microbial population of the Ronneburg heaps itself is dominated by Thiobacilli, but *Sulfolobus*/*Acidianus* spp. and *Leptospirillum ferrooxidans* occur as well (Schippers et al., 1995). Members of the genera *Pseudomonas* and *Aeromonas*, among others, could be isolated from the uranium waste piles of the neighbouring mining field in Saxony (Selenska-Pobell et al., 2001). But also members of the actinobacteria can be found in dump material, as it was shown for the metal leaching actinomycete *Nocardiopsis metallicus* (Schippers et al., 2002). The isolation campaigns during the presented studies focussed mainly on actinobacteria, due to their importance for mineralization processes in soil, the growth characteristics, the comparably high abundance and the relatively large quantities of biomass. These features illustrate the relevance of the group for bioremediation.

The investigated substrate samples of the former uranium mining area were strongly influenced by AMD containing, e.g., approximately 50 mg/L aluminum, more than 10 mg/L nickel and around 650 μg/L uranium (Haferburg et al., 2007). As long as AMD generation occurs, the metals are enriched in the soil/substrate. Only a fraction of the metals bound to the pedogenic matrix becomes easily mobilized and therefore bioavailable as it has been described for cadmium (Kothe et al., 2005). The bioavailability of different metals can be estimated by sequential extraction. It has been shown that the concentration of mobile metals in mining habitats has an influence on the sporulation of actinobacteria (Schmidt et al., 2005). The high metal burden in the habitat results in the loss of the capacity for sporulation. Nevertheless, strains obtained from these habitats displayed different grades of resistance towards nickel. Six actinobacteria isolates originating from an isolation campaign along an AMD contamination gradient were able to grow on minimal media containing up to 3000 ppm nickel – comparable to strongly nickel enriched dump material. Since isolation was achieved from the non-dormant state of the cells, metabolically active populations of actinobacteria are therefore supposed to occur in the investigated habitats.

The occurrence of actinobacteria in AMD influenced, extreme environments implies cellular resistance mechanisms. Metal resistance of a number of bacteria is well investigated and partly used in bioremediation (Lovley and Coates, 1997; Mergeay et al., 1978; Nies, 2003). In contrast to many microorganisms that were isolated from

metalliferous habitats, metal resistance of actinobacteria is a rather recent research topic and not comprehensively outlined yet.

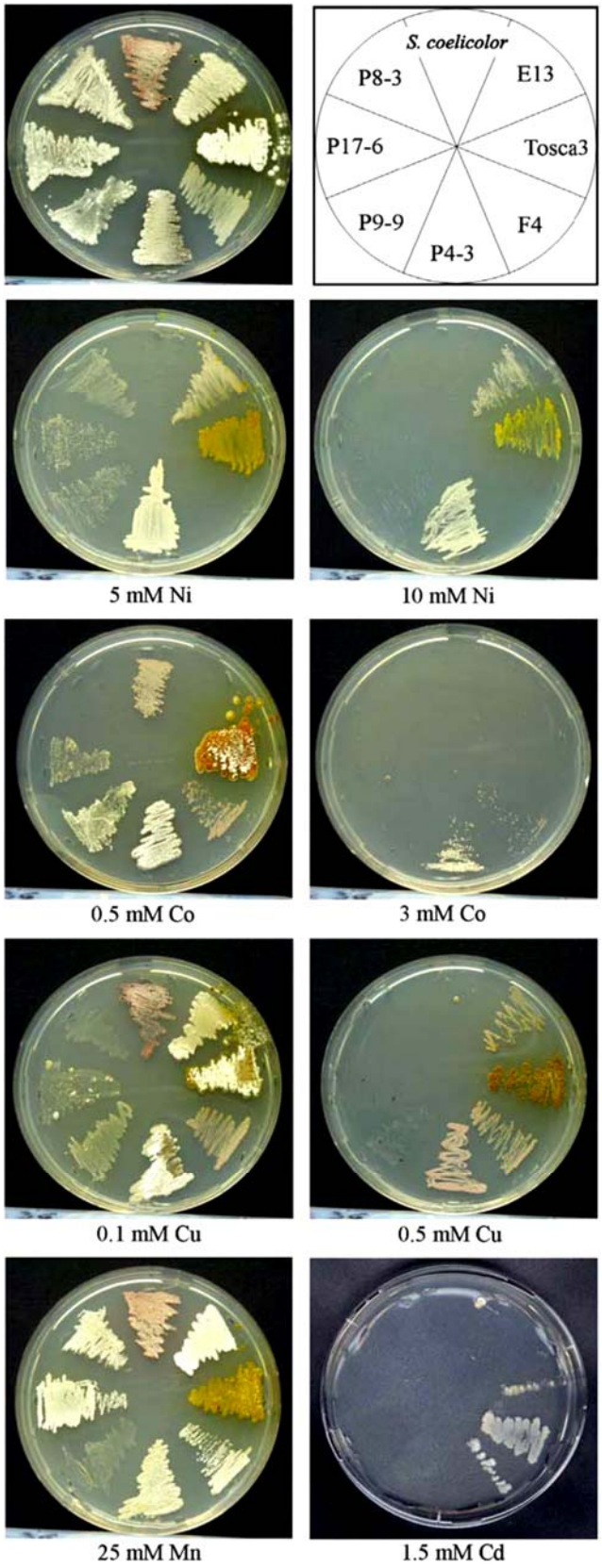

Figure 11: Plate assay for heavy metal resistance. Four strains from an isolation campaign and four control strains are shown with / without indicated concentrations of heavy metals (Manuscript III).

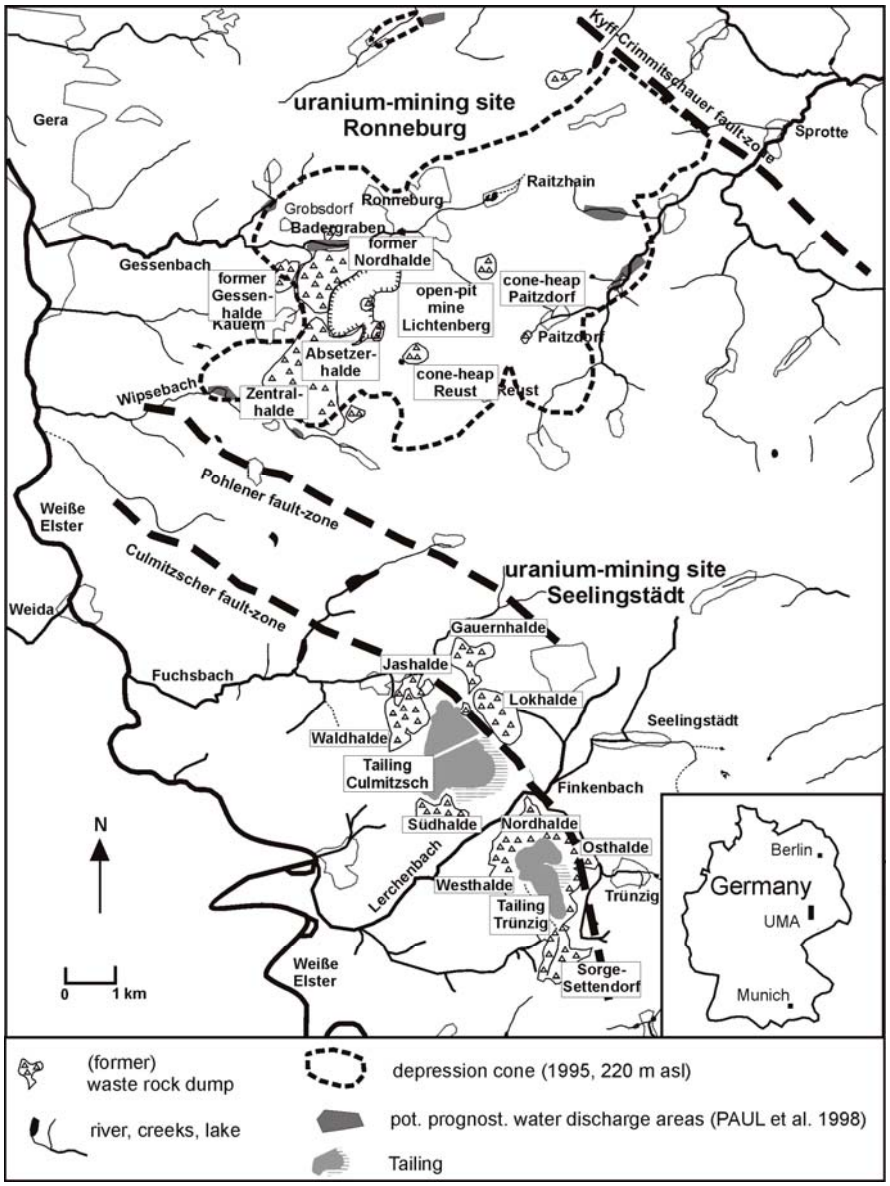

Figure 12: The former uranium mining site of Ronneburg and Seelingstädt, Germany (1950-1990), UMA: uranium mining area Ronneburg and Seelingstädt (map taken from: Merten et al., 2004; Manuscript II).

4.1.2 Abundance and distribution of nickel resistance among members of the taxon actinobacteria

Nickel is both an essential trace nutrient in nanomolar concentrations and a toxicant to microbial metabolism if over-concentrated, i.e. in the micro- or milimolar range. Enzymes like, e.g., hydrogenases, ureases, CO dehydrogenases and some superoxide dismutases depend in their function (and partially structural composition) on sufficient nickel supply. A surplus of intracellular nickel results in protein damaging especially by complexation of thiol groups, and can furthermore interfere with nucleic acids. The uptake of nickel is accomplished by the Mg^{2+} transport system or, in case of nickel deficiency, by specific nickel uptake systems. Intoxication of the cell with nickel can only marginally be prevented by regulation of the uptake systems since magnesium is an essential

macronutrient and required in larger quantities than nickel. The cellular counteractions are either performed by expression of active nickel efflux transport systems, or by complexation with nickel chelating substances, limiting free Ni^{2+} within the cell.

Resistance of actinobacteria towards cadmium, copper and mercury has been reported and is well investigated in the case of mercury (Amoroso et al., 1998; Ravel et al., 1998). For nickel resistance, knowledge has been accumulated regarding various isolates of, e.g., *Cupriavidus metallidurans* (Grass et al., 2000), *Achromobacter xylosoxidans* (Schmidt and Schlegel, 1989), *Hafnia alvei* (Park et al., 2004) and *Pseudomonas aeruginosa* (Sar et al., 1998), but only limited literature is available on nickel resistance of members of the group of actinobacteria. Nevertheless, as has been reported for the actinobacterial genus *Microbacterium*, the accumulation of nickel by nickel hyperaccumulator plants is influenced by the microflora of the rhizosphere (Abou-Shanab et al., 2003). *Microbacterium arabinogalactanolyticum* survives in nickel rich soils and promotes the nickel accumulation of the nickel accumulator *Alyssum murale*. Another example of nickel tolerating actinobacteria of the rhizosphere is *Frankia*. Members of the nitrogen-fixing genus *Frankia*, living in symbiosis with alder (*Alnus glutinosa*), were found to tolerate more than 2.0 mM nickel and to increase yield when nickel is added. This has been correlated with an enhanced hydrogenase synthesis (Wheeler et al., 2001). The increased synthesis of nickel containing hydrogenases is thought to be due to energy conservation by re-oxidising of the hydrogen liberated by nitrogenase.

In order to survey the behaviour of actinobacteria – isolated from different habitats – in presence of nickel, an isolation campaign was conducted. The distribution of nickel resistance among 100 *Streptomyces* strains originating from contaminated and non-contaminated sites was tested (Fig. 13). Special emphasis was laid on the genus *Streptomyces*, with over 500 taxa the genus most abundant in species within the class *actinobacteria*. Habitats for investigation were chosen from (1) a serpentine soil of Tuscany, Italy, naturally rich in nickel; (2) a mining site in Thuringia, Germany, that was approximately 40 years in operation, characterised by patches of high concentrations of nickel among a set of accompanying metals; (3) an area inside the province San Miguel de Tucumán, Argentina polluted by effluents of both a copper filter plant and sugar mill industries. Subsequently the received isolates were screened on resistance towards nickel and thereby classified as (a) sensitive, if growth ceased in presence of 0.2 mM nickel; (b) tolerant, if growth still occurred at 0.5 mM nickel; (c) resistant, if isolates could grow on plates containing 5.0 mM nickel and (d) highly resistant, if growth was possible on 10.0 mM nickel. The results could show adaptation of the actinobacteria isolates to metalliferous environments. Strong resistance has been observed for the strains isolated from the soils contaminated with metals and organic compounds. It is known for a variety of environmental bacterial isolates – but not yet for actinobacteria – that a co-resistance against metal and organic loading can be expressed. Besides the high resistance for some isolates especially of the grossly polluted soils originating from San Miguel de Tucumán,

the screening revealed the comparably wide distribution of a basic nickel tolerance up to 0.5 mM Ni^{2+} (Haferburg et al., 2004). This basic tolerance might be attributed to the taxon-specific characteristics and the distinct plasticity of microorganisms living in highly inconstant habitats.

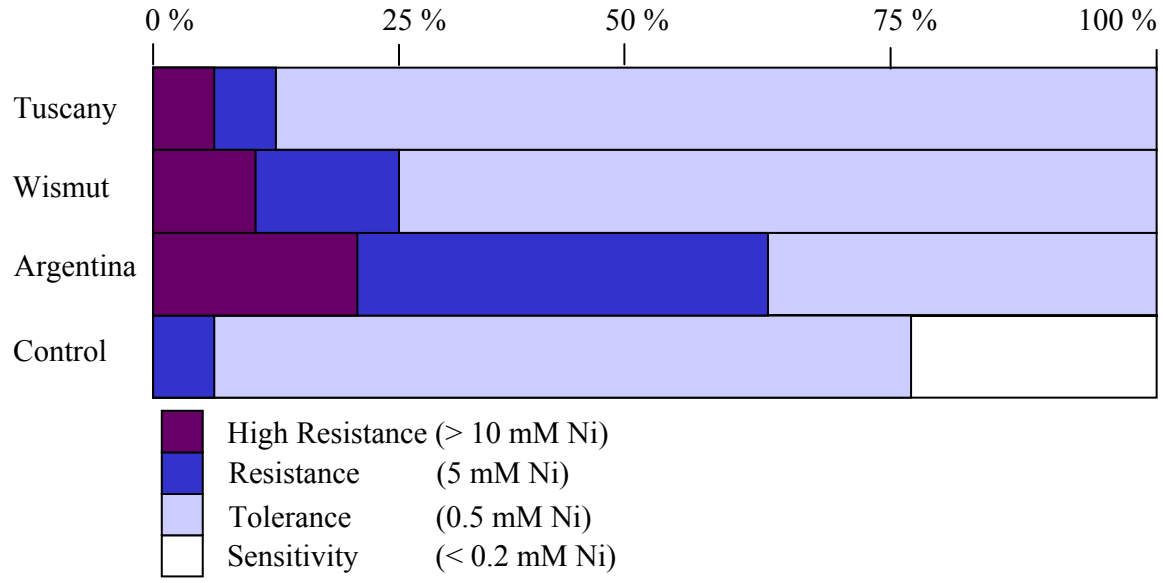

Figure 13: Nickel resistance levels of streptomycetes isolated from contaminanted and non-contaminated soils. Tuscany: naturally nickel-rich serpentine soils, Wismut: soil samples of a former uranium mining site, Argentina: site in province San Miguel de Tucumán polluted with sugar mill and mining effluents, Control: soil samples of the Botanical Garden and the public park of Jena (Manuscript I).

Nickel with its well investigated toxic effects on the metabolism of microbe, plant and human-being is reported to be a common heavy metal released to the biosphere due to mining operations and AMD generation. As has been studied at different sites at the former uranium mining area in Eastern Thuringia, the nickel concentrations are comparably high for many locations. High nickel concentrations in the habitat in combination with the fact of good solubility at even less acidic pH values is the reason for a mobile and fairly bioavailable fraction of nickel. Since the property of resistance mechanisms seems to be the precondition for survival, monitoring of both, the bioavailable fraction of nickel and the composition of the microbial community in the polluted area could be one approach to track the success of a particular bioremediation strategy. The loss of well characterized nickel resistance mechanisms inside a microbial population during the time of remediation could be considered a hint at a decline of microbe-metal interactions.

4.2 Biosorption capacity of mining isolates

It is the pragmatic goal of current bioprocess research on metal removal from treatable sources to identify species of microorganisms that are capable of efficient uptake of environmentally and economically important metals (Unz and Shuttleworth, 1996). As a result of metal toxicity, living cells may be inactivated; therefore most living-cell systems exploited to date have been used to decontaminate effluents containing metals at subtoxic concentrations (Gadd and White, 1993). However, the studies introduced in the following focus on metal resistant microorganisms to investigate the possible exploitation of both the purely physical sorption capacity and the active metal uptake with a subsequent intracellular sequestration. Both mechanisms could support strategies applicable to bioremediation when effluents contain toxic concentrations of metals. Decontamination of AMD containing high concentrations of numerous metals requires vital microbial cells surviving harsh environmental conditions. Therefore, especially strains from metal contaminated habitats like the former uranium mining site in Eastern Thuringia were investigated for resistance mechanisms and biosorption characteristics.

4.2.1 Using rare earth element patterns to analyze biosorption

Rare earth elements, or lanthanides, comprise a group of 15 elements with fairly similar geochemical properties and an approximately similar content in different soils of various regions. However, the concentrations of REE in mining areas can reach high levels (Tab 2). During weathering processes, the REE are fractionated (Kabata-Pendias and Pendias, 2001b). The differences in the fractionation of REE are dependent of the sorption characteristics of the substrate.

Table 2: Physicochemistry and rare earth element concentrations of surface water and seepage water from sampling points close to a creek and two dumps of a former uranium mining area in Eastern Thuringia (Manuscript II).

Sample	Date	pH	El. conductivity ($\mu S\,cm^{-1}$)	Redox potential (mV)	T(°C)	Σ Rare earth elements ($\mu g\,l^{-1}$)
G16	07–11–2001	4.88	6470	480	6.4	712
Q4 S	24–10–2000	3.55	11,900	440	12.2	2490
Q4 N	26–06–2001	2.77	11,900	580	16.2	2200
G13	26–06–2001	7.82	1970	140	18.2	< 0.1
G14B	26–06–2001	3.41	11,200	480	14.5	1845
G14A	26–06–2001	5.94	3100	310	17.2	67
G18	26–06–2001	5.70	2850	290	13.9	41
G7	26–06–2001	4.51	2700	490	15.5	41

Numerous studies refer to the sorption of REE by microorganisms (Johnson and Kyker, 1961; Philip et al., 2000). But only few literature reports reflect on the mechanisms of REE sorption. The potential role of bacteria on REE fractionation processes is not yet clearly understood (Takahashi et al., 2005; Ozaki et al., 2005). However, if REE fractionation is planned to be applied as a monitoring tool for remediation, the reason of the different fractionation behaviour of geogenic and biogenic substrates has to be elucidated. In contrast to a geogenic matrix, the microbial REE sorption comprises the two subprocesses of passive cation attachment to the cell surface and metal uptake with intracellular accumulation. For a *Pseudomonas aeruginosa* strain endowed with a high metal uptake capacity it was shown that around 85% of the REE can be desorbed using citrate buffer (Philip et al., 2000). The fractionation analysis of a *Bacillus subtilis* strain and an *Escherichia coli* strain showed an enrichment of especially heavy REE on the cell surface with at least two binding sites, i.e. carboxylate and phosphate groups (Takahashi et al., 2005). Even so here it has been hypothesized that REE uptake in conjunction with adsorption is responsible for the particular microbial fractionation patterns in some strains. The strong differences between the fractionation patterns of several strains are very likely not explainable solely with adsorption processes of cell envelopes. The composition of the REE binding sites of the cell envelope is supposed to be rather similar for the different Gram-positive strains. It remains to be shown how accumulation of specific elements of the chemically fairly similar set of REE, resulting in fractionation, proceeds. Incubation of AMD containing comparably high concentrations of REE with different microbial strains does not necessarily always lead to an alteration in the fractionation pattern (Fig. 14). In our screening only the fungal strain F1 (*Cordyceps sinensis*) displayed the capacity to alter the REE pattern of the tested AMD water. The investigation of the REE fractionation pattern of isolates of the genera *Bacillus*, *Micrococcus*, *Streptomyces* could show small differences in fractionation at the genus level (Manuscript V). Nevertheless, the time dependent differences in the fractionation behaviour of the strains are remarkable. The capacity of some microorganisms to fractionate the REE pattern by uptake and sorption (including precipitation) can be used as information of the particular strain on its remediation abilities.

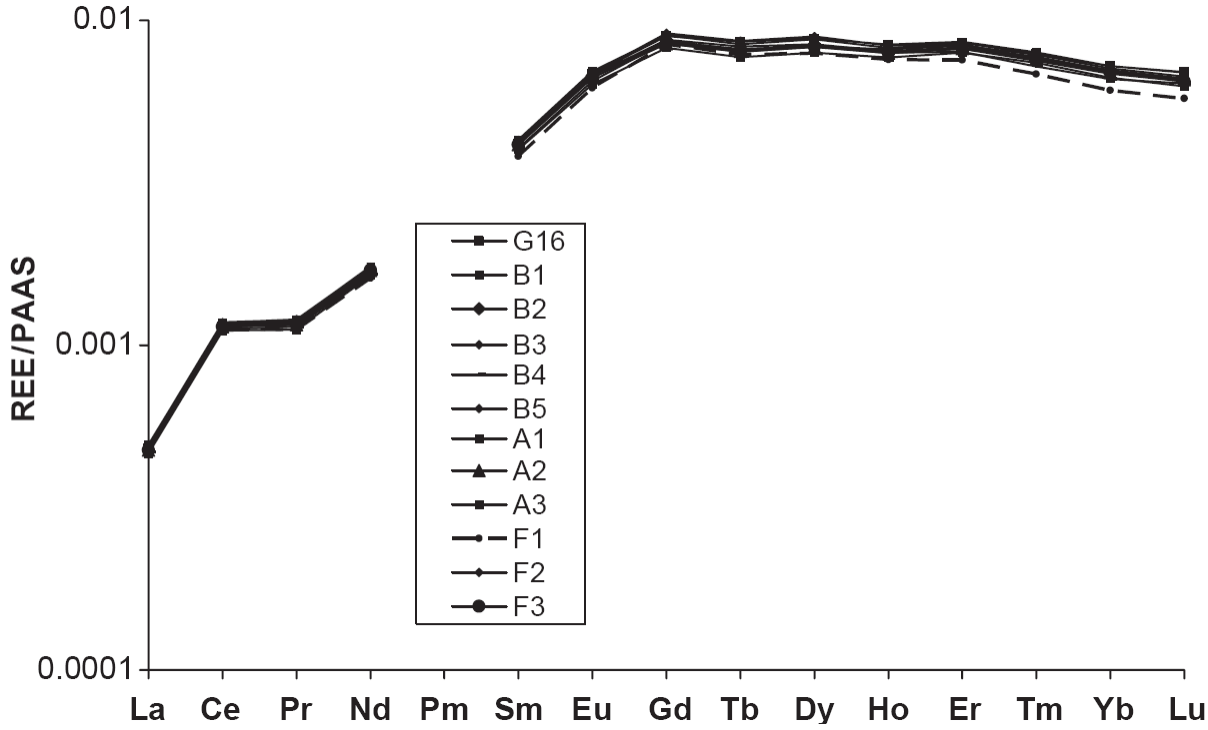

Figure 14: Normalized REE patterns of AMD G16 incubated with actinobacteria (A), bacteria (B) and fungi (F), isolated from the Gessental area (Manuscript II).

The concept of how to make use of the REE fractionation as a monitoring strategy is based on the comparison of the geological and microbial fractionation patterns. In the case that REE analysis of environmental samples, e.g. pore water, displays a fractionation that can be classified as geologically caused, the mobility is thought to be comparably limited and the environmental stress should be low. If the fractionation pattern is microbiologically caused, it is concluded that a pool of mobile REE influence the habitat. This means that bioavailability and mobility of both, REE and chemically similar behaving metals, is high. This situation would display an ecological burden to an endangered biota and demands a variation of the remediation course.

4.2.2 Screening on microorganisms active in biosorption of heavy metals from acid mine drainage

The capacity of microbial cells for biosorption is considerable. Bacteria, for instance, have a cell volume of 1.5-2.5 μm^3 resulting in a high surface to volume ratio. If a cube with the edge length of 1 cm is divided into 10^{12} cubes with an edge of 1 μm, the entire surface of the 10^{12} small cubes is 10,000 times larger than the surface of the big cube. The large surface area permits not only the efficient uptake of nutrients and the release of metabolic waste products, but the interaction with the mobile metal fraction of the environment as well. Although Gram-positive and Gram-negative bacteria differ markedly in the structure of the cell envelope, the potential for biosorption is comparable, due to the similar

composition of functional groups appropriate to bind (metal) cations (Jiang, 2004). Nevertheless, microbial isolates from contaminated environments behave remarkably different in biosorption of metals from AMD. In order to test the differences of biosorption, a set of 15 microbial isolates distinguished by a pronounced resistance towards the "in-situ" metal cocktail of seepage water from a former uranium mining site in Eastern Thuringia were selected and tested for their survival in AMD. Subsequently, the metal biosorption of those strains that survived the treatment was investigated. Of the tested isolates (5 single-celled bacteria, 5 actinobacteria and 5 fungi), 11 could survive extended incubation in AMD. One fungal isolate, later identified as *Cordyceps sinensis* F1, and an actinobacterium, subsequently identified as *Nocardia carnea* A2, displayed a strong biosorption capacity for uranium. Both strains extracted approximately 50% of the uranium from the AMD solution (Tab. 3). An isolate of the single-celled bacteria group showed a noticeable aluminum biosorption by extracting more than 10% of this highly toxic and metabolically afunctional element. Many parameters besides metal toxicity, metal composition of the mix and total metal content of the solution influence the biosorption. The age of the culture determines to a great extent the biosorption capacity of the cells as it was tested for uranium uptake of *Streptomyces longwoodensis* (Friis and Myers-Keith, 1986). Older cells (> 14 h) accumulated about double the amount of uranium of younger cells (< 14 h). *Streptomyces albus* in contrast to *Pseudomonas stutzeri* accumulates metals from a mixed metal solution preferentially at the cell wall. However, the electron-microscopic analysis revealed that the cell walls of neighbouring cells contained metals to a strongly varying degree (Mattuschka et al., 1994). The differing sorption by cells of one isolate hint at the dependence of the biosorption on the stage in the life cycle. Biosorption of uranium from a solution by *Streptomyces* strains seems to be a fast process. Within seconds or only few minutes, the tested dead and vital biomass is saturated (Golab et al., 1990; Golab et al., 1991; Horikoshi et al., 1981). In comparison, the efficiency of the removal of uranium from solution is similar in the presented publication (Haferburg et al., 2007) and the literature data, although the applied incubation times differed to a great extent (one week vs. minutes). This means the fixation of the uranium containing compounds from solution onto the biomass seems to be stable for at least one week. In the context of bioremediation the outstanding importance of research on both biosorption and resistance of microorganisms that can thrive in metalliferous environments cannot be overlooked.

Table 3: Concentrations of metals in medium containing 50% AMD after 7 days of incubation of selected isolates (Manuscript IV).

	^{27}Al (µg/L)	^{53}Cr (µg/L)	^{55}Mn (µg/L)	^{59}Co (µg/L)	^{60}Ni (µg/L)	^{63}Cu (µg/L)	^{66}Zn (µg/L)	^{86}Sr (µg/L)	^{111}Cd (µg/L)	^{208}Pb (µg/L)	^{238}U (µg/L)
Medium	22,770	4.983	35,080	1008	5648	636.8	1497	356.2	20.5	2.255	582.2
Incubated	23,770	5.081	37,100	1100	6041	693.9	1595	346.2	22.25	9.754	612.2
Bacteria:											
B1	24,410	5.376	36,650	1080	5967	625.6	1646	362.3	22.26	4.343	536.7
B2	23,220	4.952	34,810	1033	5701	678	1574	358.1	20.87	5.375	542.3
B3	23,500	5.406	36,340	1080	6002	675.5	1574	370.5	21.44	3.404	496.5
B4	22,960	5.04	34,890	1019	5695	622	1555	358.8	20.46	3.605	564
B5	**18,710**	5.27	37,660	1125	6207	652.9	1524	368.9	22.02	12.22	469.1
Actinobacteria:											
A1	23,780	5.472	36,610	1081	5988	675.7	1588	372.4	22.26	9.04	474.2
A2	22,070	4.918	36,990	1080	5960	688.5	1598	364.4	20.94	4.419	**301.4**
A3	22,940	5.499	36,430	1084	6021	635.1	1683	362.2	20.47	5.348	445.8
Fungi:											
F1	22,360	5.253	36,600	1076	5952	602.2	1520	372.3	21.83	4.593	**319.5**
F2	23,130	5.416	36,400	1081	5958	667.1	1592	366.6	21.63	5.695	539.8
F3	20,960	5.106	35,780	1057	5911	648	1577	356.6	20.62	2.337	548.8

4.2.3 Time course of metal sorption from acid mine drainage

The sorption of metals from AMD by microbial cells is a time-dependent and (partially) reversible process. The three investigated isolates from a mining area, *Streptomyces acidiscabies*, *Micrococcus luteus* and *Bacillus* sp. are characterized by a fast sorption. After incubation of the vital biomass for one hour more than 50% of the content of most of the metals in the AMD solution is already sorbed onto the cells or cell-induced precipitated, respectively (Fig. 15). The percentage of the bioprecipitated metal fraction is supposed to be low, due to a pH of 2.8 in the AMD. Low pH is not favourable for crystallization. Therefore it is concluded that sorption comprises essential adsorption onto the cell envelope and uptake with subsequent cytosolic accumulation. After one week of incubation 85 % of the content of almost all metals is sorbed. The three investigated strains display similar results. Among the strains *Bacillus* sp. W-28 shows the strongest sorption capacity for uranium with approximately 95 % (Fig. 15). Interestingly, the sorbed metals are not stably fixed at/inside the cells. After four weeks of incubation in AMD the sorption is less strong compared to the incubation for one hour. Even if cells lyse the amount of constituents of the cell envelope that are prone to complex metals should not be markedly reduced. Therefore it is concluded that a relatively high proportion of sorbed metals is cytosolically accumulated. After cell lysis, which seems to have occurred after the first week, the cytosol is not compartmentalized from the AMD any further and the intracellularly accumulated metal fraction is released again. Thus, the metal concentration of the supernatant is comparably high again after four weeks of incubation. The number of vital, metabollicaly active cells does not change significantly within the first week of incubation. Later on cells start to lyse and the biosorption activity is lost. Nevertheless, from all three sampling dates aliquots of the incubated AMD were distributed on agar

plates containing rich medium and the strains could be regrown to each point of time. Consequently, the strains survived four weeks of incubation in AMD with pH 2.8 and a decidedly high metal content. It is known that the genera *Streptomyces*, *Micrococcus* and *Bacillus* possess the ability to survive adverse environmental conditions by the formation of the resistant stages exospores, dormant cells, and endospores. This characteristic can be seen as part of the overall resistance towards metals in conjunction with the influx preventing sorption of metals onto the cell envelope as important resistance mechanism in both habitats of mining areas and treatment plants like wetlands.

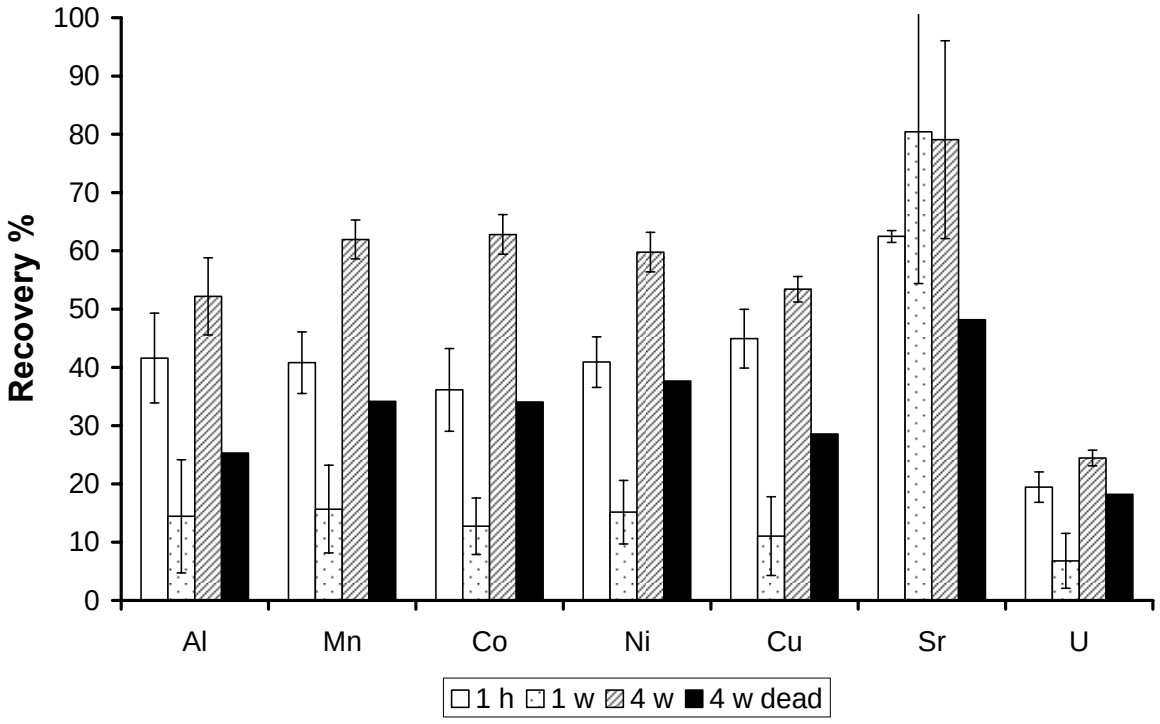

Figure 15: Recovery of metals from AMD after incubation with *Bacillus* sp. W-28 for 1 hour (1h), 1 week (1w) and 4 weeks (4w) with living or dead biomass (4w dead), respectively. (Manuscript V).

4.3 Extracellular sequestration of heavy metals

Metals can – as part of the microbial resistance response – be extracellularly immobilized. Metals are immobilized by either complexation with released chelating compounds or precipitation across the cell envelope depending on structural features. Particular structures of the cell envelope as, e.g., S-layer proteins (Beveridge et al., 1997), act as nucleation centre and facilitate crystal growth resulting in the formation of biominerals. However, precipitation and biosorption are sometimes overlapping phenomena and it can be difficult to assign the contribution of each to metal immobilisation (Glasauer et al., 2001).

4.3.1 Release of chelators as possible resistance strategy

The genus *Streptomyces* is known to be the largest antibiotic-producing group and is still of central importance in the identification of medically relevant natural compounds. With the discovery of streptothricin and streptomycin in the 1940ies, a systematic screening of antibiotics on isolates of this genus and related taxa was initiated (Berdy 1974, Waksman, 1963). Later on, the screening efforts started to decrease due to the difficulties to obtain novel compounds. This is in contrast to the continued demand for new antibiotics against resistant pathogens (Chopra et al., 1997). Interestingly, it was calculated that only a tiny fraction of the antimicrobial compounds the genus is capable to produce has been discovered so far. A total number in the order of 100,000 antibiotics was calculated using a conservative estimate (Watve et al., 2001). It can be concluded that the decline in the discovery of new metabolites is due to limitations in the screening efforts rather than an exhaustion of potentially produced compounds raising the question of developing new screening strategies.

Could the metabolic response of actinobacteria on metals be used to trace new secondary metabolites or could metal supply even direct and support metabolite synthesis? Metals facilitate secondary metabolism not only in actinobacteria, but in some other prokaryotic taxa and fungal groups as well (Weinberg, 1990; Chakrabarty and Roy, 1964). The complex effect of metals on secondary metabolism can be seen best with results of product research: A *Streptomyces galbus* strain producing an antifungal antibiotic is enhanced in production if the fermentation medium is supplemented with copper, zinc or iron, whereas nickel and cadmium addition lead to a reduction of antibiotic concentration in the same strain (Paul and Banerjee, 1983; Raytapadar et al., 1995). The addition of as little as 0.01 μg/mL of cobalt to the medium results in the production of over 93% coumermycin A_1 by *Streptomyces rishiriensis* compared to 40-75% production in the control (Claridge et al., 1966). Chromium is known to have a stimulatory effect on both actinorhodin production and growth yield of the model streptomycete *S. coelicolor* (Abbas and Edwards, 1990). However, there is no generalization that could be made for the effects that metals exert on metabolite production. The isolates FSU-E13 and FSU-JE12 are two of the tested strains in this thesis (manuscript VI) displaying a remarkable antibiotic activity towards *Staphylococcus aureus* and *Mycobacterium smegmatis* after addition of nickel to the medium with a final concentration of 0.3 mM (Tab. 4). There was no antibiotic effect in the metal free control culture. Therefore we concluded that during a common screening procedure the strains would have been discarded as non-producer strains and the capacity of antibiotic production would have remained undiscovered.

Table 4: Inhibition assay of extracts of supernatant (S) and mycelial fraction (M) of actinobacteria from contaminated (c), non-contaminated (nc) and serpentine (ser) environments. 1 – inhibition, 2 – moderate inhibition, 3 – strong inhibition, M1 – rich medium, MM – minimal medium (Manuscript VI).

isolate	production culture	*S. aureus*		*E. coli*		*M. smegmatis*		*C. albicans*	
		S	M	S	M	S	M	S	M
FSU-F4	M1+Cd		**3**				**3**		
(c)	M1		**3**				**3**		
	MM+Cd								
	MM								
FSU-E13	M1+Ni		**2**				**3**		
(c)	M1								
	MM+Ni								
	MM								
FSU-PT01	M1+Ni		**2**				**3**		**3**
(c)	M1		**2**				**3**		
	MM+Ni								
	MM								
FSU-PT05	M1+Ni	**3**						**3**	
(c)	M1	**3**					**1**	**2**	
	MM+Ni								
	MM	**2**	**2**	**3**			**1**		
FSU-PT13	M1+Ni						**3**		
(c)	M1						**3**	**2**	
	MM+Ni								
	MM						**2**		
FSU-Tosca2	M1+Ni	**3**	**3**	**2**		**3**	**3**		
(ser)	M1	**3**	**3**			**3**	**3**		
	MM+Ni	**3**	**3**			**2**	**2**		
	MM	**3**	**3**			**3**	**3**		
FSU-Tosca3	M1+Ni	**3**	**3**				**3**		
(ser)	M1	**3**					**3**		
	MM+Ni		**3**						
	MM								
FSU-Tosca4	M1+Ni						**3**	**3**	
(ser)	M1						**3**	**3**	
	MM+Ni								
	MM								
FSU-JE12	M1+Ni						**3**		
(nc)	M1								
	MM+Ni		**2**						
	MM								
FSU-Wip14	M1+Ni	**3**		**1**		**2**		**2**	
(nc)	M1	**3**				**3**		**2**	
	MM+Ni						**2**		
	MM	**3**				**2**	**2**		

Metals often affect secondary metabolism in actinobacteria indirectly via induction of signal molecules (gamma-butyrolactones) as it was found for the inducing effect of cobalt on A-factor in *Streptomyces griseus* successively stimulating anthracycline production (Gräfe et al., 1985). The signal molecules, although already considered as secondary metabolites, are crucial for the linkage of primary and secondary metabolism. Thus, their

regulation by metals can affect morphological differentiation and control production of antibiotics and pigments. Besides the effect of metals on antibiotic production, the stimulation of pigment production by metals has been investigated with some actinobacteria. It could be shown that the rare earth element ytterbium induces at a concentration of 25 ppm the production of a reddish pigment with a naphthoquinone-like structure in a *Streptomyces* isolate (Kamijo et al., 1999).

Melanins, pigments produced by a wide variety of microorganims, are characterized by a strong metal affinity (Nosanchuk and Casadevall, 2003). The induction of the synthesis of a melanin-like pigment after nickel supplementation was shown for strain *Streptomyces acidiscabies* E13. The metal sequestering characteristics of melanin are supposed to play an important role in the nickel resistance of this strain. Additionally, the release of nickel chelating compounds into the supplemented medium could be shown by thin layer chromatography (Fig. 16). A number of secondary metabolites, among them antibiotics like isatin (Gräfe and Radics, 1986) and pigments like actinorhodin (Coisne et al., 1999) or melanin (Beausejour and Beaulieu, 2004), are known for their metal chelating behaviour and participate very likely in extracellular metal resistance processes.

The influence that metals have on antibiotic production and secondary metabolism in general is highly complex and investigated only in traces. Metals often display intense effects even in minute concentrations. Impurities at negligible quantities can have intense effects on product yield as has been found for iron leached from fermentation vessels (Mueller, 1941). Therefore, the impact of metals on metabolite production is difficult to analyze. Nevertheless, the application of research results on metal-microbe-interaction regarding secondary metabolism could probably support fermentation control with optimized product yield or even be applied to future screening programs.

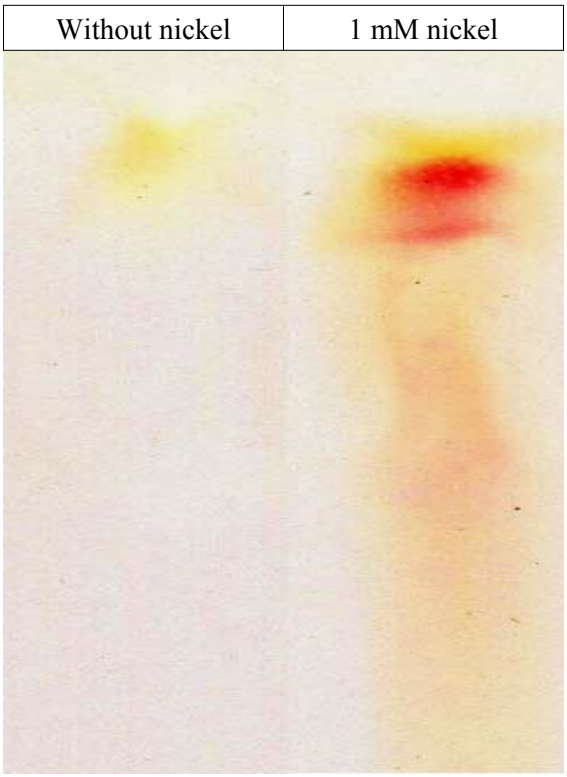

Figure 16: Exuded nickel sequestering compounds of metal resistant *S. acidiscabies* E13, separated by thin-layer chromatography and stained with di-methyl-glyoxime. The uppermost, brown-coloured band is assumed to be melanin (Manuscript I).

4.3.2 Biomineralization as putative metal resistance mechanism

The most basic processes in biomineralization operate at the nanometer scale and involve macromolecules, like proteins, directly controlling the nucleation and growth of the mineral phase (Mann and Weiner, 1999). The cell-mediated deposition of crystalline materials can proceed both within and outside the cell, but always involves anionic matrix molecules which function to sequester the relevant ions (Boskey, 2003).

During the investigations on the strain *Streptomyces acidiscabies* E13 to elucidate the resistance mechanisms towards nickel, aggregates of greenish crystals associated with the substrate mycelium of the colonies appeared in plate cultures containing high concentrations of solute $NiCl_2$ (Fig.17). Using dead biomass of the strain distributed on nickel containing solid media in a control, no such phenomenon could be observed. Therefore it was concluded that biogenic processes are responsible for nickel precipitation and crystal formation. Active biomineralization is well studied for a great number of microorganisms (Mann, 1988). Electron probe microanalysis and X-ray powder diffractometry revealed the grown bioliths as $Ni(NH_4)(PO_4)\bullet 6H_2O$, preliminary named nickel-struvite. If nickel can be hindered from entering the microbial cell by extracellular precipitation it becomes understandable how growth is maintained on media

supplemented with toxic concentrations of up to 15 mM nickel. In nature where streptomycetes are typical soil microbes, such processes may be of eminent importance to colonize a metalliferous habitat. This is the first report on nickel-struvite as biogenic mineral. Whether the biomineralization of $Ni(NH_4)(PO_4)\bullet 6H_2O$ from $NiCl_2$ containing medium by the nickel resistant *Streptomyces* strain could be applied for the recovery of nickel remains to be tested. Nickel is a valuable metal and several attempts have been made for the recovery from concentrates (Dew and Miller, 1997; Rawlings et al., 2003). The use of mycelia of strain *S. acidiscabies* E13 for the nickel recovery from collected metal containing mine drainage could probably be both an approach of bioremediation and the possibility of metal recycling.

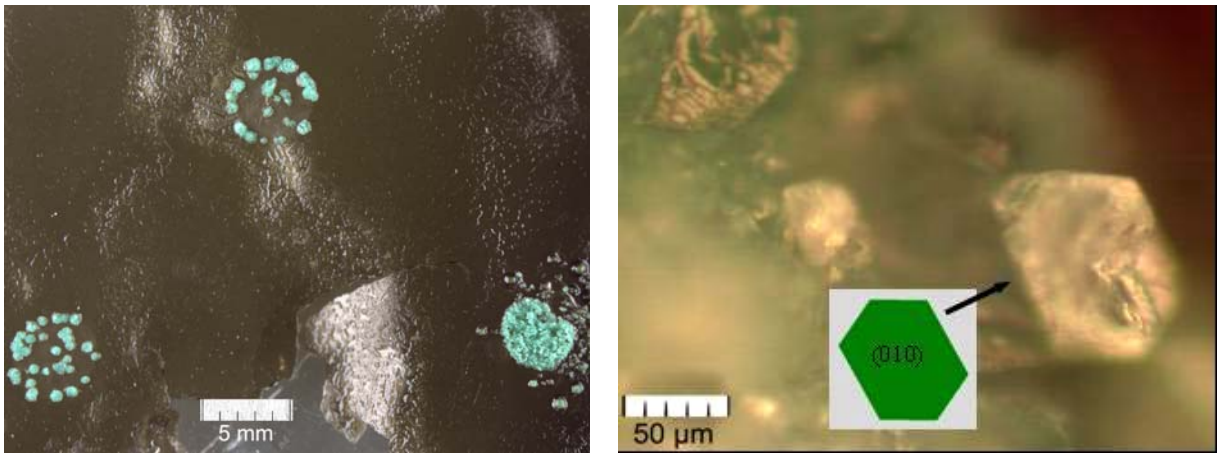

Figure 17: Crystalline aggregates of $Ni(NH_4)(PO_4)\bullet 6H_2O$ covering the area of colonies of *Streptomyces acidiscabies* **E13 in plate culture (left). Habit of a single nickel-struvite crystal (right), (Manuscript VII).**

5 Conclusions

Research on the behaviour of microorganisms in metalliferous environments is an integral part of geomicrobiology. The microbial impact on the fate of minerals and geologically significant compounds of mining areas is of major interest since its investigation leads to an understanding of the cycling (or accumulation) of metallic elements, the formation/stability of biotopes and the formation of altered compounds.
How inseparable geological processes and microbial activity in the lithosphere are allied has been expressed with the term geosymbiosis (Caldwell and Caldwell, 2004). Geosymbiosis describes the reciprocal relationship between restructuring/proliferation of a mineral and a microorganism. In the microbe-mineral interaction, both geosymbiotic partners affect and are affected in return.

Table 5: Examples for the formation and dissolution of ore deposits by metabolic processes of microorganisms.

Formation of ore deposits	← **Microbial activity** →	**Dissolution of ore deposits**	**Reference**
Banded iron formation	Iron-oxidizing phototrophs		Widdel et al., 1993
Sulfide ores (e.g. ZnS)	Sulfate reducing bacteria		Labrenz et al., 2000
Magnetite (Fe_3O_4), uraninite (UO_2) ores	Hypertermophilic microbes		Kashefi and Lovley, 2000
	Siderophore producing microbes	Iron leaching from minerals	Macrellis et al., 2001 Cocozza et al., 2002
	Anaerobic respiration	Reductive dissolution of Fe(III) oxides	Cummings et al., 1999
	Generation of acid mine drainage	Pyrite oxidation	Blake et al., 1993
Biomineralization and bioaccumulation	Heavy metal resistance mechanisms	Chelator production	this study (Manuscripts I-VII)

Metabolic processes of microorganisms have not only been identified as cause for the dissolution of pyrite and other rocks often resulting in the generation of AMD. In contrast, reverse effects of the microbial metabolism can also contribute to the formation of certain ore deposits over geological time (Table 5). The microbial formation of ore deposits is based on the capability of organisms to precipitate metals from solution. Thus, microbes participate constitutively in shaping the physical world by precipitating ore deposits (Newmann and Banfield, 2002).

Of course, on the opposite, the influence metals have on microorganisms is of the same importance for the development of a habitat. Biosorption, bioprecipitation, extracellular sequestration, transport mechanisms, cytosolic chelation are constituents of the overall process called “microbial resistance towards metals”. Which particular processes in a resistance reaction participate – and to which degree they cooperate – depends on the organism, the environment, and the (artificial) metal burden they are exposed to. Due to the high complexity of the cellular response, resistance is studied best on a molecular base. One example from this work is the strain *Streptomyces acidiscabies* E13. Nickel resistance of *S. acidiscabies* E13 can be characterized as synergistic interplay of intra- and extracellular processes which are based on the functional properties of the strain. The extracellular processes comprise metal complexation with chelating compounds like melanin (manuscript I) and precipitation of metals in form of crystalline nickel-struvite (manuscript VII).

The entire branch of the molecular geomicrobiology is attributed to the search for molecular-level understanding of coupled biological and geochemical processes (Macalady and Banfield, 2003). Since microbiologists seem to know only about 1% of the worldwide occurring prokaryotes, the understanding of the role bacteria play in a geogenic and pedogenic context is very likely to be changed deeply as soon as more habitat relevant microbial functions can be described. To gain insight into the comprehensive geomicrobial processes, molecular techniques have to be combined with classical approaches including research on yielding isolation techniques and culture media (Oremland et al., 2005). Especially for the strongly interdisciplinary field of geomicrobiology spanning from the microorganism to the mineral a broad set of methods and techniques is necessary to be applied, as this thesis might have shown.

6 Summary

The objective of this thesis is to contribute to the understanding of the interactions between metals and microorganisms with respect to

1. Isolation, taxonomic characterization and metal resistance potential of actinobacterial isolates derived from (a) the former uranium mining site in Eastern Thuringia, (b) serpentine outcrops in central Italy and (c) non-metalliferous habitats of the surrounding of Jena
2. Adaptability towards Ni and habitat-dependent variability of Ni tolerance
3. Resistance mechanisms of streptomycetes enabling growth at high Ni concentration
4. Biosorption capacity (Al, Cu, Ni, U, rare-earth elements) of various mining isolates
5. Changes in secondary metabolism of actinobacteria under the influence of Ni or Cd
6. Biomineralization of a nickel containing compound

Few studies exist on metal resistance of Gram-positive bacteria, and moreover on the ecologically very important group of actinobacteria. Most previous studies, reported in literature, on metal resistance mechanisms focussed on single processes, like cation efflux transport systems, despite the fact that microorganisms often antagonize stressors with a multiplicity of responses. The presented resistance studies were therefore aiming at three different responses of actinobacteria under conditions of metal stress: intracellular metal sequestration, metal immobilization at the cell envelope, and excretion of metal chelating compounds into the extracellular milieu.

Areas in which ore mining has been performed for many decades are ideal sites to investigate the influence of metals on bacteria. The production of 124 million tons of uranium ore within the 74 km^2 large Ronneburg district in Eastern Thuringia, Germany, led to the formation of metalliferous soil habitats on a grand scale. Nickel, commonly associated with uranium ores, has been found in concentrations of up to 2000 ppm in the dump material. The high number of waste dumps with a total volume of 140 million tons of mine spoil caused the generation of acid mine drainage which, in turn, interferes with the microbial populations of the habitats via the water path.

The presented study combines investigations of ecological and physiological effects of metals on growth and survival of actinobacteria with the analysis of molecular processes that make the settlement of actinobacteria in the metalliferous habitats of mining sites feasible. The toxicity of nickel towards several members of the actinobacteria, including *Kitasatospora*, *Lentzea*, *Nocardia*, and *Streptomyces* isolated from metalliferous and non-metalliferous habitats was evaluated. It could be shown that a high degree of nickel tolerance occurs among isolates from the serpentinite outcrops of central Italy. Millions of

years of pedogenesis above metal rich base rocks resulted in a strong adaptation to high metal contents. However, it could also be shown that actinobacterial isolates with a nickel resistance up to 10 mM can be derived even from non-metalliferous habitats. In presence of nickel, growth of the mining isolate *Streptomyces acidiscabies* E13 used to study resistance mechanisms, can be adapted from originally 8 mM to 25 mM in complex medium. This illustrates the importance of considering varied metal detoxification mechanisms when evaluating results from different resistance studies.

Nickel can bind heterogeneous cellular compounds, e.g., the phosphate groups of nucleic acids and thiol groups of proteins. Also components of the cell wall, like teichoic acids, are targets for nickel binding. Analysis of the nickel distribution within different cellular fractions of *S. acidiscabies* E13 displayed the largest amount of nickel in the cytosol. Only a minor quantity of nickel was associated with the cell wall. TEM micrographs revealed the increase of intracellular storage bodies under nickel stress. These cytosolic granules have not been identified as volutin, which would hint at a metal sequestering mechanism known from *Staphylococcus aureus*.

During biosorption studies it could be shown that *S. acidiscabies* E13 can survive four weeks of incubation in acid mine drainage waters characterized by pH 2.8 and high metal concentrations. In this environment, the strain was shown to remove more than 80% of Al, Cu and U from solution after one week of treatment. Additionally, crystallisation of a mineral associated with the colonies of *S. acidiscabies* E13 could be observed during cultivation experiments. Structure and composition of the mineral could be elucidated to be $Ni(NH_4)(PO_4)\cdot 6H_2O$. The nickel analogue of the mineral struvite was previously not found in nature. These findings demonstrate the great impact that actinobacteria dwelling in metalliferous habitas can have on the distribution of metals between mobile and stationary phases.

Using thin-layer chromatography, *S. acidiscabies* E13 was shown to release several nickel chelating compounds in the presence of nickel. Large amounts of a brownish, melanin-like pigment are excreted under conditions of nickel stress. The results suggest that the released compounds mediate an extracellular sequestration and thus impede the influx of nickel. Ten actinobacteria originating from diverse habitats were analyzed chromatographically on their metabolite pattern and in bioassays after growth in presence of nickel or cadmium. Some of the strains produced certain metabolites only in presence of the heavy metal. It is assumed that the release of these compounds can lead to extracellular sequestration, thus allowing growth at comparatively high nickel concentrations.

In conclusion, this study stresses the importance of investigating metal resistance mechanisms of actinobacteria in conjunction with bioremediation of contaminated biotopes. Evidently, there is need for interdisciplinary research involving soil sciences, microbiology and hydrogeology in order to gain a more complete understanding of microbial activity at metal polluted sites. Ideally, this thesis can make a contribution to the development of bioremediation strategies for the treatment of metal contaminated soils and substrates that originate from mining operations.

7 Zusammenfassung

Ziel der vorliegenden Arbeit ist es, einen Beitrag zum Verständnis der Wechselwirkungen zwischen Metall und Mikroorganismus im Bodenhabitat zu liefern. Die Arbeit umfasst dabei:

1. Isolation, taxonomische Beschreibung und Testung des Resistenzpotentials von Actinobakterien, die von (a) Flächen des ehemaligen Uranbergbaus in Ostthüringen, (b) Serpentinit-Standorten in Zentralitalien und (c) nicht-metallhaltigen Habitaten der Umgebung Jenas isoliert wurden
2. Anpassungsfähigkeit eines ausgewählten Bergbau-Isolates an zunehmende Nickel-Belastung und standortabhängige Ni-Toleranz verschiedener Actinobakterien-Stämme
3. Resistenzmechanismen von Streptomyceten, die Wachstum bei hohen Ni-Konzentrationen ermöglichen
4. Biosorption (Al, Cu, Ni, U, Seltenerdmetalle) verschiedener Bergbau-Isolate
5. Einfluss von Ni und Cd auf den Sekundärmetabolismus einiger Actinobakterien
6. Biomineralisation einer Ni-haltigen Verbindung durch einen Ni-resistenten Stamm

Es gibt nur wenige Untersuchungen, die sich mit der Metallresistenz von Gram-positiven Bakterien und insbesondere mit der ökologisch sehr wichtigen Gruppe der Actinobakterien befassen. Häufig zielen Studien, die sich mit Resistenzmechanismen beschäftigen, auf Einzelprozesse wie bspw. Kationenefflux-Transport ab. Häufig lösen Stressoren aber eine Vielzahl von Reaktionen aus. Die vorgestellten Untersuchungen zur Metallresistenz von Aktinobakterien verbinden aus diesem Grunde drei verschiedene Mechanismen der Detoxifizierung: Intrazelluläre Sequestrierung von Metallen, Immobilisierung von Metallen an der Zellwand und Exkretion von Chelatbildnern in das Millieu.

Bergbaugebiete, in denen jahrzehntelang Erzgewinnung erfolgt ist, stellen ideale Untersuchungsflächen für Forschungsprojekte über Metall-Mikroorganismus-Interaktionen dar. Die Gewinnung von 124 Mio t Uranerz innerhalb einer Fläche von 74 km^2 der Ronneburger Lagerstätte in Ostthüringen führte zur großflächigen Entstehung von metallhaltigen Habitaten. Nickel ist ein häufiges Begleitmetall von Uranerzen; es kommt in Konzentrationen bis zu 2000 ppm im Haldenmaterial dieses Bergbaugebietes vor. Die große Anzahl von Abraumhalden mit einem Gesamtvolumen von 140 Mio t verursachte die Bildung saurer Bergbauabwässer, die über den Wasserpfad in Verbindung mit den Mikrobenpopulationen der Habitate stehen.

Die vorliegende Arbeit verbindet Untersuchungen ökologischer und physiologischer Effekte von Metallen auf Wachstum und Überdauerung von Actinobakterien mit der Identifizierung molekularer Mechanismen, die die Besiedlung metallreicher Habitate ermöglichen. Die Toxizität von Nickel auf verschiedene Actinobakterien, einschließlich *Kitasatospora*, *Lentzea*, *Nocardia* und *Streptomyces* wurde evaluiert. Getestet wurden Isolate aus metallbelasteten und -unbelasteten Habitaten. Es konnte gezeigt werden, dass Nickel-Toleranz unter Isolaten aus Serpentinitböden Zentralitaliens weit verbreitet ist. Eine Millionen von Jahren währende Pedogenese über einem metallreichen Ausgangsgestein führte zu starker Anpassung an hohe Metallgehalte im Habitat. Es konnte jedoch ebenfalls gezeigt werden, dass Actinobakterien mit einer Resistenz bis zu 10 mM Ni vereinzelt auch aus nicht-metallhaltigen Habitaten isoliert werden können. Das Haldenisolat *Streptomyces acidiscabies* E13, das ausgewählt wurde, um Resistenzdeterminanten zu untersuchen, kann von einer ursprünglichen 8 mM Ni-Resistenz auf Wachstum in Gegenwart von 25 mM Ni adaptiert werden. Das verdeutlicht, dass ein Stamm verschiedenartige Mechanismen der Ni-Detoxifizierung besitzt, die in Abhängigkeit von den Umgebungsbedingungen exprimiert werden.

Nickel kann verschiedenartige Verbindungen der Zelle komplexieren und damit konstitutiv in Struktur und Funktion von Zellbestandteilen eingreifen. Zielorte für Bindungen sind bspw. Phosphatgruppen der Nukleinsäuren, Thiolgruppen von Proteinen und Teichonsäuren der Zellwand. Die Analyse der Nickelverteilung innerhalb der Zelle ergab für den Stamm *S. acidiscabies* E13 den größten Nickelgehalt im Cytosol. Nur eine vergleichsweise kleine Menge von Nickel war mit der Zellwand assoziiert. TEM-Aufnahmen zeigten die starke Zunahme an intrazellulären Granula unter Nickelstress. Die cytosolischen Granula wurden nicht als Volutin identifiziert, das als Matrix zur Sequestrierung von Metallen aus *Staphyloccus aureus* bekannt ist.

Biosorptionsstudien haben gezeigt, dass der Stamm *S. acidiscabies* E13 eine vierwöchige Inkubation in einem metallreichen Bergbauabwaser von pH 2,8 überdauern kann. Abwasser und Isolat entstammen demselben Bergbaugebiet. Mit Hilfe des Stammes konnten aus dem Bergbauabwasser Al, Cu und U nach einer Woche zu mehr als 80% abgetrennt werden. Aus den Ergebnissen konnte geschlossen werden, dass neben adsorptiven Prozessen auch Aufnahme und Biopräzipitation eine entscheidende Rolle spielen. Bei Kultivierungsexperimenten zur Testung der Biomineralisation von *S. acidiscabies* E13 wurde Kristallwachstum innerhalb der Kolonien beobachtet. Aufklärung von Struktur und Zusammensetzung des Minerals [$Ni(NH_4)(PO_4)\cdot 6H_2O$] zeigte, dass es sich um das bisher noch nicht in der Natur gefundene Nickel-Analogon des Minerals Struvit handelt. Die Ergebnisse zeigen, welche Bedeutung Actinobakterien, die in metallhaltigen

Habitaten vorkommen, auf die Verteilung von Metallen in der mobilen und stationären Phase haben können.

Dünnschichtchromatographisch konnte gezeigt werden, dass der Stamm *S. acidiscabies* E13 in Kulturen, die mit 1-5 mM Nickel supplementiert wurden, mehrere Nickel chelatisierende Verbindungen abgibt. Unter Bedingungen des Nickelstress' ist in komplexen Medien die Exkretion eines braunen, Melanin-ähnlichen Pigments charakteristisch für den Stamm. Einige von Pilzen produzierte Melanine sind bekannt für ihre metallbindenden Eigenschaften. Die Ergebnisse legen nahe, dass die Bildung extrazellulär nickelbindender Metabolite durch Nickelstress induziert werden kann und Bestandteil der Resistenzantwort ist. Zehn aus verschiedenen Habitaten isolierte Stämme der Gruppe Actinobacteria wurden nach Kultur in Nickel- oder Cadmium-haltigen Medien chromatographisch auf ihr Sekundärmetabolit-Muster untersucht und in einem Bioassay gegen vier Teststämme geprüft. Es konnten einige Stämme identifiziert werden, die bestimmte Metabolite nur in der metallhaltigen Kultur produzierten. Es wird angenommen, dass die Exkretion dieser Verbindungen zu einer extrazellulären Sequestrierung der Metalle führt und damit Wachstum bei vergleichsweise hohen Metallkonzentrationen ermöglicht.

Die vorliegende Arbeit unterstreicht die Bedeutung der Untersuchung von Mechanismen der Metallresistenz bei Actinobakterien unter dem Blickwinkel der Bioremediation kontaminierter Biotope. Um ein besseres Verständnis der mikrobiellen Aktivität auf metallbelasteten Flächen zu entwickeln, ist interdisziplinäre Forschung von u. a. Bodenkundlern, Mikrobiologen und Hydrogeologen unabdingbar. Wenn diese Dissertation einen Beitrag zur Entwicklung von Bioremediationsstrategien für die Behandlung belasteter Böden und Substrate liefern kann, wäre damit ihr Anliegen erfüllt.

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10 Eigenständigkeitserklärung

Ich erkläre, dass ich die Dissertation “STUDIES ON HEAVY METAL RESISTANCE OF BACTERIAL ISOLATES FROM A FORMER URANIUM MINING AREA” selbständig und nur mit der darin angegebenen Hilfe verfasst habe. Die Dissertation wurde in keiner anderen Fakultät oder Universität eingereicht.

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Götz Haferburg